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RESEARCH ARTICLE

Nodal and Hedgehog synergize in gill slit formation during development of the cephalochordate *Branchiostoma floridae*

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ABSTRACT

The larval pharynx of the cephalochordate *Branchiostoma* (amphioxus) is asymmetrical. The mouth is on the left, and endostyle and gill slits are on the right. At the neurula, *Nodal* and *Hedgehog* (*Hh*) expression becomes restricted to the left. To dissect their respective roles in gill slit formation, we inhibited each pathway separately for 20 min at intervals during the neurula stage, before gill slits penetrate, and monitored the effects on morphology and expression of pharyngeal markers. The results pinpoint the short interval spanning the gastrula/neurula transition as the critical period for specification and positioning of future gill slits. Thus, reduced Nodal signaling shifts the gill slits ventrally, skews the pharyngeal domains of *Hh*, *Pax1/9*, *Pax2/5/8*, *Six1/2* and *IrxC* towards the left, and reduces *Hh* and *Tbx1/10* expression in endoderm and mesoderm, respectively. Nodal auto-regulates. Decreased Hh signaling does not affect gill slit positions or *Hh* or *Nodal* expression, but it does reduce the domain of *Gli*, the Hh target, in the pharyngeal endoderm. Thus, during the neurula stage, Nodal and Hh cooperate in gill slit development – Hh mediates gill slit formation and Nodal establishes their left-right position.

KEY WORDS: Pharyngeal patterning, Gill slit development, Nodal signaling, Hedgehog signaling, *Branchiostoma*

INTRODUCTION

In bilateral animals, some organs are often arrayed asymmetrically about the midline. For example, in vertebrates, the viscera are asymmetrical. In the invertebrate chordate amphioxus, the larval pharynx is highly asymmetrical, with gill slits, the endostyle (homologous to the vertebrate thyroid gland) and a larval secretory organ, the club-shaped gland, forming on the right, and the mouth developing on the left. In many organisms, the specification of left-right asymmetry is mediated by the secreted signaling protein Nodal on the left (Boorman and Shimeld, 2002). In amphioxus, Nodal is initially expressed symmetrically but, by the early neurula, expression becomes restricted to the left side (Yu et al., 2002, 2007). Inhibition of Nodal signaling during the neurula stage, when pharyngeal structures are being specified, causes a duplicate endostyle, which is normally on the right side, to develop on the left, and also prevents formation of the first gill slit and mouth (Soukup et al., 2015). As in other organisms, *Nodal*, *Cerberus*, *Lefty* and *Pitx* cooperate (Li et al., 2017). However, Nodal is not the only signaling protein that is asymmetrically expressed during development. In both vertebrates and amphioxus, Hedgehog (Hh) genes also become asymmetrically expressed on the left side during early development (Shimeld, 1999; Dyer and Kirby, 2009; Tsiairis and McMahon, 2009; Tsikolia et al., 2012; Otto et al., 2014), and the Hh target *Gli* is expressed in the developing gill slit primordia (characterized by a thickening of the endoderm where each gill slit will form) (Shimeld, 2007). In the mouse, knockout of one Hh gene, sonic hedgehog (*Shh*), results in the first pharyngeal arches being fused in the midline (Moore-Scott and Manley, 2005; Yamagishi et al., 2006; Swartz et al., 2012) and in the failure of the neural tube to fuse (holoprosencephaly) (Xavier et al., 2016). Similarly, in amphioxus, knockout of *Hh* eliminates the mouth; the gill slit primordia are either absent, or aberrant and ventralized (Wang et al., 2015; Hu et al., 2017). Thus, the effects of inhibiting either Nodal or Hh signaling on gill slit formation in amphioxus embryos are much the same, raising the issue of whether Nodal and Hh act together or independently in patterning the amphioxus pharynx.

Signaling by both Nodal and Hh proteins is complex. In the canonical pathway, Nodal, which is in the TGFβ family, binds to Activin receptors 1 and 2, leading to phosphorylation of Smad2 and Smad3 that then complex with Smad4. This Smad complex then translocates to the nucleus where it participates in activation of Nodal target genes. In canonical Hh signaling, Hh binds to the transmembrane protein Patched (Ptch), relieving repression by a second transmembrane protein Smoothened, thereby converting *Gli* from a repressor to an activator of target sequences (Jenkins, 2009). In addition, non-canonical signaling by Hh and by TGFβ proteins, as well as context dependency, have been described (Bertrand and Dahmane, 2006; Jenkins, 2009; Massagué, 2012; Wang et al., 2016; Szczepny et al., 2017).

The focus on possible interactions of Nodal and Hh signaling in vertebrates has been on the CNS and the lateral plate mesoderm. In some contexts, Nodal and Hh appear to act in parallel, whereas in other contexts one may regulate the other. For example, Nodal and Shh signaling are both involved in the development of the vertebrate neural tube (Luo, 2017). Separate or simultaneous inhibition of *Shh* and *Nodal* causes holoprosencephaly, indicating that the two proteins probably act synergistically (Monuki, 2007; Mercier et al., 2013). Moreover, they may be co-regulated: for example, knockdown of the gene coding for the transcription factor Zic1 reduces signaling by both Nodal and Hh (Maurus and Harris, 2009). Specification of ventral domains in the vertebrate CNS also requires signaling by both Nodal and Hh; in this instance, Nodal is upstream of Hh. In addition, non-canonical Nodal signaling in the prechordal mesoderm acts to maintain expression of *Shh* (Ellis et al., 2015), whereas canonical Hh signaling in the lateral plate mesoderm is

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indirectly responsible for initiation and propagation of Nodal signaling (Tsiairis and McMahon, 2009). Consequently, mutations in either *Hh* or *Nodal* can also result in laterality defects (Roessler and Muenke, 2001). In animals other than vertebrates, *Hh* may also regulate *Nodal*. For example, in sea urchins, *Hh* regulates the late asymmetric expression of *Nodal* (Warner et al., 2016). This is reminiscent of the late role of *Hh* in activating Nodal signaling in the lateral plate mesoderm of vertebrates (Tsiairis and McMahon, 2009).

It is unclear whether *Hh* and *Nodal* interact in pharyngeal patterning. In vertebrates, *Hh* is expressed in the pharyngeal endoderm, where it is involved in patterning the pharyngeal arches. It is upstream of, and positively regulates, *Tbx1* (Garg et al., 2001), but negatively regulates *Fgf8* (Haworth et al., 2007; Billmyre and Klingensmith, 2015), *Bmp4* and *Pax1* (Moore-Scott and Manley, 2005). Nodal signaling, upstream of *Pax2*, *Nkx2.1* and *Hex*, is essential for development of the thyroid gland, a derivative of the pharyngeal endoderm (Elsalini et al., 2003; Porazzi et al., 2009). This raises the issue of whether Nodal and Hh act independently to pattern the pharynx or whether they interact and, if so, to what extent.

To investigate possible relationships between Nodal and Hh in pharyngeal patterning, we used amphioxus as a simple chordate model. Amphioxus has little genetic redundancy, making it highly suitable for understanding the role of specific genes in embryonic patterning. A recent study has reported that expression of the Nodal antagonist *Cerberus* is absent in amphioxus *Hh*-null mutants and, therefore, concluded that Hh signaling regulates Nodal indirectly (Hu et al., 2017). However, this interpretation is equivocal, as overexpression of *Hh* had no effect on either *Cerberus* expression or on left-right asymmetry of amphioxus embryos (Hu et al., 2017). To determine whether Nodal and Hh act together or independently in pharyngeal patterning in amphioxus, we inhibited each pathway separately for a limited time span at intervals from the late gastrula through to the neurula stage and determined the effect on pharyngeal morphology and on the expression of a variety of genes expressed in the pharyngeal endoderm and/or developing gill slits. Our results reveal a narrow window at the late gastrula/early neurula stage in which Nodal signaling establishes the left-right position of gill slits, whereas Hh functions to mediate gill slit formation. Although Nodal auto-regulates, and to some extent may also regulate Hh, the two pathways act largely in parallel in gill-slit patterning and formation in amphioxus.

