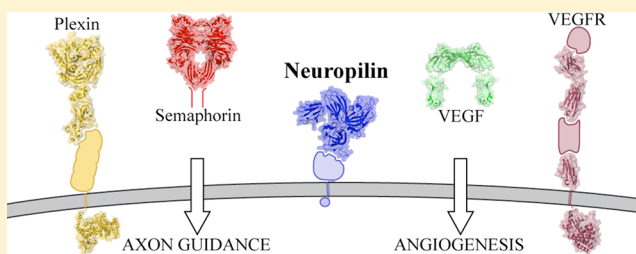


Function of Members of the Neuropilin Family as Essential Pleiotropic Cell Surface Receptors

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ABSTRACT: The neuropilin (Nrp) family consists of essential multifunctional vertebrate cell surface receptors. Nrps were initially characterized as receptors for class III Semaphorin (Sema3) family members, functioning in axon guidance. Nrps have also been shown to be critical for vascular endothelial growth factor-dependent angiogenesis. Intriguingly, recent data show that Nrp function in these seemingly divergent pathways is critically determined by ligand-mediated cross-talk, which underlies Nrp function in both physiological and pathological processes. In addition to functioning in these two pathways, Nrps have been shown to specifically function in a number of other fundamental signaling pathways as well. Multiple general mechanisms have been found to directly contribute to the pleiotropic function of Nrp. Here we review critical general features of Nrps that function as essential receptors integrating multiple molecular cues into diverse cellular signaling.



■ NRP ARCHITECTURE

Nrp was first identified in the optic tectum of *Xenopus* and termed A5 antigen.^{1,2} There are two conserved Nrp family members in vertebrates, Nrp1 and Nrp2, which share the same overall domain structure and are 44% identical at the amino acid level.^{3,4} Nrp family members are type I transmembrane proteins that possess an N-terminal signal peptide, two calcium-binding C1r/C1s/Uegf/Bmp1 (CUB) domains (a1a2),⁵ two coagulation factor V/VIII-like discoidin domains (b1b2),^{6,7} a Meprin/A5-antigen/ptp-Mu (MAM) domain (c), a single transmembrane helix, and a short intracellular domain. The four N-terminal extracellular domains are necessary and sufficient for ligand binding. The a1 domain has been shown to interact with the sema domain of Sema3 ligands.⁸ The b1 domain contains a specific C-terminal arginine binding pocket and is essential for binding to the C-terminal domain of both VEGF and Sema3 ligands.^{9–14} Additionally, the b1 and b2 domains physically interact to form an extended patch of basic residues that is involved in binding heparin, a highly sulfated member of the glycosaminoglycan (GAG) family of carbohydrates.¹⁰ The c domain is dispensable for ligand binding but essential for ligand-dependent signaling.¹⁵ The transmembrane helix possesses a conserved GXXXG repeat and shows strong inherent dimerization potential.¹⁶ The intracellular domain of Nrp interacts with PSD-95/Dlg/ZO-1 (PDZ) domain proteins, such as GAIP interacting protein, C-terminus (GIPC), via its C-terminal residues.¹⁷ Deletion of the Nrp1 intracellular domain does not result in embryonic lethality, as seen with the whole gene, but instead causes defects in vascular patterning.¹⁸

There are alternative splice forms of Nrp1 and Nrp2 that have been shown to have functional implications. The most commonly identified alternative splice forms of both Nrp1 and Nrp2 result from the inclusion of introns in the region between

the b2 and c domains.^{19,20} The included intron contains a stop codon resulting in production of secreted soluble proteins that preserve ligand binding and thus function as endogenous pathway inhibitors. Alternative splicing of Nrp2 also produces two species with divergent C-terminal intracellular domains. While multiple splice forms of both Nrp receptors exist, the functional significance of these divergent sequences is largely unknown. In addition to splice variants, there are a number of post-translational modifications that alter Nrp function. Nrp1 and Nrp2 can be modified by both N- and O-linked glycans. Nrp1 has been shown to be modified by heparan or chondroitin sulfate, which results in enhanced ligand binding.²¹ Thus, this modification may poise or preactivate Nrp for signaling. In contrast, Nrp2 is modified by polysialic acid, resulting in enhanced dendritic cell migration.^{22,23}

