Syndecan 4 interacts genetically with Vangl2 to regulate neural tube closure and planar cell polarity

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SUMMARY

Syndecan 4 (Sdc4) is a cell-surface heparan sulfate proteoglycan (HSPG) that regulates gastrulation, neural tube closure and directed neural crest migration in Xenopus development. To determine whether Sdc4 participates in Wnt/PCP signaling during mouse development, we evaluated a possible interaction between a null mutation of Sdc4 and the loop-tail allele of Vangl2. Sdc4 is expressed in multiple tissues, but particularly in the non-neural ectoderm, hindgut and otic vesicles. Sdc4;Vangl2Lp compound mutant mice have defective neuronal tube closure, disrupted orientation of the stereocilia bundles in the cochlea and delayed wound healing, demonstrating a strong genetic interaction. In Xenopus, co-injection of suboptimal amounts of Sdc4 and Vangl2 morpholinos resulted in a significantly greater proportion of embryos with defective neural tube closure than each individual morpholino alone. To probe the mechanism of this interaction, we overexpressed or knocked down Vangl2 function in HEK293 cells. The Sdc4 and Vangl2 proteins colocalize, and Vangl2, particularly the Vangl2Lp mutant form, diminishes Sdc4 protein levels. Conversely, Vangl2 knockdown enhances Sdc4 protein levels. Overall HSPG steady-state levels were regulated by Vangl2, suggesting a molecular mechanism for the genetic interaction in which Vangl2Lp/+ importance during neural tube closure in mammalian embryos.

KEY WORDS: Neural tube defects, Proteoglycans, Wnt planar cell polarity

INTRODUCTION

The Wnt/PCP pathway controls a variety of cellular and developmental processes where coordinated movement and orientation of cells within the plane of an epithelium is required. This pathway regulates the process of convergence and extension (CE) during gastrulation and neurulation (Wallingford et al., 2002; Ybot-Gonzalez et al., 2007), stereocilia orientation during ear morphogenesis, hair follicle orientation, renal tubular elongation and oriented cell division (Gray et al., 2011; Wang and Nathans, 2007). More recently, a role for Wnt/PCP signaling in epidermal wound healing has been described (Caddy et al., 2010).

The PCP pathway was originally discovered in Drosophila. Its core components include the transmembrane receptor Frizzled (Fz), the cytoplasmic proteins Disheveled (Dsh/Dvl) and Prickle (Pk), the four-pass transmembrane protein strabismus (Sbms/Vangl2), and the cadherin-like protein Flamingo/Celsr1 (Gray et al., 2011; Wang and Nathans, 2007). PCP signaling in vertebrates, but not in flies, also involves the Wnt ligands Wnt5a and Wnt11 (Gray et al., 2011; Wang and Nathans, 2007). More recently, a role for Wnt/PCP signaling in epidermal wound healing has been described (Caddy et al., 2010).

Neurulation is responsible for initial shaping of the central nervous system and formation of the neural tube, the precursor of the brain and spinal cord. Defective neurulation, where neural tube closure is incomplete, can result in neural tube defects (NTD), a group of malformations that affects ~0.5-2 per 1000 pregnancies worldwide. A breakthrough in understanding the genetic mechanism of NTDs was the finding that components of the Wnt/PCP pathway are required for neurulation in vertebrate embryos (Copp et al., 2003; Wallingford and Harland, 2002). Mice, frogs and zebrafish that are defective for Van Gogh-like 2 (Vangl2), the vertebrate ortholog of Drosophila Sbms develop NTDs (Copp et al., 2003). Moreover, recent work has identified point mutations in several genes of the PCP pathway specifically in human NTDs (Doudney et al., 2005; Fliescu et al., 2011; Kibar et al., 2007; Robinson et al., 2012), suggesting that the requirement for Wnt/PCP signaling in neural tube closure may be universal among vertebrates.

Syndecan 4 (Sdc4) is a cell surface heparan sulfate proteoglycan (HSPG) involved in cell adhesion (Couchman, 2010; Morgan et al., 2007). We have demonstrated that Sdc4 regulates gastrulation, neural tube closure and neural crest-directed migration in Xenopus embryos (Muñoz et al., 2006; Matthews et al., 2008). Sdc4 interacts biochemically with Fz7 and Dsh, and is necessary and sufficient to translocate Dsh to the membrane in a fibronectin-dependent manner, supporting its role in non-canonical Wnt signaling (Muñoz et al., 2006). Sdc4-null mice have delayed wound healing, impaired angiogenesis and defects in muscle satellite cells, but no apparent early developmental defects have been described (Cornelison et al., 2004; Echtermeyer et al., 2001; Ishiguro et al., 2000).

Here, we studied the expression of Sdc4 and its interaction with Vangl2 in different biological processes. We find that Sdc4 is expressed in the non-neural ectoderm adjacent to the neural tube, in the gut and in the otic vesicle. Sdc4 interacts genetically with...
Vangl2/Lp+/- to affect spinal neural tube closure, morphogenesis of the cochlea stereocilia and wound healing. Biochemical and cellular experiments demonstrate that Vangl2 regulates Sdc4 steady-state levels, and also affects total levels of HSPG, providing a molecular explanation for the genetic interaction between these two genes. Heparan sulfate residues could mediate the effect of this interaction, as Vangl2/Lp+/- embryos develop craniorachischisis when sulfation of the glycosaminoglycan chains is inhibited.

