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Opposing Nodal/Vg1 and BMP signals mediate axial patterning in embryos of the basal chordate amphioxus

Takayuki Onai[a], **Jr-Kai Yu**[b], **Ira L. Blitz**[c], **Ken W. Y. Cho**[c], and **Linda Z. Holland**[a,*]

[a]Marine Biology Research Division, Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA 92093-0202 USA

[b]Institute of Cellular and Organismic Biology, Academia Sinica,128 Academia Road, Sec., Nankang, Taipei 11529, Taiwan

[c]Department of Developmental and Cell Biology, and Developmental Biology Center, University of California Irvine, Irvine CA 92697-2300

Abstract

The basal chordate amphioxus resembles vertebrates in having a dorsal, hollow nerve cord, a notochord and somites. However, it lacks extensive gene duplications, and its embryos are small and gastrulate by simple invagination. Here we demonstrate that Nodal/Vg1 signaling acts from early cleavage through the gastrula stage to specify and maintain dorsal/anterior development while, starting at the early gastrula stage, BMP signaling promotes ventral/posterior identity. Knockdown and gain-of-function experiments show that these pathways act in opposition to one another. Signalling by these pathways is modulated by dorsally and/or anteriorly expressed genes including *Chordin*, *Cerberus*, and *Blimp1*. Overexpression and/or reporter assays in *Xenopus* demonstrate that the functions of these proteins are conserved between amphioxus and vertebrates. Thus, a fundamental genetic mechanism for axial patterning involving opposing Nodal and BMP signaling is present in amphioxus and probably also in the common ancestor of amphioxus and vertebrates or even earlier in deuterostome evolution.

Introduction

The anterior/posterior, dorsal/ventral and left/right axes of the basal chordate amphioxus (*Branchiostoma floridae*) are established by the end of the gastrula stage. The anterior/posterior axis lies close to the animal/vegetal axis, which is established during oogenesis. Amphioxus eggs are small and relatively yolk-poor. The oocyte nucleus is offset toward the animal pole, while sheets of endoplasmic reticulum, which will form the pole plasm, are

*Author for correspondence Marine Biology Research Division, Scripps Institution of Oceanography, University of California San Diego, La Jolla CA 92093-0202 USA Tel. 01-858-534-5607 Fax. 01-858-534-7313 lzholland@ucsd.edu.

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located near the vegetal pole (Holland and Holland, 1991; Holland and Holland, 1992). Additional asymmetries develop soon after fertilization. As the pole plasm coalesces, the sperm nucleus migrates to the vegetal pole and then, together with a cloud of mitochondria, back to the animal hemisphere to join the female chromosomes (Holland and Holland, 1992). Development is regulative with at least the first four blastomeres capable of giving rise to a gastrula (Wilson, 1893). The hollow blastula is just one cell thick with the vegetal blastomeres slightly larger than the animal ones. Gastrulation is by invagination with virtually no involution (Zhang et al., 1997). The first sign of the posterior pole of the embryo is expression of *Wnt8* and *Brachyury* in a ring around the equator of the blastula, which marks the future blastoporal lips (Holland and Holland, 2007). Although the first morphological indication of dorsal/ventral polarity is the flattening of the neural plate at the late gastrula stage, gene expression patterns show that dorsal identity is specified by the onset of gastrulation. At this stage, although several genes are broadly expressed throughout the nascent mesendoderm (*BMPs, Dkk1/2/4*) or ectoderm (*Distalless*), others have restricted expression dorsally in either the mesoderm (*Gsc*) or in both mesoderm and ectoderm (*Nodal, Lefty, Tsg, ADMP, Chordin*) (Holland et al., 1996; Yu et al., 2007). How or exactly when dorsal/ventral polarity is established in amphioxus embryos is unknown.

For other chordates, dorsal identity in at least the *Xenopus* and zebrafish blastula is mediated by nuclear β-catenin in dorsal nuclei (Yost et al., 1996; Schneider et al., 1996; Wylie et al., 1996; Kelly et al., 2000; Schier and Talbot, 2005). However, Wnt/β-catenin signaling is not required for patterning pre-implantation mouse embryos (Marikawa, 2006), while in the chick, *Wnt* expression in the marginal zone is necessary for Vg1 to induce a primitive streak, but nuclear localization of β-catenin has not clearly been demonstrated (Skromne and Stern, 2001; Schmidt et al., 2004). Moreover, in amphioxus, while Wnt/β-catenin signaling is important for specification of posterior identity during the gastrula stage, nuclear β-catenin, which is translocated to all nuclei at the 8-cell stage, is not involved in establishing dorsal identity (Holland et al., 2005).

The expression of *Nodal* and its antagonist *Lefty* dorsally in the amphioxus gastrula and its earlier asymmetric expression suggest that Nodal signaling may play a key role both in specification of dorsal identity during cleavage stages and in its maintenance later during the gastrula stage. At the onset of gastrulation, *Nodal* and *Lefty* are expressed throughout the presumptive mesoderm, which is entirely dorsal in character during gastrulation, and in the overlying presumptive neuroectoderm (Yu et al., 2002; 2007). If *Nodal* were comparably expressed earlier in development, it might function in the initial steps in specification of dorsal identity. Nodal genes in other chordates are zygotically, and in some instances maternally, expressed. However, while roles in anterior/posterior (A/P) patterning are known for at least the mouse (Varlet et al., 1997; Tian and Meng, 2006), roles in specification of dorsal identity are controversial. In the zebrafish, the Nodal genes *Squint* and *Cyclops* were suggested to participate early in induction of the dorsal organizer. Maternal *Squint* is localized to two dorsal blastomeres in the four-cell zebrafish embryo, and its knockdown can eliminate dorsal structures (Gore et al., 2005). However, mutation of maternal *Cyclops* and *Squint* showed that they are not essential for specification of the dorsal axis before the mid-blastula transition (Bennett et al., 2007; Hagos et al., 2007). In the

chick, the nodal-related gene Vg1 functions in primitive streak induction. However, Nodal has several other roles in vertebrates. In both zebrafish and *Xenopus*, *Nodal* and, at least in *Xenopus*, endodermal *Vg1* function in mesoderm induction (Agius et al., 2000; Birsoy et al., 2006; Tian and Meng, 2006). In *Xenopus*, Nodal signals are important in axial patterning after the mid-blastula transition, the onset of zygotic transcription when Nodal-related factors are expressed in dorsal-vegetal cells destined to form anterior endoderm (Takahashi et al., 2000; De Robertis and Kuroda, 2004). Adding to the rather confusing picture are reports that absence of Nodal signaling before gastrulation in the mouse results in premature neural differentiation (Camus et al., 2006), while maintenance of the neuroectoderm requires signals from the axial mesendoderm previously induced by Nodal. Moreover, in the sea urchin, which is basal to amphioxus in the deuterostomes, Nodal expression in the oral (ventral) ectoderm of the larva regulates larval aboral/oral (dorsal/ventral) polarity (Duboc et al., 2004; 2008; 2010).

To study possible roles of Nodal signaling in the establishment and maintenance of dorsal identity in amphioxus embryos, we took advantage of the structural simplicity of early amphioxus embryos, which allows gene expression patterns to be clearly followed from the egg to the blastula into the gastrula, and of the relative lack of genetic redundancy, which means that knockdown of a single gene usually gives a clear phenotype. Therefore, we first determined maternal expression of amphioxus *Nodal* and the related *Vg1* and *Activin* genes as well as that of *Cerberus*, which in *Xenopus* can antagonize ligands in the Nodal, BMP and Wnt families (Piccolo et al., 1999), and that of *Blimp1*, a transcription factor expressed in the anterior endoderm in *Xenopus* that positively regulates *Cerberus* (de Souza et al., 1999). We then upregulated signaling by Nodal/Vg1/Activin receptors by application of exogenous Activin, and downregulated it with SB505124, which blocks Type I receptor signaling downstream of the ligands (DaCosta Byfield et al., 2004). In addition, because Nodal and BMPs have been reported to antagonize one another (Tian and Meng, 2006), we overexpressed *BMP2/4* and *Cerberus* mRNA and knocked down the endogenous functions of *Blimp1* and *Chordin*. Finally, to determine how functionally conserved these proteins are we performed cross-species assays in *Xenopus* embryos and animal caps. Taken together, our results indicate that from very early cleavage stages, Nodal/Vg1 signaling probably specifies dorsal/anterior identity and that, beginning at the early gastrula stage, dorsal Nodal/Vg1 signaling is opposed by BMP signaling, which ventralizes and posteriorizes embryos. We propose that axial patterning by opposing Nodal/Vg1 and BMP signals, augmented by blastoporal Wnt/β-catenin signaling, is fundamental to chordate embryos and may have arisen even earlier in the deuterostome lineage.