■ NRP FUNCTION IN SEMAPHORIN-DEPENDENT AXON GUIDANCE

Nrp was initially identified and functionally characterized as a receptor for Sema3 family ligands in neurons. Neurons function in cooperative neural networks connected by dendrites and axons. The number and type of axonal connections are tightly regulated by guidance cues. One family of essential axon guidance molecules consists of the Semaphorins. There are seven Sema3 family members (Sema3A–G) that are secreted, diffuse through tissues, and provide guidance cues.²⁴ Indeed, axon guidance cues mediated by Sema3 family members are essential during the development for correct neuronal

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patterning in dorsal root ganglia, facial, vagal, olfactory-sensory, cortical, hippocampal, and cerebellar nerves, and others.²⁵

Sema3 family members bind Nrp receptors on the cell surface^{3,26} (Figure 1). The N-terminal a1 domain of Nrp1 and

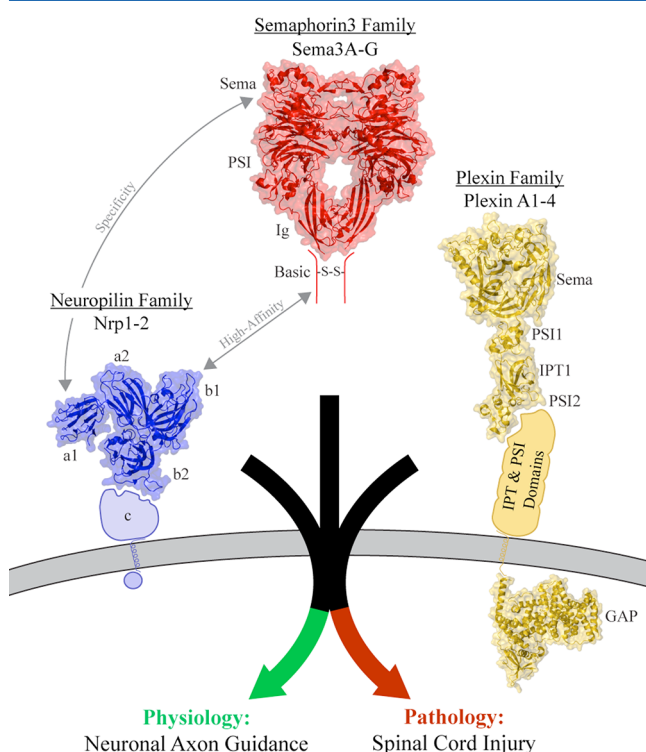


Figure 1. Nrp function is essential for Sema3-dependent axon guidance. Nrps specifically bind to Sema3 family members through a bivalent binding mechanism allowing both specific and high-affinity binding. Engagement and activation of Plexin family receptors activate signaling in both physiological axon guidance and pathological spinal cord injury. Structures of proteins or homologues with known structures were utilized: Semaphorin N-terminus (PDB entry 1OLZ),¹⁴⁰ neuropilin N-terminal domains (PDB entry 2QOQ),¹⁰⁹ Plexin extracellular domain N-terminus (PDB entry 3OKT),¹⁴¹ and Plexin intracellular domain (PDB entry 3IG3).¹⁴²

Nrp2 selectively binds the Sema domain of different Sema3 family members.^{27–29} Notably, Sema3A specifically signals via Nrp1^{3,26} and Sema3F via Nrp2.³⁰ The Nrp b1 domain allows high-affinity nonselective binding to the Sema3 C-terminal domain.^{13,15,30} Following binding of Sema3 by Nrp, Plexin family signaling receptors (PlexinA1–4) are then engaged and activated to directly guide axonal growth.^{31,32} Nrp functions by coupling specific high-affinity Sema3 binding to Plexin-dependent regulation of cytoskeleton dynamics in the axonal growth cone.³¹ Collapse of the actin cytoskeleton results in axon repulsion, and this appears to represent the most common modality for Nrp-dependent Sema3 function. However, Sema3 signaling can also be attractive rather than repulsive, such as when cGMP levels are high.³³

■ NRP IN SPINAL CORD INJURY

Nrp function in Sema3 signaling is important not only for physiological axon guidance but also for signaling in spinal cord injury^{34,35} (Figure 1). Following spinal cord injury, a glial scar forms to stabilize and seal the wound. However, the glial scar also serves as a barrier to regenerating axons due to the

production of axon repulsion molecules. Sema3 expression significantly contributes to the inhibitory nature of the glial scar, so inhibitors of Nrp–Sema3 signaling hold promise as therapeutics for the treatment of spinal cord injury.^{36,37}

