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(54) Title: ANTAGONISTS OF LIGANDS AND USES THEREOF

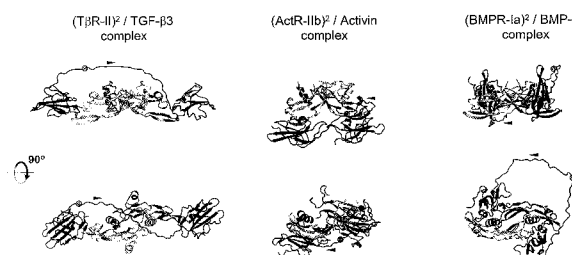


Figure 4. Feasibility of trap constructs with natural linkers from three-dimensional structural models. Shown are molecular mechanics energy-minimized natural linkers for (TβR-II)², (ActR-IIb)² and (BMPR-IIa)² homo-bivalent single-chain traps in complex with the TGF-β3, Activin and BMP-2 growth factors, respectively. Each growth factor covalent dimer is rendered in black, and consists of two folded binding domains and an intervening unstructured linker. Each single-chain trap is rendered in gray, and consists of two folded binding domains and an intervening unstructured linker. Each dot indicates the point of fusion in the linker region between two receptor ectodomains to generate the single-chain trap. Arrowheads indicate polypeptide chain direction in the trap's linker. Two 90°-rotated views are provided for each complex.

(57) Abstract: The invention provides multivalent ligand binding agents (traps) for members of the TGF-β superfamily and polypeptide linkers and methods for making and using such constructs. In an embodiment of the invention there is provided a multivalent binding agent with affinity for a member of the TGF-β superfamily, said agent comprising the general structure I: (<bd1>-linker1)_k-[(<bd1>-linker2-<bd2>-linker3)_n-(<bd3>)_m-(linker4-<bd4>)]_h, where: - n and h are independently greater than or equal to 1; - d, f, m and k are independently equal to or greater than zero; - bd1, bd2, bd3 and bd4 are polypeptide binding domains having an affinity for the same member of the TGF-β superfamily, with bd1, bd2, bd3, and bd4 being independently the same or different from each other; and, -linker1, linker2, linker3 and linker4 are unstructured polypeptide sequences; wherein the number of amino acids in each linker is determined independently and is greater than or equal to X/2.5; where, X equals the shortest linear distance between: (a) the C-terminus of an isolated form of the binding domain that is located at the N-terminus of the linker and that is specifically bound to its ligand; and, (b) the N-terminus of an isolated form of the binding domain that is located at the C-terminus of the linker and that is specifically bound to its ligand.



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Title: Antagonists of Ligands and Uses Thereof

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Field of Invention

The invention relates to the field of antagonists and, more specifically, to polypeptide antagonists capable of use as single chain multivalent ligand traps.

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Background of Invention

Many undesirable biological processes occur via ligand binding to cell surface receptors. Thus, it is sometimes desirable to have compounds and methods to reduce or modulate such binding.

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The TGF- β superfamily includes a number of ligands of biological significance.

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TGF- β and Activin play critical pathogenic roles in many diseases including the progression of cancer and uncontrolled fibrosis and scarring of tissues, e.g. kidney, lung and liver fibrotic diseases. Furthermore, Myostatin/GDF8 is another ligand which is related to Activin and which shares binding to the same Type II receptor (ActivinRIIb). Myostatin is a powerful inhibitor of skeletal muscle growth and is a validated therapeutic target for muscle wasting diseases such as muscular dystrophy. Bone morphogenetic proteins (BMP), which are other ligands in the TGF- β family, have been implicated in cardiovascular diseases. For example, high levels of both BMP2 and BMP4 have been found in calcified atherosclerotic plaques and diseased aortic valves.

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Principal agents that target these ligands are ligand traps/antagonists that bind and sequester ligand. Two examples are: 1) anti-ligand antibodies and 2) soluble receptor ectodomains.

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Efforts have been made to identify methods to reduce ligand binding by trapping ligand and preventing its interaction with the cell surface receptors. Inhibition of certain ligands has been reported using anti-ligand antibodies that trap and neutralize the ligand directly. For therapeutic and diagnostic applications, however, antibodies are problematic, particularly due to issues arising from their immunogenicity (and the

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danger of adverse immune response in patients) and their large size (restricting their ability to reach targets outside the bloodstream).

Soluble versions of receptor ectodomains antagonize ligands directly by binding to them and preventing them from interacting with cell surface receptors. In the case of TGF- β ,
5 in animal models, expression of a TGF- β receptor type II (T β RII) ectodomain (ED) partially restored host immunity and promoted tumor clearance, indicating that receptor ectodomain-mediated neutralization of TGF- β inhibits tumor progression. It has been shown, however, that the efficacy of monovalent T β RII to antagonize TGF- β is less than
10 could be desired. Attempts to overcome this led to the production of an artificially dimerized form of versions of T β RII-ED, dimerized, via fusion to either coiled-coil domains or the Fc domain of IgG. This dimerization improved the antagonist effect.

Bivalent receptor-based traps/neutralizers that antagonize multimeric ligand activity
15 have the potential to act as therapeutic or diagnostic (imaging or non-imaging) agents for diseases/disorders caused by over-production/activity of the target ligand. It has been demonstrated that non-covalent dimerization of T β RII-ED (for example, via fusion to heterodimerizing coil strands (coiled-coil T β RII-ED)), greatly enhances the antagonist potency of T β RII-ED (De Crescenzo et al., 2004, J. Biol. Chem. 279: 26013).
20 A significant disadvantage of the coiled-coil fused dimer is that the non-covalent nature of the dimerization domain limits its potency, i.e. it dissociates at low concentrations such that a large portion of the coil-fused receptor ectodomain will be acting as a monomer rather than a dimer. Use of the Fc domain of IgG provides a covalent interaction, but at the cost of large size and increased probability of immunogenicity.

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Brief Description of the Drawings

Figure 1A. Depicts embodiments of amino-acid sequences corresponding to intrinsically unstructured regions in the extracellular portions of select TGF- β -
30 superfamily receptors.

Figure 1B. Depicts embodiments of amino-acid sequences corresponding to structured ligand-binding domain regions in the extracellular portions of select TGF- β -superfamily receptors.

Figure 2A. Depicts examples of embodiments of in-line fused receptor ectodomains as homo-bivalent single-chain traps of several TGF- β -family growth factors. The “/” sign indicates the point of fusion.

Figure 2B. Depicts examples of sequences corresponding to natural linkers of
5 embodiments of homo-bivalent single-chain traps resulting from fusion of the entire extracellular portions of select TGF- β -superfamily receptors.

Figure 2C. Depicts examples of sequences corresponding to embodiments of artificial linkers for homo-bivalent single-chain traps at varying sequence identity to natural linker sequences.

10 **Figure 2D.** Depicts examples of sequences corresponding to varying the linker length for embodiments of homo-bivalent single-chain traps by deleting or repeating of natural sequences, or by inserting of artificial sequences, into the natural linker sequence.

Figure 3. Depicts an illustration of an embodiment of the (T β R-II)² single-chain trap construct on a three-dimensional molecular mechanical model of the (T β R-II)² single-chain trap bound to the TGF- β 3 growth factor. Two 90°-rotated views are provided.
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Figure 4. Depicts diagrams relating to the feasibility of specific embodiments of trap constructs with natural linkers from three-dimensional structural models. Shown are molecular mechanics energy-minimized natural linkers for embodiments of (T β R-II)₂, (ActR-IIb)₂ and (BMPR-Ia)₂ homo-bivalent single-chain traps in complex with the TGF- β 3, Activin and BMP-2 growth factors, respectively. Each growth factor covalent dimer is rendered in gray. Each single-chain trap is rendered in black, and consists of two folded binding domains and in intervening unstructured linker. Each dot indicates the point of fusion in the linker region between two receptor ectodomains to generate the single-chain trap. Arrowheads indicate polypeptide chain direction in the trap's linker. Two 90°-rotated views are provided for each complex.
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Figure 5A. Depicts molecular dynamics (MD) model for an embodiment of the (T β R-II)₂ homo-bivalent single-chain trap bound to the TGF- β 3 growth factor (right images). An initial model with energy-minimized linker and with ligand-binding domains in crystallographic positions bound onto the growth factor is also shown for reference (left images, see also Figs. 3 and 4). The single-chain trap is rendered in black and the growth factor covalent dimer is rendered in gray. Ten time-averaged structures (each over 1 ns) covering 10 ns timeframe of MD simulation are overlaid. Two 90°-rotated views are provided
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Figure 5B. is a graphical representation of per residue root-mean-square (RMS) fluctuations of an embodiment of the $(T\beta RII)^2$ / TGF- β 3 complex, time-averaged over the last 10 ns of MD simulation.

Figure 5C. is a graphical representation of solvated Interaction Energy (SIE) between
5 an embodiment of a single-chain $(T\beta RII)^2$ trap and the TGF- β 3 ligand over the last 10 ns of MD simulation of their complex, with an average value of -25.4 kcal/mol.

Figure 6. Depicts a schematic of embodiments of prototype $(T\beta RII)^2$ and modified N-His $(T\beta RII)^2$ traps.

Figure 7A. Depicts surface plasmon resonance (SPR)-based biosensor (Biacore™)
10 sensograms showing an embodiment of a prototype $(T\beta RII)^2$ (in diluted conditioned media from different % transfections) binding to surface-immobilized TGF- β 3 ligand.

Figure 7B. Depicts surface plasmon resonance sensograms comparing binding of embodiments of bivalent prototype $(T\beta RII)^2$, bivalent $T\beta RII$ -Fc and monovalent $T\beta RII$ to 270 RUs surface-immobilized TGF- β 3 ligand.

Figure 8. Is a photographic depiction of a gel showing high level production and
15 purification yield of an embodiment of N-His $(T\beta RII)^2$ protein from 500 ml culture of transfected 293 cells.

Figure 9A. Is a graphical depiction of inhibition of TGF- β signaling in Mv1Lu luciferase reporter cells by an embodiment of prototype $(T\beta RII)^2$ compared to $T\beta RII$ -Fc.

Figure 9B. Is a graphical depiction of SPR-based determination of trap binding of TGF- β
20 β in solution by an embodiment of prototype $(T\beta RII)^2$ and $T\beta RII$ -Fc compared to monomeric $T\beta RII$ -ED.

Figure 9C. Is a graphical depiction of inhibition of TGF β 1-induced 4T1 cell invasion *in vitro* by an embodiment of prototype $(T\beta RII)^2$ and $T\beta RII$ -Fc traps.

Figure 10A. Is a Biacore™ sensogram showing direct binding of embodiments of N-His
25 $(T\beta RII)^2$ and monomeric N-His $T\beta RII$ to different isoforms of TGF- β .

Figure 10B. Is a graphical depiction of a Biacore™ comparison of performance of
embodiments of 100 nM N-His $(T\beta RII)^2$ and $T\beta RII$ -Fc to bind to 500 RUs each of TGF- β 1 or TGF- β 3

Figure 10C. Is a graphical depiction of SPR-based determination of IC50 for trap
30 binding to TGF- β 1 (5 nM) in solution. The graph shows efficient binding of TGF- β 1 by an embodiment of a N-His $(T\beta RII)^2$ trap and $T\beta RII$ -Fc trap versus reduced binding by monomeric $T\beta RII$ (293 cell-produced or E.coli-produced).

Figure 10D. Is a graphical depiction showing efficient inhibition of TGF- β signaling in Mv1Lu luciferase reporter cells by an embodiment of N-His (T β RII)² and T β RII-Fc compared to poor inhibition by monomeric T β RII (293 cell-produced and E-Coli-produced).

- 5 **Figure 11.** (A) is a photographic depiction and (B) is a graphical depiction of results showing that an embodiment of N-His (T β RII)² exhibits long-term stability and activity in 10% serum at 37°C

- Figure 12.** Provides graphical depictions showing efficient neutralization of TGF- β 1 (A) and binding of TGF- β 1 in solution (B) by an embodiment of a (T β RII)² trap (ligand binding agent) having a 60 amino acid linker.
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Figure 13. Is a graphical depiction showing efficient inhibition of Myostatin signaling in A204 cells by an embodiment of an (ActRIIB)² trap (ligand binding agent) compared to the less potent inhibition of ActRIIB-Fc and monomeric ActRIIB.

- Figure 14.** Is a graphical depiction of results showing that an embodiment of a bivalent (BMPR1a)² trap (ligand binding agent) is more potent than monovalent BMPR1a trap for neutralization of BMP2.
- 15

Figure 15A. Provides schematic diagrams exemplifying embodiments of in-line fusions of receptor ectodomains leading to embodiments of heterovalent single-chain traps of TGF- β -superfamily growth factors.

- 20 **Figure 15B parts 1 and 2.** Depict embodiments of amino-acid sequences exemplifying embodiments of heterovalent single-chain traps (ligand binding agents) of TGF- β -superfamily growth factors, and corresponding to the domain organization diagrams depicted in Figure15A.

25 **Summary of Invention**

- The invention relates to ligand binding agents capable of permitting modulation of cellular response to members of the TGF- β superfamily by binding one or more members of the TGF- β superfamily and preventing interaction with cellular receptors, and methods of
- 30 designing and using such agents. The ligand binding agents taught herein are preferably single chain multivalent ligand binding agents. However, it would be possible to link such single-chain constructs to other uni- or multivalent molecules and/or to combine two or more such single chain traps using multimerization domains known in the art (e.g. coiled-

coil domains, Fc domains, pentabodies) to form a multimeric trap if so desired and any such trap having a multivalent single chain portion falls within the scope of the present invention.

In an embodiment of the invention there is provided methods and processes to engineer multivalent receptor ectodomains using a single-chain approach.

The ligand binding agents of the invention are preferably multivalent ligand traps, having at least two binding domains (bd) which recognize different sites on (or the same site of different portions of) the same member of the TGF- β superfamily. The binding domains may be modified, for example to facilitate purification, so long as such modifications do not reduce binding affinity to unacceptable levels.

The binding domains (bd) of the ligand traps are preferably joined by a flexible polypeptide linker region. This linker should preferably include an unstructured amino acid sequence which in some embodiments is either the same as or derived from conservative modifications to the sequence of a natural unstructured region in the extracellular portion of the receptor for the ligand of interest or another receptor in the TGF- β superfamily. In other instances, such linkers may be entirely artificial in composition and origin but will contain amino acids selected to provide an unstructured flexible linker with a low likelihood of encountering electrostatic or steric hindrance complications when brought into close proximity to the ligand of interest.

In some instances, the linker will include regions to facilitate purification (e.g. His tags) or to facilitate the addition of cargo or accessory molecules. When such additions affect the unstructured nature of the linker or introduce potential electrostatic or steric concerns, appropriate increases to the linker length will be made to ensure that the two binding domains are able to bind their respective sites on the ligand. In light of the methods and teachings herein, such determinations could be made routinely by one skilled in the art.

In an embodiment of the invention there are provided ligand traps having the general Structure I:

$$(<\text{bd}1>-\text{linker}1)_k-[\{<\text{bd}1>-\text{linker}2-<\text{bd}2>-\text{linker}3-\}^n-(<\text{bd}3>)_m-(\text{linker}4-<\text{bd}4>)_d]_h,$$

where:

- n and h are independently greater than or equal to 1;
- d, f, m and k are independently equal to or greater than zero;

- bd1, bd2, bd3 and bd4 are polypeptide binding domains having an affinity for the same member of the TGF- β superfamily, with bd1, bd2, bd3, and bd4 being independently the same or different from each other; and,

-linker1, linker2, linker3 and linker4 are unstructured polypeptide sequences;

- 5 wherein the number of amino acids in each linker is determined independently and is greater than or equal to $X/2.5$; where,

X equals the shortest linear distance between:

- (a) the C-terminus of an isolated form of the binding domain that is located at the N-terminus of the linker and that is specifically bound to its ligand; and,
 10 (b) the N-terminus of an isolated form of the binding domain that is located at the C-terminus of the linker and that is specifically bound to its ligand.

As used herein "an isolated form" of a binding domain is a form of that binding domain acting as a monovalent monomer.

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Subject to the constraints described herein, linkers 1, 2, 3, and 4 may be the same or different. In certain embodiments the linker is between 25 and 60 amino acids in length. Also provided are nucleic acid sequences encoding such ligand traps.

- 20 Depending on the values selected for d, f, h, k, m, and n, the ligand trap structure may comprise a large number of repeating units in various combinations or may be a relatively simple structure such as Structure II <bd1>-linker-<bd2>.

- In certain embodiments of the invention, the member of the TGF- β superfamily to which the binding domains (bd) have affinity is selected from the group consisting of: TGF- β 1, TGF- β 2, TGF- β 3, activin β A, activin β B, activin β C, activin β E, bone morphogenic protein (BMP) 2, BMP 3, BMP4, BMP 5, BMP 6, BMP 7, BMP 8, BMP 9, BMP 10, BMP 11, BMP 12, BMP 13, BMP 14, BMP 15, growth differentiation factor (GDF) 1, GDF 3, GDF 8, GDF 9, GDF 15, Nodal, Inhibin α , anti-Mullerian Hormone, Lefty 1, Lefty 2,
 25
 30 artaman, Persephin and Neurturin.

In an embodiment of the invention there is provided a binding agent wherein one or more of bd1, bd2, bd3, and bd4 is selected from one of SEQ ID NO 43-48.

In an embodiment of the invention the binding agent comprises one or more of SEQ ID No 75 to 82.

5 In an embodiment of the invention the binding agent comprises one or more of SEQ ID NO 31-42 or 49-74 as a linker sequence.

The invention also provides a method of designing a multivalent binding agent useful in modulating responsiveness of a cell to a member of the TGF- β superfamily, said method comprising:

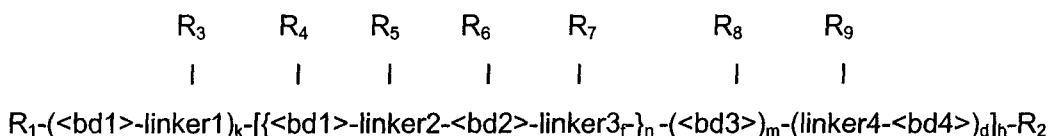
- 10 a) identifying a member of the TGF- β superfamily of interest;
- b) obtaining two polypeptide binding domains having affinity for different sites on the member of the TGF- β superfamily member;
- c) obtaining an unstructured polypeptide linker of at least a number of amino acids equal to $(X/2.5)$ where
- 15 X equals the shortest linear distance between:
 - (i) the C-terminus of an isolated form of the binding domain that is located at the N-terminus of the linker and that is specifically bound to its ligand; and,
 - (ii) the N-terminus of an isolated form of the binding domain that is located at the C-terminus of the linker and that is specifically bound to its ligand; and,
- 20 d) modelling the linker between the binding domains and carrying out molecular dynamics simulations to substantially minimize molecular mechanics energy and reduce steric and electrostatic incompatibility between the linker and the member of the TGF- β superfamily.
- 25 The design method can optionally be expanded to further include a step e) of producing a fusion protein comprising the two polypeptide binding domains joined by the unstructured polypeptide linker.

30 The ligand binding agents disclosed herein are also useful in purification of ligand, for example, by immobilization on an inert matrix on a solid support, on, for example, to nanoparticles to concentrate levels of ligand in a sample.

The invention also provides novel polypeptide sequences useful in a variety of applications. These sequences include SEQ ID NOs 53 to 74. Also provided are nucleic acid sequences encoding these polypeptide sequences.

- 5 Also provided is a method of modulating the response of a cell to TGF- β in its environment, said method comprising exposing the cell to a multivalent ligand trap comprising a ligand binding agent (ligand trap) disclosed herein.

10 In an embodiment of the invention there is provided a binding agent having the general structure V:



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Wherein R_1 , R_2 , R_3 , R_4 , R_5 , R_6 , R_7 , R_8 , R_9 , may be the same or different, may not be present and when present, may independently be one or more of a fusion protein for targeting, a single domain antibody, a radiotherapy agent, an imaging agent, a fluorescent dye, a fluorescent protein tag, a cytotoxic agent for chemotherapy a nano particle-based carrier, a polymer-conjugated to drug, nanocarrier or imaging agent, a stabilizing agent, a drug a nanocarrier and a dendrimer and a support for use in purification or concentration of ligand; and wherein bd1, bd2, bd3, bd4, linker1, linker2, linker3, linker4, k, f, n, m, d, and h are defined as in Structure I. In light of the disclosure herein, one skilled in the art can select suitable R-groups for diagnostic therapeutic or other applications.

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In an embodiment of the invention there is provided an isolated polypeptide having at least 80%, 85%, 90%, 95%, 98%, 99% and 100% sequence identity to a natural unstructured region in the extracellular portion of a receptor for a member of the TGF- β superfamily and being substantially free of structured regions capable of specific binding to a member of the TGF- β superfamily. In some instances, this isolated polypeptide has at least 80%, 85%, 90%, 95 %, 98%, 99 % sequence identity to one or more of SEQ ID NO 31-42 and SEQ ID NOs 49-74.

30

In an embodiment of the invention there is provided a polypeptide comprising a region having at least 80%, 85%, 90%, 95 %, 98%, 99 % sequence identity to one or more of SEQ ID NOs 53-74 and SEQ ID NOs 82-118. In some instances this polypeptide has a region with at least 90 %, 95 %, 98%, 99 % sequence identity to one or more of SEQ ID
5 NOs 53-74.

In an embodiment of the invention there is provided a polypeptide having between 43 % and 99 % sequence identity to a naturally unstructured region in the ectodomain of a receptor for a member of the TGF- β superfamily.
10

In an embodiment of the invention there is provided a nucleic acid sequence encoding a polypeptide disclosed herein.

In an embodiment of the invention there is provided a method of modulating the response of a cell to a TGF- β superfamily member in its environment, said method
15 comprising exposing the cell to a ligand binding agent disclosed herein.

In an embodiment of the invention there is provided a data storage medium comprising instructions for determining the minimum linker length when designing a ligand binding
20 agent.

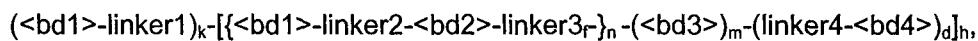
In an embodiment of the invention there is provided a data storage medium comprising a means for identifying acceptable minimal linker length when designing a ligand
binding agent.
25

Linker length will be considered acceptable when it permits binding of binding domains located on each of the N- and C-termini of the linker to bind their natural binding sites on their natural ligand such that, with both binding domains so bound, the ligand is bound with a higher affinity than it would be bound by binding of only one of the binding
30 domains.

Brief Description of the Invention

In an embodiment of the invention there is provided a single-chain non-naturally occurring polypeptide useful as a ligand binding agent. The ligand binding agent comprises structured ligand-binding domains (denoted bd) derived from or based on the extracellular portion of a natural receptor or receptors, joined by one or more polypeptide linkers. The ligand binding agent provides a multivalent binding agent and does not require fusion to any conventional dimerizing or multimerizing moieties such as coiled-coil domains of Fc domains in order to be multivalent.

In an embodiment of the invention, there is provided a multivalent binding agent with affinity for a member of the TGF- β superfamily, said agent comprising the general structure I:



where:

- n and h are independently greater than or equal to 1;
- d, f, m and k are independently equal to or greater than zero;
- bd1, bd2, bd3 and bd4 are polypeptide binding domains having an affinity for the same member of the TGF- β superfamily, with bd1, bd2, bd3, and bd4 being independently the same or different from each other; and,
- linker1, linker2, linker3 and linker4 are unstructured polypeptide sequences; wherein the number of amino acids in each linker is determined independently and is greater than or equal to X/2.5; where,
X equals the shortest linear distance between:
 - (a) the C-terminus of an isolated form of the binding domain that is located at the N-terminus of the linker and that is specifically bound to its ligand; and,
 - (b) the N-terminus of an isolated form of the binding domain that is located at the C-terminus of the linker and that is specifically bound to its ligand.

As used herein "an isolated form" of a binding domain is a form of that binding domain acting as a monovalent monomer.

The length of the linker is considered to be the number of amino acids between:

- (a) the C-terminal main chain carbon atom of the binding domain located at the linker's N-terminal end; and

(b) the N-terminal main-chain nitrogen atom of binding domain located at the linker's C-terminal end.

