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(54) Title: NOVEL INHIBIN VARIANTS

(57) Abstract: This invention relates to polypeptides, including variants of inhibin, and related therapeutics and compositions. Further, the invention relates to the use in the treatment or prevention of conditions associated with reduced levels of inhibin-mediated signalling. Specifically, the invention relates to inhibins and inhibin subunits with one or more mutations of modifications to increase the activity and/or reduce the formation of activin.



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Novel inhibin variants

Field of the invention

This invention relates to polypeptides, including variants of inhibin, and related therapeutics and compositions. Further, the invention relates to the use in the treatment
5 or prevention of conditions associated with reduced levels of inhibin-mediated signalling.

Cross-reference to related application

This application claims priority from Australian provisional application AU 2020901684, the entire contents of which are hereby incorporated by reference.

10 Background of the invention

Inhibin A and B are unique members of the transforming growth factor- β (TGF- β) superfamily as they: (i) are heterodimers composed of α - and β (β_A or β_B)-subunits, whereas most other family members are homodimers; (ii) act as antagonists, rather than agonists, inhibiting signalling of activin-related proteins; and (iii) function in an
15 endocrine rather than autocrine/paracrine manner. These aspects of inhibin biology are crucial for suppressing the release of follicle stimulating hormone (FSH) by gonadotrope cells of the anterior pituitary, but they also endow these hormones with the potential to regulate additional physiological processes.

In females, inhibin A (α/β_A) and inhibin B (α/β_B) heterodimers are produced in a
20 discordant manner across the menstrual cycle from granulosa cells within developing ovarian follicles. In males of most species, only the inhibin B form is produced continuously by the Sertoli cells of the testis. The granulosa and Sertoli cells transcribe the inhibin α -subunit in response to FSH stimulation. The inhibin α - and both β -subunits are initially synthesised as extended polypeptides, each comprising an N-terminal
25 prodomain and C-terminal mature domain. Following prodomain-guided inhibin α/β dimerisation, the prodomains are enzymatically cleaved by furin-like proprotein convertases. However, the prodomains remain non-covalently associated with the mature inhibin heterodimers, and appear to facilitate interactions with target receptors.

Inhibins lack signalling activity and function purely as antagonists of activin-mediated signalling. Typically, activins form a complex with type II (ActRIIA or ActRIIB) and type I (ALK4 or ALK7) receptors, which initiate an intracellular phosphorylation cascade that results in nuclear transfer of SMAD2/3 transcription factors. In the presence of co-receptors, inhibin A and B form inert complexes with ActRIIA/B and, thus, block activin receptor access and downregulate SMAD2/3-target genes, including *FSHB*. Early *in vitro* studies identified betaglycan as the obligate co-receptor for inhibin A and inhibin B's antagonism of activin. However, a recent study revealed that betaglycan was only required for inhibin A suppression of FSH secretion by pituitary gonadotrope cells. Inhibin B was functional even in the absence of betaglycan and, thus, likely acts through an as-yet unidentified co-receptor.

Aspects of inhibins' mode of action, including the widespread expression of inhibin receptors and the fact that activins target multiple tissues, presuppose functions beyond the negative regulation of FSH. Indeed, serum inhibin A and B levels correlate inversely with markers of bone formation and bone resorption in women across the menopause transition, and it has been proposed that these decreases in inhibin contribute to the initial bone loss during this period. A transgenic model of inducible human inhibin A expression has been used to test whether inhibin A could regulate bone mass *in vivo*. Inhibin A increased total body bone mineral density (BMD), increased bone volume and improved biomechanical properties at the proximal tibia of intact mice, and also prevented the loss of BMD and bone volume associated with gonadectomy. As activin A, one of the most highly expressed TGF- β proteins in bone, potently suppresses osteoblast differentiation, it is likely that inhibins' anabolic effect is *via* inhibition of this growth factor. Thus, gonadal inhibins are likely components of the normal endocrine repertoire that regulate bone quality, and the loss of inhibins at menopause may play a significant role in osteoporosis progression.

Efforts to delineate the biological roles for inhibin A and inhibin B beyond the pituitary have been largely constrained by activin interference. The shared requirement for the β -subunit means that in every cell that synthesises inhibins, there is an accompanied production of activins, which not only impedes the generation of pure inhibin A and B agonists, but also the study of inhibin physiology. Indeed, in inhibin (α -subunit) deficient mice, circulating activin levels increase more than 100-fold, triggering

tumorigenesis in the gonads and cachectic wasting from as early as 6 weeks of age. The elevated activin activity in this model has made it extremely difficult to study the physiological consequences of the loss of inhibin, as occurs in women at menopause. An alternative approach to identify inhibin target tissues has been to express inhibin A transgenically in healthy mice. Intriguingly, these experiments support that inhibin A activity extends beyond the regulation of pituitary FSH, and includes anabolic activity in bone. However, the physiological activities of inhibin B have been far less explored. Pure inhibin A and inhibin B agonists are needed to determine the full extent of the physiological roles of these distinct ligands.

Therefore there is a need to develop a new and/or improved system to generate inhibin A and inhibin B for clinical use.

Reference to any prior art in the specification is not an acknowledgment or suggestion that this prior art forms part of the common general knowledge in any jurisdiction or that this prior art could reasonably be expected to be understood, regarded as relevant, and/or combined with other pieces of prior art by a skilled person in the art.

Summary of the invention

In one aspect, the present invention provides an inhibin protein comprising an α -subunit and a β -subunit, wherein the α -subunit comprises a mutation or modification of a residue at, or at a position equivalent to, S344 (numbering as per SEQ ID NO: 1).

Preferably, the mutation or modification is any that increase the activity of the inhibin protein compared to an inhibin protein that does not contain a mutation or modification of a residue at, or at a position equivalent to, S344.

The β -subunit may be a β_A - or β_B -subunit, including any described herein. When a β_A -subunit is present the inhibin protein is an inhibin A protein. When a β_B -subunit is present the inhibin protein is an inhibin B protein.

As used herein, the inhibin protein may be a mammalian protein, preferably human. Typically, the amino acid sequence of the wildtype inhibin α -subunit is shown in SEQ ID NO: 1 comprising an N-terminal prodomain (SEQ ID NO: 2) and a C-terminal

mature domain (SEQ ID NO: 3). The wildtype inhibin α -subunit may be the C-terminal mature domain (SEQ ID NO: 3).

In one embodiment, the mutation is a replacement or substitution mutation with a non-conservative amino acid, more preferably the mutation is a replacement or substitution with an amino acid with a hydrophobic side chain. Amino acids with a hydrophobic side chain include alanine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine and valine. Preferably, the mutation is a replacement or substitution with isoleucine.

In this aspect, the inhibin protein may also contain one or more mutations or modifications in the α - and/or β -subunits, for example a mutation or modification in β -subunit at residues in the predicted β/β homodimerization interface required for formation of an activin. These sites include F326, Y345, A347, H369, I373, Y376 and V392 or equivalent position, in β_A -subunit (using the wild-type amino acid sequence in SEQ ID NO:4). Sites in β_B -subunit include F308, Y327, G329, H351, V355, Y358 and I373, or equivalent position (using the wild-type amino acid sequence in SEQ ID NO:7).

The one or more mutations or modifications may be at the interface site for homodimerization to occur for $\beta_A + \beta_A$ dimerization into activin A and $\beta_B + \beta_B$ dimerization into activin B. Such mutations or modifications reduce or inhibit altogether functional activin molecules being produced. Examples include A347X₁, Y345X₂, F326X₃, H369X₄, I373X₅, Y376X₆ and V392X₇, or equivalent position, wherein each of X₁, X₂, X₃, X₄, X₅, X₆ and X₇ is any amino acid except A, Y, F, H, I, Y and V, respectively (based on numbering in SEQ ID NO:4) in subunit β_A ; and F308X₈, Y327X₉, G329X₁₀, H351X₁₁, V355X₁₂, Y358X₁₃ and I373X₁₄ or equivalent position, wherein each of X₇, X₈, X₉, X₁₀, X₁₁, X₁₂, X₁₃ and X₁₄ is any amino acid except F, Y, G, H, V, Y and I, respectively (using numbering in SEQ ID NO:7).

Preferably, the mutations or modifications in the β_A subunit are made at one, two or three of F326, Y345, and A347, or equivalent positions. In one embodiment, the mutation is a replacement or substitution mutation with a non-conservative amino acid, more preferably the mutation is a replacement or substitution with an amino acid with a basic side chain. Preferably, the mutation at Y345 is to a glycine residue, i.e. the mutation is Y345G. Preferably, the mutation at A347 is to a phenylalanine, glycine,

histidine, glutamate or glutamine residue (i.e. A347F, A347G, A347H, A347E or A347Q). Most preferably, the mutation at A347 is A347F, A347G or A347H. A preferred double mutation is A347H + Y345G.

Preferably, the mutations or modifications in the β_B subunit are made at G329, or equivalent position. In one embodiment, the mutation is a replacement or substitution mutation with a non-conservative amino acid, more preferably the mutation is a replacement or substitution with an amino acid with a non-polar or acidic side chain. Preferably, the mutation at G329 is a leucine, valine, glutamate or aspartate (i.e. G329L, G329V, G329E or G329D). Most preferably, the mutation at G329 is to a glutamate or aspartate.

In any aspect, the inhibin protein β_A - or β_B -subunit may further comprise a FLAG tag comprising the amino acid sequence DYKDDDK (SEQ ID NO: 19) between amino acids 27 and 28 of β_A or 28 and 29 of β_B . The α -subunit may comprise a poly-histidine tag. These aid in purification of inhibin from conditioned medium by affinity chromatography as well as assist in distinguishing wild-type protein from modified protein.

In this aspect, the inhibin protein may comprise a heterodimer of α -subunit and β -subunit precursors each having at least one proprotein convertase cleavage site wherein at least one proprotein convertase cleavage site in the α -subunit and/or β -subunit is modified by an amino acid substitution mutation to render it more efficiently cleaved by the proprotein convertase. This enables the efficient generation of an inhibin in bioactive form. Thus, inhibin protein and its precursors as described herein are useful in the treatment of a range of diseases and conditions such as arising from reduced production of an inhibin or where antagonism of an activin would ameliorate a particular disease or condition. Reduced serum levels of inhibin in post-menopausal women, for example, can exacerbate bone disorders such as osteoporosis. Consequently, a condition is a bone disorder, preferably osteoporosis. Further, a subject in need thereof is someone with a bone disorder, preferably a post-menopausal woman having osteoporosis.

30

In an embodiment, a proprotein convertase cleavage site in each of the α -subunit and β -subunit precursors is modified by the amino acid substitution mutation. In an

embodiment, the modified proprotein convertase site is defined by the amino acid sequence set forth in SEQ ID NO: 21 (ISSRKRSVSS). The β -subunits may be β_A -subunit leading to an inhibin A analog or a β_B -subunit leading to an inhibin B analog when either subunit forms a heterodimer with the α -subunit.

5 The β -subunit of the inhibin protein may further comprise a single mutation within a type I receptor (ALK4) binding epitope of the mature domain leading to inactive activin. Activin is generally co-formed during the synthesis of inhibins and comprises β -subunit homodimers. The mutation in the β -subunit renders inactive any activin formed. A mutation at a homodimerization interface site further facilitates an absence of or
10 reduce amount of active activin. For the human β_A -subunit, the mutation includes a M418A (based on numbering in SEQ ID NO: 4) substitution mutation (numbering from start of prodomain; equivalent to M108A from start of the mature domain as shown in SEQ ID NO: 6). This means the resident methionine residue is replaced by an alanine residue. For the human β_B -subunit, the mutation includes a M399A substitution mutation
15 (from start of prodomain); numbering from SEQ ID NO: 7; equivalent to M107A from the start of the mature domain as shown in SEQ ID NO: 9.

 The modified proprotein convertase sites improve the efficiency of cleavage of the inhibin analog precursor protein relative to enzymatic cleavage of the wild-type protein. In addition, there is a concomitant reduction in bioactive activin. This is further
20 enhanced by the introduction of the mutation in the ALK4 binding epitope in the β -subunit mature domain and/or mutation at a site required for homodimerization of a β_A -subunit on β_B -subunit.

 In another aspect, the present invention provides an inhibin protein comprising an α -subunit and a β_A -subunit, wherein the β_A -subunit comprises one or more of the
25 following mutations F326A, F326G, F326L, F326Q, Y345A, A347E, A347F, A347G, and A347Q.

 In another aspect, the present invention provides an inhibin protein comprising an α -subunit and a β_A -subunit, wherein the β_A -subunit comprises one or more mutations or modifications at H369, I373 or Y376, or an equivalent position. Preferably, the β_A -
30 subunit comprises one or more of the following mutations H369X₄, I373X₅ and Y376X₆,

or equivalent position, wherein each of X_4 , X_5 and X_6 is any amino acid except H, I and Y, respectively (based on numbering in SEQ ID NO: 4).

In another aspect, the present invention provides an inhibin protein comprising an α -subunit and a β_B -subunit, wherein the β_B -subunit comprises one or more mutations or modifications at G329 or an equivalent position. In one embodiment, the mutation is a replacement or substitution mutation with a non-conservative amino acid, more preferably the mutation is a replacement or substitution with an amino acid with a non-polar or acidic side chain. Preferably, the mutation at G329 is to a leucine, valine, glutamate or aspartate residue (i.e. G329L, G329V, G329E or G329D). Most preferably, the mutation at G329 is to a glutamate or aspartate.

In another aspect, the present invention provides an inhibin protein comprising an α -subunit and a β_B -subunit, wherein the β_B -subunit comprises one or more mutations or modifications at H351, V355 or Y358, or an equivalent position. Preferably, the β_B -subunit comprises one or more of the following mutations H351 X_{11} , V355 X_{12} and Y358 X_{13} , or equivalent position, wherein each of X_{11} , X_{12} and X_{13} is any amino acid except H, V and Y, respectively (using numbering in SEQ ID NO: 7).

In another aspect, the present invention provides an inhibin protein α -subunit comprising a mutation or modification of a residue at, or at a position equivalent to, S344.

In this aspect, the inhibin protein α -subunit may further comprise:

(i) a modified proprotein convertase site ("super-cut site1" SEQ ID NO: 21);

optionally together with a poly-histidine tag to assist with purification

In another aspect, the present invention provides an inhibin protein β_A -subunit comprising any mutation or modification described herein that reduces or inhibits formation of activin A.

In this aspect, the inhibin protein β_A -subunit may further comprise a modified proprotein convertase site ("super-cut site"), optionally with a mutation disrupting the ALK4 binding epitope in the mature domain and optionally with a FLAG tag to assist in

affinity chromatography purification and optionally with a mutation disrupting homodimerization.

In another aspect, the present invention provides an inhibin protein β_B -subunit comprising any mutation or modification described herein that reduces or inhibits
5 formation of activin B.

In this aspect, the inhibin protein β_B -subunit may further comprise a modified proprotein convertase site ("super-cut site"), optionally with a mutation disrupting the ALK4 binding epitope in the mature domain and optionally with a FLAG tag to assist in affinity chromatography purification and optionally with a mutation disrupting
10 homodimerization.

In any aspect, the inhibin protein, or subunit thereof, may be of human or non-human mammalian origin. Hence, the inhibin protein, or subunit thereof, has therapeutic potential in humans and non-human mammals.

In any aspect, the inhibin protein α -subunit, may comprise an amino acid
15 sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with SEQ ID NO: 1 or 3, including any one or more of the mutations or modification as described herein.

In any aspect, the inhibin protein β_A -subunit, may comprise an amino acid
20 sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with SEQ ID NO: 4 or 6, including any one or more of the mutations or modification as described herein.

In any aspect, the inhibin protein β_B -subunit, may comprise an amino acid
25 sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with SEQ ID NO: 7 or 9, including any one or more of the mutations or modification as described herein.

In any aspect, the inhibin protein may be a heterodimer of an inhibin protein α -subunit and inhibin protein β_A -subunit as described herein (this heterodimer also referred to as inhibin protein A or inhibin A), or a may be a heterodimer of an inhibin protein α -subunit and inhibin protein β_B -subunit as described herein (this heterodimer also referred to as inhibin protein B or inhibin B).

In another aspect, the invention provides a pharmaceutical composition for treating or preventing a disease or condition arising from reduced production of an inhibin or where antagonism of an activin would ameliorate a particular disease or condition, the composition comprising an inhibin protein as described herein and a pharmaceutically acceptable diluent, excipient or carrier. In one embodiment, the only active ingredient present in the composition is an inhibin protein.

In another aspect, the invention provides a pharmaceutical composition for treating or preventing a disease or condition arising from reduced production of an inhibin or where antagonism of an activin would ameliorate a particular disease or condition, the composition comprising as an active ingredient an inhibin protein as described herein and a pharmaceutically acceptable diluent, excipient or carrier. In one embodiment, the only active ingredient present in the composition is an inhibin protein as described herein.

In another aspect, the invention provides a pharmaceutical composition for treating or preventing a disease or condition arising from reduced production of an inhibin or where antagonism of an activin would ameliorate a particular disease or condition, the composition comprising as a main ingredient an inhibin protein as described herein and a pharmaceutically acceptable diluent, excipient or carrier. In one embodiment, the only active ingredient present in the composition is an inhibin protein as described herein.

In another aspect, the invention also provides an inhibin protein as described herein in the treatment of an inflammatory disease or condition.

In another aspect, the invention also provides a pharmaceutical composition comprising an inhibin protein as described herein and a pharmaceutically acceptable diluent, excipient or carrier for use in the treatment or prevention of disease or condition

arising from reduced production of an inhibin or where antagonism of an activin would ameliorate a particular disease or condition.