■ NRP FUNCTION IN VEGF-DEPENDENT ANGIOGENESIS

Angiogenesis is the process by which new vasculature is formed by branching or splitting from existing vasculature. Physiological angiogenesis is critical during development, for homeostatic maintenance of vasculature, and for wound healing. Among the most potent pro-angiogenic cytokine families is the VEGF family. VEGF-A was the first identified family member,^{38,39} which also includes VEGF-B–D and placental growth factor (PlGF).⁴⁰ The different family members have both unique and partially overlapping functions. VEGF-A, VEGF-B, and PlGF regulate hemangiogenesis by Nrp1.^{41–43} VEGF-C and -D regulate lymphangiogenesis by Nrp2.⁴⁴ Also, VEGF-B has recently been shown to control lipid transport in endothelial cells by Nrp1.⁴⁵ To add to the complexity, VEGF-A, VEGF-B, and PlGF all have numerous alternative splice forms with varying physical and functional properties,^{46,47} whereas VEGF-C and -D require proteolytic processing from a preprotein for activation.⁴⁸ Nrp1 was initially identified as a VEGF-A₁₆₅ splice form specific receptor.⁴¹ More recent work has demonstrated that Nrp1 can also bind other VEGF-A isoforms^{49,50} yet uniquely and specifically physically engages VEGF-A₁₆₅.¹⁴

VEGF-A is secreted by an initiating tissue, typically in response to hypoxia, and diffuses to nearby vessels where it binds to two families of essential endothelial cell surface receptors: VEGFR receptor tyrosine kinases⁴⁰ and Nrps^{41,43} (Figure 2). VEGF-A binding causes the initiation of the angiogenic cascade, which involves activation of endothelial cells, basement membrane remodeling, proliferation of endothelial cells, and directional guidance leading to the growth of new vasculature toward the initiating tissue. Importantly, angiogenic signaling is accomplished only through the coordinated activity of VEGF, VEGFR, and Nrp.⁵¹ While binding of VEGF to VEGFR weakly activates its intracellular kinase activity, Nrp is required for strong and sustained kinase activation, leading to the initiation of the pro-angiogenic cascade.^{52,53} Nrp1 null mice die in utero because of defective vasculature formation, thus emphasizing the critical role of Nrp in angiogenesis.⁵⁴ Importantly, abnormalities associated with the loss of Nrp1 are caused by defective endothelial cell migration rather than proliferation.⁵⁵

■ LIGAND-MEDIATED CROSS-TALK

Intriguingly, it has been noted that blood vessels and nerves utilize similar signals and principles for guidance.^{56–58} Indeed, recent studies have identified an embryonic stem cell-derived population of cells that differentiate toward either vascular or neuronal cell type depending on the microenvironment.⁵⁹ Nrp receptors are expressed in nerves and endothelial cells, and both cell types are responsive to secreted ligands of the Sema3 and VEGF families.^{60–62} Not only are both cell types responsive to each family of ligands, but the different ligands can compete for Nrp binding (Figure 3). As previously discussed, the b1 domain of Nrp is responsible for binding the C-terminal domain of both VEGF and Sema3. Three coagulation factor loops of the b1 domain form a conserved

