

# Cerberus–Nodal–Lefty–Pitx signaling cascade controls left–right asymmetry in amphioxus

Guang Li<sup>a,1</sup>, Xian Liu<sup>a,1</sup>, Chaofan Xing<sup>a</sup>, Huayang Zhang<sup>a</sup>, Sebastian M. Shimeld<sup>b,2</sup>, and Yiquan Wang<sup>a,2</sup>

<sup>a</sup>State Key Laboratory of Cellular Stress Biology, School of Life Sciences, Xiamen University, Xiamen, Fujian 361102, China; and <sup>b</sup>Department of Zoology, University of Oxford, Oxford OX1 3PS, United Kingdom

Edited by Marianne Bronner, California Institute of Technology, Pasadena, CA, and approved February 21, 2017 (received for review December 14, 2016)

Many bilaterally symmetrical animals develop genetically programmed left–right asymmetries. In vertebrates, this process is under the control of Nodal signaling, which is restricted to the left side by *Nodal* antagonists *Cerberus* and *Lefty*. *Amphioxus*, the earliest diverging chordate lineage, has profound left–right asymmetry as a larva. We show that *Cerberus*, *Nodal*, *Lefty*, and their target transcription factor *Pitx* are sequentially activated in *amphioxus* embryos. We then address their function by transcription activator-like effector nucleases (TALEN)-based knockout and heat-shock promoter (HSP)-driven overexpression. Knockout of *Cerberus* leads to ectopic right-sided expression of *Nodal*, *Lefty*, and *Pitx*, whereas overexpression of *Cerberus* represses their left-sided expression. Overexpression of *Nodal* in turn represses *Cerberus* and activates *Lefty* and *Pitx* ectopically on the right side. We also show *Lefty* represses *Nodal*, whereas *Pitx* activates *Nodal*. These data combine in a model in which *Cerberus* determines whether the left-sided gene expression cassette is activated or repressed. These regulatory steps are essential for normal left–right asymmetry to develop, as when they are disrupted embryos may instead form two phenotypic left sides or two phenotypic right sides. Our study shows the regulatory cassette controlling left–right asymmetry was in place in the ancestor of *amphioxus* and vertebrates. This includes the *Nodal* inhibitors *Cerberus* and *Lefty*, both of which operate in feedback loops with *Nodal* and combine to establish asymmetric *Pitx* expression. *Cerberus* and *Lefty* are missing from most invertebrate lineages, marking this mechanism as an innovation in the lineage leading to modern chordates.

*amphioxus* | *Nodal* | left–right asymmetry | TALEN | embryonic development

**B**ilaterians share three primary developmental axes. The anterior–posterior (AP) and dorsal–ventral (DV) axes define bilaterally symmetrical organization. The third axis, orthogonal to these, is known as the medial–lateral or left–right (LR) axis and displays mirror-image symmetry. However, many bilaterian species deviate consistently from true symmetry, raising fundamental questions of how symmetry is broken and how different developmental programs can unfold on the left and right sides of an organism (1). In vertebrates, this includes asymmetric development of the heart and viscera, disruption of which during embryogenesis causes a range of human disorders (2).

Correct LR organization in vertebrates is regulated by a gene cassette in which right-sided *Cerberus* (*Cer*) and left-sided *Nodal* and *Lefty* regulate left-sided expression of the *Pitx* family gene *Pitx2* and hence morphological LR asymmetry (3). *Cer* expression on the right of the embryonic node is required to repress *Nodal* signaling, which happens by *Cer* protein binding directly to *Nodal* protein. This restricts the ability of *Nodal* protein to activate the expression of the *Nodal* gene, leading to an up-regulation of *Nodal* on the left of the embryo. *Lefty* expression is also up-regulated by *Nodal* and, like *Cer*, acts as an extracellular inhibitor of *Nodal*. Coexpression of *Nodal* and *Lefty* can act as an activator–inhibitor system in the sense originally described by Turing (4). *Nodal* on the left of the embryo activates expression of *Pitx2*, which directs the development of left-sided morphology (3).

Several studies have sought to dissect the evolutionary history of *Nodal* signaling and its regulation of LR asymmetry. Notably, asymmetric expression of *Nodal* and *Pitx* in gastropod mollusc embryos plays a role in the development of LR asymmetry, including the coiling of the shell (5, 6). Asymmetric expression of *Nodal* and/or *Pitx* has also been reported in some other lophotrochozoans, including *Pitx* in a brachiopod and an annelid and *Nodal* in a brachiopod (7, 8). These data can be interpreted to suggest an ancestral role for *Nodal* and *Pitx* in regulating bilaterian LR asymmetry, however the picture is complicated by data from other lineages. First, in ecdysozoan lineages, *Nodal* appears to have been lost (7, 9), whereas *Pitx* is generally symmetrically expressed (8) and, where studied, does not function in LR asymmetry (10, 11). Second, some lophotrochozoans do not show asymmetric *Pitx* expression (8, 12, 13). Third, functional studies on protostome *Nodal* signaling are currently restricted to molluscs (5), so it is unclear whether the regulatory connection between *Nodal* and *Pitx* is conserved even in those species that show asymmetric expression. An additional complication is that *Cer* and *Lefty* are also absent from many invertebrate bilaterian lineages (9, 14). The extracellular inhibition of *Nodal* by the proteins encoded by these two genes is a critical component of *Nodal* signaling in vertebrates (4, 15) and is required for both the generation and maintenance of asymmetric *Nodal* and *Pitx2*.

To examine the evolutionary history of *Nodal* signaling and its regulation, we turned to *amphioxus*, the basal chordate lineage and one that forms a typical chordate body plan while also developing pronounced LR asymmetries (16). During *amphioxus* embryogenesis, the mouth opens on the left side of the head,

## Significance

Some bilaterally symmetrical animals show genetically programmed differences between their left and right sides, for example the placement of the heart and viscera in humans and other vertebrates. We dissect the regulation of this in embryos of *amphioxus*, a close relative of vertebrates that develops extraordinary asymmetries as a larva. We demonstrate a system in which asymmetric expression of the signal gene *Nodal* is controlled by positive and negative feedback loops with its own inhibitors. When this system is disrupted, embryos develop mirror-image symmetry with two “left” sides or two “right” sides. Comparison with other animals shows how this complex regulatory mechanism has evolved by addition of new feedback regulation to a more ancient signaling interaction.

