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Plexin-B3 is a functional receptor for semaphorin 5A

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Semaphorins are a large family of molecular cues implicated in neural development and in a variety of functions outside the nervous system. Semaphorin 5A (Sema5A) is a transmembrane semaphorin, containing seven thrombospondin type-1 repeats, which was recently found to control axon guidance. Here we show that plexin-B3 is a high-affinity receptor specific for Sema5A. We further demonstrate that plexin-B3 activation by Sema5A mediates functional responses in plexin-B3-expressing cells (either fibroblasts, epithelial and primary endothelial cells). In addition, Sema5A can trigger the intracellular signalling of the hepatocyte growth factor/scatter factor receptor, Met, associated in a complex with plexin-B3. We thus conclude that Sema5A is able to elicit multiple functional responses through its receptor plexin-B3.

Keywords: cell migration; Met; HGF; axon guidance; plexin; semaphorin

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INTRODUCTION

Semaphorins comprise a large family of secreted and membrane-bound ligands, characterized by the presence of a conserved sema domain. They are best known as repelling and attracting cues in axon guidance, regulating the wiring of neural networks (He *et al*, 2002). Furthermore, semaphorins have been implicated in a range of functions, from cell migration and morphogenesis to angiogenesis, and from immune response to tumour progression (Tamagnone & Comoglio, 2000). Semaphorins fall into eight subclasses on the basis of sequence similarity and structural features. Class 5 comprises transmembrane semaphorins, identified both in vertebrates and in invertebrates, which are characterized by a unique extracellular domain containing seven thrombospondin type-1 (TSP-1) repeats, in addition to the sema domain (Adams *et al*, 1996; Bahri *et al*, 2001). The two vertebrate genes *Sema5A* (previously known as *SemF*) and *Sema5B* (*SemG*) share 58% sequence identity. Sema5A transcripts were found in muscle, heart, lung, spleen, and at lower levels in brain. *In situ* hybridization analysis of mouse embryos revealed Sema5A expression in mesodermal cells, as well as in the developing brain. Recently, *Sema5A* was found in neuroepithelial cells ensheathing retinal axons, and was shown to inhibit neurite outgrowth and to induce growth cone collapse (Oster *et al*, 2003). In addition, *Sema5A* is one of the genes lost in the Cri-du-chat gene deletion syndrome, associated with severe mental retardation (Simmons *et al*, 1998), further suggesting the role of this semaphorin in brain development.

Two major families of semaphorin receptors are known: neuropilins and plexins. Plexins are large transmembrane proteins, containing a sema domain and a highly conserved cytoplasmic domain (Winberg *et al*, 1998; Tamagnone *et al*, 1999). Neuropilins, only found in vertebrates, associate in receptor complexes with plexins, which are responsible for signal transduction (reviewed by Tamagnone & Comoglio, 2000). Other transmembrane molecules have been implicated in semaphorin receptor complexes, including tyrosine kinase receptors Met, Otk and KDR (Winberg *et al*, 2001; Giordano *et al*, 2002; Toshihiko *et al*, 2004). Interestingly, the extracellular domain of Met, which is the high-affinity receptor for hepatocyte growth factor/scatter factor (HGF/SF), contains a sema domain and shares other structural homologies with plexins (Artigiani *et al*, 1999). Moreover, we have shown that Sema4D, by activating its specific receptor plexin-B1, triggers the intracellular signalling of Met tyrosine kinase, associated with the plexin in a receptor complex (Giordano *et al*, 2002).

Although many reports have addressed the function of secreted semaphorins, much less is known about the role of membrane-bound semaphorins, largely due to the fact that only a few receptors for these have, as yet, been identified. In the present study, we identify plexin-B3 as the specific and high-affinity receptor for Sema5A. Moreover, we show that, after binding to plexin-B3, Sema5A triggers the collapsing response, consistent with its function as a repelling cue in axon guidance, and can activate the signalling of Met.