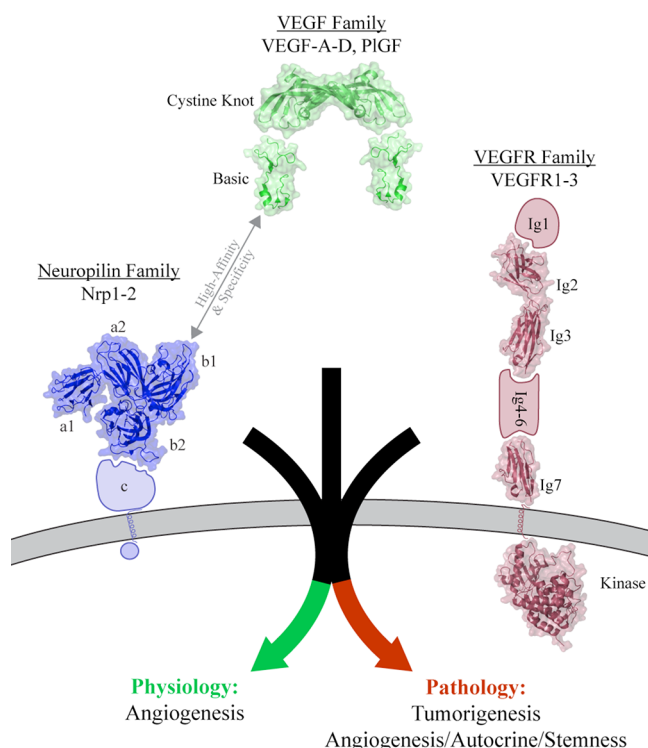


Figure 2. Nrp function is essential for VEGF-dependent angiogenesis. Nrps specifically bind to the C-terminal basic domain of VEGF family members. Cooperative binding of VEGF by VEGFR and Nrp activates the angiogenic cascade necessary for developmental and homeostatic angiogenesis and also pathological signaling associated with tumorigenesis and other types of aberrant signaling. Structures of proteins with known structures were utilized: VEGF-A cystine knot domain (PDB entry 2VPF),¹⁴³ VEGF-A basic domain (PDB entry 4DEQ),¹⁴ neuropilin N-terminal domains (PDB entry 2QKQ),¹⁰⁹ VEGFR2 Ig-like domain 2–3 (PDB entry 2X1X),¹⁴⁴ VEGFR2 Ig-like domain 7 (PDB entry 3KVQ),¹³⁷ and VEGFR2 intracellular domain (PDB entry 1VR2).¹⁴⁵

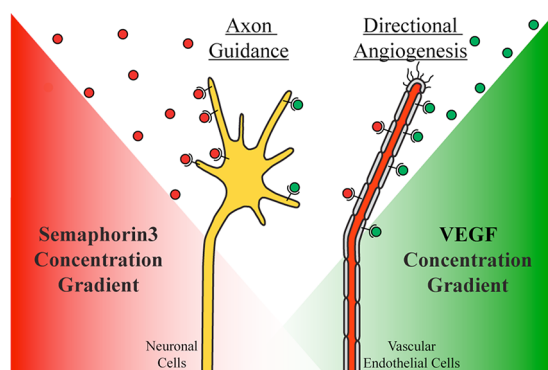


Figure 3. Cross-talk between Nrp ligands allows coordinated regulation of neuronal and vascular tissues. Both neurons and endothelial cells express Nrp, which can respond to either Sema3 or VEGF family guidance cues. Regulation of competitive Nrp binding between different ligands allows for an additional level of dominant control of Nrp function.

binding pocket specific for C-terminal arginine-containing ligands.^{10,14,63} All VEGF family members possess a C-terminal arginine, which is necessary for binding to Nrp. In contrast, no Sema3 family members possess a C-terminal arginine. However, all Sema3 family members contain furin-like protease

RXXR consensus sites in their C-terminal domain.⁶⁴ Indeed, Sema3F has been identified to possess potent anti-angiogenic activity.^{65,66} This anti-angiogenic activity was shown to result from direct competition between Sema3F and VEGF-A for binding to their shared C-terminal arginine binding pocket in the Nrp b1 domain.¹³ The interplay of VEGF and Sema3 is important for diseases associated with angiogenesis. Aberrant hypovascularization was recently demonstrated in the retina, where expression of Sema3A prevents revascularization of ischemic neural tissue.⁶⁷ In contrast, aberrant hypervascularization is associated with VEGF upregulation or Sema3 down-regulation in pathological angiogenesis of solid tumors.^{68–70}

■ NRP IN TUMOR ANGIOGENESIS

Nrp function is important not only for physiological angiogenesis but also in VEGF-dependent pathological hypervascularization in tumor angiogenesis.⁷¹ It has long been known that the extent of tumor vascularization is correlated to tumor size⁷² and that diffusible factors are able to induce neovascularization in solid tumors.⁷³ Anti-angiogenesis therapies⁷⁴ are now used against a range of solid tumors. Avastin, a monoclonal antibody targeting VEGF-A, has been among the most successful angiogenesis inhibitors.⁷⁵ While this provides a significant benefit, it has been noted that when patients relapse, those that received anti-angiogenesis treatment often have more aggressive and metastatic tumors.^{76,77} Aberrant Nrp expression and function promote tumorigenesis and metastasis in vivo in a variety of solid tumors.^{51,78–80} Surprisingly, in addition to expression in tumor vasculature, direct Nrp overexpression in malignant cells has been widely reported.

