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Evolution of Developmental Control Mechanisms

Retinoic acid signaling targets *Hox* genes during the amphioxus gastrula stage: Insights into early anterior–posterior patterning of the chordate body plan

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ABSTRACT

Previous studies of vertebrate development have shown that retinoic acid (RA) signaling at the gastrula stage strongly influences anterior–posterior (A–P) patterning of the neurula and later stages. However, much less is known about the more immediate effects of RA signaling on gene transcription and developmental patterning at the gastrula stage. To investigate the targets of RA signaling during the gastrula stage, we used the basal chordate amphioxus, in which gastrulation involves very minimal tissue movements. First, we determined the effect of altered RA signaling on expression of 42 genes (encoding transcription factors and components of major signaling cascades) known to be expressed in restricted domains along the A–P axis during the gastrula and early neurula stage. Of these 42 genes, the expression domains during gastrulation of only four (*Hox1, Hox3, HNF3-1* and *Wnt3*) were spatially altered by exposure of the embryos to excess RA or to the RA antagonist BMS009. Moreover, blocking protein synthesis with puromycin before adding RA or BMS009 showed that only three of these genes (*Hox1, Hox3* and *HNF3-1*) are direct RA targets at the gastrula stage. From these results we conclude that in the amphioxus gastrula RA signaling primarily acts via regulation of *Hox* transcription to establish positional identities along the A–P axis and that *Hox1*, *Hox3*, *HNF3-1* and *Wnt3* constitute a basal module of RA action during chordate gastrulation.

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Introduction

Retinoic acid (RA) is a morphogen that acts via heterodimers of the retinoic acid receptor (RAR) and retinoid X receptor (RXR), which bind to retinoic acid response elements (RAREs) in the promoter regions of target genes. The RA signal is mediated by a positive feedback loop involving the direct regulation of *RAR* by RAR/RXR heterodimers (Heyman et al., 1992; Rudert and Gronemeyer, 1993; Blomhoff and Blomhoff, 2006; Campo-Paysaa et al., 2008; Casci, 2008). RA has been extensively studied for its effects on cell cultures, embryonic development, adult growth, regeneration and carcinogenesis in chordates (McCaffery et al., 2003; Mongan and Gudas, 2007; Dann et al., 2008; Niederreither and Dollé, 2008). In embryos of amphioxus and vertebrates, RA has numerous pleiotropic effects. RA signaling is permissive for some tissues–like forelimbs and somites–allowing previously specified structures to complete differentiation, but instructive for other tissues–like hindbrain and foregut–conferring positional information along the anterior–posterior (A–P) axis and specifying tissue identity (Stafford et al., 2006; Duester, 2008).

The earliest known instructive signaling by RA begins at the gastrula stage and mediates patterning of the germ layers along the A–P axis of the embryo (Durston et al., 1989; Sive et al., 1990; Holland and Holland, 1996; Roelen et al., 2002; Grapin-Botton, 2005; White et al., 2007). Even a transitory perturbation of RA signaling in the gastrula can affect A–P patterning during the post-gastrula stages of development. There are few studies on the effects of RA signaling on gene transcription at the gastrula stage, and these are largely limited to *Hox* genes and to vertebrates (Kudoh et al., 2002; Roelen et al., 2002; Li et al., 2008a). Such studies are complicated by the mechanics of gastrulation in vertebrates. The germ layers at the gastrula stage are often more than one cell thick (Delarue et al., 1998; Li et al., 2008b), and the constituent cells undergo complex migrations, either individually or in coherent groups during gastrulation (Schoenwolf and Smith, 2000; Kimura et al., 2006). It is, therefore, difficult to establish the A–P limits of the domains of gene expression in vertebrate gastrulae, raising the possibility that there are early immediate targets of RA signaling at the gastrula stage that act in parallel to *Hox* genes in mediating A–P patterning.

To test this hypothesis, we used the invertebrate chordate amphioxus. Amphioxus resembles vertebrates in using RA signaling for axial patterning (Holland and Holland, 1996; Escriva et al., 2002; Marlétaz et al., 2006), but has the advantage of an early development

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that is morphologically uncomplicated: the spherical blastula has a single layer of cells surrounding a hollow blastocoel. Gastrulation begins with invagination from the posterior pole of the embryo. The resulting gastrula has an outer ectoderm and an inner mesendoderm, both only one cell thick. The invaginated mesendoderm largely obliterates the blastocoel and creates a new space, the archenteron, which opens to the exterior via a blastopore. Cell movements during invagination of the mesendoderm are minimal: the cells at the blastoporal lip do not converge towards the dorsal midline, and there is little involution over the lip of the blastopore (Zhang et al., 1997; Holland and Holland, 2007). Amphioxus is also more amenable for such a study than tunicates, where RA signaling of the A–P axis is either highly modified (ascidians) or absent (appendicularians) (Ishibashi et al., 2003; Nagatomo and Fujiwara, 2003; Fujiwara, 2005; Cañestro and Postlethwait, 2007; Imai et al., 2009).

For vertebrates, except for the study of Kudoh et al. (2002), the effects of altering RA signaling during the gastrula stage have not been tallied until the neurula and larval stages (reviewed by Duester, 2008). Similarly, for amphioxus, previous studies altering RA signaling during the gastrula stage only looked at the effects of this treatment at later stages. Therefore, although these studies documented effects of RA on expression of a number of genes (i.e. several *Hox* genes, *Cdx*, *Hedgehog*, *HNF3-1*, *Nodal*, *Notch*, *Otx*, *Pitx*, *Wnt3* and *Wnt5*) at the neurula and larval stages (Holland and Holland, 1996; Schubert et al., 2004, 2005, Schubert et al., 2006; Osborne et al., 2009), they could not differentiate direct targets of RA signaling from indirect ones.

