Development 130, 1989-1999

doi:10.1242/dev.00409

LvTbx2/3: a T-box family transcription factor involved in formation of the oral/aboral axis of the sea urchin embryo

Jeffrey M. Gross*, Robert E. Peterson, Shu-Yu Wu and David R. McClay†

Development, Cell and Molecular Biology Group, Box 91000 LSRC, Duke University, Durham, NC 27710, USA
*Present address: Department of Molecular and Cellular Biology, Harvard University, BioLabs 2094, Cambridge, MA 02138, USA
†Author for correspondence (e-mail: david.mcclay@duke.edu)

Accepted 13 December 2002

SUMMARY

T-box family transcription factors have been identified in many organisms and are frequently associated with patterning events during embryonic development. With an interest in the molecular basis of patterning in the sea urchin embryo, we identified several members of the T-box family in *Lytechinus variegatus*. Here, we report the cloning and characterization of an ortholog of the Tbx2/3 subfamily, *LvTbx2/3*. To characterize the spatial distribution of LvTbx2/3 protein throughout sea urchin embryogenesis, a polyclonal antiserum was generated. Nuclear localization of LvTbx2/3 initiated at the mesenchyme blastula stage and protein was present into the pluteus stage. Localization was asymmetric throughout this period and costaining with marker genes indicated that asymmetry was about the oral/aboral (O/A) axis. Asymmetric distribution of LvTbx2/3 was observed in the aboral territories of all three germ layers. In the skeletogenic mesoderm lineage, LvTbx2/3 expression was dynamic because expression appeared initially in all skeletogenic mesenchyme cells (PMCs) but, subsequently, became refined solely to the aboral ones during skeletogenesis. To determine if the aboral expression of *LvTbx2/3* is linked between germ layers, and to place *LvTbx2/3* in the sequence of events that specifies the O/A axis, the effects of a series of perturbations to O/A polarity on *LvTbx2/3* expression in each germ layer were examined. Preventing the nuclear localization of β-catenin, pharmacological disruption of the O/A axis with $NiCl_2$, overexpression of *BMP2/4* and disruption of the extracellular matrix all blocked *LvTbx2/3* expression in all germ layers. This indicates that expression of *LvTbx2/3* in the aboral territories of each germ layer is a common aspect of O/A specification, downstream of the molecular events that specify the axis. Furthermore, blocking the nuclear localization of β-catenin, overexpression of *BMP2/4* and disruption of the extracellular matrix also prevented the oral (stomodael) expression of LvBrachyury (LvBrac) protein, indicating that the O/A axis is established by a complex series of events. Last, the function of LvTbx2/3 in the formation of the O/A axis was characterized by examining the phenotypic consequences of ectopic expression of *LvTbx2/3* mRNA on embryonic development and the expression of marker genes that identify specific germ layers and tissues. Ectopic expression of *LvTbx2/3* produced profound morphogenetic defects in derivatives of each germ layer with no apparent loss in specification events in those tissues. This indicates that LvTbx2/3 functions as a regulator of morphogenetic movements in the aboral compartments of the ectoderm, endoderm and mesoderm.

Key words: Sea urchin, T-box, Tbx2/3, Oral/aboral, Morphogenesis

INTRODUCTION

Cell-fate specification in the sea urchin embryo is achieved through an initial polarization of the unfertilized egg along the animal/vegetal (A/V) axis and, after fertilization, through cell-cell interactions within and between blastomere layers during cleavage and blastula stages. Such polarizations, signaling events and cell-cell interactions activate region-specific genes and segregate the 60-cell stage embryo into five embryonic territories: the small micromeres; the skeletogenic mesenchyme; the vegetal plate endomesoderm; the oral ectoderm; and the aboral ectoderm (Davidson, 1998). Although there is much data about the development of the primary, A/V axis of the embryo (reviewed in Angerer and Angerer, 2000), less is known about the specification of the secondary, O/A axis in the sea urchin. In some species of sea urchins, the O/A axis can be predicted by the plane of first cleavage, whereas in others, the future axis cannot be related to the position of cleavage planes until the third cleavage or later (Cameron et al., 1989; Henry et al., 1992). Signaling events seem also to be involved (Wikramanayake et al., 1995; Wikramanayake and Klein, 1997).

Several genes are expressed asymmetrically about the O/A axis in the endoderm, mesoderm and ectoderm, but no genes have yet been identified that reflect the oral/aboral polarity extending between all three. In the endoderm, asymmetrically

localized gene products include apical LvNotch protein, which is enriched on the aboral side (Sherwood and McClay, 1997). In the nonskeletogenic mesoderm, CyII actin is distributed orally, and OrCT, CAPK and Pl103 aborally (Miller et al., 1996; Rast et al., 2002). O/A patterning has been studied most in the ectoderm and, thus, many gene products with asymmetric distribution about the axis have been identified. Aboral genes include *Spec1* and *Spec2* (Lynn et al., 1983), *CyIIIa* actin (Cox et al., 1986), *arylsulfatase* (Sasaki et al., 1988), *Hox8/Hbox1* (Angerer et al., 1989), *SpMTA* (Nemer et al., 1995) and *P3A2* (Calzone et al., 1991); whereas oral genes include EctoV (Coffman and McClay, 1990) *Otx* (Li et al., 1997a; Yuh et al., 2002), *SpCOUP-TF* (Vlahou et al., 1996), *BMP2/4* (Angerer et al., 2000), *PlOtp* (Di Bernardo et al., 1999), *Brachyury* (Gross and McClay, 2001) and *goosecoid* (Angerer et al., 2001).

Based on the expression patterns and likely function of members of the family of T-box genes in regionalization of body plans in other organisms, we hypothesized that they might play a similar role in the sea urchin embryo. Members of the T-box family of transcription factors have been identified in all metazoan organisms in which they have been sought (reviewed by Papaioannou and Silver, 1998; Smith, 1999). T-box genes are characterized by homology to the DNA-binding domain of Brachyury, the founding member of the T-box gene family. The T-box encompasses ~180 amino acids and can be located anywhere in the protein (Kispert and Hermann, 1993). T-box proteins share little homology outside this region and it is in the T-box that the specificity for target promoters resides (Conlon et al., 2001). The T-box family includes transcriptional activators such as *Brachyury*, *Tbx5*, *VegT* and *Eomesodermin* (Kispert et al., 1995; Horb and Thomsen 1997; Horb and Thomsen, 1999; Ryan et al., 1996) as well as transcriptional repressors such as *Tbx2* and *Tbx3* (Carreira et al., 1998; He et al., 1999). The importance of T-box genes in development is underscored by their involvement in a variety of human pathologies, including that of *Tbx5* in Holt-Oram syndrome (Basson et al., 1997; Li et al., 1997b), *Tbx1* in DiGeorge syndrome (Jerome and Papaioannou, 2001; Merscher et al., 2001), *Tbx3* in ulnar-mammary syndrome (Bamshad et al., 1997) and, possibly, *Tbx2* in breast cancer (Jacobs et al., 2000).

Here, we report the identification and characterization of *LvTbx2/3*, a member of the *Tbx2/3* subfamily of T-box genes, during development of the sea urchin embryo. LvTbx2/3 protein is concurrently expressed in the aboral territories of the endoderm, mesoderm and ectoderm. A series of perturbations to the molecular components that are thought to be involved in specifying the O/A axis revealed that the aboral distribution of LvTbx2/3 appears to be a common aspect of O/A specification in each of these tissues. Specifically, LvTbx2/3 expression is dependent on either β-catenin or genes downstream of β-catenin, and is prevented by ventralization with $NiCl_2$, overexpression of *LvBMP2/4* and disruption of the extracellular matrix (ECM). Thus, LvTbx2/3 is expressed downstream of, or relatively late in, the sequence of events that serve to specify this axis. That LvTbx2/3 expression can not be separated between the different tissues after perturbation indicates that O/A axis specification is linked in all three germ layers of the sea urchin embryo at the level of *LvTbx2/3* and may occur in parallel to the distinct specification events that give rise to the ectoderm, endoderm and mesoderm of the embryo. Ectopic expression of *LvTbx2/3* supports this conclusion in that universal expression of *LvTbx2/3* profoundly affects the morphogenesis of ectoderm, endoderm and mesoderm without altering specification events of embryonic territories. Combined with the loss of expression of *LvTbx2/3* after perturbation of O/A specification, these results indicate that *LvTbx2/3* may be a downstream component of the O/A axis program that is involved specifically in morphogenesis of aboral territories in the embryo.

MATERIALS AND METHODS

Animals

Sea urchins (*L. variegatus*) were obtained from either Susan Decker (Hollywood, FL) or Jennifer Keller (Duke University Marine Laboratory). Gametes were harvested and cultured at 23°C as described (Hardin et al., 1992).

Cloning an *LvTbx2/3* fragment

Degenerate primers were designed that corresponded to the amino acids YIHPDSP (forward)/AVTAYQN (reverse) and used in a PCR reaction with cDNA template prepared from mid gastrula poly(A)$^+$ mRNA. PCR conditions were 45 cycles of 96°C for 60 seconds, 40°C for 60 seconds, 72°C for 2 minutes 45 seconds. The amplified, 234 bp products were gel purified, cloned into the pGEMT vector (Promega) and sequenced bidirectionally (Duke Sequencing Core). Clones were identified as PCR products of *LvTbx2/3* by BLAST search.

cDNA library screens

Screens were performed essentially as described (Gross and McClay, 2001) with hybridizations performed at 55°C in 0.5 M $NaHPO_4$ pH 7.2, 1 mM EDTA, 7% SDS, after Church and Gilbert (1984). After rescreens, nine clones were excised, sequenced and identified as *LvTbx2/3* fragments. A full-length open-reading frame was defined by overlapping individual fragments.