Non-limiting examples of useful linkers are found in the amino acid sequences in SEQ ID NOs 53 to 74 which should be read conventionally with the N-terminus on the left and the C-terminus on the right, and in corresponding reverse sequences having the same amino acids but wherein the C-terminus is on the left and the N-terminus is on the right as the sequences are written in full. In some embodiments, such reverse sequences will preferably be produced using D-amino acids. Where immunogenicity is of concern, it will generally be desired to screen such reverse sequences for immunogenicity at an early stage. (For examples of reverse sequences, see SEQ ID NOs 82-118). All amino acids sequences in this document are written N-terminus to C-terminus unless otherwise noted. All sequences disclosed herein except SEQ ID NOs 82-118 are disclosed as using L-amino acids and the use of a D-amino acid is considered a variant affecting the percent sequence identity to the sequences as stated.

In an embodiment of the invention, the ligand binding agent has the general Structure II:
 $\text{<bd1>-linker2-<bd2>}$.

In an embodiment of the invention, the ligand binding agent has the general Structure III
 $\text{<bd1>-}(\text{linker2-<bd2>})_n$.

In an embodiment of the invention, the polypeptide has the general Structure IV:
 $([\text{bd1}]-[\text{linker1}]-[\text{bd1}])_f-[\text{linker2}]-([\text{bd2}]-[\text{linker3}]-[\text{bd3}])_g$,

when f and g are greater than or equal to one.

In an embodiment where bd2 and bd3 are the same, and f and g are the same number, this can result in a substantially mirror symmetric structure around linker 2, subject to differences in the linkers. In instances where bd3 is different from bd2 and and/or where f and g are different numbers, different structures will be produced. It is within the capacity of one of ordinary skill in the art to select suitable binding domains, linkers, and repeat frequencies in light of the disclosure herein.

In some instances, the binding domain region of the single-chain polypeptide will be selected for its ability to bind a growth-factor ligand having a covalently-stabilized dimeric quaternary structure, and may be selected from a list of growth factors from within the TGF- β family, e.g., transforming growth factor beta (TGF- β , bone morphogenetic protein (BMP), activin, myostatin, and including their naturally occurring isoforms.

In some instances, the polypeptide is designed to bind simultaneously to equivalent but spatially distinct sites on a multimeric ligand. As used herein "multimeric" includes dimeric, trimeric, and greater numbers of units, and "multivalent" includes bivalent, trivalent, and greater numbers of binding domains.

In some instances, the linker is independently selected to have varying degrees of sequence identity to naturally occurring unstructured amino acid sequences found in the native receptor sequence in the regions flanking the ligand binding domain, for example 70%, 80%, 90%, 95%, 98 %, 99% or 100% sequence identity, whereas for entirely artificial linkers (e.g. poly-Gly or poly-Ser linkers, sequence identity will be even lower. Examples of linker sequences of varying degree of identity to the natural receptor sequence are given in **Fig. 2C**. and SEQ ID NOs 82-118 (Table II).

In some instances, the number of amino acid residues in the linker of either natural or artificial origin is selected to be equal to or greater than the minimum required distance for simultaneous (bridged) binding to two binding sites on the growth factor to be bound by the relevant binding domains. An example of an embodiment of such a determination is given in the section "Feasibility assessment procedure for designed single-chain bivalent traps". Examples of natural and artificial linker sequences of varying length are given in Fig. 2D and SEQ ID NOs 82-118. In some instances, linker length is between 18-80 a.a., 25-60 a.a., 35-45 a.a.

In some instances, the overall molecular mass of bivalent agents disclosed herein before glycosylation is between about 29 kDa and 37 kDa, and the overall mass following typical glycosylation is between about 40 kDa and 60 kDa. Thus, there is provided herein, multivalent ligand traps having a pre-glycosylation size of between about 12 kDa and 19 kDa per binding domain.

The ligand traps disclosed herein will generally have a lower molecular mass than comparable multimeric ligand traps constructed using known multimerization domains.

5 Example of Selected Ligand Trap Sizes

Agent	Predicted for protein	Actual (with glycosylation) based on SDS-PAGE
(T β RII) ²	34kDa	50-60kDa
(T β RIIb) ²	37kDa	50-60kDa
(ActRIIB) ²	30kDa	50-60kDa
(BMPRIa) ²	29kDa	40-50kDa
RIIEcoil+RIIKcoil		37Kd+40kDa = 77kDa
T β RII-Fc		60Kd+60kDa = 120kDa

Polypeptides of the invention can be useful as therapeutic agents that neutralize the action of disease-associated covalently-stabilized dimeric ligands such as growth factors. They may also have commercial potential for use as diagnostic agents to detect the presence of disease-associated covalently-stabilized dimeric ligands such as growth factors in imaging and non-imaging diagnostic applications. They can also be useful in the purification and/or concentration or segregation of ligand *in vitro*.

Detailed Description of Invention

Although the invention is described with reference to specific examples, it will be understood that it is not so limited.

Experiment #1: Design strategy of single-chain bivalent traps for TGF- β -family ligands

1. Single-chain recombinant traps were designed against growth factors that belong to the transforming growth factor TGF- β superfamily of cysteine-knot cytokines according to SCOP (Andreeva et al., 2008, Nucl. Acid Res. 36: D419) and Pfam (Finn et al., 2006,

- Nucl Acid Res. 34: D247) structural classifications. More specifically, these growth factors including, for example, TGF- β s, activins and BMPs, share the same 3D architecture and form covalent disulfide-linked homodimers. The method disclosed herein is applicable to all members of the TGF- β superfamily, including TGF- β 1, - β 2, - β 3; activin β A, β B, β C, β E; bone morphogenetic proteins (BMP) 2-15; growth differentiation factors (GDF) 1, 3, 8 (myostatin), 9 and 15; Nodal; Inhibin α ; anti-Mullerian hormone (AMH); Lefty 1 and 2; Arteman, Persephin and Neurturin.
2. Single-chain recombinant traps against TGF- β superfamily growth-factors were designed from the extracellular portion of their cognate natural receptors. The extracellular segment of all these TGF- β superfamily receptors contain a single structured domain that belongs to the snake-toxin family according to SCOP (Andreeva et al., 2008, Nucl. Acid Res. 36: D419) and Pfam (Finn et al., 2006, Nucl Acid Res. 34: D247) structural classifications. The complete extracellular portion of these receptors typically includes unstructured segments flanking their folded ligand-binding domain. These unstructured extracellular portions were apparent from the experimentally determined 3D structures available from the PDB database (Berman et al., 2000, Nucl. Acid Res. 28: 235), e.g., crystal structures for type II TGF- β receptor ectodomain (Hart et al., 2002 Nat. Struct. Biol. 9: 203; Boesen et al., 2002, Structure 10: 913; Groppe et al., 2008, Mol. Cell 29: 157), type I TGF- β receptor ectodomain (Groppe et al., 2008, Mol. Cell 29:157), type IIa activin receptor ectodomain (Allendorph et al., 2006, Proc. Natl. Acad. Sci. USA 103: 7643), type IIb activin receptor ectodomain (Thompson et al., 2003, EMBO J. 22: 1555; Greenwald et al., 2004, Mol. Cell 15: 485), type I BMP receptor ectodomain (Kirsch et al., 2000, Nat. Struct. Biol. 7: 492), or the NMR structure of the type II TGF- β receptor ectodomain (Deep et al., 2003, Biochemistry 42: 10126)]. In the absence of experimental data, as for example in the case the extracellular region of the IIb splicing variant of the TGF- β type II receptor, unstructured extracellular segments were defined by: (i) sequence portions falling outside of the folded ligand-binding domain boundaries located by comparative analysis against structurally characterized homologs, and (ii) predictions based on knowledge-based algorithms, e.g., DISOPRED (Ward et al., 2004, J. Mol. Biol. 337: 635). Amino acid sequences corresponding to the unstructured (i.e., flexible) and structured (i.e., folded, ligand-

binding domain) regions from the ectodomains of several receptors of TGF- β -superfamily growth factors, are given in **Figs. 1A and 1B**, respectively..

3. Homo-bivalent single-chain recombinant traps hereby designed against TGF- β -superfamily growth factors disclosed herein were designed with regard to the
5 experimentally determined binding mode between TGF- β -family ligands and the extracellular portion of their cognate natural receptors. The ligand-receptor binding mode was provided at atomic level by the high-resolution 3D structures available for several members of the TGF- β -superfamily ligands in complex with their cognate receptor ectodomains. Examples of experimental molecular structures for TGF- β -
10 superfamily-growth-factor/receptor ectodomain complexes include TGF- β 3 bound to T β R-II-ED (Hart et al., 2002 Nat. Struct. Biol. 9: 203), activin bound to ActR-IIb-ED (Thompson et al., 2003, EMBO J. 22: 1555; Greenwald et al., 2004, Mol. Cell 15: 485), BMP-2 bound to type Ia BMP receptor ectodomain BMPR-Ia-ED (Kirsch et al., 2000, Nat. Struct. Biol. 7: 492) and ActR-IIa-ED (Allendorph et al., 2006, Proc. Natl. Acad. Sci.
15 USA 103: 7643), BMP-7 bound to ActR-IIa-ED (Greenwald et al., 2003, Mol. Cell 11: 605). These structures provided the relative spatial orientation between two separate receptor ectodomain chains (molecules) binding simultaneously onto one covalently homodimerized ligand molecule, i.e., 2:1 receptor:ligand stoichiometry. Higher-order ligand-receptor assemblies between a particular TGF- β -superfamily growth factor and
20 ectodomains from different receptor types have also been determined, for example the ternary complexes between TGF- β 3, T β R-II-ED and T β R-I-ED (Groppe et al., 2008, Mol. Cell 29:157) or between BMP-2, ActR-IIa-ED and BMPR-Ia-ED (Allendorph et al., 2006, Proc. Natl. Acad. Sci. USA 103: 7643). These structures provide the relative spatial orientation between four separate receptor ectodomain chains (molecules)
25 binding simultaneously onto one covalently homodimerized ligand molecule, i.e., 2:2:1 high-affinity-receptor:low-affinity-receptor:ligand stoichiometry. Such structures were used as guides to design hetero-bivalent, hetero-trivalent and hetero-tetravalent single-chain traps of TGF- β -superfamily growth factors and are useful in designing single-chain traps for other suitable ligands of interest involving the TGF- β superfamily.

30

4. Homo-bivalent single-chain traps of TGF-B-family ligands were therefore designed as unnatural fusion proteins consisting of the sequence (excluding the signal peptide) of the natural extracellular portion of the receptor repeated twice. **Fig. 2A** presents schematically homo-bivalent single-chain traps with natural linkers for three TGF-B-

family ligands, where structured and unstructured regions are based on experimental data for single-domain extracellular portions, as presented in **Figs. 1A and 1B**. This resulted in constructs with two structured domains for binding to select TGF- β -superfamily ligand(s), spaced by an unstructured flexible linker formed by fusing the
5 unstructured C-terminus of the first domain to the unstructured N-terminus of the second domain. The natural linker was also progressively substituted by artificial sequences as well as varied in length (**Figs. 2B-D**). From thermodynamic and kinetic considerations, it was expected that divalent receptor ectodomains would provide increased ligand-binding affinities and slower ligand-dissociation rates relative to single-
10 domain receptor ectodomains.

Experiment #2: Feasibility assessment procedure for designed single-chain bivalent traps

15 To the extent to which the structures of various TGF- β -superfamily growth factors are conserved, the structures of their cognate receptor ectodomains are conserved, and the 2:1 receptor-ligand binding stoichiometry is conserved, the concept of fusing two natural receptor ectodomain sequences to produce single-chain homo-bivalent traps with improved *in vitro* ligand binding affinity and cellular ligand neutralizing activity relative to
20 respective monovalent receptor ectodomains, is applicable to the entire family of TGF- β family. The feasibility of these ligand traps can be theoretically assessed routinely by following the stepwise procedure outlined below. Although the procedure is presented for homo-bivalent single-chain traps, it also applies to other designs covered here, e.g., hetero-bivalent and hetero-
25 tetravalent single-chain traps.

1. The linear distance is measured between the C-terminal main-chain carbon atom of one domain and the N-terminal main-chain nitrogen atom of the other domain when bound to the covalently-dimerized ligand. Alternate structures of the complex reflecting
30 internal geometrical flexibility in the homodimerization mode of the disulfide-stabilized ligand when bound to the receptor ectodomains, as reported in several cases (Greenwald et al., 2004,

Mol. Cell 15: 485), can be included in the design process. A computer hardware equipped with commercial/public software appropriate for manipulating molecular structures on an available graphics device can be routinely employed to this end.

- 5 2. The linear distance (in Å units, $1 \text{ Å} = 10^{-10} \text{ m}$) is divided by a factor of 2.5 to calculate the minimum number of amino acid residues that the flexible linker should possess (Table 1) in order to allow simultaneous binding of the folded domains to their binding sites on the homodimeric ligand. The 2.5 factor is based on the C α -C α extent of fully extended linkers, which peaks at 3.0 Å (George and Heringa, 2002, Protein Eng. 15: 871), minus an average tolerance of 0.5 Å per amino acid residue to allow for deviations of the linker path from linearity.
- 10 (Table 1. Linker characteristics for select examples of single-chain traps of TGF- β -family growth factors. Minimum number of residues required for linkage represents the structure-based linear distance for linkage (Å) divided by a factor of 2.5.)
- 15 3. The number of amino acid residues in the unstructured linker portion of the bivalent single-chain trap should be at least equal to the estimated minimum number of linker residues required. Receptor isoforms that differ in the length of the extracellular unstructured segments, such as the TGF- β receptor isoforms II and IIb (Fig. 2B), can be included in the design process. The natural sequence-based linker can also be shortened up to the estimated minimum number of amino acid residues without significantly impairing the ligand binding affinity and neutralizing activity of the trap. A preferable location for shortening the unstructured linker is from the point of fusion (see Fig. 3) in either or both directions relative to the amino acid sequence. Example of shortened natural linkers that can be utilized in single-chain trap design are given in Fig. 2D. As listed in Table 1, the required minimal length of the linker varies between various single-chain traps of TGF- β -superfamily growth factors. An upper limit for the length of the unstructured linker is not defined. Hence, ligand binding agent (trap) constructs with linkers comprising unstructured sequence segments repeated in whole or in part are envisioned to comply with bivalent design and preserve the desired characteristics of the trap. The natural linker can be progressively substituted by artificial sequences, which may or may not result in different linker lengths. Examples of linkers longer than the natural linker designed by repeating of natural sequence or by introducing of artificial sequence are given in Fig. 2D.
- 20
- 25
- 30

4. Finally, atomic-level theoretical analysis is to be carried out, where the linker is modeled between the structured domains and the molecular structure of the trap-ligand complex is refined by minimizing the molecular mechanics energy and by carrying out
- 5 molecular dynamics simulations (Cornell et al., 1995, J. Am. Chem. Soc. 117: 5179). This may, in some cases, highlight regions of steric and/or electrostatic incompatibility between the trap's linker and the growth-factor, and suggest that the length and/or composition of the linker may be incompatible with the bivalent design, even if the linker complies with the minimum number of amino acids requirement as per step (3.) above.
- 10 If the linker can be accommodated without affecting the simultaneous binding of the structured domains to their binding sites on the ligand, then the trap construct is deemed feasible for the proposed application. Computer hardware equipped with commercial/public software appropriate for manipulating molecular structures on an available graphics device, and for performing energy calculation and simulation based
- 15 on molecular mechanics force fields, e.g., the AMBER force field (Cornell et al., 1995, J. Am. Chem. Soc. 117: 5179), can be routinely employed by one skilled in the art in order to carry out this structural modeling analysis. A detailed molecular modeling analysis of the $(T\beta R-II)^2$ homo-bivalent single-chain trap is provided as an example in the following section and includes molecular dynamics simulation. Examples of molecular mechanics
- 20 energy-refined models of three single-chain homo-bivalent traps: $(T\beta R-II)^2$, $(ActR-IIb)^2$ and $(BMPR-Ia)^2$, bound to their respective growth factors are shown in **Figs. 3 and 4**. These atomic-level models represent starting points for further computer-based optimization of linker composition and length.
- 25 This process is explained in greater detail in the example below:

Modeling Example A, Experiment #2

- i. In one example, the atomic-level solution structure of the single-chain homo-bivalent trap $(T\beta R-II)^2$ was simulated in complex with the growth factor TGF- $\beta 3$.
- 30

The starting point for molecular design of the $(T\beta R-II)^2$ trap was the 2.15 Å-resolution crystal structure of the disulfide-linked dimeric human TGF- $\beta 3$ complexed with two TGF- β type II receptor ectodomains (Hart et al., 2002 Nat. Struct. Biol. 9: 203), deposited in the Protein Data Bank (Berman et al., 2000, Nucl. Acid Res. 28: 235) under

the code 1KTZ. Because this structure displays the growth factor in a non-canonical conformation probably due to the low pH used in the crystallization conditions (Hart et al., 2002 Nat. Struct. Biol. 9: 203; Groppe et al., 2008, Mol. Cell 29:157), the binary complex structure was first reconstructed with the ligand in canonical conformation as reported previously (Hinck et al., 1996, Biochemistry 35: 8517; Mittl et al., 1996, Protein Sci. 5: 1261), which was also recently confirmed by the ternary structure of TGF- β ligand-receptor assembly (Groppe et al., 2008, Mol. Cell 29:157). An initial 3D molecular model of the (T β R-II)² trap incorporating an inter-domain natural linker of 35 amino-acid residues (as per sequence listed in **Fig. 2B**) was constructed from standard geometries followed by conjugate-gradient energy minimization of the molecular mechanics force field energy using an AMBER all-atom force field (Cornell et al., 1995, J. Am. Chem. Soc. 117: 5179) and the AMBER 9 suite of programs (Case et al., 2005, J Comput. Chem. 26: 1668). During energy minimizations, only the linker regions of the traps were allowed to move, while the coordinates of the growth factors and of the folded domains of the traps were fixed. The resulting 3D molecular model of the homo-bivalent single-chain trap (T β RII)² bound to TGF- β 3 is depicted in **Fig. 4**.

This initial model of the complex was used as input for molecular dynamics (MD) simulation carried out together with AMBER FF03 force field (Duan et al., 2003, J. Comput. Chem. 24: 1999; Lee & Duan, 2004, Proteins 55: 620) within the AMBER 9 suite of programs (Case et al., 2005, J. Comput. Chem. 26: 1668). The molecular system consisting of 245 amino-acid residues from the single-chain trap (from the full-length (T β R-II)² trap with 272 amino-acid residues, 21 unstructured residues from the N-terminus and 6 flexible residues from the C-terminus were not included in the MD simulation), 224 amino-acid residues of the TGF- β 3 dimer and 14 Na⁺ counterions (added to maintain electroneutrality) was solvated in rectangular water box using the Xleap program in the AMBER 9 software. The distance between the wall of the box and the closest atom of the solute was 12.0 Å, and the closest distance between the solute and solvent atoms was 0.8 Å. 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 over 25 ps in the isothermal-isobaric ensemble (NPT) simulation. The harmonic restraints were then gradually reduced to zero with four rounds of 25 ps NPT simulations. After additional 25 ps simulation, a 15 ns production run was obtained with snapshots collected every 1 ps.

For all simulations, 2 fs time-step and 9 Å non-bonded cutoff were used. The Particle Mesh Ewald method (Darden et al., 1993, J. Chem. Phys. 98: 10089) was used to treat long-range electrostatics, and bond lengths involving bonds to hydrogen atoms were constrained by SHAKE (Ryckaert et al. 1977, J. Compt. Phys. 23: 327). No other
5 constraints were imposed during the MD simulation.

As seen from **Fig. 5A**, the single-chain trap (TβR-II)² bound to TGF-β3 attains a stable MD solution structure that preserves the simultaneous binding of the two ligand-binding domains onto the dimeric growth factor as observed in the crystal structure of
10 unlinked receptor ectodomains (Hart et al., 2002 Nat. Struct. Biol. 9: 203, Groppe et al., 2008, Mol. Cell 29:157). This substantiates the feasibility of the designed single-chain TGF-β trap in terms of the length of the linker. The MD analysis also reveals that in the complex, the linker region of the single-chain trap becomes relatively rigid, with only 6 residues experiencing greater mobility (expressed as per-residue and time-averaged root-mean-square fluctuations) than the rest of the trap's amino acid residues (**Fig. 5B**).
15 In addition, the single-chain trap established favorable interaction with the growth factor, as evaluated by the solvated interaction energy function for scoring protein-ligand binding affinity (SIE) (Naim et al., 2007, J. Chem. Inf. Model. 47: 122). A highly favorable SIE value of -25.4 kcal/mol was calculated as an average over the last 10 ns of MD simulation (**Fig. 5C**). This further indicates the feasibility of the employed natural
20 linker in terms of amino acid composition, that is, there were no significant unfavorable steric and electrostatic contacts predicted between the trap's linker and the growth factor.

25 ii. In one example, structure-based design leads to a divalent molecule consisting of two human TβRII ectodomains that are fused in tandem into a single polypeptide chain (schematically shown in **Fig. 6**). In this construct, an intervening linker sequence is formed from the unstructured natural C-terminal sequence of one ectodomain (black, 10 residues) and the unstructured natural N-terminal sequence of the other ectodomain
30 (white, 25 residues). This linker bridges between the two structured TGF-β-binding domains. This TGF-β trap is hereby named prototype (TβRII)². The construct also contains an N-terminal myc tag and C-terminal 6xHis tag for ease of detection and protein purification. In the prototype (TβRII)² the native IPP sequence is replaced by GGR within the linker due to a NotI restriction site inserted during construction of the

(T β RII)² gene. Also shown is another construct with a 35 amino acid residues linker with native IPP restored, and having a N-terminal His tag. This construct is termed "modified N-His" (T β RII)² and features a native linker sequence. Predicted molecular models of (T β RII)² bound to TGF- β are given

5 Figs. 3-5.

Experiment #3: Small scale production of prototype (T β RII)² and demonstration of TGF- β -binding activity

10 Fig. 6 shows a schematic of prototype (T β RII)². The prototype (T β RII)² gene was cloned into mammalian expression vector pTT and increasing amounts were transiently transfected into HEK293 cells. The conditioned media from these transfected cells were collected after 5 days and tested via SPR Biacore analysis for the binding of secreted (T β RII)² to a TGF- β 3 surface (Fig. 7A). The sensogram shows increasing levels of
15 binding that correlates with cells transfected with increasing levels of (T β RII)² plasmid (ranging from 1% to 95% transfected cells), indicating a dosage effect and specific binding. The binding characteristics of (T β RII)² (produced from 95%-transfected cells) was compared with dimerized T β RII-Fc and monomeric T β RII (Fig. 7B). The sensogram of prototype
20 (T β RII)² was similar to the T β RII-Fc interaction (slow off rate), and both were distinct from monomeric T β RII interaction (fast off rate), indicating that (T β RII)² interacts with the TGF- β 3 surface in a high-affinity, bivalent manner.

25 **Experiment #4: Production and purification of prototype and modified N-His (T β RII)²**

Scaleup production of prototype (T β RII)² in 293 cells resulted in variable yields of protein (1-3 mgs per 1 liter culture) upon purification via cobalt column, perhaps due to a less accessible His tag at the C-terminus. A modified version was constructed having
30 a N-terminal His tag, termed N-His (T β RII)², as shown in Fig. 6. N-His (T β RII)²-transfected HEK293 cells were grown in 500 ml culture. The media was collected, concentrated 5-fold by 10 kDa Centricon filtration and then passed through a 10 ml Fractogel Cobalt column. Fig. 8 shows a SDS-PAGE analysis of N-His (T β RII)² at the

various stages of purification. The N-His (T β RII)² in the eluted fractions (lane 6) is relatively pure and migrates as a smear (likely due to glycosylation) in the 50-60 kDa range. The total yield from 500 ml culture was 7-8 mgs, indicating that the N-His (T β RII)² protein is amenable to large-scale production.