In the present study, we administered RA or an RA antagonist (BMS009) continuously to cultures of developing amphioxus from the beginning of the gastrula stage and assayed for effects later in the gastrula stage on expression of 42 genes known to be transcribed in restricted anterior–posterior patterns at the gastrula stage (listed in Supplementary Table S1). In addition, we inhibited protein synthesis with puromycin to distinguish between direct and indirect targets of RA signaling. Our results show that only 3 of these 42 genes (*Hox1*, *Hox3* and *HNF3-1*) are direct targets of RA at the gastrula stage, while only one (*Wnt3*) is an indirect target. Moreover, we present evidence suggesting that, in the amphioxus early neurula, RA may also directly regulate *Hox4* and *Hox6*. Since, in amphioxus, the control regions of at least *Hox1* and *Hox3* include functional RAREs (Manzanares et al., 2000; Wada et al., 2006), we conclude that RA acts and acted primarily through *Hox* genes as direct targets in patterning the A–P body axis at the gastrula stage not only in amphioxus, but probably also in the last invertebrate chordate ancestor of vertebrates.

Materials and methods

Sexually mature males and females of the Florida amphioxus (*Branchiostoma floridae*) were collected in Tampa Bay, Florida, USA, during the summer breeding season. The animals were stimulated to spawn electrically (Holland and Holland, 1993). After fertilization, the embryos were raised in filtered seawater at 28 °C and staged according to Holland and Yu (2004) as very early, early, mid and late gastrulae (respectively, 3 h, 4 h, 5 h and 6 h after fertilization) or early neurulae (9 h after fertilization).

In a first series of experiments, RA or the RA antagonist BMS009, each dissolved in DMSO (for a final concentration of 1×10^{-6} M), were added to cultures of very early gastrulae. DMSO at a 1:1000 dilution alone (Holland and Holland, 1996; Escriva et al., 2002) had no detectable effect. Samples of gastrulae at 4 h, 5 h or 6 h of development were fixed in 4% paraformaldehyde in MOPS buffer (0.1 M MOPS, 0.5 M NaCl, 2 mM MgSO4, 1 mM EGTA, pH 7.4) (Holland et al., 1996). This fixation solution is referred to hereafter simply as PFA. After fixation overnight at 4 °C, the specimens were transferred to 70% ethanol and stored at −20 °C until subjected to *in situ* hybridization. For each gene tested, antisense riboprobes were synthesized according to Holland et al. (1996) from the originally described clones or matching EST clones.

The effects of RA or BMS009 were studied for the following genes expressed at the gastrula stage as well as for two *Hox* genes expressed very shortly thereafter (the normal expression of all of these genes was already known from previous studies, as listed in Supplementary Table S1): *FoxD* (AF512537), *FoxQ2* (AY163864), *HNF3-2* (Y09236), *HNF3-1* (X96519), *Pax3/7* (AF165886), *Hex* (EU296398), *Pitx* (AJ438768), *Otx* (AF043740), *Cdx* (AF052465), *EvxA* (AF374191), *Gbx* (DQ416766), *Lim1/5* (DQ399521), *Six1/2* (EF195742), *Six3/6* (EF195743), *Six4/5* (EF195741), *Sox1/2/3* (AF271787), *Blimp1* (EU708968), *Neurogenin* (AF271788), *Brachyury* (X91903), *Eya* (EF195740), *Delta* (BW899056), *Notch* (Y12539), *Nodal* (AY083838), *Lefty* (EST clone bfne107n04), *Fgf8/17/18* (FJ266460), *Hedgehog* (Y13858), *Wnt1* (AF061974), *Wnt3* (AF361013), *Wnt4* (AF061973), *Wnt5* (AF361014), *Wnt6* (AF361015), *Wnt7* (AF061975), *Wnt8* (AF190470), *Wnt11* (AF187553), *Dkk1/2/4* (EST clone bfga017h15), *Dkk3* (EST clone bflv049h10), *sFRP2-like* (EST clone bfga018e02), *sFRP3/4* (EST clone bfad036d02), *Hox1* (AB028206), *Hox3* (X68045), *Hox4* (AB028208), *Hox6* (Z35146). The last two genes in this list, although not conspicuously transcribed at the gastrula stage, have expression domains known to be influenced by RA signaling at the early neurula stage (Schubert et al., 2004, 2005, 2006).

For blocking protein synthesis, puromycin (Sigma-Aldrich, Saint Louis, MO, USA) was added to gastrula cultures to a final concentration of 200 μg/ml at 3 h, 4 h and 5 h of development. After 5 min, RA or BMS009 (1×10^{-6}M) or DMSO was added and after 1 h embryos were fixed for *in situ* hybridization. The effectiveness of the puromycin concentration was verified by its ability to block synthesis of endogenous alkaline phosphatase in the amphioxus gut endoderm (Supplementary Fig. S1) (Holland et al., 1996).

