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**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1558276> since 2016-03-18T14:57:03Z

*Published version:*

DOI:10.1007/s00018-016-2137-x

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# Transmembrane semaphorins, forward and reverse signaling: have a look both ways

Chiara Battistini, Luca Tamagnone

## Abstract

Semaphorins are signaling molecules playing pivotal roles not only as axon guidance cues, but are also involved in the regulation of a range of biological processes, such as immune response, angiogenesis and invasive tumor growth. The main functional receptors for semaphorins are plexins, which are large single-pass transmembrane molecules. Semaphorin signaling through plexins—the “classical” forward signaling—affects cytoskeletal remodeling and integrin-dependent adhesion, consequently influencing cell migration. Intriguingly, semaphorins and plexins can interact not only *in trans*, but also *in cis*, leading to differentiated and highly regulated signaling outputs. Moreover, transmembrane semaphorins can also mediate a so-called “reverse” signaling, by acting not as ligands but rather as receptors, and initiate a signaling cascade through their own cytoplasmic domains. Semaphorin reverse signaling has been clearly demonstrated in fruit fly *Sema1a*, which is required to control motor axon defasciculation and target recognition during neuromuscular development. *Sema1a* invertebrate semaphorin is most similar to vertebrate class-6 semaphorins, and examples of semaphorin reverse signaling in mammals have been described for these family members. Reverse signaling is also reported for other vertebrate semaphorin subsets, e.g. class-4 semaphorins, which bear potential PDZ-domain interaction motifs in their cytoplasmic regions. Therefore, thanks to their various signaling abilities, transmembrane semaphorins can play multifaceted roles both in developmental processes and in physiological as well as pathological conditions in the adult.

## Keywords

Bidirectional signaling Cancer Neuron Heart Retina CNS Spinal cord

## Introduction

Semaphorins are a large family of evolutionary conserved secreted as well as membrane-bound proteins, initially identified as axon guidance cues, and later found to be involved in the regulation of different biological processes, such as angiogenesis, bone homeostasis, immune response and cancer [1, 2]. Semaphorins can control cell–cell interactions, cell adhesion and migration, and are known to mediate both repulsive and attractive cues. Around 20 semaphorins have been identified, and were divided into eight classes on the basis of structural and amino acid sequence similarity. Class-3 semaphorins are secreted molecules, thereby they can diffuse in the extracellular environment and act in an autocrine and paracrine manner. Most semaphorins, however, are transmembrane proteins, so they can functionally interact with receptors exposed by adjacent cells (*in trans*), or sometimes be shed in the extracellular space. Semaphorins can thus mediate both long-range and short-range signals. Semaphorin receptors are mainly found in the Plexin family, although other relevant co-receptors have been identified. For instance, neuropilins are required co-receptors for most secreted semaphorins, but they do not seem to have a role for transmembrane members of semaphorin family.

The best characterized biological functions of semaphorins are mediated by so-called “forward” signaling, through the activity of the cytoplasmic portion of plexins, which contains a GTPase-activating protein (GAP) domain [2]. This gets activated upon ligand binding to the receptor ecto-domain and can exert its inhibitory activity on R-Ras, M-Ras and Rap1, a mechanism which can explain the observed negative regulation of integrin function. Certain plexins have also

been implicated in triggering other pathways in response to semaphorin stimulation, including the trans-activation of plexin-associated receptor tyrosine kinases [3].

In addition to this classical forward signaling, transmembrane semaphorins can also act as bi-directional cues, as has been described for other receptor–ligand systems, such as ligands of the TNF superfamily [4] or ephrins and Eph receptors [5]. In this so-called “reverse” signaling, semaphorins act not as ligands but rather as receptors, and they trigger a signaling cascade through their own cytoplasmic domains. Indeed, within the cytosolic regions of some of these semaphorins we can find domains for protein–protein interaction. For example, the cytoplasmic tail of most of class-4 semaphorins presents potential PDZ-domain interaction motifs [6], while class-6 semaphorins have large proline-rich cytoplasmic domains that may interact with Src-homology 3 (SH3) domains, as reported for Sema6D, interacting with Abl [7], or Sema6B, interacting with Src [8]. Moreover, the C-terminal region of Sema6A contains a zyxin-like domain, which allows the interaction with the enabled/vasodilator-stimulated phosphoprotein-like protein (EVL) [9]. Semaphorin reverse signaling was clearly demonstrated to play a pivotal role in the regulation of motor axon defasciculation [10] and target recognition [11] during fruit fly neuromuscular development. Recently, many examples of semaphorin reverse signaling have been reported also for vertebrates, in neuronal development and beyond [12–15].

In this review, we will provide an overview of the diverse known functions and signaling modes of transmembrane semaphorins, especially focusing on processes in which both forward and reverse signaling mechanisms have been implicated. Moreover, we will analyze non-conventional signal regulatory mechanisms due to *in cis* interactions between transmembrane semaphorins and plexin receptors co-expressed on the cell surface.

## Forward and reverse signaling of transmembrane Sema1a in fruit fly development

The fruit fly *Drosophila melanogaster*, similar to other invertebrates, contains four semaphorin genes: two of them are transmembrane molecules (Sema1a and Sema1b), while the others are of the secreted type (Sema2a and Sema2b). Sema1a was originally identified as a repulsive ligand controlling motor axon guidance during fly development. In fact, Sema1a and its receptor, PlexinA, are co-expressed by almost all motor axons, and their interaction *in trans* is crucial for defasciculation of nerve bundles at specific choice points, with individual axons projecting out toward their appropriate target muscles [16]. Notably, Sema1a–PlexinA forward repulsive signaling in motor axons is modulated by an extracellular matrix component, perlecan; this is abundant at motor axon defasciculation choice points, where it can enhance Sema1a–PlexinA signals eliciting FAK dephosphorylation and down-regulating integrin adhesive functions, which results in motor axon defasciculation [17] (Fig. 1a). Interestingly, Sema1a has been found to mediate motor axon defasciculation not only as a ligand of PlexinA, but also through reverse signaling mechanisms. In fact, the Sema1a cytoplasmic domain can interact with two major antagonistic regulators of the GTPase Rho1: the RhoGEF GDP/GTP exchanger Pebble and the inhibitor RhoGAP p190. The GEF activates Rho1 and promotes axon–axon repulsion and defasciculation, while p190-RhoGAP antagonizes this mechanism allowing axonal attraction. The extracellular Sema1a-binding molecule responsible for triggering this mechanism is still unclear [18].

