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Interplay between Lefty and Nodal signaling is essential for the organizer and axial formation in amphioxus embryos

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ABSTRACT

The organizer is an essential signaling center required for axial formation during vertebrate embryonic development. In the basal chordate amphioxus, the dorsal blastopore lip of the gastrula has been proposed to be homologous to the vertebrate organizer. *Lefty* is one of the first genes to be expressed in the organizer. The present results show that *Lefty* overexpression abolishes the organizer; the embryos were severely ventralized and posteriorized, and failed to develop anterior and dorsal structures. In *Lefty* knockouts the organizer is enlarged, and anterior and dorsal structures are expanded. Different from *Lefty* morphants in vertebrates, amphioxus *Lefty* mutants also exhibited left-right defects. Inhibition of Nodal with SB505124 partially rescued the effects of *Lefty* loss-of-function on morphology. In addition, while SB505124 treatment blocked *Lefty* expression in the cleavage stages of amphioxus embryos, activation of Nodal signaling with Activin protein induced ectopic *Lefty* expression at these stages. These results show that the interplay between Lefty and Nodal signaling plays an essential role in the specification of the amphioxus organizer and axes.

1. Introduction

Axis specification is crucial for normal embryonic development. In most bilaterians, while the anterior–posterior axis is determined during oogensis by differential localization of cytoplasmic factors, the dorsal-ventral (DV) and left-right axes are typically specified after fertilization. During vertebrate embryonic development, a specialized region, termed the organizer in the frog (Spemann and Mangold, 1924), the embryonic shield in the zebrafish (Oppenheimer, 1936) or the primitive streak in the mouse (hereafter called the organizer) (Beddington, 1994), plays an essential role in specifying the DV axis (Boettger et al., 2001; De Robertis, 2006; Langdon and Mullins, 2011). When transplanted into the ventral side of host embryos, the organizer can induce surrounding cells to generate at least a partial new body axis (Beddington, 1994; Oppenheimer, 1936; Spemann and Mangold, 1924).

The transforming growth factor-β superfamily (TGF-β) member Nodal, is a major signaling factor in the organizer in all studied vertebrates (Zinski et al., 2018). In the absence of Nodal, the organizer does not form and embryos develop defects of DV axial patterning (Zinski et al., 2018). *Nodal* activation in the organizer requires maternal Vg1, VegT and Wnt/β catenin signaling, and its later expression involves both positive and negative feedback with several genes including Lefty (Zinski et al., 2018). *Lefty* encodes a divergent member of the TGF-β family that antagonizes Nodal by competing for the Nodal co-receptor (Chen and Shen, 2004; Cheng et al., 2004). The inhibition of Nodal by Lefty is essential for limiting the range of Nodal signaling. Without Lefty function, *Nodal* expression in mouse, frog or zebrafish expands to the surrounding regions, resulting in an enlarged organizer and dorsal structures (Branford and Yost, 2002; Feldman et al., 2002; Meno et al., 1999). By contrast, when *Lefty* is mis-expressed, *Nodal* expression is abolished and, the organizer and dorsal structures do not form (Agathon et al., 2001; Branford and Yost, 2002; Cheng et al., 2000).

Structures more or less equivalent to the vertebrate organizer have also been indicated in invertebrate deuterostomes. Studies in sea urchins suggested that the oral ectoderm may function as an embryonic organizer. Like the vertebrate organizer, the oral ectoderm of sea urchins expresses *Nodal* and *Lefty* (Molina et al., 2013). Nodal activity in the oral ectoderm is indispensable for activation of *Nodal* itself, and for normal oral-aboral (equivalent to the DV axis in chordates) axial specification (Duboc et al., 2004). Moreover, as in the vertebrate organizer, *Nodal* expression in the sea urchin oral ectoderm requires maternal Vg1 and Wnt/β catenin signaling, as well as positive and negative feedback loops

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with Lefty (Duboc et al., 2008; Range et al., 2007). Finally, activation of Nodal signaling in two opposite blastomeres of *Nodal* morphants at the four-cell stage induced Siamese twins, although whether the oral ectoderm, when transplanted, can induce host cells to generate a new oral-aboral axis has not been examined (Lapraz et al., 2015). Despite these similarities, the oral ectoderm of sea urchin embryos differs in several respects from the organizer in vertebrates. First, Nodal signaling in the oral ectoderm directs cell specification not only on the oral side but also on the aboral side (Duboc et al., 2004). Second, the sea urchin embryo lacks typical vertebrate dorsal structures like the neural tube, notochord and somites–structures that are induced and patterned by the vertebrate organizer (Beddington, 1994; Boettger et al., 2001; De Robertis, 2006). Third, while the oral ectoderm is on the ventral side of sea urchin embryos, the organizer is on the dorsal side of vertebrate gastrulae. These findings suggest that a primitive organizer-like structure with a role in axial patterning may have emerged before the divergence of deuterosotmes (Lapraz et al., 2015), with a bona fide organizer evolving during vertebrate evolution.

Cephalochordates (amphioxus or lancelets) are the most basal living chordates. Like vertebrates, amphioxus has a dorsal neural tube, notochord and segmented somites (Holland et al., 2004). Evidence for the dorsal blastopore lip functioning as an organizer in amphioxus is three-fold. First, grafting of the dorsal blastopore lip into the blastocoel of host embryos can induce partial secondary axes (Le Petillon et al., 2017; Tung et al., 1961). Secondly, the dorsal blastoporal lip expresses organizer genes such as *Nodal* and *Lefty* in patterns reminiscent of those of their vertebrate orthologues (Yu et al., 2007). Third. blocking or overactivating Nodal signaling in early amphioxus embryos disrupts expression of organizer genes and alters the specification of cell fates along the DV axis (Onai et al., 2010). However, unlike sea urchins and vertebrates, in amphioxus, expression of most organizer genes is detectable only after the onset of gastrulation (Yu et al., 2007). In addition, *Nodal* is maternally expressed in amphioxus (Morov et al., 2016; Onai et al., 2010), unlike in vertebrates and sea urchin where it is zygotically activated by Vg1 and/or Wnt/β-catenin signaling (Duboc et al., 2004; Range et al., 2007; Zinski et al., 2018). These results suggest a certain degree of divergence in the regulatory mechanisms controlling organizer formation and DV axial development between amphioxus and vertebrates or sea urchin.

Due to lack of efficient genetic methods, molecular mechanisms directing the development of the dorsal blastoporal lip and their roles in amphioxus DV axial specification remain largely uninvestigated (Bertrand and Escriva, 2011; Garcia-Fernandez and Benito-Gutierrez, 2009). However, experiments using chemical inhibitors or recombinant proteins have been conducted frequently, and shed important insights on roles of Nodal and Bmp signaling in the development of amphioxus DV axis (reviewed in (Kozmikova and Yu, 2017)). Gene expression analysis has also identified several genes of regionalized expression patterns during cleavage stages of amphioxus embryos (reviewed in (Kozmikova and Yu, 2017)). Among these genes, *Lefty* is specifically transcribed in the left side of amphioxus embryos from early neurula stages (Yu et al., 2007; Soukup et al., 2015), and is also one of the first genes to be expressed in the dorsal blastopore lip (Morov et al., 2016; Onai et al., 2010). Using a Talen-mediated knockdown method, we recently showed that Lefty is required for the development of left-right asymmetry in amphioxus through inhibiting Nodal signaling (Li et al., 2017). Here we further examined the roles of *Lefty* in amphioxus early development using knockout and overexpression methods. Our results showed that Lefty is essential for the proper patterning of the organizer and the dorsal-ventral and anterior-posterior (AP) axes of amphioxus embryos. In *Lefty* mutants, the organizer and the dorsal and anterior structures were enlarged; but in embryos injected with *Lefty* mRNA, the organizer and the dorsoanterior structures were abolished. The results also demonstrated that while Nodal signaling is necessary and sufficient for *Lefty* expression in the presumptive organizer, feedback inhibition by Lefty is required for restricting Nodal activity in the organizer.

2. Materials and methods

2.1. Animal and embryo

Branchiostoma floridae amphioxus animals were obtained from Jr-Kai Yu's lab (Academia Sinica, Taipei, Taiwan) and cultured as described previously (Li et al., 2012; Molina et al., 2013). Sexually mature males and females were separately induced to spawn as described in (Li et al., 2013). The eggs were fertilized in vitro and the embryos were raised in dishes and fixed at desired stages with 4% PFA in MOPS buffer as described (Liu et al., 2013). All neurula and larva in the study are staged according to (Hirakow and Kajita, 1994).