**MATERIALS AND METHODS**

**Animal procedures**

Genotyping of the targeted Sdc4 alleles was performed by PCR of genomic DNA using the following primers: wild-type allele (forward, 5'-CGAG- GCAGCAACATCTTTGAGAGAAC-3'; reverse, 5'-TCCATCCCCATT- CACAGAGC-3'); and the null allele (forward, 5'-CGCTTCTTG- ACGATCTTCT-3'; reverse, 5'-GGATCCACTGTCCTCCAATCAA-3'). Vangl2/Lp+/- and embryos were genotyped as described (Copp et al., 1994). Sdc4lacZ+/+; Vangl2/Lp+/- mice were obtained by natural matings between Vangl2/Lp+/- males and Sdc4lacZ+/- females. From the F1 offspring, compound heterozygous mice were selected by genotyping and intercrossed with Sdc4lacZ+/+ females to obtain F2 embryos. Xenopus in vitro fertilization and microinjection were performed as previously described (Muñoz et al., 2006). The morpholinos used to knockdown Sdc4 were the same as those used previously in our own studies and their specificity has been clearly demonstrated (Muñoz et al., 2006). For knockdown of Vangl2, the morpholino oligonucleotide sequence was 5'-AGTACGCCGT- TTTGTTGCCA-3'. All animal procedures and experiments were performed in accordance with protocols approved by the Pontificia Universidad Católica de Chile Animal Ethics Committee and the Animals (Scientific Procedures) Act 1986 of the UK Government.

**Embryo cultures**

Embryos from timed matings between Vangl2/Lp+/- and wild-type mice (CBA/Ca background) were explanted at E8.5 into Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Culture was in undiluted rat serum, in a roller incubator maintained at 38°C and gassed with a mixture of 5% CO2/5% O2/90% N2, as described previously (Copp et al., 2000). Cultures were stabilized for 1 hour, and then sterile aqueous sodium chlorate was added to a final concentration of 30 mM (1% volume addition) (Yip et al., 2002). The same volume of distilled water was added to control cultures. Cultures were allowed to heal in the open air and photographs were taken every 24 hours. Data were plotted as percentage of open area per day.

**Western blot analysis**

For overexpression and knockdown experiments, xSdc4-Flag (Muñoz et al., 2006), mVangl2-HA, mVangl2Lp-HA (Gao et al., 2011) and siVangl2 (sc-45595, Santa Cruz Biotechnologies) were used. Cells were grown to 70% confluence in six-well plates and transfected with the calcium phosphate method. After transfection (48 hours), the cells were lysed with 200 μl lysis buffer A [10 mM Tris (pH 7.5), 150 mM NaCl; 0.5% Triton X-100] containing protease inhibitors and separated by SDS-PAGE. The proteins were transferred to PVDF membranes and subjected to immunoblot analysis as described previously (Carvallo et al., 2010). For anti-Stub analysis, experiments were performed as described previously (Carrasco et al., 2005). To determine the half-life of Scd4, Sdc4-Flag cells were transfected and after 2 days were incubated for 0, 1, 3, 5 and 8 hours with 40 µg/ml of cycloheximide. Scd4 levels were then evaluated by western blot. These experiments were performed in triplicate.

**Statistical analysis**

Data analyses and statistical analysis for wound healing experiments were performed using Prism 5 software (Graph Pad Software). P-values below 0.05 were considered statistically significant. All other statistical analysis was performed using Sigmastat version 3.5.

**RESULTS**

**Syndecan 4 expression during early mouse development**

Sdc4 expression during early mouse development was analyzed by whole-mount in situ hybridization. At E8.5, Sdc4 is expressed in the cranial neural folds (Fig. 1A) mainly in the cephalic mesenchyme (Fig. 1D). More caudal sections revealed additional expression in the foregut diverticulum and the presumptive cardiac tissue (supplementary material Fig. S1A). At E9.5, the expression in the cephalic mesenchyme is maintained and Sdc4 transcripts also appear in the neural tube, otic vesicle and hindgut (Fig. 1B, C, E; supplementary material Fig. S1B-D).

For further analysis, we performed staining for β-galactosidase activity in heterozygous embryos (Sdc4lacZ+/-) at E9.0-9.5 and confirmed the expression of Sdc4 in the neural tube, otic vesicle and hindgut (Fig. 1F, G; supplementary material Fig. S2). Importantly,
lacZ staining was also prominent in the non-neural ectoderm, mainly on the outside of the open spinal neural folds prior to, and during, closure of the posterior neuropore (Fig. 1I,H). This expression in the non-neural ectoderm was also found by immunofluorescence detection of Sdc4 protein on transverse sections at E9.0 (Fig. 1I), demonstrating expression of Sdc4 in the neural fold during spinal neural tube closure. In addition, Sdc4 protein is also present in the fore and hindgut of E9.5 embryos (Fig. 1J).

