
Structure of Functional Neuropilin-Centred Class 3 Semaphorin and VEGF Receptors

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Abstract

Neuropilin functions as a co-receptor for multiple cell surface signalling systems; in particular it regulates VEGF and semaphorin signalling through their respective receptors. The molecular characteristics of neuropilin, the VEGF and semaphorin signalling complexes and their multiple interaction modes have been extensively investigated by structural and biophysical analyses. Much has been learned about the molecular mechanisms by which neuropilin acts as an interaction hub, but the complexities of neuropilin function still pose many questions.

2.1 Introduction

Human neuropilin 1 and 2 (Nrp1 and Nrp2) mediate multiple biological effects though their interactions with the ligands and receptors of the semaphorin and vascular endothelial growth factor (VEGF) signalling systems. The role of neuropilin in these systems is that of a regulator or modulator. Although the neuropilins have the N-terminal ectodomain, single transmembrane region and C-terminal

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cytoplasmic region typical of type 1 cell surface receptors, they lack the classic enzymatic domains required for direct signal transduction, merely containing a cytoplasmic PSD-95/Dlg/ZO-1 (PDZ)-binding motif. Thus, signalling activity is dependent on the binding of the semaphorin or VEGF ligands with their cognate cell surface receptors. Albeit the neuropilins serve as co-receptors, their influence on signalling outcomes can be profound. This chapter reviews our current understanding of how the structural characteristics and interactions of the neuropilins determine biological outcomes in semaphorin and VEGF signalling.

2.2 Neuropilin: Structure of a Co-receptor

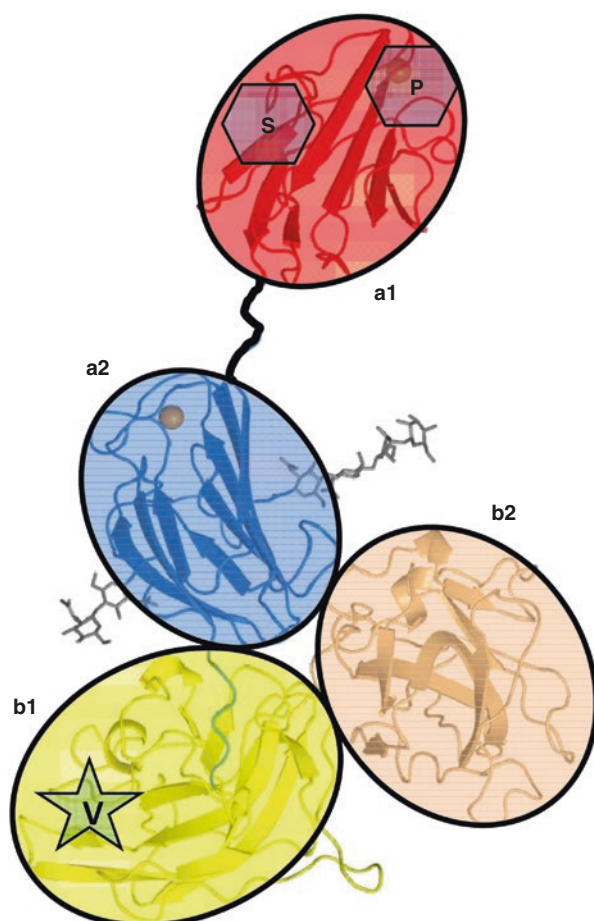
The neuropilin ectodomain consists of five distinct domains classically referred to as the a1, a2, b1, b2 and c domains. Crystal structures have been determined for the a1-a2-b1-b2 regions of Nrp1 and Nrp2 [4, 33]. These structures reveal the a1 domain is flexibly linked to a tightly clustered unit of a2-b1-b2 domains (Fig. 2.1). The a1 and a2 domains have the β -sandwich topology of CUB domains. The b1 and b2 domains also have a β -strand-based fold and belong to the family of coagulation factor 5/8 type C domains. To date no structures have been determined for the c domain; however, sequence analysis indicates that it has the β -sandwich-type fold of a meprin/A5/mu (MAM) domain, similar to the structurally characterised N-terminal domain of the receptor protein tyrosine phosphatase RPTP μ [5, 6]. This modular composition of the neuropilin ectodomain, based on structurally stable β -strand folds, provides multiple possible binding surfaces for interactions with ligands and receptors (Fig. 2.1).

