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Regulation of midline development by antagonism of *lefty* and *nodal* signaling

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SUMMARY

The embryonic midline is crucial for the development of embryonic pattern including bilateral symmetry and left-right asymmetry. In zebrafish, *lefty1* (*lft1*) and *lefty2* (*lft2*) have distinct midline expression domains along the anteroposterior axis that overlap with the expression patterns of the *nodal*-related genes *cyclops* and *squint*. Altered expression patterns of *lft1* and *lft2* in zebrafish mutants that affect midline development suggests different upstream pathways regulate each expression domain. Ectopic expression analysis demonstrates that a balance of *lefty* and *cyclops* signaling is required for normal mesendoderm patterning and *goosecoid*, *no tail* and *pitx2* expression. In late somite-stage embryos, *lft1* and *lft2* are expressed asymmetrically in the left diencephalon and left lateral plate respectively, suggesting an additional role in laterality development. A model is proposed by which the vertebrate midline, and thus bilateral symmetry, is established and maintained by antagonistic interactions among co-expressed members of the *lefty* and *nodal* subfamilies of TGF-β signaling molecules.

Key words: *lefty*, *nodal*, *cyclops*, Embryonic midline, TGF-β, Axis formation, Zebrafish

INTRODUCTION

The vertebrate midline is essential for generating dorsoventral and anteroposterior pattern, and regulating left-right asymmetry. Initial embryonic patterning is established by localized maternal signals and inductive cell interactions. These signals establish organizing centers that coordinate the body plan. Spemann and Mangold (1924) identified a population of cells from the dorsal margin of amphibian gastrulae which can organize a second embryonic axis when transplanted into the ventral side of a host embryo. The node in birds (Waddington, 1932) and mice (Beddington, 1994) and the embryonic shield in fish (Oppenheimer, 1936) provide analogous organizing functions. In amphibians and zebrafish, TGF-β signals from the endoderm induce overlying marginal cells to adopt mesodermal fates while *wnt* pathway signaling promotes organizer formation. Mesoderm is then patterned along the dorsal-ventral axis in response to a gradient of BMP signaling (reviewed by Harland and Gerhart, 1997; Thomsen, 1997; Schier and Talbot, 1998). At the onset of gastrulation, zebrafish embryonic shield cells are already specified to develop into specific tissue types including the notochord (Shih and Fraser, 1995; Melby et al., 1996). The notochord influences development of overlying neural floorplate. Together these midline tissues serve as a polarizing center, patterning the anteroposterior axis and establishing dorsoventral polarity in adjacent tissues (reviewed by Placzek, 1995).

Secreted signaling molecules of the *nodal* subfamily of TGF-βs are important in gastrulation, axial development and establishment of laterality in vertebrates (reviewed by Varlet et al., 1997; Ramsdell and Yost, 1998). In zebrafish, *nodal*-related proteins (Erter et al., 1998; Rebagliati et al., 1998a) are encoded by *squint* (Feldman et al., 1998) and *cyclops* (Rebagliati et al., 1998b; Sampath et al., 1998). Loss of gene function results in embryos with deficiencies in midline mesendoderm, ventral neuraxis (Hatta et al., 1991; Yan et al., 1995, Feldman et al., 1998) and laterality defects (Chen et al., 1997).

Recently, a novel subfamily of TGF-β proteins encoded by *lefty-1* and *lefty-2* was identified in mice (Meno et al., 1996, 1997). *lefty* RNA is expressed in the primitive streak during gastrulation and in the midline and left lateral plate mesoderm during somitogenesis. *lefty* and *nodal* expression patterns are strikingly similar. Asymmetric expression of *lefty* and *nodal* are similarly perturbed in mouse mutants with laterality defects (reviewed by Varlet and Robertson, 1997), and a knockout mutation of *lefty-1* causes misexpression of *lefty-2* and *nodal* during somitogenesis (Meno et al., 1998). These observations suggest the regulation and functions of *lefty-1*, *lefty-2* and *nodal* are related.

To better understand the roles of *lefty*-related genes in vertebrate development, we identified two zebrafish *lefty* homologs. *lefty1* and *lefty2* are expressed in distinct domains along the anteroposterior axis and the margin. Together, the spatial and temporal expression patterns of these genes coincide with those of *cyclops* and *squint*. Analysis of *lefty*

expression in mutants defective for midline development suggests individual domains of *lefty* expression are differentially regulated. Ectopic expression of *lefty* and *cyclops* have opposing effects on mesendoderm development and suggests that Lefty suppresses *cyclops* expression while Cyclops induces *lefty* expression. Co-injection of *lefty* and *cyclops* results in normal mesendoderm formation, suggesting that Lefty and Cyclops are antagonistic. We propose a model in which antagonism between co-expressed members of the TGF-β family of signaling molecules regulates midline mesendoderm formation and establishes bilateral symmetry.

MATERIALS AND METHODS

Cloning zebrafish *lefty1* and *lefty2* cDNAs

RT-PCR was used to identify zebrafish *lefty* genes using degenerate oligonucleotides designed from conserved regions of mouse *lefty* and human *endometrial bleeding associated factor*. PCR templates were produced by reverse transcription of zebrafish gastrula (80-95% epiboly) mRNA. RNA isolated by Trizol (BRL) extraction and poly(A) selected with Oligotex spin columns (Qiagen) was reverse transcribed with Superscript (BRL) and amplified using Pfu (Stratagene) in the presence of 2 μM degenerate oligonucleotides. A 270 bp PCR product was cloned into pBlueScript KS- (Stratagene) and sequenced. This PCR fragment was labeled with α-^{32}P-dCTP (Amersham) using the Decaprime kit (Ambion) and used to screen a zebrafish gastrula-stage cDNA library cloned in λ pHybriZap (Stratagene). Plaques were lifted to Magnalift membranes (MSI) and hybridized at 42°C overnight in 40% formamide, 5× SSPE, 5× Denhardt's solution, 0.5% SDS, 100 μg/ml yeast RNA. Filters were washed at a final stringency of 0.5× SSPE/0.1% SDS at 50°C. 20 positive clones were subjected to three rounds of purification and excised from λ phage into plasmids using Excist helper phage (Stratagene).

Embryo culture and zebrafish stocks

Zebrafish, *Danio rerio*, were maintained at 28.5°C on a 14 hour/10 hour light/dark cycle. Embryos were collected from natural spawnings, cultured and staged by developmental time and morphological criteria (Westerfield, 1995). Wild-type embryos were descendants of outbred stocks obtained from Ekkwill Breeders (Gibsonton, Fl). Zebrafish carrying mutations were obtained from stocks originally produced at the University of Oregon (*cyc^{b229}*, *ntl^{b160}*), MGH/Harvard (*oep^{m134}*) or Newcastle (*flh^{n1}*).

