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Development of a chordate anterior–posterior axis without classical retinoic acid signaling

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Abstract

Developmental signaling by retinoic acid (RA) is thought to be an innovation essential for the origin of the chordate body plan. The larvacean urochordate *Oikopleura dioica* maintains a chordate body plan throughout life, and yet its genome appears to lack genes for RA synthesis, degradation, and reception. This suggests the hypothesis that the RA-machinery was lost during larvacean evolution, and predicts that *Oikopleura* development has become independent of RA-signaling. This prediction raises the problem that the anterior–posterior organization of a chordate body plan can be developed without the classical morphogenetic role of RA. To address this problem, we performed pharmacological treatments and analyses of developmental molecular markers to investigate whether RA acts in anterior–posterior axial patterning in *Oikopleura* embryos. Results revealed that RA does not cause homeotic posteriorization in *Oikopleura* as it does in vertebrates and cephalochordates, and showed that a chordate can develop the phylotypic body plan in the absence of the classical morphogenetic role of RA. A comparison of *Oikopleura* and ascidian evidence suggests that the lack of RA-induced homeotic posteriorization is a shared derived feature of urochordates. We discuss possible relationships of altered roles of RA in urochordate development to genomic events, such as rupture of the *Hox*-cluster, in the context of a new understanding of chordate phylogeny.
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Keywords: Retinoic acid; Chordate development; Axial patterning; Chordate evolution; ALDH1A; RALDH; CYP26; RAR; RXR; ALDH2; HOX; OTX; PAX; DEAB; *Oikopleura*; Larvacean; Appendicularian; Ascidian; Tunicate; Urochordate; Amphioxus; Vertebrate; Olfactores

Introduction

The phylum Chordata, including its three subphyla (vertebrates, cephalochordates and urochordates (or tunicates)), is characterized by a notochord, dorsal neural tube, a muscular post-anal tail and pharyngeal slits. The morphogenetic role of all-*trans*-retinoic acid (RA) in anterior–posterior (AP) axial patterning of the chordate embryo is considered crucial for the development of the chordate body plan (reviewed in Fujiwara and Kawamura, 2003; Fujiwara, 2006; Marletaz et al., 2006; Schilling and Knight, 2001; Shimeld, 1996). The axial morphogenetic role of RA-signaling is in part mediated by the regulation of *Hox* gene expression (Marshall et al., 1994; Yan et al., 1998). *Hox* genes are expressed in a spatial and temporal collinear fashion based on their organization in a gene cluster, and provide an AP developmental code for the embryo (Gavalas, 2002; Kiecker and Lumsden, 2005; Kmita and Duboule, 2003; Krumlauf, 1994; Lumsden and Krumlauf, 1996). Excess RA in vertebrates induces a rostral expansion of the *Hox1* expression domain that transforms anterior structures into more posterior fates by a homeotic posteriorization (Durston et al., 1989).

Developing embryos regulate RA action at two levels: metabolism and signaling. In vertebrates, the metabolic level includes the enzymes that synthesize and degrade RA. Retinol dehydrogenases and alcohol dehydrogenases (Rdh and Adh) convert retinol into retinaldehydes; retinaldehyde dehydrogenases (Aldh1a, formerly Raldh) catalyze the conversion of retinaldehydes into retinoic acid; and 4-hydroxylases of the cytochrome P450 family-26 (Cyp26) degrade RA (reviewed in Ross et al., 2000). The regulation of these enzymes controls the spatio-temporal distribution of RA during vertebrate embryogenesis (Maden et al., 1998; Reijntjes et al., 2005 and references

Abbreviations: RA, retinoic acid; AP, anterior–posterior; CNS, central nervous system.

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therein). In contrast to vertebrates, the metabolism of RA in cephalochordates and urochordates is currently not well understood. Extensive independent gene duplications and losses affecting the *Adh*, *Rdh*, *Aldh1a* and *Cyp26* families have occurred during the evolution of non-vertebrate chordates, and probably modified the spatio-temporal distribution of RA in these organisms (Belyaeva and Kedishvili, 2006; Cañestro et al., 2000, 2002, 2003, 2006; Dalfó et al., 2001, 2002; Gonzàlez-Duarte and Albalat, 2005; Irie et al., 2004; Nagatomo and Fujiwara, 2003).

At the signaling level, the regulation of classical RA action on target genes in vertebrates is mediated by the RA-binding nuclear hormone receptors (Rars), which form heterodimers with the Rxr nuclear hormone receptors and recognize retinoic acid response elements (RAREs) of target genes (Dupé et al., 1999; Mark et al., 2006; Nolte et al., 2003, 2006). As with RA synthesizing and degrading enzymes, *Rar*s and *Rxr*s have undergone extensive lineage-specific gene amplification (Bastien and Rochette-Egly, 2004; Bertrand et al., 2004; Hale et al., 2006; Tallafuss et al., 2006). Cephalochordates and vertebrates share homologous mechanisms of classical Rar-mediated RA-signaling (Escrivà et al., 2002, 2006), and RAREs from amphioxus *Hox* genes can drive homologous expression of reporter genes in transgenic vertebrates (Manzanares et al., 2000; Wada et al., 2006a,b). Ascidian urochordates also have *Rar* and *Rxr* genes, but the mechanism of RA-signaling in these animals seems to have important differences with other chordates, for example, *Rar* is not up-regulated by RA as it is in other chordates (Devine et al., 2002; Escrivà et al., 2006; Hisata et al., 1998; Kamimura et al., 2000; Nagatomo et al., 2003).

In vertebrates, in addition to the classical Rar-mediated RA-signaling, RA can also play important physiological roles independent of Rar by the direct binding of RA to other proteins (Notario et al., 2003; Ochoa et al., 2003). For example, RA modulates the activity of protein kinase C alpha (PRKCA), a key enzyme regulating cell growth, differentiation and apoptosis (Radominska-Pandya et al., 2000). RA can bind directly to the C2-domain of PRKCA, and binding competition between all-*trans*-RA and acidic phospholipids can regulate the activity of PRKCA (Ochoa et al., 2003). In non-vertebrate chordates, we do not yet know whether RA can play similar non-Rar mediated roles.

Because the main components of the RA genetic machinery (i.e. *Aldh1a*, *Cyp26* and *Rar*) had been described only in chordates, it had been hypothesized that the acquisition of these gene families was a key step for the innovation of the chordate body plan (reviewed in Fujiwara and Kawamura, 2003; Fujiwara, 2006). In a recent genomic survey, however, *Aldh1a*, *Cyp26* and *Rar* genes were identified in non-chordate deuterostomes, thus questioning whether chordates 'invented' RA-signaling (Cañestro et al., 2006; Marletaz et al., 2006; Simoes-Costa et al., in press). Another unexpected finding was the absence of *Aldh1a*, *Cyp26* and *Rar* orthologs in the deep genome database (9-fold redundancy) of the larvacean *Oikopleura dioica*, suggesting the hypothesis that the RA genetic machinery was lost during larvacean evolution (Cañestro et al., 2006). This hypothesis predicts that, despite its chordate body plan, *Oikopleura* development might occur independent of the classical morphogenetic role that RA plays in other chordates.

Larvaceans, together with ascidians and thaliaceans, belong to the urochordate subphylum. Larvacean and ascidian embryos share an overall similar developmental program (Bassham and Postlethwait, 2000, 2005; Cañestro et al., 2005; Seo et al., 2004), but larvaceans, in contrast to other urochordates, do not undergo a drastic metamorphosis and maintain a chordate body plan throughout their life (Willey, 1894). The hypothesis that the RA-machinery has been lost during larvacean evolution raises the problem that the AP axial patterning of a chordate body plan can be developed and maintained in the absence of the classical morphogenetic role of RA, which has been assumed to be a chordate synapomorphy. To test this hypothesis and address this problem, we have investigated whether exogenous RA perturbs AP axial patterning during the development of *Oikopleura* embryos in a fashion similar to some other chordates.

Materials and methods

Animals and pharmacological treatments

Oikopleura dioica was collected from the Pacific Ocean at the Oregon Institute of Marine Biology (Charleston, OR, USA) and animals were cultured in the laboratory at the University of Oregon (Eugene, OR, USA) at 13 °C in 10-μm filtered sea-water (FSW) for several generations. Multi-individual matings were performed, with occasional out-crossing of animals from the natural population to decrease inbreeding. Animal culture and embryo staging was as described (Cañestro et al., 2005).

Stock solutions of 0.1 M all-*trans*-retinoic acid (RA) (Sigma), 9-*cis*-retinoic acid (9-*cis*-RA)(Sigma) and 4-(Diethylamino)-benzaldehyde DEAB (Sigma) were prepared in DMSO and stored at −80 °C, avoiding exposure to light. To minimize precipitation, stocks were diluted in FSW to working concentrations by vigorous vortexing at room temperature. After in vitro fertilization, pharmacological treatments were performed by immediately transferring the zygotes into 4 mL containing the drugs at working concentrations in 35 × 10 mm polystyrene petri dishes (Falcon #353001). Treatments were stopped by diluting 20 μL with the treated embryos into 4 mL of FSW (200 fold dilution). Control embryos received DMSO. To avoid the trapping of swimming hatchlings by surface tension, we placed one pearl of 1-Hexadecanol (Sigma) floating on the water surface.

Gene cloning, whole-mount in situ hybridization and detection of β-galactosidase activity

In a genomic survey, we found *Oikopleura* orthologs of *Aldh2*, *Rxr1* and *Rxr2* in the sequence trace file database generously made publicly available at NCBI (ftp.ncbi.nih.gov/pub) by D. Chourrout. Fragments from each gene were amplified by PCR, using gene specific primers (*Aldh2*-F: 5′TGGAACTTC-CCTCTCCTCATGCA3′, *Aldh2*-R: 5′TTATTTGGCGTATTGAGGAAGTTT-CAT3′; *Rxr1*-F: 5′GCCAGTGGAAAACATTATGGTGT3′, *Rxr1*-R: 5′ CCAATTGATCGCAGGGCTGG3′; *Rxr2*-F: 5′GCCTCTGGAAAACAC-TACGG3′, *Rxr2*-R: 5′CCAACGGTTTTGAACAGGAACAT3′) and genomic DNA or cDNA as template. The PCR-amplified gene fragments were cloned into pCR4-TOPO (Invitrogen) and used to synthesize riboprobes for whole-mount in situ hybridization as described (Bassham and Postlethwait, 2000) with these minor modifications: animals were dechorionated manually with glass needles before re-hydration; Tween-20 concentration was increased from 0.1% to 0.15% in the hybridization buffer, in the PBT solution and in the post-hybridization washing buffers; and embryos were mounted in 80% glycerol for microscopy. Gene sequences are available as Genbank accession numbers: *Aldh2,* DQ841186; *Rxr1*, DQ841187; and *Rxr2,* DQ841188. Riboprobes for

detecting the expression of *Otxa*, *Otxb*, *Otxc*, *Hox1*, *Pax2/5/8a* and *Pax2/5/8b* genes are described in (Cañestro et al., 2005). Animals for detection of β-galactosidase activity were fixed in 1 mM EGTA, 2 mM $MgSO_4$, 0.1 M MOPS, 0.5 M NaCl, and 0.2% glutaraldehyde for 30 min at room temperature. Detection of β-galactosidase activity and imaging were performed as described (Cañestro et al., 2001, 2005).