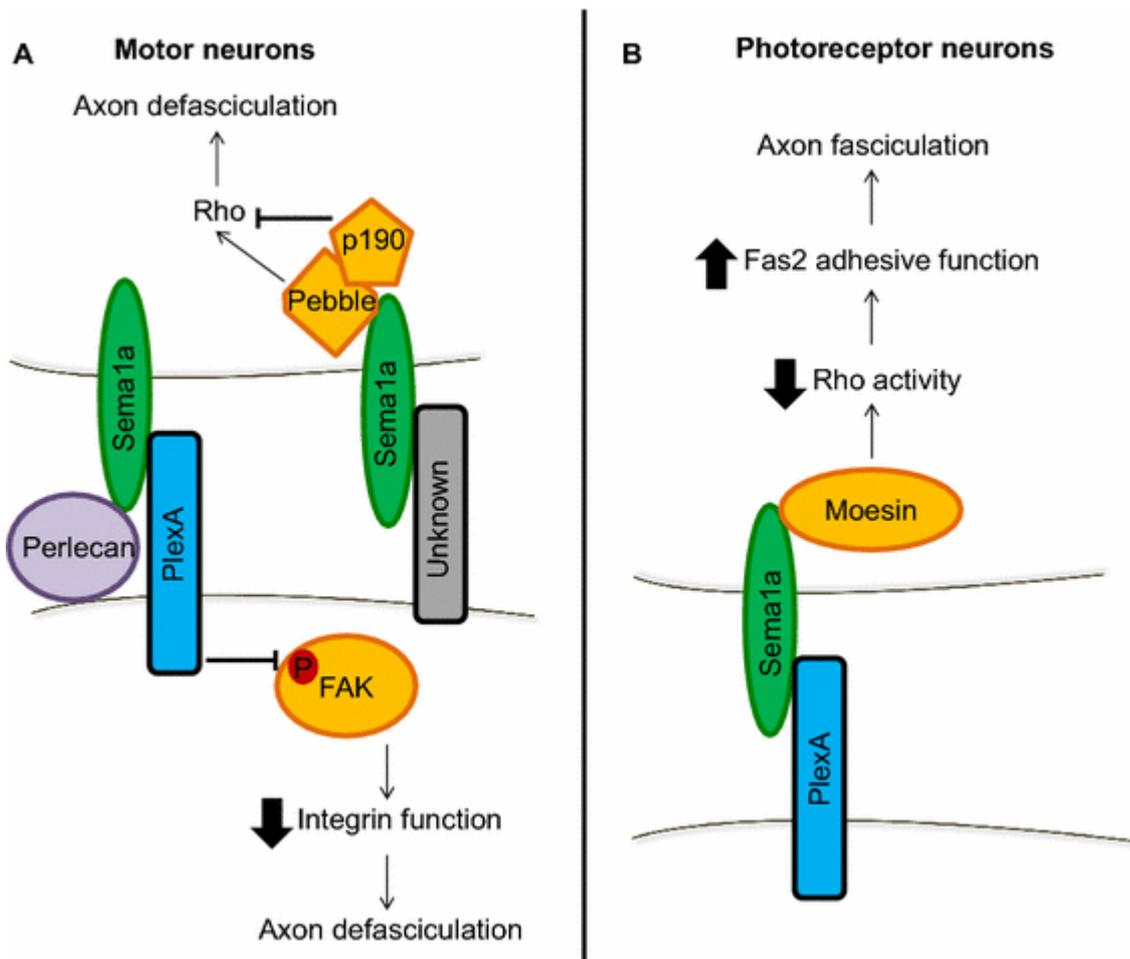


Fig. 1

Forward and reverse signaling in *Drosophila* neuromuscular development. a In motor neurons, the interaction of Sema1a with PlexinA—enhanced by perlecan—elicits FAK dephosphorylation and decreased integrin function, resulting in axon defasciculation. This process can be mediated also by Sema1a reverse signaling, through the modulation of Rho activity [17, 18]. b In photoreceptor neurons, Sema1a, acting as PlexinA receptor and interacting with Moesin, inhibits Rho activity, thus enhancing Fas2 adhesive function, resulting in axon fasciculation [20]

Sema1a reverse signaling is furthermore involved in the development of *Drosophila's* visual system. During this process, axons of the photoreceptor neurons (R-cells) navigate through the optic stalk to reach their appropriate topographic target in the ganglia of the optic lobe. Sema1a is expressed on the surface of R-cell axons and in their growth cones, and its deletion leads to aberrant axonal termination in the lamina layer of the optic lobe, as a consequence of impaired axonal interaction within nerve bundles. Conversely, when modified to overexpress Sema1a, the axons of R-cells are hyper-fasciculated; however, the expression of a mutant semaphorin lacking the cytoplasmic domain is unable to induce this effect, suggesting that Sema1a may function as a guidance receptor [10]. Interestingly, the depletion of PlexinA induces the same axonal navigation defects observed upon the knock-out of Sema1a, while axons overexpressing PlexinA are hyper-fasciculated. However, the cytoplasmic domain of PlexinA is dispensable for this effect, consistent with the finding that PlexinA acts as a trigger for Sema1a reverse signaling in this context [19]. It was then elucidated that this pathway promotes R-cell axon-axon attraction increasing the adhesive function of Fasciclin-2 (Fas2); this is achieved by the interaction between the Sema1a cytoplasmic domain and Moesin (Moe), a member of the ezrin/radixin/moesin (ERM) family, and subsequently by the inactivation of Rho1, which is a negative regulator of Fas2-mediated cell-cell adhesion [20] (Fig. 1b).

Sema1a signaling has a role also in the development of the giant fiber system, which is a small group of neurons mediating the light-off escape response in flies. Each giant interneuron sends an axon from the brain to the second

thoracic segment, where it forms a synapse with a motor neuron. In this system, *Sema1a* can act both as a ligand and as a receptor. In fact, while *Sema1a* forward signals are required for the correct pathfinding of giant fiber axons, the appropriate formation of the giant synapse in the thorax requires the reverse signaling of the semaphorin on the pre-synaptic membrane, acting as a receptor for undetermined counterparts on the post-synaptic side. This function indeed requires the cytoplasmic domain of *Sema1a* and likely its association with Enabled protein [21]. Enabled family proteins are known to regulate actin dynamics and have been involved in the modulation of both axonal and dendritic growth cone motility. In the giant fiber system, this interaction may serve to relay growth cone navigation and allow the establishment of a functional giant synapse.

*Sema1a* furthermore plays an important role during *Drosophila*'s olfactory system development. Here, axons of a single class of olfactory receptor neurons (ORNs) form synapses with a single class of projection neurons (PNs). *Sema1a* knock-out in PNs causes severe defects in dendrite (and axon) targeting, which are not rescued by the re-expression of a mutant form of *Sema1a* lacking the cytoplasmic domain, thereby suggesting that in this context the semaphorin is acting as a receptor [11]. Notably, at a later stage of development, *Sema1a* acts instead as a repulsive ligand for PlexinA to mediate interaction between different classes of ORN axons [22].

These examples show how many different outputs can be mediated by a single semaphorin signal in different cellular contexts. Within the same system, *Sema1a* can act both as a ligand and as a receptor, depending on the cellular type and/or on the stage of development. Moreover, since the intracellular partners of *Sema1a* can vary from one cell type to another, the signaling output can be either repulsion or attraction, depending on the modulated pathway.

