

Frizzled5/8 is required in secondary mesenchyme cells to initiate archenteron invagination during sea urchin development

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Wnt signaling pathways play key roles in numerous developmental processes both in vertebrates and invertebrates. Their signals are transduced by Frizzled proteins, the cognate receptors of the Wnt ligands. This study focuses on the role of a member of the Frizzled family, Fz5/8, during sea urchin embryogenesis. During development, Fz5/8 displays restricted expression, beginning at the 60-cell stage in the animal domain and then from mesenchyme blastula stage, in both the animal domain and a subset of secondary mesenchyme cells (SMCs). Loss-of-function analyses in whole embryos and chimeras reveal that Fz5/8 is not involved in the specification of the main embryonic territories. Rather, it appears to be required in SMCs for primary invagination of the archenteron, maintenance of endodermal marker expression and apical localization of Notch receptors in endodermal cells. Furthermore, among the three known Wnt pathways, Fz5/8 appears to signal via the planar cell polarity pathway. Taken together, the results suggest that Fz5/8 plays a crucial role specifically in SMCs to control primary invagination during sea urchin gastrulation.

Key words: Sea urchin, Frizzled, Wnt, PCP pathway, Notch signaling, Gastrulation, Primary invagination

INTRODUCTION

During embryogenesis, gastrulation is the crucial step when the three germ layers become organized through morphogenetic movements that require changes in cell polarity, motility and adhesion. In sea urchin, gastrulation begins at the vegetal pole with the ingression of primary mesenchyme cells (PMC), followed by the development of the archenteron through three successive phases. First, the vegetal plate, which is composed of specified endodermal cells and secondary mesenchyme cells (SMCs), bends inwards to form a primitive archenteron (primary invagination). Next, this short archenteron extends by rearrangements of endodermal cells that display convergent-extension movements (primary elongation). Finally, the archenteron is pulled to the stomodeal area by traction exerted by the SMCs at the tip of the archenteron (secondary elongation). It is widely accepted that the SMCs are the trigger of primary invagination (for a review, see Kominami and Takata, 2004); however, information about the molecular mechanisms that control archenteron invagination and elongation in sea urchin embryos is limited. Nevertheless, in other organisms, Wnt pathways have been shown to play central roles in gastrulation movements, suggesting that Wnt signaling might also be important in that process in sea urchin embryos.

Wnt ligands signal by binding to Frizzled receptors. Frizzled proteins belong to a family of seven-pass cell-surface receptors that are generally coupled with G proteins (Wang et al., 1996; Wang and Malbon, 2004). Frizzled genes were first identified in *Drosophila* as regulators of tissue polarity (Adler, 1992), before being recognized as Wnt receptors (Bhanot et al., 1996). Subsequently, at least 20 Frizzled genes have been found in metazoans (Adell et al., 2003;

Wang et al., 1996). Together with their Wnt ligands, Frizzled receptors regulate diverse cellular processes, ranging from cell fate decisions and control of proliferation to cytoskeletal rearrangements, cell adhesion, planar polarity and apoptosis (Huang and Klein, 2004).

Upon activation, Frizzled receptors can signal via three distinct signaling pathways: the canonical (or β -catenin) pathway, the Wnt/ Ca^{2+} (Wnt/ Ca^{2+}) pathway and the planar cell polarity (PCP) pathway. Of these, the canonical pathway is currently the best understood. This pathway includes β -catenin as a key intermediate and frequently controls cell fate specification in both vertebrates and invertebrates (Cadigan, 2002; Giles et al., 2003). Although less well-characterized, the non-canonical pathways appear clearly to be playing important roles in cell polarity and migration, rather than modulating cell fate decisions (Torres et al., 1996). Furthermore, these pathways signal in a β -catenin-independent manner, even though they use Dishevelled (Kühl et al., 2000b; Sheldahl et al., 2003), and the pathways frequently antagonize the β -catenin pathway (for a review, see Weidinger and Moon, 2003).

The Wnt/ Ca^{2+} pathway acts by means of a G protein to stimulate intracellular Ca^{2+} release and activate Ca^{2+} -dependent enzymes, including protein kinase C (PKC) and calmodulin-dependent kinase II (CAMKII) (Sheldahl et al., 2003). This pathway has been shown among other roles to function during dorsoventral patterning and to regulate cell migration during gastrulation and heart development (Kühl et al., 2000a; Kühl et al., 2000b; Kühl et al., 2001; Pandur et al., 2002). The PCP pathway functions through small GTPases, including RhoA and Rac, that activate effectors such as Rho-associated-kinase (ROCK) and Jun-N-terminal-kinase (JNK). It was first identified in *Drosophila* for its role in planar polarity determination (Shulman et al., 1998) but is now also recognized in vertebrates as a regulator of convergent-extension movements during gastrulation (Heisenberg et al., 2000; Kilian et al., 2003; Wallingford et al., 2000; Wallingford et al., 2001).

In sea urchin, little is known about the non-canonical pathways, whereas several components of the canonical pathway have been characterized. Functional analyses have demonstrated that the

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canonical pathway controls cell fate determination along the animal-vegetal axis (Emily-Fenouil et al., 1998; Logan et al., 1999; Range et al., 2005; Vonica et al., 2000; Weitzel et al., 2004). Furthermore, it appears that this pathway acts early and autonomously, independently of an upstream Wnt ligand or Frizzled receptor (Logan et al., 1999).

Four sea urchin Wnts have been reported to date. Wnt1, Wnt4 and Wnt5 have been identified in several species, though their functional role was not investigated (Ferkowicz et al., 1998). Wnt8 is expressed in the vegetal pole area of the early embryo and is required for the progression of endomesoderm specification downstream of the maternally nuclear β -catenin signaling (Wikramanayake et al., 2004). Two proteins related to the Frizzled family – a secreted-Frizzled related protein (Illies et al., 2002) and a partial Frizzled-like protein (Ransick et al., 2002) – have been described, although no functional data for these proteins has been obtained thus far.

In this study, we report the characterization of a sea urchin Frizzled receptor Fz5/8. Functional analyses show that Fz5/8 is required in the SMCs to control primary invagination of the archenteron via signaling through the PCP pathway.

MATERIALS AND METHODS

Animals, cultures and treatments

Paracentrotus lividus was collected in the bay of Villefranche-sur-Mer (France). Embryos were cultured in Millipore filtered sea water at 18°C. *Lytechinus variegatus* animals were obtained from the Duke University Marine Laboratory (Beaufort, NC) and Sea Life (Tavernier, FL). Embryos were cultured in artificial sea water at 23°C. Treatments were performed on *P. lividus* embryos with LiCl or ZnCl₂, as described by Ghigliione et al. (Ghigliione et al., 1993) or with Y27632, JNK Inhibitor I (L)-Form or Bisindolylmaleimide I (Calbiochem) diluted from stocks in H₂O or DMSO and used at 75 μ M, 50 μ M or 3 μ M, respectively.

Cloning of Fz5/8

A 2 kb fragment encoding a Frizzled receptor protein was isolated during a random screen by whole-mount in situ hybridization on a cDNA library from *P. lividus* 60-cell stage embryos. This fragment was used to probe a gastrula stage cDNA library. The longest clone isolated was identified as a 5199 bp cDNA encoding an ORF of 1668 bp.

Expression analysis

Northern blot analysis was performed as described by Croce et al. (Croce et al., 2003). The probe was the Fz5/8 full-length cDNA labeled with ³²P by random priming using the Prime-a-Gene Labeling System (Promega).

Whole-mount in situ hybridization was carried out as reported by Croce et al. (Croce et al., 2003). Fz5/8 probe corresponds to the Fz5/8 full-length cDNA. Other probes were synthesized from *HE* (Hatching enzyme) (Lepage and Gache, 1990), *Goosecoid*, *Coquille*, *AA29* (Croce et al., 2003), *ske-T* (Croce et al., 2001a), *L36* (T. Lepage, unpublished), *Brachyury* (Croce et al., 2001b) and *LvDelta* (Sweet et al., 2002) genes.

