Electronic Supplementary Material (ESI) for Chemical Science.

Supplementary Information

Semi-synthesis of a HGF/SF kringle one (K1) domain scaffold generates a potent in vivo MET receptor agonist.

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Supplementary Figures.

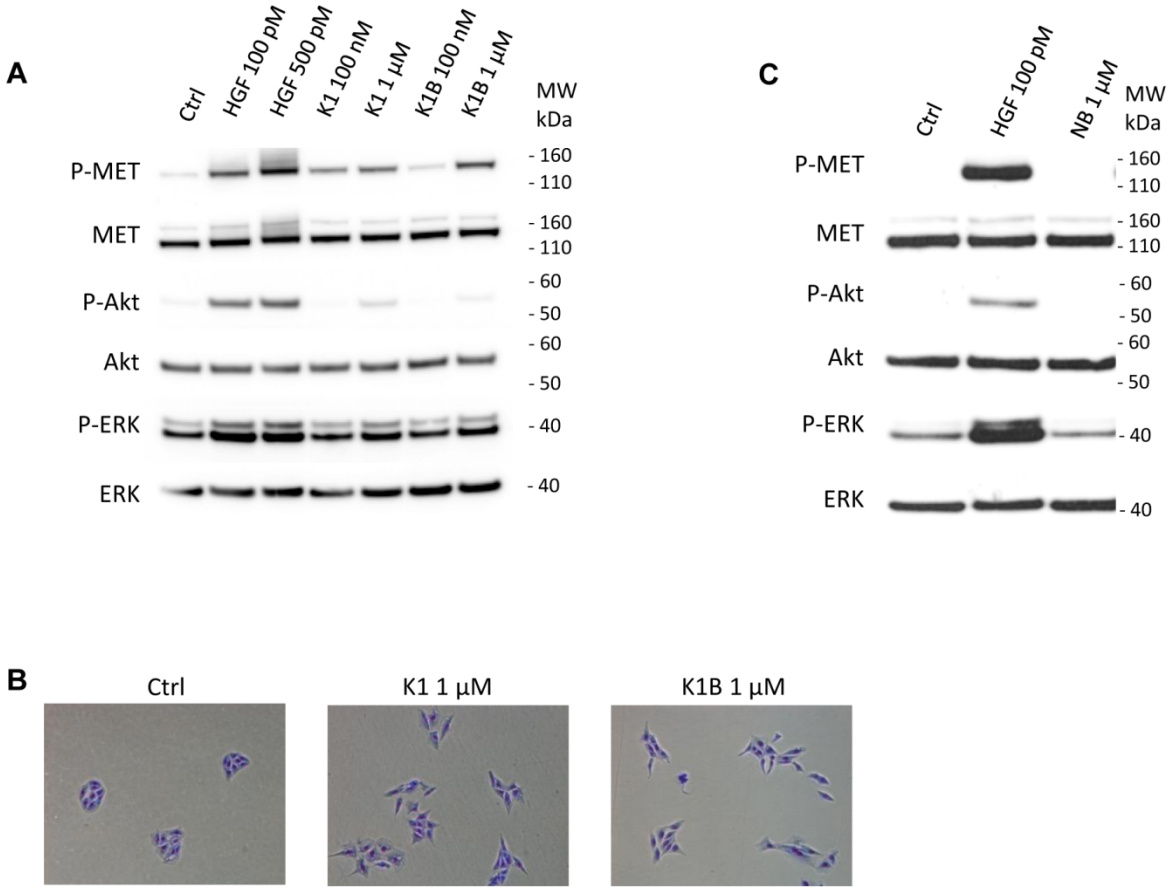

Fig. S1. (*A*) HeLa cells were treated for 7 min with 100 pM or 500 pM HGF/SF (HGF), 100 nM or 1 µM K1 and 100 nM or 1 µM K1B. Cell lysates were then analyzed by specific total MET, Akt and ERK or phospho-MET, phospho-Akt and phospho-ERK western blot. (*B*) Cell scattering assay. MDCK isolated cell islets were incubated for 18 h in presence of culture media (Ctrl) with 1 µM K1 or 1 µM K1B. Cells were then stained and observed under microscope (40x). (*C*) NB MET activation analysis. HeLa cells were treated for 7 min with 100 pM HGF/SF (HGF), 1 µM NB. Ctrl: vehicle. Cell lysates were then analyzed by specific total MET, Akt and ERK or phospho-MET, phospho-Akt and phospho-ERK western blot.

