Supplementary Materials and Methods

Molecular modeling. Molecular models of (TβRII)2/TGF-β3, (ActRIIb)2/activin and (BMPRIa)2/BMP-2 trap-ligand complexes were based on the crystal structures of the ligand complexed with two unlinked receptor ectodomains. The PDB entries 1KTZ (1), 1S4Y (2) and 2GOO (3) were used to build the (TβRII)2/TGF-β3, (ActRIIb)2/activin and (BMPRIa)2/BMP-2 complexes, respectively. In each case, a polypeptide linker between the folded ligand-binding domains was constructed using the Biopolymer module in SYBYL 6.9 (Tripos, Inc., St. Louis, MO) by fusing the natural N-terminal and C-terminal IDR sequences of the receptor ectodomains (Supplementary Tables S1 and S2). The resulting trap-ligand complexes were refined by conjugate-gradient energy-minimization using the AMBER molecular mechanics force field (4). The IDRs at the N- and C-termini of the traps were not modeled. These IDRs were inferred from 3D-structures in the Protein Data Bank (5): 2GOO (3) and 1LX5 (6) for ActRIIa-ED, 1S4Y (2) and 1NYU (7) for ActRIIb-ED, 2GOO (3) and 1ES7 (8) for BMPRIa-ED, and 1KTZ (1), 2PJY (9), 1M9Z (10) and 1PLO (11) for TβRII-ED and TβRIIb-ED.

Molecular Dynamics Simulation. The (TβRII)2/TGF-β3 molecular mechanics model was used for molecular dynamics (MD) simulation together with AMBER FF03 force field within AMBER 9 software (12). The molecular system consisting of 245 residues from the (TβRII)2 trap (21 disordered N-terminal residues and 6 flexible C-terminal residues were not included in the MD simulation), 224 amino-acid residues of the TGF-β3 dimer and 14 Na+ counterions, was solvated in a rectangular water box using the XLEAP in AMBER 9.The entire system was energy-minimized by applying harmonic restraints with force constants of 10 kcal/mol/Å2 to all solute atoms, followed by heating from 100K to 300K over 25 ps in the canonical ensemble

(NVT), and by equilibrating to adjust the solvent density under 1 atm pressure in the isothermal-isobaric ensemble (NPT) simulation. The Particle Mesh Ewald method (13) was used to treat long-range electrostatics, and bond lengths involving bonds to hydrogen atoms were constrained by SHAKE (14). Standard analysis of MD trajectories was carried out with PTRAJ in AMBER 9. The average solvated interaction energy (SIE) between (TβRII)2 and TGF-β3 was estimated with SIETRAJ (15) based on the rigid separation of the trap-ligand complex on 500 snapshots at 20 ps intervals from the last 10 ns of the MD trajectory.

[^{3}H]-Thymidine incorporation assays. 4T1 cells were seeded at 15x10^3 cells/well, respectively, in 24-well plates in 0.9 mL of growth medium. The next day serial dilutions of TGF-β were added to the cells in 100 μL DMEM containing 50 mM N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) and 0.1% BSA (Sigma-Aldrich). The cells were incubated for 20 hours at 37°C and then 0.5 μCi of [^{3}H]-TdR (PerkinElmer) was added in 100 μL nutrient mixture F12 followed by incubation for 4 hours at 37°C. The cells were rinsed with PBS and then treated with 0.05% trypsin (Hyclone) at 37°C. Cell suspensions were harvested and counted in a β-counter (LKB-Wallac). Values are calculated as (cpm TGF-β treated sample/cpm non-treated control) X 100 ± SEM.

Real-time PCR (Q-PCR). Primer sets for 28S mRNA, PAI-1 and Rae-1γ transcripts are listed in Supplemental Table S5. Mouse primer set for GZMB was purchased from SABioscience. Total RNA was extracted from cells or frozen tumor samples using an RNeasy Plus Mini-Kit including a DNase step (Qiagen). cDNA was prepared using SuperScript II RT (Invitrogen) and random primers (Invitrogen). Q-PCR was performed on a Mx3005P QPCR System (Agilent) using a QuantiTect SYBR Green PCR master mix (Qiagen). For amplification (in triplicate), cycles of

10 minutes at 95°C were followed by 40 cycles (95°C, 30 s; 55°C, 30 s; 72°C, 30 s). Fold changes were calculated using the ΔΔCt method (SABiosciences).

