Retinoic acid signaling acts via Hox1 to establish the posterior limit of the pharynx in the chordate amphioxus

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Summary

In the invertebrate chordate amphioxus, as in vertebrates, retinoic acid (RA) specifies position along the anterior/posterior axis with elevated RA signaling in the middle third of the endoderm setting the posterior limit of the pharynx. Here we show that *AmphiHox1* is also expressed in the middle third of the developing amphioxus endoderm and is activated by RA signaling. Knockdown of *AmphiHox1* function with an antisense morpholino oligonucleotide shows that *AmphiHox1* mediates the role of RA signaling in setting the posterior limit of the pharynx by repressing expression of pharyngeal markers in the posterior foregut/midgut endoderm. The spatiotemporal expression of these endodermal genes in embryos treated with RA or the RA antagonist BMS009 indicates that *Pax1/9*, *Pitx* and *Notch* are probably more upstream than *Otx* and *Nodal* in the hierarchy of genes repressed by RA signaling. This work highlights the potential of amphioxus, a genomically simple, vertebrate-like invertebrate chordate, as a paradigm for understanding gene hierarchies similar to the more complex ones of vertebrates.

Key words: Anterior/posterior patterning, Endoderm, RA signaling pathway, Gene cascade, Evolution, Lancelet, *Branchiostoma floridae*

Introduction

In developing vertebrates and their sister group, the cephalochordates (amphioxus), the vitamin-A derivative retinoic acid (RA) specifies regional identities along the anterior/posterior axis (Dupé et al., 1999; Dupé and Lumsden, 2001; Escriva et al., 2002; Holland and Holland, 1996; Matt et al., 2003; Schubert et al., 2004). Although RA patterns all three tissue layers, most studies have focused on its role in patterning the neuroectoderm and mesoderm (Allan et al., 2001; Blumberg et al., 1997; Dupé and Lumsden, 2001; Gavalas and Krumlauf, 2000; Grandel et al., 2002). The role of RA in endodermal morphogenesis was long ignored because it was believed that the effect of exogenous RA on the vertebrate pharynx was due solely to mispatterning of neural crest migrating into the branchial arches (Alexandre et al., 1996). However, it is now known that the influence of neural crest-derived mesenchyme is subordinate to patterning within the pharyngeal endoderm itself (Couly et al., 2002; Graham, 2003; Le Douarin, 1982; Mark et al., 2004). Ablation of neural crest in the chick does not inhibit formation of the pharyngeal arches and pouches (Veitch et al., 1999). Conversely, in the zebrafish, function of *tbx1* in the pharyngeal endoderm is required for normal development of neural crest-derived pharyngeal structures (Piotrowski et al., 2003; Piotrowski and Nüsslein-Volhard, 2000). Moreover, studies in amphioxus, which lacks definitive neural crest, leave no doubt that pharyngeal patterning is mediated primarily by the endoderm (Escriva et al., 2002; Holland and Holland, 1996).

Throughout the chordates, RA signaling specifies anterior/posterior position of pharyngeal structures such as the gill slits (aquatic chordates) or pharyngeal arches and pouches (non-aquatic chordates). Excess RA prevents formation of the gill slits (branchial basket) in tunicates (Hinman and Degnan, 2000). In amphioxus the pharynx is absent; the mouth (thought to be a modified gill slit) and gill slits do not form and the pharyngeal endoderm remains thin (Escriva et al., 2002; Holland and Holland, 1996). Conversely, in embryos treated with a RA antagonist, the pharynx with its thickened endoderm is expanded posteriorly (Escriva et al., 2002). Similarly, in vertebrates, excess RA prevents pharyngeal development in lampreys and causes fusion of the first two branchial arches in gnathostomes (Kuratani et al., 1998; Lee et al., 1995; Mulder et al., 1998), while decreased RA signaling has the opposite effect, expanding pharyngeal structures posteriorly. Consequently, in vertebrates with reduced RA signaling, the first pharyngeal pouch and the first two pharyngeal arches are normal, the second pouch is expanded posteriorly and the remaining pouches do not form (Niederreither et al., 2003; Quinlan et al., 2002; Wendling et al., 2000). Similarly, in amphioxus, the mouth is enlarged and the gill slit primordia are either elongated or absent altogether; presumably a low

level of RA signaling is essential for gill slit penetration (Escriva et al., 2002).

RA signaling in chordates is directly mediated by the RA receptors (RARs), that heterodimerize with the retinoid X receptors (RXRs) (Laudet and Gronemeyer, 2001) In general, vertebrates have three RARs and three RXRs, whereas amphioxus and tunicates have one each (Bertrand et al., 2004; Nagatomo et al., 2003). In chordates other than tunicates (Ishibashi et al., 2003), RARs are autoregulated, and their expression generally reflects the level of RA signaling. In vertebrates, RAR gene expression is generally high in the foregut endoderm (Matt et al., 2003; Smith, 1994), while in amphioxus, *AmphiRAR* is most intensely expressed in the middle third of the endoderm, just posterior to the mouth and the first three gill slits (Escriva et al., 2002).

The effects of altered RA signaling on endodermal expression of RARs are similar in amphioxus and vertebrates. For example, in the mouse, treatment with an RA agonist induces ectopic expression of *RARβ* in the first two pharyngeal pouches (Matt et al., 2003). Correspondingly, in amphioxus, excess RA expands expression of *AmphiRAR* into the anteriormost pharyngeal endoderm, while treatment with an RA antagonist downregulates *RAR* (Escriva et al., 2002; Wendling et al., 2000). However, little is known of the molecular mechanisms downstream of RAR/RXR that underlie pharyngeal patterning. In addition to RARs, only a few genes are known that exhibit altered expression in the pharyngeal endoderm in response to altered levels of RA signaling. In vertebrates, these include *Hoxa1* and *Hoxb1*, expressed in the caudal pharynx, *Pax1* and *Pax9* expressed in pharyngeal pouches 1-3 and 1-4, respectively, and *Fgf3* and *Fgf8* expressed in the endoderm of the pharyngeal arches and caudal-lateral pharynx respectively (Neubüser et al., 1995; Wallin et al., 1996; Wendling et al., 2000). Expression of the single *Pax1/9* gene in amphioxus is also affected by increased RA (Holland and Holland, 1996), while in tunicates, expression of *Otx* in the pharynx is decreased by RA treatment (Hinman and Degnan, 2000).

Hox1 genes in both vertebrates and amphioxus are direct targets of RA signaling (Arcioni et al., 1992; Balmer and Blomhoff, 2002; Manzanares et al., 2000; Ogura and Evans, 1995). Expression of *Hoxa1* and *Hoxb1* in the pharyngeal endoderm of vertebrates is expanded by treatment with RA or an RA agonist, while treatment with an RA antagonist or mutation of the RA response element (RARE) markedly decreases *Hoxa1* expression and eliminates that of *Hoxb1* (Alexandre et al., 1996; Li and Lufkin, 2000). Ectopic expression of *Hoxa1* results in a similar phenotype to that found with application of RA. However, since loss of *Hoxa1* and *Hoxb1* affects hindbrain patterning and migration of neural crest into the pharyngeal arches (Gavalas et al., 1998; McClintock et al., 2002; Pasqualetti et al., 2001; Rossel and Capecchi, 1999), some authors reasoned that the effects of altered expression of these genes on pharyngeal patterning are probably due primarily to abnormal neural crest (Gavalas et al., 1998; Rossel and Capecchi, 1999). Others, however, have emphasized a more direct role of *Hoxa1* and *Hoxb1* in mediating RA signaling in the pharyngeal endoderm (Matt et al., 2003; Wendling et al., 2000).

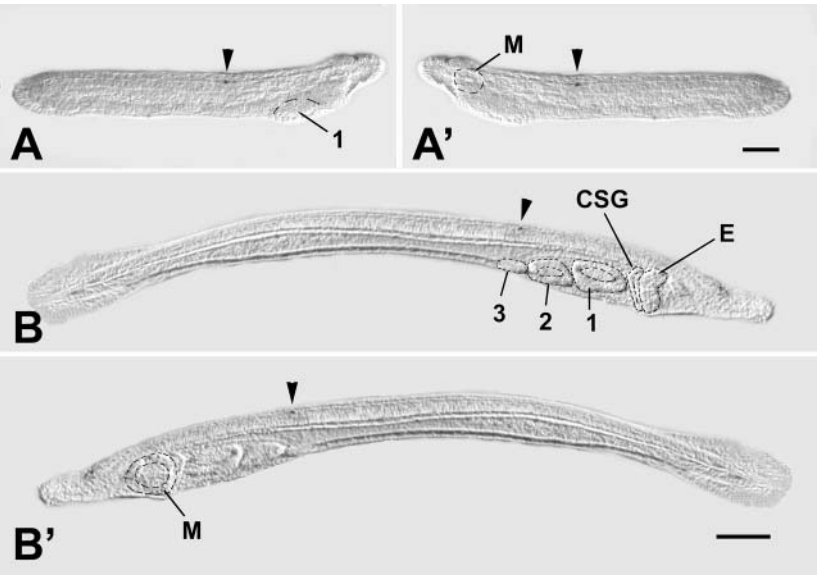

Fig. 1. Right (A) and left (A′) side views of 24-hour larvae of amphioxus (*Branchiostoma floridae*) showing the mouth (M) on the left and the primordium of the first gill slit (1) on the ventral right side. The first pigment spot (arrowhead) in the nerve cord is at the level of the primordium of the future third gill slit. Right (B) and left (B′) views of a 3-day larva showing gill slits 1 and 2 opening on the right (gill slit 3 has not yet opened) and the mouth (M) opening on the left. Just anterior to the first gill slit is the club-shaped gland (CSG) and the endostyle (E). Scale bars: A, A′=50 μm; B, B′=100 μm.