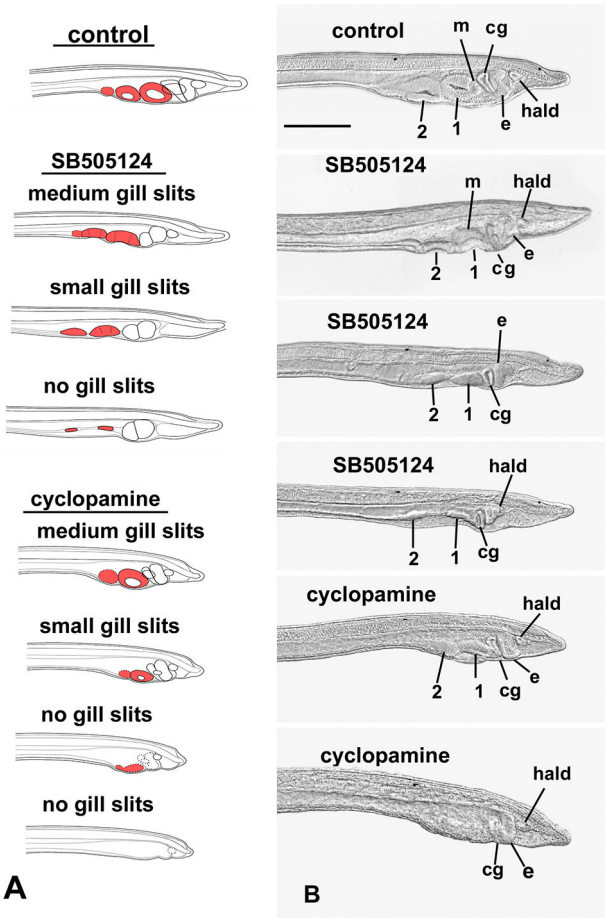

Fig. 1. The Nodal inhibitor SB505124 (15 µM) or the Hh inhibitor cyclopamine (1.75 µM) applied for 20 min at the late gastrula stage of *Branchiostoma floridae* results in a range of pharyngeal phenotypes at the early larval stage. (A) Diagrams with the gill slit primordia in red. Anterior to the right. From top to bottom: control with two gill slits (the third gill slit has not yet penetrated); SB505124-treated, medium gill slits are ventralized, small gill slits are ventralized, no gill slits and very small gill slit primordia; cyclopamine treated, development is somewhat retarded, two medium gill slits, one small gill slit, no gill slits with two small gill slit primordia, no gill slits and no gill slit primordia. (B) Photographs of living larvae. Anterior to the right. SB505124 treatment shifts gill slit primordia and gill slits to the left. Cyclopamine does not affect the lateral position of the gill slits. 1, first gill slit; 2, second gill slit; m, mouth; cg, club-shaped gland; e, endostyle; hald, Hatsheck's anterior left diverticulum, destined to form part of the adenohypophyseal homolog. Scale bar: 50 µm.

RESULTS

In normal embryos of the Florida amphioxus (*Branchiostoma floridae*), the larval gill slits form in the center of the gill slit primordia (thickened regions of the endoderm overlain by a thin layer of ectoderm). After ∼30-34 h of development at 25°C, the ectoderm and endoderm fuse to form the first gill slit on the right side. Within the next 2 days, two more gill slits are added posterior to the first (Fig. 1). Genes associated with gill slit formation begin to be expressed from the early neurula, at ∼10 h post fertilization (hpf), well before there is any morphological indication of a gill slit or even a gill slit primordium. For example, initially *Pax1/9* is broadly expressed in the pharyngeal endoderm at the early neurula, at ∼9 hpf, and becomes downregulated at ∼10-11 hpf in the primordium of the first gill slit (Holland et al., 1995). Therefore, to determine the roles of Nodal and Hh signaling in gill slit positioning and penetration, we focused our experiments on the late gastrula through neurula stages.

To inhibit Nodal and Hh signaling, we used the chemical inhibitors SB505124 {2-[5-benzo(1,3)dioxol-5-yl-2-tert-butyl-3H-imidazol-4-yl]-6-methylpyridine hydrochloride}, which inhibits the Alk4, Alk5 and Alk7 Activin/Nodal receptors, and cyclopamine, which specifically binds to Smoothened and prevents transduction of the Hh signal (Chen et al., 2002a). When embryos are continuously exposed to either 15 µM SB505124 or 1.5 µM cyclopamine from the early gastrula, the resulting larvae are severely deformed and essentially lack a pharynx: gill slits and gill slit primordia are absent, although there may be some small domains of expression of pharyngeal markers at the anteriormost region of the larva (Fig. S1). To confirm that cyclopamine blocks Hh signaling, we treated embryos at the early gastrula with 1.75 µM cyclopamine and fixed aliquots for *in situ* hybridization at the late gastrula/very early neurula and at the early to mid-neurula. *In situ* hybridization for Ptch, a target of Hh signaling in amphioxus (Hu et al., 2017), as in other organisms (Chen et al., 2002a,b),

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showed that cyclopamine inhibits *Ptch*, and thus blocks Hh signaling in amphioxus (Fig. S3). At high concentrations of cyclopamine, long exposures cause the ectoderm to dissociate into individual cells. Therefore, to determine the optimum conditions for affecting pharyngeal patterning without completely eliminating the gill slit primordia or killing the larvae, we first did pilot experiments, with each inhibitor added at the following concentrations: 1.5 µM, 1.75 µM, 2.0 µM and 4.0 µm cyclopamine for 20 min at 30 min intervals (the newly hatched late gastrula, the early neurula, the early-mid neurula and the mid-neurula; see Fig. 2), and 1.0 µM, 10 µM and 50 µM SB505124 for 20 min added at the same times plus mid/late neurula and late neurula (Table S1). The optimal concentrations of inhibitors for additional experiments were determined as those resulting in a lateral shift in the position of the gill slit primordia and/or the failure of the gill slits to open (Table S1). The results of these experiments showed that 10 µM SB505124 added at late gastrula or early neurula had a very mild effect with slightly smaller gill slits, whereas 50 µm had a severe effect, with most embryos either lacking gill slits or dying. We, therefore, chose 15 µM SB505124 for subsequent experiments. Cyclopamine also eliminated gill slits at the highest concentration we used (4 µM). Therefore, for subsequent experiments, we used 1.5 µM or 1.75 µM, which produced smaller gill slits.

As is typical when either inhibitors or activators of signaling pathways are added to amphioxus embryos in DMSO or ethanol, at a given concentration, there is a range of severe to mild phenotypes because of slightly uneven mixing. Fig. 1 shows the range of phenotypes resulting from treatment with 15 µM SB505124 or 1.75 µM cyclopamine for 20 min at the late gastrula stage. The effects on gill slit size of adding these inhibitors at three stages (late gastrula, early neurula and early-mid neurula) are shown in Fig. 2. In controls, at 46 hpf, the first two gill slits have opened on the right (Fig. 1, top). In the slightly milder phenotype resulting from addition of 15 µM SB505124 at the late gastrula stage, the gill slit primordia are present, but smaller than normal and shifted somewhat ventrally; in the most severe phenotype, gill slits are imperforate and the gill slit primordia are small (Fig. 1B). With 1.75 µM cyclopamine added at the late gastrula stage, the first two gill slit primordia appear to be on the right, but are much smaller than normal; the first gill slit has opened (Fig. 1B). In the more severe phenotype, neither the gill slits nor the gill slit primordia are detectable (Fig. 1). Fig. 2 shows that, for 15 µM SB505124, small differences in the time of treatment had major effects on the size of gill slits, with most gill slits failing to penetrate with the earliest treatment, and most being about half the normal size at the latest treatment (Fig. 2A). In contrast, most of the larvae treated with 1.75 µM cyclopamine had very small gill slits regardless of the time of treatment (Figs 1 and 2B). Thus, the effect of altered Nodal signaling on gill slit size is restricted to a narrow time window, whereas Hh regulates gill slit size through to at least mid-neurula.

Pharyngeal gene expression in embryos and larvae treated with SB505124 and cyclopamine

To determine the respective roles of Nodal and Hh in regulating expression of pharyngeal markers during gill slit formation in amphioxus, we performed *in situ* hybridization for seven genes