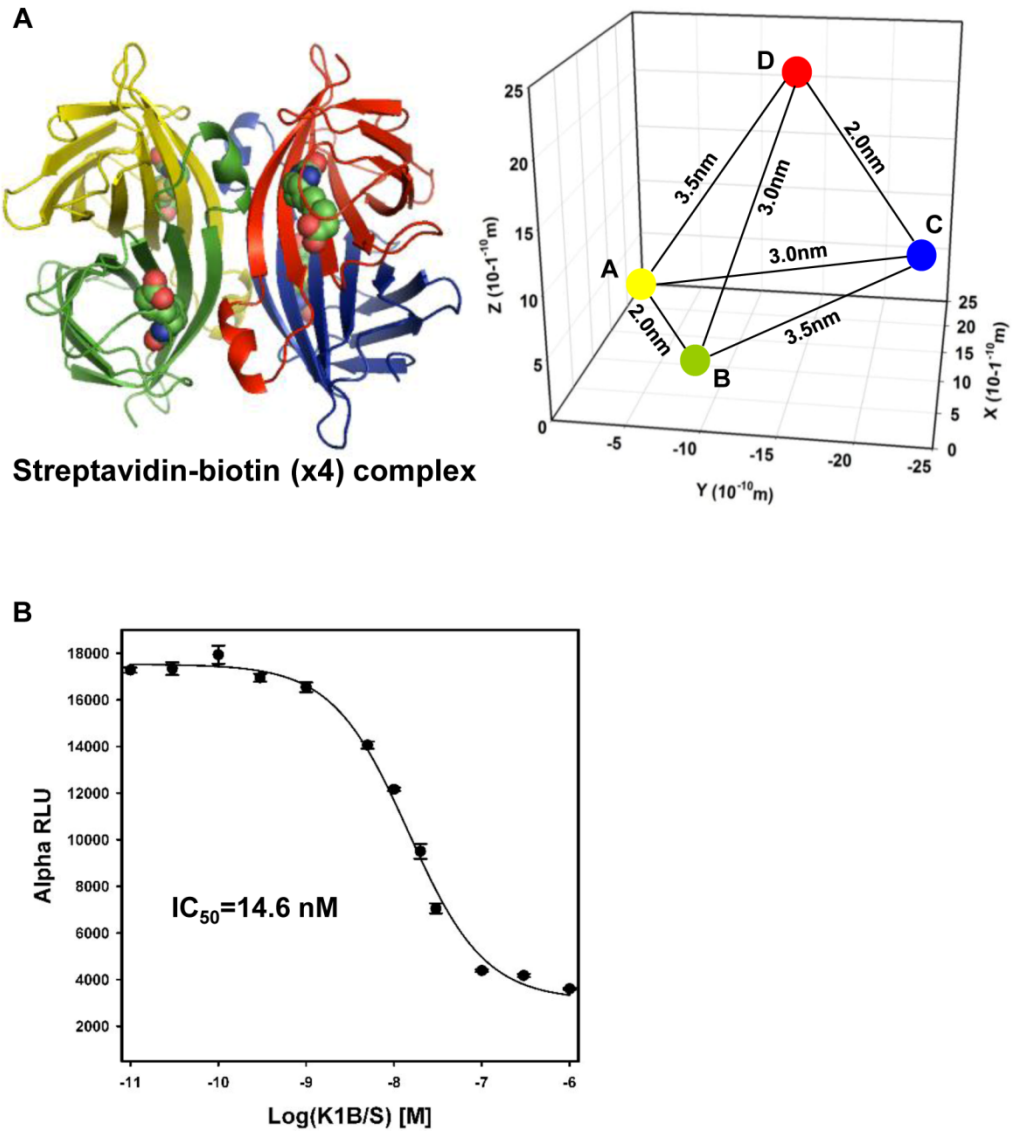

Fig. S2. (*A*) Structure of a streptavidin homotetramer with 4 bound biotins (left, PDB 1SWE) and distances between binding sites (right). (*B*) AlphaScreen competition assay. Increasing concentrations of K1B/S complex (competitor, ratio 2:1) were added to a mixture of K1B (20 nM), MET-Fc (2 nM) and donor and acceptor Alpha beads (10 µg/mL for each) pre-equilibrated at 22°C for 20 min. IC_{50} of Alpha signal was measured after incubating the mixtures at 22°C for 1 h. In these particular conditions, when K1B (Ligand, L) and MET-Fc (Tracer, T) concentrations are under donor and acceptor Alpha beads maximal binding capacities, i.e. 30 and 3 nM respectively, IC_{50} is linked to K_D based on Cheng and Prusoff equation[1]: $K_D = IC_{50} / (1+([L+T]/K_D)$, where [L+T] is for the concentration of K1B/S-MET-Fc complexes at equilibrium). Therefore, if [T]<<[L] (10 fold in this experiment) and if [L+T] < K_D, IC_{50} approximates K_D. In our case, [L+T] is obligatory less than 2 nM, the concentration of MET-Fc in the assay, making this approximation realistic. The error bars correspond to standard error (+/-SD) of triplicates. The graph is representative of three independent experiments.

