Reception of Slit requires only the chondroitin–sulphate-modified extracellular domain of Syndecan at the target cell surface

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Syndecan (Sdc) is a conserved transmembrane heparan sulfate proteoglycan (HSPG) bearing additional chondroitin sulfate (CS) modifications on its extracellular domain. In vertebrates, this extracellular domain of Sdc is shed and acts as a soluble effector of cellular communication events, and its cytoplasmic domain participates in intracellular signaling needed to maintain epithelial integrity. In *Drosophila*, Sdc has been shown to be necessary for Slit signaling-dependent axon and myotube guidance during CNS development and muscle pattern formation. We report that Sdc acts in a cell-autonomous manner in Slit-receiving cells and that its membrane-anchored extracellular domain is sufficient to mediate Slit signaling. Sdc activity can be replaced by the human homolog *hsdc2*. However, the HSPG Dally-like protein (Dlp), which lacks CS modifications at its extracellular domain, can only partially substitute for Sdc function, and its activity is not restricted to the Slit target cells. Our results suggest that Sdc and Dlp act in a cooperative but nonredundant fashion in axon and myotube guidance. We propose that Dlp, which lacks CS modifications, participates in the transfer of Slit from its site of expression to the target cells, where CS-modified Sdc concentrates and presents the ligand.

Drosophila | heparan sulfate | Slit signal transduction | axon guidance

Heparan sulfate proteoglycans (HSPGs) are secreted or cell-associated ECM proteins that are modified with specific linear heparan sulfate (HS) glycosaminoglycan polymers (1, 2). Studies of mutants with impaired HS synthesis and of HSPGs themselves have revealed their essential role in the transport and reception of secreted factors, including Wingless, Hedgehog, Decapentaplegic, Fibroblast Growth Factor (3), and Slit (4, 5). *Drosophila* contains 4 HSPGs: Perlecan (6), Division abnormally delayed (Dally), Dally-like protein (Dlp; 7, 8), and Syndecan (Sdc; 4, 5). The requirement for Sdc has been established for vertebrates, showing that the HSPG acts as an independent signaling receptor and has a number of functional features assigned to its cytoplasmic and extracellular domains, respectively. Its cytoplasmic domain functions in intracellular signal transduction and plays a role in the maintenance of epithelial integrity by linking the ECM to the actin cytoskeleton (9–12). Furthermore, the transmembrane domain of Sdc not only serves to localize Sdc at the cell membrane but to mediate protein-protein interactions (13). Finally, the extracellular domain of vertebrate Sdc is proteolytically shed (14–17) and acts as an extracellular effector in cell communication events (15, 16).

In *Drosophila*, Sdc was shown to regulate Slit signaling (4, 5). Slit, a secreted ligand produced in ventral midline cells, acts as a repellent in both axon and myotube guidance during embryogenesis, 2 processes that are mediated by Robo receptors in the target cells (18). Loss of Slit signaling causes axons and muscle fibers to cross the ventral midline of the embryo (18), a mutant phenotype that is also observed in the absence of Sdc activity (4, 5) [supporting information (SI) Fig. S1]. Here, we show that Sdc is specifically required in the target cells. The membrane-anchored chondroitin sulfate (CS)-modified extracellular domain of *Drosophila* Sdc is both necessary and sufficient to mediate proper Slit signaling.

Results and Discussion

To understand Sdc function both in molecular and functional terms, to identify its cellular requirement, and to elucidate the mechanism by which Sdc mediates Slit/Robo signaling, we asked which portions of the Sdc protein are required for that signaling process and how Sdc function compares with that in vertebrates. Reduced Slit activity is evident in ventral midline crossovers of axon fascicles and muscles never observed in WT embryos (18, 19) (Fig. S1). To test various portions of Sdc to determine whether they are required for Slit signaling, we generated Sdc deletion mutants and expressed them in specific subsets of cells in *sdc* mutant embryos (5). For these experiments, we used the Gal4/UAS expression system (20) and conditions used in previous experiments (5) showing that expression of WT Sdc [Sdc-PA] can fully rescue the *sdc* mutant phenotype (Fig. 1*A*).

