Role of glypican 4 in the regulation of convergent extension movements during gastrulation in *Xenopus laevis*

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SUMMARY

Coordinated morphogenetic cell movements during gastrulation are crucial for establishing embryonic axes in animals. Most recently, the non-canonical Wnt signaling cascade (PCP pathway) has been shown to regulate convergent extension movements in *Xenopus* and zebrafish. Heparan sulfate proteoglycans (HSPGs) are known as modulators of intercellular signaling, and are required for gastrulation movements in vertebrates. However, the function of HSPGs is poorly understood. We analyze the function of *Xenopus* glypican 4 (Xgly4), which is a member of membrane-associated HSPG family. In situ hybridization revealed that *Xgly4* is expressed in the dorsal mesoderm and ectoderm during gastrulation. Reducing the levels of Xgly4 inhibits cell-membrane accumulation of Dishevelled (Dsh), which is a transducer of the Wnt signaling cascade, and thereby disturbs cell movements during gastrulation. Rescue analysis with different Dsh mutants and Wnt11 demonstrated that Xgly4 functions in the non-canonical Wnt/PCP pathway, but not in the canonical Wnt/β-catenin pathway, to regulate gastrulation movements. We also provide evidence that the Xgly4 protein physically binds Wnt ligands. Therefore, our results suggest that Xgly4 functions as positive regulator in non-canonical Wnt/PCP signaling during gastrulation.

Key words: Heparan sulfate proteoglycan, Wnt signaling pathway, Gastrulation movements, *Xenopus*

INTRODUCTION

Most animals undergo gastrulation to establish germ layers and embryonic axes. The dynamic morphogenetic cell movements that take place during gastrulation are highly coordinated. In *Xenopus* embryos, these cell movements, which include involution and convergent extension, are driven predominantly by mesodermal cells (Keller, 1991; Keller et al., 1992). This is particularly obvious during convergent extension, when polarized axial mesodermal cells intercalate in radial and mediolateral directions to cause dramatic elongation of the dorsal marginal zone along the anteroposterior (AP) axis (Shih and Keller, 1992; Wilson and Keller, 1991). However, the molecular mechanisms by which mesodermal cells become polarized and drive these movements are poorly understood.

Recently, it has been reported that a non-canonical Wnt signaling cascade, which is known to regulate planar cell polarity (PCP) in *Drosophila* (Adler, 1992; Mlodzik, 1999), also participates in the regulation of convergent extension movements in *Xenopus*, as well as in the zebrafish embryo (Djiane et al., 2000; Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000). The zebrafish *silberblick* (*slb*) locus encodes Wnt11, and Slb/Wnt11 activity is required for cells to undergo correct convergent extension movements during gastrulation. The signal transducer Dishevelled (Dsh) acts downstream of Slb/Wnt11 through domains specific to the non-canonical Wnt/PCP signaling cascade, and directly regulates cell polarity within cells undergoing convergent extension (Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000). In addition, the relocalization of Dsh to the cell membrane is required for convergent extension movements in *Xenopus* gastrulae, in the same way as recruitment of Dsh to the membrane through the Frizzled (Fz) receptor is required for the PCP pathway in *Drosophila* (Axelrod, 2001; Wallingford et al., 2000). Although many genetic experiments have demonstrated downstream effectors of the non-canonical PCP Fz/Dsh signal in *Drosophila* (Shulman et al., 1998; Tree et al., 2002), and some of these effectors (e.g. JNK) function to regulate the convergent extension movements (Darken et al., 2002; Yamanaka et al., 2002), the precise signaling mechanism that regulates the convergent extension movements remains unclear.

In addition to the intracellular signaling mechanism, intercellular modulators are involved in regulating coordinated movements of large cell populations. Heparan sulfate proteoglycans (HSPGs) have been implicated in the modulation of intercellular signaling in vertebrates and in *Drosophila*, and have been shown to be required for gastrulation movements in the *Xenopus* embryo (Brickman and Gerhart, 1994; Itoh and Sokol, 1994). Glypican, a member of the membrane-associated

HSPG family, is known to regulate Wnt signaling in *Drosophila* (Baeg et al., 2001; Tsuda et al., 1999) and zebrafish embryos (Topczewski et al., 2001). However, the molecular function of the HSPGs is poorly understood. Therefore, we cloned a gene encoding *Xenopus* glypican 4 (Xgly4), a member of the HSPG family, and analyzed its role in the non-canonical Wnt/PCP signaling pathway during gastrulation. In situ hybridization revealed that *Xgly4* is expressed in the dorsal mesoderm and ectoderm during gastrulation. Xgly4 overexpression and translational inhibition by a morpholino oligonucleotide inhibited the gastrulation movements of the embryo and the convergent extension (elongation) of activin-treated animal caps, but did not affect mesoderm induction. Rescue analysis with different Dsh mutants and Wnt11 demonstrated that Xgly4 functions in the non-canonical Wnt/PCP pathway, but not in the canonical Wnt/β-catenin pathway for convergent extension movements. Furthermore, we show that reducing the levels of Xgly4 inhibits the cell-membrane accumulation of Dsh, as does the inhibition of Wnt11 in the dorsal marginal zone. Finally, we provide evidence that the Xgly4 protein physically binds Wnt ligands.

MATERIALS AND METHODS

Plasmid constructs and morpholino oligonucleotides
The entire coding region of Xgly4, in clone 11o13 (pBS SK–) from the NIBB Mochii normalized *Xenopus* neurula library, was inserted into the *Eco*RI and *Xho*I sites of the CS2+ vector. For the immunoprecipitation assay, Xgly4-Flag, encoding amino acids 1-539 of Xgly4 with a Flag-tagged amino acid sequence at the C-terminal end, was used. We also used ΔCRD and ΔC, which lack amino acids 59-421 and 353-539 of the encoding region of Xgly4-Flag, respectively, and ΔGAG where the three putative glycosylated Ser (492, 496 and 498) residues of Xgly4-Flag were replaced with Ala residues. *Xenopus* activin (Thomsen et al., 1990), Xnr1 (Jones et al., 1995), XeFGF (a gift from Dr H. Okamoto), Xwnt11 (Tada and Smith, 2000), Xdsh with Myc-tag (Sokol, 1996), Xdsh-ΔDEP and Xdsh-ΔDIX (Wallingford et al., 2000) have been reported previously. Each construct was linearized, and capped mRNA was synthesized in vitro using the mMESSAGE mMACHINE SP6 kit (Ambion).

The Xgly4 morpholino sequence was 5′-TGCATGGTGAGAAC-GAAAGCGATC-3′. We used the morpholino oligonucleotide 5′-CATAGATGTATAAACGCTACGAAAG-3′ for the ascidian gene HrTT-1 (Hotta et al., 1998) as a negative control (Gene Tools, LLC)

Manipulation of embryos, microinjection of synthetic mRNA and morpholino oligonucleotide
Unfertilized eggs were collected and fertilized in vitro as described previously (Suzuki et al., 1997). Embryos were de-jellied using 3% cysteine and washed with water several times. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Synthesized RNA was injected into the animal pole or marginal zone of two- or four-cell-stage embryos, respectively, and the injected embryos were kept in 3% Ficoll/0.1× Steinberg's solution as described (Yamamoto et al., 2000). The animal cap (AC) and dorsal marginal zone (DMZ) were dissected at stage 8 and stage 10.5, respectively, and cultured in 0.1% BSA/1× Steinberg's solution (Asashima et al., 1990). For the standard *Xenopus* AC assay, ACs were cultured until stage 18, and scored for elongation.