Early studies sought to dissect the functional contributions of the a, b and c domains of neuropilin. The interactions of class 3 semaphorins with neuropilins were mapped to the a1-a2-b1-b2 segment [29, 39] as were VEGF-neuropilin interactions [61]. The c (MAM) domain was reported to mediate neuropilin homodimerisation [10, 19, 47]. Interestingly, the MAM domain in RPTP μ contributes to receptor-receptor adhesive interactions [6]; however, the interaction characteristics of the neuropilin MAM domain have not been further characterised. Subsequent structural and functional studies have focused attention on the roles of protein binding sites on the neuropilin a1 and b1 domains. In addition, the interaction properties of the membrane-spanning α helix have been investigated.

2.3 Neuropilin and VEGF Signalling

VEGF signalling occurs through VEGF receptors (VEGFRs), which are type 1 single membrane-spanning receptors belonging to the receptor tyrosine kinase superfamily. VEGFR ectodomains consist of seven Ig-like domains (D1-D7). The VEGFs are homodimeric ligands that can be cleaved to generate an N-terminal dimer which retains VEGFR-binding activity and two monomeric C-terminal tails that bind heparin [34]. The subunits of the N-terminal dimer have cystine knot growth factor-type

Fig. 2.1 Neuropilin structure and binding sites. Structure of Nrp1 *a1-a2-b1-b2* with interaction sites on the *a1* domain indicated by hexagons (*S* semaphorin binding, *P* plexin binding) and on the *b1* domain indicated by a star (*V* VEGF binding) (Adapted from Janssen et al. [33])



architectures and are tightly interfaced with inter-subunit disulphide bonds [46]. The C-terminal tail comprises two, flexibly linked, subdomains [14]. As is common for the receptor tyrosine kinases, ligand binding results in VEGFR dimerisation with consequent activation of the cytoplasmic kinase domains [41]. The VEGF dimer cross-links two VEGFR ectodomains to generate the 2:2 complex (Fig. 2.2a). Structural analyses have detailed the various contributions of VEGFR domains D1 to D7. VEGFR D2 makes a major contribution to VEGF binding [70]. The ligand binding site also includes contributions from the D2-D3 inter-domain linker region and D3 [42], consistent with earlier mapping of domain function [15]. Low-resolution (negative stain electron microscopy and small-angle solution X-ray scattering, SAXS) studies of full-length VEGFR ectodomains in complex with VEGF suggest that VEGF-mediated VEGFR dimerisation at the membrane distal D2-D3 site triggers direct VEGFR-VEGFR interactions involving the middle segment of the ectodomain (D4-D5) as well as the membrane proximal domain, D7 [36, 43, 56]. Functional data support the importance of these homotypic interactions for