Results

Altered RA signaling affects gene expression domains in amphioxus gastrulae

To identify potential direct targets of RA signaling during the gastrula stage in amphioxus, we first determined the effect of altered RA signaling on 40 genes with limited domains of expression along the A–P axis of the gastrula (Fig. 1; Supplementary Table S1) plus *Hox4* and *Hox6*, although expression of the last two only begins at the very end of (*Hox4*) or shortly after (*Hox6*) gastrulation (Schubert et al., 2004, 2005, 2006). Expression of *Hox2* at the gastrula stage was too weak to allow proper interpretation and was therefore excluded from our analysis. The other sampled genes fall into several categories: Notch, Nodal, Wnt and FGF signaling, forkhead and homeobox genes as well as transcription factors (*Neurogenin*, *Sox1/2/3*). About half have a single expression domain in a single tissue layer (either the outer ectoderm or the inner mesendoderm), and about half have domains in both tissue layers and/or two domains in a given tissue layer. Expression of thirteen of these 42 genes (*Cdx*, *Hedgehog*, *HNF3-1*, *Hox1*, *Hox3*, *Hox4*, *Hox6*, *Nodal*, *Notch*, *Otx*, *Pitx*, *Wnt3* and *Wnt5*) at the neurula and later stages was already known to be affected by RA applied during the gastrula stage, but it was not known if their expression in the gastrula itself was also affected (Holland and Holland, 1996; Schubert et al., 2004, 2005, 2006; Osborne et al., 2009). Therefore, it was not known whether any or all are direct targets of RA signaling. Administration of RA or the RA antagonist BMS009 from the onset of gastrulation did not alter the expression patterns of *Pitx*, *Otx*, *Cdx*, *Notch*, *Nodal*, *Hedgehog* and *Wnt5* during the gastrula stage (Figs. 1I–K, X, Y, B′, F′), even though their expression was affected by altered RA signaling in later embryos and larvae (Schubert et al., 2005, 2006; Osborne et al., 2009). *Nodal*, *Hedgehog* and *Wnt5* encode secreted signaling proteins involved in axial patterning in early development, and these results suggest that they are acting in parallel to RA signaling at the gastrula stage, with any crosstalk occurring only later in development. Moreover, none of

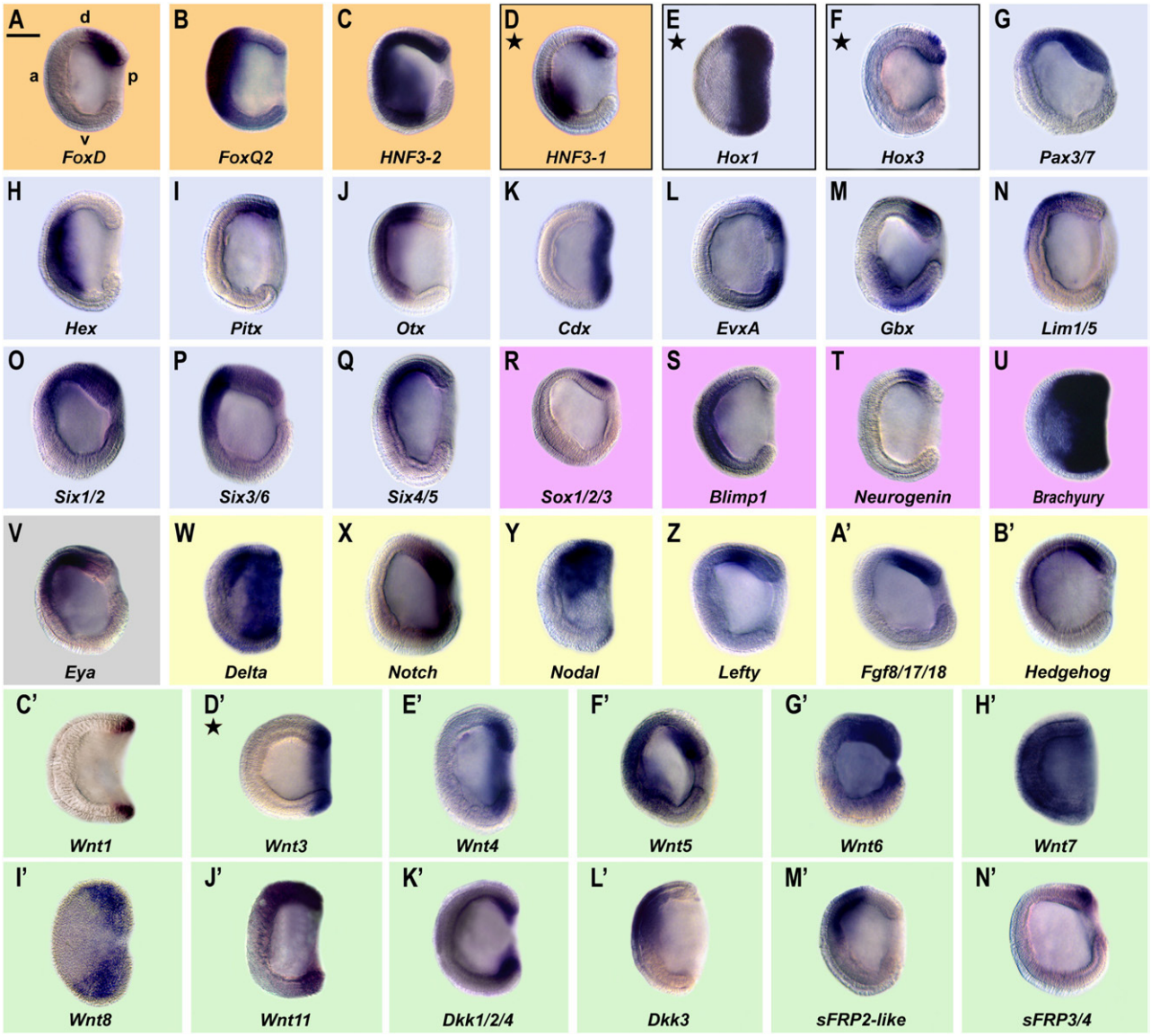

Fig. 1. Genes assayed for regulation by retinoic acid (RA) in amphioxus gastrulae. Side views of 40 whole mounts of embryos of the Florida amphioxus (*Branchiostoma floridae*) at the mid to late gastrula stage. In A, the anterior, posterior, dorsal, and ventral sides of the embryo are indicated, respectively by a, p, d and v. The 50-μm scale line in A is applicable to the entire figure. Each embryo shows the normal expression pattern of a developmental gene. These genes were selected, because they are transcribed in spatially discrete patterns at the gastrula stage. Gene families are indicated by the background color as follows: Forkhead genes (orange); Homeobox genes (blue); Sox domain, zinc finger, bHLH and T-box transcription factor genes (purple); Notch/Delta, Tgfβ, Fgf and Hedgehog signaling genes (yellow); Eya, a protein tyrosine phosphatase (grey); Wnt signaling and Wnt modulator genes (green). Expression of four genes (indicated by stars) is affected in gastrulae when exogenous RA or the RA antagonist BMS009 is administered from very early gastrulation (D–F and D'). Only three of them (boxed) are direct targets of RA signaling (D–F).