2.2. Probe preparation and in situ hybridization

Total RNA was extracted from gastrulae and neurulae embryos using the RNeasy mini kit (Qiagen, Valencia, CA, USA) and transcribed to cDNA using the PrimescriptTM RT reagent kit (Takara, Shiga, Japan). Primers used for amplifying the coding sequences of amphioxus *Lefty*, *Vg1*, *Nodal*, *Gsc*, *Cer*, *SoxB1a*, *Chordin*, *Six1/2*, *AP2*, *Evx*, *Brachyury*, *Wnt3*, *Actin*, *FoxQ2*, *Otx*, *Hex*, *Bmp2/4* and *Wnt8* genes were designed according to their mRNA sequences deduced from our transcriptome dataset. PCR products of expected sizes were subcloned into pGEM-T easy vector (Promega, USA) and confirmed by sequencing analysis. The resultant plasmids were linearized with restriction enzymes, extracted by phenol-chloroform and used for synthesizing digoxigenin-labeled (Roche, USA) antisense probes with Sp6 or T7 RNA polymerase (Promega, USA). Whole-mount *in situ* hybridization was performed according to (Yu and Holland, 2009). After staining, the embryos were post-fixed with 4% PFA in PBST and mounted in 80% glycerol for photographing under an inverted microscope (Olympus IX71).

2.3. Embryo microinjection

Amphioxus *Lefty* coding sequences were subcloned into the pXT7 vector. The recombinant pXT7 plasmids were linearized with *Sac*I, extracted by phenol-chloroform and dissolved in RNase-free water. *Lefty* mRNAs were in vitro transcribed using T7 mMESSAGE mMACHINE kit (Ambion Co.). Embryo microinjection was performed as previously described (Liu et al., 2013).

2.4. Lefty mutants

A previously described *Lefty* Talen pair was used to generate amphioxus *Lefty* mutants (Li et al., 2017). The Talen binding sites are 5′-TCTCGTGTGTTCTGGC-3' (upstream arm) and 5′-GACGTGGTCCGCCGAG-3' (downstream arm). There is a *Sty*I restriction site in the middle of the spacer sequence (5′-CAGCGCCTTGGCGTT-3′, underlined). *Lefty* heterozygous mutants were screened and cultured as described previously (Li et al., 2017), and the homozygouos embryos were generated by crossing the heterozygotes.

2.5. Embryo treatments with SB505124 and activin protein

To test if Nodal signaling is required and sufficient for initiating *Lefty* expression, wild type embryos were treated with 75 μM SB-505124 (Sigma, USA) or 75 ng/mL human Activin protein (R&D, USA) from the one-cell stage in a 4-well dishes coated with 1% agarose. We also tried a relatively lower concentration of SB-505124 (50 μM) for this experiment, but no apparent effect was observed. Before the treatments, fertilization envelopes were removed about 20 min after fertilization by pipetting them with a 10 μL micropipette tip in a dish coated with 1% agarose, to improve access of reagents to the embryo. The treated embryos were collected and fixed with 4% PFA-MOPS at the 64-cell, 128-cell and 256-cell stages for *in situ* analysis. To see if inhibiting Nodal signaling could rescue phenotype of *Lefty*$^{-/-}$ mutants, embryos from

crossing of *Lefty* heterozygotes were treated with 6 μM SB-505124 at the late blastula and fixed at the mid-gastrula for *in situ* analysis. Fertilization envelopes were also removed as described above before the drug was added in this experiment.

2.6. Histological sections

Embryos injected with *Lefty* mRNA, *Lefty* mutants and control embryos were fixed in 4% PFA-MOPS at desired stages. The fixed embryos were double embedded by agar-paraffin, and serially sectioned at 5 μm, stained with hematoxylin and eosin.

3. Results

3.1. Lefty expression during amphioxus embryogenesis

Lefty expression in amphioxus embryos before the eight-somite neurula stage has been addressed by several previous studies (Li et al., 2017; Morov et al., 2016; Onai et al., 2010; Yu et al., 2007), but its expression in the following stages has not been examined. To provide a complete view of *Lefty* expression pattern during amphioxus embryonic development, we performed whole-mount *in situ* hybridization on amphioxus embryos spanning from one-cell stage to mouth-opening larval stage with *Lefty* anti-sense probes. The result confirmed that *Lefty* is not maternally expressed and its zygotic expression is activated in vegetal blastomeres at 32- to 64-cell stages (Fig. 1A–D). After that, the expression becomes more conspicuous in blastomeres destined to form the dorsal blastoporal lip at 128-cell and 256-cell stages (Fig. 1E and F). At the early and mid-gastrula stages, *Lefty* transcripts are detected in both ectoderm and endomesoderm of the dorsal blastoporal lip (Fig. 1G and H). In the late gastrula, *Lefty* is expressed weakly in nearly all neuro-ecdoderm and strongly in the mesoderm localized in the middle one-third of the embryo (Fig. 1I and J). After this, *Lefty* expression begins to restrict to the left side of the embryo. By the N2 stage (neurulae with 3–5 somites), the expression is present in the left side of neural plate, diverticulum, somites, dorsal-most axial mesoderm, lateral endoderm and few epidermal cells of the anterior two-third of the embryo (Fig. 1K-O). In the N3 stage of neurula (with 9–10 somites), *Lefty* expression on the left side expands posteriorly to the