Author contributions: G.L., X.L., S.M.S., and Y.W. designed research; G.L., X.L., C.X., and H.Z. performed research; G.L., X.L., C.X., H.Z., S.M.S., and Y.W. analyzed data; and G.L., X.L., S.M.S., and Y.W. wrote the paper.

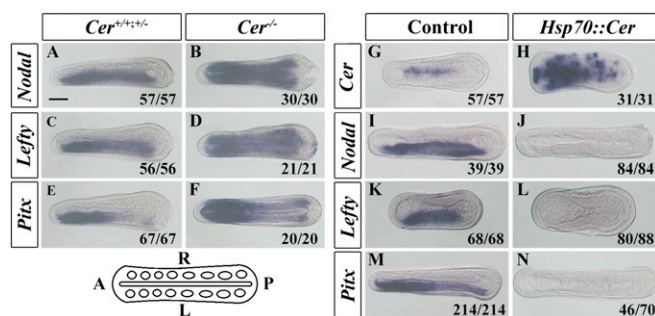
The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

<sup>1</sup>G.L. and X.L. contributed equally to this work.

<sup>2</sup>To whom correspondence may be addressed. Email: wangyq@xmu.edu.cn or sebastian.shimeld@zoo.ox.ac.uk.

This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1620519114/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1620519114/-DCSupplemental).



**Fig. 1.** Cer regulates *Nodal*, *Lefty*, and *Pitx* expression in amphioxus. (A–F) Expression of *Nodal*, *Lefty*, and *Pitx* in *Cer* wild-type/heterozygous ( $Cer^{+/+}$ ,  $Cer^{+/-}$ ) or homozygous mutant ( $Cer^{-/-}$ ) embryos. (G–N) Expression of *Cer*, *Nodal*, *Lefty*, and *Pitx* in Control (uninjected but heat-shocked) and *Cer* misexpressed (*Hsp70::Cer*-injected and heat-shocked) embryos. Numbers at the bottom right of each panel indicate the number of times the phenotype shown was observed out of the total number of manipulated embryos. (Scale bar, 50  $\mu$ m.) All embryos are in dorsal view with anterior to left as indicated in the sketch at the bottom left.

while the gill slits, which initiate opening at the ventral midline, later extend up the right side. Other endodermal organs are similarly asymmetric, and somites also show pronounced LR asymmetry. *Cer*, *Nodal*, *Lefty*, and *Pitx* are asymmetrically expressed in amphioxus, and pharmacological manipulation of the Alk4/5/7 Tgfb receptor disrupts LR development, suggesting a function for *Nodal* in amphioxus LR asymmetry (17, 18). However, *Nodal* function has not been directly tested, and it is not known if *Pitx* regulates LR asymmetric morphology. The functions of *Cer* and *Lefty* are also untested, so it is not clear if and how they regulate *Nodal* signaling.

To test these regulatory interactions in amphioxus, we developed transcription activator-like effector nucleases (TALEN)-based methods of removing gene function and heat-shock promoter (HSP)-driven methods of overexpression and deployed them to address the function of all four genes. We show that right-sided *Cer* represses *Nodal* expression and find that when *Cer* function is impaired *Nodal* becomes bilateral. *Nodal* activates its own expression and also that of *Lefty*. *Lefty* feeds back to repress *Nodal*, restricting its expression to the left side. This results in left-sided activation of *Pitx*. We further show that these regulatory steps are essential for normal LR asymmetry to develop. The knockdown or overexpression of *Cer*, *Nodal*, *Lefty*, or *Pitx* results in embryos with either two phenotypic left sides or two phenotypic right sides, with bilateral duplication of most normally lateralized structures in each case. These data show the full regulatory cassette, with both inhibitors acting to restrict *Nodal* expression to the left side, is present in amphioxus and controls LR morphology. We conclude this pathway represents an ancient chordate character, with the full antagonist-based regulation of *Nodal* already present in the common ancestor of the chordates.

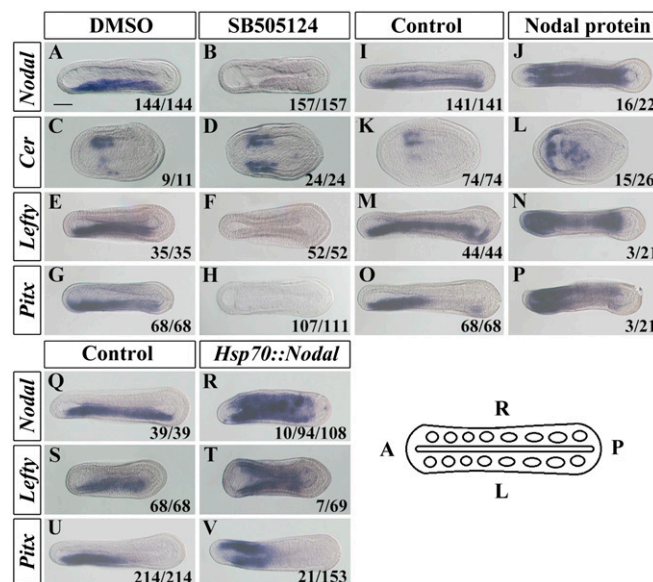
## Results

*Cer*, *Nodal*, *Lefty*, and *Pitx* are known to be asymmetrically expressed in amphioxus (18–21), however the relative timing of their expression has yet to be elucidated. We therefore examined whether these genes are spatiotemporally activated in patterns consistent with a regulatory hierarchy. Staged in situ hybridization demonstrated that right-sided *Cer* preceded lateral restriction of *Nodal*, *Lefty*, and *Pitx* (SI Appendix, Fig. S1). Furthermore, lateralized expression of *Lefty* and *Pitx* preceded that of *Nodal* (SI Appendix, Fig. S1).

These temporal expression patterns are consistent with *Cer* regulating the lateralization of the other genes. To test this, we generated a TALEN knockout heterozygous *Cer* line (SI Appendix, Figs. S2 and S3). Crossing  $Cer^{+/-}$  animals yielded  $Cer^{+/-}$ ,

$Cer^{+/-}$ , and  $Cer^{-/-}$  genotypes in the expected ratio (SI Appendix, Fig. S4), and we assessed the expression of *Nodal*, *Lefty*, and *Pitx* in these embryos. Although  $Cer^{+/-}$  and wild-type embryos maintained wild-type expression,  $Cer^{-/-}$  embryos showed bilateral expression of *Nodal*, *Lefty*, and *Pitx* (Fig. 1A–F). This indicates *Cer* is necessary to restrict the expression of *Nodal*, *Lefty*, and *Pitx* to the left side of amphioxus embryos. To test whether *Cer* was also sufficient to regulate these genes, we induced overexpression of *Cer* using a heat-regulated *HSP::Cer* construct (SI Appendix, Fig. S5). This generated widespread ectopic *Cer* expression in 100% (31/31) of embryos (Fig. 1H) and abolished left-sided expression of *Nodal*, *Lefty*, and *Pitx* in 100% (84/84), 95% (80/88), and 66% (46/70) of embryos, respectively (Fig. 1J, L, and N). Combined, these data show *Cer* is necessary and sufficient for regulating the lateral expression of *Nodal*, *Lefty*, and *Pitx*.