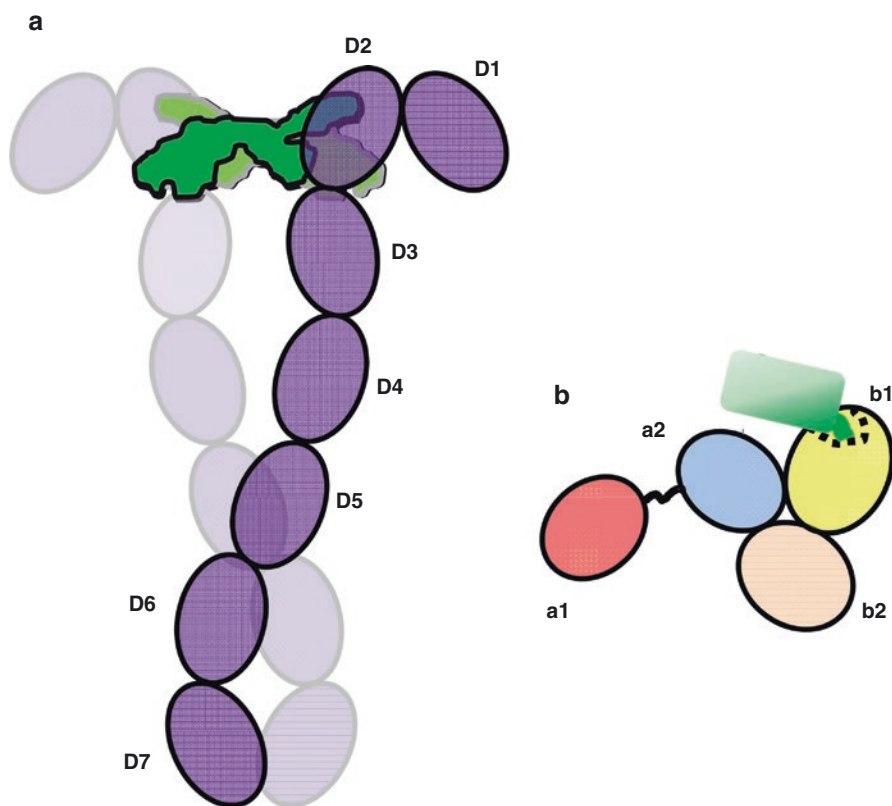


Fig. 2.2 VEGF-VEGFR structure and interaction with neuropilin. (a) A composite model of the 2:2 VEGF-VEGFR complex. The VEGF N-terminal dimer is shown in *green* and the VEGFR ectodomains in shades of mauve. (b) Schematic of VEGF C-terminal tail region (*green*) binding to neuropilin (domain colouring as in Fig. 2.1)

receptor activation and crystal structures of D7 and D4-D5 have detailed D7-D7 and D5-D5 interaction interfaces [43, 72]. Thus a series of structural studies has built up a model for the extracellular arrangement of the dimeric VEGF ligand and two VEGFR ectodomains in the 2:2 signalling complex (Fig. 2.2a). Neuropilin can modulate this core VEGF-VEGFR signalling complex in a number of ways.

In terms of structural analyses, the VEGF-neuropilin interaction is the best characterised mechanism for interplay between neuropilin and the VEGF-VEGFR system (Fig. 2.2b). The ability of neuropilin to interact directly with VEGF is primarily dependent on the isoform or proteolytically activated state of the ligand. Nrp1 binds specific VEGF-A isoforms, classically VEGF-A₁₆₅ [51, 61]. Nrp2 has been shown to bind to proteolytically activated VEGF-C [53] as well as to particular VEGF-A isoforms [21]. These binding specificities can be understood in terms of the atomic level details of the neuropilin-VEGF interface. Structural studies have revealed that a surface groove in the b1 domain of neuropilin selectively binds linear epitopes that

have a C-terminal arginine [67]. The neuropilin-binding isoforms of VEGF-A and the proteolytically activated form of VEGF-C all have in common a tail region ending in a C-terminal arginine. The specificity of the interaction is fine-tuned by some additional side-chain specific interactions with the neuropilin b1 domain, but the multiple interactions of the C-terminal arginine render this residue the key structural determinant for ligand binding [51]. Typically VEGF may be expected to bind to cell surface-attached neuropilin; however, secreted splice variants have been identified [17, 55]. A secreted Nrp1 isoform comprising $\alpha 1$ - $\alpha 2$ -b1-b2 has been shown to be able to act as an antagonist of VEGF-A₁₆₅ [17]. A splice variant of Nrp2 truncated within the b2 domain forms a disulphide-linked dimer, each subunit presenting a functional VEGF-binding site, prompting the suggestion that this covalently stabilised dimer may serve as a particularly potent inhibitor of VEGF-C binding to cell-attached Nrp2 [53].

The VEGFR and neuropilin binding sites on VEGF are independent. VEGF can therefore serve as a cross-linker to recruit neuropilin into a heterocomplex with VEGFR [61, 62]. Interestingly, formation of the neuropilin-VEGF-VEGFR complex appears possible either with neuropilin and VEGFR attached to the same cell (i.e. in *cis*) or with neuropilin and VEGFR attached to different cells (i.e. in *trans*), albeit *cis* complex formation is kinetically favoured [38, 62]. The ability to form *cis* and *trans* complexes clearly requires a substantial degree of freedom in the relative orientation of the VEGF-bound neuropilins and VEGFRs. This orientational flexibility might be facilitated by the nature of the VEGF-neuropilin interaction; rather than involving an extended molecular surface, only the C-terminal tail region of VEGF is required to bind to the b1 domain of neuropilin.

The interplay of neuropilin with VEGF and VEGFR is further complicated by the potential for interactions with the glucosaminoglycans (GAGs) of heparan sulphate proteoglycans (HSPGs) and heparin. Both VEGF and neuropilin bind heparin, and heparin has been reported to enhance the VEGF-neuropilin interaction [16]. The heparin binding site on neuropilin has been mapped to a positively charged surface extending over the b1 and b2 domains [67]. VEGFRs also bind heparin, consistent with the notion that cell surface-attached HSPGs can promote the formation and stability of signalling complexes [11–13, 20, 49, 71]. The HSPGs can be presented in *cis* (i.e. on the same cell surface as the VEGFR) or in *trans* on neighbouring cells [31]. Most recently detailed biophysical analyses have shown that VEGF, VEGFR and neuropilin bind synergistically to HSPG, suggesting that the multiple binding modalities of HSPG GAG chains modulate the composition, stability and output of VEGFR-based signalling complexes [65].

