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RESEARCH ARTICLE

# Complexes of plexin-A4 and plexin-D1 convey semaphorin-3C signals to induce cytoskeletal collapse in the absence of neuropilins

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## ABSTRACT

Class-3 semaphorin guidance factors bind to receptor complexes containing neuropilin and plexin receptors. A semaphorin may bind to several receptor complexes containing somewhat different constituents, resulting in diverse effects on cell migration. U87MG glioblastoma cells express both neuropilins and the four class-A plexins. Here, we show that these cells respond to Sema3A or Sema3B by cytoskeletal collapse and cell contraction but fail to contract in response to Sema3C, Sema3D, Sema3G or Sema3E, even when class-A plexins are overexpressed in the cells. In contrast, expression of recombinant plexin-D1 enabled contraction in response to these semaphorins. Surprisingly, unlike Sema3D and Sema3G, Sema3C also induced the contraction and repulsion of plexin-D1-expressing U87MG cells in which both neuropilins were knocked out using CRISPR/Cas9. In the absence of neuropilins, the EC50 of Sema3C was 5.5 times higher, indicating that the neuropilins function as enhancers of plexin-D1-mediated Sema3C signaling but are not absolutely required for Sema3C signal transduction. Interestingly, in the absence of neuropilins, plexin-A4 formed complexes with plexin-D1, and was required in addition to plexin-D1 to enable Sema3C-induced signal transduction.

**KEY WORDS: Semaphorins, Neuropilins, Plexins, Receptor complexes**

## INTRODUCTION

Signal transduction is frequently initiated following the binding of extracellular ligands to cell surface receptors. Initially, it was thought that each cell surface receptor binds a specific ligand that then activates a unique signaling cascade. However, subsequent studies revealed that many receptors can bind and transduce signals in response to multiple ligands, as in the case of the epidermal growth factor receptor family (Yarden and Pines, 2012). Nevertheless, cells need to be able to differentiate between signals conveyed by ligands that bind to shared receptors. This problem is perhaps most acute in the case of guidance factors such as those belonging to the diffusible class-3 semaphorin subfamily (Neufeld and Kessler, 2008). During embryonic development, migrating cells or extending axons may simultaneously encounter several gradients of semaphorins that bind to shared receptors, and misinterpretation of these signals may result in misdirection.

Semaphorins were initially characterized as axon guidance factors (Huber et al., 2003) but have emerged as repulsive guidance factors that direct the migration of many types of cells during development (Alto and Terman, 2017; Neufeld et al., 2016; Valdembri et al., 2016; Yoshida, 2012). The seven members of the class-3 semaphorin subfamily are the only secreted vertebrate semaphorins. Class-3 semaphorins bind to one of the two receptors of the neuropilin family or to both. In addition, the neuropilins also function as receptors for several growth factors such as VEGF, TGF-β, HGF and PDGF family members to name but a few (Neufeld and Kessler, 2017). The neuropilins associate with class-A plexin receptors or with plexin-D1 to transduce class-3 semaphorin signals because their short intracellular domains render them unable to transduce signals on their own (Tamagnone et al., 1999). Sema3A binds specifically to neuropilin-1 (NRP1) and Sema3F and Sema3G to NRP2, whereas Sema3B, Sema3C and Sema3D bind to both neuropilins (Neufeld et al., 2016). Sema3E is an exception since it binds directly to plexin-D1 and does not bind to neuropilins (Gu et al., 2005). However, NRP1 can associate with plexin-D1 in response to stimulation by Sema3E, and when associated, can turn the response to Sema3E from a repulsive to an attractive one (Chauvet et al., 2007).

The simplest explanation regarding the mechanism by which cells distinguish between signals of class-3 semaphorins that bind to shared neuropilins is that different class-3 semaphorins induce associations of neuropilins with different plexins. It was indeed observed that the affinity of specific semaphorins for their neuropilin receptors is enhanced in the presence of specific plexins (Gitler et al., 2004; Rohm et al., 2000) suggesting that the binding site of functional high affinity class-3 semaphorin receptors is formed by a complex of plexins and neuropilins (Janssen et al., 2012). However, we have found that this model is also a bit simplistic since under physiological conditions more than one plexin seems to be required in addition to a neuropilin in order to form functional, signal transducing receptors for given class-3 semaphorins. Thus, Sema3A signal transduction requires the simultaneous presence of NRP1, plexin-A1 and plexin-A4 while Sema3B signaling requires the presence of either NRP1 or NRP2, plexin-A2 and plexin-A4 (Kigel et al., 2011; Sabag et al., 2014). There is, however, a fair degree of plasticity built into the composition of these receptor complexes. For example, when plexin-A2 is artificially overexpressed it can compensate for a lack of plexin-A4 and plexin-A1 to enable Sema3A signaling (Janssen et al., 2012; Sabag et al., 2014). However, under these conditions, cells lose their ability to distinguish between Sema3A and Sema3B (Sabag et al., 2014).

In order to gain a deeper understanding of the composition of the functional receptor complexes that convey signals of additional class-3 semaphorins, we concentrated here on a group of class-3

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semaphorins that are not able to induce the cytoskeletal collapse of U87MG cells. U87MG cells express the four class-A plexins and both neuropilins and respond well to Sema3A, Sema3B and Sema3F (Kigel et al., 2011; Sabag et al., 2014). They do not respond to stimulation with Sema3E since they only express marginal amounts of plexin-D1 if any at all, and they also fail to respond to Sema3C, Sema3D and Sema3G, despite the presence of neuropilins. We find that signal transduction by these three semaphorins required plexin-D1, and even when overexpressed, class-A plexins could not compensate for a lack of plexin-D1. Surprisingly, expression of recombinant plexin-D1 in U87MG cells in which we have knocked out both NRP1 and NRP2 using CRISPR/Cas9 was sufficient to enable Sema3C-induced signal transduction, but not Sema3D or Sema3G signaling, indicating that Sema3C behaves like Sema3E and can transduce signals utilizing plexin-D1 directly in the absence of neuropilins. However, unlike Sema3E, in the absence of neuropilins, Sema3C signaling also depended on the presence of plexin-A4, since silencing expression of plexin-A4 inhibited Sema3C-induced signal transduction despite the presence of plexin-D1.

## RESULTS

### Signal transduction induced by Sema3C, Sema3D and Sema3G requires plexin-D1

U87MG glioblastoma cells express the four class-A plexins as well as both neuropilins (Kigel et al., 2011; Sabag et al., 2014). They also express very small amounts of plexin-D1 mRNA as determined by RT-PCR. However, we have been unable to detect plexin-D1 in these cells using western blot analysis (Fig. 1A), and the amounts of plexin-D1 produced, if produced at all, are not sufficient to enable signal transduction induced by Sema3E, a class-3 semaphorin that signals using exclusively plexin-D1 (Fig. 1B) (Gu et al., 2005). Interestingly, Sema3C, Sema3D and Sema3G also failed to induce contraction and collapse of the cytoskeleton of U87MG cells. This failure was not due to a lack of neuropilins or class-A plexins since U87MG cells express both neuropilins as well as all the four class-A plexins, and contract efficiently in response to other class-3 semaphorins such as Sema3A, Sema3B and Sema3F, which require various neuropilins as well as various class-A plexins to transduce signals (Kigel et al., 2011; Sabag et al., 2014). Similar results were also obtained when we examined the response of HT1080 fibrosarcoma cells, which also do not express plexin-D1, to these class-3 semaphorins (Fig. S1A). These results suggested that plexin-D1 is required for signal transduction by Sema3C, Sema3D and Sema3G, and in addition, suggest that class-A plexins cannot compensate for the absence of plexin-D1. To determine if this is indeed the case, we expressed in both cell types the cDNA encoding full-length plexin-D1. Indeed, both U87MG and HT1080 cells expressing recombinant plexin-D1 (Fig. 1A) gained the ability to respond by cell contraction to these three class-3 semaphorins, as well as to purified UNCL-Sema3E, a point-mutated form of Sema3E that is not cleaved by furin-like pro-protein convertases (Casazza et al., 2012) (Fig. 1B,C and Fig. S1A,B). Furthermore, when HEK293 cells expressing Sema3C and stained with the fluorescent dye DiI were seeded on top of such U87MG cells expressing recombinant plexin-D1 they repelled the cells, whereas HEK293 cells containing empty expression vector did not (Fig. 1D).

