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Down syndrome critical region protein 5 regulates membrane localization of Wnt receptors, Dishevelled stability and convergent extension in vertebrate embryos

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The Glypican family of heparan sulfate proteoglycans regulates Wnt signaling and convergent extension (CE) in vertebrate embryos. They are predicted to be glycosylphosphatidylinositol (GPI)-tethered membrane-bound proteins, but there is no functional evidence of their regulation by the GPI synthesis complex. Down syndrome critical region protein 5 (Dscr5, also known as Pigp) is a component of the GPI-N-acetylglucosaminyltransferase (GPI-GnT) complex, and is associated with specific features of Down syndrome. Here we report that Dscr5 regulates CE movements through the non-canonical Wnt pathway. Both *dscr5* overexpression and knockdown impaired convergence and extension movements. Dscr5 functionally interacted with Knypek/Glypican 4 and was required for its localization at the cell surface. Knockdown of *dscr5* disrupted Knypek membrane localization and caused an enhanced Frizzled 7 receptor endocytosis in a Caveolin-dependent manner. Furthermore, *dscr5* knockdown promoted specific Dishevelled degradation by the ubiquitin-proteosome pathway. These results reveal a functional link between Knypek/Glypican 4 and the GPI synthesis complex in the non-canonical Wnt pathway, and provide the new mechanistic insight that Dscr5 regulates CE in vertebrate embryos by anchoring different Wnt receptors at the cell surface and maintaining Dishevelled stability.

KEY WORDS: Zebrafish, Wnt signaling, Convergent extension, Dscr5, Glypicans, Frizzled, Dishevelled

INTRODUCTION

Gastrulation in vertebrate embryos is the fundamental morphogenetic movement that gives rise to the three germ layers: ectoderm, mesoderm and endoderm. During gastrulation, the narrowing and lengthening of a group of cells, termed CE, represents one of the main engines producing the driving force and making an important contribution to the elongation of the anteroposterior axis (Keller et al., 2000; Solnica-Krezel, 2005). The cellular and molecular bases of this process are well described in *Xenopus* and in zebrafish (Wallingford et al., 2002). In particular, the non-canonical Wnt pathway, also called the planar cell polarity (PCP) pathway, plays an important role in CE and is well conserved (Smith et al., 2000; Myers et al., 2002; Kühl, 2002; Heisenberg and Tada, 2002; Tada et al., 2002). It is triggered by Wnt11 through interaction with its seven-pass transmembrane Frizzled receptors (Tada and Smith, 2000; Heisenberg et al., 2000; Djiane et al., 2000; Ulrich et al., 2003) and coreceptors of the Glypican family, including zebrafish Knypek and its ortholog *Xenopus* Glypican 4 (Topczewski et al., 2001; Ohkawara et al., 2003). The signal is then relayed to the cytoplasmic protein Dishevelled, whose relocalization to the plasma membrane and interaction with membrane components of the PCP pathway are required for CE (Wallingford et al., 2000; Kinoshita et al., 2003; Park et al., 2005; Carreira-Borbosa et al., 2009). In addition, a number of downstream components for this pathway have been identified, including the small GTPases RhoA and Rac, and Jun N-terminal kinase (reviewed by Klein and Mlodzik, 2005; Wallingford and Habas, 2005). Activation of this pathway regulates cell movements through modification of the actin cytoskeleton (Winter et al., 2001; Habas et al., 2001; Sato et al., 2006).

Down syndrome is one of the most frequent human birth defects, occurring in 1 out of 600 to 1000 births. It is caused by trisomy of chromosome 21 and is associated with mental retardation and various facial and physical anomalies. Phenotypic and molecular analyses of patients have led to the identification of a region in chromosome 21, which is central to the pathogenesis of Down syndrome (Delabar et al., 1993; Pritchard et al., 2008). This region is called Down syndrome critical region (DSCR), and several genes have been identified in this region, including *dscr5* (Togashi et al., 2000; Shibuya et al., 2000). However, the function of most of them in key early developmental events remains largely unexplored. Thus, functional analysis of these genes during early development represents an essential step in understanding the pathogenesis of the birth defects in Down syndrome patients.

Here we report the implication of Dscr5 in the regulation of CE movements in the zebrafish embryo. Dscr5 protein, also called Pigp (phosphatidylinositol glycan, class P), is a component of the GPI-GnT complex required for GPI biosynthesis (Watanabe et al., 2000), and GPI, a complex glycolipid, acts as a membrane anchor of many surface proteins, including Glypicans. Although the importance of Glypicans in regulating Wnt signaling has been well established, and Knypek and Glypican 4 are potential GPI-anchored proteins (Topczewski et al., 2001; Ohkawara et al., 2003), whether and how their membrane localization and activity are regulated by the GPI biosynthesis complex remain unclear. Thus, it remains to be determined if there is a functional link between these potential GPI-anchored proteins and the GPI biosynthesis complex. GPI biosynthesis is essential for embryonic development, and loss-of-

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function of individual components in the GPI biosynthesis complex results in distinct abnormality (Nozaki et al., 1999; Inoue et al., 2003; Almeida et al., 2006), suggesting that different components may have tissue-specific function and may be involved in some specific developmental events. In the present study, we examined the mechanism through which the Dscr5 homolog is involved in CE. Our results demonstrate that Dscr5 regulates CE movements both in zebrafish and *Xenopus* embryos through the PCP pathway. It is required for the membrane localization of both Knypek and the Frizzled 7 receptor. Knockdown of *dscr5* causes an increase of free extracellular Knypek and an enhanced Frizzled 7 receptor endocytosis in a Caveolin-dependent manner, and leads to specific Dishevelled degradation by the ubiquitin-proteosome pathway. These findings thus provide novel insight into the regulation of non-canonical Wnt signaling and CE movements.

MATERIALS AND METHODS

Zebrafish and *Xenopus* embryos

Wild-type zebrafish embryos were produced by natural matings and maintained at 28.5°C. Experiments using *Xenopus* embryos were as described previously (Djiane et al., 2000).

Plasmid constructs

Zebrafish and *Xenopus dscr5* coding sequences were cloned into the *pCS2* vector (Rupp and Weintraub, 1991). Myc-tagged *dscr5* (*dscr5MT*), tagged to the carboxyl terminus, was obtained by cloning *dscr5* into the *pCS2MT* vector. Mutant forms of *dscr5* (referred to here as *mut-dscr5* and *mut-dscr5MT*) were created by introducing five nucleotide substitutions, without a change of amino acid residues, in the first 25 nucleotides. *Xdsh-myc* (Sokol, 1996), *dshΔDIX* (Tada and Smith, 2000), *wnt11-myc* (Djiane et al., 2000), *Xfz7-myc* (Carron et al., 2003), *dsh-GFP* (Axelrod et al., 1998), *gly4ΔC* (Ohkawara et al., 2003), dominant negative JNK (Carron et al., 2005) and AP1-luciferase reporter (Wong et al., 2004) were described previously. Myc-tagged *knypek* (*kny-myc*) was obtained by inserting six myc epitopes between the signal peptide and the remaining sequence. *caveolin-GFP* was generated by inserting *caveolin* before the *GFP* sequence. Dominant negative *caveolin* (referred to here as *dn-caveolin*) lacking the first 53 amino acid residues was obtained by PCR and cloned in the *pCS2* vector.

Morpholinos and RNA synthesis for microinjections

Two morpholinos (MOs) designed over the initiation methionine of zebrafish *dscr5* (MO1: 5′-GAGGAGAAGGAGAGTTCTCCACCAT-3′ and MO2: 5′-CATCTCCAGCAGCGACACATGTCTT-3′), one MO designed over the splice acceptor site of exon 3 (MO3: 5′-GACCACATACAGCACTGATGGAGAA-3′), the control MO (CoMO: 5′-GTGGAGATGGGGAATTCTCTACCAT-3′) with five mismatches with respect to MO1 (underlined), and *knypek* MO (*kny*MO: 5′-AACACAACGATCATCTTCATCGCTG-3′) were synthesized by Gene Tools. A BLAST search against the zebrafish genome indicated that these sequences are not present elsewhere in the genome. All MOs were diluted in sterile water and stored at –20°C as small aliquots. Capped RNAs were synthesized by in vitro transcription as described (Djiane et al., 2000). For some experiments, injected embryos were cultured in the presence of 10 μM of the proteosome inhibitor MG132 (Sigma) or 25 mM of the lysosome inhibitor NH_4Cl.

Analysis of CE movements by cell transplantations and UV-mediated photo-activation

Donor embryos were injected with rhodamine-lysine-dextran (RLDx), along with CoMO or *dscr5*MO at the one-cell stage. At shield stage, a small group of cells at the lateral margin were transplanted into the corresponding region of wild-type recipients. For UV-mediated photo-activation, *Kaede* RNA (200 pg) was coinjected with CoMO or *dscr5*MO at the one-cell stage. At the shield stage, UV irradiation was performed with a DAPI filter using the smallest pinhole under the 20× objective of a Nikon microscope. Images were taken at shield and bud stages for analysis of convergence and extension as described (Sepich and Solnica-Krezel, 2005).

Zebrafish embryonic cell culture

Thirty embryos from each condition were cultured until 30% epiboly; they were then collected into a 1.5 ml microtube and dechorionated through Pronase E treatment as described (Link et al., 2006). The yolk was mechanically disrupted by gentle pipeting and vortexing. After several washes in E2 buffer to completely remove the yolk, the remaining small cell aggregates and individual cells were cultured in 30 μl E2 buffer for 3 hours at 28.5°C. After a brief centrifugation, the culture medium and cell pellets were subjected to western blot.

Western blot and immunofluorescence microscopy

Protein extraction and western blot using anti-Myc (Santa Cruz), anti-JNK (Santa Cruz) and anti-β-Catenin (Abcam) antibodies and the ECL chemiluminescence kit (Amersham Biosciences) were described previously (Carron et al., 2005). Whole-mount immunostaining was performed as previously described (Umbhauer et al., 2000). The embryos were mounted in Mowiol and analyzed by confocal microscopy.