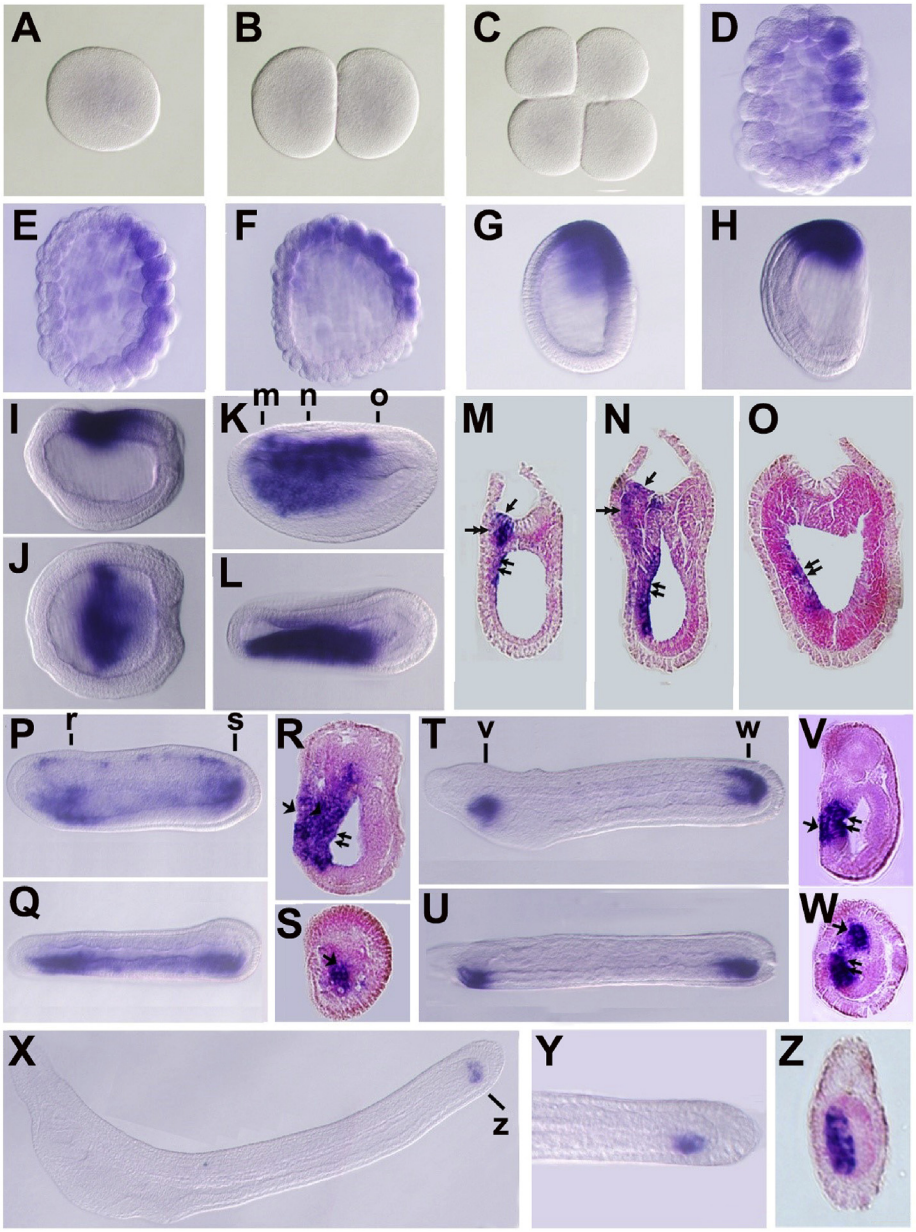

Fig. 1. Expression of *Lefty* gene in amphioxus embryos and larva. A. One-cell embryo. **B**. Two-cell embryo. **C**. Four-cell embryo. **D**. 64-cell embryo. **E**. 128-cell embryo. **F**. 256-cell embryo. **G**. Early gastrula. **H**. Mid-gastrula. **I** and **J**. Late gastrula. **K and L**. N2 neurula. **M-O**. Cross-sections through level m, n and o in panel **K**. Single arrow shows the expression in left-side neural plate; tandem arrows show the expression in left somite; twin arrows indicate expression left-side endoderm. **P and Q**. N3 neurula. **R and S**. Sections through level r and s in panel **P**. Single arrow shows the expression in the left-side ectoderm while twin arrows show the expression in the left-side endoderm. **T and U**. L1 larva. **V**. Section through level v in panel T. Single arrow and twin arrows respectively show the expression in the left-side ectoderm and endoderm wheres the pre-oral pit will form. **W**. Section through level w in panel **T**. Single arrow and twin arrows respectively show the expression in the neural ectoderm and left-side endoderm and mesoderm. **X**. L2 larva. **Y**. Dorsal view of the posterior part of the preceding larvae. **Z**. Section through level z in panel **X** showing the expression in the left-side endomesoderm. Embryos in **D-I**, **K**, **P**, **T** and **X** are lateral view, dorsal to the up-side and anterior to the left; embryos in **J**, **L**, **Q**, **P** and **Y** are dorsal views, anterior to the left; sections are dorsal to the up-side viewed from the posterior end.

tailbud region, but declines in the dorsal- and middle region of the embryo (Fig. 1P, Q). A section through the first somite shows the expression in the ventral somitic mesoderm, notochordal mesoderm, pharygeal endoderm, and a small region of epidermis where the mouth will form later (Fig. 1R). Another section through the posterior end shows transcription in the left side hindgut endoderm (Fig. 1S). At the L1 stage (larvae of knife-shaped body), the expression is more confined and present in a small anterior left region where the mouth is perforating, and the left side of the tail bud (Fig. 1T–W). By the L2 stage (mouth is opened, but the first gill is not perforated), the expression in the anterior pharynx disappears, and that in the posterior end is confined to the left side of nascent somites and the hindgut (Fig. 1X–Z).

3.2. *Lefty* and dorsal/anterior structure development in amphioxus

To reveal the role of *Lefty* gene in the development of amphioxus organizer and dorsal-ventral axis, we generated a *Lefty* mutant line using the Talen method. The mutant carried an 8 bp deletion at 46-bp downstream of the start codon. *Lefty*$^{-/-}$ homozygous embryos were generated by intercrossing *Lefty*$^{+/-}$ heterozygotes. Phenotype examination revealed no obvious difference between *Lefty*$^{+/+}$/*Lefty*$^{+/-}$ and *Lefty*$^{-/-}$ embryos before the gastrula stage. But by the N1 stage (neurulae just begin to rotate), the *Lefty*$^{-/-}$ embryos curved mid-dorsally (compare Fig. 2A to 2A′), and developed an enlarged head and expanded dorsal structures at the N3 stage (compare Fig. 2B to B′). At the L1 stage, *Lefty*$^{-/-}$ mutants were shorter and thicker comparing with WT and *Lefty*$^{+/-}$ siblings (compare Fig. 2C–C’). Cross-sections of embryos at this stage revealed that the anterior structures of *Lefty*$^{-/-}$ mutants such as cerebral vesicle, Hatschek’s left diverticulum, pharynx, dorsal structures including neural tube, somites and notochord were all enlarged (compare Fig. 2C1-C3 to C1’-C3’), while the gut at the posterior region was not formed (compare Fig. 2C4 to C4’). At this time, the mutants also showed defects in left-right asymmetry with the Hatschek’s left diverticulum and left-side part of endostyle presented on both sides (compare Fig. 2C1, C2 to C′1, C′2). At the L3 stage (larvae with two formed and one forming gill slits), the *Lefty*$^{-/-}$ mutant formed an enlarged cerebral vesicle, as evidenced by expanded pigment of the frontal eye, and enlarged pre-oral pits and mouths on both sides, but their rostral end did not form properly (compare Fig. 2D–G to H–K). In addition, the mutant had no anus and the gut was present only in the anterior half of their body (Fig. 2H).

To define *Lefty*$^{-/-}$ phenotypes further, expression patterns of several marker genes were examined at the N3 stage. *FoxQ2* expression in the anterior ectoderm was somewhat enlarged and shifted to the ventral side in the *Lefty*$^{-/-}$ mutant (compare Fig. 3A and B), and the expressions of *Otx* in the cerebral vesicle (Fig. 3C and D) and Wnt3 in the hindbrain and spinal cord (Fig. 3E and F) were also expanded, covering respectively the anterior 1/3 and the posterior 2/3 of the ectoderm. *Brachyury* expression domain in the notochord (Fig. 3G and H), that of *m-actin* and *Mrf2* gene in the somites (Fig. 3I and J) (Fig. 3K and L) seemed to be expanded somewhat in the mutants. In contrast, *Hex* expression in the anterior-ventral endoderm was slightly reduced after depletion of *Lefty* gene (Fig. 3M and N). Moreover, the expression of *Otx* in the posterior endoderm was almost eliminated in the *Lefty* mutants (compare Fig. 3C and D), and that of *Hex* in this region was completely lost in the mutants (compare Fig. 3M to N), consistent with the finding that the mutant lacked posterior gut at the early larva stage shown above.

To further dissect the role of *Lefty* gene, we then injected amphioxus eggs with *Lefty* mRNA. The injected embryos developed normally before the mid-gastrula stage. But at the late gastrula stage, they failed to flatten at the dorsal side like WT embryos (Fig. 4A and B), indicating a loss of dorsal identity. As development proceeded, the injected embryos were shorter relative to the control (Fig. 4C, D, E and F). Transverse sections of N3 (Fig. 4C1-D2) and L1 embryos (Fig. 4E1-F2) showed that after *Lefty* mRNA injection the embryo lost all anterior structures including the cerebral vesicle, Hatsheck’s left diverticulum, the rostral coelom, the endostyle, the club-shaped gland and the gill slits. In addition, the injected embryos lacked dorsal structures like somites, notochord and neural tube although some residues appeared in the posterior half of the embryo (compare Fig. 4C1, C2 to D1, D2, and E1, E2 to F1, F2). However, the injection seemed to have no effect on the development of the most