Sequence alignment and phylogenetic analysis

Rxr sequence alignments were generated with clustalX (Thompson et al., 1997). A neighbor-joining phylogenetic tree corrected by a Poisson distribution of amino acid substitutions was constructed with the MEGA package (Kumar et al., 2001), using the segment of the alignment covered by the two *Oikopleura* Rxr paralogs, which corresponds to the least variable and most reliable part of the Rxr alignment, with a thousand bootstrap repetitions. Mollusk Rxr proteins were used to root the deuterostome Rxr phylogenetic tree.

Results

Morphological effects of exogenous retinoic acid treatment in Oikopleura

To investigate the possible effects of all-*trans*-retinoic acid (RA) on *Oikopleura* development, we exposed embryos to different RA-treatment regimens and scored for animals that successfully completed development by counting the number of embryos that could develop to tailshift stage (tail at an acute angle relative to trunk) and inflate their first house. Although control DMSO-treatments did not alter development of *Oikopleura* embryos compared to untreated controls, exogenous RA-treatments produced a range of morphological abnormalities, varying from mild to severe, depending on the developmental stage, duration, and concentration of RA exposure (Fig. 1).

The RA dose–response curve of developing *Oikopleura* is comparable to that for ascidians (Hinman and Degnan, 1998). Treatments at 10^{-5} M RA were toxic for both urochordate taxa and embryos did not develop beyond a few rounds of abnormal cleavage. Treatments at 10^{-6} M RA induced a consistent set of developmental abnormalities in both urochordate taxa (Fig. 2). Treatments at 10^{-7} M RA did not affect *Oikopleura* development, and only induced minor and variable defects in ascidians (Hinman and Degnan, 1998). To readily compare *Oikopleura* results with those for ascidians, we used standard working concentrations of 1×10^{-6} M to 2.5×10^{-6} M RA to induce mild or more severe phenotypes.

Embryos continuously cultured at 2.5×10^{-6} M RA from 1-cell stage did not display any obvious morphological abnormality until hatchling stages (for a detail description of *Oikopleura* morphology and developmental stages see Cañestro et al., 2005). The development of RA-treated embryos was heterogeneously delayed in comparison to DMSO-treated controls. At 24 h after fertilization, all continuously RA-treated embryos were unable to inflate their first house and fewer than

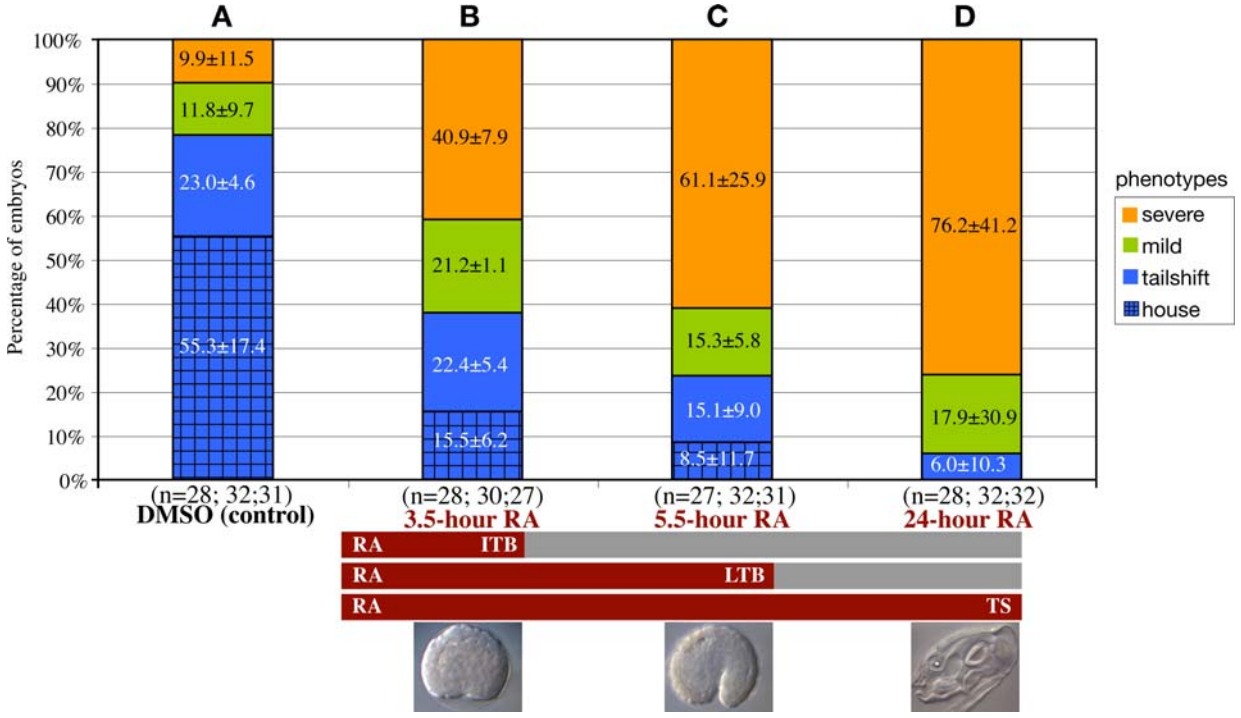


Fig. 1. Effects of exogenous RA treatments on *Oikopleura* development. Embryos were cultured in 2.5×10^{-6} M RA from fertilization until specific developmental stages (ITB, incipient-tailbud stage; LTB, late-tailbud stage; and tailshift-stage) and then transferred to filtered sea water. Phenotypes were scored 24 h after fertilization when the majority of the DMSO-controls were at tailshift stage and had inflated their houses. RA treatment experiments were repeated three times. The average numbers of animals showing different phenotypes are represented in this stacked column graph: blue grid, apparently normal tailshift larva residing in a successfully constructed house; blue, normal tailshift larva that either have not yet inflated their first house, or that have already abandoned a house and are in the process of inflating the next one; green, larvae with a body morphology slightly disrupted that have not achieved the tailshift stage; orange, larvae with severely perturbed developmental abnormalities. Numbers in each category are the percentage plus or minus the standard deviation. *n* = number of animals scored in each condition for the three repetitions.

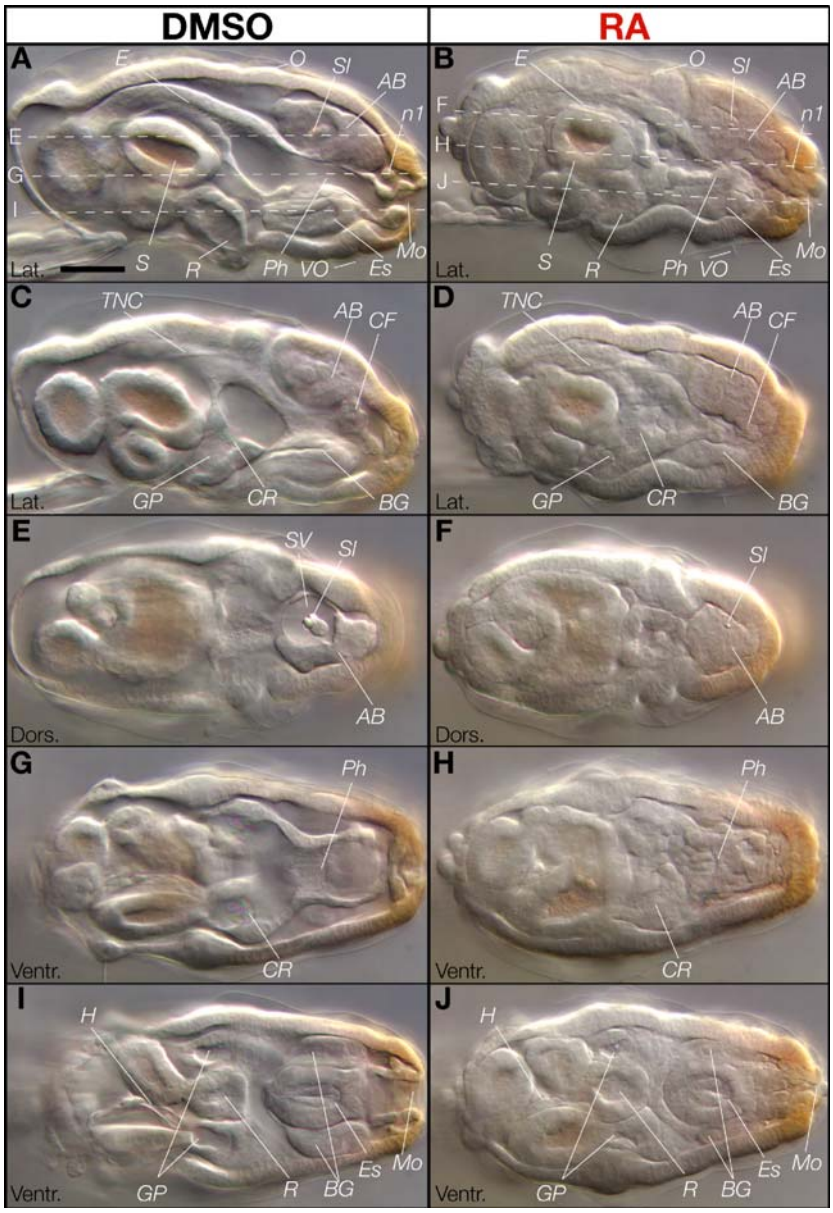


Fig. 2. Morphological alterations induced by exogenous RA-treatment during *Oikopleura* development: the compact trunk phenotype. Differential interference contrast (DIC) images of live late-hatchlings showing normal development in the presence of DMSO (left panels: A, C, E, G and I) and a mildly affected late-hatchling showing the characteristic compact trunk phenotype, including a short trunk depleted of internal cavities induced by exogenous treatment at 10^{-6} M RA (right panels: B, D, F, H and J). Lateral (A–D), dorsal (E, F) and ventral (G–J) views at different focal planes show various aspects of the internal trunk cavities. Despite some malformations, most organs can be clearly recognized. Dotted lines in panels A and B indicate approximate levels for dorsal and ventral panels below. Abbreviations (in italics): *AB*, anterior brain; *BG*, buccal gland; *CF*, ciliary funnel; *CR*, ciliary ring; *E*, esophagus; *Es*, endostyle; *GP*, gill pouch; *H*, heart; *Mo*, mouth; *O*, oikoblast; *n1*, anterior paired-nerve; *Ph*, pharynx; *R*, rectum; *S*, stomach; *St*, statolith; *SV*, sensory vesicle; *TNC*, trunk nerve cord; *VO*, ventral organ. Scale bar (20 μm) for all images is as in panel A.