At E18.5, Sdc4 expression was detected in the sensory hair cells of the inner ear, particularly in the organ of Corti (Fig. 1K). Detailed analysis suggests that the expression is stronger in the row of three outer hair cells compared with the inner hair cell (Fig. 1K, inset). Immunofluorescence analysis at E9.0 showed expression of Sdc4 protein in the apical pole of the cells in the otic epithelium (Fig. 1L, white arrows).

To compare the expression of Sdc4 with Vangl2, we performed in situ hybridization analysis for this component of the PCP pathway. At E9.5, Vangl2 was expressed in the neural tube (mainly in the ventral side), the hindgut and the otic vesicle (Fig. 1M,N), demonstrating that Sdc4 and Vangl2 are co-expressed, at least in the hindgut and otic vesicle.

In summary, Sdc4 has a dynamic expression pattern during development and is detected in tissues, including the neural tube during and following closure, the hindgut and the cochlea. Co-expression with Vangl2 is consistent with a possible role for Sdc4 in Wnt/PCP signaling during morphogenesis.

**Sdc4 and Vangl2 interact genetically to regulate neural tube closure**

Although Sdc4 knockdown resulted in defective neural tube closure in Xenopus embryos (Muñoz et al., 2006), no apparent neural tube defect was detected in Sdc4-null mice (Echtermeyer et al., 2001; Ishiguro et al., 2000). To investigate whether total levels of HSPGs might be altered in Sdc4 knockouts, we used an anti-Stub antibody that recognizes a neo-epitope generated in all HSPG core proteins after treatment with heparitinase. Cell homogenates from mouse embryonic fibroblasts isolated from Sdc4+/− mice showed increased levels of a least three other HSPGs (Fig. 2A, see arrows), suggesting that redundancy and compensation could explain the absence of a neural tube closure phenotype in Sdc4-null mice.

Thus, we evaluated a possible role for Sdc4 in PCP signaling and neural tube closure by generating Sdc4-Vangl2 compound mutants. For these studies, we decided to use the loop-tail (Vangl2+/-) allele as this has a stronger PCP phenotype than other Vangl2 alleles, including hypomorphic point mutations and a total knockout (Guyot et al., 2011; Yin et al., 2012). Moreover, heterozygotes (Vangl2+/-) have a weak PCP phenotype that has been extensively used as a sensitized background to detect genes that interact with the PCP pathway (Gao et al., 2011; Lu et al., 2004; Merte et al., 2010; Qian et al., 2007;
Yamamoto et al., 2008). Therefore, a genetic interaction with Vangl2<sup>Δp/+</sup> would demonstrate a role for Sdc4 in Wnt/PCP signaling.

To obtain Sdc4<sup>ΔaZ/Lp/+</sup>;Vangl2<sup>ΔaZ/Lp/+</sup> compound mutants, we crossed double heterozygous males (Sdc4<sup>ΔaZ/Lp;ΔaZ/Lp</sup> with Sdc4<sup>ΔaZ/LaZ/LaZ</sup> females. This protocol was followed to avoid using Vangl2<sup>ΔaZ/+</sup> females, almost half of which exhibit imperforate vagina (Murdoch et al., 2001). The number of newborn mice with Sdc4<sup>ΔaZ/LaZ/LaZ</sup> genotype showed a statistically significant reduction from the expected Mendelian distribution (<I>P</I> = 0.01) suggesting that loss of Sdc4 function is detrimental to mouse survival (supplementary material Table S1). Importantly, we found that whereas 17% (six out of 35) of Sdc4<sup>ΔaZ/LaZ</sup>;Vangl2<sup>ΔaZ/Lp</sup> mice were born with a sacral spina bifida (Fig. 2B,C), Sdc4<sup>ΔaZ/LaZ</sup>;Vangl2<sup>ΔaZ/LaZ</sup> mice exhibited a threefold higher frequency of this defect (55%; 11 out of 20), a statistically significant difference (<I>P</I> = 0.006).

Moreover, in two Sdc4<sup>ΔaZ/LaZ/LaZ</sup>;Vangl2<sup>Δp/+</sup> mice, a more severe lumbosacral spina bifida was detected (supplementary material Fig. S3). Most of the defective mice also have a looped tail (Fig. 2B,C), as is routinely observed in mice of genotype Vangl2<sup>ΔaZ/+</sup> (Copp et al., 1994). In agreement with published results (Echtermeyer et al., 2001; Ishiguro et al., 2000), no defective neural tube closure was observed in mice of Sdc4<sup>ΔaZ/LaZ</sup>;Vangl2<sup>ΔaZ/+</sup> and Sdc4<sup>ΔaZ/LaZ</sup>;Vangl2<sup>Δp/+</sup> genotypes (Fig. 2C).

Although the open spina bifida heals at postnatal days 10-15, most of these animals have posterior locomotor defects and are unable to move properly. Importantly, only 12% (2 out of 17) of the Sdc4<sup>ΔaZ/LaZ/LaZ</sup>;Vangl2<sup>ΔaZ/Lp</sup> mice survived beyond one postnatal month, compared with 78% survival for Sdc4<sup>ΔaZ/LaZ/LaZ</sup>;Vangl2<sup>ΔaZ/+</sup> mice (supplementary material Table S2).

