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(57) Abstract: Compositions and methods are provided for modulating the signaling intensity of receptors that are activated by di-
merization, which receptors include without limitation cytokine receptors. A bivalent ligand that binds to the receptor and enforces
receptor inter-subunit distances is brought into contact with the receptor. The bivalent ligand binding results in a degree of separa-
tion between the receptor subunits, which degree determines the signaling intensity. Signaling can be modulated through subunit
separation to produce intermediate signaling intensities, as well as "on" and "off" signaling. The enforcement of certain distances can
further be used to prevent ligand-independent downstream signaling, e.g. Jak/STAT signaling.



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TUNING DIMERIC RECEPTOR SIGNALING WITH EXTRACELLULAR LIGANDS THAT ALTER RECEPTOR ORIENTATION AND PROXIMITY UPON BINDING

GOVERNMENT RIGHTS

[0001] This invention was made with Government support under contract AI051321 awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND

[0002] Phosphorylation of target proteins by kinases is an important mechanism in signal transduction and for regulating enzyme activity. Tyrosine kinases (TK) are a class of over 100 distinct enzymes that transfer a phosphate group from ATP to a tyrosine residue in a polypeptide. Tyrosine kinases phosphorylate signaling, adaptor, enzyme and other polypeptides, causing such polypeptides to transmit signals to activate (or inactive) specific cellular functions and responses. Receptor tyrosine kinases are high affinity receptors for hormones, growth factors and cytokines. The binding of hormones, growth factors and/or cytokines generally activates these kinases to promote cell growth and division. Exemplary kinases include insulin-like growth factor receptor, epidermal growth factor receptor, platelet-derived growth factor receptor, etc. Most receptor tyrosine kinases are single subunit receptors but some, for example the insulin receptor, are multimeric complexes.

[0003] Cytokine receptors are cell-surface glycoproteins that bind specifically to cytokines and transduce their signals. Receptor dimerization is a universal mechanism that is shared by many classes of growth factors to initiate signal transduction. Cytokines, which are a large class of secreted glycoproteins that contribute to regulating the fate and function of most cell types, bind to the extracellular domains of their cell surface receptors and signaling is initiated by homo- or hetero- dimerization. Cytokines such as erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), thrombopoietin (TPO), and human growth hormone (hGH) homodimerize their receptors.

[0004] Multi-subunit receptors may consist of two subunit types such as the receptors for granulocyte-macrophage CSF (GM-CSF), interleukin-3 (IL-3), and IL-5 where an α subunit is specific for each ligand and a β subunit is common to all three (β_c), with both chains participating in signaling. The IL-6 receptor also contains two subunit types, IL-6R α and gp130. Receptors that contain three different subunits include the CNTF receptor (CNTFR), formed by the CNTFR α chain, gp130, and the leukemia inhibitory factor (LIF) receptor, and the IL-2 receptor (IL-2R) which consists of the IL-2R α chain or α (which is not a typical member of the cytokine receptor family), IL-2R β , and IL-2R γ , with the latter two being the signaling molecules, while other cytokines, such as interleukin-2 (IL-2), heterodimerize a shared receptor (common gamma chain) with a cytokine-specific subunit.

[0005] Cytokine receptor dimerization principally results in activation of the JAK/STAT pathway to modulate gene expression and ultimately determine cell fate. The JAK-STAT system consists of three main components: a receptor; Janus kinase (JAK); and Signal Transducer and Activator of Transcription (STAT). When the receptor is activated, it turns on the kinase function of JAK, which autophosphorylates itself. The STAT protein then binds and is phosphorylated by JAK.

[0006] Over the last twenty years, structures of cytokine-receptor extracellular domain (ECD) complexes from different systems have revealed a diverse range of molecular architectures and receptor dimer topologies that are compatible with signaling. Furthermore, monoclonal antibodies that dimerize cytokine receptor extracellular domains can act as agonists in some cases, but the signaling geometries of such dimers are unknown. This apparent permissiveness in dimer architecture raises the question of whether structurally distinct receptor- ligand dimer geometries can fine-tune the degree and nature of receptor activation, or if dimerization alone of the extracellular domains is sufficient regardless of the structural features.

SUMMARY OF THE INVENTION

[0007] Compositions and methods are provided for modulating the signaling intensity of receptors that are activated by dimerization, which receptors include without limitation cytokine receptors. A bivalent ligand that binds to the receptor and enforces receptor inter-subunit distances is brought into contact with the receptor. The bivalent ligand binding results in a degree of separation between the receptor subunits, which degree determines the signaling intensity. Such bivalent ligands can remodel receptor dimer topology, i.e. geometry and/or distance, to activate differential signaling pathways and gene expression from one another and from the parent cytokine signaling. Such biased agonists provide therapeutically useful variants that may exhibit reduced toxicity or distinct functional activities. Signaling can be modulated through subunit separation to produce intermediate signaling intensities, as well as “on” and “off” signaling. The enforcement of certain distances can further be used to prevent ligand-independent downstream signaling, e.g. JAK/STAT signaling.

[0008] In some embodiments the receptor is a cytokine receptor, e.g. a receptor present on a cell, which may be present *in vivo* or in an *in vitro* culture system. Cytokine receptors include without limitation type I receptors and type II receptors. Of interest are included homodimeric receptors, for example EPO receptor, G-CSF receptor, TPO receptor, hGH receptor, and the like.

[0009] In some embodiments the bivalent ligand is a diabody that induces topological changes in the receptor upon binding. The activity of a diabody on cytokine receptor activation can result from changes in orientation and proximity of the receptor subunits. Diabody molecules of

the invention comprises a first and a second polypeptide chain, which first polypeptide chain comprises (i) a first domain comprising a binding region of a light chain variable domain of a first immunoglobulin (VL1) specific for a first epitope, and (ii) a second domain comprising a binding region of a heavy chain variable domain of a second immunoglobulin (VH2) specific for a second epitope, which first and second domains are linked such that the first and second domains do not associate to form an epitope binding site; which second polypeptide chain comprises (i) a third domain comprising a binding region of a light chain variable domain of the second immunoglobulin (VL2), (ii) a fourth domain comprising a binding region of a heavy chain variable domain of the first immunoglobulin (VH1), which third and fourth domains are linked such that the third and fourth domains do not associate to form an epitope binding site. The two polypeptide chains associate to form a rigid bivalent binding structure where the VH1 and VL1 domains associate to form an epitope binding site; and the VH2 and VL2 domains associate to form an epitope binding site. The epitope binding sites may be the same or different. Identical binding sites can be used, e.g. where the cytokine receptor forms a homodimer. Alternatively, the VH1/VL1 and VH2/VL2 are different, e.g. to generate a greater diversity of signaling modulation by mixing binding domains of varying topological constraint; by binding to heterodimeric receptors; and the like.

[0010] The two domains present on each polypeptide chain of the diabody are separated by a short linker. The peptide linker may be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids in length, and is of sufficient length and amino acid composition to enforce the distance between receptor subunits when bound to the diabody. In some embodiments the linker is glycine and/or serine, and is 3, 4, 5, 6, 7 amino acids in length, and can be 4, 5, 6 amino acids in length. It will be understood by one of skill in the art that the exemplary diabody sequences provided herein can be substituted in the linker region with any suitable polypeptide.

[0011] One feature of the invention is the provision of a diabody that, when bound to a cytokine receptor, can reduce or prevent ligand-independent signaling, e.g. signaling by a JAK kinase operably linked to the cytokine receptor. JAK kinases of interest include JAK1, JAK2, JAK3 and TYK2, and oncogenic mutants thereof. It is shown herein that extracellular ligands that enforce large receptor dimer separation can counteract intracellular ligand-independent JAK/STAT activation. Without being limited by the theory, it is believed that the enforced receptor dimer separation exceeds the accessible distance that the JAK2 kinase domain can extend to transphosphorylate the opposing JAK2 and receptor. For example, several mutations in JAK kinases are known to cause immune disorders and cancer by rendering activation ligand-independent, which signaling can be blocked by contacting to external cytokine receptor, e.g. EPO receptor, thrombopoietin receptor, etc. with a diabody that enforces a large distance between subunits. The diabody molecules DA10 and DA330 provided herein are exemplary of such activity.

[0012] In certain embodiments, methods are provided for inhibiting ligand independent JAK signaling, by contacting a receptor operably linked to the JAK protein with a diabody that enforces a large receptor dimer separation. In some such embodiments the receptor is EPO receptor. The contacting may be performed in vivo, and includes the treatment of hematologic malignancies, including acute lymphoblastic leukemia, myeloproliferative neoplasms, e.g. AML, polycythemia vera, essential thrombocytopenia, myelofibrosis, etc. In some embodiments, treatment of an individual with such a hematologic malignancy by inhibiting ligand-independent JAK signaling, comprises the steps of administering an effective dose of a diabody that enforces a large distance between the receptor subunits, e.g. the EPO receptor subunits, where the dose is effective to substantially decrease JAK/STAT signaling in the malignant cells, i.e. to decrease by greater than about 20%, to decrease by greater than about 30%, to decrease by greater than about 40%, to decrease by greater than about 50%, to decrease by greater than about 75%, to decrease by greater than about 90%, to decrease by greater than about 95%, to decrease by greater than about 99% or more relative to an untreated individual. Alternatively the effect can be monitored by decreases in STAT activity, cellular proliferation, and any other suitable signaling and functional metric showing reduction in intensity. The receptor subunit distance thus enforced may be greater than about 140 Å. Diabodies for such purpose include without limitation DA10 and DA330 provided herein.

[0013] In one embodiment, one or more epitope binding site(s) comprising VL and a VH domains, which epitope is present on a receptor, e.g. a cytokine receptor, is formatted as a diabody. The one or more diabodies are bound to the cognate receptor, and the degree of separation between receptor subunits when bound to the diabody is determined. The degree of separation is correlated with receptor signaling intensity, and is used to select for a diabody that provides for the desired level of signaling from the receptor. In some such embodiments structural determination, e.g. NMR, X-ray crystallography, and the like is used to determine the topography of the complex between receptor and diabody.

[0014] In yet another embodiment of the present invention, the diabodies of the invention can be used to treat a variety of diseases and disorders. Accordingly, the present invention is directed to a method for treating a disease or disorder comprising administering to a patient in need thereof an effective amount of a diabody of the invention, in which the diabody has been selected to provide a specific level of signaling correlated with the degree of separation enforced on the receptor subunits upon binding to the diabody. In further such embodiments of the invention, the receptor is a cytokine receptor, including without limitation the EPO receptor; the thrombopoietin receptor, etc. Administration of a diabody comprising a heavy chain variable region and a light chain variable region from DA5, DA10 and DA330 joined by a peptide linker is exemplary for modulating activity of the EPO receptor. Administration of a diabody comprising a heavy chain variable region and a light chain variable region from AK119,

AK113 and AK111 joined by a peptide linker is exemplary for modulating the activity of the TPO receptor.

[0015] In one such embodiment, an effective dose of a diabody that activates the EPO receptor is provided to an individual in need of EPO activity, e.g. kidney dialysis patients, anemia patients, etc. The effective dose is sufficient to increase signaling from the EPO receptor, e.g. to increase at least about 50%, at least about 75%, at least about 100%, at least about 150%, at least about 200%, at least about 3-fold, at least about 5-fold, at least about 10-fold or more relative to an untreated individual. In some such embodiments, the diabody is selected for a desired level of EPO signaling based on a correlation with the degree of separation the diabody enforces between the EPO receptor subunits. In some such embodiments the degree of separation is from about 50 Å to about 135 Å. In some embodiments the diabody comprises a heavy and light variable region from DA5, provided herein, where the heavy and light variable regions are joined by a peptide linker.

[0016] In one such embodiments, an effective dose of a diabody that activates the TPO receptor is provided to an individual in need of TPO activity, e.g. to stimulate megakaryocyte and platelet production and reduce thrombocytopenia. In another such embodiment, an effective dose of a diabody that activates TPO receptor is provided in an in vitro culture to induce megakaryocyte differentiation; to promote self-renewal of hematopoietic stem cells, e.g. in combination with stem cell factor; etc. The effective dose is sufficient to increase signaling from the TPO receptor, e.g. to increase at least about 50%, at least about 75%, at least about 100%, at least about 150%, at least about 200%, at least about 3-fold, at least about 5-fold, at least about 10-fold or more relative to an untreated individual or cell population. In some such embodiments, the diabody is selected for a desired level of TPO signaling. In some such embodiments, the diabody comprises a heavy and light variable region from AK119, AK113 and AK111 diabodies provided herein, where the heavy and light variable regions are joined by a peptide linker.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] Fig. 1. EpoR dimerization and signaling potencies induced by EMPs and diabodies: (A,B) Levels of EpoR dimerization (A) and phosphorylation (B) promoted by EMPs at the indicated doses. Data (mean +/- SD) are from four independent replicates (C) Schematic view of a bivalent diabody molecule. V_H is connected to the V_L domain by a short Gly-linker. EpoR binding sites in the diabody are highlighted with a yellow circle. (D,E) Levels of EpoR dimerization (D) and phosphorylation (E) promoted by diabodies at the indicated doses. Data (mean +/- SD) are from four independent replicates (F) Percentage of pSTAT5 activation induced by the indicated doses of EPO or the four diabodies in Ba/F3 EpoR cells. Data (mean +/- SD) are from two independent experiments. (G) Ba/F3 proliferation in response to EPO or

the four diabodies. Data (mean $\pm$ SD) are from two independent replicates (H) Number of CFU-E colonies derived from mouse bone marrow induced by EPO and the four diabodies. Data (mean $\pm$ SD) are from three different experiments.

[0018] Fig. 2. “Biased” signaling activation induced by the diabodies: (A) Bubble plot representation of the signaling pathways activated by EPO and the three diabodies at the indicated times in UT-7-EpoR cells. The size of the bubble represents the intensity of the signal activated. (B) The levels of signal activation induced by the three diabodies at 15 min of stimulation were normalized to those induced by EPO and order based on signaling potency. The red line represents the EPO signaling activation potency normalized to 100 %. Data (mean $\pm$ SD) are from three independent replicates. (C) pSTAT1 and pSTAT5 dose-response experiments performed in UT-7-EpoR cells stimulated with EPO or DA5 for 15 min. Data (mean $\pm$ SD) are from two independent replicates. (D) Bubble plot representation of genes induced by EPO and the three diabodies after stimulation of MEP cells for two hours. The size of the bubble represents the fold of gene induction. See also Figure S3 and Table S1.

[0019] Fig. 3. Crystal structures of DA5, DA10 and DA330 in complex with EpoR: (A) Overlay of the three diabody_EpoR complexes. EpoR binding to DA5 is colored green, EpoR binding to DA10 is colored red and EpoR binding to DA330 is colored purple. The DA330 crystal lattice appears to contain domain-swapped diabodies as scFv in similar but not identical subunit relationships. (B) Diabodies binding footprint on the EpoR surface. Amino acids on EpoR interacting with the diabodies are colored white. DA5 CDRs are colored green; DA10 CDRs are colored red and DA330 CDRs are colored purple. Vectors connecting the V_H CDR1 and the V_L CDR1 in the diabodies define the binding topology of the three diabodies_EpoR complexes. (C-D) Diabodies and EPO binding footprint on the EpoR surface. Hot-spot interaction on EpoR are colored lime and are shared by the diabodies and EPO. Diabodies use Y34, R98 (DA5), Y105 (DA10), Y32, R101 (DA330) to interact with the amino acids forming the two hot-spots on EpoR. EPO uses similar chemistry with F43 and K45 filling the two hot-spots pockets on EpoR. (E-H) Crystal structures of DA5 (E), DA10 (F), DA330 (G), and EPO (H) dimerizing two EpoR are shown in top (left) and side (right) views. In the side view representation, EpoR is depicted as surface. Yellow spheres represent the C-terminal region of the SD2 EpoR domain.

[0020] Fig.4. Diabodies dimerize EpoR in the surface of living cells: (A) Cell surface labeling of EpoR using dye-labeled anti-GFP nanobodies. (B) Relative co-localization of RHO11EpoR and DY647EpoR in absence and presence of ligand. As a negative control, co-localization of maltose binding protein fused to an indifferent transmembrane domain is shown. (C) Trajectories (150 frames, ~4.8 s) of individual Rho11-labeled (red) and DY647-labeled EpoR (blue) and co-trajectories (magenta) for unstimulated cells as well as after stimulation with EPO (5 nM) and DA5 (250 nM). (D) Relative amount of co-trajectories for unstimulated EPOR and after stimulation with EPO and diabodies (DA5, DA330, DA10). (E) Diffusion properties of EpoR

represented as trajectory step-length distribution for unstimulated cells and after dimerization with EPO or DA5. The curves correspond to fitted data from >10 cells (~1500 trajectories each). (F) Diabody-induced dimerization of EpoR demonstrated by dual-step bleaching analysis. Upper panel: A pseudo-3D kymograph illustrating dual-color single-step bleaching for an individual DA5-induced EpoR co-trajectory. Bottom left panel: The corresponding pixel-intensity profiles are shown for both acquisition channels. Bottom right panel: The fraction of signals within co-trajectories that decay within a single step vs. multiple steps. Comparison for complexes obtained with EPO (from 154 co-trajectories) and DA5 (from 186 co-trajectories).

[0021] Fig. 5. DA10 and DA330 inhibit JAK2V617F constitutive activity: (A) Model depicting the mechanism by which the diabodies affect signaling activation potencies. The large dimer intersubunit distances exhibited by the diabodies may alter the position of JAK2 upon ligand binding, decreasing its ability to transactivate each other and start downstream signaling amplification. (B) Kinetics of pSTAT5 in Ba/F3 cells expressing the JAK2V617F mutant after stimulation with EPO or the four diabodies. DA10, DA307 and DA330 induce a decrease on the basal pSTAT5 levels in a time dependent manner. Data (mean +/- SD) are from two independent experiments. (C) pSTAT5, pErk and pAkt levels induced by 1 μ M of the four diabodies in Ba/F3 cells expressing the JAK2V617F mutant after 3 hrs of stimulation. (D) EpoR surface levels after 1 hr stimulation with EPO or the four diabodies. (E) Proliferation of Ba/F3 cells expressing JAK2 wt or JAK2 V617F in response to 1 μ M of each of the four diabodies after 5 days of stimulation. Data (mean +/- SD) are from three independent experiments.

[0022] Fig. 6. DA10 and DA330 inhibit erythroid colony formation in JAK2V617F-positive patients samples: (A) Number of erythroid BFU-E (EPO-dependent) colonies in heterozygous JAK2V617F positive myeloproliferative neoplasm patient samples after stimulation with the indicated ligands. Data (mean +/- SD) are from three different donors. (B) Number of myeloid colonies in heterozygous JAK2V617F positive myeloproliferative neoplasm patient samples after stimulation with the indicated ligands. (C) Overview pictures highlight EPO-independent BFU-E colonies (no drug and DA5) which are significantly diminished with DA330 and DA10 treatment. * P < 0.05; ** P < 0.01; *** P < 0.001; paired Student's t-test was used to determine significant changes. (D) The genotype of 109 erythroid colonies derived from sorted CD34+ cells derived from PMF cases was determined by multiplexed custom TaqMan SNP assay for JAK2V617F and JAK2 wild type. Each colony is represented by a single dot in the graph and colored according to different treatment regimens. Grey dots represent colonies derived from conditions without treatment or treatment with an agonist (dark grey with EPO, light grey without EPO), orange and green dots represent few residual colonies treated with DA330, and blue and red dots very rare residual colonies treated with DA10. (E) Number of erythroid colonies (Burst-forming units-erythroid (BFU) or endogenous erythroid colonies (EEC)) and myeloid colonies (EPO-independent) in a Polycythemia Vera (PV) (top panel) and primary

Myelofibrosis (PMF) patient (bottom panel) homozygous for JAK2V617F. SI: SCF + IL-3; SIE: SCF +IL-3 + EPO. Data (mean +/- SD) are from three different donors. (F) Morphology of EEC colonies after treatment with the indicated conditions is shown.

[0023] Fig. 7. EMP-33 binds more than 50 fold weaker to EpoR than EMP-1. (A) The EMP-1 and EMP-33 complexes structures were superimposed and are shown in side and top view. (B) Mass-spectrometry profiles of EMP-1 and EMP-33 peptides (C) EMP-1 and EMP-33 K_D EpoR binding affinities measured by Surface plasmon resonance.

[0024] Fig. 8. Diabodies CDR3 sequences and EpoR binding affinities. (A) V_H and V_L CDR3 sequences of DA5, DA10, DA307 and DA330. (B) Structure of the construct for affinity screening. (C) EpoR dose/response binding curves of the four diabodies and EPO displayed on the yeast surface.

[0025] Fig. 9. Functional characterization of the EpoR Diabodies. (A) STAT5 transcriptional activity induced by the diabodies and EPO in γ 2A cells transfected with a STAT5 luciferase reporter. Data (mean +/- SD) are from three different experiments (B) Kinetics of EpoR downregulation in Ba/F3 EpoR cells stimulated with EPO or the four diabodies for the indicated times. Data (mean +/- SD) are from three different experiments (C) Levels of *CISH* and *Pim1* gene induction by EPO and the indicated diabodies measured by qPCR in UT-7 EpoR cells. Data (mean +/- SD) are from three different experiments.

[0026] Fig. 10. Biophysical and structural characterization of the DA_EpoR complexes. (A) Multi Angle light scattering chromatography experiments show that the DA_EpoR complex run with a predicted molecular weight of 97-98 kDa, in agreement with a stoichiometry of 2EpoR bound to 1diabody in each complex. (B) V_H and V_L CDR loops overlay of the three diabodies on the EpoR surface. (C) Amino acids on EpoR used by EPO to form the high and low affinity binding interfaces. Amino acids shared by the two sites are colored red. Amino acids only used by the high affinity site I are colored in salmon; and amino acids used by the low affinity site II interface are colored green. (D) Amino acids on EpoR contacting residues on the V_H CDR loops of the three diabodies. (E) Amino acids on EpoR contacting residues on the V_L CDR loops of the three diabodies.

[0027] Fig. 11. Diabodies_EpoR binding interface. (A, C and E) Position of the V_H and V_L CDR loops of DA5 (A), DA10 (C) and DA330 (E) on the EpoR surface. The side chains of the amino acids contributing to the binding interface formation are shown. The EpoR surface interacting with the diabodies is colored in grey. (B, D and F) Two dimensional interactions maps of the V_H and V_L CDR_EpoR binding interfaces. Amino acids are depicted as nodes in the interaction map (rectangles: EpoR; ellipses: diabodies). Interactions between side chains are represented by lines. Van der Waals interactions and hydrophobic contacts are shown as solid lines, H-bonds are shown as dashed lines and electrostatic interaction as red lines.

[0028] Fig. 12. Simulating the linker flexibility in DA5/EpoR and DA10/EpoR complexes. (A, B) Each image column shows the original X-ray structure (opaque) overlaid with the representative structure (translucent) from one out of the three major conformational clusters from the structural ensembles that were generated by Parallel Tempering Monte Carlo simulations. Each row presents a new orientation of the structures. The density plots present the probability of conformations as a function of the distances between the EpoR C-termini of diabody/EpoR. Blue and light blue arrows mark the distances in the X-ray and simulated structures, respectively. The left, middle and right image columns are presenting the structures that have corresponding distances indicated by the left, middle and right light blue arrows on the conformational probability density plots. (A) DA5/EpoR. (B) DA10/EpoR.

[0029] Fig.13. Signaling potencies induced by TpoR diabodies. (A) Percentage of pSTAT5 activation induced by the indicated doses of TPO or the three TpoR diabodies in Ba/F3 TpoR cells. (B) Bubble plot representation of the signaling pathways activated by TPO and the three TpoR diabodies at the indicated times in UT-7-TpoR cells. The size of the bubble represents the intensity of the signal activated. (C) Ba/F3-TpoR cells proliferation in response to TPO or the three TpoR diabodies. Data (mean +/- SD) are from two independent replicates.

[0030] Fig. 14. Gene expression signatures induced by TPO and TpoR Dbs. UT-7-TpoR cells were grown in the presence of saturating amounts of TPO or the three TpoR Dbs for 21 days and the levels of Megakaryocytic (A) and Erythroid (B) specific genes were analyzed by Real-time PCR. Data (mean +/- SD) are from three independent replicates.

[0031] Fig. 15. DB-treated and TPO-treated cells have different morphology. (A) UT-7-TpoR cells were grown in the presence of the indicated doses of TPO or the three TpoR Dbs for three days and their morphology was assayed by microscopy. Only cells treated with AK113 and AK111 formed very characteristics cell clusters. (B) Giemsa staining of AK113 or AK119 UT-7-TpoR treated cells. AK119-treated cells differentiate into Megakaryocytes, denoted as big cells with several nucleuses. AK113-treated cells, on the other hand, aggregate and form big undifferentiated clusters.

[0032] Fig. 16. AK113 promotes the growth of CD42b-negative colonies. (A) Megakaryocyte-Erythroid (MEP) progenitors were sorted from bone marrow samples and plated in semi-solid cultures in the presence of saturating doses of TPO and the three TpoR Dbs. Colonies were characterized for morphology (A-B) and CD42b (Megakaryocyte marker) expression (C) two weeks later. Only AK113-treated MEP differentiated into a non-megakaryocytic CD42b-negative colony.

[0033] Fig. 17. AK111 and AK113 stimulation favor self-renewal of hematopoietic stem cells. (A) Schematic representation of a hematopoietic stem cell (HSC) life cycle. HSC can indefinitely replicate and maintain an undifferentiated state or differentiate into the different blood cell types, depending on the cytokine milieu where they are embedded. TPO plays a

critical role in maintaining HSC homeostasis by acting both at the level of self-renewal and differentiation stages. (B) Dot-plot showing the percentage of negative/positive CD34+CD45RA-cells after 15 days of in vitro culture. CD34+CD45RA- subset denotes HSC and early precursors populations. (C) Percentage of HSCs and Megakaryocytes after treatment of TPO or the three TpoR Dbs for the indicated times. HSCs stimulated with AK111 or AK113 survive and remain undifferentiated longer than HSCs treated with TPO or AK119 (left panel). On the other hand, TPO and AK119 treatment yield higher number of Megakaryocytes (right panel).

Definitions

Unless otherwise defined, all terms of art, notations and other scientific terms or terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, nucleic acid chemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, Current Protocols in Immunology (J. E. Coligan et al., eds., 1999, including supplements through 2001); Current Protocols in Molecular Biology (F. M. Ausubel et al., eds., 1987, including supplements through 2001); Molecular Cloning: A Laboratory Manual, third edition (Sambrook and Russel, 2001); PCR: The Polymerase Chain Reaction, (Mullis et al., eds., 1994); The Immunoassay Handbook (D. Wild, ed., Stockton Press NY, 1994); Bioconjugate Techniques (Greg T. Hermanson, ed., Academic Press, 1996); Methods of Immunological Analysis (R. Masseyeff, W. H. Albert, and N. A. Staines, eds., Weinheim: VCH Verlags gesellschaft mbH, 1993), Harlow and Lane Using Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1999; and Beaucage et al. eds., Current Protocols in Nucleic Acid Chemistry John Wiley & Sons, Inc., New York, 2000).

[0034] It is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, animal species or genera, and reagents described, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

[0035] As used herein the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the culture" includes reference to one or more cultures and equivalents thereof known to those skilled in the art, and so forth. All technical and

scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs unless clearly indicated otherwise.

[0036] *Receptor tyrosine kinase*. In some embodiments of the invention a receptor, e.g. a cell surface receptor, targeted by an artificial ligand of the invention is a receptor tyrosine kinase. Such receptors may include c-Fms, PDGFR α , PDGFR β , c-Kit, Flt-3, VEGFR1, VEGFR2, VEGFR3, FGFR family, etc. Also included is the JAK family, which are linked to cytoplasmic receptors of interest.