These experiments suggest that at physiological levels of expression class-A plexins cannot compensate for lack of plexin-D1 to enable signal transduction induced by Sema3D, Sema3C and Sema3G. In order to determine if class-A plexins can replace plexin-D1 when expressed at levels that exceed their physiological levels of expression, we infected U87MG cells with lentiviruses directing expression of plexin-A1, plexin-A2 and plexin-A4 (Fig. 2A). Wild-type U87MG cells and U87MG cells overexpressing each of these class-A plexins (Fig. 2A) or plexin-D1 were seeded on fibronectin-coated wells of the E-plate of the xCELLigence machine and stimulated with purified FR-Sema3C/Fc, a point-mutated form of Sema3C stabilized against degradation and inactivation by furin-like pro-protein convertases (Mumblat et al., 2015) or with elution buffer (Control). Cell contraction was then measured using the xCELLigence machine essentially as previously described (Camillo et al., 2017; Mumblat et al., 2015). Decreased cell index values in these experiments correlate with lower impedance and with enhanced cell contraction. None of the cells overexpressing class-A plexins were able to contract in response to FR-Sema3C/Fc, even though Sema3A and Sema3B, used as positive controls, induced cell contraction efficiently (Fig. 2B-D,F). In contrast, cells expressing recombinant plexin-D1 contracted efficiently in response to purified FR-Sema3C/Fc (Fig. 2E,F) or conditioned medium containing recombinant wild-type Sema3C (Fig. 2E). We therefore concluded that even when overexpressed in the presence of neuropilins, these class-A plexins cannot compensate for a lack of plexin-D1 to enable Sema3C signal transduction.

### Generation of U87MG cells lacking functional neuropilin receptors using CRISPR/Cas9

The plexin-D1 receptor binds Sema3E and transduces Sema3E signals independently of neuropilins. However, NRP1 can form complexes with plexin-D1 and this association can modulate significantly Sema3E signal transduction (Chauvet et al., 2007; Gu et al., 2005). In order to determine if neuropilins are required for signal transduction by other class-3 semaphorins that transduce their signals using plexin-D1, we first abolished the expression of NRP1 in U87MG cells by the introduction of frame-shift mutations into each of the alleles encoding NRP1 in U87MG cells using a *NRP1*-specific guide RNA and CRISPR/Cas9 (Fig. S2A) (Ran et al., 2013). These experiments resulted in the isolation by limiting dilution of several single-cell-derived clones that do not express NRP1 and which, as a result, are no longer able to contract in response to Sema3A, such as clone 18 and clone 25 (Fig. 3C,D, Fig. S2B) and in which NRP1 can no longer be detected using western blot analysis (Fig. 3A). These clones lacking NRP1 still express functional NRP2 and are still able to contract in response to Sema3B, a class-3 semaphorin that can utilize both NRP1 and NRP2 for signal transduction (Fig. 3B,C) (Sabag et al., 2014). To generate U87MG cells in which the genes encoding both neuropilins are dysfunctional, we used a similar procedure employing a guide RNA targeting *NRP2* (Fig. S2A) and CRISPR/Cas9 to introduce frame-shift mutations into the *NRP2* alleles of clone 25 cells in which we had already knocked out *NRP1*. Following limiting dilution, we identified three clones of cells as NRP2 knockout clones, which no longer expressed any NRP2 as determined by western blot analysis (clones 25/1, 25/20 and 25/23) and one clone (clone 25/10) in which one allele contained a frame-shift mutation and the other remained intact. Cells of clone 25/10 expressed as a result reduced levels of NRP2 (Fig. 3B). We characterized in clone 25/23 the frame-shift mutations in both alleles (Fig. S2C). In clone 25/1 we found a frame-shift mutation due to a single base insertion in one of the alleles whereas in the other allele, we identified a large insertion at the PAM cleavage area, which rendered the gene dysfunctional (Fig. S2C). In clone 20, we could identify only one allele with a frame-shift mutation. We do not

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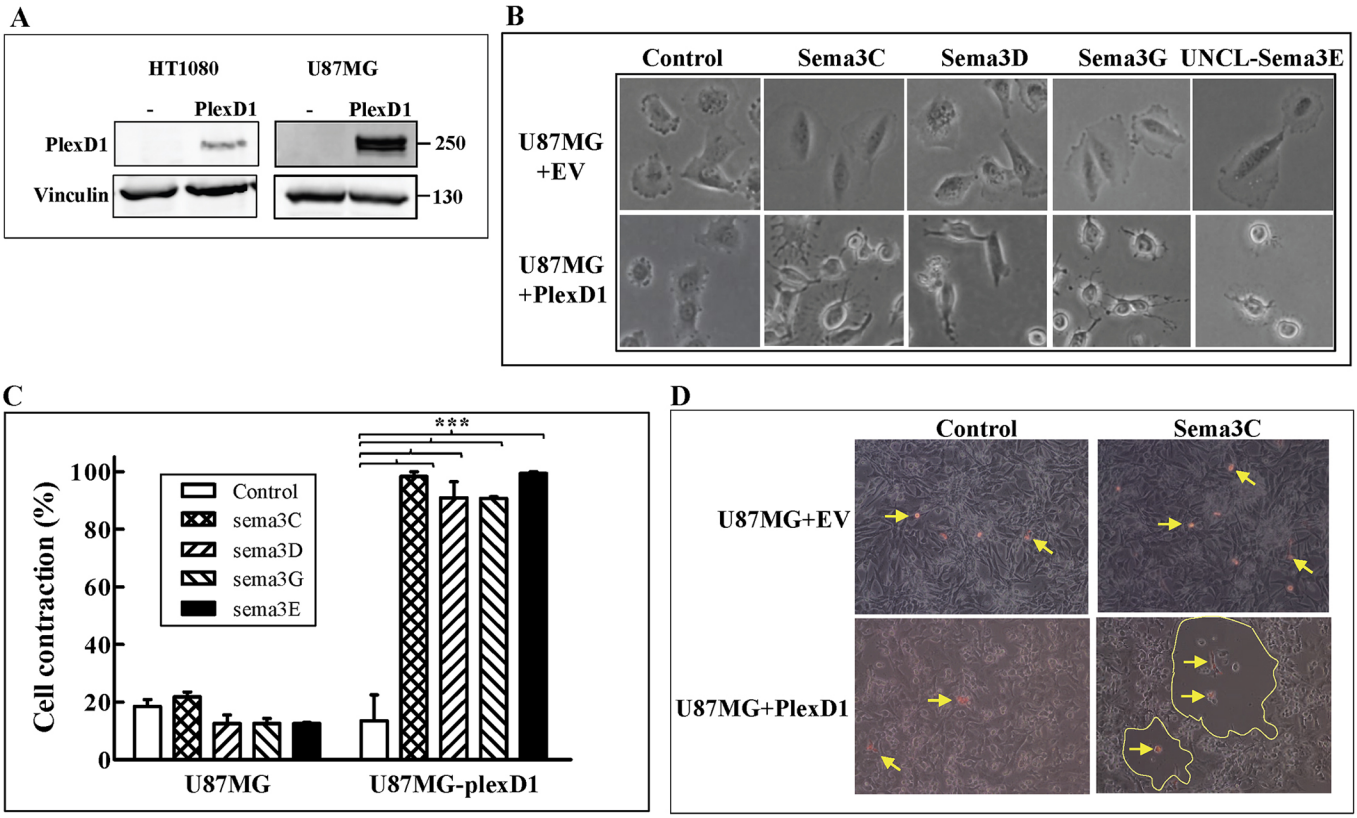


**Fig. 1. Expression of plexinD1 is required for contraction of U87MG cells induced by Sema3C, Sema3E, Sema3D and Sema3G.** (A) Western blot analysis of plexin-D1 expression in U87MG and HT1080 cells infected with empty lentiviral expression vector (–) and U87MG cells infected with lentiviruses directing plexin-D1 expression (PlexD1). Molecular mass is indicated on the right in kDa. (B) U87MG cells infected with empty lentiviral expression vector (U87MG+EV) as well as U87MG cells expressing plexinD1 (U87MG+PlexD1), were stimulated with conditioned medium containing Sema3C, Sema3D or Sema3G or with 1 µg/ml purified UNCL-Sema3E/Fc (Casazza et al., 2012). Phase-contrast pictures were taken 30 min after stimulation at 10× magnification. (C) The percentage of contracted cells was determined in eight microscopic fields derived from two replicate wells in each of three independent experiments similar to the experiment described in B. *N*=6, data represent mean±s.e.m. One-way ANOVA followed by Bonferroni's multiple comparison test was used to determine statistical significance; ****P*<0.001. (D) U87MG cells, infected with empty lentiviruses (U87MG+EV) or lentiviruses directing expression of recombinant plexin-D1 (U87MG +PlexD1) were grown to subconfluence. Control HEK293 cells (control) or HEK293 cells expressing Sema3C were stained with DiI and seeded at clonal density on top of the U87MG cells. Shown are merged phase-contrast and fluorescence images taken after 24 h. The borders of clearings produced in the U87MG monolayer by the Sema3C-expressing cells are marked by a yellow line. Arrows indicate Sema3C-expressing HEK293 cells.

know if this clone contains only one *NRP2* encoding chromosome or whether the *NRP2* genes on both chromosomes contain identical CRISPR/Cas9-induced mutations (Fig. S2C). We therefore concluded that these three clones no longer expressed NRP1 or NRP2. Indeed, contraction assays performed on these three double-knockout clones revealed that they are unable to contract in response to either Sema3A or Sema3B, confirming that cells of these three clones do not express functional neuropilins (Fig. 3C,D). In further experiments, we used clone 25/1. We excluded clone 25/23 and did not use it in subsequent experiments because in contraction experiments it displayed a higher background of contracted cells, and because it still displayed a very low but statistically significant response to Sema3B which we cannot currently explain (Fig. 3D).

