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Frizzled1/2/7 signaling directs β-catenin nuclearisation and initiates endoderm specification in macromeres during sea urchin embryogenesis

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SUMMARY

In sea urchins, the nuclear accumulation of β-catenin in micromeres and macromeres at 4th and 5th cleavage activates the developmental gene regulatory circuits that specify all of the vegetal tissues (i.e. skeletogenic mesoderm, endoderm and non-skeletogenic mesoderm). Here, through the analysis of maternal Frizzled receptors as potential contributors to these processes, we found that, in *Paracentrotus lividus*, the receptor Frizzled1/2/7 is required by 5th cleavage for β-catenin nuclearisation selectively in macromere daughter cells. Perturbation analyses established further that Frizzled1/2/7 signaling is required subsequently for the specification of the endomesoderm and then the endoderm but not for that of the non-skeletogenic mesoderm, even though this cell type also originates from the endomesoderm lineage. Complementary analyses on Wnt6 showed that this maternal ligand is similarly required at 5th cleavage for the nuclear accumulation of β-catenin exclusively in the macromeres and for endoderm but not for non-skeletogenic mesoderm specification. In addition, Wnt6 misexpression reverses Frizzled1/2/7 downregulation-induced phenotypes. Thus, the results indicate that Wnt6 and Frizzled1/2/7 are likely to behave as the ligand-receptor pair responsible for initiating β-catenin nuclearisation in macromeres at 5th cleavage and that event is necessary for endoderm specification. They show also that β-catenin nuclearisation in micromeres and macromeres takes place through a different mechanism, and that non-skeletogenic mesoderm specification occurs independently of the nuclear accumulation of β-catenin in macromeres at the 5th cleavage. Evolutionarily, this analysis outlines further the conserved involvement of the Frizzled1/2/7 subfamily, but not of specific Wnts, in the activation of canonical Wnt signaling during early animal development.

KEY WORDS: Canonical Wnt signaling, Early embryonic development, Sea urchin

INTRODUCTION

The canonical Wnt pathway plays several key roles in early development of various organisms. It is involved, for example, in the establishment of embryonic axis polarities and germ layer specification (e.g. Logan et al., 1999; Petersen and Reddien, 2009; Thorpe et al., 1997). One of the main effectors of this signaling is the protein β-catenin. The binding of a Wnt ligand onto its cognate Frizzled receptor leads in the cytoplasm to a cascade of molecular events that comprises activation of the cytoplasmic effector Dishevelled, silencing of the β-catenin inhibitory complex APC/Axin/GSK3β and, as a consequence, the non-degradation of β-catenin. In turn, β-catenin proteins accumulate in the nucleus, where, associated with a member of the TCF/Lef family, they positively regulate transcription of canonical Wnt signaling target genes (for review, see Logan and Nusse, 2004). This nuclear accumulation of β-catenin has been reported in a wide range of animals, extending from cnidarians to vertebrates. It usually takes place within the first cleavage stages, and always asymmetrically within the embryo (e.g. Darras et al., 2011; Henry et al., 2008; Larabell et al., 1997; Logan et al., 1999; Miyawaki et al., 2003; Momose and Houliston, 2007; Nakamura et al., 2005; Schneider and Bowerman, 2007; Wikramanayake et al., 2003). Despite the number of studies relating to this event, the identity of the Wnt ligands and Frizzled receptors responsible for initiating these nuclear accumulations of β-catenin remains fragmentary.

In sea urchins, β-catenin nuclearisation is first detected at the 4th cleavage (16-cell stage) in the most vegetal cells of the embryo, the micromeres. Then, at 5th cleavage, β-catenin is also found in the nuclei of the adjacent cells above them: the eight macromere daughter cells (Logan et al., 1999). Cell dissociation experiments, during which blastomeres were separated following each cleavage, previously established that these β-catenin nuclear entries occurred independently of a paracrine cell-cell signaling (Logan et al., 1999). Additionally, these polarized nuclear accumulations were shown later to require the asymmetrical enrichment of the Dishevelled protein at the vegetal cortex (Weitzel et al., 2004). More recently, however, two reports suggested the involvement of a Wnt ligand in the regulation of the nuclear entry of β-catenin into the macromeres (Croce et al., 2011; Smith et al., 2007), adding further detail to the nature of the molecular events culminating in β-catenin nuclearisation. Nevertheless, the activation mechanism of the canonical Wnt pathway during sea urchin embryogenesis remains, to date, incompletely described.

One of the earliest recognized functions for nuclear β-catenin during animal development is the regulation of germ layer specification, in particular that of the endoderm and the endomesoderm. In the diploblastic cnidarians *Nematostella vectensis* and *Clytia hemisphaerica*, nuclear β-catenin plays a crucial role in endoderm formation (Momose and Houliston, 2007; Wikramanayake et al., 2003). Similarly in various triploblastic

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metazoans, including ascidians, hemichordates and nemerteans, embryos depleted of nuclear β-catenin fail to develop endoderm and mesoderm (Darras et al., 2011; Henry et al., 2008; Imai et al., 2000; Kawai et al., 2007). Likewise, in sea urchins, nuclear β-catenin represents a required input for the specification of all vegetal tissues, for the micromeres to become skeletogenic mesoderm and for the vegetal descendants of the macromeres (the Veg2 cells) to produce an endomesoderm lineage that will later segregate into endoderm and non-skeletogenic mesoderm descendants (Logan et al., 1999; Wikramanayake et al., 1998). Embryos depleted of nuclear β-catenin lack all these vegetal tissues (Logan et al., 1999; Wikramanayake et al., 1998), and many of the genes present in the skeletogenic mesoderm and the endomesoderm gene regulatory circuits have been identified experimentally as transcriptional targets of the β-catenin/TCF-Lef complex (e.g. Davidson et al., 2002; Oliveri and Davidson, 2004).

Here, in an effort to determine whether Wnt and Frizzled molecules are involved in initiating the nuclear accumulation of β-catenin in the sea urchin vegetal blastomeres early in cleavage, we focused on Frizzled receptors. During this analysis, we discovered that of the four Frizzled genes present in the sea urchin genome, Frizzled1/2/7 is expressed maternally and is required by 5th cleavage (32-cell stage) to initiate the nuclear entry of β-catenin selectively in the macromeres. Loss-of-function assays revealed that Frizzled1/2/7 signaling is subsequently required for endoderm specification, but not for non-skeletogenic mesoderm development. Complementary analyses on the maternal Wnt6 protein showed, further, that this ligand has the same activity as Frizzled1/2/7, suggesting that it is the likely inducer of this receptor; titration experiments also reinforced this observation. Our findings therefore provide new insights into the molecular mechanism underlying β-catenin nuclearisation during sea urchin embryogenesis, as well as into the role of the canonical Wnt pathway in endoderm and mesoderm development in this model organism.

MATERIALS AND METHODS

Animals and embryos

Adults *Paracentrotus lividus* were collected in the bay of Villefranche-sur-Mer, France. Gametes were obtained as previously described (Lepage and Gache, 1990) and embryos were cultured in filtered seawater at 18°C.

Micromanipulations

Animal-vegetal half recombinations were executed between the 16- and the 32-cell stage as previously described (Croce and McClay, 2010) using control embryos microinjected with a rhodamine-lysine dextran (RLDX) (Sigma) and embryos co-microinjected with DnFz7 mRNA and a fluorescein-lysine dextran (FLDX) (Sigma). Forty-eight hours post-fertilization the embryos were imaged using a Zeiss Imager M2 upright fluorescent microscope to record morphology and the presence of the dyes. The embryos were then fixed individually and immunostained to reveal the presence of an archenteron.