10% were able to reach tailshift stage (blue and grided bars in Fig. 1 column D). Experiments in which RA-treatments starting at 1-cell stage were stopped at incipient-tailbud stage – more than 2 h before hatching – showed that the proportion of larvae that achieved tailshift stage was significantly reduced, and only about 15% were capable of inflating the first house (Fig. 1 column B). This observation is important because it indicates that the chorion is not a barrier that blocks response to RA. In general, longer RA treatments cause more severe phenotypes (Figs. 1B–D). Animals treated with RA from incipient-tailbud or late-tailbud stage until tailshift stage presented the same type of developmental abnormalities, following the trend that shorter treatments produced fewer severe phenotypes (data not shown).

After hatching, embryos continuously treated with 2.5×10^{-6} M RA from the one-cell stage displayed a shortened trunk and aberrant tail phenotype (some examples of body shape malformations are shown in Figs. 3 and 8). During the normal development of *Oikopleura*, internal body cavities expand in late-hatchling stages (Cañestro et al., 2005; Fenaux, 1998), but RA-treated animals had reduced or absent internal

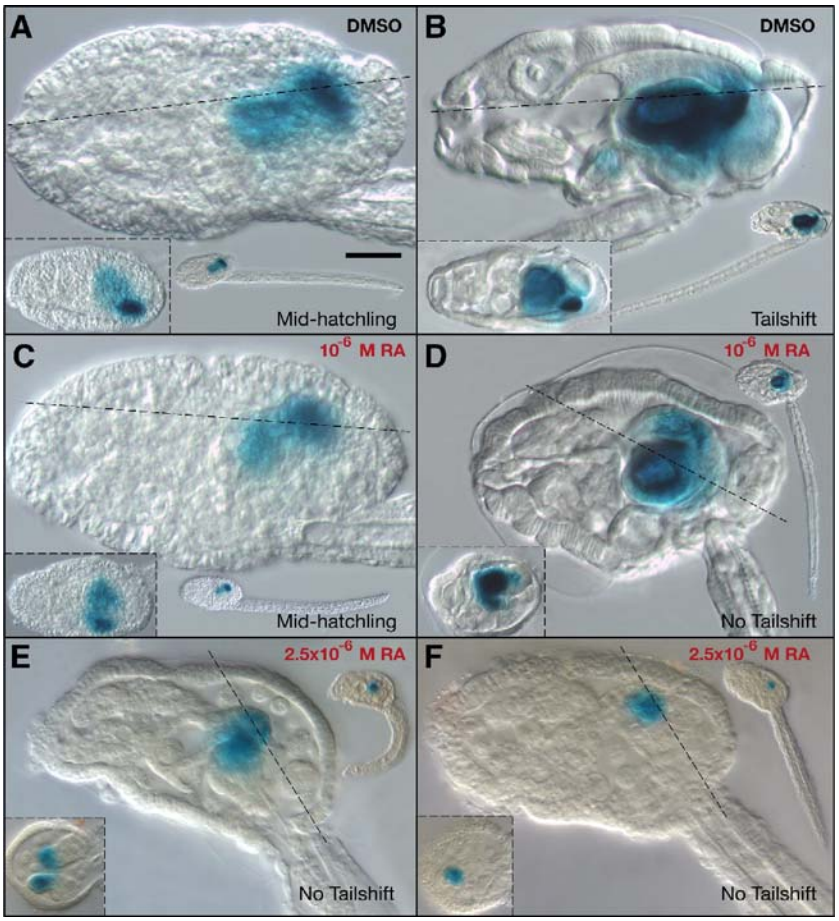


Fig. 3. Endogenous β-galactosidase activity, a marker of AP position along the digestive tract of *Oikopleura*. (A–B) DMSO-treated controls (C–F) RA-treated embryos. (A) Mid-hatchling and (B) tailshift stage of DMSO-control embryos showing β-galactosidase activity in the right stomach lobe and in the junction between the esophagus and the left stomach lobe during normal development. (C) 10^{-6} M RA-treated larva at mid-hatchling stage, (D) 10^{-6} M RA and (E–F) 2.5×10^{-6} M RA-treated larva 24 h after fertilization that have not achieved the tailshift stage (no tailshift) and display different degrees of *compact trunk* phenotypes. No embryos showed anterior expansion of the β-galactosidase activity domain. All panels show left lateral views of the trunk in the central image, and include an inset with a dorsal view (posterior in panels E and F) in the bottom-left corner corresponding to the level of the dashed line in the central image. A small view of the entire animal to visualize the shape and overall phenotypic severity induced by the RA-treatment is also shown. Scale bar (20 μm) for all central images is as in panel A.

cavities in the trunk (compare the morphology of DMSO-control in the left panels in Fig. 2 to an RA-treated animal in the right panels): the mouth cavity (*Mo*) was reduced, the roof and floor of the pharynx (*Ph*) collapsed together, and the walls of the gill pouches (*GP*) at both sides of the ciliary rings were also collapsed. In some severe phenotypes, the volume of the sensory vesicle (*SV*) within the anterior brain (*AB*) was also drastically reduced, coupled with altered formation of the statolith (*Sl*) (Figs. 2E, F). Despite RA-induced cavity malformations of the trunk, most organs were recognizable morphologically, suggesting that RA did not affect the differentiation and structural diversity of organs during *Oikopleura* development (Fig. 2). We designate the RA-induced shortened trunk depleted of internal cavities as the *compact trunk* phenotype, and it is the most characteristic developmental defect induced by exogenous RA in *Oikopleura* (Fig. 2). This phenotype is similar to that reported in ascidians treated with RA, called *shortened head* or *moonfaced* (Hinman and Degnan, 1998; Katsuyama and Saiga, 1998).

Does retinoic acid induce homeotic posteriorization of the endoderm in Oikopleura?

In vertebrates and cephalochordates, exogenous RA alters patterning of the AP axis both in endodermal derivatives (i.e. digestive tract and pharynx) and in ectodermal derivatives (i.e. nervous system and epidermis). Does RA have the same effect on *Oikopleura* development? To answer this question, we analyzed whether exogenous RA alters histochemical markers and gene expression patterns along the AP axis.

Endogenous β-galactosidase activity is a useful marker for the functional organization of the digestive system in amphioxus (Cañestro et al., 2001). During normal amphioxus development, β-galactosidase activity appears in the posterior half of the digestive tract (from the anterior part of the mid-gut to the hind-gut), but after RA treatments, β-galactosidase activity expands to the most anterior part of the amphioxus digestive system (Cañestro et al., 2001), showing that RA induces a homeotic transformation of the anterior endoderm

(the pharynx) into a more posterior fate (mid- or hindgut) in amphioxus (Holland and Holland, 1996; Schubert et al., 2005). In *Oikopleura,* as in amphioxus, we found that the digestive system possesses endogenous β-galactosidase activity that serves as a specific histochemical marker for the posterior half of the digestive tract, the stomach and gut (Figs. 3A, B). In contrast to amphioxus, however, RA-treated *Oikopleura* embryos did not show any anterior expansion of β-galactosidase activity in the digestive system (Figs. 3C, D), even in severe phenotypes in which the organs were barely recognizable (Figs. 3E–F).

β-galactosidase staining of *Oikopleura* showed that the function of the anterior endoderm was not posteriorized by RA treatments, but we wondered whether RA otherwise changed cell fates in the digestive tract. To test for additional possible fate changes, we analyzed the expression patterns of developmental genes that are expressed during the specification and differentiation of the pharynx and associated structures. In addition to the previously described expression patterns of three *Otx* paralogs (*Otxa*, *Otxb* and *Otxc*) and two *Pax2/5/8* paralogs (*Pax2/5/8a* and *Pax2/5/8b*) in the *Oikopleura* central nervous system (CNS) (Cañestro et al., 2005), we describe here for the