### Plexin-D1 transduces Sema3C signals in the absence of neuropilins

The availability of U87MG-derived clones of cells lacking functional neuropilin-encoding genes enabled us to determine if neuropilins are indeed required for signal transduction by semaphorins such as Sema3C, Sema3D or Sema3G that signal using the plexin-D1 receptor. To answer this question, we infected U87MG clone 25/1 cells lacking neuropilins with lentiviruses containing plexin-D1 cDNA and isolated clones that express plexin-D1 by limiting dilution. Plexin-D1 expression was verified in these clones using western blot analysis and one of these plexin-D1-expressing clones (clone 7) was picked and used in further experiments (Fig. 4A) because the expression level of plexin-D1 was very similar to that found in a clone (clone 15) derived from wild-type U87MG cells in which we expressed plexin-D1 (Fig. 5D). Indeed, while U87MG clone 25/1 cells lacking neuropilins were unable to contract in response to Sema3E similarly to parental U87MG cells, U87MG clone 25/1-derived clone 7 cells expressing recombinant plexin-D1 contracted in response to Sema3E (Fig. 4C,D). Unexpectedly, these cells also contracted in response to stimulation with purified FR-Sema3C/Fc (Fig. 4C,D) (Mumblat et al., 2015), as well as in response to conditioned medium derived from HEK293 cells expressing recombinant FR-Sema3C (Fig. 4E,F) and in response to conditioned medium derived from HEK293 cells expressing recombinant wild-type Sema3C (Fig. S3A). The contraction of these cells was accompanied by the collapse of the actin cytoskeleton and by the disappearance of vinculin from focal adhesions (Fig. 4E and Fig. S3B). These observations suggest that the signaling cascades activated by Sema3C using plexin-D1-dependent signal

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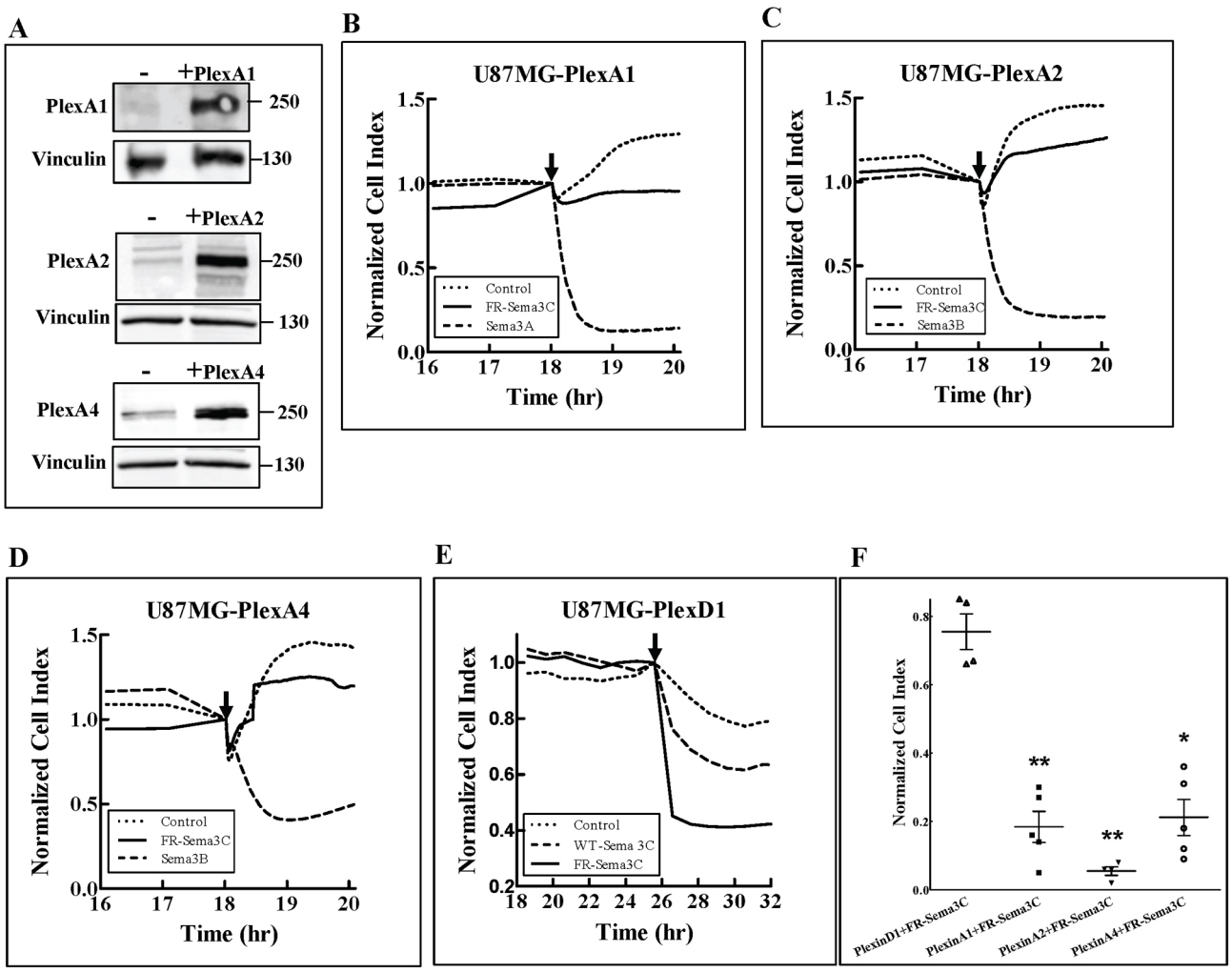


**Fig. 2. Class-A plexins do not enable Sema3C signal transduction in the absence of plexin-D1 even when they are overexpressed.** (A) U87MG cells were infected with lentiviruses directing overexpression of plexinA1, plexinA2 or plexinA4. The expression levels of the plexins before and after infection were monitored by western blot. (B-D) U87MG cells expressing different recombinant class-A plexins were seeded at $2\times10^4$ cells/well in xCELLigence E-plates. After 24 h, the cells were stimulated with 1 µg/ml purified FR-Sema3C/Fc or elution buffer (Control). Conditioned medium derived from HEK293 cells expressing Sema3A or Sema3B was added as a positive control to cells expressing class A plexins. Shown are the normalized cell index values before and after addition of semaphorins. Arrows indicate the time points at which stimulation was initiated. Shown are representative experiments out of four performed for each plexin with similar results. (E) Conditioned medium containing wild-type Sema3C was added to cells expressing plexin-D1 to compare its effect with that of FR-Sema3C/Fc. Shown are the normalized cell index values before and after addition of semaphorins. Arrows indicate time points at which stimulation was initiated. Decreased cell index values correlate with lower impedance and enhanced cell contraction (Camillo et al., 2017). (F) The average effect of FR-Sema3C on the contraction of U87MG cells overexpressing plexin-A1, plexin-A2, plexin-A4 or plexin-D1 as derived from four independent experiments is shown. Shown are the differences in normalized cell index units between the start point at which the semaphorins were added and the maximal decline in the normalized cell index which corresponds to maximal contraction. The small declines seen following addition of controls were not subtracted. Statistical significance was assessed using the Mann-Whitney one-tailed test; *$P$<0.05, **$P$<0.01.

transduction in the absence of neuropilins are in all likelihood not very different from the signaling cascades activated by Sema3C using plexin-D1 in the presence of neuropilins since the biological responses are very similar (Fig. 4E). Taken together, these observations suggest that neuropilins are not absolutely required for plexin-D1-mediated signal transduction induced by Sema3C.