Cloning of Frizzled1/2/7 and related constructs of Frizzled1/2/7 and Wnt6

The *P. lividus* Frizzled1/2/7 (Fz7) open reading frame (570 amino acid) was isolated by PCR using oligonucleotides designed against the *Strongylocentrotus purpuratus* Fz7 sequence, available on SpBase (http://www.spbase.org/SpBase/index.php). The 5′UTR region of Pl-Fz7 was analyzed by 5′ RACE PCR. The Fz7 sequence submitted to GenBank (HQ322502) includes both 5′UTR and ORF sequences. *Pl*-Wnt6 (HQ322504) was generously provided by Thierry Lepage (CNRS, Villefranche-sur-Mer, France). pCS2+Fz7 (entire ORF), pCS2+DnFz7 (1-255 amino acids), pCS2+Wnt6 (entire ORF) and pCS2+DnWnt6 (1-107 amino acids) constructs were obtained by subcloning the corresponding coding sequences into the pCS2+ vector (Turner and Weintraub, 1994). pCS2+DnFz7-Flag was produced through the insertion of DnFz7 (1-255 amino acids) into the pCS2+-Flag vector kindly provided by Muriel Umbhauer (CNRS, Paris, France). pCS2+DnFz7ΔCRD-Flag was made by PCR reaction using as template pCS2+DnFz7-flag. In this construct, only the N-terminal cysteine-rich Wnt-binding domain was eliminated, whereas the upstream peptide signal for membrane localization and the first downstream transmembrane domain were maintained. pCS2+Fz7mut was generated by site-directed mutagenesis to exchange within the C-terminal sequence KTXXXW the tryptophan (W) residue for a glycine (G).

mRNA microinjections

5′ capped mRNAs for Fz7, DnFz7, DnFz7-Flag, DnFz7ΔCRD-Flag, Fz7mut, Wnt6 and DnWnt6 were generated using the mMessage mMachine kit (Ambion). After dilution into DEPC H_2O, they were microinjected into unfertilized eggs as previously described (Emily-Fenouil et al., 1998) at the following concentrations: Fz7 up to 1.5 μg/μl; DnFz7 and DnFz7-Flag at a final concentration of 0.7 μg/μl (note that even when microinjected at 1.1 μg/μl DnFz7 mRNA produced identical developmental defects; supplementary material Fig. S1); DnFz7ΔCRD-Flag at 0.7 μg/μl and 1.4 μg/μl; Fz7mut at 2 μg/μl; Wnt6 at 0.012 μg/μl or 0.05 μg/μl; and DnWnt6 at 0.2 μg/μl. In all experiments, but the animal-vegetal swap and the TopFlash assays, control embryos correspond to uninjected embryos.

Expression analysis

Northern blot investigations were performed as previously described (Croce et al., 2003). ^{32}P-labeled probes for Fz7 (HQ322502), Fz9 (JN712908) and Fz4 (JN712907) ORF were synthesized using the Prime-a-Gene Labeling System (Promega).

Whole-mount in situ hybridization analyses were carried out as previously reported (Croce et al., 2003). Accession Numbers of all probes used in this study are as follows: Blimp1, HQ322503; Brachyury, CAD11971; Coquillette (Tbx2/3), CAD48605; Delta, ABG00198; FoxA, ABX71819; Fz7, HQ322502; GataC, ACZ62636; Gcm, ABG66953; NLK, AAS00536; Nodal, AAS00534; Papss, DQ531774; Ske-T (Tbr), CAC51029; Wnt8, HQ322501.

Immunostaining analyses were carried out as previously described (Sherwood and McClay, 1997) using the endoderm marker EndoI (Wessel and McClay, 1985), the skeletogenic mesoderm marker 1d5 (Hardin et al., 1992), SoxB1 as a counterstain (Kenny et al., 1999) and β-catenin antibodies (Logan et al., 1999). Images were acquired using a Leica SP2 or SP5 confocal microscope.

For flag-tagged protein immunolabeling, the embryos were fixed 20 minutes in cold methanol at the 60-cell stage. After rehydration in TBST (1×), the embryos were incubated 30 minutes in blocking solution (2% heat-inactivated sheep serum, 5 mg/ml BSA in TBST 1×), before a 1-hour incubation at room temperature with the anti-flag antibodies [monoclonal anti-flag M2-peroxidase (Sigma)] diluted at 1:1000 in blocking solution. The peroxidase-conjugated antibodies were revealed using the TSA-plus kit (Perkin Elmer), following the manufacturer's recommended protocol. Images were obtained on a Zeiss Imager M2 upright fluorescent microscope.

TOP-Flash reporter assay

Sea urchin eggs were microinjected with 5 ng/μl linearized TOP-Flash reporter plasmid together with a linearized carrier DNA (25 ng/μl). In some cases, these embryos were further treated with lithium chloride (30 mM) or co-injected with DnFz7 mRNA (0.7 μg/μl). Reporter activity was determined with the Luciferase Assay System (Promega) and using a GloMax luminometer. For each experiment, 200 embryos were collected, reporter activity was measured twice and each experiment was repeated three times. Supplementary material Fig. S2 displays the luciferase activity measured in whole embryos at successive developmental stages. Note that, even though nuclear β-catenin accumulation starts in the micromeres at the 16-cell stage (Logan et al., 1999), the activity of nuclear β-catenin remains undetectable up to the 60-cell stage (almost 2 hours later). Therefore, as β-catenin nuclearisation in the macromeres occurs between the 32- and the 60-cell stage (5 and 6 hpf, respectively), the activity of nuclear β-catenin both in micromeres and macromeres is most likely to become detectable only by 8 hpf, the stage at which the luciferase activity further seems to

reach a plateau (supplementary material Fig. S2). For this reason, the level of nuclear β-catenin activity in DnFz7 embryos was assessed at 8 hpf. The relative level of luciferase activity in Fig. 5 was normalized to the level detected in Top-Flash untreated control embryos.

RESULTS

Frizzled1/2/7 is expressed maternally and dynamically throughout embryogenesis, with only a transient allocation to the vegetal hemisphere

In the EST database of *Paracentrotus lividus* (http://goblet.molgen.mpg.de/cgi-bin/webapps/paracentrotus.cgi?url=main), all four Frizzled genes annotated in the genome of *Strongylocentrotus purpuratus* (Croce, J. C. et al., 2006) were identified. A northern blot analysis indicated that one of these molecules was strongly expressed in unfertilized eggs: Frizzled1/2/7 (Fig. 1A; supplementary material Fig. S3) (Croce, J. et al., 2006).

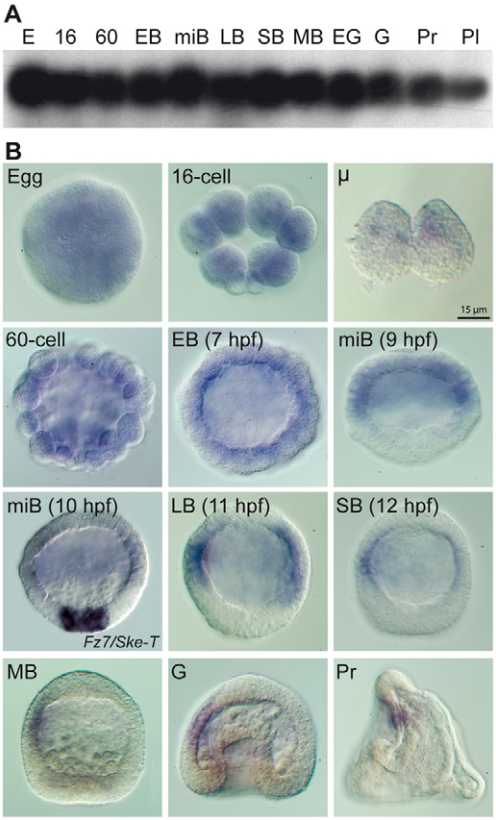

Fig. 1. Characterization of Frizzled1/2/7 expression during sea urchin embryogenesis. (**A**) Temporal expression profile of Fz7 analyzed by northern blot. Fz7 is expressed throughout embryogenesis. (**B**) Spatial distribution of Fz7 mRNAs revealed by in situ hybridization. At the 16-cell stage, even though the micromeres seemed to be devoid of Fz7 mRNA, examination of isolated micromeres (μ) confirmed that these cells were positive for Fz7 transcripts. Stages are as follows: E and Egg, fertilized egg; 16 and 16-cell, 16-cell stage; 60 and 60-cell, 60-cell stage; EB, early blastula; miB, mid-blastula; LB, late blastula; SB, swimming blastula; MB, mesenchyme blastula; EG, early gastrula; G, gastrula; Pr, prism; Pl, pluteus. In B, all embryos are about 90 μm in diameter, except μ (scale bar: 15 μm). All embryos are in lateral view and display solely Fz7 expression, except for the mid-blastula stage embryo labeled Fz7/Ske-T, which is labeled for both Fz7 and Ske-T (T-Brain), a skeletogenic mesoderm marker.

Whole-mount in situ hybridization experiments revealed that Fz7 transcripts were distributed ubiquitously in eggs and early embryos, at least up to 7 hours post-fertilization (hpf) (Fig. 1B) [similar to that previously reported in *S. purpuratus* eggs and 16-cell stage embryos (Stamateris et al., 2010)]. At 9 hpf or mid-blastula stage, Fz7 mRNAs were observed in only one half of the embryo, identified as the animal half through a double staining performed with the micromere descendants marker Ske-T (or T-Brain) (Croce et al., 2001; Fuchikami et al., 2002) (Fig. 1B). By late blastula stage (11 hpf), Fz7 expression was also extinguished from the apical pole domain, and from that stage onwards, Fz7 staining was progressively restricted to the stomodeum region of the embryo where it was maintained at least up to the prism stage (Fig. 1B). Thus, Fz7 is expressed maternally and its spatial distribution is dynamic throughout embryogenesis, with only a transient allocation to the vegetal hemisphere during an early ubiquitous phase.

