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# Cis-regulatory analysis of *nodal* and maternal control of dorsal-ventral axis formation by Univin, a TGF-β related to Vg1

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The TGF-β family member Nodal is essential for specification of the dorsal-ventral axis of the sea urchin embryo, but the molecular factors regulating its expression are not known. Analysis of the *nodal* promoter is an excellent entry point to identify these factors and to dissect the regulatory logic driving dorsal-ventral axis specification. Using phylogenetic footprinting, we delineated two regulatory regions located in the 5′ region of the *nodal* promoter and in the intron that are required for correct spatial expression and for autoregulation. The 5′ regulatory region contains essential binding sites for homeodomain, bZIP, Oct, Tcf/Lef, Sox and Smad transcription factors, and a binding site for an unidentified spatial repressor possibly related to Myb. Soon after its initiation, *nodal* expression critically requires autoregulation by Nodal and signaling by the maternal TGF-β Univin. We show that Univin is related to Vg1, that both Nodal and Univin signal through Alk4/5/7, and that zygotic expression of *univin*, like that of *nodal*, is dependent on SoxB1 function and Tcf/β-catenin signaling. This work shows that Tcf, SoxB1 and Univin play essential roles in the regulation of *nodal* expression in the sea urchin and suggests that some of the regulatory interactions controlling *nodal* expression predate the chordates. The data are consistent with a model of *nodal* regulation in which a maternal TGF-β acts in synergy with maternal transcription factors and with spatial repressors to establish the dorsal-ventral axis of the sea urchin embryo.

**KEY WORDS: *nodal*, *vg1*, *univin*, GDFs, bZIP, Smad, SoxB1, Dorsal-ventral axis, Oral-aboral axis, Cis-regulatory analysis, Phylogenetic footprint, Gene regulatory network, *Paracentrotus lividus***

## INTRODUCTION

In echinoderms, as in vertebrates, the dorsal-ventral axis is specified after fertilisation by mechanisms that rely on cell-cell interactions and TGF-β signals of the Nodal family (Duboc and Lepage, 2006; Duboc et al., 2004). In the sea urchin embryo, expression of *nodal* is initiated around the 60-cell stage in most cells of the presumptive ectoderm and becomes rapidly restricted to the presumptive ventral ectoderm by mechanisms that remain unknown.

*nodal* is the earliest zygotic gene displaying a restricted expression along the dorsal-ventral axis during sea urchin development and Nodal function is absolutely required for establishment of dorsal-ventral polarity. When translation of the *nodal* transcript is prevented, specification of both the ventral and the dorsal ectoderm fails and most of the ectoderm differentiates into a thickened ciliated ectoderm that represents the default state in the absence of Nodal. Functional studies have shown that overexpression of Nodal is sufficient to specify most ectodermal cells with a ventral fate. Furthermore, rescue experiments revealed that Nodal-expressing cells have a long-range organizing activity and are capable of restoring dorsal-ventral polarity over the whole embryo (Duboc et al., 2005; Duboc et al., 2004). Nodal activates a regulatory network of genes necessary for dorsal-ventral axis formation, encoding key transcription factors such as Goosecoid, Brachyury and FoxA, as well as signaling molecules such as Bmp2/4 and the Nodal antagonist Lefty (Duboc and Lepage, 2006; Duboc et al., 2005). Therefore, the *nodal* gene is a crucial component of dorsal-ventral axis formation in the sea urchin and understanding the molecular mechanisms responsible for the initiation of *nodal* expression is important for understanding how the body plan of this organism is established.

Classical bisection experiments on sea urchin embryos performed by Hörstadius and more recently by Wilkrayamanake et al. and Yaguchi et al. demonstrated that in the absence of vegetal signaling, animal-half explants develop into neurogenic ectoderm that lacks dorsal-ventral polarity. However, when these explants are recombined with vegetal blastomeres or when they are treated with lithium, dorsal-ventral polarity and stomodeum formation are rescued (Hörstadius, 1973; Wikramanayake et al., 1995; Yaguchi et al., 2006). Together with our previous finding that expression of *nodal* also requires a functional Tcf/β-catenin pathway (Duboc et al., 2004), these experiments strongly suggest that a β-catenin-dependent vegetal signal is required for induction of *nodal* expression in the animal hemisphere.

Another molecular pathway implicated in the activation of *nodal* expression is the p38 MAP kinase signaling pathway. p38 signaling is activated around the 60-cell stage ubiquitously and is transiently inactivated on the presumptive dorsal side. When p38 signaling is inhibited, *nodal* expression is not initiated (Bradham and McClay, 2006). The function of p38 during the establishment of *nodal* expression is not known.

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A possible link between p38 signaling and the transcriptional activation of *nodal* is suggested by the known function of p38 in the transcriptional responses downstream of oxidative stress (Torres and Forman, 2003). Experiments performed some years ago implicated oxidative gradients in the formation of the dorsal-ventral axis of sea urchin embryos (Czihak, 1971). Intriguingly, these respiratory gradients, visualized by mitochondrial cytochrome oxidase activity, prefigure the dorsal-ventral axis of the early embryo as early as the 8-cell stage (Czihak, 1963). Even more enigmatic is the finding that orientation of the dorsal-ventral axis can be biased by using respiratory inhibitors or by culturing embryos in hypoxic conditions (Child, 1948; Coffman and Davidson, 2001; Pease, 1941). Recent studies reported that mitochondria are asymmetrically distributed in the egg of *Strongylocentrotus purpuratus* and that microinjection of purified mitochondria can bias orientation of the dorsal-ventral axis (Coffman et al., 2004). However, the links between the mitochondria, redox gradients, p38 signaling and the transcriptional machinery responsible for initiating *nodal* expression remain to be established. Since the *nodal* gene is a key regulator of dorsal-ventral axis formation and the earliest zygotic gene showing a restricted expression along this axis, it provides an excellent entry point to dissect these relationships.

In this study, we identified the cis-regulatory elements of the sea urchin *nodal* gene and used them to dissect the regulatory interactions involved in the control of *nodal* expression.

## MATERIALS AND METHODS

### Phylogenetic and linkage analyses

Phylogenetic trees were calculated using the maximum likelihood method with PhyML using the WAG substitution model (http://atgc.lirmm.fr/phyml/) (Guindon et al., 2005). A consensus tree with a 50% cut-off value was derived from a 500 bootstrap analysis using Mega 3.1 (http://www.megasoftware.net/). Numbers above branches represent posterior probabilities, calculated from this consensus.

Linkage analysis was performed by searching the zebrafish (http://www.ensembl.org/Danio_rerio/index.html) and sea urchin (http://www.ncbi.nlm.nih.gov/genome/guide/sea_urchin/index.html) genomes for *dvr1/bmp2* and for *univin/bmp2/4*.

### In situ hybridization and quantitative (Q) PCR

The *nodal* and *univin* probes have been described previously (Duboc et al., 2004; Lapraz et al., 2006). For QPCR, total RNA was prepared from 100 embryos using Trizol (Invitrogen) and reverse transcribed using the TaqmanR PCR Kit (Applied Biosystems) after first removing DNA with DNaseI. Cycling was performed using a Lightcycler 480 (Roche) and Fast Start SYBR Green PCR Kit (Roche) with optimized primer pairs. Relative quantification of *nodal* expression between experimental samples and controls was obtained by subtracting the sample Ct (threshold cycle) from the control Ct and using *ubiquitin* mRNA as an internal control.

### Treatments, constructs, RNA and morpholino injections

Treatment with 10 μM SB431542 was performed as described previously (Duboc et al., 2005). Dominant-negative *dnTcf* mRNA was used at 500 μg/ml and *univin* mRNA at 800 μg/ml.

The specificity of the Nodal morpholino used in this study has been demonstrated previously (Duboc et al., 2004). Two oligonucleotides, directed against different regions of the SoxB1 5′ UTR were used and produced similar phenotypes including radialization, absence of spicules and gut as described previously (Kenny et al., 2003). Similarly, two different morpholino oligonucleotides directed against the *univin* transcript produced identical phenotypes. A single morpholino directed against the first eight codons of the *alk4/5/7* transcript produced a phenotype extremely consistent with the presumed role of the protein encoded by this transcript as a receptor for Nodal. The specificity of this morpholino was demonstrated by performing a rescue experiment. Sequences of morpholino oligonucleotides are:
NodalMo, 5′-ACTTTGCGACTTTAGCTAATGATGC;
UnivinMo1, 5′-ACGTCCATATTTAGCTCGTGTTTGT;
UnivinMo2, 5′-GTTAAACTCACCTTTCTAAACTCAC;
SoxBMo1, 5′-GACAGTCTCTTTGAAATTAGACGAC;
SoxB1Mo2, 5′-GAAATAAAGCCAAAGTCTTTTGATG; and
Alk4/5/7Mo, 5′-TAAGTATAGCACGTTCCAATGCCAT.

All injections were repeated three times and for each experiment 50-100 embryos were analyzed. Only representative phenotypes present in at least 80% of the injected embryos are presented.

### Isolation of BAC clones

*Paracentrotus lividus* and *Lytechinus variegatus* BAC libraries were screened with a radioactive probe corresponding to the 5′ UTR of *nodal* and ten positive clones were further characterized by pulse-field electrophoresis, PCR and restriction analysis. 15 kb of upstream sequence were obtained for the longest *L. variegatus* clone (120 kb) and for the *Paracentrotus* (60 kb) clone by subcloning and sequencing restriction fragments. The entire *P. lividus* BAC clone was subsequently sequenced by the Marine Genomics Europe technology platform. Sequence from the *Strongylocentrotus nodal* locus was obtained from the Sea Urchin Genome Assembly.

### Comparison of genomic sequences

Sequence comparisons were performed with the Vista platform (http://genome.lbl.gov/vista/index.shtml). The window size used varied between 50 and 100 bp, with a window of 50 bp with 75% conservation.

### Reporter constructs and mutagenesis

A GFP construct containing the *Endo16* basal promoter, EpGFP (Arnone et al., 1997), was used for spatial expression analysis. For quantitative analyses, the luciferase expression vector pGL3 Basic was modified by introducing the *endo16* promoter in front of the luciferase start site (henceforth referred to as EpGluc). The relevant conserved regions were PCR amplified from the *Paracentrotus* BAC clone using the Long Expand PCR System (Roche) and appropriate primers (see Table 1). Each relevant fragment was introduced into the appropriate vector using standard molecular biology techniques and each construct was verified by sequencing. The conserved predicted binding sites for Smad, homeodomain, TCF, Oct, Sox and bZIP factors were identified using TransFac and MatInspector software (Genomatix). Primers containing between four and eight mutated bases were generated for each of these sites (see Table 1). Mutations were introduced by PCR using Pfx polymerase (Invitrogen). All mutations were confirmed by restriction digestion and sequencing.