In the above experiments, we have used U87MG-derived cells in which we have expressed recombinant plexin-D1, raising the possibility that neuropilin-independent Sema3C signal transduction may only be possible when the concentration of plexin-D1 is abnormally high, and outside of the range of physiological concentrations. We therefore compared the concentration of the recombinant plexin-D1 in U87MG clone 25/1-derived clone 7 cells with the plexin-D1 concentration in cultured primary human umbilical vein-derived endothelial cells (HUVECs). We found that the concentration of the recombinant plexin-D1 was very similar to that found in HUVECs (Fig. 4B). These observations suggest that Sema3C may also be able to affect the migration of plexin-D1-expressing cells that lack neuropilins *in vivo*, and it should be remembered that it was reported that endothelial cells of tumor-associated blood vessels have been found to upregulate plexin-D1 expression about threefold (Roodink et al., 2005, 2009). Since we have observed that the expression level of the recombinant plexin-D1 can decline somewhat over time, we conducted concomitantly contraction experiments using the clone 7 cells to show that they were still able to contract in response to Sema3C (Fig. S4A,B).

To find out if cells expressing plexin-D1 in the absence of neuropilins can be repelled by Sema3C, we seeded HEK293 cells

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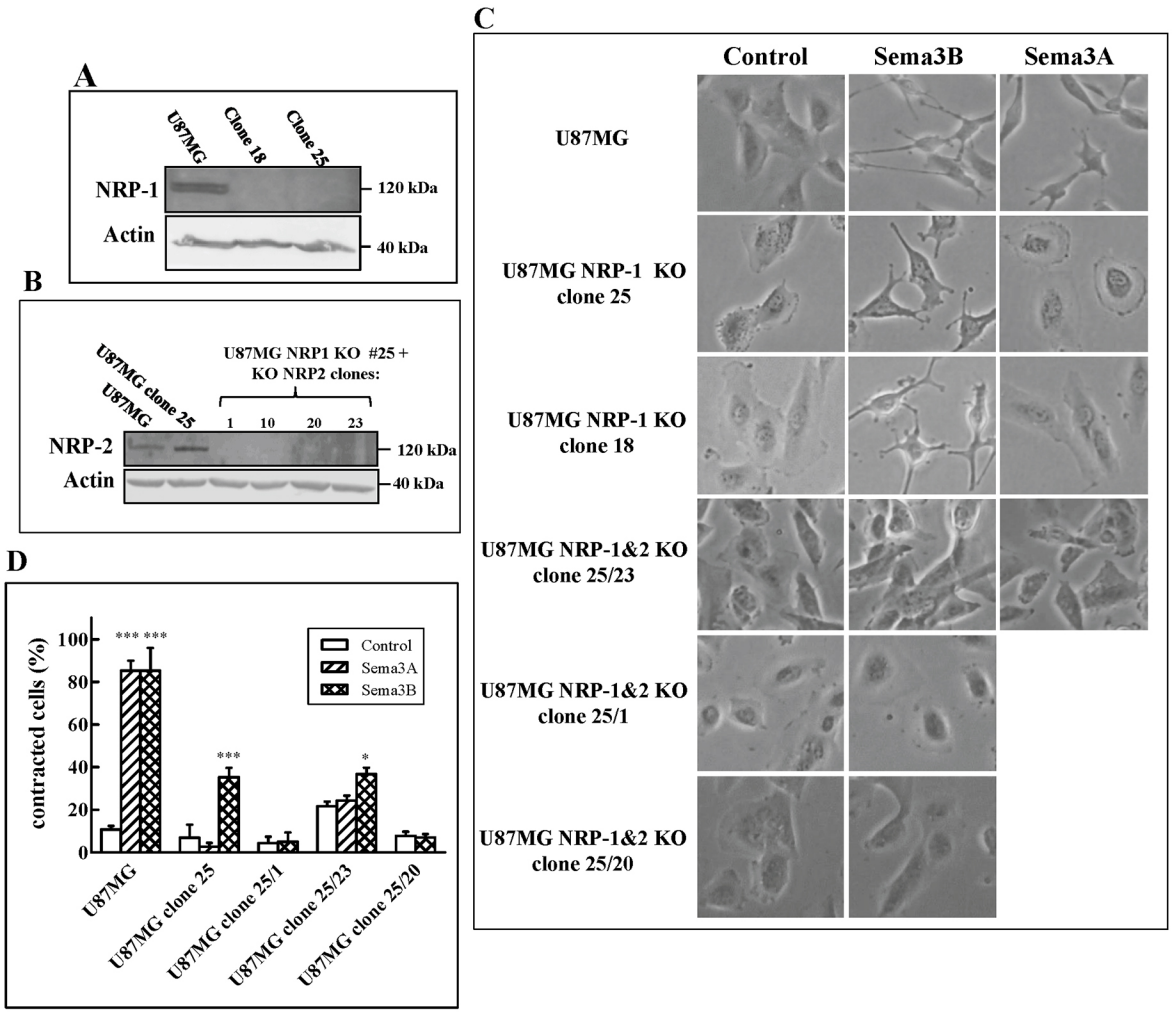


**Fig. 3. Cells in which *NRP1* and *NRP2* are knocked out fail to contract in response to Sema3A and Sema3B.** (A) Western blot analysis of NRP1 expression in the two U87MG NRP1-knockout clones (18 and 25) compared with parental U87MG cells. (B) Western blot analysis of NRP2 expression in NRP2-knockout clones derived from the NRP1-knockout clone 25. Shown is expression of NRP2 in clone 25 cells and in the clone 25-derived NRP2-knockout clones 25/1, 25/10, 25/20 and 25/23. Clone 10 contained one mutated and one wild-type allele of *NRP2*. (C) Control conditioned medium from HEK293 cells infected with empty lentiviral expression vector (Control) or conditioned medium from HEK293 cells expressing recombinant Sema3A or Sema3B (300 µl) (Sabag et al., 2014) were added to parental U87MG cells or to various U87MG-derived clones of cells in which *NRP1* or both *NRP1* and *NRP2* were knocked out. Shown are representative images after a 30 min incubation at 37°C. The experiment was repeated three times with similar results. (D) Quantification of the percentage of contracted cells in three repeats of the experiment in C, each of which was done in duplicate wells as described in Fig. 1C. One-way ANOVA followed by Bonferroni's multiple comparison post test was used to determine statistical significance. *N*=6, data represent mean±s.e.m.; **P*<0.05, ****P*<0.001.

expressing FR-Sema3C/Fc which were stained with the fluorescent dye DiI at a clonal concentration on top of either U87MG clone 15 cells expressing recombinant plexin-D1 and both neuropilins, or on top of U87MG clone 25/1-derived plexin-D1-expressing clone 7 cells, which lack neuropilins. These experiments indicated that the FR-Sema3C-producing cells repel both cell types similarly. In contrast, when HEK293 cells containing empty expression vector were seeded on top of these cells they were unable to repulse the cells and create 'holes' in the cell monolayer (Fig. 5A and Movies 1-4). Activation of plexin-D1 by Sema3E is reported to be associated with inhibition of R-ras activity due to activation of the GAP activity located in the cytoplasmic domain of the plexin-D1 receptor (Uesugi et al., 2009). However, stimulation of either U87MG clone 25/1-derived clone 7 cells or U87MG clone 15 cells with FR-Sema3C did not result in increased hydrolysis of R-ras-associated GTP (data not shown) even though the cells did contract in response to stimulation. Further experiments revealed that, in these cells, Sema3E was also unable to induce hydrolysis of R-ras-associated GTP, even in U87MG clone 15 cells that express both neuropilins and plexin-D1 (Fig. S4C). Additional experiments will be required in order to find out if activation of plexin-D1 by Sema3C in the absence of neuropilins activates different signaling pathways to those activated in the presence of neuropilins. These experiments also suggested that additional class-3 semaphorins, such as Sema3D and Sema3G, that transduce signals using the plexin-D1 receptor may also be able to transduce signals in the absence of neuropilins. However, U87MG clone 25/1-derived clone 7 cells, which lack neuropilins but express recombinant plexin-D1, were unable to contract in response to either Sema3D or Sema3G, whereas wild-type U87MG clone 15 cells that express recombinant plexin-D1 at similar levels of expression contracted in response to both of these semaphorins (Fig. 5B,C). These observations suggest that the ability to transduce signals