the other 29 other genes we tested was affected by RA at the gastrula stage, excluding them as potential direct targets (Fig. 1). However, expression of seven of these was affected at the neurula stage, indicating that they are probably indirect targets of RA signaling (data not shown). Thus, only six of the 42 genes expressed in limited patterns in the early amphioxus embryo, namely *HNF3-1*, *Hox1*, *Hox3*, *Hox4*, *Hox6* and *Wnt3* respond to the administration of RA or BMS009 at the start of the gastrula stage (Figs. 1D–F, D′; Figs. 2A–O), and are, therefore, potential early immediate targets of RA signaling in the amphioxus gastrula.

Genes with expression domains responding rapidly to RA signaling at the gastrula stage

In untreated embryos, *HNF3-1* expression is detectable from the mid gastrula stage (Fig. 2A) in two domains, the posterior–dorsal and anterior–ventral mesendoderm. By the end of gastrulation (Fig. 2B), *HNF3-1* is expressed throughout both the dorsal and ventral mesendoderm, except in an anterior gap (between the arrowheads in Fig. 2A). In RA-treated embryos, both domains are expanded at the mid-gastrula stage with the anterior gap between them (between the arrowheads in Fig. 2A) being markedly reduced compared to the controls. At the end of gastrulation, the gap is eliminated as *HNF3-1* becomes expressed throughout the mesendoderm of RA-treated embryos (Fig. 2B). Conversely, BMS009 treatments result in a posterior shift in expression, resulting in an increase in the anterior gap between the two domains at the mid-gastrula stage (Fig. 2A) and a posterior restriction of ventral mesendodermal expression compared to controls at the end of gastrulation (Fig. 2B).

Hox1 expression is first detectable in the early gastrula of controls as a small patch of weak staining in the dorsal blastoporal lip (Fig. 2C). As gastrulation proceeds, expression expands first throughout the blastopore lip and then spreads anteriorly in both the ectoderm and mesendoderm. Treatment with RA upregulates *Hox* expression. The