5

Experiment #5: Demonstration that (T β RII)² is a potent TGF- β trap

The ability of purified prototype (T β RII)² to neutralize TGF- β was tested on Mv1Lu cells having a TGF- β -responsive luciferase reporter gene and compared with T β RII-Fc from
10 two sources, commercial R&D and collaborator H. Lin (**Fig. 9A**). The resulting inhibition curves indicated the average IC₅₀ for prototype (T β RII)² is 0.58 nM (S.D. 0.64) which is in the same range as for T β RII-Fc Lin (0.45 nM) and slightly higher than T β RII-Fc R&D (0.1 nM). Purified prototype (T β RII)² was also compared with dimeric T β RII-Fc and monomeric T β RII-ED for their ability to competitively bind TGF- β in solution via Biacore
15 analysis (**Fig. 9B**). Increasing amounts of each binder was added separately to a constant amount of TGF- β 1 or - β 3 (5 nM) followed by coinjection of this mixture over a TGF- β -specific antibody surface. The level of unbound TGF- β at equilibrium is assessed by the maximum/plateau level of the surface binding curve (**Fig. 9B**)
Prototype (T β RII)² and T β RII-Fc have similarly low IC₅₀s in the range of 5-8 nM, as
20 would be expected for intra-molecular, divalent binding of TGF- β . In contrast, the IC₅₀ for monovalent T β RII-ED is 10-20 fold higher. One might predict, for full avidity, that the IC₅₀ for dimeric (T β RII)² could be at least 100-fold greater than for monomeric T β RII-ED. In order to augment avidity, variable linker lengths may be sampled for (T β RII)² (see **Figs. 2C and 2D**). These results (**Figs. 9A and 9B**) indicate that (T β RII)² is an
25 excellent trapping/neutralizing reagent for TGF- β and hence is a good candidate therapeutic and/or diagnostic agent for diseases in which TGF- β is causative and overexpressed/overactive (e.g. breast tumors). To this end we examined the ability of prototype (T β RII)² to prevent TGF- β -induced invasion of 4T1 breast cancer cells in vitro (**Fig. 9C**). Similar to T β RII-Fc, prototype (T β RII)² reduced 4T1 cell invasion to
30 approximately 20% of the non-trap treated (+ TGF- β) control.

Experiment #6: Assessment of binding characteristics and efficacy of N-His (T β RII)²

The two different SPR Biacore assays that were utilized in Figure 7 and Figure 9B were used again to characterize the N-His (T β RII)² – TGF- β ligand interaction. First, the direct binding assay was utilized where the TGF- β trap was injected over various immobilized TGF- β isoform surfaces. While this assay can verify binding to different TGF- β isoform surfaces, it cannot verify that a 1:1 trap: TGF- β homodimer interaction is occurring in solution due to the nature of using an immobilized TGF- β surface. In order to show trap binding enhancement to soluble TGF- β ligand, indirect binding assays were carried out in which a constant TGF- β concentration was preincubated with various trap or T β RII monomer concentrations and then injected over a 1D11 antibody surface (anti-TGF- β 1 to 3). In this manner, the 1D11 surface measures the amount of free (or unbound) TGF- β . A lower IC₅₀ indicates binding enhancement due solely to avidity. In the direct binding assay, the binding of bivalent N-His (T β RII)² to immobilized TGF- β 1 and 3 was compared to that of the monomeric N-His T β RII construct (**Fig. 10A**). N-His (T β RII)² bound to all TGF- β isoforms (1-3), showing a fast on rate and significantly slower off rate of binding to TGF- β 1 and β 3 compared with monomeric N-His T β RII, as is expected for a bivalent binding interaction. In addition, N-His (T β RII)² showed binding to TGF- β 2 whereas monomeric T β RII binding to this isoform was undetectable. We also compared binding of N-His (T β RII)² and T β RII-Fc to TGF- β 1 and β 3 (**Fig. 10B**). Both traps showed similar binding kinetics with characteristic fast on rates and slow off rates. In order to assess trap binding to ligand in solution, the indirect binding assay to determine IC₅₀s as carried out using 5nM TGF- β 1. IC₅₀ curves for bivalent N-His (T β RII)², T β RII-Fc and monovalent T β RII (produced either in 293 cells or E.coli) were generated (**Fig. 10C**). N-His (T β RII)² and T β RII-Fc both showed efficient binding, having IC₅₀s of 1.1 and 1.6 nM, respectively. The IC₅₀s of N-His T β RII (293 cells) and T β RIIED (E.coli) were approximately 8 and 70 fold higher (respectively) than that of N-His (T β RII)². Similar differences between bivalent and monovalent traps were observed in neutralization assays using Mv1Lu luciferase reporter cells (**Fig. 10D**). The IC₅₀s for N-His (T β RII)² and T β RII-Fc in this assay were in the sub nM range whereas monomeric N-His T β RII (293 cells) showed only partial neutralization in the 10-100 nM range, and monomeric T β RII (E.coli) was unable to neutralize TGF- β . The results also show that, compared to prototype (T β RII)², the modified N-His (T β RII)² was most efficient in neutralizing TGF- β (compare **Figs 9A and 10D**).

Experiment #7: N-His (T β RII)² exhibits long-term stability and activity

The susceptibility of the N-His (T β RII)² to proteolytic degradation was assessed by incubating N-His (T β RII)² in the presence of 10% fetal bovine serum at 37°C for a period of 7 days (**Fig. 11**). The western blot on the left shows that N-His (T β RII)² protein remains intact throughout the 7 day period. In addition, the neutralization curves on the right demonstrate that N-His (T β RII)² retains its activity. These results show that N-His (T β RII)² is not adversely sensitive to proteolysis and therefore is a good candidate therapeutic and/or imaging agent for animal studies.

Experiment #8: The N-His (T β RIIb)², which has a long linker (60 amino acids, see Figs. 1A and 2A) is more potent than N-His (T β RII)²

The IC₅₀ for N-His (T β RIIb)² for neutralization of TGF β 1 was 0.04 nM (**Fig. 12A**), which is 4-fold more potent than N-His (T β RII)² (IC₅₀ = 0.16 nM, **Fig. 10D**). Similarly, when tested by SPR analysis (Biacore) for binding TGF- β 1 in solution, N-His (T β RIIb)² was more potent than N-His (T β RII)² (**Fig. 12B**). These results illustrate that modification of linker length is at least one parameter whereby trap efficiency can be improved.

Experiment #9: (ActRIIb)²: another example of a single-chain receptor trap within the TGF- β family

In order to show that the single-chain bivalent receptor strategy taught herein can be applied to other ligands of the TGF- β family, (ActRIIb)² (shown schematically in **Fig. 2A**) was constructed from the human ActRIIb receptor using this strategy. ActRIIb is the high affinity receptor for both myostatin and activin B. (ActRIIb)² and monomeric ActRIIb were produced in 293 cells and their ability to neutralize myostatin was tested using human rhabdomyosarcoma A204 cells. These cells have the ActRIIb receptor and were transfected with (CAGA)₁₂-luciferase reporter gene (responsive to activin and myostatin) (**Fig. 13**). (ActRIIb)² exceeded the neutralization potency of monomeric ActRIIb (IC₅₀ of 0.1 and 0.38 nM, respectively), thus demonstrating the better binding efficiency of this bivalent trap. In addition, (ActRIIb)² was 10-fold more potent than dimeric ActRIIb-Fc. These results therefore indicate that the single-chain receptor

strategy taught herein can be used as a platform technology to develop effective trapping reagents of other ligands within the TGF- β family.

Experiment #10: (BMPR1a)²: another example of a single-chain receptor trap within the TGF- β family

Another example of a TGF- β family member trap is (BMPR1a)², shown schematically in **Fig. 2A**. The (BMPR1a)² trap was compared with monomeric BMPR1a for neutralization of BMP2 (**Fig. 14**). The bivalent (BMPR1a)² trap was clearly able to neutralize BMP2 whereas monomeric BMPR1a showed poor neutralization.

The multivalent polypeptide ligand binding agents described herein allow for high affinity and specificity by single-chain multivalency. This single-chain attribute is fundamentally different from existing multi-chain agents such as Fc-based fusions (covalent dimer), E/K-coiled-coil-based fusions (non-covalent dimer), or described cytokines and ligand traps that include fused multimerizing moieties. The present design can facilitate tissue penetration, thereby increasing access to sites of interest. The present design can also provide a shorter half life in systemic circulation, which can be desirable for certain applications such as imaging and other diagnostic applications, as well as where ongoing abundant systemic distribution of the antagonist is not desirable. In addition, the present design permits linkage of other cargo molecules (for example imaging agents like fluorescent molecules), toxins, etc.

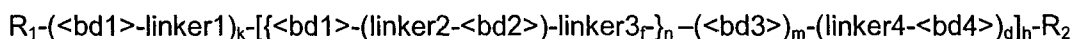
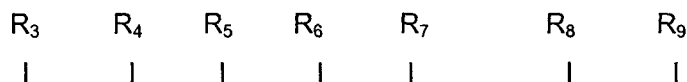
Linkers can be designed to facilitate purification of the linker and/or ligand binding agent. The exact purification scheme chosen will determine what modifications are needed, for example, additions of purification "tags" such as His tags is contemplated.

The general Structure I

$$(<\text{bd1}>\text{-linker1})_k\text{-}[\{<\text{bd1}>\text{-(linker2-}<\text{bd2}>\text{)-linker3}_r\}_n\text{-}(<\text{bd3}>)_m\text{-(linker4-}<\text{bd4}>)_d]_h$$

can be modified to add one or more cargo and/or accessory molecules (referred to collectively herein by R₁, R₂, R₃, R₄, etc.).

For example, to provide Structure V:



5

Where bd1, bd2, bd3, bd4, linker1, linker2, linker3, linker4, k, f, n, m, d, and h are defined as in Structure I.

Without limiting the generality of R substituents available, R₁, R₂, R₃, R₄, R₅, R₆, R₇, R₈,
 10 R₉, may be the same or different, may not be present and when present, may independently be one or more of:

A fusion protein for targeting such as an antibody fragment (e.g. single chain Fv) and/or a single domain antibody (sdAb).

15

A radiotherapy and/or imaging agent such as a radionuclide (e.g. ¹²³I, ¹¹¹In, ¹⁸F, ⁶⁴C, ⁶⁸Y, ¹²⁴I, ¹³¹I, ⁹⁰Y, ¹⁷⁷Lu, ⁶⁷Cu, ²¹³Bi, ²¹¹At), a fluorescent dye (e.g. Alexa Fluor, Cy dye) and/or a fluorescent protein tag (e.g. GFP, DsRed).

20 A cytotoxic agent for chemotherapy such as doxorubicin, calicheamicin, a maytansinoid derivatives (e.g. DM1, DM4), a toxin (eg. truncated Pseudomonas endotoxin A, diphtheria toxin).

25 A nano particle-based carrier such as polyethylene glycol (PEG), a polymer-conjugated to drug, nanocarrier or imaging agent (e.g. of a polymer N-(2-hydroxypropyl)methacrylamide (HPMA), glutamic acid, PEG, dextran).

A drug (e.g. doxorubicin, camptothecin, paclitaxel, palatinat).

30 A nanocarrier such as a nanoshell or liposome.

An imaging agent such as Supermagnetic Iron Oxide (SPIO)

A dendrimer

A solid support for use in ligand purification, concentration or sequestration (e.g. nanoparticles, inert resins, suitable silica supports).

- 5 In general, it will not be preferable to have cargo or accessory molecules in all possible positions, as this may cause steric or electrostatic complications. However, the effects of adding a cargo or accessory molecule to any given position or positions on the structure can be determined routinely in light of the disclosure herein by modeling the linker between the binding domains and carrying out molecular dynamics simulations to
- 10 substantially minimize molecular mechanics energy and reduce steric and electrostatic incompatibility between the linker and the member of the TGF- β superfamily as taught herein.

It will frequently be preferable to add the cargo or accessory molecule to the linker portion of the agent, rather to the binding domain, to reduce the likelihood of

15 interference in binding function. However, addition to the binding domain is possible and could be desirable in some instances and the effect of such an addition can be determined routinely in advance by modeling the binding agent and the linker with the proposed addition as described herein.

- 20 In certain embodiments of conjugation to cargo molecules and accessory molecules, the following structures will be produced:



- 25 $[bd]-(R-linker-[bd])_n$



30



Conjugation methodologies are somewhat diverse but typically can be performed using commercial kits that enable conjugation via common reactive groups such as primary amines, succinimidyl (NHS) esters and sulfhydryl-reactive groups. Some examples are; Alexa Fluor 488 protein labeling kit (Molecular Probes, Invitrogen detection technologies) and PEGylation kits (Pierce Biotechnology Inc.).

Many embodiments of the binding agents taught herein will have a lower molecular mass, as compared with competing multivalent receptor-based neutralizing agents.

In an embodiment of the invention there is provided ligand binding agents wherein the intervening linker sequence, between the ligand-binding domains, is composed of native amino acids, the sequence of which is based on the receptor ectodomains (e.g. the various linkers shown in Fig. 2B and the "repeat" and "delete" linkers shown in Fig. 2D) or conservative substitutions of natural or unnatural amino acids into such regions or reversal of such natural or modified sequences. It will frequently be considered preferable to use unstructured regions from these receptor ectodomains as the template for linker design. Once linkers have been designed, it will generally be preferred to test their effectiveness using the procedures described herein or other substantially functionally equivalent procedures. Routine testing for immunogenicity may be desired for *in vivo* use.

In some instances, it will be desirable to subject the polypeptide-based linking design of the ligand binding agents disclosed herein to optimization of characteristics desired for a particular application. For example, the linker may be modified in length and composition based on atomic-level simulations and knowledge-based design in order to improve binding affinity, specificity, immunogenicity and stability. This is applicable to a wide range of molecular systems exhibiting homomeric, heteromeric, dimeric and multimeric ligand-receptor structural characteristics

Additional different binding domains can be incorporated to generate multivalent traps with even higher binding potency.

In an embodiment of the invention, a non-naturally occurring single-chain hetero-bivalent polypeptide is produced by the inline fusion of two or more different structured

ligand-binding domains (denoted <bd1>, <bd2>, <bd3> and <bd4>) from the extracellular portion of distinct natural receptors, and which is not fused to any dimerizing or multimerizing moieties. In some instances, this polypeptide will have the general structure <bd1>-linker2-<bd2>. In some instances, the binding domains will be selected from the ectodomains of the T β R-II and T β RI receptors, and fused to produce hetero-bivalent single-chain traps active against TGF- β isoforms. In other instances, the binding domains will be selected from the ectodomains of the ActR-IIa and BMPR-Ia receptors and fused to generate single-chain hetero-bivalent traps active against activin, myostatin and BMP isoforms. In other embodiments, the binding domains are selected from other receptors to members of the TGF- β superfamily.

In another embodiment of the invention a non-naturally occurring single-chain hetero-trivalent polypeptide is produced by the inline fusion of two or more different structured ligand-binding domains (denoted bd1 and bd2) from the extracellular portion of distinct natural receptors, and which is not fused to any dimerizing or multimerizing moieties. In some instances, this polypeptide will have the general structure [bd1]-linker1-[bd2]-linker2-[bd2]. In other instances, this polypeptide will have the general structure [bd1]-linker1-[bd1]-linker2-[bd2]. In some instances, [bd1] and [bd2] will be selected from the ectodomains of the T β R-II and T β RI receptors, and fused to produce hetero-bivalent single-chain traps active against TGF- β isoforms. In other instances, bd1 and bd2 will be selected from the ectodomains of the ActR-IIa and BMPR-Ia receptors and fused to generate single-chain hetero-bivalent traps active against activin, myostatin and BMP isoforms.

In another embodiment of the invention a non-naturally occurring single-chain hetero-tetravalent polypeptide is produced by the inline fusion of two or more identical or different structured ligand-binding domains from the extracellular portion of natural receptors repeated twice or more times in various orders. In an embodiment to the invention this hetero-tetravalent polypeptide is not fused to any dimerizing or multimerizing moieties. In one embodiment, this polypeptide will have the general structure [bd1]-linker1-[bd2]-linker2-[bd1]-linker1-[bd2]. In other instances, this polypeptide will have the general structure [bd1]-linker1-[bd1]-linker2-[bd2]-linker3-[bd2]. In one embodiment, this polypeptide will have the general structure [bd1]-linker1-[bd2]-linker2-[bd2]-linker3-[bd1]. In some instances, [bd1] and [bd2] will be selected from the

ectodomains of the T β R-II and T β R-I receptors, and fused to produce single-chain hetero-tetravalent traps active against TGF- β isoforms. In other instances, [bd1] and [bd2] will be selected from the ectodomains of the ActR-IIa and BMPR-Ia receptors and fused to generate single-chain hetero-tetravalent traps active against activin, myostatin and BMP isoforms.

Specific non-limiting examples of embodiments of heteromeric single-chain traps against TGF- β are represented schematically as well as with full sequence details in **Figs. 15A and 15B.**

A nucleotide sequence encoding a single-chain protein produced according to the teachings herein can be cloned and inserted into any suitable vector and therefore is very amenable to production (i.e. there is no requirement for two vectors, or one vector with two promoters, to express two receptor ectodomains).

The linker region provides a segment that is distinct from the structured ligand binding domains and thus can be used for conjugation to accessory molecules (for example, molecules useful in increasing stability such as PEGylation moieties) or cargo molecules such as contrast agents (for imaging) without having to chemically modify the binding domains.

In an embodiment of the invention in which the ligand-binding domains and the linker contain primarily natural sequences they would not ordinarily be expected to be severely immunogenic or toxic in a typical patient.

Smaller size (for example, 50-60 kDa for (T β RII)² compared to 100-120 kDa for T β RII-Fc or 150 kDa for monoclonal antibodies) will generally be expected to increase access to target tissues.

Large scale production is an attainable goal. One 500 ml scale-up of N-His (T β RII)² in 293 cells yielded 7 mg of purified protein.

In some instances, it may be desirable to permit a computer or other machine capable of calculation to determine linker length according to the disclosure herein. Thus, in an embodiment of the invention there is provided a data storage medium comprising

instructions for determining the minimum linker length. In an embodiment of the invention there is provided a data storage medium comprising a means for identifying acceptable minimal linker length.

- 5 Linker length will be considered acceptable when it permits binding of binding domains located on each of the N- and C-termini of the linker to bind their natural binding sites on their natural ligand such that, with both binding domains so bound, the ligand is bound with a higher affinity than it would be bound by binding of only one of the binding domains.

10

Methods

Construction and cloning of TGF β family traps

15

1) Prototype (T β RII)²

- Step 1:* The mammalian expression vector pTT2-RII E (De Crescenzo et al., 2003, J. Mol. Biol. 328: 1173), which contains a myc-tagged ectodomain of the human type II TGF- β receptor (T β RII) was cut with NotI and BamHI to eliminate E-coil/His regions.
- 20 *Step 2:* A second ectodomain of T β RII was PCR amplified from plasmid huTGF β RII/pCDNA3 as template and using primers R2ECD3'Bamrev2 and R2ECD 5'Not to incorporate a 3' 6-His tag+Bam HI site, and a 5' Not I restriction site, respectively.

25

R2ECD3'Bamrev2: SEQ ID NO 1

GACAGGATCCTAGTGATGATGGTGGTGGTGCAGGATTGCTGGTGTTATATTC

R2ECD 5'Not: SEQ ID NO 2

CACGGCGGCCGCCACGTTTCAGAAGTCGGTTAATAAC

30

This PCR product was ligated to pCDNA3 cut with NotI and BamHI. The insert was verified by sequencing, re-excised by Not I /Bam HI digestion and then cloned into the vector from step 1, resulting in the assembly of two T β RII ectodomains in tandem. The

sequence of this construct was verified by sequencing and the amino acid sequence of the prototype (T β RII)² protein is shown below and is presented schematically in **Fig. 6**.

Prototype (T β RII)²: SEQ ID NO 3

5 MGRLRLGLWFLHMLWTRIASTIPPEQKLISEEDLLHVQKSVNNDMIVTDNNGAVKF
 PQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCAVWRKNDENITLETVCHDPK
 LPYHDFILEDAAAPKCMKEKKKPGETFFMCSCSSDECNDNIIFSEEYNTSNPDGGRHV
 QKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCA
 VWRKNDENITLETVCHDPKLPYHDFILEDAAAPKCMKEKKKPGETFFMCSCSSDECND
 10 NIIFSEEYNTSNPDHHHHHH

* The gray sequence within brackets denotes the huT β RII signal peptide which is cleaved upon processing in 293 cells. The boxed sequences (EQK...LL and HH...HH), are Myc and His tags, respectively.

15

2) Modified N-His (T β RII)² and N-His T β RII monomer

Step 1: Reversion of non-native amino acids GGR to native amino acids IPP in the linkers of prototype (T β RII)²

20 The Not1 site within the linker coding sequence of prototype (T β RII)² created a GGR sequence (underlined in the amino acid sequences shown above). This was reverted back to the native IPP sequence by PCR-based mutagenesis. Internal primers 2XR2mutfor and 2Xmutrev span the region to be mutated and two flanking primers pTT2 5' and pTT2 3' contain the flanking regions.

25

2XR2mutfor : SEQ ID NO 4

CCTGACATCCCACCGCAGGTTCAGAAG

2Xmutrev: SEQ ID NO 5

GAACGTGCGGTGGGATGTCAGGATTGC

30 **pTT2 5' SEQ ID NO 6**

ATACACTTGAGTGACAATGACA

pTT2 3' SEQ ID NO 7

AAAATTCCAACACACTACTTTGCAATCT

The template used was pTT2-prototype(T β RII)². Primers pTT2 5' and 2XR2mutrev were paired to create PCR fragment 1. Primers 2XR2mutfor and pTT2 3' were paired to create PCR fragment 2. The PCR fragments 1 and 2 were heated at 95°C and allowed
 5 to anneal together. The flanking primers were then used to amplify the assembled fragment 1+2. The amplified fragment 1+2 was then cut with HindIII and BamHI and inserted into pTT vector also cut with HindIII and BamHI. The resultant plasmid was designated pTT2-native(T β RII)².

10 **Step 2:** Elimination of C-terminal His and N-terminal Myc tags and fusion with N-terminal His tag/thrombin cleavage site.

Two primers were designed, incorporating the desired sequence change (restriction sites, thrombin cleavage site, and eliminating the mycMyc tag, and the His tag).

15

BamHI-Thr-IPP-R2ECD_for: SEQ ID NO 8

GGATCCTTCAACCCGCGTATTCCGCCGCACGTTTCAGAAAGTCGGTT

BstBI stop R2ECD rev : SEQ ID NO 9

GCGTTCGAACTAGTCAGGATTGCTGGTGTTATATTC

20

These primers were used to generate two fragments by PCR using pTT2-native(T β RII)² as a template: fragment 1XECD (monomer) and 2XECD (dimer). Both fragments were digested with BstBI and BamHI and cloned separately into plasmid vector pTTVH8G (unpublished, derived from pTT vector; Durocher et al., 2002, Nucl. Acid. Res. 30: No.
 25 2 e9) which has the human VEGF signal sequence/10 N-terminal amino acids of VEGF and 8Xhis tag. The protein sequences of the resulting constructs are as follows:

N-His (T β RII)²: SEQ ID NO 10

30 [MNFLESWVHWSLALLLYLHHAKWSQA]APMAEGGGQN[HHHH]
 [HHHH]GGSFNPRIPP HVQKSVNNDMIVTDNNGAVKFPQLCKF
 CDVRFSTCDNQKSCMSNCSITSICEKPQEVCAVVRKND
 NITLETVCHDPKLPYHDFILEDASPCKIMKEKKKPGETFFM
 CSCSSDECNDNIIFSEYNTSNPD[IPP]HVQKSVNNDMIVTD
 NNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQ

EVCVAVWRKNDENITLETVCHDPKLPYHDFILED AASPKCIM
KEKKKPGETFFMCSCSSDECNDNIIFSEEYNTSNPD

- * The gray sequence within brackets denotes the VEGF signal peptide. The boxed
5 sequences; (HH...HH and IPP), are the His tag and reverted IPP sequence,
respectively.

N-His TβRII monomer: SEQ ID NO 11

[~~MTNFI~~SWVHWSLATLLYLHHAKWSQA]APMAEGGGQNHHHH
10 HHHHGGSFNPRIIP HVQKSVNNDMIVTDNNGAVKFPQLCKF
CDVRFSTCDNQKSCMSNCSITSICEKPQEVAVWRKNDEN
ITLETVCHDPKLPYHDFILED AASPKCIMKEKKKPGETFFM
CSCSSDECNDNIIFSEEYNTSNPD

15 **3) N-His (TβRIIb)²**

Step 1: Assembly of (TβRIIb)² gene.

