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# The expression and distribution of Wnt and Wnt receptor mRNAs during early sea urchin development

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ABSTRACT

The protein β-catenin plays a critically important role in establishing axial polarity during early animal development. In many organisms, β-catenin is degraded preferentially on one side of the cleavage stage embryo. On the opposite side of the embryo, β-catenin is stabilized and accumulates in the nucleus, where it functions in concert with members of the LEF/TCF family to activate the transcription of diverse target genes. Genes that are activated by β-catenin play an essential role in the specification of endomesoderm and in the establishment of key signaling centers in the early embryo. In several organisms, the asymmetric distribution of maternal components of the canonical Wnt pathway has been shown to be responsible for the polarized stabilization of β-catenin. In this study, we identified all Wnt and Wnt receptor mRNAs that are present in unfertilized sea urchin eggs and early embryos and analyzed their distributions along the primary (AV) axis. Our findings indicate that the asymmetric distribution of a maternal Wnt or Wnt receptor mRNA is unlikely to be a primary determinant of the polarized stabilization of β-catenin along the AV axis. This contrasts sharply with findings in other organisms and points to remarkable evolutionary flexibility in the molecular mechanisms that underlie this otherwise very highly conserved patterning process.



## 1. Results and discussion

The protein β-catenin plays an essential role in axis specification (reviewed by Ettensohn, 2006; Heasman, 2006; Momose et al., 2008). This developmental function of β-catenin is likely to be very ancient, as it operates in phylogenetically diverse metaozoans, including anthozoan and hydrozoan cnidarians (Lee et al., 2007; Momose and Houliston, 2007; Momose et al., 2008; Wikramanayake et al., 2003), the lophotrochozoan *Cerebratulus lacteus* (Henry et al., 2008), echinoderms (Emily-Fenouil et al., 1998; Wikramanayake et al., 1998; Logan et al., 1999; Miyawaki et al., 2003), ascidians (Imai et al., 2000), and several vertebrates (Kelly et al., 2000; Roeser et al., 1999; Schneider et al., 1996). Despite the critical importance of the polarized degradation of β-catenin and the remarkable evolutionary conservation of this general patterning process, the molecular mechanisms of the asymmetry remain poorly understood, and the extent to which these mechanisms are conserved across various metazoan taxa is unknown. Although it is widely accepted that the asymmetric distribution of maternal components of the canonical Wnt pathway underlies the polarized degradation of β-catenin, few common features have emerged from the experimental models that have been studied most intensively. In *Xenopus*, a vegetally-localized, maternal Wnt mRNA (*wnt11* mRNA) is the primary determinant of axial polarity (Tao et al., 2005) and there is no evidence that Wnt receptors are expressed asymmetrically in the egg. In cnidarians, in contrast, the asymmetric localization of maternal frizzled receptor mRNAs is thought to be critical in the establishment of polarity (Momose and Houliston, 2007; Momose et al., 2008). A maternal Wnt (*wnt3*) mRNA also exhibits an asymmetric distribution along the animal-vegetal (AV) egg axis, but this does not appear to be a primary determinant of axial polarity. In sea urchins, the spatial distributions of maternal Wnt and Wnt receptor mRNAs have not been analyzed systematically. A different Wnt signaling molecule, Dishevelled protein, is localized in the vegetal cortex of the unfertilized egg and is required to stabilize β-catenin in that region, although the localization of Dsh does not appear to be sufficient to fully account for the observed asymmetry in β-catenin degradation (Weitzel et al., 2004; Leonard and Ettensohn, 2007; Kumburegama and Wikramanayake, 2008). In this study, we identified all Wnt and Wnt receptor mRNAs that are present in unfertilized sea urchin eggs and early embryos and analyzed their distributions along the primary (AV) axis.

