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/Vangl2Lp² mutant embryos show strong defects in neurulation, with neural tube closure initiated in the prospective hindgut and in the otic vesicle. 20%-60% of embryos from co-injection of Sdc4 and Vangl2 morpholinos showed co-localization. Conversely, knockdown of Sdc4 enhances Vangl2Lp² mutants, while knockdown of Vangl2 enhances Sdc4-null embryos, but not to levels comparable to the double mutant. Synergizing 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 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 Vangl2Lp² enhances the Sdc4-null phenotype. This could be mediated via heparan sulfate residues, as Vangl2Lp² embryos fail to initiate neural tube closure and develop craniorachischisis (usually seen only in Vangl2Lp²) when cultured in the presence of chlorate, a sulfation inhibitor. These results demonstrate that Sdc4 can participate in the Wnt/PCP pathway, unveiling its 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 (Stbm/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). 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

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Vangl2Lp/+ embryos 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 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'-CGAGG-GACGCAAACATTTTTAGAGAAC-3'; reverse, 5'-CTCTTTCCATTTCACAGAGC-3'); and the null allele (forward, 5'-CGCTTTCTTG-AAGTTTCTTT-3'; reverse, 5'-GGAATCTACTGTCCCTTCAA-3'). Vangl2Lp/+ and Vangl2Lp/+ embryos were genotyped as described (Copp et al., 1994). Sdc4lacZ/lacZ; 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'-AGTACCGGCTT-TGTGCGGCAFTCCA-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). All animal procedures and experiments were performed with the approval of the Pontificia Universidad Católica de Chile Animal Ethics Committee and the Animals (Scientific Procedures) Act 1986 of the UK Government.

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 Sigmatstat 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 E8.5-8.5, Sdc4 is expressed in the cranial neural folds (Fig. 1A) mainly in the cephalic mesenchyme (Fig. 1D). More caudal sections revealed additional transcripts also in the cranial neural folds (Fig. 1A) mainly in the cephalic mesenchyme (Fig. 1D). More caudal sections revealed additional transcripts also in the cranial neural folds (Fig. 1A) mainly in the cephalic mesenchyme (Fig. 1D). More caudal sections revealed additional transcripts also in the cranial neural folds (Fig. 1A) mainly in the cephalic mesenchyme (Fig. 1D).
**Fig. 1. Sdc4 and Vangl2 expression during mouse development.** (A-E) Whole-mount *in situ* hybridization with an antisense RNA probe for Sdc4. Embryos at E8.5, dorsal view (A), and E9.5, lateral view (B) and rostral view (C). Transverse sections (DE) are at the levels indicated on embryos in A and B, respectively. (F-H) β-Galactosidase activity in *Sdc4lacZ/+* embryos at E9.0. Lateral view (F) shows expression in otic vesicle (arrowhead) and hindgut (arrow). Dorsal view of caudal region (G) shows expression in non-neural ectoderm on the outside of the neural fold of the posterior neuropore (arrowhead) and overlying recently closed neural tube (arrow). Section (H) is from the level indicated by the line in G. Arrowhead indicates the non-neural ectoderm. (I,J) Immunostaining of transverse sections through wild-type E9.0 embryos at the hindbrain level using anti-Sdc4 (green, arrows) and anti-pan-cadherin (red). Expression is detected in the neural folds (I, arrow), foregut (I, arrowhead) and hindgut (J, arrow). (K) X-Gal staining of the whole cochlea from *Sdc4lacZ/+* embryo at E18.5. Inset: section showing *lacZ* expression in the outer hair cells (bracket) and in the inner hair cell (arrow). (L) Immunostaining of *Sdc4* (green) on transverse section through the otic vesicle in wild-type E9.0 embryo. (M,N) Sections through the closed neural tube (M) and open PNP (N) of an E9.5 embryo subjected to whole-mount *in situ* hybridization using an antisense RNA probe for *Vangl2*. cm, cephalic mesenchyme; hf, head folds; hg, hindgut; nt, neural tube; ov, otic vesicle; PNP, posterior neuropore. Scale bars: 500 μm in A,B; 300 μm in G; 10 μm in I,L; 250 μm in K.

*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*-null mice showed increased levels of at 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 during morphogenesis.

**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 during and following closure, the hindgut and the otic vesicle.

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.
Yamamoto et al., 2008). Therefore, a genetic interaction with Vangl2<sup>+/−</sup> would demonstrate a role for Sdc4 in Wnt/PCP signaling.

To obtain Sdc4<sup>−/−</sup>;Vangl2<sup>−/−</sup> compound mutants, we crossed double heterozygous males (Sdc4<sup>lacZ/lacZ</sup>;Vangl2<sup>Lp/+</sup>) with Sdc4<sup>lacZ/lacZ</sup>;Vangl2<sup>Lp/+</sup> females. This protocol was followed to avoid using Vangl2<sup>+/−</sup> females, almost half of which exhibit imperfecta vagina (Murdoch et al., 2001). The number of newborn mice with Sdc4<sup>lacZ/lacZ</sup>;Vangl2<sup>Lp/+</sup> 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 Sdc4<sup>lacZ/lacZ</sup>;Vangl2<sup>Lp/+</sup> mice were born with a sacral spina bifida (Fig. 2B,C), Sdc4<sup>lacZ/lacZ</sup>;Vangl2<sup>Lp/−</sup> mice exhibited a threefold higher frequency of this defect (55%; 11 out of 20), a statistically significant difference (P=0.006).