Immunohistochemistry of 4T1 tumors. Paraffin-embedded tumors sections (sliced at 5 µm thickness) were placed on Superfrost Plus microscope slides (Fisher Scientific) and,dried at 60°C. Sections were de-waxed 3x 3 minutes in Xylene and re-hydrated twice in 100% EtOH, 95% EtOH, 75% EtOH (2 minutes each) and finally in dH_2O (2 minutes). Antigen retrieval was carried out by heat-induced epitope retrieval in sodium citrate pH6.0 (for CD31 and Ki67) or Tris-EDTA pH9.0 (for CD3). The REVEAL Polyvalent HRP-AEC Detection System was used (Spring Biosciences) with the following antibodies: CD3 (1:50, 1 hour RT; Rabbit, DAKO), CD31 (1:50, overnight 4°C; Rabbit, Clone SP38, Spring Biosciences), and anti-Ki67 (1:150, 30 minutes RT; Rabbit, Clone SP6, ThermoFisher Sci.). Sections were counter-stained in Surgipath hematoxylin 560 (5 seconds, Leica Microsystems). Slides were coverslipped using aqueous mounting media, (R&D Systems), signals were visualized with a Carl Zeiss Axioskop 2.0 microscope, images were processed using Photoshop CS4 (Adobe) and ImageJ (http://rsbweb.nih.gov/ij/) and quantified using Graphpad Prism.

Supplementary Figure Legends

Supplementary Figure S1. (A) Examples of in-line fused activin and BMP receptor ectodomains as single-chain bivalent traps against TGF-β-family growth factors. The point of fusion is indicated (slash). (B) Energy-minimized molecular models for (ActRIIb)2 and (BMPRIa)2 single-chain traps in complex with activin and BMP-2, respectively. The red dot indicates the point of fusion and grey arrowhead indicates polypeptide chain direction. Structured domains (in magenta) and intervening linker (in black). Each ligand dimer is rendered with its monomers in yellow and orange.

Supplementary Figure S2. (A) The MD solution structure of the (TβRII)2 trap/TGF-β3 complex. Shown are the single-chain trap (tones of magenta) and the ligand dimer with its monomers in tones of yellow and orange. Ten time-averaged structures (each over 1 ns) covering 10 ns timeframe of MD simulation are overlaid. (B) Analysis of the MD simulation of the (TβRII)2/TGF-β3 complex. Per residue root-mean-square (RMS) fluctuations, time-averaged over the last 10 ns of MD simulation. (C) Trap-ligand solvated interaction energy (SIE) calculated over the last 10 ns of MD simulation of the (TβRII)2/TGF-β3 complex. Molecular dynamics simulation was performed as described in Supplementary Methods.

Supplementary Figure S3. TGF-β1 neutralization using a (TβRII)2 trap having either 35 amino acid natural (SEEYNTSNPDIPPHVQKSVNNDMIVTDNNGAVKFP) or artificial linker sequence (GGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGG).

Supplementary Figure S4. Assessment of the effects of TGF-β and (TβRII)2 trap on 4T1 cells *in vitro*. (A) 4T1 cells treated *in vitro* with TGF-β1, -β2 or -β3 are not growth inhibited, as determined by [^{3}H]-TdR incorporation assay. (B) Relative quantities (RQ) of PAI-1 transcript (TGF-β response marker) in 4T1 cells treated *in vitro* with TGF-β1, -β2 or -β3 and (TβRII)2 trap or 1D11, as determined by Q-PCR. (C) (TβRII)2 trap and 1D11 inhibition of TGF-β mediated Smad-2 phosphorylation in 4T1 cells. Shown are western blots of lysates from cells treated with TGF-β1, -β2 or -β3 in the absence or presence of 1D11 or trap. The blots were probed with either phospho-Smad2 (Smad2-P) or Smad2 antibody, as indicated on the left.

Supplementary Figure S5. Quantification of proliferating cells in 4T1 tumors identified through Ki67 staining.

Supplementary Figure S6. Improved neutralization of BMP-2 using single-chain bivalent trap (BMPRIa)2, compared to monovalent receptor BMPR1a-ED. Neutralization of the BMP-2 was assayed in C2C12BRA cells stably transfected with a BMP-responsive luciferase reporter.

Supplementary Table S1. Structured/unstructured(IDR) sequences in the extracellular regions of TGF-β-superfamily receptors.