Northern analysis

Northern blotting (RNA gel blot hybridization) for *LvTbx2/3* was performed as described (Gross and McClay, 2001). Blots were given two 5 minute washes with 6× SSPE, 0.5% SDS at room temperature, one 45 minute wash with 1× SSPE, 0.1% SDS at 37°C, and one 45 minute wash with 1× SSPE, 0.1% SDS at 50°C. After washing, the blot was wrapped in plastic wrap and placed on film for 72 hours at –70°C with an intensifying screen. It was then stripped in 50% formamide, 6× SSPE for 30 minutes at 65°C and reprobed as above with an *L. pictus* ubiquitin fragment as a loading control.

Antibody production

LvTbx2/3 fusion protein was expressed following PCR amplification of a *Bam*HI-*Xho*I fragment of *LvTbx2/3* (encoding amino acids 11-339) and subcloning into the pGEX4T-1 glutathione S-transferase (GST) expression system (AmershamPharmacia Biotech). Expressed, affinity-purified protein (80 μg) was mixed 1:1 with Freund's complete adjuvant and injected into each of three guinea pigs (Charles River, Raleigh, NC). Animals were boosted with 80 μg protein mixed 1:1 with incomplete Freund's adjuvant after 21, 42 and 70 days. Bleeds were performed 31, 53 and 80 days after the last injection and serum isolated as described (Harlow and Lane, 1988).

Western analysis

1500 late-gastrula embryos were homogenized in the presence of protease inhibitors, boiled and run on a 10% SDS-PAGE gel. Protein was blotted onto nitrocellulose, blocked overnight at 4°C

in 2% milk, 1% bovine serum albumin (BSA) TBST and probed for 1.5 hours at room temperature with a 1:1000 dilution of either α-Tbx2/3 or preimmune serum in 2% milk, 1% BSA TBST. The blot was washed three times with TBS before applying goat α-guinea pig HRP-tagged secondary antibody (Jackson Immunoresearch Laboratories) at 1:5000 for 1 hour at room temperature. Labeled proteins were visualized by ECL (AmershamPharmacia Biotech).

Immunolocalization and image analysis

Embryos were fixed in 2% paraformaldehyde, 60% artificial sea water (ASW) for 10-12 minutes at room temperature, before being permeablized for 60 seconds with ice cold, 100% methanol. They were then washed three times with PBS, blocked 10-20 minutes in PBS, 4% normal goat serum (NGS; GibcoBRL) and incubated overnight at 4°C in primary antibody, 4% NGS. After washing four times in PBS, they were blocked as above and incubated for 60 minutes at room temperature in secondary antibody, 4% NGS (either Cy3 or Cy5-conjugated; Jackson Immunoresearch Laboratories). Embryos were then washed four times in PBS and mounted in 70% glycerol. LvTbx2/3 and LvBrac sera were diluted 1:500 for all images. Undiluted supernatants of monoclonal antibodies (mABs) 5a7 (EctoV), 5c7 (Endo1) and 295 were used with the above fixation and incubation conditions. All images were obtained using a 40× Plan-Neofluar oil-immersion objective (NA=1.3) on a Zeiss laser-scanning confocal microscope (Carl Zeiss, Thornwood, NY) mounted on a Zeiss Axiovert inverted microscope. Where necessary, 1 μm sections from single label images were rendered into 3D projections using Zeiss confocal software. Double labeled images were taken sequentially using appropriate filters and subsequently overlayed using Adobe Photoshop 5.0.

Chemical treatments

Treatment of embryos with either $NiCl_2$ or β-aminopropionitrile (βAPN) were performed as described (Hardin et al., 1992; Wessel and McClay, 1987).

Generation of LvTbx2/3 constructs

Full-length *LvTbx2/3* was generated by subcloning fragments from individual excised cDNA clones obtained in library screening (details available on request). For ectopic overexpression studies, an *SpOtx* 5′ UTR plus the first five amino acids of *SpOtx* was cloned in frame, 5′ to the *LvTbx2/3* translation-start site. This leader sequence has been demonstrated to provide an excellent translation start site for mRNA constructs in the sea urchin (Sherwood and McClay, 1999). All clones were sequenced bidirectionally to verify fidelity.

mRNA preparation and injection

ΔLvG-cadherin and *LvBMP2/4* were linearized and injected as described (Logan et al., 1999; Angerer et al., 2000). *LvTbx2/3* was linearized with *XhoI* and used as a template to generate in vitro-transcribed 5′ capped mRNA using the T3 mMessage mMachine kit (Ambion). Concentrations of mRNA were determined by spectrophotometry, and by comparison to known amounts of RNA using both gel electrophoresis and dotting onto a 0.6% agarose gel.

Quantitative PCR (QPCR)

RNA was isolated using Trizol (Invitrogen). Reverse transcription reactions were performed using oligo dT priming and MMLV-reverse transcriptase (Gibco). Reactions were purified using a PCR-purification kit (Qiagen). QPCRs were performed using Roche LightCycler Fast Start Master SYBR as manufacturers instructions. Primers used were ubiquitin (Rast et al., 2000) and LvTbx2/3. A Tbx2/3 plasmid was used to generate a standard curve for quantification, and ubiquitin was used to normalize the cDNA samples. Each time point was determined from two independent batches, and each reaction was confirmed by gel electrophoresis.

RESULTS

Identification of a *Tbx2/3* subfamily member in the sea urchin

LvTbx2/3 was PCR amplified from a mid-gastrula stage cDNA pool using degenerate oligonucleotides that correspond to evolutionarily conserved regions of the DNA-binding domain of other T-box proteins. Cloning and sequencing of the amplified fragment identified it as a *L. variegatus Tbx2/3* ortholog (*LvTbx2/3*). A mid-gastrula cDNA library was then screened and nine *LvTbx2/3* cDNA clones recovered. Alignment of the sequences of these clones defined the full coding region of the gene. *LvTbx2/3* encodes a 637 amino acid protein, based on the predicted open reading frame from the primary sequence data (Fig. 1A; GenBank accession number AY120889). Supporting *LvTbx2/3* as a member of this T-box subfamily, LvTbx2/3 aligns in a phylogenetic tree of Tbx2/3 subfamily proteins (Fig. 1B).

Northern-blot analysis of *LvTbx2/3* mRNA revealed that a 5.37 kb message appears first at the mesenchyme blastula stage and that it is present throughout the pluteus stage (Fig. 2). The highest concentrations of mRNA were observed during gastrula stages. Quantitative PCR analysis of the *LvTbx2/3* RNA corresponds well with the Northern-blot data. No *LvTbx2/3* mRNA is present in the egg but low concentrations start to be detected at the hatched blastula stage. In the mesenchyme blastula there are ~200 copies of *LvTbx2/3* mRNA per aboral cell and this level is retained until the early prism stage when the number of copies per aboral cell drops. To characterize the temporal and spatial distribution of LvTbx2/3 protein, a polyclonal antiserum was generated in guinea pigs against recombinant LvTbx2/3 (amino acids 11-339 fused to GST). This serum was tested for immunoreactivity by protein analysis on SDS-PAGE gels and whole-mount immunofluorescent staining of fixed embryos. Western blots of protein extracts from late gastrula were probed with LvTbx2/3 polyclonal and preimmune sera to ascertain specificity (Fig. 3). Two immunoreactive bands were observed when blots were probed with LvTbx2/3 serum, one of ~70 kDa and one of 35 kDa. The 70 kDa band corresponds with the predicted size of LvTbx2/3 from primary sequence data (637 amino acids). The 35 kDa band was also recognized when blots were probed with preimmune serum, indicating that it is a nonspecific antigen. Whole-mount immunofluorescent analysis of fixed embryos stained with preimmune serum did not result in any distinct staining pattern at any stage examined (Fig. 4C). Additionally, embryos stained with LvTbx2/3 serum after preincubation with recombinant LvTbx2/3 protein did not stain positively at any stage examined (Fig. 4D).

Throughout development, the spatial distribution of LvTbx2/3 is asymmetric about the O/A axis in the endoderm, mesoderm and ectoderm

Fig. 4A,B shows two different orientations of prism-stage embryos costained with anti-LvTbx2/3 antiserum (red) and 5a7 mAB (green). 5a7 recognizes EctoV, a protein that is localized to the foregut and oral ectoderm (Coffman and McClay, 1990). LvTbx2/3 was localized to the nucleus, as expected given its role as a transcription factor. A striking asymmetry of LvTbx2/3 distribution was observed in the ectoderm, endoderm and skeletogenic mesoderm at this stage of

development. EctoV and LvTbx2/3 were present in complementary patterns, indicating that this asymmetry is about the O/A axis of the ectoderm and that LvTbx2/3 is restricted solely to the aboral territories of the embryo. The aboral distribution of LvTbx2/3 in the endoderm and mesoderm is apparent in Fig. 4B where the protein is clearly localized to the aboral regions of the archenteron and skeletogenic mesoderm. As such, LvTbx2/3 is the first marker of O/A polarity expressed in the derivatives of all three germ layers of the sea urchin embryo. Additionally, the LvTbx2/3 characterization reported here is, to our knowledge, the first report of protein expression for a non-Brachyury T-box gene in any organism.