In situ hybridization
Whole-mount in situ hybridization was performed with digoxigenin (DIG)-labeled probes, as described by Harland (Harland, 1991). Hybridization was detected with an alkaline phosphatase-coupled anti-DIG antibody and visualized by using BM purple (Roche Molecular Biochemicals). Antisense in situ probes against Xgly4 and Xwnt11 were generated by linearizing the pBS-KS-Xgly4 and pBS-KS-Xwnt11 constructs with *Not*I, and transcribing them with T7 RNA polymerase. Xbra (Smith et al., 1991), XmyoD (Hopwood et al., 1989), Xgsc (Blumberg et al., 1991; Cho et al., 1991), Xvent1 (Nishimatsu and Thomsen, 1998) and Xnot (von Dassow et al., 1993) have been reported previously.

RT-PCR analysis
Profiles of *Xbra* and *Xwnt11* expression in the animal cap injected with each mRNA or morpholino oligonucleotide were examined semi-quantitatively using RT-PCR (Yamamoto et al., 2000). ACs were collected when sibling embryos had developed to stage 11. The total RNA was then isolated using TRIzol (GIBCO/BRL), and cDNA was synthesized as previously reported (Yamamoto et al., 2000). PCR was performed with oligonucleotide primers: *Xbra*, 5′-GGATCATCTT-CTCAGCGCTGTGGA-3′ (upsteam) and 5′-GTTGTCGGCTGCCA-CAAAGTCCA-3′ (downstream); and *Xwnt11*, 5′-AAGTGCCACG-GAGTGTCTGG-3′ (upstream) and 5′-CTCAGACTCTCTCACTG-GCC-3′ (downstream). The primer sequence of histone, an internal input control, was as previously described (Iemura et al., 1998).

Northern blot analysis
PolyA+ RNA was extracted from whole oocytes and staged embryos using TRIzol reagent (GIBCO/BRL) and oligotex (Roche). Oocytes and embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). RNA was run on a 1% agarose gel containing formaldehyde, blotted onto nylon membranes, and hybridized with a DNA probe in 50% formamide, 5×SSPE, 5% SDS and 100 μg/ml denatured salmon sperm DNA. The entire Xgly4 cDNA was used as the probe. Blots were hybridization overnight at 55°C and then washed twice in 0.1×SSPE, 0.1% SDS at 55°C for 1 hour each.

Immunofluorescence
The DMZ and AC were dissected, fixed in MEMFA and incubated with anti-Myc antibody (9E10, Santa Cruz Biotechnology; 1:200) for 16 hours at 4°C. Subsequently, these tissues were incubated with an FITC-labeled secondary antibodies (anti-mouse IgG goat serum, 1:200) for 2 hours at room temperature, and examined using fluorescence microscopy (Larabell et al., 1997). To quantitate the localization of Xdsh in cells, five cells were randomly chosen from the mRNA- or morpholino oligonucleotide-injected area, which was marked by fluorescence, and the intensity of Xdsh staining in the cytoplasm and membrane in each cell was quantitated by using NIH image. The closest four among five sets of data were processed for graphical presentation.

Immunoprecipitation
HEK293T cells were transiently transfected with the indicated constructs using the calcium phosphate method. Forty hours after transfection, the cells were lyzed in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF] in the presence of protease inhibitors. Immunoprecipitation was performed by incubating the extracts with the M2-Flag monoclonal antibody (Sigma) coupled to protein A Sepharose CL 4B at 4°C for 1 hour. The precipitates were then washed with lysis buffer and subjected to western blot analysis using an anti-HA antibody (Santa Cruz Biotechnology).

RESULTS

A novel *Xenopus* glypican is the ortholog of mammalian glypican 4
Proteoglycans have recently been shown to be a key

component in regulating the action of polypeptide growth factors in a number of developmental processes (Bernfield et al., 1999). To investigate the role of HSPGs during early *Xenopus* development, we searched our *Xenopus laevis* EST database (http://xenopus.nibb.ac.jp) for proteoglycan-related genes. We found a cDNA containing an open reading frame of 556 amino acids that displays 69% amino acid identity to human and mouse glypican 4, and 57% identity to zebrafish *knypek* (*kny*; Fig. 1A). Thus, the new *Xenopus* glypican-related gene was named *Xenopus* glypican 4 (*Xgly4*). The deduced protein has a typical glypican structure, with an N-terminal signal peptide, an extracellular cysteine-rich domain and three putative O-glycosylation sites (Fig. 1B), which further supports the idea that Xgly4 is a member of the glypican family (Fig. 1A).

Expression of Xglyp4 is temporally and spatially regulated

As revealed by northern blotting, Xgly4 mRNA appears to be maternally expressed at low levels, and its transcripts of 7.3, 4.8, 3.0 and 2.5 kb were detected throughout early development. Interestingly, the two smallest transcripts were relatively abundant in gastrula embryos (Fig. 2A). The spatial expression pattern produced by whole-mount in situ hybridization showed that *Xgly4* was preferentially expressed in the animal hemisphere at the cleavage stages of the early blastula (data not shown) and that its expression was later confined to the deep layer of dorsal ectoderm and mesoderm of gastrula stage 11 embryos (Fig. 2B; upper left and right). In later embryogenesis, *Xgly4* was expressed along the neuroectoderm in the neurula, and then in the head and somites in tail bud embryos (Fig. 2B; bottom left and right).

The intriguing graded expression of Xgly4 along the dorsoventral (DV) axis of the early gastrula encouraged us to investigate how the expression was regulated. Consistent with the preferential dorsal expression in normal gastrulae, injection of mRNA for Xnr1, β-catenin or a dominant-negative type I BMP-receptor ALK3 (DN-BR) into the ventral blastomeres (to dorsalize embryo) induced ectopic expression of Xgly4 in the ventral region (Fig. 3B-D). In contrast to its expression in these dorsalized embryos, the expression of Xgly4 was diminished in ventralized embryos that had received an injection of Bmp4 mRNA in the dorsal region (Fig. 3F). These results suggest that, even if it is indirect, the expression of Xgly4 is positively regulated by the dorsalizing signal in DV axis formation.

Loss-of-function and gain-of-function analyses of Xgly4 causes defective convergent extension movements

To clarify the in vivo function of Xgly4, we performed loss-of-function and gain-of-function analyses. For the loss-of-
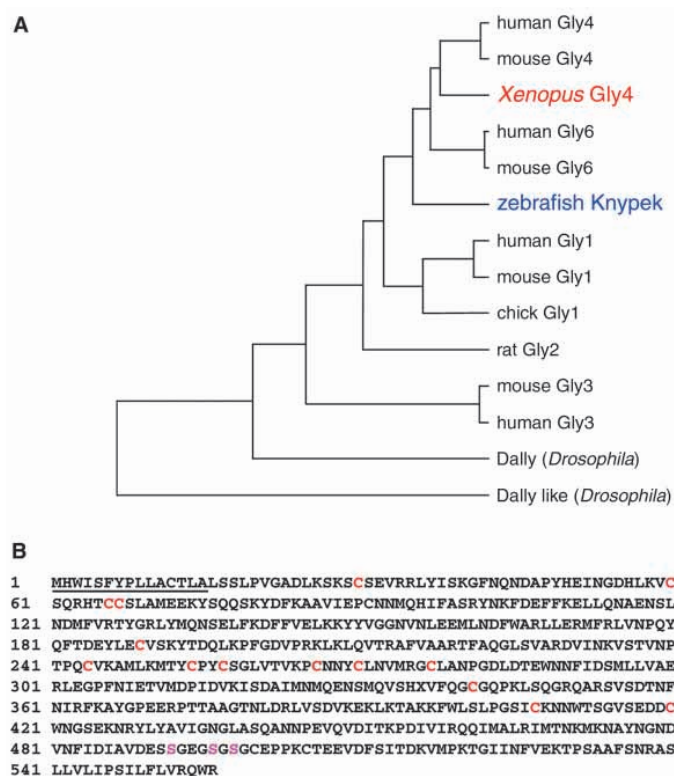