In another aspect, the invention also provides a method of treating or preventing a disease or condition arising from reduced production of an inhibin or where antagonism of an activin would ameliorate a particular disease or condition in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an inhibin protein as described herein, or a pharmaceutical composition as described, therein thereby treating or preventing the disease or condition.

10 In another aspect, the invention also provides use of a therapeutically effective amount of a inhibin protein as described herein or pharmaceutical composition as described herein in the manufacture of a medicament for the treatment or prevention of a disease or condition arising from reduced production of an inhibin or where antagonism of an activin would ameliorate a particular disease or condition in a subject
15 in need thereof.

In another aspect, the present invention provides a method for the treatment of a disease or condition arising from reduced production of an inhibin or where antagonism of an activin would ameliorate a particular disease or condition in a subject, the method comprising the steps of

20 identifying a subject having a disease or condition arising from reduced production of an inhibin or where antagonism of an activin would ameliorate a particular disease or condition; and

administering to the subject in need thereof a therapeutically effective amount of an inhibin protein as described herein or pharmaceutical composition as described
25 herein,

thereby treating the disease or condition in the subject.

In another aspect, the invention also provides a nucleic acid molecule encoding an inhibin protein, α -subunit, β_A -subunit or β_B -subunit as described herein. The nucleic acid may be operably linked to a promoter and if necessary a terminator sequence or

other regulatory sequence. The nucleic acid molecule is used to transfect cells or cell lines to coproduce α - and either β_A - or β_B -subunits.

In another aspect, the invention also provides a vector comprising a nucleic acid molecule described herein.

5 In another aspect, the invention also provides a cell comprising a vector or nucleic acid molecule described herein. The cell may be a cell line, transiently or stably expressing the nucleic acid molecule. In an embodiment, an equal amount of α -subunit encoding nucleic acid and β -subunit encoding are transfected into the cell or cell line. In another embodiment, the ratio of α -subunit to β -subunit encoding nucleic acid is $\alpha > \beta$
10 including the ratio 3:2 ($\alpha:\beta$, respectively). In another embodiment, the ratio is $\alpha < \beta$ including 2:3 ($\alpha:\beta$, respectively).

In another aspect, the invention also provides an animal or tissue derived therefrom comprising a cell described herein.

In another aspect, the invention also provides a method for generating an inhibin
15 protein, the method comprising co-expressing in a cell or cell line a nucleic acid encoding the α -subunit and a β_A -subunit or β_B -subunit as defined herein for a time and under conditions sufficient for an inhibin precursor protein to be produced, cleaved by a proprotein convertase and secreted from the cell or cell line as an inhibin protein. This leads to the generation of inhibin A protein or inhibin B protein as described herein. In
20 an embodiment, minimal bioactive activin A or activin B is produced.

In any aspect, the subject in need of a treatment has reduced serum levels of inhibin compared to the level in a pre-menopausal healthy subject or has a disease or condition exacerbated by activin-mediated signaling.

As used herein, except where the context requires otherwise, the term
25 "comprise" and variations of the term, such as "comprising", "comprises" and "comprised", are not intended to exclude further additives, components, integers or steps. "Comprises" and "includes", and variations thereof, may be used interchangeably. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed
30 elements are required or mandatory, and that no other elements may be present. By

“consisting essentially of” is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase “consisting essentially of” indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

Further aspects of the present invention and further embodiments of the aspects described in the preceding paragraphs will become apparent from the following description, given by way of example and with reference to the accompanying drawings.

Brief description of the drawings

Figure 1. Effect of β_A -subunit mutations on inhibin/activin A production and activity. (A) Inhibin α - and β_A -subunit modifications were introduced via site-directed mutagenesis. (B) Variant inhibin α - and β_A -subunits were co-transfected into HEK293 cells and changes to processing and production of inhibin/activin were assessed by Western blot, relative to wild type (WT) forms, using α - (R1) and β_A -subunit (E4) specific antibodies, respectively. Band densitometry was performed to quantify changes in 31/34 kDa mature inhibin A (C) and 26 kDa mature activin A (D) formation, relative to WT (n=3, *p<0.05, **p<0.01, ***p<0.001). (E) Inhibin A activity measured by its ability to suppress activin A-induced luciferase response in a COV434 cell line (n=3). (F) Activin variant activity measured by way of luciferase response in a COV434 cell line (n=3). *p<0.05, **p<0.01, ***p<0.001.

Figure 2. Effect of β_B -subunit mutations on inhibin/activin B production and activity. (A) Inhibin α - and β_B -subunit modifications introduced via site-directed mutagenesis. (B, C) Variant inhibin α - and β_B -subunits were co-transfected into a mammalian HEK293T cell line and changes to processing and production of inhibin/activin B was assessed by Western blot, relative to the wild type (WT) forms, using α - (R1) and β_B -subunit (C5) specific antibodies, respectively. Band densitometry was performed to quantify changes in 31/34 kDa mature inhibin B (D) and 26 kDa mature activin B (E) formation, relative to WT (n=3, **p<0.01, ***p<0.001). (F) Inhibin B activity measured by its ability to suppress activin B-induced luciferase response in a

COV434 cell line. (G) Activin B variant activity measured by way of luciferase response in a COV434 cell line (n=3). *p<0.05, **p<0.01, ***p<0.001.

Figure 3. Effect of α -subunit mutations on inhibin A bioactivity. (A) Variant inhibin α - and β_A -subunits were co-transfected into a mammalian HEK293T cell line and changes to processing and production of inhibin A were assessed by Western blot, relative to the wild type form, using an α -specific antibody (R1). (B) Inhibin A variant activity measured by its ability to suppress activin A-induced luciferase response in a COV434 cell line.

Figure 4. Variant inhibin α - and β_A -subunits were co-transfected into a mammalian HEK293T cell line and changes to processing and production of inhibin/activin A were assessed by Western blot, relative to wild type (WT) forms, using a β_A -subunit specific antibody (E4).

Figure 5. Variant β_A -subunits were co-transfected into a mammalian HEK293T cell line and changes to processing and production of activin A was assessed by Western blot, relative to wild type (WT) forms, using a β_A -subunit specific antibody (E4). (B) Band densitometry was performed to quantify changes in 26 kDa mature activin B formation, relative to WT (n=3).

Detailed description of the embodiments

It will be understood that the invention disclosed and defined in this specification extends to all alternative combinations of two or more of the individual features mentioned or evident from the text or drawings. All of these different combinations constitute various alternative aspects of the invention.

Reference will now be made in detail to certain embodiments of the invention. While the invention will be described in conjunction with the embodiments, it will be understood that the intention is not to limit the invention to those embodiments. On the contrary, the invention is intended to cover all alternatives, modifications, and equivalents, which may be included within the scope of the present invention as defined by the claims.

One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present

invention. The present invention is in no way limited to the methods and materials described. It will be understood that the invention disclosed and defined in this specification extends to all alternative combinations of two or more of the individual features mentioned or evident from the text or drawings. All of these different
5 combinations constitute various alternative aspects of the invention.

All of the patents and publications referred to herein are incorporated by reference in their entirety.

For purposes of interpreting this specification, terms used in the singular will also include the plural and vice versa.

10 As used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a variant" or "a mutation" includes a single variant or mutation, as well as two or more variants or mutations; reference to "an inhibin" includes a single inhibin, as well as two or more types of inhibins; reference to "the disclosure" includes a single and
15 multiple aspects taught by the disclosure; and so forth. Aspects taught and enabled herein are encompassed by the term "invention". All such aspects are enabled within the width of the present invention. Any variants and derivatives contemplated herein are encompassed by "forms" of the invention.

Gonadal-derived inhibin A and inhibin B are components of the normal endocrine
20 repertoire that regulated bone quality, and the loss of inhibins at menopause likely contributes to the accompanying decrease in bone mass. Therefore, inhibins have been touted as potential therapeutics for osteoporosis in post-menopausal women as well as the treatment of male and female subjects having a disease or condition exacerbated by low levels of inhibins and/or activin-mediated signaling. However, as
25 heterodimeric proteins of α - and β - (β_A or β_B)-subunits, inhibins are difficult to produce recombinantly, they are poorly processed to their mature bioactive forms and their expression is always accompanied by production of activins (β -subunit homodimers).

An inhibin protein or subunit that contains one or more mutations or modifications may also be referred to as an "analog", "variant", "mutant" or "modified protein".

Reference to an "inhibin" or "inhibin protein" includes inhibin A comprising a heterodimer of an α -subunit and a β_A -subunit and inhibin B comprising a heterodimer of an α -subunit and a β_B -subunit. Unless otherwise specified, or the context indicates otherwise, reference to an "inhibin protein" may be a reference to an inhibin A protein or an inhibin B protein. An inhibin protein may be a precursor inhibin that comprises a heterodimer of an α -subunit and a β -subunit where those subunits have not undergone any cleavage, for example enzymatic cleavage. In this form, the α -subunits and β -subunits comprise at least the prodomain and mature subunit covalently associated, for example as a contiguous polypeptide. In this form the inhibin protein may also be referred to as an inhibin precursor protein. Alternatively, an inhibin protein may be a pro-mature inhibin that comprises a heterodimer of an α -subunit and a β -subunit where those subunits have undergone any cleavage, for example enzymatic cleavage, and where the cleavage releases the prodomains. In this form, the prodomains may be non-covalently associated with the α -subunit and β -subunit heterodimer. In this form, the inhibin protein may also be referred to as an inhibin pro-mature protein. An inhibin protein may be a mature inhibin that comprises a heterodimer of an α -subunit and a β -subunit where those subunits have undergone any cleavage, for example enzymatic cleavage, and where the cleavage releases the prodomains of each subunit. In this form, the prodomains are not non-covalently associated with the α -subunit and β -subunit heterodimer. In this form, the inhibin protein may also be referred to as a mature inhibin.

Unless otherwise specified, reference to an "inhibin protein α -subunit" or " α -subunit" includes an inhibin α -subunit precursor or a mature inhibin α -subunit. In other words, throughout the specification reference to "inhibin protein α -subunit" or " α -subunit" may be substituted with inhibin α -subunit precursor or a mature inhibin α -subunit.

Unless otherwise specified, reference to an "inhibin protein β_A -subunit" or " β_A -subunit" includes an inhibin β_A -subunit precursor or a mature inhibin β_A -subunit. In other words, throughout the specification reference to "inhibin protein β_A -subunit" or " β_A -subunit" may be substituted with inhibin β_A -subunit precursor or a mature inhibin β_A -subunit.

Unless otherwise specified, reference to an "inhibin protein β_B -subunit" or " β_B -subunit" includes an inhibin β_B -subunit precursor or a mature inhibin β_B -subunit. In other

words, throughout the specification reference to “inhibin protein β_B -subunit” or “ β_B -subunit” may be substituted with inhibin β_B -subunit precursor or a mature inhibin β_B -subunit.

The inhibin protein is of mammalian origin including a human or non-human
5 primate, a laboratory test animal such as a mouse, rat, rabbit, guinea pig or hamster, a farm animal such as a sheep, cow, pig, horse or deer or a companion animal such as a dog or cat. In an embodiment, the inhibin is of human origin. Reference hereinafter to amino acid positions in a human inhibin includes the equivalent position in a non-human inhibin.

10 Unless otherwise specified, numbering herein is made with reference to the wildtype human precursor amino acid sequence. Reference to a particular mutation or modification with reference to numbering of the precursor amino acid sequence should be taken as a disclosure of the same mutation or modification in the prodomain or mature domain as the case may be.

15 As used herein an amino acid residue at the position equivalent to position, for example in SEQ ID NOs: 1, 4 or 7, can be determined by any means known to a person skilled in the art. For example, an alignment of one or more sequences with an amino acid sequence of SEQ ID NO: 1 would allow a person skilled in the art to determine the amino acid at the position equivalent to position in SEQ ID NO: 1. A person skilled in the
20 art can compare the three dimensional structure or model of a protein with the three dimensional structure or model of a protein having the amino acid sequence of SEQ ID NO: 1 and determine the amino acid residue that is at an equivalent position to that in SEQ ID NO: 1.

In any aspect, the inhibin protein, or subunit, precursor protein does not contain a
25 signal sequence. Typically, the signal sequence is any one underlined in SEQ ID NOs: 1, 4 or 7. Therefore, in any aspect, the precursor protein, or subunit, precursor protein does not contain a signal sequence as shown in SEQ ID NOs: 1, 4 or 7.

As described herein, an inhibin protein with a mutation or modification in the α -subunit may exhibit an increased activity. Preferably, the activity, and reference to
30 inhibin activity anywhere herein, is the ability to suppress activin activity. An exemplary method to determine the ability of an inhibin protein to suppress activin activity, either

activin A and/or B activity, is the luciferase assay described herein using a COV434 cell line. The increased activity of the inhibin protein may be relative to an inhibin protein without the mutation(s) or modification(s). Preferably, the inhibin protein has an equal to, or greater than, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12-fold increase in activity compared to
5 an inhibin protein without the mutation(s) or modification(s).

As described herein, inhibin protein with one or more mutations or modifications in the β -subunit may exhibit one or more of the following properties: an improved processing efficiency resulting in a higher proportion of inhibin formation (or a reduced proportion of activin formation), exhibit enhanced α/β heterodimerization, and reduced
10 or silenced activin receptor binding or activation activity. Any one of these properties may be determined by methods known in the art, including exemplary methods described herein. Preferably, the mutation or modification in the β -subunit results in an equal to, or greater 50%, 55%, 60%, 65% 70%, 75%, 80%, 85%, 90%, 95%, or 100% reduction in the level of activin formation compared to a β -subunit that does not contain
15 the one or more mutations or modifications. Preferably, the mutation or modification does not result in any more than 1%, 5%, 10%, 15%, 20% or 25% reduction in inhibin yield or activity.

"Isolated," when used to describe the various polypeptides disclosed herein, means the polypeptide that has been identified and separated and/or recovered from a
20 component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or
25 internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated protein includes polypeptide *in situ* within recombinant cells, since at least one component of the polypeptide natural environment will not be present. Ordinarily, however, isolated polypeptide will be
30 prepared by at least one purification step.

A "fragment" is a portion of a polypeptide of the present invention that retains substantially similar functional activity or substantially the same biological function or activity as the polypeptide, which can be determined using assays described herein.

Any protein or subunit as described herein may be isolated, recombinant,
5 synthetic, purified or substantially purified. It may also contain one or more non-native amino acids.

The terms "polypeptide," "proteinaceous molecule," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues and to variants and synthetic analogues of the same. Thus, these terms apply to amino acid polymers
10 in which one or more amino acid residues is a synthetic non-naturally-occurring amino acid, such as a chemical analogue of a corresponding naturally-occurring amino acid, as well as to naturally-occurring amino acid polymers. These terms do not exclude modifications, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, proteins containing one or more analogs
15 of an amino acid including, for example, unnatural amino acids or polypeptides with substituted linkages.

As used herein, the terms "inhibit," "inhibiting" and the like are used interchangeably herein to refer to blocking, stopping, diminishing, reducing, impeding or impairing an activity, function or characteristic, in any setting including *in vivo*, *ex vivo* or
20 *in vitro*. By way of example, "inhibit" can refer to a decrease of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% in an activity, function or characteristic

The present invention provides an inhibin protein comprising an α -subunit and a β -subunit, wherein the α -subunit comprises a mutation or modification of a residue at, or at a position equivalent to, S344. Preferably, the mutation or modification is any that
25 increase the activity of the inhibin protein compared to an inhibin protein that does not contain a mutation or modification of a residue at, or at a position equivalent to, S344.

The β -subunit may be a β_A - or β_B -subunit, including any described herein. When a β_A -subunit is present the inhibin protein is an inhibin A protein. When a β_B -subunit is present the inhibin protein is an inhibin B protein.

The terms "wild-type" and "naturally occurring" are used interchangeably to refer to a gene or gene product that has the characteristics (e.g., nucleotide sequence, amino acid sequence, etc.) of that gene or gene product when isolated from a naturally occurring source. A wild type gene or gene product (e.g., a polypeptide) is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene. Typically, the amino acid sequence of the wildtype inhibin α -subunit is shown in SEQ ID NO: 1 comprising an N-terminal prodomain (SEQ ID NO: 2) and a C-terminal mature domain (SEQ ID NO: 3).

In one embodiment, the mutation is a replacement or substitution mutation with a non-conservative amino acid, more preferably the mutation is a replacement or substitution with an amino acid with a hydrophobic side chain. Amino acids with a hydrophobic side chain include alanine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine and valine. Preferably, the mutation is a replacement or substitution with isoleucine.

The inhibin α -subunit protein convertase cleavage site is at ²²⁹RARR²³² in human inhibin A (site 1). When modified, a more efficient proprotein cleavage site is substituted comprising ISSRKKRSVSS (SEQ ID NO: 21). At site 1 of α -subunit, the amino acid sequence is ²²⁹ISSRKKRSVSS²³⁹. The substituted cleavage site may further be modified by one or more amino acid substitutions, additions and/or deletions to the amino acid sequence set forth in SEQ ID NO: 21. Hence, the substituted proprotein cleavage site may comprise SEQ ID NO: 21 or an amino acid sequence having at least 80% similarity to SEQ ID NO: 21 after optimal alignment. Reference to "at least 80%" includes 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 and 100%, or a percentage in between.

The modification to change the cleavage site is also referred to herein as "super-cut" variant. Generally, the cleavage site is referred to as super-cut site1 and the modified sequence is SEQ ID NO: 21. The present invention may be practised with a wild-type α -subunit precursor, an α -subunit variant precursor with a modified site 1.