Frizzled1/2/7 signaling is involved in archenteron formation

To assess the function of Fz7, we engineered a dominant-negative form of the receptor (DnFz7). This strategy was adopted because of a concern with the possible presence of maternal proteins that would render a morpholino antisense oligonucleotide (MASO) approach ineffective. DnFz7 proteins encode a truncated form of Fz7 that retains only the N-terminal peptide signal, the Wnt-binding cysteine-rich domain (CRD) and the first transmembrane domain of the receptor, similar to dominant-negative forms of Frizzled used in previous studies (Croce, J. et al., 2006; Kim et al., 2002). Microinjection of DnFz7 mRNAs into *P. lividus* eggs led to embryos at 24 hpf that failed to gastrulate and displayed only a blastopore-like structure at the vegetal pole (Fig. 2A). At 72 hpf, the DnFz7 larvae were still missing an archenteron, yet they exhibited an oral-aboral polarity, visible both by the presence of a thick and a thin epithelium and an enlarged stomodeum and had developed pigment cells and skeleton rods, though these were mispatterned (Fig. 2A). Thus, downregulation of Fz7 signaling mainly affected archenteron formation, with no noticeable effects on ectoderm or mesoderm development.

Several control experiments were next carried out to challenge the specificity of the DnFz7-induced defects. First, overexpression of a wild-type form of Fz7, which does not induce any developmental defects by itself (data not shown), rescued DnFz7-associated phenotypes. As previously performed in the Frizzled5/8 investigation (Croce, J. et al., 2006), twice the concentration of Fz7 mRNA (1.4 μg/μl) was co-injected with DnFz7 (0.7 μg/μl). In 92% of the embryos scored, this co-injection rescued formation of apparently normal embryos with a tripartite gut and a bilateral skeleton (Fig. 2B). Thus, this result supported the notion that DnFz7 effects were indeed caused by out-competing endogenous Fz7 proteins. Second, based on the knowledge that dominant-negative Frizzled molecules act by sequestering the Wnt ligands (e.g. Deardorff et al., 1998), we tested whether a mutated form of DnFz7, deleted of its extracellular Wnt-binding domain (DnFz7ΔCRD), would affect development. As negative results were anticipated, an additional internal control was required, and flag-tagged-DnFz7ΔCRD and -DnFz7 constructs were generated to monitor protein presence. When microinjected at a similar concentration to DnFz7, the DnFz7-flag construct caused defects comparable with DnFz7 (compare Fig. 2C with 2A), establishing that the presence of the flag tag at the C-terminal end of the truncated receptor did not interfere with its ability to inhibit Fz7

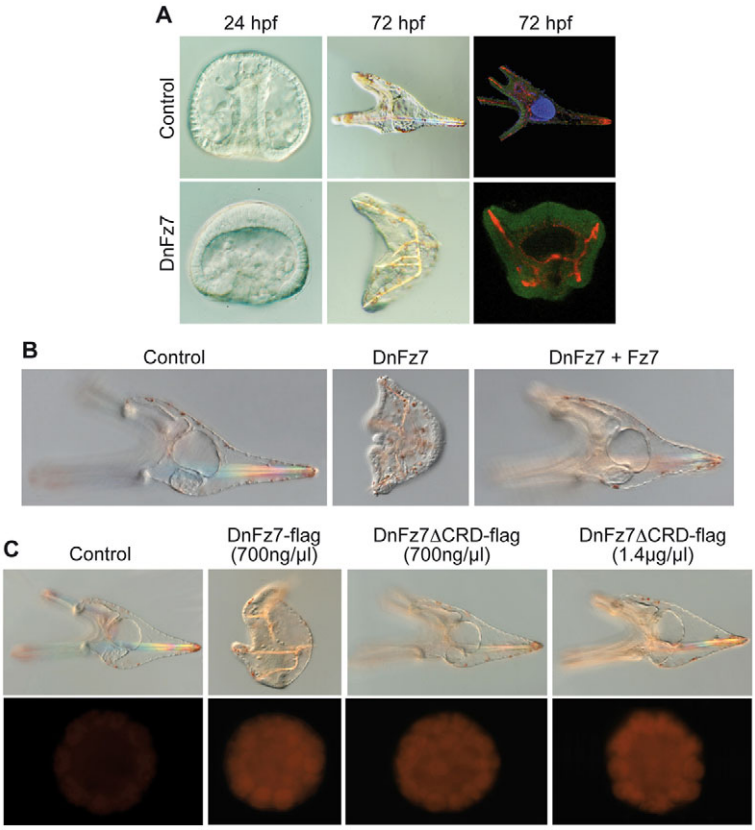

Fig. 2. Frizzled1/2/7 is required for archenteron development. (**A**) Phenotypes resulting from Fz7 signaling inhibition at indicated time points. Two first columns are DIC images. The last column shows immunostained embryos at 72 hours post-fertilization (hpf) using an endoderm marker (blue), a skeletogenic mesoderm marker (red) and a green counterstain. (**B**) Rescue experiment showing that overexpression of wild-type Fz7 mRNA counteracts DnFz7 development defects. (**C**) Microinjection of a mutated form of DnFz7, depleted of its Wnt cysteine-rich binding domain (CRD) (DnFz7ΔCRD), does not induce any developmental defects when injected at the same concentration or at twice the concentration of DnFz7. The bottom line displays 60-cell stage embryos microinjected with DnFz7-flag and DnFz7ΔCRD-flag mRNA, and immunostained with an anti-flag antibody (red) to ascertain production of the exogenous proteins.

signaling. By contrast, expression of mRNA encoding DnFz7ΔCRD-flag at the same or twice the concentration of DnFz7-flag did not affect development (Fig. 2C), even though antibodies against the flag tag indicated that the proteins were present (Fig. 2C). Thus, these results reinforced the hypothesis that DnFz7 acts as a dominant-negative form of its related receptor and validated our functional analysis strategy.

Frizzled1/2/7 is required for endoderm specification

To characterize the DnFz7-induced phenotypes, an in situ hybridization analysis was conducted using several molecular markers, each specific for a major embryonic territory. First, the expression profile of four endoderm genes, Wnt8, Blimp1, Brachyury and FoxA, was examined. In control mesenchyme blastulae (15 hpf), each of these genes is expressed exclusively in the endoderm territory at the periphery of the vegetal plate, except for Brachyury, which is also transcribed in the oral ectoderm (Fig. 3A). In DnFz7 embryos, all endoderm markers were absent in the vegetal plate, while Brachyury expression in the oral ectoderm was unaffected (Fig. 3A). The expression of FoxA at 24 hpf was also missing in the DnFz7 embryos, highlighting that the blastopore-like structure observed at that stage was not made up of endoderm cells. Consistent with Brachyury oral expression, the restricted transcriptional domain of Nodal and Coquillette (or Tbx2/3) on either side of the ectoderm territory (oral and aboral, respectively) was unaltered by the loss of the Fz7 signal (Fig. 3B). Similarly, none of the skeletogenic or non-skeletogenic mesoderm markers tested was impaired by downregulation of Fz7, whether analyzed early in development (60-cell stage) or late (mesenchyme blastula or gastrula) (Fig. 3C,D). This analysis, when performed at 24 hpf with non-skeletogenic mesoderm markers, further testified that the nature of the blastopore-like structure was formed of mesoderm cells. Thus, this molecular analysis showed that loss of Fz7 signaling has no noticeable effect on ectoderm markers, oral-aboral polarity, or skeletogenic mesoderm or non-skeletogenic mesoderm markers. By contrast, these data strongly support a crucial role for the Fz7 pathway in endoderm specification, providing a functional clue to the absence of an archenteron in the DnFz7 embryos (Fig. 2A).

