## **SUPPLEMENTARY INFORMATION**

Structural basis for cell surface patterning through NetrinG-NGL interactions

Elena Seiradake, Charlotte H. Coles, Pavel Perestenko, Karl Harlos, Jeff McIlhinney, A. Radu Aricescu & E. Yvonne Jones



**Supplementary figure 1.** Family tree of Netrin and Laminin N-terminal Lam domains. Branch distances are based on sequence alignment and were calculated with programs from the PHYLIP package (Felsenstein, 1993). h=human, m=mouse, c= chicken, f= frog, z= zebrafish. The N-terminal domain of Netrin5 differs from the Lam consensus sequence and was not included in the analysis.

hNetrinG2			<u>1</u> β0	β0' 1	$\rightarrow 0 0 0 0 - \beta 1$
hNetrinG2 mNetrinG2 cNetrinG2 fNetrinG2 fNetrinG1 mNetrinG1 cNetrinG1 fNetrinG1 zNetrinG1 zNetrinG1 hNetrin1 hLamin1nB1 mLamin1nA5 mLamin1nA5	1111111111111	MLHLLAIFLHCIPLASGDY MLRLLAIFLHCIPLASGDY MLYLLAILHRIPLAMGQY MLYLAILLHCIPLALGQY MLSQALILLQCIILTLGQY MYLSRFISIHAIWVTVSSVMQPYLVWGHY MYLSRFISIHAIWVTVSSVMQPYLFVWGHY MYLSRFISIHAIWVTVSSMQHYPSVWGHY MYLFMVIYLQTIWVTVSSAMQHGHY MYLFMVIYLQTIWVTVSSAMQHGHY MHFLLAFVLQAIWVNWCHATQHYLASWGHY MMRAVWBALAAIAAVACLVGAVRGGF MGLLQLIAFSFIA MAKRGGQLCAGBAPGALGPRSPAPRPLLLI	DICKSWVTTDEG DICKSWVTTDEG DICKSWVTTDEG DICKSWVTTDEG DICKSIVTSDEG DICKSLYTEEG DVCKTQIYTEEG DVCKTQIYTEEG DVCKTQIYTEEG DVCKSQVHAEDR GLSMFAGQAAQP LCRARVRAQEP JLAGLALVGEART	PTWEFYACQPKVI PTWEFYACQPKAI PSWEFYACQPKAI PSWEFYACQPKPI PTWEYYACQPES' KVWDYMACQPES' KVWDYMACQPESI LWHYMACQPESI LWHYMACQPESI DPCSDENGHPR.I E.FSYGCAEGSC PGGDGFSLHPPYI	MRLKDYVK.VKVEPSG MRLKDYVK.VKVEPSG MRLKDYVT.VRVDPAG MIMKEYTM.VKVDPPG MSMKEYMQ.IRVEPPD TDMTKYLK.VKLDPPD TDMTKYLK.VKLDPPD IDMTKYMR.VKLDPPD IDMTQYLSMVSLDPPM RCIPDFVNAAFGKDVR YPATGDLLIGRAQKLS FNLAEGARITASA β1
		Πα			
hNetrinG2		$\beta^{1}$		<sup>3</sup> β1"2 	α1 α
hNetrinG2 mNetrinG2 CNetrinG2 fNetrinG2 hNetrinG1 cNetrinG1 cNetrinG1 zNetrinG1 zNetrinG1 hNetrinG1 hNetrinG1 mLamininB1 mLamininA5 mLamininA5	59 59 59 59 70 70 65 52 68	I. PECGDP. PERFCSHEN.   I. PECGDP. PERFCSHEN.   I. PECGNP. PERFCTHEN.   I. PECGNP. PERFCTHEN.   I. PECGDP. PERFCTHEN.   I. PECGDP. PERFCTMEN.   I. PECGDP. PERFCAMGN.   I. PECGDP. PETFCAMGN.   I. PECGDP. PENYCAMGN.   I. PECGDP. PENYCALEN.   VSTCGDP. PENYCALEN. VVSTCGRP.   VSTCGCRP. PARYCVVSERG. VTSTCGLH.   VTSTCGEAPTRSVSRPTEDLYCKLVGGPV VGPVCYNENT.	PY PY PY PY PY PY PY PY PY PY PY AGGDPNQTIQGQ	LCSNECDASNP. LCSNECDASNP. LCSDECDASNP. LCSDECDASTK. MCNNECDASTP. MCNNECDASTP. MCNNECDASTQ. MCNNECTASTQ. MCNNE	DLAEPPRLMF DLAEPPRLMF DLAEPPRLMF DLAEPPSLMF ELAEPPELMQ ELAEPPELMF ELAEPPELMF ELAEPPELMF ELAEPPELMF ELAEPPELMF ELAEPPELMF ELAEPPLMF KAEPVSMAI