RESULTS AND DISCUSSION

Sema5A binds specifically to plexin-B3

To identify novel plexin receptors of membrane-bound semaphorins, we exploited molecular probes consisting of the extracellular domain of several semaphorins (from subclasses 4–7) fused to secreted alkaline phosphatase (Sema–AP; for details, see supplementary Fig S1 online). We assayed the binding of these chimeric

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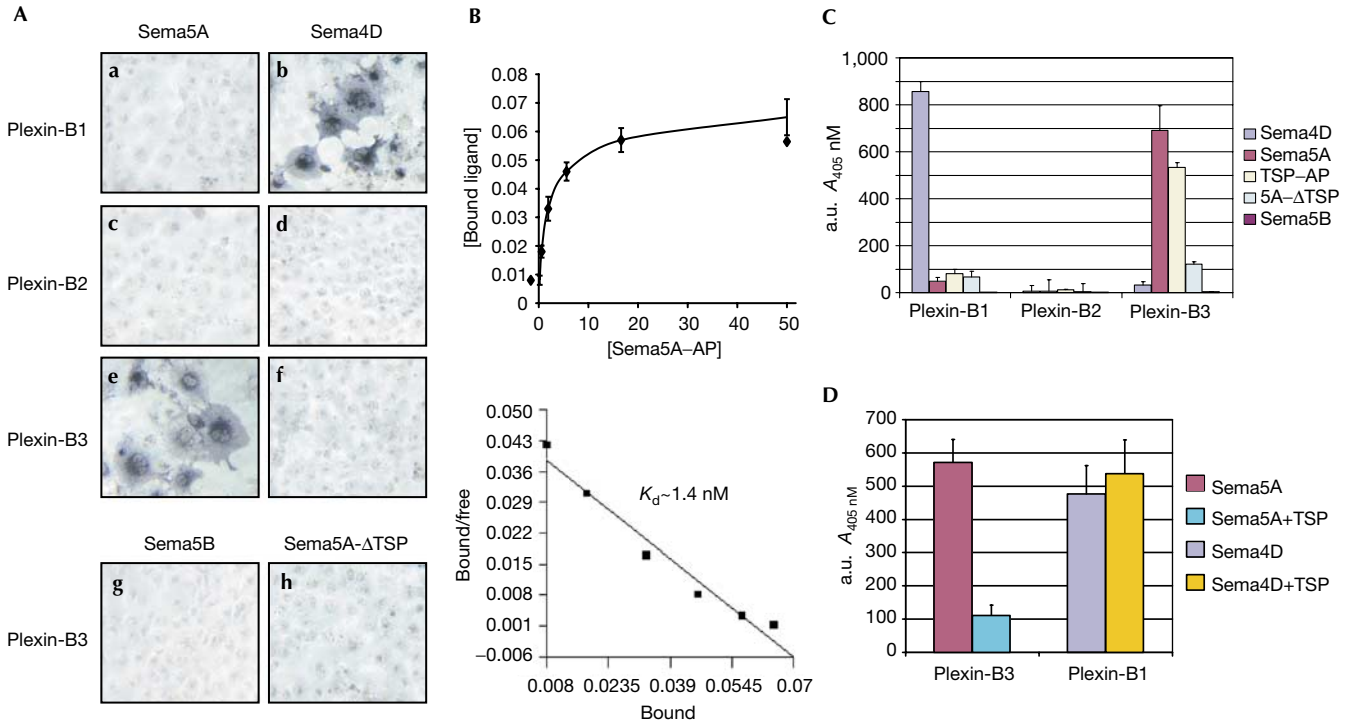