Fig. 1. Xenopus glypican 4 (Xgly4) is a novel member of the glypican family. (A) Phylogenetic tree of the glypican family. (B) The deduced 556 amino acid protein has a typical glypican structure with an N-terminal signal peptide (underline), an extracellular cysteine (red)-rich domain and three putative O-glycosylation sites (purple).
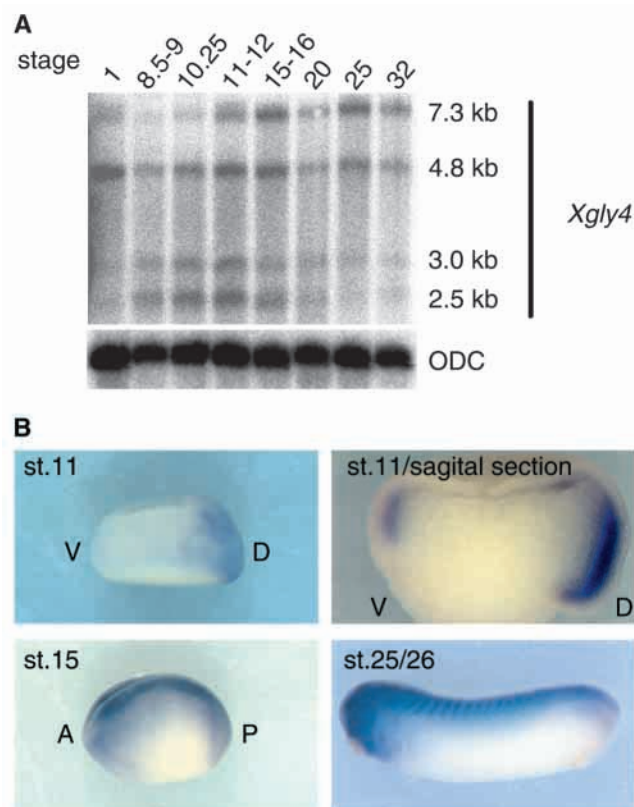

Fig. 2. Xgly4 is expressed in the dorsal mesoderm and ectoderm. Expression pattern of Xgly4 during *Xenopus* early development was analyzed by northern blot analysis (A) and whole-mount in situ hybridization (B). Numbers indicate developmental stage. In addition to expression in the deep layer of dorsal ectoderm, expression of Xgly4 was detected in the dorsal mesoderm and also weakly detected in the ventral mesoderm (cross-section along DV axis at stage 11; upper right in B). V, ventral; D, dorsal; A, anterior; P, posterior.
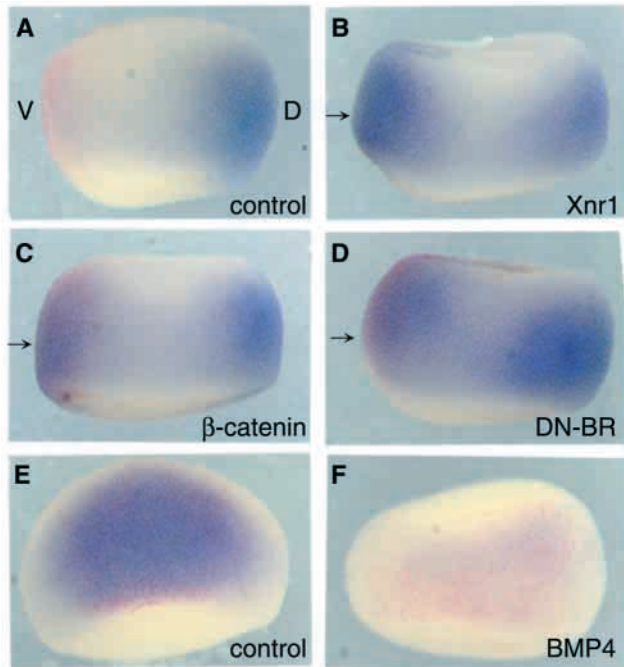
Fig. 3. Expression of Xgly4 is induced by dorsalizing signals during gastrulation. Injections of β-gal (100 pg; red) mRNA alone into ventral (A) or dorsal (E) regions did not affect Xgly4 expression pattern. However, expression of Xgly4 (purple) was detected by whole-mount in situ hybridization in the ventral side of embryos dorsalized with mRNA injection of Xnr1 (100 pg; B), β-catenin (100 pg; C) or the dominant-negative BMP receptor ALK3 (100 pg; D) with β-gal mRNA into the ventral region. Expression of Xgly4 was suppressed in embryo ventralized with Bmp4 mRNA (100 pg; F) and β-gal mRNA injected into the dorsal region. Arrows indicate the injection sites.

function analysis, the translation of Xgly4 mRNA was blocked by a specific antisense morpholino oligonucleotide (Xgly4Mo) directed against the 5′ untranslated region (5′ UTR) and the first methionine region. To confirm the specificity and efficacy of Xgly4Mo, western blot analysis was performed using a Flag-tagged Xgly4 mRNA that included the native 5′ UTR. Xgly4Mo specifically targeted the Xgly4 5′ UTR and did not reduce the protein level in either of the constructs: the Xgly4-Flag without the 5′ UTR and the Xglobin-Flag (Fig. 4A). Interestingly, a dorsal injection of Xgly4Mo or wild-type Xgly4 mRNA at the four-cell stage caused severe defects. Embryos at the tail-bud stage typically showed spina bifida, shortening of the AP axis and a defective head that specifically affected eye development (Fig. 4B). To examine the qualitative difference in phenotype between gain- or loss-of-function of Xgly4, we injected several doses of its mRNA or morpholino oligonucleotide. The observed phenotype of embryos injected with 1 ng of Xgly4 mRNA was similar to that of embryos injected with 21 ng Xgly4Mo (data not shown). Therefore, we believe there is no qualitative difference between gain- and loss-of-function of Xgly4. Compare with the effects of dorsal injection, ventral injection of either Xgly4Mo or wild-type Xgly4 mRNA resulted in minor defects in gastrulation movements (data not shown). Importantly, these defects were extremely similar to those of the zebrafish mutant *kny*.

