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RESEARCH ARTICLE

The role of the plexin-A2 receptor in Sema3A and Sema3B signal transduction

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ABSTRACT

Class 3 semaphorins are anti-angiogenic and anti-tumorigenic guidance factors that bind to neuropilins, which, in turn, associate with class A plexins to transduce semaphorin signals. To study the role of the plexin-A2 receptor in semaphorin signaling, we silenced its expression in endothelial cells and in glioblastoma cells. The silencing did not affect Sema3A signaling, which depended on neuropilin-1, plexin-A1 and plexin-A4, but completely abolished Sema3B signaling, which also required plexin-A4 and one of the two neuropilins. Interestingly, overexpression of plexin-A2 in plexin-A1- or plexin-A4-silenced cells restored responses to both semaphorins, although it nullified their ability to differentiate between them, suggesting that, when overexpressed, plexin-A2 can functionally replace other class A plexins. By contrast, although plexin-A4 overexpression restored Sema3A signaling in plexin-A1-silenced cells, it failed to restore Sema3B signaling in plexin-A2-silenced cells. It follows that the identity of plexins in functional semaphorin receptors can be flexible depending on their expression level. Our results suggest that changes in the expression of plexins induced by microenvironmental cues can trigger differential responses of different populations of migrating cells to encountered gradients of semaphorins.

KEY WORDS: Cell guidance, Neuropilin, Plexin, Receptor complex, Semaphorin

INTRODUCTION

During embryonic development, cells migrate extensively to distant locations. Similar migrations, although much less extensive, take place in adult animals and in diseases such as in the various forms of cancer (Trepat et al., 2012). Migrating cells are guided to their correct destination by a variety of guidance cues, which include gradients of secreted repulsive and attractive guidance factors. Essential to the understanding of the mechanisms by which cells interpret guidance cues is the characterization of the receptors that interact with these guidance cues and of mechanisms that modulate the activity and expression of such receptors, because the activity of these receptors determines which signal transducing cascades will be activated downstream.

The semaphorins were identified as repulsive axon guidance factors (Kolodkin et al., 1993) but have also emerged as important regulators of angiogenesis, immune responses and tumor progression (Tamagnone, 2012; Kumanogoh and Kikutani, 2013; Sakurai et al., 2012). Class 3 semaphorins are a subfamily of secreted semaphorins containing seven members, of which six, with the exception of Sema3E (Gu et al., 2005), bind to one of the two neuropilin receptors. Neuropilins contain short intracellular domains that limit their signal transducing ability, and they associate with members of the class A plexin receptor subfamily or with plexin-D1 to transduce class 3 semaphorin signals. These plexins also function in addition as direct binding and signal transducing receptors for some membrane-anchored members of the class 4, class 5 and class 6 semaphorin subfamilies (reviewed in Neufeld et al., 2012; Gu and Giraudo, 2013).

Sema3A is an anti-angiogenic semaphorin that binds to neuropilin-1 (NP1) but not to neuropilin-2 (NP2) (Kolodkin et al., 1997; Chen et al., 1997; Acevedo et al., 2008). Sema3B is a lung cancer tumor suppressor (Tomizawa et al., 2001) that binds to both neuropilins and functions, in addition, as an inhibitor of angiogenesis (Varshavsky et al., 2008). The roles of specific class A plexins in Sema3B signal transduction have not been well characterized. Plexin-A1 was found to be required, along with the adhesion receptor NRCAM, for the transduction of Sema3B signals guiding commissural projections at the vertebrate ventral midline during the development of the nervous system (Nawabi et al., 2010), but the possible roles of additional plexins in Sema3B signaling have not been investigated. By contrast, Sema3A signal transduction has been studied more extensively. Studies conducted using knockout mice lacking functional plexin-A1, plexin-A3 or plexin-A4 receptors found evidence suggesting that these three receptors all play a role in Sema3A signal transduction (Yaron et al., 2005; Suto et al., 2005; Schwarz et al., 2008; Moretti et al., 2008; Bouvrée et al., 2012; Katayama et al., 2013). Interestingly, another study has suggested that plexin-A3 transduces exclusively pro-apoptotic signals of Sema3A but not signals affecting cytoskeletal organization (Ben-Zvi et al., 2008). Additional studies in which plexin-A2 was ectopically expressed at higher expression levels in cells have suggested that plexin-A2 can also transduce Sema3A signals (Janssen et al., 2012; Takahashi and Strittmatter, 2001). Taken together, these observations seem to suggest that all four class A plexins can transduce Sema3A signals, which, in turn, seems to suggest that for Sema3A signaling the class A plexins might be interchangeable. By extension, these studies suggest that the class A plexins might not display high specificity for any of the class 3 semaphorins. However, it should be noted that in most studies

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Received 2 May 2014; Accepted 13 October 2014

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Journal of Cell Science (2014) 127, 5240–5252 doi:10.1242/jcs.155960

using gene-targeted mouse models it is unclear whether the observed loss of responsiveness to Sema3A is due to a direct effect on Sema3A signaling, or whether the targeting affects the expression of a secondary modulator. Likewise, much of the information obtained from cell culture experiments is derived from experiments in which specific plexins were overexpressed, and thus might not be relevant physiologically (Takahashi and Strittmatter, 2001; Janssen et al., 2012).

In all these studies it is assumed that successful Sema3A signaling requires NP1 and a single plexin. However, we have recently observed that, in endothelial cells and in U87MG glioblastoma cells, Sema3A signaling affecting cytoskeletal organization requires the simultaneous presence of plexin-A1 and plexin-A4, which form spontaneous heterocomplexes. Inhibition of the expression of any one of these plexins but not of plexin-A3 is sufficient to completely inhibit the cytoskeletal collapse induced by Sema3A, suggesting that physiologically relevant functional Sema3A receptors consist of a complex containing NP1, plexin-A1 and plexin-A4 (Kigel et al., 2011). These observations suggest that specific combinations of plexins and neuropilins determine the specificity of receptor complexes to specific semaphorins and that the plexins are not interchangeable.

To gain a better understanding of the roles of the class A plexins in class 3 semaphorin signal transduction, and to clarify the possible role of plexin-A2 in Sema3A signaling, we studied in more detail the contribution of plexin-A2 as well as additional class A plexins to Sema3A and Sema3B signaling. We provide evidence suggesting that, in endothelial cells and glioblastoma cells, plexin-A4 is a required component of both Sema3A and Sema3B receptor complexes and inhibition of its expression nullifies both Sema3A and Sema3B signaling. The specificity for Sema3A or Sema3B is determined by the presence of plexin-A1 in Sema3A receptors and plexin-A2 in Sema3B receptors, and silencing each abrogates signaling by the appropriate semaphorin. Interestingly, when plexin-A2 was overexpressed in cells silenced for plexin-A1 or plexin-A4 expression it restored both Sema3A and Sema3B signaling, suggesting that the requirement for specific plexins might be abolished if the expression levels of some of the plexins exceed certain threshold levels and further suggesting that when highly expressed the different plexins are functionally interchangeable. This comes at a price, as such cells lost their ability to distinguish between Sema3A and Sema3B. However, this might not always be true, given that plexin-A4 overexpression failed to restore Sema3B signaling in plexin-A2-silenced cells.

RESULTS

Plexin-A2 is not required for Sema3A signal transduction but is required for that of Sema3B and Sema6A in U87MG glioblastoma cells and in endothelial cells

U87MG glioblastoma multiforme cells and human umbilical-vein-derived endothelial cells (HUVECs) respond to stimulation by semaphorins such as Sema3A with localized disassembly of their actin cytoskeleton, which is associated with the loss of focal contacts followed by cell contraction and with the appearance of ‘spikes’ that remain attached to the substrate around the contracted cell (Fig. 1C,E; arrows). In both cell types, the number of mRNA molecules per cell encoding NP2, type A and type D plexins is similar with some variations, whereas the number of mRNA molecules per cell encoding NP1 is two to fivefold higher (supplementary material Fig. S1A). We have previously observed that NP1, plexin-A1 and plexin-A4, but not plexin-A3, are required for the transduction of Sema3A-induced signals resulting in cytoskeletal collapse in HUVECs and in U87MG cells. Inhibition of the expression of any of these genes using specific short hairpin (sh)RNA species that do not cross-inhibit the expression of the other plexins completely inhibits signal transduction by Sema3A (Kigel et al., 2011). These observations suggest that the Sema3A signaling complex contains these three receptors, an assumption that is supported by co-immunoprecipitation experiments. Because plexin-A2 has also been reported to transduce Sema3A signals and to participate in the formation of functional Sema3A receptors (Takahashi and Strittmatter, 2001; Janssen et al., 2012), we sought to determine whether plexin-A2 also participates in the transduction of Sema3A signals in these cells. To examine this possibility, we inhibited the expression of plexin-A2 in HUVECs and in U87MG cells using two specific shRNA species that do not inhibit the expression of the other class A plexins or the expression of neuropilins (Fig. 1A; supplementary material Fig. S1B–D). However, even though the shRNA silenced the expression of plexin-A2 effectively in HUVECs and U87MG cells, the silencing did not inhibit Sema3A-induced cell contraction (Fig. 1C,D; supplementary material Fig. S1E) as does plexin-A1 or plexin-A4 silencing (Kigel et al., 2011).

To gain a better understanding of the role of plexin-A2 in the transduction of class 3 semaphorin signals, we determined whether plexin-A2 is required for the transduction of the signals of other class 3 semaphorins. Silencing plexin-A2 expression had no effect on Sema3F-induced contraction of U87MG cells (Fig. 1C,D), whereas, in agreement with prior reports, silencing plexin-A3 inhibited Sema3F signaling completely (Fig. 2B) (Yaron et al., 2005; Schwarz et al., 2008). However, the collapse of the actin cytoskeleton, the disassembly of vinculin-containing focal contacts and the subsequent cell contraction induced by Sema3B were strongly inhibited in the plexin-A2-silenced cells (Fig. 1C–E). The silencing also completely inhibited Sema6A-induced contraction of these cells (Fig. 1C,D), a surprising result considering that these cells also express plexin-A4, a plexin that is known to function as a signal-transducing Sema6A receptor (Suto et al., 2007; Okada et al., 2007; Kigel et al., 2011), indicating that the concentration of plexin-A4 in these cells, which seems rather low (supplementary material Fig. S1A), is probably not sufficient to enable Sema6A signaling on its own. The contractile response of the U87MG cells to Sema3B and Sema6A was rescued following re-expression of recombinant plexin-A2 in the silenced cells (Fig. 1B–D). These observations attest to the specificity of the silencing and indicate that plexin-A2 is essential for the transduction of Sema3B and Sema6A signals in these cells. Likewise, silencing the expression of plexin-A2 in HUVECs completely inhibited Sema3B- and Sema6A-induced cytoskeletal collapse (supplementary material Fig. S2A–C).