Materials and methods

Amphioxus methods

Sexually mature adults of the Florida amphioxus (*Branchiostoma floridae*) were collected in Old Tampa Bay, Florida. Embryos were raised in a laboratory on site. Fixation and in situ hybridizations were as described (L. Z. Holland et al., 1996; Holland and Yu, 2004). Gene markers included AmphiChordin, AmphiSox1/2/3, AmphiBMP2/4, AmphiGsc, AmphiEvx, *AmphiWnt3* (Schubert et al., 2001), *Amphibra2*, *AmphiHex*, *AmphiNodal* (Yu et al., 2007),

AmphiOtx (Schubert et al., 2006), *AmphiFoxQ2* (Yu et al., 2003) and *AmphiCer* (EU670254). Synteny analysis confirmed the identity of *AmphiCer* (Figs. S1, S2). *AmphiVg1* (EU670255), *AmphiBlimp1* (EU708968) were identified from EST sequences (Yu et al., 2008). Human activin protein was purchased from R&D Systems, Minneapolis, MN. Controls included equal concentrations of bovine serum albumin. SB505124 (Sigma/Aldrich, St. Louis, MO) which inhibits the Nodal/Vg1/Activin receptor inhibitor (DaCosta Byfield et al., 20004) was dissolved in DMSO at a concentration of 20 mM and added to embryos at a final concentration of 50 μM.

Microinjection was performed as previously described (Holland and Yu, 2004), either with the control antisense morpholino oligonucleotide (MO) (5′-CCTCCTACCTCAGTTACAATTTATA-3′), *Chordin* MO-A (5′-GCACAACGTGCGAGGAACAACATCC-3′) or MO-B (5′-CGGCCAGGACTTCAGAGAATGTT-3′), *Blimp1* MO-A (5′-TCTGTCATCGTTGTCCCTCGCATTG–3′(Gene Tools, LLC, Philomath, OR, USA), or with mRNA of *amphiBMP2/4* or *amphiCerberus*. Approximately 2 pl was injected into amphioxus eggs (140 μm in diameter) prior to their fertilization. MOs were dissolved at 1mM and mRNAs at 1μg/μl in 15% glycerol, 5 mg/ml Texas Red dextran (Molecular Probes, Inc., Eugene, OR, USA). In vitro translation confirmed that MOs blocked translation (Fig. S6). cDNAs were generated by PCR and cloned into the pCS2+ vector. Injection of mRNA coding for tandem dimer Tomato, derived from pCS2+tdTomato demonstrated effective translation in vivo (Fig. S3).

Xenopus assays

Embryos were staged according to the table of Nieuwkoop and Faber (1956) and transferred into 0.1X modified Barth's saline (MBS). For animal cap assays, tissues were excised at stage 9 and cultured in 1X low calcium magnesium Ringer's with 0.2% BSA. Injections were in 1x MBS. The *xVent2* reporter vector -385*xVent2*-luc (Candia et al., 1997) was used to assay BMP signaling (50 pg/cell), and the *xMix2* reporter vector (Huang et al., 1995) for Nodal signaling (50 pg/cell). Unless otherwise indicated, injections were into the animal 4 blastomeres at the 8 cell stage. Animal caps were excised at stage 9, cultured until siblings reached stage 10 and assayed for luciferase (Watabe et al., 1995).

Results

The present study focuses on the combinatorial roles of the TGFβ superfamily genes Nodal/Vg1/Activin in axial patterning in amphioxus. These three proteins signal via the same Smad2/3 proteins and have similar, although not entirely identical, functions (Reissmann et al., 2001; Ramis et al., 2007; Schmierer and Hill, 2007). In particular, all three can antagonize BMP signaling and vice versa (Dale et al., 1992; Jones et al., 1992; Candia et al., 1997; Lagna and Hemmati-Brivanlou, 1999; Tian and Meng, 2006), and the effects of mis-expressing any one are typically interpreted as reflecting the roles of the other two in vivo (Hoodless et al., 1999). Because expression of some key genes in these signaling pathways has either not been studied at all in amphioxus embryos or not studied before the early gastrula, we first investigated expression of *Nodal*, *Vg1*, *Activin* and the Nodal/Vg1

antagonist *Lefty* as well as *Cerberus* and *Blimp1*. Each of these genes is single copy in amphioxus.

Although *Activin* is present in the amphioxus genome sequence (http://genome.jgi-psf.org/Brafl1/Brafl1.info.html), there were no *Activin* clones in our EST libraries from unfertilized egg, gastrula, neurula, early larva and adults, and an attempt to amplify *Activin* by RT-PCR was unsuccessful (data not shown), indicating that if *Activin* is expressed at all during amphioxus early development, it is at a very low level. In contrast, the EST libraries contained cDNAs for the other four genes. Both *Nodal* and *Vg1*, but not *Lefty*, *Cerberus* or *Blimp1*, are maternally expressed (Fig. 1). However, maternal expression differs in that *Vg1* mRNA is ubiquitous (Fig. 1A-C), while that of *Nodal* is lower toward the vegetal pole (Fig. 1I-N). At the mid-blastula stage, *Vg1* expression is reduced but still ubiquitous, while that of *Nodal* remains largely restricted to the animal two-thirds of the embryo (Fig. 1C, L, M). However, by the late blastula/early gastrula, mRNA of both genes becomes restricted to the dorsal ectoderm and mesoderm of the gastrula, including the dorsal blastopore lip, the presumed gastrula organizer (Fig. 1D, O, P) (Yu et al., 2002; 2007). The *Nodal* domain in both ectoderm and mesoderm extends somewhat more anteriorly than that of *Vg1*. However as dorsal views show, by the mid-late gastrula, neither gene is expressed in the rim of the blastopore (Fig. 1E, Q). By the mid-neurula, expression of *Vg1*, like that of *Nodal*, is detected only in the anterior most left somites (Fig. 1F-H) (Yu et al., 2002).

The Nodal/Vg1 antagonist, *Lefty*, is not maternally expressed. Zygotic transcription as shown by nuclear localization of transcripts, begins at the early blastula (Fig. 1R, S). Expression is restricted to a subset of blastomeres at one side of the vegetal pole, which in some embryos is marked by a space between the blastomeres, the vegetal pore (Fig. 1V). Although there is no morphological difference between the dorsal, ventral, left or right blastomeres during the blastula stage, since *Lefty* is clearly co-expressed with *Vg1* and *Nodal* by the mid-gastrula stage (Fig. 1W-Y), *Lefty* expression during cleavage appears to mark presumptive dorsal cells. Like *Nodal* and *Vg1*, by the mid-late gastrula, *Lefty* is not expressed in the posterior one-third of the embryo (Fig. 1Z). Since we found no evidence for *Activin* expression in the early amphioxus embryo, we conclude that Smad2/3-mediated Activin/Nodal/Vg1 signaling occurs via by Nodal and Vg1 ligands and will be hereafter be referred as Nodal/Vg1 signaling.

Cerberus, a member of the cystine-knot superfamily of secreted proteins, can inhibit signaling by Nodal, BMPs and Wnts (Piccolo et al., 1999; reviewed in Tian and Meng, 2006). Since our amphioxus EST libraries lack *Cerberus* clones, we used PCR to clone the entire intronless coding sequence from genomic DNA. *Cerberus* has very limited expression. It begins to be transcribed at the mid-gastrula in a small region of dorsal/anterior mesoderm, fated to become notochord and somites (Fig. 1A'-B'). By early neurula, expression becomes restricted to the anteriormost right somites (Fig. 1C'-D'). This pattern is consistent with a role for Cerberus in antagonism of Nodal/Vg1 and/or Wnt and/or BMP and suggests that *Cerberus* does not function in the earliest steps of embryonic patterning, but in the maintenance of dorsal/anterior identity.

In contrast to *Vg1*, *Nodal* and *Cerberus*, *Blimp1* is a transcription factor that is essential for head formation in *Xenopus* (de Souza et al., 1999; John and Garrett-Sinha, 2009). Amphioxus*Blimp1* is first expressed in the anterior endoderm of the mid-gastrula (Fig. 1F'), shifting ventrally at later stages (Fig. 1G', H'). The evident lack of substantial overlap between the *Blimp1* domain and those of *Cerberus* and *Chordin* suggests that *Blimp1* probably does not directly regulate either gene in amphioxus, but might function in limiting their expression to the dorsal mesoderm.

Dorsal/anterior development in amphioxus is promoted by Nodal/Vg1/Activin signaling

To investigate the role of Nodal/Vg1/Activin in axial patterning in amphioxus, we both up- and down-regulated the signaling pathway. In addition, to determine whether Nodal/Vg1/Activin and BMP signaling oppose one another, we tested whether Activin protein could overcome the effect of overexpressing *BMP2/4*. For upregulation of Nodal/Vg1/Activin signaling, embryos were exposed to 10 ng/ml human Activin protein from the early blastula stage (Fig. 2). Activin, Nodal, and Vg1 signal via the same receptors (ActRIIB/ActRII, ALK4, ALK7) and downstream transcription factors (Smad2 and Smad3) (Feng and Derynck, 2005; Tian and Meng, 2006). Consequently, signaling by Activin protein is generally assumed to closely approximate that of Nodal and/or Vg1.

