# The Sema domain of Met is necessary for receptor dimerization and activation

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**Summary**

**Hepatocyte growth factor (HGF) binds the extracellular domain and activates the Met receptor to induce mitogenesis, morphogenesis, and motility. The extracellular domain of Met is comprised of Sema, PSI, and four IPT subdomains. We investigated the contribution of these subdomains to Met receptor dimerization. Our observations indicate that the Sema domain is necessary for dimerization in addition to HGF binding. Treatment of Met-overexpressing tumor cells with recombinant Sema in the presence or absence of HGF results in decreased Met-mediated signal transduction, cell motility, and migration, behaving in a manner similar to an antagonistic anti-Met Fab. These data suggest that the Sema domain of Met may not only represent a novel anticancer therapeutic target but also acts as a biotherapeutic itself.**

## Introduction

HGF binds the extracellular domain of the Met receptor tyrosine kinase (RTK) and regulates diverse biological processes such as cell scattering, proliferation, and survival. HGF-Met signaling is essential for normal embryonic development, especially in migration of muscle progenitor cells and development of the liver and nervous system (Bladt et al., 1995; Hamanoue et al., 1996; Maina et al., 1996; Schmidt et al., 1995; Uehara et al., 1995). Developmental phenotypes of Met and HGF knockout mice are very similar, suggesting that HGF is the cognate ligand for the Met receptor (Schmidt et al., 1995; Uehara et al., 1995). HGF and Met also play a role in liver regeneration, angiogenesis, and wound healing (Bussolino et al., 1992; Matsumoto and Nakamura, 1993; Nusrat et al., 1994). Met undergoes proteolytic cleavage into an extracellular $\alpha$ subunit and membrane spanning $\beta$ subunit linked by disulfide bonds (Tempest et al., 1988). The $\beta$ subunit contains the cytoplasmic kinase domain and harbors a multisubstrate docking site at the C terminus where adaptor proteins bind and initiate signaling (Bardelli et al., 1997; Nguyen et al., 1997; Pelicci et al., 1995; Ponzetto et al., 1994; Weidner et al., 1996). Upon HGF binding, activation of Met leads to tyrosine phosphorylation and downstream signaling through Gab and Grb2/Sos-mediated PI3 kinase and Ras/MAPK activation, respectively, which drives cell motility and proliferation (Furge et al., 2000; Hartmann et al., 1994; Ponzetto et al., 1996; Royal and Park, 1995).

Met was shown to be transforming in a carcinogen-treated osteosarcoma cell line (Cooper et al., 1984; Park et al., 1986). Met overexpression or gene amplification has been observed in a variety of human cancers. For example, Met protein is overexpressed at least 5-fold in colorectal cancers and reported to be gene amplified in liver metastasis (Di Renzo et al., 1995; Liu et al., 1992). Met protein is also reported to be overexpressed in oral squamous cell carcinoma, hepatocellular carcinoma, renal cell carcinoma, breast carcinoma, and lung carcinoma (Jin et al., 1997; Morello et al., 2001; Natali et al., 1996; Olivero et al., 1996; Suzuki et al., 1994). In addition, overexpression of mRNA has been observed in hepatocellular carcinoma, gastric carcinoma, and colorectal carcinoma (Boix et al., 1994; Kuniyasu et al., 1993; Liu et al., 1992).

A number of mutations in the kinase domain of Met have been found in renal papillary carcinoma, which leads to constitutive receptor activation (Olivero et al., 1999; Schmidt et al., 1997, 1999). These activating mutations confer constitutive Met tyrosine phosphorylation and result in MAPK activation, focus formation, and tumorigenesis (Jeffers et al., 1997). In addition, these mutations enhance cell motility and invasion (Giordano et al., 2000; Lorenzato et al., 2002). HGF-dependent Met activation in transformed cells mediates increased motility, scattering,

SIGNIFICANCE

**Activation of the Met receptor tyrosine kinase induces multifunctional cellular responses. Aberrant Met activation, reported in several cancers, has been shown to drive oncogenesis. This paper defines the Met Sema domain, which is shared by semaphorins and plexins and is important for dimerization and ligand interaction, as necessary for receptor dimerization. Met-overexpressing tumor cells treated with recombinant Sema or a Sema-recognizing anti-Met Fab inhibits ligand-dependent and -independent receptor activation and downstream signaling. Our observations address the relevance of inhibiting Met receptor dimerization and elucidate the domains important for this inhibition. Furthermore, our results illustrate the potential for targeting the Met Sema domain to generate novel antineoplastic agents.**

and migration, which eventually leads to invasive tumor growth and metastasis (Birchmeier et al., 2003; Jeffers et al., 1996; Meiners et al., 1998).

Recently, Met has been shown to interact with other proteins that drive receptor activation, transformation, and invasion. In neoplastic cells, Met is reported to interact with $\alpha 6\beta 4$ integrin, a receptor for extracellular matrix components such as laminins, to promote HGF-dependent invasive growth (Trusolino et al., 2001). In addition, the extracellular domain of Met has been shown to interact with a member of the semaphorin family, plexin B1, and to enhance invasive growth (Giordano et al., 2002). Furthermore, CD44v6, which has been implicated in tumorigenesis and metastasis, is also reported to form a complex with Met and HGF and result in Met receptor activation (Orian-Rousseau et al., 2002).

Met is a member of the subfamily of RTKs that includes Ron and Sea (Maulik et al., 2002). Prediction of the extracellular domain structure of Met suggests shared homology with the semaphorins and plexins. The N terminus of Met contains a Sema domain of approximately 500 amino acids that is conserved in all semaphorins and plexins. The semaphorins and plexins belong to a large family of secreted and membrane bound proteins first described for their role in neural development (Van Vactor and Lorenz, 1999). However, more recently semaphorin overexpression has been correlated with tumor invasion and metastasis. A cysteine-rich PSI domain (also referred to as a Met Related Sequence domain) found in plexins, semaphorins, and integrins lies adjacent to the Sema domain followed by four IPT repeats that are immunoglobulin-like regions found in plexins and transcription factors. A recent study suggests that the Met Sema domain is sufficient for HGF and heparin binding (Gherardi et al., 2003).

Although the role of the Met kinase domain has been investigated in detail, the extracellular domain of Met is poorly characterized. Since HGF binds the extracellular domain of Met, resulting in receptor activation, we wished to examine which subdomain(s), if any, contributes to receptor dimerization. Deletion mutants of the extracellular subdomains upon crosslinking demonstrate that the Sema domain is not only necessary for Met receptor association but is also essential for HGF binding. In addition, we demonstrate that the Sema domain can inhibit HGF-dependent and -independent receptor phosphorylation, downstream signaling, and functional receptor activation. These observations are the first to address the importance of the Sema domain in Met receptor dimerization and potential therapeutic applications in Met-overexpressing cancers.