The mechanism of action of neuropilin function in VEGF-VEGFR signalling is still not fully understood. As discussed above the extracellular interactions with HSPG and neuropilin can serve to modulate VEGF-VEGFR binding and hence signalling output [25]. However, the PDZ-binding domain in the neuropilin cytoplasmic region can also influence complex formation with VEGFR [54]. Furthermore, although Nrp1 and Nrp2 do not bind VEGF₁₂₁, Nrp1 has been shown to be able to modulate VEGF₁₂₁-induced VEGFR2 signalling, and Nrp2 has been found to interact with VEGFR1 on endothelial cells stimulated by this ligand isoform [22, 59].

Mice have been generated that have Nrp1 bearing a mutation in the b1 groove that selectively abolishes VEGF binding [18]. Intriguingly, these studies suggest that, for developmental angiogenesis, the VEGF-neuropilin interaction is not central to a co-receptor function in the signalling complex, but rather than Nrp1 regulates the cell surface expression of VEGFR2 [18].

2.4 Neuropilin and Class 3 Semaphorin Signalling

Classically, semaphorin signalling is mediated by members of the plexin family of cell surface receptors [64]. Semaphorin ligands are secreted or cell surface attached, either by a single membrane-spanning helix or a GPI anchor. The class 3 semaphorins are the secreted members of the semaphorin family in mammals, namely, *Sema3A*, *Sema3B*, *Sema3C*, *Sema3D*, *Sema3E*, *Sema3F* and *Sema3G*. Whilst the cell surface-attached classes of semaphorins are able to trigger signalling by direct binding to their cognate plexin receptors, the class 3 semaphorins are not. All of the *Sema3s* interact with neuropilin and, apart from *Sema3E*, require a holoreceptor complex, comprising plexin and neuropilin, for signalling [24, 63, 64].

The semaphorins are now well characterised structurally. The eponymous sema domain has at its core a seven-blade β -propeller topology; however, the main chain fold is elaborated by some extensive insertions in the loops linking the β -strands in the so-called blades of the propeller (Fig. 2.3a) [3; 45]. The various classes of semaphorins vary in domain composition, but, immediately after the N-terminal sema domain, all contain a PSI (for plexin-semaphorin-integrin) domain. The PSI domain is a cysteine knot and interfaces with the sema domain to form a relatively rigid unit of some 700 residues (Fig. 2.3a) [45]. The semaphorins are functionally active as dimers [37; 40]. A sema domain to sema domain interface lies at the heart of semaphorin dimerisation, although inter-subunit interactions and disulphide bonds from additional, class-specific domains can also make important contributions to dimer stability [60]. The class 3 semaphorins comprise a sema domain, PSI domain and Ig-like (β -sandwich) domain followed by a basic, C-terminal region. The Ig-like domain contributes an additional homotypic interaction, and the basic tail provides inter-subunit disulphide bonds to stabilise *Sema3* dimers [2; 37; 40; 33].

The plexins are type 1 cell surface receptors with N-terminal ectodomain, single transmembrane α -helix and C-terminal cytoplasmic region. The ectodomain contains an N-terminal sema domain, followed by multiple PSI and IPT (Ig-like, plexins, transcription factors) domains. The cytoplasmic region chiefly comprises a distinctive GTPase-activating protein (GAP) domain. The plexin GAP domain is unusual in that it has a Ras GAP-type topology but preferentially functions as a GAP for Rap [68]. The plexin GAP architecture is also novel in having inserted into a loop, midway through the GAP topology, a Rho GTPase-binding domain that protrudes as distinct additional domain [28; 66]. Structural and functional analyses suggest that the plexin GAP domain requires activation by conformational changes that are triggered in the juxtamembrane region as a result of extracellular ligand