Fig 1 | Sema5A binds to plexin-B3 with high affinity and specificity. (**A**) Binding of the indicated recombinant semaphorins fused to AP (Sema–AP) to COS cells expressing either plexin-B1 (**a**,**b**), plexin-B2 (**c**,**d**) or plexin-B3 (**e**–**h**). Cell-bound AP activity was revealed *in situ* using NBT–BCIP substrates, as described in Methods. (**B**) Scatchard analysis of Sema5A binding to cells expressing plexin-B3. COS cells expressing plexin-B3 or vector alone were incubated with increasing concentrations of Sema5A–AP for 1 h. Cell-bound semaphorin was then measured as AP activity on the chromogenic substrate pNPP, as described in Methods. The results are the mean of triplicate data points, and are representative of three independent experiments. (**C**) The binding of the indicated proteins (fused to AP) to cells expressing plexin-B1, plexin-B2 and plexin-B3 was quantified using the chromogenic substrate pNPP. Results are representative of three experiments performed in duplicate with consistent results. (**D**) Plexin-B3-expressing cells were incubated with Sema5A–AP alone (10 nM) or in the presence of equimolar amounts of soluble TSP-1 domain, and the binding was quantified as in (**C**). TSP-1 repeats markedly reduced the binding of Sema5A to plexin-B3, whereas they did not interfere with the interaction between Sema4D and its receptor plexin-B1.

ligands to COS cells expressing different candidate receptors among plexin family members, by revealing cell-bound AP activity as described (Tamagnone *et al*, 1999). We thereby found that Sema5A interacted specifically with cells expressing plexin-B3 (Fig 1A,C). Other ligands, including the highly homologous Sema5B, did not bind to plexin-B3. Moreover, Sema5A did not bind to cells expressing either plexin-B1, plexin-B2 (Fig 1A) or plexin-A1 (not shown). The interaction does not depend on neuropilins, as it was unchanged when neuropilin 1 or 2 were co-transfected with plexin-B3 (not shown). This agrees with the recent report by Oster *et al* (2003), suggesting that Sema5A-mediated functions do not require neuropilins. By performing quantitative analysis of the binding with increasing ligand concentrations, we demonstrated that Sema5A binds to plexin-B3 with high affinity (K_d ~1.4 nM; see Fig 1B).

The unique feature of class 5 semaphorins is that they contain seven TSP-1 repeats in their extracellular portion. Intriguingly, we observed that a truncated form of Sema5A comprising the sema domain but lacking TSP-1 repeats (Sema5A-ΔTSP) interacts weakly, although specifically, with plexin-B3 (shown in Fig 1A,C). To investigate further the functional relevance of this region, we probed plexin-B3-expressing cells with the isolated TSP-1 repeats of Sema5A fused to AP (TSP–AP). Surprisingly, TSP–AP efficiently bound to plexin-B3, but not to plexin-B1 or plexin-B2 (Fig 1C). Thus, although TSP-1 repeats can be found in other unrelated molecules, this interaction is specific. Moreover, in the presence of comparable amounts of a soluble form of TSP-1 repeats of Sema5A fused to Myc tag (TSP–Myc), the binding of full-size Sema5A–AP to its receptor is markedly reduced (Fig 1D). These data demonstrate that TSP-1 repeats of Sema5A, in association with the sema domain, are crucially implicated in the interaction with plexin-B3. Conversely, plexin-B3 seems to be required to interact with both the regions of the ligand. On the basis of current literature, high-affinity receptors of semaphorins comprise neuropilins and plexins. Therefore, the fact that Sema5A could interact exclusively with plexin-B3 among potential receptor molecules seems to be rather indicative. Of course, our data cannot rule out the possibility that other unrelated receptors might have a role in Sema5A signalling, independently from plexin-B3.

To gain insights into the role of plexin-B3 as a specific Sema5A receptor, we studied its tissue distribution. Using northern blot analysis, we found that its transcript was below detection levels in fetal tissues as well as in several immortalized cell lines (not shown). However, by using semiquantitative reverse transcription

(RT)–PCR, we could reveal plexin-B3 mRNA in several fetal tissues (see supplementary Fig S2 online). The expression of Sema5A was measured in parallel in the same samples, and indicated that the two genes are coexpressed in tissues during development, consistent with the hypothesis of their functional interaction *in vivo*.