A

```
1    ATG AAA CCG GCA TCG AAC GAC CAC CAC ACG ATG GCC TAT GCA CCT ATA CTG CCG CCT CGT CTC
       M   K   P   A   S   N   D   H   H   T   M   A   Y   A   P   I   L   P   P   R   L
64   AGC GAC TTC TCC GTC AAC TCC CTC CTC ACA CCG CCC CAA TTC TTC CCG GGG ATG TTC CGC GGA
       S   D   F   S   V   N   S   L   L   T   P   P   Q   F   F   P   G   M   F   R   G
127  CAG GCC TGT TTG CCC GGC GCA GGG CTG CCG GGC TTT CCC CTG CCG AAA TTC GGT GAA CAT CCA
       Q   A   C   L   P   G   A   G   L   P   G   F   P   L   P   K   F   G   E   H   P
190  GCG GGG TAC TCC CCG CAT GAC TTG TTG GCA GCA CAC GCT CAT CGC TCA GCA TTG GGC CCC TTA
       A   G   Y   S   P   H   D   L   L   A   A   H   A   H   R   S   A   L   G   P   L
253  CAC CCC ATG GAA ACA CAG AGC GAC GAT TCG GAT GAT CCA CAA GTT ACA CTT GAA TCT AAA GAA
       H   P   M   E   T   Q   S   D   D   S   D   D   P   Q   V   T   L   E   S   K   E
316  CTT TGG GAG AAA TTT CAC AAA AGA GGA ACG GAA ATG GTC ATC ACA AAA TCA GGC CGG CGG ATG
       L   W   E   K   F   H   K   R   G   T   E   M   V   I   T   K   S   G   R   R   M
379  TTC CCT TCT TTC AAA GTC CGT GTA TCT GGG CTG GAC AAG AAG GCC AAA TAC ATC CTT TTA ATG
       F   P   S   F   K   V   R   V   S   G   L   D   K   K   A   K   Y   I   L   L   M
442  GAC ATC GTC GCC GCC GAC GAC TGC CGG TAC AAG TTT CAC AAT TCC CGC TGG ATG GTC GCT GGC
       D   I   V   A   A   D   D   C   R   Y   K   F   H   N   S   R   W   M   V   A   G
505  AAG GCC GAT CCC GAG ATG CCC AAA CGG ATG TAT ATA CAC CCG GAT TCT CCG AGC ACA GGG GAA
       K   A   D   P   E   M   P   K   R   M   Y   I   H   P   D   S   P   S   T   G   E
568  CAA TGG ATG CAG AAA TGT GTT TCA TTC CAT AAG CTC AAA CTC ACC AAT AAC ATC TCC GAC AAG
       Q   W   M   Q   K   C   V   S   F   H   K   L   K   L   T   N   N   I   S   D   K
631  CAT GGA TTC CAG ACC ATT CTG AAT TCG ATG CAC AAG TAC CAA CCT CGT TTC CAC ATT GTC AAG
       H   G   F   Q   T   I   L   N   S   M   H   K   Y   Q   P   R   F   H   I   V   K
694  GCC AAT GAC ATC CTC AAG CTT CCC TGG AGT CAA TTC AGG ACC TTC GTA TTC GTC GAG ACC GTC
       A   N   D   I   L   K   L   P   W   S   Q   F   R   T   F   V   F   V   E   T   V
757  TTC ATC GCT GTC ACT GCC TAT CAA AAC GAA AAG ATT ACG CAA CTT AAA ATA GAC TAC AAC CCA
       F   I   A   V   T   A   Y   Q   N   E   K   I   T   Q   L   K   I   D   Y   N   P
820  TTC GCT AAA GGT TTC AGA GAT ACT GGC GCA GGG AAA AGG GAA AAG AGG AAA TAC ATT GGT GCA
       F   A   K   G   F   R   D   T   G   A   G   K   R   E   K   R   K   Y   I   G   A
883  ACT GGT ACC TAT GAA ATC GAC CAT CGA GAC GGC GAT GAC ATC CCA AGC GAC CAG GAG GCC GAG
       T   G   T   Y   E   I   D   H   R   D   G   D   D   I   P   S   D   Q   E   A   E
946  GCC GCC GAG GTC AGC ACA ACC AGC AAC GAC AGG CAT GAC GAA AGA GGT CAT TCG TCA CAC GAG
       A   A   E   V   S   T   T   S   N   D   R   H   D   E   R   G   H   S   S   H   E
1009 CTT GCA AGG CTA GCC AGC GAG GGC CGC CTG AAC GGA CCT GGC CTG AAC AAG TGT AAA CCC TCG
       L   A   R   L   A   S   E   G   R   L   N   G   P   G   L   N   K   C   K   P   S
1072 GAC ATG AAG GAA GGG CCA CAT GGA AGT TCA AGC TCC AAA GAT GAC GTA GAG ATG AGG GAT GTG
       D   M   K   E   G   P   H   G   S   S   S   S   K   D   D   V   E   M   R   D   V
1135 AGC TGT AAA GAC CAC GAG AGG AGG ATG GAG GGT AAA CAT AGA TTA AGT CAG GAT GAC AGT TCA
       S   C   K   D   H   E   R   R   M   E   G   K   H   R   L   S   Q   D   D   S   S
1198 ATT GAC AAG AAA ACC GAT CAC AAT GAG CGA TCA GAT TCG CGG AAA AGT GAC GGG CCC AGT TCA
       I   D   K   K   T   D   H   N   E   R   S   D   S   R   K   S   D   G   P   S   S
1261 AGA CTT TCT CCT CCA AGT CTA CAC CTT GGT TCT GCC GGC TCA TCC TTC TCG TCA TTG CAC GGT
       R   L   S   P   P   S   L   H   L   G   S   A   G   S   S   F   S   S   L   H   G
1324 TCC CAT CCA CCT GTT GTG ACG CCA ATC TAC CCC ACA CCT CAG CAG TTA TTT CTC AAT CCC CAT
       S   H   P   P   V   V   T   P   I   Y   P   T   P   Q   Q   L   F   L   N   P   H
1387 GCG CTA CAT GGT GCT GTA CCA GGA CTT GGA GCA ATG CAT CAC ATG TTA CCC CTC CCA AGC AGC
       A   L   H   G   A   V   P   G   L   G   A   M   H   H   M   L   P   L   P   S   S
1450 TCC TCC CAT TCC CCT TCA GGA CAT CCT AGT TAT TTA GAC GCC CAT CCT TTC GCG TTT GGA GCA
       S   S   H   S   P   S   G   H   P   S   Y   L   D   A   H   P   F   A   F   G   A
1513 GCT CAT GCT TCA GGA CTC CTT TCC TCG CAA GGT GGC GCC GCC AGC TTT GGC AGC CTC TAT TCA
       A   H   A   S   G   L   L   S   S   Q   G   G   A   A   S   F   G   S   L   Y   S
1576 GAG GCC GCC GCC CTT AGC TCA ATG TAT GCC AGC AAC CCG TGT ACT AGT GCA ATA TTA AAT GGA
       E   A   A   A   L   S   S   M   Y   A   S   N   P   C   T   S   A   I   L   N   G
1639 CAT CCA AGA TTA AGG TTC TCA CCT TAT CAC CTA CCA GTC ACC AGC ACG ACT ATG GTC ACC ACT
       H   P   R   L   R   F   S   P   Y   H   L   P   V   T   S   T   T   M   V   T   T
1702 GCT AAC CCT CTA GCC ACA CCT ATC CCA TAC GAA AGC GCG TTA CAT TCA TCA CTC TCA GCG TTT
       A   N   P   L   A   T   P   I   P   Y   E   S   A   L   H   S   S   L   S   A   F
1765 GGT GGG TCA TCA CTG CTC ACG CCG GCC TCG GCA TCC ACT TCA CCG ACA TCG TCG TCA TCT TCA
       G   G   S   S   L   L   T   P   A   S   A   S   T   S   P   T   S   S   S   S   S
1828 CTT CCA GCC AGT AAA GAC GTG CCG ACG TCG CCT GCG AGG TCG GTC AGC GCT GCC ACA AAC GAA
       L   P   A   S   K   D   V   P   T   S   P   A   R   S   V   S   A   A   T   N   E
1891 CTT CAA AGC ATA CAA AAG ATG GTC AGT GGA CTC GAT AAA ACA CAA AAA TGA
       L   Q   S   I   Q   K   M   V   S   G   L   D   K   T   Q   K   .
```

The ectoderm, endoderm and mesoderm are all specified prior to LvTbx2/3 expression. Because LvTbx2/3 was distributed in a subset of cells in each of these tissues, we next characterized the temporal details of LvTbx2/3 protein expression (Fig. 5). LvTbx2/3 was localized to the nucleus at all stages examined. At mesenchyme blastula stage, LvTbx2/3 protein was observed in cells of the presumptive endoderm and ectoderm but not the mesoderm, as neither the ingressed skeletogenic nor the presumptive nonskeletogenic mesoderm expressed LvTbx2/3 protein when these territories were defined by marker genes (data not shown). A view of the vegetal surface of an early-gastrula stage embryo is shown in Fig. 5D. LvTbx2/3 was present at high concentrations in the presumptive endoderm and the ectoderm that surrounds the blastopore, whereas invaginated tissues contained much less protein. Asymmetric distribution in the endoderm and ectoderm continued through mid-gastrula stage (Fig. 5E,F). Between mid-gastrula and late-gastrula stages, LvTbx2/3 started to be expressed in cells of the skeletogenic mesenchyme lineage and the asymmetric localization in the invaginated endoderm became more apparent (Figs 5, 6). LvTbx2/3 protein in early and late-plutei embryos is shown in Fig. 5I-K. From an animal view of an early pluteus embryo that has been optically sectioned and

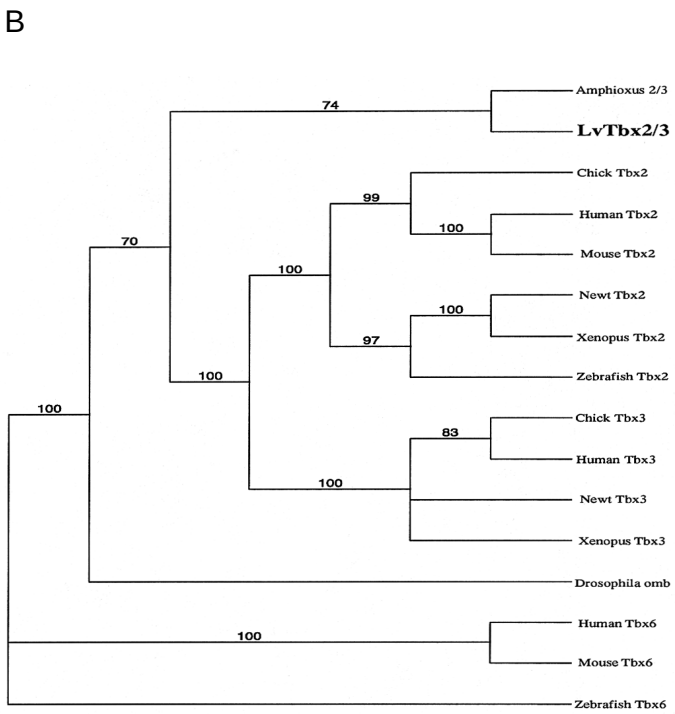

Fig. 1. (A) Nucleotide and predicted amino acid sequences of *LvTbx2/3*. (B) Phylogenetic tree of LvTbx2/3, Tbx2, Tbx3 and Tbx2/3 orthologs from other organisms generated by the neighbour-joining method. Bootstrap values indicated on nodes.

projected so that the animal-most ectoderm is removed, asymmetric distribution of LvTbx2/3 was observed in the ectoderm of the embryo and in the archenteron (Fig. 5I). A vegetal projection of a similarly staged pluteus embryo revealed that LvTbx2/3 is present in the ectoderm that surrounds the anus and was very strong in the distal-most portions of the extending embryonic arms (Fig. 5J). Although the concentration of LvTbx2/3 began to decline at the late pluteus stage, it was still observed asymmetrically in the ectoderm, endoderm and skeletogenic mesenchyme (Fig. 5K).