[0037] Cytokine receptors include, without limitation, the following receptors:

Cytokine	Receptor subunits
IL-1-like	
IL-1 α	CD121a, CDw121b
IL-1 β	CD121a, CDw121b
IL-1RA	CD121a
IL-18	IL-18R α , β
IL-2	CD25, 122, 132
IL-4	CD124, 213a13, 132
IL-7	CD127, 132
IL-9	IL-9R, CD132
IL-13	CD213a1, 213a2, CD1243, 132
IL-15	IL-15Ra, CD122, 132
IL-3	CD123, CDw131
IL-5	CDw125, 131
GM-CSF	CD116, CDw131
IL-6	CD126, 130
IL-11	IL-11Ra, CD130
G-CSF	CD114
IL-12	CD212
LIF	LIFR, CD130
OSM	OSMR, CD130
IL-10	CDw210
IL-20	IL-20R α , β
IL-14	IL-14R
IL-16	CD4
IL-17	CDw217
IFN- α	CD118
IFN- β	CD118
IFN- γ	CDw119
CD154	CD40
LT- β	LT β R
TNF- α	CD120a, b

TNF- β	CD120a, b
4-1BBL	CDw137 (4-1BB)
APRIL	BCMA, TACI
CD70	CD27
CD153	CD30
CD178	CD95 (Fas)
GITRL	GITR
LIGHT	LTbR, HVEM
OX40L	OX40
TALL-1	BCMA, TACI
TRAIL	TRAILR1-4
TWEAK	Apo3
TRANCE	RANK, OPG
TGF- β 1	TGF- β R1
TGF- β 2	TGF- β R2
TGF- β 3	TGF- β R3
Epo	EpoR
Tpo	TpoR
Flt-3L	Flt-3
SCF	CD117
M-CSF	CD115
MSP	CDw136

[0038] Signaling subunits can be shared between receptors. The IL-2 receptor common gamma chain (also known as CD132) is shared between: IL-2 receptor, IL-4 receptor, IL-7 receptor, IL-9 receptor, IL-13 receptor, IL-15 receptor. The common beta chain (CD131 or CDw131) is shared between: GM-CSF receptor, IL-3 receptor, IL-5 receptor. The gp230 receptor common gamma chain (also known as gp130, IL6ST, IL6-beta or CD130) is shared between: IL-6 receptor, IL-11 receptor, IL-12 receptor, leukemia inhibitory factor receptor, Oncostatin M receptor.

[0039] *Janus kinases.* Janus kinases (JAKs) are non-receptor tyrosine kinases. In mammals, the family has four members, JAK1, JAK2, JAK3 and Tyrosine kinase 2 (TYK2). Seven JAK homology (JH) domains have been identified, numbered from the carboxyl to the amino terminus. The JH1 domain at the carboxyl terminus has all the features of a typical eukaryotic tyrosine kinase domain. Adjacent to the JH1 domain is a catalytically inactive pseudokinase or kinase-like domain (JH2), which is distantly related to other tyrosine kinase domains. This tandem architecture of kinase domains is the hallmark of JAK kinases and gives them their name. Although the pseudokinase domain lacks catalytic activity, it has an essential regulatory function. A number of mutations within this domain abrogate kinase activity, or conversely activate the kinase leading to oncogenesis.

[0040] In mammals JAK1, JAK2 and TYK2 are ubiquitously expressed. At the cellular level, JAKs can be found in the cytosol when they are experimentally expressed, but because of their intimate association with cytokine receptors, they ordinarily localize to endosomes and the plasma membrane, along with their cognate receptors. A large number of cytokines are dependent upon JAK1, including a family that use a shared receptor subunit called common γ chain (γ_c), which includes interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15 and IL-21. These cytokines are also dependent upon Jak3, because Jak3 binds γ_c . Jak1 is also essential for another family that uses the shared receptor subunit gp130 (IL-6, IL-11, oncostatin M, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNF)) as well as granulocyte colony-stimulating factor (G-CSF) and IFNs. JAK2 is essential for the hormone-like cytokines such as growth hormone (GH), prolactin (PRL), erythropoietin (EPO), thrombopoietin (TPO) and the family of cytokines that signal through the IL-3 receptor (IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor, GM-CSF). JAK2 is also important for cytokines that use the gp130 receptor and for some IFNs.

[0041] It is believed that ligand binding promotes a conformational change in the receptor, which promotes JAK activation through reciprocal interaction of two juxtapositioned JAK kinases and auto- and/or trans-phosphorylation of tyrosine residues on the activation loop of the JAK kinase domain.

[0042] Oncogenic JAK mutations include JAK proteins with ligand-independent activity. Surprisingly, diabodies that enforce a large intersubunit distance in a cytokine receptor, prevent downstream ligand-independent JAK signaling. Activating mutations can promote autonomous cell proliferation, e.g. in ALL, AML, and other myeloproliferative disorders, and can also confer resistance to ATP-competitive inhibitors. Mutants include JAK2V617F, JAK2 K607N, JAK1F635V, JAK1S646F, JAK1Y652H, JAK1V658I/L/F, JAK1K1026E, JAK1Y1035C, JAK1S1043I, JAK1F958V, JAK1F958C, JAK1F958L, JAK1D895H, JAK1E897K, JAK1T901R, JAK1L910Q, etc. The JAK2V617F mutation is of particular interest.

[0043] As used herein, the terms "antibody" and "antibodies" refer to monoclonal antibodies, multispecific antibodies, human antibodies, humanized antibodies, synthetic antibodies, chimeric antibodies, polyclonal antibodies, camelized antibodies, single-chain Fvs (scFv), single chain antibodies, Fab fragments, F(ab') fragments, disulfide-linked bispecific Fvs (sdFv), intrabodies, and anti-idiotypic (anti-Id) antibodies (including, e.g., anti-Id and anti-anti-Id antibodies to antibodies of the invention), and epitope-binding fragments of any of the above. In particular, antibodies include immunoglobulin molecules and immunologically active fragments of immunoglobulin molecules, i.e., molecules that contain an epitope binding site. Immunoglobulin molecules can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG₁, IgG₂, IgG₃, IgG₄, IgA₁, and IgA₂) or subclass.

[0044] As used herein, the terms "immunospecifically binds," "immunospecifically recognizes," "specifically binds," "specifically recognizes" and analogous terms refer to molecules that specifically bind to an antigen (e.g., epitope or immune complex) and do not specifically bind to another molecule. A molecule that specifically binds to an antigen may bind to other peptides or polypeptides with lower affinity as determined by, e.g., immunoassays, BIAcore, or other assays known in the art. Preferably, molecules that specifically bind an antigen do not cross-react with other proteins. Molecules that specifically bind an antigen can be identified, for example, by immunoassays, BIAcore, or other techniques known to those of skill in the art.

[0045] As used herein, the terms "heavy chain," "light chain," "variable region," "framework region," "constant domain," and the like, have their ordinary meaning in the immunology art and refer to domains in naturally occurring immunoglobulins and the corresponding domains of synthetic (e.g., recombinant) binding proteins (e.g., humanized antibodies, single chain antibodies, chimeric antibodies, etc.). The basic structural unit of naturally occurring immunoglobulins (e.g., IgG) is a tetramer having two light chains and two heavy chains, usually expressed as a glycoprotein of about 150,000 Da. The amino-terminal ("N") portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal ("C") portion of each chain defines a constant region, with light chains having a single constant domain and heavy chains usually having three constant domains and a hinge region. The variable regions of an immunoglobulin consist of the complementarity determining regions (CDRs), which contain the residues in contact with antigen and non-CDR segments, referred to as framework segments, which in general maintain the structure and determine the positioning of the CDR loops (although certain framework residues may also contact antigen). Thus, the V_L and V_H domains have the structure n-FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4-c. When referring to antibodies (as broadly defined herein), the assignment of amino acids to each domain may be made in accordance with the definitions of Kabat, Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, Md., 1987 and 1991).

[0046] As used herein, the term "derivative" in the context of polypeptides or proteins refers to a polypeptide or protein that comprises an amino acid sequence which has been altered by the introduction of amino acid residue substitutions, deletions or additions. The term "derivative" as used herein also refers to a polypeptide or protein which has been modified, i.e., by the attachment of any type of molecule to the polypeptide or protein. For example, but not by way of limitation, an antibody may be modified, e.g., by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular an antigen or other protein, etc. A derivative polypeptide or protein may be produced by chemical modifications using techniques known to those of skill in

the art, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Further, a derivative polypeptide or protein derivative possesses a similar or identical function as the polypeptide or protein from which it was derived.

[0047] As used herein, the term "diabody molecule" refers to a complex of two or more polypeptide chains or proteins, each comprising at least one VL and one VH domain or fragment thereof, wherein both domains are comprised within a single polypeptide chain. The polypeptide chains in the complex may be the same or different, i.e., the diabody molecule may be a homo-multimer or a hetero-multimer. In specific aspects, "diabody molecule" includes dimers or tetramers or said polypeptide chains containing both a VL and VH domain.

[0048] "Identical polypeptide chains" as used herein also refers to polypeptide chains having almost identical amino acid sequence, for example, including chains having one or more amino acid differences, preferably conservative amino acid substitutions, such that the activity of the two polypeptide chains is not significantly different.

[0049] As used herein, the term "epitope" refers to a fragment of a polypeptide or protein or a non-protein molecule having antigenic or immunogenic activity in an animal, preferably in a mammal, and most preferably in a human. An epitope having immunogenic activity is a fragment of a polypeptide or protein that elicits an antibody response in an animal. An epitope having antigenic activity is a fragment of a polypeptide or protein to which an antibody immunospecifically binds as determined by any method well-known to one of skill in the art, for example by immunoassays.

[0050] As used herein, the term "fragment" refers to a peptide or polypeptide comprising an amino acid sequence of at least 5 contiguous amino acid residues, at least 10 contiguous amino acid residues, at least 15 contiguous amino acid residues, at least 20 contiguous amino acid residues, at least 25 contiguous amino acid residues, at least 40 contiguous amino acid residues, at least 50 contiguous amino acid residues, at least 60 contiguous amino acid residues, at least 70 contiguous amino acid residues, at least contiguous 80 amino acid residues, at least contiguous 90 amino acid residues, at least contiguous 100 amino acid residues, at least contiguous 125 amino acid residues, at least 150 contiguous amino acid residues, at least contiguous 175 amino acid residues, at least contiguous 200 amino acid residues, or at least contiguous 250 amino acid residues of the amino acid sequence of another polypeptide. In a specific embodiment, a fragment of a polypeptide retains at least one function of the polypeptide.

[0051] As used herein, the terms "nucleic acids" and "nucleotide sequences" include DNA molecules (e.g., cDNA or genomic DNA), RNA molecules (e.g., mRNA), combinations of DNA and RNA molecules or hybrid DNA/RNA molecules, and analogs of DNA or RNA molecules. Such analogs can be generated using, for example, nucleotide analogs, which include, but are not limited to, inosine or tritylated bases. Such analogs can also comprise DNA or RNA molecules comprising modified backbones that lend beneficial attributes to the molecules such as, for example, nuclease resistance or an increased ability to cross cellular membranes. The nucleic acids or nucleotide sequences can be single-stranded, double-stranded, may contain both single-stranded and double-stranded portions, and may contain triple-stranded portions, but preferably is double-stranded DNA.

[0052] As used herein, a "therapeutically effective amount" refers to that amount of the therapeutic agent sufficient to treat or manage a disease or disorder. A therapeutically effective amount may refer to the amount of therapeutic agent sufficient to delay or minimize the onset of disease, e.g., delay or minimize the spread of cancer, or the amount effect to decrease or increase signaling from a receptor of interest. A therapeutically effective amount may also refer to the amount of the therapeutic agent that provides a therapeutic benefit in the treatment or management of a disease. Further, a therapeutically effective amount with respect to a therapeutic agent of the invention means the amount of therapeutic agent alone, or in combination with other therapies, that provides a therapeutic benefit in the treatment or management of a disease.

[0053] As used herein, the terms "prevent", "preventing" and "prevention" refer to the prevention of the recurrence or onset of one or more symptoms of a disorder in a subject as result of the administration of a prophylactic or therapeutic agent.

[0054] As used herein, the term "in combination" refers to the use of more than one prophylactic and/or therapeutic agents. The use of the term "in combination" does not restrict the order in which prophylactic and/or therapeutic agents are administered to a subject with a disorder. A first prophylactic or therapeutic agent can be administered prior to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks 6 weeks, 8 weeks, or 12 weeks before), concomitantly with, or subsequent to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of a second prophylactic or therapeutic agent to a subject with a disorder.

Detailed Description of the Embodiments

[0055] Artificial bivalent ligands are provided that bind to dimeric cell surface receptors, particularly cytokine receptors, and that further have sufficient rigidity to enforce a defined distance between the receptor subunits. Suitable artificial ligands include antibody variable regions reformatted into diabodies, which have two binding sites that are the same or different. Each peptide of the diabody comprises two domains separated by a short linker. Upon binding of the diabody to a cell surface receptor, e.g. a cytokine receptor, signaling by the receptor is 'tuned', i.e. the intensity of signaling is changed by binding to the diabody, where a range of signaling intensities is obtained based on the distance between receptor subunits.

[0056] The diabodies of the invention are useful in therapeutic and prophylactic methods where a desired level of signaling from the receptor is desired. In some embodiments the distance enforced between the receptor subunits is determined, and used to correlate with the signaling intensity. The distance can also be adjusted to achieve a desired signaling intensity, for example by grafting CDR sequences onto a variable region framework that provides a suitable distance for the desired signal intensity. Alternatively, variable regions can be mixed such that the diabody has two different variable regions, thereby providing a distance intermediate between the distance of the homodimeric diabodies. Variable regions of interest can be reformatted into diabodies, and the distances enforced by binding determined, and used to design diabodies that elicit a desired signaling intensity from a receptor of interest. Diabodies that provide for a tuned signaling intensity can be used in therapeutic and prophylactic methods by providing for the desired receptor-mediated signaling.

[0057] The at least two binding sites of the diabody molecule can recognize the same or different epitopes. Different epitopes can be from the same antigen or epitopes from different antigens. In one embodiment, the epitopes are from different subunits of the receptor. In another embodiment, the epitopes are on the same receptor subunit, but the variable regions have different framework residues with the same CDR sequences in order to specify a desired degree of receptor subunit separation upon binding. In another embodiment, the epitopes are on the same receptor subunit, but the variable regions have different framework residues and CDR sequences in order to specify a desired degree of receptor subunit separation upon binding.

[0058] The invention further encompasses incorporation of unnatural amino acids to generate the diabodies of the invention. Such methods are known to those skilled in the art such as those using the natural biosynthetic machinery to allow incorporation of unnatural amino acids into proteins, see, e.g., Wang et al., 2002 Chem. Comm. 1: 1-11; Wang et al., 2001, Science, 292: 498-500; van Hest et al., 2001. Chem. Comm. 19: 1897-1904, each of which is incorporated herein by reference in its entirety. Alternative strategies focus on the enzymes responsible for the biosynthesis of amino acyl-tRNA, see, e.g., Tang et al., 2001, J. Am. Chem.

123(44): 11089-11090; Kiick et al., 2001, FEBS Lett. 505(3): 465; each of which is incorporated herein by reference in its entirety.

[0059] In some embodiments, the invention encompasses methods of modifying a VL, VH or Fc domain of a molecule of the invention by adding or deleting a glycosylation site. Methods for modifying the carbohydrate of proteins are well known in the art and encompassed within the invention, see, e.g., U.S. Pat. No. 6,218,149; EP 0359096 B1; U.S. Publication No. US 2002/0028486; WO 03/035835; U.S. Publication No. 2003/0115614; U.S. Pat. No. 6,218,149; U.S. Pat. No. 6,472,511; all of which are incorporated herein by reference in their entirety.

[0060] A diabody that enforces large receptor-dimer separation can counteract intracellular ligand-independent JAK/STAT activation. Without being limited by the theory, it is believed that the enforced receptor dimer separation exceeds the accessible distance that the JAK2 kinase domain can extend to transphosphorylate the opposing JAK2 and receptor. Methods are provided for inhibiting ligand independent JAK signaling, by contacting a receptor operably linked to the JAK protein with a diabody that enforces a large receptor dimer separation. In some such embodiments the receptor is EPO receptor. The contacting may be performed in vivo, and includes the treatment of hematologic malignancies, including acute lymphoblastic leukemia, myeloproliferative neoplasms, e.g. AML, polycythemia vera, essential thrombocytopenia, myelofibrosis, etc.

[0061] The large receptor-dimer separation, for example with respect to two EPO-R subunits, at the furthest points is from about 50 Å to 200 Å. For example, an antagonist diabody, which can have the additional property of inhibiting intracellular ligand-independent JAK/STAT activation, may enforce a distance of from about 140 Å to about 200 Å, from about 145 Å to about 175 Å, from about 145 Å to about 150 Å. In contrast, a fully agonistic diabody may enforce a distance of from about 50 Å to about 135 Å, and may be from about 100 Å to about 130 Å, from about 110 Å to about 130 Å, from about 120 Å to about 130 Å, from about 125 Å to about 130 Å. A partial agonist may enforce a distance of from about 130 Å to about 145 Å, from about 135 Å to about 145 Å, from about 140 Å to about 145 Å.

[0062] In some embodiments, treatment of an individual with such a hematologic malignancy by inhibiting ligand-independent JAK signaling, comprises the steps of administering an effective dose of a diabody that enforces a large distance between the receptor subunits, e.g. the EPO receptor subunits, where the dose is effective to substantially decrease JAK signaling in the malignant cells, i.e. to decrease by greater than about 20%, to decrease by greater than about 30%, to decrease by greater than about 40%, to decrease by greater than about 50%, to decrease by greater than about 75%, to decrease by greater than about 90%, to decrease by greater than about 95%, to decrease by greater than about 99% or more relative to an untreated individual. The receptor subunit distance thus enforced may be greater than

about 140 Å. Diabodies for such purpose include without limitation DA10 and DA330 provided herein.

Variable regions

- [0063] The diabodies of the present invention comprise antigen binding domains generally derived from immunoglobulins or antibodies. The antibodies from which the binding domains used in the methods of the invention are derived may be from any animal origin including birds and mammals (e.g., human, non-human primate, murine, donkey, sheep, rabbit, goat, guinea pig, camel, horse, or chicken). Preferably, the antibodies are human or humanized monoclonal antibodies. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or libraries of synthetic human immunoglobulin coding sequences or from mice that express antibodies from human genes.
- [0064] The antibodies typically bind specifically to a cell surface receptor, including the receptor tyrosine kinase receptors described above, and particularly the cytokine receptors, as defined herein, including homomultimers and heteromultimers. In some embodiments the cytokine receptor is one of EPO receptor, TPO receptor, FLT3, CD117, CD115, CDw136. In some embodiments the receptor is EPO receptor, where the diabodies DA5, DA10, DA307 and DA330 are examples. In some embodiments the receptor is the TPO receptor, where the diabodies AK119, AK111 and AK113 are examples.
- [0065] The diabodies used in the methods of the invention include derivatives that are modified, i.e., by the attachment of any type of molecule to the diabody. For example, but not by way of limitation, the diabody derivatives include diabodies that have been modified, e.g., by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to, specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the derivative may contain one or more non-classical amino acids.
- [0066] Framework residues and CDR residues can be substituted to alter the specific angle of separation. These framework substitutions are identified by methods known in the art, e.g., by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. (See, e.g., U.S. Pat. No. 5,585,089; and Riechmann et al., 1988, Nature 332:323, which are incorporated herein by reference in their entireties.)
- [0067] Monoclonal antibodies from which binding domains of the diabodies of the invention can be prepared using a wide variety of techniques known in the art including the use of hybridoma,

recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, et al., in: *Monoclonal Antibodies and T-Cell Hybridomas*, pp. 563-681 (Elsevier, N.Y., 1981) (both of which are incorporated by reference in their entireties). The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

[0068] Antibodies can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. In a particular embodiment, such phage can be utilized to display antigen binding domains, such as Fab and Fv or disulfide-bond stabilized Fv, expressed from a repertoire or combinatorial antibody library (e.g., human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, e.g., using labeled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage, including fd and M13. The antigen binding domains are expressed as a recombinantly fused protein to either the phage gene III or gene VIII protein. Examples of phage display methods that can be used to make the immunoglobulins, or fragments thereof, of the present invention include those disclosed in Brinkman et al., *J. Immunol. Methods*, 182:41-50, 1995; Ames et al., *J. Immunol. Methods*, 184:177-186, 1995; Kettleborough et al., *Eur. J. Immunol.*, 24:952-958, 1994; Persic et al., *Gene*, 187:9-18, 1997; Burton et al., *Advances in Immunology*, 57:191-280, 1994; PCT Application No. PCT/GB91/01134; PCT Publications WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Pat. Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108; each of which is incorporated herein by reference in its entirety.

[0069] Standard techniques known to those skilled in the art can be used to introduce mutations in the nucleotide sequence encoding an antibody, or fragment thereof, including, e.g., site-directed mutagenesis and PCR-mediated mutagenesis, which results in amino acid substitutions.

[0070] The diabody molecules of the invention (i.e., polypeptides) can be fused to marker sequences, such as a peptide to facilitate purification. In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, Calif., 91311), among others, many of which are commercially available. As described in Gentz et al., 1989, *Proc. Natl. Acad. Sci. USA*,

86:821-824, for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the hemagglutinin "HA" tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson et al., Cell, 37:767 1984) and the "flag" tag (Knappik et al., Biotechniques, 17(4):754-761, 1994).

[0071] The present invention also encompasses diabody molecules of the invention conjugated to or immunospecifically recognizing a diagnostic or therapeutic agent or any other molecule for which serum half-life is desired to be increased/decreased and/or targeted to a particular subset of cells. The molecules of the invention can be used diagnostically to, for example, monitor the development or progression of a disease, disorder or infection as part of a clinical testing procedure to, e.g., determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the molecules of the invention to a detectable substance or by the molecules immunospecifically recognizing the detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron emitting metals, and nonradioactive paramagnetic metal ions. The detectable substance may be coupled or conjugated either directly to the molecules of the invention or indirectly, through an intermediate (such as, for example, a linker known in the art) using techniques known in the art, or the molecule may immunospecifically recognize the detectable substance: immunospecifically binding said substance. See, for example, U.S. Pat. No. 4,741,900 for metal ions which can be conjugated to antibodies for use as diagnostics according to the present invention. Such diagnosis and detection can be accomplished designing the molecules to immunospecifically recognize the detectable substance or by coupling the molecules of the invention to detectable substances including, but not limited to, various enzymes, enzymes including, but not limited to, horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; prosthetic group complexes such as, but not limited to, streptavidin/biotin and avidin/biotin; fluorescent materials such as, but not limited to, umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; luminescent material such as, but not limited to, luminol; bioluminescent materials such as, but not limited to, luciferase, luciferin, and aequorin; radioactive material such as, but not limited to, bismuth (^{213}Bi), carbon (^{14}C), chromium (^{51}Cr), cobalt (^{57}Co), fluorine (^{18}F), gadolinium (^{153}Gd , ^{159}Gd), gallium (^{68}Ga , ^{67}Ga), germanium (^{68}Ge), holmium (^{166}Ho), indium (^{115}In , ^{113}In , ^{112}In , ^{111}In), iodine (^{131}I , ^{125}I , ^{123}I , ^{121}I), lanthanum (^{140}La), lutetium (^{177}Lu), manganese (^{54}Mn), molybdenum (^{99}Mo), palladium (^{103}Pd), phosphorous (^{32}P), praseodymium (^{142}Pr), promethium (^{149}Pm), rhenium (^{186}Re , ^{188}Re), rhodium (^{105}Rh), ruthenium (^{97}Ru), samarium (^{153}Sm), scandium (^{47}Sc), selenium (^{75}Se), strontium (^{85}Sr), sulfur (^{35}S), technetium (^{99}Tc), thallium (^{201}Tl), tin (^{113}Sn , ^{117}Sn), tritium (^3H), xenon (^{133}Xe), ytterbium (^{169}Yb , ^{175}Yb), yttrium (^{90}Y), zinc (^{65}Zn); positron

emitting metals using various positron emission tomographies, and nonradioactive paramagnetic metal ions.

[0072] Techniques for conjugating such therapeutic moieties to polypeptides are well known; see, e.g., Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in *Monoclonal Antibodies And Cancer Therapy*, Reisfeld et al. (eds.), 1985, pp. 243-56, Alan R. Liss, Inc.); Hellstrom et al., "Antibodies For Drug Delivery", in *Controlled Drug Delivery* (2nd Ed.), Robinson et al. (eds.), 1987, pp. 623-53, Marcel Dekker, Inc.); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in *Monoclonal Antibodies '84: Biological And Clinical Applications*, Pinchera et al. (eds.), 1985, pp. 475-506); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in *Monoclonal Antibodies For Cancer Detection And Therapy*, Baldwin et al. (eds.), 1985, pp. 303-16, Academic Press; and Thorpe et al., *Immunol. Rev.*, 62:119-58, 1982.

[0073] The diabody molecules may be characterized in a variety of ways. In particular, molecules of the invention may be assayed for the ability to immunospecifically bind to an antigen. Such an assay may be performed in solution (e.g., Houghten, *Bio/Techniques*, 13:412-421, 1992), on beads (Lam, *Nature*, 354:82-84, 1991), on chips (Fodor, *Nature*, 364:555-556, 1993), on bacteria (U.S. Pat. No. 5,223,409), on plasmids (Cull et al., *Proc. Natl. Acad. Sci. USA*, 89:1865-1869, 1992) or on phage (Scott and Smith, *Science*, 249:386-390, 1990; Devlin, *Science*, 249:404-406, 1990; Cwirla et al., *Proc. Natl. Acad. Sci. USA*, 87:6378-6382, 1990; and Felici, *J. Mol. Biol.*, 222:301-310, 1991) (each of these references is incorporated by reference herein in its entirety).

[0074] The affinities and binding properties of the molecules of the invention for an antigen may be initially determined using in vitro assays (biochemical or immunological based assays) known in the art for antigen-binding domain interactions, including but not limited to ELISA assay, surface plasmon resonance assay, immunoprecipitation assays. In some embodiments, screening and identifying molecules comprising epitope binding domains are done with functional based assays, preferably in a high throughput manner. The functional based assays can be any assay known in the art for characterizing cytokine signaling.

[0075] BIAcore kinetic analysis can be used to determine the binding on and off rates of molecules of the present invention to an antigen comprises analyzing the binding and dissociation of an antigen from chips with immobilized molecules (e.g., molecules comprising epitope binding domains, respectively) on their surface. Fluorescence activated cell sorting (FACS), using any of the techniques known to those skilled in the art, can be used for immunological or functional based assay to characterize molecules of the invention. Flow sorters are capable of rapidly examining a large number of individual cells that have been bound, e.g., opsonized, by molecules of the invention (e.g., 10-100 million cells per hour)

(Shapiro et al., Practical Flow Cytometry, 1995). Additionally, specific parameters used for optimization of diabody behavior, include but are not limited to, antigen concentration, kinetic competition time, or FACS stringency, each of which may be varied in order to select for the diabody molecules comprising molecules of the invention which exhibit specific binding properties, e.g., concurrent binding to multiple epitopes. Flow cytometers for sorting and examining biological cells are well known in the art. Known flow cytometers are described, for example, in U.S. Pat. Nos. 4,347,935; 5,464,581; 5,483,469; 5,602,039; 5,643,796; and 6,211,477; the entire contents of which are incorporated by reference herein.

[0076] Characterization of target antigen binding affinity, and assessment of target antigen density on a cell surface may be made by methods well known in the art such as Scatchard analysis or by the use of kits as per manufacturer's instructions. The one or more functional assays can be any assay known in the art for characterizing cell function as known to one skilled in the art or described herein. I

Methods of Producing Diabody Molecules of the Invention

[0077] The diabody molecules of the present invention can be produced using a variety of methods well known in the art, including de novo protein synthesis and recombinant expression of nucleic acids encoding the binding proteins. The desired nucleic acid sequences can be produced by recombinant methods (e.g., PCR mutagenesis of an earlier prepared variant of the desired polynucleotide) or by solid-phase DNA synthesis. Usually recombinant expression methods are used. Because of the degeneracy of the genetic code, a variety of nucleic acid sequences encode each immunoglobulin amino acid sequence, and the present invention includes all nucleic acids encoding the binding proteins described herein.

[0078] Once the nucleotide sequence of the molecules that are identified by the methods of the invention is determined, the nucleotide sequence may be manipulated using methods well known in the art, e.g., recombinant DNA techniques, site directed mutagenesis, PCR, etc. (see, for example, the techniques described in Sambrook et al., 2001, Molecular Cloning, A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.; and Ausubel et al., eds., 1998, Current Protocols in Molecular Biology, John Wiley & Sons, NY, which are both incorporated by reference herein in their entireties), to generate, for example, antibodies having a different amino acid sequence, for example by generating amino acid substitutions, deletions, and/or insertions. In one embodiment, human libraries or any other libraries available in the art, can be screened by standard techniques known in the art, to clone the nucleic acids encoding the molecules of the invention.

[0079] Once a molecule of the invention (i.e., diabodies) has been recombinantly expressed, it may be purified by any method known in the art for purification of polypeptides, polypeptides or diabodies (e.g., analogous to antibody purification schemes based on antigen selectivity) for

example, by chromatography (e.g., ion exchange, affinity, particularly by affinity for the specific antigen, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of polypeptides, polyproteins or diabodies.

METHODS OF USE

[0080] The invention encompasses methods and compositions for treatment or prevention of disease in a subject comprising administering to the subject a therapeutically effective amount of one or more tuned diabodies of the invention. For example, a subject requiring EPO activity can be provided with an effective dose of an EPO-R agonist diabody that provides for a desired level of signaling from the EPO-R. A subject requiring TPO activity, e.g. in the treatment of thrombopoiesis, can be provided with an effective dose of a TPO-R agonist diabody.