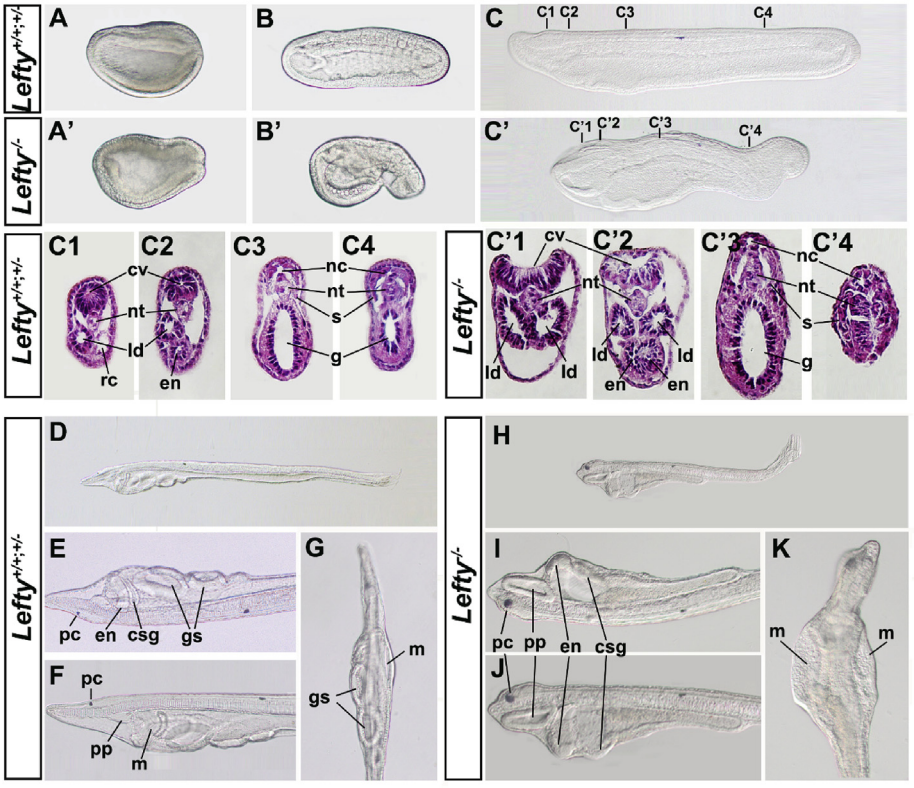

Fig. 2. Phenotype of amphioxus *Lefty* mutant. *Lefty*$^{+/+;+/-}$ marks wild type or heterozygous mutants and *Lefty*$^{-/-}$represents homozygous mutants. **A′**. N1 neurula. **B′**. N3 neurula. **C and C′**. L1 neurula. **C1–C4**. Sections through level C1–C4 in panel **C**. **C′1-C′4**. Sections through level C′1-C′4 in panel **C**. **D-K**. L3 larva. **E-G**. Magnifications of the anterior part of the larvae in **D**. **I–K**. Magnifications of the anterior part of the larvae in **H**. Embryos in all panels except **G** and **K** are lateral view, anterior to the left and dorsal to the up-side (**A-C′**, **D**, **F**, **H** and **J**) or bottom (**E** and **I**); embryos in **G** and **K** are dorsal view, anterior to the up-side; sections are dorsal to the up-side viewed from the posterior end. Abbreviations: csg, club shaped gland; cv, cerebral vesicle; en, endostyle; g, gut; gs, gill slit; ld, left diverticula; m, mouth; nc, nerve cord; nt, notochord; pc, pigment cell of the front eye; pp, preoral pit; rc, rostral coelom; s, somite.

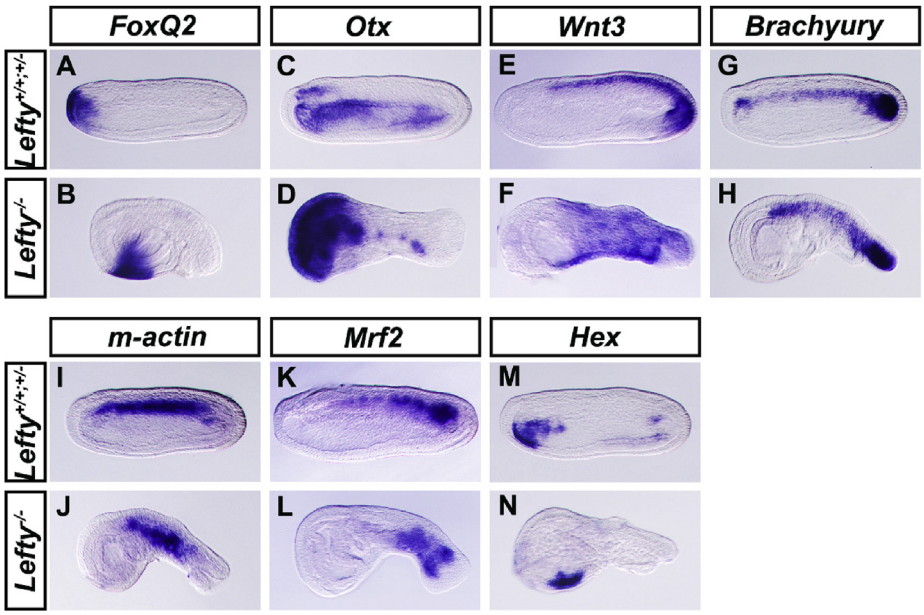

Fig. 3. Expression of marker genes in amphioxus *Lefty* mutants. Embryos at the N3 neurula stage were examined. All panels are lateral view with anterior to the left and dorsal to the up-side.

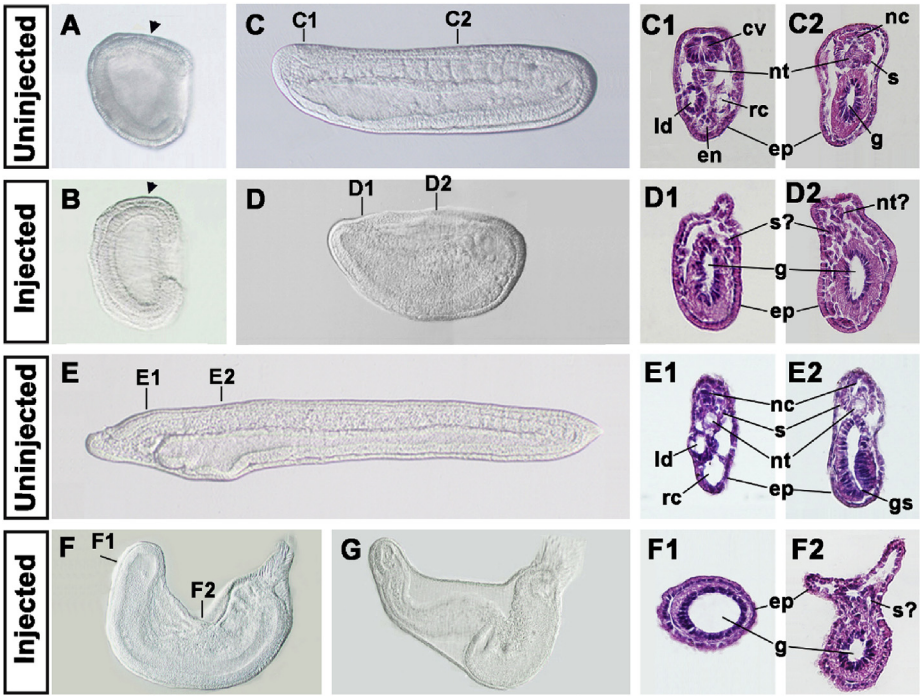

Fig. 4. Phenotype of embryos injected with *Lefty* mRNA. Embryos in line one and three were uninjected control and embryos in line two and four were embryos injected with *Lefty* mRNA. **A and B.** Late gastrula. Uninjected embryos at this stage flatten their dorsal side (arrowhead), but embryos injected with Lefty mRNA fail to do it (arrowhead). **C and D.** N3 neurula. **C1 and C2.** Sections through level C1 and C2 in panel **C**. **D1 and D2.** Sections through level D1 and D2 in panel **D**. **E and F.** L1 larvae. **G.** An injected embryo at stage when control developed two gill slits. **F1 and F2.** Sections through level F1 and F2 in panel **F**. Embryos are lateral view, posterior to the right and dorsal to the up; sections are dorsal to the up viewed from the posterior end. Abbreviations: cv, cerebral vesicle; en, endostyle; ep, epidermis; g, gut; gs, gill slit; ld, left diverticula; nc, nerve cord; nt, notochord; rc, rostral coelom; s, somite.

posterior end, since after injection the embryo could develop tail fin normally at the larval stage (Fig. 4F and G). This phenotype is highly reproducible and was obtained after injection of *Lefty* mRNA ranging from 5 ng/μL to 42 ng/μL (Supplementary Fig. 1). Consistent with these morphological defects, marker gene expression assay revealed that the expression of *Hex* in anterior-ventral endoderm (Fig. 5A and A′), *FoxQ2* in anterior ectoderm (Fig. 5B and B′), *Wnt3* in neural tube (Fig. 5C and C′), *Brachyury* in notochord (Fig. 5D and D′), *Mrf2* and m-actin in somites (Fig. 5E-G′), and *Otx* in cerebral vesicle and anterior pharyngeal endoderm (Fig. 5H-I′) were all disappeared in the injected embryos. But *Wnt3* and *Brachyury* expression in tailbud were remained and became wider in the injected embryos (Fig. 5C and D), compared to that in the controls (Fig. 5C', D′). Together, these experiments demonstrated that *Lefty* is essential for the proper patterning of dorsal and anterior structures in amphioxus with its loss-of-function expanding dorsal and anterior structures and overexpression abolishing development of dorsal-anterior structures.