### GFP and luciferase reporter assays

Microinjections of purified and linearized plasmids were carried out by established protocols (Arnone et al., 2004). In the case of GFP reporters, the linearized construct was injected at 5 ng/μl together with carrier DNA (*Hin*dIII-digested sea urchin genomic DNA) at 30 ng/μl. In the case of luciferase reporters, the linearized plasmid was injected at 5 ng/μl, together with pRL-TK (*Renilla* luciferase) or Endo16-Renilla DNA at 7.5 ng/μl and carrier DNA at 30 ng/μl. For each experiment, more than 50 embryos were mounted and the position of GFP-expressing cells within individual embryos was scored. For luciferase analysis, between 100 and 150 injected embryos were collected and the level of luciferase and Renilla determined with the Dual Luciferase Kit (Promega) according to the manufacturer's instructions using a GloMax luminometer with an integration of 10 seconds. The level of luciferase activity was normalized to the level of Renilla activity for each experiment. All experiments were repeated three times using separate batches of embryos.

**Table 1. Oligonucleotides (5′-3′) used in this study**

| Site/primer | | Sequence |
|---|---|---|
| **R-module PCR primers** | | |
| 5′ proximal region A For | | CGGGTACCCTAGGCCTATACAGATACGTG |
| 5′ proximal region A Rev | | TCCCCCGGGCATTAGTCTATCTCTTTTTTC |
| R-module For | | CGGAATTTCGAAAAAAGAGATAGACTAATG |
| R-module Rev | | TCCCCCGGGGACACTACCTGCCCCTTAAC |
| **Intron PCR primers** | | |
| Intron For | | CGGGGTACCATTATCATCTTATATTTTCG |
| Intron Rev | | TCCCCCGGGGAAAGTGAAGGCTTATATTT |
| R-1 module For | | CGGGGTACCTCGCTGATCTTGGTGAATAC |
| A-module For | | CGGAATTCGAAGTCTATTCGCTCTCCTC |
| A-module Rev | | TCCCCCGGGCTGAAAGCTCGAACTTCAGC |
| **R-module mutagenesis primers*** | | |
| M1 | *Xho*I Fw | GAAAAAAGAGATAGACCTCGAGTTAACACTTAGAAGG |
| | *Xho*I Rev | CCTTCTAAGTGTTAACTCGAGGTCTATCTCTTTTTTC |
| M2 | *Xho*I Fw | ATAGACTAATGCTTACTCGAGAGAAGGACTACCC |
| | *Xho*I Rev | GGGTAGTCCTTCTCTCGAGTAAGCATTAGTCTATC |
| M3 | *Bgl*II Fw | CTTAACACTTAGAAGGAAGATCTTTTTATTGTCGTTTGG |
| | *Bgl*II Rev | CCAAACGACAATAAAAAGATCTTCCTTCTAAGTGTTAAG |
| M4 | *Xba* Fw | GAAGGACTACCCTTTTTCTAGAATTGTCGTTTGGTTAAAAC |
| | *Xba* Rev | GTTTTAACCAAACGACAATTCTAGAAAAAGGGTAGTCCTTC |
| M5 | *Nsi*I Fw | CCCTTTTATTGTCGTTATGCATAAACTAATTATAGTGTG |
| | *Nsi*I Rev | CACACTATAATTAGTTTATGCATAACGACAATAAAAGGG |
| M6 | *Pst*I Fw | GTCGTTTGGTTAAAACCTGCAGGCTTAGGGTGTTACAAAAG |
| | *Pst*I Rev | CTTTTGTAACACCCTAAGCCTGCAGGTTTTAACCAAACGAC |
| M7 | *Cla*I Fw | GTTAAAACTAATTATAGTATCGATAGGGTGTTACAAAAG |
| | *Cla*I Rev | CTTTTGTAACACCCTATCGATACTATAATTAGTTTTAAC |
| M8 | *Bam*HI Fw | GTGCTTAGGGTGTTAGGATCCAGCCGATTCATTGTTAATTAG |
| | *Bam*HI Rev | CTAATTAACAATGAATCGGCTGGATCCTAACACCCTAAGCAC |
| M9 | *Not*I Fw | CTTAGGGTGTTACAAAAGAGCGCGGCCGCTGTTAATTAGGAAGAC |
| | *Not*I Rev | GTCTTCCTAATTAACAGCGGCCGCGCTCTTTTGTAACACCCTAAG |
| M10 | *Sph*I Fw | CAAAAGAGCCGATTCATTGGCATGCAGGAAGACGGGTTGGG |
| | *Sph*I Rev | CCCAACCCGTCTTCCTGCATGCCAATGAATCGGCTCTTTTG |
| M11 | *Sac*I Fw | GAAGACGGGTTGGGAGGAGCTCTCCTTGTGTAGTGGG |
| | *Sac*I Rev | CCCACTACACAAGGAGAGCTCCTCCCAACCCGTCTTC |
| M12 | *Bam*HI Fw | CTTGTGTAGTGGGCCGAGGGATCCTGCTTCTATTCAACGTG |
| | *Bam*HI Rev | CACGTTGAATAGAAGCAGGATCCCTCGGCCCACTACACAAG |
| M13 | *Xho*I Fw | GGGTGAAAGGTTTTTACTCGAGATGCTTCGATTGCTAAAC |
| | *Xho*I Rev | GTTTAGCAATCGAAGCATCTCGAGTAAAAACCTTTCACCC |
| M14 | *Eco*RI Fw | GTTTTTAGCATGAATGCTTCGGAATTCAAACAATGCGCGCGCATG |
| | *Eco*RI Rev | CATGCGCGCGCATTGTTTGAATTCCGAAGCATTCATGCTAAAAAC |
| M15 | *Bam*HI Fw | CATGAATGCTTCGATTGCTGGATCCTGCGCGCGCATGTGGGG |
| | *Bam*HI Rev | CCCCACATGCGCGCGCAGGATCCAGCAATCGAAGCATTCATG |
| M16 | *Xho*I Fw | CGATTGCTAAACAATGCCTCGAGATGTGGGGTTAGGAG |
| | *Xho*I Rev | CTCCTAACCCCACATCTCGAGGCATTGTTTAGCAATCG |
| M17 | *Hin*dIII Fw | GTGGGGTTAGGAGTTTTTAAGCTTGGTGTCTGATTAGACTG |
| | *Hin*dIII Rev | CAGTCTAATCAGACACCAAGCTTAAAAACTCCTAACCCCAC |
| S2 | *Xho*I Fw | GATTCATTGTTAATTAGGACTCGAGGTTGGGAGATGGGTTC |
| | *Xho*I Rev | GAACCCATCTCCCAACCTCGAGTCCTAATTAACAATGAATC |
| S3 | *Xho*I Fw | GTTAGGAGTTTTTGAAAAGGGCTCGAGATTAGACTGTTAAGGGGC |
| | *Xho*I Rev | GCCCCTTAACAGTCTAATCTCGAGCCCTTTTCAAAAACTCCTAAC |
| S4 | *Bgl*II Fw | GAAAAGGGCTCGAGATAGATCTGTTAAGGGGCAGG |
| | *Bgl*II Rev | CCTGCCCCTTAACAGATCTATCTCGAGCCCTTTTC |
| **Intron Smad mutagenesis primers†** | | |
| S1 | *Xba*I Fw | GCGTGTTTGCTCAATCTAGAAAAATGGTCATAAAAATG |
| | *Xba*I Rev | CATTTTTATGACCATTTTTCTAGATTGAGCAAACACGC |
| S2 | *Hin*dIII Fw | CTCATTGACTTTGTTTTGAAAAGCTTTCGCTCTCCTCTAACACC |
| | *Hin*dIII Rev | GGTGTTAGAGGAGAGCGAAAGCTTTTCAAAACAAAGTCAATGAG |
| S3 | *Bam*HI Fw | CCAAATTGTTTTCTAATTAACTCGGATCCGCTATTCCGTTATCGGATAA |
| | *Bam*HI Rev | TTATCCGATAACGGAATAGCGGATCCGAGTTAATTAGAAAACAATTTGG |
| S4 | *Xho*I Fw | CAGATCAAAGGGATGGGACCTCGAGCTTTTGTTCAGAAGACCG |
| | *Xho*I Rev | CGGTCTTCTGAACAAAAGCTCGAGGTCCCATCCCTTTGATCTG |
| S5 | *Bam* Fw | CTCTTTTGTTCAGAAGACCGGATCCATGCTGAAGTTCGAGCTTTC |
| | *Bam* Rev | GAAAGCTCGAACTTCAGCATGGATCCGGTCTTCTGAACAAAAGAG |

Table continued on next page.

**Table 1. Continued**

| Site/primer | | Sequence |
|---|---|---|
| **Intron Smad mutagenesis primers†** | | |
| S6 | *Bgl*II Fw | CTCGAGCTTTTGTTCAGAGATCTGGATCCATGCTGAAGTTC |
| | *Bgl*II Rev | GAACTTCAGCATGGATCCAGATCTCTGAACAAAAGCTCGAG |
| S7 | *Eco*RI Fw | CTTTTGTTGCGGATTGAATTCCAGTGTGTTAATGG |
| | *Eco*RI Rev | CCATTAACACACTGGAATTCAATCCGCAACAAAAG |
| S8 | *Sac*II Fw | GTGTGTTAATGGAACCGCGGCTTGAAAAGAGGGG |
| | *Sac*II Rev | CCCCTCTTTTCAAGCCGCGGTTCCATTAACACAC |
| S9 | *Hind*III Fw | GGCAAGTCAGAACAGTTAACAAAAGTGATACGTC |
| | *Hind*III Rev | GACGTATCACTTTTGTTAACTGTTCTGACTTGCC |
| S10 | *Xho*I Fw | GTTAATAATATTCGCTCGAGGCATCGACCTTGGG |
| | *Xho*I Rev | CCCAAGGTCGATGCCTCGAGCGAATATTATTAAC |
| S11 | *Xba*I Fw | CATCGACCTTGGGCGTCTAGATAACTATTAATTAC |
| | *Xba*I Rev | GTAATTAATAGTTATCTAGACGCCCAAGGTCGATG |
| S12 | *Eco*RI Fw | GATTTGCGATTTTGAATTCTACAATGCAAAACC |
| | *Eco*RI Rev | GGTTTTGCATTGTAGAATTCAAAATCGCAAATC |

Restriction sites are underlined.
*Correspond to mutant site numbers (M1-M17; Smad mutants S1-S4) as shown in Fig. 4.
†Correspond to site numbers (S1-12) as shown in Fig. S1 in the supplementary material.