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The assembly and annotation of the complete genome of the purple sea urchin, *Strongylocentrotus purpuratus* (Sea Urchin Genome Sequencing Consortium, 2006) facilitated the identification of canonical Wnt signaling genes in the sea urchin (Croce et al., 2006a). There are 11 different Wnt genes in the *S. purpuratus* genome (*Sp-wntA*, *Sp-wnt1*, *Sp-wnt3*, *Sp-wnt4*, *Sp-wnt5*, *Sp-wnt6*, *Sp-wnt7*, *Sp-wnt8*, *Sp-wnt9*, *Sp-wnt10*, and *Sp-wnt16*). Significantly, *S. purpuratus* lacks an orthologue of *wnt11*, which plays a pivotal role in the polarized stabilization of β-catenin in *Xenopus* (Tao et al., 2005). There are four frizzled receptor genes in *S. purpuratus* (*Sp-fz*, *Sp-fz4*, *Sp-fz5/8*, and *Sp-fz9/10*) and a single orthologue of the Wnt co-receptor *arrow*/*lrp6* (*Sp-lrp6*).

We first isolated total RNA from the unfertilized eggs of three different female sea urchins and used conventional reverse transcription-polymerase chain reaction (RT-PCR) to assess the maternal expression of each of the 16 Wnt and Wnt receptor genes (Fig. 1 and Table 1). We reliably detected maternal mRNAs corresponding to seven different Wnt genes (*Sp-wntA*, *Sp-wnt3*, *Sp-wnt4*, *Sp-wnt5*, *Sp-wnt7*, *Sp-wnt8*, and *Sp-wnt16*). *Sp-wnt16* was clearly the most abundant of these maternal Wnt mRNAs. It consistently provided the most robust signal in our qualitative RT-PCR studies (Fig. 1A) and was the only *wnt* mRNA detected in unfertilized eggs in a genome-wide analysis of temporal patterns of gene expression in the sea urchin (Wei et al., 2006). Based on Northern blot analyses and RNase protection assays, it was previously concluded that transcripts encoded by *Sp-wnt5*, and *Sp-wnt8* were not expressed maternally (Ferkowicz et al., 1998; Wikramanayake et al., 2004). The greater sensitivity of PCR analysis, however, allowed us to detect maternal transcripts encoding these and other Wnts in unfertilized eggs. Two Wnt mRNAs, *Sp-wnt1* and *Sp-wnt6*, yielded variable results; these mRNAs were detected in some, but not all, RNA preparations by RT-PCR. We do not know the source of this variability, but repeated trials with the same RNA samples yielded consistent results, suggesting that this variability reflected inherent differences among the three different females. It is important to note that in subsequent QPCR studies (see below and Table 2), which were carried out using RNA isolated from a different set of three female sea urchins, the levels of *Sp-wnt1* and *Sp-wnt6* transcripts were extremely low (<2 transcripts/egg) all cases. Together, our findings indicate that in the eggs of most (and probably all) females, *Sp-wnt1* and *Sp-wnt6* mRNAs are present at extremely low levels, but in a small minority of females the numbers of these transcripts are sufficiently high to be detected by qualitative RT-PCR, which is an exceptionally sensitive technique.

Transcripts corresponding to each of the five Wnt receptor genes were detected in all three egg mRNA preparations (Fig. 1B

**Table 1**
RT-PCR analysis of the maternal expression of Wnt and Wnt receptor mRNAs. Unfertilized eggs were collected from three different female sea urchins and total RNA was extracted. For each RNA preparation, the presence (+) or absence (–) of each of the 16 Wnt and Wnt receptor mRNAs was analyzed by RT-PCR.

| mRNA | Egg RNA preparation | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| *Sp-wntA* | + | + | + |
| *Sp-wnt1* | + | – | – |
| *Sp-wnt3* | + | + | + |
| *Sp-wnt4* | nd | + | + |
| *Sp-wnt5* | + | + | + |
| *Sp-wnt6* | + | – | – |
| *Sp-wnt7* | + | + | + |
| *Sp-wnt8* | + | + | + |
| *Sp-wnt9* | – | – | – |
| *Sp-wnt10* | – | – | – |
| *Sp-wnt16* | + | + | + |
| *Sp-fz* | + | + | + |
| *Sp-fz4* | + | + | + |
| *Sp-fz5/8* | + | + | + |
| *Sp-fz9/10* | + | + | + |
| *Sp-lrp6* | + | + | + |