hNetrinG2		$\beta 1^{m}$ $\beta 2$	— <b>→</b> ·	β3	β4 β5
hNetrinG2 mNetrinG2 cNetrinG2 fNetrinG2 fNetrinG1 mNetrinG1 cNetrinG1 fNetrinG1 fNetrinG1 hNetrinG1 hNetrinG1 hNetrinG1 hLamininB1 mLamininA5	97 97 97 108 108 108 103 109 110 102	DKEEEGLATYWOSITWSRYPSPLEANITIS DREDEGLATYWOSITWSRYPSPLEANITIS DSEDEGLATYWOSVTWSRYPSPLEANITIS DSERTGLITYWOSVTWSRYPEPLLANITIS DSERTGLITYWOSVTWSRYPEPLLANITIS DFEGRHPSTFWOSATWSPRYPEPLLANITIS DFEGRHPSTFWOSATWSPRYPEPLLANITIS DFEGRHPSTFWOSATWSPRPLQVNITIS DSERTSNPSTFWOSATWSPRPLQVNITIS DVEGRNPSTFWOSSTWSRYPRPLQVNITIS DSEGRNPTTFWOSSTWSRYPRPLQVNITIS DSEGRNPTTFWOSSTWSRYPRPLQVNITIS DSEGRNPTTFWOSSTWSRYPRPLQVNITIS DSEGRNPTTFWOSSTWSRYPRPLQVNITIS DSEGRNPTTFWOSSTWSRYPRPLQVNITIS DSEGRNPTTFWOSSTWSRYPRPLQVNITIS DSEGRNPTTFWOSSTWSRYPRPLQVNITIS DSEGRNPTTFWOSSTWSRYPRPLQVNITIS DSEGRNPTTFWOSSTWSRYPRPLQVNITIS DSEGRNPTTFWOSSTWSRYPRPLQVNITIS DSEGRNPTTFWOSSTWSRYPRPLQVNITIS DSEGRNPTTFWOSSTWSRYPRPLQVNITIS DSEGRNPTTFWOSSTWSRYPRPLQVNITIS DSEGRNPTFFWOSSTWSRYPRPLQVNITIS DSEGRNPTFFWOSSTWSRYPRPLQVNITIS DSEGRNPTFFWOSSTWSRYPRPLQVNITIS DSEGRNPTFFWOSSTWSRYPRPLQVNITIS DSEGRNPTFFWOSSTWSRYPRPLQVNITIS DSEGRNPTFFWOSSTWSRYPRPLQVNITIS DSEGRNPTFFWOSSTWSRYPRPLQVNITIS DSEGRNPTFFWOSSTWSRYPRPLQVNITIS DSEGRNPTFFWOSSTWSRYPRPLQVNITIS DSEGRNPTFFWOSSTWSRYPRPLQVNITIS	WMKSVELTDD WMKSVELTDD WMKSVELTDD WMKSLELTDD WMKIELTDD WMKTIELTDN WMKTIELTDN WMKTIELTDN UMKTIELTDN UMKTIELTDD LSLGKKFEVT.Y LDLEABFH.FTH LGQVFHVAYV	VVVTFEYGRPTVI IVITFEYGRPTI IVITFEYGRPTI IVITFEYGRPTI IVITFESGRPDQI IVITFESGRPDQI IVITFESGRPDQI IVITFESGRPE IVITFES	wvlbksldngrtwopy     wvlbksldngrtwopy     wwlbksldngrtwopy     wwlbksldngrtwopy     wilbksldngrtwopy     wilbksld
		4			<b>a</b> 3
hNetrinG2		$\rightarrow$ $\alpha^2$ $\alpha^2$ $\alpha^2$ $\alpha^2$ $\alpha^2$ $\alpha^2$ $\alpha^2$ $\alpha^2$			reviere ée