3.3. *Lefty* and specification of organizer and DV axis in amphioxus

To address how Lefty affects axial development, we analyzed the expression of axial marker genes in *Lefty* mutants at mid- and/or late

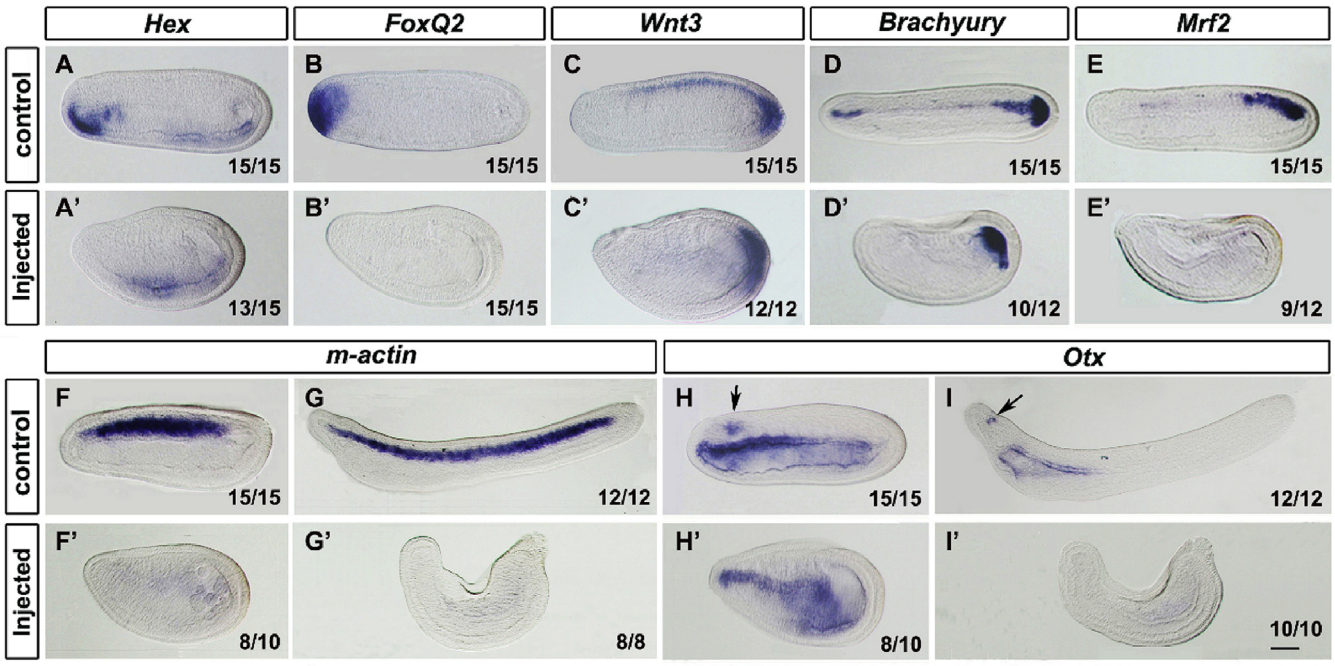

Fig. 5. Expression of marker genes in *Lefty* mRNA injected embryos. Embryos at N3 neurula stage (A-E, A′-E′, F, F′, H and H′) and L1 larval stage (G, G′, I and I′) were examined. Embryos in all panels are lateral view, anterior to the left and dorsal to the up-side.

gastrula stage. In normal embryos, the transcription factor *Gsc* and signaling molecules *Nodal*, *Vg1*, *Chordin* and *Lefty* are specifically expressed in the dorsal organizer of the amphioxus gastrula (Fig. 6A–I) (Onai et al., 2010; Yu et al., 2007). In *Lefty*$^{-/-}$ mutants, however, their expressions were all expanded ventrally (Fig. 6A'-I′). Likewise, the expressions of *Cer* in the dorsal-anterior endomesoderm (Fig. 6J), and *Bmp2/4* in the anterior dorsolateral endomesoderm (Fig. 6K and L) were both expanded in *Lefty*$^{-/-}$ mutants (Fig. 6J-L). Different from these genes, the expressions of ventral/posterior marker genes *Evx* and *Wnt8* (Fig. 6M and N) were greatly reduced due to *Lefty* loss-of function (Fig. 6M′ and N′). *SoxB1a* expression markers the dorsal presumptive neural ectoderm of the embryo (Fig. 6O). After *Lefty* mutation, its expression was expanded ventrally (Fig. 6O′). The expression of *Six1/2*, a marker of paraxial mesoderm (Fig. 6P and Q), was also expanded due to *Lefty* loss-of-function (Fig. 6P′ and Q′). In contrast, the expression of *AP2*, a marker of the ventral/lateral epithelia ectoderm (Fig. 6R), was significantly reduced in *Lefty* mutant embryos (Fig. 6R').

We further analyzed above gene expressions in *Lefty*-mRNA injected embryos at mid- and/or late gastrula stage. The expressions of *Gsc*, *Nodal*, *Vg1* and *Chordin* in the dorsal blastoporal lip (Fig. 7A–H), and that of *Cer* in the dorsal-anterior endomesoderm (Fig. 7I) were all abolished due to injection of *Lefty* mRNA (Fig. 7A'-I′). *Bmp2/4* expression in the anterior dorsolateral endomesoderm was reduced but not erased by the injection (compare Fig. 7J and K to J′, K′). In contrast, the expressions of *Evx* and *Wnt8* in the ventral and lateral blastopore lip were both expanded throughout the blastoporal lip after *Lefty* mRNA injection (compare Fig. 7L–O to L′-O′). However, *Brachyury* expression in the posterior endomesoderm was not significantly affected by the injection (compare Fig. 7P to P′). After *Lefty* mRNA injection, the expressions of *SoxB1a* in the presumptive neural ectoderm (Fig. 7Q), and *Six1/2* in the paraxial mesoderm (Fig. 7R) were both eliminated (Fig. 7Q′ and R′). In contrast, the expression of *AP2*, a marker of the ventral/lateral epithelia ectoderm, was expanded throughout the ectoderm due to *Lefty* mRNA injection (compare Fig. 7S, T to 7S′, T′). As for the anterior ectoderm maker *FoxQ2*, its expression was reduced in *Lefty* mRNA-injected embryos (compare Fig. 7U–U′). These results together demonstrate that Lefty is required for organizer development and organizer size control in amphioxus: its overexpression disrupts organizer formation, while knockout of it expands the size of organizer.

3.4. Inhibiting Nodal signaling partially rescues the defects of Lefty mutation

The phenotype and gene expression pattern of *Lefty* mutants resemble those when Nodal signaling is overactivated (Onai et al., 2010). This indicates that Lefty probably functions as a Nodal inhibitor in amphioxus like its cognates in vertebrates and sea urchins (Agathon et al., 2001; Duboc et al., 2008; Meno et al., 1999). To test this, we treated *Lefty* mutant embryos and their siblings with the Nodal signaling inhibitor SB505124 (6 μM) from late blastula stage to mid-gastrula stage, and asked if the treatment could counteract loss of *Lefty* function. SB505124 treatment caused a dorsal expansion of *Evx* expression in the WT and *Lefty*$^{+/-}$ embryos (compare Fig. 8A, A′ to G, G′), and a recovery of *Evx* expression in the ventral/lateral blastoporal lip of the *Lefty*$^{-/-}$ embryos (compare Fig. 8B, B′ to H, H′). *Nodal* expression was greatly down-regulated in both *Lefty*$^{-/-}$ mutants and their siblings, but the expression domain was unaffected compared to the untreated control embryos (compare Fig. 8C, C′, D, D′ to I, I′, J, J′). Probably due to the down-regulation of *Nodal* expression, *Lefty* expression in either the dorsal blastoporal lip of WT and *Lefty*$^{+/-}$ embryos or the ectoderm and endoderm of *Lefty*$^{-/-}$ mutants was abolished (compare Fig. 8E, E′, F, F′ to 8K, K′). This result indicates that expression of *Lefty*, and probably other Nodal target genes as well, requires a high level of Nodal activity. Consistent with this, all treated embryos showed a uniform ventralized phenotype (compare Fig. 8L, M, L′, M′ to 8L″, M″). Together, these results demonstrate that inhibition of Nodal signaling can partially rescue or bypass the defects of *Lefty*-depleted embryos and that Lefty inhibits Nodal signaling in early amphioxus embryonic development.

3.5. Nodal signaling is necessary and sufficient for initial Lefty expression in early cleavage stages of amphioxus embryos

Lefty is one of the genes showing very early expression in the presumptive organizer domain in amphioxus embryos (Fig. 9A–C) (Morov