■ NRP TUMOR CELL EXPRESSION AND AUTOCRINE ACTIVATION

The initial identification of Nrp as a receptor for VEGF noted its expression in both endothelial and tumor cells.⁴¹ Since then, aberrant Nrp expression in a wide variety of malignant cells has been observed.^{69,81–84} The function of Nrp in tumor cells, and its contribution to tumorigenesis are sources of considerable interest. Recently, Nrp expression was demonstrated to be critical for autocrine activation of tumor cells, influencing carcinoma survival,⁸⁵ growth,⁸⁶ and migration.^{87,88} Nrp-dependent autocrine activation provides a basis for understanding why Nrp expression and function directly correlate to both tumor aggressiveness⁸⁹ and the metastatic potential of a variety of solid tumors.^{90,91} Further, Nrp function in tumor cells has been tied to dedifferentiation and stemness.⁹² It has recently been shown that Nrp1 directly contributes to the self-renewal of cancer stem cells in skin cancer.⁹³ Indeed, Nrp1 deletion blocked the ability of VEGF to promote cancer stem cell self-renewal. Further, an autocrine activation pathway involving Nrp1 was shown to contribute to stemlike cell viability and tumor growth in glioma.⁹⁴ The multifunctional role for Nrp in tumor initiation and progression has motivated research focusing on the development of novel Nrp inhibitory modalities.

■ GENERAL MODES OF NRP ACTION

Nrps are normally expressed in a variety of tissue types, including the previously discussed nerves and vascular tissues along with immune and hematopoietic cells.^{95,96} In addition to the critical role for Nrp in the Sema3 and VEGF pathways, interactions with a number of other ligands and receptors have

been reported. Other ligands identified include members of the fibroblast growth factor (FGF) family, platelet-derived growth factor (PDGF), transforming growth factor β 1 (TGF- β 1), and hepatocyte growth factor/scatter factor (HGF/SF) functioning via receptors in the FGFR, PDGFR, T β Rs, and c-Met families, respectively.^{89,97–104} In light of the diverse expression profile and functional interactions, determining the general mechanism(s) of Nrp action is critical to further our understanding of physiological and pathological functions of Nrp.

■ NRP FUNCTIONS BY PHYSICALLY ORGANIZING THE FULL SIGNALING COMPLEX

As a primary function, Nrps promote pathway activation by recruiting ligands to the cell surface through high-affinity interactions (Figure 4, A). This is particularly important in the

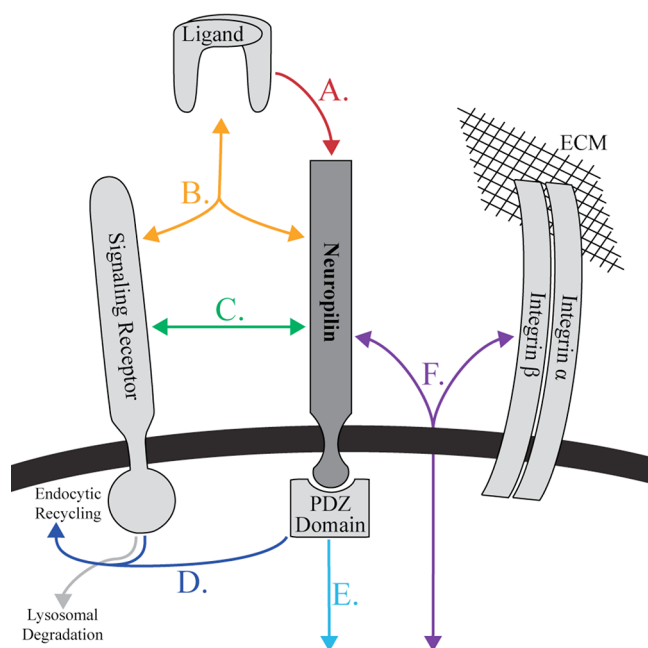


Figure 4. Nrp utilizes common mechanisms to regulate diverse signaling pathways. (A) Specific high-affinity ligand binding initiates Nrp-mediated signaling. (B) Engagement and organization of an active signaling complex are accomplished through specific receptor–ligand and coreceptor contacts. (C) Ligand-independent receptor association forms a preactivated receptor complex poised for signaling. (D) Nrp regulates dynamic trafficking of signaling complexes. (E) Binding of intracellular PDZ domain proteins, including GIPC, allows direct coupling between Nrp ligand binding and signaling functions. (F) Direct engagement of integrins allows Nrp to couple molecular events with cellular cues.