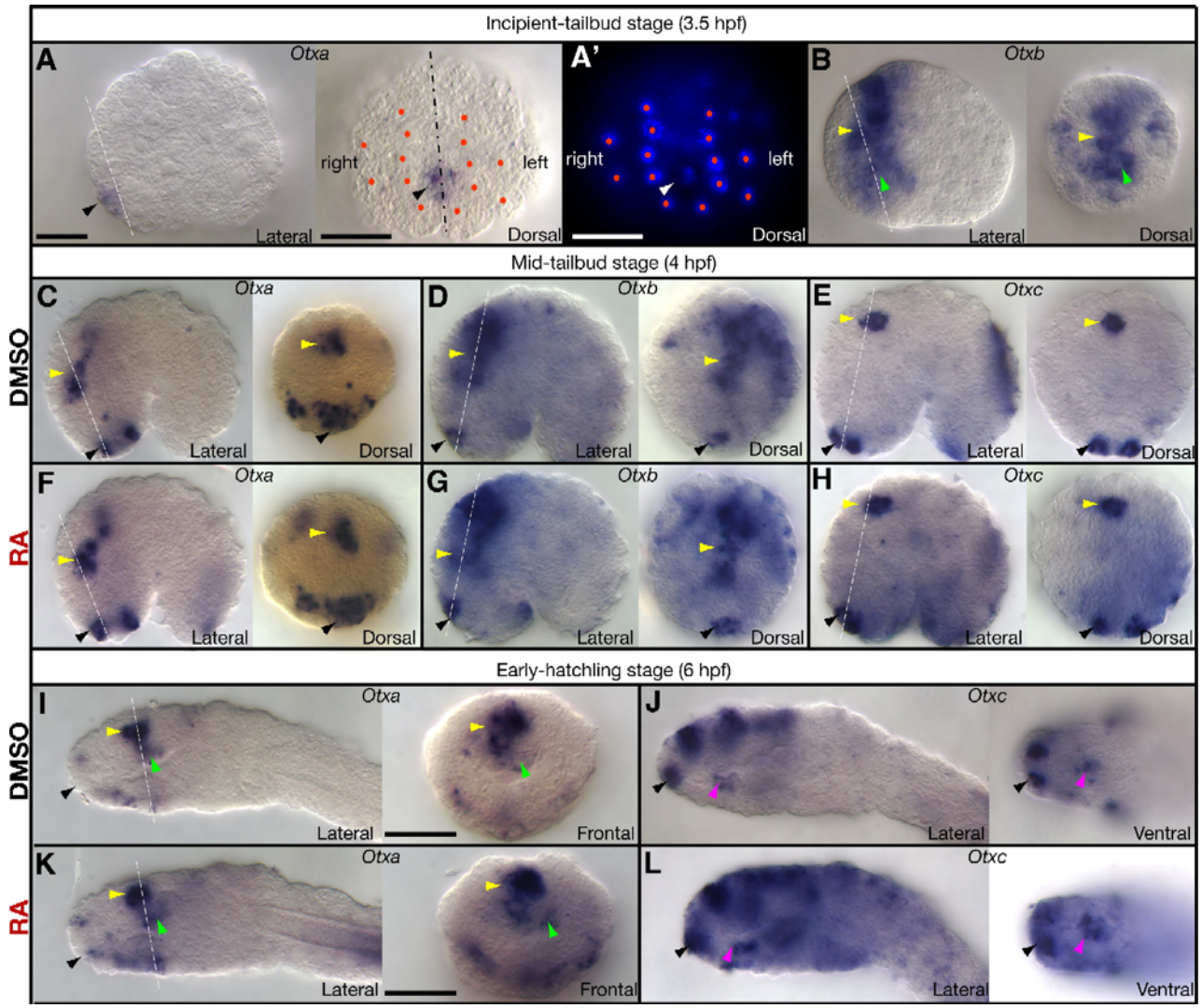


Fig. 4. Analysis of the expression patterns of *Oikopleura*'s three *Otx* paralogs (*Otxa*: A, C, F, I and K; *Otxb*: B, D and G; *Otxc*: E, H, J and L) in the absence or presence of RA. The left part of each panel shows a left lateral view of the embryos, and the right panel shows details of the *Otx* expression patterns in dorsal views (anterior is down), frontal views at the level of the white dashed line in the lateral views (dorsal is up) or ventral views (anterior is left). By incipient-tailbud stage, the stomodeal component of the pharynx was labeled by the expression of *Otxa* (black arrowhead) (A), while the presumptive pharyngeal endoderm was labeled by *Otxb* expression (green arrowhead) (B). (A′) Analysis of cell distribution (red dots) by nuclear staining helps to visualize the right–left symmetry (dashed line), and suggests that the stomodeum in *Oikopleura* does not have a bilateral paired origin, but derives from a single cell in the midline (arrowhead) expressing *Otxa*. At mid-tailbud stage, the anterior endoderm down-regulated *Otxb* expression, and the presumptive stomodeum expressed all three *Otx* paralogs, but in different patterns for each gene (C–E). At the early-hatchling stage, *Otxc* (but not *Otxb* or *Otxa*) expression appeared in the stomodeal region, a new *Otxa* expression domain appeared in the roof of the pharynx (I) and *Otxc* was expressed in the anterior part of the endostyle (J). Expression of each *Otx* paralog was maintained from early to late hatchling stages (Cañestro et al., 2005). 2.5×10^{-6} M RA-treated embryos (F–H, K–L) do not show obvious differences from DMSO-controls (C–E, I–J). Colored arrowheads point to the stomodeum (black), presumptive pharyngeal endoderm (green), endostyle (pink) and anterior brain (yellow). Scale bar (20 μm) for all panels is as in panel A, unless another bar is indicated.

first time the expression of *Otx* paralogs in the stomodeal component of the primordial pharynx (black arrowheads in Figs. 4A–E) and anterior endoderm (green arrowhead in Fig. 4B) at tailbud stages, and in the pharynx at mid-hatchling stages (green arrowhead in Fig. 4I). RA-treatments at 2.5×10^{-6} M RA did not perturb the expression pattern of any of the three *Oikopleura Otx* paralogs. As a control for the effectiveness of these RA treatments, we allowed some of the embryos from each experimental batch to develop to hatchling stages, and found the compact trunk phenotype in each case, confirming the efficacy of the RA treatments despite normal gene expression patterns.

To help understand mechanisms leading to the compact trunk phenotype, we followed organogenesis in live animals from late-hatchling stage to tailshift stage and examined expression patterns of two *Oikopleura Pax2/5/8* paralogs that are expressed in specific compartments of the digestive system (Cañestro et al., 2005; and Bassham S., Cañestro C. and Postlethwait J.H., unpublished). Observation of live animals revealed that, although the differentiation and morphogenesis of embryonic organs, including lips, ciliary rings, gill slits and ciliary epithelia of the pharynx, appeared to be the same in DMSO-treated controls and RA-treated animals, the normal expansion of the internal cavities (black dots in the posterior part of the digestive system and asterisks in the anterior half of the trunk of DMSO-controls Figs. 5A–C) was severely diminished in RA-treated animals (Figs. 5D–F). The opening of the gill slits was also affected, probably as a result of the failure to expand the gill pouch cavity (see inserts in Fig. 5 corresponding to a lateral focal plane showing the gill pouches). *Pax2/5/8a* and *Pax2/5/8b* expression domains in the endostyle, gill pouches, rectum and pharynx, were similar in DMSO-treated controls and RA-treated animals despite the apparent compact trunk phenotype (Figs. 5G–J). These results show that, while RA-treatments affect the formation of body cavities, they do not change the fate of anterior structures of the digestive system nor do they induce homeotic posteriorization of the endoderm in *Oikopleura*.

Does retinoic acid induce homeotic posteriorization of the CNS and epidermis in Oikopleura embryos?

Although the experiments reported above show that exogenous RA does not posteriorize the endoderm in *Oikopleura* embryos, it remained possible that it posteriorizes ectodermal derivatives (i.e., CNS and epidermis), as it does in vertebrates and cephalochordates (Conlon, 1995; Escrivà et al., 2002; Holland and Holland, 1996; Kiecker and Lumsden, 2005; Schubert et al., 2004, 2005, 2006). To test whether excess RA can alter the AP organization of the *Oikopleura* CNS or

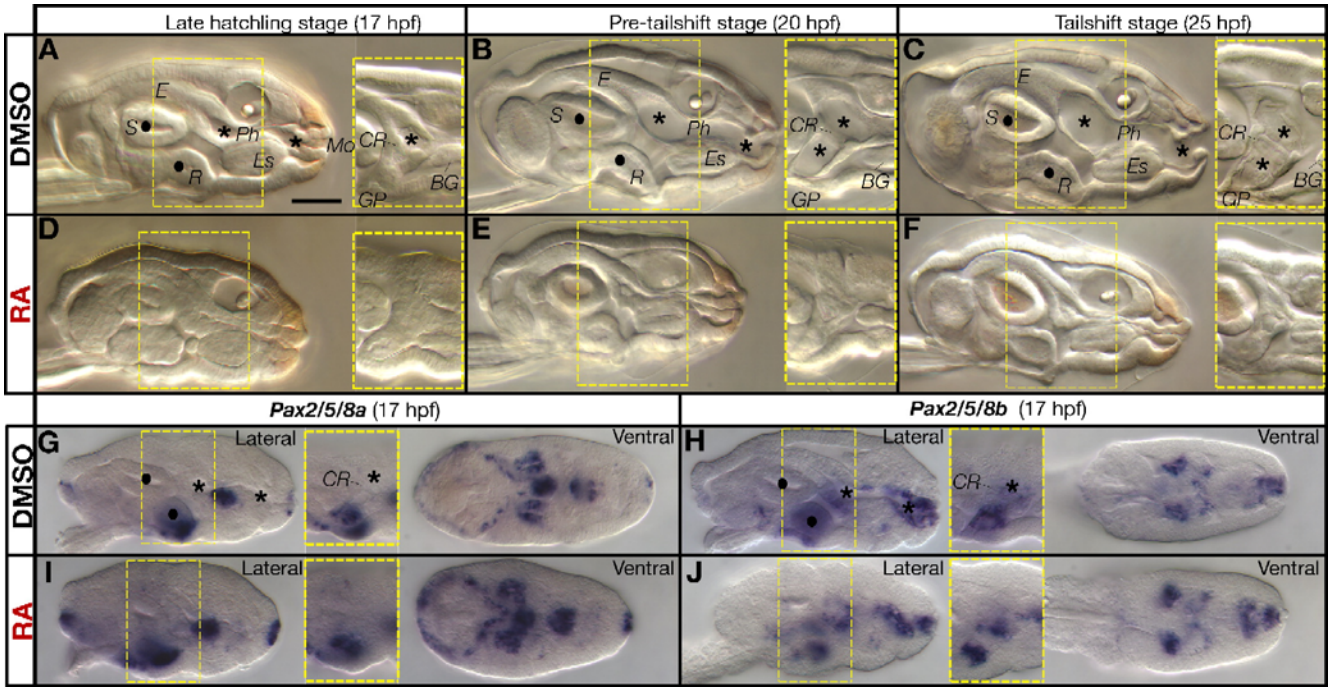


Fig. 5. Analysis of living embryos and gene expression patterns. (A–F) Developmental series showing the formation of internal cavities within the trunk in the absence (A–C) or presence of RA (D–F), at 17 h post-fertilization (hpf) (A, D), 20 hpf (B, E) and 25 hpf (C, F) in live specimens. Anterior is to the right and dorsal to the top. Insets to the right of each panel show a more lateral focal plane at the AP level of the dotted squares showing the formation of the gill slit. In general, the cavities of the anterior half of the trunk (asterisks in the mouth, pharynx and gill slits) are more sensitive to the presence of exogenous RA than the cavities of the posterior half of the digestive system (solid circles in stomach and rectum). Abbreviations are as in Fig. 2. (G–J) Analysis of the expression pattern of *Oikopleura Pax2/5/8a* (G–I) and *Pax2/5/8b* (H–J) paralogs during the initiation of the expansion of the internal cavities at late hatchling stage in DMSO-controls (G–H) and RA-treated animals (I–J). During normal development *Pax2/5/8a* is expressed in the stomodeum, dorsal part of the endostyle, distal part of the gill pouches, and ventral epidermis, and *Pax2/5/8b* is expressed in the pharynx, ventral part of the endostyle, heart, and proximal and distal part in the gill pouches. Animals treated with RA displayed obvious compact trunk phenotypes with shorter trunks and absence of internal cavities, but normal *Pax2/5/8a* and *Pax2/5/8b* expression patterns, suggesting that RA does not induce posteriorization in the pharynx or associated structures, in contrast to vertebrates and cephalochordates. Scale bar is 20 μm.