Whole-mount in situ hybridization and RT-PCR

In situ hybridization was performed as described (Thisse and Thisse, 2008). RNA extraction, reverse transcription and PCR primers for different *Xenopus* markers were described previously (Djiane et al., 2000). PCR primers for detecting *dscr5* splicing defects are as follows: 5′-GAGATGGTGATGGTGGAGAACT-3′ and 5′-CAGCGGAGCCGT-GTTCAT-3′.

Luciferase assay

The AP1-luciferase reporter plasmid (200 pg) was injected alone or coinjected with 500 pg *gly4ΔC* RNA in the animal pole region of the two-cell stage *Xenopus* embryo, or with 5 ng MO1 or MO3 at the one-cell stage zebrafish embryo. Cell lysates were prepared from ten *Xenopus* early gastrula ectodermal explants or 30 zebrafish embryos at 70% epiboly. Luciferase activity was measured using the Luciferase Assay System (Promega).

RESULTS

Overexpression and knockdown of *dscr5* inhibit axis extension

Dscr5 proteins are well conserved among different species, for example, zebrafish and human Dscr5 proteins show 64% overall identity and 85% similarity (see Fig. S1A in the supplementary material). Using in situ hybridization, *dscr5* transcripts were detected as early as the eight-cell stage (see Fig. S1B in the supplementary material), suggesting that they are maternally supplied and might play an early role during development. At sphere and 50% epiboly stages, *dscr5* transcripts were present in the whole embryo. At 12.5 hours post fertilization (hpf), they were found in both the neural tissue and the somites (see Fig. S1B in the supplementary material).

To analyze the function of Dscr5 during early development, we first performed overexpression and knockdown experiments by injecting different amounts of *dscr5* RNA (30-500 pg), translation-blocking (MO1 and MO2) or splice-inhibiting (MO3) MOs (0.3-5 ng) at the one- to four-cell stage. The results showed that both *dscr5* overexpression and knockdown caused gastrulation defects. When the expression pattern of *dlx3* (*dlx3b* – ZFIN), which marks the neural plate boundaries and has been used as a marker to reflect CE movements (Topczewski et al., 2001; Marlow et al., 2002; Huang et al., 2007), was examined at 100% epiboly, we found that embryos injected with *dscr5* RNA or different MOs exhibited a broader neural plate (Fig. 1A, upper panel), indicating that CE of the neural plate is impaired. Overexpression and knockdown of *dscr5* also caused CE defects of axial mesoderm, as the prechordal plate cells were positioned posterior to the neural plate border and the notochord was wider, as revealed by the expression pattern of *hgg1*

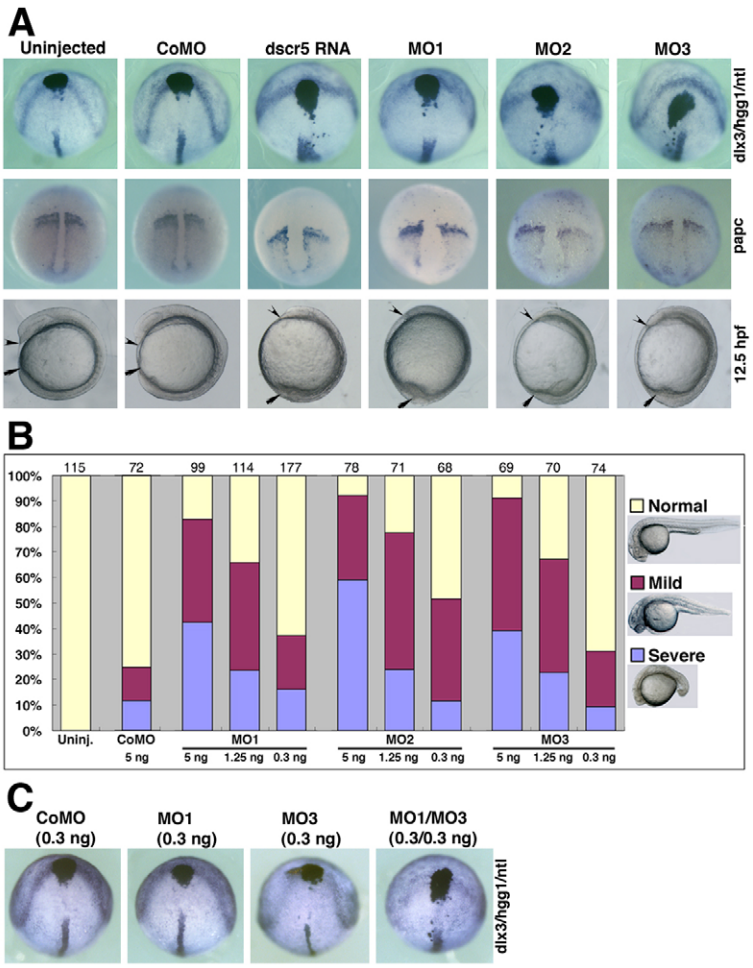

Fig. 1. Overexpression or knockdown of *dscr5* impairs CE movements. (**A**) Embryos were injected with *dscr5* RNA or different MOs, as indicated. In situ hybridization was performed at 100% epiboly using the indicated probes, and live images were taken at 12.5 hpf. Upper panel, dorsoanterior view shows embryos simultaneously hybridized with *dlx3*, *hgg1* and *ntl* probes; middle panel, dorsal view shows the expression domain of *papc*; bottom panel, lateral view shows the phenotypes of embryos at 12.5 hpf, with anterior to the top and dorsal side to the right. Arrowheads and arrows denote the anterior tip of the hypoblast and the tail region, respectively. (**B**) Summary of the dose-dependent effects of *dscr5* knockdown using different MOs. Numbers at the top indicate total embryos scored at 27 hpf from three independent experiments. (**C**) Embryos were injected with a low dose of the indicated MOs and simultaneously hybridized with *dlx3*, *hgg1* and *ntl* probes at 100% epiboly.

(*ctsl1b* – ZFIN) and *ntl* (*ntla* – ZFIN) (Fig. 1A, upper panel). In addition, the paraxial mesoderm was compressed along the anteroposterior axis and widened mediolaterally, as evidenced by the expression pattern of *papc* (*pcdh8* – ZFIN) (Fig. 1A, middle panel). At 12.5 hpf, when control embryos developed a long anteroposterior axis around the yolk, embryos injected with *dscr5* RNA or different MOs exhibited a shortened anteroposterior axis (Fig. 1A, bottom panel), suggesting impaired extension movements. A dose-response analysis indicated that all three MOs had similar efficiency in producing CE defects (Fig. 1B), we thus used MO1 (designated as *dscr5*MO) in the majority of the experiments, unless stated otherwise. Since a low dose of MO1 or MO3 (0.3 ng) elicited little or no CE defects, we coinjected MO1 and MO3 at these 'subthreshold' concentrations and observed similar CE defects, as monitored by the expression pattern of *dlx3*, *hgg1* and *ntl* at 100% epiboly (Fig. 1C). These observations suggest that Dscr5 activity might be important for cell movements during gastrulation.

To further analyze *dscr5* function, we determined the specificity of *dscr5*MO (MO1) and MO3 using different approaches (Eisen and Smith, 2008). We first performed rescue experiments using *mut-dscr5* RNA with five degenerate base substitutions in the *dscr5*MO-targeted sequence. When *mut-dscr5* RNA (125 pg) was coinjected with *dscr5*MO (5 ng), we obtained a significant rescue of the axis extension defects, with a notable increase in the percentage of normal phenotype embryos at 25 hpf (Fig. 2A). We then tested the efficiency of *dscr5*MO in blocking the translation of *dscr5MT* and *mut-dscr5MT* RNAs. Western blot analysis showed that the translation of *dscr5MT* RNA, but not *mut-dscr5MT* RNA, was efficiently blocked by *dscr5*MO (Fig. 2B). A more efficient rescue of the CE defects was obtained by coinjecting 40 pg *dscr5* RNA with 10 ng splice-inhibiting MO3 (Fig. 2C-F). RT-PCR and sequence analysis of the altered splice product showed that MO3, but not control MO (CoMO) or MO1, indeed induced defective splicing of *dscr5* pre-mRNA, resulting in the loss of exon 3 and a frameshift at amino acid 27 (Fig. 2G; data not shown). These results suggest that the targeting effects of different MOs should be specific. Thus, we conclude that *dscr5* knockdown using different MOs specifically affects CE movements.

Knockdown of *dscr5* disrupts convergence of lateral cells and extension of dorsal cells

To see how cell movement is disrupted during gastrulation in *dscr5* morphants, we first transplanted small groups of lineage-labeled non-axial mesendodermal cells into the corresponding region of wild-type embryos (Fig. 3A,C) and followed their movements. At the bud stage, RLDx-labeled cells from CoMO-injected embryos (n=12) formed strings of cells extended along the anteroposterior axis (Fig. 3B,B′). By contrast, cells from *dscr5*MO-injected donors (n=11) remained scattered and failed to elongate along the anteroposterior axis (Fig. 3D,D′). This indicates that mediolateral cell intercalations underlying CE are disrupted in *dscr5* morphants in a cell autonomous manner. We further analyzed the convergence of lateral cells and extension of dorsal cells using photo-conversion of a small group of cells in embryos previously coinjected with