When LvTbx2/3 first appeared in the skeletogenic mesoderm, it was present in all of the PMCs (Fig. 5G,H and data not shown). However, as development proceeded to the prism and pluteus stages, LvTbx2/3 became restricted to the aboral PMCs (Figs 4, 5). The spatial and temporal aspects of LvTbx2/3 expression in the skeletogenic mesenchyme were further characterized to determine precisely when this restriction occurs (Fig. 6). Fig. 6A shows the initial, panskeletogenic mesoderm distribution of LvTbx2/3. Two different levels of confocal projections from the same prism-stage embryo are shown (Fig. 6B,C). The asymmetric distribution of LvTbx2/3 protein is clearly confined to the aboral PMCs and not present in the ventrolateral clusters. An oblique view of another late-prism stage embryo stained for LvTbx2/3 (red) and 5a7 (green) reinforced this observation, because LvTbx2/3 persists in the aboral ectoderm and endoderm of the embryo whereas no expression is observed in the ventrolateral clusters of PMCs (Fig. 6D). In early-pluteus stage embryos, asymmetric distribution of LvTbx2/3 persists in the PMCs, endoderm and ectoderm (Fig. 6E,F). Thus, in the skeletogenic mesoderm LvTbx2/3 is restricted to the aboral PMCs between late gastrula and early prism stages at the time when skeletal patterning begins to shape the spicule skeleton.

LvTbx2/3 in the sequence of O/A axis specification and patterning

The striking asymmetry of the distribution of LvTbx2/3 about the O/A axis in the endoderm, ectoderm and mesoderm raises the possibility that O/A polarity might be either established or maintained by the same molecular component(s) in all three germ layers of the embryo. To place LvTbx2/3 in the framework of specification pathways and patterning events that impinge on the formation of the O/A axis, and to gain further insights into the mechanisms of O/A axis specification, the distribution of LvTbx2/3, 5a7 (Fig. 7A) and LvBrac (Fig. 7B) were examined under a variety of perturbations to this axis.

β-catenin/vegetal signaling in O/A patterning

The influence of β-catenin and β-catenin-dependent signaling on LvTbx2/3 expression was assayed first. Injection of a construct that encodes the cytoplasmic tail of the sea urchin ortholog of E-cadherin, *LvG-cadherin* (*ΔLvG-cadherin*) (Logan et al., 1999), serves as a 'sink' for cytoplasmic β-catenin by binding to it and preventing nuclear translocation and gene activation. Such embryos develop without endoderm

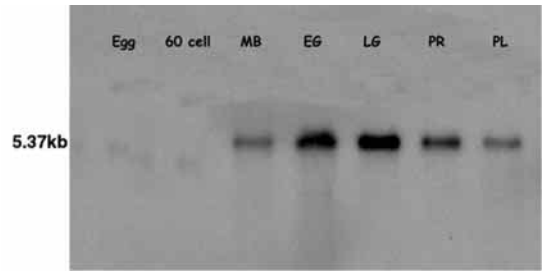

Fig. 2. Developmental northern blot of *LvTbx2/3* expression. Poly(A)$^+$ RNA (3 μg per lane) was loaded (calculated by OD_{260}). Loading was verified by probing the blot with a ubiquitin fragment from *L. pictus* (data not shown). Egg; 60 cell stage; MB, mesenchyme blastula; EG, early gastrula; LG, late gastrula; PR, prism; PL, pluteus larva.

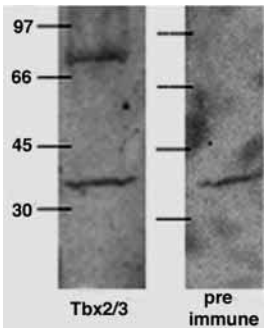

Fig. 3. Tbx2/3 polyclonal and preimmune sera controls. Western-blot analysis of protein extracts from late gastrula (1500 embryos) using polyclonal LvTbx2/3 and preimmune serum. In blots probed with LvTbx2/3, two immunoreactive bands appear, one of ~70 kDa and one of 35 kDa. A 35 kDa band was also recognized by preimmune serum, indicating the presence of a nonspecific antigen.

or mesoderm, and β-catenin depletion eliminates the O/A axis in the ectoderm (Wikramanayake et al., 1998; Logan et al., 1999). Expression of this construct resulted in uniform expression of EctoV (Fig. 7C) and prevented the expression of LvTbx2/3 (Fig. 7D). LvBrac and *SpGsc* (*Goosecoid*), two gene products that normally localize to the stomodaeum, are also not expressed when the nuclear translocation of β-catenin is prevented (Gross and McClay, 2001; Angerer et al., 2001). This indicates that the blockage of β-catenin nuclear localization prevents both aboral and oral (stomodael) specification.

Pharmacological perturbation of O/A patterning

Treatment of embryos with $NiCl_2$ at any point between the hatched blastula and early gastrula stages disrupts O/A patterning events (Hardin et al., 1992). Embryos perturbed in this manner are oralized, displaying defects in ectodermal patterning manifested by the formation of a circumferential stomodaeum around the animal pole, rather than at a localized

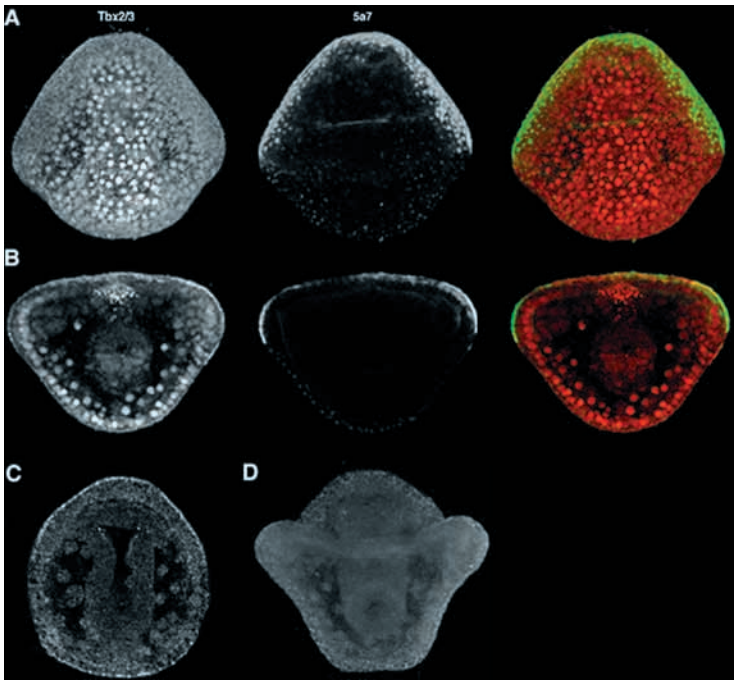

Fig. 4. Aboral distribution of LvTbx2/3 protein (red) in prism-stage embryos, demonstrated by co-staining with 5a7 (EctoV; green). The EctoV antigen is expressed from late gastrula stages onward, solely in the oral ectoderm and foregut. Prism-stage embryos viewed aborally (A) and in a vegetal cross-section (B). Complementary expression is observed, indicating that LvTbx2/3 is distributed asymmetrically about the oral/aboral axis, and localized in aboral territories of the endoderm, ectoderm and mesoderm. In many prism and pluteus-stage embryos, a patch of cilia on the oral surface crossreacts with Cy3 secondary antibodies nonspecifically, as observed in the LvTbx2/3 (red) images. (C) Whole-mount, immunofluorescent analysis of fixed embryos probed with preimmune serum. No nuclear staining is observed at any stage (early prism stage shown). (D) Whole-mount, immunofluorescent analysis of fixed embryos probed with with polyclonal LvTbx2/3 serum that had been preincubated with recombinant fusion protein. No nuclear staining is observed at any stage examined (early pluteus stage shown).

