Structural basis of semaphorin–plexin cis interaction

Daniel Rozbesky¹, Marieke G Verhagen², Dimple Karia¹, Gergely N Nagy¹, Luis Alvarez³, Ross A Robinson¹, Karl Harlos¹,§, Sergi Padilla-Parra¹,³,§, R Jeroen Pasterkamp²,* and Edith Yvonne Jones¹,±,§

Abstract

Semaphorin ligands interact with plexin receptors to contribute to functions in the development of myriad tissues including neurite guidance and synaptic organisation within the nervous system. Cell-attached semaphorins interact in trans with plexins on opposing cells, but also in cis on the same cell. The interplay between trans and cis interactions is crucial for the regulated development of complex neural circuitry, but the underlying molecular mechanisms are uncharacterised. We have discovered a distinct mode of interaction through which the Drosophila semaphorin Sema2b and mouse Sema6A mediate binding in cis to their cognate plexin receptors. Our high-resolution structural, biophysical and in vitro analyses demonstrate that monomeric semaphorins can mediate a distinctive plexin binding mode. These findings suggest the interplay between monomeric vs dimeric states has a hereto unappreciated role in semaphorin biology, providing a mechanism by which Sema6s may balance cis and trans functionalities.

Keywords axon guidance; cis interaction; plexin; semaphorin; semaphorin signalling

Subject Categories Neuroscience; Structural Biology

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Introduction

Semaphorins and plexins are one of the classical cell guidance ligand-receptor families first characterised by their ability to steer axon growth cones in the developing nervous system (Kolodkin et al, 1992, 1993; Luo et al, 1993; Tamagnone et al, 1999). Beyond axon guidance, semaphorin–plexin signalling is implicated in a plethora of physiological functions including other aspects of neural development, angiogenesis, vascularisation, organogenesis and regulation of immune responses (Tran et al, 2007; Pasterkamp, 2012; Takamatsu & Kumanogoh, 2012). Conversely, deregulation of semaphorin–plexin signalling is associated with tumour progression and other diseases (Tamagnone, 2012). Exquisite control of the local level and biological consequence of signalling is characteristic of the semaphorin–plexin system and essential for many of its functions.

Semaphorins are secreted, transmembrane or GPI-anchored proteins (Kolodkin et al, 1993). Membrane-attached semaphorins and plexins commonly function through cell-to-cell trans interactions in which the semaphorin ligands and plexin receptors are presented on opposing cells. However, when ligand and receptor are present on the same cell surface there is potential for ligand-receptor binding in cis at the same plasma membrane. An increasing body of evidence points to the importance of cis interactions in the regulation of diverse cell guidance signalling systems (Seiradake et al, 2016). In the semaphorin–plexin signalling system, cis interactions were first described between class 6 semaphorins (Sema6s) and their cognate plexin class A (PlxnA) receptors. Studies in migrating granule cells suggest that binding of Sema6A and PlxnA2 in cis inhibits the binding of PlxnA2 by Sema6A in trans as the absence of Sema6A in cis causes over-activation of PlxnA2 (Renaud et al, 2008). The cis interaction of Sema6A–PlxnA2 has been further reported to be essential for proper development of lamina-restricted projection of hippocampal mossy fibres (Suto et al, 2007; Tawarayama et al, 2010). Finally, the inhibitory effect of cis interaction has been demonstrated between Sema6A and PlxnA4 (Haklai-Topper et al, 2010), and Sema6B and PlxnA2 (Andermatt et al, 2014). Contrary to these inhibition effects, the cis interaction between semaphorin SMP-1 and the PlxnA4 homolog, PLX-1, in C. elegans has been shown to result in plexin activation (Mizumoto & Shen, 2013). Similarly, mouse Sema5A signals through PlxnA2 co-expressed on hippocampal dentate granule cells to regulate synaptogenesis (Duan
et al., 2014). Perhaps the most exquisite interplay of semaphorin–plexin cis and trans interactions reported to date is that of Sema6A and PlexA2 in the elaboration of dendritic arbors during retinal circuit assembly (Sun et al., 2013). Intriguingly, it has been suggested that the cis and trans interaction modes of semaphorins andplexins require distinct binding sites (Haklai-Topper et al., 2010; Perez-Branguli et al., 2016).

The first crystal structures of semaphorins revealed that the hallmark N-terminal sema domain is a seven-bladed β-propeller with a propensity to dimerise (Antipenko et al., 2003; Love et al., 2003) and the homodimeric architecture has long been reported as essential for semaphorins to function as repulsive guidance cues (Klostermann et al., 1998; Koppel & Raper, 1998). In a recent study, we have demonstrated that semaphorins can also form heterodimers and monomers, and thus, their architecture is not restricted to homodimers (Rozbesky et al., 2019). Plexins are type I transmembrane proteins containing an N-terminal sema domain followed by multiple PSI and IPT domains in their extracellular segment (Bork et al., 1999; Tamagnone et al., 1999). The plexin intracellular region has a distinctive GAP domain architecture (He et al., 2009; Tong et al., 2009; Bell et al., 2011; Wang et al., 2013), which structural and functional studies suggest is activated by dimerisation (He et al., 2009; Tong et al., 2009; Wang et al., 2012, 2013). Recent crystal structures of full-length mouse PlexA ectodomains (comprising ten domains) revealed a ring-like overall shape, which is presumably orientated parallel to the plane of the plasma membrane at the cell surface (Kong et al., 2016). The ring-like structure is consistent with an observed PlexA-to-PlexA “head-to-stalk” cis interaction being able to maintain pre-ligand bound plexins in a clustered, but autoinhibited, state on the cell surface, presumably by favouring separation, and thus preventing spontaneous dimerisation, of the transmembrane and intracellular regions (Kong et al., 2016). The existence of inactive dimers of pre-ligand bound plexin is further supported by data from fluorescence cross-correlation spectroscopy experiments on mouse PlexA4 (Marita et al., 2015). Crystal structures have been reported for complexes formed between semaphorin ectodomains and fragments comprising up to four of the N-terminal domains of the cognate plexin ectodomain. These semaphorin–plexin complexes all show a bivalent 2:2 architecture that comprises a semaphorin dimer interacting with two copies of the plexin consistent with receptor activation by ligand-mediated dimerisation, a conclusion supported by structure-guided biophysical and cell-based assays (Janssen et al., 2010; Liu et al., 2010; Nogi et al., 2010). In all semaphorin–plexin complexes analysed to date, the semaphorins and plexins bind in a head-to-head (semaphorin sema domain-to-plexin sema domain) orientation suitable for a trans interaction between ligands and receptors attached to opposing cell surfaces triggering receptor activation (Kong et al., 2016). No molecular interaction surfaces have been characterised in terms of their ability to mediate semaphorin–plexin binding modes in cis; thus, the structural basis and molecular mechanism(s) governing the divergent outcomes of cis and trans binding remain elusive.

The ectodomain of Sema6A forms a weak dimer with monomeric and dimeric forms present in solution (Janssen et al., 2010; Nogi et al., 2010). The interplay of monomeric and dimeric Sema6A at the plasma membrane is likely relevant to cis interactions with the cognate PlexA receptors. Structural and biophysical analyses at high concentrations have provided detailed insight into the interaction of dimeric Sema6A with PlexA2; however, because of the monomer–dimer equilibrium, the binding properties of wild-type monomeric Sema6A have eluded direct analysis. In structural and biophysical studies of the Drosophila semaphorin system, we recently discovered a wild-type monomeric semaphorin, Sema1b (Rozbesky et al., 2019). This unexpected discovery provided us with a system in which we could dissect the interaction surfaces, and contributions to plexin binding in cis, of a semaphorin that is purely in the monomeric state. The class 1 (Sema1a and Sema1b) and class 2 (Sema2a and Sema2b) Drosophila semaphorins are membrane-attached and secreted, respectively. Sema1a and Sema1b are most closely related to the mammalian class 6 semaphorins and interact with the sole Drosophila class A plexin, PlexA (Pasterkamp, 2012). In previous studies, we have shown that the secreted Drosophila semaphorins, Sema2a and Sema2b, and also the ectodomain of membrane-attached Sema1becto are disulphide-linked dimers. All three of these semaphorins contain an intermolecular sema-to-sema disulphide bridge. Conversely, we found the ectodomain of membrane-attached Sema1becto to be a monomer in solution due to an amino acid substitution in the intermolecular disulphide bridge at position 254 (Rozbesky et al., 2019). Here, we show that Drosophila Sema1b is a monomer on the cell surface and can interact in cis with PlexA. We further report two crystal structures of Sema1b complexed with the semaphorin-binding region of PlexA. The crystal structures, along with biophysical and cell-based assays, show that monomeric Sema1b binds PlexA at two independent binding sites. One interaction mode corresponds to the canonical head-to-head orientation described previously for semaphorin–plexin binding. The second mode uses an interactive surface on Sema1b that is occluded in dimeric semaphorins. We were able to demonstrate that this novel “side-on” binding mode perturbs the ring-like structure of the PlexA ectodomain. In cell collapse assays, we found that the side-on mode of monomeric Sema1b–PlexA binding in cis was sufficient to inhibit PlexA signalling by dimeric Sema1a binding in trans. In dorsal root ganglion neurons, we also confirmed that mouse Sema6A utilises the same molecular mechanism for cis interaction with its cognate plexin receptor as its Drosophila homolog, Sema1b. Based on our findings, we propose models for semaphorin–plexin cis interactions which incorporate a distinctive role for monomeric semaphorin binding in the regulation of plexin signalling.