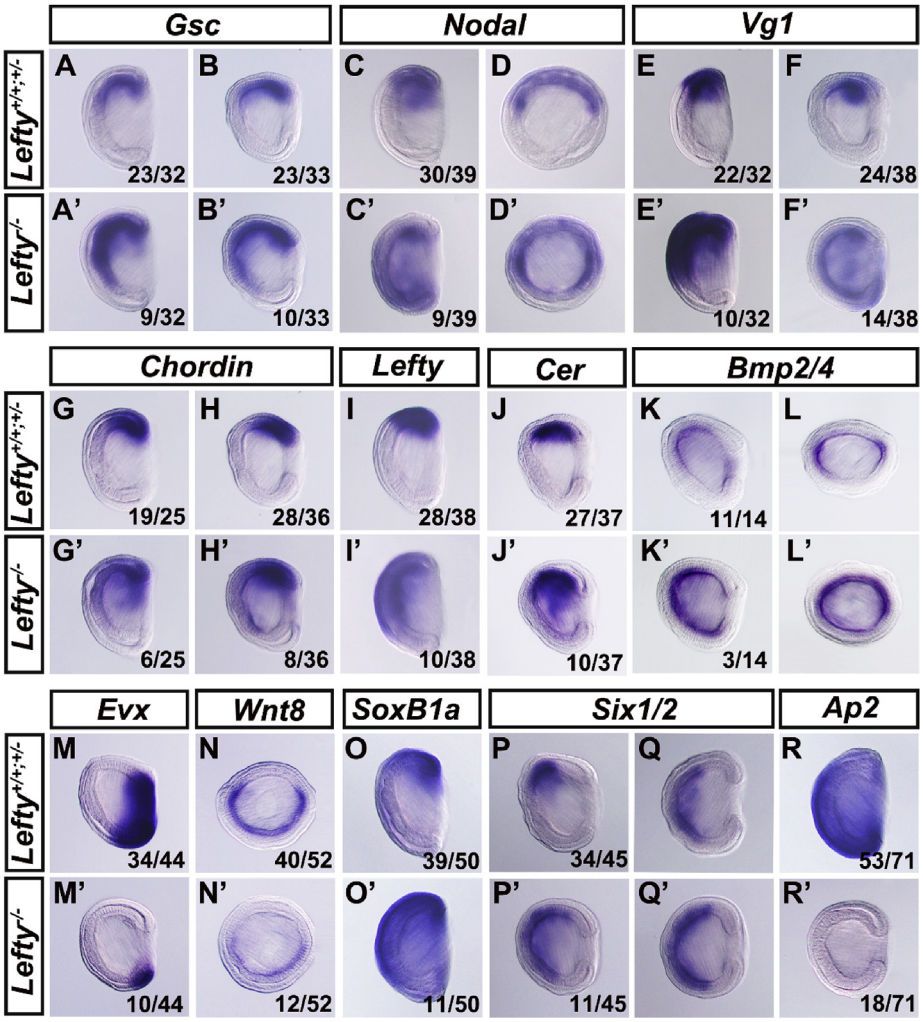

Fig. 6. *Lefty* loss-of-function expands organizer size and dorsal/anterior territory. A-R. Gene expressions in *Lefty* wild type/heterozygous mutant embryos (*Lefty*$^{+/+;+/-}$). **A′-R′.** Gene expressions in *Lefty* homozygous mutant embryos (*Lefty*$^{-/-}$). Embryos in panel A, A′, C, C′, D, D′, E, E′, G, G′, I, I′, O, O′ are mid-gastrulae, and embryos in panel B, B′, F, F′, H, H′, J, J′, K, K′, L, L′, M, M′, N, N′, P-R and P′-R′ are late gastrulae. The embryos in D, D′, L, L′, N and N′ are blastopore view (dorsal to the up), the embryos in Q and Q′ are dorsal view (anterior to the left), and the embryos in all other panels are lateral view (dorsal to the up and anterior to the left). 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.

et al., 2016; Onai et al., 2010). It has been demonstrated that *Lefty* expression at and after gastrula stages requires Nodal signal in amphioxus as in vertebrates and sea urchin (Duboc et al., 2005; Morov et al., 2016; Onai et al., 2010; Saudemont et al., 2010; Zinski et al., 2018). To determine whether initial *Lefty* expression also depends on Nodal signaling, we treated the embryo with 75 μM SB505124 from the one-cell stage and fixed samples at the 64-cell, 128-cell and 256-cell stages. The result showed that inhibition of Nodal signaling eliminated *Lefty* expression in the presumptive organizer region of 67% (12/18) of examined 64-cell embryos, 55.56% (10/18) of examined 128-cell embryos, and 94.12% (16/17) of examined 256-cell embryos (Fig. 9D–F). This suggests that Nodal signaling is required for the initial *Lefty* expression in amphioxus. To further test whether Nodal signaling is sufficient to activate *Lefty* expression at the early cleavage stage, we treated embryos with 75 ng/mL recombinant Activin protein from the one-cell stage and examined *Lefty* expression at the 64-cell, 128-cell and 256-cell stages. Activin signals through the same pathway as Nodal and has previously been used as a substitute for Nodal protein (Onai et al., 2010). The result revealed that increasing Nodal signaling activity by Activin led to a global transcription of *Lefty* mRNA in over 72% of embryos at each examined stages (Fig. 9G–H). The results therefore implies that all blastomeres of amphioxus early cleavage stage embryos are competent to transduce Nodal signaling by Activin protein, and that activation of Nodal signaling is able to induce ectopic *Lefty* expression at these stages of amphioxus embryos.

4. Discussion

4.1. Regulation of Lefty expression by nodal signaling in amphioxus

Lefty expression is regulated by Nodal signaling in vertebrates and sea urchin (Duboc et al., 2005; Saudemont et al., 2010; Zinski et al., 2018). This study and previous results indicate this is also true in amphioxus. First, *Lefty* expression follows that of *Nodal* in amphioxus embryos (Fig. 1 and (Morov et al., 2016; Onai et al., 2010; Yu et al., 2002; Yu et al., 2007)): they both express in the dorsal blatoporal lip of early and mid-gastrula, the first somites of early neurula, and the left side somites and lateral endoderm of 3-somite neurula; their expression begins to concentrate in the left side of the pharynx and tail bud from the mid-neurula stage, and remains only on the left side of the tail bud from the early larval stage. Second, blocking Nodal signaling with SB505124 at either cleavage stage, blastula stage or gastrula stage abolishes *Lefty* expression (Figs. 8 and 9, and (Morov et al., 2016)). Third, overactivating Nodal signaling by incubating the cleavage stages of embryos with Activin protein leads to ubiquitous ectopic expression of *Lefty* at the 32-cell stage (Fig. 9). A similar result was also observed in embryos injected with an *Hsp70-Nodal* construct and heat-shocked at the mid-gastrula stage (Li et al., 2017). These results indicate that in amphioxus as in vertebrates and sea urchins, Nodal signaling is necessary and sufficient for *Lefty* expression. Interestingly, *Lefty* genes exist only in deuterostomes while *Nodal* genes emerged before the divergence of bilaterians (Kenny et al., 2014; Watanabe et al., 2014). This suggests that