- a. Plasmid pTT2-native(TβRII)² was cut with NotI and BamHI to eliminate the second
TβRIIECD.
20 b. A PCR fragment was generated using plasmid pRC/CMV-huTβRIIb (containing the
human TβRIIb gene) as a template with the following primers:

R2ECD-3'Bamrev-2: SEQ ID NO 12

GACAGGATCCTAGTGATGATGGTGGTGATGGTCAGGATTGCTGGTGTTATATTC
and

25 **R2bECD 5'Not for: SEQ ID NO 13**

CACGGCGGCCGCCACGTTCAGAAAGTCGGATGTGG

- The resulting fragment comprised the TβRIIb with a 3' 6-His tag , Bam HI site and stop
codon, and Not I at the 5' end. This fragment was cut with Not I and Bam HI and then
30 cloned into the the vector from step a.

Step 2:

Using the plasmid from step 1b as template a PCR fragment was generated (that eliminates the N-terminal Myc tag and C-terminal His tags) with the following primers:

BamHI-Thr-IPP-R2-ECD-for: SEQ ID NO 14

GGATCCTTCAACCCGCGTATTCCGCCGCACGTTTCAGAAGTCGGTT

5 **BstBI stop R2ECD rev : SEQ ID NO 15**

GCGTTCGAACTAGTCAGGATTGCTGGTGTTATATTC

The resulting PCR fragment was digested with the appropriate enzymes and subcloned into pTTVH8G. The protein sequence of the this trap is as follows:

10

N-His (TβRIIb)²: SEQ ID NO 16

[MNFILSWVHWGALLEYLHAKWSCA]APMAEGGGQN[HHHHHHHH]GGSF

NPRIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICE
KP

15 QEVCAVWRKNDENITLETVCHDPKLPYHDFILEDAAAPKCIMKEKKKPGETFFMCSCS
S

DECNDNIIFSEEYNTSNPDIPPHVQKSDVEMEAQKDEIICPSCNRTAHPLRHINNDMIVT
DNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPEVCAVWRKNDENITL
ET

20 VCHDPKLPYHDFILEDAAAPKCIMKEKKKPGETFFMCSCSSDECNDNIIFSEEYNTSNP
D

* The gray sequence within brackets denotes the VEGF signal peptide. The boxed sequence is the His tag.

25

30 **4) (ActRIIB)² and ActRIIB monomer**

For construction of (ActRIIB)², three primer pairs (1+2, 3+4, 5+6) were used to generate 3 PCR fragments (A, B and C) using a plasmid containing the human ActRIIB sequence as a template.

Primer1: SEQ ID NO 17

cgcagatctgcggccgcATGACGGCGCCCTGGGTGGCCCTCGCCCTCCTCTGGGGATCG
CTGTGCGCAGGATCAGGATCAGAACAGAAGCTGATCaGCG

5 **Primer2: SEQ ID NO 18**

CCGtGATCAGCTTCTGTTCTGATCCTGATCCTGCGCACAGCGATCCCCAGAGGAGG
GCGAGGGGCCACCCAGGGCGCCGTCATgcggccgcagatctggc

Primer3: SEQ ID NO 19

GGCaGATCTCCGAGGAAGATTACTAGGGCGTGGGGAGGCTGAGACACGGGAGT
10 G CATC

Primer4: SEQ ID NO 20

ccgactagtGGGGGCTGTCTGGGGGTGGCTC

Primer5: SEQ ID NO 21

ccgactagtGGGCGTGGGGAGGCTGAGAC

15 **Primer6: SEQ ID NO 22**

cgctggaaccCTAATGGTGATGATGGTGATGGGTGGGGGCTGTCTGGGGGTGGC

PCR Fragment A contains 5' Bgl II and NotI sites, the ATG start codon, the signal peptide and the 5' half of the Myc tag and Bcl I site. PCR fragment B contains the first
20 ECD, with a BglII site and the 3' half of the Myc tag and SpeI site. Fragment C contains the second ECD, SpeI site at the 5' end, and a BamHI site, stop codon, the 6Xhis tag at the 3' end. These fragments were subcloned into pGemT vector (Promega), digested with the appropriate enzymes, and ligated together. The resulting A+B+C fragment, which encodes the (ActRIIB)² single chain dimer, was cut using NotI and BamHI and
25 inserted into pTT expression vector. The ActRIIB monomer was assembled in a similar manner using primer pairs 1+2 and 3+6. The resulting constructs have the following protein sequences:

(ActRIIB)²: SEQ ID NO 23

30 **[MTAPWVALALLWGSICAG]SGS[EQKLISEEDLL]GRGEAETRECIYYNANWELERTNQ**
SG
LERCEGEQDKRLHCYASWRNSSGTIELVKKGCWLDDFNCYDRQECVATEENPQVYF
CCCE

GNFCNERFTHLPEAGGPEVTYEPPTAPTSGRGEAETRECIYYNANWELERTNQSGL
 ERC
 EGEQDKRLHCYASWRNSSGTIELVKKGCWLDDFNCYDRQECVATEENPQVYFCCCE
 GNFC

5 NERFTHLPEAGGPEVTYEPPTAPT[HHHHHH]

ActRIIB monomer: SEQ ID NO 24

[MTAPWVAALWGSICAG]SGS[EQKLISEEDLL]GRGEAETRECIYYNANWELERTNQ
 SGLE

10 RCEGEQDKRLHCYASWRNSSGTIELVKKGCWLDDFNCYDRQECVATEENPQVYFCC
 CEGN
 FCNERFTHLPEAGGPEVTYEPPTAPT[HHHHHH]

*The gray sequence within brackets denotes the human ActRIIB signal peptide. The
 15 boxed sequences; (EQK...LL and HH...HH), are Myc and His tags, respectively.

5) (BMPR1a)² and BMPR1a monomer

For construction of (BMPR1a)², 2 primer pairs (1+2, 5+6) were used to generate two
 20 PCR fragments (A, B) using a plasmid containing the human BMPR1a (ALK3)
 sequence as a template.

Primer1for: SEQ ID NO 25 GCG AAG CTT ATG CCT CAG CTA TAC ATT TAC ATC

Primer4rev: SEQ ID NO 26 CGGC CTC CGG ATG CTG CCA TCA AAA AAC GG

25 **Primer5for:** SEQ ID NO 27 CCGCG CGC CGG CAG AAT CTG GAT AGT ATG CTT
 C

Primer6rev: SEQ ID NO 28 CGAC AGG ATC CTA GTG ATG ATG GTG GTG ATG
 TCG AAT GCT GCC ATC AAA AAA CGG

30 PCR fragment A contains a 5'Hind III site, start codon, signal peptide and first
 BMPR1aECD. PCR fragment B contains the second BMPR1aECD, 6Xhis tag, stop
 codon and BamHI site.

These fragments were subcloned into pGemT vector (Promega), digested with the
 appropriate enzymes, and ligated together. The resulting A+B fragment, which encodes

the (BMPR1a)² single-chain dimer, was cut with HindIII and BamH1 and inserted into pTT2 expression vector. The BMPR1a monomer was assembled using primer pairs 1+6. The resulting constructs have the following protein sequences:

5 (BMPR1a)²: SEQ ID NO 29

[MPQLYIMRLGAYLEISRVQG]QNLD SMLHGTGMKSDSDQKKSENGVT
LAPEDTL PFLKCYCSGHCPDDAINNTCITNGHCF AII EDDQGETTLASGCMKYEGSDF
Q
CKDSPKAQLRRTIECCRTNLCNQYLQPTLPPVIGPFFDGSIRQNLD SMLHGTGMKSD
10 SD
QKKSENGVT LAPEDTL PFLKCYCSGHCPDDAINNTCITNGHCF AII EDDQGETTLASG
C
MKYEGSDFQCKDSPKAQLRRTIECCRTNLCNQYLQPTLPPVIGPFFDGSIR[HHHHHH]

BMPR1a monomer: SEQ ID NO 30

15 [MPQLYIMRLGAYLEISRVQG]QNLD SMLHGTGMKSDSDQKKSENGVT LAPEDTL PFL
K
CYCSGHCPDDAINNTCITNGHCF AII EDDQGETTLASGCMKYEGSDFQCKDSPKAQL
RR
TIECCRTNLCNQYLQPTLPPVIGPFFDGSIR[HHHHHH]

- 20 * The gray sequence within brackets denotes the human BMPR1a signal peptide. The boxed sequence is the His tag.

Expression and purification of ligand binding agents

- 25 Modified human embryonic kidney cells (293-EBNA1 clone 6E) stably expressing EBNA1 were transfected using 25kDa linear polyethylenimine (PEI) (Poysciences, Warrington, PA) as described below (and Durocher et al., 2002, Nucl. Acid Res. 30: e9)). The cells growing as suspension cultures in Freestyle medium (Invitrogen) were transfected at 1×10^6 cells/ml with variable amounts of pTT vector plasmid DNA (for
30 small scale cultures), or a fixed amount of plasmid DNA (for large scale culture), and 2 ug/ml PEI.

1. Small-scale transient transfections:

Five hundred microliters of the suspension culture was distributed per well in a 12-well plate. DNA was diluted in Freestyle medium (in a volume equivalent to one-tenth of the culture to be transfected), PEI was added, and the mixture immediately vortexed and incubated for 10 min at room temperature prior to its addition to the cells. Following 3 h
5 incubation with DNA-PEI complexes, culture medium was completed to 1 ml. The culture was harvested 5 days after transfection and the media was clarified by centrifugation at 3500 g for 10 min and sterile filtered. Aliquots of conditioned media were analyzed for TGF- β binding activity via SPR analyses (see below and **Fig. 7A**)

10 **2. Large-scale cultures and protein purification:**

Large scale cultures were processed as per (Pham et al., 2005: Biotechnol. Bioeng. 90: 332). Bioreactors of 1L (Biostat Q, B. Braun, Germany) were equipped with 45° pitched blade impellers and stirring speed was maintained at 100 rpm. Surface aeration was
15 applied with a gas mixture of nitrogen, carbon dioxide and oxygen at a gas-flow rate of 100 standard cubic cm/min). The dissolved oxygen tension was controlled at 40% air saturation. The temperature was maintained at 37°C and the pH was maintained at 7.15 with CO₂ at the beginning of the run and with NaHCO₃ (7.5% w/v) during the cell growth phase. A feed with 0.5% (w/v) TN1 peptone (OrganoTechnie) was done 24 hours post-
20 transfection. The culture medium was harvested 120 hours post transfection and trap protein was purified by immobilized metal affinity chromatography on Fractogel-Cobalt column as previously described (Cass et al., 2005, Protein Expr. Purif. 40: 77) except that wash and elution steps contained 25 mM and 300 mM imidazole respectively. A 10 ml column packed with 5 cm Talon Metal Affinity Resin (BD Biosciences, Mississauga,
25 Ont.) and was equilibrated with 10 column bed volumes (CVs) of Talon Wash Buffer (TWB: 50 mM sodium phosphate, 300 mM NaCl, pH 7). The conditioned medium was passed through a 0.22 μ m filter, and then loaded by gravity. The column was washed with 10 CVs of TWB and (T β RII)² was eluted in 1 ml fractions using 300 mM imidazole in TWB. Eluted trap protein was then desalted in PBS using a HiPrep 26/10 desalting
30 column (GE-Healthcare) as recommended by the manufacturer. Protein concentration was determined by Bradford using BSA as a standard. The progress of the various stages of purification for N-His (T β RII)² is seen in **Fig. 8**.

Surface Plasmon Resonance (SPR) experiments**Analyses of conditioned media of transfected 293-EBNA1 clone 6E cells for TGF β -binding activity**

5

Conditioned media from cells, transfected with increasing amounts of pTT2-prototype (T β RII)² plasmid (to generate increasing percentages of transfected cells ranging from 1-95%), were collected 5 days post-transfection and sterile filtered (0.22 μ m). The samples were diluted to 1:100 or 1:20 using HBS buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.02% Tween 20) prior to surface plasmon resonance (SPR) analysis of the (T β RII)² interaction with TGF- β 3. SPR data was generated using a Biacore 3000 instrument (G.E. Healthcare Inc.) at 25°C using HBS as running buffer. Ligand was prepared by covalently immobilizing 2000 resonance units (RUs) of TGF- β 3, along with a mock blank control surface, onto a Biacore CM-5 sensor chip using standard amine coupling methods. Samples were injected simultaneously over the TGF- β 3-immobilized and blank surfaces for 240 s followed by a 240 s dissociation time at a flow rate of 10 μ l/min. Specific (T β RII)²-TGF- β 3 interaction sensograms were generated by subtracting the sensogram generated from the blank surface from the one generated from the TGF- β 3-immobilized surface. The sensograms were aligned to the injection start points using BiaEval software version 4.1 (Biacore Inc.), as shown in Fig. 7A.

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Association and dissociation of T β RII, prototype (T β RII)² and T β RII-Fc with TGF β

Sensograms comparing (T β RII)² with T β RII-Fc and E.coli-produced monomeric T β RII ectodomain were generated. The ligand surface and injection conditions were the same as described above except injection times were for 120 s. Solutions containing 200 nM purified T β RII and 25 nM T β RII-Fc in HBS buffer were used for analysis. (T β RII)²-conditioned media (from 95% transfected cells) was diluted 1:20 in HBS buffer. The SPR sensograms generated from these sample injections were aligned to their injection start point and normalized to a maximum RU response of 100 using Biacore BiaEval software version 4.1 (Biacore Inc.), as shown in Fig. 7B.

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Comparison of the TGF β -binding efficacies of purified (T β RII)², T β RII-Fc and T β RII monomer

Solutions containing a T β RII variant ((T β RII)², T β RII-Fc or E.coli-produced T β RII) and 5 nM of TGF- β (β 1 or β 3) were pre-incubated and then injected over covalently immobilized 1D11 anti-TGF- β antibody (R&D Systems) under mass-transport limiting conditions to measure free TGF- β . SPR data was generated using a Biacore 3000 instrument (G.E. Healthcare Inc.) using HBS as running buffer. A high density 1D11 surface (approximately 10,000 RUs) and matching blank control surface were created on a Biacore CM-5 sensorchip using standard amine coupling methods. A twenty-fold stock concentration of TGF- β (100 nM in 10mM acetic acid) was used. This gave the final 1-fold assay concentration of TGF- β (5 nM) when 10 μ l was added to 190 μ l HBS containing a T β RII variant at a 1.05 times final concentration. Blank injection samples were made from 10 μ l 10 mM acetic acid mixed with 190 μ l HBS. TGF- β was added to the T β RII variant solution using the TRANSFER command, mixed, and incubated for 120 s at 4°C prior to injection over the 1D11 surface. Using the KINJECT command, samples were simultaneously injected for 5 min over the 1D11 and control surfaces with a 30 s dissociation time at a flow rate of 5 μ l/min at 25°C. The 1D11 surface was regenerated for the next cycle by injecting 10 mM HCl for 15 s at 20 μ l/min using the INJECT command. All sensogram analysis was carried out using Biacore BiaEvaluation software v4.1 (G.E. Healthcare Inc.). T β RII variant sensograms were aligned to the injection start point, and double-referenced using the control surface and blank injection sensograms. The plateau levels (which measure the amount of free TGF β) were taken from the average value of the stabilized dissociation phase of each double-referenced sensogram. Examples are shown in **Figs. 9B, 10C and 12B.**

Comparison of the antagonistic/inhibitor potencies of various binding agents by luciferase reporter assays

1. Luciferase assay for a TGF- β binding agent in mink lung epithelial (Mv1Lu) cells.

Mink lung epithelial cells, stably transfected with the TGF- β -responsive *PAI-1* promoter fused to the firefly luciferase reporter gene (Abe et al., 1994, Anal. Biochem. 216: 276),

were used. These cells were plated in 96-well tissue culture plates (2×10^4 cells/well) in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and were allowed to attach for at least 6 h at 37°C. Cells were then washed with phosphate buffered saline (PBS), and the medium was replaced by Dulbecco's modified Eagle's medium containing 1.0% fetal bovine serum and 0.1% bovine serum albumin (DMEM-1, 0.1% BSA). Various concentrations of purified (T β RII)² or (T β RIIb)² trap or T β RII-Fc (either from R&D Systems or collaborator Dr. Herbert Lin) were mixed with 20 pM TGF- β 1 in DMEM-1, 0.1% BSA) and added to the cells. After 16 hr. incubation at 37°C, the medium was removed, and the cells were washed once with PBS. Cells were then lysed with 25 μ l reporter lysis buffer (Promega Corp.) and assayed for luciferase activity using the Promega luciferase assay kit according to the manufacturer's instructions. Luminescence was measured in a MRX (Dynex Inc.) or Lumioskan RS (Global Medical Instrumentation, Inc.) microplate reader. The activity is expressed as the percentage of the maximum TGF- β 1 activity (i.e. in the absence of any antagonist) or relative luciferase units (RLU) (see examples shown in Figs. 9A, 10D and 12A).

2. Luciferase assay for ActRIIB binding agents in A204 cells.

A204 cells (rhabdomyosarcoma, ATCC) were plated in 48-well culture plates (5×10^4 cells/well) in McCoy's 5A Media (ATCC) supplemented with 10% Fetal bovine serum. After 24 hrs. the cells were transfected with (CAGA)₁₂MLP-Luc (luciferase reporter responsive to Activin and myostatin; Dennler et al., 1998, EMBO J. 17: 3091) and pRL-CMV (constitutive renilla reporter for normalization of transfections, Promega Corp.) using Lipofectamine 2000 transfection reagent, according to the manufacturer's specifications (Gibco-BRL). After 24 hours the cells were washed once with DMEM-1, 0.1% BSA and then treated with 4 nM human myostatin (GDF8, R&D Systems) without or with increasing concentrations of ActRIIb trap or ActRIIb-Fc (R&D Systems) for 6 hrs, 37°C. The cells were then washed once with PBS and lysed with 50 μ l 1X Passive lysis buffer. The lysates were measured by a dual firefly/renilla luciferase reporter kit, according to the manufacturer's (Promega Corp). The activity is expressed as firefly RLU normalized to renilla (see example shown in Fig. 13).

3. Luciferase assay for BMPR1a binding agents in C2C12BRA cells.

C2C12BRA cells (mouse myoblast cells stably transfected with a BMP-luciferase reporter; Zilderberg et al., 2007, BMC Cell Biology 8: 41) were plated onto 96-well culture plates (5×10^3 cells/well) in DMEM supplemented with 10% fetal bovine serum.

5 After 24 hrs. the cells were washed once with DMEM-1, 0.1% BSA and then treated with 1 nM human BMP2 with or without increasing amounts of BMPR1a trap or BMPR1a-Fc (R&D Systems) for 24

hrs. at 37°C. The cells were then washed once with PBS and lysed with 50 μ l 1X Reporter lysis buffer. The lysates were measured by a firefly luciferase reporter kit,

10 according to the manufacturer's (Promega Corp). The activity is expressed as firefly RLU (see example Fig. 14).

Neutralization/Inhibition of TGF- β -induced 4T1 cell invasion by TGF β binding agents

15 4T1 cells (mouse mammary carcinoma, ATCC) were seeded onto BD BioCoat Matrigel invasion chambers (BD Biosciences) at 1×10^5 cells/chamber in DMEM containing no serum, without or with 100 pM TGF- β , and with 400 nM prototype (T β RII)² trap or T β RII-Fc. The cells were allowed to invade through the matrigel into the bottom chamber for

20 18 hrs at 37°C. The cells on the upper side of matrigel membrane were removed by scraping and invaded cells were stained/fixed with 0.2% crystal violet, 100% ethanol. The number of invaded cells was quantified for 4 fields of equal size via light microscopy. The example shown in Fig. 9C shows the average % invasion (relative to + TGF- β control) from 3 experiments.

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Western blot to determine N-His (T β RII)² protein stability in serum

Equal amounts N-His (T β RII)² protein were incubated for 1-7 days at 37°C in DMEM+10% fetal bovine serum. Equal aliquots were electrophoresed in a 8% SDS-

30 reducing gel followed by western blotting and probing with anti-T β RII antibody (R&D Systems). The result is shown in Fig. 11A.

Single-chain trap	Targeted ligand(s)	Receptor ectodomain used	Reference structures (PDB entries)	Residues in "natural" linker	Linear distance (Å) for linkage	Minimum residues required for linkage
(ActR-IIa) ²	BMP-7	ActR-IIa-ED	2GOO, 1LX5	28	70	28
(ActR-IIb) ²	Activin Myostatin	ActR-IIb-ED	1S4Y, 1NYU	25	45, 50	18
(BMPR-Ia) ²	BMP-2	BMPR-Ia-ED	2GOO, 1ES7	41	60	24
(TβR-II) ²	TGF-β1 TGF-β3	TβR-II-ED	1KTZ, 1PLO, 1M9Z	35	80	32
(TβR-IIb) ²	TGF-β1 TGF-β3	TβR-IIb-ED	1KTZ, 1PLO, 1M9Z	60	80	32

Table 1. Linker characteristics for select examples of single-chain traps of TGF-β-family growth factors. Minimum number of residues required for linkage represents the structure-based linear distance for linkage (Å) divided by a factor of 2.5.

Table II:

In addition to linkers disclosed elsewhere herein, the following polypeptide sequences may be useful as linkers or components thereof. These polypeptides may be useful
 5 when produced using either L- or D-amino acids. However, with respect to SEQ ID NOs 82 to 118, use of D-amino acids will frequently be preferred.

	COOH -IPPHVQKSVNNDMIVTDNNGAVKFP- NH2	SEQ ID NO 82
	COOH -SEYNTSNPD NH2	SEQ ID NO 83
10	COOH IPPHVQKSDVEMEAQKDEIICPSCNRTAHPLRHINNDMIVTDNNGAVKFP-NH2	SEQ ID 84
	COOH -SEYNTSNPD NH2	SEQ ID NO 85
	COOH -AALLPGAT NH2	SEQ ID NO 86
	COOH -PTTVKSSPGLGPVE NH2	SEQ ID NO 87
15	COOH -AILGRSE NH2	SEQ ID NO 88
	COOH -EM EVTQPTSNPVTPKPPYYNI NH2	SEQ ID NO 89
	COOH -SGRGEAET NH2	SEQ ID NO 90
	COOH -EAGGPEVTYEPPTAPT NH2	SEQ ID NO 91
	COOH -1QNLD SMLHGTGMKSDSDQKKSENGVTLAPED NH2	SEQ ID NO 92
20	COOH -PVVIGPFFDGSIR N	SEQ ID NO 92
	COOH -SEYNTSNPDIPPHVQKSVNNDMIVTDNNGAVKFP NH2	SEQ ID NO 93
	COOH -SEYNTSNPDIPPHVQKSDVEMEAQKDEIICPSCNRTAHPLRHINNDMIVT	DNNGAVKFP NH2 SEQ ID NO 94
	COOH -EAGGPEVTYEPPTAPTSGRGEAET NH2	SEQ ID NO 95
25	COOH -PVVIGPFFDGSIRQNLD SMLHGTGMKSDSDQKKSENGVTLAPED N	SEQ ID NO 96
	COOH -PVVIGPFFDGSIRGNLD SMLHGTGMKSDSDQKKSENGVTLAPED NH2	SEQ ID NO 97
	COOH -SEYNTSNPDGPPHVQKSVNNDMIVTDNNGAVKFP NH2	SEQ ID NO 98
30	COOH -EAGGPEVTGEPPTAPTSGRGEAET NH2	SEQ ID NO 99
	COOH -SEYNTSNPDGGRHVQKSDVEMEAQKDEIICPSCNRTAHPLRHINND	MIVTDNNGAVKFP NH2 SEQ ID NO 100
	COOH -SEYNTSNPDGGPHVQKSVNNDMIVTDNNGAVKFP NH2	SEQ ID NO 101
	COOH -SEYNTSNPDGGRHVQKSVNNDMIVTDNNGAVKFP N -	SEQ ID NO 102

- COOH-SEEYNTSNPSGGGSGGGSGGGMEAQKDEIICPSCNRRTAHPLRHINND
MIVTDNNGAVKFP NH2 SEQ ID NO 103
- COOH -SEEYNTSNPSGGGSGGGSGGKSVNNDMIVTDNNGAVKFP NH2 SEQ ID NO 104
- COOH -SEEYNTSNPSGGGSGGGSGGGSGGDMIVTDNNGAVKFP NH2 SEQ ID NO 105
- 5 -SEEYNTSNPDIPPHVQKSGGGSGGGSGGGSGGGSGGGSGGGSGGGNNDMI
VTDNNGAVKFP NH2 SEQ ID NO 106
- COOH -SEEYNTSNPDGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGNND
MIVTDNNGAVKFP NH2 SEQ ID NO 107
- COOH -SEEYNTSNPDIPPHVQKSVNNDMIVTDNNGAVKFP NH2 SEQ ID NO 108
- 10 COOH -SEEYNTSNPDIPPHVQKSVNNDMIPPHVQKSVNNDMIVTDNNGAVKFP NH2
SEQ ID NO 109
- COOH -SEEYNTSNPPHVQKSVNNDMIVTDNNGAVKFP NH2 SEQ ID NO 110
- COOH -SEEYNTSNPDGGGGGGGGGIPPHVQKSVNNDMIVTDNNGAVKFP NH2 SEQ
ID NO 111
- 15 COOH -SEEYNTSNPDGGGSGGGSGGGSIIPPHVQKSVNNDMIVTDNNGAVKFP NH2
SEQ ID NO 112
- COOH -SEEYNTSNPDIPPHVQKSDVEMEAQKDEIICPSCNRRTAHPLRHINNDM
IVTDNNGAVKFP NH2 SEQ ID NO 113
- COOH -SEEYNTSNPDIPPHVQKSDVEMEAQKDERTAHPLRHINNDMIVTDNNGAVK
20 FP NH2 SEQ ID NO 114
- COOH -EAGGPEVTYEPPTAPTSGRGEAET NH2 SEQ ID NO 115
- COOH -EAGGPEVTYEPPTAPTGGGGGGGGGGSGRGEAET NH2 SEQ ID NO 116
- COOH -PVVIGPFFDGSIRQNLDSMLHGTGMKSDSDQKKSENGVTLAPED NH2
SEQ ID NO 117
- 25 COOH -PVVIGPDGSIRQNLDSHGTGMKSDSDQKKSENGVTLAPED NH₂ SEQ ID NO
118

Also contemplated are nucleic acid sequences encoding such linkers.