An α -subunit may further comprise a poly-histidine tag comprising the amino acid sequence HHHHHHHHH (SEQ ID NO: 23). Such a tag is useful in the purification by affinity chromatography and to distinguish wild-type and modified inhibins.

Additional mutation(s) may also be added at the interface site for homodimerization to occur for $\beta_A + \beta_A$ dimerization into activin A and $\beta_B + \beta_B$ dimerization into activin B. Such mutations or modifications reduce or inhibit altogether functional activin molecules being produced. Examples include A347X₁, Y345X₂,
 5 F326X₃, H369X₄, I373X₅, Y376X₆ and V392X₇, or equivalent position, wherein each of X₁, X₂, X₃, X₄, X₅, X₆ and X₇ is any amino acid except A, Y, F, H, I, Y and V, respectively (based on numbering in SEQ ID NO:4) in subunit β_A ; and F308X₈, Y327X₉, G329X₁₀, H351X₁₁, V355X₁₂, Y358X₁₃ and I373X₁₄ or equivalent position, wherein each of X₇, X₈, X₉, X₁₀, X₁₁, X₁₂, X₁₃ and X₁₄ is any amino acid except F, Y, G, H, V, Y and I,
 10 respectively (using numbering in SEQ ID NO:7). Particular examples including A347H, Y345G and A347H + Y345G in β_A -subunit. As above, reference to "at least 80%" includes 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 and 100% or a percentage inbetween. Any mutation which reduces or inhibits homodimerization may be introduced into β_A or β_B .

15 Hence, enabled herein is an inhibin α -subunit precursor comprising:

(i) a modified proprotein convertase site ("super-cut site1");

optionally together with a poly-histidine tag to assist with purification.

Further enabled herein is an inhibin β_A -subunit precursor comprising a modified proprotein convertase site ("super-cut site");

20 optionally with a mutation disrupting the ALK4 binding epitope in the mature domain and optionally with a FLAG tag to assist in affinity chromatography purification and optionally with a mutation disrupting or eliminating homodimerization.

The inhibin β_A -subunit carries a proprotein convertase cleavage site at ³⁰⁶RRRRR³¹⁰ in human inhibin A. In an embodiment this is modified to ISSRKKRSVSS
 25 (SEQ ID NO: 21) which is a more efficient cleavage site. In β_A -subunit, the amino acid sequence is ³⁰⁶ISSRKKRSVSS³¹⁶. As in the α -subunit, the substituted cleavage site may be further modified by one or more amino acid substitutions, additions and/or deletions to the amino acid sequence set forth in SEQ ID NO: 21. Hence, the substituted proprotein cleavage site in β_A -subunit may comprise SEQ ID NO: 21 or an
 30 amino acid sequence having at least 80% similarity to SEQ ID NO: 21 after optimal

alignment. As above, reference to "at least 80%" includes 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 and 100% or a percentage in between.

The β_A -subunit variant precursor may further comprise a poly-histidine tag comprising the amino acid sequence HHHHHHHHH (SEQ ID NO: 23) such a tag is
5 useful in the purification by affinity chromatography.

In addition, the β_A -subunit mature domain may further contain a mutation to disrupt the type I receptor (ALK4) binding epitope. This results in inactive activin A (comprising the homodimers β_A - β_A). In an embodiment, the mutation is a single point mutation comprising an M418A substitution (numbering from SEQ ID NO: 4); equivalent
10 to M108A from start of mature domain (SEQ ID NO: 6); in β_B -subunit, the site is M399A (numbering from SEQ ID NO: 7). The effect of more efficient cleavage is an at least a 4 to 9-fold increase in cleavage compared to the wild-type protein. This includes a 4, 5, 6, 7, 8, or 9-fold increase. As indicated above, a mutation eliminating or reducing homodimerization of β_A or β_B may also be introduced (referred to as a dimerization
15 interface mutation).

Taught herein is an inhibin β_B -subunit precursor comprising a modified proprotein convertase site ("super-cut site");

optionally with a mutation disrupting the ALK4 binding epitope in the mature domain and optionally with a FLAG tag to assist in affinity chromatography
20 purification and optionally with a mutation disrupting or eliminating homodimerization.

The inhibin β_B -subunit carries a proprotein convertase cleavage site at ²⁸⁸RIRKR²⁹² in human inhibin B. In an embodiment this is modified to ISSRKKRSVSS (SEQ ID NO: 21) which is a more efficient cleavage site. In β_B -subunit, the amino acid sequence is ²⁸⁸ISSRKKRSVSS²⁹⁸. As in the α -subunit, the substituted cleavage site
25 may be further modified by one or more amino acid substitutions, additions and/or deletions to the amino acid sequence set forth in SEQ ID NO: 21. Hence, the substituted proprotein cleavage site in β_B -subunit may comprise SEQ ID NO: 21 or an amino acid sequence having at least 80% similarity to SEQ ID NO: 18 after optimal alignment. Reference to the "at least 80%" is as defined above.

The β_B -subunit variant precursor may further comprise a poly-histidine tag comprising the amino acid sequence HHHHHHHHHH (SEQ ID NO: 23) such a tag is useful in the purification by affinity chromatography.

In addition, the β_B -subunit may further contain a mutation to disrupt the type I
5 receptor (ALK4) binding epitope. This results in inactive Activin A (comprising the homodimers β_B - β_B). In an embodiment, the mutation is a single point mutation comprising an M399A substitution (see numbering in SEQ ID NO: 7). A dimerization interface mutation may also be introduced to reduce or eliminate homodimerization.

The expression "at least 80%" has the same meaning as above.

10 Hence, to summarize the modifications, the α -subunit may comprise:

- (i) a mutation or modification at S344 or equivalent position;
- (ii) a modified proprotein convertase site ("super-cut site 1"); and/or
- (iii) a poly-histidine tag to assist with purification;

which α -subunit forms a heterodimer with a β_A - or β_B -subunit wherein the β_A - or
15 β_B -subunit may comprise:

- (i) a modified proprotein convertase site ("super-cut site");
- (ii) a mutation disrupting the ALK4 binding epitope in the mature
domain;
- (iii) a FLAG tag to assist in affinity chromatography purification; and/or
- 20 (iv) a mutation disrupting or eliminating homodimerization.

In an embodiment, the α -subunit has (i), (ii), and/or (iii). In an embodiment, the β_A - or β_B -subunit has (i), (ii), (iii) and (iv). In an embodiment, all mutations are present in the α - and β -subunits together.

As indicated above, the amino acid sequence of α - and/or β -subunit precursor
25 and/or mature form may have one or more amino acid substitutions, additions and/or deletions which do not render the inhibin inactive. Such changes may enhance activity

or render the inhibin more stable. It is well understood in the art that some amino acids may be changed to others with broadly similar properties without changing the nature of the activity of the polypeptide. Variant polypeptides encompassed by the present invention are those which are biologically active, that is, they continue to possess the antagonistic biological activity towards an activin or the ability to confer higher affinity binding as described herein. Experimental methods to determine antagonistic biological activity towards an activin or the ability to confer higher affinity binding as known in the art and exemplary methods are described herein including the Examples. Methods also for determining a reduction in activin formation are also known in the art and exemplary methods are described herein including the Examples. Amino acid modifications are preferably conservative amino acid substitutions although additions and/or deletions may also be made.

Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the polypeptides can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA*. 82:488-492; Kunkel *et al.* (1987) *Methods in Enzymol*, 154:367-382; U.S. Patent No. 4,873,192; Watson *et al.* (1987) "Molecular Biology of the Gene", Fourth Edition, Benjamin/Cummings, Menlo Park, Calif. and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure*, Natl. Biomed. Res. Found., Washington, D.C.

Variant propeptides may contain conservative amino acid substitutions at various locations along their sequence, as compared to a parent (e.g. naturally-occurring or reference) amino acid sequence. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, which can be generally sub-classified as follows:

Acidic: The residue has a negative charge due to loss of H ion at physiological pH and the residue is attracted by aqueous solution so as to seek the surface positions in the conformation of a peptide in which it is contained when the peptide is in aqueous

medium at physiological pH. Amino acids having an acidic side chain include glutamic acid and aspartic acid.

Basic: The residue has a positive charge due to association with H ion at physiological pH or within one or two pH units thereof (e.g. histidine) and the residue is attracted by aqueous solution so as to seek the surface positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium at physiological pH. Amino acids having a basic side chain include arginine, lysine and histidine.

Charged: The residues are charged at physiological pH and, therefore, include amino acids having acidic or basic side chains (i.e. glutamic acid, aspartic acid, arginine, lysine and histidine).

Hydrophobic: The residues are not charged at physiological pH and the residue is repelled by aqueous solution so as to seek the inner positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium. Amino acids having a hydrophobic side chain include tyrosine, valine, isoleucine, leucine, methionine, phenylalanine and tryptophan.

Neutral/polar: The residues are not charged at physiological pH, but the residue is not sufficiently repelled by aqueous solutions so that it would seek inner positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium. Amino acids having a neutral/polar side chain include asparagine, glutamine, cysteine, histidine, serine and threonine.

This description also characterizes certain amino acids as "small" since their side chains are not sufficiently large, even if polar groups are lacking, to confer hydrophobicity. With the exception of proline, "small" amino acids are those with four carbons or less when at least one polar group is on the side chain and three carbons or less when not. Amino acids having a small side chain include glycine, serine, alanine and threonine. The gene-encoded secondary amino acid proline is a special case due to its known effects on the secondary conformation of peptide chains. The structure of proline differs from all the other natural-occurring amino acids in that its side chain is bonded to the nitrogen of the α -amino group, as well as the α -carbon. Several amino

acid similarity matrices (e.g. PAM120 matrix and PAM250 matrix as disclosed for example by Dayhoff *et al.* (1978) *supra*, A model of evolutionary change in proteins.

The degree of attraction or repulsion required for classification as polar or non-polar is arbitrary and, therefore, amino acids specifically contemplated by the invention
5 have been classified as one or the other. Most amino acids not specifically named can be classified on the basis of known behaviour.

Amino acid residues can be further sub-classified as cyclic or non-cyclic, and aromatic or non-aromatic, self-explanatory classifications with respect to the side-chain
10 substituent groups of the residues, and as small or large. The residue is considered small if it contains a total of four carbon atoms or less, inclusive of the carboxyl carbon, provided an additional polar substituent is present; three or less if not. Small residues are, of course, always non-aromatic. Dependent on their structural properties, amino acid residues may fall in two or more classes.

Conservative amino acid substitution also includes groupings based on side
15 chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic
20 side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. For example, it is reasonable to expect that 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 will not have a major effect on the properties of the
25 resulting variant polypeptide. Whether an amino acid change results in a functional peptide polypeptide can readily be determined by assaying its activity. Amino acid substitutions falling within the scope of the invention, are, in general, accomplished by selecting substitutions that do not differ significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, (b) the charge or
30 hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. After the substitutions are introduced, the variants are screened for biological activity as described herein.

Alternatively, similar amino acids for making conservative substitutions can be grouped into three categories based on the identity of the side chains. The first group includes glutamic acid, aspartic acid, arginine, lysine, histidine, which all have charged side chains; the second group includes glycine, serine, threonine, cysteine, tyrosine, glutamine, asparagine; and the third group includes leucine, isoleucine, valine, alanine, proline, phenylalanine, tryptophan, methionine, as described in Zubay (1993) *Biochemistry*, third edition, Wm.C. Brown Publishers.

Thus, a predicted non-essential amino acid residue in a precursor inhibin polypeptide subunit is typically replaced with another amino acid residue from the same side chain family. A "non-essential" amino acid residue is a residue that can be altered from the reference sequence of an embodiment polypeptide without abolishing or substantially altering one or more of its activities. Suitably, the alteration does not substantially alter one of these activities, for example, the activity is at least 60%, 70%, 80%, 90% or 100% of the reference sequence. By contrast, an "essential" amino acid residue is a residue that, when altered from the wild-type sequence of a reference polypeptide, results in abolition of an activity of the parent molecule such that less than 20% of the activity of the reference polypeptide is present. In an embodiment, essential amino acid residues include those that are conserved in inhibin polypeptides across different species.

The propeptides of the present invention may be prepared by any suitable procedure known to those of skill in the art. Recombinant propeptides can be conveniently prepared using standard protocols as described for example in Sambrook *et al. Molecular Cloning: A Laboratory Manual, 2nd ed.*, Cold Spring Harbor, in particular Sections 13, 16 and 17; Ausubel *et al.* (1994) *Current Protocols in Molecular Biology*, John Wiley & Sons Inc, in particular Chapters 10 and 16; and Coligan *et al.* (1995-1997) *Current Protocols in Protein Science*, John Wiley & Sons, Inc., Chapters 1, 5 and 6. Methods of purification include size exclusion, affinity or ion exchange chromatography/separation. The identity and purity of peptides is determined for example by SDS-polyacrylamide electrophoresis or chromatographically such as by high performance liquid chromatography (HPLC). Alternatively, the propeptides or parts of the propeptides may be synthesized by chemical synthesis, e.g. using solution synthesis or solid phase synthesis as described, for example, in Chapter 9 of Atherton

and in Roberge *et al.* (1995) *Science*, 269:202). In some embodiments, the propeptides are prepared by recombinant techniques. For example, the inhibin analogs of the present invention may be prepared by a procedure including the steps of: (a) preparing a construct comprising a nucleic acid sequence that encodes an α - or β -subunit modified as herein described and that is operably linked to a regulatory element; (b) introducing (e.g. transfecting) the construct into a host cell or cell line; (c) culturing the host cell to express the nucleic acid to thereby produce the encoded subunit precursors; and (d) isolating the processed inhibin or its precursor from conditioned medium.

The invention also contemplates variants of the nucleic acid molecules encoding the subject modified propeptides including the dimerization domain. Nucleic acid variants can be naturally-occurring (native), such as allelic variants (same locus), homologs (different locus), and orthologs (different organism) or can be non-naturally-occurring. Naturally-occurring nucleic acid variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as known in the art. Non-naturally occurring polynucleotide variants can be made by mutagenesis techniques, including those applied to polynucleotides, cells, or organisms. The variants can contain nucleotide substitutions, deletions, inversions and insertions. Variation can occur in either or both the coding and non-coding regions. The variations can produce both conservative and non-conservative amino acid substitutions (as compared in the encoded product) and glycosylation variants. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of a reference polypeptide. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis but which still encode a polypeptide. Variants of a particular nucleic acid sequence will have at least about 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 more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs known in the art using default parameters.

The term "sequence identity" as used herein refers to the extent that sequences are identical on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis

over a window of comparison. Thus, a "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g. A, T, C, G, I, U) or the identical amino acid residue (e.g. Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e. the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

A comparison window may comprise additions or deletions (i.e. gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerized implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA) or by inspection and the best alignment (i.e. resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as for example disclosed by Altschul *et al.* (1997) *Nucl. Acids Res.* 25:3389. A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel *et al.* (1994-1998) "Current Protocols in Molecular Biology", John Wiley & Sons Inc, Chapter 15.

In another aspect, the invention provides a purified nucleic acid molecule that comprises a nucleotide sequence encoding the herein described inhibin subunit precursors including variants having substantial sequence identity or ability to cross hybridise under stringent hybridization conditions, synonymous codon variants and codon optimized variants thereof.

As known in the art, "stringency" refers to the temperature and ionic strength conditions, and presence or absence of certain organic solvents, during hybridization and washing procedures. The higher the stringency, the higher will be the degree of complementarity between nucleic acid sequences that remain hybridized after washing. The term "*high stringency*" etc. refers to temperature and ionic conditions under which only nucleotide sequences having a high frequency of complementary bases will hybridize. The stringency required is nucleotide sequence dependent and depends

upon the various components present during hybridization. The stringency conditions may be high, medium or low.

The nucleotide sequence may comprise codon substitution with a synonymous codon. The term "synonymous codon" as used herein refers to a codon having a different nucleotide sequence compared to another codon but encoding the same amino acid as that other codon. Codon optimization is standard in the art and is contemplated herein.

The present invention provides a method for generating bioactive inhibin proteins as described herein. The method comprises:

- 10 (i) mutating or modifying S344 or equivalent position;
- (ii) optimally modifying a proprotein convertase cleavage site on one or both of the α -subunit precursor and/or a β -subunit precursor thereby making proprotein convertase cleavage more efficient;
- (iii) optionally introducing a mutation to eliminate the type I receptor
15 (ALK4) binding epitope in the β -subunit mature domain thereby rendering inactive any activin formed;
- (iv) optionally introducing a mutation to disrupt homodimerization;
- (v) transfecting a cell or cell line with nucleic acid molecules encoding the α -subunit and β -subunit precursors as defined in (i) and optionally (ii) and optionally
20 (iii) and optionally (iv);
- (vi) culturing the transfected cells or cell line for a time and under conditions sufficient for the inhibin precursor protein to be processed by a proprotein convertase and the processed protein released from the cell into conditioned medium; and
- 25 (vii) isolating the inhibin or its precursor form from the conditioned medium.

In another aspect the present specification provides nucleic acid constructs encoding a inhibin protein or subunit as described herein or a functional fragment thereof.

The invention extends to vectors and other constructs comprising isolated nucleic acid molecules including those capable of expressing (producing) the subject inhibin subunit precursors and to isolated host cells or cell lines comprising same.

The terms "expression" or "gene expression" refer to either production of RNA message or translation of RNA message into proteins or polypeptides.

By "expression vector" is meant any genetic element capable of directing the transcription of a polynucleotide contained within the vector and suitably the synthesis of a peptide or polypeptide encoded by the polynucleotide. Such expression vectors are known to practitioners in the art.