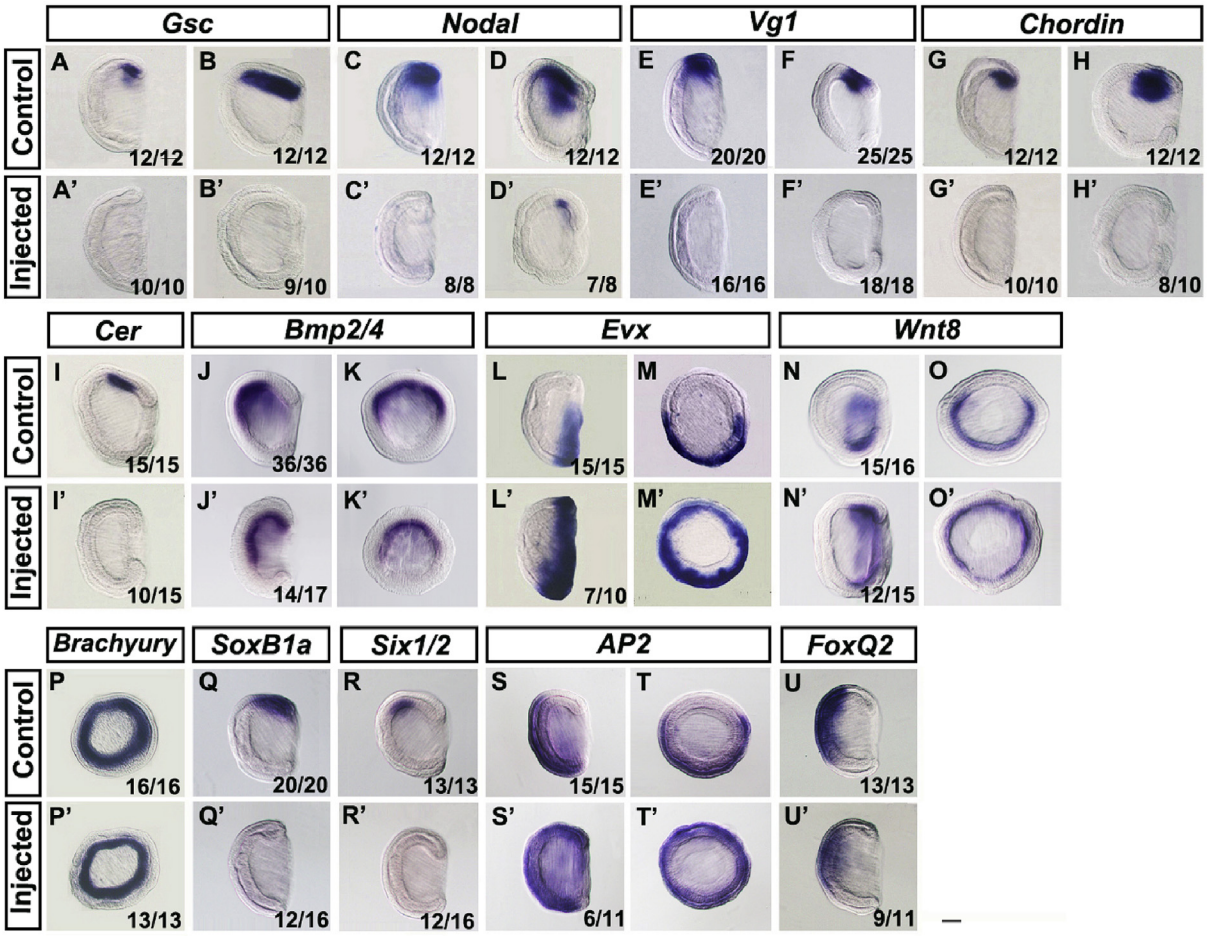

Fig. 7. ***Lefty*** **overexpression abolishes organizer formation and dorsal identity. A-U.** Gene expressions in uninjected control embryos. A′-U′. Gene expressions in *Lefty* mRNA injected embryos. Embryos in panel A, A′, C, C′, E, E′, G, G′, L, L′, M, M′, P, P′, Q, Q′, S, S′, T, T′, U and U′ are mid-gastrulae, and embryos in panel B, B′, D, D′, F, F′, H, H′, I, I′, J, J′, K, K′, N, N′, O, O′, R and R′ are late gastrulae. The embryos in K, K′, M, M′, O, O′, P, P′, T and T′ are blastopore view, dorsal to the up, and ones in all other panels are lateral view, dorsal to the up-side and anterior to the left.

since *Lefty* gene diverged, its expression had been regulated by Nodal.

4.2. Effects of Lefty-mediated inhibition of Nodal signaling on the size of the organizer in deuterostomes

The organizer is a gastrular signaling center that mediates dorsal-ventral axial patterning in vertebrates by secreting antagonists of Wnt and Bmp signaling (De Robertis, 2006). The organizer was first identified in frog embryos and then in other vertebrates, e.g. the shield of fish and primary streak of mouse (Beddington, 1994; Oppenheimer, 1936; Spemann and Mangold, 1924). Based on gene expression and functional or transplantation experiments, recent studies indicated that the ventral ectoderm of the sea unchin gastrula and the dorsal blastoporal lip of the amphioxus gastrula are probably equivalent to the organizer of vertebrates (Lapraz et al., 2015; Le Petillon et al., 2017; Saudemont et al., 2010; Tung et al., 1961; Yu et al., 2007). Consistent with this, as in vertebrates (Zinski et al., 2018), *Nodal* is specifically expressed in the ventral ectoderm of sea unchin embryos and the dorsal blastoporal lip of amphioxus embryos (Duboc et al., 2004; Yu et al., 2002), and blocking Nodal results in defects in the formation of these regions and the patterning of the dorsal-ventral (or oral-aboral in sea urchin) axis (Duboc et al., 2004; Onai et al., 2010). As a direct target of Nodal signaling, *Lefty* is transcribed strongly in the organizer of the above animals (Duboc et al., 2008; Meno et al., 1997, 1999; Thisse and Thisse, 1999; Yu et al., 2007). It has been demonstrated that *Lefty* expression in these domains is required to restrict Nodal activity to the organizer in both sea urchin and vertebrate embryos. Indeed, loss of Lefty function in vertebrates or sea urchins results in expanded *Nodal* expression and an enlarged organizer (Agathon et al., 2001; Branford and Yost, 2002; Duboc et al., 2008; Feldman et al., 2002; Meno et al., 1999), while overexpression of *Lefty* abolishes *Nodal* expression and organizer formation (Cheng et al., 2000; Duboc et al., 2008; Thisse and Thisse, 1999). In a recent study, we showed that inhibition of Nodal signaling by Lefty is required for proper left-right patterning in amphioxus (Li et al., 2017). The present study further demonstrates a similar regulatory relationship between Lefty and Nodal during organizer formation in amphioxus embryos. We show that while expression of *Nodal* and other organizer marker genes like *Gsc*, *Chordin*, *Vg1* and *Lefty*, is expanded both anteriorly and ventrally at the gastrula stages in amphioxus *Lefty* mutants (Fig. 6), their expression was entirely abolished following overexpression of *Lefty* mRNA (Fig. 7). As a result, the mutant embryos formed enlarged dorsal structures at the expense of ventral structures (Figs. 2 and 3), and embryos overexpressing *Lefty* gene develop a tube-like morphology with no anterior structures and very few remnants of dorsal structures at the posterior end of their body (Figs. 4 and 5).

4.3. Phenotype variation of Lefty loss-of-function among deuterostomes

Although *Lefty* loss-of-function expands organizer gene expression in amphioxus, sea urchin and vertebrates, its effects on subsequent development differ among species. First, in sea urchin and amphioxus knockdown or knockout of *Lefty* has no effect on invagination of

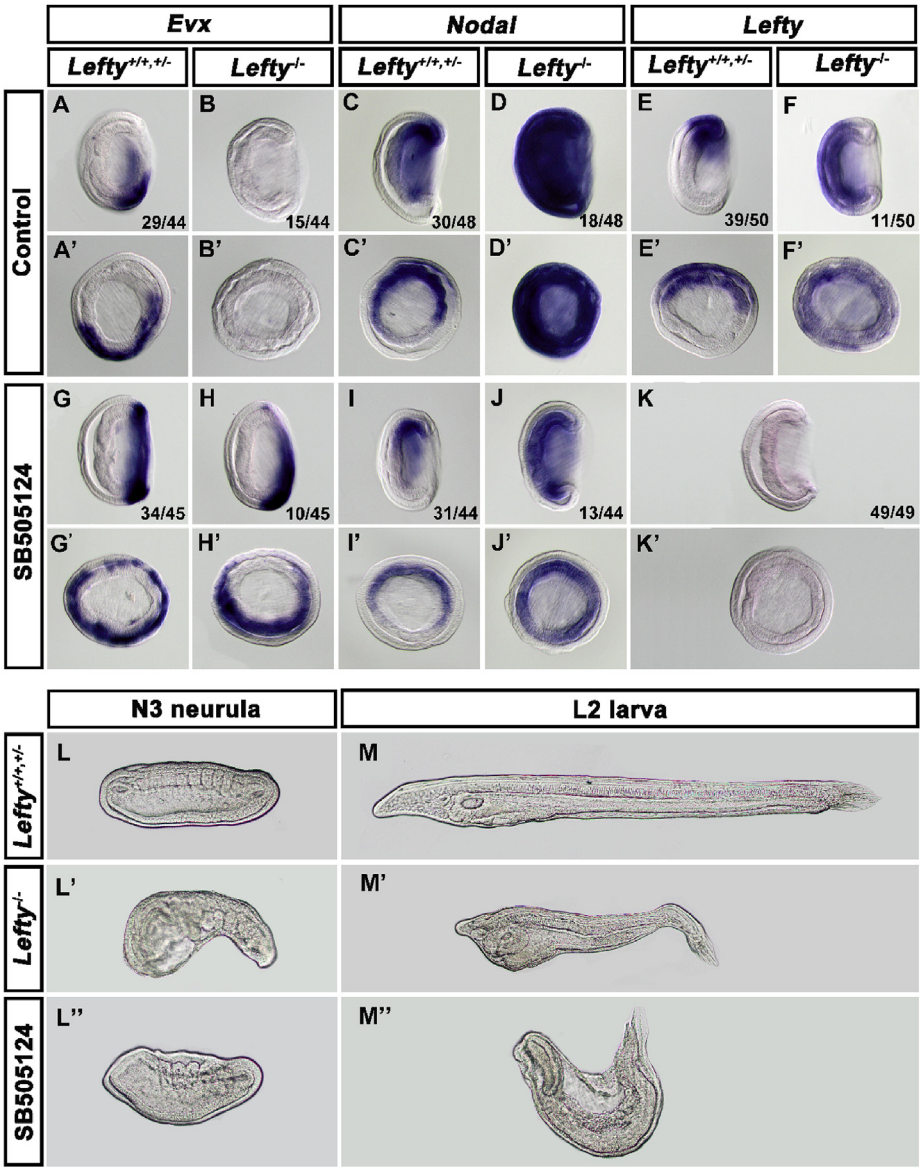

Fig. 8. Inhibition of Nodal signal with SB505124 partially rescued *Lefty* mutant phenotype. The embryos were generated by crossing *Lefty* heterozygotes and then treated with 6 μM SB505124 or equal volume of DMSO (control) from late blastula stage. **A-F** and **A′-F′**. Expression of *Evx*, *Nodal* and *Lefty* in DMSO-treated *Lefty* wild-type/heterozygous (*Lefty*$^{+/+}$, *Lefty*$^{+/-}$) or homozygous mutant (*Lefty*$^{-/-}$) embryos at the mid-gastrula stage. **G-K** and **G′-K′**. Expression of *Evx*, *Nodal* and *Lefty* in SB505124-treated *Lefty* wild-type/heterozygous or homozygous mutant embryos at the mid-gastrula stage. **L** and **M**. DMSO-treated *Lefty* wild-type/heterozygous embryo and larva. **L′** and **M′**. DMSO-treated *Lefty* homozygous embryo and larva. **L″** and **M″**. SB505124-treated *Lefty* wild-type/heterozygous/homozygous embryo and larva. Numbers at the bottom right of panel A–E′ indicate the number of times the phenotype shown was observed out of the total number of manipulated embryos. Embryos in panel A–K and L-M″ are lateral view with anterior to the left and dorsal to the up-side, and embryos in panel A′- K′ are blastopore view with dorsal to the up.