Results

Sema1b is a monomer on the cell surface and fails to mediate PlexA dimerisation

We considered the oligomeric state of Sema1b on the membrane of live cells. COS-7 cells were transiently transfected with Sema1b–F254C-mClover (a mutant which provides Sema1a-like disulphide-linked dimer formation) or with the wild-type Sema1b-mClover. Both constructs encompassed the ectodomain followed by a native transmembrane segment, short cytoplasmic linker and the C-terminal fluorescent protein mClover. mClover is a monomeric bright yellow-green fluorescent protein commonly used for the analysis of dimerisation or protein–protein interactions in live cells (Lam et al., 2012). Using Number and Brightness analysis, we determined a molecular brightness (c) in live cells, which is directly related to the
olfomeric state. Number and Brightness analysis is a fluorescence fluctuation spectroscopy technique to measure the average number and oligomeric state of labelled entities in each pixel of a stack of fluorescently labelled images (Digman et al., 2008). We have recently developed the method further by implementing a novel detrending algorithm to detect monomers and dimers in live cells (Nolan et al., 2017, 2018a; Lioiopoulos et al., 2018b) or in vitro (Nolan et al., 2018b). Here, we calculated the molecular brightness of Sema1b-F254C-mClover to be double that of the molecular brightness of Sema1b-mClover consistent with Sema1b-mClover molecules being present on the membrane of COS-7 cells as monomers (Fig EV1A and B).

Our previous studies have shown that although monomeric, Sema1b ecto maintains PlexA binding in the nanomolar range (Rozbesky et al., 2019). To investigate whether Sema1b ecto dimers or clusters of PlexA on live cell surfaces, we probed the molecular brightness of PlexA-mClover on the membrane of COS-7 cells before and after stimulation with purified wild-type Sema1b ecto or the disulphide-linked dimer Sema1b ecto-F254C. The PlexA-mClover construct contained the ectodomain followed by a transmembrane segment and the C-terminal fluorescent protein mClover. The addition of Sema1b ecto-F254C resulted in a significant 3.0 ± 1.8 fold increase of the average molecular brightness which is likely related to a change of the PlexA-mClover oligomeric state. Conversely, the addition of wild-type Sema1b ecto, at the same concentration had no noticeable effect on the average molecular brightness (1.2 ± 0.7 fold increase) (Fig EV1C and D). Thus, though Sema1b binds PlexA in the nanomolar range, it fails to mediate PlexA dimerisation on the cell surface, presumably due to its monomeric state.

A novel binding mode revealed by the crystal structure of the PlexA-Sema1b complex

We next determined crystal structures of the PlexA1-4-Sema1b1-2 complex from two different crystal forms (1:1 complex and 2:2 complex) at 3.0 and 4.8 Å resolution (Fig 1A–C, Table 1). The 1:1 complex crystal lattice contains one PlexA1-4 monomer and one Sema1b1-2 monomer per asymmetric unit. The crystal packing provides no Sema1b1-2 dimerisation resembling that of the generic homodimeric architecture. The Sema1b1-2 bound in the 1:1 complex with PlexA1-4 is very similar to the unbound Sema1b1-2 with the Cα rmsd of 0.81 Å indicating no large conformational changes upon complex formation. Only small differences in loop orientations at the ligand–receptor interface are apparent. The ectodomain of Drosophila PlexA has not been structurally characterised previously. The PlexA1-4 structure in the 1:1 complex contains a sema domain composed of a seven-bladed β-propeller fold, which is followed by a PSI domain; however, we were not able to locate the IPT1-PSI2 domain segment. The sema domain of PlexA is most similar to mouse PlxnA2 with an rmsd of 1.43 Å over 424 matched Cα positions. In the 1:1 complex crystal structure, PlexA1-4 and Sema1b1-2 interact through their sema domains in a head-to-head orientation similar to the generic architecture shared by all reported structures of semaphorin–plexin complexes (Janssen et al., 2010; Liu et al., 2010; Nogi et al., 2010). The PlexA1-4-Sema1b1-2 interface buries a total solvent-accessible area of 1,837 Å2. This extensive interface is composed of a mixture of hydrophobic and hydrophilic interactions similar to that of PlxnA2-Sema6A.

In the crystal of 2:2 complex, two pairs of 1:1 complexes are packed together in the asymmetric unit with a relative orientation of 168.8° to form a pseudo tetramer. Each Sema1b molecule binds to both PlexA molecules in the pseudo tetramer. There are therefore two independent interaction sites, A and B (Fig 1C), involving two different Sema1b-PlexA orientations. The first, head-to-head orientation is equivalent to that observed in the 1:1 complex (interaction site A; Fig 1B). In the second interaction mode, termed side-on (Fig 1D), Sema1b1-2 and PlexA1-4 bind through the site B or B’ with their carboxy-terminal PSI1 domains oriented in parallel. The B and B’ binding sites are not identical within the pseudo tetramer. While the B interaction site is extensive, the B’ interaction site is formed through distant contacts between three residues only. Although the two PlexA molecules in the pseudo tetramer form a substantial interface, we did not observe any propensity for PlexA1-4 to dimerise in solution to a concentration at least of 33 μM (Fig EV1E and F).

The structure of individual Sema1b1-2 and PlexA1-4 molecules in the 2:2 complex is very similar to those observed in the 1:1 complex, showing no significant conformational changes with the exception of the Exβ1-β2 loop in the extrusion of Sema1b1-2, which adopts a different orientation in order to avoid steric clashes with PlexA1-4. Unfortunately, we were not able to model the Exβ1-β2 loop completely because of fragmentary electron density; however, the loop’s position is consistent with it making interactions to the PlexA1-4. As well as undergoing this large-scale reorientation to accommodate PlexA1-4, the Exβ1-β2 loop may also stabilise the complex.

In the side-on orientation, the sema domains of Sema1b1-2 and PlexA1-4 are bound in a configuration in which the bottom face of PlexA1-4 is oriented perpendicularly to the side edge of Sema1b1-2 (Fig 1D). The position of the B binding site between Sema1b and PlexA in the 2:2 complex is different to the binding site of the coreceptor neuropilin in the previously reported mouse Sema3A-PlexinA2-Nrp1 ternary complex (Janssen et al., 2012); however, they are positioned in very close proximity (Fig EV2A–C). Interface B can be divided into three main binding sites (Fig EV1G). The most prominent, site 1, is formed by the extrusion of Sema1b and blade 6 of PlexA. Site 2 is composed of the β4B-β4C loop of Sema1b and the β4D-β5A loop of PlexA. In site 3, the N-linked glycan at residue N289 of Sema1b forms contacts with the PSI1 domain of PlexA. The Exβ1-β2 and β4B-β4C loops are involved in semaphorin homodimerisation (Siebold & Jones, 2013); however, for Sema1b1-2 in the 2:2 complex these loops mediate interaction with PlexA suggesting that a B-type interaction can only be mediated by a monomeric semaphorin molecule (Fig EV1H).

To confirm both interaction interfaces observed in the 2:2 complex, we produced three mutants of Sema1b ecto, termed A, B or A+B, and analysed plexin binding using microscale thermophoresis (MST). In Sema1b-mutaB, we mutated interface residues F203E, Q219R and K223E in order to disrupt the head-to-head interaction at site A. The single point mutations were designed to introduce electrostatic repulsions or reduction of surface hydrophobicity. Given the low resolution of the 2:2 complex and consequent lack of detailed information on residue-to-residue interactions, we decided to test the side-on interface by replacing each residue in the Exβ1-β2 loop by alanine rather than simple point mutations. Sema1b mutA+B combined the modifications to potentially abolish both head-to-head and side-on interactions. All three Sema1b mutants were expressed and secreted at similar levels to the Sema1b wild-
type. Furthermore, wild-type and all mutants were eluted at the same time from the size-exclusion column suggesting there is no problem with folding. We found using MST that both Sema1b-mutA and Sema1b-mutB maintained PlexA1-4 binding while Sema1b-mutA+B did not provide any measurable indication of PlexA 1-4 binding at concentrations up to 66.3 μM (Figs 1E and EV2D–L). These data indicate that in solution Sema1b ecto can interact with PlexA 1-4 using either the head-to-head (site A) or side-on (site B) binding modes. We further assessed the stoichiometry of interaction between Sema1b1-2 and PlexA1-4 in solution using SEC-MALS. The unliganded Sema1b1-2 or PlexA1-4 eluted as a single peak corresponding to monomer (Fig EV2M and N); no propensity to dimerise was observed. SEC-MALS analysis of a sample containing an equimolar mixture of Sema1b1-2 and PlexA1-4 revealed three peaks corresponding to unliganded Sema1b1-2 and PlexA1-4 and a Sema1b1-2-PlexA1-4 complex in 1:1 stoichiometry (Fig EV2O).