In Xenopus embryos, alteration of Wnt/PCP signaling results in CE defects in the mesoderm and neuroectoderm (Wallingford and Harland, 2001). Gain and loss of function of Sdc4 and Vangl2 in Xenopus embryos disrupts gastrulation and neural tube closure (Goto and Keller, 2002; Muñoz et al., 2006). To analyze whether Sdc4 and Vangl2 also interact functionally in Xenopus embryos, we reduced the endogenous levels of both genes using morpholinos. The effects of these morpholinos could be rescued by overexpression of the respective synthetic mRNA, demonstrating their specificity (Muñoz et al., 2006) (supplementary material Fig. S4). In order to reduce gastrulation defects of the mesoderm (type I phenotype, exogastrulation), the two dorsal animal blastomeres at the eight-cell stage (Wallingford and Harland, 2001) were injected to target the neuroectoderm and produced mainly a defective closure of the neural tube (type II phenotype). Co-injection of suboptimal amounts of Sdc4 and Vangl2 morpholinos (xSdc4-Mo, xVangl2-Mo) resulted in a significantly greater proportion of embryos with defective neuonal tube closure than the individual morpholinos alone (<I>P</I> < 0.001; Fig. 2D).

Taken together, these findings demonstrate a genetic interaction between Sdc4 and Vangl2. Based on the role of both genes in Wnt/PCP signaling, we suggest they likely interact in this pathway.

**Sdc4 and Vangl2 regulate PCP pathway in cochlear sensory hair cells**

Proper polarization and tissue organization of the organ of Corti is one of the clearest examples of PCP in vertebrates (Jones and Chen, 2008). As Sdc4 is expressed in the cochlea, specifically in the hair cells of the organ of Corti (Fig. 1K,L), we analyzed the orientation of the stereocilia in the sensory hair cells. Cochleae were isolated at E18.5, stained with phallolidin (stereocilia) and acetylated tubulin (kinocilium), and analyzed by confocal microscopy. Normal organization of the hair cells was observed in Sdc4<sup>ΔaZ/Lp</sup>;Vangl2<sup>Δp/+</sup> embryos.
Delayed wound healing in Sdc4 and Vangl2 mutant mice

Sdc4 is upregulated in the epidermis and dermis after wounding (Gallo et al., 1996) and is required for proper wound repair (Bass et al., 2011; Echtermeyer et al., 2001). Moreover, a role for PCP signaling in wound healing has been unveiled by crossing PCP mutants such as Vangl2 with mice lacking the transcription factor Grhl3 (Caddy et al., 2010). Based on this, we evaluated a possible interaction between Sdc4 and Vangl2 in wound healing.

Excisional wounds were made in the shaved back of 2-month-old mice and the wound area was measured daily for up to 12 days. As reported by Echtermeyer et al. (Echtermeyer et al., 2001), Sdc4 heterozygous mice showed a small delay in wound closure (Fig. 4C, compare black circles and blue triangles). A much stronger delay in wound healing was detected in Sdc4;Vangl2 compound mutants (Fig. 4A-C): at 5 days, only 45% of the wound was closed in Sdc4lacZ+/+;Vangl2Lp/+ compared with 75% closure in Sdc4lacZ+/+;Vangl2Lp/Lp. Moreover, the wound remained open in the double heterozygous mice at day 9, when it was already closed in the Sdc4lacZ+/+;Vangl2Lp/Lp mice. The fact that even homozygous Vangl2Lp/Lp mice do not have defective wound healing (Caddy et al., 2010), indicates a strong genetic interaction between Sdc4 and Vangl2 in wound healing.

Vangl2 regulates Sdc4 steady-state levels

Cellular and biochemical experiments have demonstrated that the Vangl2Lp mutation affects the ability of Vangl2 to interact with Dvl2, and adversely affects the subcellular localization and stability of several PCP proteins (Gravel et al., 2010; Iliessen et al., 2011; Merte et al., 2010). Immunofluorescence analysis in HeLa cells with antibodies against endogenous Sdc4 and Vangl2 showed precise colocalization of both proteins (Fig. 5A). To study the effect of Vangl2 on Sdc4 steady-state levels, HEK293 cells were co-transfected with increasing amounts of Sdc4-Flag DNA and small interfering RNAs designed to inactivate Vangl2 (siVangl2). Western blots were performed after 48 hours. Knockdown of Vangl2 resulted in an increased steady-state level of Sdc4-Flag (Fig. 5B, compare lanes 1, 2 and lanes 4, 5), an effect that was dependent on the
amount of Sdc4-Flag expressed (Fig. 5B, compare lane 3 and lane 6). Co-transfection of a mouse version of Vangl2 restored normal levels of Sdc4, indicating that the effects of siVangl2 were specific (supplementary material Fig. S5A).