*Nodal* signaling is transduced by Tgfb receptors of the Alk4/5/7 family (22). Pharmacological inhibition of Alk4/5/7 in amphioxus has been reported to block left-sided *Nodal*, *Lefty*, and *Pitx* expression (18), and we found the same in our experiments (Fig. 2B, F, and H). However, the Alk4/5/7 receptor may also mediate signaling by other Tgfb family ligands, including Activin and Tgfb (23), raising uncertainty as to the ligand involved in its activation in amphioxus in vivo. *Nodal* in amphioxus also has an earlier developmental role (24), precluding its inhibition by the TALEN method as embryos would not develop to the stage where LR asymmetry can be addressed. We therefore tested the effect of inducible and ectopic *Nodal* on amphioxus LR asymmetry. Overexpression of *Nodal* using an *HSP::Nodal* construct (Fig. 2R, T, and V and SI Appendix, Fig. S5), or administration of *Nodal* protein to developing embryos (Fig. 2J, N, and P), induced ectopic



**Fig. 2.** *Nodal* regulates *Cer*, *Nodal*, *Lefty*, and *Pitx* expression in amphioxus. (A–H) Expression patterns of *Nodal*, *Cer*, *Lefty*, and *Pitx* in control (DMSO) and *Nodal* pathway inhibitor (SB505124)-treated embryos. Embryos were treated from the midgastrula stage until they were collected for gene expression analysis (the 3-somite stage for *Cer* and around the 8-somite stage for other genes). (I–P) Expression patterns of *Nodal*, *Cer*, *Lefty*, and *Pitx* in Control (untreated) and *Nodal* protein-treated embryos. Embryos were cultured with 8  $\mu$ g/mL of mouse recombinant *Nodal* protein for the same times as above. (Q–V) Expression patterns of *Nodal*, *Lefty*, and *Pitx* in control (uninjected but heat-shocked) and *Nodal* misexpressed (*Hsp70::Nodal*-injected and heat-shocked) embryos. Numbers at the bottom right of each panel indicate the number of times the phenotype shown was observed, out of the total number of embryos examined. (Scale bar, 50  $\mu$ m.) All images are dorsal views with anterior to left as indicated in the sketch at the bottom right.



right-sided expression of *Nodal*, *Lefty*, and *Pitx*. We also found that Nodal manipulation fed back on *Cer* expression, with *Alk4/5/7* inhibition inducing ectopic left-sided *Cer* (as previously reported in ref. 18) and Nodal overexpression inhibiting *Cer* expression in the first somites (Fig. 2 *D* and *L*). These results confirm previous inference of the necessity of Nodal for lateralized *Pitx* expression in amphioxus, demonstrate that it is also sufficient, and further show that Nodal also negatively regulates *Cer* expression. Because we have shown that *Cer* inhibits *Nodal* expression, this suggests a negative feedback loop in which Nodal represses the expression of its own inhibitor.

In vertebrate embryos, *Lefty* also acts as an extracellular inhibitor of Nodal protein, and experiments in an echinoderm suggest this might also be the case in this lineage (25). To test the function of *Lefty* in amphioxus, we generated embryos in which the *Lefty* gene was targeted by injection of a TALEN mRNA (*SI Appendix*, Fig. S6). In 82.4% (98/118) of injected embryos, *Nodal* expression became bilateral (Fig. 3*D*). Furthermore, *Pitx* and *Lefty* itself also became bilateral, in 49.3% (102/207) and 36.7% (40/109) of embryos, respectively (Fig. 3 *H* and *F*). To test the sufficiency of *Lefty* to inhibit normal left-sided gene expression, we also injected embryos with an inducible *HSP::lefty* construct (*SI Appendix*, Fig. S5). In the majority of heat-shocked embryos, left-sided expression of *Nodal* and *Pitx* was lost (Fig. 3 *L* and *N*). These data demonstrate that, in amphioxus, *Lefty* can act to inhibit *Nodal* expression. In the absence of *Lefty*, *Nodal* is ectopically expressed on the right-hand side (Fig. 3*D*). As Nodal up-regulates

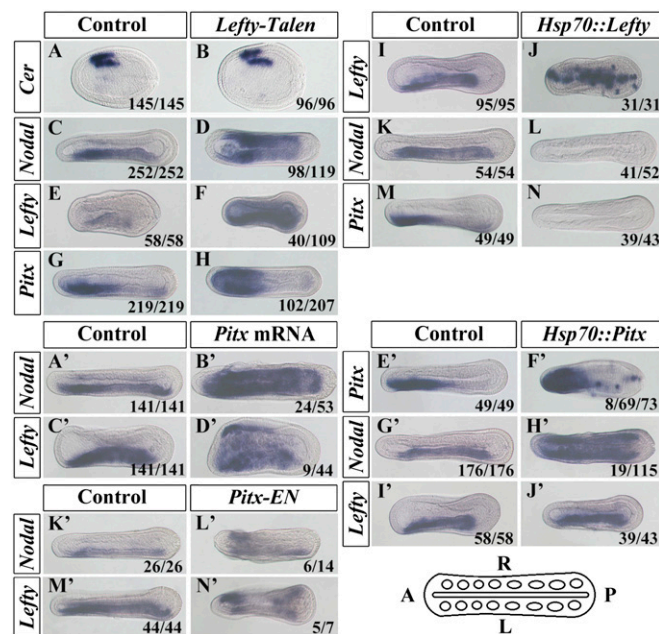
*Lefty* and *Pitx* (Fig. 2 *N*, *P*, *T*, and *V*), this probably in turn leads to the ectopic expression of *Lefty* and *Pitx* seen in *Lefty* TALEN-injected embryos (Fig. 3 *F* and *H*).