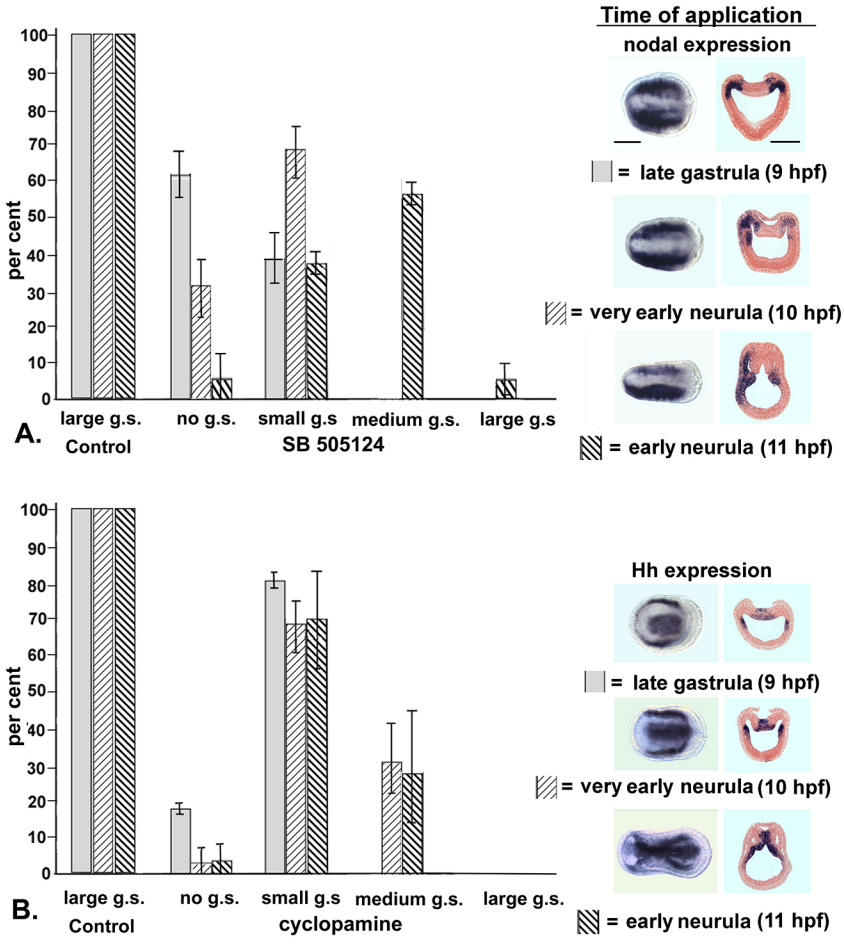

Fig. 2. Effects on gill slit formation of Nodal or Hh inhibition at different stages of development. (A,B) Left hand panels show effects on gill slit formation of amphioxus (*B. floridae*) embryos with treatment of 15 µM SB505124 (A) or 1.75 µM cyclopamine (B) applied for 20 min at the late gastrula (9 h), very early neurula (10 h) and early neurula (11 h) stages. Control larvae were treated with DMSO (for SB505124) or ethanol (for cyclopamine). Gill slit formation was assayed at the early larval stage (43-45 hpf). At this stage, control larvae have formed two or three gill slits. For SB505124, sample numbers ranged from 54 to 154, with an average of 87 for controls and 105 for each experiment. For cyclopamine, sample numbers averaged 100 for controls and 108 for each experiment. Data are mean±s.d. Right hand panels show expression patterns of Nodal (A) and Hh (B) in normal larvae at the late gastrula, very early neurula and early neurula stages. Dorsal views are on the left and cross-sections are on the right. g.s., gill slit. Scale bars: 50 µm.

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expressed in the developing pharynx (*Nodal*, *Hh*, *Gli*, *Pax1/9*, *Pax2/5/8*, *IrxC* and *Tbx1/10*). As a high percentage of larvae treated with 15 µM SB505124 at the early-mid neurula stage had nearly normal gill slits, and the effects of adding 1.75 µM cyclopamine for 20 min at the early neurula and early-mid neurula stages were the same (Fig. 1), we restricted these experiments to treatments at the late gastrula (9 hpf) and early neurula (10 hpf) stages. Fig. 3 shows that addition of 15 µM SB505124 for 20 min at either late gastrula or early neurula severely reduces *Nodal* expression (Fig. 3A-L,A′-L′). In addition, when SB505124 is applied at the late gastrula stage, downregulation of *Nodal* in the dorsal-lateral endoderm on the right, which normally occurs at the early/mid-neurula stage, is retarded (compare Fig. 3D with J). In line with a lesser effect on morphology if Nodal inhibition is delayed until the early neurula stage, the reduction in *Nodal* expression is not as pronounced in embryos

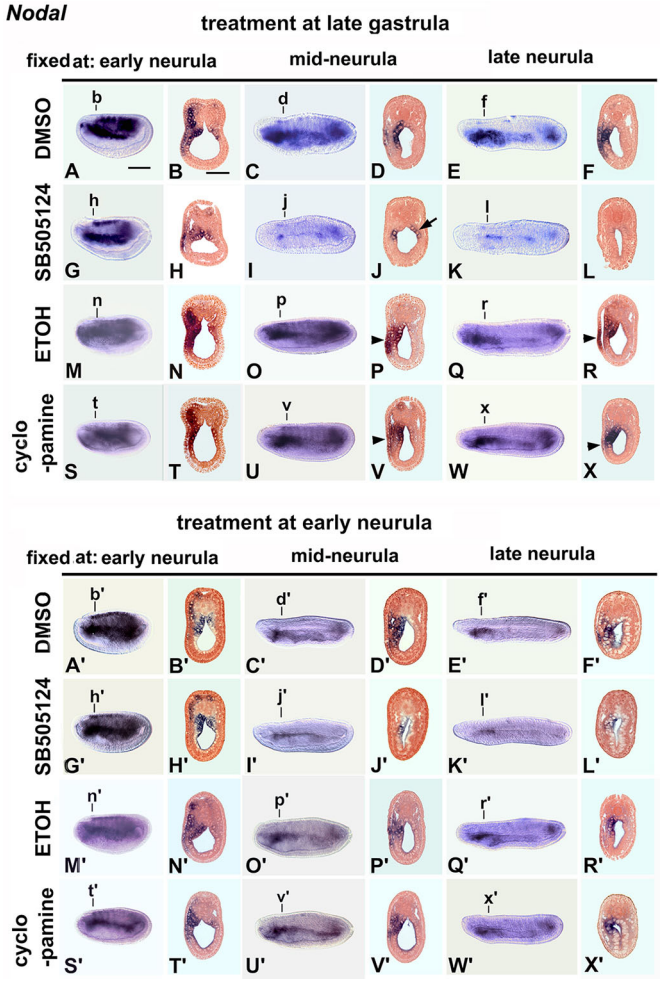

Fig. 3. Transient inhibition of Nodal, but not Hh, reduces *Nodal* expression in amphioxus embryos. Expression of *Nodal* in control embryos (A-F,M-R,A′-F′,M′-R′) and in those treated for 20 min with the Nodal inhibitor SB505124 (15 µM) at the late gastrula (G-L) and very early neurula (G′-L′) stages or with the Hh inhibitor cyclopamine (1.75 µM) at the late gastrula (S-X) and very early neurula (S′-X′) stages. Control larvae were treated with DMSO (for SB505124) or ethanol (ETOH) (for cyclopamine). Embryos were fixed for *in situ* hybridization at the stages indicated. Anterior to the left in wholemounts. Cross-sections (pink), viewed from the posterior, are at the levels indicated with the lowercase letters in the preceding wholemounts (blue). Arrow in J indicates persistent expression of *Nodal* in SB505124-treated embryos in dorsal/lateral endoderm on the right at the mid-neurula stage. Arrowheads in P, R,V and X indicate the ectodermal domain on the left. Scale bars: 50 µm.

treated at the early neurula stage compared with those treated at the late gastrula stage (Fig. 3A′-L′). In contrast, cyclopamine, when added at either the late gastrula stage (Fig. 3M-X) or the early neurula stage (Fig. 3M′-X′), has little effect on Nodal expression, except that when it is applied at late gastrula stage, the ectodermal domain on the left side is reduced or eliminated (arrows Fig. 3P,R and V,X). These results show that, whereas Nodal likely auto-regulates, Hh does not regulate Nodal, except perhaps to a slight extent in the ectoderm.

To investigate whether Hh expression is also auto-regulated or is regulated by Nodal signaling (Fig. 4), we assayed for *Hh* expression in embryos treated with either SB505124 or cyclopamine at the late gastrula and early neurula stages (Fig. 4). The major effects of SB505124 on *Hh* expression are a reduction of the domain in the ventral endoderm and a shift of the endodermal domain to the left. This is in line with the first gill slit primordium being shifted ventrally (compare Fig. 4G,H, and J,K with R,S and U,V) and suggests that Nodal may regulate the ventral endodermal domain of *Hh*. In addition, in embryos treated with SB505124 at the late gastrula stage, downregulation of *Hh* on the right side (arrows, Fig. 4O) compared with the control (Fig. 4D) was retarded, and upregulation of *Hh* ventrally and on the left side was reduced (compare Fig. 4G,J with R,U). *Hh* expression was not reduced on the left when Nodal inhibition was delayed until early neurula (Fig. 4A″-V″). Inhibition of Hh signaling at either the late gastrula or early neurula stage had little effect on *Hh* expression (Fig. 4W-R′, W″-R‴). Therefore, it is unlikely that there is a feedback loop whereby Hh regulates itself.

Gli is a zinc-finger transcription factor that transduces the Hh signal. In early amphioxus embryos, the two genes are expressed in largely complementary patterns, with *Hh* expressed in the notochord and dorsal-lateral endoderm and *Gli* in the developing somites and anterior neural plate (Figs 4A,B and 5A,B). At the mid-neurula stage, both genes also become expressed in the ventral endoderm, although the domain of *Hh* is skewed to the left, whereas that of *Gli* is more medial (Figs 4D,G and 5D,F,T,V). Inhibition of Nodal signaling for 20 min at the late gastrula stage has little effect on *Gli* expression, except that by the early larval stage the ventral endodermal domain in the second gill slit is greatly reduced in severely affected larvae with imperforate gill slits (Fig. 5O), whereas *Gli* expression persists in the gill slit primordia of control embryos (Fig. 5G,H). Inhibition of Nodal signaling for 20 min at the early neurula has no clear effect on *Gli* expression (Fig. 5A″-P″). In contrast, when Hh signaling is inhibited at the late gastrula stage, the ventral endodermal domain of *Gli* is reduced at the mid and late neurula stages (Fig. 5S-V,A′-E′). Whether this is because of slightly retarded development or inhibition of *Gli* per se is not clear. As seen in normal embryos, the ventral domain of *Gli* only appears between the early and mid-neurula stages (compare Fig. 5Q,R with S,T). There is no apparent effect on *Gli* expression when the addition of cyclopamine is delayed to the early neurula stage (Fig. 5Q″-F‴). These results indicate that although Nodal signaling has little, if any, effect on *Gli* expression, at the late gastrula stage Hh signaling regulates the ventral endodermal domain of *Gli*.