## **Regulation of axon guidance in vertebrates: complex interplay between transmembrane semaphorins and plexin molecules, *intrans* and in *cis* regulatory mechanisms**

In vertebrates, there are 13 different transmembrane semaphorins, divided into 3 subclasses. As seen in flies, also in this case transmembrane semaphorins are involved in the regulation of different processes during neural development. For instance, developmental analyses of mice deficient for *Sema5A* and *5B* revealed that both semaphorins provide repulsive guidance signals for extending neurites, critically involved in retinal neural circuit formation [23]. Moreover, class-5 semaphorins differentially guide axonal navigation of dorsal root ganglia (DRG) sensory neurons. In particular, *Sema5B* is reported to act as a barrier in the spinal cord for extending DRG axons, preventing their premature entry in the dorsal grey matter [24]. A recent work by Browne and colleagues reports that *Sema5B* can be cleaved and shed from the plasma membrane by metalloproteases; notably, the soluble molecule is even more repulsive than the transmembrane one, which indicates a forward signaling mechanism [25]. On the other hand, Masuda and colleagues showed that *Sema5A* is an attractive cue for DRG axons at early stages of embryonic development [26]. Actually, *Sema5A* can also act as a bifunctional guidance cue, exerting both attractive and repulsive effects on certain developing axons. This has been described by Kantor and colleagues in the development of the fasciculus retroflexus (FR) in the diencephalon [27]. In this context, *Sema5A* activity is conditioned by its interaction with either chondroitin sulfate proteoglycans (CSPGs) or heparan sulfate proteoglycans (HSPGs). In fact, HSPGs act as *Sema5A* co-receptors on FR axons, mediating an attractive function, whereas—in a different diencephalic district—CSPGs expression converts *Sema5A* to an inhibitory guidance cue [27].

Axonal extension in the spinal cord is also regulated by *Sema6A*. In fact, the latter is expressed by boundary cap cells (BCCs), and it is required for their clustering in entry and exit sites of the spinal cord, where they form the boundary cap (a transient structure acting as a gate keeper between PNS and CNS). In particular, motor axons leaving the ventral spinal cord express PlexinA1, which is recognized by *Sema6A* expressed on BCCs. In this context, it was reported that *Sema6A* acts as a receptor and induces the aggregation of BCCs at the appropriate ventral motor axon exit point, in order to form the boundary cap and preventing motor neuron mislocalization in the periphery [12]. Moreover, *Sema6A* expressed by BCCs could provide a forward repulsive signal for motor axons, acting as the ligand for PlexinA2. One of the downstream effectors involved in this pathway is MICAL3, a protein belonging to a conserved family of cytoskeletal proteins, known to interact with PlexinAs [28].

Given its precise laminar pattern, the hippocampus is a complex system in which repulsive and attractive cues play an important role to control lamina-restricted axonal termination (thereby afferents from various brain regions will project to specific laminae). Here we can find an example of a complex interaction between Sema6A and 6B on one hand, and PlexinA2 and A4 on the other, in the regulation of lamina-restricted projection of hippocampal mossy fibers. Mossy fibers are the axons of granule cells of the dentate gyrus, that project into specific subdivisions of the hippocampal area CA3. In PlexinA2 knock-out mice, mossy fibers invade the infrapyramidal region of CA3c and the stratum pyramidale of CA3ab instead of growing into the suprapyramidal region. In PlexinA4 knock-out mice, mossy fibers invade most parts of CA3, including their normal target site in the suprapyramidal region. The knock-out of Sema6A, which is known as ligand for both PlexinA2 and PlexinA4, does not result in abnormalities in the projection of mossy fibers, and surprisingly it rescues the phenotype of the PlexinA2 knock-out phenotype. These results suggested a non-conventional signaling mechanism, which was elucidated taking into account the expression patterns of these genes in the hippocampus [29]. PlexinA2 is preferentially expressed on pyramidal cell dendrites, while PlexinA4 is present on extending mossy fibers (Fig. 2). Sema6A is highly expressed in the suprapyramidal and infrapyramidal regions of CA3, where it acts as a repulsive cue for PlexinA4-expressing mossy fibers, which explains why PlexinA4-deficient fibers invade all CA3 regions. However, Sema6A-mediated repelling signals are attenuated by PlexinA2 co-expression in the proximal part of suprapyramidal CA3 area, allowing mossy fibers to invade this region. It remains unclear whether the interaction between PlexinA2 and Sema6A mainly takes place in *cis* (on the surface of the same cell), or in *trans* between different pyramidal cells (leading to Sema6A internalization); either mechanism could in fact override Sema6A repelling activity on incoming PlexinA4-expressing mossy fibers [29]. Notably, this modulatory function of PlexinA2 is only seen in the presence of Sema6A, and the Sema6A/PlexinA2 double knock-out does not show aberrant mossy fibers projections, which suggests the presence of additional PlexinA4-binding repellents in CA3. One potential candidate is Sema6B, which is reported to repel sympathetic axons through the interaction with PlexinA4 [30]. The analysis of additional knock-out mice has demonstrated that Sema6B expressed in CA3 pyramidal cells plays an important role as repellent for mossy fibers, and this function is mediated by PlexinA4. Differently from what is seen for Sema6A, PlexinA2 expression is unable to modulate Sema6B activity, probably due to a lower binding affinity [31]. Taken together, these studies demonstrate that Sema6A and Sema6B provide repulsive cues for PlexinA4 expressing mossy fibers, and they are fundamental for their projection to the appropriate target laminae. Moreover, they provide a clear example of how a plexin molecule (PlexinA2, in this case), instead of mediating intracellular signaling, could associate *in cis* with a semaphorin and prevent its interaction in *trans* with receptors expressed by adjacent cells.

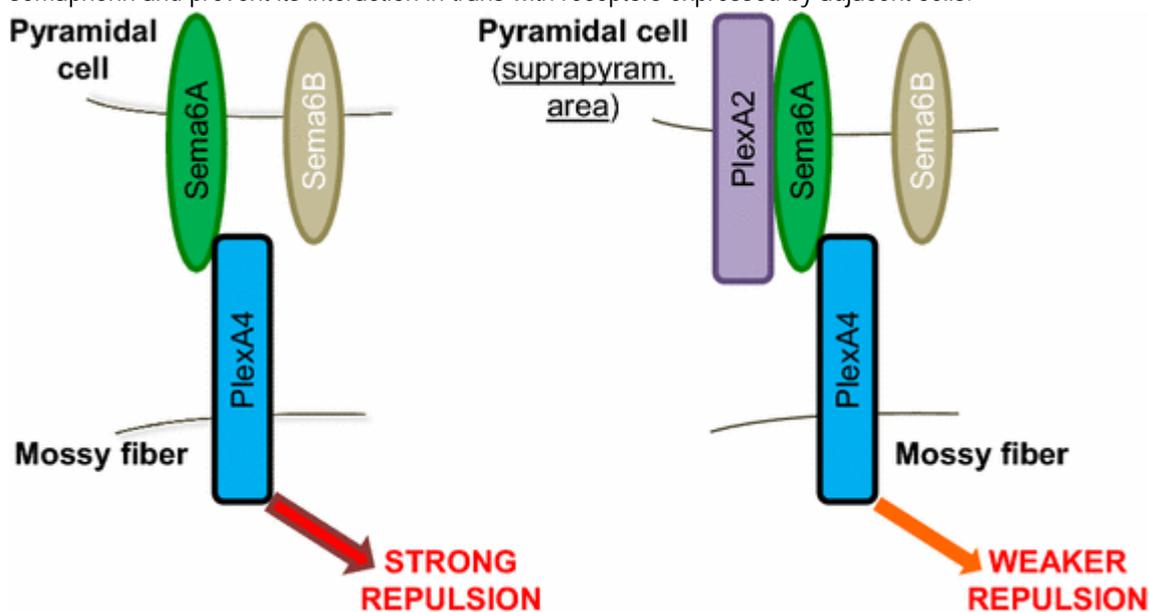


Fig. 2

PlexinA2 co-expression suppresses Sema6A-induced repulsion of extending axons in the hippocampus. Sema6A is highly expressed in dendrites of pyramidal neurons of the hippocampus, where it acts in *trans* as a repellent for extending PlexinA4-expressing axons (mossy fibers). However, Sema6A-mediated repelling signals are attenuated by PlexinA2 co-expression in

suprpyramidal CA3 area, allowing mossy fibers to invade this region [29, 30]. This modulatory function of PlexinA2 is only seen in the presence of *Sema6A*; the homologous semaphorin *Sema6B* expressed in pyramidal areas is considered a likely alternative repulsive signal, also acting via the PlexinA4 receptor, but independently of PlexinA2 regulation [31]