Frizzled1/2/7 is required in the vegetal hemisphere for archenteron formation

From the results described above, even though Fz7 transcripts are ubiquitously distributed at the 32-cell stage, they are no longer detected in the vegetal half of the embryo starting at the mid-blastula stage (9 hpf); hence, many hours before archenteron invagination begins (usually around 18-19 hpf) (Fig. 1B). This suggested therefore two possibilities: (1) the signal transduced by Fz7 prior to 9 hpf during its ubiquitous phase is crucial for archenteron development or (2) somehow it is the animal Fz7 transcripts that are involved in archenteron formation. To distinguish between these two possibilities, a mosaic analysis was conducted. Chimeras were produced in which Fz7 signaling was downregulated only in the animal half or in the vegetal half of the embryos (Fig. 4). When Fz7 signaling was downregulated only in the animal half, 93% of the chimeras (n=14) produced complete pluteus larvae with a normal tripartite archenteron and a normal skeleton (Fig. 4). By contrast, when the Fz7 pathway was impaired in the vegetal half, 71% of the chimeras (n=17) developed without production of an archenteron (Fig. 4), an outcome that is similar to that observed with microinjection of DnFz7 in whole embryos. Thus, the deficit in archenteron formation in absence of Fz7

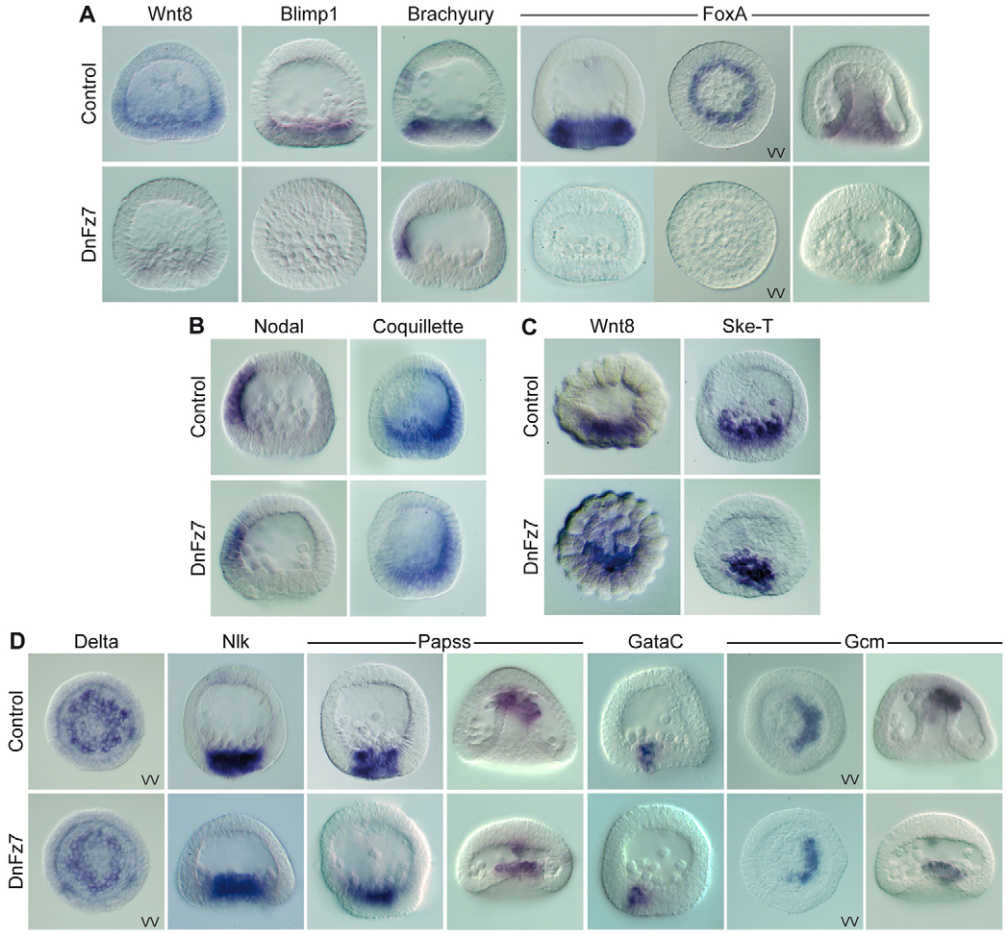

Fig. 3. Frizzled1/2/7 is required for endoderm specification. In situ distribution of mRNA encoding germ layer markers in control and DnFz7-injected embryos. (**A**) Four endoderm specification genes, including Brachyury, which is expressed both in the vegetal plate and the oral ectoderm. (**B**) Two ectoderm markers staining, respectively, the oral (Nodal) and aboral (Coquillette-Tbx2/3) ectoderm. (**C**) Two skeletogenic mesoderm markers. (**D**) Five distinct non-skeletogenic mesoderm markers that either label all (Delta, Nlk, Papss) or a subset (GataC, Gcm) of the non-skeletogenic mesoderm cells. Expression patterns of all genes are shown in control and DnFz7 embryos, as indicated. All embryos are in lateral view unless otherwise indicated as 'vv' for vegetal view. All embryos are at the mesenchyme blastula stage, except the Wnt8 section (analyzed also at the 60-cell stage in C), and FoxA, Papss and Gcm sections [shown also at 24 hpf (gastrula stage)].

signaling was due to the vegetal component of the initial, transient, ubiquitous expression of Fz7 from the egg to the mid-blastula stage.

Frizzled1/2/7 signaling regulates the nuclear entry of β-catenin in the macromeres

The cytoplasmic C-terminal motif KTXXXW is crucial for Frizzled activation of the canonical Wnt pathway (Umbhauer et al., 2000). To determine whether Fz7 acts through this pathway, another dominant-negative mutated form of Fz7 was generated that exhibited a KTXXXG domain (Fz7mut). At 24 hpf, embryos microinjected with Fz7mut mRNA did not exhibit an elongated archenteron. By 72 hpf, these embryos still failed to gastrulate, although they had developed pigment cells, skeletal rods, an oral-aboral polarity and an enlarged stomodeum (Fig. 5A). The Fz7mut phenotype was thus identical to the DnFz7 phenotype (compare Fig. 5A with Fig. 2A), strongly suggesting that Fz7 acts through the canonical Wnt pathway. To further test this hypothesis, immunostaining experiments were performed using a sea urchin β-catenin antibody (Logan et al., 1999), allowing assessment of whether Fz7 triggers β-catenin nuclearisation. Embryos were fixed at the 32-cell stage (5th cleavage) when controls exhibit β-catenin proteins in the nuclei of both the micromeres (large and small) and the macromere progeny, but not in those of the mesomere progeny, the animal pole blastomeres (Fig. 5B). This pattern was observed in 84.5% of the embryos analyzed by confocal microscopy (n=13), as previously described in *Lytechinus variegatus* (Logan et al., 1999). In embryos microinjected with DnFz7, β-catenin was observed at the 5th cleavage solely in the nuclei of the micromeres (large and small) and not in nuclei of the eight macromere daughter cells (Fig. 5B) (80%, n=10). These data therefore support the hypothesis that Fz7 is required at the 32-cell stage to initiate β-catenin nuclear entry selectively into the macromeres. Finally, a quantitative analysis of nuclear β-catenin activity carried out using a TOP-Flash reporter plasmid corroborated this result. At 8 hpf, when both micromere and macromere nuclear β-catenin activity can be analyzed (see Materials and methods for details), the level of nuclear β-catenin activity determined in DnFz7 embryos was half the level identified in control embryos (Fig. 5C). This result again supports the notion that the initial function of Fz7 is to activate the canonical Wnt pathway selectively in the macromeres, without affecting micromeres in which canonical Wnt signaling is evidently activated by another mechanism.

Frizzled1/2/7 signaling is required for gene expression in the endomesoderm precursor cells

After their emergence at the 4th cleavage and a doubling at the 5th cleavage, the macromeres divide then at the 6th cleavage equatorially, giving rise to the Veg1 and the Veg2 cell tiers. Nuclear β-catenin continues in the Veg2 cells, at least up to hatching, whereas it quickly disappears from the Veg1 cells after their formation (Logan et al., 1999). The Veg2 cells later produce both endoderm and non-skeletogenic mesoderm descendants that segregate from one another at around hatching in both *L. variegatus* and *S. purpuratus* sea urchin species (Croce and