Amphioxus is particularly useful for deciphering the molecular mechanism whereby RA signaling in the endoderm regulates anterior/posterior patterning of the pharynx, because it lacks neural crest and has single genes for *RAR*, *Hox1* and most other endodermal markers. Moreover, at the neurula stage, the pharynx of the small, transparent embryos consists of only two cell layers – an inner endoderm and an outer ectoderm. The pharynx is asymmetrical with the first three gill slits forming ventrally on the right in an anterior/posterior series and the mouth, thought to be a modified gill slit, on the left (Fig. 1). Metamorphosis, resulting in a bilaterally symmetrical adult, occurs at 9-11 gill slits.

Previously, we showed that a high level of RA signaling establishes the posterior limit of the amphioxus pharynx, a relatively low level being required for gill slit formation (Escriva et al., 2002). In the present work, we investigate the mechanism whereby a high level of RA signaling in the middle third of the endoderm sets the posterior limit of the amphioxus pharynx. We first characterize the effects of both increased and decreased RA signaling on the spatio-temporal expression of 11 genes in the pharyngeal endoderm. Since these results reveal a new domain of *AmphiHox1* in the endoderm that is congruent with that of *AmphiRAR*, we then tested whether AmphiHox1 mediates the effects of RA in establishing the posterior limit of the pharynx by knocking down its function

with an antisense morpholino-oligonucleotide. Our results suggest a hierarchy of pharyngeal markers with anterior/posterior limits regulated by RA signaling and show that *AmphiHox1* mediates the role of RA in establishing the posterior extent of the pharynx, but not the role of RA in gill slit penetration. We thus propose that a *RAR-Hox1* gene hierarchy regulates endoderm fate by promoting posterior foregut/midgut formation.

Materials and methods

Embryo culture and treatments with RA and BMS009 (a RA antagonist)

Adult amphioxus (*Branchiostoma floridae*) were collected in Old Tampa Bay, Florida, during summer and induced to spawn by electric stimulation (Holland and Yu, 2004). After fertilization, the embryos were raised in filtered seawater at 25°C. At the late blastula stage, RA (dissolved in DMSO), the RA antagonist BMS009 (dissolved in DMSO) or DMSO alone was added to the embryonic cultures to a final concentration of 1×10^{-6} M (Escriva et al., 2002; Holland and Holland, 1996). After hatching at the early neurula stage, embryos were transferred to untreated seawater. All the DMSO-treated control embryos developed normally. Embryos were fixed for in situ hybridization at various developmental stages (Holland et al., 1996).

In situ hybridization, light microscopy and photography

In situ hybridizations were performed as previously described (Holland et al., 1996). Clones used as templates for riboprobes were as follows: *AmphiPax1/9* (U20167) (Holland et al., 1995); *AmphiNotch* (Y12539) (Holland et al., 2001); *AmphiWnt3* (AF361013) (Schubert et al., 2001); *AmphiNodal* (AY083838) (Yu et al., 2002); *AmphiHox1* (AB028206) and *AmphiOtx* (AF043740) (both provided by J. Garcia-Fernàndez and P. W. H. Holland); *AmphiIslet* (AF226616) (provided by W. R. Jackman); *AmphiFoxA2* (*HNF3β*) (Y09236), *AmphiPitx* (AJ438768) and amphioxus *hedgehog* (*AmphiHh*) (Y13858) (all three provided by Sebastian M. Shimeld). After in situ hybridization, the embryos were first photographed as whole mounts and subsequently counterstained in Ponceau S, embedded in Spurr's resin and prepared as sections for light microscopy (Holland et al., 1996).

Microinjection of antisense morpholino-oligonucleotides

Microinjection of amphioxus eggs was as described by (Holland and Yu, 2004). Unfertilized eggs were injected with either the control morpholino (5′-CCTCTTACCTCAGTTACAATTTATA-3′) or one specific for *AmphiHox1* from *B. floridae* (5′-ATTCTTGCCGTGTC-CATTTGCTCCA-3′) (Gene Tools, Philomath, OR, USA). Approximately 2 pl of a solution containing 15% glycerol, 2 mg/ml Texas Red dextran (Molecular Probes, Eugene, OR, USA) and 500 μM morpholino was injected. The morpholinos were heated to 65°C for 5 min prior to use. After injection, the eggs were fertilized and fixed at either the late neurula stage (24-30 hours) or the early larval stage (36-40 hours). Fixed embryos showing clear fluorescence of the Texas Red dextran were analyzed by in situ hybridization (Holland et al., 1996).

In vitro translation assay

For in vitro translation, the *AmphiHox1* coding region cDNA was amplified by PCR and cloned into the pCS2+ vector (Rupp et al., 1994; Turner and Weintraub, 1994). In vitro translation was with the TnT Quick Coupled Transcription/Translation System. 200 ng of plasmid DNA containing the *AmphiHox1* coding region was assayed together with different amounts of control or *AmphiHox1*-specific morpholino (100 ng, 500 ng, 1000 ng or 5000 ng). After the reactions, the samples were subjected to electrophoresis on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane. AmphiHox1 protein was detected by the Transcend Non-Radioactive Translation Detection Systems (Promega, Madison, WI, USA).

Results

To investigate how RA establishes the posterior limit of the amphioxus pharynx, we first determined the expression of 11 endodermal markers in amphioxus embryos treated with RA or the RA antagonist BMS009 at final concentrations of 1×10^{-6} M. The effects of altered RA signaling on the spatio-temporal expression of these genes revealed which genes are downstream of RAR/RXR and their approximate hierarchy. These markers included *AmphiRAR* (Escriva et al., 2002), *AmphiHox1* (Holland and Holland, 1996), *AmphiWnt3* (Schubert et al., 2001), *AmphiPax1/9* (Holland et al., 1995), *AmphiPitx* (Boorman and Shimeld, 2002b), *AmphiNotch* (Holland et al., 2001), *AmphiNodal* (Yu et al., 2002), *AmphiOtx* (Williams and Holland, 1996), *AmphiIslet* (Jackman et al., 2000), *AmphiFoxA2* (*HNF3β*) (Shimeld, 1997) and *AmphiHh* (Shimeld, 1999). In normal embryos, three of these (*AmphiRAR*, *AmphiHox1* and *AmphiWnt3*) are expressed in the middle third of the endoderm at the early neurula stage, three (*AmphiPax1/9*, *AmphiPitx* and *AmphiNotch*) have expression limited to the pharyngeal endoderm from an early stage, and five (*AmphiNodal*, *AmphiOtx*, *AmphiIslet*, *AmphiFoxA2* and *AmphiHh*) are initially expressed throughout the length of the endoderm (Figs 2-7). The limits of expression are measured from the anterior end of the embryo and given as a percentage of the total body length.

AmphiHox1 is a probable direct target of RA signaling

Although expression of *AmphiHox1* in ventrolateral regions of amphioxus embryos was thought to be entirely ectodermal (Wada et al., 1999), frontal sections show that there is also a corresponding endodermal domain (Fig. 2C,G,K). Endodermal expression of *AmphiHox1* generally parallels that of *AmphiRAR* (see Fig. S1 in the supplementary material) (Escriva et al., 2002), although, especially at early stages, the anterior limit of expression of *AmphiHox1* (Fig. 2A) is somewhat posterior to that of *AmphiRAR* (Escriva et al., 2002). By 20 hours, expression domains of *AmphiHox1* and *AmphiRAR* in the endoderm are approximately congruent and remain similar during later development (Fig. 2B,D) (Escriva et al., 2002). For example, at 20-24 hours of development, the anterior limits of *AmphiRAR* and *AmphiHox1* in the endoderm are 38% and 40% respectively. Treatment with RA shifts expression of *AmphiHox1* anteriorly in the endoderm (Fig. 2E-H). Correspondingly, treatment with BMS009 downregulates expression of *AmphiHox1* and shifts expression posteriorly by an additional 10% of the length of the embryo (Fig. 2I-L). Overlapping expression of *AmphiHox1* and *AmphiRAR* plus the presence of a RARE in the regulatory region of *AmphiHox1* (Manzanares et al., 2000), to which AmphiRAR/AmphiRXR binds in vitro (H. Escriva, H. Wada and V. Laudet, unpublished), suggest that *AmphiHox1* is a direct target of RA signaling in the endoderm as well as in nerve cord and mesoderm.

