Atsushi Kumanogoh *Editor*

Semaphorins

A Diversity of Emerging Physiological and Pathological Activities

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Preface

Semaphorins were originally identified in the early 1990s as axon-guidance molecules that function during neuronal development. The name "semaphorin" is derived from "semaphore", which refers to a method of visual signaling, usually with flags or lights, often used in rail and maritime transportation. The semaphorin family is structurally characterized by the presence of the Sema domain in their extracellular regions. The genes encoding semaphorins are highly conserved from invertebrates to mammals, including humans, and more than 20 members of the semaphorin family have been found in vertebrates. Importantly, two groups of proteins, the neuropilins and plexins, have been identified as the main semaphorin receptors.

The evidence accumulated to date has revealed that semaphorins are involved in many crucial processes beyond axon guidance, including angiogenesis, tumor progression through a micro-environment, immune responses, cardiovascular development, bone homeostasis, and retina homeostasis. From a clinical point of view, semaphorins have been implicated in various human diseases, including immune disorders, neurodegenerative diseases, and retinal degenerative diseases. Furthermore, recent studies using crystal structure analysis have established the structural basis of the associations between semaphorins and their receptors. Based on these significant molecular findings, it is likely that drugs targeting semaphorin-mediated signaling will be developed in the near future.

In light of this situation, I decided to publish this book about semaphorins in order to encourage a greater understanding of semaphorins and their activities. I welcomed a number of experts in each field as co-authors and have included the latest advances in each of the book's 11 chapters. Chapter 1 is a general introduction to semaphorins, their receptors, and the signaling mediated by these molecules. Chapter 2 presents the structure of semaphorins and their receptors. The critical roles of semaphorins in the nervous system, angiogenesis, tumor micro-

environment, immune system, bone homeostasis, heart development, pathogenesis of neurodegenerative diseases, and retina homeostasis are introduced in Chaps. 3, 4, 5, 6, 7, 8, 9, 10, and 11, respectively.

I hope that this book will help a wide range of researchers and students to understand the emerging physiological and pathological activities of semaphorins.

Osaka, Japan Atsushi Kumanogoh M.D., Ph.D.

Contents

Chapter 1
Semaphorin Receptors and Their Signaling

Manabu Negishi and Izumi Oinuma

Abstract Semaphorins, which are a large family of secreted and membrane-bound molecules, were initially identified as neuronal axon-guidance signaling molecules but are now known as important key regulators for cell adhesion and motility in a wide range of organ systems, such as angiogenesis and immune response. The semaphorin receptors, neuropilins and plexins, are expressed in a variety of cell types, including neurons, endothelial cells, and cancer cells. Plexins are primarily receptors responsible for intracellular semaphorin signalings. Plexins possess an intrinsic GAP (glyceraldehyde-3-phosphate) activity for R-Ras subfamily GTPases, and this GAP activity is one of the crucial signals of semaphorins. In addition, plexins associate with a variety of signaling molecules, such as Rho GEFs and Rho GAP, and these associated molecules determine the characters of semaphorin signals. On the other hand, their signalings are critically modulated by their associated co-receptor molecules, including tyrosine kinase receptors. Semaphorins provide attractive and repulsive responses in a variety of cells, but associated co-receptors of plexins frequently hold the key to conversion between attraction and repulsion. In this chapter, we focus attention on the molecular signaling systems of plexins.

Keywords Semaphorin • Plexin • GAP • Small GTPase

1.1 Introduction

The semaphorins were initially identified as axon-guidance factors that induce axonal growth cone collapses. However, it is now clear that semaphorins and their receptors are widely expressed in a variety of cells, including neurons, endothelial cells, and various tumor cells, and they are implicated in a variety of physiological functions, including control of nervous and endothelial development and immune systems. Semaphorins display their functions through direct binding to either

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their specific receptors, plexins, or their co-receptors, neuropilins, which form complexes with plexins. Plexins function as crucial receptors of semaphorins, and their intracellular domains are responsible for initiating cellular signal transduction inducing cellular functions of semaphorins. Initially, Plexin-B1, the receptor for Sema4D, has been shown to activate RhoA through binding to RGS-RhoGEF, but the plexin family has been identified to display GAP activity for R-Ras and Rap small GTPases through intrinsic GAP domains within the intracellular tails of plexins (Negishi et al. 2005; Zhou et al. 2008). In this chapter we focus attention on the molecular signaling systems of plexins.

1.2 Semaphorins and Their Receptors, Plexins and Neuropilins

Semaphorins are a large family of secreted or membrane-bound proteins. The semaphorin family contains 21 vertebrate genes and 8 additional genes found in invertebrates, and they are now classified into 8 subclasses on the basis of amino acid sequence similarity and distinctive structural features (Neufeld and Kessler 2008). Classes 1 and 2 are invertebrate semaphorins, classes 3 through 7 are vertebrate semaphorins, and class V are viral-encoded semaphorins, found in the genome of non-neurotrophic DNA viruses. Among these, classes 1, 4, 5, and 6 are transmembrane molecules, and class 7 is a membrane-associated form through a glycosylphosphatidylinositol-anchor motif. In the case of Sema4D, membrane-bound Sema4D is cleaved proteolytically, generating a biologically active soluble Sema4D fragment in some conditions (Zhu et al. 2007). On the other hand, classes 2, 3, and V are secreted proteins. Semaphorins are characterized by the sema domains and PSI (plexins, semaphorins, and integrins) domains, located in amino-terminal regions. In contrast, the carboxyl-terminal regions are more variable, and some of them have immunoglobulin-like loops. Class 3 and 4 semaphorins are homodimerized through cysteine disulfide bonds, and dimerization appears to be critical for their biological function. Semaphorins of classes 4 to 7 as well as Sema3E bind directly to specific plexins and produce plexin-mediated signal transductions. Other class 3 semaphorins do not bind to plexins but instead bind to neuropilins.

The function of semaphorins is mediated by their receptors, the plexins, and mammalian plexins are classified into four subfamilies: Plexin-A1-4, Plexin-B1-3, Plexin-C1, and Plexin-D1 (Tamagnone et al. 1999). Plexins are single-pass transmembrane receptors, and they contain sema domains, PSI domains, and G-P (glycine-proline)-rich motifs in the extracellular regions. On the other hand, in the cytoplasmic regions, plexins directly encode GAP domains for Ras family small GTPases split by the docking site for several Rho family small GTPases such as Rnds, RhoD, and Rac1. In addition, B-type plexins contain a C-terminal PDZ-binding motif for RGS-RhoGEFs. The neuropilins, neuropilin-1 and neuropilin-2, are another type of receptor and were initially identified as direct binding receptors

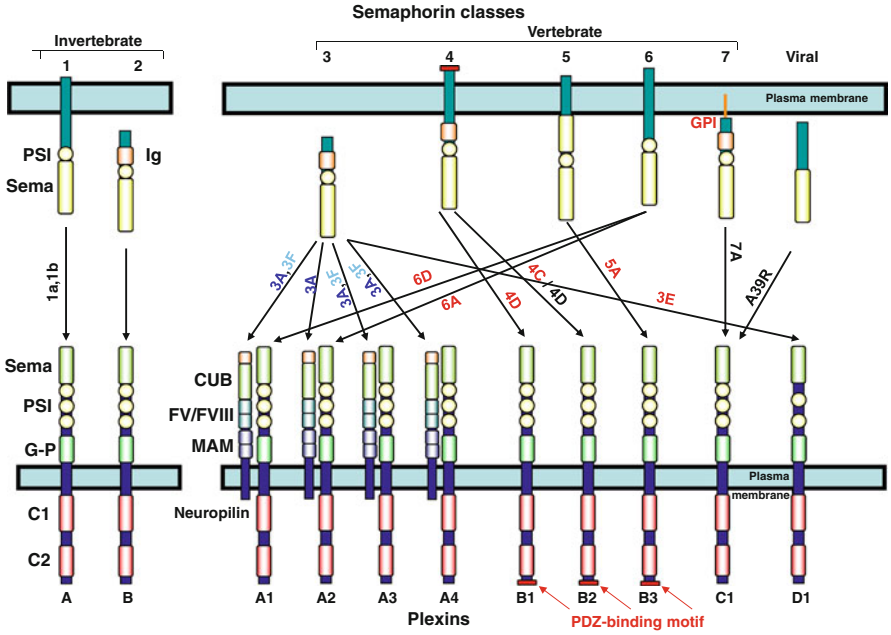

Fig. 1.1 The structures of semaphorins and their receptors. The structural features of the various classes of semaphorins and their neuropilin and plexin receptors are depicted. Semaphorins exist as the secreted, transmembrane, or GPI-anchored form shown *above*. The two neuropilins are single-pass transmembrane receptors. The nine vertebrate plexins are subdivided into four type A and three type B plexins and plexins C1 and D1. Plexins are single-pass transmembrane receptors distinguished by the presence of a split cytoplasmic GAP domain. Domains: *PSI* plexin, semaphoring, and integrin, *Sema* semaphorin, *Ig* immunoglobulin-like, *G-P* glycine-proline-rich, *CUB* complement binding, *FV/FVIII* FV/FVIII coagulation factor-like, *MAM* Meprin, A5, Mu

for class 3 semaphorins. The B-type plexins also contain a conserved cleavage site in their extracellular domains for furin-like pro-protein convertases that is not found in other plexins. The two neuropilins are single-pass transmembrane receptors, and they have two CUB (complement-binding) domains, two FV/FVIII coagulation factor-like domains, and a MAM domain in their extracellular regions. However, neuropilins have very short cytoplasmic tails, and they alone have no ability to transduce the signals of Sema3. Neuropilins form complexes with Plexin-As, and associated Plexin-As are signaling moieties. Classification of semaphorins and their receptors are depicted in Fig. 1.1.

1.3 Invertebrate Plexins

Invertebrate semaphorins have been characterized in *Drosophila melanogaster. D. melanogaster* semaphorins consist of Sema-1a, Sema-1b, and Sema-2a. Sema-1a and Sema-1b are transmembrane semaphorins and bind to PlexA, whereas Sema-

2a is a secreted semaphorin and binds to PlexB, inducing axon repulsion, dendritic targeting, and synapse formation (Fig. 1.2). PlexA forms a complex with Off-track, a transmembrane-type putative receptor tyrosine kinase, and this association has been thought to be required for axon repulsion (Winberg et al. 2001). In addition, PlexA probably associates with Gyc76C, a transmembrane-type guanylate cyclase, and Gyc76C-mediated cyclic GMP production has been thought to function in PlexA signaling (Ayoob et al. 2004). Downstream signaling systems of PlexA have been studied and several associated molecules have been identified. PlexA associates with Nervy, a member of the A-kinase anchoring proteins, and this association links to A-kinase, suggesting the involvement of cyclic AMP-dependent phosphorylation in the functions of Sema-1a/PlexA (Terman and Kolodkin 2004). Another important association molecule of PlexA is MICAL (molecule interacting with CasL). MICAL is a flavoprotein monooxygenase, and this enzyme activity has been shown to be required for Sema-1a-mediated axon repulsion (Terman et al. 2002). Recently, it has been shown that MICAL oxidizes actin, inducing the severing of actin

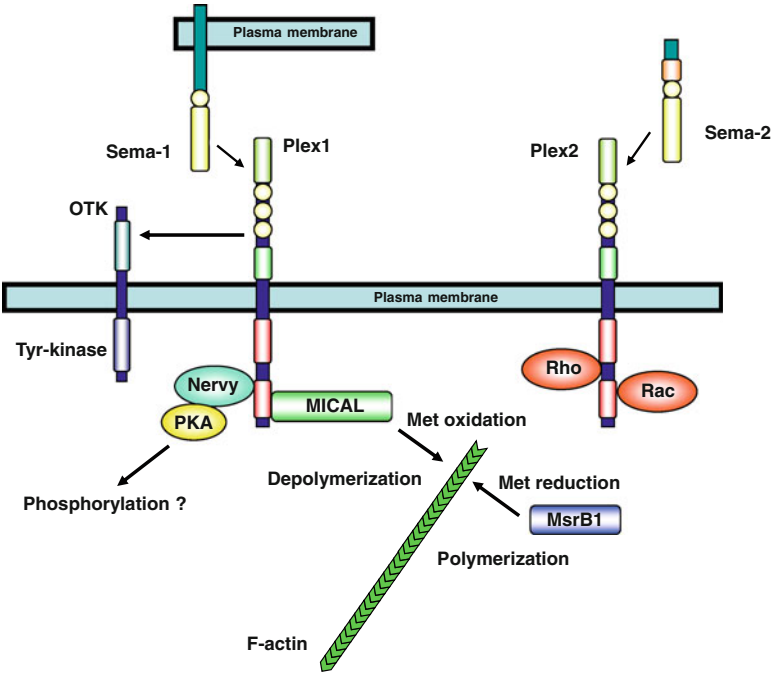

Fig. 1.2 Model for signal transduction of invertebrate semaphorin receptors. Two *Drosophila melanogaster* plexins have been described, PlexA and PlexB. Sema-1 directly binds to PlexA. A novel protein, MICAL, directly associates with the C2 domain of PlexA, inducing repulsive response through actin cytoskeleton reorganization. MICAL and MsrB1 are an oxidase and a reductase, respectively, and promote depolymerization and polymerization of acin filament, respectively, through regulation of actin oxidation. PlexB is a functional Sema-2a receptor and interacts directly with both Rac and Rho

filaments, and decreasing polymerization (Hung et al. 2011), and this redox activity of MICAL links semaphorins to F-actin disassembly (Hung et al. 2010), proposing a striking novel mechanism for semaphorin-mediated cytoskeletal reorganization. Furthermore, methionine-R-sulfoxide reductase B1 (MsrB1) has been shown to reduce the methionine residues of actin oxidized by MICAL and support actin assembly, indicating that MICAL and MsrB1 regulate actin assembly via oxidation and reduction of the methionine residues of actin (Lee et al. 2013). In contrast to PlexA, the signaling systems of PlexB are not yet understood. It has been reported that PlexB associates with the Rho family small GTPases, Rac and Rho, but this signaling system is not known (Hu et al. 2001).

1.4 Plexin-As

The Sema3 subfamily does not directly bind to Plexin-As but does bind to neuropilins, and the Sema3-bound Plexin-A/neuropilin complex triggers downstream signaling. Among the Sema3 subfamily, Sema3A and Sema3F selectively associate with the neuropilin-1/Plexin-A4 complex and the neuropilin-2/Plexin-A3 complex, respectively.

Semaphorins induce guidance signaling through regulation of cytoskeletal reorganization. One of the key regulators of cytoskeletal reorganization is Rho family small GTPases (Burridge and Wennerberg 2004). The Rho family GTPases serve as molecular switches by cycling between an inactive GDP-bound state and an active GTP-bound state, and, once activated, they can interact with their specific effectors, leading to a variety of biological functions. Activation of the Rho family GTPases requires GDP–GTP exchange catalyzed by various guanine nucleotide exchange factors (GEFs) in response to a variety of extracellular stimuli, and their activation is turned off by GTPase-activating proteins (GAPs), which promote the intrinsic GTPase activities of the G proteins. Extracellular stimuli regulate the activities of Rho family GTPases through control of either GEFs or GAPs. Sema3A-induced growth cone collapse has been known to require Rac1 activity in dorsal root ganglion (DRG) neurons and spinal motoneurons (Jin and Strittmatter 1997). Plexin-A1 has been shown to associate with FARP2 (FERM, Rho GEF, and pleckstrin domain protein 2), a Rac GEF, and Sema3A binding to the receptor triggers the dissociation of FARP2 from Plexin-A1 and induces Rac1 activation (Toyofuku et al. 2005) (Fig. 1.3). Concerning the role of Rac1 activity in Sema3A signaling, it was reported that Rac1 is required for Sema3A-mediated endocytosis of the growth cone plasma membranes and reorganization of F-actin in DRG neurons (Jurney et al. 2002). Endocytosis of plasma membranes is supposed to be an important step for growth cone collapse. Sema3A-induced Rac1 activation may drive endocytosis of the plasma membranes instead of membrane protrusions, resulting in growth cone collapse. In addition, Rac1 activation transduces signaling through PAK (p21-activated kinase)-LIM kinase-cofilin to reorganization of actin filaments. Another role of Rac1 in the signaling of Plexin-A is the regulation of

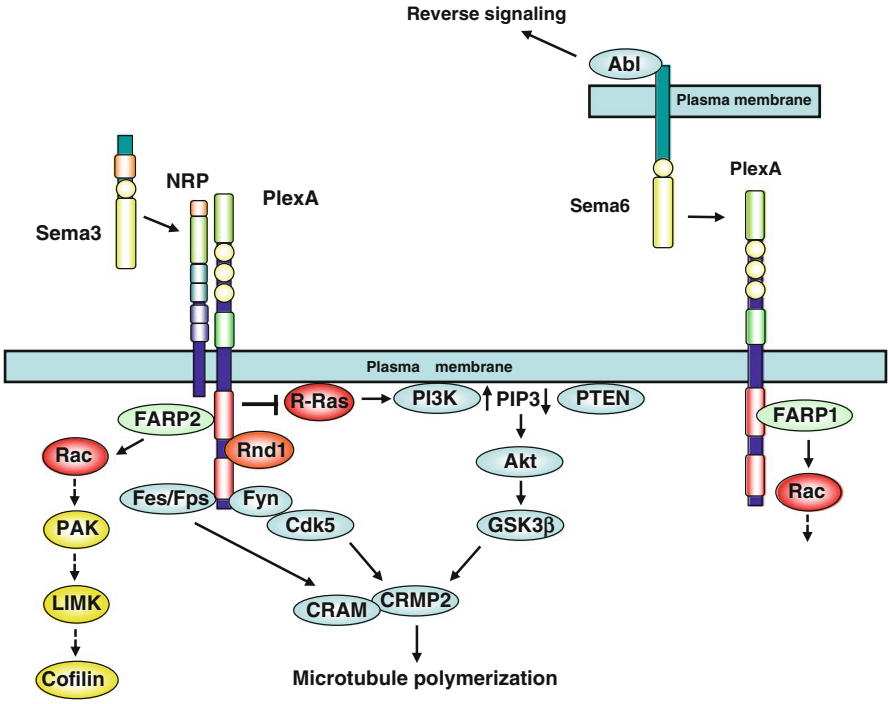

Fig. 1.3 Model for Plexin-A signal transduction. Sema3A and Sema3F bind to neuropilin-1 and -2, respectively, which associate with Plexin-A, and Plexin-A transduces repulsive signals. Several tyrosine and serine/threonine protein kinases, including Fes/Fps, Fyn, Cdk5, and GSK3β, are activated by Sema3A, and eventual phosphorylation of CRMP2 is involved in Sema3A-induced repulsive response. Sema3A stimulates R-Ras GAP activity of Plexin-A, suppressing R-Ras activity, whereas it activates Rac through a Rac GEF, FARP2. Sema6A directly binds to Plexin-A2 and -A4, whereas Sema6C binds to Plexin-A1 to elicit axon repulsion

Plexin-A GAP activity. Plexins have a GAP domain in the intracellular tail, and the GAP domain is split by the Rho family GTPase-binding domain (Negishi et al. 2005; Zhou et al. 2008). Rac1 activated by FARP2 facilitates the binding of Rnd1, a member of Rho family GTPases, to the inserted region between the split GAP domains, inducing GAP activity for R-Ras (Toyofuku et al. 2005). Dissociated FERP2 also inhibits type 1 phosphatidylinositol phosphate kinase (PIPKIγ661), resulting in suppression of integrin activation.

Tyrosine phosphorylation is involved in Sema3A/Plexin-A signaling, and Fes/Fps tyrosine kinase has been implicated in Sema3A-induced growth cone collapse (Mitsui et al. 2002). Fes/Fps is a non-receptor-type tyrosine kinase and directly binds to the cytoplasmic tail of Plexin-A1. In the unstimulated condition, neuropilin associates with Plexin-A1 and blocks the binding of Fes/Fps to Plexin-A1. Sema3A binding to neuropilin permits Fes/Fps to associate with and phosphorylate Plexin-A1. This tyrosine phosphorylation stimulates a repulsive

response in the receptor. Collapsin response mediator protein 2 (CRMP2) was originally identified as an intracellular signaling molecule of Sema3A (Goshima et al. 1995). CRAM was identified as another member of CRMPs and was shown to associate with CRMP2, forming a CRMP2–CRAM complex (Inatomi et al. 2000). Fes/Fps tyrosine-phosphorylates the CRMP2/CRAM complex as well as Plexin-A1. In addition to Fes/Fps, another kinase signaling is involved in Sema3A-mediated repulsive response. Fyn, a member of the Src family of tyrosine kinases, associates with and phosphorylates Plexin-A2 in response to Sema3A (Sasaki et al. 2002). Furthermore, serine/threonine kinase Cdk5 associates with Plexin-A2 and is activated through Sema3A-mediated Fyn; activated Cdk5 initially phosphorylates CRMP2. Then, another Ser/Thr kinase, GSK3β, subsequently phosphorylates the Cdk5-phosphorylated CRMP2, and this dually phosphorylated CRMP2 is essential for the Sema3A-induced growth cone collapse (Brown et al. 2004). CRMP2 binds to tubulin heterodimers, promoting microtubule assembly (Fukata et al. 2002). It is thus plausible that Cdk5 and GSK3β participate in Sema3A signaling through regulation of microtubule dynamics by phosphorylation of CRMP2.

In addition to Sema3s, Sema6s are ligands for Plexin-As. In contrast to Sema3s, secreted semaphorins, membrane-associated Sema6s directly bind to Plexin-As and do not require neuropilins (Fig. 1.3). Sema6A binds to Plexin-A2, controlling cerebellar granule cell migration (Kerjan et al. 2005). Sema6A binds to both Plexin-A2 and Plexin-A4, controlling lamina-restricted projection of hippocampal mossy fibers (Suto et al. 2007). Sema6A has been shown to associate with Plexin-A4 and promote the dendritic growth of spinal motor neurons through FARP1, a member of the Rho GEFs (Zhuang et al. 2009). In optic chiasm retinal ganglion cells, Sema6D has been shown to regulate axon guidance through Plexin-A1, switching the responses between repulsion and attraction dependent on the combination with Nr-CAM (Kuwajima et al. 2012). Interestingly, it has been shown that *cis* interaction between Sema6A and Plexin-A4 modulates the repulsive response to Sema6A (Topper et al. 2010). Further, reverse signaling of Sema6D has been reported, meaning that Sema6D is a Plexin-A1 ligand and also a receptor for Plexin-A1 (Toyofuku et al. 2004). Sema6D/Plexin-A1 regulates ventricular expansion, whereas Sema6D reverse signaling induces the expansion of the outer myocardial layer and trabeculation through the recruitment of Abl to the C-terminal domain of Sema6D. Thus, Sema6s/Plexin-As functions are diverse and complicated, and their downstream signaling pathways are not well characterized.

In addition to the aforementioned molecular systems, several other signaling molecules have been reported to associate with Plexin-As, including RanBPM (Togashi et al. 2006), DAP12 (Takegahara et al. 2006), and p75 neurotrophin receptor (Zvi et al. 2007). Furthermore, it has been reported that Sema3A induces intra-axonal translation of RhoA mRNA, and this local translation of RhoA is necessary and sufficient for Sema3A-mediated axonal growth cone collapse, indicating regulation of the neuronal cytoskeleton by local RhoA translation (Wu et al. 2005). These signaling molecules may contribute to many functions of the Sema3 subfamily.

1.5 Plexin-Bs

Plexin-Bs are responsible for a wide range of Sema4 subfamily functions, including axon guidance, dendrite remodeling, and angiogenesis, and Sema4s directly bind to Plexin-Bs, transducing signals. Plexin-B1 is the receptor for Sema4D, and it has been well characterized in its signaling pathways. Rho family small GTPases are also thought to be required for Plexin-B1 signaling. Active Rac1 was first reported to directly bind to the CRIB-like domain located between two GAP domains in the cytoplasmic tail of Plexin-B1 (Driessens et al. 2001). Plexin-B suppresses Rac activity by sequestering active Rac from its downstream effector PAK, inducing repulsive response in both *Drosophila* and mammalian cells (Hu et al. 2001; Vikis et al. 2002). Inhibition of Rac-mediated PAK activation by Plexin-B may suppress Rac-induced membrane protrusions, supporting the repulsive response. Furthermore, active Rac was shown to stimulate the localization of Plexin-B1 to the cell surface, enhancing Sema4D binding to the receptor (Vikis et al. 2002). Thus, signaling of Rac and Plexin-B1 appears to be bidirectional; Rac modulates Plexin-B1 activity, and Plexin-B1 modulates Rac function.

Among plexin families, the Plexin-B subfamily has a PSD-95/Dlg/ZO-1 (PDZ) domain-binding motif at the C-terminus (Fig. 1.4). Plexin-B1 directly interacts with two Rho GEFs containing PDZ domains, PDZ-RhoGEF and leukemia-associated Rho GEF (LARG), through the C-terminal PDZ binding motif (Swiercz et al. 2002; Aurandt et al. 2002; Perrot et al. 2002). They have PDZ domains at the N-terminal region and are members of the RGS-RhoGEF family, which has G12-family binding domains and specifically activates RhoA through G12-family binding. Sema4D binding to Plexin-B1 regulates the activity of PDZ-RhoGEF/LARG, leading to activation of RhoA, and this RhoA activation is involved in the Plexin-B1-induced repulsive response. It was shown that Sema4D/Plexin-B1-induced repulsive response is inhibited by an inhibitor of Rho-kinase, a well-studied Rho effector (Swiercz et al. 2002). Rho-kinase is known to phosphorylate the myosin-binding subunit of myosin phosphatase and myosin light chain, causing contraction of actomyosin (Fukata et al. 2001). The activation of myosin by Rho-kinase and the resultant contraction of actomyosin trigger cellular contraction and neurite retraction. In angiogenic systems, Sema4D/Plexin-B1 promotes endothelial cell motility through RhoA/Rho-kinase activation, and this action is mediated by integrin-dependent Pyk2 activation and the resultant stimulation of PI3kinase, Akt, and ERK through RhoA/Rho-kinase activation (Basile et al. 2007). Plexin-B1 constitutively interacts with PDZ-RhoGEF through its C-terminal PDZ domain-binding motif. How does Sema4D trigger the activation of associated PDZ-RhoGEF? It is known that ErbB-2, a tyrosine kinase receptor, associates with Plexin-B1 and tyrosine-phosphorylates Plexin-B1 in response to Sema4D (Swiercz et al. 2004). It has been shown that this ErbB-2-mediated tyrosine phosphorylation of Plexin-B1 promotes the interaction of phosphorylated Plexin-B1 with phospholipase Cγ through its SH2 domain and that phospholipase Cγ activates PDZ-RhoGEF via its SH3 domain

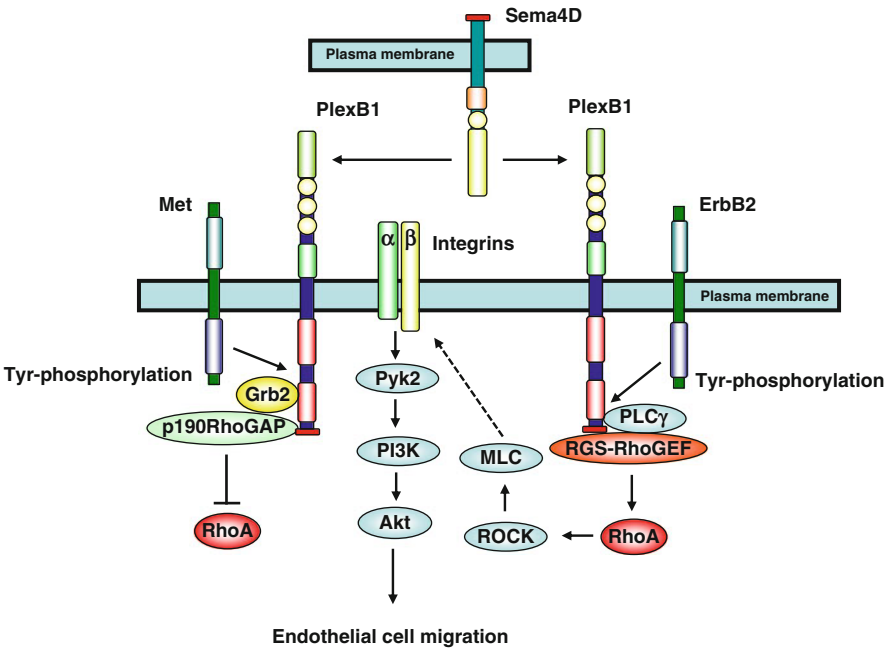

Fig. 1.4 Model for signal transduction of regulation of Rho activity by Plexin-B1. The C-terminal tail of Plexin-B1 has a PDZ-binding motif and stably associates with Rho-specific GEF, PDZ-RhoGEF, and LARG through this motif. Tyrosine phosphorylation of Plexin-B1 by ErbB2 in response to Sema4D induces phospholipase Cγ association with Plexin-B1, inducing RGS-RhoGEF and resultant RhoA activation. On the other hand, tyrosine phosphorylation of Plexin-B1 by Met triggers the association of Grb2/p190RhoGAP with Plexin-B1, activating p190RhoGAP and inhibition of RhoA activity

(Swiercz et al. 2009). This phospholipase Cγ-mediated activation of PDZ-RhoGEF is independent of its lipase activity.

Sema4D/Plexin-B1 stimulation frequently evokes different and sometimes opposing cellular responses depending on the cellular context; Sema4D/Plexin-B1 inhibits integrin-mediated cell attachment and cell migration in NIH-3T3 cells, whereas Sema4D/Plexin-B1 stimulates migration of SK-BR3 cells and endothelial cells through RhoA activation (Zhou et al. 2008). In contrast to RhoA activation by Plexin-B1 through PDZ-RhoGEF, Plexin-B1 has been shown to associate with p190 RhoGAP, leading to inhibition of cell migration (Barberis et al. 2005). Furthermore, it has been shown that interaction of Plexin-B1 with Met, another tyrosine kinase receptor, promotes the association of Plexin-B1 with p190 RhoGAP and subsequent RhoA inactivation, leading to Sema4D-mediated inhibition of cell migration (Swiercz et al. 2008). The activation of Plexin-B1 by Sema4D induces tyrosine phosphorylation of Plexin-B1 by Met, creating a docking site for the SH2 domain of Grb2, and associated Grb2 recruits p190 RhoGAP into the Plexin-B1/Grb2 complex through its SH3 domain, inducing RhoA inactivation (Sun et al. 2012).

Therefore, Plexin-B1 associates with two types of tyrosine kinase receptors, ErbB-2 and Met, and reciprocally regulates RhoA activity; the Plexin-B1/ErbB-2 complex activates RhoA through PDZ-RhoGEF, whereas Plexin-B1/Met complex inactivates RhoA through p190 RhoGAP, explaining how Sema4D exerts differential activities through governing different signaling pathways.

Plexin-B1 activates RhoA through association of PDZ-RhoGEF with the C-terminal PDZ-binding motif of Plexin-B1. However, among the entire plexin family, the C-terminal PDZ-binding motif is found only in the Plexin-B subfamily, and it is not found in invertebrate Plexin-B. Thus, it is unlikely that PDZ-RhoGEF-mediated RhoA activation is a common signaling pathway for the plexin family. The cytoplasmic tails of plexins have GAP domains. Plexin-B1 was first identified to display GAP activity for R-Ras (Oinuma et al. 2004a, b), and Plexin-A1, -D1, and -C1 have been shown to have R-Ras GAP activity in succession (Uesugi et al. 2009) (Fig. 1.5). Plexin-B1 has two subdomains, C1 and C2, showing GAP activity within the C-terminal tail, and these domains contain primary and secondary arginine motifs, respectively, which are critical motifs for GAP activity for small GTPases. Rnd1, a constitutively active atypical Rho family GTPase, binds to the region between C1 and C2, and this Rnd1 binding is essential for the expression of R-Ras GAP activity. Both Rnd1 binding to Plexin-B1 and Sema4D stimulation are indispensable for this activity (Oinuma et al. 2004a, b). In the absence of Rnd1, C1 and C2 domains interact intramolecularly, rendering the receptor inactive for R-Ras GAP activity. Rnd1 binding to the region between C1 and C2 domains disrupts the interaction of C1 and C2 domains, indicating that Rnd1 relieves the closed conformation of the C-terminal tail of Plexin-B1. This Rnd1-bound open conformation acquires an ability to associate with GTP-bound R-Ras. The Rnd1–Plexin-B1 complex can hold the GTP-bound R-Ras but not promote GTPase activity. Sema4D is homodimerized through cysteine disulfide bonds. Sema4D ligand binding to Plexin-B1 induces clustering of the Rnd1-bound monomeric receptor, and this clustering triggers the hydrolysis of GTP on R-Ras. Therefore, GAP activation of Plexin-B1 consists of two steps, interaction with GTP-bound R-Ras and promotion of GTP hydrolysis by R-Ras; the former is stimulated by Rnd1, and the latter is a process induced by the clustering by Sema4D. In addition to Plexin-B1, interaction of C1 and C2 domains has also been reported in Plexin-A1 and Plexin-D1, suggesting that this activation mechanism is a common system for the plexin family (Uesugi et al. 2009). The expression of R-Ras GAP activity of Plexin-B1 in response to Sema4D is required for the Sema4D/Plexin-B1-mediated axonal growth cone collapse. Sema4D/Plexin-B1 inhibits integrin-mediated cell migration in a variety of cells through R-Ras GAP activity (Oinuma et al. 2006). R-Ras has been shown to play a key role in cell adhesion and its activation is known to promote cell adhesion and neurite outgrowth through integrin activation (Kinbara et al. 2003). Therefore, downregulation of R-Ras activity by Sema4D/Plexin-B1 suppresses integrin activation and thereby reduces cell adhesiveness, leading to growth cone collapse and inhibition of cell migration.

Downstream signaling pathways of R-Ras GAP activity of Plexin-B1 were characterized. PI3-kinase is one of the prominent downstream effectors of R-Ras. It

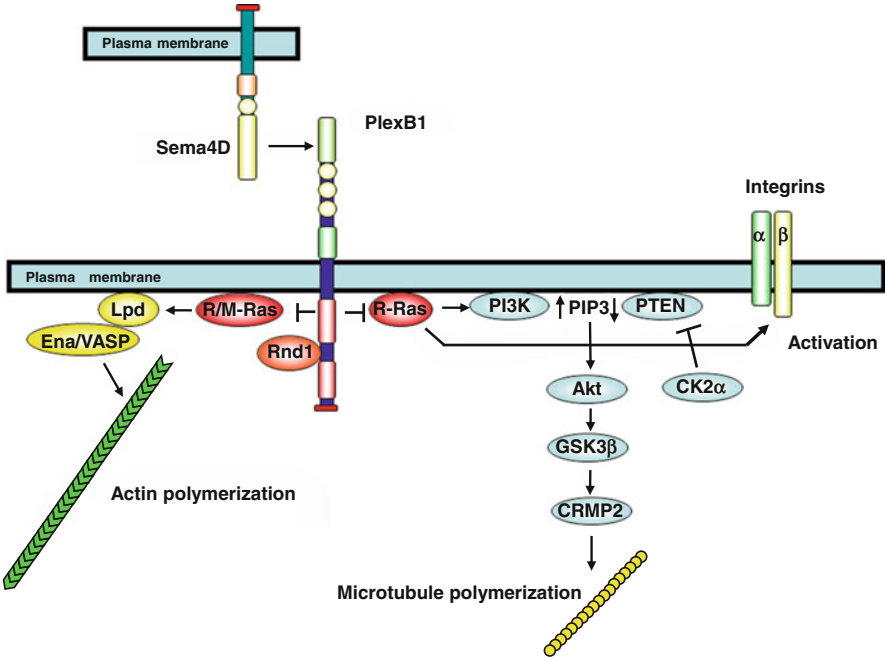

Fig. 1.5 Model for signal transduction of R-Ras GAP activity of Plexin-B1. The C1 and C2 domains of the cytoplasmic tail of Plexin-B1 encode R-Ras GAP. The C1 and C2 domains interact with each other, and Rnd1 binding to the region between C1 and C2 domains disrupts this interaction, allowing the receptor to associate with GTP-bound R-Ras. Sema4D-induced clustering of the Plexin-B1/Rnd1 complex promotes the hydrolysis of GTP by R-Ras. Downregulation of R-Ras activity inhibits integrin activity, reducing cell adhesion. Sema4D/Plexin-B1 suppresses the PI3-kinase pathway through both inhibition of R-Ras activity and PTEN activation, leading to CRMP2 phosphorylation and resultant microtubule depolymerization. R-Ras/M-Ras GAP activity suppresses R-Ras/M-Ras-mediated lamellipodin activation, leading to inhibition of actin filament polymerization

has been shown that Sema4D stimulation of Plexin-B1 inactivates PI3-kinase and Akt and activation of GSK3β through R-Ras GAP activity, and that active GSK3β phosphorylates and inactivates CRMP2, inducing axonal growth cone collapse (Ito et al. 2006). On the other hand, the phosphatidylinositol-3-phosphate level is critically regulated by PI3-kinase and PTEN (phosphatase and tensin homologue deleted chromosome 10). Phosphorylation of PTEN is known to suppress its phosphatase activity. It has been shown that Sema4D stimulation of Plexin-B1 induces dephosphorylation and activation of PTEN through R-Ras GAP activity and that this PTEN activation is also involved in Sema4D/Plexin-B1-mediated axonal growth cone collapse (Oinuma et al. 2010). Furthermore, Sema4D-induced PTEN activation via R-Ras GAP activity is mediated by the inhibition of casein kinase 2α. Thus, R-Ras GAP activity of Plexin-B1 dually regulates the phosphatidylinositol-3-phosphate level, inhibition of PI3-kinase activity, and stimulation of PTEN activity,

leading to Akt activity suppression, GSK3β activation, and CRMP2 phosphorylation and then induces repulsive response, probably through suppression of microtubule polymerization mediated by CRMP2.

The R-Ras subfamily consists of three G proteins: R-Ras, M-Ras, and TC21. Among these, Plexin-B1 displays GAP activity for R-Ras and M-Ras but not for TC21 (Saito et al. 2009). During neuronal development, R-Ras is expressed in the stage of axon specification and elongation, whereas M-Ras expression is upregulated during dendritic development, and R-Ras and M-Ras localize at the axon and dendrite, respectively (Saito et al. 2009). Sema4D/Plexin-B1 induces axonal growth cone collapse through inhibition of R-Ras activity, whereas Sema4D/Plexin-B1 induces reduction of dendrite growth through inhibition of M-Ras activity (Saito et al. 2009). Thus, Plexin-B1 is a dual functional GAP for R-Ras and M-Ras, remodeling axon and dendrite morphology, respectively. Concerning downstream signaling of M-Ras, lamellipodin was identified to be a novel effector of M-Ras (Tasaka et al. 2012). Lamellipodin is a ligand for Ena/VASP, which is an actin polymerization-promoting factor (Legg and Machesky 2004). Furthermore, Sema4D/Plexin-B1 has been shown to induce inhibition of lamellipodin action and the resultant disappearance of F-actin from distal dendrites through M-Ras GAP activity, reducing dendrite outgrowth (Tasaka et al. 2012). Thus, the M-Ras GAP activity of Plexin-B1 induces repulsive response through suppression of actin polymerization mediated by lamellipodin. Recently, plexins, including Plexin-B1, have been reported to display GAP activity for Rap1, and Rnd1 binding to plexins does not contribute to the activation of Rap GAP activity (Wang et al. 2012). However, it is not well characterized how both GAP activities, Rap GAP and R-Ras GAP, coordinately contribute to functions of plexins.

In contrast to Plexin-B1, signaling systems of the other Plexin-B subfamily receptors, Plexin-B2 and -B3, are not well characterized. Sema5A is a ligand for Plexin-B3, and Sema5A induces cell collapse of NIH3T3 fibroblasts through Plexin-B3, whereas Sema5A elicits attractive responses for epithelial and endothelial cells through the Plexin-B3/Met receptor complex (Artigiani et al. 2004). Thus, Sema5A/Plexin-B3 appears to provide reciprocal responses in cell contexts and Met may convert the Sema5A signaling.

1.6 Plexin-C1 and -D1

Plexin-D1 is a receptor for Sema3E. In contrast to the other Sema3s, Sema3E directly binds to Plexin-D1. Plexin-D1 is expressed in vascular endothelium, and Sema3E acts as a repulsive cue for the endothelial cells (Gu et al. 2005). Plexin-C1, also named VESPR for its viral origin, serves as a receptor for the virally encoded SemaVA, and Plexin-C1 stimulation induces inhibition of integrin-mediated adhesion and the chemokine-induced migration of dendritic cells (Walzer et al. 2005). Plexin-D1 and -C1 also display GAP activity for R-Ras and M-Ras, but differ from Plexin-A and -B subfamilies in the requirement for Rnd GTPases of R-

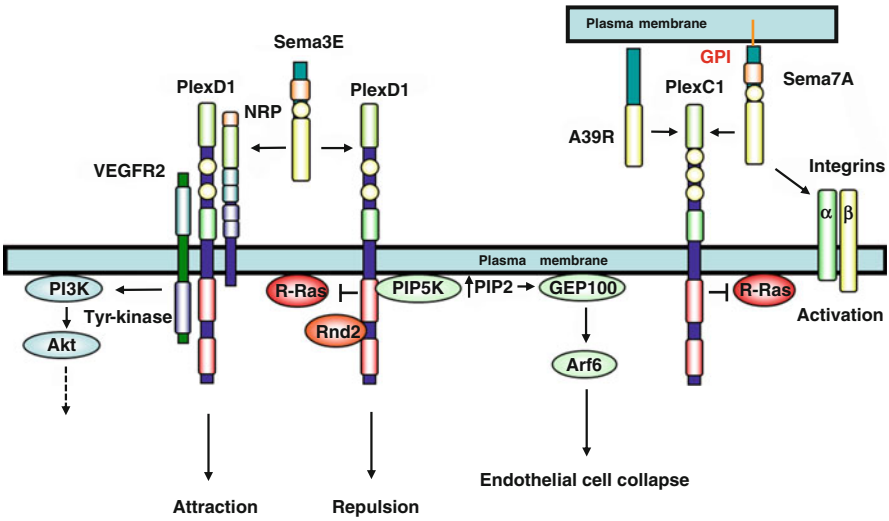

Fig. 1.6 Models for signal transduction of Plexin-C1 and D1. Sema3E directly binds to Plexin-D1 to either induce repulsive response through R-Ras GAP activity or attract response through the Plexin-D1/neuropilin/VEGF receptor complex. Plexin-C1 is a receptor for viral SemaVA or Sema7A. Plexin-C1 encodes R-Ras GAP, inhibiting R-Ras activity. Sema7A also binds to and activates integrins

Ras GAP activity (Fig. 1.6). Although Plexin-A and -B subfamilies require Rnd1 for displaying R-Ras GAP activity among Rnd subfamily GTPases, Plexin-C1 exhibits R-Ras GAP activity without the Rnd subfamily, whereas Plexin-D1 requires Rnd2 for displaying R-Ras GAP activity (Uesugi et al. 2009). Therefore, R-Ras GAP activity is a common signaling of plexin subfamilies, but the regulation of R-Ras GAP activity of plexins by Rnd proteins differs among plexin subfamilies.

Concerning antiangiogenic signaling through Plexin-D1, Sema3E/Plexin-D1 has been reported to initiate a two-pronged mechanism, R-Ras inactivation and Arf6 stimulation, which affect the status of activation of integrins and their intracellular trafficking, respectively (Sakurai et al. 2010). Furthermore, Sema3E stimulation has been shown to recruit phosphatidylinositol-4-phosphate-5-kinase to Plexin-D1, and its product, phosphatidylinositol-4,5-bis-phosphate, binds to GEP100/Brag2, a GEF for Arf6, stimulating Arf6 activity and inducing endothelial cell collapse (Sakurai et al. 2011). Sema3E/Plexin-D1 acts as the critical regulator for angiogenesis through cooperative linkage of two small GTPase signaling pathways. In contrast, Sema3E/Plexin-D1 provides reciprocal responses; Sema3E acts as a repellent for corticofugal and striatonigral neurons, expressing Plexin-D1 but not neuropilin-1, and Sema3E acts as an attractant for subiculomammillary neurons, coexpressing Plexin-D1 and neuropilin-1 (Chauvet et al. 2007). Furthermore, the latter neurons express VEGFR2 (vascular endothelial growth factor receptor type 2), and VEGFR2 associates with the Plexin-D1/neuropilin-1 receptor complex (Bellon et al. 2010). Sema3E triggers VEGFR2-dependent activation of the PI3-kinase/Akt signaling

pathway, leading to axonal growth; this activation is independent of VEGF, a ligand for VEGFR2. Thus, gating of Sema3E/Plexin-D1 signaling by neuropilin-1 switches axonal repulsion to attraction, controlling the formation of select forebrain projections.

Sema7A was reported to associate with Plexin-C1, influencing immune cell functions, such as cytokine production. However, it was clearly shown that Sema7A promotes axon outgrowth through integrin activation and initiates T-cell-mediated inflammatory responses through the integrin receptor, indicating that integrin is a receptor for Sema7A (Pasterkamp et al. 2003; Suzuki et al. 2007). On the other hand, Sema7A has been shown to suppress melanoma progression through activation of Plexin-C1-mediated R-Ras GAP activity (Scott et al. 2009). Further work is needed for characterization of the endogenous ligands of Plexin-C1.

1.7 Concluding Remarks

Here we have summarized great advances in our understanding of the molecular mechanisms for intracellular signalings of semaphorin receptors. The main receptors for semaphorins are plexins, which directly encode R-Ras GAP and exert guidance signaling through R-Ras GAP activity and the resultant regulation of integrin activity, and this GAP activity is probably a common feature of semaphorin signaling. In addition, plexins differentially regulate Rho family GTPase activities through associating with either Rho GEF or Rho GAP. Identifying various molecules involved in semaphorin signal transduction pathways provides a good understanding of the diverse molecular mechanisms for the intricate guidance functions of semaphorins. In addition to semaphorins, many guidance molecules, such as netrins, ephrins, and slits, have been identified, and their signaling systems are extensively studied. Navigation of many cells is frequently determined by the integration of various guidance cues, and the combined actions of guidance molecules influence the outcome of guidance (Dudanova and Klein 2013). To unravel the complicated signaling systems, studies of the signaling by each guidance molecule and their combined actions are required. It seems certain that semaphorins are important guidance molecules in a wide range of cellular functions. Future research will undoubtedly reveal the entire picture of the signaling cascades of semaphorins and plexins for their diverse functions.

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Chapter 2
Semaphorin Regulation of Neural Circuit Assembly in the Central Nervous System

Randal A. Hand and Alex L. Kolodkin

Abstract The assembly of neural circuits requires a wide array of molecular cues. These cues include secreted and transmembrane ligands and also the signaling receptors that together modulate axonal and dendritic morphology to promote functional neural connectivity during development. The semaphorin family of proteins and their various receptors serve this function. Many experiments demonstrate in vivo, and in multiple neural systems, how semaphorin-mediated regulation of neuronal morphology is critical for the regulation of neuronal connectivity. This system is nicely illustrated by recent work on semaphorin function in establishing neural connections in the olfactory and visual systems in both flies and mice. Further, semaphorins and their receptors regulate the elaboration of axon trajectories and precise targeting of these projections in the mammalian central nervous system, in addition to mediating axon pruning and also excitatory and inhibitory synaptogenesis. Taken together, these investigations into how semaphorins regulate neural connectivity provide insight into developmental mechanisms critical for neurite targeting, laminar-specific innervation, selective synapse formation, and neural circuit refinement.

Keywords Neural circuits • Semaphorin • Neural development • Plexin • Axon guidance • Dendritic morphology • Visual system • Olfactory system • Hippocampus • Cerebral cortex

2.1 Introduction

The establishment of a central nervous system during neural development requires a diverse and complex array of neurons to be selectively assembled into functioning neural circuits. This assembly depends upon neurons precisely responding to environmental cues to form neural circuits, both locally within a given structure and distantly among neurons residing in distinct structures. A wide range of

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neuronal guidance cues and their receptors mediate neuronal migrations, influence the elaboration of distinct neuronal morphologies, guide axons and dendrites to their appropriate targets, and also regulate synaptogenesis. Semaphorins, a large group of extracellular signaling molecules, are essential for the assembly of neural circuits in both invertebrates and vertebrates. Many studies show that semaphorins are essential for mediating short- and long-range axon guidance, sculpting dendritic morphology, facilitating synapse formation, and directing axon pruning. Here, we explore recent findings that demonstrate roles for semaphorin signaling during neural circuit formation and refinement in the central nervous system.

2.2 Semaphorins Direct Circuit Assembly in the Developing Visual System

A fully functional visual system requires precise wiring within the highly stratified neural retina (Fig. 2.1a) and also accurate central projections by retinal ganglion cells (RGCs) to appropriate central nervous system targets. Recent studies demonstrate the importance of semaphorins and their receptors for the development of neural circuits in the vertebrate and invertebrate visual systems. Here, we consider these new findings, which advance our understanding of laminar organization within the retina and of the specificity of central projections from the eye to central visual system targets in the brain.

Membrane-bound semaphorins have recently been identified as key regulators of neuronal process stratification in both vertebrate and invertebrate visual systems. The *Drosophila melanogaster* visual system is composed of several interconnected neuronal structures. The fly retina contains eight photoreceptor neuronal subtypes (R1–R8) that send axonal projections to either the lamina (R1–R6) or the medulla (R7 and R8), the first- and second-order neuropils, respectively, that initially process visual information in this system. Lamina neurons (L1–L5), in turn, form stereotypic connections with neuronal targets in the medulla (Fig. 2.1b) (Sanes and Zipursky 2010). Several recent studies show that the transmembrane semaphorin Sema-1a is a key regulator of laminar-specific connectivity within the *Drosophila* visual system.

In Sema-1a mutant flies, R1–R6 photoreceptor (PR) cell axons fail to fasciculate in a tight bundle and stray from their normal target area in the lamina. This defect in *Sema-1a* mutants can be rescued by providing wild-type Sema-1a to mutant PRs; however, Sema-1a lacking its cytoplasmic domain fails to rescue Sema-1a mutant PR axon-targeting defects, suggesting that Sema-1a functions here not as a ligand but as a receptor, so-called "reverse signaling," to regulate the stereotypic R1–R6 PR lamina innervation (Cafferty et al. 2006). In support of this finding, mutations in the *Sema-1a* receptor gene, *PlexA*, phenocopy PR axonal-targeting defects observed in *Sema-1a* mutants, and in gain-of-function (GOF) experiments full-length PlexA and also PlexA lacking its cytoplasmic domain are functionally equivalent (Yu et al. 2010). Taken together, these results suggest PlexA serves as a ligand for Sema-1a reverse signaling, a function also observed in embryonic *Drosophila* motor neurons (Jeong et al. 2012).

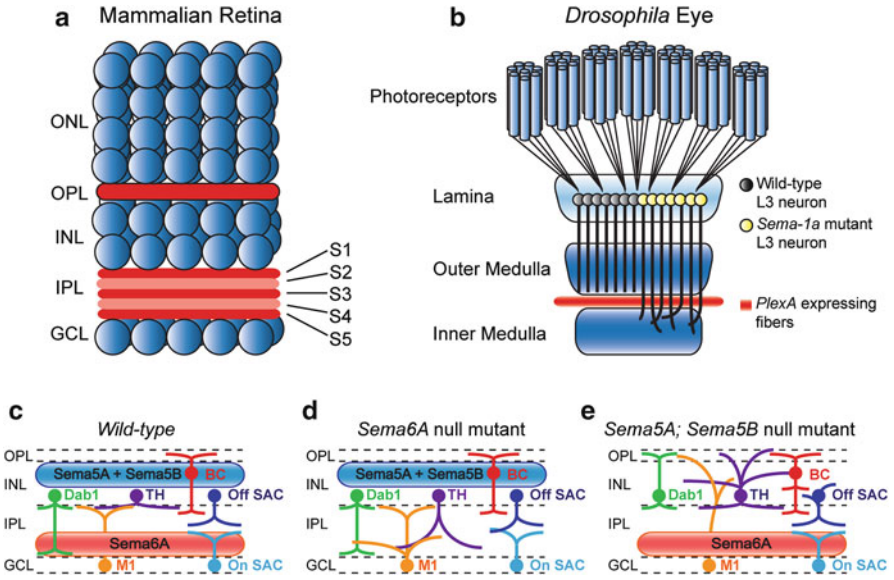

Fig. 2.1 Schematic diagrams of the mammalian retina and *Drosophila* visual system. (**a**) The mammalian retina consists of three nuclear layers (*blue*) containing neuronal cell bodies and two plexiform layers containing their neurites (*red*). (**b**) The *Drosophila* visual system includes photoreceptors that send axons to the lamina and the medulla. Lamina neurons (*grey circles*) have axons that innervate the medulla. In the Sema-1a null mutant, L3 neuron (*yellow circles*) axons overshoot their normal laminar targets in the outer medulla and extend into the inner medulla. PlexA-expressing fibers located between the outer and inner medulla serve to repel L3 axons through Sema-1a reverse signaling. (**c**) A diagram of Sema5A, Sema5B, and Sema6A expression patterns in the mouse retina, and the wild-type innervation patterns of retinal neurons: Dab1-positive amacrine cell (*Dab1*); TH-positive amacrine cell (*TH*); M1-type melanopsin intrinsically photosensitive RGC (*M1*); bipolar cells (BP); Off starburst amacrine cell (*Off SAC*); and On starburst amacrine cell (*On SAC*). (**d**) In Sema6A-null mutants, the sublaminar targeting in the IPL is disrupted in Dab1- and TH-positive amacrine cells, M1-type melanopsin intrinsically photosensitive RGCs, Off SACs, and On SACs. (**e**) Sema5A and Sema5B serve to repel neurites from the INL. In Sema5A–Sema5B double-null mutants, Dab1- and TH-positive amacrine cells and M1-type melanopsin intrinsically photosensitive RGCs and bipolar cells, and ON SACs aberrantly innervate the INL

In addition to regulating *Drosophila* PR axon guidance, Sema-1a plays a critical role in targeting axons from lamina (L) neurons to select strata within the medulla (Pecot et al. 2013). L3 neurons selectively innervate the M3 layer of the outer medulla within the developing *Drosophila* visual system. Interestingly, Sema-1a localizes to growth cones of L3 neuron axons. Following either RNAi-mediated knockdown of Sema-1a in L neurons, or single-cell genetic ablation of Sema-1a in L3 neurons using the MARCM (mosaic analysis with a repressible cell marker) technique, L3 axons project beyond the M3 layer and into the inner medulla (Fig. 2.1b). The outer region of the inner medulla contains fibers that express high levels of PlexA, and ubiquitous RNAi-mediated knockdown of PlexA results in the

mistargeting of L3 neuron axons into the inner medulla. Interestingly, these results again suggest that Sema-1a reverse signaling is important for the precise wiring of the *Drosophila* visual system, and this signaling apparently is repulsive. Taken together, these results show that transmembrane semaphorin reverse signaling is necessary for the assembly of multiple neural circuits within the invertebrate visual system.

Transmembrane semaphorins are also essential for establishing connectivity within the mammalian visual system. The mammalian retina is organized into a highly stratified structure that includes several nuclear layers: the outer nuclear layer (ONL), composed of rod and cone photoreceptor cell bodies; the inner nuclear layer (INL), composed of cell bodies belonging to bipolar cells, horizontal cells, and amacrine cells; and the ganglion cell layer (GCL), which includes cell bodies of retinal ganglion cells (RGCs) and displaced amacrine cells. Between these nuclear layers exist plexiform layers: dense networks of neurites and synapses that serve to interconnect neurons whose cell bodies reside in the nuclear layers. The outer plexiform layer (OPL) is located between the ONL and the INL and is where photoreceptors, bipolar cells, and horizontal cell establish contacts with one another. The inner plexiform layer (IPL) is located between the INL and the GCL, contains multiple sublamina (S1–S5, S5 being located closest to the GCL), and serves to interconnect neurites extending from bipolar cells, amacrine cells, and RGCs (Fig. 2.1a) (Sanes and Zipursky 2010). RGCs are the projection neurons of the retina, and their axons extend into the central nervous system.

Transmembrane semaphorins Sema5A, Sema5B, and Sema6A are essential for establishing neural circuit formation within the plexiform layers of the mammalian retina (Fig. 2.1c–e). Sema5A and Sema5B are both expressed within the INL, where they serve to organize and constrain neurites that normally stratify within the IPL from extending into outer retina regions (Matsuoka et al. 2011a). In *Sema5A*; *Sema5B* double-null mutants, neurites from amacrine cells, bipolar cells, and RGCs fail to target in appropriate IPL sublamina and instead extend toward the INL and into the INL. Some of these neurites even extended all the way into the OPL. Type 1 and 2 Off cone-bipolar cells normally innervate both the OPL and IPL, but in *Sema5A*; *Sema5B* double mutants bipolar cell neurites were also observed innervating the INL. Sema5A and Sema5B in the developing retina serve as ligands for the PlexA1 and PlexA3 receptors, and *PlexA1*; *PlexA3* double-null mutants phenocopy the neurite targeting errors observed in *Sema5A*; *Sema5B* double-null mutants (Matsuoka et al. 2011a). Therefore, transmembrane Sema5 proteins play a general role in establishing correct neurite arborization in IPL sublaminae through their ability to constrain neurites to this plexiform layer. But how is specific laminar targeting by neurites from individual neuronal subtypes in the retina achieved?