In order to verify these results by a different method, we also expressed in the U87MG cells a truncated form of the plexin-A2 receptor lacking the intracellular domain of plexin-A2, which was tagged at its C-terminus with a FLAG tag (A2ExTm) (supplementary material Fig. S3A). It has been observed previously that a similarly truncated form of plexin-A1 functions as a dominant-negative inhibitor of Sema3A (Serini et al., 2003). The truncated plexin-A2 formed complexes with full-length plexin-A2 (supplementary material Fig. S3B), as well as with plexin-A4 (supplementary material Fig. S3C).

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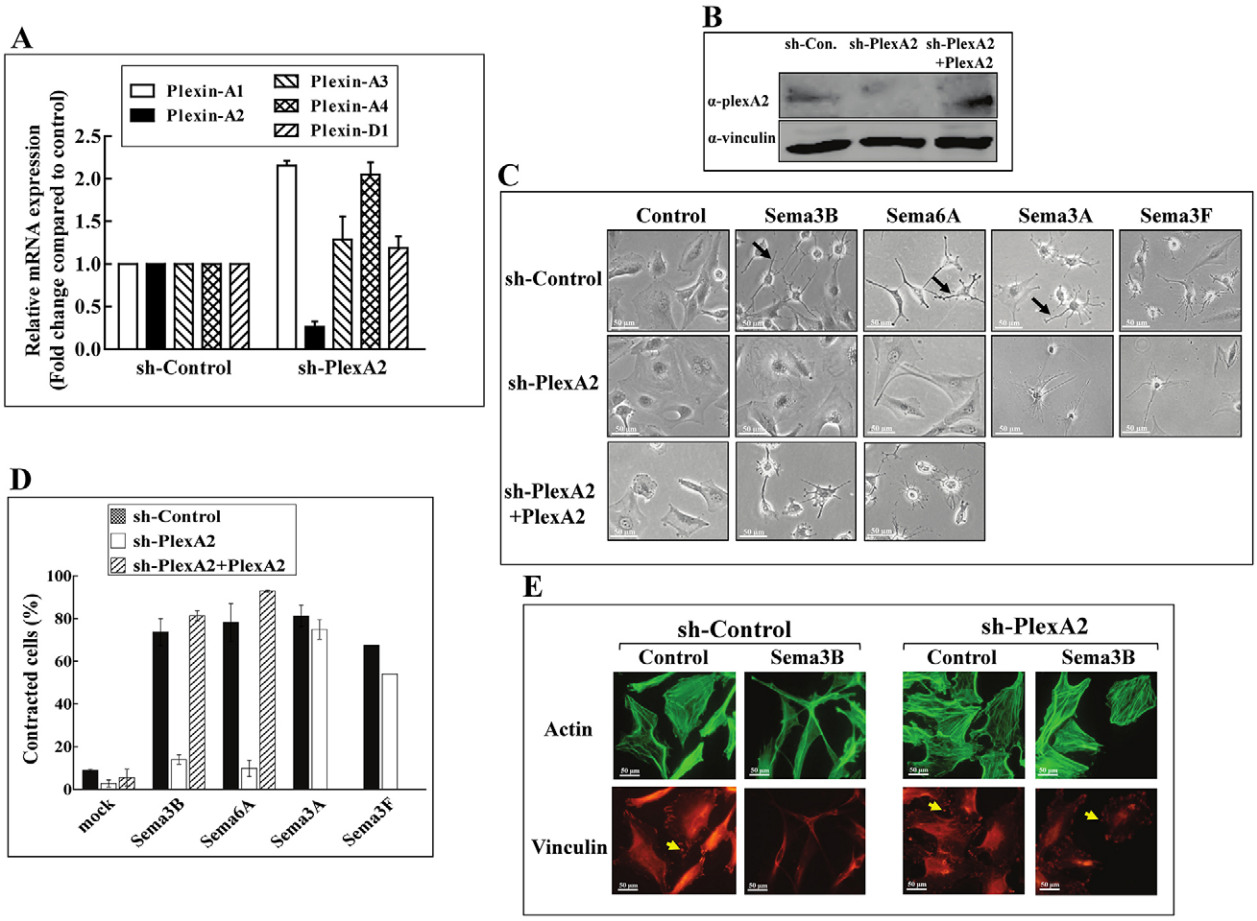

Fig. 1. Plexin-A2 is required for Sema3B signaling but not for Sema3A signaling in U87MG cells. (A) Lentiviruses were used to express control (Sh-Control) or plexin-A2-targeting (sh-PlexA2) shRNAs. The expression of the indicated class A plexins and plexin-D1 was then examined using qRT-PCR. Data represent the mean±s.e.m (two independent experiments). (B) Recombinant plexin-A2 was expressed (Sh-PlexA2+PlexA2) or not in cells silenced for plexin-A2 expression. Western blots prepared from cell lysates were probed with antibodies directed against plexin-A2 or vinculin. (C) Cells silenced for plexin-A2 expression, control cells expressing a non-targeting shRNA or cells silenced for plexin-A2 in which plexin-A2 expression was restored were stimulated with the indicated semaphorins for 30 minutes and photographed. Arrows show attached cytoplasm 'spikes' left behind after cell contraction. Scale bars: 50 μm. (D) Cells were counted in eight microscopic fields obtained from two independent experiments similar to the experiment shown in C, and the percentage of contracted cells was determined. Data represent the mean±s.e.m. (E) Cells silenced for plexin-A2 expression or control cells expressing a non-targeting shRNA were stimulated or not with Sema3B. After 30 minutes, the cells were stained with fluorescent phalloidin to visualize actin fibers or with an antibody specific for vinculin. Arrows show focal contacts stained by using anti-vinculin antibodies. Scale bars: 50 μm.

Overexpression of A2ExTm in U87MG cells inhibited Sema3B- and Sema6A-induced contraction of the U87MG cells independently, suggesting that plexin-A2 is indeed required for Sema3B and Sema6A signal transduction in these cells (supplementary material Fig. S3D,E).

Sema3A transduces signals exclusively using NP1, whereas sema3B utilizes both neuropilins for signal transduction

HUVECs and U87MG cells express the two neuropilins as well as the four class A plexins and plexins belonging to additional plexin subclasses (Shraga-Heled et al., 2007; Kigel et al., 2011; Basile et al., 2005; Artigiani et al., 2004; Gu et al., 2005). The primary binding receptors of class 3 semaphorins are the neuropilins, which cannot transduce signals on their own and associate with class A plexins or plexin-D1 to transduce semaphorin signals. We have previously observed that Sema3B can utilize both neuropilins for signal transduction. However, in HUVECs, Sema3B transduces signals using primarily NP1, and NP2 only plays a secondary role (Varshavsky et al., 2008). By contrast, in U87MG cells, only inhibition of the expression of both neuropilins completely and efficiently abrogated Sema3B signal transduction, whereas inhibition of either NP1 or NP2 expression alone was not sufficient to inhibit Sema3B signaling (supplementary material Fig. S4A,B). By contrast, silencing NP1 expression alone completely inhibited Sema3A-induced cell contraction, whereas silencing NP2 alone was sufficient to abrogate Sema3F-induced cell contraction (supplementary material Fig. S4A,B). Thus, plexin-A2 seems to associate with both neuropilins to transduce Sema3B signals in U87MG cells.

Plexin-A2 and plexin-A4 are required simultaneously for the transduction of Sema3B signals in HUVECs and U87MG cells

Our previous work on Sema3A demonstrated that more than one plexin is required, in addition to NP1, for the transduction of Sema3A signals (Kigel et al., 2011). To find out whether any additional plexins are required besides plexin-A2 for Sema3B-induced cell contraction, we silenced the expression of additional class A plexins in HUVECs and U87MG cells (Fig. 2A) using