Transplantation experiments

Animal and vegetal half transplantations were performed at the 16- or 32-cell stage with *L. variegatus* embryos as described (Logan et al., 1999).

Antibody staining

Immunofluorescent analyses were carried out as described (Sherwood and McClay, 1997). Bright-field and epifluorescence images were acquired with a Zeiss confocal laser microscope, while immunolabeling was imaged with a Zeiss confocal laser microscope.

Plasmid constructions and in vitro transcription

After PCR amplification, Fz (entire Fz5/8 ORF) and FzE (codons 1-230) were cloned in pCS²⁺ (Turner and Weintraub, 1994) to give pCS²⁺Fz and pCS²⁺FzE, respectively. pCS²⁺FzTM1 was obtained by deletion of codons 261 to 556 from pCS²⁺Fz. A point mutation changing W into G in the sequence KTXXXW was introduced by PCR to give pCS²⁺Fz-W521G.

pCS²⁺FzLRP5/6 was obtained by PCR as reported by Tolwinski et al. (Tolwinski et al., 2003). The 5' capped mRNAs were generated with the mMessage mMachine kit (Ambion). pCS²⁺GSK3 β has been described by Emily-Fenouil et al. (Emily-Fenouil et al., 1998). pBsNotch activated form was used as indicated by Sherwood and McClay (Sherwood and McClay, 1999).

mRNA microinjection

After dilution in double distilled H₂O, Fz, Fz-W521G and FzLRP5/6 constructs were injected up to 2.5 μ g/ μ l. Others mRNAs were used at a final concentration of 0.3 μ g/ μ l for FzE; 0.55 μ g/ μ l for FzTM1; 0.5 μ g/ μ l for GSK3 β ; 0.7 μ g/ μ l for Notch activated form; and 0.026 μ g/ μ l for RhoA activated form. Double injections were performed by simultaneous injection of FzTM1 mRNA with mRNA from Fz, Notch activated form or RhoA activated form at the concentrations indicated above.

RESULTS

Cloning and sequence analysis of Fz5/8

During a random screen by in situ hybridization (T. Lepage, unpublished), followed by a cDNA library screen, a Fz5/8 clone was isolated that encodes a 556 amino acids protein (GenBank Accession Number AM084899) with the characteristic domain organization of the Frizzled receptors: a signal peptide, a cysteine-rich domain (CRD), seven transmembrane domains and a C-terminal cytoplasmic domain, which contains the conserved sequence KTXXXW (Fig. 1A).

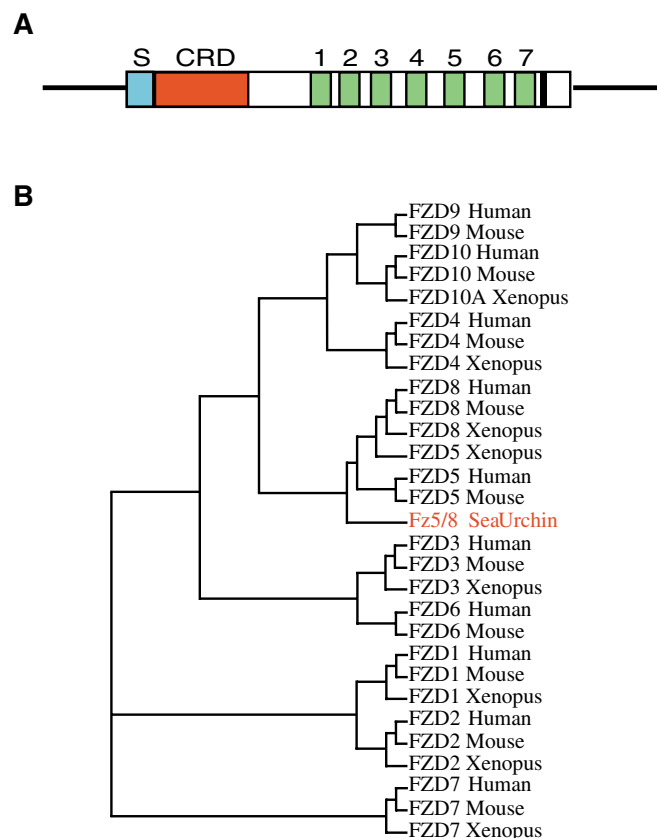


Fig. 1. Fz5/8 protein. (A) Schematic representation of Fz5/8. Blue, signal peptide; red, cysteine rich domain; green, transmembrane domain; black, KTXXXW motif. (B) Cladogram of representative members of the Frizzled family. Amino acid sequences of Frizzled proteins from sea urchin and other organisms were aligned using ClustalX and the tree was drawn with Tree View.

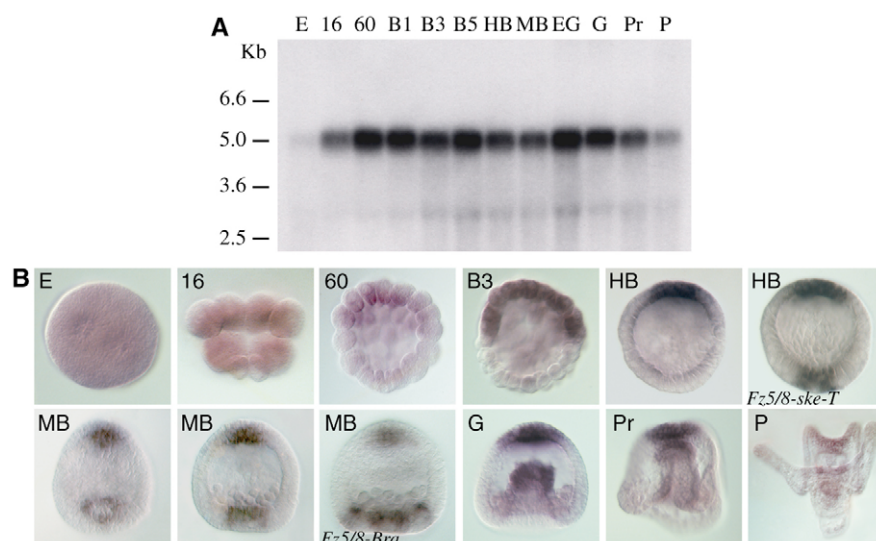


Fig. 2. Spatiotemporal expression pattern of Fz5/8 during embryogenesis.

(A) Temporal analysis by northern blotting with total RNA isolated from embryos at indicated stages. (B) Spatial distribution of Fz5/8 transcripts revealed by whole-mount in situ hybridization. All embryos were fixed at indicated stages and hybridized with Fz5/8 antisense probe, except for double labeling (where Fz5/8 probe was used simultaneously with ske-T or Brachyury probes). Lateral views with the animal pole at the top. E, unfertilized egg; 16, 16-cell stage; 60, 60-cell stage; B1-5, blastula stages; HB, hatched blastula; MB, mesenchyme blastula; EG, early gastrula; G, gastrula; Pr, prism; P, pluteus.

Alignment of the predicted amino acid sequence with Frizzled sequences from human, mouse and *Xenopus* showed that the sea urchin protein has strong similarity to Frizzled 5 and Frizzled 8. Phylogenetic analysis shows that Fz5/8 branches at the base of those two subfamilies, suggesting that Fz5/8 might be the ortholog of an ancestral gene Frizzled5/8 that has been duplicated later during evolution to create the paralogs Frizzled 5 and Frizzled 8 (Fig. 1B).

Fz5/8 expression pattern during sea urchin embryogenesis

To assess the temporal expression of Fz5/8, northern blot analysis was performed. A single, appropriately sized Fz5/8 transcript (~5 kb) was detected (Fig. 2A). Present at low levels in unfertilized eggs, Fz5/8 transcripts are detectable at higher levels throughout embryogenesis, with two major phases of expression during cleavage and gastrulation.