**Table 2**
Numbers of Wnt and Wnt receptor transcripts in unfertilized eggs, as determined by QPCR. Each QPCR reaction was run in triplicate and the average of the three $C_t$ values for each gene was compared with the average $C_t$ value for *Sp-z12*. To calculate transcript numbers, we used the difference in $C_t$ value between the test gene and *Sp-z12* and assumed an amplification efficiency of 1.9. We used a value of 2300 for the number of Sp-z12 transcripts in the egg (Wang et al., 1995). Values shown are the averages (and standard deviations) obtained from such measurements made on three separate batches of eggs obtained from different adult females. mRNAs are listed in order of relative abundance, from most abundant (top) to least abundant (bottom).

| mRNA | No. transcripts/egg |
|---|---|
| *Sp-lrp6* | 1338 (290) |
| *Sp-fz5/8* | 911 (140) |
| *Sp-fz* | 882 (296) |
| *Sp-wnt16* | 413 (202) |
| *Sp-fz9/10* | 32 (32) |
| *Sp-fz4* | 17 (10) |
| *Sp-wnt5* | 8 (5) |
| *Sp-wntA* | 4 (5) |
| *Sp-wnt1* | <2 (<1) |
| *Sp-wnt3* | <2 (<1) |
| *Sp-wnt4* | <2 (<1) |
| *Sp-wnt6* | <2 (<1) |
| *Sp-wnt7* | <2 (<1) |
| *Sp-wnt8* | <2 (<1) |

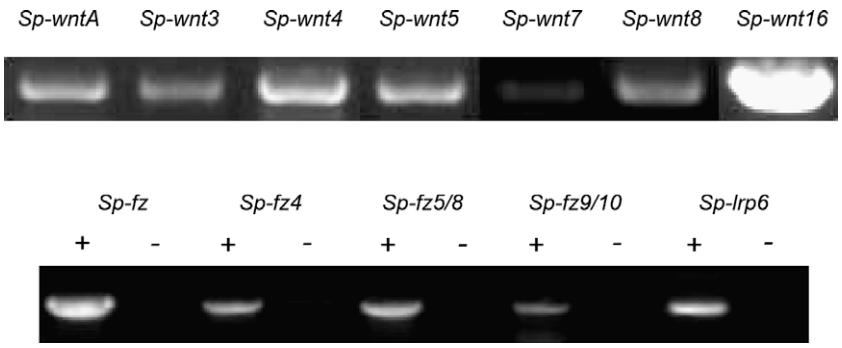


**Fig. 1.** RT-PCR analysis of Wnt and Wnt receptor mRNA expression in unfertilized eggs. We reliably detected maternal mRNAs corresponding to seven different Wnt ligands (*Sp-wntA*, *Sp-wnt3*, *Sp-wnt4*, *Sp-wnt5*, *Sp-wnt7*, *Sp-wnt8*, and *Sp-wnt16*) and all five Wnt receptors (*Sp-lrp6*, *Sp-fz*, *Sp-fz4*, *Sp-fz5/8*, and *Sp-fz9/10*). Primers for all Wnt ligands spanned large introns. In the case of Wnt receptors, samples with and without reverse transcriptase are shown. Although all Wnt ligand PCR products were aligned for this figure, each product was of a unique molecular weight. The predicted sizes of the four frizzled receptor PCR products were nearly equivalent; therefore, each of the bands shown was cloned and sequenced to verify that it corresponded to the appropriate protein.

and Table 1). Of these maternal Wnt receptor mRNAs, *Sp-fz*, *Sp-fz5/8*, and *Sp-lrp6* mRNAs appeared to be the most abundant in unfertilized eggs. This is consistent with a genome-wide analysis of temporal patterns of gene expression in *S. purpuratus*, which detected these same three frizzled receptor mRNAs, but not *Sp-fz4* or *Sp-fz9/10* mRNAs, in unfertilized eggs (Wei et al., 2006). Because the expected sizes of the four frizzled PCR products were almost identical, we cloned and sequencing the relevant PCR products shown in Fig. 1. These studies confirmed that each of the four products corresponded to the expected mRNA.