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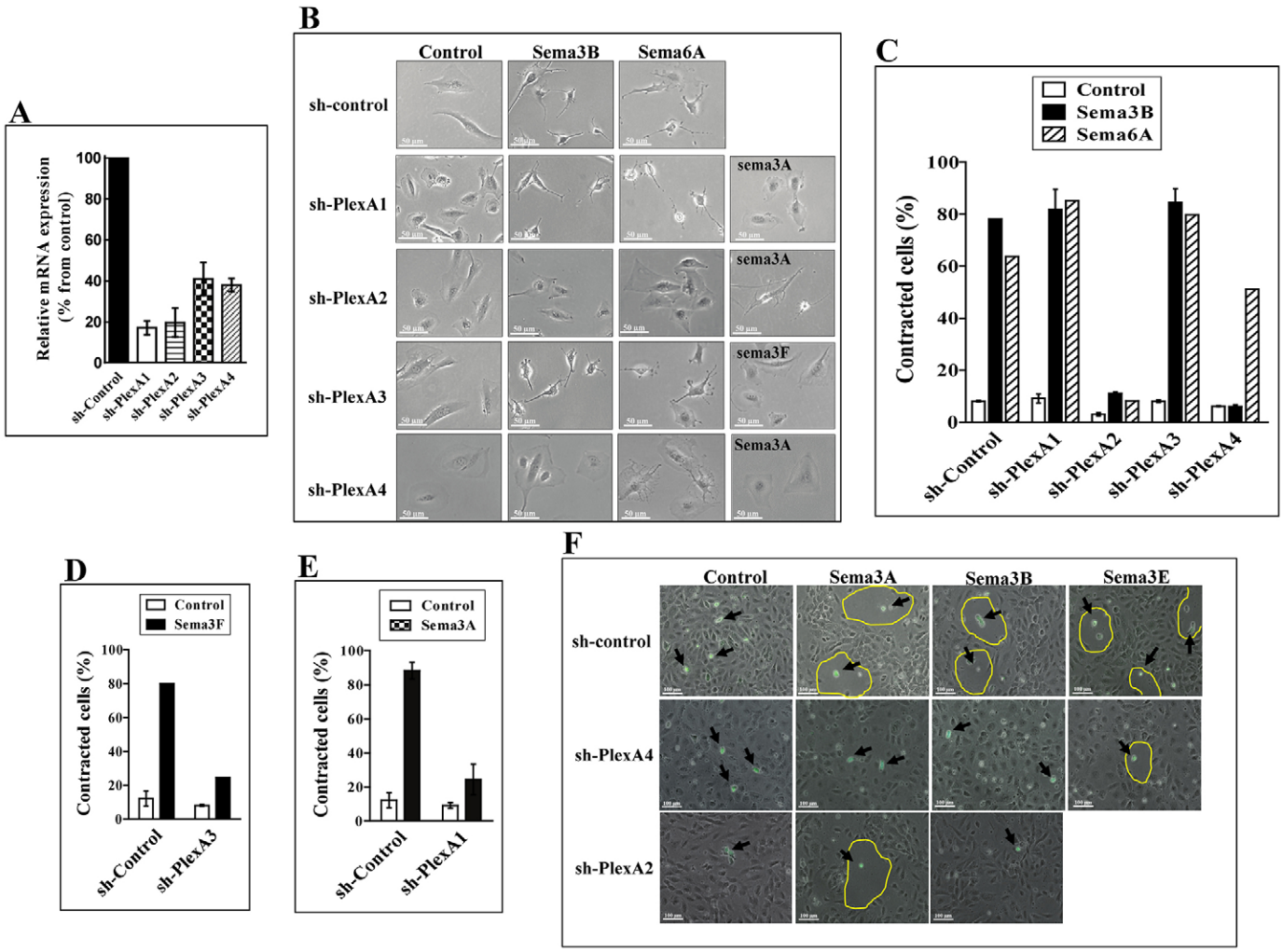

Fig. 2. Plexin-A4 is required in addition to plexin-A2 for Sema3B-induced contraction of U87MG cells. (A) The expression of class A plexin mRNAs was determined by qRT-PCR following infection with lentiviruses encoding shRNA targeting each of the class A plexins or with a control shRNA (sh-control). The mean expression of each plexin in the control cells was taken as 100%. Data represent the mean±s.e.m. (B) U87MG cells were infected with non-targeting shRNA or with shRNAs targeting the different class A plexins (sh-PlexA1–4). Following selection, the cells were stimulated with the indicated semaphorins for 30 minutes and photographed. Scale bars: 50 μm. (C–E) The percentage of contracted cells in a microscopic field was determined under each of the experimental conditions shown in B as described for Fig. 1D. (F) HUVECs infected with lentiviruses expressing a non-targeting control shRNA or shRNAs targeting plexin-A2 or plexin-A4 were grown to subconfluency following selection. Control HEK293 cells expressing GFP or cells expressing the indicated semaphorins in addition to GFP were seeded at clonal density on top of the HUVECs. Cells were photographed after 24 hours by phase-contrast and fluorescence microscopy. Shown are merged pictures. The borders of clearings created in the HUVEC monolayer by semaphorin-expressing cells are marked by a yellow line. Arrows show GFP-expressing HEK293 cells. Scale bars: 100 μm.

shRNA species that do not affect the expression of neuropilins or the expression of other class A plexins (Kigel et al., 2011). Silencing plexin-A1 or plexin-A3 did not inhibit Sema3B- or Sema6A-induced contraction of U87MG cells, suggesting that they do not play an essential role in Sema3B- or Sema6A-induced signal transduction (Fig. 2B,C). This lack of an effect was not due to insufficient silencing because silencing plexin-A1 expression completely inhibited the response to Sema3A, and silencing plexin-A3 completely inhibited cell contraction induced by Sema3F (Fig. 2B–E). As before, silencing plexin-A2 completely aborted responses to Sema3B and Sema6A but had no effect on Sema3A-induced cell contraction. However, cells in which we silenced the expression of plexin-A4 failed to contract in response to Sema3B and to Sema3A (Fig. 2B,C). Identical results were observed when the expression of plexin-A4 was silenced in HUVECs (supplementary material Fig. S2B,C). These results suggest that plexin-A4 is an essential shared component of the Sema3A and Sema3B receptor complexes and that in its absence Sema3A and Sema3B signaling are inhibited despite the presence of plexin-A1 and plexin-A2. These observations were also confirmed by observing the response of cultured HUVECs to gradients of semaphorins produced by the seeding of a low concentration of semaphorin-producing HEK293 cells on top of monolayers of HUVECs. Although cells expressing Sema3A, Sema3B or Sema3E repulsed HUVECs infected with lentiviruses inducing the expression of a control non-targeting shRNA, HUVECs in which the expression of plexin-A4 was silenced were only repulsed by Sema3E, whereas HUVECs in which plexin-A2 was silenced were repulsed by cells expressing Sema3A but not by cells expressing Sema3B (Fig. 2F). We hypothesize that plexin-A4 facilitates the association of plexin-A2 with neuropilins, and that silencing plexin-A4 inhibits responses to

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Sema3B because plexin-A2 is no longer able to associate with neuropilins. Silencing plexin-A4 did not seem to inhibit the association of overexpressed plexin-A2 with NP1 in co-immunoprecipitation experiments (supplementary material Fig. S4C). However, when plexin-A2 is overexpressed it might be able to associate with neuropilins independently of plexin-A4, and we have not been able to perform a similar experiment in cells that express only endogenous receptors.

In contrast to the very strong inhibitory effect that the silencing of plexin-A4 had on Sema3B-induced cell contraction, the silencing of plexin-A4 expression only produced a modest ~20% reduction in the percentage of cells that contracted in response to Sema6A. This is a much weaker effect compared with the effect produced by plexin-A2 silencing, which results in almost complete inhibition of Sema6A-induced cell contraction (Fig. 2B,C). Taken together, these results suggest that the main signal-transducing receptor of Sema6A in these cells is plexin-A2 and that plexin-A4 functions as a less effective Sema6A receptor in these cells.

Sema3B induces the association of NP1 with plexin-A2 but not the association of plexin-A2 with plexin-A4

The experiments described above suggested that the functional Sema3B receptor in HUVECs and in U87MG cells consists of a complex containing NP1 or NP2, plexin-A2 and plexin-A4. In order to map in more detail the associations formed by the plexin-A2 receptor in response to Sema3B and Sema6A, we expressed recombinant plexin-A2 tagged with a C-terminal Strep/FLAG epitope tag (Gloeckner et al., 2007) in U87MG cells. We then labeled the cells metabolically with arginine and lysine containing heavy or light nitrogen isotopes following established stable isotope labeling by amino acids in cell culture (SILAC) protocols (Walther and Mann, 2010; Pimienta et al., 2009). Cells that incorporated amino acids labeled with the