By "vector" is meant a polynucleotide molecule, suitably a DNA molecule derived, for example, from a plasmid, bacteriophage, yeast or virus, into which a nucleic acid molecule can be inserted or cloned. A vector may contain one or more unique restriction sites and can be capable of autonomous replication in a defined host cell including a target cell or tissue or a progenitor cell or tissue thereof, or be integral with the genome of the defined host such that the cloned sequence is reproducible. Accordingly, the vector can be an autonomously replicating vector, i.e. a vector that exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, e.g. a linear or closed circular plasmid, an extra-chromosomal element, a mini-chromosome, or an artificial chromosome. The vector can contain any means for assuring self-replication. Alternatively, the vector can be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. A vector system can comprise a single vector or plasmid, two or more vectors or plasmids, which together contain the total DNA to be introduced into the genome of the host cell, or a transposon. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. In the present case, the vector is preferably a viral or viral-derived vector, which is operably functional in animal and preferably mammalian cells. Such vector may be derived from a poxvirus, an

adenovirus or yeast. The vector can also include a selection marker such as an antibiotic resistance gene that can be used for selection of suitable transformants. Examples of such resistance genes are known to those of skill in the art and include the *nptII* gene that confers resistance to the antibiotics kanamycin and G418 [Geneticin
5 (Registered Trade Mark)] and the *hph* gene that confers resistance to the antibiotic hygromycin B.

Vectors useful for expressing the subject nucleotide sequence in subject host cells *in vivo* are known to those of skill in the art and are expressly contemplated. They include adenoviral vectors and adeno-associates virus vectors. Illustrative vectors
10 include AAV8 or AAV6 described for example in Qiao *et al.* (2008) *Human Gene Therapy* 19:000-000.

In another embodiment host cells are provided comprising a nucleic acid construct encoding a modified inhibin subunit precursor as described herein, wherein the host cell or cell line expresses the precursor.

15 The term "host cell" includes an individual cell or cell culture which can be or has been a recipient of any recombinant vector(s) or isolated nucleic acid of the invention. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation and/or change. A host cell includes
20 cells transfected or infected *in vivo* or *in vitro* with a recombinant vector or a polynucleotide of the invention. A host cell which comprises a recombinant vector of the invention is a recombinant host cell.

Host cells are conveniently eukaryotic cells include mammalian, plant, yeast and insect cells as known in the art. Recombinant proteins are produced by culturing the
25 host cells for a period of time sufficient to allow for expression of the modified polypeptide inhibin subunits in the host cells or, more preferably, secretion of the protein into the culture medium in which the host cells are grown. Suitable mammalian cell lines include, but are not limited to, HEK293T, HEK293, HEK293T-Rex, BHK, VERO, HT1080, RD, COS-7, CHO, Jurkat, HUT, SUPT, C8166, MOLT4/clone8, MT-2, MT-4,
30 H9, PM1, CEM, myeloma cells (e.g. SB20 cells) and CEMX174 are available, for example, from the ATCC. Other host cells include without limitation yeast, e.g. *Pichia*

pastoris, or insect cells such as Sf9 cells although such molecules would not ordinarily be glycosylated.

In another aspect the present invention provides a method of treating or preventing activin-induced conditions, such as muscle wasting, fibrosis or inflammation
5 in a subject, the method comprising administering to the subject an inhibin protein as described herein or a nucleic acid construct encoding same which provides the α - and β -subunits to the subject.

In another aspect the present invention provides a method of treating or preventing activin-induced conditions, such as muscle wasting, fibrosis or inflammation
10 in a subject, the method comprising administering to the subject an inhibin protein as described herein or a nucleic acid construct encoding same which provides the modified α - and β -subunits to the subject.

In these embodiments, minimal bioactive activin A or B is generally produced. In an embodiment, any similar inhibin generally comprises a β -subunit chain with a
15 mutation in the ALK4 binding epitope. In an embodiment, homodimerization interface mutations are included in β_A - or β_B -subunits.

"Subjects" contemplated in the present invention are humans or mammals including laboratory or art accepted test or vehicle animals. "Subjects" include human subjects in need of treatment or prophylaxis.

20 Usefully, the present invention further provides compositions comprising an inhibin protein, subunit, or its precursor or nucleic acid encoding inhibin subunits as herein described. The term "compound" includes "medicament", "agent", "therapeutic", "pharmacologically acceptable compound" and "pharmaceutical composition" and the like. In another embodiment, the composition comprises a pharmaceutically or
25 physiologically acceptable carrier or diluent. In an embodiment, the inhibin analog or its precursor are for use in the treatment or prevention of conditions or symptoms of conditions promoted or exacerbated by activin signaling.

Pharmaceutical compositions are conveniently prepared according to conventional pharmaceutical compounding techniques. See, for example, Remington's
30 Pharmaceutical Sciences (1990) 18th Ed., Mack Publishing, Company. These

compositions may comprise, in addition to one of the active substances (inhibin analog or its precursor), a pharmaceutically acceptable excipient, carrier, buffer, stabilizer or other materials well known in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The carrier may take a wide
5 variety of forms depending on the form of preparation desired for administration, e.g. intravenous, oral or parenteral.

The inhibin proteins, subunits, or their precursors or compositions comprising same are administered in an effective amount. The terms "effective amount" includes "therapeutically effective amount" and "prophylactically effective amount" and mean a
10 sufficient amount of active either in a single dose or as part of a series or slow release system which provides the desired therapeutic, preventative, or physiological effect in some subjects. Undesirable effects, e.g. side effects, may sometimes manifest along with the desired therapeutic effect; hence, a practitioner balances the potential benefits against the potential risks in determining an appropriate "effective amount". The exact
15 amount of composition required will vary from subject to subject, depending on the species, age and general condition of the subject, mode of administration and the like. Thus, it may not be possible to specify an exact "effective amount". However, an appropriate "effective amount" in any individual case may be determined by one of ordinary skill in the art using routine skills or experimentation.

20 The term "treatment" refers to any measurable or statistically significant amelioration in at least some subjects in one or more symptoms of a condition associated with dysregulated or overactive activin signaling in a subject. Prophylactic administration of the compound serves to prevent or attenuate onset of symptoms of a condition associated with dysregulated or overactive activin signaling in a subject. In an
25 embodiment, the subject is a post-menopausal female subject with reduced levels of serum inhibin and has or is at risk of developing a bone disorder such as osteoporosis.

A "pharmacologically acceptable" composition is one tolerated by a recipient patient. A "pharmaceutically acceptable carrier and/or a diluent" is a pharmaceutical vehicle comprised of a material that is not otherwise undesirable i.e. it is unlikely to
30 cause a substantial adverse reaction by itself or with the active composition. Carriers may include all solvents, dispersion media, coatings, antibacterial and antifungal agents, agents for adjusting tonicity, increasing or decreasing absorption or clearance

rates, buffers for maintaining pH, chelating agents, membrane or barrier crossing agents. A pharmaceutically acceptable salt is a salt that is not otherwise undesirable. The agent or composition comprising the agent may be administered in the form of pharmaceutically acceptable non-toxic salts, such as acid addition salts or metal
5 complexes.

For oral administration, the compositions can be formulated into solid or liquid preparations such as capsules, pills, tablets, lozenges, powders, suspensions or emulsions. In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils,
10 alcohols, flavoring agents, preservatives, coloring agents, suspending agents, and the like in the case of oral liquid preparations (such as, for example, suspensions, elixirs and solutions); or carriers such as starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations (such as, for example, powders, capsules and tablets). Because of their
15 ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. Tablets may contain a binder such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate. If desired, tablets may be sugar-coated or enteric-coated by standard techniques. The active
20 composition can be encapsulated to make it stable to passage through the gastrointestinal tract. See for example, International Patent Publication No. WO 96/11698.

For parenteral administration, the composition may be dissolved in a carrier and administered as a solution or a suspension. For transmucosal or transdermal (including
25 patch) delivery, appropriate penetrants known in the art are used for delivering the composition. For inhalation, delivery uses any convenient system such as dry powder aerosol, liquid delivery systems, air jet nebulizers, propellant systems. For example, the formulation can be administered in the form of an aerosol or mist. The compositions may also be delivered in a sustained delivery or sustained release format. For example,
30 biodegradable microspheres or capsules or other polymer configurations capable of sustained delivery can be included in the formulation. Formulations can be modified to alter pharmacokinetics and biodistribution. For a general discussion of

pharmacokinetics, see, e.g., Remington's Pharmaceutical Sciences, 1990 (*supra*). In some embodiments the formulations may be incorporated in lipid monolayers or bilayers such as liposomes or micelles. Targeting therapies known in the art may be used to deliver the agents more specifically to certain types of cells or tissues.

5 **Table 1**

Summary of sequence identifiers

SEQ NO:	DESCRIPTION	Amino acid or nucleotide sequence
1	Amino acid sequence of wildtype human inhibin α -subunit precursor	<u>M V L H L L L F L L L T P Q G G H</u> S C Q G L E L A R E L V L A K V R A L F L D A L G P P A V T R E G G D P G V R R L P R R H A L G G F T H R G S E P E E E E D V S Q A I L F P A T D A S C E D K S A A R G L A Q E A E E G L F R Y M F R P S Q H T R S R Q V T S A Q L W F H T G L D R Q G T A A S N S S E P L L G L L A L S P G G P V A V P M S L G H A P P H W A V L H L A T S A L S L L T H P V L V L L L R C P L C T C S A R P E A T P F L V A H T R T R P P S G G E R A R R S T P L M S W P W S P S A L R L L Q R P P E E P A A H A N C H R V A L N I S F Q E L G W E R W I V Y P P S F I F H Y C H G G C G L H I P P N L S L P V P G A P P T P A Q P Y S L L P G A Q P C C A A L P G T M R P L H V R T T S D G G Y S F K Y E T V P N L L T Q H C A C I

SEQ NO:	DESCRIPTION	Amino acid or nucleotide sequence
2	Amino acid sequence of the prodomain of wildtype human inhibin α -subunit	C Q G L E L A R E L V L A K V R A L F L D A L G P P A V T R E G G D P G V R R L P R R H A L G G F T H R G S E P E E E E D V S Q A I L F P A T D A S C E D K S A A R G L A Q E A E E G L F R Y M F R P S Q H T R S R Q V T S A Q L W F H T G L D R Q G T A A S N S S E P L L G L L A L S P G G P V A V P M S L G H A P P H W A V L H L A T S A L S L L T H P V L V L L L R C P L C T C S A R P E A T P F L V A H T R T R P P S G G E R A R R
3	Amino acid sequence of the mature wildtype human inhibin α -subunit	S T P L M S W P W S P S A L R L L Q R P P E E P A A H A N C H R V A L N I S F Q E L G W E R W I V Y P P S F I F H Y C H G G C G L H I P P N L S L P V P G A P P T P A Q P Y S L L P G A Q P C C A A L P G T M R P L H V R T T S D G G Y S F K Y E T V P N L L T Q H C A C I
4	Amino acid sequence of wildtype human inhibin β_A -subunit precursor	<u>M P L L W L R G F L L A S C W I I</u> <u>V R S S</u> P T P G S E G H S A A P D C P S C A L A A L P K D V P N S Q P E M V E A V K K H I L N M L H L K K R P D V T Q P V P K A A L L N A I R K L H V G K V G E N G Y V E I E D D I G R R A E M N E L M E Q T S E I I T F A E S G T A R K T L H F E

SEQ NO:	DESCRIPTION	Amino acid or nucleotide sequence
		I S K E G S D L S V V E R A E V W L F L K V P K A N R T R T K V T I R L F Q Q Q K H P Q G S L D T G E E A E E V G L K G E R S E L L L S E K V V D A R K S T W H V F P V S S S I Q R L L D Q G K S S L D V R I A C E Q C Q E S G A S L V L L G K K K K K E E E G E G K K K G G G E G G A G A D E E K E Q S H R P F L M L Q A R Q S E D H P H R R R R R G L E C D G K V N I C K K Q F F V S F K D I G W N D W I I A P S G Y H A N Y C E G E C P S H I A G T S G S S L S F H S T V I N H Y R M R G H S P F A N L K S C C V P T K L R P M S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S
5	Amino acid sequence of the prodomain of wildtype human inhibin β_A -subunit	S P T P G S E G H S A A P D C P S C A L A A L P K D V P N S Q P E M V E A V K K H I L N M L H L K K R P D V T Q P V P K A A L L N A I R K L H V G K V G E N G Y V E I E D D I G R R A E M N E L M E Q T S E I I T F A E S G T A R K T L H F E I S K E G S D L S V V E R A E V W L F L K V P K A N R T R T K V T I R L F Q Q Q K H P Q G S L D T G E E A E E V G L K G E R S E L L L S E K V V D A R K S T W H V F P V S S S I Q R L L D Q G K S S L D V R I A C E

SEQ NO:	DESCRIPTION	Amino acid or nucleotide sequence
		Q C Q E S G A S L V L L G K K K K K E E E G E G K K K G G G E G G A G A D E E K E Q S H R P F L M L Q A R Q S E D H P H R R R R R
6	Amino acid sequence of the mature wildtype human inhibin β_A -subunit	G L E C D G K V N I C C K K Q F F V S F K D I G W N D W I I A P S G Y H A N Y C E G E C P S H I A G T S G S S L S F H S T V I N H Y R M R G H S P F A N L K S C C V P T K L R P M S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S
7	Amino acid sequence of wildtype human inhibin β_B -subunit precursor	<u>M D G L P G R A L G A A C L L L L</u> <u>A A G W L G P E A W G S P T P P P</u> T P A A P P P P P P G S P G G S Q D T C T S C G G F R R P E E L G R V D G D F L E A V K R H I L S R L Q M R G R P N I T H A V P K A A M V T A L R K L H A G K V R E D G R V E I P H L D G H A S P G A D G Q E R V S E I I S F A E T D G L A S S R V R L Y F F I S N E G N Q N L F V V Q A S L W L Y L K L L P Y V L E K G S R R K V R V K V Y F Q E Q G H G D R W N M V E K R V D L K R S G W H T F P L T E A I Q A L F E R G E R R L N L D V Q C D S C Q E L A V V P V F V D P G E E S H R P F V V V Q A R L G D S R H R I R K R G L E C D G R T N L C C R Q Q F F I D

SEQ NO:	DESCRIPTION	Amino acid or nucleotide sequence
		F R L I G W N D W I I A P T G Y Y G N Y C E G S C P A Y L A G V P G S A S S F H T A V V N Q Y R M R G L N P G T V N S C C I P T K L S T M S M L Y F D D E Y N I V K R D V P N M I V E E C G C A
8	Amino acid sequence of the prodomain of wildtype human inhibin β_B -subunit	S P T P P P T P A A P P P P P P P G S P G G S Q D T C T S C G G F R R P E E L G R V D G D F L E A V K R H I L S R L Q M R G R P N I T H A V P K A A M V T A L R K L H A G K V R E D G R V E I P H L D G H A S P G A D G Q E R V S E I I S F A E T D G L A S S R V R L Y F F I S N E G N Q N L F V V Q A S L W L Y L K L L P Y V L E K G S R R K V R V K V Y F Q E Q G H G D R W N M V E K R V D L K R S G W H T F P L T E A I Q A L F E R G E R R L N L D V Q C D S C Q E L A V V P V F V D P G E E S H R P F V V V Q A R L G D S R H R I R K R
9	Amino acid sequence of the mature wildtype human inhibin β_B -subunit	G L E C D G R T N L C C R Q Q F F I D F R L I G W N D W I I A P T G Y Y G N Y C E G S C P A Y L A G V P G S A S S F H T A V V N Q Y R M R G L N P G T V N S C C I P T K L S T M S M L Y F D D E Y N I V K R D V P N M I V E E C G C A

SEQ NO:	DESCRIPTION	Amino acid or nucleotide sequence
10	Nucleotide sequence of wildtype human inhibin α -subunit precursor	atggtgctgcacactactgctcttctgctgctgacccacagggg gggcacagctgccaggggctggagctggcccggaactgtt ctggccaaggtgagggccctgttcttgatgcctggggccccc cgcggtgaccaggaaggtggggaccctggagtcaggcgg ctgccccgaagacatgccctgggggcttcacacacagggg ctctgagcccgaggaagaggaggatgtctccaagccatcct ttcccagccacagatgccagctgtgaggacaagtcagctgcc agagggctggccaggaggctgaggaggcctcttcagata catgttccggccatcccagcatacacgcagccgacagtgac ttcagcccagctgtggtccacaccgggctggacaggcaggg cacagcagcctccaatagctctgagcccctgtaggcctgctg gcactgtcaccgggaggaccggtggctgtgccatgtctttgg gccatgtccccctcactgggcccgtgctgcacctggccacct gtctctctctgctgacccaccccgtcctggtgctgctgctg gtcccctctgtacctgtcagcccggcctgaggccacgcccttc ctggtggccacactcggaccagaccacccagtgaggggga gagagcccgcgctcaactcccctgatgtcctggccttggtctc cctctgctctgcgctgctgcagaggcctccggaggaaccgg ctgcccattgccaactgccacagagtagcactgaacatctcctt ccaggagctgggctgggaacggtggatcgtgtacctcccag ttcatcttccactactgtcatggtggtgtgggctgcacatccac caaacctgtccctccagtcctggggctccccctacccagcc cagccctactccttgctgccagggggccagccctgctgtgctgc tctccagggaccatgaggcccctacatgtccgcaccacctg gatggaggttactcttcaagtatgagacagtgcccaaccttctc acgcagcactgtgctgtatc
11	Nucleotide sequence of the prodomain of wildtype human inhibin α -subunit	tgccaggggctggagctggcccggaactgttctggccaag gtgagggccctgttcttgatgcctggggcccccccgcggtgac caggaaggtggggaccctggagtcaggcggctgccccga agacatgccctgggggcttcacacacaggggctctgagccc