The spatial expression of Fz5/8 was investigated by whole-mount in situ hybridization. In egg and during early cleavage, Fz5/8 transcripts were uniformly distributed (Fig. 2B). At the 60-cell stage, Fz5/8 expression was restricted to a region of the embryo opposite to the micromeres, and thus to the animal hemisphere. During cleavage, this expression domain was progressively reduced to a small region of the embryo at hatched blastula stage (HB). At this stage, a double labeling with ske-T, a specific marker of the presumptive primary mesenchyme cell (PMC) (Croce et al., 2001a; Fuchikami et al., 2002), established that Fz5/8 is expressed in the animal-most region of the embryo, which corresponds to the animal (or apical) pole domain (ApD) (Angerer and Angerer, 2003; Takacs et al., 2004). At mesenchyme blastula stage (MB), Fz5/8 begins to be expressed in a second domain that forms a ring of cells centered on the vegetal pole. To determine the identity of these cells, another double labeling was performed with Brachyury, which is expressed at the ectoderm/endoderm boundary at this stage (Croce et al., 2001b; Gross and McClay, 2001). In double-stained embryos, the Fz5/8 ring was concentric with, and internal to, the Brachyury ring, with the two rings separated by two or three rows of cells. As the secondary mesenchyme cells (SMCs) are internal to the endodermal cells and external to the small micromeres, these results indicate that Fz5/8 is expressed in an inner subset of the SMCs. Finally, throughout gastrulation and until pluteus stage,

Fz5/8 transcripts are detectable both in the ApD and at the tip of the archenteron. Thus, during embryogenesis, Fz5/8 is expressed in two restricted areas, first in the ApD and then in the SMC territory.

Fz5/8 expression pattern is sensitive to axis patterning

To assess whether the spatial restrictions of Fz5/8 are linked to the establishment of the animal-vegetal polarity, Fz5/8 expression was evaluated in animalized and vegetalized embryos. Animalized embryos that completely lack endomesoderm were produced by zinc treatment or by overexpression of the wild-type form of GSK3 β (Emily-Fenouil et al., 1998). At HB, in both Zn²⁺-treated and GSK3 β -injected embryos, the animal expression domain of Fz5/8 extended toward the vegetal pole, covering approximately two-thirds of the embryo (Fig. 3A). This expression pattern persisted at later stages (equivalent of MB and gastrula) (not shown), establishing that

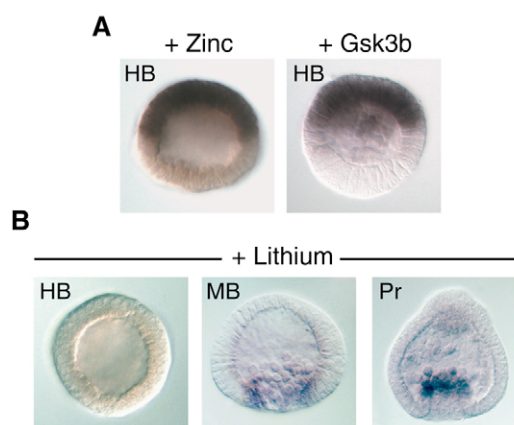


Fig. 3. Expression of Fz5/8 during perturbed development.

(A) Fz5/8 expression in animalized embryos obtained either by treatment with 0.1 mM zinc chloride or by overexpression of the wild-type form of GSK3 β . (B) Fz5/8 expression in embryos vegetalized with 30 mM lithium chloride. All embryos were fixed at indicated stages and hybridized with Fz5/8 antisense probe. HB, hatched blastula; MB, mesenchyme blastula; Pr, prism. Lateral views with the animal pole at the top.

Fz5/8 transcription in the vegetal hemisphere did not occur in these embryos. Thus, animalization perturbed both domains of Fz5/8 expression, expanding the animal component and suppressing the vegetal one.

Vegetalization of the embryos was accomplished by lithium chloride treatment, which induces an increase in endoderm at the expense of the ectoderm, but without apparent alteration of the mesodermal territories. In vegetalized embryos, the animal expression of Fz5/8 was undetectable from HB to prism stage (Fig. 3B). By contrast, the vegetal expression of Fz5/8 was unaffected. At MB, Fz5/8 transcripts were detectable in a ring of cells surrounding the vegetal pole, and then, at prism stage, in delaminating cells present at the vegetal pole (Fig. 3B). Although at prism stage exogastrulation is not easily detectable at the morphological level, these embryos are and at pluteus stage will display the well-known LiCl phenotype (Croce et al., 2001b; Croce et al., 2003) (not shown). This indicates thus that Fz5/8 is not expressed in endoderm but rather in SMCs. Thus, whereas vegetalization of the embryos completely abolished Fz5/8 animal expression, it did not affect vegetal expression. Together, these data suggest that the two Fz5/8 expression domains are regulated by distinct transcription regulatory modules that respond differently to perturbations along the animal-vegetal axis, and strengthen the previous conclusion that at the vegetal pole Fz5/8 is transcribed in SMCs.

Loss of Fz5/8 function affects gastrulation and skeletogenesis

To investigate the function of Fz5/8 during embryogenesis, two C-terminal truncated forms of Fz5/8, FzE and FzTM1, were employed (Fig. 4A), that by analogy with similar truncated forms of xFz8 and fz8a, should act in a dominant-negative fashion (Deardorff et al., 1998; Kim et al., 2002). FzE corresponds to the extracellular region of the receptor, including the peptide signal and the CRD domain. FzE protein is secreted and binds the Wnt ligand, preventing it from interacting with the endogenous receptor. FzTM1 is composed of the same extracellular region plus the first transmembrane domain. Unlike the secreted version, FzTM1 protein is retained in the membrane and will thus compete with endogenous receptors in the same cellular compartment.

Overexpression of FzE and FzTM1 cause the same morphological defects (Fig. 4B). At late gastrula stage when control embryos have full-length archenterons (Fig. 4B1), FzE- or FzTM1-injected embryos have not gastrulated (Fig. 4B2,B3). These embryos have PMCs within the blastocoel, but the vegetal plate fails to invaginate. At pluteus stage (Fig. 4B4), injected embryos are spherical with a thick epithelium covered by long cilia, and a thin epithelium within which pigment cells are inserted. They have few or no spicules and lack both archenterons and blastopores (Fig. 4B5,B6).

To ascertain whether these effects were specific, the ability of wild-type Fz5/8 (Fz) to rescue the FzTM1 phenotype was tested. In controls, mRNA encoding Fz was injected at increasingly higher concentrations, until compromising embryo viability, and no developmental defects were observed. In experimental injections, mRNA encoding FzTM1, at levels that inhibit gastrulation, were co-injected with Fz mRNA and the resulting embryos develop normally to larvae in 95% of the injected cases (Fig. 4C). These rescued embryos contained full tripartite archenterons and normal skeletons, indicating that the perturbations caused by FzTM1 overexpression resulted specifically from the loss of Fz5/8 signaling. Taken together, these results strongly suggest that Fz5/8 signaling is specifically required for gastrulation and skeletogenesis.