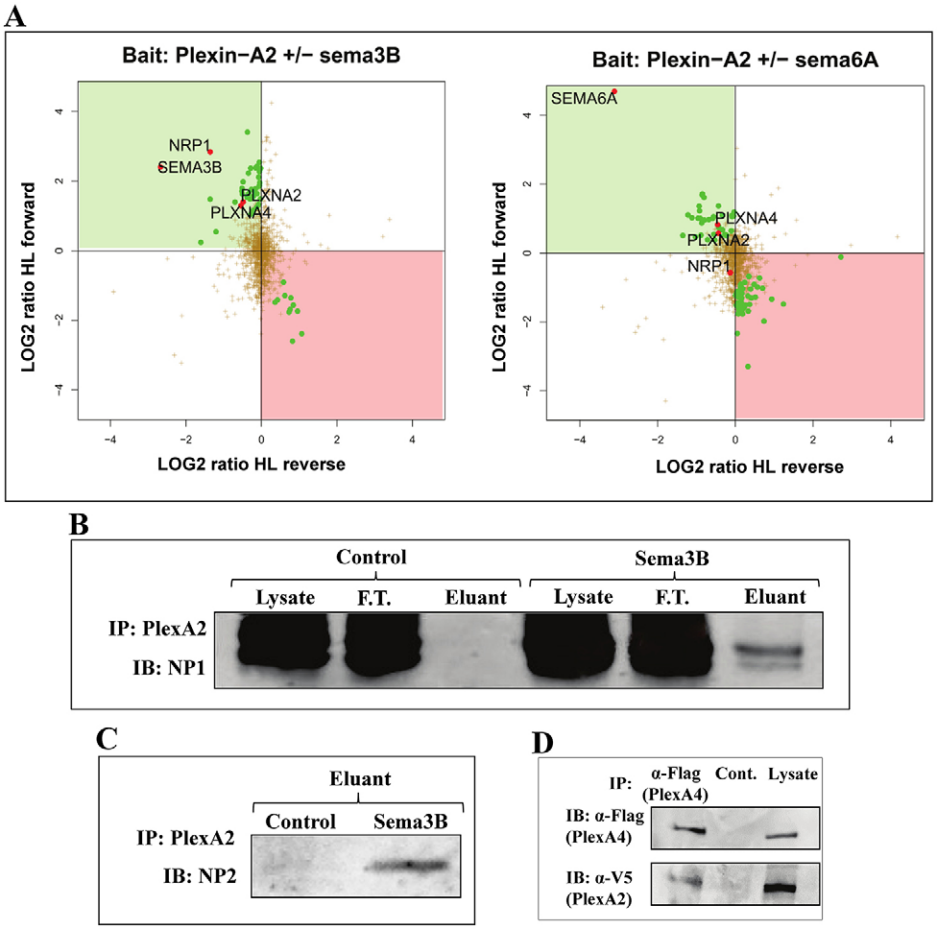

Fig. 3. Sema3B induces the formation of complexes between NP1 and plexin-A2. (A) U87MG cells expressing plexin-A2 tagged with a Strep/FLAG tag were labeled with amino acids containing light or heavy isotopes according to SILAC protocols. Cells labeled with heavy amino acids were incubated with conditioned medium derived from heavy-isotope-labeled HEK293 cells expressing recombinant Sema3B (left panel) or Sema6A (right panel) (forward experiment). Labels were switched for the reverse experiment. The plots show heavy:light (HL) isotope ratios for proteins co-purified with the plexin-A2 bait protein (PLXNA2). *x*-axis, H:L ratios for the reverse experiment (HL reverse); *y*-axis, H:L ratios for the forward experiment (HL forward). Proteins enriched in the presence of the ligand appear in the second quadrant (green), proteins decreased in presence of the ligand appear in the fourth quadrant (red). The bait protein (PLXNA2) as well as the co-purified proteins Sema3B, Sema6A, plexin-A4 (PLXNA4) and NP1 (NRP1) are indicated. (B,C) U87MG cells expressing Strep/FLAG-tagged plexin-A2 were stimulated with control conditioned medium (control) or conditioned medium containing recombinant Sema3B. Cells were stimulated for 5 minutes at 37°C and lysed, and the lysates were purified on an anti-FLAG column. NP1 or NP2 were then detected by western blotting. IP, immunoprecipitated; IB, immunoblotted; F.T., flow through, i.e. the fraction of the cell lysate that did not bind to the affinity column. (D) Plexin-A2 and plexin-A4–FLAG were expressed in PAE cells. Co-immunoprecipitation was performed using an anti-FLAG antibody or a control antibody (Cont.). Precipitates were probed with anti-plexin-A2 antibodies or antibodies against the FLAG tag of plexin-A4.

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heavy isotopes were then stimulated with conditioned medium derived from heavy-isotope-labeled HEK293 cells secreting recombinant Sema3B or Sema6A. Cells containing amino acids labeled with the light isotopes served as controls. In order to exclude false-positive hits, labels were switched in a second experiment. The cells were then solubilized using NP-40, and, following centrifugation to remove insoluble debris, cell lysates from the control and stimulated cells were independently affinity purified on an anti-FLAG affinity resin. The eluants from columns loaded with conditioned medium from control and Sema3B-stimulated cells were mixed and analyzed by mass spectroscopy. These experiments revealed that Sema3B strongly enhances the association of NP1 with plexin-A2 (Fig. 3A,B). As expected, Sema6A did not promote the association of plexin-A2 with neuropilins (Fig. 3A). Surprisingly, we could not detect in the SILAC experiments a similar Sema3B-induced association of NP2 with plexin-A2 (Fig. 3A). This might be due to a weaker association between these receptors, because when eluants recovered from the anti-FLAG affinity column were examined by western blot analysis, we found that NP2 co-purified with plexin-A2 only in cell extracts purified from Sema3B-stimulated cells but not in those of control cells (Fig. 3C). These observations suggest that Sema3B is nevertheless able to induce the association of NP2 with plexin- although this association seems to be less stable than the Sema3B-induced association of plexin-A2 with NP1.

We have shown previously that plexin-A4 forms spontaneous complexes with plexin-A1 (Kigel et al., 2011). We found that plexin-A4 is also able to form complexes with plexin-A2, as revealed by experiments in which plexin-A4 was co-immunoprecipitated with plexin-A2 from cell lysates prepared from porcine aortic endothelial (PAE) cells in which we expressed the two receptors (Fig. 3D). However, the formation of plexin-A2–plexin-A4 complexes was not enhanced by Sema3B or by Sema6A (Fig. 3A).

Overexpression of plexin-A2 in cells silenced for plexin-A4 expression restores responsiveness to Sema3B but not vice versa

Silencing plexin-A2 or plexin-A4 completely inhibited the Sema3B-induced collapse of the cytoskeleton of U87MG cells and HUVECs. A likely explanation is that each plexin provides a unique property essential for the function of the Sema3B receptor complex that the other plexin cannot contribute. Alternatively, it is possible that the identity of the plexins that participate in the formation of the Sema3B signaling complex is not important so long as the total concentration of the plexins exceeds a certain threshold required to transduce Sema3B signals. To differentiate between these two possibilities, we expressed in U87MG cells a plexin-A2 shRNA under the control of doxycycline. In these cells, the expression of plexin-A2 was inhibited following stimulation with doxycycline (Fig. 4A). Following exposure to doxycycline, these cells lost their contractile response to Sema3B and Sema6A (Fig. 4B,C). When recombinant plexin-A4 was ectopically overexpressed in the silenced cells (Fig. 4A) it restored the response to Sema6A but not the response to Sema3B (Fig. 4B,C). It should be noted that overexpression of plexin-A4 did not restore plexin-A2 expression (Fig. 4A). This experiment therefore suggests that plexin-A2 and plexin-A4 are not interchangeable with respect to Sema3B signaling but can be interchanged to restore Sema6A signal transduction. We also conducted the reciprocal experiment, in which we ectopically

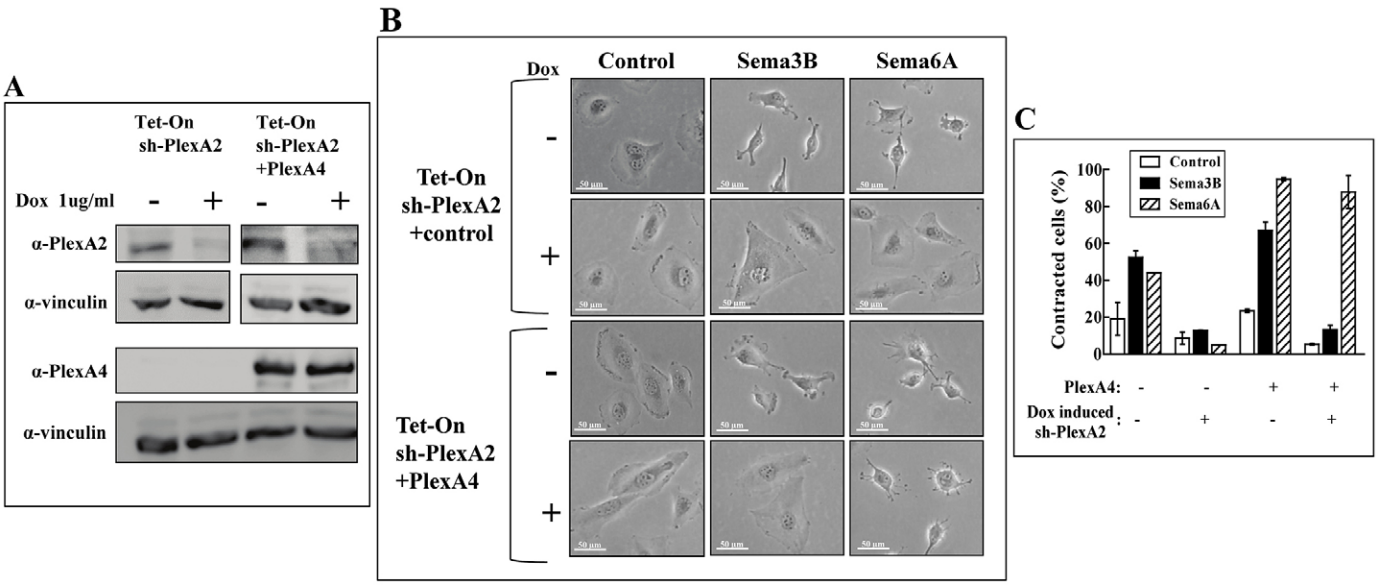

Fig. 4. Ectopic overexpression of plexin-A4 does not restore responsiveness to Sema3B in cells silenced for plexin-A2 expression. (A) U87MG cells infected with lentivirus carrying a doxycycline (Dox)-induced shRNA targeting plexin-A2 (Tet-on sh-plexA2) as well as such cells that additionally overexpress plexin-A4 (Tet-on sh-plexA2+plexA4) were stimulated or not by addition of doxycycline. Shown are western blots probed with antibodies directed against plexin-A2, plexin-A4 or vinculin. (B) U87MG cells expressing an shRNA targeting plexin-A2 under the control of the tetracycline repressor were infected with an empty lentiviral expression vector (control) or with a lentiviral vector directing expression of plexin-A4. Cells were grown in the absence or presence of doxycycline (1 μg/ml) for 72 hours and then seeded for the contraction assay. Contraction was induced by the addition of either control conditioned medium or conditioned medium containing saturating concentrations of either Sema3B or the soluble extracellular domain of Sema6A. Scale bars: 50 μm. (C) The percentage of contracted cells was determined as described for Fig. 1D. Data show the mean±s.e.m.