In the developing chick retina, GOF and LOF experiments show that adhesion molecules belonging to the immunoglobulin (IG) superfamily provide short-range cues important for specific stratification events among BPs, RGCs, and ACs that co-stratify within the IPL (Sanes and Yamagata 2009). Recent studies demonstrate the importance of repulsive guidance in regulating laminar-specific targeting and retinal neuron morphology, showing that the transmembrane semaphorin Sema6A

and its receptors, PlexA2 and PlexA4, serve key roles in establishing the sublaminar organization of the IPL and regulating the arborization of horizontal cell and On starburst amacrine cell (SAC) neurites. Reminiscent of Sema-1a and PlexA expression patterns in the outer and inner medulla of the *Drosophila* eye (Pecot et al. 2013), Sema6A is highly expressed in the IPL of the mouse retina in neurites within sublaminae S4 and S5, whereas PlexA4 is highly expressed in a small subset of neurites that stratify their projections in sublaminae S1 and S2. In either *Sema6A* or *PlexA4* null mutants, neurites from calbindin-positive amacrine cells, dopaminergic amacrine cells, and M1-type melanopsin intrinsically photosensitive RGCs (ipRGCs) innervate inappropriate sublaminae within the IPL, consistent with Sema6A serving to constrain a small subset of amacrine cell and RGC neurites to laminar targets in the outer IPL (Matsuoka et al. 2011b). Sema6A and PlexA4 protein expression is complementary, even very early in the development of IPL stratification (for example, at postnatal day 2), suggesting that the initial establishment of repulsive IPL regions sets the stage for specific laminar targeting observed later in retinal development. Sema6A also regulates the sublaminar targeting of SAC neurites in the IPL (Sun et al. 2013). Interestingly, Off SAC neurites innervate the S2 IPL lamina and express PlexA2, whereas On SAC neurites innervate S4 and express both Sema6A and PlexA2. In *Sema6A* and *PlexA2* null mutants, neurites from both Off and On SACs often fail to stratify into their appropriate sublaminae and exhibit "crossovers" as they extend between S2 and S4, normally the exclusive targets of Off SACs and On SACS, respectively (Sun et al. 2013) (Fig. 2.2).

In addition to regulating the sublaminar targeting of IPL amacrine cells and RGC neurites, Sema6A also controls the arborization of horizontal cell neurites in the OPL and On, but not Off, SACs in the IPL. Horizontal cells express both Sema6A and PlexA4, and in Sema6A and PlexA4 mutants horizontal cell neurites exhibit a reduction in self-avoidance, leading to an increase in dendrite self-crossing and also increased fasciculation among horizontal cell neurites; this results in an overall reduction in horizontal cell neurite coverage in the OPL (Matsuoka et al. 2012). A more dramatic dendritic arborization phenotype is observed in On SAC neurites of *Sema6A* and *PlexA2* mutants (Fig. 2.2). In these mutant On SACs, dendritic fields of individual SACs are dramatically reduced, accompanied by a loss of SAC dendritic arbor symmetry, a phenotype that suggests reduced dendrite self-avoidance during the course of On SAC dendritic elaboration during postnatal retinal development is regulated by Sema6A–PlexA2 signaling (Sun et al. 2013). Interestingly, On–Off direction-selective (DS) RGCs in *Sema6A* mutants exhibit normal Off DS tuning responses, however, On direction selective tuning responses are compromised (Fig. 2.2). Therefore, the On SAC morphological defects observed in *Sema6A* and *PlexA2* mutants correlate with defective Sema6A On DS responses, demonstrating how semaphorin–plexin signaling is critical for establishing distinct On and Off DS responses by On–Off DS RGCs. These On SAC dendritic arborization phenotypes appear quite distinct from those observed in mice harboring mutations that affect protocadherin expression (Lefebvre et al. 2012), suggesting that distinct repulsive signaling mechanisms together contribute to the

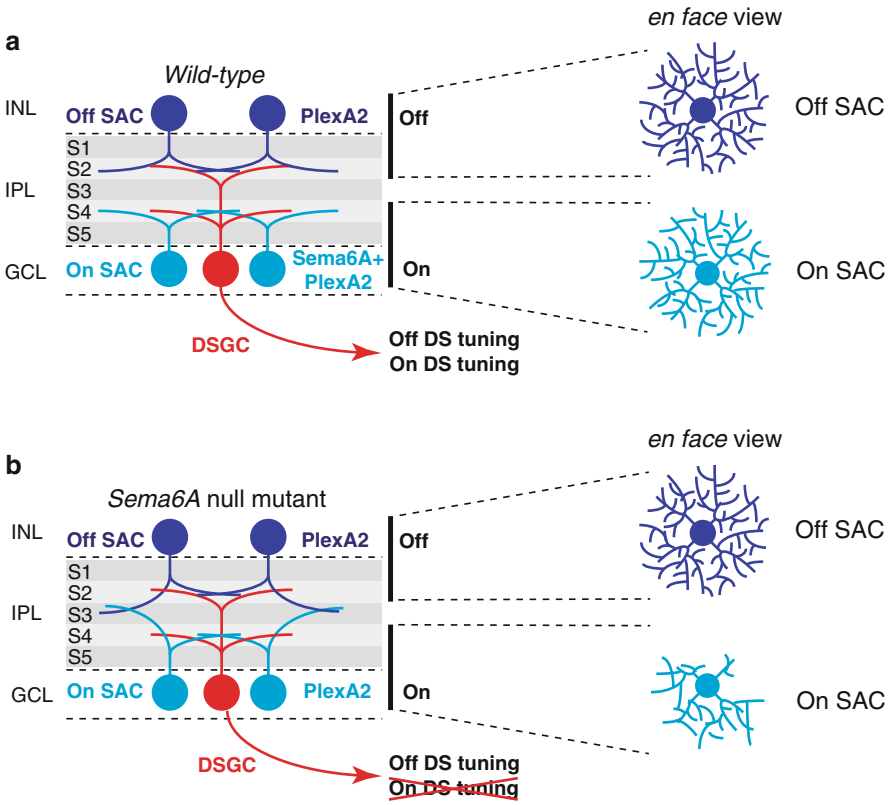

Fig. 2.2 Sema6A ensures On–Off SAC stratification and On SAC dendrite self-avoidance. (**a**) Schematic diagram shows sublaminar targeting in wild-type mouse retinas of Off SACs in S2, of On SACs in S4, and of direction-selective retinal ganglion cells (*DSGC*) in both S2 and S4. An en face view of Off SACs and On SACs reveals dendrite elaboration within their respective sublaminae. (**b**) In *Sema6A* null mutants, Off and On SACs fail to stratify, and On SACs neurites display self-avoidance defects and hyperfasciculation of dendritic processes. These defects ultimately lead to defects in DSGC On, but not Off, direction-selective (*DS*) tuning responses by On-Off DSGCs

radially symmetrical nature of nonoverlapping SAC dendritic morphology. These results, taken together, demonstrate the importance of transmembrane semaphorins in critical short-range repulsive guidance events that contribute to the establishment of proper laminar stratification, neuronal morphology, and connectivity within the retina.

Semaphorins and their receptors also are important in the guidance of central RGC projections. As RGC axons leave the eye and project centrally, they must navigate through the optic chiasm, a site where the majority of RGC axons cross the central nervous system (CNS) midline. The transmembrane semaphorin Sema6D, its PlexA1 receptor, and the NrCAM Ig superfamily adhesion molecule are key

mediators of midline crossing by RGC axons (Kuwajima et al. 2012). Sema6D is expressed by glial cells located at the CNS midline within the optic chiasm. PlexA1, a known Sema6D receptor (Toyofuku et al. 2004), and NrCAM, which binds Sema6D (Kuwajima et al. 2012), are both expressed in RGC axons. In either *Sema6D* mutants or *PlexA1*; *NrCAM* double mutants, RGC axons that normally cross the midline at the optic chiasm and project to central targets were found to either defasciculate at the optic chiasm or to fail to cross the midline altogether and instead project toward ipsilateral targets (Kuwajima et al. 2012). These phenotypes result from a loss not only of Sema6A-mediated midline repulsion, but also additional Sema6A attractive effects on RGC axons that are dependent upon PlexA1 and NrCAM, both of which are also expressed in the chiasm. In the zebrafish, secreted semaphorins also regulate RGC axon CNS midline crossing. Sema3D and Sema3E are expressed in tissue surrounding the optic chiasm. In zebrafish where either Sema3D or Sema3E is knocked down, or in *Sema3D* null mutants, the percentage of ipsilaterally projecting RGC axons is dramatically increased (Sakai and Halloran 2006; Dell et al. 2013). Neuropilin-1a (Nrp1a), a receptor for Sema3D and Sema3E, is expressed in zebrafish RCGs, and knockdown of Nrp1a results in an increase in the number of ipsilaterally projecting RGC axons (Dell et al. 2013). Interestingly, in the mouse visual system Nrp1 is required for RGC axons to successfully navigate through the optic chiasm; however, this is apparently achieved through the action of VEGF164, not class three secreted semaphorins (Erskine et al. 2011). These findings further demonstrate the importance of both transmembrane and secreted semaphorins for the guidance of RGC axons as they navigate toward their central nervous system targets.

2.3 Semaphorins Direct Wiring of the Developing Olfactory System

The secreted semaphorins Sema3A and Sema3F and their receptors Nrp1 and Nrp2, respectively, serve well-established functions during the formation of neural circuits within the mouse olfactory system (Schwarting et al. 2000, 2004; Cloutier et al. 2002, 2004; Walz et al. 2002, 2007; Matsuo et al. 2012). These studies demonstrate the importance of secreted semaphorins in the guidance and selective fasciculation of olfactory receptor neuron (ORN) axons that target both the main olfactory bulb (MOB) and the accessory olfactory bulb (AOB) of mice. Here, we explore recent findings showing that both secreted and transmembrane semaphorins regulate olfactory system innervation through axon–axon interactions and dendritic targeting.

The olfactory systems of vertebrates and invertebrates share a common neural circuit blueprint (Imai et al. 2010). Each ORN is located in a sensory organ and expresses a single functional odorant receptor (OR). ORNs project an axon to specialized central targets called glomeruli, regions where ORN axons connect to

dendrites of projection neurons. Interestingly, multiple ORNs expressing the same OR innervate the same specific glomerulus, and this is conserved from animal to animal. Together, all these observations lead to the principle that each ORN expresses a single OR, and OR expression defines innervation of specific individual glomeruli during olfactory system wiring. Although the mouse olfactory system, with more than 2 million ORNs, 1,000 ORs, and 2,000 glomeruli, is larger in scale than that of *Drosophila*, which contains 1,300 ORNs, 62 ORs, and 50 glomeruli, both have evolved somewhat similar strategies for ORN axon segregation to ensure the fidelity of ORN axon targeting to specific glomeruli.

Several studies from the Sakano laboratory have elucidated essential functions for secreted semaphorins in regulating the segregation of ORN axons as they project from the mouse olfactory epithelium to their targets in the MOB. With respect to ORN projections to the MOB along the anterior-posterior axis, graded levels of ORN intracellular cAMP, low anterior to high posterior with respect to MOB targeting, are generated from non-ligand-induced OR activity; this results in defined gradients of Nrp1 and Sema3a expression in ORNs (Imai et al. 2006). ORs are G protein-coupled receptors (GPCRs) that utilize the α-G-protein subunit G_{olf} to induce neural signaling in postnatal animals; however, during embryogenesis it is the α-G-protein subunit G_s that is critical for the generation of cAMP through basal, agonist-independent, activity of ORs (Nakashima et al. 2013). ORNs with high levels of cAMP, which induce high levels of Nrp1 expression, innervate glomeruli in more posterior regions of the dorsal MOB. Interestingly, ORNs with low levels of cAMP, which results in high Sema3a expression, innervate glomeruli in more anterior regions of the MOB (Imai et al. 2006, 2009). Mutant ORNs lacking Nrp1 or G_s have an altered trajectory and innervate glomeruli anterior to their normal target glomerulus. A striking phenotype observed in mouse mutants where Nrp1 or Sema3A is removed only in ORNs occurs within the ORN axon bundle itself, and this can be observed as ORN axons project toward the MOB. Within the ORN axon bundle, the Nrp1-expressing axons, which are destined to innervate posterior MOB glomeruli, are fully segregated from Sema3A-expressing ORN axons, which will innervate the anterior glomeruli. This segregation is dependent on Sema3A expression in ORNs, and it occurs before ORN axons reach the MOB (Fig. 2.3). Further, this segregation still occurs in mice that lack their MOB (Imai et al. 2009). Taken together, these results suggest that axon–axon interactions mediated by Sema3A and Nrp1 are critical for correct innervation of MOB glomeruli by ORNs. In addition to Sema3A, the secreted semaphorin Sema3F and its holoreceptor complex Nrp2/PlexA3 also play an essential, but distinct, role in topographic innervation of the MOB, here along the dorsal-ventral axis. Sema3F expression is limited to a subset of ORN axons that first arrive in the dorsal MOB; later-arriving ORNs do not express Sema3F at high levels but do express high levels of Nrp2 (Fig. 2.3). In *Sema3F*, *Nrp2*, and *PlexA3* mutants there is a dorsal shift in glomeruli innervation. These results implicate Sema3F release from ORNs as being required for topographic ORN innervation along the dorsal-ventral axis in the MOB. Taken together, these results demonstrate the importance of secreted semaphorins

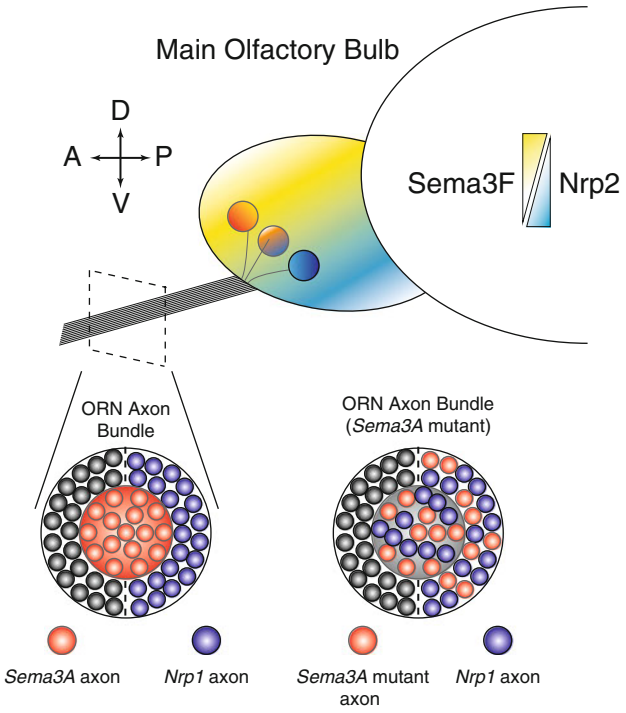

Fig. 2.3 Sema3A and Sema3F regulate olfactory neuron axon glomerular innervation in the mouse main olfactory bulb. Olfactory receptor neuron (ORN) axons fully segregate as they project toward the main olfactory bulb (MOB) in a Sema3A/Nrp1-dependent manner. This pre-target sorting of ORN axons results in axons that express high levels of Sema3A-innervating glomeruli in the anterior MOB and axons that express high levels of Nrp1-innervating posterior MOB glomeruli. ORN axons that innervate dorsal MOB glomeruli arrive in the MOB first and express high levels of Sema3F. ORN axons expressing high levels of Nrp2 arrive later and innervate the ventral glomeruli, a targeting event that requires ORN-expressed Sema3F

in regulating topographic innervation of the mouse MOB through ORN axon–axon interaction mechanisms.

Semaphorins also are critical for establishing neural connectivity in the invertebrate olfactory system. Interestingly, semaphorin regulation of axon–axon interactions is essential for regulating topographic innervation of glomeruli in the antennal lobe by ORNs residing in the third antennal segment and also in the maxillary palp of the developing *Drosophila* olfactory system. The transmembrane semaphorin Sema-1a and its PlexA receptor are required for proper topographic innervation of the antennal lobe by maxillary palp ORNs. Using a molecular mechanism that is reminiscent of Sema3F regulation of ORN axon targeting in the mouse MOB (see above), Sema-1a expression on axons from ORNs in the third antenna segment is essential for proper targeting of glomeruli by axons from ORNs in the maxillary palp (Fig. 2.4a). Sema-1a-expressing ORN axons from the third segment of the

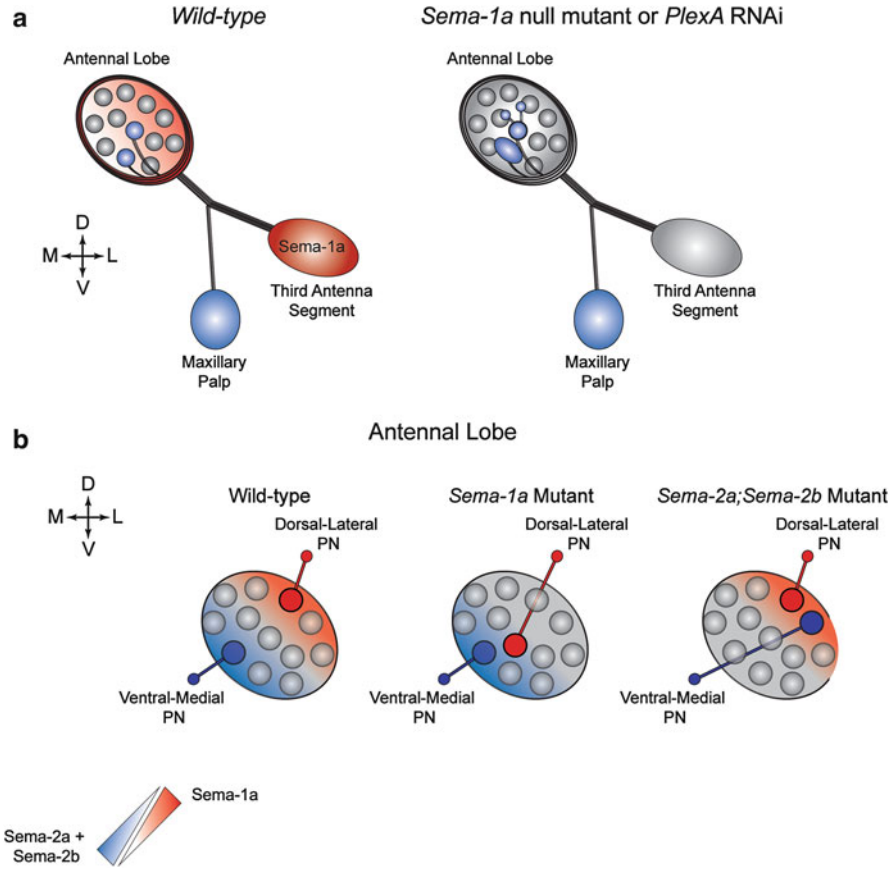

Fig. 2.4 Semaphorins regulate glomerular targeting by olfactory receptor neuron axons and projection neuron dendrites in the *Drosophila* antennal lobe. (**a**) In wild-type animals, ORN axons from the third antennal segment express Sema-1a and innervate the antennal lobe before the arrival of ORN axons from the maxillary palp. Sema-1a-expressing axons from the third antennal segment serve to constrain maxillary palp ORN axons, allowing them to project to their appropriate glomeruli. In *Sema-1a* null mutant pupae, or pupae expressing PlexA RNAi, ORN axons from the maxillary palp fail to innervate their appropriate target glomeruli. (**b**) Projection neurons (PNs) selectively target their dendrites to appropriate antennal lobe locations. Within the antennal lobe there is a high dorsolateral to low ventromedial gradient of Sema-1a expression and a high ventromedial to low dorsolateral Sema2a–Sema2b gradient. In *Sema-1a* null mutant animals, PNs that typically target the dorsolateral antennal lobe innervate ventromedial regions. Further, in *Sema2a*; *Sema2b* double-null mutants, PNs that normally target ventromedial antennal lobe regions instead innervate more dorsolateral target areas

antenna are the first to arrive in the antennal lobe, and they serve to constrain later-arriving ORN axons from the maxillary palp through Sema-1a-mediated axon–axon interactions within the antennal lobe (Lattemann et al. 2007; Sweeney et al. 2007). The secreted semaphorin Sema2b and its receptor PlexB also regulate axon–axon

interactions during ORN innervation of the antennal lobe. ORN axons extending from the antenna take either a dorsolateral or ventromedial trajectory to innervate the antennal lobe. Only those ORN axons that take the ventromedial trajectory express Sema2b. Sema2b and PlexB expression in antenna ORN axons are both required for proper trajectory and glomerular targeting by axons in the ventromedial region of the antennal lobe; they are dispensable for innervation of the dorsolateral region of the antennal lobe. Sema2b functions as an attractive cue, and in addition to being expressed on ventromedial ORN axons it is also expressed in the ventromedial target region of the antennal lobe. Therefore, Sema2b mediates both ORN axon–axon interactions and ORN axon–target interactions, demonstrating how a single guidance cue can function at multiple stages of axon targeting to drive select neural circuit assembly (Joo et al. 2013). These results in both *Drosophila* and mouse olfactory systems demonstrate highly conserved molecular mechanisms by which semaphorins regulate topographic mapping through a series of axon–axon interactions, both along ORN trajectories and between ORNs and their targets.

Recent work also illuminates a role for semaphorins in regulating dendritic targeting in the *Drosophila* antennal lobe. Projection neurons (PNs) are located proximal to the antennal lobe, target their dendrites to individual olfactory glomeruli, and have an axon that targets both the mushroom body and the lateral horn. PN dendrites target individual glomeruli before they are innervated by pupal ORNs. Both secreted and transmembrane semaphorins establish overlapping gradients in the developing antennal lobe that regulate PN dendritic targeting (Komiyama et al. 2007; Sweeney et al. 2011). Sema-1a is expressed in a high dorsolateral to low ventromedial gradient across the antennal lobe in PN dendrites. In the absence of Sema-1a, PN dendrites that would normally target the dorsolateral antennal lobe instead innervate more ventral regions (Fig. 2.4b). Expression of full-length Sema-1a in PN neurons was sufficient to rescue this defect, but rescue experiments using Sema-1a lacking its intracellular domain failed to rescue the dendritic targeting phenotype (Komiyama et al. 2007). This work provided the initial example of transmembrane semaphorin reverse signaling. In the antennal lobe, the secreted semaphorins Sema2a and Sema2b are expressed in a high ventromedial to low dorsolateral gradient, complementary to the Sema-1a expression pattern. In *Sema2a*; *Sema2b* double-null mutants, dendrites from PNs that normally target the ventromedial regions of the antennal lobe innervate dorsal regions, suggesting that Sema2a and Sema2b serve to constrain targeting of ventromedial projecting PN dendrites away from the dorsal antennal lobe (Sweeney et al. 2011) (Fig. 2.4b). Although Sema2a expression in larval ORN axons was sufficient to rescue the Sema2a–Sema2b double-mutant PN dendritic targeting phenotype, a striking result from this study was that the known semaphorin receptors PlexA and PlexB were dispensable for ventromedial PN dendrite targeting. Interestingly, the *Sema2a*; *Sema2b* double-mutant PN dendritic targeting phenotype is qualitatively similar to that observed following loss of Sema-1a in PNs, Sema2a/2b expression is opposite to that of Sema-1a in the developing antennal lobe, and the extracellular domain of Sema-1a binds to Sema2a exogenously expressed by cells in fly wing disc and in brain neurons that overexpress Sema2a. This observation suggests the intriguing

possibility that Sema2a and Sema2b signal through Sema-1a to control dendritic targeting, and future work will confirm the identity of the Sema2a/2b receptor or receptor complex that serves to guide PN dendrites to their appropriate antennal lobe regions. Taken together, these studies show an important role for both secreted and transmembrane semaphorins in directing topographic dendritic targeting in the developing *Drosophila* olfactory system.

2.4 Semaphorin Regulation of Neural Circuit Formation in the Brain

Semaphorins direct the formation of a wide range of neural circuits within the mammalian brain. Semaphorin signaling is required for many conserved aspects of neural circuit assembly, including the successful guidance of axons to their innervation sites, formation of synapses, and the refinement of neural circuits through pruning of axons and synapses. In recent years, many studies have identified roles for semaphorins in these processes, and novel molecular mechanisms of semaphorin signaling have been uncovered. Here, we consider some of these findings that provide new insights into the molecular mechanisms utilized by semaphorins during neural circuit formation in the mammalian brain.

To regulate axon guidance, class three secreted semaphorins typically signal through a holoreceptor complex by binding directly to either Nrp1 or Nrp2 and forming a complex with a PlexA receptor family member that mediates signal transduction (Tran et al. 2007). However, there are exceptions to this rule, and there is growing evidence that interactions between semaphorin receptors and additional transmembrane receptors modulate axon guidance responses to class three semaphorins. One notable exception is the secreted semaphorin Sema3E, which can bind directly to the PlexD1 receptor independent of Nrp1 or Nrp2 (Gu et al. 2005). Although Sema3E and PlexD1 were originally characterized for their roles in regulating vascular development, they are now known to play critical roles in the development of several neural circuits in the brain. In a recent study, Sema3E expression in the striatum was shown to be required for the convergence of cortical thalamic axons (CTAs) and thalamocortical axons (TCAs); this is achieved by stalling PlexD1-expressing CTAs in the striatum until TCAs arrive to converge and guide the CTAs along their appropriate trajectory (Deck et al. 2013). Similar to many other axon guidance cues, Sema3E can serve as either an attractive or repulsive guidance cue for axons navigating through the brain. For example, Sema3E was shown to mediate attraction through a novel signaling complex. In the brain, the cortical fugal and subiculomammillary tracts run in close proximity to one another, and they both express PlexD1. The cortical neurons are repelled by Sema3E; however, the subicular neurons are attracted to Sema3E (Chauvet et al. 2007). How is this differential response achieved? PlexD1 is required for both responses, and PlexD1 mutants have defects in both the cortical fugal and

subiculomammillary tracts. Interestingly, subicular neurons also express Nrp1, and the application of exogenous Nrp1 ectodomain is sufficient to convert Sema3E from a repellent to an attractant for cortical neurons (Chauvet et al. 2007; Bellon et al. 2010). The vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) is also necessary for Sema3E-mediated attraction of subicular neurons, and Sema3E can form a complex with PlexD1/Nrp1/VEGFR2 in a heterologous system (Bellon et al. 2010). These studies demonstrate the importance of Sema3E as a bifunctional guidance cue for establishing neural circuits within the brain, and they provide evidence of unconventional semaphorin signaling.

The secreted semaphorin Sema3F was also shown to have a bifunctional role in regulating innervation in the dopaminergic system. Mesodiencephalic dopaminergic (mdDA) neurons located in the medial ventral tegmental area (mVTA) project axons into the prefrontal cortex (PFC). In either Sema3F or Nrp2 mutants, mdDA neuron axons display both fasciculation and targeting defects along the trajectory of this neural circuit (Kolk et al. 2009). An interesting result in this study is an apparent change in response to Sema3F by mdDA neurons in the mVTA. At early embryonic time points Sema3F is attractive to mdDA neurons, but as the embryos developed this attractive response to Sema3F shifts to repulsion. The precise molecular mechanism that mediates this transition from attraction to repulsion remains to be elucidated.

In the spinal cord and in the brain, L1 Ig superfamily adhesion molecule family members have been shown to be modulators of class three semaphorin signaling (Castellani et al. 2000; Falk et al. 2005). Since these studies were presented, additional evidence supporting the importance of Ig superfamily-mediated adhesion in neuronal guidance has been obtained in the context of TCA topographic projections into the cortex. The *Close homologue of L1 (CHL1)* gene is expressed in thalamic neurons along with Nrp1. CHL1 was found to form a complex with Nrp1 and mediate Sema3A growth cone collapse in cultured thalamic neurons. In either *CHL1* mutants or $Nrp1^{Sema}$ mutants (an *Nrp1* allele deficient in secreted semaphorin binding) (Gu et al. 2003), somatosensory TCAs become misrouted and innervate the visual cortex (Wright et al. 2007). In a related study, another L1 adhesion family member, NrCAM, was found to be coexpressed with Nrp2 in thalamic neurons and to form an NrCAM/Nrp2 protein complex. NrCAM was also found to be necessary for Sema3F-mediated growth cone collapse of cultured thalamic neurons. In vivo, motor and somatosensory TCAs from both NrCAM and Nrp2 mutants aberrantly innervate the visual cortex (Demyanenko et al. 2011). These studies provide further evidence that L1 adhesion molecule family members are important modulators of semaphorin-regulated axon guidance.

In addition to sculpting the overall trajectories that define a wide range of axon tracts in the mammalian brain, semaphorins also play instrumental roles in controlling the precise innervation of laminated structures in the brain. In the cerebellum, basket cells located in the molecular layer densely innervate the axon hillock of Purkinje cells located in the Purkinje cell layer (PCL). Basket cells express Nrp1, but Sema3A is expressed specifically in the PCL. In either Sema3A or $Nrp1^{Sema}$ mutants, a dramatic reduction in basket cell axon branching occurs

in the PCL, resulting in a reduction in innervation of the Purkinje cell axon hillock (Cioni et al. 2013). In contrast to class three secreted semaphorin promotion of laminar innervation in the cerebellum, transmembrane semaphorins serve to constrain laminar innervation in the hippocampus. In the hippocampus, axons from dentate gyrus (DG) granule cell (GC) neurons project within the two mossy fiber tracts into the CA3 region. The main bundle, or suprapyramidal tract, innervates the apical dendrites of CA3 pyramidal neurons, whereas the much less organized infrapyramidal tract (IPT) innervates the basal dendrites of CA3 pyramidal neurons. The transmembrane semaphorins Sema6A and Sema6B are expressed in CA3 pyramidal neurons, and they serve to constrain the main mossy fiber tract to the surprapyramidal region (Suto et al. 2007; Tawarayama et al. 2010). Interestingly, both *PlexA2* and *PlexA4* single mutants exhibit a more severe phenotype than is observed in the *Sema6A*; *Sema6B* double-null mutant, suggesting that additional ligands may serve to regulate mossy fiber laminar innervation in CA3. Future studies will determine whether other semaphorins also contribute to hippocampal laminar innervation.

Semaphorins also regulate the precise innervation of target tissues through the refinement of synapses following initial innervation events. In the mouse hippocampus, a signaling cascade that regulates pruning of the infrapyarmidal tract (IPT) was recently elucidated. During postnatal development, DG axons in the IPT extend along the basal dendrites of CA3 pyramidal neurons. However, as the mice approach adulthood (between postnatal days 20 and 40), the IPT undergoes retraction-mediated axon pruning and is significantly shortened (Bagri et al. 2003). This response is dependent on Sema3F signaling through the Nrp2/PlexA3 holoreceptor complex (Bagri et al. 2003; Liu et al. 2005; Riccomagno et al. 2012). β2-Chimaerin (β2Chn) is a Rho GTPase-activating protein (GAP) specific for Rac, and Sema3F robustly activates its GTPase activity. β2Chn is required for Sema3F/Nrp2/PlexA3 IPT pruning, and this IPT pruning is achieved through the downregulation of Rac activity (Riccomagno et al. 2012). Remarkably, β2Chn is dispensable for Sema3F-mediated repulsive axon guidance, and also for Sema3F-mediated dendritic spine constraint, and therefore these observations provide the first example of a semaphorin-mediated signaling mechanism that selectively regulates axon pruning. In addition to retraction-mediated pruning, Nrp2 and PlexA3 are necessary for degeneration-like pruning of mouse cortical neurons. Layer V pyramidal neurons in the visual cortex initially innervate regions of both the motor and visual systems through the formation of extensive collateral axon branches. During postnatal development, collateral axons from visual cortex layer V pyramidal neurons that innervate the motor system undergo Nrp2/PlexA3-dependent degeneration-like pruning (Low et al. 2008). Nrp2 and PlexA3 mutants fail to prune axons extending from the visual cortex into areas devoted to motor systems. It remains to be determined exactly which semaphorin signaling events govern these cortical axon pruning events. These examples demonstrate the importance of semaphorin signaling in the refinement of neural circuit formation in the brain.

In addition to regulating axon guidance and pruning in the CNS, semaphorins also modulate neural circuit formation by selectively regulating synapse formation

in several systems within the brain. For example, Sema3F signaling through its Nrp2/PlexA3 holoreceptor complex constrains dendritic spine elaboration and synaptogenesis in layer V pyramidal neurons of the cerebral cortex and hippocampal DG cells (Tran et al. 2009). In *Sema3F*, *Nrp2*, and *PlexA3* mutants, a significant increase in the density of spines and glutamatergic excitatory synapses is observed. Strikingly, in layer V pyramidal neurons the increase in dendritic spine density is observed only along the apical dendrite in vivo, and in cultured deep layer cortical neurons the Nrp2 co-receptor is also observed to be localized only on apical dendrites and not on basal dendritic processes (Tran et al. 2009). Therefore, Sema3F signaling through Nrp2/PlexA3 selectively constrains excitatory synapses and dendritic spines to a subdomain within cortical pyramidal neuron dendrites. Sema3E and PlexD1 also selectively limit excitatory synapse formation. Within the adult striatum, PlexD1 is exclusively expressed within the medium spiny neurons (MSNs) in the direct dopaminergic pathway. These MSNs receive excitatory glutamatergic input from both cortical neurons and thalamic neurons. In *Sema3E* and *PlexD1* mutants, MSNs in the direct pathway exhibit an increase in excitatory synaptic input from thalamic neurons, and MSNs display an increase in synapses formed selectively on their somas and dendritic shafts (Ding et al. 2012). The thalamic axons are a source of Sema3E in the adult striatum, and Sema3E/PlexD1 signaling apparently plays an important feedback role in balancing excitatory synaptic input in direct pathway MSNs. Although class three semaphorins are important for constraining excitatory input, there is now growing evidence that the transmembrane semaphorin Sema4D promotes inhibitory synapse formation. In cultured hippocampal neurons, siRNA-mediated knockdown of Sema4D reduces GABAergic inhibitory synaptic formation (Paradis et al. 2007). Remarkably, the application of exogenous Sema4D rapidly induces the formation of inhibitory synapses in a matter of hours. This effect is completely abolished in cultured neurons or hippocampal sections prepared from mice lacking the Sema4D receptor PlexB1 (Kuzirian et al. 2013). These studies, taken together, demonstrate that semaphorins are required for the selective establishment of excitatory and inhibitory synapses in the mammalian brain.

2.5 Conclusion

More than 20 years have passed since semaphorins were first identified as regulators of axon growth cone guidance (Kolodkin et al. 1992). However, new neuronal functions for these proteins are still being identified. We have highlighted recent and novel findings regarding semaphorin function in the central nervous system. Semaphorins play critical roles in all aspects of neural circuit assembly, including axon targeting, laminar-specific innervation, selective synapse formation, and refinement of neural circuits through axon and synapse pruning. We expect that new roles for semaphorins in neural circuit establishment, maintenance, and plasticity

remain to be discovered, and it seems likely that novel semaphorin signaling mechanisms will be elucidated that underlie these functions.

Recent work demonstrates that Sema6A also plays a critical role in assembly of accessory optic system RGC circuits (Sun et al. 2015). Sema6A is expressed in On direction selective retinal ganglion cells and serves as a receptor in these neurons to establish connectivity with their brainstem target, the medial terminal nucleus.

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Chapter 3
Axon Guidance in the Spinal Cord

Fumiyasu Imai and Yutaka Yoshida

Abstract Neural circuit assembly requires the coordination of various developmental processes, including axon guidance and synapse formation. Growth cones, the leading edges of axons, navigate by interacting with different kinds of attractive and repulsive axon guidance cues along their trajectories and at target areas.

Spinal cords consist of different types of neural circuits, such as sensory-motor, commissural, and sympathetic circuits. Each neural circuit provides an unique model system to understand the cellular and molecular mechanisms underlying neural circuit formation. Studies of sensory-motor circuits have outlined the basic events leading to circuit formation. First, both sensory and motor axons project to the appropriate peripheral muscles. Sensory axons then form precise monosynaptic connections with a select group of motor neuron pools in the ventral spinal cord, and these pools of motor neurons ultimately develop their appropriate dendrite morphologies. Examination of commissural neurons and their axon trajectories in the spinal cord has contributed important data on how attractants and repellents influence the midline crossing of axons. Finally, studies of sympathetic neurons have led toward a greater overall understanding of the cellular and molecular mechanisms underlying neuronal survival, retrograde signaling, neuronal migration, and axon guidance.

Semaphorin signaling in neural circuit assembly has been studied extensively in the spinal cord. Importantly, the first mammalian semaphorin, Sema3A, was identified by monitoring growth cone collapse in sensory neurons in vitro. Here, we review the current knowledge on semaphorin signaling in various steps of vertebrate neural circuit assembly in the spinal cord, including axon guidance, synaptic specificity, synapse formation, and dendrite development, highlighting the different neural circuit systems that have helped to broaden our understanding of semaphorins and their functions in the developing nervous system.

Keywords Spinal cord • Dorsal root ganglia • Sensory neurons • Motor neurons • Commissural neurons • Sympathetic neurons

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3.1 Identification of Semaphorins

The first semaphorin was originally cloned in 1992 as Fasciclin IV (Sema-I) in the grasshopper using monoclonal antibody (MAb) screens (Kolodkin et al. 1992). Subsequently, the *Drosophila* homologues D-Sema-I (Sema-1a) and D-Sema-II (Sema-1b) were identified a year later (Kolodkin et al. 1993). In vertebrates, Sema3A was first discovered as collapsin in chicks (Luo et al. 1993) using a functional cloning assay developed by the same group to detect growth cone collapse in sensory neurons in vitro (Raper and Kapfhammer 1990). When tested for activity, recombinant collapsin-1 (Sema3A) caused growth cones of sensory neurons to collapse but had no effect on retinal ganglion cell growth cones (Luo et al. 1993). Other collapsin members (collapsin-2 to -5; corresponding to Sema3C–Sema3E and Sema4D) were subsequently cloned in the chick (Luo et al. 1995).

Mammalian semaphorins were first cloned by two groups led by Messersmith et al. (1995) and Puschel et al. (1995), followed by other teams shortly thereafter (Inagaki et al. 1995; Wright et al. 1995; Zhou et al. 1997). Sema3A, the first mammalian semaphorin, was shown to be expressed in the ventral region of the developing spinal cord in rodents (Messersmith et al. 1995; Puschel et al. 1995). Because cutaneous and proprioceptive sensory neurons, whose cell bodies are both located in the dorsal root ganglia (DRG), project axons to the dorsal and ventral spinal cord regions, respectively (Fig. 3.1a) (Koerber and Mendell 1992; Brown 1981), the expression of *Sema3A* in the ventral region suggested that it may play a role in inhibiting cutaneous, but not proprioceptive, axons in the developing spinal cord. In fact, coculture assays using DRG neurons and COS cells expressing Sema3A in collagen gels showed that Sema3A repels nerve growth factor (NGF)-responsive axons, which are likely to be cutaneous axons, whereas it does not repel NT3-responsive axons, which are presumably proprioceptive axons (Fig. 3.1b)

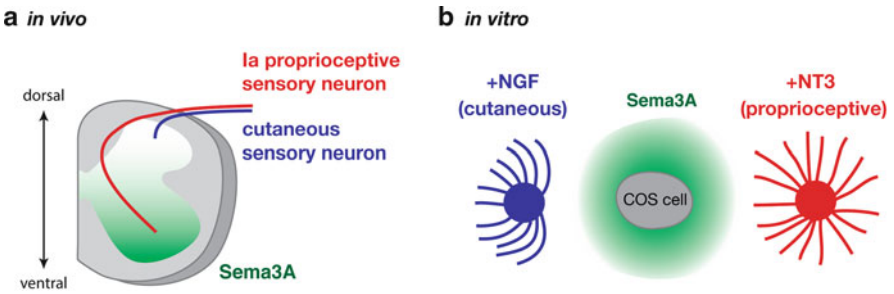

Fig. 3.1 Sema3A repels cutaneous sensory axons. (**a**) Axons of Ia proprioceptive sensory neurons (*red*) penetrate the spinal cord, extend to the ventral spinal cord, and form synapses with motor neurons. Cutaneous axons (*blue*) form synapses with dorsal spinal cord neurons. Sema3A (*green*) is expressed in the ventral spinal cord. (**b**) Schematic drawing of an in vitro dorsal root ganglia (DRG) culture experiment. Exogenous Sema3A (*green*) acts as a repulsion cue for nerve growth factor (NGF)-induced cutaneous axons (*blue*, *left*) but not NT3-induced proprioceptive axons (*red*, *right*)

(Messersmith et al. 1995). This finding suggests that repellent Sema3A may inhibit specifically the ventral projections of cutaneous sensory axons in the spinal cord.

3.2 Identification of Semaphorin Receptors

The cloning of the first semaphorin receptors, plexin and neuropilin, was accomplished by Fujisawa's group in Japan using MAb screens to search for molecules involved in axon interaction and growth cone navigation in *Xenopus* tadpoles. Two antibodies, MAb-A5 and MAb-B2, showed specific axon staining in their assay (Takagi et al. 1987). The protein recognized by the MAb-A5 antibody was identified (Takagi et al. 1991) and named neuropilin from the term neuropile (Satoda et al. 1995), whereas the MAb-B2 antibody was found to bind a different protein, identified as plexin-A1 from the term plexiform layer (Ohta et al. 1995). Although plexin-A1 and neuropilin were discovered concurrently by Fujisawa's group, their interactions with one other as co-receptors for semaphorins were completely unknown at that time.

Human plexins were identified independently by molecular homology searches for the ectodomain of the Met receptor (Maestrini et al. 1996). Meanwhile, neuropilin-1 and neuropilin-2 (Npn1 and Npn2) were identified as class 3 semaphorin receptors by different groups using varying approaches: an expression cloning assay using COS cells (He and Tessier-Lavigne 1997; Kolodkin et al. 1997); a candidate molecule approach (Feiner et al. 1997); and RT-PCR cloning of Npn1-related molecules (Chen et al. 1997).

The first interactions between semaphorins and plexins were observed in the binding of viral semaphorin with a novel transmembrane protein that happened to be a virus-encoded semaphorin protein receptor (VESPR; later named plexin-C1) (Maestrini et al. 1996). Corroborating evidence for semaphorin–plexin interactions arose when plexin-A was shown to bind to Sema-1a and Sema-1b in the *Drosophila* nervous system (Winberg et al. 1998). The complex of class 3 semaphorin/neuropilin/plexin-A was ultimately demonstrated by three groups (Takahashi et al. 1999; Tamagnone et al. 1999; Rohm et al. 2000), who showed that plexin-As are required for Sema3A-mediated growth cone collapse of DRG or spinal neurons using dominant-negative forms of plexin-As (Takahashi et al. 1999; Tamagnone et al. 1999; Rohm et al. 2000).

3.3 Sensory and Motor Neuron Projections

As already mentioned, Sema3A was identified as a potential chemorepellent for cutaneous sensory axons (Luo et al. 1993). During development, cutaneous sensory neurons project axons to the dorsal root entry zone, then wait for several days before innervating the spinal cord. Once these axons reach the dorsal spinal cord

region, they stop and do not extend to the ventral spinal cord. There are two regions where *Sema3A* is expressed in the spinal cord during development. First, *Sema3A* is expressed in the dorsal spinal cord before any afferents begin to grow into the spinal gray matter (Shepherd et al. 1997; Fu et al. 2000). Expression then declines (Fu et al. 2000), suggesting that Sema3A may inhibit sensory axon ingrowth to the spinal gray matter during the waiting period. *Sema3A* is also expressed in the embryonic ventral spinal cord (Wright et al. 1995; Puschel et al. 1996; Shepherd et al. 1997; Luo et al. 1995; Messersmith et al. 1995), suggesting that it may also prevent cutaneous axons from extending to the ventral region of the spinal cord during embryonic development.

Since the initial in vitro culture experiments by Messersmith et al. (1995), many in vitro and in vivo studies have corroborated the finding that Sema3A inhibits axons of cutaneous sensory neurons. For example, anti-Sema3A (collapsin-1) function-blocking antibodies neutralize repulsion of chick sensory axons by ventral spinal cord explants (Shepherd et al. 1997). In addition, recombinant Sema3A proteins injected into the explants of chick spinal cords with DRGs reduces the ingrowth of $TrkA^+$ (presumably cutaneous) axons, whereas $TrkC^+$ (presumably proprioceptive) axons, which do not express Npn1, are not affected (Fu et al. 2000). Adenoviral vector-mediated ectopic expression of Sema3A in rat dorsal spinal cords with DRGs also reveals that Sema3A strongly inhibits NGF-responsive afferent fibers, and these afferents fail to reach their appropriate target regions (Pasterkamp et al. 2000).

Interestingly, Sema3A can inhibit not only developing but also adult sensory afferents. Using in vitro culture systems, Sema3A has been shown to induce growth cone collapse of only small-diameter (likely cutaneous) sensory afferents from adult rats, and this effect is inhibited by anti-Npn1 function-blocking antibodies (Reza et al. 1999), suggesting that Sema3A inhibits adult sensory axons through Npn1. Moreover, gene gun-mediated ectopic expression of Sema3A in vivo repulses adult sensory afferents in adult rabbits (Tanelian et al. 1997). Sema3A was also found to inhibit NGF-induced sprouting of sensory afferents in adult rat spinal cords (Tang et al. 2004).

Many attempts to elucidate the physiological roles of semaphorins and their receptors in sensory and motor axons in vivo have been undertaken using loss-of-function approaches. Using $Npn1^{Sema-}$ mice, which express a mutant Npn1 protein that fails to bind class 3 semaphorins, Gu et al. showed that $TrkA^+$ cutaneous fibers enter the spinal cord gray matter earlier than those in wild-type mice (2003), indicating that Sema3A-Npn1 repulsive signaling is involved in eliciting the waiting period before sensory ingrowth into the spinal gray matter. Studies using single-knockout mice (mutant mice) have shown some surprising results. Despite the hypothesis that Sema3A inhibits ventral projections of cutaneous sensory neurons through the Npn1 receptor, *Sema3A, Npn1*, *plexin-A3*, and *plexin-A4* mutant mice do not show strong defects in central projections of cutaneous sensory neurons in the spinal cord (Taniguchi et al. 1997; Kitsukawa et al. 1997; Cheng et al. 2001; Suto et al. 2005). Only a minor population of cutaneous axons reach the ventral spinal cord in *Sema3A* mutant mice (Behar et al. 1996) as well as $Npn1^{Sema-}$ mice (Gu et al. 2003). Therefore, Sema3A-Npn1 signaling does not appear to have a major

role in preventing cutaneous axons from entering the ventral spinal cord in vivo, even though Npn1 and co-receptors plexin-A3/A4 have been shown to be relevant receptors for mediating Sema3A-mediated growth cone collapse in vitro (Kitsukawa et al. 1997; Yaron et al. 2005; Suto et al. 2005).

In addition to showing temporally segregated expression in the dorsal and ventral spinal cord, Sema3A has also been shown to be expressed in the peripheral tissues where sensory and motor axons meet (Taniguchi et al. 1997), suggesting that Sema3A-Npn1 signaling through plexin-A3/A4 receptors may be important in regulating peripheral projections of both sensory and motor neurons. In fact, *Sema3A* null, *Npn1* null, *plexin-A3/A4* null, and *Npn1*$^{Sema-}$ mice all showed defasciculation, disorganization, branching defects, and overshooting phenotypes of peripheral projections in both sensory and motor neurons during early embryogenesis [embryonic day (E) 10.5–12.5] (Kitsukawa et al. 1997; Yaron et al. 2005; Suto et al. 2005; Taniguchi et al. 1997; Cheng et al. 2001). Interestingly, the aberrant peripheral projections in these mutants are mostly eliminated by E15.5 (White and Behar 2000), indicating that axon pruning to remove aberrant axons occurs during this stage in embryonic development.

Motor neuron axonal trajectories have been extensively studied to understand the cellular and molecular mechanisms underlying the establishment of functional motor circuitry. Vertebrate motor neurons extend axons out of the spinal cord and innervate target skeletal muscles. Along these trajectories, motor axons encounter many axon guidance cues and ultimately find their appropriate muscle targets, including those that reside within the developing limb. In the ventral spinal cord, cohorts of motor neurons are arrayed in longitudinal columns and project their axons to distinct peripheral regions. For example, lateral motor column (LMC) neurons, which are generated only at limb levels, extend their axons into the limb mesenchyme, whereas median motor column (MMC) neurons, which are generated at all spinal cord levels, extend their axons specifically to axial muscles.

As already described, Sema3A-Npn1 signaling is required for peripheral nerve projections during mouse embryogenesis (Taniguchi et al. 1997; Kitsukawa et al. 1997; Yaron et al. 2005; Cheng et al. 2001; Suto et al. 2005). More recent detailed analyses of the roles of Sema3A-Npn1 and Sema3F-Npn2 signaling in motor axon pathfinding have shown how semaphorin signaling participates in motor axon targeting (Huber et al. 2005; Huettl et al. 2011). At E10.5, *Npn1* mRNA is expressed by most LMC neurons, whereas *Npn2* is expressed by only a subset of the medial LMC (LMCm) motor neurons but not by motor neurons in the lateral division of the LMC (LMCl) (Huber et al. 2005). Expression of *Sema3A* is detected in the limb at E10.5, when spinal nerves have extended into the plexus region but have not yet entered the limb (Huber et al. 2005), suggesting that Sema3A regulates the timing of motor axon ingrowth into the limb. Supporting this idea, precocious extensions of motor and sensory projections toward distal forelimb regions have been observed in *Sema3A*-mutant mice and in *Npn1*$^{Sema-}$ mice (Gu et al. 2003; Huber et al. 2005). *Sema3A* is also expressed adjacent to peripheral nerve tracts within the forelimb at E11.5 (Huber et al. 2005), raising the possibility that Sema3A may regulate motor axon fasciculation at later embryonic stages. Peripheral nerves

in *Npn1*$^{Sema-}$ and *Sema3A* mutants also show marked defasciculation and aberrant growth when compared to wild-type mice (Fig. 3.2a, b) (Huber et al. 2005). *Sema3F*, which encodes a ligand for the Npn2 receptor, is expressed in the dorsal limb bud and participates in directing *Npn2*-expressing LMCm motor neuron axons along a ventral trajectory in the forelimb (Huber et al. 2005). Loss-of-function and gain-of-function experiments reveal that Sema3F-Npn2 signaling is required to direct LMCm motor neuron axons along their ventral trajectories (Fig. 3.2c, d; Huber et al. 2005).

A more recent study examined the reciprocal interactions between sensory and motor axons as they navigate along their trajectories and investigated the roles

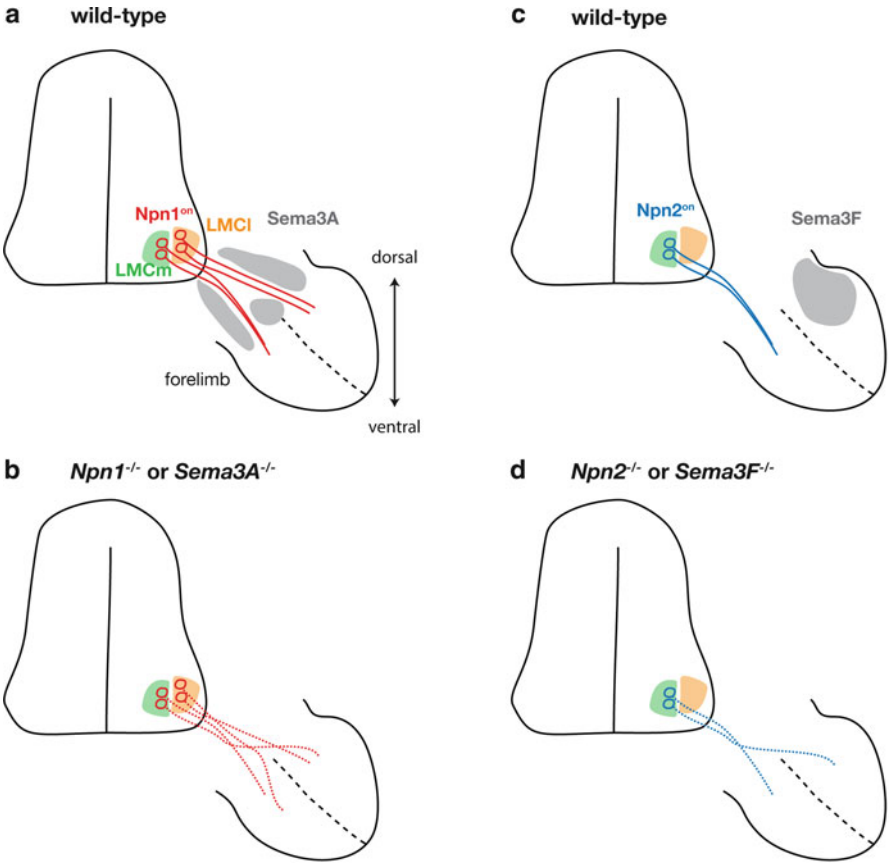

Fig. 3.2 Semaphorins and neuropilins are essential for motor axon innervation of the forelimb. (**a**) *Sema3A* is expressed in developing forelimbs (*gray*) and *Npn1* is expressed by lateral motor column (LMC) motor neurons (*red*). LMCm (*green*) and LMCl (*orange*) axons innervate the ventral and dorsal forelimb regions, respectively. (**b**) Dorsoventral LMC axon innervation patterns were perturbed in *Npn1* mutant and *Sema3A* mutant embryos. (**c**) *Sema3F* is expressed in the dorsal forelimb (*gray*) and Npn2 is expressed by only LMCm motor neurons (*blue*). (**d**) LMCm axon projections were affected in *Npn2* mutant and *Sema3F* mutant embryos

played by Npn1 signaling in these axon–axon interactions (Huettl et al. 2011). Deletion of *Npn1* solely in motor neurons reveals that peripheral sensory projections are still established correctly, even though motor projections in the distal limbs may be severely defasciculated (Huettl et al. 2011). Genetic elimination of motor neurons demonstrates that sensory axons require only minimal motor axon scaffolding to establish their projections in the distal limb (Huettl et al. 2011). In contrast, defects in sensory axonal trajectories caused by sensory neuron-specific *Npn1* deletion are accompanied by defasciculation of motor axons (Huettl et al. 2011). Thus, motor axons are dependent on sensory axons, and they interact, in part, through Npn1-mediated fasciculation in the developing limb.

In addition to peripheral tissues, *Sema3A* is also expressed by motor neurons. Because motor neurons also express the Sema3A receptor, Npn1, it was a mystery why both *Npn1* and *Sema3A* are present on motor neurons. Using gain-of-function and knockdown approaches in chicks, Moret et al. (2007) found that coexpression of *Npn1* and *Sema3A* in motor neurons regulates axon sensitivity to environmental *Sema3A* sources encountered along their motor axon trajectories. In chicks, *Sema3A* is expressed by MMC motor neurons at both early and late developmental stages, whereas *Sema3A* is not expressed by LMC motor neurons at early stages but is expressed when the axons reach the base of the limb (Moret et al. 2007). In contrast, *Npn1* is expressed by both MMC and LMC motor neurons in the developing chick spinal cord (Moret et al. 2007). Premature expression of *Sema3A* in LMC motor neurons leads motor axons to defasciculate and invade territories they normally avoid, suggesting that Npn1 becomes insensitive to early ectopic environmental expression of Sema3A (Moret et al. 2007). Moreover, knockdown studies show that *Sema3A* expression in motor neurons is required for correct spinal nerve compaction and dorsal motor axon extension (Moret et al. 2007). Therefore, *Sema3A* in chick motor neurons sets the level of sensitivity of their growth cones to exogenous *Sema3A* exposure (Moret et al. 2007), and this regulation is associated with posttranslational and local control of Npn1 expression levels at the growth cone surface (Moret et al. 2007). This interplay between intrinsic and extrinsic *Sema3A* may represent a fundamental mechanism in the accurate specification of axon pathways. Future studies will explore this proposed role for nonneuronal Sema3A and the regulation of motor axon guidance.

3.4 Sensory-Motor Reflex Circuit in the Spinal Cord

In the mammalian somatosensory system, peripheral stimuli are conveyed by sensory neurons in the DRG. As mentioned earlier, DRG sensory neurons are subdivided into two major groups: those transducing proprioceptive stimuli and those transducing cutaneous sensory stimuli (Brown 1981; Koerber and Mendell 1992). Proprioceptive sensory neurons convey information about the state of muscle contraction and limb position, whereas cutaneous sensory neurons mediate a wide range of noxious and innocuous stimuli (Brown 1981; Koerber and Mendell 1992).