Ectopic expression assays

Synthetic RNAs for injection were generated from *lft1*, *lft2* and *myc* epitope-tagged versions of both genes. Coding sequences from *lft1* and *lft2* were amplified using PCR with Pfu polymerase (Stratagene) and cloned into pCS2+ and pCS2+MT expression plasmids (Rupp et al., 1994; Turner and Weintraub, 1994).

Capped RNA was synthesized from Lefty expression constructs and from the Cyclops expression construct pCS2+Cyclops (Rebagliati et al., 1998b) using the mMessage mMachine SP6 transcription kit (Ambion). Zebrafish embryos at 1- to 8-cell stages were pressure injected with RNA in 0.1 M KCl containing 24 mg/ml FITC-dextran. Following injection embryos with widespread lineage tracer dye were selected, allowed to develop to desired stages, then photographed or fixed for in situ hybridization and immunohistochemistry.

RNA in situ localization and immunolocalization

For in situ hybridizations, embryos were fixed in 4% parafomaldehyde in sucrose buffer (Westerfield, 1995), rinsed in PBS, dehydrated into absolute methanol and stored at −20°C. Riboprobes were synthesized from linearized DNA templates using T3 or T7 polymerases and digoxigenin labeling mixes (BMB). In situ hybridizations were carried out as described by Stachel et al. (1993).

To identify clones of cells expressing *myc* epitope-tagged RNAs, embryos were refixed following in situ hybridization and were processed for immunohistochemistry. The *myc* epitope was detected with 9E10 monoclonal antibody diluted 1/500 (Santa Cruz Biotechnology), HRP-conjugated goat anti-mouse diluted 1/200 (Jackson ImmunoResearch) and 3,3′-diaminobenzidine as development substrate.

Embryos were cleared in 70% glycerol/PBS or benzyl benzoate:benzyl alcohol (2:1), and photographed with a Leica MZ12 microscope. Images were collected on Kodak T160 film, scanned and processed using Adobe Photoshop.

RESULTS

Identification of *lefty* homologs in zebrafish

To understand the roles of *lefty*-related genes in development, *lefty* homologs expressed during zebrafish gastrulation were identified and characterized. Two genes, *lefty1* (*lft1*) and *lefty2* (*lft2*), were identified as members of the TGF-β superfamily of signaling polypeptides (GenBank AF132444, AF132445). Amino acid sequence alignments (Fig. 1) place *lft1* and *lft2* within the TGF-β subfamily that includes mouse and human *lefty*. One hallmark of the *lefty* subfamily is the absence of one of seven cysteines conserved in other TGF-βs. Both Lft1 and Lft2 lack this cysteine (Fig. 1). While Lft1 and Lft2 are 70% identical, there is only 32-35% identity between Lft1 or Lft2 and mouse or human Lefties. *lefty* genes in each organism are more similar to one another than to their homologs in other species; thus orthology of *lefty* homologs cannot be established between species by sequence comparison. Nomenclature for the zebrafish genes has been assigned based on conservation of late somite-stage expression patterns when compared to mouse *lefty-1* and *lefty-2* (Meno et al., 1997).

lft1 and *lft2* are expressed in distinct domains in mesendodermal precursors during gastrulation

Early expression patterns of zebrafish *lft1* and *lft2* are dynamic, with co-localized expression at some stages and distinct expression domains at others. Initiation of expression at sphere stage differs between the two genes (Fig. 2A,B). *lft1* is localized to the dorsal blastoderm margin (Fig. 2A), as confirmed by double-labeling for *goosecoid* (*gsc*) RNA (Stachel et al., 1993) (not shown). In contrast, *lft2* is expressed in patches along the blastoderm margin without bias to the dorsal side (Fig. 2B). By dome stage both genes are expressed around the margin (Fig. 2C,D) in cells that will contribute to mesendodermal tissues of the embryo (Kimmel et al., 1990).

Expression of *lft1* in the margin is maintained late in gastrulation, while expression of *lft2* is downregulated after 50% epiboly. At shield stage, both genes are expressed in involuted cells of the dorsal hypoblast (Fig. 2E,F). At 85-95% epiboly, *lft1* and *lft2* are expressed in the anterior midline in the polster and prechordal plate (Fig. 2G,H). Posteriorly, expression patterns of *lft1* and *lft2* diverge. *lft1* is absent from the posterior midline, which gives rise to notochord and floorplate. Dorsal forerunner cells (Cooper and D'Amico, 1996; Melby et al., 1996), which express high levels of *lft1* mRNA, are readily seen as marginal *lft1* expression begins to