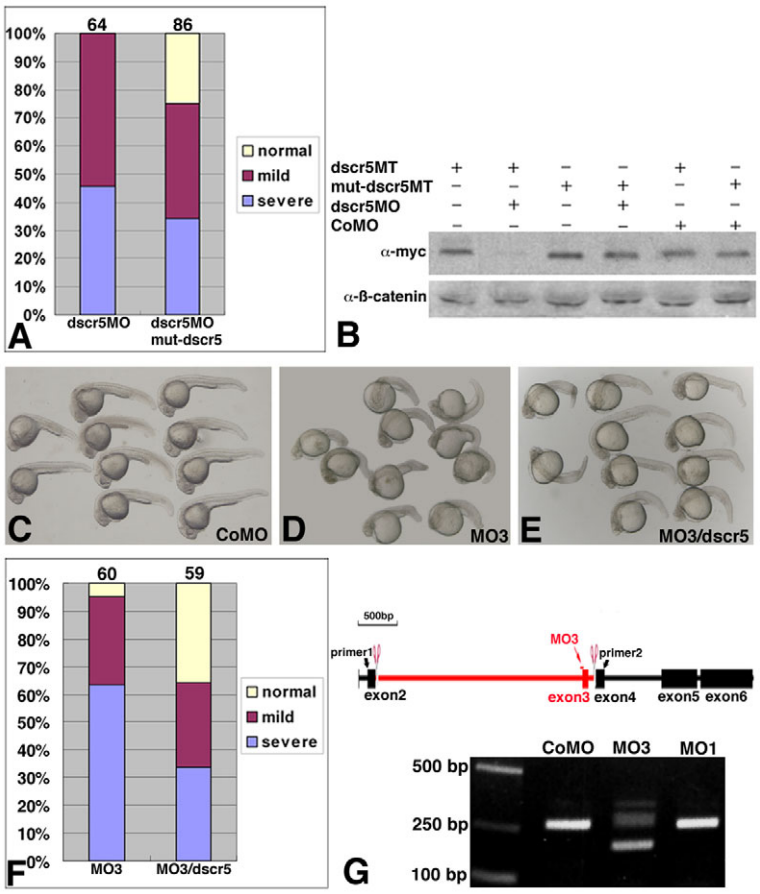

Fig. 2. Specificity and effectiveness of *dscr5* MOs. (**A**) Rescue of CE defects in *dscr5* morphants by *mut-dscr5* RNA. Summary of the phenotypes at 25 hpf with numbers at the top indicating total embryos scored from four independent experiments. (**B**) Western blot analysis of the effect of *dscr5*MO on the translation of *dscr5MT* and *mut-dscr5MT* RNAs. Endogenous β-Catenin was used as a loading control. (**C-F**) Rescue of CE defects in embryos injected with MO3 and cultured to 27 hpf. (C) CoMO-injected embryos. (D) MO3-injected embryos. (E) Embryos coinjected with MO3 and *dscr5* RNA. (F) Summary of the rescue from three experiments, with the number of total embryos indicated at the top. (**G**) Schematic representation and RT-PCR analysis at 12.5 hpf of MO3-induced mis-splicing of *dscr5* pre-mRNA.

Kaede-GFP RNA and CoMO or *dscr5*MO at the one-cell stage. Compared with CoMO-injected embryos, the extent of convergence of lateral cells and of extension of dorsal cells was significantly reduced in *dscr5*MO-injected embryos (Fig. 3E-N). This result clearly shows that *dscr5* knockdown disrupts convergence and extension. Furthermore, when cell morphology was examined by confocal microscopy at 100% epiboly, we found that notochord cells from CoMO-injected embryos were elongated in shape and aligned mediolaterally (Fig. 3O). By contrast, the notochord was significantly wider and the cells exhibited a rounder shape in *dscr5*MO-injected embryos (Fig. 3P). This further suggests that *dscr5* knockdown disrupts mediolateral cell intercalations.

Overexpression or knockdown of *dscr5* does not affect embryonic patterning

The axis extension defects might be due to a requirement of *dscr5* for cell movements or for embryonic patterning, as reported for other genes (Myers et al., 2002; Caneparo et al., 2007; Huang et al., 2007). To address this question, we analyzed the expression pattern of a set of dorsoventral markers. At the shield stage, injection of *dscr5* RNA (500 pg) or *dscr5*MO (1.25 ng) did not change the expression pattern of the dorsal gene *chordin* (Fig. 4A-C), the lateroventral gene *eve1* (Fig. 4D-F) or the pan-mesoderm gene *ntl* (Fig. 4G-I). The same result was observed in *Xenopus* embryos in which *dscr5* overexpression strongly blocked CE, resulting in embryos with spina bifida and shortening of the anteroposterior axis (see Fig. S2A,B in the supplementary material). In animal cap explants, *dscr5* overexpression also inhibited activin-induced elongation, which mimics CE (see Fig. S2C-E in the supplementary material). RT-PCR analysis indicated that *dscr5* overexpression did not affect the expression level of different dorsoventral mesoderm genes, including *chordin* and *wnt8* (*wnt8a* – ZFIN), in these explants (see Fig. S2F in the supplementary material). Since Dally-like, a *Drosophila* Knypek homolog, was shown to regulate hedgehog signaling (Desbordes and Sanson, 2003; Gallet et al., 2008), we examined by in situ hybridization the expression of hedgehog target genes *nk2.1b*, *nk2.2* (*nkx2.2a* – ZFIN) and *patched 1* (Barth and Wilson, 1995; Lewis et al., 1999; Rohr et al., 2001). The results indicate that expression of these genes was not affected in *dscr5* RNA- or *dscr5*MO-injected embryos (Fig. 4J-R). Together, these results suggest that, at least at the dose injected, both *dscr5* overexpression and knockdown affect CE movements rather than mesoderm and neural patterning.

Dscr5 interacts with the PCP pathway in CE movements

CE movements are regulated by the PCP pathway, which involves Dishevelled, small GTPases and the Jun N-terminal kinase (JNK) cascade (Klein and Mlodzik, 2005). To examine whether Dscr5 regulates CE movements through this pathway, we first tested if *dishevelled* mutant RNA, which lacks the DIX domain and only activates the PCP pathway (referred to here as *dshΔDIX*) (Tada and Smith, 2000), could rescue the effect of *dscr5*MO. We found that coinjection of 200 pg *dshΔDIX* RNA with 5 ng *dscr5*MO significantly reduced the extent of CE defects (Fig. 5A), suggesting that *dscr5*MO inhibits activation of the PCP pathway and that Dscr5 protein should be upstream of Dishevelled. We next tested the functional interaction between Dscr5 and JNK, which is activated by non-canonical Wnt

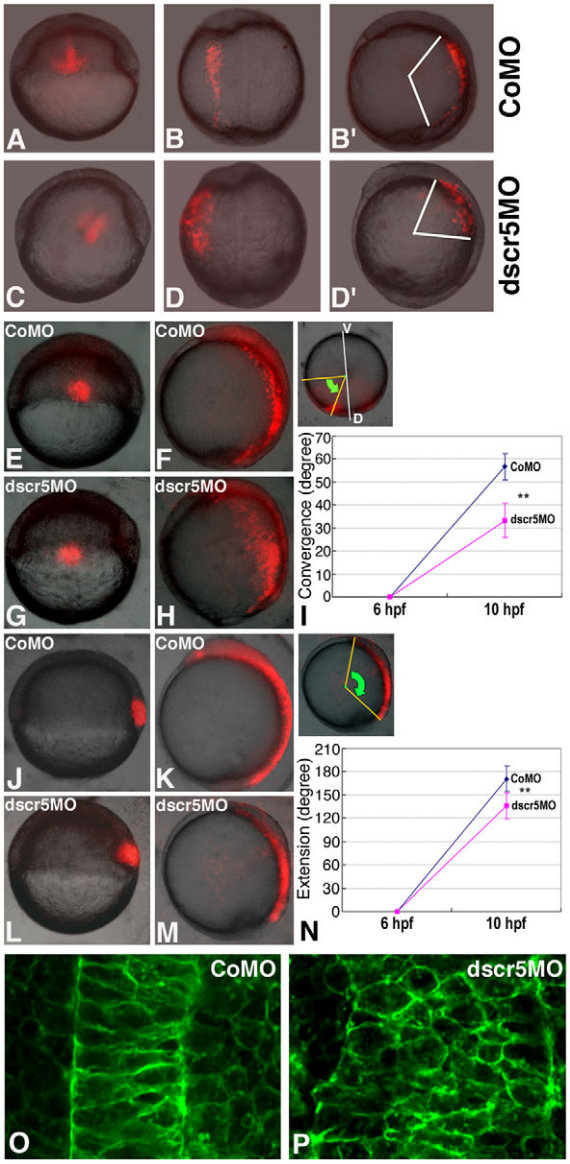

Fig. 3. Convergence and extension movements are affected in *dscr5* morphants. (**A-D'**) Transplantations of RLDx-labeled cells from the margin of shield stage embryos injected with CoMO or *dscr5*MO to the corresponding region in wild-type recipients. (A) A 6 hpf recipient embryo with CoMO-injected cells. (B,B′) Dorsal (B) and lateral (B′) views of the embryo in A developed to 10 hpf. (C) A 6 hpf recipient embryo with *dscr5*MO-injected cells. (D,D′) Dorsal (D) and lateral (D′) views of the embryo in C developed to 10 hpf. (**E-N**) Analysis of the convergence of lateral mesendodermal cells (E-I) and the extension of axial mesendodermal cells (J-N) in embryos injected with CoMO (E,F,J,K) or *dscr5*MO (G,H,L,M) using a photo-conversion strategy. (E-I) Labeled lateral mesendodermal cells at the shield stage in embryos injected with CoMO (E) or *dscr5MO* (G) were analyzed at the bud stage (F,H,I). Lateral view for all embryos except the inset in I, which is an animal pole view showing the angle between the labeled cells and the line perpendicular to the dorsoventral axis (green arrow). (J-N) Labeled axial mesendodermal cells at the shield stage in embryos injected with CoMO (J) or *dscr5MO* (L) were analyzed at the bud stage (K,M,N). Lateral view for all embryos; the inset in N shows the angle between the most anterior and the most posterior labeled cells (green arrow). Green dot represents the center of the images. Quantification of the convergence of lateral cells (I) and the extension of dorsal cells (N) was performed using five embryos for each condition. Asterisks indicate statistically significant differences ($P<0.001$, Student's *t*-test), error bars indicate standard deviation. D, dorsal region; V, ventral region. (**O**,**P**) Analysis of cell intercalation in the notochord by confocal microscopy. Representative images from a control embryo (O) and a *dscr5* morphant (P) at the bud stage.

signaling during CE movements (Yamanaka et al., 2002), using a dominant negative mutant RNA (referred to here as *dnJNK*). When a low dose of *dscr5*MO (0.3 ng) or *dnJNK* RNA (100 pg) was injected alone, the majority of embryos developed essentially normally or with mild CE defects, as shown by the expression pattern of *dlx3* at the eight-somite stage (Fig. 5B-D). Coinjection of *dscr5*MO with *dnJNK* RNA strongly enhanced axis extension defects (Fig. 5E,F), indicating a synergistic effect. Furthermore, both MO1 and MO3 significantly inhibited JNK activation, as monitored by the AP1-luciferase reporter assay (Fig. 5G). These results suggest that Dscr5 regulates CE through the PCP pathway.