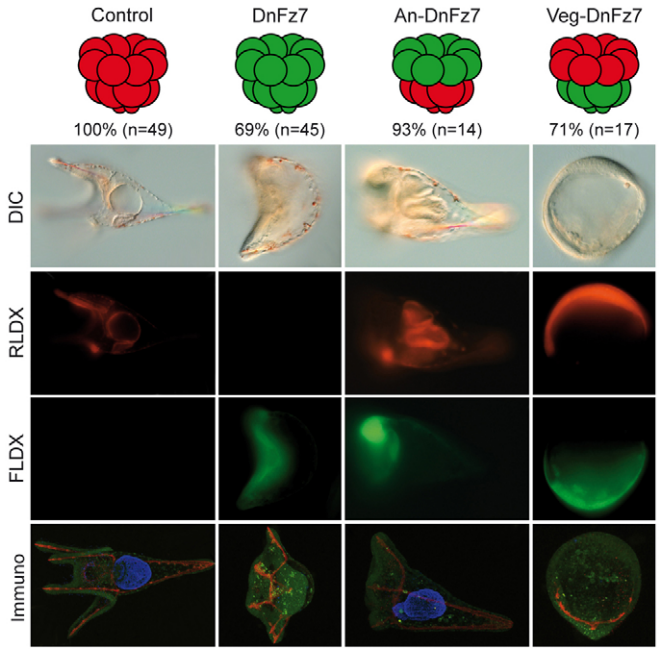

Fig. 4. Frizzled1/2/7 is necessary within the vegetal hemisphere to ensure archenteron development. Animal-vegetal chimeric swaps carried out between the 16- and 32-cell stages and that recombine RLDX-injected control embryos (red) with DnFz7-FLDX-injected embryos (green). First row, schematic representation of the experiment. Second row, DIC images of unperturbed embryos and chimeras. Third and fourth rows, fluorescent images of the same embryos shown in row two to reveal the RLDX and FLDX staining. Fifth row, immunostaining of the same embryos displayed in row two to highlight the presence of endoderm (blue) and skeletogenic mesoderm (red), plus a green counterstain to reveal the shape of the larvae. Embryos were all examined at 48 hours post-fertilization.

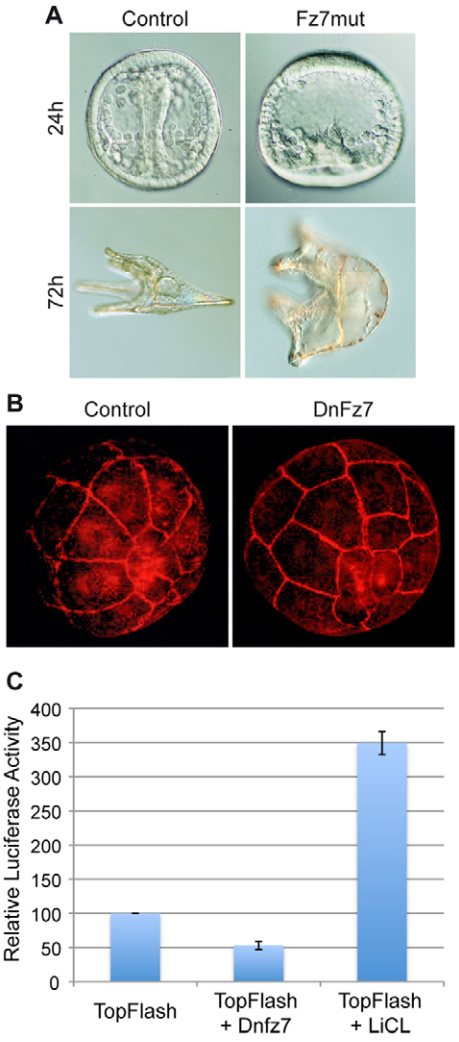

Fig. 5. Frizzled1/2/7 acts through the canonical Wnt pathway and triggers β-catenin nuclearisation specifically in the macromeres. (**A**) Phenotypes associated with the use of the KTXXXG mutated form of Fz7 (Fz7mut). Fz7mut produced phenotypes similar to DnFz7. (**B**) Nuclear distribution of endogenous β-catenin proteins at the 32-cell stage in control and DnFz7 embryos. Fz7 signaling is required for nuclear translocation of β-catenin in macromeres but not in micromeres. (**C**) Nuclear β-catenin activity 8 hours post-fertilization assessed in control, DnFz7 or LiCl-treated embryos using a TOP-Flash reporter assay. Data are mean±s.e.m. of three experiments.

McClay, 2010; Peter and Davidson, 2010). So far, our results establish that Fz7 is necessary for early nuclear entry of β-catenin into the macromeres (Fig. 5), and subsequently for endoderm but not for non-skeletogenic mesoderm specification, at least when assessed at the mesenchyme blastula and gastrula stages (Fig. 3). The next experiments addressed molecular information to determine how these two observations were related. At the 7th cleavage, immediately after the emergence of the Veg2 tier, two of the earliest genes detected in these cells are Wnt8 and Blimp1, which at that stage are also expressed in the micromeres (Fig. 6A,B). In DnFz7 embryos, although neither Wnt8 nor Blimp1 expression was affected in the micromeres, expression of both genes was absent in the Veg2 cells (Fig. 6B). These results therefore reinforced the non-involvement of Fz7 in skeletogenic mesoderm specification (Fig. 3C) and showed that Fz7 signaling is necessary within the Veg2 cells to positively regulate transcription of at least Wnt8 and Blimp1.

A few cell cycles later, but prior to the endoderm/non-skeletogenic mesoderm segregation, the progeny of the Veg2 cells co-express FoxA and Gcm, which are later expressed separately in endoderm (FoxA) and non-skeletogenic mesoderm (Gcm) descendants (Fig. 6A). In *P. lividus* embryos, FoxA and Gcm are both detected in a ring of cells that surround the micromere progeny at the mid-blastula stage (9 hpf) (Fig. 6C). In DnFz7 embryos, at that stage, Gcm expression was unaltered whereas FoxA expression was undetectable (Fig. 6C). Thus, FoxA and Gcm respond differently to Fz7 perturbations, which appear to influence only expression of endomesoderm genes that will later function in endoderm.

Wnt6 mediates nuclear entry of β-catenin into the macromeres and is required for endoderm gene expression

In a recent study involving the maternal Wnt ligand Wnt6, we observed that embryos depleted of Wnt6 activity [either through the use of a dominant-negative form (DnWnt6) or of an antisense morpholino oligonucleotides] failed to develop an archenteron or express endoderm markers, whereas transcription of skeletogenic and non-skeletogenic mesoderm genes was seemingly unaffected (Croce et al., 2011). The striking similarities between the developmental defects induced by Wnt6 knockdown and Fz7

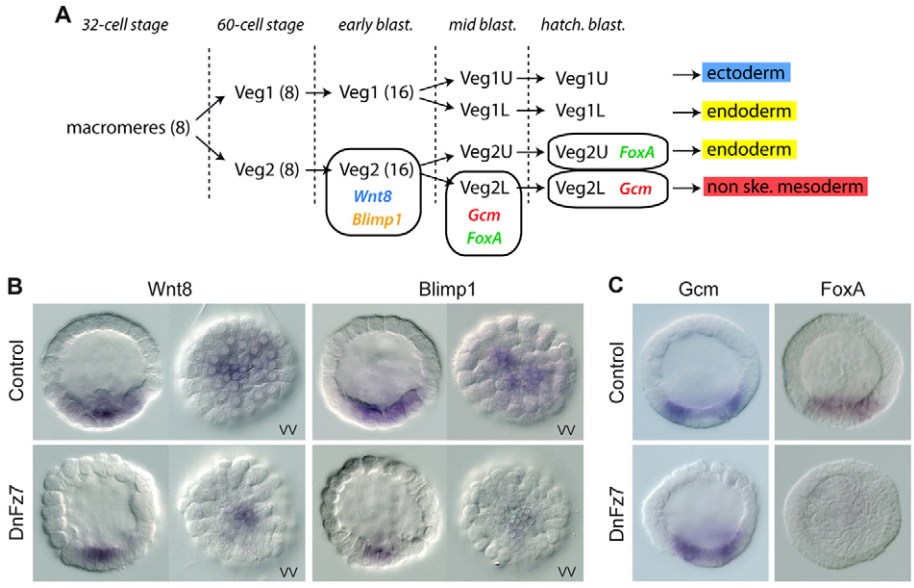

Fig. 6. Frizzled1/2/7 regulates gene expression in endomesoderm cells. (**A**) Schematic representation of the endomesoderm lineage from its emergence to its segregation into endoderm and non-skeletogenic mesoderm during sea urchin embryogenesis. Adapted, with permission, from Croce and McClay (Croce and McClay, 2010). (**B**) Expression profile of Wnt8 and Blimp1 in control and DnFz7-injected embryos at the early blastula stage (7 hpf). (**C**) Expression of Gcm and FoxA, a non-skeletogenic mesoderm and an endoderm specification gene, respectively, in control and DnFz7-injected embryos at the mid-blastula stage (9 hpf). All embryos are in lateral view unless indicated 'vv' for vegetal view.