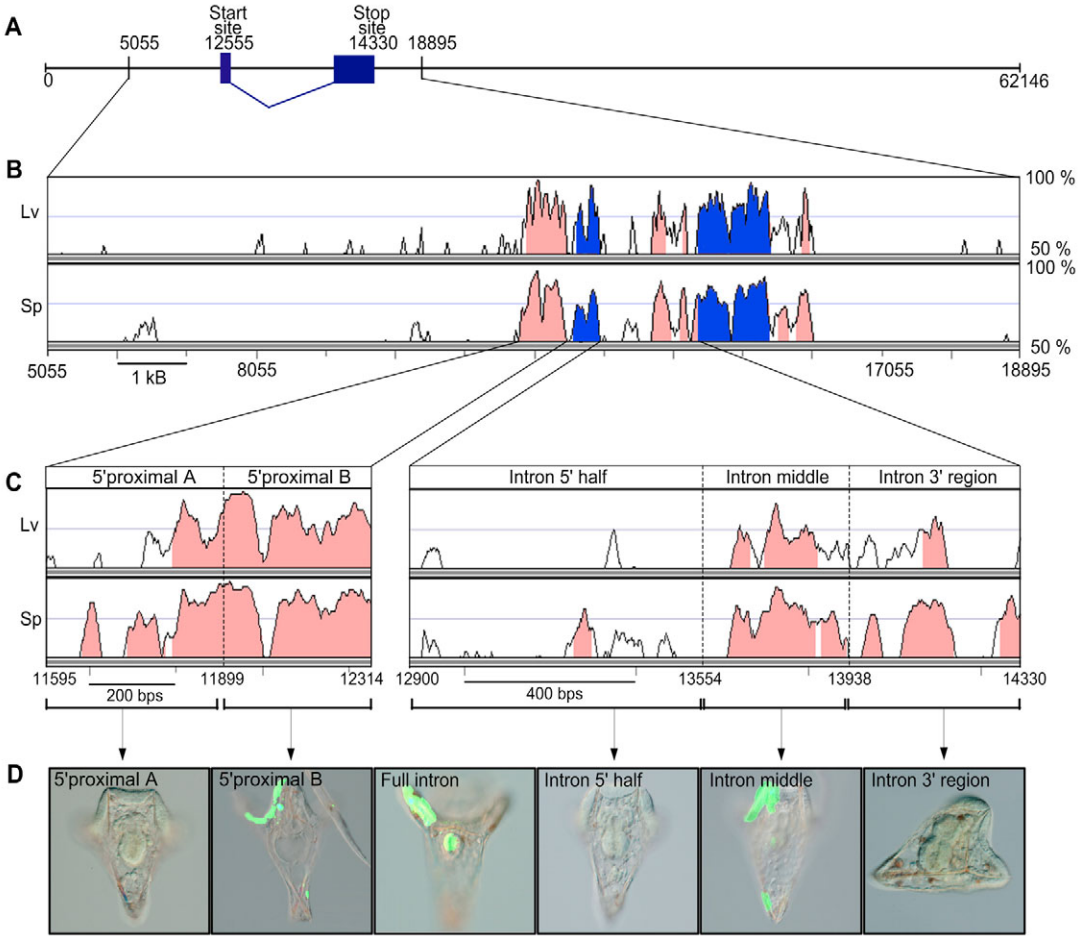


**Fig. 1. Phylogenetic footprinting and regulatory element expression analysis of *nodal*.** (**A**) Diagram showing the position of the *P. lividus nodal* gene in the BAC (top) and the region used for the phylogenetic footprinting analysis (brackets). (**B**) 15 kb of sequence surrounding the *P. lividus nodal* gene was aligned with 15 kb of *S. purpuratus* (Sp) and 9 kb of *L. variegatus* (Lv) genomic sequence using Vista. The two exons are labeled in blue and non-coding sequence showing more than 75% homology in pink. (**C**) Enlargement of the two non-coding conserved regions used for the expression and deletion analyses. Broken lines delineate the sequences used for the deletion analysis in D. (**D**) *P. lividus* genomic DNA sequence was used to drive GFP expression in *P. lividus* embryos. Merged GFP-fluorescent and DIC images of pluteus-stage embryos illustrating the results of the deletion analysis.

## RESULTS

### Identification of the cis-regulatory elements driving *nodal* expression

In order to identify cis-regulatory regions responsible for the control of *nodal* expression, we used phylogenetic footprinting. Previous studies have shown that comparisons of genomic sequences between *Lytechinus variegatus* and *Strongylocentrotus purpuratus* allow efficient identification of the highly conserved regions expected to contain regulatory elements (Revilla-i-Domingo et al., 2004; Yuh et al., 2002). BAC recombinant clones containing the *nodal* gene were isolated from *P. lividus* and *L. variegatus*. The sequence of the *S. purpuratus nodal* locus was obtained in silico from the sea urchin genome sequence. We scanned the genomic sequences of the three *nodal* genes for short conserved motifs with the Vista program using as parameters a 70% identity in a 50 bp window. In addition to regions corresponding to the coding sequences, this comparison identified three highly conserved non- coding regions (Fig. 1A,B). These consist of a 700 bp region just 5′ of the transcriptional start site, a 650 bp region within the intron, and a 600 bp region corresponding to the 3′ UTR (Fig. 1B,C).

In order to test the regulatory function of these conserved regions, we created GFP constructs with the 5′ proximal region and the full intron region, injected these into fertilized embryos and scored GFP expression at various developmental stages. Both the 5′ proximal region and the intron GFP constructs drove robust GFP expression in embryos at blastula stage (data not shown). By contrast, the region corresponding to the 3′ UTR of the transcript did not drive significant expression of the reporter gene.

In order to determine which areas of the conserved 5′ and intronic sequences contained sites involved in the transcriptional activation and/or spatial repression of *nodal* expression, we performed a deletion analysis (Fig. 1C,D). A fragment containing the first 350 bp of the 5′ proximal region did not drive detectable expression of the GFP reporter gene (Fig. 1D). By contrast, a fragment containing the last 300 bp of the proximal region efficiently drove expression of the reporter gene (Fig. 1D). Finally, a deletion that removed 600 bp of the proximal region, leaving only the last 50 bp and the predicted TATA box, did not show any expression (data not shown). This indicates that cis-regulatory elements driving *nodal* expression are located within a 300 bp region just upstream of the transcriptional start site.

Deletion analysis of the intron region showed that neither the first half nor the last 464 bp of the intron is able to drive GFP expression, whereas a small conserved region of 350 bp is capable of driving strong expression (Fig. 1D). These results indicate that regulatory elements sufficient to drive *nodal* expression reside in a short region just upstream of the first exon (R-module) and in a small, highly conserved region within the intron.

### Spatial regulatory activity of the conserved *nodal* cis-regulatory sequences

In order to determine whether the identified regulatory modules are sufficient for the proper spatial expression of *nodal* on the oral side, we examined the spatial expression profiles of GFP reporter genes driven by the R-module (the 300 bp 5′ of exon 1) or by various regions derived from the intron (Fig. 2A and Table 2).

Embryos injected with the R-module GFP construct (R-module GFP) and the whole intron GFP construct showed different spatial expression profiles. Overall, 57% of the embryos injected with R-module GFP displayed expression and of these, 87% displayed GFP expression in the oral ectoderm, whereas 35% displayed ectopic expression in other territories. The whole, highly conserved 5′ proximal region (700 bp 5′ of exon 1) showed a similar spatial expression profile (data not shown). Similar to the R-module, the whole intron drove oral expression in 85% of the GFP-expressing embryos; however, the percentage expressing GFP ectopically rose to 52% (Table 2). When these two regulatory regions were fused together into a mini-gene, the construct showed an oral bias similar to that of the R-module alone (Fig. 2B; Table 2).

Taken together, these data suggest that both the R-module and the intron contain regulatory elements involved in the spatial restriction of *nodal* and that the combinatorial activity of these elements may restrict *nodal* expression to the oral territory. However, the relatively

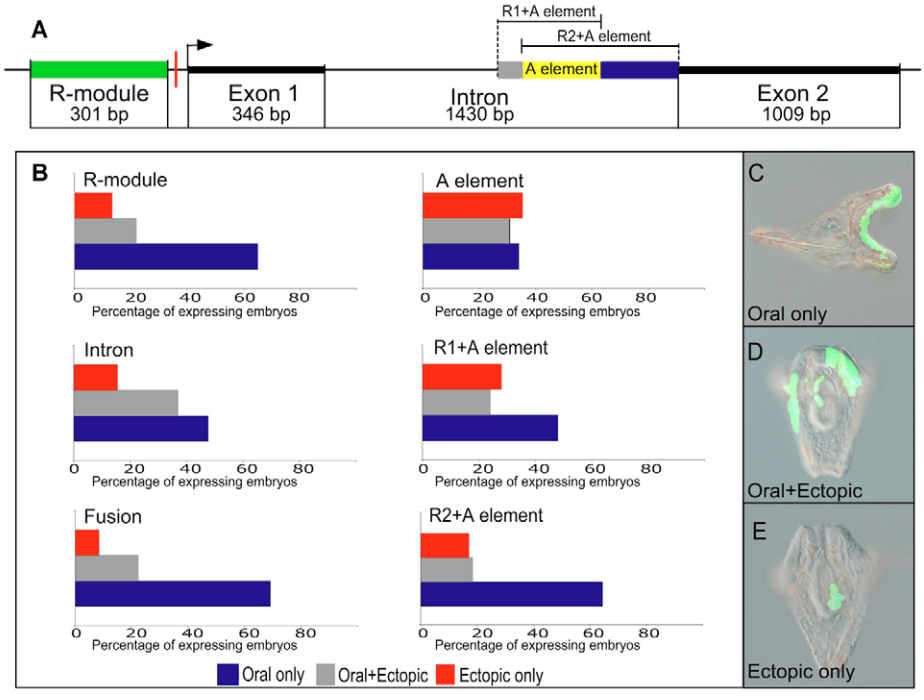


**Fig. 2. Spatial GFP expression driven by the R-module and intron GFP reporters.** (**A**) Diagram of the five regulatory elements from sea urchin *nodal* used for the spatial expression analysis. (**B**) Expression data summary for GFP constructs containing the various regulatory elements. Embryos with expression clones just in the oral ectoderm were scored as oral only; clones in either aboral ectoderm or endoderm were scored as ectopic only; and embryos that contained clones in both oral and ectopic territories were scored as oral+ectopic. (**C-E**) Examples of embryos that fit into these categories: (C) oral only, (D) oral+ectopic, (E) ectopic only.

