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(71) Applicant(s)  
**Acceleron Pharma, Inc.**

(72) Inventor(s)  
**Kumar, Ravindra; Suragani, Naga Venkata Sai Rajasekhar; Knopf, John**

(74) Agent / Attorney  
**Shelston IP Pty Ltd., L 9 60 Margaret St, Sydney, NSW, 2000, AU**

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(71) Applicant: **ACCELERON PHARMA, INC.** [US/US];  
128 Sidney Street, Cambridge, MA 02139 (US).

(72) Inventors: **KUMAR, Ravindra**; 421 Arlington Street, Acton, MA 01720 (US). **SURAGANI, Naga Venkata, Sai Rajasekhar**; 301 Buckminster Drive, Apt#05-201, Norwood, MA 02062 (US). **KNOPF, John**; 147 Robbins Drive, Carlisle, MA 01741 (US).

(74) Agents: **VARMA, Anita** et al.; Ropes & Gray LLP, Prudential Tower, 800 Boylston Street, Boston, MA 02199 (US).

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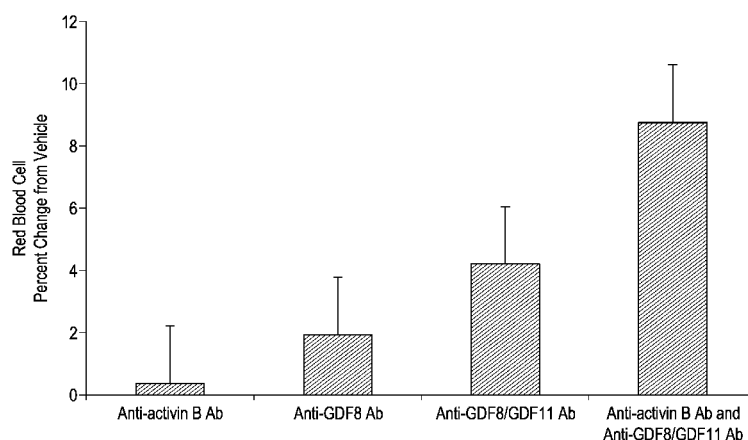


FIG. 30

(57) Abstract: In certain aspects, the present disclosure provides compositions and methods for increasing red blood cell and/or hemoglobin levels in a subject in need thereof comprising administering an effective amount of an agent/s that inhibits activin B and/or GDF11. Subjects in need include, for example, subject having an anemia and subjects have an ineffective erythropoiesis disorder.

# METHODS FOR INCREASING RED BLOOD CELL LEVELS AND TREATING INEFFECTIVE ERYTHROPOIESIS BY INHIBITING ACTIVIN B AND/OR GDF11

## CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application Ser. No. 61/969,073, filed March 21, 2014, and U.S. Provisional Application Ser. No. 62/021,923, filed July 8, 2014. All the teachings of the above-referenced applications are incorporated herein by reference.

## BACKGROUND OF THE INVENTION

[0002] The mature red blood cell, or erythrocyte, is responsible for oxygen transport in the circulatory systems of vertebrates. Red blood cells contain high concentrations of hemoglobin, a protein that binds to oxygen in the lungs at relatively high partial pressure of oxygen ( $pO_2$ ) and delivers oxygen to areas of the body with a relatively low  $pO_2$ .

[0003] Mature red blood cells are produced from pluripotent hematopoietic stem cells in a process termed erythropoiesis. Postnatal erythropoiesis occurs primarily in the bone marrow and in the red pulp of the spleen. The coordinated action of various signaling pathways controls the balance of cell proliferation, differentiation, survival, and death. Under normal conditions, red blood cells are produced at a rate that maintains a constant red cell mass in the body, and production may increase or decrease in response to various stimuli, including increased or decreased oxygen tension or tissue demand. The process of erythropoiesis begins with the formation of lineage committed precursor cells and proceeds through a series of distinct precursor cell types. The final stages of erythropoiesis occur as reticulocytes are released into the bloodstream and lose their mitochondria and ribosomes while assuming the morphology of mature red blood cell. An elevated level of reticulocytes, or an elevated reticulocyte:erythrocyte ratio, in the blood is indicative of increased red blood cell production rates.

[0004] Erythropoietin (EPO) is widely recognized as the most significant positive regulator of postnatal erythropoiesis in vertebrates. EPO regulates the compensatory erythropoietic response to reduced tissue oxygen tension (hypoxia) and low red blood cell levels or low hemoglobin levels. In humans, elevated EPO levels promote red blood cell formation by stimulating the generation of erythroid progenitors in the bone marrow and spleen. In the mouse, EPO enhances erythropoiesis primarily in the spleen.

[0005] Effects of EPO are mediated by a cell-surface receptor belonging to the cytokine receptor superfamily. The human EPO receptor gene encodes a 483 amino acid transmembrane protein; however, the active EPO receptor is thought to exist as a multimeric complex even in the absence of ligand [see, *e.g.*, U.S. Pat. No. 6,319,499]. The cloned full-length EPO receptor expressed in mammalian cells binds EPO with an affinity similar to that of the native receptor on erythroid progenitor cells. Binding of EPO to its receptor causes a conformational change resulting in receptor activation and biological effects including increased proliferation of immature erythroblasts, increased differentiation of immature erythroblasts, and decreased apoptosis in erythroid progenitor cells [see, *e.g.*, Liboi *et al.* (1993) Proc Natl Acad Sci USA 90:11351-11355 and Koury *et al.* (1990) Science 248:378-381].

[0006] Various forms of recombinant EPO are used by physicians to increase red blood cell levels in a variety of clinical settings, particularly in the treatment of anemia. Anemia is a broadly-defined condition characterized by lower than normal levels of hemoglobin or red blood cells in the blood. In some instances, anemia is caused by a primary disorder in the production or survival of red blood cells (*e.g.*, a thalassemia disorder or sickle cell anemia). More commonly, anemia is secondary to diseases of other systems [see, *e.g.*, Weatherall & Provan (2000) Lancet 355, 1169-1175]. Anemia may result from a reduced rate of production or increased rate of destruction of red blood cells or by loss of red blood cells due to bleeding. Anemia may result from a variety of disorders that include, for example, acute or chronic renal failure or end stage renal disease, chemotherapy treatment, a myelodysplastic syndrome, rheumatoid arthritis, and bone marrow transplantation.

[0007] Treatment with EPO typically causes a rise in hemoglobin by about 1-3 g/dL in healthy humans over a period of weeks. When administered to anemic individuals, this treatment regimen often provides substantial increases in hemoglobin and red blood cell levels and leads to improvements in quality of life and prolonged survival. However, EPO is not uniformly effective, and many individuals are refractory to even high doses [see, *e.g.*, Horl *et al.* (2000) Nephrol Dial Transplant 15, 43-50]. For example, over 50% of patients with cancer have an inadequate response to EPO, approximately 10% with end-stage renal disease are hyporesponsive to EPO [see, *e.g.*, Glaspy *et al.* (1997) J Clin Oncol 15, 1218-1234 and Demetri *et al.* (1998) J Clin Oncol 16, 3412-3425], and less than 10% with myelodysplastic syndrome respond favorably to EPO [see Estey (2003) Curr Opin Hematol 10, 60-670]. Several factors, including inflammation, iron and vitamin deficiency,

inadequate dialysis, aluminum toxicity, and hyperparathyroidism may predict a poor therapeutic response. The molecular mechanisms of resistance to EPO are as yet unclear. Recent evidence suggests that higher doses of EPO may be associated with an increased risk of cardiovascular morbidity, tumor growth, and mortality in some patient populations [see, *e.g.*, Krapf *et al.* (2009) Clin J Am Soc Nephrol 4:470-480 and Glaspy (2009) Annu Rev Med 60:181-192]. It has been therefore recommended that EPO-based therapeutic compounds (*e.g.*, erythropoietin-stimulating agents, ESAs) be administered at the lowest dose required to avoid red blood cell transfusions [see, *e.g.*, Jelkmann *et al.* (2008) Crit Rev Oncol. Hematol 67:39-61].

[0008] Ineffective erythropoiesis is a term used to describe a group of erythroid disorders in which erythrocyte production is decreased despite increased numbers of earlier-stage erythroid cells [see, *e.g.*, Tanno (2010) Adv Hematol 2010:358283]. Ineffective erythropoiesis often gives rise to anemia, elevated erythropoietin levels, formation of excessive numbers of red blood cell precursors, and iron overload. If they persist, these conditions can lead to splenomegaly, liver and heart disorders, and bone damage as well as other complications. As endogenous erythropoietin levels are commonly very high in patients with ineffective erythropoiesis, EPO-based therapeutics often will not treat the anemia in these patients and/or may cause an aggravation of other aspects of the disease, such as splenomegaly and iron overload.

[0009] Thus, in one aspect, the present disclosure provides alternative methods for increasing red blood cell levels and/or addressing other disorders in the context of ineffective erythropoiesis.

[0009a] It is an object of the present invention to overcome or ameliorate at least one of the disadvantages of the prior art, or to provide a useful alternative.

[0009b] Any discussion of the prior art throughout the specification should in no way be considered as an admission that such prior art is widely known or forms part of the common general knowledge in the field.

## SUMMARY OF THE INVENTION

[0009c] Unless the context clearly requires otherwise, throughout the description and the claims, the words “comprise”, “comprising”, and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of “including, but not limited to”.

[0009d] According to a first aspect, the present invention provides a method for increasing red blood cell levels or treating or preventing an anemia in a subject comprising administering to a subject in need thereof an effective amount of a combination of agents that inhibit activin B and GDF11; wherein the combination comprises an antibody or antigen binding fragment thereof that binds to GDF11, and an antibody or antigen binding fragment thereof that binds to activin B, wherein the combination of agents does not substantially bind to activin A.

[0009e] According to a second aspect, the present invention provides the use of a combination of agents that inhibit activin B and GDF11A for the manufacture of a medicament for increasing red blood cell levels or treating or preventing an anemia in a subject; wherein the combination comprises an antibody or antigen binding fragment thereof that binds to GDF11, and an antibody or antigen binding fragment thereof that binds to activin B, wherein the combination of agents does not substantially bind to activin A.

[0010] In certain aspects, the disclosure provides methods for increasing red blood cell levels, treating or preventing an anemia, and/or treating or preventing ineffective erythropoiesis in a subject comprising administering to a subject in need thereof an effective amount of an agent, or combination of agents, that antagonizes (inhibits) at least activin B and/or GDF11. In some embodiments, the agent, or combination of agents, that inhibits activin B further inhibits one or more additional ligands that bind to ActRIIB and signal through Smad 2/3. In some embodiments, the agent, or combination of agents, that inhibits GDF11 further inhibits one or more additional ligands that bind to ActRIIB and signal

through Smad 2/3. For example, the agent, or combination of agents, that inhibits activin B and/or GDF11 may further inhibit GDF8. Optionally, the agent, or combination of agents, that inhibits activin B and/or GDF11 does not inhibit activin A. In certain embodiments, the agent, or combination of agents, that inhibits activin B and/or GDF11 further inhibits one or more of GDF8, activin A, activin E, activin C, and BMP6. In certain embodiments, the agent, or combination of agents, may further inhibit one or more of GDF15, Nodal, GDF3, BMP3, and BMP3B. In certain embodiments, the agent, or combination of agents, that inhibits activin B and/or GDF11 further inhibits BMP9 from interacting with a type II receptor of the TGF $\beta$  superfamily (*e.g.*, ActRIIA and/or ActRIIB) and/or BMP10 from interacting with a type II receptor of the TGF $\beta$  superfamily (*e.g.*, ActRIIA and/or ActRIIB). Preferably, the agent, or combination of agents, to be used in accordance with the methods of the present disclosure do not inhibit, or substantially inhibit, interaction (*e.g.*, binding, activation of Smad 2/3 signaling, *etc.*) between BMP9 and ALK1 and/or BMP10 and ALK1. Inhibition may be assessed by a variety of biochemical assays known in the art as well as those provided herein (*e.g.*, protein-based assays, cell-based assays, *etc.*).

[0011] In some embodiments, the agent, or combination of agents, inhibits at least activin B and/or GDF11 signaling (Smad 2/3 signaling) in a cell-based assay. In some embodiments, the agent, or combination of agents, binds to activin B and/or GDF11. In some embodiments, the agent, or combination of agents, does not substantially inhibit activin A signaling in a cell-based assay. In some embodiments, the agent, or combination of agents, does not substantially bind to activin A. In some embodiments, the agent, or combination of agents, that inhibits GDF11 and/or activin B signaling in a cell-based assay further inhibits one or more of GDF8, BMP6, activin C, activin A, activin E, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10 signaling in a cell-based assay. In some embodiments, the agent, or combination of agents, that binds to GDF11 and/or activin B further binds to one or more of GDF8, BMP6, activin C, activin A, activin E, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10.

[0012] In some embodiments, the agent is a multispecific antibody, or combination of multispecific antibodies, that binds to and/or inhibits at least GDF11 and activin B. In some embodiments, the multispecific antibody, or combination of multispecific antibodies, does not substantially bind to and/or inhibit activin A. In some embodiments, the multispecific antibody, or combination of multispecific antibodies, further binds to and/or inhibits GDF8. In some embodiments, the multispecific antibody, or combination of multispecific antibodies,

that binds to and/or inhibits GDF11 and/or activin B further binds to and/or inhibits one or more of activin C, activin E, activin A, GDF8, ActRIIA, ActRIIB, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10.

[0013] In some embodiments, the multispecific antibody is a bispecific antibody, or combination of bispecific antibodies. In some embodiments, the bispecific antibody, or combination of bispecific antibodies, binds to and/or inhibits at least GDF11 and activin B. In some embodiments, the bispecific antibody, or combination of bispecific antibodies, does not substantially bind to and/or inhibit activin A.

[0014] In some embodiments, bispecific antibody comprises two different monospecific antibodies that are associated with one another. In some embodiments, the antibody is a chimeric antibody. In some embodiments, the antibody is a humanized antibody. In some embodiments, the antibody is a human antibody.

[0015] In some embodiments, the antibody is a single-chain antibody. In some embodiments, the antibody is an F(ab')<sub>2</sub> fragment. In some embodiments, the antibody is a single-chain diabody, a tandem single-chain Fv fragment, a tandem single-chain diabody, a or a fusion protein comprising a single-chain diabody and at least a portion of an immunoglobulin heavy chain constant region.

[0016] In some embodiments, the antibody is a dual variable-domain immunoglobulin.

[0017] In some embodiments, the antibody comprises a heterologous moiety. In some embodiments, the heterologous moiety is a sugar, a detectable label, or a stabilization moiety.

[0018] In some embodiments, the agent is a GDF11/activin B trap comprising an amino acid sequence that is at least 80% (*e.g.* at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100%) identical to amino acids 29-109 of SEQ ID NO:1. In some embodiments, the GDF11/activin B trap does not substantially bind to and/or inhibit activin A. In some embodiments, the GDF11/activin A trap does not comprise an acidic amino acid at the position corresponding to position 79 of SEQ ID NO:1. In some embodiments, the agent is a GDF11/activin B trap comprising an amino acid sequence that is at least 80% (*e.g.* at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100%) identical to amino acids 29-109 of SEQ ID NO:1, and wherein the GDF11/activin B trap binds to activin B with a K<sub>D</sub> of less than 100 pM, less than 10pM, or less than 1pM. In some embodiments, the GDF11/activin B trap binds to activin B with a K<sub>D</sub> of 1 nM-750 pM, 750 pM-500 pM, 500 pM-250 pM, 250pM-100 pM, 100 pM – 50 pM, 50-25 pM, 25-10 pM, or 10-1 pM. In some embodiments, the

agent is a GDF11/activin B trap comprising an amino acid sequence that is at least 80% identical (*e.g.* at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) to amino acids 29-109 of SEQ ID NO:1, and wherein the GDF11/activin B trap has a binding affinity for GDF11 that is 3-fold higher, 4-fold higher, 5-fold higher, 6-fold higher, 7-fold higher, 8-fold higher, 9-fold higher, 10-fold higher, 15-fold higher, or 20-fold higher than the binding affinity of a wild-type ligand binding domain of a ActRIIB receptor. In some embodiments, the agent is a GDF11/activin B trap comprising an amino acid sequence that is at least 80% (*e.g.* at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to amino acids 29-109 of SEQ ID NO:1, and wherein the GDF11/activin B trap has a binding affinity for activin B that is 3-fold less, 4-fold higher, 5-fold higher, 6-fold higher, 7-fold higher, 8-fold higher, 9-fold higher, 10-fold higher, 15-fold higher, or 20-fold higher than the binding affinity of a wild-type ligand binding domain of a ActRIIB receptor.

[0019] In some embodiments, the GDF11/activin B trap comprises an amino acid sequence that is at least 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 29-109 of SEQ ID NO:1. In some embodiments, the GDF11/activin B trap comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 25-131 of SEQ ID NO:1. In some embodiments, the GDF11/activin B trap comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence of SEQ ID NO: 3 or 4. In some embodiments, the GDF11/activin B trap comprises a polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100% identical to the amino acid sequence of SEQ ID NO: 5 or 6.

[0020] In some embodiments, the GDF11/activin B trap comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence of SEQ ID NO:23.

[0021] In some embodiments, the GDF11/activin B trap comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence of SEQ ID NO:48.

[0022] In some embodiments, the GDF11/activin B trap comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence of SEQ ID NO:49.

[0023] In some embodiments, the agent is a GDF11/activin B trap comprising an amino acid sequence that is at least 80% identical to amino acids 30-110 of SEQ ID NO:9. In some embodiments, the agent is a GDF11/activin B trap comprising an amino acid sequence that is at least 80% identical to amino acids 30-110 of SEQ ID NO:9, and wherein the

5 GDF11/activin trap has a binding affinity for GDF11 that is 3-fold higher, 4-fold higher, 5-fold higher, 6-fold higher, 7-fold higher, 8-fold higher, 9-fold higher, 10-fold higher, 15-fold higher, or 20-fold higher than the binding affinity of a wild-type ligand-binding domain of a ActRIIA receptor. In some embodiments, the agent is a GDF11/activin B trap comprising an amino acid sequence that is at least 80% identical to amino acids 30-110 of SEQ ID NO:9,  
10 and wherein the GDF11/activin B trap has a binding affinity for activin B that is 3-fold higher, 4-fold higher, 5-fold higher, 6-fold higher, 7-fold higher, 8-fold higher, 9-fold higher, 10-fold higher, 15-fold higher, or 20-fold higher than the binding affinity of a wild-type ligand-binding domain of a ActRIIA receptor. In some embodiments, the GDF11/activin B trap comprises an amino acid sequence that is at least 85%, 90%, 95%, 96%, 97%, 98%, 99%, or  
15 100% identical to amino acids 30-110 of SEQ ID NO:9.

[0024] In some embodiments, the GDF11/activin B trap comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence of SEQ ID NO:10.

[0025] In some embodiments, the GDF11/activin B trap comprises a polypeptide  
20 comprising an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence of SEQ ID NO:11.

[0026] In some embodiments, the GDF11/activin B trap comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence of SEQ ID NO:20.

25 [0027] In some embodiments, the agent may be any one of the GDF11, activin B and GDF11/activin B traps disclosed herein, or a combination thereof.

[0028] In some embodiments, the GDF11/activin B trap is a fusion protein comprising, in addition to a GDF11/activin B trap polypeptide domain, one or more heterologous polypeptide domains that enhance one or more of: *in vivo* half-life, *in vitro* half-life,  
30 uptake/administration, tissue localization or distribution, formation of protein complexes, multimerization of the fusion protein and/or purification. In some embodiments, the GDF11/activin B trap fusion protein comprises a heterologous polypeptide domain selected

from: an immunoglobulin Fc domain and a serum albumin. In some embodiments, the immunoglobulin Fc domain is an IgG1 Fc domain. In some embodiments, the immunoglobulin Fc domain comprises an amino acid sequence selected from SEQ ID NO: 16 or 17. In some embodiments, fusion protein further comprises a linker domain positioned  
5 between the Trap polypeptide domain and the immunoglobulin Fc domain. In some embodiments, the linker domain is a TGGG linker (SEQ ID NO: 45). In some embodiments, the linker domain may be any of the linker domains disclosed herein. In some embodiments, a fusion protein may include a purification subsequence, such as an epitope tag, a FLAG tag, a polyhistidine sequence, and a GST fusion. In certain embodiments, a GDF11/activin B trap  
10 fusion comprises a leader sequence. The leader sequence may be a native leader sequence or a heterologous leader sequence. In certain embodiments, the leader sequence is a tissue plasminogen activator (TPA) leader sequence. In an embodiment, a GDF11/activin B trap fusion protein comprises an amino acid sequence as set forth in the formula A-B-C. The B portion is a GDF11/activin B trap of the disclosure. The A and C portions may be  
15 independently zero, one or more than one amino acids, and both A and C portions are heterologous to B. The A and/or C portions may be attached to the B portion via a linker sequence. In some embodiments, the GDF11/activin B trap fusion protein may comprise any of the fusion proteins disclosed herein.

[0029] In some embodiments, the GDF11/activin B trap comprises one or more amino  
20 acid modifications selected from: a glycosylated amino acid, a PEGylated amino acid, a farnesylated amino acid, an acetylated amino acid, a biotinylated amino acid, an amino acid conjugated to a lipid moiety, an amino acid conjugated to an organic derivatizing agent. In some embodiments, the GDF11/activin B trap is glycosylated and has a mammalian glycosylation pattern. In some embodiments, the GDF11/activin B trap has a glycosylation  
25 pattern obtainable from a Chinese hamster ovary cell line. In general, it is preferable that a GDF trap be expressed in a mammalian cell line that mediates suitably natural glycosylation of the GDF trap so as to diminish the likelihood of an unfavorable immune response in a patient. Human and CHO cell lines have been used successfully, and it is expected that other common mammalian expression vectors will be useful. In some embodiments, the  
30 GDF11/activin B trap may comprise any of the amino acid modifications disclosed herein, or a combination thereof.

[0030] In certain aspects, the disclosure provides nucleic acids encoding a GDF11/activin B trap polypeptide. An isolated polynucleotide may comprise a coding sequence for a

soluble GDF trap polypeptide, such as described above. Nucleic acids disclosed herein may be operably linked to a promoter for expression, and the disclosure provides cells transformed with such recombinant polynucleotides. Preferably the cell is a mammalian cell such as a CHO cell. In some embodiments, the host cell may be selected from any of the cells

disclosed herein for such purpose.

[0031] In certain aspects, the disclosure provides methods for making a GDF11/activin B trap polypeptide. Such a method may include expressing any of the nucleic acids disclosed herein in a suitable cell, such as a Chinese hamster ovary (CHO) cell. Such a method may comprise: a) culturing a cell under conditions suitable for expression of the GDF11/activin B trap polypeptide, wherein said cell is transformed with a GDF11/activin B trap expression construct; and b) recovering the GDF11/activin B trap polypeptide so expressed.

GDF11/activin B trap polypeptides may be recovered as crude, partially purified or highly purified fractions using any of the well-known techniques for obtaining protein from cell cultures.

[0032] In certain aspects, the disclosure provides a method for increasing red blood cell levels or treating or preventing an anemia in a subject comprising administering to a subject in need thereof an effective amount of a combination of agents that inhibit signaling of activin B and GDF11. In some embodiments, the combination of agents inhibits activin B and GDF11 signaling in a cell based assay. In some embodiments, the combination of agents does not substantially inhibit activin A signaling. In some embodiments, the combination of agents does not substantially inhibit activin A signaling in a cell based assay. In some embodiments, the combination of agents does not substantially bind to activin A. In some embodiments, one or more of the agents that inhibit GDF11 and/or activin B further inhibits the signaling of one or more of GDF8, BMP6, activin A, activin C, activin E, GDF15, Nodal, GDF3, BMP3B, BMP9, and BMP10.

[0033] In some embodiments, the combination of agents comprises at least one agent that is an antibody or antigen-binding fragment thereof that binds to GDF11. In some embodiments, the combination of agents comprises at least one agent that is an antibody or antigen-binding fragment thereof that binds to activin B. In some embodiments, the combination of agents comprises a combination of two or more antibodies or antigen-binding fragments thereof directed against two or more targets disclosed herein (*e.g.*, activin A, activin B, activin C, activin E, GDF11, GDF8, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10).

[0034] In some embodiments, the antibody or antigen-binding fragment thereof is a chimeric antibody or fragment thereof. In some embodiments, the antibody or antigen-binding fragment thereof is a humanized antibody or fragment thereof. In some embodiments, the antibody or antigen-binding fragment thereof is a human antibody or fragment thereof. In some embodiments, the antibody or antigen-binding fragment thereof is a single-chain antibody. In some embodiments, the antigen-binding fragment is selected from the group consisting of: Fab, Fab', F(ab')<sub>2</sub>, F(ab')<sub>3</sub>, Fd, Fv, domain antibody. In some embodiments, the antibody or antigen-binding fragment thereof comprises a heterologous moiety. In some embodiments, the heterologous moiety is a sugar, a detectable label, or a stabilization moiety.

[0035] In some embodiments, the combination of agents comprises at least one agent that is a GDF11 trap, and wherein the GDF11 trap comprises a polypeptide comprising an amino acid sequence that is at least 80% (*e.g.* at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to amino acids 29-109 of SEQ ID NO:1. In some embodiments, the combination of agents comprises at least one agent that is an activin B trap, and wherein the activin B trap comprises a polypeptide comprising an amino acid sequence that is at least 80% (*e.g.* at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to amino acids 29-109 of SEQ ID NO:1.

[0036] In some embodiments, the combination of agents comprises at least one GDF11 trap or activin B trap comprising an amino acid sequence that is at least 80% (*e.g.* at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to amino acids 29-109 of SEQ ID NO:1, and wherein the GDF11 trap or activin B trap does not comprise an acidic amino acid at the position corresponding to position 79 of SEQ ID NO:1. In some embodiments, the agent is a GDF11 trap comprising an amino acid sequence that is at least 80% (*e.g.* at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to amino acids 29-109 of SEQ ID NO:1, and wherein the GDF11 trap binds to activin B with a  $K_D$  of less than 100 pM, less than 10pM, or less than 1pM. In some embodiments, the GDF11 trap binds to activin B with a  $K_D$  of 1 nM-750 pM, 750 pM-500 pM, 500 pM-250 pM, 250pM-100 pM, 100 pM – 50 pM, 50-25 pM, 25-10 pM, or 10-1 pM. In some embodiments, the agent is a GDF11 trap comprising an amino acid sequence that is at least 80% identical (*e.g.* at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) to amino acids 29-109 of SEQ ID NO:1, and wherein the GDF11 trap has a binding affinity for GDF11 that is 3-fold higher, 4-fold higher, 5-fold higher, 6-fold higher, 7-fold higher, 8-fold higher, 9-fold higher, 10-fold higher, 15-fold higher, or 20-fold higher than the binding affinity of a wild-type

ligand binding domain of a ActRIIB receptor. In some embodiments, the agent is a activin B trap comprising an amino acid sequence that is at least 80% (*e.g.* at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to amino acids 29-109 of SEQ ID NO:1, and wherein the GDF11 trap has a binding affinity for activin B that is 3-fold higher, 4-fold higher, 5-fold higher, 6-fold higher, 7-fold higher, 8-fold higher, 9-fold higher, 10-fold higher, 15-fold higher, or 20-fold higher than the binding affinity of a wild-type ligand binding domain of a ActRIIB receptor.

[0037] In some embodiments, the GDF11 trap or activin B trap comprises an amino acid sequence that is at least 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 29-109 of SEQ ID NO:1. In some embodiments, the GDF11 trap or activin B trap comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 25-131 of SEQ ID NO:1. In some embodiments, the GDF11 trap or activin B trap comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence of SEQ ID NO: 3 or 4. In some embodiments, the GDF11 trap or activin B trap comprises a polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence of SEQ ID NO: 5 or 6.

[0038] In some embodiments, the GDF11 trap or activin B trap comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence of SEQ ID NO:23.

[0039] In some embodiments, the combination of agents comprises at least one agent that is a GDF11 trap, and wherein the GDF11 trap comprises a polypeptide comprising an amino acid sequence that is at least 80% (*e.g.* at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to amino acids 30-110 of SEQ ID NO:9. In some embodiments, the combination of agents comprises at least one agent that is an activin B trap, and wherein the activin B trap comprises a polypeptide comprising an amino acid sequence that is at least 80% (*e.g.* at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to amino acids 30-110 of SEQ ID NO:9. In some embodiments, the agent is a GDF trap comprising an amino acid sequence that is at least 80% identical to amino acids 30-110 of SEQ ID NO:9, and wherein the GDF11 trap has a binding affinity for GDF11 that is 3-fold higher, 4-fold higher, 5-fold higher, 6-fold higher, 7-fold higher, 8-fold higher, 9-fold higher, 10-fold higher, 15-fold higher, or 20-fold higher than the binding affinity of a wild-type ligand-binding domain of a ActRIIA receptor. In some embodiments, the agent is an activin B trap

comprising an amino acid sequence that is at least 80% identical to amino acids 30-110 of SEQ ID NO:9, and wherein the activin B trap has a binding affinity for activin B that is 3-fold higher, 4-fold higher, 5-fold higher, 6-fold higher, 7-fold higher, 8-fold higher, 9-fold higher, 10-fold higher, 15-fold higher, or 20-fold higher than the binding affinity of a wild-type ligand-binding domain of a ActRIIA receptor. In some embodiments, the GDF11 trap or  
5    activin B trap comprises an amino acid sequence that is at least 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 30-110 of SEQ ID NO:9.

[0040]     In some embodiments, the GDF11 trap or activin B trap comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to  
10   the amino acid sequence of SEQ ID NO:10.

[0041]     In some embodiments, the GDF11 trap or activin B trap comprises a polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence of SEQ ID NO:11.

[0042]     In some embodiments, the GDF11 trap or activin B trap comprises an amino acid  
15   sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence of SEQ ID NO:20.

[0043]     In some embodiments, the GDF11 trap or activin B trap is a fusion protein comprising, in addition to a GDF trap or activin B trap polypeptide domain, one or more heterologous polypeptide domains that enhance one or more of: *in vivo* half-life, *in vitro* half-  
20   life, uptake/administration, tissue localization or distribution, formation of protein complexes, multimerization of the fusion protein and/or purification. In some embodiments, the GDF11 trap or activin B trap fusion protein comprises a heterologous polypeptide domain selected from: an immunoglobulin Fc domain and a serum albumin. In some embodiments, the immunoglobulin Fc domain is an IgG1 Fc domain. In some embodiments, the  
25   immunoglobulin Fc domain comprises an amino acid sequence selected from SEQ ID NO: 16 or 17. In some embodiments, fusion protein further comprises a linker domain positioned between the trap polypeptide domain and the immunoglobulin Fc domain. In some embodiments, the linker domain is a TGGG linker. In some embodiments, the linker domain may be any of the linker domains disclosed herein. In some embodiments, a fusion protein  
30   may include a purification subsequence, such as an epitope tag, a FLAG tag, a polyhistidine sequence, and a GST fusion. In certain embodiments, a GDF11 trap or activin B trap fusion comprises a leader sequence. The leader sequence may be a native leader sequence or a

heterologous leader sequence. In certain embodiments, the leader sequence is a tissue plasminogen activator (TPA) leader sequence. In an embodiment, a GDF11 trap or activin B trap fusion protein comprises an amino acid sequence as set forth in the formula A-B-C. The B portion is a GDF11 trap or activin B trap of the disclosure. The A and C portions may be independently zero, one or more than one amino acids, and both A and C portions are heterologous to B. The A and/or C portions may be attached to the B portion via a linker sequence. In some embodiments, the GDF11 trap or activin B trap fusion protein may comprise any of the fusion proteins disclosed herein.

[0044] In some embodiments, the GDF11 trap or activin B trap comprises one or more amino acid modifications selected from: a glycosylated amino acid, a PEGylated amino acid, a farnesylated amino acid, an acetylated amino acid, a biotinylated amino acid, an amino acid conjugated to a lipid moiety, an amino acid conjugated to an organic derivatizing agent. In some embodiments, the GDF11 trap or activin B trap is glycosylated and has a mammalian glycosylation pattern. In some embodiments, the GDF11 trap or activin B trap has a glycosylation pattern obtainable from a Chinese hamster ovary cell line. In general, it is preferable that a GDF11 trap be expressed in a mammalian cell line that mediates suitably natural glycosylation of the GDF11 trap so as to diminish the likelihood of an unfavorable immune response in a patient. Human and CHO cell lines have been used successfully, and it is expected that other common mammalian expression vectors will be useful. In some embodiments, the GDF11 trap or activin B trap may comprise any of the amino acid modifications disclosed herein, or a combination thereof.

[0045] In certain aspects, the disclosure provides nucleic acids encoding a GDF11 trap or activin B trap polypeptide. An isolated polynucleotide may comprise a coding sequence for a soluble GDF11 trap polypeptide or activin B trap polypeptide, such as described above.

Nucleic acids disclosed herein may be operably linked to a promoter for expression, and the disclosure provides cells transformed with such recombinant polynucleotides. Preferably the cell is a mammalian cell such as a CHO cell. In some embodiments, the host cell may be selected from any of the cells disclosed herein for such purpose.

[0046] In certain aspects, the disclosure provides methods for making a GDF11 trap or activin B trap polypeptide. Such a method may include expressing any of the nucleic acids disclosed herein in a suitable cell, such as a Chinese hamster ovary (CHO) cell. Such a method may comprise: a) culturing a cell under conditions suitable for expression of the GDF11 trap or activin B trap polypeptide, wherein said cell is transformed with a GDF11 trap

or activin B trap expression construct; and b) recovering the GDF11/activin B trap polypeptide so expressed. GDF11/activin B trap polypeptides may be recovered as crude, partially purified or highly purified fractions using any of the well-known techniques for obtaining protein from cell cultures.

5 [0047] In some embodiments, two or more of any of the GDF11, activin B and GDF11/activin B traps disclosed herein may be combined.

[0048] In some embodiments, the GDF11 and/or activin B antagonist is a small-molecule antagonist, or combination of small-molecule antagonists. In some embodiments, the combination of agents comprises at least one agent that is a small-molecule antagonist of  
 10 activin B. In some embodiments, the combination of agents comprises at least one agent that is a small-molecule antagonist of GDF11. In some embodiments, the GDF11 and/or activin B small-molecule antagonist, or combination of small-molecule antagonists, does not bind to and/or inhibit activin A. In some embodiments, the GDF11 and/or activin B small-molecule antagonist, or combination of small-molecule antagonists, further bind to and/on inhibit  
 15 GDF8. In some embodiments, the GDF11 and/or activin B small-molecule antagonist, or combination of small-molecule antagonists, further bind to and/or inhibit one or more of activin A, activin C, activin E, GDF8, BMP6, GDF15, Nodal, GDF3, BMP3 and BMP10.

[0049] In another aspect, an antagonist agent, or combination of agents, of the present disclosure is a polynucleotide antagonist that inhibits at least GDF11 and/or activin B. In  
 20 some embodiments, an antagonist polynucleotide of the disclosure inhibits the expression (*e.g.*, transcription, translation, and/or cellular secretion) of at least GDF11 and/or activin B. Optionally, a polynucleotide antagonist, or combinations of polynucleotide antagonists, of the disclosure does not inhibit activin A (*e.g.* inhibits expression and/or activity of activin A). Optionally, a polynucleotide antagonist, or combinations of polynucleotide antagonists, of the  
 25 disclosure further inhibit GDF8 (*e.g.* inhibits expression and/or activity of GDF8). In some embodiments, a polynucleotide antagonist, or combinations of polynucleotide antagonists, of the disclosure that inhibits GDF11 and/or activin B (*e.g.* expression and/or activity of GDF11 and/or activin B) further inhibits (*e.g.*, inhibits expression and/or activity) one or more of activin E, activin C, activin A, GDF8, BMP6, GDF15, Nodal, GDF3, BMP3, and BMP3B.

30 [0050] In some embodiments, the polynucleotide molecule is an antisense oligonucleotide that hybridizes to a transcript of a gene selected from: activin B, activin C, activin E, GDF11, and GDF8, activin A, GDF15, GDF3, Nodal, BMP3, and BMP3B to

inhibit expression of the gene. In some embodiments, the combination of agents comprises an antisense oligonucleotide that hybridizes to a transcript of activin B and inhibits activin B expression. In some embodiments, the combination of agents comprises an antisense oligonucleotide that hybridizes to a transcript of GDF11 and inhibits GDF11 expression. In some embodiments, the combination of agents comprises a combination of two or more antisense oligonucleotides that inhibit the expression of two or more targets of the disclosure (e.g., activin A, activin B, activin C, activin E, GDF11, GDF8, BMP6, GDF15, Nodal, GDF3, BMP3, and BMP3B).

[0051] In some embodiments, the polynucleotide molecule comprises an RNAi molecule that targets the transcript of a gene selected from: activin A, activin B, activin C, activin E, BMP6, GDF11, GDF8, GDF15, Nodal, GDF3, BMP3, and BMP3B. In some embodiments, the polynucleotide molecule comprises an RNAi molecule that targets the transcript of GDF11. In some embodiments, the polynucleotide molecule comprises an RNAi molecule that targets the transcript of activin B. In some embodiments, the combination of agents comprises a combination of two or more RNAi molecules that inhibit the expression of two or more targets of the disclosure (e.g., activin A, activin B, activin C, activin E, GDF11, GDF8, BMP6, GDF15, Nodal, GDF3, BMP3, and BMP3B).

[0052] In some embodiments, the RNAi molecule comprises an siRNA. In some embodiments, the siRNA is from about 19 to about 45 nucleotides in length. In some embodiments, the siRNA is from about 25 to about 30 nucleotides in length. In some embodiments, the siRNA is from about 10 to about 20 nucleotides in length. In some embodiments, the RNAi molecule comprises an shRNA. In some embodiments, the shRNA molecule has a stem length of 19-29 nucleotides. In some embodiments, the shRNA molecule has a stem length of 19-23 nucleotides. In some embodiments, the loop region of the shRNA has a length of 5-9 nucleotides.

[0053] In some embodiments, any of the disclosed GDF11 antagonists herein (e.g., GDF11 trap polypeptide, anti-GDF11 antibody, small-molecule antagonist, polypeptide or polynucleotide antagonist) can be combined with an activin B antagonist of the disclosure (e.g., activin B trap polypeptide, anti-activin B antibody, small-molecule antagonist, polypeptide or polynucleotide antagonist) to inhibit both a GDF11 and an activin B activity (e.g., the ability to bind to and/or activate an ActRIIA and/or ActRIIB receptor).

[0054] In some embodiments, methods of the disclosure are for increasing red blood cells in a subject in need thereof. In some embodiments, the method is for treating or preventing an anemia in a subject in need thereof. In some embodiments, the anemia is associated with one or more of: multiple myeloma, chronic or acute renal disease or failure,

5 chemotherapeutic treatment of the subject, a myelodysplastic syndrome, and a thalassemia. In some embodiments, the thalassemia is beta-thalassemia. In some embodiments, the renal failure is end-stage renal failure. In some embodiments, the subject has sickle cell anemia.

#### BRIEF DESCRIPTION OF THE DRAWINGS

10 [0055] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0056] Figure 1 shows an alignment of the extracellular domains of human ActRIIA (SEQ ID NO: 36) and human ActRIIB (SEQ ID NO: 46) with the residues that are deduced  
15 herein, based on composite analysis of multiple ActRIIB and ActRIIA crystal structures to directly contact ligand (the ligand binding pocket) indicated with boxes.

[0057] Figure 2 shows a multiple sequence alignment of various vertebrate ActRIIB proteins and human ActRIIA (SEQ ID NOs: 37-44).

[0058] Figures 3A and 3B depict the human GDF11 cDNA sequence (NCBI Reference  
20 Sequence No. NM\_005811.3) (SEQ ID NO: 24).

[0059] Figure 4 depicts the amino acid sequence of the human GDF11 precursor protein sequence (NCBI Reference Sequence No. NP\_005802.1) (SEQ ID NO: 25).

[0060] Figures 5A and 5B depict the human activin B cDNA sequence (NCBI Reference Sequence No. NM\_002193.2) (SEQ ID NO: 26).

25 [0061] Figure 6 depicts the amino acid sequence of the human activin B precursor protein (NCBI Reference Sequence No. NP\_002184.2) (SEQ ID NO: 27).

[0062] Figure 7 depicts the human activin E cDNA sequence (GenBank Accession No. NM\_031479.3) (SEQ ID NO: 28).

[0063] Figure 8 depicts the amino acid sequence of the human activin E precursor protein  
30 (GenBank Accession No. NP\_113667.1) (SEQ ID NO: 29).

[0064] Figures 9A and 9B depict the human activin C cDNA sequence (NCBI Reference Sequence No. NM\_005538.3) (SEQ ID NO: 30).

[0065] Figure 10 depicts the amino acid sequence of the human activin C precursor protein (NCBI Reference Sequence No. NP\_005529.1) (SEQ ID NO: 31).

5 [0066] Figure 11 depicts the human GDF8 cDNA sequence (NCBI Reference Sequence No. NM\_005259.2) (SEQ ID NO: 32).

[0067] Figure 12 depicts the amino acid sequence of the human GDF8 precursor protein (NCBI Reference Sequence No. NP\_005250.1) (SEQ ID NO: 33).

10 [0068] Figure 13 depicts the ligand binding profile for ActRIIB(L79D 25-131)-Fc (see, *e.g.*, U.S. Patent No. 8,058,229) characterized with respect to various ActRII ligands (GDF11, GDF8, activin A, activin B, BMP10, BMP6, and BMP9) as determined by surface plasmon resonance.

[0069] Figure 14 depicts the human BMP6 cDNA sequence (NCBI Reference Sequence No. NM\_001718.4) (SEQ ID NO: 34).

15 [0070] Figure 15 depicts the amino acid sequence of the human BMP6 precursor protein (NCBI Reference Sequence No. NP\_001709.1) (SEQ ID NO: 35).

[0071] Figure 16 depicts the amino acid sequence of human GDF15 precursor protein (NCBI Reference Sequence No. NP\_004855.2) (SEQ ID NO: 50).

20 [0072] Figure 17 depicts the human GDF15 cDNA sequence (NCBI Reference Sequence No. NM\_004864.2) (SEQ ID NO: 51).

[0073] Figure 18 depicts the amino acid sequence of human Nodal precursor protein (NCBI Reference Sequence No. NP\_060525.3) (SEQ ID NO: 52).

[0074] Figure 19 depicts the human Nodal cDNA sequence (NCBI Reference Sequence No. NM\_018055.4) (SEQ ID NO: 53).

25 [0075] Figure 20 depicts the amino acid sequence of human GDF3 precursor protein (NCBI Reference Sequence No. NP\_065685.1) (SEQ ID NO: 54).

[0076] Figure 21 depicts the human GDF3 cDNA sequence (NCBI Reference Sequence No. NM\_020634.1) (SEQ ID NO: 55).

30 [0077] Figure 22 depicts the amino acid sequence of human BMP3 precursor protein (NCBI Reference Sequence No. NP\_001192.2) (SEQ ID NO: 56).

[0078] Figures 23A and 23B depict the human BMP3 cDNA sequence (NCBI Reference Sequence No. NM\_001201.2) (SEQ ID NO: 57).

[0079] Figure 24 depicts the amino acid sequence of human BMP3B precursor protein (NCBI Reference Sequence No. NP\_004953.1) (SEQ ID NO: 58).

5 [0080] Figure 25 depicts the human BMP3B cDNA sequence (NCBI Reference Sequence No. NM\_004962.3) (SEQ ID NO: 59).

[0081] Figure 26 depicts the amino acid sequence of human BMP9 precursor protein (NCBI Reference Sequence No. NP\_057288.1) (SEQ ID NO: 60).

10 [0082] Figure 27 depicts the human BMP9 cDNA sequence (NCBI Reference Sequence No. NM\_016204.2) (SEQ ID NO: 61).

[0083] Figure 28 depicts the amino acid sequence of human BMP10 precursor protein (NCBI Reference Sequence No. NP\_055297.1) (SEQ ID NO: 62).

[0084] Figure 29 depicts the human BMP10 cDNA sequence (NCBI Reference Sequence No. NM\_014482.1) (SEQ ID NO: 63).

15 [0085] Figure 30 shows the effect of treatment with an anti-activin B antibody (Ab), an anti-GDF8 Ab, a bispecific anti-GDF8/GDF11 Ab, or a combination of an anti-activin B Ab and a bispecific anti-GDF8/GDF11 Ab on red blood cell levels in C57BL6 mice (n = 5 mice per group). Data is shown as the percent increase in red blood cell levels over that observed in vehicle (PBS) treated subjects.

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## DETAILED DESCRIPTION OF THE INVENTION

### 1. Overview

[0086] The transforming growth factor-beta (TGF- $\beta$ ) superfamily contains a variety of growth factors that share common sequence elements and structural motifs. These proteins are known to exert biological effects on a large variety of cell types in both vertebrates and invertebrates. Members of the superfamily perform important functions during embryonic development in pattern formation and tissue specification and can influence a variety of differentiation processes, including adipogenesis, myogenesis, chondrogenesis, cardiogenesis, hematopoiesis, neurogenesis, and epithelial cell differentiation. By manipulating the activity of a member of the TGF- $\beta$  family, it is often possible to cause significant physiological

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changes in an organism. For example, the Piedmontese and Belgian Blue cattle breeds carry a loss-of-function mutation in the GDF8 (also called myostatin) gene that causes a marked increase in muscle mass [see, *e.g.*, Grobet *et al.* (1997) Nat Genet. 17(1):71-4]. Furthermore, in humans, inactive alleles of GDF8 are associated with increased muscle mass and,

5 reportedly, exceptional strength [see, *e.g.*, Schuelke *et al.* (2004) N Engl J Med, 350:2682-8.

[0087] TGF- $\beta$  signals are mediated by heteromeric complexes of type I and type II serine/threonine kinase receptors, which phosphorylate and activate downstream Smad proteins (*e.g.*, Smad proteins 1, 2, 3, 5, and 8) upon ligand stimulation [see, *e.g.*, Massagué (2000) Nat. Rev. Mol. Cell Biol. 1:169-178]. These type I and type II receptors are

10 transmembrane proteins, composed of a ligand-binding extracellular domain with cysteine-rich region, a transmembrane domain, and a cytoplasmic domain with predicted serine/threonine specificity. Type I receptors are essential for signaling. Type II receptors are required for binding ligands and for expression of type I receptors. Type I and II activin receptors form a stable complex after ligand binding, resulting in phosphorylation of type I

15 receptors by type II receptors.

[0088] Activins are dimeric polypeptide growth factors belonging to the TGF- $\beta$  superfamily. There are three principal activin forms (A, B, and AB) that are homo/heterodimers of two closely related  $\beta$  subunits ( $\beta_A\beta_A$ ,  $\beta_B\beta_B$ , and  $\beta_A\beta_B$ , respectively). The human genome also encodes an activin C and an activin E, which are primarily

20 expressed in the liver, and heterodimeric forms containing  $\beta_C$  or  $\beta_E$  are also known.

[0089] Two related type II receptors for activins have been identified, ActRIIA (encoded by the *ACVR2A* gene) and ActRIIB (encoded by the *ACVR2B* gene) [see, *e.g.*, Mathews and Vale (1991) Cell 65:973-982; and Attisano *et al.* (1992) Cell 68: 97-108]. Besides activins, ActRIIA and ActRIIB can interact biochemically with several other TGF- $\beta$  family proteins

25 including, for example, BMP7, Nodal, GDF8, and GDF11 [see, *e.g.*, Yamashita *et al.* (1995) J. Cell Biol. 130:217-226; Lee and McPherron (2001) Proc. Natl. Acad. Sci. 98:9306-9311; Yeo and Whitman (2001) Mol. Cell 7: 949-957; and Oh *et al.* (2002) Genes Dev. 16:2749-54]. Activin-like kinase-4 (ALK4) is the primary type I receptor for activins, particularly for activin A, and ALK7 may serve as a receptor for other activins as well, particularly for

30 activin B. In certain embodiments, the present disclosure relates to antagonizing a ligand of an ActRIIA or ActRIIB receptor (also referred to as an ActRIIA ligand or an ActRIIB ligand) with one or more agents disclosed herein, particularly agents that can antagonize GDF11 and/or activin B.

[0090] As described herein, agents that bind to “activin B” are agents that specifically bind to the  $\beta_B$  subunit, whether in the context of an isolated  $\beta_B$  subunit or as a dimeric complex (e.g., a  $\beta_B\beta_B$  homodimer or a  $\beta_A\beta_B$  heterodimer). In the case of a heterodimer complex (e.g., a  $\beta_A\beta_B$  heterodimer), agents that bind to “activin B” are specific for epitopes present within the  $\beta_B$  subunit, but do not bind to epitopes present within the non- $\beta_B$  subunit of the complex (e.g., the  $\beta_A$  subunit of the complex). Similarly, agents disclosed herein that antagonize (inhibit) “activin B” are agents that inhibit one or more activities as mediated by a  $\beta_B$  subunit, whether in the context of an isolated  $\beta_B$  subunit or as a dimeric complex (e.g., a  $\beta_B\beta_B$  homodimer or a  $\beta_A\beta_B$  heterodimer). In the case of  $\beta_A\beta_B$  heterodimers, agents that inhibit “activin B” are agents that specifically inhibit one or more activities of the  $\beta_B$  subunit, but do not inhibit the activity of the non- $\beta_B$  subunit of the complex (e.g., the  $\beta_A$  subunit of the complex). This principle applies also to agents that *bind to* and/or *inhibit* “activin A”, “activin C”, and “activin E”.

[0091] In the TGF- $\beta$  superfamily, activins are unique and multifunctional factors that can stimulate hormone production in ovarian and placental cells, support neuronal cell survival, influence cell-cycle progress positively or negatively depending on cell type, and induce mesodermal differentiation at least in amphibian embryos [DePaolo *et al.* (1991) *Proc Soc Ep Biol Med.* 198:500-512; Dyson *et al.* (1997) *Curr Biol.* 7:81-84; and Woodruff (1998) *Biochem Pharmacol.* 55:953-963]. Moreover, an erythroid differentiation factor (EDF) isolated from the stimulated human monocytic leukemic cells was found to be identical to activin A [Murata *et al.* (1988) *PNAS*, 85:2434]. It has been suggested that activin A promotes erythropoiesis in the bone marrow. In several tissues, activin signaling is antagonized by its related heterodimer, inhibin. For example, during the release of follicle-stimulating hormone (FSH) from the pituitary, activin promotes FSH secretion and synthesis, whereas inhibin prevents FSH secretion and synthesis. Other proteins that may regulate activin bioactivity and/or bind to activin include follistatin (FS), follistatin-related protein (FSRP), and  $\alpha_2$ -macroglobulin.