Activin-treated embryos were strongly dorsalized and anteriorized. In gastrulae, which were somewhat foreshortened but otherwise grossly normal (Fig. 2H), the domains of two dorsal mesoderm markers (*Chordin* and *Gsc*) were expanded ventrally (Fig. 2A, B, E, F), while *Evx* expression in ventral ectoderm and endoderm was eliminated (Fig. 2C, G), and that of the anterior ectodermal marker *FoxQ2* was expanded (Fig. 2D, H). By the mid-neurula stage, the effects of Activin protein were obvious. The neurulae comprised an expanded anterior end lacking somites and notochord plus a narrow somite and notochord-containing trunk (Fig. 2M-P). The somites and notochord are expanded ventrally (Fig. 2O, U). Cross-sections show that the neural tube is absent (Fig. 2R-U). Gene expression shows that all of the ectoderm is neural, with the anterior neural marker *Otx* and anterior ectodermal marker *FoxQ2* expressed throughout the ectoderm of the expanded portion (Fig. 2I,M,L,P), and the hindbrain and spinal cord marker *Wnt3* expressed in the entire ectoderm of the narrow posterior part (Fig. 2J, N). *Brachyury* is expressed throughout the shortened notochord (Fig. 2K, O). These results indicate that Nodal/Vg1/activin signaling promotes dorsal/anterior (i.e. head) development. At higher concentrations of Activin protein, the embryos were more severely anteriorized with complete absence of the notochord and somites (data not shown).

To reduce Nodal/Vg1/Activin signaling, we first attempted to knock-down *Nodal* function with an antisense morpholino oligonucleotide (MO). However, although it effectively blocked *Nodal* translation in an in vitro assay, the MO-injected embryos were normal (data not shown) presumably because of a large store of maternal *Nodal* mRNA and/or because *Vg1*, which signals through the same Smads as Nodal (Birsoy et al., 2006; White and Heasman, 2008) can compensate for lack of *Nodal* (Fig. 1). Therefore, we blocked the function of the single amphioxus homolog of vertebrate *Alk4*, *Alk5* and *Alk7*, which are receptors for Nodal, Vg1 and Activin, with 50 μM SB505124 added at the 16-cell stage, early blastula or early gastrula (Fig. 3). Although Alk5 is also a receptor for TGFβ, no TGFβ

clones were present in our EST libraries from unfertilized egg through the neurula stage, suggesting that it does not function together with Nodal and Vg1 during those stages. All the inhibitor-treated embryos gastrulated. Gastrulae treated from the early blastula stage looked grossly normal (Fig. 3 A-J). However, they lost dorsal identity and partially lost anterior identity. Thus, expression of dorsal markers, including the BMP2/4 antagonist, *Chordin*, which is normally expressed in both the axial mesoderm and overlying neural plate, and the neural plate specifier, *Sox1/2/3*, was eliminated (Fig. 3A-D), and that of the anterior endodermal marker *Blimp1* is severely reduced (Fig. 3G,H). Although *FoxQ2* expression was relatively normal at the gastrula stage (Fig. 3E, F), by the late neurula stage, it was completely eliminated (Fig. 3O, P). Conversely, the posterior ventral marker *Evx*, was upregulated and radialized (Fig. 3I, J). By the late neurula, it is clear that if SB505124 is added at either the 16-cell stage or early blastula, the embryos are severely foreshortened and completely lack dorsal/anterior identity, but have retained posterior identity. *Brachyury* was still expressed in the tailbud, but expression was absent from the notochord (Fig. 3K-M). *Otx* remained expressed in the endoderm, but not in the forebrain (Fig. 3S-U). Similarly, expression of the forebrain marker *Tcf* was eliminated (Fig. 3W-Y), as was *Wnt3* expression in the hindbrain, spinal cord and tailbud (Fig. 3A'-C'). Moreover, expression of the somite marker, muscle actin (*BFMA1*) was eliminated if SB505124 is added at the 16-cell stage (Fig. 3F'), but expressed at a low level when addition was delayed until the early blastula (Fig. 3G'). The embryos were much less severely ventralized by addition of SB505124 at the early gastrula in that expression of muscle actin together with Wnt3 was not blocked if addition was postponed until the early gastrula (Fig. 3N, R, V, Z, D', H'). These results show that blocking signaling of both maternal and zygotic Nodal and Vg1 inhibits dorsal/anterior development, whereas delaying blocking Nodal/Vg1 signaling until the gastrula stage, when transcription is all zygotic allows some development of the dorsal neural tube and somites, but not of the notochord or anterior structures. Taken together, these gain- and loss-of-function experiments with Activin protein and SB505124 are consistent with an early role for maternal Nodal/Vg1 signaling in specification of dorsal/anterior identity and a continuing requirement for zygotic Nodal/Vg1 signaling in maintenance of dorsal/anterior identity in the early gastrula.

BMP signaling opposes that of Nodal/Vg1/Activin

Because neither *BMP2/4* nor *BMP5-8* is highly expressed before the onset of gastrulation (Yu et al., 2007), they are probably not required for axial patterning at the blastula stage. However, since BMPs are known to oppose signaling by Nodal/Vg1/Activin (Dale et al., 1992; Jones et al., 1992; Candia et al., 1997) and have overlapping expression with Nodal and Vg1 in the early amphioxus gastrula, we overexpressed amphioxus *BMP2/4* mRNA in embryos and tested whether it reduced the effect of added Activin (Fig. 4). Injection of amphioxus *BMP2/4* mRNA alone severely ventralized and posteriorized embryos, eliminating expression of the dorsal neural plate marker *Sox1/2/3* and the notochord and medial neural plate marker *Chordin* (Fig. 4A, B, E, F), as was expression of *Otx* in the forebrain and pharynx (Fig. S3). Expression of *Brachyury* in the notochord was also eliminated, but the domain around the blastopore was expanded (Fig. 4C, G). Moreover, the domain of *Hex* in the endoderm was expanded dorsally (Fig. 4D, H). These effects are comparable to those of zebrafish BMP4 protein added from the early blastula stage (Yu et

al., 2007). Embryos injected with *BMP2/4* mRNA and treated with human Activin protein at the blastula stage had phenotypes intermediate between those of either single treatment. The embryos were less severely foreshortened than those injected with BMP2/4 alone, and one-third of them retained the notochordal domain of *Brachyury* (Fig. 4I-K). These results show first, that the phenotype of embryos overexpressing BMP2/4 is the opposite (posteriorized/ventralized) of that of embryos treated with Activin protein (anteriorized/dorsalized) and suggest that at the early gastrula stage BMP signaling antagonizes that of Nodal and Vg1 to maintain dorsal/anterior and posterior/ventral identities.

In a second strategy to test the role of BMP signaling in early amphioxus development, we knocked down function of the BMP antagonist *Chordin*. Amphioxus *Chordin* is first expressed in the dorsal mesendoderm and adjacent dorsal ectoderm at the onset of gastrulation, before *Sox1/2/3* expression is detectable in the future neural plate (Yu et al., 2007). In the gastrula and neurula, the *Chordin* and *Cerberus* domains overlap with the former extending more posteriorly, and the latter more anteriorly (Figs. 1A'-B', 5A) (Yu et al., 2007). *Chordin* knockdown impaired anterior development, but the phenotype was milder than from overexpressing *BMP2/4* (Figs. 5A-F', S5). The MO-injected embryos were foreshortened with truncated heads, as shown by the down-regulation of *Otx* (Fig. 5B, C, E, F), *FoxQ2* (Fig. 5H, I, K, L), the anterior mesodermal marker *Cerberus* and anterior endodermal marker *Hex* (Fig. 5G, J, T, U, W, X). However, the embryos still had dorsal structures such as a CNS and notochord as shown by normal expression of *Chordin* itself (Fig. 5A, D), *Sox1/2/3* (Fig. 5M, P) and *Wnt3* (Fig. 5N, O, Q, R), while the *Brachyury* domain curved ventrally around the anterior tip of the embryo (Fig. 5A'-F'). Down-regulation of *BMP2/4* in neuroectoderm, which normally occurs in the early neurula, was only partially inhibited (Fig. 5S, V), suggesting either incomplete knockdown of *Chordin* or the presence of other BMP antagonists. Even so, in line with loss of the forebrain/midbrain, *Chordin* knockdown strongly suppressed *Cerberus* expression in the dorsal/anterior mesoderm (Fig. 5G, J). In contrast, expression of the ventral marker *Evx* was unaffected (Fig. S5). In early larvae, *Hex* expression in the anterior endoderm is normally downregulated, but a new domain appears in the endostyle (Fig. 5T, U). In embryos in which *Chordin* is knocked down, the latter domain shifted anteriorly (Fig. 5W, X), in agreement with loss of the anterior part of the head. Identical results were obtained with a second *Chordin* morpholino (Fig. S7). In sum, the results suggest that specification of anterior identity in all tissue layers --forebrain, non-neural ectoderm, endoderm and mesoderm-- requires *Chordin* function.