inhibition suggested that Wnt6 could be the ligand of Fz7 during the initial activation of the endoderm gene regulatory circuit. To test this hypothesis, we perturbed the Wnt6 input and determined the effects on Wnt8 and Blimp1 expression at the 7th cleavage, on FoxA and Gcm expression at the mid-blastula stage (9 hpf), and on β-catenin nuclearisation at the 5th cleavage. In each case, DnWnt6 microinjection had a similar result to that seen with DnFz7, whereas Wnt6 overexpression produced the exact opposite outcome (compare Fig. 7 with Figs 5 and 6; supplementary material Fig. S4) (Croce et al., 2011). Importantly, DnWnt6 embryos at 5th cleavage displayed nuclear β-catenin only in the micromeres (large and small) and not in the macromeres (Fig. 7C) (87.5%, *n*=8), whereas in Wnt6-overexpressing embryos, β-catenin was found in the nuclei of the micromeres, the macromeres and the mesomeres (Fig. 7C) (73.3%, *n*=15), strengthening the notion that Wnt6 is able to convey β-catenin nuclearisation. In addition, in embryos misexpressing Wnt6, the expression domains of Wnt8, Blimp1 and FoxA were greatly enlarged towards the animal pole compared with controls, whereas Gcm expression was unaffected by this perturbation (Fig. 7B). Thus, Wnt6, like Fz7, is necessary in Veg2 cells to initiate expression of endoderm but not non-skeletogenic mesoderm markers, and it must be present to initiate the nuclear entry of β-catenin selectively in the macromeres.

Frizzled1/2/7 and Wnt6 most probably act as a Wnt/Frizzled pair

Our final set of experiments tested whether Wnt6 and Fz7 may act as a ligand/receptor pair. Although it is unclear to date how dominant-negative Wnt ligands inhibit their related pathways, we assumed it may be by interacting with their targeted Frizzled receptors but without activating them, just as dominant-negative Frizzled proteins bind ligands but fail to transduce the signal (e.g. Deardorff et al., 1998). Accordingly, two reciprocal rescue experiments were performed to determine whether the developmental defects induced by the inhibition of one protein could be rescued by titration with the wild-type form of the other molecule. When increasing concentrations of Fz7 mRNAs were co-injected with a constant amount of DnWnt6, a progressive recovery of archenteron formation and of the overall shape of the larvae was observed (Fig. 8A). Similarly, when higher concentrations of Wnt6 mRNA were used in combination to a constant amount of DnFz7, a complete rescue of archenteron formation was obtained, up to the point where a normal pluteus larva with a tripartite gut and a correctly patterned skeleton developed (Fig. 8B). These results therefore support the notion that Wnt6 and Fz7 are binding partners, and that they most probably function together to activate canonical Wnt signaling and endoderm specification during sea urchin embryogenesis.

DISCUSSION

Frizzled1/2/7 and Wnt6 behave as a Wnt/Frizzled pair to activate canonical Wnt signaling in the macromeres

Here, several results suggest that Wnt6 and Fz7 behave as a ligand/receptor pair: (1) downregulation of either Fz7 signaling or loss of the Wnt6 input similarly impair β-catenin nuclearisation solely in the macromeres (Figs 5, 7); (2) both perturbations impede Wnt8 and Blimp1 expression only in Veg2 cells (Figs 6, 7); (3) both perturbations prevent also endoderm specification but not skeletogenic or non-skeletogenic mesoderm fate establishment (Figs 3, 6, 7) (Croce et al., 2011); (4) overexpression of the wild-type form of Wnt6 or Fz7 is sufficient to reverse the developmental defects induced by the dominant-negative form of the other molecule (DnFz7 or DnWnt6, respectively) (Fig. 8). Thus, with the caveat that these results do not provide a direct biochemical demonstration of an endogenous interaction between the Wnt6 ligand and the Fz7 receptor, they nevertheless strongly support that relationship. Furthermore, although Wnt6 may not be the only ligand that binds to Fz7 [as 11 Wnt ligands are present in the genome to activate a total of only four Frizzled receptors (Croce, J. C. et al., 2006)], that particular combination does appear to act at the 5th cleavage to activate canonical Wnt signaling in the macromeres and to steer their progeny towards an endoderm fate.

Throughout early embryogenesis Wnt6 and Fz7 mRNAs are ubiquitously distributed (Fig. 1) (Croce et al., 2011), yet the data demonstrate that they control β-catenin nuclearisation selectively in macromeres (Figs 5, 7). This is puzzling, but several explanations could account for that selective activation of the canonical Wnt pathway. For example, even though Wnt6 and Fz7 are uniformly transcribed in early embryos, Wnt6 proteins may well be produced

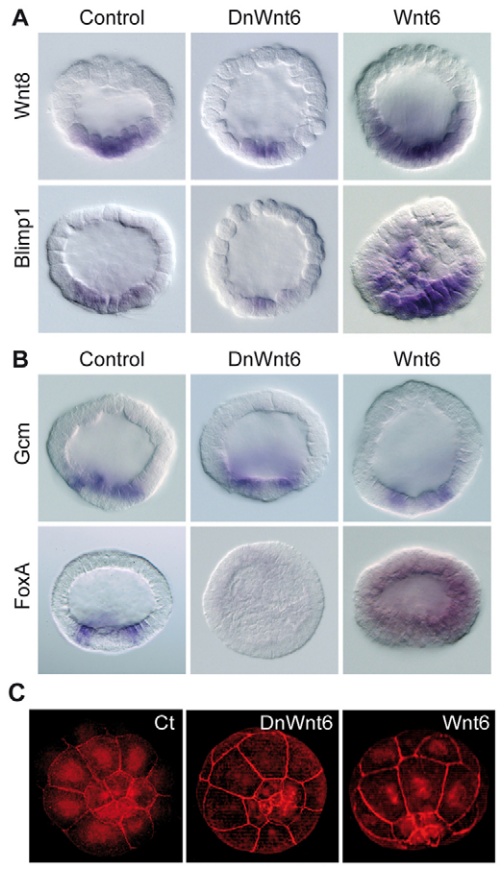

Fig. 7. Wnt6 is required for gene expression in endomesoderm cells and for the initial nuclear entry of β-catenin in macromeres. (**A**) Expression profile of Wnt8 and Blimp1 in controls, DnWnt6 and Wnt6 overexpressing embryos at the early blastula stage (7 hpf). (**B**) Expression pattern of FoxA and Gcm at the mid-blastula stage (9 hpf) in control, DnWnt6 and Wnt6-overexpressing embryos. (A,B) All embryos are in lateral view. (**C**) Immunostaining for endogenous β-catenin proteins in 32-cell stage control, DnWnt6 and Wnt6-overexpressing embryos.

solely in the vegetal blastomeres. Supporting this hypothesis, misexpression of Wnt6, and not of Fz7, leads to ectopic nuclear β-catenin and endoderm specification in the animal hemisphere (Fig. 7) (Croce et al., 2011). Alternatively, a system that represses Wnt6 could be present in the animal blastomeres that would be overcome by Wnt6 misexpression. Whatever the mechanism may turn out to be, the data here support the conclusion that Wnt6 signal transduction operates specifically on macromeres, most probably through Fz7, to initiate eventually endoderm specification.

In addition, we showed here that nuclear entry of β-catenin in macromeres, but not in micromeres, depends upon the Wnt6/Fz7 signal transduction. Minimally, this suggests that β-catenin nuclearisations in macromeres and micromeres are driven by distinct mechanisms. Superficially, this finding is in agreement with the activation mechanism model proposed by Smith and collaborators in 2007 (Smith et al., 2007). In their model, the authors suggested that after the cell-autonomous entry of β-catenin into the micromere nuclei, the micromeres produce Wnt8 that in turn activates the canonical Wnt pathway in macromeres. Our work clearly indicates that, in *P. lividus*, it is Wnt6 and not Wnt8 that is responsible for β-catenin nuclear accumulation in the macromeres. In the absence of Wnt6 or Fz7, even if Wnt8 is produced by the micromeres, β-catenin nuclearisation does not take place in macromeres, nor does expression of Wnt8 and Blimp1 (two genes thought to be direct targets of canonical Wnt signaling) in the Veg2 cells at the 7th cleavage (Figs 6, 7). Thus, even though in *P. lividus*, we do not know yet from where the Wnt6 input originates, it is clear that the endoderm specification sequence mediated by the activation of nuclear β-catenin is triggered by Wnt6.