Besides the hippocampus, another vertebrate structure that is organized in laminae is the retina. Here two distinct synaptic regions, the outer and the inner plexiform layers, are interposed with three retinal cell body layers. The Inner Plexiform Layer (IPL) contains the synapses between the axons of bipolar cells and the dendrites of ganglion and amacrine cells. The IPL displays a further sublaminal organization, in order to spatially separate the ON pathway (that responds to an increment in illumination) and the OFF pathway (that responds to decreased illumination). Notably, *Sema6A* also acts as repulsive cue in retinal development via its receptors PlexinA2 and PlexinA4, selectively expressed by specific subsets of amacrine cells. In fact, *Sema6A* expression is restricted to cells of the ON sublamina, while PlexinA4 is expressed in the OFF sublamina, and PlexinA2 is expressed in both. Thus, *Sema6A* act as a repulsive barrier for dopaminergic amacrine cells expressing PlexinA4 and constrains their dendritic arborization in the OFF sublaminae [32]. In this model, PlexinA2 also mediates *Sema6A*-dependent repulsion of dendritic stratification of OFF lamina starburst amacrine cells away from the adjacent layer. Remarkably, *Sema6A*-expressing cells of the ON lamina also express PlexinA2; however, they are insensitive to the repulsive activity of this semaphorin presented by other cells in the same layer, possibly because PlexinA2 receptor signaling is blocked by the interaction in *cis* with its own ligand [33] (Fig. 3).

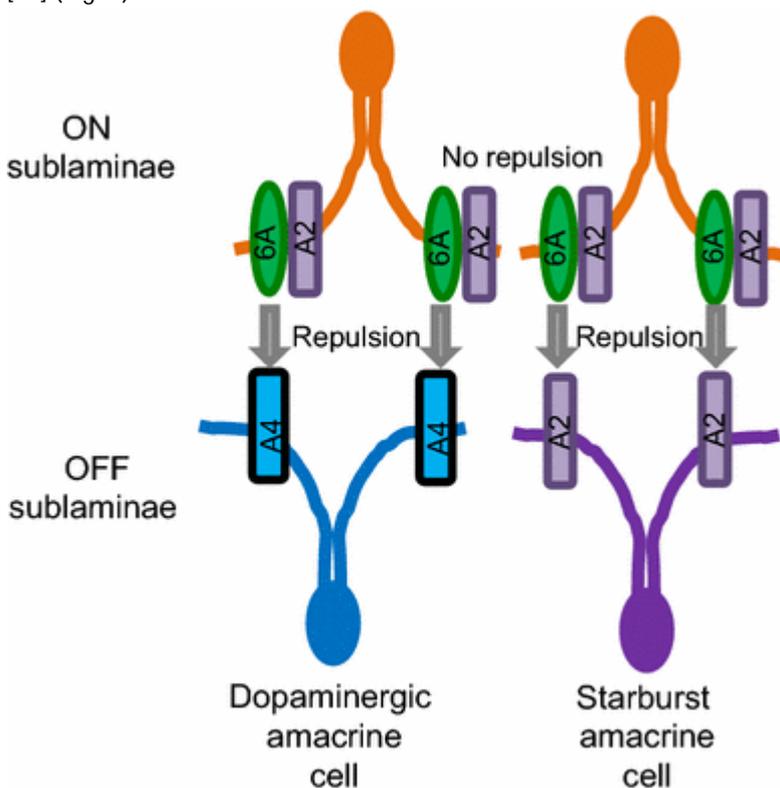


Fig. 3

In *cis* interaction between *Sema6A* and PlexinA2 blocks the repulsive response to *Sema6A*. During retinal development, *Sema6A* expressed by starburst amacrine cells of the ON sublamina (represented in *orange*) acts as a repulsive barrier for amacrine cells expressing PlexinA4 or PlexinA2, constraining their dendrite arborization in the OFF sublamina. However, neural projections of cells within the ON sublamina, characterized by co-expression of *Sema6A* and PlexinA2, are not sensitive to the repelling activity of the semaphorin [32, 33]

In a complementary setting, also a transmembrane semaphorin was found to modulate the function of a plexin in *cis*. In fact, although both express PlexinA4 receptor, sympathetic neurons are repelled by *Sema6A* while DRG sensory

neurons are not. Notably, unlike sympathetic neurons, DRG neurons strongly co-express Sema6A with its receptor. Moreover, upon Sema6A knock-out, DRG neurons become responsive to exogenous Sema6A [34]. This suggests that Sema6A expression on the axonal membrane of DRG neurons can attenuate the inhibitory response to Sema6A presented in *trans* by surrounding cells (Fig. 4). Studies conducted in heterologous systems demonstrated that Sema6A and PlexinA4 can form a complex in *cis* on the cell surface, which prevents the binding of soluble Sema6A to the receptor. Interestingly, Haklai-Topper and colleagues showed that also Sema6B can associate in *cis* with PlexinA4, and block the response to Sema6A. Furthermore, they showed that in *cis* and in *trans* interactions with Semas require different plexins domains [34].

