Supplementary information for

Structure and Functional Relevance of the Slit2 Homodimerization Domain

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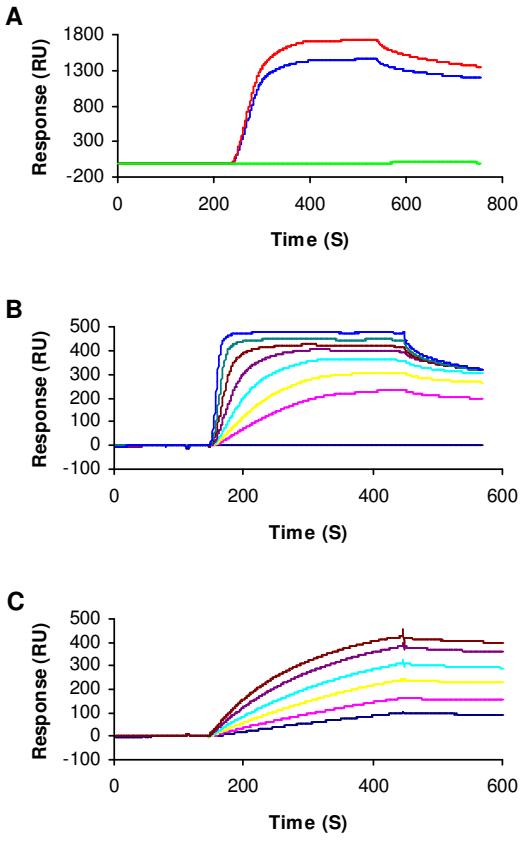

Figure S5. Analysis of Slit binding to heparin (HP), heparan sulfate (HS) and dermatan sulfate (DS)**. A.** SPR sensorgrams measured when 100 nM of Slit2 D2 was injected over HP (blue), HS (red) or DS (green) activated sensorchips. **B**. Overlay of sensorgrams showing the binding of Slit2 D2, at (from top to bottom) 112.5, 75, 50, 33.3, 22.2, 14.8, 9.9 and 0 nM, to immobilized HS. **C.** Overlay of sensorgrams showing the binding of Slit2 D4, at (from top to bottom) 1000, 750, 500, 300, 200 and 100 nM, to immobilized HS.

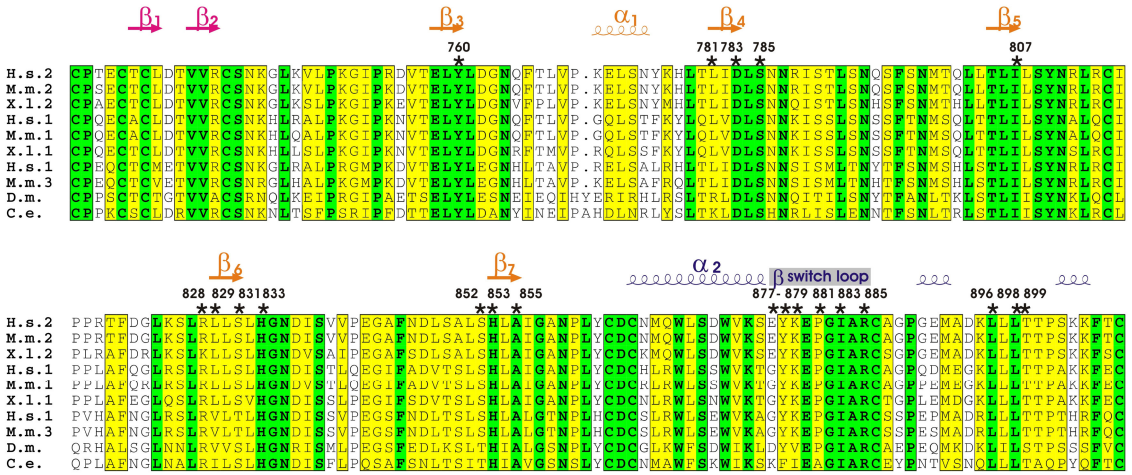

Figure S6. Slit2 D4 sequence conservation and colored as in Fig 3B. Secondary structure elements as found in hSlit2 D4 are colored as in Fig 1A and the β–switch loop are indicated. Residues involved in dimerisation are marked with a star. H.s.2: *Homo sapiens* Slit2; M.m.2: *Mus musculus* Slit2; X.l.2: *Xenopus laevis* Slit2; H.s.1: *Homo sapiens* Slit1; M.m.1: *Mus musculus* Slit1; X.l.1: *Xenopus laevis* Slit1; H.s.3: *Homo sapiens* Slit3; M.m.3: *Mus musculus* Slit3; D.m.: *Drosophila melanogaster* Slit; C.e.: *Caenorhabditis elegans* Slit.