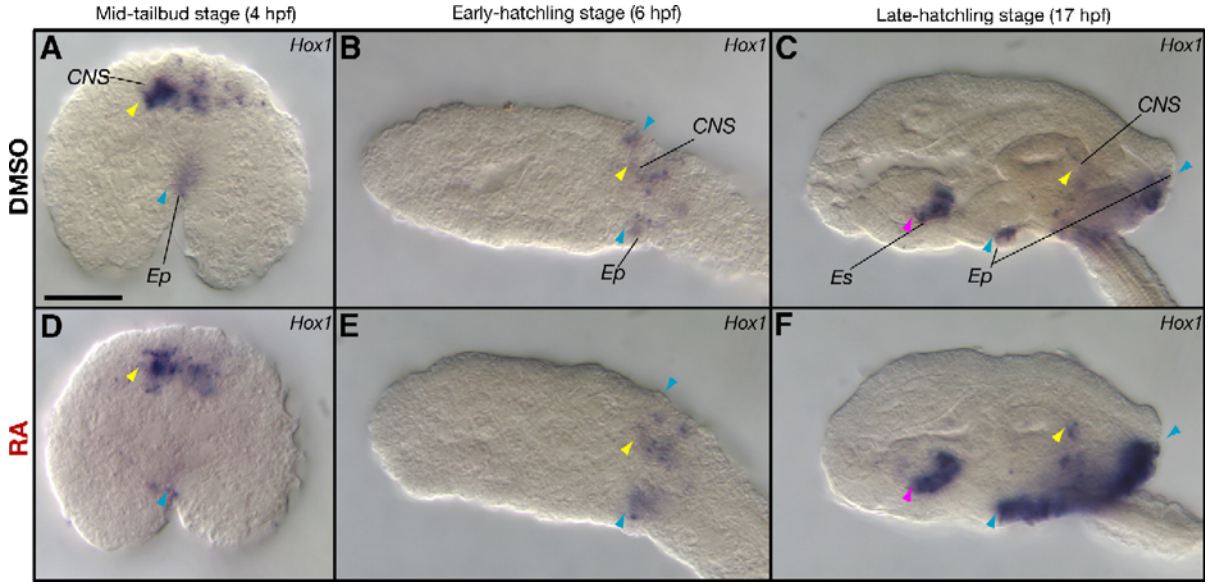


Fig. 6. RA treatments do not cause *Hox1* expression domains to expand rostrally. Analysis of the *Hox1* expression pattern in DMSO controls (A–C) and 2.5×10^{-6} M RA-treated (D–F) animals during *Oikopleura* development (A, D: mid-tailbud stage; B, E: early-hatchling stage; and C–F: late-hatchling stage). RA did not induce rostral expansion of the anterior boundaries of any of the *Hox1* expression domains. All images correspond to a left lateral view. Arrowheads label expression in the hindbrain (yellow), posterior endostyle (pink) and the epidermis at the trunk-tail transition area (blue). Scale bar is 20 μm.

epidermis, we compared the expression patterns of *Otx* and *Hox1* in animals treated with RA and in DMSO controls at various developmental stages (Figs. 4, 6). Experimental animals showed no expansion, reduction, or anterior displacement of *Otx* or *Hox1* expression domains (even after prolonged staining) that would be predicted by the hypothesis that RA perturbs the AP regionalization of the CNS (yellow arrowheads in Figs. 4, 6) and epidermis (blue arrowheads in Figs. 4, 6) in *Oikopleura*. Thus, these critical tests revealed no evidence for RA-induced posteriorization in either the ectoderm or endoderm.

Characterization of Oikopleura Aldh2, Rxr1 and Rxr2

Although the main components of the RA genetic machinery – *Aldh1a, Cyp26* and *Rar* – appear to be absent from the deep *Oikopleura* genome database (Cañestro et al., 2006), genes closely related to two of these gene families – *Aldh2* and *Rxr* – should be considered to evaluate the hypothesis that they could be playing a role in either the production or binding of RA in *Oikopleura*. To test this possibility, we cloned and analyzed the expression patterns of *Aldh2*, *Rxr1* and *Rxr2* during *Oikopleura* development in the presence and absence of RA (Fig. 7), and performed pharmacological treatments with 9-*cis*-RA and with an inhibitor of retinaldehyde dehydrogenase activity (Fig. 8).

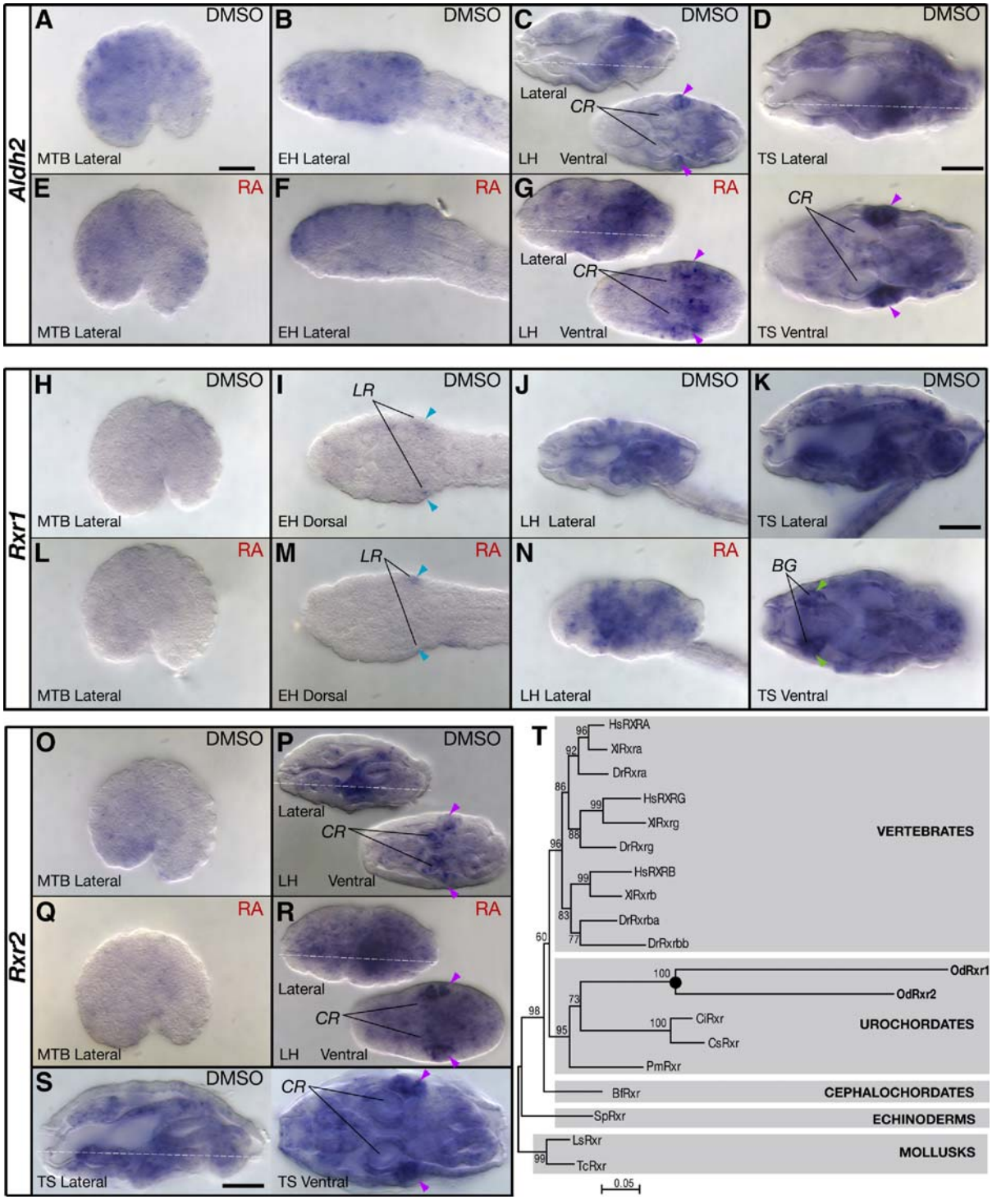
Oikopleura Aldh2 expression pattern

The *Oikopleura* gene that has a sequence most similar to the vertebrate *Aldh1a* retinaldehyde dehydrogenase family is actually more similar to vertebrate *Aldh2* than it is to *Aldh1a* (Cañestro et al., 2006). Aldh2 is an enzyme located in the mitochondria of all eukaryotes so far analyzed and it functions in the degradation of acetaldehyde (Landin et al., 1996). PSORT-II analysis of mitochondrial targeting signals in the N-terminus of the *Oikopleura* Aldh2 protein provides a 95% probability that this protein has a subcellular localization within the mitochondria, and therefore it is likely that *Oikopleura* Aldh2 might function as a typical acetaldehyde dehydrogenase (Cañestro et al., 2006). Phylogenetic analysis, however, suggests that the Aldh1a and Aldh2 in extant deuterostomes originated by the duplication of an ancestral Aldh2-like enzyme (Cañestro et al., 2006). The hypothetical possibility that *Oikopleura* Aldh2 plays a role similar to Aldh1a in vertebrates predicts that *Oikopleura Aldh2* should be expressed temporally and spatially along the AP axis in restricted domains to generate RA gradients as the Aldh1a genes do in vertebrates, especially in the vicinity of the *Hox* expression domain in the posterior part of the body (Dupé and Lumsden, 2001; Niederreither et al., 1997; Reijntjes et al., 2005; Wilson et al., 2003). In addition, *Oikopleura Aldh2* expression should be down-regulated by exogenous RA as RA-synthesizing enzymes are in vertebrates (Niederreither et al., 1997). In situ hybridization experiments revealed that *Aldh2* is broadly expressed from mid-tailbud to early-hatchling stages (Figs. 7A, B) and it is highly expressed in the posterior oikoblast from late-hatchling to tailshift stages, especially in two bilateral domains at the level of the ciliary rings (Figs. 7C, D). The absence of temporally and spatially restricted AP expression domains of *Aldh2* during tailbud stages after patterning has taken place, and the lack of correlation between the expression of *Oikopleura Aldh2* and *Hox* genes (Fig. 6 and (Cañestro et al., 2005; Seo et al., 2004)) argues against the hypothesis that *Aldh2* plays a morphogenetic role in axial patterning of *Oikopleura* comparable to the role of *Aldh1a* enzymes in vertebrates. The fact that excess RA did not perturb the expression pattern of *Aldh2* in *Oikopleura* as RA perturbs *Aldh1a1* expression in vertebrates

suggests also that *Aldh2* might not be functioning as a primary retinaldehyde dehydrogenase (Figs. 7E–G).