## Results

### The Sema domain is necessary for Met receptor crosslinking

To determine the contribution made by the extracellular domain of Met to receptor dimerization and activation, subdomain deletions of Met were made as shown in Figure 1. Each deletion mutant is flanked by the signal peptide (S.P.) at the N terminus and a C-terminal transmembrane region carrying a V5/His tag. We tested the Met deletion mutants individually for the ability to crosslink using sulfo-EGS. Transfections of the V5/His-tagged deletion mutants treated with increasing concentrations of sulfo-EGS were lysed and analyzed on 4%–12% SDS-PAGE. In samples not treated with sulfo-EGS, EC-WT-V5/His appears as processed monomer (EC-M, ~90 kDa) and unprocessed (*, ~120 kDa) proteins. EC-WT crosslinking is detected by a shift from the lower (EC-M, ~90 kDa) to an upper (EC-D, ~180 kDa) migratory dimer as shown in Figure 2A. Met mutants lacking the Sema domain did not display a similar shift. We note that as expected, unprocessed EC-WT (*) Met did not display crosslinking, as sulfo-EGS is cell membrane impermeable. The intra-

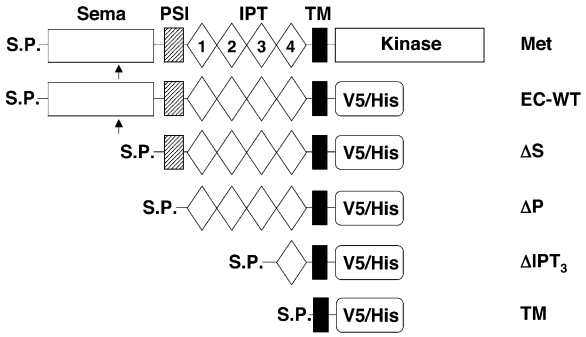


**Figure 1.** Schematic representation of Met deletion mutants

Subdomain deletions of the Met extracellular domain were made from the N terminus and tagged after the TM region with V5/His. The Met signal peptide (S.P.) sequence was appended to the N terminus of each mutant. Abbreviations: Sema, semaphorin; PSI, plexin, semaphorin, integrin; IPT, immunoglobulin-like regions in plexins and transcription factors; and TM, transmembrane. The arrow on the Sema region points to the Met proteolytic site.

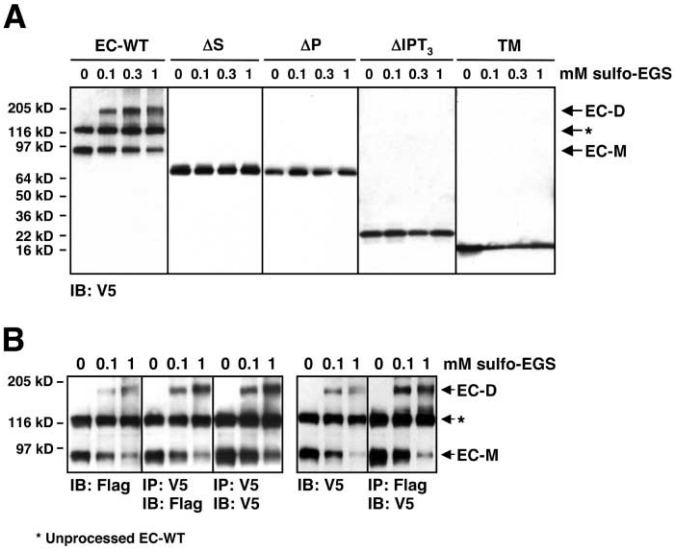


**Figure 2.** The Sema domain is necessary for crosslinking Met

**A:** The Met deletion mutants were transfected into 293 cells and exposed to increasing concentrations of sulfo-EGS. 10 μg of lysates were analyzed by 4%–12% SDS-PAGE and immunoblotted with V5 antibody. EC-M and EC-D represent EC-WT monomer and dimer, respectively. The asterisk indicates the presence of unprocessed EC-WT.

**B:** EC-WT-Flag and EC-WT-V5/His were cotransfected in 293 cells, subjected to increasing concentrations of sulfo-EGS, lysed, immunoprecipitated with either V5 or Flag antibodies, and analyzed by 8% SDS-PAGE. The immunoprecipitated samples were blotted with either Flag or V5 antibodies.

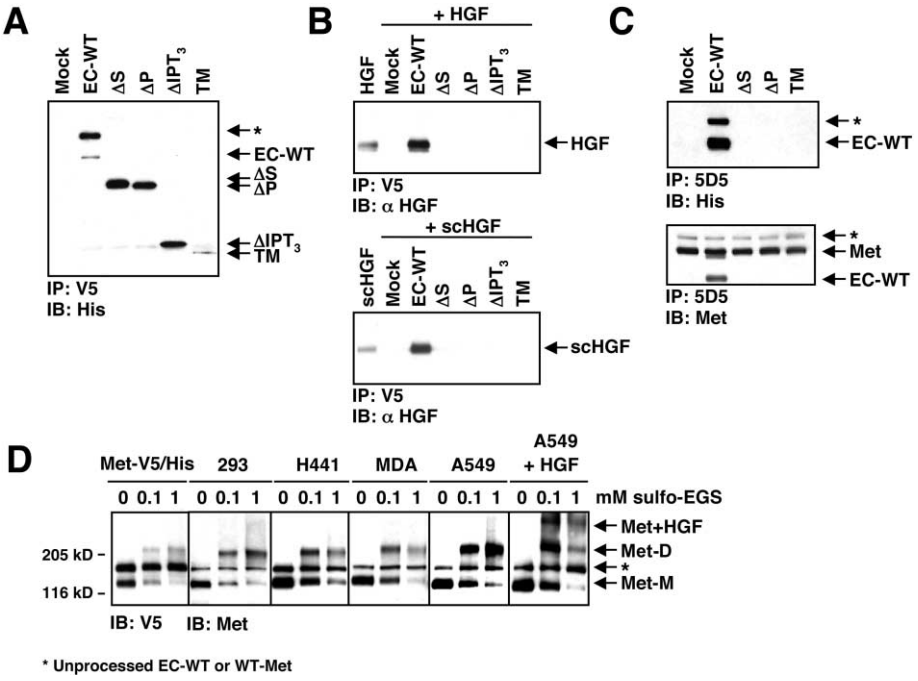


**Figure 3.** HGF, scHGF (R494E), and anti-Met 5D5 antibody bind the Sema domain of Met

**A:** 293 cells were transfected with the indicated Met-V5/His tagged construct, immunoprecipitated with V5 antibody, analyzed by 4%–12% SDS-PAGE, and immunoblotted with His antibody.