hNetrinG2 cNetrinG2 fNetrinG2 fNetrinG2 fNetrinG1 nNetrinG1 fNetrinG1 fNetrinG1 kNetrinG1 hNetrinG1 hNetrinG1 hNetrinG1 hLamininB1 mLamininA5	165 165 165 176 176 177 177 175 164 191	QFYAEDCMEAFGMSARRARDMSSSSAH QFYAEDCMEAFGMSARRARDMSPSSAH QYYADDCMEAFGMPARVVRDLSTTSAN QYYADDCMEAFGMPARVVRDLSTTSAN QYYADCCMEAFGMLAKRVQDLSATNVT QYYATDCLDAFHMDPKSVKDLSQHTVL QYYATDCLDAFHMDPKSVKDLSQHTVL QYYATDCLDAFHMDPKSVKDLSQHTVL QFYAADCLDAFHMDPKSVKDLSQHTVL QFYAADCLDAFHMPKSVKDLSHTVI QFYAADCLDAFHMPKSVKDLSHTVI QFYAADCLDAFMSVKDLTSTML QFYSTQCRKMYNRPHRAPITKQNSQ RYFAYDCEASFPGISTGPMKKVD QFFASSSKRDCLEFFGPRTLERITQDD 0000000 022	RVLCTEEYSRWA RVLCTEEYSRWA RVLCTEEYSRWA RVLCTEEYSRWA RVLCTEEYSRWA RVLCTEEYSRWA EIICTEEYSRWA DIICTEEYSTGY BIICTEEYSTGY EIICTEEYSTGY EAVCTDSHTDMR DIICTEEYSRU DOVICTEYSRU BS'	SKKEKHVRFEV GSKKEKHVRFEV GSKKEKTVRFEV GSKKEKDVRFEV GSKKEKNVRFEV GSKKEKNVRFEV GSKKEKNVRFEV GSKKEKNVRFEV GSKNEKNVFET STN.SKIIHFEI MTN.SKIIHFEI MTN.SKIIHFEI AAH.AKTLSFEI PLS.GGLIAFST PLS.GGLIAFST PLS.GGLIAFST PLS.GGLIAFST PLS.GGLIAFST FA	RDRFAIFAGPDLRNMD RDRFAIFAGPDLRNMD RDRFAIFAGPDLKNMD RDRFAIFAGPDLKNME RDRFAFFAGPRLRNMA KDRFAFFAGPRLRNMA KDRFAFFAGPRLRNMA KDRFAFFAGPRLHNMA KDRFAILAGPRLHNMA KDRFAILAGPRLHNMA KDRFAILAGPRLHNMA KDRFAILAGPRLHNMA KDRFAILAGPRLHNMA
		α3' α3'' β7	Loop II	α4	β8
nNetrinG2 hNetrinG2 cNetrinG2 cNetrinG2 zNetrinG2 zNetrinG1 mNetrinG1 cNetrinG1 fNetrinG1 fNetrinG1 fNetrinG1 hNetrinG1 hNetrinG1 hNetrinG1 hNetrinG1 hNetrinG1 hNetrinG1 hNetrinG1 hNetrinG1 hNetrinG1 hNetrinG1	232 232 232 232 242 242 237 244 237 244 229 216 246	NLYTRLESAKGLKEFFTLTDLRMRLLRPAL NLYTRMESAKGLKEFFTTTDLRMRLLRPAL NLYTRMESAKGLKEFFTTTDLRMRLLRPAL NLYTRMESAKGLKDFFTTTDLRLRLLRPAL SLYGQLDTTKKLRDFFTTTDLRIRLLRPAT SLYGQLDTTKKLRDFFTTTDLRIRLLRPAT SLYGQLDTTKKLRDFFTTTDLRIRLLRPAT SLYGQLDTTKKLRDFFTTTDLRIRLLRPAT SLYSQLDTTKKLRDFFTTTDLRIRLLRPAT SLYSQLDTTKKLRDFFTTTDLRIRLLRPAT SLYSQLDTTKKLRDFFTTTDLRIRLRPAT SLYSQLDTTKKLRDFFTTTDLRIRLRPAT SLYSQLDTTKKLRDFFTTTDLRIRLRPAT SLYSQLDTTKKLRDFFTTTDLRIRLRPAT SLYSQLDTTKKLRDFFTTTDLRIRLRPAT SLYSQLDTTKKLRDFFTTTDLRIRLRPAT SLYSQLDTTKKLRDFFTTTDLRIRLRPAT SLYSQLDTTKKLRDFFTTTDLRIRLRPAT SLYSQLDTTKKLRDFFTTTDLRIRLRPAT	GGTYVQRE GGTYVQRE GGTYVQRE GGTYVQRD GEIYVQRD GEIFVDEL GEIYVDEL GEIYVDEL GATMYDEX FGDEMEDDS LGDNLLDSR LGDNLLDSR LGDNLLDSR	VIYKYFYAIS NIYKYFYAIS NIYKYFYAS NIYKYFYASS NIXKHFYRSS NIXKYFYAIS ILARYFYAIS HLARYFYAIS NIGRYFYAIS NIGRYFYAIS NIGR.YFYASIS NIGR.YFYASIS NIGR.YYYAYS ELARDSYFYAVS MEIREKYYYAVY OCO 035	VIEVIGR VIEVIGR VIEVTGR CLAVSCR VIDIPAR DIKVRGR DIKVRGR DIKVQGR DIKVQGR DIKVQGR DISIGGR