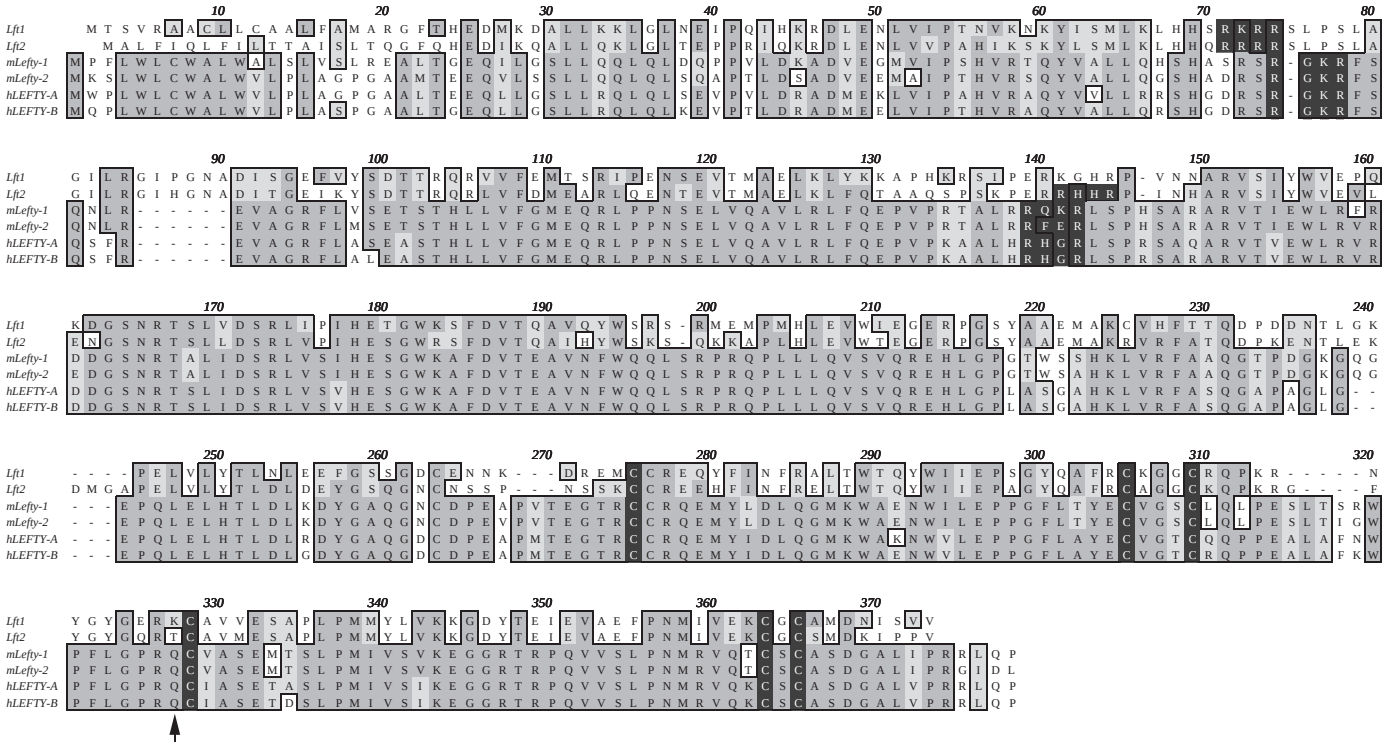

Fig. 1. Amino acid sequence alignment of *lefty* genes in zebrafish, mouse and human. Regions of amino acid identity are shaded in mid-gray, those with conservative amino acid substitutions are shaded in light gray. Putative cleavage sites (RXXR) and conserved cysteines are shaded in black, and the position of the cysteine absent in the *lefty* subfamily is indicated by an arrow.

be downregulated at 90% epiboly (Fig. 2G). In contrast, *lft2* is expressed along the axis from polster to margin, but is absent from dorsal forerunner cells (Fig. 2H).

During early somitogenesis, both genes are expressed in the polster and prechordal plate. *lft1* is also expressed in Kupffer's vesicle, a dorsal forerunner cell derivative, while *lft2* is localized to presumptive neural floorplate cells (Fig. 2I,J). Expression of *lft2* is downregulated after the 3-somite stage (Fig. 2L,N) while *lft1* expression is maintained in the prechordal plate through 6-8 somites (Fig. 2K). During mid-somitogenesis *lft1* is expressed in the left habenula of the diencephalon and in the posterior notochord (Fig. 2M). Expression domains of *lft1* and *lft2* extensively overlap those of the *nodal*-related genes *squint* (*sqt*) and *cyclops* (*cyc*) (aka *ndr1*, *ndr2*, Rebagliati et al., 1998a).

lft1 and *lft2* are asymmetrically expressed during late somitogenesis

During late somitogenesis, domains of *lefty* expression exhibit left-right asymmetry (Fig. 2O,P). *lft1* is expressed strongly in the left habenula and weakly in left lateral plate mesoderm. *lft1* is also expressed in anterior and posterior notochord. *lft1* expression is extinguished by 24 hours postfertilization (hpf). *lft2* is strongly expressed in left lateral plate mesoderm from the 19-somite stage through 30 hpf. Low levels of *lft2* RNA are present in the left habenula. The asymmetric *lefty* expression domains are similar to those of *cyc* (Rebagliati et al., 1998a,b; Sampath et al., 1998) and *nodal*-related genes in other vertebrates (Levin et al., 1995; Lowe et al., 1996; Lustig et al., 1996). Analysis of the roles of *lft1* and *lft2* in left-right development will be discussed elsewhere (unpublished data).

Ift expression domains are differentially affected in midline mutants

Domains of *lft1* and *lft2* expression are divergent, suggesting that distinct regulatory pathways direct expression of each gene. To assess upstream regulatory pathways, *lefty* expression patterns were assessed in four recessive lethal mutants that affect midline tissue differentiation. Mutations in *cyclops*, a *nodal* homolog (Hatta et al., 1991), and *one-eyed pinhead* (*oep*) (Schier et al., 1996), a member of the EGF-CFC gene family (reviewed in Salomon et al., 1999), cause defects in prechordal plate mesendoderm and floorplate neurectoderm. These genes encode secreted and membrane-bound signaling molecules, respectively (Rebagliati et al., 1998b; Sampath et al., 1998; Zhang et al., 1998). Mutations in *no tail* (*ntl*) (Schulte-Merker et al., 1994) and *floating head* (*flh*) (Talbot et al., 1995), which are homologs of the transcription factors *Brachyury* and *Xnot* respectively, result in loss of notochord and alterations in floorplate (Halpern et al., 1993, 1995).

flh^{-} embryos show no alteration in *lft1* or *lft2* expression through early somitogenesis (Fig. 3A,B), indicating that genetic changes in midline cells do not necessarily lead to altered *lefty* expression. In contrast, domain-specific *lefty* expression was altered in *cyc*, *oep* and *ntl* mutants. In *cyc*$^{-}$ embryos, *lft1* expression was absent from the polster and prechordal plate but wild-type in dorsal forerunner cells and the margin (Fig. 3C). Prior to shield stage, *lft2* expression in the blastoderm margin was unaffected. In late gastrulae, *lft2* was expressed only in a few involuting cells at the dorsal margin (Fig. 3D). This suggests that Cyclops is essential for maintenance of *lft1* and *lft2* expression in the anterior midline and floorplate but not the margin. In contrast, expression of *lft1*