**Sema1b binds PlexA in cis**

We then investigated whether Sema1b binds PlexA in cis on live cell surfaces by FRET-FLIM (Padilla-Parra et al, 2008; Padilla-Parra & Tramier, 2012; Kong et al, 2016). COS-7 cells were transiently co-transfected with PlexA-mClover and Sema1b-mRuby2. For cells co-expressing PlexA-mClover and Sema1b-mRuby2, we observed an average lifetime ($t_{av}$) of $2.51 \pm 0.06$ ns, while $t_{av}$ of $2.72 \pm 0.03$ ns was detected for cells expressing PlexA-mClover alone (Figs 2A and...
The average fraction of the interacting donor (f_D) for cells co-expressing PlexA-mClover and Sema1b-mutA-mRuby2 was 0.26 ± 0.09 (Fig EV3C). This result clearly demonstrates that Sema1b indeed binds PlexA in cis.

Based on the architecture of our 2:2 complex, the cis interaction may be mediated by the head-to-head or side-on orientation between PlexA and Sema1b. Thus, to elucidate the structural basis for the PlexA-Sema1b cis interaction, we measured τ_av for FRET-FLIM between PlexA-mClover and Sema1b-mRuby2 mutants, (site A, B and A+B mutants as described in the previous section). In order to assay the level of surface expression of the wild-type and mutant proteins, we measured fluorescence intensity on the membrane of COS-7 cells by TIRF microscopy. We observed comparable intensities for all constructs (Fig EV3D and E) indicating that Sema1b wild-type and all three mutants are expressed at similar levels on the cell surface. As a negative control, we used COS-7 cells co-expressing PlexA-mClover and Sema1b-mutA-B-mRuby2 because Sema1b-muta-B abolished both the head-to-head and side-on interactions in MST. These cells showed a τ_av of 2.65 ± 0.03 ns, which is similar to the lifetime of donor alone with τ_av of 2.72 ± 0.03 ns indicating no or very low FRET. A statistically significant (calculated by ANOVA test) shortening of the average lifetime due to FRET was observed for cells co-expressing PlexA-mClover and Sema1b-mutA-mRuby2 (τ_av = 2.43 ± 0.10 ns) as well as for cells co-expressing PlexA-mClover and Sema1b-mutB-mRuby2 (τ_av = 2.53 ± 0.04 ns) indicating that both head-to-head and side-on orientations can mediate cis interactions (Fig 2A). The side-on orientation appears to be more populated than the head-to-head as the average fraction of the interacting donor for cells co-expressing PlexA-mClover and Sema1b-mutB-mRuby2 (f_D = 0.37 ± 0.15) is higher than for cells co-expressing PlexA-mClover and Sema1b-mutB-mRuby2 (f_D = 0.21 ± 0.07) (Fig EV3C). Intriguingly, FRET-FLIM data indicate that the side-on orientation dominates over the canonical head-to-head orientation in the Sema1b-PlexA cis interaction on the cell surface. This observation appears to be counterintuitive to the structural data suggesting the side-on interface is weaker than the head-to-head interface. One possible explanation might be that partial deglycosylation of Sema1b2 weakened the interaction mediated by the N-linked glycan at residue N289 of Sema1b in the 2:2 complex.

We then interrogated whether cis interaction occurs between PlexA and dimeric Sema1a, which has also been shown to bind PlexA (Winberg et al, 1998). Cells co-expressing PlexA-mClover and Sema1a-mRuby2 showed τ_av of 2.70 ± 0.03 ns, which is similar to the lifetime of donor alone indicating no cis interaction (Fig 2A). Sema1a is tethered, from PSI domain to the membrane, by a linker that is some 35 residues shorter than in Sema1b, which is possibly insufficient to allow site A (head-to-head) mediated binding in cis. The lack of the PlexA-Sema1a cis interaction through an interaction site B on Sema1a is consistent with the inaccessibility of the sema domain loop Exβ1–β2 in dimeric semaphorins. Indeed, in all previously reported semaphorin crystal structures apart from Sema7A and the viral semaphorin A39R, the Exβ1–β2 loop is involved in the homodimerisation and is therefore not accessible for the side-on interaction with plexin (Fig EV3F). Thus, semaphorin–plexin binding through site B appears to be a unique feature of monomeric semaphorin molecules.

### PlexA ectodomain architecture and interactions

How does site B mediated interaction sit in the context of the structure and binding characteristics of full-length PlexA ectodomains? Recently, it has been shown that the ectodomains of human and mouse PlxnAs can adopt two distinct conformations: a preferred ring-like conformation and a less frequent chair-like conformation. In the ring-like conformation, the ectodomain forms a ring, which is nearly or fully closed by an intramolecular head-to-tail interaction (Kong et al, 2016; Suzuki et al, 2016). In light of the mammalian PlxnA ring-like conformation, we set out to assess the possible conformations of the "Drosophila" PlexA full ectodomain (PlexA_{ecto}) using negative stain EM. In the micrographs, PlexA_{ecto} was monomeric. Single particle 2D class averages showed PlexA in the

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### Table 1. Data collection and refinement statistics.

|                        | PlexA_{α+}•Sema1b_{β,2:2} complex | PlexA_{α+}•Sema1b_{β2:2} complex |
|------------------------|------------------------------------|----------------------------------|
| **Data collection**    |                                    |                                  |
| Space group            | C 2 2 2, 2                       | P 6_3 , 2 2                     |
| Cell dimensions        |                                    |                                  |
| a, b, c (Å)            | 130.9, 195.1, 124.8               | 153.6, 153.6, 425.4             |
| α, β, γ (°)            | 90, 90, 90                       | 90, 90, 120                     |
| Resolution (Å)         | 76.86–2.96 (3.07–2.96)            | 127–4.80 (4.97–4.80)            |
| Unique reflections     | 31,584 (2,791)                    | 15309 (1,485)                   |
| Multiplicity           | 4.6 (2.2)                         | 20.9 (19.8)                     |
| Completeness (%)       | 93.78 (83.79)                     | 99.90 (100.00)                  |
| l/σ(l)                 | 8.86 (1.75)                       | 7.41 (1.19)                     |
| Wilson B-factor (Å²)   | 69.89                              | 217.35                          |
| R-merge (%)            | 17.4 (63.4)                       | 33.9 (224.1)                    |
| CC1/2                  | 0.99 (0.548)                      | 0.998 (0.492)                   |
| CC²                    | 0.997 (0.841)                     | 1 (0.812)                       |
| **Refinement**         |                                    |                                  |
| Resolution (Å)         | 76.86–2.96 (3.06–2.96)            | 127–4.80 (4.97–4.80)            |
| Reflections used in    | 31,582                            | 15303                           |
| refinement             |                                    |                                  |
| Rwork/Rfree (%)        | 18.62/24.63                       | 28.66/30.68                     |
| Number of atoms        | 8,119                             | 15,728                          |
| Protein                | 7,941                             | 15,350                          |
| Ligands                | 178                               | 378                             |
| B-factor (Å²)          |                                    |                                  |
| Protein                | 73.66                             | 260.8                           |
| Ligand                 | 129.39                            | 297.52                          |
| R.m.s. deviations      |                                    |                                  |
| Bond lengths (Å)       | 0.005                             | 0.01                            |
| Bond angles (°)        | 0.76                              | 1.43                            |
| Ramachandran           |                                    |                                  |
| Favoured (%)           | 95.28                             | 95.31                           |
| Allowed (%)            | 4.72                              | 4.69                            |
| Outliers (%)           | 0                                  | 0                               |

*Highest resolution shell is shown in parenthesis.*
ring-like conformation with overall shapes ranging from the nearly closed to the predominant, fully closed ring, which matches the mouse PlxnA ectodomain crystal structures and major 2D class averages (Figs 2B and EV3G and H).