Knockdown of *dscr5* disrupts the membrane localization of Wnt receptors

Since Dscr5 protein is a component of GPI-GnT required for GPI biosynthesis, and both Knypek and Glypican 4 contain a potential GPI moiety attachment site (Topczewski et al., 2001; Ohkawara et al., 2003), we tested whether Dscr5 is involved in their membrane localization using Myc-tagged Knypek (referred to here as Kny-Myc) and confocal microscopy. Synthetic *kny-myc* RNA (500 pg) was injected alone or coinjected with *dscr5*MO (5 ng) at the four-cell stage and immunostaining was performed at 50% epiboly. When *kny-myc* RNA was injected alone, the protein accumulated at the cell membrane and in the cytoplasm (Fig. 6A), as previously described for FLAG-tagged Knypek (Topczewski et al., 2001). This membrane and cytoplasmic distribution was severely disrupted when *dscr5*MO was coinjected. In this case, weak fluorescence staining was found uniformly distributed in the cytoplasm (Fig. 6B). We then used cultured embryonic cells previously injected with *kny-myc* RNA alone, or coinjected with *kny-myc* RNA and *dscr5*MO or *dscr5* RNA, to examine by western blot whether the disrupted Knypek membrane localization correlated with an enhanced secretion to the extracellular medium. We found that the level of Kny-Myc protein was markedly increased in the culture medium of *dscr5* knockdown cells. Conversely, *dscr5* overexpression caused a decrease or absence of Kny-Myc protein in the culture medium (Fig. 6C). This result is consistent with the function of Dscr5 in GPI synthesis and with the sequence prediction that Knypek is GPI-tethered. It indicates that Dscr5 is required for the membrane localization of Knypek and functions upstream of Knypek in CE movements.

Since *Xenopus* Glypican 4 physically interacts with Frizzled 7 (Ohkawara et al., 2003), it is possible that *dscr5* knockdown also affects the membrane localization of Frizzled 7, at least indirectly, and then prevents it from interacting with and activating downstream components. To test this possibility, we injected 500 pg *Xenopus frizzled7-myc* (referred to here as *Xfz7-myc*) RNA alone or coinjected with *dscr5*MO. In the absence of *dscr5*MO, XFz7-Myc was mainly localized to the plasma membrane, with a low level in the cytoplasm (Fig. 6D). In sharp contrast, *dscr5* knockdown disrupted XFz7-Myc membrane localization and caused its accumulation in the cytoplasm

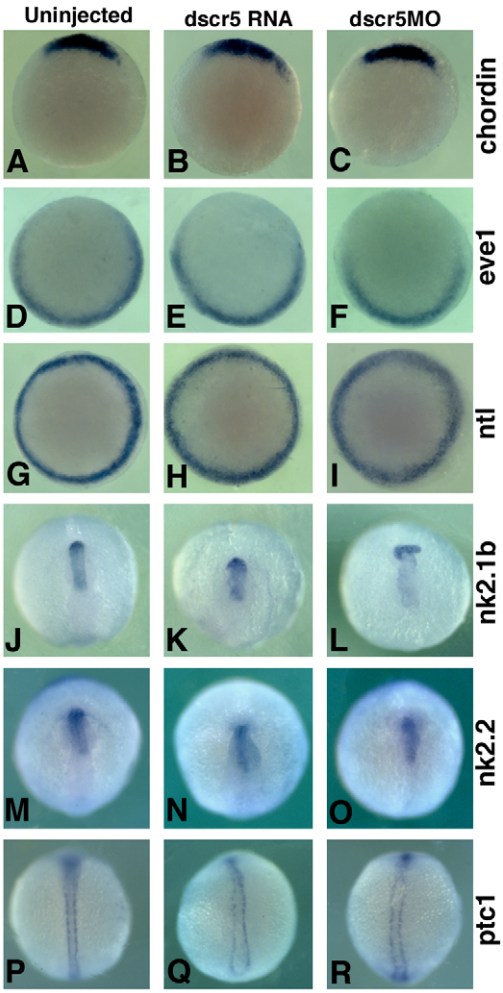

Fig. 4. Overexpression or knockdown of *dscr5* has no effect on mesoderm and neural patterning. Embryos were injected as indicated and in situ hybridization was performed using the indicated markers. (**A-I**) Animal pole view shows the expression pattern of *chordin* (A-C), *eve1* (D-F) and *ntl* (G-I) at 50% epiboly. (**J-R**) Dorsoanterior view (J-O) and dorsal view (P-R), with anterior to the top, show the expression pattern of *nk2.1b* (J-L), *nk2.2* (M-O) and *patched 1* (*ptc1*) (P-R) at 12.5 hpf. Note the broader neuroectoderm stained by *ptc1* in *dscr5* RNA- and *dscr5*MO-injected embryos.

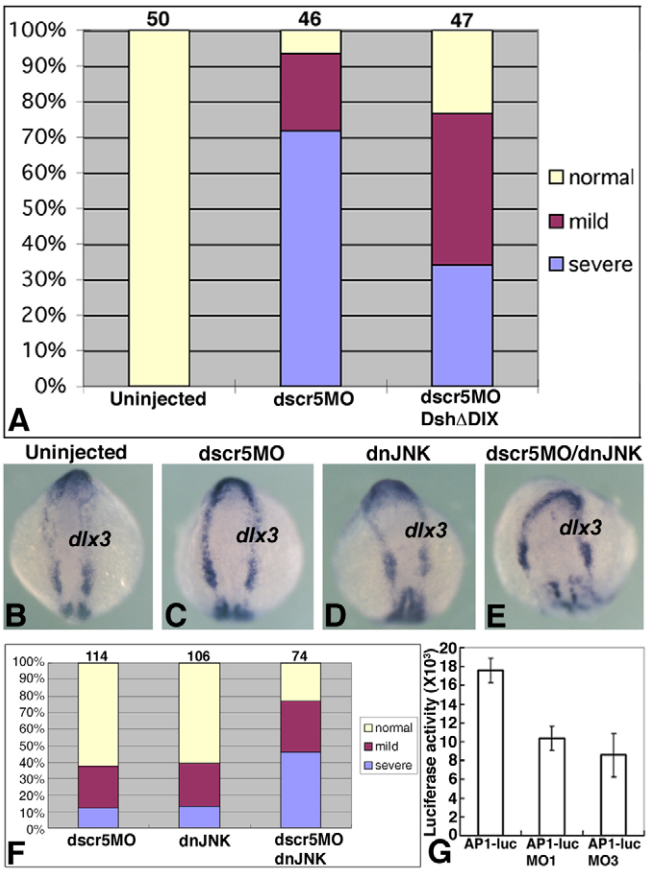

Fig. 5. Interaction between Dscr5 and the PCP pathway in CE movements. (**A**) Rescue of CE movements by *dshΔDIX* in *dscr5* morphants. Summary of three independent experiments of phenotypic analysis at 27 hpf with total numbers indicated at the top. (**B-F**) Inhibition of the JNK pathway and *dscr5* knockdown enhances CE defects. (B-E) Control (B) and injected (C-E) embryos at the eight-somite stage simultaneously hybridized with *myoD* and *dlx3* probes. Dorsoanterior view of *dlx3* expression pattern. *myoD* expression pattern was used to control the stage of injected embryos and was not shown. (F) Summary of the phenotypes at 27 hpf. Numbers at the top represent total embryos scored from four independent experiments. (**G**) Knockdown of *dscr5* by MO1 or MO3 inhibits JNK activation. The AP1-luciferase reporter assay was performed in triplicate ($P<0.03$, Student's *t*-test). Error bars indicate standard deviation.

(Fig. 6E,H). In addition, we found that this cytoplasmic distribution of XFz7-Myc colocalized with Caveolin-GFP (Fig. 6E-G), indicating that *dscr5* knockdown leads to an enhanced endocytosis of Frizzled 7, which might be Caveolin-dependent. This was confirmed by using *dn-caveolin*, which lacks the first 53 residues and acts as a dominant negative mutant (Pol et al., 2001). We found that coinjection of *dn-caveolin* RNA (500 pg) efficiently rescued XFz7-Myc membrane localization in *dscr5*MO-injected cells (Fig. 6I). We then directly tested the requirement of Knypek/Glypican 4 on Frizzled 7 membrane localization using *gly4ΔC*, which lacks the GPI moiety attachment site and potently blocks CE in *Xenopus* and in zebrafish (Ohkawara et al., 2003) (see Fig. 7J-L). We further confirmed that it indeed inhibits JNK activation using the AP1-luciferase reporter (see Fig. S3 in the supplementary material). As in *dscr5* knockdown cells, overexpression of *gly4ΔC* RNA (100 pg) strongly disrupted Frizzled 7 membrane localization (Fig. 6J), and this was also rescued by *dn-caveolin* (Fig. 6K), indicating that interfering with Knypek/Glypican 4 activity disrupts Frizzled 7 membrane localization. Together, these observations suggest that *dscr5* knockdown disrupts the membrane accumulation of Knypek and Frizzled 7 receptors and that the enhanced Frizzled 7 endocytosis in *dscr5* morphants is Caveolin-dependent.

Functional interaction between Dscr5 and Knypek/Glypican 4

To directly analyze the functional interaction between Dscr5 and Knypek/Glypican 4, we first compared the CE defects induced by interfering with their activity. Injection of *dscr5*MO (5 ng), *kny*MO (5 ng) and *gly4ΔC* RNA (100 pg) induced CE defects in a similar manner, as revealed by in situ hybridization using *myoD* (*myod1* – ZFIN) (Fig. 7A,D,G,J) and *dlx3* (Fig. 7A′,D′,G′,J′) at the eight-somite stage. In the live images at 12.5 hpf (Fig. 7B,E,H,K) and 27 hpf (Fig. 7C,F,I,L), severe axis extension defects were evident in *dscr5* and *knypek* morphants, and in *gly4ΔC*-injected embryos.