**Table 2. Spatial expression profiles of GFP reporter genes driven by the R-module or various regions derived from the *nodal* intron**

| | | | Specific domain expression % (*n*) | | | Overall expression % (*n*) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total | Expressing | Oral only | Oral+ectopic | Ectopic only | Oral | Ectopic | Aboral | Endoderm |
| R-module | 531 | 57 (302) | 65 (196) | 22 (66) | 13 (40) | 87 (262) | 35 (106) | 26 (80) | 13 (39) |
| Intron | 322 | 52 (168) | 48 (80) | 37 (62) | 25 (26) | 85 (142) | 52 (88) | 35 (58) | 27 (45) |
| Fusion | 202 | 64 (130) | 69 (90) | 22 (29) | 8 (11) | 92 (119) | 31 (40) | 22 (28) | 13 (17) |
| A-module | 264 | 56 (147) | 34 (50) | 31 (45) | 35 (52) | 65 (95) | 66 (97) | 50 (74) | 46 (67) |
| R1+A-module | 220 | 55 (118) | 48 (57) | 27 (32) | 25 (29) | 75 (89) | 52 (61) | 36 (42) | 37 (44) |
| R2+A-module | 200 | 56 (111) | 61 (68) | 20 (22) | 19 (21) | 81 (90) | 39 (43) | 22 (24) | 26 (29) |
| R-module Myb mutant | 233 | 57 (133) | 47 (63) | 26 (35) | 26 (22) | 74 (98) | 53 (70) | 32 (43) | 33 (44) |

high level of ectopic expression of the R-module- and intron-driven reporter constructs suggests that additional repressor elements might be involved in the spatial control of *nodal* expression.

## Deletion analysis of the intron

Deletion analysis of the intron revealed that it contains a central module involved in global activation (the A-element), flanked by two restriction elements (R1 and R2) involved in spatial restriction (Fig. 2A,B). The A-element drives robust GFP expression evenly throughout the embryonic territories (overall: oral 66%, ectopic 66%), suggesting that this element responds to broadly distributed factors that regulate *nodal* expression. Embryos injected with an R1+A-element GFP construct displayed GFP expression similar to embryos injected with the full intron GFP reporter (overall: 72% oral, 52% ectopic). Embryos injected with an R2+A-module GFP construct also showed preferential expression of GFP in the oral territory (overall: 83% oral, 36% ectopic). Both of these constructs showed a decrease in the overall aboral and endoderm ectopic expression and a concomitant rise in the oral expression compared with the A-module. The effect of the R1-module on the spatial restriction of the reporter is modest (R1+A, 14% aboral decrease, 8% endoderm decrease), suggesting that it acts as a weak repressor element. The R2-module is more effective than R1 in restricting the spatial expression of the reporter (R2+A, 28% aboral decrease, 20% endoderm decrease), suggesting that R2 can exert negative control of *nodal* expression (Table 2). The extent of this effect is in keeping with other negative-regulatory spatial elements characterized in several sea urchin genes (Minokawa et al., 2005; Ransick and Davidson, 2006). Thus, the 3′ half of the intron sequence contains regulatory elements that are able to respond to a positive regulator(s) that is globally expressed, as well as elements that are necessary to repress *nodal* expression in the endoderm.

## The proximal R-module and the intron both contain Smad binding sites involved in autoregulation by Nodal

Studies on the regulation of *nodal* gene expression in vertebrates have shown that regulatory elements upstream of the first exon control activation of *nodal* and that elements within the introns contain binding sites that are necessary for the autoregulation by Smad2/3 (Norris et al., 2002; Osada et al., 2000). To examine the regulatory architecture of the sea urchin *nodal* gene, we compared the kinetics of luciferase expression driven by the R-module and by the intron (Fig. 3A). When the R-module was fused to a luciferase reporter construct carrying the *endo16* basal promoter (EpGluc), it activated luciferase expression as early as the 60-cell stage (Fig. 3A) and levels of expression rose until the mesenchyme blastula stage, similar to endogenous *nodal* activation (Duboc et al., 2004). However, the intron region did not activate luciferase expression until the very early blastula stage and the level of expression was reduced when compared with R-module expression at this stage. The activity of the intron-driven luciferase reporter continued to rise, with a sharp increase between early blastula and mesenchyme stages, perhaps indicating the time when autoregulation by Nodal signaling is strongly influencing the promoter (Fig. 3A). These data suggest that the R-module contains transcription factor binding sites necessary for the activation of *nodal* around the 60-cell stage, whereas the function of the intronic modules might be to respond to autoregulation by Nodal signaling. We reasoned that mutating the predicted Smad binding sites

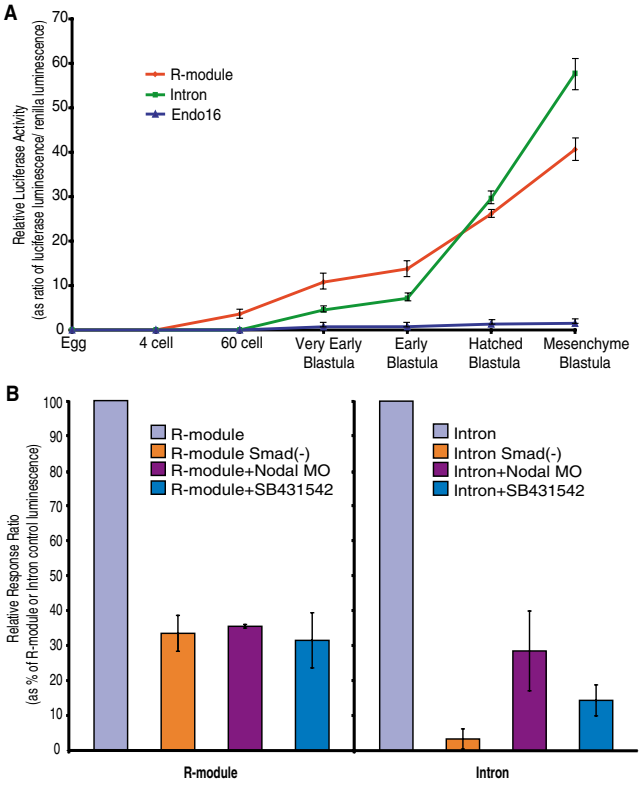


**Fig. 3. The R-module and intron have different temporal expression profiles and are both influenced by autoregulation by Nodal signaling.** (**A**) Kinetics of luciferase expression levels driven by the R-module EpGluc, intron EpGluc and control EpGluc constructs. (**B**) Comparison at the hatched blastula stage of the transcriptional activities of R-module, intron, R-module Smad(–) (i.e. lacking the binding sites for Smads) and intron Smad(–) in normal sea urchin embryos and embryos injected with the Nodal morpholino or treated with the Alk4/5/7 inhibitor SB431542. The data are presented as the ratio of luciferase expression between EpGluc and the wild-type module.

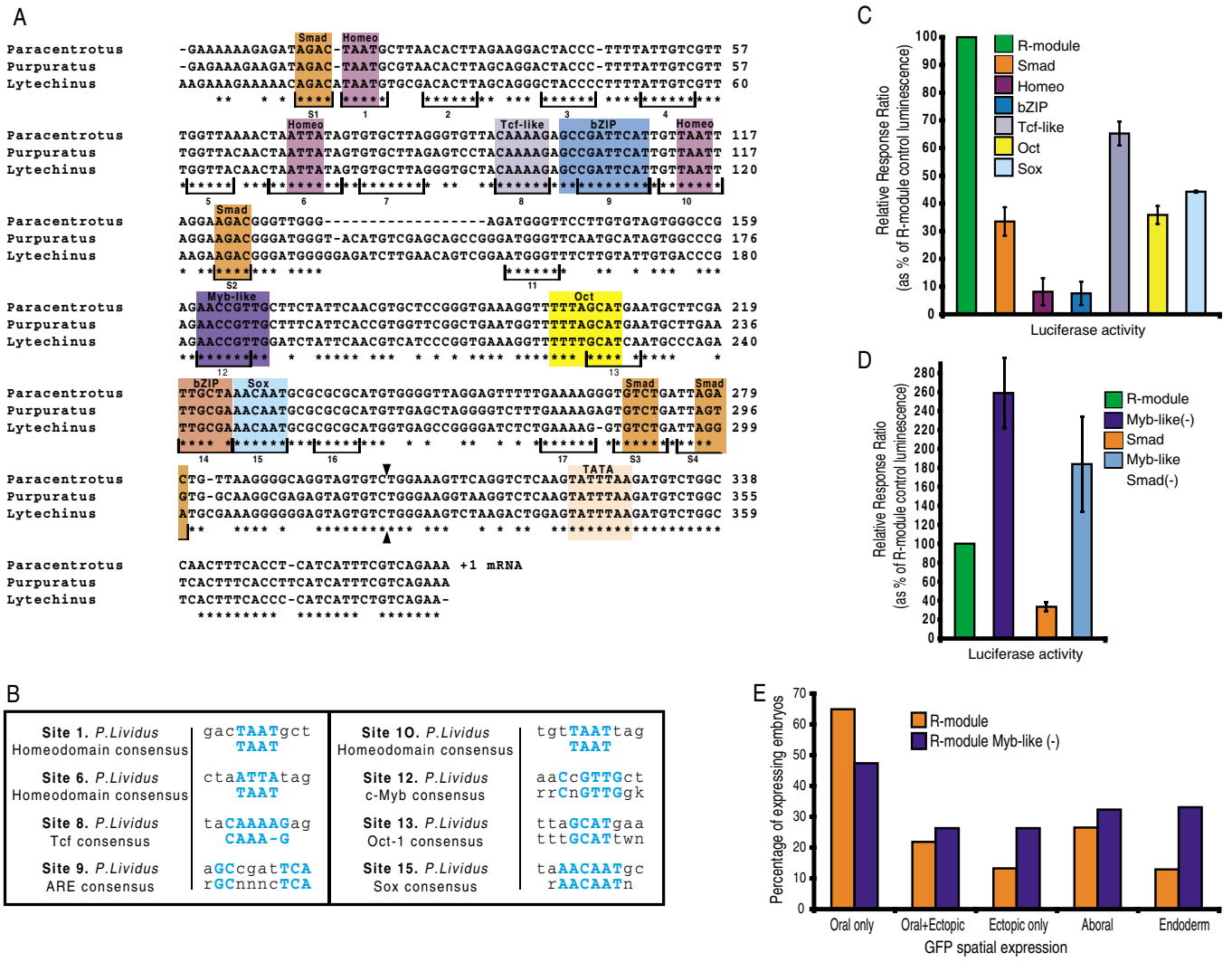


**Fig. 4. Homeodomain, bZIP, TCF-like, Oct, Sox, Smad and Myb-like binding sites are important for R-module transcriptional activity.** (**A**) Alignment of the *P. lividus* R-module with the corresponding *S. purpuratus* and *L. variegatus* sequences. The conserved sites mutated in the functional analysis are underlined in black and the colored boxes indicate putative transcription factor binding sites identified using MatInspector. Arrowheads indicate the limit of the R-module. (**B**) Sequence comparisons of predicted binding sites within the R-module with known consensus sequences for transcription factors. (**C**) Effects of Smad, homeodomain, bZIP, TCF-like, Oct and Sox site mutations on the transcriptional activity of the R-module at the hatched blastula stage. The homeodomain site mutation data are from a triple mutant, whereas TCF-like, bZIP, Oct and Sox correspond to single mutations within the R-module. (**D**) Mutation of a putative Myb-like site increases the transcriptional activity of the R-module and R-module Smad(–) constructs. The data are presented as the ratio of luciferase expression between EpGluc and the wild-type module. (**E**) Effect of the Myb-like binding site mutation on the spatial expression of a GFP reporter driven by the R-module.

present within these regions should uncover which module is involved in autoregulation, as the mutated construct would no longer be able to respond to Nodal autoregulation. We mutated all four Smad binding sites present within the R-module and all 12 sites present within the intron (Fig. 4 and see Fig. S1 in the supplementary material). In both cases, mutation of the Smad binding sites caused a sharp decrease of the activity of the reporters at the hatched blastula stage. However, the R-module Smad mutant still retained 35% of its original expression when compared with the normal R-module, whereas the intron Smad mutant had only 3% residual expression compared with the normal intronic region (Fig. 3B). Similarly, injection of the Nodal morpholino or treatments with the Alk4/5/7 receptor inhibitor SB431542 (Inman et al., 2002), which should both inhibit autoregulation by Nodal, severely decreased the activity of the intronic reporter gene, whereas it only moderately affected the activity of the R-module reporter (Fig. 3B). Taken together, these data suggest that both the R-module and the intron act as autoregulatory elements. However, when compared with the intron, the R-module still drove relatively high levels of luciferase expression in the absence of autoregulatory Smad binding sites, suggesting that it contains other transcription factor binding sites necessary for full *nodal* expression.