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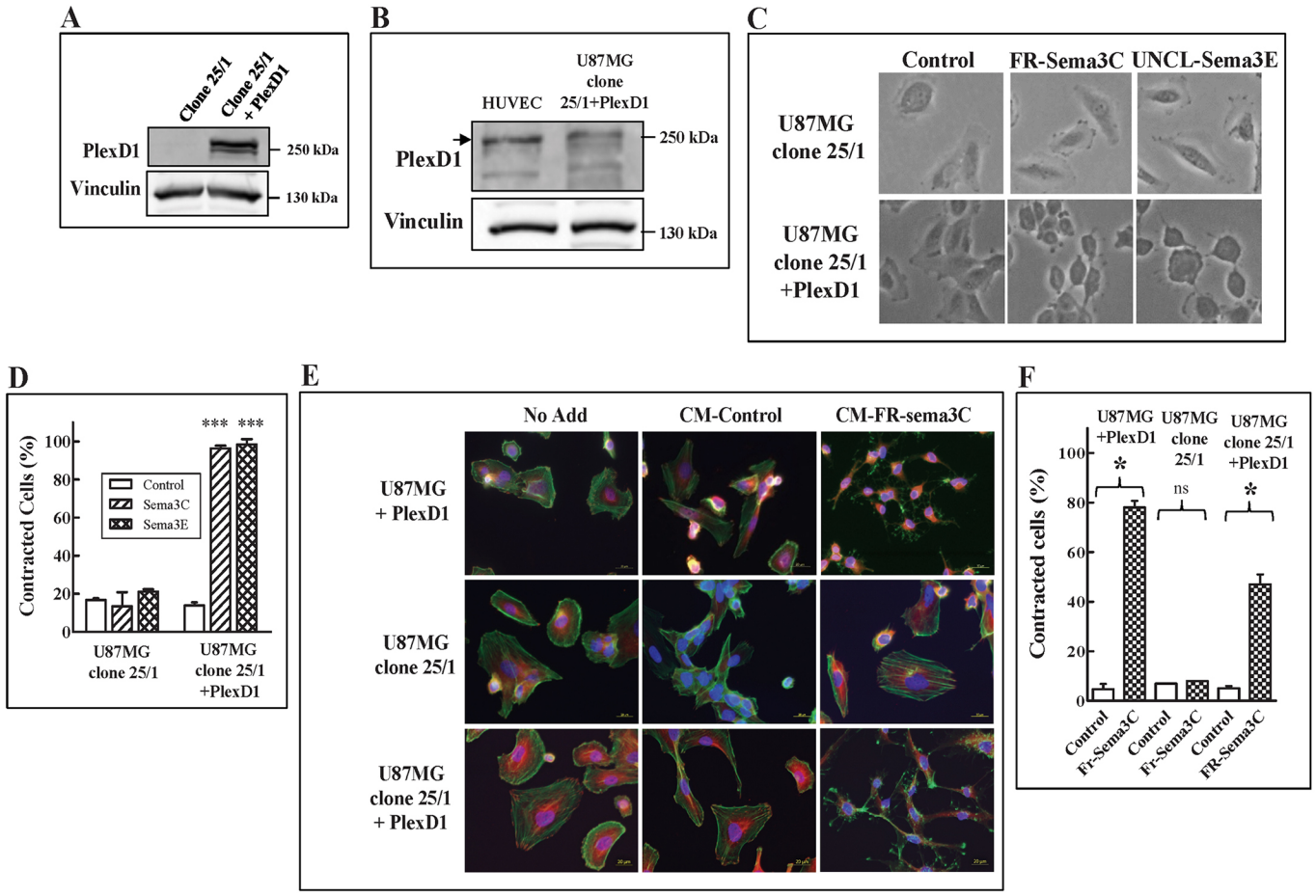


**Fig. 4. Sema3C induces cytoskeletal contraction using plexin-D1 in the absence of neuropilins.** (A) Western blot analysis of plexinD1 expression in a clone of U87MG cells in which the genes encoding neuropilins were knocked out (U87MG clone 25/1) and in a clone derived from these cells expressing recombinant plexin-D1 (U87MG clone 25/1+plexin-D1 clone 7). (B) Equal concentrations of cell lysates prepared from HUVECs or U87MG clone 25/1+plexin-D1 clone 7 cells were subjected to western blot analysis using antibodies directed against plexin-D1 or vinculin. (C) The contractile response to elution buffer (Control) or to 1 µg/ml purified FR-Sema3C/Fc or UNCL-Sema3E/Fc was compared between U87MG clone 25/1 and U87MG clone 25/1+plexin-D1 clone 7 cells. Shown are representative images of cells 30 min after stimulation. (D) Comparison of the percentage of contracted cells in three independent repeats performed in triplicate wells of the experiment shown in C. One-way ANOVA followed by Bonferroni's multiple comparison post test was used to determine statistical significance. *N*=9, data represent mean±s.e.m. ****P*<0.001. (E) The growth medium of U87MG cells expressing plexin-D1, U87MG clone 25/1 cells lacking both neuropilins, and U87MG clone 25/1 cells expressing recombinant plexin-D1 (clone 7) grown on glass coverslips was exchanged with conditioned medium derived from HEK293 cells infected with empty lentiviral expression vector (CM-Control) or a lentiviral expression vector for FR-Sema3C/myc (Mumblat et al., 2015). After 30 min, the cells were stained with DAPI to visualize cell nuclei (blue), with fluorescent Phalloidin (green) to visualize actin fibers, and with an antibody specific for vinculin (red). Shown are merged confocal photographs generated using ZEN 2.3 lite software. (F) Comparison of the percentage of contracted cells in two independent repeats of the experiment shown in E. Data represent mean±s.e.m.; **P*<0.05; ns, not significant.

independently of neuropilins via the plexin-D1 receptor is specific to Sema3E and Sema3C.

To verify these results by a different method we also inhibited the expression of both neuropilins in U87MG clone 15 cells expressing recombinant plexin-D1 using specific siRNA species. Although siRNAs do not cause complete inhibition of gene expression like CRISPR/Cas9-mediated knockout, the expression of both neuropilins was strongly inhibited by these siRNAs (Fig. S5A). Such plexin-D1-expressing cells in which the expression of both neuropilins was silenced using siRNAs contracted when they were stimulated by conditioned medium from HEK293 cells expressing wild-type Sema3C (Fig. S5B,C), but failed to contract in response to either Sema3D or Sema3G, supporting the conclusions obtained using the knockout cells. Further experiments in which only one of the two neuropilins was silenced revealed that Sema3D utilizes both neuropilins in addition to plexin-D1 for signal transduction, whereas Sema3G utilizes NRP2 exclusively and is not able to transduce signals using NRP1 (Fig. S5B,C). To verify our results using a cell line that expresses plexin-D1 endogenously, we silenced the expression of NRP1 in primary HUVECs that express plexin-D1. Despite the silencing of NRP1, the HUVECs were still able to contract in response to FR-Sema3C, although the response was not as robust as that in control cells (Fig. S6A,B).

### Sema3C-induced signal transduction mediated by plexin-D1 is enhanced in the presence of neuropilins

In order to better understand the roles of neuropilins in plexin-D1-mediated signaling induced by Sema3C, we compared the effect of increasing concentrations of purified FR-Sema3C/Fc on the contraction of wild-type U87MG-derived clone 15 cells in which we expressed recombinant plexin-D1 and U87MG clone 25/1-derived clone 7 cells, which lack neuropilins but express similar concentrations of plexin-D1 (Fig. 5D). These experiments revealed that in the absence of neuropilins the FR-Sema3C/Fc concentration

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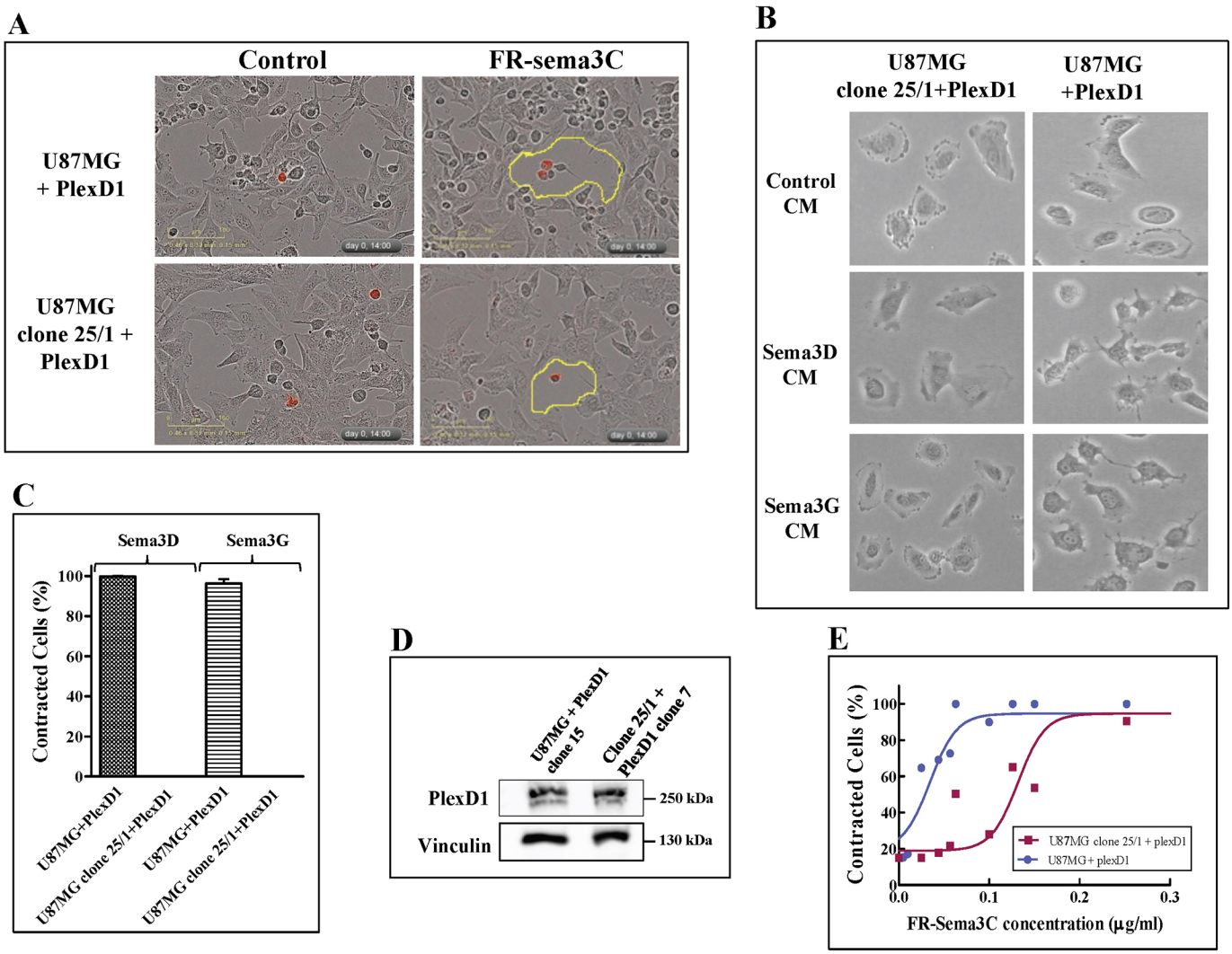