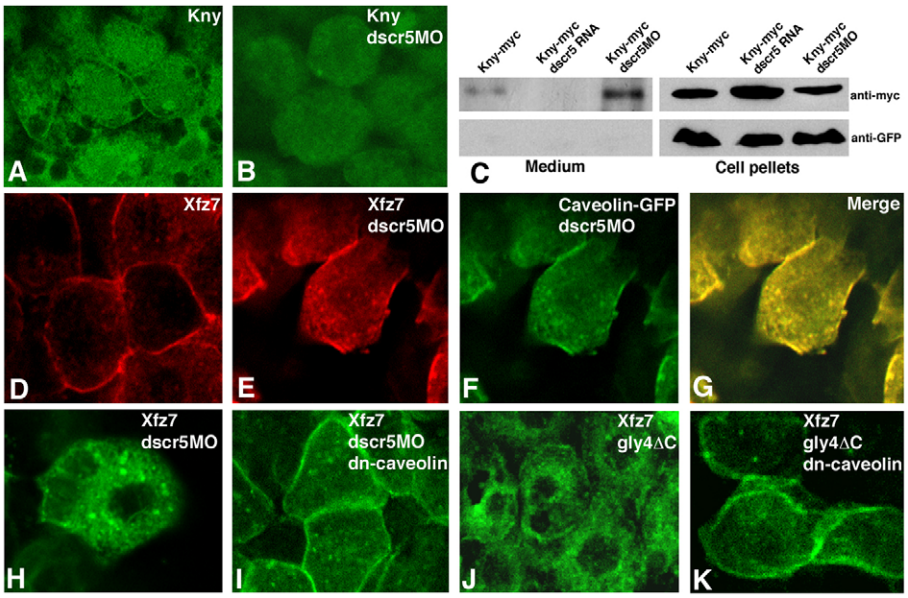

Fig. 6. Disruption of the membrane localization of Knypek and Frizzled 7 receptors in *dscr5* morphants. (**A**) Localization of Kny-Myc at the cell surface and in the cytoplasm. (**B**) Kny-Myc distribution in *dscr5*-knockdown cells. (**C**) Western blot analysis of Kny-Myc levels in the culture medium (left) and cell pellets (right) of *dscr5* RNA- or *dscr5*MO-injected cells. GFP protein was used as a loading control in the cell pellets and to monitor the integrity of the cultured cells. (**D**,**E**) XFz7-Myc localization revealed by Cy3-conjugated secondary antibody. (D) Localization of XFz7-Myc to the cell membrane of control cells. (E) XFz7-Myc distribution in *dscr5*-knockdown cells. (**F**) Caveolin-GFP localization in *dscr5*-knockdown cells. (**G**) Merge of E and F showing the colocalization between XFz7-Myc and Caveolin-GFP. (**H-K**) Rescue of XFz7-Myc membrane localization by *dn-caveolin* in cells injected with *dscr5*MO (I) or *gly4ΔC* RNA (K).

However, we noticed that *knypek* morphants developed a shorter anteroposterior axis (Fig. 7I), which was similar to *kny* mutants (Topczewski et al., 2001), whereas *dscr5* morphants and *gly4ΔC*-injected embryos frequently had a folded tail (Fig. 7F,L). These differences can be explained by the fact that injection of *dscr5*MO or *gly4ΔC* RNA produces an excess of free extracellular Knypek/Glypican 4 protein (see Fig. 6C), whereas injection of *kny*MO leads to an absence of or decrease in the corresponding protein. Thus, the similarity in CE defects between *dscr5* morphants and *gly4ΔC*-injected embryos further suggests that *dscr5* knockdown causes CE defects through disruption of Knypek/Glypican 4 activity.

We next used *gly4ΔC* to rescue CE in *Xenopus* whole embryos and activin-treated animal cap explants overexpressing *dscr5*. If this overexpression disrupts CE through an increased Knypek/Glypican 4 membrane attachment, inhibition of Knypek/Glypican 4 activity should rescue CE movements. *Xenopus* embryos at the four-cell stage were injected at the animal pole region or in the dorsal region with *dscr5* RNA (500 pg) alone or coinjected with *gly4ΔC* RNA (100 pg). Ectodermal explants from embryos injected at the animal pole region were dissected at the early blastula stage, treated with activin and cultured until the early neurula stage. Dorsally injected embryos were allowed to develop until the tailbud stage. The results showed that *gly4ΔC* efficiently rescued explant elongation inhibited by *dscr5* overexpression (Fig. 7M-O; see Table S1 in the supplementary material). It also efficiently rescued CE defects in whole embryos with *dscr5* injected dorsally (see Table S2 in the supplementary material). The same rescue was achieved in zebrafish embryos (Fig. 7P). In addition, we found that injection of *kny*MO (0.5 ng) was also able to rescue the effect of *dscr5* overexpression, albeit to a lesser extent (Fig. 7P). These results further suggest that Dscr5 regulates non-canonical Wnt signaling and CE movements through Knypek/Glypican 4.

Knockdown of *dscr5* triggers specific Dishevelled degradation

Dishevelled membrane translocation mediated by Frizzled receptors is important for PCP signaling (Axelrod et al., 1998), and Frizzled 7 efficiently recruits Dishevelled to the plasma membrane (Rothbächer et al., 2000; Umbhauer et al., 2000), thus disruption of Frizzled 7 membrane localization following *dscr5* knockdown would also affect the membrane recruitment of Dishevelled. To examine this possibility, we injected one-cell stage embryos with *dsh-GFP* RNA (200 pg) alone or with *dscr5*MO (5 ng). Surprisingly, only weak fluorescence was observed in embryos coinjected with *dsh-GFP* and *dscr5*MO at the shield stage (see Fig. S4A,B in the supplementary material). This effect was rescued by coinjection of *mut-dscr5* RNA (see Fig. S4C in the supplementary material). In addition, overexpression of *gly4ΔC* similarly reduced Dsh-GFP expression (see Fig. S4D in the supplementary material). This implies that *dscr5* knockdown or interfering with Knypek/Glypican 4 activity affects Dishevelled stability.

To investigate whether *dscr5* knockdown also affects the stability of other components of the canonical and the non-canonical Wnt pathways, we performed western blot analysis of different components of these pathways. When *wnt11-myc*, *Xfz7-myc* or *Xenopus dishevelled-myc* (referred to here as *Xdsh-myc*) RNA was coinjected with *dscr5*MO, we found that the stability of Wnt11-Myc and XFz7-Myc proteins from injected RNA and endogenous β-Catenin was not affected (Fig. 8A). However, XDsh-Myc was degraded in *dscr5* morphants in a dose-dependent manner (Fig. 8B). To test directly the requirement of Knypek/Glypican 4 for Dishevelled stability, *gly4ΔC* RNA (200 pg) or *kny*MO (10 ng) was coinjected with *Xdsh-myc* RNA. Although both *gly4ΔC* overexpression and *knypek* knockdown affected XDsh-Myc stability, we reproducibly observed that *gly4ΔC* overexpression, which should increase the level of free extracellular Glypican 4, more efficiently caused XDsh-Myc degradation (Fig. 8C). Since *dn-caveolin* could rescue Frizzled 7 membrane localization (see Fig. 6I,J), we assayed whether it could rescue XDsh-Myc stability in *dscr5* morphants. Coinjection of *dn-caveolin* RNA efficiently prevented XDsh-Myc from *dscr5*MO-induced degradation (Fig. 8D). Thus, we conclude that disruption of Frizzled 7 membrane localization is correlated to XDsh-Myc degradation in *dscr5* morphants. To further examine how XDsh-Myc is degraded following *dscr5* knockdown, embryos injected with *XDsh-myc* and *dscr5*MO were incubated in the proteosome inhibitor MG132 or in the lysosome inhibitor

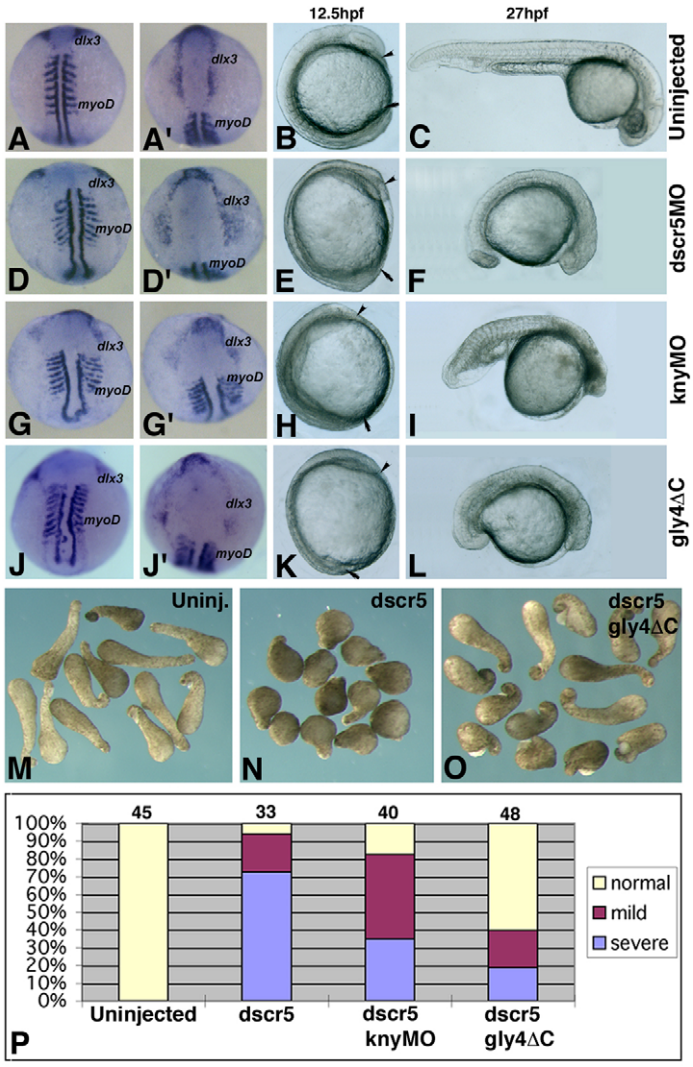

Fig. 7. Functional interaction between Dscr5 and Knypek/Glypican 4. (**A-L**) Comparison of CE defects in control embryos (A-C) and following injection of *dscr5*MO (D-F), *kny*MO (G-I) and *gly4ΔC* RNA (J-L), as indicated on the right. (A,A′,D,D′,G,G′,J,J′) Embryos at the eight-somite stage were simultaneously hybridized with *myoD* and *dlx3* probes. Dorsal view (A,D,G,J) shows the expression of *myoD*, and dorsoanterior view (A′,D′,G′,J′) shows the expression of *dlx3*. (B,E,H,K) Live images at 12.5 hpf with anterior to the top and dorsal side to the left. Arrowheads and arrows denote the anterior tip of the hypoblast and the tail region, respectively. (C,F,I,L) Live images at 27 hpf with anterior to the right. (**M-O**) Rescue by *gly4ΔC* of CE movements in *Xenopus* animal cap explants overexpressing *dscr5*. Uninjected (M), *dscr5*RNA-injected (N), and *dscr5*RNA- and *gly4ΔC*-coinjected (O) embryos. (**P**) Rescue by *knypek* knockdown or *gly4ΔC* overexpression of CE movements in zebrafish whole embryos overexpressing *dscr5*. The phenotypes were scored at 25 hpf from three independent experiments, with total numbers indicated at the top.