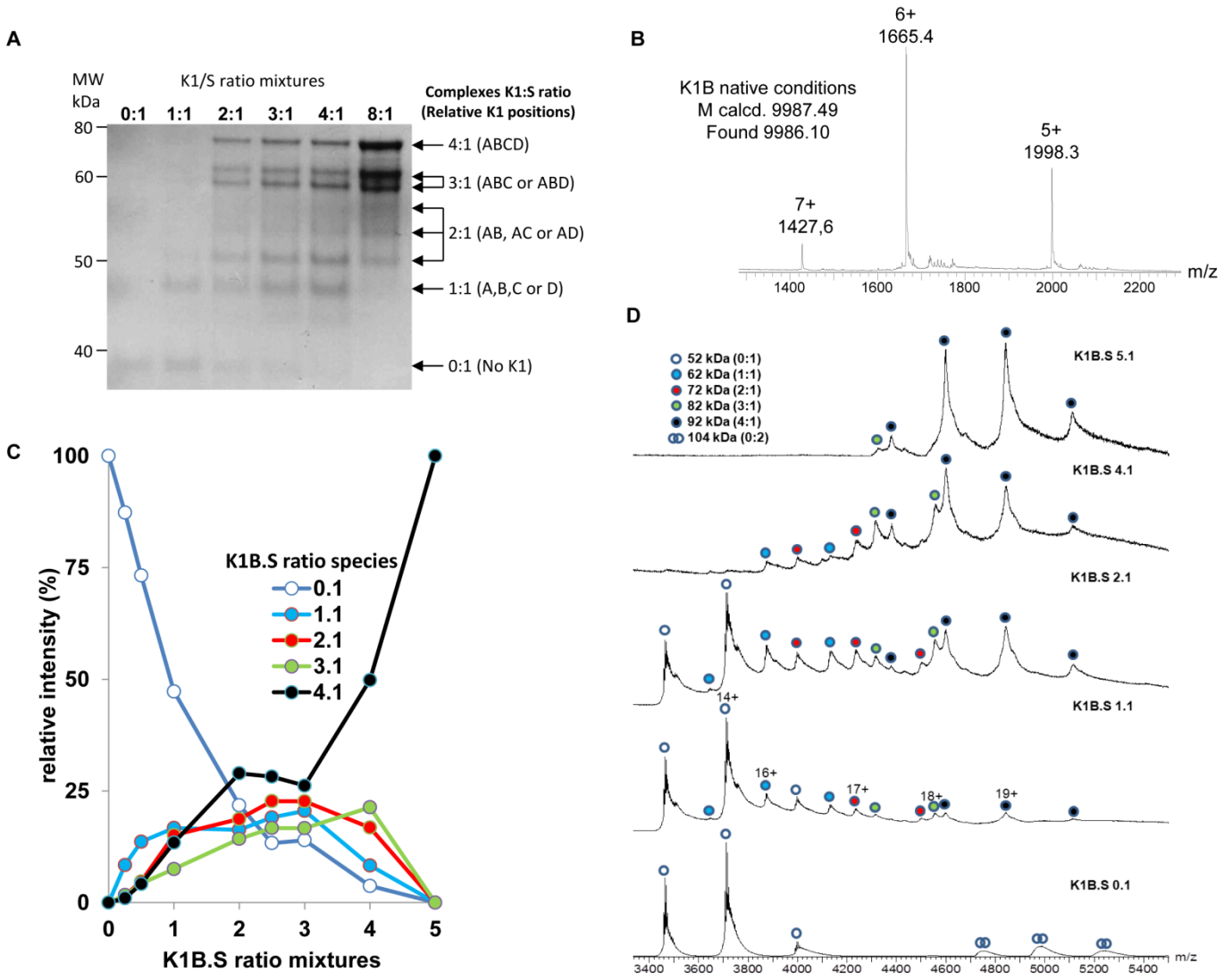

Fig. S3. (*A*) Analysis of K1B/S complexes. Increasing ratio of K1B and streptavidin (from 0:1 to 8:1) were analyzed in non-denaturing condition by SDS-PAGE on a 10% NuPage® gel in MES buffer. Gel was fixed and stained with Coomassie Brilliant Blue. K1B:S ratio for each complex composition is indicated with corresponding A,B,C and D relative biotin binding sites positioned as proposed in Supplementary Fig. 4. (*B*) Mass spectrum of K1B under native conditions. (*C*) Relative intensity of each species depending on the K1B.S ratio mixture. (*D*) Titration of streptavidin with K1B. Upon addition of K1B, new species corresponding to the binding of 1 to 4 molecules of K1B to the streptavidin are clearly visible.