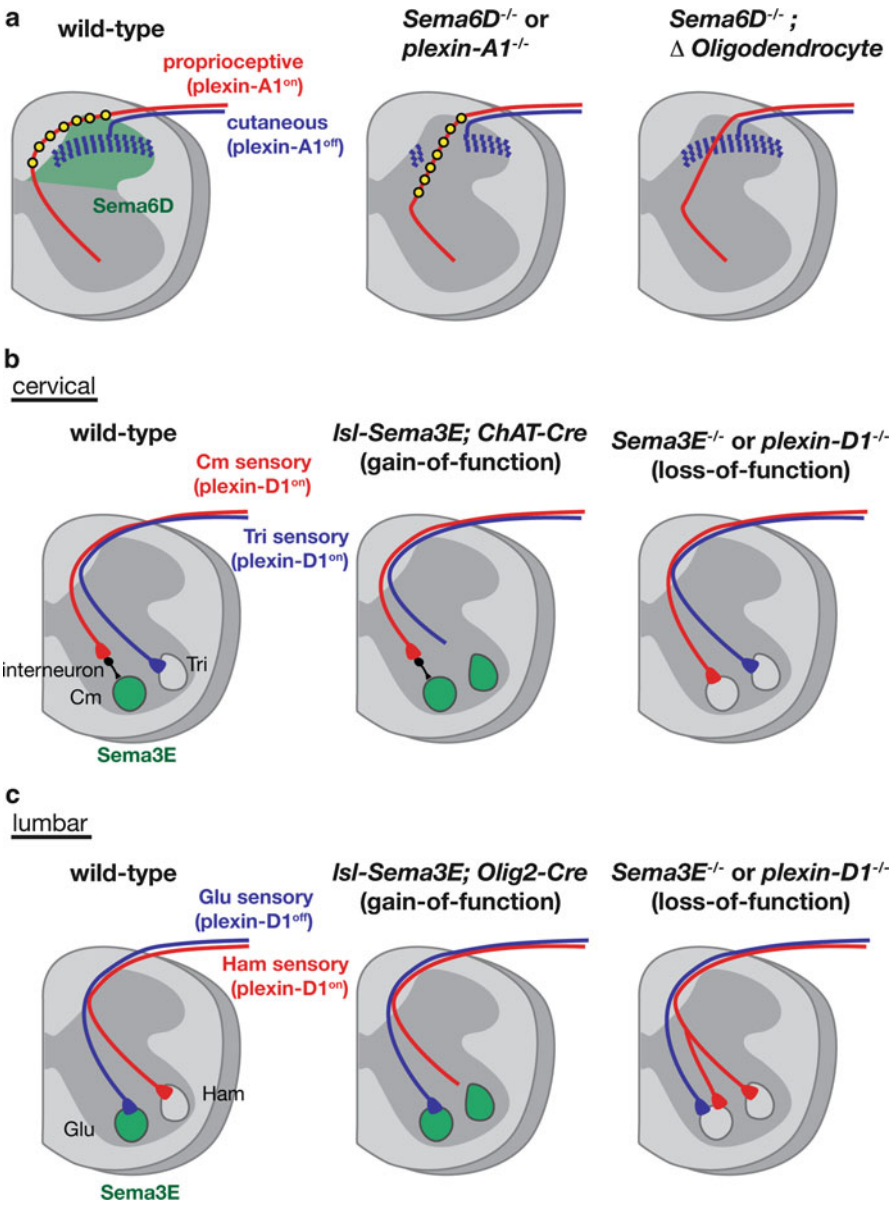

Fig. 3.3 Roles of Sema–plexin signaling in sensory-motor circuitry. (**a**) Cross-sectional diagrams depict the spinal cords of wild-type (*left*), *Sema6D* and *plexin-A1* mutant (*Sema6D*$^{-/-}$ or *plexin-A1*$^{-/-}$) (*middle*), and oligodendrocyte-deleted *Sema6D* mutant mice (*Sema6D*$^{-/-}$*; ΔOligodendrocyte*) (*right*). Plexin-A1 is expressed by proprioceptive axons (*red lines*; plexin-A1^{on}) but not cutaneous axons (*blue lines*; plexin-A1^{off}), and *Sema6D* is expressed in the dorsal spinal cord (*green shaded area*). Oligodendrocytes (*yellow circles*) and cutaneous synapses (*blue dotted lines*) are shown. (**b**, **c**) Cross-sectional diagrams depict the cervical (**b**) and lumbar (**c**) spinal cords of wild-type (*left*), motor neuron-specific *Sema3E*-expressing mice (*middle*; gain-of-function), and

The myelinated axons of proprioceptive sensory neurons avoid the superficial dorsal horn and project to the intermediate or ventral spinal cord, whereas the axons of cutaneous sensory neurons project directly into the superficial dorsal horn (Fig. 3.1a) (Brown 1981; Koerber and Mendell 1992). Signals mediated by Sema3A have been suggested to inhibit cutaneous axons in the ventral spinal cord (Messersmith et al. 1995; Fu et al. 2000). However, genetic inactivation of *Sema3A* in mice has yet to reveal a major role for these ligands in the patterning of these sensory axonal trajectories (Behar et al. 1996; Taniguchi et al. 1997; Gu et al. 2003). Nevertheless, other semaphorins are known to be expressed in the spinal cord (Cohen et al. 2005), and plexins are expressed by sensory and spinal neurons (Cheng et al. 2001; Cohen et al. 2005), raising the possibility that Sema–plexin signaling may play some role in establishing central projections of sensory afferents.

Two studies demonstrate the role of the plexin-A1 receptor and its ligand Sema6D in proprioceptive sensory axon positioning in the dorsal spinal cord and their effects on cutaneous sensory axons (Yoshida et al. 2006; Leslie et al. 2011). *Plexin-A1* is exclusively expressed by proprioceptive sensory neurons in the DRG, whereas *Sema6D* is expressed in the dorsal spinal cord (Fig. 3.3a, left) (Yoshida et al. 2006). In *plexin-A1* or *Sema6D* mutants, proprioceptive axons ectopically invade the dorsal horn (Fig. 3.3a, middle) (Yoshida et al. 2006; Leslie et al. 2011). Proprioceptive axons are heavily myelinated whereas most cutaneous axons either have no myelination or are only thinly myelinated (Fig. 3.3a, left and middle). Although the oligodendrocytes associated with the ectopic proprioceptive axons in *plexin-A1* or *Sema6D* mutants synchronously invade the dorsal spinal cord (Fig. 3.3a, middle) (Yoshida et al. 2006; Leslie et al. 2011), the axonal trajectories of cutaneous sensory neurons are unaffected. However, these cutaneous sensory neurons are unable to form appropriate synapses with dorsal spinal cord neurons (Fig. 3.3a, middle). Genetic deletion of oligodendrocytes demonstrates that it is the ectopic oligodendrocytes, not the proprioceptive axons, in the dorsal spinal cord that inhibit synapse formation in the absence of Sema6D–plexin-A1 signaling (Fig. 3.3a, right) (Leslie et al. 2011). These studies provide new insights into the roles of oligodendrocytes in synapse formation in vivo, which may be an important element regulating the overall development of neural wiring in the central nervous system (CNS).

Once proprioceptive axons reach the ventral spinal cord, most of the axons make monosynaptic connections with specific motor neurons. However, in the case of cutaneous maximus (Cm) group Ia afferents that project to the Cm muscle,

Fig. 3.3 (continued) and *plexin-D1* and *Sema3E* mutant mice (*right*; loss-of-function). At cervical levels of the spinal cord, *plexin-D1* is expressed by both cutaneous maximus (*Cm*) and triceps (*Tri*) sensory neurons (*red and blue lines* in **b**; plexin-D1^{on}) and *Sema3E* is expressed by only Cm motor neurons (*green* areas in **b**). Cm sensory neurons project to Cm motor neurons through interneurons (shown in *black* in **b**). At spinal cord lumbar levels, *plexin-D1* is expressed by many hamstring (Ham; *red lines* in **c**; plexin-D1^{on}) but by few gluteus (Glu; *blue lines* in **c**; plexin-D1^{off}) sensory neurons. *Sema3E* is only expressed by Glu motor neurons (*green* areas in **c**). *lsl* :lox-stop-lox

they form only indirect connections with Cm motor neurons through interneurons in wild-type mice (Fig. 3.3b, left) (Vrieseling and Arber 2006). This failure to develop monosynaptic inputs between Cm sensory and motor neurons was found to be regulated by plexin-D1 and its ligand Sema3E (Pecho-Vrieseling et al. 2009). Deletion of *plexin-D1* or *Sema3E* in mice results in ectopic monosynaptic connections between Cm Ia afferents and Cm motor neurons (Fig. 3.3b, right) (Pecho-Vrieseling et al. 2009). Furthermore, ectopic expression of Sema3E in triceps (Tri) motor neurons similarly prevents monosynaptic connectivity between Tri sensory and motor neurons (Fig. 3.3b, middle) (Pecho-Vrieseling et al. 2009). Thus, repulsive Sema3E–plexin-D1 signaling controls the exclusion of sensory afferent inputs on Cm motor neuron pools at cervical levels.

With the exception of Cm motor neurons, most other motor neuron pools make monosynaptic connections with proprioceptive Ia afferents in the ventral spinal cord. Although anatomical and electrophysiological analyses have revealed many details of the specificity of monosynaptic connections between Ia afferents and motor neurons, the molecular mechanisms underlying this sensory-motor specificity remained largely unknown. Recently, the specificity of these connections has been shown to be regulated by the dorsoventral positions of motor neuron pools, independently of motor neuron-derived cues (Surmeli et al. 2011). Mice in which *FoxP1* has been deleted in motor neurons exhibit scrambled motor neuron positions and defects in sensory-motor specificity (Surmeli et al. 2011). Interestingly, these *FoxP1* mutants showed that the axon terminals of proprioceptive sensory neurons depend on the dorsoventral tier positions of motor neuron pools but not on the motor neurons themselves (Surmeli et al. 2011), indicating that the relevant cues are not derived from motor neurons. Although the initial patterns of sensory-motor connectivity seems to be independent of motor neurons, it is still unclear whether motor neuron-based recognition systems are involved in consolidating and reinforcing initial tier-based connectivity restrictions.

A recent study revealed that motor neuron-derived Sema3E controls pool-by-pool specificity of sensory-motor connections at lumbar levels (Fukuhara et al. 2013), in addition to inhibiting monosynaptic Cm sensory-motor connections at cervical levels (Pecho-Vrieseling et al. 2009). First, Sema3E expression was shown to be expressed by gluteus (Glu) motor neurons, but not by hamstring (Ham) motor neurons in the lumbar spinal cord (Fig. 3.3c, left) (Fukuhara et al. 2013). Conversely, *plexin-D1* is expressed by many Ham proprioceptors but by few Glu proprioceptors. The authors focused on Glu and Ham motor neurons because these two motor neuron subtypes innervate muscles that control different leg joints and occupy overlapping rostrocaudal levels of the lumbar spinal cord. Both anatomical and electrophysiological analyses revealed that ectopic expression of Sema3E in Ham motor neurons reduces monosynaptic sensory-motor connections (Fig. 3.3c, middle) (Fukuhara et al. 2013). Furthermore, significant numbers of Ham sensory axons make strong aberrant monosynaptic connections with Glu motor neurons in the absence of Sema3E–plexin-D1 signaling (Fig. 3.3c, right) (Fukuhara et al. 2013). Taken together, these studies show that motor neuron-derived molecules can regulate the specificity of monosynaptic sensory-motor connections in the spinal cord. However, considering that there are approximately 50 different kinds

of limb muscles, other motor neuron-derived molecules as well as motor neuron-independent cues are likely to be involved in regulating sensory-motor specificity. Future studies will identify and characterize these molecules.

3.5 Midline Crossing of Commissural Axons

Commissural neurons are located in the dorsal spinal cord and project their axons to the ventral spinal cord. After these axons reach the floor plate and cross the midline, they turn rostrally, extend longitudinally on the contralateral side of the ventral spinal cord, and eventually project to the brain. Importantly, once they cross the midline, they never re-cross it. Commissural axons are attracted by netrin-1 that is produced in the floor plate. However, after commissural axons cross the floor plate, they lose their responsiveness to chemoattractants such as netrin-1. In *Drosophila*, the interaction between a Slit repellent protein and its receptor Roundabout (Robo) prevent commissural axons from re-crossing the midline (Kidd et al. 1998a, b, 1999). Robo protein expression on the surface of commissural axons is low before midline crossing; however, after crossing, Robo expression is augmented, which prevents any subsequent re-crossing (Kidd et al. 1998a, b).

In vertebrates, Zou et al. (2000) tested whether mammalian Slit and semaphorins are involved in preventing the re-crossing of commissural axons at the midline. To understand the molecular mechanisms underlying commissural axon guidance in the spinal cord, Zou et al. (2000) developed a novel in vitro explant assay in which commissural axon extensions can be observed in vitro using an "open book" preparation where the spinal cord is opened at the dorsal midline (Fig. 3.4b) (Zou et al. 2000). This assay is very useful, because both pre- and post-midline crossing axons can be examined using explants of either the spinal cord plus the floor plate or just the dorsal spinal cord alone. Using these explant culture experiments, Slit-2, Sema3B, and Sema3F were shown to inhibit the extensions of post-crossing but not pre-crossing commissural axons (Fig. 3.4b; Zou et al. 2000).

As Sema3B and Sema3F are ligands for Npn2, the authors examined *Npn2* mutant mice using their explant culture assay. Previous studies reported no defects in commissural axon trajectories during the period of initial axon growth to the floor plate in *Npn2* mutant mice; however, the axons had not been examined within or around the floor plate (Chen et al. 2000; Giger et al. 2000). Using the open-book preparation with DiI injected into the dorsal spinal cord to visualize the commissural axons, an array of defects were detected in *Npn2* mutant mice (Zou et al. 2000). Although there were no obvious defects observed in axon trajectories approaching the floor plate, some commissural axon growth cones stalled while crossing the midline in *Npn2* mutant mice, and many axons within the floor plate area seemed less straight and more wavy in *Npn2* mutant mice compared to controls. Moreover, many axons that did not cross the floor plate made aberrant turns causing axonal trajectories to be randomized along the anteroposterior axis. This observation led to the conclusion that Sema3B/3F–Npn2 interactions transduce repulsive signals for commissural axons in the floor plate (Fig. 3.4b).

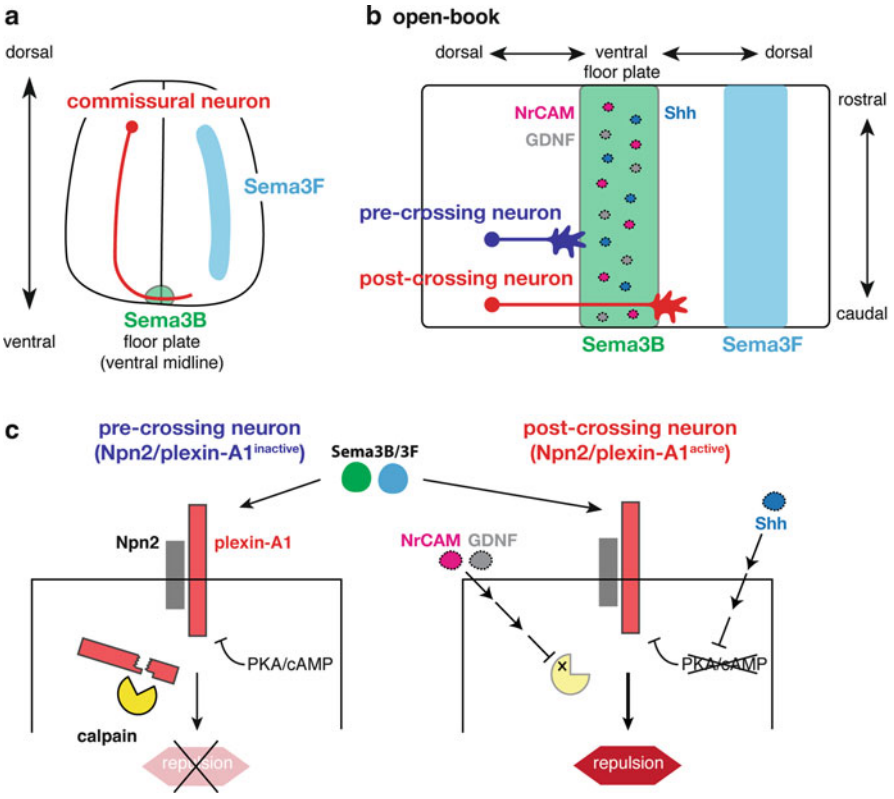

Fig. 3.4 Roles of Sema–Npn/plexin signaling in the midline. (**a**) Commissural neurons (*red*) express both Npn2 and plexin-A1. Sema3B (*green*) and Sema3F (*cyan*) are expressed in the floor plate and mantle layer, respectively, of the developing spinal cord. (**b**) An "open-book" view of the developing spinal cord. NrCAM (*magenta circles*), GDNF (*gray circles*), and Shh (*blue circles*) are expressed in the floor plate. Pre-crossing midline commissural axons (*blue line*) are not repulsed by Sema3B and Sema3F, but after crossing the midline these axons (*red line*) are actively repulsed by both Sema3B and Sema3F. (**c**) Molecular mechanism of calpain-mediated plexinA1 proteolysis in pre- (*left*; Npn2/plexin-A1^{inactive}) and post-midline crossing commissural axons (*right*; Npn2/plexin-A1^{active}). In the absence of NrCAM/GDNF, calpain (*yellow*) can cleave plexin-A1 (*left*). After crossing the midline, NrCAM and GDNF inhibit calpain-mediated plexin-A1 cleavage (*right*). Shh-mediated downregulation of PKA/cAMP signaling is essential for the response to Sema3B and Sema3B

The molecular mechanisms underlying this change of responsiveness to floor plate cues were largely unknown. Three recent studies revealed novel mechanisms that may explain how this switch occurs (Parra and Zou 2010; Nawabi et al. 2010; Charoy et al. 2012). First, the mechanisms by which semaphorin repulsion is switched on at the midline were investigated by Parra and Zou (2010). Using a collagen explant assay, sonic hedgehog (Shh) was shown to activate a novel repulsive response to Sema3B and Sema3F in pre-crossing commissural axons

(Parra and Zou 2010). Furthermore, perturbing the Shh receptors Ptch1 and Smo, or blocking Shh activity, caused defects in midline guidance, indicating that post-crossing commissural axons are no longer repulsed by Sema3B or Sema3F (Parra and Zou 2010). The involvement of cyclic nucleotides in this switching event was also examined because they are known to influence signaling in response to general guidance cues, with the ratio of cAMP/cGMP being particularly important for regulating axonal attraction and repulsion in vitro (Song et al. 1998). Consistent with previous observations, enhancing protein kinase A (PKA) activity in pre-crossing axons diminishes Shh-induced semaphorin-mediated repulsion and causes profound midline stalling, along with overshooting and wandering of post-crossing axons (Parra and Zou 2010). Thus, this study showed that Shh can alter commissural axon guidance responses to semaphorins by reducing cAMP/PKA signaling (Fig. 3.3c).

The second study presented a different mechanism for altering the responsiveness to guidance cues by commissural axons (Nawabi et al. 2010). In this study, plexin-A1 was shown to bind to Npn2 to mediate repellency to Sema3B (Nawabi et al. 2010). Interestingly, plexin-A1 protein levels in commissural axons are upregulated by floor plate signals that prevent calpain 1 from cleaving and inactivating plexin-A1 (Nawabi et al. 2010). NrCAM was identified as the floor plate cue responsible for regulating plexin-A1 levels (Nawabi et al. 2010). NrCAM was a candidate signaling molecule because of its high and restricted expression in the floor plate and its ability to regulate axon growth and guidance during the formation of various commissural tracts (Falk et al. 2005; Williams et al. 2010). A variety of in vitro and in vivo experiments revealed that floor plate-derived NrCAM indeed inhibits plexin-A1 cleavage by calpain 1 and thus increases growth cone sensitization to the repellent signals of Sema3B (Fig. 3.4c) (Nawabi et al. 2010). These results demonstrate novel mechanisms for changing commissural axon responsiveness to semaphorin guidance cues.

In the third study, the same group that investigated NrCAM also showed that glial cell line-derived neurotrophic factor (GDNF) activates midline repulsion by Sema3B during commissural axon guidance (Charoy et al. 2012). The authors first found that GDNF is strongly expressed in the floor plate at E11.5–E12.5 during commissural axon crossing; however, they also showed that GDNF is not required for commissural axons to reach the floor plate and that it does not elicit commissural growth cone collapse (Charoy et al. 2012). As others have shown that (1) the sensitivity of commissural axons to floor plate repellents is switched on after midline crossing (Evans and Bashaw 2010; Chedotal 2011; Nawabi and Castellani 2011), and (2) the local floor plate cues trigger responsiveness of commissural axons to the midline repellent, Sema3B (Nawabi et al. 2010), the authors examined whether GDNF could be a local floor plate cue. When tested, GDNF induced Sema3B-mediated collapse of commissural growth cones (Charoy et al. 2012). Interestingly, GDNF also mediates suppression of calpain 1 activity and increases plexin-A1 protein levels in commissural neurons similar to floor plate-derived NrCAM (Charoy et al. 2012). Moreover, the analysis of GDNF/NrCAM double-mutant mice showed that neither cue is redundant: both are required to ensure appropriate axon guidance across the floor plate (Charoy et al. 2012). The major

receptors for GDNF are tyrosine kinase RET and IgSFCAM NCAM, but only NCAM was found to be required for GDNF-induced regulation of plexin-A1 protein levels and capain 1 activity in commissural axons (Fig. 3.4c) (Charoy et al. 2012).

3.6 Segregation Between the Central and Peripheral Nervous System

The physical separation between the CNS and the peripheral nervous system (PNS) is evident at the ventral and dorsal root transitional zones in the spinal cord where two boundaries are observed. The first boundary exists at the motor exit point (MEP) where motor axons leave the spinal cord, and the second boundary appears at the dorsal root entry zone (DREZ) where sensory axons enter the spinal cord. Boundary cap (BC) cells at the MEP inhibit the emigration of motor neurons from the ventral spinal cord (Vermeren et al. 2003), and deletion of these BC cells leads to the ectopic positioning of motor neuron cell bodies along their axons and into the ventral nerve roots (Vermeren et al. 2003).

Semaphorin–plexin signaling aids in establishing the division between CNS and PNS (Bron et al. 2007; Mauti et al. 2007). *Sema6A* is expressed in BC cells, and deletion of *Sema6A* in mice (Bron et al. 2007) or downregulation of *Sema6A* (Mauti et al. 2007) in chicks causes ectopic motor neuron cell bodies to be distributed along the ventral roots. Because class 6 semaphorins bind to plexin-A family members (Toyofuku et al. 2004; Suto et al. 2005; Yoshida et al. 2006), *plexin-A1* or *plexin-A2* knockdown chick embryos or *Npn2* mouse mutant embryos have been studied and noted to exhibit similar defects (Mauti et al. 2007; Bron et al. 2007). It remains unclear which plexin-A is required for Sema6A signaling in mice, but these knockdown studies suggest that Sema6A/plexin-A/Npn2 signaling is involved in constraining spinal motor neuron migration at the MEP. Furthermore, knockdown of *Sema6A*, *plexin-A1*, *plexin-A4*, or *Sema6D* causes improper formation and segregation of dorsal roots (Mauti et al. 2007), indicating that semaphorin–plexin signaling functions in the DREZ as well as in the MEP.

3.7 Dendrite Development in the Spinal Cord

The development of dendrites, including growth, targeted extension, and branching, must be precisely regulated (Jan and Jan 2003). Sema3A has been shown to control dendritic growth and orientation of cortical pyramidal neuron subtypes in the developing brain (Polleux et al. 2000; Fenstermaker et al. 2004), and recently Sema signaling has been implicated in dendrite development in the spinal cord (Zhuang et al. 2009).

Specific motor neuron pools exhibit distinct dendritic arbor morphologies that are critical for their ability to receive and process various types of synaptic inputs (Landmesser 1978; Okado et al. 1990; Vrieseling and Arber 2006). Although the molecular mechanisms underlying motor pool-specific dendrite development are not well understood, it is known that the Ets class transcription factor, *Pea3,* is expressed by only a few motor neuron pools, and in *Pea3* mutants, aberrant dendrite morphology is detected in Pea3^{on} motor neuron pools (Vrieseling and Arber 2006). Interestingly, *Sema3E* mRNA expression is absent in these Pea3^{on} motor neuron pools (Livet et al. 2002), raising the possibility that semaphorin signaling may function in regulating dendrite morphologies of motor neurons. In fact, Sema6A–plexin-A4 signaling controls dendritic growth of motor neuron subtypes in the spinal cord by modulating a downstream signaling molecule, FARP1, which is a FERM Rho-GEF protein (Zhuang et al. 2009). FARP1 proteins are expressed on dendrites of LMC, but not MMC, motor neurons in chicks (Zhuang et al. 2009). Ectopic expression studies as well as knockdown approaches demonstrate that FARP1 is sufficient and essential for regulating the dendrite process length of LMC motor neurons (Zhuang et al. 2009). FARP2, a protein related to FARP1, interacts with all plexin-A family members (Toyofuku et al. 2005), whereas FARP1 strongly binds to plexin-A4 but associates only very weakly with plexin-A1 (Zhuang et al. 2009). Gain-of-function and knockdown experiments for *Sema6A* and *plexin-A4* show results similar to those observed following similar manipulations of FARP1, suggesting that FARP1 is a downstream molecule of Sema6A–plexin-A4 signaling (Zhuang et al. 2009). Sema6A has thus been shown to regulate motor neuron subtype-specific dendritic growth through the action of FARP1. Future studies will explore other potential roles of semaphorin–plexin signaling in the regulation of dendrite morphology in motor neuron pools and other CNS neurons.

3.8 Sympathetic Nervous System

The sympathetic nervous system, a major component of the autonomic nervous system, is required for organ homeostasis. Postganglionic sympathetic neurons arise from neural crest cells and eventually establish the superior cervical ganglion (SCG), whereas preganglionic neurons are generated and located within the spinal cord. Sympathetic neurons, particularly those from the SCG, have been used widely to understand the cellular and molecular mechanisms governing neuronal survival, retrograde signaling, neuronal migration, and axon guidance (Glebova and Ginty 2005).

Sema3A, Sema3C, Sema3D, and Sema3F have been shown to induce growth cone collapse or axonal repulsion of SCG neurons in vitro (Koppel et al. 1997; Adams et al. 1997; Chen et al. 1998), indicating that Npns and plexins are likely expressed by SCG neurons. In fact, Npn1, Npn2, plexin-A3, and plexin-A4 have all been shown to be expressed in the SCG (Cheng et al. 2001; Chen et al. 1998). SCG explants with COS cell aggregates expressing different class 3 semaphorins revealed

that Sema3A and Sema3F signals are mediated by Npn1 and Npn2, respectively, and both Npn1 and Npn2 can transduce Sema3C signals (Chen et al. 1998). Conversely, loss-of-function together with SCG explant culture experiments using COS cells expressing class 3 semaphorins showed that Npn2 is, indeed, required for axon repulsion of sympathetic neurons by Sema3F but not Sema3A (Chen et al. 2000).

Interestingly, *Npn1* and *Sema3A* mutant embryos showed displacement of both sympathetic precursors and neurons in vitro (Kawasaki et al. 2002), suggesting that Sema3A–Npn1 signaling controls cell migration. In fact, Sema3A suppressed neuronal migration activity of sympathetic neurons (Kawasaki et al. 2002). Further studies in which *Sema3A* or *Npn1* are deleted after the completion of neuronal migration will have to be conducted to determine whether Sema3A–Npn1 signaling is also involved in axon guidance of SCG neurons in vivo.

Because both *plexin-A3* and *plexin-A4* are strongly expressed by SCG neurons (Cheng et al. 2001), SCG axons were analyzed in *plexin-A3* single, *plexin-A4* single, and *plexin-A3/A4* double mutants (Cheng et al. 2001; Yaron et al. 2005; Suto et al. 2005). Sema3F-mediated SCG axon repulsion was completely abolished in *plexin-A3* single-mutant mice, but *plexin-A4*-deficient SCG axons responded to Sema3F in a manner similar to wild-type neurons (Cheng et al. 2001; Yaron et al. 2005; Suto et al. 2005). Thus, the Npn2–plexin-A3 receptor complex mediates Sema3F-induced SCG axonal repulsion. In contrast, both plexin-A3 and plexin-A4 transduce Sema3A signals to induce SCG axon repulsion (Cheng et al. 2001; Yaron et al. 2005). In vivo, aberrant axonal projections of SCG are observed in *plexin-A4* mutant mice (Suto et al. 2005). In contrast to *Npn1* mutant mice, *plexin-A4* mutant mice do not show displacement of SCG neurons (Suto et al. 2005). Future studies, particularly conditional targeting approaches, will reveal further roles for semaphorin signaling in the different steps of sympathetic nervous system development.

3.9 Motor Axon Guidance in Zebrafish

Three types of primary motor neurons are located in each trunk segment of zebrafish: caudal primary (CaP), middle primary (MiP), and rostral primary (RoP) motor neurons (Fig. 3.5a). Half the segments additionally contain a fourth type of motor neuron called variable primary (VaP); however, only a few VaP motor neurons survive until 36 h postfertilization (Beattie 2000). Axons of CaP, MiP, and RoP motor neurons exit the spinal cord from a defined exit point and innervate their specific targets on myotomes (Fig. 3.5a). Using transplant experiments, the myotome has been shown to be essential for motor axon targeting, suggesting that the myotome or myotome-derived factors regulate motor axon targeting (Beattie and Eisen 1997).

Zebrafish contain two Npn1s (Npn1a and Npn1b) and two Sema3As (Sema3A1 and Sema3A2). Npn1a, which is expressed in CaP motor neurons, and its ligands Sema3A1 and Sema3A2, which are expressed in the myotome, all exhibit dynamic

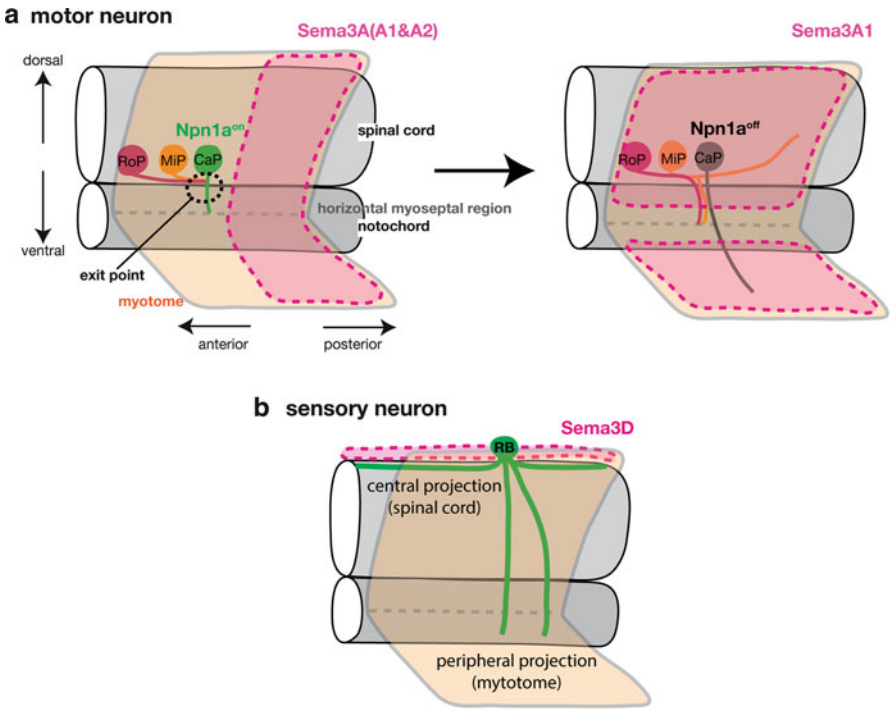

Fig. 3.5 Motor and sensory neuron development in zebrafish. (**a**) Caudal primary (CaP), middle primary (MiP), and rostral primary (RoP) motor neurons are located in the ventral spinal cord. Their axons exit the spinal cord from the exit point (*black dotted circle*) and innervate the myotome (*orange shaded area*). RoP, MiP, and CaP primary motor neurons all express *plexin-A3*. *Npn1a* is expressed by only CaP motor neurons before axons cross the horizontal myoseptal region (*gray dotted line*; *left diagram*). Expression patterns of *Sema3As* (*magenta shaded area*) also change from the posterior half of the myotome (*left diagram*) to the dorsal and ventral myotome regions with the exception of the horizontal myoseptal region (*right diagram*). At this stage CaP neurons do not express *Npn1a*, and CaP axons no longer respond to the repellent signals of Sema3A (*black line in right diagram*). (**b**) RB sensory neurons (*green*) project both centrally and peripherally. *Sema3D* (*magenta shaded area*) is expressed in the roof plate

expression patterns (Feldner et al. 2005; Sato-Maeda et al. 2006; Shoji et al. 2003). Npn1a expression in motor neurons is only observed while their axons are extending to the horizontal myoseptal region (Fig. 3.5a, left). Once this region is crossed, Npn1a expression is no longer detected (Fig. 3.5a, right). In contrast, Sema3A1 and Sema3A2 are initially expressed in the posterior half of the myotome; then, in later stages, only Sema3A1 is expressed in the myotome dorsal region with the exception of the horizontal myoseptal region (Fig. 3.5a). At these later stages, CaP neurons no longer respond to Sema3A1 as a result of diminished expression of Npn1a (Fig. 3.5a).

Npn1a morphants show aberrant axon growth and multiple nerve exit points (Feldner et al. 2005; Sato-Maeda et al. 2006, 2008). Although Npn1a–Sema3A1 signaling is essential for axon growth, Npn1a–Sema3A2 signaling has been shown

to be important for CaP positioning (Feldner et al. 2005; Sato-Maeda et al. 2006, 2008), which affects the location of CaP axons at the exit point (Sato-Maeda et al. 2008).

Another Sema3A receptor, *plexin-A3*, is expressed by primary motor neurons, and the *plexin-A3* mutants *sidetracked* and *vermicelli* both show aberrant motor neuron exit points and axonal trajectories (Tanaka et al. 2007; Palaisa and Granato 2007). Double knockdown of *plexin-A3* and *Sema3As* causes synergistic effects, but double knockdown of *plexin-A3* and *Npn1a* does not affect the branching or exit points of motor axons (Feldner et al. 2007), suggesting that plexin-A3 acts as the receptor for Sema3As rather than as a co-receptor for Npn1a. Thus, both the Sema3A–Npn1a and Sema3A–plexin-A3 signaling cascades likely act independently in the guidance of primary motor axons. Another class 3 semaphorin, Sema3F, is also expressed in the myotome, raising the possibility that it may serve as an additional plexin-A3 ligand along with Sema3A (Palaisa and Granato 2007). Plexin-A3 together with plexin-A1 also acts as a receptor for class 5 semaphorins, and this semaphorin–plexin signaling is essential for retinal lamination and function (Matsuoka et al. 2011). The myotome also expresses a class 5 semaphorin, Sema5A, which, similar to plexin-A3, is involved in determining exit points and axon trajectories of motor neurons (Hilario et al. 2009). In fact, double knockdown of *Sema5A* and plexin-A3 produces synergistic exit point defects, suggesting that Sema5A binds to plexin-A3 to regulate exit pathways. Although plexin-B1 is another potential ligand for Sema5A, the double-knockdown phenotype of plexin-B1 and Sema5A does not exhibit any synergistic effects. These data suggest that other plexins, such as plexin-A1, could potentially be involved as Sema5A receptor(s) in regulating axon branching in zebrafish. In the zebrafish cranium, trigeminal motor neurons and facial motor neurons that originate from rhombomeres project into jaw muscles. Plexin-A3 is expressed in these cranial motor neurons, whereas Sema3A1 is expressed in the brachial region. In fact, both *plexin-A3* mutants and *Sema3A1* morphants show defects in facial motor axon guidance (Tanaka et al. 2007). Another semaphorin family member that is also expressed in the brachial region, Sema4E (Xiao et al. 2003), has been shown to inhibit motor axon growth. Conversely, *Sema4E* morphants show defasciculation of motor axons. These data demonstrate that spatial expression patterns of Sema3A1 and Sema4E are essential for proper motor axon guidance in the cranial region. Further studies will identify the relevant receptors for Sema4E in these processes.

3.10 Sensory Axon Guidance in Zebrafish

Rohon-Beard (RB) sensory neurons are the primary sensory neurons in zebrafish and provide a great model system for understanding sensory development in vertebrates. Central projections of RB sensory neurons innervate the spinal cord whereas peripheral projections extend into the myotome (Fig. 3.5b). The transcription factors, Islet 1 and 2a (Isl1 and Isl2a), are expressed by RB sensory neurons to

regulate their peripheral projections. Isl2a is essential for plexin-A4 expression in RB sensory neurons (Miyashita et al. 2004). In addition, in embryos that overexpress the LIM domain of Isl1, *plexin-A4* expression was reduced and peripheral axon projections were eliminated. *Plexin-A4* knockdown also shows reduction in axon branching, suggesting that plexin-A4 is involved in regulating branching specifically, rather than affecting axon guidance. Interestingly, excessive branching of peripheral axons caused by Slit2 overexpression is rescued by *plexin-A4* knockdown (Miyashita et al. 2004). These data suggest that plexin-A4 acts as a downstream target of Slit signaling to regulate peripheral axon branching.

Isl1 is also important for regulating the expression of *collapsin-response mediator protein 4* (*Crmp4*), which is involved in the semaphorin-mediated signaling pathway controlling cytoskeleton and endocytosis regulation in RB neurons (Schmidt and Strittmatter 2007; Tanaka et al. 2011). Knockdown of *Crmp4* affects peripheral axon growth of RB neurons, and this phenotype is similar to *Sema3D* morphants (Liu and Halloran 2005; Tanaka et al. 2011). Additionally, double knockdown of *Sema3D* and *Crmp4* shows synergistic effects on peripheral axon growth. These data suggest that Sema3D and Crmp4 act on the same signaling cascade. Interestingly, both Sema3D and Crmp4 knockdown do not affect central projections of RB neurons. Only peripheral axons were shown to respond to ectopic Sema3D (Liu and Halloran 2005; Tanaka et al. 2011), indicating that Sema3D–Crmp4 signaling primarily affects the growth of peripheral axons. It has been hypothesized that the expression of Sema3D in the midline roof plate might repel peripheral axons from the spinal cord (Fig. 3.5b) (Liu and Halloran 2005; Tanaka et al. 2011). Alternatively, Sema3D might act as an initial stimulator for axon branching (Liu and Halloran 2005). In fact, Sema–plexin signaling is involved in axon branching in the fly and vertebrate retina (Campbell and Holt 2001; Winberg et al. 1998). Further studies seeking functional Sema3D receptor(s) such as plexins and neuropilins, and on the relationships between Sema3D signaling and their potential downstream targets such as Crmp4, will provide new insights into the molecular mechanisms of Sema–plexin-mediated sensory axon growth in zebrafish.

3.11 Summary

Semaphorins play various roles in neural development, such as in axon guidance, dendrite development, and synapse formation in the spinal cord through their plexin and neuropilin receptors. This chapter highlights the various combinations of semaphorins and their receptors that control different steps of neural circuit development within the spinal cord. Similar molecular mechanisms are observed in the invertebrate nervous system and the vertebrate developing brain (see earlier chapter by Kolodkin). Future studies will reveal how semaphorin signaling controls not only embryonic but also adult neural circuitry, whether it regulates neuronal degeneration/regeneration, and what upstream and downstream molecules are involved in different semaphorin signaling cascades. Furthering our knowledge of the roles of semaphorin signaling in neural circuit assembly and regeneration will

indicate some of the fundamental mechanisms involved in establishing functional neural circuits, and may one day serve as a new paradigm for understanding how signaling pathways shape and influence the developing and adult nervous system.

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Chapter 4
Semaphorins and Cell Migration in the Central Nervous System

Alain Chédotal

Abstract In the central nervous system (CNS), neuroblasts, postmitotic neurons, and glial cells migrate along stereotyped routes from their birthplace to their final destination. Two main types of neuronal migration have been distinguished: a radial one for neurons that migrate along radial glia in a direction perpendicular to the pial surface and a tangential mode for neurons which migrate along other neurons or axons, independently of radial glia (Marin O, Rubenstein JL, Annu Rev Neurosci 26:441–483, 2003; Metin et al, J Neurosci 28(46):11746–11752, 2008). The initiation of migration, the direction followed by migrating neurons or glia, and their decision at specific choice points are influenced by molecules in the environment of the neurons and by intrinsic developmental programs. Many studies in various systems have shown that semaphorins and their receptors play an essential role in this process. Semaphorins influence the motility of neurons and oligodendrocytes and also shape the pathway they follow during their migration. Here, I review these results, focusing on the vertebrate CNS and a few model systems.

Keywords Neural stem cells • Cerebellum • Granule cells • Centrosome • Oligodendrocytes • GnRH

4.1 Semaphorins Control the Radial Migration of Cortical Neurons

The six-layered mammalian neocortex contains two main types of neurons: (1) pyramidal neurons that primarily project outside the cortex and originate from the ventricular zone of the dorsal telencephalon, or pallium; and (2) interneurons, which make local connections between different layers or across layers and originate from the ganglionic eminence in the ventral telencephalon or subpallium (Rakic 2009;

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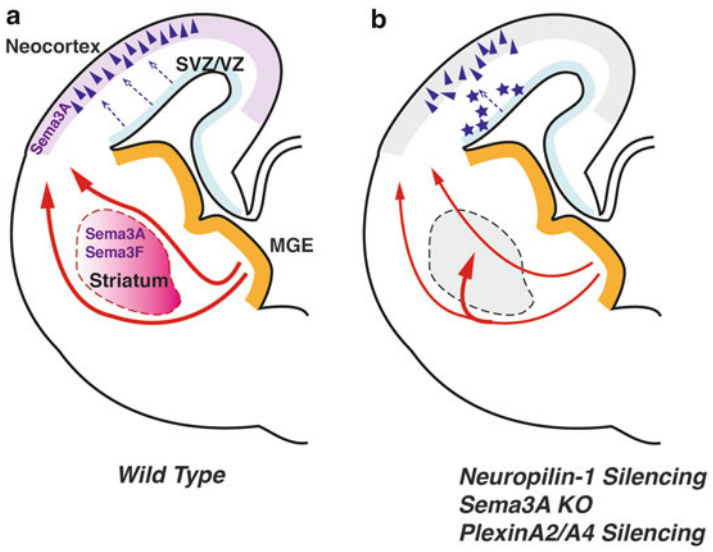

Fig. 4.1 Role of secreted semaphorins in cortical neuron migration. (**a**) In the embryonic forebrain of wild-type mice, cortical interneurons originate from the medial ganglionic eminence (*MGE*) and migrate tangentially to the cortex avoiding the striatum where Sema3A and Sema3F are expressed (*red arrows*). Sema3A is also expressed in the upper part of the cortical plate and promotes the radial migration of pyramidal neurons (*blue triangles*) from the subventricular/ventricular zone (*SVZ/VZ*) and controls their polarity. (**b**) Sema3A, neuropilin-1, and Plexin-A2 loss of function perturb the migration of both types of neurons. A large fraction of cortical interneurons invade the striatum. In the cortex, the radial migration of layer II/III neurons from the SVZ/VZ is affected and many neurons differentiate at an ectopic location. Their polarity and morphology are also abnormal

Bartolini et al. 2013). There is a variety of interneurons differing in morphology (dendritic arborization) and neurotransmitter content (Ascoli et al. 2008). Several studies have shown that semaphorins control the migration of both types of cortical neurons (Fig. 4.1). Pyramidal neurons are known to migrate along the processes of radial glia cells, which extend from the ventricular surface to the cortical surface (Metin et al. 2008). There is little dispersion of the pyramidal neurons during their radial migration, which at least partially explains the columnar organization of the cortex (Rakic et al. 2009).

Several studies showed that Sema3A is expressed in the superficial layers of the cortical plate (Bagnard et al. 1998; Polleux et al. 1998; Chen et al. 2007) in a decreasing gradient from the surface to the depth of the cortex. Cortical neurons also express the neuropilin-1 receptor during radial migration (Chen et al. 2007). In the embryonic day 16 (E16) mouse cortex, the silencing of neuropilin-1 expression using RNA interference or Sema3A overexpression strongly impaired the ability of layer II/II neurons to reach the cortical plate, although they could enter the intermediate zone and proliferated normally (Chen et al. 2007; Shelly et al. 2011) (Fig. 4.1). The same defects were seen using

recombinase-mediated inactivation of neuropilin-1 in cortical neurons. Co-culture experiments mixing cortical slices and sema3A-expressing cells showed that Sema3A has a chemoattractive activity on radially migrating neurons (Chen et al. 2007). Neuropilin-2 loss of function also significantly perturbed radial migration. Similarly, the silencing of Plexin-A4 and Plexin-A2 prevented cortical neurons from leaving the intermediate zone, suggesting that they interact with neuropilin-1 to mediate Sema3A activity. The polarity of the ectopic neuropilin-1-deficient neurons was severely affected, and they adopted a multipolar morphology with multiple neurites rather than a bipolar morphology with a single and unbranched leading process oriented toward the cortical surface (Chen et al. 2007; Shelly et al. 2011). A role for Sema3A in the polarization of newly generated neurons has been also demonstrated for hippocampal neurons, which also migrate radially (Shelly et al. 2011). This step is essential to initiate radial migration (Namba et al. 2014), and therefore Sema3A could act first to polarize neurons and initiate migration and later to attract the neurons toward the surface. Interestingly, similar migration defects were also observed after inactivation of various molecules that are known to act downstream of Sema3A/neuropilin-1 such as the cycling-dependent kinase Cdk5 (Gupta et al. 2003), the Src Kynase Fyn (Sasaki et al. 2002), or Tag-1 (Law et al. 2008; Namba et al. 2014). In addition, Sema3A inhibits the activity of protein kinase A on the phosphorylation of GSK3β and LKB1, two kinases acting on radial migration and neuronal polarity (Asada et al. 2007; Asada and Sanada 2010). However, the exact mechanism of action of Sema3A remains unknown, and the apparently normal cortical layering in the Sema3A knockout (Catalano et al. 1998) suggests that Sema3A acts redundantly with other factors. Sema3A not only controls the migration of pyramidal neurons but also regulates the growth of their apical and basal dendrites and guides their axons in the white matter (Polleux et al. 1998, 2000; Sasaki et al. 2002; Zhou et al. 2013). Surprisingly, much less is known about the function of transmembrane semaphorins in the migration of cortical neurons. A recent study (Azzarelli et al. 2014) revealed that Plexin-B2 is required for radial migration. In the mouse embryo, Plexin-B2 is expressed in the ventricular zone, subventricular zone, and cortical plate. ShRNA-mediated knockdown of Plexin-B2 prevents most neurons from leaving the ventricular zone/subventricular zone (VZ/SVZ) and perturbs the acquisition of cortical neuronal morphology. It was shown that the small GTP-binding protein, Rnd3, which inhibits RhoA signaling through p190RhoGAP (Wennerberg et al. 2003; Pacary et al. 2013), acts downstream of Plexin-B2 during radial migration, confirming previous studies that had shown that Rnds are partners of type-B plexins (Oinuma et al. 2004; Azzarelli et al. 2014). Plexin-B2 inhibits Rnd3, which results in the activation of RhoA activity. The antagonistic activity of Plexin-B2 and Rnd3 allows stabilizing the level of active RhoA during radial migration. Several class 4 semaphorins bind to Plexin-B2, and Sema4D enhances cortical neuron motility in vitro (Hirschberg et al. 2010). However, a role for transmembrane semaphorins in radial migration has not yet been confirmed in vivo. Worthy of note, the organization of the embryonic cortex is severely disturbed in the constitutive Plexin-B2 knockout, suggesting that

Plexin-B2 function in cortical development extends beyond radial migration and that it also acts on cell proliferation and neuronal differentiation (Hirschberg et al. 2010).

4.2 Semaphorin Function in the Tangential Migration of Cortical Neurons

Most inhibitory interneurons [using gamma-aminobutyric acid (GABA) as a neurotransmitter] are produced from progenitors located in the medial ganglionic eminence that migrate tangentially (between E12 and E16 in the mouse), independently of radial glia fibers, across the basal forebrain and then colonize the cortical wall (de Carlos et al. 1996; Anderson et al. 1997; Tamamaki et al. 1997). In the subpallium, cortical interneurons avoid the striatum where Sema3A and Sema3F are highly expressed (Fig. 4.1). During their migration, GABAergic interneurons express neuropilin-1 or neuropilin-2 receptors and are repelled from the nascent striatum by Sema3A and Sema3F. In the absence of neuropilin-1 or neuropilin-2 signaling, many interneurons enter the striatum (Marin et al. 2001; Tamamaki et al. 2003; Zimmer et al. 2010). Ectopic interneurons are also detected after silencing Plexin-A1 in cortical interneurons (Andrews et al. 2013). Lim kinases (Limk) control actin dynamics through cofilin and were previously seen to be involved in semaphorin signaling (Aizawa et al. 2001; Scott et al. 2009). Limk2 was detected in cortical interneurons, and loss-of-function studies using electroporation of Limk2 siRNA in forebrain slices and medial ganglionic eminence (MGE) suggest that it acts downstream of Sema3A/neuropilin-1/Plexin-A1 during migration (Andrews et al. 2013).

Migrating interneurons also express Roundabout 1 (Robo1), one of the receptors of Slit repellents, but Slits are not required for cortical interneuron migration in the subpallium (Marin et al. 2003). However, in Robo1 knockout mice, some GABAergic interneurons abnormally enter into the striatal anlage, and their density is also increased in the cortex (Hernandez-Miranda et al. 2011), a phenotype not observed in *Slit* knockouts (Marin et al. 2003; Andrews et al. 2008). It was recently shown that Robo1-deficient interneurons are less responsive to Sema3A/3F repulsion in migration assays and express a lower level of neuropilin-1 and Plexin-A1. This result suggests that there could be crosstalk between the semaphorin/plexin and Slit/Robo pathways in migrating neurons. Accordingly, Robo1 could be co-immunoprecipitated with neuropilins, suggesting that they are in the same receptor complex. Moreover, chondroitin sulfate proteoglycans (CSPGs), which bind to many extracellular matrix components, regulate the spatial distribution of Sema3A in the basal forebrain and potentiate its repulsive activity for migrating cortical interneurons (Zimmer et al. 2010).

Interestingly, neuropilin-2 expression in striatal interneurons is repressed by the transcription factor Nkx2.1 (Nobrega-Pereira et al. 2008), which directly binds to regulatory elements in the neuropilin-2 promoter. In *Nkx2.1* knockout, neuropilin-

2 is upregulated and there is a reduction in the number of striatal interneurons (Nobrega-Pereira et al. 2008). Therefore, the differential expression of neuropilin-2 by MGE-derived interneurons controls their cortical versus striatal fate. The Dlx1/2 TFs, which control MGE development, are also candidate repressors of neuropilin-2, but the possible influence of this interaction on cortical interneuron migration is unknown (Le et al. 2007).

Neuropilin-2 was also involved in the migration of another type of forebrain neurons, the "LOT cells." The lateral olfactory tract (LOT) contains axons projecting from the olfactory bulb to the pyriform cortex and conveys olfactory inputs. The LOT extends caudally along a region of the forebrain at the interface between the pallium (cortex) and subpallium (MGE) (Sato et al. 1998). It was shown that LOT axons follow a path that is delineated by a specific set of cells, called the LOT cells. It was initially thought that the LOT cells were acting as guidepost cells for LOT axons, but these can be misguided even if the LOT cells are properly positioned, suggesting that these are not sufficient for guiding LOT axons (Fouquet et al. 2007). Cell-labeling studies showed that LOT cells are among the first to be born in the dorsal telencephalon and that they migrate tangentially (between E9.5 and E11.5 in the mouse) in a ventral direction to their final position at the surface of the forebrain (Tomioka et al. 2000; Kawasaki et al. 2006). Sema3F (and not other secreted semaphorins) has a repulsive activity on migrating LOT cells, mediated by neuropilin-2 (Ito et al. 2008). In the final phase of LOT cell migration, Sema3F is expressed in the subpallium in a domain that is surrounding the LOT cells. In neuropilin-2- and Sema3F-knockout embryos, LOT cells migrate more deeply in the forebrain rather than being confined to the surface. Together, these data suggest that neuropilin-2/sema3F act at the end of LOT cell migration to confine them to their superficial position. In parallel, other guidance cues such as Netrin-1 are controlling the dorsoventral patterning of the LOT cells (Kawasaki et al. 2006).

As illustrated by these examples, semaphorins have an important function in the control of neuronal migration in the neocortex, suggesting that defects in semaphorin signaling might be linked to various human neurological diseases in which ectopic or mislocalized neurons were detected, such as schizophrenia and autism spectrum disorders. This hypothesis is supported by preliminary studies indicating increase of Sema3A expression in the cerebellum of schizophrenic patients (Eastwood et al. 2003) and the existence of single-nucleotide polymorphisms (SNPs) in a Japanese cohort of schizophrenics (Fujii et al. 2011). Similarly, there is also evidence for SNPs in Plexin-A2 (Mah et al. 2006; Allen et al. 2008) associated with schizophrenia, but this is debated (Fujii et al. 2007).

4.3 Semaphorins and the Migration of GnRH Neurons

Gonadotropin-releasing hormone (GnRH) has a key role in reproduction by controlling the production of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by the pituitary gland. Hypothalamic neuroendocrine cells secreting GnRH

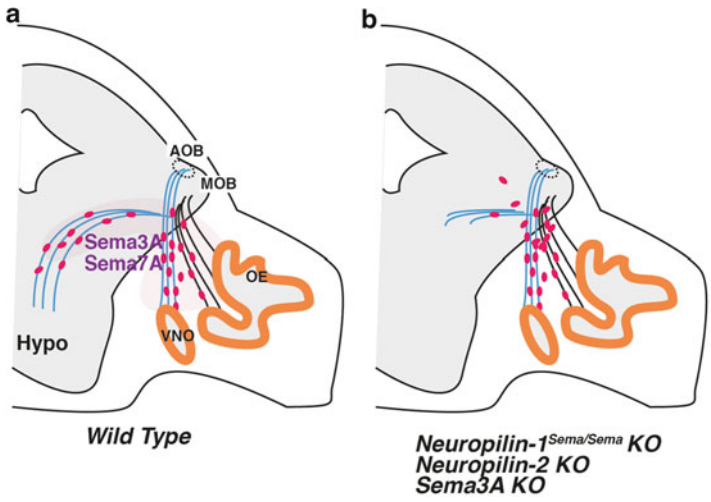

Fig. 4.2 Control of GnRH neuron migration by secreted semaphorins. (**a**) In the wild-type embryo, GnRH neurons (*red*) migrate along vomeronasal axons (*blue*) and olfactory axons (*black*) to the basal forebrain and hypothalamus. Sema3A and Sema7A are expressed along their migration pathway. (**b**) In neuropilin and Sema3A knockouts, the growth of olfactory axons and vomeronasal axons is perturbed, which results in the misrouting of GnRN neurons. Abbreviations: *VNO* vomeronasal organ, *OE* olfactory epithelium, *Hypo* hypothalamus, *MOB* main olfactory bulb, *AOB* accessory olfactory bulb

originate outside the central nervous system (CNS) in the nasal placode (Fig. 4.2) and migrate (from about E11 in the mouse) into the median eminence in the central nervous system along olfactory/vomeronasal axons (Cariboni et al. 2007b). This particular type of neuronal migration is called axophilic (Schwanzel-Fukuda and Pfaff 1989). The abnormal migration of GnrH neurons, such as in Kallmann syndrome, causes a depletion of GnRH neurons, leading to hypogonadism and infertility. Many Kallmann patients are also anosmic. So far, ten causative genes have been identified, only accounting for about one third of Kallmann cases: KAL1, which is X-linked and encodes Anosmin-1 (Legouis et al. 1991), fibroblast growth factor 8 (FGF8) and its receptor FGFR1 (Falardeau et al. 2008), HS6ST1 (Tornberg et al. 2011), WDR11 (Kim et al. 2010), CHD7 (Jongmans et al. 2009), Prokineticin-2 (PROK2, and its receptor PROKR2) (Dode et al. 2006; Pitteloud et al. 2007), and more recently SEMA3A (Hanchate et al. 2012). In mouse and human, olfactory and vomeronasal axons express neuropilin-1, neuropilin-2, and Plexin-A1 during GnRH neuron migration (Giger et al. 1996; Murakami et al. 2001; Cloutier et al. 2002; Cariboni et al. 2007a, b; Hanchate et al. 2012). Sema3A and Sema3F are found in the olfactory epithelium and vomeronasal organ (Cloutier et al. 2002). GnRH cells also express neuropilin-1/2, Plexin-A1 mRNA, Sema3A, and Sema3F (Cariboni et al. 2007a).

Many studies suggest that semaphorin/neuropilin are required for guiding vomeronasal/olfactory axons to the CNS. In the *Sema3A* knockout, olfactory axon

projections to the olfactory bulb are disorganized (Schwarting et al. 2000) and most GnRH neurons accumulate ectopically close to the olfactory bulb (OB) (Cariboni et al. 2011b), which likely explains hypogonadism in adult *Sema3A*$^{-/-}$ (Cariboni et al. 2011b; Hanchate et al. 2012). In *Neuropilin-1*$^{Sema/Sema}$ knock-in mice, a large fraction of olfactory axons fails to enter the OB, and vomeronasal axons are also misrouted (Cariboni et al. 2011b). In these mice, about half the GnRH neurons still migrate along these axons and, as a result, settle in the cortex and thalamus instead of the hypothalamus (Fig. 4.2). Puberty is delayed in *Neuropilin-1*$^{Sema/Sema}$ knock-in mice, and fertility is severely affected; this is also true for *Neuropilin-2* knockout mice, which have reduced gonadal size (Cariboni et al. 2007a). Their phenotypic analysis revealed that the number of GnRH neurons in the hypothalamus is significantly reduced (Cariboni et al. 2007a) and that many migrate into the dorsal telencephalon or accumulate at the nasal septum. In *Sema3F* knockouts, vomeronasal axons defasciculate at the surface of the CNS (Cloutier et al. 2002, 2004), but GnRH migrate normally (Cariboni et al. 2011b). Surprisingly, in this system, Sema3A seems to signal through neuropilin-1 and neuropilin-2 as a *Neuropilin-1*$^{Sema/Sema}$; *Neuropilin-2* double-mutant phenocopy of *Sema3A* knockouts (Cariboni et al. 2011b).

Interestingly, exon sequencing in Kallmann patients identified families with heterozygous nonsense and missense mutations in the *SEMA3A* gene (Hanchate et al. 2012; Young et al. 2012). These mutations either prevent Sema3A from being produced or result in the secretion of an inactive Sema3 protein (Hanchate et al. 2012; Young et al. 2012). It was also proposed that Sema3A heterozygous mutations alone are not sufficient to induce Kallmann syndrome but that it acts synergistically with other proteins or pathways also mutated in the patients (Hanchate et al. 2012). Together, these observations suggest that the inability of GnRH to enter the brain when semaphorin/neuropilin signaling is altered is probably secondary to a failure of olfactory axons to penetrate into the olfactory bulb.

However, there is also evidence for a cell-autonomous action of neuropilin-1 in GnRH neurons. In chemotactic assays, Sema3A and Sema3F have a repulsive activity on the migration of the GnRH cell line (GN11) whereas vascular endothelial growth factor (VEGF) has an attractive activity (Cariboni et al. 2007a). VEGF also controls their survival (Cariboni et al. 2011a). Interestingly, both activities appear to be mediated by neuropilin-1, at least in vitro.

The phenotypic analysis of *Neuropilin-1* null embryos (Cariboni et al. 2007a) revealed a severe decrease of the number of GnRH neurons in the forebrain, which could be explained by a combination of increased cell death and abnormal migration. This reduction was also observed following conditional deletion of neuropilin-1 in neuronal precursors (Cariboni et al. 2011a). However, it was also reported that the conditional inactivation of neuropilin-1 in GnRH neurons (using a *GnRH*:*cre* line) does not perturb their migration (Hanchate et al. 2012), which would argue in favor of the non-cell-autonomous model of action of neuropilin-1 in GnRH neurons.