NH_4Cl. Western blot analysis clearly showed that MG132, but not NH_4Cl, efficiently prevented XDsh-Myc from degradation in *dscr5* morphants (Fig. 8E). This result suggests that *dscr5* knockdown causes specific Dishevelled degradation by the ubiquitin-proteosome pathway.

DISCUSSION

We have demonstrated that Dscr5, a component of GPI-GnT complex required for GPI biosynthesis, regulates CE movements through the PCP pathway. Specifically, it is required for the membrane localization of Knypek/Glypican 4 and Frizzled receptors and, as a consequence, for Dishevelled stability. This study provides novel insights into the mechanism that regulates Wnt signaling and controls cell movements during gastrulation.

Dscr5 is involved in CE movements

The PCP pathway plays a crucial role in CE movements in vertebrate embryos. At the level of the plasma membrane, several important components have been identified, including Wnt11 and the Frizzled 7 receptor (Tada and Smith, 2000; Heisenberg et al., 2000; Djiane et al., 2000), as well as coreceptors of the Glypican family (Topczewski et al., 2001; Ohkawara et al., 2003). These Glypicans are predicted to be membrane bound as known GPI-tethered proteins and cooperate with Frizzled receptors to regulate Wnt signaling (Tsuda et al., 1999; Lin and Perrimon, 1999; Baeg et al., 2001; Topczewski et al., 2001; Ohkawara et al., 2003; Franch-Marro et al., 2005; Han et al., 2005). Dscr5 participates in the biosynthesis of GPI, which is essential for anchoring many proteins at the cell membrane (Watanabe et al., 2000). We have provided several lines of evidence suggesting that Dscr5 is involved in cell movements during gastrulation through the regulation of the PCP pathway. First, both *dscr5* overexpression and knockdown in zebrafish and *Xenopus* embryos specifically affect CE movements, without an obvious effect on embryonic patterning. The CE defects closely phenocopy those produced by interfering with Knypek/Glypican 4 activity. Knockdown of *dscr5* also enhances the CE defects caused by inhibition of the PCP pathway through the dominant negative JNK mutant. Conversely, the effect of *dscr5* knockdown is rescued by a *dishevelled* mutant, which only activates non-canonical Wnt signaling (Tada and Smith, 2000). These are reminiscent of different components of the PCP pathway in CE. Second, *dscr5* knockdown disrupts mediolateral cell intercalations underlying CE during gastrulation. Finally, and more importantly, we showed that in *dscr5* morphants, the membrane localization of Knypek and Frizzled 7 receptors was disrupted, and Dishevelled protein was partially degraded by the ubiquitin-proteosome pathway. These results strongly suggest that Dscr5 regulates CE through the PCP pathway.

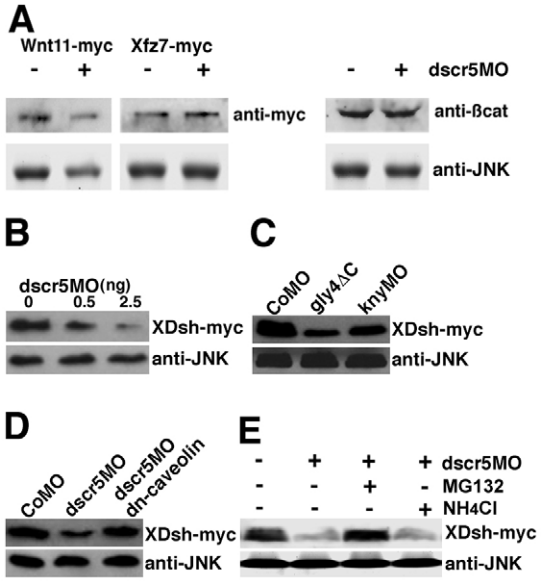

Fig. 8. Rescue of Dishevelled stability by *dn-caveolin* and proteosome inhibitor. Western blot analysis of XDsh-Myc at the shield stage of zebrafish embryos. (**A**) Absence of effect of *dscr5* knockdown on the stability of Wnt11-Myc, XFz7-Myc and endogenous β-Catenin. (**B**) Dose-dependent effect of *dscr5* knockdown on XDsh-Myc stability. (**C**) XDsh-Myc degradation in embryos injected with *gly4ΔC* RNA or *kny*MO. (**D**) Coexpression of *dn-caveolin* rescues XDsh-Myc stability in *dscr5* morphants. (**E**) MG132, but not NH_4Cl, maintains XDsh-Myc stability in *dscr5* knockdown cells. Endogenous JNK was used as a loading control.

Dscr5 is required for the membrane localization of Wnt receptors

It has been shown that GPI-anchoring is required for embryogenesis. Conditional knockout of *piga*, another component of the GPI biosynthesis complex, leads to insufficient neural tube closure and a cleft palate (Nozaki et al., 1999), and knockout of individual components of the complex in mice reveals a differential requirement for GPI-anchored proteins (Hong et al., 1999). Although we have shown that *dscr5* is required for the membrane localization of Knypek/Glypican 4, the possibility that *dscr5* knockdown also affects other GPI-anchored proteins cannot be excluded. For example, the EGF-CFC protein one-eyed pinhead (Oep) is required for nodal signaling (Gritsmann et al., 1999) and is predicted to be GPI-anchored. However, it has been demonstrated that the carboxyl-terminal region required for membrane anchorage is dispensable for the activity of EGF-CFC protein in nodal signaling, as the EGF-CFC domain is sufficient to rescue the maternal-zygotic *oep* mutant phenotype (Minchiotti et al., 2001). Therefore, even if *dscr5* knockdown disrupts the membrane anchorage of Oep, nodal signaling should not be affected because secreted Oep is functional in nodal signaling. This is consistent with our observation that early dorsoventral patterning was not affected in *dscr5* morphants.

The *Drosophila* Glypican Dally-like, a Knypek ortholog, is required for hedgehog signaling (Desbordes and Sanson, 2003; Gallet et al., 2008). This raises the question of whether *dscr5* knockdown also affects hedgehog signaling, which is required for specification and patterning of ventral cell type at all axial levels (Rubenstein and Beachy, 1998). Our result showed that the expression of hedgehog target genes *nk2.1b*, *nk2.2* and *patched 1* (Barth and Wilson, 1995; Lewis et al., 1999; Rohr et al., 2001) was not affected in *dscr5* morphants, which is consistent with a previous observation showing that *kny* mutations do not interfere with hedgehog signaling (Marlow et al., 1998). Understanding why the absence of Dscr5 or Knypek/Glypican 4 activity specifically affects non-canonical Wnt signaling will require further study. It is likely that this specificity might be dependent on cell context, as was shown for the extracellular α/β-hydrolase Notum, which modifies the ability of Dally to bind Wnt ligands at the cell surface, and the loss-of-function of which specifically leads to increased Wingless activity (Giráldez et al., 2002). The demonstration that both *knypek* knockdown and an interfering mutant of *glypican 4* were able to rescue the CE defects produced by *dscr5* overexpression, both in vivo and in vitro, supports this conclusion and argues that Dscr5 indeed functions upstream of Knypek/Glypican 4 in cell movements. Since Glypican 4 physically interacts with Frizzled 7 (Ohkawara et al., 2003), it is conceivable that *dscr5* knockdown directly affects the membrane localization of Knypek/Glypican 4; this then indirectly causes an enhanced Frizzled 7 endocytosis in a Caveolin-dependent manner. This is also consistent with our observation that overexpression of *gly4ΔC*, which lacks the GPI moiety attachment site and should increase the free extracellular level, as in *dscr5* knockdown, disrupts Frizzled 7 membrane localization.

Knockdown of *dscr5* specifically affects Dishevelled stability

The specific degradation of Dishevelled following *dscr5* knockdown further supports the conclusion that *dscr5* is involved in Wnt signaling. It is unlikely that Dscr5 is directly involved in stabilizing Dishevelled protein; however, the observed degradation might be a consequence of the disrupted membrane localization of Frizzled receptors. This blocks the recruitment of Dishevelled to the cell membrane and makes it more accessible for degradation. A similar situation was observed for Prickle, which blocks Frizzled 7-dependent membrane localization of Dishevelled and leads to its degradation (Carreira-Barbosa et al., 2003). As *dscr5* knockdown disrupts the membrane localization of the Frizzled 7 receptor, which interacts with Dishevelled (Axelrod et al., 1998; Wong et al., 2003), it is likely that this indirectly prevents the membrane recruitment of Dishevelled, which is then targeted to the ubiquitin-proteosome pathway for degradation (Fig. 9). The fact that overexpression of *gly4ΔC*, like *dscr5* knockdown, similarly caused Dishevelled degradation is consistent with a requirement of Knypek/Glypican 4 membrane localization in maintaining Dishevelled stability. It was shown previously that interfering with Glypican 4 activity prevents Dishevelled membrane recruitment (Ohkawara et al., 2003), which is consistent with our observation that overexpression of *gly4ΔC* disrupts Frizzled 7 membrane localization and affects Dishevelled stability. However, whether Dishevelled is subjected to degradation was not addressed previously. In this study, we observed that interfering with Dscr5 or Knypek/Glypican 4 activity similarly leads to Dishevelled degradation; nevertheless, complete Dishevelled degradation was not observed. This could account for, at least partially, the presence of Dishevelled protein detected by immunofluorescence staining in a previous study. Taken together, our results demonstrate that Dscr5 plays a crucial role for membrane anchoring of Knypek/Glypican 4 and is thus required for CE movements.