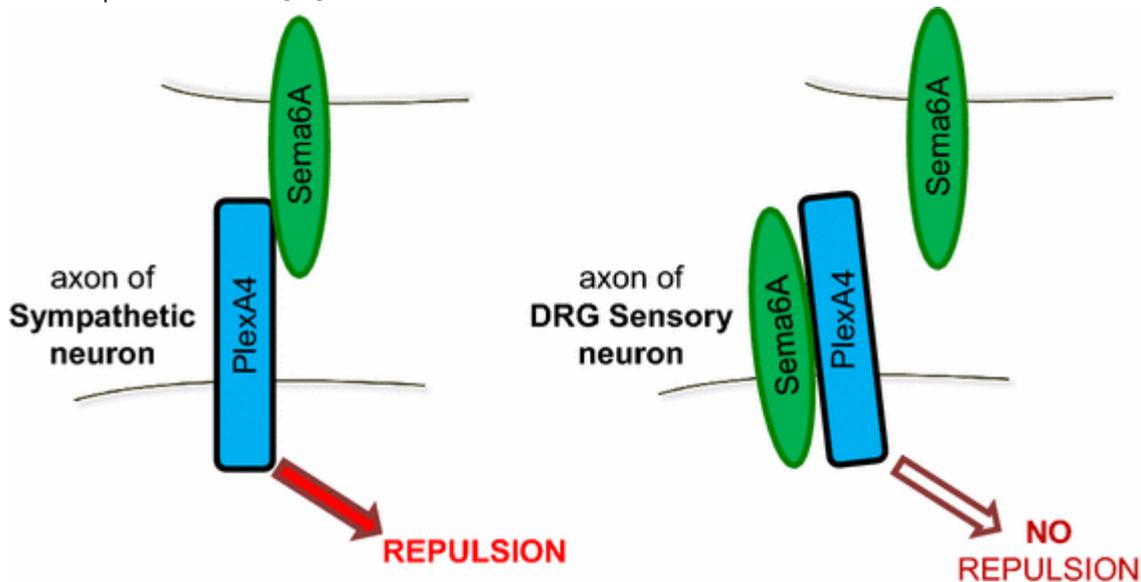


Fig. 4

Sema6A co-expression in *cis* suppresses PlexinA4 receptor signaling. Sympathetic neurons are repelled by Sema6A, signaling in *trans* via PlexinA4. By contrast, DRG neurons strongly co-express Sema6A, which forms a complex on the cell surface with its receptor in *cis*; this impairs PlexinA4 signaling in response to the ligand expressed in other cells. Notably, upon Sema6A knock-out, DRG neurons become responsive to exogenous Sema6A [34]

Recently, Andermatt and colleagues reported a dual role for Sema6B in commissural axon guidance during chick embryos development. In this context, Sema6B can act both as a receptor for PlexinA2 in post-crossing commissural axons, and as an inhibitor of repulsive class-3 semaphorin signaling. In fact, PlexinA2 is expressed by both commissural axons, and floorplate cells at the CNS midline, located where commissural axons cross to the contralateral side and turn rostrally along the spinal cord. Sema6B is transiently expressed in commissural axons during the window of axonal midline crossing and turning. In the vicinity of the floorplate, Sema6B and PlexinA2 can interact in *cis* on pre-crossing commissural axons; this might serve to prevent the premature response of PlexinA2 to repulsive class-3 semaphorins, which could interfere with midline crossing. At the floorplate, this *cis* interaction, however, is replaced by an *in trans* interaction of axonal Sema6B with floorplate-expressed PlexinA2, which induces axon turning. This effect depends on Sema6B cytoplasmic tail; in fact, the sole extracellular domain of PlexinA2 is sufficient to rescue plexin silencing effects, suggesting that the semaphorin is acting as an axon guidance receptor. Moreover, Sema6B-PlexinA2 interaction in *trans* now allows axonal response to class-3 semaphorins, facilitating post-commissural egression trajectory [13]. An alternative mechanism found to prevent response to class-3 semaphorins in the spinal cord is the calpain-dependent degradation of PlexinA1 cytoplasmic tail in pre-crossing commissural axons [35]. It is not presently known whether a similar regulatory mechanism could also apply to other plexins, such as PlexinA2 which may be involved.

From these studies, the complexity of semaphorin-plexin signaling is clearly evident in the vertebrate nervous system. In particular, it is remarkable to note how the "classical" repulsive function mediated by semaphorins can be modulated by *cis* interactions with plexins, resulting in an attenuated response. Interestingly, there is at least one case in which an

interaction in *cis* between a semaphorin and its plexin receptor results in the activation of the plexin itself: in fact, in *C. elegans* motor neuron axons, the transmembrane semaphorin SMP-1 activates in *cis* the PlexinA4 homolog PLX-1, regulating its subcellular localization and finally resulting in inhibition of synapse formation [36].

## Transmembrane semaphorins in oligodendrocyte and Schwann cells development

Oligodendrocytes are a type of neuroglia found in the CNS, mainly responsible for forming the myelin sheath which wraps neuronal projections; their homologs in the PNS are Schwann cells. Semaphorins have been shown to modulate both glial cells proliferation and their differentiation, which results in a proper myelination of axons. Moreover, interactions between axons and glial cells can influence myelination and axon guidance, and semaphorins can play an important role in this cross-talk.

For example, *Sema5A* is expressed by oligodendrocytes and their precursors among the optic glial cells. It was demonstrated that this semaphorin induces growth cone collapse and inhibits axon growth of retinal ganglion cells (RGC) [37]. This could be relevant for the guidance of navigating RGC axons, but could also mediate the function of glial cells in CNS to inhibit axonal regeneration upon injury. The receptor for *Sema5A* responsible for these effects has not been clearly identified yet, although this is likely *PlexinB3*, which is expressed in the white matter of the nervous system [38].

*Sema6A* is expressed at high levels during oligodendrocyte development, peaking during myelination. Oligodendrocytes from *Sema6A* knock-out mice show delayed differentiation both *in vivo* and *in vitro*, and they fail to properly myelinate axons. Interestingly, this delayed differentiation of *Sema6A*-deficient oligodendrocytes is not rescued by the addition of exogenous *Sema6A* *ex vivo*, indicating a potential reverse signaling mechanism, to be elucidated. Moreover, the knock-out of *PlexinA2* or *PlexinA4* has no effect on oligodendrocyte differentiation, suggesting a role for alternative *Sema6A*-interacting molecules, currently unknown [14].

For what concerns class-4 semaphorins, *Sema4D* is typically expressed by oligodendrocytes in CNS. Moreover, its expression is strongly induced upon CNS injury [39]. Notably, *Sema4D* knock-out mice display an increased number of oligodendrocytes in the adult cerebral cortex, which is not due to increased proliferation of their precursors, but rather due to decreased oligodendrocyte apoptosis [40]. This effect is reverted by the addition of soluble *Sema4D*, suggesting that the semaphorin is acting as a ligand in this context, triggering oligodendrocyte apoptosis in autocrine and juxtacrine manner, via a still undetermined receptor.

Another class-4 semaphorin, *Sema4F*, was found to preserve the physiological interaction between axons and Schwann cells in the adult stage. Schwann cells depend on signals from axons for proliferation, survival and differentiation. An impairment of this control is seen in Neurofibromatosis type 1, a genetic disorder characterized by the neoplastic transformation of Schwann cells, caused by the loss of *NF1* gene. The latter encodes neurofibromin, a protein with a regulatory RasGAP domain, and its loss results in the hyper-activation of the Ras/Raf/Erk pathway [41]. The Lloyd lab found that this pathway leads to the downregulation of *Sema4F* expression in Schwann cells. The signaling downstream to *Sema4F* normally is activated in the adhesive interaction between Schwann cells and axons (although semaphorin-binding counterpart is still unknown here); its downregulation in *NF1* syndrome results in a loss of heterotypic contact inhibition of Schwann cells proliferation [41], leading to neoplastic growth.