SEQ NO:	DESCRIPTION	Amino acid or nucleotide sequence
		gaggaagaggaggatgtctccaagccatcctttccagcc acagatgccagctgtgaggacaagtcagctgccagagggt ggcccaggaggctgaggaggcctcttcagatacatgttccg gccatcccagcatacacgcagccgccagggtgacttcagccc agctgtggttccacaccgggtggacaggcagggcacagca gcctccaatagctctgagcccctgctaggcctgctggcactgtc accgggaggaccctgtggtgtgcccattgtttggccatgtc cccctcactgggcccgtgctgcacctggccacctgtctctctc tgctgaccaccccgtcctggtgctgctgctgcgctgtcccctct gtacctgtcagcccggcctgaggccacgccctcctggtggc ccacactcggaccagaccaccagtgaggaggagagagcc cgacgc
12	Nucleotide sequence of the mature wildtype human inhibin α -subunit	tcaactcccctgatgtcctggccttggtctccctctgctctgcgct gctgcagaggcctccggagggaaccggctgcccattgccaact gccacagagtagcactgaacatctccttcaggagctgggt gggaacggtggatcgtgtaccctcccagttcatcttcactact gtcatggtggttggtggctgcacatcccacaaacctgtccctc cagtccttggggctccccctacccagcccagccctactccttg ctgccagggggcccagccctgctgtgctgtctcccaggggacc atgaggccccctacatgtccgcaccacctcggtaggaggttact cttcaagtatgagacagtgcccaaccttctcacgcagcactgt gcttgatc
13	Nucleotide sequence of wildtype human inhibin β_A -subunit precursor	atgcccttgcttggtgagaggatttctgttgcaagttgctgga ttatagtgaggagtccccccacccaggatccgaggggcaca gcgcggcccccgactgtccgtcctgtgcgctggccgccctccc aaaggatgtaccaactctcagccagagatggtggaggccgt caagaagcacattttaacatgctgcacttgaagaagagacc cgatgtcaccagccggtacccaaggcggcgcttctgaacgc gatcagaaaagcttcatgtgggcaaagtcggggagaaacgggt

SEQ NO:	DESCRIPTION	Amino acid or nucleotide sequence
		atgtggagatagaggatgacattggaaggagggcagaaatg aatgaacttatggagcagacctcggagatcatcacgtttgccg agtcaggaacagccaggaagacgctgcacttcgagattcca aggaaggcagtgacctgtcagtggtggagcgtgcagaagtct ggctcttcctaaaagtccccaaggccaacaggaccaggacc aaagtcaccatccgcctcttcagcagcagaagcaccgcga gggcagcttgacacaggggaagaggccgaggaagtggg cttaaagggggagaggagtgaactgttgctctctgaaaaagta gtagacgctcgaagagcacctggcatgtctccctgtctccag cagcatccagcggttgctggaccagggaagagctccctgg acgttcgattgcctgtgagcagtgccaggagagtggcgcca gcttggtctcctgggaagaagaagaagaagaagaggag ggggaagggaaaaagaagggcggagggtgaaggtggggc aggagcagatgaggaaaaggagcagtcgcacagaccttcc tcatgtgcaggcccgagctctgaagaccaccctcatcgcc ggcgtcggcggggcttgagtgatggcaaggtcaacatct gctgtaagaaacagttcttgtagttcaaggacatcggctgg aatgactggatcattgctccctctggctatcatgccaactactgc gagggtagtgcccgagccatatagcaggcacgtccgggtc ctcactgtccttcactcaacagtcacatcaaccactaccgatgc ggggccatagccccttgccaacctcaaatcggtgtgtgtgccc accaagctgagacctatgtccatgtgtactatgatgatgggtca aaacatcatcaaaaaggacattcagaacatgatcgtggagg agtgtgggtgctca
14	Nucleotide sequence of the prodomain of wildtype human inhibin β_A -subunit	tccccacccaggatccgaggggcacagcgcgcccccg actgtccgtcctgtgctgctggccgcctcccaaaggatgtacc caactctcagccagagatggtggaggccgtcaagaagcaca tttaaacatgctgcactgaagaagagacccgatgtcaccca gccggtaccaagggcggtctgaacgcgatcagaaagc ttcatgtgggcaaagtcgggggagaacgggtatgtggagatag

SEQ NO:	DESCRIPTION	Amino acid or nucleotide sequence
		aggatgacattggaaggagggcagaaatgaatgaacttatg gagcagacctcggagatcatcacgtttgccgagtcaggaaca gccaggaagacgctgcacttcgagattccaaggaaggcagt gacctgtcagtggtggagcgtgcagaagtctggctcttctaaa agtccccaaggccaacaggaccaggaccaaagtcaccatc cgcctcttcagcagcagaagcacccgcagggcagcttga cacaggggaagaggccgaggaagtgggctaaaggggga gaggagtgaactgttgctctgaaaaagtagtagacgctcgg aagagcacctggcatgtctccctgtctccagcagcatccagc ggttgctggaccagggcaagagctccctggacgttcggattgc ctgtgagcagtgccaggagagtggcgccagcttggttctctg ggcaagaagaagaagaagaagaggagggggaagggga aaaagaagggcgaggtgaaggtggggcaggagcagatg aggaaaaggagcagtcgcacagaccttcctcatgtgcagg cccggcagctctgaagaccacctcatcgccggcgctcgccgg
15	Nucleotide sequence of the mature wildtype human inhibin β_A -subunit	ggcttgagtgatggcaaggtcaacatctgctgaagaaac agttcttgtcagttcaaggacatcggtggaatgactggatca ttgctccctctggctatcatgccaactactgcgaggggtgagtcc cgagccatatagcaggcacgtccgggtcctactgtcctcca ctcaacagtcatcaaccactaccgcatcggggccatagccc ctttgccaacctcaaactgtgctgtgtgcccaccaagctgagac ccatgtccatgttgtactatgatgatggtaaaacatcatcaaaa aggacattcagaacatgatcgtggaggagtgtgggtgtca
16	Nucleotide sequence of wildtype human inhibin β_B -subunit precursor	atggacgggctgccggctcgggcgctggggccgcctgcctt ctgctgctggcggccggctggctggggcctgaggcctggggc tcaccacgcccccgccgacgcctgccgcgcccggccacc cccgccacccggatccccgggtggctcgcaggacacctgtac gtcgtgcgggcggtccggcgccagaggagctcgccgag tggacggcgacttctggaggcggtaagcggcacatctga

SEQ NO:	DESCRIPTION	Amino acid or nucleotide sequence
		gccgcctgcagatgcggggccggcccaacatcacgcacgc cgtgcctaaggccgccatggtcacggccctgcgcaagctgca cgcgggcaaggtgcgcgaggacggccgctggagatcccg cacctcgacggccacgccagcccggcgccgacggccagg agcgcggttcgaaatcatcagcttcgccgagacagatggcct cgctcctcccgggtccgcctatacttctcatctccaacgaagg caaccagaacctgtttgtgtccaggccagcctgtggcttacct gaaactcctgccctacgtcctggagaagggcagccggcgga aggtgcgggtcaaagtgtacttcaggagcagggccacggtg acaggtggaacatggtggagaagaggggtggacctcaagcg cagcggctggcatacctcccactcacggaggccatccaggc ctgtttgagcggggcgagcggcgactcaacctagacgtgca gtgtgacagctgccaggagctggccgtggtgccggtgttcgtg gacccaggcgaagagtcgcaccggccctttgtggtggtgcag gctcggctgggcgacagcaggcaccgcattcgaagcgag gcctggagtgcgatggccggaccaacctctgttcagggaac agttcttcattgacttcgcctcatcggtggaacgactggatca tagcaccaccggctactacgggaactactgtgagggcagct gccagcctacctggcaggggtccccggctctgcctcctcttc cacacggctgtggtgaaccagtaccgcatgcggggtctgaac cccggcacggtaactcctgctgcattcccaccaagctgagc accatgtccatgctgtacttcgatgatgagtacaacatcgtaac gcgggacgtgccaacatgattgtggaggagtgcggtgcgc c
17	Nucleotide sequence of the prodomain of wildtype human inhibin β_B -subunit	tcaccacgcccccgccgacgcctgccgcgccgccacc cccgccaccggatccccgggtggctgcaggacacctgtac gtcgtgcggcggttcggcgccagaggagctcggccgag tggaaggcgacttctggaggcggtaagcggcacatctga gccgcctgcagatgcggggccggcccaacatcacgcacgc cgtgcctaaggccgccatggtcacggccctgcgcaagctgca

SEQ NO:	DESCRIPTION	Amino acid or nucleotide sequence
		cgcgggcaaggtgcgcgaggacggccgctggagatcccg cacctcgacggccacgccagccggcgccgacggccagg agcgcgttccgaaatcatcagcttcgccgagacagatggcct cgctcctcccgggtccgcctatacttctcatctccaacgaagg caaccagaacctgtttgtgtccaggccagcctgtggcttacct gaaactctgccctacgtcctggagaagggcagccggcgga aggtgcggtcaaagtgtacttcaggagcagggccacgggtg acaggtggaacatggtggagaagaggggtggacctcaagcg cagcggctggcatacctcccactcacggaggccatccaggc ctgtttgagcggggcgagcggcgactcaacntagacgtgca gtgtgacagctgccaggagctggccgtggtgccggtgttcgtg gaccagggcgaagagtcgcaccggcccttgtggtggtgcag gctcggctgggcgacagcaggcaccgcattcgcaagcga
18	Nucleotide sequence of the mature wildtype human inhibin β_B -subunit	ggcctggagtgcgatggccggaccaacctctgttcaggcaa cagttctcattgactccgcctcatcggtggaacgactggatc atagcaccacccggctactacgggaactactgtgagggcag ctgccagcctacctggcaggggtccccggctctgcctcctct tccacacggctgtggtgaaccagtaccgcatgcggggctga acccggcacgggtgaactcctgctgcattcccaccaagctga gcaccatgtccatgctgtacttcgatgatgagtacaacatcgtc aagcgggacgtgccaacatgattgtggaggagtgcggtgc gcc
19	Amino acid sequence of FLAG tag	DYKDDDDK
20	Nucleotide sequence of FLAG tag	gactacaagacgacgacgacaaa
21	Amino acid of super cut site 1	ISSRKKRSVSS

SEQ NO:	DESCRIPTION	Amino acid or nucleotide sequence
22	Nucleotide sequence of super cut site 1	atctcatcgagaaagaaacgctcagtcctcatcg
23	Amino acid sequence of His tag	HHHHHHHHH
24	Nucleotide sequence of His tag	catcatcaccatcaccaccatcatcac

Examples

Aspects disclosed herein are further described by the following non-limiting Examples.

5 **Example 1**

MATERIALS AND METHODS

Generation of inhibin mutant variants

Previously, the human inhibin α - (sequence reference NM_002191.4), β_A - (sequence reference NM_002192.4) and β_B -subunits (sequence reference NM_002193.4) were incorporated into the mammalian expression vector, pCDNA3.1 (Life Technologies; CA, USA), and modified to include an improved processing site between their pro- and mature domains (ISSRKKRSVSS, 'supercut' or SCUT) (Walton KL, Kelly EK, Johnson KE, Robertson DM, Stanton PG, Harrison CA. *Endocrinology*. 2016;157(7):2799-2809). In addition, a poly-histidine tag was inserted into the inhibin α -subunit at the C-terminus of the prodomain to facilitate downstream inhibin purification, as previously described (Walton et al., supra). The $\beta_{A/B}$ -subunit variants comprised FLAG tags at the N-terminus of their prodomains to facilitate activin purification. These modified inhibin constructs (inhibin α -SCUT HIS, β_A -SCUT, β_B -SCUT), henceforth referred to as the wildtype forms, were used as a template in generating additional inhibin subunit variants. Mutagenic primers were developed and targeted point

mutations were introduced via the QuikChange Lightning site-directed mutagenesis kit (Stratagene; CA, USA), according to manufacturer's guidelines. The resulting plasmid DNA constructs were transformed into XL-Gold Competent *E. coli* (Integrated Sciences; NSW, Australia), according to manufacturer guidelines, and positive colonies were selected and grown in LB media (supplemented with 100 µg/mL of ampicillin) at 37°C overnight. DNA was extracted from the grown mini-cultures using the Wizard Plus SV Miniprep kit (Promega; WI, USA), as per manufacturer's instructions. Target mutations were then confirmed by DNA sequencing (Micromon DNA Sequencing Facility, Monash University, Australia).

10 *Transient expression of inhibin A and B variants in HEK293T cells*

Inhibin protein variants were generated by transient transfection in a Human Embryonic Kidney (HEK293T) cell line. In brief, HEK293T cells were plated at a density of 8×10^5 per well in a 6-well plate, and maintained at 37°C with 5% CO₂. After 24 hours, each well was transfected with 5 µg (3 µg α-subunit:2 µg β-subunit) of DNA using the transfection reagent Lipofectamine 2000 (Thermo Fisher Scientific; MA, USA) and incubated in 2 mL of serum-free OPTI-MEM (Thermo Fisher Scientific) for a further 48 hours, as per manufacturer's guidelines.

Western blotting to assess inhibin protein production

Conditioned media containing inhibin and activin proteins was harvested after 48 hours and protein production assessed by Western blotting. Equal amounts of conditioned media and 2x NuPAGE LDS sample dye (Thermo Fisher Scientific) was combined and separated by a hand-cast 10% SDS-polyacrylamide gel electrophoresis. Following electrophoresis, samples were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories; CA, USA) and later blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline with 0.05% Tween-20 (TBS-T, pH 7.5). The membranes were then probed with either an anti-α-subunit monoclonal antibody (R1; Oxford Bio-innovation, UK (Groome et al. *Clin Endocrinol (Oxf)*. 1994;40(6):717-723), which binds residues 233–264, an anti-β_A subunit monoclonal antibody (E4; Oxford Bio-innovation, (Groome et al., supra)), which binds residues 401-413, or an anti-β_B subunit monoclonal antibody (C5; Oxford Bio-innovation, (Groome NP, Illingworth PJ, O'Brien M, Pai R, Rodger FE, Mather JP, McNeilly AS. *J Clin Endocrinol Metab*. 1996;81(4):1401-1405),

which binds residues 373-405. Primary antibodies were incubated for a minimum of 2 hours in 1% BSA in TBS-T solution, with the E4 and C5 antibodies requiring co-incubation with 6% Hydrogen Peroxide. An anti-mouse secondary antibody, conjugated with horseradish peroxidase (HRP) (GE healthcare; IL, USA), was diluted in 1% BSA in TBS-T solution to detect bound primary antibodies. The ECL membranes were developed using a Lumi-Light Western blotting substrate (Roche Diagnostics; Switzerland) and detected using the Bio-Rad ChemiDoc™ detection system (Bio-Rad Laboratories). Where inhibin/activin ELISAs were found to be unsuitable for the quantification of the mature ligands (owing to disruption of the antibody epitopes), band densitometry was performed using ImageLab software (Bio-Rad Laboratories).

Production and purification of inhibin variants

To generate sufficient quantities of inhibin for bioactivity testing, inhibin production was first scaled up in HEK293T cells. Briefly, HEK293T cells were plated at a density of 11×10^6 per 15 cm plate. After 24 hours, each plate was transfected with 60 µg (3:2, α:β) of DNA using the transfection reagent Polyethylenimine (PEI MAX, Sigma-Aldrich; MO, USA) and incubated in 20 mL of serum-free OPTI-MEM (Life Technologies) for 4 hours. Following transfection, the media was changed to fresh serum-free OPTI-MEM and protein expression allowed to proceed for a further 48 hours. Conditioned media batches were concentrated down to 5 mL using Centricon-70 devices (10 kDa molecular weight cut-off, Pall Life Science; NY, USA). Concentrates (5 mL) were resuspended in phosphate buffer (50 mM PO_4 ; 300 mM NaCl, pH 8.0), in preparation for protein purification.

Immobilized metal ion affinity chromatography (IMAC), specifically targeting the poly-histidine tag in the inhibin α-subunit, was used to extract pro-inhibin from the conditioned media. Briefly, the concentrate was incubated in a HisPur cobalt-resin column (Thermo Fisher Scientific) at room temperature for 2 hours and bound inhibin was eluted using 300 mM imidazole in phosphate buffered saline. Contaminating imidazole was removed by a Slide-A-Lyser dialysis device (Thermo Fisher Scientific), as per manufacturer's guidelines, and 0.1% BSA was added to the final eluate to minimise protein loss. Following purification, inhibin A and B levels were quantified by ELISAs as previously described (Walton et al. *Mol Cell Endocrinol.* 2013;381(1-2):106-114).

Production and purification of activin variants

Activin variants were generated via transient transfection in a HEK293T cell line, as previously described for inhibin; however, in this instance, cells were only transfected with 60 µg of the β_A - or β_B -subunits. Activin was purified from concentrated conditioned media by using a FLAG-immunoaffinity approach, which specifically targets the FLAG-tag in the β -subunits. In brief, concentrated conditioned media was incubated with FLAG M2 affinity beads (Sigma) and eluted using a competitive FLAG peptide (3x FLAG peptide, Sigma) which competitively binds to the FLAG affinity beads, as per the manufacturer's guidelines. A final concentration of 0.1% BSA was added to the eluate.