[0081] Where the diabody prevents ligand independent JAK signaling, the effective dose of the antibody is useful in the treatment or prevention of malignancies associated with the JAK. Cancers and related disorders that can be treated or prevented by methods and compositions of the present invention include, but are not limited to, the following: Leukemias including, but not limited to, acute leukemia, acute lymphocytic leukemia, acute myelocytic leukemias such as myeloblastic, promyelocytic, myelomonocytic, monocytic, erythroleukemia leukemias and myelodysplastic syndrome, chronic leukemias such as but not limited to, chronic myelocytic (granulocytic) leukemia, chronic lymphocytic leukemia, hairy cell leukemia; polycythemia vera; lymphomas such as but not limited to Hodgkin's disease, non-Hodgkin's disease; multiple myelomas such as but not limited to smoldering multiple myeloma, nonsecretory myeloma, osteosclerotic myeloma, plasma cell leukemia, solitary plasmacytoma and extramedullary plasmacytoma; Waldenstrom's macroglobulinemia; monoclonal gammopathy of undetermined significance; benign monoclonal gammopathy; heavy chain disease; bone and connective tissue sarcomas such as but not limited to bone sarcoma, osteosarcoma, chondrosarcoma, Ewing's sarcoma, malignant giant cell tumor, fibrosarcoma of bone, chordoma, periosteal sarcoma, soft-tissue sarcomas, angiosarcoma (hemangiosarcoma), fibrosarcoma, Kaposi's sarcoma, leiomyosarcoma, liposarcoma, lymphangiosarcoma, neurilemmoma, rhabdomyosarcoma, synovial sarcoma; brain tumors including but not limited to, glioma, astrocytoma, brain stem glioma, ependymoma, oligodendroglioma, nonglial tumor, acoustic neurinoma, craniopharyngioma, medulloblastoma, meningioma, pineocytoma, pineoblastoma, primary brain lymphoma; breast cancer including, but not limited to, adenocarcinoma, lobular (small cell) carcinoma, intraductal carcinoma, medullary breast cancer, mucinous breast cancer, tubular breast cancer, papillary breast cancer, Paget's disease, and inflammatory breast cancer; adrenal cancer, including but not limited to, pheochromocytom and adrenocortical carcinoma; thyroid cancer such as but not limited to papillary or follicular thyroid cancer, medullary thyroid cancer and anaplastic thyroid cancer; pancreatic cancer, including

but not limited to, insulinoma, gastrinoma, glucagonoma, vipoma, somatostatin-secreting tumor, and carcinoid or islet cell tumor; pituitary cancers including but not limited to, Cushing's disease, prolactin-secreting tumor, acromegaly, and diabetes insipidus; eye cancers including but not limited to, ocular melanoma such as iris melanoma, choroidal melanoma, and ciliary body melanoma, and retinoblastoma; vaginal cancers, including but not limited to, squamous cell carcinoma, adenocarcinoma, and melanoma; vulvar cancer, including but not limited to, squamous cell carcinoma, melanoma, adenocarcinoma, basal cell carcinoma, sarcoma, and Paget's disease; cervical cancers including but not limited to, squamous cell carcinoma, and adenocarcinoma; uterine cancers including but not limited to, endometrial carcinoma and uterine sarcoma; ovarian cancers including but not limited to, ovarian epithelial carcinoma, borderline tumor, germ cell tumor, and stromal tumor; esophageal cancers including but not limited to, squamous cancer, adenocarcinoma, adenoid cystic carcinoma, mucoepidermoid carcinoma, adenosquamous carcinoma, sarcoma, melanoma, plasmacytoma, verrucous carcinoma, and oat cell (small cell) carcinoma; stomach cancers including but not limited to, adenocarcinoma, fungating (polypoid), ulcerating, superficial spreading, diffusely spreading, malignant lymphoma, liposarcoma, fibrosarcoma, and carcinosarcoma; colon cancers; rectal cancers; liver cancers including but not limited to hepatocellular carcinoma and hepatoblastoma, gallbladder cancers including but not limited to, adenocarcinoma; cholangiocarcinomas including but not limited to, papillary, nodular, and diffuse; lung cancers including but not limited to, non-small cell lung cancer, squamous cell carcinoma (epidermoid carcinoma), adenocarcinoma, large-cell carcinoma and small-cell lung cancer; testicular cancers including but not limited to, germinal tumor, seminoma, anaplastic, classic (typical), spermatocytic, nonseminoma, embryonal carcinoma, teratoma carcinoma, choriocarcinoma (yolk-sac tumor), prostate cancers including but not limited to, adenocarcinoma, leiomyosarcoma, and rhabdomyosarcoma; penile cancers; oral cancers including but not limited to, squamous cell carcinoma; basal cancers; salivary gland cancers including but not limited to, adenocarcinoma, mucoepidermoid carcinoma, and adenoidcystic carcinoma; pharynx cancers including but not limited to, squamous cell cancer, and verrucous; skin cancers including but not limited to, basal cell carcinoma, squamous cell carcinoma and melanoma, superficial spreading melanoma, nodular melanoma, lentigo malignant melanoma, acral lentiginous melanoma; kidney cancers including but not limited to, renal cell cancer, adenocarcinoma, hypemephroma, fibrosarcoma, transitional cell cancer (renal pelvis and/ or uterus); Wilms' tumor; bladder cancers including but not limited to, transitional cell carcinoma, squamous cell cancer, adenocarcinoma, carcinosarcoma. In addition, cancers include myxosarcoma, osteogenic sarcoma, endotheliosarcoma, lymphangioendotheliosarcoma, mesothelioma, synovioma, hemangioblastoma, epithelial carcinoma, cystadenocarcinoma, bronchogenic carcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma and

papillary adenocarcinomas (for a review of such disorders, see Fishman et al., 1985, Medicine, 2d Ed., J. B. Lippincott Co., Philadelphia and Murphy et al., 1997, Informed Decisions: The Complete Book of Cancer Diagnosis, Treatment, and Recovery, Viking Penguin, Penguin Books U.S.A., Inc., United States of America).

[0082] In a specific embodiment, an inhibitory diabody that enforces a large distance between receptor subunits and thereby prevents ligand independent JAK signaling inhibits or reduces the growth of cancer cells by at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, at least 50%, at least 45%, at least 40%, at least 35%, at least 30%, at least 25%, at least 20%, or at least 10% relative to the growth of cancer cells in the absence of said molecule of the invention. Exemplary is DA10 inhibition of JAK2V617F signaling.

[0083] In a specific embodiment, a molecule of the invention kills cells or inhibits or reduces the growth of cancer cells at least 5%, at least 10%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 100% better than the parent molecule.

[0084] The invention further encompasses administering the molecules of the invention in combination with other therapies known to those skilled in the art for the treatment or prevention of cancer, including but not limited to, current standard and experimental chemotherapies, hormonal therapies, biological therapies, immunotherapies, radiation therapies, or surgery. In some embodiments, the molecules of the invention may be administered in combination with a therapeutically or prophylactically effective amount of one or more agents, therapeutic antibodies or other agents known to those skilled in the art for the treatment and/or prevention of cancer, autoimmune disease, infectious disease or intoxication.

[0085] In certain embodiments, one or more molecule of the invention is administered to a mammal, preferably a human, concurrently with one or more other therapeutic agents useful for the treatment of cancer. The term "concurrently" is not limited to the administration of prophylactic or therapeutic agents at exactly the same time, but rather it is meant that a molecule of the invention and the other agent are administered to a mammal in a sequence and within a time interval such that the molecule of the invention can act together with the other agent to provide an increased benefit than if they were administered otherwise. For example, each prophylactic or therapeutic agent (e.g., chemotherapy, radiation therapy, hormonal therapy or biological therapy) may be administered at the same time or sequentially in any order at different points in time; however, if not administered at the same time, they should be administered sufficiently close in time so as to provide the desired therapeutic or prophylactic effect. In preferred embodiments, the prophylactic or therapeutic agents are administered in a

time frame where both agents are still active. One skilled in the art would be able to determine such a time frame by determining the half life of the administered agents.

[0086] When used in combination with other prophylactic and/or therapeutic agents, the molecules of the invention and the prophylactic and/or therapeutic agent can act additively or, more preferably, synergistically. In one embodiment, a molecule of the invention is administered concurrently with one or more therapeutic agents in the same pharmaceutical composition. In another embodiment, a molecule of the invention is administered concurrently with one or more other therapeutic agents in separate pharmaceutical compositions. In still another embodiment, a molecule of the invention is administered prior to or subsequent to administration of another prophylactic or therapeutic agent. The invention contemplates administration of a molecule of the invention in combination with other prophylactic or therapeutic agents by the same or different routes of administration, e.g., oral and parenteral. In certain embodiments, when a molecule of the invention is administered concurrently with another prophylactic or therapeutic agent that potentially produces adverse side effects including, but not limited to, toxicity, the prophylactic or therapeutic agent can advantageously be administered at a dose that falls below the threshold that the adverse side effect is elicited.

[0087] The dosage amounts and frequencies of administration provided herein are encompassed by the terms therapeutically effective and prophylactically effective. The dosage and frequency further will typically vary according to factors specific for each patient depending on the specific therapeutic or prophylactic agents administered, the severity and type of cancer, the route of administration, as well as age, body weight, response, and the past medical history of the patient. Suitable regimens can be selected by one skilled in the art by considering such factors and by following, for example, dosages reported in the literature and recommended in the Physician's Desk Reference (56^{sup}.th ed., 2002).

PHARMACEUTICAL COMPOSITIONS

[0088] The invention provides methods and pharmaceutical compositions comprising molecules of the invention (i.e., diabodies). The invention also provides methods of treatment, prophylaxis, and amelioration of one or more symptoms associated with a disease, disorder or infection by administering to a subject an effective amount of a molecule of the invention, or a pharmaceutical composition comprising a molecule of the invention. In a preferred aspect, the molecule of the invention is substantially purified (i.e., substantially free from substances that limit its effect or produce undesired side-effects). In a specific embodiment, the subject is an animal, preferably a mammal such as non-primate (e.g., cows, pigs, horses, cats, dogs, rats etc.) and a primate (e.g., monkey such as, a cynomolgous monkey and a human). In a preferred embodiment, the subject is a human. In yet another preferred embodiment, the binding domains of the diabody is from the same species as the subject.

[0089] Various delivery systems are known and can be used to administer a composition comprising molecules of the invention, e.g., encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the antibody or fusion protein, receptor-mediated endocytosis (See, e.g., Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a nucleic acid as part of a retroviral or other vector, etc. Methods of administering a molecule of the invention include, but are not limited to, parenteral administration (e.g., intradermal, intramuscular, intraperitoneal, intravenous and subcutaneous), epidural, and mucosal (e.g., intranasal and oral routes). In a specific embodiment, the molecules of the invention are administered intramuscularly, intravenously, or subcutaneously. The compositions may be administered by any convenient route, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent. See, e.g., U.S. Pat. Nos. 6,019,968; 5,985,320; 5,985,309; 5,934,272; 5,874,064; 5,855,913; 5,290,540; and 4,880,078; and PCT Publication Nos. WO 92/19244; WO 97/32572; WO 97/44013; WO 98/31346; and WO 99/66903, each of which is incorporated herein by reference in its entirety.

[0090] The invention also provides that the molecules of the invention, are packaged in a hermetically sealed container such as an ampoule or sachette indicating the quantity of antibody. In one embodiment, the molecules of the invention are supplied as a dry sterilized lyophilized powder or water free concentrate in a hermetically sealed container and can be reconstituted, e.g., with water or saline to the appropriate concentration for administration to a subject. Preferably, the molecules of the invention are supplied as a dry sterile lyophilized powder in a hermetically sealed container at a unit dosage of at least 5 mg, more preferably at least 10 mg, at least 15 mg, at least 25 mg, at least 35 mg, at least 45 mg, at least 50 mg, or at least 75 mg. The lyophilized molecules of the invention should be stored at between 2 and 8° C. in their original container and the molecules should be administered within 12 hours, preferably within 6 hours, within 5 hours, within 3 hours, or within 1 hour after being reconstituted. In an alternative embodiment, molecules of the invention are supplied in liquid form in a hermetically sealed container indicating the quantity and concentration of the molecule, fusion protein, or conjugated molecule. Preferably, the liquid form of the molecules of the invention are supplied in a hermetically sealed container at least 1 mg/ml, more preferably at least 2.5 mg/ml, at least 5 mg/ml, at least 8 mg/ml, at least 10 mg/ml, at least 15 mg/kg, at least 25 mg/ml, at least 50 mg/ml, at least 100 mg/ml, at least 150 mg/ml, at least 200 mg/ml of the molecules.

[0091] The amount of the composition of the invention which will be effective in the treatment, prevention or amelioration of one or more symptoms associated with a disorder can be

determined by standard clinical techniques. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the condition, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

[0092] For diabodies encompassed by the invention, the dosage administered to a patient is typically 0.0001 mg/kg to 100 mg/kg of the patient's body weight. Preferably, the dosage administered to a patient is between 0.0001 mg/kg and 20 mg/kg, 0.0001 mg/kg and 10 mg/kg, 0.0001 mg/kg and 5 mg/kg, 0.0001 and 2 mg/kg, 0.0001 and 1 mg/kg, 0.0001 mg/kg and 0.75 mg/kg, 0.0001 mg/kg and 0.5 mg/kg, 0.0001 mg/kg to 0.25 mg/kg, 0.0001 to 0.15 mg/kg, 0.0001 to 0.10 mg/kg, 0.001 to 0.5 mg/kg, 0.01 to 0.25 mg/kg or 0.01 to 0.10 mg/kg of the patient's body weight. The dosage and frequency of administration of diabodies of the invention may be reduced or altered by enhancing uptake and tissue penetration of the diabodies by modifications such as, for example, lipidation.

[0093] In one embodiment, the dosage of the molecules of the invention administered to a patient are 0.01 mg to 1000 mg/day, when used as single agent therapy. In another embodiment the molecules of the invention are used in combination with other therapeutic compositions and the dosage administered to a patient are lower than when said molecules are used as a single agent therapy.

[0094] In yet another embodiment, the compositions can be delivered in a controlled release or sustained release system. Any technique known to one of skill in the art can be used to produce sustained release formulations comprising one or more molecules of the invention. See, e.g., U.S. Pat. No. 4,526,938; PCT publication WO 91/05548; PCT publication WO 96/20698; Ning et al., 1996, "Intratumoral Radioimmunotherapy of a Human Colon Cancer Xenograft Using a Sustained-Release Gel," *Radiotherapy & Oncology* 39:179-189, Song et al., 1995, "Antibody Mediated Lung Targeting of Long-Circulating Emulsions," *PDA Journal of Pharmaceutical Science & Technology* 50:372-397; Cleek et al., 1997, "Biodegradable Polymeric Carriers for a bFGF Antibody for Cardiovascular Application," *Pro. Int'l. Symp. Control. Rel. Bioact. Mater.* 24:853-854; and Lam et al., 1997, "Microencapsulation of Recombinant Humanized Monoclonal Antibody for Local Delivery," *Proc. Int'l. Symp. Control Rel. Bioact. Mater.* 24:759-760, each of which is incorporated herein by reference in its entirety. In one embodiment, a pump may be used in a controlled release system (See Langer, supra; Sefton, 1987, *CRC Crit. Ref Biomed Eng.* 14:20; Buchwald et al., 1980, *Surgery* 88:507; and Saudek et al., 1989, *N. Engl. J. Med* 321:574). In another embodiment, polymeric materials can be used to achieve controlled release of antibodies (see e.g., *Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Fla. (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New

York (1984); Ranger and Peppas, 1983, J., *Macromol. Sci. Rev. Macromol. Chem.* 23:61; See also Levy et al., 1985, *Science* 228:190; During et al., 1989, *Ann. Neurol.* 25:351; Howard et al., 1989, *J. Neurosurg.* 71:105; U.S. Pat. No. 5,679,377; U.S. Pat. No. 5,916,597; U.S. Pat. No. 5,912,015; U.S. Pat. No. 5,989,463; U.S. Pat. No. 5,128,326; PCT Publication No. WO 99/15154; and PCT Publication No. WO 99/20253).

[0095] Treatment of a subject with a therapeutically or prophylactically effective amount of molecules of the invention can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is treated with molecules of the invention in the range of between about 0.1 to 30 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. In other embodiments, the pharmaceutical compositions of the invention are administered once a day, twice a day, or three times a day. In other embodiments, the pharmaceutical compositions are administered once a week, twice a week, once every two weeks, once a month, once every six weeks, once every two months, twice a year or once per year. It will also be appreciated that the effective dosage of the molecules used for treatment may increase or decrease over the course of a particular treatment.

[0096] The compositions of the invention include bulk drug compositions useful in the manufacture of pharmaceutical compositions (e.g., impure or non-sterile compositions) and pharmaceutical compositions (i.e., compositions that are suitable for administration to a subject or patient) which can be used in the preparation of unit dosage forms. Such compositions comprise a prophylactically or therapeutically effective amount of a prophylactic and/or therapeutic agent disclosed herein or a combination of those agents and a pharmaceutically acceptable carrier. Preferably, compositions of the invention comprise a prophylactically or therapeutically effective amount of one or more molecules of the invention and a pharmaceutically acceptable carrier.

[0097] In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant (e.g., Freund's adjuvant (complete and incomplete), excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical

excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like.

[0098] Generally, the ingredients of compositions of the invention are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachet indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

[0099] The compositions of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include, but are not limited to those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

[00100] The invention provides a pharmaceutical pack or kit comprising one or more containers filled with the molecules of the invention. Additionally, one or more other prophylactic or therapeutic agents useful for the treatment of a disease can also be included in the pharmaceutical pack or kit. The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

[00101] The present invention provides kits that can be used in the above methods. In one embodiment, a kit comprises one or more molecules of the invention. In another embodiment, a kit further comprises one or more other prophylactic or therapeutic agents useful for the treatment of cancer, in one or more containers. In certain embodiments, the other prophylactic or therapeutic agent is a chemotherapeutic. In other embodiments, the prophylactic or therapeutic agent is a biological or hormonal therapeutic.

Characterization and Demonstration of Therapeutic Utility

[00102] Several aspects of the pharmaceutical compositions, prophylactic, or therapeutic agents of the invention are preferably tested in vitro, in a cell culture system, and in an animal model

organism, such as a rodent animal model system, for the desired therapeutic activity prior to use in humans. For example, assays which can be used to determine whether administration of a specific pharmaceutical composition is desired, include cell culture assays in which a patient tissue sample is grown in culture, and exposed to or otherwise contacted with a pharmaceutical composition of the invention, and the effect of such composition upon the tissue sample is observed. The tissue sample can be obtained by biopsy from the patient. This test allows the identification of the therapeutically most effective prophylactic or therapeutic molecule(s) for each individual patient. Toxicity and efficacy of the prophylactic and/or therapeutic protocols of the instant invention can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Prophylactic and/or therapeutic agents that exhibit large therapeutic indices are preferred. While prophylactic and/or therapeutic agents that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such agents to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[00103] The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage of the prophylactic and/or therapeutic agents for use in humans. The dosage of such agents lies preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any agent used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC₅₀ (i.e., the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

[00104] The anti-cancer activity of the therapies used in accordance with the present invention can be determined by using various experimental animal models for the study of cancer such as the SCID mouse model or transgenic mice or nude mice with human xenografts, animal models, such as hamsters, rabbits, etc. known in the art and described in *Relevance of Tumor Models for Anticancer Drug Development* (1999, eds. Fiebig and Burger); *Contributions to Oncology* (1999, Karger); *The Nude Mouse in Oncology Research* (1991, eds. Boven and Winograd); and *Anticancer Drug Development Guide* (1997 ed. Teicher), herein incorporated by reference in their entireties.

[00105] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

[00106] All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

[00107] The present invention has been described in terms of particular embodiments found or proposed by the present inventor to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. For example, due to codon redundancy, changes can be made in the underlying DNA sequence without affecting the protein sequence. Moreover, due to biological functional equivalency considerations, changes can be made in protein structure without affecting the biological action in kind or amount. All such modifications are intended to be included within the scope of the appended claims.

EXPERIMENTAL

EXAMPLE 1

Tuning cytokine receptor signaling by remodeling dimer architecture with surrogate ligands

[00108] Receptor dimerization is a universal mechanism to initiate signal transduction, and is utilized by many growth factors such as cytokines, and ligands for tyrosine kinase receptors (RTK), among others. Cytokines are a large class of secreted glycoproteins that contribute to regulating the fate and function of most cell types. Cytokines bind to the extracellular domains (ECD) of their cell surface receptors, forming signaling complexes with receptor homo- or hetero-dimers. In some cases these receptors are pre-associated on the cell surface in an inactive state, and the role of cytokines is to re-orient the receptor dimers into an active state. Cytokines such as Erythropoietin (EPO) and Growth Hormone (GH) homodimerize their receptors, while other cytokines, such as Interleukin-2, heterodimerize a shared receptor (common gamma chain) with a cytokine-specific subunit to initiate signaling. Cytokine receptor dimerization principally results in activation of intracellular, non-covalently associated Janus

Kinases (JAKs) which then activate the STAT pathway to modulate gene expression and ultimately determine cell fate.

[00109] Structures of cytokine-receptor extracellular domain (ECD) complexes from different systems have revealed a diverse range of molecular architectures and receptor dimer topologies that are compatible with signaling. This topological diversity is also apparent for dimeric RTK ECD complexes with their agonist ligands. Furthermore, monoclonal antibodies, as well as other engineered agents that dimerize receptor extracellular domains, including those of dimeric RTKs, can have disparate impacts on signaling, but the topological relationships of these non-native dimers to those induced by the endogenous ligands are unknown. Prior studies have shown that cytokine receptor signaling efficiency can be influenced by extracellular domain mutations or structural perturbations. However, the apparent permissiveness in dimer architecture compatible with signaling raises the question: to what degree can modulation of receptor-ligand geometries fine-tune receptor activation?. A direct structural correlation of a single receptor-ligand complex in different dimerization topologies to differential signaling output would be highly informative in addressing this question.

[00110] On one hand, prior studies showing that cytokine-induced intracellular signaling could be activated through chimeric receptors containing alternative ECD's demonstrated that dimerization geometries compatible with signaling were permissive to some degree. On the other hand, a series of studies comparing activation of EpoR by its natural ligand EPO versus synthetic peptides concluded that very small changes in dimer orientation could modulate signal strength. However these studies left open the question of whether the observed signaling efficiency differences were due to alternative dimer topologies or simply reflected ligand affinity. In one example, it was reported that an EPO agonist peptide (EMP-1) could be converted into a non-activating, or 'antagonist' peptide (EMP-33) simply through a chemical modification (Bromination) of the EMP-1 peptide. Crystal structures of both peptide ligands bound to the extracellular domains of EpoR revealed dimeric complexes, however it was noted that the non-signaling EMP-33/EpoR ectodomain dimer angle differed by an approximate 15° rotation versus the agonist EMP-1/EpoR dimeric complex (Fig. 7A). The lack of signal initiation by the EMP-33 peptide was attributed to this small change in the EpoR ECD dimer angle.

[00111] Given the diverse range of dimer topologies evident in agonistic cytokine-receptor complexes, that in many cases exceed 15° angular differences, we revisited the striking observation seen with the EPO peptide ligands. We explored the biological activity of these peptides using EpoR reporter cells we developed that gave us the ability to test EpoR signaling by receptor phosphorylation, but importantly, also using a beta-galactosidase complementation system that is a sensitive reporter of EPO-induced EpoR oligomerization in physiologic conditions at 37 °C, which directly informs on early signaling and internalization. First, we synthesized the EMP-1 and EMP-33 peptides and found that EMP-1 binds EpoR with a K_D of

1 μ M, while EMP-33 binds EpoR with a K_D of more than 50 μ M (Fig. 7B and 7C). The low affinity of EMP-33 prompted us to ask whether its lack of receptor activation is due to low occupancy of the receptor on the cell. We measured the actions of both peptides at inducing signaling and receptor dimerization on cells at a wide range of concentrations. At 10 μ M of peptide, only EMP-1 induced dimerization and phosphorylation of EpoR at levels comparable to those achieved by EPO stimulation (Fig. 1A and 1B). At higher concentrations of peptide (100 μ M), approaching that used for co-crystallization of both the agonistic and non-signaling dimeric EpoR/peptide complexes, EMP-33 induced a similar degree of receptor dimerization and phosphorylation of EpoR as EMP-1 and EPO itself (Fig. 1A and 1B). Thus, when EMP-33 is applied at concentrations that dimerize EpoR on cells, the dimer geometry of the EMP-33/EpoR complex is competent to initiate signaling. The different signaling potencies exhibited by the EPO mimetic peptides appears to be primarily due to their relative EpoR binding affinities.

[00112] We turned our attention to developing surrogate cytokine ligands that could induce much larger topological differences in the EpoR dimer and enable a systematic study relating dimer architecture to signaling and function. We reasoned that diabodies, which are essentially 'back-to-back' antibody V_H/V_L Fv's could dimerize and possibly induce signaling of the EpoR, albeit at significantly larger inter-dimer distances than induced by EPO. Additionally, diabodies might be rigid enough to allow crystallization of their complexes with EpoR so that we can directly visualize the dimeric topologies. By comparison, whole antibodies have been shown to activate cytokine receptor signaling in many systems, presumably by dimerization. However, the flexibility of intact antibodies has precluded a structural analysis of intact dimeric agonist complexes that can be related to the biological activities.

[00113] We synthesized genes of four previously reported anti-EpoR antibodies, and reformatted their V_H and V_L domains into diabodies (Fig. 1C). The four diabodies bound EpoR with approximately similar affinities (Fig. 8) and multimerized EpoR with similar efficiency (as measured by EC_{50}), albeit less efficiently than EPO (Fig. 1D). However, they induced EpoR phosphorylation with very different relative efficacies, ranging from full agonism (DA5) to very weak partial agonism (DA10) (Fig. 1E). The four diabodies also exhibited different extents of STAT5 phosphorylation (Fig. 1F), STAT5 transcriptional activity (Fig 9A), Ba/F3 cell proliferation (Fig. 1G) and CFU (Colony forming unit)-E colony formation (Fig. 1H). These dramatic differences in diabody-induced signaling and functional activities persist at saturating ligand concentrations, so are not attributable to significantly different relative affinities for EpoR, or to a stronger EpoR internalization induced by the weak agonist diabodies (DA10, DA307 and DA330), since the internalization closely correlated with their signaling efficacies (Fig. 9B).

[00114] STAT5 is the most prominent STAT protein activated by EPO. However additional signaling pathways, including other STATs (STAT1 and STAT3), the MAPK pathway and the PI3K pathway, are also activated by this cytokine and fine-tune its responses. "Biased" signal

activation is a phenomenon that has been described for G-protein coupled receptor ligands, where one GPCR can differentially activate signaling pathways (e.g. beta-arrestin versus G-protein) depending on the ligand. Thus, we asked whether similar differential signal activation could be observed in a dimeric single-pass transmembrane receptor such as the EPO-EpoR system. We studied the activation of 78 different signaling molecules by phospho-Flow cytometry in the EPO-responsive cell line UT7-EpoR. EPO and the diabodies induced the activation of 33 signaling proteins, including members of the STAT family (STAT1, STAT3 and STAT5), MAP kinase family (MEK, p38) and PI3K family (Akt, RSK1, RPS6) (Fig. 2A). We also observed the upregulation of known EPO-induced transcription factors such as Myc, cFos, IRF1 and Elk (Fig. 2A). In agreement with our previous results, the signaling potencies exhibited by the three diabodies ranged from full agonism for DA5, to partial agonism for DA330 and non-agonism for DA10 (Fig. 2A). Interestingly, the diabodies did not activate all 33 signaling molecules to the same extent (Fig. 2B). When the signal activation levels induced by the three diabodies after 15 min stimulation were normalized to those induced by EPO we observed that, while EPO and DA5 induced similar levels of activation in the majority of the signaling pathways analyzed, DA5 activated some of them to a lower extent than EPO (Fig. 2B). Among those, STAT1 and STAT3 activation were the most affected, with DA5 inducing 30 % of the STAT1 and 40 % of the STAT3 activation levels induced by EPO (Fig. 2B). Interestingly, STAT3 S727 phosphorylation, which requires MAPK activation, was equally induced by EPO and DA5, consistent with the two ligands activating the MAPK pathway to the same extent (Fig. 2A and 2B). Dose/response studies in UT7-EpoR cells confirmed these observations and showed that DA5 activates STAT1 to a much lower extent than EPO, while still promoting comparable levels of STAT5 activation (Fig. 2C), confirming that biased signaling can be induced through the dimeric EpoR with surrogate ligands.