In agreement with this, overexpression of Vangl2 decreased Sdc4 steady-state levels, an effect that was dose dependent (Fig. 5C, compare lanes 2-4 and lane 1). A mutant form of Vangl2 that mimics the Lp mutant (mVangl2Lp-HA, Gao et al., 2011) showed the same ability to reduce Sdc4 steady-state levels (Fig. 5C, compare lanes 5-7 and lane 1). Interestingly, Vangl2Lp was even more active than wild-type Vangl2. Transfection of 1 ng of Vangl2Lp completely abrogated expression of Sdc4, whereas the same amount of Vangl2-HA showed only a partial effect (Fig. 5C, compare lanes 1, 4 and 7). This effect was even more dramatic considering that mVangl2Lp-HA expression levels were lower than the ones of wild-type protein (Fig. 5C, lower
Vangl2Lp interacts with overall HSPG expression and function

Our cell transfection and Xenopus expression results suggest a mechanism whereby Vangl2 diminishes existing levels of Sdc4 protein, exacerbating Sdc4 reduction and causing a stronger phenotype than partial loss of Sdc4 function alone. However, this explanation is not sufficient to explain the strong interaction observed in Sdc4lacZ/lacZ; Vangl2Lp/+ embryos, where Sdc4 is already completely absent because of the null mutation. Based on the fact that other syndecans, or HSPGs more generally, could compensate for removal of both Sdc4 copies (Fig. 2A), we decided to test a synthetic mRNA (Fig. 5G). These results support a mechanism whereby Vangl2 regulates Sdc4 steady-state levels.

In addition to the ability of Vangl2 to regulate Sdc4 steady-state protein level, we also observed the converse: Sdc4 was found to affect Vangl2 steady-state level, as demonstrated using siRNA against Sdc4 (supplementary material Fig. S5B). Taken together, these findings predicted that overexpression of Vangl2 should summate with a knockdown of Sdc4 in Xenopus embryos. To test this, we performed an assay similar to the one described in Fig. 2D. The frequency of defective embryos produced by injection of suboptimal amounts of Sdc4 morpholino (1-4 ng) was significantly greater when xVangl2 was overexpressed by co-injection of synthetic mRNA (Fig. 5G). These results support a mechanism whereby Vangl2 regulates Sdc4 steady-state levels.

**Vangl2Lp interacts with overall HSPG expression and function**

Panels, a finding that is in agreement with previous reports (Gravel et al., 2010; Iliescu et al., 2011).

The fact that Sdc4 was being overexpressed by transfection of HEK293 cells with an epitope-tagged Sdc4 under the control of a strong CMV promoter suggested that the effect of Vangl2Lp might be at the post-translational level. To test this prediction, cells overexpressing Sdc4 in the absence or presence of exogenous Vangl2 were incubated for different times with cycloheximide, a protein synthesis inhibitor, and the half-life of Sdc4 was estimated. We found that overexpression of Vangl2Lp levels reduced the half-life of Sdc4 from 3.2 hours to 2.2 hours (Fig. 5D,E), indicating a direct or indirect effect of Vangl2 at a post-translational level.

To assess the in vivo relevance of this observation, we analyzed the effect of Vangl2Lp on Sdc4 levels in wild-type and Vangl22/4Lp mice. By immunofluorescence, Sdc4 protein was readily detected in the otic epithelium and non-neural ectoderm of E9.0 Vangl2+/+ embryos, whereas no signal could be detected in the otic vesicles and non-neural ectoderm of Vangl22/4Lp embryos (Fig. 5F; supplementary material Fig. S6).

In our cell transfection and Xenopus expression results suggest a mechanism whereby Vangl2 diminishes existing levels of Sdc4 protein, exacerbating Sdc4 reduction and causing a stronger phenotype than partial loss of Sdc4 function alone. However, this explanation is not sufficient to explain the strong interaction observed in Sdc4lacZ/lacZ; Vangl2Lp/+ embryos, where Sdc4 is already completely absent because of the null mutation. Based on the fact that other syndecans, or HSPGs more generally, could compensate for removal of both Sdc4 copies (Fig. 2A), we decided to test a possible effect of Vangl2Lp on overall HSPG expression. Whole E14.5 fetuses were homogenized, and analyzed by western blot using the anti-Stub antibody. As shown in Fig. 5H, all HSPG core proteins were strongly reduced in Vangl22/4Lp mutants compared with Vangl22/4Lp and wild type. This suggests that Vangl2Lp has a destabilizing effect on most HSPGs, which provides a mechanistic explanation for the genetic interaction between Sdc4 and Vangl2.

The interaction between Vangl2 and HSPGs could be mediated via the proteoglycan core proteins and/or via the heparan sulfate chains. To test the latter idea, we evaluated the effects of chlorate treatment on Vangl22/4Lp embryos. Chlorate is an inhibitor of proteoglycan sulfation, a modification that is crucial for proteoglycan function. Previously, we have shown that chlorate modulates the rate of spinal neurulation, an effect specifically attributed to diminution of heparan sulfation (Yip et al., 2002). Vangl22/4Lp and wild-type embryos were cultured in vitro from E8.5 (fewer than five somites) for 24 hours in the presence or absence of 30 mM sodium chlorate. Strikingly, five out of six Vangl22/4Lp embryos failed to initiate neural tube closure (i.e. closure 1 failure) and developed craniorachischisis (entirely open neural tube). This phenotype is not usually observed in Vangl22/4Lp embryos, whereas it is always present in Vangl22/4Lp individuals. Indeed, Vangl22/4Lp embryos cultured in the absence of chlorate exhibited normal closure 1, as did wild-type embryos cultured in chlorate (Fig. 6; Table 1). Hence, there is a gene-environment interaction in which the phenotype of Vangl22/4Lp is converted to that of Vangl22/4Lp as a result of suppression of heparan sulfation by chlorate. Based on the fact that Sdc4 expression is not detected at the site of closure initiation (Fig. 1A), and that Vangl2Lp affects the levels of many HSPGs (Fig. 5H), these findings raise the possibility that Vangl2 may interact with other HSPGs in addition to Sdc4.
DISCUSSION