To analyze the functional homology of Xgly4 to *kny*, we injected Xgly4 mRNA into zebrafish *kny*-mutant embryos to examine whether Xgly4 could rescue the *kny* phenotype. The embryonic axis and organ primodia of *kny* mutants are shorter along the AP axis and broader mediolaterally compared with their wild-type siblings (Topczewski et al., 2001). These phenotypes were significantly rescued by the injection of Xgly4 mRNA, which suggests that Xgly4 is a functional homolog of the zebrafish *kny* gene (Fig. 4D). Previous studies indicate that Xbra and Xwnt11, which are immediate-early targets of mesoderm inducers, are required for gastrulation movements (Tada and Smith, 2000). Therefore, we analyzed the expression of Xbra and Xwnt11 in embryos that had been injected dorsally with Xgly4Mo or wild-type Xgly4 mRNA. In situ hybridization analysis showed that *Xbra* and *Xwnt11* were expressed normally (Fig. 4C). Using mesodermal (XmyoD, Xnot), ventral (Xvent-1), dorsal (Xgsc) and neural markers (Xen2), we found that embryos injected dorsally with Xgly4Mo exhibited defects in the positioning and morphology of the mesoendodermal and neural tissues, although they expressed normal levels of specific marker genes (Fig. 4C; data not shown). These results further suggest that both loss- and gain-of-function of Xgly4 perturb gastrulation cell movement without affecting mesodermal differentiation.

Convergent extension movements have been analyzed in a simple assay in which naive AC cells can elongate in response to activin; this is associated with the induction of cell populations that give rise to notochord and muscle (Smith and Howard, 1992). To analyze the effects of Xgly4 on activin-induced AC elongation, Xgly4 mRNA or Xgly4Mo was injected with activin mRNA into the animal pole and the AC assay was performed. Although tissue elongation occurred in ACs that had been injected with activin mRNA injection, the elongation in response to activin was strongly inhibited by co-injection of higher amounts of Xgly4 mRNA or Xgly4Mo. In addition, Xgly4Mo-induced inhibition was rescued with a low dose of wild-type Xgly4 mRNA without the native 5′ UTR region (Fig. 5A). For the analysis of mesoderm induction by activin, the expression levels of both Xbra and Xwnt11 were measured using RT-PCR. The expression of these genes was not affected by the co-injection of Xgly4 mRNA or Xgly4Mo (Fig. 5B), which suggests that Xgly4 controls cell movements without regulating the transcription of mesodermal genes (mesoderm induction).

Xgly4 regulates the non-canonical Wnt/PCP pathway during gastrulation

The *Drosophila* proteins Dally and Dally-like (Dlp), which are *Drosophila* members of the glypican family, modulate the Wingless (Wg) pathway, regulating Wg activity extracellularly (Baeg et al., 2001; Lin and Perrimon, 1999; Tsuda et al., 1999). Therefore, we examined the possible function of Xgly4 in the Wnt pathway in *Xenopus*. In situ hybridization analysis revealed that Xgly4 transcripts were co-expressed with Xwnt11 at the DMZ and Xwnt5A at the dorsal ectoderm, but were not co-expressed with Xwnt8 (Christian et al., 1991; Ku and Melton, 1993; Moon et al., 1993; Tada and Smith, 2000). Several studies have shown that Xwnt5A and Xwnt11, but not Xwnt8, have dominant effects on gastrulation movements (Hoppler et al., 1996; Kelly et al., 1995; Moon et al., 1993; Tada and Smith, 2000). Therefore, Xgly4 could be involved in
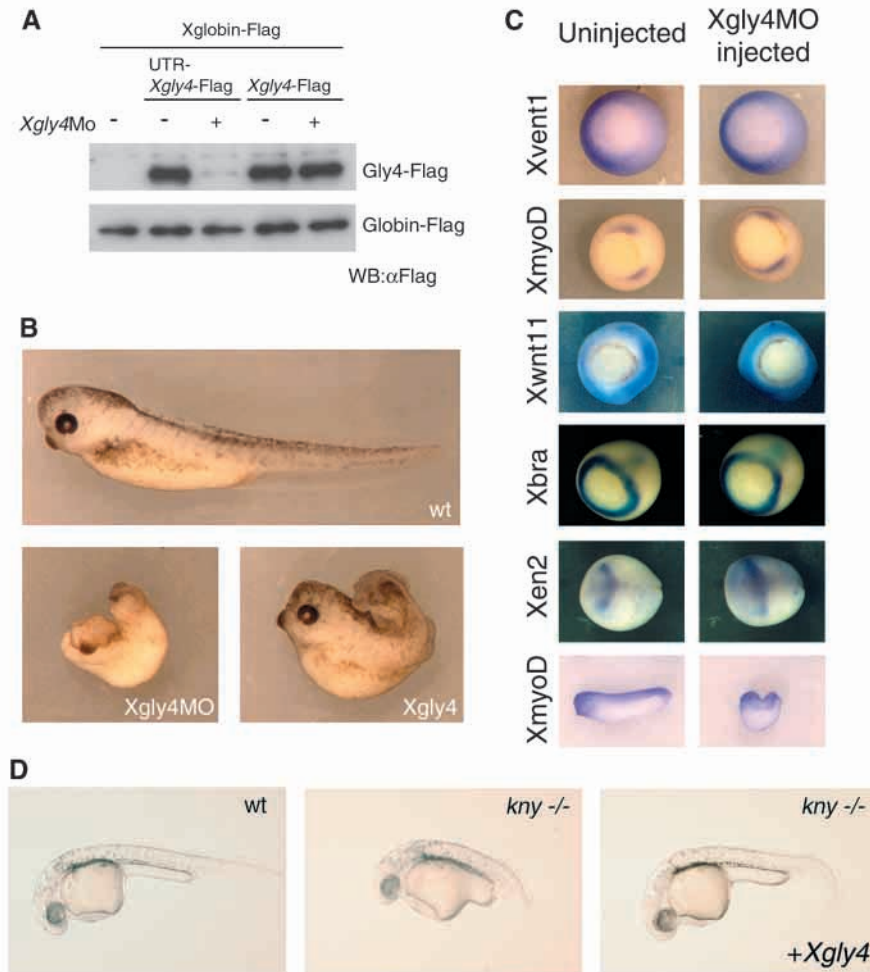
Fig. 4. Inhibition of Xgly4 translation blocks gastrulation movements. (A) Western blot analysis of Xgly4 protein tagged with a Flag epitope at the C terminus using an anti-Flag antibody. Translation of the injected globin-Flag (50 pg) and Xgly4-Flag (500 pg) mRNA lacking the 5′ UTR sequence was not inhibited by the morpholino oligonucleotide Xgly4Mo (41 ng), directed against the 5′ untranslated region and the first methionine region of Xgly4 mRNA. This finding indicates that Xgly4Mo does not inhibit translation nonspecifically. (B) Embryos injected with Xgly4Mo (41 ng) and Xgly4 mRNA (1 ng) at stage 35/36. Xgly4 morpholino oligonucleotide, which was injected into dorsal region, blocked gastrulation movements. (C) In situ hybridization for Xvent1 at stage 11, XmyoD at stage 11, Xwnt11 at stage 11, Xbra at stage 11, Xen2 at stage 13 and XmyoD at stage 25 in uninjected embryos (left) and embryos injected with XglyMo (41ng; right). (D) Injection of Xgly4 mRNA rescues the phenotypes of the knypek (*kny*) mutant. Embryos from crossing of *kny*[m119] heterozygotes were injected with 20 pg Xgly4 RNA at one-cell stage and scored at pharyngula stage. Twenty-five percent of uninjected embryos showed the *kny* mutant phenotype (*n*=64) (Topczewski et al., 2001), whereas none of injected embryos showed the phenotype. Rather, injected embryos were indistinguishable from wild-type embryos except that they occasionally exhibited a slightly curly tip of the tail (*n*=112). This result suggests that Xgly4 is a functional homolog of the zebrafish *kny* gene.