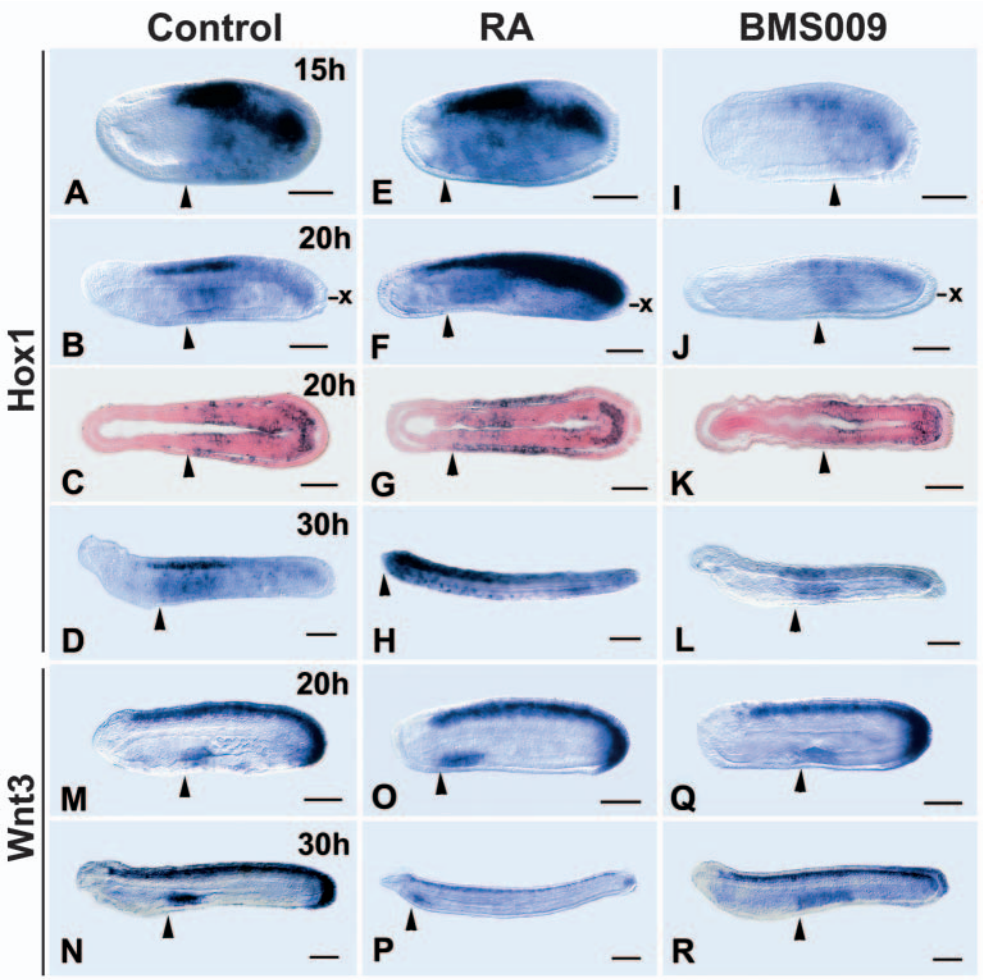

Fig. 2. Expression of *AmphiHox1* (A-L) and *AmphiWnt3* (M-R) in amphioxus embryos treated as follows: DMSO control (A-D,M,N), 1×10^{-6} M RA (E-H,O,P) and 1×10^{-6} M BMS009 (a RA antagonist) (I-L,Q,R). Anterior to left. Whole mounts viewed from the left side and frontal sections in C, G, K viewed from dorsal side. The 'x' in B,F,J shows the level of the section in C,G,K, respectively. Arrowheads indicate the anterior limit of expression in the endoderm. Scale bars=50 μm. (A) In 15-hour control neurulae, *AmphiHox1* is expressed in the posterior half of the endoderm and overlying ectoderm with the highest expression localizing to the middle third. (B-D) At 20 hours (B,C) and 30 hours (D) of development, the anterior limit of endodermal and ectodermal expression is just posterior to the pharynx. (E-H) RA upregulates expression of *AmphiHox1* in endoderm and ectoderm and expands it anteriorly. By 30 hours (H) expression in the endoderm is restricted to the extreme anterior end of the larva. (I-L) Treatments with the RA antagonist BMS009 downregulate *AmphiHox1* and shift expression posteriorly in both the ectoderm and endoderm. (M,N) In mid-neurulae and early larvae of amphioxus, *AmphiWnt3* is expressed in the ventral endoderm just posterior to the pharynx. (O,P) Treatment with RA initially shifts endodermal expression of *AmphiWnt3* anteriorly and subsequently leads to a downregulation of expression by 30 hours of development (P). (Q,R) In BMS009-treated embryos, *AmphiWnt3* expression is expanded posteriorly.

Early response genes: *AmphiWnt3*, *AmphiPax1/9*, *AmphiPitx* and *AmphiNotch* respond to RA signaling by the mid-neurula stage

At the mid-neurula stage, *AmphiWnt3* transcription begins in the ventral endoderm just posterior to the pharynx (Fig. 2M). The onset of expression is later than that of either *AmphiRAR* or *AmphiHox1* and appears to be earlier in RA-treated embryos and a little later in BMS009 embryos than in controls (Fig. 2M,O,Q). The anterior limit of *AmphiWnt3* expression (38% at 20 hours) is similar to that of *AmphiHox1* (40% at 20 hours) and is also shifted anteriorly by RA (to 20% at 20 hours) and slightly posteriorly by BMS009 (to 43% at 20 hours). In addition, BMS009 broadens the domain posteriorly (Fig. 2O-R). However, by 30 hours of development, *AmphiWnt3* is almost completely downregulated in the endoderm of RA-treated larvae (Fig. 2P), although whether it is a direct target of RA signaling remains to be determined.

AmphiPax1/9 and *AmphiPitx* are expressed in the pharyngeal endoderm with posterior limits at the mid-neurula stage (51% and 54% respectively) overlapping the anterior limits of *AmphiHox1* and *AmphiRAR* (compare Fig. 2A-D with Fig. 3A,B,G,H; Fig. 7) (Holland et al., 1995; Yasui et al., 2000). Expression of both genes is reduced where the first gill slit will form (Fig. 3A,B,G,H). Expression of *AmphiPax1/9* does not change throughout the neurula and early larval stages (Fig. 3B). After 36 hours, expression of *AmphiPitx* becomes limited to the mouth and to Hatschek's anterior left diverticulum, the precursor of Hatschek's pit, the homolog of the adenohypophysis (Fig. 3I, double arrowhead). At the mid-neurula stage, *AmphiNotch* is broadly expressed in the pharyngeal endoderm with a posterior limit (35%) somewhat rostral to those of *AmphiPax1/9* and *AmphiPitx* (Fig. 3P). Expression of *AmphiNotch* is reduced where the first gill slit will form (Fig. 3P,Q) (Holland et al., 2001). By the early larval stage (30 hours), the posterior limits of *AmphiNotch*, *AmphiPax1/9* and *AmphiPitx* are approximately the same (44-49%; compare Fig. 3Q with Fig. 3B,H).

Treatment with RA at the gastrula stage shifts the posterior limit of all three genes anteriorly in the endoderm. This shift is not marked at 18-20 hours; the posterior limits shift only from 54% in controls to 50% with RA for *AmphiPax1/9*, 51% to 40% for *AmphiPitx* and 35% to 30% for *AmphiNotch*. However, by 30 hours, the difference is obvious with the posterior limits of *AmphiPax1/9* and *AmphiNotch* shifting to