[00115] Next we studied how different signal activation amplitudes exhibited by the diabodies at the membrane-proximal level would impact their membrane-distal gene expression programs. We carried out RNA-seq studies of EPO-responsive genes in purified human primary megakaryocyte-erythroid progenitor (MEP) cells derived from bone marrow of a normal subject (Fig. 2D). Temporally, MEPs are the first progenitors to robustly express EpoR during hematopoiesis in humans. In agreement with the signaling data, the relative gene-induction potencies exhibited by the diabodies matched their signaling efficacies (i.e. DA5>DA330>DA10) (Fig. 2D). DA5 induced a very similar gene induction profile to EPO, but with some differences, with a small subset of genes (e.g. *Pim2* and *RN7SK*) being differentially regulated by DA5 when compared to EPO.

[00116] The different EPO-responsive gene induction potencies induced by the diabodies were further confirmed by qPCR experiments in the EPO-responsive cell line UT7-EpoR. UT-7-EpoR cells were stimulated with saturating doses of EPO or the three diabodies for 2, 4 and 8 hr and

the levels of *CISH* and *Pim1* gene expression were studied (Fig. 9C). Here again DA5 stimulation led to similar levels of *CISH* and *Pim1* induction as EPO; DA330 resulted in only 30-40% induction of these genes and DA10 only marginally induced *CISH* and *Pim1* in these cells (Fig. 9C). When compared to the RNA-seq experiment performed in MEP cells, DA10 induced lower level of *CISH* and *Pim1* expression in UT7-EpoR cells. These differences likely result from the use of different cell types in the two assays. Overall our signaling and gene expression data show that the diabodies exhibit various degrees of differential signaling properties from EPO and from one another.

[00117] To explore the structural basis for the differential signaling activation exhibited by the diabodies, we expressed and purified three diabody/EpoR complexes (DA5, DA10 and DA330) from baculovirus-infected insect cells. All exhibited molecular weights of 97-98 kDa as measured by Multi Angle Light Scattering (MALS) chromatography, in agreement with a 2:1 complex stoichiometry (two EpoR bound to one diabody (Fig. 10A)). We crystallized the diabody/EpoR complexes (DA5 (2.6 Å), DA10 (3.15 Å) and DA330 (2.85 Å)) and determined their structures by molecular replacement (Fig. 3 and Table S2). The diabody subunit relationships are clear for the most and least potent diabody complexes (DA5 and DA10, respectively). For the DA330/EpoR complex, the crystal appears to contain domain-swapped diabodies as “back-to-back,” single-chain Fv’s that pack in similar, but not identical subunit relationships as diabodies. The MALS data show that all of the diabodies are the expected 2:1 complexes in solution.

[00118] All three diabodies converge on the protruding “elbow” of EpoR that also serves as the EPO binding site (Fig. 3 and Fig. 10 and 11). When the diabody V_H/V_L modules are aligned, the EpoR’s ‘rotational’ binding topology is most similar between DA5 and DA330, with DA10 being markedly different (Fig. 3A). While DA5 and DA330 both bind horizontally and differ primarily in their vertical ‘tilt’ (~14°), DA10 is orthogonally disposed relative to the other two (Fig. 3B). In a striking example of chemical mimicry of EPO binding, the diabody CDR loops use two patches of basic (Arg98/Arg101 of DA5 and DA330, respectively) and hydrophobic (Tyr34/Tyr105/Tyr32 of DA5/DA10/DA330, respectively) residues in a nearly identical manner as residues presented on the EPO helices (Lys45, Phe48) in the EPO site I binding interface to engage the same regions of the EpoR binding site (Fig. 3C and 3D).

[00119] The overall architectures of the three diabody/EpoR complexes (Fig. 3E, 3F and 3G) are quite distinct from that of the EPO/EpoR complex, which dimerizes two molecules of EpoR in a classical Y-fork cytokine-receptor architecture, resulting in close proximity between the C-termini of the membrane-proximal EpoR ECD’s (Fig. 3H). In contrast, the diabodies impose much larger separation between the two EpoR molecules with distances ranging from approximately 127 Å in the case of the DA5/EpoR (full agonist) complex, to approximately 148 Å as in the case of the DA10/EpoR complex (non-agonist) (Fig. 3E, 3F and 3G). The exact EpoR

dimer separation is uncertain for the partial agonist DA330. Interestingly, the relative EpoR dimer distances observed in the full and non-agonist diabody/EpoR complexes correlate their signaling potencies in that the full agonist dimer is closer together, while the non-agonist is further. One caveat is that the diabody molecules themselves are not rigid, they exhibit some degree of flexibility in the hinge angle relating the two V_H/V_L modules, raising the question of whether we captured one of a range of dimer angles that could be enforced by crystal lattice contacts. We performed conformational sampling studies exploring the relationship between the EpoR separation distance as a function of the diabody hinge angle on the full agonist DA5 and the non-signaling DA10 (Fig. 12). The results of these studies show that the thermodynamically permitted variation in diabody hinge angles (appear to occupy a few energy minima) lead to only a small range of alternative conformations (i.e. distances) around that seen in the crystal structures (Fig. 12). The sampling of these alternative conformations has only minimal consequences on the inter-EpoR distances.

[00120] Since we observe differences in *both* the EpoR/diabody docking angles (Fig. 3A and 3B), and the distances between EpoR C-termini in the dimeric complexes, we cannot say whether distance or geometry/topology, or a combination of both factors, is responsible for the differences in signaling between the complexes. However, that the differences in signaling amplitude are due to alternative overall extracellular dimer topologies appears quite clear. Such large differences in extracellular architecture would likely influence the relative orientation and proximity of the two JAKs associated with the membrane proximal intracellular domains of the receptors, and impact their subsequent phosphorylation profiles (Fig. 5A).

[00121] An important mechanistic question is whether the diabody/EpoR complexes on the cell surface are indeed homodimers or higher order species due to clustering of preformed EpoR dimers, which has been reported previously. To explore the ability of the diabodies to dimerize EpoR in the plasma membrane we probed the assembly and dynamics of signaling complexes by dual color single molecule imaging. For this purpose, EpoR fused to an N-terminal monomeric EGFP (mEGFP) was expressed in HeLa cells and labeled by addition of anti-GFP nanobodies, which were site-specifically conjugated with DY647 and ATTO Rho11, respectively (Fig. 4A). We labeled the receptors extracellularly so as not to introduce fusion proteins to the intracellular regions that may result in artefactual dimerization behavior. Efficient dual color labeling suitable for long-term observation of individual EpoR was achieved with typical densities of ~ 0.3 molecules/ μm^2 in both channels, which was exploited for co-localization and co-tracking analysis. In the absence of an agonist, independent diffusion of EpoR molecules could be observed with no significant single molecule co-localization beyond the statistical background (Fig. 4B). Single molecule co-tracking analysis corroborated the absence of pre-dimerized EpoR at the plasma membrane (Fig. 4C,D). Upon addition of EPO, dimerization of EpoR was detectable by both, co-localization and co-tracking analysis (Fig. 4B-D). Individual

receptor dimers could be tracked (Video S3) and a clear decrease in their mobility compared to EpoR in absence of ligand was identified (Fig. 4E). Stimulation of EpoR endocytosis in presence of EPO was observed, which was accompanied by an increased fraction of immobile EpoR molecules in presence of EPO. The stoichiometry within individual complexes was analyzed by photobleaching at elevated laser power. Single step photobleaching confirmed the formation of EpoR dimers in the plasma membrane (Fig. 4F). Upon labeling the mEGFP-EpoR only with ATTO-Rho11, two-step bleaching could be observed only in presence of EPO. For all diabodies, very similar levels of receptor dimerization were obtained (Fig. 4D). A slightly increased dimerization level compared to EPO was observed, which may be due to the symmetric binding affinities of diabodies to both EpoR subunits compared to the asymmetric receptor dimer assembly observed for EPO. Importantly, the diffusion properties of receptor dimers assembled by the diabodies were comparable as shown for DA5 in Fig. 4E, confirming a comparable mode of receptor dimerization by diabodies compared to EPO. Moreover, 1:1 receptor dimers recruited by the diabodies is observed by single step photobleaching (Fig. 4F). Thus, although we do not rule out any role of EpoR pre-association in the observed signaling effects, our microscopy data indicates that the diabodies are not simply clustering quiescent EpoR dimers into higher order assemblies.

[00122] Several mutations in JAKs are known to cause immune disorders and cancer by rendering activation ligand-independent. We asked whether the large EpoR distances and different binding geometries induced by the diabodies could modulate the activity of these kinase mutants in an extracellular ligand-dependent manner by separating the two JAKs at distances where they could not undergo transactivation. The JAK2V617F mutant is the best-described example of an oncogenic JAK mutation, causing the development of hematological disorders such as Polycythemia Vera (PV) and other myeloproliferative (MPN) neoplasms. At physiologic expression levels, JAK2V617F-positive cells require EpoR to proliferate in a ligand-independent manner. Stimulation of Ba/F3 cells expressing the murine EpoR and the JAK2V617F mutant with EPO or DA5 did not significantly affect the basal phosphorylation of STAT5, Akt and Erk in these cells (Fig. 5B and 5C). However, stimulation of these cells with DA10, DA307 and DA330 decreased the STAT5, Akt and Erk phosphorylation in a time dependent manner (Fig. 5B and 5C). This decrease in signal activation induced by DA10, DA307 and DA330 was not the result of EpoR surface depletion. Only the full agonists, EPO and DA5, led to a significant decrease in the levels of EpoR on the surface (Fig. 5D). The decrease in the JAK2V617F-induced basal signaling activation promoted by the diabodies was followed by a reduction in the proliferation rate of Ba/F3 cells expressing the mutated JAK2 (Fig. 5E), suggesting that oncogenic JAK mutant activities can be modulated in an extracellular ligand-dependent manner.

[00123] The Ba/F3 cells used here are a transformed cell line engineered to overexpress EpoR and JAK2V617F, which led to transformation and to autonomous growth, so we also performed erythroid colony formation assays in primary cells from human JAK2V617F-positive patients. CD34⁺ hematopoietic stem cells and progenitors (HSPC) from heterozygous JAK2V617F-positive patients were isolated and stimulated with the indicated diabodies +/- EPO, and their ability to form erythroid colonies was assayed. In the absence of diabodies, JAK2V617F-positive CD34⁺ cells gave rise to erythroid colonies, which were further increased in numbers in the presence of EPO in the media (Fig. 6A). Stimulation with a non-specific negative control diabody did not significantly alter the number of erythroid (EpoR dependent) or myeloid colonies (EpoR independent) (Fig. 6A and 6B), ruling out possible toxic side effects induced by diabodies. Stimulation of JAK2V617F-positive CD34⁺ cells with the agonistic diabody, DA5, led to a specific increase in the number of erythroid colonies (Figs. 6A and 6C) without significantly altering the number of myeloid colonies (Fig. 6B). On the other hand, stimulation with DA330 and DA10 led to a potent and specific decrease in the number of erythroid colonies (Fig. 6A, 6B and 6C). We note that DA330, which is a partial agonist of normal JAK2 signaling, limits, but does not prevent signaling in JAK2V617F cells, giving the appearance of a structural 'governor' controlling signaling output. All the colonies analyzed in the study harbored the JAK2V617F mutation as determined by single colony genotyping (Fig. 6D). The diabody with the largest intersubunit distance, DA10, inhibited colony formation the strongest, comparably to the JAK1/2 inhibitor Ruxolitinib, which is approved and standard of care for JAK2V617F-positive MPN (Verstovsek et al., 2010) (Fig. 6A). DA10 also decreased the number of erythroid colonies from homozygous JAK2V617F-positive patients (Fig. 6E and 6F), suggesting that the binding topology imposed by this diabody dominates over the influence of the mutated JAK2 expressed in the cell. Overall these results show that extracellular ligands that enforce large receptor dimer separation and different binding geometries can counteract intracellular oncogenic ligand-independent receptor activation, presumably by exceeding the accessible distance that the JAK2 kinase domain can extend to transphosphorylate the opposing JAK2 and receptor (Fig. 5A).

[00124] Single-pass Type-I and Type-II transmembrane receptors that contain ligand-binding ECDs constitute a major percentage of all signaling receptors in the mammalian genome, and include cytokine (JAK/STAT) receptors, Tyrosine Kinase (RTK) receptors (e.g. EGF-R, Insulin-R, etc), and many others. In most cases, these receptors signal in response to ligand engagement as homo- or heterodimers). For G-protein coupled receptors, ligand binding within the TM helices induces conformational changes within the plane of the membrane, and this can be read out as differential signaling (e.g. biased signaling, inverse and partial agonism). However, it has been unclear to what extent extracellular ligands can influence signaling through dimeric Type-I and Type-II receptors by enforcing ECD orientational differences. For

this class of receptors, ligand binding ECDs are structurally autonomous, and separated from the intracellular signaling modules (e.g. Kinase domains) through juxtamembrane linkers and a TM helix. Thus, the intracellular domains presumably sense ligand mainly through ligand-induced spatial perturbations of receptor orientation and proximity that are relayed as conformational changes relayed through the membrane. We asked whether ligand-induced orientational changes of receptor dimer geometry could serve a functionally analogous role to the diverse types of conformational changes induced by GPCR ligands that result in differential signaling.

[00125] While there exists a vast literature showing that dimeric receptor signaling strength is determined by extracellular parameters such as ligand affinity and complex half-life on the cell surface, the role of orientation-specific effects has remained speculative. Studies using mutated, chimeric or genetically modified receptors have pointed to the importance of the extracellular domain structure in mediating signaling output. Nevertheless, for this parameter to be exploited in a manner that could be useful therapeutically, surrogate ligands with the capacity to induce alternative signaling outputs through naturally, non-mutated receptors on human cells are required. We used diabodies because they would presumably induce large-scale alterations in dimer geometry, and have been previously shown to have the capacity to act as agonists of c-MPL. Additionally, diabodies have more constrained structures than antibodies, which can also act as agonists, but remain elusive structural targets due to their segmental flexibility.

[00126] Our results indicate that cytokine receptor dimer architectural and spacing constraints compatible with signaling appear liberal, but with limits. This is consistent with the diverse range of dimeric ligand-receptor geometries seen in agonistic cytokine-receptor complex structures. Consequently, we find that large-scale re-orientations of receptor dimer topology with alternative ligands are required to qualitatively and quantitatively modulate signaling output. We propose that this strategy is potentially applicable to other dimeric receptor systems, such as RTK's, where the role of ligand is to bind to the ECDs, dimerize and/or re-orient receptors.

[00127] The broader implications of our results are that signaling patterns delivered by endogenous ligands only constitute one, of many possible signaling patterns that can be elicited through a dimeric receptor system using ligands that re-orient dimer topology. By using surrogate or engineered ligands to re-orient receptor dimer topology, a given dimeric receptor can be induced to deliver a wide range of signals of different amplitudes and pathway specificities. Cytokine receptor dimers have the potential to be modulated as rheostats to control signaling output, similar to partial and biased GPCR agonists. Given that many endogenous cytokines and growth factors have adverse effects as therapeutic agonists, our results portend the possibility of dimer re-orientation as a strategy to 'tune' signaling output to minimize toxicity, maximize efficacy, or elicit specific functional outcomes.

[00128] The molecular mechanisms through which the diabodies described here alter intracellular signaling by remodeling dimer geometry remain unclear, but the signal tuning effects are clearly the result of extracellular receptor dimer proximity (distance) and geometry (orientation) effects. Our single molecule fluorescence tracking shows that the assembled signaling complexes are not due to higher order assemblies that could have resulted from diabody-induced clustering of preformed EpoR receptor dimers. Even if receptor clustering were occurring to some degree, which we do not rule out, the diabodies still exert a powerful modulatory effect on signaling through repositioning receptor topology whether or not these are monovalent or polyvalent cell surface complexes. Indeed, our results can be reconciled with a recent mechanistic study of cytokine receptor activation. Growth Hormone was shown to activate its receptor (GH-R) by rotating the ECD subunits of a pre-associated, but inactive GH-R dimer, resulting in separation of the Box 1 receptor ICD motifs and removal of the JAK2 pseudokinase inhibitory domain, which collectively result in productive JAK2 kinase domain positioning for receptor activation. Diabodies could disrupt a quiescent cytokine receptor dimer to form an activated dimer topology through a related 'separation' mechanism that relieves JAK2 inhibition. For the agonist DA5, the outcome of this separation would be placement of the JAK kinase domains into productive apposition, but one that is topologically distinct from that induced by the natural cytokine. In the case of DA10, the kinase domains of JAK2 are separated such that they are not in proper position to trans-phosphorylate.

[00129] The surprisingly large EpoR dimer separation distances imposed by the agonistic diabodies may be rationalized by the fact that the intracellular, receptor-associated JAKs are long molecules that exist as a dynamic ensemble of open and closed conformations which could span >100Å distances between receptors in a dimer. Given that the kinase domain of JAK resides at its C-terminus, which is most distal to the receptor bound by the JAK FERM domain, it is likely sensitive to positioning relative to its substrates that it trans-phosphorylates. Changes in the relative positioning of the kinase domain to its substrates could influence the efficiency and patterns of phosphorylation through steric effects imposed by extracellular dimer geometry. By manipulating the dimer geometry, as seen with the non-signaling diabody DA10, such an approach can achieve complete shutoff of constitutively active signaling pathways (JAK2V617F) from the outside of the cell. This is conceptually distinct from Ankyrin repeat antagonists to ErbB2 that were shown to prevent activation of wild-type ErbB2 by distorting the receptors such that they cannot form signaling-competent dimers. Here, the role of DA10 is to dimerize EpoR, yet terminate ligand-independent signaling, possibly through enforcing a large dimer separation distance. This strategy is applicable to diseases mediated by mutated, constitutively active receptors and could offer the advantage of specificity and reduced toxicity versus broadly neutralizing kinase inhibitors.

[00130] Diabodies are a convenient surrogate ligand because they can be created from existing monoclonal antibody sequences, which exist to most human cell surface receptors. However, dimer re-orientation could be achieved by many different types of engineered scaffolds. A range of altered dimerization geometries could be screened with dimerizing scaffolds for those that induced a particular signaling profile. Using the receptor ECD's as accessible cell surface targets to induce alternative orientations of intracellular signaling motifs for many Type-I or Type-II cell surface receptors should be feasible.

Table 1

Data collection and refinement statistics	DA5_EpoR complex	DA10_EpoR complex	DA330_EpoR complex
Resolution	2.6 Å	3.1 Å	2.6 Å
Space group	C2221	P41212	P22121
Unit cell (Å)	125.73, 215.021, 171.9, 90, 90, 90	239.5, 239.5, 132.6, 90, 90, 90	78.14, 95.8, 145, 90, 90, 90
Rmerge (%)	13.7	9.2	9.2
I/sigma	13.6(1.7)	10.1(1.6)	11.1(1.1)
Completeness (%)	95.8	95.2	99.4
Redundancy	4.8	3.4	4.4
Rfree/Rwork (%)	23.33/19.08	24.2/21.11	24.2/22.4
RSMD (Bond)	0.002	0.002	0.002
RSMD (Angle)	0.56	0.464	0.493
Mean B Value (Å ²)	60.4	73.4	64.8
Ramachandran plot (%)	96.78/0	93.6/0.35	95.06/0

Materials and Methods

[00131] *Cell lines and media.* The Ba/F3 cells expressing mouse or human receptors and mouse or human JAK2, U2O2 and γ 2A cells were grown in RPMI containing 10% v/v FBS, penicillin-streptomycin, and L-glutamine (2 mM) and supplemented with IL-3. The human UT-7 cells were grown in DMEM containing 10% v/v FBS, penicillin-streptomycin and L-glutamine (2mM) and supplemented with EPO. All cell lines were maintained at 37 °C with 5% CO₂.

[00132] *Protein expression and purification.* EpoR diabodies and an EpoR glycomutant (N52Q and N164Q) were cloned into the pAcGP67-A vector (BD Biosciences) in frame with an N-terminal gp67 signal sequence and a C-terminal hexahistidine tag and produced using the baculovirus expression system, as described in. Baculovirus stocks were prepared by transfection and amplification in *Spodoptera frugiperda* (Sf9) cells grown in SF900II media (Invitrogen) and protein expression was carried out in suspension Trichoplusiani (High Five) cells grown in InsectXpress media (Lonza). Following expression, proteins were captured from High Five supernatants after 60 h by nickel-NTA agarose (Qiagen) affinity chromatography,

concentrated, and purified by size exclusion chromatography on a Superdex 200 column (GE Healthcare), equilibrated in 10 mM HEPES (pH 7.2) containing 150 mM NaCl and 15% glycerol. Recombinant diabodies and EpoR were purified to greater than 98% homogeneity. For crystallization studies, the diabodies and EpoR were nickel purified and carboxypeptidase treated overnight at a ratio 1/100 and then purified by size exclusion chromatography. 98% pure diabodies and EpoR were mixed together in a ratio 1:1 and co-purified by size exclusion chromatography to obtain the ternary complex (1 diabodies/2 EpoR). The complex was concentrated to 8-20 mg/ml for crystallization. For the DA330_EpoR complex, the complex was methylated and purified by size exclusion chromatography. Protein concentrations were quantified by UV spectroscopy at 280 nm using a Nanodrop2000 spectrometer (Thermo Scientific).

[00133] *Crystallization and data collection.* The three diabodies_EpoR complexes crystals were grown in sitting drops at 25°C by mixing 0.1 μ l protein (12 mg/ml in 10 mM HEPES (pH 7.2) and 150 mM NaCl) with an equal volume of different crystallization solutions. For the DA5_EpoR complex the buffer used was composed of 8% PEG6K, 0.1M magnesium chloride and 0.1M MES pH 6. For DA10_EpoR complex the buffer used contained 10% PEG8K, 1M sodium citrate pH 7. For DA330_EpoR complex the buffer used had 12 % PEG6K and 0.1M bicine pH 9.5. Crystals grew in 2-5 days. They were subsequently flash frozen in liquid nitrogen using mother liquor containing 25% glycerol (DA5 and DA330) and 30% glucose (DA10) as a cryoprotectant. A 2.6 Å data set for DA5_EpoR complex, 2.85 Å data set for DA10_EpoR complex and 2.6 Å data set for DA330_EpoR complex were collected at beamline 12.2, Stanford Synchrotron Radiation Lightsource (SLAC). The data were indexed, integrated with the XDS package, and scaled by Aimless in CCP4 suite. Data processing statistics are presented in Table 1.

[00134] *Structure determination and refinement.* The three DA-EpoR crystal structures were solved by molecular replacement with the program PHASER (5) using the coordinates of the 1121B Fab heavy chain (PDB ID 3S34), Fab light chain of human anti-HIV-1 Env antibody A32 (PDB ID 3TNM), and SD1 (8-119aa) and SD2 (120-220aa) domains of EpoR (PDB ID 1EER) as searching models respectively, and refined with PHENIX and COOT. For the DA330-EpoR complex, only a partial solution was obtained, which did not include the SD2 domain of EpoR. To complete the structure coordinates of the SD2 domain from PDB 1EER were directly merged to the partial model and performed one cycle of rigid body refinement. The loop and side chains were then fixed manually. Ramachandran analysis was performed with MolProbity.

[00135] *Surface plasmon resonance.* SPR experiments were conducted on a Biacore T100 instrument using a Biacore SA sensor chip (GE Healthcare). Biotinylated EpoR was captured at a low density (200-300) response units (RU)) and kinetics measurements were conducted

at 30 μ L/min. An unrelated biotinylated protein was immobilized as a reference surface for the SA sensor chip with matching RU to the experimental surface. All measurements were made using 3-fold serial dilutions of the stimulatory and non- stimulatory EPO mimetic peptides (EMP) in the running buffer (1xHBS-P (GE Healthcare), 0.1 % BSA). The EpoR bound to the chip surface did not required regeneration due to the fast off-rate kinetics exhibited by the two peptides. Kinetic parameters were determined using 120s of EMP association time and 300 s dissociation time. All data fitting was performed using the Biacore T100 evaluation software version 2.0 with a 1:1 Langmuir binding model.

[00136] *Molecular dynamic simulation.* Computational simulations were initiated from the initial structures (Figure 2E,F) of the DA5/EpoR and DA10/EpoR complexes. All missing linkers have been built using MODELLER 9.12 and the MMTSB toolset. The original structures were left unchanged and the loops between the V_H C-termini and V_L N-termini were modelled. Two hundred loops for each of the five residue stretches (GGGGS) were generated and the models with the lowest modeller scores were selected. These completed structures were coarse-grained using a simplified protein model that has been shown to predict all known fold topologies and was successfully applied to probe functionally relevant conformational changes in large molecular machines such as chaperonines.

[00137] The structures were then fed into our Natural Move Monte Carlo (NMMC) protocol. In NMMC the system is partitioned into independently moving segments (that can be part of the same chain) and melting regions. The independent (translational and rotational) motion of segments may break the molecular chain that is restored by a chain closure algorithm applied on residues in the melting region. This technique has been successfully used to study conformational changes on protein and RN assemblies and recently for the prediction of primary chromatin structure. To evaluate how diabody flexibility in the linker region will affect the distance distribution of the EpoR ligands, the melting region comprised the three center residues along the linker regions of the two diabodies. The choice of using three residues was made as a compromise between maximizing the conservation of the initial structures (e.g. experimental information) and providing sufficient flexibility in the linker regions.

[00138] To further accelerate conformational sampling efficiency, we used parallel tempering, in which NMMC simulations were performed parallel using six replicas that span a temperature range from 300K to 529K (300, 336, 376, 421, 472, 529). The simultaneous propagation of the six systems has been performed for 2,000,000 Monte Carlo iterations and the inter replica exchange probability was set to 0.1. The acceptance rate of propagating conformations within individual replicas and the inter replica exchange ratio were 0.3 and 0.1, respectively. All conformational statistics were collected from the system at 300K. The simulation can be reproduced by following the available tutorial.

[00139] *Molecular dynamic simulation videos.* A sequence of conformations that morphs between the predicted major conformational states (see Fig. 12) of DA5 and DA10 were generated using a linear morphing algorithm available in the UCSF Chimera package. A total of 39 intermediate conformational states connect the three representative structures for each system and have increasing EpoR distances. For representation purposes the experimentally measured X-ray structure domains were superimposed on each frame with exception of the linkers, which were taken from the coarse-grained trajectory and thus are depicted in stick representation. The frames were edited in Pymol and the movie was mpeg4 encoded with MEncoder and converted to the mov format with ffmpeg.

[00140] *EPO Receptor dimerization/oligomerization detected by β -galactosidase protein fragment complementation.* The full length EPO receptor (NM_000121.3) was cloned as a fusion to the EA and ProLink β -galactosidase enzyme fragments in an MMLV retroviral vector. The ProLink is a modified alpha donor peptide that exhibits a low affinity for the EA deletion mutant of β -galactosidase resulting in low levels of spontaneous complementation when the two fragments are co-expressed in mammalian cells. However when these enzyme fragments are fused to two target proteins of interest, the interaction of the two target proteins forces the complementation of the fragments resulting in a measurable increase in β -galactosidase activity. Between the receptor and the enzyme fragment, a 9 amino acid G4SG4 linker was included to ensure flexibility. The EpoR-EA construct was transduced into U2OS cells followed by the EpoR-ProLink vector. From this cell pool a clone was selected that showed a reproducible response to EPO. The functional assay was run according to manufacturer's suggested protocol (DiscoverX). Briefly, cells were plated in growth medium containing 1%FBS at 5,000 cells per well in 384-well plates and incubated overnight at 37 °C 5%CO₂. The next day the cells were treated with agonist and incubated for 3hr at 37 °C. The chemiluminescent detection reagent was then added in a single addition and the plates incubated for 30 min at room temperature. The luminescent signal was then read on an envision plate reader.

[00141] *EPO Receptor Phosphorylation assays.* The full length EpoR-ProLink plasmid was transduced into U2OS cells expressing the tandem SH2 domain from PLCG2 fused to the complementary enzyme fragment EA. After selection in antibiotic, the cells were cloned by limiting dilution. A clone was selected that exhibited a robust response to EPO and was used for these studies. The assays were run similarly to the dimerization assays according to manufacturer's protocols (DiscoverX). Briefly, the cells were plated in 384-well plates at 10,000 cells per well in growth medium containing 1%FBS. After an overnight incubation at 37°C, the cells were treated with compounds for 3hr at room temperature. The detection reagent was then added in a single addition and the plates incubated for 30min at room temperature. The luminescent signal was then read on an envision plate reader.