The degradation of Dishevelled following *dscr5* knockdown implies that Dscr5 should also have an effect on canonical Wnt signaling and thus on dorsoventral patterning. We found that a high dose of *dscr5*MO occasionally results in a dorsalized

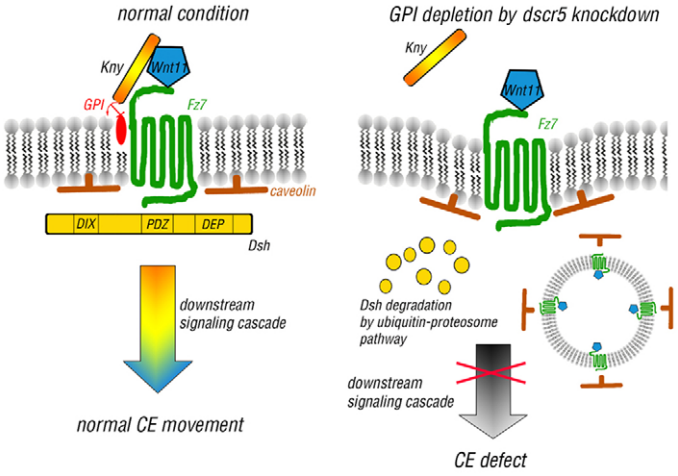

Fig. 9. Summary of the required activity of Dscr5 in Wnt signaling and CE movements. Dscr5 functions in the GPI biosynthesis complex to anchor Knypek/Glypican 4 at the cell surface. Interaction between Knypek/Glypican 4 and the Frizzled 7 receptor leads to the recruitment of Dishevelled to the cell surface and the activation of a downstream signaling cascade. The absence of Dscr5 activity disrupts the membrane localization and increases the free extracellular levels of Knypek/Glypican 4. This leads to an enhanced Frizzled 7 endocytosis in a Caveolin-dependent manner and triggers Dishevelled degradation through the ubiquitin-proteosome pathway.

phenotype (data not shown), which is reminiscent of inhibition of zygotic canonical Wnt signaling. However, at intermediate or low dose, we only observed CE defects, whereas mesoderm patterning was not affected, suggesting that the canonical Wnt/β-Catenin pathway was not, or at least not significantly, affected. This is consistent with a recent observation showing that global canonical Wnt/β-Catenin signaling was not affected in mutant mice with low Dishevelled levels, whereas the embryos exhibited a PCP phenotype in the neural tube (Etheridge et al., 2008). Since Dishevelled levels are reduced, rather than absent, in embryos lacking Dscr5 activity, it is conceivable that these embryos displayed CE defects without an obvious effect on dorsoventral patterning.

In conclusion, Glypicans are potential GPI-linking molecules and play a crucial role in the regulation of signaling by growth factors (Selleck, 2000). Our findings suggest that regulation of the membrane localization of Knypek/Glypican 4 by Dscr5 controls CE movements during early development. The specificity of the effects implies that the activity of specific subsets of proteoglycans, including Knypek/Glypican 4 in particular, requires membrane anchorage. The DSCR on chromosome 21 contains several genes including *dscr5* and is associated with specific features of Down syndrome likely to be caused by an increased gene dosage (Pritchard et al., 2008). Thus, functional analysis should also help to understand how they regulate morphogenesis and how their duplication leads to the major phenotypic features of Down syndrome.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/12/2121/DC1

References

Almeida, A. M., Murakami, Y., Layton, D. M., Hillmen, P., Sellick, G. S., Maeda, Y., Richards, S., Patterson, S., Kotsianidis, I., Mollica, L. et al. (2006). Hypomorphic promoter mutation in PIGM causes inherited glycosylphosphatidylinositol deficiency. *Nat. Med.* **12**, 846-851.

Axelrod, J. D., Miller, J. R., Shulman, J. M., Moon, R. T. and Perrimon, N. (1998). Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways. *Genes Dev.* **12**, 2610-2622.

Baeg, G. H., Lin, X., Khare, N., Baumgartner, S. and Perrimon, N. (2001). Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless. *Development* **128**, 87-94.

Barth, K. A. and Wilson, S. W. (1995). Expression of zebrafish *nk2. 2* is influenced by sonic hedgehog/vertebrate hedgehog-1 and demarcates a zone of neuronal differentiation in the embryonic forebrain. *Development* **121**, 1755-1768.

Caneparo, L., Huang, Y. L., Staudt, N., Tada, M., Ahrendt, R., Kazanskaya, O., Niehrs, C. and Houart, C. (2007). Dickkopf-1 regulates gastrulation movements by coordinated modulation of Wnt/beta catenin and Wnt/PCP activities, through interaction with the Dally-like homolog Knypek. *Genes Dev.* **21**, 465-480.

Carreira-Barbosa, F., Concha, M. L., Takeuchi, M., Ueno, N., Wilson, S. W. and Tada, M. (2003). Prickle 1 regulates cell movements during gastrulation and neuronal migration in zebrafish. *Development* **130**, 4037-4046.

Carreira-Barbosa, F., Kajita, M., Morel, V., Wada, H., Okamoto, H., Martinez Arias, A., Fujita, Y., Wilson, S. W. and Tada, M. (2009). Flamingo regulates epiboly and convergence/extension movements through cell cohesive and signalling functions during zebrafish gastrulation. *Development* **136**, 383-392.

Carron, C., Pascal, A., Djiane, A., Boucaut, J. C., Shi, D. L. and Umbhauer, M. (2003). Frizzled receptors dimerization is sufficient to activate the Wnt/β-catenin pathway. *J. Cell Sci.* **116**, 2541-2550.

Carron, C., Bourdelas, A., Li, H. Y., Boucaut, J. C. and Shi, D. L. (2005). Antagonistic interaction between IGF and Wnt/JNK signaling in convergent extension in *Xenopus* embryo. *Mech. Dev.* **122**, 1234-1247.

Delabar, J. M., Theophile, D., Rahmani, Z., Chettouh, Z., Blouin, J. L., Prieur, M., Noel, B. and Sinet, P. M. (1993). Molecular mapping of twenty-four features of Down syndrome on chromosome 21. *Eur. J. Hum. Genet.* **1**, 114-124.

Desbordes, S. C. and Sanson, B. (2003). The glypican Dally-like is required for Hedgehog signalling in the embryonic epidermis of Drosophila. *Development* **130**, 6245-6255.

Djiane, A., Riou, J. F., Umbhauer, U., Boucaut, J. C. and Shi, D. L. (2000). Role of frizzled 7 in the regulation of convergent extension movements during gastrulation in *Xenopus laevis*. *Development* **127**, 3091-3100.

Eisen, J. S. and Smith, J. C. (2008). Controlling morpholino experiments: don't stop making antisense. *Development* **135**, 1735-1743.

Etheridge, S. L., Ray, S., Li, S., Hamblet, N. S., Lijam, N., Tsang, M., Greer, J., Kardos, N., Wang, J., Sussman, D. J. et al. (2008). Murine dishevelled 3 functions in redundant pathways with dishevelled 1 and 2 in normal cardiac outflow tract, cochlea, and neural tube development. *PLoS Genet.* **4**, e1000259.

Franch-Marro, X., Marchand, O., Piddini, E., Ricardo, S., Alexandre, C. and Vincent, J. P. (2005). Glypicans shunt the Wingless signal between local signalling and further transport. *Development* **132**, 659-666.

Gallet, A., Staccini-Lavenant, L. and Thérond, P. P. (2008). Cellular trafficking of the glypican Dally-like is required for full-strength Hedgehog signaling and wingless transcytosis. *Dev. Cell* **14**, 712-725.

Giráldez, A. J., Copley, R. R. and Cohen, S. M. (2002). HSPG modification by the secreted enzyme Notum shapes the Wingless morphogen gradient. *Dev. Cell* **2**, 667-676.

Gritsman, K., Zhang, J., Cheng, S., Heckscher, E., Talbot, W. S. and Schier, A. F. (1999). The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. *Cell* **97**, 121-132.

Habas, R., Kato, Y. and He, X. (2001). Wnt/Frizzled activation of Rho regulates vertebrate gastrulation and requires a novel Formin homology protein Daam1. *Cell* **107**, 843-854.

Han, C., Yan, D., Belenkaya, T. Y. and Lin, X. (2005). Drosophila glypicans Dally and Dally-like shape the extracellular Wingless morphogen gradient in the wing disc. *Development* **132**, 667-679.

Heisenberg, C. P. and Tada, M. (2002). Zebrafish gastrulation movements: bridging cell and developmental biology. *Semin. Cell Dev. Biol.* **13**, 471-479.