endomesoderm during gastrulation (Fig. 2 and (Duboc et al., 2008)), while in zebrafish and frog, knockdown of *Lefty* via a morpholino caused gastrulation defects (Branford and Yost, 2002; Feldman et al., 2002). The reason for the difference might be that gastrulation in vertebrates involves more complicated cellular movements than that in invertebrate deuterostomes. Notably, the gastrulation defect is not observed in zebrafish *Lefty1* and *Lefty2* double mutants (Rogers et al., 2017). We speculate that this discrepancy might be caused either by off-target effects of the morpholino (Rogers et al., 2017) or by incomplete loss of function of *Lefty1* and/or *Lefty2* genes in the mutants. Studies with more conclusive methods such as completely deleting *Lefty* loci are required to distinguish between the two possibilities. Second, amphioxus *Lefty* mutants can develop a dorsal-ventral axis even though the dorsal structures are expanded (Figs. 2 and 3), while sea urchin *Lefty* morphants lack the oral/aboral axis and present a radialised phenotype (Duboc et al., 2008). The difference correlates well with the expansion of *Nodal* expression entirely around the blastopore in sea urchin *Lefty* (Duboc et al., 2008), while in amphioxus *Lefty* mutants Nodal activity does not reach the ventral-most region, as evidenced by the expression patterns of *Lefty*, one of the direct targets of Nodal signaling, and of *Evx*, a ventral/lateral marker (Fig. 6). Third, due to loss of the dorsal-ventral (or oral-aboral) axis as described above, left-right defects in sea urchins, zebrafish and frog *Lefty* morphants can not be analyzed (Duboc et al., 2008; Feldman et al., 2002; Yu et al., 2002). However, in amphioxus *Lefty* mutants a two-left-side phenotype is present at the larval stage (Fig. 2).

4.4. Regulation of Lefty expression in amphioxus early cleavage stage embryos

Lefty is one of the earliest genes known to be expressed asymmetrically in amphioxus embryos (Morov et al., 2016; Onai et al., 2010). Blocking or ectopic activation of Nodal from the one-cell stage respectively inhibits or up-regulates *Lefty* expression at the 64-128 cell stages (Fig. 9), indicating that *Lefty* expression in this domain probably depends on Nodal signaling. *Lefty* shows a similar expression pattern and dependence on Nodal signaling in sea urchin embryos (Duboc et al., 2008; Saudemont et al., 2010). However, the *Nodal* expression pattern is different between the two species. In sea urchin embryos *Nodal* is zygotically expressed from the 60-cell stage which is slightly earlier than *Lefty* at 128-cell stage (Duboc et al., 2004, 2008). But, in amphioxus *Nodal* is maternally expressed and shows no obvious restricted expression pattern at cleavage stage albeit a slightly stronger signal is detected in the

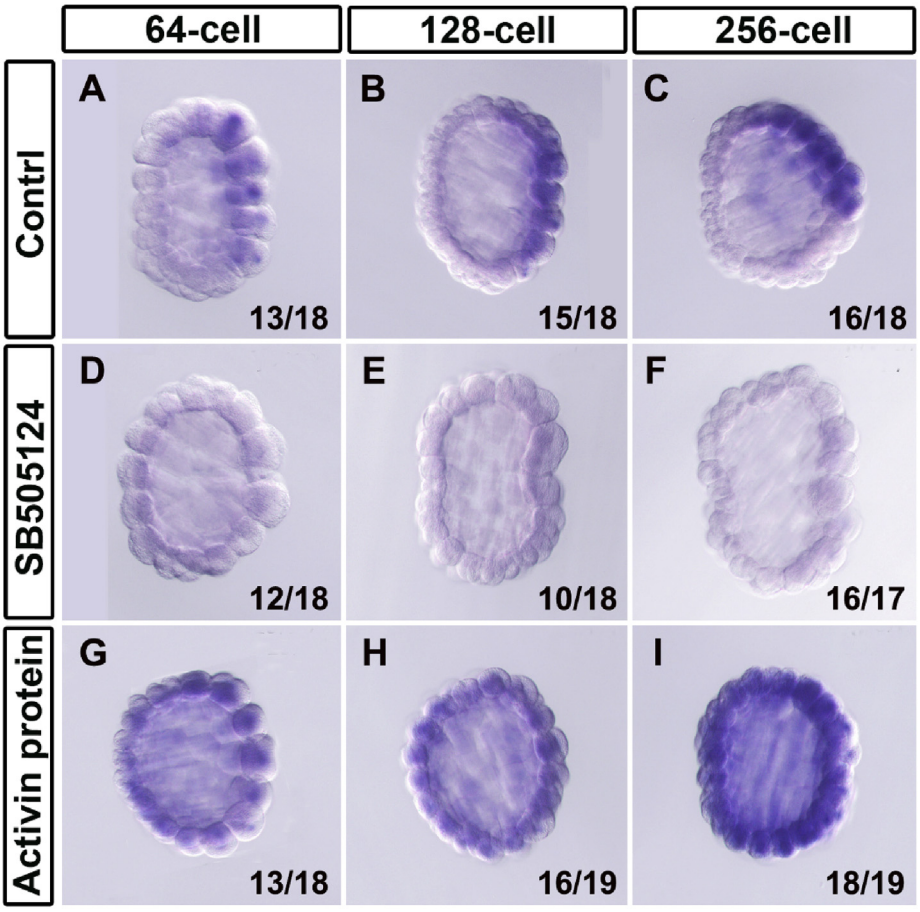

Fig. 9. *Lefty* expression in cleavage stage embryos treated with SB505124 and Activin. The embryos were treated from one-cell stage with 75 μM SB505124, 75 ng/mL recombinant Activin protein, or DMSO (control), and collected at 64-cell, 128-cell and 256-cell stage for *in situ* hybridization analysis. 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. All images are side view with animal pole toward the left.

animal hemisphere (Morov et al., 2016; Onai et al., 2010). This might indicate that other elements of Nodal signaling, but not Nodal, acts to restrict Nodal signaling activity in the dorsal vegetal blastomere to activate *Lefty* expression there in amphioxus. In vertebrates and sea urchin embryos, Vg1 is required to form heterodimer with Nodal for maximal Nodal signal transduction and Vg1 loss-of-function abolishes expression of *Lefty* and other Nodal signaling target genes (Montague and Schier, 2017; Pelliccia et al., 2017; Range et al., 2007; Tanaka et al., 2007). We examined *Vg1* expression pattern in the cleavage stage of amphioxus embryos using *in situ* hybridization and RT-qPCR methods. We found that *Vg1* is not maternally expressed as reported previously (Onai et al., 2010), and its zygotic expression is activated at the 16- to 32-cell stages in the dorsal vegetal blastomeres which is one cell cleavage earlier than *Lefty* gene begins to transcribe in this domain (unpublished data). This result indicates that Vg1 might be a key element for the initial activation of *Lefty* expression in the presumptive organizer domain by Nodal signaling, although possibility that other components of Nodal signaling contribute to the process can not be excluded.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.08.006.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: H.Z., G.L.; Methodology: H.Z., S.C., G.L.; Formal analysis: H.Z., G.L.; Investigation: H.Z., S.C., G.L., Y.W.; Data curation: H.Z., S.C., G.L.; Writing - original draft: H.Z., G.L.; Writing - review & editing: G.L., Y.W.; Supervision: G.L., Y.W.; Funding acquisition: G.L., Y.W.

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