[0092] Growth and differentiation factor-8 (GDF8) is also known as myostatin. GDF8 is a negative regulator of skeletal muscle mass. GDF8 is highly expressed in developing and adult skeletal muscle. GDF8 gene deletion in mice is characterized by a marked hypertrophy and hyperplasia of the skeletal muscle [McPherron *et al.*, *Nature* (1997) 387:83-90]. Similar increases in skeletal muscle mass are evident in naturally occurring mutations of GDF8 in cattle [see, e.g., Ashmore *et al.* (1974) *Growth*, 38:501-507; Swatland and Kieffer (1994) *J.*

Anim. Sci. 38:752-757; McPherron and Lee (1997) Proc. Natl. Acad. Sci. USA 94:12457-12461; and Kambadur *et al.* (1997) Genome Res. 7:910-915] and, strikingly, in humans [see, *e.g.*, Schuelke *et al.* (2004) N Engl J Med 350:2682-8]. Studies have also shown that muscle wasting associated with HIV-infection in humans is accompanied by increases in GDF8 protein expression [see, *e.g.*, Gonzalez-Cadavid *et al.* (1998) PNAS 95:14938-43]. In addition, GDF8 can modulate the production of muscle-specific enzymes (*e.g.*, creatine kinase) and modulate myoblast cell proliferation [see, *e.g.* international patent application publication no. WO 00/43781]. The GDF8 propeptide can noncovalently bind to the mature GDF8 domain dimer, inactivating its biological activity [see, *e.g.*, Miyazono *et al.* (1988) J. Biol. Chem., 263: 6407-6415; Wakefield *et al.* (1988) J. Biol. Chem., 263: 7646-7654; and Brown *et al.* (1990) Growth Factors, 3: 35-43]. Other proteins which bind to GDF8 or structurally related proteins and inhibit their biological activity include follistatin, and potentially, follistatin-related proteins [see, *e.g.*, Gamer *et al.* (1999) Dev. Biol., 208: 222-232].

[0093] Growth and differentiation factor-11 (GDF11), also known as bone morphogenetic protein-11 (BMP11), is a secreted protein first identified as a regulator of vertebrate development [McPherron *et al.* (1999) Nat. Genet. 22: 260-264]. GDF11 is expressed in the tail bud, limb bud, maxillary and mandibular arches, and dorsal root ganglia during mouse embryonic development [see, *e.g.*, Nakashima *et al.* (1999) Mech. Dev. 80: 185-189]. GDF11 plays a unique role in patterning both mesodermal and neural tissues [see, *e.g.*, Gamer *et al.* (1999) Dev Biol., 208:222-32] and was shown to be a negative regulator of chondrogenesis and myogenesis in the developing chick limb [see, *e.g.*, Gamer *et al.* (2001) Dev Biol. 229:407-20]. GDF11 is also implicated as a regulator of tissue homeostasis postnatally. For example, expression of GDF11 in muscle also suggests a role for this ligand in regulating muscle growth in a manner similar to that of GDF8. In addition, expression of GDF11 in brain suggests that GDF11 may also regulate nervous system function, and GDF11 has been found to inhibit neurogenesis in the olfactory epithelium [see, *e.g.*, Wu *et al.* (2003) Neuron. 37:197-207].

[0094] Bone morphogenetic protein (BMP7), also called osteogenic protein-1 (OP-1), is well known to induce cartilage and bone formation. In addition, BMP7 regulates a wide array of physiological processes. For example, BMP7 may be the osteoinductive factor responsible for the phenomenon of epithelial osteogenesis. BMP7 also plays a role in calcium regulation and bone homeostasis. Like activin, BMP7 binds to ActRIIA and

ActRIIB. However, BMP7 and activin recruit distinct type I receptors into heteromeric receptor complexes. Whereas BMP7 signals preferentially through ALK2, activin signals through ALK4. This difference allows BMP7 and activin to activate different Smad pathways and elicit distinct biological responses [see, *e.g.*, Macias-Silva *et al.* (1998) J Biol Chem. 273:25628-36].

[0095] It has been previously reported that special biological properties are exhibited *in vitro* and *in vivo* by variant ActRIIB-Fc fusion proteins, one variant comprising amino acids 20-134 of instant SEQ ID NO:1 with an acidic amino acid at position 79 with respect to SEQ ID NO:1 [referenced hereafter as the “ActRIIB(L79D 20-134)-Fc” fusion protein] and a second, truncated variant comprising amino acids 25-131 of instant SEQ ID NO: 1 and also incorporating an acidic amino acid at position 79 [referenced hereafter as “ActRIIB(L79D 25-131)-Fc”]. See, *e.g.*, U.S. Patent No. 8,058,229. In comparison to the unmodified fusion proteins ActRIIB(20-134)-Fc and ActRIIB(25-131)-Fc, the corresponding L79D variants [ActRIIB(L79D 20-134)-Fc and ActRIIB(L79D 25-131)-Fc, respectively] are characterized, in part, by substantial loss of binding affinity for activin A, and therefore significantly diminished capacity to antagonize activin A activity, but retain near wild-type levels of binding and inhibition of GDF11. The ActRIIB(L79D 20-134)-Fc and ActRIIB(L79D 25-131)-Fc variants were found to be significantly more potent in the capacity to increase red blood cell levels *in vivo* in comparison to the unmodified ActRIIB(20-134)-Fc and ActRIIB(25-131)-Fc fusion proteins, respectively. These data therefore indicate that the observed biological activity is not dependent on activin A inhibition.

[0096] The instant application is directed, in part, to the insight that the ActRIIB(L79D 20-134)-Fc and ActRIIB(L9D 25-131)-Fc variants disclosed in U.S. Patent No. 8,058,229, while having significantly reduced binding affinity for activin A, are capable of inhibiting activin B as well as GDF11. Therefore, Applicants conclude herein that erythropoiesis can be increased by an agent, or combination of agents, that antagonizes both GDF11 and activin B activity. Although inhibition of activin A is not necessary to promote red blood cell formation, it is known that an ActRII-Fc fusion protein that inhibits activin A also promotes red blood cell formation. Therefore, agents that inhibit activin A are included in the scope of the present disclosure.

[0097] As demonstrated herein, multiple ActRII ligands (*e.g.*, activin B, GDF11, and GDF8) appear to be regulators erythropoiesis as there is a trend toward increased levels of various blood parameters (*e.g.*, hematocrit, hemoglobin, and red blood cell levels) as more of

these ligands are inhibited. For example, the data presented herein demonstrate that administration of an agent that inhibits GDF8 and GDF11 activity has a more substantial effect on increasing red blood cell levels *in vivo* compared to an agent that only antagonizes GDF8. In addition, it is shown that combination therapy using agents that inhibit activin B, GDF8, and GDF11 have even a greater effect on increasing red blood cells compared to treatment with a GDF8/GDF11 antagonist. Accordingly, the data presented herein indicates that an effective strategy for promoting erythropoiesis in a subject is to target multiple (*i.e.*, two or more) ActRII ligands.

[0098] Accordingly, the present disclosure provides, in part, methods for increasing red blood cell levels, treating or preventing anemia, and/or treating or preventing ineffective erythropoiesis in a subject in need thereof with an agent, or combination of agents, that antagonizes (inhibits) GDF11 (*e.g.*, GDF11-mediated activation of Smad2/3 signaling through ActRIIA and/or ActRIIB) and/or activin B (*e.g.*, activin B-mediated activation of Smad2/3 signaling through ActRIIA and/or ActRIIB). Optionally, an agent, or combination of agents, of the disclosure that antagonizes GDF11 and/or activin B does not substantially antagonizes activin A (*e.g.*, activin A-mediated activation of Smad2/3 signaling through ActRIIA and/or ActRIIB). Optionally an agent, or combination of agents, of the disclosure that antagonizes GDF11 and/or activin B may further inhibit GDF8 activity. In certain embodiments, an agent, or combination of agents, of the disclosure that antagonizes GDF11 and/or activin B may further inhibit one or more of GDF8, BMP6, activin C, activin E, activin A, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10.

[0099] In some embodiments, an agent, or combination of agents, that inhibits GDF11 and/or activin B activity are agents that directly bind to GDF11 and/or activin B, including for example: a multispecific antibody (*e.g.*, bispecific antibody) that binds to at least GDF11 and activin B (optionally such an agent, or combination of agents, does not substantially bind to activin A); a combination of antibodies comprising an anti-GDF11 antibody and an anti-activin B antibody; a variant ActRII polypeptide (*e.g.*, a variant ActRIIA or ActRIIB polypeptide) that binds to GDF11 and activin B (optionally may not bind to activin A); a combination of variant ActRII polypeptides (*e.g.*, variant ActRIIA or ActRIIB polypeptides) comprising at least one variant ActRII polypeptide that binds to GDF11 (but does not substantially activin B) and at least one soluble, variant ActRII polypeptide that binds to activin B (but does not substantially bind to GDF11) (optionally one or both of the GDF- and activin B-binding ActRII polypeptides may not substantially bind to activin A); a small

molecule that directly binds to GDF11 and/or activin B (optionally may not substantially bind to activin A); and a combination of small molecules comprising at least one small molecule that binds to GDF11 (but does not substantially bind to activin B) and one small molecule that binds to activin B (but does not substantially bind to GDF11) (one or both of the GDF-  
5 and activin B-binding small molecules may not substantially bind to activin A).

[0100] In alternative embodiments, an agent, or combination agents, that inhibit GDF11 and/or activin B activity are indirect antagonist agents that do not directly bind to GDF11 and/or activin B. For example, an indirect antagonist agent may be an antibody that binds to a native ActRII receptor (*e.g.*, an ActRIIA or ActRIIB receptor) and prevents GDF11 and/or  
10 activin B from binding to and/or activating the ActRII receptor. Optionally, such an agent, or combination of agents, does not substantially inhibit activin A from binding to and/or activating the ActRII receptor. Other indirect antagonist agents of the present disclosure include an agent, or combination of agents, that inhibit the expression (*e.g.*, transcription, translation, and/or cellular secretion) of GDF11 and/or activin B. Optionally such an agent,  
15 or combination of agents, do not substantially affect the expression of activin A. Such indirect agents include, for example, small-molecule inhibitors of GDF11 and/or activin B expression as well as the use of various polynucleotide antagonists [*e.g.* antisense DNA, RNA or chemical analogues, and interfering RNA molecules including small interfering RNA (siRNA), small hairpin RNA (shRNA) or microRNA (miRNA) molecules, targeted to  
20 GDF11 and/or activin B mRNA] to inhibit GDF11 and/or activin B expression.

[0101] In some embodiments, an agent, or combination agents, of the disclosure that inhibit GDF11 and/or activin B activity optionally bind to and/or inhibit the activity of one or more of: GDF8, activin A, activin C, activin E, activin A, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10.

[0102] Additionally, methods of the present disclosure are directed to the use of one or  
25 more antagonist agents described herein (*e.g.*, an agent or combination of agents that inhibit GDF11 and activin B) in combination with an EPO receptor activator to increase red blood cell formation as well as to treat or prevent various anemias and ineffective erythropoiesis disorders and associated conditions. It should be noted that hematopoiesis is a complex  
30 process, regulated by a variety of factors, including erythropoietin, G-CSF and iron homeostasis. The terms “increase red blood cell levels” and “promote red blood cell formation” refer to clinically observable metrics, such as hematocrit, red blood cell counts,

and hemoglobin measurements, and are intended to be neutral as to the mechanism by which such changes occur.

[0103] EPO is a glycoprotein hormone involved in the growth and maturation of erythroid progenitor cells into erythrocytes. EPO is produced by the liver during fetal life and by the kidney in adults. Decreased production of EPO, which commonly occurs in adults as a consequence of renal failure, leads to anemia. EPO has been produced by genetic engineering techniques based on expression and secretion of the protein from a host cell transfected with the EPO gene. Administration of such recombinant EPO has been effective in the treatment of anemia. For example, Eschbach *et al.* (1987, N Engl J Med 316:73) describe the use of EPO to correct anemia caused by chronic renal failure.

[0104] Effects of EPO are mediated through its binding to, and activation of, a cell surface receptor belonging to the cytokine receptor superfamily and designated the EPO receptor. The human and murine EPO receptors have been cloned and expressed [see, *e.g.*, D'Andrea *et al.* (1989) Cell 57:277; Jones *et al.* (1990) Blood 76:31; Winkelman *et al.* (1990) Blood 76:24; and U.S. Pat. No. 5,278,065]. The human EPO receptor gene encodes a 483 amino acid transmembrane protein comprising an extracellular domain of approximately 224 amino acids and exhibits approximately 82% amino acid sequence identity with the murine EPO receptor [see, *e.g.*, U.S. Pat. No. 6,319,499. The cloned, full-length EPO receptor expressed in mammalian cells (66-72 kDa) binds EPO with an affinity ( $K_D = 100\text{-}300\text{ nM}$ ) similar to that of the native receptor on erythroid progenitor cells. Thus, this form is thought to contain the main EPO binding determinant and is referred to as the EPO receptor. By analogy with other closely related cytokine receptors, the EPO receptor is thought to dimerize upon agonist binding. Nevertheless, the detailed structure of the EPO receptor, which may be a multimeric complex, and its specific mechanism of activation are not completely understood [see, *e.g.*, U.S. Pat. No. 6,319,499].

[0105] Activation of the EPO receptor results in several biological effects. These include increased proliferation of immature erythroblasts, increased differentiation of immature erythroblasts, and decreased apoptosis in erythroid progenitor cells [see, *e.g.*, Liboi *et al.* (1993) Proc Natl Acad Sci USA 90:11351-11355; Koury *et al.* (1990) Science 248:378-381]. The EPO receptor signal transduction pathways mediating proliferation and differentiation appear to be distinct [see, *e.g.*, Noguchi *et al.* (1988) Mol Cell Biol 8:2604; Patel *et al.* (1992) J Biol Chem, 267:21300; and Liboi *et al.* (1993) Proc Natl Acad Sci USA 90:11351-11355]. Some results suggest that an accessory protein may be required for mediation of the

differentiation signal [see, *e.g.*, Chiba *et al.* (1993) *Nature* 362:646; and Chiba *et al.* (1993) *Proc Natl Acad Sci USA* 90:11593]. However, there is controversy regarding the role of accessory proteins in differentiation since a constitutively activated form of the receptor can stimulate both proliferation and differentiation [see, *e.g.*, Pharr *et al.* (1993) *Proc Natl Acad Sci USA* 90:938].

[0106] EPO receptor activators include small-molecule erythropoiesis-stimulating agents (ESAs) as well as EPO-based compounds. An example of the former is a dimeric peptide-based agonist covalently linked to polyethylene glycol (proprietary name Hematide), which has shown erythropoiesis-stimulating properties in healthy volunteers and in patients with both chronic kidney disease and endogenous anti-EPO antibodies [see, *e.g.*, Stead *et al.* (2006) *Blood* 108:1830-1834; and Macdougall *et al.* (2009) *N Engl J Med* 361:1848-1855]. Other examples include nonpeptide-based ESAs [see, *e.g.*, Qureshi *et al.* (1999) *Proc Natl Acad Sci USA* 96:12156-12161].

[0107] EPO receptor activators also include compounds that stimulate erythropoiesis indirectly, without contacting EPO receptor itself, by enhancing production of endogenous EPO. For example, hypoxia-inducible transcription factors (HIFs) are endogenous stimulators of EPO gene expression that are suppressed (destabilized) under normoxic conditions by cellular regulatory mechanisms. Therefore, inhibitors of HIF prolyl hydroxylase enzymes are being investigated for EPO-inducing activity *in vivo*. Other indirect activators of EPO receptor include inhibitors of GATA-2 transcription factor [see, *e.g.*, Nakano *et al.* (2004) *Blood* 104:4300-4307], which tonically inhibits EPO gene expression, and inhibitors of hemopoietic cell phosphatase (HCP or SHP-1), which functions as a negative regulator of EPO receptor signal transduction [see, *e.g.*, Klingmuller *et al.* (1995) *Cell* 80:729-738].

[0108] The terms used in this specification generally have their ordinary meanings in the art, within the context of this disclosure and in the specific context where each term is used. Certain terms are discussed below or elsewhere in the specification, to provide additional guidance to the practitioner in describing the compositions and methods of the disclosure and how to make and use them. The scope or meaning of any use of a term will be apparent from the specific context in w

[0109] “Homologous,” in all its grammatical forms and spelling variations, refers to the relationship between two proteins that possess a “common evolutionary origin,” including

proteins from superfamilies in the same species of organism, as well as homologous proteins from different species of organism. Such proteins (and their encoding nucleic acids) have sequence homology, as reflected by their sequence similarity, whether in terms of percent identity or by the presence of specific residues or motifs and conserved positions.

5 [0110] The term “sequence similarity,” in all its grammatical forms, refers to the degree of identity or correspondence between nucleic acid or amino acid sequences that may or may not share a common evolutionary origin.

[0111] However, in common usage and in the instant application, the term “homologous,” when modified with an adverb such as “highly,” may refer to sequence similarity and may or  
10 may not relate to a common evolutionary origin.

[0112] “Percent (%)sequence identity” with respect to a reference polypeptide (or nucleotide) sequence is defined as the percentage of amino acid residues (or nucleic acids) in a candidate sequence that are identical with the amino acid residues (or nucleic acids) in the reference polypeptide (nucleotide) sequence, after aligning the sequences and introducing  
15 gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can  
20 determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid (nucleic acid) sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has  
25 been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, Calif., or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison  
30 parameters are set by the ALIGN-2 program and do not vary.

[0113] As used herein “does not substantially bind to *X*” is intended to mean that an agent has a  $K_D$  that is greater than about  $10^{-7}$ ,  $10^{-6}$ ,  $10^{-5}$ ,  $10^{-4}$  or greater (*e.g.*, no detectable binding by the assay used to determine the  $K_D$ ) for “*X*”.

## 5 2. Antagonist Agents

### A. Antibody Antagonists

[0114] In certain aspects, antagonist agents, or combinations of agents, of the present disclosure are antibodies that bind to and/or inhibit the activity of at least activin B and/or GDF11 (*e.g.*, activation of ActRIIA- or ActRIIB-based Smad 2/3 signaling). Optionally, an antibody, or combination of antibodies, of the disclosure does not bind to and/or inhibit the activity of activin A (*e.g.*, activin A-mediated activation of ActRIIA- or ActRIIB-based Smad 2/3 signaling). Optionally, an antibody, or combination of antibodies, of the present disclosure further binds to and/or inhibits the activity of GDF8 (*e.g.*, GDF8-mediated activation of ActRIIA or ActRIIB Smad 2/3 signaling). In some embodiments, an antibody, or combination of antibodies, of the disclosure that binds to and/or inhibits the activity of at least activin B and/or GDF11 further binds to and/or inhibits the activity of one or more of GDF8, activin C, activin E, BMP6, activin A, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, or BMP10 (*e.g.*, activation of ActRIIA- or ActRIIB-based Smad 2/3 and/or Smad 1/5/8 signaling).

[0115] In another aspect, an antibody, or combination of antibodies, of the present disclosure is an anti-ActRII receptor antibody (*e.g.* an ActRIIA or ActRIIB receptor antibody) that binds to an ActRII receptor and prevents binding and/or activation of the ActRII receptor by at least activin B and/or GDF11. Optionally, an anti-ActRII receptor antibody, or combination of antibodies, of the disclosure does not substantially inhibit activin A from binding to and/or activating an ActRII receptor. Optionally, an anti-ActRII receptor antibody, or combination of antibodies, of the disclosure further prevents binding and/or activation of the ActRII receptor by GDF8. In some embodiments, an anti-ActRII receptor antibody, or combination of antibodies, of the disclosure that binds to an ActRII receptor and prevents binding and/or activation of the ActRII receptor by activin B and/or GDF11 further prevents binding and/or activation of the ActRII receptor by one or more of activin A, activin C, activin E, GDF8, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9 and BMP10.

[0116] The term antibody is used herein in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments so long as they exhibit the desired antigen-binding activity. An antibody fragment refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, F(ab')<sub>2</sub>; diabodies; linear antibodies; single-chain antibody molecules (*e.g.*, scFv); and multispecific antibodies formed from antibody fragments [see, *e.g.*, Hudson *et al.* (2003) Nat. Med. 9:129-134; Plückthun, in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenberg and Moore eds., (Springer-Verlag, New York), pp. 269-315 (1994); WO 93/16185; and U.S. Pat. Nos. 5,571,894; 5,587,458; and 5,869,046]. Antibodies disclosed herein may be polyclonal antibodies or monoclonal antibodies. In certain embodiments, the antibodies of the present disclosure comprise a label attached thereto and able to be detected (*e.g.*, the label can be a radioisotope, fluorescent compound, enzyme, or enzyme co-factor). In preferred embodiments, the antibodies of the present disclosure are isolated antibodies.

[0117] Diabodies are antibody fragments with two antigen-binding sites that may be bivalent or bispecific [see, *e.g.*, EP 404,097; WO 1993/01161; Hudson *et al.* (2003) Nat. Med. 9:129-134 (2003); and Hollinger *et al.* (1993) Proc. Natl. Acad. Sci. USA 90: 6444-6448]. Triabodies and tetrabodies are also described in Hudson *et al.* (2003) Nat. Med. 9:129-134.

[0118] Single-domain antibodies are antibody fragments comprising all or a portion of the heavy-chain variable domain or all or a portion of the light-chain variable domain of an antibody. In certain embodiments, a single-domain antibody is a human single-domain antibody [see, *e.g.*, U.S. Pat. No. 6,248,516].

[0119] Antibody fragments can be made by various techniques, including but not limited to proteolytic digestion of an intact antibody as well as production by recombinant host cells (*e.g.*, *E. coli* or phage), as described herein.

[0120] The antibodies herein may be of any class. The class of an antibody refers to the type of constant domain or constant region possessed by its heavy chain. There are five major classes of antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), for example, IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, IgG<sub>4</sub>, IgA<sub>1</sub>, and IgA<sub>2</sub>. The

heavy chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu.

[0121] In general, an antibody for use in the methods disclosed herein specifically binds to its target antigen, preferably with high binding affinity. Affinity may be expressed as a  $K_D$  value and reflects the intrinsic binding affinity (*e.g.*, with minimized avidity effects).

Typically, binding affinity is measured *in vitro*, whether in a cell-free or cell-associated setting. Any of a number of assays known in the art, including those disclosed herein, can be used to obtain binding affinity measurements including, for example, Biacore, radiolabeled antigen-binding assay (RIA), and ELISA. In some embodiments, antibodies of the present disclosure bind to their target antigens (*e.g.* GDF11, activin B, GDF8, activin E, activin C, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, or BMP10) with at least a  $K_D$  of  $1 \times 10^{-7}$  or stronger,  $1 \times 10^{-8}$  or stronger,  $1 \times 10^{-9}$  or stronger,  $1 \times 10^{-10}$  or stronger,  $1 \times 10^{-11}$  or stronger,  $1 \times 10^{-12}$  or stronger,  $1 \times 10^{-13}$  or stronger, or  $1 \times 10^{-14}$  or stronger.

[0122] In certain embodiments,  $K_D$  is measured by RIA performed with the Fab version of an antibody of interest and its target antigen as described by the following assay. Solution binding affinity of Fabs for the antigen is measured by equilibrating Fab with a minimal concentration of radiolabeled antigen (*e.g.*,  $^{125}\text{I}$ -labeled) in the presence of a titration series of unlabeled antigen, then capturing bound antigen with an anti-Fab antibody-coated plate [see, *e.g.*, Chen *et al.* (1999) J. Mol. Biol. 293:865-881]. To establish conditions for the assay, multi-well plates (*e.g.*, MICROTITER<sup>®</sup> from Thermo Scientific) are coated (*e.g.*, overnight) with a capturing anti-Fab antibody (*e.g.*, from Cappel Labs) and subsequently blocked with bovine serum albumin, preferably at room temperature (approximately 23°C). In a non-adsorbent plate, radiolabeled antigen are mixed with serial dilutions of a Fab of interest [*e.g.*, consistent with assessment of the anti-VEGF antibody, Fab-12, in Presta *et al.*, (1997) Cancer Res. 57:4593-4599]. The Fab of interest is then incubated, preferably overnight but the incubation may continue for a longer period (*e.g.*, about 65 hours) to ensure that equilibrium is reached. Thereafter, the mixtures are transferred to the capture plate for incubation, preferably at room temperature for about one hour. The solution is then removed and the plate is washed times several times, preferably with polysorbate 20 and PBS mixture. When the plates have dried, scintillant (*e.g.*, MICROSCINT<sup>®</sup> from Packard) is added, and the plates are counted on a gamma counter (*e.g.*, TOPCOUNT<sup>®</sup> from Packard).

[0123] According to another embodiment,  $K_D$  is measured using surface plasmon resonance assays using, for example a BIACORE<sup>®</sup> 2000 or a BIACORE<sup>®</sup> 3000 (BIAcore,

Inc., Piscataway, N.J.) with immobilized antigen CM5 chips at about 10 response units (RU). Briefly, carboxymethylated dextran biosensor chips (CM5, BIACORE, Inc.) are activated with N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's instructions. For example, an antigen  
5 can be diluted with 10 mM sodium acetate, pH 4.8, to 5 µg/ml (about 0.2 µM) before injection at a flow rate of 5 µl/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1 M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in PBS with 0.05% polysorbate 20 (TWEEN-20<sup>®</sup>) surfactant (PBST) at  
10 at a flow rate of approximately 25 µl/min. Association rates ( $k_{on}$ ) and dissociation rates ( $k_{off}$ ) are calculated using, for example, a simple one-to-one Langmuir binding model (BIACORE<sup>®</sup> Evaluation Software version 3.2) by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant ( $K_D$ ) is calculated as the ratio  $k_{off} / k_{on}$  [see, *e.g.*, Chen *et al.*, (1999) J. Mol. Biol. 293:865-881]. If the on-rate exceeds, for example,  
15  $10^6 \text{ M}^{-1} \text{ s}^{-1}$  by the surface plasmon resonance assay above, then the on-rate can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (*e.g.*, excitation=295 nm; emission=340 nm, 16 nm band-pass) of a 20 nM anti-antigen antibody (Fab form) in PBS in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow equipped  
20 spectrophotometer (Aviv Instruments) or a 8000-series SLM-AMINCO<sup>®</sup> spectrophotometer (ThermoSpectronic) with a stirred cuvette.

[0124] In general, an anti-GDF11 antibody refers to an antibody that is capable of binding to GDF11 with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting GDF11. In certain embodiments, the extent of binding  
25 of an anti-GDF11 antibody to an unrelated, non-GDF11 protein is less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or less than 1% of the binding of the antibody to GDF11 as measured, for example, by a radioimmunoassay (RIA). In certain embodiments, an anti-GDF11 antibody binds to an epitope of GDF11 that is conserved among orthologous GDF11 proteins from different species. In preferred embodiments, an anti-GDF11 antibody of the  
30 present disclosure is an antagonist antibody that can substantially inhibit GDF11 activity. For example, an anti-GDF11 antibody of the disclosure may substantially inhibit GDF11 from binding to a cognate receptor (*e.g.*, ActRIIA or ActRIIB receptor) and/or substantially inhibit GDF11-mediated signal transduction (activation) of a cognate receptor, such as Smad2/3

signaling by ActRIIA and/or ActRIIB receptors. In some embodiments, anti-GDF11 antibodies of the present disclosure do not substantially bind to and/or inhibit activity of activin A. It should be noted that GDF11 has high sequence identity with GDF8 on the amino acid level and therefore antibodies that bind and/or to GDF11, in many cases, may also bind to and/or inhibit GDF8.

[0125] In general, an anti-activin B antibody refers to an antibody that is capable of binding to activin B with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting activin B. In certain embodiments, the extent of binding of an anti-activin B antibody to an unrelated, non-activin B protein is less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or less than 1% of the binding of the antibody to activin B as measured, for example, by a radioimmunoassay (RIA). In certain embodiments, an anti-activin B antibody binds to an epitope of activin B that is conserved among orthologous activin B proteins from different species. In preferred embodiments, an anti-activin B antibody of the present disclosure is an antagonist antibody that can substantially inhibit activin B activity. For example, an anti-activin B antibody of the disclosure may substantially inhibit activin B from binding to a cognate receptor (*e.g.*, ActRIIA or ActRIIB receptor) and/or substantially inhibit activin B-mediated signal transduction (activation) of a cognate receptor, such as Smad2/3 signaling by ActRIIA and/or ActRIIB receptors. In some embodiments, anti-activin B antibodies of the present disclosure do not substantially bind to and/or inhibit activity of activin A.

[0126] In general, an anti-activin C antibody refers to an antibody that is capable of binding to activin C with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting activin C. In certain embodiments, the extent of binding of an anti-activin C antibody to an unrelated, non-activin C protein is less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or less than 1% of the binding of the antibody to activin C as measured, for example, by a radioimmunoassay (RIA). In certain embodiments, an anti-activin C antibody binds to an epitope of activin C that is conserved among orthologous activin C proteins from different species. In preferred embodiments, an anti-activin C antibody of the present disclosure is an antagonist antibody that can substantially inhibit activin C activity. For example, an anti-activin C antibody of the disclosure may substantially inhibit activin C from binding to a cognate receptor (*e.g.*, ActRIIA or ActRIIB receptor) and/or substantially inhibit activin C-mediated signal transduction (activation) of a cognate receptor, such as Smad2/3 signaling by ActRIIA and/or ActRIIB receptors. In some

embodiments, anti-activin C antibodies of the present disclosure do not substantially bind to and/or inhibit activity of activin A.

[0127] In general, an anti-activin A antibody refers to an antibody that is capable of binding to activin A with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting activin A. In certain embodiments, the extent of binding of an anti-activin A antibody to an unrelated, non-activin A protein is less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or less than 1% of the binding of the antibody to activin A as measured, for example, by a radioimmunoassay (RIA). In certain embodiments, an anti-activin A antibody binds to an epitope of activin A that is conserved among orthologous activin A proteins from different species. In preferred embodiments, an anti-activin A antibody of the present disclosure is an antagonist antibody that can substantially inhibit activin A activity. For example, an anti-activin A antibody of the disclosure may substantially inhibit activin A from binding to a cognate receptor (*e.g.*, ActRIIA or ActRIIB receptor) and/or substantially inhibit activin A-mediated signal transduction (activation) of a cognate receptor, such as Smad2/3 signaling by ActRIIA and/or ActRIIB receptors.

[0128] In general, an anti-activin E antibody refers to an antibody that is capable of binding to activin E with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting activin E. In certain embodiments, the extent of binding of an anti-activin E antibody to an unrelated, non-activin E protein is less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or less than 1% of the binding of the antibody to activin E as measured, for example, by a radioimmunoassay (RIA). In certain embodiments, an anti-activin E antibody binds to an epitope of activin E that is conserved among orthologous activin E proteins from different species. In preferred embodiments, an anti-activin E antibody of the present disclosure is an antagonist antibody that can substantially inhibit activin E activity. For example, an anti-activin E antibody of the disclosure may substantially inhibit activin E from binding to a cognate receptor (*e.g.*, ActRIIA or ActRIIB receptor) and/or substantially inhibit activin E-mediated signal transduction (activation) of a cognate receptor, such as Smad2/3 signaling by ActRIIA and/or ActRIIB receptors. In some embodiments, anti-activin E antibodies of the present disclosure do not substantially bind to and/or inhibit activity of activin A.

[0129] In general, an anti-GDF8 antibody refers to an antibody that is capable of binding to GDF8 with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting GDF8. In certain embodiments, the extent of binding of an

anti-GDF8 antibody to an unrelated, non-GDF8 protein is less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or less than 1% of the binding of the antibody to GDF8 as measured, for example, by a radioimmunoassay (RIA). In certain embodiments, an anti-GDF8 antibody binds to an epitope of GDF8 that is conserved among orthologous GDF8 proteins from different species. In preferred embodiments, an anti-GDF8 antibody of the present disclosure is an antagonist antibody that can substantially inhibit GDF8 activity. For example, an anti-GDF8 antibody of the disclosure may substantially inhibit GDF8 from binding to a cognate receptor (*e.g.*, ActRIIA or ActRIIB receptor) and/or substantially inhibit GDF8-mediated signal transduction (activation) of a cognate receptor, such as Smad2/3 signaling by ActRIIA and/or ActRIIB receptors. In some embodiments, anti-GDF8 antibodies of the present disclosure do not substantially bind to and/or inhibit activity of activin A. It should be noted that GDF8 has high sequence homology to GDF11 and therefore antibodies that bind and/or to GDF8, in many cases, may also bind to and/or inhibit GDF11.

[0130] In general, an anti-BMP6 antibody refers to an antibody that is capable of binding to BMP6 with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting BMP6. In certain embodiments, the extent of binding of an anti-BMP6 antibody to an unrelated, non-BMP6 protein is less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or less than 1% of the binding of the antibody to BMP6 as measured, for example, by a radioimmunoassay (RIA). In certain embodiments, an anti-BMP6 antibody binds to an epitope of BMP6 that is conserved among orthologous BMP6 proteins from different species. In preferred embodiments, an anti-BMP6 antibody of the present disclosure is an antagonist antibody that can substantially inhibit BMP6 activity. For example, an anti-BMP6 antibody of the disclosure may substantially inhibit BMP6 from binding to a cognate receptor (*e.g.*, ActRIIA or ActRIIB receptor) and/or substantially inhibit BMP6-mediated signal transduction (activation) of a cognate receptor, such as Smad2/3 signaling by ActRIIA and/or ActRIIB receptors. In some embodiments, anti-BMP6 antibodies of the present disclosure do not substantially bind to and/or inhibit activity of activin A.

[0131] In general, an anti-GDF15 antibody refers to an antibody that is capable of binding to GDF15 with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting GDF15. In certain embodiments, the extent of binding of an anti-GDF15 antibody to an unrelated, non-GDF15 protein is less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or less than 1% of the binding of the antibody to GDF15 as measured, for example, by a radioimmunoassay (RIA). In certain embodiments, an anti-

GDF15 antibody binds to an epitope of GDF15 that is conserved among orthologous GDF15 proteins from different species. In preferred embodiments, an anti-GDF15 antibody of the present disclosure is an antagonist antibody that can substantially inhibit GDF15 activity. For example, an anti-GDF15 antibody of the disclosure may substantially inhibit GDF15 from binding to a cognate receptor (*e.g.*, ActRIIA or ActRIIB receptor) and/or substantially inhibit GDF15-mediated signal transduction (activation) of a cognate receptor, such as Smad 2/3 signaling by ActRIIA and/or ActRIIB receptors. In some embodiments, anti-GDF15 antibodies of the present disclosure do not substantially bind to and/or inhibit activity of activin A.

[0132] In general, an anti-Nodal antibody refers to an antibody that is capable of binding to Nodal with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting Nodal. In certain embodiments, the extent of binding of an anti-Nodal antibody to an unrelated, non-Nodal protein is less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or less than 1% of the binding of the antibody to Nodal as measured, for example, by a radioimmunoassay (RIA). In certain embodiments, an anti-Nodal antibody binds to an epitope of Nodal that is conserved among orthologous Nodal proteins from different species. In preferred embodiments, an anti-Nodal antibody of the present disclosure is an antagonist antibody that can substantially inhibit Nodal activity. For example, an anti-Nodal antibody of the disclosure may substantially inhibit Nodal from binding to a cognate receptor (*e.g.*, ActRIIA or ActRIIB receptor) and/or substantially inhibit Nodal-mediated signal transduction (activation) of a cognate receptor, such as Smad 2/3 signaling by ActRIIA and/or ActRIIB receptors. In some embodiments, anti-Nodal antibodies of the present disclosure do not substantially bind to and/or inhibit activity of activin A.

[0133] In general, an anti-GDF3 antibody refers to an antibody that is capable of binding to GDF3 with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting GDF3. In certain embodiments, the extent of binding of an anti-GDF3 antibody to an unrelated, non-GDF3 protein is less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or less than 1% of the binding of the antibody to GDF3 as measured, for example, by a radioimmunoassay (RIA). In certain embodiments, an anti-GDF3 antibody binds to an epitope of GDF3 that is conserved among orthologous GDF3 proteins from different species. In preferred embodiments, an anti-GDF3 antibody of the present disclosure is an antagonist antibody that can substantially inhibit GDF3 activity. For example, an anti-GDF3 antibody of the disclosure may substantially inhibit GDF3 from binding to a cognate

receptor (*e.g.*, ActRIIA or ActRIIB receptor) and/or substantially inhibit GDF3-mediated signal transduction (activation) of a cognate receptor, such as Smad 2/3 signaling by ActRIIA and/or ActRIIB receptors. In some embodiments, anti-GDF3 antibodies of the present disclosure do not substantially bind to and/or inhibit activity of activin A.

5 [0134] In general, an anti-BMP3 antibody refers to an antibody that is capable of binding to BMP3 with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting BMP3. In certain embodiments, the extent of binding of an anti-BMP3 antibody to an unrelated, non-BMP3 protein is less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or less than 1% of the binding of the antibody to BMP3 as measured,  
10 for example, by a radioimmunoassay (RIA). In certain embodiments, an anti-BMP3 antibody binds to an epitope of BMP3 that is conserved among orthologous BMP3 proteins from different species. In preferred embodiments, an anti-BMP3 antibody of the present disclosure is an antagonist antibody that can substantially inhibit BMP3 activity. For example, an anti-BMP3 antibody of the disclosure may substantially inhibit BMP3 from binding to a cognate  
15 receptor (*e.g.*, ActRIIA or ActRIIB receptor) and/or substantially inhibit BMP3-mediated signal transduction (activation) of a cognate receptor, such as Smad 2/3 signaling by ActRIIA and/or ActRIIB receptors. In some embodiments, anti-BMP3 antibodies of the present disclosure do not substantially bind to and/or inhibit activity of activin A.

[0135] In general, an anti-BMP3B antibody refers to an antibody that is capable of  
20 binding to BMP3B with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting BMP3B. In certain embodiments, the extent of binding of an anti-BMP3B antibody to an unrelated, non-BMP3B protein is less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or less than 1% of the binding of the antibody to BMP3B as measured, for example, by a radioimmunoassay (RIA). In certain embodiments, an anti-  
25 BMP3B antibody binds to an epitope of BMP3B that is conserved among orthologous BMP3B proteins from different species. In preferred embodiments, an anti-BMP3B antibody of the present disclosure is an antagonist antibody that can substantially inhibit BMP3B activity. For example, an anti-BMP3B antibody of the disclosure may substantially inhibit BMP3B from binding to a cognate receptor (*e.g.*, ActRIIA or ActRIIB receptor) and/or  
30 substantially inhibit BMP3B-mediated signal transduction (activation) of a cognate receptor, such as Smad 2/3 signaling by ActRIIA and/or ActRIIB receptors. In some embodiments, anti-BMP3B antibodies of the present disclosure do not substantially bind to and/or inhibit activity of activin A.

[0136] In general, an anti-BMP9 antibody refers to an antibody that is capable of binding to BMP9 with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting BMP9. In certain embodiments, the extent of binding of an anti-BMP9 antibody to an unrelated, non-BMP9 protein is less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or less than 1% of the binding of the antibody to BMP9 as measured, for example, by a radioimmunoassay (RIA). In certain embodiments, an anti-BMP9 antibody binds to an epitope of BMP9 that is conserved among orthologous BMP9 proteins from different species. In preferred embodiments, an anti-BMP9 antibody of the present disclosure is an antagonist antibody that can substantially inhibit BMP9 activity. For example, an anti-BMP9 antibody of the disclosure may substantially inhibit BMP9 from binding to a cognate receptor (*e.g.*, ActRIIA or ActRIIB receptor) and/or substantially inhibit BMP9-mediated signal transduction (activation) of a cognate receptor, such as Smad 2/3 signaling by ActRIIA and/or ActRIIB receptors. In some embodiments, anti-BMP9 antibodies of the present disclosure do not substantially bind to and/or inhibit activity of activin A. In certain embodiments, anti-BMP9 antibodies inhibit the interaction between BMP9 and a type II receptor of the TGF $\beta$  superfamily (*e.g.*, ActRIIA and/or ActRIIB). Preferably, anti-BMP9 antibodies do not inhibit, or substantially inhibit, interaction between BMP9 and ALK1.

[0137] In general, an anti-BMP10 antibody refers to an antibody that is capable of binding to BMP10 with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting BMP10. In certain embodiments, the extent of binding of an anti-BMP10 antibody to an unrelated, non-BMP10 protein is less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or less than 1% of the binding of the antibody to BMP10 as measured, for example, by a radioimmunoassay (RIA). In certain embodiments, an anti-BMP10 antibody binds to an epitope of BMP10 that is conserved among orthologous BMP10 proteins from different species. In preferred embodiments, an anti-BMP10 antibody of the present disclosure is an antagonist antibody that can substantially inhibit BMP10 activity. For example, an anti-BMP10 antibody of the disclosure may substantially inhibit BMP10 from binding to a cognate receptor (*e.g.*, ActRIIA or ActRIIB receptor) and/or substantially inhibit BMP10-mediated signal transduction (activation) of a cognate receptor, such as Smad 2/3 signaling by ActRIIA and/or ActRIIB receptors. In some embodiments, anti-BMP10 antibodies of the present disclosure do not substantially bind to and/or inhibit activity of activin A. In certain embodiments, anti-BMP10 antibodies inhibit the interaction between BMP10 and a type II receptor of the TGF $\beta$  superfamily (*e.g.*, ActRIIA and/or ActRIIB).

Preferably, anti-BMP10 antibodies do not inhibit, or substantially inhibit, interaction between BMP10 and ALK1.

[0138] An anti-ActRIIA antibody refers to an antibody that is capable of binding to ActRIIA with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting ActRIIA. In certain embodiments, the extent of binding of an anti-ActRIIA antibody to an unrelated, non-ActRIIA protein is less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or less than 1% of the binding of the antibody to ActRIIA as measured, for example, by a radioimmunoassay (RIA). In certain embodiments, an anti-ActRIIA antibody binds to an epitope of ActRIIA that is conserved among orthologous ActRIIA proteins from different species. In preferred embodiments, an anti-ActRIIA antibody of the present disclosure is an antagonist antibody that can inhibit an ActRIIA activity. For example, an anti-ActRIIA antibody of the present disclosure may inhibit one or more ActRIIA ligands selected from activin B, activin C, activin E, GDF11, GDF8, activin A, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, or BMP10 from binding to the ActRIIA receptor and/or inhibit one of these ligands from activating ActRIIA signaling (*e.g.*, through Smad2/3 and/or Smad 1/5/8 pathways). In preferred embodiments, anti-ActRIIA antibodies of the present disclosure inhibit GDF11 and/or activin B from binding to the ActRIIA receptor and/or inhibit GDF11 and/or activin B from activating ActRIIA signaling. Optionally, anti-ActRIIA antibodies of the disclosure further inhibit GDF8 from binding to the ActRIIA receptor and/or inhibit GDF8 from activating ActRIIA signaling. Optionally, anti-ActRIIA antibodies of the present disclosure do not substantially inhibit activin A from binding to the ActRIIA receptor and/or do not substantially inhibit activin A-mediated activation of ActRIIA signaling. In some embodiments, an anti-ActRIIA antibody of the disclosure that inhibits GDF11 and/or activin B from binding to and/or activating an ActRIIA receptor further inhibits one or more of activin C, activin E, activin A, GDF8, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10 from binding to and/or activating the ActRIIA receptor.

[0139] An anti-ActRIIB antibody refers to an antibody that is capable of binding to ActRIIB with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting ActRIIB. In certain embodiments, the extent of binding of an anti-ActRIIB antibody to an unrelated, non-ActRIIB protein is less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or less than 1% of the binding of the antibody to ActRIIB as measured, for example, by a radioimmunoassay (RIA). In certain embodiments, an anti-

ActRIIB antibody binds to an epitope of ActRIIB that is conserved among orthologous ActRIIB proteins from different species. In preferred embodiments, an anti-ActRIIB antibody of the present disclosure is an antagonist antibody that can inhibit an ActRIIB activity. For example, an anti-ActRIIB antibody of the present disclosure may inhibit one or more ActRIIB ligands selected from activin B, activin C, activin E, GDF11, GDF8, activin A, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10 from binding to the ActRIIB receptor and/or inhibit one of these ligands from activating ActRIIB signaling (*e.g.*, through Smad2/3 and/or Smad 1/5/8 pathways). In preferred embodiments, anti-ActRIIB antibodies of the present disclosure inhibit GDF11 and/or activin B from binding to the ActRIIB receptor and/or inhibit GDF11 and/or activin B from activating ActRIIB signaling. Optionally, anti-ActRIIB antibodies of the disclosure further inhibit GDF8 from binding to the ActRIIB receptor and/or inhibit GDF8 from activating ActRIIB signaling. Optionally, anti-ActRIIB antibodies of the present disclosure do not substantially inhibit activin A from binding to the ActRIIB receptor and/or do not substantially inhibit activin A-mediated activation of ActRIIB signaling. In some embodiments, an anti-ActRIIB antibody of the disclosure that inhibits GDF11 and/or activin B from binding to and/or activating an ActRIIB receptor further inhibits one or more of activin C, activin E, activin A, GDF8, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10 from binding to and/or activating the ActRIIB receptor.

[0140] The nucleic acid and amino acid sequences of human GDF11, activin A, activin B, activin C, activin E, GDF8, BMP6, ActRIIB, ActRIIA, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP9 are well known in the art. In addition, numerous methods for generating antibodies are well known in the art, some of which are described herein. Therefore antibody antagonists for use in accordance with this disclosure may be routinely made by the skilled person in the art based on the knowledge in the art and teachings provided herein.

[0141] In certain embodiments, an antibody provided herein (*e.g.*, an anti-GDF11 antibody, an anti-activin B antibody, an anti-ActRIIA antibody, or an anti-ActRIIB antibody) is a chimeric antibody. A chimeric antibody refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species. Certain chimeric antibodies are described, for example, in U.S. Pat. No. 4,816,567; and Morrison *et al.*, (1984) Proc. Natl. Acad. Sci. USA, 81:6851-6855. In some embodiments, a chimeric antibody

comprises a non-human variable region (*e.g.*, a variable region derived from a mouse, rat, hamster, rabbit, or non-human primate, such as a monkey) and a human constant region. In some embodiments, a chimeric antibody is a “class switched” antibody in which the class or subclass has been changed from that of the parent antibody. In general, chimeric antibodies include antigen-binding fragments thereof.

[0142] In certain embodiments, a chimeric antibody provided herein (*e.g.*, an anti-GDF11 antibody, an anti-activin B antibody, an anti-ActRIIA antibody, or an anti-ActRIIb antibody) is a humanized antibody. A humanized antibody refers to a chimeric antibody comprising amino acid residues from non-human hypervariable regions (HVRs) and amino acid residues from human framework regions (FRs). In certain embodiments, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the HVRs (*e.g.*, CDRs) correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of a human antibody. A humanized antibody optionally may comprise at least a portion of an antibody constant region derived from a human antibody. A “humanized form” of an antibody, *e.g.*, a non-human antibody, refers to an antibody that has undergone humanization.

[0143] Humanized antibodies and methods of making them are reviewed, for example, in Almagro and Fransson (2008) *Front. Biosci.* 13:1619-1633 and are further described, for example, in Riechmann *et al.*, (1988) *Nature* 332:323-329; Queen *et al.* (1989) *Proc. Nat’l Acad. Sci. USA* 86:10029-10033; U.S. Pat. Nos. 5,821,337; 7,527,791; 6,982,321; and 7,087,409; Kashmiri *et al.*, (2005) *Methods* 36:25-34 [describing SDR (a-CDR) grafting]; Padlan, *Mol. Immunol.* (1991) 28:489-498 (describing “resurfacing”); Dall’Acqua *et al.* (2005) *Methods* 36:43-60 (describing “FR shuffling”); Osbourn *et al.* (2005) *Methods* 36:61-68; and Klimka *et al.* *Br. J. Cancer* (2000) 83:252-260 (describing the “guided selection” approach to FR shuffling).

[0144] Human framework regions that may be used for humanization include but are not limited to: framework regions selected using the “best-fit” method [see, *e.g.*, Sims *et al.* (1993) *J. Immunol.* 151:2296 ]; framework regions derived from the consensus sequence of human antibodies of a particular subgroup of light or heavy chain variable regions [see, *e.g.*, Carter *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, 89:4285; and Presta *et al.* (1993) *J. Immunol.*, 151:2623]; human mature (somatically mutated) framework regions or human germline framework regions [see, *e.g.*, Almagro and Fransson (2008) *Front. Biosci.* 13:1619-1633]; and framework regions derived from screening FR libraries [see, *e.g.*, Baca *et al.*,

(1997) J. Biol. Chem. 272:10678-10684; and Rosok et al., (1996) J. Biol. Chem. 271:22611-22618].

[0145] In certain embodiments, an antibody provided herein (*e.g.*, an anti-GDF11 antibody, an anti-activin B antibody, an anti-ActRIIA antibody, or an anti-ActRIIB antibody) is a human antibody. Human antibodies can be produced using various techniques known in the art. Human antibodies are described generally in van Dijk and van de Winkel (2008) Curr. Opin. Pharmacol. 5: 368-74 (2001) and Lonberg, Curr. Opin. Immunol. 20:450-459.

[0146] Human antibodies may be prepared by administering an immunogen (*e.g.*, a GDF11 polypeptide, an activin B polypeptide, an ActRIIA polypeptide, or an ActRIIB polypeptide) to a transgenic animal that has been modified to produce intact human antibodies or intact antibodies with human variable regions in response to antigenic challenge. Such animals typically contain all or a portion of the human immunoglobulin loci, which replace the endogenous immunoglobulin loci, or which are present extrachromosomally or integrated randomly into the animal's chromosomes. In such transgenic animals, the endogenous immunoglobulin loci have generally been inactivated. For a review of methods for obtaining human antibodies from transgenic animals see, for example, Lonberg (2005) Nat. Biotech. 23:1117-1125; U.S. Pat. Nos. 6,075,181 and 6,150,584 (describing XENOMOUSE™ technology); U.S. Pat. No. 5,770,429 (describing HuMab® technology); U.S. Pat. No. 7,041,870 (describing K-M MOUSE® technology); and U.S. Patent Application Publication No. 2007/0061900 (describing VelociMouse® technology). Human variable regions from intact antibodies generated by such animals may be further modified, for example, by combining with a different human constant region.