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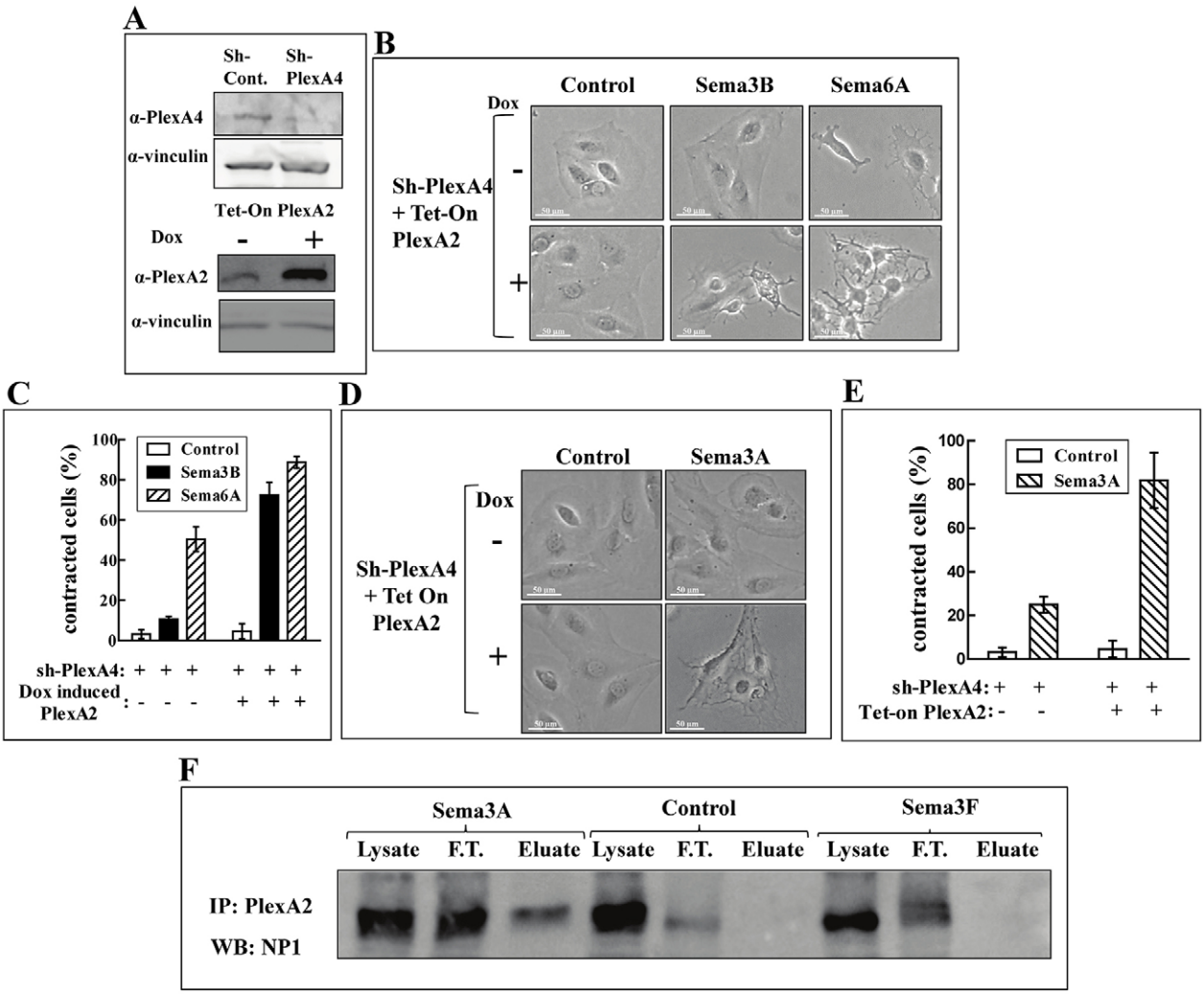

Fig. 5. Ectopic overexpression of plexin-A2 restores responsiveness to Sema3A and Sema3B in cells silenced for plexin-A4. (A) U87MG cells expressing doxycycline (Dox)-inducible plexin-A2 (Tet-on) were infected with shRNA targeting plexin-A4 or with a non-targeting shRNA (Sh-Cont.). Cells were stimulated or not with doxycycline (100 ng/ml) for 24 hours. Shown are western blots probed with anti-plexin-A2, anti-plexin-A4 or anti-vinculin antibodies. (B) The cells described in A were seeded for a contraction assay, stimulated with Sema3B or Sema6A as indicated and photographed after 30 minutes. Scale bars: 50 μm. (C) The mean (±s.e.m.) percentage of contracted cells in each of the cultures shown in B was determined as described for Fig. 1D. (D) U87MG cells expressing inducible plexin-A2 (Tet-on PlexA2) were infected with shRNA targeting plexin-A4, selected using puromycin and stimulated or not with doxycycline (100 ng/ml) for 24 hours. Cells were seeded for contraction assay, stimulated with control or Sema3A-conditioned medium and photographed after 30 minutes. Scale bars: 50 μm. (E) The mean (±s.e.m.) percentage of contracted cells shown in D was determined as described for Fig. 1D. (F) U87MG cells expressing recombinant Strep/FLAG-tagged plexin-A2 were stimulated with control, Sema3A- or Sema3F-conditioned medium from HEK293 cells. Following stimulation for 5 minutes at 37°C, cells were lysed and purified on an anti-FLAG column, and the presence of co-purified NP1 was examined using western blotting. IP, immunoprecipitated; IB, immunoblotted; F.T., flow through, i.e. the fraction of the cell lysate that did not bind to the affinity column.

overexpressed plexin-A2 under the control of doxycycline in cells that were silenced for plexin-A4 expression (Fig. 5A). Surprisingly, ectopic overexpression of plexin-A2 in the plexin-A4-silenced cells fully restored responsiveness to Sema3B (Fig. 5B,C). Thus, although at elevated expression levels plexin-A2 is able to compensate for missing plexin-A4 activity and thus restore Sema3B signal transduction in the plexin-A4-silenced cells, the opposite is not true, as plexin-A4 cannot replace plexin-A2 in plexin-A2-silenced cells to restore Sema3B signaling.

We also determined whether the overexpression of NP1 could restore responsiveness to Sema3B following the silencing of plexin-A2 expression. For this experiment, we used cells in which ectopic overexpression of NP1 is under the control of the tet repressor (Fig. 6A,B). When NP1 was not overexpressed, silencing plexin-A2 inhibited, as expected, Sema3B- and Sema6A-induced signaling but not Sema3A-induced signal transduction. When NP1 was ectopically overexpressed by addition of doxycycline, the response to Sema3B, but not the response to Sema6A, was restored (Fig. 6C,D), suggesting that upregulation of NP1 enables a higher percentage of the remaining plexin-A2 in the silenced cell to associate with NP1, thus enabling signal transduction.

Silencing plexin-A4 in the U87MG cells only minimally affected the response to Sema6A (Fig. 2C). Doxycycline-induced expression of plexin-A2 in cells silenced for plexin-A4 expression resulted in enhanced contraction in response to Sema6A (Fig. 5B,C), indicating that for Sema6A signal transduction plexin-A2 and plexin-A4 are fully interchangeable.

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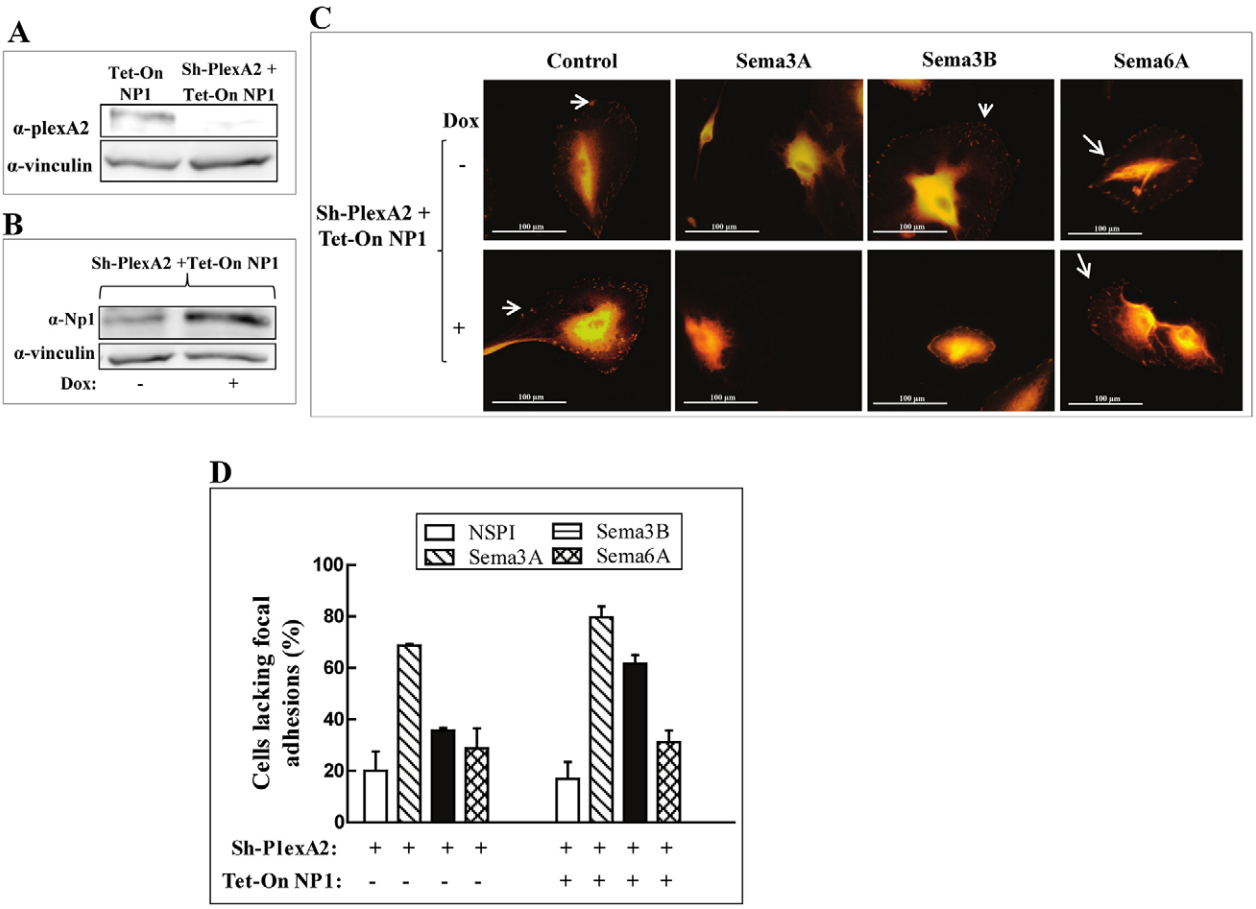

Fig. 6. Ectopic overexpression of NP1 can restore responsiveness to Sema3B in cells in which plexin-A2 expression was silenced. (A) U87MG cells expressing NP1 under the control of the Tet repressor were silenced or not for plexin-A2 expression and the effect of the silencing on plexin-A2 expression was determined using western blot analysis. (B) U87MG cells ectopically expressing NP1 under the control of the Tet repressor in which the expression of plexin-A2 was silenced were stimulated or not with doxycycline (Dox). Expression of NP1 was then determined using western blot analysis. (C) The expression of plexin-A2 was silenced in U87MG cells expressing NP1 under the control of the tetracycline repressor (Tet-On). Cells were then incubated with or without doxycycline for 24 hours. The cells were then stimulated by the addition of conditioned medium containing the indicated semaphorins for 15 minutes. The presence or absence of focal contacts (arrows) was then determined using an antibody specific for vinculin. Scale bars: 100 μm. (D) The percentage of cells lacking focal contacts per microscopic field was determined in each of the cell cultures shown under A. Data represent the mean±s.e.m.