Human Receptor (SwissProt ID)	**Full-length ectodomain sequence** [a]	**Reference Structure** (PDB ID)
TβRII (P37173-1)	IPPHVQKSVNNDMIVTDNNGAVKFP**QLCKFCDVRFSTCD NQKSCMSNCSITSICEKPQEVCVAVWRKNDENITLETVC HDPKLPYHDFILEDAASPKCIMKEKKKPGETFFMCSCSS DECNDNIIF**SEEYNTSNPD	1KTZ 2PJY 1M9Z 1PLO
TβRIIb [b] (P37173-2)	IPPHVQKS[*DVEMEAQKDEIICPSCNRTAHPLRHI*]NNDMIVTD NNGAVKFP**QLCKFCDVRFSTCDNQKSCMSNCSITSICEK PQEVCVAVWRKNDENITLETVCHDPKLPYHDFILEDAA SPKCIMKEKKKPGETFFMCSCSSDECNDNIIF**SEEYNTSN PD	1KTZ 2PJY 1M9Z 1PLO
BMPRIa (P36894)	QNLDSMLHGTGMKSDSDQKKSENGVTLAPED**TLPFLKCY CSGHCPDDAINNTCITNGHCFAIIEEDDQGETTLASGCM KYEGSDFQCKDSPKAQLRRTIECCRTNLCNQYLQPTLP**P VVIGPFFDGSIR	2GOO 1ES7
ActRIIa (P27037)	AILGRSE**TQECLFFNANWEKDRTNQTGVEPCYGDKDKR RHCFATWKNISGSIEIVKQGCWLDDINCYDRTDCVEKK DSPEVYFCCCEGNMCNEKFSYFP**EMEVTQPTSNPVTPKPP YYNI	2GOO 1LX5
ActRIIb (Q13705)	SGRGEAET**RECIYYNANWELERTNQSGLERCEGEQDKR LHCYASWRNSSGTIELVKKGCWLDDFNCYDRQECVAT EENPQVYFCCCEGNFCNERFTHLP**EAGGPEVTYEPPPTAP T	1S4Y 1NYU

[a] Structured (folded) ligand-binding domains are underlined and in bold; the rest of the sequences represent unstructured regions.

[b] For the TβRIIb isoform, the 25 a.a. insertion relative to TβRII is indicated by italics within brackets.

Supplementary Table S2. Linker characteristics for designed single-chain traps of TGF-β-family growth factors.

Single-chain trap	Targeted ligand(s)	Receptor ectodomain	Reference structures (PDB ID)	Linear distance (Å) for linkage	Residues in natural linker	Minimum residues required for linkage [a)]
(TβRII)2	TGF-β1 TGF-β3	TβRII-ED	2PJY 1KTZ 1PLO 1M9Z	80	35	32
(TβRIIb)2	TGF-β1 TGF-β3	TβRIIb-ED	2PJY 1KTZ 1PLO 1M9Z	80	60	32
(BMPRIa)2	BMP-2	BMPRIa-ED	2GOO 1ES7	60	41	24
(ActRIIa)2	BMP-7	ActRIIa-ED	2GOO 1LX5	70	28	28
(ActRIIb)2	Activin Myostatin	ActRIIb-ED	1S4Y 1NYU	45, 50 [b)]	25	18

[a)] Minimum number of residues required for linkage represents the structure-based linear distance for linkage (Å) divided by a factor of 2.5. The 2.5 factor is based on the Cα-Cα extent of fully extended linkers, which peaks at 3.0 Å (16), minus an average tolerance of 0.5 Å per amino acid residue to allow for deviations of the linker path from linearity.

[b)] The two values (45 Å and 50 Å) of the linear distance for linkage reflect activin flexibility and are based on two activin-ActRIIb-ED reference crystal structures (1S4Y and 1NYU, respectively).

Supplementary Table S3. Binding IC50s (nM) determined from competitive SPR analysis of trap binding to TGF-β in solution.

Trap	Binding IC50 for TGF-β3	Binding IC50 for TGF-β2
(TβRII)2	0.114 (0.096-0.134) [a)]	> 100
(TβRIIb)2	0.347 (0.278-0.432)	> 100
TβRII-Fc	0.218 (0.173-0.275)	> 100
TβRII-ED	2.724 (2.339-3.172)	> 1000
1D11 antibody	0.438 (0.356-0.538)	1.75 (0.587-5.219)

[a)] 95% confidence interval in brackets.

Supplementary Table S4. Trap IC50s (nM) determined from TGF-β neutralization curves.

Trap	IC50 for TGF-β1	IC50 for TGF-β2	IC50 for TGF-β3
(TβRII)2	1.359 (0.459, n=3) [a)]	No neutralization	0.336 (0.125, n=5)
(TβRIIb)2	0.098 (0.021, n=4)	No neutralization	0.045 (0.012, n=3)
TβRII-Fc	0.506 (0.128, n=4)	No neutralization	0.323 (0.067, n=3)
TβRII-ED	> 100	No neutralization	> 100
1D11 antibody	1.429 (0.676, n=4)	8.674 (0.303, n=2)	0.029 (0.022, n=2)

[a)] SEM for n experiments, each performed with triplicate samples.

Supplementary Table S5. Primer sets used in Q-PCR for the detection of 28S RNA, PAI-1 and Rae-1γ transcripts in 4T1 tumors and cells.

Transcript	Reverse primer	Forward primer
28S RNA	AGTTCTTTTCAACTTTCCCT	GGGTGGTAAACTCCATCTAA
PAI-1	GCATTCACCAGCACCAGGCGTG	GGTGAAACAGGTGGACTTCTCA
Rae-1γ	AGGTCCCCATCATCGTTCCAT	TGATTTATCCGCAAAGCCAGG

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