**B:** Top: The immunoprecipitates from **A** were incubated with 5 μg HGF. The samples were analyzed by 4%–12% SDS-PAGE and immunoblotted with HGF antibody that recognizes the α chain. 10 ng of input HGF served as a positive control. Bottom: Immunoprecipitations and immunoblotting were performed as in the top panel using scHGF (R494E). As a positive control, 15 ng scHGF (R494E) was analyzed with the samples.

**C:** 293 cells were transfected with the indicated constructs and analyzed by 4%–12% SDS-PAGE. Lysates were immunoprecipitated with anti-Met 5D5 antibody and immunoblotted with His antibody (top) and reprobed with Met C-12 antibody (bottom).

**D:** Endogenous Met is crosslinked in tumor cells. Increasing concentrations of sulfo-EGS were added to 293, H441, MDA-MB-435, and A549 cells that had been serum starved. A549 cells were also incubated with 10 μg/ml HGF prior to crosslinking. Lysates were immunoblotted with Met C-12 antibody. As a control, full-length Met-V5/His was transfected into 293 cells, crosslinked, and immunoblotted with V5 antibody.

cellular single-chain precursor of Met is detected as a proteolytically cleaved protein on the cell surface (Giordano et al., 1989). We used sulfo-EGS as a crosslinking agent because of its limited cell permeability and examined the effects of crosslinking membrane-inserted processed Met receptor.

To address if membrane bound extracellular Met formed homodimers, cells were cotransfected with EC-WT-V5/His and EC-WT-Flag and crosslinked with sulfo-EGS. Cells were lysed, immunoprecipitated with V5 antibody, analyzed on 8% SDS-PAGE, and immunoblotted with either Flag or V5 antibodies. The immunoblots display an upper migratory form (EC-D, ~180 kDa) containing EC-WT-Flag and EC-WT-V5/His (Figure 2B), indicating that both forms of EC-WT are crosslinked and coimmunoprecipitated. A reverse immunoprecipitation using Flag antibody showed similar results confirming our prior observation (Figure 2B). Residual noncrosslinked EC-WT-Flag (EC-M) is also detected in the V5 immunoprecipitates but shifts to the upper migratory form with the addition of increased sulfo-EGS (Figure 2B). Collectively, our crosslinking studies reveal that extracellular membrane bound Met homodimers are formed when the Sema domain is present.

### HGF and scHGF (R494E) bind to the Sema domain

HGF is secreted as an inactive single-chain precursor and is cleaved by extracellular proteases into an α and β chain (Naka et al., 1992). The cleaved disulfide-linked α and β chains of HGF bind the Met receptor with high affinity (Bottaro et al., 1991; Naldini et al., 1991). We tested the Met deletion mutants for their ability to bind HGF. Cells were transfected with EC-WT, ΔS, ΔP, $\Delta IPT_3$, or TM or were mock transfected (Figure 3A), and cell lysates were incubated with HGF. Immunoprecipitation with V5 antibody followed by 4%–12% SDS-PAGE and detection with α chain-HGF antibody shows that HGF bound EC-WT alone (Figure 3B, top). Since HGF did not bind the Sema-deleted mutants, our observations suggest that the Sema domain is necessary for HGF binding.

Single-chain HGF containing an R494E mutation [scHGF (R494E)] binds and inhibits activation of the Met receptor (Lokker et al., 1992). scHGF (R494E) was also examined for binding to the Met extracellular domain. Transfected Met deletion mutants were lysed, incubated with scHGF (R494E), and analyzed as above. We observed scHGF (R494E) binding only to EC-WT and not the Sema-deleted mutants (Figure 3B, bottom). These observations were corroborated with coimmunoprecipitation experiments where both HGF and scHGF (R494E) bound to recombinant EC-Met and Sema domain proteins (data not shown). Thus, we confirm that the Sema domain of Met is the HGF interaction site (Gherardi et al., 2003; Stamos et al., 2004).

### 5D5 monoclonal antibody binds the Sema domain of Met

Monoclonal antibodies were raised to the extracellular domain of Met in an attempt to identify antagonistic Met antibodies. One of these antibodies, anti-Met 5D5, bound Met and inhibited HGF binding. The Fab fragment of anti-Met 5D5 (anti-Met 5D5-Fab) was shown to inhibit HGF-driven Met phosphorylation, cell proliferation, and tumor growth (R. Schwall, personal communication). We tested binding of anti-Met 5D5 to the Met extracellular domain. Cells were transfected with the Met deletion constructs or mock transfected as indicated. These cell lysates were immunoprecipitated with anti-Met 5D5 and immunoblotted with His antibody. Only the Sema-containing EC-WT bound to anti-Met 5D5 (Figure 3C, top). All transfected proteins were detected in the lysates as seen in Figure 3A (data not shown),

and endogenous Met was immunoprecipitated by anti-Met 5D5 (Figure 3C, bottom). Thus, our data suggest that anti-Met 5D5 binds the Sema domain of Met. Since anti-Met 5D5-Fab acts as an antagonist in addition to binding the Sema domain, we used it as a control in our functional studies.

**Crosslinking of endogenous Met in tumor cell lines**

Since our results suggest that the Sema domain is associated with two functions, namely HGF binding and receptor dimerization, we next examined the role of the Met Sema domain in dimerization with and without the involvement of HGF binding. The levels of HGF expressed in 293 (embryonic kidney), A549 (lung carcinoma), H441 (lung adenocarcinoma), and MDA-MB-435 (breast carcinoma) cell lines were determined by RT-PCR and Western blot analysis. MDA-MB-435 and H441 cells did not have detectable HGF RNA or protein, while A549 and 293 cells expressed HGF RNA and had barely detectable HGF protein in lysates and conditioned media (data not shown). To determine if Met expressed in various tumor cell lines could crosslink in the absence of HGF, cells were maintained in serum-free media with 0.5% BSA for the duration of the experiment. These cell lines were crosslinked with sulfo-EGS as before and lysates were analyzed by 4%–12% SDS-PAGE followed by immunoblot with Met antibody. In the absence of crosslinking reagent, Met appears as processed (Met-M) and unprocessed (*) proteins as seen previously in crosslinking studies with EC-WT (Figure 2A). In each cell line, even at 0.1 mM sulfo-EGS, a shift from Met-M to an upper migratory form (Met-D) was observed (Figure 3D). Increased concentrations of crosslinking agent enhanced the shift of Met-M to the Met-D form. Consistent with our previous observation, intracellular unprocessed Met does not crosslink upon sulfo-EGS treatment. In comparison, A549 cells were incubated with HGF and crosslinked as described before. Immunoblotting for Met reveals a higher migratory form in the presence of HGF when compared to Met-D seen in the absence of HGF (Figure 3D). The data suggest that crosslinked Met dimers are formed in the absence of HGF and are shifted further due to HGF binding.