**Supplementary figure 2.** Sequence alignment of NetrinG and laminin N-terminal Lam domains. Secondary structure elements found in the human NetrinG2 and mouse LamininA5 (Hussain et al, 2011) structures are indicated above and below the alignment, respectively. Cysteine pairs forming disulfide bridges in human NetrinG2 are colored red and paired by numbers above the alignment. A predicted N-linked glycosylation site, which was visible in the NetrinG and LamininA5 structures, is marked with a black star. Asparagine residues (N) predicted to carry glycans are outlined with a purple box (Gupta et al, 2004, in preparation). Residues contacting NGL in the complex crystal structures are highlighted by a coloured background box: blue for loop I, yellow for loop II and orange for loop III. h=human, m=mouse, c= chicken, f= frog, z= zebrafish.



**Supplementary figure 3.** The N-terminal lam-domains of NetrinG2, Laminin A5 and HSPB11 share a similar beta-sandwich core (Ramelot et al, 2009). N-linked sugars are shown as red spheres and include a glycan on  $\beta$ 2, predicted to be conserved among netrins and laminins. A structural calcium ion conserved in HSPB11 and NetrinG2 is depicted in brown. (A) Ribbon diagram of unliganded human NetrinG2<sub>Lam-EGF1</sub>. Loop II is disordered in apo NetrinG2. Its position when NetrinG2 is in complex with NGL2 is indicated with a dotted yellow line. (B) Ribbon diagram of mouse Laminin A5 (Hussain et al, 2011). (C) Ribbon diagram of human HSPB11 (Ramelot et al, 2009).



**Supplementary figure 4.** Variation in the relative orientations of the NGL and Lingo1 LRR and Ig domains. (A) NGL1<sub>LRR-Ig</sub> (cyan), NGL2<sub>LRR-Ig</sub> (green) and NGL3<sub>LRR-Ig</sub> (orange) were superposed via the LRR domains. (B) NGL1<sub>LRR-Ig</sub> (cyan) and Lingo1<sub>LRR-Ig</sub> (grey, (Mosyak et al, 2006)) were superposed via the LRR domains.



**Supplementary figure 5**. Multiple angle light scattering results suggest NetrinG<sub>Lam-EGF</sub> and NGL<sub>LRR-Ig</sub> are monomers in solution and form 1:1 complexes. (A) UV traces (relative response units) and molar masses (kDa) are shown as thin and thick lines, respectively, for unliganded NetrinG<sub>Lam-EGF</sub> and NGL<sub>LRR-Ig</sub>. The purified proteins contain glycans added by HEK293T cells (for NGL3<sub>LRR-Ig</sub>) or GnTI-deficient HEK293S cells (Aricescu et al, 2006; Chang et al, 2007) (for NetrinG1/2 <sub>Lam-EGF</sub>, NGL1/2<sub>LRR-Ig</sub>). The measured masses are in general agreement with masses predicted based on the primary sequence plus 1.34 kDa mass per potential N-linked glycosylation site for protein produced in GnTI-deficient HEK293S cells, or 2.06 kDa per site for protein produced in HEK293T cells. Thus the predicted molecular masses for fully glycosylated proteins (protein mass + glycan mass) are NetrinG1<sub>Lam-EGF</sub> = 46.3+2.7 kDa, NetrinG2<sub>Lam-EGF</sub> = 37.8+4.0 kDa, NGL1<sub>LRR-Ig</sub> = 49.3+8.0 kDa, NGL2<sub>LRR-Ig</sub> = 48.7+6.7 kDa, NGL3<sub>LRR-Ig</sub> = 42.2+16.5 kDa. (B) UV traces and molar masses (in kDa) are shown for NetrinG<sub>Lam-EGF</sub> =  $NGL_{LRR-Ig}$  complexes.