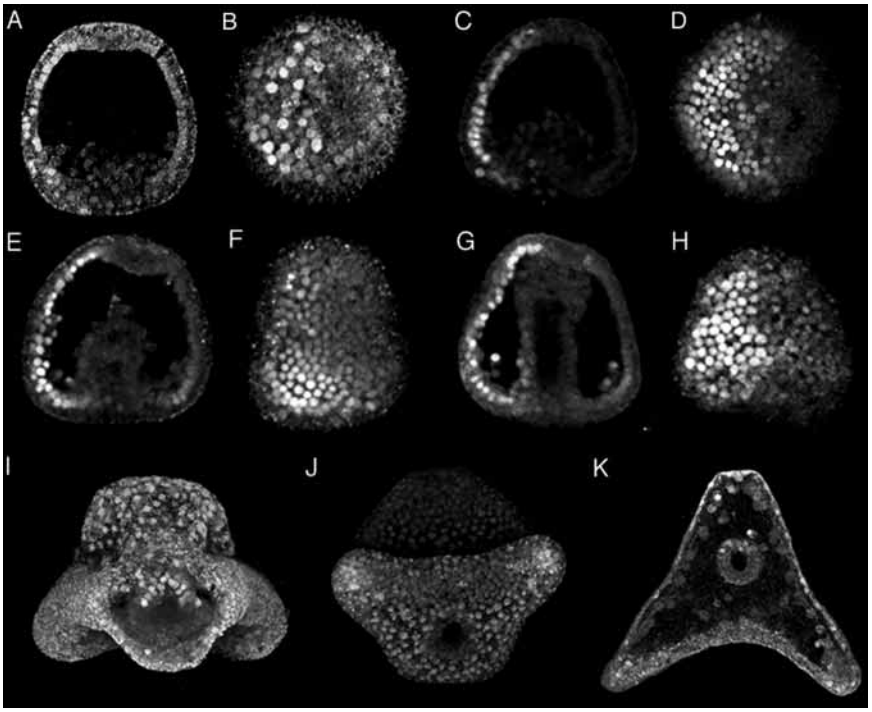

Fig. 5. LvTbx2/3 protein is asymmetric throughout embryonic development. Cross-section images (A,C,E,G,I,K) and surface projections (B,D,F,H,J). LvTbx2/3 expression first appears at the mesenchyme blastula stage and is distributed asymmetrically in the presumptive endoderm and ectoderm, as viewed in cross-section (A) and in a vegetal-surface view (B). Early-gastrula stage embryos in cross-section (C) and a vegetal view (D) maintain asymmetric distribution of LvTbx2/3 in the presumptive endoderm and ectoderm, whereas the endoderm and mesoderm that have invaginated into the blastocoel do not express protein. (E,F) Mid-gastrula stage embryo (cross-section and surface projection of the same embryo). LvTbx2/3 expression is maintained asymmetrically in the presumptive endoderm and ectoderm and is not present in invaginated endoderm or mesoderm. (G,H) Late gastrula distribution of LvTbx2/3 protein (cross-section and surface projection of the same embryo). Asymmetric expression is observed in the invaginated endoderm, the ectoderm and in all of the skeletogenic mesenchyme cells at this stage (also see Fig. 6). (I) Animal view of early pluteus embryo optically sectioned to remove the most superficial layers of ectoderm and expose the archenteron and stomodaeum. LvTbx2/3 is distributed asymmetrically in the surface ectoderm and the length of the archenteron. (J) Vegetal surface view. Distribution of LvTbx2/3 protein is asymmetric in the aboral ectoderm nuclei. High concentrations of LvTbx2/3 are also observed in the distal most nuclei of the extending pluteus arms. (K) Vegetal cross-section of a late pluteus embryo. Asymmetric distribution is maintained in cells of the ectoderm, endoderm and skeletogenic mesoderm.

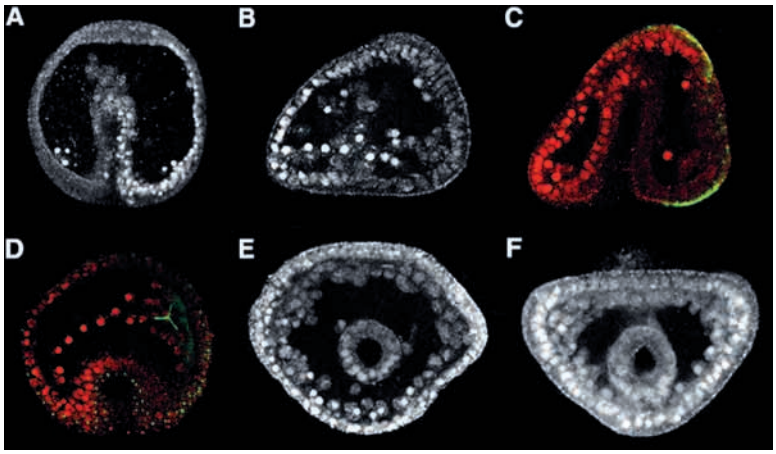

Fig. 6. Dynamic expression of LvTbx2/3 in the skeletogenic mesenchyme cells (PMCs). (A) Expression in PMCs begins at mid-late gastrula stage in all PMCs. (B,C) Two views of the same early-prism stage embryo. (B) Superficial view of the PMCs under the ectoderm. At this stage, asymmetric distribution of LvTbx2/3 is observed in the PMC lineage. (C) Deeper, cross-sectional view of the embryo in B co-stained for LvTbx2/3 (red) and EctoV (green). LvTbx2/3 localization in PMCs is in the aboral territory of the embryo. Note the clear, asymmetric distribution in the endoderm. (D) An oblique view of a late prism-stage embryo stained for LvTbx2/3 (red) and 5a7 (green). Distribution of LvTbx2/3 protein is limited to the aboral PMCs and is not present in the ventrolateral clusters that have begun to form triradiate spicules. Animal (E) and vegetal (F) cross-sectional views of early-pluteus stage embryos. LvTbx2/3 protein persists in the aboral PMCs and is not observed in the oral PMCs.

site, and the formation of ectopic spicule clusters. These animals express EctoV and LvBrac around their entire circumference except the vegetal plate (Hardin et al., 1992; Gross and McClay, 2001). Treatment of embryos with 1 mM $NiCl_2$ resulted in expression of LvBrac throughout the entire ectoderm (Fig. 7E) and elimination of LvTbx2/3 expression in all tissues (Fig. 7F).

BMP2/4 in O/A specification

Recent evidence indicates that an animally derived BMP2/4 ortholog affects O/A specification (Angerer et al., 2000). In situ analysis localizes *BMP2/4* mRNA to presumptive oral ectoderm at the hatching blastula stage. Ubiquitous overexpression of *BMP2/4* mRNA animalizes the embryo, causing it to form a ball of squamous epithelium whereas lower concentrations radialize the ectoderm of the embryo, as indicated by the formation of multiple clusters of spicules. At concentrations of *BMP2/4* mRNA that radialize the spicules, oral expression of LvBrac was prevented but vegetal LvBrac expression was normal (Fig. 7G). Aboral expression of LvTbx2/3 was not observed in the ectoderm, endoderm or skeletogenic mesoderm under such conditions (Fig. 7H). This indicates that ectopic expression of *BMP2/4* prevented the normal expression of *LvTbx2/3* in all three germ layers, and that O/A polarity in the ectoderm, mesoderm and endoderm is linked by some common genetic or molecular mechanism that is likely to be sensitive to changes in *BMP2/4* levels. The failure to observe stomodael LvBrac protein after ectopic expression of *BMP2/4* indicates that *BMP2/4* signals prevent the expression of a subset of genes in the oral ectoderm and do not uniformly oralize the embryo. Expression of LvBrac is normal in the vegetal blastopore region, indicating that, unlike in the stomodaeum, *LvBrac* regulation in this region is refractory to ectopic *BMP2/4* injected at this level.

The ECM in O/A patterning

Disruption of the ECM with βAPN, a drug that prevents collagen crosslinking implicates the ECM in O/A specification or maintenance. Embryos treated with βAPN do not gastrulate and do not express the aboral-ectoderm-specific *Spec1* gene (Wessel et al., 1989). The effects of ECM disruption on LvBrac and LvTbx2/3 expression was assayed (Fig. 7I,J). Neither stomodael LvBrac (Fig. 7I) nor aboral LvTbx2/3 (Fig. 7J) were expressed following treatment with βAPN. This indicates that an intact ECM is necessary for specification and/or maintenance of gene expression in both the oral and aboral territories of the ectoderm, not solely in the aboral territory as previously thought. Normal expression of LvBrac in the vegetal blastopore region indicates that this perturbation did not affect LvBrac regulation in this region.

Functional characterization of LvTbx2/3

The results of the perturbation studies detailed above place aboral *LvTbx2/3* expression downstream of several signals and specification events that are known to be involved in the formation of the O/A axis. To determine the role of *LvTbx2/3* in the formation of this axis, ectopic mRNA expression studies were performed. Ectopic *LvTbx2/3* expression produced drastic morphological defects in derivatives of all germ layers, suggesting that LvTbx2/3 functions in each germ layer (Fig. 8). Between 60-75% of embryos that ubiquitously express *LvTbx2/3* mRNA displayed severe morphological abnormalities 24-48 hours post-fertilization (three- to fivefold overexpression obtained following injection of 0.75-1 pg/pl of mRNA amounting to 600-1000 copies of *LvTbx2/3* mRNA per cell).