We asked whether, like in vertebrates, the *Drosophila* Sdc cytoplasmic domain is required for intracellular Slit signal transduction. To test for this feature, we generated *sdc-ΔC*, which contains GFP in place of the Sdc cytoplasmic domain. On Gal4/UAS-driven expression in neurons, where Sdc is normally expressed (4, 5), *sdc-ΔC* rescued the *sdc* mutant axon phenotype (Fig. 1*A*). Thus, the cytoplasmic domain is not essential for Sdc function in Slit signaling. To test further whether the transmembrane domain of Sdc mediates essential protein-protein interactions (13), we tested an *sdc* variant containing a heterologous GPI anchor (*sdc-GPI*) in place of both the Sdc transmembrane and cytoplasmic domains (Fig. 1*A*), allowing the extracellular domain of Sdc to associate with the target cell membrane via the GPI anchor. Fig. 1*A* shows that *sdc-GPI* expression rescues the *sdc* mutant phenotype. Taken together, these findings demonstrate that the membrane-anchored extracellular domain of Sdc is sufficient to mediate proper Slit signaling in target cells.

We next asked whether shedding of the extracellular domain is required to generate a physiologically active form of Sdc in *Drosophila* (14–16). To mimic shedding, we used a secreted extracellular domain of Sdc (Sdc-ΔTC; Fig. 1*A*). Fig. 1 *B–D* shows that Sdc-ΔTC is indeed secreted when expressed in the CNS (Fig. 1 *B* and *C*), in the embryonic hindgut (Fig. 1 *D* and *E*), in the tracheal system (Fig. S2), and in tissue culture cells (Fig. 1*F*). However, no rescue was observed in response to Sdc-ΔTC expression in the CNS or any other place of the embryo (Fig. 1*A*). The fact that the membrane-anchored extracellular domain of Sdc exhibited rescue activity, whereas the secreted extracellular domain did not could be attributable to deficient

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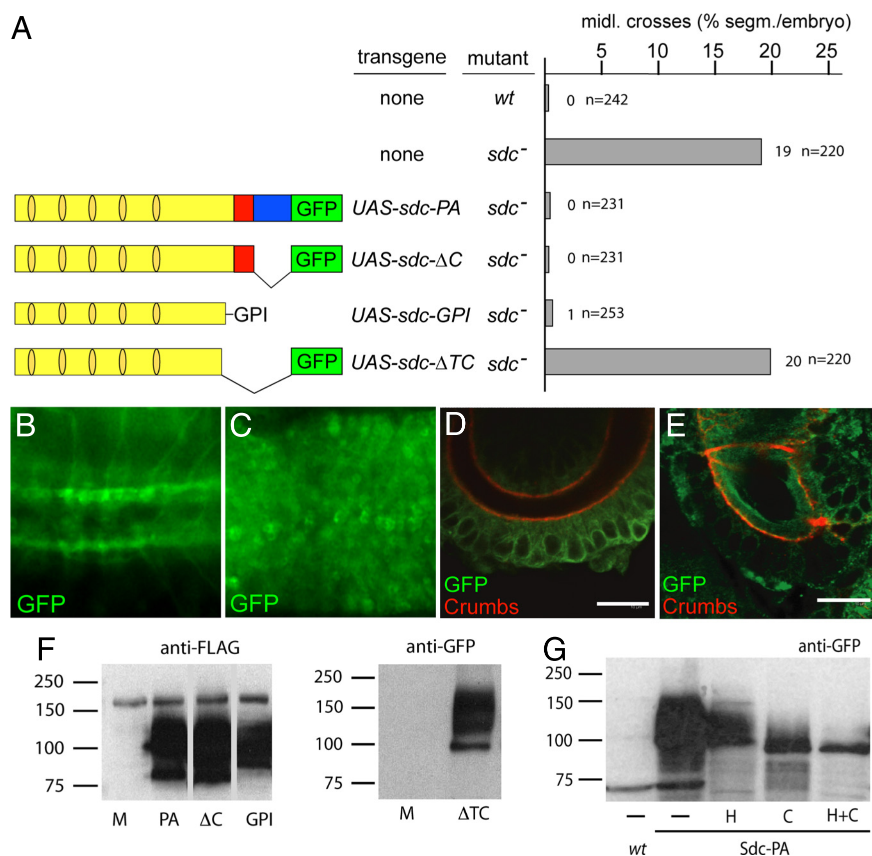