Transmembrane semaphorins have also been involved in the migration of GnRH neurons. The GPI-linked Sema7A is expressed along the migratory pathway

followed by GnRH neurons to the olfactory bulb. GnRH neurons express two of its receptors, integrin-β1 and Plexin-C1, and their migration is altered in Sema7A knockout mice, which have fertility defects (Messina et al. 2011). GnRH neurons and olfactory axons also express Plexin-B1 during their migration, and its ligand Sema4D promotes, at least in vitro, the migration of GnRH cells by coupling B1 to methylethyltryptamine (*N*-methyl, *N*-ethyltryptamine, MET), a tyrosine kinase receptor for hepatocyte growth factor (HGF) (Giacobini et al. 2008). The migration of GnRH neurons is perturbed in *Plexin-B1* knockouts although the olfactory projections look normal. However, GnRH neurons are not affected in *Sema4D* knockouts, suggesting that other class 4 semaphorins might act redundantly. Interestingly, at later stages, semaphorin signaling (such as 4D/B1) regulates cellular rearrangements that accompany the cyclic production of the pituitary hormones (see Messina and Giacobini 2013, for review).

4.4 When Semaphorins Restrain Neuronal Migration at CNS Boundaries

In the hindbrain and spinal cord, several studies showed that semaphorins act at boundaries between CNS compartments to restrict cell migration.

Boundary cap (BC) cells are neural crest cell derivatives localized at the motor axon exit points at the border between the CNS and PNS in vertebrate embryos (Vermeren et al. 2003). Following genetic or surgical ablation of BC cells, motor axons exit the brain at their normal position, but some motor neuron cell bodies migrate out of the CNS inside the motor nerves, which suggests that BC cells constitute a repulsive barrier for motor neurons (Fig. 4.3). It was later found that in mouse and chick embryos BC cells express Sema6A, Sema3B, and Sema3G (Bron et al. 2007; Mauti et al. 2007). Motor neurons express neuropilin-2 (a Sema3B receptor) and Plexin-A2. In neuropilin-2 knockouts, a significant fraction of the motor neurons enter the nerve roots (Bron et al. 2007), but this is not seen when sema3B is inactivated, suggesting that BCs express another neuropilin-2 ligand. Motor neuron emigration in the peripheral nervous system (PNS) was also observed following silencing of Sema6A expression in BC cells and of its receptor plexin-A2 in motor neurons (Bron et al. 2007).

During early development, the hindbrain is transiently segmented into cellular units called rhombomeres (Lumsden and Keynes 1989; Kiecker and Lumsden 2005). Each rhombomere expresses specific combinations of transcription factors and contain progenitors that generate various neuronal populations. There is little cell dispersion and mixing between neighboring rhombomeres, and specialized cells, with distinct molecular properties and that do not produce neurons, delineate rhombomere boundaries (Lumsden and Krumlauf 1996). In zebrafish embryos, a group of neurons expressing fibroblast growth factor 20a (fbfg20a) occupy the center of each rhombomere, where it represses neuronal differentiation (Gonzalez-

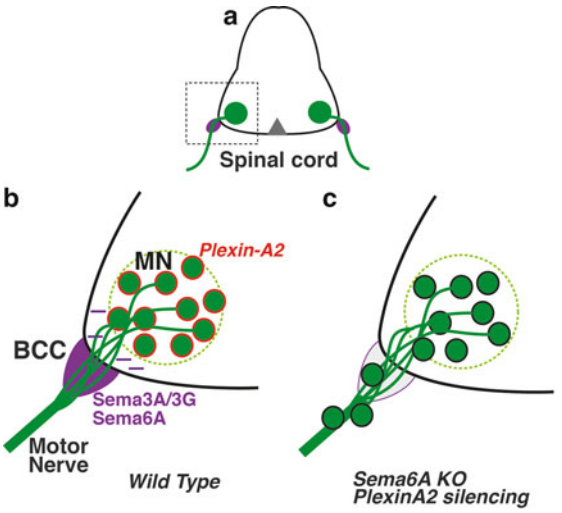

Fig. 4.3 Semaphorins and boundary cap cells. (**a**) Schematic representation of the embryonic spinal cord. Motor neurons (*green*) are localized in the ventral part of the spinal cord and send their axons outside the cord. (**b**) Region outlined in (**a**). In the wild type, motor neurons (*MN*) express Plexin-A2 and their cell bodies are prevented from leaving the spinal cord by semaphorins, expressed by boundary cap cells (*BCC*). (**c**) After Sema6A or Plexin-A2 loss of function, some MN exit the spinal cord and migrate inside motor nerves

Quevedo et al. 2010). Fgf20a neurons were found to express neuropilin-2 whereas boundary cells express its ligands, Sema3fb and Sema3gb (Terriente et al. 2012). The knockdown of either neuropilin-2 or Sema3fb/Sema3gb leads to the dispersion of Fgf20 neurons inside the rhombomere and increased neurogenesis; this suggests that Sema3fb/Sema3gb, secreted by boundary cells, repels fgf20 cells and confines them to the center of each rhombomere, thereby influencing neurogenesis.

Neuropilin-1 also influences neuronal migration between rhombomeres. In the mouse, neurons that will form the facial motor nucleus are generated in rhombomere 4 but migrate caudally to settle in rhombomere 6 (Studer et al. 1996). In neuropilin-1-null mice, the tangential migration of facial motor neurons is compromised and most fail to reach rhombomere 6 (Schwarz et al. 2004). In this case, it was found that VEGF, and not class 3 semaphorins, is the neuropilin-1 ligand.

4.5 Semaphorins and Plexins Control the Migration of Granule Cells in the Cerebellum and Olfactory Bulb

In the developing cerebellum of most vertebrates, granule cells (GCs) derive from precursors localized in the external granular layer (EGL), which covers the surface cerebellar cortex (Chedotal 2010). In mice, GCs are generated during the first 3 postnatal weeks. They first migrate tangentially in the EGL and then radially across the molecular layer to reach the internal granule cell layer (IGL) (Ramon and Cajal 1911; Altman 1972) (Fig. 4.4).

Previous studies showed that during their tangential migration GCs express the transmembrane semaphorin Sema6A (Kerjan et al. 2005), which is downregulated at the onset of radial migration. Migrating GCs also express Plexin-A2, one of

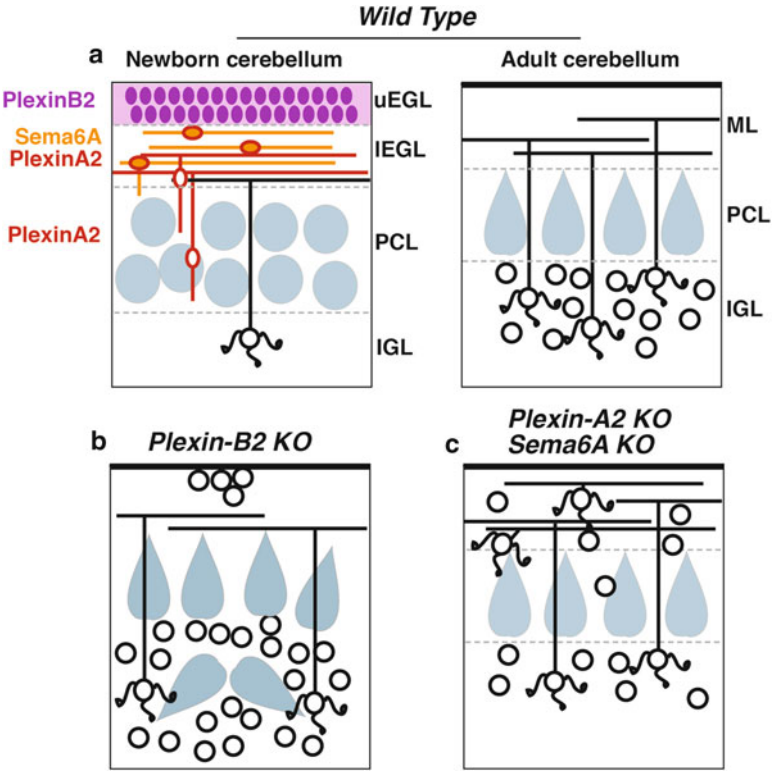

Fig. 4.4 Control of cerebellar granule cell migration by transmembrane semaphorins. (**a**) In newborn wild-type mice, granule cell progenitors (in *purple*) proliferate exclusively in the upper external granule cell layer (*uEGL*) and express Plexin-B2. Postmitotic granule cells start differentiating and migrate tangentially in the lower EGL (*lEGL*). During this phase, they express Sema6A and Plexin-A2. Next, they migrate radially through the molecular layer (*ML*) and the Purkinje cell layer (*PCL*) to the internal granule cell layer (*IGL*). Sema6A is downregulated at the onset of radial migration. At this stage Purkinje cells (in *blue*) are still distributed in multiple layers. In adult mice, Purkinje cells are aligned in a monolayer, above differentiated granule cell bodies all localized in the inner granular layer (*IGL*). (**b**) In *Plexin-B2* mutants, some ectopic granule cells are found at the top of the molecular layer and the cerebellar cortex is fragmented. Islands of Purkinje cells (*arrow*) are embedded in differentiated granule cells. (**c**) In *Sema6A* and *Plexin-A2* knockouts, about half the granule cells fail to migrate radially and are found in the molecular layer

the receptors for Sema6A (Renaud et al. 2008). Phenotypic analysis of *Sema6A* knockout and *PlexinA2* knockout mice revealed that migration from the EGL of about half of the GC population is aborted and that their cell body remains trapped inside the molecular layer (Kerjan et al. 2005; Renaud et al. 2008). Time-lapse studies of GC migration in postnatal EGL explants showed that, in both *Sema6A* and *PlexinA2* knockouts, the migration defects are probably related to an anomaly of nuclear translocation (Renaud et al. 2008). In *Sema6A* knockouts,

the centrosome appears unable to detach from the nucleus, whereas in *Plexin-A2* knockouts, the distance separating the centrosome from the nucleus becomes significantly increased. Therefore, in both cases, an abnormal coupling of the nucleus and centrosome might block migration. However, this could just illustrate a more global disorganization of the migration machinery, in particular of the actin dynamics. In the current working model, Sema6A–Plexin-A2 interaction is thought to have a role in the control of the switch from a tangential to radial mode of migration (Chédotal 2010) as this is correlated with the downregulation of Sema6A expression. The analysis of GC migration in mouse chimeras combining the wild type and either Plexin-A2 or Sema6A knockouts suggests that Sema6A is a ligand for Plexin-A2 in migrating GCs (Renaud et al. 2008). In vitro assays have also shown that GC migration is impaired on Sema6A-expressing cells (Janssen et al. 2010), suggesting that Sema6A could repel GCs away from the EGL. However, it might promote cell motility rather than simply inhibiting tangential migration. In vitro data suggest that the guanine nucleotide exchange factor (GEF) trio might act downstream of Plexin-A2/Sema6A (Peng et al. 2010). Rac1 might also be involved, because the conditional inactivation of Rac1 in migrating GCs somehow phenocopies the defects observed in *Sema6A* and *Plexin-A2* knockouts (Tahirovic et al. 2010). As Sema6A and Plexin-A2 are also coexpressed in GCs, this suggests that they could also signal in *cis* in tangentially migrating GCs, as described for Sema6A and Plexin-A4 in sensory axons (Haklai-Topper et al. 2010).

During their proliferation in the upper part of the EGL, GC precursors express Plexin-B2, which is downregulated in postmitotic GCs entering the lower EGL (Fig. 4.4). Therefore, there is a correlation between Plexin-B2 extinction and the initiation of tangential migration and cell-cycle exit (Friedel et al. 2007). In Plexin-B2 knockout mice, GC differentiation is initiated and the GCs migrate away from the cerebellar surface along radial glia. However, mutant GCs do not stop their proliferation and keep dividing outside the upper EGL, within the molecular layer, and even in the IGL, which profoundly disorganizes the structure of the adult cerebellar cortex (Deng et al. 2007; Friedel et al. 2007). The Plexin-B2 mechanism of action and its physiological ligand(s) in the developing cerebellum are still unknown, although genetic and in vitro evidence suggests that Sema4C and Sema4G are involved (Maier et al. 2011). Both semaphorins are expressed in migrating GCs; they bind to Plexin-B2, and in Sema4C/sema4G double knockouts a subset of GCs does not migrate properly. However, the cerebellar defects are much more severe in Plexin-B2 knockouts, supporting the existence of other ligands.

Interestingly, Plexin-B2 is also expressed in the postnatal and adult brain in the precursors of olfactory bulb (OB) granule cells (Saha et al. 2012). These interneurons are generated throughout life from neural stem cells located in the subventricular zone lining the cerebral ventricles. These cells produce neuroblasts that migrate tangentially to the OB along the rostral migratory stream (RMS) (Lois and Alvarez-Buylla 1994; Sanai et al. 2011). These cells differentiate and switch to a radial mode of migration on entering the OB (Bovetti et al. 2007; Snapyan et al. 2009). As in the cerebellum, the downregulation of Plexin-B2 expression is correlated with the final cell division and the initiation of the radial

migration of postmitotic granule cells. However, in this case, Plexin-B2 promotes the proliferation of SVZ neuroblasts in addition to acting on GC migration (Saha et al. 2012). It also acts on periglomerular cells, the second type of OB interneurons generated by SVZ neuroblasts. RMS neuroblasts migrate more rapidly in *Plexin-B2* knockout than in wild-type mice. Similarly, macrophages and dendritic cells purified from Plexin-B2 knockout mice migrate more rapidly (Roney et al. 2011), suggesting that in various cell types, including cerebellar cortical and OB neurons, Plexin-B2 could provide a molecular tuning mechanism allowing migrating cells to control migration/proliferation choices at specific locations and time points. Again, the Plexin-B2 ligands involved in this process remain to be characterized.

4.6 Semaphorins Control the Migration of Oligodendrocyte Precursors During Myelination and Remyelination

Oligodendrocytes are the myelinating cells in the central nervous system of jawed vertebrates (Zalc et al. 2008). These cells extend processes that wrap around the axon and form the myelin sheath, interrupted at the level of the nodes of Ranvier where voltage-gated sodium channels accumulate, which allows the rapid saltatory conduction of action potentials (Sherman and Brophy 2005 for a review).

Oligodendrocytes are generated across the CNS from precursors (or OPCs, oligodendrocyte precursors) localized in multiple neuroepithelial foci of the ventricular zone (Pringle and Richardson 1993; Vallstedt et al. 2005). Starting at embryonic ages, OPCs migrate from the ventricular zone to colonize the CNS. In rodents, OPCs start to produce postmitotic oligodendrocytes after birth, but myelination continues for several weeks. Moreover, some OPCs are still present in the adult CNS and produce new oligodendrocytes throughout life. In demyelinating diseases, such as multiple sclerosis (MS), the myelin sheath is destroyed and oligodendrocytes die, leading to myelin-poor areas called plaques. OPCs also exist in the adult CNS and can proliferate to generate new oligodendrocytes in response to injury (Franklin and Ffrench-Constant 2008). These adult OPCs appear to migrate into demyelinated regions, and their recruitment is required for myelin repair. The migration and proliferation of OPCs during development is influenced by trophic/growth factors such as platelet-derived growth factor (PDGF) and fibroblast growth factor 2 (FGF-2). However, mounting evidence indicates that axon guidance molecules, including secreted semaphorins, also pattern their migration.

OPCs invade the optic nerve (ON) from the preoptic area in the brain, around 2 weeks of gestation, and reach the optic nerve head before birth (Spassky et al. 2002). In mammals, OPCs do not enter the retina. In mouse and rat embryos, ON nerve OPCs express neuropilin-1 and neuropilin-2 (Sugimoto et al. 2001; Spassky et al. 2002) as well as postnatal rat OPCs (Cohen et al. 2003). Neuropilin-1 is still expressed in mature oligodendrocytes (Ricard et al. 2001), unlike neuropilin-2 (Xiang et al. 2012). In three-dimensional (3D) collagen gel cultures, OPCs

migrating from ON explants are repelled by cells secreting Sema3A, and this repulsive activity is blocked by anti-neuropilin-1 antibodies (Sugimoto et al. 2001; Spassky et al. 2002). By contrast, OPCs are attracted by cells secreting Sema3F via a receptor complex comprising neuropilin-2 (Spassky et al. 2002) and Plexin-A3 (Xiang et al. 2012). These chemotropic activities appear specific, as migrating OPCs do not respond to gradients of soluble Sema3C or Sema3E. However, postnatal OPCs avoid substrate-bound Sema3C and Sema3B (Cohen et al. 2003). Sema3A is enriched in cells that surround the ON and might prevent OPCs from leaving the ON. Sema3F, which is expressed by retinal ganglion cells, might attract OPCs toward their axons. Moreover, oligodendrocytes themselves express several secreted and transmembrane semaphorins (Cohen et al. 2003; Moreau-Fauvarque et al. 2003; Bernard et al. 2012), which might also act influence cell–cell interaction during oligodendrocyte migration. For instance, Sema4F is a transmembrane semaphorin (Encinas et al. 1999) expressed by OPCs in the mouse CNS (Armendariz et al. 2012). In vitro migration assays suggest that Sema4F inhibits the migration of ON OPCs, possibly in a paracrine/autocrine manner. Of note, direct in vivo support for these models is still lacking as neither the distribution of oligodendrocytes nor myelination seems to be perturbed in *Sema3A*, *Sema3F*, or *neuropilin-1/2* knockouts (Kitsukawa et al. 1997; Taniguchi et al. 1997; Chen et al. 2000; Giger et al. 2000). A recent study suggests that the distribution of OPCs is uneven in the neocortex of newborn Plexin-A4 knockout mice (Okada and Tomooka 2012). Plexin-A4 forms, with neuropilin-1, a receptor complex for Sema3A, but it is also a receptor for Sema6A. Sema6A is expressed during myelination by postnatal oligodendrocytes and could therefore influence the migration of OPCs. Accordingly, 3T3 cells expressing Sema6A have a repulsive activity for cells of an OPC line, which is mediated by Plexin-A4 (Okada et al. 2007; Okada and Tomooka 2013). However, these cells also express Sema6A and are repelled by Plexin-A4, suggesting that Sema6A might act as a receptor in OPCs. Although the analysis of *Sema6A* knockout revealed a myelination delay of CNS white matter tracts, including the ON, the recruitment of oligodendrocytes to these axonal tracts does not seem to be altered (Bernard et al. 2012), which would support a ligand function of Sema6A in OPC migration, if any.

Recent data suggest that secreted semaphorins might also influence the remyelination of demyelinating lesions by acting on the migration of adult OPCs. It has been proposed that impaired remyelination in chronic MS lesions is linked to a poor recruitment of OPCs to the plaques. Adult OPcs still express neuropilins and Plexin-A1/-A3 receptors, suggesting that these and OPCs could still be attracted or repelled by Sema3F and Sema3A, respectively (Piaton et al. 2011). It was shown that the expression of Sema3A and Sema3A transcripts is enriched and upregulated in neurons and glia (mostly astrocytes) at active inflammatory MS lesions and in demyelinating lesions induced by lysophosphatidylcholine (LPC) in the rat spinal cord (Williams et al. 2007). Therefore, abnormal semaphorin expression might perturb the ability of OPCs and new oligodendrocytes to disseminate inside the lesion, opening new therapeutic perspectives for MS treatment. A first step toward the in vivo validation of this strategy has been obtained in LPC mouse models

in which lentiviral vectors expressing Sema3A or Sema3F were injected into the demyelinated lesion. Sema3F overexpression increased significantly the number of OPCs at the lesion whereas Sema3A decreased it (Piaton et al. 2011). In addition, OPC recruitment after LPC lesions was impaired in *neuropilin-1*$^{Sema/Sema}$ knock-in mice, which are unable to bind Sema3A but still bind vascular endothelial growth factor (VEGF) (Gu et al. 2003). Although another study has confirmed the inhibitory action of Sema3A on remyelination in a model of ethidium bromide-induced demyelination (Syed et al. 2011), it was suggested that this was primarily through an action on OPC differentiation rather than on their migration. In conclusion, the physiological function of semaphorins in myelination and remyelination is still an open question. Another transmembrane semaphorin, Sema5A, is expressed by cells of the oligodendrocyte lineage (Goldberg et al. 2004; Hilario et al. 2009), as well as its receptor Plexin-B3 (Artigiani et al. 2004). However, no myelin defects were detected in Plexin-B3 knockout mice (Worzfeld et al. 2009). By contrast, Sema5A was found to inhibit the motility of glioma cells through Plexin-B3 and the inactivation of Rac1. Sema5A also promotes the glial differentiation of glioma cells, suggesting that a downregulation of Sema5A expression, as observed in human astrocytomas, might influence the dissemination of tumor cells within the CNS (Li and Lee 2010; Li et al. 2012).

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Chapter 5
Structure of Semaphorins and Their Receptors

E. Yvonne Jones

Abstract The biological activities of the semaphorins and their receptors depend on their structural properties and atomic-level interactions. This chapter reviews our current knowledge of molecular architecture and interactions with the aim of identifying features and properties that fit the semaphorins and their receptors for biological function. Structural analyses of the hallmark sema domain in semaphorins have revealed a seven-bladed β-propeller with distinctive elaborations that mediate dimerization as well as receptor binding. The plexins, the major family of semaphorin receptors, are also distinguished by the presence of an N-terminal sema domain in their ectodomain. In plexins the sema domain fold is modified to serve as a monomeric platform for interactions with semaphorin ligands and co-receptors. In the recognition complex between semaphorin and plexin, the dimeric semaphorin acts as a crosslink to juxtapose two plexin receptors. The role of extracellular dimerization in triggering signaling appears to be mirrored inside the cell by dimerization-driven activation of the plexin cytoplasmic segment. The architecture of the cytoplasmic segment is novel in comprising a small RhoGTPase-binding domain inserted into a GTPase-activating protein (GAP)-type topology. The functional properties of this unique signal transducer are beginning to emerge, but the mode of signaling is one of many aspects of the semaphorin–plexin system that still pose fascinating questions. In addition to surveying our current state of knowledge, this chapter delineates the limits of our understanding of molecular mechanism in semaphorin biology.

Keywords Crystal structure • Molecular mechanism • Cell guidance • Plexin • Neuropilin

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5.1 Introduction

The other chapters in this volume explore the multifaceted functional activities of semaphorin signaling systems. What information pertinent to this biomedically important biology can we usefully derive from detailed analyses of the three-dimensional structures and interaction characteristics of semaphorins, their receptors, and their co-receptors? This chapter focuses on structure from the point of view of how it contributes to the molecular mechanisms at work in biological function. Semaphorins interact with their plexin receptors to trigger signal transduction through direct extracellular binding events. These interactions with cell-surface plexins can variously involve secreted semaphorins, semaphorins attached to an opposing cell surface (i.e., interactions in *trans*), or semaphorins on the same cell surface (*cis* interactions). The structural characteristics of semaphorin and plexin ectodomain segments must furnish them with the appropriate specificity and binding affinities to associate ligands with cognate receptors and, when appropriate, trigger signaling. The transmembrane segments must link the extracellular events initiating signaling through to the interior of the cell. Similarly, the architecture and interactions of the cytoplasmic segment of the plexin must combine to provide appropriate fidelity and subcellular localization for signal transduction to generate the correct cellular response. In the following sections, the current state-of-play in our understanding of structure and function is explored and the challenges for future work discussed.

5.2 The Sema Domain: A Stable Platform for Protein Interactions

Semaphorins were defined as a family of glycoproteins bearing the hallmark of an approximately 500-residue N-terminal sequence of unknown structure, the so-called sema domain (Kolodkin et al. 1993). Contemporaneous reports of crystal structures for the sema domain of Sema3A (Antipenko et al. 2003) and the full ectodomain of Sema4D (Love et al. 2003) first revealed the novel architecture of this domain. Subsequent studies have provided us with structures for the sema domains of Sema6A, Sema7A (in complex with its cognate plexin receptor, PlxnC1), and that of a pox virus semaphorin, A39R (Janssen et al. 2010; Nogi et al. 2010; Liu et al. 2010). All these examples of sema domains show practically identical three-dimensional structures at the level of the protein main chain, conforming to a common type of protein fold termed a β-propeller. In this domain architecture the blades of the 'propeller' are formed by four-strand anti-parallel β-sheets that are arrayed sequentially around the central axis of the propeller (Fig. 5.1a). The circular arrangement of blades is typically locked into place by an N-terminal β-strand completing the C-terminal blade, and this is the case in the sema domain.

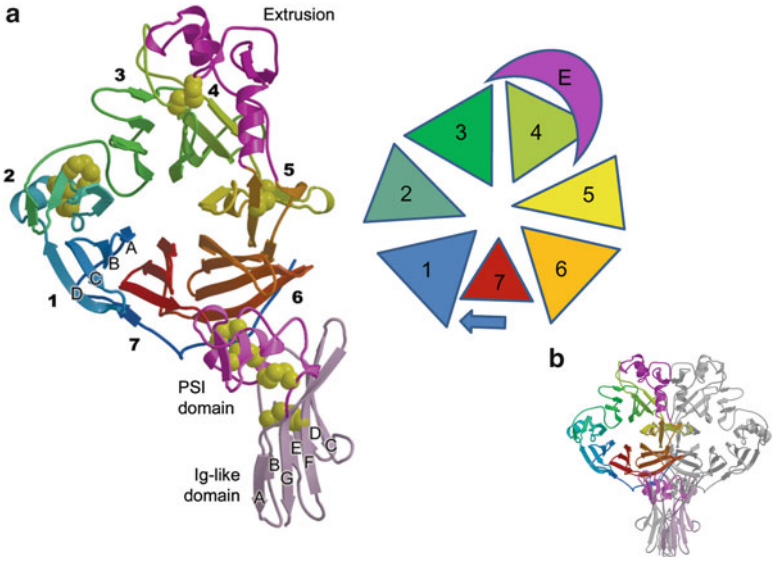

Fig. 5.1 Semaphorin ectodomain structure. (**a**) Structural cartoon representation of Sema4D ectodomain. The sema domain is colored (*blue* N-terminus, *red* C-terminus). Key elements of the semaphorin sema domain structure are highlighted by the accompanying schematic, namely, the seven blades of the β-propeller, the contribution of an N-terminal β-strand to blade 7, and the elaboration of the 'extrusion' insertion in blade 5. (**b**) Structural cartoon representation of the Sema4D ectodomain dimer (Adapted from Love et al. 2003)

The size of a β-propeller domain varies in accordance with the number of blades; the sema domain comprises seven blades, a particularly common β-propeller size (Chen et al. 2011).

What do the characteristics of the β-propeller fold imply for sema domain function? This arrangement of β-strands has the general property of providing a very stable and rigid platform (Fülöp and Jones 1999). X-ray crystallographic analyses of a protein carried out using different crystal forms provide independent three-dimensional 'snapshots' of the structure. Comparisons of such snapshots can reveal differences in structure that occur as a result of the different packing arrangement of the proteins in the crystals; for example, a loop may differ in conformation or the relative orientation of two domains may alter. Regions of inherent flexibility may thus be identified. Pairs of independently determined sema domain structures allow such comparisons to be made for Sema6A (Janssen et al. 2010; Nogi et al. 2010) and Sema3A (Antipenko et al. 2003; Janssen et al. 2012). Indeed, the structures are essentially identical in both cases, consistent with the sema domain providing a rigid scaffold. The β-propeller fold has been utilized for a broad spectrum of activities in extracellular and cytosolic proteins, often providing sites for protein interactions (Chen et al. 2011). It is of interest, given the associated cellular functions, that the seven-blade β-propeller domain in the α-chain of the integrin

family of cell-surface adhesion molecules shares closest structural similarity to that of the sema domain (Antipenko et al. 2003; Love et al. 2003). The integrin β-propeller domain provides interaction sites for ligands as well as contributing to the heterodimerization interface between the integrin α- and β-chains (Luo et al. 2007).

Although possibly evolutionarily related to the integrin β-propeller, the semaphorin sema domain defines a distinct subgroup of seven-blade β-propellers in which this robust platform is adorned with an insertion of some 70 residues. These additional residues exit after β-strand C of blade 5 and wend an elaborate path over the top, bottom, and side surfaces of the β-propeller before rejoining the canonical fold to complete the fifth blade. We have termed this rococo feature the extrusion (Love et al. 2003) (Fig. 5.1a). Comparisons between Sema3A, Sema4D, Sema6A, Sema7A, and A39R reveal that the extrusion plus the β-propeller form a single highly conserved structural unit.

Sequence alignments indicate that the sema domain is also a characteristic feature of the N-terminal regions of the plexin and MET families of cell-surface receptors (Winberg et al. 1998; Tamagnone et al. 1999). The MET family of receptor tyrosine kinases comprises MET, the receptor for HGF/SF (hepatocyte growth factor/scatter factor), and RON, the receptor for HGFl/MSP (hepatocyte growth factor-like/macrophage-stimulating protein). Gherardi and coworkers presciently deduced, from detailed analysis of mutagenesis and sequence data, the β-propeller-type topology of the MET sema domain (Gherardi et al. 2003). Crystal structures are now available to provide atomic-level detail for the sema domains of MET, RON, and representatives for three of the four classes of plexins, PlxnA2, PlxnB1, and PlxnC1 (Stamos et al. 2004; Niemann et al. 2007; Chao et al. 2012; Janssen et al. 2010; Nogi et al. 2010; Liu et al. 2010). Semaphorin, plexin, and MET sema domains group as distinctive variants of the β-propeller fold because they all include extrusion-like insertions (Gherardi et al. 2004). Pairwise structural comparisons reveal the most similarity between the insertions of the plexin and MET sema domains. Interestingly, this subgrouping echoes a marked difference in the physical characteristics of the domains; of the three types, only the semaphorin sema domain shows a strong propensity to form homodimers.

The sequence similarities between semaphorin, plexin, and MET family members extend beyond the sema domain into a cysteine-rich segment of some 50 residues that also shows homology to a segment in the β-chain of integrins, resulting in this region being defined as a PSI (plexin-semaphorin-integrin) domain (Bork et al. 1999). The structure of a PSI domain was first elucidated as part of the crystal structure of the Sema4D ectodomain (Love et al. 2003) (Fig. 5.1a). This semaphorin PSI domain and subsequent structures for MET, integrin, and plexin family members showed a compact cysteine knot-type fold defined by three conserved disulfide bridges (Kozlov et al. 2004; Xiao et al. 2004; Janssen et al. 2010; Nogi et al. 2010; Liu et al. 2010). In semaphorin and plexin ectodomains the sema and PSI domains are tightly coupled by an apparently rigid interface.

5.3 The Semaphorin Ectodomain Structure Provides a Dimeric Ligand

The first crystal structures of semaphorin sema domains revealed they formed dimers (Antipenko et al. 2003; Love et al. 2003) (Fig. 5.1b). This observation chimed with results dating back to some of the first biochemical characterizations of secreted semaphorins that associated biological activity with the dimeric forms of the proteins (Klostermann et al. 1998; Koppel and Raper 1998). All subsequent semaphorin crystal structures have shown the same dimeric arrangement. The sema–sema interface results from an off-center 'face-to-face' interaction between the top surfaces of the two β-propellers. Part of this interface is contributed by the extrusion. Some of the interface residues are hydrophobic and show some degree of conservation across semaphorin classes; however, there are also substantial inter-class variations resulting in constellations of specific hydrophobic and electrostatic interactions. Thus, although the gross architecture of sema domain dimerization is conserved, if we zoom in to inspect the interfaces at the level of individual residues we find numerous distinctive features. These variations are large enough to make it unlikely that the sema domains of different semaphorin classes can heterodimerize; however, it remains possible that the level of interface conservation within a semaphorin class may be sufficient to permit heterodimerization.

The absolute conservation of the dimer architecture across species and semaphorin classes is highly indicative of its functional importance. Detailed structural maps of sema–sema domain interfaces can be used to guide the design of mutations to disrupt dimerization while maintaining the sema domain fold. Monomeric forms of Sema4D and Sema6A have been successfully engineered using this strategy. These mutant molecules retain the ability to bind their cognate plexin receptors but show no biological activity in cellular assays (Janssen et al. 2010). Conversely, a version of Sema6A that is locked into the dimer form by addition of a disulfide bridge showed enhanced biological activity in cell collapse assays compared to the wild-type molecule (Nogi et al. 2010). These data establish that the dimer is the signal-activating form of the semaphorin molecule and that this dimer does not disassociate to bind plexin.

How stable are semaphorin dimers? Structural analyses suggest that the contribution of the sema-to-sema domain interaction to dimer stability varies substantially between semaphorins, the residues of the Sema3A interface providing fewer favorable interactions than the equivalent sites in Sema4D (Love et al. 2003). The class-specific differences in the domain composition of the semaphorin extracellular segment also impact on dimer stability. Class 1 and class 6 semaphorin ectodomains contain only the signature sema and PSI domain unit; however, in the secreted class 2 and class 3 semaphorins and the ectodomains of class 4 and 7 semaphorins, this unit is followed by an Ig-like domain. Crystal structures of Sema3A, Sema4D, and Sema7A segments constituting the sema–PSI unit plus Ig-like domain show that the Ig-like domain also provides intradimer interactions (Janssen et al. 2010; Love et al. 2003; Liu et al. 2010). This contribution to semaphorin dimer stability may be

viewed as modulatory as the extra interface is considerably smaller than the sema–sema interface; in Sema4D, some 550 Å^2 is contributed by the interface between Ig-like domains of a total of approximately 3,000 Å^2 of solvent-accessible area buried by dimer formation (Love et al. 2003).

Intersubunit disulfide bridges also contribute to dimer stability in some semaphorins. Structural analysis of the class 3 semaphorins has to date encompassed the sema-PSI-Ig segment; however, Sema3 sequences contain an additional C-terminal region rich in basic residues. The basic region provides intersubunit disulfide bridges, but also includes furin cleavage sites (Adams et al. 1997; Klostermann et al. 1998; Koppel and Raper 1998). The contribution to dimer stability of the basic region disulfides can therefore be removed by furin processing. Sema3A also contains a furin cleavage site in the PSI domain, which allows removal of the Ig-like domain (Adams et al. 1997; Antipenko et al. 2003; Janssen et al. 2012); thus, the Sema3A dimer can be progressively destabilized. This modulation mechanism may be particularly important for the control of biological activity for this class of secreted semaphorins.

All the structures of the sema-PSI-Ig-like domain containing semaphorin segments show the Ig-like domain extending away from the sema–PSI unit (Fig. 5.1), and comparisons of structures determined in different crystal packing environments reveal little evidence of interdomain flexibility (Janssen et al. 2010). Thus, the ectodomains of cell surface-attached semaphorins appear to be relatively rigid, although the C-terminal linkers that connect into the membrane-spanning regions may well provide some orientational flexibility. The ectodomains of the class 5 semaphorins are distinguished by a substantial membrane proximal segment which sequence analysis predicts to comprise seven thrombospondin type 1 repeats. These repeats contain binding sites for the glycosaminoglycans (GAGs) of heparan sulfate proteoglycans (HSPGs) and chondroitin sulfate proteoglycans (CSPGs), interactions that determine the attractive or repulsive nature of the signaling activity (Kantor et al. 2004). The structure of Sema5 ectodomains and the molecular-level characteristics of their interactions with GAGs await analysis; however, a GAG-mediated switch between inhibition (CSPG) and growth promotion (HSPG) for receptor protein tyrosine phosphatase-σ (RPTPσ) function in sensory neuron extension has been structurally characterized. Interestingly, the results suggest a model in which islands of high sulfation present in HS, but not CS, GAGs promote close packing of RPTPσ molecules and consequent modulation of phosphatase activity (Coles et al. 2011). Whether differential clustering effects play an analogous role in semaphorin signaling is as yet unknown.

5.4 Plexin Ectodomain Structure and Ligand Interactions

The plexins are so-called type 1 receptors, which means that they are composed of a N-terminal extracellular segment, a single membrane-spanning region (predicted to be α-helical), and a C-terminal cytoplasmic segment. Sequence analyses indicate

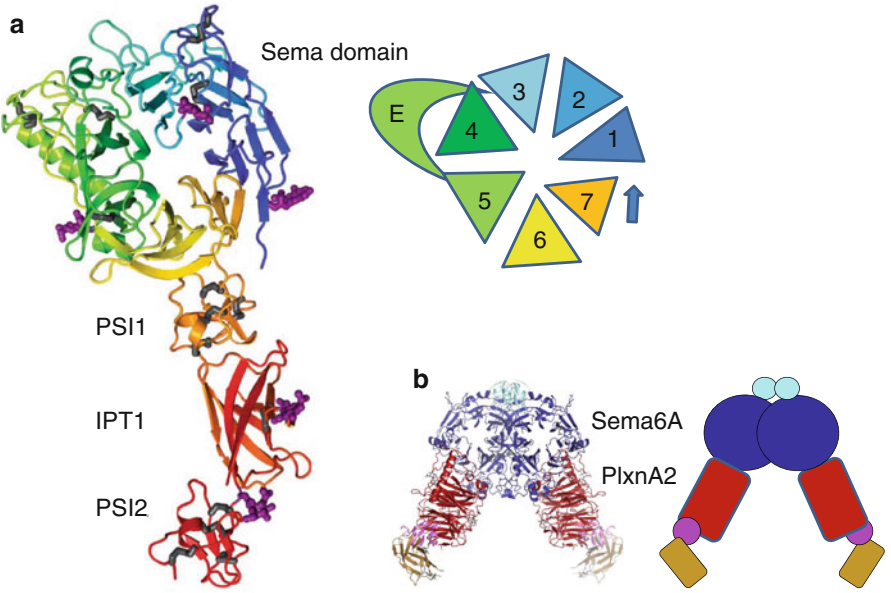

Fig. 5.2 Plexin ectodomain structure and the semaphorin–plexin complex. (**a**) Structural cartoon representation of PlxnA2 sema-PSI1-IPT1-PSI2. The plexin sema domain is flipped over by 180° relative to the view shown for the semaphorin sema domain in Fig. 5.1a. Key elements of the plexin sema domain structure are highlighted by the accompanying schematic. (**b**) The Sema6A (*blue hues*)–PlxnA2 (*red* to *tan hues*) complex in structural cartoon representation has domains highlighted by the accompanying schematic. The PlxnA2 sema domains are viewed side on (From Janssen et al. 2010)

that the ectodomains of all four classes of plexins (A, B, C, and D) comprise a sema-PSI unit followed by a combination of IPT (Ig domain shared by plexins and transcription factors) and PSI domains. To date reported crystal structures for portions of plexin ectodomains (in isolation or in complex with their semaphorin ligands) provide information on the sema-PSI region for PlxnA2, PlxnB1, and PlxnC1 (Nogi et al. 2010; Janssen et al. 2010; Liu et al. 2010) and the sema-PSI1-IPT1-PSI2 region for PlxnA2 (Janssen et al. 2010) (Fig. 5.2a). These structures show that the plexin sema domain conforms to the architecture of a seven-blade β-propeller with insertions but, as discussed in the previous section, lacks the distinctive homodimerization interface of the semaphorin sema domain. Indeed, in contrast to the data for the semaphorin sema domains, biophysical analyses of sema domain containing fragments of plexin ectodomains reveal no clear propensity for dimerization (Janssen et al. 2010; Nogi et al. 2010). Thus, although the sema domains of semphorins and plexins both serve as protein interaction platforms, their oligomerization properties are very different.

Examination of the packing arrangement in a protein crystal can reveal low-affinity biologically relevant interactions. The sema domains from two neighboring molecules in the crystal lattice of the PlxnA2 sema-PSI1 structure do pack against

each other (through an interface involved in ligand binding) (Nogi et al. 2010); however, this sema–sema interaction is not present in the lattice formed by the PlxnA2 sema-PSI1-IPT1-PSI2 crystal structure (Janssen et al. 2010), rendering it less likely to be physiologically significant. The structure of PlxnA2 sema-PSI1-IPT1-PSI2 shows the PSI1-IPT1-PSI2 portion extending away from the sema domain (Fig. 5.2a). Similar to MET receptor IPT domains (Niemann et al. 2007), the plexin IPT domain has the structure of a β-sandwich fold (Janssen et al. 2010). The robust, block-like architecture of the IPT domain is typical of the β-sandwich-based topologies of Ig-like and fibronectin type III domains, common components in the extracellular regions of cell-surface receptors. The sequential arrangement of multiple domains within a receptor ectodomain segment is commonly compared to 'beads-on-a-string.' Such arrangements may be able to switch between a number of conformations, for example, as found for integrin ectodomains (Luo et al. 2007), or be locked into a rigid rod-like structure, as seen in the cell–cell adhesive interaction of the receptor protein tyrosine phosphatase mu (RPTPμ) (Aricescu et al. 2007). As the individual PSI and IPT domains are relatively rigid, it is the properties of the linker regions and interdomain interfaces that define the overall conformation and flexibility of plexin ectodomains; however, these properties as yet remain obscure. Functional studies have suggested that in the unliganded state the sema domain of the plexin is involved in an autoinhibitory interaction (Takahashi and Strittmatter 2001). The molecular determinants of this interaction as well as any interactions mediated by the other domains of the plexin ectodomain are currently unknown. Intriguingly, biophysical characterizations of full-length PlxnA2 and PlxnC1 ectodomains have demonstrated weak clustering (Janssen et al. 2010; Liu et al. 2010), but the biological relevance of such behavior remains an open question.

The binding of semaphorin ligand is one interaction property of the plexin ectodomain that is well established as pivotal for biological function. Crystal structures have been determined for the recognition complexes involving semaphorin sema domains and the sema domains of class A, B, and C plexins, namely, two independently determined complexes of Sema6A:PlxnA2 (Janssen et al. 2010; Nogi et al. 2010), a complex of Sema4D:PlxnB1 (Janssen et al. 2010), of Sema7A:PlxnC1 (Liu et al. 2010), of the viral semaphorin A39R with PlxnC1 (Liu et al. 2010), and of Sema3A with PlxnA2 and coreceptor neuropilin 1 (Janssen et al. 2012). This gallery of structures allows us to assess which features of a semaphorin–plexin complex may be fundamental to biological activity. It is very striking that, for all the complex structures, a sema domain from each of the subunits of the dimeric semaphorin interacts essentially independently with a plexin sema domain. The dimeric semaphorin thus recruits two copies of the plexin into the complex (Fig. 5.2b). The relative orientations of ligand and receptor in this complex appear appropriate for cell-to-cell interactions between a semaphorin dimer attached to one cell surface and two plexins presented by an opposing cell. Receptor dimerization, resulting from the binding of an extracellular ligand, is a well-established mechanism for triggering

signal transduction in a number of receptor families, for example, for many of the receptor tyrosine kinases (Lemmon and Schlessinger 2010). As discussed in the previous section, dimeric forms of semaphorin are required to activate plexin signaling. The generic architecture of the semaphorin–plexin recognition complex, in which the two semaphorin subunits serve as a molecular crosslink to juxtapose two plexins, is consistent with a dimerization-driven mode of receptor triggering. The similarities between the semaphorin–plexin complexes are, however, not merely at the level of a gross crosslinking effect; the arrangement of the ligand and receptor components is highly conserved (Siebold and Jones 2013). This finding suggests that a relatively precise positioning of the plexin receptors within the complex may be necessary to trigger signaling. The role of extracellular complex geometry in governing receptor activation for the extensively studied cytokine and growth hormone signaling systems is a topic of ongoing discussion with implications for drug discovery (Atanasova and Whitty 2012; Moraga et al. 2014). It remains to be seen if this is a possible point of manipulation for the plexin signaling mechanism. Our knowledge of the extracellular arrangement of plexin receptors required to trigger signaling so far only extends to the positioning of the N-terminal four-domain segment (for PlxnA2) (Janssen et al. 2010). Insights into the architecture and degree of flexibility of full-length plexin ectodomains are required before we can properly assess the implications of the extracellular interactions with ligands for the arrangement of plexin transmembrane and cytoplasmic regions in a signaling complex.

Although the overall architecture of the semaphorin–plexin interaction is conserved across classes, the residues contributing to the recognition interface are not. The specific amino acid compositions of the interaction surfaces determine which ligand–receptor combinations of semaphorin and plexin can form an interface. Furthermore, the physical characteristics of the interaction surfaces determine the affinity and kinetics of binding for the cognate semaphorin–plexin pairs. Binding affinity and kinetics directly impact on the strength and duration of extracellular complex formation and hence signal transduction effects. Mutations of individual interface residues can have profound impact on biological activity; for example, the substitution of a single alanine residue in murine PlxnA2 (Ala396) by glutamic acid results in loss of Sema6A binding (Janssen et al. 2010; Nogi et al. 2010) and consequent changes in the migratory behavior of cerebellar granule cells in vivo (Renaud et al. 2008). Distinct functions have been associated with more subtle differences in ligand–receptor binding affinities and complex stability for cytokine signaling systems (Kalie et al. 2008; Thomas et al. 2011; Arneja et al. 2014). It is possible that similarly subtle differences in the affinity and kinetics of semaphorin–plexin binding can serve to modulate the biological outcome. Such mechanisms generally await investigation; however, some data are available concerning one important mechanism by which the properties of the semaphorin–plexin signaling complex may be modulated, the association of co-receptors.

5.5 Neuropilin, a Co-receptor Providing Molecular Glue

The neuropilins play major roles in semaphorin–plexin function. Sema3s, with the exception of Sema3E, require either neuropilin-1 or -2 (Nrp1 or Nrp2) to serve as a co-receptor for formation of a signaling complex with the class A plexins (PlxnA1, PlxnA2, PlxnA3, or PlxnA4). Furthermore, although Sema3E can activate PlxnD1 in the absence of neuropilins, the presence of Nrp1 converts Sema3E–PlxnD1 repulsive signaling to attraction (Chauvet et al. 2007). What have we learnt to date about the structure of neuropilin that can inform our understanding of its place in the function of the semaphorin–plexin system?

The neuropilins are type 1 single membrane-spanning glycoproteins, with substantial ectodomains, but only short, unstructured cytoplasmic regions that have not been associated with any signaling activity. Rather than act as biologically active receptors in their own right, the neuropilins serve as 'interaction hubs,' determining signaling outcome through association with a range of receptors and ligands in the semaphorin and vascular endothelial growth factor (VEGF) systems (Raimondi and Ruhrberg 2013). Sequence analyses subdivide the neuropilin ectodomain into five domains (termed a1, a2, b1, b2, c) classified as: two N-terminal CUB domains, two coagulation factor V/VIII homology domains, and a meprin/A5/mu (MAM) domain. A series of structural and biophysical analyses have mapped out much of the architecture and interactions of these domains.

To date no crystal structures have been determined for the neuropilin MAM domain; however, homologous structures are available in the form of the N-terminal domain of RPTPμ (Aricescu et al. 2006, 2007); these reveal a 'jelly-roll' topology of β-strands that form a compact β-sandwich-type domain. The MAM domain has a central role in the homophilic cell-to-cell (*trans*) adhesion function of the RPTPμ ectodomain (Aricescu et al. 2007), and it is noteworthy that the neuropilin MAM domain has also been reported to mediate receptor homodimerization, albeit in *cis* at the same cell surface (Chen et al. 1998; Giger et al. 1998; Nakamura et al. 1998).

Crystal structures of neuropilin-1 and -2 (Nrp1 and Nrp2) a1-a2-b1-b2 segments reveal that domain a1 is tethered by a flexible linker to a tightly clustered unit of a2-b1-b2 domains (Appleton et al. 2007; Janssen et al. 2012) (Fig. 5.3a). Although the topologies of the neuropilin a and b domains are different, both types of fold generate the β-sandwich-type architectures that so commonly serve as building blocks in receptor ectodomains. In contrast to the MAM domain, the a1-a2-b1-b2 component of the neuropilin ectodomain has been associated with heterophilic interactions, involving isoforms of VEGF (Soker et al. 1998) as well as class 3 semaphorins (He and Tessier-Lavigne 1997; Kolodkin et al. 1997). A crystal structure of Nrp1 b1-b2 in complex with Tuftsin, a tetrapeptide (sequence TKPR) mimic of the basic C-terminal tail of the VEGF165 isoform, provides details of the major VEGF interaction site on neuropilin (Vander Kooi et al. 2007). The structural data are consistent with an essentially linear epitope from the VEGF ligand docking snugly into a groove in the surface of the b1 domain. Crucially, a C-terminal arginine residue (common to Tuftsin and VEGF165) can form a dense network of

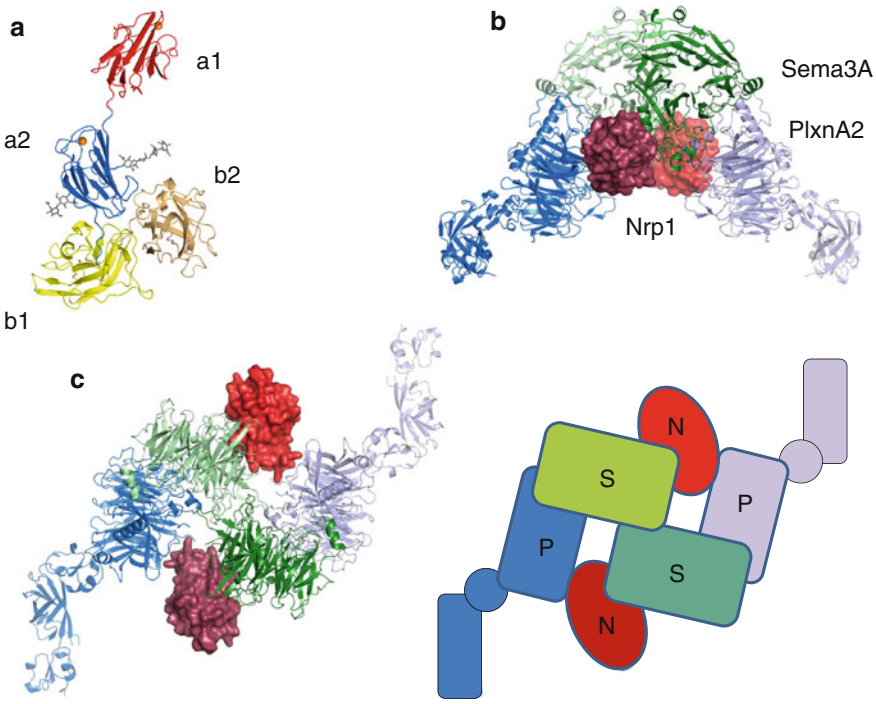

Fig. 5.3 Neuropilin structure and complex with semaphorin and plexin. (**a**) Structural cartoon representation of Nrp1 a1-a2-b1-b2. Note the extended linker region between a1 and a2. (**b**, **c**) Two orthogonal (90°) views of the Sema3A–PlxnA2–-Nrp1 complex structure. The semaphorin and plexin molecules are shown in structural cartoon representation and the neuropilins as solid surfaces. Domains are highlighted by the accompanying schematic. *S* Sema3A, *P* PlxnA2, *N* Nrp1 (Adapted from Janssen et al. 2012)

specific interactions. This observation is of relevance for semaphorin–neuropilin interactions. Early studies found that cleavage by furin can affect Sema3 activity (Adams et al. 1997). Recent work suggests that furin processing of the basic region can tune Sema3A potency (Guo et al. 2013) by generating a C-terminal arginine to facilitate binding to Nrp1 domain b1. The interaction of Sema3s with neuropilins is, however, more complex than the VEGF165 mode; it involves a major site on domain a1 (Janssen et al. 2012).

Cellular assays and in vivo studies have long pointed to the importance of a semaphorin–neuropilin interaction, rather than direct semaphorin–plexin binding for Sema3 signaling through class A plexins (Tamagnone et al. 1999; Takahashi et al. 1999; reviewed by Sharma et al. 2012). Consistent with these functional data, in vitro analysis of the interaction between purified Sema3A (minus the basic C-terminal tail region) and PlxnA2 ectodomain segments in surface plasmon resonance (SPR)-based assays detected no measurable binding for Sema3A concentrations up to 73 μM whereas addition of Nrp1 a1-a2-b1-b2 to the SPR

measurement of the Sema3A and PlxnA2 interaction generated a K_d of 6.0 μM (Janssen et al. 2012). The crystal structure of a Sema3A–PlxnA2–Nrp1 complex provides an elegant explanation for these observations, and reveals the mechanism of action of neuropilin in semaphorin–plexin biology; it is acting as molecular glue. The complex comprises dimeric semaphorin (sema domains), two copies of plexin (sema-PSI1-IPT1-PSI2 domains), and two copies of neuropilin (a1-a2-b1-b2 domains) (Janssen et al. 2012) (Fig. 5.3b, c). Each subunit of the dimeric Sema3A makes the classic sema-to-sema interface with PlxnA2; however, to compensate for the low binding affinity of Sema3A-PlxnA2, the Nrp1 a1 domain bridges between the sema domain of one semaphorin subunit and the sema domain of the plexin bound to the second semaphorin subunit (Fig. 5.3c). Interestingly, this 'crossover' arrangement is specific in stabilizing the interaction of dimeric semaphorin with plexin. The crystal structure delineates binding surfaces on the a1 domain of the Nrp1, the PlxnA2 sema domain, and the Sema3A sema domain, which are all supported by biophysical and functional data. Notably, the pivotal role of the a1 domain is consistent with its previously reported importance for semaphorin-, but not VEGF-, associated signaling (Gu et al. 2003). Although present in the complex, the a2-b1-b2 segment of the Nrp1 is not visible in the crystal structure; presumably, the flexibility of the linkage to a1 allows a2-b1-b2 to occupy a range of positions relative to the rest of the complex. This degree of conformational flexibility could allow binding between the b1 domain and furin-processed semaphorin C-terminal segments to boost the affinity of a1 domain-mediated Sema3-Nrp interactions, and, more generally, may be important for the role of neuropilin as a hub involved in multiple interactions.

Although the foregoing studies reveal a core set of neuropilin-binding modes, emerging functional data imply that this interaction hub can be cloaked in additional layers of complexity. Neufeld and coworkers have reported data that are consistent with a pro-proliferative requirement for heterodimerization of PlxnA1 and PlxnA4 within a Sema3–PlxnA–Nrp1 signaling complex in endothelial and glioblastoma cells (Kigel et al. 2011). Additional *cis* interactions and consequent alterations in plexin signaling pathways have been reported, for example, associations with VEGF receptors (Kigel et al. 2011; Casazza et al. 2013). The interactions governing the composition of such complexes have as yet not been characterized.

5.6 The Plexin Cytoplasmic Region: A Novel Signaling Machine

In the plexins a single membrane-spanning segment (predicted to be an α-helix) links the extracellular interactions of the ectodomain to the signal transduction machinery of the cytoplasmic region. Plexins are broadly similar in ectodomain architecture to members of the MET family of receptor tyrosine kinases; however, the plexin cytoplasmic segment is unique, and much concerning its mode of

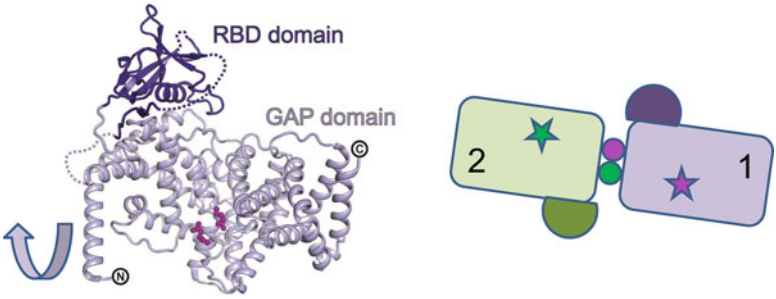

Fig. 5.4 Plexin cytoplasmic region structure. Structural cartoon representation of the PlxnA3 intracellular region. Active site arginine residues are highlighted as balls-and-sticks and colored *violet*. The structural rearrangement of the juxtamembrane helix on dimer formation is indicated by an *arrow*. Key structural elements in the context of the zebrafish PlxnC1 dimer structure are highlighted by the accompanying schematic, namely, the GAP and RBD domains, the active sites, and the rearranged juxtamembrane helices that are now orientated out of the page, that is, the view of the schematic is as if through the cell membrane (Adapted from Siebold and Jones 2013)

action remains obscure. This approximately 600-residue region lacks the canonical features of a kinase receptor; instead, structural analyses (He et al. 2009; Tong et al. 2009; Bell et al. 2011; Wang et al. 2012, 2013) reveal a juxtamembrane segment with coiled-coil propensities leading into an essentially globular structure with the topology of a classical Ras GTPase-activating protein (GAP) domain, but one bearing the distinctive insertion of a RhoGTPase-binding domain (RBD) (reviewed by Hota and Buck 2012; Siebold and Jones 2013) (Fig. 5.4a). Sequence analyses of the plexin cytoplasmic region have traditionally partitioned it into three sections, a1, the RBD insert, and a2. We can now see that in terms of the actual three-dimensional protein structure sections a1 and a2 are inseparably intertwined to form the GAP domain. This architecture is unusual on two counts. First, GAP domains are usually present in cytoplasmic proteins, not membrane-spanning receptors, and second, GAP domains do not normally incorporate a RBD. Given these distinctive features, how similar is the plexin cytoplasmic region to a canonical GAP domain in mechanism of action?

In addition to conforming to the canonical fold, the plexin cytoplasmic segment displays the key residues of a Ras GAP domain active site and has been identified as acting on R-Ras (a homologue of Ras) (Oinuma et al. 2004a). Intriguingly, a combination of in vitro and in vivo data now argue for plexin having a major function as a GAP for Rap rather than Ras homologues (Wang et al. 2012; Worzfeld et al. 2014). These results are unexpected because the three-dimensional structure of a canonical Rap GAP is completely different to that of a Ras GAP. However, a Ras GAP architecture and active site exhibiting Rap GAP activity is not entirely without precedent; GAPs belonging to the Ras GAP family have been shown to function with dual specificity, catalyzing GTP hydrolysis on Rap (Sot et al. 2010). So, how do the extracellular and cytoplasmic segments of plexins communicate and what mechanisms control signaling through GAP activity?