SUPPLEMENTARY METHODS

Production and purification of human Slit2 protein constructs. DNA fragments corresponding to human Slit2 D4 (residues 726-907) and Slit2 D2-4 (residues 271-907) were produced by PCR amplification using the full-length cDNA coding for human Slit2 (NM_004787) as a template and cloned into a modified pTT3 vector as used previously (Morlot *et al.*, 2007). Slit2 D2 (residues 271-479) was produced as previously (Morlot *et al.*, 2007). For the production of Slit2 D4 and Slit2 D2-4 human embryonic kidney cells (HEK293) stably expressing Epstein-Barr virus nuclear antigen 1 (EBNA-1) were maintained in Dulbecco's Modified Eagle's Medium (DMEM high glucose, Sigma) supplemented with 4mM L-glutamine (Sigma), penicillin/streptomycin mix (Sigma), 0.05 mg/ml G418 (Sigma), 10 mM hepes pH 7.0 and 5 % foetal bovine serum (FBS) (Sigma). Before transfection, the concentration of FBS was reduced to 2 %. 3000 cm^2 adherent cells were transfected with a mixture of 500 µg DNA vector, 1000 µg polyethylenimine (PEI) and 25 ml Optimem medium (+Glutamax, Invitrogen). 1 mg/l kifunensine (Cayman Chemical) was added to the culture medium. The culture medium was harvested 3-4 days after transfection and loaded onto a Ni sepharose column (GE Healthcare). Protein bound to the resin was washed with wash buffer 1 (20 mM Tris-HCl pH 7.5, 200-500 mM NaCl, 50 mM imidazole), wash buffer 2 (20 mM Tris-HCl pH 7.5, 1 M NaCl, 20 mM imidazole), and eluted with elution buffer (20 mM Tris-HCl pH 7.5, 200-500 mM NaCl, 400 mM imidazole). To de-glycosylate the eluted fractions, these were pooled and incubated overnight at 4˚ C with GST-tagged endoglycosidase F_1 (Grueninger-Leitch *et al.*, 1996) at a ratio of 5:1 (mg:mg). The deglycosylated protein was dialysed against wash buffer 1 to reduce the imidazole concentration, loaded onto a second Ni-NTA column, washed with wash buffer 1 and wash buffer 2, and eluted with elution buffer. The eluted fractions were concentrated to 1-2 mg/ml and loaded onto a Superdex200 column (GE Healthcare). The running buffer contained 200 mM NaCl, and 20 mM Tris pH 7.5.

Production of Slit2 D4 mutants. Three Slit2 D4 mutant proteins were generated using the QuikChange kit (Stratagene, La Jolla, CA). Two multiple mutated Slit2 D4 proteins were designed to disrupt the dimer interface by introducing the following changes (Slit2 D4 mutant 1: H853A and R885E; Slit2 D4 mutant 2: Y760A, D783A and H853A). The following mutations were introduced to disrupt the Slit2 D4 heparin binding site on the bottom face

(Slit2 D4 mutant 3: R788E, R812E and R814E). These proteins were essentially produced and purified as described above.

Crystallization of human Slit2 D4 and X-ray data processing. Purified and deglycosylated Slit2 D4 was concentrated to 3.8 mg/ml. Crystals were produced in hanging drops containing 2 µl protein solution and 1 µl well solution (0.2 M $LiSO_4$, 0.1 M Tris pH 8.5, 30% Peg 4000). After equilibration at 20˚ C for 7 days, the crystallization set up was transferred to 4˚ C, and crystals were harvested after 14 days. The crystals were frozen in a cryoprotectant solution containing all well solution components and 10-15 % glycerol. Diffraction data were collected at the European Synchrotron Radiation Facility (beamline ID14-EH4) and all data were integrated in the space group $I4_1$ using XDS (Kabsch, 1993). A model derived from human Slit2 D3 (Morlot *et al.*, 2007) was used as a search model in the molecular replacement program PHASER (McCoy *et al*., 2005). The derived model phases were then used for automatic model building in ARP/wARP (Perrakis *et al*., 1999). The model was refined with Refmac (Murshudov *et al*., 1997), all manual editing was done with COOT (Emsley and Cowtan, 2004), and MOLPROBITY (Lovell *et al*., 2003) was used for model validation. The crystallographic details are summarized in Supplementary Table 1. The analysis and graphical representation was performed with ESPRIPT (Gouet *et al*., 1999), PYMOL (Delano Scientific LLC), and the CorelDRAW graphics suite.