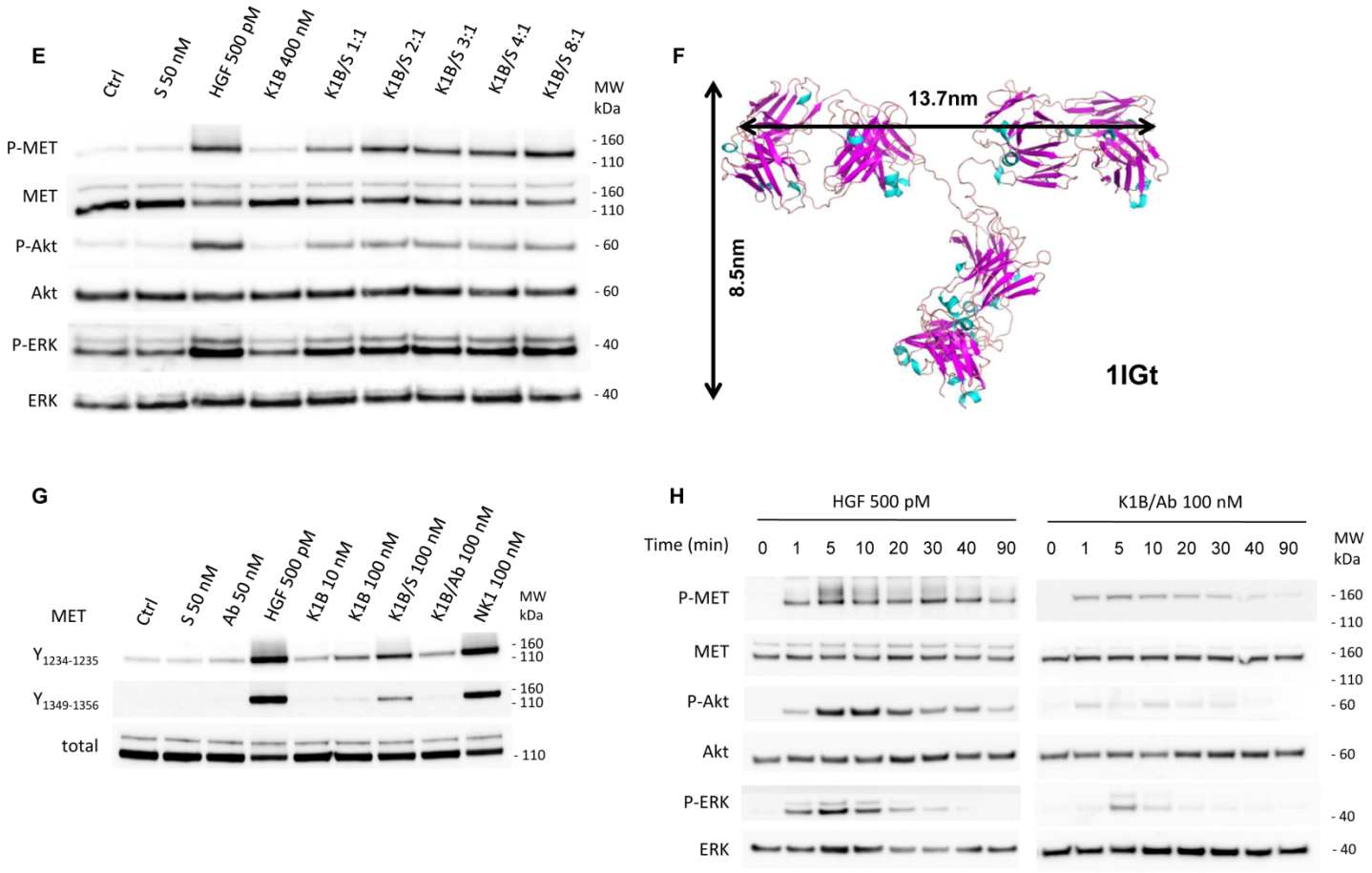

Fig. S3. (*E*) Determination of optimal K1B:S ratio. HeLa cells were treated for 7 min with 50 nM streptavidin (S), 500 pM mature HGF/SF (HGF), 400 nM K1B and an increasing ratio of K1B/S mixture (from 1:1 to 8:1) with 50 nM streptavidin. Cell lysates were then analyzed by specific total MET, Akt and ERK or phospho-MET, phospho-Akt and phospho-ERK western blot. (*F*) Structure of human IgG: distance between two paratopes is 13.7 nm (PDB 1IGt). (*G*) Analysis of MET tyrosine phosphorylation profile. HeLa cells were treated for 7 min with 50 nM streptavidin (S), 50 nM anti-biotin antibody (Ab), 500 pM mature HGF/SF (HGF), 10 or 100 nM K1B, 100 nM K1B/S, 100 nM K1B/Ab or 100 nM NK1. Cell lysates were then analyzed by western blot with total MET and phospho-specific MET Y1234-1235 and Y1349-1356 residues. (*H*) HGF/SF, K1B/Ab kinetic analysis. HeLa cells were treated with 500 pM HGF/SF or 100 nM K1B/Ab, for 1, 5, 10, 20, 30, 40 or 90 min. Cell lysates were then analyzed by specific total MET, Akt and ERK or phospho-MET, phospho-Akt and phospho-ERK western blot.