Oikopleura Rxr1 and Rxr2 expression patterns

Although we found no Rar in the *Oikopleura* genome, we did find two *Rxr* genes. Tetrapods have three Rar and three Rxr paralogs (A, B and G) that form heterodimers and mediate RA-signaling (reviewed in Mark et al., 2006). Vertebrate Rxrs can also form homodimers or heterodimers with other nuclear receptors to mediate additional functions. In vertebrates, Rxrs specifically bind 9-*cis*-RA, while Rars can bind both RA and 9-*cis*-RA (Mader et al., 1993), but the binding of different retinoids to different Rars and Rxrs is not the same in all species (Escrivà et al., 2006). Phylogenetic analysis suggests that the two *Oikopleura Rxr* paralogs arose from a gene duplication event that occurred after the separation of larvacean and ascidian lineages (Fig. 7T). This finding raises the possibility that after the duplication, one or both of the two *Oikopleura* Rxr



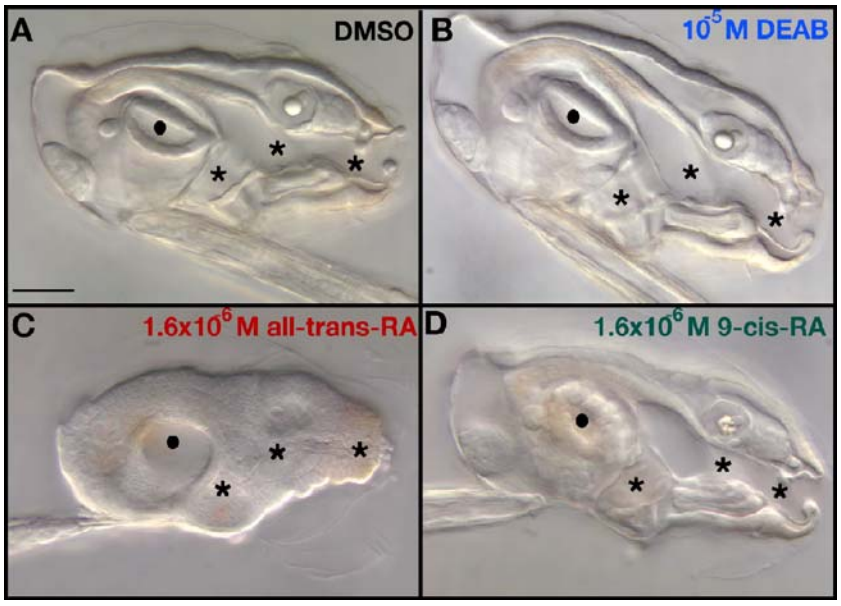


Fig. 8. Pharmacological treatments of *Oikopleura* embryos. (A) DMSO control *Oikopleura* tailshift embryo at 24 h post fertilization. (B) Continuous 24-h treatment with 10^{-5} M DEAB did not alter the AP axial organization of *Oikopleura* embryos, whereas in vertebrates concentrations of $1–5\times10^{-6}$ M DEAB produce obvious developmental defects (Maves and Kimmel, 2005). (C) The compact trunk phenotype induced by all-*trans*-RA in *Oikopleura* is not reproduced by continuous treatments with 9-*cis*-RA at the same concentration (D), ruling out the possibility that spontaneous conversion of all-*trans*-RA to 9-*cis*-RA could be inducing such phenotype. Asterisks and solid circles label internal cavities as in Fig. 5. Scale bar is 20 μm.

receptors could have evolved the ability to bind RA, and thereby could have become capable of mediating RA-signaling. To explore this possibility, we investigated the expression patterns of the two *Rxr* genes in the presence or absence of exogenous RA (Figs. 7H–R). At tailbud stage, when CNS patterning is occurring and CNS markers like *Hox1* are expressed in restricted domains along the AP axis (Fig. 6A and Cañestro et al., 2005), neither of the two *Oikopleura Rxr* paralogs was expressed over background levels even after prolonged staining (Figs. 7H, O). We conclude, therefore, that neither *Oikopleura Rxr* gene is likely to have evolved the ability to organize the AP axis during *Oikopleura* development. At the early-hatchling stage, *Rxr1* expression appeared in the epidermis at the level of the anterior part of the notochord (blue arrowheads in Fig. 7I). By late-hatchling and tailshift stages, the expression of both *Rxr1* and *Rxr2* was broadly up-regulated, especially *Rxr1* in the buccal glands (green arrowheads in Fig. 7K ventral view) and *Rxr2* in the ciliary rings and in a bilateral domain at the level of the gill slits (purple arrowheads in Figs. 7R, S). In contrast to vertebrates and cephalochordates, in which exogenous RA up-regulates *Rar* expression in a positive autoregulatory loop (Escrivà et al., 2002; Kamei et al., 1993; Sucov et al., 1990), RA did not perturb expression domains of *Oikopleura Rxr* genes (Figs. 7H–R). In conclusion, the late onset of expression of the two *Rxr* genes in *Oikopleura* is incompatible with functions related to an early AP signaling role, but instead suggests a role in morphogenetic processes shown to occur during organogenesis at hatchling stages (Cañestro et al., 2005).

Treatments with 9-cis-RA and DEAB

Because in vertebrates, Rxrs specifically bind 9-*cis*-RA, we explored the possibility that all-*trans*-RA could be spontaneously converted into 9-*cis*-RA during our treatments and act via Rxr receptors. We treated *Oikopleura* embryos with 9-*cis*-RA at concentrations that provoked the compact trunk

Fig. 7. *Aldh2* and *Rxr* expression patterns are not compatible with roles in global specification of AP patterning. Analysis of *Aldh2* (A–G), *Rxr1* (H–N) and *Rxr2* (O–S) expression patterns during *Oikopleura* development in DMSO-controls (A–D, H–K, O–P, S) and RA-treated embryos (E–G, L–N, Q–R). Lateral and ventral views (labeled in each panel) show different aspects of the expression patterns (anterior is to the left). A paired epidermal domain at the level of the ciliary rings (CR) co-expressing *Aldh2* and *Rxr2* are labeled with purple arrowheads (C and P). *Rxr1* expression in the presumptive Langerhans receptors (*LR*) precursors and the buccal glands (*BC*) are labeled with blue and green arrowheads, respectively. Scale bar (20 μm) for all panels is as in panel A, except as otherwise indicated. MTB: mid-tailbud stage; EH, early-hatchling stage; LH, late-hatchling stage; TS, Tailshift stage. (T) Evolutionary tree of deuterostome Rxr proteins, inferred by a Poisson-corrected neighbor joining method, rooted with mollusk Rxr proteins, suggesting that the two *Oikopleura* Rxr paralogs arose from a gene duplication event (black solid dot) that occurred during the evolution of the larvacean lineage. Numbers indicate bootstrap support of each node ($n=1000$ repetitions). Genbank accession numbers, Vertebrates: *Homo sapiens*: HsRXRA, AAH63827; HsRXRB, CAI95622; HsRXRG: NP_008848. *Xenopus laevis*: XlRxra, P51128; XlRxrb, AAH72132; XlRxrg, P51129. *Danio rerio* (nomenclature according to Tallafuss et al., 2006): DrRxra, NP_571228; DrRxrba, AAH54649, DrRxrbb, NP_571313, DrRxrg, AAH59576. Urochordates: Larvaceans: *Oikopleura dioica*: OdRxr1, DQ841187; OdRxr2, DQ841188. Ascidians: *Polyandrocarpa misakiensis*, PmRxr, BAA82618; *Ciona intestinalis*, CiRxr, BAE06678; *Ciona savignyi*, CsRxr, ENSCSAVG00000007831 (accession number from EMSEMBL CSAV 2.0 www.ensembl.org/Ciona_savignyi). Cephalochordates: *Branchiostoma floridae*, BfRxr, AAM46151. Echinoderms: *Strongylocentrotus purpuratus*: SpRxr, XP_784246. Mollusks: *Lymnaea stagnalis*, LsRxr, AAW34268; *Thais clavigera*, TcRxr, AAU12572.

phenotype when using all-*trans*-RA (Figs. 8A, C–D). These experiments showed that 9-*cis*-RA did not generate the compact trunk phenotype (Fig. 8D), and therefore ruled out the possibility that spontaneous conversion from all-*trans*-RA into 9-*cis*-RA could be causing the compact trunk phenotype. Moreover, these experiments suggest that it is improbable that the compact trunk phenotype induced by all-*trans*-RA is due to unspecific toxicity produced by the generic chemical properties of just any retinoic acid-related molecule.

In vertebrates, the pharmacological inhibition of retinaldehyde dehydrogenase activity with DEAB alters axial patterning (Maves and Kimmel, 2005 and references therein). Results from our all-*trans*-RA and 9-*cis*-RA treatments show that RA does not play a role in anterior–posterior patterning in *Oikopleura* and predict that a pharmacological inhibitor of retinaldehyde dehydrogenase activity should not alter developmental patterning either. To test this possibility, we treated *Oikopleura* embryos continuously with DEAB at 10 μM, a concentration at which DEAB perturbs AP patterning in vertebrates (Maves and Kimmel, 2005). Results showed that this inhibitor did not affect *Oikopleura* developmental patterning (Figs. 8A–B), and therefore, allow us to conclude that *Oikopleura* does not possess the ability to synthesize and respond to retinoic acid in a vertebrate fashion.