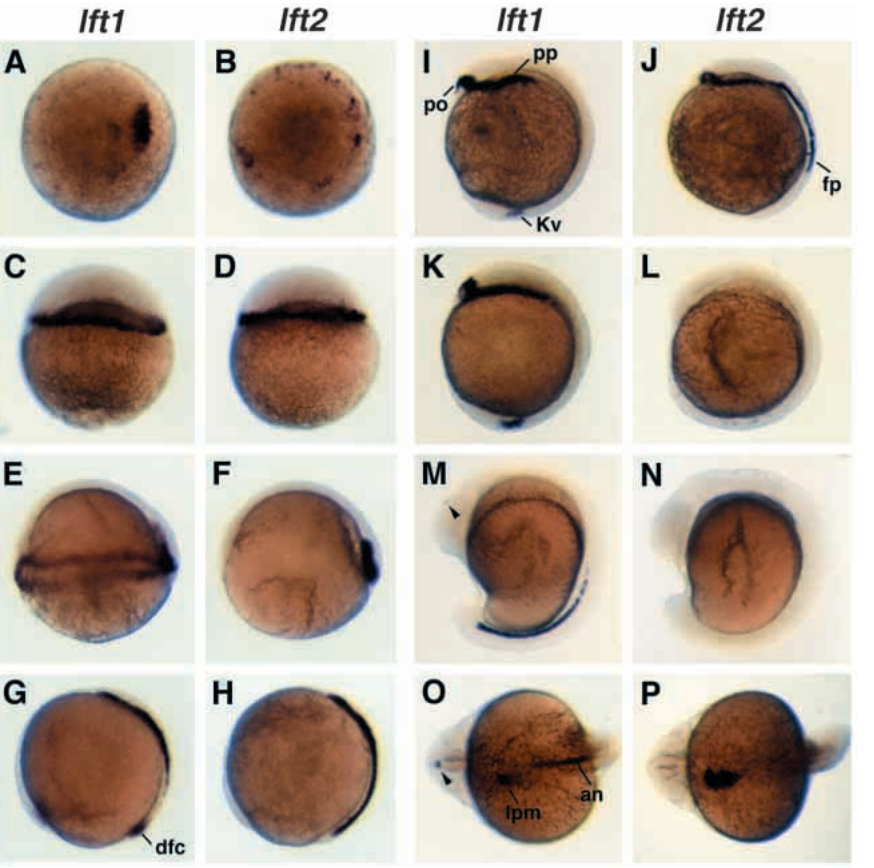

Fig. 2. *lefty* expression during early zebrafish development. (A-P) In situ localization of *lft1* and *lft2* transcripts at blastula through late somite stages (A,B, animal pole views; C-N, lateral views, dorsal at right; O,P, dorsoanterior views, anterior at left). *lft1* and *lft2* localize to the blastoderm margin at sphere (A,B) and dome stages (C,D). At shield stage (E,F), and at 90% epiboly (G,H) both genes localize to the dorsal hypoblast; *lft1* is also expressed in dorsal forerunner cells (dfc). 1- to 3-somite embryos express both genes in the polster (po) and prechordal plate (pp, I,J); *lft1* is uniquely expressed in Kupffer's vesicle (Kv) (I), *lft2* is uniquely expressed in floorplate precursors (fp) (J). At 6-8 somites *lft1* expression is maintained (K) while *lft2* is downregulated (L). 13-15 somite embryos express *lft1* in the posterior notochord and left habenula (arrowhead) (M); *lft2* is not expressed (N). 22-24 somite embryos showing *lft1* in the anterior notochord (an), left habenula (arrowhead) and left lateral plate mesoderm (lpm) (O); *lft2* is expressed in left lateral plate mesoderm (P).

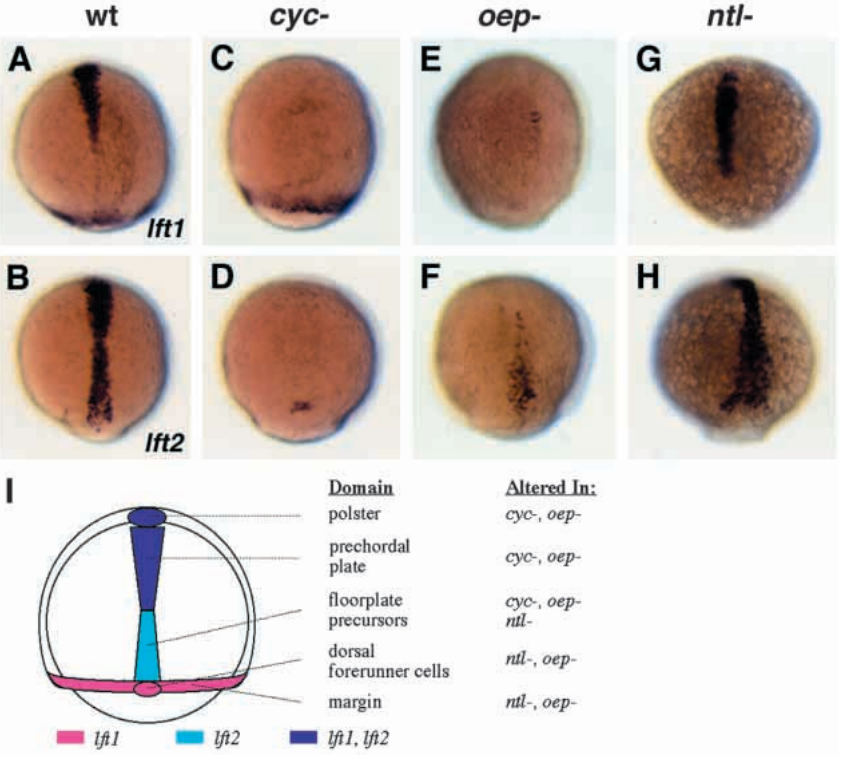

Fig. 3. *lefty1* and *lefty2* expression domains are disrupted in zebrafish midline mutants. (A-H) In situ localization of *lft1* and *lft2* in mutant embryos at 85-90% epiboly (dorsal views). Embryos from heterozygous *flh* parents show wild-type expression of *lft1* (A) and *lft2* (B). *cyc* mutant embryos do not express *lft1* or *lft2* in the anterior midline (C,D) and *lft2* is only expressed in a few cells near the dorsal margin. In *oep* mutants *lft1* and *lft2* expression in the prechordal plate is limited to a few cells (E,F); *lft2* expression is also reduced in floorplate precursor cells (F). In *ntl* mutants *lft1* expression is lost in dorsal forerunner and marginal cells (G), and *lft2* expression in floorplate precursors is expanded laterally (H). (I) *lefty* expression domains at 85% epiboly. *lft1* is expressed at the margin, and in dorsal forerunner cells. *lft2* is expressed in the posterior midline in floorplate precursors. Both genes are expressed in the anterior midline in the polster and prechordal plate. Expression domains affected in different midline mutants are summarized at right.