The anterior mesendodermal markers Cerberus and Blimp1 have opposite roles in axial patterning in amphioxus

To further investigate the roles of Nodal/Vg1 and BMP signaling in axial patterning in amphioxus, we first manipulated levels of two anterior mesendodermal markers, *Cerberus* and *Blimp1*, which have both been implicated as regulating Nodal and/or BMP signaling. Although Cerberus was found to inhibit Nodal, BMP and Wnt signaling in *Xenopus* (Piccolo et al., 1999), it can also promote BMP signaling in the chick (Yu et al., 2008). *Blimp1* can induce *Cerberus* expression in *Xenopus* embryos and ventralize zebrafish embryos, decreasing *Chordin* expression and potentiating BMP signaling (reviewed in John and

Garrett-Sinha, 2009). Overexpression of amphioxus *Cerberus* strongly ventral/posteriorized embryos with loss of the CNS, notochord and anterior identity (Fig. 6A-H). Expression of anterior and dorsal markers was suppressed, and the embryos were shortened. *FoxQ2* expression, *Otx* expression in the forebrain plus midbrain (termed the cerebral vesicle) and that of *Wnt3* in the hindbrain and spinal cord was lost, together with *Brachyury* expression in the developing notochord (Fig. 6). The *Brachyury* and *Wnt3* domains in the tailbud remained. This phenotype resembles, but is less severe than, that from overexpressing amphioxus *BMP2/4* mRNA (Fig. 4A-H), and is the expected phenotype if Cerberus were either a Nodal/Vg1/Activin antagonist or BMP2/4 agonist.

To further test the function of amphioxus *Cerberus*, we performed comparative experiments in *Xenopus*. Injection of mRNA for amphioxus *Cerberus*, into the ventral vegetal D4 blastomere of *Xenopus* embryos at the 32-cell stage induced a secondary axis with a head (sometimes partial) and a trunk (Fig. 7B), similar to injection of *Xenopus Cerberus*, although the latter induces a head but no trunk (Bouwmeester et al., 1996). In *Xenopus* animal cap assays, amphioxus Cerberus effectively repressed the ability of *XBMP4* to activate an *XVent2*- luciferase reporter construct and blocked activation from the *XMix2* reporter activated by amphioxus Nodal (Fig. 7G, I), but was only weakly effective in blocking activation driven by *Xenopus* Activin (Fig. 7J). Although amphioxus Cerberus inhibits both Nodal and BMP signaling in these assays, suppression of anterior development in amphioxus by overexpressing *Cerberus* indicates that in amphioxus, Cerberus probably preferentially antagonizes Nodal/Vg1 signaling.

Amphioxus embryos injected with a *Blimp1* MO had a similar, but milder phenotype than those overexpressing *Cerberus*, suggesting that the two genes have opposite functions in axial patterning. Embryos were foreshortened with either no head or a very small one (Fig. 6M-P, U-X) and with a bent notochord (Fig. 6N, P, V, X). Although the MO eliminated *FoxQ2* expression, by larval stages, that of the forebrain/midbrain and pharyngeal marker *Otx*, although greatly reduced, was still present (Fig. 6I-P). Expression of *Wnt3* was expanded to the anterior tip of the CNS (Fig. 6U), and *Brachyury* expression in the notochord was expanded ventrally (Fig. 6W). To further test the function of amphioxus *Blimp1*, we injected *Blimp1* mRNA into the marginal zones of four-cell *Xenopus* embryos. The A/P axis was shortened and the ventral region enlarged (Fig. 7C, D) similar to *Xenopus* embryos injected with *Xenopus blimp1* (de Souza et al., 1999). Moreover, in *Xenopus* animal cap assays, amphioxus *Blimp1* promoted transcription from the *XMix2* reporter (Fig. 7H). Taken together, these results indicate that *Blimp1* is necessary for proper head development in amphioxus, and are consistent with a role for *Blimp1* in both amphioxus and *Xenopus* in either promotion of Smad2/3-mediated (Activin/Nodal) signaling cascades or in inhibition of BMP signaling.

Discussion

Establishment of dorsal/anterior identity by asymmetric localization of Nodal signaling

Our previous work has indicated that maternal Wnt/β-catenin signaling probably does not establish the D/V axis in amphioxus (Holland et al., 2005), as it does in frogs and fish (Kofron et al., 2001; Schier and Talbot, 2005). In fact, amphioxus lacks genes for *bozozok*,

siamois and *twin*, which mediate the pre-gastrular role of Wnt/β-catenin in establishing the D/V axis in both vertebrates (Schier and Talbot, 2005; Heasman, 2006). Instead, in amphioxus, nuclear β-catenin is localized to all nuclei during the blastula stage (Holland, 2002). Starting at the early gastrula, nuclear β-catenin becomes largely restricted to ectoderm around the blastopore, where it has an evolutionarily conserved role in specification of posterior identity (Onai et al., 2009).

The present results show that dorsal identity in amphioxus is probably initially specified during early cleavage stages by asymmetric localization and co-expression of maternal *Nodal* and *Vg1* mRNAs, modulated from the early-mid blastula stage by zygotic *Lefty* (Figs. 1A-C, I-M, R-V; 8) (Yu et al., 2002; 2007). Uregulation of Nodal/Vg1 signaling from the blastula stage by addition of Activin protein, presumably mediated by Smad2/3, severely anteriorizes and dorsalizes the embryos to the extent that all of the ectoderm is neural (i.e. *Otx* and *Wnt3* expression is radialized) and the domains of anterior markers such as *FoxQ2* are expanded. Conversely, inhibition of the pathway has the opposite effect (e.g. expression of dorsal markers such as *Chordin* and *Sox1/2/3* and of anterior markers such as *FoxQ2* is eliminated). The requirement for Nodal/Vg1 signaling for dorsal development of amphioxus embryos gradually decreases until, by the early gastrula, inhibition of Nodal signaling fails to eliminate dorsal structures, although the embryos do not elongate normally and anterior identity is still lost (Fig. 3). Maternal Nodal mRNA may be localized by cytoplasmic movements occurring shortly after fertilization in which large numbers of mitochondria accompany the sperm nucleus as it migrates from the vegetal pole to one side of the animal pole (Holland and Holland, 1992). It has been proposed for other deuterostomes that asymmetry of mitochondria sets up a respiration gradient specifying dorsal/ventral polarity (Coffman, 2009; Coffman and Denegre, 2007; Coffman et al., 2004). In sea urchins, dispersal of a concentration of mitochondria localized on the oral side of the embryo, where *Nodal* is normally expressed, disrupts oral/aboral polarity. Moreover, quenching mitochondrial H_2O_2 also inhibits *Nodal* activation, which is required for specification of oral identity, although increasing H_2O_2 does not activate *Nodal* (Coffman et al. 2009). To clarify a possible relationship between mitochondria, Nodal and Vg1 mRNAs in amphioxus it would be useful in the future to determine if Nodal and Vg1 proteins co-localize with their respective mRNAs and with their receptors and how localization of these proteins correlates with localization of mitochondria.

Maternal Nodal/Vg1 may also function in establishing dorsal/anterior identity in vertebrates, although this role has been lost in tunicates, the sister group of vertebrates (Hudson and Yasuo, 2005). In the zebrafish, a major role of the nodal-related genes *Squint* and *Cyclops* is in animal/vegetal patterning of the mesoderm (Dougan et al., 2003), although a role in D/V patterning has also been proposed (Harvey and Smith, 2009). Maternal *Squint* mRNA is localized to blastomeres giving rise to dorsal cells, and its knockdown in oocytes ventralized embryos (Gore et al., 2005). In addition, mutants in both maternal and zygotic *Squint* have both dorsal and anterior defects (Hagos et al., 2007), although mutants of zygotic *Squint* and *Cyclops* do not (Bennett et al., 2007; Pei et al., 2007). If localized maternal *Nodal* does help specify dorsal identity in zebrafish, it probably acts in parallel with maternal β-catenin, which establishes dorsal identity both in zebrafish and *Xenopus* (Moon and Kimelman,

1998; Kelly et al., 2000; Schier and Talbot, 2005). While currently there is no evidence for maternal expression of any of the several *Nodal* genes in *Xenopus*, mRNAs for both *Vg1* and *VegT*, a T-box transcriptional regulator of *Nodal* genes, are maternal, and Vg1 is essential for head development (Birsoy et al., 2006; Heasman, 2006). These data, together with those from amphioxus and sea urchins, indicate that a central role for Nodal/Vg1 in dorsal specification was present at the base of the deuterostomes and has been conserved in amphioxus and possibly also in teleosts among the vertebrates.