Polarity of FzTM1-injected larvae

At pluteus stage, FzTM1-injected embryos are spherical and possess a pronounced ectodermal polarity, with a thick epithelium bearing long non-motile cilia and a thin epithelium covered with short motile cilia (Fig. 5A) (cilia motility was observed by stroboscopy, not shown). Based on morphology, it was unclear whether this represented a polarization about the animal-vegetal axis or the oral-aboral axis, each of which possess an asymmetry of cilia. To distinguish these possibilities, FzTM1 mRNA was injected into eggs, then at the two-cell stage, a lineage tracer was introduced in one of the two blastomeres. In control plutei, the tracer was asymmetric either along the oral-aboral (about 30% of embryos) or the left-right axes (about 70% of embryos), but not along the animal-vegetal axis, as expected (Fig. 5B). In FzTM1-injected embryos, the tracer was observed in either the thick or the thin epithelium of the non-gastrulating spheres, but never in both (Fig. 5B), indicating that the morphological polarity is not along the animal-vegetal axis. To

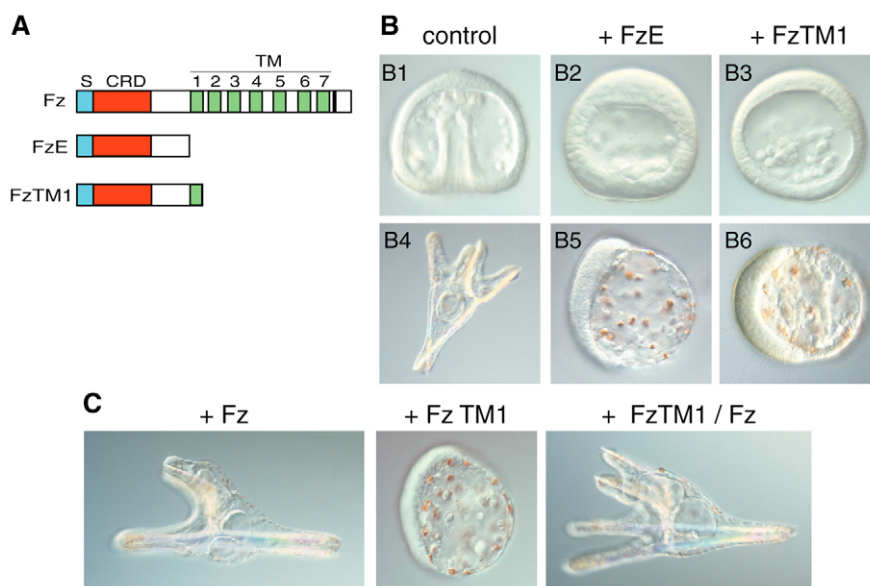


Fig. 4. Perturbation of Fz5/8 function during embryogenesis. (A) Structure wild-type or dominant-negative forms of Fz5/8 encoded by cDNA constructs. S, signal peptide; CRD, cysteine rich domain; TM, transmembrane domain. (B) Phenotypes associated with the overexpression of FzE or FzTM1. B1-B3, gastrula stages; B4-B6, pluteus. (C) Rescue of FzTM1 phenotype. Control (left), injection of FzTM1 alone (middle), and co-injection of FzTM1 and Fz in a 1:2 ratio (right).

determine whether this polarity is oral-aboral or left-right, embryos were immunostained for the neural marker 1e11. In control larvae, 1e11-stained nerve cells that are in the ciliary band, and their axons that are present only in the oral ectoderm (Fig. 5C) (Nakajima et al., 2004). In FzTM1-injected larvae, 1e11 staining revealed nerve cells and axons only in the thick epithelium. Thus, because in larvae the thick epithelium includes neurons, it is likely to be related to a ciliated band. These observations indicate that FzTM1-injected larvae display oral-aboral polarity. Intriguingly enough, an effect of the FzTM1 expression is also to induce 100% of the embryos to display oral/aboral entrainment instead of obtaining the 30% versus 70% oral/aboral versus left/right fate predicted by the first cleavage. At present, we have no explanation for this observation, other than to suggest that it is an indirect consequence of Fz5/8 expression in

the vegetal hemisphere, as it is known that oral/aboral polarity depends on signals from the vegetal hemisphere (Wikramanayake et al., 1997).

Fz5/8 signaling is not involved in the specification of the main territories but is required for maintenance of endodermal marker expression

To determine whether Fz5/8 is required for cell specification, the expression of molecular markers specific for the main embryonic territories was studied by whole-mount in situ hybridization after Fz5/8 function was impaired (Fig. 6). The *HE* gene labels the presumptive ectoderm (Lepage and Gache, 1990), while *goosecoild* and *coquillet* (Tbx2/3) label oral and aboral ectoderm, respectively (Angerer et al., 2001; Croce et al., 2003; Gross et al., 2003). Expression patterns of those three genes were unchanged in FzE- or FzTM1-injected embryos, indicating that Fz5/8 is not required for ectoderm specification or patterning (Fig. 6A-F).

For SMCs and their precursors, AA29 was used. At gastrula stage, AA29 transcripts are normally localized to the SMCs at the tip of the archenteron (Fig. 6G). At the same stage, injected embryos also have AA29-positive cells that are present within the blastocoel (Fig. 6H). Thus, Fz5/8 does not have a required function in SMC specification or differentiation, consistent with the presence of SMC-derived pigment cells in FzTM1-expressing larvae (Fig. 4B).

The T-box transcription factor ske-T (T-brain) is specifically expressed in PMCs and their precursors from blastula to gastrula stage (Croce et al., 2001a; Fuchikami et al., 2002). In blastulae, the expression of ske-T is not altered by FzE or FzTM1 overexpression (not shown). However, at gastrula stage, injected embryos have fewer cells expressing ske-T than controls, and these cells also fail to become organized in comparison with the PMCs within control embryos (Fig. 6I,J). Thus, although Fz5/8 loss-of-function blocks skeletogenesis, Fz5/8 does not appear to be involved in PMC specification.

Finally, the expression of two endodermal markers, L36 and Brachyury, was tested. L36 is expressed throughout gastrulation in the endoderm (Fig. 6K,M,O). During the same period, Brachyury is expressed in the stomodeum and, in a dynamic pattern, first within the vegetal plate and then around the blastopore (Croce et al., 2001b; Gross and McClay, 2001) (Fig. 6Q,S). At MB, not surprisingly the expression of both L36 and Brachyury was not modified by Fz5/8 loss of function (Fig. 6L,N,R), indicating that Fz5/8 is not required to trigger endoderm specification. These results were expected as Fz5/8 is expressed in the vegetal hemisphere beginning only at MB and therefore well after endoderm specification had been launched by the maternal Wnt/ β -catenin pathway (Davidson et al., 2002; Logan et al., 1999). However, at late gastrula stage, no vegetal staining was observed in FzE- or FzTM1-injected embryos (Fig. 6P,T), and only the oral expression of Brachyury was detected (Fig. 6T). Thus, although Fz5/8 is not required for endoderm specification, it appears to be necessary to maintain endodermal marker expression. Together, these data suggest that Fz5/8 is not involved in the specification of the main territories, including the endoderm territory, but show that it is required for the maintenance of expression of late endodermal markers associated with the formation of the archenteron.

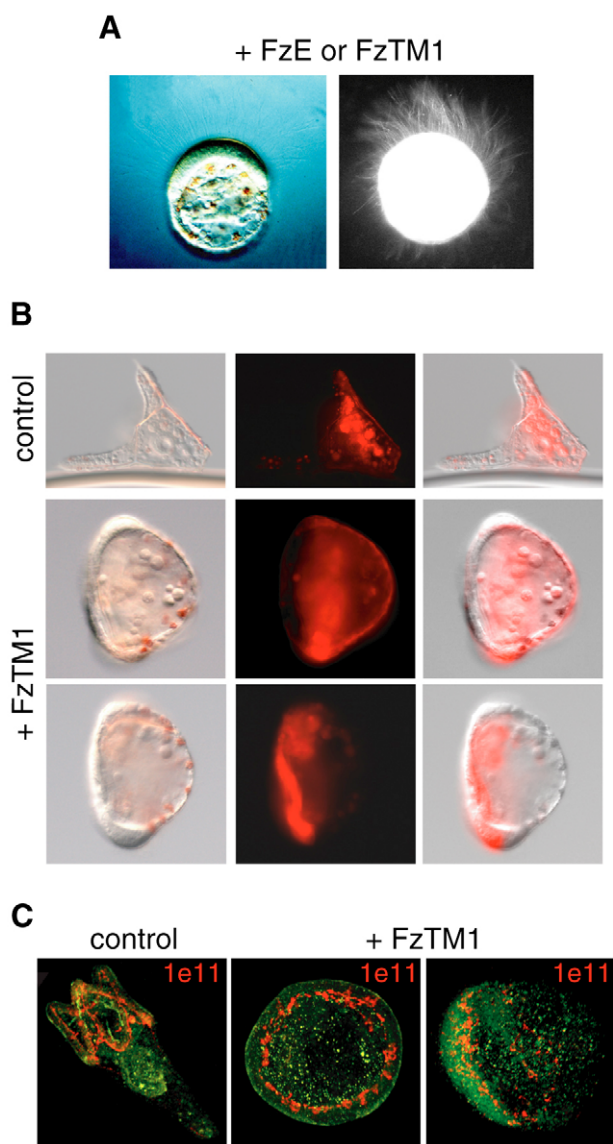


Fig. 5. Polarity of FzTM1-injected larvae. (A) DIC (left) and dark-field (right) images of FzTM1-injected larvae. (B) Determination of the morphological polarity of FzTM1-injected larvae by labeling one of the two blastomeres at the two-cell stage. (C) Confocal images of neurons immunostaining on FzTM1-injected larvae. Red, 1e11 antibody (Nakajima et al., 2004); green, counterstain. Control embryo, animal pole view (left); FzTM1-injected embryos, oral view (middle) and transverse view (right).