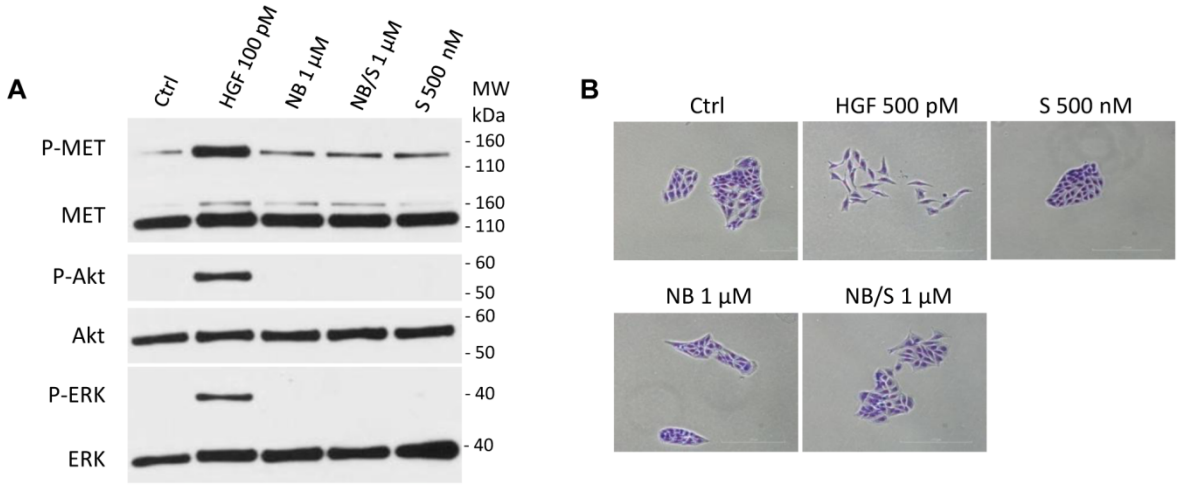

Fig. S4. (*A*) HeLa cells were treated for 7 min with 100 pM HGF/SF (HGF), 1 µM NB and 1 µM NB/S (2:1 ratio) and 500 nM streptavidin (S). Ctrl: vehicle. Cell lysates were then analyzed by specific total MET, Akt and ERK or phospho-MET, phospho-Akt and phospho-ERK western blot. (*B*) Cell scattering assay. MDCK isolated cell islets were incubated for 18 h in culture media (Ctrl), 500 pM HGF/SF (HGF) 500 nM streptavidin (S), 1 µM NB or 1 µM NB/S. Cells were then stained and observed under microscope (40x).