Overexpression of plexin-A2 in cells silenced for plexin-A1 or plexin-A4 expression restores responsiveness to Sema3A

Silencing plexin-A1 or plexin-A4 in U87MG cells or in HUVECs inhibits Sema3A-induced cell contraction, whereas silencing plexin-A2 has no effect (Fig. 2B). However, conditional ectopic overexpression of plexin-A2 in cells silenced for plexin-A4 or plexin-A1 expression completely restored their responsiveness to Sema3A (Fig. 5D,E; Fig. 7A,B). Similar results were obtained when recombinant plexin-A2 was expressed in PAE cells expressing either NP1 or NP2. In the absence of plexin-A2, PAE cells expressing NP1 contracted weakly in response to either Sema3A or Sema3B, whereas PAE cells expressing NP2 did not contract at all in response to these semaphorins (supplementary material Fig. S2D). When recombinant plexin-A2 was expressed in these cells in addition to NP1, the cells contracted strongly in response to both Sema3A and Sema3B, whereas cells co-expressing NP2 and plexin-A2 only contracted in response to Sema3B (supplementary material Fig. S2D). These results suggest again that when plexin-A2 is highly expressed it can transduce Sema3A and Sema3B signals independently of plexin-A4. These results are also in agreement with previous reports showing that at high plexin-A2 expression levels Sema3A can transduce signals through receptors containing complexes of NP1 and plexin-A2 (Janssen et al., 2012). Indeed, we found that Sema3A was able to induce the association of plexin-A2 with NP1 in cells overexpressing plexin-A2, whereas Sema3F was not able to promote such an association (Fig. 5F). These observations imply that the ability to distinguish between Sema3A and Sema3B can be lost if the concentration of plexin-A2 in the cell membrane exceeds a certain threshold. These observations also predict that overexpression of A2ExTm in the U87MG cells should also inhibit Sema3A activity. Indeed, A2ExTm overexpression in U87MG cells also inhibited Sema3A signaling in addition to inhibition of Sema3B and Sema6A signaling (supplementary material Fig. S3D,E). Finally, we ectopically overexpressed plexin-A4 in cells silenced for plexin-A1 expression. To our surprise, we found that although plexin-A4 was not able to restore Sema3B signaling owing to loss of plexin-A2 expression, it was able to restore responsiveness to Sema3A in cells silenced for plexin-A1 expression (Fig. 7C,D).

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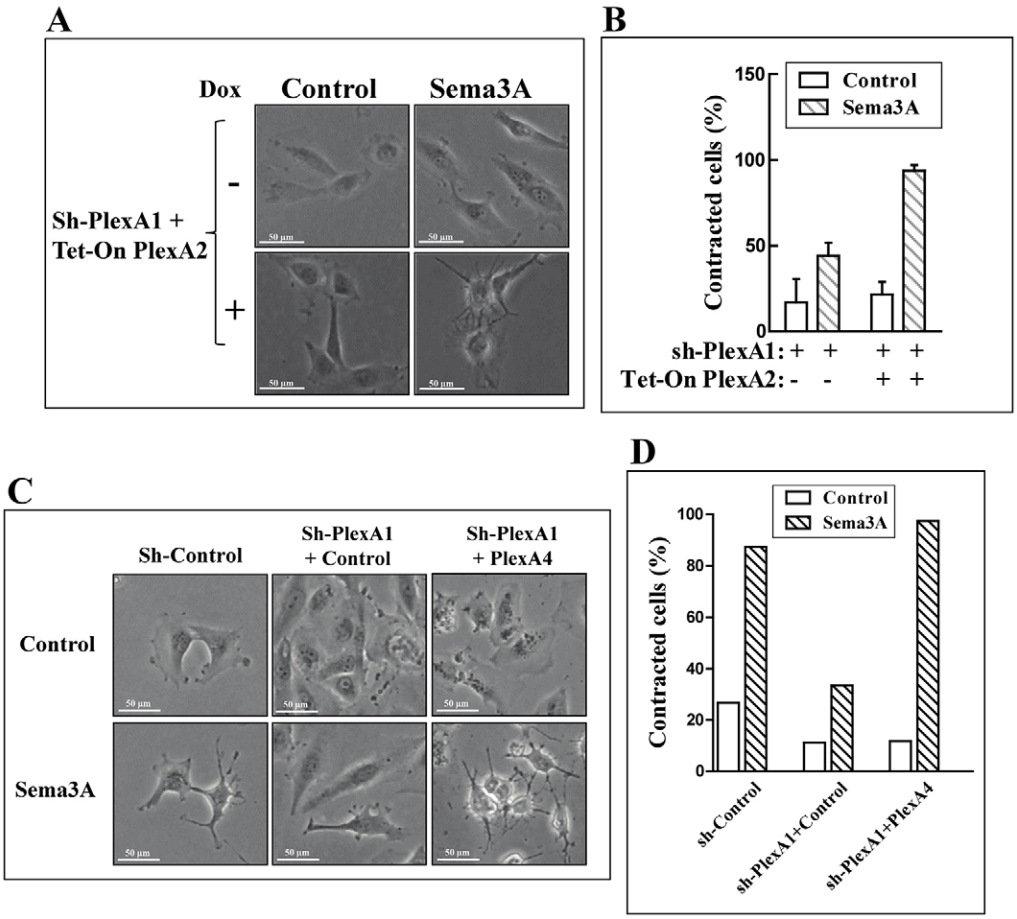

Fig. 7. Ectopic overexpression of plexin-A2 or plexin-A4 can restore responsiveness to Sema3A in cells silenced for plexin-A1. (A) U87MG cells expressing doxycycline (Dox)-induced plexin-A2 (Tet-on PlexA2) were infected with shRNA targeting plexin-A1, selected using puromycin and grown in the absence or presence of doxycycline (100 ng/ml) for 24 hours. Cells were stimulated with control or Sema3A-conditioned medium and photographed after 30 minutes. Scale bars: 50 μm. (B) The mean (±s.e.m.) percentage of contracted cells in each of the cell cultures shown in A was determined as described for Fig. 1D. (C) U87MG cells were infected with shRNA targeting plexin-A1 (Sh-plexA1) or control non-targeting shRNA (Sh-Control), selected with puromycin and infected with empty lentiviral expression vector or with lentivirus expressing plexin-A4 as indicated. The cells were then stimulated with control or Sema3A-conditioned medium and photographed. Scale bars: 50 μm. (D) The percentage of contracted cells per microscopic field in the cell cultures shown in C was determined as described for Fig. 1D.

DISCUSSION

Cells employ cell surface receptors in order to sense changes in their environment. These receptors include receptors for guidance factors such as semaphorins. When cells transverse gradients of semaphorins in the course of their migrations, the activation of semaphorin receptors triggers local changes in the actin cytoskeleton that are translated into changes in the direction of their movement. The characterization of the semaphorin receptors and of the molecular mechanisms that ensure that cells correctly interpret guidance instructions delivered by semaphorins is thus important for the understanding of the mechanisms that regulate cell migration *in vivo*.

Research conducted in the last two decades has revealed that functional receptors are frequently complexes that contain a ligand-binding receptor as well as additional membrane-anchored proteins that can affect the specificity of binding and can modulate signal transduction. In endothelial cells and in U87MG glioblastoma cells, Sema3A induces the collapse of the actin cytoskeleton and consequent cell contraction. Our results and the results of a previous study (Kigel et al., 2011) indicate that the Sema3A-induced collapse of the actin cytoskeleton in these cells is completely inhibited if the expression of NP1, plexin-A1 or plexin-A4, but not plexin-A3 or plexin-A2, is silenced. These results suggest that the minimal functional Sema3A receptor in these cells consists of a complex containing these three receptors. Although silencing plexin-A2 expression did not affect Sema3A signaling, it completely inhibited Sema3B signaling in these cells, indicating that the level of expression of endogenous plexin-A2 in these cells is sufficiently high to enable signal transduction, and further suggesting that plexin-A2 is an essential component of Sema3B receptors but not of Sema3A receptors.

Further examination of the composition of the functional Sema3B receptor revealed that, unlike Sema3A, which signals exclusively using the NP1 receptor, Sema3B utilizes both NP1 and NP2 for signal transduction. These observations suggest that signal transduction by semaphorins that utilize NP2 as their exclusive or primary binding receptor might require the presence of plexin-A2. However, cell contraction induced by the specific NP2 ligand Sema3F was not inhibited following plexin-A2 silencing and was inhibited only following the silencing of plexin-A3. We conclude from these experiments that when plexin-A2 is expressed at its physiological level of expression in endothelial cells and in U87MG cells it functions as an essential component of Sema3B receptors, but not of functional Sema3A or Sema3F receptors. This was supported by observations showing that upon stimulation with Sema3B both NP1 and NP2 form stable complexes with plexin-A2. Taken together, these experiments suggest that plexin-A1 and plexin-A2 and not the neuropilins confer specificity to Sema3A and Sema3B within Sema3A and Sema3B receptor complexes.