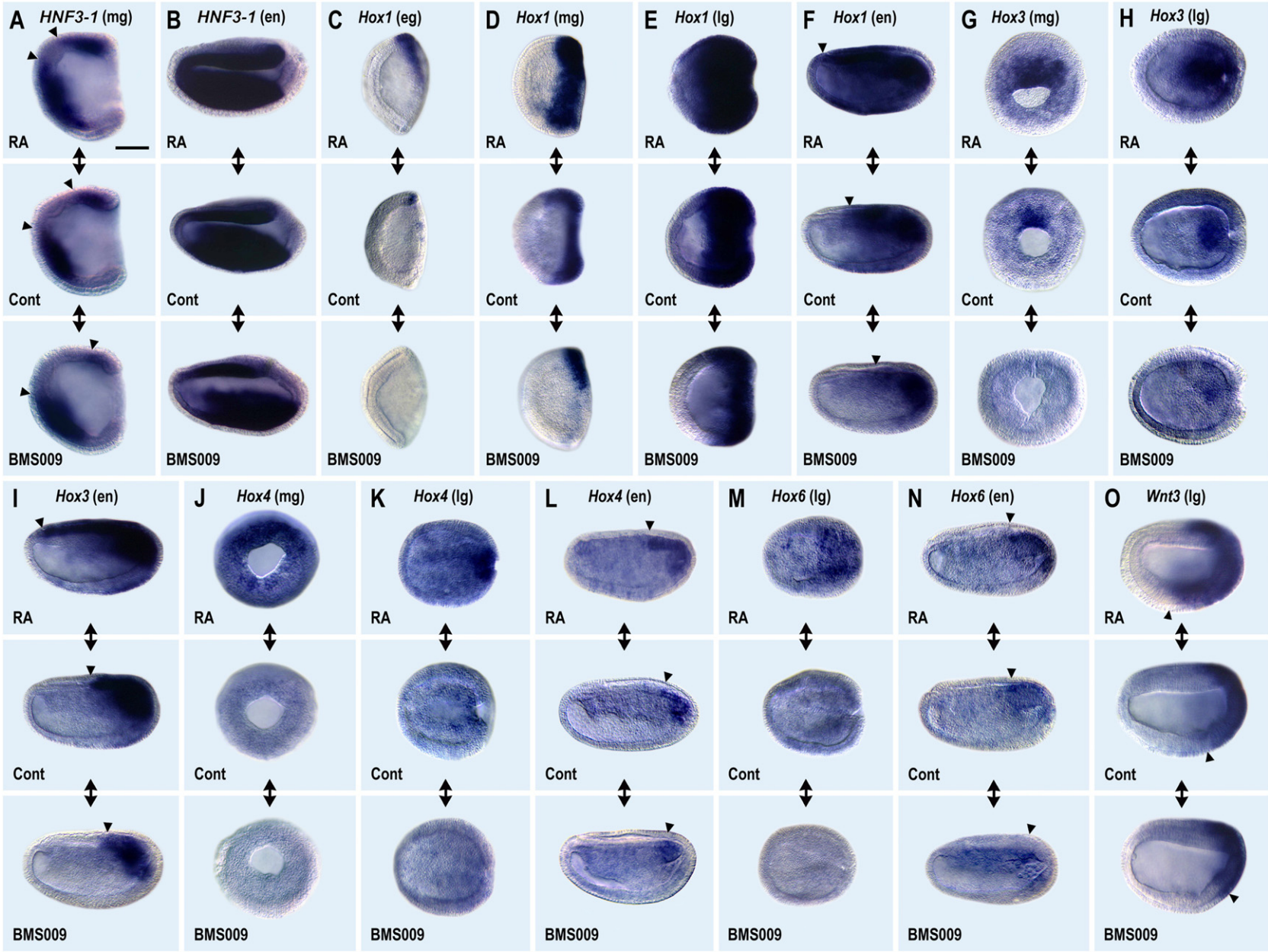

Fig. 2. Identification of putative retinoic acid (RA) signaling target genes during amphioxus gastrulation. Whole mounts of amphioxus embryos in side view (A–F, I, L, N and O) with anterior to the left and dorsal up, in dorsal view (H, K and M) with anterior to the left and in blastopore view (G and J) with dorsal up. The 50-μm scale line in A is applicable to the entire figure. The figure included four genes (*HNF3-1*, *Hox1*, *Hox3* and *Wnt3*), whose domains during the gastrula stage are altered by exposure to RA or to the RA antagonist BMS009. In addition, the effects of RA and BMS009 treatments on *Hox4* and *Hox6*, expressed at the very end of (*Hox4*) or just after (*Hox6*) gastrulation, show that all *Hox* genes tested are affected by altered RA signaling from the onset of expression. The arrowheads indicate the anterior limits of gene expression in mesendoderm (A and O) or nerve cord (F, I, L and N). For each gene, the vertical double-headed arrows facilitate aligning the control and experimental embryos. Abbreviations: (eg) = early gastrula; (mg) = mid gastrula; (lg) = late gastrula; (en) = early neurula; Cont = control; RA = retinoic acid; BMS009 = RA antagonist.

Hox1 domain in the dorsal blastopore lip is considerably enlarged at the early gastrula, and by the mid-gastrula stage it is expanded anteriorly compared to controls (Figs. 2C, D). By the late gastrula and early neurula, the *Hox1* domain is expanded almost to the anterior tip of the embryo (Figs. 2E, F). Conversely, in BMS009-treated embryos, *Hox1* expression is undetectable at the early gastrula and is restricted to the dorsal lip of the blastopore at the mid and late gastrula stages and to the tail bud, posterior-most nerve cord and somites at the neurula stage (Figs. 2C–F).

Hox3 turns on later than *Hox1* with a more posterior anterior limit. In controls, *Hox3* is first detectable in the dorsal blastopore lip of the late gastrula (Fig. 2G). RA expands the *Hox3* domain, while BMS009 completely downregulates expression. In the late gastrula treated with RA (Fig. 2H), *Hox3* expression is expanded anteriorly compared to controls, in BMS009-treated embryos expression of *Hox3* is restricted to the dorsal blastopore lip Fig. 2H. By the early neurula, *Hox3* is expressed throughout the nerve cord of RA treated embryos, and the mesodermal and endodermal domains are also expanded anteriorly (Fig. 2I). Conversely, BMS009 treatment restricts *Hox3* expression to the posterior third of the embryo Fig. 2I.

The effect of altered RA signaling on *Hox4* expression is similar to that on *Hox1* and *Hox3*. In embryos treated with RA, *Hox4* expression is first detectable in the dorsal blastopore lip of the mid gastrula (Fig. 2J). However in controls, expression is only first apparent in a few dorsal cells at the very end of gastrulation (Fig. 2K). At this stage and at the early neurula stage, the *Hox4* domain is expanded anteriorly in RA-treated embryos, while expression is undetectable in BMS009-treated ones (Figs. 2K, L). In RA-treated embryos, *Hox6* expression is first detectable at the late gastrula stage (Fig. 2M), but in controls not until the neurula stage (Fig. 2N). At this stage, expression in RA-treated embryos is expanded anteriorly, especially in the nerve cord, compared to controls, while expression is only weakly detectable in BMS009-treated amphioxus embryos.

The only other gene we found to be affected by altered RA signaling at the gastrula is *Wnt3*. However, the effects were minimal. In normal embryos, expression of *Wnt3* is detectable around the blastopore from the early gastrula stage onwards. We saw no effect of RA or BMS009 until the late gastrula stage (Fig. 2O). At this stage in controls, *Wnt3* is expressed posteriorly in the ectoderm and ventral endoderm. RA treatment leads to an anterior expansion of the ventral endodermal expression domain, while treatment with BMS009 results in a posterior restriction. There was no effect on expression dorsally in the embryo.

Direct RA target genes in early amphioxus embryos

To determine which of the genes affected by RA treatments at the gastrula stage are direct targets of RA signaling, and which are indirect, we used puromycin to block protein synthesis before adding RA or BMS009. We did not test *Hox4* and *Hox6* because they are not normally expressed until the very late gastrula or early neurula, respectively. In the presence of puromycin, of the four genes tested, only *Hox1*, *Hox3* and *HNF3-1* were still affected by altered RA signaling levels (Figs. 3A–E). However, when embryos were treated with puromycin and either RA or BMS009, the expression pattern of *Wnt3* in the ventral mesendoderm was not noticeably different from that in controls at the late gastrula stage (Fig. 3F). This is in contrast to the effects of treating solely with RA or BMS009, which resulted, respectively, in anterior and posterior shifts in expression. Thus, protein synthesis is not necessary for altered RA signaling to affect expression of *Hox1*, *Hox3* and *HNF3-1*, but is required for *Wnt3* expression to be affected. These results indicate that *Hox1*, *Hox3* and *HNF3-1* are direct targets of RA signaling at the gastrula stage, while *Wnt3* is likely an indirect target.