Functional response to Sema5A is mediated by plexin-B3

In our experiments, we observed that Sema5A–AP could not induce the collapsing response in COS cells, as shown for other semaphorins. However, it was recently reported by Oster *et al* (2003) that antibody-mediated oligomerization of the extracellular domain of Sema5A is required for its axon-repelling activity. Therefore, we tested the functional response mediated by oligomerizable Sema5A–Fc in cells expressing plexin-B3. We have previously shown that plexin signalling in fibroblasts induces a spectrum of functional responses, including cellular collapse and inhibition of integrin-dependent cell adhesion and cell migration (Barberis *et al*, 2004). We therefore induced plexin-B3 activation in fibroblasts, by addition of 30 nM of antibody-oligomerized Sema5A–Fc. As shown in Fig 2A, this induced the cellular collapse within 30 min of stimulation. In contrast, Sema7A–Fc, used as a control in the same conditions, was ineffective. Cells expressing the homologous receptor plexin-B1 did not respond to Sema5A–Fc stimulation, but collapsed in the presence of its ligand Sema4D, as described (Artigiani *et al*, 2003). As expected, non-oligomerized Sema5A–Fc elicited a markedly lower functional response in plexin-B3-expressing cells, only evident at later times (not shown). This may explain why Sema5A–AP used for binding experiments could not induce the collapsing response, and suggests that transmembrane Sema5A molecules may be required to cluster on the cell surface to activate the receptor, as reported for another family of guidance cues, the ephrins. Notably, in analogy to that reported following plexin-B1 activation (Barberis *et al*, 2004), the treatment of plexin-B3-expressing fibroblasts with Sema5A also led to inhibition of integrin-based adhesions (not shown).

Sema5A activates Met signalling via plexin-B3

Plexins share high similarity with Met and Ron, members of the SF receptor family (Artigiani *et al*, 1999). We have previously demonstrated that the Sema4D receptor, plexin-B1, is associated with Met in a complex (Giordano *et al*, 2002). Moreover, in cells where the two receptors are endogenously expressed, Sema4D can trigger Met signalling, leading to the 'invasive growth' programme. We have recently shown that plexin-B2 and plexin-B3 also, but not other family members, can associate with Met in receptor complexes (Conrotto *et al*, 2004). We therefore investigated the potential role of Sema5A and its receptor plexin-B3 in the activation of Met signalling. Fig 3A shows that, after 15 min stimulation with Sema5A–Fc, Met becomes tyrosine phosphorylated in the presence of plexin-B3, whereas Met phosphorylation induced by its cognate ligand HGF is independent of plexin-B3 expression. It is known that the invasive growth programme mediated by Met, in response to HGF or Sema4D, includes increased chemotactic migration (Giordano *et al*, 2002). Therefore, we tested the ability of Sema5A–Fc to induce the migration of MLP-29 cells in plexin-B3- and Met-dependent manner. As shown in Fig 3B, Sema5A–Fc induced significant