## Class-6 semaphorin forward and reverse signaling in cardiac development

Heart morphogenesis is a complex developmental process that implicates reciprocal interaction of different cell types. In fact, the heart forms from a tube of endocardial cells, derived from a section of the primitive endothelial vascular plexus, which is then wrapped by an outer layer of myocardial cells. Then, upon a bending of this primary tube, it is possible to identify atrial and ventricular chambers, together with an outflow tract, deriving from the distal conotruncal segment of the developing heart; this will originate the arterial trunks emerging from both ventricles. At this point complex events

contribute to chamber formation, a process facilitated by migration of neural crest cells from the dorsal neural tube into the cardiac outflow tract. After septation of the tube, the four cardiac chambers are formed and progressively acquire their definitive functional morphology. In the ventricle, the two major maturational steps are the expansion of the myocardial layer and its trabeculation at the endocardial side [42, 43].

The role of class-6 semaphorin signaling in cardiac development is addressed by studies of Toyofuku and colleagues, which show that knockdown of either *Sema6D* or *PlexinA1* affects cardiac morphogenesis in chick [7, 44]. In fact, *Sema6D* plays a dual role in modulating cell migration during heart development, acting as a ligand in endothelial cells (which form the endocardial layer and heart chambers) and as both receptor and ligand in myocardial cells (which are recruited to build heart muscular structure). Moreover, *Sema6A* and *6B*, and their receptor *PlexinA2*, were found to provide a forward repulsive cue required for the precise migration of cardiac neural crest cells in the heart outflow tract [45].

*Sema6D*, acting as a ligand, controls endothelial cell migration in opposite ways depending on the region of the developing cardiac tube: it is an attractive cue for cells of the conotruncal segment, while it is repulsive for cells of the ventricle. *PlexinA1* is the receptor for *Sema6D* in both cases, but the divergent effects on cells from different regions are accounted by different transmembrane molecules associated in complex with *PlexinA1*. In particular, the repulsive activity is mediated by a receptor complex formed by *PlexinA1* and OTK (Off-Track Kinase), while in the conotruncal segment *PlexinA1* is associated with VEGFR2 kinase; here, the binding of *Sema6D* elicits VEGFR2 signaling, which promotes endothelial cell migration [44].

The role of *Sema6D*–*PlexinA1* signaling in myocardial cells is demonstrated by the fact that knockdown of either gene in chick embryos results in a reduced expansion of the primitive ventricle and poor trabeculation of muscular layer [7]. By means of rescue experiments, Toyofuku and colleagues demonstrated that forward signaling through *PlexinA1* is required for ventricular expansion, while trabecular formation (a process that requires the migration of myocardial cells from the compact layer towards the endocardium) is regulated by *Sema6D* reverse signaling. Using *in vitro* heterologous systems, it has been demonstrated that in absence of *PlexinA1* the cytoplasmic portion of *Sema6D* can bind both Abl kinase (thanks to a proline-rich sequence recognized by SH3 domains) and Mena (through a zyxin-like domain). The interaction of *Sema6D* with *PlexinA1* enhances the binding of Abl kinase to *Sema6D*, resulting in the phosphorylation of this kinase. Abl can then phosphorylate Mena, inducing its dissociation from *Sema6D*. In this way, the suppressive effect of Mena on cell migration is removed, resulting in an increased migration of myocardial cells that contribute to trabeculae formation [7] (Fig. 5). It is important to note that the above works focused on chick embryo phenotypes. However, in mice, *Sema6D* or *PlexinA1* deficiency does not cause cardiac defects during development [46], suggesting that other class-6 semaphorins and plexins could substitute for their functions. For example, it was shown that the phenotype of *PlexinA2* knock-out in chick cardiac neural crest cells differs from what observed in the corresponding knock-out in mice due to the compensatory function of *PlexinA4* [45].

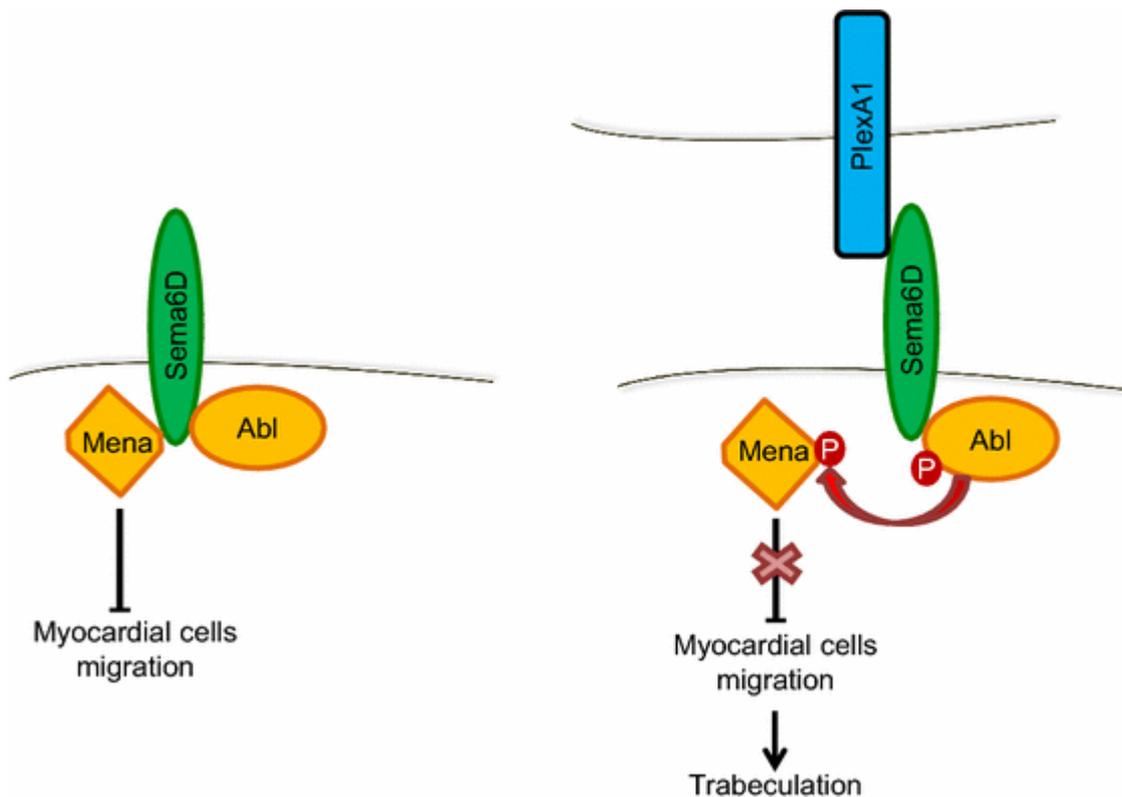


Fig. 5

In myocardial cells, the Sema6D cytoplasmic tail can bind to Abl and Mena. The interaction with PlexinA1 enhances Abl binding and activation, which results in Mena phosphorylation and dissociation from Sema6D. This removes the inhibitory effect of Mena on cell migration, which is fundamental for heart wall trabeculation [7]

### Transmembrane semaphorin functions in adult organisms

While the importance of transmembrane semaphorins in embryo development is well established, their functional role in the adult stage needs further investigation, though these molecules have been shown to play diverse roles in the regulation of angiogenesis, immune responses and cancer. In these processes, semaphorin-mediated forward signaling has mainly been implicated (though the underlying mechanisms have been elucidated only in part), while the potential role of reverse signaling has not been thoroughly addressed. Here we will provide an overview of the main transmembrane semaphorins involved in these functions, describing some examples of their downstream signaling pathways.

