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

Noelia Escobedo1, Osvaldo Contreras1, Rosana Muñoz1, Marjorie Farias1, Héctor Carrasco1, Charlotte Hill1, Uyen Tran2, Sophie E. Pryor3, Oliver Wessely2, Andrew J. Copp3 and Juan Larraín1,*

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/Lp compound mutant mice have defective spinal neural 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 presence of chlorate, a sulfation inhibitor. These results demonstrate that Sdc4 can participate in the Wnt/PCP pathway, unveiling its role in multiple tissues, but particularly in the non-neural ectoderm, hindgut and otic vesicles

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 Fzfrzzled (Fz), the cytoplasmic proteins Disheveled (Dsh/Dvl) and Prickle (Pk), the four-pass transmembrane protein strabismus (Sibm/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; Wallingford et al., 2002).

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 Sibm 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; Iliescu 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.

Syndecan4 (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; Echtelermeyer 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 Vangl2Lp/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 colocalizes in multiple tissues, but particularly in the non-neural ectoderm, hindgut and otic vesicles.
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 Vangl2Lp/+ 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'-CAGG-GGACGCAACATTTTGGAGAGAC-3'; reverse, 5'-TCCTCCTCAGGGT- CACAGGC-3'); and the null allele (forward, 5'-CGCTCTTCTTGGAGGTTTCTT-3'; reverse, 5'-GGACTCCACTGTCCTTCCAA-3'). Vangl2Lp/+ mice and embryos were genotyped as described (Copp et al., 1994). Sdc4lacZ/+; Vangl2Lp/+ mice were obtained by natural matings between Vangl2Lp/+ males and Sdc4lacZ/lacZ females. From the F1 offspring, compound heterozygous mice were selected by genotyping and intercrossed with Sdc4lacZ/lacZ 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'-AGATCCGGCT-TTGTGGCGATCCA-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 Vangl2Lp/+ 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 chloride 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. After 24 hours, embryos were harvested from culture and yolk sacs were processed for genotyping. Embryos were inspected for presence/absence of closure 1, somites were counted and PNP length was measured. Embryos were fixed overnight in ice-cold 4% paraformaldehyde in PBS, before wax embedding and preparation of 7 µm transverse sections, which were stained with Haematoxylin and Eosin.

Immunohistochemistry

Frozen sections (10 µm) were fixed for 10 minutes with 4% paraformaldehyde, blocked for 1 hour at room temperature with 5% rat serum in PBS, and then incubated in mouse monoclonal anti-syndecan 4 antibody (Santa Cruz, sc-12766) at a 1:100 dilution or rabbit anti-pan-cadherin antibody (Sigma C3678) at a 1:200 dilution. Slides were then washed in PBS and four times for 15 minutes each and primary antibody was detected with appropriate secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594 (1:1000 dilution; Invitrogen). Sections were mounted in Vectashield and imaged with a Fluoview FV1000 confocal microscope.

Whole-mount in situ hybridization

Embryos were obtained from timed-mated pregnant females and processed for whole mount in situ hybridization according to standard protocols. To generate a probe for Sdc4, a 543 bp partial cDNA fragment containing a small region of the cytoplasmic domain and the 3' UTR region was subcloned into pGEM-T easy vector. The Vangl2 probe was as described previously (Doudney et al., 2005). Antisense and sense cRNA probes were generated by in vitro transcription using SP6 RNA polymerase and a DIG RNA Labeling Kit. Whole-mount Sdc4 embryos were embedded in paraffin wax and sectioned with a microtome at 10 µm. Whole-mount Vangl2 embryos were embedded in 2% agarose in PBS and sectioned at 50 µm using a vibratome.

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 neural tube, otic vesicle and hindgut (Fig. 1B,C,E; 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. 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. 1G,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., 2007; Yamamoto et al., 2008). Therefore, a genetic interaction would demonstrate a role for Sdc4 in Wnt/PCP signaling.
To obtain Sdc4; Vangl2 compound mutants, we crossed double heterozygous males (Sdc4lacZ/+; Vangl2Lp/) with Sdc4lacZ/lacZ females. This protocol was followed to avoid using Vangl2Lp/+ females, almost half of which exhibit imperforate vagina (Murdoch et al., 2001). The number of newborn mice with Sdc4lacZ/lacZ genotype showed a statistically significant reduction from the expected Mendelian distribution (P<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 Sdc4lacZ/+; Vangl2Lp/+ mice were born with a sacral spina bifida (Fig. 2B, C), Sdc4lacZ/lacZ; Vangl2Lp/+ mice exhibited a threefold higher frequency of this defect (55%; 11 out of 20), a statistically significant difference (P=0.006).

Moreover, in two Sdc4lacZ/lacZ; Vangl2Lp/+ 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 Vangl2Lp/+ (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 Sdc4lacZ/+; Vangl2Lp/+ and Sdc4lacZ/lacZ; Vangl2Lp/+ 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 Sdc4lacZ/lacZ; Vangl2Lp/+ mice survived beyond one postnatal month, compared with 78% survival for Sdc4lacZ/lacZ; Vangl2Lp/+ mice (supplementary material Table S2).