During the gastrula stage, BMP signaling opposes Nodal/Vg1 signaling to ventral/posteriorize amphioxus embryos

In amphioxus, the role of Nodal/Vg1 signaling during the gastrula stage appears to be in maintenance of dorsal/anterior identity—a direct continuation of its role in specification of dorsal identity at earlier stages. By the onset of gastrulation, mRNAs of *BMPs* and their modulators such as *Chordin* are detectable by in situ hybridization (Fig. 8), and our experiments indicate that BMP signaling is a ventralizing and posteriorizing influence opposing Nodal/Vg1 signaling from the gastrula organizer (Fig. 4) (Yu et al., 2007). In vertebrates, Nodal/Vg1 signaling also acts to maintain dorsal/anterior identity in opposition to BMP signaling (Birsoy et al., 2006; Tian and Meng, 2006). Moreover, similar to up- and down-regulation of amphioxus Nodal/Vg1 signaling, overexpression of *Xenopus nodal-related (Xnr)-2* in animal caps induces expression of anterior markers such as *Cerberus* as does injection of *Vg1*, *Xnr1* and *Xnr2* into two ventral blastomeres, while injection of a dominant-negative *Xnr2* causes anterior deletions (Osada and Wright, 1999; Zorn et al., 1999). In addition, amphioxus embryos treated with human Activin strongly resemble *Xenopus* embryos in which function of BMP2, BMP4, BMP7 and ADMP, which has BMP activity, has been knocked down; the entire ectoderm is neural, expressing *Otx2* in the swollen anterior part and the neural marker *Sox2* throughout the ectoderm (Fig. 2) (Reversade and De Robertis, 2005), suggesting that in both, antagonism between Nodal/Vg1 and BMP signaling plays a major role in axial patterning. However, in vertebrates, Nodal genes also have roles in establishment of mesoderm and endoderm (De Robertis and Kuroda, 2004; Heasman, 2006), and suppression of both Nodal and BMP signals is essential for neural induction (Chang and Harland, 2007). As in *Xenopus*, the lack of Nodal signaling also results in premature neural differentiation in the mouse (Camus et al., 2006). In fact, in the mouse, BMP is expressed before gastrulation, maintaining Nodal signaling and preventing premature neural differentiation (Di-Gregorio et al., 2007). Roles for Nodal in mesoderm induction are unlikely in amphioxus as it is expressed in both presumptive mesoderm and neuroectoderm at the blastula stage. Thus, although roles for opposing Nodal/Vg1 and BMP signaling in axial patterning are evidently a conserved feature of chordates, Nodal/Vg1 signaling may have acquired additional ones in vertebrates.

Nodal/Vg1 and BMP signaling is modulated by dorsal/anteriorly expressed antagonists in amphioxus and vertebrates

Several potential modulators and regulators of Nodal/Vg1 and BMP signaling have localized expression in the amphioxus gastrula. In addition to *Cerberus*, *Chordin* and *Blimp1*, they include the BMP antagonists *Bambi*, *Tsg* and *Tob*, the nodal antagonist *Lefty*, as well as *Tolloid-like*, which encodes a metalloprotease that may degrade Chordin (Fig. 8) (Holland et

al., 1997; Yu et al., 2007). Most of these genes are expressed in comparable patterns as their vertebrate homologs suggesting that regulation of Nodal/Vg1 and BMP signaling is conserved in amphioxus and vertebrates. Our experiments with *Blimp1*, *Cerberus* and *Chordin* are consistent with this idea.

In *Xenopus*, as in amphioxus, *Blimp1* is required for development of the head and anterior endomesoderm (de Souza et al., 1999). However, although *Xenopus Blimp1* positively regulates *Cerberus*, the same is not likely to be true in amphioxus as the domains of *Blimp1* and *Cerberus* do not appear to overlap (Fig. 1A'-H') and *Blimp1* knockdown in amphioxus, like *Cerberus* overexpression, eliminates *FoxQ2* expression (Fig. 6A, E, I, M). Consistent with these data, amphioxus *Blimp1* activates transcription from a Nodal-responsive promoter (*XMix2*) in *Xenopus* animal cap assays (Fig. 7H) indicating that it can potentiate Nodal signaling. Since neither *Nodal* nor *Vg1* is co-expressed with *Blimp1*, this effect is likely to be indirect. In the zebrafish, unlike *Xenopus* and amphioxus, overexpression of *Blimp1* inhibits dorsal/anterior structures and reduces *Chordin* expression (Wilm and Solnica-Krezel, 2005). Thus, *Blimp1* function in head formation may be conserved to some extent between amphioxus and *Xenopus*, but appears to have been modified in teleosts. Interestingly, *Blimp1* is expressed in the invaginating mesendoderm not only in amphioxus, but also in sea urchins, where it activates *Wnt8* expression and represses itself, ultimately causing downregulation of *Wnt8* (reviewed in Smith et al., 2007). The same could be true of amphioxus since *Wnt8* is initially co-expressed with *Blimp1* in the invaginating mesendoderm but is soon down-regulated except immediately around the blastopore (Schubert et al., 2000; Yasui et al., 2001).

Expression of *Cerberus* in the anterior mesoderm of amphioxus, the anterior endomesoderm in *Xenopus* and the anterior visceral endoderm in the mouse is comparable (Fig. 1A'-C') (Bouwmeester et al., 1996; Perea-Gomez et al., 2002). Moreover, both amphioxus *Cerberus* and *Xenopus Cerberus* induce respectively a complete secondary axis or secondary head when ventro-vegetally expressed in frog embryos (Bouwmeester et al., 1996; Piccolo et al., 1999) (Fig. 7B). Although experiments in *Xenopus* animal caps indicate that amphioxus Cerberus, like its vertebrate counterparts is multifunctional, overexpression in vivo suggests that during gastrulation in amphioxus, the function of Cerberus as a suppressor of Nodal/Vg1 signaling dominates its function as a BMP suppressor (Fig. 6). Later in development, like *Cerl-2* expression in the mouse node (Marques et al., 2004), and Kupffer's vesicle in the zebrafish (Hashimoto et al., 2004), expression of amphioxus *Cerberus* shifts to the right (Fig. 1C',D'), where it presumably suppresses Nodal/Vg1 signals from the left (Yu et al., 2002; 2007).

Unlike Cerberus, Chordin is not multifunctional. It is only known to antagonize signaling by vertebrate BMP2, 4 and 7 (Gazzerro and Canalis, 2006). Compared to overexpressing BMP2/4, knockdown of *Chordin* function in amphioxus gives a relatively mild phenotype which could be due to residual Chordin translation or redundancy with another BMP antagonist, or because Chordin only antagonizes BMP2/4 and not BMP5-8, which is co-expressed with BMP2/4 (Yu et al., 2007). The emerging picture is that opposing BMP and Nodal/Vg1 signals are major players in head induction. Signaling by these pathways is modulated by secreted antagonists, most of which are expressed anteriorly and dorsally.

Although these interactions are doubtlessly complex, the amphioxus embryo offers an excellent system for unraveling them because of its structural simplicity and comparative lack of genetic redundancy.

The evolution of axial patterning in chordates

The model we propose for head specification in amphioxus (and, by extension, in the common chordate ancestor of amphioxus and vertebrates) involves evolutionarily ancient roles of BMP2/4 and Wnt/β-catenin signaling plus the more recent ones for Nodal/Vg1 signaling. In cnidarians and the two major groups of bilaterians, BMPs and their antagonists are expressed asymmetrically (Matus et al., 2006a; Rentzsch et al., 2006), while Wnt genes are expressed around the blastopore (reviewed in Holland, 2002; Tanaka and Weidinger, 2008). Asymmetrical expression of these genes led to the suggestion that the cnidarian ancestor had both A/P and D/V polarity, although the latter is not mediated by BMPs (Matus et al., 2006a,b). In contrast, it is well documented that opposing BMP and Chordin signals mediate D/V patterning in both protostomes and deuterostomes (De Robertis, 2008; Mizutani and Bier, 2008) while at least in most deuterostomes, Wnt/β-catenin signaling specifies posterior identity.

Roles for Nodal, Vg1 and Lefty in axial patterning evolved at the base of the deuterostomes or possibly at the base of the Bilateria. Cnidarians lack these genes, although *Nematostella* has genes related to *inhibin/activin* (Miyazawa et al., 2002; Herpin et al., 2004). In protostomes, Nodal signaling also functions in left-right patterning, but apparently not in dorsal/ventral patterning (Grande and Patel. 2009). However, among the invertebrate deuterostomes, *Nodal* and *Vg1* mediate D/V patterning in sea urchins, as they do in amphioxus and vertebrates. In both sea urchins and amphioxus, the *Vg1*, *Nodal* and *Lefty* domains overlap with one another (Fig. 8) (Duboc et al., 2008; 2010), and with that of *BMP2/4*. There are two minor differences. The first is that in the sea urchin, the expression domain of *BMP2/4* is restricted to the oral (ventral) side, although the protein is localized to the aboral side (Duboc et al., 2010), but initially includes the entire embryo in amphioxus, being downregulated dorsally at the onset of neurulation. The second is that *Nodal* is expressed on the oral (ventral) side in sea urchins but dorsally in amphioxus, probably indicative of a D/V inversion at the base of the chordates. In sea urchin embryos, *univin/Vg1*, like amphioxus *Vg1*, is maternally expressed and is required for *Nodal* expression (Flowers et al., 2004; Duboc and Lepage, 2008). Moreover, like amphioxus embryos, those of sea urchins are radialized and ventralized by human Activin B (Flowers et al., 2004). In addition, antagonism between Nodal and BMP2/4 patterns the sea urchin embryonic D/V axis, while *Nodal* overexpression transforms the entire ectoderm into oral (ventral) ectoderm (Duboc et al., 2004). This is comparable to conversion of the entire amphioxus ectoderm to neuroectoderm by human Activin. Therefore, we propose that the fundamental mechanism mediating D/V polarity in deuterostomes involves two steps—first maternal Nodal/Vg1 establishes the ventral (oral) side of the sea urchin embryo or the dorsal side of the amphioxus embryo and then, opposition of Nodal/Vg1 signaling by BMPs specifies the opposite side of the embryo, thus establishing the gastrula D/V axis.