The anatomical similarities between the nervous and vascular systems suggests that the same factors playing a role as axon guidance cues may also direct the migration of endothelial cells and guide angiogenesis. Indeed, this is well established for soluble class-3 semaphorins [47, 48]; moreover, among transmembrane family members, Sema4D, Sema5A and certain class-6 semaphorins have been found to regulate angiogenesis, both in physiological context and in cancer [49–55]. In case of tumor-induced angiogenesis, neoplasms can produce (and also release in soluble form) Sema4D, which can then activate PlexinB1 expressed by endothelial cells. This results in RhoA activation and in a downstream signaling cascade which finally leads to Akt and Erk1/2 activation, a pro-angiogenic response necessary for endothelial cell migration [53]. Moreover, PlexinB1 has been shown to form a complex with Met receptor on the cell surface, and Sema4D-mediated PlexinB1 activation can elicit Met tyrosine kinase activity, resulting in a pro-angiogenic effect [54].

Also Sema6A and Sema6B have been described as pro-angiogenic factors. In particular, Sema6A can sustain VEGF/VEGFR2 signaling in endothelial cells, thereby promoting their survival and exerting a pro-angiogenic effect

during development and in tumor angiogenesis [51]. Sema6B was found to promote endothelial cell proliferation, through the PlexinA4 receptor and its association with FGFR1, FGFR2, and VEGFR2 tyrosine kinases, enhancing bFGF and VEGF pro-survival signaling pathways [52].

Cell-to-cell contacts are fundamental for the establishment of proper interactions among immune cells, and several studies have revealed that semaphorins and plexins can regulate the outcome of immune responses. Transmembrane semaphorins involved in this process are mainly Sema4A, Sema4D and Sema6D, and their functions have been recently reviewed by Roney and colleagues [56]. The receptors involved are Tim-2 and PlexinD1 for Sema4A [57, 58], CD72, PlexinB1 and B2 for Sema4D [59–61], PlexinA1 for Sema6D [62].

Recent studies have also focused on the pathological aspects of semaphorin signaling connected with hallmarks of cancer, such as tumor cell survival and invasiveness, as well as tumor angiogenesis mentioned above [1]. In particular, the role of Sema4D forward signaling in cancer progression has been extensively studied, as recently reviewed by Ch'ng and Kumanogoh [63]. Sema4D activity in cancer has been mainly linked to PlexinB1 signaling, not only through its cytoplasmic domain, but also via plexin-associated oncogenic receptor tyrosine kinases, such as ErbB2 and Met [64]. In these studies, a clear role of Sema4D reverse signaling was not seen, and in most cases the effects could be mediated by diffusible Sema4D ectodomain released by proteolytic cleavage.

Other transmembrane semaphorins with emerging roles in the cancer field are Sema5A, Sema5B, Sema6A, Sema6B and Sema6D [65–75]. Sema5A expression has been associated with increased growth and metastatic potential in pancreatic and gastric cancer [65, 67], while it was found to inhibit glioma cell motility [70]; these divergences may be accounted by distinctive receptor complexes. Ectopic expression of Sema5A in a pancreatic cancer cell line enhanced tumorigenesis and metastasis *in vivo*, as well as proliferation and invasiveness *in vitro* [65]. Intriguingly, the Sema5A extracellular domain was sufficient to drive cancer cell invasion and metastasis, possibly through an induction of Erk activity; however, it was unable to induce tumor growth, suggesting in this case the potential involvement of a reverse signaling mechanism [66]. Also in gastric cancer, Sema5A can promote invasion and metastasis possibly via the receptor PlexinB3 [67]. However, a Sema5A-dependent inhibition of glioma cell motility mediated by PlexinB3 was also demonstrated, which involved the disassembly of stress fibers and focal adhesions [69]. These effects are mediated by fascin-1, an actin-binding and bundling protein that can interact with the cytoplasmic domain of PlexinB3 and become activated upon Sema5A binding [70].

As concerning class-6 semaphorins in cancer, in a recent work it was shown that Sema6A is overexpressed in BRAF\_V600E-mutated melanomas, but not in NRAS\_Q61R-mutated ones [72]. Moreover, in BRAF-mutated melanoma cells, Sema6A is required to sustain the activity of Akt and Erk pathways, and its depletion induces cell apoptosis, suggesting a role in BRAF-mutant melanoma survival. Sema6A silencing reduces anchorage-independent growth and invasion of BRAF\_V600E cells, whereas its overexpression in NRAS\_Q61R melanoma cells leads to acquisition of the ability to grow in soft agar, to invade and metastasize *in vivo*. The Sema6A receptors and the downstream signaling which would account for this function are still unclear [72]. Also Sema6D and its receptor PlexinA1 have been shown to exert pro-tumorigenic effects in malignant pleural mesothelioma. In this context, PlexinA1 is associated with VEGFR2, and the presence of Sema6D triggers its tyrosine phosphorylation, inducing NF- $\kappa$ B transcriptional activity, that ultimately mediates tumor cell survival [73]. Thus, also in this case, a semaphorin forward signaling cascade can be postulated.

## **Multifaceted signaling of class-4 semaphorins**

Most transmembrane semaphorins fall into class 4. Compared to other family members, the developmental role of these semaphorins is less understood, but they have been implicated in a range of functions in the adult. Class-4 semaphorins have been found to act mainly through PlexinB1 and PlexinB2 receptors in a forward signaling mode. Notably, these plexins can also form receptor complexes with transmembrane tyrosine kinases (Met, Ron and ErbB2) and elicit their transactivation [3]; as a consequence, class-4 semaphorins can mediate multiple signaling and functional outputs. These include inhibiting integrin function and cell migration [76, 77], as well as promoting immune responses, angiogenesis, tumor cell invasiveness and metastasis [63, 78, 79]. The extracellular moiety of some of these transmembrane

semaphorins was found to be shed in the extracellular milieu due to proteolytic cleavage (possibly mediated by metalloproteases), and it was demonstrated that this secreted fragment is still competent to mediate a number of functions, consistent with forward signaling mechanisms.

Class-4 semaphorins, however, also contain a relevant cytoplasmic domain. In four out of six members, the C-terminus of the protein ends with PDZ-domain interaction motifs. PDZ domains were originally described in post-synaptic density protein PSD-95/SAP90; these protein-protein interaction domains mediate the clustering and organization of neurotransmitter receptors and regulators in neuronal post-synaptic membranes, and -in general- serve as scaffolds for the assembly of multi-molecular signaling complexes. Notably, three different class-4 semaphorins have been shown to co-localize and interact with PSD-95/SAP90: Sema4C in cerebral cortical neurons [80], Sema4B and Sema4F in hippocampal neurons [6, 81]; the C-terminal consensus sequence is required for this localization, but clear evidence for a functional role of the semaphorins in this context is still lacking. Notably, Sema4C has been found to interact with another PDZ domain-containing protein, SEMCAP1/GIPC, which is not typically found in synaptic sites; however, also in this case, the association has merely been demonstrated to control semaphorin localization, so far [82].