binding of Sema3, which is the only diffusible Sema family and requires Nrp for cell surface binding.³⁴ Additionally, Nrps show selective binding to different members within the VEGF and Sema3 families. The basis for specific Nrp1–VEGF-A interactions in hemangiogenesis has been described biochemically and structurally,^{14,105} and other family members are actively being pursued.

Ligand binding is followed by assembly of the active signaling complex, where Nrp functions to promote and stabilize the active signaling holoenzyme^{31,52,106} (Figure 4, B). Assembly of the active signaling complex requires both receptor recruitment and binding-associated conformational changes, providing the physical mechanism required for signal transduction. Import-

tantly, Nrp ligands are dimeric, with conserved intermolecular disulfide bonds. Thus, the minimal signaling holocomplex is thought to involve a dimeric ligand bound to two Nrp molecules and two signaling receptors. Energetically, it appears that highly favorable ligand binding drives the formation of specific homo- and heterotypic receptor contacts that do not occur spontaneously.^{107,108} This is consistent with optimal receptor function, with physical mechanisms strongly inhibiting spontaneous ligand-independent activation.

Understanding Nrp function in holocomplex assembly requires knowledge of the structure and interactions of the receptor both before and after ligand binding. The a2b1b2 domains of Nrp have been reported to adopt a stably associated trimeric core with a loosely tethered a1 domain.¹⁰⁹ The c/MAM domain, which is dispensable for ligand binding but essential for ligand-induced signaling,¹⁵ has been reported to form homo- and heterodimers between Nrp1 and Nrp2.²⁷ However, experiments with purified protein have reported that there is no self-association or dimerization propensity that can be observed for the Nrp ectodomain, leaving the structure and physical coupling of the MAM domain within Nrp an open question.¹⁰⁹

GAG binding also plays a critical role in Nrp-dependent signaling. GAGs make up a diverse family of naturally occurring sulfated polysaccharides that orchestrate numerous processes, including the function of endothelial cells in angiogenesis.¹¹⁰ GAG binding dramatically enhances Nrp–ligand interactions and drives dimerization of Nrp.^{10,107} Thus, GAG binding likely plays a dual role by bridging and stabilizing the dimer holocomplex assembly.

Intriguingly, a number of receptors have been shown to associate with Nrp in a ligand-independent fashion. A direct and specific interaction has been demonstrated between Nrp1 and VEGFR1.¹⁰⁷ Nrp and Plexin family receptors have also been shown to associate in a ligand-independent manner.¹¹¹ In these cases, rather than having sequential receptor binding, the preassociated receptor complex is poised to bind and signal and requires only conformational changes (Figure 4, C).

■ NRP TRAFFICKING

Following ligand binding and assembly of the active signaling complex, Nrp has been demonstrated to have a critical function in receptor trafficking (Figure 4, D). Intriguingly, it was shown that Sema3 and VEGF induce Nrp1 endocytosis via distinct pathways.¹¹² Further, Nrp promotes the partitioning of VEGFR into vesicles that are recycled back to the cell surface, whereas in the absence of Nrp, VEGFR2 is targeted for degradation instead of recycling.¹¹³ The intracellular GIPC-binding domain of Nrp was demonstrated to be essential for the observed VEGFR recycling, suggesting the involvement of a Nrp-associated cytoplasmic protein.

■ NRP INTRACELLULAR DOMAIN

The discovery of the Nrp-binding PDZ protein GIPC provided a mechanism by which Nrp may directly couple to intracellular adaptors and pathways¹⁷ (Figure 4, E). The critical portion of the Nrp intracellular domain consists of the three C-terminal amino acids, conserved in Nrp1 and Nrp2, that constitute a PDZ domain-binding motif and allow binding to PDZ domain proteins. A growing body of evidence implicates the intracellular domain of Nrp in a variety of biological processes. Mice expressing a mutant Nrp receptor with a deleted cytoplasmic