Heisenberg, C. P., Tada, M., Rauch, G. J., Saude, L., Concha, M. L., Geisler, R., Stemple, D. L., Smith, J. C. and Wilson, S. W. (2000). Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. *Nature* **405**, 76-81.
Hong, Y., Ohishi, K., Watanabe, R., Endo, Y., Maeda, Y. and Kinoshita, T. (1999). GPI1 stabilizes an enzyme essential in the first step of glycosylphosphatidylinositol biosynthesis. *J. Biol. Chem*. **274**, 18582-18588.
Huang, H., Lu, F. I., Jia, S., Meng, S., Cao, Y., Wang, Y., Ma, W., Yin, K., Wen, Z., Peng, J. et al. (2007). Amotl2 is essential for cell movements in zebrafish embryo and regulates c-Src translocation. *Development* **134**, 979-988.
Inoue, N., Murakami, Y. and Kinoshita, T. (2003). Molecular genetics of paroxysmal nocturnal hemoglobinuria. *Int. J. Hematol.* **77**, 107-112.
Keller, R., Davidson, L., Edlund, A., Elul, T., Ezin, M., Shook, D. and Skoglund, P. (2000). Mechanisms of convergence and extension by cell intercalation. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **355**, 897-922.
Kinoshita, N., Iioka, H., Miyakoshi, A. and Ueno, N. (2003). PKC delta is essential for Dishevelled function in a noncanonical Wnt pathway that regulates Xenopus convergent extension movements. *Genes Dev.* **17**, 1663-1676.
Klein, T. J. and Mlodzik, M. (2005). Planar cell polarization: an emerging model points in the right direction. *Annu. Rev. Cell Dev. Biol*. **21**, 155-176.
Kühl, M. (2002). Non-canonical Wnt signaling in *Xenopus*: regulation of axis formation and gastrulation. *Semin. Cell Dev. Biol*. **3**, 243-249.
Lewis, K. E., Concordet, J. P. and Ingham, P. W. (1999). Characterisation of a second patched gene in the zebrafish Danio rerio and the differential response of patched genes to Hedgehog signalling. *Dev. Biol*. **208**, 14-29.
Lin, X. and Perrimon, N. (1999). Dally cooperates with Drosophila Frizzled 2 to transduce Wingless signalling. *Nature* **400**, 281-284.
Link, V., Shevchenko, A. and Heisenberg, C. P. (2006). Proteomics of early zebrafish embryos. *BMC Dev. Biol.* **6**, 1.
Marlow, F., Zwartkruis, F., Malicki, J., Neuhauss, S. C. F., Abbas, L., Weaver, M., Driever, W. and Solnica-Krezel, L. (1998). Functional interactions of genes mediating convergent extension, *knypek* and *trilobite*, during partitioning of the eye primordium in zebrafish. *Dev. Biol*. **203**, 382-399.
Marlow, F., Topczewski, J., Sepich, D. and Solnica-Krezel, L. (2002). Zebrafish Rho kinase 2 acts downstream of Wnt11 to mediate cell polarity and effective convergence and extension movements. *Curr. Biol*. **12**, 876-884.
Minchiotti, G., Manco, G., Parisi, S., Lago, C. T., Rosa, F. and Persico, M. G. (2001). Structure-function analysis of the EGF-CFC family member Cripto identifies residues essential for nodal signalling. *Development* **128**, 4501-4510.
Myers, D. C., Sepich, D. S. and Solnica-Krezel, L. (2002). Convergence and extension in vertebrate gastrulae: cell movements according to or in search of identity? *Trends Genet*. **18**, 447-455.
Nozaki, M., Ohishi, K., Yamada, N., Kinoshita, T., Nagy, A. and Takeda, J. (1999). Developmental abnormalities of glycosylphosphatidylinositol-anchor-deficient embryos revealed by Cre/loxP system. *Lab. Invest*. **79**, 293-299.
Ohkawara, B., Yamamoto, T. S., Tada, M. and Ueno, N. (2003). Role of glypican 4 in the regulation of convergent extension movements during gastrulation in Xenopus laevis. *Development* **130**, 2129-2138.
Park, T. J., Gray, R. S., Sato, A., Habas, R. and Wallingford, J. B. (2005). Subcellular localization and signaling properties of dishevelled in developing vertebrate embryos. *Curr. Biol*. **15**, 1039-1044.
Pol, A., Luetterforst, R., Linsday, M., Heino, S., Ikonen, E. and Parton, R. G. (2001). A caveolin dominant negative mutant associates with lipid bodies and induces intracellular cholesterol imbalance. *J. Cell Biol*. **152**, 1057-1070.
Pritchard, M., Reeves, R. H., Dierssen, M., Patterson, D. and Gardiner, K. J. (2008). Down syndrome and the genes of human chromosome 21, current knowledge and future potentials. Report on the Expert workshop on the biology of chromosome 21 genes: towards gene-phenotype correlations in Down syndrome. *Cytogenet. Genome Res*. **121**, 67-77.
Rohr, K. B., Barth, K. A., Varga, Z. M. and Wilson, S. W. (2001). The nodal pathway acts upstream of hedgehog signaling to specify ventral telencephalic identity. *Neuron* **29**, 341-351.
Rothbächer, U., Laurent, M. N., Deardorff, M. A., Klein, P. S., Cho, K. W. and Fraser, S. E. (2000). Dishevelled phosphorylation, subcellular localization and multimerization regulate its role in early embryogenesis. *EMBO J*. **19**, 1010-1022.
Rubenstein, J. L. and Beachy, P. A. (1998). Patterning of the embryonic forebrain. *Curr. Opin. Neurobiol.* **8**, 18-26.
Rupp, R. and Weintraub, H. (1991). Ubiquitous MyoD transcription at the midblastula transition precedes induction-dependent MyoD expression in presumptive mesoderm of *X. laevis*. *Cell* **65**, 927-937.
Sato, A., Khadka, D. K., Liu, W., Bharti, R., Runnels, L. W., Dawid, I. B. and Habas, R. (2006). Profilin is an effector for Daam1 in non-canonical Wnt signaling and is required for vertebrate gastrulation. *Development* **133**, 4219-4231.
Selleck, S. B. (2000). Proteoglycans and pattern formation: sugar biochemistry meets developmental genetics. *Trends Genet*. **16**, 206-212.
Sepich, D. S. and Solnica-Krezel, L. (2005). Analysis of cell movements in zebrafish embryos: morphometrics and measuring movement of labeled cell populations *in vivo*. *Methods Mol. Biol*. **294**, 211-233.
Shibuya, K., Kudoh, J., Minoshima, S., Kawasaki, K., Asakawa, S. and Shimizu, N. (2000). Isolation of two novel genes, DSCR5 and DSCR6, from Down syndrome critical region on human chromosome 21q22.2. *Biochem. Biophys. Res. Commun*. **271**, 693-698.
Sokol, S. Y. (1996). Analysis of Dishevelled signalling pathways during *Xenopus* development. *Curr. Biol*. **6**, 1456-1467.
Solnica-Krezel, L. (2005). Conserved patterns of cell movements during vertebrate gastrulation. *Curr. Biol*. **15**, R213-R228.
Smith, J. C., Conlon, F. L., Saka, Y. and Tada, M. (2000). *Xwnt11* and the regulation of gastrulation in *Xenopus*. *Philos. Trans. R. Soc. Lond. B Biol. Sci*. **355**, 923-930.
Tada, M. and Smith, J. C. (2000). *Xwnt-11*, a target of *Xenopus* Brachyury, regulates gastrulation movements via Dishevelled, but not through the canonical Wnt pathway. *Development* **127**, 2227-2238.
Tada, M., Concha, M. L. and Heisenberg, C. P. (2002). Non-canonical Wnt signalling and regulation of gastrulation movements. *Semin. Cell Dev. Biol.* **3**, 251-260.
Thisse, C. and Thisse, B. (2008). High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat. Protoc*. **3**, 59-69.
Togashi, T., Choi, D. K., Taylor, T. D., Suzuki, Y., Sugano, S., Hattori, M. and Sakaki, Y. (2000). A novel gene, DSCR5, from the distal Down syndrome critical region on chromosome 21q22.2. *DNA Res*. **7**, 207-212.
Topczewski, J., Sepich, D. S., Myers, D. C., Walker, C., Amores, A., Lele, Z., Hammerschmidt, M., Postlethwait, J. and Solnica-Krezel, L. (2001). The zebrafish glypican knypek controls cell polarity during gastrulation movements of convergent extension. *Dev. Cell* **1**, 251-264.
Tsuda, M., Kamimura, K., Nakato, H., Archer, M., Staatz, W., Fox, B., Humphrey, M., Olson, S., Futch, T., Kaluza, V. et al. (1999). The cell-surface proteoglycan Dally regulates Wingless signalling in Drosophila. *Nature* **400**, 276-280.
Ulrich, F., Concha, M. L., Heid, P. J., Voss, E., Witzel, S., Roehl, H., Tada, M., Wilson, S. W., Adams, R. J., Soll, D. R. et al. (2003). Slb/Wnt11 controls hypoblast cell migration and morphogenesis at the onset of zebrafish gastrulation. *Development* **130**, 5375-5384.
Umbhauer, M., Djiane, A., Goisset, C., Penzo-Méndez, 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/beta-catenin signalling. *EMBO J*. **19**, 4944-4954.
Wallingford, J. B. and Habas, R. (2005). The developmental biology of Dishevelled: an enigmatic protein governing cell fate and cell polarity. *Development* **132**, 4421-4436.
Wallingford, J. B., Rowning, B. A., Vogeli, K. M., Rothbacher, U., Fraser, S. E. and Harland, R. M. (2000). Dishevelled controls cell polarity during *Xenopus* gastrulation. *Nature* **405**, 81-85.
Wallingford, J. B., Fraser, S. E. and Harland, R. M. (2002). Convergent extension: the molecular control of polarized cell movement during embryonic development. *Dev. Cell* **2**, 695-706.
Watanabe, R., Murakami, Y., Marmor, M. D., Inoue, N., Maeda, Y., Hino, J., Kangawa, K., Julius, M. and Kinoshita, T. (2000). Initial enzyme for glycosylphosphatidylinositol biosynthesis requires PIG-P and is regulated by DPM2. *EMBO J*. **19**, 4402-4411.
Winter, C. G., Wang, B., Ballew, A., Royou, A., Karess, R., Axelrod, J. D. and Luo, L. (2001). Drosophila Rho-associated kinase (Drok) links Frizzled-mediated planar cell polarity signaling to the actin cytoskeleton. *Cell* **105**, 81-91.
Wong, C. K., Luo, W., Deng, Y., Zou, H., Ye, Z. and Lin, S. C. (2004). The DIX domain protein coiled-coil-DIX1 inhibits c-Jun N-terminal kinase activation by Axin and dishevelled through distinct mechanisms. *J. Biol. Chem*. **279**, 39366-39373.
Wong, H. C., Bourdelas, A., Krauss, A., Lee, H. J., Shao, Y., Wu, D., Mlodzik, M., Shi, D. L. and Zheng, J. (2003). Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled. *Mol. Cell* **12**, 1251-1260.
Yamanaka, H., Moriguchi, T., Masuyama, N., Kusakabe, M., Hanafusa, H., Takada, R., Takada, S. and Nishida, E. (2002). JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. *EMBO Rep*. **3**, 69-75.