Fig. 1. Anchorage of the Sdc extracellular domain is a prerequisite for function. (*A*) Percentage of segments with ectopic ventral midline crossover of ipsilateral axon fascicles stained with anti-FASII antibody in *wt*, *sdc*, and *sdc* homozygous mutants rescued with *UAS-sdc-PA*, *UAS-sdc-ΔC*, *UAS-sdc-GPI*, and *UAS-sdc-ΔTC* driven by *elavG4*. *n*, number of segments analyzed. (*Left*) Schematic representation of transgenes. Light yellow indicates *sdc* extracellular domain, dark yellow indicates HS attachment sites, red indicates *sdc* transmembrane domain, and blue indicates *sdc* cytoplasmic domain. Sdc-PA and Sdc-ΔC contain FLAG and GFP tags, whereas Sdc-GPI and Sdc-ΔTC contain a FLAG tag and a GFP tag, respectively. For details, see *Methods*. Ventral view of 3 segments (*Left*, anterior) showing GFP expression in first instar larvae in response to *UAS-sdc-PA* (*B*) and *UAS-sdc-ΔTC* (*C*) expression using *elavG4*. Sdc-PA is localized along CNS axon trajectories, whereas Sdc-ΔTC is not localized. Cross sections of stage 16 hindgut showing Sdc-PA (*D*) and Sdc-ΔTC (*E*) expression in response to *daG4*. Anti-Crumbs antibody labels the apical membrane of the hindgut bordering the lumen. Note that Sdc-PA is attached to cell membranes (*D*), whereas Sdc-ΔTC is secreted into the lumen (*E*). (Scale bar: 10 μm.) (*F*) Western blot of Kc167 cell extracts expressing Sdc-PA, Sdc-ΔC, or Sdc-GPI and the supernatant of cells expressing Sdc-ΔTC to confirm their expression and modification. M, mock transfected cells. Sdc-PA, Sdc-ΔC, and Sdc-GPI were detected with anti-FLAG antibody, and Sdc-ΔTC was detected with anti-GFP antibody. (*G*) Sdc-PA is modified by HS and CS. Extracts of embryos expressing Sdc-PA (using *tubPG4*) were either mock treated (—) or treated with heparinase (H), chondroitinase (C), or a combination of heparinase and chondroitinase (H+C). Proteins were detected with anti-GFP antibody. Treatment with chondroitinase dramatically reduced molecular weight, but only treatment with both enzymes released unmodified Sdc.

glycosaminoglycan modifications of the protein that were shown to be critical for ligand binding (21). To test this possibility, we performed modification-specific enzyme degradation assays with heparinase and/or chondroitinase, showing that WT Sdc was modified by HS and CS (Fig. 1*G*) and that Sdc-ΔC, Sdc-GPI, and the secreted Sdc-ΔTC were modified by glycosaminoglycans (Fig. 1*F*). Thus, an absence of glycosaminoglycan modifications of the Sdc extracellular domain cannot be the reason why Sdc-ΔTC is functionally inactive. We also found that expression of Sdc-ΔTC in WT embryos had no dominant negative effect on Slit signaling, suggesting that it does not interfere with Slit activity when released from the cell membrane. The simplest explanation for this finding is that Slit binding by Sdc requires one or several components that are present in the ECM of the target cells. In fact, a complex composed of Sdc, Slit, and Robo has been reported recently (4). Thus, it is possible that Slit only interacts with Sdc in conjunction with, for example, Slit receptors. These results support the conclusion that shedding of Sdc is not a prerequisite for its function in vivo. To demonstrate the lack of significance of shedding in vivo further, we marked the N-terminus of the Sdc extracellular domain with a 2X-FLAG tag and the C-terminus of the cytoplasmic domain of Sdc with GFP and then expressed the double-marked protein in embryonic neurons. We found that the two markers, 2X-FLAG and GFP, always colocalize wherever Sdc is expressed (Fig. 2), indicating that there is no notable shedding of *Drosophila* Sdc. The lack of shedding is consistent with the reported surface staining by antibodies directed against the Sdc extracellular domain (17). Taken together, the in vivo results support the conclusion that Sdc-dependent Slit signaling requires only the extracellular portion of the protein to be attached to the target cell membrane.