**Fig. 5. Neuropilins enhance plexin-D1-dependent FR-Sema3C/Fc-induced signal transduction.** (A) HEK293 cells expressing FR-Sema3C/myc or control cells infected with empty expression vector were stained with DiI and implanted at clonal density on top of semi-confluent cultures of U87MG clone 15 cells expressing plexin-D1 or on top of U87MG clone 25/1-derived clone 7 cells expressing plexin-D1 but lacking neuropilins. Shown are merged phase contrast and fluorescent images taken 14 h after seeding that were taken from Movies 1-4. The borders of clearings produced in the U87MG monolayer by the Sema3C-expressing cells are marked by a yellow line. (B) Clone 7 cells (U87MG clone 25/1+PlexD1) or clone 15 cells (U87MG+PlexD1) were stimulated with conditioned medium from HEK293 cells infected with empty lentiviral vector (control cm) or with conditioned medium from HEK293 cells expressing Sema3D (Sema3D CM) or Sema3G (Sema3G CM) and photographed after 30 min. (C) The average percentage of contracted cells in three repeats of the experiment shown in B was determined. (D) Western blot analysis comparing Plexin-D1 expression levels in U87MG+plexin-D1 clone 15 cells and clone 7 cells lacking neuropilins and expressing recombinant plexin-D1. (E) The percentage of contracted cells in cultures stimulated with increasing concentrations of FR-Sema3C/Fc was compared between clone 7 cells and clone 15 cells. The cells were seeded in PBS-gelatin-coated 12-well plates and after 24 h stimulated with increasing concentrations of purified FR-Sema3C/Fc. The percentage of contracted cells in each of the wells was determined after 30 min. Shown are the pooled results from three independent experiments. Dose response curves were fitted using the EC50 equation of the GraphPad Prism program and used to derive the EC50 values.

required for the induction of half-maximal contraction (EC50) is 5.5 times higher compared with values measured in the presence of neuropilins (Fig. 5E). We conclude that even though the neuropilins are not strictly required for plexin-D1-mediated Sema3C-induced signal transduction, they nevertheless serve as potent amplifiers that enhance plexin-D1-mediated signal transduction in response to Sema3C. This is a role similar to that played by neuropilins in VEGF-induced signal transduction (Soker et al., 1998). To find out if the reduced potency is also reflected in the ability to bind Sema3C, we used Sema3C fused at the C-terminal to alkaline-phosphatase (AP-Sema3C) in binding experiments. We compared the binding of AP-Sema3C to clone 15 cells, to clone 25/1 cells lacking neuropilins and plexin-D1 and to clone 7 cells. In agreement with the contraction experiments, we found that AP-Sema3C binds specifically to clone 7 cells. In the absence of neuropilins, the binding was less robust than in their presence, suggesting that the higher EC50 value observed in contraction experiments using cells lacking neuropilins is probably due to lower binding affinity (Fig. S3C,D).

### Plexin-A4 is required in addition to plexin-D1 to enable Sema3C signal transduction in the absence of neuropilins

Binding experiments performed using COS-7 cells expressing recombinant plexin-D1 suggested that Sema3C is not able to bind directly to plexin-D1 (Gitler et al., 2004). These observations seem to contradict the experiments shown above which suggest that plexin-

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D1 can transduce Sema3C signals on its own. We have previously shown that functional Sema3A and Sema3B receptors contain two plexins and a neuropilin (Kigel et al., 2011; Sabag et al., 2014). We therefore suspected that it was possible that in the absence of neuropilins, plexin-D1 may interact with another plexin to enable neuropilin-independent Sema3C signal transduction. We therefore silenced each of the class-A plexins in turn in U87MG cells in which we had knocked out both neuropilins and in which we expressed recombinant plexin-D1 (U87MG clone 25/1-derived clone 7 cells) using specific shRNA species that we have previously used to silence expression of these plexins in U87MG cells (Kigel et al., 2011; Sabag et al., 2014). Following the silencing of plexin-A4 in these cells (Fig. 6A), FR-Sema3C/Fc-induced cell contraction, but not UNCL-Sema3E-induced cell contraction (Casazza et al., 2012), was almost completely inhibited (Fig. 6B,C). This experiment was independently repeated three times using two different shRNAs targeting plexin-A4 with similar results, suggesting that in the absence of neuropilins, both plexin-A4 and plexin-D1 need to be present to enable Sema3C-induced signal transduction. Interestingly, it was previously shown that COS-7 cells which do not express plexin-A4 (Suto et al., 2003) and in which recombinant plexin-D1 was expressed fail to bind Sema3C (Gitler et al., 2004). These results suggested that in the absence of neuropilins plexin-A4 may form complexes with plexin-D1 to generate a functional Sema3C receptor complex. Indeed,