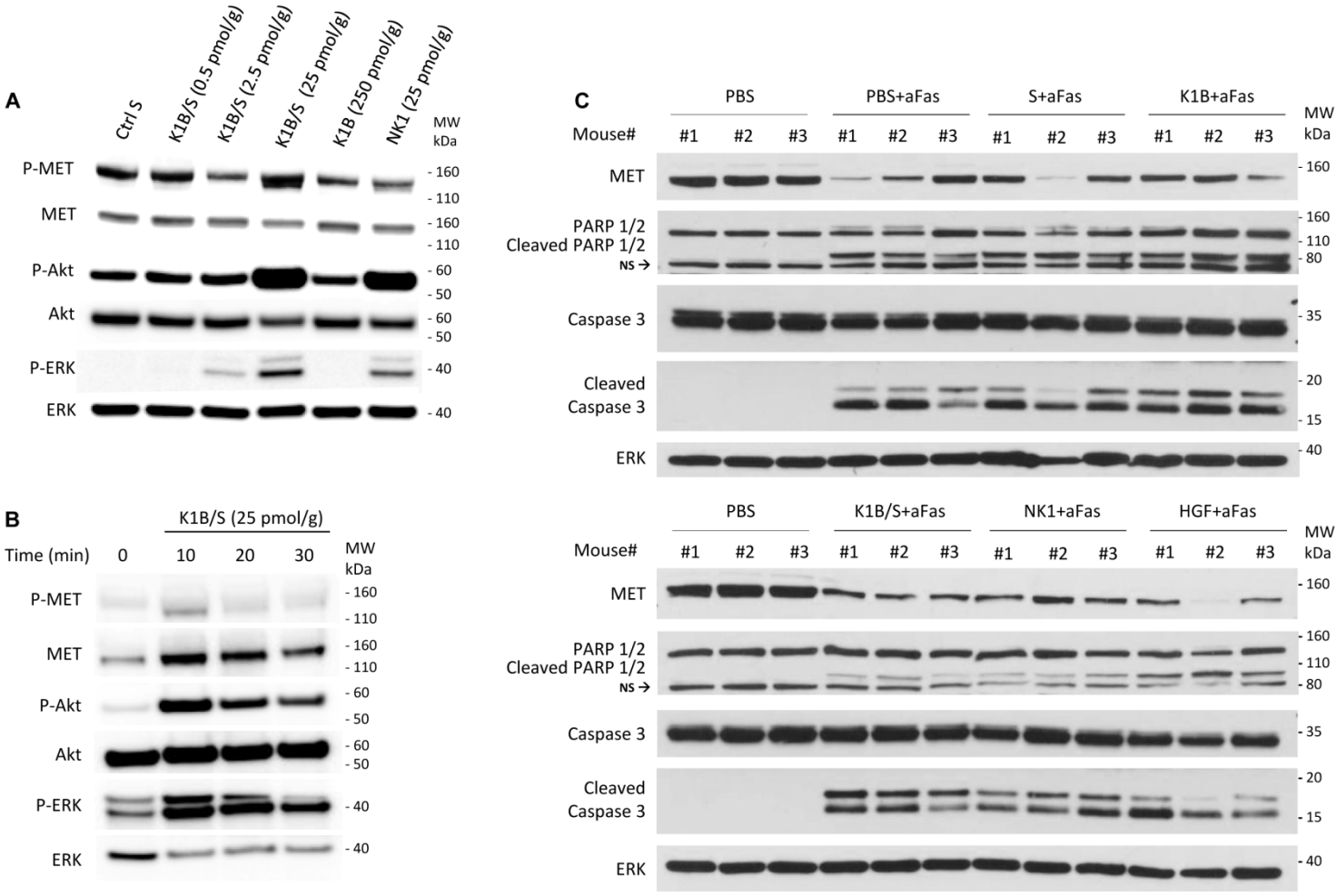

Fig. S5. (*A*) Mice were injected with an increased concentration of K1B/S complex (0.5, 2.5 or 25 pmol g, corresponding to 5 ng K1B/14 ng S, 25 ng K1B/70 ng S and 250 ng K1B/700 ng S), 25 pmol K1B/g (250 ng/g) or 25 pmol/g NK1 (500 ng/g). After 10 min, livers were extracted, snap frozen and crushed. Cell lysates were analyzed by specific total MET, Akt and ERK or phospho-MET, phospho-Akt and phospho-ERK western blot. (*B*) *In vivo* MET activation kinetics. Mice were injected with 25 pmol K1B/S (250 ng/700 ng) per g of body weight, and livers were extracted after 0, 10, 20 or 30 min, snap frozen and crushed. Cell lysates were analyzed by specific total MET, Akt and ERK or phospho-MET, phospho-Akt and phospho-ERK western blot. (*C*) Fas-induced fulminant hepatitis. FVB mice were injected intravenously with 125 ng anti-Fas monoclonal antibody (aFas) mixed with 25 pmol K1B, 25 pmol K1B/S complex, 25 pmol NK1, 12.5 pmol streptavidin (S) or 2.5 pmol mature HGF/SF per g of body weight, or PBS. A second injection without anti-Fas was performed 90 min later. Livers were extracted, snap frozen and crushed. Proteins were analyzed by specific total MET, PARP 1/2, Caspase 3, cleaved Caspase 3 and total ERK western blot.

ESI Materials and Methods.

Reagents and Antibodies. Recombinant human HGF/SF was purchased from Invitrogen (Breda, Netherlands), recombinant VEGF-A from R&D Systems (Minneapolis, MN, USA), Streptavidin (*Streptomyces avidinii*) from ProZyme (Hayward, CA, USA) and Anisomycin (*Streptomyces griseolus*) from CalbioChem (Germany). Recombinant human NK1 protein (residues 28-209) was kindly provided by Prof. Ermanno Gherardi (University of Pavia (Italy). Antibodies directed against the kinase domain of MET were purchased from Invitrogen, anti-phospho-MET (Tyr1234/1235), anti-phospho-MET (Tyr1349), anti-total