domain were generated.¹⁸ While capable of binding ligand, these Nrp receptors are decoupled from signal transduction via its PDZ domain-binding motif and GIPC and result in vascular abnormalities distinct from those of full Nrp1 knockout. Angiogenesis was unaffected, but vascular patterning was disrupted as evidenced by frequent crossing of arteries and veins in the retina. A similar approach was applied in zebrafish, demonstrating that knockdown of Nrp1 does disrupt angiogenesis and only full length Nrp, not a Nrp1 construct lacking the three C-terminal amino acids, is able to rescue disrupted blood vessel growth.¹¹⁴ Further, deletion of the PDZ domain-binding C-terminal amino acids of Nrp1 disrupted stable association of the full angiogenic signaling complex with VEGFR-2.¹¹⁵ There is also a growing body of evidence of Nrp function in the absence of other recognized receptor families. Specifically, Nrp is capable of mediating adhesiveness¹¹⁶ and migration¹¹⁷ of endothelial cells in the absence of VEGFR2, functions that require the C-terminal PDZ domain-binding motif of the Nrp intracellular domain.

Interaction with a common intracellular adaptor, such as GIPC, suggests that Nrp function in physiologically diverse pathways may be due to coupling to basic cellular signaling pathways. Intriguingly, integrin subunits $\alpha 6$ and $\alpha 5$ have been reported to interact with GIPC via their C-termini.^{118,119}

■ NRP COUPLING WITH INTEGRINS

Integrins mediate interactions with the extracellular matrix and as a result are important for cellular adhesion and migration. Indeed, the biological function first associated with Nrp1 was its ability to mediate heterophilic cellular adhesion.¹²⁰ A variety of studies have now demonstrated that both Nrp1 and Nrp2 physically and functionally associate with integrins^{121–123} (Figure 4, F).

Exogenous Semaphorin 3 was able to inhibit the adhesion of endothelial cells to the integrin ligands fibronectin and vitronectin, while VEGF enhanced the adhesive strength of endothelial cells.¹²¹ Furthermore, a recent study demonstrated that Nrp1 was necessary for the adhesion of endothelial cells to fibronectin.¹²⁴ Surprisingly, rescue of fibronectin binding following Nrp1 knockdown was only seen using vectors carrying full-length Nrp but not Nrp constructs lacking the C-terminal PDZ domain-binding motif, suggesting coupling between integrin and PDZ domain proteins. $\alpha v\beta 3$ integrin has been shown to interact with Nrp1 in a VEGF-dependent fashion, and this serves to sequester Nrp1 from the active VEGF–VEGFR2 signaling complex, thereby limiting angiogenesis.¹²⁵ The function of integrins in controlling cellular migration led to an investigation of the interplay between Nrp1 and integrin $\beta 1$, demonstrating that blockade of either Nrp1 or $\beta 1$ resulted in reduced invasiveness and weakened adhesion in a pancreatic cancer cell line.¹²² Recently, an important specific role for Nrp2 in integrin-mediated adhesion has also been defined. Nrp2 makes specific interactions with integrin $\alpha 6\beta 1$ at focal adhesion sites. Depleting the level of Nrp2 expression in breast carcinoma cells inhibited focal adhesion assembly of these cells and regulated the downstream targets of $\alpha 6\beta 1$ integrin that ultimately modulate laminin adhesion.¹²³ These data demonstrate that Nrp and integrin receptors function cooperatively to regulate cellular adhesion, and there is considerable interest in understanding the precise nature of the physical coupling and extent of biological function.

■ INHIBITORY MODALITIES TARGETING NRP

In light of Nrp function in diverse diseases, significant effort has been devoted to developing potent specific Nrp inhibitors for use either alone or in combination with other inhibitory modalities, such as Avastin.

Inhibition of Semaphorin 3 signaling has been actively pursued for regenerative therapy following spinal cord injury.¹²⁶ A selective inhibitor of binding of Semaphorin 3A to Nrp1¹²⁷ showed significant benefit in recovery from spinal cord injury.¹²⁸ However, the entire Semaphorin 3 family is upregulated in the glial scar,³⁷ indicating that broad-spectrum inhibition of Semaphorin 3 signaling is desired. Initial work has demonstrated that Nrp2 inhibition allows penetration of axons into a model glial scar.¹²⁹ Currently, efforts are focusing on developing potent, broad-spectrum, Nrp inhibitory modalities suitable for use in the central nervous system.