We used quantitative PCR (QPCR) to determine the numbers of Wnt ligand and Wnt receptor transcripts in unfertilized eggs (Table 2). To calculate numbers of Wnt and Wnt receptor transcripts, we compared $C_t$ values for the various Wnt and Wnt receptor mRNAs to those obtained for *z12*, an mRNA encoding a zinc-finger transcription factor, the abundance of which has been quantified in the egg and early embryo by independent methods (Wang et al., 1995). These QPCR studies confirmed that the most abundant maternal transcripts were those encoded by *Sp-lrp6*, *Sp-fz5/8*, *Sp-fz* and *Sp-wnt16* (>400 transcripts/egg). *Sp-fz9/10*, *Sp-fz4* and *Sp-wnt5* transcripts were present at intermediate levels (~10–30 transcripts/egg), while several Wnt and Wnt receptor mRNAs (*Sp-wnt1*, *Sp-wnt3*, *Sp-wnt4*, *Sp-wnt6*, *Sp-wnt7*, *Sp-wnt8*) were extremely rare in unfertilized eggs (<2 transcripts/egg). With respect to this latter class of very rare transcripts, in our qualitative RT-PCR analysis (Fig. 1), we used intron-spanning primers to detect PCR products that corresponded to some of these mRNAs (*Sp-wnt3*, *Sp-wnt4*, *Sp-wnt7*, and *Sp-wnt8*). This supports the notion that at least some members of this class mRNAs are present in unfertilized eggs, albeit at extremely low levels. Nevertheless, it seems unlikely that the polarized degradation of β-catenin is regulated by members of this very rare class of mRNAs, which, even if present, are expressed at levels that correspond to <1 transcript/micromere. It is important to note that our QPCR studies were carried out on mature eggs, and it is possible that some Wnt or Wnt receptor mRNAs are expressed at higher levels earlier in oogenesis.

We also used QPCR to determine the numbers of Wnt and Wnt receptor transcripts at the 16-cell stage, when the asymmetry in β-catenin nuclear localization first becomes apparent (Logan et al., 1999). All of the Wnt and Wnt receptor mRNAs were found to be present at levels that were equal to or slightly lower than their levels in the unfertilized egg, with the exception of *Sp-wnt8* mRNA, which showed a ~30-fold increase in abundance at the 16-cell stage. This increase in *Sp-wnt8* transcripts reflects zygotic expression of the *Sp-wnt8* gene, which begins at the 16-cell stage (Wikramanayake et al., 2004). We found no evidence of early zygotic activation of any of the other Wnt or Wnt receptor genes. Our QPCR analysis measured only steady-state levels of transcripts, however, and therefore it remains possible that a low level of zygotic synthesis of a particular mRNA might be balanced by the degradation of maternal transcripts.

The spatial distributions of the most abundant, maternal Wnt mRNA (*Sp-wnt16*) and two of the most abundant Wnt receptor mRNAs (*Sp-fz*, and *Sp-lrp6*) were examined by WMISH. The expression of *fz5/8* in eggs and early embryos was examined previously by WMISH and no asymmetry in localization was observed (Croce et al., 2006b). Our estimates of the abundance of the remaining Wnt ligand and Wnt receptor transcripts (Table 2) indicated that these other mRNAs were almost certainly too rare to be detected by WMISH. Two developmental stages were examined: fertilized eggs (<5 min post-fertilization) and 16-cell stage embryos. We tested several fixation conditions (both formaldehyde and glutaraldehyde fixation) and eggs/embryos from several different females. We consistently found that *Sp-wnt16*, *Sp-lrp6*, and *Sp-fz* transcripts were distributed uniformly at both developmental stages examined (Fig. 2). As a control for non-specific staining of eggs and early embryos, we used an antisense RNA probe complementary to *Sp-P19*, a PMC-specific transcript that is expressed only zygotically (Illies et al., 2002). As expected, we observed almost no signal with this probe (Fig. 2).