Fz5/8 function is required in SMC for archenteron invagination

As Fz5/8 transcripts are present in animal and vegetal domains, we assessed Fz5/8 function in each domain by transplantation experiments. Chimeric embryos were generated such that Fz5/8 function was blocked only in the animal or the vegetal hemisphere

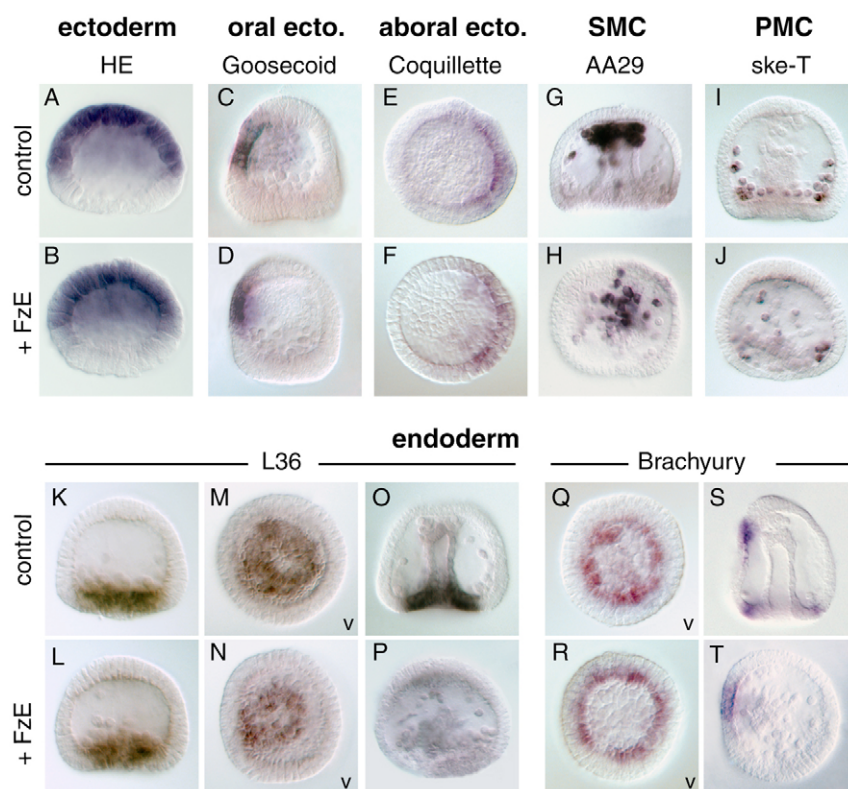


Fig. 6. Molecular characterization of the phenotype associated with the disruption of Fz5/8 signaling. (A–T) Embryos injected with FzTM1 were fixed at the appropriated stages and processed for whole-mount in situ hybridization with probes for specific markers of embryonic territories, as indicated. (A,B) Early blastula (B3), (C,D,K,N,Q,R) mesenchyme blastula, (E,F) hatched blastula, (G–J,O,P,S,T) gastrula. Lateral views with the animal pole at the top except M,N,Q,R (viewed from the vegetal pole).

(Fig. 7A). mRNA encoding FzTM1 were injected into unfertilized eggs with a red lineage tracer. Then, between 16 and 32-cell stage, animal and vegetal halves from those embryos were isolated and recombined with the complementary animal or vegetal half from a normal embryo (Fig. 7A). Resulting chimeras were observed at pluteus stage (Fig. 7B).

When FzTM1 was expressed exclusively in animal halves, embryos gastrulated and developed into normal pluteus larvae (20/24; Fig. 7B7). Descendants of transplanted mesomeres formed the ectoderm (Fig. 7B8). Immunostaining with endodermal and skeletogenic markers further confirmed that these embryos produced normal tripartite archenterons and skeletons (Fig. 7B9). Thus, Fz5/8 is not required in the animal half for endodermal or skeletal development.

By contrast, the reciprocal chimeric embryos, in which Fz5/8 function is inhibited in the vegetal hemisphere, produced the same phenotype as that observed with ubiquitous FzTM1 overexpression (23/27; Fig. 7B10,B4). Immunostaining demonstrated that these larvae did not produce endodermal tissue, and, while PMCs were produced, skeletons were not (Fig. 7B12). Thus, these findings reveal that inhibition of Fz5/8 in the vegetal hemisphere, where Fz5/8 is normally expressed in SMCs, is sufficient to block archenteron invagination and prevent skeletogenesis. In addition, chimeras were produced with FzTM1 present only in micromeres to test whether the skeletogenic defect arise from the PMCs. Such chimeras gave rise to embryos that were indistinguishable from controls (not shown), indicating that the skeletogenic defect originates not in the PMCs, but elsewhere, and therefore is non-autonomous.

Relationship between Fz5/8 and Notch signaling pathway

In the sea urchin, Notch is involved in three distinct events. The first two are required in the SMCs to mediate, via Delta activation, SMC specification and differentiation (Sherwood and McClay, 1999;

Sweet et al., 2002), whereas the third one is important for gastrulation (Peterson and McClay, 2005; Sherwood and McClay, 2001). As Fz5/8 is expressed in SMCs and is required for endoderm morphogenesis, the relationship between Fz5/8 and Notch signaling was assessed.

During development, Notch receptors present a dynamic expression pattern within both SMCs and endodermal cells. At MB and throughout gastrulation, Notch proteins are no longer detectable in SMCs, whereas they are expressed at high levels at the apical surface of endodermal cells (Fig. 8A) (Sherwood and McClay, 1997). Overexpression of FzTM1 eliminated the apical expression of Notch (Fig. 8A), indicating that Fz5/8 signaling is required for the mesenchymal apical localization of Notch in endodermal cells.

To test whether this loss of apical Notch was responsible to the lack of invagination in FzTM1-injected embryos, the ability of an activated form of Notch protein (Nact) to rescue the FzTM1 phenotype was tested. When overexpressed alone, Nact induces an increase of SMC derivatives cell types, including pigment cells (Sherwood and McClay, 1999) (Fig. 8B). Co-expression of FzTM1 and Nact gave rise to embryos that present the FzTM1 phenotype, with no endodermal structures, although pigment cells were produced in excess (Fig. 8B). These results show first that although Fz5/8 is expressed in SMCs, it is not required for the first Notch-mediated SMC specification signal, as was suggested by the presence of pigment cells in the FzTM1 phenotype. Second, ectopic activation of Notch pathway using the Nact construct is not sufficient to rescue archenteron invagination downstream of Fz5/8 signaling.