Although the focus on D/V patterning of bilaterian embryos has been on the suppression of BMP signaling by Chordin, our results indicate that antagonism between Nodal/Vg1 and BMP signaling is perhaps of more fundamental importance. In sea urchins, BMP/Nodal antagonism is required for maintenance of the aboral-oral (D/V) axis (Christiaen et al., 2007; Duboc et al., 2010), but there are conflicting reports as to whether manipulating Chordin levels affects D/V patterning. Although both Bradham et al. (2009) and Lapraz et al. (2009), report that chordin opposes BMP2/4 signaling in sea urchins, the former found no effect of altered chordin levels on D/V patterning, while the latter found that overexpression of *Chordin* mRNA strongly inhibited dorsal development in 30% of the embryos while the remaining 70% lacked expression of *Tbx2/3* on the dorsal side. Additional experiments are needed to clarify the discrepancy.

In hemichordate embryos, which have little or no CNS and are the sister group of echinoderms, *BMP2/4* and *Chordin* are expressed on opposite sides of the embryo (Lowe et al., 2006), but it is not known if antagonism between Nodal and BMPs is involved in D/V patterning. If *Nodal* expression is co-localized with *Chordin*, it could indicate that coupling of opposing Nodal and BMP signals to partition the ectoderm into neural and non-neural territories evolved at the base of the chordates. This would add fuel to the argument concerning whether or not the CNS of protostomes and chordates evolved independently (Holland, 2003; De Robertis, 2008).

The emerging model for axial patterning is that in the deuterostome ancestor, Nodal/Vg1 signaling specified dorsal identity, and opposition between Nodal/Vg1 and BMP signaling was fundamental to both D/V and A/P patterning. In chordates, head formation requires modulation of these signaling pathways by antagonists that are secreted both anteriorly and dorsally. Thus a fundamental mechanism for D/V and A/P patterning with opposing gradients of BMP and Nodal/Vg1 signaling along both the D/V and A/P axes and of Wnt/β-catenin along the A/P axis was probably present in the embryo of the ancestral chordate (Fig. 8) and has been modified to a greater or lesser extent in various chordate lineages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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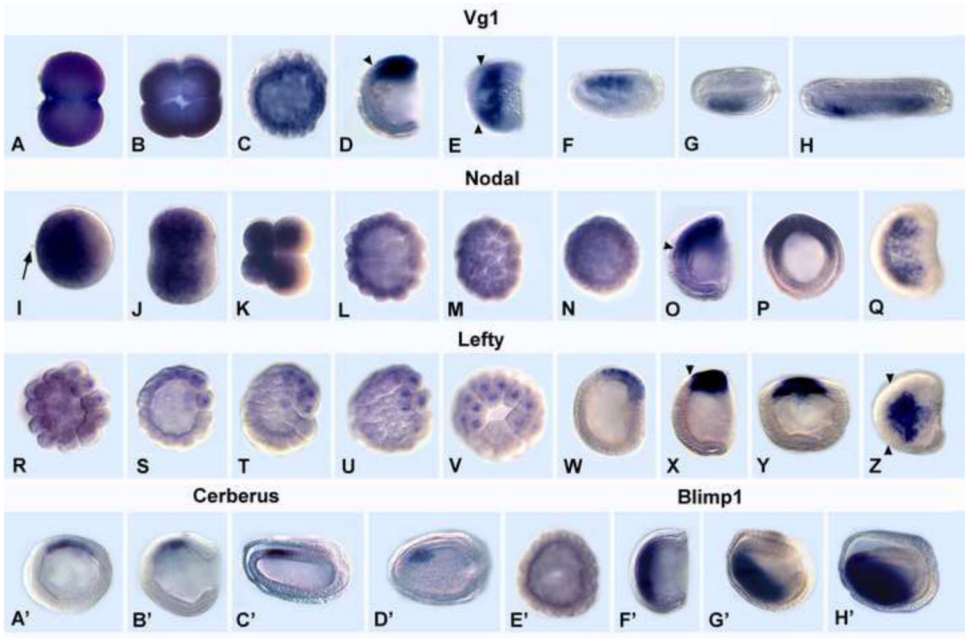

Fig. 1.

Expression of *Vg1*, *Nodal*, *Lefty*, *Cerberus* and *Blimp1* in normal amphioxus embryos. Animal pole or anterior to left except as noted. Arrowheads = anterior limits of expression. (A-H) *Vg1*. (A) 2-cell stage. (B) 4-cell stage; dorsal view. (C) mid-blastula. (D) mid-gastrula; side view, blastopore at right. (E) Dorsal view of mid-gastrula in D. (F) Mid-neurula (15 hrs); side view. (G) Dorsal view of embryo in F. Expression in 4 left anterior somites; blastopore at right. (H) Late neurula (20 hrs); dorsal view; expression in left somites. (I-Q). *Nodal*. (I) Fertilized egg. Arrow shows second polar body. (J) 2-cell stage. (K) 8-cell stage. (L) Mid-blastula. (M) Surface view of blastula in K. (N) Late blastula; optical cross section. (O) Mid-gastrula. Side view; blastopore at right. (P). Blastopore view of the gastrula in O. (Q) Dorsal view of the gastrula in O. (R-Z) *Lefty*. (R) Very early blastula. (S) early-mid blastula. (T) Mid-blastula. (U) surface view of the embryo in T. (V) Vegetal pole view of the embryo in V. (W) Very early gastrula. (X) Mid-gastrula. (Y) Blastoporal view of late gastrula. (Z) Dorsal view of late gastrula. (A'-D'). *Cerberus*. (A') Mid-gastrula; optical cross-section; animal pole at top. (B') Mid-gastrula; blastopore at right. (C') Early neurula; side view and (D') dorsal view. *Cerberus* expression in anterior right paraxial mesoderm. (E'-H') *Blimp1*. (E') Mid-blastula. (F'). Mid-gastrula. Blastopore at right. (G') Late gastrula. (H') Early neurula.

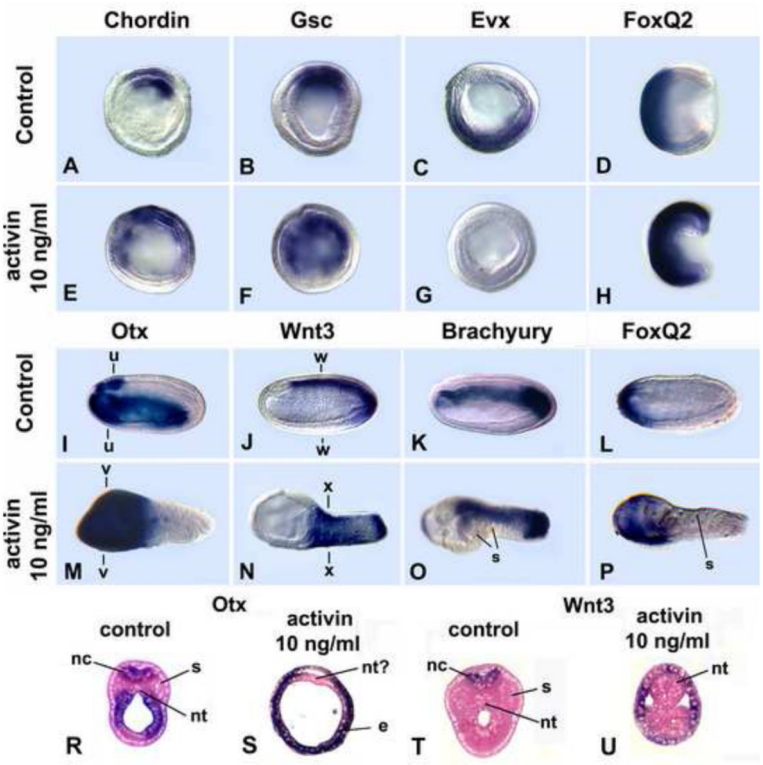

Fig. 2.