In this study, we report the expression of Sdc4 during mouse development, its genetic interaction with Vangl2, and the finding that Vangl2 regulates Sdc4 steady-state protein levels. These data suggest that Sdc4 can function within the non-canonical Wnt/PCP signaling pathway. This conclusion is supported by published observations showing that Sdc4 regulates skeletal muscle regeneration through a PCP/Vangl2-dependent mechanism (Cornelison et al., 2004; Le Grand et al., 2009; Bentzinger et al., 2013).

It is important to consider whether the phenotypes obtained in this study can be attributed solely to the presence of a single copy of the Vangl2Lp allele. Although looped tails, occasional spina bifida aperta and a low frequency of defective cochlear hair cell orientation are all found in Vangl2Lp/+ mice (Copp et al., 1994; Yin et al., 2012), we detected a much enhanced frequency and severity of this phenotypic combination in compound Sdc4;Vangl2Lp/+ mutants. In particular, Sdc4lacZ/lacZ;Vangl2Lp/+ embryos had a much stronger phenotype than Sdc4lacZ/lacZ;Vangl2Lp/+, littermates, arguing for a dose-dependent interaction between Sdc4 and Vangl2.

The phenotypes obtained in the Sdc4lacZ/lacZ;Vangl2Lp/+ compound mice are consistent with the partially overlapping expression patterns of Sdc4 and Vangl2. Our results and other studies showed that Sdc4 and Vangl2 are both expressed in the cochlea (Torban et al., 2007) and in the epidermis (Gallo et al., 1996; Murdoch et al., 2003; Devenport and Fuchs, 2008), supporting the defective orientation of sensory hairs in the cochlea and delayed wound healing observed in Sdc4lacZ/lacZ;Vangl2Lp/+ compound mice. During organogenesis, Sdc4 and Vangl2 are also expressed in the epithelia of many other tissues, including the kidney (N.E., O.C., R.M., F.H., C.H., U.T., S.E.P., O.W., A.J.C. and J.L., unpublished) (Torban et al., 2007). Interaction at these levels producing defective organ formation could provide an explanation for the diminished survival of Sdc4;Vangl2 compound mice.

With regard to the neural tube defect phenotypes we observed, the fact that Sdc4 is not expressed at the site of closure initiation (closure 1) at E8.5, whereas Vangl2 is precisely expressed at this site (Ybot-Gonzalez et al., 2007), can explain why Sdc4lacZ/lacZ;Vangl2Lp/+ compound mice do not develop craniorachischisis, unlike homozygous Lp mutants. Indeed, when heparan sulfate was inhibited more generally by chlorate, we then observed closure 1 failure in Vangl2Lp/+ embryos.

This leaves unresolved the issue of how Sdc4 and Vangl2 interact to produce the spina bifida phenotype exhibited by the compound mutants. It is interesting to note that the spina bifida in Sdc4lacZ/lacZ;Vangl2Lp/+ mice is reminiscent of that observed in the curly tail mutant, which carries a hypomorphic mutation of the transcription factor grainyhead-like 3 (Grhl3) (Brouns et al., 2011; Gustavsson et al., 2007). In curly tail, there is a decrease in cell proliferation specifically in the hindgut (Copp et al., 1988), with a consequent increase in ventral curvature causing mechanical obstruction of PNP closure that results in spina bifida (Brook et al., 1991). The co-expression of Sdc4 and Vangl2 in the hindgut raises the possibility that imperfect gut morphogenesis could also explain the defective neural tube closure observed in Sdc4;Vangl2Lp/+ compound mice. An alternative possibility is that the expression of Sdc4 in the non-neural ectoderm of the spinal neural folds might be important for the development of spina bifida in compound Sdc4;Vangl2Lp/+ mutants. A key role for non-neural ectoderm in the process of neural tube closure has been demonstrated in both mice and Xenopus (Pyrgaki et al., 2010; Pyrgaki et al., 2011; Morita et al., 2012). However, as Vangl2 expression is not detected in non-neural ectoderm, a non-cell autonomous interaction mechanism would likely be involved in Sdc4;Vangl2Lp/+ mutants.