Four Wnt and Wnt receptor mRNAs (*Sp-fz9/10*, *Sp-fz4*, *Sp-wnt5*, and *Sp-wntA*) were present at the 16-cell stage at levels that were >2 transcripts/embryo, but were too rare to be detected by WMISH. As an alternative means of assessing the possible axial polarity of these mRNAs, we isolated micromeres and (mesomeres + macromeres) from a single batch of 16-cell stage embryos (Wilt and Benson, 2004) and used QPCR to compare the relative levels of expression of transcripts in the two cell populations (Fig. 3). We

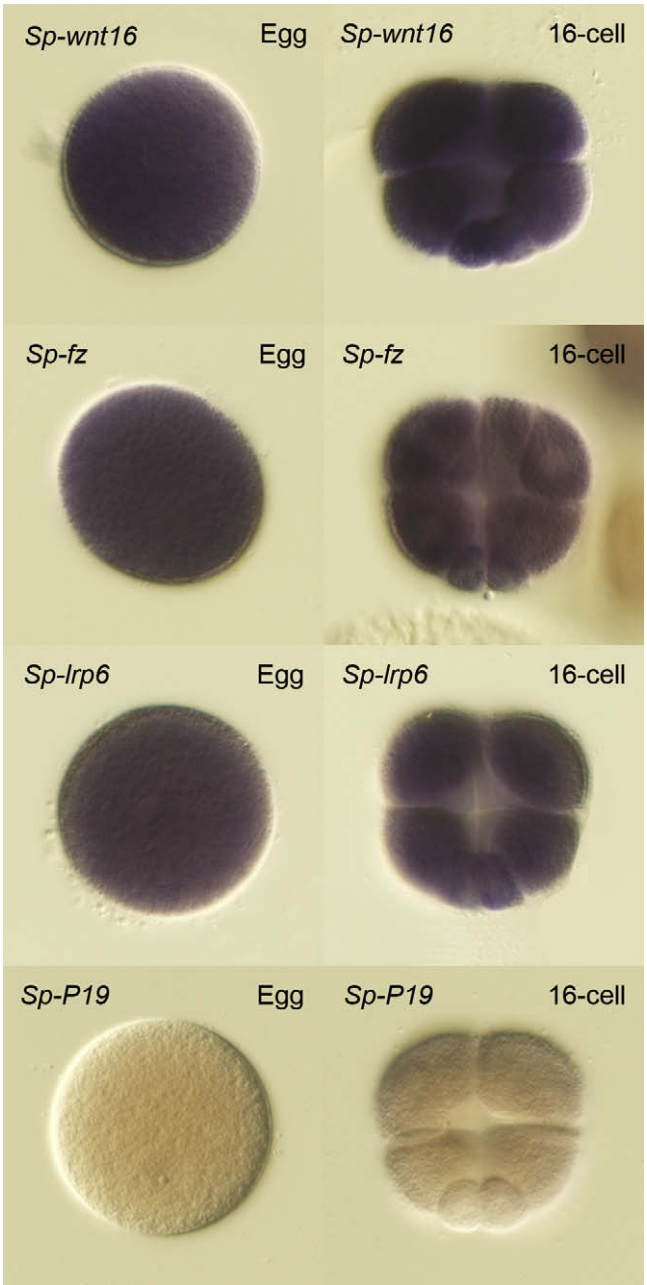


**Fig. 2.** Whole-mount in situ hybridization analysis of the most abundant maternal Wnt and Wnt receptor mRNAs (*Sp-lrp6*, *Sp-wnt16*, and *Sp-fz*) in fertilized eggs and at the 16-cell stage, when the AV asymmetry in β-catenin levels is first apparent. As a control, we used a probe complementary to *Sp-P19*, a mRNA that is not expressed until the blastula stage. *Sp-lrp6*, *Sp-wnt16*, and *Sp-fz* transcripts were distributed uniformly along the AV axis at both developmental stages examined. Note that the spatial expression of *fz5/8* was reported previously (Croce et al., 2006a).

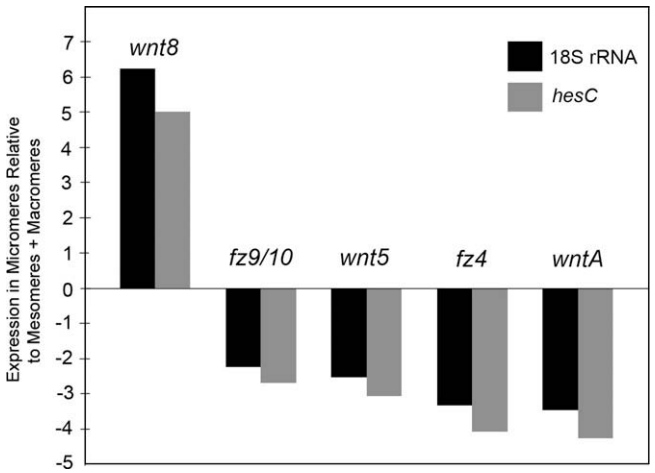


**Fig. 3.** Relative levels of expression of selected Wnt and Wnt receptor mRNAs in micromeres and (mesomeres + macromeres) at the 16-cell stage. Levels of input cDNA in the two samples were standardized by comparing the levels of expression of two different ubiquitous mRNAs, 18S rRNA (black bars) and *Sp-hesC* (gray bars). These two reference mRNAs yielded very similar results. Values on