We Claim:

1. A multivalent binding agent with affinity for a member of the TGF- β superfamily, said agent comprising the general structure I:
5 (<bd1>-linker1)_k-[$\{\text{<bd1>-linker2-<bd2>-linker3-}$]_n-(<bd3>)_m-(linker4-<bd4>)_d]_h,
where:
- n and h are independently greater than or equal to 1;
- d, f, m and k are independently equal to or greater than zero;
- bd1, bd2, bd3 and bd4 are polypeptide binding domains having an affinity for the same
10 member of the TGF- β superfamily, with bd1, bd2, bd3, and bd4 being independently the same or different from each other; and,
-linker1, linker2, linker3 and linker4 are unstructured polypeptide sequences;
wherein the number of amino acids in each linker is determined independently and is greater than or equal to X/2.5; where,
15 X equals the shortest linear distance between:
(a) the C-terminus of an isolated form of the binding domain that is located at the N-terminus of the linker and that is specifically bound to its ligand; and,
(b) the N-terminus of an isolated form of the binding domain that is located at the C-terminus of the linker and that is specifically bound to its ligand.
20
2. The agent of claim 1 wherein the member of the TGF- β superfamily to which the binding domains have affinity is selected from the group consisting of: TGF- β 1, TGF- β 2, TGF- β 3, activin β A, activin β B, activin β C, activin β E, bone morphogenic protein (BMP) 2, BMP 3, BMP4, BMP 5, BMP 6, BMP 7, BMP 8, BMP 9, BMP 10, BMP 11, BMP 12,
25 BMP 13, BMP 14, BMP 15, growth differentiation factor (GDF) 1, GDF 3, GDF 8, GDF 9, GDF 15, Nodal, Inhibin α , anti-Mullerian Hormone, Lefty 1, Lefty 2, arteman, Persephin and Neurturin.
3. The agent of claim 2 wherein the member of the TGF- β superfamily to which the binding domains have affinity is selected from the group consisting of: TGF- β 1, TGF- β 2, TGF- β 3, BMP2, GDF 8, and activin.
30
4. The agent of any preceding claim wherein the linker is between 25 and 60 amino acids in length

5. The agent of any preceding claim wherein bd4 is the same as bd1, bd 2 is the same as bd3, h>0, and d, f, m, and n=1.

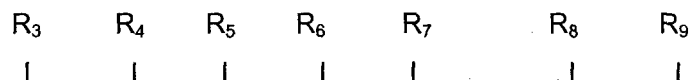
5 6. The agent of any preceding claim comprising any one or more of SEQ ID No 75 to 82.

7. The agent of any preceding claim comprising one or more of SEQ ID NO 31-42 or 49-74 as a linker sequence.

10

8. The agent of any preceding claim wherein one or more of bd1, bd2, bd3, and bd4 is selected from one of SEQ ID NO 43-48.

15 9. The agent of any preceding claim having the general structure V:



$R_1-(\text{<bd1>-linker1})_k-[\{\text{<bd1>-linker2-<bd2>-linker3}\}_n-(\text{<bd3>})_m-(\text{linker4-<bd4>})_d]_h-R_2$

20

Wherein R_1 , R_2 , R_3 , R_4 , R_5 , R_6 , R_7 , R_8 , R_9 , may be the same or different, may not be present and when present, may independently be one or more of a fusion protein for targeting, a single domain antibody, a radiotherapy agent, an imaging agent, a fluorescent dye, a fluorescent protein tag, a cytotoxic agent for chemotherapy, a nano particle-based carrier, a polymer-conjugated to drug, nanocarrier or imaging agent, a stabilizing agent, a drug, a nanocarrier, a support, and a dendrimer.

25

10. A method of designing a multivalent linker useful in modulating responsiveness of a cell to a member of the TGF- β superfamily, said method comprising:

30

- a) identifying a member of the TGF- β superfamily of interest;
- b) obtaining two polypeptide binding domains having affinity for different sites on the member of the TGF- β superfamily member;
- c) obtaining an unstructured polypeptide linker of at least a number of amino acids equal to $(X/2.5)$,

wherein the number of amino acids in each linker is determined independently and is greater than or equal to $X/2.5$; where,

X equals the shortest linear distance between:

- (i) the C-terminus of an isolated form of the binding domain that is located at the N-terminus of the linker and that is specifically bound to its ligand; and,
 - (ii) the N-terminus of an isolated form of the binding domain that is located at the C-terminus of the linker and that is specifically bound to its ligand; and
- d) modeling the linker having one of the binding domains covalently attached at each end, and carrying out molecular dynamics simulations to substantially minimize molecular mechanics energy and reduce steric and electrostatic incompatibility between the linker and the member of the TGF- β superfamily.

11. The method of claim 10 further including a step e) producing a fusion protein comprising the two polypeptide binding domains joined by the unstructured polypeptide linker.

12. An isolated polypeptide having at least 80% sequence identity to a natural unstructured region in the extracellular portion of a receptor for a member of the TGF- β superfamily and being substantially free of structured regions capable of specific binding to a member of the TGF- β superfamily.

13. The isolated polypeptide of claim 12 having at least 80% sequence identity to one or more of SEQ ID NO 31-42 and SEQ ID NOs 49-74.

14. A polypeptide comprising a region having at least 80% sequence identity to one or more of SEQ ID NOs 53-74 and SEQ ID NOs 82-118.

15. The polypeptide of claim 14 having a region with at least 90 % sequence identity to one or more of SEQ ID NOs 53-74.

16. A polypeptide having between 43 % and 99 % sequence identity to a naturally unstructured region in the ectodomain of a receptor for a member of the TGF- β superfamily.

17. A nucleic acid sequence encoding a polypeptide of any preceding claim.
18. A method of modulating the response of a cell to a TGF- β superfamily member in its environment, said method comprising exposing the cell to an agent of any one of
5 claims 1 to 9.
19. A data storage medium comprising instructions for determining the minimum linker length in step c of claim 10.
- 10 20. A data storage medium comprising a means for identifying acceptable minimal linker length in step c of claim 10.
21. Use of an agent of claim 1 to concentrate ligand in a sample.
- 15 22. Use of an agent of claim 1 to purify ligand.
23. Use of an agent of claim 9 in diagnosis of a condition characterized in whole or part by an abnormality in levels of one or more TGF- β superfamily members in the body or a portion thereof.
20
24. Use of an agent of claim 9 in targeting delivery of a compound to a site of interest within a body.

Figure 1A. Amino-acid sequences corresponding to intrinsically unstructured regions in the extracellular portions of select TGF-β-superfamily receptors.

TGF-β-superfamily receptor	Sequences corresponding to unstructured extracellular regions	
Human TβR-II	¹ IPPHVQKSVNNDMIVTDNNGAVKFP ²⁵	SEQ ID NO 31
	¹²⁷ SEYNTSNPD ¹³⁶	SEQ ID NO 32
Human TβR-IIb	¹ IPPHVQKSDVEMEAQKDEIICPSCNRTAHPLRHINNDMIVTDNNGAVKFP ⁵⁰	SEQ ID NO 33
	¹⁵² SEYNTSNPD ¹⁶¹	SEQ ID NO 34
Human TβR-I	¹ AALLPGAT ⁸	SEQ ID NO 35
	⁸⁸ PTTVKSSPGLGPE ¹⁰¹	SEQ ID NO 36
Human ActR-IIa	¹ AILGRSE ⁷	SEQ ID NO 37
	⁹⁹ EMEVQTQPTSNPVTPKPPYYNI ¹²⁰	SEQ ID NO 38
Human ActR-IIb	¹ SGRGEAET ⁸	SEQ ID NO 39
	⁹⁹ EAGGPEVTEPPPTAPT ¹¹⁶	SEQ ID NO 40
Human BMPR-Ia	¹ QNLDSDLHGTGMKSDSDQKKSENGVTLAPED ³¹	SEQ ID NO 41
	¹¹⁷ PVVGPFDDGSIR ¹²⁹	SEQ ID NO 42

Note: Residue numbering starts after signal peptide.

Figure 1B. Amino-acid sequences corresponding to structured ligand-binding domain regions in the extracellular portions of select TGF-β-superfamily receptors.

TGF-β-superfamily receptor	Sequences corresponding to structured extracellular regions (ligand-binding domains)
Human TβR-II	26QLCKFCDVRFSTCDNQKSCMSNCISITICEKPEVCVAVWRKNDENITLETVCHDPKLPYHDFILE DAASPKCIMKEKKKPGETFFMCSDECDNDNIIIF¹²⁶ SEQ ID NO 43
Human TβR-IIb	51QLCKFCDVRFSTCDNQKSCMSNCISITICEKPEVCVAVWRKNDENITLETVCHDPKLPYHDFILE DAASPKCIMKEKKKPGETFFMCSDECDNDNIIIF¹⁵¹ SEQ ID NO 44
Human TβR-I	9ALQCFCCHLCTKDNFTCVTDGLCFVSVTETTDKVIHNSMCIAEIDLIPDRPFVFCAPSSKGTGSVTITY CCNQDHCNKIEL⁸⁷ SEQ ID NO 45
Human ActR-IIa	8TQECLFFNANWEKDRTNQTGVEPCYGDKDKRRHCFATWKNISGSIEIVKQGCWLDINCYDRTDCVE KKDSPVYFCCCEGNMCNEKFSYFP⁹⁸ SEQ ID NO 46
Human ActR-IIb	9RECIYYNANWELERTNQSLERCEGEQDKRLHCYASWRNSSGTIELVKKGCWLDNFNCYDRQECVAT EENPQVYFCCCEGNFCNERFTHLPEAGGPEVTYEPPTAPT⁹⁸ SEQ ID NO 47
Human BMPR-Ia	32TLPFLKCYCSGHCPPDDAINNTCITNGHCFATIEEDDQGETTLASGCMKYEGSDFQCKDSPKAQLRR TIECCRTNLCNQYLQPTLPPVVGPFDDGSIR¹¹⁶ SEQ ID NO 48

Note: Residue numbering starts after signal peptide.

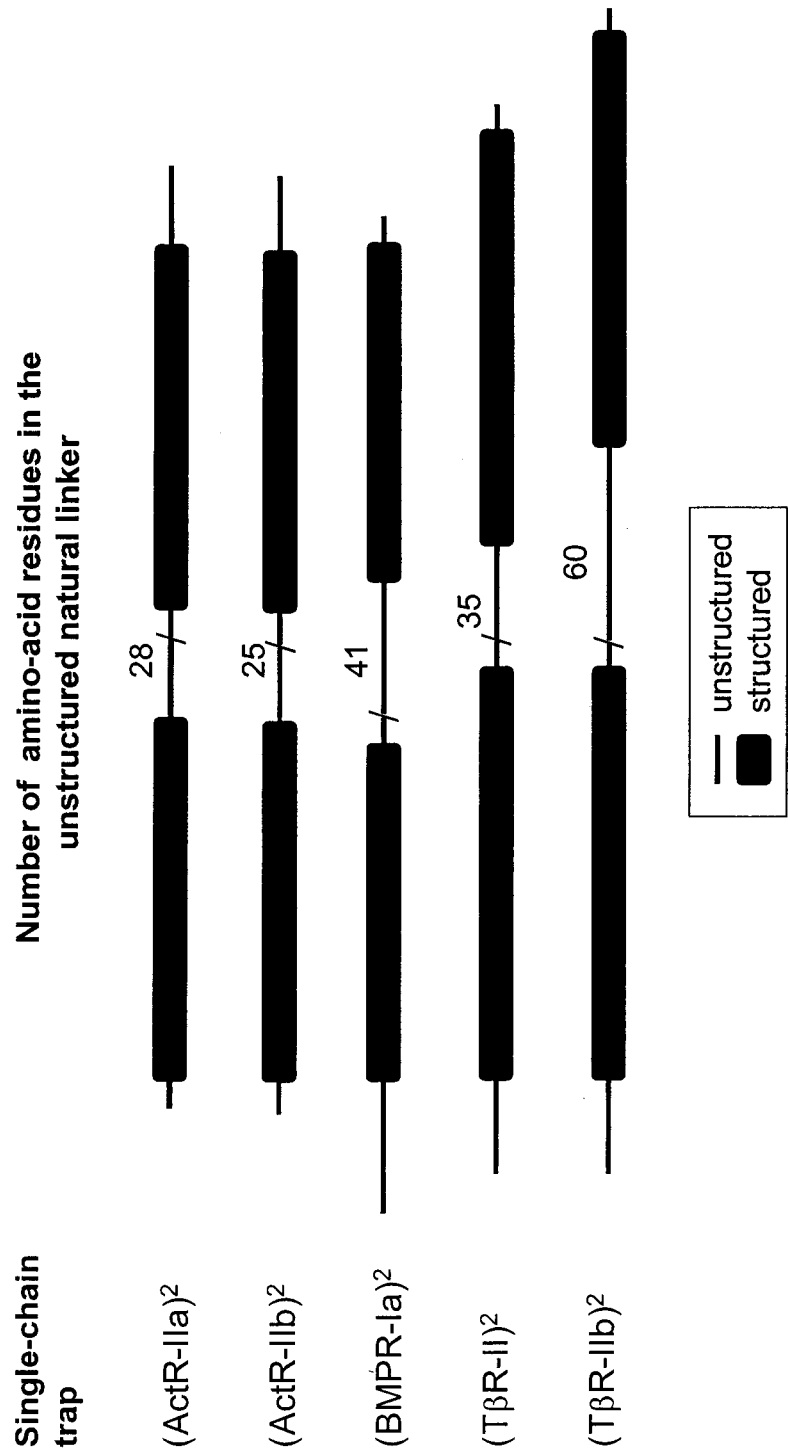


Figure 2A. Examples of in-line fused receptor ectodomains as homo-bivalent single-chain bivalent traps of several TGF-β-family growth factors. The “/” sign indicates the point of fusion.

Figure 2B. Examples of sequences corresponding to natural linkers of homo-bivalent single-chain traps resulting from fusion of the entire extracellular portions of select TGF- β -superfamily receptors.

Single-chain trap	Linker length (a.a.)	Linkers of natural sequence	
(T β R-II) ²	35	¹²⁷ SEEYNTSNPD:IPPHVQKSVNNDMIVTDNNGAVKFP ¹⁶¹	SEQ ID NO 49
(T β R-IIb) ²	60	¹²⁷ SEEYNTSNPD:IPPHVQKSDVEMEAQKDEIICPSCNRRTAHPRLRHINNDMIVTDNNGAVKFP ¹⁸⁷	SEQ ID NO 50
(ActR-IIb) ²	25	⁹⁹ EAGGPEVTYEPPTAPT:SGRGEAET ¹²⁴	SEQ ID NO 51
(BMPR-Ia) ²	44	¹¹⁷ PVVIGPFFDGSIR:QNLDMSMLHGTGMKSDSDQKKSENGVTIAPED ¹⁶⁰	SEQ ID NO 52

Notes: Residue numbering corresponds to trap construct and starts after N-terminal tag. Fusion position is indicated by (:).

Figure 2D. Examples of sequences corresponding to varying the linker length for homo-bivalent single-chain traps by deleting or repeating of natural sequences, or by inserting of artificial sequences, into the natural linker sequence.

Single-chain trap	Linker length (a.a.)	Linker sequences	
(TβR-II) ²	35	¹²⁷ SEEYNTSNPDIPPHVQKSVNNDMIVTDNNGAVKFP ¹⁶¹	SEQ ID NO 64
(TβR-II) ² -repeat	48	¹²⁷ SEEYNTSNPDIPPHVQKSVNNDMIPPHVQKSVNNDMIVTDNNGAVKFP ¹⁷⁴	SEQ ID NO 65
(TβR-II) ² -delete	33	¹²⁷ SEEYNTSN--PPHVQKSVNNDMIVTDNNGAVKFP ¹⁵⁹	SEQ ID NO 66
(TβR-II) ² -G8	43	¹²⁷ SEEYNTSNPDGGGGGGGIPPHVQKSVNNDMIVTDNNGAVKFP ¹⁶⁹	SEQ ID NO 67
(TβR-II) ² -(G3S) ₃	47	¹²⁷ SEEYNTSNPDGGGGGGGGSGIPPHVQKSVNNDMIVTDNNGAVKFP ¹⁷³	SEQ ID NO 68
(TβR-IIb) ²	60	¹²⁷ SEEYNTSNPDIPPHVQKSDVEMEAQKDEICPSCNRHTAHLRHINNDMIVTDNNGAVKFP ¹⁸⁷	SEQ ID NO 69
(TβR-IIb) ² -delete	53	¹²⁷ SEEYNTSNPDIPPHVQKSDVEMEAQKDE-----RTAHLRHINNDMIVTDNNGAVKFP ¹⁸⁰	SEQ ID NO 70
(ActR-IIb) ²	25	⁹⁹ EAGGPEVTYEPPTAPTSGRGEAET ¹²⁴	SEQ ID NO 71
(ActR-IIb) ² -G10	35	⁹⁹ EAGGPEVTYEPPTAPTGGGGGGGGSGRGEAET ¹³⁴	SEQ ID NO 72
(BMPR-Ia) ²	44	¹¹⁷ PVVGPFDFDGSIRQNLDMLHGTGMKSDSDQKKSNGVTLAPED ¹⁶⁰	SEQ ID NO 73
(BMPR-Ia) ² -delete	40	¹¹⁷ PVVGPF--DGSIRQNLD--HGTGMKSDSDQKKSNGVTLAPED ¹⁵⁶	SEQ ID NO 74

Notes: Residue numbering corresponds to trap construct and starts after N-terminal tag. Added amino-acid sequences, either natural or artificial, are underlined. Deletions are denoted by dashes. Natural linker sequences are also included as reference.

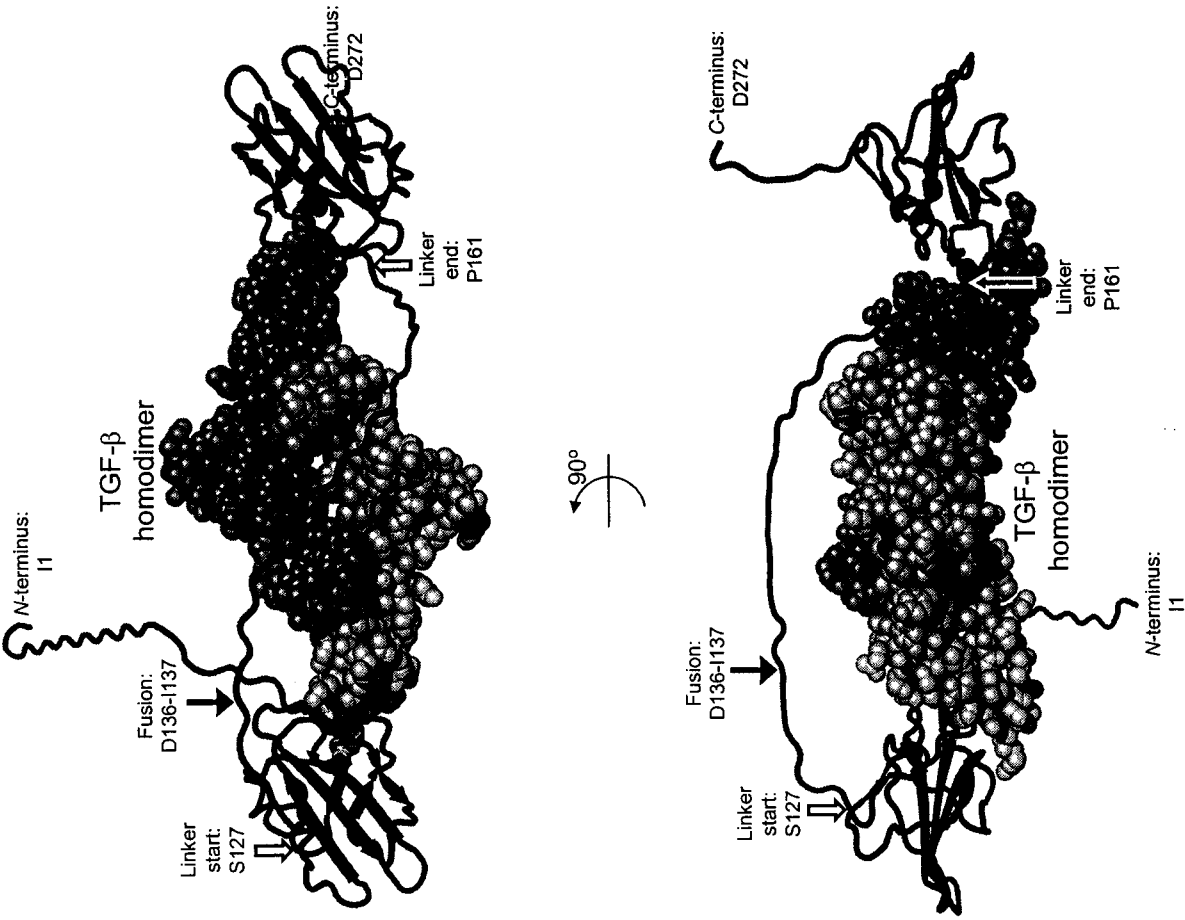
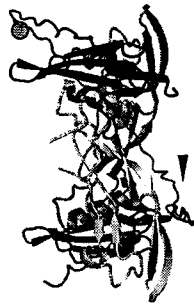
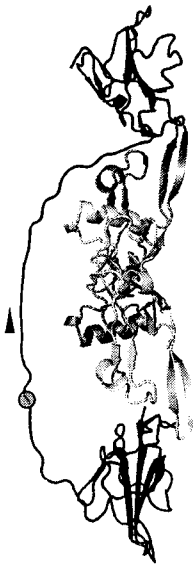


Figure 3. Illustration of the (TβR-II)² single-chain trap construct on a three-dimensional molecular mechanical model of the (TβR-II)² single-chain trap bound to the TGF-β₃ growth factor. Two 90°-rotated views are provided.