GAP domains are present in a plethora of different multidomain protein architectures, and this diversity is echoed in the variety of the mechanisms utilized to control GAP activity (Scheffzek and Ahmadian 2005; Bernards and Settleman 2004): protein–protein interaction, multimerization, and structural rearrangements. We can now begin to assess the mechanism that is at work in the case of the plexins. Analyses of the cytoplasmic regions of PlxnA3 and PlxnB1 in isolation reveal monomeric structures (He et al. 2009; Tong et al. 2009), and biophysical assays of the oligomeric behaviour of these regions in solution have provided scant evidence of dimerization or higher-order association in solution (Tong et al. 2009; He et al. 2009; Bell et al. 2011; Wang et al. 2012). The crystal structures of these monomeric PlxnA3 and PlxnB1 GAP domains show active sites that are in an apparent 'closed' state which may be expected to bar substrate entry (although molecular dynamics simulations do not rule out the possibility of some degree of access) (Tong et al. 2009). Of course, interactions involving the extracellular and transmembrane segments of the plexin have the potential to influence the oligomeric state of the cytoplasmic region. As detailed here, there is now a wealth of data to demonstrate that the dimeric form of semaphorin is necessary to trigger plexin signaling. It appears plausible that the effects of ligand binding on the extracellular and transmembrane segments may trigger coiled-coil formation in the juxtamembrane region to initiate GAP domain activity in the cytoplasmic segment (Bell et al. 2011). Indeed, in vitro assays support a coiled-coil, dimerization-driven activation mechanism for the Rap GAP function of the PlxnA1 cytoplasmic region (Wang et al. 2012). Zhang and colleagues have mimicked this type of mechanism, engineering a dimeric form of the cytoplasmic region of zebrafish PlxnC1 by fusing an additional coiled-coil motif to the N-terminal in the juxtamembrane segment (Wang et al. 2013). The crystal structure of this dimerized plexin GAP domain does indeed show a more 'open' active site. Conversely, a structure of the zebrafish PlxnC1 cytoplasmic region captured with human Rap1B bound at the active site (through judicious engineering of a Rap-linked PlxnC1 to favor formation of an otherwise weak interaction complex) is dimeric (Wang et al. 2013). Thus, structural and functional data for the plexin extracellular and cytoplasmic regions are now beginning to coalesce in support of a dimerization-driven activation mechanism that harnesses structural changes involving the juxtamembrane segment (Fig. 5.4b), although our insights into the multilayered complexities of plexin signaling are still very hazy. For example, what is the role of the RBD?

Plexin signaling has been reported to require two separate ligand-binding events: extracellular binding of a semaphorin and intracellular binding of a RhoGTPase (Turner et al. 2004; Oinuma et al. 2004b). The distinctive RBD insertion of the plexin cytoplasmic region has been extensively studied because of its role as a site for RhoGTPase binding. The RBD is a discrete domain, of about 120 residues, with a compact ubiquitin-like fold (Tong and Buck 2005; Tong et al. 2007). Structures of this domain (in isolation or in complex with the Rho GTPase Rnd1) for representative class A, B, C, and D plexins show essentially identical

architectures with subtle variations in Rho GTPase binding surfaces (Tong and Buck 2005; Tong et al. 2007, 2009; Wang et al. 2011), and a detailed comparison of Rho GTPase binding has been reported for Rac1 and Rnd1 to PlxnB1 RBD (Hota and Buck 2009). Clearly the challenge now is to integrate these data into an understanding of the role of Rho GTPase binding in the signaling behavior of the full plexin cytoplasmic region, and this has proved difficult. In vitro studies of plexin Rap GAP activity show no dependency on Rho GTPase binding (Wang et al. 2012). The site of RhoGTPase binding on the RBD is some distance from the GAP domain active site and structures of complexes between plexin cytoplasmic regions, and RhoGTPases show no evidence of communication between these two sites, the structure of the plexin cytoplasmic region appearing essentially unaltered on binding RhoGTPase (Bell et al. 2011; Wang et al. 2012). However, in one crystal structure of a PlxnB1–Rac1 complex, the complex forms trimeric clusters within the crystal lattice. In this cluster the Rac1 binds at the expected site on the RBD but also mediates the trimeric arrangement by simultaneously binding to a neighboring plexin at a second, novel site (Bell et al. 2011). Site-directed mutagenesis combined with cellular and biophysical assays demonstrated that the second site is essential for signaling. Interestingly, in the recent structures of dimerized plexin cytoplasmic regions (discussed earlier) (Wang et al. 2013), the structural changes in the juxtamembrane segment, and the associated dimer formation, are coupled to GAP activation through interactions in this region. The available data thus raise the intriguing possibility that interplay can occur between RhoGTPase binding and the clustering state of the plexin cytoplasmic regions.

In addition to GAP-based signaling activity, the cytoplasmic regions of many plexins contain specific sites for interaction with other signaling pathways (reviewed by Zhou et al. 2008). To date we lack direct structural insights into these interactions; however, some unexpected features of one novel signaling system associated with plexin biology have emerged. Members of the MICAL family of proteins have been implicated in class A plexin-mediated cell repulsion effects (Terman et al. 2002). MICALs are large (more than 1,000 residues), multidomain cytoplasmic proteins that have activities characteristic of monooxygenases. The structure of the N-terminal, approximately 490-residue domain of MICAL1 confirmed that it was a NADPH-dependent flavoprotein monooxygenase (Nadella et al. 2005; Siebold et al. 2005). The fold of the MICAL monooxygenase domain is closely related to that of the hydroxylase class, but close inspection of the structure prompted the hypothesis that rather than act on small molecule substrates this monooxygenase might specifically target a protein side chain for oxidative modification, a likely candidate being actin (Siebold et al. 2005). Subsequent studies have demonstrated that MICAL can indeed function to destabilize F-actin filaments by oxidizing two specific methionine residues (Hung et al. 2011), but the role of plexin in the activation of this system remains elusive.

5.7 Conclusion

We now have structures for many of the component parts of the semaphorin–plexin system. We also have structures of some complexes, notably of the recognition complexes of semaphorins with representative class A, B, and C plexins. The multiple interactions and overlapping expression patterns of these molecules often make it very difficult to separate out the specific contribution to biological effects of any one interaction. The detailed residue-level information on interaction surfaces, and the GAP domain active site, provided by the structural analyses can now guide the engineering of reagents and inform the design of in vivo experiments to dissect semaphorin–plexin biology. In terms of molecular-level understanding of functional mechanisms, structural studies have highlighted the importance of the dimeric architecture of the semaphorin in triggering plexin signaling and have begun to relate this to the role of dimerization in the GAP activity of the cytoplasmic region. There remain, however, many gaps in our knowledge. Even for the relatively well-characterized semaphorin–plexin interaction surface, we cannot model semaphorin–plexin interfaces for which we lack crystal structures with sufficient confidence to predict differences in specificity and binding affinity. We have some insights into the mechanisms by which extracellular semaphorin–plexin interactions trigger intracellular Rap GAP activity, but our snapshots are of fragments and we have yet to join up all the pieces to reveal the full transmembrane signaling machinery in action. We have uncovered the cross-bridging role of neuropilin in stabilizing Sema3–PlxnA complexes, but we know no molecular-level detail concerning its mode of action in other complexes. Is the biological activity of the plexin signaling system simply dependent on dimerization? or do higher-order clustering effects play a role as in some other cell-surface receptor systems? (for example, the ephrin-Eph system) (Seiradake et al. 2010, 2013; reviewed by Janes et al. 2012). The semaphorin–plexin system has emerged as central in myriad activities of clinical relevance. Structural studies have already provided a toolkit of reagents and concepts that can aid in the unraveling of the complex biology of the system. However, if we are to understand and manipulate this signaling system for therapeutic purposes, much structural biology work remains to be done.

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Chapter 6
Regulation of Angiogenesis and Tumor Progression by Semaphorins

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Abstract The semaphorins constitute a large family of secreted and membrane-bound proteins that were initially characterized as axon-guidance factors. In addition, several semaphorins, such as sema3B, sema3E, sema3F, sema4D, and a few additional less studied, were found to function as suppressors or inducers of tumor progression. These semaphorins were found to modulate tumor progression by a variety of mechanisms that include direct effects on tumor cells expressing semaphorin receptors, promotion or inhibition of tumor angiogenesis or tumor lymphangiogenesis, and recruitment or inhibition of the recruitment of various nonmalignant cell types such as bone marrow-derived cells capable of modulating tumor progression into the tumor microenvironment. In this review, we focus on the mechanisms by which semaphorins modulate tumor progression, excluding the effects of the semaphorins on the immune system, which obviously also contributes to the regulation of tumor progression.

Keywords Review • Angiogenesis • Lymphangiogenesis • Neuropilin • Plexin • Semaphorin • Tumor progression • Metastasis

6.1 The Semaphorin Family of Axon-Guidance Factors

The semaphorin family members are divided into eight subclasses. Subclasses 1 and 2 contain invertebrate semaphorins, subclasses 3 through 7 contain the 22 vertebrate semaphorins, and the eighth subclass contains viral semaphorins. In early publications, semaphorins were assigned confusing names. This situation was rectified by the adoption of a unified nomenclature in which sema is followed by the subclass number and by alphabetic designation within the subclass. Thus, sema3E is the fifth class-3 semaphorin (Goodman et al. 1999). Semaphorins are characterized by the presence of a sema domain approximately 500 amino acids long located close to their N-termini and by a plexin-semaphorin-integrin (PSI) domain located down-

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stream to the sema domain. The sema domain is essential for semaphorin activity and has a role in the determination of the receptor-binding specificity (Feiner et al. 1997). The sema domains of several different semaphorins were characterized by X-ray crystallography revealing a β-propeller topology (Love et al. 2003; Antipenko et al. 2003; Liu et al. 2010). Different semaphorin subclasses are characterized by class-specific structural motifs. Thus, the vertebrate semaphorins belonging to classes 4 and 7 contain immunoglobulin-like domains, class-5 semaphorins contain thrombospondin repeats, and class-3 semaphorins contain a basic domain. Class-3 semaphorins are the only vertebrate semaphorins produced as secreted proteins; other vertebrate semaphorins are membrane-anchored or transmembrane proteins that can be further processed into soluble proteins by proteolytic cleavage. Interestingly, some of the transmembrane semaphorins are apparently able to function as signal-transducing proteins themselves (Toyofuku et al. 2012; Segarra et al. 2012). The active form of several class-3 and class-6 semaphorins consists of homodimers (Klostermann et al. 1998; Liu et al. 2010; Janssen et al. 2010; Nogi et al. 2010), suggesting that the active forms of all semaphorins are homodimers.

6.2 Semaphorin Receptors

Most semaphorins bind to one or to several of the nine receptors that constitute the plexin gene family (Hota and Buck 2012). However, six of the seven class-3 semaphorins are an exception as they do not bind to plexins but instead bind one of the two receptors that constitute the neuropilin receptor family or to both neuropilins (Gu et al. 2005; Neufeld and Kessler 2008). In contrast with the plexins, the neuropilins possess short intracellular domains and are apparently unable to transduce semaphorin signals independently. However, the intracellular domain of the neuropilins contains a PDZ-binding domain that binds GICP (Cai and Reed 1999). Mice lacking functional neuropilin-1 displayed, in addition to defects in the organization of their nervous systems, major defects in the organization of their blood vessels that resulted in embryonic lethality. These defects were even more severe in mice lacking both neuropilin-1 and neuropilin-2 (Kawasaki et al. 1999; Takashima et al. 2002). Mice in which full-length neuropilin-1 was replaced with a neuropilin-1 lacking the intracellular domain were viable and displayed only minor changes in the organization of their blood vessels as compared with mice lacking functional neuropilin-1 (Fantin et al. 2011). Recent experiments, however, reveal that the intracellular domain of neuropilin-1 plays a major role in the process of tubulogenesis and that mice in which the neuropilin-1 gene was replaced with neuropilin-1 lacking the intracellular domain have a defect in arteriogenesis (Lanahan et al. 2013). The intracellular domain of neuropilin-1 was also found to be important for the interaction of myofibroblasts with soluble fibronectin, an interaction that promotes alpha5/beta1 integrin-dependent fibronectin fibril assembly (Yaqoob et al. 2012). To transduce signals of class-3 semaphorins that bind to neuropilins, both neuropilins form complexes either with one of the type-A plexins

(plexins-A1–A4) or with plexin-D1 (Tamagnone et al. 1999; Takahashi et al. 1999; Gitler et al. 2004). It should be noted that the neuropilins function as extracellular "scaffold" receptors that can bind, in addition to class-3 semaphorins, several angiogenic growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) (Soker et al. 1998; West et al. 2005; Sulpice et al. 2007). Neuropilins also form complexes, in addition to their interaction with plexins, with diverse tyrosine kinase receptors such as VEGF receptors (Soker et al. 1998; Gluzman-Poltorak et al. 2001), platelet-derived growth factor (PDGF) receptors (Cao et al. 2010), neurotrophin receptors (Ben-Zvi et al. 2007), and epidermal growth factor (EGF) receptors (Rizzolio et al. 2012) and modulate signal transduction by the respective ligands of these receptors. Neuropilins also form complexes with adhesion receptors such as L1-CAM, which associates with neuropilin-1, and Nr-CAM, which associates with neuropilin-2, and these interactions were found to modulate signal transduction induced by class-3 semaphorins (Castellani et al. 2002; Falk et al. 2005). It should be noted that the VEGF-A-binding domain of neuropilin-1 is distinct from the semaphorin-binding domain (Appleton et al. 2007).

The nine receptors of the plexin family are segregated into four groups consisting of four type-A plexins, three type-B plexins, and single C- and D-plexins (Negishi et al. 2005a; Gutmann-Raviv et al. 2006). Plexins serve as direct binding receptors for most semaphorins. Thus, plexin-B1 is a receptor for sema4D (Tamagnone et al. 1999), plexin-B3 is a receptor for sema5A (Artigiani et al. 2004), plexin-A1 is a binding receptor for sema6D, sema5A, and sema5B (Toyofuku et al. 2004), plexin-A2 and plexin-A4 function as sema6A and sema6B receptors (Suto et al. 2007, 2005), plexin-A3 functions as a receptor for sema5A and sema5B (Matsuoka et al. 2011), plexin-C1 is the receptor for sema7A (Liu et al. 2010), and plexin-D1 is a receptor for sema3E and sema4A (Gu et al. 2005; Toyofuku et al. 2007), to name but a few examples (Fig. 6.1). The extracellular domains of all plexins contain a sema domain that serves as an autoinhibitory domain in the basal, nonactivated state of the receptor (Takahashi and Strittmatter 2001). Plexins contain a split cytoplasmic SP (sex-plexin) domain (also known as the C1 and C2 domains). The intracellular domain contains putative tyrosine phosphorylation sites but no tyrosine kinase domain. The intracellular parts of the plexins are characterized by the presence of a GTPase-activating protein (GAP) domain. This GAP domain is conserved quite highly throughout the plexin family (Oinuma et al. 2004; He et al. 2009; Sakurai et al. 2010). Type-A plexins associate spontaneously to form homodimers (Janssen et al. 2010; Nogi et al. 2010) or heterodimers (Kigel et al. 2011). Recent data indicate that activation of plexin signaling by semaphorins that bind directly to plexins such as sema6A is likely to be associated with a change in the spatial organization of plexin dimers, shifting the conformation from the inactive to the active form (Liu et al. 2010; Nogi et al. 2010). In the case of the class-3 semaphorin sema3A there is functional and structural evidence suggesting that the receptor for sema3A is a tetramer composed of two plexin-A2 receptors and two neuropilin-1 receptors (Takahashi and Strittmatter 2001; Janssen et al. 2012). However, another study suggests that functional sema3A receptors may consist of

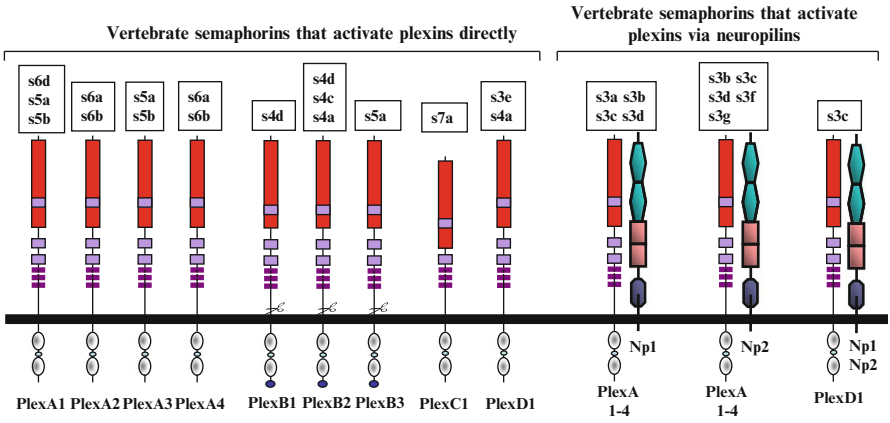

Fig. 6.1 Interactions of vertebrate semaphorins with semaphorin receptors belonging to the neuropilin and plexin receptor families with their neuropilin and plexin receptors are summarized. It should be noted that semaphorins belonging to the semaphorin subclasses 4 through 7 are membrane bound, although proteolytic cleavage can release, in some cases, soluble active extracellular domains, as in the case of sema4D. The semaphorins are designated by a shortened code; thus, *s3e* is shorthand for sema3E

complexes containing neuropilin-1, plexin-A1, and plexin-A4 (Kigel et al. 2011). This observation is supported by studies in which it was observed that sema3A signaling is impaired in mice lacking functional plexin-A4 or plexin-A1 receptors (Schwarz et al. 2008; Wen et al. 2010; Bouvree et al. 2012; Moretti et al. 2007). Further studies are required to clarify the reason for these differences.

Activation of plexin signaling by semaphorins such as sema4D activates the GAP domain of the sema4D receptor plexin-B1, leading to the inactivation of R-ras, resulting in the subsequent inactivation of beta-1-integrin, and finally reduced adhesion (Negishi et al. 2005b). Similar effects on cell adhesion and integrin function are also associated with the activation of type-A plexins and plexin-D1 (Toyofuku et al. 2005; Sakurai et al. 2010, 2011). The activation of type-A plexins also leads to the activation of enzymes of the Mical family. These enzymes perform reduction-oxidation (redox) enzymatic reactions and oxidize actin subunits, leading to the disassembly of actin fibers and to the localized collapse of the actin cytoskeleton of axonal growth cones, thereby contributing to growth cone guidance (Terman et al. 2002; Hung et al. 2010, 2011). Activation of plexin signaling by semaphorins also results in the activation of various intracellular tyrosine kinases (Franco and Tamagnone 2008) and to the inactivation of small GTPases that control the polymerization of the actin cytoskeleton such as Rho as a result of the activation of regulators of Rho activity such as the p190 Rho-GTPase (Puschel 2007). However, semaphorin-induced signal transduction is far from being completely understood, and a thorough description of this is beyond the scope of the present review.

Some semaphorins activate additional types of receptors besides plexins. Thus, sema4A also signals using the Tim-2 receptor, a member of the family of T-cell immunoglobulin domain and mucin domain (Tim) proteins that is expressed on activated T cells (Kumanogoh et al. 2002). Sema4D was also found to bind to the lymphocyte receptor CD-72 (Kumanogoh et al. 2000), and sema5A was observed to interact with chondroitin sulfate proteoglycans, an interaction that can convert it from an attractive to an inhibitory guidance cue (Kantor et al. 2004).

6.3 A Very Short Overview of Physiological Functions of Semaphorins

Semaphorins were initially identified as axon-guidance factors that function primarily as repellants of axon growth cones (Mey et al. 2013; Luo et al. 1993), although it is now recognized that under certain circumstances they can also function as attractants of axonal growth cones (Ding et al. 2007; Wolman et al. 2004; Toyofuku et al. 2008). Semaphorins have subsequently been characterized as guidance factors for additional types of cells such as neural crest cells (Schwarz et al. 2009; Feiner et al. 2001; Lepore et al. 2006) and to play important roles in organogenesis (Hinck 2004). Several semaphorins have been found to function as regulators of vascular development (Ruhrberg and Bautch 2013; Zygmunt et al. 2011; Gu et al. 2005). Finally, semaphorins have been found to have important roles in immune recognition and in the regulation of immune responses (Kumanogoh et al. 2000, 2002; Shi et al. 2000; Takamatsu and Kumanogoh 2012).

6.4 Semaphorins as Regulators of Tumor Progression

Following their identification as axon-guidance factors, it was realized that several semaphorins also function either as inhibitors or as promoters of tumor progression. For example, sema3B and sema3F had been characterized as tumor suppressor genes that inhibit the progression of lung cancer (Xiang et al. 1996; Sekido et al. 1996; Tomizawa et al. 2001; Tse et al. 2002). Subsequently, all the class-3 semaphorins, with the exception of sema3C and sema3G, have been found to function as inhibitors of tumor progression (Kigel et al. 2008; Sabag et al. 2012). Other semaphorins such as sema3E (Christensen et al. 1998; Casazza et al. 2010), sema4D (Giordano et al. 2002), and sema5A (Pan et al. 2012; Sadanandam et al. 2010) have been identified as semaphorins that promote tumor progression, although other studies have revealed that sema3E and sema5A may also exert opposite effects on tumor progression (Casazza et al. 2012; Li and Lee 2010).

6.5 Semaphorins as Regulators of Angiogenesis

6.5.1 Antiangiogenic Semaphorins

Vascular endothelial growth factor (VEGF-A) was discovered in 1989 (Gospodarowicz et al. 1989; Leung et al. 1989; Keck et al. 1989) and has since been extensively characterized as a major angiogenesis-promoting factor. It is produced in several forms as a result of alternative splicing. VEGF-A signals are transduced by two tyrosine kinase receptors (VEGFR-1 and VEGFR-2), which bind all the VEGF-A splice forms. We hypothesized that receptors able to differentially recognize VEGF-A splice forms may also exist, and this resulted in the identification of such receptors in endothelial cells (Gitay-Goren et al. 1996) and subsequently in breast cancer-derived tumor cells (Soker et al. 1996). These receptors were later identified as the products of the neuropilin-1 gene (Soker et al. 1998), which encodes a receptor that had been previously characterized as a receptor for sema3A (Kolodkin et al. 1997; He and Tessier-Lavigne 1997), and of the neuropilin-2 gene (Gluzman-Poltorak et al. 2000), which was already known to function as a sema3F receptor (Giger et al. 1998; Chen et al. 1997). Both neuropilin-1 and neuropilin-2 were found to associate with VEGF tyrosine kinase receptors and to enhance VEGF-induced signal transduction (Soker et al. 1998; Shraga-Heled et al. 2007). These findings suggested that class-3 semaphorins may modulate the behavior of endothelial cells and function as regulators of angiogenesis. This hypothesis was verified when it was found that sema3F functions as an inhibitor of angiogenesis in-vivo (Kessler et al. 2004; Bielenberg et al. 2004). These findings were subsequently followed by the discovery of several additional class-3 semaphorins that function as inhibitors of angiogenesis, including sema3A, sema3B, sema3D, and sema3E (Acevedo et al. 2008; Varshavsky et al. 2008; Sabag et al. 2012; Gu et al. 2005). In addition, several semaphorins belonging to other semaphorin subfamilies such as sema6A and sema4A have also been characterized as inhibitors of angiogenesis (Toyofuku et al. 2007; Dhanabal et al. 2005). It was initially assumed that class-3 semaphorins simply compete with factors of the VEGF family for binding to neuropilins (Miao et al. 1999). However, it was subsequently observed that semaphorins such as sema3A and sema3F are unable to inhibit VEGF- and bFGF-induced phosphorylation of bFGF and VEGF tyrosine kinase receptors, but in spite of that they are able to inhibit bFGF- and VEGF-induced activation of ERK1/2 and to induce apoptosis of endothelial cells. These observations suggest that the inhibition does not occur because of direct competition between angiogenic growth factors that bind to neuropilins and semaphorins (Guttmann-Raviv et al. 2007). This conclusion is also supported by structural studies in which it was observed that the binding domains of VEGF and sema3A on neuropilin-1 do not overlap (Appleton et al. 2007). These data suggest that the activation of plexins by semaphorins activates inhibitory signaling cascades that intersect and inhibit signal transduction cascades which are activated by pro-angiogenic growth factors such as VEGF

(Fig. 6.2). These conclusions are also supported by observations that indicate that semaphorins such as sema3E or sema6A, which do not bind to neuropilins but instead bind directly to plexins, can also function as inhibitors of angiogenesis (Dhanabal et al. 2005; Urbich et al. 2012; Casazza et al. 2012). However, it is not clear if this conclusion holds true for all semaphorins. It was recently reported that sema3F, a class-3 semaphorin that normally binds with low affinity to neuropilin-1 and does not activate neuropilin-1-mediated signal transduction (Chen et al. 1997), can compete with VEGF for binding to neuropilin-1 following posttranslational cleavage by furin-like pro-protein convertases (Parker et al. 2010; Guo et al. 2013). Thus, it is possible that direct competition with angiogenic factors for binding to neuropilins may also contribute to the anti-angiogenic properties of some class-3 semaphorins.

The induction of angiogenesis is of critical importance for the development of solid tumors (Folkman 1990; Hanahan and Folkman 1996). Because class-3 semaphorins are secreted proteins, these findings suggest that semaphorins displaying antiangiogenic properties could perhaps be utilized as drugs for the treatment of diseases associated with uncontrolled angiogenesis. This hypothesis

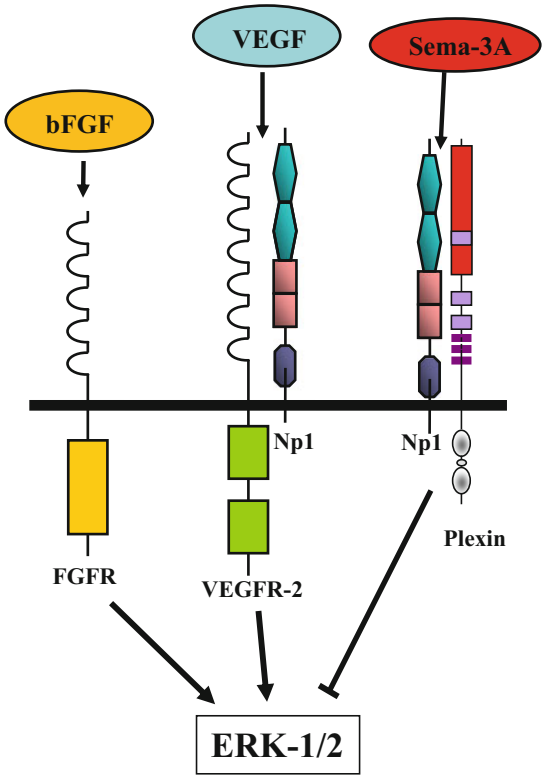

Fig. 6.2 Proposed mechanism employed by semaphorins to inhibit angiogenesis. The proposed mechanism by which class-3 semaphorins such as sema3A inhibit angiogenesis does not depend on binding competition with vascular endothelial growth factor (VEGF) or other neuropilin-1-binding growth factors such as basic fibroblast growth factor (bFGF). Rather, semaphorin receptors composed of plexins or complexes of neuropilins and plexins trigger the activation of inhibitory signals that intersect stimulatory signaling cascades, which result in the activation of ERK1/2 (Lepore et al. 2006)

was recently verified in principle (Casazza et al. 2011, 2012; Fukushima et al. 2011). These findings also suggest that semaphorins may function as endogenous regulators of developmental as well as pathological angiogenesis. In the case of class-3 semaphorins that signal through neuropilin-1, it was observed that the vascular system of knock-in mice expressing a neuropilin-1 mutant that binds $VEGF_{165}$ but is incapable of binding class-3 semaphorins develops normally (Gu et al. 2003). However, there is evidence suggesting that semaphorins, including some class-3 semaphorins, nevertheless contribute importantly to the shaping of the vascular system. Thus, sema3F expression inhibits vascularization of the outer layers of the retina whereas sema3E plays a critical role in the regulation of VEGF-induced vascularization of the early retina (Buehler et al. 2013; Kim et al. 2011). Sema3E also plays a critical role in the formation of the early dorsal aorta (Meadows et al. 2012), and there is evidence suggesting that additional semaphorins such as sema6A and sema5B also function as regulators of the formation of the vascular system (Grundmann et al. 2013; Urbich et al. 2012).

6.5.2 Pro-angiogenic and Pro-tumorigenic Semaphorins

Some semaphorins, such as sema4D (Basile et al. 2004; Conrotto et al. 2005), sema5A (Sadanandam et al. 2012), sema6A (Segarra et al. 2012), sema4A (Meda et al. 2012), and sema7A (Ghanem et al. 2011), have been described as promoters of angiogenesis. The mechanism by which these semaphorins induce angiogenesis is not clear, but in several cases, such as in the case of sema4D, it was found that the mechanism involves semaphorin-induced complex formation between plexin receptors and tyrosine kinase receptors, which subsequently results in the activation of the tyrosine kinase receptor (Giordano et al. 2002). It should be noted that some semaphorins such as sema6A and sema4A have been described as both inhibitors and inducers of angiogenesis. Sema4A inhibits angiogenesis as a result of its interaction with plexin-D1, but was also reported to promote angiogenesis indirectly by enhancing the expression of VEGF in macrophages, and may also induce angiogenesis by a mechanism similar to that of sema4D as sema4A also binds to the sema4D receptor plexin-B1 (Meda et al. 2012; Toyofuku et al. 2007; Nkyimbeng-Takwi and Chapoval 2011). The extracellular domain of sema6A functions as an inhibitor of angiogenesis (Dhanabal et al. 2005), but it was recently reported that full-length sema6A functions as a promoter of angiogenesis and that loss of the expression of full-length sema6A in endothelial cells can compromise VEGF/VEGFR-2 signal transduction, apparently as a result of the inhibition of VEGFR-2 expression (Segarra et al. 2012). Surprisingly, the secreted class-3 semaphorin sema3C was also described as possessing pro-angiogenic rather than the anti-angiogenic properties that are associated with the rest of the members of the class-3 semaphorin su-family (Banu et al. 2006; Miyato et al. 2012).

6.6 The Mechanisms Utilized by Semaphorins to Regulate Angiogenesis and Tumor Progression

As detailed in the previous two paragraphs, there is ample evidence indicating that many semaphorins can modulate tumor progression or angiogenesis or both processes. The mechanisms by which these diverse semaphorins affect tumor progression and angiogenesis have not been studied in depth for most of these semaphorins. However, a few semaphorins have received more attention than others, and their effects on angiogenesis and tumor progression have been studied more extensively. The data gathered on their activity and on the mechanisms by which these relatively better characterized semaphorins modulate tumor progression and angiogenesis is summarized in the following paragraphs.

6.6.1 Pro-angiogenic and Pro-tumorigenic Semaphorins

6.6.1.1 Sema4D

Sema4D is a single-pass transmembrane protein that forms homodimers (Love et al. 2003) that binds to the plexin-B1 and to the CD-72 receptors (Tamagnone et al. 1999; Kumanogoh et al. 2000). The extracellular domain of sema4D can be cleaved and released from producing cells by membrane type-1 matrix metalloproteinase (MT1-MMP) and by the metalloprotease ADAM17 (TACE), two metalloproteases that are upregulated in many types of malignant cells (Strongin 2010; Zhu et al. 2007; Arribas and Esselens 2009). Sema4D is also stored in platelets, and its extracellular domain can be released from platelets (Zhu et al. 2007). The soluble cleaved extracellular domain of sema4D retains the biological activity of full-length sema4D (Basile et al. 2007b) and was used extensively to study the role of sema4D in tumor progression and in the control of angiogenesis.

The tyrosine kinase receptor Met is the receptor for hepatocyte growth factor/scatter factor (HGF/SF), a potent inducer of tumor cell invasiveness and angiogenesis (Bussolino et al. 1992; Trusolino et al. 2010). The soluble extracellular domain of sema4D was found to induce the association of the sema4D receptor plexin-B1 with Met. Furthermore, it was observed that this association promotes "in-trans" autophosphorylation of the Met receptor and induction of tumor cell invasiveness following the binding of sema4D to plexin-B1 (Giordano et al. 2002). It was subsequently observed that sema4D can also transactivate the related macrophage-stimulating protein (MSP) receptor Ron (Yao et al. 2013), and that all three type-B plexins are able form complexes with the Met and Ron receptors (Conrotto et al. 2004, 2005). Furthermore, sema5A, a semaphorin that binds to the plexin-B3 receptor, can also transactivate Met similarly to sema4D (Artigiani et al. 2004). The "in-trans" activation of Met by sema4D is also important for the

regulation of developmental processes such as the migration of GnRH-1 neurons during brain development (Giacobini et al. 2008). Because HGF is also a potent inducer of angiogenesis, these observations suggested that sema4D may also function as a pro-angiogenic factor and thereby further promote tumor progression, as is indeed the case (Conrotto et al. 2005).

However, there are also observations suggesting that activation of Met may not be required for the pro-angiogenic activity of sema4D. Sema4D was found to induce angiogenesis independently of Met utilizing plexin-B1-induced Rho-dependent mechanisms (Basile et al. 2004). This mechanism involves the activation of the PI3K/Akt pathway following the binding of sema4D to plexin-B1. Activated plexin-B1 activates in turn an intracellular tyrosine kinase cascade that involves the sequential activation of PYK2 and Src, which results in the tyrosine phosphorylation of Plexin-B1, recruitment of a multimeric signaling complex that includes PYK2, Src, and PI3K to Plexin-B1, and the activation of the Akt signaling pathway (Basile et al. 2005, 2007a). It was recently reported that this Met-independent activity is mediated in addition by Rho/Rho kinase- (ROK-)dependent generation of PI(4,5)P(2) upon treatment of endothelial cells with Sema4D (Binmadi et al. 2011, 2012). In addition, activation of plexin-B1 by sema4D in endothelial cells can result in the activation of NF-kappaB and subsequently in the induction of the expression of the angiogenesis-inducing factor interleukin (IL)-8 (Yang et al. 2011; Strieter et al. 1992). Because sema4D activates angiogenesis using the plexin-B1 receptor and not the VEGFR-2 receptor used by VEGF, it follows that sema4D can act additively with VEGF and that inhibition of sema4D signaling can represent an alternative antiangiogenic treatment strategy (Zhou et al. 2012).

Regardless of the mechanism involved, there are numerous observations suggesting that sema4D contributes a stimulatory role in the progression of several types of tumors, including head and neck tumors, breast carcinomas, ovarian cancinomas, pancreatic carcinoma, and soft tissue sarcomas (Valente et al. 2009; Basile et al. 2006; Campos et al. 2013; Kato et al. 2011; Sierra et al. 2008). However, there are also opposite observations. In melanocytes and in malignant melanoma cells, sema4D inhibits HGF-induced activation of Met, and the inhibition of plexin-B1 expression in these cells leads to the activation rather than to the inactivation of Met (McClelland et al. 2011; Soong et al. 2012; Soong and Scott 2012). In breast carcinoma cells, plexin-B1 and plexin-B2 also form complexes with the ERBB2 tyrosine kinase receptor, and sema4D and sema4C are both able to induce ERBB2 phosphorylation “in trans” following their binding to plexin-B1 or plexin-B2 receptors (Swiercz et al. 2004). In these cells, the binding of sema4D to plexin-B1 associated with ErbB2 induces cell migration and metastasis whereas the binding of sema4D to plexin-B1 associated with Met inhibits cell migration, indicating that the exchange of the two receptor tyrosine kinases is sufficient to convert the cellular response of Sema4D from pro- to anti-migratory and vice versa (Swiercz et al. 2008). Similar observations were also reported in prostate cancer cells (Damola et al. 2013). It was recently also observed that the activation of plexin-B1 by Sema4D in breast cancinoma cells results in tyrosine phosphorylation of plexin-B1 by Met, thus creating a docking site for the SH2 domain of growth

factor receptor-bound 2 (Grb2). Grb2 is thereby recruited into the plexin-B1 receptor complex and, through its SH3 domain, interacts with p190 RhoGAP and mediates RhoA deactivation and leads to the subsequent inhibition of breast carcinoma cell motility (Sun et al. 2012).

6.6.2 Antiangiogenic and Antitumorigenic Class-3 Semaphorins

6.6.2.1 Sema3A

Sema3A is the only class-3 semaphorin that transduces signals exclusively using the neuropilin-1 receptor (Kolodkin et al. 1997; He and Tessier-Lavigne 1997). Neuropilin-1 and neuropilin-2 function in addition as VEGF receptors (Soker et al. 1998; Gluzman-Poltorak et al. 2000). Sema3A was observed to inhibit developmental angiogenesis in chick embryo forelimbs (Bates et al. 2003) and vascular branching in the developing chick brain (Serini et al. 2003). Similar to other class-3 semaphorins, sema3A can induce apoptosis of endothelial cells, suggesting that induction of apoptosis is a part of the mechanism by which it inhibits angiogenesis (Guttmann-Raviv et al. 2007). In agreement with these observations, it was noted that downregulation of sema3A expression in tumor cells promotes tumor angiogenesis and tumor progression (Vacca et al. 2006; Maione et al. 2009; Barresi and Tuccari 2010; Song et al. 2012), suggesting that it functions as an endogenous negative regulator of the angiogenic switch (Hanahan and Folkman 1996). Indeed, overexpression of sema3A in tumor cells or addition of exogenous sema3A can inhibit angiogenesis in vivo and in tumor progression (Kigel et al. 2008; Acevedo et al. 2008; Casazza et al. 2011; Chakraborty et al. 2012; Sabag et al. 2012). Sema3A was recently reported to inhibit tumor development and tumor metastasis and to enhance the antiangiogenic effects of VEGF receptor inhibitors such as DC101 and sunitinib. Moreover, sema3A counteracted the pro-metastatic side effects of these VEGF receptor inhibitors and drove sunitinib- or DC101-treated tumors back from a pro-metastatic phenotype to a benign phenotype (Maione et al. 2012). However, in contrast with all these aforementioned observations, in glioblastoma multiforme and in pancreatic cancer an opposite role was reported for sema3A, suggesting that sema3A promotes rather than inhibits the metastatic dissemination of tumor cells (Bagci et al. 2009; Muller et al. 2007). Sema3A can also affect tumor cells directly. Thus, it was found to inhibit the migration and spreading of MDA-MB-231 breast cancer cells and to inhibit the invasiveness of prostate cancer cells by in vitro assays (Bachelder et al. 2003; Herman and Meadows 2007).

Bone marrow-derived cells can be recruited to sites of active angiogenesis by factors such as SDF-1 that are produced at sites of active angiogenesis, and these cells then promote angiogenesis by the secretion of angiogenic factors such as VEGF (Grunewald et al. 2006). Interestingly, sema3A produced by tumor cells is also able to recruit bone marrow-derived cells to tumors. These recruited

bone marrow cells consist of a special subpopulation of monocytes that express neuropilin-1. Interestingly, these cells were found to contribute to the stabilization and normalization of tumor vessels by promoting mural cell coverage of tumor vessels and by decreasing vascular leakiness, resulting in smaller but better perfused and less hypoxic tumors (Zacchigna et al. 2008; Carrer et al. 2012). Thus, sema3A seems to affect tumor angiogenesis and tumor progression by several concomitant mechanisms.

6.6.2.2 Sema3B

The sema3B gene was identified as a tumor suppressor gene whose function is lost in small cell lung carcinoma cells by a variety of mechanisms (Tomizawa et al. 2001; Kuroki et al. 2003; Tischoff et al. 2005; Nair et al. 2007; Campioni et al. 2008). In addition, sema3B also functions as an endogenous inhibitor of endometrial cancer (Nguyen et al. 2011), and single-nucleotide polymorphisms in the sema3B gene were found to be associated with poor prognosis of prostate cancer (Beuten et al. 2009). Differing from sema3A, which only binds to neuropilin-1, sema3B binds to both neuropilins. Sema3B inhibits the anchorage-independent growth of responsive lung cancer cells and induces apoptosis, indicating that it exerts direct inhibitory effects on tumor cells (Tomizawa et al. 2001). The pro-apoptotic effects of sema3B were inhibited by $VEGF_{165}$ but not by $VEGF_{121}$ (Castro-Rivera et al. 2004). Because both neuropilins bind the $VEGF_{165}$ splice form of VEGF but not the $VEGF_{121}$ form (Soker et al. 1998; Gluzman-Poltorak et al. 2000), these results indicate that the pro-apoptotic effects of sema3B are mediated by neuropilins and that sema3B may compete with $VEGF_{165}$ for binding to neuropilins. The pro-apoptotic and antiproliferative effects of sema3B were linked to decreased Akt phosphorylation, increased cytochrome *c* release, and caspase-3 activation, as well as phosphorylation of several additional pro-apoptotic proteins including glycogen synthase kinase-3-beta (GSKβ3), FKHR, and MDM-2 (Castro-Rivera et al. 2008). As do other class-3 semaphorins, sema3B functions as an inhibitor of angiogenesis (Varshavsky et al. 2008). Interestingly, it was also observed that sema3B can indirectly induce opposite effects and potentiate tumor metastasis as well as tumor angiogenesis in many types of tumors as a result of sema3B-induced expression of interleukin-8, which, in turn, induces the recruitment of tumor-associated macrophages and metastatic dissemination to lungs (Rolny et al. 2008). Because interleukin-8 is a well-characterized angiogenic factor (Huang et al. 2002), it is also likely that when expressed it may counteract the direct antiangiogenic effects of sema3B.

In humans, a single-nucleotide alteration in the sema3B gene (T415I) resulted in decreased sema3B function and was associated with increased susceptibility to lung cancer in African-Americans and Latino-Americans, indicating that sema3B is involved in the determination of predisposition to lung cancer (Marsit et al. 2005). Sema3B single-nucleotide polymorphisms are also associated with increased

prostate cancer risk and poor prognosis (Beuten et al. 2009), whereas sema3B expression is decreased in stage 3 ovarian tumors and in breast cancer, suggesting a contribution to the development of these types of cancer as well (Joseph et al. 2010; Staton et al. 2011).

6.6.2.3 Sema3F

Similarly to sema3B, sema3F was also initially identified as a gene encoding a tumor suppressor of lung cancer (Xiang et al. 1996; Roche et al. 1996). Sema3F binds to the neuropilin-2 receptor and, with very low affinity, also to the neuropilin-1 receptor. However, it seems that it is unable to transduce signals using the neuropilin-1 receptor (Chen et al. 1997; Giger et al. 1998). Sema3F suppressed the formation of tumors derived from several types of xenografted lung cancer cells when it was ectopically expressed in such cells. Expression of sema3F in these cells suppressed their anchorage-independent growth, suggesting that sema3F can directly affect the behavior of tumor cells (Xiang et al. 2002; Kigel et al. 2008). The expression of sema3F in lung cancer cells was recently found to be downregulated by the transcription repressor ZEB-1, which is highly active in lung cancer cells (Clarhaut et al. 2009). In metastatic tumor cells, *myc*-driven expression of the transcription factor Id2 was also found to downregulate sema3F expression, resulting in the induction of tumor metastasis (Coma et al. 2010). In H157 lung cancer cells, sema3F inhibited multiple signaling pathways, including AKT/STAT3 signaling, resulting in the loss of activated αvβ3-integrin (Potiron et al. 2007; Kusy et al. 2005). Sema3F also inhibited the attachment and spreading of MCF-7 breast cancer cells and inhibited the expression of E-cadherin (Nasarre et al. 2003, 2005). The transcription factor retinoid orphan nuclear receptor-alpha (RORα) functions in breast cancer cells as a tumor suppressor, and this inhibitory activity is mediated at least in part through its control of sema3F expression (Xiong et al. 2012). In endometrial cancer it was found that progesterone and 1,25-dihydroxyvitamin D(3) inhibit endometrial cancer cell growth by upregulating sema3B and sema3F (Nguyen et al. 2011). The progression of ovarian cancer was also associated with the downregulation of sema3F and its neuropilin-2 receptor, whose expression in these cells was regulated by estrogen (Joseph et al. 2010; Drenberg et al. 2009). Sema3F also inhibits the growth and metastasis of tumors derived from colorectal carcinoma cells and inhibits the proliferation and the migration of these cells (Wu et al. 2011). Sema3F inhibited integrin-β1-mediated attachment of A375 melanoma cells by a neuropilin-2-mediated mechanism and suppressed the metastatic spread of cells from tumors derived from these cells (Bielenberg et al. 2004). As in the case of sema3B, in prostate cancer, single-nucleotide polymorphisms of sema3F were associated with increased prostate cancer risk and poor prognosis (Beuten et al. 2009). Taken together, it can be concluded that sema3F has an inhibitory function in many types of tumors, and that the inhibition is caused, at least in part, by sema3F-induced inhibition of the migration and proliferation of responsive tumor cell types.

In addition to its direct effects on tumor cells expressing sema3F receptors, sema3F also functions as an inhibitor of angiogenesis as well as lymphangiogenesis and was the first class-3 semaphorin to be characterized as an antiangiogenic factor (Kessler et al. 2004; Bielenberg et al. 2004). Sema3F promotes apoptosis of endothelial cells and can act additively with sema3A to inhibit the proliferation of endothelial cells (Guttmann-Raviv et al. 2007). As a result, sema3F can also inhibit tumor progression as a result of its antiangiogenic properties. Indeed, sema3F also inhibited the development of tumors from tumor cell types that do not respond to stimulation by sema3F (Kessler et al. 2004). Sema3F inhibits the expression of HIF-1α and consequently VEGF expression, resulting in the inhibition of hypoxia-induced angiogenesis (Potiron et al. 2007; Kusy et al. 2005). Interestingly, the expression of sema3F is induced by wild-type p53, and loss of functional p53 in tumor cells can thus result in reduced sema3F expression and consequently cause the induction of tumor angiogenesis (Futamura et al. 2007). Similarly, in neurofibromatosis type 2 (NF2), an autosomal-dominant multiple neoplasia resulting from mutations in the NF2 tumor suppressor gene, the expression of sema3F is downregulated. Reintroduction of sema3F into schwannoma cells lacking a functional NF2 gene resulted in the normalization of tumor blood vessels, reduced tumor burden, and extended survival, suggesting that the product of the NF2 gene regulates angiogenesis via sema3F (Wong et al. 2012).

6.6.2.4 Sema3E

In contrast with other class-3 semaphorins, sema3E was initially identified as a pro-metastatic semaphorin (Christensen et al. 1998). Sema3E is unique among the class-3 semaphorins in that it is the only class-3 semaphorin that does not bind to a neuropilin and utilizes instead the plexin-D1 receptor as a binding and signal-transducing receptor (Gu et al. 2005). It should be noted, however, that plexin-D1 can associate with neuropilins to transduce signals of other class-3 semaphorins such as sema3A and sema3C (Gitler et al. 2004), and that this association can change responses of cells to sema3E (Chauvet et al. 2007). Similar to other class-3 semaphorins, sema3E functions as a repulsive factor for endothelial cells and as an inhibitor of angiogenesis (Sakurai et al. 2010, 2011). Opposing sema3E gradients originating from the lateral plate mesoderm and the notochord repulse endothelial progenitor cells during early development, inducing them to concentrate, and enabling the subsequent formation of the early dorsal aorta (Meadows et al. 2012). Sema3E is also highly expressed in somites in the early embryo and inhibits, possibly as a result of sema3E-induced repulsion of blood vessels, the growth of blood vessels into the somites (Gu et al. 2005).

The development of the retinal vasculature serves as a major model in which to study developmental angiogenesis as well as eye diseases associated with abnormal angiogenesis, because the whole network of blood vessels can be easily observed in retinal whole mounts. During the development of the retina, the growth of the vascular network is driven by VEGF that is produced by astrocytes in response to

local hypoxia (Stone et al. 1995). Tip cells are endothelial cells located at the tips of the growing angiogenic sprouts. These cells send out filopodia and lamellipodia to guide the growing sprout although the stalk cells that are the endothelial cells that form the main body of the growing sprout do not extend such filopodia (Siekmann et al. 2008; Eilken and Adams 2010). VEGF signals via the VEGFR-2 receptor of the tip cells and its activation by VEGF induce the expression of the notch ligand Dll4. Dll4 activates notch receptors in adjacent stalk cells, which in response downregulate the expression of VEGFR-2, resulting in the maintenance of their stalk cell identity (Hellstrom et al. 2007; Lobov et al. 2007). Interestingly, VEGF also induces the expression of plexin-D1 in the tip cells of sprouting angiogenic retinal blood vessels. Sema3E produced by retinal ganglion cells acts specifically on the plexin-D1-expressing tip cells to inhibit the VEGF-induced expression of Dll4, causing a cell fate shift that favors tip cell identity. Thus, sema3E expression is part of a feedback mechanism by which neuronal cells of the retina regulate the formation of the developing vascular network (Kim et al. 2011). Sema4A, a membrane-anchored semaphorin that also utilizes plexin-D1 as its receptor, also functions as an antiangiogenic factor (Toyofuku et al. 2007), although it is not known if it is also part of a similar feedback mechanism.

Newborn babies as well as newborn mouse pups exposed to high partial oxygen pressure develop blindness when shifted back to normoxia (retinopathy of prematurity, ROP) because of the wild growth of new blood vessels that is driven by the acute hypoxia experienced by astrocytes following the sudden drop in oxygen partial pressure, which induces the astrocytes to express high levels of VEGF (Alon et al. 1995). Interestingly, these new vessels are misdirected toward the vitreous and fail to vascularize the developing retina because they are repelled by sema3A expressed by hypoxic neuronal cells (Joyal et al. 2011). However, these abnormalities can be partially remedied by intravitral injection of sema3E, which was observed to suppress the extraretinal vascular outgrowth without affecting the desired regeneration of the retinal vasculature (Fukushima et al. 2011). In addition, it was recently observed that the avascular characteristic of the outer layers of the retina is caused by the expression of sema3F in cells in these outer layers (Buehler et al. 2013), suggesting that several different semaphorins acting in concert regulate the distribution of blood vessels in the normal and diseased retina.

In the context of cancer, it was observed that ectopic overexpression of sema3E in a variety of tumor cell types inhibits tumor development from such cells (Kigel et al. 2008; Sabag et al. 2012). However, inhibition of tumor growth that occurs primarily because of the antiangiogenic effects of sema3E is accompanied by sema3E-induced induction of tumor metastasis (Christensen et al. 1998). A recent report suggested that sema3E can actually inhibit apoptosis that is induced by the sema3E receptor plexin-D1 in the absence of sema3E, suggesting that sema3E can contribute to tumor progression in this way too (Luchino et al. 2013). Furthermore, it was recently found that sema3E can induce inflammation that is mediated by macrophages (Schmidt and Moore 2013). Inflammation is recognized as a major contributor to tumor progression as are macrophages that are recruited to the tumor microenvironment

(Schmidt and Moore 2013; Pikarsky et al. 2004), suggesting that semaphorins such as sema3E may be able to influence tumor progression by modulation of the chronic inflammation that is a hallmark of many types of tumors.

Similar to other class-3 semaphorins, sema3E contains conserved cleavage sites for furin such as pro-protein convertases. Interestingly, it was found that the major furin cleavage product, p61-sema3E, is responsible for the induction of tumor metastasis rather then full-length sema3E (Christensen et al. 2005). It was observed that p61-sema3E induces the association of plexin-D1 with the ErbB2 tyrosine kinase receptor and induces "in trans" the autophosphorylation of ErbB2, which in turn enhances the invasiveness of plexin-D1- and ErbB2-expressing tumor cells (Casazza et al. 2010). Because furins are upregulated in most metastatic cells (Bassi et al. 2005), the balance between the full-length and cleaved forms of sema3E is tilted toward the cleaved form in most types of tumor cells, thus promoting tumor metastasis. A point-mutated sema3E that resists cleavage by furin like pro-protein convertases is still able to bind to plexin-D1 and to activate plexin-D1 mediated inhibition of angiogenesis, but fails to induce tumor metastasis or phosphorylation of ErbB2. Furthermore, this cleavage resistant point mutated sema3E inhibits the pro-metastatic activity of p61-sema3E because it competes with p61-sema3E for binding to plexin-D1 (Fig. 6.3) (Casazza et al. 2012). However, in cases in which tumor cells do not express plexin-D1 or tyrosine kinase receptors that associate with plexin-D1, it is possible that wild-type sema3E, which in the tumor microenvironment may contain a high proportion of p61-sema3E, may also display antimetastatic properties as a result of its antiangiogenic effects. Indeed, overexpression of wild-type sema3E, which is susceptible to furin-like pro-protein convertases, nevertheless potently inhibited metastasis of melanoma cells (Roodink et al. 2008). It is not known at this point in time if sema3E activities such as the induction of inflammation (Shimizu et al. 2013; Schmidt and Moore 2013) or inhibition of tumor cell apoptosis (Luchino et al. 2013) can be induced by the full-length form of sema3E or the p61-cleaved form or by both.

6.7 Future Research Directions and Conclusions

Research conducted in the past 10 years has revealed both pro-angiogenic as well as antiangiogenic semaphorins. Initially, the thought was that antiangiogenic semaphorins would function exclusively as antitumorigenic factors. However, it turns out that interactions between semaphorin receptors and apparently unrelated receptors such as various tyrosine kinase receptors, in addition to posttranslational modifications of the semaphorins and their receptors, can profoundly affect their biological activities, as exemplified in the case of sema3E (Casazza et al. 2010; Chauvet et al. 2007; Luchino et al. 2013; Rizzolio et al. 2012). These interactions and modifications can in turn profoundly affect the course of diseases such as cancer, and a better understanding of these interactions is required if one considers the development of antitumorigenic and antiangiogenic therapeutic agents that

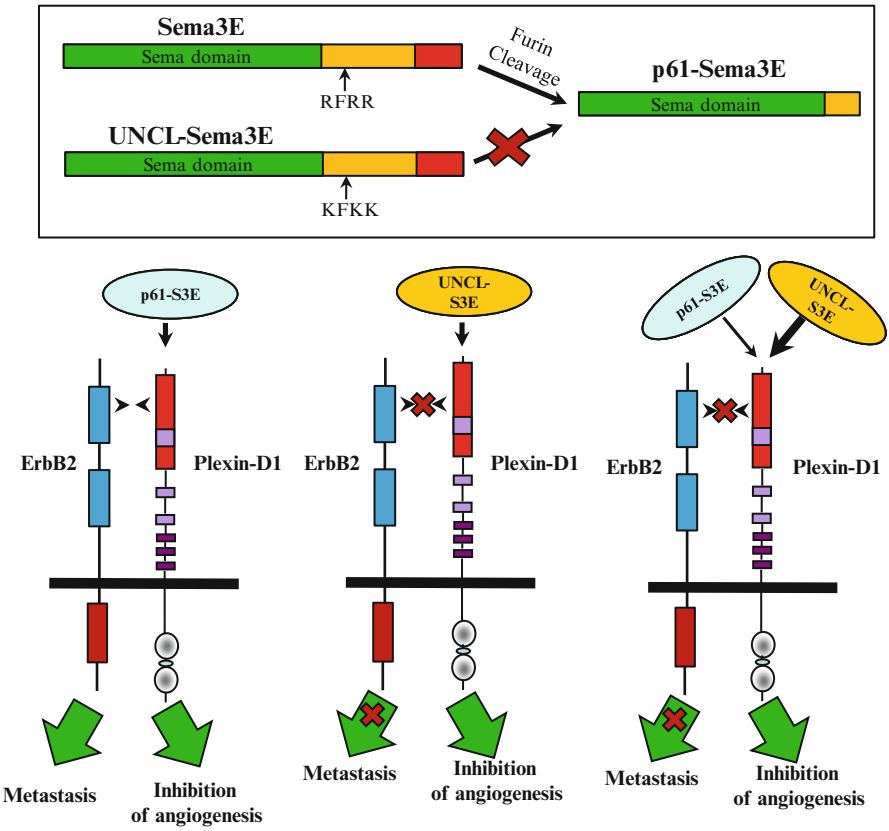

Fig. 6.3 Dual role of sema3E in tumor progression. Similar to all class-3 semaphorins, sema3E contains a conserved cleavage site for furin such as pro-protein convertases, which when cleaved generates the active N-terminal fragment p61-Sema3E (*upper panel*). Introduction of point mutations into this site resulted in the generation of UNCL-Sema3E, a sema3E mutant that is resistant to cleavage by furin, as are pro-protein convertases. The three sema3E forms bind to plexin-D1 and inhibit angiogenesis as a result. However, in contrast with uncleaved sema3E or UNCL-Sema3E, p61-Sema3E induces the association of its plexin-D1 receptor with ErbB2 and enables the activation of ErbB2. Both uncleaved sema3E and UNCL-Sema3E compete with p61-Sema3E for binding to plexin-D1 and inhibit p61-Sema3E induced activation of ErbB2 in tumor cells, thereby inhibiting tumor metastasis induced as a result of the activation of ErbB2 (Casazza et al. 2010, 2012)

target or utilize semaphorin signal transduction. Thus, research aimed at a better understanding of the processing of semaphorins and their receptors and better characterization of the crosstalk between semaphorins and their receptors and other signal transduction systems is likely to be a focus of research for some time to come. In addition to cancer, it seems that semaphorins play major regulatory roles in the development and maintenance of the vascular and neuronal networks of organs such as the retina and the kidney, and it is likely that the study of the role of the

semaphorins in the development of vascular diseases, including complications of diabetes such as diabetic retinopathy or diabetic nephropathy, will become a focus of intensive research in the near future.

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Chapter 7
Semaphorins in the Immune System

Satoshi Nojima and Atsushi Kumanogoh

Abstract Although semaphorins were originally identified as axon-guidance molecules that function during neuronal development, several semaphorins play a crucial role in various phases of the immune responses, including immune cell activation, differentiation, trafficking and migration, and cell–cell interaction. These semaphorins are called immune semaphorins. Immune semaphorins participate in immune responses via their primary receptors, plexins and neuropilins (Nrps). Secreted semaphorins generally require neuropilins as obligate co-receptors for interacting semaphorins, whereas most membrane-associated semaphorins bind plexins directly. Furthermore, immune semaphorins are considered to be potential diagnostic and/or therapeutic targets for various immunological diseases, including autoimmune and allergic diseases. If the molecular basis of immune semaphorin signaling could be determined more clearly, critical and powerful semaphorin-targeted therapy could be established. In this chapter, we discuss current knowledge of immune semaphorins from the perspective of both physiological and pathological immune responses.