Moreover, in two Sdc4<sup>lacZ/lacZ</sup>;Vangl2<sup>Lp/−</sup> mice, a more severe lumbar sacral 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>−/−</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>lacZ/lacZ</sup>;Vangl2<sup>−/−</sup> and Sdc4<sup>lacZ/lacZ</sup>;Vangl2<sup>+/−</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>lacZ/lacZ</sup>;Vangl2<sup>Lp/−</sup> mice survived beyond one postnatal month, compared with 78% survival for Sdc4<sup>lacZ/lacZ</sup>;Vangl2<sup>Lp/−</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. Most 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 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. 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>lacZ/lacZ</sup>;Vangl2<sup>+/−</sup>
mice (Fig. 3A), whereas significant disruption of stereociliary bundle orientation was detected in $Sdc4^{lacZ/-}; Vangl2^{Lp/-} \text{ and } Sdc4^{lacZ/lacZ}; Vangl2^{Lp/-}$ 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 $Sdc4^{lacZ/lacZ}; Vangl2^{Lp/-}$ 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 $Sdc4^{lacZ/-}; Vangl2^{Lp/-}$ compound mutants (Fig. 4A-C): at 5 days, only 45% of the wound was closed in $Sdc4^{lacZ/-}; Vangl2^{Lp/-}$ compared with 75% closure in $Sdc4^{lacZ/-}; Vangl2^{Lp/-}$. Moreover, the wound remained open in the double heterozygous mice at day 9, when it was already closed in the $Sdc4^{lacZ/-}; Vangl2^{Lp/-}$ mice. The fact that even homozygous $Vangl2^{Lp/-}$ 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 $Vangl2^{Lp}$ 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 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 4-7 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}

Fig. 5. Vangl2 regulates Sdc4 steady-state levels. (A) Sdc4 and Vangl2 colocalize at the subcellular level. Confocal analysis of HeLa cells by double immunofluorescence using antibodies against endogenous Sdc4 (green) and Vangl2 (red). (B) siVangl2 increases Sdc4 steady-state levels. HEK293 cells were co-transfected as indicated and xSdc4-Flag steady-state levels were evaluated by western blot. (C) Vangl2 reduces Sdc4 steady-state levels. HEK293 cells were co-transfected with the indicated DNAs and xSdc4-Flag steady-state levels were evaluated by western blot. Vangl2Lp is more active than wild-type Vangl2 in this assay. (D,E) Cells transfected with xSdc4-Flag (D) without or (E) with mVangl2 were incubated with cycloheximide (40 μg/ml) for different times. The half-life of xSdc4 protein in the different conditions was estimated from experiments in triplicate. (F) Sdc4 is absent from otic vesicles of Vangl2Lp/Lp mice. Double immunofluorescence using antibodies against Sdc4 (green) and pan-cadherin (red) was performed on transverse sections from wild-type and Vangl2Lp/Lp mutant mice at E9.5. (G) Interaction of Sdc4 and xVangl2 in Xenopus embryos. Eight-cell stage Xenopus embryos were co-injected in the two dorsal-animal blastomeres with the indicated amounts of Sdc4 morpholino and xVangl2 synthetic mRNA. Phenotypes were classified at stage 20 as type I (severe gastrulation and neural tube closure defects; red) and type II (impairment of neural tube closure; green). The graph summarizes three independent experiments, with numbers of embryos given at the top of each bar. Co-injection of xSdc4-Mo + xVangl2 mRNA resulted in a significantly greater proportion of embryos with defective neural tube closure than the individual suboptimal amounts of xSdc4-Mo and xVangl2 mRNA (Chi-square test, P<0.001). (H) Reduced levels of HSPG in Vangl2Lp mice. Homogenates from E14.5 wild-type, Vangl2Lp/+ and Vangl2Lp/Lp mice were analyzed by western blot using anti-Stub and anti-tubulin antibodies. Scale bars: 10 μm in A; 20 μm in F.
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 expression on Sdc4 levels in wild-type and Vangl2 knockout mice. By immunofluorescence, Sdc4 protein was readily detected in the otic epithelium and non-neural ectoderm of E9.0 Vangl2 knockout embryos, whereas no signal could be detected in the otic vesicles and non-neural ectoderm of Vangl2 knockout 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 (supplementary material Fig. S5B). Taken together, these findings predicted that overexpression of Vangl2 should summate with a knockdown of Sdc4 to affect Vangl2 steady-state level, as demonstrated using siRNA (supplementary material Fig. S6).

Vangl2 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-/-; Vangl2-/-, where Sdc4 is already completely absent because of the null mutation. Based on the fact that other syndecans, or HSPGs more generally, could compensate it is always present in Sdc4-/- embryos, whereas it is 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 expression 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-/- mutants compared with Vangl2+/- and wild type. This suggests that Vangl2 expression 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-/- 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+/- 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+/- embryos failed to initiate neural tube closure (i.e. closure 1 failure) and developed craniarctischisis (entirely open neural tube). This phenotype is not usually observed in Vangl2+/- embryos, whereas it is always present in Vangl2-/- individuals. Indeed, Vangl2+/- 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 Vangl2+/- is converted to that of Vangl2-/- 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+/- affects the levels of many HSPGs (Fig. 5H), these findings raise the possibility that Vangl2 may interact with other HSPGs in addition to Sdc4.
Syndecan 4 interacts with Vangl2

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;Vangl2 mutants. In particular, Sdc4lacZ/lacZ;Vangl2Lp/+ embryos had a much stronger phenotype than Sdc4lacZ+/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 (Galvo 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 craniorchiaschisis, 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 (Grhli3) (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.

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

<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 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. C.H. performed the biochemistry experiments. C.H. helped with the genotyping. U.T. helped set up mouse experiments and procedures. S.E.P. performed the embryo culture experiments, participated in the design of the study, data analysis and revised and commented on the manuscript. A.J.C. performed the embryo culture experiments, participated in the design of the study and revised and commented on the manuscript. J.L. participated in the design of the study, supervised the project and wrote the manuscript.

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