In addition to determining the effects of Nodal and Hh inhibition on these genes themselves, and on the direct Hh target Gli, we examined the effects of their inhibition on five other genes expressed in the amphioxus pharynx at the neurula and early larval stages. These include *Pax1/9*, normally expressed throughout the pharyngeal endoderm at the early neurula and subsequently downregulated in the gill slit primordia; *Pax2/5/8*, expressed where

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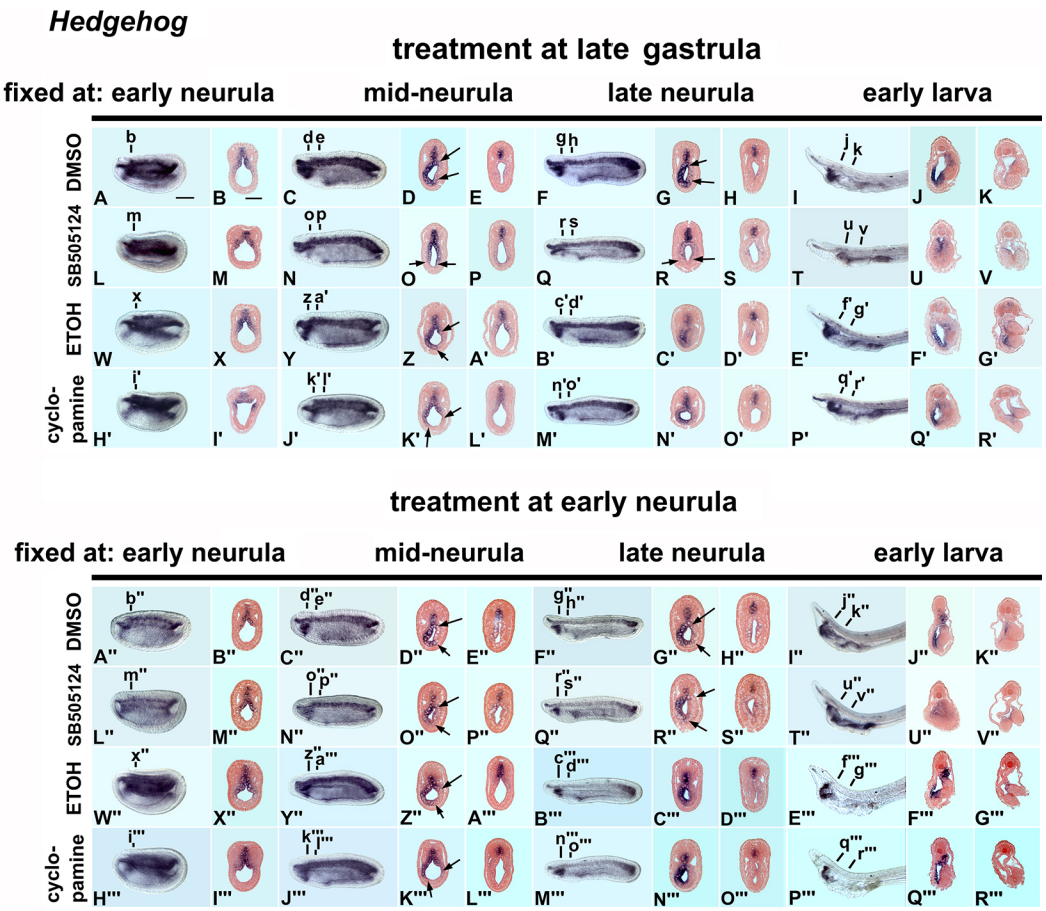

Fig. 4. Transient inhibition of Nodal, but not Hh, affects *Hh* expression in amphioxus embryos. Expression of *Hh* in control embryos treated with DMSO for SB505124 (A-K, W-G′) or ethanol (ETOH) for cyclopamine (A″-K″, W″-G‴) and in those treated for 20 min with the Nodal inhibitor SB505124 (15 µM) at the late gastrula (L-V) and very early neurula (L″-V″) stages or with the Hh inhibitor cyclopamine (1.75 µM) at the late gastrula (H′-R′) and very early neurula (H‴-R‴) stages. Embryos were fixed for *in situ* hybridization at the stages indicated. Anterior to the left in wholemounts. Cross-sections (pink), viewed from the posterior, are at the levels indicated with the lowercase letters in the preceding wholemounts (blue). Arrows indicate the limits of the endodermal expression domain. Scale bars: 50 µm.

the gill slits will penetrate; *Six1/2*, expressed in the ventral and lateral endoderm; *IrxC*, co-expressed in the pharyngeal endoderm with *Six1/2*; and *Tbx1/10*, expressed in the endoderm and mesoderm of the developing branchial bars. The effect of Nodal inhibition on the domain of *Pax1/9* is in proportion to the effect on the size and position of the gill slit primordia (Fig. 6A-P,A″-P″). Just as Nodal inhibition shifts the gill slits ventrally, it shifts the endodermal domain of *Pax1/9* to the left so that the region in which *Pax1/9* is downregulated continues to coincide with the region in which the gill slit primordia will develop. The degree of shifting to the left is similar whether SB505124 is added at the late gastrula or early neurula stages. These results suggest that Pax1/9 is downstream of Nodal and functions to maintain the undifferentiated state of the pharyngeal endoderm outside the gill slit primordia. In contrast, the primary effect of cyclopamine, as it does not change the position of the gill slits, is to delay development such that *Pax1/9* is downregulated in the endoderm somewhat later than in the controls (compare Fig. 6S,T with A′,B′, and S″,T″ with A‴,B‴).

Pax2/5/8 is first expressed in the pharyngeal endoderm at mid-neurula in a pattern complementary to that of *Pax1/9* (Figs 6C,D and 7C,D). In embryos treated with SB505124 at either the late gastrula or early neurula stages, the pharyngeal domain of *Pax2/5/8* expression is reduced (Fig. 7A-P,A″-P″), although the reduction is more pronounced when SB505124 is added at the earlier time. For example, when the inhibitor is added at the late gastrula stage, the domain of *Pax2/5/8* associated with the mouth (Fig. 7G,H,O,P) is reduced and those associated with the first two gill slits are eliminated (arrows in Fig. 7G,H,O,P). When the Nodal inhibitor is added at the early neurula stage, there is only a slight reduction of the *Pax2/5/8* domain in the pharynx (Fig. 7A″-P″). The addition of cyclopamine at either time has no clear effect on the Pax2/5/8 domain, except that, as development is delayed, initial expression of *Pax2/5/8* in the pharynx is also delayed. These results indicate that expression of *Pax2/5/8* in the pharynx probably requires Nodal signaling, but that Hh may not regulate *Pax2/5/8*.

The endodermal domains of *Six1/2* and *IrxC* in normal neurulae largely overlap (compare Figs 8A-P,Q″-F‴ with 9A-P,Q″-F‴). By the early larva stage, however, the pharyngeal domains of both genes become restricted to the mouth and gill slit primordia (Figs 8G,H and 9G,H). In fact, by mid to late neurula, the pharyngeal domain of *IrxC* is congruent with the area in which *Pax1/9* becomes downregulated around the gill slits, although that of *Six1/2* is somewhat broader than this region. The pharyngeal domains of both genes are shifted to the left when SB505124 is added at the late gastrula stage (Figs 8A-P and 9A-P). However, there is no clear effect on expression of either gene when SB505124 is added at early neurula (Figs 8A″-P″ and 9A″-P″). There is also no clear effect of cyclopamine when it is added at late gastrula or early

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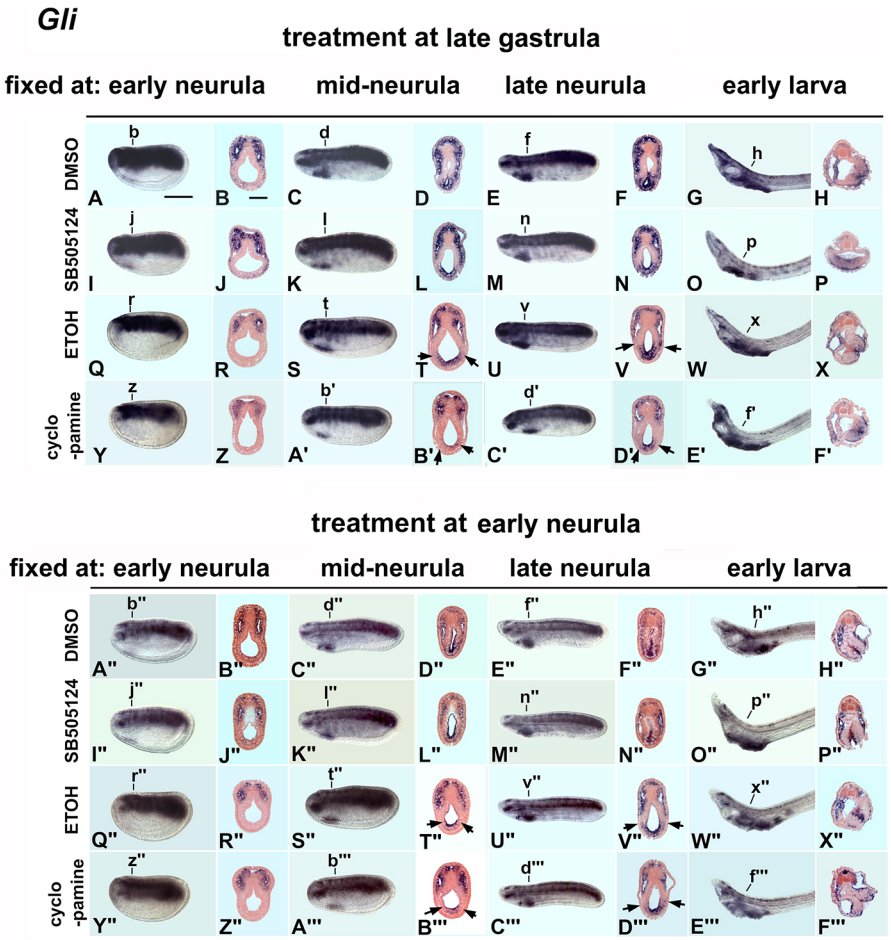