To determine whether the protein sequence of the extracellular domain of Sdc, the HS and/or CS modifications, are of functional relevance, we performed rescue experiments with human *sdc2* (*hsdc2*), which carries both HS and CS modifications on its otherwise highly divergent extracellular domain (22) (Fig. S3). In addition, we performed the same kind of rescue experiments with Dlp, a different membrane-anchored HSPG. Dlp lacks CS modifications but contains more (i.e., 9 canonical HS attachment sites compared with 5 for Sdc). These sites are defined by a serine residue followed by glycine (1). Gal4/UAS-driven *hsdc2* expression in neurons resulted in a complete rescue

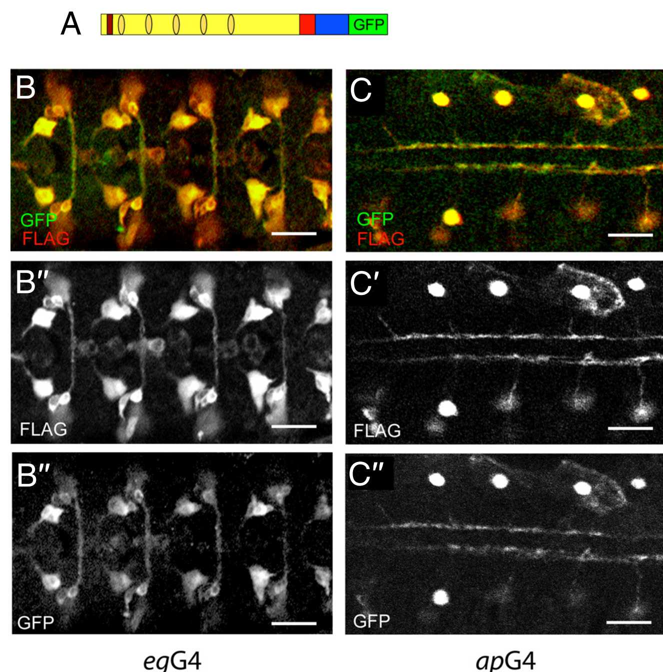

Fig. 2. Sdc extracellular domain is not shed in vivo. (*A*) Schematic representation of the double-tagged *sdc-PA*: *FLAG-sdc-GFP*. The FLAG tag is shown in red, and the others are as described in Fig. 1. *UAS-FLAG-sdc-GFP* expression using *egG4* (*B*) and *apG4* (*C*). The amino and carboxy termini of Sdc were detected with anti-FLAG and anti-GFP antibodies, respectively. Note colocalization of the N- (B′ and C′) and C- (B″ and C″) termini. (Scale bar: 20 μm.)

Table 1. Cooperation of Sdc and Dlp in Slit signalling

Driver	Mutant	Axon midline crossing, % segment/embryo
None	*wt*	0 (*n*=209)
None	sdc^{-}	19 (*n*=198)
None	ttv^{-}	0 (*n*=220)
None	dlp^{-}	0 (*n*=220)
None	$sdc^{-};ttv^{-}$	39 (*n*=198)
None	$sdc^{-};dlp^{-}$	50 (*n*=176)
None	*wt*	0 (*n*=220)
None	sdc^{-}	19 (*n*=176)
simG4:UAS-sdc	sdc^{-}	19 (*n*=198)
repoG4:UAS-sdc	sdc^{-}	16 (*n*=231)
elavG4:UAS-sdc	sdc^{-}	0 (*n*=209)
simG4:UAS-dlp	sdc^{-}	11 (*n*=231)
repoG4:UAS-dlp	sdc^{-}	5 (*n*=220)
elavG4:UAS-dlp	sdc^{-}	9 (*n*=198)

The percentages of segments with ventral midline crossover of ipsilateral axon fascicles stained with anti-FASII antibody are listed. *n*, number of segments analyzed. Analyzed embryos were *wt*, *sdc*, *ttv*, *dlp*, *sdc;ttv*, and *sdc;dlp* homozygous mutants as well as *sdc* homozygous mutants rescued with *UAS-sdc-PA* or *UAS-dlp* expressed in Slit-secreting midline cells (*simG4*), intermediate glia tissue (*repoG4*), or axonal target tissue (*elavG4*).