Keywords Immune semaphorin • B cell • T cell • Regulatory T (Treg) cell • Dendritic cells (DCs) • Basophil • Mast cell • Virus semaphorin

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7.1 Introduction

Semaphorins function in a wide range of important physiological processes, including neuronal system development, angiogenesis, tumor microenvironment, cardiogenesis, bone homeostasis (see other chapters), and immune responses, which are the focus of this chapter. Knowledge of the so-called immune semaphorins has advanced considerably over the past decade. Semaphorins and their receptors, plexins, and neuropilins (Nrps), have distinct biological activities in the immune system, with functions in innate immunity, lymphocyte development, acquired immunity, leukocyte trafficking, and effector/memory responses. Moreover, semaphorin–plexin signaling plays an important role in cell movement and morphology by regulating cytoskeletal dynamics.

Semaphorins are secreted, or membrane-associated proteins characterized, by a conserved amino-terminal 'Sema' domain. Vertebrate semaphorins, including immune semaphorins, are grouped into classes III–VII (Fig. 7.1), and invertebrate semaphorins are grouped into classes I and II (Pasterkamp and Kolodkin 2003). Semaphorins in classes IV–VII are membrane associated, whereas those in class III are secreted. Both secreted and membrane-associated semaphorins play important roles in the immune system. Secreted semaphorins regulate many aspects of immune responses, including trafficking of thymocytes during differentiation and migration of dendritic cells (DCs) from peripheral tissues to secondary lymphoid organs. In addition, membrane-associated semaphorins play a crucial role in regulating interactions between immune cells, immune homeostasis, and other processes.

Two groups of proteins, plexins and neuropilins (Nrps), have been identified as the main receptors for semaphorins (Zhou et al. 2008; Suzuki et al. 2007; Pasterkamp et al. 2003, Kumanogoh et al. 2000, 2002a). As in other physiological processes, in the immune system most membrane-bound semaphorins bind plexins directly, whereas class III semaphorins require neuropilins as obligate co-receptors. Other types of molecules are also involved in the semaphorin–plexin axis (Zhou et al. 2008). For example, CD72 (Kumanogoh et al. 2000) and TIM-2 (T cell, immunoglobulin, and mucin domain protein 2) (Kumanogoh et al. 2002a), which are unrelated to plexins and neuropilins, functionally interact with Sema4D and Sema4A, respectively, in the immune system. In addition, Sema3E signals are transduced independently of neuropilins through plexin-D1 (Gu et al. 2005), whereas Sema7A signals are transduced via integrins in the nervous and immune systems (Suzuki et al. 2007; Pasterkamp et al. 2003).

To date, studies of the immune semaphorins have revealed that several semaphorin family members are intimately involved in diverse immune cell interactions. Here we review the present knowledge of immune semaphorins in the context of immune responses.

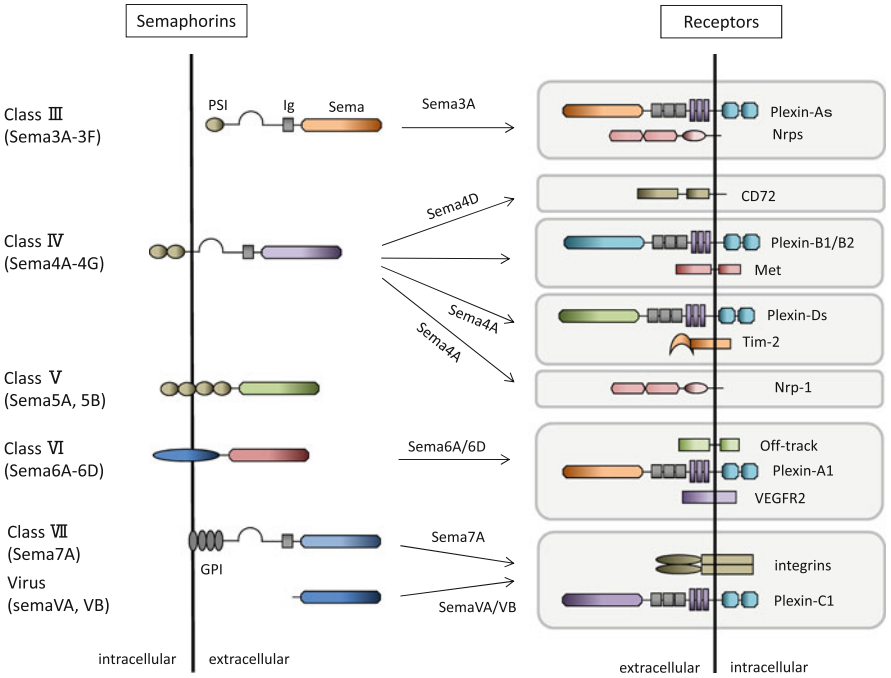

Fig. 7.1 Semaphorins and their receptors. Representative immune semaphorins and their receptors are shown graphically *above*. Sema3A binds to complexes of Nrp-1 and class A plexins. Class IV semaphorins can bind to class B and class D plexins. Sema4A binds to TIM-2 (T cell, immunoglobulin, and mucin domain protein 2), and Sema4D binds to CD72. Class VI semaphorins can bind to class A plexins in a neuropilin-independent manner. Sema6A binds to Plexin-A4. Sema6D binds to Plexin-A1 and exerts different biological activities depending on the presence of its co-receptor, VEGFR2 (vascular endothelial growth factor receptor 2), or off-track. Sema7A and viral semaphorins, including A39R, bind to Plexin-C1. Sema7A also associates with integrins. *Sema* Sema domain, *Ig* immunoglobulin-like domain, *PSI* PSI domain, *GPI* GTPase-activating domain

7.2 Semaphorins and B-Cell Function

7.2.1 Sema4D and B-Cell Function

Sema4D, also known as CD100, was the first semaphorin molecule shown to have immunoregulatory functions (Delaire et al. 1998; Bougeret et al. 1992). In immune cells, Sema4D is expressed in activated B cells, mature dendritic cells (DCs), and T cells. In B cells, Sema4D is expressed basally at a low level, but is upregulated significantly by stimuli such as lipopolysaccharide (LPS) and anti-CD40 antibody (Kumanogoh et al. 2000). Recombinant mouse Sema4D and cells expressing Sema4D augment CD40- or LPS-induced proliferation and antibody production by mouse B cells in vitro (Kumanogoh et al. 2000). Consistent with

this, *Sema4d*-deficient mice have abnormal B cells and altered antibody responses (Oinuma et al. 2004). The receptors for Sema4D in the immune system include proteins of the plexin-B subfamily (B1, B2, and B3) and CD72 (Kumanogoh et al. 2000; Oinuma et al. 2004). In particular, plexin-B1 mediates Sema4D-induced signaling in B cells. The interaction of plexin-B1 on B cells with Sema4D induces increased B-cell proliferation and lifespan (Granziero et al. 2003). The signaling pathways downstream of plexin-B1 that are triggered by Sema4D have been delineated in the context of axonal growth cone collapse, a process in which small GTPases have been implicated as mediators of the biological functions of semaphorins (Swiercz et al. 2002; Oinuma et al. 2003; Basile et al. 2004). For example, plexin-B1 activates RhoA through the interaction of the C-terminal PDZ (postsynaptic density protein 95 kDa/Discs-large/zona occludens-(1)-binding domains of plexin-B1 with PDZ-Rho-GEF and leukemia-associated Rho-GEF (LARG). Sema4D also transduces signals via CD72, which acts as an additional receptor in the immune system (Kumanogoh et al. 2000; Shi et al. 2000). CD72 has two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in its cytoplasmic region. Sema4D–CD72 interactions between B cells help maintain certain B-cell subsets by 'fine tuning' B-cell receptor (BCR) signals (Fig. 7.2a). Sema4D is strongly expressed in germinal center B cells (Hall et al. 1996), and the Sema4D–CD72 axis may promote robust population expansion of these cells (Fig. 7.2b). As discussed next, Sema4D is also produced in CD4+ T cells and is also involved in B-cell-mediated immunity. After germinal center formation, T cell-derived Sema4D may participate in the interaction between helper T cells and germinal center B cells, leading to Sema4D signals that promote the survival of germinal center B cells and support efficient selection of high-affinity B cells. Consistently, *Sema4d*-deficient mice immunized with T cell-dependent antigens exhibit defective antibody affinity maturation and poor generation of antigen-specific germinal center B cells (Shi et al. 2000).

7.3 Semaphorins and T-Cell Function

7.3.1 Sema3E/Plexin-D1 and Thymocyte Development

In the immune system, the Sema3E–plexin-D1 axis has a role in thymocyte development (Choi et al. 2008). The expression of plexin-D1 on thymocytes decreases during thymic T-cell development, from CD4+CD8+ double-positive (DP) to single-positive (SP) thymocytes, and Sema3E is preferentially expressed in the medulla of the thymus (Fig. 7.3a). In vitro chemotaxis assays have shown that Sema3E binds CD69+ DP cells and inhibits their CCR9-mediated migration. Consistent with this, the thymus of *plexin-d1*-deficient embryos is disorganized in comparison to wild-type control embryonic thymus. In addition, when fetal liver cells derived from *plexin-d1*-deficient embryos are transferred to *Sema3e*-deficient mice, the boundary between DP and SP thymocytes at the corticomedullary junction is disrupted (Choi et al. 2008). These findings indicate that plexin-D1 is involved in the development of the thymus.

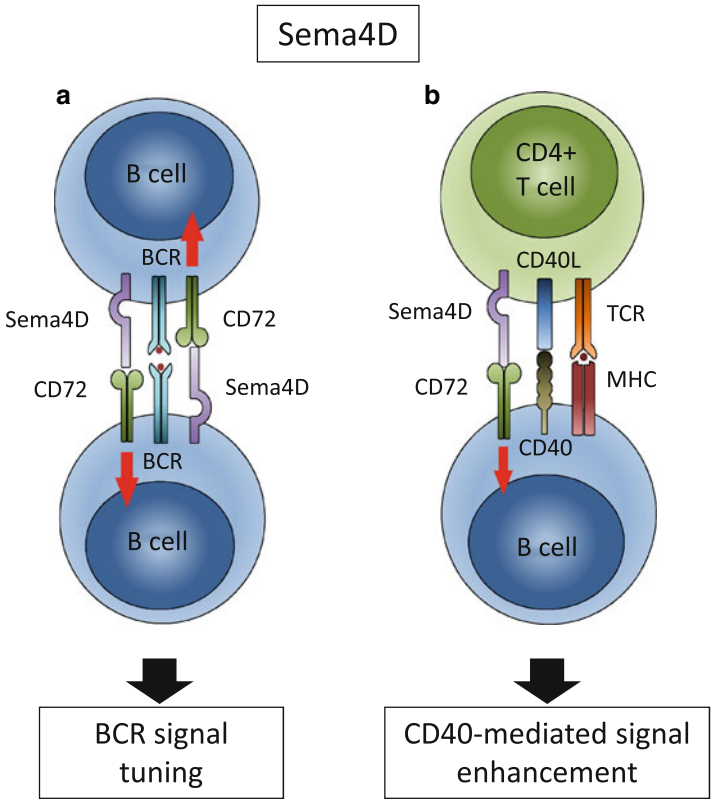

Fig. 7.2 Involvement of Sema4D in B-cell-mediated immunity. (**a**) Sema4D transduces signals via B cell–B cell interactions. The expression of Sema4D is weak in resting B cells. Under such homeostatic conditions, Sema4D might contribute to the 'fine tuning' of B-cell receptor (BCR) signaling through its association with CD72. Sema4D is significantly upregulated in germinal center B cells, where it binds to CD72 and strongly promotes the robust population expansion of germinal center B cells. (**b**) Sema4D is also involved in T cell–B cell interaction. Sema4D expressed on CD4+ T cells interacts with CD72 expressed on B cells, such as follicular B cells in extrafollicular areas of secondary lymphoid organs, and activates them. This mechanism might aid the survival of germinal center B cells and efficient selection of high-affinity B cells. *BCR* B-cell receptor, *TCR* T-cell receptor, *MHC* major histocompatibility complex

7.3.2 Sema4A and T-Cell Function

Sema4A is constitutively expressed in polarized T-helper type 1 (Th1) cells and is crucial for helper T-cell differentiation (Fig. 7.3b) (Kumanogoh et al. 2005). Indeed, *Sema4a*-deficient mice have impaired Th1 responses to heat-killed *Propionibacterium acnes*, a Th1-inducing bacterium, in vivo. Conversely, *Sema4a*-deficient mice exhibit augmented T-helper type 2 (Th2) responses against *Nippostrongylus brasiliensis*, a Th2-inducing intestinal nematode (Kumanogoh et al. 2005).

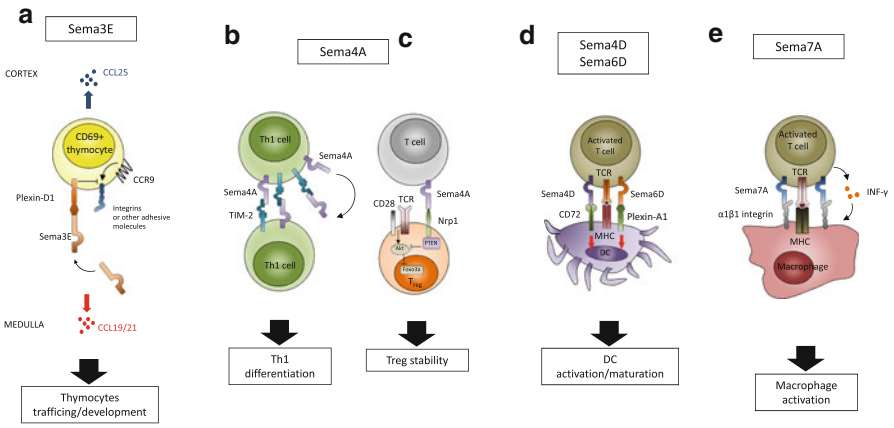

Fig. 7.3 Immune semaphorins in T-cell-mediated immunity. (**a**) Secreted Sema3E binds to Plexin-D1 expressed on CD69+ thymocytes, leading to repression of CCL25 chemokine signaling via its receptor CCR9. This selective inhibition might involve other adhesion molecules, such as integrins. Thus, the Sema3E–plexin-D1 axis inhibits migration toward the cortex induced by CCL25–CCR9 signaling; consequently, CD69+ thymocytes migrate toward the medulla in response to CCL19/21. (**b**) Sema4A is expressed on Th1 during the differentiation of CD4+ T cells into effector cells. Induced Sema4A is shed and secreted, and promotes further Th1 differentiation via TIM-2. This mechanism might be mediated in an autocrine manner. (**c**) Sema4A expressed on conventional T cells ligates to Nrp-1 on Treg cells, leading to inhibition of Akt activation via recruitment of PTEN. Akt inactivation results in nuclear exclusion of FoxO molecules, promoting the stability and increased function of Treg. (**d**) T cell-derived Sema4D mediates the reciprocal stimulation of DCs through CD72, resulting in augmented DC activation and maturation. Sema6D expressed on T cells also enhances activation and maturation of DCs via the plexin-A1–TREM2–DAP12 complex. (**e**) Sema7A expressed on tissue-infiltrating effector T cells binds to α1β1 integrin recruited on macrophages. The signals triggered by Sema7A-α1β1 integrin ligation stimulate macrophages to promote proinflammatory cytokines, thereby initiating inflammatory events in peripheral tissues. Consequently, IFN-γ and CD40L are highly upregulated, leading to further augmented inflammatory responses

Consistent with these findings, *Sema4a*-deficient mice are resistant to experimental autoimmune encephalomyelitis (EAE), which is induced by Th1 and Th17 cells, and anti-Sema4A antibody can block the development of EAE. Furthermore, Sema4A is involved in human multiple sclerosis pathogenicity, as discussed next. TIM-2 has been suggested to be a functional receptor for Sema4A in this context, in which TIM-2 is preferentially upregulated on Th2 cells during polarization. Furthermore, it appears that Sema4A in Th1 cells negatively regulates Th2 cells via TIM-2 (Kuchroo et al. 2003). However, there are some phenotypic discrepancies between *Sema4a*-deficient and *Tim2*-deficient mice, raising the possibility that Sema4A or TIM-2 have other binding partners. Indeed, members of the plexin-B subfamily and plexin-D1 also bind Sema4A (Toyofuku et al. 2007). Further studies are required to elucidate the details of the Sema4A-mediated pathway.

7.3.3 Sema4A/Nrp-1 and Regulatory T-Cell Stability

A recent study revealed a novel function of Sema4A associated with maintenance of regulatory T (Treg) cells (Fig. 7.3c) (Delgoffe et al. 2013). Sema4A interacts with receptor neuropilin-1 (Nrp-1) expressed by Treg cells, and this interaction potentiates Treg cell function and survival at inflammatory sites. In this pathway, Sema4A ligation of Nrp-1 restrains Akt phosphorylation in the cell body and at the immunological synapse by recruiting phosphatase and tensin homologue (PTEN); this in turn promotes nuclear localization of the transcription factor Foxo3a, which is important in the development and programming of Treg cells (Fontenot et al. 2003; Hori et al. 2003). This Sema4A/Nrp-1-dependent pathway is crucial for maintenance of immune homeostasis and is involved in inflammatory colitis and Treg cell stability in tumor tissues in vivo. These findings indicate that Sema4A is a potential therapeutic target that could limit Treg cell-mediated tumor-induced tolerance without inducing autoimmunity.

7.3.4 Sema4D and T Cell-Mediated Immunity

Sema4D is highly expressed by T cells and is crucially involved in T-cell activation, which requires DC maturation (Fig. 7.3d) (Kumanogoh et al. 2000, 2002b). Extensive analyses using *Sema4d*-deficient mice have shown that Sema4D is also important in T cell-mediated immunity. After immunization with protein antigens, CD4+ T cells from the draining lymph nodes of *Sema4d*-deficient mice exhibit extremely impaired proliferative responses and cytokine production following antigen restimulation (Kumanogoh et al. 2002b). *Sema4d*-deficient mice are resistant to EAE induced by myelin oligodendrocyte glycoprotein (MOG)-derived peptide, a phenotype that has been ascribed to the defective generation of MOG-specific T cells (Kumanogoh et al. 2002b). Conversely, transgenic mice that overexpress soluble Sema4D have enhanced T-cell responses (Watanabe et al. 2001). These observations indicate that Sema4D is crucially involved in the initial activation and differentiation of T cells, which are the main Sema4D-producing cells in the immune system. However, *Sema4d*-deficient T cells respond normally to CD3-specific monoclonal antibodies or mitogens such as concanavalin A (Shi et al. 2000), indicating that Sema4D does not act in a T cell-autonomous manner. Moreover, soluble recombinant Sema4D does not affect T-cell activation (Shi et al. 2000), suggesting that Sema4D has no direct effect on T cells. By contrast, recombinant Sema4D increases the surface expression of CD80, CD86, and major histocompatibility complex (MHC) class II molecules on DCs, as well as their immunogenicity induced by CD40 stimulation (Kumanogoh et al. 2002b). Sema4D expressed on T cells seems to act on DCs in a manner that promotes their activation and maturation, which in turn enhances T-cell activation.

Recent studies have revealed that Sema4D is functionally important in γδ T cells (Holl et al. 2012; Witherden et al. 2012), and that it regulates the morphology of these cells. In the context of epithelial repair, Sema4D-mediated signaling is transduced via the functional receptor plexin-B2. Thy1+ dendritic epidermal T cells (DETCs), a type of γδ T cell, express Sema4D, and plexin-B2 is expressed in keratinocytes. Furthermore, *Sema4d*-deficient mice have defective DETC responses to keratinocyte damage, resulting in delayed healing of cutaneous wounds.

7.3.5 *Sema6D/Plexin-A1 and Interaction Between T Cells and DCs*

Sema6D is expressed in T cells, B cells, and natural killer (NK) cells. Plexin-A1, the main receptor of Sema6D, is highly expressed in mature DCs (Wong et al. 2003; Takegahara et al. 2006). Plexin-A1 on DCs and osteoclasts forms a receptor complex with the triggering receptor expressed on myeloid cell 2 (TREM-2) and the adaptor molecule DNAX-activating protein 12 (DAP12), which contains an ITAM in its cytoplasmic region (Takegahara et al. 2006).

Ligation of T cell-derived Sema6D to the plexin-A1–TREM2–DAP12 receptor complex on DCs promotes DC activation and maturation (Fig. 7.3d). Studies using recombinant Sema6D protein have demonstrated that Sema6D binds and activates DCs and increases IL-12 production through plexin-A1. Consistent with those findings, *Plexin-a1*-deficient mice exhibit impaired generation of antigen-specific T cells and are resistant to the development of EAE (Takegahara et al. 2006).

7.3.6 *Sema7A and T Cell-Mediated Inflammatory Responses*

Sema7A, also known as CD108, is a membrane-associated GPI-linked semaphorin protein that contains arginine-glycine-aspartate (RGD), a well-conserved integrin-binding motif, in its Sema domain. β1 integrin transduces Sema7A signaling during olfactory nerve outgrowth (Pasterkamp et al. 2003). Integrin-mediated signaling is a common mechanism by which Sema7A functions in both the nervous and immune systems. In the immune system, activation of T cells induces them to express Sema7A (Fig. 7.3e) (Suzuki et al. 2007). Recombinant Sema7A protein stimulates monocytes/macrophages through α1β1 integrin and increases the production of proinflammatory cytokines, such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α. Sema7A is recruited to lipid rafts that accumulate at the immunological synapse between T cells and macrophages, where it interacts with α1β1 integrin (Suzuki et al. 2007). Subsequently, clustering of α1β1 integrin is induced on macrophages, resulting in firm adhesion between T cells and macrophages and the efficient secretion of proinflammatory cytokines. Consistent with this, in vivo

analysis revealed that *Sema7a*-deficient mice are resistant to T cell-mediated immune responses, including hapten-induced contact hypersensitivity (CHS) and EAE (Suzuki et al. 2007). Sema7A accumulates at the T-cell–macrophage interface and promotes effector immune responses during delayed-type hypersensitivity (DTH) and CHS (Suzuki et al. 2007).

7.4 Semaphorins and Dendritic Cell Functions

7.4.1 Sema3A/Plexin-A1 and Dendritic Cell-Mediated Immune Responses

In the immune system, Sema3A, a class III (secreted) semaphorin, is expressed in activated DCs and T cells. In other immune cells such as macrophages, B cells, and T cells, plexin-A1 is expressed at low or undetectable levels. The functional significance of plexin-A1 in DC-mediated immune responses was first described in studies using RNA interference. Using short-hairpin RNA against plexin-A1, it was shown that plexin-A1 is involved in the activation of T cells by DCs (Wong et al. 2003). Consistent with those findings, *plexin-a1*-deficient mice exhibit impaired generation of antigen-specific T cells (Takegahara et al. 2006; Takamatsu et al. 2010). Sema3A-induced signaling is also involved in immune cell migration. For example, plexin-A1 is involved in sensing Sema3A during DC migration, in particular during transmigration across the lymphatics (Fig. 7.4a) (Takamatsu et al. 2010). During DC migration, plexin-A1 is localized at the rear of migrating DCs. Sema3A produced by lymphatic endothelial cells binds plexin-A1 on DCs and induces myosin light-chain phosphorylation, resulting in squeezing of the cell body and thereby allowing cells to pass through narrow gaps between endothelial cells. Adoptive-transfer experiments have also shown that Sema3A secreted by lymphatic endothelial cells is involved in the regulation of DC trafficking from peripheral tissues to draining lymph nodes. It is worthy of special mention that semaphorins produced by vascular endothelial cells (Gu and Giraudo 2013) are also involved in immune cell migration by regulating contractility and adhesion activities in a plexin-dependent manner.

7.4.2 Sema4A and Dendritic Cell Functions

Sema4A is constitutively expressed in dendritic cells (DCs). Sema4A derived from DCs, as well as from T cells, is important for T cell-mediated immunity (Fig. 7.4b). Soluble Sema4A proteins and anti-Sema4A mAb bound to Sema4A on the surface of DCs enhance T-cell activation (Kumanogoh et al. 2002a). Consistent with this, DCs derived from *Sema4a*-deficient mice stimulate allogeneic T cells poorly

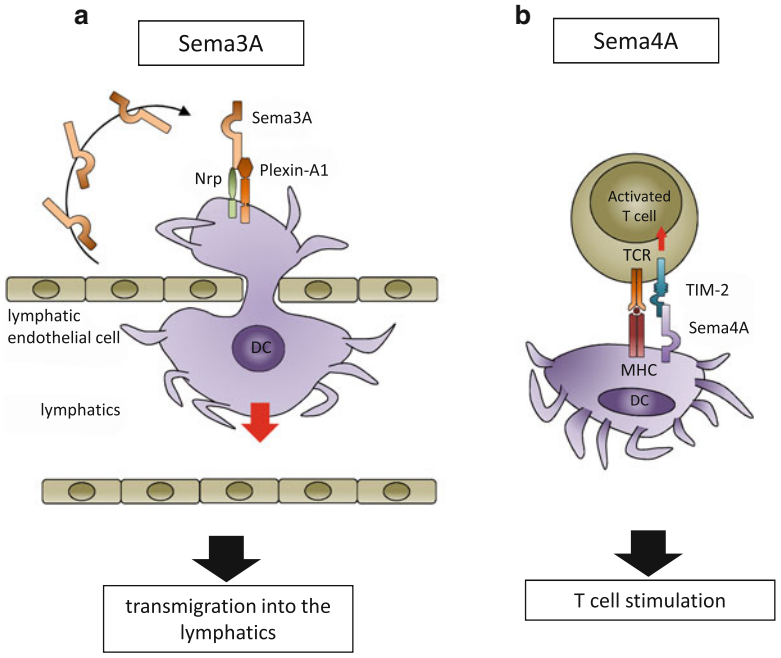

Fig. 7.4 Immune semaphorins in dendritic cell-mediated immunity. (**a**) During transmigration of dendritic cells (DCs) into the lymphatics, Sema3A secreted by lymphatic epithelial cells (ECs) binds to the plexin-A1–neuropilin 1 (Nrp-1) receptor complex expressed on the rear sides of DCs. This interaction promotes actomyosin contraction in DCs and their detachment from ECs, resulting in elevated DC transmigration into the lymphatics. (**b**) When DCs encounter T cells, Sema4A expressed on DCs directly stimulates T cells via TIM-2, leading to the optimal activation of antigen-specific T cells

compared to wild-type DCs (Kumanogoh et al. 2002a). By contrast, when CD4+ T cells from *Sema4a*-deficient mice or wild-type littermates were cultured with allogeneic DCs from wild-type mice, no differences in mixed lymphocyte reactions were observed (Kumanogoh et al. 2002a). These results suggest that DC-derived Sema4A is directly and critically involved in the activation of T cells reactive to alloantigens on DCs (Table 7.1).

7.5 Semaphorins and the Functions of Other Immune Cells

7.5.1 Sema4B and Basophil Functions

Sema4B, which contains a PDZ-binding motif in its cytoplasmic tail, is expressed by T cells and B cells and negatively regulates basophil function by promoting T cell–basophil interactions (Nakagawa et al. 2011). Basophils are strong mediators

Table 7.1 Immune semaphorins, their receptors, associated diseases, and immunological phenotypes of the corresponding knockout mice

Semaphorins	Expression	Receptor	Activities (species)	Phenotypes of KO mice	Related-diseases
Sema3A	T cells	Plexin A proteins	*Stimulatory*: differentiation of osteoblasts (M)	Impaired T-cell priming (Takamatsu et al. 2010)	Atopic dermatitis (Yamaguchi et al. 2008)
	Tumor cells		*Inhibitory*: monocyte migration (M, H)	Impaired migration of DCs to the lymph nodes (Takamatsu et al. 2010)	Allergic rhinitis (Sawaki et al. 2011)
	Endothelial cells		T-cell activation (M, H)		Osteoporosis (Hayashi et al. 2012)
			Tumor angiogenesis (M, H)		Rheumatoid arthritis (Takagawa et al. 2013)
			Osteoclast differentiation (M)		Multiple sclerosis (Williams et al. 2007)
					SLE (Vadasz et al. 2012; Kong et al. 2010)
					Cardiac dysrhythmia (Ieda et al. 2007)
					Cancer (Catalano et al. 2006)
Sema3E	Thymus (especially in the medulla)	Plexin D1	*Stimulatory*: migration of thymocytes into the medulla (M)	Impaired antigen-specific activation of T cells (Holl et al. 2011)	
				Impaired development of thymocytes within the thymus (Gu et al. 2005)	

(continued)

Table 7.1 (continued)

Semaphorins	Expression	Receptor	Activities (species)	Phenotypes of KO mice	Related-diseases
Sema4A	DCs	Plexin B proteins	*Stimulatory*: T-cell activation (M)	Impaired Th1 responses induced by *Propionibacterium acnes* (Kumanogoh et al. 2005)	EAE/multiple sclerosis (Kumanogoh et al. 2002a; Nakatsuji et al. 2012)
	Activated T cells	Plexin-D1		Enhanced Th2 responses induced by *Nippostrongylus brasiliensis* (Kumanogoh et al. 2005)	Atopic dermatitis (Morihana et al. 2013)
	T_H1 cells	TIM2	T_H1 cell differentiation (M)	Impaired antigen presentation of DCs (Kumanogoh et al. 2005)	Pigmentary retinopathy (Toyofuku et al. 2012)
Sema4B	T cells	Not known	*Inhibitory*: basophil-mediated Th2 skewing (M)	Enhanced basophil-mediated responses. (Nakagawa et al. 2011)	
	B cells				
Sema4D	T cells	Plexin-B1	*Stimulatory*: B-cell activation (M, H)	Impaired activation of DCs (Kumanogoh et al. 2002b)	EAE (Okuno et al. 2010)
	Activated B cells	CD72	DC activation (M, H)	Impaired activation of B cells (Kumanogoh et al. 2000)	HAM (Giraudon et al. 2004)
	DCs		Microglial activation (M)	Impaired migration of monocytes induced by chemokines (Delaire et al. 2001)	Immunodeficiency syndrome (Shi et al. 2000)
			Injury of oligodendrocytes (M)	Impaired secretion of iNOS from microglia (Okuno et al. 2010)	Osteopetrosis (Negishi-Koga et al. 2011)
				Impaired platelet responses to vascular injury (Zhu et al. 2009)	
Sema6A	DCs, Langerhans cells	Not known	*Stimulatory*: granuloma formation (H)?		LC histiocytosis, dermatopathic lymphadenitis (Gautier et al. 2006), GPA (Xie et al. 2013)

Sema6D	T cells	Plexin-A1	*Stimulatory*: DC activation (M)	(Sema6D−/− mice do not exhibit defects in T-cell priming) (Takamatsu et al. 2010)	Osteopetrosis (Takegahara et al. 2006)
	B cells				
	NK cells				
Sema7A	Activated T cells	Plexin-C1	*Stimulatory*: monocyte and macrophage	Impaired activation of macrophages (Suzuki et al. 2007)	Contact hypersensitivity (Suzuki et al. 2007)
		Integrin α1β1			
			activation (M, H)	Resistance to experimental autoimmune encephalomyelitis (EAE) (Suzuki et al. 2007)	EAE (Suzuki et al. 2007)
				Hypersensitive to experimental autoimmune encephalomyelitis (EAE) (Czopik et al. 2006)	Pulmonary fibrosis (Kang et al. 2007)
				Resistance to experimental contact dermatitis (Suzuki et al. 2007)	
				Enhanced responses to dextran sodium sulfate (DSS)-induced colitis (Kang et al. 2012 Feb 1)	
				Resistance to lung fibrosis (Kang et al. 2007)	

M demonstrated in mouse, *H* demonstrated in human, *VEGF* vascular endothelial growth factor, *SLE* systemic lupus erythematosus, *DC* dendritic cell, *EAE* experimental autoimmune encephalomyelitis, *HAM* HTLV-1 associated myelopathy, *GPA* granulomatosis with polyangiitis (Wegener's)

of Th2 skewing and humoral memory responses, and these cells secrete IL-4 during helminthic infections (Karasuyama et al. 2011). Basophils have also been proposed to function as antigen-presenting cells (APCs) (Sokol and Medzhitov 2010). *Sema4b*-deficient mice have considerably elevated serum IgE concentrations, despite having normal lymphocyte and DC functions. Sema4B inhibits interleukin (IL)-4 production from basophils, and T cell-derived Sema4B suppresses basophil-mediated Th2 skewing. Furthermore, Sema4B-deficient mice exhibit enhanced basophil-mediated memory IgE production (Nakagawa et al. 2011). Thus, Sema4B negatively regulates basophil-mediated Th2 and humoral memory responses. The receptor of Sema4B has not yet been identified; however, Sema4B inhibits the phosphorylation of extracellular regulated kinase (Erk) mediated by immunoreceptor tyrosine-based activating motif (ITAM)-containing molecules (Hida et al. 2009). Thus, it is possible that Sema4B regulates basophil functions via ITIM-containing molecules.

7.5.2 Sema4D and Mast Cell Functions

Sema4D is also involved in the KIT-mediated signaling pathway in mast cells (Kataoka et al. 2010). In human mast cells, Sema4D negatively regulates proliferation and CCL2 production via CD72. The Sema4D–CD72 axis may be important in negatively regulating KIT-mediated mast cell responses.

7.6 Virus Semaphorins

Concurrently with the first report of Sema3A, it was revealed that semaphorin-like proteins were encoded in several DNA viruses, including vaccinia, human smallpox, fowlpox, mousepox, and alcelaphine herpes virus type 1 virus (AHV). These proteins came to be called viral semaphorins. The structures of viral semaphorins are highly similar to those of class VII semaphorins. Next, we discuss A39R as a representative viral semaphorin.

7.6.1 Semaphorin A39R

Vaccinia virus protein A39R is a viral semaphorin with similarity to the extracellular domain of Sema7A. A39R protein upregulates expression of CD54 (ICAM-1), IL-6, and IL-8 in monocytes in vitro (Comeau et al. 1998). In addition, A39R secreted from poxvirus inhibits phagocytosis by DCs and neutrophils (Walzer et al. 2005). These findings suggest that semaphorins expressed by viruses induce a disturbance

in host immunity. Moreover, the structural basis for the mimicry of mammalian Sema7A by the viral semaphorin A39R has been elucidated by structural modeling (Liu et al. 2010).

7.7 Immune Semaphorins and Diseases

7.7.1 Multiple Sclerosis

Multiple sclerosis (MS) is a demyelinating autoimmune disease of the central nervous system. Experimental autoimmune encephalomyelitis (EAE), induced by immunization with myelin oligodendrocyte glycoprotein-derived peptides (MOG), is commonly used as an animal model for MS. The involvement of several immune semaphorins, including Sema4A, Sema4D, and Sema7A, has been established based on evidence accumulated from studies using this model. The pathogenesis of EAE has been attributed to impaired T-cell priming and reduced effector immune responses (Suzuki et al. 2007; Kumanogoh et al. 2005; Okuno et al. 2010).

As already discussed, Sema4A plays important roles in immunoregulatory functions in T cells and DCs, and also in the pathogenesis of MS. *Sema4a*-deficient mice are resistant to EAE (Kumanogoh et al. 2002a, 2005), and blocking antibodies against Sema4A significantly attenuate EAE. The resistance of *Sema4a*-deficient mice to EAE has been attributed to impaired generation of MOG peptide-specific CD4+ T cells. Furthermore, the significance of Sema4A in the pathogenesis of MS has been confirmed in studies of human MS patients (Nakatsuji et al. 2012). The level of Sema4A in serum is elevated in patients with MS, and DCs in these patients express and shed high levels of the protein. In addition, MS patients with high Sema4A levels exhibit Th17 skewing, suggesting that DC-derived Sema4A is critical for Th17, as well as Th1, differentiation. Furthermore, patients with high Sema4A levels have more severe disabilities and are unresponsive to interferon (IFN)-β treatment (Nakatsuji et al. 2012). These results suggest that Sema4A could be useful as a diagnostic or prognostic marker of MS.

Sema4D is also associated with the pathogenesis of MS. The resistance of *Sema4d*-deficient mice to EAE has been attributed to impaired T-cell priming (Shi et al. 2000; Kumanogoh et al. 2002b). However, when T cells derived from wild-type mice are adoptively transferred into *Sema4d*-deficient mice, the development of EAE is considerably attenuated. Given that Sema4D also contributes to neuroinflammation in the central nervous system, and that Sema4D expression is induced in infiltrating mononuclear cells and microglia, the results of the adoptive transfer suggest that Sema4D expressed not only on the cell surface of T cells, but also on microglia, is crucial for EAE development. Sema4D induces activation of microglia and death of immature neural cells (Okuno et al. 2010); thus, Sema4D regulates neuroinflammation through plexin-B1 expressed on microglia. Because blocking antibodies specific for Sema4D significantly inhibit neuroinflammation during EAE

development, Sema4D could represent a potential therapeutic target for treatment of MS. Indeed, clinical trials of drugs manufactured from Sema4D-blocking antibodies to treat MS have been initiated in the United States (NCT01313065).

Sema7A is also involved in the effector phase of EAE, through an interaction with its binding partner, α1β1 integrin (Suzuki et al. 2007). *Sema7a*-deficient mice are highly resistant to EAE, and their spinal cords contain very few infiltrating cells. Wild-type mice that receive T cells from MOG-immunized *Sema7a*-deficient mice develop attenuated EAE, suggesting that the poor reactivity to EAE of *Sema7a*-deficient mice is caused by impaired antigen-specific T-cell priming.

7.7.2 Rheumatoid Arthritis and Systemic Lupus Erythematosus

Rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are autoimmune connective tissue diseases that typically affect small- and medium-sized peripheral joints. Massive destruction of articular cartilage and surrounding bones and the increased proliferation of synovial cells are caused in the joints of RA and SLE patients.

Recent work has revealed the association between Sema3A and RA/SLE pathogenesis. CD4+ T cells derived from RA patients express very low levels of Sema3A, but elevated levels of neuropilin-1 (Nrp-1), the receptor of Sema3A (Catalano 2010). Additionally, Sema3A-blocking antibodies inhibit the ability of CD4+Nrp-1+ T cells to suppress immune cell responses. Consistent with this, several studies of RA/SLE patients have shown that serum levels of Sema3A are lower in SLE patients than in RA patients, whose serum levels are in turn lower than those of normal individuals (Takagawa et al. 2013; Vadasz et al. 2012). Altered serum Sema3A levels are inversely correlated with SLE disease activity, including renal damage and the presence of serum anti-cardiolipin antibodies (Vadasz et al. 2012). Furthermore, it was recently reported that anti-Nrp-1 peptide suppresses VEGF-induced synoviocyte proliferation and survival, thereby blocking the development of experimental arthritis. These findings suggest that anti-Nrp-1 peptide could be useful in regulating rheumatoid inflammation (Kong et al. 2010), and that the Sema3A–Nrp-1 axis represents a potential target for drugs aimed at treating autoimmune arthritis.

7.7.3 Allergic Dermatitis

Immune semaphorins, including Sema3A and Sema4A, are associated with allergic dermatitis, especially atopic dermatitis (AD). AD, a chronic dermatitis characterized by relapsing itching that mainly occurs in childhood, significantly lowers patient quality of life.

The therapeutic effects of Sema3A have been demonstrated in AD mouse models in the context of neuroimmune crosstalk (Goshima et al. 2012). Recombinant Sema3A exerted dose-dependent therapeutic effects when it was intracutaneously injected into the skin lesions of NC/Nga mice, an animal model of AD. The skin of NC/Nga mice treated with Sema3A injection ameliorated the histological characteristics observed in AD, for example, decreased epidermal thickness, the density of invasive nerve fibers in the epidermis, and the number of infiltrating cells (Yamaguchi et al. 2008). Unexpectedly, Sema3A treatment induced not only neuronal effects, such as a reduction in nerve fiber density, but also immunological effects, such as a reduction in the number of immune cells including CD4+ T cells, IL-4-positive cells, and mast cells. Sema3A is also involved in other skin diseases, in particular in the pathogenesis of psoriasis (Kou et al. 2012). The expression of Sema3A is lower in psoriatic skin than in healthy skin, whereas expression of nerve growth factor (NGF), which is important for regulating C-fiber extension, was higher in psoriatic skin. Given these findings, Sema3A might be a potential therapeutic target not only for AD, but also for itching dermatitis in general.

Sema4A is another immune semaphorin associated with the pathogenesis of allergic dermatitis (Nkyimbeng-Takwi et al. 2012). As already described, Sema4A is crucial for helper T-cell differentiation and polarization, and Sema4A deficiency causes disruption of Th1/Th2 balance (Kumanogoh et al. 2005). Indeed, *Sema4a*-deficient mice exhibit a phenotype of severe allergic dermatitis that resembles AD (Morihana et al. 2013).

7.7.4 Allergic Rhinitis and Asthma

Similar to allergic dermatitis, both Sema3A and Sema4A are involved in the pathogenesis of allergic airway hyperreactivity. Decreased Sema3A expression has been observed in the nasal mucosa during allergic rhinitis (Sawaki et al. 2011). In addition, intranasal administration of recombinant Sema3A attenuates severity of allergic symptoms, such as sneezing and nasal rubbing, in rhinitis model mice (Sawaki et al. 2011). In a study that used a model of ovalbumin (OVA)-specific experimental asthma, *Sema4a*-deficient mice exhibited enhanced airway hyperreactivity. Further analysis of these mice revealed increased pulmonary eosinophil infiltration and increased levels of Th2 cytokines and IgE in bronchoalveolar lavage (BAL) fluid (Morihana et al. 2013; Nkyimbeng-Takwi et al. 2012).

7.7.5 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, is a group of inflammatory conditions of the intestinal tract and is considered to be an autoimmune disease. Recently, the significance of Sema7A in the pathogenesis

of IBD has been determined (Kang et al. 2012). Sema7A is expressed in intestinal epithelial cells (IECs) and induces the production of IL-10, which negatively regulates intestinal immunological homeostasis, by intestinal macrophages. Consistent with this, *Sema7a*-deficient mice exhibit severe signs of dextran sodium sulfate-induced colitis caused by reduced intestinal IL-10 levels, and the administration of recombinant Sema7A protein to these mice ameliorates the severity of colitis; these effects are diminished by IL-10-blocking antibodies. Regarding the receptor of Sema7A in intestinal macrophages, Sema7A-triggered signaling is transduced through αvβ1 integrin. These findings suggest that Sema7A represents a target for treatment of IBD.

7.7.6 Lung Fibrosis

Sema7A also plays an important role in the pathogenesis of lung fibrosis induced by transforming growth factor (TGF)-β and bleomycin (Kang et al. 2007). TGF-β induces the expression of Sema7A and its receptor. In TGF-β1 transgenic mice, which are prone to develop pulmonary fibrosis, deletion of the *Sema7a* gene significantly diminishes parenchymal and alveolar fibrosis and decreases alveolar remodeling. In addition, TGF-β-induced fibrosis can be ameliorated by administering an anti-β1 integrin antibody, suggesting that Sema7A exacerbates pulmonary fibrosis via β1 integrin (Gan et al. 2011).

7.8 Concluding Remarks

As discussed, immune semaphorins and their receptors constitute crucial axes in multiple aspects of immune responses. The accumulated evidence has demonstrated that dysfunctions of immune semaphorins result in various immunological disorders, including autoimmune and allergic diseases. This greater understanding should facilitate development of therapies for immunological diseases. Indeed, drugs targeted against certain immune semaphorins and their receptors, including Sema4D and plexin-A1, are already under development. However, because semaphorins exert a wide range of functions in other tissues, drugs designed to treat immunological diseases might have unexpected side effects, especially in the central nervous and vascular systems. To develop safer drugs, further experimental data using gene-targeted mice and binding assays should be accumulated.

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Chapter 8
Semaphorins in Bone Homeostasis

Mikihito Hayashi, Tomoki Nakashima, and Hiroshi Takayanagi

Abstract Intercellular communication between cells within bone is essential for the regulation of bone homeostasis. Growing evidence reveals that semaphorins have crucial roles in this process, including osteoclastic bone resorption and osteoblastic bone formation. Semaphorin 4D (Sema4D), derived from osteoclasts, has a potent inhibitory effect on osteoblast differentiation without hampering osteoclastic bone resorption. Sema3A, which is highly expressed in osteoblast lineage cells, maintains bone homeostasis by simultaneously inhibiting osteoclast differentiation and promoting osteoblast differentiation. Sema3A also has a role in the regulation of innervation, indicating the importance of future studies on the interactions among bone cells and neurons. Other semaphorins and their receptors have also been implicated in bone metabolism. These studies provide a scientific basis for future therapeutic approaches to bone diseases.

Keywords Osteoclast • Osteoblast • Bone remodeling

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8.1 Introduction

Bone has multiple functions, including locomotive activity, the storage of minerals, and the harboring of hematopoietic stem cells. Because of the nature of bone as a rigid supporting tissue, bone can appear to be unchangeable and inert. However, the bony skeleton is in fact an organ that is dynamically changing over the human lifespan, characterized predominantly by skeletal growth during childhood and subsequently continuous turnover, termed bone remodeling, throughout life. Bone remodeling is a finely balanced activity that is carried out by specialized groups of cells, including osteoclasts, which are multinucleated cells that resorb bone, and osteoblasts, which refill the resorption cavities created by osteoclasts (Zaidi 2007; Henriksen et al. 2011). An imbalance between bone resorption and formation results in metabolic bone disorders such as osteoporosis, a disease of low bone mass with increased susceptibility to bone fractures (Seeman and Delmas 2006; Takayanagi 2007). Antiresorptive agents, including bisphosphonates, are currently most commonly used for the treatment of osteoporosis (Rachner et al. 2011). However, treatment with these antiresorptive agents has certain limitations, for example, a low turnover state in which bone resorption decreases along with a simultaneous decrease in bone formation, with the result that efficacy is compromised (Lewiecki 2011; Kawai et al. 2011). Each antiresorptive agent approach is based on a different molecular mechanism, but a number of reports suggest that the level of formation is coupled to that of resorption through "coupling factors" (Sims and Martin 2014). Thus, understanding the mechanisms underlying bone remodeling and coupling is important for a better understanding of both the skeletal system and the development of novel osteoprotective agents.

Semaphorins, initially identified as an evolutionally conserved guidance for developing axons, are a family of secreted and cell membrane-anchored proteins (Tran et al. 2007; Pasterkamp 2012). The predominant receptors for semaphorins are the two groups of proteins known as the plexins and neuropilins, although some semaphorins do independently function through other receptors. Recently, it has been demonstrated that semaphorins play an important role in diverse biological processes outside the nervous system, including cardiovascular development, immune response, and tumor progression, as had been suggested by their diverse expression profiles in a wide variety of tissues (Gu and Giraudo 2013; Kumanogoh and Kikutani 2013). It has also recently been suggested that the semaphorins are involved in bone homeostasis through the regulation of cell–cell communication between osteoclasts and osteoblasts during bone remodeling (Negishi-Koga and Takayanagi 2012; Kang and Kumanogoh 2013). This review summarizes the current knowledge of semaphorin-mediated regulation of bone metabolism based on recent studies.

8.2 Bone Cells and Bone Remodeling

Osteoclasts are derived from monocyte/macrophage lineage precursor cells, and their differentiation is under the control of mesenchymal cells, mainly osteoblast lineage cells, that express receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) (Fig. 8.1) (Takayanagi 2007; Henriksen et al. 2011). Osteoprotegerin (Opg), a decoy receptor for RANKL, is also expressed by osteoblast lineage cells to counterbalance the action of RANKL (Nakashima et al. 2012). Active osteoclasts enclose resorption pits on the bone surface (Teitelbaum 2011). The ring-shaped structure referred to as the sealing zone is essential for the development of resorption pits, into which osteoclasts secrete hydrochloric acid for the purpose of creating an acidic environment to decalcify the bone matrix (Sobacchi et al. 2013). After the bone mineral content has been dissolved, metalloproteinases and cathepsins, which are secreted by osteoclasts, remove the collagenous bone matrix to complete the process of resorption (Henriksen et al. 2011). Osteoclasts undergo apoptosis after bone resorption is complete (Manolagas 2000; Tanaka et al. 2006).

Following resorption, pre-osteoblasts of the mesenchymal lineage move into the resorbed area and start to proliferate and differentiate into mature osteoblasts (Fig. 8.1) (Dirckx et al. 2013; Crane and Cao 2014). These cells produce and deposit organic matrix, called osteoid, a substance predominantly composed of collagen, to fill in the resorbed cavities. Osteoid forms a scaffold in which minerals, including calcium and phosphate, begin to crystallize. During this process, a large amount of various growth factors, such as transforming growth factor (TGF)-β and insulin-like growth factor 1 (IGF-1), are synthesized and stored in the bone matrix (Sims and Martin 2014; Crane and Cao 2014). When osteoclasts resorb the bone matrix, growth factors embedded in the bone are released and activated in the resorption pits and are thus able to induce the recruitment and differentiation of osteoblast precursor cells (Fig. 8.1). After the resorbed areas are replenished with newly synthesized bone, some of the osteoblasts undergo apoptosis, whereas others become quiescent as lining cells, which cover the surface of bone (Manolagas 2000). Some of the active osteoblasts are trapped within the matrix they secreted, thereby becoming osteocytes (Fig. 8.1). Osteocytes communicate with themselves and other cells in the bone marrow, including osteoclasts and osteoblasts, via long cytoplasmic extensions (Dallas et al. 2013). The extensive connectivity of the lacuno-canalicular system allows osteocytes to transmit signals to and from tissues throughout the body. Osteocytes play an important role in sensing local changes in mechanical strain or microdamage, leading to the recruitment of osteoclast precursor cells to the bone surface (Dallas et al. 2013). Recent studies have demonstrated that osteocytes are an essential source of RANKL to control osteoclast differentiation in adult bone remodeling, whereas osteoblasts or hypertrophic chondrocytes are an important source during skeletal development (O'Brien et al. 2013). It has also been shown that osteocytes also have a key role in mineral homeostasis, functioning as the endocrine organ secreting fibroblast growth factor (FGF)-23 (Fukumoto and Martin 2009; DiGirolamo et al. 2012).

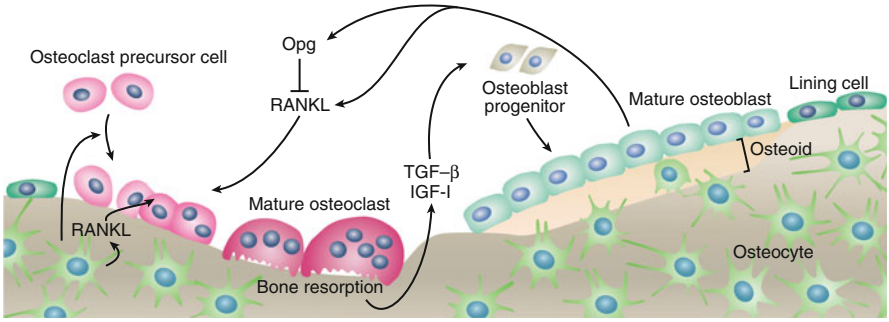

Fig. 8.1 Bone remodeling is a process that ensures the turnover of old bone and the repair of damaged bone, while keeping bone mass intact and allowing for maintenance of mechanical integrity by the requirements of calcium and phosphate metabolism in adults. New bone formation occurs at the site of resorbed bone, suggesting a tight coupling between osteoclasts and osteoblasts. The remodeling cycle can be triggered from an accumulation of microdamage or changes in mechanical loading sensed by osteocytes under the influence of hormones, cytokines, and other factors. Osteocytes communicate with surface or marrow cells to promote the recruitment of osteoclast precursor cells and the differentiation into mature osteoclasts, which is induced by RANKL derived from osteoblast lineage cells, especially osteocytes, and bone resorption starts. Opg, a soluble decoy receptor for RANKL, is produced by osteoblast lineage cells to inhibit the action of RANKL, thereby suppressing any excess active osteoclasts. In response to osteoclastic bone resorption, primary coupling factors, including IGF-1 and TGF-β, are released and stimulated to initiate the migration of osteoblast progenitors to the resorbed sites, coupling new bone formation to bone resorption spatiotemporally. Active osteoblasts replenish the resorbed area with new bone. Some of the osteoblasts become embedded within the matrix and differentiate into osteocytes

Recently, it has been made clear that the bone remodeling process is modulated by numerous humoral and coupling factors in the endocrine, immune, and nervous systems (Sims and Martin 2014). These factors are expressed or secreted from osteoclasts or osteoblasts in the bone matrix. The communication between osteoclasts and osteoblasts is regulated by a wide variety of local and systemic factors to maintain bone homeostasis adequately. As bone remodeling is a multicellular process, a better understanding of the cellular crosstalk that takes place between and among the cells in bone is important for ultimately modulating this process. Recently, there have been significant advances in our understanding of the role of semaphorins in the regulation of bone homeostasis (Negishi-Koga and Takayanagi 2012; Kang and Kumanogoh 2013), which is discussed in this review.

8.3 The Regulation of Osteoclastogenesis by Plexin-A1

Plexin-A1, a receptor for class 5 and 6 semaphorins,is also known to form a receptor complex with neuropilins for class 3 semaphorins (Pasterkamp 2012). It has been suggested that Plexin-A1 has important functions in the immune system, as shown by its high expression in dendritic cells (DCs), in addition to its role in the nervous system. *Plxna1*$^{-/-}$ mice have abnormalities in antigen-specific T-cell responses and

DC trafficking into draining lymph nodes (Takegahara et al. 2006; Takamatsu et al. 2010). Interestingly, these mice also display a high bone mass phenotype as a result of impaired osteoclast differentiation (Takegahara et al. 2006). It has been reported that the Plexin-A1 expressed on osteoclast precursor cells binds a complex composed of the triggering receptor expressed on myeloid cells 2 (TREM2) and DNAX activation protein 12 (DAP12), in response to Sema6D ligation (Fig. 8.2a).

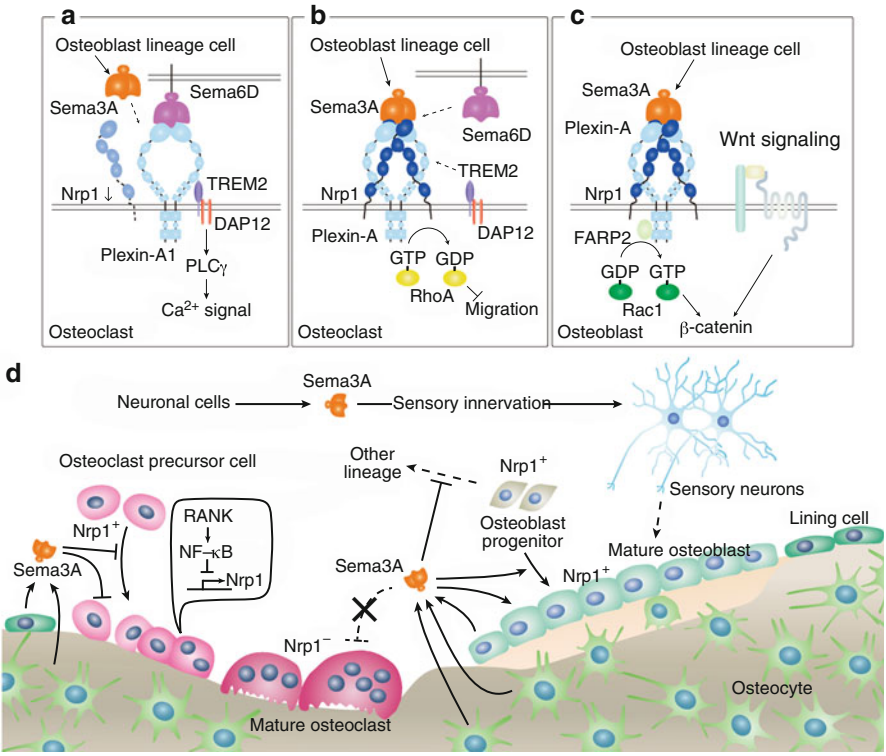

Fig. 8.2 Sema3A–Nrp1–Plexin-A1 regulates bone metabolism. (**a**) After RANKL stimulation, Nrp1 expression is blocked by NF-κB signaling, with the result that the Sema3A-induced inhibition is no longer effective and allowing Plexin-A1 to associate with Sema6D. The binding of Sema6D to Plexin-A1 facilitates the formation of a complex with TREM2 and DAP12, which activates osteoclast differentiation through the ITAM-mediated calcium signaling pathway. (**b**) In the absence of RANKL, Sema3A inhibits RhoA activation via the Nrp1–Plexin-A receptor complex and therefore suppresses the migration of osteoclast precursor cells. Furthermore, the Sema3A–Nrp1–Plexin-A1 complex inhibits osteoclast differentiation by sequestering Plexin-A1 from TREM2 and Sema6D. (**c**) During bone formation, Sema3A activates Rac1 through RacGEF FARP2 downstream of the Nrp1–Plexin-A1 complex. Rac1 activation enhances the nuclear localization of the β-catenin induced by canonical Wnt signaling, which is essential for osteoblast differentiation. (**d**) Osteoblast lineage cells produce and secrete Sema3A to sequester osteoclast precursor cells away from osteoblastic cells and inhibit the differentiation into osteoclasts during bone formation. Concomitantly, Sema3A promotes osteoblast differentiation and bone formation in an autocrine/paracrine manner, thereby shifting the balance of bone turnover from resorption to formation. The Sema3A produced in neurons is implicated in bone metabolism through the regulation of sensory innervation, not a direct effect on osteoblasts

The immunoglobulin-like receptor TREM2 and DAP12 activate the immunoreceptor tyrosine-based activation motif (ITAM) signaling pathway, which is critical for the subsequent activation of Ca^{2+} signaling and osteoclast differentiation (Fig. 8.2a) (Koga et al. 2004). In addition, FERM, RhoGEF, and pleckstrin domain protein 2 (FARP2), a Rac-specific guanyl nucleotide exchange factor (GEF) (Toyofuku et al. 2005), were shown to be involved in mature osteoclasts in vitro in actin rearrangement and podosome formation, in response to the class 6 semaphorins, including Sema6B and Sema6D (Takegahara et al. 2010). As the growing evidence suggested that immune and nervous systems share molecules that are implicated in the maintenance of bone metabolism, the discovery of Plexin-A1 as a regulator of osteoclast differentiation highlights the start of semaphorin studies in bone biology.