Fig. 5. Transient inhibition of Hh, but not Nodal, reduces *Gli* expression in amphioxus embryos. Expression of *Gli* in control embryos treated with DMSO for SB505124 (A-H, A″-H″) and ethanol (ETOH) for cyclopamine (Q-X, Q″-X″) and in those treated for 20 min with the Nodal inhibitor SB505124 (15 µM) at the late gastrula (I-P) and very early neurula (I″-P″) stages or with the Hh inhibitor cyclopamine (1.75 µM) at the late gastrula (Y-F′) and very early neurula (Y″-F‴) stages. Embryos were fixed for *in situ* hybridization at the stages indicated. Anterior to the left in wholemounts. Cross-sections (pink), viewed from the posterior, are at the levels indicated with the lowercase letters in preceding wholemounts (blue). Arrows indicate the extent of the ventral endodermal domain in controls (T,V,T″,V″) and in embryos treated with cyclopamine at the late gastrula (B′,D′) and early neurula (B‴,D‴) stages. Scale bars: 50 µm.

neurula (Figs 8Q-F′,Q″-F‴ and 9Q-F′,Q″-F‴). Thus, both *Six1/2* and *IrxC* appear to be downstream of Nodal signaling, but are not downstream of Hh.

The final gene we examined was *Tbx1/10*, normally expressed in the ventral endoderm and lateral mesoderm on the left side that will give rise to the mesoderm of the branchial bars. The only effect of Nodal inhibition at the late gastrula stage is the disappearance of the domain of *Tbx1/10* in the lateral mesoderm (Fig. 10D-L,P-X). There is no effect on *Tbx1/10* expression when SB505124 addition is delayed until the early neurula stage (Fig. 10A″-X″) and no clear effect on *Tbx1/10* expression of cyclopamine added at either late gastrula or early neurula (Fig. 10Y-V′,Y″-V‴).

Taken together, these results show that the precise left-right position of the gill slits is largely mediated by levels of Nodal signaling at the late gastrula/early neurula stages, with higher levels of Nodal shifting gill slits to the right and lower levels shifting them to the left. A Nodal feedback loop reinforces Nodal signaling. The expression domains of genes that are either downregulated in the future gill slit primordia (*Pax1/9*) or expressed in the gill slit primordia (*Six1/2*, *IrxC*, *Pax2/5/8* and, at the larval stage, *Gli*) are shifted to the left as Nodal signaling is reduced. Surprisingly, a reduction in Nodal signaling at the late gastrula stage strongly reduces *Tbx1/10* expression in the branchial arches. Thus, all of these genes are downstream of Nodal signaling (Fig. 11). In addition, Nodal signaling is required for gill slit penetration and may act by positively regulating *Pax2/5/8* where the ectoderm and endoderm will fuse to form gill slits, and/or by regulating *Hh*, which is also required for gill slit penetration.

In contrast, although inhibition of Hh signaling at either the late gastrula or early neurula strongly inhibits gill slit formation, the effects on expression of pharyngeal markers (for example *Pax1/9*) were very mild and could be because of a general developmental delay. The ventral endodermal domain of *Gli* is somewhat reduced. However, even in the most severe phenotype, with imperforate gill slits, generated by cyclopamine treatment, reducing Hh signaling has no effect on the position of the gill slits. Even when gill slits completely fail to penetrate, the expression of pharyngeal markers in the gill slit primordia is essentially unchanged (Fig. S2, Fig. 10). Thus, at the late gastrula and early neurula stages, Hh appears to play little, if any, role in establishing the position of the gill slits and gill slit primordia, but does affect gill slit penetration.

DISCUSSION

Stage-specific signaling during embryogenesis

The above results on the roles of Nodal and Hh signaling in early embryonic patterning of the amphioxus pharynx emphasize the rapidly changing roles of signaling pathways in early development. For example, during cleavage stages through to the mid-gastrula stage of amphioxus, *Nodal* is expressed dorsally and acts in opposition to BMPs to specify neuroectoderm and dorsal-anterior identity (Onai et al., 2010). Thus, inhibition of Nodal with SB505124 from cleavage stages onward results in loss of dorsal and anterior identity (Onai et al., 2010). Not surprisingly, Nodal has been identified as a neural inducer in amphioxus (Le Petillon et al., 2017). However, as represented in Fig. 11, at the late gastrula/early neurula stage, the role of Nodal changes; *Nodal* expression is downregulated on the right. From then into the neurula

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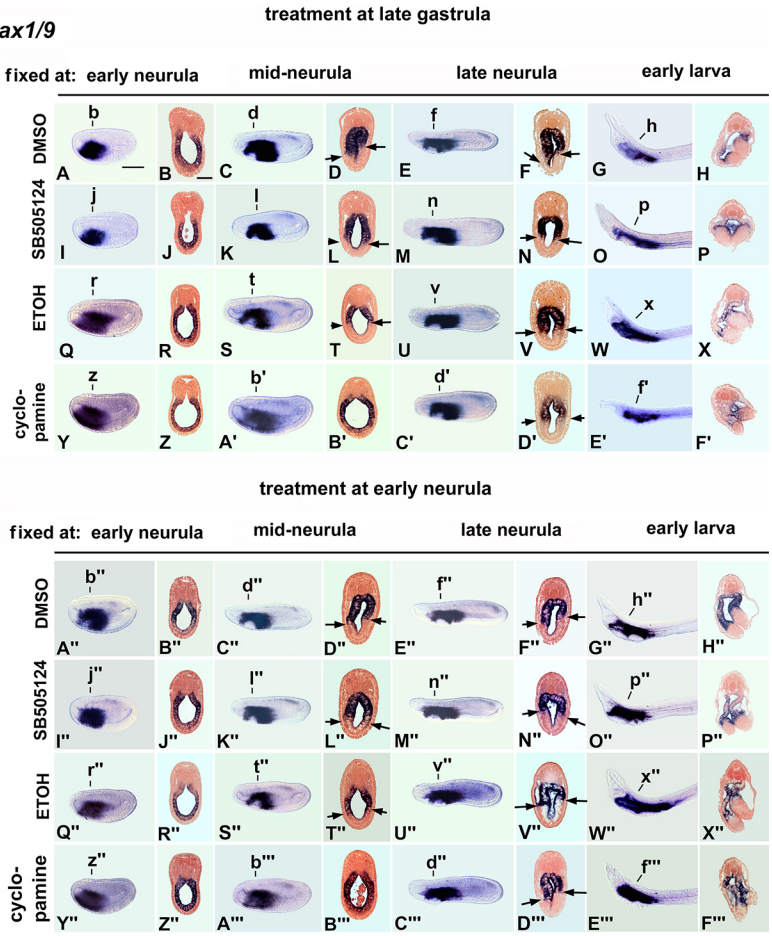

Fig. 6. Transient inhibition of Nodal, but not Hh, shifts expression of *Pax1/9* in amphioxus embryos. Expression of *Pax1/9* in control embryos treated with DMSO for SB505124 (A-H, A″-H″) or ethanol (ETOH) for cyclopamine (Q-X, Q″-X″) and in those treated for 20 min with the Nodal inhibitor SB505124 (15 µM) at the late gastrula (I-P) and early neurula (I″-P″) stages or with the Hh inhibitor cyclopamine (1.75 µM) at the late gastrula (Y-F′) and early neurula (Y″-F‴) stages. Embryos were fixed for *in situ* hybridization at the stages indicated. Anterior to the left in wholemounts. Cross-sections (pink), viewed from the posterior, are at the levels indicated with the lowercase letters in preceding wholemounts (blue). Arrows indicate boundaries of *Pax1/9* expression in the pharyngeal endoderm. Scale bars: 50 µm.

stage, Nodal signaling regulates left-right asymmetry (Soukup et al., 2015; Li et al., 2017). Correspondingly, in very early embryos, overexpression of the Nodal inhibitor *Cerberus*, normally expressed on the right, causes the loss of dorsal and anterior structures (Onai et al., 2010). A mutation of *Cerberus* results in a milder phenotype, with the right side of amphioxus embryos adopting a left-side identity and the gill slits shifted ventrally (Li et al., 2017). Null mutants of *Hh*, similar to the embryos in which Nodal is inhibited during the neurula stage, have ventralized gill slits and lack *Cerberus* expression, suggesting that *Hh* may indirectly regulate *Nodal* via *Cerberus* (Hu et al., 2017). However, our experiments, carried out at the late gastrula through to the neurula stage, after *Cerberus* ceases to be expressed on the right, indicate that if Nodal does regulate left-right asymmetry, in part through Hh, it must do so very early in development. By the early neurula stage, when inhibiting Nodal affects left-right asymmetry (Soukup et al., 2015), inhibiting Hh signaling for 20 min has little or no effect on either *Nodal* expression or on the left-right position of the gill slits (Fig. 3). Instead, it is the opposite: inhibiting Nodal signaling for 20 min at late gastrula/very early neurula inhibits expression of *Hh* in the pharyngeal endoderm (Fig. 4).