The intermolecular head-to-stalk interaction previously reported to occur for mouse PlxnA ectodomains involves sema domain and domain 4 and 5 residues that are conserved across the A class plexins in vertebrates (Kong et al., 2016). This conservation extends to Drosophila PlexA. We therefore used analytical ultracentrifugation sedimentation velocity experiments to examine whether Drosophila PlexAecto forms a dimer in solution. As seen in the equivalent experiments for mouse PlxnA1 (Kong et al., 2016),

Figure 2. Binding of Sema1b to PlexA in cis perturbs the PlexA ring-like conformation.
A FRET-FLIM in live COS-7 cells shows cell surface PlexA-Sema1b cis interaction. Sema1b mutants reveal that both head-to-head and side-on orientations of the PlexA-Sema1b complex are involved in the cis interaction. The cis interaction between Sema1a and PlexA is not observed. The boxcharts represent the average lifetime. Box limits indicate the 25th and 75th percentiles, centred lines show the median, squares represent sample means, whiskers extend 1.5-fold the interquartile range from the 25th and 75th percentiles, and P-value was calculated by one-way analysis of variance (ANOVA). *P < 0.05, ***P < 0.001.  
B Representative negative stain class averages of PlexAecto correlate well with the ring-like conformation of mouse PlxnA1. The ring-like conformation of mouse PlxnA1 is shown in surface representation rainbow colour ramped from blue (N-terminus) to red (C-terminus). Scale bar, 10 nm. All class averages of PlexAecto are shown in Fig EV3H. 
C Superposition of the side-on orientation derived from the 2.2 complex with the mouse PlexinA1 (pdb 5L56) ring structure (green) based on the Drosophila and mouse PlexinA1 sema domains. 
D Design of PlexAecto-FRET Biosensor for monitoring conformational changes. In the ring-like conformation, the fluorescent protein mClover and mRuby2 at N- and C- termini, respectively, are in close proximity generating FRET. Opening the ring-like conformation results in a FRET decrease. 
E In vitro FRET-FLIM measurement of the PlexA-FRET Biosensor shows that the PlexA ectodomain undergoes a conformational change upon treatment with Sema1b resulting in more open conformation. The boxcharts represent the average lifetime. Box limits indicate the 25th and 75th percentiles, centred lines show the median, squares represent sample means, whiskers extend 1.5-fold the interquartile range from the 25th and 75th percentiles, P-value was calculated by one-way analysis of variance (ANOVA). ***P < 0.001. 
F Relative change of COS-7 cells surface area upon treatment with Sema1a-Fc. Cells expressing PlexAFL-mClover or PlexAFL-mCover and Sema1b-mRuby2 wild-type or mutants were treated with purified Sema1a-Fc at a final concentration of 5.8 μM. Images were acquired every minute for 30 min. Cell surface area was calculated using ImageJ before and after stimulation. Data are presented as means ± sem. *P < 0.05, ***P < 0.001, P-value calculated by one-way analysis of variance (ANOVA).
exists in a heterogeneous mixture encompassing monomer up to tetramer (Fig EV3I). Since *Drosophila* PlexA1-4 is a monomer in solution (Fig EV1E and F), these data suggest that, similar to mammalian class A plexins, PlexAeclo can form an intermolecular head-to-tail interaction on the cell membrane to provide pre-ligand bound autoinhibition.

**PlexA-Sema1b cis interaction leads to opening of the PlexA ring-like conformation**

In the mouse PlxnA11-10 ring-like conformation, the ring is closed by the intramolecular head-to-tail interaction between the sema domain (domain 1) and IPT5 domain (domain 9) (Kong et al., 2016). Structural superposition of the mouse PlxnA11-10 ring-like crystal structure and *Drosophila* PlexA1-4 in the 2:2 complex (Fig 2C) revealed that the position of the head-to-tail interaction site in the mouse PlxnA11-10 is very close to that used for side-on interaction in the 2:2 complex between PlexA and Sema1b. Sema1b-PlexA interaction through site B binding appears sterically incompatible with the PlexA ectodomain maintaining a fully closed ring-like conformation. This observation suggests that a side-on interaction with monomeric semaphorin in cis may provide a mechanism for opening the plexin ectodomain ring.

We hypothesised that Sema1b and the IPT5 domain of PlexA compete for PlexA sema domain binding. Thus interaction between PlexA and Sema1b in cis might move the IPT5 domain out to open the ring and make the binding site on the PlexA sema domain accessible for the side-on interaction with Sema1b. To test our hypothesis, we constructed a PlexA-FRET-Biosensor (PlexA-FB) containing the PlexA1-10 ectodomain fused with the fluorescent proteins mClover and mRuby2 at N- and C-termini, respectively. In PlexA-FB, ring opening of the PlexA1-10 ectodomain would lead to a change of FRET efficiency between donor and acceptor (Fig 2D). Apart from PlexA-FB, we expressed and purified PlexA-mClover and tandem mClover-mRuby2 protein as control samples. In *vitro* measurement of PlexA-mClover by FRET-FLIM revealed the average lifetime ($\tau_{av}$) of donor alone of 3.01 ± 0.02 ns. For the PlexA-FB, we observed a $\tau_{av}$ of 2.68 ± 0.01 ns indicating FRET consistent with mClover and mRuby2 being held in close proximity by the PlexA ring-like conformation (Fig 2E). The calculated apparent interfluorophore distance in the PlexA-FB was 80.8 Å assuming random interfluorophore orientation. Addition of Sema1beto led to an increase of $\tau_{av}$ to 2.83 ± 0.01 ns; however, this effect was not observed when using the Sema1beto-mutantB that is unable to bind PlexA by side-on interaction. The increase of the average lifetime upon Sema1beto treatment is likely a result of lower FRET efficiency because, on average, the distance between the mClover and mRuby2 has lengthened. Assuming random interfluorophore orientation, the calculated apparent interfluorophore distance in the PlexA-FB was 90.2 Å upon Sema1beto treatment. These data are consistent with Sema1b side-on binding to PlexA perturbing the ring-like PlexA ectodomain to a more open conformation.

**PlexA-Sema1b cis interaction prevents cis-engaged PlexA from interacting with Sema1a in trans**

*Drosophila* Sema1a has been shown to bind PlexA and their interaction in *trans* has been reported to be crucial for controlling axon guidance (Winberg *et al.*, 1998). Therefore, we next investigated whether the PlexA-Sema1b cis interaction can serve as a competitive inhibitor for Sema1a binding in *trans*. We used a COS-7 cell-based assay as a heterologous system that can mimic growth cone collapse (Turner & Hall, 2006). COS-7 cells transiently expressing full-length PlexA1-4, mClover and Sema1b-mRuby2, indicating that dimeric Sema1a-Fc binding to PlexA in *trans* is blocked by the monovalent PlexA-Sema1b cis interaction. We also examined the ability of Sema1b mutants to inhibit collapse. Incubation with Sema1a-Fc did not significantly alter the morphology of COS-7 cells that co-expressed PlexA1-4, mClover and Sema1b-mutA-mRuby2 or Sema1b-mutB-mRuby2. Conversely, co-expression of PlexA1-4, mClover and Sema1b-mutA+B-mRuby2 resulted in COS-7 cells showing collapse on incubation with Sema1a-Fc similar to that observed for COS-7 cells expressing PlexA1-4, mClover alone. Taken together, these results show that the interaction of monomeric Sema1b with PlexA in cis can inhibit dimeric Sema1a signalling through PlexA in *trans*. Furthermore, our data support a model in which monovalent Sema1b-PlexA cis interaction can be mediated by two distinct binding sites, A and B, using head-to-head or side-on orientations, respectively.

**Mouse Sema6A and *Drosophila* Sema1b utilise the same head-to-head and side-on binding modes to interact with plexins in cis**

We next addressed whether this model was of relevance to vertebrate semaphorin function. Based on our results for Sema1b, we designed three mutants of mouse Sema6A, termed A, B or A+B, and analysed their PlxnA2 binding using FRET-FLIM. Instead of point mutations, we introduced N-linked glycosylation sites to potentially abolish both head-to-head and side-on interaction through site B. Sema6A-muta revealed a lifetime statistically similar to the lifetime site Sema6A H212N in order to disrupt the head-to-head interaction at site A. In Sema6A-mutB, we designed the N-linked glycosylation site Sema6A E345N, K347T to target the putative side-on interaction through site B. Sema6A-muta+B combined both N-linked glycosylation sites to potentially abolish both head-to-head and side-on interactions.