Fz5/8 signals through the PCP/noncanonical Wnt pathway

Frizzled proteins can transduce Wnt signals through three distinct pathways: the Wnt/ β -catenin or canonical pathway, the Wnt/ Ca^{2+} pathway and the planar cell polarity (PCP) pathway. To identify

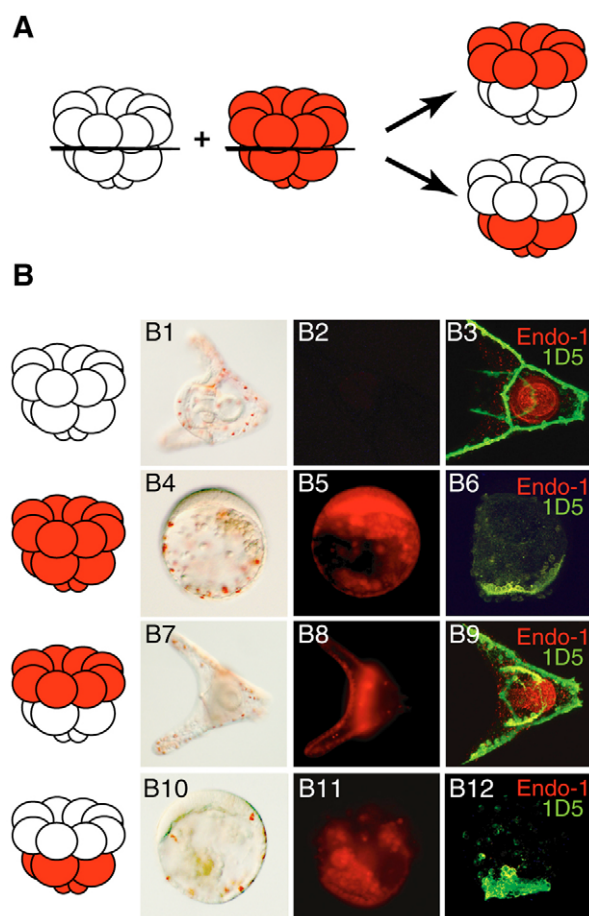


Fig. 7. Requirement of Fz5/8 signaling in SMC for archenteron and skeleton formation. (A) Experimental design. At the 16-cell stage, animal and vegetal halves of FzTM1-injected embryos (red) were isolated and transplanted to the complementary half of a normal embryo (white). (B) Experimental results. B1-B3, control glycerol-injected embryo; B4-B6, control embryo injected with FzTM1 and a red lineage tracer; B7-B9, injected animal half recombined with control vegetal half; B10-B12, injected vegetal half transplanted with control animal half. (B1, B4, B7, B10) DIC images of resulting larvae. (B2, B5, B8, B11) Epifluorescence images of the same embryos, showing the contribution of the transplanted halves. (B3, B6, B9, B12) Confocal images of the larvae stained with Endo1 (endoderm antibody) in red (Wessel and McClay, 1985) and with 1d5 (PMC marker) in green (Hardin et al., 1992).

which of these pathways acts downstream of Fz5/8, specific inhibitors were tested for the ability to reproduce the Fz5/8 loss-of-function phenotype.

In sea urchin, the role of the maternal canonical pathway has been well reported in the past decade. This pathway is required early, beginning at the 16-cell stage, to specify the endomesoderm territory and to pattern the embryo along the animal-vegetal axis. The activation of this maternal signal causes the nuclear accumulation of its key components β -catenin in the most vegetal blastomeres. This accumulation has been described as being cell-autonomous, suggesting that no Frizzled receptors are involved (Logan et al., 1999), although to date this possibility cannot be eliminated. Fz5/8 mRNA is first detected in the vegetal hemisphere starting only at MB, when both nuclearization of β -catenin and endomesoderm specification had already occurred. Fz5/8, therefore, is not necessary for the canonical pathway that initiates endomesoderm specification. This conclusion is reinforced by the observation that FzTM1, when expressed in micromeres only, has no effect (not shown). However, the possibility remained that Fz5/8 could be the receptor used for activation of a later zygotic form of the canonical pathway, for which no role has been yet described. Thus, in order to allow endomesoderm specification to arise normally and to interfere only with the zygotic form of the canonical pathway, mutated forms of two different receptors specific to the canonical pathway were used, rather than a direct inhibition of β -catenin. In *Drosophila*, it has been shown that an Fz/LRP5 fusion is a constitutively active canonical signal (Tolwinski et al., 2003). Accordingly, a murine LRP5/6-Fz5/8 fusion construct was made and found to be active in sea urchin embryos, as expression of the LRP5/6-Fz5/8 protein had a strongly vegetalizing effect (not shown). Next, a truncated form of the mouse Frizzled co-receptor LRP5/6 was then used for its specific dominant-negative effect on the β -catenin signaling (Tamaï et al., 2000). Second, Fz5/8 was also mutated at the conserved sequence KTXXXW that is essential for activation of the canonical pathway (Umbhauer et al., 2000). Expression of the mutated Fz5/8 construct alone, or of the truncated LRP5/6 construct, had no effect on sea urchin specification, or on gastrulation. In other organisms, these have dominant-negative effects on signaling when the Fz is used in the canonical pathway (He et al., 2004; Umbhauer et al., 2000). Thus, although Fz5/8 can be forced to operate through the canonical Wnt pathway if fused to murine LRP5/6, mutation of the Fz5/8 potential canonical Wnt receptive activity has no effect, indicating, again, that Fz5/8 is not used in the sea urchin for reception of the canonical Wnt signal.

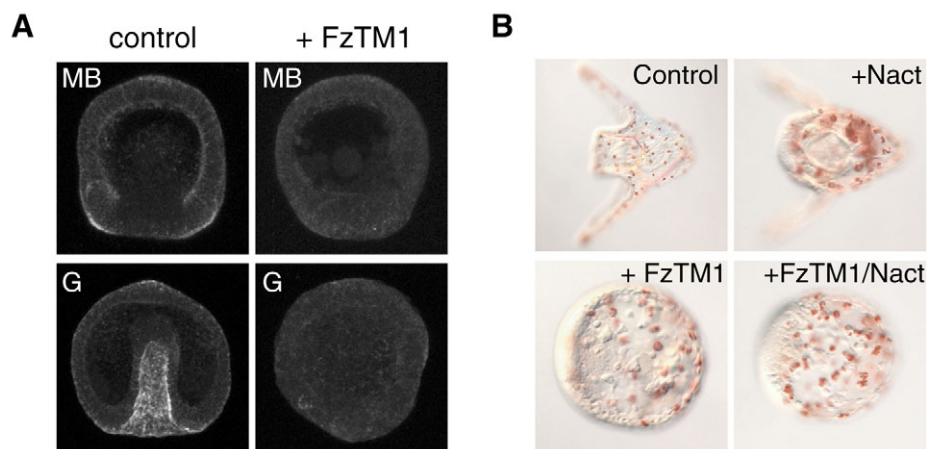


Fig. 8. Relationship between Fz5/8 and Notch signaling. (A) Confocal images of Notch protein localization revealed by antibodies against the intracellular domain of the receptor (Sherwood and McClay, 1999). Immunolocalization performed in control and FzTM1-injected embryos at mesenchyme blastula (MB) and gastrula (G) stages. (B) Larvae obtained after injection or co-injection of mRNA encoding FzTM1 and Notch activated form (Nact).

The Wnt/ Ca^{2+} pathway was inhibited with Bisindolylmaleimide I, a highly specific PKC inhibitor (Chou and Howard, 2002; Koyanagi et al., 2005). Treatments were started at HB, shortly before Fz5/8 transcription begins in the SMCs. This drug did not perturb the development of the tripartite archenteron or the ingression and organization of the PMCs, although skeletogenesis was inhibited (Fig. 9A). Thus, Fz5/8 does not seem to function via the Wnt/ Ca^{2+} pathway.

The PCP pathway involves two independent effectors, Rho-associated-kinase (ROCK) and Jun-N-terminal-kinase (JNK). In order to completely inhibit the PCP pathway, drugs specific for each effector were used. Treatment with the ROCK inhibitor (Y27632) (Riento and Ridley, 2003) alone interfered with skeletogenesis without impairing gastrulation. By contrast, inhibition with JNK Inhibitor I (L) form (Bonny et al., 2001) blocked archenteron elongation but not blastopore invagination or skeletogenesis. Combined inhibition gave rise to embryos with pigment cells but lacking skeletons and blastopores (Fig. 9A), similar to Fz-inhibited embryos. Thus, Fz5/8 may signal through the PCP pathway.