- [00142] *Phospho-Flow cytometry staining and antibodies.* Intracellular phospho-STAT5 staining was performed after ice-cold methanol (100% v/v) permeabilization. Antibodies to pSTAT5 Alexa647 were purchased from BD Biosciences and used at a 1:50 dilution. The induction of STAT5 phosphorylation was calculated by subtracting the Mean Fluorescence Intensity (MFI) of the stimulated samples from that of the unstimulated sample. The normalized values were plotted against cytokine concentration to yield dose-response curves that were fitted to a sigmoidal curve.
- [00143] *Dual Luciferase Transcriptional Assays.* Transcriptional activation of STAT5 was analyzed in γ -2A cells co-transfected with EpoR, JAK2wt, STAT5 and pGRR5 luciferase reporter. pGRR5-luc contains five copies of STAT-binding site of the Fc γ RI gene inserted upstream of the luciferase gene under transcriptional control of the thymidine kinase (TK) promoter. The pRL-TK vector (Promega, Madison, WI) containing the renilla luciferase gene under control of the TK promoter was used as an internal control. Luminescence was measured in the cell lysates 24 hours after transfection using dual luciferase reporter kit (Promega) on a Perkin-Elmer Victor X Light analyzer.
- [00144] *Cell Proliferation Assay.* Transduced and sorted Ba/F3 cells expressing mEpoR, mEpoR/JAK2, mEpoR/JAK2V617F were expanded in IL-3, washed 3 times in PBS and 5000 cells/well were placed in 96-well plate in RPMI containing 10% fetal bovine serum with either EPO or diabodies or left untreated. After 72 hours the cell proliferation assay was performed using a CellTiter 96 NonRadioactive Proliferation Assay (Promega). Absorbance was measured at 570nm, which is directly proportional to the number of living cells.
- [00145] *Immunoblots.* Ba/F3 cells expressing EpoR were starved from cytokines and serum for 4 hours in RPMI containing 1mg/ml bovine serum albumin. Cells were stimulated with 5U/ml of EPO, 1 μ M diabodies for 15 min (Ba/F3 EpoR) or 3 hr (Ba/F3 EpoR JAK2V617F) at 37 °C or left untreated. Cells were lysed in 1% NP-40 lysis buffer and protein concentration was determined by Bradford. The samples were separated by 10% SDS-PAGE and blotted onto cellulose membrane. The antibodies used for Western blotting against JAK2 (D1E2), phospho-STAT5 (Tyr694), phospho-Akt (Ser473), phospho-Erk1/2 (Tyr202/204), Erk1/2 were purchased from Cell Signaling Technology.
- [00146] *Colony Assays.* 150000 cells/ml of bone marrow cells from JAK2wt C57B16 mice were plated in MethoCult M3234 (Stem Cell Technologies) according to the manufacture recommendations. 3U/ml EPO (Eprex) and diabodies DA5, DA10, DA307, DA330 at the concentrations 1 μ M were added to assess the colony- forming units CFU-E. CFU-E count was read after 48 hours in culture.
- [00147] *Human samples.* Human myeloproliferative disease (MPN) samples were obtained from patients at the Stanford Medical Center with informed consent, according to Institutional Review Board (IRB)-approved protocols (Stanford IRB 76935 and 6453). Similarly

patient samples were obtained from the Hôtel- Dieu Hospital (Paris, France) with informed consent and the study was approved by the «Local Research Ethics Committee » and by «the Programme Hospitalier de Recherche Clinique (PHRC study). Mononuclear cells or CD34⁺ progenitors from each sample were isolated by Ficoll separation and CD34⁺ progenitors were isolated by a positive selection using an immunomagnetic cell sorting system (AutoMacs; Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were then cryopreserved in liquid nitrogen.

[00148] *HSC and progenitor derived colony genotyping assay.* Lin-CD34⁺ cells were sorted from freshly thawed cryopreserved human JAK2V617F heterozygous myeloproliferative samples. 5×10^3 CD34⁺ cells were plated in methylcellulose with and without erythropoietin (MethoCult H4434 and H4535; STEMCELL Technologies). Colony formation was assessed after 14 days in culture by microscopy and scored on the basis of morphology. Methylcellulose colony types were scored as follows: BFU-e, CFU-GM, CFU-G, CFU-M, CFU- GEMM. Photographs of colonies were obtained on day 14 with a Nikon C-FMC stereo zoom. c-DNA was isolated from each colony with TaqMan Sample-to-SNP kit (Applied Biosystems). JAK2V617F and JAK2 WT TaqMan SNP Genotyping Assay (Applied Biosystems) were designed as published recently (Levine et al., Blood 2006) and details are available upon request. The genotype of each colony was determined by Custom TaqMan SNP Genotyping Assay (Applied Biosystems) according to the manufacturer's specification.

[00149] Homozygous JAK2V617F colony assay was performed on purified CD34 positive progenitors from homozygous for JAK2V617F derived from PV and MF patients. CD34 positive progenitors from a healthy donor were used as a negative control. 500 cells per culture dish in triplicate were seeded in H4100 Methocult media (StemCell) supplemented with 12% BSA, 37% of fetal bovine serum, 2-β- mercaptoethanol (1μM), 1% L-glutamine, in the presence of cytokines: SCF – 25 ng/ml, IL3-100 U/ml, EPO- 3 U/ml and DA10 diabody at two different concentrations - 0.3 μM or 1.0 μM. Burst- forming units-erythroid (BFU-E) or endogenous erythroid colonies (EEC) were counted after 14 days in culture. EPO and SCF were generous gifts from Amgen (Thousand Oaks, CA) and Biovitrum AB (Stockholm, Sweden), respectively. IL-3 was from Miltenyi Biotec (Paris, France).

[00150] *Isolation of primary human MEP cells for RNA sequencing.* For the isolation of normal human bone marrow cells 200 Mio mononuclear ficolled cells (32K) from a single healthy donor (donor ID #: 6024, lot#: BM4871) were purchased from Allcells, Alameda, CA 94502. Cells were subsequently treated with DNAase. Milteny CD34 magnetic beads were used to enrich for CD34⁺ cells with columns following exactly manufacturers recommendations. Cells were pelleted and resuspended in PBS 2% FCS. Fc block was performed for 20 minutes and the mononuclear cells were stained with directly conjugated anti mouse CD34, CD38, CD45RA, CD123, CD3, CD4, CD8, CD10, CD19, CD20, CD11b, CD14, CD56, GPA, CD71, CD105 and

MEP were isolated as PI-lin-CD34+CD38+CD45RA-CD123- cells on a BD FACS ARIA sorter. All antibodies were purchased from eBiolegend, BD or Invitrogen. Subsequently 1000 MEP cells were set up in triplicate/condition, starved for 1.5h in RPMI 1% BSA and then treated with Epo 5 U/ml, DA5 5 μ M, DA330 5 μ M, DA10 5 μ M and not treated in RPMI 10% FCS.

[00151] *RNA isolation and library preparation.* RNA was isolated with trizol as per the manufacturer's recommendations and was further facilitated by using linear polyacrylamide as a carrier during the procedure. We treated the total RNA samples with RQ1 RNase free DNase (Promega) to remove minute quantities of genomic DNA if present. DNase treated samples were cleaned up using RNAeasy minelute columns (Qiagen). 1-10 ng of total RNA was used as input for cDNA preparation and amplification using Ovation RNA-Seq System V2 (NuGEN). Amplified cDNA was sheared using Covaris S2 (Covaris) using the following settings: duty cycle 10%, intensity 5, cycle/burst 100, total time 5 minutes. The sheared cDNA was cleaned up using Agencourt Ampure XP (Beckman Coulter). 500 ng of sheared cDNA were used as input for library preparation using NEBNext Ultra DNA Library Prep Kit for Illumina (New England BioLabs) as per manufacturer's recommendations.

[00152] *NASeq and data analysis.* Libraries were sequenced using HiSeq 2500 (Illumina) to obtain 2x150 base pair paired-end reads. We mapped the reads using Olego and used cuffdiff to estimate the genes induced by either EPO or diabody treatment by comparing them to untreated MEPs. Genes significantly modulated by EPO and/or the three diabodies treatments were sorted and their fold of induction was obtained by normalizing their expression levels with those obtained in untreated cells.

Example 2

Thrombopoietin Modulation

[00153] Using the technology described in Example 1, antibodies that bind to thrombopoietin were reformatted as diabodies and shown to provide variable levels of signaling upon binding to TPO-R. Diabody-induced signaling amplitude exhibited rheostat behavior, varying from full to minimal agonism.

[00154] We synthesized genes of three previously reported anti TpoR antibodies, and reformatted their V_H and V_L domains into diabodies by linking them through a five amino acid linker that prevents their association as single-chain Fv's, but promotes anti-parallel association into dimeric diabodies (sequences are provided as SEQ ID NO:9, 11 and 13 for nucleotide and SEQ ID NO:10, 12 and 14 for protein. The three diabodies exhibited different extents of STAT5 phosphorylation (Fig. 13A), cell proliferation (Figure 13C) and activation of 33 different signaling molecules (Fig. 13B).

[00155] The TpoR diabodies elicited different gene expression (Figure 14) and differentiation (Figure 15) programs in the acute myeloid leukemia cell line UT-7-TpoR and in primary Megakaryocyte-erythroid progenitor (MEP) cells (Figure 16).

[00156] The diabodies further varied in their ability to sustain hematopoietic stem cells in an in vitro culture, Figure 17. Hematopoietic stem cells treated with AK111 or AK113 survive longer than TPO-treated HSCs in culture without undergoing significant differentiation.

[00157] These studies demonstrate tuning signaling through dimer remodeling by extracellular ligands. Furthermore, the diabodies exhibit activities that have not been observed in dimeric, single-pass receptor systems, including partial agonism. These data demonstrate the general applicability of this approach to cytokine receptors that operate by a dimerization paradigm. Cytokine receptor dimers can be modulated as rheostats to control signaling output, both quantitatively (signaling amplitude) and qualitatively (differential signaling), with extracellular ligands that reorient receptor dimers into architectures not found in nature.

Materials and Methods

[00158] *Cell lines and media.* The cells expressing human TPO receptors were grown in RPMI containing 10% v/v FBS, penicillin-streptomycin, and L-glutamine (2 mM).

[00159] *Protein expression and purification.* TpoR diabodies were cloned into the pAcGP67-A vector (BD Biosciences) in frame with an N-terminal gp67 signal sequence and a C-terminal hexahistidine tag and produced using the baculovirus expression system, as described in. Baculovirus stocks were prepared by transfection and amplification in *Spodoptera frugiperda* (Sf9) cells grown in SF900II media (Invitrogen) and protein expression was carried out in suspension Trichoplusiani (High Five) cells grown in InsectXpress media (Lonza). Following expression, proteins were captured from High Five supernatants after 60 h by nickel-NTA agarose (Qiagen) affinity chromatography, concentrated, and purified by size exclusion chromatography on a Superdex 200 column (GE Healthcare), equilibrated in 10 mM HEPES (pH 7.2) containing 150 mM NaCl and 15% glycerol. Recombinant diabodies were purified to greater than 98% homogeneity. Protein concentrations were quantified by UV spectroscopy at 280 nm using a Nanodrop2000 spectrometer (Thermo Scientific).

[00160] *Phospho-Flow cytometry staining and antibodies.* Intracellular phospho-STAT5 staining was performed after ice-cold methanol (100% v/v) permeabilization. Antibodies to pSTAT5 Alexa647 were purchased from BD Biosciences and used at a 1:50 dilution. The induction of STAT5 phosphorylation was calculated by subtracting the Mean Fluorescence Intensity (MFI) of the stimulated samples from that of the unstimulated sample. The normalized values were plotted against cytokine concentration to yield dose-response curves that were fitted to a sigmoidal curve.

[00161] *Dual Luciferase Transcriptional Assays.* Transcriptional activation of STAT5 was analyzed in γ -2A cells co-transfected with TpoR, JAK2wt, STAT5 and pGRR5 luciferase reporter (Demoutier et al., 2000). pGRR5-luc contains five copies of STAT-binding site of the Fc γ RI gene inserted upstream of the luciferase gene under transcriptional control of the thymidine kinase (TK) promoter. The pRL-TK vector (Promega, Madison, WI) containing the renilla luciferase gene under control of the TK promoter was used as an internal control. Luminescence was measured in the cell lysates 24 hours after transfection using dual luciferase reporter kit (Promega) on a Perkin-Elmer Victor X Light analyzer.

[00162] *Cell Proliferation Assay.* Transduced and sorted Ba/F3 cells expressing TpoR, JAK2, JAK2V617F were expanded in IL-3, washed 3 times in PBS and 5000 cells/well were placed in 96-well plate in RPMI containing 10% fetal bovine serum with either TPO or diabodies or left untreated. After 72 hours the cell proliferation assay was performed using a CellTiter 96 NonRadioactive Proliferation Assay (Promega). Absorbance was measured at 570nm, which is directly proportional to the number of living cells.

[00163] *HSC and progenitor derived colony genotyping assay.* Lin-CD34⁺ cells were sorted from freshly thawed cryopreserved human samples. 5×10^3 CD34⁺ cells were plated in methylcellulose. Colony formation was assessed after 14 days in culture by microscopy and scored on the basis of morphology.

Diabody Sequences

		VH	Linker	VL
SEQ ID NO:1	DA5 polynucleotide			
SEQ ID NO:2	DA5 amino acid	1-118	119-123	124-233
SEQ ID NO:3	DA10 polynucleotide			
SEQ ID NO:4	DA10 amino acid	1-124	15-129	130-239
SEQ ID NO:5	DA307 polynucleotide			
SEQ ID NO:6	DA307 amino acid	1-118	119-123	124-234
SEQ ID NO:7	DA330 polynucleotide			
SEQ ID NO:8	DA330 amino acid	1-118	119-124	125-234
SEQ ID NO:9	AK119 polynucleotide			
SEQ ID NO:10	AK119 amino acid	1-118	119-123	124-230
SEQ ID NO:11	AK113 polynucleotide			
SEQ ID NO:12	AK113 amino acid	1-123	124-128	129-235
SEQ ID NO:13	AK111 polynucleotide			
SEQ ID NO:14	AK111 amino acid	1-122	123-127	128-235

WHAT IS CLAIMED IS:

1. A method for modulating the signaling intensity of a receptor in response to binding a bivalent ligand, the method comprising:
binding a bivalent ligand that enforces a selected receptor inter-subunit distance when bound to the receptor, wherein the inter-subunit distance correlates with the intensity of signaling.
2. The method of Claim 1, wherein the receptor is a cytokine receptor.
3. The method of Claim 1, wherein the receptor is a dimeric receptor.
4. The method of Claim 3, wherein the receptor is a homodimer.
5. The method of Claim 4, wherein the receptor is one of erythropoietin (EPO) receptor, thrombopoietin (TPO) receptor, FLT3, CD117, CD115, CDw136.
6. The method of Claim 1, wherein the bivalent ligand is a diabody.
7. The method of Claim 6, wherein the diabody fully activates signaling from the receptor.
8. The method of Claim 6 wherein the diabody partially activates signaling from the receptor.
9. The method of Claim 6 wherein the diabody induces biased signaling.
10. The method of Claim 6, wherein the diabody blocks signaling from the receptor.
11. The method of Claim 6, wherein the diabody inhibits ligand independent JAK/STAT signaling.
12. The method of Claim 11, wherein the diabody inhibits JAK2V617F signaling.
13. The method of any one of Claims 1-12, wherein the enforced receptor inter-subunit distance is modified by altering the sequence of the diabody variable region to achieve a desired level of signaling.

14. The method of any one of Claims 1-12, wherein the diabody variable regions are the same.
15. The method of any one of Claims 1-12, wherein the diabody variable regions are different.
16. The method of any one of Claims 1-15, wherein the two domains present on each polypeptide chain of the diabody are separated by a short linker of from 4-6 amino acids in length.
17. The method of any one of Claims 1-15, wherein the diabody is administered therapeutically to a subject in need thereof.
18. The method of Claim 17, wherein the diabody inhibits ligand independent JAK/STAT signaling and the subject suffers from a proliferative disorder associated with the ligand independent JAK/STAT signaling.
19. The method of Claim 18, wherein the diabody comprises the variable region sequences of DA10 or DA307, separated by a peptide linker.
20. The method of Claim 19 wherein the individual suffers from a myeloproliferative disorder associated with JAK2V617F signaling.
21. The method of Claim 17, wherein the diabody activates EPO signaling and the individual suffers from anemia.
22. The method of Claim 21, wherein the diabody comprises the variable region sequences of DA5 or DA330, separated by a peptide linker.
23. The method of Claim 17, wherein the diabody activates the TPO receptor.
24. The method of Claim 23, wherein the diabody comprises the variable regions of any one of AK111, AK111 or AK113, separated by a peptide linker.
25. A bivalent ligand for use in the method of any one of Claims 1-24.

26. The bivalent ligand of Claim 25, wherein the bivalent ligand is formulated with a pharmaceutically acceptable excipient.

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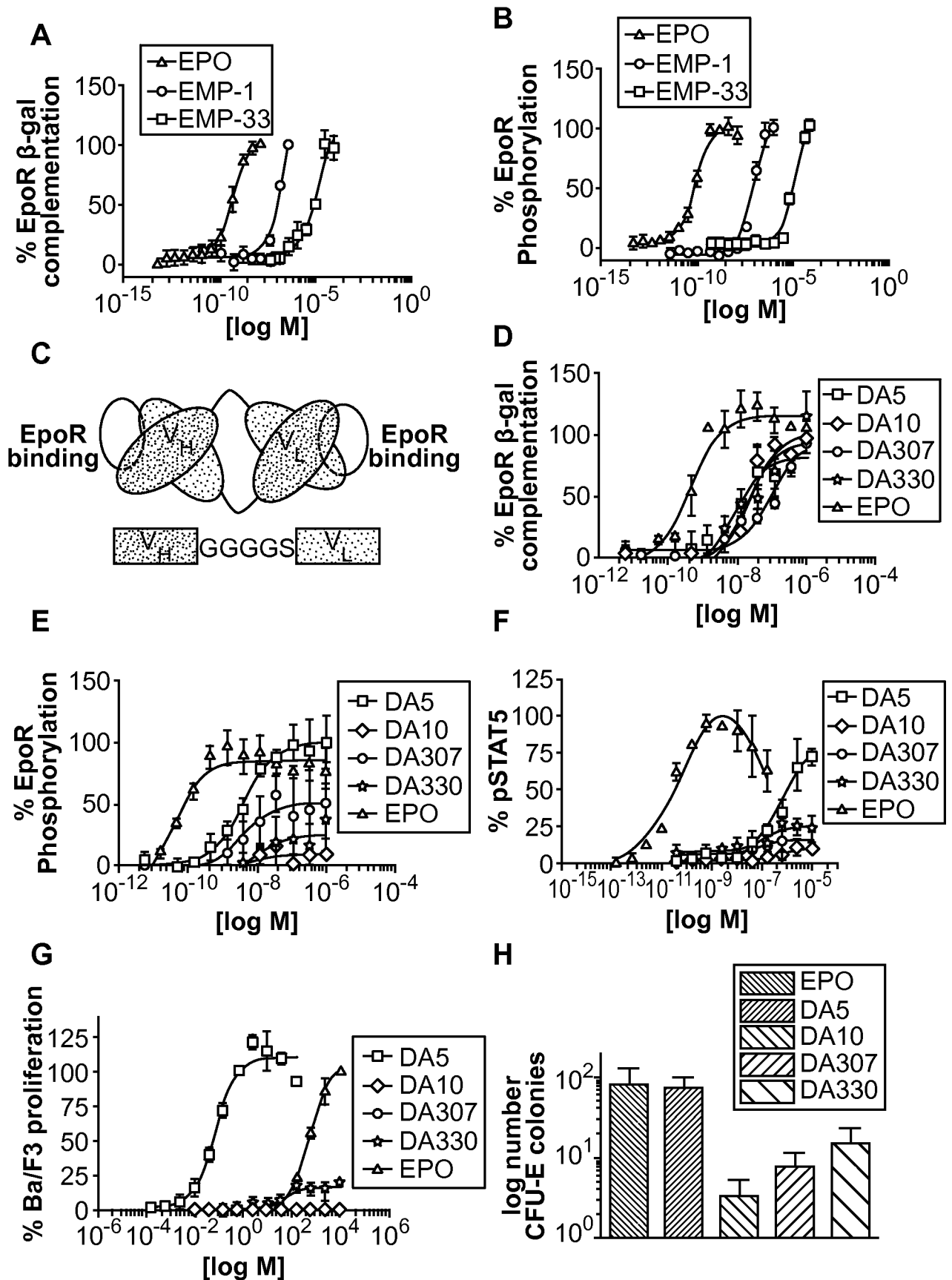


FIG. 1

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A

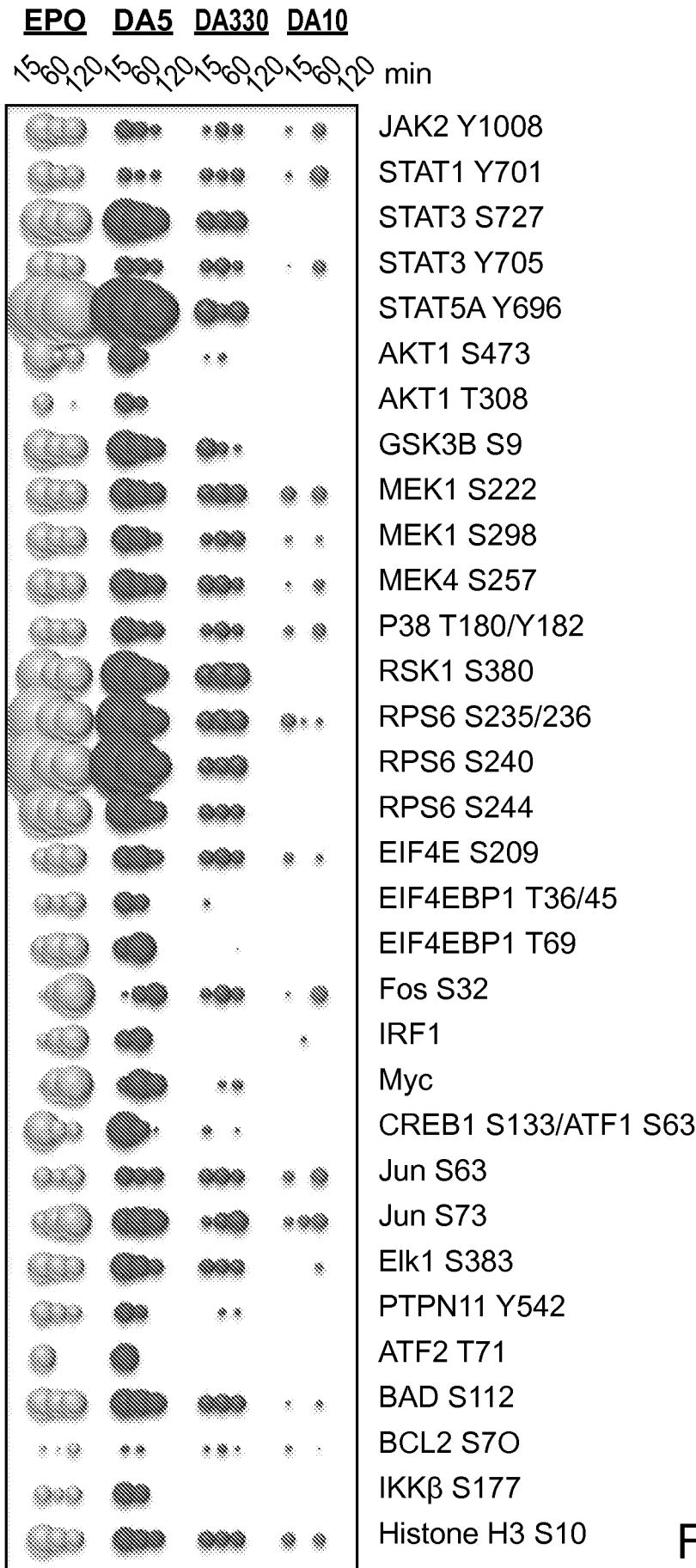


FIG. 2

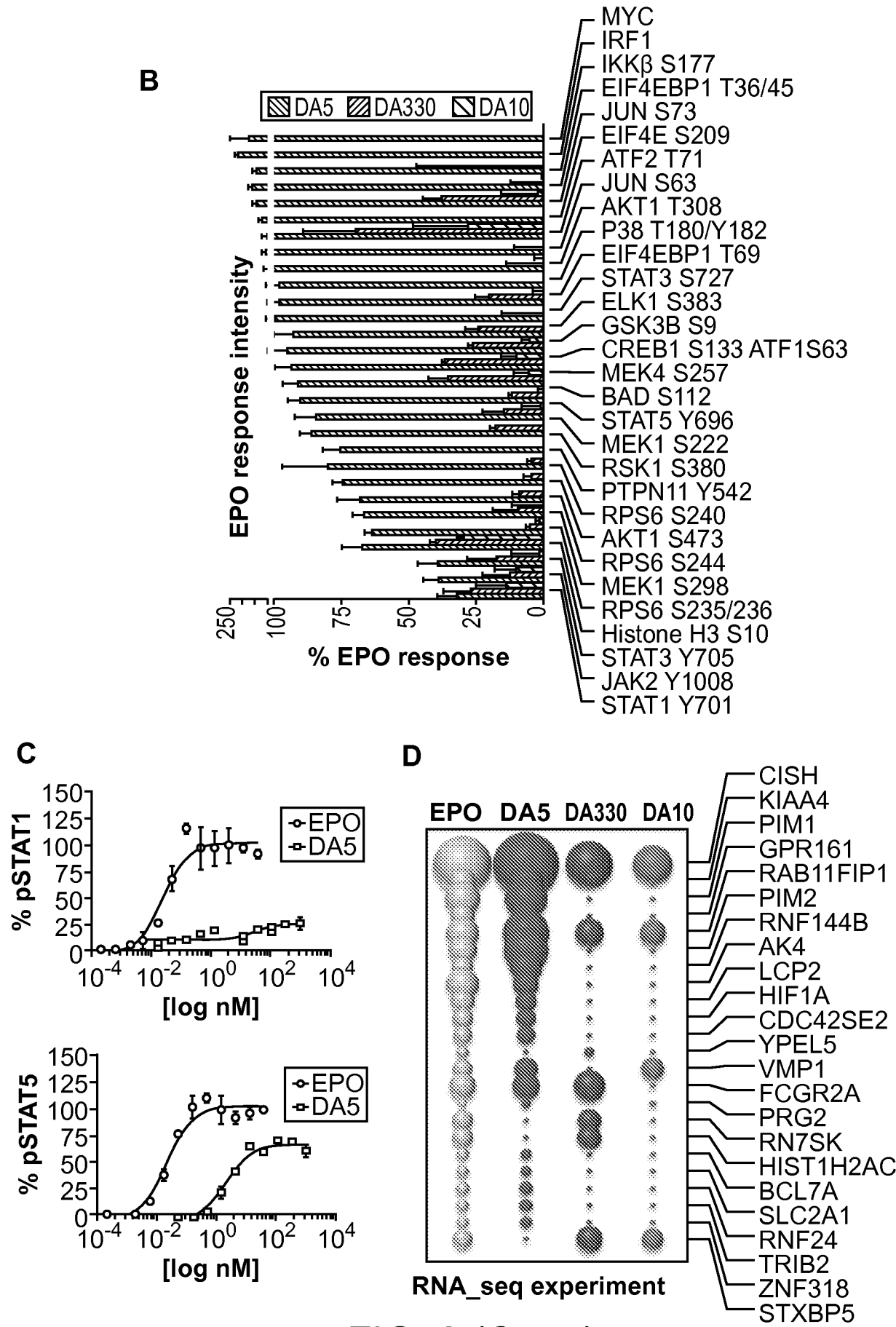


FIG. 2 (Cont.)