For FRET-FLIM, we used mouse PlxnA2 fused with mClover while Sema6A wild-type or mutants were fused with mRuby2. We observed statistically significant (calculated by ANOVA test) shortening of the average lifetime due to FRET for cells co-expressing mouse PlxnA2 and Sema6A wild-type or Sema6A-muta or Sema6A-mutB (Fig EV4A and B). Cells co-expressing mouse PlxnA2 and Sema6A-muta+B revealed a lifetime statistically similar to the lifetime of donor alone. Sema6A and its three mutants showed similar fluorescence intensities as measured in TIRF microscopy (Fig EV4C and D) confirming that they had comparable expression levels on the surface of live COS7 cells. Consistent with our findings for *Drosophila* Sema1b, these data suggest that mouse Sema6A can bind PlxnA2 in cis using both head-to-head and side-on interaction. For mouse PlxnA2 and Sema6A, we did not observe such dramatic changes in the average lifetime as those observed for their *Drosophila* counterparts, presumably because Sema6A potentially exists
on the cell surface as a mixture of monomers and non-covalent dimers while Sema1b is present exclusively as the monomer.

**Cis interaction between mouse Sema6A and PlxnA4 serves as an inhibitory mechanism to signalling in trans**

We further investigated whether mouse Sema6A and *Drosophila* Sema1b utilise the same molecular mechanism to inhibit plexin function using a growth cone collapse assay. Here, we used dorsal root ganglion (DRG) neurons, which have been previously reported to endogenously express Sema6A and PlxnA4 (Suto et al., 2005; Haklai-Topper et al., 2010). Further, binding of Sema6A to PlxnA4 in cis has been shown to inhibit growth cone collapse induced by Sema6A presented in trans in DRG neurons (Haklai-Topper et al., 2010). We transfected cultured DRG neurons from *Sema6A* knockout embryos with Sema6A-EGFP wild-type or Sema6A-EGFP mutants (mutA, mutB or mutA+mutB) or EGFP alone. Three days post-transfection, DRG neurons were treated with purified Sema6A-Fc, and growth cone collapse was scaled from one (uncollapsed) to eight (fully collapsed) using a previously established growth cone morphology matrix (Fig 3A and B) (van Erp et al., 2015; Kong et al., 2016).

In a pilot study, GFP control transfection and treatment with different concentrations of Sema6A-Fc were tested. Following treatment with 75 nM purified Sema6A-Fc, we observed a robust growth cone collapse for cells expressing EGFP, as compared to a low level of collapse in control-treated growth cones (0 nM; Fig EV5). In contrast, Sema6A knockout cells transfected with a Sema6A wild-type construct were unresponsive to addition of purified Sema6A-Fc (at 1, 10 and 75 nM) indicating that interaction of Sema6A with its PlxnA4 receptor in cis serves as a competitive inhibitor for Sema6A-Fc binding in trans. A similar effect was observed for cells expressing Sema6A-mutA or Sema6A-mutB suggesting that both mutants maintained PlxnA4 binding in cis using the side-on or head-to-head sites, respectively. Conversely, cells expressing Sema6A-mutA+mutB showed modest (10 nM) or robust (75 nM) growth cone collapse similar to that observed for cells expressing EGFP alone (as shown for 75 nM). On basis of these data, a larger number of growth cones was analysed in 3 independent experiments that used 75 nM Sema6A-Fc (Fig 3A and B). This analysis confirmed the pilot data showing strong Sema6A-Fc-induced growth cone collapse in EGFP-transfected Sema6A knockout neurons, a rescue effect by transfection of Sema6A wild-type, and a failure to rescue by Sema6A-mutA+mutB. These findings are in agreement with the Sema1b cell-based assay. Overall, these results support our model that Sema6A-PlxnA4 cis interaction can be mediated by two distinct binding sites and also demonstrate that Sema6A-PlxnA4 cis interaction directly inhibits the plexin receptor’s ability to respond to ligand binding in trans.

**Discussion**

In the immune system, cis interactions provide a mechanism to fine tune the level of signalling at which a biological response is triggered (Held & Mariuzza, 2008). Similarly, cis interactions have been proposed to act as threshold-generating mechanisms for semaphorin-plexin, ephrin–Eph and Notch-Delta signalling during the development of the nervous system (Yaron & Sprinzak, 2012). For semaphorin–plexin signalling, there is particular abundance of evidence for cis interactions between vertebrate class 6 semaphorins and their plexin A receptors. Inhibitory cis interactions between Sema6s and PlxnAs have been reported to modulate repulsive cell guidance signalling in a number of neuronal cell types: dorsal root ganglion neurons, spinal cord, starburst amacrine cells in the retina and granule cell axons (mossy fibres) in the hippocampus (Suto et al., 2007; Renaud et al., 2008; Haklai-Topper et al., 2010; Tawaraya et al., 2010; Sun et al., 2013; Andermatt et al., 2014). In these examples, either cis interaction with a semaphorin ligand directly inhibits the plexin receptor’s ability to respond to ligand binding in trans or plexin binding in cis sequesters the semaphorin ligand so that it cannot interact with a plexin receptor in trans. Both these scenarios require that the cis interaction between ligand and receptor does not activate the receptor. This poses a conundrum; how does the inhibitory interaction between ligand and receptor in cis differ from the activating interaction in trans? To date, research into the molecular mechanism underlying plexin activation by semaphorin binding in trans has highlighted the role of the dimeric ligand in cross-linking two receptors (Siebold & Jones, 2013; Jones, 2015). In this paper, we present structural, biophysical and cell-based analyses that delineate the distinctive properties of monomeric semaphorins interacting in cis with their plexin receptors. Our data suggest a mechanism by which cell-attached semaphorin molecules in the monomeric state can contribute to the inhibition of trans interactions through a side-on interaction in cis. This side-on interaction requires the semaphorin to be in the monomeric state, causes conformational change in the ectodomain of the plexin receptor and, independent of the well-established head-to-head semaphorin–plexin binding mode, can inhibit the activation of plexin receptor by dimeric semaphorin. Below we discuss these data.
Figure 3.

A

Growth cone morphology

B

GFPLI Photostability

Intensity

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and based on them propose molecular mechanisms that can provide distinct functional outcomes for cis and trans binding between a semaphorin ligand and plexin receptor.

Monomeric semaphorins with distinctive side-on binding properties

We showed that Drosophila Sema1b exists on the cell surface in a monomeric state. Whereas Drosophila Sema1a, Sema2a and Sema2b can be locked into a dimeric state through formation of a sema-to-sema domain disulphide bond, Sema1b lacks this bond (Rozbesky et al., 2019). Some, but not all, of the mammalian semaphorins can form inter-chain disulphide bonds at various points in their ectodomains, however, we are not aware of any wild-type semaphorin that lacks a measureable propensity for sema-to-sema domain dimerisation other than Sema1b. In particular, the ectodomains of Sema6s, the vertebrate homologues of the class 1 semaphorins, do not form covalently stabilised dimers, and we and others have reported secreted forms of Sema6A to be in monomeric–dimeric equilibrium (Janssen et al., 2010; Nogi et al., 2010).

We analysed the atomic level determinants of Sema1b-PlexA binding in crystal structures of the complex to address whether the monomeric state of Sema1b per se provides this semaphorin ligand with distinctive properties promoting cis interaction. In addition to the head-to-head interaction, which for dimeric semaphorin binding has been shown to mediate receptor activation in trans (Janssen et al., 2010; Liu et al., 2010; Nogi et al., 2010), our X-ray crystallographic analysis revealed a putative side-on binding mode. This side-on binding mode only becomes possible if a semaphorin is in the monomeric state, because the interaction site is otherwise occluded by the dimer interface. Notably, all previously reported crystal structures of semaphorin–plexin complexes have involved semaphorin dimers, presumably because, even when a monomer-dimer equilibrium is present in solution, crystallisation favours the dimeric state. We were able to use structure-guided mutants of Sema1b and Sema6A in FRET–FLIM experiments to reveal that cis interactions between the semaphorins and their cognate plexins can be mediated by either the, more favoured, side-on orientation or, less frequently, the head-to-head orientation. These findings were supported by the results from collapse assays, which confirmed that both interaction modes were inhibitory. Thus, monomeric Sema1b and Sema6A can utilise a side-on mode of interaction to inhibit plexin activation. Interestingly, side-on cis interaction with plexin could also serve to sequester semaphorin from monomer-dimer equilibrium into an “inert” monomeric state, inhibiting dimeric engagement and activation of plexin in trans. This mechanism would be consistent with the observation that interaction of PlxnA2 and Sema6A in cis functions to inhibit activation of PlxnA4 in trans (Suto et al., 2007). In previous work, ectopic expression of Sema1b in muscle subsets revealed that Sema1b can act as a repulsive guidance cue (Winberg et al., 1998). Although elegant, a caveat of this study is that for this Sema1b gain-of-function experiment, Sema1b was expressed at high levels. It is possible that such high expression may lead to Sema1b dimerisation or multimerisation and thereby allow this semaphorin to act as a ligand. Evidence to support the idea that Sema1b acts as a repellent under physiological conditions is currently lacking.