In Xenopus embryos, alteration of Wnt/PCP signaling results in CE defects in the mesoderm and neuroectoderm (Wallenfird 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 (Wallenfird 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 neural tube closure than the individual morpholinos alone (P<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. Cochlear tissues were isolated at E18.5, stained with phalloidin (stereocilia) and acetylated tubulin (kinocilium), and analyzed by confocal microscopy. Normal organization of the hair cells was observed in Sdc4lacZ/lacZ; Vangl2Lp/+ mice (Fig. 3A), whereas significant disruption of stereociliary bundle orientation was detected in Sdc4lacZ/lacZ; Vangl2Lp/+ and
Sdc4lacZ/lacZ;Vangl2Lp/+ mice (Fig. 3B,C). In agreement with the Sdc4 expression pattern (Fig. 1K), the outer hair cell rows 2 and 3 (OHC2, 3) showed more disruption. As with spina bifida, the strongest effect on cochlear morphogenesis was observed in the Sdc4lacZ/lacZ;Vangl2Lp/+ mice (Fig. 3C), suggesting a dose dependent effect of Sdc4. In addition, supernumerary hair cells, which are also a recognized PCP phenotype (Montcouquiol et al., 2003), were observed in the compound mice and in Sdc4-null mice (Fig. 3C and data not shown).

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/+;Vangl2+/+. Moreover, the wound remained open in the double heterozygous mice at day 9, when it was already closed in the Sdc4lacZ/+;Vangl2+/+ 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 Dvl3, and adversely affects the subcellular localization and stability of several PCP proteins (Gravel et al., 2010; Iliescu 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 lane 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

Fig. 3. Sdc4 and Vangl2 interact in cochlear stereociliary bundle orientation. (A-C) The interaction between Sdc4 and Vangl2 disrupts planar cell polarity in the cochlea. Stereociliary bundles were stained with phalloidin (red) and kinocilia with anti-acetylated-tubulin (green) in cochleae isolated from E18.5 embryos with the indicated genotypes. Arrow indicates supernumerary cells outside the inner hair cell (IHC). Middle panels depict schematic representations of the hair bundle orientation based on the top panel. Bottom panel summarizes the distribution of hair bundle orientation in the IHC, outer hair cell (OHC) 1, OHC2 and OHC3 for each of the three genotypes. Scale bar: 10 μm in A.

Fig. 4. Sdc4 and Vangl2 interact in wound healing. Wound healing is delayed in Sdc4;Vangl2 compound mutant mice. (A,B) Representative macroscopic views of wound healing in the back skin of mice with the indicated genotypes. (C) Summary of the data from a total of at least 9-12 wounds (three or four mice) for each genotype. Data are represented as mean±s.e.m. *P<0.001. Scale bar: 3 mm in A.
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 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 Vangl2 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 Vangl2 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 Vangl2^{lp} on Sdc4 levels in wild-type and Vangl2^{lp/Lp} 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 Vangl2^{Lp/Lp} embryos (Fig. 5F; supplementary material Fig. S6).

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 protein level, we also observed the converse: Sdc4 was found to affect Vangl2 steady-state level, as demonstrated using siRNA (Fig. 2B). 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.

**Vangl2^{Lp} 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 Sdc4^{lacZ/lacZ}; Vangl2^{Lp/Lp}, 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 Vangl2^{lp} 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 Vangl2^{Lp/Lp} mutants compared with Vangl2^{Lp/+} and wild type. This suggests that Vangl2^{Lp} 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 Vangl2^{Lp/+} 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). Vangl2^{Lp/+} 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 Vangl2^{Lp/+} 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 Vangl2^{Lp/+} embryos, whereas it is always present in Vangl2^{Lp/Lp} individuals. Indeed, Vangl2^{Lp/Lp} embryos cultured in the absence of chlorate exhibited normal closure 1, as did wild-type embryos cultured in chlorate (Fig. 6A, Table 1). Hence, there is a gene-environment interaction in which the phenotype of Vangl2^{Lp/+} is converted to that of Vangl2^{Lp/Lp} 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 Vangl2^{Lp} 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 Sdc4lacZ/lacZ;Vangl2Lp/+ mutants. It is interesting to note that the spina bifida in Sdc4lacZ/lacZ;Vangl2Lp/+ mice (closure 1) at E8.5, whereas Vangl2 is precisely expressed at this fact that Sdc4 is not expressed at the site of closure initiation (Cornelison et al., 2004; Le Grand et al., 2009; Bentzinger et al., 2013).

With regard to the neural tube defect phenotypes we observed, the strong phenotype of the curly tail mutant, which carries a hypomorphic mutation 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 Sdc4lacZ/lacZ;Vangl2Lp/+ mutants. It is interesting to note that the spina bifida in Sdc4lacZ/lacZ;Vangl2Lp/+ mice (closure 1) at E8.5, whereas Vangl2 is precisely expressed at this fact that Sdc4 is not expressed at the site of closure initiation (Cornelison et al., 2004; Le Grand et al., 2009; Bentzinger et al., 2013).

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., M.F., H.C., 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 sulfation 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 (Grii3) (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;Vangl2 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;Vangl2 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;Vangl2 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), glypican 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.

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

<table>
<thead>
<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 chloride</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 ($\chi^2=17.3; P<0.001$).

§Posterior neuropore (PNP) length (mean in mms±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 chloride 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 experiments 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 maintenance and mouse crosses. H.C. performed the biochemistry experiments. A.J.C. helped with 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
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