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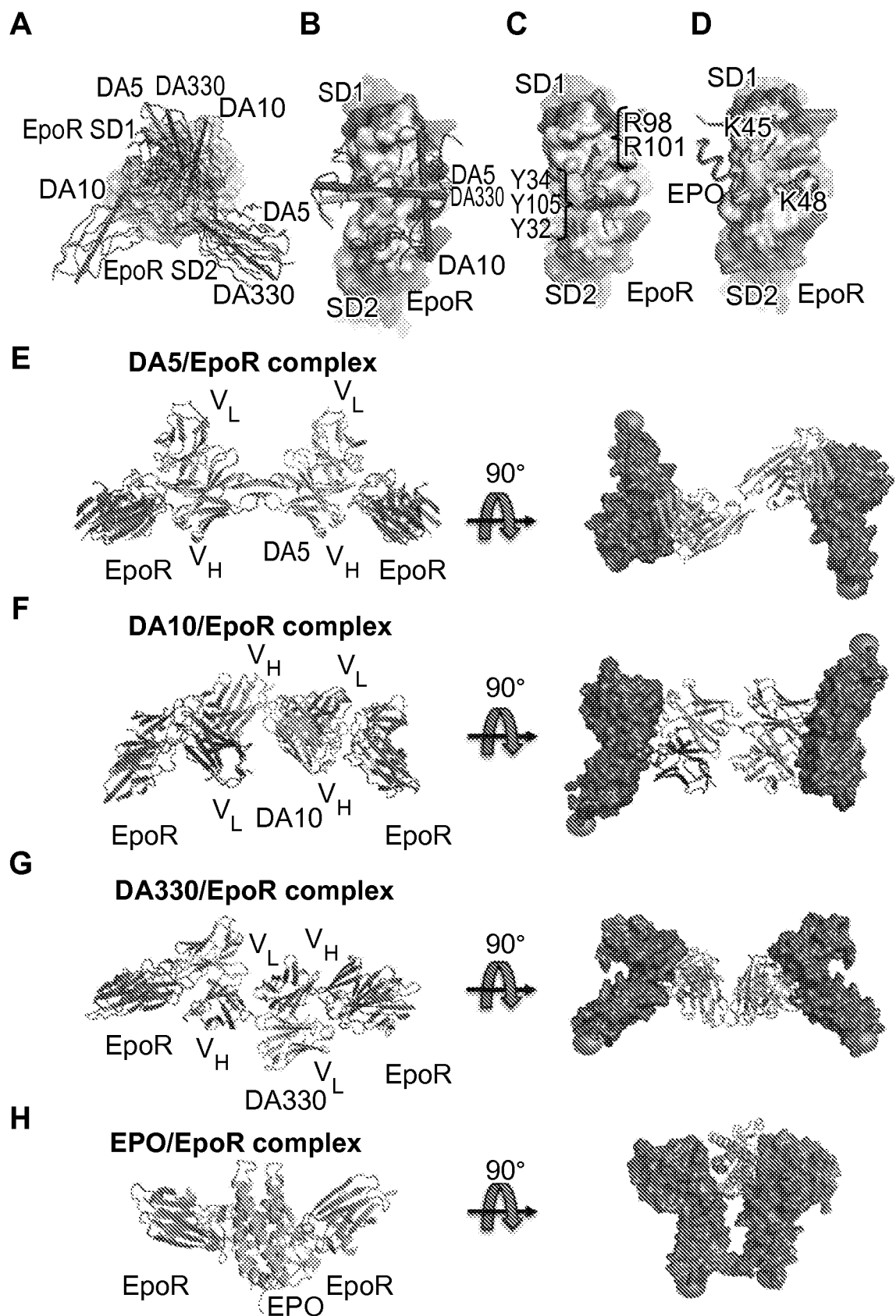


FIG. 3

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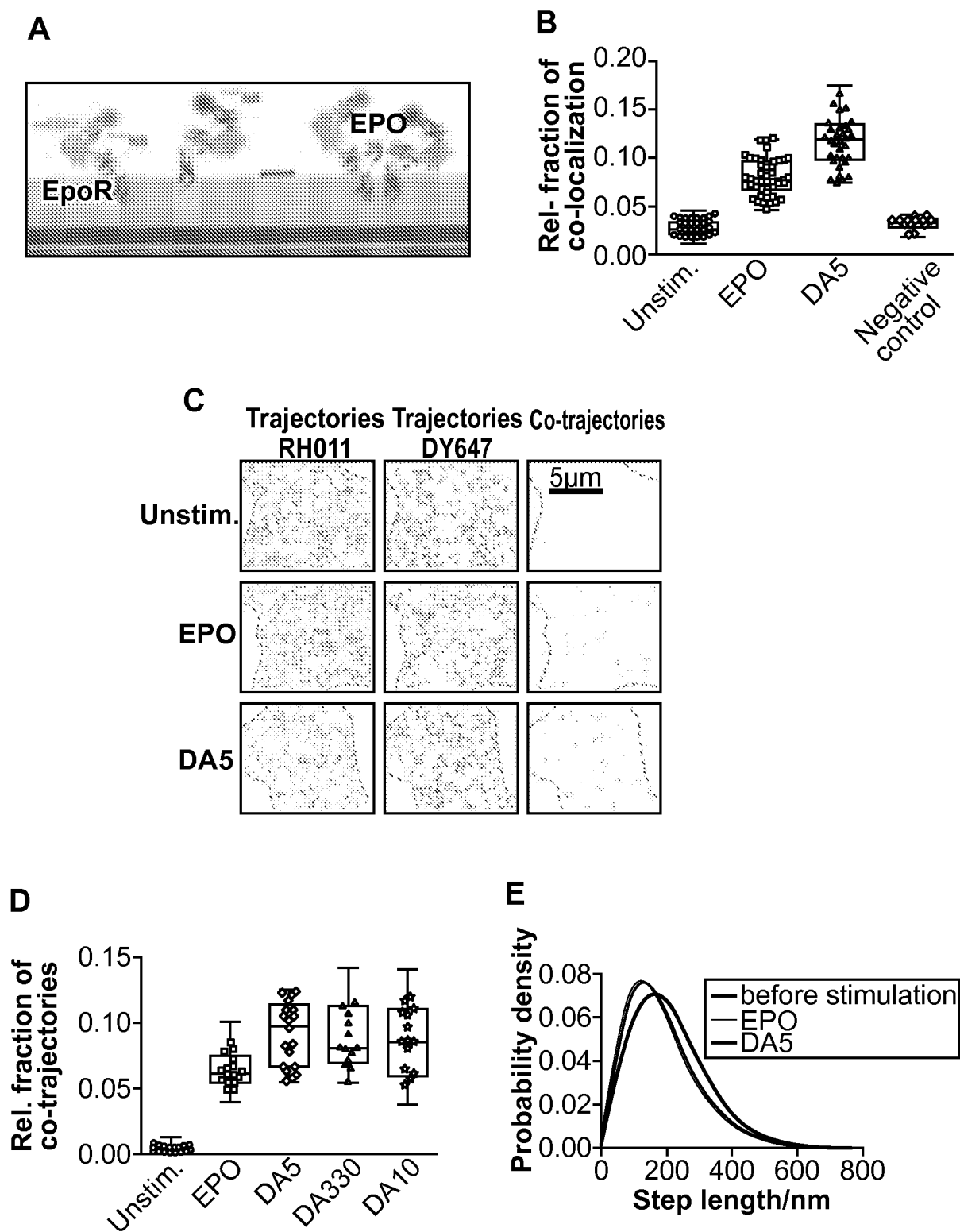


FIG. 4

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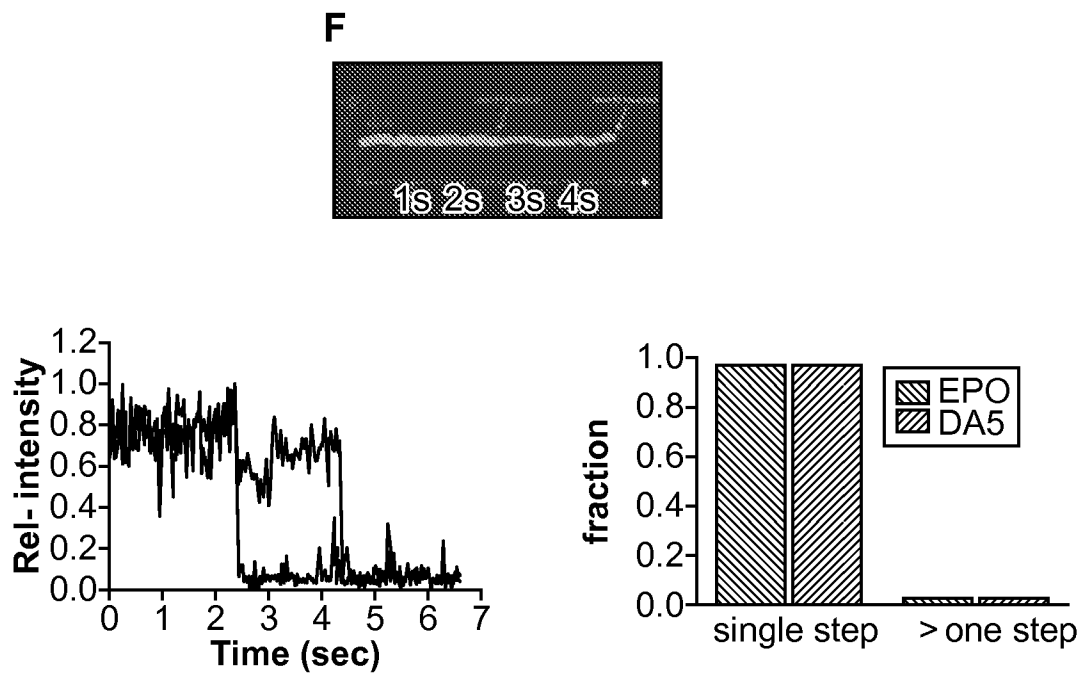


FIG. 4 (Cont.)

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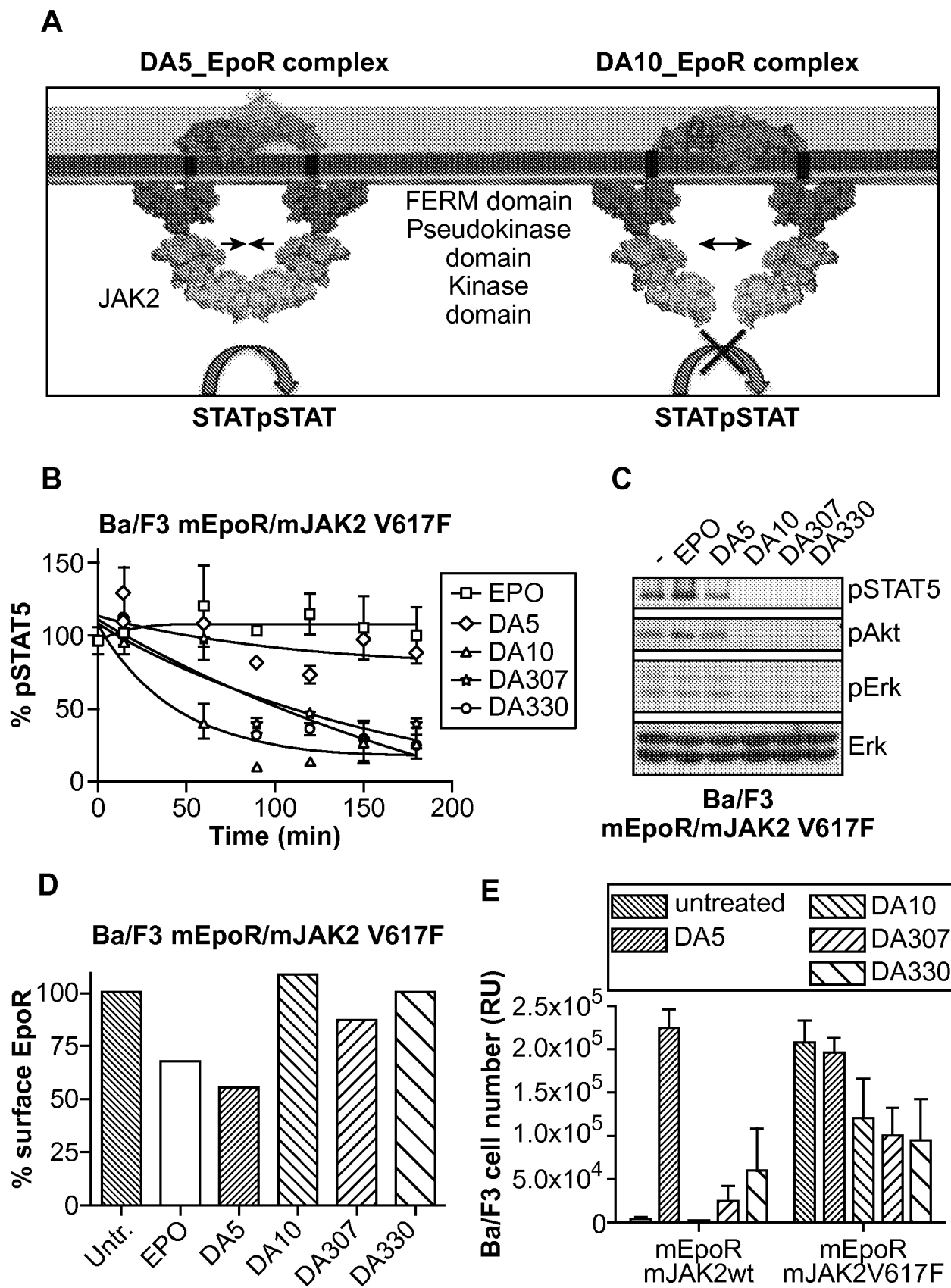


FIG. 5

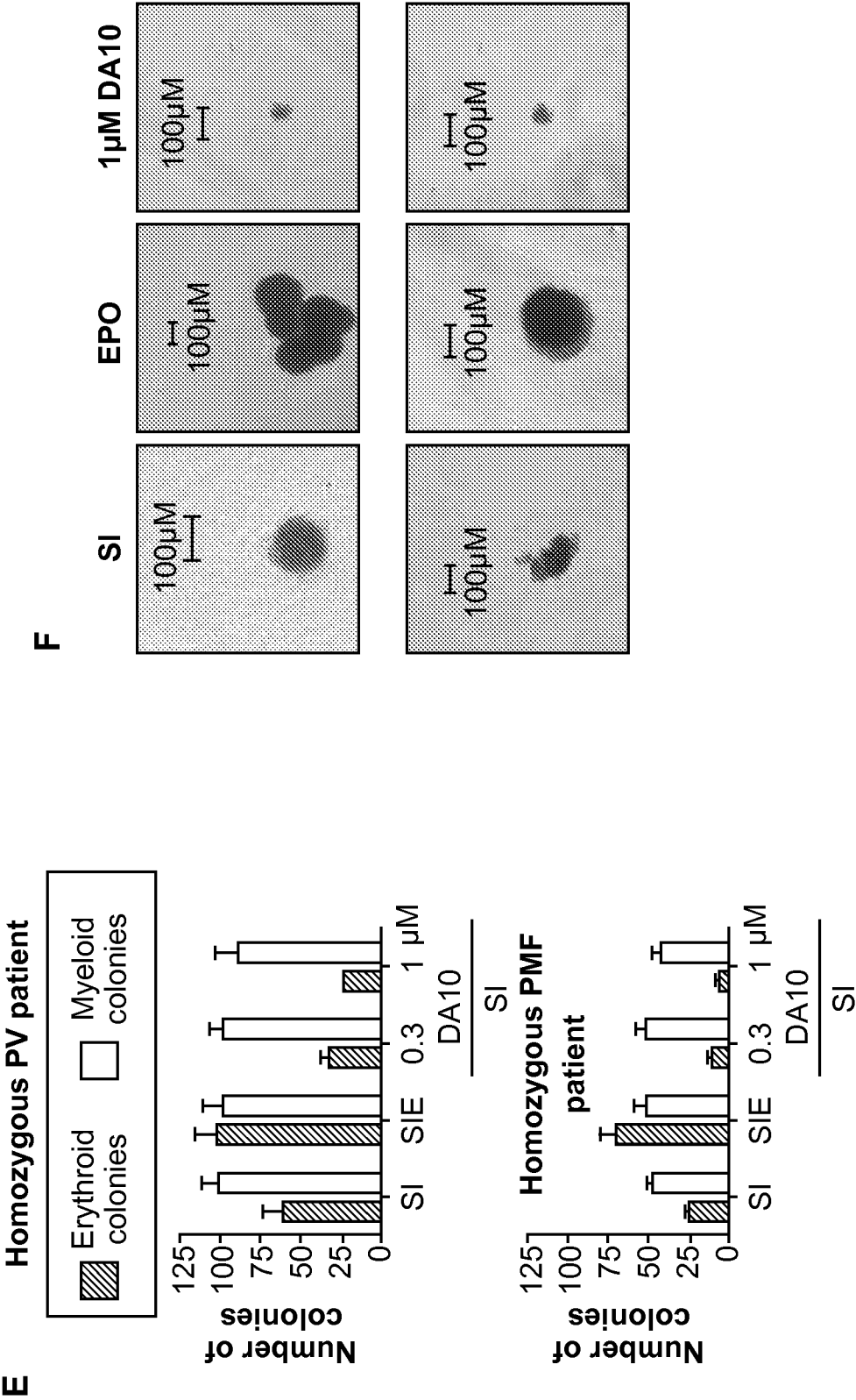


FIG. 6 (Cont.)

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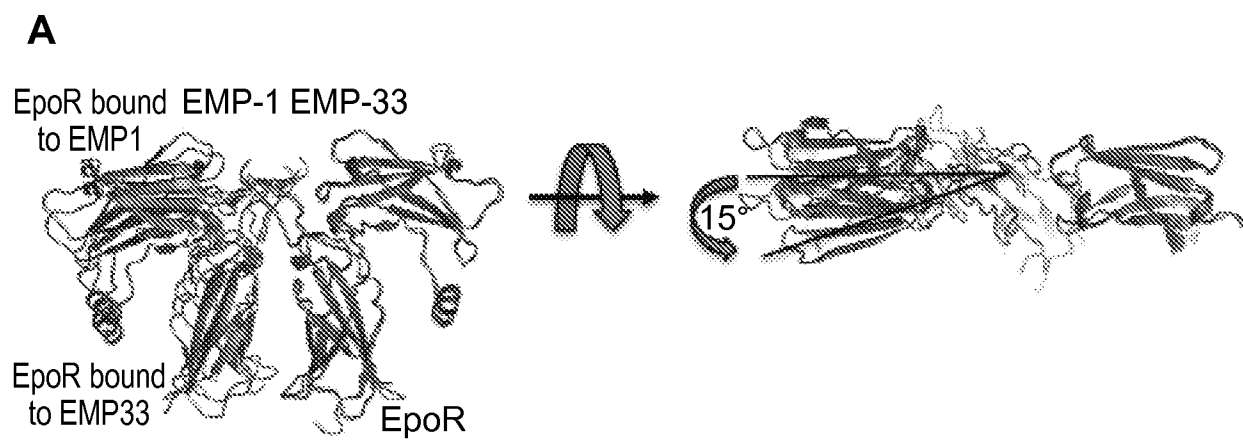


FIG. 7

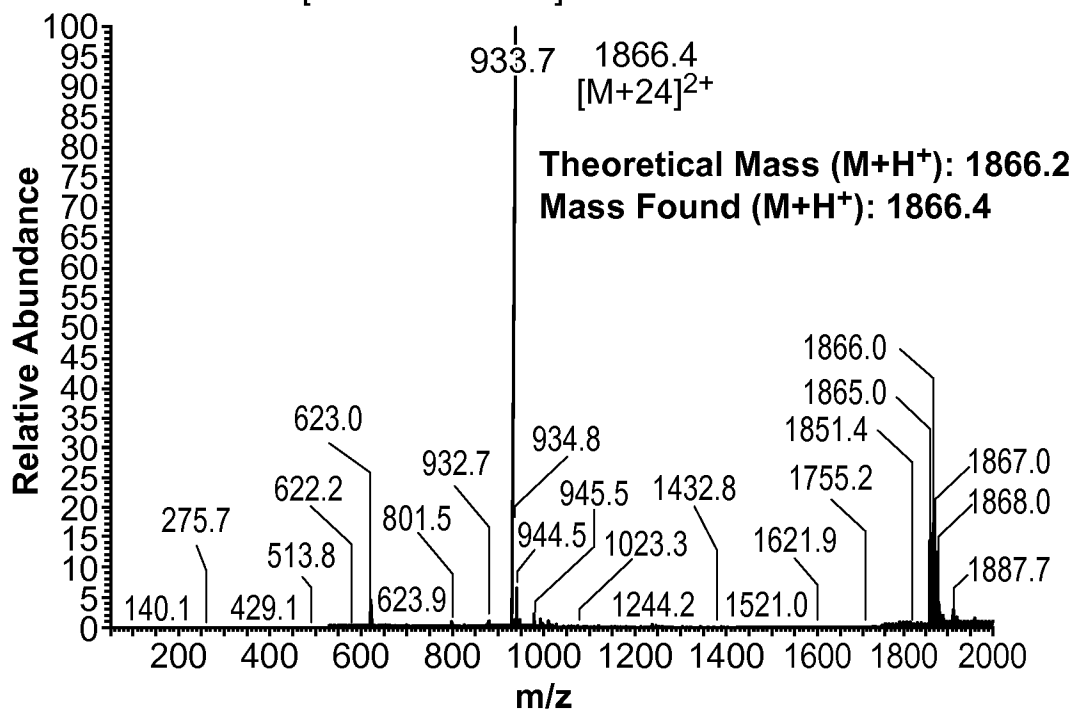
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B**EMP-1 peptide**

C:\Xcalibur\Data\Peptide\91259FC 6/21/2012 8:59:44 AM 91259 06/26/12

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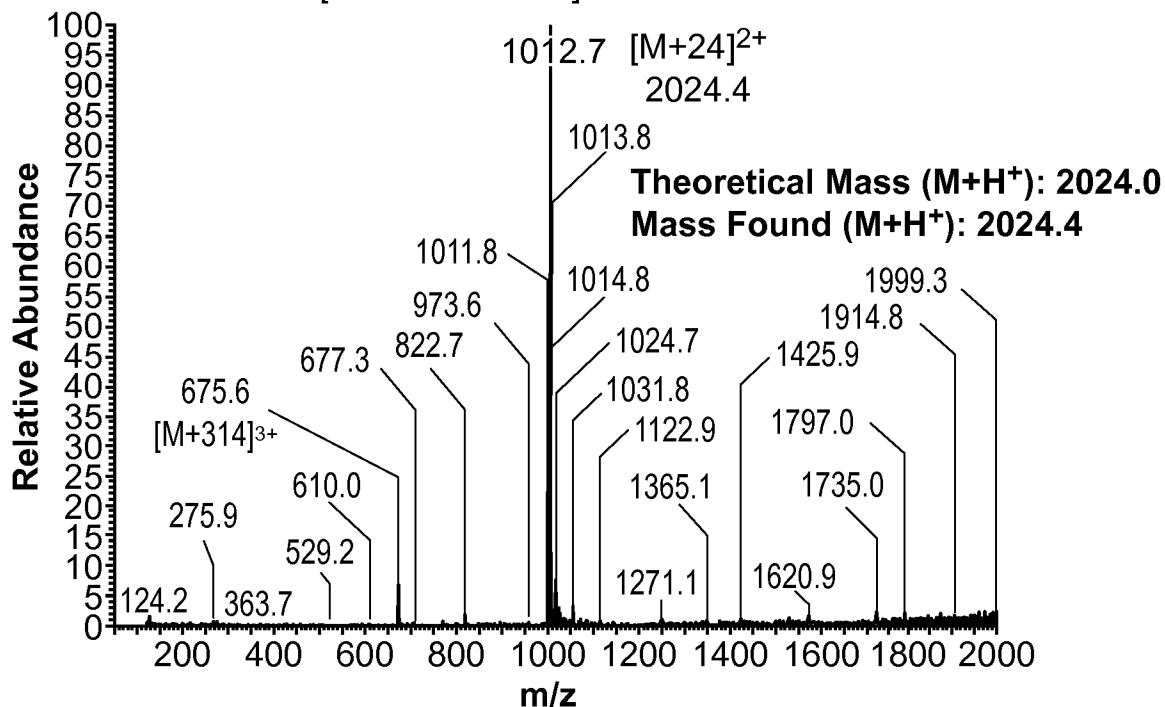
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**EMP-33 peptide**

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F: + c ESI Full ms [100.00-2000.00]

**FIG. 7 (Cont. 1)**

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C

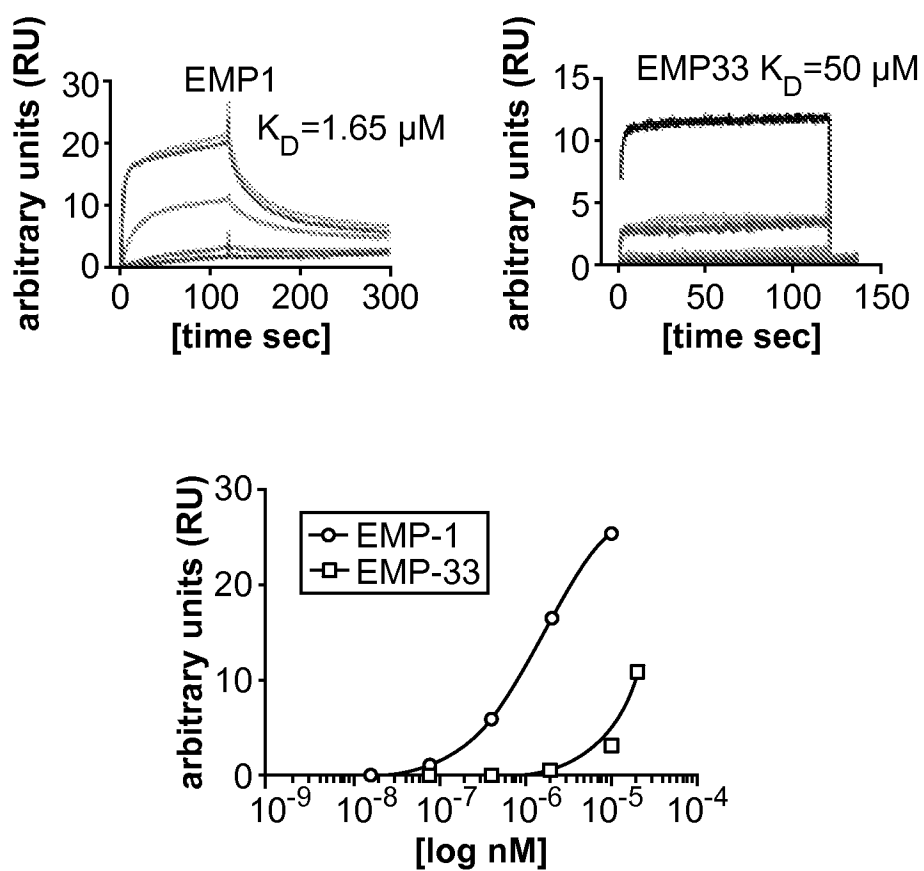
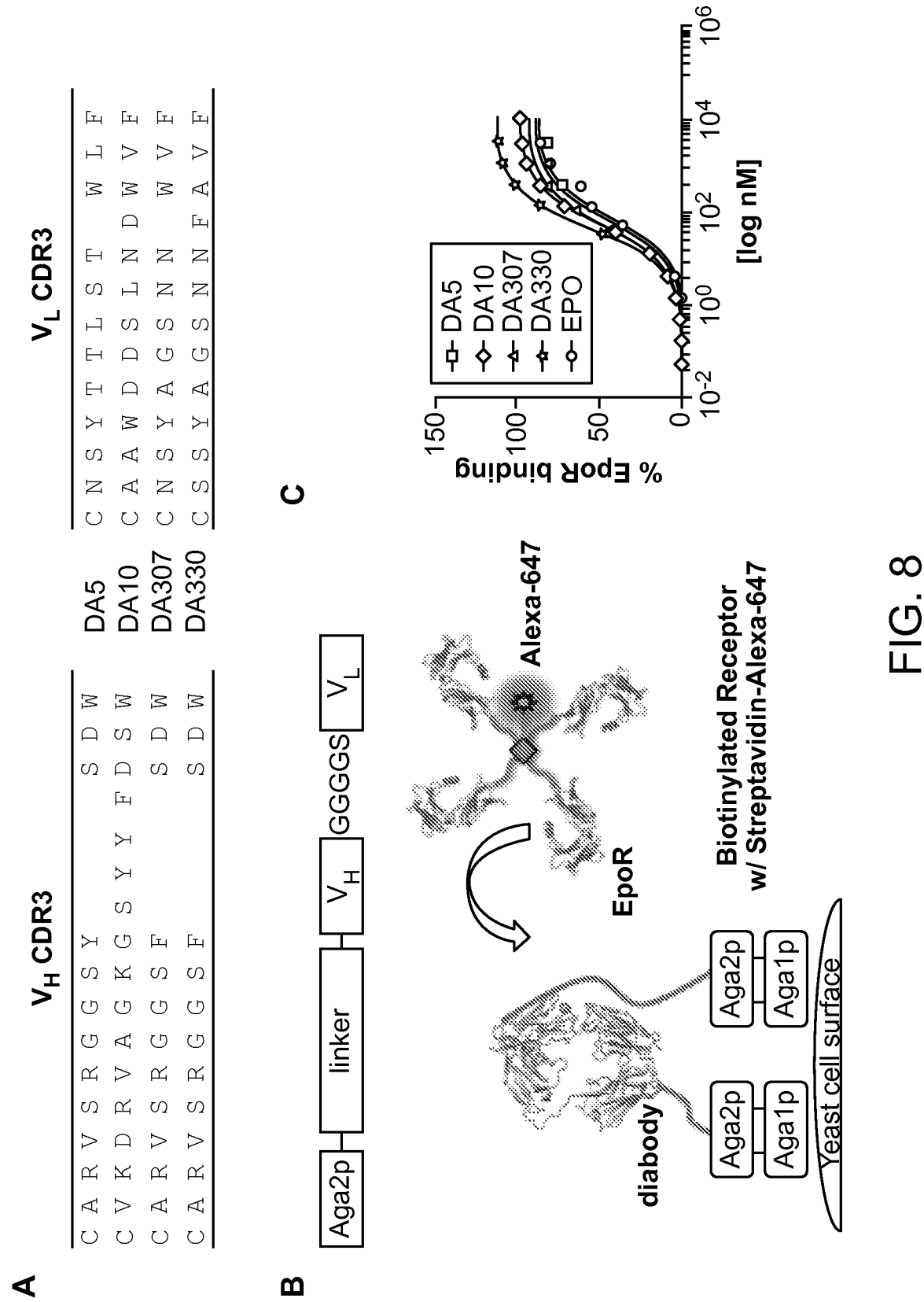