*Pitx* homeobox genes are conserved targets of Nodal signaling in vertebrates (3), echinoderms (26), and probably also molluscs (5). In vertebrates, asymmetric *Pitx2* is required for correct LR morphogenesis, but studies suggest that *Pitx2* does not feed back to regulate *Nodal* expression (27). Although the experiments described above show that *Cer*, *Lefty*, and Nodal can regulate *Pitx* expression in amphioxus, they do not establish whether *Pitx* might also regulate their expression. To test this, we sought to up- and down-regulate *Pitx* expression, while monitoring its impact on the expression of *Nodal* and *Lefty*. Up-regulation of *Pitx* by mRNA injection caused significant disruption to early development, although many of the embryos that did survive through to the neurula stage and beyond displayed ectopic right-sided expression of *Nodal* (45.3%: 24/53) and *Lefty* (20.5%: 9/44) (Fig. 3 *B'* and *D'*). To abrogate this early developmental impact of *Pitx* mRNA injection, we injected an inducible *HSP::Pitx* construct (*SI Appendix*, Fig. S5). In heat-induced *HSP::Pitx*-injected embryos, 16.5% (19/115) showed ectopic right-sided *Nodal* expression (Fig. 3*H'*). None showed ectopic *Lefty* expression (Fig. 3*J'*). To block *Pitx* function, we designed a fusion construct linking amphioxus *Pitx* DNA-binding domain to the Engrailed Repressor (EnR) domain (*SI Appendix*, Fig. S5) and injected the mRNA encoding this into amphioxus embryos. As with *Pitx* mRNA injection, many such embryos developed with significant abnormalities before the neurula stage, precluding their analysis for LR defects. However, in embryos that reached the neurula stage and displayed approximately normal AP and DV axial organization, ectopic activation of both *Lefty* and *Nodal* was observed on the right side in 71.4% (5/7) and 42.9% (6/14) of cases, respectively (Fig. 3 *L'* and *N'*).

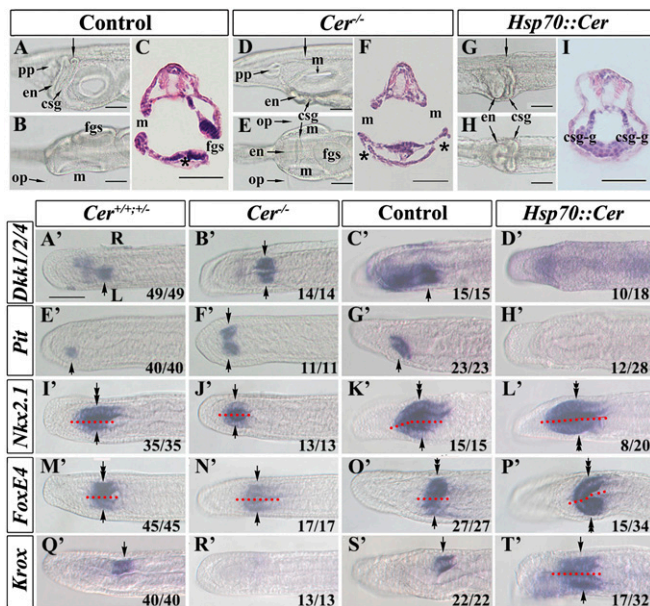
In some cases, LR asymmetry phenotypes derived from manipulating gene expression early in development might be explained by disturbed AP/DV organization, which in vertebrates may affect the midline and hence increase the incidence of laterality defects (28). Although this consideration might apply to embryos injected with *Pitx* mRNA or *Pitx-EnR* mRNA, which would be translated very early in development, the *HSP::Pitx* construct was only activated by heat shock after the midgastrula stage, and this also induced ectopic right-sided *Nodal* expression (Fig. 3*H'*). These data suggest that, unlike vertebrates, in amphioxus *Pitx* feeds back to regulate Nodal signaling.

The experiments described above show that *Cer* represses *Nodal*, that Nodal represses *Cer* and activates *Lefty* and *Pitx*, that *Lefty* represses *Nodal*, and that *Pitx* activates *Nodal*. An assumption is that left-sided *Pitx* will drive the development of left-sided morphology. To test this, we allowed embryos in which *Cer*, *Nodal*, *Lefty*, or *Pitx* expression had been manipulated to grow to the late embryonic or larval stages, so we could monitor the impact on the development of LR asymmetric characters.

Amphioxus larvae are extremely asymmetric, with the mouth positioned on the left side of the head and the gill slits, which initiate opening at the ventral midline, expanding to spread up the right side. Several internal systems are also distinctly organized across the LR axis of the head and pharynx, including the club-shaped gland, preoral pit, and endostyle (Fig. 4 *A–C*). A suit of asymmetrically expressed marker genes (18) including *Krox*, *FoxE4*, *Nkx2.1*, *Pit*, and *Dkk1/2/4* mark the LR displacement of these structures (Fig. 4 *A'*, *E'*, *I'*, *M'*, and *Q'*). To determine the impact of manipulating *Cer* expression on LR morphology, we allowed manipulated embryos to grow to the larval stage before analyzing morphology and LR marker gene expression. *Cer*<sup>−/−</sup> embryos develop a consistent two-left-side (2-left) phenotype, with a mouth opening on each side, duplication of the left-sided structures such as the preoral pit and proximal club-shaped gland, and loss of the right-sided distal club-shaped gland (Fig. 4 *D–F* and



**Fig. 3.** *Lefty* inhibits *Nodal*, *Lefty*, and *Pitx* expression, and *Pitx* overexpression or knockdown induces bilateral expression of *Nodal* and *Lefty*. (*A–H*) Expression patterns of *Cer*, *Nodal*, *Lefty*, and *Pitx* in control (uninjected) and *Lefty* TALEN mRNA-injected embryos. (*I–N*) Expression patterns of *Nodal*, *Lefty*, and *Pitx* in control (uninjected but heat-shocked) and *Lefty* misexpressed (*Hsp70::Lefty*-injected and heat-shocked) embryos. (*A'–D'*) Expression patterns of *Nodal* and *Lefty* in control (uninjected) and *Pitx* mRNA-injected embryos. (*E'–J'*) Expression patterns of *Nodal*, *Lefty*, and *Pitx* in control (uninjected but heat-shocked) and *Pitx* misexpressed (*Hsp70::Pitx*-injected and heat-shocked) embryos. (*K'–N'*) Expression patterns of *Nodal* and *Lefty* in control (uninjected) and *Pitx-En* mRNA-injected embryos. Numbers at the bottom right of each panel indicate the number of times the phenotype shown was observed, out of the total number of embryos examined. (Scale bar, 50  $\mu$ m.) All images are dorsal views with anterior to left as indicated in the sketch at the bottom right.