Discussion

Development of a chordate body plan in the absence of RA genetic machinery

A widely accepted idea is that the acquisition of the genetic machinery for producing, degrading, and binding the morphogen RA was an important event for the innovation of the chordate body plan (reviewed in Fujiwara and Kawamura, 2003; Fujiwara, 2006). A recent genomic survey, however, discovered that genes that encode the main components of the RA genetic machinery (*Aldh1a*, *Cyp26*, and *Rar*) occur in the genomes of non-chordate deuterostomes, thus calling into question the 'invention' of the RA genetic machinery as a basis for the innovation of the chordate body plan (Cañestro et al., 2006). The unexpected absence of *Aldh1a*, *Cyp26* and *Rar* orthologs from the deep *Oikopleura* genome database (9-fold redundancy), led to the hypothesis that the RA genetic machinery was secondarily lost during larvacean evolution (Cañestro et al., 2006). This hypothesis raises the problem that the AP organization of a chordate body plan can be developed and maintained in the absence of classical RA-signaling, which has been assumed to be a chordate synapomorphy. In the work presented here, evidence from markers for the anterior–posterior patterning of pharynx, digestive tract, CNS and epidermis converge to show that exogenous retinoic acid does not induce homeotic posteriorization of anterior structures in *Oikopleura* as it does in cephalochordates and vertebrates. These results together with the absence of developmental perturbations after treatment with an inhibitor of retinaldehyde dehydrogenase activity are consistent with the hypothesis that the RA genetic machinery has been lost during larvacean evolution, and show that embryonic axial patterning in *Oikopleura* has become independent of the morphogenetic role that RA plays in other chordates. Thus, *Oikopleura* offers a unique opportunity to study the development of chordate features without the influence of classical RA-mediated developmental signaling.

RA-signaling in urochordates: a comparative perspective

The overall high similarity of gene expression patterns during the development of larvaceans and ascidians suggests that a basic genetic developmental program is shared between them (Bassham and Postlethwait, 2000, 2005; Cañestro et al., 2005; Nishino et al., 2001; Seo et al., 2004). In contrast to *Oikopleura*, however, ascidian genomes possess all three major components of the RA-machinery (Cañestro et al., 2006; Devine et al., 2002; Hisata et al., 1998; Kamimura et al., 2000; Nagatomo and Fujiwara, 2003; Nagatomo et al., 2003; Yagi et al., 2003). Detailed comparison of the effects of RA in ascidians and larvaceans provides insights for understanding how *Oikopleura* can maintain the AP axial organization of a chordate body plan similar to ascidian larvae, despite the loss of RA genetic machinery.

RA-signaling and anterior–posterior axial patterning in the endoderm in Urochordates

The morphology of ascidian embryos treated with RA is generally similar to the morphology of RA-treated larvacean embryos. In the ascidian *Ciona intestinalis*, the morpholino antisense oligonucleotide knockdown of the expression of the only *Rar* gene present in the genome did not perturb development (Imai et al., 2006). The fact that ascidians lacking Rar function and *Oikopleura* lacking any *Rar* gene develop normally would be predicted by the hypothesis that *Rars* play no essential role in patterning early urochordate development.

Common features between the phenotypes induced by RA treatments in *Oikopleura* (compact trunk) and ascidians (called shortened head or moonfaced), include shortened trunk and abnormal development of the stomodeum, pharynx and gill slits (Hinman and Degnan, 1998; Katsuyama and Saiga, 1998; Nagatomo et al., 2003). Our results on *Oikopleura* show that the RA-induced morphogenetic changes in the stomodeum, pharynx and gill slits are not caused by a homeotic posteriorization, but rather by perturbed organogenesis. In ascidians, despite the fact that RA down-regulates *Otx* expression in the pharynx (Hinman and Degnan, 2000), there is no conclusive evidence that RA induces homeotic posteriorization of the pharynx because expression of posterior markers has not yet been reported in anterior structures of the digestive system of RA-treated ascidians.

In *Oikopleura*, the absence of *Rar* orthologs in the genome suggests that the pharyngeal malformations and the compact trunk phenotype induced by the RA-treatments could be due to Rar-independent but RA-sensitive pathways. In ascidians, no evidence has been reported that Rar mediates RA-induced malformations of the pharynx. In the case of *Oikopleura*, the

fact that 9-*cis*-RA treatments cannot reproduce the compact trunk phenotype induced by all-*trans*-RA suggests that this phenotype is not due to unspecific toxicity caused by the generic chemical properties of the retinoic acid molecule. We can also discard the possibility that spontaneous conversion from all-*trans*-RA to 9-*cis*-RA could be interfering with *Oikopleura's* Rxr receptors, which in vertebrates can bind 9-*cis*-RA (Mader et al., 1993). We cannot discard, however, the possibility that an *Oikopleura Rxr* paralog could have evolved the ability to bind all-*trans*-RA. The late and ubiquitous expression of *Oikopleura's* two *Rxr* genes, however, is incompatible with the global specification of AP patterning, although it is consistent with roles during organogenesis at late-hatchling stages.

The fact that in vertebrates, all-*trans*-RA can act independently of Rar receptors (Notario et al., 2003; Ochoa et al., 2003) opens the possibility that the RA-induced compact trunk phenotype of *Oikopleura* might be due to interactions between all-*trans*-RA and Rar-independent pathways. One possibility is that RA in *Oikopleura* alters the activity of protein kinase C alpha (PRKCA), which in vertebrates is a key enzyme regulating cell growth, differentiation and apoptosis (Radominska-Pandya et al., 2000). All-*trans*-RA can bind directly to the C2-domain of PRKCA, and binding competition between all-*trans*-RA and acidic phospholipids can modulate PRKCA activity (Ochoa et al., 2003). It has been suggested that various proteins that interact with all-*trans*-RA (e.g. CRABP, RAR, PRKCA) share a common core of residues that allows this interaction (Radominska-Pandya et al., 2000). We found that *Oikopleura* has a PRKCA homolog (NCBI Trace Archive accession number gnl|ti|475513348) that conserves the core of residues that have been predicted to bind to all-trans-RA in human. The high degree of sequence conservation of *Oikopleura* PRKCA (**R**LL**I**EVWD**W**D**R**LNT**ND**F**M**GSMSFG**I-SEL**KKNS**A**, with amino acids shown to be essential for RA binding in bold face) and the fact that we found it to be expressed in the trunk at RA-sensitive hatchling stages (data not shown) is consistent with the hypothesis that all-trans-RA could perturb PRKCA activity in *Oikopleura* as it does in human (Ochoa et al., 2003). In agreement with this hypothesis, we found that PRKCA is over-expressed in RA-treated embryos, suggesting that RA might interfere with PRKCA function (unpublished results).

Oikopleura can be considered as a "classical RA-signaling pathway knock-down mutant" naturally produced by evolution, providing an opportunity to study the development of chordate structures, such as the pharynx and the CNS, without the influence of Rar-mediated RA-signaling. Future large-scale genomic investigations by microarray techniques in *Oikopleura* might reveal Rar-independent pathways affected by RA treatments, which could be tested in other chordates.

RA-signaling and anterior–posterior axial patterning of the CNS in urochordates

Despite the fact that the posteriorizing effect of RA in AP axial patterning of the CNS has been assumed to be common to vertebrates, cephalochordates and urochordates (reviewed in Schilling and Knight, 2001), evidence in the literature, however, can be reasonably interpreted as indicating that RA-treated ascidian embryos do not show homeotic transformation. Embryos of the ascidian *Herdmania curvata* treated with RA did not show AP shifts of *Pax2/5/8* or *Otx* expression domains in the CNS (Hinman and Degnan, 2000). In addition, RA-treated embryos of the ascidian *Halocynthia roretzi* failed to show anterior expansion of the *Hox1* expression domain in the visceral ganglion (hindbrain) (Katsuyama et al., 1995; Nagatomo and Fujiwara, 2003). These results with ascidians are similar to ours in *Oikopleura*, and would be expected if RA-signaling is not a major factor in the development of the CNS in urochordates, as it is in vertebrates and cephalochordates. If the mechanisms for axial patterning of the CNS had already become independent of RA-signaling in ancestral urochordates before the separation of larvacean and ascidian lineages, we would easily understand why the loss of genes encoding RA synthesizing and degrading enzymes and RA receptors during larvacean evolution would not have generated a dramatic impact on the development of the *Oikopleura* CNS.

RA-signaling and anterior–posterior axial patterning in the epidermis in Urochordates

Normal ascidian and *Oikopleura* embryos share a strikingly similar *Hox1* expression pattern, including a "belt-shape" domain in the epidermis of the trunk-tail transition region at tailbud and hatchling stages (blue arrowheads in Fig. 6; Cañestro et al., 2005; Katsuyama et al., 1995; Nagatomo and Fujiwara, 2003; Seo et al., 2004). *Hox1* expression domains respond to RA-treatments differently, however, in the two classes of urochordate. While the boundaries of *Hox1* expression domains are not altered by RA-treatments in *Oikopleura*, in ascidians exogenous RA causes *Hox1* to be ectopically expressed throughout the entire epidermis, not only rostral to its normal domain, but caudal as well (Katsuyama et al., 1995; Katsuyama and Saiga, 1998; Nagatomo and Fujiwara, 2003). Phenotypes caused by RA treatments in ascidians, however, "can hardly be regarded as examples of homeotic transformation" (Fujiwara, 2006). For instance, although the failure to form palp cells in the ascidian *H. roretzi* treated with RA was initially interpreted as a homeotic posteriorization caused by the ectopic expression of *Hox1* (Katsuyama et al., 1995; Katsuyama and Saiga, 1998), recent investigations, however, have shown that the expression of gene markers specific for palp cells and surrounding sensory neurons are not abolished by RA-treatments (Nagatomo et al., 2003; Yagi and Makabe, 2002). These gene expression data demonstrate that RA does not alter the cellular identity of anterior cells during ascidian development, but rather acts merely to alter palp cell morphogenesis (Nagatomo et al., 2003; Yagi and Makabe, 2002). Therefore, results from *Oikopleura* and ascidians converge on the conclusion that RA does not induce homeotic transformations in the epidermis, suggesting that the AP axial patterning of the epidermis is independent of the classical morphogenetic role of RA in both larvaceans and ascidians. The apparent absence of RARE elements in the vicinity of *Oikopleura Hox1* (unpublished data) and the