Mechanisms for inhibition in cis resulting from the monomeric side-on interaction

Previous studies on PlxnA4-Sema6A (Haklai-Topper et al., 2010) and PlxnA2-Sema6A (Perez-Branguli et al., 2016) have suggested that cis and trans interactions are mediated by two distinct modes. In Fig 4, we propose models for the molecular mechanisms underlying inhibitory semaphorin–plexin cis interactions that are consistent with the data we report in this paper.

First, we consider the likely conformation and interactive state of the PlexA in isolation at the cell surface. Our results show that, similar to PlxnA ectodomains (Kong et al., 2016), the PlexA ectodomain forms a ring-like structure and exists as a heterogeneous mixture of oligomeric states encompassing monomer up to tetramer in solution. As the PlexA ectodomain has high sequence similarity to the mammalian PlxnA ectodomains and shares the same structural and biophysical characteristics, it appears plausible that the PlexA ectodomain can also maintain a level of pre-ligand bound autoinhibition using the intermolecular head-to-stalk interaction reported for the PlxnAs (Kong et al., 2016). Bivalent ligand binding in trans
This altered PlexA ectodomain conformation may itself prevent activation by head-to-head interaction forces the ring-like structure of PlexA through the side-on interaction mode. It is possible that PlexA by head-to-head interaction could presumably serve to activate the plexin receptor. If a dimeric semaphorin is able to make mode A interactions with both its sema domains, this form of cis interaction could presumably serve to activate the plexin receptor, consistent with the reports of cis activation for the semaphorin SMP-1 and plexin PLX-1 in C. elegans (Mizumoto & Shen, 2013) and for mouse Sema5A signalling through PlxnA2 co-expressed on hippocampal dentate granule cells (Duan et al., 2014).

Mode B is the previously unobserved side-on interaction mode revealed in our studies of Sema1b. Interestingly, FRET-FLIM measurements suggest that this side-on interaction mode is more populated than the head-to-head cis interaction of mode A. The side-on interaction does not directly involve the head-to-head ligand binding site on PlexA, but it is inhibitory in cell collapse assays. We propose that side-on binding by monomeric semaphorin prevents formation of the 2:2 arrangement of semaphorin dimer complexed with two ring-like plexin ectodomains required to trigger a repulsive signal (Fig 4C). One subunit of the dimeric Sema1a could form a 2:1 complex with PlexA by head-to-head trans binding, but side-on Sema1b binding in cis would sterically hinder the engagement of a second PlexA in the orientation seen for canonical 2:2 complexes. Furthermore, our PlexA-FRET biosensor data suggest that cis binding of monomeric Sema1b to PlexA through the side-on interaction forces the ring-like structure of the PlexA ectodomain into more open conformation. It is possible that this altered PlexA ectodomain conformation may itself prevent activation of signalling for cell collapse.

Our studies on Sema1b have provided the first insights into the cis binding modes of monomeric semaphorins. Furthermore, our biophysical and cellular assays have demonstrated that Sema6A utilises the same molecular mechanism to occlude plexin function. The molecular mechanisms for semaphorin–plexin cis interaction we propose here suggest that the balance between monomeric and dimeric states is central to the biological functions of the Sema6s. Further investigation will be required to tease out how the interplay between monomeric and dimeric states of Sema6 ligands, expression levels of Sema6s and their PlxnA receptors, and cis and trans binding affinities in the context of cell membranes, combine to set signalling thresholds.

Materials and Methods

Protein production

Constructs encoding Drosophila melanogaster Sema1b_ecto, Sema1b1-2, PlexA1-4 and PlexA_ecto (residues 37–659, 37–602, 28–730 and 28–1,272, respectively) were cloned into pHLsec vector (Aricescu et al., 2006) in-frame with a C-terminal hexahistidine (His6) tag. A human IgG1 hinge and Fe-fusion construct of Sema1a (residues 21–602) or mouse Sema6A (residues 19–571) was constructed using pHL-FChi vector (Aricescu et al., 2006). For MST experiments, PlexA1-4 was cloned in frame with a C-terminal monovenus (mVenus) followed by a C-terminal (His6) tag in pHLsec (Aricescu et al., 2006).

For crystallisation experiments, proteins were produced by transient transfection in HEK 293T cells (ATCC CRL-3216) in the presence of the α-mannosidase inhibitor kifunensine (Chang et al., 2007). For all other experiments, proteins were produced in HEK 293T cells without kifunensine. Five days post-transfection, the conditioned medium was collected and buffer exchanged using a QuixStand diafiltration system (GE Healthcare) and subjected to immobilised metal-affinity chromatography using a HisTrap FF column (GE Healthcare) and further purified by size-exclusion chromatography using a Superdex 200 16/60 column (GE Healthcare).

To investigate conformational changes of PlexA ectodomain, we produced a PlexA-FRET-Biosensor (PlexA-FB). First, based on pHlsec vector, we developed a novel pHlsec-FRET-Biosensor vector (pHLsec-FB), in which inserts can be cloned between Agel and KpnI sites allowing the protein to be secreted with the fluorescent proteins mClover and mRuby2, at the N-terminus and the C-terminus, respectively, followed by the C-terminal octahistidine tag. We produced PlexA-FB, PlexA-mClover and tandem mClover-mRuby2 by transient transfection in HEK 293T cells as described above. PlexA-mClover and tandem mClover-mRuby2 were used as control samples. For PlexA-FB, a construct encoding Drosophila PlexA1-10 (residues 67–1,274) was cloned into pHLsec-FB. For the tandem mClover-mRuby2, an insert containing GGGGGA sequence encoding Gly-Gly was cloned into pHLsec-FB. For PlexA-mClover, a construct encoding PlexA1-10 (residues 67–1,274) was C-terminally tagged with mClover followed by the C-terminal His6-tag.

Site-directed mutagenesis

Site-directed mutagenesis of Sema1b was carried out by overlap-extension PCR and the resulting PCR products were cloned into the pHlsec vector as described above. In Sema1b-mutant A (mutA), we mutated interface residues F203E, Q219R and K223E. For Sema1b-mutant B (mutB), in which the loop 350–367 was replaced with 18 alanine residues, a synthetic clone was commercially synthesised (Invitrogen-GeneArt). Sema1b mutant A+B (mutA+B) combined the previous modifications. All mutant Sema1b proteins were secreted at similar levels to the wild-type protein.

Crystallisation and data collection

Proteins for crystallisation were prepared in a buffer consisting of 15 mM Tris-HCl (pH 8.0) and 50 mM NaCl. For the Sema1b1-2–PlexA1-4 complex crystallisation, proteins were mixed at a 1:1 molar ratio and concentrated to 7.6 mg/ml. Prior to complex formation, Sema1b1-2 was treated with endoglycosidase F1 (1:100 w/w) for 1 h at 37°C, whereas PlexA1-4 was not deglycosylated. Sitting drop vapour diffusion crystallisation trials were set up using a Cartesian Technologies pipetting robot and consisted of 100 nl protein solution and 100 nl reservoir solution (Walter et al., 2005). Plates were maintained at 20°C in a Formulatrix storage

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and imaging system. The Sema1b1–PlexA1–4 complex crystallised in two different crystal forms. One crystal form, with space group C2221, was grown in 0.1 M HEPES (pH 7.0) and 8% (w/v) PEG 8000, the other crystal form, with space group P6522, crystallised in 0.1 M MES (pH 6.5) and 12% (w/v) PEG 20,000. Crystals were cryoprotected by soaking in reservoir solution supplemented with 25% (v/v) glycerol and then flash-cooled in liquid nitrogen. Diffraction data were collected at 100 K at Diamond Light Source beamlines I03 and I24 and indexed, integrated and scaled using the automated XIA2 expert system (Winter, 2010). For PlexA1–4–Sema1b1–2 1:1 complex, due to radiation sensitivity, diffraction data from two separate isomorphic crystals were merged together to increase completeness.

Structure determination and refinement

The structure of the PlexA1–4–Sema1b1–2 1:1 complex was solved by molecular replacement in PHASER with the Sema1b1–2 structure (PDB 6FFK) and the PlexinA2–4 structure (PDB 3OKY) (Janssen et al., 2010) as search models. This solution was re-built automatically by BUCCANEER and completed by several cycles of manual rebuilding in COOT and refinement in PHENIX. PlexA domains IPT1–PSI2 were omitted from the model due to disorder. The structure of the PlexA1–4–Sema1b1–2 2:2 complex was solved by molecular replacement in Phaser with the Sema1b1–2 and PlexA1–4 structures as search models. This solution was refined by Rosetta (DiMaio et al., 2013) and rigid-body in PHENIX with each domain as a rigid group and a single B factor per domain and using global NCS restraints. Structure validation was performed using MolProbity (Davis et al., 2007). Refinement statistics are given in Table 1.