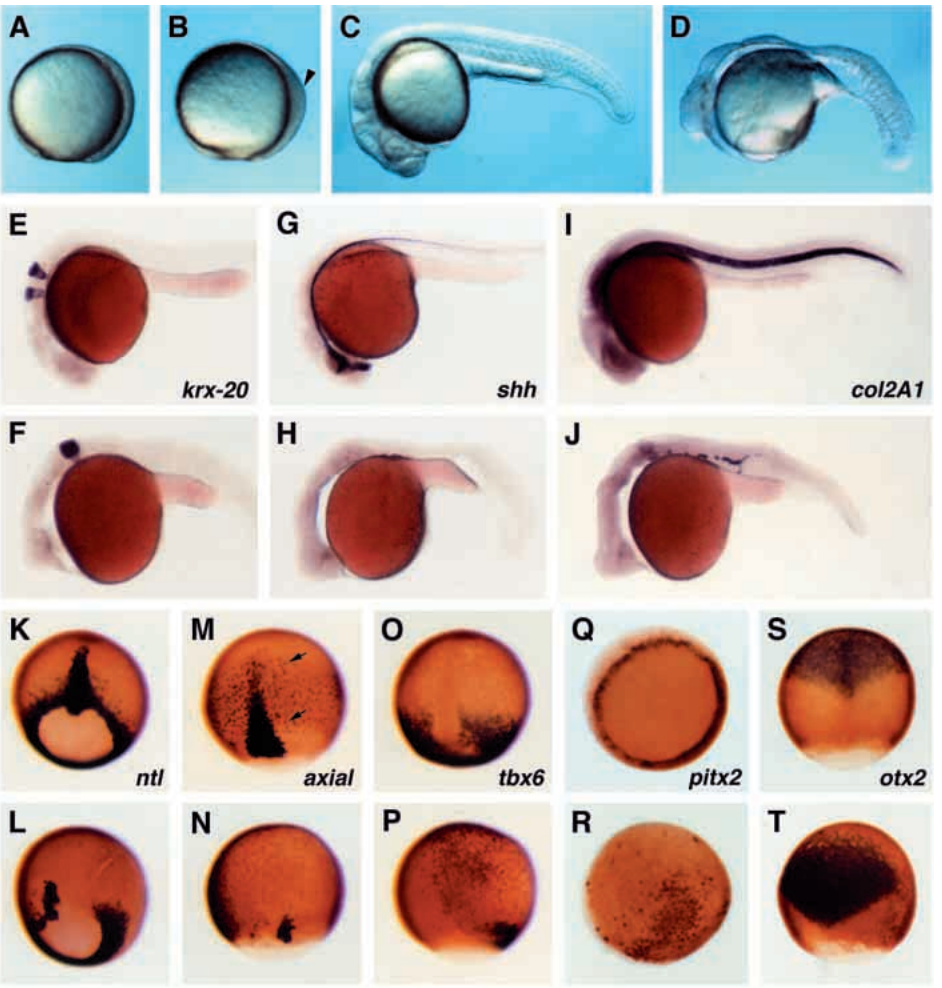

Fig. 4. Ectopic expression of Lefty1 or Lefty2 results in loss of anterior mesendoderm and ventral neurectoderm. (A-D) Morphological phenotypes of embryos injected with 25 pg Lft2-MT RNA (lateral views). At 95% epiboly, injected embryos accumulate cells on the dorsal side (B, arrowhead) compared to uninjected controls (A). At 24 hpf, injected embryos (D) lack anterior mesendoderm, notochord, and trunk somites and the anteroposterior axis is shortened relative to uninjected controls (C). The neural tube is present and retains some anteroposterior pattern. (E-T) Effect of ectopic Lefty expression on gene expression in 24 hpf (E-J, lateral views) and gastrula-stage embryos (K-P, S,T, 90% epiboly, dorsal view; Q,R, 50% epiboly, animal pole views). Upper panels in each series are uninjected controls, lower panels are embryos injected with 25 pg RNA encoding Lft1-MT (J,L,P,R) or Lft2-MT (F,H,N,T). Embryos were processed by in situ hybridization (E-T), followed by anti-*myc* immunohistochemistry to identify clones of cells expressing Lft1-MT or Lft2-MT (K-T). At 24 hpf, *krx-20* (E,F) is expressed at the normal anteroposterior level in the hindbrain of injected embryos although the bands of expression within rhombomeres 3 and 5 are compressed. *shh* expression (G,H) is abolished from the ventral brain and floorplate of injected embryos. *col2a*, expression in the floorplate, notochord and hypochord (I) is reduced to a few cells in injected embryos (J). In gastrulae, *ntl* expression is reduced in the midline and dorsal margin (K,L) and *axial* expression is reduced in the midline and abolished in endodermal precursors (arrows, M,N) of injected embryos. *tbx6* expression is suppressed in much of the ventrolateral margin (O,P), and *pitx2* expression in the margin is abolished (Q,R). The *otx2* expression domain is shifted toward the margin (S,T) in injected embryos.

and *lft2* in the margin was reduced in *oep*$^{-}$ embryos at 50% epiboly. By 90% epiboly, *lft1* expression was detected in only a few cells of the posterior prechordal plate (Fig. 3E); *lft2* was weakly expressed in floorplate precursors, and was absent elsewhere (Fig. 3F). In *ntl*$^{-}$ embryos, *lft1* and *lft2* were expressed normally in the margin at 50% epiboly. However, *lft1* was absent in the margin and in dorsal forerunner cells of late gastrula stages (Fig. 3G) and in Kupffer's vesicle subsequently. *lft2* expression in the floorplate precursor domain was widened in late gastrulae (Fig. 3H), correlating with an expansion in the medial floorplate of later stage *ntl*$^{-}$ embryos (Odenthal et al., 1996). These results identify distinct regulatory domains along the anteroposterior axis of the zebrafish midline (Fig. 3I).

Ectopic Lefty expression inhibits mesendoderm development

To investigate downstream responses to Lefty protein, synthetic RNAs were injected into 2- to 8-cell embryos. Injection of 25 pg of RNA encoding Lft1, Lft2 or *myc* epitope-tagged variants (Lft1-MT or Lft2-MT) produced embryos with identical phenotypes. Injected embryos were indistinguishable from controls until shield stage. In injected embryos, epiboly and dorsal convergence occurred normally during gastrulation but cells failed to involute, resulting in embryos with a dorsal accumulation of cells and deficient axial development (Fig. 4A,B). At 24 hpf, injected embryos lacked anterior mesendodermal derivatives including cephalic mesoderm, anterior somites and notochord, but had some mesoderm in the tail (Fig. 4C,D). Given the apparent absence of axial mesoderm, *lefty*-injected embryos had surprisingly normal anteroposterior patterning and neural axis development including the presence of developing eyes and otic vesicles.

Analysis of injected 24 hpf embryos with molecular markers indicated a loss of ventral neural floorplate and absence of anterior mesendoderm. Approximately 50% of embryos overexpressing Lefty had wild-type *krx-20* expression in rhombomeres 3 and 5 (Oxtoby and Jowett, 1993) (Fig. 4E). The remainder displayed a shortening of the distance between the bands (extreme case shown in Fig. 4F), suggesting that although anteroposterior patterning is largely normal, the neural tube is slightly compressed. *sonic hedgehog* expression in the ventral brain and floorplate (Krauss et al. 1993; Fig. 4G) is abolished in embryos injected with *lefty* RNAs (Fig. 4H),

confirming the absence of ventral neural tissue. *col2a1* (Yan et al. 1995) is expressed in head mesenchyme, notochord, hypochord and floorplate (Fig. 4I). In *lefty*-injected embryos, *col2a1* is abolished anteriorly and is expressed in only a few cells of unknown fate in the trunk (Fig. 4J). Vascular endoderm that expresses *flk-1* (Liao et al., 1997) is absent in *lefty*-injected embryos (not shown). These results suggest that, with the exception of neural floorplate, neural ectoderm can develop fairly normal anteroposterior pattern in the absence of underlying mesendoderm.

To address whether neural development observed in 24 hpf embryos might be due to earlier mesodermal induction, expression of mesendodermal and neurectodermal markers was assessed in late gastrulae. All mesendodermal markers tested including *cyc* (Rebagliati et al., 1998a,b; Sampath et al., 1998), *ntl* (Schulte-Merker et al., 1994), *gsc* (Stachel et al., 1993), *axial* (Strahle et al., 1993) and *tbx6* (Hug et al., 1997) were downregulated in response to ectopic Lefty.