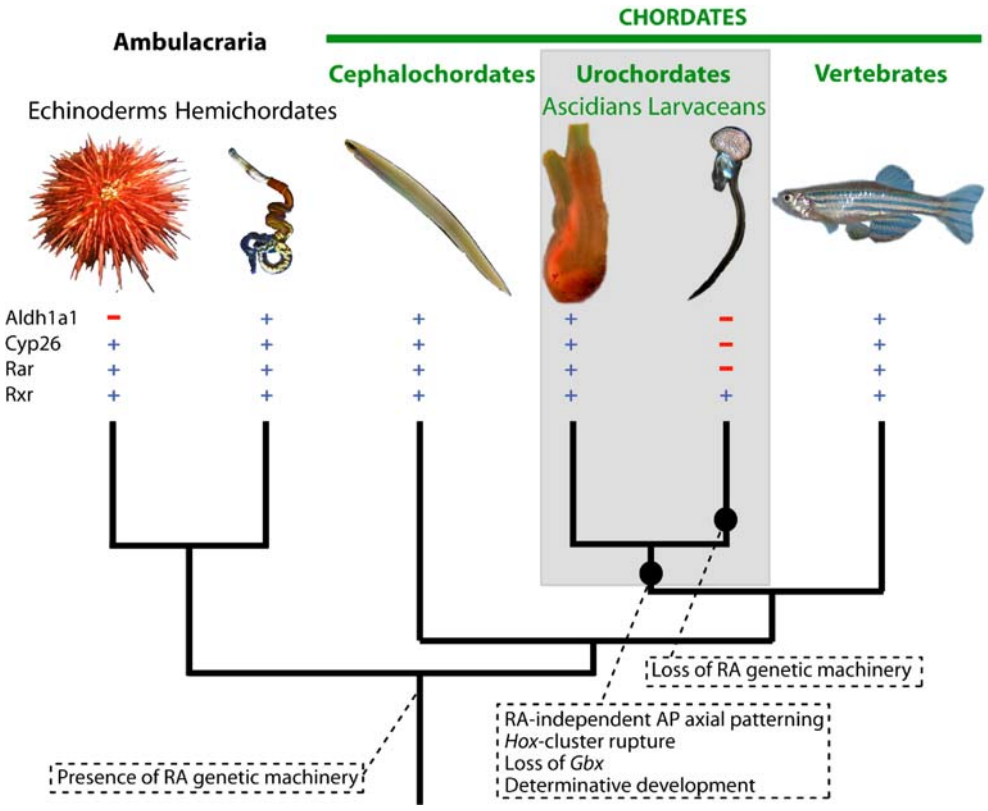


Fig. 9. Schematic representation of the newly proposed deuterostome phylogeny (Bourlat et al., 2006; Delsuc et al., 2006) highlighting the presence of RA genetic machinery and important events for the evolution of urochordates. Analysis of the RA genetic machinery (*Aldh1a1*, *Cyp26*, *Rar* and *Rxr*) in extant deuterostomes reveals that these genes were already present before the origin of chordates (Cañestro et al., 2006). Gene presence is indicated with "+" and absence with "−". Urochordates (=tunicates, grey background) include larvaceans and ascidians. Four important events that probably occurred in the stem urochordates might have been crucial for the evolution of this subphylum: AP axial patterning became independent of RA-signaling, the *Hox* cluster was disrupted, *Gbx* was lost, and a determinative mode of development was adopted. Larvaceans appear to have lost most of the components of RA genetic machinery.

ascidian *Ciona intestinalis Hox1* (Wada et al., 2006a,b), and the recent detection of RA-sensitive cis-regulatory elements with epidermal activity in *Ciona Hox1* led to the hypothesis that the ascidian epidermis possesses a secondarily derived RA-regulatory mechanism for *Hox1* expression that is different from the RARE-mediated RA-signaling described in vertebrates and cephalochordates (Wada et al., 2006a,b). This hypothesis is consistent with the fact that we do not observe RA-induced ectopic over-expression of *Hox1* in the epidermis of *Oikopleura*.

RA-independent axial patterning, rupture of the Hox cluster, and urochordate evolution

Recent phylogenetic studies based on the analysis of hundreds of homologous genes suggest that, contrary to previous belief, urochordates are the group most closely related to vertebrates (Blair and Hedges, 2005; Bourlat et al., 2006; Delsuc et al., 2006; Wada et al., 2006a,b). In this new phylogenetic context, Fig. 9 highlights some key events for the evolution of urochordate development that can help us to understand the divergence of this chordate subphylum. Because RA has been shown to regulate both spatial and temporal *Hox* gene expression (Maconochie et al., 1996; Marshall et al., 1994), and because RA-signaling and *Hox* clusters are functionally interconnected in AP axial patterning in vertebrates (Krumlauf, 1994) and cephalochordates (Holland and Holland, 1996), it is plausible to propose that the alteration of the *Hox* cluster and RA-signaling during urochordate evolution is not just coincidental. In vertebrates, the spatial and temporal collinearity of *Hox* cluster gene expression provides an AP code to pattern the segmented hindbrain during development (Gavalas, 2002; Kiecker and Lumsden, 2005; Kmita and Duboule, 2003; Krumlauf, 1994; Lumsden and Krumlauf, 1996). While spatial collinearity seems to depend on cis-regulatory elements and has been found in most bilaterians, temporal collinearity seems to occur only in animals that have maintained an intact *Hox* cluster (reviewed in Garcia-Fernàndez, 2005). Thus, temporal collinearity is missing in echinoderms (Arenas-Mena et al., 1998), *Drosophila* (Negre et al., 2005), *Bombyx mori* (Yasukochi et al., 2004) and *Caenorhabditis elegans* (Aboobaker and Blaxter, 2003). It had been proposed that a time-based activation of the *Hox* genes in the clusters might derive from the gradual accessibility of genes to the transcription machinery (reviewed in Kmita and Duboule, 2003). It has been recently shown that RA can regulate the temporal expression of *Hox* cluster genes in a choreographed manner by inducing different parts of the cluster to become differentially poised outside chromosome territories involving histone acetylation and methylation (Chambeyron and Bickmore, 2004). This finding supports previous observations in

which the position of *Hox* genes within the cluster correlates with their response to RA (i.e. early-expressed *Hox* genes positioned 3′ of the cluster show high RA response, while late-expressed *Hox* genes positioned 5′ of the cluster show low RA response; Hunt and Krumlauf, 1992; Maconochie et al., 1996; Marshall et al., 1994). This mode of regulation is probably responsible for the temporal, but probably not spatial, collinear regulation of the *Hox* cluster, and it is likely a major force conserving the integrity of *Hox* clusters (Ferrier and Holland, 2002; Ferrier and Minguillón, 2003; Garcia-Fernàndez, 2005; Monteiro and Ferrier, 2006).

An important genomic characteristic common to larvaceans and ascidians is the disruption of the *Hox* cluster during urochordate evolution (Ikuta et al., 2004; Seo et al., 2004). Comparative analysis of gene loss and chromosomal distribution of *Hox* genes suggests that the rupture of the cluster was an early event that preceded the divergence of larvacean and ascidian lineages, followed by independent evolution and further disintegration in each taxon (Ikuta and Saiga, 2005). Disintegration of the *Hox* cluster probably disrupted the choreographed regulation of *Hox* genes, and may help explain the breakdown of the temporal collinearity of *Hox* expression in larvaceans and ascidians (Ikuta et al., 2004; Seo et al., 2004). The spatial collinearity of *Hox* gene expression that remains in urochordates exists probably because it is cis-promoter-dependent and does not rely on the integrity of *Hox* cluster organization (Seo et al., 2004).

At least two possible scenarios can explain the relationship between the rupture of the *Hox* cluster and the alteration of RA-signaling during urochordate evolution. In the first hypothesis, the disintegration of the *Hox* cluster in stem urochordates might have led to the loss of temporal collinearity, which consequently relaxed functional restraints on RA-signaling imposed by the nature of the cluster. If AP patterning by *Hox* genes could occur without RA-signaling, then the selective pressure to maintain the RA genetic machinery (*Aldh1a*, *Cyp26* and *Rar*) would be relieved and maybe even selected against due to the rapid life cycle adapted by larvaceans. In the ascidian lineage, the RA-machinery might have been maintained due to the involvement of RA-signaling in biological functions other than AP patterning, such as asexual reproduction (Hara et al., 1992; Kawamura et al., 1993).

In the second hypothesis relating *Hox* cluster rupture and RA-signaling, the requirement for RA-signaling was usurped by some other mechanism, such as determinative development (Seo et al., 2004), which made the temporal collinearity of *Hox* gene expression unnecessary. This hypothesized new developmental program may have led to the relaxation of evolutionary restraints to maintain RA-signaling and hence to maintain the contiguous structure of the *Hox* cluster. Better understanding of the mechanisms of CNS patterning and *Hox* regulation in larvaceans and ascidians will help us to evaluate the *Hox*-cluster-loss-first hypothesis and the RA-patterning-loss-first hypothesis, and might also help to explain differences in the organization of the CNS in urochordates compared to cephalochordates and vertebrates, including the loss of the midbrain, the midbrain–hindbrain boundary, and a segmented hindbrain in urochordates (Cañestro et al., 2005; Castro et al., 2006; Dufour et al., 2006). Other genomic events that occurred in stem urochordates, such as the loss of *Gbx* (Edvardsen et al., 2005; Wada et al., 2003), which has been proposed to be linked to the divergence of the AP organization of the CNS in urochordates (Cañestro et al., 2005), can also be connected to the evolution of RA-signaling and the rupture of the *Hox* cluster. In vertebrates, because *Gbx*, RA-signaling, and Hox code information play a crucial role in the positioning and development of the anterior vertebrate hindbrain (Raible and Brand, 2004; Rhinn and Brand, 2001), it is also possible that the loss of *Gbx* in the urochordate lineage was linked to the changes derived from the rupture of the *Hox* cluster and the modification of the morphogenetic role of RA in the mechanisms of development of anterior–posterior axial patterning in urochordates.

Conclusions

The experiments described here show that *Oikopleura* develops and maintains the AP axial patterning of a chordate body plan despite the absence of the classical morphogenetic role of retinoic acid. In contrast to cephalochordates and vertebrates, *Oikopleura* embryos do not experience homeotic transformation after RA treatments, and comparison of our data with published evidence from ascidians suggests that this is a shared derived feature of the urochordate subphylum. Phenotypes induced by RA excess in *Oikopleura*, and probably ascidians, appear to be due to alterations of organ morphogenesis rather than cell fate changes. We propose that genome evolution in stem urochordates, including the rupture of the *Hox* cluster, could be causally connected to the evolution of the RA-signaling pathway in urochordates. This work raises the intriguing question of how *Oikopleura* can construct a chordate body plan in the absence of both classical Rar-mediated RA-signaling and an intact *Hox* cluster, and provides a new perspective to our understanding of the developmental evolution and divergence of urochordates within the chordate phylum.

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