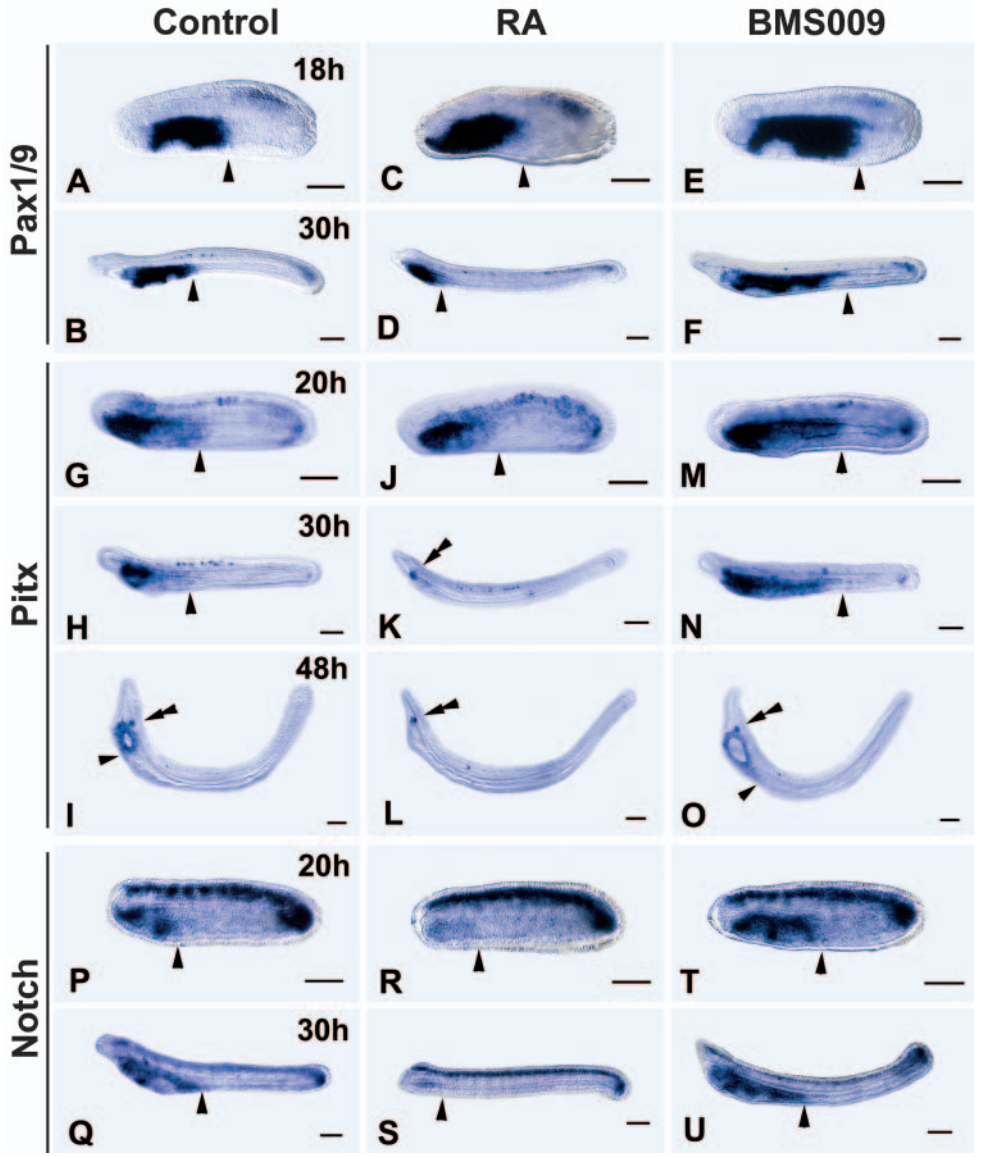

Fig. 3. The posterior limit of pharyngeal expression of *AmphiPax1/9* (A-F), *AmphiPitx* (G-O) and *AmphiNotch* (P-U) is shifted anteriorly by RA (C,D,J-L,R,S) and posteriorly by BMS009 (E,F,M-O,T,U) compared to expression in control animals treated with DMSO (A,B, G-I,P,Q). Anterior to left. Arrowheads indicate the posterior limit of endodermal expression. Double arrowheads in I,K,L,O point to Hatschek's anterior left diverticulum, the precursor of Hatschek's pit, which is the amphioxus homolog of the vertebrate adenohypophysis. Scale bars=50 μm. Mid-neurula (18 hours)=A,C,E. Late neurula (20 hours)=G,J,M,P,R,T. Early larva (30 hours)= B,D,F,H,K,N,Q,S,U. Mid-larva (48 hours)=I,L,O.

17% in RA-treated larvae and that of *AmphiPitx* to 0%. RA-treatment also eliminates the zones of reduced expression where the gill slits would normally have formed (Fig. 3C,D,J,R,S). BMS009 has the opposite effect – the pharyngeal expression domains are expanded posteriorly (to about 71% for *AmphiPax1/9*, 60% for *AmphiPitx* and 53% for *AmphiNotch* at 20 hours; Fig. 3E,F,M-O,T,U). Expression of *AmphiPitx* in Hatschek's anterior left diverticulum is not affected by changes in RA signaling (Fig. 3I,L,O). Although the level of expression of *AmphiPax1/9* is not obviously affected by levels of RA signaling (Fig. 3C-F), *AmphiPitx* and *AmphiNotch* appear to be downregulated by RA and upregulated by BMS009 (Fig. 3J-O,R-U).

The relatively early response of these three genes to altered levels of RA signaling that together with *AmphiWnt3*, they are comparatively high up in the hierarchy of the RA signaling pathway. Their expression patterns and response to altered levels of RA signaling indicate that that high levels of RA signaling suppress *AmphiPax1/9*, *AmphiPitx* and *AmphiNotch* expression in the middle third of the endoderm. Moreover, the posterior limits of *AmphiPax1/9* and *AmphiPitx* are just posterior to the anterior limits of *AmphiRAR* and *AmphiHox1*, suggesting that *AmphiRAR* acting via *AmphiHox1* (see below) may set the posterior limit of expression of these genes as well as the anterior/posterior extent of the endodermal domain of *AmphiWnt3*.

Late response genes: the posterior limit of *AmphiNodal* and *AmphiOtx* is not affected by altered RA signaling until the late neurula/early larval stage

During amphioxus development, *AmphiNodal* and *AmphiOtx* are normally expressed throughout all or most of the length of the endoderm at the early to mid-neurula stage (Fig. 4A-C,M). For both genes, expression is reduced ventrally in the primordia of the first two gill slits (Fig. 4A,C,M). By the late neurula (24 hours), expression of *AmphiNodal* becomes largely restricted to the anterior endoderm (posterior limit at 42%), although there is still weak expression in the midgut and hindgut (Fig. 4C). Expression of *AmphiOtx* similarly becomes restricted to the pharyngeal endoderm, but later than

that of *AmphiNodal*. By the early larval stage (30 hours), *AmphiOtx* remains expressed in the pharyngeal endoderm (Fig. 4N), but *AmphiNodal* is largely downregulated (Fig. 4D) (Williams and Holland, 1996; Williams and Holland, 1998; Yu et al., 2002). RA treatment at the gastrula stage has little effect on endodermal expression of either gene at the mid-neurula stage (Fig. 4E,O). However, by 24 hours, RA treatment restricts the endodermal expression of *AmphiNodal* to the anterior pharynx (posterior limit at 37%; Fig. 4G). Expression is downregulated somewhat sooner in RA-treated embryos than in controls (Fig. 4D,H). The effect of RA on the posterior limit of *AmphiOtx* is not apparent until the early larva. At 30 hours, the pharyngeal expression domain of *AmphiOtx* is reduced and the posterior limit is shifted anteriorly compared to controls (posterior limit at 26%; Fig. 4N,P). BMS009 has the opposite effect. The posterior limits of the strong pharyngeal expression domains of both *AmphiNodal* and *AmphiOtx* are shifted posteriorly at the late neurula and early larval stages respectively (Fig. 4I,K,L,Q,R), and downregulation of *AmphiNodal* in the endoderm is delayed (Fig. 4L). Since, unlike *AmphiPax1/9*, the anterior/posterior extent of expression of both genes in the endoderm is not regionalized at the mid-neurula stage and is affected rather late by RA and BMS009 treatments, it is likely that they are farther downstream than *AmphiPax1/9* in the hierarchy of RA signaling.

AmphiNodal and *AmphiPitx* together with *AmphiHh* are the only known amphioxus genes with pharyngeal expression limited to the left side of the endoderm (Fig. 4B) (Shimeld, 1999; Yasui et al., 2000; Yu et al., 2002). However, neither RA nor BMS009 induces expression of *AmphiNodal* (Fig. 4F,J), *AmphiPitx* or *AmphiHh* (data not shown) on the right side of the endoderm in amphioxus. We conclude that RA signaling does not control left/right asymmetry of the amphioxus pharynx.

The posterior limits of endodermal expression of *AmphiIslet*, *AmphiFoxA2 (HNF3β)* and *AmphiHh* are not substantially changed by levels of RA signaling

The endodermal expression domain of amphioxus *AmphiIslet* at the mid-neurula stage is similar to that of *AmphiOtx* but does not extend as far posteriorly (74% versus 91%; Fig. 5A)