[0147] Human antibodies provided herein can also be made by hybridoma-based methods. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described [see, *e.g.*, Kozbor J. Immunol., (1984) 133: 3001; Brodeur *et al.* (1987) Monoclonal Antibody Production Techniques and Applications, pp. 51-63, Marcel Dekker, Inc., New York; and Boerner *et al.* (1991) J. Immunol., 147: 86]. Human antibodies generated via human B-cell hybridoma technology are also described in Li *et al.*, (2006) Proc. Natl. Acad. Sci. USA, 103:3557-3562. Additional methods include those described, for example, in U.S. Pat. No. 7,189,826 (describing production of monoclonal human IgM antibodies from hybridoma cell lines) and Ni, Xiandai Mianyixue (2006) 26(4):265-268 (2006) (describing human-human hybridomas). Human hybridoma technology (Trioma technology) is also described in Vollmers and Brandlein

(2005) *Histol. Histopathol.*, 20(3):927-937 (2005) and Vollmers and Brandlein (2005) *Methods Find Exp. Clin. Pharmacol.*, 27(3):185-91.

[0148] Human antibodies provided herein (*e.g.*, an anti-GDF11 antibody, an anti-activin B antibody, an anti-ActRIIA antibody, or an anti-ActRIIB antibody) may also be generated by isolating Fv clone variable-domain sequences selected from human-derived phage display libraries. Such variable-domain sequences may then be combined with a desired human constant domain. Techniques for selecting human antibodies from antibody libraries are described herein.

[0149] For example, antibodies of the present disclosure may be isolated by screening combinatorial libraries for antibodies with the desired activity or activities. A variety of methods are known in the art for generating phage display libraries and screening such libraries for antibodies possessing the desired binding characteristics. Such methods are reviewed, for example, in Hoogenboom *et al.* (2001) in *Methods in Molecular Biology* 178:1-37, O'Brien *et al.*, ed., Human Press, Totowa, N.J. and further described, for example, in the McCafferty *et al.* (1991) *Nature* 348:552-554; Clackson *et al.*, (1991) *Nature* 352: 624-628; Marks *et al.* (1992) *J. Mol. Biol.* 222:581-597; Marks and Bradbury (2003) in *Methods in Molecular Biology* 248:161-175, Lo, ed., Human Press, Totowa, N.J.; Sidhu *et al.* (2004) *J. Mol. Biol.* 338(2):299-310; Lee *et al.* (2004) *J. Mol. Biol.* 340(5):1073-1093; Fellouse (2004) *Proc. Natl. Acad. Sci. USA* 101(34):12467-12472; and Lee *et al.* (2004) *J. Immunol. Methods* 284(1-2): 119-132.

[0150] In certain phage display methods, repertoires of VH and VL genes are separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be screened for antigen-binding phage as described in Winter *et al.* (1994) *Ann. Rev. Immunol.*, 12: 433-455. Phage typically display antibody fragments, either as single-chain Fv (scFv) fragments or as Fab fragments. Libraries from immunized sources provide high-affinity antibodies to the immunogen (*e.g.*, GDF11, activin B, ActRIIA, or ActRIIB) without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned (*e.g.*, from human) to provide a single source of antibodies to a wide range of non-self and also self-antigens without any immunization as described by Griffiths *et al.* (1993) *EMBO J*, 12: 725-734. Finally, naive libraries can also be made synthetically by cloning unrearranged V-gene segments from stem cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement *in vitro*, as described by Hoogenboom and Winter (1992) *J. Mol. Biol.*, 227:

381-388. Patent publications describing human antibody phage libraries include, for example: U.S. Pat. No. 5,750,373, and U.S. Patent Publication Nos. 2005/0079574, 2005/0119455, 2005/0266000, 2007/0117126, 2007/0160598, 2007/0237764, 2007/0292936, and 2009/0002360.

5 [0151] In certain embodiments, an antibody provided herein is a multispecific antibody, for example, a bispecific antibody. Multispecific antibodies (typically monoclonal antibodies) that have binding specificities for at least two different epitopes (*e.g.*, two, three, four, five, or six or more) on one or more (*e.g.*, two, three, four, five, six or more) antigens.

[0152] In certain embodiments, multispecific antibodies of the present disclosure  
10 comprise two or more binding specificities, with at least one of the binding specificities being for a GDF11 epitope, and optionally one or more additional binding specificities being for an epitope on a different ActRII ligand (*e.g.*, GDF8, activin B, activin C, activin E, activin A, GDF15, Nodal, GDF3, GDF3B, BMP9, BMP10, and/or BMP6) and/or an ActRII receptor (*e.g.*, an ActRIIA and/or ActRIIB receptor). In certain embodiments, multispecific antibodies  
15 may bind to two or more different epitopes of GDF11. Preferably, a multispecific antibody of the disclosure that has binding affinity, in part, for an GDF11 epitope can be used to inhibit an GDF11 activity (*e.g.*, the ability to bind to and/or activate an ActRIIA and/or ActRIIB receptor), and optionally inhibit the activity of one or more different ActRII ligands (*e.g.*, GDF8, activin B, activin C, activin E, activin A, GDF15, Nodal, GDF3, GDF3B,  
20 BMP9, BMP10, and/or BMP6) and/or an ActRII receptor (*e.g.*, an ActRIIA or ActRIIB receptor). In preferred embodiments, multispecific antibodies of the present disclosure that bind to and/or inhibit GDF11 further bind to and/or inhibit at least activin B. Optionally, multispecific antibodies of the disclosure that bind to and/or inhibit GDF11 do not bind to and/or inhibit activin A. In some embodiments, multispecific antibodies of the disclosure  
25 that bind to and/or inhibit GDF11 further bind to and/or inhibit at least GDF8.

[0153] In certain embodiments, multispecific antibodies of the present disclosure comprise two or more binding specificities, with at least one of the binding specificities being for an activin B epitope, and optionally one or more additional binding specificities being for an epitope on a different ActRII ligand (*e.g.*, GDF8, GDF11, activin C, activin E, activin A, GDF15, Nodal, GDF3, GDF3B, BMP9, BMP10, and/or BMP6) and/or an ActRII receptor (*e.g.*, an ActRIIA and/or ActRIIB receptor). In certain embodiments, multispecific antibodies  
30 may bind to two or more different epitopes of activin B. Preferably, a multispecific antibody of the disclosure that has binding affinity, in part, for an activin B epitope can be used to

inhibit an activin B activity (*e.g.*, the ability to bind to and/or activate an ActRIIA and/or ActRIIB receptor), and optionally inhibit the activity of one or more different ActRII ligands (*e.g.*, GDF8, GDF11, activin C, activin E, activin A, GDF15, Nodal, GDF3, GDF3B, BMP9, BMP10, and/or BMP6) and/or an ActRII receptor (*e.g.*, an ActRIIA or ActRIIB receptor). In preferred embodiments, multispecific antibodies of the present disclosure that bind to and/or inhibit activin B further bind to and/or inhibit at least GDF11. Optionally, multispecific antibodies of the disclosure that bind to and/or inhibit activin B do not bind to and/or inhibit activin A. In some embodiments, multispecific antibodies of the disclosure that bind to and/or inhibit activin B further bind to and/or inhibit at least GDF8.

[0154] In certain embodiments, multispecific antibodies of the present disclosure comprise two or more binding specificities, with at least one of the binding specificities being for a GDF8 epitope, and optionally one or more additional binding specificities being for an epitope on a different ActRII ligand (*e.g.*, GDF11, activin B, activin C, activin E, activin A, GDF15, Nodal, GDF3, GDF3B, BMP9, BMP10, and/or BMP6) and/or an ActRII receptor (*e.g.*, an ActRIIA and/or ActRIIB receptor). In certain embodiments, multispecific antibodies may bind to two or more different epitopes of GDF8. Preferably, a multispecific antibody of the disclosure that has binding affinity, in part, for an GDF8 epitope can be used to inhibit an GDF8 activity (*e.g.*, the ability to bind to and/or activate an ActRIIA and/or ActRIIB receptor), and optionally inhibit the activity of one or more different ActRII ligands (*e.g.*, GDF11, activin B, activin C, activin E, activin A, GDF15, Nodal, GDF3, GDF3B, BMP9, BMP10, and/or BMP6) and/or an ActRII receptor (*e.g.*, an ActRIIA or ActRIIB receptor). In preferred embodiments, multispecific antibodies of the present disclosure that bind to and/or inhibit GDF8 further bind to and/or inhibit at least GDF11 and/or activin B. Optionally, multispecific antibodies of the disclosure that bind to and/or inhibit GDF8 do not bind to and/or inhibit activin A.

[0155] In certain embodiments, multispecific antibodies of the present disclosure comprise two or more binding specificities, with at least one of the binding specificities being for an activin C epitope, and optionally one or more additional binding specificities being for an epitope on a different ActRII ligand (*e.g.*, GDF8, GDF11, activin B, activin E, activin A, GDF15, Nodal, GDF3, GDF3B, BMP9, BMP10, and/or BMP6) and/or an ActRII receptor (*e.g.*, an ActRIIA and/or ActRIIB receptor). In certain embodiments, multispecific antibodies may bind to two or more different epitopes of activin C. Preferably, a multispecific antibody of the disclosure that has binding affinity, in part, for an activin C epitope can be used to

inhibit an activin C activity (*e.g.*, the ability to bind to and/or activate an ActRIIA and/or ActRIIB receptor), and optionally inhibit the activity of one or more different ActRII ligands (*e.g.*, GDF8, GDF11, activin B, activin E, activin A, GDF15, Nodal, GDF3, GDF3B, BMP9, BMP10, and/or BMP6) and/or an ActRII receptor (*e.g.*, an ActRIIA or ActRIIB receptor). In preferred embodiments, multispecific antibodies of the present disclosure that bind to and/or inhibit activin C further bind to and/or inhibit at least GDF11 and/or activin B. Optionally, multispecific antibodies of the disclosure that bind to and/or inhibit activin C do not bind to and/or inhibit activin A. Optionally, multispecific antibodies of the disclosure that bind to and/or inhibit activin C further bind to and/or inhibit GDF8.

[0156] In certain embodiments, multispecific antibodies of the present disclosure comprise two or more binding specificities, with at least one of the binding specificities being for an activin E epitope, and optionally one or more additional binding specificities being for an epitope on a different ActRII ligand (*e.g.*, GDF8, GDF11, activin C, activin B, activin A, GDF15, Nodal, GDF3, GDF3B, BMP9, BMP10, and/or BMP6) and/or an ActRII receptor (*e.g.*, an ActRIIA and/or ActRIIB receptor). In certain embodiments, multispecific antibodies may bind to two or more different epitopes of activin E. Preferably, a multispecific antibody of the disclosure that has binding affinity, in part, for an activin E epitope can be used to inhibit an activin E activity (*e.g.*, the ability to bind to and/or activate an ActRIIA and/or ActRIIB receptor), and optionally inhibit the activity of one or more different ActRII ligands (*e.g.*, GDF8, GDF11, activin C, activin A, GDF15, Nodal, GDF3, GDF3B, BMP9, BMP10, and/or BMP6) and/or an ActRII receptor (*e.g.*, an ActRIIA or ActRIIB receptor). In preferred embodiments, multispecific antibodies of the present disclosure that bind to and/or inhibit activin E further bind to and/or inhibit at least GDF11 and/or activin B. Optionally, multispecific antibodies of the disclosure that bind to and/or inhibit activin E do not bind to and/or inhibit activin A. Optionally, multispecific antibodies of the disclosure that bind to and/or inhibit activin E further bind to and/or inhibit GDF8.

[0157] In certain embodiments, multispecific antibodies of the present disclosure comprise two or more binding specificities, with at least one of the binding specificities being for a BMP6 epitope, and optionally one or more additional binding specificities being for an epitope on a different ActRII ligand (*e.g.*, GDF11, activin B, activin C, activin E, GDF8, activin A, GDF15, Nodal, GDF3, GDF3B, BMP9, and BMP10) and/or an ActRII receptor (*e.g.*, an ActRIIA and/or ActRIIB receptor). In certain embodiments, multispecific antibodies may bind to two or more different epitopes of BMP6. Preferably, a multispecific antibody of

the disclosure that has binding affinity, in part, for a BMP6 epitope can be used to inhibit a BMP6 activity (*e.g.*, the ability to bind to and/or activate an ActRIIA and/or ActRIIB receptor), and optionally inhibit the activity of one or more different ActRII ligands (*e.g.*, GDF11, activin B, activin C, activin E, GDF8, activin A, GDF15, Nodal, GDF3, GDF3B, BMP9, and BMP10) and/or an ActRII receptor (*e.g.*, an ActRIIA or ActRIIB receptor). In preferred embodiments, multispecific antibodies of the present disclosure that bind to and/or inhibit BMP6 further bind to and/or inhibit at least GDF11 and/or activin B. Optionally, multispecific antibodies of the disclosure that bind to and/or inhibit BMP6 do not bind to and/or inhibit activin A. Optionally, multispecific antibodies of the disclosure that bind to and/or inhibit BMP6 further bind to and/or inhibit GDF8.

[0158] In certain embodiments, multispecific antibodies of the present disclosure comprise two or more binding specificities, with at least one of the binding specificities being for a BMP9 epitope, and optionally one or more additional binding specificities being for an epitope on a different ActRII ligand (*e.g.*, GDF11, activin B, activin C, activin E, GDF8, activin A, GDF15, Nodal, GDF3, GDF3B, and BMP10) and/or an ActRII receptor (*e.g.*, an ActRIIA and/or ActRIIB receptor). In certain embodiments, multispecific antibodies may bind to two or more different epitopes of BMP9. Preferably, a multispecific antibody of the disclosure that has binding affinity, in part, for a BMP9 epitope can be used to inhibit a BMP9 activity (*e.g.*, the ability to bind to and/or activate an ActRIIA and/or ActRIIB receptor), and optionally inhibit the activity of one or more different ActRII ligands (*e.g.*, GDF11, activin B, activin C, activin E, GDF8, activin A, GDF15, Nodal, GDF3, GDF3B, and BMP10) and/or an ActRII receptor (*e.g.*, an ActRIIA or ActRIIB receptor). In preferred embodiments, multispecific antibodies of the present disclosure that bind to and/or inhibit BMP9 further bind to and/or inhibit at least GDF11 and/or activin B. Optionally, multispecific antibodies of the disclosure that bind to and/or inhibit BMP9 do not bind to and/or inhibit activin A. Optionally, multispecific antibodies of the disclosure that bind to and/or inhibit BMP9 further bind to and/or inhibit GDF8. In certain embodiments, multispecific antibodies that bind to BMP9 inhibit interaction between BMP9 and a type II receptor of the TGF $\beta$  superfamily (*e.g.*, ActRIIA and/or ActRIIB). Preferably, multispecific antibodies that bind to BMP9 do not inhibit, or substantially inhibit, interaction between BMP9 and ALK1.

[0159] In certain embodiments, multispecific antibodies of the present disclosure comprise two or more binding specificities, with at least one of the binding specificities being

for a BMP10 epitope, and optionally one or more additional binding specificities being for an epitope on a different ActRII ligand (*e.g.*, GDF11, activin B, activin C, activin E, GDF8, activin A, GDF15, Nodal, GDF3, GDF3B, and BMP9) and/or an ActRII receptor (*e.g.*, an ActRIIA and/or ActRIIB receptor). In certain embodiments, multispecific antibodies may  
5 bind to two or more different epitopes of BMP10. Preferably, a multispecific antibody of the disclosure that has binding affinity, in part, for a BMP10 epitope can be used to inhibit a BMP10 activity (*e.g.*, the ability to bind to and/or activate an ActRIIA and/or ActRIIB receptor), and optionally inhibit the activity of one or more different ActRII ligands (*e.g.*, GDF11, activin B, activin C, activin E, GDF8, activin A, GDF15, Nodal, GDF3, GDF3B, and  
10 BMP9) and/or an ActRII receptor (*e.g.*, an ActRIIA or ActRIIB receptor). In preferred embodiments, multispecific antibodies of the present disclosure that bind to and/or inhibit BMP10 further bind to and/or inhibit at least GDF11 and/or activin B. Optionally, multispecific antibodies of the disclosure that bind to and/or inhibit BMP10 do not bind to and/or inhibit activin A. Optionally, multispecific antibodies of the disclosure that bind to  
15 and/or inhibit BMP10 further bind to and/or inhibit GDF8. In certain embodiments, multispecific antibodies that bind to BMP10 inhibit interaction between BMP10 and a type II receptor of the TGF $\beta$  superfamily (*e.g.*, ActRIIA and/or ActRIIB). Preferably, multispecific antibodies that bind to BMP10 do not inhibit, or substantially inhibit, interaction between BMP10 and ALK1.

[0160] In certain embodiments, multispecific antibodies of the present disclosure comprise two or more binding specificities, with at least one of the binding specificities being for a GDF11 epitope, and one other binding specificity being for an activin B epitope. Preferably, a multispecific antibody of the disclosure that has binding affinity, in part, for a GDF11 epitope and an activin B epitope can be used to inhibit both a GDF11 and an activin  
25 B activity (*e.g.*, the ability to bind to and/or activate an ActRIIA and/or ActRIIB receptor). In some embodiments, a multispecific antibody that binds to and/or inhibits GDF11 and activin B activity further binds to and/or inhibits one or more of GDF8, activin A, activin C, activin E, GDF15, Nodal, GDF3, GDF3B, BMP9, BMP10, and/or BMP6. Optionally, a multispecific antibody that binds to and/or inhibits GDF11 and activin B does not  
30 substantially bind to and/or inhibit activin A.

[0161] Techniques for making multispecific antibodies include, but are not limited to, recombinant co-expression of two immunoglobulin heavy-chain/light-chain pairs having different specificities [see, *e.g.*, Milstein and Cuello (1983) *Nature* 305: 537; International

patent publication no. WO 93/08829; and Traunecker *et al.* (1991) EMBO J. 10: 3655, and U.S. Pat. No. 5,731,168 (“knob-in-hole” engineering)]. Multispecific antibodies may also be made by engineering electrostatic steering effects for making antibody Fc-heterodimeric molecules (see, *e.g.*, WO 2009/089004A1); cross-linking two or more antibodies or fragments [see, *e.g.*, U.S. Pat. No. 4,676,980; and Brennan *et al.* (1985) Science, 229: 81]; using leucine zippers to produce bispecific antibodies [see, *e.g.*, Kostelny *et al.* (1992) J. Immunol., 148(5):1547-1553]; using “diabody” technology for making bispecific antibody fragments [see, *e.g.*, Hollinger *et al.* (1993) Proc. Natl. Acad. Sci. USA, 90:6444-6448]; using single-chain Fv (sFv) dimers [see, *e.g.*, Gruber *et al.* (1994) J. Immunol., 152:5368]; and preparing trispecific antibodies (see, *e.g.*, Tutt *et al.* (1991) J. Immunol. 147: 60. Multispecific antibodies can be prepared as full-length antibodies or antibody fragments.

[0162] Engineered antibodies with three or more functional antigen-binding sites, including “Octopus antibodies,” are also included herein [see, *e.g.*, US 2006/0025576A1].

[0163] In certain embodiments, an antibody disclosed herein (*e.g.*, an anti-GDF11 antibody, an anti-activin B antibody, an anti-ActRIIA antibody, or an anti-ActRIIB antibody) is a monoclonal antibody. Monoclonal antibody refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variant antibodies, *e.g.*, containing naturally occurring mutations or arising during production of a monoclonal antibody preparation, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different epitopes, each monoclonal antibody of a monoclonal antibody preparation is directed against a single epitope on an antigen. Thus, the modifier “monoclonal” indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present methods may be made by a variety of techniques, including but not limited to the hybridoma method, recombinant DNA methods, phage-display methods, and methods utilizing transgenic animals containing all or part of the human immunoglobulin loci, such methods and other exemplary methods for making monoclonal antibodies being described herein.

[0164] For example, by using immunogens derived from GDF11 or activin B, anti-protein/anti-peptide antisera or monoclonal antibodies can be made by standard protocols

[see, *e.g.*, Antibodies: A Laboratory Manual ed. by Harlow and Lane (1988) Cold Spring Harbor Press: 1988]. A mammal, such as a mouse, hamster, or rabbit, can be immunized with an immunogenic form of the GDF11 or activin B polypeptide, an antigenic fragment which is capable of eliciting an antibody response, or a fusion protein. Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. An immunogenic portion of a GDF11 or activin B polypeptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibody production and/or level of binding affinity.

[0165] Following immunization of an animal with an antigenic preparation of GDF11 or activin B, antisera can be obtained and, if desired, polyclonal antibodies can be isolated from the serum. To produce monoclonal antibodies, antibody-producing cells (lymphocytes) can be harvested from an immunized animal and fused by standard somatic cell fusion procedures with immortalizing cells such as myeloma cells to yield hybridoma cells. Such techniques are well known in the art, and include, for example, the hybridoma technique [see, *e.g.*, Kohler and Milstein (1975) *Nature*, 256: 495-497], the human B cell hybridoma technique [see, *e.g.*, Kozbar *et al.* (1983) *Immunology Today*, 4:72], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole *et al.* (1985) *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. pp. 77-96]. Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with a GDF11 or activin B polypeptide, and monoclonal antibodies isolated from a culture comprising such hybridoma cells.

[0166] In certain embodiments, one or more amino acid modifications may be introduced into the Fc region of an antibody provided herein (*e.g.*, an anti-GDF11 antibody, an anti-activin B antibody, an anti-ActRIIA antibody, or an anti-ActRIIB antibody), thereby generating an Fc region variant. The Fc region variant may comprise a human Fc region sequence (*e.g.*, a human IgG1, IgG2, IgG3 or IgG4 Fc region) comprising an amino acid modification (*e.g.*, a substitution, deletion, and/or addition) at one or more amino acid positions.

[0167] For example, the present disclosure contemplates an antibody variant that possesses some but not all effector functions, which make it a desirable candidate for applications in which the half-life of the antibody *in vivo* is important yet certain effector

functions [*e.g.*, complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC)] are unnecessary or deleterious. *In vitro* and/or *in vivo* cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the antibody lacks Fc $\gamma$ R binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express Fc $\gamma$ RIII only, whereas monocytes express Fc $\gamma$ RI, Fc $\gamma$ RII and Fc $\gamma$ RIII. FcR expression on hematopoietic cells is summarized in, for example, Ravetch and Kinet (1991) *Annu. Rev. Immunol.* 9:457-492. Non-limiting examples of *in vitro* assays to assess ADCC activity of a molecule of interest are described in U.S. Pat. No. 5,500,362; Hellstrom, I. *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:7059-7063; Hellstrom, I *et al.* (1985) *Proc. Natl. Acad. Sci. USA* 82:1499-1502; U.S. Pat. No. 5,821,337; Bruggemann, M. *et al.* (1987) *J. Exp. Med.* 166:1351-1361. Alternatively, non-radioactive assays methods may be employed (*e.g.*, ACTI™, non-radioactive cytotoxicity assay for flow cytometry; CellTechnology, Inc. Mountain View, Calif.; and CytoTox 96® non-radioactive cytotoxicity assay, Promega, Madison, Wis.). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and natural killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, for example, in an animal model such as that disclosed in Clynes *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95:652-656. C1q binding assays may also be carried out to confirm that the antibody is unable to bind C1q and hence lacks CDC activity [see, *e.g.*, C1q and C3c binding ELISA in WO 2006/029879 and WO 2005/100402]. To assess complement activation, a CDC assay may be performed [see, *e.g.*, Gazzano-Santoro *et al.* (1996) *J. Immunol. Methods* 202:163; Cragg, M. S. *et al.* (2003) *Blood* 101:1045-1052; and Cragg, M. S, and M. J. Glennie (2004) *Blood* 103:2738-2743]. FcRn binding and *in vivo* clearance/half-life determinations can also be performed using methods known in the art [see, *e.g.*, Petkova, S. B. *et al.* (2006) *Intl. Immunol.* 18(12):1759-1769].

[0168] Antibodies of the present disclosure (*e.g.*, an anti-GDF11 antibody, an anti-activin B antibody, an anti-ActRIIA antibody, or an anti-ActRIIB antibody) with reduced effector function include those with substitution of one or more of Fc region residues 238, 265, 269, 270, 297, 327 and 329 (U.S. Pat. No. 6,737,056). Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including the so-called "DANA" Fc mutant with substitution of residues 265 and 297 to alanine (U.S. Pat. No. 7,332,581).

[0169] In certain embodiments, it may be desirable to create cysteine engineered antibodies, *e.g.*, “thioMAbs,” in which one or more residues of an antibody are substituted with cysteine residues. In particular embodiments, the substituted residues occur at accessible sites of the antibody. By substituting those residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the antibody and may be used to conjugate the antibody to other moieties, such as drug moieties or linker-drug moieties, to create an immunoconjugate, as described further herein. In certain embodiments, any one or more of the following residues may be substituted with cysteine: V205 (Kabat numbering) of the light chain; A118 (EU numbering) of the heavy chain; and S400 (EU numbering) of the heavy chain Fc region. Cysteine engineered antibodies may be generated as described, for example., in U.S. Pat. No. 7,521,541.

[0170] In addition, the techniques used to screen antibodies in order to identify a desirable antibody may influence the properties of the antibody obtained. For example, if an antibody is to be used for binding an antigen in solution, it may be desirable to test solution binding. A variety of different techniques are available for testing interaction between antibodies and antigens to identify particularly desirable antibodies. Such techniques include ELISAs, surface plasmon resonance binding assays (*e.g.*, the Biacore binding assay, Biacore AB, Uppsala, Sweden), sandwich assays (*e.g.*, the paramagnetic bead system of IGEN International, Inc., Gaithersburg, Maryland), western blots, immunoprecipitation assays, and immunohistochemistry.

[0171] In certain embodiments, amino acid sequence variants of the antibodies and/or the binding polypeptides provided herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody and/or binding polypeptide. Amino acid sequence variants of an antibody and/or binding polypeptides may be prepared by introducing appropriate modifications into the nucleotide sequence encoding the antibody and/or binding polypeptide, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of residues within the amino acid sequences of the antibody and/or binding polypeptide. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, *e.g.*, target-binding (GDF11 and/or activin B binding).

[0172] Alterations (*e.g.*, substitutions) may be made in HVRs, for example, to improve antibody affinity. Such alterations may be made in HVR “hotspots,” *i.e.*, residues encoded

by codons that undergo mutation at high frequency during the somatic maturation process [see, *e.g.*, Chowdhury (2008) *Methods Mol. Biol.* 207:179-196 (2008)], and/or SDRs (a-CDRs), with the resulting variant VH or VL being tested for binding affinity. Affinity maturation by constructing and reselecting from secondary libraries has been described in the art [see, *e.g.*, Hoogenboom *et al.*, in *Methods in Molecular Biology* 178:1-37, O'Brien *et al.*, ed., Human Press, Totowa, N.J., (2001). In some embodiments of affinity maturation, diversity is introduced into the variable genes chosen for maturation by any of a variety of methods (*e.g.*, error-prone PCR, chain shuffling, or oligonucleotide-directed mutagenesis). A secondary library is then created. The library is then screened to identify any antibody variants with the desired affinity. Another method to introduce diversity involves HVR-directed approaches, in which several HVR residues (*e.g.*, 4-6 residues at a time) are randomized. HVR residues involved in antigen binding may be specifically identified, *e.g.*, using alanine scanning mutagenesis or modeling. CDR-H3 and CDR-L3 in particular are often targeted.

[0173] In certain embodiments, substitutions, insertions, or deletions may occur within one or more HVRs so long as such alterations do not substantially reduce the ability of the antibody to bind to the antigen. For example, conservative alterations (*e.g.*, conservative substitutions as provided herein) that do not substantially reduce binding affinity may be made in HVRs. Such alterations may be outside of HVR "hotspots" or SDRs. In certain embodiments of the variant VH and VL sequences provided above, each HVR either is unaltered, or contains no more than one, two or three amino acid substitutions.

[0174] A useful method for identification of residues or regions of the antibody and/or the binding polypeptide that may be targeted for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (1989) *Science*, 244:1081-1085. In this method, a residue or group of target residues (*e.g.*, charged residues such as arg, asp, his, lys, and glu) are identified and replaced by a neutral or negatively charged amino acid (*e.g.*, alanine or polyalanine) to determine whether the interaction of the antibody-antigen is affected. Further substitutions may be introduced at the amino acid locations demonstrating functional sensitivity to the initial substitutions. Alternatively, or additionally, a crystal structure of an antigen-antibody complex is determined to identify contact points between the antibody and antigen. Such contact residues and neighboring residues may be targeted or eliminated as candidates for substitution. Variants may be screened to determine whether they contain the desired properties.

[0175] Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue. Other

5 insertional variants of the antibody molecule include the fusion of the N- or C-terminus of the antibody to an enzyme (*e.g.*, for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

[0176] In certain embodiments, an antibody and/or binding polypeptide provided herein may be further modified to contain additional nonproteinaceous moieties that are known in

10 the art and readily available. The moieties suitable for derivatization of the antibody and/or binding polypeptide include but are not limited to water soluble polymers. Non-limiting

examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane,

15 ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(*n*-vinyl pyrrolidone)polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (*e.g.*, glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol

propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number

20 of polymers attached to the antibody and/or binding polypeptide may vary, and if more than one polymer are attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on

25 antibody and/or binding polypeptide to be improved, whether the antibody derivative and/or binding polypeptide derivative will be used in a therapy under defined conditions.

[0177] Any of the antibodies disclosed herein (*e.g.*, an anti-GDF11 antibody, an anti-activin A antibody, an anti-activin B antibody, an anti-activin C antibody, an anti-activin E antibody, an anti-GDF8 antibody, an anti-BMP6 antibody, an anti-ActRIIA antibody, an anti-30 GDF15 antibody, an anti-Nodal antibody, an anti-GDF3 antibody, an anti-BMP3 antibody, an anti-BMP3B antibody, an anti-BMP9 antibody, an anti-BMP10 antibody, or an anti-ActRIIB antibody) can be combined with one or more additional antagonist agents of the disclosure to achieve the desired effect. An antibody disclosed herein (*e.g.*, an anti-GDF11 antibody, an

anti-activin A antibody, an anti-activin B antibody, an anti-activin C antibody, an anti-activin E antibody, an anti-GDF11 antibody, an anti-GDF8 antibody, an anti-BMP6 antibody, an anti-ActRIIA antibody, an anti-GDF15 antibody, an anti-Nodal antibody, an anti-GDF3 antibody, an anti-BMP3 antibody, an anti-BMP3B antibody, an anti-BMP9 antibody, an anti-BMP10 antibody, or an anti-ActRIIB antibody) can be combined with another antibody disclosed herein, or a ligand trap polypeptide disclosed herein (*e.g.*, a GDF11 trap polypeptide, an activin B trap polypeptide, or a GDF11/activin B trap polypeptide), or a small-molecule antagonist directed to any of the targets of the disclosure (*e.g.*, an activin A small-molecule antagonist, an activin B small-molecule antagonist, a GDF11 small-molecule antagonist, an activin C small-molecule antagonist, an activin E small-molecule antagonist, a GDF8 small-molecule antagonist, a BMP6 small-molecule antagonist, a GDF15 small-molecule antagonist, a Nodal small-molecule antagonist, a GDF3 small-molecule antagonist, a BMP3B small-molecule antagonist, a BMP3B small-molecule antagonist, a BMP9 small-molecule antagonist, or a BMP10 small-molecule antagonist), or a polynucleotide antagonist of the disclosure (*e.g.*, a polynucleotide antagonist of activin A, activin B, activin C, activin E, GDF11, GDF8, GDF15, Nodal, GDF3, BMP3, BMP3B, or BMP6), or a non-antibody binding polypeptide disclosed herein (*e.g.*, a GDF11 binding polypeptide, an activin A binding polypeptide, an activin B binding polypeptide, an activin E binding polypeptide, an activin C binding polypeptide, a GDF8 binding polypeptide, a GDF15 binding polypeptide, a Nodal binding polypeptide, a GDF3 binding polypeptide, a BMP3 binding polypeptide, a BMP3B polypeptide, a BMP9 binding polypeptide, a BMP10 binding polypeptide, or a BMP6 binding polypeptide). For example, an anti-GDF11 antibody can be combined with an activin B antagonist of the disclosure (*e.g.*, an activin B trap polypeptide, an anti-activin B antibody, a small-molecule antagonist of activin B, a polynucleotide antagonist of activin B, or a non-antibody polypeptide antagonist of activin B) to inhibit both a GDF11 and an activin B activity (*e.g.*, the ability to bind to and/or activate an ActRIIA and/or ActRIIB receptor). In an alternative embodiment, an anti-activin B antibody can be combined with a GDF11 antagonist of the disclosure (*e.g.*, a GDF trap polypeptide, an anti-GDF11 antibody, a small-molecule antagonist of GDF11, a polynucleotide antagonist of GDF11, or a non-antibody polypeptide antagonist of GDF11) to inhibit both a GDF11 and an activin B activity.

## **B. GDF11, Activin B, and GDF11/Activin B Trap Polypeptides**

[0178] In one aspect, the antagonist (inhibitory) agents of the present disclosure are ActRII polypeptides and variants thereof. As used herein the term “ActRII” refers to the family of type II activin receptors. This family includes both the activin receptor type IIA (ActRIIA) and the activin receptor type IIB (ActRIIB). In some embodiments, the methods of the present disclosure relate to the use of one or more variant ActRII polypeptides (*e.g.*, variant ActRIIA and ActRIIB polypeptides) that bind to and/or antagonize (inhibit) the activity of one or more of: GDF11, GDF8, activin B, activin C, activin E, activin A, BMP6, GDF15, Nodal, GDF3, BMP3, and BMP3B (*e.g.*, activation of ActRIIA and/or ActRIIB Smad2/3 and/or Smad 1/5/8 signaling). In some embodiments, variant ActRII polypeptides are ligand “trap” polypeptides (*e.g.*, GDF11 trap polypeptides, activin B trap polypeptides, GDF11/activin B trap polypeptides). In some embodiments, variant ActRII polypeptides, and fusion constructs thereof, have reduced binding affinity for BMP9 and/or BMP10.

[0179] As used herein, the term “ActRIIB” refers to a family of activin receptor type IIB (ActRIIB) proteins from any species and variants derived from such ActRIIB proteins by mutagenesis or other modification. Reference to ActRIIB herein is understood to be a reference to any one of the currently identified forms. Members of the ActRIIB family are generally transmembrane proteins, composed of a ligand-binding extracellular domain with a cysteine-rich region, a transmembrane domain, and a cytoplasmic domain with predicted serine/threonine kinase activity. An alignment of the amino acid sequences of human ActRIIB soluble extracellular domain and the related ActRIIA soluble extracellular domain are illustrated in Figure 1.

[0180] The term “ActRIIB polypeptide” includes polypeptides comprising any naturally occurring polypeptide of an ActRIIB family member as well as any variants thereof (including mutants, fragments, fusions, and peptidomimetic forms) that retain a useful activity [see, *e.g.*, international patent application publication no. WO 2006/012627]. Numbering of amino acids for all ActRIIB-related polypeptides described herein is based on the numbering for SEQ ID NO:1, unless specifically designated otherwise.

[0181] Applicants have ascertained that that an Fc fusion protein having the sequence disclosed by Hilden *et al.* [Blood (1994) 83(8): 2163-2170], which has an alanine at the position corresponding to amino acid 64 of SEQ ID NO:1 (A65), has a relatively low affinity for activin and GDF11. By contrast, the same Fc fusion protein with an arginine at position (R65) has an affinity for activin and GDF11 in the low nanomolar to high picomolar range.

Therefore, a sequence with an R64 is used as the “wild-type” reference sequence for human ActRIIB in this disclosure.

[0182] The human ActRIIB precursor protein sequence is as follows:

[0183] MTAPWVALALLWGSLWPGSGRGEAETRECIYYNANWELERTNQSGLE  
 5 **RCEGEQDKRLHCYASWRN**SSGTIELVKKGCWLDDFNCYDRQECVATEENPQV  
**YFCCCEGNFCNERFTHLPEAGGPEVTYEPPTAPTLLTVLAYSLPIGGLSLIVLLA**  
 FWMYRHRKPPYGHVDIHEDPGPPPPSPLVGLKPLQLLEIKARGRFGCVWKAQLMND  
 FVAVKIFPLQDKQSWQSEREIFSTPGMKHENLLQFIAAEKRGSNLEVELWLITAFHDK  
 GSLTDYLGKNIITWNECHVAETMSRGLSYLHEDVPWCRGEGHKPSIAHRDFKSKN  
 10 VLLKSDLTAVLADFGLAVRFEPGKPPGDTHGQVGTRRYMAPEVLEGAINFQRDAFL  
 RIDMYAMGLVLWELVSRCKAADGPVDEYMLPFEEEIGQHPSLEELQEVVHKKMRP  
 TIKDHWLKHPLAQLCVTIEECWDHDAEARLSAGCVEERVSLIRRSVNGTTSDCLV  
 LVTSVTNVDLPPKESSI (SEQ ID NO:1)

[0184] The signal peptide is indicated with single underlined; the extracellular domain is  
 15 indicated in **bold** font; and the potential, native N-linked glycosylation sites are indicated  
 with double underlining.

[0185] A form with an alanine at position 64 is also reported in the literature, as follows:

[0186] MTAPWVALALLWGSLWPGSGRGEAETRECIYYNANWELERTNQSGLE  
**RCEGEQDKRLHCYASWAN**SSGTIELVKKGCWLDDFNCYDRQECVATEENPQV  
 20 **YFCCCEGNFCNERFTHLPEAGGPEVTYEPPTAPTLLTVLAYSLPIGGLSLIVLLA**  
 FWMYRHRKPPYGHVDIHEDPGPPPPSPLVGLKPLQLLEIKARGRFGCVWKAQLMND  
 FVAVKIFPLQDKQSWQSEREIFSTPGMKHENLLQFIAAEKRGSNLEVELWLITAFHDK  
 GSLTDYLGKNIITWNECHVAETMSRGLSYLHEDVPWCRGEGHKPSIAHRDFKSKN  
 VLLKSDLTAVLADFGLAVRFEPGKPPGDTHGQVGTRRYMAPEVLEGAINFQRDAFL  
 25 RIDMYAMGLVLWELVSRCKAADGPVDEYMLPFEEEIGQHPSLEELQEVVHKKMRP  
 TIKDHWLKHPLAQLCVTIEECWDHDAEARLSAGCVEERVSLIRRSVNGTTSDCLV  
 LVTSVTNVDLPPKESSI (SEQ ID NO:2).

[0187] The human ActRIIB soluble (extracellular), processed polypeptide sequence is as  
 follows:

30 [0188] GRGEAETRECIYYNANWELERTNQSGLER**CCEGEQDKRLHCYASWRN**SSG  
TIELVKKGCWLDDFNCYDRQECVATEENPQVYFCCCEGNFCNERFTHLPEAGGPEV  
TYEPPTAPT (SEQ ID NO:3).

[0189] The alternative form with an A64 is as follows:

[0190] GRGEAETRECIYYNANWELERTNQSGLERCEGEQDKRLHCYASWANSSG  
TIELVKKGCWLDDFNCYDRQECVATEENPQVYFCCCEGNFCNERFTHLPEAAGGPEV  
TYEPPPTAPT (SEQ ID NO:4).

5 [0191] In some embodiments, the protein may be produced with an “SGR...” sequence at the N-terminus. The C-terminal “tail” of the extracellular domain is indicated by single underlining. The sequence with the “tail” deleted (a  $\Delta 15$  sequence) is as follows:

[0192] GRGEAETRECIYYNANWELERTNQSGLERCEGEQDKRLHCYASWRNSSG  
TIELVKKGCWLDDFNCYDRQECVATEENPQVYFCCCEGNFCNERFTHLPEA (SEQ ID  
10 NO:5).

[0193] The alternative form with an A64 is as follows:

[0194] GRGEAETRECIYYNANWELERTNQSGLERCEGEQDKRLHCYASWANSSG  
TIELVKKGCWLDDFNCYDRQECVATEENPQVYFCCCEGNFCNERFTHLPEA (SEQ ID  
NO:6).

15 [0195] The nucleic acid sequence encoding a human ActRIIB precursor protein is as follows: (nucleotides 5-1543 of Genbank entry NM\_001106; the sequence as shown provides an alanine at position 64, and may be modified to provide an arginine instead)

[0196] ATGACGGCGCCCTGGGTGGCCCTCGCCCTCCTCTGGGGATCGCTGTGG  
CCCGGCTCTGGGCGTGGGGAGGCTGAGACACGGGAGTGCATCTACTACAACGCC  
20 AACTGGGAGCTGGAGCGCACCAACCAGAGCGGCCTGGAGCGCTGCGAAGGCGA  
GCAGGACAAGCGGCTGCACTGCTACGCCTCCTGGGCCAACAGCTCTGGCACCAT  
CGAGCTCGTGAAGAAGGGCTGCTGGCTAGATGACTTCAACTGCTACGATAGGCA  
GGAGTGTGTGGCCACTGAGGAGAAACCCCAAGGTGTACTTCTGCTGCTGTGAAGG  
CAACTTCTGCAACGAGCGCTTCACTCATTGTCAGAGGCTGGGGGGCCCGGAAGTC  
25 ACGTACGAGCCACCCCGACAGCCCCACCCTGCTCACGGTGCTGGCCTACTCAC  
TGCTGCCCATCGGGGGCCTTTCCCTCATCGTCCTGCTGGCCTTTTGGATGTACCGG  
CATCGCAAGCCCCCTACGGTCATGTGGACATCCATGAGGACCCTGGGCCTCCAC  
CACCATCCCCTCTGGTGGGCCTGAAGCCACTGCAGCTGCTGGAGATCAAGGCTCG  
GGGGCGCTTTGGCTGTGTCTGGAAGGCCAGCTCATGAATGACTTTGTAGCTGTC  
30 AAGATCTTCCCACTCCAGGACAAGCAGTCGTGGCAGAGTGAACGGGAGATCTTC  
AGCACACCTGGCATGAAGCACGAGAACCTGCTACAGTTCATTGCTGCCGAGAAG  
CGAGGCTCCAACCTCGAAGTAGAGCTGTGGCTCATCACGGCCTTCCATGACAAG

GGCTCCCTCACGGATTACCTCAAGGGGAACATCATCACATGGAACGAACTGTGTC  
 ATGTAGCAGAGACGATGTCACGAGGCCTCTCATACCTGCATGAGGATGTGCCCTG  
 GTGCCGTGGCGAGGGCCACAAGCCGTCTATTGCCACAGGGACTTTAAAAGTAA  
 GAATGTATTGCTGAAGAGCGACCTCACAGCCGTGCTGGCTGACTTTGGCTTGGCT  
 5 GTTCGATTTGAGCCAGGGAAACCTCCAGGGGACACCCACGGACAGGTAGGCACG  
 AGACGGTACATGGCTCCTGAGGTGCTCGAGGGAGCCATCAACTTCCAGAGAGAT  
 GCCTTCCTGCGCATTGACATGTATGCCATGGGGTTGGTGCTGTGGGAGCTTGTGT  
 CTCGCTGCAAGGCTGCAGACGGACCCGTGGATGAGTACATGCTGCCCTTTGAGG  
 AAGAGATTGGCCAGCACCCCTTCGTTGGAGGAGCTGCAGGAGGTGGTGGTGCACA  
 10 AGAAGATGAGGCCCACCATTAAAGATCACTGGTTGAAACACCCGGGCCTGGCCC  
 AGCTTTGTGTGACCATCGAGGAGTGCTGGGACCATGATGCAGAGGCTCGCTTGTC  
 CGCGGGCTGTGTGGAGGAGCGGGTGTCCCTGATTTCGGAGGTTCGGTCAACGGCAC  
 TACCTCGGACTGTCTCGTTTCCCTGGTGACCTCTGTCACCAATGTGGACCTGCCCC  
 CTAAAGAGTCAAGCATCTAA (SEQ ID NO: 7).

- 15 [0197] The nucleic acid sequence encoding a human ActRIIB soluble (extracellular) polypeptide is as follows (the sequence as shown provides an alanine at position 64, and may be modified to provide an arginine instead):

[0198] GGGCGTGGGGAGGCTGAGACACGGGAGTGCATCTACTACAACGCCAA  
 CTGGGAGCTGGAGCGCACCAACCAGAGCGGCCTGGAGCGCTGCGAAGGCGAGC  
 20 AGGACAAGCGGCTGCACTGCTACGCCTCCTGGGCCAACAGCTCTGGCACCATCG  
 AGCTCGTGAAGAAGGGCTGCTGGCTAGATGACTTCAACTGCTACGATAGGCAGG  
 AGTGTGTGGCCACTGAGGAGAACCCCCAGGTGTACTTCTGCTGCTGTGAAGGCA  
 ACTTCTGCAACGAGCGCTTCACTCATTTGCCAGAGGCTGGGGGCCCCGGAAGTCAC  
 GTACGAGCCACCCCCGACAGCCCCCACC (SEQ ID NO:8).

- 25 [0199] In certain embodiments, the present disclosure relates to ActRIIA polypeptides. As used herein, the term "ActRIIA" refers to a family of activin receptor type IIA (ActRIIA) proteins from any species and variants derived from such ActRIIA proteins by mutagenesis or other modification. Reference to ActRIIA herein is understood to be a reference to any one of the currently identified forms. Members of the ActRIIA family are generally  
 30 transmembrane proteins, composed of a ligand-binding extracellular domain with a cysteine-rich region, a transmembrane domain, and a cytoplasmic domain with predicted serine/threonine kinase activity.

[0200] The term “ActRIIA polypeptide” includes polypeptides comprising any naturally occurring polypeptide of an ActRIIA family member as well as any variants thereof (including mutants, fragments, fusions, and peptidomimetic forms) that retain a useful activity (see, *e.g.*, international patent application publication no. WO/2006/012627).

- 5 Numbering of amino acids for all ActRIIA-related polypeptides described herein is based on the numbering for SEQ ID NO:9, unless specifically designated otherwise.

[0201] The human ActRIIA precursor protein sequence is as follows:

[0202] MGAAAKLAFAVFLISSSGAILGRSETQECLFFNANWEKDRTNQTGVE  
**PCYGD****KDKRRHCFATWKN**ISGSIEIVKQGCWLDDINCYDRTDCVEKKDSPEVYF  
 10 **CCCEGNMCNEKFSYFP**EMEVTQPTSNPVTPKPPYYNILLYSLVPLMLIAGIVICAF  
WVYRHHKMAYPPVLVPTQDPGPPPSPLLGLKPLQLLEVKARGRFGCVWKAQLLNE  
YVAVKIFPIQDKQSWQNEYEVYSLPGMKHENILQFIGAEKRGTSVDVDLWLITAFHE  
KGSLSDFLKANVVSWNELCHIAETMARGLAYLHEDIPGLKDGHKPAISHRDIKSKNV  
LLKNNLTACIADFGLALKFEAGKSAGDTHGQVGTRRYMAPEVLEGAINFQRDAFLRI  
 15 DMYAMGLVLWELASRCTAADGPVDEYMLPFEEEIGQHPSLEDMQEVVVHKKKRPV  
LRDYWQKHAGMAMLCETIEECWDHDAEARLSAGCVGERITQMQRLTNITTEDIVT  
VVTMVTNVDFPPKESSL (SEQ ID NO:9)

[0203] The signal peptide is indicated by single underlined; the extracellular domain is indicated in **bold** font; and the potential N-linked glycosylation sites are indicated by double underlined.  
 20 underlined.