of the *sdc* mutant axon phenotype, as had been observed with *Drosophila* Sdc (Fig. 3*A*). This finding suggests that the apparently nonconserved amino-acid sequence of the extracellular domain is not essential for Slit signaling, and thus emphasizes the potential importance of HS and CS modifications. In contrast, the corresponding expression of Dlp, which lacks CS modifications, could only partially rescue the *sdc* mutant phenotype (4) (Table 1), supporting the argument that the CS modifications of Sdc are required for Slit signaling. We addressed this point more directly by expressing Sdc mutants bearing gradually decreasing numbers of canonical HS attachment sites (21) (Fig. 3*A*). Expression of these Sdc mutant proteins, including one that lacked all canonical HS modifications (Sdc-SG5; Fig. 3*A*), in neurons resulted in a complete or nearly complete rescue of the axon phenotype. The Sdc-SG5 mutant protein shows a dramatic reduction of sugar modifications (Fig. 3*B*) and consists mostly of remaining CS modifications as revealed by modification-specific enzyme assays with heparinase and chondroitinase (Fig. S4). Low-level HS modifications may still occur at noncanonical HS modification sites (Fig. S4). The complete rescue in response to Sdc-SG5 and hSdc2 expression in contrast to the comparatively weak rescue activity in response to Dlp further supports the conclusion that Sdc-dependent Slit signaling requires either CS modifications or a combination of CS and HS modifications and is not based on HS modifications of the HSPG only. Furthermore, it seems unlikely that the protein core of the extracellular domain plays a role, because the corresponding domain of *hsdc2* contains a highly divergent amino acid sequence (Fig. S3) that can fully compensate for Sdc function in the fly.

The different rescue abilities of Sdc and Dlp suggest that they play different roles in the Slit signaling process. In fact, *dlp* is expressed in the CNS when axons grow toward their target (4). To test whether Dlp acts in an Sdc-like fashion, we examined whether the lack of Dlp and the absence of HS biosynthetic enzyme activity, as in *tout-velu* (*ttv*) mutants (23), affects axon and muscle guidance. A lack of Dlp or Ttv activity had no effect on axon and muscle patterns. However, in double-mutant combinations such as *sdc;ttv* and *sdc;dlp*, the *sdc* mutant phenotype was strongly enhanced, with a 2-fold increase in ventral midline crossovers of axon fascicles (2.0- and 2.6-fold, respectively; Table 1). Because *sdc* is essential for Slit signaling (4, 5), its genetic interaction with *ttv* and *dlp* suggests that the products of the 2 genes are also involved in Slit signaling, acting either in conjunction with or parallel to Sdc.

Finally, we asked whether Dlp can replace Sdc function and which cells of the embryo require Sdc, Dlp, or both of these HSPGs for proper Slit signaling. We performed rescue experiments in which Sdc or Dlp was expressed ubiquitously or in distinct sets of cells using the Gal4/UAS system (20). Ubiquitous expression was achieved by a UAS-driven transgene in response to *da*-Gal4 (5) in the Slit-secreting ventral midline cells by *sim*-Gal4, in cells between midline and Slit target cells by

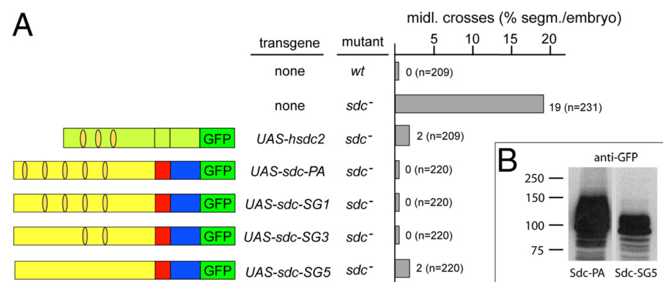

Fig. 3. CS modifications contribute to Sdc activity. (*A*) Percentage of segments with ventral midline crossover of ipsilateral axon fascicles stained with anti-FASII antibody in *wt*, *sdc*, and *sdc* homozygous mutants rescued with *UAS-hsdc2*, *UAS-sdc-PA*, *UAS-sdc-SG1*, *UAS-sdc-SG3*, and *UAS-sdc-SG5*. *n*, number of segments analyzed. (*Left*) Schematic representation of transgenes. Light green indicates human *sdc2*, and the others are as described in Fig. 1. (*B*) Western blot to compare Sdc-PA and Sdc-SG5. Sdc-PA and Sdc-SG5 were expressed in embryos (using *tubPG4*) and visualized by anti-GFP antibody in embryo extracts. The mutation of all HS attachment sites results in a dramatically reduced apparent molecular weight of Sdc-SG5 compared with Sdc-PA.