By 24 hours, control embryos injected with glycerol had formed a tripartite gut, characteristic skeletal structures and the embryonic shape appropriate for these stages of development (Fig. 8A,B). Embryos that expressed *LvTbx2/3* ectopically were often delayed in gastrulation but did invaginate endoderm and gastrulate normally several hours after controls (data not shown). At 24 hours post-injection, embryos injected with *LvTbx2/3* mRNA lacked normal skeletal rods and had a grossly mispatterned skeleton with several spicule clusters forming around the circumference of the embryo (Fig. 8C,D). Consistent with the observation that *LvTbx2/3* is downstream of both germ-layer specification and O/A patterning events, embryos that ectopically expressed *LvTbx2/3* had endoderm, ectoderm, skeletogenic mesoderm, pigment and blastocoelar cells. This indicates that germ-layer specification was not perturbed noticeably, rather, it is likely that aspects of patterning and later morphogenesis were affected. Because ectopic expression of T-box-family members might affect the function of other T-box proteins, these results must be considered cautiously. However, the interpretation that the

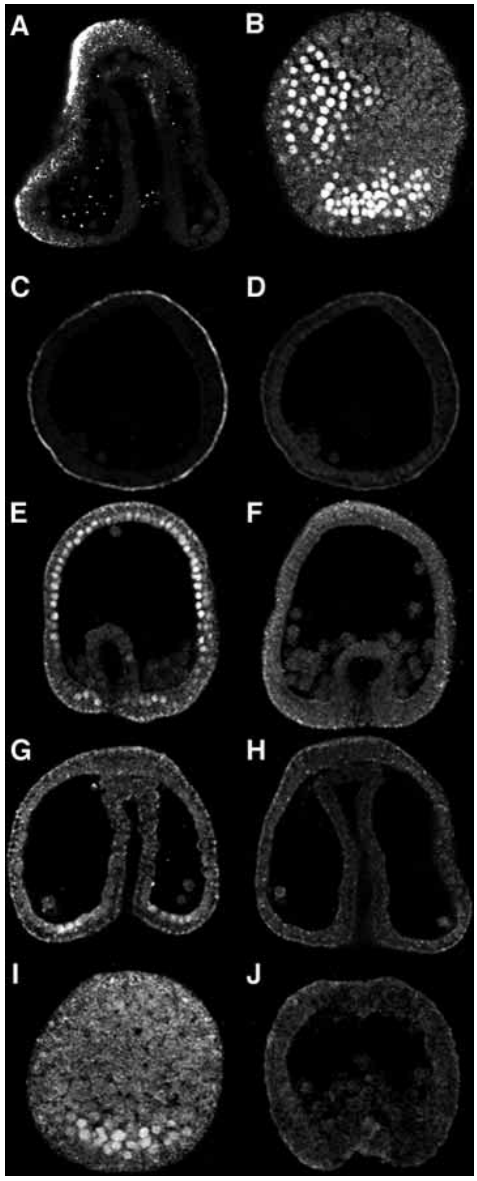

Fig. 7. Perturbation of the oral/aboral axis and the consequences for LvTbx2/3 expression. (A,B) Control embryos depicting normal expression of EctoV and LvBrac. (A) Cross-sectional view of EctoV oral ectoderm distribution in the late gastrula. (B) Mid-gastrula surface view of normal blastopore and somodael LvBrac expression. (C,D) Injection of *ΔLvG-cadherin* mRNA animalizes the embryo by binding to endogenous β-catenin and preventing its nuclear localization. These embryos lack endoderm and mesoderm and, as previously reported, express the EctoV antigen uniformly (C). They do not express aboral LvTbx2/3 (D). (E,F) $NiCl_2$ ventralization. (E) LvBrac expression expands to all ectoderm cells after ventralization with $NiCl_2$. (F) LvTbx2/3 is not expressed in any germ layer of these embryos. (G,H) Ectopic expression of *BMP2/4* radializes the ectoderm of the embryo, as indicated by the formation of multiple triradiate spicules (Angerer et al., 2000). Such embryos express normal levels of vegetal LvBrac around the blastopore but do not express oral LvBrac (G) and aboral LvTbx2/3 in the tissue of any germ layer (H), indicating that ectopic expression of *BMP2/4* antagonizes normal specification events along the A/V axis and in the O/A axis in all germ layers. (I,J) Disruption of the extracellular matrix with βAPN, a drug that prevents collagen crosslinking, results in the failure to express oral LvBrac, but vegetal expression of LvBrac is, apparently, unaffected (I). (J) βAPN also prevents LvTbx2/3 expression.

disruption is at the level of patterning and not at the level of specification appears to be conservative.

At 48 hours post-injection, the skeletons of embryos injected with *LvTbx2/3* lacked a consistent pattern, with each embryo elaborating a different, abnormal skeletal phenotype. Two such embryos are presented in Fig. 8, and it is clear that, when compared to a normal pluteus-stage embryo (Fig. 8A,B), patterning of the skeletogenic mesoderm was grossly perturbed (Fig. 8E-H). Embryos that expressed *LvTbx2/3* ectopically also had severe endodermal defects. In a few cases, exogastrulae were observed following ectopic *LvTbx2/3* expression (data not shown) but, most often, defects were manifest in an archenteron that had multiple 'chambers' rather than a typical tripartite structure. Despite their abnormal morphology embryos stained positively for the Endo1 antigen (5c7), which is normally expressed in the midgut and hindgut (Fig. 8I). Vegetal (blastopore) *LvBrac* expression in embryos that ectopically express *LvTbx2/3* was also normal, indicating that the endodermal defect was independent of LvBrac in the vegetal plate. In other words, it occurred after gastrulation (Fig. 8J).

It is well established that, in the sea urchin embryo, the skeletogenic mesoderm uses spatial and temporal patterning cues that are localized to the ectoderm to form appropriate skeletal structures (reviewed by McClay, 1999). The morphological skeletal abnormalities observed in embryos that express *LvTbx2/3* ectopically could result from inappropriate expression of either oral-specific or aboral-specific genes in the ectoderm that are induced by ectopic *LvTbx2/3* expression. Thus, downstream patterning cues would also be misexpressed or absent. Embryos were stained either 24 hours (data not shown) or 48 hours after ectopic expression of *LvTbx2/3* using antibodies against the two markers of oral ectoderm, EctoV and LvBrac (Fig. 8K,L). EctoV expression was confined to one region of the embryo, which indicates that the ectoderm contained an oral territory (Fig. 8K). LvBrac was expressed in a stomodael domain, indicating that substructures in the oral ectoderm were also specified (Fig. 8L). mAb 295 is an antibody that recognizes the ciliary band, a neurogenic region composed of both oral and aboral cells (Cameron et al., 1989). In embryos injected with *LvTbx2/3*, mAb 295 stained an amorphous region around the embryo, indicating that although oral and aboral territories have been specified and subdivided in the ectoderm, the boundary is not tightly localized (Fig. 8M).

DISCUSSION

LvTbx2/3: A T-box family transcription factor distributed asymmetrically in derivatives of all three embryonic germ layers

Here, we report the identification and characterization of a novel sea urchin T-box gene, an ortholog belonging to the *Tbx2/3* subfamily. The spatial restriction of LvTbx2/3 protein to the aboral regions of each germ layer demonstrates that there is polarized gene expression about the O/A axis in the ectoderm, endoderm and mesoderm of the sea urchin embryo. Because all three tissues share this molecular component, O/A specification in each does not involve totally unique sets of proteins. Perturbations of either molecules or pathways involved in O/A axis formation indicate that LvTbx2/3 acts

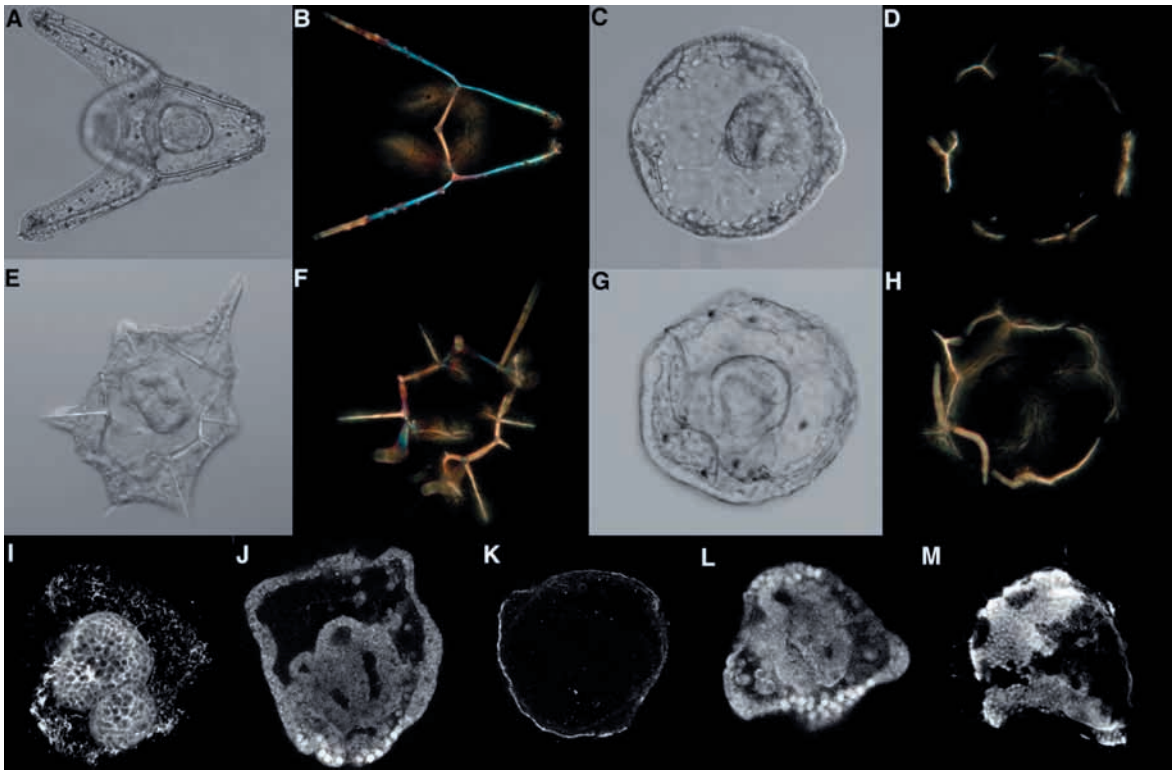

Fig. 8. Ectopic expression of *LvTbx2/3* mRNA causes profound morphological defects in embryonic development but does not prevent expression of markers of ectoderm, mesoderm and endoderm specification. Nomarski (A) and polarized light (B) images of 24 hour control, glycerol-injected embryos. These embryos exhibit the morphology, skeletal pattern and tripartite gut characteristic of the pluteus stage of development. Embryos that ectopically express *LvTbx2/3* mRNA (0.75-1.0 pg/pl; three to five times the endogenous copy number per nucleus, but in all nuclei) and imaged under Nomarski (C,E,G) or polarized light (D,F,H) optics are positioned to show a vegetal view. Twenty-four hours after injection, ectopic *LvTbx2/3*-expressing embryos appear radialized with multiple spicule clusters forming around the circumference of the embryo (D). They are, in many cases, delayed in gastrulation compared with control embryos as the archenteron has not yet reached the animal pole. The embryos contain derivatives of all germ layers, including pigment and blastocoelar cells derived from the nonskeletogenic mesenchyme, indicating that early specification events have not been eliminated. (E-H) embryos ectopically expressing *LvTbx2/3* for 48 hours. These embryos exhibit severe morphological defects in tissues derived from all three germ layers. They lack the typical pluteus form, have drastically mispatterned skeletons and have archenterons composed of multiple chambers rather than the normal three. Two embryos (F,H) display the variability in the skeletal phenotypes. No two embryos that ectopically express *LvTbx2/3* display identical defects in their skeletons, although all are severely mispatterned. When stained for terminal markers of pattern formation in these tissues, these embryos express markers for each known cell lineage. In the endoderm, the mid/hindgut marker, Endo1, is expressed and, in many cases, is localized to several of the additional chambers that have formed (I). LvBrac is normally expressed in two domains, a blastopore/hindgut domain and an oral/stomodael domain. Within the endoderm of injected embryos, LvBrac expression remains around the blastopore (J). EctoV is normally expressed in a refined domain corresponding to the oral ectoderm. In injected embryos, EctoV expression is still refined, indicating an oral axis has formed (K). (L) LvBrac is also expressed normally in the stomodael domain, indicating that substructures have been specified in the oral ectoderm and that domain is not ‘aboralized’. (M) mAb 295, which recognizes the ciliated band, a structure at the boundary between oral and aboral cells, is also expressed in these embryos. However, instead of being a tight band, in many cases the ciliary band is broadly dispersed, indicating a loss of a refined O/A boundary.