**Inhibition of Met signaling in tumor cells with rSema and anti-Met 5D5-Fab**

Since our data suggest that the Sema domain is necessary for Met dimerization, we generated recombinant Sema (rSema) protein, which included the Sema and PSI domains of the Met receptor (Stamos et al., 2004), and examined the functional consequences on Met signaling. Human tumor cells were treated with increasing concentrations of rSema and analyzed for Met tyrosine phosphorylation and downstream activation of mitogen-activated protein kinase (MAPK) upon HGF stimulation. A549 cells were serum starved and incubated with increasing concentrations of rSema in the presence of HGF. Cell lysates were harvested, immunoprecipitated with a C-terminal Met antibody, and immunoblotted with phospho-tyrosine antibody. A decrease in Met phosphorylation was observed with increasing amounts of rSema in the presence of HGF compared to HGF stimulation alone (Figures 4A and 4B). Similarly, phosphorylation of MAPK decreases upon treatment with increasing concentrations of rSema in the presence of ligand. H441 and MDA-MB-435 cell lines were also treated with rSema and HGF in a similar manner, and a dose-dependent decrease in phospho-MAPK is observed (Figures 4A and 4B).

In addition, we examined inhibition of Met activation by rSema in the absence of HGF. For comparison, anti-Met 5D5-Fab that acts as a Met receptor antagonist was used in our functional studies. A549 cells were serum starved and treated with anti-Met 5D5-Fab or rSema. Treatment with rSema or anti-Met 5D5-Fab caused an inhibition of ligand-independent Met phosphorylation and phospho-MAPK levels compared to mock-treated control (Figures 4C and 4D). Similarly, H441 and MDA-MB-435 cell lines were also treated with anti-Met 5D5-Fab and rSema in the absence of HGF (Figures 4C and 4D); the decrease in MAPK phosphorylation followed a trend similar to that of our previous observations with A549 cells. These results suggest that rSema not only inhibits ligand-dependent and -independent Met phosphorylation but also affects downstream MAPK signaling.

Met deletion mutants were also examined for their ability to attenuate endogenous Met signal transduction. Cells were transfected with the Met deletion mutants (Figure 5A); lysates were immunoprecipitated with a C-terminal Met antibody and immunoblotted with phospho-tyrosine antibody. EC-WT transfectants showed attenuation of Met tyrosine phosphorylation and phospho-MAPK compared to mock-treated controls (Figures 5B and 5C). Sema-deleted transfectants, while showing some attenuation of phospho-Met, did not show a decrease in downstream MAPK phosphorylation (Figures 5B and 5C). Together, these observations confirm that the Sema domain is necessary for Met activation, corroborating our previous results with rSema inhibition of Met signaling (Figure 4).

**Cell migration is inhibited by rSema and anti-Met 5D5-Fab**

H441 cells were used in a scrape assay as a measure of Met-mediated cell motility (Lorenzato et al., 2002). H441 cells grown to high density were scraped in each well on Day 0 and maintained in serum-free media with 0.5% BSA for the duration of the experiment. To examine the effect of rSema on cell motility in the presence of ligand, cells were treated with 0, 0.1, 0.5, or 5 μg/ml rSema plus 20 ng/ml HGF. Cells treated with HGF alone closed the gap completely by Day 1. In comparison, cells treated with rSema in the presence of HGF retained the gap in a dose-dependent manner (Figure 6A). The data suggest that rSema inhibits Met-mediated cell motility in the presence of HGF. We also examined the effect on cell motility with rSema or anti-Met 5D5-Fab in the absence of HGF. Serum-starved cells were treated with either 10 μg/ml rSema or anti-Met 5D5-Fab. As controls, cells were also mock treated or treated with 100 ng/ml HGF. On Day 2, the gap in mock-treated wells closed completely while a gap remained in the rSema-treated cells (Figure 6B). The gap in anti-Met 5D5-Fab-treated cells also remained visible, to a lesser extent. HGF-treated cells had closed the gap by Day 1 as seen in Figure 6A and remained as such on Day 2 (data not shown). The data suggest that cell motility mediated by endogenous Met activation is inhibited by both rSema and anti-Met 5D5-Fab in the absence of HGF.

A transwell assay with MDA-MB-435 cells was used to measure Met-driven cell migration (Coltella et al., 2003) in the presence and absence of HGF. Addition of HGF to these cells results in ~4-fold increase in migration over mock-treated wells. A dose-dependent inhibition of HGF-driven cell migration is observed with the addition of rSema (Figure 7A). In the absence of HGF, migration is consistently decreased by the addition of