**Fig. 4.** *Cer* mutation or misexpression affects development of amphioxus LR asymmetry and the expression of organ-specific marker genes. (A–C) Asymmetrical morphologies of control (wild-type, *Cer*<sup>+/+</sup>, or *Hsp70::Cer*-uninjected but heat-shocked) larvae. (A) Left lateral view of the pharyngeal region focused on the right side. (C) Transverse section of larva from A (arrow). (D–F) Symmetrical morphologies of *Cer* mutant larvae. (D) Left lateral view of the pharyngeal region focused on the ventral side. (F) Transverse section of larva from D (arrow). (G–I) Symmetrical morphologies of *Cer* misexpressed larvae. (G) Left lateral view of the pharyngeal region focused on the sagittal plane. (H) Dorsal view of the pharyngeal region focused on the ventral side. (I) Transverse section of larva from G (arrow). (A'–D') Expression of *Dkk1/2/4* in the region destined to develop into the mouth opening (arrows). The expression domain is detected on the left side of *Cer*<sup>+/+</sup> or *Cer*<sup>+/+</sup> siblings or control embryos and on both sides of *Cer*<sup>+/+</sup> mutant embryos but is lost in *Cer* misexpressed embryos. (E'–H') Expression of *Pitx* in the prospective preoral pit (arrows). *Pitx* transcripts are detected on the left side of *Cer*<sup>+/+</sup> or *Cer*<sup>+/+</sup> siblings or control embryos and on both sides of *Cer*<sup>+/+</sup> mutants but are undetectable in *Cer* misexpressed embryos. (I'–L') Expression of *Nkx2.1* in the prospective endostyle. Expression of *Nkx2.1* in the endostyle can be divided into the left-side part (arrows) and the right-side part (tandem arrows) along the sagittal plane (indicated by red dotted lines). Although its expression in the left side is duplicated in *Cer*<sup>+/+</sup> mutants and lost in *Cer* misexpressed embryos, its expression in the right side is lost in *Cer*<sup>+/+</sup> mutants and duplicated in *Cer* misexpressed embryos. (M'–P') Expression of *FoxE4* in the prospective csg. The csg comprises two parts: the left-sided duct (arrows) and the right-sided glandular region (tandem arrows). *FoxE4* expression in the duct is duplicated in *Cer*<sup>+/+</sup> mutants and lost in *Cer* misexpressed embryos, but its expression in the glandular region is duplicated in *Cer* misexpressed embryos and lost in *Cer*<sup>+/+</sup> mutants. (Q'–T') Expression of *Krox* in the duct of the prospective csg (arrows). Its expression is lost in *Cer*<sup>+/+</sup> mutants but duplicated in *Cer* misexpressed embryos. Embryos in A'–T' are in dorsal view with anterior to left. On A', L marks the left side and R the right side, and this applies to all panels from A' to T'. Numbers at the bottom right of each panel indicate the number of times the phenotype shown was observed, out of the total number of embryos examined. In some images, red dotted lines separate the left and right of the embryos along the midline. Genotypes as depicted in Fig. 1. Asterisks in C and F denote the external opening of the csg. csg, club-shaped gland; csg-g, right-sided glandular region of csg; en, endostyle; fgs, first gill slit; m, mouth; op, oral papillae; pp, preoral pit. (Scale bar, 50 μm). The scale bar in A' also applies to B'–T'.

*SI Appendix, Fig. S7*). The gill slits, while still initiating and opening at the ventral midline, were also affected as they failed to extend up the right side (Fig. 4 D–F and *SI Appendix, Fig. S7*). Conversely, *HSP::Cer* larvae developed the reciprocal phenotype, with two-right-side (2-right) and concomitant duplication of most right-sided structures (Fig. 4 G–I and *SI Appendix, Fig. S7*). The gill slits were

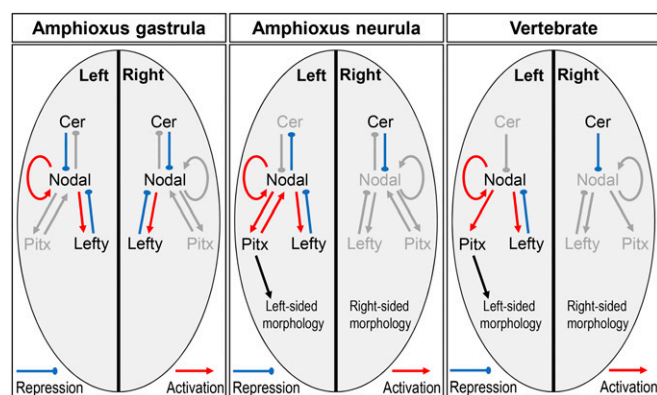
also affected: The first slit appeared to initiate at the ventral midline and like the *Cer*<sup>+/+</sup> phenotype failed to extend up either side, however unlike *Cer*<sup>+/+</sup> embryos, it also failed to properly open. LR marker gene expression precisely matched this morphology, confirming the duplication and loss of reciprocal structures in the 2-left and 2-right phenotypes (Fig. 4 A'–T'). We also allowed embryos in which *Nodal* or *Lefty* had been up- or down-regulated to grow to the larval stage. These manipulations also produced embryos with asymmetry defects, with *Nodal* overexpression and *Lefty* knockdown resulting in 2-left phenotypes and *Nodal* knockdown or *Lefty* overexpression resulting in 2-right phenotypes (*SI Appendix, Fig. S7*). As discussed above, injection of *Pitx* mRNA caused early developmental malformations, however a small number of such embryos did pass through to the larval stage and among them 6.1% exhibited 2-left phenotypes. Likewise, *Pitx* overexpression via the *HSP::Pitx* construct, which did not affect early development, also produced 2-left phenotypes in 10.2% of cases. No 2-right phenotypes were observed in either manipulation. Embryos injected with *Pitx-EnR* were severely malformed by the larval stage, and few pharyngeal structures could be discerned. However, in 7% of cases, paired bilateral mouths were observed, approximating the 2-left phenotype. This is consistent with the ectopic right-sided activation of *Nodal* in *Pitx-EnR*-injected embryos (Fig. 3 L' and N'). These data indicate that the asymmetric expression of the *Cer*–*Nodal*–*Lefty*–*Pitx* cassette regulates the LR morphology of the larva in a predictable way: Any side on which *Nodal*–*Pitx* are activated becomes phenotypically left. If they are not activated, it becomes phenotypically right.