Inhibitory modalities targeting Nrp-dependent VEGF-induced angiogenesis have been extensively explored. A soluble Nrp splice form, containing only the ligand binding region of the extracellular domain of Nrp, has been employed and found to inhibit tumorigenesis.¹⁹ A number of peptides and a synthetic peptidomimetic inhibitor of Nrp have been described previously.^{9,13,130,131} Methods of overexpressing inhibitory molecules, for example, Semaphorin 3A, have been reported with promising activity in tumor angiogenesis.¹³² Finally, antibodies targeting both Nrp1 and Nrp2 that show promising activity in animal models with observed devascularization of solid tumors and decreases in levels of metastasis have been developed.^{133,134} These anti-Nrp antibodies are currently being tested in clinical trials. Surprisingly, one of the observed side effects of administration of the Nrp1 antibody, MNRP1685A, was platelet depletion.¹³⁵ This was shown to be due to specific binding of MNRP1685A to platelet cell surface Nrp1, modest platelet activation, aggregation, and clearance.

■ FUTURE DIRECTIONS OF NRP RESEARCH

Because of the complex interplay of Nrp with other molecules, a number of critical areas regarding the mechanism of Nrp function remain to be explored. Of particular interest is the specific roles for Nrp in distinct signaling cascades and tissue types. Connected to this is the extent to which Nrp functions by the same general mechanism in highly diverse pathways or if there are distinct mechanisms employed in different signaling cascades.

The basis for ligand binding specificity has begun to come into focus and continues to be explored. In particular, the contribution of physical mechanisms governing ligand binding selectivity versus tissue specific expression remains to be determined. The physical basis for binding of divalent Semaphorin 3 to Nrp remains an important unanswered question. Specifically, the contribution and coupling between the a1 and b1 interaction sites and the effect of post-translational modification by furin on binding and signaling remain outstanding questions.

The nature of coupling of Nrp to signaling receptors to form a functional signaling holocomplex represents a major area of research. In particular, unique heterophilic receptor–receptor contacts are likely critical to the formation and stability of the complex. Although the Nrp MAM domain is necessary for signaling, its role is largely unknown. Thus, the MAM domain might physically couple to cognate receptors. Indeed, the membrane proximal seventh Ig-like domain of VEGFR-2 has been shown to form a dimer required for activation,^{136,137}

which could be coupled to MAM domain function in the holo complex. Further, the specific function of the transmembrane domain of Nrp may be to directly couple to signaling receptors or to allow the precise physical arrangement of the intracellular domain. The transmembrane domain of VEGFR-2 has been demonstrated to facilitate the correct orientation of homodimers to couple ligand binding to receptor activation.¹³⁸ Nrp may require a similar specific alignment of the transmembrane domain and may, in fact, directly couple to the transmembrane domain of cognate signaling receptors. Indeed, it is possible that the extracellular juxtamembrane, transmembrane, and intracellular juxtamembrane domains may coordinately function to physically couple ligand binding to receptor activation.

The function of the intracellular domain of Nrp and the extent to which Nrp can function independently of a canonical signaling receptor, for example, VEGFR or Plexin, remain to be determined. Of particular interest are the molecules that can form direct interactions or indirectly bridge with Nrp. While GIPC is clearly a critical Nrp adaptor protein, other PDZ domain proteins have also been suggested to function with Nrp. In particular, Nrp was recently identified as a positive regulator of hedgehog signaling,¹³⁹ and the PDZ domain protein required for this activity is currently being pursued. Connected to this is the extent to which autocrine signaling represents a fundamental mode of Nrp activation in disease, with particular interest in the connection to cancer stem cell maintenance. The continued development of novel inhibitory modalities targeting the different fundamental mechanisms of Nrp action will be both mechanistically informative and directly relevant to human health.

In summary, Nrp family receptors utilize multiple general mechanisms by which they serve pleiotropic functions integrating distinct fundamental pathways essential to physiological and pathological function.

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ABBREVIATIONS

Nrp, neuropilin; Sema3, class III Semaphorin family; VEGF, vascular endothelial growth factor; MAM, Meprin/AS-antigen/ptp-Mu; PDZ, PSD-95/Dlg/ZO-1; GIPC, GAIP interacting protein C-terminus; GAG, glycosaminoglycan; VEGFR, vascular endothelial growth factor receptor; PlGF, placental growth factor; PDB, Protein Data Bank.

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