[0204] The human ActRIIA soluble (extracellular), processed polypeptide sequence is as follows:

[0205] ILGRSETQECLFFNANWEKDRTNQTGVEPCYGD**KDKRRHCFATWKNISGS**  
IEIVKQGCWLDDINCYDRTDCVEKKDSPEVYFCCCEGNMCNEKFSYFPEMEVTQPTS  
 25 NPVTPKPP (SEQ ID NO:10)

[0206] The C-terminal “tail” of the extracellular domain is indicated by single underlining. The sequence with the “tail” deleted (a  $\Delta 15$  sequence) is as follows:

[0207] ILGRSETQECLFFNANWEKDRTNQTGVEPCYGD**KDKRRHCFATWKNISGS**  
IEIVKQGCWLDDINCYDRTDCVEKKDSPEVYFCCCEGNMCNEKFSYFPEM (SEQ ID  
 30 NO:11)

[0208] The nucleic acid sequence encoding human ActRIIA precursor protein is as follows (nucleotides 164-1705 of Genbank entry NM\_001616):

[0209] ATGGGAGCTGCTGCAAAGTTGGCGTTTGCCGTCTTTCTTATCTCCTGTT  
CTTCAGGTGCTATACTTGGTAGATCAGAACTCAGGAGTGTCTTTTCTTTAATGCT  
5 AATTGGGAAAAAGACAGAACCAATCAAACCTGGTGTGTAACCGTGTTATGGTGAC  
AAAGATAAACGGCGGCATTGTTTTGCTACCTGGAAGAATATTTCTGGTTCCATTG  
AAATAGTGAAACAAGGTTGTTGGCTGGATGATATCAACTGCTATGACAGGACTG  
ATTGTGTAGAAAAAAAAGACAGCCCTGAAGTATATTTTTGTTGCTGTGAGGGCAA  
TATGTGTAATGAAAAGTTTTCTTATTTTCCAGAGATGGAAGTCACACAGCCCACT  
10 TCAAATCCAGTTACACCTAAGCCACCCTATTACAACATCCTGCTCTATTCCTTGGT  
GCCACTTATGTTAATTGCGGGGATTGTCATTTGTGCATTTTGGGTGTACAGGCATC  
ACAAGATGGCCTACCCTCCTGTACTTGTTCCTCAACTCAAGACCCAGGACCACCCCC  
ACCTTCTCCATTACTAGGGTTGAAACCACTGCAGTTATTAGAAGTGAAAGCAAGG  
GGAAGATTTGGTTGTGTCTGGAAAGCCCAGTTGCTTAACGAATATGTGGCTGTCA  
15 AAATATTTCCAATACAGGACAAACAGTCATGGCAAAATGAATACGAAGTCTACA  
GTTTGCCTGGAATGAAGCATGAGAACATATTACAGTTCATTGGTGCAGAAAAAC  
GAGGCACCAGTGTTGATGTGGATCTTTGGCTGATCACAGCATTTCATGAAAAGGG  
TTCCTATCAGACTTTCTTAAGGCTAATGTGGTCTCTTGGAATGAACTGTGTCATA  
TTGCAGAAACCATGGCTAGAGGATTGGCATATTTACATGAGGATATACCTGGCCT  
20 AAAAGATGGCCACAAACCTGCCATATCTCACAGGGACATCAAAAGTAAAAATGT  
GCTGTTGAAAAACAACCTGACAGCTTGCTGACTTTGGGTTGGCCTTAAAA  
TTTGAGGCTGGCAAGTCTGCAGGCGATACCCATGGACAGGTTGGTACCCGGAGG  
TACATGGCTCCAGAGGTATTAGAGGGTGCTATAAACTTCCAAAGGGATGCATTTT  
TGAGGATAGATATGTATGCCATGGGATTAGTCCTATGGGAACTGGCTTCTCGCTG  
25 TACTGCTGCAGATGGACCTGTAGATGAATACATGTTGCCATTTGAGGAGGAAATT  
GGCCAGCATCCATCTCTTGAAGACATGCAGGAAGTTGTTGTGCATAAAAAAAG  
AGGCCTGTTTTAAGAGATTATTGGCAGAAACATGCTGGAATGGCAATGCTCTGTG  
AAACCATGGAAGAATGTTGGGATCACGACGCAGAAGCCAGGTTATCAGCTGGAT  
GTGTAGGTGAAAGAATTACCCAGATGCAGAGACTAACAAATATTATTACCACAG  
30 AGGACATTGTAACAGTGGTCACAATGGTGACAAATGTTGACTTTCCTCCCAAAGA  
ATCTAGTCTATGA (SEQ ID NO:12)

[0210] The nucleic acid sequence encoding a human ActRIIA soluble (extracellular) polypeptide is as follows:

ATACTTGGTAGATCAGAACTCAGGAGTGTCTTTTCTTTAATGCTAATTGGGAAA  
 AAGACAGAACCAATCAAACCTGGTGTGTAACCGTGTATGGTGACAAAGATAAAC  
 GGCGGCATTGTTTTGCTACCTGGAAGAATATTTCTGGTTCCATTGAAATAGTGAA  
 ACAAGGTTGTTGGCTGGATGATATCAACTGCTATGACAGGACTGATTGTGTAGAA  
 5 AAAAAAGACAGCCCTGAAGTATATTTTTGTTGCTGTGAGGGCAATATGTGTAATG  
 AAAAGTTTTCTTATTTTCCAGAGATGGAAGTCACACAGCCCACTTCAAATCCAGT  
 TACACCTAAGCCACCC (SEQ ID NO:13).

[0211] In certain embodiments, the present disclosure relates to ActRII polypeptides (ActRIIA and ActRIIB polypeptides) which are soluble ActRII polypeptides. As described  
 10 herein, the term “soluble ActRII polypeptide” generally refers to polypeptides comprising an extracellular domain of an ActRII protein. The term “soluble ActRII polypeptide,” as used herein, includes any naturally occurring extracellular domain of an ActRII protein as well as any variants thereof (including mutants, fragments, and peptidomimetic forms) that retain a useful activity (*e.g.*, a GDF11 trap, an activin B trap, and a GDF11/activin B trap as  
 15 described herein). Other examples of soluble ActRII polypeptides comprise a signal sequence in addition to the extracellular domain of an ActRII protein. For example, the signal sequence can be a native signal sequence of an ActRIIA or ActRIIB protein, or a signal sequence from another protein including, for example, a tissue plasminogen activator (TPA) signal sequence or a honey bee melittin (HBM) signal sequence.

20 [0212] An example of a HBM signal sequence is as follows:

[0213] MKFLVNVALVFMVVYISYIYA (SEQ ID NO:14).

[0214] An example of a TPA signal sequence is as follows:

[0215] MDAMKRGLCCVLLLCGAVFVSP (SEQ ID NO:15).

[0216] In certain embodiments, the present disclosure contemplates making mutations in  
 25 the extracellular domain (also referred to as ligand-binding domain) of an ActRII polypeptide such that the ActRII polypeptide has an altered ligand-binding activity (*e.g.*, binding specificity). In certain aspects, such “ligand trap” polypeptides have altered (elevated or reduced) binding affinity for one or more specific ActRII ligands. In other aspects, ligand trap polypeptides have altered binding specificity for one or more ActRII ligands.

30 [0217] For example, the present disclosure provides methods of using ligand trap polypeptides that preferentially bind to at least GDF11 and/or activin B, particularly methods for increasing red blood cell levels and/or treating anemia in a subject in need thereof.

Optionally, trap polypeptides of the disclosure do not bind to and/or inhibit activin A.

Optionally, trap polypeptides of the disclosure further bind to GDF8. In some embodiments, trap polypeptides of the disclosure that bind to and/or inhibit GDF11 and/or activin B further bind to and/or inhibit one or more of activin C, activin E, activin A, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, and GDF8. Optionally, trap polypeptides of the disclosure have reduced to no binding affinity for BMP9 and/or BMP10.

[0218] As disclosed herein, GDF11/activin B traps are variant ActRII polypeptides (*e.g.* variant ActRIIA or ActRIIB polypeptides) that bind to and/or antagonize (inhibit) the activity of both GDF11 and activin B (*e.g.*, GDF11- and activin B-mediated activation of the ActRIIA or ActRIIB Smad2/3 signaling pathway). Thus, the present disclosure provides GDF11/activin B traps that comprise an altered ligand-binding domain of an ActRII receptor [*e.g.*, comprising one or more amino acid mutations (*e.g.*, amino acid substitutions, additions, or deletions)] which are characterized, in part, by increased selectivity for GDF11 and activin B relative to an unmodified (wild-type) ligand-binding domain of an ActRII receptor.

Optionally, GDF11/activin B traps do not substantially bind to and/or inhibit activity of activin A (*e.g.*, activin A-mediated activation of the ActRIIA or ActRIIB Smad2/3 signaling pathway).

[0219] In some embodiments, GDF11/activin B traps of the disclosure have equivalent or increased binding affinity (*e.g.*, 2-, 3-, 10-, 100-, 1000-fold increased binding affinity) for GDF11 and/or activin B relative to an unmodified (wild-type) ligand-binding domain of an ActRII receptor. Optionally, the GDF11/activin B trap comprises an altered ActRII ligand-binding domain that has a ratio of  $K_D$  for activin A binding to  $K_D$  for GDF11 binding that is at least 2-, 5-, 10-, 100-, or even 1000-fold greater relative to the ratio for the unmodified (wild-type) ActRII ligand-binding domain. Optionally, the GDF11/activin B trap comprises an altered ActRII ligand-binding domain that has a ratio of  $K_D$  for activin A binding to  $K_D$  for activin B binding that is at least 2-, 5-, 10-, 100-, or even 1000-fold greater relative to the ratio for the unmodified (wild-type) ActRII ligand-binding domain. Optionally, the GDF11/activin B trap comprises an altered ActRII ligand-binding domain that has a ratio of  $IC_{50}$  for inhibiting activin A to  $IC_{50}$  for inhibiting GDF11 that is at least 2-, 5-, 10-, 100- or even 1000-fold greater relative to the unmodified (wild-type) ActRII ligand-binding domain. Optionally, the GDF11/activin B trap comprises an altered ActRII ligand-binding domain that has a ratio of  $IC_{50}$  for inhibiting activin A to  $IC_{50}$  for inhibiting activin B that is at least 2-, 5-, 10-, 100- or even 1000-fold greater relative to the unmodified (wild-type) ActRII ligand-

binding domain. Optionally, the GDF11/activin B trap comprises an altered ligand-binding domain that inhibits GDF11 with an  $IC_{50}$  at least 2-, 5-, 10-, or even 100-times less than the  $IC_{50}$  for inhibiting activin A. Optionally, the GDF11/activin B trap comprises an altered ligand-binding domain that inhibits activin B with an  $IC_{50}$  at least 2-, 5-, 10-, or even 100-  
5 times less than the  $IC_{50}$  for inhibiting activin A.

[0220] In some embodiments, a GDF11/activin B trap of the present disclosure optionally further binds to and/or inhibits activity of one or more of BMP6, activin C, activin E, activin A, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10, and GDF8.

[0221] In some embodiments, a GDF11/activin B trap of the present disclosure comprises  
10 an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 29-109 of SEQ ID NO:1 or 2. In preferred embodiments, GDF11/activin B traps of the present disclosure do not comprise or consist of amino acids 29-109 of SEQ ID NO:1 or 2. In other preferred embodiments, GDF11/activin B traps of the present disclosure do not comprise an acidic amino acid [*e.g.*, aspartic (D) or glutamic (E)  
15 acid] at position 79 with respect to SEQ ID NO: 1 or 2.

[0222] In other embodiments, the GDF11/activin B trap comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% to any one of SEQ ID Nos: 3, 4, 5, 6, 21, 23, 48, and 49. In preferred embodiments, GDF11/activin B traps of the present disclosure do not comprise or consist of the amino acid sequence of any one of  
20 SEQ ID Nos: 3, 4, 5, 6, 21, 23, 48, and 49.

[0223] In some embodiments, a GDF11/activin B trap of the present disclosure comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 30-110 of SEQ ID NO:9. In preferred embodiments, a GDF11/activin B trap of the present disclosure does not comprise or consist of amino acids  
25 30-110 of SEQ ID NO:9. In other embodiments, the GDF11/activin trap comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% to any one of SEQ ID Nos: 10, 11, 18, and 20. In preferred embodiments, GDF11/activin B traps of the present disclosure do not comprise or consist of the amino acid sequence of any one of SEQ ID Nos: 10, 11, 18, and 20.

[0224] As disclosed herein, GDF11 traps are variant ActRII polypeptides (*e.g.* variant ActRIIA or ActRIIB polypeptides) that bind to and/or antagonize (inhibit) the activity of GDF11 (*e.g.*, GDF11-mediated activation of the ActRIIA or ActRIIB Smad2/3 signaling  
30

pathway), but that do not substantially bind to and/or inhibit activity of activin B (*e.g.*, activin B-mediated activation of the ActRIIA or ActRIIB Smad2/3 signaling pathway). Thus, the present disclosure provides GDF11 traps that comprise an altered ligand-binding domain of an ActRII receptor [*e.g.*, comprising one or more amino acid mutations (*e.g.*, amino acid substitutions, additions, or deletions)] which are characterized, in part, by increased selectivity for GDF11 relative to an unmodified (wild-type) ligand-binding domain of an ActRII receptor. Optionally, GDF11 traps do not bind to and/or inhibit activin A activity (*e.g.*, activin A-mediated activation of the ActRIIA or ActRIIB Smad2/3 signaling pathway).

[0225] In some embodiments, GDF11 traps of the disclosure have equivalent or enhanced increased binding affinity (*e.g.*, 2-, 3-, 10-, 100-, 1000-fold increased binding affinity) binding affinity for GDF11 relative to an unmodified (wild-type) ligand-binding domain of an ActRII receptor. Optionally, the GDF11 trap comprises an altered ActRII ligand-binding domain that has a ratio of  $K_D$  for activin A binding to  $K_D$  for GDF11 binding that is at least 2-, 5-, 10-, 100-, or even 1000-fold greater relative to the ratio for the unmodified (wild-type) ActRII ligand-binding domain. Optionally, the GDF11 trap comprises an altered ActRII ligand-binding domain that has a ratio of  $K_D$  for activin B binding to  $K_D$  for GDF11 binding that is at least 2-, 5-, 10-, 100-, or even 1000-fold greater relative to the ratio for the unmodified (wild-type) ActRII ligand-binding domain. Optionally, the GDF11 trap comprises an altered ActRII ligand-binding domain that has a ratio of  $IC_{50}$  for inhibiting activin A to  $IC_{50}$  for inhibiting GDF11 that is at least 2-, 5-, 10-, 100- or even 1000-fold greater relative to the unmodified (wild-type) ActRII ligand-binding domain. Optionally, the GDF11 trap comprises an altered ActRII ligand-binding domain that has a ratio of  $IC_{50}$  for inhibiting activin B to  $IC_{50}$  for inhibiting GDF11 that is at least 2-, 5-, 10-, 100- or even 1000-fold greater relative to the unmodified (wild-type) ActRII ligand-binding domain. Optionally, the GDF11 trap comprises an altered ligand-binding domain that inhibits GDF11 with an  $IC_{50}$  at least 2-, 5-, 10-, or even 100-fold less than the  $IC_{50}$  for inhibiting activin A. Optionally, the GDF11 trap comprises an altered ligand-binding domain that inhibits GDF11 with an  $IC_{50}$  at least 2-, 5-, 10-, or even 100-fold less than the  $IC_{50}$  for inhibiting activin B.

[0226] In some embodiments, a GDF11 trap of the present disclosure optionally further binds to and/or inhibits activity of one or more of BMP6, activin C, activin E, activin A, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, BMP10, and GDF8.

[0227] In some embodiments, a GDF11 trap of the present disclosure comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical

to amino acids 29-109 of SEQ ID NO:1 or 2. In preferred embodiments, GDF11 traps of the present disclosure do not comprise or consist of amino acids 29-109 of SEQ ID NO:1 or 2.

In other preferred embodiments, GDF11 traps of the present disclosure do not comprise an acidic amino acid [*e.g.*, aspartic (D) or glutamic (E) acid] at position 79 with respect to SEQ

5 ID NO: 1 or 2.

[0228] In other embodiments, the GDF11 trap comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% to any one of SEQ ID Nos: 3, 4, 5, 6, 21, 23, 48 and 49. In preferred embodiments, GDF11 traps of the present disclosure do not comprise or consist of the amino acid sequence of any one of SEQ ID Nos: 3, 4, 5, 6,

10 21, 23, 48, and 49.

[0229] In some embodiments, a GDF11 trap of the present disclosure comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 30-110 of SEQ ID NO:9. In preferred embodiments, a GDF11 trap of the present disclosure does not comprise or consist of amino acids 30-110 of SEQ ID NO:9. In other embodiments, the GDF11 trap comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% to any one of SEQ ID Nos: 10, 11, 18, and 20. In preferred embodiments, GDF11 traps of the present disclosure do not comprise or consist of the amino acid sequence of any one of SEQ ID Nos: 10, 11, 18, and 20.

[0230] As disclosed herein, activin B traps are variant ActRII polypeptides (*e.g.* variant ActRIIA or ActRIIB polypeptides) that bind to and/or antagonize (inhibit) the activity of activin B (*e.g.*, activin B-mediated activation of the ActRIIA or ActRIIB Smad2/3 signaling pathway), but that do not substantially bind to and/or inhibit activity of GDF11 (*e.g.*, GDF11-mediated activation of the ActRIIA or ActRIIB Smad2/3 signaling pathway). Thus, the present disclosure provides activin B traps that comprise an altered ligand-binding domain of an ActRII receptor [*e.g.*, comprising one or more amino acid mutations (*e.g.*, amino acid substitutions, additions, or deletions)] which are characterized, in part, by increased selectivity for activin B relative to an unmodified (wild-type) ligand-binding domain of an ActRII receptor. Optionally, activin B traps do not substantially bind to and/or inhibits activin A activity (*e.g.*, activin A-mediated activation of the ActRIIA or ActRIIB Smad2/3 signaling pathway)

[0231] In some embodiments, activin B traps of the disclosure have equivalent or increased binding affinity (*e.g.*, 2-, 3-, 10-, 100-, 1000-fold increased binding affinity) for

activin B relative to an unmodified (wild-type) ligand-binding domain of an ActRII receptor. Optionally, the activin B Trap comprises an altered ActRII ligand-binding domain that has a ratio of  $K_D$  for activin A binding to  $K_D$  for activin B binding that is at least 2-, 5-, 10-, 100-, or even 1000-fold greater relative to the ratio for the unmodified (wild-type) ActRII ligand-

5 binding domain. Optionally, the activin B trap comprises an altered ActRII ligand-binding domain that has a ratio of  $K_D$  for GDF11 binding to  $K_D$  for activin B binding that is at least 2-, 5-, 10-, 100-, or even 1000- fold greater relative to the ratio for the unmodified (wild-type) ActRII ligand-binding domain. Optionally, the activin B trap comprises an altered ActRII ligand-binding domain that has a ratio of  $IC_{50}$  for inhibiting activin A to  $IC_{50}$  for inhibiting  
10 activin B that is at least 2-, 5-, 10-, 100- or even 1000-fold greater relative to the unmodified (wild-type) ActRII ligand-binding domain. Optionally, the activin B trap comprises an altered ActRII ligand-binding domain that has a ratio of  $IC_{50}$  for inhibiting GDF11 to  $IC_{50}$  for inhibiting activin B that is at least 2-, 5-, 10-, 100- or even 1000-fold greater relative to the unmodified (wild-type) ActRII ligand-binding domain. Optionally, the activin B trap  
15 comprises an altered ligand-binding domain that inhibits activin B with an  $IC_{50}$  at least 2-, 5-, 10-, or even 100-fold less than the  $IC_{50}$  for inhibiting activin A. Optionally, the activin B trap comprises an altered ligand-binding domain that inhibits activin B with an  $IC_{50}$  at least 2-, 5-, 10-, or even 100-fold less than the  $IC_{50}$  for inhibiting GDF11.

[0232] In some embodiments, an activin B trap of the present disclosure optionally  
20 further binds to and/or inhibits activity of one or more of BMP6, activin C, activin E, activin A, GDF15, Nodal, BMP3, GDF3, BMP3B, BMP9, BMP10, and GDF8.

[0233] In some embodiments, an activin B trap of the present disclosure comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 29-109 of SEQ ID NO:1 or 2. In preferred embodiments, activin B  
25 traps of the present disclosure do not comprise or consist of amino acids 29-109 of SEQ ID NO:1 or 2. In other preferred embodiments, GDF11 traps of the present disclosure do not comprise an acidic amino acid [*e.g.*, aspartic (D) or glutamic (E) acid] at position 79 with respect to SEQ ID NO: 1 or 2.

[0234] In other embodiments, the activin B trap comprises an amino acid sequence that is  
30 at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% to any one of SEQ ID Nos: 3, 4, 5, 6, 21, 23, 48, and 49. In preferred embodiments, activin B traps of the present disclosure do not comprise or consist of the amino acid sequence of any one of SEQ ID Nos: 3, 4, 5, 6, 21, 23, 48, and 49.

[0235] In some embodiments, an activin B trap of the present disclosure comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 30-110 of SEQ ID NO:9. In preferred embodiments, an activin B trap of the present disclosure does not comprise or consist of amino acids 30-110 of SEQ ID NO:9. In other embodiments, the activin B trap comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% to any one of SEQ ID Nos: 10, 11, 18, and 20. In preferred embodiments, activin B traps of the present disclosure do not comprise or consist of the amino acid sequence of any one of SEQ ID Nos: 10, 11, 18, and 20.

[0236] As will be recognized by one of skill in the art, most of the described mutations, variants or modifications may be made at the nucleic acid level or, in some cases, by post translational modification or chemical synthesis. Such techniques are well known in the art and described herein.

[0237] ActRII proteins have been well characterized in the art in terms of structural and functional characteristics, particularly with respect to ligand-binding [see, *e.g.*, Attisano *et al.* (1992) *Cell* 68(1):97-108; Greenwald *et al.* (1999) *Nature Structural Biology* 6(1): 18-22; Allendorph *et al.* (2006) *PNAS* 103(20): 7643-7648; Thompson *et al.* (2003) *The EMBO Journal* 22(7): 1555-1566; and U.S. Patent Nos: 7,709,605; 7,612,041; and 7,842,663]. For example, Attisano *et al.* showed that a deletion of the proline knot at the C-terminus of the extracellular domain of ActRIIB reduced the affinity of the receptor for activin. An ActRIIB-Fc fusion protein containing amino acids 20-119 of SEQ ID NO:1, "ActRIIB(20-119)-Fc", has reduced binding to GDF-11 and activin relative to an ActRIIB(20-134)-Fc, which includes the proline knot region and the complete juxtamembrane domain (see, *e.g.*, U.S. Patent No. 7,842,663). However, an ActRIIB(20-129)-Fc protein retains similar but somewhat reduced activity relative to the wild type, even though the proline knot region is disrupted. Thus, ActRIIB extracellular domains that stop at amino acid 134, 133, 132, 131, 130 and 129 (with respect to SEQ ID NO:1 or 2) are all expected to be active, but constructs stopping at 134 or 133 may be most active. Similarly, mutations at any of residues 129-134 (with respect to SEQ ID NO:1 or 2) are not expected to alter ligand-binding affinity by large margins. In support of this, mutations of P129 and P130 (with respect to SEQ ID NO:1 or 2) do not substantially decrease ligand binding. Therefore, an ActRIIB trap polypeptide of the present disclosure may end as early as amino acid 109 (the final cysteine), however, forms ending at or between 109 and 119 are expected to have reduced ligand-binding. Amino acid 119 (with respect to SEQ ID NO:1 or 2) is poorly conserved and so is readily altered or

truncated. ActRIIB-based ligand traps ending at 128 (with respect to SEQ ID NO:1 or 2) or later retain ligand-binding activity. ActRIIB-based ligand traps ending at or between 119 and 127 (with respect to SEQ ID NO:1 or 2) will have an intermediate binding ability. Any of these forms may be desirable to use, depending on the clinical or experimental setting.

5 [0238] At the N-terminus of ActRIIB, it is expected that a protein beginning at amino acid 29 or before (with respect to SEQ ID NO:1 or 2) will retain ligand-binding activity. Amino acid 29 represents the initial cysteine. An alanine to asparagine mutation at position 24 (with respect to SEQ ID NO:1 or 2) introduces an N-linked glycosylation sequence without substantially affecting ligand-binding (see, *e.g.*, U.S. Patent No. 7,842,663). This  
10 confirms that mutations in the region between the signal cleavage peptide and the cysteine cross-linked region, corresponding to amino acids 20-29 are well tolerated. In particular, ActRIIB-based ligand trap constructs beginning at position 20, 21, 22, 23, and 24 (with respect to SEQ ID NO:1 or 2) will retain general ligand-binding activity, and ActRIIB-based ligand trap constructs beginning at positions 25, 26, 27, 28, and 29 (with respect to SEQ ID  
15 NO:1 or 2) are also expected to retain ligand-binding activity. Data shown in, *e.g.*, U.S. Patent No. 7,842,663 demonstrates that, surprisingly, an ActRIIB construct beginning at 22, 23, 24, or 25 will have the most activity.

[0239] Taken together, an active portion (*e.g.*, ligand binding activity) of ActRIIB comprises amino acids 29-109 of SEQ ID NO:1 or 2. Therefore ActRIIB-based ligand trap  
20 constructs of the present disclosure may, for example, comprise an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to a portion of ActRIIB beginning at a residue corresponding to amino acids 20-29 (*e.g.*, beginning at amino acid 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29) of SEQ ID NO: 1 or 2 and ending at a position corresponding to amino acids 109-134 (*e.g.*, ending at amino acid 109, 110, 111, 112, 113,  
25 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, or 134) of SEQ ID NO: 1 or 2. In preferred embodiments, ActRIIB-based ligand trap polypeptides of the present disclosure do not comprise an acidic amino acid [*e.g.*, aspartic (D) or glutamic (E) acid] at the position corresponding to position 79 of SEQ ID NO: 1 or 2. In  
30 other preferred embodiments, ActRIIB-based ligand trap polypeptides of the present disclosure do not comprise or consist of amino acids 29-109 of SEQ ID NO:1 or 2. Other examples include ActRIIB-based ligand trap constructs that begin at a position from 20-29 (*e.g.*, position 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29) or 21-29 (*e.g.*, position 21, 22, 23, 24, 25, 26, 27, 28, or 29) and end at a position from 119-134 (*e.g.*, 119, 120, 121, 122, 123, 124,

125, 126, 127, 128, 129, 130, 131, 132, 133, or 134), 119-133 (*e.g.*, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, or 133), 129-134 (*e.g.*, 129, 130, 131, 132, 133, or 134), or 129-133 (*e.g.*, 129, 130, 131, 132, or 133) of SEQ ID NO: 1 or 2. Other examples include constructs that begin at a position from 20-24 (*e.g.*, 20, 21, 22, 23, or 24), 21-24 (*e.g.*, 21, 22, 23, or 24), or 22-25 (*e.g.*, 22, 23, 24, or 25) and end at a position from 109-134 (*e.g.*, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, or 134), 119-134 (*e.g.*, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, or 134) or 129-134 (*e.g.*, 129, 130, 131, 132, 133, or 134) of SEQ ID NO: 1 or 2. Variants within these ranges are also contemplated, particularly those having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the corresponding portion of SEQ ID NO: 1 or 2, optionally such ActRIIB-based ligand trap polypeptides i) do not comprise an acidic amino acid [*e.g.*, aspartic (D) or glutamic (E) acid] at the position corresponding to position 79 of SEQ ID NO: 1 or 2, and ii) do not comprise or consist of amino acids 29-109 of SEQ ID NO: 1 or 2. In certain embodiments, the ActRIIB-based ligand trap polypeptide comprises a polypeptide having an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acid residues 25-131 of SEQ ID NO: 1 or 2. In preferred embodiments, ActRIIB-based ligand trap polypeptides do not comprise or consist of amino acids 25-131 of SEQ ID NO: 1 or 2. In certain embodiments, the ActRIIB-based trap polypeptide comprises a polypeptide having an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NOs: 3, 4, 5, 6, 21, and 23, provided that: i) the ActRIIB-based trap polypeptide does not comprise an acidic amino acid [*e.g.*, aspartic (D) or glutamic (E) acid] at the position corresponding to position 79 of SEQ ID NO: 1 or 2, and ii) the ActRIIB-based trap polypeptide does not comprise or consist of amino acids 29-109 of SEQ ID NO: 1 or 2.

[0240] The disclosure includes the results of an analysis of composite ActRIIB structures, shown in Figure 1, demonstrating that the ligand-binding pocket is defined, in part, by residues Y31, N33, N35, L38 through T41, E47, E50, Q53 through K55, L57, H58, Y60, S62, K74, W78 through N83, Y85, R87, A92, and E94 through F101. At these positions, it is expected that conservative mutations will be tolerated, although a K74A mutation is well-tolerated, as are R40A, K55A, F82A and mutations at position L79. R40 is a K in *Xenopus*, indicating that basic amino acids at this position will be tolerated. Q53 is R in bovine ActRIIB and K in *Xenopus* ActRIIB, and therefore amino acids including R, K, Q, N and H

will be tolerated at this position. Thus, a general formula for an ActRIIB-based ligand trap protein is one that comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 29-109 of SEQ ID NO: 1 or 2, optionally beginning at a position ranging from 20-24 (*e.g.*, 20, 21, 22, 23, or 24) or 22-25(*e.g.*, 22, 23, 24, or 25) and ending at a position ranging from 129-134 (*e.g.*, 129, 130, 131, 132, 133, or 134), and comprising no more than 1, 2, 5, 10 or 15 conservative amino acid changes in the ligand-binding pocket, and zero, one or more non-conservative alterations at positions 40, 53, 55, 74, 79 and/or 82 in the ligand-binding pocket. In preferred embodiments, ActRIIB-based trap polypeptides of the present disclosure do not comprise an acidic amino acid [*e.g.*, aspartic (D) or glutamic (E) acid] at the position corresponding to position 79 of SEQ ID NO: 1 or 2. In other preferred embodiments, ActRIIB-based ligand trap polypeptides of the present disclosure do not comprise or consist of amino acids 29-109 of SEQ ID NO:1 or 2. Sites outside the binding pocket, at which variability may be particularly well tolerated, include the amino and carboxy termini of the extracellular domain (as noted above), and positions 42-46 and 65-73 (with respect to SEQ ID NO:1). An asparagine to alanine alteration at position 65 (N65A) actually improves ligand-binding in the A64 background, and is thus expected to have no detrimental effect on ligand-binding in the R64 background (see, *e.g.*, U.S. Patent No. 7,842,663). This change probably eliminates glycosylation at N65 in the A64 background, thus demonstrating that a significant change in this region is likely to be tolerated. While an R64A change is poorly tolerated, R64K is well-tolerated, and thus another basic residue, such as H may be tolerated at position 64 (see, *e.g.*, U.S. Patent No. 7,842,663).

[0241] ActRIIB is well-conserved across nearly all vertebrates, with large stretches of the extracellular domain conserved completely. Many of the ligands that bind to ActRIIB are also highly conserved. Accordingly, comparisons of ActRIIB sequences from various vertebrate organisms provide insights into residues that may be altered. Therefore, an active, human ActRIIB variant polypeptide useful in accordance with the presently disclosed methods may include one or more amino acids at corresponding positions from the sequence of another vertebrate ActRIIB, or may include a residue that is similar to that in the human or other vertebrate sequence. The following examples illustrate this approach to defining an active ActRIIB variant. L46 is a valine in *Xenopus* ActRIIB, and so this position may be altered, and optionally may be altered to another hydrophobic residue, such as V, I or F, or a non-polar residue such as A. E52 is a K in *Xenopus*, indicating that this site may be tolerant

of a wide variety of changes, including polar residues, such as E, D, K, R, H, S, T, P, G, Y and probably A. T93 is a K in *Xenopus*, indicating that a wide structural variation is tolerated at this position, with polar residues favored, such as S, K, R, E, D, H, G, P, G and Y. F108 is a Y in *Xenopus*, and therefore Y or other hydrophobic group, such as I, V or L should be tolerated. E111 is K in *Xenopus*, indicating that charged residues will be tolerated at this position, including D, R, K and H, as well as Q and N. R112 is K in *Xenopus*, indicating that basic residues are tolerated at this position, including R and H. A at position 119 is relatively poorly conserved, and appears as P in rodents and V in *Xenopus*, thus essentially any amino acid should be tolerated at this position.

[0242] It has been previously demonstrated that the addition of a further N-linked glycosylation site (N-X-S/T) is well-tolerated relative to the ActRIIB(R64)-Fc form (see, *e.g.*, U.S. Patent No. 7,842,663). By introducing an asparagine at position 24 (A24N construct; with respect to SEQ ID NO:1), an NXT sequence is created that may confer a longer half-life. Other NX(T/S) sequences are found at 42-44 (NQS) and 65-67 (NSS), although the latter may not be efficiently glycosylated with the R at position 64. N-X-S/T sequences may be generally introduced at positions outside the ligand binding pocket defined in Figure 1. Particularly suitable sites for the introduction of non-endogenous N-X-S/T sequences include amino acids 20-29, 20-24, 22-25, 109-134, 120-134 or 129-134 (with respect to SEQ ID NO:1). N-X-S/T sequences may also be introduced into the linker between the ActRIIB sequence and an Fc domain or other fusion component. Such a site may be introduced with minimal effort by introducing an N in the correct position with respect to a pre-existing S or T, or by introducing an S or T at a position corresponding to a pre-existing N. Thus, desirable alterations that would create an N-linked glycosylation site are: A24N, R64N, S67N (possibly combined with an N65A alteration), E105N, R112N, G120N, E123N, P129N, A132N, R112S and R112T (with respect to SEQ ID NO:1). Any S that is predicted to be glycosylated may be altered to a T without creating an immunogenic site, because of the protection afforded by the glycosylation. Likewise, any T that is predicted to be glycosylated may be altered to an S. Thus the alterations S67T and S44T (with respect to SEQ ID NO:1) are contemplated. Likewise, in an A24N variant, an S26T alteration may be used. Accordingly, an ActRIIB-based ligand trap polypeptide of the present disclosure may be an ActRIIB variant having one or more additional, non-endogenous N-linked glycosylation consensus sequences as described above.

[0243] The variations described may be combined in various ways. Additionally, the results of the mutagenesis program described herein indicate that there are amino acid positions in ActRIIB that are often beneficial to conserve. With respect to SEQ ID NO:1, these include position 64 (basic amino acid), position 80 (acidic or hydrophobic amino acid), position 78 (hydrophobic, and particularly tryptophan), position 37 (acidic, and particularly aspartic or glutamic acid), position 56 (basic amino acid), position 60 (hydrophobic amino acid, particularly phenylalanine or tyrosine). Thus, in each of the traps disclosed herein, the disclosure provides a framework of amino acids that may be conserved. Other positions that may be desirable to conserve are as follows: position 52 (acidic amino acid), position 55 (basic amino acid), position 81 (acidic), 98 (polar or charged, particularly E, D, R or K), all with respect to SEQ ID NO:1.

[0244] A general formula for an active ActRIIA polypeptide is one that comprises a polypeptide that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 30-110 of SEQ ID NO:9. Accordingly, ActRIIA-based ligand traps of the present disclosure may comprise a polypeptide that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids 30-110 of SEQ ID NO:9. In preferred embodiments, ActRIIA-based ligand traps of the present disclosure do not comprise or consist of amino acids 30-110 of SEQ ID NO:9. Optionally, ActRIIA-based ligand trap polypeptides of the present disclosure comprise a polypeptide that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to amino acids amino acids 12-82 of SEQ ID NO:9 optionally beginning at a position ranging from 1-5 (*e.g.*, 1, 2, 3, 4, or 5) or 3-5 (*e.g.*, 3, 4, or 5) and ending at a position ranging from 110-116 (*e.g.*, 110, 111, 112, 113, 114, 115, or 116) or 110-115 (*e.g.*, 110, 111, 112, 113, 114, or 115), respectively, and comprising no more than 1, 2, 5, 10 or 15 conservative amino acid changes in the ligand binding pocket, and zero, one or more non-conservative alterations at positions 40, 53, 55, 74, 79 and/or 82 in the ligand-binding pocket (with respect to SEQ ID NO:9).

[0245] Functionally active fragments of ligand traps of the disclosure (*e.g.* GDF11 traps, activin B traps, or GDF11/activin B traps) can be obtained by screening polypeptides recombinantly produced from the corresponding fragment of the nucleic acid encoding an ligand trap polypeptide. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments that can function as antagonists (inhibitors) of

GDF11 and/or activin B but that do not substantially bind to and/or inhibit the activity of activin A.

[0246] Functional variants may be generated by modifying the structure of a ligand trap of the present disclosure (*e.g.* a GDF11 trap, activin B trap, or GDF11/activin B trap) for such purposes as enhancing therapeutic efficacy, or stability (*e.g.*, shelf-life and resistance to proteolytic degradation *in vivo*). Such modified ligand trap polypeptides when selected to retain GDF11 and/or activin B binding, are considered functional equivalents of the naturally-occurring ActRII polypeptides. Modified ligand trap polypeptides can also be produced, for instance, by amino acid substitution, deletion, or addition. For instance, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (*e.g.*, conservative mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Whether a change in the amino acid sequence of an ligand trap polypeptide results in a functional homolog can be readily determined by assessing the ability of the variant ligand trap polypeptide to produce a response in cells in a fashion similar to the wild-type ActRII polypeptide, or to bind to one or more ligands, such as GDF11, activin A, activin B, activin C, activin E, GDF8, BMP6, BMP9, BMP10, GDF3, BMP3, BMP3B, Nodal, and GDF15, as compared to the unmodified ActRII polypeptide or a wild-type ActRII polypeptide.

[0247] In certain embodiments, the present disclosure contemplates specific mutations of the ligand trap polypeptides of the present disclosure (*e.g.* GDF11 traps, activin B traps, or GDF11/activin B traps) so as to alter the glycosylation of the polypeptide. Such mutations may be selected so as to introduce or eliminate one or more glycosylation sites, such as O-linked or N-linked glycosylation sites. Asparagine-linked glycosylation recognition sites generally comprise a tripeptide sequence, asparagine-X-threonine or asparagine-X-serine (where "X" is any amino acid) which is specifically recognized by appropriate cellular glycosylation enzymes. The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the ligand trap polypeptide (for O-linked glycosylation sites). A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence. Another means of increasing the number of carbohydrate moieties on an

ligand trap polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine; (b) free carboxyl groups; (c) free sulfhydryl groups such as those of cysteine; (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline; (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan; or (f) the amide group of glutamine. Removal of one or more carbohydrate moieties present on an ActRII polypeptide may be accomplished chemically and/or enzymatically. Chemical deglycosylation may involve, for example, exposure of the ligand trap polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the amino acid sequence intact. Enzymatic cleavage of carbohydrate moieties on ligand trap polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al.* [Meth. Enzymol. (1987) 138:350]. The sequence of an ligand trap polypeptide may be adjusted, as appropriate, depending on the type of expression system used, as mammalian, yeast, insect and plant cells may all introduce differing glycosylation patterns that can be affected by the amino acid sequence of the peptide. In general, ligand trap proteins for use in humans may be expressed in a mammalian cell line that provides proper glycosylation, such as HEK293 or CHO cell lines, although other mammalian expression cell lines are expected to be useful as well.

[0248] This disclosure further contemplates a method of generating mutants, particularly sets of combinatorial mutants of ligand trap polypeptide (*e.g.* a GDF11 trap, activin B trap, or GDF11/activin B trap), as well as truncation mutants; pools of combinatorial mutants are especially useful for identifying ligand trap sequences. The purpose of screening such combinatorial libraries may be to generate, for example, ligand trap polypeptides which bind to activin B, GDF11, and optionally other ligands but do not substantially bind to activin A. A variety of screening assays are provided below, and such assays may be used to evaluate variants. For example, a ligand trap may be screened for ability to bind to an ActRII ligand (*e.g.*, GDF11 and/or activin B), to prevent binding of an ActRII ligand to an ActRII polypeptide or to interfere with signaling caused by an ActRII ligand.

[0249] The activity of a ligand trap (*e.g.* a GDF11 trap, activin B trap, or GDF11/activin B trap) or its variants may also be tested in a cell-based or *in vivo* assay. For example, the effect of a ligand trap on the expression of genes involved in hematopoiesis may be assessed. This may, as needed, be performed in the presence of one or more recombinant ActRII ligand

proteins (*e.g.*, GDF11 and/or activin B), and cells may be transfected so as to produce a ligand trap polypeptide and/or variants thereof, and optionally, an ActRII ligand. Likewise, a ligand trap may be administered to a mouse or other animal, and one or more blood measurements, such as an RBC count, hemoglobin, or reticulocyte count may be assessed using art recognized methods.

[0250] Combinatorial-derived ligand traps (*e.g.* a GDF11 trap, activin B trap, or GDF11/activin B trap) can be generated which have a selective or generally increased potency relative to a reference ligand trap. Such variants, when expressed from recombinant DNA constructs, can be used in gene therapy protocols. Likewise, mutagenesis can give rise to variants which have intracellular half-lives dramatically different than the corresponding unmodified ligand trap. For example, the altered protein can be rendered either more stable or less stable to proteolytic degradation or other cellular processes which result in destruction of, or otherwise inactivation of an unmodified ligand trap. Such variants, and the genes which encode them, can be utilized to ligand trap polypeptide levels by modulating the half-life of the ligand trap. For instance, a short half-life can give rise to more transient biological effects and, when part of an inducible expression system, can allow tighter control of recombinant ligand trap polypeptide levels within the cell. In an Fc fusion protein, mutations may be made in the linker (if any) and/or the Fc portion to alter the half-life of the protein.

[0251] A combinatorial library may be produced by way of a degenerate library of genes encoding a library of polypeptides which each include at least a portion of potential ActRII polypeptide sequences. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential ActRII polypeptide nucleotide sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (*e.g.*, for phage display).

[0252] There are many ways by which the library of potential homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes can then be ligated into an appropriate vector for expression. The synthesis of degenerate oligonucleotides is well known in the art [see, *e.g.*, Narang, SA (1983) *Tetrahedron* 39:3; Itakura *et al.* (1981) *Recombinant DNA, Proc. 3rd Cleveland Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura *et al.* (1984) *Annu. Rev. Biochem.* 53:323; Itakura *et al.* (1984) *Science* 198:1056; Ike *et al.* (1983) *Nucleic Acid Res.* 11:477]. Such techniques have been employed in the directed evolution of other proteins [see, *e.g.*,

Scott *et al.*, (1990) Science 249:386-390; Roberts *et al.* (1992) PNAS USA 89:2429-2433; Devlin *et al.* (1990) Science 249: 404-406; Cwirla *et al.*, (1990) PNAS USA 87: 6378-6382; as well as U.S. Patent Nos: 5,223,409; 5,198,346; and 5,096,815)].

[0253] Alternatively, other forms of mutagenesis can be utilized to generate a combinatorial library. For example, ligand traps of the present disclosure can be generated and isolated from a library by screening using, for example, alanine scanning mutagenesis [see, *e.g.*, Ruf *et al.* (1994) Biochemistry 33:1565-1572; Wang *et al.* (1994) J. Biol. Chem. 269:3095-3099; Balint *et al.* (1993) Gene 137:109-118; Grodberg *et al.* (1993) Eur. J. Biochem. 218:597-601; Nagashima *et al.* (1993) J. Biol. Chem. 268:2888-2892; Lowman *et al.* (1991) Biochemistry 30:10832-10838; and Cunningham *et al.* (1989) Science 244:1081-1085], by linker scanning mutagenesis [see, *e.g.*, Gustin *et al.* (1993) Virology 193:653-660; and Brown *et al.* (1992) Mol. Cell Biol. 12:2644-2652; McKnight *et al.* (1982) Science 232:316)], by saturation mutagenesis [see, *e.g.*, Meyers *et al.*, (1986) Science 232:613]; by PCR mutagenesis [see, *e.g.*, Leung *et al.* (1989) Method Cell Mol Biol 1:11-19]; or by random mutagenesis, including chemical mutagenesis [see, *e.g.*, Miller *et al.* (1992) A Short Course in Bacterial Genetics, CSHL Press, Cold Spring Harbor, NY; and Greener *et al.* (1994) Strategies in Mol Biol 7:32-34]. Linker scanning mutagenesis, particularly in a combinatorial setting, is an attractive method for identifying truncated (bioactive) forms of ActRII polypeptides.

[0254] A wide-range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations and truncations, and, for that matter, for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of ligand traps of the disclosure. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Preferred assays include GDF11, activin B, and/or activin binding assays and GDF11-, activin-B-, and/or activin-mediated cell signaling assays.

[0255] In certain embodiments, the ligand traps (*e.g.* a GDF11 trap, activin B trap, or GDF11/activin B trap) of the present disclosure may further comprise post-translational modifications in addition to any that are naturally present in the ActRII polypeptide (*e.g.*, an

ActRIIA or ActRIIB polypeptide). Such modifications include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. As a result, the modified ligand trap polypeptides may contain non-amino acid elements, such as polyethylene glycols, lipids, poly- or mono-saccharide, and phosphates. Effects of such non-amino acid elements on the functionality of a ligand trap polypeptide may be tested as described herein for other ligand trap polypeptide variants. When a ligand trap polypeptide is produced in cells by cleaving a nascent form of the ligand trap polypeptide, post-translational processing may also be important for correct folding and/or function of the protein. Different cells (*e.g.*, CHO, HeLa, MDCK, 293, WI38, NIH-3T3 or HEK293) have specific cellular machinery and characteristic mechanisms for such post-translational activities and may be chosen to ensure the correct modification and processing of the ligand trap polypeptides.

[0256] In certain aspects, ligand traps of the present disclosure (*e.g.* a GDF11 trap, activin B trap, or GDF11/activin B trap) include fusion proteins having at least a portion (domain) of an ActRII polypeptide (*e.g.*, an ActRIIA or ActRIIB polypeptide) and one or more heterologous portions (domains). Well known examples of such fusion domains include, but are not limited to, polyhistidine, Glu-Glu, glutathione S transferase (GST), thioredoxin, protein A, protein G, an immunoglobulin heavy chain constant region (Fc), maltose binding protein (MBP), or human serum albumin. A fusion domain may be selected so as to confer a desired property. For example, some fusion domains are particularly useful for isolation of the fusion proteins by affinity chromatography. For the purpose of affinity purification, relevant matrices for affinity chromatography, such as glutathione-, amylase-, and nickel- or cobalt- conjugated resins are used. Many of such matrices are available in “kit” form, such as the Pharmacia GST purification system and the QIAexpress<sup>TM</sup> system (Qiagen) useful with (HIS<sub>6</sub>) fusion partners. As another example, a fusion domain may be selected so as to facilitate detection of the ligand trap polypeptides. Examples of such detection domains include the various fluorescent proteins (*e.g.*, GFP) as well as “epitope tags,” which are usually short peptide sequences for which a specific antibody is available. Well known epitope tags for which specific monoclonal antibodies are readily available include FLAG, influenza virus haemagglutinin (HA), and c-myc tags. In some cases, the fusion domains have a protease cleavage site, such as for Factor Xa or thrombin, which allows the relevant protease to partially digest the fusion proteins and thereby liberate the recombinant proteins therefrom. The liberated proteins can then be isolated from the fusion domain by subsequent chromatographic separation. In certain preferred embodiments, a ligand trap is fused with a

domain that stabilizes the ligand trap polypeptide *in vivo* (a “stabilizer” domain). By “stabilizing” is meant anything that increases serum half-life, regardless of whether this is because of decreased destruction, decreased clearance by the kidney, or other pharmacokinetic effect. Fusions with the Fc portion of an immunoglobulin are known to confer desirable pharmacokinetic properties on a wide range of proteins. Likewise, fusions to human serum albumin can confer desirable properties. Other types of fusion domains that may be selected include multimerizing (*e.g.*, dimerizing, tetramerizing) domains and functional domains (that confer an additional biological function, such as further stimulation of muscle growth).

10 [0257] In certain embodiments, the present disclosure provides ligand trap fusion proteins comprising a variant extracellular domain (*e.g.*, a ligand-binding domain) of ActRII protein fused to the following Fc domain:

[0258] THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD(A)VSHPEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK(A)VS  
15 NKALPVPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWE  
SNGQPENNYKTTTPVLDSDGPFLLYSKLTVDKSRWQQGNVFSCSVMHEALHN(A)HY  
TQKSLSLSPGK (SEQ ID NO:16).

[0259] In other embodiments, the present disclosure provides a ligand trap fusion protein comprising a variant extracellular domain (*e.g.*, a ligand-binding domain) of ActRIIB fused  
20 to an Fc domain:

[0260] SGRGEAETRECIYYNANWELERTNQSLERCEGEQDKRLHCYASWRNSS  
GTIELVKKGCWLDDFNCYDRQECVATEENPQVYFCCCEGNFCNERFTHLPEAGGPE  
VTYEPPTAPTGGGTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS  
HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC  
25 KVS NKALPVPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAV  
EWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH  
YTQKSLSLSPGK (SEQ ID NO:47)

[0261] An alternative form with an A64 substitution is as follows:

[0262] SGRGEAETRECIYYNANWELERTNQSLERCEGEQDKRLHCYASWANSS  
30 GTIELVKKGCWLDDFNCYDRQECVATEENPQVYFCCCEGNFCNERFTHLPEAGGPE  
VTYEPPTAPTGGGTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS  
HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC

KVSNKALPVPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAV  
EWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNH  
YTQKSLSLSPGK (SEQ ID NO:17).

[0263] Optionally, the Fc domain has one or more mutations at residues such as Asp-265,  
lysine 322, and Asn-434. In certain cases, the mutant Fc domain having one or more of these  
mutations (e.g., Asp-265 mutation) has reduced ability of binding to the Fcγ receptor relative  
to a wild-type Fc domain. In other cases, the mutant Fc domain having one or more of these  
mutations (e.g., Asn-434 mutation) has increased ability of binding to the MHC class I-  
related Fc-receptor (FcRN) relative to a wildtype Fc domain.

[0264] It is understood that different elements of the fusion proteins may be arranged in  
any manner that is consistent with the desired functionality. For example, a ligand trap (e.g. a  
GDF11 trap, activin B trap, or GDF11/activin B trap) may be placed C-terminal to a  
heterologous domain, or alternatively, a heterologous domain may be placed C-terminal to a  
ligand trap domain. The ligand trap domain and the heterologous domain need not be  
adjacent in a fusion protein, and additional domains or amino acid sequences may be  
included C- or N-terminal to either domain or between the domains.

[0265] In certain embodiments, the ligand traps (e.g. a GDF11 traps, activin B traps, or  
GDF11/activin B traps) of the present disclosure contain one or more modifications that are  
capable of stabilizing the ligand trap polypeptides. For example, such modifications enhance  
the *in vitro* half-life of the ligand trap polypeptides, enhance circulatory half-life of the ligand  
trap polypeptides, and/or reducing proteolytic degradation of the ligand trap polypeptides.  
Such stabilizing modifications include, but are not limited to, fusion proteins (including, for  
example, fusion proteins comprising an ligand trap and a stabilizer domain), modifications of  
a glycosylation site (including, for example, addition of a glycosylation site to a ligand trap  
polypeptide), and modifications of carbohydrate moiety (including, for example, removal of  
carbohydrate moieties from a ligand trap polypeptide). As used herein, the term “stabilizer  
domain” not only refers to a fusion domain (e.g., an immunoglobulin Fc domain) as in the  
case of fusion proteins, but also includes nonproteinaceous modifications such as a  
carbohydrate moiety, or nonproteinaceous moiety, such as polyethylene glycol.

[0266] An example of an ActRIIA-Fc fusion protein comprising a TPA linker is provided  
below:

[0267] MDAMKRGLCCVLLLCGAVFVSPGAAILGRSETQECLFFNANWEKDRTNQ  
 TGVEPCYGDKDKRRHCFATWKNISGSIEIVKQGCWLDDINCYDRTDCVEKKDSPEV  
 YFCCCEGNMCNEKFSYFPEMEVTQPTSNPVTPKPPTTGGG**THTCPPCPAPELLGGPS**  
**VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR**  
 5 **EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPVPIEKTISKAKGQPREP**  
**QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD**  
**DGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK** (SEQ ID  
 NO:18).

[0268] The TPA leader sequence is indicated by single underlining; the TGGG linker  
 10 domain is indicated by double underlining; and the immunoglobulin Fc domain is indicated  
 with **bold** font.