**Supplementary figure 6.** NGL-NetrinG intra-class binding is >100 times stronger than cross-class binding. Sensograms and fitted data (1:1 Langmuir binding model) are presented for binding of wild type NGL1<sub>LRR-Ig</sub> and NGL2<sub>LRR-Ig</sub> to NetrinG1<sub>Lam-EGF1/4</sub> and NetrinG2<sub>Lam-EGF1</sub> constructs. Using a shorter version of NetrinG1, which lacks EGF4 and therefore is equivalent to NetrinG2 Lam-EGF1 in its domain composition, resulted in the same NGL1- and NGL2 binding affinities as measured for NetrinG1<sub>lam-</sub> EGF1/4, demonstrating that EGF4 does not affect binding (unpublished observation). Depending on the experimental set-up, previous measurements of NetrinG-NGL affinities had shown some variation in the calculated  $K_d$  values. In a solid-phase binding assay (Lin et al, 2003), performed prior to the discovery of the NetrinG2-NGL2 interaction, binding of soluble NGL1 to microtitre wells coated with NetrinG1 was measured as giving a  $K_d$ = 1.6 nM. Similarly, in an ELISA assay using immobilized NetrinG2, an affinity of 2.5 nM was reported for the NetrinG2-NGL2 interaction (Zhang et al, 2008) and no affinity was detected between immobilized NetrinG1 and NGL2. In an SPR assay, where the ligands were directly immobilized on the sensor chip, the affinity measured for the NetrinG1-NGL1 interaction was K<sub>d</sub>= 155 nM, and K<sub>d</sub>= 377 nM for the NetrinG2-NGL2 interaction (Nishimura-Akiyoshi et al, 2007). Again, cross-class interactions were not detected, presumably in this case due to the overall lower level of binding measured in the alternative experimental conditions chosen (Nishimura-Akiyoshi et al, 2007).