Not only does Nodal signaling at the late gastrula/early neurula stage control the lateral position of the gill slits, it also regulates the size of the gill slits. In severe phenotypes caused by inhibition of Nodal signaling, gill slits do not penetrate; gill slit primordia can be fused or be completely absent. These effects could be mediated by reduced *Hh* expression in the pharyngeal endoderm and/or by reduced *Tbx1/10* expression in the pharyngeal mesoderm. Reduced Nodal signaling at the late gastrula stage reduces the endodermal domain of *Hh* and shifts it to the left (Fig. 4), and a reduction in *Hh* signaling can completely eliminate the gill slit primordia (Fig. 2D). Inhibition of Nodal signaling at the late gastrula stage also eliminates mesodermal expression of *Tbx1/10* (Fig. 10F,I,R,U), and knockdown of *Tbx1/10* eliminates the gill slits and branchial bars (Koop et al., 2014).

Surprisingly, the *Hh*-null mutants, as in the milder phenotype of cyclopamine-treated larvae, have been reported to develop gill slits (Hu et al., 2017). Although we would have expected the null mutants to lack gill slits, as in the more severe phenotype resulting from cyclopamine treatment at the late gastrula/very early neurula stages (Fig. S2), it could be that the most severely affected *Hh* mutants failed to develop to the larval stage. Alternatively, although cyclopamine has been shown to be a very specific inhibitor of Hh signaling (Chen et al., 2002a,b), off-target effects cannot be completely ruled out (Meyers-Needham et al., 2012).

In addition to Nodal and Hh, other signaling pathways also function in early embryonic patterning. For example, during the gastrula stage, when Nodal and BMP act in opposition to establish dorsoventral identity in amphioxus embryos, retinoic acid (RA) signaling establishes position along the anterior-posterior axis (Koop et al., 2010), and Wnt signaling concomitantly specifies the posterior end of the amphioxus embryo. RA signaling also has a late role in pharyngeal patterning; for gill slits to penetrate, RA levels are kept low in the gill slit primordia by the RA antagonist *Tr2/4* (Escriva et al., 2002; Koop et al., 2014). Fgf and Wnt genes are also expressed in the developing amphioxus pharynx, but their roles in pharyngeal patterning have not yet been reported (Bertrand et al., 2011, 2015; Schubert et al., 2000, 2001).

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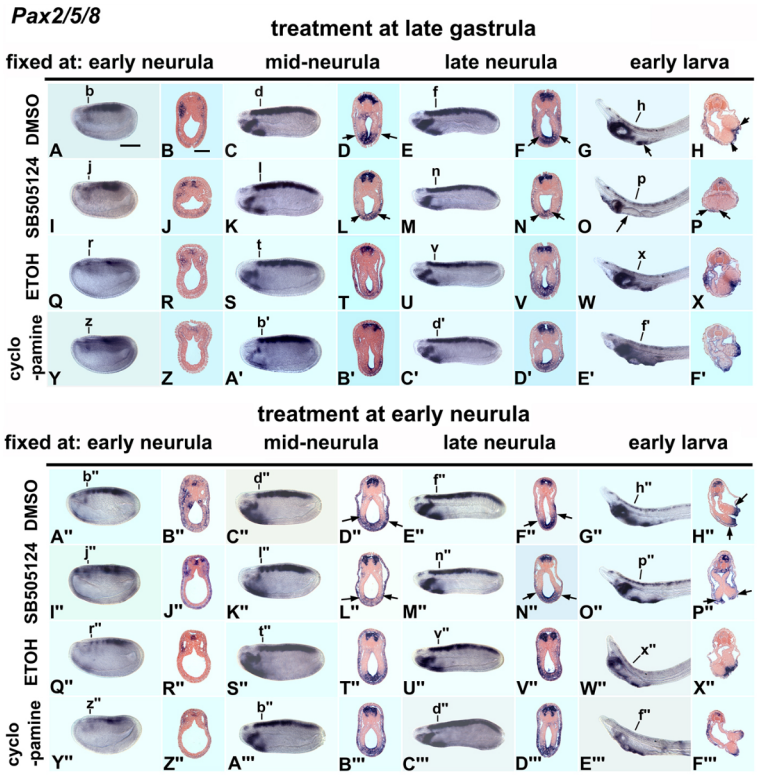

Fig. 7. Transient inhibition of Nodal, but not Hh, reduces *Pax2/5/8* expression in amphioxus embryos. Expression of *Pax2/5/8* in control embryos treated with DMSO for SB505124 (A-H, A″-H″) and ethanol (ETOH) for cyclopamine (Q-X, Q″-X″) and in those treated for 20 min with the Nodal inhibitor SB505124 (15 µM) at the late gastrula (I-P) and early neurula (I″-P″) stages or with the Hh inhibitor cyclopamine (1.75 µM) at the late gastrula (Y-F′) and early neurula (Y″-F‴) stages. Embryos were fixed for *in situ* hybridization at the stages indicated. Anterior to the left in wholemounts. Cross-sections (pink), viewed from the posterior, are at the levels indicated with the lowercase letters in preceding wholemounts (blue). Arrows indicate the extent of the ventral endodermal domain that marks the future edges of the gill slits and is reduced in SB505124-treated larvae. Scale bars: 50 µm.

Nodal and Hh function in pharyngeal patterning in vertebrates

Nodal and Hh genes are both involved in patterning vertebrate embryos, but whether they interact in pharyngeal patterning is not clear. In vertebrates, Nodal signaling is required for endoderm specification and development of the thyroid, a derivative of the pharyngeal endoderm (Elsalini et al., 2003; Grapin-Botton and Constam, 2007; Porazzi et al., 2009). Similar to amphioxus, in which expression of *Hh* in the pharynx is highest immediately anterior to the first gill slit primordium (Fig. 4), in vertebrates, *Shh* is strongly expressed in the endoderm and ectoderm of the first pharyngeal arch, where it is essential for development of mandibular arch derivatives (Brito et al., 2006, 2008; Haworth et al., 2007; Gillis et al., 2009; Swartz et al., 2012; Billmyre and Klingensmith, 2015; Dworkin et al., 2016). Shh signaling is low in the posterior pharyngeal pouches, allowing the parathyroid marker *Gcm2* to be expressed (Grevellec et al., 2011).

Shh interacts with a variety of genes in pharyngeal patterning (Yamagishi et al., 2006; Billmyre and Klingensmith, 2015; Xavier et al., 2016). For example, ectopic expression of *Shh* in the endoderm of the pharyngeal pouches of the mouse induces expression of *Tbx1* (Yamagishi et al., 2003; Bain et al., 2016). In $Shh^{-/-}$ mice, the pharynx is initially patterned correctly, but the first arch atrophies and the first pharyngeal pouch does not form. The second, third and fourth pharyngeal arches are small (Moore-Scott and Manley, 2005). Like Nodal, Shh signaling is also required for normal development of the thyroid (Fagman et al., 2004; Moore-Scott and Manley, 2005; Bain et al., 2016; Figueiredo et al., 2016). Nodal and Hh cooperate in patterning the mesoderm, but whether they act together or in parallel in thyroid development has not been addressed. In some models, initial Nodal asymmetry was proposed to be downstream of Shh. For example, in the chick, Shh inhibition in early development represses Nodal in lateral plate and paraxial mesoderm (Otto et al., 2014). In contrast, proNodal, but not mature Nodal, acts indirectly to maintain Shh expression in the prechordal mesoderm (Ellis et al., 2015). However, it has also been suggested that other factors may regulate asymmetry of both genes in the paraxial mesoderm (Tsikolia et al., 2012).

Interactions of Nodal and Hh in development of the neural tube have been well documented in vertebrates. Mutations or chemical inhibition of either *Nodal* or *Hh* genes results in holoprosencephaly (Monuki, 2007; Mercier et al., 2013). In patterning the ventral neural tube, Nodal is upstream of Shh, which contains a Nodal-responsive enhancer (Muller et al., 2000; Ito et al., 2001; Rohr et al., 2001; Lupo et al., 2006). In addition, Nodal and Shh both function in the development of the hypothalamus (Mathieu et al., 2002), and both Nodal and Hh signaling in the ventral diencephalon of the zebrafish are regulated by Zic1 (Maurus and Harris, 2009).