Interestingly, Sema3B-induced signaling was also completely inhibited by the silencing of plexin-A4 expression, suggesting that plexin-A4 is an essential shared component of both Sema3A and Sema3B receptors. In agreement with these results, we have observed that plexin-A4 forms spontaneous complexes with plexin-A2 as well as with plexin-A1 (Kigel et al., 2011), suggesting that heterocomplexes of plexin-A1 and plexin-A2 with plexin-A4 pre-exist in HUVECs and U87MG cells. We

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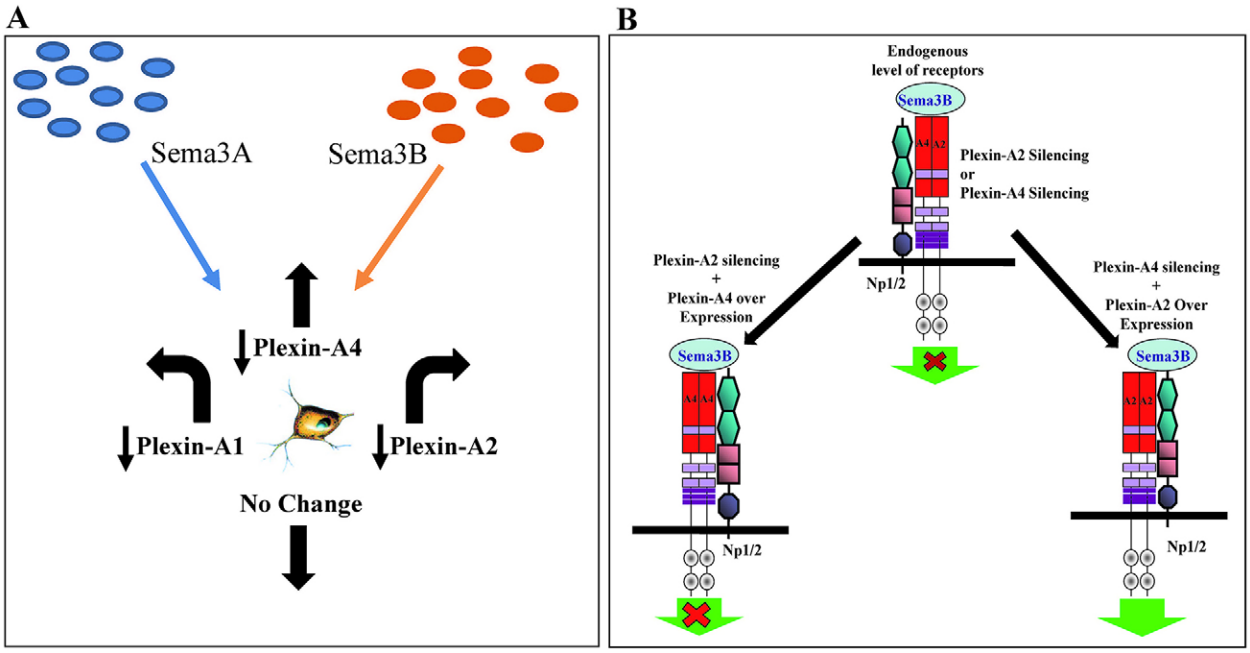

Fig. 8. A schematic representation of how changes in the composition of Sema3A and Sema3B receptor complexes affect biological responses to these semaphorins. (A) This diagram depicts a U87MG cell that normally expresses all the neuropilins and class A plexins and that is migrating upwards and encounters two intersecting gradients of Sema3A and Sema3B. Normally the cell would be repelled in the opposite direction by the semaphorins (No Change). If a local signal would downregulate plexin-A4 expression the cell will not be able to respond to either of the semaphorins and will continue to migrate upwards. Downregulation of plexin-A1 or plexin-A2 would cause the cell to veer left or right, as the cell will be rendered blind to the repulsive effects of Sema3A or Sema3B, respectively. Thus, variation in the expression of a component contained in the multicomponent receptors of Sema3A or Sema3B can have a profound effect on the way in which cells interpret the encountered semaphorin gradients. (B) Reduced expression of either plexin-A2 or plexin-A4 causes endothelial cells and glioblastoma cells to completely lose their responsiveness to Sema3B. Overexpression of recombinant plexin-A2 in cells in which plexin-A4 was silenced restores responsiveness to Sema3B, whereas overexpression of recombinant plexin-A4 in cells silenced for plexin-A2 expression is not sufficient to restore responsiveness to Sema3B.

postulate that stimulation with Sema3B recruits such pre-formed complexes to NP1 or NP2. It is possible that when plexin-A4 is in complex with either plexin-A1 or plexin-A2 it is able to increase the affinity of these two plexins for neuropilins, and this might be of crucial importance when plexin-A1 or plexin-A2 are expressed at their physiological levels.

These observations have profound implications for our understanding of the mechanisms by which responses to guidance factors such as semaphorins are interpreted by migrating cells, and highlight the advantages of multicomponent receptors. They suggest that local factors encountered in the microenvironments traversed by the migrating cells might modulate the expression of essential constituents of semaphorin receptor complexes and, as a result, change the way in which the migrating cells interpret signals of encountered gradients of semaphorins. For example, a migrating cell that encounters two intersecting gradients of Sema3A and Sema3B might also encounter local factors that affect the expression of specific plexins. Such changes might render cells ‘blind’ to a specific semaphorin or to several semaphorins and, as a result, change the direction of migration (Fig. 8A). In cases in which two populations of migrating cells that are destined to migrate to different targets encounter several intersecting gradients of guidance factors this mechanism might enable each cell population to differently interpret semaphorin signals. Thus, multicomponent receptor complexes provide plasticity and flexibility that is not available in simple single component receptors.

Previous studies have provided evidence suggesting that all of the four class A plexins, including plexin-A2, might be able to transduce Sema3A signals (Takahashi and Strittmatter, 2001; Bouvrée et al., 2012; Janssen et al., 2012; Katayama et al., 2013; Yaron et al., 2005). These results seemingly contradict our findings, which suggest that functional receptor complexes for Sema3A and Sema3B contain specific essential plexins. We hypothesize that these apparent discrepancies might be the result of abnormally high plexin expression levels, given that many of these studies were conducted using ectopically overexpressed plexins. Indeed, when we overexpressed plexin-A2 in U87MG cells in which plexin-A4 or plexin-A1 expression was silenced, the overexpressed plexin-A2 was able to restore both Sema3A and Sema3B signaling, and the cells lost their ability to differentiate between Sema3A and Sema3B. These observations suggest that at higher levels of expression all the class A plexins might be interchangeable. However, further experiments revealed that this assumption might not be general, because plexin-A4 was unable to restore Sema3B signaling in cells silenced for plexin-A2 expression even when it was strongly overexpressed (Fig. 8B).

We hypothesize that the association of plexin-A4 with plexin-A2 increases the affinity of plexin-A2 for neuropilins and thus enables the formation of the functional signaling receptor complexes. This might also explain why overexpression of plexin-A2 can substitute for plexin-A4 but not vice versa. When plexin-A2 is overexpressed it can form complexes with neuropilins without plexin-A4. We hypothesize that the reason plexin-A4 cannot replace plexin-A2 even when highly expressed is because it does not play a real functional role in Sema3B signal

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transduction and its only role is to facilitate the recruitment of plexin-A2 to neuropilins when the concentration of plexin-A2 is below a threshold at which it cannot associate with neuropilins on its own.

It remains to be determined whether similar combinations of plexins regulate signal transduction induced by other class 3 semaphorins in a similar manner, and if signal transduction by these semaphorins is gated similarly by changes in the expression levels of specific plexins. Semaphorins have the potential to be used as therapeutic agents for the treatment of diseases such as cancer (Tamagnone, 2012). Our observations suggest that changes in the expression levels of plexins in the tumor microenvironment might change the sensitivity of tumor cells and endothelial cells found in the tumor microenvironment to specific semaphorins, indicating that successful future use of semaphorins as therapeutics might require the monitoring of the expression levels of plexins in the tumors.

MATERIALS AND METHODS

Antibodies and reagents

The following antibodies were used: Goat anti-mouse-IgG peroxidase conjugate (Sigma; A4416), goat anti-rabbit-IgG peroxidase conjugate (Sigma; A6154), bovine anti-goat-IgG peroxidase conjugate (Santa Cruz Biotechnology; sc-2350), mouse anti-actin clone AC-74 (Sigma; A5316), mouse anti-FLAG (Sigma; F3165), mouse anti-FLAG agarose beads (Sigma; A2220), goat anti-human NP1 (Santa Cruz Biotechnology; sc-7239), mouse anti-human NP2 (Santa Cruz Biotechnology; sc-13117), Cy3-conjugated donkey anti-mouse-IgG antibody (Jackson Immunoresearch Laboratories; 705-165-147), Cy2-conjugated anti-rabbit-IgG antibody (Jackson Immunoresearch Laboratories), mouse anti-V5 (Invitrogen; R960-25), rabbit anti-plexin-A1 (Cell Signaling Technology; 3813s), rabbit anti-plexin-A2 (Santa Cruz Biotechnology; sc-25640), rabbit anti-plexin-A2 (Abcam; ab39357), rabbit anti-plexin-A4 (Sigma; R30914), rabbit anti-Sema6A (Abcam; ab72369), anti-HA (Boeringer Manheim; 1583-816), mouse anti-c-myc (Santa Cruz Biotechnology sc-789) and mouse anti-vinculin (Chemicon; 3574).

Kits

The RNA reverse PCR kit 5-Prime was from PerfectPure (Gaithersburg, MD). The Verso cDNA kit was from Thermo Scientific, Fugene-6 was purchased from Roche Ltd (Switzerland) and the Block-it Inducible H1 Lentiviral RNAi System was from Invitrogen.

Plasmids

The NSPI-CMV-MCS-myc-His lentiviral expression vector was given to us by Gal Akiri (Mount Sinai School of Medicine, NY). The PcDNA3-Sema6A plasmid and the Plexin-A2/myc cDNAs were provided by Oded Behar (Hebrew University, Jerusalem, Israel). The ΔNRF (pCMV dR 8.74) and pMD2-VSV-G vectors for lentivirus production were obtained from Tal Kafri (University of North Carolina at Chapel Hill, NC). The pDonor221, pLenti6/V5-DEST, pLenti6.3/TO/V5-DEST, pENTR/H1/TO and the pLenti4/Block-iT-DEST plasmids were purchased from Invitrogen. The pENTR1A-GFP-N2, pEF-1α/pENTRA, pLenti-CMV-GFP-DEST, pLenti-CMV-Puro-DEST and pLenti-CMV-Neo-DEST plasmids were from Addgene. The pLKO-Tet-On plasmid was kindly provided by Ayoub Nabieh (Faculty of Biology, Technion, Israel). The shRNAs containing lentiviral vectors were purchased from Sigma Aldrich.