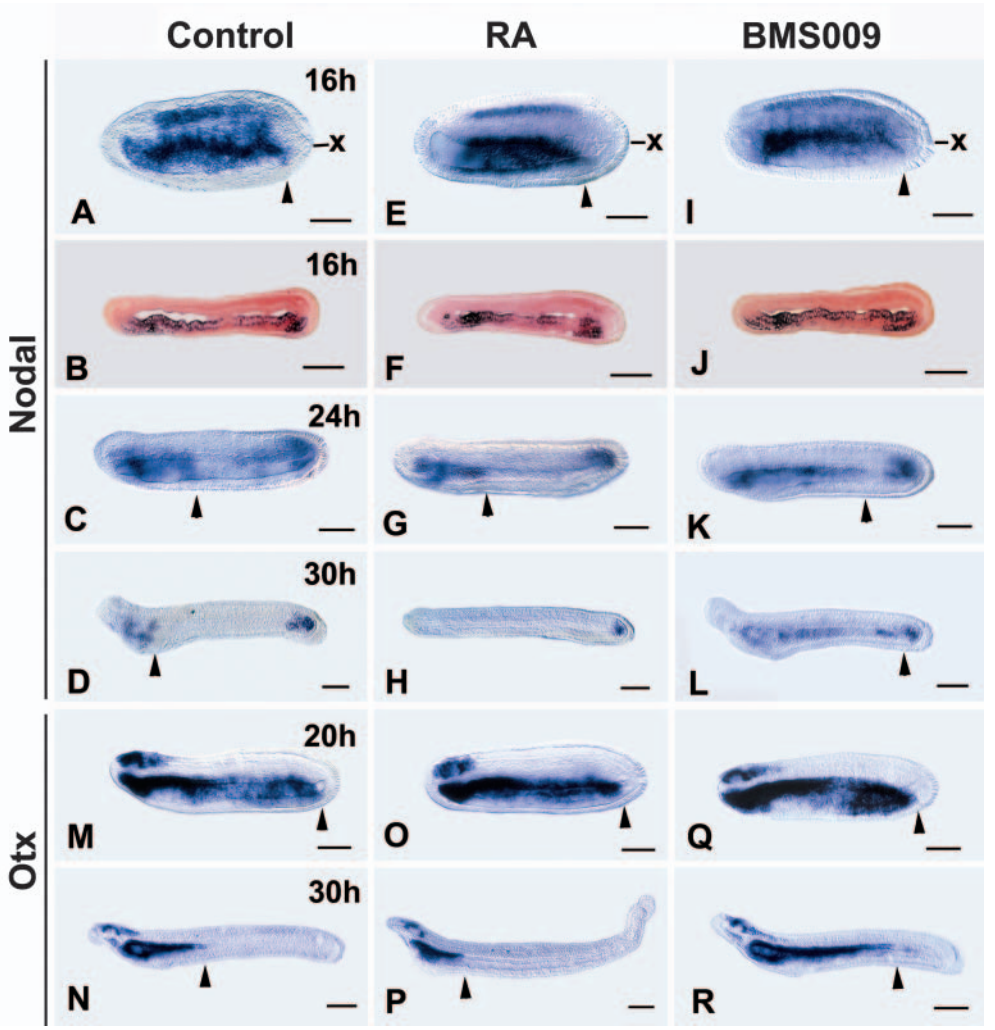

Fig. 4. The posterior limit of expression of *AmphiNodal* (A-L) and *AmphiOtx* (M-R) in the endoderm is not affected by altered levels of RA signaling until the late neurula/early larval stage. Anterior to left. Whole mounts viewed from the left side and frontal sections in B,F,J viewed from dorsal side. The 'x' in A,E,I shows the level of the section in B,F,J, respectively. Arrowheads indicate the posterior limit of expression in the endoderm. Scale bars=50 μm. (A) At mid-neurula (16 hours), *AmphiNodal* is expressed throughout the length of the endoderm. The posterior limit of expression in the endoderm at this stage is not affected by either RA (E) or by BMS009 (I). The frontal sections show that expression of *AmphiNodal* is restricted to the left side of the pharynx in controls (B), RA-treated (F), and BMS009-treated (J) embryos. By the late (24-hour) neurula, expression of *AmphiNodal* is restricted to the tail bud and pharyngeal endoderm in DMSO-treated controls (C), and is shifted slightly anteriorly by RA (G) and posteriorly by BMS009 (K). In early (30-hour) larvae, pharyngeal expression of *AmphiNodal* is reduced in the pharynx of controls (D), eliminated in RA-treated larvae (H) and expanded posteriorly in animals treated with BMS009 (L). (M) At the late (20-hour) neurula, *AmphiOtx* is expressed throughout the length of the pharynx in DMSO-treated controls. At this stage, the posterior limit of *AmphiOtx* expression is not affected by either RA (O) or BMS009 (Q). In 30-hour larvae, endodermal expression of *AmphiOtx* is restricted to the pharyngeal endoderm in DMSO-treated controls (N) and its posterior limit is shifted anteriorly by RA (P) and posteriorly by BMS009 (R).

(Jackman et al., 2000). However, unlike *AmphiOtx*, *AmphiIslet* remains expressed along much of the length of the endoderm through the early larval stage (Fig. 5B,C). As with *AmphiOtx*, RA treatment inhibits downregulation of *AmphiIslet* where the first gill slit would normally have formed (Fig. 5D-F). However, neither RA- (Fig. 5D,F) nor BMS009 treatment (Fig. 5G-I) has a marked effect on the posterior limit of expression.

In control embryos, *AmphiFoxA2* is expressed throughout the pharynx at the mid- to late-neurula stages (Fig. 5J) (Shimeld, 1997). Unlike *AmphiOtx* and *AmphiIslet*, *AmphiFoxA2* remains expressed where the first gill slit will form, although expression is reduced anteriorly in the pharyngeal endoderm (Fig. 5J-L). The only evident effect of altered RA signaling on endodermal expression of *AmphiFoxA2* is that in RA-treated embryos and larvae, it is not downregulated where the gill slits and mouth would have formed (Fig. 5M-O), while in larvae treated with BMS009, *AmphiFoxA2* is largely downregulated in an expanded region of the anterior endoderm (Fig. 5P-R).

AmphiHh is weakly expressed throughout the length of the endoderm on the left side (Fig. 5S,T) (Shimeld, 1999). Expression is particularly high anterior to the mouth (Fig. 5S). As development proceeds, *AmphiHh* becomes upregulated around the first gill slit (Fig. 5T). Altered RA signaling does not substantially affect either the left/right asymmetry or the anterior/posterior extent of endodermal expression. However, treatment with RA reduces the size of the strong expression domain in the anteriormost pharyngeal endoderm at the mid-neurula and almost completely downregulates endodermal expression by the early larval stage (Fig. 5U,V). In contrast, while BMS009 does not alter the expression domain of *AmphiHh* substantially, it does appear to upregulate the gene somewhat (Fig. 5W,X). Failure of altered levels of RA signaling to change the posterior limit of expression of *AmphiIslet*, *AmphiFoxA2* and *AmphiHh* suggests that they are involved in specification of posterior foregut/midgut structures as well as pharyngeal structures. This is not surprising in light of the roles of their homologs in specification of the foregut and its derivatives in

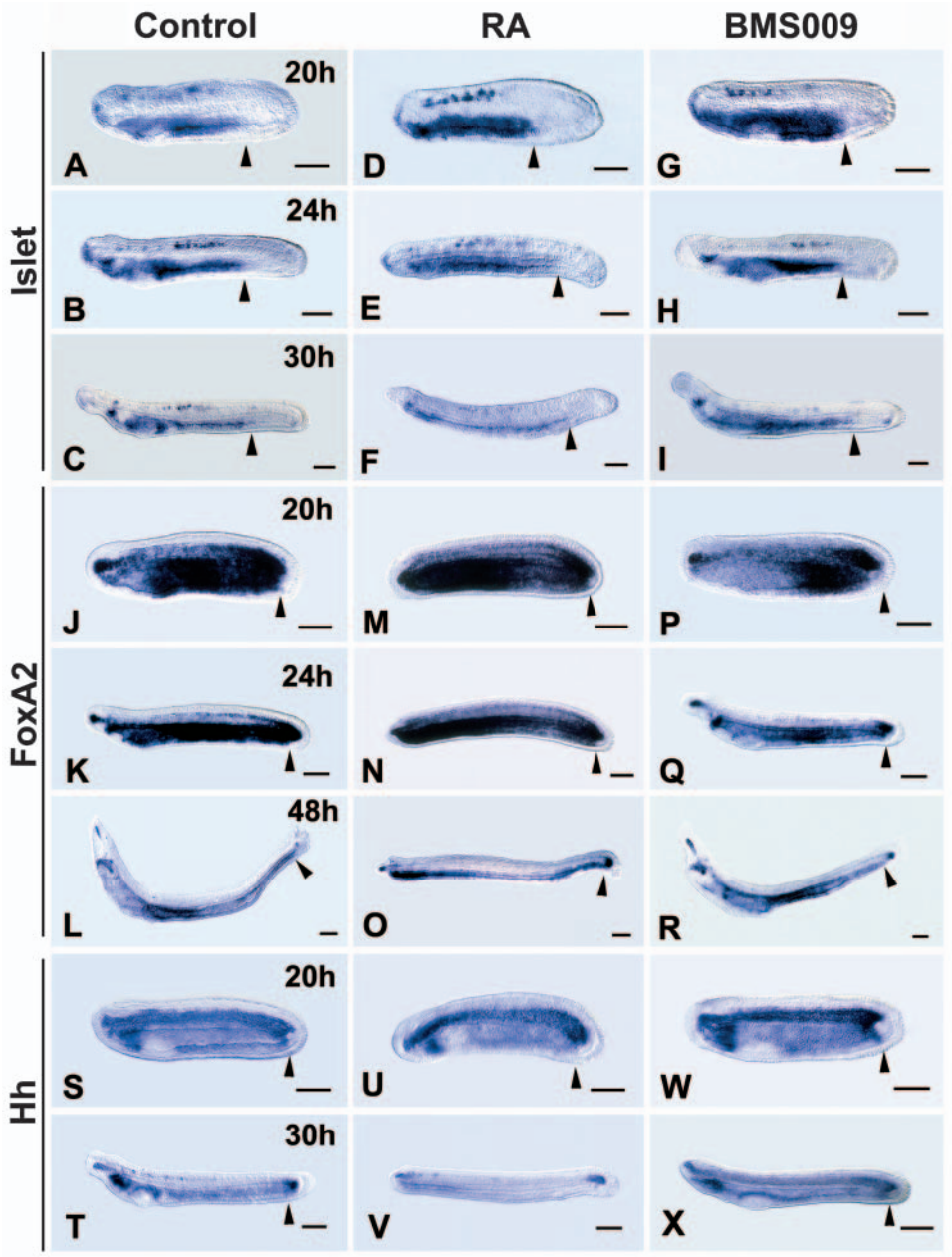

Fig. 5. The posterior limit of endodermal expression of *AmphiIslet* (A-I), *AmphiFoxA2* (J-R), and *AmphiHh* (S-X) is not affected by altered levels of RA signaling. Anterior to left. Arrowheads indicate the posterior limit of endodermal expression. Scale bars=50 μm. (A-I) *AmphiIslet* is expressed in the anterior three-quarters of the endoderm in controls at all stages. Expression is not changed by RA or BMS009. (J-R) The posterior limit of *AmphiFoxA2* is at the posterior end of the endoderm in DMSO controls and in RA- and BMS009-treated embryos at all stages. (S-X) *AmphiHh* is expressed strongly in the extreme anterior endoderm and more weakly throughout the remainder of the endoderm in DMSO controls at all stages (S,T). In mid-neurula embryos, the posterior limit of the region of weak endodermal expression is not affected by RA (U) or BMS009 (W). At this stage, *AmphiHh* expression in the endoderm of RA-treated embryos is largely downregulated (V), while expression in embryos treated with BMS009 appears to be slightly upregulated. A,D,G,J,M,P,S,U,W=20 hours. B,E,H,K,N,Q=24 hours. C,F,I,T,V,X=30 hours. L,O,R=48 hours.

vertebrates (Chen et al., 2004; Gauthier et al., 2002; Yuan and Schoenwolf, 2000).

