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-acetylgalcosaminyltransferase (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-proteasome 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 an essential step in understanding the pathogenesis of 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 (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 Bernard, 2000; Tada et al., 2002). 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). 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-proteasome 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 carboxy 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. Xshy-myc (Sokol, 1996), dshΔDIX (Tada and Smith, 2000), wt11-myc (Djiane et al., 2000), Xf7-myc (Carron et al., 2003), dsh-GFP (Axelrod et al., 1998), gly4AC (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 (knymyc) 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.

**Morphinolinos and RNA synthesis for microinjections**  Two morphinolinos (MOs) designed over the initiation methionine of zebrafish dscr5 (M01: 5'-GAGGAGAAGGAGATCTTCATCGCTG-3' and M02: 5'-CATCTCCACGACGCACATGTCTT-3'), one MO designed over the splice acceptor site of exon 3 (M03: 5'-GACCCATAACGACCTGTAAAGAGAAGA-3'), the control MO (CoMO: 5'-GTGGAGATGGGAGAGTGTCTCTACCAT-3') with five mismatches with respect to M01 (underlined), and knypek MO (knymyc: 5'-AACACAAGCTCATCTCTACGTG-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 NH4Cl.

**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 dscr5MO 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 dscr5MO 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 Thissee, 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'-GAGATGGGTAGGTGGAGAAGC-3' and 5'-CACGCGAGCCGT-GTTCAT-3'.

**Luciferase assay**  The AP1-luciferase reporter plasmid (200 pg) was injected alone or coinjected with 500 pg gly4AC 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 (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 expression domain at the one- to four-cell stage.
(cts1b – 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 dscr5MO (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 dscr5MO (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 dscr5MO in blocking the translation of dscr5MT RNA, but not mut-dscr5MT RNA, was efficiently blocked by dscr5MO (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. 3A,B). By contrast, cells from dscr5MO-injected embryos (n=11) remained scattered and failed to elongate along the anteroposterior axis (Fig. 3D). This indicates that mediatolateral 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
Kaede-GFP RNA and CoMO or dscr5MO 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 dscr5MO-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 dscr5MO-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 dscr5MO (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 dscr5MO-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 dscr5MO. We found that coinjection of 200 pg dshΔDIX RNA with 5 ng dscr5MO significantly reduced the extent of CE defects (Fig. 5A), suggesting that dscr5MO 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
signaling during CE movements (Yamanaka et al., 2002), using a dominant negative mutant RNA (referred to here as dnJNK). When a low dose of dscr5MO (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 dscr5MO 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 dscr5MO (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 dscr5MO 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 dscr5MO 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 (Okawara 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 dscr5MO. In the absence of dscr5MO, 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.
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 gly-4AC 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), knyMO (5 ng) and gly-4AC RNA (100 pg) induced CE defects in a similar manner, as revealed by in situ hybridization using myoD (myod1–ZFIN) (Fig. 7A,D,G) and dlx3 (Fig. 7A,D,G) 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 kny morphants, and in gly-4AC-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.
However, we noticed that knypek morphants developed a shorter anteroposterior axis (Fig. 7I), which was similar to kny mutants (Topeczowski 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 dscr5MO or gly4ΔC RNA produces an excess of free extracellular Knypek/Glypican 4 protein (see Fig. 6C), whereas injection of knyMO 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 co-injected 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 knyMO (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 dscr5MO (5 ng). Surprisingly, only weak fluorescence was observed in embryos co-injected with dsh-GFP and dscr5MO 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 asXdsh-myc) RNA was co-injected with dscr5MO, 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 knyMO (10 ng) was co-injected 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. 6J), we assayed whether it could rescue XDsh-Myc stability in dscr5 morphants. Coinjection of dn-caveolin RNA efficiently prevented XDsh-Myc from dscr5MO-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 dscr5MO were incubated in the proteosome inhibitor MG132 or in the lysosome inhibitor.
NH₄Cl. Western blot analysis clearly showed that MG132, but not NH₄Cl, 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.
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 carboxy-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 gly4AC, 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 gly4AC, 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 gly4AC 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 smo occasionally results in a dorsalmost
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 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 pathway was not, or at least not significantly, affected. This is from the Ministry of Science and Technology of China, the Association Française contre les Myopathies, the Ligue Nationale Contre le Cancer and the Association pour la Recherche sur le Cancer. H.Y.L. was supported by a CNRS post-doctoral fellowship.

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

References


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Dscr5 in convergent extension


Huang, H., Li, F., Jia, S., Meng, S., Cao, W., Yang, W., Ma, W., Yin, K., Wen, Z., Peng, J. et al. (2007). Atoz2 is essential for cell movements in zebrafish embryo and regulates c-src translocation. Development 134, 979-988.


**Table S1. Rescue of explant elongation by gly4ΔC in activin-treated Xenopus animal cap explants overexpressing dscr5**

<table>
<thead>
<tr>
<th>Construct injected</th>
<th>Elongated (%)</th>
<th>Rounded (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjected/activin</td>
<td>77%</td>
<td>23</td>
<td>119</td>
</tr>
<tr>
<td>dscr5/activin</td>
<td>21</td>
<td>79</td>
<td>113</td>
</tr>
<tr>
<td>dscr5/gly4ΔC/activin</td>
<td>67</td>
<td>33</td>
<td>120</td>
</tr>
</tbody>
</table>

**Table S2. The effect of dscr5 overexpression in Xenopus whole embryos is rescued by coexpression of gly4ΔC**

<table>
<thead>
<tr>
<th>Construct injected</th>
<th>Normal (%)</th>
<th>Bent axis (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjected</td>
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<td>5</td>
<td>135</td>
</tr>
<tr>
<td>dscr5</td>
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<td>67</td>
<td>116</td>
</tr>
<tr>
<td>dscr5/gly4ΔC</td>
<td>73</td>
<td>27</td>
<td>154</td>
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