[0269] This polypeptide is encoded by the following nucleic acid sequence:

[0270] ATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGCTGTGTGGA  
 GCAGTCTTCGTTTCGCCCGGCCGCTATACTTGGTAGATCAGAACTCAGGAGT  
 15 GTCTTTTTTTAATGCTAATTGGGAAAAAGACAGAACCAATCAAACCTGGTGTGAA  
 CCGTGTTATGGTGACAAAGATAAACGGCGGCATTGTTTTGCTACCTGGAAGAATA  
 TTTCTGGTTCCATTGAATAGTGAAACAAGGTTGTTGGCTGGATGATATCAACTGC  
 TATGACAGGACTGATTGTGTAGAAAAAAAAGACAGCCCTGAAGTATATTTCTGTT  
 GCTGTGAGGGCAATATGTGTAATGAAAAGTTTTCTTATTTTCCGGAGATGGAAGT  
 20 CACACAGCCCACTTCAAATCCAGTTACACCTAAGCCACCCACCGGTGGTGGAACT  
 CACACATGCCACCGTGCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCC  
 TCTTCCCCCCTAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCAC  
 ATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTA  
 CGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGT  
 25 ACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCT  
 GAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGTCCCCAT  
 CGAGAAAACCATCTCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACAC  
 CCTGCCCCCATCCCGGGAGGAGATGACCAAGAACCAGGTCAGCCTGACCTGCCT  
 GGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCA  
 30 GCCGGAGAACAACCTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTC  
 TTCCTCTATAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTC  
 TTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCC  
 TCTCCCTGTCTCCGGGTAAATGAGAATTC (SEQ ID NO:19)

[0271] An example of a mature ActRIIA-Fc fusion protein as purified from a CHO cell line is provided below:

[0272] ILGRSETQECLFFNANWEKDRTNQTGVEPCYGDKDKRRHCFATWKNISGS  
IEIVKQGCWLDDINCYDRTDCVEKKDSPEVYFCCCEGNMCNEKFSYFPEMEVTQPTS  
5 NPVTPKPPTTGGGTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV  
SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE  
YKCKVSNKALPVPPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGF  
YPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS  
VMHEALHNHYTQKSLSLSPGK (SEQ ID NO:20)

10 [0273] The TGGG linker domain is indicated by double underlining; and the immunoglobulin Fc domain is indicated with **bold** font.

[0274] An example of an ActRIIB-Fc fusion protein comprising a TPA linker is provided below:

[0275] MDAMKRGLCCVLLLCGAVFVSPGASGRGEAETRECIYYNANWELERTNQ  
15 SGLERCEGEQDKRLHCYASWRNSSGTIELVKKGCWLDDFNCYDRQECVATEENPQV  
YFCCCEGNFCNERFTHLPEAGGPEVTYEPPTAPTTGGGTHTCPPCPAPELLGGPSVF  
LFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE  
QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPVPPIEKTISKAKGQPREPQ  
VYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSD  
20 GSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID  
NO: 21)

[0276] The TPA leader sequence is indicated by single underlining; the TGGG linker domain is indicated by double underlining; and the immunoglobulin Fc domain is indicated with **bold** font.

25 [0277] This polypeptide is encoded by the following nucleic acid sequence:

[0278] ATGGATGCAAT GAAGAGAGGG CTCTGCTGTG TGCTGCTGCT  
GTGTGGAGCA GTCTTCGTTT CGCCCGGCGC CTCTGGGCGT GGGGAGGCTG  
AGACACGGGA GTGCATCTAC TACAACGCCA ACTGGGAGCT GGAGCGCACC  
AACCAGAGCG GCCTGGAGCG CTGCGAAGGC GAGCAGGACA AGCGGCTGCA  
30 CTGCTACGCC TCCTGGCGCA ACAGCTCTGG CACCATCGAG CTCGTGAAGA  
AGGGCTGCTG GCTCGATGAC TTCAACTGCT ACGATAGGCA GGAGTGTGTG  
GCCACTGAGG AGAACCCCCA GGTGTACTTC TGCTGCTGTG AAGGCAACTT

CTGCAACGAG CGCTTCACTC ATTTGCCAGA GGCTGGGGGC CCGGAAGTCA  
 CGTACGAGCC ACCCCCGACA GCCCCACCG GTGGTGGAAAC TCACACATGC  
 CCACCGTGCC CAGCACCTGA ACTCCTGGGG GGACCGTCAG TCTTCCTCTT  
 CCCCCAAAA CCCAAGGACA CCCTCATGAT CTCCCGGACC CCTGAGGTCA  
 5 CATGCGTGGT GGTGGACGTG AGCCACGAAG ACCCTGAGGT CAAGTTCAAC  
 TGGTACGTGG ACGGCGTGGA GGTGCATAAT GCCAAGACAA AGCCGCGGGA  
 GGAGCAGTAC AACAGCACGT ACCGTGTGGT CAGCGTCCTC ACCGTCCTGC  
 ACCAGGACTG GCTGAATGGC AAGGAGTACA AGTGCAAGGT CTCCAACAAA  
 GCCCTCCCAG TCCCCATCGA GAAAACCATC TCCAAAGCCA AAGGGCAGCC  
 10 CCGAGAACCA CAGGTGTACA CCCTGCCCCC ATCCCGGGAG GAGATGACCA  
 AGAACCAGGT CAGCCTGACC TGCCTGGTCA AAGGCTTCTA TCCCAGCGAC  
 ATCGCCGTGG AGTGGGAGAG CAATGGGCAG CCGGAGAACA ACTACAAGAC  
 CACGCCTCCC GTGCTGGACT CCGACGGCTC CTTCTTCCTC TATAGCAAGC  
 TCACCGTGGA CAAGAGCAGG TGGCAGCAGG GGAACGTCTT CTCATGCTCC  
 15 GTGATGCATG AGGCTCTGCA CAACCACTAC ACGCAGAAGA GCCTCTCCCT  
 GTCTCCGGGT AAATGA (SEQ ID NO:22).

[0279] An example of a mature ActRIIB-Fc fusion protein [referenced herein as ActRIIB(20-134)-Fc] as purified from a CHO cell line is provided below:

[0280] GRGEAETRECIYYNANWELERTNQSGLERCEGEQDKRLHCYASWRNSSG  
 20 TIELVKKGCWLDDFNCYDRQECVATEENPQVYFCCCEGNFCNERFTHLPEAGGPEV  
 TYEPPPTAPTGGGTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH  
EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK  
VSNKALPVPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE  
WESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH  
 25 YTQKSLSLSPGK (SEQ ID NO:48)

[0281] The immunoglobulin Fc domain is indicated by single underlining.

[0282] An alternative form with an L79D substitution [referenced herein as ActRIIB(L79D 20-134)-Fc] is as follows:

[0283] GRGEAETRECIYYNANWELERTNQSGLERCEGEQDKRLHCYASWRNSSG  
 30 TIELVKKGCWDDDFNCYDRQECVATEENPQVYFCCCEGNFCNERFTHLPEAGGPEV  
 TYEPPPTAPTGGGTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH  
EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK

VSNKALPVPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE  
WESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH  
YTQKSLSLSPGK (SEQ ID NO:23)

[0284] The immunoglobulin Fc domain is indicated with single underlining.

- 5 [0285] An alternative form comprising a double-truncated ActRIIB domain and an L79D substitution [referenced herein as ActRIIB(L79D 25-131)-Fc] is as follows:

[0286] ETRECIYYNANWELERTNQSGLERCEGEQDKRLHCYASWRNSSGTIELVK  
KGCWDDDFNCYDRQECVATEENPQVYFCCCEGNFCNERFTHLPEAGGPEVTYEP  
TGGGTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN  
10 WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPA  
PIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPE  
NNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS  
PGK (SEQ ID NO:49)

[0287] The immunoglobulin Fc domain is indicated with single underlining.

- 15 [0288] In certain embodiments, a ligand trap fusion protein (*e.g.*, a GDF11 trap, an activin B trap, or a GDF11/activin B trap) of the present disclosure comprises an amino acid sequence that is at least 80%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence of any one of SEQ ID Nos: 18, 20, 21, 23, 48, and 49. In preferred  
20 embodiments, ligand trap fusion proteins of the present disclosure do not comprise or consist of the amino acid sequence of any one of SEQ ID Nos: 18, 20, 21, 23, 48 and 49. In other preferred embodiments, ActRIIB-based ligand trap fusion proteins comprising an amino acid sequence that is at least 80%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence of SEQ ID NO: 21 or 23 do not comprise an acidic amino acid at the position corresponding to position 79 of SEQ ID NO: 1 or 2.

- 25 [0289] In certain embodiments, the present disclosure makes available isolated and/or purified forms of the ActRII polypeptides, which are isolated from, or otherwise substantially free of, other proteins.

- [0290] In certain embodiments, ligand trap polypeptides of the disclosure can be produced by a variety of art-known techniques. For example, ligand trap polypeptides can be  
30 synthesized using standard protein chemistry techniques such as those described in Bodansky, M. Principles of Peptide Synthesis, Springer Verlag, Berlin (1993) and Grant G. A. (ed.), Synthetic Peptides: A User's Guide, W. H. Freeman and Company, New York (1992). In

addition, automated peptide synthesizers are commercially available (*see, e.g.*, Advanced ChemTech Model 396; Milligen/Bioscience 9600). Alternatively, the ligand trap polypeptides, fragments or variants thereof may be recombinantly produced using various expression systems (*e.g.*, *E. coli*, Chinese Hamster Ovary (CHO) cells, COS cells, baculovirus) as is well known in the art. In a further embodiment, the modified or unmodified ligand trap polypeptides may be produced by digestion of recombinantly produced full-length ligand trap polypeptides by using, for example, a protease, *e.g.*, trypsin, thermolysin, chymotrypsin, pepsin, or paired basic amino acid converting enzyme (PACE). Computer analysis (using a commercially available software, *e.g.*, MacVector, Omega, PCGene, Molecular Simulation, Inc.) can be used to identify proteolytic cleavage sites. Alternatively, such ligand trap polypeptides may be produced from recombinantly produced full-length ligand trap polypeptides such as standard techniques known in the art, such as by chemical cleavage (*e.g.*, cyanogen bromide, hydroxylamine).

[0291] In preferred embodiments, all proteins and polypeptides of the present disclosure (*e.g.*, GDF11/activin B traps, GDF11 traps, and activin B traps) to be used in accordance with the methods described herein are isolated proteins and polypeptides. As used herein, an isolated protein or polypeptide is one which has been separated from a component of its natural environment. In some embodiments, a protein or polypeptide is purified to greater than 95%, 96%, 97%, 98%, or 99% purity as determined by, for example, electrophoretic (*e.g.*, SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (*e.g.*, ion exchange or reverse phase HPLC). Methods for assessment of antibody purity are well known in the art [see, *e.g.*, Flatman *et al.*, (2007) *J. Chromatogr. B* 848:79-87].

[0292] Any of the ligand trap polypeptides disclosed herein (*e.g.*, a GDF11 trap, an activin B trap, or a GDF11/activin B trap) can be combined with one or more additional antagonist agents of the disclosure to achieve the desired effect. A ligand trap polypeptide disclosed herein (*e.g.*, a GDF11 trap polypeptide, an activin B trap polypeptide, or a GDF11/activin B trap polypeptide) can be combined with another ligand trap polypeptide disclosed herein, or an antibody directed to any of the targets of the disclosure (*e.g.*, an anti-GDF11 antibody, an anti-activin A antibody, an anti-activin B antibody, an anti-activin C antibody, an anti-activin E antibody, an anti-GDF8 antibody, an anti-BMP6 antibody, an anti-ActRIIA antibody, an anti-ActRIIB antibody, an anti-GDF15 antibody, an anti-Nodal antibody, an anti-GDF3 antibody, an anti-BMP3 antibody, an anti-BMP3B antibody, an anti-BMP9 antibody, or an anti-BMP10 antibody), or a small-molecule antagonist directed to any

of the targets of the disclosure (*e.g.*, an activin B small-molecule antagonist, an activin B small-molecule antagonist, a GDF11 small-molecule antagonist, an activin C small-molecule antagonist, an activin E small-molecule antagonist, a GDF8 small-molecule antagonist, a BMP6 small-molecule antagonist, a GDF15 small-molecule antagonist, a Nodal small-molecule antagonist, a GDF3 small-molecule antagonist, a BMP3 small-molecule antagonist, a BMP3B small-molecule antagonist, BMP9 small-molecule antagonist, or a BMP10 small-molecule antagonist), or a polynucleotide antagonist of the disclosure (*e.g.*, a polynucleotide antagonist of activin A, activin B, activin C, activin E, GDF11, GDF8, or BMP6), or a non-antibody binding polypeptide disclosed herein (*e.g.*, a GDF11 binding polypeptide, an activin B binding polypeptide, activin B binding polypeptide, an activin E binding polypeptide, an activin C binding polypeptide, a GDF8 binding polypeptide, a BMP6 binding polypeptide, a GDF15 binding polypeptide, a Nodal binding polypeptide, a BMP3 binding polypeptide, GDF3 binding polypeptide, a BMP3B binding polypeptide, a BMP9 binding polypeptide, or a BMP10 binding polypeptide). For example, a GDF11 trap polypeptide can be combined with an activin B antagonist of the disclosure (*e.g.*, an activin B trap polypeptide, an anti-activin B antibody, a small-molecule antagonist of activin B, a polynucleotide antagonist of activin B, or a non-antibody polypeptide antagonist of activin B) to inhibit both GDF11 and activin B activity (*e.g.*, the ability to bind to and/or activate an ActRIIA and/or ActRIIB receptor). In an alternative embodiment, an activin B trap polypeptide can be combined with a GDF11 antagonist of the disclosure (*e.g.*, a GDF trap polypeptide, an anti-GDF11 antibody, a small-molecule antagonist of GDF11, a polynucleotide antagonist of GDF11, or a non-antibody polypeptide antagonist of GDF11) to inhibit both a GDF11 and activin B activity.

### C. Nucleic Acids Encoding Trap Polypeptides and Recombinant Methods

[0293] In certain embodiments, the present disclosure provides isolated and/or recombinant nucleic acids encoding the ActRII polypeptides (*e.g.*, soluble ActRIIA polypeptides and soluble ActRIIB polypeptides), including fragments, functional variants, and fusion proteins disclosed herein. For example, SEQ ID NO:12 encodes the naturally occurring human ActRIIA precursor polypeptide, while SEQ ID NO:13 encodes the processed extracellular domain of ActRIIA. For example, SEQ ID NO:7 encodes a naturally occurring human ActRIIB precursor polypeptide (the A64 variant described above), while SEQ ID NO:8 encodes the processed extracellular domain of ActRIIB (the A64 variant described above). The subject nucleic acids may be single-stranded or double stranded. Such

nucleic acids may be DNA or RNA molecules. These nucleic acids may be used, for example, in methods for making ActRII-based ligand trap polypeptides of the present disclosure.

[0294] As used herein, isolated nucleic acid(s) refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

[0295] In certain embodiments, nucleic acids encoding ActRIIA-based ligand trap polypeptides of the present disclosure are understood to include nucleic acids that are variants of SEQ ID NO: 12 or 13. In certain aspects, nucleic acids encoding ActRIIB-based ligand trap polypeptides of the present disclosure are understood to include nucleic acids that are variants of SEQ ID NO: 7 or 8. Variant nucleotide sequences include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as allelic variants.

Nucleic acids of the disclosure encode ActRIIA- and ActRIIB-based ligand trap polypeptides that bind to and/or antagonize the activity of at least GDF11 and/or activin A. Optionally, nucleic acids of the disclosure encode ActRIIA- and ActRIIB-based ligand trap polypeptides that do not substantially bind to and/or inhibit activin A. In some embodiments, nucleic acids of the disclosure that encode ActRIIA- and ActRIIB-based ligand trap polypeptides that bind to and/or inhibit GDF11 and/or activin B further bind to and/or inhibit one or more of: activin A, activin C, activin E, GDF8, GDF15, Nodal, GDF3, BMP3, BMP3B, and BMP6.

[0296] In certain embodiments, ActRIIA- and ActRIIB-based ligand traps of the present disclosure are encoded by isolated or recombinant nucleic acid sequences that are at least 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% identical to SEQ ID NOs: 8, 13, 19, and 22.

In preferred embodiments, ActRIIA- and ActRIIB-based ligand traps of the present disclosure are not encoded by nucleic acid sequences that comprise or consist of any one of: SEQ ID NOs: 8, 13, 19, and 22. One of ordinary skill in the art will appreciate that nucleic acid sequences that are at least 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% identical to the sequences complementary to SEQ ID NOs: 8, 13, 19, and 22, and variants thereof, are also within the scope of the present disclosure, provided that the sequences do not comprise or consist of sequences complementary to SEQ ID NOs 8, 13, 19, and 22. In further embodiments, the nucleic acid sequences of the disclosure can be isolated, recombinant, and/or fused with a heterologous nucleotide sequence, or in a DNA library.

[0297] In other embodiments, nucleic acids of the present disclosure also include nucleotide sequences that hybridize under highly stringent conditions to the nucleotide sequence designated in SEQ ID NOs: 8, 13, 19, and 22, complement sequence of SEQ ID NOs: 8, 13, 19, and 22, or fragments thereof, provided that they do not comprise or consist of the nucleotides of SEQ ID NOs: 8, 13, 19, and 22. As discussed above, one of ordinary skill in the art will understand readily that appropriate stringency conditions which promote DNA hybridization can be varied. For example, one could perform the hybridization at 6.0 x sodium chloride/sodium citrate (SSC) at about 45 °C, followed by a wash of 2.0 x SSC at 50 °C. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50 °C to a high stringency of about 0.2 x SSC at 50 °C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22 °C, to high stringency conditions at about 65 °C. Both temperature and salt may be varied, or temperature or salt concentration may be held constant while the other variable is changed. In one embodiment, the disclosure provides nucleic acids which hybridize under low stringency conditions of 6 x SSC at room temperature followed by a wash at 2 x SSC at room temperature.

[0298] Isolated nucleic acids which differ from the nucleic acids as set forth in SEQ ID NOs: 8, 13, 19, and 22 due to degeneracy in the genetic code are also within the scope of the disclosure. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC are synonyms for histidine) may result in “silent” mutations which do not affect the amino acid sequence of the protein. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject proteins will exist among mammalian cells. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-5% of the nucleotides) of the nucleic acids encoding a particular protein may exist among individuals of a given species due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of this disclosure.

[0299] In certain embodiments, the recombinant nucleic acids of the present disclosure may be operably linked to one or more regulatory nucleotide sequences in an expression construct. Regulatory nucleotide sequences will generally be appropriate to the host cell used for expression. Numerous types of appropriate expression vectors and suitable regulatory sequences are known in the art for a variety of host cells. Typically, said one or more

regulatory nucleotide sequences may include, but are not limited to, promoter sequences, leader or signal sequences, ribosomal binding sites, transcriptional start and termination sequences, translational start and termination sequences, and enhancer or activator sequences. Constitutive or inducible promoters as known in the art are contemplated by the disclosure.

5 The promoters may be either naturally occurring promoters, or hybrid promoters that combine elements of more than one promoter. An expression construct may be present in a cell on an episome, such as a plasmid, or the expression construct may be inserted in a chromosome. In some embodiments, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selectable marker genes are well known in  
10 the art and will vary with the host cell used.

[0300] In certain aspects of the present disclosure, the subject nucleic acid is provided in an expression vector comprising a nucleotide sequence encoding an ActRII polypeptide and operably linked to at least one regulatory sequence. Regulatory sequences are art-recognized and are selected to direct expression of the ActRII polypeptide. Accordingly, the term  
15 regulatory sequence includes promoters, enhancers, and other expression control elements. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology*, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express DNA sequences encoding an  
20 ActRII polypeptide. Such useful expression control sequences, include, for example, the early and late promoters of SV40, *tet* promoter, adenovirus or cytomegalovirus immediate early promoter, RSV promoters, the lac system, the *trp* system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda, the control regions for fd coat protein, the promoter for  
25 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast  $\alpha$ -mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the  
30 choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

[0301] A recombinant nucleic acid of the present disclosure can be produced by ligating the cloned gene, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells (yeast, avian, insect or mammalian), or both. Expression vehicles for production of a recombinant ActRII polypeptide include plasmids and other  
5 vectors. For instance, suitable vectors include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

[0302] Some mammalian expression vectors contain both prokaryotic sequences to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription  
10 units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and  
15 eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papilloma virus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. Examples of other viral (including retroviral) expression systems can be found below in the description of gene therapy delivery systems. The various methods employed in the preparation of the plasmids and in transformation of  
20 host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures [see, e.g., Molecular Cloning A Laboratory Manual, 3rd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press, 2001)]. In some instances, it may be desirable to express the recombinant polypeptides by the use of a baculovirus expression system.  
25 Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the  $\beta$ -gal containing pBlueBac III).

[0303] In some embodiments, a vector will be designed for production of the subject ActRII polypeptides in CHO cells, such as a Pcmv-Script vector (Stratagene, La Jolla, Calif.),  
30 pcDNA4 vectors (Invitrogen, Carlsbad, Calif.) and pCI-neo vectors (Promega, Madison, Wisc.). As will be apparent, the subject gene constructs can be used to cause expression of the subject ActRII polypeptides in cells propagated in culture, e.g., to produce proteins, including fusion proteins or variant proteins, for purification.

[0304] This disclosure also pertains to a host cell transfected with a recombinant gene including a coding sequence for one or more of the subject ActRII polypeptides. The host cell may be any prokaryotic or eukaryotic cell. For example, an ActRII polypeptide of the may be expressed in bacterial cells such as *E. coli*, insect cells (e.g., using a baculovirus expression system), yeast, or mammalian cells. Other suitable host cells are known to those skilled in the art.

[0305] Accordingly, the present disclosure further pertains to methods of producing the subject ActRII polypeptides. For example, a host cell transfected with an expression vector encoding an ActRIIA or ActRIIB polypeptide can be cultured under appropriate conditions to allow expression of the ActRII polypeptide to occur. The ActRII polypeptide may be secreted and isolated from a mixture of cells and medium containing the ActRII polypeptide. Alternatively, the ActRII polypeptide may be retained cytoplasmically or in a membrane fraction and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The subject ActRII polypeptides can be isolated from cell culture medium, host cells, or both, using techniques known in the art for purifying proteins, including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, immunoaffinity purification with antibodies specific for particular epitopes of the ActRII polypeptides and affinity purification with an agent that binds to a domain fused to the ActRII polypeptide (e.g., a protein A column may be used to purify an ActRIIA-Fc or ActRIIB-Fc fusion). In some embodiments, the ActRII polypeptide is a fusion protein containing a domain which facilitates its purification. In some embodiments, purification is achieved by a series of column chromatography steps, including, for example, three or more of the following, in any order: protein A chromatography, Q sepharose chromatography, phenylsepharose chromatography, size exclusion chromatography, and cation exchange chromatography. The purification could be completed with viral filtration and buffer exchange. An ActRIIB-hFc or ActRIIA-hFc protein may be purified to a purity of >90%, >95%, >96%, >98%, or >99% as determined by size exclusion chromatography and >90%, >95%, >96%, >98%, or >99% as determined by SDS PAGE. The target level of purity should be one that is sufficient to achieve desirable results in mammalian systems, particularly non-human primates, rodents (mice), and humans.

[0306] In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence at the N-terminus of the desired

portion of the recombinant ActRII polypeptide, can allow purification of the expressed fusion protein by affinity chromatography using a  $\text{Ni}^{2+}$  metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase to provide the purified ActRII polypeptide. *See, e.g., Hochuli et al. (1987) J. Chromatography 411:177; and Janknecht et al. PNAS USA 88:8972).*

[0307] Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence [see, e.g., Current Protocols in Molecular Biology, eds. Ausubel *et al.*, John Wiley & Sons: 1992].

#### **D. Other Binding Polypeptides**

[0308] In another aspect, an antagonist agent, or combination of agents, of the present disclosure is a non-antibody binding polypeptide that binds to and/or inhibits the activity of at least GDF11 and/or activin B (*e.g., activation of ActRIIA or ActRIIB Smad2/3 signaling*). Optionally, a non-antibody binding polypeptide, or combinations of non-antibody-binding polypeptides, of the disclosure does not bind to and/or inhibit the activity of activin A (*e.g., activin A-mediated activation of ActRIIA or ActRIIB Smad2/3 signaling*). Optionally, a non-antibody binding polypeptide, or combinations of non-antibody-binding polypeptides, of the disclosure further binds to and/or inhibits the activity of GDF8 (*e.g., GDF8-mediated activation of ActRIIA or ActRIIB Smad2/3 signaling*). In some embodiments, a non-antibody binding polypeptide, or combinations of non-antibody-binding polypeptides, of the disclosure that binds to and/or inhibits the activity of GDF11 and/or activin B further binds to and/or inhibits activity of one or more of activin E, activin C, activin A, GDF8, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10 (*e.g., activation of ActRIIA or ActRIIB Smad2/3 and/or Smad 1/5/8 signaling*). In certain embodiments, non-antibody-binding polypeptides that bind to BMP9 and/or BMP10 inhibit interaction between BMP9

and a type II receptor of the TGF $\beta$  superfamily (*e.g.*, ActRIIA and/or ActRIIB) and/or BMP10 and a type II receptor of the TGF $\beta$  superfamily (*e.g.*, ActRIIA and/or ActRIIB). Preferably, non-antibody-binding polypeptides that bind to BMP9 and/or BMP10 do not inhibit, or substantially inhibit, interaction between BMP9 and ALK1 and/or BMP10 and ALK1.

[0309] Binding polypeptides of the present disclosure may be chemically synthesized using known polypeptide synthesis methodology or may be prepared and purified using recombinant technology. Binding polypeptides are usually at least about 5 amino acids in length, alternatively at least about 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 amino acids in length or more, wherein such binding polypeptides that are capable of binding, preferably specifically, to a target as described herein (*e.g.*, GDF11, activin B, activin E, activin C, GDF8, activin A, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10). Binding polypeptides may be identified without undue experimentation using well known techniques. In this regard, it is noted that techniques for screening polypeptide libraries for binding polypeptides that are capable of specifically binding to a polypeptide target are well known in the art including, for example, U.S. Pat. Nos. 5,556,762; 5,750,373; 4,708,871; 4,833,092; 5,223,409; 5,403,484; 5,571,689; and 5,663,143; PCT Publication Nos. WO 84/03506 and WO84/03564; Geysen *et al.* (1984) Proc. Natl. Acad. Sci. U.S.A., 81:3998-4002; Geysen *et al.* (1985) Proc. Natl. Acad. Sci. U.S.A., 82:178-182; Geysen *et al.* (1986) in Synthetic Peptides as Antigens, 130-149; Geysen *et al.* (1987) J. Immunol. Meth, 102:259-274; Schoofs *et al.* (1988) J. Immunol., 140:611-616, Cwirla, S. E. *et al.* (1990) Proc. Natl. Acad. Sci. USA, 87:6378; Lowman, H. B. *et al.* (1991) Biochemistry, 30:10832; Clackson, T. *et al.* (1991) Nature, 352: 624; Marks, J. D. *et al.* (1991), J. Mol. Biol., 222:581; Kang, A. S. *et al.* (1991) Proc. Natl. Acad. Sci. USA, 88:8363, and Smith, G. P. (1991) Current Opin. Biotechnol., 2:668.

[0310] In this regard, bacteriophage (phage) display is one well known technique which allows one to screen large polypeptide libraries to identify member(s) of those libraries which are capable of specifically binding to a target polypeptide (*e.g.*, GDF11, activin B, activin E, activin C, GDF8, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10). Phage display is a technique by which variant polypeptides are displayed as fusion proteins to

the coat protein on the surface of bacteriophage particles [see, *e.g.*, Scott, J. K. and Smith, G. P. (1990) *Science*, 249: 386]. The utility of phage display lies in the fact that large libraries of selectively randomized protein variants (or randomly cloned cDNAs) can be rapidly and efficiently sorted for those sequences that bind to a target molecule with high affinity.

5     Display of peptide [see, *e.g.*, Cwirla, S. E. *et al.* (1990) *Proc. Natl. Acad. Sci. USA*, 87:6378] or protein [Lowman, H. B. *et al.* (1991) *Biochemistry*, 30:10832; Clackson, T. *et al.* (1991) *Nature*, 352: 624; Marks, J. D. *et al.* (1991), *J. Mol. Biol.*, 222:581; Kang, A. S. *et al.* (1991) *Proc. Natl. Acad. Sci. USA*, 88:8363] libraries on phage have been used for screening millions of polypeptides or oligopeptides for ones with specific binding properties [see, *e.g.*,  
10     Smith, G. P. (1991) *Current Opin. Biotechnol.*, 2:668]. Sorting phage libraries of random mutants requires a strategy for constructing and propagating a large number of variants, a procedure for affinity purification using the target receptor, and a means of evaluating the results of binding enrichments (see, *e.g.*, U.S. Pat. Nos. 5,223,409; 5,403,484; 5,571,689; and 5,663,143).

15     [0311]     Although most phage display methods have used filamentous phage, lambdoid phage display systems (see, *e.g.*, WO 95/34683; and U.S. Pat. No. 5,627,024), T4 phage display systems [see, *e.g.*, Ren *et al.* (1998) *Gene*, 215: 439; Zhu *et al.* (1998) *Cancer Research*, 58(15): 3209-3214; Jiang *et al.* (1997) *Infection & Immunity*, 65(11): 4770-4777; Ren *et al.* (1997) *Gene*, 195(2):303-311; Ren (1996) *Protein Sci.*, 5: 1833; Efimov *et al.*  
20     (1995) *Virus Genes*, 10: 173] and T7 phage display systems [see, *e.g.*, Smith and Scott (1993) *Methods in Enzymology*, 217: 228-257; and U.S. Pat. No. 5,766,905] are also known.

[0312]     Additional improvements to enhance the ability of display systems to screen peptide libraries for binding to selected target molecules and to display functional proteins with the potential of screening these proteins for desired properties are known in the art.

25     Combinatorial reaction devices for phage display reactions have been developed (see, *e.g.*, WO 98/14277) and phage display libraries have been used to analyze and control bimolecular interactions (see, *e.g.*, WO 98/20169; and WO 98/20159) and properties of constrained helical peptides (see, *e.g.*, WO 98/20036). International patent publication no. WO 97/35196 describes a method of isolating an affinity ligand in which a phage display library is  
30     contacted with one solution in which the ligand will bind to a target molecule and a second solution in which the affinity ligand will not bind to the target molecule, to selectively isolate binding ligands. International patent publication no. WO 97/46251 describes a method of biopanning a random phage display library with an affinity purified antibody and then

isolating binding phage, followed by a micropanning process using microplate wells to isolate high affinity binding phage. The use of *Staphylococcus aureus* protein A as an affinity tag has also been reported [see, e.g., Li *et al.* (1998) Mol. Biotech., 9:187.

[0313] WO 97/47314 describes the use of substrate subtraction libraries to distinguish enzyme specificities using a combinatorial library, which may be a phage display library. A method for selecting enzymes suitable for use in detergents using phage display is described in international patent publication no. WO 97/09446. Additional methods of selecting specific binding proteins are described in, for example, U.S. Pat. Nos. 5,498,538; 5,432,018; and international patent publication WO 98/15833.

[0314] Methods of generating peptide libraries and screening these libraries are also disclosed in, for example, U.S. Pat. Nos. 5,723,286; 5,432,018; 5,580,717; 5,427,908; 5,498,530; 5,770,434; 5,734,018; 5,698,426; 5,763,192; and 5,723,323.

[0315] Any of the non-antibody binding polypeptides disclosed herein (e.g., a GDF11 binding polypeptide, an activin B binding polypeptide, an activin B binding polypeptide, an activin E binding polypeptide, an activin C binding polypeptide, a GDF8 binding polypeptide, a BMP6 binding polypeptide, a GDF15 binding polypeptide, a Nodal binding polypeptide, a GDF3 binding polypeptide, a BMP3binding polypeptide, a BMP3B binding polypeptide, a BMP9 binding polypeptide, or a BMP6 binding polypeptide) can be combined with one or more additional antagonist agents of the disclosure to achieve the desired effect.

A non-antibody binding polypeptide disclosed herein (e.g., a GDF11 binding polypeptide, an activin A binding polypeptide, an activin B binding polypeptide, an activin E binding polypeptide, an activin C binding polypeptide, a GDF8 binding polypeptide, a BMP6 binding polypeptide, a GDF15 binding polypeptide, a Nodal binding polypeptide, a GDF3 binding polypeptide, a BMP3binding polypeptide, a BMP3B binding polypeptide, a BMP9 binding polypeptide, or a BMP6 binding polypeptide) can be combined with another non-antibody binding polypeptide of the disclosure, or with an antibody directed to any of the targets of the disclosure (e.g., an anti-GDF11 antibody, an anti-activin B antibody, an anti-activin B antibody, an anti-activin C antibody, an anti-activin E antibody, an anti-GDF11 antibody, an anti-GDF8 antibody, an anti-BMP6 antibody, an anti-ActRIIA antibody, an anti-ActRIIB antibody, an anti-GDF15 antibody, an anti-Nodal antibody, an anti-GDF3 antibody, an anti-BMP3 antibody, an anti-BMP3B antibody, an anti-BMP9 antibody, or an anti-BMP10 antibody) or a ligand trap polypeptide disclosed herein (e.g., a GDF11 trap polypeptide, an activin B trap polypeptide, or a GDF11/activin B trap polypeptide), or a small molecule

directed to any of the targets of the disclosure or a small-molecule antagonist directed to any of the targets of the disclosure (*e.g.*, an activin B small-molecule antagonist, an activin B small-molecule antagonist, a GDF11 small-molecule antagonist, an activin C small-molecule antagonist, an activin E small-molecule antagonist, a GDF8 small-molecule antagonist, a BMP6 small-molecule antagonist, a GDF15 small-molecule antagonist, a Nodal small-molecule antagonist, a GDF3 small-molecule antagonist, a BMP3 small-molecule antagonist, a BMP3B small-molecule antagonist, a BMP9 small-molecule antagonist, or a BMP10 small-molecule antagonist), or a polynucleotide antagonist of the disclosure (*e.g.*, a polynucleotide antagonist of activin B, a polynucleotide antagonist of activin B, activin C, activin E, GDF11, GDF8, or BMP6). For example, a GDF11-binding polypeptide can be combined with an activin B antagonist of the disclosure (*e.g.*, an activin B trap polypeptide, an anti-activin B antibody, a small-molecule antagonist of activin B, a polynucleotide antagonist of activin B, or a non-antibody polypeptide antagonist of activin B) to inhibit both a GDF11 and an activin B activity (*e.g.*, the ability to bind to and/or activate an ActRIIA and/or ActRIIB receptor). In an alternative embodiment, an activin-B-binding polypeptide can be combined with a GDF11 antagonist of the disclosure (*e.g.*, a GDF-trap polypeptide, an anti-GDF11 antibody, a small-molecule antagonist of GDF11, a polynucleotide antagonist of GDF11, or a non-antibody polypeptide antagonist of GDF11) to inhibit both a GDF11 and an activin B activity.

#### **E. Small-Molecule Antagonists**

[0316] In another aspect, an antagonist agent, or combination of agents, of the present disclosure is a small-molecule antagonist that inhibits the expression (*e.g.*, transcription, translation, and/or cellular secretion) of at least GDF11 and/or activin B. Optionally, a small-molecule antagonist, or combinations of small-molecule antagonists, of the disclosure does not inhibit the expression of activin A. Optionally, a small-molecule antagonist, or combinations of small-molecule antagonists, of the disclosure further inhibits expression of GDF8. In some embodiments, a small-molecule antagonist, or combinations of small-molecule antagonists, of the disclosure that inhibits expression of GDF11 and/or activin B further inhibits expression of one or more of activin E, activin C, activin A, GDF8, GDF15, Nodal, GDF3, BMP3, and BMP3B.

[0317] In another aspect, an antagonist agent, or combination of agents, of the present disclosure is a small-molecule agent that binds to and inhibits the activity of at least GDF11

and/or activin B (*e.g.*, activation of ActRIIA and/or ActRIIB Smad2/3 signaling). Optionally, a small-molecule antagonist, or combinations of small-molecule antagonists, of the disclosure does not bind to and/or inhibit activin A activity (*e.g.*, activin A-mediated activation of ActRIIA and/or ActRIIB Smad2/3 signaling). Optionally, a small-molecule antagonist, or combinations of small-molecule antagonists, of the disclosure further binds to and inhibits the activity of GDF8 (*e.g.*, GDF8-mediated activation of ActRIIA and/or ActRIIB Smad 2/3 signaling). In some embodiments, a small-molecule antagonist, or combinations of small-molecule antagonists, of the disclosure that binds to and inhibits an activity of GDF11 and/or activin B further binds to and inhibits an activity of one or more of GDF8, activin E, activin C, activin A, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10 (*e.g.*, activation of ActRIIA and/or ActRIIB Smad2/3 and/or Smad 1/5/8 signaling). In certain embodiments, small molecules that bind to BMP9 and/or BMP10 inhibit interaction between BMP9 and a type II receptor of the TGF $\beta$  superfamily (*e.g.*, ActRIIA and/or ActRIIB) and/or BMP10 and a type II receptor of the TGF $\beta$  superfamily (*e.g.*, ActRIIA and/or ActRIIB). Preferably, small molecules that bind to BMP9 and/or BMP10 do not inhibit, or substantially inhibit, interaction between BMP9 and ALK1 and/or BMP10 and ALK1.

[0318] In a further aspect, an antagonist agent, or combination of agents, of the present disclosure is a small-molecule agent that indirectly antagonizes at least GDF11 and/or activin B activity. In some embodiments, an indirect small-molecule antagonist, or combination of indirect small-molecule antagonists, of the present disclosure is a small molecule that binds to an ActRII receptor (*e.g.*, an ActRIIA and/or an ActRIIB receptor) and inhibits at least GDF11 and/or activin B from binding to and/or activating an ActRII receptor (*e.g.*, activation of ActRIIA and/or ActRIIB Smad2/3 signaling). Optionally, an indirect small-molecule antagonist, or combination of indirect small-molecule antagonists, of the present disclosure does not substantially inhibit activin A from binding to and/or activating an ActRII receptor (*e.g.*, activin A-mediated ActRIIA and/or ActRIIB Smad 2/3 signaling). Optionally, an indirect small-molecule antagonist, or combination of indirect small-molecule antagonists, of the present disclosure binds to an ActRII receptor and further inhibits binding to and/or activation of the ActRII receptor by GDF8. In some embodiments, an indirect small-molecule antagonist, or combination of indirect small-molecule antagonists, of the present disclosure that bind to an ActRII receptor and inhibit at least GDF11 and/or activin B from binding to and/or activating an ActRII receptor further inhibit one or more of activin C, activin E, GDF8, BMP6, activin A, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and

BMP10 from binding to and/or activating the ActRII receptor (*e.g.*, activation of ActRIIA and/or ActRIIB Smad 2/3 and/or Smad 1/5/8 signaling).

[0319] Binding organic small-molecule antagonists of the present disclosure may be identified and chemically synthesized using known methodology (*see, e.g.*, PCT Publication Nos. WO 00/00823 and WO 00/39585). In general, small-molecule antagonists of the disclosure are usually less than about 2000 daltons in size, alternatively less than about 1500, 750, 500, 250 or 200 daltons in size, wherein such organic small molecules that are capable of binding, preferably specifically, to a polypeptide as described herein (activin B, activin C, activin E, GDF11, GDF8, and BMP6). Such small-molecule antagonists may be identified without undue experimentation using well-known techniques. In this regard, it is noted that techniques for screening organic small-molecule libraries for molecules that are capable of binding to a polypeptide target are well known in the art (*see, e.g.*, international patent publication Nos. WO00/00823 and WO00/39585).

[0320] Binding organic small molecules of the present disclosure may be, for example, aldehydes, ketones, oximes, hydrazones, semicarbazones, carbazides, primary amines, secondary amines, tertiary amines, N-substituted hydrazines, hydrazides, alcohols, ethers, thiols, thioethers, disulfides, carboxylic acids, esters, amides, ureas, carbamates, carbonates, ketals, thioketals, acetals, thioacetals, aryl halides, aryl sulfonates, alkyl halides, alkyl sulfonates, aromatic compounds, heterocyclic compounds, anilines, alkenes, alkynes, diols, amino alcohols, oxazolidines, oxazolines, thiazolidines, thiazolines, enamines, sulfonamides, epoxides, aziridines, isocyanates, sulfonyl chlorides, diazo compounds, and acid chlorides.

[0321] Any of the small-molecule antagonists disclosed herein (*e.g.*, a small-molecule antagonist of activin B, activin C, activin E, GDF11, GDF8, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, or BMP10) can be combined with one or more additional antagonist agents of the disclosure to achieve the desired effect. A small-molecule antagonist disclosed herein (*e.g.*, a small-molecule antagonist of activin A, activin B, activin C, activin E, GDF11, GDF8, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, or BMP10) can be combined with another small-molecule antagonist of the disclosure, or an antibody directed to any of the targets of the disclosure (*e.g.*, an anti-GDF11 antibody, an anti-activin B antibody, an anti-activin B antibody, an anti-activin C antibody, an anti-activin E antibody, an anti-GDF8 antibody, an anti-BMP6 antibody, an anti-ActRIIA antibody, an anti-ActRIIB antibody, an anti-ActRIIB antibody, an anti-GDF15 antibody, an anti-Nodal antibody, an anti-GDF3 antibody, an anti-BMP3 antibody, an anti-BMP3B antibody, an anti-BMP9 antibody, or an

anti-BMP10 antibody), or a non-antibody binding polypeptide disclosed herein (*e.g.*, a GDF11-binding polypeptide, an activin-B-binding polypeptide, an activin-B-binding polypeptide, an activin-E-binding polypeptide, an activin-C-binding polypeptide, a GDF8-binding polypeptide, a BMP6-binding polypeptide, a GDF15-binding polypeptide, a Nodal-binding polypeptide, a GDF3-binding polypeptide, a BMP3-binding polypeptide, a BMP3B-binding polypeptide, a BMP9-binding polypeptide, or a BMP10-binding polypeptide), or a ligand trap polypeptide disclosed herein (*e.g.*, a GDF11-trap polypeptide, an activin-B trap polypeptide, or a GDF/activin-B trap polypeptide), or a polynucleotide antagonist of the disclosure (*e.g.*, a polynucleotide antagonist of activin A, a polynucleotide antagonist of activin B, activin C, activin E, GDF11, GDF8, BMP6, GDF15, Nodal, GDF3, BMP3, or BMP3B). For example, a small-molecule antagonist of GDF11 can be combined with an activin-B antagonist of the disclosure (*e.g.*, an activin-B trap polypeptide, an anti-activin-B antibody, a small-molecule antagonist of activin B, a polynucleotide antagonist of activin B, or a non-antibody polypeptide antagonist of activin B) to inhibit both a GDF11 and an activin B activity (*e.g.*, the ability to bind to and/or activate an ActRIIA and/or ActRIIB receptor). In an alternative embodiment, a small-molecule antagonist of activin-B antibody can be combined with a GDF11 antagonist of the disclosure (*e.g.*, a GDF-trap polypeptide, an anti-GDF11 antibody, a small-molecule antagonist of GDF11, a polynucleotide antagonist of GDF11, or a non-antibody polypeptide antagonist of GDF11) to inhibit both a GDF11 and an activin B activity.

## F. Antagonist Polynucleotides

[0322] In another aspect, an antagonist agent, or combination of agents, of the present disclosure is a polynucleotide antagonist that inhibits at least GDF11 and/or activin B. In some embodiments, an antagonist polynucleotide of the disclosure inhibits the expression (*e.g.*, transcription, translation, and/or cellular secretion) of at least GDF11 and/or activin B. Optionally, a polynucleotide antagonist, or combinations of polynucleotide antagonists, of the disclosure does not inhibit activin A (*e.g.* inhibits expression and/or activity of activin A). Optionally, a polynucleotide antagonist, or combinations of polynucleotide antagonists, of the disclosure further inhibit GDF8 (*e.g.* inhibits expression and/or activity of GDF8). In some embodiments, a polynucleotide antagonist, or combinations of polynucleotide antagonists, of the disclosure that inhibits GDF11 and/or activin B (*e.g.* expression and/or activity of GDF11

and/or activin B) further inhibits (*e.g.*, inhibits expression and/or activity) one or more of activin E, activin C, activin A, GDF8, BMP6, Nodal, GDF3, BMP3, and GDF3B.

[0323] The polynucleotide antagonists of the disclosure may be an antisense nucleic acid, an RNAi molecule [*e.g.*, small interfering RNA (siRNA), small-hairpin RNA (shRNA), microRNA (miRNA)], an aptamer and/or a ribozyme. The nucleic acid and amino acid sequences of human GDF11, activin B, activin C, activin E, GDF8, activin A, BMP6, Nodal, GDF3, BMP3, and GDF3B are known in the art. In addition, many different methods of generating polynucleotide antagonists are well known in the art. Therefore polynucleotide antagonists for use in accordance with this disclosure may be routinely made by the skilled person in the art based on the knowledge in the art and teachings provided herein.

[0324] Antisense technology can be used to control gene expression through antisense DNA or RNA, or through triple-helix formation. Antisense techniques are discussed, for example, in Okano (1991) *J. Neurochem.* 56:560; Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, Fla. (1988). Triple-helix formation is discussed in, for instance, Cooney *et al.* (1988) *Science* 241:456; and Dervan *et al.*, (1991) *Science* 251:1300. The methods are based on binding of a polynucleotide to a complementary DNA or RNA. In some embodiments, the antisense nucleic acids comprise a single-stranded RNA or DNA sequence that is complementary to at least a portion of an RNA transcript of a gene disclosed herein (*e.g.*, GDF11, activin B, activin C, activin E, GDF8, activin A, BMP6, Nodal, GDF3, BMP3, and GDF3B). However, absolute complementarity, although preferred, is not required.

[0325] A sequence “complementary to at least a portion of an RNA,” referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded antisense nucleic acids of a gene disclosed herein (*e.g.*, GDF11, activin A, activin B, activin C, activin E, GDF8, activin A, BMP6, Nodal, GDF3, BMP3, and GDF3B), a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the larger the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

[0326] Polynucleotides that are complementary to the 5' end of the message, for example, the 5'-untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3'-untranslated sequences of mRNAs have been shown to be effective at inhibiting translation of mRNAs as well [see, *e.g.*, Wagner, R., (1994) *Nature* 372:333-335]. Thus, oligonucleotides complementary to either the 5'- or 3'-non-translated, non-coding regions of a gene of the disclosure (*e.g.*, GDF11, activin A, activin B, activin C, activin E, GDF8, BMP6, Nodal, GDF3, BMP3, and GDF3B), could be used in an antisense approach to inhibit translation of an endogenous mRNA (*e.g.*, GDF11, activin A, activin B, activin C, activin E, GDF8, activin A, BMP6, Nodal, GDF3, BMP3, and GDF3B). Polynucleotides complementary to the 5'-untranslated region of the mRNA should include the complement of the AUG start codon. Antisense polynucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the methods of the present disclosure. Whether designed to hybridize to the 5'-, 3'- or coding region of an mRNA of the disclosure (*e.g.*, GDF11, activin A, activin B, activin C, activin E, GDF8, activin A, BMP6, Nodal, GDF3, BMP3, and GDF3B), antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

[0327] In one embodiment, the antisense nucleic acid of the present disclosure (*e.g.*, a GDF11, activin B, activin C, activin E, GDF8, activin A, BMP6, Nodal, GDF3, BMP3, and GDF3B and/or antisense nucleic acid) is produced intracellularly by transcription from an exogenous sequence. For example, a vector or a portion thereof, is transcribed, producing an antisense nucleic acid (RNA) of a gene of the disclosure. Such a vector would contain a sequence encoding the desired antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in vertebrate cells. Expression of the sequence encoding desired genes of the instant disclosure, or fragments thereof, can be by any promoter known in the art to act in vertebrate, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include, but are not limited to, the SV40 early promoter region [see, *e.g.*, Benoist and Chambon (1981) *Nature* 290:304-310], the promoter contained in the 3' long-

terminal repeat of Rous sarcoma virus [see, *e.g.*, Yamamoto *et al.* (1980) Cell 22:787-797], the herpes thymidine promoter [see, *e.g.*, Wagner *et al.* (1981) Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445], and the regulatory sequences of the metallothionein gene [see, *e.g.*, Brinster, *et al.* (1982) Nature 296:39-42].

5 [0328] In some embodiments, the polynucleotide antagonists are interfering RNA (RNAi) molecules that target the expression of one or more of: GDF11, activin B, activin C, activin E, GDF8, activin A, BMP6, Nodal, GDF3, BMP3, and GDF3B. RNAi refers to the expression of an RNA which interferes with the expression of the targeted mRNA. Specifically, RNAi silences a targeted gene via interacting with the specific mRNA through a  
10 siRNA (small interfering RNA). The ds RNA complex is then targeted for degradation by the cell. An siRNA molecule is a double-stranded RNA duplex of 10 to 50 nucleotides in length, which interferes with the expression of a target gene which is sufficiently complementary (*e.g.* at least 80% identity to the gene). In some embodiments, the siRNA molecule comprises a nucleotide sequence that is at least 85, 90, 95, 96, 97, 98, 99, or 100% identical to the  
15 nucleotide sequence of the target gene.

[0329] Additional RNAi molecules include short-hairpin RNA (shRNA); also short-interfering hairpin and microRNA (miRNA). The shRNA molecule contains sense and antisense sequences from a target gene connected by a loop. The shRNA is transported from the nucleus into the cytoplasm, and it is degraded along with the mRNA. Pol III or U6  
20 promoters can be used to express RNAs for RNAi. Paddison *et al.* [Genes & Dev. (2002) 16:948-958, 2002] have used small RNA molecules folded into hairpins as a means to effect RNAi. Accordingly, such short-hairpin RNA (shRNA) molecules are also advantageously used in the methods described herein. The length of the stem and loop of functional shRNAs varies; stem lengths can range anywhere from about 25 to about 30 nt, and loop size can  
25 range between 4 to about 25 nt without affecting silencing activity. While not wishing to be bound by any particular theory, it is believed that these shRNAs resemble the double-stranded RNA (dsRNA) products of the DICER RNase and, in any event, have the same capacity for inhibiting expression of a specific gene. The shRNA can be expressed from a lentiviral vector. An miRNA is a single-stranded RNA of about 10 to 70 nucleotides in  
30 length that are initially transcribed as pre-miRNA characterized by a “stem-loop” structure, which are subsequently processed into mature miRNA after further processing through the RISC.