Injection of an *AmphiHox1*-specific morpholino mimics the effect of BMS009 treatments in setting the posterior limit of the pharynx

To test whether *AmphiHox1* mediates RA signaling in setting the posterior limit of the pharynx, we knocked-down *AmphiHox1* function by injection of an antisense morpholino oligonucleotide. In vitro translation showed that the *AmphiHox1*-specific morpholino effectively blocks translation of *AmphiHox1* mRNA (see Fig. S2 in the supplementary material). Injected embryos were fixed at late neurula and early larval stages and hybridized with riboprobes for three genes: *AmphiHox1*, *AmphiPax1/9* and *AmphiOtx*. Although the *AmphiHox1*-specific morpholino does not affect expression of *AmphiHox1* in the nerve cord (where expression of *AmphiOtx* is expanded posteriorly), expression of *AmphiHox1* in the endoderm is shifted somewhat posteriorly as in animals treated with the RA antagonist BMS009 (anterior limit shifted from 30% to 36%; Fig. 6A,B). In addition, pharyngeal expression of both *AmphiPax1/9* (Fig. 6C,D) and *AmphiOtx* (Fig. 6E-H) is expanded posteriorly in embryos and larvae injected with the *AmphiHox1*-specific morpholino (posterior limits changed from 37% to 67% and 43% to 55%, respectively), showing that both genes are downstream of *AmphiHox1* in the RA signaling hierarchy. However, gill slits form normally in embryos injected with the *AmphiHox1*-specific morpholino (Fig. 6D,H). These results indicate that like RA antagonist treatment, the injection of an *AmphiHox1*-specific morpholino expands the pharyngeal region posteriorly. We conclude that AmphiHox1 probably mediates RA signaling in the amphioxus endoderm to establish the posterior limit of the pharynx, but probably does not mediate the role of RA in gill slit penetration.

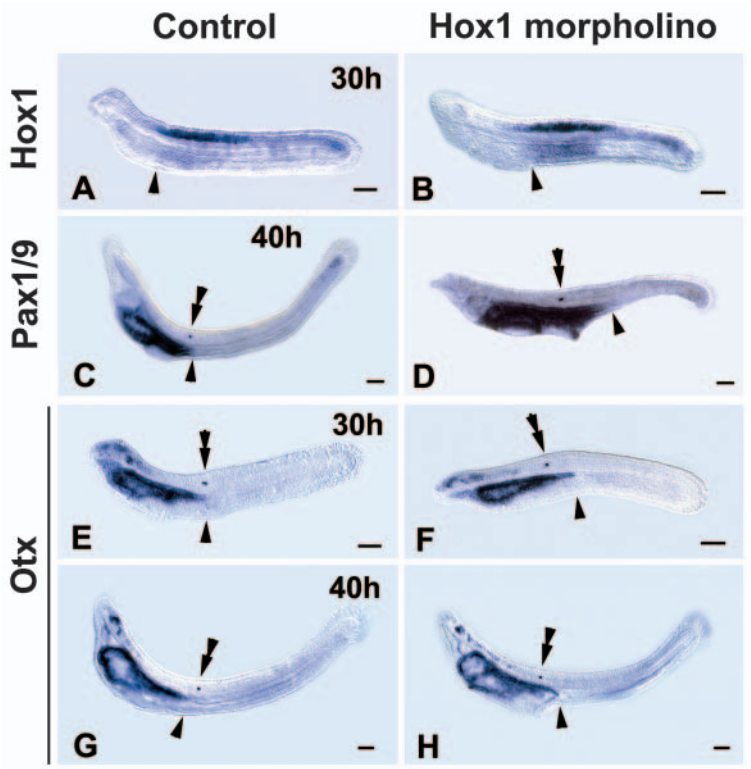

Fig. 6. Knockdown of *AmphiHox1* function with an antisense morpholino oligonucleotide mimics the effect of BMS009 treatments on the posterior limit of the pharynx in amphioxus. Scale bar=50 μm. (A,C,E,G) Embryos injected with the control morpholino. (B,D,F,H) Embryos injected with the *AmphiHox1*antisense morpholino-oligonucleotide. (A,B) *AmphiHox1* expression. In controls (A), the anterior limit of *AmphiHox1* expression in the endoderm (arrowhead) coincides with the first pigment spot to form in the nerve cord, and is shifted slightly posteriorly in embryos injected with the *AmphiHox1* morpholino (B). (C,D) *AmphiPax1/9* expression. The posterior limit of *AmphiPax1/9* expression in the endoderm (arrowhead) of an amphioxus larva injected with the control morpholino (C) coincides with the first pigment spot to form in the nerve cord (double arrowhead), whereas in larvae injected with the *AmphiHox1*-specific morpholino (D), the pharynx is expanded posteriorly and the posterior limit of *AmphiPax1/9* expression in the pharyngeal endoderm (arrowhead) is posterior to the first pigment spot in the nerve cord (double arrowhead). (E-H) *AmphiOtx* expression. In control embryos (E,G), the posterior limit of *AmphiOtx* expression (arrowhead) coincides with the first pigment spot in the nerve cord (double arrowhead). *AmphiOtx* expression is expanded posteriorly in embryos injected with the *AmphiHox1*-specific morpholino (F,H). A,B,E,F=30 hours. C,D,G,H=40 hours.

Discussion

AmphiRAR and *AmphiHox1* are probable direct targets of RA signaling in the endoderm

The present research has taken advantage of the uncomplicated body plan and relatively unduplicated genome of amphioxus to begin elucidating the developmental mechanism whereby RA signaling in the posterior foregut/midgut endoderm establishes the posterior limit of the pharynx. *AmphiHox1* (Manzanares et al., 2000) and *AmphiRAR*, like their vertebrate homologs, are probably both direct targets of RA signaling in the endoderm. Although the regulatory elements of *AmphiRAR* have not been studied, we have previously shown that *AmphiRAR* is strongly upregulated by RA and downregulated by the RA antagonist BMS009 (Escriva et al., 2002) (and Fig. S1 in the supplementary material). Moreover, the effects of altered RA signaling on expression of *AmphiRAR* are much like those on expression of vertebrate RARs, which are known to be autoregulated (Sucov et al., 1990). Both *AmphiRAR* and vertebrate *RARβ* are expressed in the posterior foregut endoderm (Escriva et al., 2002; Mollard et al., 2000) and are ectopically expressed in the pharyngeal endoderm in embryos treated with RA and/or RA agonists (Wendling et al., 2000). Conversely, RA antagonists downregulate expression of RARs in the endoderm of both vertebrates and amphioxus (Escriva et al., 2002). These parallels suggest that *AmphiRAR*, like vertebrate RARs, is probably autoregulated in the pharyngeal endoderm.

Similarly, the *AmphiHox1* gene contains a RA response element (RARE) 3′ of the coding region (Manzanares et al., 2000) to which the AmphiRAR/AmphiRXR heterodimer can bind in vitro (H. Escriva, H. Wada and V. Laudet, unpublished). In addition, the effects of altered RA signaling on expression of *AmphiHox1* are similar to those on vertebrate *Hoxa1* and *Hoxb1*, which are also expressed