hard 1							β1 β2
hNGL1	1	MINEME LUDOOTMICH		ג ו דייע אייד אייר א	T. OF		
mNGL1	i	.MLNKMT.LHPQQIMIGP	FNRALF	DPLLVVLLA.	LQ	LVVAGLVRAQT	CPSVCSCSNQFSKV
CNGL1	1	.MLNKMT.LHPQQIMIGP	FNRALF	DPLLVVLLA	LQ	LUVAGLVRAQT	CPSVCSCSNQFSKV
INGLI ZNGL1	1	.MINKMTSSLMOOTMRGP	RWNRALS	DPLFVLSLA.	LQ	LUVAGLVRAQT	CPSVCSCSNOFSKV
hNGL3	1	MARAR.GSPCPPLPPG	RMSWPHG	ALLFLWLFSPI	LGAGGGGVAVT	<b>S</b> A <b>A</b> GGGSPPATS	CPVACSCSNQASRV
mNGL3	1	MAQAHIR.GSPCPLLPPG	RMSWPHG	ALLLWLFSPI	LRAGGGGVAVT	SAAGGGSPPATS	CPAACSCSNQASRV CPAACTCSNQASRV
ZNGL3	i	MRIT.TVTSLPSPSP	LLLLVQ	LLLRLLPG.	Q	GANGAAST	CPAVCSCSNQASRV
hNGL2	1	MKLLWQVTVHHHTWNA	LLPFVY	LTAQVWILC	A	IAAAASAGPON	CPSVCSCSNQFSKV
fNGL2	1	. MNFLWLVTVHH. TWKA	LFSAIY	LMVHMWISC.	A	NIMAAASAGPON	CPSVCSCSNOFSKV CPSVCSCSNOFSKV
zNGL2	1	MSLLWQVTVHR.AWNA	LLCAVY	LMVRSWSVS	A	PSGQLT	CPSVCFCSNVSNKV
							Сар
			β3	β4	β5	β6 α1	β7
<b>bNGL1</b>		2	<b>→</b>	. →		· - 200	₽. <b>→</b> .
hNGL1	61	ICVRENLREVPDGISTNTI	RLLNLHB	NQIQIIKVNSE	KHLRHLEILQL	SRNHIRTIBIGA	FNGLANLNTLELFD
CNGL1	61	ICVRKNLRDVPDGISTNT	RLLNLHE	NOIGIIKVNSE	KHLRHLBILOL	SRNHIRTIEIGA	FNGLANLNTLELFD
fNGL1	61	ICTRRNLREVPDGISTNTI	RQLNLHE	NQIQIIKVDSF	KHLRHLEVLQL	SRNHIRTIEIGA	FNGLANLNTLELFD
ZNGL1 hNGL3	62 71	ICTRRGLRDVPDGISTNTI ICTRRDI.ARVPASIPVNTI	RYLNLOB	NGIOVIKVDSE	KHLRHLBILQL KHLRHLBILOL	SKNHIRNIBIGA Sknivrkirvga	FNGLANLNTLELFD
mNGL3	73	ICTRRELABVPASIPVNT	RYLNLQE	NSIQVIRTOTE	KHLRHLBILQL	KNLVRKIEVGA	FNGLPSLNTLELFD
fNGL3	56	ACTRRELMEVPESISVNTI	RYLNLQE	NITOVIKTOTE	KHLRHLEILQL	SKNLIRKIEVGA	FNGLPNLSTLELFD
hNGL2	60	VCTRRGLSEVPOGIPSNTI	RYLNLME	NNIQMIQADTE	RHLHHLEVLQL	GRNSIRQIEVGA	FNGLASLNTLELFD
mNGL2	59	VCTRRGLSEVPQGIPSNT	RYLNLME	NNIQMIQADTE	RHLHHLEVLQL	GRNSIRQIEVGA	FNGLASLNTLELFD
INGL2	54	VCTRRGLSEVPQGIPSNT	RHLNLME	NSIBTIBAGTE	OHLRHLEVLOL	GRNSIRQIEVGA	FSGLNSLNTLELFD
				Irr1		Irr2	
			*				
			68	89	<b>B10</b>	<b>B</b> 11	<b>B12</b>
hNGL1					· · ·	<b>→</b>	
hNGL1	134	NRLTTIPNGAFVYLSKLK	LWLRNN	PIESIPSYAF	RIPSLRRLDLG	LKRLSYISEGAL	FEGLSNLRYLNLAM
mNGL1	134	NRLTTIPNGAFVYLSKLKI	LWLRNN	PIESIPSYAFS	RIPSLRRLDLG	ELKRLSYISEGAL	FEGLSNLRYLNLAM
fNGL1	134	NRLTTIPNGAFEYLSKLK	LWLRNN	PIESIPSYAF	RIPSLRRLDLG	EMERLSYISEGA	FEGLSNLKYLNLGM
ZNGL1	135	NRLTTIPNGAFEYLSKLKI	LWLRNN	PIESIPSYAFS	RVPSLRRLDLG	<b>LKRLSYISE</b> GAI	FEGLSNLRYLNLGM
mNGL3	144	NRLTTVPTOAFEYLSKLRI	SLWLRNN BLWLRNN	PIESIPSYAFE	IRVPSLRRLDLG	ELKRLEYISEAA)	FEGLUNLRYLNLGM
fNGL3	129	NRLTTVPTQAFEYLSKLRI	BLWLRNN	PIESIPSYAF	RVPSLRRLDLG	ELKKLEYISEAAI	FEGLVNLRYLNLGM