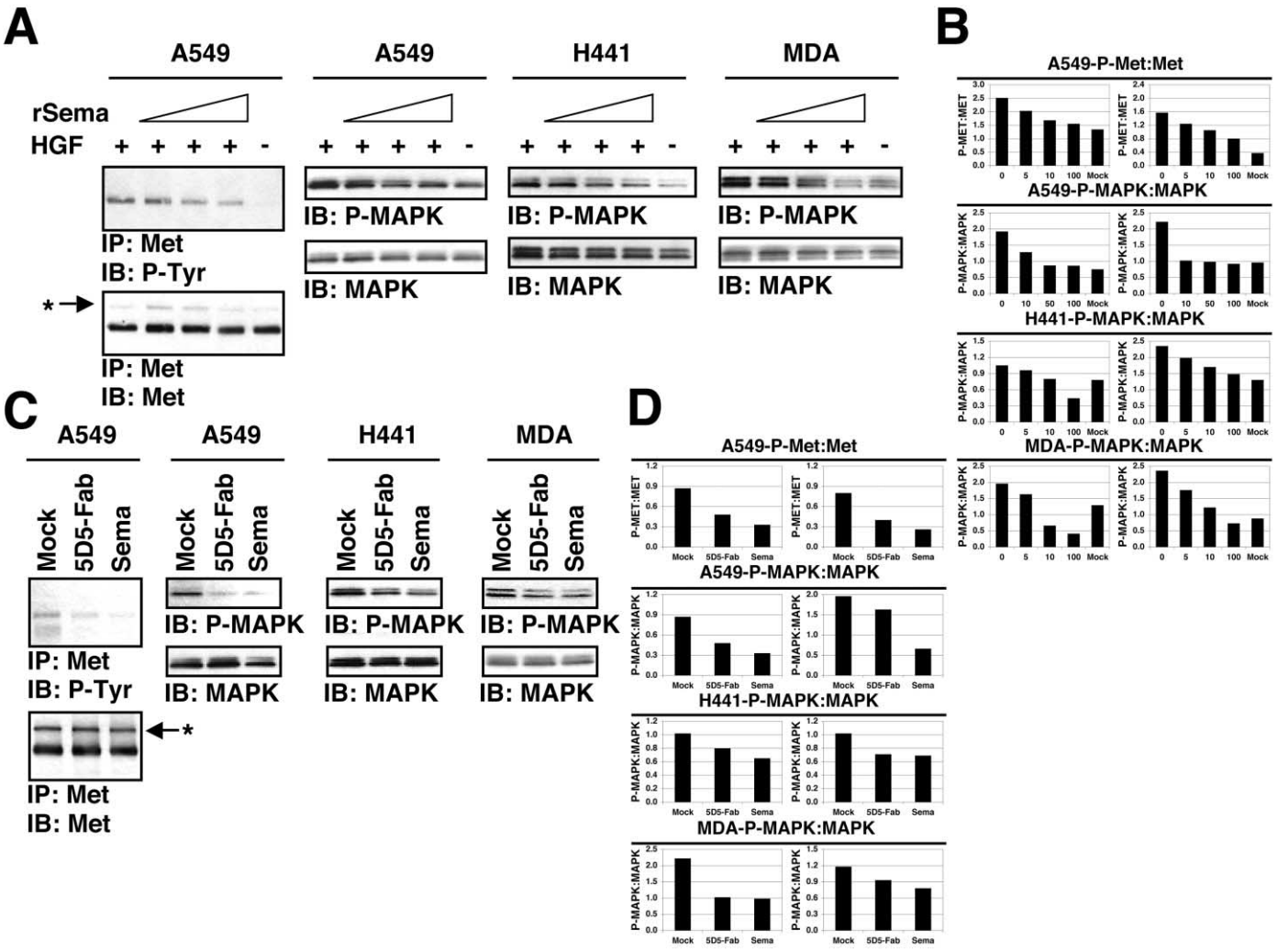


**Figure 4.** Met signal transduction is inhibited by rSema and anti-Met 5D5-Fab

**A:** A549 cells were kept serum-free and treated with 0, 5, 10, 50, or 100 μg/ml of rSema with 10 ng/ml HGF for 10 min for phospho-Met and phospho-MAPK analysis. Lysates were immunoprecipitated with Met C-12 antibody and immunoblotted with phospho-tyrosine antibodies to detect phosphorylated Met followed by immunoblotting with Met antibody. H441 and MDA-MB-435 cells were treated with 0, 5, 10, or 100 μg/ml rSema plus 5 or 10 ng/ml HGF, respectively, for 5 min. Proteins were detected with phospho-MAPK and reprobed with MAPK antibodies.

**B:** The data in **A** were quantified using NIH Image software and are represented as bar graphs in the left panels comparing the ratio of phosphorylated to unphosphorylated proteins between each sample. The right panels represent data from another experiment.

**C:** A549, H441, and MDA-MB-435 cells were kept serum-free and treated with 10 μg/ml rSema or anti-Met 5D5-Fab. The samples were immunoblotted with phospho-tyrosine, Met, phospho-MAPK, or MAPK antibodies.

**D:** The data in **C** were quantified and presented as in **B**.

rSema and anti-Met 5D5-Fab to a lesser extent (Figure 7B). The data correlate with the scrape assay results in the H441 cell line. Activation of Akt was also examined in H441 and MDA-MB-435 cells used in the cell motility and migration assays. We observed a dose-dependent decrease in Akt phosphorylation with increasing concentrations of rSema in the presence of HGF (data not shown). The data correlate with our observations that rSema can inhibit cell motility in the presence of ligand. Taken together, these observations suggest that the Sema domain is capable not only of inhibiting Met activation, but also of blocking downstream effects of cell motility and migration in both ligand-dependent and -independent contexts.

## Discussion

The Sema domain resides in the extracellular region of semaphorins and their receptors, plexins, and serves as a receptor/ligand recognition site (Tamagnone et al., 1999). Previous publications of the crystal structures of Sema3A and Sema4D suggest that these Sema domains form seven-bladed β-propeller structures that are important for homodimerization (Antipenko et al., 2003; Love et al., 2003). Recently, the crystal structure of the Sema domain of Met in complex with the β chain of HGF was determined and confirmed that the Sema domain of Met forms a seven-bladed β-propeller structure that binds HGF (Stamos et al., 2004). We report here experimental evidence

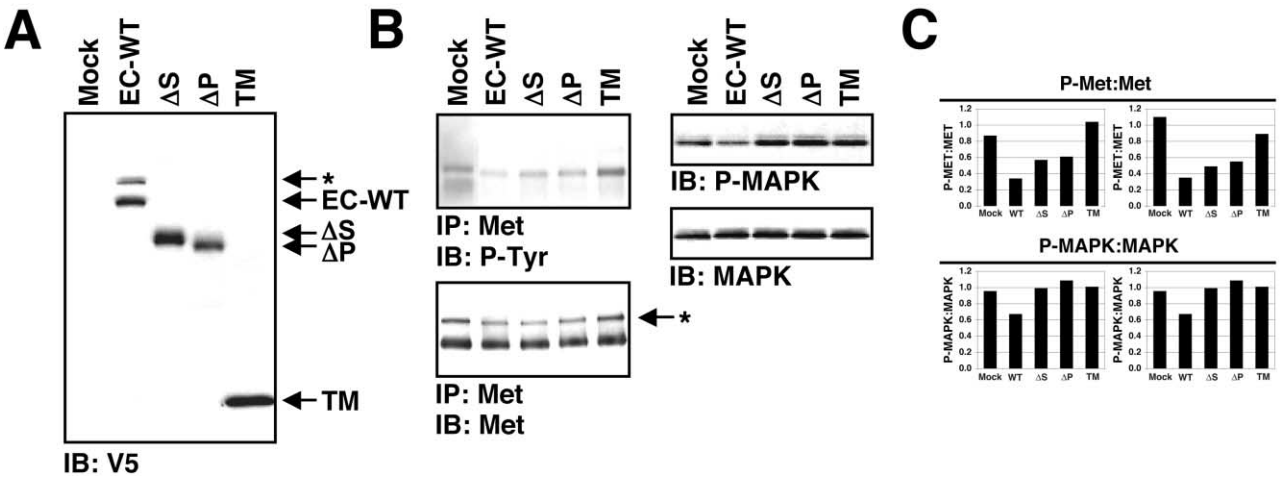


**Figure 5.** The extracellular domain of Met attenuates Met signal transduction

**A:** A549 cells were transfected with the indicated constructs. The expression of the Met mutants was detected by immunoblotting with V5 antibody.
**B:** Left: Lysates from **A** were immunoprecipitated with Met C-12 antibody and immunoblotted with phospho-tyrosine antibody. The membrane was reprobed with Met antibody. Right: Lysates were immunoblotted with phospho-MAPK and then reprobed with MAPK.
**C:** The data in **B** were quantified using NIH Image software and are represented as bar graphs in the left panels comparing the ratio of phosphorylated to unphosphorylated proteins for each sample. The right panels represent data from another experiment.

that strongly support a role for the Sema domain in Met receptor dimerization. Our data show that crosslinking of Met occurs only in the presence of the Sema domain, suggesting a necessary role for the Sema domain in receptor dimerization. In addition, crosslinking of Met was observed in the absence of HGF, indicating that Met receptor in these tumor cell lines may dimerize without ligand stimulation.