Mechanistically, we have found that Vangl2 can regulate Sdc4 stability. More importantly, the Vangl2Lp mutant protein is extremely potent in reducing Sdc4 levels in embryos as well as in cell culture. Experiments with cycloheximide indicate that the effect of Vangl2 on Sdc4 is at the post-transcriptional level, although whether this is a direct or indirect effect remains an open question. In addition, we found that the levels of other HSPGs are also reduced in Lp mutant mice. This coincides with recent findings that the Lp mutation disrupts Vangl2 protein trafficking from the endoplasmic reticulum to the plasma membrane (Merte et al., 2010), and that the presence of the Vangl2Lp protein alters the normal localization of other PCP proteins, including Vangl1 and the putative Vangl2-interacting protein Prickle-like2 (Yin et al., 2012). Hence, the Vangl2Lp mutation may disturb PCP signaling through an adverse effect on several key interacting proteins, producing a more profound disturbance than loss of Vangl2 alone. Indeed, this putative effect on protein trafficking could also mediate effects of Vangl2Lp through PCP-independent pathways, as suggested by the finding that Vangl2 regulates the cell-surface availability and levels of MMP14 in migrating cells during gastrulation (Williams et al., 2012).

Taken together, our findings provide a potential explanation for the absence of phenotype in the Sdc4 mutant mice: we suggest that a compensatory mechanism, likely mediated by functional redundancy among HSPGs, may be responsible. Because of its role in non-canonical Wnt signaling, and its interaction with Vangl2 (Marlow et al., 1998), glypicans could be a candidate HSPG for this compensatory relationship with Sdc4 loss of function. Although many other HSPGs are upregulated in Sdc4-null fibroblasts, Lp mutant mice have reduced HSPG levels. Thus, as Vangl2 is required to regulate the stability of HSPGs, it probably also affects these levels in Sdc4 mice, offering a possible molecular mechanism for the strong phenotype of Sdc4lacZ/lacZ;Vangl2Lp/+ mutant mice.

Table 1. Vangl2Lp/+ embryos develop severe NTDs when cultured from E8.5 for 24 hours in the presence of sodium chlorate

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<tr>
<th>Treatment</th>
<th>Genotype</th>
<th>Number of embryos</th>
<th>Somite number*</th>
<th>Embryos with CRN (%)‡</th>
<th>PNP length§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>Wild type</td>
<td>6</td>
<td>13.5±1.3</td>
<td>0</td>
<td>0.59±0.05</td>
</tr>
<tr>
<td></td>
<td>Lp/+</td>
<td>5</td>
<td>13.8±1.0</td>
<td>0</td>
<td>0.54±0.02</td>
</tr>
<tr>
<td>Na chlorate</td>
<td>Wild type</td>
<td>5</td>
<td>12.2±0.4</td>
<td>0</td>
<td>0.78±0.05</td>
</tr>
<tr>
<td></td>
<td>Lp/+</td>
<td>6</td>
<td>11.2±0.4</td>
<td>83.3</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*Somite number at the end of the culture period (mean±s.e.m.) does not differ significantly between the groups (one-way analysis of variance; P>0.05).

†Proportion of embryos with craniorachischisis (CRN) varies significantly between the four groups (χ²=17.3; P<0.001).

‡Posterior neuropore (PNP) length (mean in mm±s.e.m.) varies significantly among the three groups whose embryos did not exhibit CRN (one-way analysis of variance; P=0.008). Wild-type embryos treated with sodium chlorate had significantly longer PNPs than either wild-type or Lp/+ embryos exposed to water addition only (P<0.05).
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Competing interests statement
The authors declare no competing financial interests.

Author contributions
N.E. participated in the design of the study, carried out most of the experiments, analyzed data and commented on the manuscript. O.C. carried out the cellular and biochemical experiments and participated in wound healing experiments. R.M. performed all the experiments in Xenopus embryos. M.F. carried out the embedding and sectioning of embryos, colony maintenance and mouse crosses. H.C. performed the biochemistry experiments. C.H. helped with the genotyping. U.T. helped set up mouse experiments. S.E.P. performed Vangl2 in situ hybridization. O.W. participated in the design of the study and revised and commented on the manuscript. A.J.C. performed the embryo culture experiments, participated in the design of the study, data analysis and revised and commented on the manuscript. J.L. participated in the design of the study, supervised the project and wrote the manuscript.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.091173/-/DC1

References


Fig. S1. *Sdc4* expression during mouse development. (A-D) Transverse sections through E8.5 and E9.5 embryos after whole-mount *in situ* hybridization with an antisense RNA probe for *Sdc4*. (A) At E8.5, *Sdc4* is expressed in the foregut diverticulum (arrowhead) and in the developing heart tube (arrow). At E9.5, *Sdc4* is strongly expressed in the cephalic mesenchyme (arrowhead in B) and in the hindgut (arrowhead in C and D). Scale bars: 100 μm in A; 200 μm in B,C; 50 μm in D.