β-Catenin is not required at the 32-cell stage in macromeres for non-skeletogenic mesoderm specification

In sea urchins, some of the endoderm and all of the non-skeletogenic mesoderm arise from the Veg2 cells. Segregation of this lineage into endoderm and non-skeletogenic mesoderm descendants can be revealed over embryogenesis either through lineage analyses or at the molecular level through the investigation of the expression profiles of endoderm and non-skeletogenic mesoderm specification genes, including FoxA and Gcm, respectively (Croce and McClay, 2010; Peter and Davidson, 2010; Ruffins and Ettensohn, 1996). Here, we show that activation of the canonical Wnt pathway in the macromeres at the 32-cell stage is not required to initiate or then maintain Gcm expression, at least up to the gastrula stage (Figs 3, 6 and 7). This may therefore indicate that non-skeletogenic mesoderm solely relies on the Delta/Notch signal originating from the micromeres starting at the 7th cleavage (Croce and McClay, 2010), which is unaffected by loss of Fz7 signaling. However, it has been shown previously that embryos depleted of nuclear β-catenin selectively in the mesomeres and the macromeres, but not in micromeres, by the microinjection of cadherin mRNA followed by chimeric swap, did not develop SMC1-positive cells, hence non-skeletogenic mesoderm cells (McClay et al., 2000). That set of experiments suggested thus that canonical Wnt signaling was required at some point during embryogenesis (most likely in the macromeres), to ensure non-skeletogenic mesoderm development. Here, although we have clearly established the loss of nuclear β-catenin selectively in the macromeres at the 32-cell stage in absence of Wnt6/Fz7 signaling, we have not determined whether or not this impairment continues throughout embryogenesis, as is the case with the microinjection of the β-catenin-binding cadherin. It is possible, therefore, that a later Wnt pathway input is required after the 32-cell stage, but prior to the mid-blastula stage (stage at which Gcm expression starts to be detected), to initiate non-skeletogenic mesoderm specification, or that it is involved later on to maintain this fate and/or contribute to its differentiation; these are issues that we will assess in the future.

Evolutionary perspectives on the role of Wnt6 and Frizzled1/2/7 in activating canonical Wnt signaling

Because of the important role of canonical Wnt signaling during animal development, this pathway has been investigated in a wide range of organisms (e.g. Darras et al., 2011; Henry et al., 2008; Imai et al., 2000; Wikramanayake et al., 2003). In all these animals, the nuclear accumulation of β-catenin in early embryogenesis has been described. However, in only a few model systems has the identity of the upstream activators of the pathway (Wnt and Frizzled molecules) been disclosed. In the ascidian *Halocynthia roretzi*, for example, the maternal Wnt ligand, Wnt5, was found to act upstream of β-catenin (Kawai et al., 2007). Similarly, in the zebrafish, a recent report highlighted the role of Wnt8a in the control of β-catenin nuclearisation (Lu et al., 2011). Comparatively, in the cnidarian *Clytia hemisphaerica*, the amphibian *Xenopus*

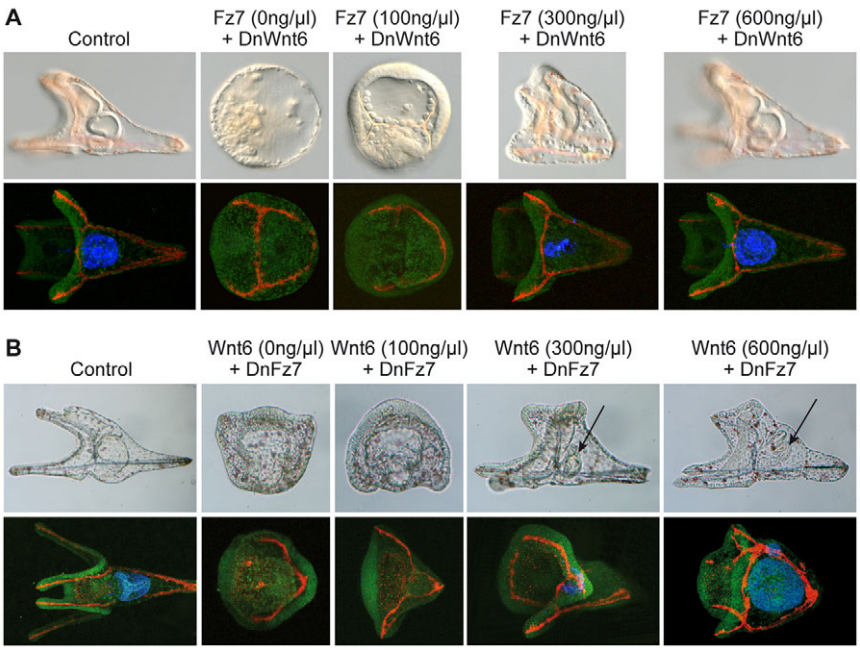

Fig. 8. Wnt6 and Frizzled1/2/7 rescue knockdowns of the other. (**A**) Rescue of DnWnt6 (0.2 µg/µl) by co-injection of increasing concentrations of Fz7. (**B**) Rescue of the DnFz7 phenotype by co-injection of DnFz7 mRNA (0.7 µg/µl) with rising amounts of Wnt6 mRNA. Top rows are DIC images and bottom rows are embryos immunostained with an endoderm marker (blue), a skeletogenic mesoderm marker (red) and a green counterstain. Arrows indicate the presence of an archenteron.

laevis and the nematode *Caenorhabditis elegans*, both maternal Wnt proteins (CheWnt3, Wnt-11 and mom-2, respectively) and Frizzled receptors (CheFz1, Fz7 and mom-5, respectively) were discovered to regulate expression of canonical Wnt signaling target genes and in some cases to induce β-catenin nuclear entry (Momose et al., 2008; Momose and Houliston, 2007; Nakamura et al., 2005; Sumanas et al., 2000; Tao et al., 2005). Here, we establish in yet another animal phylum, the echinoderms, the involvement of a Wnt/Frizzled pair in conducting a β-catenin nuclear localization event. Thus, a comparable activation mechanism of the canonical Wnt pathway using upstream maternal Wnt and Frizzled molecules is shared by at least five distinct animal phyla. Additionally, a comparison of the identities of the different Wnt and Frizzled molecules involved in this activation mechanism indicates that different Wnt ligands regulate β-catenin nuclearisation in these various animals [Wnt5, Wnt8a, Wnt3, Wnt11, Wnt6 and mom-2 (homologous with Wnt2, Wnt4 and Wnt7)], whereas a member of the same Frizzled receptor subfamily (Frizzled1/2/7) is involved in each case (supplementary material Fig. S5). Therefore, the conserved component of the canonical Wnt pathway activation mechanism appears primarily to be the receptor, a finding that may facilitate subsequent analyses on the nature of this mechanism in additional animal phyla.

Future directions

Although Wnt6 and Fz7 specifically direct β-catenin nuclear accumulation in macromeres, information is still missing about how this signal operates so specifically on these cells. In addition, it is still unclear to date whether or not canonical Wnt signaling is required at some point over embryogenesis for non-skeletogenic mesoderm fate development. These issues will be addressed in the future in order to reach a deeper understanding of the complex mechanisms of endomesoderm activation and segregation, and the role of the canonical Wnt pathway in early specification.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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References

Croce, J., Lhomond, G., Lozano, J. C. and Gache, C. (2001). ske-T, a T-box gene expressed in the skeletogenic mesenchyme lineage of the sea urchin embryo. *Mech. Dev.* **107**, 159-162.
Croce, J., Lhomond, G. and Gache, C. (2003). Coquillette, a sea urchin T-box gene of the Tbx2 subfamily, is expressed asymmetrically along the oral-aboral axis of the embryo and is involved in skeletogenesis. *Mech. Dev.* **120**, 561-572.
Croce, J., Duloquin, L., Lhomond, G., McClay, D. R. and Gache, C. (2006). Frizzled5/8 is required in secondary mesenchyme cells to initiate archenteron invagination during sea urchin development. *Development* **133**, 547-557.
Croce, J. C. and McClay, D. R. (2010). Dynamics of Delta/Notch signaling on endomesoderm segregation in the sea urchin embryo. *Development* **137**, 83-91.
Croce, J. C., Wu, S. Y., Byrum, C., Xu, R., Duloquin, L., Wikramanayake, A. H., Gache, C. and McClay, D. R. (2006). A genome-wide survey of the evolutionarily conserved Wnt pathways in the sea urchin *Strongylocentrotus purpuratus*. *Dev. Biol.* **300**, 121-131.
Croce, J. C., Range, R., Shu-Yu, W., Miranda, E., Lhomond, G., Peng, C. F., Lepage, T. and McClay, D. R. (2011). Wnt 6 activates endoderm in the sea urchin gene regulatory network. *Development* **138**, 3297-3306.
Darras, S., Gerhart, J., Terasaki, M., Kirschner, M. and Lowe, C. J. (2011). β-Catenin specifies the endomesoderm and defines the posterior organizer of the hemichordate *Saccoglossus kowalevskii*. *Development* **138**, 959-970.
Davidson, E. H., Rast, J. P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C. H., Minokawa, T., Amore, G., Hinman, V., Arenas-Mena, C. et al. (2002). A genomic regulatory network for development. *Science* **295**, 1669-1678.
Deardorff, M. A., Tan, C., Conrad, L. J. and Klein, P. S. (1998). Frizzled-8 is expressed in the Spemann organizer and plays a role in early morphogenesis. *Development* **125**, 2687-2700.
Emily-Fenouil, F., Ghiglione, C., Lhomond, G., Lepage, T. and Gache, C. (1998). GSK3β/shaggy mediates patterning along the animal-vegetal axis of the sea urchin embryo. *Development* **125**, 2489-2498.
Fuchikami, T., Mitsunaga-Nakatsubo, K., Amemiya, S., Hosomi, T., Watanabe, T., Kurokawa, D., Kataoka, M., Harada, Y., Satoh, N.,