## Discussion

This study shows that the *Cer*–*Nodal*–*Lefty*–*Pitx* cassette functions in amphioxus to regulate LR asymmetry. Our data allow us to construct a regulatory model for the left and right side of amphioxus embryos (Fig. 5). The model suggests competitive feedback on the left side of the amphioxus embryo, with *Nodal* repressing the expression of its upstream repressor (*Cer*), while activating the expression of its downstream repressor (*Lefty*) and activator (*Pitx*). In vertebrates, both *Cer* and *Lefty* repress *Nodal* signaling by directly binding to *Nodal* protein, whereas *Nodal* up-regulates its own expression and that of *Lefty* (4, 15). Thus, in both lineages, the establishment of LR identity is dependent on the appropriate regulation of *Nodal* via positive and negative feedback mediated by extracellular factors. Their focus is establishing asymmetric *Pitx* as the downstream effector, and disruption of this process in amphioxus or vertebrates leads to embryos with disrupted LR asymmetry. The morphological outcome in amphioxus is predictable based on the gene expression; essentially, if *Pitx* expression is activated on a side of the embryo, it will develop left-sided morphology, whereas if *Pitx* is absent, right-sided morphology will develop. Manipulations that yield bilateral *Pitx* distributions generate unviable embryos with 2-left- or 2-right-sided organization, with duplication of the respective pharyngeal and endodermal structures including the mouth, endostyle, and club-shaped gland. These morphologies show that, once handedness is established in amphioxus, the two sides develop independently and autonomously according to their gene expression and are not noticeably influenced by what is happening on the other side of the embryo.

Although the core *Cer*–*Nodal*–*Lefty*–*Pitx* pathway appears conserved between amphioxus and vertebrates, we also note a difference: In amphioxus, *Pitx* feeds back on the regulation of *Nodal* expression. Our model (Fig. 5) suggests that in amphioxus the propagation of “leftness” downstream of asymmetric *Nodal* may therefore depend on competition between positive feedback from *Pitx* and negative feedback from *Lefty*. This regulatory link is not seen in vertebrates or echinoderms (where *Nodal* regulation of *Pitx* expression has been demonstrated) (26), suggesting it may be an amphioxus novelty.





**Fig. 5.** A gene regulatory network model for the control of LR asymmetry in amphioxus. At the gastrula stage, bilateral expression of *Cer*, *Nodal*, and *Lefty* are observed, but *Pitx* is not expressed, indicating *Nodal* regulation of *Pitx* is blocked. During the late gastrula and early neurula stages, *Cer* expression is maintained on the right side of the embryo. *Cer* continues to represses *Nodal* expression, and as a consequence, downstream activation of *Nodal* and *Lefty* is lost, and *Pitx* is not activated. In the absence of *Pitx*, right-sided morphology develops. On the left of the embryo, *Cer* expression is lost during the late gastrula to early neurula stage. *Nodal* is therefore not inhibited and can activate the expression of itself, *Pitx*, and *Lefty*. Left-sided *Pitx* expression results, and hence left-sided morphology develops. An equivalent diagram summarizing the interactions in vertebrates is shown for comparison.

There are also some aspects of amphioxus morphology on which our work sheds light. The amphioxus mouth is unusual in that it opens on the left side of the head, rather than the midline as in most animal lineages, including vertebrates. This has led to speculation that the amphioxus mouth is a derived structure, possibly evolving from the equivalent of a left-sided coelomic pore and not homologous to mouths in other chordates (17). Our demonstration of its dependence on the Cer–Nodal–Lefty–Pitx pathway supports this theory, as the mouth is regulated as a left-sided rather than a midline structure.

Amphioxus gill slits are also unusual. The first gill slit openings, known as the primary gill slits, appear first at the ventral midline and then extend only up the right side of the head (29). Through larval development, gill slits are hence right-sided only. However, at metamorphosis, another set of gill slit openings appear, known as the secondary gill slits. These initiate also on the right side of the larva, dorsal to the primary slits. As these secondary slits extend, the primary slits move ventrally and eventually come to lie on the left side of the head, such that the final adult morphology has symmetrical gill slits. This has led to an assumption, dating from the 1800s, that the slits that lie on the right side of the larval head are in fact homologous to the left-sided slits in other deuterostomes (30). In our experiments, the primary slits initiate but fail to extend in 2-left embryos or to open at all in 2-right embryos. That they initiate is consistent with their positioning at the ventral midline, suggesting this does not require LR axial information. Failure to extend in either sort of embryo suggests subsequent morphogenesis requires both left- and right-sided information. Furthermore, that the slits develop further in 2-left embryos than in 2-right embryos is in keeping with the historical view that the primary slits are, primitively, left-sided structures.

We do not know what breaks symmetry in amphioxus. Studies in some vertebrates, in the urochordate *Halocynthia*, and in echinoderms suggest a role for cilia in symmetry breaking (31–33). Although the amphioxus embryo is heavily ciliated at the stages at which the model suggests symmetry is broken, as yet there is no evidence for a functional role for cilia in amphioxus. We consider the timing of expression and regulatory interactions consistent with the proposal by Blum et al. for a cilia-based mechanism

localized to the gastrocoel roof (31). Whatever the symmetry-breaking mechanism, our data suggest it might work in two ways. Symmetric *Cer* precedes asymmetric right-sided *Cer*. This in turn precedes asymmetric *Nodal* expression, but asymmetric *Lefty* and *Pitx* expression are detectable before asymmetric *Nodal* expression yet are regulated by *Nodal*. Thus, the symmetry-breaking mechanism might act to repress *Cer* expression on the left, allowing *Nodal* protein to activate *Lefty* and *Pitx*. *Nodal* autoregulates, explaining the lag in asymmetric *Nodal* expression compared to *Lefty* and *Pitx*. Under this model, *Nodal* negative feedback on *Cer* would act to lock in this state. We do not know precisely how *Nodal* feeds back on *Cer*, although inhibition of the Alk4/5/7 receptor shows it works through this pathway.

The second possibility is that, as *Nodal* feeds back negatively on *Cer*, the symmetry-breaking mechanism might act to liberate *Nodal* from *Cer* repression. Because *Cer* represses *Nodal* by direct protein–protein binding (15), this could be via some additional extracellular factor preventing or disrupting this interaction. Under this model, *Nodal* would then repress *Cer* and regulate *Lefty* and *Pitx* as above. Although compatible with much of our data, this does not easily account for the early onset of asymmetric *Cer* compared with *Lefty* and *Pitx*. It is also not how the system is thought to work in vertebrates (Fig. 5), where there is so far no evidence for *Nodal* feeding back on *Cer*. We hence consider it a less parsimonious explanation.