repo-Gal4, and in the Slit target neurons in response to *elav*-Gal4. In addition, Sdc was expressed in myotubes in response to *mef2*-Gal4 and in the cells between the Slit-expressing and Slit-responding muscle cells by *elav*-Gal4. Ubiquitous expression of WT Sdc (Sdc-PA) restored both axon and muscle patterns (5). Sdc expression in the Slit-secreting cells had no rescue effect on either axons or muscles (Table 1 and Fig. S5), indicating that Sdc is not required for Slit production and/or Slit secretion. Furthermore, Sdc expression in cells connecting the Slit-secreting and Slit-responding target cells also failed to rescue the *sdc* mutant phenotype (Table 1 and Fig. S5). Thus, Sdc is not required for the transport of secreted Slit to the Slit-responding cells. In contrast, Sdc expression in the Slit target cells [i.e., neurons (Table 1) and muscles (Fig. S5)] resulted in a complete rescue, indicating that Sdc is required in these cells only and that Sdc acts in a cell-autonomous manner. Corresponding expression of Dlp caused only a partial rescue of the *sdc* mutant phenotype, irrespective of its site of expression (Table 1). Thus, Dlp can only partially compensate for the loss of Sdc activity, irrespective of its site of expression, in Slit-secreting cells, the target cells, or the cells in between. Hence, the genetic interaction between Sdc and Dlp is based on independent functions of the 2 HSPGs in Slit signaling. Sdc functions exclusively and in a cell-autonomous manner in Slit-receiving cells, where it is coexpressed with Robo (4, 5). This finding suggests that Sdc plays a role in the concentration or presentation of Slit to the Robo receptors and that this specific activity depends on CS modifications of the extracellular domain. Dlp, which lacks such CS modifications and acts in a non-cell autonomous manner, could participate in the transport of Slit, a function that was already established in conjunction with a different signaling molecule, Hedgehog, in wing imaginal discs (7, 8). Alternatively or in addition, Dlp might be required for the concentration or presentation of Slit from neighboring cells *in trans*.

Our results provide evidence that only the extracellular domain of Sdc, in association with the target cell membrane, is both necessary and sufficient to promote Slit signaling. The human homolog hSdc2 and the Sdc-SG5 mutant exert complete Sdc WT activity when expressed in *sdc* lack-of-function mutants, whereas Dlp does not. HSdc2 carries both CS and HS modifications on its otherwise highly diverged extracellular domain, Sdc-SG5 is modified by CS but has all canonical HS modification sites deleted, and Dlp is modified by HS but lacks CS modifications. Thus, it is reasonable to conclude that the CS modifications of the Sdc extracellular domain are specifically necessary to concentrate the ligand Slit at the receiving cells and to receive and/or present the ligand to the Robo receptors. The observed non-cell autonomous requirement for Dlp, combined with the results of the genetic interaction studies on *sdc* and *dlp* mutants, prompts us to propose that Dlp participates in the transport of Slit from its site of expression in the ventral midline cells toward the receiving cells. The different cellular requirements for Sdc and Dlp as revealed by the cell-specific expression studies suggest a model in which the 2 HSPGs participate in different aspects of Slit signaling (i.e., the transport and the proper reception of Slit, respectively).

The functional properties established for vertebrate Sdc, such as intracellular signaling, linking the ECM to the actin cytoskeleton (11, 12), and the proteolytic release of the extracellular domain that promotes cell communication events (15, 16) are clearly not essential for *Drosophila* Sdc to promote Slit signaling. The results unambiguously establish that only the extracellular domain of Sdc participates in the Slit signaling process, provided that it is attached to the Slit target cell membrane. At this location, Sdc can serve as a tether, or coreceptor, to facilitate Slit binding to the Robo receptors (18). This conclusion is consistent with the observation that shedded Sdc has no dominant-negative effect when overexpressed and that Sdc and Robo receptors are coexpressed in both axons and myotubes and are capable of physical interaction (4, 5). This cooperation of 2 differently modified HSPGs in the transport and reception of ligands could explain how a small number of HSPGs can shape multiple ligand/receptor interactions as suggested by the analysis of mutants that fail to synthesize HS (2, 3).