FIG. 7 (Cont. 2)



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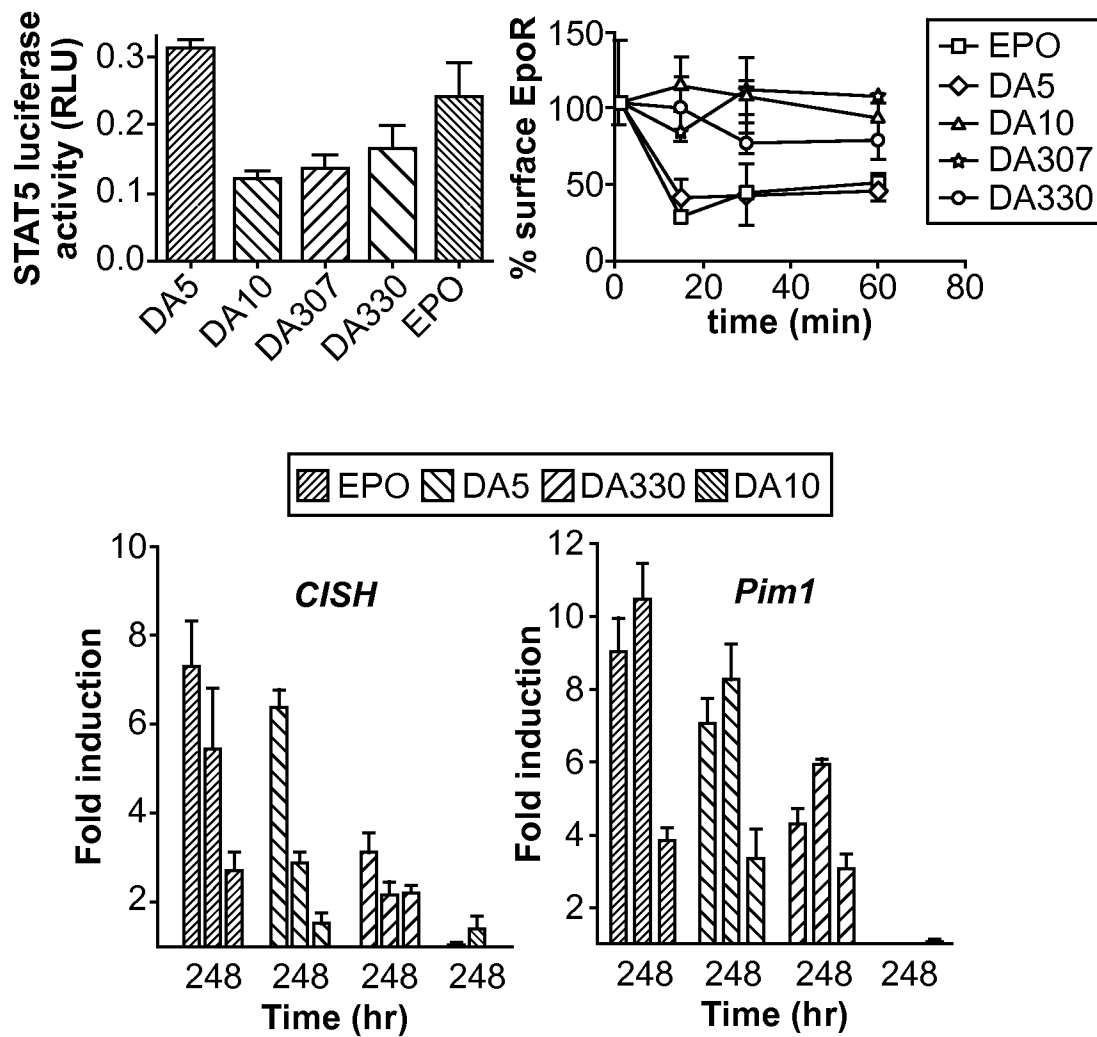


FIG. 9

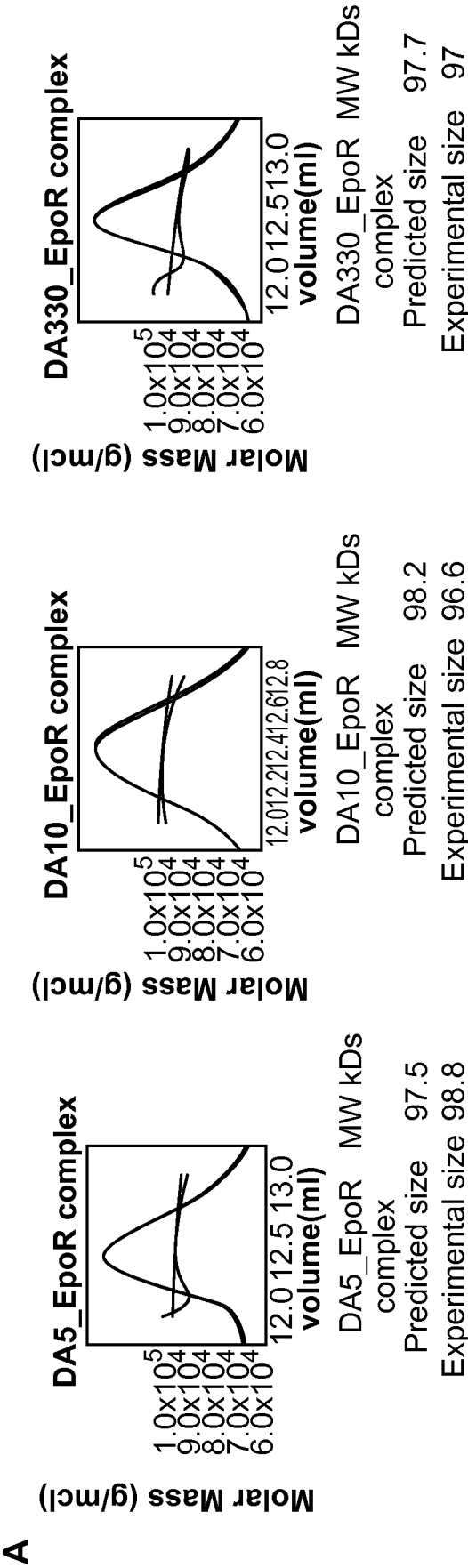


FIG. 10

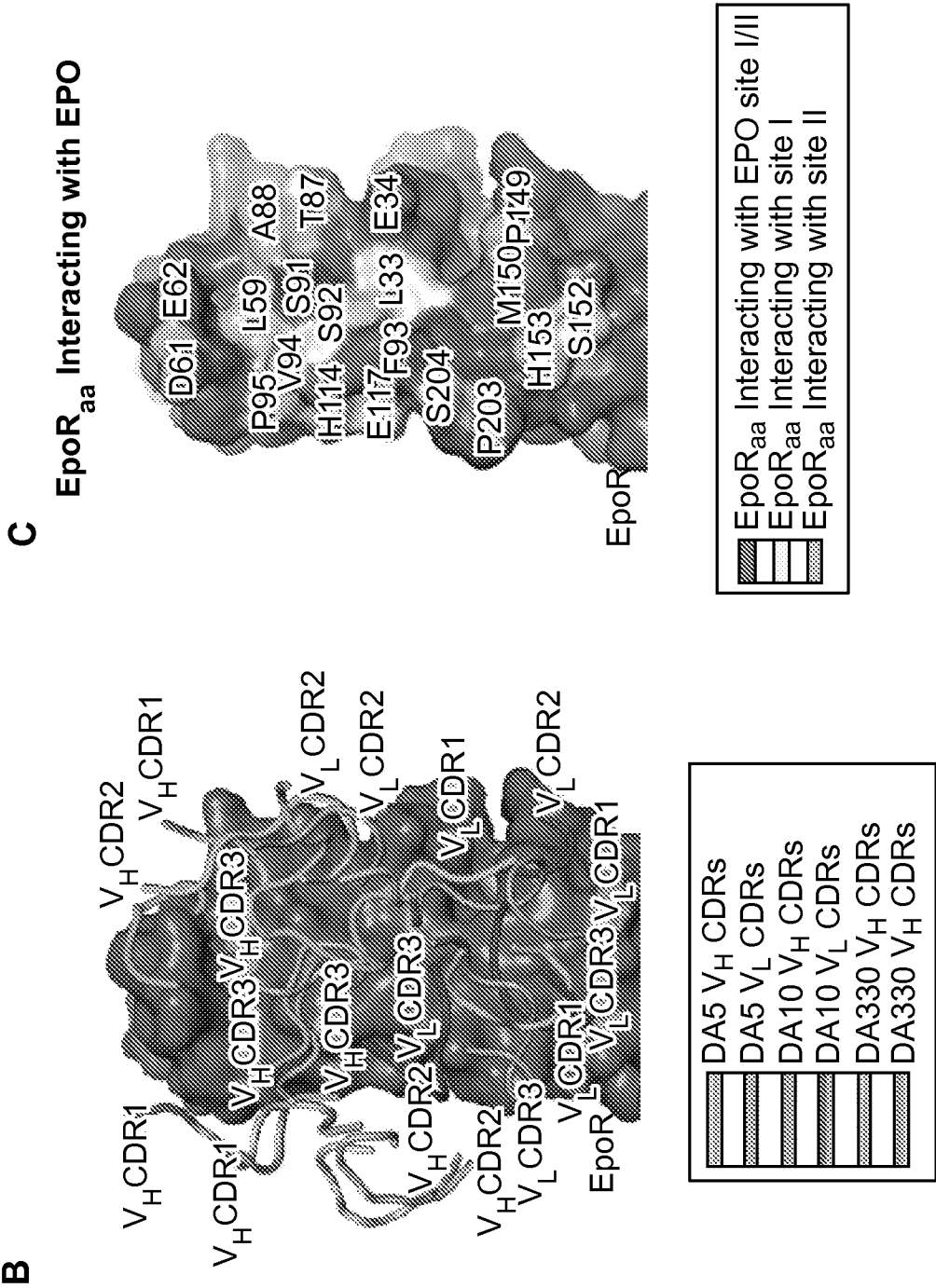


FIG. 10 (Cont. 1)

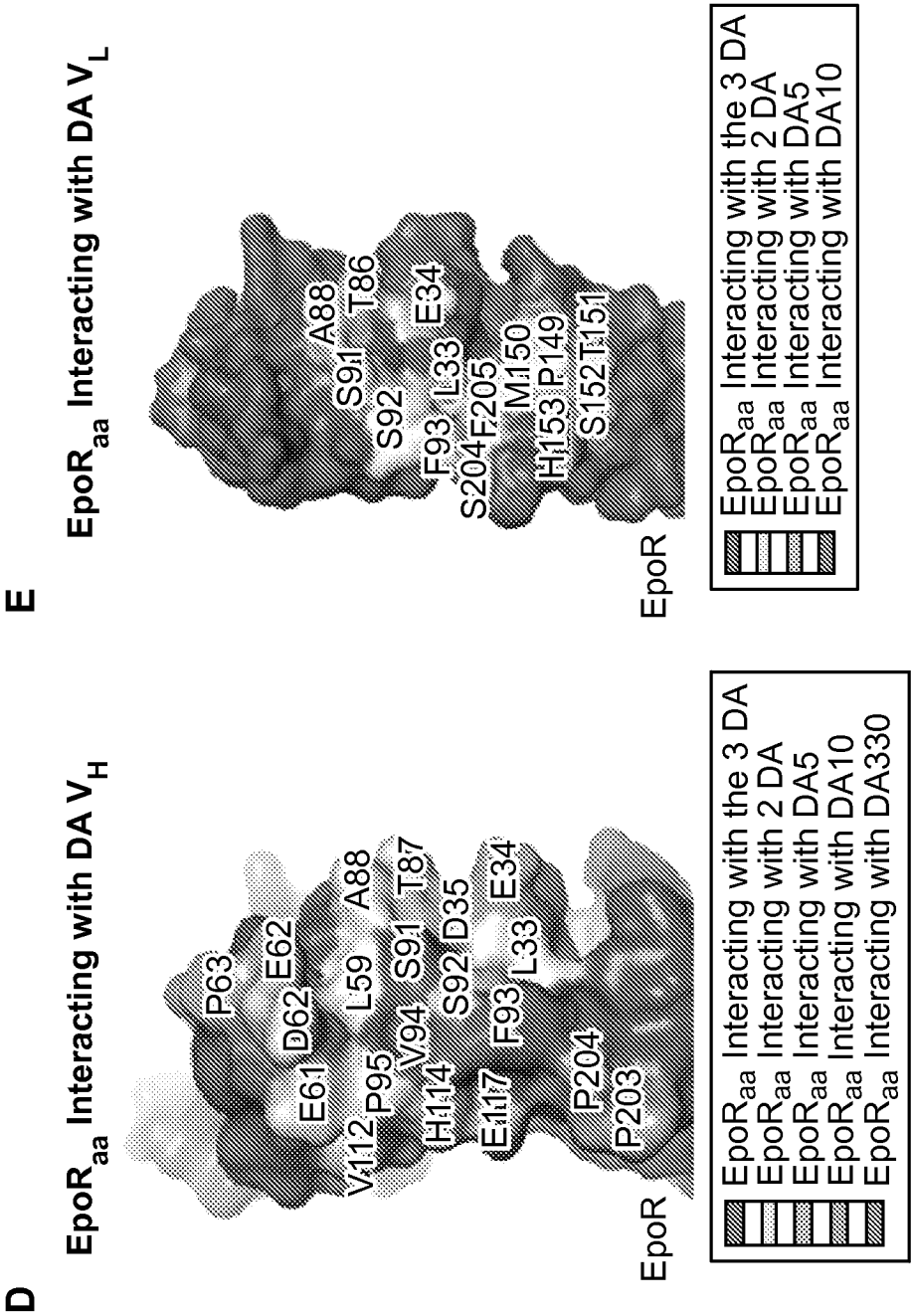
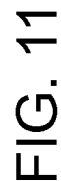


FIG. 10 (Cont. 2)



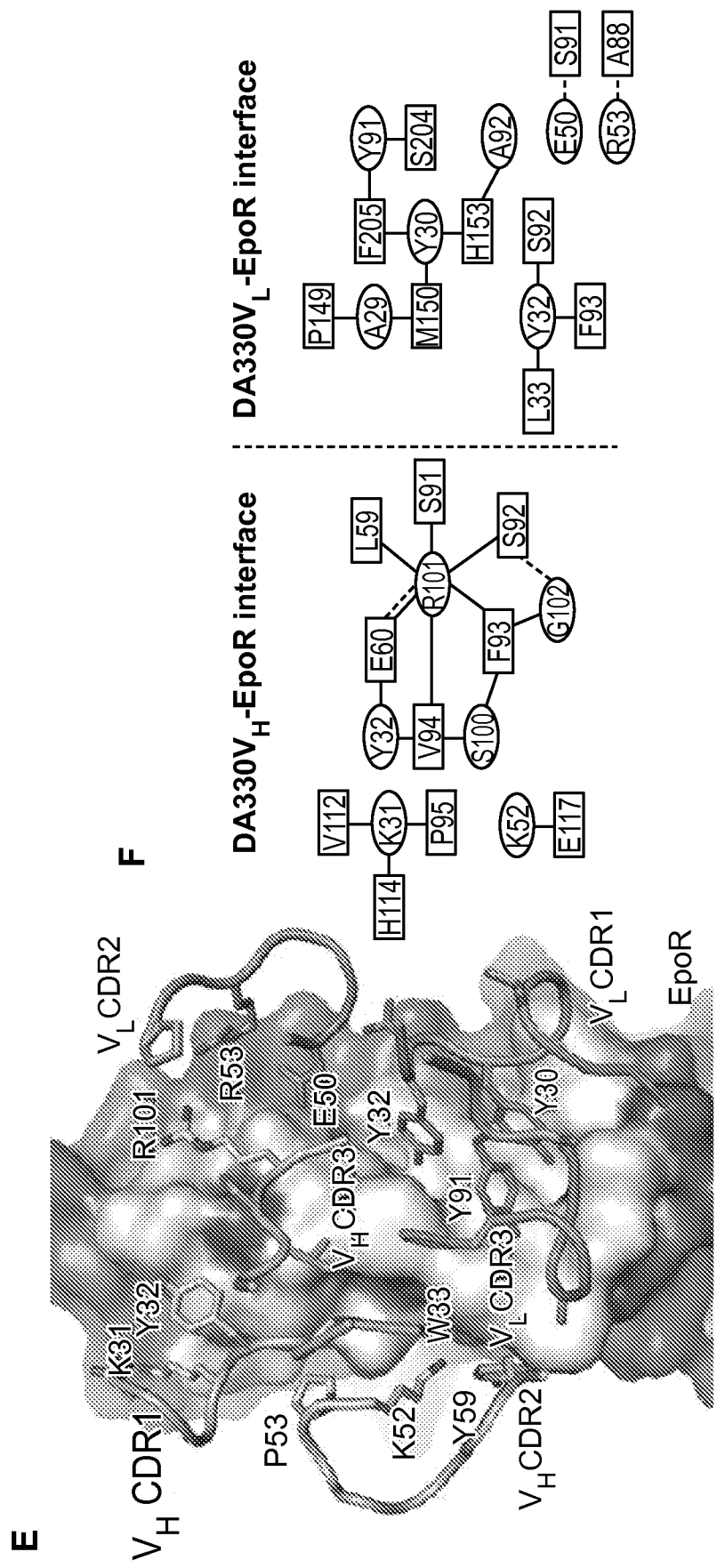
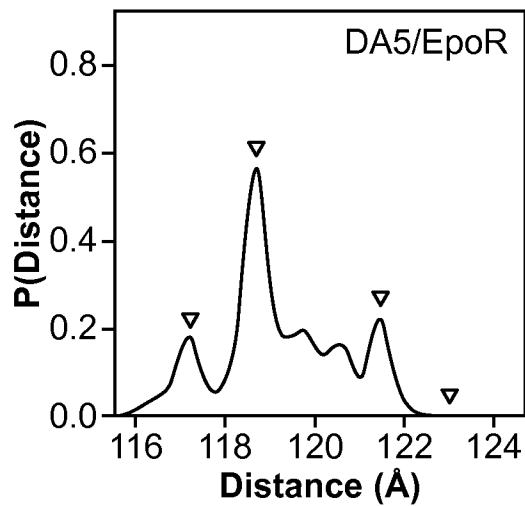
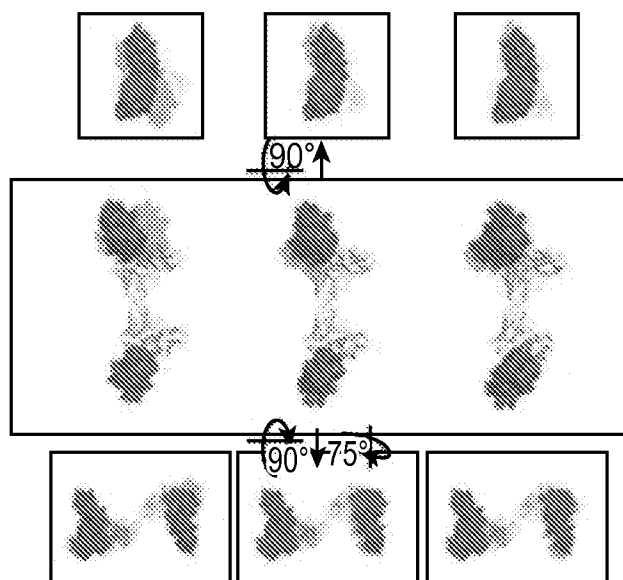


FIG. 11 (Cont.)

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A



B

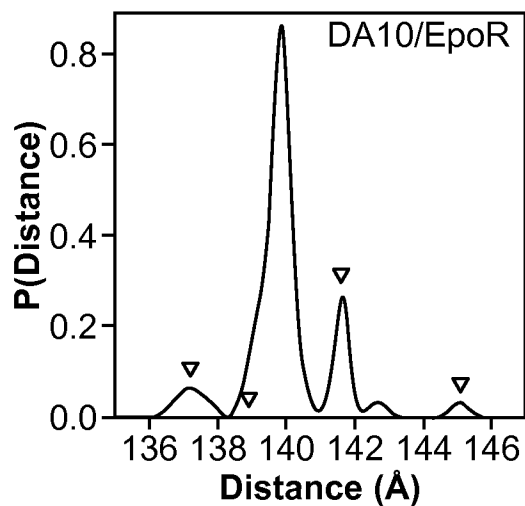
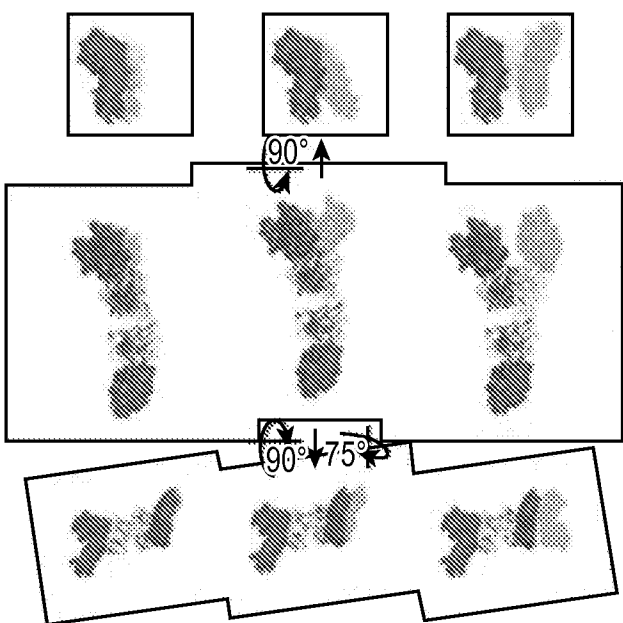
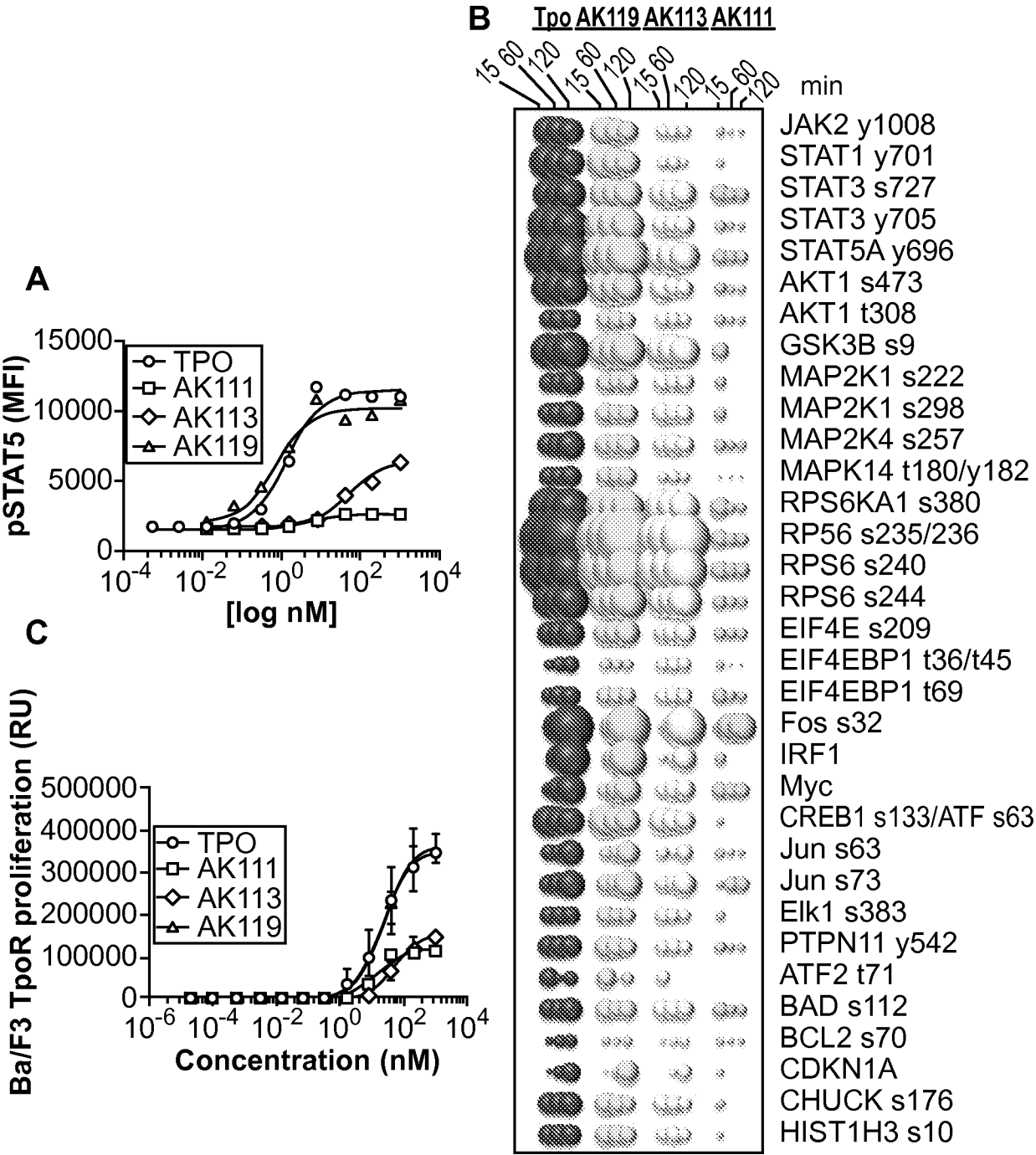
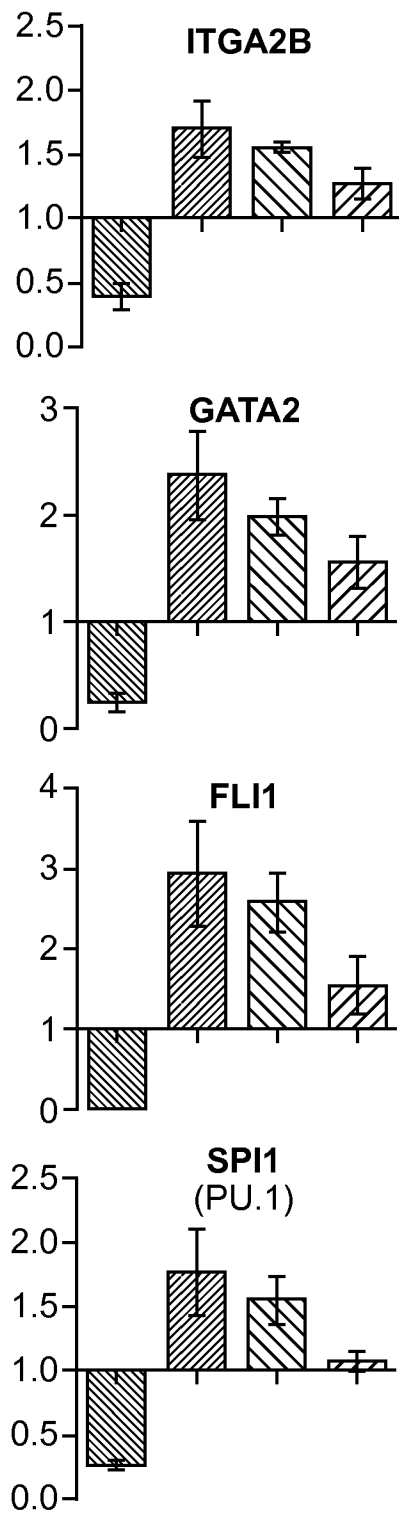
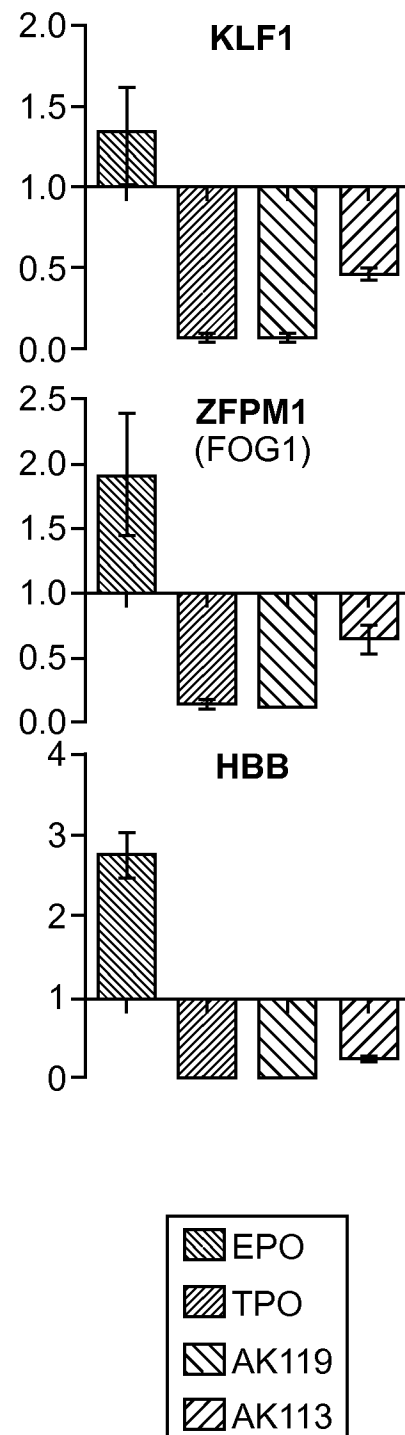