To reinforce these results, first the expression of Fz5/8 was assessed in double-inhibited embryos to assess whether their combined effects were due to repression of Fz5/8 transcription. In situ analyses ruled out this hypothesis, as the combined treatment did not block Fz5/8 expression in either the ApD or SMCs (not shown). Second, as RhoA is an activator of both JNK and ROCK

(Kim and Han, 2005), the ability of activated RhoA (actRhoA) to rescue the FzTM1-associated phenotype was tested. Overexpression of actRhoA alone produces embryos that form a precocious invagination before hatching (W. S. Beane, unpublished); however, such embryos were indistinguishable from controls at pluteus stage (Fig. 9B). Co-expression of FzTM1 and actRhoA rescued FzTM1 phenotype in 42% of the cases ($n=129$) (Fig. 9B). In 14% of the cases, the rescue was partial and embryos produced a reduced archenteron and some spicules. In 28% of the cases, the rescue was complete; embryos developed into normal pluteus larvae with a normal tripartite archenteron and a normal skeleton (Fig. 9B). The percentage of complete rescue (28%) is equivalent to the penetrance of actRhoA mRNA (W. S. Beane, unpublished). Together, these results strongly indicate that Fz5/8 signals through the PCP pathway in its role as an initiator of archenteron invagination.

DISCUSSION

In this study, we have characterized the first identified member of the Frizzled receptor family in the sea urchin. Sequence comparisons indicate that this protein has strong similarities with the vertebrate Fz5 and Fz8 and a phylogenetic analysis suggests further that it is related to a common ancestor of these two families. During sea urchin embryogenesis, Fz5/8 displays restricted expression in two separate domains, the animal domain and the SMCs. Although loss-of-function analyses could not establish a Fz5/8 function in the animal domain, they clearly indicate that Fz5/8 is required in the SMC lineage to control primary invagination, while the other phenotypic defects observed are probably due to the absence of blastopore formation (see below). Finally, Fz5/8 signals through the PCP pathway, which is involved in cell polarity and cell shape changes in other organisms.

Fz5/8 in the animal domain

During cleavage, Fz5/8 is expressed in the animal-most region of the blastula (Fig. 2), an area previously defined as the animal domain (ApD) (Angerer and Angerer, 2003; Takacs et al., 2004). The ApD is a specific area of the ectoderm territory that has been first identified by the lack of expression of several markers mRNAs found in the oral or aboral pre-ectoderm, its resistance to vegetalizing perturbations (for a review, see Angerer and Angerer, 2003), and then by the unique case of restricted expression of the SpNK2.1 factor (Takacs et al., 2004). The ApD has been previously thought to be specified at the end of cleavage (Angerer and Angerer, 2003). However, Fz5/8 expression starts being restricted to the animal hemisphere much earlier, at the 60-cell stage. Considering this, the ApD may not emerge suddenly at the end of cleavage, but rather be broadly defined as early as the 60-cell stage and then progressively delimited during cleavage, as is the Fz5/8 expression domain. Alternatively, establishment of this territory may also occur in different ways among sea urchin species. For both *Strongylocentrotus purpuratus* (SpNK2.1) (Takacs et al., 2004) and *Paracentrotus lividus* (Fz5/8, this study), animalizing perturbations extended the expression domain of the ApD genes. By contrast, although in *S. purpuratus* the ApD is not sensitive to enhanced vegetal signaling (Takacs et al., 2004), in *P. lividus*, ApD expression of Fz5/8 completely disappeared in LiCl-treated embryos (Fig. 3) and a GSK3- β overexpression completely erased the HE expression domain, including in the ApD (Emily-Fenouil et al., 1998). Thus, even though the ApD appears morphologically identical in the different sea urchin species, its establishment or its regulation may not be identical in the two species. Despite that, the symmetrical responses of Fz5/8 to

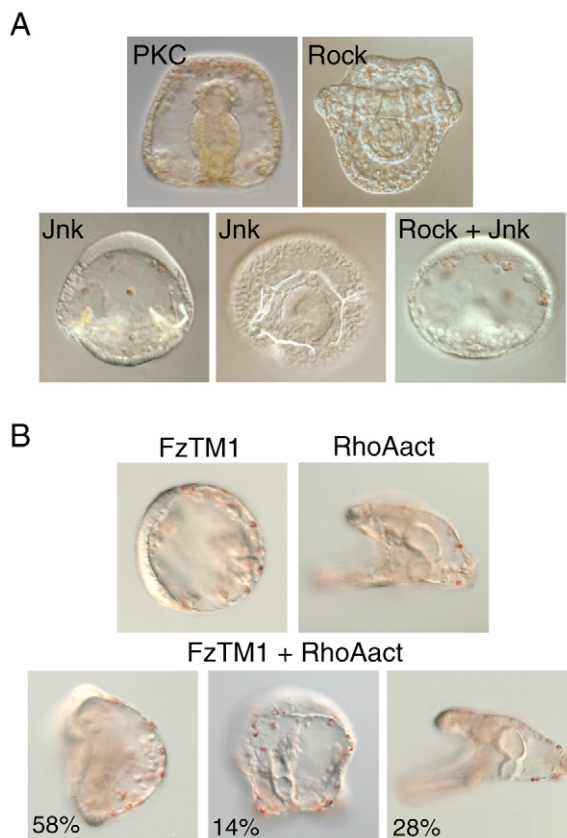


Fig. 9. Relationship between Frizzled and the PCP pathway. (A) Representative phenotypes of larvae treated with inhibitors for PKC (Bisindolylmaleimide I), ROCK (Y27632) or JNK [JNK inhibitor I (L-form)], added at HB separately or in combination. (B) Larvae obtained after injection or co-injection of mRNA encoding FzTM1 and an activated form of LvRhoA protein (actRhoA).

vegetalization and animalization, together with the progressive restriction of its expression to the animal pole area, starting at the 60-cell stage, suggest that during early cleavage Fz5/8 may be controlled by maternal nuclear β -catenin signaling.

The functional role of Fz5/8 in the ApD remains enigmatic. During embryogenesis, the ApD first extends a tuft of long cilia, then becomes the part of the oral hood of the larva in which the neurons develop (Nakajima et al., 2004). Suppression of Fz5/8 function throughout the embryo, or in animal halves alone, did not perturb neurogenesis (Fig. 5C). Furthermore, in the suppressed embryos, oral-aboral polarization was present, neurogenesis continued to occur at the boundary of the oral and aboral territories, and IE11 is expressed in neurons in the oral territory (Figs 5, 6), indicating that establishment of that axis does not require Fz5/8 signaling. Thus, no function of the ApD was revealed by elimination of Fz5/8. Potentially, the role of Fz5/8 in the ApD is too subtle to be observed with currently available tools, or there is some redundancy between Fz5/8 and other signals in this area. Alternatively Fz5/8 ligand or other essential components of Fz5/8 pathway could also be limited at the animal pole. Furthermore, overexpression of Fz5/8 alone has no phenotype and the rescued embryos co-injected with FzTM1 and Fz mRNAs similarly displayed a normal phenotype. This was despite the fact that with overexpression Fz5/8 is present throughout the embryo. Thus, the embryo must have a way to restrict the function of the receptor, most probably via the distribution of other components of its pathway.