the *y*-axis indicate relative levels of expression, shown as fold-differences. Positive values indicate that a particular mRNA was enriched in micromeres (observed for *Sp-wnt8* only), while negative values indicate enrichment in (mesomeres + macromeres). For example, a value of −2 on the *y*-axis indicates that a given mRNA was twice as abundant in (mesomeres + macromeres) as in micromeres.

used two different reference mRNAs, 18S rRNA and *Sp-hesC*, to correct for variations in the amount of starting material in the PCR reactions. We made the working assumption that 18S rRNA transcripts are distributed uniformly along the AV axis. *Sp-hes*C transcripts are present at equivalent levels in all blastomeres of the 16-cell embryo, as shown by quantitative, fluorescent WMISH (Sharma and Ettensohn, unpublished observations). As a control for our cell isolation and QPCR methods, we included *Sp-wnt8*, which is expressed selectively in micromeres at the 16-cell stage. The zygotic activation of *Sp-wnt8* in the micromeres is a consequence, rather than a cause, of the vegetal accumulation of β-catenin (Wikramanayake et al., 2004; Minokawa et al., 2005). As shown in Fig. 3, our analysis confirmed that *Sp-wnt8* transcripts are enriched in micromeres. In contrast, the four other Wnt and Wnt receptor mRNAs that we analyzed (*Sp-fz9/10*, *Sp-fz4*, *Sp-wnt5*, and *Sp-wntA*) all showed a low level of enrichment in (mesomeres + macromeres). Results obtained using the two different reference transcripts, 18S rRNA and *Sp-hesC*, were in close agreement with one another (Fig. 3).