Our study shows that full complexity of the Cer–Nodal–Lefty–Pitx cassette was established by the common ancestor of the chordates and inherited by their vertebrate descendants. *Pitx* and *Nodal* are also involved in LR specification in molluscs (5) and may be asymmetrically expressed in some other lophotrochozoans (7, 8), leading to speculation that the role of *Nodal* signaling in LR development was acquired early in animal evolution. However, the roles of *Cer* and *Lefty* may be more recent innovations. *Cer* is a member of the larger Cer/DAN/Gremlin family of Tgfb signaling regulators. Clear *Cer* genes have only been identified in chordates, although one study suggests they may be older and lost by many lineages (9). *Lefty* is a divergent member of the Tgfb family. It is so far found only in chordates and ambulacrarians and in the latter does function in LR asymmetry (9, 25). Therefore, it is unlikely that *Cer* and *Lefty* are more broadly involved in LR asymmetry across protostomes, and we suggest their incorporations into regulation of LR asymmetry are innovations of chordates and deuterostomes, respectively, with negative feedback providing more nuanced control of *Nodal* signaling in these lineages.

## Materials and Methods

**Animal and Embryo Cultivation and Embryo Microinjection.** Amphioxus (*Branchiostoma floridae*) were originally acquired from Jr-Kai Yu, Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan, and the colony was maintained under previously described conditions (34, 35). Gametes were obtained using the thermal-shock method (from 20 °C to 26 °C) (34). Fertilization and subsequent culturing of the embryos were carried out essentially as described previously (36) at 27–28 °C unless otherwise stated. Injection solution was prepared containing 20% (vol/vol) glycerol, 5 mg/mL Texas Red dextran (Life Technology Co.), and 0.25 µg/µL plasmid DNA or 0.15–1.5 µg/µL synthesized mRNA. Embryo microinjection was performed as previously described (36).

## Larval Cultivation and Detection of Cer Mutations in Founder and F1 Amphioxus.

Cer TALEN mRNA-injected embryos and larvae were reared at 24 °C and fed with unicellular algae *Dicrateria zhanjiangensis*, and juveniles and adults were reared in a same way as a previous description (34, 35). Each F0 founder was crossed with a wild-type animal, respectively. About 30–50 embryos from each cross were collected and lysed with Animal Tissue Direct PCR Kit (Foregene Co.) for mutation efficiency and mutation site analysis (34). Progeny of crosses carrying frame-shift mutations were maintained. F1 genotypes were determined in a similar way as described for F0 by cutting a tiny tip of the tail of each animal. The F1 animals carrying identical frame-shift mutations were maintained and intercrossed to generate F2 generation. The F2 embryos were fixed at different developmental stages with 4% (wt/vol) PFA–Mops–EGTA for in situ hybridization

analysis or morphological examination. Animal husbandry and mutation detection are described in detail in *SI Appendix, SI Materials and Methods*.

**Plasmid Construct Preparation.** The full length of coding sequences of *Cer*, *Nodal*, *Lefty*, and *Pitx* genes was amplified from a gastrula cDNA library and then ligated into a pGEM-T-Easy vector (Promega Co.) separately. Further, they were recombined into expression vectors pXT7 or P1. The P1 is a construct initially derived from vector pAcGFP1-1 (Clontech Co.) by integrating a 790-bp regulatory region of *B. belcheri* *Hsp70* gene upstream of the GFP coding sequence (37). A *Pitx-EN*-dominant negative construct was made by fusing the *Pitx* DNA-binding domain and the *Drosophila* EnR domain in the pXT7 vector. TALENs targeting *Cer* and *Lefty* genes were designed and assembled as previously described (38). TALEN binding sites and primer sequences used for cloning and TALEN efficiency assay are shown in *SI Appendix, Tables S1–S5*. The details for the preparation are described in *SI Appendix, SI Materials and Methods*.

**In Vitro Synthesized mRNA and Plasmid DNA Preparation.** All plasmid DNAs were prepared using Plasmid Mini Kit (Omega Co.) and linearized with either *Bam*HI (for pXT7-derived constructs) or *Sac*I (for TALEN constructs), extracted by phenol-chloroform, and dissolved in RNase-free water. In vitro mRNA synthesis was conducted using either T7 (for pXT7-derived constructs) or T3 (for TALEN constructs) mMESAGE mMACHINE kit (Ambion Co.).

**Heat-Shock Experiment.** Embryos injected with *Hsp70::Cer*, *Hsp70::Nodal*, *Hsp70::Lefty*, or *Hsp70::Pitx* DNA plasmids were raised at 25 °C, and heat-shocked at 34 °C in a water bath for 30 min at the early gastrula stage. Uninjected embryos at the same stage were also heat-shocked and used as controls. After heat shock, the embryos were returned to 25 °C and allowed to develop until they were fixed with 4% (wt/vol) PFA–Mops–EGTA at desired stages for in situ or morphological analysis.

**SB505124 and Nodal Protein Treatment and in Situ Hybridization.** Embryos were treated with 50 μM SB505124 (Sigma Co.) or 8 μg/mL recombinant mouse Nodal protein (R&D Co.) in four-well dishes [coated with 1.5% (wt/vol) agarose] from early gastrula, and then were fixed at required stages for in situ hybridization or cultured continuously for morphological observation. The experiments of in situ hybridization were performed essentially according to a previous description (39). All details are described in *SI Appendix, SI Materials and Methods*.

**ACKNOWLEDGMENTS.** We thank Dr. Jr-Kai Yu for providing *B. floridae* animals, and Dr. Dominic Norris for comments on the manuscript. This work was supported by National Natural Science Foundation of China Grants 31471986, 31372188, and 31672246 and Fundamental Research Funds for the Central Universities of China Grant 20720160056.