the regulation of the Wnt5A/Wnt11 pathway, which is also called the non-canonical Wnt/PCP pathway. To test whether Xgly4 functions in Xwnt11 signaling, the phenotype of embryos co-injected with Xgly4 and Xwnt11 mRNA was carefully examined. As shown in Fig. 6, embryos that had been injected with wild-type Xwnt11 mRNA showed disrupted development, including aberrations in gastrulation, neural tube closure and head formation, all of which are considered to arise from defects in gastrulation cell movements. These phenotypes were classified according to four grades of severity, and the embryos were scored using this index. Wild-type Xgly4 mRNA per se had a weak inhibitory effect on gastrulation. Interestingly, however, whereas co-injection of a low dose (0.5 pg) of Xgly4 mRNA with Xwnt11 mRNA enhanced the defective gastrulation movements caused by the Xwnt11 mRNA, co-injection of a high dose (25 pg) of Xgly4 reduced the defective phenotype. This indicates that the Xgly4 protein acts as a positive modulator of the Xwnt11 ligand but inhibits Xwnt11 signaling at a high dose (Fig. 6), as does Kny in *slb* (zebrafish wnt11) signaling (Topczewski et al., 2001).

In *Drosophila*, several experiments and additional genetic data have led to the prediction that, in vivo, Dsh protein may localize to the membrane in response to Fz signaling through the PCP pathway (planar cell polarity/non-canonical Wnt-signaling cascade), but not in response to activation of the β-catenin-dependent Wg pathway (canonical Wnt/β-catenin

pathway) (Axelrod, 2001; Axelrod et al., 1998). Consistent with this idea, in dorsal mesoderm cells of the *Xenopus* embryo, the *Xenopus* dishevelled (Xdsh) protein accumulates at the cell membrane in a manner that depends on endogenous PCP signaling and controls gastrulation movements (Wallingford and Harland, 2001; Wallingford et al., 2000) (Fig. 7A). To examine whether Xgly4 is involved in the regulation of this conserved PCP pathway, we tested whether disruption of Xgly4 function perturbed the Xdsh protein localization. As we expected, the injection of Xgly4Mo disturbed the membrane localization of Xdsh, as did injection of dominant-negative Xwnt11 mRNA (Fig. 7B,C), which suggests that Xgly4 function is required for Xdsh protein localization to the membrane. Overexpression of Xgly4 mRNA into the DMZ also inhibited the accumulation of Xdsh protein at the membrane (Fig. 7D). Staining of Xdsh associated with plasma membrane varied from cell to cell but it was clear that the membrane localization of Xdsh was inhibited in dnWnt11-, Xgly4Mo- or Xgly4mRNA-injected embryos compared with uninjected or HrTT-1 Mo-injected embryos (Fig. 7E). Taken together, these results suggest that both the gain- and loss-of-function of Xgly4 lead to dislocation of Xdsh protein, and this is consistent with previous observations that an adequately tuned level of Wnt11 signaling is necessary for normal gastrulation movements (Djiane et al., 2000; Tada and Smith, 2000).
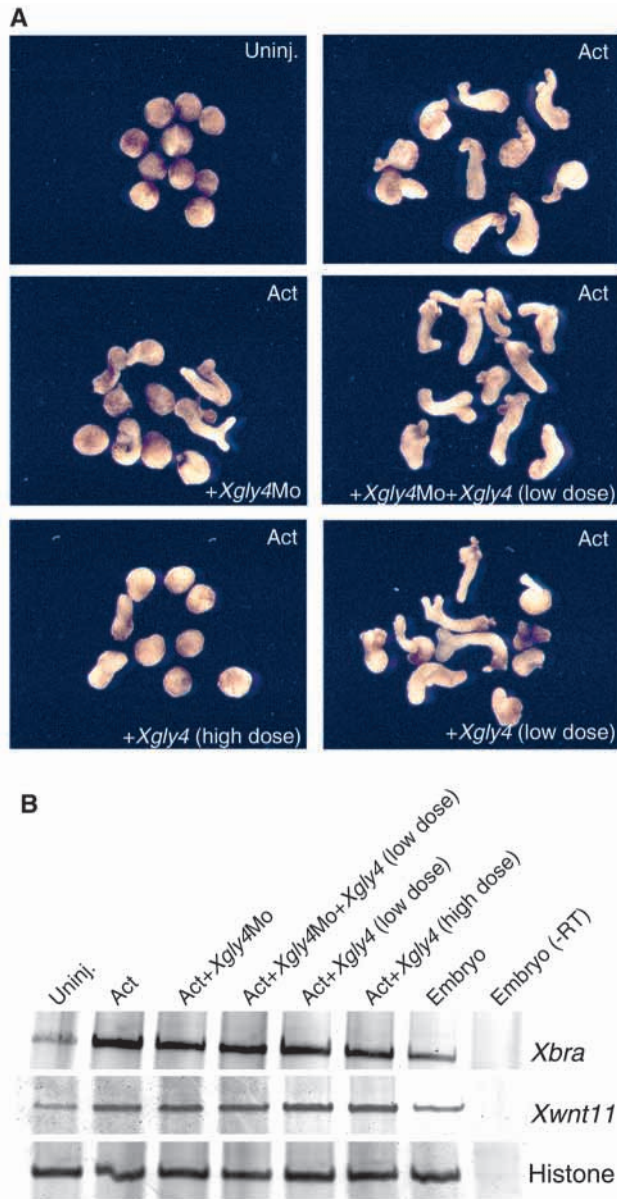
Fig. 5. Both Xgly4Mo and wild-type overexpression inhibit convergent extension movements. (A) Activin (0.25 pg)-induced elongation of AC explants (top right) mimics the convergent extension movements seen during gastrulation. This elongation was inhibited by Xgly4Mo (41 ng; middle left). The inhibition was rescued by a low dose (25 pg) injection of Xgly4 (middle right). A higher dose (500 pg) of Xgly4 inhibited activin-induced elongation (bottom left), but a low dose (25 pg) did not (bottom right). (B) Expression levels of Xbra, Xwnt11 and Histone (control) were analyzed by RT-PCR assay. The expression levels of Xbra and Xwnt11 were not affected in any of the explants shown in A.

Fig. 6. (A) Xgly4 enhances the Xwnt11-induced convergent extension movements. (B) Severity of affected embryos. The phenotypes were graded according to the index of severity from 0 (normal) to 3 (spina bifida). Number of each grade was indicated as percentage. Although co-injection of Xgly4 mRNA at a low dose (0.5 pg) enhanced the defective gastrulation movements caused by injecting Xwnt11 mRNA (1 ng), co-injection of Xgly4 mRNA at a high dose (25 pg) rescued these defects. This indicates that the Xgly4 protein acts as positive modulator, but inhibits Xwnt11 signaling at a high dose, just as Knypek does in silberblick (zebrafish wnt11) signaling (Topczewski et al., 2001).