Our analysis indicates that the asymmetric distribution of a maternal Wnt or Wnt receptor mRNA is unlikely to be a primary determinant of the polarized stabilization of β-catenin along the AV axis of the sea urchin embryo. This contrasts sharply with findings in *Xenopus* (Tao et al., 2005) and cnidarians (Momose and Houliston, 2007; Momose et al., 2008) and points to remarkable evolutionary flexibility in the molecular mechanisms that underlie this otherwise very highly conserved patterning process. Our data also argue that polarized, zygotic transcription of Wnt or Wnt receptor genes along the AV axis is unlikely to underlie the earliest asymmetry in β-catenin degradation. Only *Sp-wnt8* shows a polarized expression along the AV axis at the 16-cell stage, when the asymmetry in β-catenin nuclear localization first becomes evident, and a variety of evidence argues against the notion that *wnt8* is upstream of the initial asymmetry in β-catenin degradation (Wikramanayake et al., 2004; Minokawa et al., 2005). It remains possible that post-transcriptional mechanisms polarize the expression of Wnt ligands or receptors along the AV axis, or that other components of the pathway that function downstream of the ligand–receptor interaction are polarized along the axis. One such regulator, Dishevelled (Dsh), has already been identified in the sea urchin (Weitzel et al., 2004), but other polarized components may act in concert with Dsh.

## 2. Experimental procedures

### 2.1. Animals

Adult *S. purpuratus* were obtained from Pat Leahy (Kerchoff Marine Laboratory, Caltech). Gametes were obtained by intracoelomic injection of 0.5 M KCl and embryos were maintained at 15 °C.

### 2.2. Isolation of animal and vegetal blastomeres

Micromeres and (mesomeres + macromeres) were isolated from 16-cell stage embryos as described by Wilt and Benson (2004), with minor modifications. Briefly, *S. purpuratus* eggs were fertilized in 1.6 mg/ml para-aminobenzoic acid (PABA) in seawater (SW) and rinsed several times with a 2:1 mixture of calcium-free SW (CFSW): normal SW. The embryos were allowed to develop in 2:1 CFSW:SW until the 4-cell stage, when they were collected by gentle centrifugation and resuspended in complete CFSW. At the 16-cell stage, the embryos were passed through Nitex cloth (53 μm pore size) to remove the fertilization envelope and were suspended in calcium/magnesium-free seawater (CMFSW) for ∼15 min on ice. The embryos were pelleted gently in a clinical centrifuge and 0.5 ml embryos was resuspended in 10 ml ice-cold CFSW. The embryos were dissociated into single blastomeres by pipetting and the resulting cell suspension was layered onto a 5–25% continuous sucrose gradient. The cells were allowed to settle for ∼30 min at 4 °C, and micromeres and (mesomeres + macromeres) were removed using a Pasteur pipette. Inspection of the two cell fractions by light microscopy indicated that the both fractions were >95% pure. The isolated blastomeres were transferred to 15 ml conical glass centrifuge tubes and pelleted in a clinical centrifuge.

### 2.3. Polymerase chain reaction (PCR)

Total RNA was isolated from unfertilized eggs, 16-cell stage embryos, and isolated blastomeres using the Nucleospin RNA II kit (Clontech). Conventional reverse transcriptase-PCR (RT-PCR) was carried out using the RETROscript kit (Ambion) and HiFi Taq polymerase (Invitrogen). PCR reactions were typically carried out for 40 cycles with the following cycle parameters: 94 °C for 3 min (cycle 1 only), 94 °C (30 s), 52–58 °C (1 min), and 72 °C (2 min), with a 5 min extension at 72 °C following the final cycle. Conventional PCR primers for all Wnt ligands were designed to span at least one large intron. Frizzled receptor genes lack introns, and therefore RT-PCR were carried out in the presence or absence of reverse transcriptase to control for possible amplification of contaminating genomic DNA. We did not observe PCR amplification from genomic DNA in any of our RT-PCR studies. The predicted sizes of the four frizzled receptor RT-PCR products were almost identical; we therefore cloned and sequenced all four PCR products and confirmed that each corresponded to the expected frizzled protein. Each of the LRP6 and Wnt ligand PCR products corresponded to a unique, expected size.