## Identification and functional analysis of binding sites for transcription factors in the *nodal* R-module

To identify additional transcription factors necessary for R-module activity, we searched the R-module sequence for known transcription factor binding sites using MatInspector software (Cartharius et al.,

2005). This analysis indicated that the R-module contains multiple candidate binding sites for several families of transcription factors. We then mutated 17 conserved motifs within the R-module, including the predicted binding sites as well as other well conserved regions and analyzed the effect of each mutation alone, or in combination with other mutations, on the activity of the luciferase and GFP reporters at blastula stages (Fig. 4). These functional tests allowed the identification of six motifs which, when mutated, caused a strong decrease in reporter gene activity at the hatched blastula stage, suggesting that these sites are necessary for activation of *nodal* expression. Each of the sites found in the systematic screen corresponds to a predicted transcription factor binding site (Fig. 4A-C). Mutation of all three predicted homeodomain factor binding sites decreased luciferase expression to about 5% of that of control constructs (Fig. 4C). Similarly, a mutation that maps to one of the predicted bZIP binding sites caused a 20-fold reduction of the activity of the reporter (Fig. 4C). Interestingly, the sequence of this motif, GCCGATTCAT, resembles the antioxidant responsive element (ARE), GCNNNGTCAY, which mediates transcriptional regulation of antioxidant proteins (Rushmore et al., 1991). These data suggest that both homeodomain and bZIP transcriptional regulators are crucial for the activation and/or maintenance of *nodal* expression. In addition to homeodomain and bZIP, three mutations produced strong effects on the activity of the reporter. These mapped to a motif, TACAAAAGA, that resembles a Tcf binding site (A/TA/TCAAAG) (Giese et al., 1991; van de Wetering and Clevers, 1992), to a motif, AACAAT, which fits the consensus Sox binding site (AACAAT) (van Beest et al., 2000) and to a motif, ATGCTAAA, that resembles the Oct1 consensus binding site (ATGCAAA) (Jin and Li, 2001). Mutation of the TCF-like, Sox or Oct sites decreased the activity of the R-module-driven reporter gene to ~64%, ~40% and 30%, respectively, of its original value (Fig. 4C).

Interestingly, we identified a site which, when mutated, consistently resulted in an almost 3-fold stimulation of the expression of the reporter gene (Fig. 4D). The sequence of this motif, CAACGGT, fits the consensus binding site sequence for Myb (YAACG/TG) (Luscher and Eisenman, 1990). The Myb transcription factor is known to act as a repressor and a global regulator of chromatin structure (Coffman et al., 1997; Lipsick, 2004). To determine whether this site is involved in spatial restriction of *nodal*, we introduced the mutation into the R-module GFP construct and assayed spatial expression at the early pluteus stage (Fig. 4E). Indeed, the percentage of embryos displaying restricted expression of GFP in the oral territory decreased from 65% to 47% when this site was mutated. Furthermore, when the Myb site was mutated, the percentage of embryos expressing GFP in aboral and endoderm also increased compared with the wild-type R-module (4% and 20% increase, respectively). These variations in the spatial expression of reporter genes are somewhat modest, but they are of the same order of magnitude as those caused by mutating important transcription factor binding sites in other spatially regulated sea urchin genes (Minokawa et al., 2005; Ransick and Davidson, 2006). Thus, the Myb-like binding site is likely to bind a regulatory factor that represses *nodal* expression in the aboral and endomesodermal territory. Alternatively, the function of this repressor site might be to control the level of *nodal* expression in the oral ectoderm.

## Initiation of *nodal* expression requires TCF, SoxB1 and early TGF-β signaling

Since the R-module contains binding sites for Sox and Tcf and because both factors are expressed maternally and are broadly distributed in the early embryo (Huang et al., 2000; Kenny et al., 2003), we examined whether Tcf and SoxB1 function is required for expression of endogenous *nodal* and of the *nodal* reporter genes. Consistent with previous studies (Kenny et al., 2003), we found that interfering with SoxB1 function using antisense morpholino oligonucleotides severely affected dorsal-ventral patterning, causing embryos to develop with a strongly radialized phenotype (data not shown). Examination of endogenous *nodal* expression in the SoxB1 morpholino-injected embryos at early blastula stages revealed that *nodal* expression was abolished (Fig. 5A). Also, the activity of the R-module-driven reporter gene decreased to 18% of its normal value in embryos injected with the SoxB1 morpholino at the hatched blastula stage (Fig. 5D). Similarly, co-injection of RNA encoding a dominant-negative (dn) version of Tcf reduced the activity of the reporter gene to 11% of its original value (Fig. 5D). We conclude that TCF and SoxB1 function is essential for *nodal* expression, possibly through direct binding to the R-module.

We had shown previously that maintenance of *nodal* expression strongly depends on an autoregulatory loop and that in the absence of Nodal signaling or following overexpression of Lefty protein, *nodal* expression is lost at late blastula stages (Duboc et al., 2005; Duboc et al., 2004). To further test whether *nodal* expression also requires TGF-β signaling and to determine the period when autoregulation becomes important for maintenance of *nodal*, we microinjected morpholino oligonucleotides directed against *nodal* or *alk4/5/7*, which encodes a candidate Nodal type I receptor (Lapraz et al., 2006), and analyzed the temporal and spatial expression of *nodal* by in situ hybridization and QPCR (Fig. 5B). Strikingly, *nodal* expression was barely detectable in the MoNodal-injected embryos at the early 64/128-cell stage (Fig. 5Bf-h) and completely absent starting at the early blastula stage (Fig. 5Bi-j). In the MoAlk4/5/7-injected embryos, *nodal* expression was not detectable at any stage (Fig. 5Bk-o). Consistent with these observations, MoNodal injection or SB431542 treatment caused a 3- to 4-fold decrease of the activity of the luciferase reporter gene (Fig. 5D). These results suggest that a Nodal-dependent autoregulatory loop is active very early and its integrity is crucial to maintain *nodal* expression.

## Univin, a maternally expressed TGF-β, is the sea urchin ortholog of Vg1 and acts upstream of *nodal* expression

During the experiments described above, we noticed that *nodal* expression was more effectively downregulated in embryos treated with SB431542 or microinjected with MoAlk4/5/7 than in embryos injected with the Nodal morpholino. This suggested that another early-acting TGF-β signal might participate in the regulation of *nodal* expression. Univin is a good candidate for this additional early-acting TGF-β signal required for *nodal* expression as it is an abundant, ubiquitously expressed maternal transcript and because its zygotic expression pattern encompasses that of *nodal* (Lapraz et al., 2006).

We tested whether Univin is the early signal required for *nodal* expression. Following injection of the Univin morpholino, *nodal* transcripts could not be detected by in situ hybridization (Fig. 5Bp-t), although a residual level of *nodal* transcripts could be detected by QPCR (Fig. 5E). Injection of the Univin morpholino also caused a reduction in the activity of the R-module-driven luciferase reporter gene at the hatched blastula stage (Fig. 5D). However, as in the presence of the Alk4/5/7 inhibitor or in the absence of Smad binding sites, the activity of the R-module was not completely abolished, suggesting that initial activation of *nodal* is achieved by transcription factors that act in parallel with TGF-β signaling.

Furthermore, unlike *nodal*, which requires Nodal and Alk4/5/7 signaling early to be maintained, *univin* expression was found to be independent of Alk4/5/7 signaling (Fig. 5C) or *nodal* expression (data not shown), consistent with the idea that Univin acts very early to regulate *nodal* expression. Finally, in an attempt to link the zygotic expression of *univin* with the activity of maternal transcription factors, we examined the dependence of *univin* expression on maternal Wnt/β-catenin signaling and SoxB1. We found that zygotic expression of *univin*, like zygotic expression of *nodal*, critically requires TCF and SoxB1 function (Fig. 5A). Taken together, these results show that Univin, a maternally deposited TGF-β, is required early for *nodal* expression during sea urchin development and that both *univin* and *nodal* expression require Tcf and SoxB1 function.