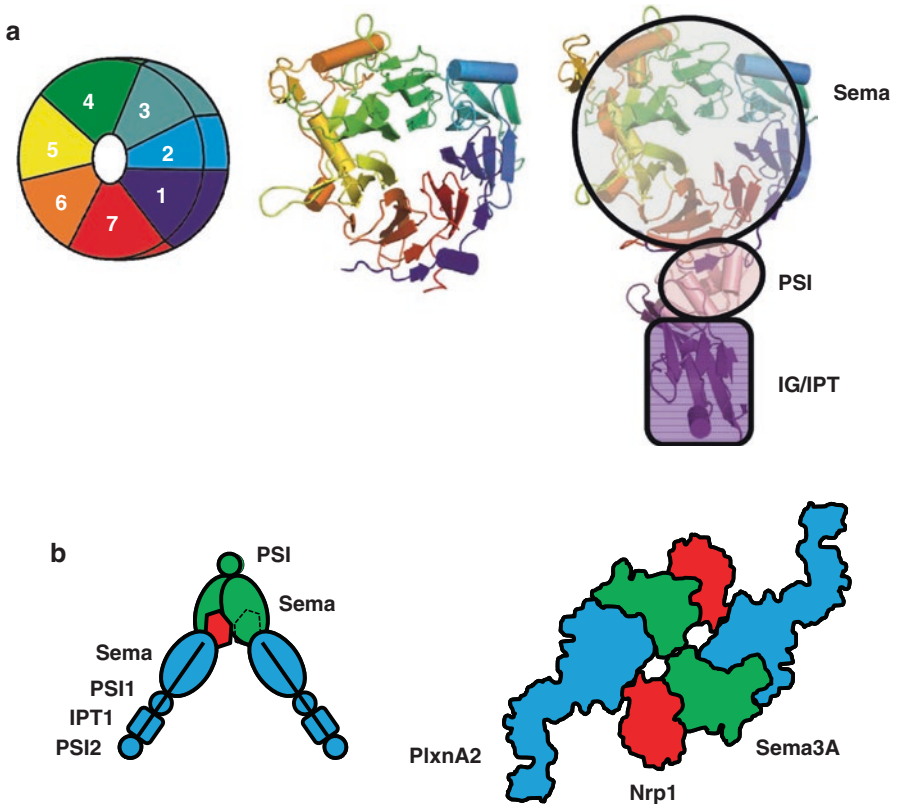


Fig. 2.3 Semaphorin-plexin structure and interaction with neuropilin. (a) The sema domain seven-blade β -propeller. The left panel shows a schematic of a seven-blade β -propeller. The middle panel depicts the Sema3A sema domain. The right panel shows a representative sema-PSI-Ig/IPT structure (based on the structure of Sema4D sema-PSI-Ig) (The panels are adapted from Siebold and Jones [60]). (b) Schematics (orthogonal views) of the Sema3A-PlxnA2-Nrp1 2:2:2 complex. Only the Nrp1 a1 domain (red) is visible

binding and consequent receptor dimerisation [8; 68; 69]. Interestingly, *in silico* studies indicate that the transmembrane α -helix and juxtamembrane regions of plexins have dimer, and in some cases trimer, forming propensities [73].

The interaction of semaphorin ligand with plexin receptor is entirely through sema-to-sema domain binding [32, 44, 48]. The sema domain of one semaphorin subunit binds ‘face to edge’ with the sema domain of one plexin ectodomain. This sema-to-sema interaction is repeated by the second subunit of the semaphorin dimer resulting in a 2:2 complex in which the semaphorin ligand cross-links two plexin receptors. The position of the sema-sema interface and 2:2 arrangement of the semaphorin-plexin complex are conserved across classes for cell surface-attached semaphorins and their cognate plexins, consistent with this architecture being central to receptor activation [32, 60]. However, although class A plexins can be

activated by class 6 semaphorins through engagement in the canonical 2:2 complex, these same plexin receptors must be associated with neuropilin for activation by the secreted class 3 semaphorins.

Neuropilin can interact directly with class 3 semaphorins as well as mediating holoreceptor-based complex formation with *Sema3s* and plexin *As*. In vitro studies, using purified *Sema3A* (minus the basic C-terminal region) in surface plasmon resonance (SPR)-based assays, showed direct *Sema3A*-Nrp1 interaction, but no measurable *Sema3A* binding to plexin A2 (PlxnA2) [33]. The ability of neuropilin to mediate a stand-alone interaction with class 3 semaphorins is consistent with the recent observation that premature repulsion of axons is prevented by the expression of neuropilin on neighbouring cells. The neuropilin acts as a developmentally regulated 'molecular sink', reducing the local levels of secreted semaphorin encountered by the axon growth cones [30]. SPR assays have also shown direct binding between Nrp1 and PlxnA2 ectodomain segments [33]. Thus for the semaphorin-plexin system, neuropilin does interact directly with both the ligand and the receptor in the extracellular region. The crystal structure of *Sema3A*-PlxnA2-Nrp1 reveals a 2:2:2 complex at the heart of which is the canonical 2:2 arrangement of dimeric semaphorin ligand cross-linking two plexin receptors (Fig. 2.3b; [33]). Although the interaction of the class 3 semaphorin with the class A plexin is too weak to form a stable 2:2 complex in isolation, two copies of the a1 domain of neuropilin 'glue' the classical ligand-receptor arrangement together. Each Nrp1 a1 domain makes an interaction with the sema domain of one *Sema3A* subunit and the sema domain of the PlxnA2 that is bound to the other *Sema3A* subunit. Thus the neuropilin a1 domain specifically acts as a cross-brace to stabilise the 2:2 complex involving semaphorin dimer and two plexins, rather than locking together the 1:1 interaction between a single semaphorin subunit and plexin ectodomain. This simple cross-brace mode of action provides a satisfying mechanism by which neuropilin can function as a co-receptor gating the formation of activated plexin signalling complexes. Interestingly, the architecture of the 2:2:2 complex can serve to homodimerise or hetero-dimerise PlxnAs consistent with functional data indicating that sometimes two different plexins are required to transduce class 3 semaphorin signals [9, 57].