Buried surface areas of protein–protein interactions were calculated using PISA (Krissinel & Henrick, 2007), alignments were generated with Clustal Omega (Sievers et al., 2011), structural alignment was performed using PDBBeFold (Krissinel & Henrick, 2004), and electrostatics potentials were generated using APBS (Baker et al., 2001). Figures were produced with PyMOL (Schrodinger, LLC), ESPRRIPT (Gouet et al., 1999) and Corel Draw (Corel Corporation).

Microscale thermophoresis (MST)

MST experiments were performed using a Nanotemper Monolith NT.115 instrument (Nanotemper) at 28°C in 15 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM CaCl2 and 0.05% (v/v) Tween-20. A dilution series were prepared and a concentration of the fluorescent PlexA1–4–mVenus was kept constant in all samples and the unlabelled Sema1b in all samples were varied in 1:1 dilution to give a titration. The samples were equilibrated 1 h at room temperature before filling into the standard capillaries (Nanotemper). To find the best thermophoretic setting, a measurement at 20, 40, 60 and 80% MST power was performed and the best signal to noise ratio was obtained by using 80% MST power. The LED power was set to 40%. The overall measurement time consisted of 5 s of cold fluorescence followed by IR-laser on and off times set at 30 and 5 s. Data were analysed using the MO Affinity Analysis v2.1.3 software (Nanotemper). The experiments were performed with three independent replicates.

Analytical ultracentrifugation

Sedimentation velocity experiments were performed using an Optima XL-1 analytical ultracentrifuge (Beckman). PlexA1–4 and PlexAecto samples in 15 mM HEPES (pH 7.4) and 150 mM NaCl were centrifuged in double sector 12 mm centriplate in an An-60 Ti rotor (Beckman) at 40,000 rpm. Protein sedimentation was monitored by an absorption optical system and Rayleigh interference system. Data were analysed using SEDFIT (Schuck, 2000). Expected sedimentation coefficients of the structural models were predicted using WinHydroPRO (Ortega et al., 2011).

Size-exclusion chromatography with multi-angle light scattering (SEC-MALS)

SEC-MALS experiments were carried out with the Superdex 200 10/300 column (GE Healthcare) connected online with a static light scattering (DAWN HELOS II, Wyatt Technology), differential refractive index (Optilab rEX, Wyatt Technology) and Agilent 1200 UV (Agilent Technologies) detectors. PlexA1–4 were injected into the column at flow rate of 0.5 ml/min in 15 mM HEPES (pH 7.8) and 150 mM NaCl. The molecular mass of glycoproteins containing N-linked oligomannose-type sugars was determined using an adapted RI increment value (dn/dc standard value, 0.185 ml/g). Data were analysed using the Astra software (Wyatt Technology).

Single particle negative stain electron microscopy

A freshly purified PlexAecto (3.5 μg/ml) was stained with 0.75% uranyl formate using the conventional negative staining protocol (Booth et al., 2011). Images were recorded using a Tecnai T12 transmission electron microscope operated at 120 kV on a 4,000 × 4,000 high-sensitivity FEI Eagle at magnification of 67,000, which corresponds to 1.68 Å/pixel sampling of the specimen. A defocus value of about −1.5 μm was used. Particles were manually selected and processed using the Eman2 (Tang et al., 2007) and Imagic (van Heel & Keegstra, 1981) software.

Fluorescence resonance energy transfer—fluorescence lifetime imaging microscopy (FRET-FLIM) in live cells

Drosophila melanogaster PlexA (residues 28–1,311) or Sema1b (residues 37–686) and Sema1a (residues 21–633) were cloned into pHsec vector in-frame with C-terminal fluorescent proteins mClover or mRuby2, respectively. For FRET-FLIM analysis of mouse Sema6A and PlxnA2, genes encoding mouse Sema6A (residues 19–675) and PlxnA2 (residues 36–1,263) were cloned into pHR-CMV-TetO2 vector in frame with C-terminal fluorescent proteins mClover and mRuby2, respectively (Elegeheet al., 2018). All constructs encompassed the ectodomain followed by a transmembrane segment and the C-terminal fluorescent protein. The same Drosophila constructs were used for Number and Brightness analysis. COS-7 cells (ATCC CRL-1651) grown on glass-bottom 35 mm Petri dishes (Mattek) were transiently co-transfected with Drosophila PlexA-mClover and Sema1b-mRuby2 or a donor-only sample (PlexA-mClover) or a fusion construct of mClover-mRuby2, which was used as a positive control. For mouse Sema6A and PlxnA2, we used lentiviral transduction of...
COS-7 cells followed by FACS to enrich subpopulations of transduced cells (Elegeheert et al., 2018).

Before imaging, a Dulbecco’s modified eagle medium was replaced with PBS equilibrated at 37°C. Multicolour images were acquired 2 days post-transfection using a Leica SP8-X-SMD confocal microscope (Leica Microsystems) with a 63×/1.40 numerical aperture oil immersion objective. mClover and mRuby2 were excited at 488 and 561 nm, respectively, and the fluorescence emission was detected using two hybrid detectors in photon counting mode at 498–551 and 573–625 nm, respectively.

FRET detection was based on the time domain FLIM experiments which were performed using a Time-Correlated Single Photon Counting (TCSPC) system operated by a PicoHarp 300 module (PicoQuant) attached to the Leica SP8-X-SMD confocal microscope (Leica Microsystems) with a 63×/1.40 numerical aperture oil immersion objective. mClover and mRuby2 were excited at 488 and 561 nm, respectively, and the fluorescence emission was detected using an external hybrid detector in photon counting mode at 498–551 and 573–625 nm, respectively.

In equations (1) and (2), τ₁ is the lifetime of the donor alone, τ₂ is the lifetime of the donor in the presence of the acceptor, A, A₁ and A₂ are amplitudes. The average donor lifetime obtained from a mono-exponential fit from the cells expressing the donor only (PlexA-mClover) was fixed in the bi-exponential model to calculate the remaining two amplitudes and the second lifetime (Padilla-Parra et al., 2008; Padilla-Parra & Tramier, 2012). The amplitude weighted average lifetime of donor (τ(av)) was calculated using the equation (3):

\[ \tau_{av} = \frac{\sum_i A_i \tau_i}{\sum_i A_i} \]  

The fraction of the interacting donor (f_D) was calculated using the equation (4):

\[ f_D = A_2/(A_1 + A_2) \]  

The fraction of the interacting donor was normalised by multiplying by a factor of 2 because we were able to detect just ~50% of the real interaction (Padilla-Parra et al., 2009).

Fluorescence resonance energy transfer—fluorescence lifetime imaging microscopy (FRET-FLIM) in solution

For FLIM measurements in vitro, the purified fluorescent proteins were diluted to a concentration of 106 nM in 15 mM HEPES (pH 7.6) and 150 mM NaCl and 30 μl of the protein sample was loaded onto the μ-Slide with 18 wells (Ibidi). FRET was measured using the time domain FLIM experiments which were performed using a Time-Correlated Single Photon Counting (TCSPC) system operated by a PicoHarp 300 module (PicoQuant) attached to the Leica SP8-X-SMD confocal microscope (Leica Microsystems) with a 63×/1.40 numerical aperture oil immersion objective at room temperature.

A 488 nm picosecond pulsed diode laser PDL 800-B (PicoQuant) tuned at 40 MHz was used to excite the donor, and the emitted photons passing through the 500–550 nm emission filter were detected using an external hybrid detector in photon counting mode for a period of 350 s. The lifetime analysis was carried out using a Symphotime (PicoQuant). The acquired fluorescent decays were fitted by mono- or bi-exponential model as described above. The apparent interfluorophore distance r was calculated from the equation (5):

\[ E_{app} = 1 - \tau_{DA,av}/\tau_{D,av} = R_0^6/(R_0^6 + r^6) \]  

In equation (5), τ₀ is the lifetime of the donor alone, τ_DA is the lifetime of the donor in the presence of the acceptor and R₀ is the Förster radius. The R₀ for the mClover/mRuby2 pair was calculated by the following equation

\[ R_0 = \left( \frac{9,000 \ln 10 \Phi_D \mu^5 J}{128 \pi^3 N_A n^2} \right)^{1/6} \]  

Where Φ_D is the fluorescence quantum yield of the donor in the absence of the acceptor, μ is the dipole orientation factor, n is the refractive index of the medium, N_A is Avogadro’s number, and J is the spectral overlap integral calculated as

\[ J = \int F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda/\int F_D(\lambda) d\lambda \]  

Where F_D is the donor emission spectrum, and ε_A is the acceptor molar extinction coefficient. To calculate the apparent interfluorophore distance, we used the Förster radius of 57 Å for the mClover/mRuby2 assuming random interfluorophore orientation. The spectral overlap integral of the mClover/mRuby2 pair was calculated using the Simpson method utilising the normalised excitation spectrum of the donor and the acceptor corrected for the published extinction coefficient (Lam et al., 2013). Knowing the value of J we calculated R₀ assuming random interfluorophore orientation (κ² = 2/3).