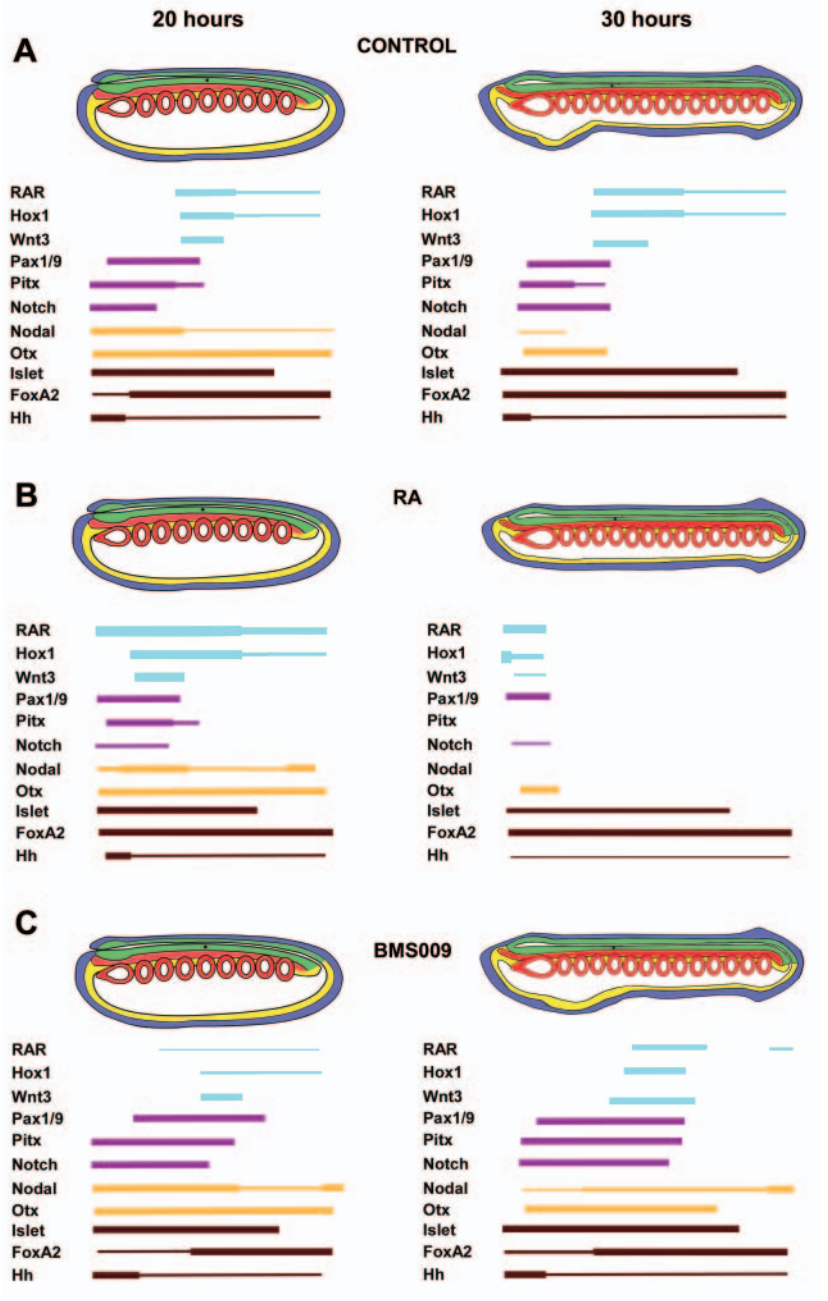

Fig. 7. Summary of expression of endodermal markers in amphioxus at the mid-neurula (20 hours) and early larval (30 hours) stages for (A) controls, (B) RA-treated embryos and (C) BMS009-treated embryos. The thickness of the lines is proportional to the relative level of expression. Changes of expression in the mouth and gill slit primordia are not shown. The position of the pigment spot in the nerve cord at the level of somite 5 is not affected by levels of RA signaling. Markers of the posterior foregut/midgut endoderm (*AmphiRAR*, *AmphiHox1* and *AmphiWnt3*) are in light blue, those with expression restricted to the pharynx from the early neurula (*AmphiPax1/9*, *AmphiPitx* and *AmphiNotch*) are in purple, markers with expression becoming restricted to the pharynx at the early larval stage (*AmphiNodal* and *AmphiOtx)* are in orange, and those expressed throughout the length of the endoderm at all stages (*AmphiIslet*, *AmphiFoxA2* and *AmphiHh*) are in brown. These groupings in controls (A) are predictive of the changes in expression in response to RA (B) and the RA antagonist BMS009 (C). In controls (A) *AmphiRAR*, *AmphiHox1* and *AmphiWnt3* are most strongly expressed in the middle third of the endoderm. RA (B) shifts their expression anteriorly. BMS009 (C) downregulates *AmphiRAR* and *AmphiHox1* and by 30 hours shifts posteriorly their anterior limits of expression and expands posteriorly the domain of *AmphiWnt3*. In controls (A), expression of genes in the second group (*AmphiPax1/9*, *AmphiPitx* and *AmphiNotch*) is restricted to the pharynx at both stages. RA (B) shifts the posterior limits of their expression anteriorly and by 30 hours downregulates expression of *AmphiPitx* and *AmphiNotch*. BMS009 (C) has the opposite effect, expanding the domains of all three posteriorly. In controls (A), expression of genes in the third group (*AmphiNodal* and *AmphiOtx*) becomes restricted to the pharynx by 30 hours and is not affected by RA (B) until 30 hours, when expression is truncated posteriorly and/or downregulated. BMS009 (C) keeps expression of both genes high throughout much of the length of the endoderm. In controls (A), genes in the fourth group (*AmphiIslet*, *AmphiFoxA2* and *AmphiHh*) are expressed throughout most of the length of the endoderm at both stages. The posterior limits of their expression are not markedly affected by either RA (B) or BMS009 (C).

in the posterior part of the pharyngeal endoderm (Matt et al., 2003; Wendling et al., 2000). These genes also contain RAREs, required to direct expression of *Hoxa1* and *Hoxb1* reporter constructs to the foregut (Huang et al., 1998). Not surprisingly, as with amphioxus, treatment of mouse embryos with a pan-RAR antagonist eliminates or greatly reduces pharyngeal expression of Hox1 genes (Matt et al., 2003). Conversely, treatment with RA or RAR agonists results in an anterior shift of Hox1 gene expression in the pharynx (Escriva et al., 2002; Wendling et al., 2000). Taken together these data clearly suggest that the general shape of the RAR-Hox1 hierarchy is conserved in vertebrates, but has become more complex due to gene duplications early in vertebrate evolution that resulted in three RAR and three Hox1 paralogs.

AmphiHox1 mediates the effect of RA signaling in setting the posterior limit of the amphioxus pharynx

Our results show that blocking function of *AmphiHox1* expands the amphioxus pharynx to the same extent as inhibiting RA signaling and demonstrate an approximate hierarchy of downstream genes (Fig. 7). In our model (Fig. 8), RA signaling activates *AmphiHox1*, which is co-expressed with the RA receptor *AmphiRAR* in the middle third of the endoderm. *AmphiHox1* in turn represses *AmphiPax1/9* and *AmphiOtx* expression posterior to the pharynx.

The order of genes in the hierarchy downstream of *AmphiRAR* and *AmphiHox1* is suggested by the time in development at which expression of endodermal markers becomes restricted to the pharynx (Fig. 7). Expression of *AmphiPax1/9*, together with *AmphiPitx* and *AmphiNotch* is

restricted to the pharynx early in development suggesting that they are high in the hierarchy of downstream genes. In fact, *AmphiPax1/9*, which first turns on at the early neurula stage, is never expressed posterior to the pharyngeal region (Holland et al., 1995), suggesting that *AmphiPax1/9* is likely to be very high up in the gene network downstream of *AmphiRAR* and *AmphiHox1*. In contrast, expression of *AmphiOtx* and *AmphiNodal* becomes restricted to the pharynx relatively late (Fig. 7). For example, expression of *AmphiOtx* does not become restricted to the pharyngeal endoderm until the late neurula (Williams and Holland, 1996; Williams and Holland, 1998), and it is only at this time that altered levels of RA signaling affect the anterior/posterior limit of expression. Thus, *AmphiOtx* is likely to be much farther downstream than *AmphiPax1/9* in the RA-/Hox1-signaling pathway. Together, our results suggest the scenario in Fig. 8 in which *AmphiHox1* is a direct target of RA signaling (Manzanares et al., 2000) and in turn sets the posterior limit of the pharynx by repressing expression of pharyngeal markers in the endoderm of the posterior foregut/midgut. Although not yet unequivocally demonstrated, it is likely that *AmphiRAR* and *AmphiHox1* turn on specific markers of the posterior foregut/midgut endoderm, such as *AmphiWnt3* (Fig. 2).

The posterior limit of the amphioxus and vertebrate pharynx may be established by a similar suite of genes

Comparison with vertebrates suggests that the model in Fig. 8 may also apply to anterior/posterior patterning of the pharyngeal endoderm in vertebrates. In vertebrates, as in amphioxus, Hox1 genes (*Hoxa1* and *Hoxb1*) are expressed in endoderm of the foregut and extreme caudal end of the pharynx (Frasch et al., 1995; Huang et al., 1998; Wendling et al., 2000). Pharyngeal expression of both genes is severely decreased by treatment with an RA antagonist (Wendling et al., 2000). Moreover, as in amphioxus embryos treated with RA, treatment of mouse embryos with an RA agonist induces strong ectopic expression of both *Hoxa1* and *Hoxb1* in the anterior pharyngeal endoderm (Matt et al., 2003). This suggests that in vertebrates as well as amphioxus, Hox1 genes mediate the effect of RA in establishing the posterior limit of the pharyngeal endoderm.

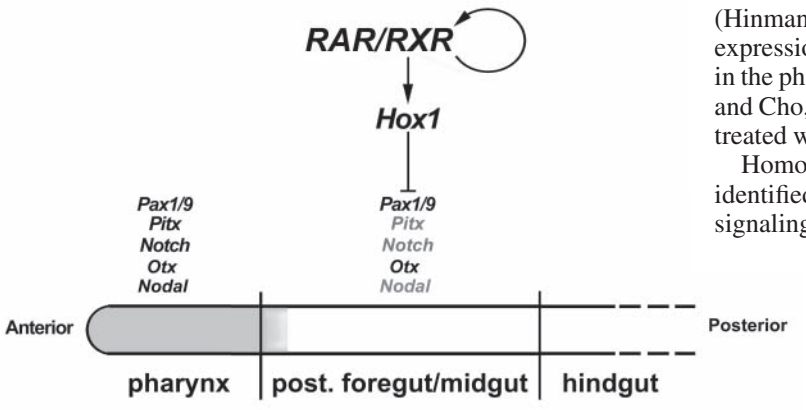

Fig. 8. Model for the mechanism whereby RA signaling establishes the posterior limit of the amphioxus pharynx. RA signaling via heterodimers of RAR/RXR in the posterior fore/midgut endoderm directly activates *Hox1*. RAR probably autoregulates its own transcription. Hox1 in turn represses expression of the pharyngeal markers *Pax1/9* and *Otx* (black type). *Pitx*, *Notch* and *Nodal* (gray) are also repressed in the posterior fore/midgut by RA signaling and may also be downstream of *Hox1*.