[0330] Molecules that mediate RNAi, including without limitation siRNA, can be produced in vitro by chemical synthesis (Hohjoh, FEBS Lett 521:195-199, 2002), hydrolysis of dsRNA (Yang *et al.*, Proc Natl Acad Sci USA 99:9942-9947, 2002), by *in vitro* transcription with T7 RNA polymerase (Donzeet *et al.*, Nucleic Acids Res 30:e46, 2002; Yu *et al.*, Proc Natl Acad Sci USA 99:6047-6052, 2002), and by hydrolysis of double-stranded RNA using a nuclease such as E. coli RNase III (Yang *et al.*, Proc Natl Acad Sci USA 99:9942-9947, 2002).

[0331] According to another aspect, the disclosure provides polynucleotide antagonists including but not limited to, a decoy DNA, a double-stranded DNA, a single-stranded DNA, a complexed DNA, an encapsulated DNA, a viral DNA, a plasmid DNA, a naked RNA, an encapsulated RNA, a viral RNA, a double-stranded RNA, a molecule capable of generating RNA interference, or combinations thereof.

[0332] In some embodiments, the polynucleotide antagonists of the disclosure are aptamers. Aptamers are nucleic acid molecules, including double-stranded DNA and single-stranded RNA molecules, which bind to and form tertiary structures that specifically bind to a target molecule, such as a GDF11, activin B, activin C, activin E, GDF8, activin A, BMP6, Nodal, GDF3, BMP3, or GDF3B polypeptide. The generation and therapeutic use of aptamers are well established in the art (see, *e.g.*, U.S. Pat. No. 5,475,096). Additional information on aptamers can be found in U.S. Patent Application Publication No. 20060148748. Nucleic acid aptamers are selected using methods known in the art, for example via the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) process. SELEX is a method for the *in vitro* evolution of nucleic acid molecules with highly specific binding to target molecules as described in, *e.g.*, U.S. Pat. Nos. 5,475,096; 5,580,737; 5,567,588; 5,707,796; 5,763,177; 6,011,577; and 6,699,843. Another screening method to identify aptamers is described in U.S. Pat. No. 5,270,163. The SELEX process is based on the capacity of nucleic acids for forming a variety of two- and three-dimensional structures, as well as the chemical versatility available within the nucleotide monomers to act as ligands (form specific binding pairs) with virtually any chemical compound, whether monomeric or polymeric, including other nucleic acid molecules and polypeptides. Molecules of any size or composition can serve as targets. The SELEX method involves selection from a mixture of candidate oligonucleotides and step-wise iterations of binding, partitioning and amplification, using the same general selection scheme, to achieve desired binding affinity and selectivity. Starting from a mixture of nucleic acids, which can comprise a segment of

randomized sequence, the SELEX method includes steps of contacting the mixture with the target under conditions favorable for binding; partitioning unbound nucleic acids from those nucleic acids which have bound specifically to target molecules; dissociating the nucleic acid-target complexes; amplifying the nucleic acids dissociated from the nucleic acid-target complexes to yield a ligand enriched mixture of nucleic acids. The steps of binding, partitioning, dissociating and amplifying are repeated through as many cycles as desired to yield nucleic acid ligands which bind with high affinity and specificity to the target molecule.

[0333] Typically, such binding molecules are separately administered to the animal [see, *e.g.*, O'Connor (1991) J. Neurochem. 56:560], but such binding molecules can also be expressed *in vivo* from polynucleotides taken up by a host cell and expressed *in vivo* [see, *e.g.*, Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, Fla. (1988)].

[0334] Any of the polynucleotide antagonists disclosed herein (*e.g.*, a polynucleotide antagonist of activin A, activin B, activin C, activin E, GDF11, GDF8, BMP6, Nodal, GDF3, BMP3, or GDF3B) can be combined with one or more additional antagonist agents of the disclosure to achieve the desired effect. A polynucleotide antagonist disclosed herein (*e.g.*, a polynucleotide antagonist of activin A, activin B, activin C, activin E, GDF11, GDF8, BMP6, Nodal, GDF3, BMP3, or GDF3B) can be combined with another polynucleotide antagonist of the disclosure, or an antibody directed to any of the targets of the disclosure (*e.g.*, an anti-GDF11 antibody, an anti-activin B antibody, an anti-activin B antibody, an anti-GDF8 antibody, an anti-activin C antibody, an anti-activin E antibody, an anti-BMP6 antibody, an anti-BMP6 antibody, an anti-GDF15 antibody, an anti-Nodal antibody, an anti-GDF3 antibody, an anti-BMP3 antibody, an anti-BMP3B antibody, an anti-BMP9 antibody, or an anti-BMP10 antibody), or a non-antibody binding polypeptide disclosed herein (*e.g.*, a GDF11 binding polypeptide, an activin B binding polypeptide, an activin B binding polypeptide, an activin E binding polypeptide, an activin C binding polypeptide, a GDF8 binding polypeptide, a BMP6 binding polypeptide, a GDF15 binding polypeptide, a Nodal binding polypeptide, a GDF3 binding polypeptide, a BMP3 binding polypeptide, a BMP3B binding polypeptide, a BMP9 binding polypeptide, or a BMP10 binding polypeptide), or a ligand-trap polypeptide disclosed herein (*e.g.*, a GDF11-trap polypeptide, an activin-B trap polypeptide, or a GDF11/activin-B trap polypeptide), or a small-molecule antagonist directed to any of the targets of the disclosure (*e.g.*, an activin-A small-molecule antagonist, an activin-B small-molecule antagonist, a GDF11 small-molecule antagonist, an activin-C

small-molecule antagonist, an activin-E small-molecule antagonist, a GDF8 small-molecule antagonist, a BMP6 small-molecule antagonist, a BMP6 small-molecule antagonist, a GDF15 small-molecule antagonist, a Nodal small-molecule antagonist, a GDF3 small-molecule antagonist, a BMP3 small-molecule antagonist, a BMP3B small-molecule antagonist, a BMP9 small-molecule antagonist, or a BMP10 small-molecule antagonist). For example, an antisense antagonist of GDF11 can be combined with an activin-B antagonist of the disclosure (*e.g.*, an activin-B trap polypeptide, an anti-activin B antibody, a small-molecule antagonist of activin B, a polynucleotide antagonist of activin B, or a non-antibody polypeptide antagonist of activin B) to inhibit both a GDF11 and an activin B activity (*e.g.*, the ability to bind to and/or activate an ActRIIA and/or ActRIIB receptor). In an alternative embodiment, an antisense antagonist of activin-B antibody can be combined with a GDF11 antagonist of the disclosure (*e.g.*, a GDF-trap polypeptide, an anti-GDF11 antibody, a small-molecule antagonist of GDF11, a polynucleotide antagonist of GDF11, or a non-antibody polypeptide antagonist of GDF11) to inhibit both a GDF11 and an activin B activity.

### 3. Screening Assays

[0335] In certain aspects, the present disclosure relates to the use of disclosed ActRII polypeptides (*e.g.*, soluble ActRIIA and ActRIIB polypeptides and variants thereof) to identify antagonists of one or more of GDF11, activin A, activin B, activin C, activin E, GDF8, activin A, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10, particularly antagonist that inhibit one or more of these ligands from binding to and/or activating an ActRII receptor (*e.g.*, an ActRIIA and/or ActRIIB receptor), but that do not inhibit activin A from binding to and/or activating an ActRII receptor. Compounds identified through the screening assays disclosed herein can be tested to assess their ability to modulate red blood cell, hemoglobin and/or reticulocyte levels *in vivo* or *in vitro*. These compounds can be tested, for example, in animal models.

[0336] There are numerous approaches to screening for therapeutic agents for increasing red blood cell or hemoglobin levels by targeting ActRII signaling. In certain embodiments, high-throughput screening of compounds can be carried out to identify agents that perturb ActRII-mediated effects on a selected cell line. In certain embodiments, the assay is carried out to screen and identify compounds that specifically inhibit or reduce binding of an ActRII polypeptide to its binding partner, such as an ActRII ligand (*e.g.*, GDF11, activin A, activin

B, activin C, activin E, GDF8, activin A, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10). Alternatively, the assay can be used to identify compounds that do not substantially affect binding of an ActRII polypeptide to its binding partner, such as an ActRII ligand (*e.g.*, activin A). In a further embodiment, the compounds can be identified by their ability to interact with an ActRII polypeptide.

[0337] A variety of assay formats will suffice and, in light of the present disclosure, those not expressly described herein will nevertheless be comprehended by one of ordinary skill in the art. As described herein, the test compounds (agents) of the disclosure may be created by any combinatorial chemical method. Alternatively, the subject compounds may be naturally occurring biomolecules synthesized *in vivo* or *in vitro*. Compounds (agents) to be tested for their ability to act as modulators of tissue growth can be produced, for example, by bacteria, yeast, plants or other organisms (*e.g.*, natural products), produced chemically (*e.g.*, small molecules, including peptidomimetics), or produced recombinantly. Test compounds contemplated by the present disclosure include non-peptidyl organic molecules, peptides, polypeptides, peptidomimetics, sugars, hormones, and nucleic acid molecules. In a specific embodiment, the test agent is a small organic molecule having a molecular weight of less than about 2,000 Daltons.

[0338] The test compounds of the disclosure can be provided as single, discrete entities, or provided in libraries of greater complexity, such as made by combinatorial chemistry.

These libraries can comprise, for example, alcohols, alkyl halides, amines, amides, esters, aldehydes, ethers and other classes of organic compounds. Presentation of test compounds to the test system can be in either an isolated form or as mixtures of compounds, especially in initial screening steps. Optionally, the compounds may be derivatized with other compounds and have derivatizing groups that facilitate isolation of the compounds. Non-limiting examples of derivatizing groups include biotin, fluorescein, digoxigenin, green fluorescent protein, isotopes, polyhistidine, magnetic beads, glutathione S transferase (GST), photoactivatable crosslinkers or any combinations thereof.

[0339] In many drug screening programs which test libraries of compounds and natural extracts, high-throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as “primary” screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound.

Moreover, the effects of cellular toxicity or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity between an ActRII polypeptide and its binding partner (*e.g.*, GDF11, activin A, activin B, *etc.*).

[0340] Merely to illustrate, in an exemplary screening assay of the present disclosure, the compound of interest is contacted with an isolated and purified ActRIIB polypeptide which is ordinarily capable of binding to an ActRIIB ligand (*e.g.*, GDF11, activin A, activin B, activin C, activin E, GDF8, BMP6, activin A, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and/or BMP10), as appropriate for the intention of the assay. To the mixture of the compound and ActRIIB polypeptide is then added to a composition containing an ActRIIB ligand. Detection and quantification of ActRIIB/ActRIIB ligand complexes provides a means for determining the compound's efficacy at inhibiting (or potentiating) complex formation between the ActRIIB polypeptide and its binding protein. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. For example, in a control assay, isolated and purified ActRIIB ligand is added to a composition containing the ActRIIB polypeptide, and the formation of ActRIIB/ActRIIB ligand complex is quantitated in the absence of the test compound. It will be understood that, in general, the order in which the reactants may be admixed can be varied, and can be admixed simultaneously. Moreover, in place of purified proteins, cellular extracts and lysates may be used to render a suitable cell-free assay system.

[0341] Complex formation between the ActRII polypeptide and its binding protein may be detected by a variety of techniques. For instance, modulation of the formation of complexes can be quantitated using, for example, detectably labeled proteins such as radiolabeled (*e.g.*,  $^{32}\text{P}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$  or  $^3\text{H}$ ), fluorescently labeled (*e.g.*, FITC), or enzymatically labeled ActRII polypeptide or its binding protein, by immunoassay, or by chromatographic detection.

[0342] In certain embodiments, the present disclosure contemplates the use of fluorescence polarization assays and fluorescence resonance energy transfer (FRET) assays in measuring, either directly or indirectly, the degree of interaction between an ActRII polypeptide and its binding protein. Further, other modes of detection, such as those based on optical waveguides (see, *e.g.*, PCT Publication WO 96/26432 and U.S. Pat. No.

5,677,196), surface plasmon resonance (SPR), surface charge sensors, and surface force sensors, are compatible with many embodiments of the disclosure.

[0343] Moreover, the present disclosure contemplates the use of an interaction trap assay, also known as the “two hybrid assay,” for identifying agents that disrupt or potentiate interaction between an ActRIIB polypeptide and its binding partner [see, *e.g.*, U.S. Pat. No. 5,283,317; Zervos *et al.* (1993) *Cell* 72:223-232; Madura *et al.* (1993) *J Biol Chem* 268:12046-12054; Bartel *et al.* (1993) *Biotechniques* 14:920-924; and Iwabuchi *et al.* (1993) *Oncogene* 8:1693-1696)]. In certain embodiments, the present disclosure contemplates the use of reverse two hybrid systems to identify compounds (*e.g.*, small molecules or peptides) that dissociate interactions between an ActRII polypeptide and its binding protein [see, *e.g.*, Vidal and Legrain, (1999) *Nucleic Acids Res* 27:919-29; Vidal and Legrain (1999) *Trends Biotechnol* 17:374-81; and U.S. Pat. Nos. 5,525,490; 5,955,280; and 5,965,368].

[0344] In certain embodiments, the subject compounds are identified by their ability to interact with an ActRII polypeptide. The interaction between the compound and the ActRII polypeptide may be covalent or non-covalent. For example, such interaction can be identified at the protein level using *in vitro* biochemical methods, including photo-crosslinking, radiolabeled ligand binding, and affinity chromatography [see, *e.g.*, Jakoby WB *et al.* (1974) *Methods in Enzymology* 46: 1]. In certain cases, the compounds may be screened in a mechanism based assay, such as an assay to detect compounds which bind to an ActRII polypeptide. This may include a solid phase or fluid phase binding event. Alternatively, the gene encoding an ActRII polypeptide can be transfected with a reporter system (*e.g.*,  $\beta$ -galactosidase, luciferase, or green fluorescent protein) into a cell and screened against the library preferably by a high-throughput screening or with individual members of the library. Other mechanism based binding assays may be used, for example, binding assays which detect changes in free energy. Binding assays can be performed with the target fixed to a well, bead or chip or captured by an immobilized antibody or resolved by capillary electrophoresis. The bound compounds may be detected usually using colorimetric or fluorescence or surface plasmon resonance.

#### 4. Exemplary Therapeutic Uses

[0345] In certain aspects, antagonist agents, or combination of agents, of the disclosure that inhibit at least GDF11 and/or activin B can be used to increase red blood cell levels, treat

or prevent an anemia, and/or treat or prevent ineffective erythropoiesis in a subject in need thereof. Optionally, antagonist agents, or combination of agents, to be used in accordance with the methods herein do not inhibit activin A. Optionally, antagonist agents, or combination of agents, to be used in accordance with the methods herein further inhibit

5 GDF8. In certain aspects, antagonist agents, or combination of agents, to be used in accordance with the methods herein, in addition to inhibiting GDF11 and/or activin B, further inhibit one or more of GDF8, activin C, activin E, activin A, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10.

[0346] The terms “subject,” an “individual,” or a “patient” are interchangeable

10 throughout the specification and generally refer to mammals. Mammals include, but are not limited to, domesticated animals (*e.g.*, cows, sheep, cats, dogs, and horses), primates (*e.g.*, humans and non-human primates such as monkeys), rabbits, and rodents (*e.g.*, mice and rats).

In certain aspects, an antagonist agent of the present disclosure may be used in combination with conventional therapeutic approaches for increasing red blood cell levels, particularly

15 those used to treat anemias of multifactorial origin. Conventional therapeutic approaches for increasing red blood cell levels include, for example, red blood cell transfusion, administration of one or more EPO receptor activators, hematopoietic stem cell

transplantation, immunosuppressive biologics and drugs (*e.g.*, corticosteroids). In certain embodiments, an antagonist agent of the present disclosure can be used to treat or prevent an

20 anemia in a subject in need thereof. In certain embodiments, an antagonist agent of the present disclosure can be used to treat or prevent ineffective erythropoiesis and/or the disorders associated with ineffective erythropoiesis in a subject in need thereof. In certain

aspects, an antagonist agent of the present disclosure can be used in combination with conventional therapeutic approaches for treating or preventing an anemia or ineffective

25 erythropoiesis disorder, particularly those used to treat anemias of multifactorial origin.

[0347] As used herein, a therapeutic that “prevents” a disorder or condition refers to a compound that, in a statistical sample, reduces the occurrence of the disorder or condition in the treated sample relative to an untreated control sample, or delays the onset or reduces the severity of one or more symptoms of the disorder or condition relative to the untreated

30 control sample.

[0348] The term “treating” as used herein includes amelioration or elimination of the condition once it has been established.

[0349] In either case, prevention or treatment may be discerned in the diagnosis provided by a physician or other health care provider and the intended result of administration of the therapeutic agent.

[0350] In general, treatment or prevention of a disease or condition as described in the present disclosure is achieved by administering one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the present disclosure in an “effective amount”. An effective amount of an agent refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result. A “therapeutically effective amount” of an agent of the present disclosure may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the agent to elicit a desired response in the individual. A “prophylactically effective amount” refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result.

[0351] In certain embodiments, one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure, optionally combined with an EPO receptor activator, may be used to increase red blood cell, hemoglobin, or reticulocyte levels in healthy individuals and selected patient populations. Examples of appropriate patient populations include those with undesirably low red blood cell or hemoglobin levels, such as patients having an anemia, and those that are at risk for developing undesirably low red blood cell or hemoglobin levels, such as those patients who are about to undergo major surgery or other procedures that may result in substantial blood loss. In one embodiment, a patient with adequate red blood cell levels is treated with one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) to increase red blood cell levels, and then blood is drawn and stored for later use in transfusions.

[0352] One or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure, optionally combined with an EPO receptor activator, may be used to increase red blood cell levels, hemoglobin levels, and/or hematocrit levels in a patient having an anemia. When observing hemoglobin and/or

hematocrit levels in humans, a level of less than normal for the appropriate age and gender category may be indicative of anemia, although individual variations are taken into account. For example, a hemoglobin level from 10-12.5 g/dl, and typically about 11.0 g/dl is

considered to be within the normal range in healthy adults, although, in terms of therapy, a lower target level may cause fewer cardiovascular side effects [see, *e.g.*, Jacobs et al. (2000) Nephrol Dial Transplant 15, 15-19]. Alternatively, hematocrit levels (percentage of the volume of a blood sample occupied by the cells) can be used as a measure for anemia.

Hematocrit levels for healthy individuals range from about 41-51% for adult males and from 35-45% for adult females. In certain embodiments, a patient may be treated with a dosing regimen intended to restore the patient to a target level of red blood cells, hemoglobin, and/or hematocrit. As hemoglobin and hematocrit levels vary from person to person, optimally, the target hemoglobin and/or hematocrit level can be individualized for each patient.

[0353] Anemia is frequently observed in patients having a tissue injury, an infection, and/or a chronic disease, particularly cancer. In some subjects, anemia is distinguished by low erythropoietin levels and/or an inadequate response to erythropoietin in the bone marrow [see, *e.g.*, Adamson (2008) Harrison's Principles of Internal Medicine, 17th ed.; McGraw Hill, New York, pp 628-634]. Potential causes of anemia include, for example, blood loss, nutritional deficits (*e.g.* reduced dietary intake of protein), medication reaction, various problems associated with the bone marrow, and many diseases. More particularly, anemia has been associated with a variety of disorders and conditions that include, for example, bone marrow transplantation; solid tumors (*e.g.*, breast cancer, lung cancer, and colon cancer); tumors of the lymphatic system (*e.g.*, chronic lymphocyte leukemia, non-Hodgkins lymphoma, and Hodgkins lymphoma); tumors of the hematopoietic system (*e.g.*, leukemia, a myelodysplastic syndrome and multiple myeloma); radiation therapy; chemotherapy (*e.g.*, platinum containing regimens); inflammatory and autoimmune diseases, including, but not limited to, rheumatoid arthritis, other inflammatory arthritides, systemic lupus erythematosus (SLE), acute or chronic skin diseases (*e.g.*, psoriasis), inflammatory bowel disease (*e.g.*, Crohn's disease and ulcerative colitis); acute or chronic renal disease or failure, including idiopathic or congenital conditions; acute or chronic liver disease; acute or chronic bleeding; situations where transfusion of red blood cells is not possible due to patient allo- or auto-antibodies and/or for religious reasons (*e.g.*, some Jehovah's Witnesses); infections (*e.g.*, malaria and osteomyelitis); hemoglobinopathies including, for example, sickle cell disease (anemia), thalassemias; drug use or abuse (*e.g.*, alcohol misuse); pediatric patients with

anemia from any cause to avoid transfusion; and elderly patients or patients with underlying cardiopulmonary disease with anemia who cannot receive transfusions due to concerns about circulatory overload [see, *e.g.*, Adamson (2008) Harrison's Principles of Internal Medicine, 17th ed.; McGraw Hill, New York, pp 628-634]. In some embodiments, one or more GDF11  
5 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, and BMP6) of the disclosure could be used to treat or prevent anemia associated with one or more of the disorders or conditions disclosed herein.

[0354] Many factors can contribute to cancer-related anemia. Some are associated with the disease process itself and the generation of inflammatory cytokines such as interleukin-1,  
10 interferon-gamma, and tumor necrosis factor [Bron *et al.* (2001) Semin Oncol 28(Suppl 8):1-6]. Among its effects, inflammation induces the key iron-regulatory peptide hepcidin, thereby inhibiting iron export from macrophages and generally limiting iron availability for erythropoiesis [see, *e.g.*, Ganz (2007) J Am Soc Nephrol 18:394-400]. Blood loss through various routes can also contribute to cancer-related anemia. The prevalence of anemia due to  
15 cancer progression varies with cancer type, ranging from 5% in prostate cancer up to 90% in multiple myeloma. Cancer-related anemia has profound consequences for patients, including fatigue and reduced quality of life, reduced treatment efficacy, and increased mortality. In some embodiments, one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15,  
20 Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure, optionally combined with an EPO receptor activator, could be used to treat a cancer-related anemia.

[0355] A hypoproliferative anemia can result from primary dysfunction or failure of the bone marrow. Hypoproliferative anemias include: anemia of chronic disease, anemia of kidney disease, anemia associated with hypometabolic states, and anemia associated with  
25 cancer. In each of these types, endogenous erythropoietin levels are *inappropriately low* for the degree of anemia observed. Other hypoproliferative anemias include: early-stage iron-deficient anemia, and anemia caused by damage to the bone marrow. In these types, endogenous erythropoietin levels are *appropriately elevated* for the degree of anemia observed. Prominent examples would be myelosuppression caused by cancer and/or  
30 chemotherapeutic drugs or cancer radiation therapy. A broad review of clinical trials found that mild anemia can occur in 100% of patients after chemotherapy, while more severe anemia can occur in up to 80% of such patients [see, *e.g.*, Groopman *et al.* (1999) J Natl Cancer Inst 91:1616-1634]. Myelosuppressive drugs include, for example: 1) alkylating

agents such as nitrogen mustards (*e.g.*, melphalan) and nitrosoureas (*e.g.*, streptozocin); 2) antimetabolites such as folic acid antagonists (*e.g.*, methotrexate), purine analogs (*e.g.*, thioguanine), and pyrimidine analogs (*e.g.*, gemcitabine); 3) cytotoxic antibiotics such as anthracyclines (*e.g.*, doxorubicin); 4) kinase inhibitors (*e.g.*, gefitinib); 5) mitotic inhibitors

such as taxanes (*e.g.*, paclitaxel) and vinca alkaloids (*e.g.*, vinorelbine); 6) monoclonal antibodies (*e.g.*, rituximab); and 7) topoisomerase inhibitors (*e.g.*, topotecan and etoposide). In addition, conditions resulting in a hypometabolic rate can produce a mild-to-moderate hypoproliferative anemia. Among such conditions are endocrine deficiency states. For

example, anemia can occur in Addison's disease, hypothyroidism, hyperparathyroidism, or

males who are castrated or treated with estrogen. In some embodiments, one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure, optionally combined with an EPO receptor activator, could be used to treat a hyperproliferative anemia.

[0356] Chronic kidney disease is sometimes associated with hypoproliferative anemia, and the degree of the anemia varies in severity with the level of renal impairment. Such anemia is primarily due to inadequate production of erythropoietin and reduced survival of red blood cells. Chronic kidney disease usually proceeds gradually over a period of years or decades to end-stage (Stage-5) disease, at which point dialysis or kidney transplantation is required for patient survival. Anemia often develops early in this process and worsens as disease progresses. The clinical consequences of anemia of kidney disease are well-documented and include development of left ventricular hypertrophy, impaired cognitive function, reduced quality of life, and altered immune function [see, *e.g.*, Levin *et al.* (1999) *Am J Kidney Dis* 27:347-354; Nissenson (1992) *Am J Kidney Dis* 20(Suppl 1):21-24; Revicki *et al.* (1995) *Am J Kidney Dis* 25:548-554; Gafter *et al.*, (1994) *Kidney Int* 45:224-231]. In some embodiments, one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10), optionally combined with an EPO receptor activator, could be used to treat anemia associated with acute or chronic renal disease or failure.

[0357] Anemia resulting from acute blood loss of sufficient volume, such as from trauma or postpartum hemorrhage, is known as acute post-hemorrhagic anemia. Acute blood loss initially causes hypovolemia without anemia since there is proportional depletion of RBCs

along with other blood constituents. However, hypovolemia will rapidly trigger physiologic mechanisms that shift fluid from the extravascular to the vascular compartment, which results in hemodilution and anemia. If chronic, blood loss gradually depletes body iron stores and eventually leads to iron deficiency. In some embodiments, one or more GDF11 and/or  
5 activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) optionally combined with an EPO receptor activator, could be used to treat anemia resulting from acute blood loss.

[0358] Iron-deficiency anemia is the final stage in a graded progression of increasing iron  
10 deficiency which includes negative iron balance and iron-deficient erythropoiesis as intermediate stages. Iron deficiency can result from increased iron demand, decreased iron intake, or increased iron loss, as exemplified in conditions such as pregnancy, inadequate diet, intestinal malabsorption, acute or chronic inflammation, and acute or chronic blood loss. With mild-to-moderate anemia of this type, the bone marrow remains hypoproliferative, and  
15 RBC morphology is largely normal; however, even mild anemia can result in some microcytic hypochromic RBCs, and the transition to severe iron-deficient anemia is accompanied by hyperproliferation of the bone marrow and increasingly prevalent microcytic and hypochromic RBCs [see, *e.g.*, Adamson (2008) Harrison's Principles of Internal Medicine, 17th ed.; McGraw Hill, New York, pp 628-634]. Appropriate therapy for iron-  
20 deficiency anemia depends on its cause and severity, with oral iron preparations, parenteral iron formulations, and RBC transfusion as major conventional options. In some embodiments, one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure, optionally combined with an  
25 EPO receptor activator, could be used to treat a chronic iron-deficiency.

[0359] Myelodysplastic syndrome (MDS) is a diverse collection of hematological conditions characterized by ineffective production of myeloid blood cells and risk of transformation to acute myelogenous leukemia. In MDS patients, blood stem cells do not mature into healthy red blood cells, white blood cells, or platelets. MDS disorders include,  
30 for example, refractory anemia, refractory anemia with ringed sideroblasts, refractory anemia with excess blasts, refractory anemia with excess blasts in transformation, refractory cytopenia with multilineage dysplasia, and myelodysplastic syndrome associated with an isolated 5q chromosome abnormality. As these disorders manifest as irreversible defects in

both quantity and quality of hematopoietic cells, most MDS patients are afflicted with chronic anemia. Therefore, MDS patients eventually require blood transfusions and/or treatment with growth factors (e.g., erythropoietin or G-CSF) to increase red blood cell levels. However, many MDS patients develop side-effects due to frequency of such therapies. For example, patients who receive frequent red blood cell transfusion can exhibit tissue and organ damage from the buildup of extra iron. Accordingly, one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, and BMP6) of the disclosure, may be used to treat patients having MDS. In certain embodiments, patients suffering from MDS may be treated using one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure, optionally in combination with an EPO receptor activator. In other embodiments, patient suffering from MDS may be treated using a combination of one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure and one or more additional therapeutic agents for treating MDS including, for example, thalidomide, lenalidomide, azacitadine, decitabine, erythropoietins, deferoxamine, antithymocyte globulin, and filgrastim (G-CSF).

[0360] Originally distinguished from aplastic anemia, hemorrhage, or peripheral hemolysis on the basis of ferrokinetic studies [see, e.g., Ricketts *et al.* (1978) Clin Nucl Med 3:159-164], ineffective erythropoiesis describes a diverse group of anemias in which production of mature RBCs is less than would be expected given the number of erythroid precursors (erythroblasts) present in the bone marrow [Tanno *et al.* (2010) Adv Hematol 2010:358283]. In such anemias, tissue hypoxia persists despite elevated erythropoietin levels due to ineffective production of mature RBCs. A vicious cycle eventually develops in which elevated erythropoietin levels drive massive expansion of erythroblasts, potentially leading to splenomegaly (spleen enlargement) due to extramedullary erythropoiesis [see, e.g., Aizawa *et al.* (2003) Am J Hematol 74:68-72], erythroblast-induced bone pathology [see, e.g., Di Matteo *et al.* (2008) J Biol Regul Homeost Agents 22:211-216], and tissue iron overload, even in the absence of therapeutic RBC transfusions [see, e.g., Pippard *et al.* (1979) Lancet 2:819-821]. Thus, by boosting erythropoietic effectiveness, a GDF11 and/or activin B antagonist of the present disclosure may break the aforementioned cycle and thus alleviate not only the underlying anemia but also the associated complications of elevated

erythropoietin levels, splenomegaly, bone pathology, and tissue iron overload. In some embodiments, one or more antagonist agents of the present disclosure (*e.g.*, an GDF11, activin A, activin B, activin C, activin E, GDF8, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and/or BMP10 antagonist) can be used to treat or prevent ineffective erythropoiesis, including anemia and elevated EPO levels as well as complications such as splenomegaly, erythroblast-induced bone pathology, iron overload, and their attendant pathologies. With splenomegaly, such pathologies include thoracic or abdominal pain and reticuloendothelial hyperplasia. Extramedullary hematopoiesis can occur not only in the spleen but potentially in other tissues in the form of extramedullary hematopoietic pseudotumors [see, *e.g.*, Musallam *et al.* (2012) Cold Spring Harb Perspect Med 2:a013482]. With erythroblast-induced bone pathology, attendant pathologies include low bone mineral density, osteoporosis, and bone pain [see, *e.g.*, Haidar *et al.* (2011) Bone 48:425-432]. With iron overload, attendant pathologies include hepcidin suppression and hyperabsorption of dietary iron [see, *e.g.*, Musallam *et al.* (2012) Blood Rev 26(Suppl 1):S16-S19], multiple endocrinopathies and liver fibrosis/cirrhosis [see, *e.g.*, Galanello *et al.* (2010) Orphanet J Rare Dis 5:11], and iron-overload cardiomyopathy [Lekawanvijit *et al.*, 2009, Can J Cardiol 25:213-218].

[0361] The most common causes of ineffective erythropoiesis are the thalassemia syndromes, hereditary hemoglobinopathies in which imbalances in the production of intact alpha- and beta-hemoglobin chains lead to increased apoptosis during erythroblast maturation [see, *e.g.*, Schrier (2002) Curr Opin Hematol 9:123-126]. Thalassemias are collectively among the most frequent genetic disorders worldwide, with changing epidemiologic patterns predicted to contribute to a growing public health problem in both the U.S. and globally [Vichinsky (2005) Ann NY Acad Sci 1054:18-24]. Thalassemia syndromes are named according to their severity. Thus,  $\alpha$ -thalassemias include  $\alpha$ -thalassemia minor (also known as  $\alpha$ -thalassemia trait; two affected  $\alpha$ -globin genes), hemoglobin H disease (three affected  $\alpha$ -globin genes), and  $\alpha$ -thalassemia major (also known as hydrops fetalis; four affected  $\alpha$ -globin genes).  $\beta$ -Thalassemias include  $\beta$ -thalassemia minor (also known as  $\beta$ -thalassemia trait; one affected  $\beta$ -globin gene),  $\beta$ -thalassemia intermedia (two affected  $\beta$ -globin genes), hemoglobin E thalassemia (two affected  $\beta$ -globin genes), and  $\beta$ -thalassemia major (also known as Cooley's anemia; two affected  $\beta$ -globin genes resulting in a complete absence of  $\beta$ -globin protein).  $\beta$ -Thalassemia impacts multiple organs, is associated with considerable morbidity and mortality, and currently requires life-long care. Although life expectancy in patients with

$\beta$ -thalassemia has increased in recent years due to use of regular blood transfusions in combination with iron chelation, iron overload resulting both from transfusions and from excessive gastrointestinal absorption of iron can cause serious complications such as heart disease, thrombosis, hypogonadism, hypothyroidism, diabetes, osteoporosis, and osteopenia [see, *e.g.*, Rund *et al.* (2005) N Engl J Med 353:1135-1146]. In certain embodiments, one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure, optionally combined with an EPO receptor activator, can be used to treat or prevent a thalassemia syndrome.

[0362] In some embodiments, one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure, optionally combined with an EPO receptor activator, can be used for treating disorders of ineffective erythropoiesis besides thalassemia syndromes. Such disorders include siderblastic anemia (inherited or acquired); dyserythropoietic anemia (Types I and II); sickle cell anemia; hereditary spherocytosis; pyruvate kinase deficiency; megaloblastic anemias, potentially caused by conditions such as folate deficiency (due to congenital diseases, decreased intake, or increased requirements), cobalamin deficiency (due to congenital diseases, pernicious anemia, impaired absorption, pancreatic insufficiency, or decreased intake), certain drugs, or unexplained causes (congenital dyserythropoietic anemia, refractory megaloblastic anemia, or erythroleukemia); myelophthistic anemias including, for example, myelofibrosis (myeloid metaplasia) and myelophthisis; congenital erythropoietic porphyria; and lead poisoning.

[0363] In certain embodiments, one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure may be used in combination with supportive therapies for ineffective erythropoiesis. Such therapies include transfusion with either red blood cells or whole blood to treat anemia. In chronic or hereditary anemias, normal mechanisms for iron homeostasis are overwhelmed by repeated transfusions, eventually leading to toxic and potentially fatal accumulation of iron in vital tissues such as heart, liver, and endocrine glands. Thus, supportive therapies for patients chronically afflicted with ineffective erythropoiesis also include treatment with one or more iron-chelating molecules to promote iron excretion in the urine and/or stool and thereby prevent, or reverse, tissue iron overload [see, *e.g.*, Hershko (2006) Haematologica 91:1307-

1312; Cao *et al.* (2011), *Pediatr Rep* 3(2):e17]. Effective iron-chelating agents should be able to selectively bind and neutralize ferric iron, the oxidized form of non-transferrin bound iron which likely accounts for most iron toxicity through catalytic production of hydroxyl radicals and oxidation products [see, *e.g.*, Esposito *et al.* (2003) *Blood* 102:2670-2677]. These agents are structurally diverse, but all possess oxygen or nitrogen donor atoms able to form neutralizing octahedral coordination complexes with individual iron atoms in stoichiometries of 1:1 (hexadentate agents), 2:1 (tridentate), or 3:1 (bidentate) [Kalinowski *et al.* (2005) *Pharmacol Rev* 57:547-583]. In general, effective iron-chelating agents also are relatively low molecular weight (*e.g.*, less than 700 daltons), with solubility in both water and lipids to enable access to affected tissues. Specific examples of iron-chelating molecules include deferoxamine, a hexadentate agent of bacterial origin requiring daily parenteral administration, and the orally active synthetic agents deferiprone (bidentate) and deferasirox (tridentate). Combination therapy consisting of same-day administration of two iron-chelating agents shows promise in patients unresponsive to chelation monotherapy and also in overcoming issues of poor patient compliance with dereroxamine alone [Cao *et al.* (2011) *Pediatr Rep* 3(2):e17; Galanello *et al.* (2010) *Ann NY Acad Sci* 1202:79-86].

[0364] As used herein, “in combination with” or “conjoint administration” refers to any form of administration such that the second therapy is still effective in the body (*e.g.*, the two compounds are simultaneously effective in the patient, which may include synergistic effects of the two compounds). Effectiveness may not correlate to measurable concentration of the agent in blood, serum, or plasma. For example, the different therapeutic compounds can be administered either in the same formulation or in separate formulations, either concomitantly or sequentially, and on different schedules. Thus, an individual who receives such treatment can benefit from a combined effect of different therapies. One or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure can be administered concurrently with, prior to, or subsequent to, one or more other additional agents or supportive therapies. In general, each therapeutic agent will be administered at a dose and/or on a time schedule determined for that particular agent. The particular combination to employ in a regimen will take into account compatibility of the antagonist of the present disclosure with the therapy and/or the desired therapeutic effect to be achieved.

[0365] In certain embodiments, one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure may be used in combination with hepcidin or a hepcidin agonist for ineffective erythropoiesis. A circulating polypeptide produced mainly in the liver, hepcidin is considered a master regulator of iron metabolism by virtue of its ability to induce the degradation of ferroportin, an iron-export protein localized on absorptive enterocytes, hepatocytes, and macrophages. Broadly speaking, hepcidin reduces availability of extracellular iron, so hepcidin agonists may be beneficial in the treatment of ineffective erythropoiesis [see, *e.g.*, Nemeth (2010) Adv Hematol 2010:750643]. This view is supported by beneficial effects of increased hepcidin expression in a mouse model of  $\beta$ -thalassemia [Gardenghi *et al.* (2010) J Clin Invest 120:4466-4477].

[0366] One or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure, optionally combined with an EPO receptor activator, would also be appropriate for treating anemias of disordered RBC maturation, which are characterized in part by undersized (microcytic), oversized (macrocytic), misshapen, or abnormally colored (hypochromic) RBCs.

[0367] In certain embodiments, the present disclosure provides methods of treating or preventing anemia in an individual in need thereof by administering to the individual a therapeutically effective amount of one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure and a EPO receptor activator. In certain embodiments, one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure may be used in combination with EPO receptor activators to reduce the required dose of these activators in patients that are susceptible to adverse effects of EPO. These methods may be used for therapeutic and prophylactic treatments of a patient.

[0368] One or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure may be used in combination with EPO receptor activators to achieve an increase in red blood cells, particularly at lower

dose ranges of EPO receptor activators. This may be beneficial in reducing the known off-target effects and risks associated with high doses of EPO receptor activators. The primary adverse effects of EPO include, for example, an excessive increase in the hematocrit or hemoglobin levels and polycythemia. Elevated hematocrit levels can lead to hypertension (more particularly aggravation of hypertension) and vascular thrombosis. Other adverse effects of EPO which have been reported, some of which relate to hypertension, are headaches, influenza-like syndrome, obstruction of shunts, myocardial infarctions and cerebral convulsions due to thrombosis, hypertensive encephalopathy, and red cell blood cell aplasia. See, *e.g.*, Singibarti (1994) *J. Clin Invest* 72(suppl 6), S36-S43; Horl *et al.* (2000) *Nephrol Dial Transplant* 15(suppl 4), 51-56; Delanty *et al.* (1997) *Neurology* 49, 686-689; and Bunn (2002) *N Engl J Med* 346(7), 522-523).

[0369] Provided that antagonists of the present disclosure act by a different mechanism than EPO, these antagonists may be useful for increasing red blood cell and hemoglobin levels in patients that do not respond well to EPO. For example, an antagonist of the present disclosure may be beneficial for a patient in which administration of a normal-to-increased dose of EPO (>300 IU/kg/week) does not result in the increase of hemoglobin level up to the target level. Patients with an inadequate EPO response are found in all types of anemia, but higher numbers of non-responders have been observed particularly frequently in patients with cancers and patients with end-stage renal disease. An inadequate response to EPO can be either constitutive (observed upon the first treatment with EPO) or acquired (observed upon repeated treatment with EPO).

[0370] In certain embodiments, the present disclosure provides methods for managing a patient that has been treated with, or is a candidate to be treated with, one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure by measuring one or more hematologic parameters in the patient. The hematologic parameters may be used to evaluate appropriate dosing for a patient who is a candidate to be treated with the antagonist of the present disclosure, to monitor the hematologic parameters during treatment, to evaluate whether to adjust the dosage during treatment with one or more antagonist of the disclosure, and/or to evaluate an appropriate maintenance dose of one or more antagonists of the disclosure. If one or more of the hematologic parameters are outside the normal level, dosing with one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A,

activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure may be reduced, delayed or terminated.

[0371] Hematologic parameters that may be measured in accordance with the methods provided herein include, for example, red blood cell levels, blood pressure, iron stores, and other agents found in bodily fluids that correlate with increased red blood cell levels, using art-recognized methods. Such parameters may be determined using a blood sample from a patient. Increases in red blood cell levels, hemoglobin levels, and/or hematocrit levels may cause increases in blood pressure.

[0372] In one embodiment, if one or more hematologic parameters are outside the normal range or on the high side of normal in a patient who is a candidate to be treated with one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure, then onset of administration of the one or more antagonists of the disclosure may be delayed until the hematologic parameters have returned to a normal or acceptable level either naturally or via therapeutic intervention. For example, if a candidate patient is hypertensive or pre-hypertensive, then the patient may be treated with a blood pressure lowering agent in order to reduce the patient's blood pressure. Any blood pressure lowering agent appropriate for the individual patient's condition may be used including, for example, diuretics, adrenergic inhibitors (including alpha blockers and beta blockers), vasodilators, calcium channel blockers, angiotensin-converting enzyme (ACE) inhibitors, or angiotensin II receptor blockers. Blood pressure may alternatively be treated using a diet and exercise regimen. Similarly, if a candidate patient has iron stores that are lower than normal, or on the low side of normal, then the patient may be treated with an appropriate regimen of diet and/or iron supplements until the patient's iron stores have returned to a normal or acceptable level. For patients having higher than normal red blood cell levels and/or hemoglobin levels, then administration of the one or more antagonists of the disclosure may be delayed until the levels have returned to a normal or acceptable level.

[0373] In certain embodiments, if one or more hematologic parameters are outside the normal range or on the high side of normal in a patient who is a candidate to be treated with one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure, then the onset of administration may not be delayed. However, the dosage amount or frequency of dosing of the one or more antagonists

of the disclosure may be set at an amount that would reduce the risk of an unacceptable increase in the hematologic parameters arising upon administration of the one or more antagonists of the disclosure. Alternatively, a therapeutic regimen may be developed for the patient that combines one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure with a therapeutic agent that addresses the undesirable level of the hematologic parameter. For example, if the patient has elevated blood pressure, then a therapeutic regimen involving administration of one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure and a blood pressure-lowering agent may be designed. For a patient having lower than desired iron stores, a therapeutic regimen of one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) and iron supplementation may be developed.

[0374] In one embodiment, baseline parameter(s) for one or more hematologic parameters may be established for a patient who is a candidate to be treated with one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure and an appropriate dosing regimen established for that patient based on the baseline value(s). Alternatively, established baseline parameters based on a patient's medical history could be used to inform an appropriate antagonist-dosing regimen for a patient. For example, if a healthy patient has an established baseline blood pressure reading that is above the defined normal range it may not be necessary to bring the patient's blood pressure into the range that is considered normal for the general population prior to treatment with the one or more antagonist of the disclosure. A patient's baseline values for one or more hematologic parameters prior to treatment with one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure may also be used as the relevant comparative values for monitoring any changes to the hematologic parameters during treatment with the one or more antagonists of the disclosure.

[0375] In certain embodiments, one or more hematologic parameters are measured in patients who are being treated with a one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure. The hematologic parameters may be used to monitor the patient during treatment and permit adjustment or termination of the dosing with the one or more antagonists of the disclosure or additional dosing with another therapeutic agent. For example, if administration of one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure results in an increase in blood pressure, red blood cell level, or hemoglobin level, or a reduction in iron stores, then the dose of the one or more antagonists of the disclosure may be reduced in amount or frequency in order to decrease the effects of the one or more antagonist of the disclosure on the one or more hematologic parameters. If administration of one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure results in a change in one or more hematologic parameters that is adverse to the patient, then the dosing of the one or more antagonist of the disclosure may be terminated either temporarily, until the hematologic parameter(s) return to an acceptable level, or permanently. Similarly, if one or more hematologic parameters are not brought within an acceptable range after reducing the dose or frequency of administration of the one or more antagonists of the disclosure, then the dosing may be terminated. As an alternative, or in addition to, reducing or terminating the dosing with the one or more antagonists of the disclosure, the patient may be dosed with an additional therapeutic agent that addresses the undesirable level in the hematologic parameter(s), such as, for example, a blood pressure-lowering agent or an iron supplement. For example, if a patient being treated with one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure has elevated blood pressure, then dosing with the one or more antagonists of the disclosure may continue at the same level and a blood pressure-lowering agent is added to the treatment regimen, dosing with the one or more antagonists of the disclosure may be reduced (*e.g.*, in amount and/or frequency) and a blood pressure-lowering agent is added to the treatment regimen, or dosing with the one or more antagonists of the disclosure may be terminated and the patient may be treated with a blood pressure-lowering agent.

## 5. Pharmaceutical Compositions

[0376] In certain embodiments, one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure can be administered alone or as a component of a pharmaceutical formulation (therapeutic composition or pharmaceutical composition). A pharmaceutical formation refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered. The subject compounds may be formulated for administration in any convenient way for use in human or veterinary medicine. For example, one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure may be formulated with a pharmaceutically acceptable carrier. A pharmaceutically acceptable carrier refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is generally nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative. In general, pharmaceutical formulations for use in the present disclosure are in a pyrogen-free, physiologically acceptable form when administered to a subject. Therapeutically useful agents other than the antagonist of the disclosure, which may optionally be included in the formulation as described above, may be administered in combination with the subject compounds in the methods of the present disclosure.

[0377] Typically, compounds will be administered parenterally [*e.g.*, by intravenous (I.V.) injection, intraarterial injection, intraosseous injection, intramuscular injection, intrathecal injection, subcutaneous injection, or intradermal injection]. Pharmaceutical compositions suitable for parenteral administration may comprise one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, and BMP6) of the disclosure in combination with one or more pharmaceutically acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use. Injectable solutions or dispersions may contain antioxidants, buffers, bacteriostats, suspending agents, thickening agents, or solutes which render the

formulation isotonic with the blood of the intended recipient. Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical formulations of the present disclosure include water, ethanol, polyols (*e.g.*, glycerol, propylene glycol, polyethylene glycol, *etc.*), vegetable oils (*e.g.*, olive oil), injectable organic esters (*e.g.*, ethyl oleate), and suitable mixtures thereof. Proper fluidity can be maintained, for example, by the use of coating materials (*e.g.*, lecithin), by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

[0378] In certain embodiments, a therapeutic method of the present disclosure includes administering the formulation systemically, or locally, from an implant or device. Further, the composition may be encapsulated or injected in a form for delivery to a target tissue site (*e.g.*, bone marrow or muscle). In certain embodiments, compositions of the present disclosure may include a matrix capable of delivering one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure to a target tissue site (*e.g.*, bone marrow or muscle), providing a structure for the developing tissue and optimally capable of being resorbed into the body. For example, the matrix may provide slow release of one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure. Such matrices may be formed of materials presently in use for other implanted medical applications.

[0379] The choice of matrix material may be based on one or more of: biocompatibility, biodegradability, mechanical properties, cosmetic appearance, and interface properties. The particular application of the subject compositions will define the appropriate formulation.

Potential matrices for the compositions may be biodegradable and chemically defined calcium sulfate, tricalciumphosphate, hydroxyapatite, polylactic acid, and polyanhydrides. Other potential materials are biodegradable and biologically well-defined including, for example, bone or dermal collagen. Further matrices are comprised of pure proteins or extracellular matrix components. Other potential matrices are non-biodegradable and chemically defined including, for example, sintered hydroxyapatite, bioglass, aluminates, or other ceramics. Matrices may be comprised of combinations of any of the above mentioned types of material including, for example, polylactic acid and hydroxyapatite or collagen and tricalciumphosphate. The bioceramics may be altered in composition (*e.g.*, calcium-

aluminate-phosphate) and processing to alter one or more of pore size, particle size, particle shape, and biodegradability.

[0380] In certain embodiments, formulations (compositions) of present disclosure can be administered orally, for example, in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis such as sucrose and acacia or tragacanth), powders, granules, a solution or a suspension in an aqueous or non-aqueous liquid, an oil-in-water or water-in-oil liquid emulsion, or an elixir or syrup, or pastille (using an inert base, such as gelatin and glycerin, or sucrose and acacia), and/or a mouth wash, each containing a predetermined amount of a compound of the present disclosure and optionally one or more other active ingredients. A compound of the present disclosure and optionally one or more other active ingredients may also be administered as a bolus, electuary, or paste.

[0381] In solid dosage forms for oral administration (*e.g.*, capsules, tablets, pills, dragees, powders, and granules), one or more compounds of the present disclosure may be mixed with one or more pharmaceutically acceptable carriers including, for example, sodium citrate, dicalcium phosphate, a filler or extender (*e.g.*, a starch, lactose, sucrose, glucose, mannitol, and silicic acid), a binder (*e.g.* carboxymethylcellulose, an alginate, gelatin, polyvinyl pyrrolidone, sucrose, and acacia), a humectant (*e.g.*, glycerol), a disintegrating agent (*e.g.*, agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, a silicate, and sodium carbonate), a solution retarding agent (*e.g.* paraffin), an absorption accelerator (*e.g.* a quaternary ammonium compound), a wetting agent (*e.g.*, cetyl alcohol and glycerol monostearate), an absorbent (*e.g.*, kaolin and bentonite clay), a lubricant (*e.g.*, a talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate), a coloring agent, and mixtures thereof. In the case of capsules, tablets, and pills, the pharmaceutical formulation (composition) may also comprise a buffering agent. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using one or more excipients including, *e.g.*, lactose or a milk sugar as well as a high molecular-weight polyethylene glycol.