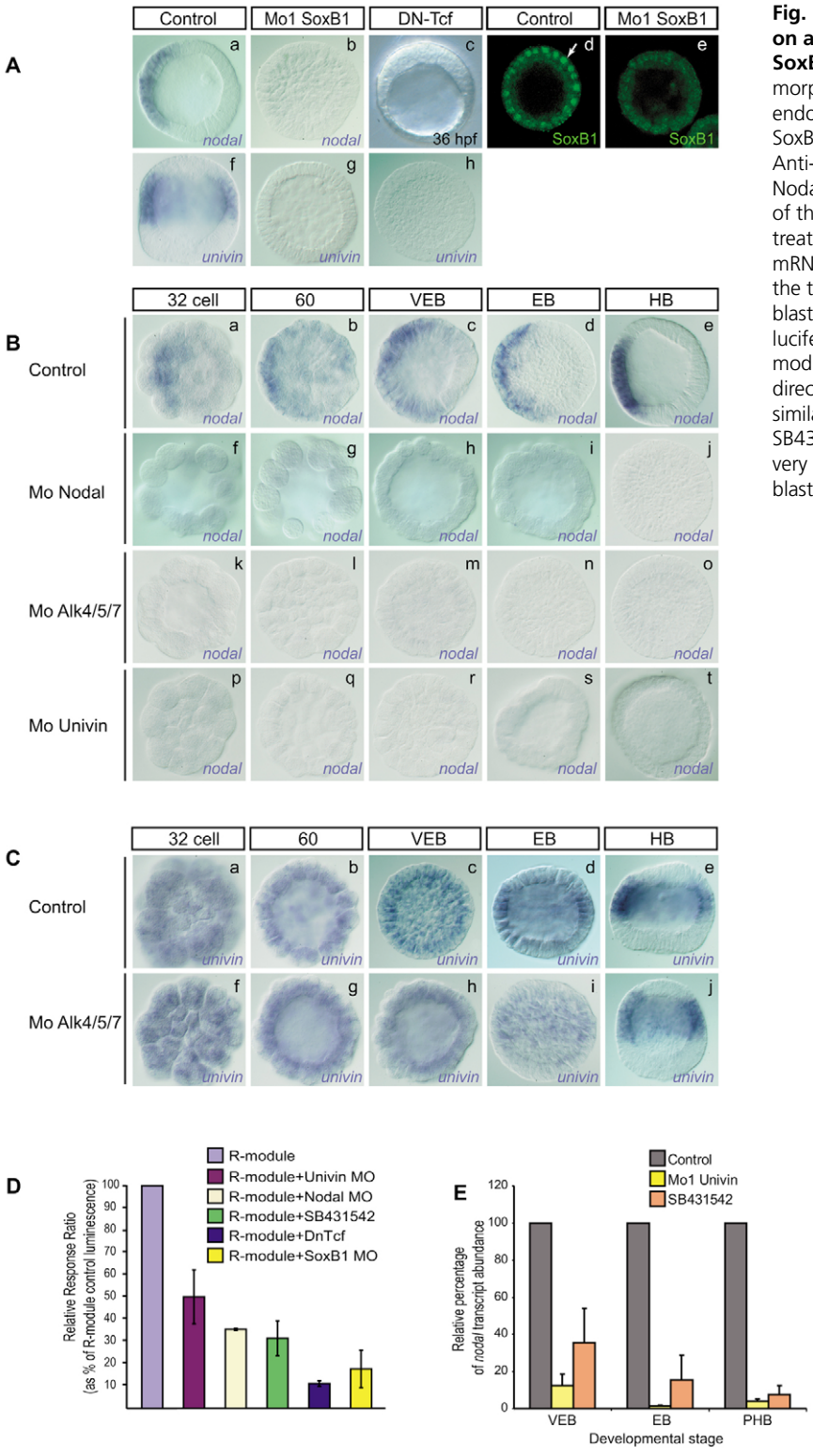


**Fig. 5. *nodal* expression in the sea urchin relies early on autoregulation and Univin signaling and requires SoxB1 and TCF function.** (**Aa-h**) Effects of SoxB1 morpholino and *dnTcf* mRNA on the expression of the endogenous *nodal* and *univin* genes. (d,e) Injection of SoxB1 morpholino diminishes SoxB1 protein expression. Anti-SoxB1 in green (arrow). (**Ba-t**,**Ca-j**) Effects of the Nodal, Alk4/5/7 and Univin morpholinos on the expression of the endogenous *nodal* and *univin* genes. (**D**) Effects of treatment with SB431542, of microinjection of *dnTcf* mRNA and of Nodal, SoxB1 and Univin morpholinos on the transcriptional activity of the R-module at the hatched blastula stage. The data are presented as the ratio of luciferase expression between EpGluc and the wild-type module. Two different morpholino oligonucleotides directed against *univin* and *soxB1* were used and gave similar results. (**E**) QPCR analysis of *nodal* expression in SB431542-treated or MoUnivin-injected embryos. VEB, very early blastula; EB, early blastula; PHB, pre-hatching blastula.

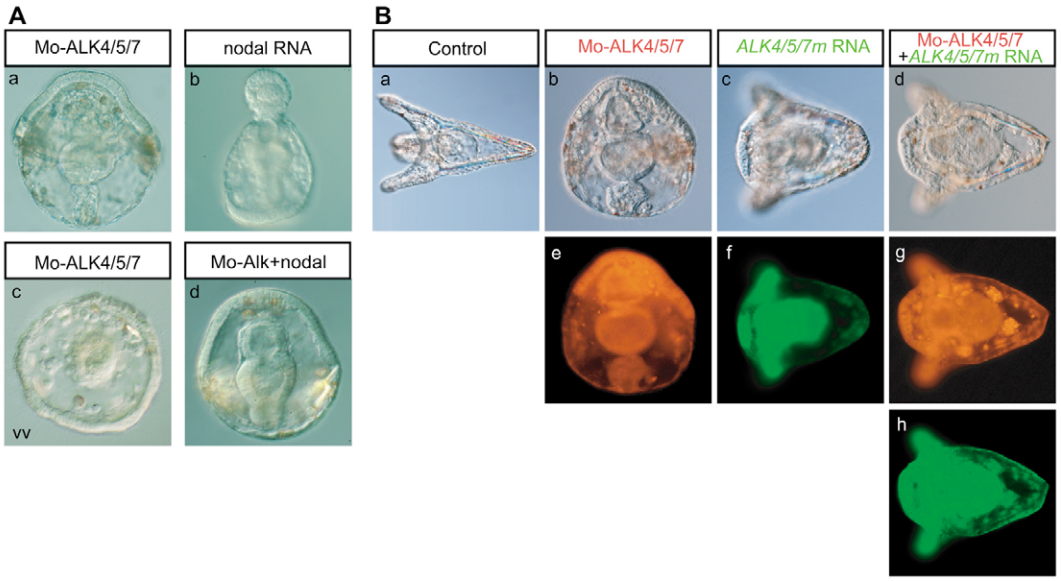


**Fig. 6. Sea urchin Alk4/5/7 is required for dorsal-ventral axis formation and Nodal signaling.** (**Aa-d**) MoAlk4/5/7 disrupts dorsal ventral axis formation and blocks the response to *nodal* overexpression. (**Ba-h**) Rescue experiment to demonstrate the specificity of the Alk4/5/7 morpholino. Embryos were injected with Alk4/5/7 morpholino (b,e) or *alk4/5/7* mRNA (c,f) alone, or successively injected with the Alk4/5/7 morpholino then with a synthetic *alk4/5/7* mRNA containing eight mismatches over the region recognized by the morpholino (d,g,h). Whereas embryos injected with the Alk4/5/7 morpholino alone developed with a radialized phenotype indicative of inhibition of Nodal signaling, embryos co-injected with the Alk4/5/7 morpholino and the modified wild-type *alk4/5/7* mRNA developed into normal pluteus larvae.

## Loss of Univin function phenocopies the loss of Nodal

To further document the function of Univin in the regulation of *nodal* expression, we performed gain- and loss-of-function analyses. As described above, two different antisense morpholino oligonucleotides directed against Univin were similarly effective in suppressing *nodal* expression (Figs 5, 7). Consistent with these observations, the Univin morphants obtained with either morpholino developed with a fully penetrant, extremely severe phenotype: numerous spicule rudiments formed around the archenteron that remained straight, the mouth never formed, and the whole ectoderm differentiated into a thick, ciliated epithelium without any apparent dorsal-ventral polarity (Fig. 7Ab,c,g,h). This phenotype is indistinguishable from that caused by inhibition of Nodal function (Fig. 7Aa-c,f-h). Consistent with the loss of *nodal* expression in these embryos, expression of the aboral marker 29D was suppressed in most of the ectoderm, except the sub-anal region, and the ciliary band marker gene *tubulin* was expressed in a large apical domain, exactly as in the *nodal* morphants (Fig. 7Ba,b,f,g) (see also Duboc et al., 2004).

Overexpression of Univin by microinjection of synthetic mRNA into the egg perturbed dorsal-ventral patterning and produced radialized embryos that only partially resembled those obtained by overexpression of *nodal* (Fig. 7Ad). Molecular analysis revealed that *nodal* was expressed ectopically in most of the Univin-overexpressing embryos (Fig. 7Bd,i), half of them displaying a completely radial expression (Fig. 7Be,j). This indicates that when overexpressed, *univin* is able to induce *nodal* expression in the aboral ectoderm, possibly by overwhelming a repression mechanism operating in this territory. Simultaneous overexpression of Univin and inhibition of Alk4/5/7 with SB431542 (Fig. 7Ae) produced embryos with a phenotype identical to that caused by MoAlk4/5/7 (Fig. 6) or SB431542 treatment (Fig. 7Ai) and not resembling the phenotype of Univin-overexpressing embryos (Fig. 7Ad), suggesting that Univin signals through the same receptor as nodal, namely Alk4/5/7.

The striking effects on *nodal* expression resulting from overexpression or downregulation of Univin prompted us to re-examine the phylogenetic relationships between this TGF-β and Nodal. Previous phylogenetic comparisons indicated that Univin is most closely related to BMPs and Vg1 (Stenzel et al., 1994), whereas more recent comparisons indicated a close evolutionary relationship with Gdf factors (Lapraz et al., 2006). To precisely determine the orthology relationship of Univin, we performed a phylogenetic analysis using a set of sequences that included several *vg1* members (Fig. 8A). This analysis confirmed that Univin is more closely related to Vg1 from *Xenopus*, Dvr1 from zebrafish, and to Gdf1 and Gdf3 from mouse, than to BMPs. This strong phylogenetic relationship is further supported by genomic linkage data (Fig. 8B). In the zebrafish genome, the *dvr1* gene is located 8 kb from *bmp2a*. Similarly, the *univin* transcription unit lies only 32 kb from *bmp2/4*. The synteny of *univin/bmp2/4* and *dvr1/bmp2a* in the sea urchin and zebrafish genomes strongly suggests that these two genes evolved by gene duplication before emergence of the chordates.