Genes expressed in the anterior midline were more susceptible to downregulation by *lefty*-injection than those with posterior expression domains. For example, *gsc* expression in the anterior midline at 80-90% epiboly was abolished in 90% (*n*=61) of injected embryos (not shown, see Fig. 6B). Genes expressed more posteriorly were less frequently abolished by Lefty (*ntl*: 42%, *n*=64; *axial*: 38%, *n*=58), but were reduced when clones of Lefty-expressing cells were nearby (Fig. 4K-N). *axial* expression in endodermal precursors was abolished in all *lefty*-injected embryos (Fig. 4M,N, arrows). The absence of mesendodermal gene expression from the anterior midline in late gastrulae suggests that neural tube development observed at 24 hpf did not require mesodermal induction earlier in development.

Mesendodermal genes expressed in the margin vary in their sensitivity to ectopic Lefty. *ntl* was downregulated only when clones of Lefty expression contacted the dorsal margin (Fig. 4K,L). Expression of *tbx6* in the ventrolateral margin was inhibited considerable distances from clones expressing Lefty (Fig. 4O,P). In the most extreme case, expression of the paired homeobox gene *pitx2* (GenBank AF132446) was abolished from the blastoderm margin regardless of the site of ectopic Lefty expression (Fig. 4Q,R). The distinct responses of *ntl*, *tbx6* and *pitx2* to ectopic Lefty suggests these genes are differentially regulated and that misexpression of Lefty does not result simply in loss of all marginal mesendoderm.

Presumptive anterior neurectoderm marked by *otx2* expression (Li et al., 1995) extends across the animal pole to the equator at 80-90% epiboly (Fig. 4S). In *lefty*-injected embryos, the *otx2* expression domain appeared unchanged in dimension but was shifted dorsovegetally to extend almost to the margin (Fig. 4T), suggesting that early mesendoderm formation is required for complete axis elongation. That *otx2* expression is not expanded suggests ectopic Lefty does not cause cells that lose expression of mesendodermal markers to transfate to neurectoderm.

lefty and *cyclops* affect expression of each other

Two observations suggest *lefty* and *nodal* subfamilies of TGF-βs act in a common signaling pathway. First, the combined temporal and spatial expression patterns of *lft1* and *lft2* are coincident with those of *cyc* and *sqt*. Second, morphological phenotypes and alterations of gene expression resulting from *lefty* RNA injection are very similar to those observed in *sqt*;*cyc* double-mutant embryos (Feldman et al., 1998).

To test whether these TGF-βs affect expression of one another, we injected RNAs encoding Lft1, Lft2 or Cyc and examined expression of the others. *cyc* expression was abolished by ectopic Lft1-MT or Lft2-MT in 76% (*n*=72) of embryos (Fig. 5B,C). Injection of RNAs encoding Lft1-MT or Lft2-MT abolished expression of its paralog in 76% and 71% of embryos, respectively (Fig. 5E, and not shown). Partial downregulation of midline *cyc*, *lft1* and *lft2* expression occurred when Lefty-expressing clones did not encompass the midline, as observed for *ntl* and *axial* (Fig. 4L,N). In contrast, injection of *cyc* RNA induced ectopic expression of *lft1* and *lft2* in all embryos (Fig. 5F, and not shown). At 50% epiboly, *sqt* expression was induced by ectopic Cyc and suppressed by Lft2-MT (not shown). These results indicate that *nodal* subfamily members induce expression of themselves and *lefty* subfamily members. In contrast, *lefty* subfamily members suppress expression of themselves and *nodal* subfamily members.

Competitive interaction of Lefty and Cyclops control mesendoderm induction

As shown above, ectopic expression of Lft1 or Lft2 causes downregulation of *cyc* and other mesendodermal genes. Conversely, ectopic expression of Cyc expands the expression domains of *lft1*, *lft2* and other mesendodermal genes including *gsc* and *lim1* (this study, Rebagliati et al., 1998a; Sampath et al., 1998). These results suggest that *lefty* and *cyclops* have opposite effects, with *lefty* driving differentiation pathways away from mesendoderm and *cyclops* promoting differentiation of mesendoderm.

To assess whether *lefty* and *cyclops* could counteract each other, we injected RNAs encoding Lft2-MT and Cyc singly or in combination into 1- to 4-cell embryos and assessed mesendodermal gene expression by in situ hybridization. Injection of 25 pg *lft2-MT* RNA abolished *gsc* expression in 40-50% epiboly embryos (Fig. 6A,B) and caused slight downregulation of *ntl* expression in the blastoderm margin relative to uninjected embryos (Fig. 6E,F). Injection of cyc RNA had the opposite effect. Injection of 5 pg or 10 pg *cyc* RNA expanded *gsc* expression from a 90 degree marginal arc to encompass 25-60% or 50-100% of the blastoderm, respectively (Fig. 6C), and resulted in ectopic *ntl* expression in 25-60% to 50-100% of the blastoderm, respectively (Fig. 6G).

Co-injection of 25 pg *lft2-MT* RNA with 5 pg or 10 pg *cyc* RNA suppressed the phenotypes that result from overexpression of either gene, yielding embryos with wild-type *gsc* expression (Fig. 6D). Similarly, ectopic *ntl* expression was reduced in embryos co-injected with 25 pg *lft2-MT* RNA and 10 pg *cyc* RNA, and was nearly extinguished in embryos co-injected with 25 pg *lft2-MT* RNA and 5 pg *cyc* RNA (Fig. 6H). Wild-type expression patterns of *lft1* and *sqt* were also restored by co-expression of Lft2-MT and Cyc (not shown). Thus, the regulation of several mesendodermal genes can be normalized by co-expression of Lft2 and Cyc. This suggests that *lft2* and *cyc*, which are co-expressed in many tissues, function as mutual antagonists.

DISCUSSION

We have identified two highly related members of the *lefty*

subfamily of TGF-β signaling molecules that likely play important roles in the generation of embryonic pattern in zebrafish. The *lefty* genes are expressed early in development in several distinct domains, some of which subdivide the midline along the anteroposterior axis. As revealed by analysis of expression patterns in midline mutants *lefty* expression domains are regulated by distinct upstream pathways. Ectopic expression of Lefty1 and Lefty2 indicates both are equally capable of inhibiting mesendodermal gene expression. This inhibition appears to arise from antagonism of signals from the *nodal* subfamily of TGF-βs. Thus a balance of mutual antagonists expressed in the midline likely regulates embryonic mesendoderm formation.