[0382] Liquid dosage forms for oral administration of the present formulations (compositions) may include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and elixirs. In addition to the active ingredient(s), the liquid dosage form may contain an inert diluent commonly used in the art including, for example, water or other solvent, a solubilizing agent and/or emulsifier [*e.g.*, ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, or

1,3-butylene glycol, an oil (*e.g.*, cottonseed, groundnut, corn, germ, olive, castor, and sesame oil), glycerol, tetrahydrofuryl alcohol, a polyethylene glycol, a fatty acid ester of sorbitan, and mixtures thereof]. Besides inert diluents, the oral formulation can also include an adjuvant including, for example, a wetting agent, an emulsifying and suspending agent, a sweetening agent, a flavoring agent, a coloring agent, a perfuming agent, a preservative agent, and combinations thereof.

[0383] Suspensions, in addition to the active compounds, may contain suspending agents including, for example, an ethoxylated isostearyl alcohol, polyoxyethylene sorbitol, a sorbitan ester, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar, tragacanth, and combinations thereof.

[0384] Prevention of the action and/or growth of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents including, for example, paraben, chlorobutanol, and phenol sorbic acid.

[0385] In certain embodiments, it may be desirable to include an isotonic agent including, for example, a sugar or sodium chloride into the compositions. In addition, prolonged absorption of an injectable pharmaceutical form may be brought about by the inclusion of an agent that delay absorption including, for example, aluminum monostearate and gelatin.

[0386] It is understood that the dosage regimen will be determined by the attending physician considering various factors which modify the action of the one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure. The various factors include, but are not limited to, the patient's red blood cell count, hemoglobin level, the desired target red blood cell count, the patient's age, the patient's sex, the patient's diet, the severity of any disease that may be contributing to a depressed red blood cell level, the time of administration, and other clinical factors. The addition of other known active agents to the final composition may also affect the dosage. Progress can be monitored by periodic assessment of one or more of red blood cell levels, hemoglobin levels, reticulocyte levels, and other indicators of the hematopoietic process.

[0387] In certain embodiments, the present disclosure also provides gene therapy for the *in vivo* production of one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure. Such therapy would

achieve its therapeutic effect by introduction of the antagonist sequences into cells or tissues having one or more of the disorders as listed above. Delivery of the antagonist sequences can be achieved, for example, by using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system. Preferred therapeutic delivery of one or more GDF11 and/or  
5 activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure is the use of targeted liposomes.

[0388] Various viral vectors which can be utilized for gene therapy as taught herein include adenovirus, herpes virus, vaccinia, or an RNA virus (*e.g.*, a retrovirus). The  
10 retroviral vector may be a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous sarcoma virus (RSV). A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer  
15 or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. Retroviral vectors can be made target-specific by attaching, for example, a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using an antibody. Those of skill in the art will recognize that specific polynucleotide sequences can be inserted into the retroviral genome or attached to a viral envelope to allow target specific delivery of the  
20 retroviral vector containing one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the disclosure.

[0389] Alternatively, tissue culture cells can be directly transfected with plasmids encoding the retroviral structural genes (*gag*, *pol*, and *env*), by conventional calcium  
25 phosphate transfection. These cells are then transfected with the vector plasmid containing the genes of interest. The resulting cells release the retroviral vector into the culture medium.

[0390] Another targeted delivery system for one or more GDF11 and/or activin B antagonist agents (optionally further antagonists of one or more of GDF8, activin A, activin C, activin E, BMP6, GDF15, Nodal, GDF3, BMP3, BMP3B, BMP9, and BMP10) of the  
30 disclosure is a colloidal dispersion system. Colloidal dispersion systems include, for example, macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. In certain embodiments, the preferred colloidal system of this disclosure is a liposome. Liposomes are

artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. RNA, DNA, and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form [see, *e.g.*, Fraley, et al. (1981) Trends Biochem. Sci., 6:77]. Methods for efficient gene transfer using a liposome vehicle are known in the art [see, *e.g.*,  
5 Mannino, *et al.* (1988) Biotechniques, 6:682, 1988].

[0391] The composition of the liposome is usually a combination of phospholipids, which may include a steroid (*e.g.* cholesterol). The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations. Other phospholipids or other lipids may also be used including, for example, a phosphatidyl compound (*e.g.*,  
10 phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, a sphingolipid, a cerebroside, and a ganglioside), egg phosphatidylcholine, dipalmitoylphosphatidylcholine, and distearoylphosphatidylcholine. The targeting of liposomes is also possible based on, for example, organ-specificity, cell-specificity, and organelle-specificity and is known in the art.

#### EXEMPLIFICATION

[0392] The invention now being generally described, it will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain embodiments and embodiments of the present invention, and are not  
20 intended to limit the invention.

#### [0393] Example 1: Characterization of Ligand Binding Specificity for ActRIIB(L79D 20-134)-Fc and ActRIIB(L79D 25-131)-Fc

[0394] It has been previously reported that a variant ActRIIB-Fc fusion protein  
25 comprising amino acids 20-134 of instant SEQ ID NO:1 with an acidic amino acid at position 79 with respect to SEQ ID NO:1 [referenced herein as the "ActRIIB(L79D 20-134)-Fc" fusion protein, construct, variant, *etc.*; see SEQ ID NO:23 of the present disclosure] is characterized by unique biological properties *in vitro* and *in vivo* (see, *e.g.*, U.S. Patent No. 8,058,229). In comparison to a corresponding sample of an unmodified fusion protein (an  
30 ActRIIB(20-134)-Fc fusion protein), the ActRIIB(L79D 20-134)-Fc variant is characterized, in part, by substantial loss of binding affinity for activin A, and therefore significantly diminished capacity to antagonize activin A activity, but retains near wild-type levels of

binding and inhibition of GDF11. In *vivo*, the ActRIIB(L79D 20-134)-Fc variant was found to be significantly more potent in the capacity to increase red blood cell levels in comparison to the unmodified ActRIIB(20-134)-Fc fusion protein. These data therefore indicate the observed biological activity is not dependent on activin A inhibition.

5 [0395] Similar results were previously reported for the double-truncated variant ActRIIB(L79D 25-131)-Fc (see, *e.g.*, SEQ ID NO:49 of the present disclosure as well as U.S. Patent No. 8,058,229), and the ligand binding profile for ActRIIB(L79D 25-131)-Fc was further characterized with respect to various ActRII ligands (*e.g.*, GDF11, GDF8, activin A, activin B, BMP10, BMP6, and BMP9) by surface plasmon resonance. The results are  
10 depicted in Figure 13.

[0396] Upon consideration of this and other data, Applicants have determined that the ActRII ligands that should be inhibited to promote increased red blood cell levels are GDF11 and activin B. This data further suggests that it is also permissible to antagonize (inhibit)  
15 activin A, activin C, activin E, BMP6, and GDF8 in the context of a method for increasing red blood cell levels in a subject.

[0397] Example 2: Bioassay for GDF-11, GDF-8, Activin B, Activin C, and Activin E-Mediated Signaling

[0398] An A-204 reporter gene assay is used to evaluate the effects of an anti-  
20 GDF11/activin B bispecific antibody on signaling by GDF11, activin B, activin A and/or GDF8. This assay has been previously described in the art (see, *e.g.*, U.S. Patent Application No. 2013/0243743). In brief, the A-204 reporter gene assay uses a human rhabdomyosarcoma cell line, which has been derived from muscle, and the reporter vector pGL3(CAGA)12 as described in Dennler *et al.* (1998) EMBO 17: 3091-3100. The CAGA12  
25 motif is present in TGF- $\beta$  responsive genes (*e.g.*, PAI-1 gene), so this vector is of general use for factors signaling through Smad2 and 3 (*e.g.*, GDF11, activin B, activin A, and GDF8).

[0399] At day 1, A-204 cells are transferred into one or more 48-well plates. At day 2, the A-204 cells are transfected with 10  $\mu$ g pGL3(CAGA)12 or pGL3(CAGA)12(10  $\mu$ g) + pRLCMV (1  $\mu$ g) and Fugene. At day 3, ligand factors (*e.g.*, GDF11, activin B, activin A),  
30 which are diluted into medium + 0.1% BSA, are added to the cells along with the anti-GDF11/activin B bispecific antibody. Typically, the anti-GDF11/activin B bispecific

antibody needs to be preincubated with factors for 1 hr before adding to cells. Approximately six hour later, the cells are rinsed with PBS and lysed.

[0400] The cell lysate is then subjected to a luciferase assay to determine the extent of Smad2/3 activation. The extent of inhibition of the anti-GDF11/activin B bispecific antibody is determined relative to appropriate controls.

[0401] Example 3: Treatment with an Anti-GDF11/Anti-Activin B Bispecific Antibody

[0402] Nineteen-week-old male C57BL/6NTac mice are randomly assigned to one of two groups. Mice are dosed with vehicle (10 mM Tris-buffered saline, TBS) or an anti-GDF11/anti-activin B bispecific antibody by subcutaneous injection twice per week for three weeks. Blood is collected at baseline and after three weeks of dosing. The blood samples will be analyzed for cell distribution using a hematology analyzer (*e.g.*, HM2, Abaxis, Inc.). In particular, mice will be monitored for changes in red blood cell parameters including, for example, red blood cell count (RBC), hemoglobin (HGB), and hematocrit (HCT).

[0403] Example 4: Conjoint Administration of an Anti-GDF11 Antibody and an Anti-Activin B Antibody

[0404] Nineteen-week-old male C57BL/6NTac mice are randomly assigned to one of four groups: mice are dosed with vehicle (10 mM TBS), an anti-GDF11 antibody, an anti-activin B antibody, or both an anti-GDF11 and an anti-activin B antibody. Blood is collected at baseline and after three weeks of dosing. The blood samples will be analyzed for cell distribution using a hematology analyzer (*e.g.*, HM2, Abaxis, Inc.). In particular, mice will be monitored for changes in red blood cell parameters including, for example, red blood cell count (RBC), hemoglobin (HGB), and hematocrit (HCT).

[0405] Example 5: Effects of Anti-Activin B, Anti-GDF8, and Anti-GDF8/GDF11 Antibodies on Erythropoiesis

[0406] Three antibodies, an anti-activin B, an anti-GDF8 antibody, and a bispecific anti-GDF8/GDF11 antibody, were evaluated for their ability to stimulate erythropoiesis (*e.g.*, increase red blood cell, hemoglobin, and hematocrit levels) in mice. C57BL6 mice (8-10 weeks old) were divided into one of five treatment groups: i) treatment with vehicle (TBS,

twice weekly; subcutaneously), ii) treatment with an anti-activin B antibody (10 mg/kg; twice weekly; subcutaneously), iii) treatment with an anti-GDF8 antibody (10 mg/kg, twice weekly; subcutaneously), iv) treatment with a bispecific anti-GDF8/GDF11 antibody (10 mg/kg, twice weekly; subcutaneously), and v) treatment with a combination of the anti-activin B antibody (10 mg/kg, twice weekly; subcutaneously) and the bispecific anti-GDF8/GDF11 antibody (10 mg/kg, twice weekly; subcutaneously). After two weeks of treatment, mice were euthanized and complete blood count parameters were measured.

[0407] Compared to vehicle treated (control) subjects, there was a slight increase in red blood cell levels in mice treated with the anti-activin B antibody alone and the anti-GDF8 antibody alone. See Figure 16. A more substantial effect on red blood cell levels was observed in mice treated with the bispecific anti-GDF8/GDF11 antibody. See Figure 16. However, the greatest effect on erythropoiesis was observed in mice treated with a combination of the anti-activin B antibody and the bispecific anti-GDF8/GDF11 antibody, e.g., the combination therapy significantly increased red blood cell levels (8.8%) compared to control subjects. See Figure 16. In addition, it was observed that combination treatment with the anti-activin B antibody and the bispecific anti-GDF8/GDF11 antibody resulted in increased levels of hemoglobin (9.6%) and hematocrit (9.1%) compared to control subjects.

[0408] Accordingly, among the ligands examined, there is a clear trend toward increased blood cell, hematocrit, and hemoglobin levels as additional ActRII ligand antagonists (inhibitors) are administered to the animals. Interestingly, inhibition of a single ActRII ligand did not have a significant effect on red blood cells levels. However, it appears that inhibition of at least two ActRII ligands can stimulate erythropoiesis and an even greater effect on red blood cell levels, as well as hemoglobin and hematocrit levels, is observed when three ActRII ligands are inhibited. Taken together, these data demonstrate that multiple ActRII ligands contribute erythropoiesis and further indicate that inhibiting just one of these ligands may not be sufficient to achieve significant increases in hematocrit, hemoglobin, and/or red blood cell levels. Therefore, from these experiments, it appears that an effective strategy for promoting erythropoiesis in a subject is to target multiple (*i.e.*, at least two) ActRII ligands.

## INCORPORATION BY REFERENCE

[0409] All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

- 5 [0410] While specific embodiments of the subject matter have been discussed, the above specification is illustrative and not restrictive. Many variations will become apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

We claim:

1. A method for increasing red blood cell levels or treating or preventing an anemia in a subject comprising administering to a subject in need thereof an effective amount of a combination of agents that inhibit activin B and GDF11; wherein the combination comprises an antibody or antigen binding fragment thereof that binds to GDF11, and an antibody or antigen binding fragment thereof that binds to activin B, wherein the combination of agents does not substantially bind to activin A.
2. The method of claim 1, wherein the combination of agents inhibits activin B and GDF11 signaling in a cell-based assay.
3. The method of claim 1 or 2, wherein the combination of agents does not substantially inhibit activin A.
4. The method of any one of claims 1-3, wherein the combination of agents does not substantially inhibit activin A signaling in a cell-based assay.
5. The method of any one of claims 1-4, wherein one or more of the agents further inhibits one or more of GDF8, activin C, activin E, GDF15, Nodal, BMP3, BMP3B, BMP9, and BMP10.
6. The method of any one of claims 1-5, wherein at least one antibody or antigen-binding fragment thereof of the combination of agents is a humanized antibody or fragment thereof.
7. The method of any one of claims 1-5, wherein at least one antibody or antigen-binding fragment thereof of the combination of agents is a human antibody or fragment thereof.
8. The method of any one of claims 1-5, wherein at least one antibody or antigen-binding fragment thereof of the combination of agents is a single-chain antibody.
9. The method of any one of claims 1-8, wherein at least one antigen-binding fragment of the combination of agents is selected from the group consisting of: Fab, Fab', F(ab')<sub>2</sub>, F(ab')<sub>3</sub>, Fd, and Fv.
10. The method of any one of claims 1-9, wherein the antibody or antigen-binding fragment thereof comprises a heterologous moiety.

11. The method of claim 10, wherein the heterologous moiety is a sugar, a detectable label, or a stabilization moiety.
12. The method of any one of claims 1-11, wherein the method is for increasing red blood cell in a subject in need thereof.
13. The method of any one of claims 1-12, wherein the method is for treating or preventing an anemia in a subject in need thereof.
14. The method of claim 13, wherein the anemia is associated with one or more of: multiple myeloma, chronic or acute renal disease or failure, chemotherapeutic treatment of the subject, a myelodysplastic syndrome, and a thalassemia.
15. The method of claim 14, wherein the anemia is associated with myelodysplastic syndrome.
16. The method of claim 14, wherein the anemia is associated with thalassemia.
17. The method of claim 16, wherein the thalassemia is beta-thalassemia.
18. The method of claim 14, wherein the renal failure is end-stage renal failure.
19. The method of any one of claims 1-12, wherein the subject has sickle cell anemia.
20. Use of a combination of agents that inhibit activin B and GDF11A for the manufacture of a medicament for increasing red blood cell levels or treating or preventing an anemia in a subject; wherein the combination comprises an antibody or antigen binding fragment thereof that binds to GDF11, and an antibody or antigen binding fragment thereof that binds to activin B, wherein the combination of agents does not substantially bind to activin A.

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ActRIIa ILGRSETQEC IEFNANWEKD RTNQTGVEPC YGDKDKRRHC FATWKNISGS  
ActRIIb GRGEAETREC IYYNANWELE RTNQSGLERC EGEQDKRLHC YASWRNSSGT

IEIVRQGCWL DDINCYDRTD CVEKKDSPEV YFCCCEGNMC NEKFSYFPEN  
IELVRKGCWL DDFNCYDRQE CVATEENPQV YFCCCEGNFC NERFTHLPEA

EVTQPTSNPV TEKPPT  
GGPEVTYEPP PTAPT

FIG. 1

	10										20										30										40										50									
Rat Ilt	M	A	A	A	A	L	A	L	L	G	L	C	A	G	G	G	A	A	A	G	L	A	A	A	L	R	R	L	G	L	R	A	C	G	A	A	A													
Pig Ilt	M	A	A	A	A	L	A	L	L	G	L	C	A	G	G	G	A	A	A	G	L	A	A	A	L	R	R	L	G	L	R	A	C	G	A	A	A													
Mouse Ilt	M	A	A	A	A	L	A	L	L	G	L	C	A	G	G	G	A	A	A	G	L	A	A	A	L	R	R	L	G	L	R	A	C	G	A	A	A													
Human Ilt	M	A	A	A	A	L	A	L	L	G	L	C	A	G	G	G	A	A	A	G	L	A	A	A	L	R	R	L	G	L	R	A	C	G	A	A	A													
Bovine Ilt	M	A	A	A	A	L	A	L	L	G	L	C	A	G	G	G	A	A	A	G	L	A	A	A	L	R	R	L	G	L	R	A	C	G	A	A	A													
Rengus Ilt	M	G	A	A	A	L	L	L	L	A	A	C	A	G	A	G	A	A	A	A	G	L	A	A	A	L	R	R	L	G	L	R	A	C	G	A	A													
Human IIA	M	G	A	A	A	L	L	L	L	A	A	C	A	G	A	G	A	A	A	A	G	L	A	A	A	L	R	R	L	G	L	R	A	C	G	A	A													
Consensus	M	A	A	A	A	L	A	L	L	G	L	C	A	G	G	G	A	A	A	G	L	A	A	A	L	R	R	L	G	L	R	A	C	G	A	A														

	70										80										90										100										110									
Rat Ilt	L	H	C	A	A	R	L	L	G	L	L	V	K	I	G	C	L	L	C	A	G	A	A	L	V	V	V	V	V	V	V	V	V	V	V	V														
Pig Ilt	L	H	C	A	A	R	L	L	G	L	L	V	K	I	G	C	L	L	C	A	G	A	A	L	V	V	V	V	V	V	V	V	V	V	V	V														
Mouse Ilt	L	H	C	A	A	R	L	L	G	L	L	V	K	I	G	C	L	L	C	A	G	A	A	L	V	V	V	V	V	V	V	V	V	V	V	V														
Human Ilt	L	H	C	A	A	R	L	L	G	L	L	V	K	I	G	C	L	L	C	A	G	A	A	L	V	V	V	V	V	V	V	V	V	V	V	V														
Bovine Ilt	L	H	C	A	A	R	L	L	G	L	L	V	K	I	G	C	L	L	C	A	G	A	A	L	V	V	V	V	V	V	V	V	V	V	V	V														
Rengus Ilt	L	H	C	A	A	R	L	L	G	L	L	V	K	I	G	C	L	L	C	A	G	A	A	L	V	V	V	V	V	V	V	V	V	V	V	V														
Human IIA	L	H	C	A	A	R	L	L	G	L	L	V	K	I	G	C	L	L	C	A	G	A	A	L	V	V	V	V	V	V	V	V	V	V	V	V														
Consensus	L	H	C	A	A	R	L	L	G	L	L	V	K	I	G	C	L	L	C	A	G	A	A	L	V	V	V	V	V	V	V	V	V	V	V	V														

	120										130										140										150									
Rat Ilt	H	L	P	A	G	G	G	V	T	L	P	R	A	A	A	L	L	V	L	A	A	L	L	V	L	A	L	L	V	G	L	L	-	-	-					
Pig Ilt	H	L	P	A	G	G	G	V	T	L	P	R	A	A	A	L	L	V	L	A	A	L	L	V	L	A	L	L	V	G	L	L	-	-	-					
Mouse Ilt	H	L	P	A	G	G	G	V	T	L	P	R	A	A	A	L	L	V	L	A	A	L	L	V	L	A	L	L	V	G	L	L	-	-	-					
Human Ilt	H	L	P	A	G	G	G	V	T	L	P	R	A	A	A	L	L	V	L	A	A	L	L	V	L	A	L	L	V	G	L	L	-	-	-					
Bovine Ilt	H	L	P	A	G	G	G	V	T	L	P	R	A	A	A	L	L	V	L	A	A	L	L	V	L	A	L	L	V	G	L	L	-	-	-					
Rengus Ilt	H	L	P	V	-	-	-	-	-	-	-	-	-	-	A	V	L	L	L	L	L	L	L	L	V	V	L	L	V	G	L	L	-	-	-					
Human IIA	H	L	P	M	V	-	-	-	-	-	-	-	-	-	A	V	L	L	L	L	L	L	L	L	V	V	L	L	V	G	L	L	-	-	-					
Consensus	H	L	P	A	G	G	G	V	T	L	P	R	A	A	A	L	L	V	L	A	A	L	L	V	L	A	L	L	V	G	L	L	-	-	-					

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1  tccccgcccc ccagtcctcc ctccccctccc ctccagcatg gtgctcgcgg cccgctgct
61  gctgggcttc ctgctcctcg ccctggagct gcggccccgg ggggaggcgg ccgagggccc
121 cgcgggcgcg gcggcgggcg cgcgggcggc ggcagcggcg ggggtcgggg gggagcgctc
181 cagccggcca gccccgtccg tggcgcccga gccggacggc tgccccgtgt gcgtttggcg
241 gcagcacagc cgcgagctgc gcctagagag catcaagtcg cagatcttga gcaaactgcg
301 gctcaaggag gcgccaaca tcagccgcga ggtggtgaag cagctgctgc ccaaggcgcc
361 gccgctgcag cagatcctgg acctacacga cttccagggc gacgcgctgc agcccagga
421 cttcctggag gaggacgagt accacgccac caccgagacc gtcattagca tggcccagga
481 gacggacca gcagtacaga cagatggcag ccctctctgc tgccattttc acttcagccc
541 caaggtgatg ttcacaaagg tactgaaggg ccagctgtgg gtgtacctac ggcctgtacc
601 ccgcccagcc acagtctacc tgcagatctt gcgactaaaa cccctaactg ggggaaggac
661 cgcaggggga gggggcgagg gccggcgctc catccgtatc cgctcactga agattgagct
721 gcactcacgc tcaggccatt ggcagagcat cgacttcaag caagtgtctac acagctggtt
781 ccgccagcca cagagcaact ggggcatcga gatcaacgcc tttgatccca gtggcacaga
841 cctggctgtc acctccctgg ggccgggagc cgaggggctg catccattca tggagcttcg
901 agtcctagag aacacaaaac gttcccggcg gaacctgggt ctggactgcg acgagcactc
961 aagcgagtcc cgctgctgcc gatatcccct cacagtggac tttgaggctt tcggctggga
1021 ctggatcatc gcacctaaagc gctacaaggc caactactgc tccggccagt gcgagtacat
1081 gttcatgcaa aaatatccgc ataccatttt ggtgcagcag gccaatccaa gaggctctgc
1141 tgggcccgtg tgtaccccca ccaagatgtc cccaatcaac atgctctact tcaatgacaa
1201 gcagcagatt atctacggca agatccctgg catggtggtg gatcgctgtg gctgctctta
1261 aggtggggga tagaggatgc ctcccccaca gaccctaccc caagaccctt agccctgccc
1321 ccatccccc aagccctaga gctccctcca ctcttcccg cgaacatcaca ccgttccccg
1381 accaagccgt gtgcaatata acagagggag gcaggtggga attgaggggt aggggttttg
1441 gggaaagggg aagcaggggc atagtcaggg tggggagtgt ttgaagtttg cagatgagaa
1501 ggtttgacaa aaagacagag agatgtagag acagtgtatg agacagagga aaaaaagag
1561 cagcagttag aaggcaaaaga gagaggcaga agagacagag gaggcagaga caaacactg
1621 agaaagagac tgaaatggag taataaatga aagccccaca ccaagcctcc tttcttccac
1681 tggcaagggt aggggcttgg tatagtttgg ggagatcccc tgactattca gtaggagaag
1741 aaatcaaaaa tccattcttt tctccttctc tccctccaac agtggccagg ggaaggggaa
1801 gtgagggcag gggcaaaaag atttgggaat ttttatttat ttatttattg tgacttttca
1861 tttttttggt atttggcttt actggaatag gagggcccct gccactgtg ccccgtttat
1921 cccttattcc ccaaaccctg ctctcccaa cacctactca cttaagcact tgtataaagc
1981 ctccaggggt gggaatggga gtaaagggca agagggcgga cacatgaagt ttagtttcta
2041 acccatcatc accctaactc aaccttttct gagccaaatg gcttgaattg aagccagttg
2101 tcatggaaat agtaagaggt tagggtttaa gagctgggga tgcgggggtg ggagagagaa
2161 ccctcaacat ccaggatcta tataatgaga gctactttaa accctcaggt ccaccctcat
2221 gatgctgagt tatttagcca gaggggtgcag cctgcttatg cccaaattcc ctgagccaag
2281 agagagacca aagagcctct ggaatggccc tgctcccagc ctctatcttc aggtcaatta
2341 gagagagtat agagacccca gagtcccctg ggtctggaaa gcgttaggag aggtcaagaa
2401 aggagcagta aggaggctga aggttacagg gcatttgaat ccaaatacct gctctgggct
2461 agggaaataga gccagcagac caaggtggga aggattctgg aagggggaca ttttagtctc
2521 ctaaccccaa agctcagggt ggaagaggag agaacaagga agcagagtgat ataattttt
2581 tttcctttta tttttggaat ctaacagtag ctggcagcag ggaggggaaa gtacagtggg
2641 gaaaagcatc tgacaaggcc agttagaaca gaggatggga aggatggaga ctcccgggct
2701 tgggaaggcta ggaagcaggc agagactggt tgccatttca agtcactagc tagggccatt
2761 cattcctccc acaaccctga cccattctcc tctggactca ctgtgcctca gtttcttccc
2821 ctcaatggaa tgagaaatga cagcaccgcg cacagccaag agatgaattc tgagcactta
2881 ccacgggcac tttatggaca taaaatacct ctgcgtgtgg gacagataac cagggcacca
2941 gagtagtggt gaagagatgt gaggtttaag aggagtcaca ggcttcagag tacaagttcc
3001 cctctgcctc ccagctggac agtgcctaga agccaaggag ttgagaatct cctgatccac
3061 accctatcct tacttcacca ccaggcctct tggctccagg caagagctta gaggatgtca
3121 ggagaggtgg gggtaagaat cttcagcaaa actgtcactc taagtagagc cagcagttac

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FIG. 3A

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3181 ggggtctgata aaaacagtac tgaactaaag taaagcccaa gctgggtgagc aaactggat
3241 ggctcattct tcccaagagc atgactctcc cccttggcca gttgggtggaa ggggcaaagg
3301 tatgtgacca cccttgagaa ggtgatgttg gtgagcttta acatcttatt cctattctta
3361 tagtgagaaa gtgaaacaag atctttcagt agaggaatgg gcagggctgt aggctcttc
3421 agcttgccct caccatata gcagctatgc taaccccaag cctctctggc cctgttcttc
3481 atccttcctt ctgccccaat cctgaaggac aagacacacc cggccatcaa accactcac
3541 atttccttgg tggaaggaaa ggaacagaga agtgaagaac agatacctcc ctccaaggtc
3601 aaatgcctcg tgatcttggc agagtaggga ttgggcaata agcatcaggt atcttcctc
3661 tacagattct agagagctgg ggcattaaat atgggggaca cttagaatac agctccttaa
3721 ataccaccaa ataaagacct ttgtgtgtgt gtgggtgggtg gggggggggc aggggtcttt
3781 ctcttatgaa cataaatctg tgagctgaag tctcattccc ctgttcctcc ctaccccaa
3841 agaggcacag agtgaaggga cttggggggc acagctcagc aaccagtggt gagttagcac
3901 cccctccac cttatgatgt gtgtggacct ggccagtgcc cctctgaaca tatcattatt
3961 agtgtaatta tcatttattt tgtgtatttg tcacattgtg tgcattgacag cctttgttaa
4021 ggggtgtctga ggagtatgga gctgacaggg gcattggaat gccaggaaag aacttcttca
4081 actgagatca aggcttcctg gagggaaacca ctgcaaaaag gccatcaggc agttttcaag
4141 ttatgtgaca gagggcaaag acggccatag ggtgctctga gttttgggt ggtcacatga
4201 cacaatccag cacttgaacc tgaaaaaaaa aataaaagcg gtcaaagagt ttagaattca
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FIG. 3B

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1 mvlaapl1lg fl11alelrp rgeaaegpaa aaaaaaaaaa agvggerssr apsvapepd
61 gcpvcvwrqh sre1rlesik sqilsklrlk eapnisrevv kqllpkappl qqildlhdfq
121 gdalqpedfl eedeyhatte tvismagetd pavqtdgspl cchfhfspkv mftkvlkaql
181 wvylrpvprp atvylqilrl kpltgegtag gggggrrhir irslkielhs rsghwqsidf
241 kqvlhswfrq pqsngiein afdpsgtdla vtslgpgaeg lhpfmelrvl entkrstrnl
301 gldcdehsse srccrypltv dfeafgwdwi iapkrykany csgqceymfm qkypthlvq
361 qanprgsagp cctptkmspi nmlyfndkqq iiygkipgmv vdrcgcs
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FIG. 4

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1  actcggctcg cctcgcggcg ggcgcctctg tcgccagcgg cgcaccatgg acgggctgcc
61  cggtcggggcg ctggggggcgg cctgccttct gctgctggcg gccggctggc tggggcctga
121  ggccctggggc tcacccacgc ccccgccgac gcctgccgcg ccgcccacac ccccgccacc
181  cggatccccg ggtggctcgc aggacacctg tacgtcgtgc ggcggtcttc ggcgccaga
241  ggagctcggc cgagtggacg gcgacttcct ggaggcgggt aagcggcaca tcttgagccg
301  cctgcagatg cggggccggc ccaacatcac gcacgccgtg cctaaggccg ccatggtcac
361  ggccctgcgc aagctgcacg cgggcaaggt gcgcgaggac ggccgctgg agatcccgca
421  cctcgacggc cagccagcc cgggcgccga cggccaggag cgcgtttccg aaatcatcag
481  cttcgccgag acagatggcc tcgcctcctc ccgggtccgc ctatacttct tcacttccaa
541  cgaaggcaac cagaacctgt ttgtggtcca ggccagcctg tggctttacc tgaaactcct
601  gccctacgtc ctggagaagg gcagccggcg gaaggtgcgg gtcaaagtgt acttccagga
661  gcagggccac ggtgacagg ggaacatggt ggagaagagg gtggacctca agcgcagcgg
721  ctggcatacc ttcccactca cggaggccat ccaggccttg tttgagcggg gcgagcggcg
781  actcaacctc gacgtgcagt gtgacagctg ccaggagctg gccgtggtgc cgggtgtcgt
841  ggaccacggc gaagagtcgc accggccctt tgtggtggtg caggctcggc tgggcgacag
901  caggcaccgc attcgcaagc gaggcctgga gtgcgatggc cggaccaacc tctgttgag
961  gcaacagttc ttcattgact tccgcctcat cggctggaac gactggatca tagcaccac
1021  cggctactac gggaactact gtgagggcag ctgccagcc tacctggcag gggctcccgg
1081  ctctgcctcc tccttccaca cggctgtggt gaaccagtac cgcagtcggg gtctgaacct
1141  cggcacggtg aactcctgct gcattcccac caagctgagc accatgtcca tgctgtactt
1201  cgatgatgag tacaacatcg tcaagcggga cgtgcccaac atgattgtgg aggagtgcgg
1261  ctgcgcctga cagtgcgaag caggggcacg gtggtggggc acggagggca gtcccgggtg
1321  ggcttcttcc agcccccgcg ggaacggggg tacacggtgg gctgagtaca gtcattctgt
1381  tgggctgtgg agatagtgcc aggtgcggc ctgagatatt tttctacagc ttcatagagc
1441  aaccagtcaa aaccagagcg agaaccctca actgacatga aatactttaa aatgcacacg
1501  tagccacgca cagccagacg catcctgcca cccacacagc agcctccagg ataccagcaa
1561  atggatcgcg tgacaaatgg cagcttagct acaaatgcct gtcagtcgga gagaatgggg
1621  tgagcagcca ccattcccac cagctggccc ggccactctg aattgcgcct tccgagcaca
1681  cataaaagca caaagacaga gacgcagaga gagagagaga gccacggaga ggaaaagcag
1741  atgcaggggt ggggagcgca gctcggcgga ggctgcgtgt gcccgtggc ttttaccagg
1801  cctgctctgc ctggctcgat gtctgcttct tcccagcct gggatccttc gtgcttcaag
1861  gcctggggag cctgtccttc catgcccttg tcgagggaaa gagaccaga aaggacacaa
1921  cccgtcagag acctgggagc aggggcaatg accgtttgac tgtttgtggc ttgggcctct
1981  gacatgactt atgtgtgtgt gtgttttttg ggtggggagg gagggagaga agagggggct
2041  aaatttgatg ctttaactga tctccaacag ttgacaggtc atccttgcca gttgtataac
2101  tgaaaaagga cttttctacc aggtatgacc ttttaagtga aaatctgaat tgttctaaat
2161  ggaaagaaaa aaagttgcaa tctgtgccct tcattgggga cattcctcta ggactggttt
2221  ggggacgggt gggaatgacc cctaggcaag gggatgagac cgcaggagga aatggcgggg
2281  aggaggcatt cttgaactgc tgaggatggg ggggtgtccc tcagcggagg ccaaggagg
2341  ggagcagcct agttggtctt ggagagatgg ggaaggcttt cagctgattt gcagaagttg
2401  cccatgtggg ccccgccat cagggtggc cgtggacgtg gccctgccc actcacctgc
2461  ccgctgccc gcccgcccgc atagcacttg cagacctgcc tgaacgcaca tgacatagca
2521  cttgccgatc tgctgtgtgc cagaagtggc ccttggccga gcgcgaact cgtgcacct
2581  ctgatgtcc aagtgccacg tgaactatgc aatttaaagg gttgacctaa actagacgaa
2641  actggactcg tacgactctt tttatatatt ttatacttga aatgaaatcc tttgcttctt
2701  ttttaagcga atgattgctt ttaatgtttg cactgattta gttgcatgat tagtcagaaa
2761  ctgccatttg aaaaaagtt atttttatag cagcaaaaaa aaaaaaaaaa gaatacagtt
2821  aaatgtatta tacataattt tggaacccaa gagccaaca gatcagtttt aattttatta

```

FIG. 5A

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```
2881 gacggtgagg ccatctgaga tgagggtggac gttctgagca gtcccttgag ggcttgcca
2941 acgtttcagg gtatgaatgg attttggtta ttcggtttga tgtgtctttt ccatccttac
3001 acacccagaa ggtagagtaa aaatgactat gatagaatgc aggtgtgtat ccttaaatcc
3061 tcatctttat gtttatttaa taaagctccc cttagattct gtttcataat aatttaaac
3121 caaacaattt tcccatagac ttgctgttaa agtattgtac gtttgtgtac agtttaagaa
3181 aataaaagat tgagtgccac gggaaaaaaaa aaaaaaaaa
```

FIG. 5B

```
1 mdglpgralg aacllllaag wlgpeawgsp tppptpaapp pppppgspgg qdtctscgg
61 frrpeelgrv dgdfleavkr hilsrlqmrq rpnithavpk aamvtalrkl hagkvredgr
121 veiphldgha spgadgqerv seiisfaetd glassrvrly ffisnegnqn lfvvqaslwl
181 ylkllpyvle kgsrrkvrvk vyfqeqghgd rwnmvekrvd lkrsgwhthp lteaiqalfe
241 rgerrlnldv qcdscqelav vpvfvdpgge shrpfvvvqa rlgdsrhrir krglecdgrt
301 nlccrqqffi dfrligwndw iiaptgyygn ycegscpayl agvpgsassoc htavvnqyrm
361 rglnpgtvns cciptklstm smlyfddeyn ivkrdvpnmi veecgca
```

FIG. 6

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```

1  cagacatgag ctgtgagggg caagcacagc tatccatcag atgatctact ttcagccttc
61  ctgagtccca gacaatagaa gacaggtggc tgtacccttg gccaaaggta ggtgtggcag
121 tgggtgtctgc tgtcactgtg ccctcattgg ccccagcaa tcagactcaa cagacggagc
181 aactgccatc cgaggctcct gaaccagggc cattcaccag gagcatgcgg ctccctgatg
241 tccagctctg gctggtgctg ctgtgggcac tgggtgcgagc acaggggaca gggctctgtg
301 gtccctcctg tgggggctcc aaactggcac cccaagcaga acgagctctg gtgctggagc
361 tagccaagca gcaaatcctg gatgggttgc acctgaccag tcgtcccaga ataactcatc
421 ctccacccca ggcagcgctg accagagccc tccggagact acagccaggg agtgtggctc
481 cagggaaatg ggaggagggtc atcagctttg ctactgtcac agactccact tcagcctaca
541 gctccctgct cactttttcac ctgtccactc ctcggtccca ccacctgtac catgcccgcc
601 tgtggctgca cgtgctcccc acccttcctg gcactctttg cttaggagtc ttccgatggg
661 gaccaaggag gaggcgcaa ggggtccgca ctctcctggc tgagcaccac atcaccaacc
721 tgggctggca taccttaact ctgccctcta gtggcttgag gggtgagaag tctggtgtcc
781 tgaaactgca actagactgc agaccctag aaggcaacag cacagt tact ggacaaccga
841 ggcggtctt ggacacagca ggacaccagc agcccttcct agagcttaag atccgagcca
901 atgagcctgg agcaggccgg gccaggagga ggacccccac ctgtgagcct gcgacccctc
961 tatgttgagc gcgagaccat tacgtagact tccaggaact gggatggcgg gactggatac
1021 tgcagcccga ggggtaccag ctgaattact gcagtgggca gtgccctccc cacctggctg
1081 gcagcccagg cattgctgcc tctttccatt ctgccgtctt cagcctcctc aaagccaaca
1141 atccttggcc tgccagtacc tcctgttgtg tccctactgc ccgaaggccc ctctctctcc
1201 tctacctgga tcataatggc aatgtggtca agacggatgt gccagatatg gtggtggagg
1261 cctgtggctg cagctagcaa gaggacctgg ggctttggag tgaagagacc aagatgaagt
1321 ttcccaggca caggcatct gtgactggag gcatcagatt cctgatccac accccaacct
1381 aacaaccacc tggcaatatg actcacttga cccctatggg acccaaatgg gcactttctt
1441 gtctgagact ctggcttatt ccaggttggc tgatgtgttg ggagatgggt aaagcgtttc
1501 ttctaaaggg gtctacccag aaagcatgat ttccctgccct aagtccgtgt agaagatgtc
1561 agggactagg gagggaggga gggaaaggcag agaaaaatta cttagcctct cccaagatga
1621 gaaagtcctc aagtgagggg agggaggaagc agatagatgg tccagcaggc ttgaagcagg
1681 gtaagcaggc tggcccaggg taagggctgt tgaggtagct taaggggaag tcaagaggga
1741 gatgggcaag gcgctgaggg aggatgctta ggggaccccc agaaacagga gtcaggaaaa
1801 tgaggcacta agcctaagaa gttccctggg ttttcccagg ggacaggacc cactgggaga
1861 caagcattta tactttcttt cttctttttt atttttttga gatcgagtct cgctctgtca
1921 ccaggctgga gtgcagtgac acgatcttgg ctactgcaa cctccgtctc ctgggttcaa
1981 gtgattcttc tgcctcagcc tcccagagcag ctgggattac aggcgcccac taatttttgt
2041 attcttagta gaaacgaggt ttcaacatgt tggccaggat ggtctcaatc tcttgacctc
2101 ttgatccacc cgacttggcc tcccgaagtg atgagattat aggcgtgagc caccgcgcct
2161 ggcttatact ttcttaataa aaaggagaaa gaaaatcaac aaatgtgagt cataaagaag
2221 ggttaggggtg atgggtccaga gcaacagttc ttcaagtgtg ctctgtaggc ttctgggagg
2281 tcccttttca ggggtgtcca caaagtcaaa gctattttca taataatact aacatgttat
2341 ttgccttttg aattctcatt atcttaaaat tgtattgtgg agttttccag aggccgtgtg
2401 acatgtgatt acatcatctt tctgacatca ttgttaaaaa aaaaaaaaaa aaa

```

FIG. 7

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```
1 mrlpdvqlwl vllwalvraq gtgsvcpscg gsklapqaer alvlelakqq ildglhltsr
61 prithppppqa altralrrlq pgsvapgng evisfatvtd stsayssllt fhlstprshh
121 lyharlwlhv lptlpgtlcl rifrwgprrr rggstrllae hhitnlgwht ltlpssglrg
181 eksgvlklql dcrplegnst vtgqprrrld taghqqpfle lkiranepga grarrtptc
241 epatplccrr dhyvdfqelg wrdwilqpeg yqlnycsgqc pphlagspgi aasfhsavfs
301 llkannpwa stscvptar rplslllydh ngnvvktvdp dmvveacgcs
```

FIG. 8

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```

1 agattctgcc aagttctacc tgtaactggc ttcattttca agtcagacgt ttggctgctg
61 ctctgtcccc tgcaacaagg agccatgcca gctggacaca cacttcttcc agggcctctg
121 gcagccagga cagagttgag accacagctg ttgagaccct gagccctgag tctgtattgc
181 tcaagaaggg ccttccccag caatgacctc ctcattgtct ctggccttcc tctcctggc
241 tccaaccaca gtggccactc ccagagctgg cggtcagtgt ccagcatgtg gggggccac
301 cttggaactg gagagccagc gggagctgct tcttgatctg gccaagagaa gcaccttgga
361 caagctgcac ctcacccagc gcccaacact gaaccgccct gtgtccagag ctgctttgag
421 gactgcactg cagcacctcc acgggggtccc acagggggca cttctagagg acaacaggga
481 acaggaatgt gaaatcatca gctttgctga gacaggcctc tccaccatca accagactcg
541 tcttgatttt cacttctcct ctgatagaac tgctggtgac agggaggtcc agcaggccag
601 tctcatgttc tttgtgcagc tcccttccaa taccacttgg accttgaaag tgagagtcct
661 tgtgctgggt ccacataata ccaacctcac cttggctact cagtacctgc tggaggtgga
721 tgccagtggc tggcatcaac tccccctagg gcctgaagct caagctgcct gcagccaggg
781 gcacctgacc ctggagctgg tacttgaagg ccaggtagcc cagagctcag tcactcctggg
841 tggagctgcc cataggcctt ttgtggcagc ccgggtgaga gttgggggca aacaccagat
901 tcaccgacga ggcacgact gcccaaggagg gtccaggatg tgctgtcgac aagagttttt
961 tgtggacttc cgtgagattg gctggcacga ctggatcatc cagcctgagg gctacgccat
1021 gaacttctgc atagggcagt gccactaca catagcaggc atgcctggta ttgctgcctc
1081 ctttcacact gcagtgtca atcttctcaa ggccaacaca gctgcaggca ccactggagg
1141 gggctcatgc tgtgtaccca cggcccggcg cccctgtct ctgctctatt atgacaggga
1201 cagcaacatt gtcaagactg acatacctga catggtagta gaggcctgtg ggtgcagtta
1261 gtctatgtgt ggtatgggca gcccaagggt gcatgggaaa acacgccctc acagaagtgc
1321 acttccttga gaggagggaa tgacctcatt ctctgtccag aatgtggact cctcttctct
1381 gagcatctta tggaaattac cccacctttg acttgaagaa accttcatct aaagcaagtc
1441 actgtgccat cttcctgacc actaccctct ttcctagggc atagtcctac ccgctagtc
1501 atcccgtag cccactcca gggactcaga cccatctcca accatgagca atgccatctg
1561 gttcccaggc aaagacaccc ttagctcacc ttaataagac ccataaccc actatgcctt
1621 cctgtccttt ctactcaatg gtcccccact caagatgagt tgacacaacc ccttcccca
1681 atttttgtgg atctccagag aggcccttct ttggattcac caaagtttag atcactgctg
1741 cccaaaatag aggccttacc acccccctct ttgttgtgag cccctgtcct tcttagttgt
1801 ccagggtgaac tactaaagct ctctttgcat accttcatcc atttttgtc cttctctgcc
1861 tttctctatg cccttaagggt ctgacttgcc tgagctctat cacctgagct cccctgcct
1921 ctggcttctt gctgaggtca gggcatttct tatccctgtt ccctctctgt ctaggtgtca
1981 tggttctgtg taactgtggc tattctgtgt ccctacacta cctggctacc cccttccatg
2041 gccccagctc tgcctacatt ctgatataac tgcttcaaca ctaggggggt ctaaaggcct
2101 tctatcttgc tagtccctgg ggcctcaaca tctcactact gttcccttaa ctctgcctat
2161 acctctgtaa ataattcctt cactaagttc tcttgatgaa gcaaaaacag acagctgaaa
2221 agtcctctat ctctacaag ggccttaact ggcaccccag atgacacaga gctgcctgc
2281 ttatgtctga gtctgcctac tctgtgtct cttcacatgg tctctcaga actgaactat
2341 tgtatccatc tcacacttta tgctcttct ttcttaggca ccccgctcct ccactcttcc
2401 agaaccatct ttgaggtctc atggctaata aaaacctagg ctttacctgt tccctctgta
2461 atccctccaa aagatgagac agatctatgc ttggtcatcc agtaaacatga ccagctgtgg
2521 gcacgcaagt gtgggaggca gaggcattgt cagagctggc tgccaggacc tctgacttgc
2581 cttcctttca cccaccccca gtgctccacc caggagtcct gcctggaagc tggaatgggc
2641 aagggtctgt ggagtgggac agggagaaga ggaaggcctg gatgaggaga ggggtggcatt
2701 tgctctgaga ctgggtcctt tttagacctt tgcccgtcct cccccacatc tctcctttt
2761 ggctggacag tctgaacca tgaggtcgat aatgtctgca gccaaggcc gagtttgcgc
2821 aaaacccatg tgttcttttg taaacgtgat gtctgtgttt gctcagttta tgacccctc
2881 ctatgagggt aagaggtccc tgaaatagga accctagagg agaaagtctg aaaaggactg

```

FIG. 9A

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```
2941 cctggggggac tgtaaactctg agcttgaggg cttcctgagc aacccatgga agttatccca
3001 cctttgactt gaggagacct tcatctaagg agaatctaag gaggccttct ggtgtctccc
3061 ccacacatcc ccgaccccca gatctaacct ctttcccaat tacagcttag tctccagggc
3121 taggactggg gttaaagcaaa gtgagtcatt cacctggggg ggctaaattt taaggggggtg
3181 gtgaacaatt tattaatcaa gataggactt taatgcaata ttatttttaa tcaaaatta
3241 atgcaaaaaa tccatgatga acaaaatagc ctacttttaa ataaaaacag gatcagcatt
3301 aaaaaaaaaa aaaaa
```

FIG. 9B

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```
1 mtsslllafl llapttvatp ragggcpacg gptlelesqr ellldlakrs ldklhltqr
61 ptlnrpvsra alrtalqhlh gvpqgalled nregeceiis faetglstin qtrldfhfss
121 drtagdrevq qaslmffvql psnttwtlkv rvlvlgphnt nltlatqyll evdasgwhql
181 plgpeaqaac sqghltlelv legqvaqssv ilggaahrfp vaarvrvvgk hqihrrgidc
241 qggssrmccrq effvdfreig whdwiiqpeg yamnfciqgc plhiagmpgi aasfhtavln
301 llkantaagt tgggscvpt arrplsllly drdsnivktd ipdmvveacg cs
```

FIG. 10

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```

1 agattcactg gtgtggcaag ttgtctctca gactgtacat gcattaaaaat tttgcttggc
61 attactcaaa agcaaaaagaa aagtaaaaagg aagaaaacaag aacaagaaaa aagattatat
121 tgatttttaa atcatgcaaa aactgcaact ctgtgtttat atttacctgt ttatgctgat
181 tgttgctggg ccagtggaac taaatgagaa cagtgagcaa aaagaaaatg tggaaaaaga
241 ggggctgtgt aatgcatgta cttggagaca aaacactaaa tcttcaagaa tagaagccat
301 taagatacaa atcctcagta aacttcgtct ggaaacagct cctaacatca gcaaagatgt
361 tataagacaa cttttaccca aagctcctcc actccgggaa ctgattgatc agtatgatgt
421 ccagagggat gacagcagcg atggctcttt ggaagatgac gattatcacg ctacaacgga
481 aacaatcatt accatgccta cagagtctga ttttctaata caagtggatg gaaaacccaa
541 atgttgcttc tttaaattta gctctaaaat acaatacaat aaagtagtaa aggcccaact
601 atggatata tttgagaccg tctgagactcc tacaacagtg tttgtgcaaa tcctgagact
661 catcaaacct atgaaagacg gtacaaggta tactggaatc cgatctctga aacttgacat
721 gaaccaggc actggtattt ggcagagcat tgatgtgaag acagtgttgc aaaattggct
781 caaacaacct gaatccaact taggcattga aataaaaagc ttagatgaga atggatcatg
841 tcttgctgta accttcccag gaccaggaga agatgggctg aatccgtttt tagaggtcaa
901 ggtaacagac acaccaaaaa gatccagaag ggatttttgt cttgactgtg atgagcactc
961 aacagaatca cgatgctgtc gttaccctct aactgtggat tttgaagctt ttggatggga
1021 ttggattatc gctcctaaaa gatataaggc caattactgc tctggagagt gtgaatttgt
1081 atttttacaa aaatatcctc atactcatct ggtacaccaa gcaaacccca gaggttcagc
1141 aggcccttgc tgtactccca caaagatgtc tccaattaat atgctatatt ttaatggcaa
1201 agaacaata atatatggga aaattccagc gatggtagta gaccgctgtg ggtgctcatg
1261 agatttata taagcgttca taacttccca aaacatggaa ggttttcccc tcaacaattt
1321 tgaagctgtg aaattaagta ccacaggcta taggcctaga gtatgtctca gtcacttaag
1381 cataagctac agtatgtaaa ctaaaagggg gaatatatgc aatggttggc atttaacctat
1441 ccaaacaata catacaagaa agttttatga tttccagagt ttttgagcta gaaggagatc
1501 aaattacatt tatgttccca tatattacaa catcggcgag gaaatgaaag cgattctcct
1561 tgagtctgta tgaattaaag gagtatgctt taaagtctat ttctttaaag ttttgtttaa
1621 tatttacaga aaaatccaca tacagtattg gtaaaatgca ggattgttat ataccatcat
1681 tcgaatcatc cttaaactct tgaatttata ttgtatggta gtatacttgg taagataaaa
1741 ttccacaaaa atagggatgg tgcagcatat gcaatttcca ttctattat aattgacaca
1801 gtacattaac aatccatgcc aacggtgcta atacgataag ctgaatgtct gaggctacca
1861 ggtttatcac ataaaaaaca ttcagtaaaa tagtaagttt ctcttttctt caggtgcatt
1921 ttctacacac tccaaatgag gaatggattt tctttaatgt aagaagaatc atttttctag
1981 aggttggctt tcaattctgt agcatacttg gagaaactgc attatcttaa aaggcagtc
2041 aatgggtgtt gtttttatca aaatgtcaaa ataacatact tggagaagta tgtaattttg
2101 tctttggaaa attacaacac tgcctttgca aactgacagt ttttatggta aaataataga
2161 aatgatcgac tctatcaata ttgtataaaa agactgaaac aatgcattta tataatatgt
2221 atacaatatt gttttgtaaa taagtgtctc cttttttatt tactttggta tttttttaca
2281 ctaaggacat ttcaaattaa gtactaaggc acaaagacat gtcatgcatc acagaaaagc
2341 aactacttat atttcagagc aaattagcag attaaatagt ggtcttaaaa ctccatattg
2401 taatgattag atggttatat tacaatcatt ttatatTTTT ttacatgatt aacattcact
2461 tatggattca tgatggctgt ataaagtga tttgaaaatt caatggttta ctgtcattgt
2521 gtttaaatct caacgttcca ttattttaat acttgcaaaa acattactaa gataccaaa
2581 ataattgact ctattatctg aaatgaagaa taaactgatg ctatctcaac aataactgtt
2641 acttttattt tataatttga taatgaatat atttctgcat ttatttactt ctgttttgta
2701 aattgggatt ttgttaatca aatttattgt actatgacta aatgaaatta tttcttacat
2761 ctaatttgta gaaacagtat aagttatatt aaagtgtttt cacatttttt tgaaagacaa
2821 aaa