# DISCUSSION

## Nodal expression and Wnt/β-catenin signaling

The regulation of expression of *nodal* genes by the Wnt/β-catenin pathway is well documented in zebrafish (Bellipanni et al., 2006; Kelly et al., 2000), *Xenopus* (Agius et al., 2000; Hyde and Old, 2000; Rex et al., 2002; Schohl and Fagotto, 2003; Takahashi et al., 2000; Xanthos et al., 2002; Yang et al., 2002) and mouse (Ben-Haim et al., 2006; Chazaud and Rossant, 2006; Huelsken et al., 2000). Studies performed in *Xenopus* and mouse identified the regulatory elements responsible for regulation of *nodal* genes and, in the case of *Xnr1* and *Xnr3*, Wnt-responsive elements containing consensus binding sites for the Lef-1/Tcf proteins have been identified (Hyde and Old, 2000; Kofron et al., 1999; McKendry et al., 1997; Osada et al., 2000). Inspection of the sea urchin *nodal* promoter detected one such motif and mutational analysis indicated that it is indeed

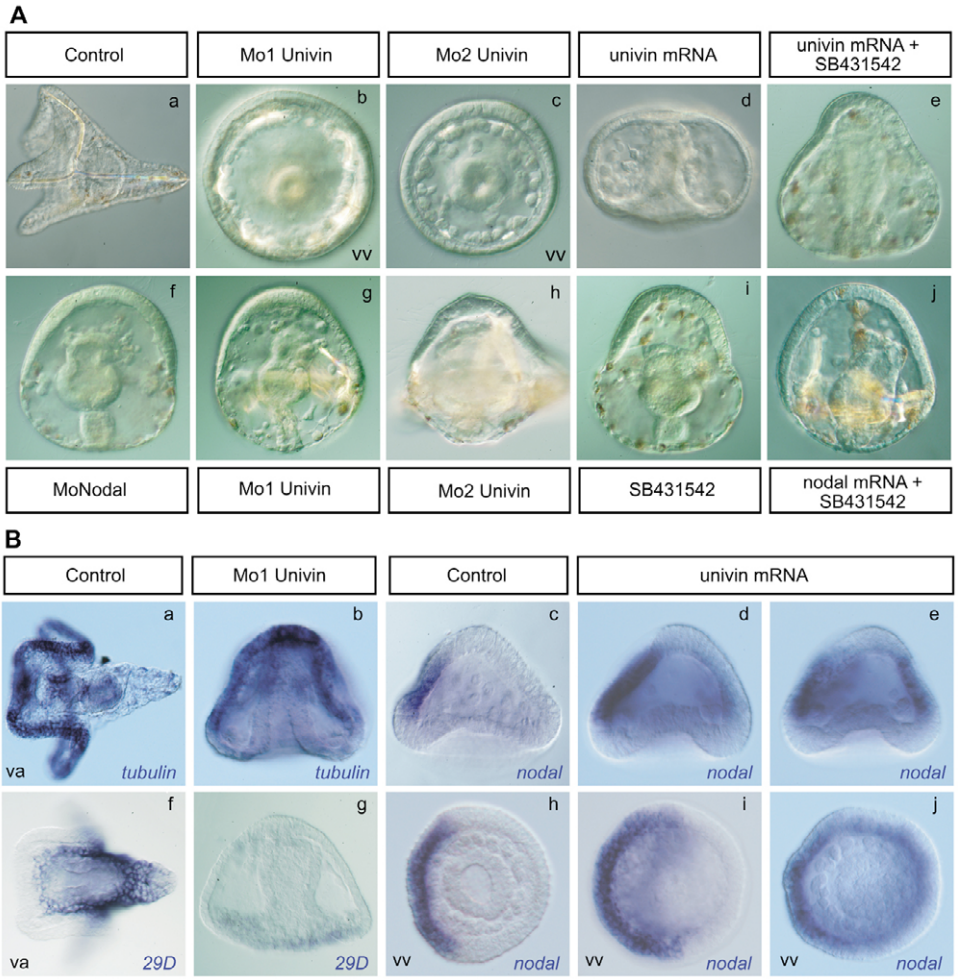


**Fig. 7. Univin functions in the Nodal signaling pathway.** (**Aa-j**,**Ba-j**) Functional analysis of sea urchin *univin*. Inhibition of Univin function phenocopies the loss-of-function of Nodal, whereas overexpression of Univin causes ectopic expression of *nodal*.

essential for high-level promoter activity. Furthermore, inhibition of Tcf function severely affected expression of a *nodal* reporter gene. Thus, expression of *nodal* (Duboc et al., 2004) and Univin (this work) requires Tcf activity, further reinforcing the idea that in the sea urchin, as in vertebrates, initiation of *nodal* expression critically relies on prior activation of the Wnt/β-catenin pathway. In the future, it will be important to determine whether the Tcf/β-catenin complex binds directly to the *nodal* promoter, or if a relay molecule produced at the level of the vegetal pole mediates this effect.

## Sox factors: conserved regulators of *nodal* expression with opposing functions in sea urchin and vertebrates

Recently, SoxB1-type factors have emerged as evolutionarily conserved maternal/early zygotic regulators of germ layer specification and axis formation in deuterostomes (Kenny et al., 2003; Zhang et al., 2005; Zhang et al., 2004; Zhang et al., 2003). In *Xenopus*, the SoxB1 family member Sox3 is expressed maternally in the animal hemisphere, where it restricts mesendoderm induction (Penzel et al., 1997). Studies in *Xenopus* and zebrafish have also shown that Sox3 acts as a transcriptional repressor of *Xnr5* (Zhang et al., 2003). Based on the conservation of Sox3 function in vertebrates, Zhang et al. proposed that SoxB1 proteins might fulfil a phylogenetically conserved role in regulating cell fate specification along the animal-vegetal axis through the regulation of *nodal*-like genes.

During sea urchin early development, SoxB1 is expressed in the presumptive animal hemisphere, where it prevents the early β-catenin-dependent vegetal signaling necessary for specification of the mesendoderm (Kenny et al., 1999; Kenny et al., 2003). Our finding that SoxB1 is required for expression of both *nodal* and *univin*, confirms that SoxB1 factors do indeed play a phylogenetically conserved role as regulators of *nodal* expression in deuterostomes. However, it appears that the function of SoxB1 factors has diverged in the two phyla as they serve as positive regulators of *nodal* in the sea urchin but act as repressors of *nodal* expression in vertebrates.

## An ancient and conserved positive regulatory interaction between *univin/vg1* and *nodal*

*vg1* was discovered as a maternal mRNA localized at the vegetal pole of *Xenopus* eggs. Overexpression of mature Vg1 mimics ectopic expression of *nodal* resulting in strong induction of mesoderm and endoderm (Thomsen and Melton, 1993). Conversely, when Vg1 signaling is blocked, or in embryos depleted of endogenous *vg1* transcript, endomesoderm development and formation of the organizer fail (Birsoy et al., 2006; Joseph and Melton, 1998). Transcripts encoding Dvr1, the zebrafish ortholog of Vg1, are also deposited maternally and are distributed throughout the dorsal-ventral axis (Dohrmann et al., 1996; Helde and Grunwald, 1993). Mature Dvr1 is a potent inducer of dorsal mesoderm, but loss-of-function experiments

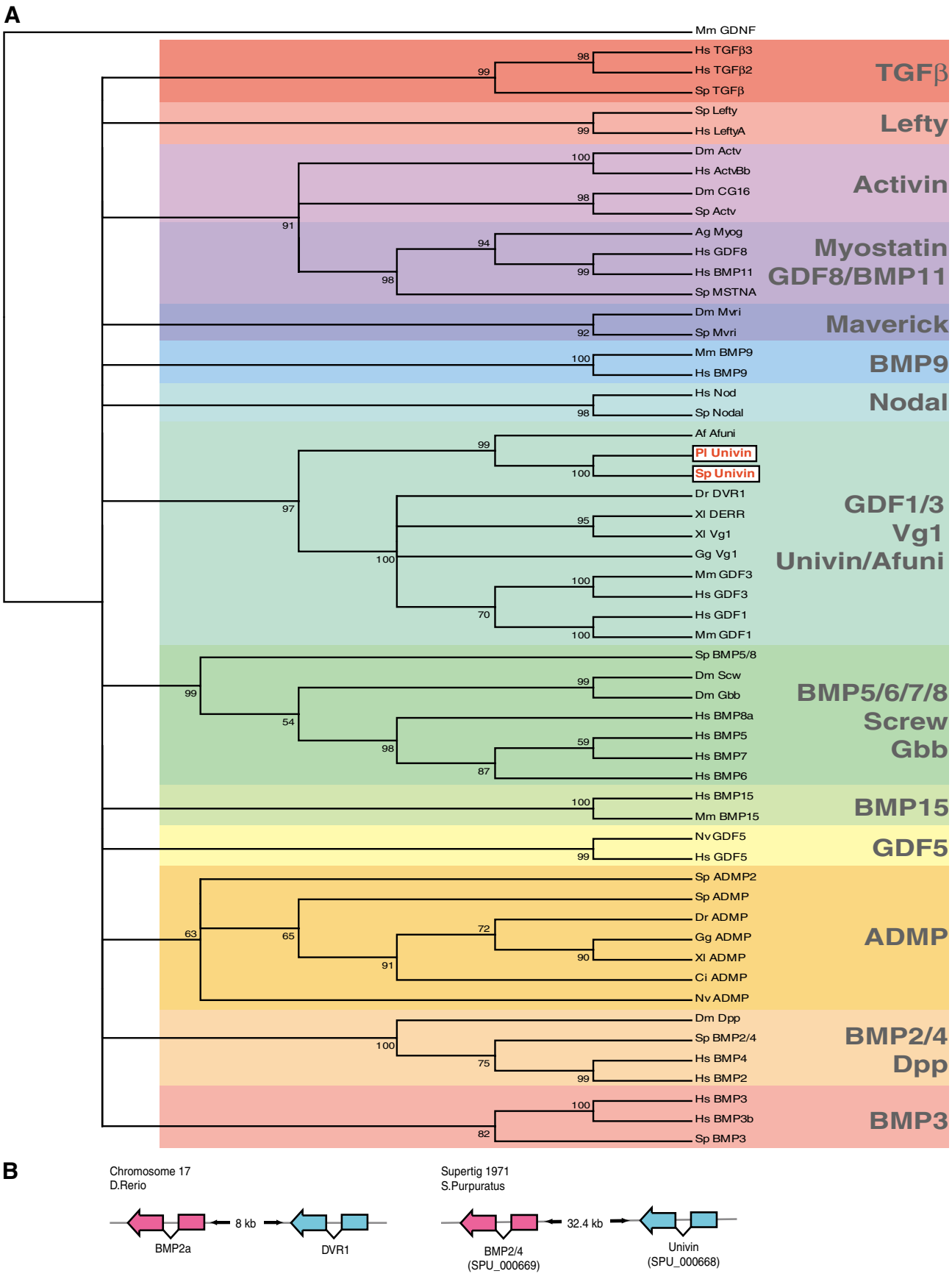


**Fig. 8. Univin is related to vertebrate Vg1/Dvr1.** (**A**) Molecular phylogeny of GDF/Vg1/Univin family members. (**B**) Synteny between sea urchin *univin/bmp2/4* and zebrafish *bmp2a/dvr1*.