Quantitative PCR (QPCR) was performed using an ABI 7300 real-time PCR system and SYBR-Green/ROX master mix (Bio-Rad). Three pairs of primers that were complementary to mRNA coding sequences were designed using the Primer3Plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Primers were synthesized by Integrated DNA Technologies (Coralville, IA). One primer pair for each gene was selected for QPCR experiments based on the following criteria: (1) the primer pair yielded a single, abundant product of the expected size on

an agarose gel when used to amplify cDNA from unfertilized eggs; (2) the primer pair yielded a single PCR product as assessed by an automated dissociation curve of the QPCR reaction products. QPCR primers were:

| | |
|---|---|
| *Sp-wntA* | Forward- CTCGTGGAACTCACCAGAGA |
| | Reverse- CTTGGGTCACACTTGGAATG |
| *Sp-wnt1* | Forward- ACGAAGCAGGCAGAAAAACT |
| | Reverse- GTCGAAACGCTCTTTGATGA |
| *Sp-wnt3* | Forward- GACGCAAGGCAGAGAGTGTA |
| | Reverse- ACTTTGCAACACCAGACGAA |
| *Sp-wnt4* | Forward- GAACAGTGCAGATGGGTTTG |
| | Reverse- GCTTCGTTGTTGTGCAAGTT |
| *Sp-wnt5* | Forward- AACCCAGAGCTGTTCATCCT |
| | Reverse- ACTCGTCGATGCTCATCTTG |
| *Sp-wnt6* | Forward- AGCGAAGGAGCGATATAGGA |
| | Reverse- GCCATGGCATTTACATTCAC |
| *Sp-wnt7* | Forward- ACTCGGAGCCAACGTAATCT |
| | Reverse- CCTTCTCCAATGGCCACTAT |
| *Sp-wnt8* | Forward- TGGGTGAAGCAGAGCTGTAG |
| | Reverse- ACCTGACAACACCAAACGAA |
| *Sp-wnt16* | Forward-CTTACCGAATGTGGATGTGC |
| | Reverse-TATCGCCAGAGTCGACAAAC |
| *Sp-fz* | Forward-CCGGGTCCAGACTATCTTGT |
| | Reverse-AAAACTGGGTCCACGATCTC |
| *Sp-fz4* | Forward- CAAGCGGAATGTGGATAATG |
| | Reverse- TTCTGTCACGTCCGTTCTTC |
| *Sp-fz5/8* | Forward- TGTACCGATGTGTCGTGATG |
| | Reverse- CCGGAGAACATTGGATCTCT |
| *Sp-fz9/10* | Forward- CAACGTGGAGCCTTATCCTT |
| | Reverse- GCAATGTCCCTGTGTGTTTC |
| *Sp-lrp6* | Forward- GGACCTCCCGTAAGATGCTA |
| | Reverse- GGTTTGCACGCTCAATCTTA |

Transcript abundances were calculated relative to *Sp-z12* mRNA, which is present at 2300 and 2100 copies/embryo, respectively, at the 1-cell and 16-cell stages (Wang et al., 1995). The abundance of each mRNA relative to *Sp-z12* was determined based upon differences in their $C_t$ values and assuming an amplification efficiency of 1.9. All samples were run in triplicate.

### 2.4. Whole-mount in situ hybridization (WMISH)

WMISH using digoxigenin-labeled probes was carried out as described previously (Zhu et al., 2001). Eggs were fertilized in 1.6 mg/ml PABA in SW and then washed into normal SW after ~5 min. Fertilization envelopes were removed prior to fixation by passing the zygotes through Nitex cloth, as described above. Fixation was carried out overnight at 4 °C in a mixture of 1 part formalin (37% formaldehyde solution) + 7 parts SW, or for 2–4 h at 4 °C in 2.5% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.4) with 0.14 M NaCl added. Probes were tested at a range of concentrations from 0.1 to 1 ng/ml.

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