To analyze the pathway-specific contribution of Xgly4 to Wnt signaling, we tried to rescue the inhibition of activin-induced elongation of ACs that is caused by injection of Xgly4Mo with pathway-specific Xdsh mutants. The inhibition of elongation caused by Xgly4Mo (Fig. 4) was rescued by wild-type Xdsh (data not shown) or by a Xdsh mutant lacking the DIX, but not the DEP, domain (Wallingford et al., 2000)
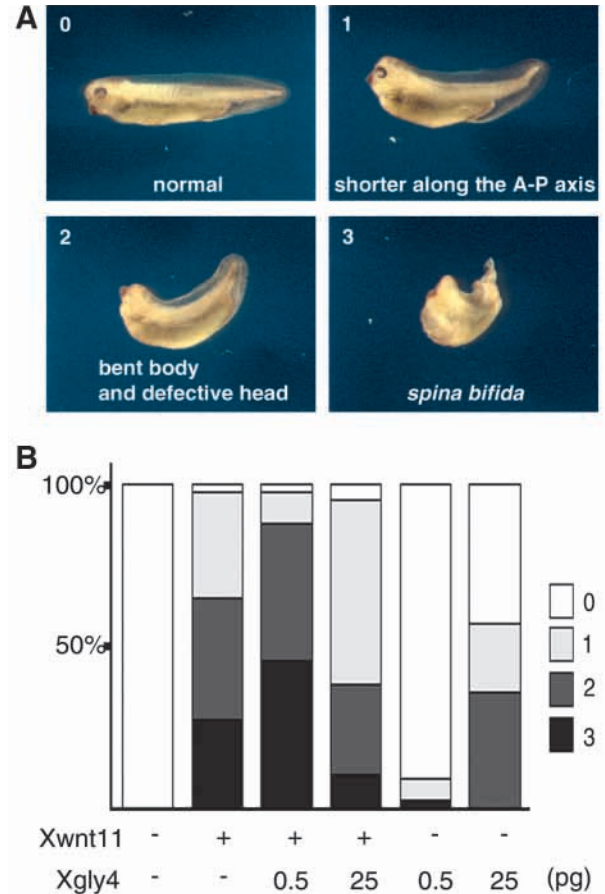
(Fig. 8). Because the DIX domain of Xdsh protein is required for the canonical Wnt/β-catenin pathway and the DEP domain is required for the non-canonical Wnt/PCP pathway (Axelrod et al., 1998; Boutros and Mlodzik, 1999; Boutros et al., 1998; Tada and Smith, 2000; Wallingford et al., 2000), it was shown that Xgly4 would participate in activin-induced elongation movements through the non-canonical Wnt/PCP pathway. Our results support the idea that Xgly4 affects gastrulation movement through the non-canonical Wnt/PCP pathway upstream of Dsh.

Based on the results of these rescue experiments, it seems likely that Xgly4 plays a role as a positive modulator of Xwnt11. Consistent with this functional interaction, we found that the HA-tagged Wnt11 ligand co-immunoprecipitated with the extracellular domain of Xgly4 protein (Fig. 9A). These results suggest that the Xgly4 protein in extracellular matrices binds the Wnt11 ligand to modulate its action on the Frizzled7 (Fz7) receptor (Djiane et al., 2000). We also found that HA-
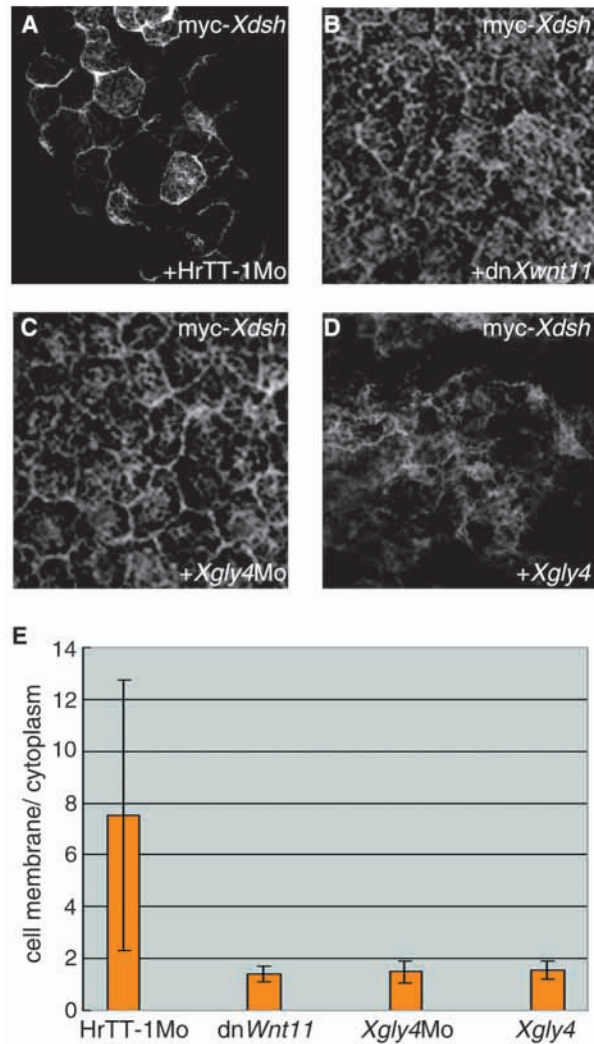
Fig. 7. Inhibition of Xgly4 translation disturbs the membrane accumulation of the Xdsh protein in the dorsal mesoderm. (A-D) The Xdsh protein accumulated at the cell membrane of dorsal mesoderm cells during gastrulation (data not shown). Injection of the dominant-negative Xwnt11 mRNA (dn-Xwnt11; 500 pg; B), Xgly4Mo (41 ng; C), or Xgly4 mRNA (500 pg; D) disturbed the Xdsh protein localization. The accumulation of Xdsh protein was not affected by the control morpholino oligonucleotide (A). The smeared staining in cytoplasm was observed only in co-injected cells but not in cells injected with Xdsh alone, which had previously been injected with Xgly4Mo only in one side of dorsal blastmeres at the four-cell-stage (data not shown). (E) The intensity of Xdsh staining in the cytoplasm and membrane in each cell was quantitated by using NIH image.

Fig. 8. Xgly4 inhibition of elongation is rescued by the activation of the non-canonical, but not the canonical, Wnt pathway. It has previously been shown that XdshΔDIX cannot activate the canonical Wnt pathway, whereas ΔDEP cannot activate the non-canonical pathway. Inhibition of activin-induced elongation (top left) by Xgly4Mo (41 ng; top right) was rescued by ΔDIX (50 pg; middle right) but not by ΔDEP (50 pg; middle left), which suggests that Xgly4 regulates the convergent extension movements by acting as a positive modulator of the non-canonical Wnt pathway upstream of Dsh. When injected alone, ΔDEP (bottom left) and ΔDIX (bottom right) had no effect.