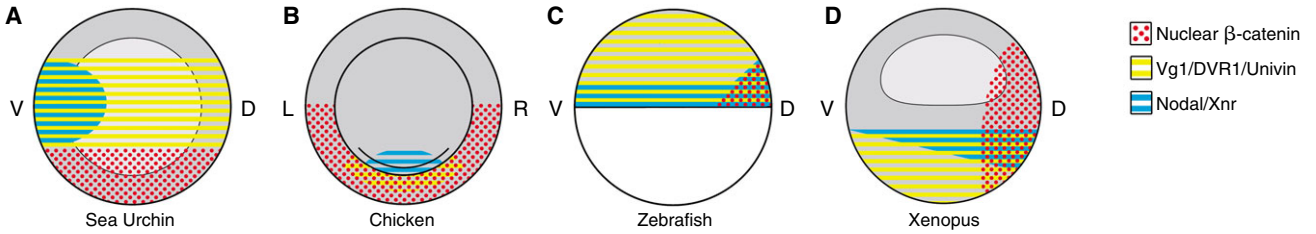


**Fig. 9. Expression territory comparisons of Vg1/Univin signals with Nodal and nuclear β-catenin in sea urchin, chick, zebrafish and *Xenopus*.** Embryos are depicted before gastrulation. (**A**) In the early sea urchin embryo, nuclear β-catenin is present in the vegetal pole region while zygotic *univin* and *nodal* are expressed in the overlying presumptive ectoderm. (**B**) In the chick embryo, Vg1 and Wnt8c are expressed in the posterior marginal zone and cooperate to induce *nodal* expression in the adjacent epiblast and primitive streak. (**C**) In the zebrafish embryo, maternal Vg1 transcripts are expressed ubiquitously, while β-catenin accumulates in nuclei of the dorsal marginal zone and zygotic *nodal* transcripts at the blastoderm margin. (**D**) In *Xenopus* embryos, β-catenin is stabilized on the dorsal side while *Xnr* transcripts and Vg1 are expressed in a dorsal-to-ventral gradient. Data taken from the published literature (Skromne and Stern, 2001; Agius et al., 2000; Helde and Grunwald, 1993; Schier and Talbot, 2005) and this study.

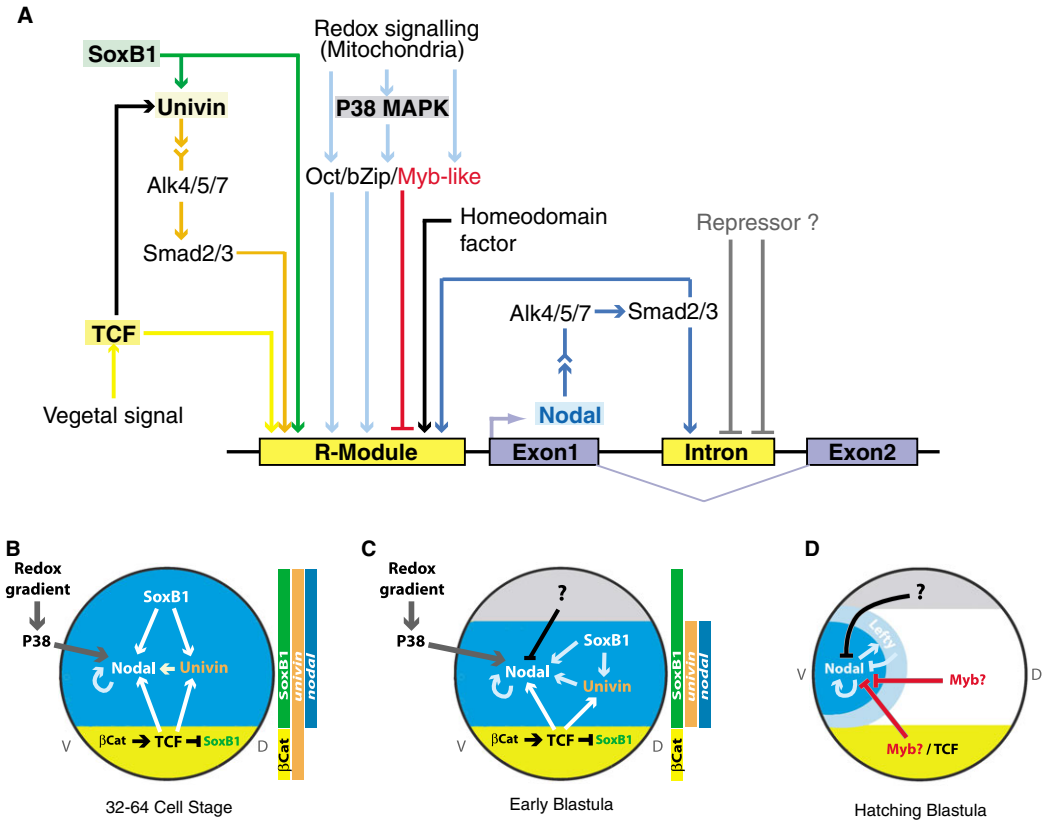


**Fig. 10. Model of *nodal* regulation and dorsal-ventral axis specification in the sea urchin embryo.** (**A**) Integration of signaling and maternal transcription factor inputs by cis-regulatory elements of the *nodal* gene. (**B**) Starting at the 32-64 cell-stage, *nodal* expression is initiated throughout most of the presumptive ectoderm by combinatorial maternal inputs from p38 MAP kinase and maternal Univin, as well as from signals emanating from the vegetal pole. Unidentified repressors prevent expression of *nodal* and zygotic expression of *univin* in the animal pole domain. These signals are transduced by maternal transcription factors such as Smad, homeodomain, Oct, bZIP families and require SoxB1 and TCF, resulting in a broad initial expression of *nodal* in the ectoderm. Expression of *nodal* and zygotic expression of *univin* at the vegetal pole is prevented by the β-catenin-mediated downregulation of SoxB1. (**C**) Starting at the very early blastula stage, an endogenous ventral-dorsal redox gradient, possibly related to an asymmetric distribution of mitochondria in the egg and/or early embryo and acting on bZIP and other redox-sensitive transcription factors, results in a slightly increased expression of *nodal* on the presumptive ventral side, thereby reinforcing the Nodal autoregulatory loop. This slight asymmetry in the expression of *nodal* is translated into a corresponding asymmetry in the expression of Lefty, which starts downregulating Nodal signaling on the presumptive dorsal side. (**D**) During blastula stages, Nodal autoregulation and Lefty-mediated lateral inhibition then establish a robust reaction diffusion system which results in the sharp restriction of *nodal* expression to the ventral territory.

have not been performed to demonstrate that Dvr1 is required for axis formation. A chick ortholog of *vg1* has also been characterized (Seleiro et al., 1996; Shah et al., 1997). Overexpression of chick Vg1 is capable of initiating formation of an ectopic embryonic axis and organizer by cooperating with canonical Wnt signals, such as Wnt1 and Wnt8, to induce *nodal* (Bertocchini et al., 2004; Skromne and Stern, 2001; Skromne and Stern, 2002). The mouse genome contains two genes, *Gdf1* and *Gdf3*, that are closely related to *vg1* (Jones et al., 1992; Lee, 1991), and functional experiments demonstrated that they act in a Nodal signaling pathway and share several properties with Nodal factors (Andersson et al., 2006; Chen et al., 2006; Cheng et al., 2003). For example, *Gdf1* and *Gdf3*, like *nodal*, can induce an ectopic embryonic axis when overexpressed. Gdf1 and Gdf3 also form a complex with activin receptors, require the EGF-CFC co-receptor cripto (also known as cryptic) and, like Nodal, their activity is inhibited by Lefty. Furthermore, embryos mutant for Gdf3 frequently display abnormal expression of *Nodal*, indicating that Gdf factors participate in the regulation of *Nodal* expression in the mouse (Chen et al., 2006). Taken together, these experiments suggest that Gdf1 and Gdf3 are indeed functional homologs of Vg1 in the mouse (Shen, 2007).

Examination of the recently sequenced sea urchin genome revealed that *univin* is the only gene related to *Gdf1/3* (Lapraz et al., 2006). The functional relationship between Univin and other TGF-β proteins, such as Vg1 and Nodal, escaped attention in previous studies partly because both GDFs and Univin were initially described as related to BMPs. The subsequent finding that *univin* lies only a few kb from *Bmp2/4* in the sea urchin genome further reinforced the idea that *univin* and *bmp2/4* were closely related and recently duplicated genes (Lapraz et al., 2006). However, the functional analysis of Univin led us to reinvestigate the relationships between Univin and other TGF-β proteins and to discover that the function of Univin is much more closely related to that of Nodal than of Bmp2/4. Overexpression of Univin induces ectopic expression of *nodal* in the aboral ectoderm, whereas blocking translation of *univin* transcripts prevents initiation of *nodal* expression. *univin* is expressed maternally throughout the dorsal-ventral axis, then zygotically in a large belt of cells surrounding the equatorial region starting at the early blastula stage (Lapraz et al., 2006; Stenzel et al., 1994). Therefore, in the sea urchin, as in vertebrates, the territories expressing *univin/vg1* and *nodal/xnr* are overlapping, consistent with the finding that Univin and Vg1 factors act upstream of *nodal/Xnr* expression (Fig. 9). Furthermore, the linkage between Univin and Bmp2/4 suggests that one gene derived from the other by gene duplication. Finally, the strong regulatory interaction between Univin and *nodal* suggests that an ancestral function of Vg1/Univin might have been to regulate expression of *nodal* genes and that this regulatory interaction may have been an evolutionary conserved early step in the establishment of the dorsal-ventral axis of deuterostomes.

## Homeodomain factors, bZIP, Oct and Myb: candidate regulators of *nodal* expression

SoxB1, TCF and Smads have already been implicated in patterning of the ectoderm along the animal-vegetal axis of the sea urchin embryo (Huang et al., 2000; Kenny et al., 2003; Yaguchi et al., 2007). In this study, we showed that these maternal factors are also required for dorsal-ventral axis formation upstream of *nodal* expression. In addition, we identified several binding sites for potential novel regulators of *nodal* expression including activating binding sites for homeodomain, Oct and bZIP factors, along with a binding site for a repressor, possibly Myb (Coffman et al., 1997). It is intriguing to note that several of the candidate transcription factors predicted to bind to this promoter are known to be regulated by redox signaling, including bZIP (Liu et al., 2005), Oct (Guo et al., 2004; Zheng et al., 2003) and Myb (Bergholtz et al., 2001; Brendeford et al., 1997; Myrset et al., 1993). In particular, a wealth of data is available on the role of bZIP transcription factors as sensors of redox signaling downstream of MAP kinases (Amoutzias et al., 2006; Liu et al., 2005). It is therefore tempting to speculate that in the sea urchin embryo, bZIP, Oct and Myb act downstream of p38 and redox gradients to regulate *nodal* expression (Fig. 10). Further studies will be required to identify and characterize these transcription factors. These studies are warranted because *nodal* is, to our knowledge, the earliest gene displaying a restricted expression along the dorsal-ventral axis in the sea urchin embryo. Analysing the regulatory circuit driving *nodal* expression might therefore help to understand how maternal information is integrated at the level of the promoter sequence of regulatory genes to specify the secondary axis of polarity of the embryo.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/20/3649/DC1

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