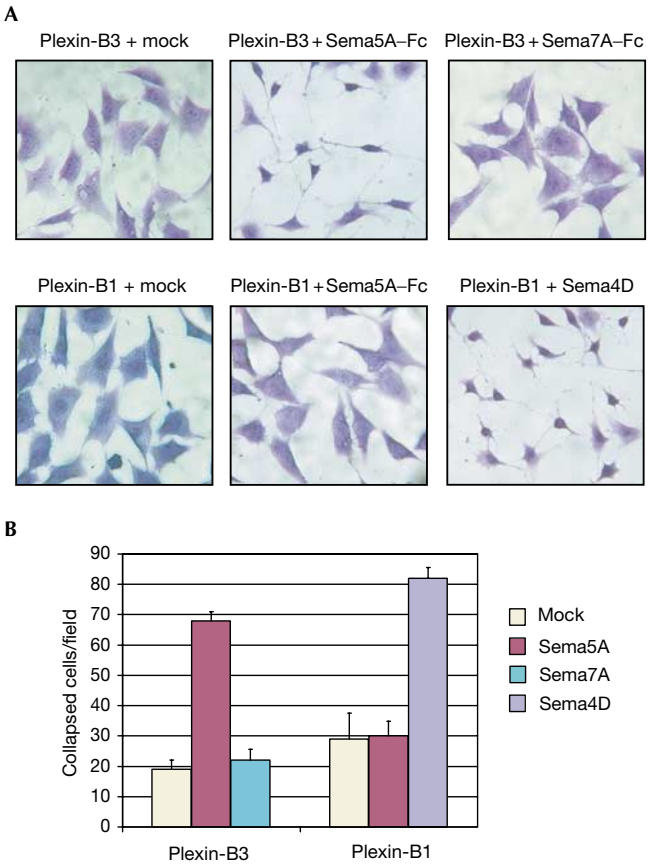

Fig 2 | Sema5A triggers the collapsing response in fibroblasts expressing plexin-B3. (**A**) NIH-3T3 fibroblasts expressing plexin-B3 were incubated with 30 nM of oligomerized Sema5A–Fc or Sema7A–Fc (see Methods). Plexin-B1-expressing cells were treated in the same conditions with oligomerized Sema5A–Fc or with Sema4D. Cells were stained with crystal violet and examined using a Leica DMIL microscope. (**B**) The collapsing response shown in (**A**) was quantified by photographing at least four representative ×20 microscope fields from two independent experiments and counting the number of cells that underwent contraction (defined as rounded cells having a diameter equal to or less than 20 μm; see Barberis *et al*, 2004). The results are the means of cell counts per field.

chemotactic migration of plexin-B3-expressing cells but not wild-type (WT) cells. To demonstrate the role of Met in this functional response, we submitted plexin-B3-expressing cells to short interfering RNA (siRNA)-mediated technology, targeting m-Met transcripts. Interestingly, siRNA-targeted MLP-29 cells expressed barely detectable Met protein (Fig 3C) and showed markedly reduced ability to migrate towards gradients of both HGF and Sema5A, but not to fetal bovine serum (FBS; Fig 3B). We verified further the role of plexin-B3 signalling in primary human umbilical vein endothelial cells (HUVECs). These cells express Met, and their treatment with HGF leads to increased cell migration and angiogenesis (Bussolino *et al*, 1992). As shown in Fig 3D, oligomerized Sema5A–Fc specifically increased endothelial cell migration in a plexin-B3-dependent manner, consistent with the activation of the endogenous Met receptor. Taken together,

the results shown in this paper and in our previous reports indicate that two structurally different semaphorins (i.e. Sema5A and Sema4D) can elicit Met activation, through interaction with specific receptors falling in the plexin-B subfamily.

Importantly, Met expression is neither required to mediate the binding of Sema5A to plexin-B3, as demonstrated by knocking down Met expression in COS cells (see supplementary Fig S3 online), nor is it required for the functional response elicited in NIH-3T3 fibroblasts (which do not express Met; see Giordano *et al*, 1993). Therefore, we propose here that the plexin-B family can elicit two distinct signalling pathways: one dependent specifically on the cytoplasmic domain of the plexins that negatively regulates integrin function and leads to cellular collapse, and another which depends on their ability to trigger the tyrosine kinase activity of Met, and leads to the invasive growth programme. Interestingly, we report here for the first time that, on Met knock-down, MLP-29 cells do not respond any more with chemotactic attraction to Sema4D. Instead, they now show cell repulsion, consistent with the activation of plexin-specific signalling (see supplementary Fig S4 online).

In summary, in this paper, we have demonstrated that Sema5A specifically interacts with plexin-B3, alone or in association with Met tyrosine kinase, through which it can elicit multiple functional responses in cells, featuring either repelling or attracting signals.

METHODS

Expression of recombinant semaphorins in mammalian cells. A detailed description of mammalian expression constructs used in this paper is provided in the supplementary data online. Recombinant semaphorins were produced by transient transfection in COS cells, and enriched preparations were obtained from cell-conditioned media, as described previously (Tamagnone *et al*, 1999). Fc-tagged Sema5A and Sema7A proteins were purified by protein-A chromatography and protein concentrations were determined by Coomassie blue staining and comparison with bovine serum albumin (BSA) standards.