Inhibitory role of *lefty* in mesoderm induction

Similar functions of Lefty1 and Lefty2 proteins in ectopic expression assays suggests that both genes function in embryonic patterning, by antagonizing mesendoderm induction. Injection of *lefty* RNA in zebrafish embryos inhibits expression of early dorsal mesoderm markers such as *goosecoid* and suppresses formation of the embryonic shield, resulting in impaired cellular involution during gastrulation. These results and the localization of *lft1* transcripts in the presumptive shield at sphere stage suggest an early zygotic function for Lefty in dorsal mesoderm induction and organizer development. Ectopic Lefty expression also perturbs genes expressed later in the posterior midline, including *axial* and *no tail*. These perturbations correlate with a later loss of mesendodermal derivatives including notochord, somites and vascular endoderm. These results, and the observation that the combined expression domains of *lft1* and *lft2* encompass the midline and margin during gastrulation, suggest an essential role for Lefty signaling in the regulation of mesendoderm formation during early development.

Early dorsoventral polarity is preserved in embryos overexpressing Lefty, indicating that other positional information signals act in parallel to Lefty. Dorsoventral polarity in zebrafish is likely established by the BMP subfamily of TGF-β signaling molecules (Schulte-Merker et al., 1997; Nguyen et al., 1998). Thus, although Lefty signaling may play a role in mesoderm induction, it is not responsible for establishing the dorsal organizer per se.

Although ectopic Lefty expression suppresses mesendodermal differentiation, embryos retain relatively normal anteroposterior patterning of the neuraxis, as indicated by correct positioning of eyes and otic vesicles and molecular markers such as *krx-20*. The extensive development of neural tissue in embryos lacking significant mesendoderm indicates vertical signals from dorsal mesoderm are not required for anteroposterior patterning of the neuraxis, concurring with the view that planar signals from other organizing centers induce anteroposterior patterning (reviewed by Schier and Talbot, 1998). Ectopic Lefty expression greatly diminishes *shh* expression and formation of neural floorplate, indicating that development of the dorsoventral axis of the neural tube is dependent on underlying mesoderm as has been reported (reviewed by Tanabe and Jessel, 1996).

Diverse functions of *lefty* family members may be regulated by distinct expression domains

Mature Lefty proteins are very similar in amino acid sequence, and ectopic expression of either protein has equivalent effects on embryogenesis. We therefore suggest that, if *lft1* and *lft2* have distinct functions during embryogenesis, this results from divergent expression patterns, not divergent protein functions. For example, *lft2* expression in floorplate precursors of late gastrulae might make this tissue refractory to mesoderm-inducing signals, including Cyclops (Rebagliati et al., 1998a; Sampath et al., 1998), emanating from the underlying notochord and allow these cells to progress toward a neural fate. Tissue-specific regulation of *lefty* expression is also evident in the asymmetric expression of these genes during late somitogeneis. Comparable to *lefty-1* and *lefty-2* expression in mice (Meno et al., 1996, 1997), *lft1* is expressed at high levels in the neural tube while *lft2* is expressed at high levels in the lateral plate mesoderm, suggesting they may play different roles in neural or mesodermal patterning. Recent work has shown that distinct regulatory mechanisms also underlie the asymmetric expression of mouse *lefty-1* and *lefty-2* (Saijoh et al., 1999).

Expression domains of *lft1* and *lft2* are differentially affected in midline mutants, indicating that individual domains of *lefty* expression are under control of distinct, tissue-specific, regulatory pathways (Fig. 3). Domains of *lefty* expression and those of *cyc* and *sqt* show considerable overlap. Interestingly, alterations in the patterns of *cyc* expression reported in midline mutants are similar to the alterations of *lefty* expression seen in this study. *cyc* expression is normal in *flh*$^{-}$ embryos (Sampath et al., 1998), fails to be maintained in *cyc*$^{-}$ (Sampath et al., 1998) and *oep*$^{-}$ (Schier et al., 1996) late gastrulae, and is widened in the posterior axis of *ntl*$^{-}$ late gastrulae (Rebagliati et al., 1998a). Thus, both *cyc* and *lefty* genes are under similar regulatory controls, suggesting coordinated functions of these related signaling molecules.

Antagonism among co-expressed TGF-β family members

Strikingly, the expression domains of the *lefty* and *nodal* subfamilies in zebrafish are coincident, and embryos in which Lefty is overexpressed (Fig. 4) are remarkably similar to *nodal*-deficient *cyc*;*sqt* embryos (Feldman et al., 1998). *lefty* and *cyclops* appear to regulate each other's expression (Fig. 5). Ectopic expression of Lefty suppresses both *lefty* and *cyclops* expression, whereas ectopic expression of Cyclops induces *lefty* expression. Furthermore, ectopic co-expression of Lefty and Cyclops drives the embryo back toward normal development (Fig. 6). From these results, we propose that Lefty functions during mesendoderm induction and midline differentiation by antagonizing Cyclops signaling, and that a balance between these co-expressed mutually antagonistic signals results in a normally proportioned embryo (Fig. 7).

Recently, a zebrafish TGF-β family member, *antivin*, has been characterized (Thisse and Thisse, 1999). The protein sequences of Antivin and Lefty1 differ by only a single amino acid suggesting they are alleles of the same gene. The reported expression pattern of *antivin* is similar to the combined patterns of *lft1* and *lft2* suggesting that the *antivin* probe recognized both *lefty* transcripts. Ectopic expression of Antivin greatly reduced mesendoderm development, similar to our results, but did not alter its own expression, in contrast to our results where ectopic Lft1 and Lft2 suppressed one another. Co-injection experiments indicated that Activin can antagonize the effects

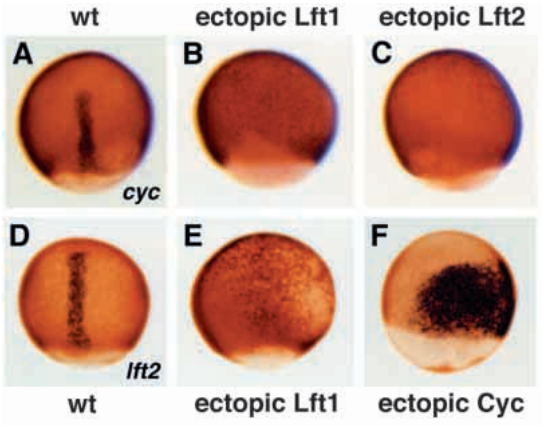

Fig. 5. Ectopic expression of Lefty1, Lefty2 or Cyclops affects the endogenous expression of each other. (A-C) *cyc* expression at 85% epiboly. In uninjected embryos *cyc* is expressed in the midline (A). Expression is abolished in embryos injected with 25 pg of RNA encoding either Lft1-MT (B) or Lft2-MT (C). (D-F) *lft2* expression at 85% epiboly. In control embryos *lft2* is expressed in the midline (D). Expression of *lft2* is abolished in embryos injected with 25 pg of RNA encoding Lft1-MT (E) and is expanded in embryos injected with 10 pg of *cyc* RNA (F). Embryos (dorsal views) were processed by in situ hybridization followed by anti-*myc* immunohistochemistry to identify clones of cells expressing Lft1-MT or Lft2-MT.

of Antivin (Thisse and Thisse, 1999). However, *activin* appears to have a restricted maternal role in development (Wittbrodt and Rosa, 1994), and its expression does not overlap extensively that of the *lefty* genes. In contrast, *lefty* expression domains described here co-localize with *nodal* (*cyc* and *sqt*) expression domains in the margin, embryonic midline, left habenula and the left lateral plate mesoderm. The multiple endogenous co-expression domains throughout embryogenesis suggest that *lefty* serves to antagonize *nodals* rather than *activin*.