Akt, anti-phospho-Akt (Ser473), anti-phospho-ERK1/2 (Thr202/Tyr204) and anti-Caspase-3 from Cell Signaling (Massachusetts, USA), anti-ERK2 (C-14) and anti-PARP1/2 from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-biotin monoclonal antibody and horseradish peroxidase (HRP)-conjugated antibodies directed against rabbit or mouse IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Characterization of K1B/S Complex. K1B and streptavidin complex ratios were analyzed by SDS-PAGE using 10% NuPage precast gels run in MES buffer (Life Technologies) without heating the samples. Gels were fixed in 20% methanol and 5% acetic acid for 30 min, and stained in Coomassie Brilliant Blue solution.

Native Mass Spectrometry. Streptavidin and K1B were first buffer exchanged in 200 mM ammonium acetate pH 7.4, using Zeba™ bench-top spin desalting columns (Thermo Scientific). Protein concentrations were determined by measuring the absorbance at 280 nm and using extinction coefficients of 16,500 and 165,000 M^{-1} cm^{-1} for K1B and streptavidin, respectively. Titration was performed by adding 0 to 5 molar equivalents of K1B to streptavidin. A 10 µl volume was prepared per sample, and final concentrations ranged from 1 to 20 µM. Noncovalent MS analysis was performed on a Synapt G2 HDMX (Waters, Manchester, UK) coupled to an automated chip-based nanoelectrospray device (Triversa Nanomate, Advion Biosciences, Ithaca, USA) operating in the positive ion mode. Instrument parameters were as follows: capillary, sample cone and extraction cone voltages were set at 1.55 kV, 65 V and 5 V, respectively. The backing pressure was increased to 6 mbar to improve the transmission of high molecular weight species by collisional cooling. Calibration was performed with a 2 mg/ml cesium iodide solution and data were analyzed with MassLynx software v.4.1 (Waters, Manchester, UK).