Materials and Methods

Molecular Biology. All transgenes were cloned in pUAST. *Drosophila sdc* domains were amplified from cDNA LD08230, *hsdc2* from cDNA MGC:14,928, and *dlp* GPI anchor sequence from a *Drosophila* cDNA library. Protein analysis tools (available at: www.expasy.org) were used to predict the Sdc signal peptide (1–29 aa), extracellular (1–339 aa), transmembrane (340–364 aa), and cytoplasmic (365–399 aa) domains and Dlp GPI anchor sequence (terminal 66aa common to *dlpRA* and *dlpRB*). Five putative HS attachment sites (serine glycine motifs) were identified in dSdc (Ser-62, Ser-79, Ser-81, Ser-109, and Ser-194), 3 were identified in hSdc2 (Ser-41, Ser-55, and Ser-57), and 9 were identified in Dlp (Ser-147, Ser-380, Ser-463, Ser-504, Ser-625, Ser-629, Ser-630, Ser-643, Ser-686). Sequential mutation of dSdc HS attachment sites was performed to generate *sdc-SG1* (Ser62Ala), *sdc-SG3* (Ser62Ala, Ser79Ala, and Ser81Ala) and *sdc-SG5* (Ser62Ala, Ser79Ala, Ser81Ala, Ser109Ala, and Ser194Ala). *sdc-PA* (1–399 aa), *sdc-ΔC* (1–364 aa), *sdc-ΔTC* (1–339 aa), *hsdc2*, *sdc-SG1*, *sdc-SG3*, and *sdc-SG5* carried a C-terminal GFP in frame with their ORF. A 2X-FLAG tag with an internal SpeI restriction site was inserted after the *sdc* signal peptide sequence, after Glu-29, to create *FLAG-sdc-GFP* and *FLAG-sdcΔC-GFP*. FLAG-tagged *sdc* extracellular domain was fused in frame with the Dlp-GPI anchor sequence to generate *sdc-GPI*. All plasmids used for generation of transgenic flies were sequenced before injection. Control experiments employing Western blot analysis with protein extracts of transfected cells and of embryos expressing WT Sdc and Sdc mutant proteins, respectively, revealed similar protein expression levels (within a 2-fold range; Fig. 1*F*).

Fly Strains. The *sdc* mutant used in this study, sdc^{23}, had been generated previously (5). w^{1118}, *elavG4*, *simG4*, *repoG4*, *daG4*, *tubulinPG4*, and ttv^{00681b} were obtained from the Bloomington Stock Centre. The *apG4* and *egG4* fly stocks were kindly provided by B. Dickson (Institute of Molecular Pathology, Vienna), dlp^{A187} and *UAS-dlp* were provided by X. Lin (Children's Hospital Medical Center, Cincinnati), and *mef2G4* was provided by M. Taylor (Cardiff University, UK). Transgenic flies were generated by P-element–mediated germline transformation.

Immunohistochemistry. Whole-mount antibody staining was performed as described previously (5). β-galactosidase (1:1,000; Promega), rabbit anti-β-galactosidase (1:1,000; Cappel), mouse (anti-Fasciclin II) 1D4 [1:5; Developmental Studies Hybridoma Bank (DSHB)], mouse 2A12 (1:5; DSHB), mouse (anti-Crumbs) Cq4 (1:1,000; DSHB), mouse anti-FLAG M2 (1:1,000; Sigma-Aldrich), rabbit anti-GFP (1:1,000; Synaptic Systems), and rabbit anti-MHC (1:2,000; kindly provided by D. Kiehart, Duke University, Durham, NC) were used as primary antibodies. Goat anti-mouse IgG and anti-rabbit IgG (coupled to Alexa 488 or 568, 1:400; Molecular Probes) and donkey anti-mouse IgM (coupled to Cy3, 1:400; Jackson Labs) were used as secondary antibodies. Stained embryos were analyzed on either a Zeiss epifluorescence microscope or Leica TCS SP2 confocal laser scanning microscope.

Cell Culture. *Drosophila* Kc167 cells were cultured in Gibco's *Drosophila* medium supplemented with 10% FCS (vol/vol) and streptomycin (100 μg/mL). To determine protein expression and modification, cells were transfected (Effectene reagent; Qiagen) with the respective UAS-transgene and *actinG4* plasmids. Cell lysates were analyzed; to test secretion, the cell supernatant was concentrated 10 times (Vivaspin columns; Sartorius) and analyzed by Western blotting.

Enzymatic Assay. Embryos squashed in the respective enzyme buffers following the supplier's instructions were incubated for 3 h at 37 °C with heparinase III (1.75 U/mL), Chondroitinase ABC (1.5 U/mL) (Sigma-Aldrich), or a combination of the 2 and analyzed by Western blotting.

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