Discussion

RA-targeted Hox genes compared between amphioxus and vertebrate embryos

In both amphioxus and vertebrates, A–P patterning is most sensitive to perturbations in RA signaling at the gastrula stage. However, the effects of perturbations have usually been assayed as alterations in gene expression, especially in the central nervous system (CNS) and endoderm, at developmental stages *subsequent to* the gastrula (Holland and Holland, 1996; Balmer and Blomhoff, 2002; Escriva

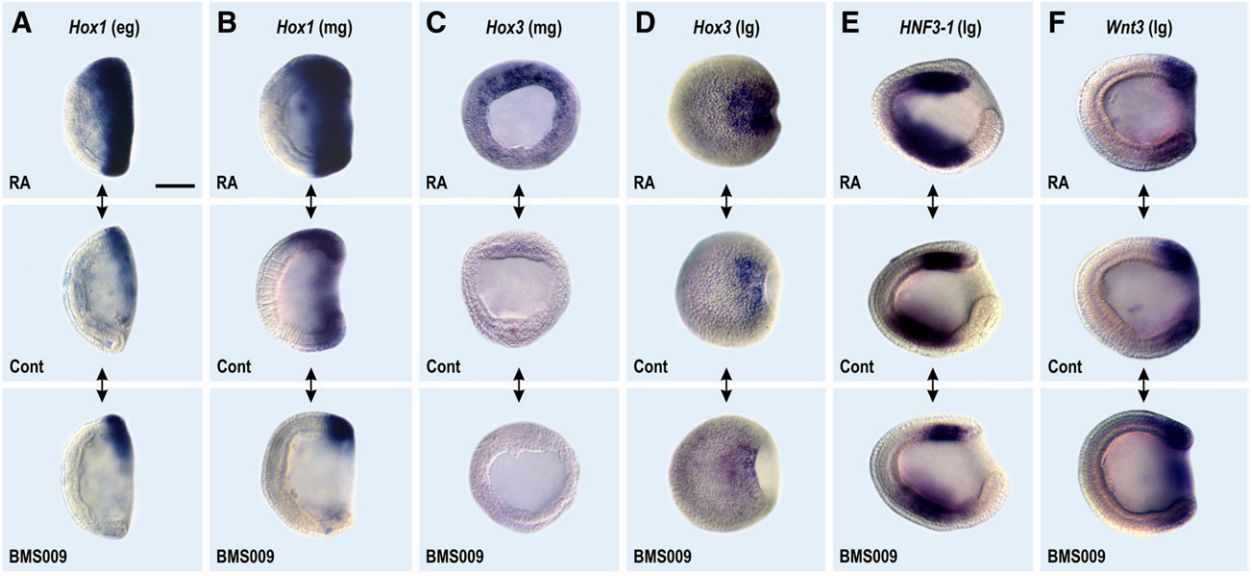

Fig. 3. Identification of direct retinoic acid (RA) signaling target genes in the amphioxus gastrula using puromycin assays. To block translation, amphioxus embryos were treated with puromycin from the start of gastrulation—prior to exposure with RA or RA antagonist (BMS009). For side views (A, B and E, F), anterior is to the left and dorsal is up, for blastopore views (C), dorsal is up and for dorsal views (D), anterior is to the left. This figure shows the expression of *Hox1* (A, B), *Hox3* (C, D), *HNF3-1* (E) and *Wnt3* (F) in controls as well as in embryos treated with either RA or BMS009 after exposure to puromycin. *Hox4* and *Hox6* were not appropriate for this experiment, because their expression begins at the very end of (*Hox4*) or just after (*Hox6*) the gastrula stage. Puromycin does not block the effects of RA or BMS009 on expression of *Hox1*, *Hox3* and *HNF3-1*. In contrast, puromycin blocks the effects of RA or BMS009 on *Wnt3* expression. For each gene, the vertical double-headed arrows facilitate aligning the control and experimental embryos. The 50-μm scale line in A is applicable to the entire figure. Abbreviations: (eg) = early gastrula; (mg) = mid gastrula; (lg) = late gastrula; Cont = control; RA = retinoic acid; BMS009 = RA antagonist.

et al., 2002; Schubert et al., 2005, 2006; Duester, 2008; Osborne et al., 2009). To date, very few studies in vertebrates have considered the effects of altered RA signaling on gene expression during the gastrula stage itself. Furthermore, these studies have focused on the effects of altered RA signaling on only a few genes, such as anterior *Hox, Cdx*, *Otx*, *Raldh2*, *Cyp26*, *Chordin*, *Iroquois*, *Meis* or *Sizzled* (Kudoh et al., 2002; Roelen et al., 2002; Li et al., 2008a; Ribes et al., 2009). In the present study, we considered the effects of altered RA signaling on 42 genes and covered both temporal and spatial changes in transcription (Supplementary Table S1). From our data and the relatively incomplete data for vertebrates, we conclude that the anterior *Hox* genes are the major components of the RA-sensitive gene network involved in the initial A–P patterning of amphioxus and vertebrate embryos (Fig. 4). The importance of the *Hox* genes is emphasized in our study by failure of altered RA signaling to change the expression of several dozen non-*Hox* genes (Supplementary Table S1).