tagged Wnt5A and Wnt8 ligands co-immunoprecipitated with Xgly4 protein (Fig. 9A). Therefore, Xgly4 may also regulate the activity of Xwnt5A in vivo because these two molecules are co-localized during early *Xenopus* development. However, Xgly4 is not likely to affect Xwnt8 signaling in a dominant manner given that their expression patterns are complementary to each other (data not shown). We were surprised to find that Xgly4 also bound to a structurally unrelated growth factor activin (data not shown). This may suggest that Xgly4 has a rather broad range of binding specificity to soluble ligands. However, activin activity was not affected by Xgly4 because
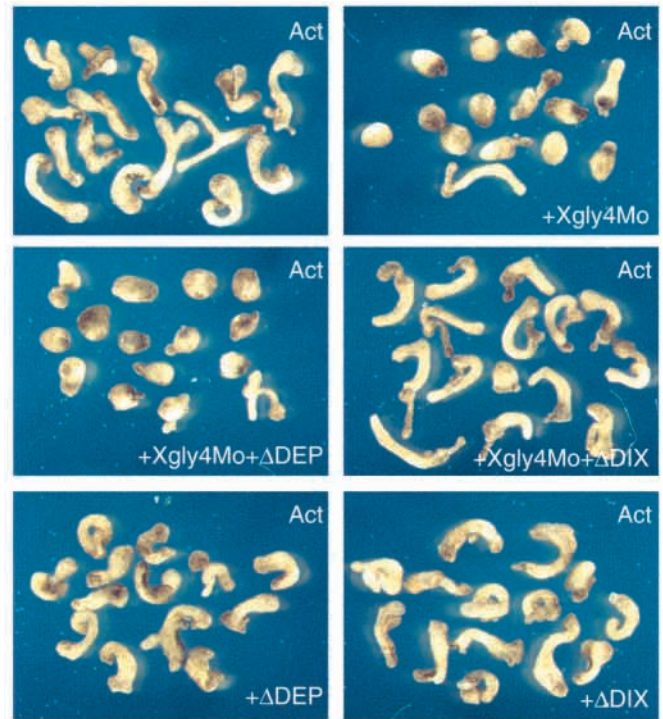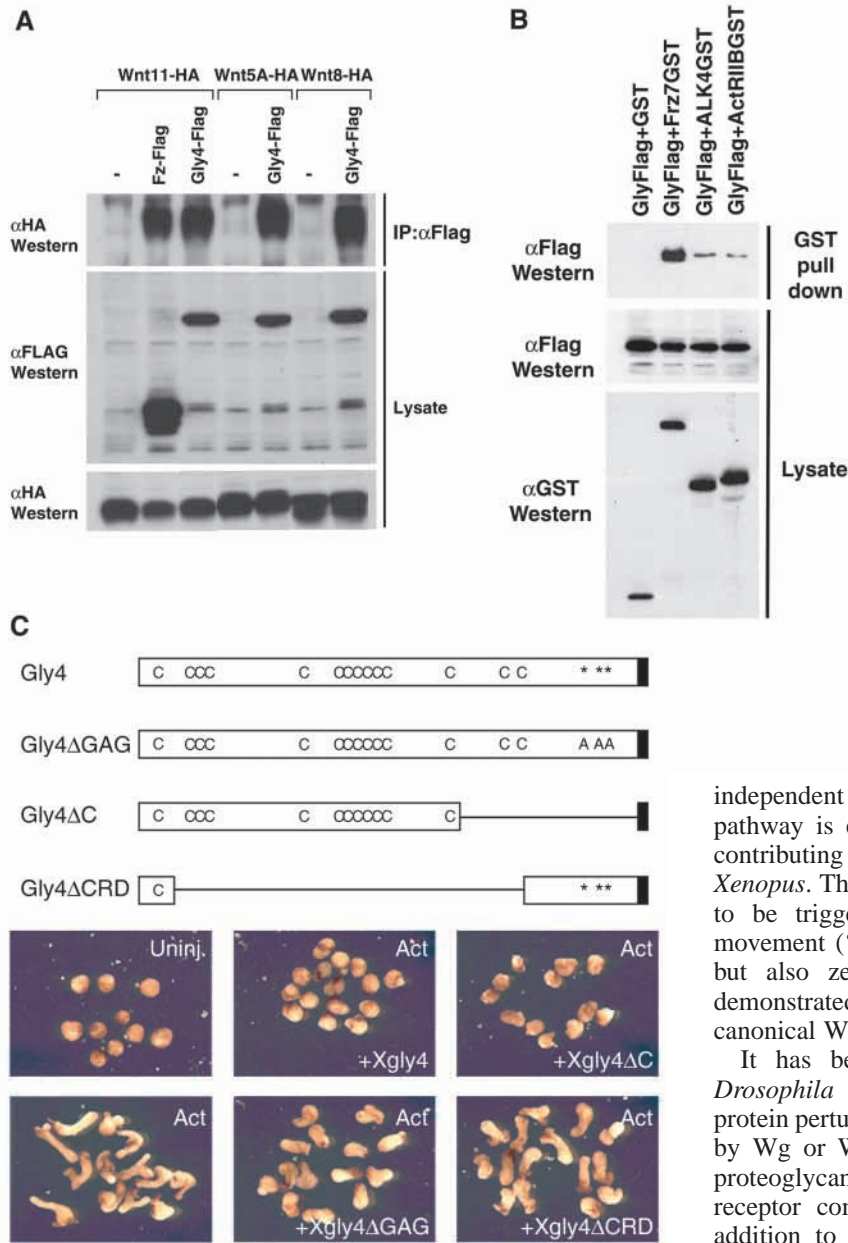
Xgly4 did not interfere with mesoderm induction in the AC elongation assay, as revealed by Xbra expression (Fig. 5). To ascertain functional significance of the binding to these ligands, we examined Xgly4 binding to its receptor(s). Interestingly, we found that Xgly4 showed a preferential binding to Fz7 rather than to activin type I (ALK4) or type II (ActRIIA) receptors (Fig. 9B). Therefore, we propose that functional interaction of Xgly4 with growth factors depends on the physical interaction of Xgly4 with receptors. This further suggests that one of molecular mechanisms of the ligand presentation involves the physical interaction of glypicans and ligand-binding signaling receptors. We next examined which domain is required for binding to Wnt11. To address this question, we constructed *Xgly4*-mutant cDNAs designed to encode Gly4 proteins lacking the C-terminal region (Gly4ΔC) and the cystein-rich domain (Gly4ΔCRD), and replaced the three putative glycosylated Ser residues (Gly4ΔGAG) with Ala residues (Fig. 9C). We found that activin-induced elongation was strongly inhibited by the injection of Xgly4ΔC or Gly4ΔGAG, as it was by wild-type Gly4, but it was not inhibited by the injection of Xgly4ΔCRD mRNA (Fig. 9C). Because we also found that HA-tagged Wnt11 ligand co-immunoprecipitated with Gly4ΔC, Gly4 may interact with

A

B

Fig. 9. Physical and functional interaction of Xgly4 with Wnt11. (A) Xgly4 binds and co-precipitates Wnt11, Wnt5a or Wnt8. HA-tagged Wnts expressed in HEK293T cells were efficiently co-precipitated with Flag-tagged full-length Xgly4 as well as a Flag-tagged extracellular domain of Fz7. (B) Xgly4 interacts efficiently with the signaling receptor Fz7, but barely with ActRIB (ALK4) or with ActRIIA. (Top) Pull-down of each GST-fused receptor expressed in 293T cells with glutathione-Sepharose, followed by western blotting with anti-Flag antibody. (Middle) Expression levels of Xgly4 revealed with anti-Flag antibody. (Bottom) Expression levels of receptors revealed with anti-GST antibody. (C) The cysteine-rich domain (CRD) of Xgly4 protein functionally interacts with Wnt11 signaling. (Top) The construction of wild-type and mutant Xgly4 proteins. Line represents internal deletion, black box designates epitope flag-tag sequence, asterisks indicate the putative glycosylation sites and C represents cysteine residues. (Bottom) Activin-induced elongation (bottom left) was inhibited by co-injection with wild-type Xgly4 (500 pg; top middle), Xgly4ΔGAG (500 pg; bottom middle), or Xgly4ΔC (500 pg; top right), but not with Xgly4ΔCRD (500 pg; bottom right) mRNA.