Fig. S2. *Sdc4* expression during mouse development. (A-C) β-Galactosidase activity in *Sdc4lacZ/+* embryos at E9.0 showing staining throughout the gut in the lateral view of whole embryo (A), and in the hindgut (arrowheads in B,C) in the transverse sections at the embryo levels indicated by black lines in A. hg, hindgut; nt, neural tube.
Fig. S3. Lumbosacral spina bifida in a Sdc4lacZ/lacZ;Vangl2Lp/+ mouse. Compared with the sacral spina bifida (see Fig. 2B), this more severe neural tube defect extends further rostrally, into the lumbar level of the body axis. Open vertebrae are visible extending across the midline (arrows in expanded view), indicating that the overlying spinal cord tissue has completely degenerated at this level. A sharply flexed tail is associated with the spina bifida.
**Fig. S4. Specificity of morpholinos against Vangl2.** The specificity of the morpholinos against Vangl2 was demonstrated by rescue experiments using synthetic mRNA for Vangl2. Eight-cell stage *Xenopus* embryos were co-injected with the indicated amounts of morpholinos against xVangl2-MO and synthetic mRNA for an epitope-tag Vangl2 and neural tube closure defects were quantified at stage 20. Phenotypes were classified as type I (severe gastrulation and neural tube closure defects; red) and type II (impairment of neural tube closure; green). The graph summarizes two independent experiments, with numbers of embryos given at the top of each bar.

**Fig. S5. Effect of Sdc4 on Vangl2 steady-state levels.** (A) Vangl2 rescues the effect of siVangl2. HEK293 cells were transfected with xSdc4-Flag and Vangl2-HA plus siCtrl or siVangl2 and Sdc4 protein levels were evaluated. Knockdown of Vangl2 (middle lane) dramatically upregulates Sdc4 protein, and this is rescued by simultaneous expression of HA-mVangl2 (right lane), indicating a specific effect of siVangl2. (B) Sdc4 regulates Vangl2 protein level. HEK293 cells were transfected with Vangl2-HA and siCtrl or siSdc4, and Vangl2 protein levels were evaluated. Knockdown of Sdc4 resulted in increased steady-state levels of Vangl2 protein.
Fig. S6. Absence of Sdc4 expression in the non-neural ectoderm of Vangl2<sup>Lp/Lp</sup> embryos. (A-C′) Double immunofluorescence using antibodies against Sdc4 (green) and pan-cadherin (red) was performed on transverse sections of E9.0 wild-type and Vangl2<sup>Lp/Lp</sup> embryos. Sdc4 is expressed in the non-neuronal ectoderm of wild-type embryos; however, no signal is detected in Vangl2<sup>Lp/Lp</sup> embryos. Bracket in A′ indicates the non-neuronal ectoderm; arrowhead in B′ indicates lack of Sdc4 expression in this tissue.
Table S1. Genotype distribution and incidence of spina bifida in the *Sdc4*/*Vangl2* interaction

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total pups</th>
<th>Expected (%)</th>
<th>Experimental (%)</th>
<th>Spina bifida</th>
<th>Phenotype (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sdc4</em> lacZ/+</td>
<td>33</td>
<td>25</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>lacZ</em>+/+</td>
<td>35</td>
<td>25</td>
<td>32</td>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td><em>lacZ</em>/lacZ</td>
<td>21</td>
<td>25</td>
<td>19</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>lacZ</em>/lacZ Lp/+</td>
<td>20</td>
<td>25</td>
<td>18</td>
<td>11</td>
<td>55</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>109</strong></td>
<td><strong>100</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A total of 109 mice were born in 17 separate litters from matings between *Sdc4*^lacZ/+;*Vangl2*^Lp/+ males and *Sdc4*^lacZ/lacZ^ females. Offspring were analyzed daily from P0 to P30. There is a significant deviation from the expected Mendelian ratio of genotypes (Chi-square test; *P*<0.01), suggesting that absence of *Sdc4* on a *Vangl2*^Lp/+^ background adversely affects pre/postnatal survival.

Chi-square analysis of proportion of each genotype versus 25% expectation: significant; *P*<0.01 (chi-square = 6.77, dof = 1).
Table S2. Postnatal viability of different genotypes in litters from matings between
Sdc4lacZ/Vangl2+/+ males and Sdc4lacZlacZ females

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Sdc4lacZ/Vangl2+/+</th>
<th>Sdc4lacZ/Vangl2Lp/+</th>
<th>Sdc4lacZlacZ/Vangl2+/+</th>
<th>Sdc4lacZlacZ/Vangl2Lp/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>21 (100%)</td>
<td>21 (100%)</td>
<td>14 (100%)</td>
<td>17 (100%)</td>
</tr>
<tr>
<td>P15</td>
<td>21 (100%)</td>
<td>18 (86%)</td>
<td>14 (100%)</td>
<td>12 (70%)</td>
</tr>
<tr>
<td>P30</td>
<td>18 (86%)</td>
<td>12 (57%)</td>
<td>11 (78%)</td>
<td>2 (12%)</td>
</tr>
</tbody>
</table>

Individual pups were identified at birth (P0) and then followed up at P15 and P30 to determine viability (percentage values show the proportion of P0 pups that survive to the later time point). There is a dramatic decrease in survival of Sdc4lacZlacZ/Vangl2Lp/+ pups beyond P15 (significantly different survival at P30 from Sdc4lacZlacZ/Vangl2Lp/+ littermates, P<0.01).

Fisher’s exact test of proportion of pups surviving to P30: 12/21 (Sdc4lacZlacZ) versus 2/17 (Sdc4lacZlacZ). Significant; P=0.006