downstream of these events and may be proximal to the morphogenetic events that shape aboral structures. To our knowledge, no member of the T-box gene family, or of any transcription factor family, has been described that is distributed in such a strikingly polarized manner in the three germ layers of any organism.

O/A polarity in the sea urchin embryo

That *LvTbx2/3* is expressed in the aboral territories of the endoderm, ectoderm and mesoderm provides a point of entry to examine the regulation of gene expression along the O/A axis in each of these tissues. To this end, we perturbed several events that are thought to be involved in patterning this axis and examined the effects on the expression of LvTbx2/3 and LvBrac, markers of aboral and oral gene expression, respectively. Results of these experiments suggest that either β-catenin or the expression of genes downstream of β-catenin is necessary for the expression of both proteins and, thus, gene expression along both the A/V and the O/A axes of the sea urchin embryo. Pharmacologically blocking formation of the aboral axis with $NiCl_2$ prevented LvTbx2/3 expression in all tissues, suggesting that gene expression along this axis is uniformly sensitive to this perturbation. In addition, it is possible that gene expression of axial information is controlled by mechanisms common to each germ layer rather than through different pathways in each.

The results on protein distribution after ectopic expression of *BMP2/4* are particularly interesting because previous experiments in *Strongelocentrotus purpuratus* embryos demonstrated that oral expression of EctoV was blocked by ectopic expression of *BMP2/4* but the aboral domain of Spec1 expression increased (Angerer et al., 2000). In our study, ectopic expression of *BMP2/4* in *L. variegatus* prevented the expression of both aboral LvTbx2/3 and oral LvBrac (Fig. 7G,H). Thus, two aboral genes, *Spec1* and *LvTbx2/3* differ in their response to ectopic expression *of BMP2/4*. The ectoderm of these embryos may be a locked in some sort of pre-aboral ectoderm state in which some aboral proteins are expressed but others are not because the signals necessary for their expression are inhibited by increased concentrations of BMP2/4. The most obvious explanation is that BMP2/4 might be a component of aboral specification, but, given the different *Spec1* and *LvTbx2/3* responses to *BMP2/4* perturbation, other, aboral-specification mechanisms must also exist. Evidence for a veg1-derived signal to overlying animal tissues has been observed recently (D.R.M. and J.M.G., unpublished observations), and this signal is sensitive to ectopic BMP2/4 expression. Thus, the defects in O/A gene expression described here might result from a perturbation to this signal. Further characterization of the BMP2/4 pathway, and the identification of more markers for O/A-axis formation in the ectoderm will likely clarify this issue. It is also possible that species-specific differences in specification of the oral and aboral axes might explain this discrepancy. *S. purpuratus* embryos at least partially differentiate aboral ectoderm autonomously, whereas *L. pictus* embryos require vegetal signaling to do so (Wikramanayake et al., 1995). Therefore, the loss of *LvTbx2/3* expression in embryos of *L. variegatus* following overexpression of *BMP2/4* could reflect a slightly different role of this pathway in specifying structures along the O/A axis in *Lytechinus* species of urchins than that in *S. purpuratus*.

LvTbx2/3 patterning and morphogenesis in the sea urchin embryo

Based on perturbation studies, *LvTbx2/3* expression is downstream of the specification of endoderm, mesoderm and ectoderm, including initial O/A specification events in these tissues. When ectopically expressed, *LvTbx2/3* consistently produces abnormal morphological phenotypes and patterning deficiencies in derivatives of each tissue. Nevertheless, markers for specific germ layers and axial regions are expressed (Fig. 8). Thus, what is the role of *LvTbx2/3* in the aboral territories? Genes downstream of LvTbx2/3 may be involved directly in patterning and morphogenesis, as suggested by the skeletal and endodermal phenotypes that result from the ectopic *LvTbx2/3* expression studies presented here. Several other T-box genes have also been noted to have distinct functions during morphogenesis. These include *Brachyury* in gastrulation movements (Kimmel et al., 1989; Conlon et al., 1996; Wilson and Beddington 1997; Gross and McClay, 2001), *Eomesodermin* in the formation of bottle cells and initiation of gastrulation (Ryan et al., 1996; Russ et al., 2000), *spadetail* in paraxial mesoderm migration (Griffin et al., 1998; Yamamoto et al., 1998), and *Tbx24* in somite segmentation (Nikaido et al., 2002). It will be of great interest to refine the position of *LvTbx2/3* in a network of O/A specification when more genes are identified in this gene-regulatory network. Also, using the differential screening technologies that are available currently, it should be possible to identify downstream targets of LvTbx2/3 and determine their roles in patterning and morphogenesis along this axis.

We are indebted to Drs. Dave Sherwood (CalTech) and Cyndi Bradham for discussion of these studies and invaluable comments and criticisms on the text of this manuscript. Specifically, we are grateful to Cyndi Bradham for advice on QPCR experiments. We would also like to thank Robert E. Keen for technical support throughout the injection phase of this work. This work was supported by NIH grants HD14483 and GM64164 to D.R.M. and Department of Defense Breast Cancer Research Program grant BC990657 to J.M.G.

REFERENCES

Angerer, L. M. and Angerer, R. C. (2000). Animal-vegetal axis patterning mechanisms in the early sea urchin embryo. *Dev. Biol.* **218**, 1-12.

Angerer, L. M., Dolecki, G. J., Gagnon, M. L., Lum, R., Wang, G., Yang, Q., Humphreys, T. and Angerer, R. C. (1989). Progressively restricted expression of a homeo box gene within the aboral ectoderm of developing sea urchin embryos. *Genes Dev.* **3**, 370-383.

Angerer, L. M., Oleksyn, D. W., Levine, A. M., Li, X., Klein, W. H. and Angerer, R. C. (2001). Sea urchin goosecoid function links fate specification along the animal-vegetal and oral-aboral embryonic axes. *Development* **128**, 4393-4404.

Angerer, L. M., Oleksyn, D. W., Logan, C. Y., McClay, D. R., Dale, L. and Angerer, R. C. (2000). A BMP pathway regulates cell fate allocation along the sea urchin animal-vegetal embryonic axis. *Development* **127**, 1105-1114.

Bamshad, M., Lin, R. C., Law, D. J., Watkins, W. C., Krakowiak, P. A., Moore, M. E., Franceschini, P., Lala, R., Holmes, L. B., Gebuhr, T. C. et al. (1997). Mutations in human TBX3 alter limb, apocrine and genital development in ulnar-mammary syndrome. *Nat. Genet.* **16**, 311-315.

Basson, C. T., Bachinsky, D. R., Lin, R. C., Levi, T., Elkins, J. A., Soults, J., Grayzel, D., Kroumpouzou, E., Traill, T. A., Leblanc-Straceski, J. et al. (1997). Mutations in human TBX5 cause limb and cardiac malformation in Holt-Oram syndrome. *Nat. Genet.* **15**, 30-35.

Calzone, F. J., Hoog, C., Teplow, D. B., Cutting, A. E., Zeller, R. W., Britten, R. J. and Davidson, E. H. (1991). Gene regulatory factors of the sea urchin embryo. I. Purification by affinity chromatography and cloning of P3A2, a novel DNA-binding protein. *Development* **112**, 335-350.

Cameron, R. A., Fraser, S. E., Britten, R. J. and Davidson, E. H. (1989). The oral-aboral axis of a sea urchin embryo is specified by first cleavage. *Development* **106**, 641-647.

Church, G. M. and Gilbert, W. (1984). Genomic sequencing. *Proc. Natl. Acad. Sci. USA* **81**, 1991-1995.

Coffman, J. A. and McClay, D. R. (1990). A hyaline layer protein that becomes localized to the oral ectoderm and foregut of sea urchin embryos. *Dev. Biol.* **140**, 93-104.

Conlon, F. L., Fairclough, L., Price, B. M., Casey, E. S. and Smith, J. C. (2001). Determinants of T box protein specificity. *Development* **128**, 3749-3758.

Conlon, F. L., Sedgwick, S. G., Weston, K. M. and Smith, J. C. (1996). Inhibition of Xbra transcription activation causes defects in mesodermal patterning and reveals autoregulation of Xbra in dorsal mesoderm. *Development* **122**, 2427-2435.

Cox, K. H., Angerer, L. M., Lee, J. J., Davidson, E. H. and Angerer, R. C. (1986). Cell lineage-specific programs of expression of multiple actin genes during sea urchin embryogenesis. *J. Mol. Biol.* **188**, 159-172.