ZNGL3 hNGL2	127	NRLTLVPSQAFEYLSKLRI NWI.TVIPSGAFEYI.SKI.R	ELWLRNN ELWLRNN	PIETLPGYAFE	IRVPSLRRLDLG	ELKKLDYISDAA) ELKKLEYISEGAI	FVGLINLRYLNLGM FRGLENLKYLNLGM
mNGL2	132	NWLTVIPSGAFEYLSKLR	LWLRNN	PIESIPSYAF	RVPSLMRLDLG	LKKLEYISEGA	FEGLFNLKYLNLGM
fNGL2	127	NWLTVIPSGAFEYLSKLRI MDLTVIPSGAFEYLSKLRI	ELWLRNN ELWLRSN	PIESIPSYAFN	RVPSLMRLDLG	ELKKLEYISEGA	FEGLYNLKYLNLGM
ANGUA		Irr3		Irr4		Irr5	- BODIN DAT DA DOR
		813		<b>B14</b>	B15	<b>B16</b>	817
hNGL1			•	<b>→</b>	→.	-	
hNGL1	207	CNLREIPNLTPLIKLDEL	LSGNHL	SAIRPGSFQGI	MHLQKLWMIQS	DIQVIERNAFON	LQSLVEINLAHNNL
mNGL1	207	CNLREIPNLTPLIKLDEL	LSGNHL	SAIRPGSFQGI	MHLOKLWMIQS	DIQVIERNAFON	LOSLVEINLAHNNL
fNGL1	207	CNLRBIPNLTPLVKLDEL	DLSGNHL	SVLRPGSFOGI	THLOKLWMIQS	DIOVIERNAFD	LOSLVEINLAHNNL
zNGL1	208	CNLEEIPNLIPLVRLDELI	BMSGNQL	SIIRPGSFŘGI	VHLOKLWMMHA	<b>DIQTIERNAFD</b> D	LÕSLVELNLAHNNL
mNGL3	217	CNLKDIPNLTALVRLEELI CNLKDIPNLTALVRLEELI	ELSGNRL BLSGNRL	DLIRPGSFQGI	TSLRKLWLMHA	OVATIERNAFOD OVATIERNAFOD	LKSLEELNLSHNNL LKSLEELNLSHNNL
fNGL3	202	CNLEDIPNLTALVRLEEL	LSGNRL	EMIRPGSFQGI	TSLRKLWLMHA	<b>IVTIIERNAFD</b> D	LKSLEELNLSHNNL
ZNGL3	200	COLKDIPNLTPLVRLEEL	ELSGNRL MSGNHF	EIIRPGSFQGI	ESLRKLWLMHS	OMSVIERNAFDD	LKNLEELNLSHNSL
mNGL2	205	CNIEDMPNLTPLVGLEBL	BMSGNHF	PEIRPGSFHGI	SSLKKLWVMNS	VSLIERNAFDG	LASLVELNLAHNNL
fNGL2	200	CNIRDMPNLTPLVGLEEL	BISGNNF	PEIKPGSFHGI	RSLKKLWIMNS	QINTIERNAFDD	LTSLVELNLAHNNV
ZNGLZ	200	Irr6	SROAD IF	Irr7	T SUKKUWIMNS	Irr8	VIALVELALARAAL
							<b>A</b>
		818	819	α2		820	
hNGL1					200		
hNGL1	280	TLLPHDLFTPLHHLERIH	HHNPWN	CNCDILWLSWW	IKDMAPSNTAC	ARCNTPPNLKG	RYIGELDONYFTC
mNGL1	280	TLLPHDLFTPLHHLERIH	HHNPWN	CNCDILWLSWW	IRDMAPSNTAC	CARCNTPPNLKG	RYIGELDQNYFTC
ENGL1	280	TLLPHDLFTPLR.LERIH	LHHNPWN	CNCDILWLSWW	LKBIVTTGSTC	CARCSTPPSLKG	KIIGBIDLNYFTC THIABIDHNYFTC
zNGL1	281	TLLPHDLFTPLHHLERVH	HHNPWN	CNCDILWLSWW	LKEMVPANTSC	CARCSSPTSEKG	RYIGELDQNYFHC
hNGL3	290	MSLPHDLFTPLHRLERVH	LNHNPWH	CNCDVLWLSWW	LKETVPSNTTC	CARCHAPAGLKG	RYIGELDQSHFTC RYIGELDQSHFTC
fNGL3	275	MSLPHDLFTPLHRLERVHI	LNHNPWH	CNCDVLWLSW	LKETVPNNTTC	CARCHSPPNLKMI	RYIGELDQSHFTC
ZNGL3	273	HSLPHDLFTPLQKLERVHI	NHNPWV	CNCDVLWLSWW	LKETVPSNTTC	CARCHAPPYLKG	KYIGELDOSHFTC
mNGL2	278	SSLPHDLFTPLRYLVELH	LHHNPWN	CDCDILWLAWW	LREYIPTNSTC	CGRCHAPMHMRGI	RYLVEVDOAAFOC
fNGL2	273	TSLPHDLFAPLKYLVELH	HHNPWD	CDCDVLWLSWW	LREYIPTNSTC	GRCHSPPHMRG	ĸyvvevdhsmfqc
zNGL2	273	SSLPHDLFAPLSYLVELH	LHHNPWR	CDCDVVWLAWW	LREYIPTNSTC	CGRCHTPAYLRGI	RYLVEVDQSTFQC