8.4 Sema4D Regulates Osteoblastic Bone Formation

During bone resorption, various coupling factors are secreted that recruit osteoblast precursor cells to the site of future bone formation. However, osteoblast recruitment and differentiation need to be suppressed to complete the resorption of damaged or aged bone adequately. Axon-guidance molecules, such as semaphorins and ephrins, are involved in cell–cell communication outside the nervous system. From the transcriptomic analysis of genes encoding axon-guidance molecules during osteoclast differentiation, Sema4D was found to be highly and selectively induced after RANKL stimulation (Negishi-Koga et al. 2011). Sema4D, a membrane protein, which is also functional in the soluble form after proteolytic cleavage, regulates the activation and survival of B cells and DCs and inhibits monocyte migration, mainly through CD72 (Kumanogoh and Kikutani 2013). Recent studies have demonstrated that the binding of Sema4D to class B plexins induces tumor angiogenesis, progression, and metastasis (Gu and Giraudo 2013). *Sema4d*$^{-/-}$ mice have increased bone mass caused by the activation of osteoblast differentiation and bone formation without any change in bone resorption. Among the bone cells examined, Sema4D is exclusively expressed in premature and mature osteoclasts, whereas Plexin-B1, a receptor for class 4 semaphorins, is expressed in osteoblasts, indicating that the Sema4D expressed on osteoclasts directly acts on the Plexin-B1 expressed on osteoblasts (Fig. 8.3a). Indeed, *Plxnb1*$^{-/-}$ mice recapitulate the high bone phenotype observed in *Sema4d*$^{-/-}$ mice (Negishi-Koga et al. 2011).

The binding of Sema4D to Plexin-B1 leads to the recruitment of PDZ-RhoGEF and leukemia-associated Rho guanine nucleotide exchange factor (LARG), both of which are GEF for RhoA GTPases (Fig. 8.3b). Activated RhoA and its effector, Rho-associated coiled-coil-containing protein kinase (ROCK), negatively regulate osteoblast differentiation through the inhibition of the phosphorylation of insulin receptor substrate 1 (IRS-1), which is downstream of the IGF-1 receptor (Fig. 8.3b).

Semaphorins are known to be involved in the regulation of cellular motility via the modulation of Rho family GTPases (Tran et al. 2007). Indeed, Sema4D-induced

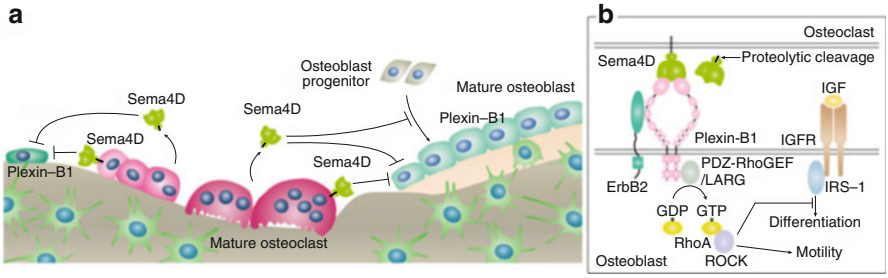

Fig. 8.3 Inhibition of osteoblastic bone formation by osteoclast-derived Sema4D. (**a**) Osteoclastic expression of Sema4D is amplified in response to RANKL stimulation, which is supplied by osteoblastic cells. Sema4D inhibits osteoblast differentiation in the proximity of bone-resorbing osteoclasts and repels osteoblast progenitors by increasing their motility, thus acting as a negative feedback loop during bone resorption. (**b**) Binding of Sema4D to the Plexin-B1–ErbB2 receptor complex activates RhoA via PDZ-RhoGEF and LARG. RhoA activates ROCK so as to inhibit IRS-1 downstream of IGF-1 signaling, which is essential for osteoblast differentiation. Activated RhoA-ROCK also promotes the motility of osteoblasts, thereby repulsing bone-forming osteoblasts

RhoA activation promotes osteoblast motility. In normal bone, it is frequently observed that bone-forming osteoblasts are sequestered from bone-resorbing osteoclasts. In *Sema4d*$^{-/-}$ and *Plxnb1*$^{-/-}$ mice, as well as RhoA DN^{OB} (an osteoblast-specific dominant-negative form of RhoA transgenic) mice, osteoblasts were observed to be in close proximity to osteoclasts. These findings suggest that osteoclast-derived Sema4D suppresses RhoA activation in osteoblasts, thereby inhibiting the differentiation of osteoblasts and keeping osteoblasts away from osteoclasts to enable efficient osteoclastic bone resorption (Fig. 8.3a, b).

8.5 Sema3A Synchronously Promotes Osteoblastogenesis and Inhibits Osteoclastogenesis

During osteoblastic bone formation, the recruitment and differentiation of osteoclasts must be tightly restricted. It is likely that osteoblasts contribute to the regulation of osteoclasts by producing pro- and anti-osteoclastogenic factors. However, an inhibitory factor of osteoclast differentiation derived from osteoblasts has not been identified except for Opg (Takayanagi 2007; Nakashima et al. 2012). Opg-deficient osteoblastic cell-conditioned medium inhibited RANKL-induced osteoclast differentiation, suggesting the presence of other osteoblast-secreted protein(s) involved in the negative regulation of osteoclast differentiation. Based on the anti-osteoclastic activity of Opg-deficient osteoblastic cell-conditioned medium, an axon-guidance molecule, Sema3A, was determined to be a novel negative regulator of osteoclast differentiation (Hayashi et al. 2012). Among the various cells tested, Sema3A mRNA and protein were predominantly expressed in osteoblast lineage cells but were not detected in osteoclasts. The receptor complex for Sema3A is

composed of the ligand-binding subunit neuropilin-1 (Nrp1) and one of the class A plexins. These proteins are expressed on osteoblast lineage and osteoclast precursor cells, respectively.

As already discussed, Sema6D–Plexin-A1 promotes osteoclast differentiation through the activation of ITAM signaling through the formation of the Plexin-A1–TREM2–DAP12 complex (Fig. 8.2a). In the absence of RANKL, the binding of Sema3A to the Nrp1–Plexin-A1 complex on osteoclast precursor cells blocked the interaction between Plexin-A1 and the TREM2–DAP12 complex so as to suppress ITAM signaling as well as RANKL and Sema6D-induced osteoclast differentiation (Fig. 8.2b). On the other hand, the expression of Nrp1 was downregulated by stimulation with RANKL (Fig. 8.2a). Therefore, RANKL-induced downregulation of Nrp1 expression is important for proper osteoclast differentiation to overcome the inhibitory effect of Sema3A. This RANKL-induced inhibition of Nrp1 expression is dependent on the NF-κB transcription factor, but not NFATc1 or c-Fos. The NF-κB-mediated inhibition of Nrp1 expression was also observed in tumor-associated macrophages via the Hif2-mediated activation of NF-κB in a hypoxic condition (Casazza et al. 2013).

It is well established that semaphorin–plexin signaling regulates cell migration via the modulation of the Rho family of small GTPases (Tran et al. 2007). The migration of osteoclast precursor cells toward M-CSF was suppressed by the presence of Sema3A via the abrogation of RhoA activation in response to M-CSF, but not Rac activation (Fig. 8.2b). Taken together, in the absence of RANKL, Sema3A binding to Nrp1–Plexin-A receptor inhibits RhoA activation and therefore suppresses migration of osteoclast precursor cells. In addition, the Sema3A–Nrp1 axis inhibits osteoclast differentiation by sequestering Plexin-A1 from TREM2. After the RANKL stimulation by which Nrp1 is rapidly downregulated, Plexin-A1 forms a complex with TREM2 and DAP12 that facilitates osteoclast differentiation (Fig. 8.2a, b).

Sema3a$^{-/-}$ mice, as well as *Nrp1*$^{\text{Sema}-}$ mice, in which the semaphorin-binding site of Nrp1 is genetically disrupted, are severely osteopenic because of both enhanced osteoclastic bone resorption and reduced osteoblastic bone formation. In both *Sema3a*$^{-/-}$ and *Nrp1*$^{\text{Sema}-}$ mice, a massive increase in adipocytes in the bone marrow was observed. These results suggest that Sema3A is also important for the differentiation of mesenchymal progenitors into osteoblasts in bone marrow and inhibits the commitment of mesenchymal cells to adipocytes.

What are the mechanisms by which Sema3A promotes bone formation and inhibits adipocyte differentiation? Canonical Wnt/β-catenin signaling has been considered to have osteogenic and anti-adipogenic effects and to control skeletal development and integrity (Monroe et al. 2012; Baron and Kneissel 2013). Sema3A induces Rac1 activation, which was reported to control nuclear localization of β-catenin during canonical Wnt signaling (Wu et al. 2008), through FARP2 (Fig. 8.2c). The activation of Rac, the nuclear localization of β-catenin and the mRNA expression of transcriptional targets of β-catenin in response to the canonical Wnt ligand were considerably reduced in *Sema3a*$^{-/-}$ osteoblasts. Taken together, Sema3A not only inhibits RANKL-induced osteoclast differentiation, but also activates osteoblast differentiation (Fig. 8.2c, d).

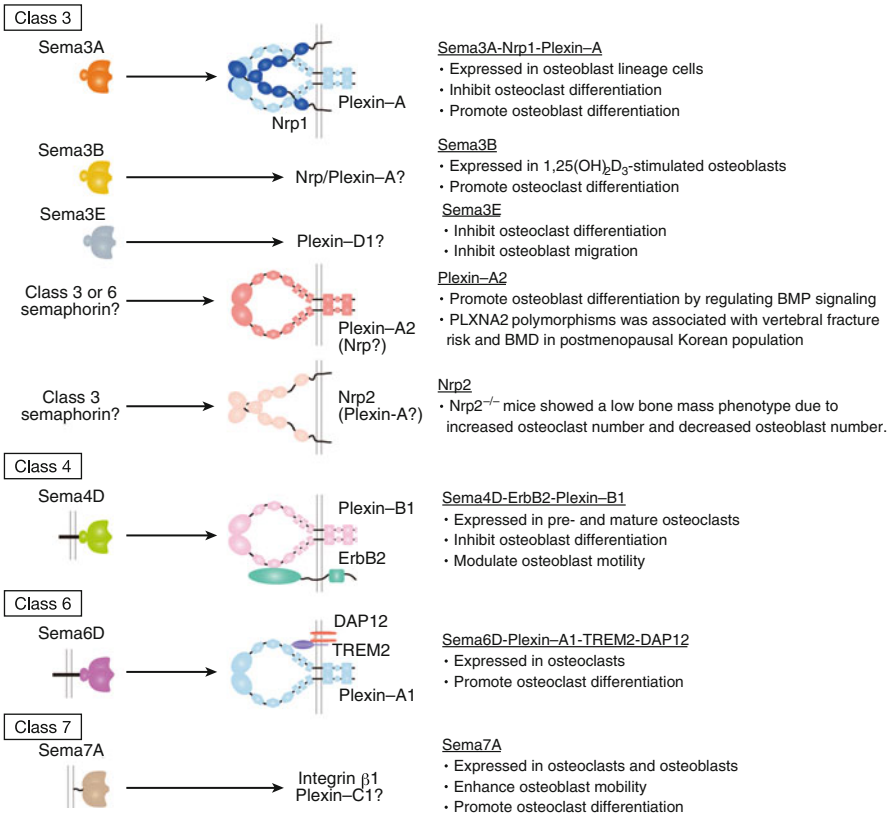

Fig. 8.4 Semaphorins in bone remodeling. A summary of the function of the semaphorin family and its receptors in bone remodeling. Various semaphorins and their receptors are involved in the regulation of osteoclasts and osteoblasts

The importance of Sema3A signaling in bone is supported by the finding that polymorphisms in *PLXNA2* (encoding Plexin-A2, which is a member of the type A plexins and a component of the receptor complex for Sema3A), are associated with low bone mineral density and an increased risk of bone fracture in postmenopausal women of a Korean cohort (Hwang et al. 2006). Furthermore, another group showed that Plexin-A2 expression in osteoblastic cells was induced by an osteogenic medium, whereas BMP-2 treatment and Plexin-A2 increased osteoblastic differentiation through the regulation of Smad and Akt signaling downstream of BMP2 (Fig. 8.4) (Oh et al. 2012).

Hofmann et al. recently reported that the biallelic mutations in the *SEMA3A* gene (a combination of a nonsense mutation in exon 1 and a splice site mutation in exon 9) resulted in a novel disorder characterized by skeletal abnormalities, including postnatal short stature, barrel thorax, funnel chest, and flattened vertebrae of the upper thoracic region, along with multiple minor congenital anomalies (Hofmann

et al. 2013). It is interesting that some of the phenotypes in *Sema3a*$^{-/-}$ mice are recapitulated in the patient, but intellectual development and heart abnormalities were not prominent, possibly because the approximately 20 % remaining *SEMA3A* expression is sufficient to maintain some organs and physiological processes.

8.6 Sema3A-Mediated Regulation of Sensory Innervation of Bone

After the first report of Sema3A function in bone homeostasis, another source of Sema3A in the regulation of bone mass accrual was reported (Fukuda et al. 2013). They confirmed that osteoblastic differentiation from calvarial cells derived from *Sema3a*$^{-/-}$ mice was impaired in a cell-autonomous manner and that Sema3A inhibits osteoclast differentiation in vitro, in agreement with the earlier study.

However, osteoblast lineage cell-specific *Sema3a* conditional knockout mice generated by crossing *Col1a1-Cre*$^{+}$ mice with *Sema3a*$^{flox/flox}$ mice had normal bone mass, bone formation rate, and osteoblast/osteoclast numbers in the lumbar vertebrae, although the *Sema3a* level in bone was significantly decreased and osteoblastic differentiation from calvarial cells derived from *Col1a1-Cre*$^{+}$ *Sema3a*$^{flox/flox}$ mice was impaired in vitro (Fukuda et al. 2013). The *Sp7* (encoding Osterix)-*Cre*$^{+}$ *Sema3a*$^{flox/flox}$ mice were shown to have a normal bone volume, at least in the lumbar vertebra. These results suggest that the mechanism by which Sema3A controls bone homeostasis is more complex than originally hypothesized, and Sema3A expressed in cells other than osteoblasts might be involved.

The neuron-specific *Sema3a* knockout mice generated by crossing *Nes* (encoding Nestin)-*Cre*$^{+}$ *Sema3a*$^{flox/flox}$ and *Syn1* (encoding synapsin I)-*Cre*$^{+}$ *Sema3a*$^{flox/flox}$ mice developed a 25 % reduction in bone mass phenotype because of decreased osteoblastic bone formation without any change in osteoblast number. Sensory innervation into bone, but not sympathetic innervation, was significantly reduced in mice lacking *Sema3a* in the neurons. In comparison, sensory innervation into bone in mice lacking *Sema3a* in osteoblasts was normal. It was proposed that sensory innervation was involved in the regulation of bone mass accrual in mice during development (Fig. 8.2d).

There findings give rise to interesting questions: How do sensory nerves promote bone formation? Why does the loss of Sema3A, a chemorepulsive factor, selectively affect sensory innervation into bone? Why is sympathetic innervation into bone unaffected in *Sema3a*$^{-/-}$ mice, although sympathetic nerves normally express Sema3A receptors and sympathetic innervation into other organs, become impaired in *Sema3a*$^{-/-}$ mice? (Elefteriou 2013). In addition, recent reports showed that Sema3A is involved in the development of gonadotropin-releasing hormone neurons in the hypothalamus (Cariboni et al. 2011; Giacobini et al. 2014). Therefore, the effect on the production of pituitary hormones and sex steroids should be considered in both global and neuron-specific Sema3A knockout mice.

8.7 Other Semaphorins and Their Receptors in Bone Cell Regulation

Several other semaphorins and their receptors are involved in the regulation of bone homeostasis (Fig. 8.4). It is reported that the expression of Sema3B, a member of the class 3 semaphorins, was induced in osteoblastic cells by treatment with $1,25(OH)_2D_3$, a key regulator of bone and mineral homeostasis (Sutton et al. 2008). Osteoclast differentiation supported by osteoblastic cells derived from *Sema3b* transgenic mice under the control of *Col1a1* promoter was significantly increased compared with a culture with osteoblastic cells derived from wild-type (WT) mice. Therefore, osteoblast-derived Sema3B may promote osteoclast differentiation. These transgenic mice display an osteopenic phenotype as a result of increased osteoclast differentiation along with normal osteoblastic parameters. However, a detailed analysis of $Sema3b^{-/-}$ mice is required to further determine the role of Sema3B in vivo.

The mRNA and protein expression of Nrp2, which is another family member of the neuropilins and a receptor for the class 3 semaphorins except for Sema3A, Sema3D, and Sema3E, were significantly induced during RANKL-induced osteoclast differentiation under the control of NF-κB (Verlinden et al. 2013). Nrp2 expression in osteoblasts and osteoclasts, but not in growth plate chondrocytes or osteocytes, was confirmed by immunofluorescence of the murine long bones. $Nrp2^{-/-}$ mice exhibited a mild low bone mass phenotype accompanied by an increased osteoclast number and a decreased osteoblast number, without any significant changes in the parameters of the function of osteoclasts and osteoblasts. Sema3B, one of the ligands of Nrp2, is a positive regulator of osteoclast differentiation, as previously stated, and the addition of recombinant Sema3C or Sema3F had no effect on osteoclast differentiation. Therefore, other class 3 semaphorins or the vascular endothelial growth factor (VEGF) family of ligands may have an influence on osteoclast and osteoblast differentiation via Nrp2.

Sema3E is expressed in osteoblasts and its receptor, Plexin-D1, is expressed in both osteoblasts and osteoclasts (Hughes et al. 2012). RANKL-induced osteoclast differentiation was shown to be decreased by treatment with the recombinant Sema3E protein. It was also shown that osteoblast migration, but not differentiation, was inhibited by Sema3E in a wound-healing assay. Thus, Sema3E derived from osteoblasts may have a role in the regulation of bone homeostasis through an effect exerted on osteoblast migration in an autocrine/paracrine manner and the inhibition of osteoclast differentiation in a paracrine manner, although the in vivo importance of Sema3E should be thoroughly investigated in the future. It was also reported that the class 3 semaphorins, except for Sema3F, are expressed in osteoblasts and that their expression is differentially regulated by differentiation and diverse signaling pathways, including $1,25(OH)_2D_3$ and canonical Wnt/β-catenin signaling.

Sema7A, a glycosyl phosphatidyl inositol-anchored semaphorin that is a ligand for Plexin-C1 and integrins, is expressed in osteoblasts and osteoclasts (Delorme et al. 2005). In contrast to Sema3E, Sema7A promotes osteoblast migration through integrin β1 and osteoclast differentiation.

Collectively, these reports suggest that the semaphorins are pivotal in the regulation of bone homeostasis through several distinct mechanisms (Fig. 8.4).

8.8 Potential for Therapeutic Targeting of Semaphorin

Bisphosphonates are currently the drugs most widely used for the treatment of osteoporosis. However, the prolonged use of antiresorptive agents often leads to the suppression of bone formation coupled with the target of inhibited bone resorption, resulting in the accumulation of low-quality bone (Lewiecki 2011; Kawai et al. 2011). Consequently, there is a strong need for anabolic therapies that positively increase bone mass by stimulating new bone formation instead of antiresorptive treatments, but such agents have been essentially unavailable except for parathyroid hormone or an anti-sclerotin antibody. Therefore, it would be desirable to develop new therapeutic approaches that effectively increase bone mass independently of a coupling mechanism.

To test whether the inhibition of Sema4D signaling would be a useful approach to the treatment of osteoporosis, the effect of anti-Sema4D antibody administration on bone loss in ovariectomized mice, a model of postmenopausal osteoporosis, was examined (Negishi-Koga et al. 2011). The administration of anti-Sema4D antibody prevented bone loss after ovariectomy through the activation of bone formation without affecting osteoclastic bone resorption. Importantly, the anti-Sema4D antibody stimulated bone formation and increased bone mass, even after the osteoporotic condition had developed in ovariectomized mice. Thus, the inhibition of the Sema4D–Plexin-B1 pathway is a potentially valuable therapeutic strategy for the treatment of osteopenic diseases.

Sema3A functions as a potent osteoprotective factor by decreasing bone resorption and increasing bone formation. To investigate the in vivo effect of recombinant Sema3A administration on bone metabolism, wild-type mice were injected with recombinant Sema3A once a week (Hayashi et al. 2012). The trabecular bone volume and osteoblastic parameters were significantly increased and osteoclastic parameters were decreased, indicating a combined bone-increasing effect of recombinant Sema3A through both the stimulation of osteoblastic bone formation and the inhibition of osteoclastic bone resorption. The therapeutic potential of Sema3A treatment was further investigated in a bone regeneration model of mouse cortical bone defects and a mouse model of postmenopausal osteoporosis. Sema3A treatment promoted the regeneration of cortical bone after drill-hole injury and prevented bone loss after ovariectomy, accompanied by significant increases in osteoblast number and bone formation and decreases in osteoclast number and bone resorption. Remarkably, the action of Sema3A as an anti-resorptive agent is not

accompanied by reduction in bone formation. Therefore, the activity of Sema3A, which uncouples the process of bone remodeling, is exceptional in that almost all the drugs that inhibit bone degradation also decrease bone formation.

Other semaphorin family members may also be therapeutic targets in bone diseases, although more detailed analyses are required to develop such strategies.

8.9 Concluding Remarks

Remarkable progress has been made in our understanding of the molecular basis underlying cell–cell communication among bone cells. Since the function of Plexin-A1 in bone metabolism was reported, the semaphorin–plexin system has been shown to have various important roles in the regulation of bone metabolism and is emerging as a target for future bone and joint disease therapeutics. Other axon-guidance molecules, including ephrins, are reported to be important in the regulation of bone metabolism, raising the question of why a number of axon-guidance molecules play key roles in bone. Further studies are required to establish safe, efficient, and (hopefully) groundbreaking therapies for bone diseases through a rebalancing of bone remodeling by modulating semaphorin–plexin signaling.

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Chapter 9
Semaphorin in the Heart

Masaki Ieda and Keiichi Fukuda

Abstract Semaphorin was cloned as a potent neural chemorepellent and is known as a directional guidance molecule for nerve fibers. However, recent studies demonstrated that semaphorin family members have important roles in cardiovascular development and diseases. The cardiovascular system consists of many types of cells, including neural cells, and is extensively innervated and tightly regulated by the autonomic nervous system. To maintain cardiac function properly, innervation density is strictly determined by the balance between neural chemoattractant and chemorepellent. Nerve growth factor, a potent chemoattractant, is abundantly synthesized from cardiomyocytes, but the chemorepellent for cardiac nerves was not determined until recently. We found that Sema3a is strongly expressed in the trabecular layer in early-stage embryos, but is reduced after birth, forming an epicardial-to-endocardial transmural sympathetic innervation patterning. Both Sema3a-deficient and -overexpressed mice showed sudden cardiac death and lethal arrhythmias from disruption of proper innervation patterning. More recently, Nakano et al. reported that a nonsynonymous polymorphism in Sema3a DNA is a risk factor for human cardiac arrest with ventricular fibrillation. Sympathetic innervation patterning was disrupted in these patients, suggesting proper expression of Sema3a and innervation patterning are critical to maintain arrhythmia-free hearts. Sema3c is expressed in the cardiac outflow tract during development and is important in cardiovascular patterning. Gata6 regulates Sema3c expression, and disruption of the Gata6/Sema3c pathway leads to congenital heart disease in mouse and human. This review focuses on the recent progress of our understanding of heart development and disease, regulated by semaphorin family members, in mouse and human.

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9.1 Introduction

The heart consists of many types of cells, including cardiomyocytes, vascular cells, neural cells, and cardiac fibroblasts. Heart function is tightly regulated by the interactions between cardiomyocytes and other type of cells through secreting molecules. Understanding the cell–cell interactions and their molecular mechanisms during heart development might provide new therapeutic and preventive approaches for heart disease.

The relationship between autonomic nerve activity and arrhythmias is well recognized, but the molecular mechanisms determining the innervation densities and the consequence of its disruption were unclear. Compared with other organs, the heart is extensively innervated via the autonomic nervous system, which comprises the sympathetic and parasympathetic nerves. The sympathetic nervous system produces norepinephrine, and increases heart rate, conduction velocity, and myocardial contraction and relaxation. It is well known that sympathetic innervation density, which is abundant in the subepicardium and the central conduction system, is strictly determined within the heart (Ito and Zipes 1994; Crick et al. 1994, 1999a, b). The regional difference in sympathetic innervation influences specific cardiac functions to a different extent, effectively controlling heart rate and myocardial contraction and relaxation. Unbalanced sympathetic innervation might trigger lethal arrhythmia through ion channel modulation in cardiomyocytes (Cao et al. 2000; Dae et al. 1997; Qu and Robinson 2004). *Sema3a*, a class 3 secreted semaphorin, was cloned as a potent neural chemorepellent and a directional guidance molecule for nerve fibers. We found that Sema3a is expressed in cardiac tissues and determines sympathetic innervation patterning in the developing heart. Disruption of Sema3a expression in mouse hearts lead to fetal arrhythmias and sudden cardiac death. More recently, Nakano et al. reported that a nonsynonymous polymorphism in Sema3a is a risk factor for human unexplained cardiac arrest with ventricular fibrillation. They showed that sympathetic innervation patterning was disrupted in these patients, suggesting proper function of Sema3a is critical for arrhythmia-free hearts in humans.

Congenital heart diseases (CHD) occur in nearly 1 % of all live births and are the major cause of infant mortality and morbidity. Cardiac outflow tract (OFT) defects are estimated to account for approximately 30 % of CHD, but the genetic etiology of most OFT defects remains unknown. Increasing evidence has demonstrated that semaphorin 3C, a member of the semaphorin family of secreted factors, is expressed in the cardiovascular system during development. The semaphorin 3C knockout (KO) mouse revealed impaired migration of cardiac neural crest cells to the developing outflow tract, leading to OFT defects and perinatal death. Gata6 directly

regulates semaphorin 3C expression during development, and neural crest-specific Gata6 mutant mice revealed OFT defects, recapitulating the phenotype of the semaphorin 3C KO mouse. Kodo et al. found two mutations in GATA6 in their series of 21 Japanese patients with persistent truncus arteriosus (PTA), a major disease in OFT defects. They demonstrated that both mutations disrupted the transcriptional activity of the GATA6 protein, leading to failure of transactivation of SEMA3C and PLXNA2. The results in human OFT defects are consistent with those observed in Gata6 and Sema3c mutant mice, demonstrating that the GATA6/Sema3c pathway is conserved among species and is critical for development of the cardiovascular system.

In this chapter, we review the function of semaphorin family members, demonstrated as important for cardiovascular development and disease in both mouse and human.

9.2 Cardiac Sympathetic Innervation and Nerve Growth Factor

The heart is innervated by sympathetic, parasympathetic, and sensory nerves derived from neural crest cells. Trunk neural crest cells migrate and form sympathetic ganglia by midgestation, subsequently proliferating and differentiating into mature neurons. The cardiac sympathetic nerves extend from the sympathetic neurons in stellate ganglia, which are located bilateral to the thoracic vertebrae. Sympathetic nerve fibers project from the base of the heart into the myocardium, and are located predominantly in the subepicardium of the ventricle (Fig. 9.1). The central conduction system, which includes the sinoatrial node, atrioventricular node, and His bundle, is abundantly innervated compared with the working myocardium. The regional difference in cardiac sympathetic innervation is highly conserved among mammals.

In general, the growth cone behavior of nerves is modulated by coincident signaling between neural chemoattractant and chemorepellent synthesized in the innervated tissue. Nerve growth factor (NGF), a potent neural chemoattractant, is a prototypic member of the neurotrophin family, members of which are critical for the differentiation, survival, and synaptic activity of the peripheral sympathetic and sensory nervous systems (Snider 1994; Lockhart et al. 1997; Brennan et al. 1999). We and others have reported that NGF expression in cardiomyocytes is critical as a sympathetic chemoattractant in normal and diseased hearts. Among several cardiac hypertrophic factors, we found that ET-1 is a key regulator of NGF expression in cardiomyocytes, and that the ET-1/NGF pathway is critical for sympathetic innervation in developing and right ventricular hypertrophy hearts (Ieda et al. 2004; Kimura et al. 2007). Given that ET-1 is strongly induced in myocardial infarction, the ET-1/NGF pathway might also be involved in NGF upregulation and nerve regeneration after myocardial infarction.

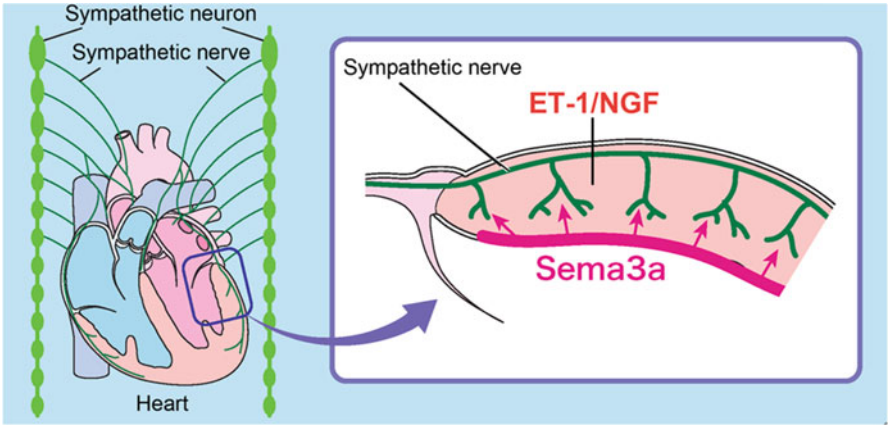

Fig. 9.1 Inverse expression pattern of Sema3a and sympathetic innervation in developing hearts The cardiac sympathetic nerves (*green*) extend from the sympathetic neurons in stellate ganglia, which are located bilateral to the thoracic vertebra (*left*). Cardiac sympathetic innervation shows an epicardial-to-endocardial transmural gradient (*right*). This patterning is established by the balance between ET-1/NGF and Sema3a expression in the heart. Note that NGF is abundantly expressed in the working myocardium, whereas Sema3a is specifically expressed in the subendocardium (Ieda et al. 2007, 2008)

9.3 Sema3a Is Critical for Cardiac Sympathetic Innervation Patterning

As already discussed, NGF, a neural chemoattractant, has critical functions in cardiac nerve development. In contrast, the neural chemorepellent that induces growth cone collapse and repels nerve axons was not identified in the heart. *Sema3a*, a class 3 secreted semaphorin, was cloned as a potent neural chemorepellent and a directional guidance molecule for nerve fibers (Puschel et al. 1995; Tanelian et al. 1997; Kawasaki et al. 2002). However, it was unknown whether cardiomyocytes produce Sema3a, and if so, whether this protein affects sympathetic neural patterning and cardiac performance.

We analyzed the time course and distribution of cardiac sympathetic innervation in developing mice ventricles (Ieda et al. 2007). TH-immunopositive sympathetic nerve endings appeared at embryonic day (E) 15 in the epicardial surface, and gradually increased in the myocardium after postnatal day (P) 7 and 42. In the ventricular myocardium, sympathetic nerves were richer in the subepicardium than the subendocardium, showing an epicardial-to-endocardial gradient (Crick et al. 1994; Ito and Zipes 1994; Chow et al. 1993; Hansson et al. 1998). To identify *Sema3a* expression and its relationship with innervation patterning in the heart, we analyzed the heterozygous *Sema3a* knocked-in *lacZ* mouse ($Sema3a^{lacZ/+}$). At E12, *lacZ* expression was strongly detected in the heart, especially in the trabecular

component of both ventricles. In E15 hearts, *lacZ* expression was observed at the subendocardium but not at the subepicardium in the atria and ventricles. At P1 and P42, *lacZ* expression was reduced in places and highlighted the Purkinje fiber network along the ventricular free wall (Tago et al. 1986; Kupershmidt et al. 1999). Quantitative RT-PCR of *Sema3a* in developing hearts also revealed *Sema3a* was detected from E12, and then decreased linearly in contrast to the sympathetic innervation. These results indicated that Sema3a has the opposite time-course and distribution of expression from sympathetic innervation in developing hearts, suggesting Sema3a might regulate cardiac innervation negatively (Fig. 9.1) (Ieda et al. 2008).

To investigate whether Sema3a is critical for cardiac sympathetic nerve development, we analyzed *Sema3a*-deficient mice ($Sema3a^{-/-}$) (Behar et al. 1996; Taniguchi et al. 1997). The wild-type (WT) hearts showed a clear epicardial-to-endocardial gradient of sympathetic innervation; in contrast, sympathetic nerve density was reduced in the subepicardium but increased in the subendocardium in $Sema3a^{-/-}$ mice, resulting in disruption of the innervation gradient in $Sema3a^{-/-}$ ventricles. The $Sema3a^{-/-}$ mice also exhibited malformation of stellate ganglia that extended sympathetic nerves to the heart. To address whether the abnormal sympathetic innervation patterning in $Sema3a^{-/-}$ hearts was a secondary effect of the stellate ganglia malformation, we generated transgenic mice overexpressing *Sema3a* specifically in the heart (*SemaTG*) (Gulick et al. 1991). *SemaTG* mice were associated with reduced sympathetic innervation and attenuation of the epicardial-to-endocardial innervation gradient. These results indicated that cardiomyocyte-derived Sema3a has a critical role in cardiac sympathetic innervation by inhibiting neural growth. As cardiomyocyte-derived NGF acts as a chemoattractant, it might be the balance between NGF and Sema3a synthesized in the heart that determines cardiac sympathetic innervation patterning.

9.4 Sema3a Maintains Arrhythmia-Free Hearts Through Sympathetic Innervation Patterning in Mouse

Most $Sema3a^{-/-}$ died within the first postnatal week; only 20 % remained viable until weaning (Taniguchi et al. 1997; Behar et al. 1996). To identify the cause of death and the effects of abnormal sympathetic neural distribution in $Sema3a^{-/-}$ hearts, we performed telemetric electrocardiography and heart rate variability analysis (Shusterman et al. 2002; Saba et al. 2003). In addition to multiple premature ventricular contractions, $Sema3a^{-/-}$ mice developed sinus bradycardia and abrupt sinus arrest as the result of sympathetic neural dysfunction.

The *SemaTG* mice died suddenly without any symptoms at 10 months of age. Sustained ventricular tachyarrhythmia was induced only in *SemaTG* mice but not in WT mice after epinephrine administration, and programmed electrical

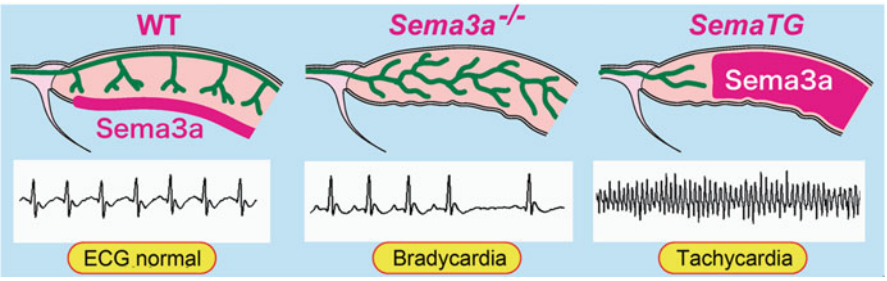

Fig. 9.2 Regulation of cardiac innervation patterning and arrhythmias. Sema3a-mediated proper sympathetic innervation patterning is critical to maintain arrhythmia-free hearts. *Sema3a*$^{-/-}$ mice exhibited sinus bradycardia, and *SemaTG* mice were highly susceptible to ventricular tachyarrhythmias (Ieda et al. 2007, 2008)

stimulation also revealed that *SemaTG* mice were highly susceptible to ventricular tachyarrhythmia (Wehrens et al. 2004; Kannankeril et al. 2006). The β-adrenergic receptor density was upregulated and the cAMP response after catecholamine injection was exaggerated in *SemaTG* ventricles. Action potential duration was significantly prolonged in hypoinnervated *SemaTG* ventricles, presumably via ion channel modulation. These results suggested that the higher susceptibility of *SemaTG* mice to ventricular arrhythmia was at least in part caused by catecholamine supersensitivity and action potential duration prolongation, either or both of which might augment triggered activity in cardiomyocytes (Kuo et al. 2001; Opthof et al. 1991; Priori and Corr 1990; Brunet et al. 2004; Costantini et al. 2005). Thus, Sema3a-mediated proper sympathetic innervation patterning is critical to maintain arrhythmia-free hearts (Fig. 9.2) (Ieda et al. 2008).

Sympathetic nerves modulate the function of ion channels and trigger various arrhythmias in diseased hearts (Qu and Robinson 2004; Dae et al. 1997). However, the relationship between sympathetic innervation and arrhythmogenicity in structurally normal hearts was unclear. *Sema3a*$^{-/-}$ mice exhibited sinus bradycardia, abrupt sinus slowing, and stellate ganglia defects. Consistent with our data, right stellectomy induced sinus bradycardia and sudden asystolic death in dogs (Sosunov et al. 2001). Stramba-Badiale et al. also reported that a developmental abnormality in cardiac innervation might be involved role in the genesis of some cases of sudden infant death syndrome (Stramba-Badiale et al. 1992). The *SemaTG* hearts were also highly susceptible to ventricular arrhythmias, although without contractile dysfunction or structural defects. Given that catecholamine augments systolic function, it is surprising that *SemaTG* mice showed normal cardiac function. However, patients who underwent heart transplantation and had denervated hearts did not show heart failure, whereas about 10 % of the patients developed sudden cardiac death, presumably because of arrhythmias (Chantranuwat et al. 2004). These results suggested that regulation of cardiac nerves should be a new paradigm in the management of sudden cardiac death.

9.5 The Crosstalk Between Neurotrophin and Semaphorin Signaling in Nerve Development

Because cardiomyocyte-derived NGF acts as a chemoattractant, it is possible that the balance between NGF and Sema3a synthesized in the heart determines cardiac sympathetic innervation patterning. The growth cone behavior of somatic sensory axons is modulated by coincident signaling between NGF and Sema3a (Tang et al. 2004; Kitsukawa et al. 1997), both of which are expressed within the developing spinal cord and influence pathway guidance of sensory axons during development. Sema3a is specifically expressed in the ventral half of the spinal cord and mediates NGF-responsive sensory axons to terminate at the dorsal part of the spinal cord (Puschel et al. 1995; Wright et al. 1995). In addition, targeted inactivation of *Sema3a* disrupts neural patterning and projections in the spinal cord, further highlighting the importance of Sema3a signaling for the directional guidance of nerve fibers (Taniguchi et al. 1997; Behar et al. 1996).

However, the interaction of NGF and Sema3A signaling is further complicated as the low-affinity NGF receptor p75 can partner with the Sema3A receptors neuropilin-1 and plexin A4 to attenuate their combined ability to repel growing axons (Ben-Zvi et al. 2007). Lorentz et al. found that the subendocardium innervation of adult p75 knockout ventricles was disrupted, with the left ventricle essentially devoid of sympathetic nerve fibers, whereas innervation density of the subepicardium was normal (Lorentz et al. 2010). This neural patterning defect is similar to that seen in mice overexpressing Sema3A, such that the sympathetic axons lacking p75 are highly sensitive to Sema3A-mediated inhibition of neurite outgrowth. The heterogeneous innervation was also associated with altered cardiac β1-adrenergic receptor expression and sensitivity, and a significant increase in spontaneous ventricular arrhythmias, which were also observed in Sema3A-overexpressing mice. These results suggested that as sympathetic neurons are innervating the heart, p75, a receptor for neurotrophins, acts to blunt the repulsive effects of Sema3A from the subendocardium, thereby allowing axonal arborization and orchestrating a highly organized epicardial-to-endocardial innervation gradient in the ventricle (Carter et al. 2010).

9.6 A Nonsynonymous Polymorphism in Sema3a Is a Risk Factor for Human Unexplained Cardiac Arrest with Ventricular Fibrillation

Unexplained cardiac arrest (UCA) with documented ventricular fibrillation is defined as spontaneous ventricular fibrillation (VF) that is not associated with known structural or electrical heart diseases and is one of the major causes of sudden cardiac death. The relationship between abnormal autonomic nerve activity and lethal ventricular arrhythmias is well known, but the molecular mechanism deter-

mining the innervation densities in patients with lethal arrhythmia was not clarified because of the high mortality rate and difficulty of diagnosis. Nakano et al. reported that UCA patients with spontaneous VF had a high incidence of a polymorphism in the semaphorin 3A gene (Nakano et al. 2013). A nonsynonymous polymorphism (I334V, rs138694505A>G) in exon 10 of the SEMA3A gene identified through resequencing was significantly associated with UCA (combined $P = 0.0004$, OR 3.08, 95% CI 1.67–5.7). Overall, 15.7 % of UCA patients carried the risk genotype G, whereas only 5.6 % of controls did so. In patients with SEMA3AI334V, VF predominantly occurred at rest during the night. They showed sinus bradycardia and sinus dysfunction, and their RR intervals on electrocardiography tended to be longer than those in patients without SEMA3AI334V ($P = 0.039$), which is consistent with the Sema3A KO mouse phenotype (Ieda et al. 2007). Immunofluorescence staining of cardiac biopsy specimens revealed that sympathetic nerves were absent in the subendocardial layer in normal hearts and in patients without SEMA3AI334V. In patients with SEMA3AI334V, in contrast, sympathetic nerves were distributed in the subendocardial layer. This finding was consistently observed in patients with SEMA3AI334V ($n = 4$) but not in those without SEMA3AI334V ($n = 8$), suggesting disruption of the epicardial-to-endocardial transmural sympathetic innervation patterning in the heart of UCA patients with SEMA3AI334V. On the other hand, NGF, a neural attractant factor, was similarly expressed in the subendocardial layer in patients with and without SEMA3AI334V. The dorsal root ganglion (DRG) repulsion assay revealed that the axon-repelling and axon-collapsing activities of mutant SEMA3AI334V genes were significantly weaker than those of wild-type SEMA3A genes. These results indicate that SEMA3AI334V disrupts the SEMA3A function of inhibiting neural growth and impaired appropriate innervation patterning, and that SEMA3AI334V is a risk factor for human UCA and contributes to the pathogenesis of UCA.

9.7 The Gata6/Sema3c Pathway Is Critical for Cardiovascular Morphogenesis in the Mouse

The cardiac neural crest plays a critical role in patterning the vertebrate vascular system (Creazzo et al. 1998). During early embryogenesis (E8.0–9.0 in the mouse), the cardiac neural crest arises from the dorsal neural tube and migrates ventrally to populate the aortic arch arteries and cardiac outflow tract. These neural crest-derived cells respond to poorly characterized developmental cues to differentiate into vascular smooth muscle cells (VSMCs) and contribute to vascular formation during development. Semaphorin 3C is a member of the semaphorin family of secreted factors that are known to be important in regulating neuronal as well as vascular patterning. During murine embryonic development, semaphorin 3C is expressed in the mesenchyme surrounding the developing aortic arch arteries and in neural crest-derived SMCs. These cells also express neuropilin 1 and plexin-

A2, the components of a potential heterodimeric semaphorin 3C receptor. Targeted inactivation of semaphorin 3C causes impaired migration of neural crest cells to the developing cardiac outflow tract, resulting in interruption of the aortic arch and persistent truncus arteriosus (Feiner et al. 2001; Brown et al. 2001). It has been proposed that semaphorin 3C acts as a guidance molecule, regulating migration of neural crest cells that express semaphorin receptors such as plexin-A2, but the molecular mechanisms regulating Sema3c expression and disruption of this pathway in human hearts were not clear until recently. Toyofuku et al. reported that the coordination of repulsive cues provided by Sema6A/Sema6B through plexin-A2 paired with the attractive cue by Sema3C through plexin-D1 is required for the precise navigation of migrating cardiac neural crest cells to the cardiac outflow tract (Toyofuku et al. 2008).

Lepore et al. demonstrated that GATA-6 regulates semaphorin 3C expression in the neural crest cells, and this pathway regulates morphogenetic patterning of the aortic arch and cardiac outflow tract during development in the mouse (Lepore et al. 2006). Conditional inactivation of GATA-6 in vascular smooth muscle cells resulted in perinatal mortality from a spectrum of cardiovascular defects, including interrupted aortic arch and persistent truncus arteriosus. Inactivation of GATA-6 in neural crest cells recapitulated these abnormalities, demonstrating a cell-autonomous requirement for GATA-6 in neural crest-derived SMCs. Importantly, deletion of GATA-6 in neural crest cells resulted in an absence of detectable semaphorin 3C- or plexin-A2-expressing cardiac neural crest cells within the conotruncal endocardial cushions that contribute to aorticopulmonary septation. These results are consistent with the phenotypes observed in semaphorin 3C-deficient mice (Feiner et al. 2001; Brown et al. 2001), the impairment of neural crest cell migration into the cardiac outflow tract.

9.8 Gata6 Mutations Cause Human Congenital Heart Disease by Disrupting Sema3c/Plexin-A2 Signaling

Congenital heart diseases (CHD) occur in nearly 1 % of all live births and are the major cause of infant mortality and morbidity (Hoffman and Kaplan 2002). Although an improved understanding of the genetic causes of CHD would provide insight into the underlying pathobiology, the genetic etiology of most CHD remains unknown. Cardiac outflow tract (OFT) defects are estimated to account for approximately 30 % of CHD and usually require an intervention during the first year of life. Kodo et al. reported the identification and characterization of two mutations of GATA6 DNA (9.5 %) in their series of 21 Japanese patients with persistent truncus arteriosus (PTA), a form of OFT defect (Kodo et al. 2009). Both mutations disrupted the transcriptional activity of the GATA6 protein on downstream target genes involved in the development of the outflow tract. They also confirmed that the expression of SEMA3C and PLXNA2 in the developing OFT was regulated directly

through the consensus GATA binding sites conserved between human and mouse, in vitro and in vivo. Mutant GATA6 proteins failed to transactivate SEMA3C and PLXNA2, and mutation of the GATA sites on enhancer elements of Sema3c and Plxna2 abolished their activity, suggesting that mutations of GATA6 cause specific forms of human CHDs. These results are consistent with the mouse phenotype observed in semaphorin 3C-deficient mice and conditional inactivation of GATA-6 in neural crest cells and VSMCs, as previously discussed.

9.9 Conclusions

The heart consists of many types of cells, including cardiomyocytes, vascular cells, and neural cells, which tightly regulate heart development and cardiac function. Semaphorin family members are critical in the interactions between cardiomyocytes and other types of cells (Fig. 9.3). Cardiac nerves are highly plastic, and innervation patterning is strictly controlled by the balance between NGF and Sema3a synthesized in the heart. Sema3a inhibits neural growth and establishes proper innervation patterning in the heart. The disruption of sympathetic innervation patterning by Sema3a misexpression may lead to sudden cardiac death and fetal arrhythmias in both mouse and human (Ieda et al. 2007; Nakano et al. 2013). Cardiac outflow tract formation is organized by the interaction of migrating cardiac neural crest cells and surrounding tissues. The GATA6/Sema3c signaling pathway is conserved among mammals, and its disruption results in congenital heart diseases in humans.

		Sema3a	Ref.	Gata6/Sema3c	Ref.
Mouse	Function	Sympathetic innervation patterning	Ieda et al Lorentz et al	Cardiac outflow tract development	Feiner et al. Brown et al.
	Mutant mice	Disruption of sympathetic innervation Fetal arrhythmias and sudden death	Ieda et al	Cardiac outflow tract defects (interrupted aortic arch and persistent truncus arteriosus)	Lepore et al.
Human		A polymorphism in Sema3A Cardiac arrest and ventricular fibrillation	Nakano et al.	Two mutations in Gata6 Persistent truncus arteriosus	Kodo et al.

Fig. 9.3 Summary of cardiac defects mediated by Sema3a and Sema3c signaling. Sema3a is important for cardiac sympathetic innervation and maintains arrhythmia-free hearts, and Gata6/sema3c is critical for proper cardiac outflow tract formation (*Ref.* indicates references in the text)

Understanding of the divergent effects of semaphorin family members on cardiac innervation and cardiovascular patterning might thus represent a novel step toward potential therapies for heart diseases.

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Chapter 10
Semaphorins and Neurodegenerative Disorders

Tatsusada Okuno and Yuji Nakatsuji

Abstract Neurodegenerative disorders are characterized by progressive dysfunction or death and structural abnormalities of neurons. A number of diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) that result from neurodegenerative processes are included in this category. Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). However, accumulating evidence indicates that a neurodegenerative process participates in the pathogenesis, particularly in the progressive stage of the disease. Although various causes of neurodegenerative disorders, including genetic mutations, protein abnormalities, and inflammation, have been reported, changes in neural connectivity, loss of synaptic contacts and activity, and inflammatory reactions in glial cells are common features of these disorders. It has been suggested that aberrant semaphorin expression may result in altered neuronal connectivity or synaptic function and inflammation associated with a number of degenerative neuronal disorders. This role of semaphorins has been currently suggested in the pathogenesis of AD, ALS, and MS (Table 10.1).

Keywords Alzheimer's disease • Parkinson's disease • Amyotrophic lateral sclerosis • Multiple sclerosis • Experimental autoimmune encephalomyelitis

10.1 Semaphorins and Amyotrophic Lateral Sclerosis (ALS)

10.1.1 ALS

Amyotrophic lateral sclerosis (ALS) is a devastating disease characterized by progressive loss of motor neurons in the brain and spinal cord. Initial symptoms include weakness in the limbs and/or difficulties with speech and swallowing caused by weakness in the bulbar region (Rowland 1998). Patients eventually become paralyzed and die of respiratory failure if they are not maintained on a ventilator approximately 3 years after the onset of symptoms (Rowland and Shneider 2001).

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Table 10.1 The roles of semaphorins and their receptors in neurodegenerative diseases

Semaphorins	Binding partners	Related disease	Expression	Functions
Sema3A	NP-1/Plexin-As	ALS	TSC in NMJ	Repulsion of motor axons in NMJ
		AD	Neurons in CA1 and subiculum	Phosphorylation of CRMP2
		MS	Neurons, astrocytes, microglia	Inhibition of OPC recruitment toward the lesions
		EAE	EC, T cells	Migration of immune cells
Sema3F	NP-2/Plexin-As	MS	Neurons, astrocytes, microglia	Recruitment of OPCs toward the lesions
Sema4A	Plexin-Bs, TIM2	MS/EAE	DC, Th1	Th1/Th17 differentiation
Sema4D	Plexin-B1, CD72	EAE/MS	T cells	Activation of DC and microglia
Sema5A	Plexin-B3	PD	Unknown	Unknown
Sema6D	Plexin-A1	EAE	T cells	Activation of DC
Sema7A	α1β1 integrin	EAE	T cells	Activation of macrophages

NP neuropilin, *ALS* amyotrophic lateral sclerosis, *AD* Alzheimer's disease, *MS* multiple sclerosis, *EAE* experimental autoimmune encephalomyelitis, *PD* Parkinson's disease, *TSC* terminal Schwann cell, *NMJ* neuromuscular junction, *EC* endothelial cell, *DC* dendritic cell, *OPC* oligodendrocyte precursor cell

Approximately 5 % to 10 % of patients with ALS inherit the disease, which is described as familial ALS (FALS) (Beghi et al. 2006; Mitchell and Borasio 2007). Currently, riluzole, a putative glutamate receptor antagonist, is the only available drug approved for the treatment of ALS, yet its efficacy is limited (Radunović et al. 2007). Sporadic ALS (SALS) is considered as a complex multifactorial disease with an interaction of genetic and environmental factors affecting disease susceptibility and clinical expression (Siddique and Siddique 2008). Risk factors include age, sex, smoking, mechanical and electrical trauma, professional and environmental exposure to metals and herbicides, and heavy physical activity (Horner et al. 2003; Beghi et al. 2006; Sutedja et al. 2007). In addition, several pathways have been implicated in the pathogenesis of SALS, such as glutamate-mediated excitotoxicity, mitochondrial dysfunction, neuroinflammation, oxidative stress, protein aggregation, aberrant axonal transport, and abnormality in RNA-binding proteins (Wang et al. 2004; Shaw 2005; Pasinelli and Brown 2006; Van Deerlin et al. 2008; Yokoseki et al. 2008). Up to 20 % to 25 % of FALS patients exhibit mutations in the Cu/Zn superoxide dismutase-1 (SOD1) gene (Rosen et al. 1993; Cudkowicz et al. 1997). At present, more than 120 mutations in SOD1 have been reported in FALS patients. Transgenic mice overexpressing the mutant human SOD1 gene develop progressive motor neuron degeneration that resembles ALS; therefore, these mice serve as an appropriate animal model for the disease (Gurney et al. 1994). By utilizing this model, the pathogenesis of ALS has been analyzed.

10.1.2 ALS and the Neuromuscular Junction (NMJ)

Interestingly, recent data have suggested that pathological changes in the lower motor axons and nerve terminals precede motor neuron loss and onset of clinical symptoms (Frey et al. 2000; Fischer et al. 2004; Pun et al. 2006). These findings suggest that the pathological change may be initiated distally at the nerve terminal or NMJ and spread toward the cell body. In addition, aberrant expression of axon-guidance proteins has recently been thought to contribute to the pathological changes in motor neuron connectivity in ALS. Axon guidance proteins are important in regulating motor axon pathfinding during development, and they also influence axonal transport and synaptic function (Pasterkamp and Giger 2009). The expression of several different axon guidance molecules is changed in ALS patients and model mice (Lesnick et al. 2007, 2008; Pradat et al. 2007). Consistently, single-nucleotide polymorphisms (SNPs) in semaphorins and other genes encoding axon-guidance molecules in ALS patients are reported to be of diagnostic value for disease susceptibility, onset, and severity (Lesnick et al. 2007, 2008; Pradat et al. 2007). Therefore, semaphorins may be genetic risk factors for ALS. Together, these data suggest that abnormal axon-guidance protein expression or function may contribute to the pathological changes in motor axons and nerve terminals associated with ALS.

10.1.3 ALS and Sema3A

Progressive loss of motor neurons and subsequent muscle innervation are pathological hallmarks in the mSOD1 transgenic mouse model for ALS (Gurney et al. 1994; Wong et al. 1995). In this process, terminal Schwann cells (TSC) in NMJs come to express Sema3A NMJs in the muscles of G93A-hSOD1 mice (transgenic mice overexpressing the familial ALS-associated G93A SOD1 mutation harboring a single glycine-to-alanine substitution at codon 93) (De Winter et al. 2006). The expression of Sema3A in NMJ is not ubiquitous throughout the entire muscle sections; it is mainly located in the lateral regions of the gastrocnemius muscle. Intriguingly, most Sema3A-expressing TSCs are located on type IIb/x fibers in the gastrocnemius muscles of G93A-hSOD1 mice. When the number of Sema3A-positive TSC clusters per muscle fiber subtype was quantified, Sema3A expression was increased but restricted to IIb and IIx muscle fibers throughout the course of G93A-hSOD1 mice. The number of Sema3A-positive endplates on type IIb and IIx muscle fibers was significantly higher just before and during the onset of the disease than in the more progressive and end stages. Type IIb and IIx motor units are fast-fatigable fiber types, and fast-fatigable neuromuscular synapses are susceptible to early loss in motor neuron diseases. Type IIb and IIx muscle fibers are the first muscle subtype that is lost in ALS because nerve sprouting after injury does not occur in these subtypes of muscle fibers (Pinter et al. 1995; Frey et al. 2000;

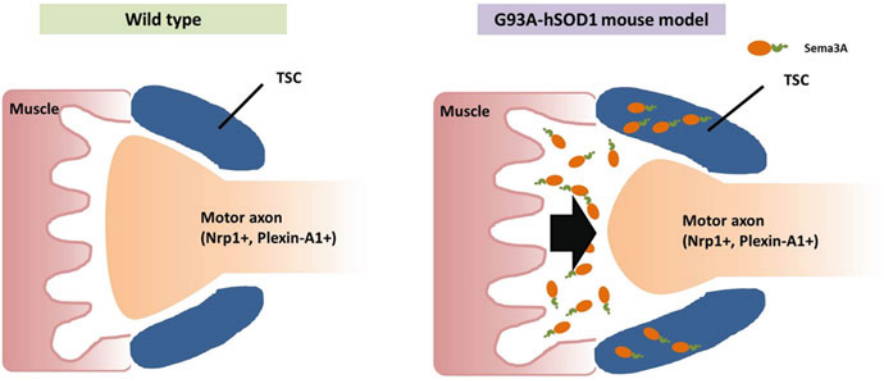

Fig. 10.1 Neuromuscular junction (*NMJ*) of ALS model mice (G93A-hSOD1 mice). Sema3A expression in terminal Schwann cells (*TSC*) is markedly increased. This increase is specifically limited to endplates on type IIb and IIx muscle fibers. Increased expression of Sema3A in TSCs may cause the dissociation or repulsion of motor axons at the NMJ, eventually resulting in axonal denervation and motor neuron degeneration

Pun et al. 2006). Therefore, it is plausible that increased expression of Sema3A in TSCs causes the dissociation or repulsion of motor axons at the NMJ, eventually resulting in axonal denervation and motor neuron degeneration (Fig. 10.1).

10.2 Semaphorins and Alzheimer's Disease (AD)

10.2.1 AD

Alzheimer's disease (AD) is the most common cause of senile dementia, affecting 35 million individuals worldwide. The most common initial manifestation is the disturbance of recent memory (Waldemar et al. 2007; Blennow et al. 2006). As the disease progresses, patients exhibit various symptoms, including deficits in language and executive function. Psychotic behavior, delusions, and hallucinations increase according to disease progression. Cholinesterase inhibitors have been approved to improve these symptoms; however, drug efficacy remains limited and is not sufficient to cure AD (Hansen et al. 2008). Accordingly, the brain function of AD patients is gradually lost, ultimately leading to death (Molsa et al. 1986, 1995).