C

Wnt11 through the CRD domain. Together, these results suggest that CRD domain is essential for the ability of Xgly4 to produce convergent extension movements. It is interesting to note that the Wnt11 receptor Fz and co-receptor Gly4 share a common structural feature (CRD) for binding the ligand.

DISCUSSION

Like other polypeptide growth factors, the Wnt family proteins have essential roles in many developmental processes in vertebrates, including limb formation and posteriorization of the central nervous system. Recent studies have revealed that, depending on the ligands and receptors involved, the Wnt proteins signal through three distinct intracellular signaling pathways: the canonical Wnt/β-catenin pathway; the non-canonical Wnt/PCP pathway; and the non-canonical Dsh-independent Wnt/Ca^{2+} pathway. The canonical Wnt/β-catenin pathway is essential for DV axis formation and patterning, contributing to the establishment of Spemann's organizer in *Xenopus*. The two non-canonical pathways, which are thought to be triggered by Wnt5A/Wnt11, are required for cell movement ('convergent extension') during not only *Xenopus* but also zebrafish gastrulation. In this report, we have demonstrated that Xgly4 acts as a modulator in the non-canonical Wnt/PCP pathway during *Xenopus* gastrulation.

It has been shown genetically that mutations of the *Drosophila* Dally and Dlp proteins, or the zebrafish Kny protein perturb normal patterning or cell movements controlled by Wg or Wnt, respectively. It has been proposed that the proteoglycans Dally, Dlp and Kny act as components of a receptor complex that serves as a co-receptor for Fz. In addition to genetic evidence, we have been able to show successfully that Xgly4 physically interacts with the Wnt11 ligand (Fig. 9A) and the Fz7 receptor (Fig. 9B), which activate the non-canonical Wnt/PCP pathway. These results suggest that Xgly4 may present Wnt ligands to Fz receptors, in the same way as the FGF low-affinity HSPG receptor does with FGF high-affinity signalling receptors (Pellegrini et al., 2000; Schlessinger et al., 1995). We also found that Xgly4 physically interacts with Wnt8. The question arising from these results is whether Xgly4 affects the Xwnt8 signaling pathway. In fact, the overexpression of Xgly4 inhibits the Xwnt8-induced second axis. However, because Otx2, the anterior marker gene, was expressed in Xgly4Mo-injected embryos (data not shown), head formation, except eye formation (Fig. 4B), was not affected by reducing Xgly4 levels using Xgly4Mo; this lack of affect on head formation is similar to that in the zebrafish *kny* mutant (Topczewski et al., 2001). The expression of *XmyoD* gene, which is regulated by Xwnt8, was also unperturbed (Fig. 4C), and we also found that Xgly4 transcripts were not co-

expressed with Xwnt8 in gastrula (Christian et al., 1991; Ku and Melton, 1993; Moon et al., 1993; Tada and Smith, 2000). In addition, given that Xwnt8 is an endogenous ligand of Xgly4 and that Xgly4 acts as a co-receptor for Wnt8, depletion of Xgly4 by morpholino oligonucleotides is expected to result in a loss of Wnt8 activity, and in anteriorization of embryo, because Wnt8 is considered to be an inhibitor of head formation. These results suggest that Xgly4 is not involved in the regulation of the Xwnt8 canonical pathway in vivo during *Xenopus* gastrulation, but that it inhibits Xwnt8 activity only when it is overexpressed beyond the physiological level. Nevertheless, as several HSPGs have been shown to act as modulators of the canonical Wg/Wnt pathway in different model systems (Alexander et al., 2000; Baeg et al., 2001; Tsuda et al., 1999), we cannot rule out the possibility that Xgly4 could potentially act as a modulator of the canonical Wnt/β-catenin pathway if it is co-expressed with the corresponding ligands.

Although embryos in which Xgly4 protein levels were reduced exhibited defects of the dorsal convergent movements, embryos with overexpressed Xgly4 also exhibited these defects. We found that overexpression of Xgly4 inhibited the non-canonical Wnt/PCP pathway in the same way as the *kny* gene product does (Topczewski et al., 2001). In the *Drosophila* wing disc, glypicans can regulate the Wg protein distribution in the extracellular spaces and, in some contexts at least, block Wg signaling (Baeg et al., 2001). Taken together, these findings suggest that high levels of glypicans may serve to prevent Wg/Wnt ligands from interacting with the signaling receptor. This is supported by our finding that Xgly4 forms a complex with Fz7 (Fig. 9A,B). It is also known that cell-cell adhesion, by the interaction of integrin and fibronectin, disturbs Dsh localization and then convergent extension movements (Marsden and DeSimone, 2001). Because Xgly4 is an extracellular matrix component, Xgly4 may play a role in cell-cell interactions. In addition, we also speculate that glypicans may affect the non-canonical Dsh/Wnt signaling pathway by regulating the distribution of Wnt ligands in extracellular spaces during gastrulation in vertebrates.

A growing body of evidence indicates that HSPGs also have crucial roles in the regulation of other growth factors, including FGF, TGFβ and the BMPs (Bernfield et al., 1999; Tumova et al., 2000). It is interesting to note that *Drosophila dally* interacts genetically with *dpp*, a fly ortholog of vertebrate BMP, in the formation of the genital organ. Thus, we suspected that Xgly4 might regulate BMP function as well as the function of Wnt5A/Wnt11 during *Xenopus* development. However, we found no indication that gain- or loss-of-function of Xgly4 affected the DV patterning regulated by BMP, which is consistent with our observation that Xgly4 does not affect Bmp4-induced gene expression in whole embryos (Xvent-1 expression pattern; Fig. 4C) and in the AC (data not shown). This observation is supported by an independent observation by Topczewski et al., from cell tracing experiments, that the fates of lateral cells in *kny* mutants are comparable to the fates of the cells in wild-type embryos (Topczewski et al., 2001). In addition to the BMP ligands, we investigated the functional interactions of Xgly4 with eFGF and *Xenopus* nodal-related ligand 1, which are known to be expressed in *Xenopus* gastrulae. Because neither reducing the levels of nor overexpressing Xgly4 affected BMP, FGF or nodal signals

(data not shown), it appears that impaired gastrulation movements are not a consequence of the regulation of BMP, FGF or nodal signals. We speculate that the functional interaction of Xgly4 with Wnt and other ligands depends on the extracellular environment, which is altered in a temporally and spatially controlled manner.

In *Xenopus* gastrulae, Xwnt5A, which can also activate the Dsh-independent Wnt/Ca^{2+} signaling pathway, physically binds to Xgly4 protein (Fig. 9A) and is co-expressed with Xgly4 in the dorsal ectoderm (Moon et al., 1993). In zebrafish, the phenotype of *kny* is similar to *slb* and also to *pipetail*, which represents a mutation of zebrafish Wnt5A (Rauch et al., 1997). The mediators of the Ca^{2+}-dependent Wnt signaling cascade act in convergent extension movements (Kuhl et al., 2001; Wallingford et al., 2001) and in tissue separation (Winklbauer et al., 2001). Given that *Xgly4* is the *Xenopus* ortholog of *kny*, one might expect that Xgly4 would affect the Ca^{2+}-dependent Wnt signaling pathway. However, this may be unlikely because, in the AC assay, the inhibition of activin-induced elongation by Xgly4Mo was efficiently rescued by Xdsh alone. This suggests that, at least in the AC, Xgly4 affects only the non-canonical Wnt/PCP pathway.

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