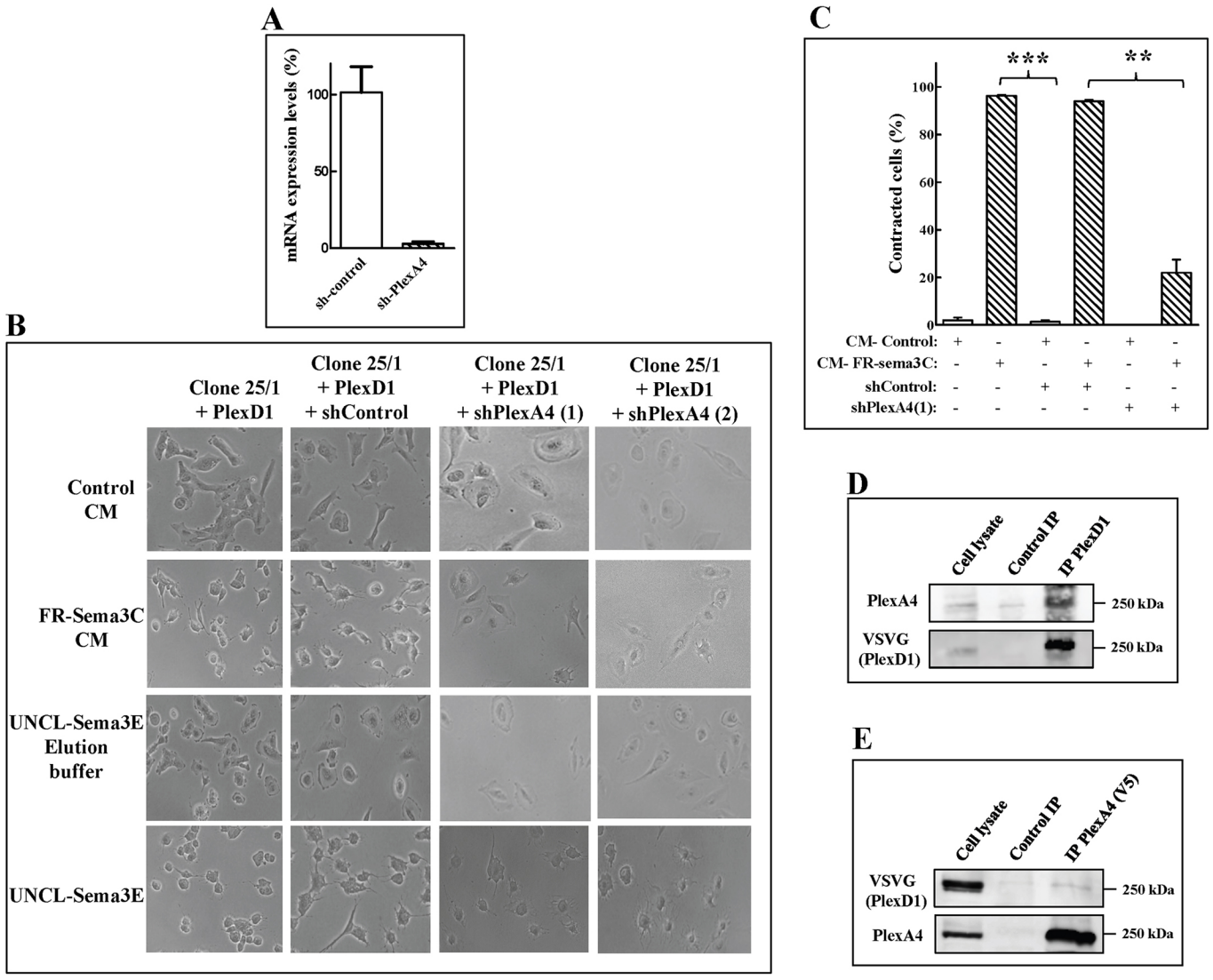


**Fig. 6. Plexin-A4 is required in addition to plexin-D1 for Sema3C signal transduction in the absence of neuropilins.** (A) Real-time RT-PCR assay showing the expression level of plexin-A4 RNA in cells infected with non-specific shRNA (sh-Control) and shRNA targeting plexin-A4 (sh-PlexA4). StepOne Software v.2.3 was used to analyze results. (B) U87MG clone 25/1+plexin-D1 clone 7 cells lacking neuropilins but expressing plexin-D1 were infected with lentiviruses encoding an shRNA targeting plexin-A4. Following selection with puromycin, cells were stimulated with 1 µg/ml purified FR-Sema3C/Fc or UNCL-Sema3E/Fc and photographed after 30 min. Elution buffer was used as a control. Shown are representative images of microscopic fields. The experiment was repeated twice with similar results. (C) Comparison of the percentage of contracted cells in two independent repeats performed in triplicate wells for each condition of the experiment shown in B. Statistical analysis was performed as described in the Materials and Methods. *N*=6, data represent mean±s.e.m.; ***P*<0.01, ****P*<0.001. (D) U87MG clone 25/1+plexin-D1 clone 7 cells knocked out for both neuropilins and overexpressing VSVG-tagged plexin-D1 were lysed, and plexin-D1 was immunoprecipitated using either an anti-VSVG antibody or a non-related control anti-Ramp3 antibody (Brekhman et al., 2011). Western blot analysis was then used to detect co-immunoprecipitated plexin-A4. (E) cDNA encoding plexin-A4 fused in-frame to a V5 tag at the C-terminus was expressed in U87MG clone 25/1 +plexin-D1 clone 7 cells. The cells were lysed and immunoprecipitated using an antibody directed against plexin-A4. Western blots were then probed with antibodies directed against VSVG or plexin-A4.

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immunoprecipitation of plexin-D1 from U87MG clone 25/1-derived clone 7 cells using antibodies directed against a VSVG epitope tag fused to the C-terminus of plexin-D1 co-immunoprecipitated plexin-A4 (Fig. 6D). Likewise, antibodies against plexin-A4 co-immunoprecipitated plexin-D1 from these cells (Fig. 6E), indicating that plexin-A4 and plexin-D1 can associate to form complexes.

## DISCUSSION

U87MG glioblastoma cells respond to stimulation by the class-3 semaphorins Sema3A, Sema3B and Sema3F by cytoskeletal collapse and cell contraction (Kigel et al., 2011; Sabag et al., 2014). In contrast, we found that these cells do not respond by cell contraction to Sema3C, Sema3D, Sema3E and Sema3G. U87MG cells express both neuropilins as well as the four class-A plexins that are known to mediate, in collaboration with either NRP1 or NRP2 signal transduction of class-3 semaphorins (Neufeld et al., 2016). However, even when we overexpressed class-A plexins in these cells at non-physiological concentrations, we could not induce contraction of U87MG cells by these semaphorins. These cells express very little, if any, plexin-D1, a receptor that transduces Sema3E signals independently of neuropilins (Gu et al., 2005). We therefore determined if these semaphorins required plexin-D1 for signal transduction. Indeed, expression of recombinant plexin-D1 in U87MG cells enabled these cells to contract upon stimulation with these semaphorins, suggesting that these semaphorins require plexin-D1 for signal transduction. These conclusions are in agreement with several prior publications that have not excluded class-A plexins as participants in signal transduction by these semaphorins, but have implicated plexin-D1 as a major signal transducing component of Sema3C, Sema3D and Sema3G receptors (Aghajanian et al., 2014; Gitler et al., 2004; Hamm et al., 2016; Liu et al., 2016; Mumblat et al., 2015).

To examine the role of the neuropilins in plexin-D1-dependent signal transduction induced by these semaphorins, we knocked out the genes that encode both neuropilins in U87MG cells using CRISPR/Cas9 to introduce frame-shift mutations into both alleles of each of the neuropilin-encoding genes. We then expressed recombinant plexin-D1 in these knockout cells, which are completely devoid of NRP1 or NRP2 expression. As expected, we found that Sema3D and Sema3G cannot transduce signals in the absence of neuropilins. We did not expect to detect Sema3C-induced signal transduction in these plexin-D1-expressing knockout cells either, since it was previously observed that COS-7 cells expressing recombinant plexin-D1 are not able to bind Sema3C (Gitler et al., 2004). However, to our surprise, we found that these cells, which express recombinant plexin-D1 at physiological levels that are comparable to those observed in endothelial cells, were able to transduce Sema3C signals in the absence of neuropilins. Indeed, Sema3C bound specifically to these cells, but not to cells lacking both neuropilins and plexin-D1, despite the absence of the neuropilins. However, in the presence of neuropilins, Sema3C was able to induce signal transduction at a five-fold lower concentration, indicating that, in the case of Sema3C, the neuropilins function as enhancers of signal transduction but are not absolutely necessary for signal transduction. We have also observed that HUVECs, which naturally express plexin-D1, are still able to contract in response to FR-Sema3C even after the expression of NRP1 is silenced, indicating that Sema3C can utilize plexin-D1 expressed at physiological levels under conditions at which the levels of NRP1 are reduced significantly. These observations imply that migrating cells that express plexin-D1 but no neuropilins may still respond to Sema3C guidance cues provided that the concentration of Sema3C is sufficiently high, which is likely to be the case in close proximity to Sema3C-producing cells. Such responses may not necessarily affect cell migration but could inhibit, for example, cell-cell communication mediated by cell-anchored ligands and their receptors. It remains to be determined if the nature of the responses to Sema3C *in vivo* is affected by the absence or presence of neuropilins as for Sema3E, which in the presence of NRP1 can be transformed from a repulsive to an attractive guidance factor (Chauvet et al., 2007).

We have previously found that more than one plexin is required for the successful transduction of Sema3A and Sema3B signals in U87MG cells and in endothelial cells (Kigel et al., 2011; Sabag et al., 2014). To determine if an additional plexin is involved in Sema3C signal transduction in U87MG cells that lack neuropilins but express plexin-D1, we silenced in these cells the expression of the four class-A plexins using specific siRNAs, and determined if the silencing affected Sema3C signal transduction. Indeed, we found that expression of plexin-D1 alone is not sufficient to enable Sema3C signal transduction in the absence of neuropilins since signal transduction was completely abolished in these cells following plexin-A4 silencing using a specific shRNA. This was not due to a non-specific effect since the silenced cells still responded to Sema3E. These observations suggest that plexin-A4 may form complexes with plexin-D1 in the absence of neuropilins to form a functional Sema3C receptor. Indeed, co-immunoprecipitation experiments indicate that plexin-A4 and plexin-D1 associate to form complexes. These observations apparently contrast with previous findings that suggested that Sema3C does not bind to plexin-D1 expressed in Cos-7 cells, which have low, if any, expression of neuropilins (Gitler et al., 2004). However, plexin-A4 is apparently not expressed in COS-7 cells or is expressed at levels that are too low to enable Sema3A signal transduction even when recombinant NRP1 is expressed in the cells (Suto et al., 2003). It is thus possible that COS-7 cells expressing plexin-D1 are unable to bind Sema3C because of a lack of plexin-A4 (Gitler et al., 2004). In contrast, the endogenous expression level of plexin-A4 in U87MG cells is sufficient to enable signal transduction by Sema3A (Kigel et al., 2011), as well as by Sema3C, in U87MG cells that lack neuropilins but express recombinant plexin-D1.