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(BMPR-Ia)² / BMP-2
complex(ActR-IIb)² / Activin
complex(TβR-II)² / TGF-β3
complex

90°

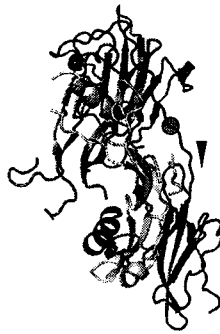


Figure 4. Feasibility of trap constructs with natural linkers from three-dimensional structural models. Shown are molecular mechanics energy-minimized natural linkers for (TβR-II)², (ActR-IIb)² and (BMPR-Ia)² homo-bivalent single-chain traps in complex with the TGF-β3, Activin and BMP-2 growth factors, respectively. Each growth factor covalent dimer is rendered in gray. Each single-chain trap is rendered in black, and consists of two folded binding domains and in intervening unstructured linker. Each dot indicates the point of fusion in the linker region between two receptor ectodomains to generate the single-chain trap. Arrowheads indicate polypeptide chain direction in the trap's linker. Two 90°-rotated views are provided for each complex.

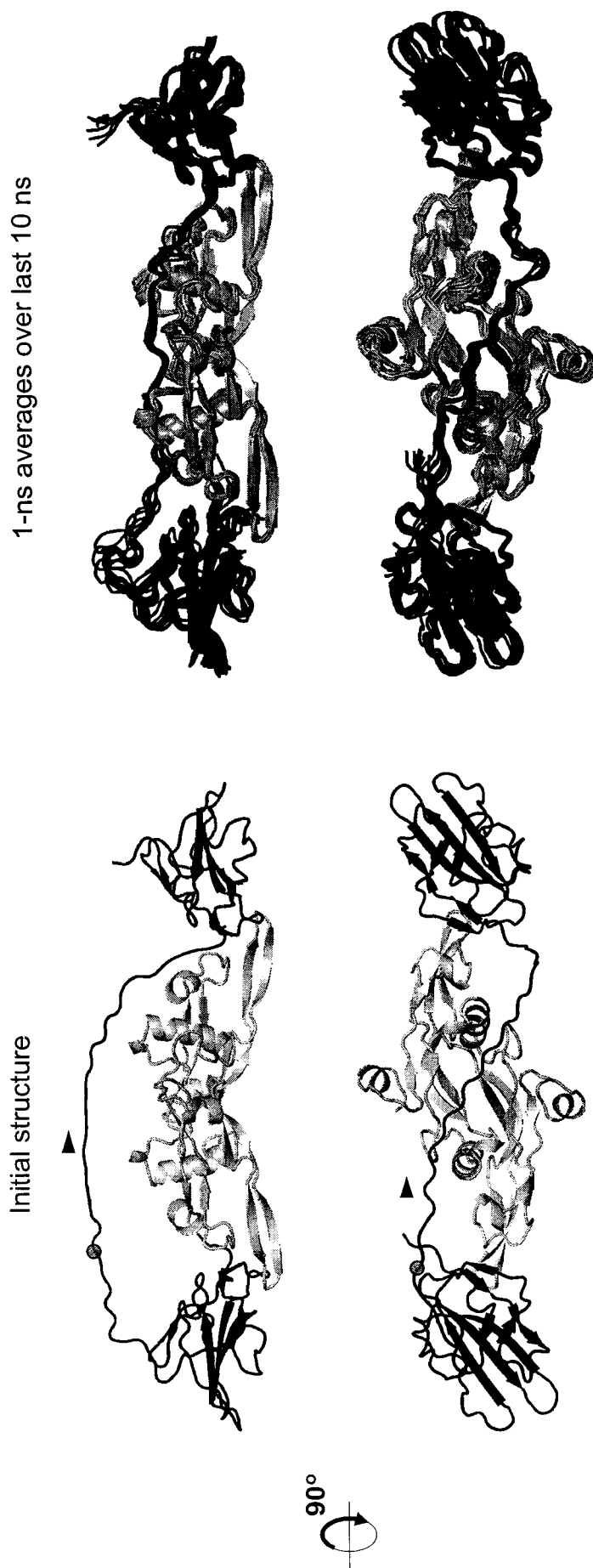
(T β R-II)² / TGF- β 3
complex

Figure 5A. Molecular dynamics (MD) model for the (T β R-II)² homo-bivalent single-chain trap bound to the TGF- β 3 growth factor (right images). An initial model with energy-minimized linker and with ligand-binding domains in crystallographic positions bound onto the growth factor is also shown for reference (left images, see also Figs. 3 and 4). The single-chain trap is rendered in black and the growth factor covalent dimer is rendered in gray. Ten time-averaged structures (each over 1 ns) covering 10 ns timeframe of MD simulation are overlaid. Two 90°-rotated views are provided.

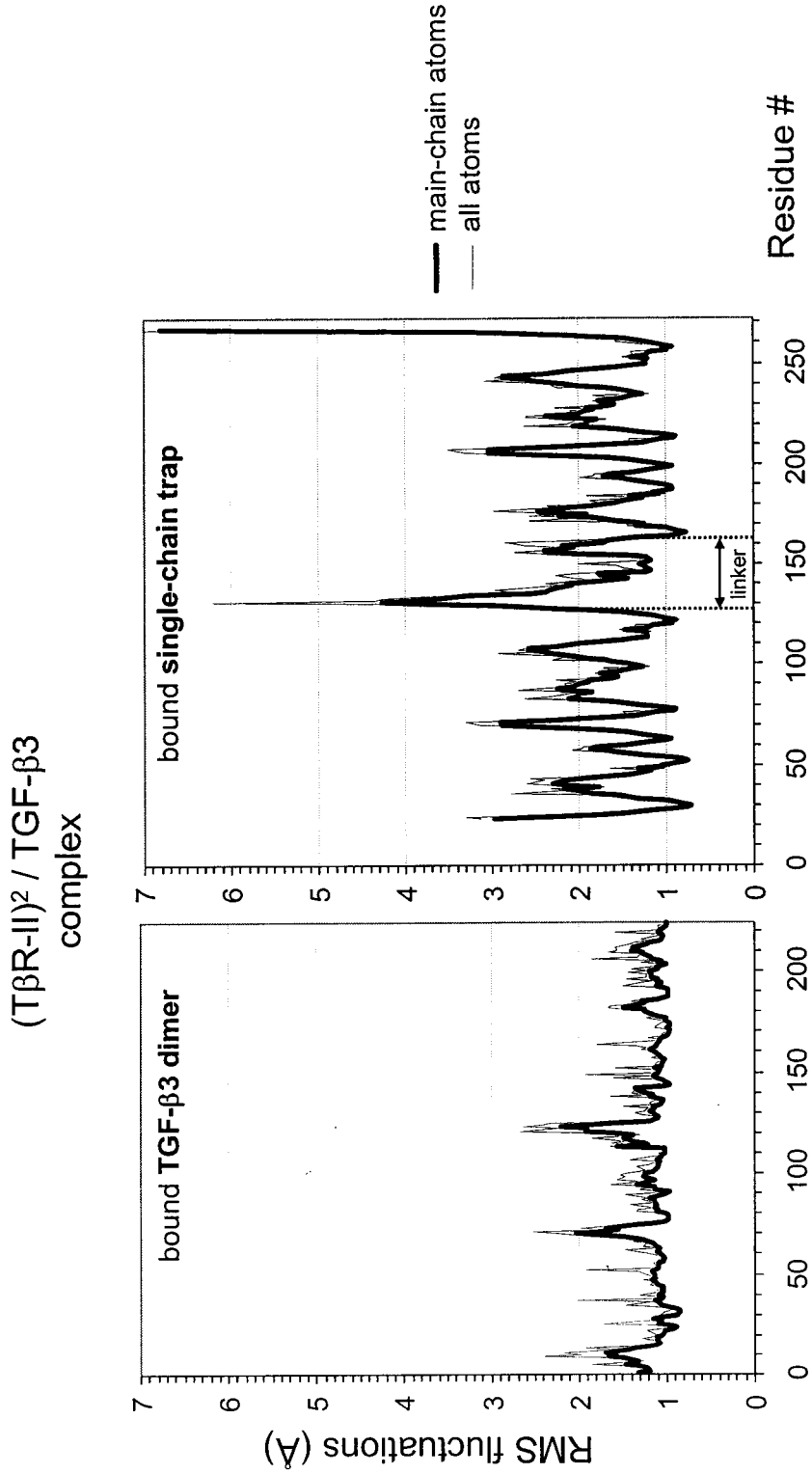


Figure 5B. Per residue root-mean-square (RMS) fluctuations of the $(T\beta R-II)^2 / TGF-\beta 3$ complex, time-averaged over the last 10 ns of MD simulation.

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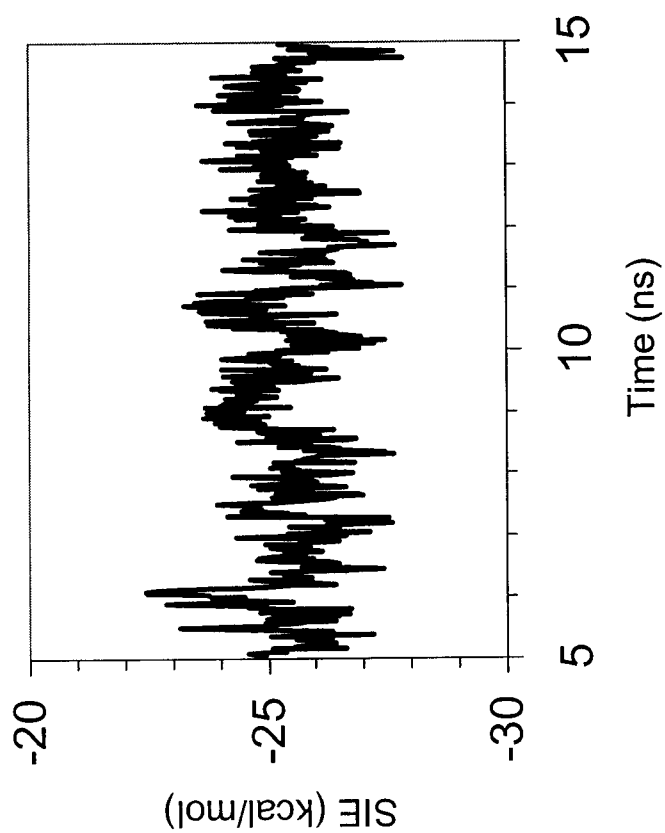


Figure 5C. Solvated Interaction Energy (SIE) between the single-chain (T β R-II)² trap and the TGF- β 3 ligand over the last 10 ns of MD simulation of their complex, with an average value of -25.4 kcal/mol.

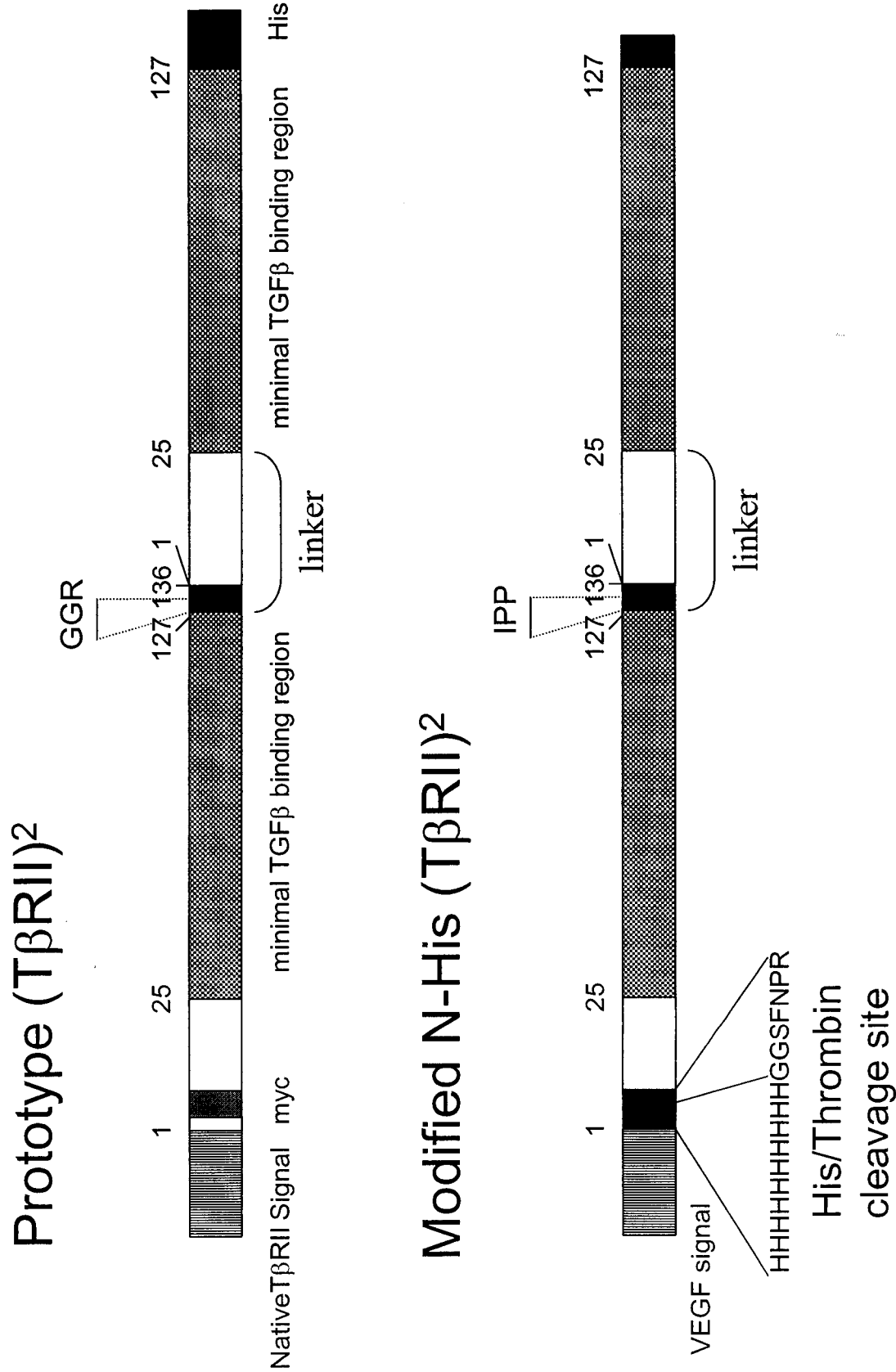


Figure 6. Schematic of prototype (TβRII)² and modified N-His (TβRII)² traps.

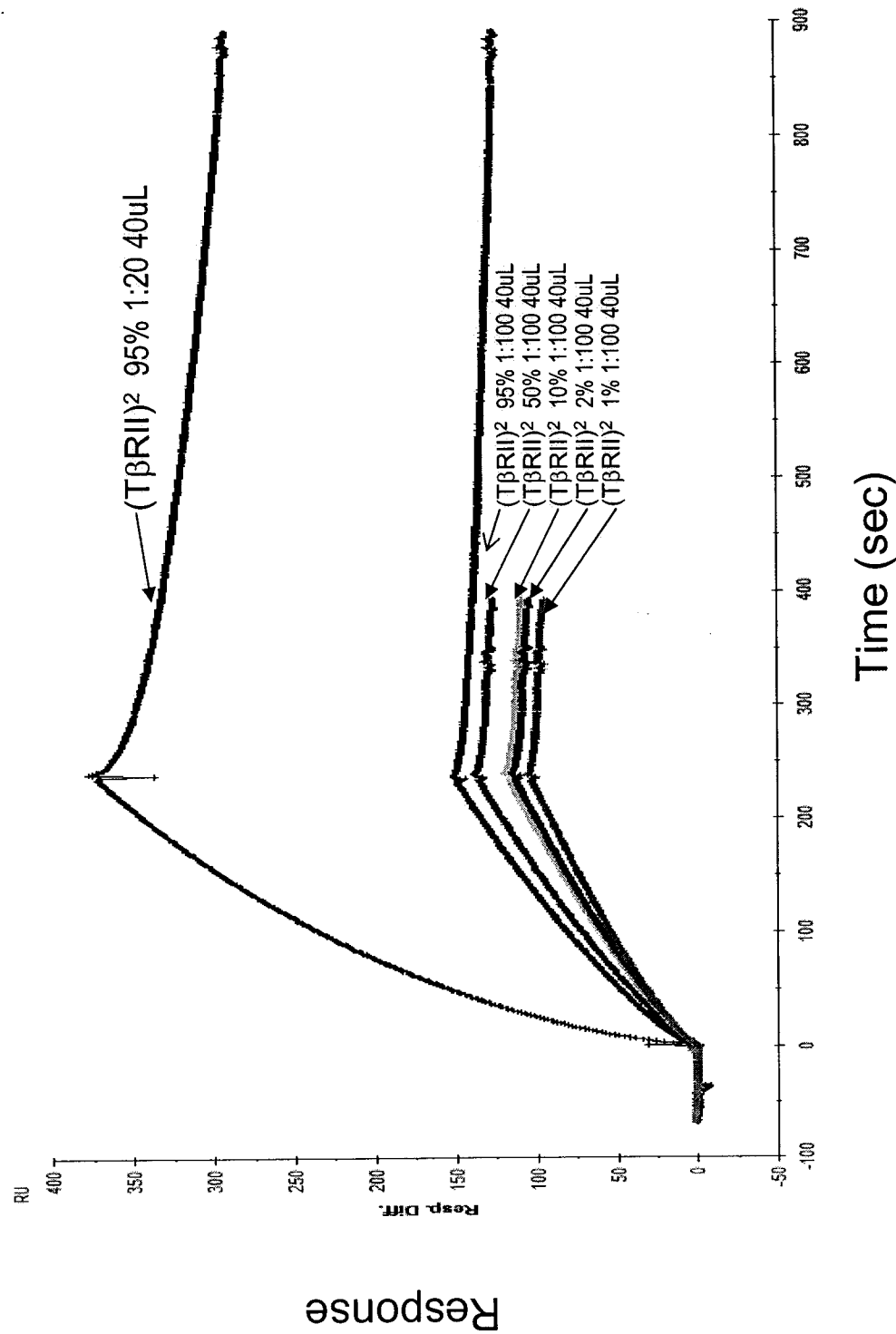
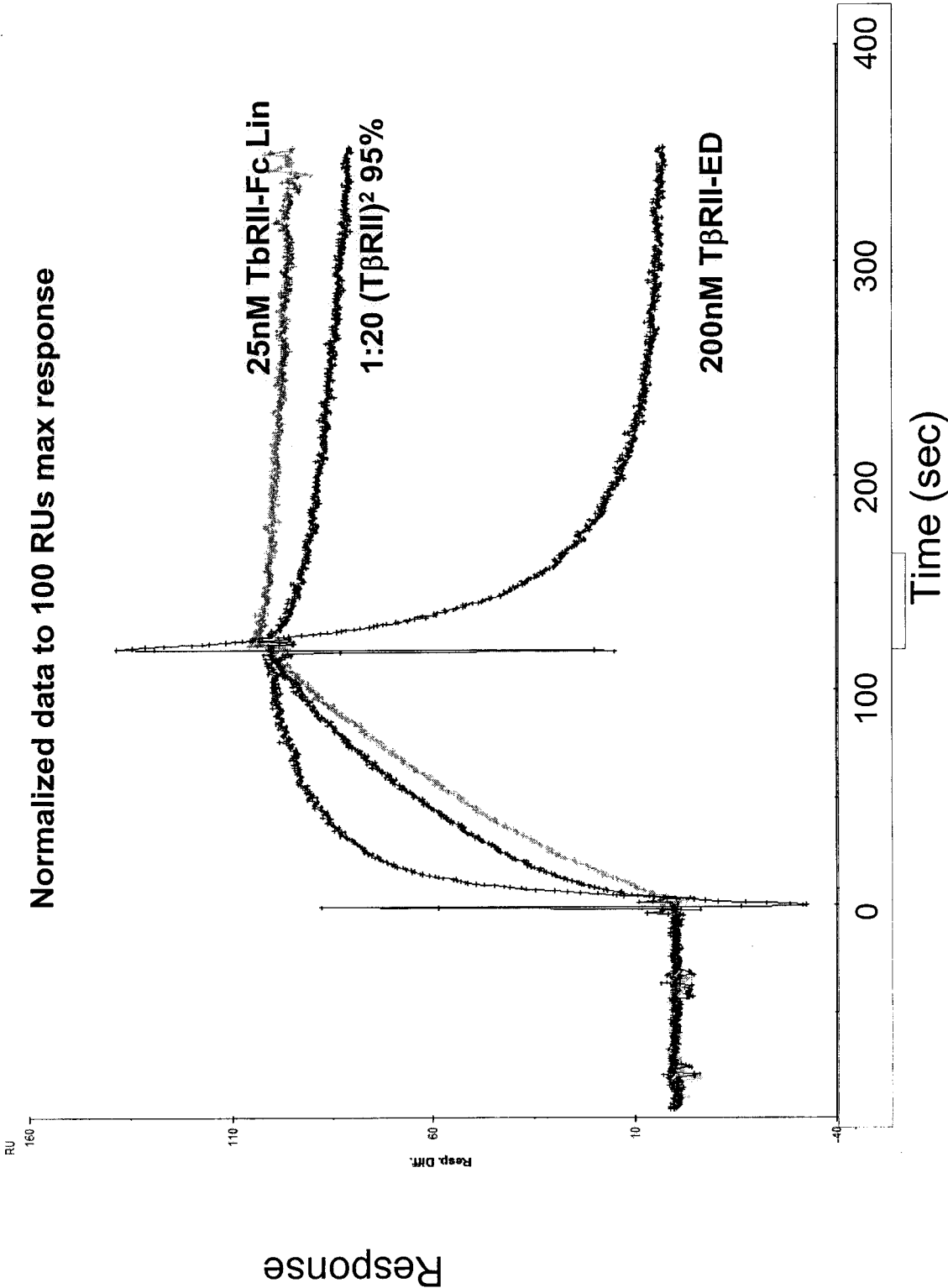


Figure 7A. Surface plasmon resonance sensogram showing prototype (TβRII)² (in diluted conditioned media from different % transfections) binding to surface-immobilized TGF-β3 ligand.

Figure 7B. Surface plasmon resonance sensograms comparing binding of bivalent prototype (TβRII)², bivalent TβRII-Fc and monovalent TβRII to 270 RUs surface-immobilized TGF-β3 ligand.