The crystal structure of Sema3A suggests that four interaction "loops" in the Sema domain are important to establish the interface between Sema3A dimers (Antipenko et al., 2003). Alignment of the Sema3A sequence with Met reveals that one loop resides close to the proteolytic cleavage site between R307 and S308 in the Met Sema domain. Further studies will be required to determine the precise role of these loops and their potential involvement in Met dimerization.

In our current cell-based study, we observe Sema domain-mediated homophilic interactions of the Met receptor in the absence of HGF. However, previous reports suggest that Met also interacts with CD44v6, $\alpha 6\beta 4$ integrin, and plexin B1 (Giordano et al., 2002; Orian-Rousseau et al., 2002; Trusolino et al., 2001). Based on our studies, it is tempting to speculate that Met association with plexin B1 could occur through these highly conserved Sema domains. In addition, overexpression of both Met and semaphorins has been described in cancers and invasive metastases, tempting further speculation that any potential interactions could be mediated through the Sema domains. However, the role of these molecules and the mechanisms that mediate heteromeric interactions in these pathological contexts need further elucidation. CD44v6 and $\alpha 6\beta 4$ are not known to contain Sema domains. Therefore, interactions with other protein motifs mediated through the Sema and/or the PSI or IPT domains may be important in initiating specific biological responses (Bertotti and Comoglio, 2003) and cannot be excluded as a possibility.

In RTKs such as fibroblast growth factor receptor 2 (FGFR2) and RET, disulfide bonding between cysteines in the extracellular domain of each receptor have been implicated in receptor dimerization (Robertson et al., 2000). In the Met receptor, our study shows that neither the cysteine-rich PSI domain nor the four IPT domains exhibit crosslinking in the absence of the Sema domain, suggesting that these regions may not be necessary for Met dimerization. Furthermore, Met interaction with plexin B1 was unaltered despite deletion of the PSI domain of Met (Giordano et al., 2002). Although the rSema used in our functional studies contained a Sema and PSI domain (Stamos et al., 2004), our own crosslinking studies and the reported PSI domain deletion in plexin B1 (Giordano et al., 2002) strongly suggest that the Sema domain plays a dominant role in these interactions in the absence of the PSI domain. Attempts to generate internal PSI or IPT deletions resulted in expression of nonprocessed Met that could not be used in our studies. Therefore, we cannot formally exclude a role for the PSI and IPT domains in Met dimer formation or some other mechanism such as auto-inhibition of dimerization as reported for EGFR (Ferguson et al., 2003). Resolution of the crystal structure of extracellular Met would shed more light on these complex interactions.

Crystallographic studies of ligand-receptor complexes of RTKs have revealed several structural insights. Both Flt-1 and TrkA utilize dimerization of ligands for receptor dimerization and activation (Wiesmann et al., 1997, 1999). In FGFR, heparin bound FGF ligand drives receptor dimerization (Plotnikov et al., 1999). In contrast, the EGF:EGFR (epidermal growth factor receptor) complex homodimerizes through the receptor, independent of ligand-ligand interaction (Garrett et al., 2002; Ogiso et al., 2002). The interaction of Met and HGF is less clear, although a recent report suggests that Met, HGF, and heparin exist as a 1:1:1 complex (Gherardi et al., 2003). Our cell-based studies differ from these reported in vitro observations since cellular components such as the extracellular matrix (ECM) are present in our study and may have considerable influence on receptor dimerization in vivo. Since the ECM contains laminins

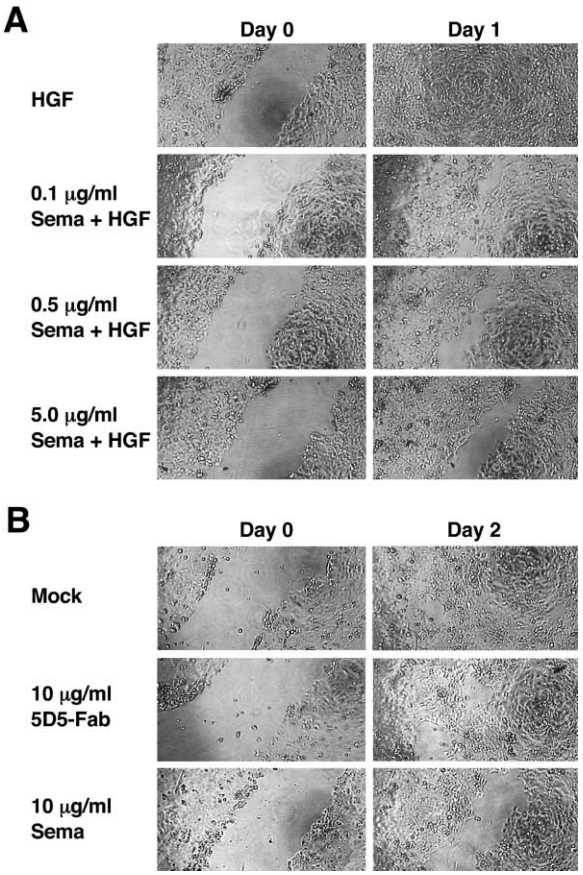


**Figure 6.** rSema and anti-Met 5D5-Fab inhibit cell motility

**A:** H441 cells were scraped on Day 0 and treated with increasing concentrations of rSema plus HGF in serum-free media. The scrapes were photographed on Day 0 and Day 1. A representative data set of three independent experiments is shown.

**B:** H441 cells were scraped and treated with mock, rSema, or anti-Met 5D5-Fab in serum-free media over the course of 2 days. Photographs were taken on Day 0 and Day 2. Four independent experiments were carried out and a representative data set is shown.