Number & brightness analysis

COS-7 cells grown on μ-Slides (chambered coverslips) with 8 wells (Ibidi) were transiently transfected with Drosophila melanogaster PlexA-mClover (residues 28–1,311), Sema1b-mClover (residues 37–686) or Sema1b-F254C-mClover (residues 37–686). Before imaging, the cells were washed with PBS and Dulbecco’s modified eagle medium was replaced with a phenol red-free Dulbecco’s modified eagle medium equilibrated at 37°C.

Images were acquired using a Leica SP8-X-SMD confocal microscope (Leica Microsystems) with a 63×/1.40 numerical aperture oil immersion objective at 37°C. For each studied cell, a single plane stack of 500 images was acquired at a resolution of 256 × 256 pixels and pixel size of 481 nm, with a pixel dwell time 2.43 μs. mClover was excited at 488 nm with the same laser power for each cell, and
the fluorescence emission was detected using a hybrid detector in photon counting mode at 498–551 nm. The data were analysed using a nandb—an R package for performing N&B analysis (Nolan et al., 2017).

**Total internal reflection microscopy**

COS-7 cells grown on bottom 35 mm Petri dishes (Mattek) were transiently co-transfected with PlexA-mClover, wild-type Sema1b-mRuby2 and all three Sema1b mutants. The same constructs were used for FRET-FLIM or Number and Brightness analysis. Two days post-transfection, the cells were washed with PBS and Dulbecco’s modified eagle medium was replaced with a phenol red-free Dulbecco’s modified eagle medium equilibrated at 37°C. For mouse Sema6A and PlxnA2, we used the same stable COS-7 cell lines as we used for FRET-FLIM. The images were acquired by a Zeiss Elyra TIRF microscope equipped with a 100× oil objective (1.46 NA). mRuby2 was excited at 561 nm, and the images were acquired at a resolution of 512 × 512 pixels (image size 49.7 × 49.7 μm). The average fluorescence intensity was calculated using ImageJ (Schindelin et al., 2012). In particular, TIRF micrographs were background subtracted to get rid of the EM-CCD camera noise. After this, each cell was profiled utilising a mask that only contained the signal coming from each cell and non-attributed-numbers for the background. The average grey value per each profiled cell was obtained and plotted as a box plot for each condition.

**Collapse assay of COS-7 cells**

COS-7 cells grown on μ-Slides (chambered coverslips) with 8 wells (Ibidi) were transiently transfected with *Drosophila melanogaster* full-length PlexA-mClover (residues 28–1,945), wild-type Sema1b-mRuby2 (residues 37–686) or Sema1b-mutA-mRuby2 (residues 37–686) or Sema1b-mutB-mRuby2 (residues 37–686) or Sema1b-mutA+B-mRuby2 (residues 37–686). Two days post-transfection, the cells were washed with PBS and Dulbecco’s modified eagle medium was replaced with a phenol red-free Dulbecco’s modified eagle medium equilibrated at 37°C.

Images were acquired using a Leica SP8-X-SMD confocal microscope (Leica Microsystems) with a 63 × 1.40 numerical aperture oil immersion objective at 37°C. mClover and mRuby2 were excited at 488 and 561 nm, respectively, and the fluorescence emission was detected using two hybrid detectors in photon counting mode at 498–551 and 573–625 nm, respectively. Tiled positions (3 × 3) were scanned in 512 × 512 format every minute for 30 min. The pinhole was set at 3.0 Airy units, and an automatic adaptive autofocus was used to prevent z-drifting while imaging. After 2 min of imaging, a recombinant Sema1a-Fc was added to a final concentration of 5.8 μM. Cell surface area was calculated using ImageJ (Schindelin et al., 2012) before and after stimulation with recombinant Sema1a-Fc.

**DRG cultures**

Culture methods were as previously described (Van Battum et al., 2014). In short, DRG neurons were dissected from E12.5 Sema6A knockout mouse embryos (Lilley et al., 2019). DRGs were collected in 1× Krebs medium (0.7% NaCl, 0.04% KCl, 0.02% KH₂PO₄, 0.2% NaHCO₃ and 0.25% glucose) and dissociated by incubation with 0.25% trypsin in Krebs/EDTA for 10 min at 37°C. The reaction was halted by adding 2 mg soybean trypsin inhibitor, followed by trituration with a fire-polished Pasteur pipette in Krebs medium containing soybean trypsin inhibitor and 20 μg/ml DNAse. Dissociated cells were resuspended in neurobasal medium supplemented with B-27, l-glutamine, penicillin/streptomycin, β-mercaptoethanol and nerve growth factor 2.5S (50 ng/ml, Alomone labs). Cells were plated onto poly-D-lysine (20 μg/ml) and laminin (10 μg/ml) coated glass coverslips in 12 wells plates in a humidified incubator at 37°C and 5% CO₂. Cultures were fixed by adding equal volume of 8% PFA in PBS containing 30% sucrose for 10–30 min at room temperature.

**Growth cone collapse assay**

For growth cone collapse assays, DRG neurons were transfected at 1 day in vitro (DIV1) with Sema6A-WT-EGFP-pCAG, Sema6A-mutA-EGFP-pCAG, Sema6A-mutB-EGFP-pCAG or Sema6A-mutA+B-EGFP-pCAG mutant constructs or empty EGFP-pCAG vector using lipofectamine 2000 for 45 min at 37°C. At DIV4, vehicle (medium) or purified Sema6A-Fc was added to the cultures at a concentration of 1, 10 or 75 nM for 1 h at 37°C. Cultures were fixed and processed for immunocytochemistry with anti-GFP antibodies and counterstained with phalloidin to visualise F-actin in filopodia and lamellipodia to determine growth cone morphology. The following antibodies were used in this experiment: Rabbit anti GFP (Thermo Fisher Scientific, catalog # A-11122), Donkey anti-Rabbit IgG (H+L) Alexa Fluor 488 (Thermo Fisher Scientific, catalog # A-21206) and Alexa Fluor 568 Phalloidin (Invitrogen, catalog # A-12380). Coverslips were mounted and scored for growth cone morphology using a scale from one to four (uncollapsed) and five to eight (fully collapsed) according to a matrix of growth cones with different morphologies, allowing the detection of even subtle changes in growth cone morphology (van Erp et al., 2015; Kong et al., 2016). Assigning each growth cone to its appropriate category was based on the following criteria: Categories 1-4 show uncollapsed growth cones, with category 1 showing multiple extending filopodia that are reduced in number in category 2. Category 3 and 4 growth cones both lack filopodia with category 4 growth cones additionally showing F-actin reduction. Categories 5–8 show collapsed growth cones, with growth cone size and shape reducing to no discernible growth cone at all in category 8. Images were acquired on an epifluorescence microscope (Zeiss Axioscope A1). A categorical analysis using Fisher’s exact test was used to test for statistical significance of uncollapsed or fully collapsed growth cones for each mutant or control construct. Statistical tests were performed using IBM SPSS Statistics 23.

**Animals**

All animal use and care was in accordance with institutional guidelines and approved by the animal experimentation committee (DEC). The mouse strain was Sema6A (Sema6aGt[KST069]Byg) kept on a C57/B6J background. Timed-pregnant females were 3–6 months of age. Timed-pregnant mice were euthanised by means of cervical dislocation. The morning on which a vaginal plug was detected was considered embryonic day 0.5 (E0.5).
Data availability

Structure factors and coordinates have been deposited in the Protein Data Bank with identification numbers PDB: 6FKM (https://www.rcsb.org/structure/6FKM) and 6FKN (https://www.rcsb.org/structure/6FKN).

Expanded View for this article is available online.

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Author contributions
Conceptualisation, DR, RJP and EYJ; Methodology, DR,RAR, LA, SP-P and EYJ; Investigation, DR, MGV, DK, GNN, LA and KH, Writing, DR and EYJ, Funding Conceptualisation, DR, RJP and EYJ; Methodology, DR, RAR, LA, SP-P and EYJ; Investigation, DR, MGV, DK, GNN, LA and KH, Writing, DR and EYJ; Funding Acquisition, DR, SP-P, RJP and EYJ; Supervision, SP-P, RJP and EYJ.

Conflict of interest
The authors declare that they have no conflict of interest.

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