High level production of N-His (TβRII)²
by transient transfection of 293 cells

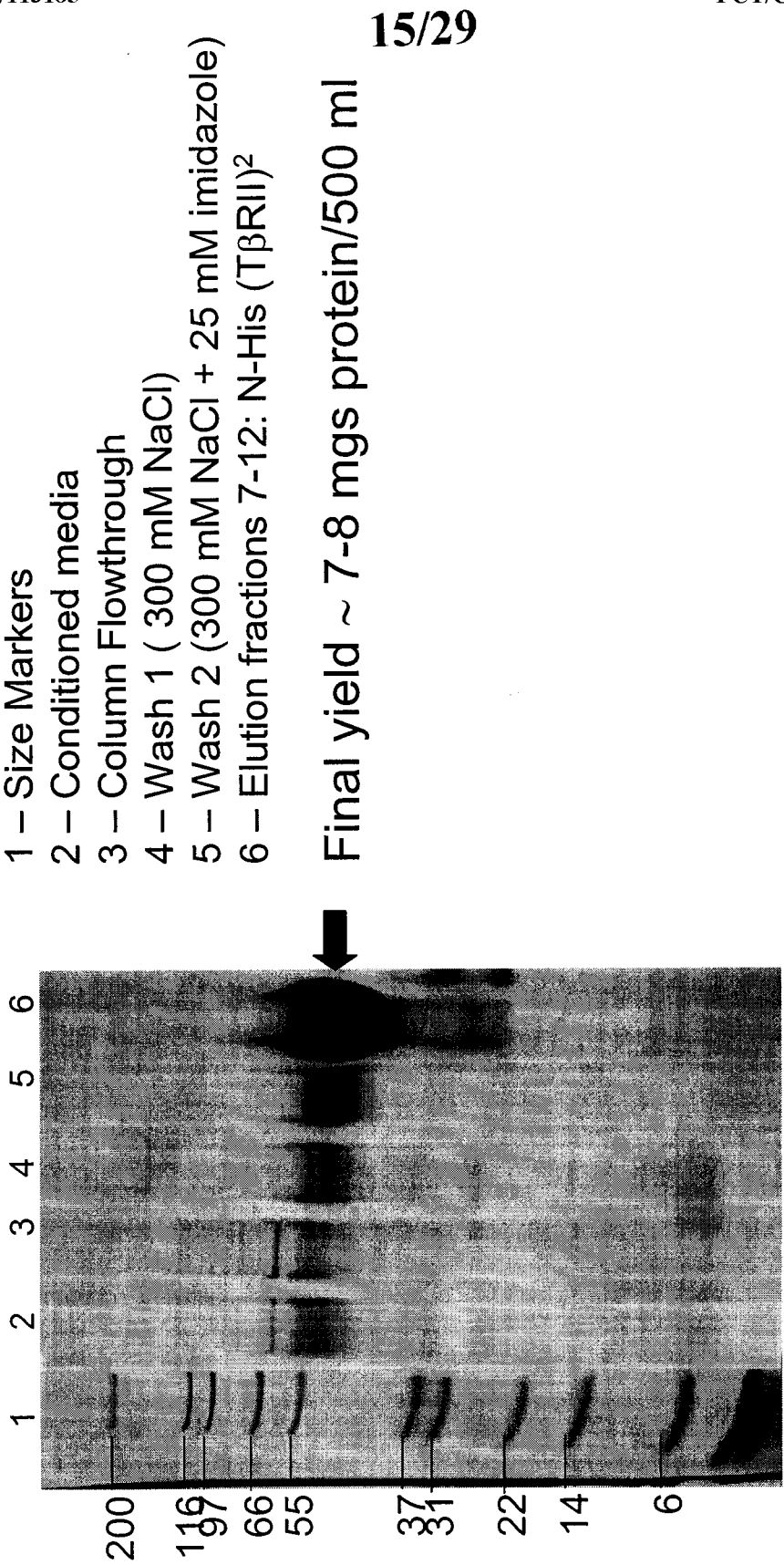


Figure 8. High level production and purification yield of N-His (TβRII)² protein from 500 ml culture of transfected 293 cells.

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	IC50 [nM]
Prototype (TβRII) ²	0.58 (±0.64)
TβRII-Fc (H. Lin)	0.45 (±0.42)
TβRII-Fc (R&D)	0.10 (±0.02)

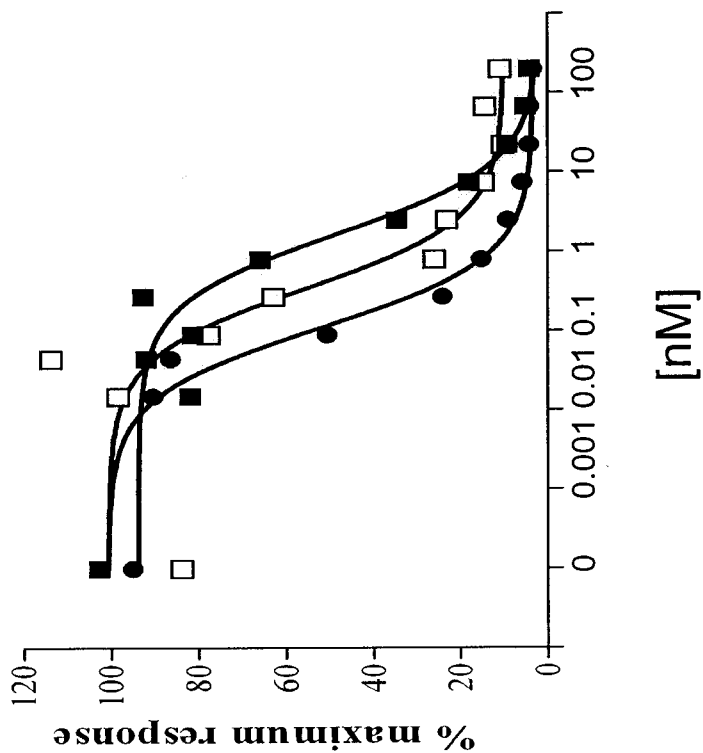


Figure 9A. Inhibition of TGF-β signaling in Mv1Lu luciferase reporter cells by prototype (TbRII)² compared to TβRII-Fc.

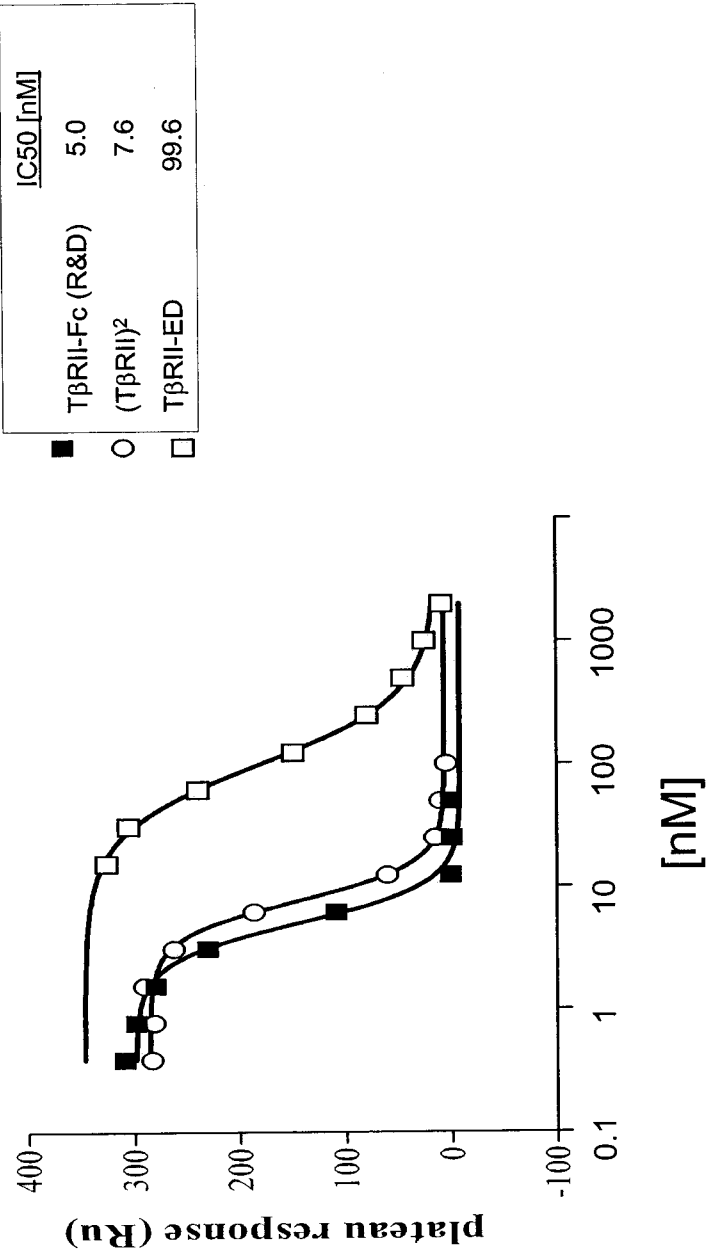


Figure 9B. SPR-based determination of trap binding of TGF- β in solution by prototype (T β RII)² and T β RII-Fc compared to monomeric T β RII-ED.

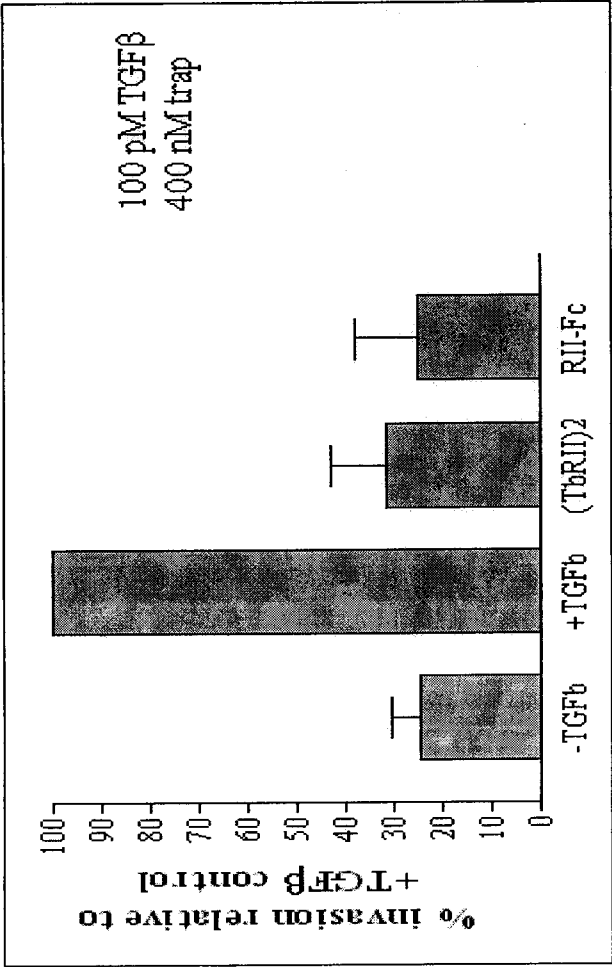
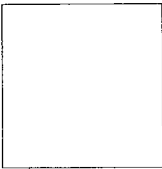


Figure 9C. Inhibition of TGFβ1-induced 4T1 cell invasion *in vitro* by prototype (TβRII)² and TβRII-Fc traps.

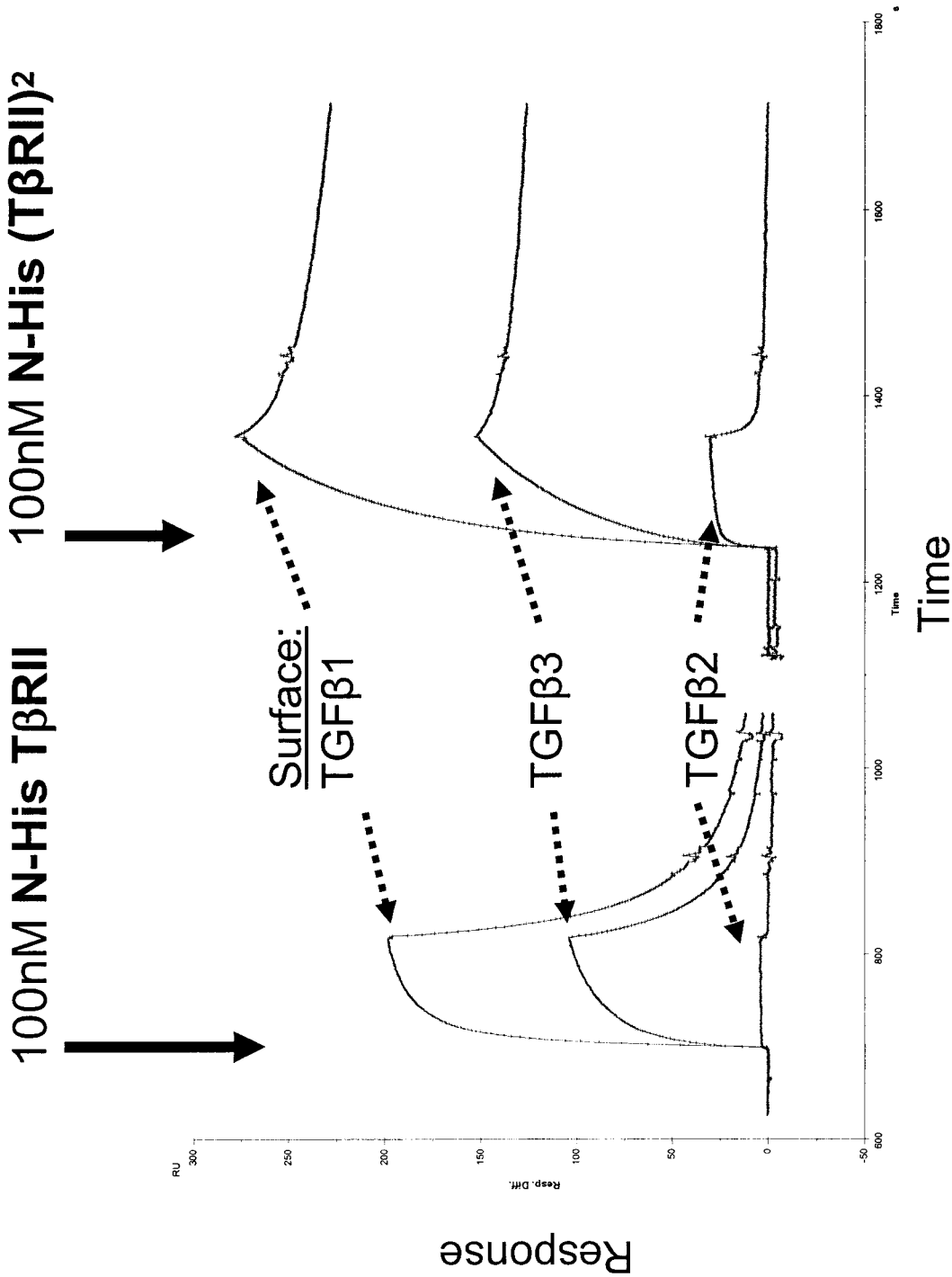


Figure 10A. Biacore sensogram showing direct binding of N-His (TβRII)² and monomeric N-His TβRII to different isoforms of TGF-β.

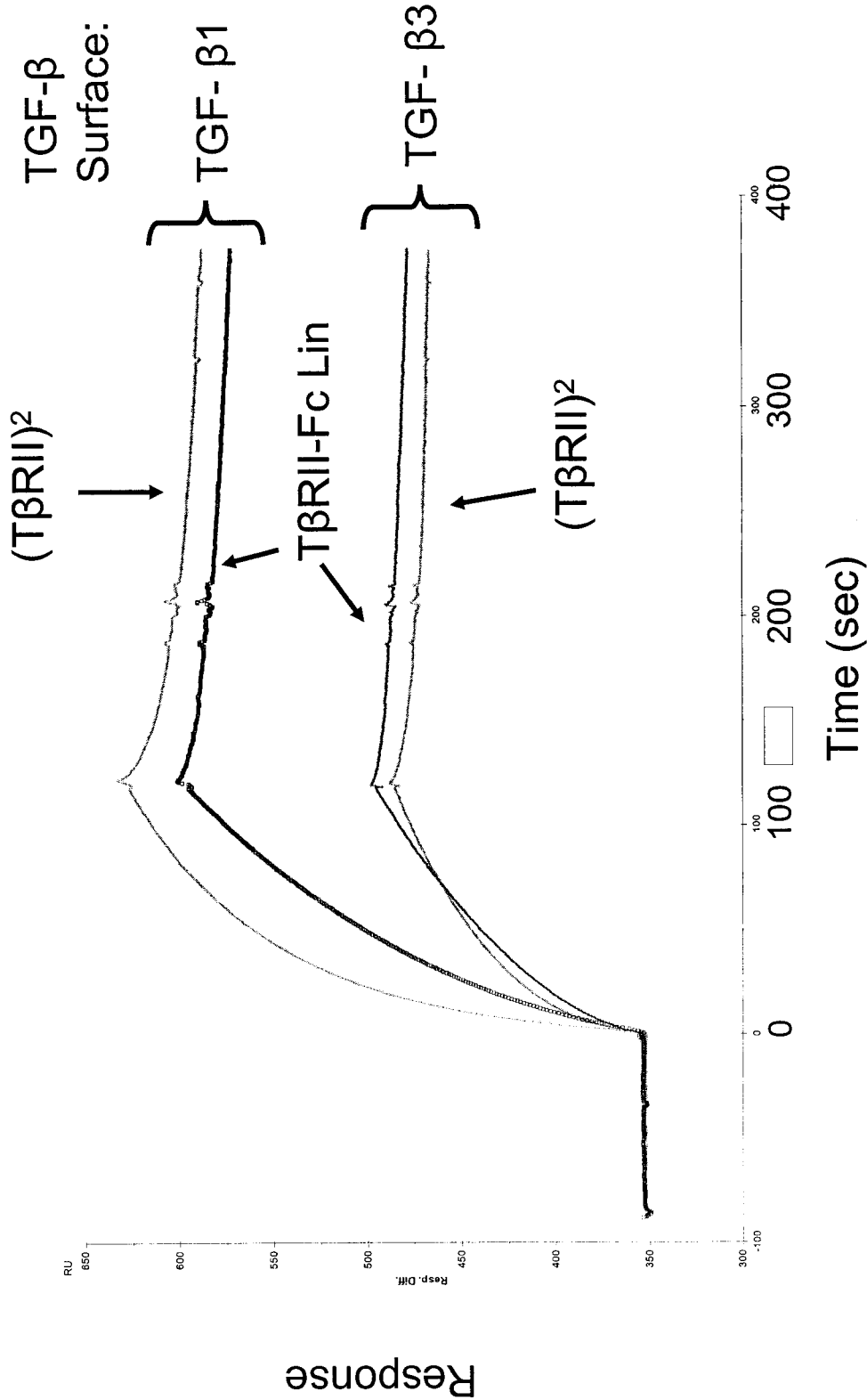


Figure 10B. Comparison of 100 nM N-His (TβRII)² and TβRII-Fc binding to 500 RUs each of TGF-β1 or TGF-β3

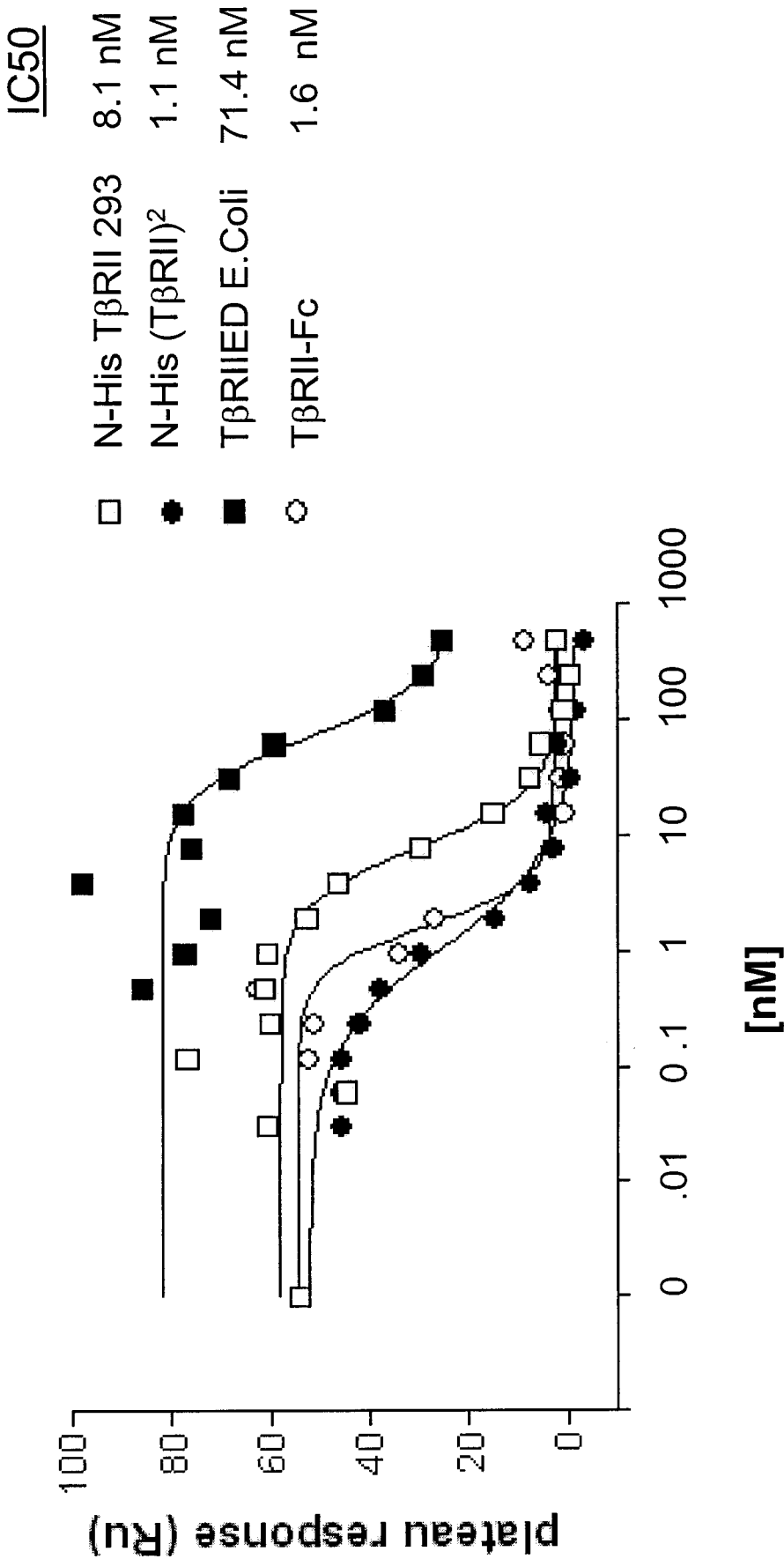


Figure 10C. SPR-based determination of IC50 for trap binding of TGF-β1 (5 nM) in solution. The graph shows efficient binding of TGF-β1 by N-His (TβRII)² trap and TβRII-Fc trap versus reduced binding by monomeric TβRII (293 cell-produced or E.coli-produced).

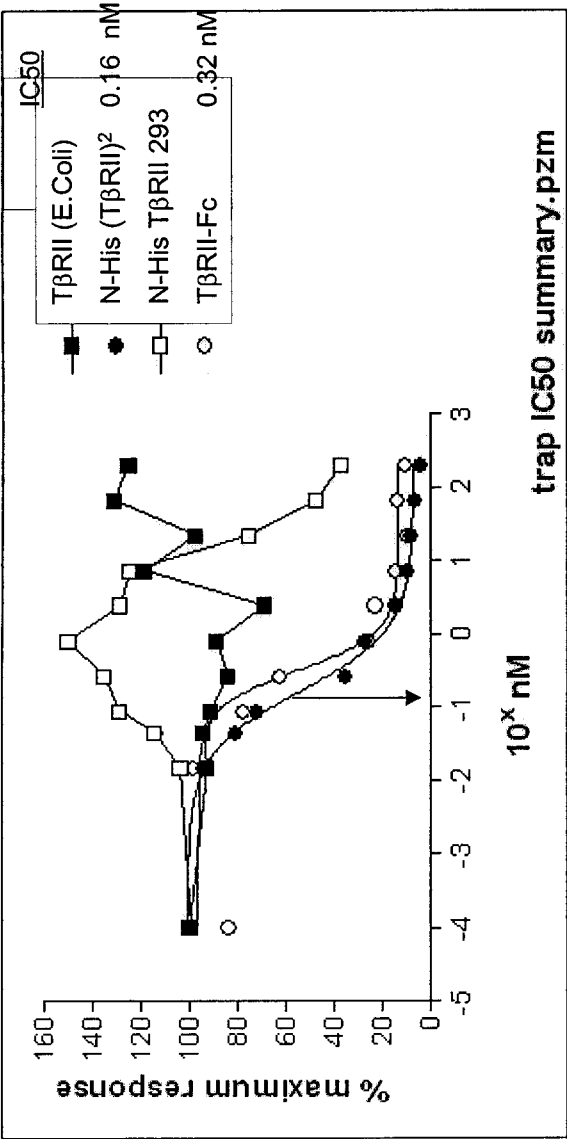


Figure 10D. Efficient inhibition of TGF- β signaling in Mv1Lu luciferase reporter cells by N-His (TbRII)² and T β RII-Fc compared to poor inhibition by monomeric T β RII (293 cell-produced and E-Coli-produced).