Davidson, E. H., Cameron, R. A. and Ransick, A. (1998). Specification of cell fate in the sea urchin embryo: summary and some proposed mechanisms. *Development* **125**, 3269-3290.

Di Bernardo, M., Castagnetti, S., Bellomonte, D., Oliveri, P., Melfi, R., Palla, F. and Spinelli, G. (1999). Spatially restricted expression of PlOtp, a Paracentrotus lividus orthopedia-related homeobox gene, is correlated with oral ectodermal patterning and skeletal morphogenesis in late-cleavage sea urchin embryos. *Development* **126**, 2171-2179.

Griffin, K. J., Amacher, S. L., Kimmel, C. B. and Kimelman, D. (1998). Molecular identification of spadetail: regulation of zebrafish trunk and tail mesoderm formation by T-box genes. *Development* **125**, 3379-3388.

Gross, J. M. and McClay, D. R. (2001). The role of Brachyury (T) during gastrulation movements in the sea urchin Lytechinus variegatus. *Dev. Biol.* **239**, 132-147.

Hardin, J., Coffman, J. A., Black, S. D. and McClay, D. R. (1992). Commitment along the dorsoventral axis of the sea urchin embryo is altered in response to NiCl2. *Development* **116**, 671-685.

Harlow, E. and Lane, D. (1988). Antibodies: A Laboratory Manual. Cold Spring Harbour: Cold Spring Harbour Laboratory.

He, M., Wen, L., Campbell, C. E., Wu, J. Y. and Rao, Y. (1999). Transcription repression by Xenopus ET and its human ortholog TBX3, a gene involved in ulnar-mammary syndrome. *Proc. Natl. Acad. Sci. USA* **96**, 10212-10217.

Henry, J. J., Klueg, K. M. and Raff, R. A. (1992). Evolutionary dissociation between cleavage, cell lineage and embryonic axes in sea urchin embryos. *Development* **114**, 931-938.

Horb, M. E. and Thomsen, G. H. (1997). A vegetally localized T-box transcription factor in Xenopus eggs specifies mesoderm and endoderm and is essential for embryonic mesoderm formation. *Development* **124**, 1689-1698.

Horb, M. E. and Thomsen, G. H. (1999). Tbx5 is essential for heart development. *Development* **126**, 1739-1751.

Jacobs, J. J., Keblusek, P., Robanus-Maandag, E., Kristel, P., Lingbeek, M., Nederlof, P. M., van Welsem, T., van de Vijver, M. J., Koh, E. Y., Daley, G. Q. et al. (2000). Senescence bypass screen identifies TBX2, which represses Cdkn2a (p19(ARF)) and is amplified in a subset of human breast cancers. *Nat. Genet.* **26**, 291-299.

Jerome, L. A. and Papaioannou, V. E. (2001). DiGeorge syndrome phenotype in mice mutant for the T-box gene, Tbx1. *Nat. Genet.* **27**, 286-291.

Kimmel, C. B., Kane, D. A., Walker, C., Warga, R. M. and Rothman, M. B. (1989). A mutation that changes cell movement and cell fate in the zebrafish embryo. *Nature* **337**, 358-362.

Kispert, A. and Hermann, B. G. (1993). The Brachyury gene encodes a novel DNA binding protein. *EMBO J.* **12**, 4898-4899.

Kispert, A., Koschorz, B. and Herrmann, B. G. (1995). The T protein encoded by Brachyury is a tissue-specific transcription factor. *EMBO J.* **14**, 4763-4772.

Li, Q. Y., Newbury-Ecob, R. A., Terrett, J. A., Wilson, D. I., Curtis, A. R., Yi, C. H., Gebuhr, T., Bullen, P. J., Robson, S. C., Strachan, T. et al. (1997a). Holt-Oram syndrome is caused by mutations in TBX5, a member of the Brachyury (T) gene family. *Nat. Genet.* **15**, 21-29.

Li, X., Chuang, C. K., Mao, C. A., Angerer, L. M. and Klein, W. H. (1997b). Two Otx proteins generated from multiple transcripts of a single gene in Strongylocentrotus purpuratus. *Dev. Biol.* **187**, 253-266.

Logan, C. Y., Miller, J. R., Ferkowicz, M. J. and McClay, D. R. (1999). Nuclear beta-catenin is required to specify vegetal cell fates in the sea urchin embryo. *Development* **126**, 345-357.

Lynn, D. A., Angerer, L. M., Bruskin, A. M., Klein, W. H. and Angerer, R. C. (1983). Localization of a family of mRNAS in a single cell type and its precursors in sea urchin embryos. *Proc. Natl. Acad. Sci. USA* **80**, 2656-2660.

McClay, D. R. (1999). The role of thin filopodia in motility and morphogenesis. *Exp. Cell. Res.* **253**, 296-301.

Merscher, S., Funke, B., Epstein, J. A., Heyer, J., Puech, A., Lu, M. M., Xavier, R. J., Demay, M. B., Russell, R. G., Factor, S. et al. (2001). TBX1 is responsible for cardiovascular defects in velo-cardio-facial/DiGeorge syndrome. *Cell* **104**, 619-629.

Miller, R. N., Dalamagas, D. G., Kingsley, P. D. and Ettensohn, C. A. (1996). Expression of S9 and actin CyIIa mRNAs reveals dorso-ventral polarity and mesodermal sublineages in the vegetal plate of the sea urchin embryo. *Mech. Dev.* **60**, 3-12.

Nemer, M., Stuebing, E. W., Bai, G. and Parker, H. R. (1995). Spatial regulation of SpMTA metallothionein gene expression in sea urchin embryos by a regulatory cassette in intron 1. *Mech. Dev.* **50**, 131-137.

Nikaido, M., Kawakami, A., Sawada, A., Furutani-Seiki, M., Takeda, H. and Araki, K. (2002). Tbx24, encoding a T-box protein, is mutated in the zebrafish somite-segmentation mutant fused somites. *Nat. Genet.* **31**, 195-199.

Papaioannou, V. E. and Silver, L. M. (1998). The T-box gene family. *Bioessays* **20**, 9-19.

Rast, J. P., Amore, G., Calestani, C., Livi, C. B., Ransick, A. and Davidson, E. H. (2000). Recovery of developmentally defined gene sets from high-density cDNA macroarrays. *Dev. Biol.* **228**, 270-286.

Rast, J. P., Cameron, R. A., Poustka, A. J. and Davidson, E. H. (2002). *brachyury* target genes in the early sea urchin embryo isolated by differential macroarray screening. *Dev. Biol.* **246**, 191-208.

Russ, A. P., Wattler, S., Colledge, W. H., Aparicio, S. A., Carlton, M. B., Pearce, J. J., Barton, S. C., Surani, M. A., Ryan, K., Nehls, M. C. et al. (2000). Eomesodermin is required for mouse trophoblast development and mesoderm formation. *Nature* **404**, 95-99.

Ryan, K., Garrett, N., Mitchell, A. and Gurdon, J. B. (1996). Eomesodermin, a key early gene in *Xenopus* mesoderm differentiation. *Cell* **87**, 989-1000.

Sasaki, H., Yamada, K., Akasaka, K., Kawasaki, H., Suzuki, K., Saito, A., Sato, M. and Shimada, H. (1988). cDNA cloning, nucleotide sequence and expression of the gene for arylsulfatase in the sea urchin (Hemicentrotus pulcherrimus) embryo. *Eur. J. Biochem.* **177**, 9-13.

Sherwood, D. R. and McClay, D. R. (1997). Identification and localization of a sea urchin Notch homologue: insights into vegetal plate regionalization and Notch receptor regulation. *Development* **124**, 3363-3374.

Sherwood, D. R. and McClay, D. R. (1999). LvNotch signaling mediates secondary mesenchyme specification in the sea urchin embryo. *Development* **126**, 1703-1713.

Smith, J. (1999). T-box genes: what they do and how they do it. *Trends Genet.* **15**, 154-158.

Vlahou, A., Gonzalez-Rimbau, M. and Flytzanis, C. N. (1996). Maternal mRNA encoding the orphan steroid receptor SpCOUP-TF is localized in sea urchin eggs. *Development* **122**, 521-526.

Wessel, G. M. and McClay, D. R. (1987). Gastrulation in the sea urchin embryo requires the deposition of crosslinked collagen within the extracellular matrix. *Dev. Biol.* **121**, 149-165.

Wessel, G. M., Zhang, W., Tomlinson, C. R., Lennarz, W. J. and Klein, W. H. (1989). Transcription of the Spec 1-like gene of Lytechinus is selectively inhibited in response to disruption of the extracellular matrix. *Development* **106**, 355-365.

Wikramanayake, A. H., Brandhorst, B. P. and Klein, W. H. (1995). Autonomous and non-autonomous differentiation of ectoderm in different sea urchin species. *Development* **121**, 1497-1505.

Wikramanayake, A. H., Huang, L. and Klein, W. H. (1998). beta-Catenin is essential for patterning the maternally specified animal-vegetal axis in the sea urchin embryo. *Proc. Natl. Acad. Sci. USA* **95**, 9343-9348.

Wikramanayake, A. H. and Klein, W. H. (1997). Multiple signaling events specify ectoderm and pattern the oral-aboral axis in the sea urchin embryo. *Development* **124**, 13-20.

Wilson, V. and Beddington, R. (1997). Expression of T protein in the primitive streak is necessary and sufficient for posterior mesoderm movement and somite differentiation. *Dev. Biol.* **192**, 45-58.

Yamamoto, A., Amacher, S. L., Kim, S. H., Geissert, D., Kimmel, C. B. and De Robertis, E. M. (1998). Zebrafish paraxial protocadherin is a downstream target of spadetail involved in morphogenesis of gastrula mesoderm. *Development* **125**, 3389-3397.

Yuh, C. H., Brown, C. T., Livi, C. B., Rowen, L., Clarke, P. J. and Davidson, E. H. (2002). Patchy interspecific sequence similarities efficiently identify positive cis-regulatory elements in the sea urchin. *Dev. Biol.* **246**, 148-161.