Fz5/8 in the vegetal hemisphere

Starting at MB, Fz5/8 is expressed in a subset of the SMC territory. This restricted vegetal expression in the SMC territory is supported by double in situ comparisons with Brachyury expression (Fig. 2). Fz5/8 is entirely inside the Brachyury ring, which is known to be in the endoderm at the endoderm-SMC boundary. Furthermore, Fz5/8 expression is not expanded in LiCl-treated embryos (Fig. 3). However, loss of function of Fz5/8 has no effect on SMC cell fate because that specification precedes Fz5/8 expression; in FzTM1-injected embryos, SMCs are normally specified, correctly execute their epithelial-mesenchyme transition, as revealed by AA29 staining (Fig. 6), and they correctly give rise to pigment cells, a normal SMC derivative (Fig. 4). Notch signaling is known to be required in SMCs to mediate SMC specification and differentiation, and this occurs independently of Fz5/8, according to this study. However, Notch receptors are also expressed on endodermal cells where, starting at MB, they are localized at a high level on apical membranes. In FzTM1-injected embryos, this appearance of apical Notch protein is absent from MB onwards (Fig. 8), indicating that Fz5/8 is required for this particular distribution. Similarly, Brachyury is expressed, beginning at hatched blastula, in endoderm; however, at MB, when Fz5/8 normally is expressed, expression of FzTM1 eliminates the normally continuing expression of Brachyury. Although Notch signaling is clearly important for gastrulation (Peterson and McClay, 2005; Sherwood and McClay, 2001), the mechanism by which it does so has not yet been characterized. However, ectopic activation of Notch signaling downstream of FzTM1 was not sufficient to rescue archenteron formation, even though an excess of pigment cells was produced. These results are consistent with previous data that show that Notch acts via two distinct processes to mediate SMC formation and to regulate ectoderm-endoderm boundary position (Sherwood and McClay, 2001). Thus, although this study does not clarify the functional relationship between

Fz5/8 and Notch, it establishes that Fz5/8 is required, indirectly, for the apical localization of Notch proteins in endodermal cells, an event for which no upstream mediator is known to date.

Another defect of the Fz5/8 loss of function is the lack of spicules. Again, Fz5/8 is not expressed in PMC or their precursors, and in FzTM1-injected embryos, PMCs ingress and differentiate normally (Figs 6, 7). The FzTM1 dominant-negative effect is not due to nonspecific inhibition of other Frizzled signaling in PMCs or ectoderm as FzTM1 expression exclusively in micromeres or in the animal half did not affect skeletogenesis (Fig. 7). Thus, we suggest that the lack of skeletogenesis is a secondary effect of the absence of invaginated cells. A previous study reported that a transplanted archenteron could induce an ectopic bilaterally symmetric skeleton around it using the host PMCs (Benink et al., 1997), suggesting that the archenteron plays a role in skeletogenesis. Alternatively, this defect can also be caused by a lack of a Fz5/8-dependent signal emitted directly by the SMCs to the PMCs. However, as the behavior of the SMCs appears to occur normally, this seems less probable.

Fz5/8 and the PCP pathway

Frizzled proteins can transduce three distinct Wnt pathways: the canonical pathway, the PCP pathway and the Wnt/ Ca^{2+} pathway. Several pieces of evidence indicate that Fz5/8 does not use either the maternal or the zygotic canonical pathway. First, Fz5/8 expression occurs in SMC at MB, many hours after the first β -catenin nuclear accumulation (Logan et al., 1999). Furthermore, when Fz5/8 is expressed in SMCs, β -catenin is no longer nuclear in that territory (Logan et al., 1999), indicating that Fz5/8 is unlikely to be responsible of this characteristic event of the canonical Wnt pathway activation. Second, the FzTM1 phenotype is different from an animalization obtained by downregulation of the canonical pathway. FzTM1-injected embryos have both PMCs and pigment cells, which are absent in animalized embryos. Third, overexpression of the mutants of Fz5/8 or of the co-receptor LRP5/6, the dominant-negative effects of which are each specific to the canonical pathway (He et al., 2004; Umbhauer et al., 2000), and used to block the potential zygotic form of the pathway, has no impact on the development of the archenteron. Finally, LiCl treatment, which when applied early enhances endoderm formation, has no effect on vegetal plate specification or invagination when it is added after blastula stage (Livingston and Wilt, 1992; Nocente-McGrath et al., 1991). Thus, together those observations strongly suggest that Fz5/8 does not signal through the Wnt canonical pathway.

Comparison of the FzTM1 phenotype with those obtained with specific inhibitors for the Wnt/ Ca^{2+} or the PCP pathway eliminated the Wnt/ Ca^{2+} pathway as a candidate, and left the PCP pathway as the likely mechanism (Fig. 9). The FzTM1 phenotype is mimicked by the dual inhibition of JNK activity and ROCK inhibition. Alone, inhibition of JNK activity suppresses archenteron elongation and affects oral-aboral polarity causing formation of a radialized skeleton. However, skeletogenesis was not affected. By contrast, ROCK inhibition disrupts skeletogenesis, showing that in sea urchin embryo the two downstream components of the PCP pathway are independently required in distinct important events of embryogenesis. The ability of an activated form of RhoA (actRhoA) to rescue the FzTM1 phenotype further supports the hypothesis that initiation of gastrulation operates through the PCP pathway and is activated by Fz5/8. In other organisms, the PCP pathway is known to control cell polarity and cell shape changes during gastrulation (Barrett et al., 1997; Boutros et al., 1998; Hacker and Perrimon, 1998; Harden et al., 1995; Strutt et al., 1997; Tahinci and Symes, 2003). At the cellular level, the PCP pathway controls cells polarity

through effects on cytoskeletal organization (Wallingford, 2005; Marlow et al., 2002; Winter et al., 2001). Thus, as Fz5/8 appears to signal through the PCP pathway, it is more likely to control cell properties than cell fate decisions.

Fz5/8 is required in SMCs for archenteron invagination

A dramatic alteration resulting from disruption of Fz5/8 function is the absence of a blastopore and archenteron. Previous studies have shown that endoderm specification occurs early during cleavage, while the commitment period is between late blastula and early mesenchyme blastula stage (Chen and Wessel, 1996; Godin et al., 1997). As Fz5/8 is expressed in SMCs beginning at MB, it is therefore unlikely to be involved either in endoderm specification or commitment; normal expression of endodermal markers at MB in FzTM1-injected embryos supports this conclusion (Fig. 6). Thus, it seems most likely that Fz5/8 is required in SMCs to control archenteron morphogenesis, following endoderm specification.

Fz5/8 is required for invagination and is expressed in the SMCs. At the onset of invagination, a subset SMCs located near the center of the vegetal plate modifies their shape to acquire the so-called bottle shape. These cells form a one- or two-cell wide ring located between the eight small micromere derivatives and a tier of elongated cells that will form the lip of the early blastopore (Kimberly and Hardin, 1998). The bottle cells are the first cells to invaginate and ablation studies indicate that they are crucial for primary invagination (Kimberly and Hardin, 1998). At MB, Fz5/8 is expressed in a small ring of one or two layers of cells surrounding the unlabeled small micromeres, and so are likely to be the bottle cells. Thus, we hypothesize that Fz5/8 serves as a modulator of the morphogenetic movements responsible for bottle cell formation. Fz5/8 would therefore control the initiation of primary invagination by regulating SMC adhesion, shape and polarity by activating RhoA via the PCP pathway. Fz5/8 would thus serve to connect inductive molecular signals to the morphogenetic machinery. Furthermore, to explain the loss of late endodermal markers expression in FzTM1-injected embryos, we propose that Fz5/8 pathway activates in SMCs a paracrine signal that acts directly on endodermal cells, as it does in segmentation polarity in *Drosophila* for which cells receiving Frizzled signal synthesize Hedgehog which acts on the neighboring cells (Ingham et al., 1991).

Identification of the Wnt ligand that binds to Fz5/8 will aid in further understanding of the inductive signaling process responsible for archenteron invagination. Of particular interest will be identifying the cellular source of the signal. At present, none of the Wnt proteins reported in sea urchin have a spatial and temporal expression pattern that abut or overlap Fz5/8 expression. Nevertheless, completion of the sequencing of the sea urchin genome will provide the complete set of sea urchin Wnt genes, providing additional candidates for the ligand of Fz5/8.

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