Cell lines

HUVECs were isolated and cultured as described previously (Gospodarowicz et al., 1978). Porcine aortic endothelial (PAE) cells and HEK293 cells were cultured as described previously (Gluzman-Poltorak et al., 2000; Kigel et al., 2011). U87MG cells were purchased from the American Type Culture Collection (ATCC) and were maintained in MEM-Eagle Earl's medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 μg/ml gentamicin, 2.5 μg/ml fungizone, 1 mM sodium pyruvate and non-essential amino acids (Biological Industries, Beth Haemek, Israel). All shRNA-expressing stable cell lines were grown in this medium, which was supplemented with 10% Tet-approved FBS (Biological Industries). Expression of the shRNA was induced by addition of 1 μg/ml doxycycline (Sigma) dissolved in deionized water for 72 hours.

Expression and production of semaphorins

Class 3 semaphorin cDNAs were subcloned into the NSPI-CMV-myc-his lentiviral expression vector as described previously (Kigel et al., 2008; Varshavsky et al., 2008). An expression plasmid containing the extracellular domain of the Sema6A cDNA was kindly provided by Oded Behar (Hebrew University, Jerusalem, Israel). The production of lentiviruses and the generation of conditioned media containing various semaphorins were performed as described previously (Kigel et al., 2008). For repulsion assays, HEK293 cells expressing various semaphorins were also infected with a lentiviruses generated from the PGK-eGFP lentiviral vector (Chrenek et al., 2011).

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed using the StepOne Plus Real Time PCR System with TaqMan Gene Expression Master Mix, according to the instructions of the manufacturer (Applied Biosystems). The normalizing gene was RPLP0. Data were analyzed with the StepOne Software (Applied Biosystems) using the relative Quantification-comparative C_T method. The following assays were used: NP1, Hs00826128; NP2, Hs00187290; plexin-A1, Hs00413698; plexin-A2, Hs00300697; plexin-A3, Hs00250178; plexin-A4, Hs00297356; plexin-D1, Hs00892410; RPLP0, Hs99999902.

Quantification of the number of mRNA molecules per cell by real-time reverse PCR

The number of mRNA molecules per cell of the plexins and neuropilins expressed in non-stimulated HUVECs and U87MG cells was quantified using the TaqMan gene expression assay. Plasmids containing the cDNA of each plexin or neuropilin were used to build a standard curve of cycle threshold (Ct) versus plasmid concentration. RNA from HUVECs and U87MG cells was extracted and converted to cDNA. Real-time PCR was performed in triplicate as described above. The amount of each mRNA (ng/well) was calculated from the standard curve and converted to moles/well taking into consideration the dilutions made in the process to derive the number of mRNA molecules per cell for each plexin and neuropilin in HUVECs and U87MG cells.

Inhibition of gene expression with shRNA-expressing lentiviruses

Lentiviral vectors directing the expression of various shRNAs were purchased from Sigma Aldrich. The following shRNA-encoding DNA sequences were used: plexin-A1, 5′-CCGGGCACTTCTTCACGTCCA-AGATCTCGAGATCTTGGACGTGAAGAAGTGCTTTTTG-3′; plexin-A2 first shRNA, 5′-CCGGCGGCAATTTCATCATTGACAACTCG-AGTTGTCAATGATGAAATTGCCGTTTTTG-3′; plexin-A2 second shRNA, 5′-CCGGCGCCCAGATGAGTTTGGATTCTCGAGAATCC-AAACTCATCTGGGCGTTTTTG-3′; plexin-A3, 5′-CCGGGCTGTATT-TCTATGTCACCAACTCGAGTTGGTGACATAGAAATACAGCTTTT-TG-3′; plexin-A4, 5′-CCGGGCAGATAAATGACCGCATTAACTCGA-GTTAATGCGGTCATTTATCTGCTTTTTG-3′. The production of the lentiviruses, infection of cells and the selection of shRNA expression cells in HEK293 cells were performed as described previously (Varshavsky et al., 2008).

Inducible expression of shRNA species and downregulation of neuropilin expression using siRNA

To generate inducible shRNA-expressing plasmids, double-stranded oligonucleotides encoding the desired shRNAs were generated and cloned into the single-vector inducible shRNA expression vector pLKO-Tet-On (gift of Ayoub Nabieh; Faculty of Biology, Technion, Israel) as described previously (Wiederschain et al., 2009). Inhibition of NP1 and NP2 expression in U87MG cells using transfection of small interfering (si)RNAs was performed as described previously (Varshavsky et al., 2008).

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Expression of recombinant plexins

The full-length cDNAs of plexin-A2 and plexin-A4 were cloned into the gateway pDonor221 plasmid and then transferred by recombination into the pLenti6/V5-DEST or pLenti6.3/TO/V5-Dest lentiviral expression vector in frame with a C-terminal V5 tag according to the instructions of the manufacturer (Invitrogen). A deletion mutant of plexin-A2 containing the extracellular and transmembrane domains (amino acids 1–1323) of the protein fused to a C-terminal FLAG tag was constructed according to the same procedures. Production of lentiviruses using these plasmids and stable infection of target cells were performed essentially as described previously (Brekhman and Neufeld, 2009). The Sema6A expression vector was transfected into target cells using FuGENE6 transfection reagent (Roche) as described previously (Guttmann-Raviv et al., 2007).

Cell contraction and repulsion assays, co-immunoprecipitation and immunofluorescence

Contraction of HUVECs and U87MG cells in response to semaphorins as well as repulsion assays of endothelial cells were performed as described previously (Varshavsky et al., 2008; Guttmann-Raviv et al., 2007). Co-immunoprecipitation assays were performed as described previously (Shraga-Heled et al., 2007). For immunofluorescence experiments, U87MG cells were plated on glass coverslips coated with PBS-gelatin. Detection of vinculin and actin was performed as described previously (Kigel et al., 2011).

SILAC and affinity purification of plexin-A2 tagged with a Strep/FLAG epitope tag

U87MG cells expressing plexin-A2 fused in-frame with a Strep/FLAG epitope tag (Gloeckner et al., 2007), as well as HEK293 cells expressing recombinant Sema3B (Varshavsky et al., 2008) or a secreted soluble extracellular domain of Sema6A (Kigel et al., 2011) were metabolically labeled with lysine and arginine containing light (K0/R0) (Sigma; control cells) or heavy (K8/R10) carbon, nitrogen or hydrogen isotopes (Cambridge Isotope Laboratories) for ten passages in order to achieve complete labeling of cell proteins with these amino acids according to standard SILAC protocols (Pimienta et al., 2009). To prepare the ligands, the cells labeled with amino acids containing heavy isotopes were then stimulated with conditioned medium containing saturating concentrations of recombinant Sema3B or Sema6A for 5 minutes at 37℃ (five 20-cm diameter confluent plates for each treatment). Control cells were incubated with conditioned medium from HEK293 cells labeled with light isotopes. For reverse experiments, labels were switched. Both the control cells and the cells stimulated with Sema3B or Sema6A were then washed briefly with ice-cold Tris-buffered saline (TBS), scraped, lysed quickly with lysis buffer [TBS supplemented with a protease inhibitor cocktail (Roche) and 0.5% NP-40]. Equal amounts of protein from each treatment were loaded onto an anti-FLAG affinity column (150-µl bed volume). The column was washed briefly with 1.5 ml of TBS, and bound proteins were eluted using a buffer containing 100 mM glycine (pH 2.5) and snap-frozen in liquid nitrogen.

Proteomic analysis of proteins co-purified with plexin-A2

Mass spectrometric analysis was performed as described previously (Gloeckner et al., 2010). Briefly, light-isotope- and heavy-isotope-labeled eluants from the anti-FLAG columns were mixed in a 1:1 ratio based on protein content, proteolytically cleaved with trypsin and subsequently analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a nano-flow high-performance liquid chromatography (HPLC) system (Dionex Ultimate 3000 RSLC, Thermo Fisher) coupled to an Orbitrap Velos (Thermo Fisher) tandem mass spectrometer. To exclude false-positive hits caused by labeling artifacts, reverse labeling was performed, where heavy and light labels were switched. In order to identify proteins that were induced to bind to plexin-A2 following the stimulation, quantification of the resulting mass spectrometry data was performed by using the MaxQuant Software v.1.3.0.2 (Cox et al., 2009). Downstream analysis and graphical representation was performed using in-house R scripts. We only considered those hits that were identified by at least one peptide in both forward and reverse experiments.

Statistical analysis

All experiments were repeated at least twice with similar results unless otherwise stated. The one-tailed unpaired Student's *t*-test with Welch's correction was used in most experiments. In cell proliferation experiments in which we normalized the data across several experiments, we used the paired Student's *t*-test, taking care that the differences between the controls did not vary by >50%. Cell proliferation experiments were performed in triplicate. Variations between replicates within single experiments did not exceed 10%. Unless otherwise stated, error bars represent the standard error of the mean. Statistical significance is presented in the following manner: $*P<0.05$, $**P<0.01$ and $***P<0.001$.

Competing interests

The authors declare no competing interests.

Author contributions

A.D.S. and T.S. carried out most of the experiments, and were involved in their planning and interpretation. Y.M. conducted the SILAC experiments. M.U. and C.J.G. planned the SILAC experiments and planned and carried out mass spectroscopy analyses of these experiments. O.K. helped with the planning and execution of experiments and with their analysis, and helped with the writing. G.N. planned the experiments, interpreted the results and wrote the manuscript.

Funding

This work was funded by grants from the Israel Science Foundation (ISF), the Rappaport Family Institute for Research in the Medical Sciences of Technion (to G.N.), and the Tistou und Charlotte Kerstan Foundation (to M.U.).

Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.155960/-/DC1

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