10 *COV434 in vitro luciferase assay for assessment of inhibin activity*

Inhibin *in vitro* bioactivity was determined using an activin-responsive luciferase reporter assay in COV434 cells. COV434 cells were first plated at a density of 75,000 cells per well in a 48-well plate. The following day, the cells were transfected with 200 ng per well of DNA, consisting of an A3-luciferase reporter construct (50 ng), the transcription factor FAST2 (100 ng) (Walton et al. *The Journal of biological chemistry*. 2009;284(14):9311-9320.), and betaglycan (50 ng), using Lipofectamine 3000 (Thermo Fisher Scientific) according to manufacturer's guidelines). At 24 hours post transfection, cells were treated in triplicate with 150 pM activin A or activin B (R&D Systems), and increasing doses of purified inhibins (0-10 nM). The following day, the conditioned media was removed and the cells were lysed in a solubilisation buffer (1% Triton X-100, 25 mmol/l glycylglycine, pH 7.8, 15 mmol/l MgSO₄, 4 mmol/l EGTA, and 1 mmol/l dithiothreitol) and incubated on ice for 20 minutes. The lysates were transferred to a 96-well plate and combined with an equal amount of D-luciferin (Thermo Fisher Scientific) to measure luciferase activity; luminescence was quantified using a CLARIOstar microplate reader (BMG Labtech; Germany).

Statistics

Statistical differences between wildtype and mutant inhibin/activin isoforms was assessed by a one-way ANOVA, followed by a Dunnett's multiple comparisons test, using GraphPad Prism 7.0. Data was presented as mean $\pm$ SEM. Significance was defined by *p<0.05, **p<0.01, ***p<0.001.

Example 2

Modification of the β_A -subunit disrupts activin formation during inhibin synthesis

The inventors identified three residues (Phe³²⁶, Tyr³⁴⁵ and Ala³⁴⁷) in the β_A -subunit that were central to activin A homo-dimerisation, but were likely dispensable for inhibin A hetero-dimerisation. Targeted mutations (e.g. F326G, Y345G or A347H) were introduced into the β_A -subunit, and the effect on inhibin A/activin A expression was determined following co-transfection of α - and β_A -subunits in HEK293T cells (Fig. 1A). Western blot analysis of conditioned media revealed that single point mutations Y345G, A347G and A347H (Fig 1B, lanes 3, 5 and 6, respectively) significantly reduced the levels of activin A between 70% and 95%, but had much less effect on the production of 31/34 kDa inhibin A glycosylated forms. Combining the β_A -subunit mutations Y345G and A347H completely abolished the formation of activin A, but also resulted in a marked loss (up to 75%) in inhibin A yield (Fig 1B, lane 7; Fig. 4). Additional modification of β_A -subunit residues (F326A, F326G, F326Q, F326L, Y345A, A347Q, A347E and A347F) did not reduce activin levels below those observed for the Y345G or A347H mutations (Fig 5B). Densitometry analysis across five Western blots (Fig. 1C-D) indicated that the β_A -subunit mutation, A347H, was the most advantageous in minimising activin A production, while having little impact on inhibin A heterodimer formation.

Example 3

Modification of the β_A -subunit dimerization interface silences activin A, but not inhibin A, activity

Subsequently, the inventors produced and purified wild type inhibin A and inhibin A variants with β_A -subunit mutations (Y345G, A347H or Y345G/A347H) and tested activity using an activin-responsive luciferase assay in COV434 granulosa cells. Transfected cells were treated with 100 pM activin A and increasing doses of the inhibin A variants. Luciferase activity was used as a direct measure of activation or inhibition of the canonical activin A SMAD2/3 signalling pathway. These assays revealed that β_A -subunit mutations Y345G and A347H (alone or in combination) had no effect on inhibin A's ability to suppress activin A signalling (Fig 1E), supporting that β_A -subunit residues Tyr³⁴⁵ and Ala³⁴⁷ are dispensable for inhibin A activity. Although the Y345G and A347H

mutations were highly disruptive for β_A -subunit dimerisation, some activin A contamination remained in the inhibin A preparations (Fig. 1B and D). Therefore, the inventors assessed whether the residual activin retained activity. To do this, HEK293T cells were transfected with wild type or mutant β_A -subunits alone and activin A variants were purified from the conditioned media (Fig. 5A-B). In the SMAD2/3-responsive reporter assay, wild type activin A induced a dose-dependent response, whereas the activin A Y345G variant displayed >90-fold reduction in potency (Fig. 1F). Significantly, the A347H and Y345G/A347H variants displayed no activin-like activity in this assay (Fig. 1F). These results indicate that Ala³⁴⁷ in the finger region of the β_A -subunit is required for activin A dimerisation and activity, but is largely dispensable for inhibin A formation and bioactivity.

Example 4

Generation of inhibin B free of activin B interference

The inventors next applied this approach to inhibin B, targeting β_B -subunit residue Gly³²⁹ (corresponds to Ala³⁴⁷ in the β_A -subunit) with the goal of minimising activin B interference. Gly³²⁹ at the predicted β_B/β_B dimerization interface was mutated to various residues (Fig. 2B), and the effect on inhibin B/activin B production was examined following transfection in HEK293T cells. Western blot analysis and densitometry revealed that point mutations G329L, G329E and G329D reduced activin B production by 55%, 70% and 80%, respectively, compared to wildtype (Fig. 2B-C), without altering inhibin B formation (Fig. 3D-E). In the luciferase assay, the G329E variant (IC₅₀ 0.25 nM, Fig. 2F) retained near wild-type inhibin B activity (IC₅₀ 0.1 nM), but the G329D mutation surprisingly reduced activity >10-fold (IC₅₀ 1.2 nM).

The G329E and G329D β_B -subunit mutations markedly reduced activin B expression in the inhibin B preparations, but they also affected migration of the remaining mature activin B forms through polyacrylamide electrophoresis (Fig. 2B). These shifts in molecular weight, relative to wild type activin B, suggested that these point mutations may disrupt the folding (and therefore migration) of mature activin B. Therefore, the inventors generated wild type activin B, together with activin B variants (G329E and G329D) and tested their activity in the Smad2/3-responsive luciferase assay. The G329D mutation reduced activin B activity by >90%, whereas the G329E

mutation completely abolished activin B activity (Fig 2G). Together, these results indicate that incorporation of the G329E substitution into the β_B -subunit essentially removes activin B interference during inhibin B production.

Example 5

5 *Increasing potency of inhibin A analogues*

Once the inventors had determined a means to produce inhibin in the absence of activin, the inventors then sought to improve inhibin potency by targeted mutagenesis of the receptor binding sites for betaglycan. The inventors generated the inhibin α -subunit variant (S344I) and assessed its effects on expression and activity. The S344I variant
 10 was expressed at similar levels to wild type inhibin A (Fig. 3A), but it displayed a 4.5-fold increase in potency (Fig. 3B), supporting that a hydrophobic residue in this position increases affinity for betaglycan. Surprisingly, when the S344I α -subunit mutation was combined with the A347H β_A -subunit mutation, which favours inhibin A formation, potency was increased a further 2-fold (inhibin A IC_{50} 180 pM vs. inhibin A $^{\alpha:S344I/\beta:A347H}$
 15 IC_{50} 15 pM; Fig. 3B).

The inventors have identified a means to increase both the purity and potency of inhibin A and B. First, the inventors performed a comprehensive analysis of the dimer interface of activin A. Numerous residues, including Phe³⁶⁸, His³⁶⁹, Thr³⁷¹, Ile³⁷³, His³⁷⁵, Tyr³⁷⁶, Pro³⁸³, Phe³⁸⁴, Leu³⁸⁷, Val³⁹² and Ser⁴²⁶ from one monomer, and Phe³²⁶, Val³²⁸,
 20 Ile³³³, Trp³³⁵, Tyr³⁴⁵, Ala³⁴⁷, Tyr³⁴⁹, Asn⁴¹⁷, Met⁴¹⁸ and Val⁴²⁰ from the opposing monomer, contribute to activin A dimerisation. In particular, finger domain residues (Phe³²⁶, Tyr³⁴⁵, Ala³⁴⁷, Met⁴¹⁸) interact with helix residues from the other monomer (His³⁶⁹, Ile³⁷³, Tyr³⁷⁶), while residues closer to the centre of the molecule (Asn⁴¹⁷, Val⁴²⁰, Ser⁴²⁶, Val³⁹²) provide additional stability and Cys³⁹⁰ forms an interchain disulfide bond.
 25 Interestingly, although the inhibin α -subunit evolved from the β_A -subunit, it lacks the wrist-region helix. Phylogenomic analysis revealed a complex series of indels through the wrist region of the α -subunit throughout vertebrate evolution. First the helix region was lost in non-mammalian species and then a proline-rich insertion occurred around the time eutherian mammals evolved. Biochemical analysis suggests that the proline-rich wrist region ensures that the mammalian α -subunit cannot homodimerize, but can
 30 only heterodimerize with a β -subunit.

Based on these insights, the inventors identified two finger residues in the β_A -subunit (Tyr³⁴⁵ and Ala³⁴⁷) that are central to activin A homodimer formation, but are superfluous for inhibin A heterodimerisation. Indeed, a single point mutation in the β_A -subunit, A347H, reduced activin A expression by >90%, while only marginally affecting inhibin A production. Strikingly, this same mutation completely abrogated the activity of the residual activin A produced, but had no effect on inhibin A bioactivity. Mutation of the corresponding residue in the β_B -subunit, G329E, similarly eliminated activin B interference during inhibin B production. Replacing Ala³⁴⁷ in the β_A -subunit and Gly³²⁹ in the β_B -subunit with basic or acidic residues, respectively, likely disrupts the formation/conformation of the activin A and activin B dimers. Because these residues are distant to the α/β -dimer interface, they have little to no impact on inhibin A or B formation and activity. These inactivating point mutations in the $\beta_{A/B}$ -subunits will greatly facilitate the production of pure inhibin A and inhibin B analogues.

In this study, the inventors also used targeted mutagenesis to improve inhibin potency by increasing its affinity for the co-receptor, betaglycan. In the presence of betaglycan, inhibin A/B bind and sequester activin type II receptors (ActRIIA/IIB) and, thereby, inhibit activin-induced SMAD2/3 signalling. *In vivo*, betaglycan mediates inhibin A's ability to suppress FSH production by gonadotrope cells of the pituitary, but inhibin B may utilise a distinct co-receptor. The inventors generated the α -subunit variant (S344I) and assessed the effect of this mutation on inhibin bioactivity. Inhibin A (S344I) was significantly more potent (4.5-fold) than wild type inhibin A at suppressing activin A activity, presumably via increased affinity for betaglycan. Surprisingly, combining the mutations that favoured inhibin production (β_A :A347H) and activity (α :S344I) led to a further increase in potency, such that Inhibin A (α :S344I/ β_A :A347H) was 12-fold more potent than the wild type protein. Future studies could determine whether inhibin affinity for ActRIIA/IIB could also be enhanced.

In conclusion, the inventors have identified single point mutations in the β_A - (A347H) and β_B - (G329E) subunits that eliminate activin interference during inhibin production. In addition, the inventors have identified an α -subunit point mutation (S344I) that can increase the potency of inhibin A ~12-fold. The methodology described here will enable the generation of pure and potent inhibin A and inhibin B agonists, which are useful to define the discrete and/or overlapping roles of these hormones in both

reproductive and non-reproductive tissues, and in therapeutic and preventative applications.

It will be understood that the invention disclosed and defined in this specification extends to all alternative combinations of two or more of the individual features
5 mentioned or evident from the text or drawings. All of these different combinations constitute various alternative aspects of the invention.

CLAIMS

1. An inhibin protein comprising an α -subunit and a β -subunit, wherein the α -subunit comprises a mutation or modification of a residue at, or at a position equivalent to, S344 (numbering as per SEQ ID NO: 1).
- 5
2. The inhibin protein of claim 1, wherein the mutation or modification is any that increase the activity of the inhibin protein compared to an inhibin protein that does not contain a mutation or modification of a residue at, or at a position equivalent to, S344.
- 10
3. The inhibin protein of claim 1 or 2, wherein the β -subunit is a β_A - or β_B -subunit.
4. The inhibin protein of any one of claims 1 to 3, wherein the mutation is a replacement or substitution mutation with a non-conservative amino acid.
- 15
5. The inhibin protein of claim 4, wherein the mutation is a replacement or substitution with an amino acid with a hydrophobic side chain.
6. The inhibin protein of claim 5, wherein the amino acid with a hydrophobic side chain is selected from the group consisting of alanine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine and valine.
- 20
7. The inhibin protein of claim 6, wherein the mutation is a replacement or substitution with isoleucine.
8. The inhibin protein of any one of claims 1 to 7, wherein the modification increases the hydrophobicity of the side chain of the serine residue at, or at a position equivalent to, 344.
- 25
9. The inhibin protein of claim 8, wherein the residue at, or at a position equivalent to, S344 (numbering as per SEQ ID NO: 1) is modified.
10. The inhibin protein of any one of claims 1 to 9, wherein the inhibin protein also contains one or more mutations or modifications in the β -subunit at residues in the predicted β/β homodimerization interface required for formation of an activin.

11. The inhibin protein of claim 10, wherein residues in the β_A -subunit and the residues the β_A/β_A homodimerization interface include A347, Y345, F326, H369, I373, Y376 and V392, or equivalent position (numbering as per SEQ ID NO: 4).

5 12. The inhibin protein of claim 10, wherein residues in the β_B -subunit and the residues the β_B/β_B homodimerization interface include F308, Y327, G329, H351, V355, Y358 and I373, or equivalent position (numbering as per SEQ ID NO: 7).

10 13. The inhibin protein of claim 11, wherein one or more mutations are selected from A347X₁, Y345X₂, F326X₃, H369X₄, I373X₅, Y376X₆ and V392X₇, or equivalent position, wherein each of X₁, X₂, X₃, X₄, X₅, X₆ and X₇ is any amino acid except A, Y, F, H, I, Y and V, respectively.

15 14. The inhibin protein of claim 12, wherein one or more mutations are selected from F308X₈, Y327X₉, G329X₁₀, H351X₁₁, V355X₁₂, Y358X₁₃ and I373X₁₄, or equivalent position, wherein each of X₈, X₉, X₁₀, X₁₁, X₁₂, X₁₃ and X₁₄ is any amino acid except F, Y, G, H, V, Y and I, respectively.

15 15. The inhibin protein of claim 13, wherein the mutations or modifications in the β_A subunit are made at one, two or three of F326, Y345, and A347, or equivalent position.

20 16. The inhibin protein of claim 13, wherein the mutation is a replacement or substitution mutation with a non-conservative amino acid.

17. The inhibin protein of claim 14, wherein the mutation is a replacement or substitution mutation with a non-conservative amino acid.

25 18. The inhibin protein of claim 16, wherein the mutation is a replacement or substitution with an amino acid with a basic side chain.

19. The inhibin protein of claim 13, 15 or 16, wherein the protein includes a mutation Y345G.

19. The inhibin protein of claim 16 or 19, wherein the protein includes a mutation at A347 selected from the group consisting of phenylalanine, glycine, histidine, glutamate or glutamine.
20. The inhibin protein of claim 19, wherein the protein includes one or more
5 mutations selected from the group consisting of A347F, A347G or A347H.
21. The inhibin protein of claim 14 or 17, wherein the mutations or modifications in the β_B subunit are made at G329, or equivalent position.
22. The inhibin protein of claim 21, wherein the mutation is a replacement or substitution mutation with an amino acid with a non-polar or acidic side chain.
- 10 23. The inhibin protein of claim 22, wherein the mutation at G329 is selected from the group consisting of a leucine, valine, glutamate or aspartate.
24. The inhibin protein of claim 23, wherein the protein includes a mutation of G329E.
25. The inhibin protein of any one of claims 1 to 24, wherein the inhibin is in
15 the form of a precursor inhibin.
26. The inhibin protein of any one of claims 1 to 24, wherein the inhibin is in the form of a pro-mature inhibin.
27. The inhibin protein of any one of claims 1 to 24, wherein the inhibin is in the form of a mature inhibin.
- 20 28. The inhibin protein of any one of claims 1 to 25, wherein the inhibin protein comprises α -subunit and β -subunit precursors each having at least one proprotein convertase cleavage site wherein at least one proprotein convertase cleavage site in the α -subunit and/or β -subunit is modified by an amino acid substitution mutation to render it more efficiently cleaved by the proprotein convertase.
- 25 29. The inhibin protein of claim 28, wherein the modified proprotein convertase cleavage site is ISSRKKRSVSS (SEQ ID NO: 21).
30. The inhibin protein of claim 28 or 29, wherein a proprotein convertase

cleavage site in each of the α -subunit and a β -subunit precursor is modified by the amino acid substitution mutation.

31. The inhibin protein of any one of claims 28 to 30, wherein the inhibin is of human origin.

5 32. The inhibin protein of claim 31, wherein a proprotein convertase site in the α -subunit at ²²⁹RARR²³² is replaced by ²²⁹ISSRKKRSVSS²³⁹.

33. The inhibin protein of claims 28 to 32, further comprising a poly-his tag.

34. An inhibin protein of claim 30, wherein the β -subunit is a β_A subunit.

36. The inhibin protein of claim 34, wherein the cleavage site ³⁰⁶RRRRR³¹⁰ in
10 the β_A subunit is replaced with the amino acid sequence ³⁰⁶ISSRKKRSVSS³¹⁶.

37. The inhibin protein of claim 30, wherein the β subunit is a β_B subunit.

38. The inhibin protein of claim 37, wherein the cleavage site ²⁸⁸RIRKR²⁹² in the β_B subunit is replaced by the amino acid sequence ²⁸⁸ISSRKKRSVSS²⁹⁸.