Our puromycin experiments show that *Hox1* and *Hox3* are direct targets of RA. This result is not surprising in the light of the presence of RAREs in the control regions of *Hox1* and *Hox3* in amphioxus (Manzanares et al., 2000; Wada et al., 2006; Amemiya et al., 2008) and vertebrates (Balmer and Blomhoff, 2002; Mainguy et al., 2003; Oosterveen et al. 2003; Glover et al., 2006; Wada et al., 2006; Su and Gudas, 2008). The RAREs near amphioxus *Hox1* and *Hox3* are functional and, when linked to a reporter construct, can direct expression in chicken and mice in an RA-dependent manner (Manzanares et al., 2000; Wada et al., 2006). Moreover, the region of the amphioxus *Hox* cluster comprising *Hox1* through *Hox6* contains several potential RAREs (Supplementary Fig. S2) (Amemiya et al., 2008). Combined with the effects of RA or RA antagonist treatment on initial *Hox4* and *Hox6* expression, this suggests that, like *Hox1* and *Hox3*, *Hox4* and *Hox6* might also be directly regulated by RA. Likewise, in vertebrates, several *Hox* genes (i.e. *Hoxa1*, *Hoxb1*, *Hoxa3*, *Hoxa4*, *Hoxb4, Hoxd4, Hoxb5* and *Hoxb8*) are directly regulated by RA signaling (Balmer and Blomhoff, 2002; Mainguy et al., 2003; Oosterveen et al., 2003; Glover et al., 2006; Wada et al., 2006; Su and Gudas, 2008). Since both RA signaling and *Hox* genes were probably present in the last common ancestor of bilaterians (Deschamps, 2007; Campo-Paysaa et al., 2008; De Robertis, 2008), it will be interesting to assess, when in evolution the direct regulation of *Hox* genes by RA signaling first appeared.

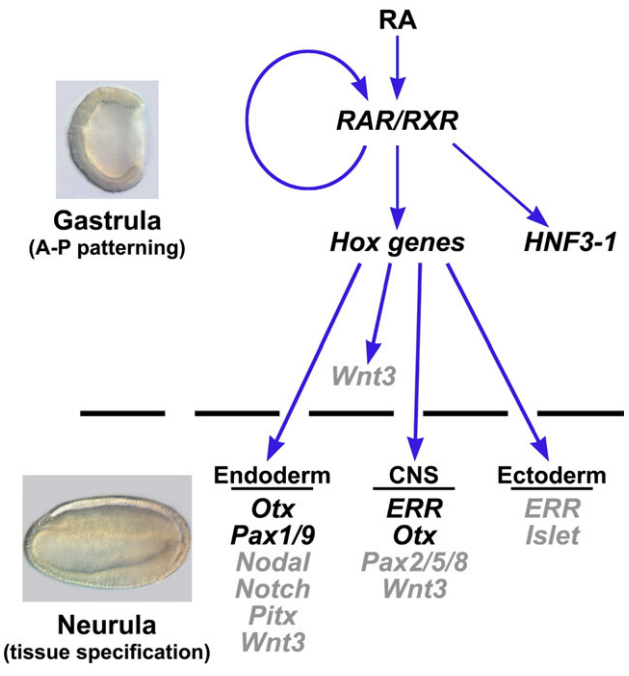

Fig. 4. The retinoic acid (RA) signaling network in the developing amphioxus embryo. Experimentally confirmed regulatory interactions are indicated in black and putative ones in grey. RA binds to RAR, whose expression is controlled by an autoregulatory loop (data not shown). RA patterns the anterior–posterior (A–P) axis during gastrulation by directly regulating expression of *Hox* genes and of *HNF3-1*. These genes are in turn involved in conferring positional identity during the neurula stage with *Hox* genes regulating genes, like *ERR*, *Otx* and *Pax2/5/8* in the central nervous system (CNS) or *ERR* and *Islet* in the general ectoderm (Schubert et al., 2004, 2006). In the endoderm, *Hox1* is involved in establishing the posterior limit of the pharynx by downregulating expression of pharyngeal genes, such as *Otx*, *Pax1/9*, *Nodal*, *Notch* and *Pitx*, in posterior foregut endoderm (Schubert et al., 2005). *Wnt3*, an indirect RA signaling target, might act downstream of *Hox* genes, such as *Hox1*, both in the gastrula and during later development in the endoderm and the CNS (Schubert et al., 2005, 2006).

Genes other than Hox that may be involved in RA signaling during A–P patterning

The present study included nine non-*Hox* genes (i.e. *Cdx*, *Hedgehog*, *HNF3-1*, *Nodal*, *Notch*, *Otx*, *Pitx*, *Wnt3* and *Wnt5*) known to have their expression altered in the amphioxus neurula and later stages by perturbations of RA signaling at the gastrula stage (Schubert et al., 2005, 2006; Osborne et al., 2009). However, we found that expression of only two of these genes (*HNF3-1* and *Wnt3*) was influenced by RA signaling at the gastrula stage (Fig. 4).

Our puromycin experiments indicate that, although amphioxus *Wnt3* is probably involved in early A–P patterning of the embryo, it is an indirect, not a direct, target of RA signaling. During gastrulation, the *Wnt3* domain overlaps that of *Hox1*, with *Hox1* being expressed earlier and more broadly than *Wnt3*. Similar patterns have previously been reported in the posterior foregut endoderm of amphioxus neurulae (Schubert et al., 2005) suggesting that *Wnt3* might act downstream of *Hox* genes, such as *Hox1* (Fig. 4). The expression patterns of seven other amphioxus *Wnt* genes (*Wnt1*, *Wnt4*, *Wnt5*, *Wnt6*, *Wnt7*, *Wnt8* and *Wnt11*), which signal through both the canonical and the Wnt/Ca^{++} pathways (Holland et al., 2000; Schubert et al., 2000a,b,c, 2001), as well as those of several Wnt antagonists (*Dkk1/2/4*, *Dkk3*, *sFRP2-like*, *sFRP3/4*) were unaffected by changing RA levels. In contrast, in vertebrates RA is known to affect *Wnt* gene expression and to suppress Wnt signaling possibly by competition of RAR and TCF for binding to β-catenin (Easwaran et al., 1999; Shum et al., 1999; Balmer and Blomhoff, 2002; Halilagic et al., 2007; Li et al., 2008a). Taken together, our results support the hypothesis that the complex interactions during early development between RA and *Wnt* genes may be vertebrate innovations (Onai et al., 2009).