Human Activin protein dorsal/anteriorizes amphioxus embryos. 10 ng/ml human activin applied at the early blastula eliminates expression of the ventral marker *Evx* and expands those of the anterior marker *FoxQ2* and dorsal markers *Chordin* and *Gsc*. The entire ectoderm is specified as neural; the forebrain expressing *Otx* is expanded. (A-C, E-G) Mid-gastrulae; optical cross-sections. (D, H) Mid-gastrulae; side views, blastopore at right. (I-P) Mid-neurulae (15 hr); side view. Anterior at left. (R-U) Cross-sections through levels indicated in I, J, M, N. nc = nerve cord, nt = notochord, s = somites, e = endoderm. Gene markers as indicated.

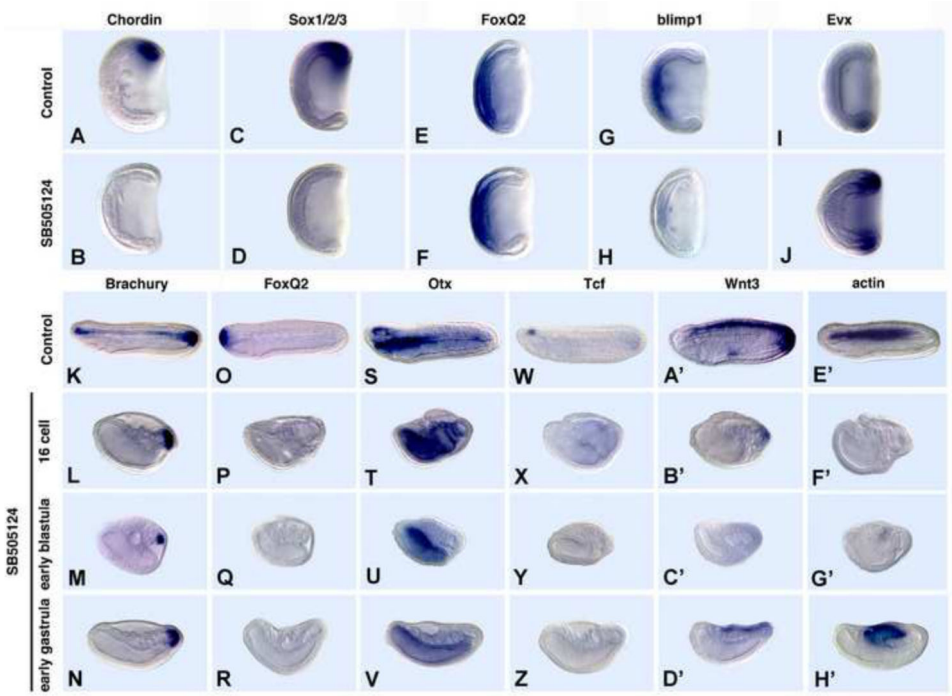

Fig. 3.

Inhibition of *Nodal/Vg1* signaling ventral/posteriorizes amphioxus embryos. 50 μM SB505124, which inhibits signaling by Nodal, Vg1 and Activin, was added at the early blastula unless otherwise indicated. Side views. Anterior to left. (A-J) Mid-gastrula stage. Expression of dorsal and anterior markers except for *FoxQ2* is eliminated. (K-H') Mid-late neurulae. The earlier SB505124 is added, the more severe the ventralization and the greater the foreshortening.

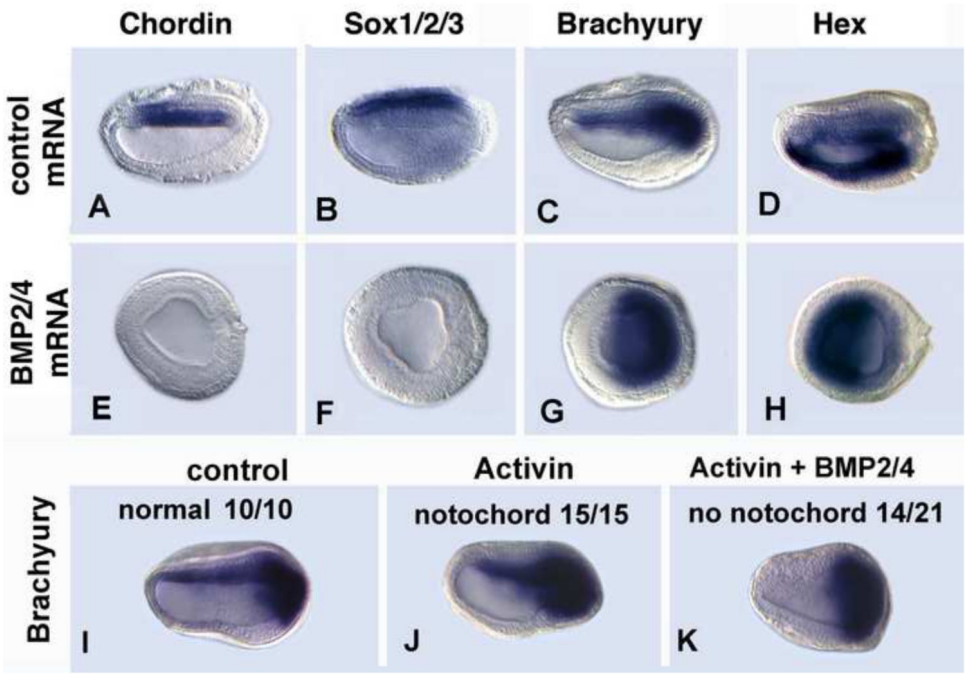

Fig. 4.

Activin protein rescues the ventral/posteriorizing defects in embryos overexpressing *BMP2/4*. Early mid-neurulae (14 hrs). Gene markers as indicated (A-H) Injection of amphioxus *BMP2/4* mRNA eliminates dorsal and anterior structures. Embryos are severely foreshortened. (I-K). Human activin protein (10 ng/ml) added to blastulae dorsal/anteriorizes embryos, causing an expanded anterior end (J). (K) Embryos injected with amphioxus *BMP2/4* mRNA and treated with human Activin protein at the blastula stage are either partially rescued with restoration of a narrower anterior end and a decreased domain of posterior *Brachyury* expression, but no notochord (14/21 embryos) or nearly completely rescued with restoration of the notochord as well (7/21).

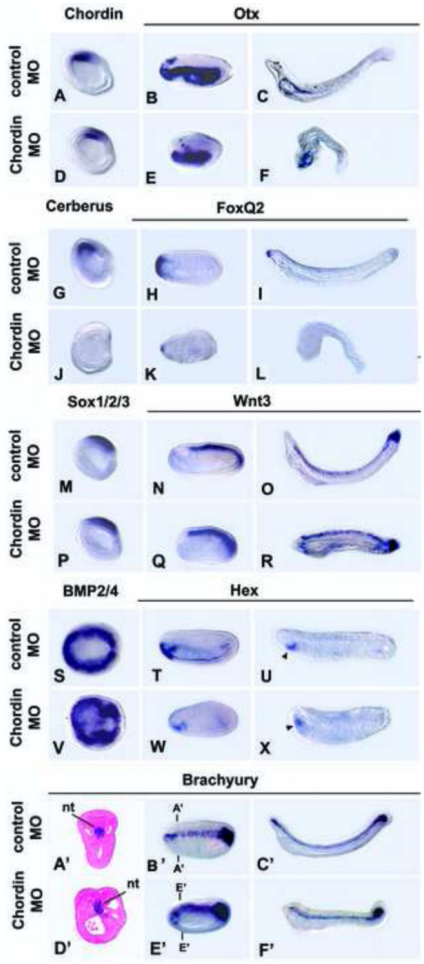

Fig. 5.

Knockdown of Chordin truncates the head. Side views, anterior to left, except dorsal views in S,V and cross sections in A', D'. (A, D, G, J, M, P) Mid gastrulae. (B, E, H, K, N, Q, T, W, A', B',D', E') Mid-neurulae (15 hrs). (U, X) Late neurulae (22 hrs). (C, F, I, L, O, R, U, X, C' F') Early larvae (36 hrs). (A,D) *Chordin* expression is unaffected. (B,C,E,F) *Otx* expression in the CNS is reduced (arrow). (G,J) *Cerberus* expression is eliminated. (H,K,I,L) *FoxQ2* expression eliminated. (M, P) *Sox1/2/3* expression in CNS unaffected. (N, O, Q, R) *Wnt3* expression unaffected, but the forebrain/midbrain, which normally does not express *Wnt3*, is reduced. (S,V) Late gastrula; dorsal views. *BMP2/4* partially down regulated in neural plate. (T,W,U,X) *Hex* expression in anterior endoderm reduced. Arrowhead in U,X indicates presumptive endostyle (A', F') Brachyury expression in notochord largely unaffected (A'-F'). In some neurulae (D', E'), the domain extends anteriorly. A'-A' and D', D' indicate levels of cross sections in A' and D'.