Cell culture and transfections. COS, MLP-29 and 293T cells were grown in standard culture media supplemented with of 10% FBS (Gibco). NIH-3T3 fibroblasts were cultured in the presence of 10% heat-inactivated calf serum. Primary HUVECs were prepared as described previously (Bussolino *et al*, 1992), and cultured in 1% gelatin-coated dishes in M199 medium (Sigma), supplemented with 20% FBS, heparin sodium salt (50 mg/l; Sigma) and endothelial growth factors (100 μg/ml). DNA transfections in COS cells were carried out by the DEAE–dextran method. NIH-3T3, MLP-29 and HUVEC cells expressing plexin-B3 and plexin-B1 were obtained by transduction with lentiviral vectors, according to published protocols (Follenzi *et al*, 2000).

Immunoprecipitation and western blot analysis. Cells were lysed in the presence of 1% Triton X-100 and a cocktail of protease inhibitors, as described (Artigiani *et al*, 2003). Immunoprecipita-

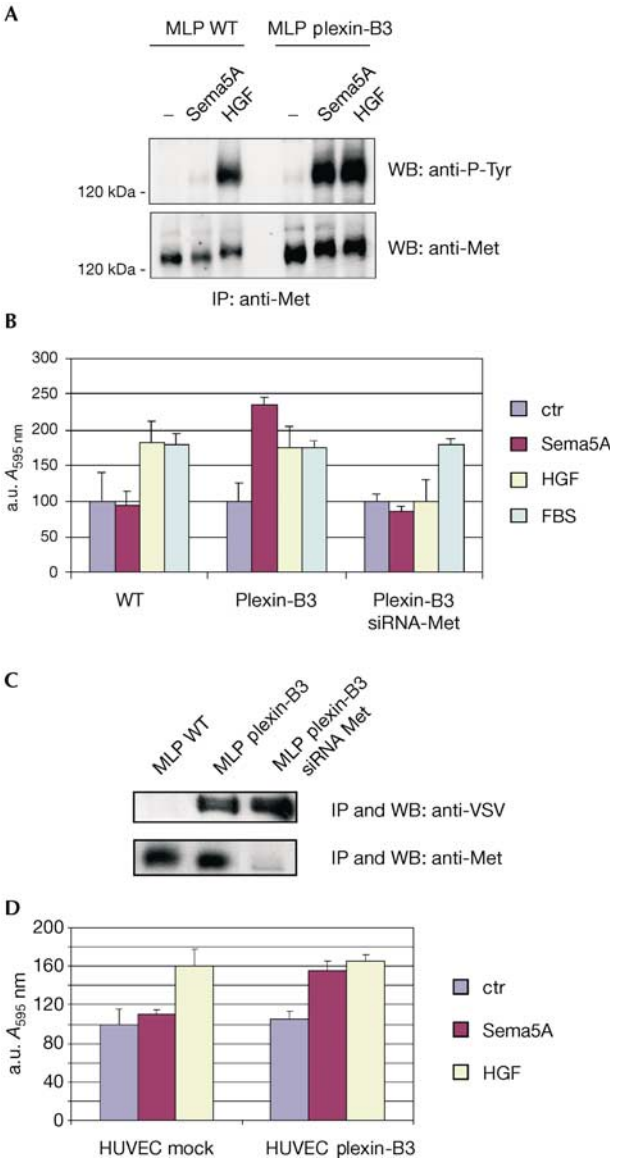

Fig 3 | Sema5A triggers Met signalling by interaction with plexin-B3. (**A**) Western blotting of immunopurified endogenous Met from parental MLP-29 cells and cells engineered to express plexin-B3. Sema5A triggers Met activation and autophosphorylation in a plexin-B3-dependent manner. This is also indicated by a slight band shift of phosphorylated proteins, as expected. (**B**) MLP-29 cell migration assay using Transwell® inserts (see Methods). The indicated cell types were allowed to migrate towards oligomerized 10 nM Sema5A or 1 nM HGF in the presence of 5% FBS, or towards 20% FBS alone. After 16 h, migrated cells were fixed and stained with crystal violet. Cell migration was quantified by reading dye absorbance at 595 nm, and values were normalized to migration in control (ctr) medium (= 100). Results are representative of three experiments performed in duplicate with consistent results. (**C**) Immunoblot analysis to demonstrate the expression of plexin-B3 (VSV-tagged) and Met in cells used for the experiments shown in (**A**,**B**). Met expression is barely detectable in cells subjected to targeted siRNA technology. (**D**) The chemotactic migration of primary HUVECs, either mock or infected to express plexin-B3, in response to either 4 nM HGF or 10 nM Sema5A added to the lower chamber of Transwell® insert. Cell migration was quantified as in (**B**). The data are the means of two independent experiments.