Growth cone collapse assays and stripe assays. Retinal ganglion cells (RGCs) were dissected and cultured according to the previously described method (Walter *et al*., 1987). The retina of chicks from embryonic day 6-7 were dissected and cut into 275 µm wide strips. Two strips were cultivated overnight (at 37 ^{o}C, 4% CO_2) in F12 medium on a laminin coated coverslip. During that time, the RCGs extend axons (up to 1 mm long) tipped with growth cones. To keep the conditions for the collapse assay consistent with the experiments using heparin sulphate (see below), the medium was carefully exchanged on a heating block and cultures were incubated for a further 15 min at 37 ^{o}C. To test the effects of the Slit2 fragments, the culture medium was again replaced with medium containing the Slit2 fragments. The protein concentrations tested were (10-20 µg/ml Slit2 D2-4, 50 µg/ml Slit2 D2 and 50 µg/ml Slit2 D4) and the cultures were incubated for 30 mins at 37 ^{o}C. To test the inhibitory effect of heparan sulphate (HS) on Slit2 D2 and D4, the RCGs were first incubated for 15 min with fresh medium containing 100 µg/ml of a HS (12-mer) oligo, followed by

incubation with medium containing 100 µg/ml of a HS (12-mer) oligo and 50 µg/ml Slit2 D2 or D4 for 30 min at 37 ^{o}C. Afterwards, the cultures were fixed in 4% paraformaldehyde containing 0.1% glutaraldehyde, stained with fluorescently labeled phalloidin for actin, mounted in Mowiol and coverslipped. Since growth cones and axons are rich in actin, the phalloidin staining shows both collapsed and uncollapsed axons. The cultures were photographed and growth cone collapse is evaluated. For the statistical analysis at least three biological replicates (with 4 retinal explants) were used for each construct and the controls. We usually performed the whole set (D4, D4+HS, D2, D2+HS, HS, buffer-control) in a single experiment. As described in Figure 4B, only growth cones with several filopodia but without any lamellipodia were counted as 'bare branch' morphology. Fully collapsed growth cones are defined as having no lamellipodia and filopodia and show the typical beaded appearance of the remaining axon shaft. The statistical analyses were performed using a special chi-square test (*Brand-Snedecor* method).

For the stripe assay, the protein was not added to the culture medium after outgrowth, but rather bound in a striped pattern onto the cover slip on which the axons were cultivated. To produce a striped pattern, a silicone matrix with small channels was placed onto a coverslip and filled with protein solution. During an incubation period, protein from solution adheres to the glass surface as previously described in (Vielmetter et al., 1990). The pattern was then coated with laminin (a protein promoting axon outgrowth). To make the patterns visible, the protein of interest was mixed with a fluorescently labeled inactive protein (4 µg/ml of Fc clustered with Alexa Flour 594 anti-Fc antibody) and mixed with 10 µg/ml or 20 µg/ml Slit2 constructs in PBS. Alternatively, Fc or laminin containing strips were stained with corresponding antibodies. In the stripe assay, outgrowth axons can decide between protein coated strips and strips solely coated with laminin. The axonal avoidance reaction toward strips coated with repulsive guidance molecules was classified according to Walter *et al*., 1987.

SPR spectroscopy binding assay. Analyses were performed by using a BIAcore 3000 instrument (BIAcore AB, Uppsala, Sweden). HS was biotinylated at the reducing end and immobilized on a Biacore sensorchip. For this purpose, the flow cells of a CM4 sensorchip were functionalized with 2500 to 2800 resonance units (RU) of streptavidin and biotinylated HS (2.5 µg/ml) was injected across the flow cells for immobilization. One flow cell was left

untreated and served as a negative control. For binding assays, 250 µl of Slit2 D2, Slit2 D4 or Slit2 D4 mutant 3 were simultaneously injected over the control and the HS, at a flow rate of 50 µl/min, after which the formed complexes were washed with running buffer for 5 min. Sensorchip surfaces were regenerated with a 4 minute pulse of 2 M NaCl.

For Robo1-Slit2 D2 or D4 binding assays, analyses were performed using a BIAcore X instrument (BIAcore AB, Uppsala, Sweden). Robo1 ectodomain was immobilized (2500 RU) on the surface of a CM5 sensor chip (BIAcore) using the amine-coupling chemistry (Biacore amine coupling kit) according to the manufacturer's instructions. Binding of Slit2 D2 and Slit2 D4 were measured at a flow rate of 20 µl/min in 145 mM NaCl and 10 mM Hepes (pH 7.4), containing 0.005% surfactant P20 (BIAcore). Equivalent volumes of each protein sample were injected over a surface with no protein immobilized to serve as blank sensorgrams for subtraction of the bulk refractive index background. Regeneration of the surfaces was achieved by injections of 20 µl of 2 M NaCl.

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Supplementary Table 1. Data collection and refinement statistics

Space Group	$I4_1$
Cell Dimensions	a = b = 128.66 Å
	c = 48.15 Å
	α = β =γ = 90°
Resolution Range	30-1.8 Å
Completeness (last shell)	94.2 (88.6)
R-factor (last shell)	0.184 (0.266)
R-free (last shell)	0.219 (0.308)
RMS deviations from ideal values	
Bond distances (Å)	0.013
Angles	1.461°