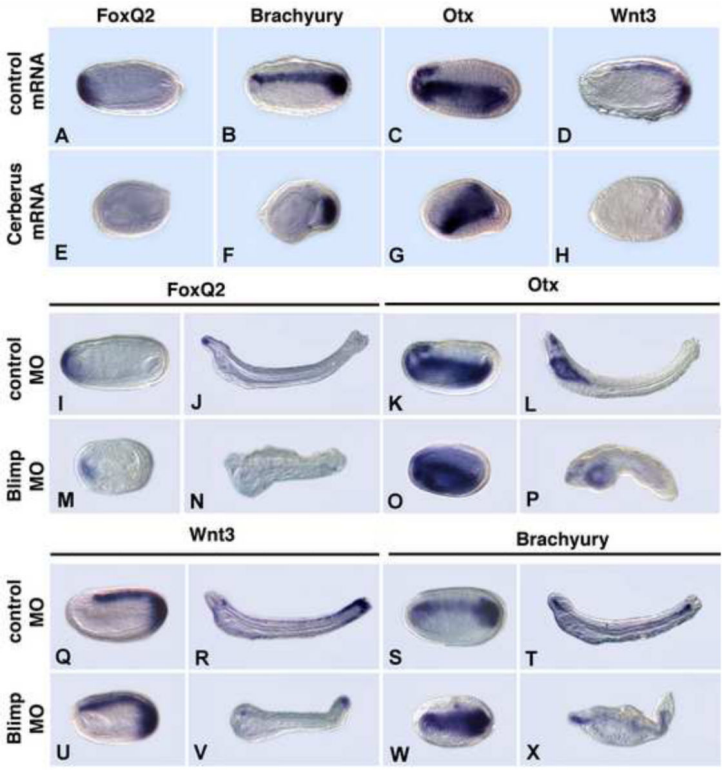

Fig. 6.

Overexpression of *Cerberus* mRNA or knockdown of *Blimp1* ventralizes and posteriorizes amphioxus embryos. Side views. Anterior at left. Expression of gene markers as noted. (A-H) Overexpression of *Cerberus* eliminates dorsal, anterior structures. Early mid-neurulae (15 hrs). (A-D) Injection of control mRNA. (E-H) Injected with *Cerberus* mRNA. (I-X) MO knockdown of *Blimp* eliminates the anterior part of the head. (I,M,K,O,Q,U,S,W) Early mid-neurulae (15 hrs). (J,N,L,P,R,V,T,X) Early larva (36 hrs). (I-L,Q-T) Embryos injected with control MO. (M-P,U-X) Injected with *Blimp1* MO.

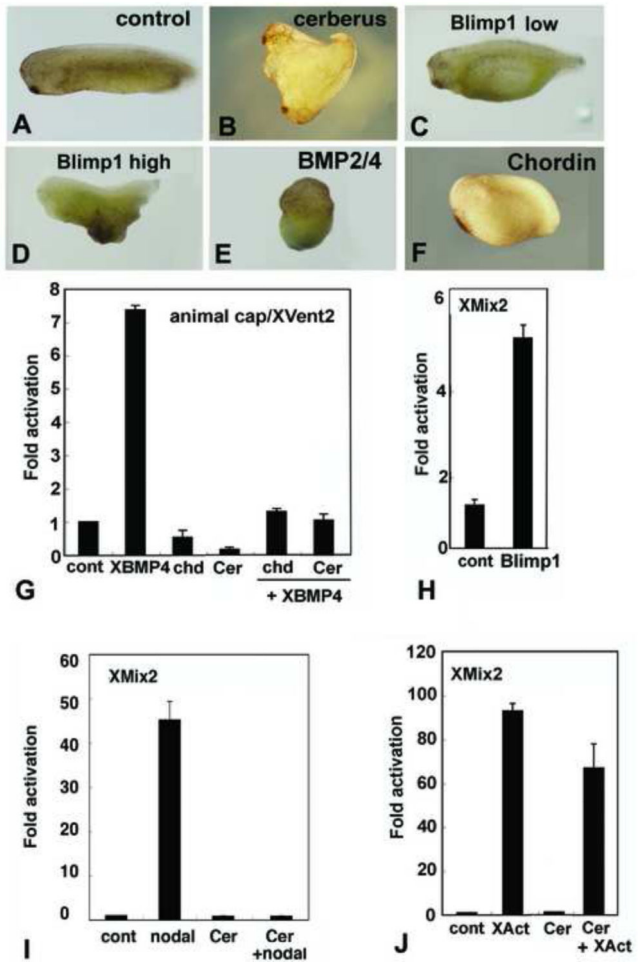

Fig. 7.

Experiments in *Xenopus* show conserved protein functions. (A-F) Injections into marginal zones of 4-cell *Xenopus* embryos except for B. (A) Uninjected control. (B) 10pg/cell amphioxus *Cerberus* mRNA in D4 blastomere at 32 cell stage induces secondary axis. (C) 100pg/cell amphioxus *Blimp1* mRNA expands ventral structures. (D) 500 pg/cell amphioxus *Blimp1* mRNA reduces head structures and expands ventral ones. (E) 100pg/cell amphioxus *BMP2/4* mRNA ventralizes. (F) 10pg/cell amphioxus *Chordin* mRNA enlarges the cement gland. (G-J) Assays of amphioxus proteins in *Xenopus* animal caps. (G) BMP responsive reporter assay. Amphioxus Chordin and Cerberus suppress signaling by *Xenopus* BMP4, as monitored by transcription from an *Xvent2* reporter. (H) Amphioxus Blimp1 induces transcription from a Nodal-responsive (Xmix2) reporter construct. (I) Nodal-responsive assay. Amphioxus Cerberus suppresses amphioxus Nodal. (J) Activin response assay. Amphioxus Cerberus suppresses *Xenopus* Activin signaling. Experiments done twice with comparable results; error bars ± 1 s.d.

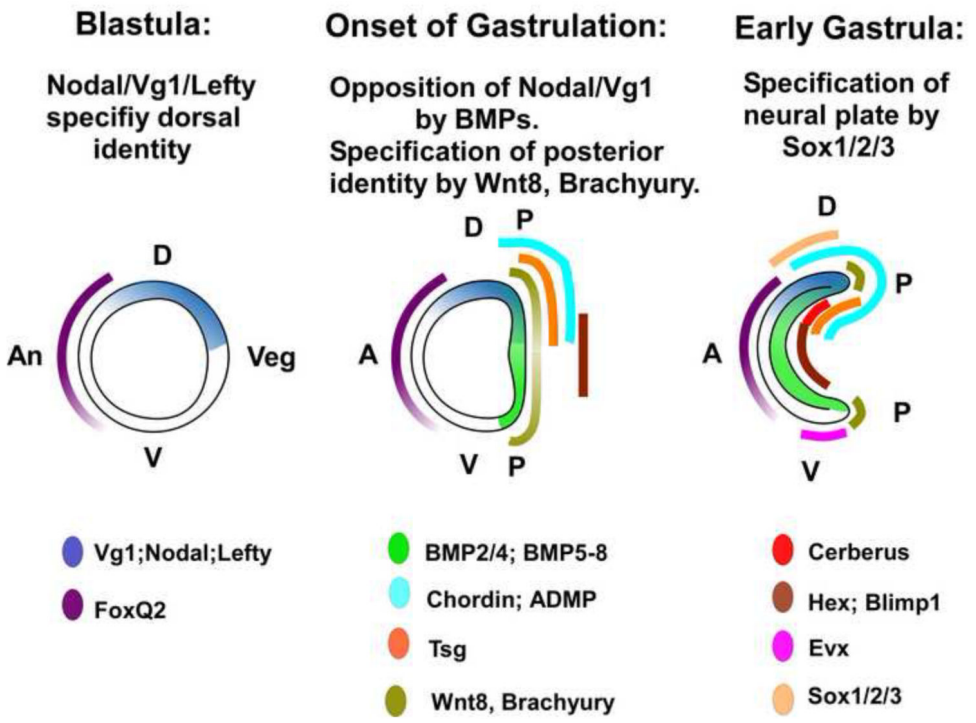

Fig. 8.

Diagram of axial patterning genes in early amphioxus development of amphioxus. In the late blastula, Nodal/Vg1 signaling, presumably opposed by Lefty marks the future dorsal side (D) of the embryo, while *FoxQ2* marks the anterior ectoderm. At the onset of gastrulation, dorsal Nodal/Vg1 signaling becomes opposed by BMP signaling in the mesendoderm. BMP signaling is modulated by several dorsally and anteriorly expressed genes. *Wnt8* is expressed throughout the future mesendoderm, most strongly in the rim of the forming blastopore, which will be the posterior pole of the embryo. By the early gastrula, additional modulators of Nodal/Vg1 and BMP signaling, *Evx*, a marker of ventral ectoderm, and the neuroectoderm marker *Sox1/2/3* are first expressed. A= anterior pole, An = animal pole, P= posterior pole, V= ventral.