The targets of Hox1 genes in the pharyngeal endoderm of vertebrates have not been described. However, they are probably very much the same as in amphioxus since the pharyngeal endoderm of both amphioxus and vertebrates expresses similar suites of genes. Vertebrates have two homologs of *AmphiPax1/9*, *Pax1* and *Pax9*. Both are expressed throughout the length of the pharyngeal endoderm that will give rise to the pharyngeal pouches and later in the endoderm of the definitive pharyngeal pouches themselves (Müller et al., 1996; Ogasawara et al., 2000). As in amphioxus embryos treated with BMS009, reduced RA signaling extends expression of *Pax1* posteriorly (Dupé et al., 1999; Quinlan et al., 2002; Wendling et al., 2000). Conversely, RA treatment reduces *Pax1* expression in the endoderm of the third pharyngeal pouch in the mouse in connection with a greatly reduced third pharyngeal arch or fusion of the third and fourth arches (Mulder et al., 1998). Altered levels of RA signaling have similar effects on *Pax9*. In the mouse, the domain of *Pax9* expression in the second pouch is expanded in embryos treated with a pan-RA antagonist, and expression where the third pouch would normally form is nearly eliminated (Wendling et al., 2000). Mouse knockouts of both *RARα* and *RARβ* have a somewhat less severe phenotype, but even so, *Pax1* expression in the third pouch is reduced (Dupé et al., 1999). Together, these results suggest that in vertebrates, as in amphioxus, high levels of RA signaling may activate RAR and Hox1 expression in the endoderm and that Hox1 expression in turn represses, directly or indirectly, transcription of *Pax1/9* genes in the endoderm posterior to the pharynx.

Similarly, expression of Otx genes in the pharyngeal endoderm is common to tunicates and vertebrates as well as amphioxus. The effects of loss of Hox1 gene function on *Otx* expression in chordates other than amphioxus has not been studied. However, RA treatment has a similar effect on *Otx* expression in these organisms as in amphioxus. In ascidian tunicates, reduction of the pharynx in RA-treated embryos correlates with reduced expression of *Otx* in the pharynx (Hinman and Degnan, 2000). In addition, in vertebrates, expression of Otx genes in the anterior mesendoderm and later in the pharyngeal endoderm of the first pharyngeal pouch (Blitz and Cho, 1995; Tomsa and Langeland, 1999) is lost in embryos treated with RA (Bally-Cuif et al., 1995; Simeone et al., 1995).

Homologs of the remaining pharyngeal markers we have identified with their posterior limits set by a high level of RA signaling are also expressed in the pharyngeal endoderm of vertebrates and other chordates, although the effects of RA signaling on expression of these genes in vertebrates is not known. For example, expression of *AmphiPitx* in the endoderm around the mouth and Hatschek's anterior left diverticulum, the homolog of the adenohypophysis (Yasui et al., 2000), is comparable to that of *Pitx2* in the pituitary and *Pitx1* in the stomodaeum and rostral foregut endoderm in the mouse and chick (Lanctot et al., 1997). Similarly, in the lamprey, Pitx genes are expressed in the stomodaeum, neurohypophyseal duct and pharyngeal endoderm among other

locations (Boorman and Shimeld, 2002a). In larval tunicates, *Pitx* is also expressed in the nascent pharynx (Boorman and Shimeld, 2002b).

Vertebrate Notch genes are expressed in the pharynx as in amphioxus, although their roles in pharyngeal development are not well understood. For example, in the mouse, *Notch2* is expressed in the anterior part of the first branchial arch (Williams et al., 1995), but it is not known if this is in the endodermal portion or not. *Notch1* is also expressed in the epibranchial placodes associated with branchial arches 1-3, near the fourth arch, in neural crest migrating into first and second arches (Williams et al., 1995) and in the thymus (Weinmaster et al., 1991; Weinmaster et al., 1992). Notch genes are also expressed in the developing pancreas and the lung, which are both endodermal derivatives (Kim and Hebrok, 2001; Lammert et al., 2000; Post et al., 2000). In the zebrafish blastula, Notch signaling appears to regulate the number of endodermal cells; overexpression reduces the number of cells expressing the endodermal marker *foxa2* (Kikuchi et al., 2004). However, whether Notch genes have a later role in development of the pharyngeal endoderm is unknown.

Expression of *Nodal* in amphioxus and vertebrates is also similar. In amphioxus, *AmphiNodal* is expressed at the dorsal lip of the blastopore in the early gastrula and throughout the length of the endoderm at the mid-neurula stage, subsequently becoming restricted to the pharyngeal endoderm (Yu et al., 2002). In vertebrates, *Nodal* expression in mesendodermal precursors is required for endoderm formation, in particular for the foregut endoderm where it is upstream of *Pitx2* (Faucourt et al., 2001; Tam et al., 2003). Taken together, these data suggest that the gene networks specifying anterior endoderm are similar in amphioxus and vertebrates, and that in both, a RAR/Hox1 signaling cascade determines fore/midgut identity and restricts expression of anterior endodermal genes to the pharynx. However, in vertebrates, extensive gene duplications have evidently conferred added complexity on these gene networks.

The RA and WNT signaling cascades may interact during regionalization of the amphioxus and vertebrate endoderm

AmphiWnt3 is expressed ventrally in the endoderm just posterior to the pharynx with anterior/posterior limits coinciding approximately with those of *AmphiRAR* and *AmphiHox1*. Like *AmphiRAR* and *AmphiHox1*, *AmphiWnt3* expression is shifted anteriorly by RA, and expanded posteriorly by BMS009. *AmphiWnt3* is expressed relatively late in the gut and is therefore probably acting downstream of RA signaling. In vertebrates, *Wnt3a* is expressed in the vertebrate foregut endoderm of the chick (Theodosiou and Tabin, 2003), and is downregulated by RA in an embryonic carcinoma cell line (Katoh, 2002) as well as in the tail bud (Shum et al., 1999). This suggests that a role of *Wnt3a* in regionalization of the gut in the chick may have its antecedents in an amphioxus-like ancestor. However, whether there is cross-talk between RA signaling and WNT signaling in patterning the vertebrate foregut remains to be demonstrated.

Left/right asymmetry in amphioxus involves the same genes (*Hh*, *Nodal* and *Pitx*) as in vertebrates, but is independent of RA signaling

Specification of left/right position involves the evolutionarily conserved series of *Shh*, *Nodal* and *Pitx2* (Cooke, 2004). In the vertebrates, high concentrations of RA randomize heart looping and can induce bilateral expression of *Nodal* and *Pitx2* on the right side (Chazaud et al., 1999; Smith et al., 1997; Wasiak and Lohnes, 1999). However, expression of *Shh* is not affected (Smith et al., 1997), and it appears to act either in parallel to or downstream of RA signaling (Tsuki et al., 1999). In amphioxus, *AmphiHh*, *AmphiNodal* and *AmphiPitx* are all expressed on the left side of the pharyngeal endoderm. Expression of these genes on the left side of the body is not affected by altering the levels of RA signaling. Thus RA signaling is not required for establishment of left/right asymmetry in amphioxus, or apparently, in tunicates (Hinman and Degnan, 1998), and its role in left/right asymmetry may be a vertebrate innovation.

Conclusions

Our results show that amphioxus is particularly advantageous for understanding developmental mechanisms and that it can serve as a simplified model for comparable patterning in vertebrate embryos. Because many amphioxus genes are present in single copies (including RAR and the Hox genes), functional knockdowns are relatively easy to interpret. Moreover, since amphioxus lacks definitive neural crest, the model we present for patterning of the pharynx applies unequivocally to the endoderm, thereby giving insights into the separate roles of the endoderm and neural crest in pharyngeal patterning of vertebrates. It is likely that similar regulatory cascades involving Hox1-mediated RA signaling help to direct pharyngeal patterning in both amphioxus and vertebrates. In addition, in vertebrates, the evolution of neural crest evidently led to the elaboration of novel pharyngeal structures, which were superimposed on the already existing pharyngeal patterning intrinsic to the endoderm.

A role for Hox genes in patterning the endoderm is widespread in the animal kingdom (Brunschwig et al., 1999; Irvine and Martindale, 2000; Marty et al., 2001). Recent evidence suggests that the RAR genes may be more ancient than previously thought, having been secondarily lost in *Drosophila* and nematodes (Bertrand et al., 2004). Thus, endodermal patterning by RAR/Hox1 may not be limited to chordates, and the model we present here for regionalization of the amphioxus endoderm may provide a framework for understanding endodermal patterning in a wide spectrum of bilaterian animals.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/1/61/DC1

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