Although the cause of AD remains incompletely understood, the pathological hallmarks of AD are amyloid plaques and neurofibrillary tangles (NFTs) in the brain (Tiraboschi et al. 2004; Wenk 2003). Amyloid plaques comprise dense, mostly insoluble deposits of beta-amyloid (Aβ), a fragment of amyloid precursor protein (APP), outside and around neurons (Hardy and Allsop 1991; Mudher and Lovestone 2002; Hsiao et al. 1996). NFTs are aggregates of the microtubule-associated

protein tau, which becomes hyperphosphorylated and accumulates inside the cells. Although Aβ and tau are the components in plaque and NFT, respectively, they have also been postulated to be the fundamental cause of AD. Mutations in genes that alter Aβ protein production, aggregation, or clearance, such as APP and presenilins, cause the early-onset forms of AD, suggesting that Aβ contributes to AD pathogenesis (Waring and Rosenberg 2008; Hsiao et al. 1996). In addition, Aβ at low doses inhibits activity-dependent synaptic transmission and is neurotoxic at higher doses. However, tau protein abnormalities supposedly initiate the disease cascade (Mudher and Lovestone 2002; Haass and Selkoe 2007). Hyperphosphorylated tau causes dysfunction of the neuronal network and neuronal death by causing disintegration of microtubules and collapse of the transport system (Goedert et al. 1991; Iqbal et al. 2005; Chun and Johnson 2007).

Presumably because of the neurotoxic effects of Aβ and hyperphosphorylated tau, there is loss of neurons and synapses in the cerebral cortex and certain subcortical regions in AD. Among various fields in the brain, the hippocampal formation is known as a selectively vulnerable subfield (Braak and Braak 1991; Price et al. 1991) in the early pathogenesis of AD. CA1 and the subiculum initially show neurodegenerative changes during the incipient phases of AD. The most vulnerable neurons of the hippocampus are the pyramidal cells of the CA1 field and subiculum, although neurons in the CA3 field are resistant to neurodegeneration. Furthermore, the dentate gyrus rarely exhibits any degenerative changes in AD. This differential vulnerability of neurons underlies the mechanisms leading to neuronal degeneration in AD.

10.2.2 AD and Sema3A

Evidence of the involvement of semaphorins in AD is provided by the fact that an isolated multiprotein complex from the brain tissue of AD patients contains the phosphorylated microtubule-associated protein (MAP) 1B, Sema3A, CRMP-2, plexin-A1, and plexin-A2 (Good et al. 2004). Interestingly, AD brain tissue includes a hyperphosphorylated form of CRMP-2 that shows increased phosphorylation on both Ser522 and Thr509 residues (Uchida et al. 2005). Phosphorylation of CRMP-2 at Ser522 and Thr509 by cyclin-dependent kinase-5 (Cdk-5) and glycogen synthase kinase-3 beta (GSK-3b), respectively, decreases its interaction with tubulin and is required for repulsion of neurites by Sema3A (Uchida et al. 2005; Yoshimura et al. 2005; Good et al. 2004). Because Sema3A is aberrantly produced and released in the subiculum, and then taken up and transported to CA1 in the early stage of AD (Good et al. 2004) (Fig. 10.2), Sema3A may contribute to the neurodegeneration of AD by inducing neural collapse or enhancing phosphorylation of CRMP2. Interestingly, Aβ, the most important component of senile plaques in the AD brain, decreases neurite length in vitro and regulates the phosphorylation of CRMP-2 through a RhoA GTPase-dependent mechanism (Petratos et al. 2008). Therefore, there may

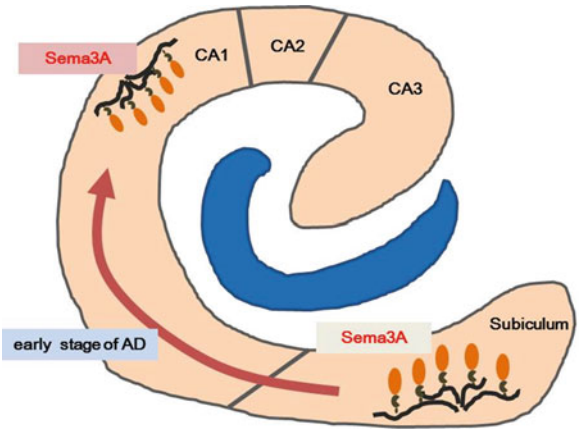

Fig. 10.2 Aberrant expression of Sema3A in AD hippocampal formation. In the early stage of Alzheimer's disease (AD), Sema3A is aberrantly produced and released in the subiculum, and then taken up and transported to CA1. As a result, Sema3A accumulates in the soma of CA1 neurons. Sema3A may contribute to the neurodegeneration of AD by inducing neural collapse or enhancing phosphorylation of CRMP2

be crosstalk between Sema3A and Aβ in the context of neural repulsion. In AD patients, Aβ aggregation induces hyperphosphorylation of CRMP-2, which may then lead to changes in Sema3A-induced modulation of microtubule dynamics (Fukata et al. 2002).

10.3 Semaphorins and Parkinson's Disease (PD)

10.3.1 PD

Parkinson's disease (PD) is a common neurodegenerative disorder, and its prevalence is approximately 160 per 100,000. PD is biochemically characterized by the degeneration of the nigrostriatal dopamine system, which results in a marked loss of striatal dopamine content, and is pathologically characterized by the loss of dopaminergic neurons in the substantia nigra and the presence of neuronal intracellular Lewy bodies. The clinical features of PD are manifested by resting tremors, rigidity, bradykinesia, and postural instability (Calne et al. 1992). Both environmental and genetic factors contribute to the development of PD, but the exact mechanism has not yet been identified. Mutations of several genes such as α-synuclein, parkin, LRRK2, and PINK1 have been identified as causes of familial PD (Polymeropoulos et al. 1997; Valente et al. 2001; Nichols et al. 2005).

10.3.2 PD and Sema5A

Semaphorin 5A is a transmembrane protein belonging to the semaphorin protein family. The extracellular domain of Sema5A contains seven thrombospondin (TSP)

type-1 repeats in addition to the sema domain (Adams et al. 1996). It is essential for the development of extra-embryonic tissues and the cardiovascular system, and it can elicit multiple differentiation, cell–cell signaling, and nervous system development through its functional receptor plexin-B3 (Oster et al. 2003; Pineda et al. 2005; Artigiani et al. 2004). In the nervous system, SEMA5A is expressed by oligodendrocytes and inhibits axonal growth (Goldberg et al. 2004).

Analysis of SNPs in semaphorins and other genes encoding axon-guidance molecules in PD patients was reported to be of diagnostic value for disease susceptibility, onset, and severity. These data suggest that mechanisms involved in axonal maintenance and repair can participate in the pathogenesis of PD. Therefore, semaphorins have been suggested to be associated with PD. In this context, one study recently showed that an SNP, rs7702187, within Sema5A was associated with the disease susceptibility of PD (Maraganore et al. 2005). Other groups evaluated rs7702187 and other SNPs of Sema5A in two independent case-control series from Finland and Taiwan and found that rs7702187 was associated with a decreased risk whereas rs3798097 was associated with an increased risk of PD in the Taiwanese population, but not in the Finland population (Clarimon et al. 2006). However, conflicting results were obtained from a case-control study of Polish Caucasians and Asians from Singapore. They concluded that rs7702187 was not a marker of PD risk (Bialecka et al. 2006). Another genome-wide association study, wherein most participants were of Caucasian ethnicity, did not support the data of Maraganore et al. (2005) and Elbaz et al. (2006). Further investigations are required to clarify the significance of Sema5A as a disease-susceptible gene in PD.

10.4 Semaphorins and Multiple Sclerosis (MS)

10.4.1 Multiple Sclerosis (MS)

Multiple sclerosis (MS) is a demyelinating autoimmune disease and a leading cause of neurological disabilities in young adults (Noseworthy et al. 2000; Compston and Coles 2002). Both genetic and environmental factors are supposed to participate in the pathogenesis of MS, and it is developed when genetically predisposed individuals are exposed to an environmental trigger that stimulates myelin-specific T cells (McFarland and Martin 2007). Genome-wide association studies revealed that the IL-2 receptor, IL-7 receptor, and HLA class II are associated with disease susceptibility (Lincoln et al. 2005; Hafler et al. 2007; Fugger et al. 2009). Recent evidence suggests that Th17 lymphocytes play crucial roles in MS in addition to Th1 cells (Bettelli et al. 2007; Tzartos et al. 2008; Sospedra and Martin 2005). Therefore, antigen presentation and subsequent $CD4^+$ T-cell activation and differentiation are essential steps for the development of MS, and it has been characteristically classified as an immune-mediated disorder. However, it also has characteristics of a neurodegenerative disease. MS is a heterogeneous disease and is

pathologically classified into four patterns (Lucchinetti et al. 2000, 2004). Although patterns I and II show T-cell-mediated or T-cell plus antibody-mediated autoimmune pathology, patterns III and IV are suggestive of oligodendrocyte apoptosis or primary oligodendrocyte dystrophy rather than autoimmunity. Axonal damage is observed in a relatively early phase of the disease course (Trapp et al. 1998; Trapp and Nave 2008). In the chronic phase of the disease, the disability persistently progresses without recovery. These features are observed in secondary progressive MS and are thought to arise primarily from neuronal or axonal damage (Bjartmar et al. 2000). Therefore, the neurodegenerative process seems to participate in MS pathology, and its involvement increases in accordance with disease duration.

10.4.2 Experimental Autoimmune Encephalomyelitis (EAE)

Experimental autoimmune encephalomyelitis (EAE) is a representative animal model for MS. It is believed to reproduce many of the clinical and histopathological features of the human disease and is induced by immunizing myelin proteins such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and proteolipid protein (PLP) in susceptible animals together with an adjuvant. EAE can also be induced by the passive transfer of myelin antigen-reactive T cells (Mix et al. 2008).

10.4.2.1 Sema3A

Sema3A directly binds to neuropilin-1, which induces the activation of plexin-A proteins and the transduction of axon-guidance signals. In the immune system, plexin-A1 is expressed in dendritic cells (DCs), and Sema3A/neuropilin-1 (NP-1)/plexin-A1 interactions promote DC migration to the draining lymph nodes after immunization. Because of impaired migration of DC, antigen-specific T-cell priming is inhibited in plexin-A1-deficient mice (Takamatsu et al. 2010). Accordingly, plexin-A1-deficient mice exhibit less severe EAE induced by MOG peptide, with impaired MOG peptide-specific CD4+ T-cell responses (Takegahara et al. 2006).

10.4.2.2 Sema4D

Sema4D is highly expressed in T cells, playing an important role in the activation of T cells by causing maturation of DCs. Regarding the Sema4D receptors, plexin-B1 and CD72 have been identified in the nervous and immune systems (Tamagnone et al. 1999; Kumanogoh et al. 2000). Sema4D-deficient mice exhibit attenuated EAE with impaired antigen-specific T-cell responses (Kumanogoh et al. 2002b). In addition to the priming phase, Sema4D on encephalitogenic T cells also directly

activates microglia through plexin-B1. When MOG-reactive CD4+ T cells prepared from wild-type mice were adoptively transferred into plexin-B1-deficient mice or bone marrow chimera mice with plexin-B1-deficient central nervous system (CNS) resident cells, the development of EAE was considerably improved (Okuno et al. 2010). Consistent with this, anti-Sema4D blocking antibodies were effective after the onset of EAE (Okuno et al. 2010). In addition, Sema4D in T cells causes the collapse of process extensions in immature oligodendrocytes and the death of immature neural cells (Giraudon et al. 2004). Collectively, these findings indicate that T cell-derived Sema4D is important in both the priming and effector phases of EAE.

10.4.2.3 Sema4A

Sema4A is expressed in dendritic and Th1 cells and is important in the activation of Th cells and the differentiation of Th1 and Th17 cells (Kumanogoh et al. 2002a, 2005). Consistently, the development of MOG-induced EAE in wild-type mice can be improved by intravenous injection of an anti-Sema4A monoclonal antibody concurrently with MOG immunization. The infiltration of mononuclear inflammatory cells into the spinal cord is diminished in anti-Sema4A antibody-treated mice, in which CD4+ T cells isolated from the draining lymph nodes have markedly decreased responses to the MOG peptide (Kumanogoh et al. 2002a).

10.4.2.4 Sema6D

Sema6D directly interacts with plexin-A1 independently of NP-1 and activates the plexin-A1–DAP12–TREM2 complex (Toyofuku et al. 2004; Steinman 2004; Takegahara et al. 2006). Plexin-A1-deficient mice exhibit milder severity when EAE is induced (Takegahara et al. 2006). Similarly, DAP12-deficient mice exhibit attenuated development of MOG-induced EAE and impaired generation of MOG-specific T cells (Bakker et al. 2000). Therefore, Sema6D is suggested to contribute to the resistance to EAE in plexin-A1-deficient mice by activating signals downstream of the DAP12/TREM2 complex.

10.4.2.5 Sema7A

Sema7A is a membrane-associated glycosyl phosphatidylinositol (GPI)-linked protein. In the nervous system, Sema7A has been shown to promote olfactory bulb axon outgrowth and is required for the appropriate formation of the lateral olfactory tract during embryonic development. Although plexin-C1 was initially identified as a receptor for Sema7A (Tamagnone et al. 1999), Sema7A has an arginine-glycine-aspartate sequence in its Sema domain, which is a well-conserved integrin-binding

motif, and Sema7A attracts axons through the β1-integrin receptor, not through plexin-C1, by activating the downstream mitogen-activated protein kinase pathway (Pasterkamp et al. 2003).

T cell-derived Sema7A is involved in inflammation by activating macrophages in the inflammatory lesion (Suzuki et al. 2007). Sema7A on antigen-primed effector T cells plays a role in inducing inflammation in EAE through interactions with α1β1 integrin and contributes to the exacerbation of EAE. These data indicate that Sema7A is involved in the pathogenesis of EAE in the effector phase.

10.4.3 Sema3A and Sema3F in MS

The presence of demyelinated plaques in the CNS is the central pathology of MS. One of the most important causes of permanent damage is the disturbance of remyelination. A possible reason is the lack of migration of oligodendrocyte precursor cells (OPCs) to the lesion. Sema3A and Sema3F transcripts are upregulated in grey matter neurons, astrocytes, and the microglial cells around active inflammatory lesions in patients with MS (Williams et al. 2007). Piaton et al. reported the role of Sema3A and Sema3F in remyelination in the adult CNS, showing that they exert opposite effects in the recruitment of adult OPCs to CNS lesions in a toxin-induced demyelination model (Piaton et al. 2011). The expression of Sema3F and its receptor Np-2 is upregulated following demyelination, guiding the recruitment of OPCs toward the lesion. In this context, the overexpression of Sema3F enhances early remyelination and accelerates myelin repair. In contrast, the expression of Sema3A and Np-1 is delayed and inhibits remyelinating precursor cell recruitment (Piaton et al. 2011). These data suggest that Sema3A may act as a stop signal for OPC recruitment after an adequate number of precursor cells have been attracted to the lesion by Sema3F. Accordingly, an inhibitory role for Sema3A in remyelinating processes is identified (Syed et al. 2011), indicating a block of OPC differentiation in the presence of Sema3A. These observations support the idea that aberrant expression of Sema3A and Sema3F in the CNS may underlie impaired OPC recruitment and differentiation in MS lesions, ultimately limiting myelin repair.

10.4.4 Sema4A in MS

It is not surprising that Sema4A deeply participates in MS development in addition to EAE because both Th1 and Th17 cells are suggested to be involved in human MS pathogenesis (Stromnes et al. 2008).

Serum Sema4A levels are significantly higher in patients with MS than in those with other neurological diseases (ONDs) and healthy volunteers when assayed by enzyme-linked immunosorbent assay (ELISA) (Nakatsuji et al. 2012). Approximately one fourth of patients with relapsing-remitting (RR) MS have extremely

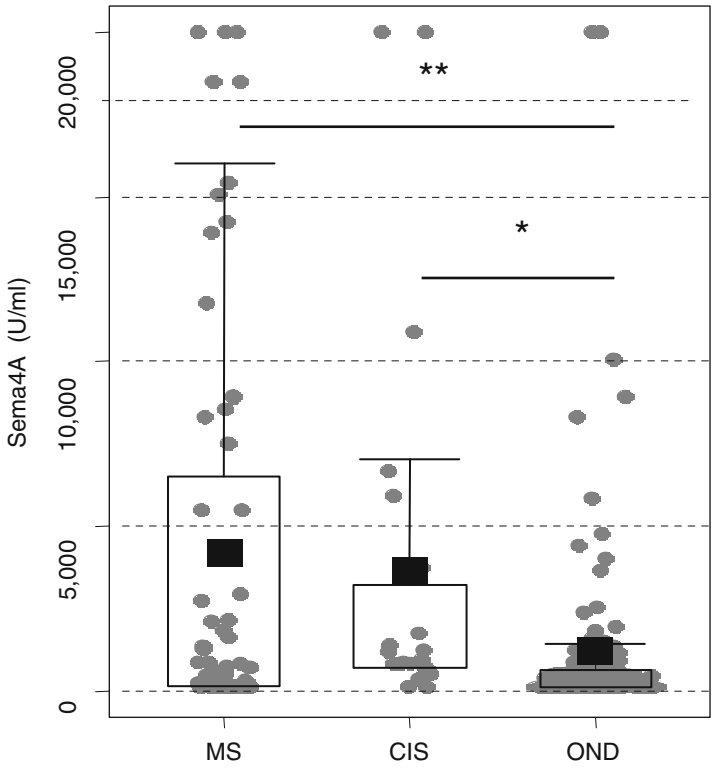

Fig. 10.3 Elevated serum Sema4A levels in multiple sclerosis (MS) patients. Serum Sema4A levels were significantly increased in MS and clinically isolated syndrome (CIS) patients compared to OND patients. The levels of serum Sema4A were assayed by ELISA in relapsing-remitting MS (*RRMS*) patients in the remitting phase, CIS patients, and age- and gender-matched other neurological disease (OND) patients. The *black squares* show the means. The *top* and *bottom* of the box in the box-and-whisker plot indicate the 25th and 75th percentiles, respectively, and the end of the whisker represents 1.5 times the interquartile range from the *top* of the box or the maximum point of all the data. $*p < 0.05$; $**p < 0.01$

high Sema4A levels. Furthermore, serum Sema4A levels of patients with clinically isolated syndrome (CIS), which is considered to be an early stage of MS, are as high as those in patients with MS and significantly higher than those in patients with ONDs. Therefore, Sema4A levels are increased in the early stage of MS (Fig. 10.3).

With regard to the source of serum Sema4A, T and B cells show very low expression. $CD11c^+$, $HLADR^+$ monocytes, and dendritic cells (DCs) from healthy donors express moderate amounts of Sema4A, and significantly increased expression is observed on these cells from patients with MS.

Sema4A is suggested to be released from the cell surface by metalloproteinases because its release can be inhibited by protease inhibitors such as the metalloproteinases ADAM and matrix metalloproteinases (MMPs). An MMP inhibitor,

phosphoramidon, and light metal chelators also inhibit Sema4A shedding (Nakatsuji et al. 2012). The mRNAs for metalloproteinases, including ADAM 10 and MMPs, are increased in peripheral blood mononuclear cells (PBMCs) from MS patients with high serum Sema4A levels compared with those in MS patients with low serum Sema4A levels and healthy controls. These facts collectively suggest that Sema4A, which is abundantly expressed on monocytes and DCs from patients with MS, is enzymatically shed in a subpopulation of these patients.

With regard to the characteristics of MS patients with high Sema4A levels, these are very important findings. One important feature is that MS patients with high serum Sema4A levels have a significantly higher proportion of IL-17-positive cells among their $CD4^+$ T cells compared with those with low serum Sema4A levels or healthy controls (Nakatsuji et al. 2012). With regard to the serum cytokine levels of patients with high Sema4A levels, their IL-2 levels are higher, which is compatible with the observation that DC-derived Sema4A activates T cells. Serum IL-10 levels are lower in patients with high Sema4A levels, which is compatible

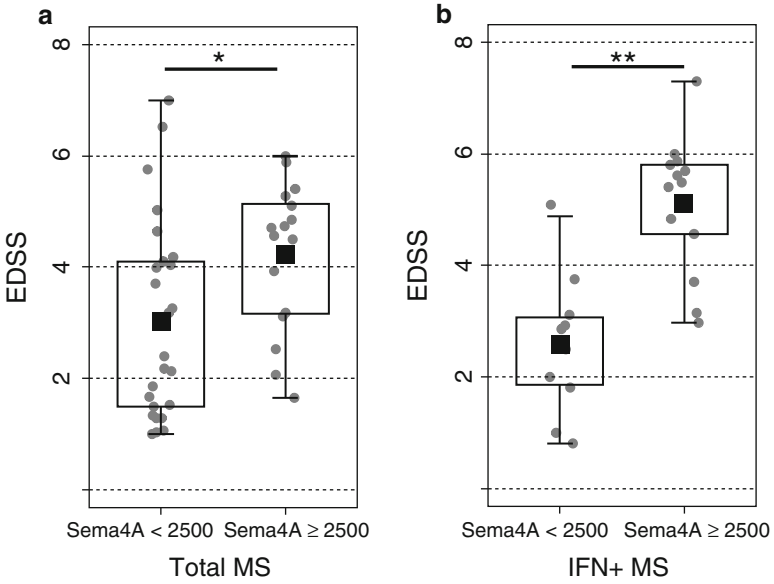

Fig. 10.4 Unresponsiveness of MS patients with high Sema4A to IFN-β treatment. (**a**) Correlation between Sema4A levels and neurological disabilities (EDSS score). RRMS patients were divided into two groups based on Sema4A titer. MS patients with higher Sema4A levels showed worse EDSS scores than patients with lower Sema4A levels. (**b**) Correlation between serum Sema4A levels and the EDSS scores of MS patients treated with IFN-β. MS patients with higher Sema4A levels had worse EDSS scores than patients with lower Sema4A levels. The *top* and *bottom* of the box in the box-and-whisker plot indicate the 25th and 75th percentiles, respectively; the end of the whisker represents 1.5 times the interquartile range from the box or the most extreme points of all the data. $*p < 0.05$; $**p < 0.01$

with the observation that Sema4A inhibits Th2 differentiation (Makino et al. 2008). Therefore, Sema4A levels in patients with MS seem to reflect an underlying Th17-mediated MS pathogenesis.

Another important feature is that MS patients with high Sema4A levels exhibit a significantly more severe disease course compared with those with low Sema4A levels as evaluated by the expanded disability status scale (EDSS) (Kurtzke 1983). More importantly, MS patients with high Sema4A levels are refractory to the first-line drug interferon (IFN)-β (Fig. 10.4). Therefore, MS patients with high Sema4A levels have some undesirable characteristics such as high disease activity and refractoriness to treatment. These facts suggest that serum Sema4A may be a biomarker of refractoriness to IFN-β in addition to being a reliable aid for arriving at an early diagnosis.

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Chapter 11
Semaphorin in the Retinal System

Toshihiko Toyofuku

Abstract The vertebrate retina is a light-sensitive layer of tissue that lines the inner surface of the eye. Light striking the retina initiates a cascade of chemical and electrical events that ultimately trigger nerve impulses, which are sent to various visual centers of the brain through the fibers of the optic nerve. Each axis of the retina is mapped independently using different mechanisms and sets of axon-guidance molecules, such as the semaphorins, which are expressed in gradients to achieve projections from points in the retina to points in the target regions of the brain. In animal models, mutations in several of the guidance molecules disrupt axonal projections at specific sites, whereas mutation of one of the semaphorins reduces photoreceptor survival. Understanding the molecular mechanisms of neural defects in a variety of animal models can provide valuable insights into the effects of each molecule in clinical disorders and may form the basis of future therapies to prevent retinal diseases.

Keywords Retina • Rhodopsin • Phosphodiesterase (PDE) • The visual cycle • The retinal pigment epithelium (RPE) • Visual map • The immunoglobulin superfamily (IgSF) • Plexin A4 (PlexA4) • Sema5A/B • Robo2 • EphB1 receptor • Neuropilin 1 (Nrp1) • VEGF • Retinitis pigmentosa • Reactive oxygen and nitrogen species (RONS) • Sema4A • FIP2 • Rab11 • 11-cis-retinal • CRALBP • CRBP1

11.1 Anatomy of the Retina

The retina is a layered structure containing several layers of neurons interconnected by synapses (Fig. 11.1). The only neurons that are directly sensitive to light are the photoreceptor cells, which are mainly of two types: rods and cones. Rods

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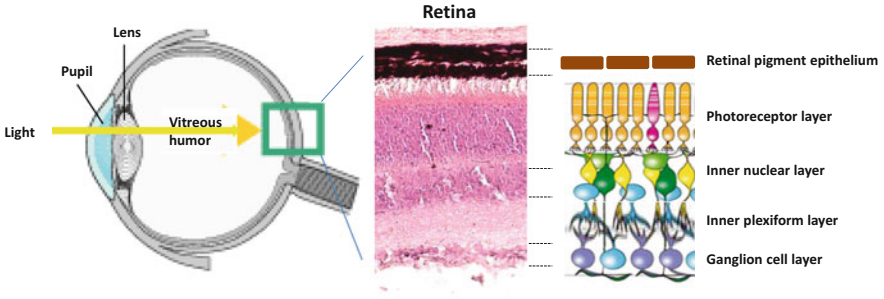

Fig. 11.1 Structure of the retina. Light passes through the pupil, lens, and vitreous cavity before reaching the light-sensitive retina. Cross section of the retina shows its laminated structure

function mainly in dim light and provide black-and-white vision, whereas cones support daytime vision and the perception of color. Neural signals from the rods and cones undergo processing by other neurons in the retina, taking the form of action potentials in retinal ganglion cells, whose axons form the optic nerve. These processes can be simplified into four main processing stages: photoreception, transmission to bipolar cells, transmission to ganglion cells, and transmission along the optic nerve. At each synaptic stage, there are also laterally connecting horizontal and amacrine cells. The optic nerve, a central tract consisting of many ganglion cell axons, connects primarily to the lateral geniculate body, a visual relay station in the diencephalon (the rear of the forebrain). It also projects to the superior colliculus, the suprachiasmatic nucleus, and the nucleus of the optic tract.

11.2 Physiology of the Retina

11.2.1 Phototransduction

In the photoreceptors, exposure to light hyperpolarizes the membrane (Fig. 11.2). The outer segment of a photoreceptor cell contains the photopigment rhodopsin. Inside the cell, normal levels of cyclic guanosine monophosphate (cGMP) keep Na^+ channels open; therefore, in the resting state, the cell is depolarized. Photons cause 11-*cis*-retinal bound to the receptor protein (opsin) to isomerize to all-*trans*-retinal; once this isomerization reaction has taken place, the receptor activates multiple G proteins; this in turn causes the G_α-subunit of the protein to activate a phosphodiesterase (PDE), which degrades cGMP, resulting in the closing of cyclic nucleotide-gated ion channels. As a consequence, the cell is hyperpolarized. The amount of neurotransmitter released is reduced in bright light and increases as light levels fall. The actual photopigment is bleached away in bright light and can only be replaced by a chemical process; therefore, in a transition from bright light to darkness, the eye can take up to 30 min to reach full sensitivity.

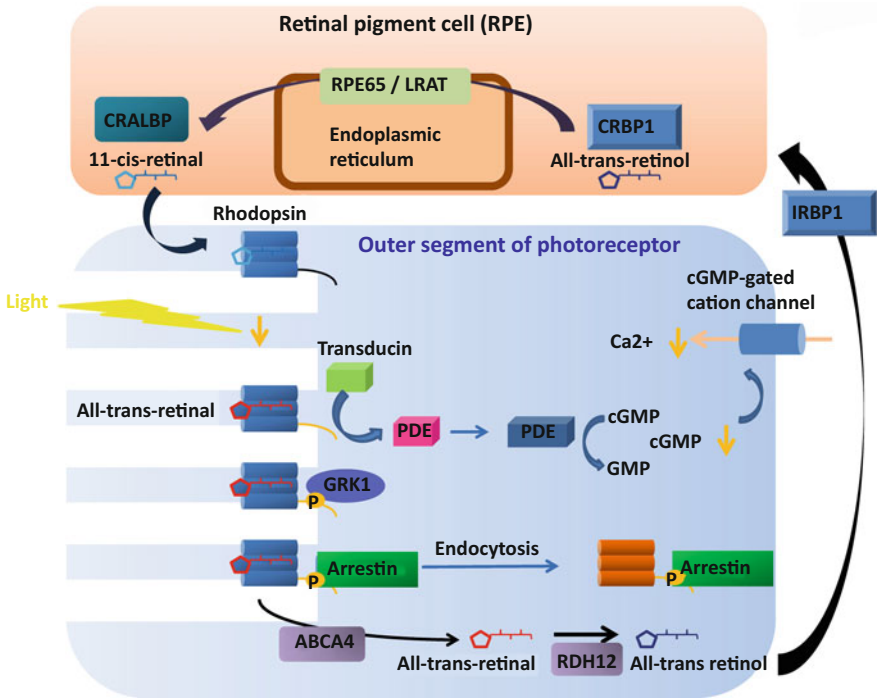

Fig. 11.2 Light-induced photoreceptor excitation and visual cycle. Phototransduction occurs in the outer segment of the photoreceptor, where the light-induced isomerization of rhodopsin triggers sequential chemical steps leading to membrane depolarization. The visual cycle is a pathway of enzymatic reactions that recycle the retinoids used during light detection by the photoreceptor

11.2.2 Visual Cycle

The visual cycle is a pathway of enzymatic reactions that recycle the retinoids that are used during light detection in photoreceptor cells (Lamb and Pugh 2004) (Fig. 11.2). The activation of the photoreceptor rhodopsin by light occurs through the isomerization of 11-*cis* retinal, bound to opsin, to all-*trans* retinal. All-*trans* retinal is released from rhodopsin, conjugated with the membrane lipid phosphatidylethanolamine, and transported to the cytoplasm by the ATP-binding cassette, subfamily A, member 4 (ABCA4) protein. After modification to all-*trans* retinol by a retinol dehydrogenase (RDH12), the molecule is transported to the retinal pigment epithelium (RPE), where it is esterified to a fatty acyl group (FA) by lecithin retinol acyltransferase (LRAT) to form all-*trans* retinyl ester. This compound is subject to *trans*-isomerization to 11-*cis* retinal through the activities of two additional enzymes (RPE65 and 11-*cis* retinol dehydrogenase). After transport back to the photoreceptor, 11-*cis* retinal binds rhodopsin, rendering it sensitive to light. Retinoid-binding proteins, such as interstitial retinol-binding protein (IRBP),

cellular retinol-binding protein (CRBP1), and cellular retinaldehyde-binding protein (CRALBP), are involved in transport of the hydrophobic retinoids in the aqueous environment of the cytoplasm.

11.3 Visual Map Development

Visual information is transferred from the retina to multiple areas in the brain, with the spatial information of the visual field maintained in each target region. Two of the main targets of the retina are the superior colliculus [SC, analogous to the optic tectum (OT) in lower vertebrates], a midbrain structure used to control head and eye movements, and the dorsal lateral geniculate nucleus (dLGN) in the dorsal thalamus. The dLGN in turn projects to the primary visual cortex (V1) in the posterior cerebral cortex, which subsequently projects to several "higher" visual areas responsible for conscious vision. Each component of the visual system is mapped topographically (Lewin 1994; Udin and Fawcett 1988).

11.3.1 Lamina-Specific Targeting of Neurites in the Retina

Retinal circuits are activated by photoreceptors (rods or cones), which transform light energy into neural signals and make contacts with both excitatory interneurons (bipolar cells) and inhibitory interneurons (horizontal cells) that process information within the retina. Subsets of bipolar cells and another class of inhibitory interneurons (amacrine cells) converge selectively onto one ganglion cell. The circuit of each type of ganglion cell determines its specific sensitivity to visual input. A fundamental organizing principle of the retina is its division into ON and OFF pathways. Bipolar cells are excited by either increases (ON cells) or decreases (OFF cells) in light; the axons of ON and OFF bipolar cells terminate in different sublayers of the inner synaptic layer of the retina (Fig. 11.3a). At least two distinct mechanisms serve to target neurites to the appropriate laminae: homophilic cell–cell adhesion via IgSF molecules, and short-range guidance by classical axon-guidance cues.

Intercellular (cell–cell) recognition is carried out by cell-surface adhesion molecules belonging to the immunoglobulin superfamily (IgSF) of transmembrane glycoproteins (Yamagata and Sanes 2008; Yamagata et al. 2002). These molecules often act in a homophilic fashion, that is, they function as reciprocal receptors and ligands on the surface of both axons and dendrites. The homophilic IgSF molecules Sidekick1 (Sdk1), Sdk2, Dscam, and DscamL are expressed in nonoverlapping subsets of bipolar cells, amacrine cells, and ganglion cells in the retina. For example, expression of Sdk1 on a neurite directs its stratification to a lamina containing high levels of Sdk1. Other classes of molecules, such as cadherins, have been implicated in cell–cell recognition (Inoue and Sanes 1997; Poskanzer et al. 2003).

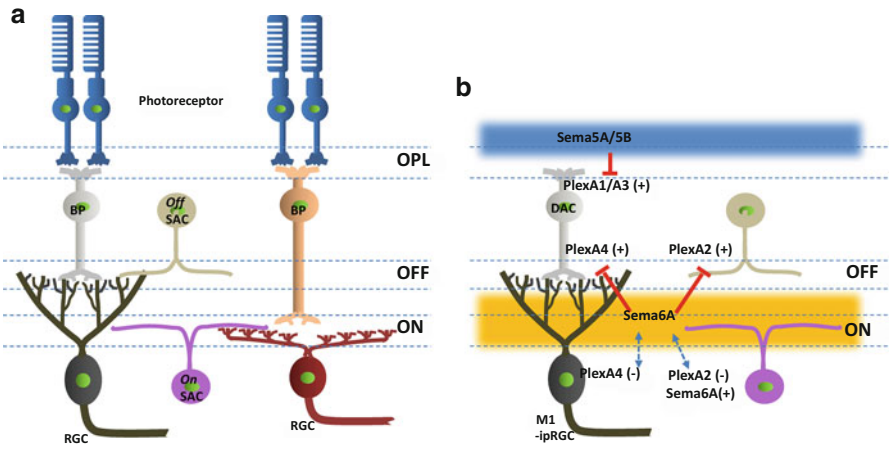

Fig. 11.3 Schematic model of semaphorin-dependent neurite stratification in the retina. (**a**) The retina is divided into ON and OFF pathways. Bipolar cells are excited by either increases (ON cells) or decreases (OFF cells) in light; the axons of ON and OFF bipolar cells terminate in different sublayers of the inner synaptic layer of the retina, and connect the layer of ganglion cells. (**b**) Semaphorins regulate the formation of synapses between bipolar cells and ganglion cells

A recent study has revealed a novel role for transmembrane semaphorin 6A (Sema6A) signaling through plexin A4 (PlexA4) in the regulation of retinal neurite stratification (Matsuoka et al. 2011b; Sun et al. 2013) (Fig. 11.3b). Restricted neurite stratification in OFF layers is controlled, at least in part, by semaphorin-mediated repulsion away from ON layers. Sema6A, which is concentrated within the ON layers of the inner plexiform layer (IPL), repels PlexA4-expressing dopaminergic amacrine cells (DACs) in the OFF region of the IPL, whereas M1-melanopsin-positive intrinsically photosensitive retinal ganglion cells (RGCs) (M1-ipRGCs) that do not express PlexA4 stratify to the OFF layer through the Sema6A region (Matsuoka et al. 2011b). Sema5A/B signaling, mediated by the PlexA1 and PlexA3 receptors (which are functionally redundant for Sema5 signaling in the retina), repels initial neurite extension of bipolar cells away from the INL (Matsuoka et al. 2011a). Stardust amacrine cells (SACs) are divided into ON and OFF subtypes, which co-stratify with distinct direction-selective ganglion cells (DSGCs) at the ON and OFF layers. This laminification is important for direction-selective responses to visual cues. The arborization of ON and OFF SACs is controlled by Sema6A (Sun et al. 2013); arborization of OFF SACs is restricted to the OFF layer through the action of Plex-A2, whereas arborization of ON SACs is restricted to the ON layer through the action of Sema6D in the absence of PlexA2. These findings provide an example of multilevel control of synaptic specificity. In the case of DACs, Sema5 signaling repels initial neurite extension away from the inner nuclear layer. Subsequently, Sema6A directs terminal arborization of DACs to OFF strata of the IPL by repelling their neurites away from ON layers; homophilic

adhesion may function to specify cell type-specific synapse formation within these layers. Furthermore, Sema6A directs arborization of ON and OFF cells to their respective layers.

11.3.2 Retinal Axon Guidance at the Optic Chiasm

The optic chiasm is the structure where partial contralateral crossover of RGC axons occurs. Netrin-1 likely exerts its attractant influence on RGC axons after they exit the eye (Fig. 11.4). However, netrin-1 is not present around the chiasm midline, where RGC axons come under the influence of repulsive molecules such as Sema5A and Slit/Robo. Sema5A is expressed at the optic disc and along the optic nerve, and blockade of Sema5A function causes retinal axons to stray out of the optic nerve bundle (Oster et al. 2003). It is possible that Slit/Robo signaling defines the site of optic chiasm formation. RGCs express Robo2, a receptor for Slit, and Slit1 and Slit2 are present in the ventral diencephalon (Plump et al. 2002; Long et al. 2004). Thus, Sema5A and Slit proteins act as a repulsive "guardrail," establishing a corridor through which RGC axons are channeled. Ephrin-B2 and EphB1 control axon divergence at the optic chiasm (Williams et al.

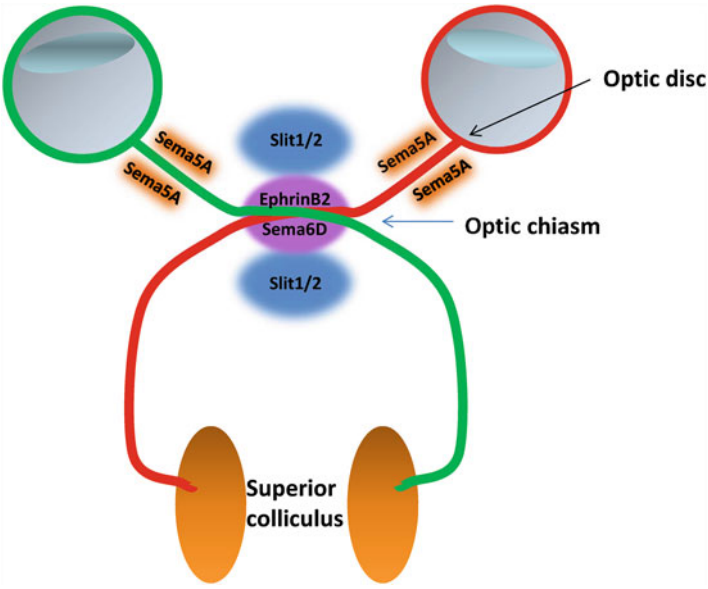

Fig. 11.4 Retinal ganglion cell (RGC) axon guidance. At the optic disc, RGC axons exit the retina into the optic nerve because of an attractive effect mediated by netrin/DCC. Within the optic nerve, RGC axons are kept within the pathway through Sema5A and by inhibitory Slit/Robo interaction. At the optic chiasm, ephrin-B2 repels EphB1-expressing axons and terminates at ipsilateral targets, whereas Sema6D in combination with Nr-CAM regulates the contralateral projection of axons

2003). Ephrin-B2 is expressed in radial glial cells at the optic chiasm concurrent with the development of the ipsilateral projections; in mice, blockade of ephrin-B2 eliminates ipsilateral projections. The EphB1 receptor is specifically expressed in RGCs in the mouse ventrotemporal (VT) retina that give rise to the ipsilateral projections. By contrast, Sema6D, which is expressed in radial glial cells at the optic chiasm, regulates the contralateral projection of PlexA1-expressing RGCs in combination with coexpressed Nr-CAM (Kuwajima et al. 2012).

11.3.3 Retinal Axon Guidance at SC and dLGN

Upon crossing the midline, RGC axons project to their major targets, the SC and dLGN. Topographic mapping of RGC axons occurs along two sets of orthogonally oriented axes. The nasal–temporal (NT) axis of the retina maps along the posteroanterior (PA) axis of the SC, and the dorsoventral (DV) retinal axis maps along the lateromedial (LM) SC axis. Accumulating evidence has revealed that ephrin-As and their EphA receptors are required for proper retinal NT mapping along the SC PA axis (Brown et al. 2000; Feldheim et al. 2000; Rashid et al. 2005). In addition, an external gradient of Engrailed-2, a homeodomain transcription factor, may also participate in the formation of the PA axis in the vertebrate SC, possibly by regulating the expression of Eph family members (Brunet et al. 2005; Itasaki and Nakamura 1996; Logan et al. 1996). By contrast, Ephrin-Bs in the SC, and their EphB receptors in the retina, are also required for proper DV mapping along the LM axis (Hindges et al. 2002).

11.3.4 Retinal Axon Guidance at Thalamocortical (TC) Projections

During development, TC axons grow into the subcortical telencephalon (ST); postnatally, they continue from the ST along their paths to the cortex. Recent studies have suggested that some guidance cues may be required in the ST, and that such cues may play a key role in controlling the initial topography of thalamic projections to the neocortex. EphAs in the thalamus and ephrin-As in the ST are involved in the regulation of TC projections in the somatosensory area in the frontal cortex (Dufour et al. 2003). Slit/Robo signaling is involved in the lamination of TC projections (Xiao et al. 2011). The optic tectum is pre-patterned by specific ECM molecules such as large glycoproteins and proteoglycans including Tenascin, Versican, and Nel; each has been shown to influence neurite growth in vitro (Yamagata and Sanes 2005; Yamagata et al. 1995; Jiang et al. 2009). Slit and Col4a5 bind directly in vitro, suggesting that Slit influences TC projections through direct binding to the ECM.

11.4 Vascular Development

The multilayered retina is initially supplied by a combination of two extraretinal vascular systems, the choroidal vasculature that supplies the outer retina and the hyaloid arteries that supply the inner retina and lens. The choroidal vasculature persists throughout the lifespan; by contrast, late in mammalian development, the hyaloid arteries are replaced with a dedicated intraretinal vascular system. In rodents, the retina is vascularized after birth to give rise to a system of three interconnected vascular plexi (Fruttiger 2007). Vascularization begins on the day of birth, when vessel sprouts emerge from the optic nerve head and spread radially over the retina, guided by a template of astrocytes, with blood vessels and astrocytes forming copatterned networks (Fruttiger 2007; West et al. 2005). During the process of radial expansion, the primary plexus undergoes arteriovenous differentiation. After the first week of life, vessel sprouts emerge from this primary retinal vessel plexus to dive into the inner retinal layers at near right angles and form the deep plexus (during week 2 after birth) and then the intermediate plexus (during week 3).

In the neonatal mouse retina, the three vascular endothelial growth factor (VEGF) isoforms are produced and displayed by an astrocytic network located beneath the expanding vascular plexus. Neuropilin 1 (Nrp1) is a noncatalytic transmembrane protein whose genetic loss, either globally or specifically in endothelial cells, severely inhibits central nervous system (CNS) vascularization. Nrp1 serves as a receptor for VEGF165 and a member of the structurally unrelated class 3 semaphorin family, Sema3A (Schwarz and Ruhrberg 2010). Tumor studies have implicated Sema3A as a modulator of pathological angiogenesis. Thus, Sema3A reduces the overall vascularity of tumors and "normalizes" tumor vessels, in part by recruiting myeloid cells that stimulate vessel maturation (Maione et al. 2009).

Sema3E is the only class 3 semaphorin that does not bind to a neuropilin receptor, but instead binds directly to PlexD1 (Gu et al. 2005). In the developing retinal vasculature, high VEGF levels emanating from the avascular retinal periphery induce PlexD1 expression in endothelial cells at the vascular front; this phenomenon is dependent on VEGFR2 (Kim et al. 2011). Furthermore, loss-of-function studies have demonstrated that Sema3E, derived from the neural layers of the retina, signals through endothelial PlexD1 to upregulate DLL4 at the vascular front. This, in turn, increases endothelial Notch signaling, resulting in a loss of tip cells and tip-cell filopodia. Consequently, normal vascular expansion into the retinal periphery is disrupted in mice lacking Sema3E. Remarkably, Sema3E normalizes VEGF-A-induced pathological vessel growth in a mouse model of oxygen-induced retinopathy, in which retinal vessels grow abnormally into the vitreous humor (Fukushima et al. 2011). In that study, intravitreal administration of Sema3E protein prevented this abnormal vessel growth. This observation suggests that Sema3E could serve as a therapeutic tool for fine-tuning VEGF-A signaling and vascular growth in the ischemic nervous system.

11.5 Semaphorin in Retinal Diseases

11.5.1 Photoreceptor Degeneration

Retinitis pigmentosa (RP) is an inherited, degenerative eye disease that causes severe vision impairment and often blindness. A form of retinal dystrophy, RP is caused by abnormalities of the photoreceptors (rods and cones) or the retinal pigment epithelium (RPE) of the retina leading to progressive sight loss. More than 100 mutations have been found in this gene, accounting for 15 % of all types of retinal degeneration. Most of those mutations are missense mutations, and the disease is mostly inherited in a dominant manner.

The most obvious factor that makes photoreceptors (PRs) vulnerable to degeneration is light exposure. Visible and ultraviolet light are insufficiently energetic to ionize most biomolecules, but oxygen enhances the ionizing effect of light. Consequently, damage can occur when reactive oxygen and nitrogen species (RONS) are generated by light acting on photosensitizing molecules such as retinoids. Light damage to PRs requires the release of all-*trans* retinal from light-activated rhodopsin (Sun and Nathans 2001; Travis et al. 2007). Photo-excitation of all-*trans* retinal generates singlet oxygen and can cause photo-oxidative damage. If mutations affecting the visual cycle block the recycling of all-*trans* retinal to 11-*cis* retinal, toxic bis-retinoids (such as the all-*trans* retinal dimer) and adducts [such as *N*-retinylidene-*N*-retinyl-ethanolamine (A2E)] build up during the course of aging.

Some types of PR degeneration are accelerated by light (Hartong et al. 2006; Cideciyan et al. 2005) (Fig. 11.5). Defects that are potentially exacerbated by light exposure are as follows. (1) Visual cycle defects, for example, mutations in *ABCA4* or retinol dehydrogenase 12 (*RDH12*) (Radu et al. 2005); ABCA4 transports toxic all-*trans*-retinal to the cytoplasm, and RDH12 dehydrogenates it. Defects in these enzymes increase the levels of toxic retinoids in photoreceptor cells. (2) RPE phagocytosis defects, for example, mutations in c-Mer proto-oncogene tyrosine kinase *(MERTK*) (Tschernutter et al. 2006); MERTK in retinal pigment epithelial cells is required to clear the light-damaged outer segments of photoreceptors. (3) PR cilia defects that slow outer segment turnover, for example, mutations in *RPGR* (Robson et al. 2008). PR cilia connect the outer and inner segments of the photoreceptor cell and transport the components required for phototransduction to the outer segment; defects in PR cilia compromise all outer-segment functions. (4) Defects in the stability of outer-segment discs, such as mutations in peripherin 2 (*PRPH2*) (Renner et al. 2009).

11.5.2 Sema4A in Retinal Degeneration

Rice et al. reported that insertion of a gene-trap vector into intron 11 of the mouse *Sema4A* gene results in the loss of retinal PRs (Rice et al. 2004). A subsequent study identified mutations in the human *Sema4A* gene in patients with

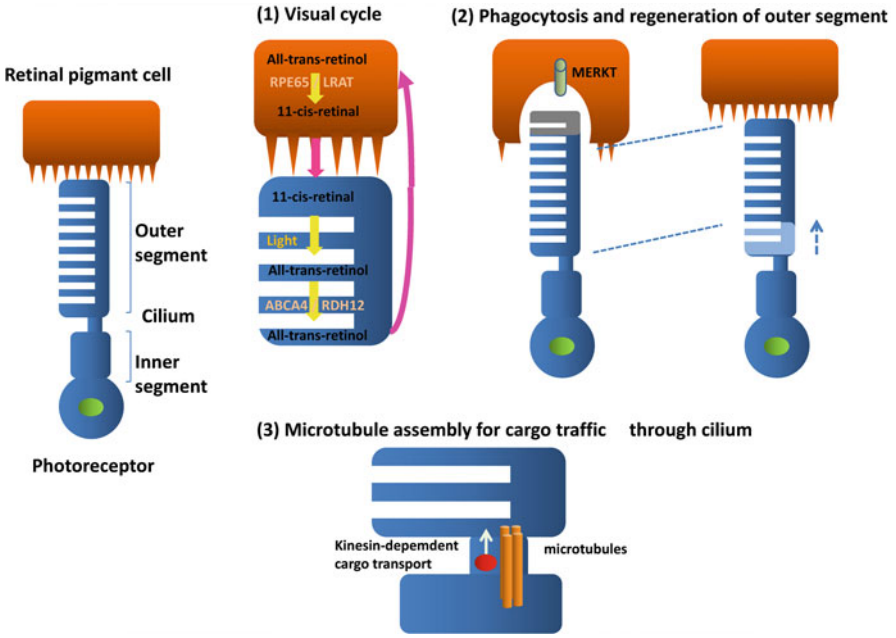

Fig. 11.5 Protection of light-induced photoreceptor degeneration. Photoreceptor (PR) homeostasis is functionally and mechanically supported by retinal pigment epithelium (RPE) cells. (*1*) RPE cells regenerate 11-*cis*-retinal from toxic all-*trans* retinal via the retinoid cycle. (*2*) RPE cells phagocytose the shed distal end of the outer segment of PR, which is constantly adding newly generated discs at its base. (*3*) The connecting cilium of the PR provides a pathway for transport of proteins and membranes from the inner segment to the outer segment

retinal degeneration (Abid et al. 2006). In Sema4A-deficient (Sema4A$^{-/-}$) mice, normal retinal development was observed at postnatal day 0 (P0), but at P14 the outer segments of PRs were disrupted, followed by a complete loss of PRs by P28 (Fig. 11.6a). In response to illumination, Sema4A$^{-/-}$ retinas exhibited a dramatic increase in the number of apoptotic cells in the outer nuclear layer before recovering to basal levels (Fig. 11.6b). Sema4A is expressed in RPE and bound to prosaposin. Prosaposin is synthesized and associated with procathepsin D in the Golgi membrane (Gopalakrishnan et al. 2004), and can be targeted to lysosomes (Kishimoto et al. 1992; Benes et al. 2008) or secreted into the extracellular space. Previous studies demonstrated that such secreted lysosomal precursor proteins are antiapoptotic for various neuronal populations (O'Brien et al. 1994; Benes et al. 2008). In those studies, oxidative stress caused by H_2O_2 treatment resulted in prosaposin transport to the cell periphery in Sema4A$^{+/+}$ RPE cells, but not in Sema4A$^{-/-}$ RPE cells. Via its intercellular region, Sema4A bound to a complex of Rab11 and the adaptor protein FIP2 more effectively than to FIP2 alone; Rab11 is involved in Sema4A-mediated prosaposin transport to the cell periphery

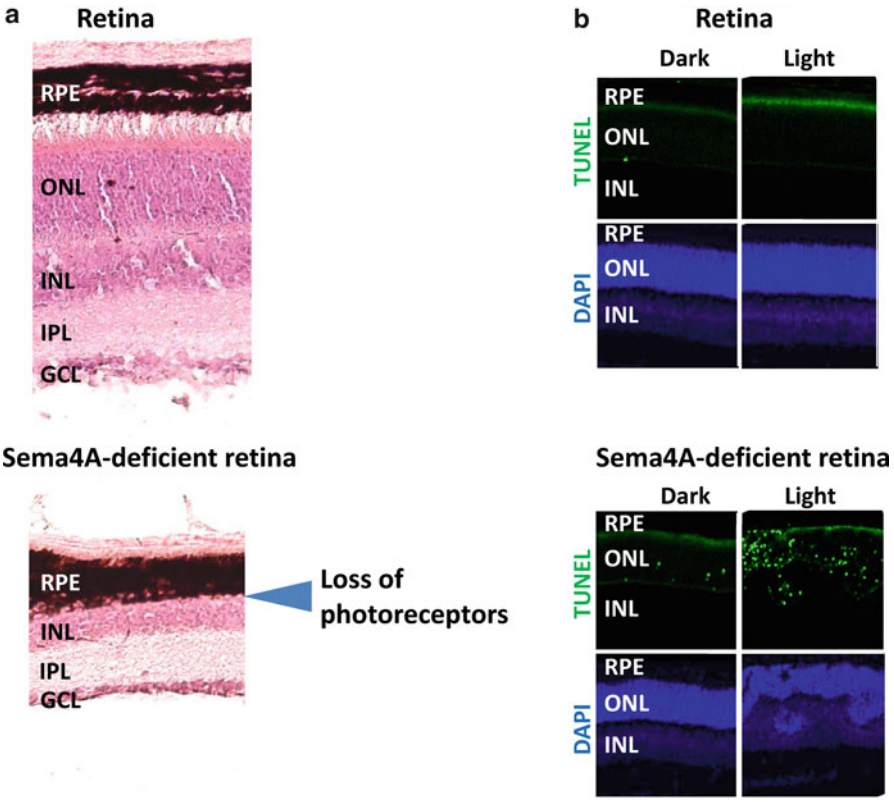

Fig. 11.6 Light-induced damage of photoreceptors in Sema4A$^{-/-}$ retinas. (**a**) Sema4A$^{-/-}$ retinas show progressive loss of the outer nuclear layer. (**b**) In neonatal mouse retinas after 60 min of light exposure, TUNEL assays reveal photoreceptor apoptosis in the Sema4A$^{-/-}$ retinas

under oxidative stress. In response to H_2O_2, prosaposin-containing vesicles were transported to the cell periphery via Sema4A/Rab11-mediated transport machinery (Fig. 11.7).

Levels of 11-*cis*-retinal are significantly increased in Sema4A$^{+/+}$ retinas at P14 and P28, whereas these levels remain low in Sema4A$^{-/-}$ retinas. Sema4A participates in the retinoid cycle by regulating the transport of retinoid-binding proteins in RPE cells. At least two proteins that bind water-insoluble retinoids in RPE cells are involved in the retinoid cycle (Lem and Fain 2004): CRALBP and CRBP1, which transport 11-*cis*-retinal and all-*trans*-retinol, respectively. These proteins are transported via the Sema4A-mediated endosomal sorting machinery; in the absence of Sema4A, they are mistargeted to different compartments. CRALBP is mistargeted to the cell periphery where it is likely unable to interact with 11-*cis*-retinal, which is generated in the endoplasmic reticulum. CRBP1 is mistargeted to the endoplasmic reticulum where it cannot interact with all-*trans*-retinol, which

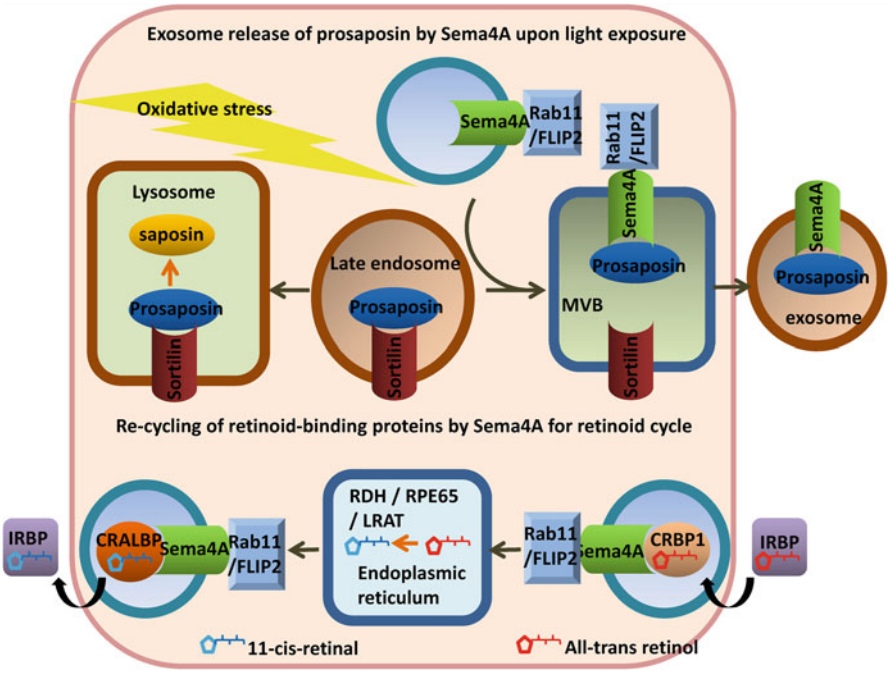

Fig. 11.7 Schematic model of Sema4A-mediated endosomal sorting in photoreceptor epithelium (PRE) after exposure to light. Sorting of prosaposin to the exosomal pathway is dependent on preferential binding of prosaposin to Sema4A and the Rab11/FIP2 endosomal sorting machinery. During dark adaptation, the recycling of retinoid-binding proteins is dependent on Sema4A-mediated transport

is imported from the extracellular space. Thus, Sema4A regulates intracellular sorting of retinoid-binding proteins to regenerate retinoids for phototransduction, an essential process in the retinoid cycle during dark adaptation (Fig. 11.7). The finding that Sema4A functions as an intracellular guide for specific molecules complements the previously known functions of semaphorins as extracellular guidance molecules (Kolodkin and Tessier-Lavigne 2011).

In patients with retinal degenerative diseases, three mutations, D345H, F350C, and R713Q, in Sema4A have been reported (Abid et al. 2006). An analysis of a series of knock-in mouse lines carrying mutated alleles of Sema4A demonstrated that expression of Sema4A(F350C) caused severe retinal degeneration (Nojima et al. 2013). In the RPE, Sema4A(F350C) tends to aggregate, and the resultant mislocalization of Sema4A protein may lead to the impaired endosomal sorting of molecules such as prosaposin and retinoid-binding proteins. Notably, virus-mediated gene transfer of Sema4A into RPE in neonatal Sema4A-deficient mice successfully prevents retinal degeneration for at least 4 months after injection. Considering the importance of the endosomal sorting function of Sema4A in

maintaining retinal homeostasis, it is possible that Sema4A replacement gene therapy might be efficacious in wider subsets of patients with retinal degenerative diseases.

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