Nodal and Hh in other invertebrate deuterostomes

In invertebrate deuterostomes other than amphioxus, Nodal and Hh sometimes interact. In sea urchin embryos, Nodal signaling establishes left-right asymmetry, and Hh signaling helps to maintain Nodal expression once asymmetry is established (Materna et al., 2013; Warner et al., 2016). Whether Nodal and Hh interact in patterning developing hemichordates is unknown. In larvae of the indirectly developing hemichordate *Ptychodera flava*, Nodal inhibition disrupts mesoderm formation and dorsoventral fates (Rottinger et al., 2015). *Hh* is expressed in the anterior region and pharyngeal endoderm of *P. flava* larvae (Arimoto and Tagawa, 2015). In another indirectly developing hemichordate, *Balanoglossus simodensis*, *Hh* is expressed in the dorsal endoderm and the stomochord (an anterior projection from the gut) (Miyamoto and Wada, 2013). In the direct developer *Saccoglossus kowalevskii*, *Hh* is expressed in the anterior ectoderm and anterior gut (Lowe et al., 2006). Expression of these genes in the developing adult pharynx of hemichordates has not been reported.

The emerging picture is that a role for Nodal in patterning the left-right axis existed at the base of the Bilateria and has been

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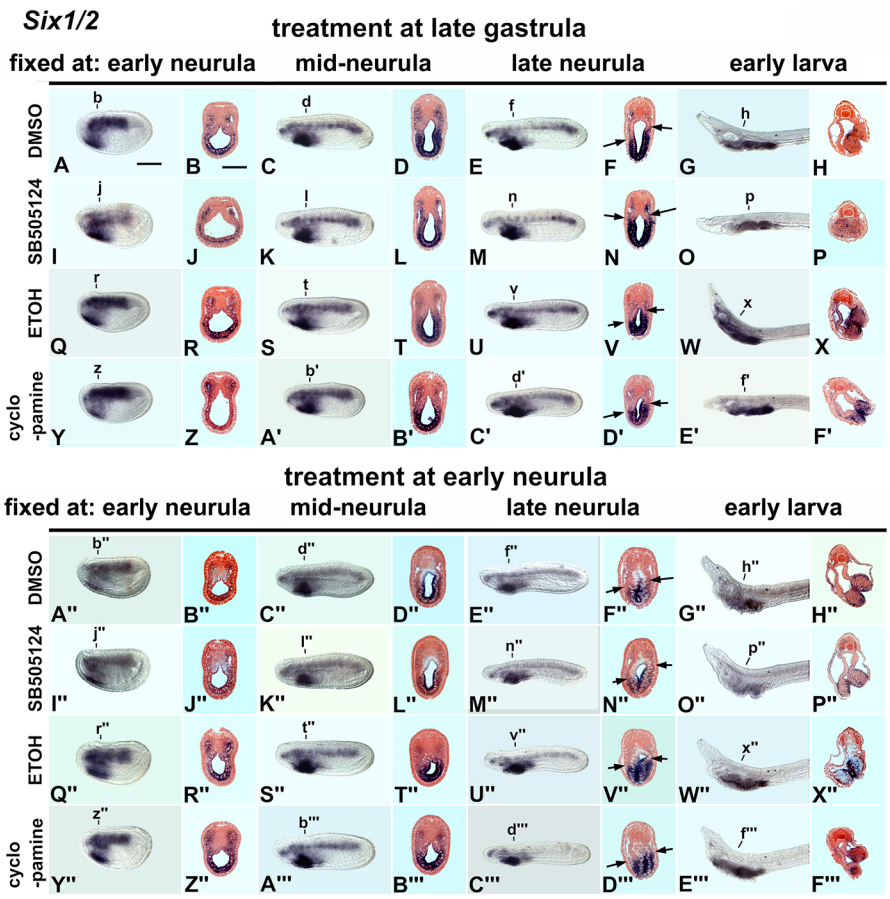

Fig. 8. Transient inhibition of Nodal, but not Hh, shifts expression of *Six1/2* in amphioxus embryos. Expression of *Six1/2* in control embryos treated with DMSO for SB505124 (A-H, A″-H″) and ethanol (ETOH) for cyclopamine (Q-X, Q″-X″) and in those treated for 20 min with the Nodal inhibitor SB505124 (15 µM) at the late gastrula (I-P) and very early neurula (I″-P″) stages or with the Hh inhibitor cyclopamine (1.75 µM) at the late gastrula (Y-F′) and early neurula (Y″-F‴) stages. Embryos were fixed for *in situ* hybridization at the stages indicated. Anterior to the left in wholemounts. Cross-sections (pink), viewed from the posterior, are at the levels indicated with the lowercase letters in preceding wholemounts (blue). Arrows indicate the borders of the ventral endodermal domain, which is skewed to the right in normal larvae, but shifted to the left in embryos treated at the early gastrula stage with the SB505124. Scale bars: 50 µm.

preserved in both spiralians and chordates (Grande and Patel, 2009; Martín-Durán et al., 2016). Subsequently, in deuterostomes, additional functions for these genes in patterning the dorsoventral and anterior-posterior axes arose. With multiple signaling pathways, including Hh and RA signaling working at the same time, and the same signaling pathways doing different things at different times, the

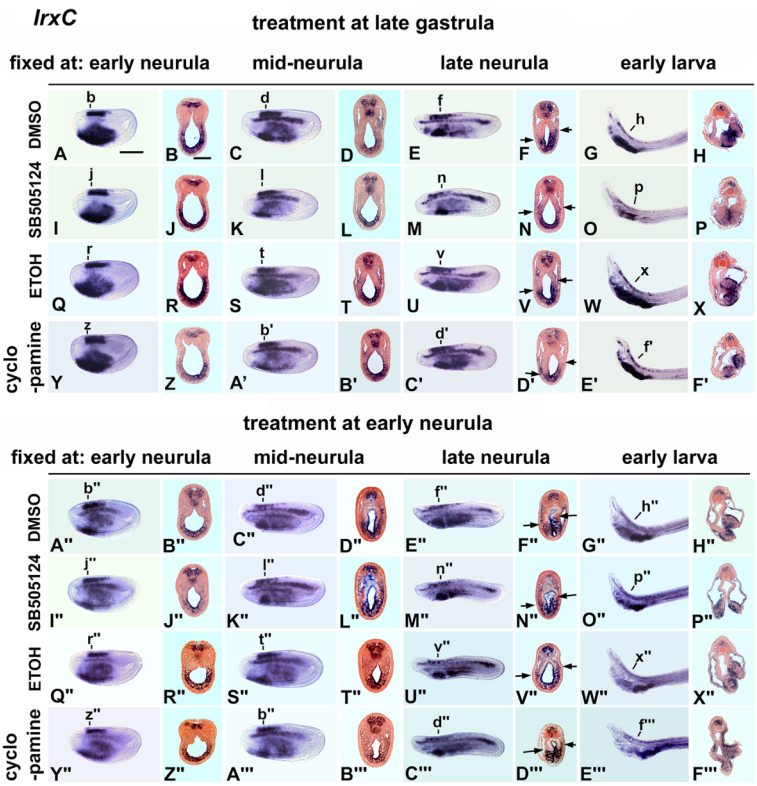

Fig. 9. Transient inhibition of Nodal, but not Hh, shifts expression of *IrxC* in amphioxus embryos. Expression of *IrxC* in control embryos treated with DMSO for SB505124 (A-H, A″-H″) and ethanol (ETOH) for cyclopamine (Q-X, Q″-X″) and in those treated for 20 min with the Nodal inhibitor SB505124 (15 µM) at the late gastrula (I-P) and early neurula (I″-P″) stages or with the Hh inhibitor cyclopamine (1.75 µM) at the late gastrula (Y-F′) and early neurula (Y″-F‴) stages. Embryos were fixed for *in situ* hybridization at the stages indicated. Anterior to the left in wholemounts. Cross-sections (pink), viewed from the posterior, are at the levels indicated with the lowercase letters in preceding wholemounts (blue). Arrows indicate boundaries of endodermal domain of IrxC, which is shifted to the left by treatment with SB505124 at the early gastrula stage but not at the early neurula stage or by treatment with cyclopamine. Scale bars: 50 µm.