39. The inhibin protein of any one of claims 1 to 38 wherein the β subunit
15 mature domain further comprises a single point mutation within a type I receptor (ALK4) binding epitope.

40. The inhibin protein of claim 39, wherein the β_A subunit comprises an M418A substitution mutation.

41. The inhibin protein of claim 39, wherein the β_B subunit comprises an
20 M410A substitution mutation.

42. An inhibin protein comprising an α -subunit and a β_A -subunit, wherein the β_A -subunit comprises one or more of the following mutations H369X₄, I373X₅ and Y376X₆, or equivalent position, wherein each of X₄, X₅ and X₆ is any amino acid except
25 H, I and Y, respectively (based on numbering in SEQ ID NO: 4).

43. An inhibin protein comprising an α -subunit and a β_A -subunit, wherein the β_A -subunit comprises one or more of the following mutations F326A, F326G, F326L,

F326Q, Y345A, A347E, A347F, A347G, and A347Q.

44. An inhibin protein comprising an α -subunit and a β_B -subunit, wherein the β_B -subunit comprises one or more of the following mutations H351X₁₁, V355X₁₂ and
5 Y358X₁₃, or equivalent position, wherein each of X₁₁, X₁₂ and X₁₃ is any amino acid except H, V and Y, respectively (using numbering in SEQ ID NO: 7)

45. An inhibin protein comprising an α -subunit and a β_B -subunit, wherein the β_B -subunit comprises one or more mutations or modifications at G329 or an equivalent position, wherein the mutation is a replacement or substitution with an amino acid with a
10 non-polar or acidic side chain.

46. The inhibin protein of claim 45, wherein the mutation at G329 is a leucine, valine, glutamate or aspartate.

47. The inhibin protein of claim 46, wherein the mutation at G329 is a glutamate or aspartate, preferably glutamate.

15 48. A pharmaceutical composition comprising an inhibin protein of any one of claims 1 to 47, and a pharmaceutically acceptable diluent, excipient or carrier.

49. A method of treating or preventing a disease or condition arising from reduced production of an inhibin or where antagonism of an activin would ameliorate a particular disease or condition in a subject in need thereof, the method comprising
20 administering to the subject a therapeutically effective amount of an inhibin protein of any one of claims 1 to 47, or a pharmaceutical composition of claim 48, thereby treating or preventing the disease or condition.

50. Use of a therapeutically effective amount of a inhibin protein of any one of claims 1 to 47, or pharmaceutical composition of claim 48, in the manufacture of a
25 medicament for the treatment or prevention of a disease or condition arising from reduced production of an inhibin or where antagonism of an activin would ameliorate a particular disease or condition in a subject in need thereof.

51. An inhibin protein of any one of claims 1 to 47, or a pharmaceutical composition of claim 48, for use in treating or preventing a disease or condition arising

from reduced production of an inhibin or where antagonism of an activin would ameliorate a particular disease or condition in a subject in need thereof.

52. A nucleic acid molecule encoding an inhibin protein of any one of claims 1 to 47.

5 53. A nucleic acid molecule encoding an α -subunit defined in any one of claims 1 to 47.

54. A nucleic acid molecule encoding a β_A -subunit defined in any one of claims 1 to 47.

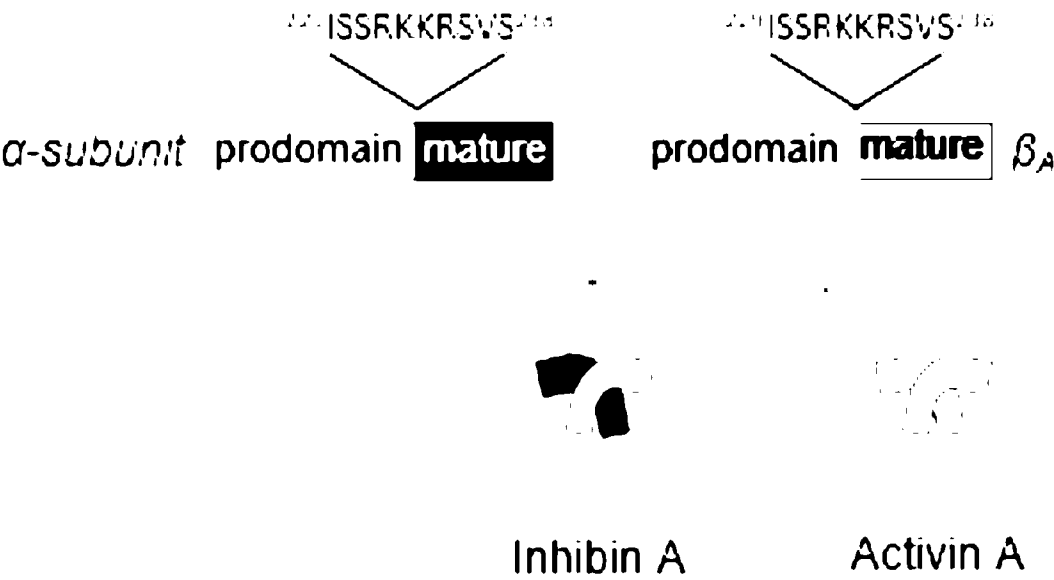
10 55. A nucleic acid molecule encoding a β_B -subunit defined in any one of claims 1 to 47.

56. A vector comprising a nucleic acid molecule of any one of claims 52 to 55.

57. An isolated cell or cell line comprising a vector of claim 56 or nucleic acid molecule of any one of claims 52 to 55.

Figure 1

A



B

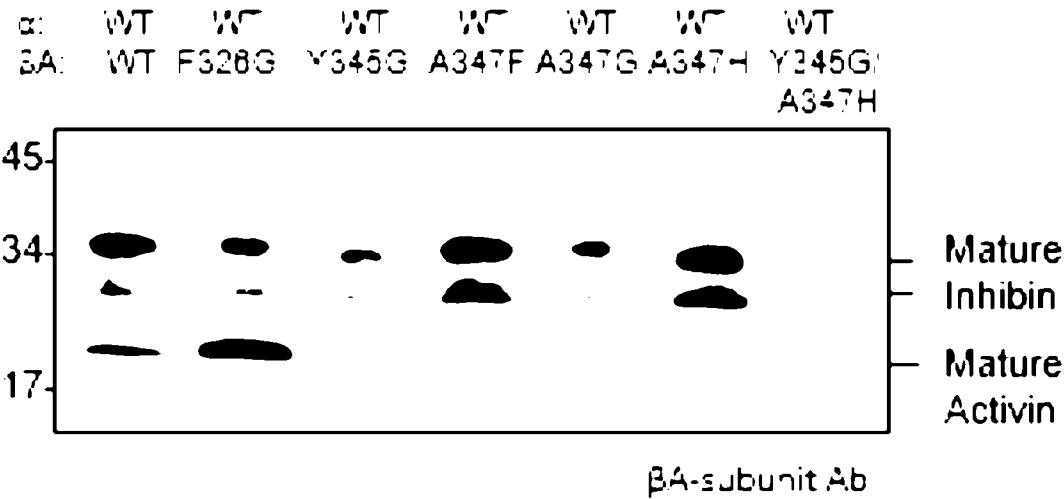
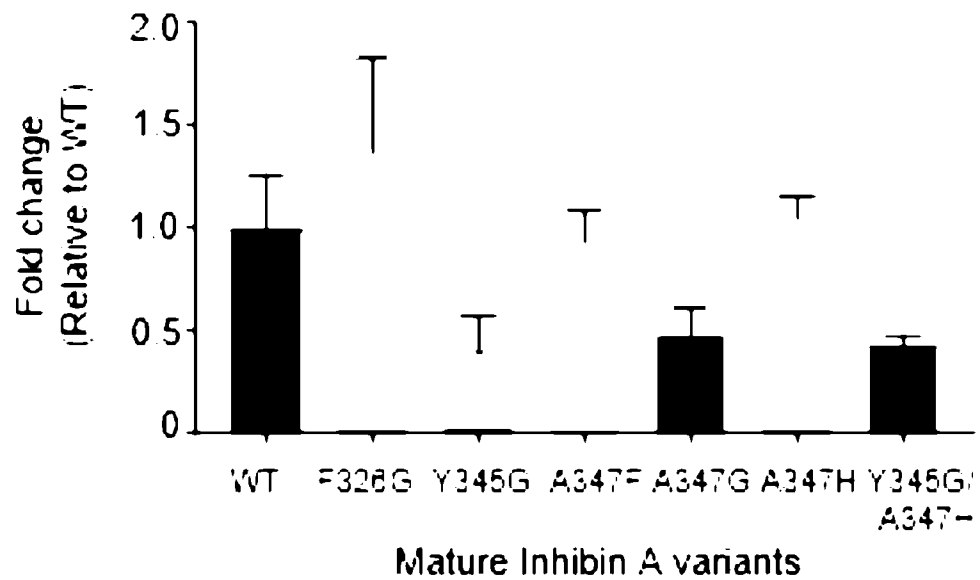


Figure 1 Continued

C



D

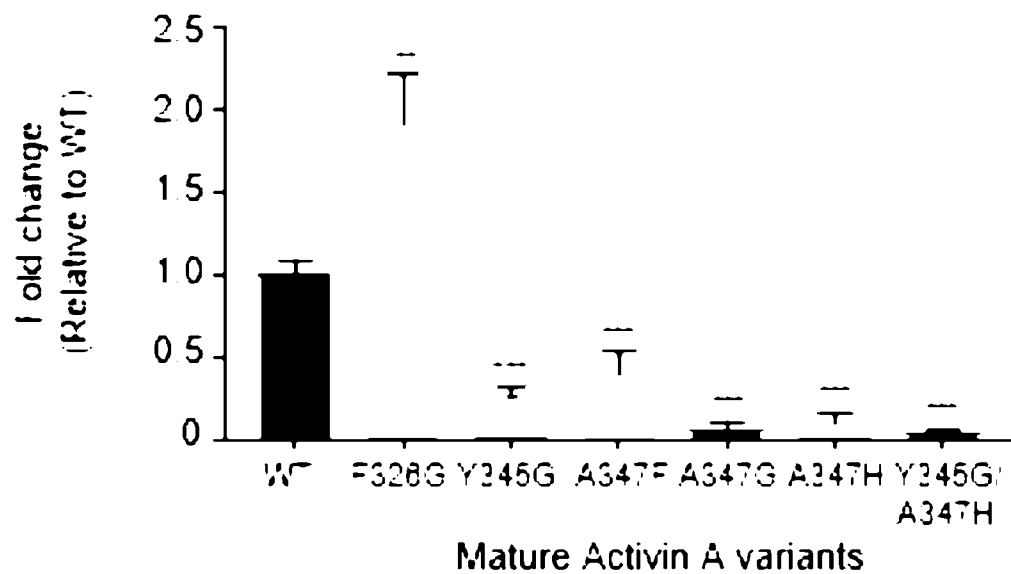
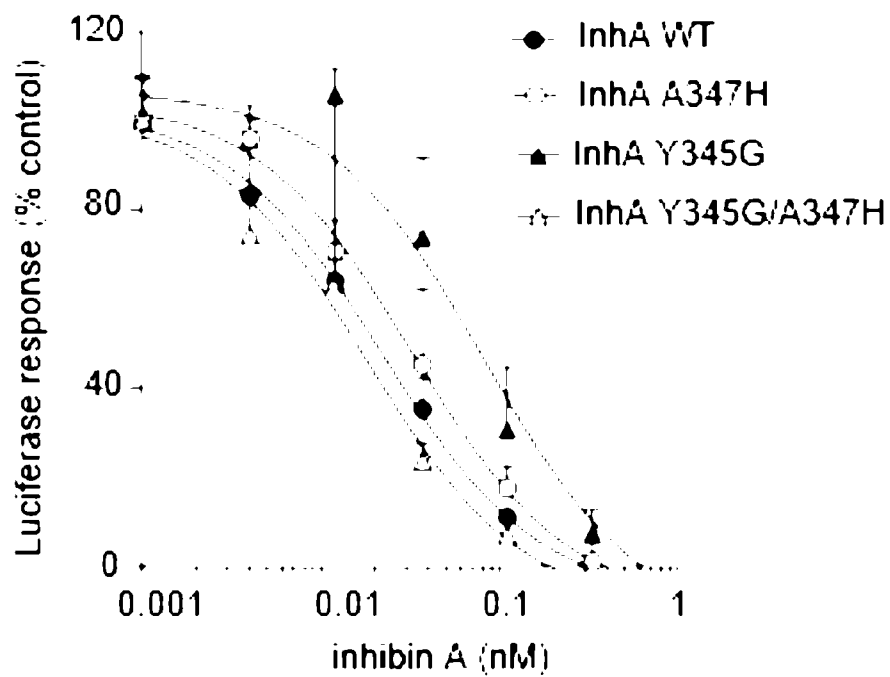


Figure 1 Continued

E



F

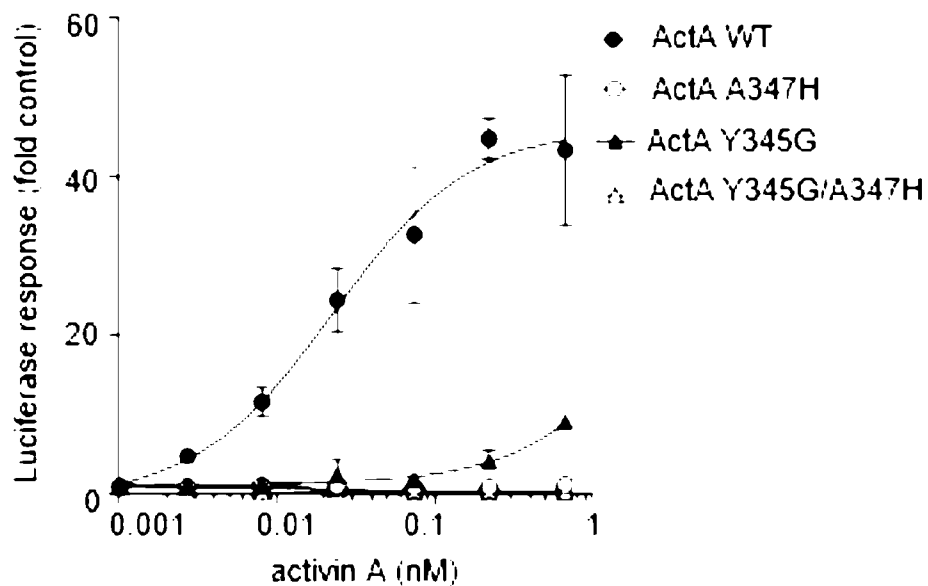
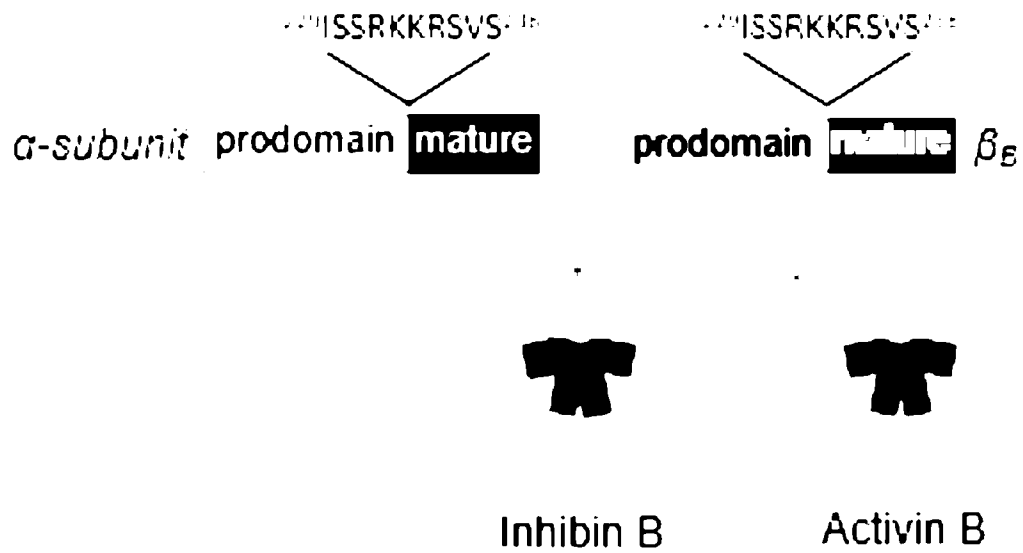