Neuropilin contains additional potential binding sites for both the semaphorin ligand and the plexin receptor. The importance of the a1 domain for neuropilin functions involving semaphorin-plexin signalling has been demonstrated in vivo [23]; however, early studies mapping domains involved in *Sema3* binding to neuropilin identified both the sema domain (the site of the a1 domain interaction) and the basic C-terminal region [29]. Class 3 semaphorins contain furin cleavage sites in their basic C-terminal regions, which on proteolysis generate *Sema3s* bearing a similar C-terminal motif to that used by VEGF for binding to the neuropilin b1 domain [2; 50; 52]. Thus *Sema3s* can use the same 'C-terminal epitope-to-b1 groove' interaction mode as VEGFs, and potentially can compete with them to bind neuropilin [26, 52], albeit this is not necessarily the only mechanism by which class 3 semaphorins can inhibit VEGF activity [27]. The contributions of the sema and basic C-terminal region to neuropilin binding appear to be additive, consistent with simultaneous

engagement through the two binding modes [29]. The crystal structure of Sema3A-PlxnA2-Nrp1 was determined using a form of Sema3A lacking the basic C-terminal region, the N-terminal four domain segment of PlxnA2 (sema-PSI1-IPT1-PSI2) and the a1-a2-b1-b2 segment of Nrp1. Only the Nrp1 a1 domain was visible in the crystal structure, consistent with the flexible linker between a1 and a2 allowing the a2-b1-b2 unit to sample multiple positions relative to the rest of the complex. This flexibility appears sufficient to allow the neuropilin b1 domain to bind the basic C-terminal region of Sema3 in addition to a1 domain interactions with the core semaphorin-plexin complex. Furin cleavage at various points in the basic tail of class 3 semaphorins (including complete removal) therefore provides two mechanisms by which ligand function can be modulated: firstly, the generation or removal of neuropilin b1 domain binding ability and, secondly, the loss of intermolecular disulphide bridges that contribute to Sema3 dimer stability [2; 37; 40; 50; 52]. Notably, the combination of two flexibly linked binding sites provides neuropilin with an intriguing ability to act as a hub involved in multiple interactions bridging the semaphorin and VEGF systems. Further work is required to explore the molecular interactions that underlie the reported effects on plexin signalling of associations with VEGF receptors [9, 35].

The transmembrane α -helix in plexin and neuropilin also provides a possible region for receptor-receptor interactions, both homophilic and heterophilic. A number of *in silico* and biophysical analyses support this possibility [1, 58; 7]. The most recent *in silico* studies have begun to shed light on the interaction properties of the plexin transmembrane plus juxtamembrane region [73] however, our understanding of the interplay of interactions in the full-length transmembrane receptors is in its infancy.

Conclusions

Structural studies have detailed the molecular interaction modes of the neuropilin a1 and b1 domains as well as the overall architecture of the ectodomain. These analyses, combined with biophysical characterisations of binding properties plus functional assays, have started to provide detailed insights into the mechanisms by which neuropilin functions as a co-receptor in the VEGF-VEGFR and semaphorin-plexin signalling systems. Excitingly, the availability of detailed information on interaction sites can guide the design of mutant neuropilins to dissect the functional contributions of selected interactions *in vivo* (e.g. [18]). The combination of mechanistic insights, gained from detailed molecular level studies, and *in vivo* analyses offers a route to understanding, and learning how to manipulate, the modes of action of this multipurpose co-receptor.

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