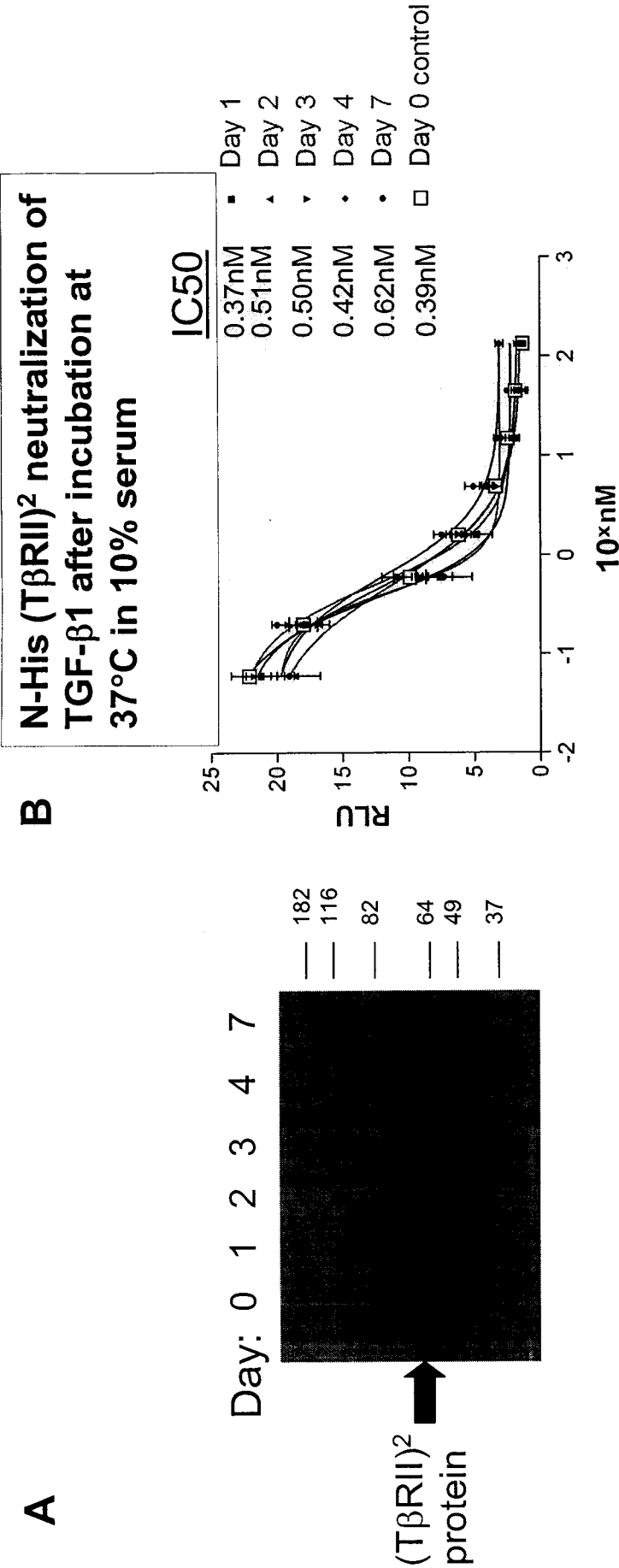
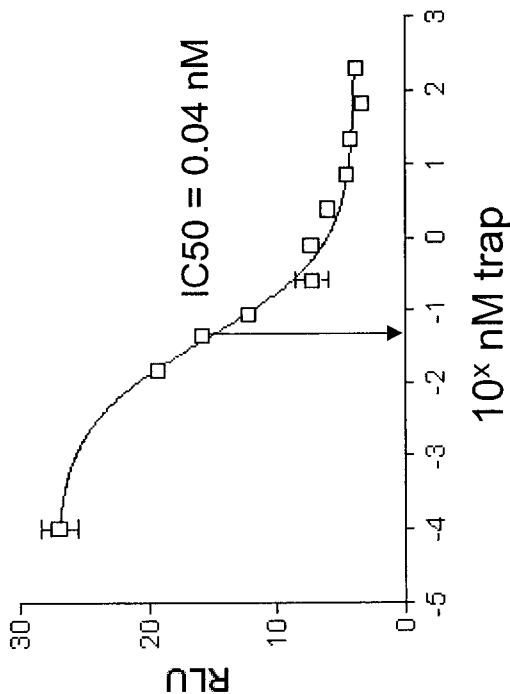


Figure 11. N-His (TβRII)² exhibits long-term stability and activity in 10% serum at 37°C

B

**Neutralization of TGF- β 1 by
N-His (T β R1Ib)² trap**



**Coinject TGF- β traps with 5nM
TGF- β 1 over 1D11 surface**

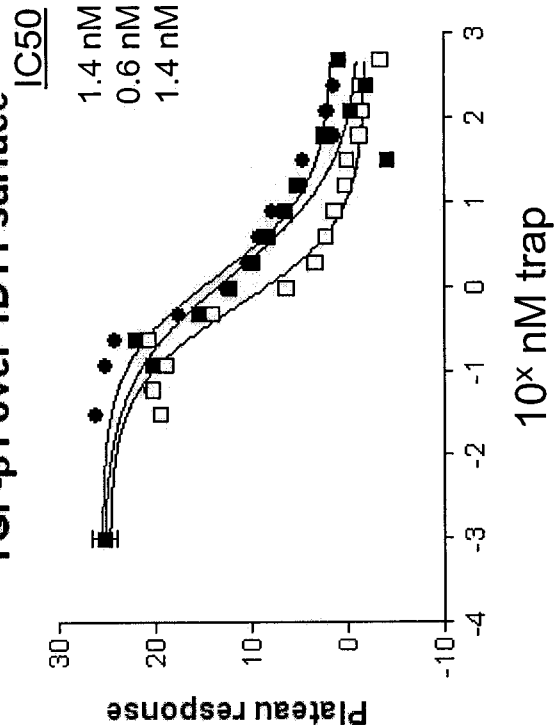


Figure 12. Efficient neutralization of TGF- β 1 (A) and binding of TGF- β 1 in solution (B) by (T β R1Ib)² trap having 60 amino acid linker.

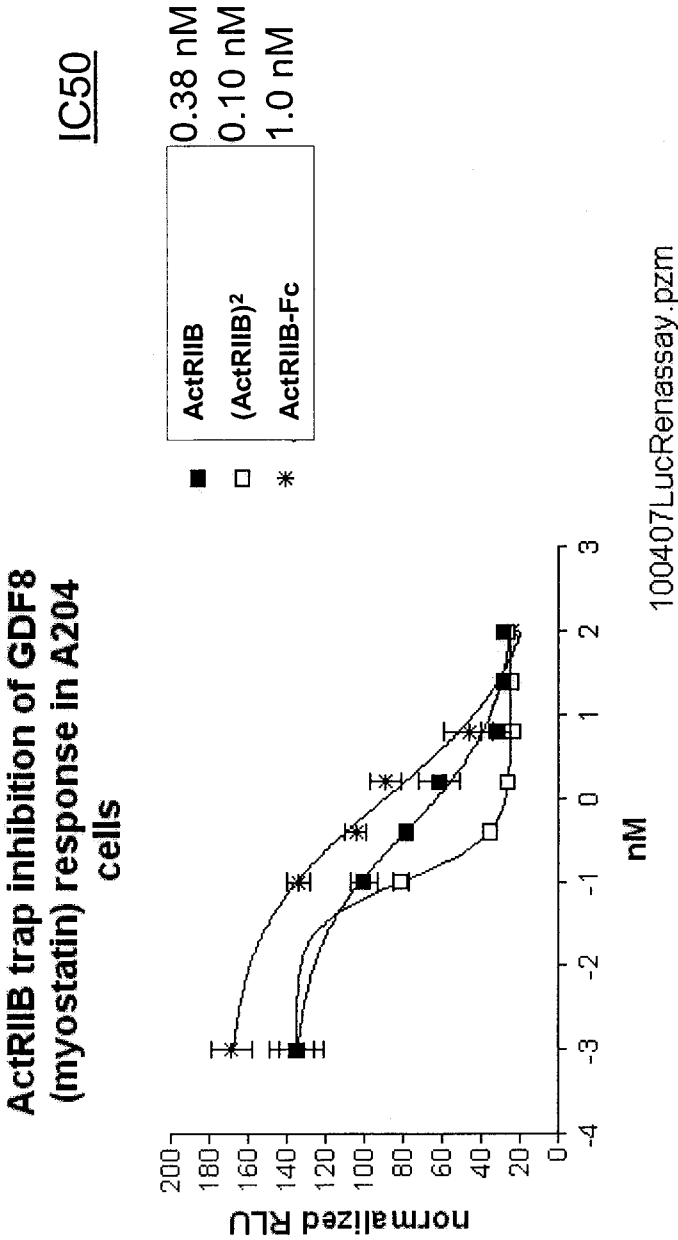


Figure 13. Efficient inhibition of Myostatin signaling in A204 cells by (ActRIIB)² trap compared to the less potent inhibition of ActRIIB-Fc and monomeric ActRIIB.

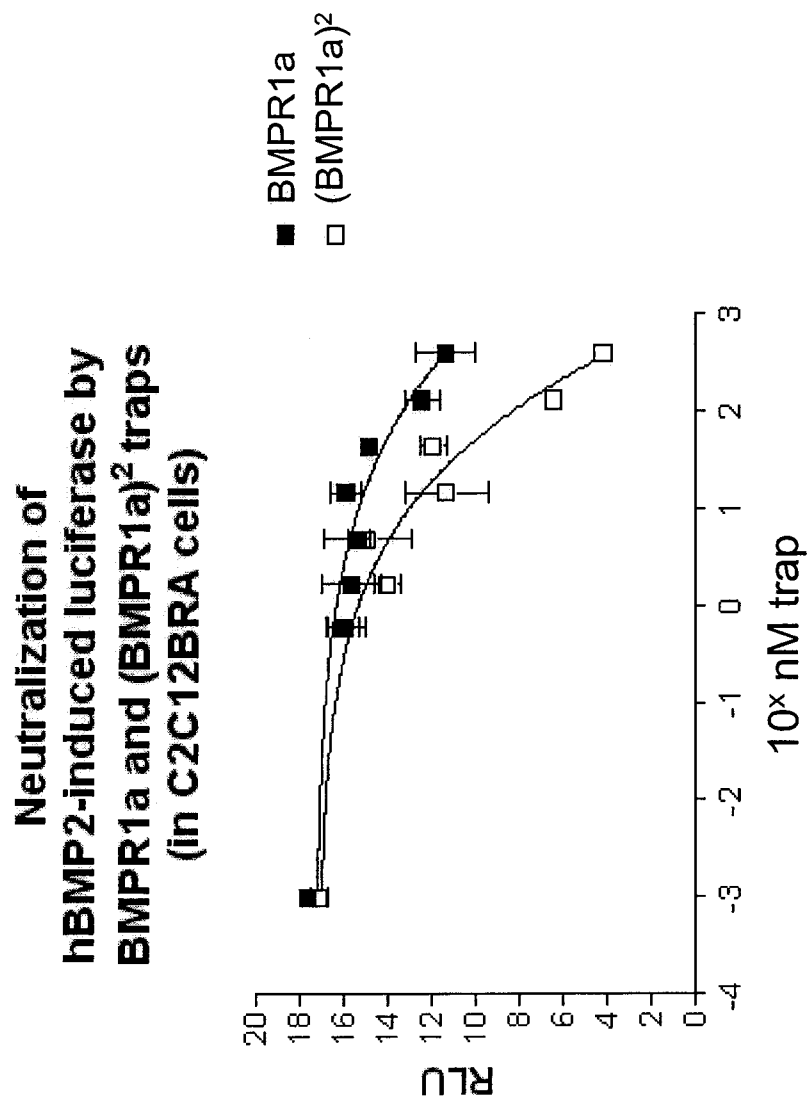


Figure 14. Bivalent (BMPR1a)² trap is more potent than monovalent BMPR1a trap for neutralization of BMP2.

Figure 15A. Schematic diagrams exemplifying in-line fusions of receptor ectodomains leading to heterovalent single-chain traps of TGF- β -superfamily growth factors.

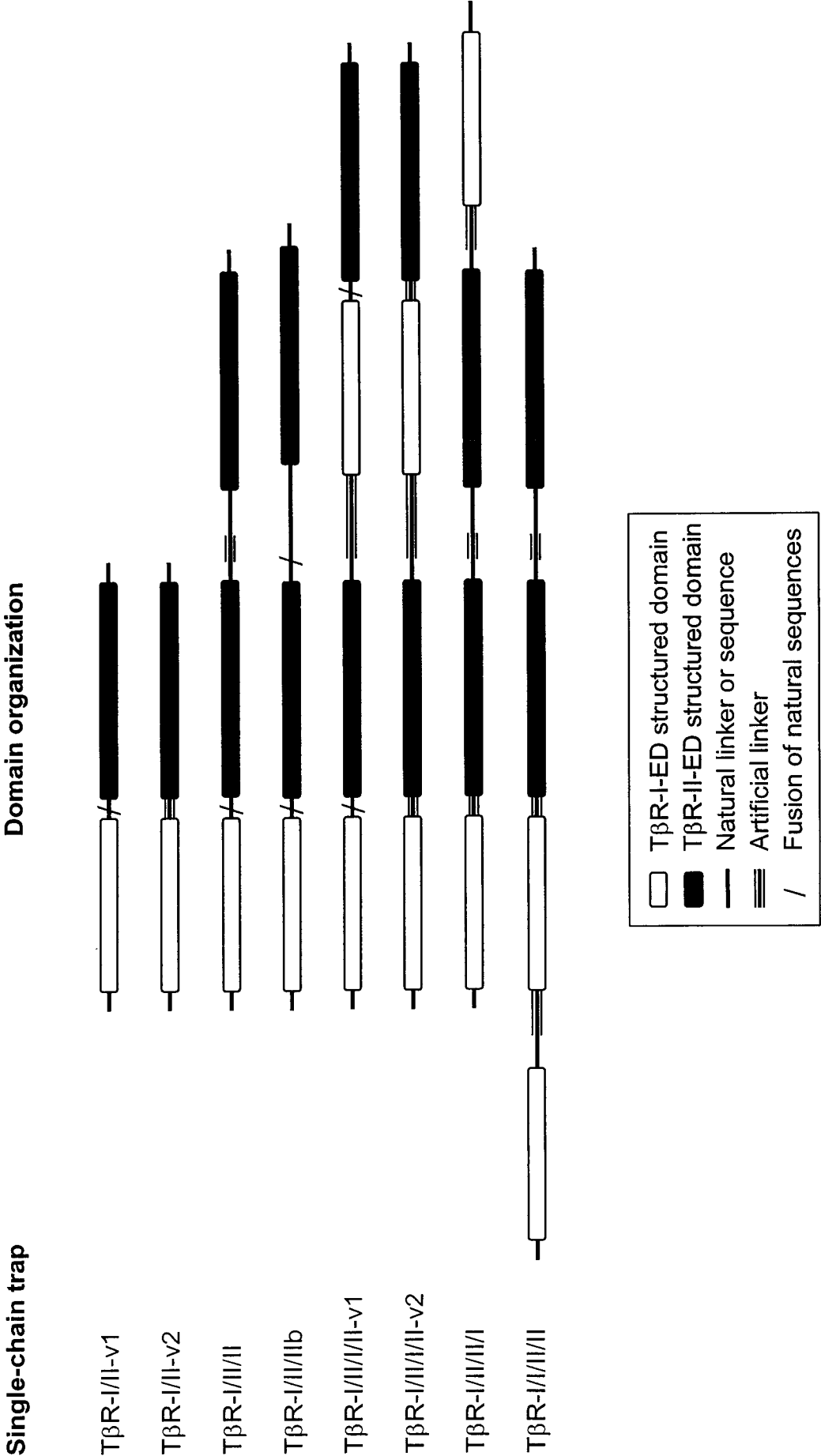


Figure 15B Part 1. Amino-acid sequences exemplifying heterovalent single-chain traps of TGF- β -superfamily growth factors, and corresponding to the domain organization diagrams depicted in panel (15A).

TβR-1/1-v1
SEQ ID NO 75

CDVRFSTCDNOKSCMNCNCSITSICEKPOEVCVAVWRKNDENITLTVCHDPKLPYHDFILEDAAAPKIMKEKKPGGETFMCSDECDNDNLIFFSEEYNTSNPD
AALLPGATALQCFCHLCTKDNFTCVTDTGLGCFVSVTETTDKVTHNSMCIAEIDLIPDRPFCAPSSKTSGSVTTTYCCNQHCKNIELEPTTVTDNNGAVKFPQLCKE

TβR-1/11-v2
SEQ ID NO 76

ALQCFCHLCTKDNFTCVTDGLCFVSVTETTKVTHNSMCIAEIDLIPDRPFVFCAPSSKTGSVTTYCCNQHCKNIELGGGGGGGNGAVKFPQLCKE

TβR-1/1/11
SEQ ID NO 77

AAALLPGATALQCFCHLCTKDNFTCVTDGLCEFSVTEETDKVTHNSMCIAEIDLIPDRPFVCAPSSKTGSVTTTYCCNQDCKNKLPTTIVTDNNGAVKFQOLCKE
CDVRFSTCDNQKSCMSNCISITICEKPQECVAVWRKNDENITLETVCHDPKLPYHDFILEDAAAPKCIIMKEKKKPGETFFCMSCSSDECDNDNIIFSEEYNTSNPD
SPKCIIMKEKKKPGETFFCMSCSSDECDNDNIIFSEEYNTSNPD

TβR-I/II/IIIb
SEQ ID NO
78

AAALLPGATALQCFCHLCTKDNFTCVTDGLCFVSVTETTDKVTHNSMCIAEIDLIPDRPFPVCAPSSKTSVTTTCNQDHCKNKLFTTTVTDDNGAVKFPQLCKFCDFRSTCDNQKSCMSNCISITICEKQPQVCVAVWRKNDENTILETVCHDPKLPYHDFILEDAAAPKICMKEKKKPGETTFMCSCSDECNNDILFSEEYNTSNPDIPPHVOKSDVEMEAQKDEIICPSCNRHTAHLRHINNDMIVTDNNGAVKFPQLCKFCDFRSTCDNQKSCMSNCISITICEKQPQVCVAVWRKNDENTILETVCHDPKLPYHDFILEDAAAPKICMKEKKKPGETTFMCSCSDECNNDILFSEEYNTSNPD

TβR-1/II/II-v1

[illegible]

Key: underlined: natural linker or sequence; **bold-italics**: TβR-I-ED structured domain; **bold**: TβR-II-ED structured domain; underlined-italics: artificial linker; **bold-italics**: TβR-I/TGF-β [Groppe et al. 2008].

Key: underlined: natural linker or sequence; **bold-italics**: *TβR-II-ED* structured domain; **bold**: *TβR-II-ED* structured domain; underlined-italics: artificial linker; **bold-italics**: *TβR-I/II/TGF-β* [Groppe et al. 2008].

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2008/000547

A. CLASSIFICATION OF SUBJECT MATTER

IPC: *C12N 15/18* (2006.01), *A61K 39/395* (2006.01), *A61K 47/48* (2006.01), *A61K 49/00* (2006.01), *C07K 1/22* (2006.01), *C07K 14/71* (2006.01), *C12N 15/12* (2006.01), *G01N 1/34* (2006.01), *G01N 1/40* (2006.01), *G01N 33/53* (2006.01), *C07K 14/495* (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 15/18 (2006.01), *A61K 39/395* (2006.01), *A61K 47/48* (2006.01), *A61K 49/00* (2006.01), *C07K 1/22* (2006.01), *C07K 14/71* (2006.01), *C12N 15/12* (2006.01), *G01N 1/34* (2006.01), *G01N 1/40* (2006.01), *G01N 33/53* (2006.01), *C07K 14/495* (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

STN (Biosis, CaPlus, Dissabs), Delphion, Scopus, NCBI, Canadian Patent Database, Genome Quest

Keywords: transforming growth factor, antagonist, inhibitor, ectodomain, (bi-, multi-)valent, ligand(s), linker(s)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ESPARZA-LOPEZ, J. Ligand binding and functional properties of betaglycan, a co-receptor of the transforming growth factor- β superfamily. Journal of Biological Chemistry. May 2001, Vol 276, No. 18, pages 14588-14596, ISSN 0021-9258. *See whole document*	1-4, 9, 17, 18, 21 and 22
X	LARRAIN, J. BMP-binding modules in chordin: a model for signalling regulation in the extracellular space. Development. February 2000, Vol. 127, No. 4, pages 821-830, ISSN 0950-1991. *See whole document*	1, 2, 4, 9, 17, 18, 21 and 22

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

26 June 2008 (26-06-2008)

Date of mailing of the international search report

18 July 2008 (18-07-2008)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
Place du Portage I, C114 - 1st Floor, Box PCT
50 Victoria Street
Gatineau, Quebec K1A 0C9
Facsimile No.: 001-819-953-2476

Authorized officer

Christine Teixeira 819- 994-0244

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. ☐ Claim Nos. :
because they relate to subject matter not required to be searched by this Authority, namely :

2. ☒ Claim Nos. : 1-9, 18 and 21-24 (wholly) and claim 17 (partially)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

Said claims are so unclear and inadequately supported by the description that no meaningful search could be carried out over the full scope of the claims. Therefore, the search was restricted to those embodiments for which there is support in the description (SEQ ID NOs: 3, 10, 16, 23 and 29) and other known TGF β binding proteins that inherently have the desired structure.

3. ☐ Claim Nos. :
because they are dependant claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

Invention 1: Claims 1-9, 18 and 21-24 (wholly) and claim 17 (partially)

Multivalent binding agent comprising the general structure (<bd1>-linker1)_k-[{<bd1>-linker2-<bd2>-linker3_f]_n-(<bd3>)_m-(linker4-<bd4>)_d]_l, wherein bd1, bd2, bd3 and bd4 are binding domains having an affinity for the same member of the TGF- β superfamily and linker1, linker2, linker3 and linker4 are unstructured polypeptide sequences, encoding nucleic acids, methods and uses thereof.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

1-9, 18 and 21-24 (wholly) and 17 (partially)

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Continuation of: Box III

Invention 2: Claims 10, 11, 19 and 20 (wholly) and claim 17 (partially)

Method of designing a multivalent linker useful in modulating responsiveness of a cell to a member of the TGF- β superfamily, nucleic acids encoding said multivalent linker and data storage medium comprising instructions for determining the acceptable minimum linker length.

Inventions 3-29: Claims 12-17 (partially)

Polypeptides and encoding nucleic acids, wherein the amino acid sequences of the polypeptides have at least 80% sequence identity to a natural unstructured region in the extracellular portion of a receptor for a member of the TGF- β superfamily or have between 43-99% sequence identity to a naturally unstructured region in the ectodomain of a receptor for a member of the TGF- β superfamily, including: SEQ ID NOs: 31 and 82 (invention 3); 32, 34, 83 and 85 (invention 4); 33 and 84 (invention 5); 35 and 86 (invention 6); 36 and 87 (invention 7); 37 and 88 (invention 8); 38 and 89 (invention 9); 39 and 90 (invention 10); 40 and 91 (invention 11); 41 and 92 (invention 12); 42 and 92 (invention 13); 43 and 44 (invention 14); 45 (invention 15); 46 (invention 16); 47 (invention 17); 48 (invention 18); 49, 54, 57, 58, 64, 66, 93, 98, 101, 102, 108 and 110 (invention 19); 50, 94 and 100 (invention 20); 51, 55, 71, 95, 99 and 115 (invention 21); 52, 53, 73, 74, 96, 97, 117 and 118 (invention 22); 56, 59, 69, 103 and 113 (invention 23); 60, 61, 104 and 105 (invention 24); 62, 63, 106 and 107 (invention 25); 65 and 109 (invention 26); 67, 68, 111 and 112 (invention 27); 70 and 114 (invention 28); and 72 and 116 (invention 29).

No special technical feature exists among the groups of inventions. The special technical feature of invention 1 is considered to be the formula of the binding agent of claim 1. The linkers in said formula are not defined by structure and cannot be considered as a special technical feature. Further, said linkers do not need to be designed by the method of claim 10. Similarly, the isolated polypeptides of invention 3-29 do not have to be designed by the method of claim 10. Finally, the polypeptide linkers and encoding nucleic acids of inventions 3-29 are classes of peptides that do not share a significant structural element. Their common functional feature as a linker is not held to be a special technical feature, as linkers are well known in the art and any polypeptide may potentially serve as a linker. Therefore, distinct linker embodiments are considered to be distinct inventions, with the exception of specific groups of peptides that exhibit extensive sequence identity and are indicated above as single inventions. Consequently, there is no common special technical feature linking the groups of inventions.