- Palmer AR (2004) Symmetry breaking and the evolution of development. *Science* 306(5697):828–833.
- Bisgrove BW, Morelli SH, Yost HJ (2003) Genetics of human laterality disorders: Insights from vertebrate model systems. *Annu Rev Genomics Hum Genet* 4:1–32.
- Vandenberg LN, Levin M (2013) A unified model for left-right asymmetry? Comparison and synthesis of molecular models of embryonic laterality. *Dev Biol* 379(1):1–15.
- Müller P, et al. (2012) Differential diffusivity of Nodal and Lefty underlies a reaction-diffusion patterning system. *Science* 336(6082):721–724.
- Grande C, Patel NH (2009) Nodal signalling is involved in left-right asymmetry in snails. *Nature* 457(7232):1007–1011.
- Kuroda R, Endo B, Abe M, Shimizu M (2009) Chiral blastomere arrangement dictates zygotic left-right asymmetry pathway in snails. *Nature* 462(7274):790–794.
- Grande C, Martín-Durán JM, Kenny NJ, Truchado-García M, Hejnal A (2014) Evolution, divergence and loss of the Nodal signalling pathway: New data and a synthesis across the Bilateria. *Int J Dev Biol* 58(6–8):521–532.
- Martin-Duran JM, Vellutini BC, Hejnal A (2016) Embryonic chirality and the evolution of spiralian left-right asymmetries. *Philos Trans R Soc Lond B Biol Sci* 371(1710):20150411.
- Kenny NJ, et al. (2014) The Lophotrochozoan TGF-β signalling cassette—Diversification and conservation in a key signalling pathway. *Int J Dev Biol* 58(6–8):533–549.
- Vorbrüggen G, et al. (1997) Embryonic expression and characterization of a Ptx1 homolog in *Drosophila*. *Mech Dev* 68(1–2):139–147.
- Westmoreland JJ, McEwen J, Moore BA, Jin Y, Condie BG (2001) Conserved function of *Caenorhabditis elegans* UNC-30 and mouse *Pitx2* in controlling GABAergic neuron differentiation. *J Neurosci* 21(17):6810–6819.
- Currie KW, Pearson BJ (2013) Transcription factors *lhx1/5-1* and *pitx* are required for the maintenance and regeneration of serotonergic neurons in planarians. *Development* 140(17):3577–3588.
- März M, Seebeck F, Bartscherer K (2013) A *Pitx* transcription factor controls the establishment and maintenance of the serotonergic lineage in planarians. *Development* 140(22):4499–4509.
- Namigai EK, Kenny NJ, Shimeld SM (2014) Right across the tree of life: The evolution of left-right asymmetry in the Bilateria. *Genesis* 52(6):458–470.
- Piccolo S, et al. (1999) The head inducer *Cerberus* is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* 397(6721):707–710.
- Boorman CJ, Shimeld SM (2002) The evolution of left-right asymmetry in chordates. *BioEssays* 24(11):1004–1011.
- Kaji T, Reimer JD, Morov AR, Kuratani S, Yasui K (2016) Amphioxus mouth after dorso-ventral inversion. *Zoological Lett* 2:2.
- Soukup V, et al. (2015) The Nodal signaling pathway controls left-right asymmetric development in amphioxus. *EvoDevo* 6:5.
- Onai T, Yu JK, Blitz IL, Cho KW, Holland LZ (2010) Opposing Nodal/Vg1 and BMP signals mediate axial patterning in embryos of the basal chordate amphioxus. *Dev Biol* 344(1):377–389.
- Yasui K, Zhang S, Uemura M, Saiga H (2000) Left-right asymmetric expression of BpPtx, a Ptx-related gene, in a lancelet species and the developmental left-sidedness in deuterostomes. *Development* 127(1):187–195.
- Yu JK, Holland LZ, Holland ND (2002) An amphioxus nodal gene (*AmphiNodal*) with early symmetrical expression in the organizer and mesoderm and later asymmetrical expression associated with left-right axis formation. *Evol Dev* 4(6):418–425.
- Reissmann E, et al. (2001) The orphan receptor ALK7 and the Activin receptor ALK4 mediate signaling by Nodal proteins during vertebrate development. *Genes Dev* 15(15):2010–2022.
- Moustakas A, Heldin CH (2009) The regulation of TGFβ signal transduction. *Development* 136(22):3699–3714.
- Yu JK, et al. (2007) Axial patterning in cephalochordates and the evolution of the organizer. *Nature* 445(7128):613–617.
- Duboc V, Lapraz F, Besnardeau L, Lepage T (2008) Lefty acts as an essential modulator of Nodal activity during sea urchin oral-aboral axis formation. *Dev Biol* 320(1):49–59.
- Duboc V, Röttinger E, Lapraz F, Besnardeau L, Lepage T (2005) Left-right asymmetry in the sea urchin embryo is regulated by nodal signaling on the right side. *Dev Cell* 9(1):147–158.
- Ryan AK, et al. (1998) *Pitx2* determines left-right asymmetry of internal organs in vertebrates. *Nature* 394(6693):545–551.
- Roessler E, Muenke M (2001) Midline and laterality defects: Left and right meet in the middle. *BioEssays* 23(10):888–900.
- Hatschek B (1882) Studien über entwicklung des amphioxus. *Arbeiten aus den Zoologischen Instituten der Universität Wien und der Zoologischen Station in Triest* 4(1):1–88.
- Willey A (1894) *Amphioxus and the Ancestry of the Vertebrates* (Macmillan and Co., New York).
- Blum M, Feistel K, Thumberger T, Schweickert A (2014) The evolution and conservation of left-right patterning mechanisms. *Development* 141(8):1603–1613.
- Nishida K, Mugitani M, Kumano G, Nishida H (2012) Neurula rotation determines left-right asymmetry in ascidian tadpole larvae. *Development* 139(8):1467–1475.
- Tisler M, et al. (2016) Cilia are required for asymmetric nodal induction in the sea urchin embryo. *BMC Dev Biol* 16(1):28.
- Li G, Shu Z, Wang Y (2013) Year-round reproduction and induced spawning of Chinese amphioxus, *Branchiostoma belcheri*, in laboratory. *PLoS One* 8(9):e75461.
- Li G, Yang X, Shu Z, Chen X, Wang Y (2012) Consecutive spawnings of Chinese amphioxus, *Branchiostoma belcheri*, in captivity. *PLoS One* 7(12):e50838.
- Liu X, Li G, Feng J, Yang X, Wang YQ (2013) An efficient microinjection method for unfertilized eggs of Asian amphioxus *Branchiostoma belcheri*. *Dev Genes Evol* 223(4):269–278.
- Li D, et al. (2012) Isolation and functional analysis of the promoter of the amphioxus *Hsp70a* gene. *Gene* 510(1):39–46.
- Li G, et al. (2014) Mutagenesis at specific genomic loci of amphioxus *Branchiostoma belcheri* using TALEN method. *J Genet Genomics* 41(4):215–219.
- Yu JK, Holland LZ (2009) Amphioxus whole-mount in situ hybridization. *Cold Spring Harb Protoc* 2009(9):pdb.prot5286.