Our puromycin experiments showed that *HNF3-1*, unlike *Wnt3*, is a likely direct target of RA signaling—a conclusion that is supported by the presence of three putative RAREs in the amphioxus *HNF3* locus (Supplementary Fig. S2). Amphioxus has a second *HNF3* gene, *HNF3-2*, due to an independent duplication (Shimeld, 1997; Wang et al., 2007), but its expression is not affected by RA signaling at the gastrula stage (data not shown). As in amphioxus, one of the *HNF3* genes in vertebrates (*HNF3α*) is a direct target of RA signaling, at least in cultured cells (Jacob et al., 1999).

Chordate *HNF3* genes are expressed in the endoderm during gastrulation and are crucial for proper expression of some genes in the anterior visceral endoderm in the mouse and for anterior endoderm development in tunicates (Olsen and Jeffery, 1997; Kimura-Yoshida et al., 2007). Furthermore, regulation of *Otx* and of several Wnt antagonists (e.g. *Dkk1* and *Cerl*) by *HNF3* in both vertebrates and tunicates suggests a conserved mechanism for *HNF3*-dependent specification of anterior identity (Lamy et al., 2006; Kimura-Yoshida et al., 2007). While it is not known whether *HNF3* genes regulate *Otx* expression in amphioxus, their domains overlap in the endoderm. In amphioxus, the effect of RA on *HNF3-1* expression is limited to the anterior mesendoderm in the gastrula and to the pharyngeal endoderm in the neurula and larval stages—domains where *Hox* genes are not expressed (Schubert et al., 2005). This suggests that RA regulation of *HNF3* in the amphioxus gastrula may be important for establishing the anterior endoderm, a feature that could be common to all chordates.

RA regulation of Hox genes in chordates: implications for evolution and mechanisms of development

Recent phylogenetic studies have placed amphioxus as the most basal chordate clade, with tunicates as sister group to the vertebrates (Philippe et al., 2005; Delsuc et al., 2008; Putnam et al., 2008). This arrangement makes amphioxus a favorable model for deciphering the evolutionary history of chordate development. The utility of amphioxus is also enhanced both by its morphological and genomic simplicity (Koop and Holland, 2008), with the unduplicated genome containing a single cluster of 15 *Hox* genes (Holland et al., 2008). Whether this *Hox15* gene arose by lineage-specific duplication or represents an ancestral chordate condition is unknown. RA signaling influences the spatial expression domains of these *Hox* genes as soon as their transcription has been initiated. In amphioxus, RA treatment results in *Hox* gene expression becoming detectable at earlier developmental stages, while maintaining temporal collinearity. Whether this is due to premature initiation of *Hox* gene expression or to upregulation of *Hox* gene expression is unclear. By the larval stage, expression of anterior *Hox* genes is expanded to the rostral tip in the CNS of RA-treated amphioxus (Schubert et al., 2004, 2006). RA sequentially induces *Hox* gene expression in human embryonal carcinoma cells (Simeone et al., 1990) and precociously initiates expression of *Hoxb1* and *Hoxb2* in mice and of *Hoxd4a* in zebrafish, while in *Raldh2* mutant mice, expression of *Hox1b* is both reduced and delayed (Niederreither et al., 1999; Roelen et al., 2002; Maves and Kimmel, 2005). Although prematurely activated *Hoxb1* expression initially followed the normal spatiotemporal pattern, a *Hoxb1* transgene did not activate prematurely in response to RA, suggesting the existence of global, *Hox* cluster-specific regulatory mechanisms controlling the initiation of *Hox* transcription (Roelen et al., 2002).

Hox genes are activated in a temporally collinear manner in amphioxus (Wada et al., 1999; Schubert et al., 2006). Expression of *Hox1*, *Hox3*, *Hox4* and *Hox6* is first detectable in the dorsal blastopore lip, most prominently in presumptive neuroectoderm. Their domains subsequently spread to the mesendoderm and expand anteriorly. In vertebrates, *Hox* gene expression is initiated in the mesodermal component of the blastopore margin (or equivalent) (Wacker et al., 2004) and then progresses anteriorly along the A–P axis. The temporal sequence of *Hox* initiation thus translates into a spatial sequence of *Hox* expression along the A–P body axis (Forlani et al., 2003; Wacker et al., 2004; Deschamps and van Nes, 2005; Jansen et al., 2007). Subsequent modification of *Hox* expression in mesoderm and neuroectoderm leads to the establishment of the definitive *Hox* codes (Forlani et al., 2003; Deschamps and van Nes, 2005). At least in the mouse, the initial temporal activation in the primitive streak is under the control of two antagonistic global regulators at either end of the *Hox* cluster, while the second phase is under the control of regulatory cues located within the *Hox* cluster (Tschopp et al., 2009).

Taken together, these results suggest that in amphioxus, as in vertebrates, temporal collinearity of *Hox* genes is initiated under the control of a global regulator, possibly sensitive to RA, in the dorsal blastopore margin, from which expression subsequently spreads anteriorly. Spatial collinearity seems to result from a temporally coordinated activation of *Hox* genes in a "*Hox* induction field" (Deschamps et al., 1999) with final spatial expression of *Hox* genes depending on regulatory cues located within the *Hox* cluster. These local cues include RAREs and hence direct regulation by RA signaling.

In sum, our data suggest that, while RA may play a role in the initiation of *Hox* expression in amphioxus, it is probably not required for the establishment and maintenance of *temporal* collinearity. In contrast, RA signaling is clearly important in controlling *spatial* collinear *Hox* expression in amphioxus after initiation of expression. It thus seems very likely that in the chordate ancestor, a "*Hox* induction field" was established in the posterior ectoderm at the onset of gastrulation, with RA signaling regulating *Hox* gene expression to establish positional identity along the developing A–P axis. RA regulation of *Hox* initiation in this ancestral chordate might have been important for the tight coordination of temporal and spatial collinearity in A–P patterning of new body segments as they were sequentially added during the posterior growth of the larva.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.11.016.

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