Endogenous MET Capture. HeLa and CaPan1 cells were collected by scraping and then lysed on ice with a lysis buffer (20 mM Tris HCl, 50 mM NaCl, 5 mM EDTA and 1% Triton X-100). Lysates were clarified by centrifugation (20,000 g x 15 min) and protein concentration was determined (BCA protein assay Kit, Pierce®, Thermo scientific, IL, USA). Streptavidin-Sepharose beads (GE Healthcare) were washed and equilibrated in PBS. Beads were loaded with 15 µg K1B or NB (100 µL beads in a 50:50 PBS:bead slurry) for 20 min at room temperature and immediately washed with PBS. Beads were incubated with 250 µg of protein cell lysates overnight at 4°C under mild agitation. Beads were quickly washed with PBS and bound proteins were eluted with 200 mM glycine buffer pH 2. Elution fractions were then analyzed by western blotting.

Western Blots. Cells were collected by scraping and then lysed on ice with a lysis buffer (20 mM HEPES pH 7.4, 142 mM KCl, 5 mM MgCl2, 1 mM EDTA, 5% glycerol, 1% NP40 and 0.1% SDS) supplemented with freshly added protease and phosphatase inhibitors (#P8340 and #P5726, respectively, Sigma). Lysates were clarified by centrifugation (20,000 g x 15 min) and protein concentration was determined (BCA protein assay Kit, Pierce®, Thermo scientific, IL, USA). The same protein amount of cell extracts was separated by either classical SDS-PAGE or NuPAGE (4-12% or 10% Bis-Tris precast gels) (Life technologies) and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore). Membranes were probed with indicated primary antibodies, followed by incubation with appropriate HRP conjugated secondary antibodies. Protein-antibody complexes were visualized by chemiluminescence with the SuperSignal® West Dura Extended Duration Substrate (Thermo scientific), using a LAS-3000 imaging system (Fujifilm, Tokyo, Japan) or X-ray films (CL-Xposure™ Film, Thermo scientific).

MTT Assay. Cells were washed with PBS to eliminate dead cells and then incubated in medium containing 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Invitrogen) for 1 h. After a washing step with PBS, the formazan crystals were solubilized and mixed thoroughly with 0.04 M HCl in isopropanol. For each condition, 60 µl of formazan solution was loaded in triplicate onto a 96-well plate. Absorbance was then measured with a microplate spectrophotometer at 550 nm and 620 nm, as test and reference wavelengths, respectively. The absorbance correlates with cell number.

Scattering Assay. Cells were seeded at low density (2,000 cells/well on a 12-well plate) to form compact colonies. After treatment, when colony dispersion was observed, the cells were fixed and colored by Hemacolor® stain (Merck, Darmstadt, Germany) according to the manufacturer's instructions. Representative images were snap-captured using a phase contrast microscope with 40x magnification (Nikon Eclipse TS100, Tokyo, Japan).

Morphogenesis Assay. Cells were seeded onto a layer of Growth Factor Reduced Matrigel™ (BD Biosciences) (100,000 cells/well of a 24-well plate), treated and observed under phase contrast microscope. Representative images were snap-captured with 40x magnification (Nikon Eclipse TS100).

Statistical Analysis. Data were obtained in triplicate from at least 3 independent experiments, and expressed either as mean values or percentages of control values +/- SD or SEM depending on the experiments performed. When indicated, differences between data groups were determined by ANOVA using Prism 5 (GraphPad Software, Inc., San Diego, CA, USA), and considered to be statistically significant for $P < 0.05$.

References

1. Y. Cheng and W. H. Prusoff, *Biochem. Pharmacol.*, 1973, **22**, 3099-3108.