Kusunoki, S. et al. (2002). T-brain homologue (HpTb) is involved in the archenteron induction signals of micromere descendant cells in the sea urchin embryo. *Development* **129**, 5205-5216.
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.
Henry, J. Q., Perry, K. J., Wever, J., Seaver, E. and Martindale, M. Q. (2008). β-catenin is required for the establishment of vegetal embryonic fates in the nemertean, *Cerebratulus lacteus*. *Dev. Biol.* **317**, 368-379.
Imai, K., Takada, N., Satoh, N. and Satou, Y. (2000). β-catenin mediates the specification of endoderm cells in ascidian embryos. *Development* **127**, 3009-3020.
Kawai, N., Iida, Y., Kumano, G. and Nishida, H. (2007). Nuclear accumulation of β-catenin and transcription of downstream genes are regulated by zygotic Wnt5alpha and maternal Dsh in ascidian embryos. *Dev. Dyn.* **236**, 1570-1582.
Kenny, A. P., Kozlowski, D., Oleksyn, D. W., Angerer, L. M. and Angerer, R. C. (1999). SpSoxB1, a maternally encoded transcription factor asymmetrically distributed among early sea urchin blastomeres. *Development* **126**, 5473-5483.
Kim, S. H., Shin, J., Park, H. C., Yeo, S. Y., Hong, S. K., Han, S., Rhee, M., Kim, C. H., Chitnis, A. B. and Huh, T. L. (2002). Specification of an anterior neuroectoderm patterning by Frizzled8a-mediated Wnt8b signalling during late gastrulation in zebrafish. *Development* **129**, 4443-4455.
Larabell, C. A., Torres, M., Rowning, B. A., Yost, C., Miller, J. R., Wu, M., Kimelman, D. and Moon, R. T. (1997). Establishment of the dorso-ventral axis in Xenopus embryos is presaged by early asymmetries in β-catenin that are modulated by the Wnt signaling pathway. *J. Cell Biol.* **136**, 1123-1136.
Lepage, T. and Gache, C. (1990). Early expression of a collagenase-like hatching enzyme gene in the sea urchin embryo. *EMBO J.* **9**, 3003-3012.
Logan, C. Y. and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **20**, 781-810.
Logan, C. Y., Miller, J. R., Ferkowicz, M. J. and McClay, D. R. (1999). Nuclear β-catenin is required to specify vegetal cell fates in the sea urchin embryo. *Development* **126**, 345-357.
Lu, F. I., Thisse, C. and Thisse, B. (2011). Identification and mechanism of regulation of the zebrafish dorsal determinant. *Proc. Natl. Acad. Sci. USA* **108**, 15876-15880.
McClay, D. R., Peterson, R. E., Range, R. C., Winter-Vann, A. M. and Ferkowicz, M. J. (2000). A micromere induction signal is activated by β-catenin and acts through notch to initiate specification of secondary mesenchyme cells in the sea urchin embryo. *Development* **127**, 5113-5122.
Miyawaki, K., Yamamoto, M., Saito, K., Saito, S., Kobayashi, N. and Matsuda, S. (2003). Nuclear localization of beta-catenin in vegetal pole cells during early embryogenesis of the starfish *Asterina pectinifera*. *Dev. Growth Differ.* **45**, 121-128.
Momose, T. and Houliston, E. (2007). Two oppositely localised frizzled RNAs as axis determinants in a cnidarian embryo. *PLoS Biol.* **5**, e70.
Momose, T., Derelle, R. and Houliston, E. (2008). A maternally localised Wnt ligand required for axial patterning in the cnidarian *Clytia hemisphaerica*. *Development* **135**, 2105-2113.
Nakamura, K., Kim, S., Ishidate, T., Bei, Y., Pang, K., Shirayama, M., Trzepacz, C., Brownell, D. R. and Mello, C. C. (2005). Wnt signaling drives WRM-1/beta-catenin asymmetries in early *C. elegans* embryos. *Genes Dev.* **19**, 1749-1754.
Oliveri, P. and Davidson, E. H. (2004). Gene regulatory network controlling embryonic specification in the sea urchin. *Curr. Opin. Genet. Dev.* **14**, 351-360.
Peter, I. S. and Davidson, E. H. (2010). The endoderm gene regulatory network in sea urchin embryos up to mid-blastula stage. *Dev. Biol.* **340**, 188-199.
Petersen, C. P. and Reddien, P. W. (2009). Wnt signaling and the polarity of the primary body axis. *Cell* **139**, 1056-1068.
Ruffins, S. W. and Ettensohn, C. A. (1996). A fate map of the vegetal plate of the sea urchin (*Lytechinus variegatus*) mesenchyme blastula. *Development* **122**, 253-263.
Schneider, S. Q. and Bowerman, B. (2007). β-Catenin asymmetries after all animal/vegetal-oriented cell divisions in *Platynereis dumerilii* embryos mediate binary cell-fate specification. *Dev. Cell* **13**, 73-86.
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.
Smith, J., Theodoris, C. and Davidson, E. H. (2007). A gene regulatory network subcircuit drives a dynamic pattern of gene expression. *Science* **318**, 794-797.
Stamateris, R. E., Rafiq, K. and Ettensohn, C. A. (2010). The expression and distribution of Wnt and Wnt receptor mRNAs during early sea urchin development. *Gene Expr. Patterns* **10**, 60-64.
Sumanas, S., Strege, P., Heasman, J. and Ekker, S. C. (2000). The putative wnt receptor Xenopus frizzled-7 functions upstream of β-catenin in vertebrate dorsoventral mesoderm patterning. *Development* **127**, 1981-1990.
Tao, Q., Yokota, C., Puck, H., Kofron, M., Birsoy, B., Yan, D., Asashima, M., Wylie, C. C., Lin, X. and Heasman, J. (2005). Maternal wnt11 activates the canonical wnt signaling pathway required for axis formation in *Xenopus* embryos. *Cell* **120**, 857-871.
Thorpe, C. J., Schlesinger, A., Carter, J. C. and Bowerman, B. (1997). Wnt signaling polarizes an early *C. elegans* blastomere to distinguish endoderm from mesoderm. *Cell* **90**, 695-705.
Turner, D. L. and Weintraub, H. (1994). Expression of achaete-scute homolog 3 in xenopus embryos converts ectodermal cells to a neural fate. *Genes Dev.* **8**, 1434-1447.
Umbhauer, M., Djiane, A., Goisset, C., Penzo-Mendez, A., Riou, J. F., Boucaut, J. C. and Shi, D. L. (2000). The C-terminal cytoplasmic Lys-thr-X-X-X-Trp motif in frizzled receptors mediates Wnt/β-catenin signalling. *EMBO J.* **19**, 4944-4954.
Weitzel, H. E., Illies, M. R., Byrum, C. A., Xu, R., Wikramanayake, A. H. and Ettensohn, C. A. (2004). Differential stability of β-catenin along the animal-vegetal axis of the sea urchin embryo mediated by dishevelled. *Development* **131**, 2947-2956.
Wessel, G. M. and McClay, D. R. (1985). Sequential expression of germ-layer specific molecules in the sea urchin embryo. *Dev. Biol.* **111**, 451-463.
Wikramanayake, A. H., Huang, L. and Klein, W. H. (1998). β-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., Hong, M., Lee, P. N., Pang, K., Byrum, C. A., Bince, J. M., Xu, R. and Martindale, M. Q. (2003). An ancient role for nuclear beta-catenin in the evolution of axial polarity and germ layer segregation. *Nature* **426**, 446-450.