The role of class-4 semaphorins, as well as of other family members, in synaptogenesis are well described in a review by Koropouli and Kolodkin [83]. For instance, an RNAi-based screening identified Sema4B and Sema4D as key regulators of synapse development [84]; in particular, these semaphorins are required for the postsynaptic specialization of glutamatergic and GABAergic synapses, respectively. Notably, Sema4D does not contain a PDZ-domain interaction motif at its C-terminus, and indeed it was later discovered that the effect of Sema4D on GABAergic synaptogenesis relies on a forward signaling mechanism [85, 86]. In fact, treatment of cultured hippocampal neurons with the soluble extracellular domain of Sema4D is sufficient to increase the density of functional GABAergic synapses, in a PlexinB1-dependent manner [85]. Moreover, a mutant form of Sema4D lacking the cytoplasmic domain is able to rescue the decrease in GABAergic synapse density induced by Sema4D silencing, while another mutant lacking the extracellular portion of the semaphorin is not [86].

Beyond the nervous system, Sema4C has been found to play a role in myogenic differentiation. Its expression is increased both during the differentiation of C2C12 murine myoblasts in myotubes, and upon muscle regeneration after injury in mice. Both Sema4C silencing and blocking its PDZ domain-binding motif results in inhibition of myogenic differentiation, thereby suggesting that Sema4C-mediated reverse signaling is an important player in the context of muscle development [87]. Notably, interaction with scaffolding PDZ domains could also serve to cluster and localize transmembrane semaphorins on the cell surface, rather than mediate their intracellular signaling. Unfortunately, the protein counterpart engaging the extracellular domain of Sema4C (possibly a plexin) has not been identified in this context.

Interestingly, although Sema4D does not contain a PDZ-domain interaction motif, there are many indications suggesting its reverse signaling mode (acting as a receptor) in particular in the immune system. In T cells, Sema4D was initially reported in association with an intracellular serine kinase, yet to be identified [88], and with CD45, a cell surface protein tyrosine phosphatase involved in TCR activation [89]. The association between Sema4D and CD45 was also reported in B cells [90]. One report describes the role of Sema4D as PlexinB1-receptor in sustaining proliferation of both normal and leukemic B lymphocytes [15]. It was experimentally demonstrated that the viability of Sema4D-expressing cells is increased in presence of PlexinB1-expressing cells, suggesting that Sema4D may act in *trans* as a receptor for the plexin, but the molecular mechanism has not yet been elucidated.

Notably, it was shown that in certain settings, Sema4D signaling does not necessarily involve interactions with plexins. For instance, in hematopoietic cells, a major interactor in *trans* of membrane-associated Sema4D is the inhibitory receptor CD72 [59]. In an analogous manner, transmembrane Sema4A expressed in dendritic cells was found to interact with the inhibitory receptor Tim2 found in lymphocytes [91].

## Open questions and concluding remarks

Transmembrane semaphorins represent the majority of family members, and yet we know relatively little about their functions and signaling mechanisms compared to secreted paralogs. This is partly accounted by the relative greater complexity and multiplicity of the signaling modes that can be deployed by transmembrane semaphorins. In addition, while experimental assays *in vitro* are well fit to test activities and study signaling cascades elicited by soluble factors added in the extracellular milieu, it is more difficult to investigate signaling events based on localized cell-to-cell contacts. Actually, certain transmembrane semaphorins are also known to undergo proteolytic cleavage, releasing the extracellular domain from the cell surface. It is commonly believed that this extracellular moiety can then diffuse and act likewise secreted semaphorins, triggering a forward signaling cascade, e.g. through specific plexin receptors. This is clearly demonstrated for Sema4D, but is actually not so well documented for other semaphorins. Moreover, even for Sema4D, it is not so clear whether the transmembrane and secreted isoforms have distinctive functional roles, or simply represent a localized and a diffusible version of the same signal. In general, it would be interesting to assess the effect of semaphorin shedding for the reverse signaling mode. In principle, the effect could be to selectively switch off this signaling pathway, especially if the cytoplasmic domain would be degraded after cleavage. This aspect could be relevant when bidirectional signals are elicited at the same time upon cell–cell contact.

As discussed in this review, when anchored in the lipid bilayer, transmembrane semaphorins have been found to associate in *cis* with plexin molecules at the cell surface, forming complexes which do not seem to trigger signaling cascades, but rather result in signaling attenuation for either of the components engaging adjacent cells in *trans*. The underlying mechanisms are not fully elucidated, however, there are at least two possible explanations: in *cis* interactions can sterically hinder ligand-receptor binding in *trans*, or they could promote endocytosis of the full complex, thereby removing signaling molecules from the plasma membrane.

Transmembrane semaphorins possess the peculiar ability to act as bidirectional cues, triggering both forward and reverse signaling. In this review, we have described several examples of semaphorin reverse signaling (summarized in Table 1), however, several issues deserve further investigation. First, the “ligand” molecule triggering semaphorin reverse signals is often unknown, and it is unclear whether only extracellular domains of plexins may be involved. For instance, it is known that class-4 semaphorins can interact with other receptor molecules beyond plexins in a forward signaling mode, and this could possibly implicate a similar setting for reverse signaling as well. Second, downstream effectors of semaphorin cytoplasmic tails are poorly understood. The cytoplasmic domains of transmembrane semaphorins show considerable divergence, even within the same subclass, and conserved sequence motifs have rarely been identified. Intriguingly, the functional role of PDZ-domain binding sequences, identified in the cytoplasmic domains of many class-4 semaphorins, remains unclear. These sequences may bind scaffolding molecules and serve to localize semaphorins in specific regions of the plasma membrane, or promote protein clustering on the cell surface, but they could also directly recruit intracellular effectors.

Table 1

## Transmembrane semaphorins' receptor functions

Receptor	Ligand	Effector	Function
Sema1a	unknown	Pebble/RhoGap p190	Motor axon defasciculation [18]
Sema1a	PlexinA	Moesin	Photoreceptor neurons axon fasciculation [19,20]
Sema1a	unknown	Enabled?	Gyant synapse formation [21]
Sema1a	unknown	unknown	Projection neurons targeting [22]
Sema4C	unknown	unknown	Myoblasts differentiation [87]
Sema4D	PlexinB1	unknown	B-lymphocytes proliferation [15]
Sema6A	PlexinA1	unknown	Boundary cap cells aggregation [12]
Sema6A	unknown	unknown	Oligodendrocytes differentiation [14]
Sema6B	PlexinA2	unknown	Post-commissural axon guidance [13]
Sema6D	PlexinA1	Abl/Mena	Trabecular formation [7]

In sum, transmembrane semaphorins are an interesting example of a complex and multifaceted signaling system which deserves better elucidation. Moreover, while the importance of transmembrane semaphorins in embryo development is well established, further studies will be needed to improve our understanding of their functional relevance in the adult, also revealing the signaling pathways that are involved.

## Acknowledgments

We are grateful to all Tamagnone lab members for advice and discussion. The work was supported by grants from Italian Association for Cancer Research (AIRC) (IG #2014-15179) and the Fondazione Piemontese per la Ricerca sul Cancro (FPRC-ONLUS) (Grant "MIUR 2010 Vaschetto-5 per mille 2010 MIUR").

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