**Supplementary figure 7.** Sequence alignment of NGL LRR domains. Secondary structure elements found in human NGL1 are indicated above the alignment, cysteine pairs forming disulfide bridges are in red and numbered above the alignment. Asparagine residues (N) predicted to carry glycans are highlighted with purple boxes (Gupta et al, 2004, in preparation). A black star indicates the position of NGL3 Q96, a residue important in the interaction with its cognate receptor, the RPTP LAR (Kwon et al, 2010); a black triangle marks a predicted N-linked glycosylation site in the concave face of NGL3 LRR. Residues contacting NetrinG loops I-III in the complex crystal structures are highlighted by a coloured background box: blue for loop I, yellow for loop II, orange for loop III, aquamarine for loops I+III, green for loops I+III, pink for loops II+III. h=human, m=mouse, c= chicken, f= frog, z= zebrafish.





Supplementary figure 8. NGL-NetrinG interaction specificity depends on distinctive binding surfaces. (A) Affinities of NGL1 (blue) and NGL2 (red) binding to immobilized NetrinG constructs were normalized using the  $K_d$  value measured for intra-class binding to the corresponding wild type NetrinG (WT). Affinities that were too low to measure using the chosen method are indicated by an asterisk (blue or red). We created NetrinG knock out mutants (KO) by introducing an N-linked glycosylation site in loop I designed to block the interaction (NetrinG1 P85A+Y86T, NetrinG2 P74A+Y75T, NGL1 A205T, NGL2 G204T). To test the contribution of NetrinG loops I-III on NetrinG-NGL binding specificity, we transplanted individual loops from NetrinG1 into NetrinG2, and, vice versa, exchanged individual NetrinG1 loops with the equivalent sequence found in NetrinG2. The data reveal that swapping loop I decreases the affinity for intra-class and cross-class binding, thereby suggesting that loop I depends on its native protein context for efficient binding to NGL. Intriguingly, swapping NetrinG1 loop II to that of NetrinG2 leads to a small increase of affinity to NGL1, and loss of affinity to NGL2. The reverse swap in NetrinG2 also increases NGL1-binding and reduces the affinity to NGL2, overall equalizing the affinity to the two NGLs. Swapping NetrinG1 loop III to that of NetrinG2 leads to an increase (~10-fold) in affinity to both NGL1 and NGL2, while the reverse swap in NetrinG2 abolishes binding to either. Taken together, these results imply that the extra interactions provided by NetrinG2 loop III are beneficial for binding either NGL receptor, regardless of the NetrinG context. Swapping NetrinG2 loop III in combination with loop II, or all three loops at once, creates proteins that still binds weakly to NGL1, but not to NGL2, and thus has a switched binding preference compared to the native protein. However, the corresponding NetrinG1 mutants did not result in switched specificity. Overall, the data suggest that NetrinG loops do not function as standalone elements and that transplanting them into a non-native protein context can affect their NGLbinding properties. (B) Fitted SPR data (1:1 Langmuir binding model) are presented for all mutant NGL<sub>LRR-Ig</sub> and NetrinG<sub>Lam-EGF</sub> constructs. Black asterisks mark mutations that lead to the introduction of an N-linked glycosylation site within the NetrinG-NGL binding interface (=KO mutants). A hash (#) marks K<sub>d</sub> values that were too low to determine accurately using this method.

## REFERENCES

Aricescu AR, Lu W, Jones EY (2006) A time- and cost-efficient system for high-level protein production in mammalian cells. *Acta Crystallogr D Biol Crystallogr* **62**: 1243-1250

Chang VT, Crispin M, Aricescu AR, Harvey DJ, Nettleship JE, Fennelly JA, Yu C, Boles KS, Evans EJ, Stuart DI, Dwek RA, Jones EY, Owens RJ, Davis SJ (2007) Glycoprotein structural genomics: solving the glycosylation problem. *Structure* **15**: 267-273

Felsenstein J. (1993) PHYLIP (Phylogeny Inference Package) version 3.5c.

Gupta R, Jung E, Brunak S (2004, in preparation) Prediction of N-glycosylation sites in human proteins.

Hussain SA, Carafoli F, Hohenester E (2011) Determinants of laminin polymerization revealed by the structure of the alpha5 chain amino-terminal region. *EMBO Rep* **11**: 11

Kwon SK, Woo J, Kim SY, Kim H, Kim E (2010) Trans-synaptic adhesions between netrin-G ligand-3 (NGL-3) and receptor tyrosine phosphatases LAR, protein-tyrosine phosphatase delta (PTPdelta), and PTPsigma via specific domains regulate excitatory synapse formation. *J Biol Chem* **285**: 13966-13978.

Lin JC, Ho WH, Gurney A, Rosenthal A (2003) The netrin-G1 ligand NGL-1 promotes the outgrowth of thalamocortical axons. *Nat Neurosci* **6**: 1270-1276.

Mosyak L, Wood A, Dwyer B, Buddha M, Johnson M, Aulabaugh A, Zhong X, Presman E, Benard S, Kelleher K, Wilhelm J, Stahl ML, Kriz R, Gao Y, Cao Z, Ling HP, Pangalos MN, Walsh FS, Somers WS (2006) The structure of the Lingo-1 ectodomain, a module implicated in central nervous system repair inhibition. *J Biol Chem* **281**: 36378-36390.

Nishimura-Akiyoshi S, Niimi K, Nakashiba T, Itohara S (2007) Axonal netrin-Gs transneuronally determine lamina-specific subdendritic segments. *Proc Natl Acad Sci U S A* **104**: 14801-14806.

Ramelot TA, Raman S, Kuzin AP, Xiao R, Ma LC, Acton TB, Hunt JF, Montelione GT, Baker D, Kennedy MA (2009) Improving NMR protein structure quality by Rosetta refinement: a molecular replacement study. *Proteins* **75**: 147-167.

Zhang W, Rajan I, Savelieva KV, Wang CY, Vogel P, Kelly M, Xu N, Hasson B, Jarman W, Lanthorn TH (2008) Netrin-G2 and netrin-G2 ligand are both required for normal auditory responsiveness. *Genes Brain Behav* **7**: 385-392.