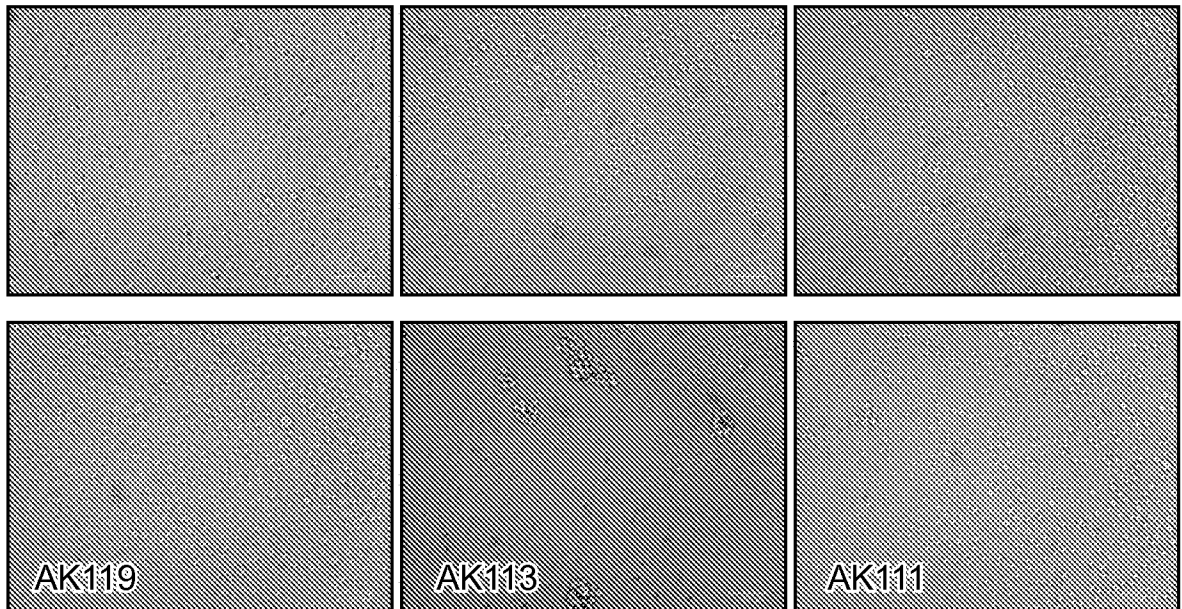
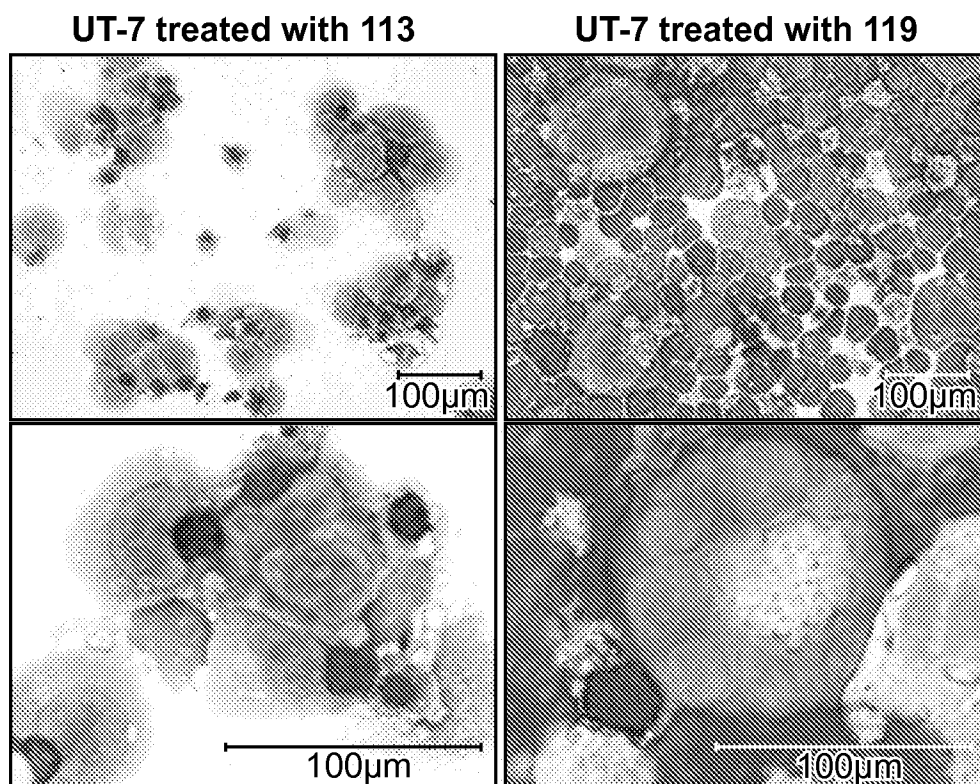
FIG. 12



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A**Megakaryocyte marker genes****B****Erythrocyte marker genes****FIG. 14**

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A**B****FIG. 15**

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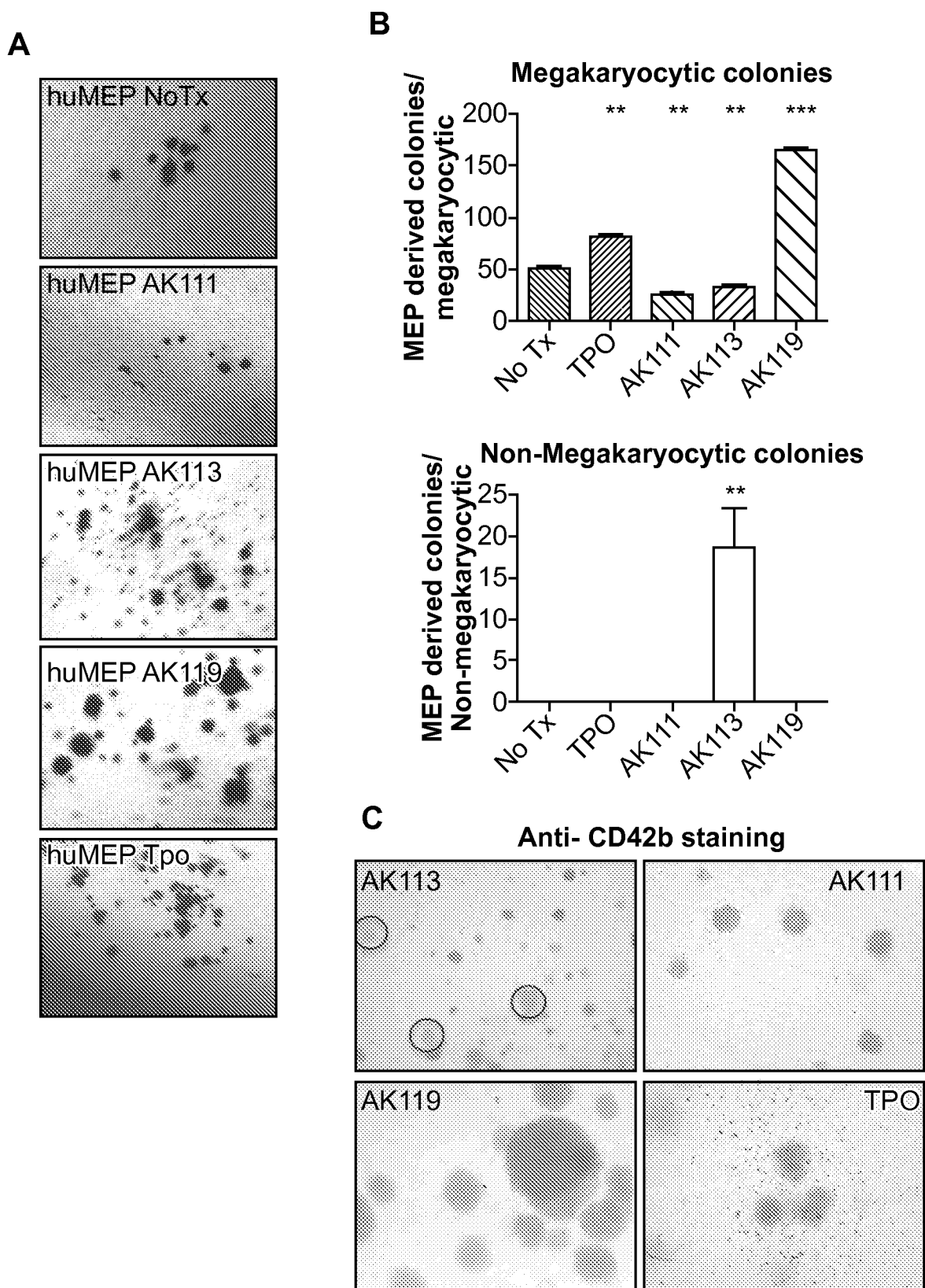
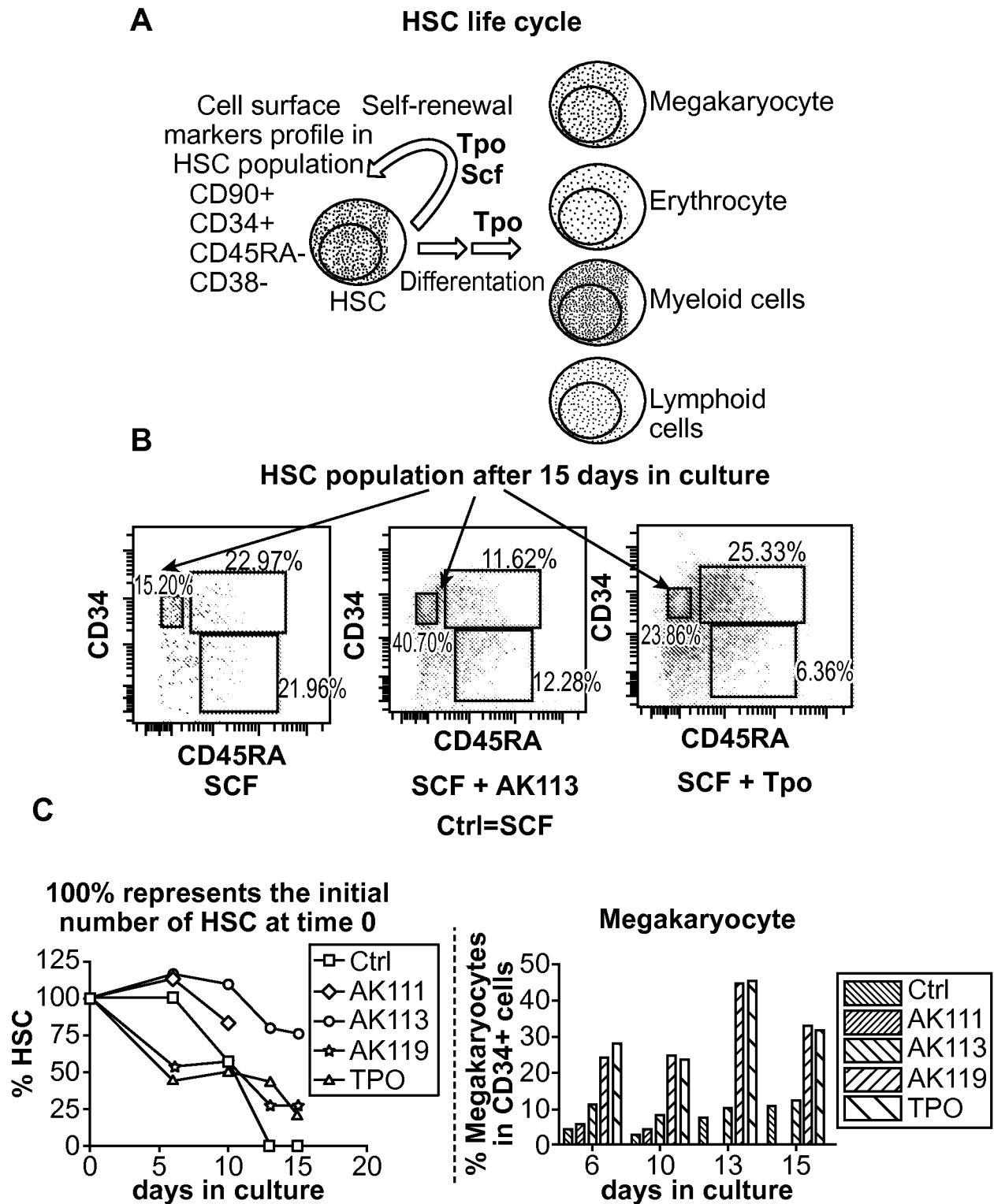


FIG. 16

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Partial TpoR Dbs agonists sustain HSC longer in culture, while the full TpoR Db agonist induces Megakaryocytes differentiation

FIG. 17

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/42406

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12P 21/04; A61K 39/395; A61K 39/00; C12P 21/08; C12N 5/02 (2015.01)

CPC - C07K 2319/00; C07K 16/2866; C07K 2317/31; C07K 2317/56; G01N 33/566

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)

IPC(8) - C12P 21/04; A61K 39/395; A61K 39/00; C12P 21/08; C12N 5/02 (2015.01) - see keyword below

CPC - C07K 2319/00; C07K 16/2866; C07K 2317/31; C07K 2317/56; G01N 33/566; USPC - 435/69.7, 375; 424/143.1, 136.1; 530/387.3

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

IPC(8) - C12P 21/04; A61K 39/395; A61K 39/00; C12P 21/08; C12N 5/02 (2015.01) - see keyword below

CPC - C07K 2319/00; C07K 16/2866; C07K 2317/31; C07K 2317/56; G01N 33/566; USPC - 435/69.7, 375; 424/143.1, 136.1; 530/387.3

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

PubWEST(USPT,PGPB,EPAB,JPAB); PatBase; Medline, Google: receptor, erythropoietin, EpoR, dimerization, bivalent, antibody, diabody, binding, inter-subunit, intersubunit, signaling, Erythropoietin, EpoR, EPO, cytokine, homodimer, dimer, dimeric, fully, anti-EpoR, antibody, activate, cross, distance, subunit, rHuEPO, heavy-chain, light chain, variable

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2008/0182976 A1 (Elliott) 31 July 2008 (31.07.2008), para [0003], [0005], [0009], [0010], [0011], [0019], [0020], [0023], [0027], [0031], [0037], [0051], [0057], [0075], [0076], [0077], Fig 2, and Fig 5	1-5 ----- 6-7, 9, 13-15/(6-7, 9)
Y	US 2006/0018902 A1 (REILLY et al.) 26 January 2006 (26.01.2006), Abstract, para [0009], [0036], [0041], [0043], [0048], [0050], and [0090]	6-7, 9, 13-15/(6-7, 9)
X	ELLIOTT et al., Activation of the Erythropoietin (EPO) Receptor by Bivalent Anti-EPO Receptor Antibodies. J Biol Chem. 1996, Vol. 271(40), p. 24691-7. Abstract; pg 24691, col 2, para 2; pg 24693, col 2, para 1, Table I, and Fig 1; pg 24694, Fig 3 and Fig 4; and pg 24695, col 2, para 1	1-5
A	JIANG et al., Receptor signaling: When dimerization is not enough. Curr Biol. 1999, Vol. 9(15), p. R568-71. Entire documentation, especially pg R569, Fig 1	1-7, 9, 13-15/(6-7, 9)
A	US 2008/0124340 A1 (BORGES et al.) 29 May 2008 (29.05.2008), para [0026], and SEQ ID NO: 46, the regions between amino acid residues 1-118 and 135-244	1-7, 9, 13-15/(6-7, 9)
A	REMY et al., Erythropoietin Receptor Activation by a Ligand-Induced Conformation Change. Science. 1999, Vol. 283(5404), p. 990-3. Entire documentation, especially Abstract; and pg 991, Fig 1	1-7, 9, 13-15/(6-7, 9)
A	VOLKEL et al., Optimized linker sequences for the expression of monomeric and dimeric bispecific single-chain antibodies. Protein Eng. 2001, Vol. 14(10), p. 815-23. Entire documentation, especially Abstract; pg 815, col 2, para 1; pg 818, Fig 2, and Table IV; and pg 821, Fig 5 and Fig 6	6-7, 9, 13-15/(6-7, 9)

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

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"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)

"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

"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

"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

"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

"&" document member of the same patent family

Date of the actual completion of the international search

28 September 2015

Date of mailing of the international search report

29 DEC 2015

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/42406

Box No. 1 Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☒ forming part of the international application as filed:

☒ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13ter. 1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13ter. 1(a)).

☐ on paper or in the form of an image file (Rule 13ter. 1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/42406

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of 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. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
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:

3. ☒ Claims Nos.: 16-26
because they are dependent 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:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+: Claims 1-15, directed to a method for modulating the signaling intensity of a receptor in response to binding a bivalent ligand, wherein the bivalent ligand is a diabody. The receptor will be searched to the extent that the receptor encompasses the first named erythropoietin (EPO) receptor, and the diabody induced signaling from the receptor will be searched to the extent wherein the diabody fully activates signaling from the receptor; additional diabody signaling will be searched wherein the diabody induces biased signaling (Specification: para [00114] - "Biased" signal activation ... diabodies ranged from full agonism for DA5...did not activate all 33 signaling molecules to the same extent (Fig. 2B)'). It is believed that claims 1-4, 5(EPO receptor), 6-7, 9, 13-15/(6-7, 9) encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass the first named erythropoietin (EPO) receptor, wherein the diabody fully activates signaling from the receptor, and wherein the diabody induces biased signaling.

*****Continued in the extra sheet*****

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 claims 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 claims Nos.: claims 1-4, 5(EPO receptor), 6-7, 9, 13-15/(6-7, 9), limited to erythropoietin (EPO) receptor, wherein the diabody fully activates signaling from the receptor and induces biased signaling

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 No III (unity of invention is lacking)

(Continuation of Groups I+) Additional receptor, the diabody induced signaling from the receptor, and/or additional diabody signaling will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected receptor, the diabody induced signaling from the receptor, and/or additional diabody signaling, which is/are supported by the disclosure. Applicants must further indicate, if applicable, the claims which only encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be the receptor encompasses thrombopoietin (TPO) receptor, wherein the diabody fully activates signaling from the receptor (Specification: para [0016] - a diabody that activates TPO receptor... AK119, AK113 and AK111 diabodies) [Claims 1-4, 5(TPO receptor), 6-7, 13-15/(6-7)]. [Please Note: I) claim 9 is not included in the exemplary election, because there is no clear disclosure supporting a diabody to TPO receptor induces biased signaling in the disclosure, even though inducing biased signaling by a ligand might be a common phenomenon to all receptors that are involved in different signal transduction pathways. If the applicants can point out clear disclosure in the Specification that supports a diabody to TPO receptor induces biased signaling, claim 9 will be included in the exemplary election if it is elected; II) claim 5 is directed to different cytokine receptors, and claims 7-8 and 10, each is directed to a diabody which induces a specific signaling from a receptor. Applicants are advised to select a receptor and a diabody that induces a specific signaling from the receptor, that is supported by the Specification, because different signaling induced by a diabody from a specific receptor is determined by the diabody structure, as evident by the disclosure; Specification: para [00114] - 'three diabodies ranged from full agonism for DA5, to partial agonism for DA330 and non-agonism for DA10'; para [00163] - Diabody Sequences - SEQ ID NO: 2, DA5 amino acid; SEQ ID NO: 4, DA10 amino acid; SEQ ID NO: 8, DA333 amino acid).

The inventions listed as Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Feature

Among Groups I+, each receptor is structurally and functionally different from all others; and each specific signaling induced by a diabody to a specific receptor is determined by the structure of the diabody (Please see discussion above, and Specification: para [00114], and para [00163] - Diabody Sequences, quotations as above), and further additional diabody induced signaling is also determined by diabody structure (Please see unsearchable claims 18-19 for diabody DA10 or DA307 that inhibits JAK/STAT signaling; Specification: para [00114] - 'non-agonism for DA10'; para [00122] - 'stimulation of these cells with DA10, DA307... decreased the STAT5, Akt and Erk phosphorylation'; para [00163] - Diabody Sequences - SEQ ID NO: 4, DA10 amino acid; SEQ ID NO: 6, DA307).

Common Technical Features

The inventions of Groups I+ share the technical feature of a method for modulating the signaling intensity of a receptor in response to binding a bivalent ligand, the method comprising: binding a bivalent ligand that enforces a selected receptor inter-subunit distance when bound to the receptor, wherein the inter-subunit distance correlates with the intensity of signaling (claim 1), and -- wherein the bivalent ligand is a diabody (claim 6)

However, these shared technical features do not represent a contribution over prior art as being anticipated by US 2008/0182976 A1 to Elliott, or being obvious over Elliott, in view of US 2006/0018902 A1 to REILLY et al. (hereinafter 'Reilly') as follows:

Elliott discloses a method for modulating the signaling intensity of a receptor (para [0037] - 'modulating... activity of an EPO receptor', which is 'modulating the signaling intensity of a receptor', because 'activity' determines 'the signaling intensity', Please see Fig 2; Fig 2; para [0005] - 'erythropoietin receptor (EPO-R)'; para [0009] - 'Activation of the EPO receptor .. may result in a proliferative signal') -- in response to binding a bivalent ligand (para [0009] - 'Activation of the EPO receptor may be due to its dimerization ...dimerization may result in a proliferative signal but not a differentiation signal', wherein 'Activation of the EPO receptor' is resulted from a bivalent ligand binding to 'the EPO receptor', that causes 'the EPO receptor ... dimerization', which is necessary for activating the receptor; para [0076] - 'both rHuEPO and Mab 71 stimulated thymidine uptake...the antibodies must be bivalent in order to activate the receptor'; para [0027] - 'recombinant human EPO (rHuEPO)'; Fig 2; para [0031] - 'antibodies or other bivalent molecular ... crosslinking two EPO receptors ... resulting in dimerization and activation of the EPO receptor') the method comprising: --binding a bivalent ligand that enforces a selected receptor inter-subunit distance when bound to the receptor, wherein the inter-subunit distance correlates with the intensity of signaling (para [0023] - 'FIG. 2 shows the effect of varying amounts of rHuEPO protein and purified Mabs 71 and 73 on .sup.3H thymidine uptake of UT7-EPO cells'; Fig 2, wherein the signal 'CPM' level is 'correlates with the intensity of signaling' of the EPO receptor activation, which is determined by the inter-subunit distance, which is further determined by the bivalent ligand binding, as seen in the Fig that Epo induced signaling is stronger than Mab 71; para [0010] - 'An anti-EPOR antibody can bind two EPOR molecules and could bring them into close proximity to each other to allow dimerization', wherein each of 'two EPOR molecules' is 'a selected receptor inter-subunit', and wherein 'anti-EPOR antibody can bind two EPOR molecules and could bring them into close proximity' is 'a bivalent ligand that enforces a selected receptor inter-subunit distance when bound to the receptor'; para [0076] - 'both rHuEPO and Mab 71 stimulated thymidine uptake. However the monovalent Fab 71 fragment did not.... This indicates that the antibodies must be bivalent in order to activate the receptor'; para [0027] - 'recombinant human EPO (rHuEPO)'; para [0031] - 'an antibody ... activates an erythropoietin receptor...FIG. 5, stimulation of 3H-thymidine uptake in UT7-EPO cells by Mab 71.... replacement of the intact, bivalent antibody with a corresponding monovalent fragment eliminates the proliferative response. ...support the dimerization model of activation for the EPO receptor....molecules that cross-link two EPO-R ... antibodies or other bivalent molecular entities that have the property of crosslinking two EPO receptors by binding to residues contained within the region between residues 49 and 78 thereby resulting in dimerization and activation of the EPO receptor'; Fig 5; Please also see an article entitled 'Receptor signaling: When dimerization is not enough' by JIANG et al.; Curr Biol. 1999, Vol. 9(15), p. R568-71 for dimerization associated with receptor inter-subunit distance: pg R569, Fig 1, shows different distance between activated dimer and partially activated dimer; Also see Specification for EPO receptor subunit; Specification: para [0037], under Receptor subunits; pg 12, EPO-R).

*****Continued in the next extra sheet*****

Continuation of:

The previous extra sheet - Box No III (unity of invention is lacking)

Elliott further discloses wherein the receptor is an erythropoietin receptor (para [0037] - 'modulating...activity of an EPO receptor'; para [0005] - 'erythropoietin receptor (EPO-R)'), and wherein the bivalent ligand is an antibody comprising two heavy-chains and two light-chains (para [0031] - 'an antibody ... activates an erythropoietin receptor... by Mab 71...cross-link two EPO-R ... antibodies or other bivalent molecular entities that have the property of crosslinking two EPO receptors ... resulting in dimerization and activation of the EPO receptor'; para [0075] - 'mobilities on nonreducing SDS gels for Mabs 71 and 73 ... indicates that these Mabs are bivalent, there are two each of the heavy and light chains'), as well as a method for screening bivalent antibodies (para [0051] - 'EIAs were initially performed to determine serum antibody (Ab) titres of individual animals, and later for screening of potential hybridomas'; para [0057] - 'Real-time biospecific interaction analysis ...was used to screen the ELISA positive monoclonal antibodies'; para [0031] - 'antibodies or other bivalent molecular ... resulting in dimerization and activation of the EPO receptor').

Elliott does not specifically teach wherein the bivalent ligand is a diabody. Reilly discloses a method for producing antibodies and antigen binding fragments thereof for binding to and activating erythropoietin receptor (Abstract - 'antibodies and antigen-binding portions thereof that bind to and activate an erythropoietin receptor'; para [0009] - 'provides antibodies, or an antigen-binding portion thereof that specifically bind to and activate human erythropoietin receptor (EpoR)'), wherein the antigen binding fragments comprising bivalent diabody, which is a common approach for generating a bivalent antibody (para [0043] - 'antigen-binding portion.... refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (e.g. human EpoR)... include...(iv) an Fv fragment consisting of the VL and VH domains of a single arm of an Ab, ...such as diabodies ... Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites').

It would have been obvious to one of ordinary skill in the art at the time the invention was made to combine the teachings of Elliott and Reilly, to obtain a method for modulating the signaling intensity of a receptor in response to binding a bivalent ligand, the method comprising: binding a bivalent ligand that enforces a selected receptor inter-subunit distance when bound to the receptor, wherein the inter-subunit distance correlates with the intensity of signaling, as taught by Elliott, and further wherein the bivalent ligand is a diabody, based on the combination of Reilly and Elliott, in order to include the fragments of the bivalent ligands available in the art in the method of generating a bivalent diabody for facilitating obtaining bivalent ligands for modulating the signaling intensity of a receptor in response to binding a bivalent ligand with a desired signaling intensity and without undue experimentation.

Without a shared special technical feature, the inventions lack unity with one another.

Groups I+ therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Continuation of item 4: Claims 16-26 are not drafted in accordance with the second and third sentences of Rule 6.4 (a). These claims are improper multiple dependent claims.

Note:

I) Claims 13-15 are objected to as partially lacking a proper antecedent basis for the "the diabody" limitation. For the purposes of this ISR, it is assumed that claims 13-15 depend from any one of Claims 6-12.

II) Claims 16-17 are objected to as partially lacking a proper antecedent basis for the "the diabody" limitation. For the purposes of this ISR, it is assumed that claims 16-17 depend from any one of Claims 6-15.