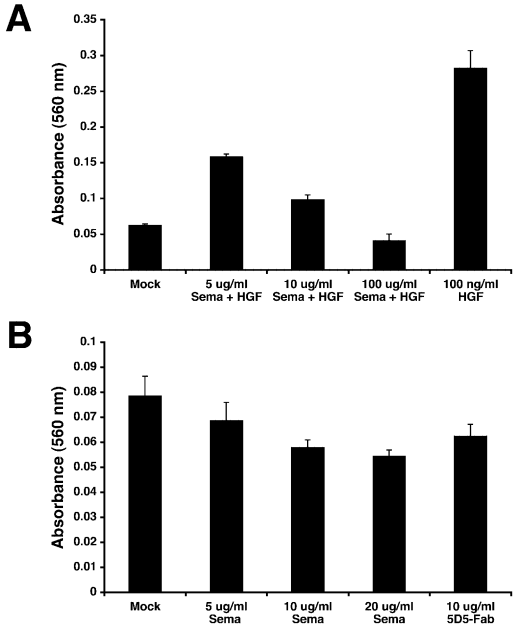


**Figure 7.** Cell migration is inhibited by rSema and anti-Met 5D5-Fab

**A:** MDA-MB-435 cells were pre-incubated with increasing concentrations of rSema and seeded into the upper chambers of a transwell plate in serum-free media. The lower chambers contained 100 ng/ml HGF with the exception of mock-treated wells.

**B:** Cells were pre-incubated with 10 μg/ml anti-Met 5D5-Fab or increasing concentrations of rSema and then seeded into the upper chamber of each transwell in serum-free media. The lower chamber contained serum-free media. The data are representative of three independent experiments performed in duplicate. The migrating cells were stained with crystal violet and the absorbance of eluted cells was measured at 560 nm.

and other components responsible for cellular interactions (Giancotti and Ruoslahti, 1999), it is likely that all proteins that facilitate Met dimerization may not yet be identified.

We report that the Sema domain is sufficient for interaction with HGF, confirming previous observations (Gherardi et al., 2003) and demonstrate that rSema inhibits ligand-driven Met activation. Thus, it is likely that rSema effectively inhibits HGF-dependent Met activation by binding HGF. Furthermore, it suggests that HGF-dependent Met-expressing tumors could also be targeted by rSema in a similar manner to Ron Sema domain-mediated inhibition of ligand-dependent receptor activation (Angeloni et al., 2004). We speculate that the Sema-HGF interaction site differs from the Met dimerization interface, since our cross-linking studies show that ligand-independent Met dimers shift further when bound to HGF (Figure 3D). These data imply that the dimerization interface and the HGF interaction site are non-overlapping.

In addition, we demonstrate that the Sema domain is necessary for binding anti-Met 5D5. Similar observations of Met 5D5 binding to rSema by surface plasmon resonance experiments corroborate our data (M. Zhang, personal communication). Since anti-Met 5D5-Fab also inhibits ligand-independent receptor activation as observed in our findings, we speculate that in addition to HGF competition, anti-Met 5D5-Fab may block receptor dimerization by steric hindrance. Likewise, rSema also inhibits ligand-independent and -dependent Met activity, suggesting a role for rSema as an effective Met inhibitor. Similar inhibitory activity has been described for the c-kit receptor (Lev et al., 1992). The role of the Met Sema domain in dimerization in a ligand-independent context addressed in this study differs from the role of the Ron Sema domain. While the Ron Sema domain competes for binding with MSP to inhibit Ron activity, it does not appear to inhibit Ron receptor dimerization (Angeloni et al., 2004). Our functional studies carried out in the presence and absence of HGF demonstrates that the Met Sema domain inhibits both ligand-dependent and –independent receptor activation. These data suggest that although the structural motifs and binding interactions of Met and Ron may be similar, the in vivo contexts may specify differing homophilic interactions.

In this report, we identify the Met Sema domain as a potential inhibitor of not only ligand-dependent but also ligand-independent Met receptor activation in a manner similar to anti-Met 5D5-Fab. Antagonistic antibodies that target the extracellular domain of Met prevent growth of Met-driven tumors in vivo (R. Schwall, personal communication). Likewise, our data suggest that the Sema domain of Met blocks receptor activation in tumor

cells by inhibiting MAPK phosphorylation, cell motility, and migration. These observations point to the exciting prospect of treating Met-overexpressing tumors not only by targeting the Sema domain of Met but also by utilizing the Sema domain itself as a biotherapeutic.

## Experimental procedures

### Constructs and recombinant proteins

Extracellular subdomain deletions of cMet were constructed using conventional PCR methods. N-terminal primers containing the start of Sema, PSI, first IPT, or fourth IPT domains flanked by a KpnI site were paired with a C-terminal primer up to Met residue 959 flanked by a StuI site. cMet was used as template and the PCR fragments for each clone were inserted into pCR-Blunt II-TOPO vector using the Zero Blunt TOPO PCR cloning kit (Invitrogen) according to manufacturer's instructions. The clones were confirmed by DNA sequencing. The constructs were then subcloned into pcDNA3.1 V5/His vector (Invitrogen) via KpnI and EcoRV to add a tag at the C terminus. The signal peptide of Met was added via the HindIII and KpnI sites at the N terminus of each clone. Each clone was digested with HindIII and EcoRV and subcloned into pRK5TKneo vector via HindIII and PmeI. For EC-WT-Flag and EC-WT-V5/His clones, an N-terminal primer containing a HindIII site was paired with a C-terminal primer up to Met residue 959 flanked by a StuI site. EC-WT-V5/His was subcloned via HindIII/StuI into the HindIII/EcoRV sites of pcDNA3.1 V5/His and then subcloned into pRK5TKneo as described above. For EC-WT-Flag, the PCR fragment was ligated to pCR-Blunt II-TOPO vector. Oligonucleotides containing a Flag tag sequence were inserted between StuI and KpnI. EC-WT-Flag was then subcloned into pRK5TKneo via HindIII and EcoRV/ScaI. Full-length Met containing a V5/His tag was constructed by PCR primers flanked by BsrGI and XhoI sites using Met as template. The BsrGI/XhoI-digested PCR product was then inserted into the vector of EC-WT-V5/His to produce full-length WT Met-V5/His. Recombinant HGF and anti-Met 5D5-Fab were provided by R. Schwall (Genentech). scHGF(R494E) was produced in mammalian cells by S. Avella as described previously (Peek et al., 2002). Recombinant Sema (and PSI domain) was prepared as described previously (Stamos et al., 2004). Attempts to make recombinant Sema domain alone did not yield protein as reported previously for Sema3A (Antipenko et al., 2003).