```

FIG. 11

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```
1  mqklqlcvyi ylfmlivagp vdlnenseqk envekeglcn actwrqntks srieaikiqi
61  lsklrletap niskdvirql lpkapplrel idqydvqrdd ssdgsleddd yhattetiit
121 mptesdflmq vdgkpkccff kfsskiqynk vvkaqlwiyl rpvetpttvf vqilrlikpm
181 kdgtrytgir slkldmnpgt giwqsidvkt vlqnlwkqpe snlgieikal denghdlavt
241 fpgpgedgln pflevkvtdt pkrsrrdfgl dcdehstesr ccrypltvdf eafgwdwiia
301 pkrykanycs gecefvflqk yphthlvhqa nprgsagpcc tptkmspinm lyfngkeqii
361 ygkipamvvd rcgcs
```

FIG. 12

Type	Ligand	kon (1/Ms)	koff (1/s)	KD (pM)
Smad2/3	GDF11	3.46e6	2.56e-4	74
	GDF8	1.18e6	2.42e-4	200
	Activin B	3.52e6	2.19e-3	620
	Activin A	5.35e6	1.23e-1	23000
Smad1/5/8	BMP10	1.67e7	1.45e-3	87
	BMP6	6.05e6	5.46e-4	90
	BMP9	5.04e6	1.37e-2	2700

FIG. 13

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```

1 caactggggg cgccccggac gaccatgaga gataaggact gagggccagg aaggggaagc
61 gagcccgccg agaggtggcg gggactgctc acgccaaggg ccacagcggc cgcgctccgg
121 cctcgctccg ccgctccacg cctcgcggga tccgcggggg cagcccggcc gggcggggat
181 gccggggctg gggcgaggag cgcatgtggt gtgctggtgg tgggggctgc tgtgcagctg
241 ctgcgggccc ccgcccgtgc ggcccgcctt gcccgtgccg gcggccgcgc cgccgggggg
301 gcagctgctg ggggacggcg ggagccccgg ccgcacggag cagccgcgcg cgtcgccgca
361 gtctctctcg ggcttcctgt accggcggct caagacgcag gagaagcggg agatgcagaa
421 ggagatcttg tcggtgctgg ggctcccga ccggccccgg ccctgcacg gcctccaaca
481 gccgcagccc ccggcgctcc ggcagcagga ggagcagcag cagcagcagc agctgcctcg
541 cggagagccc cctcccgggc gactgaagtc cgcgcccctc ttcagtctgg atctgtacaa
601 cgccctgtcc gccgacaacg acgaggacgg ggcgtcggag ggggagaggc agcagtcctg
661 gccccacgaa gcagccagct cgtcccagcg tcggcagccg cccccggcg ccgcgcaccc
721 gctcaaccgc aagagccttc tggccccgg atctggcagc ggcggcgcgt cccactgac
781 cagcgcgcag gacagcgctt tcctcaacga cgcggacatg gtcatgagct ttgtgaacct
841 ggtggagtag gacaaggagt tctcccctcg tcagcgacac cacaagagt tcaagttcaa
901 cttatcccag attcctgagg gtgaggtggt gacggctgca gaattccgca tctacaagga
961 ctgtgttatg gggagtttta aaaaccaaac ttttcttatc agcatttatc aagtcttaca
1021 ggagcatcag cacagagact ctgacctgtt tttgttggac acccgtgtag tatgggcctc
1081 agaagaaggc tggctggaat ttgacatcac ggccactagc aatctgtggg ttgtgactcc
1141 acagcataac atggggcttc agctgagcgt ggtgacaagg gatggagtcc acgtccaccc
1201 ccgagccgca ggcctggtgg gcagagacgg cccttacgac aagcagccct tcatggtggc
1261 tttcttcaaa gtgagtgagg tgcacgtgcg caccaccagg tcagcctcca gccggcgccg
1321 acaacagagt cgtaatcgct ctaccagtc ccaggacgtg gcgcgggtct ccagtgttc
1381 agattacaac agcagtgaat tgaaaacagc ctgcaggaag catgagctgt atgtgagttt
1441 ccaagacctg ggatggcagg actggatcat tgcacccaag ggctatgctg ccaattactg
1501 tgatggagaa tgctccttcc cactcaacgc acacatgaat gcaaccaacc acgcgattgt
1561 gcagaccttg gttcacctta tgaaccccga gtatgtcccc aaaccgtgct gtgcgccaac
1621 taagctaaat gccatctcgg ttctttactt tgatgacaac tccaatgtca ttctgaaaaa
1681 atacaggaat atggttgtaa gagcttggtg atgccactaa ctcgaaacca gatgctgggg
1741 acacacattc tgccttggtt tcctagatta catctgcctt aaaaaaacac ggaagcacag
1801 ttggagggtg gacgatgaga ctttgaaaact atctcatgcc agtgccctat taccaggaa
1861 gattttaaag gacctatta ataatttgct cacttggtta atgacgtgag tagttgttgg
1921 tctgtagcaa gctgagtttg gatgtctgta gcataaggtc tggtaactgc agaaacataa
1981 ccgtgaagct ctctctaccc tcctccccc aaaaaccacc aaaattagtt ttagctgtag
2041 atcaagctat ttggggtgtt tgttagtaaa tagggaaaat aatctcaaag gagttaaatg
2101 tattcttggc taaaggatca gctgggtcag tactgtctat caaaggtaga ttttacagag
2161 aacagaaatc ggggaagtgg ggggaacgcc tctgttcagt tcattcccag aagtccacag
2221 gacgcacagc ccaggccaca gccagggtc cagggggcgc cctgtctca gtcattgctg
2281 ttgtatgttc gtgctggagt tttgttggtg tgaaaaataca cttatttcag ccaaaacata
2341 ccatttctac acctcaatcc tccatttgct gtactctttg ctagtaccaa aagtagactg
2401 attacactga ggtgaggcta caaggggtgt gtaaccgtgt aacacgtgaa ggcaatgctc
2461 acctcttctt taccagaacg gttctttgac cagcacatta acttctggac tgccggtctc
2521 agtacctttt cagtaaagtg gttctctgcc tttttactat acagcatacc acgccacagg
2581 gttagaacca acgaagaaaa taaaatgagg gtgcccagct tataagaatg gtgttagggg
2641 gatgagcatg ctgtttatga acggaatca tgatttccct tgtagaaagt gaggtcaga
2701 ttaaatttta gaatatattc taaatgtctt tttcacaatc atgtactggg aaggcaattt
2761 cataactaac tgattaaata atacatttat aatctacaac tgtttgactt tacagctttt
2821 tttgtaataa taaactataa tttattgtct attttatatac tgttttgctg taacattgaa
2881 ggaaagacca gacttttaaa aaaaaagagt ttatttagaa agtatcatag tgtaacaaa
2941 caaattgtac cactttgatt ttcttggaat acaagactcg tgatgcaaag ctgaagtgtg
3001 gtgtacaaga ctcttgacag ttgtgcttct ctaggaggtt gggttttttt aaaaaaagaa
3061 ttatctgtga accatacgtg attaataaag atttccttta aggca

```

FIG. 14

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```
1  mpglgrraqw lcwwwglles ccgppplrpp lpaaaaaaag gqllgdggsp grteqppppsp
61  qsssgflyrr lktqekremq keilsvlglp hrprplhglq qpqppalrqq eeqqqqqqqlp
121 rgepppgrrk saplfmldly nalsadnded gasegerqqq wpheaasssq rrqpppgaah
181 plnrksllap gsgsggaspl tsaqdsafln dadmvmsfvn lveydkefsp qqrhhkefkf
241 nlsqiwegev vtaafriyk dcvmgsfknq tflisiyqvl gehqhrdsdl flldtrvvwa
301 seegwlefdi tatsnlwvvt pqhnmglqls vvtrdgvhvh praaglvgrd gpydkqpfmv
361 affkvsevhv rttrsassrr rqqsrnrstq sqdvarvssa sdynsselkt acrkhelyvs
421 fqdlgwqdw iapkgyaany cdgecsfpln ahmnatnhai vqtlvhlmp eyvpkpccap
481 tklnaisvly fddnsnvilk kyrnmvvrac gch
```

FIG. 15

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```
1  mpgqelrtvn  gsmllvllv  lswlphggal  slaeasrasf  pgpselhsed  srfrelrkry
61  edlltrlrn  qswedsntdl  vpapavrilt  pevrlgsggh  lhlrisraal  peglpeasrl
121 hralfrlspt  asrswdvtrp  lrrqlslarp  qapalhlrls  pppsqsqdll  aesssarpql
181 elhlrpqaar  grrrararng  dhcplgpgrc  crlhtvrasl  edlgwadwvl  sprevqvtmc
241 igacpsqfra  anmhaqikts  lhrlkpdtvp  apccvpasyn  pmvliqktdt  gvslqtyddl
301 lakdchci
```

FIG. 16

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```
1 agtcccagct cagagccgca acctgcacag ccatgcccgg gcaagaactc aggacggtga
61 atggctctca gatgctcctg gtgttgctgg tgctctcgtg gctgccgcat gggggcgccc
121 tgtctctggc cgaggcgagc cgcgcaagtt tcccgggacc ctgagagttg cactccgaag
181 actccagatt ccgagagttg cggaaacgct acgaggacct gctaaccagg ctgcgggcca
241 accagagctg ggaagattcg aacaccgacc tcgtcccggc ccctgcagtc cggataactca
301 cgccagaagt gcggtgga tccggcgggc acctgcacct gcgtatctct cgggcccgcc
361 ttcccagagg gctccccgag gcctcccgcc ttcaccgggc tctgttcagg ctgtccccga
421 cggcgtcaag gtcgtgggac gtgacacgac cgctgcggcg tcagctcagc cttgcaagac
481 cccaggcgcc cgcgctgcac ctgcgactgt cgccgccgcc gtgcgagtcg gaccaactgc
541 tggcagaatc ttcgtccgca cggccccagc tggagttgca cttgcggccg caagccgcca
601 gggggcgccg cagagcgctg gcgcgcaacg gggaccactg tccgctcggg cccgggcgtt
661 gctgccgtct gcacacggtc cgcgcgtcgc tggaagacct gggctgggcc gattgggtgc
721 tgtcgccacg ggaggtgcaa gtgacctgt gcacgcggcg gtgcccagac cagttccggg
781 cggcaaacat gcacgcgcag atcaagacga gcctgcaccg cctgaagccc gacacggtgc
841 cagcgccctg ctgcgtgccc gccagctaca atcccatggt gctcattcaa aagaccgaca
901 ccggggtgtc gctccagacc tatgatgact tgtagccaa agactgccac tgcatatgag
961 cagtccctgg ccttccactg tgcacctgcg cgaggagcgc gacctcagtt gtccctgcct
1021 gtggaatggg ctcaagggtc ctgagacacc cgattcctgc ccaaacagct gtatttatat
1081 aagtctgtta ttattatta atttattggg gtgaccttct tggggactcg ggggctggtc
1141 tgatggaact gtgtatttat ttaaaactct ggtgataaaa ataaagctgt ctgaactgtt
1201 aaaaaaaaaa aaaaaaaaaa
```

FIG. 17

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1 mhahclpfll hawwallqag aatvatallr trgqpsspsp laymlslyrd plpradiirs  
61 lqaedvavdg qnwtfafdfs flsqqedlaw aelrlqlssp vdlptegsla ieifhqpkpd  
121 teqasdscl e rfqmdlftvt lsqvtfslgs mvlevtrpls kwlkhpgale kqmsrvagec  
181 wprpptppat nvllmlysnl sqeqrqlggs tllweaessw raqegqlswe wgkrhrrhhl  
241 pdrsqlcrkv kfqvdfnlig wgswwiypkq ynayrcegec pnpvgeefhp tnhayiqsll  
301 kryqphrvps tccapvktkp lsmlyvdngr vlldhhdmi veecgcl

FIG. 18

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```

1  ataagggctg gaggtgctgc tttcaggcct ggccagccca ccatgcacgc ccactgcctg
61  cccttccttc tgcacgcctg gtgggcccta ctccaggcgg gtgctgcgac ggtggccact
121 ggcctcctgc gtacgcgggg gcagccctcg tcgccatccc ctctggcgta catgctgagc
181 ctctaccgag acccgctgcc gagggcagac atcatccgca gcctacaggc agaagatgtg
241 gcagtggatg ggcagaactg gacgtttgct tttgacttct ccttcctgag ccaacaagag
301 gatctggcat gggctgagct ccggctgcag ctgtccagcc ctgtggacct cccactgag
361 ggctcacttg ccattgagat tttccaccag ccaaagcccg acacagagca ggcttcagac
421 agctgcttag agcggtttca gatggacctt ttactgttca ctttgtccca ggtcaccttt
481 tccttgggca gcatggtttt ggaggtgacc aggcctctct ccaagtggct gaagcaccct
541 gggggccctg agaagcagat gtccagggtg gctggagagt gctggccgag gccccccaca
601 ccgcctgcca ccaatgtgct ccttatgctc tactccaacc tctcgagga gcagaggcag
661 ctgggtgggt ccaccttgct gtgggaagcc gagagctcct ggcgggccca ggagggacag
721 ctgtcctggg agtggggcaa gaggcaccgt cgacatcact tgccagacag aagtcaactg
781 tgtcggaagg tcaagttcca ggtggacttc aacctgatcg gatggggctc ctggatcatc
841 taccccaagc agtacaacgc ctatcgctgt gagggcgagt gtcctaattc tgttggggag
901 gagtttcatc cgaccaacca tgcatacatc cagagtctgc tgaaacgtta ccagccccac
961 cgagtccctt ccacttggtg tgccccagtg aagaccaagc cgctgagcat gctgtatgtg
1021 gataatggca gagtgctcct agatcaccat aaagacatga tcgtggaaga atgtgggtgc
1081 ctctgatgac atcctggagg gagactggat ttgctgacac tctggaaggc tgggaaactc
1141 ctggaagaca tgataaccat ctaatccagt aaggagaaac agagaggggc aaagtgtgctc
1201 tgcccaccag aactgaagag gaggggctgc ccactctgta aatgaagggc tcagtggagt
1261 ctggccaagc acagaggctg ctgtcaggaa gagggaggaa gaagcctgtg cagggggctg
1321 gctggatggt ctctttactg aaaagacagt ggcaaggaaa agcacaagtg catgagttct
1381 ttactggatt ttttaaaaac ctgtgaaccc cccgaaactg tatgtgaaag ttgagacata
1441 tgtgcatgta ttttggagggt gggatgaagt cacctatagc tttcatgtat tctccaaagt
1501 agtctgtgtg tgacctgtcc ccctccccaag atattaagga tcaactgtata gattaaaaag
1561 agtccgtcaa tctcattgcc tcaggctggg ttggggggagc cccacagctt tctggctggc
1621 cagtggcaat ctactggcct tgtccagagg ctcaactggg tggttctctg ctaatgagct
1681 gtacaacaat aaagccattg tctagtcttc ctgggccagc tgggtgcctgt gaaggcagag
1741 gcaggaaactc atccaagagg accggccatg ttgggttaca gaagacatcc ctgcgtcagt
1801 ctgcttcggc agacacagcc tgagtgtgtt aaagtgtgtg acaatccacc tcagtctctc
1861 aatgtgtgct attaattgagg cctctgagct tcctatccag cagtgggtgaa ggccttgccc
1921 tgggtggcaa gatacttgct ctatggtcac agctcagcca ctggaagctg tgcgacctca
1981 ggtgagcaat tcaactgtcca gtctccactt gtaaaaggaa cgctgggtgaa tcctaattgca
2041 ttcataattaa atgtctgttg tcaggctcag aagagccatg agcttt

```

FIG. 19

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1 mlrflpdlaf sflililalgq avqfqeyvfl qflgldkaps pqkfqpvpvyi lkkifqdrea  
61 aattgvsrdl cyvkelgvrg nvlrflpdqg fflypkkisq assclqkllly fnlsaikere  
121 qltlaqlgld lgpnsyyngl pelelalflv qephvwgqtt pkpgkmfvlr svpwpqgavh  
181 fnlldvakdw ndnprknfgl fleilvkedr dsgvnfqped tcarlrcslh asllvvtlnp  
241 dqchpsrkr r aaipvpklsc knlchrhqlf infrdlgwhk wiiapkgfma nychgecpfs  
301 ltisl nssny afmqalmhav dpeipqavci ptklspisml yqdndndvil rhyedmvvde  
361 cgcg

FIG. 20

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```
1 ggagctctcc ccggtctgac agccactcca gaggccatgc ttcgtttctt gccagatttg
61 gctttcagct tcctgttaat tctggctttg ggccaggcag tccaatttca agaatatgtc
121 tttctccaat ttctgggctt agataaggcg ccttcacccc agaagttcca acctgtgcct
181 tatactcttga agaaaatttt ccaggatcgc gaggcagcag cgaccactgg ggtctcccga
241 gacttatgct acgtaaagga gctgggcgctc cgcggaatg tacttcgctt tctcccagac
301 caaggtttct ttctttaccc aaagaaaatt tccaagctt cctcctgcct gcagaagctc
361 ctctacttta acctgtctgc catcaaagaa agggaaacagt tgacattggc ccagctgggc
421 ctggacttgg ggcccaattc ttactataac ctgggaccag agctggaact ggctctgttc
481 ctgggttcagg agcctcatgt gtggggccag accacccta agccaggtaa aatgtttgtg
541 ttgcggtcag tcccatggcc acaagggtgct gttcacttca acctgctgga tgtagctaag
601 gattggaatg acaacccccg gaaaaatttc gggttattcc tggagatact ggtcaaagaa
661 gatagagact caggggtgaa ttttcagcct gaagacacct gtgccagact aagatgctcc
721 cttcatgctt ccctgctggt ggtgactctc aaccctgatc agtgccaccc ttctcgaaa
781 aggagagcag ccatccctgt cccaagctt tcttgtaaga acctctgcca ccgtcaccag
841 ctattcatta acttccggga cctgggttgg cacaagtgga tcattgcccc caaggggttc
901 atggcaaatt actgccatgg agagtgtccc ttctcactga ccctctctct caacagctcc
961 aattatgctt tcatgcaagc cctgatgcat gccgttgacc cagagatccc ccaggctgtg
1021 tgtatcccca ccaagctgtc tcccatttcc atgctctacc aggacaataa tgacaatgtc
1081 attctacgac attatgaaga catggtagtc gatgaatgtg ggtgtgggta ggatgtcaga
1141 aatgggaata gaaggagtgt tcttagggta aatcttttaa taaaactacc tatctgggtt
1201 atgaccactt agatcgaaat gtca
```

FIG. 21

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1 magasrllfl wlgcfcvsla qgerpkppfp elrkavpgdr tagggpdsel qpqdkvsehm  
61 lrlydrystv qaartpgsle ggsqpwrprl lregntvrsf raaaaetler kglyifnlts  
121 ltksenilsa tlyfcigelg nislscpvsg gcshhaqrkh iqidlsawtl kfsrnqsqll  
181 ghlsvdmaks hrdimswlsk ditqllrkak eneeffligfn itskgrqlpk rrlpfpepyi  
241 lvyandaais epesvvsslq ghrnfptgtv pkwdshiraa lsierrkkrs tgvllplqnn  
301 elpgaeyqyk kdevweerkp yktlqaqape ksknkkkqrk gphrksqtlq fdeqtlkkr  
361 rkqwieprnc arrylkvdfa digwsewiis pksfdayycs gacqfmpks lkpsnhatiq  
421 sivravgvvp gipepccvpe kmsslsilff denknvvlkv ypnmtvesca cr

FIG. 22

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```

1  agatccttgaa aacacccggg ccacacacgc cgcgacctac agctctttct cagcgttgga
61  gtggagacgg cgcccgacgc gccctgcgcg ggtgaggtcc gcgcagctgc tggggaagag
121  cccacctgtc aggtctgcgt gggtcagcgc agcaagtggg gctggccgct atctcgtgc
181  acccgggcgc gtcccgggct ccgtgcgccc tcgccccagc tggtttgag ttcaaccctc
241  ggctccgccc ccggtcctt gcgccttcgg agtgtcccgc agcgacgccg ggagccgacg
301  cgccgcgcgg gtacctagcc atggctgggg cgagcaggct gctctttctg tggctgggct
361  gcttctgcgt gagcctggcg cagggagaga gaccgaagcc acctttcccg gagctccgca
421  aagctgtgcc aggtgaccgc acggcagggt gtggcccggg ctccgagctg cagccgcaag
481  acaaggtctc tgaacacatg ctgcggctct atgacaggta cagcacggct caggcgcccc
541  ggacaccggg ctccctggag ggaggctcgc agccctggcg ccctcggctc ctgcgcgaag
601  gcaacacggg tcgcagcttt cgggcggcag cagcagaaac tcttgaaaga aaaggactgt
661  atatcttcaa tctgacatcg ctaaccaagt ctgaaaacat tttgtctgcc aactgtatt
721  tctgtattgg agagctagga aacatcagcc tgagttgtcc agtgtctgga ggatgtccc
781  atcatgtctc gaggaacac attcagattg atctttctgc atggaccctc aaattcagca
841  gaaaccaaag tcaactcctt ggccatctgt cagtggatat ggccaaatct catcgagata
901  ttatgtcctg gctgtctaaa gatatactc aactcttgag gaaggccaaa gaaaatgaag
961  agttcctcat aggatttaac attacgtcca agggacgcca gctgccaaag aggaggttac
1021  cttttccaga gccttatatc ttggtatatg ccaatgatgc cgccatttct gagccagaaa
1081  gtgtggtatc aagcttacag ggacaccgga attttccac tggaactgtt cccaaatggg
1141  atagccacat cagagctgcc ctttccattg agcggaggaa gaagcgctct actggggtct
1201  tgctgcctct gcagaacaac gagcttcctg ggcagaata ccagtataaa aaggatgagg
1261  tgtgggagga gagaaagcct tacaagacc ttccaggctc ggccctgaa aagagtaaga
1321  ataaaaagaa acagagaaag gggcctcatc ggaagagcca gacgtccaa tttgatgagc
1381  agaccctgaa aaaggcaagg agaaagcagt ggattgaacc tcggaattgc gccaggagat
1441  acctcaagg agactttgca gatattggct ggagtgaatg gattatctcc cccaagtctt
1501  ttgatgccta ttattgctct ggagcatgcc agttcccat gccaaagtct ttgaagccat
1561  caaatcatgc taccatccag agtatagtga gagctgtggg ggtcgttctt gggattcctg
1621  agccttgctg tgtaccagaa aagatgtcct cactcagtat tttattcttt gatgaaaata
1681  agaatgtagt gcttaaagta taccctaaca tgacagtaga gtcttgcgct tgcagataac
1741  ctggcaaaga actcatttga atgcttaatt caatcattag tttattttta tggacttctt
1801  cctgtttttt ttttttttt ttttgcactg ccaatgcatt ttgtttcaaa agattatttc
1861  tatagtcaga ggggaatgag caaatagact gaagattgcc accaaggaaa agaactgtat
1921  ttgtttctga atgtaactta aagcaagatt tttagtaa atggacatct atttctcttt
1981  ttgtaatcaa acacaacaac ttatcaaact gtttttagaa ctgttagaga acacactggg
2041  ttatttttgt aatgttcttt gaaaacagaa tggagaagca gcaatagctt gtcatttatc
2101  tcatttaatg actaatggga aatagagaac aatttcgcgt tttgaattag gcttattgcc
2161  ttagaatcct gagaaagtgc taaataatca actctgatgt tttcttaag ttcttgagac
2221  tcttgtttat ccttgttttt cctccacaag tcattgtcta agtgtaatgg aaagtttatg
2281  ctgagcggtt gtgtgtatgt atgtgcgtac atgcgccagg tgcctgtgcc ctctgtagga
2341  tggtttgctt aatatgggtt tataattcag ttacacagg attctttatt tttttaatt
2401  ttgtattttg gcaaacacca ttcagttata agaactttgc caaatatgat agaataattc
2461  aagagcatat acagagagtt accacttgac ccagctattt aattgcaaat acagttgttt
2521  tcatttcatt tcctaccaga aaaaggaatc agaaacctag tttttgaaaa cacaagtgtt
2581  attcctcttt tgtacttctt tttcacaaat gcttttattt attctaaatt gaatttaaaa
2641  atccttctta aagccattaa ctctttaatt ctctgatat gcctttactt cctatgaagt
2701  tattggtaga tggtgaggcc caaaaactgg tagaatattg aagatcttct taaatgacca
2761  atttaaccat aaccaaatat tgaatatcat tcttcagtca catctaagtc aggcactttt
2821  tcacatagat cagggtcttt ggctcagtca cgaaatctac aagttagcaa agcttacaaa

```

Fig. 23A

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2881 acattatttcg tcaggtatgg gaatcaaata tagacacttg tttgtctttg tttccattt
2941 ctatgtgtca catacatata tgtgtcctct tataacttta gtcttcaaaa ttatttcaat
3001 atccttcttc tcactatatt tatttgtgtg atggaaatgc tttcaggccg tagatcattg
3061 ttggtgttaa tctgtggtta atcctcattt tagttccgtc ttatctgata cttagaaata
3121 tctcagccat tttggaggct gtgcagtatc agaagacgtg gagtttggtc tgtctctgcc
3181 tgtagctaata tatggtggtt cagtcattta ataaaatgtt tttgagcatc tattttgtga
3241 aaggcactgt gttacctgtg tgtctttagt gtcctcactg gtaaaatgaa gaggctggcc
3301 atgagctgga agggttaagt ttataattcc agctatttca caccgtctt ccttgaagga
3361 atgatagtga tagatataaa aacactgtaa gtccctcttt taataaacta aatgaaagaa
3421 catcctatac ttcgctgttt gtaaattagt atggcattcg ctttggttta agtggtattt
3481 tattgcaaac ccattaaaag aataactcat gaaaagaagc tctttgacac cttggggtac
3541 acaaatgttg gtgtgggtgt gtgttaattc tgtgagttag acacaccagt tctaaaaaaa
3601 atgagtgaag ttctgggtgcc tgagttacca tgctttcttc tagttcttac agtagcataa
3661 aattaaagat tcaaagttag atggaggata aaattacttt ttaatacatg ttctcaaaca
3721 tttgaaaata aaagtatatg atagaagggg gccagagtgt ggccaccatc ctgactgtac
3781 tgtttttcaa taaagaaaac tttttcattg gtagatttgg tgaaattcta aatttaggtt
3841 tttttctaga gctgtatcaa ccaaaacttc tggcaattcc cagtatcact tcttagcctt
3901 cttatatcca aatgcctgtt tattaccttt cttaatttga atcaatgcct agttattaca
3961 gattgcaccc cacaatggcc aaaaaccacac tacataataa aatttacagg tactaactag
4021 ttaagattat attttaagta gcaattgata taaaattaca acacaatgaa agaacttggg
4081 taatctctta gcaatggaaa taggttttaa ccagcagttt ttctgggtgc tttgtaacta
4141 tcattttact aatgaattga ggatgtatta tggtttaaat tggaagagtt ttattcccaa
4201 agaataaagc aagattatct ttcagtagta gagattgaag taaatgtatt aatattttaa
4261 ttaatacaga ttactaaga gtagttagaa aatttagtaa gtgcctgttt tacaaattgt
4321 taggtactag tttctgtata attcctacac agaagcttta gaaatctcct gatattaaat
4381 tattaaattg gcattcatga aaagagaagc tacaattata aactccattt gctaaatcat
4441 gcataatact ctctctctct cttccccacac aagtaatctc tctaccccat gcagtgtgca
4501 cacacacaca cacacagtca gttactgaaa aaaataattc tttttctttt tttttttaa
4561 tggagtttca ctcttgctgc ccaggctgga gtgcagtggc gtgatctcgg ccactgcaa
4621 cctccacctc ccaggttcaa gcagttctcc tgcctcagcc tcctgagtag ctgggattac
4681 aggtgtccgc caccatgcct ggctaatttt tttatttgat aaaaagaatt ctttttctca
4741 ataactgttc tcttgaattc aaattaaggg actgccaaag tcaattagaa tattttaaaa
4801 atactttgtt gtaacctgtg taaataatat acaatttaca ggatttggga ttgtagaact
4861 taaactggaa gactggattc ctcagatctc aggactataa cattccagat aaatttttac
4921 attccctttg ctgtatatata actgatgatc atttatatgt taagattttt taccttaata
4981 tttctgaata aaactcttat tgccatttta atattttcat aggcaatcaa atgtgagtaa
5041 tactgctaag agtctgattt attaaaaata tttgtataat tcattcagtt tagtttttca
5101 gtttagtctt tctgctttca cttttctctg tgctaacaag taactaatgt ctgggcattg
5161 acttcttatt gaatcaaagt tgggttaggc atagctatgc acacctgatg tgtaagatta
5221 aagaagagat taaataagaa atcttgggta agttggactt ttctgtatag ctcttttttc
5281 ctctgagttg tattttaatg tagtttataa gtgataaaat gatccttggt ttctaaaagc
5341 cagtccttcc cttcagcttt ccacagtttc tgtaaatgtt taatacttgt acagtcaatg
5401 gcaattttta atatatatat atatatataa tatgtatatg gaaaagggtc aaagatgctt
5461 ttaattttatt taatgactat tgccttccta taataataa tttcatcctt aattatgata
5521 atacttttag caagaaaaat tccttttttac tacagttttt agatgcaaaa tgcagtttgg
5581 ttcttttagtc aaatccactt agagggtata ttgcagttaa actgtgaagg atacttcact
5641 accaatgtat aagctttgtt gaatttgtat ctttttcttt cagtaatgaa aagctattca
5701 ttatacagta tggaaataaa aattgcttca ttga

```

FIG. 23B

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1 mahvpartsp gpgpqlllll lplfllllrd vagshrapaw salpaaadgl qgdrdlqrhp  
61 gdaaatlgps aqdmvavhnh rlyekysrqg arpgggntvr sfrarlevvd qkavyffnlt  
121 smqdsemilt atfhfysepp rwpralevlc kpraknasgr plplgpptrq hllfrslsqn  
181 tatqgllrga malappprgl wqakdispiv kaarrdgell lsaqldseer dpgvprpspy  
241 apyilvyand laisepnsva vtlqrydpfp agdpepraap nnsadprvrr aaqatgplqd  
301 nelpglderp prahaqfhk hqlwpspfra lkprpgrkdr rkkggevfma asqvldfdek  
361 tmqkarrkqw deprvcsrry lkvd fadigw newiispksf dayycagace fmpkivrps  
421 nhatiqsivr avgiipgipe pccvpdkmns lgvlfldenr nvvlkvypnm svdtcacr

FIG. 24

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```

1  gggccaggga  cgaccctgtc  agctgcagcc  ccagagggtcc  ggggcgcgca  gccgggtccc
61  ctcgagggcg  cagccggccg  ccccgccccg  cccctcgaag  cagccgggcc  gggcgcgcag
121  tgggctacaa  actttcgcgg  cgcgagtccg  ccaaggcagc  gcgccgactc  gggctcggct
181  cggctctgcg  ctgctccgga  cggctgtgac  cgctggccgg  gggctcgggc  cgccggtacc
241  cacggaccgc  gcgcccgggt  gcctgctccg  ctaagcccct  cgccccgcgc  ggacctcggg
301  atccagcgcc  ctgctgcccg  ggctctcccc  gcgcgcccta  ctgccgcgag  gtcagtccgc
361  agcctccggt  gcgccagcgc  tcgccttcct  cctcctggac  ttcggccctt  tgccgcctc
421  accacgccat  ggctcatgtc  cccgctcggg  ccagcccggg  acccggggcc  cagctgctgc
481  tgctgctgct  gccgttggtt  ctgctgttgc  tccgggatgt  ggccggcagc  cacagggcc
541  ccgcctggtc  cgcactgccc  gcggcccgcc  acggcctgca  gggggacagg  gatctccagc
601  ggcaccctgg  ggacgcggcc  gccacgttgg  gccccagcgc  ccaggacatg  gtcgctgtcc
661  acatgcacag  gctctatgag  aagtacagcc  ggcagggcgc  gcggccggga  gggggcaaca
721  cggctccgag  cttcagggcc  aggctggaag  tggtcgacca  gaaggccgtg  tatttcttca
781  acctgacttc  catgcaagac  tcggaaatga  tccttacggc  cactttccac  ttctactcag
841  agccgcctcg  gtggcctcga  gcgctcgagg  tgctatgcaa  gccgcggggc  aagaacgctt
901  caggccgccc  gctgcccctg  ggcccgccca  cacgccagca  cctgctcttc  cgcagcctct
961  cgcagaacac  ggccacacag  gggctaactc  gcggggccat  ggccctggcg  cccccaccgc
1021  ggggcctgtg  gcaggccaag  gacatctccc  ccatcgtcaa  ggcgggccgc  cggtatggcg
1081  agctgctcct  ctccgcccag  ctggattctg  aggagaggga  cccgggggtg  ccccgcccca
1141  gcccctatgc  gccctacatc  ctagtctatg  ccaacgatct  ggccatctcg  gagcccaaca
1201  gcgtggcagt  gacgtgcag  agatacgacc  cttccctgc  cgagacccc  gagccccgcg
1261  cagcccccaa  caactcagcg  gacccccgcg  tgcgccgagc  cgcgcaggcc  actgggcccc
1321  tccaggacaa  cgagctgccg  gggctggatg  agaggccgcc  gcgcgccac  gcacagcact
1381  tccacaagca  ccagctgtgg  cccagcccct  tccgggcgct  gaaaccccg  ccagggcgca
1441  aagaccgcag  gaagaagggc  caggaggtgt  tcatggccgc  ctgcaggtg  ctggactttg
1501  acgagaagac  gatgcagaaa  gcccggagga  agcagtggga  tgagccgagg  gtgtgctccc
1561  ggaggtacct  gaaggtggac  ttcgcagaca  tcggctggaa  tgaatggata  atctcaccca
1621  aatcttttga  tgcctactac  tgcgcgggag  catgtgagtt  ccccatgcct  aagatcggtc
1681  gtccatccaa  ccatgccacc  atccagagca  ttgtcagggc  tgtgggcatc  atccctggca
1741  tcccagagcc  ctgctgtgtt  cccgataaga  tgaactccct  tggggtcctc  ttcttggtg
1801  agaatcggaa  tgtggttctg  aaggtgtacc  ccaacatgtc  cgtggacacc  tgtgcctgcc
1861  ggtgagacca  ctccagggtg  gaaagaagcc  acgcccagca  gagctgcctt  ctccgagcct
1921  tctgcaacca  ggacttgtgg  tgcagctgca  gacacagagc  acagctcatg  ggcaacatca
1981  ctggggccca  gagagagctg  tccgccagtg  catcattagg  gggctcttca  ttgctagtga
2041  ctagcccctt  aaatgccagc  ctgagtacct  gaaggaaatc  gggaattagc  cctggcctga
2101  aagtggccca  tcattcatac  ccactgttct  gaaggcttga  aaacaaaaca  tatccacaac
2161  attggcttga  tgtgatcatc  atctcataac  tgagcaagaa  gactatgcaa  atcttagggc
2221  gctcgctccc  tgcacacgga  aagaactctg  tttaaatgct  cagttcagaa  cactttgggc
2281  cacatagtga  ttttgaaaaa  caggataatc  gtggtgtaaa  tgagtgtttc  ctttcaaagt
2341  ccactgcaga  gcttttatcc  atatggtatg  cacatgtagc  caatattggt  ttctttttct
2401  taatatatat  attttatttt  aaaacaacaa  aaaggagggg  cgttgacacc  attccccaca
2461  gagatagtca  tgctgagtgt  gggttgttta  aacatgcata  ttgaaataac  acatatagta
2521  acgtgggaat  actaaaaaat  aaccaagatt  ttatatTTTT  gtaaattata  ctttctatac
2581  tgtagattgt  gtatgttatg  tgTTTTtatg  gaaagctaata  aaattaaagg  tacagtggta
2641  tcttgaaaaa  aaaaaaaa

```

FIG. 25

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```
1 mcpgalwval pllsllagsl qgkplqswgr gsaggnahsp lgvpggglpe htfnlkmfle
61 nvkvdfllrsl nlsgvpsqdk trveppqymi dlynrytsdk sttpasnivr sfsmedaisi
121 tatedfpfqk hillfnisip rheqitrael rlyvscqnhv dpshdlkgsv viydvldgtd
181 awdsatetkt flvsqdiqde gwetlevssa vkrwvrsdst ksknklevtv eshrkgcdtl
241 disvppgsrn lpffvvfsnd hssgtketrl elremisheq esvlkklskd gsteagessh
301 eedtdghvaa gstlarrkrs agagshcqkt slrvnfedig wdswwiapke yeayeckggc
361 ffpladdvtp tkhaivqtlv hlkfptkvgk accvptklsp isvlykddmg vptlkyhyeg
421 msvaecgcr
```

FIG. 26

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```

1  aagcacaagt  ggaggacaat  ccagcccggc  agcgggtgag  agtgggtgct  ggccaggacg
61  gttccttcag  agcaaacagc  agggagatgc  cggcccgcctc  cttccagct  cctccccgtg
121  cccgctaaca  cagcacggcc  gcctgcagtc  tcctctctgg  gtgattgcgc  gggcctaaga
181  tgtgtcctgg  ggcactgtgg  gtggccctgc  ccctgctgtc  cctgctggct  ggctccctac
241  aggggaagcc  actgcagagc  tggggacgag  ggtctgctgg  gggaaacgcc  cacagcccac
301  tgggggtgcc  tggaggtggg  ctgcctgagc  acaccttcaa  cctgaagatg  tttctggaga
361  acgtgaaggt  ggatttcctg  cgcagcctta  acctgagtgg  ggtcccttcg  caggacaaaa
421  ccagggtgga  gccgccgcag  tacatgattg  acctgtacaa  caggtagacg  tccgataagt
481  cgactacgcc  agcgtccaac  attgtgcgga  gcttcagcat  ggaagatgcc  atctccataa
541  ctgccacaga  ggacttcccc  ttccagaagc  acatcttgct  cttcaacatc  tccattccta
601  ggcattgagc  gatcaccaga  gctgagctcc  gactctatgt  ctctgtcaa  aatcacgtgg
661  acccctctca  tgacctgaaa  ggaagcgtgg  tcatttatga  tgttctggat  ggaacagatg
721  cctgggatag  tgctacagag  accaagacct  tcctggtgtc  ccaggacatt  caggatgagg
781  gctgggagac  cttggaagtg  tccagcgccg  tgaagcgctg  ggtccggtcc  gactccacca
841  agagcaaaaa  taagctggaa  gtgactgtgg  agagccacag  gaagggctgc  gacacgctgg
901  acatcagtgt  cccccaggt  tccagaaacc  tgcccttctt  tgttgtcttc  tccaatgacc
961  acagcagtg  gaccaaggag  accaggctgg  agctgaggga  gatgatcagc  catgaacaag
1021  agagcgtgct  caagaagctg  tccaaggacg  gctccacaga  ggcaggtag  agcagtcacg
1081  aggaggacac  ggatggccac  gtggctgcgg  ggtcgacttt  agccaggcgg  aaaaggagcg
1141  ccggggctgg  cagccactgt  caaaagacct  ccctgcgggt  aaacttcgag  gacatcggct
1201  gggacagctg  gatcattgca  cccaaggagt  atgaagccta  cgagtgtgag  ggcggctgct
1261  tcttccccct  ggctgacgat  gtgacgccga  cgaaacacgc  tatcgtgcag  accctgggtg
1321  atctcaagtt  cccacaaaag  gtgggcaagg  cctgctgtgt  gccaccaa  ctgagcccca
1381  tctccgtcct  ctacaaggat  gacatggggg  tgcccaccct  caagtacat  tacgagggca
1441  tgagcgtggc  agagtgtggg  tgcaggtagt  atctgcctgc  ggggctggg  aggcaggcca
1501  aaggggctcc  acatgagagg  tcctgcatgc  ccctgggcac  aacaaggact  gattcaatct
1561  gcatgccagc  ctggaggagg  aaaggagacc  tgctctccct  cccacaccc  caccaaaagc
1621  atacaccgct  gagctcaact  gccagggaag  gctaaggaaa  tggggatttg  agcacaacag
1681  gaaagcctgg  gagggttgtt  gggatgcaag  gaggtgatga  aaaggagaca  gggggaaaaa
1741  taatccatag  tcagcagaaa  acaacagcag  tgagccagag  gagcacaggc  gggcagggtc
1801  ctgcagagac  tgatggaagt  tagagaggtg  gaggaggcca  gctcgctcca  aaacccttgg
1861  ggagtagagg  gaaggagcag  gccgcgtgtc  acaccatca  ttgtatgtta  tttcccacaa
1921  ccagttgga  ggggcatggc  ttccaattta  gagac

```

FIG. 27

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1 mgslvltlca lfclaaylvs gspimnleqs pleedmslfg dvfseqdgvd fntllqsmkd  
61 eflktlnlsd iptqdsakvd ppeymlelyn kfatdrtsmp saniirsfkn edlfsqpvsf  
121 nglrkypllf nvsiphheev imaelrlytl vqrdrmiydg vdrkitifev leskgdnege  
181 rnmlvlvsge iygtnewet fdvtdairrw qksgssthql evhieskhde aedassgrle  
241 idtsaqnkhn pllivfsddq ssdkerkeel nemisheqlp eldnlgl dsf ssgpgeeall  
301 qmrsniiyds tarirrnakg nyckrtplyi dfkeigwds w iiappgyeay ecr gvcnypl  
361 aehltpkha iiqalvhlkn sqkaskaccv ptklepisil yldkgvvtyk fkyegmavse  
421 cgcr

FIG. 28

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```

1  ggggagagga agagtggtag ggggagggag agagagagga agagtttcca aacttgtctc
61  cagtgcacagg agacatttac gttccacaag ataaaactgc cacttagagc ccaggggaagc
121 taaaccttcc tggcttggcc taggagctcg agcggagtca tgggctctct ggtcctgaca
181 ctgtgcgctc ttttctgcct ggcagcttac ttggtttctg gcagcccat catgaaccta
241 gagcagtctc ctctggaaga agatatgtcc ctctttggtg atgttttctc agagcaagac
301 ggtgtcgact ttaacacact gctccagagc atgaaggatg agtttcttaa gacactaaac
361 ctctctgaca tccccacgca ggattcagcc aaggtggacc caccagagta catgttggaa
421 ctctacaaca aatttgcaac agatcggacc tccatgccct ctgccaacat cattaggagt
481 ttcaagaatg aagatctgtt ttcccagccg gtcagtttta atgggctccg aaaatacccc
541 ctctcttca atgtgtccat tcctcaccat gaagaggatca tcatggctga acttaggcta
601 tacacactgg tgcaaaggga tcgtatgata tacgatggag tagaccgaa aattaccatt
661 tttgaagtgc tggagagcaa aggggataat gagggagaaa gaaacatgct ggtcttggtg
721 tctggggaga tatatggaac caacagtga tgggagactt ttgatgtcac agatgccatc
781 agacgttggc aaaagtcagg ctcatccacc caccagctgg aggtccacat tgagagcaaa
841 cacgatgaag ctgaggatgc cagcagtgga cggctagaaa tagataccag tgcccagaat
901 aagcataacc ctttgctcat cgtgttttct gatgaccaa gcagtgacaa ggagaggaag
961 gaggaactga atgaaatgat ttcccatgag caacttccag agctggacaa cttgggcctg
1021 gatagctttt ccagtggacc tggggaagag gctttgttgc agatgagatc aaacatcatc
1081 tatgactcca ctgcccgaat cagaaggaac gccaaaggaa actactgtaa gaggaccccg
1141 ctctacatcg acttcaagga gattgggtgg gactcctgga tcatcgctcc gcctggatac
1201 gaagcctatg aatgccgtgg tgtttgtaac taccctctgg cagagcatct cacaccaca
1261 aagcatgcaa ttatccaggc cttggtccac ctcaagaatt ccagaaaagc ttccaaagcc
1321 tgctgtgtgc ccacaaagct agagcccatc tccatcctct atttagacaa aggcgtcgtc
1381 acctacaagt ttaaatacga aggcattggc gtctccgaat gtggctgtag atagaagaag
1441 agtcctatgg cttatttaat aactgtaaat gtgtatatat ggtgttccta tttaatgaga
1501 ttatttaata aggtgtgata gtaatagagg cttgctgcct tcaggaaatg gacaggtcag
1561 tttgttgtag gaaatgcata tttt

```

FIG. 29

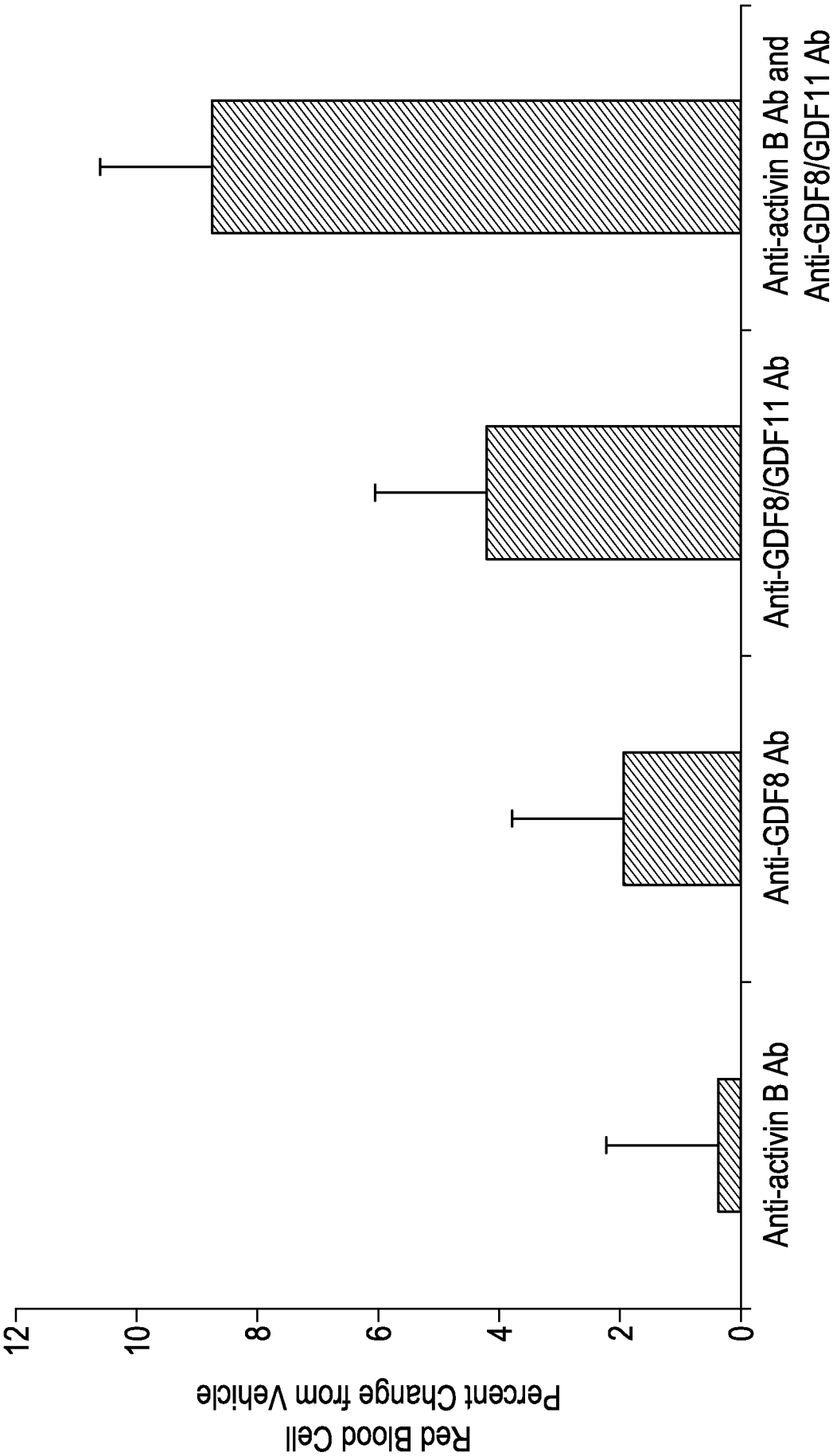


FIG. 30