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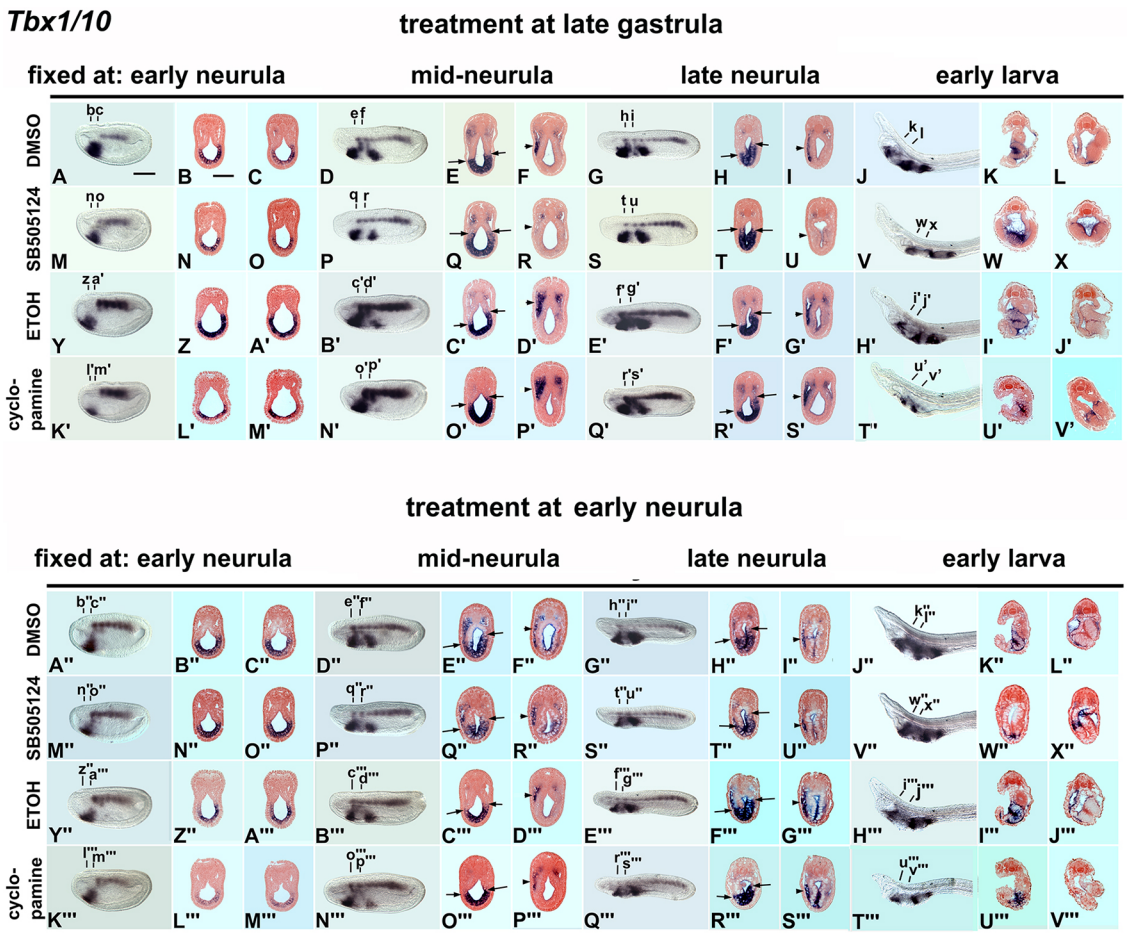

Fig. 10. Transient inhibition of Nodal, but not Hh, affects mesodermal expression of *Tbx1/10*. Expression of *Tbx1/10* in control embryos treated with DMSO for SB505124 (A-L, A″-L″) and ethanol (ETOH) for cyclopamine (Y-J′, Y″-J‴) and in those treated for 20 min with the Nodal inhibitor SB505124 (15 µM) at the late gastrula (M-X) and early neurula (M″-X″) stages or with the Hh inhibitor cyclopamine (1.75 µM) at the late gastrula (K′-V′) and early neurula (K‴-V‴) stages. Embryos were fixed for *in situ* hybridization at the stages indicated. Anterior to the left in wholemounts. Cross-sections (pink), viewed from the posterior, are at the levels indicated with the lowercase letters in preceding wholemounts (blue). Arrows indicate boundaries of endodermal domain of Tbx1/10, which is shifted to the left by treatment with SB505124 at the early gastrula stage but not at the early neurula stage or by treatment with cyclopamine. Arrowheads indicate the lateral mesoderm on the left. Scale bars: 50 µm.

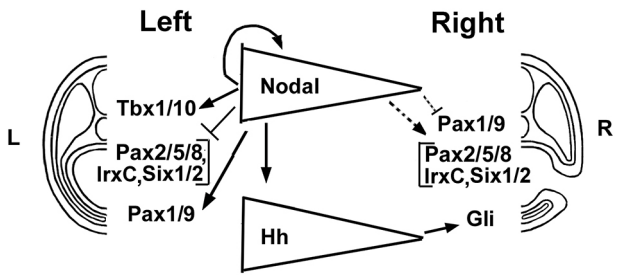

Fig. 11. Diagram of gene interactions at the neurula stage in development of the amphioxus gill slits. The interactions shown may be either indirect or direct. Nodal expression is restricted to the left in all three germ layers. Nodal auto-regulates. Secreted Nodal protein diffuses towards the right. A high level of Nodal activates Hh expression in endoderm on the left. Very low levels of Nodal protein on the right are required for expression of *Pax2/5/8*, *IrxC* and *Six1/2* in the gill slit primordia. Expression of these genes is inhibited on the left by high levels of Nodal protein. Hh protein secreted on the left diffuses toward the right. Hh in turn regulates the size of the *Gli* domain in the pharyngeal endoderm. *Tbx1/10* expression in mesoderm on the left requires Nodal signaling. Nodal signaling maintains expression of *Pax1/9* in the pharyngeal endoderm, except where gill slits will form.

developing picture of the genetic mechanisms that pattern the pharynx and establish the gill slits is increasingly complex. However, in the absence of whole-genome duplications, amphioxus is optimally positioned for understanding the fundamentals of pharyngeal patterning in chordates. Comparisons between amphioxus and vertebrates have shown that the ancestral chordate had a pharynx with gill slits and an endostyle that was patterned by signaling pathways including Nodal, Hh, RA and Wnt. Importantly, the roles of these pathways in embryogenesis change with time. First, they act simultaneously to specify the anterior-posterior and dorsoventral axes, then during the gastrula stage they divide the embryo into regions along these axes, and finally, during the neurula stage, they specify discrete tissues and organs. An important step towards understanding how the amphioxus genome generates a phenotype would be to further dissect the gene networks downstream of these pathways.

MATERIALS AND METHODS

Embryos and pharmacological treatments

Breeding cultures of the Florida amphioxus (*Branchiostoma floridae*) are maintained in L.Z.H.'s laboratory. Larvae and adults are fed on brown

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phytoplankton (*Isochrysis*, *Pavlova* and *Tisochrysis*) originally obtained from NCMA at the Bigelow Laboratory for Ocean Sciences (East Boothbay, ME, USA). Animals are raised at 25°C. Adults are kept on a 10 h dark/14 h light cycle. Ripe animals are then maintained at 17°C for at least 2 weeks. When returned to 25°C, 20-80% of the animals usually spawned 24-28 h later when the light was turned off. Nodal was inhibited with SB505124 (Medchem Express). A stock solution of 50 mM was made in DMSO and used at final concentrations of 10-50 µM. Hh signaling was inhibited with cyclopamine (LC Laboratories). A stock solution of 50 mM was made in 100% ethanol (ETOH) and added to embryos at concentrations of 1.5-4.0 µM. Embryos were treated for 20 min with each inhibitor at the late gastrula (9 hpf) and early neurula stages (10 hpf). Controls were treated with DMSO or ETOH alone. To confirm that cyclopamine inhibits Hh signaling in amphioxus, we treated with 1.75 µM cyclopamine at the very early gastrula stage and fixed for *in situ* hybridization at the late gastrula/early neurula, early to mid-neurula and early larval stages. The experiment was repeated three times. Aliquots of embryos were fixed with 4% paraformaldehyde, 0.1 M MOPS, 1 mM EGTA, 2 mM $MgSO_4$ and 0.5 M NaCl, and stored in 70% ETOH at −20°C as previously described (Holland et al., 1996). Pilot experiments to determine the optimal concentrations of SB505124 and cyclopamine, and the times of treatment, were carried out in duplicate. Experiments for determining the effects of these inhibitors on gene expression were carried out in triplicate. For each replicate, control and experimental samples were treated in parallel on embryos from the same egg batch.

In situ hybridization

Methods for *in situ* hybridization were as previously described (Yu and Holland, 2009) with the following modification: 50 µM EDTA was added to the PBST solution. Antisense riboprobes of *AmphiNodal* (AY083838), *AmphiHedgehog* (Y13858), *AmphiPax1/9* (U20167), *AmphiIrxC* (EU754750), *AmphiGli* (CAB96572), *AmphiPax2/5/8* (AF053762), *AmphiTbx* (AF262562) and *AmphiSix1/2* (EF195742) were synthesized. Genbank accession numbers are in brackets. Templates for riboprobe synthesis of *AmphiNodal*, *AmphiIrxCi*, and *AmphiPtch* were amplified from EST clones (bfga04g04, CAXF10761 and GA035K03, respectively) in the pDONR222 vector. Vector-specific primers contained sites for T7 and Sp6 RNA polymerase (pDONR222-T7-reverse: 5′-TAATACGACTCACTAT-AGGGAGGGGATATCAGCTGGATG-3′; pDONR222-Sp6-forward: 5′-ATTTAGGTGACACTATAGAAGACGGCAGTCTTAAGCTC-3′). Controls and experimental samples were hybridized in parallel with the same riboprobes at the same concentrations, and color development was for the same period of time. For each *in situ* hybridization, 10 embryos of each stage were used. After *in situ* hybridization, the embryos were photographed in wholemount, and were stained with Ponceau-S, embedded in Spurr's resin and 3 µm sections cut with a glass knife as previously described (Holland et al., 1996). For each sample, three embryos of each stage were sectioned.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.Z.H.; Formal analysis: L.Z.H.; Investigation: H.O., D.K., L.Z.H.; Resources: L.Z.H.; Writing - original draft: L.Z.H.; Writing - review & editing: D.K.; Supervision: L.Z.H.; Project administration: L.Z.H.; Funding acquisition: L.Z.H.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.162586.supplemental

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