tion was performed according to standard protocols. Purified proteins were resolved by 8% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions, transferred onto Hybond C nitrocellulose membranes (Amersham Biosciences) and analysed by immunoblotting. Anti-VSV monoclonal antibody (clone P5D4) was purchased from Sigma, antiphosphotyrosine clone 4G10 was from Upstate Biotech., and anti-Met SP260 and B-2 were from Santa Cruz. Final detection was performed with an enhanced chemiluminescence system (Amersham).

Semaphorin binding assay and Scatchard analysis. *In situ* binding assays were performed as described previously (Tamagnone *et al*, 1999). Briefly, plexin-expressing cells were incubated for 1 h with the recombinant secreted semaphorins fused to AP (Sema–AP). After five washes, cells were fixed, heated for 10 min at 65 °C to inactivate endogenous phosphatases, and incubated with NBT–BCIP (nitro blue tetrazolium–5-bromo-4-chloro-3-indolyl-phosphate) AP substrate (Promega). Alternatively, cell-bound semaphorins were quantified by measuring AP activity on *p*-nitrophenylphosphate (pNPP), and absorbance was measured at 405 nm (as described in Artigiani *et al*, 2003). Binding kinetics was determined by incubating plexin-expressing cells with increasing concentrations of the ligand and measuring cell-bound semaphorin (as described in Artigiani *et al*, 2003). Scatchard plot analysis was performed using Equilibrate (by GertJan C. Veenstra).

Cell collapse assay. NIH-3T3 plexin-expressing fibroblasts were grown in 48-multiwell dishes, previously coated with 10 μg/ml fibronectin (Sigma). Fc-tagged Sema5A or Sema7A proteins were diluted in serum-free medium supplemented with 0.2% BSA, at a final concentration of about 30 nM, and preincubated for 1 h with 7.5 μg/ml of anti-human Fc antibody (Jackson Immunochemical) at 25 °C. Cells were then incubated with antibody-oligomerized semaphorins for 30 min–1 h, fixed with 4% paraformaldehyde for 15 min, stained with crystal violet and finally examined using a Leica DMIL microscope equipped with a Leica DC300F camera.

siRNA technology. Met expression was downmodulated by cell transfection with a pSUPER plasmid (Brummelkamp *et al*, 2002) engineered to carry the required sequence for synthesizing Met-targeted siRNA (5′-ACUCUAGAUGCUCAGACUUTT-3′), together with an empty vector carrying the *neo* resistance gene. G418 resistant clones were isolated and assayed for the expression of Met protein.

Migration assays. MLP-29 cells (5×10^4), either WT or expressing plexin-B3, were seeded in the upper chamber of a Transwell® insert (carrying a polycarbonate 8 μm porous membrane). The medium in both the chambers was supplemented with 5% FBS, and chemotactic factors were added to the lower chamber. After overnight incubation, nonmigrated cells adhering to the upper side of the membrane were mechanically removed. Migrated cells were then fixed in 11% glutaraldehyde and stained with crystal violet. Cells were then photographed and solubilized in 10% acetic acid, and A_{595} was measured in a microplate reader.

HUVEC cells (4×10^4 per each well) were detached with 15 mM EDTA to avoid trypsin digestion, and seeded in the upper chamber of a Transwell® insert, the membrane of which had been previously coated on both sides with 3 μg/ml of fibronectin. M199 medium with 2% FBS was added to both the chambers, and chemoattractants were included in the lower chamber. After 6 h, the inserts were treated as above, to reveal migrated cells.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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