## MATERIALS AND METHODS

### Antibodies

The following antibodies were used. Goat anti-mouse IgG peroxidase conjugate (A4416, 1:20,000), goat anti-rabbit IgG peroxidase conjugate (A6154, 1:3000), mouse anti-actin clone AC-74 (A5316, 1:5000), rabbit anti-plexin-A4 (R30914, 1:500) and rabbit anti-VSVG (V-4888, 1:2000) were all obtained from Sigma. Bovine anti-goat IgG peroxidase conjugate (sc-2350, 1:3000), goat anti-human NRP1 (sc-7239, 1:500), mouse anti-human NRP2 (sc-13117, 1:100), goat anti-Sema3C (sc-27796, 1:1000) and mouse anti-c-myc (sc 789, 1:1000) were from Santa Cruz. Rabbit anti-plexin-A2 (ab39357, 1:700) and goat anti-plexin-D1 (ab28762, 1:700) were from AbCam; mouse anti-V5 (R960-25, 1:2000) was from Chemicon and mouse anti-vinculin (3574, 1:3000) was from Invitrogen.

### Kits and reagents

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 (Switzerland) and DiI from Life Technologies (USA).

### Plasmids

The cDNA encoding human plexin-D1 tagged with a VSVG epitope tag was kindly provided by Dr Luca Tamagnone (Institute for Cancer Research, University of Torino, Italy). The NSPI-CMV-MCS-myc-His lentiviral expression vector was previously described (Akiri et al., 2009). 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 (#19364), pEF-1α/pENTRA (#17427), pLenti-CMV-GFP-DEST (#19732), pLenti-CMV-Puro-DEST (#17452) and pLenti-CMV-Neo-DEST (#17392) plasmids were from Addgene [deposited by Eric Campeau (Campeau et al., 2009)]. The pLKO-Tet-On plasmid was kindly provided by Dr Ayoub Nabieh (Faculty of Biology, Technion, Israel). The shRNA-containing lentiviral vectors were purchased from Sigma Aldrich. The siRNAs targeting *NRP1* and *NRP2* have been previously described (Guttmann-Raviv et al., 2007). The pcDNA1.1 expression plasmid containing *SEMA3C* cDNA fused at the N-terminal to alkaline phosphatase was generously provided by Dr Stephen Strittmatter (Yale University, New Haven, CT, USA) (Takahashi et al., 1998).

### Cell lines

HUVECs were isolated and cultured as previously described (Gospodarowicz et al., 1978). HEK293 cells were cultured as previously described (Kigel et al., 2011). Parental U87MG cells were purchased from the American Type Culture Collection (ATCC), authenticated recently, and confirmed to be free of mycoplasma contamination. They 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). U87MG cells stably overexpressing plexin-A1, plexin-A2 and plexin-A4 were described previously (Sabag et al., 2014). HT1080 cells were purchased from the ATCC and were cultured similarly to HEK293 cells.

### Expression of recombinant semaphorins and plexins

Class-3 semaphorin cDNAs were subcloned into the NSPI-CMV-myc-his lentiviral expression vector as previously described (Kigel et al., 2008; Varshavsky et al., 2008). The production of lentiviruses and the generation of conditioned media containing various semaphorins as well as the production and purification of FR-Sema3C/Fc and UNCL-Sema3E/Fc were performed as previously described (Casazza et al., 2012; Mumblat et al., 2015). SnapGene software was used to design constructs and primers for CRISPR.

### Quantitative real-time PCR

Quantitative real-time PCR assays were performed as previously described (Sabag et al., 2014).

### R-ras GTPase assay

The Cytoskeleton Ras activation assay kit (BK008) was used according to the instructions of the vendor in conjunction with an antibody specific for human R-ras (ab-57650). The ratio between total R-ras and R-ras associated with GTP was determined by densitometry.

### Expression of recombinant plexins

Full-length cDNAs encoding human plexin-A1, 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). Production of lentiviruses using these plasmids and stable infection of target cells were performed essentially as described previously (Varshavsky et al., 2008).

### Co-immunoprecipitation

Co-immunoprecipitation assays were performed as previously described (Shraga-Heled et al., 2007). Western blots were imaged using an ImageQuant LAS4000 machine.

### Cytoskeletal collapse and cell repulsion experiments

Cytoskeleton collapse assays using HUVEC or U87MG cells were performed and quantified essentially as previously described (Kigel et al., 2011; Sabag et al., 2014) using either HEK293 cell-derived conditioned medium containing various recombinant semaphorins or control conditioned medium from cells containing empty expression vectors. In the case of FR-Sema3C/Fc and UNCL-Sema3E/Fc, we either used conditioned medium as above, or purified FR-Sema3C/Fc and UNCL-Sema3E/Fc which were obtained as previously described (Casazza et al., 2012; Mumblat et al., 2015). To stabilize pH we also added HEPES buffer (10 mM, pH 7.2). Cells were photographed after a 30 min incubation in a humidified incubator at 37°C using a phase-contrast inverted microscope. Quantification of the percentage of contracted cells was evaluated as previously described (Sabag et al., 2014). For repulsion assays, HEK293 cells expressing various semaphorins were stained with DiI and seeded on top of densely packed U87MG cells essentially as previously described (Sabag et al., 2014).

### Immunofluorescence

Staining cytoskeletal components of U87MG cells was done essentially as previously described (Sabag et al., 2014).

### siRNA-mediated silencing of neuropilin expression

Inhibition of *NRP1* and *NRP2* expression in U87MG cells using specific siRNAs was performed as previously described (Guttmann-Raviv et al., 2007).

### Knockout of NRP1 and NRP2 in U87MG cells

U87MG cells were transfected with a pSpCas9(BB)-2A-GFP plasmid containing the guide RNA sequences for either NRP1 or NRP2 (Fig. S2A). Fluorescence-activated cell sorting (FACS) was performed 48 h after the transfection, for selection of Cas9- and GFP-expressing cells. Single cell clones derived from the sorted cells were isolated by limiting dilution. Clones containing mutations in the areas adjacent to the sgRNA target area were identified by PCR and the area adjacent to the DNA region containing the sgRNA target sequence was then sequenced. The sequences obtained were compared with the wild-type sequence of the gene and thoroughly examined in order to find those with insertion/deletion mutations causing frame-shift disruption in both alleles. Clones found to have null mutations in both alleles were submitted to western blot analysis for confirmation of the protein absence and later for contraction assay with Sema3A (to test Np1 KO) or Sema3B (to test both Np1 and Np2 KO).

### Cell contraction assay using the xCELLigence Machine

Cells were seeded in fibronectin-coated wells of E-plates at a concentration of $2\times10^4$ cells/well and incubated in an xCELLigence dual plate of a Real-Time Cell Analyzer (RTCA) machine (Roche) for 24 h at 37°C and 5% $CO_2$ in a humidified incubator. Semaphorins were then added and changes in cell contraction measured and analyzed with RTCA software according to the instructions of the vendor (Roche) essentially as described (Camillo et al., 2017; Mumblat et al., 2015).

### Software and statistical analysis

ImagePro premier software was used to quantify the average intensity of staining in binding experiments. Since we could not predict the outcome of experiments, we did not perform power analysis but performed sufficient repetitions to make sure results were statistically significant. In the case of the cell contraction experiments, the assessment was performed in a blinded fashion. Images of cells from individual wells (at least 6 individual wells from at least 3 different experiments) were assessed for contraction by individuals unaware of the source. *N* represents the number of wells counted in each type of experiment. Statistical significance was determined in most cases using the Mann-Whitney one-tailed non-parametric test unless otherwise stated. Non-linear line fitting employing the dose-response EC50 equation of the GraphPad Prism software was used in order to obtain the average EC50 shift due to lack of neuropilins and to assess statistical significance of results. Experiments in which the percentage of contracted cells was evaluated were evaluated by blinded examination of photographs. Statistical significance is presented in the following manner: $*P<0.05$, $**P<0.01$ and $***P<0.001$. All experiments were repeated independently at least three times unless otherwise stated

#### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: T.S., I.N.-Z., G.N.; Methodology: T.S., I.N.-Z., N.D.; Validation: T.S., I.N.-Z., N.D., G.N.; Formal analysis: T.S., I.N.-Z., N.D., O.K.; Investigation: T.S., I.N.-Z., N.D., Y.M., O.K.; Data curation: O.K.; Writing - original draft: G.N.; Writing - review & editing: O.K., G.N.; Visualization: T.S., I.N.-Z., Y.M., O.K.; Supervision: O.K., G.N.; Project administration: G.N.; Funding acquisition: G.N.

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### Supplementary information

Supplementary information available online at
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