Antagonistic interactions among members of the TGF-β family could arise by several mechanisms. *lefty* and *cyclops* could function in two separate pathways whose signaling readouts had opposing effects. Alternatively, interaction between *lefty* and *cyclops* could be more direct: First, Lefty and Cyclops might compete for a common receptor. A receptor that could function in Lefty/Cyclops signaling in the midline has been identified in zebrafish. The spatial and temporal expression patterns of *TARAM-A*, a serine/threonine kinase related to TGF-β type I receptor (Renucci et al., 1996), overlap extensively those of *lft1*, *lft2*, *cyc* and *sqt*. Additionally, ectopic expression of TARAM-A causes expansion only of dorsal mesoderm, suggesting it is activated only by dorsally localized TGF-β signaling molecules. Binding of Lefty might inhibit signal transduction while binding of Cyclops might initiate signal transduction, inducing mesodermal gene expression. Second, Lefty and Cyclops might form an inactive heterodimeric ligand. Dorsoventral axis specification in amphibians occurs *via* antagonism of TGF-β signaling, however, the antagonists are not TGF-β family members. During gastrulation, organizer cells secrete noggin, chordin and follistatin, which bind and inactivate BMP4 secreted from ventral mesoderm. These interactions establish fates in marginal mesoderm and pattern dorsal ectoderm (reviewed by Sasai and De Robertis, 1997; Thomsen 1997). Third, an equilibrium between Lefty and Cyclops homodimers and the heterodimer might provide a range of signaling functions arising from different activities of each homodimer and the heterodimer. Heterodimeric forms of BMPs 2 through 7 are more potent than the corresponding homodimers at inducing alkaline phosphatase in vitro and inducing ectopic bone formation in vivo (Isreal et al., 1996). Also, BMP4/7 heterodimers can convey signals for ventral mesoderm induction and patterning in *Xenopus* while the homodimers show no inductive activity (Nishimatsu and Thomsen, 1998).

A model for the establishment of bilaterality

Previous models of embryonic axis formation invoke antagonistic interactions between signaling components; signals and 'sinks' are usually proposed to exist on opposite sides of a cellular field or embryo (Lemaire and Yasuo, 1998). While antagonists positioned on opposite sides likely participate in dorsoventral specification (described above), once the midline is established, it is unlikely that signals and sinks on opposite sides could give refined and proportionate development across the midline. In the proposed model, positive and negative signals are emitted from the same source, the embryonic midline, so cells at fixed distances from the midline on the right or left side receive identical ratios of agonistic and antagonistic signals. The ratio between the signals would then define the proportions of the embryo so that bilateral symmetry is established and maintained.

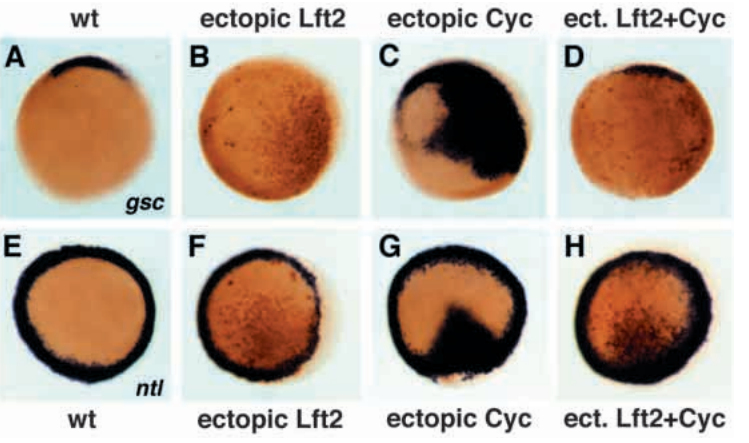

Fig. 6. Cyclops and Lefty2 have opposite and mutually antagonistic effects on mesendodermal gene expression. (A-D) *gsc* expression at 50% epiboly. Control embryos (A) express *gsc* in a 90 degree arc at the margin. *gsc* expression is abolished by injection of 25 pg *lft2-MT* RNA (B) and is expanded by injection of 10 pg *cyc* RNA (C). Co-injection of 25 pg *lft2-MT* RNA and 10 pg *cyc* RNA results in wild-type *gsc* expression (D). (E-H) *ntl* expression at 50% epiboly. Uninjected embryos (E) express *ntl* throughout the margin. In response to injection of 25 pg *lft2-MT* RNA, *ntl* expression is slightly downregulated at the site of ectopic expression (F). *ntl* expression is expanded in embryos injected with 5 pg cyc RNA (G). Embryos co-injected with 25 pg *lft2-MT* RNA and 5 pg *cyc* RNA (H) express *ntl* in a similar pattern to uninjected embryos. Embryos (animal pole view) were processed by in situ hybridization followed by anti-*myc* immunohistochemistry to identify clones of cells expressing Lft2-MT.

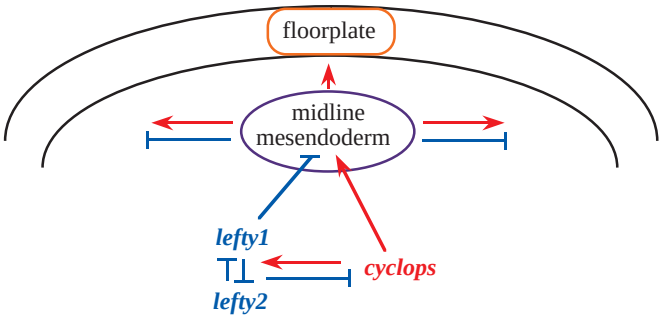

Fig. 7. Model of the potential roles of *lefty* and *cyclops* signaling in establishment of the embryonic midline and bilateral symmetry. Interaction of inhibitory *lefty* signals and positive *cyclops* signals regulates mesendoderm induction within the embryonic midline. Signals emanating from the midline (which may include *lefty* and *cyclops*) induce floorplate in the overlying neural tube and pattern lateral mesoderm, thereby establishing bilateral symmetry.

Evolutionary changes in the ratio between the agonist and antagonists emitted from the midline would allow alterations in the proportions of the embryo. This could occur via subtle changes in the regulation of the agonist or antagonist. Furthermore, by altering the ratios of agonist and antagonist in individual midline domains, specific regions of mesendoderm along the axis of the embryo could be expanded or contracted. Throughout this proposed evolutionary process, bilateral symmetry is maintained regardless of how proportions at specific points along the anteroposterior axis are altered.

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