Figure 2

A

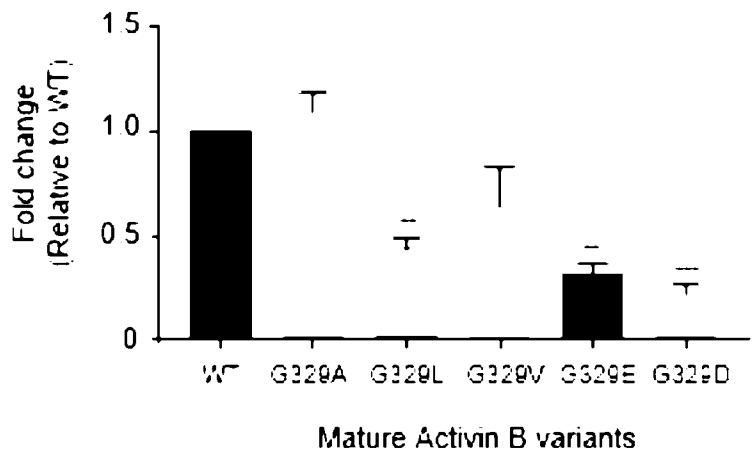


B

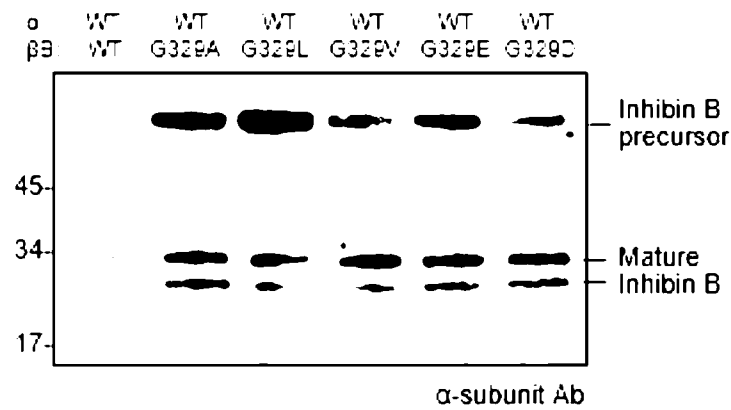


Figure 2 Continued

C



D



E

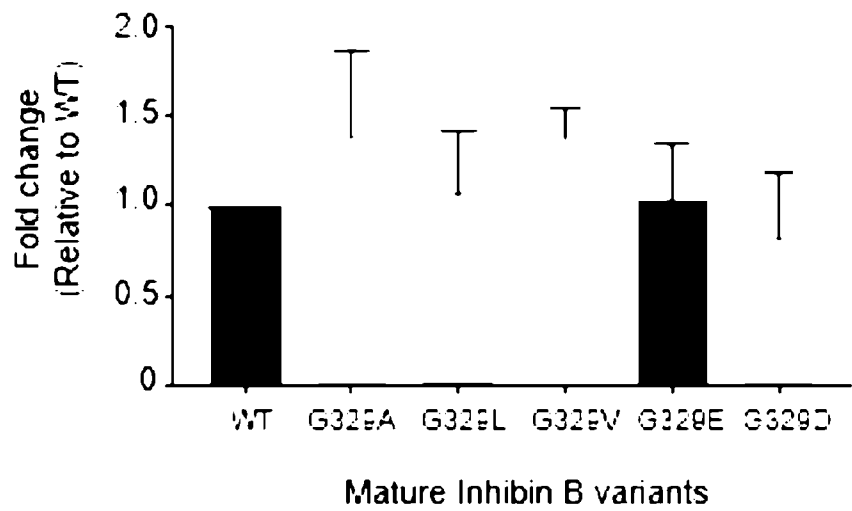
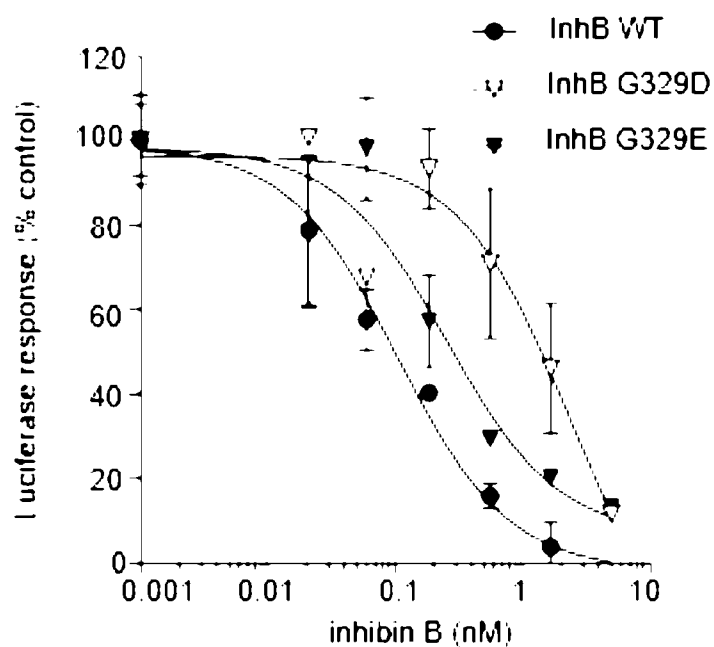


Figure 2 Continued

F



G

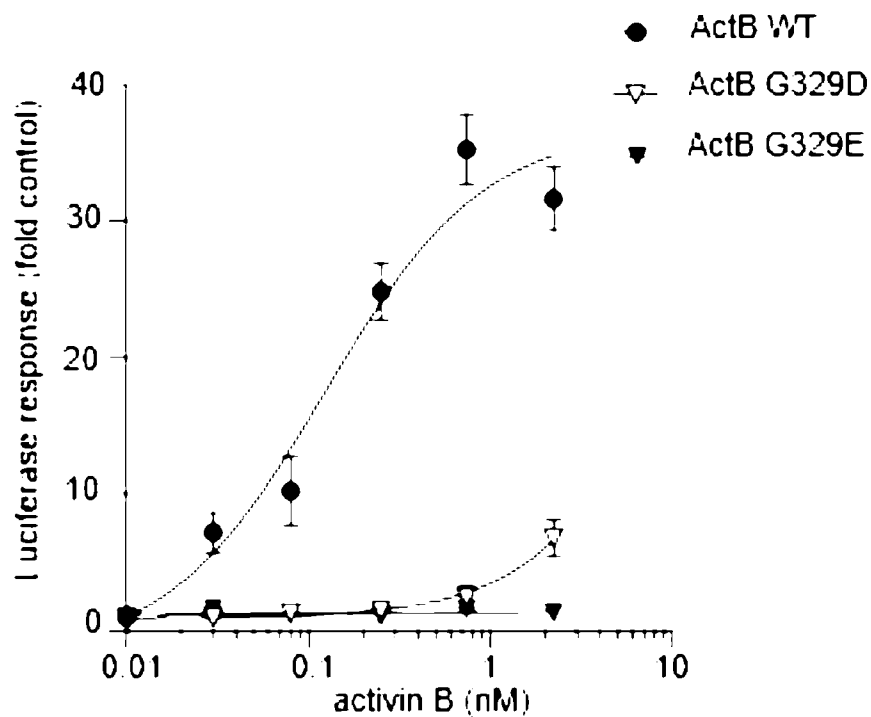
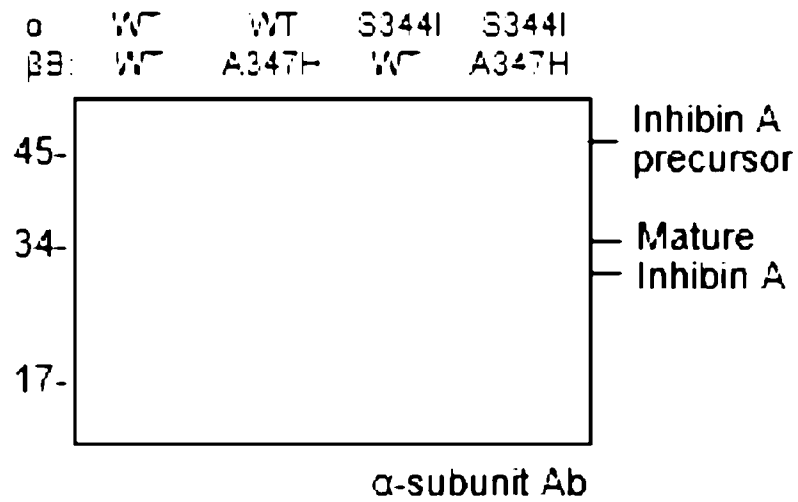


Figure 3

A



B

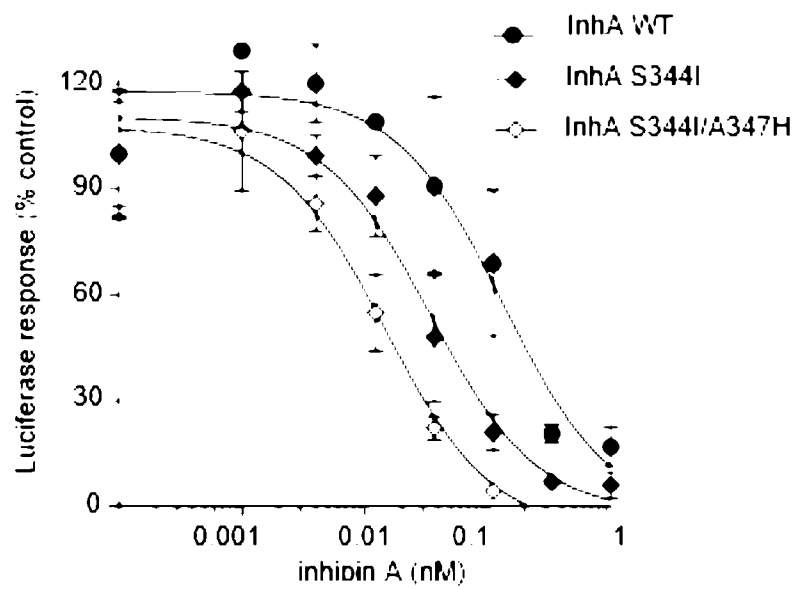


Figure 4

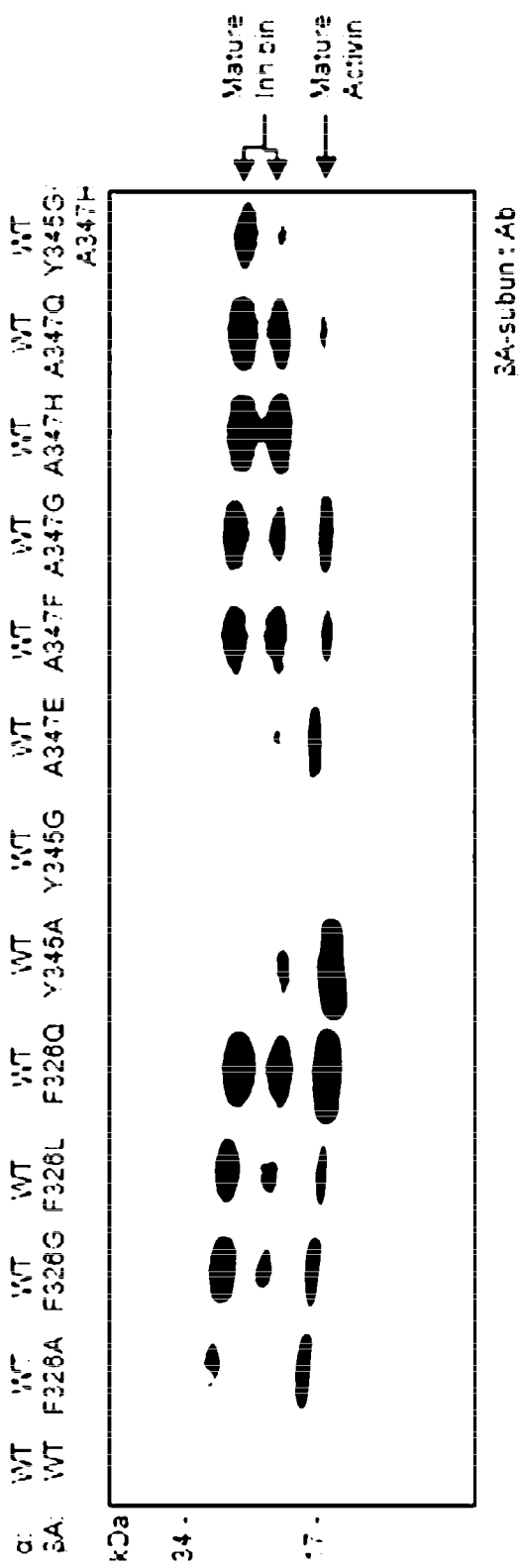
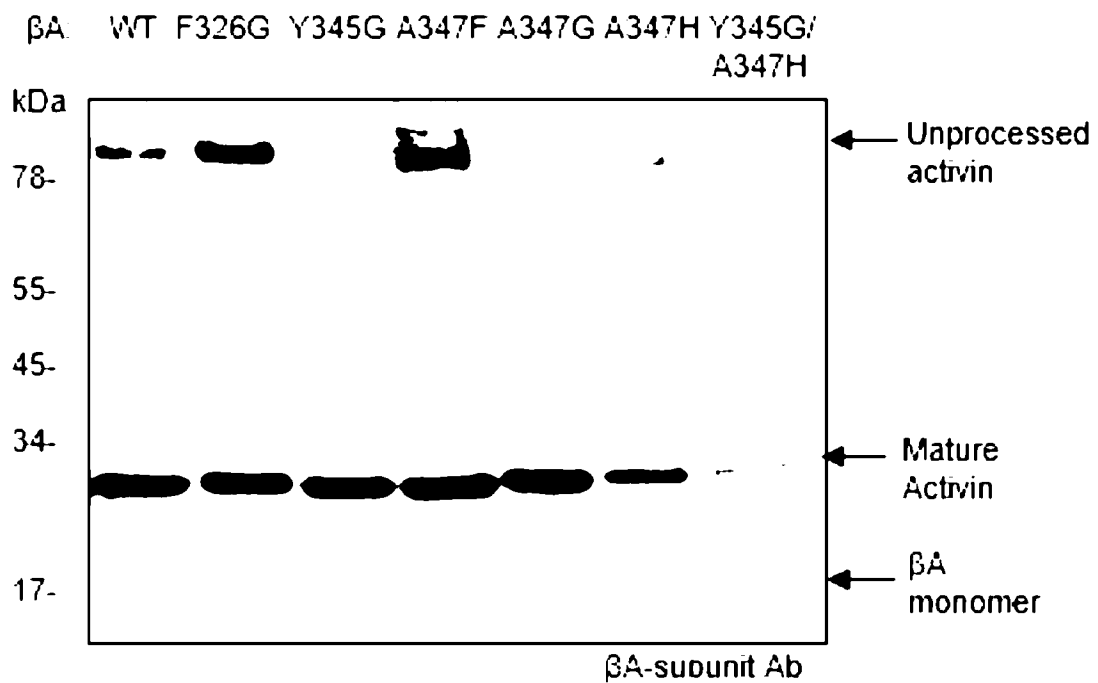
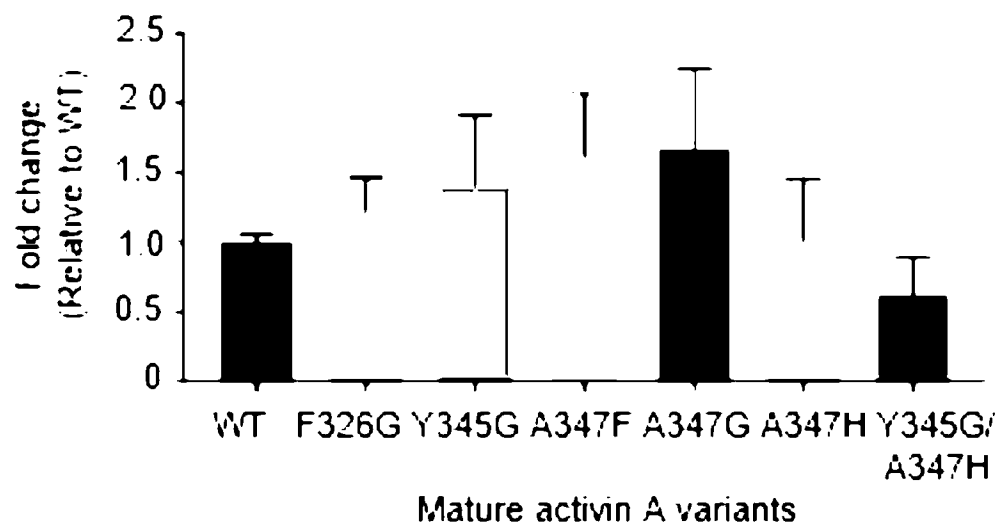


Figure 5

A



B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2021/050493

A. CLASSIFICATION OF SUBJECT MATTER

C07K 1/00 (2006.01) C07K 14/00 (2006.01) C07K 14/475 (2006.01) C07K 17/00 (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

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

Genomequest Sequence Search based on the SEQ ID NOs. 1, 4, 7; STN Registry CPlus: searched a small region around S344 i.e. TMRPLHVRTT[^S]HVRTT[^S]DGGYS[^S]DGGYSFKYET and Applicant and Inventor search in External Databases - Patentscope and Epspacenet and Internal Databases provided by IP Australia: THE UNIVERSITY OF MELBOURNE & DONNELLY, Paul Stephen; Kimberly J. NOOR, Asif.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

☒ Further documents are listed in the continuation of Box C

☒ See patent family annex

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

Date of the actual completion of the international search 7 July 2021	Date of mailing of the international search report 07 July 2021
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Authorised officer Ricky Fung AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61262223648

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/AU2021/050493
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Walton, K.L., Kelly, E.K., Chan, K.L., Harrison, C.A. and Robertson, D.M., 2015. "Inhibin biosynthesis and activity are limited by a prodomain-derived peptide" <i>Endocrinology</i> , 156(8), pp.3047-3057. See whole document especially abstract and last paragraph on right hand column on page 3056	1-9, 25-28 and 48-57
A	Makanji, Y., Walton, K.L., Wilce, M.C., Chan, K.L., Robertson, D.M. and Harrison, C.A., 2008. "Suppression of inhibin A biological activity by alterations in the binding site for betaglycan." <i>Journal of Biological Chemistry</i> , 283(24), pp.16743-16751. See whole document	1-54
A	Chand, A.L., Harrison, C.A. and Shelling, A.N., 2010. "Inhibin and premature ovarian failure." <i>Human Reproduction Update</i> , 16(1), pp.39-50. See whole document	1-54

INTERNATIONAL SEARCH REPORT Information on patent family members		International application No. PCT/AU2021/050493	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2019)			