### Immunoprecipitation and Western blot analysis

Cell lines were obtained from American Type Culture Collection (ATCC). 293 cells were maintained in DMEM supplemented with 10% FBS (Sigma), penicillin/streptomycin (GIBCO), and 2 mM glutamine. A549, H441, and MDA-MB-435 cells were maintained in 50:50 DMEM/F12 (Cellgro) supplemented with 10% FBS, penicillin/streptomycin, and 2 mM glutamine. Cells were transfected with 1–12 μg of the indicated constructs using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. 24 hr later, cells were harvested with 1% NP40 lysis buffer (50 mM Tris [pH 7.45], 150 mM NaCl, and 1% Nonidet 40) with Complete protease inhibitor cocktail tablet and phosphatase inhibitor cocktail 2 (Roche and Sigma, respectively). Cell debris was centrifuged and the supernatant was precleared with Protein A Sepharose (Sigma) or Protein G-Plus agarose beads (Santa Cruz) for 1 hr. For HGF binding studies, 500 μg of lysates were incubated with 5 μg scHGF (R494E) or HGF and immunoprecipitated with 1 μg of V5 antibody overnight at 4°C with rotation followed by incubation with Protein G-Plus beads for 2 hr. For anti-Met 5D5 binding studies, 10 μg/ml of anti-Met 5D5 was incubated with 500 μg of transfected lysates overnight followed by incubation with Protein G-Plus beads as before. For Met tyrosine phosphorylation and MAPK phosphorylation studies, cells were serum starved for 2 hr with 0.5% BSA in the medium. In HGF-dependent studies, cells were incubated with 0, 5, 10, 50, or 100 μg/ml rSema for 1 hr prior to addition of 5 or 10 ng/ml HGF for 5 or 10 min. For HGF-independent studies, cells were treated with 10 μg/ml anti-Met 5D5-Fab or rSema for 1 hr. To detect phosphorylated Met, lysates were immunoprecipitated with 20–40 μl of Met (C-28) agarose conjugate (Santa Cruz) overnight at 4°C. For all samples, 2× sample buffer (Invitrogen) with 10 mM DTT was added, boiled for 2 min, and analyzed on 4%–12% Tris-glycine gels (Invitrogen). Proteins were transferred to nitrocellulose membranes, blocked with 5% nonfat milk or BSA for 1 hr, and then probed with 1:5,000 V5 (Invitrogen), 1:10,000 C-terminal Met (C-12) (Santa Cruz), 1:1,000 MAPK (Cell Signaling), 1:500 HGF-α (145) (Santa Cruz), 1:1,000 P-MAPK (Cell Signaling), 1:1,000 His (Cell Signaling), or 1:1,000 phospho-tyrosine 4G10 (Upstate) antibodies overnight at 4°C with rocking. Secondary antibodies conjugated to HRP against mouse or rabbit IgG were used at 1:5,000–10,000 dilution (Amersham). Proteins were detected by enhanced chemiluminescence (ECL-Plus, Amersham). For membranes that were stripped and reprobed, stripping buffer was added (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl [pH 6.7]) for 30 min at 65°C with occasional agitation prior to re-incubation of primary antibodies. Densitometry analysis of Western blots was performed using NIH Image 1.63 program (http://rsb.info.nih.gov/nih-image). The data are represented as a ratio of phosphorylated to nonphosphorylated protein.

### Covalent affinity crosslinking analysis

Crosslinking studies were performed as previously described with modifications (Blechman et al., 1995). A549, H441, MDA-MB-435, and 293 cells were serum starved for 2 hr in media with 0.5% BSA. The media was removed and replaced with PBS. Cells were then treated with the crosslinking agent sulfo-EGS (Pierce) at increasing concentrations according to the manufacturer's instructions. Sulfo-EGS is known to predominantly crosslink reactive groups in lysines that are present throughout the extracellular domain of Met. Similarly, 293 cells were plated into 6-well plates and transfected with 0.1–4 μg of the indicated constructs using Lipofectamine 2000 (Invitrogen). 24 hr later, the media was replaced with PBS and cells were crosslinked with sulfo-EGS. Cells were then lysed with 1% NP40 lysis buffer with protease and phosphatase inhibitors. 10 μg of lysates in reducing sample buffer were then analyzed immediately on 8% or 4%–12% Tris-glycine gels, transferred to nitrocellulose membranes, and blocked with 5% milk for 1 hr. Membranes were probed with 1:1,000 C-terminal Met C-12 or 1:5,000 V5 antibody overnight at 4°C with rocking. After incubation of 1:5,000 secondary antibodies, proteins were detected with ECL-Plus. Full-length Met-V5/His, EC-WT-Flag, and EC-WT-V5/His constructs were cotransfected and crosslinked as described. For EC-WT binding studies, 500 μg of crosslinked lysate was immunoprecipitated with either V5 or Flag antibody, analyzed on 8% Tris-glycine gels, transferred to nitrocellulose membranes, and immunoblotted with either 1:5,000 V5 or 1:1,000 Flag (Sigma) antibodies. Proteins were detected with ECL-Plus.

### Scrape assay

H441 cells were seeded at a density of $4.5 \times 10^4$ cells/well in a 96-well plate with 10% FBS in 50:50 DMEM:F12. The next day (Day 0), a single scrape was made in the confluent monolayer in each well as described previously (Lorenzato et al., 2002). The media was replaced with media containing 0.5% BSA. For HGF-dependent studies, 0, 0.1, 0.5, or 5 μg/ml rSema was pre-incubated with HGF for 1 hr prior to addition to cells. Photographs were taken on Day 0 and Day 1, when the gap in HGF-treated cells had closed completely. For HGF-independent studies, cells were treated with mock, 10 μg/ml anti-Met 5D5-Fab, or 10 μg/ml rSema. The scrape was monitored and photographed daily. Representative results are shown for Day 0 and Day 2.

### Migration assay

Transwell migration assays were carried out as previously described with modifications (Coltella et al., 2003). MDA-MB-435 cells were seeded at $1.2 \times 10^5$ cells in 50:50 DMEM:F12 media containing 0.5% BSA to the upper chamber of each transwell (Costar) that had been previously coated with 10 μg/ml collagen type IV (Sigma). Cells were pre-incubated for 10 min with 0, 5, 10, or 20 μg/ml rSema. As control, some samples were pre-incubated with 10 μg/ml anti-Met 5D5-Fab. The cells were distributed to the upper chambers of the transwell plate. The lower chambers contained media with 0.5% BSA. Similarly, migration assays were carried out with rSema in the presence of HGF. Cells were pre-incubated for 10 min with 0, 5, 10, or 100 μg/ml rSema and added to the upper chambers as before. Each lower chamber contained media supplemented with 100 ng/ml HGF with the exception of mock-treated wells. The next day, the cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Cells that migrated to the lower chamber were stained and solubilized with 10% acetic acid and the absorbance was measured at 560 nm in a microplate reader.

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