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(54) Title: MEMBRANE SPAN-KINASE FUSION PROTEIN AND THE USES THEREOF

(57) **Abrégé/Abstract:**

The present invention relates to a recombinant membrane span protein complex, comprising (1) a fusion protein, comprising a membrane span protein fused to a kinase domain, preferably a constitutive kinase and (2) a reporter construct comprising a polypeptide, interacting with the membrane span protein, fused to a reporter phosphorylation domain. The invention relates further to the uses of such membrane span protein complex for the detection of compounds that interact with the membrane span protein and for the screening and/or detection of inhibitors of said compound-membrane span protein interactions. In a preferred embodiment, said membrane span protein is a G protein coupled receptor (GPCR) and the method is used for the screening and/or detection of inhibitors of the ligand-receptor binding.

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(57) **Abstract:** The present invention relates to a recombinant membrane span protein complex, comprising (1) a fusion protein, comprising a membrane span protein fused to a kinase domain, preferably a constitutive kinase and (2) a reporter construct comprising a polypeptide, interacting with the membrane span protein, fused to a reporter phosphorylation domain. The invention relates further to the uses of such membrane span protein complex for the detection of compounds that interact with the membrane span protein and for the screening and/or detection of inhibitors of said compound-membrane span protein interactions. In a preferred embodiment, said membrane span protein is a G protein coupled receptor (GPCR) and the method is used for the screening and/or detection of inhibitors of the ligand-receptor binding.



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Membrane span-kinase fusion protein and the uses thereof

The present invention relates to a recombinant membrane span protein complex, comprising (1) a fusion protein, comprising a membrane span protein fused to a kinase domain, preferably a constitutive kinase and (2) a reporter construct comprising a polypeptide, interacting with the membrane span protein, fused to a reporter phosphorylation domain. The invention relates further to the uses of such membrane span protein complex for the detection of compounds that interact with the membrane span protein and for the screening and/or detection of inhibitors of said compound-membrane span protein interactions. In a preferred embodiment, said membrane span protein is a G protein coupled receptor (GPCR) and the method is used for the screening and/or detection of inhibitors of the ligand-receptor binding.

Several methods have been developed to detect protein-protein interactions, all with their advantages and limitations. Co-purification of proteins and co-immunoprecipitation were amongst the first techniques used. However, these methods are tedious and do not allow high throughput screening. Moreover, they require lysis corrupting the normal cellular context. A major breakthrough was obtained by the introduction of the genetic approaches, of which the yeast two-hybrid (Fields and Song, 1989) is the most important one. Although this technique became widely used, it has several drawbacks. The fusion proteins need to be translocated to the nucleus, which is not always evident. Proteins with intrinsic transcription activation properties may cause false positives. Moreover, interactions that are dependent upon secondary modifications of the protein such as phosphorylation cannot be easily detected.

Several alternative systems have been developed to solve one or more of these problems.

Approaches based on phage display do avoid the nuclear translocation. WO9002809 describes how a binding protein can be displayed on the surface of a genetic package, such as a filamentous phage, whereby the gene encoding the binding protein is packaged inside the phage. Phages, which bear the binding protein that recognizes the target molecule, are isolated and amplified. Several improvements of the phage display approach have been proposed, as described e.g. in WO9220791, WO9710330 and WO9732017.

However, all these methods suffer from the difficulties that are inherent at the phage display methodology: the proteins need to be exposed at the phage surface and are so exposed to an environment that is not physiological relevant for the in vivo interaction. Moreover, when screening a phage library, there will be a competition between the phages that results in a selection of the high affinity binders.

US5637463 describes an improvement of the yeast two-hybrid system, whereby can be screened for modification dependent protein-protein interactions. However, this method relies on the co-expression of the modifying enzyme, which will exert its activity in the cytoplasm and may modify other enzymes than the one involved in the protein-protein interaction, which may
5 on its turn affect the viability of the host organism.

An interesting evolution is described in US5776689, by the so-called protein recruitment system. Protein-protein interactions are detected by recruitment of a guanine nucleotide exchange factor (Sos) to the plasma membrane, where Sos activates a Ras reporter molecule. This results in the survival of the cell that otherwise would not survive in the culture conditions
10 used. Although this method has certainly the advantage that the protein-protein interaction takes place under physiological conditions in the submembranary space, it has several drawbacks. Modification-dependent interactions cannot be detected. Moreover, the method is using the pleiotropic Ras pathway, which may cause technical complications. Most of these drawbacks were solved by the Mammalian Protein-Protein Interaction Trap (MAPPIT)
15 described in WO0190188, using recruitment of a prey to a cytokine type of receptor, fused to a bait. However, although this method allows to study protein-protein interactions under physiological conditions, it is not suitable to study interactions involving integral membrane proteins, particularly multispan membrane proteins, including GPCR's.

Methods for studying the interaction of proteins with a GPCR are mainly focused on ligand-receptor binding. WO9834948 discloses a GPCR wherein the amino terminus is replaced by
20 the amino-terminus of a self-activating receptor, and the use of this construct for the detection of agonists and antagonists. WO2004099419 discloses a ligand upregulatable GPCR, and the use of this construct to screen ligands. WO0158923 describes methods for detecting GPCR activity, methods for assaying GPCR activity and methods for screening GPCR ligands, G-
25 protein-coupled receptor kinase activity and compounds that interact with the GPCR regulatory process, by an enzyme complementation assay. However, this system is rather insensitive, with a maximal window of a factor 2 at the highest concentrations of agonist or antagonist used. Moreover, the system needs a mutation in arrestin, to improve arrestin binding, in order to obtain the required sensitivity.

30 Surprisingly we found that, by replacing the enzyme complementation by a detection system of a reporter phosphorylation polypeptide by a kinase, preferably a mutant kinase, even more preferably a constitutive mutant kinase, or an inactive mutant kinase that is activated by addition of an exogenous small molecule, the detection window could be increased significantly. Moreover, using a specific signaling pathway starting from the reporter
35 phosphorylation site, several reporter systems can be used.

A first aspect of the invention is a recombinant membrane span protein complex, comprising (1) a first fusion protein, comprising a membrane span protein fused to either a kinase domain or a reporter phosphorylation domain, and (2) a second fusion protein comprising a polypeptide, interacting with the membrane span protein, fused to either a reporter phosphorylation domain or a kinase domain, complementary to the first fusion protein.

“Complementary to the first fusion protein” as used here, means that in case the first fusion protein is a fusion to a kinase domain, the second fusion protein is a fusion to a reporter phosphorylation domain and vice versa: if the first fusion protein is a fusion to a reporter phosphorylation domain, the second fusion protein is a fusion to a kinase domain. In the normal two hybrid technology, the membrane span protein acts as a first interaction protein and is indicated as “bait” and the second fusion protein acts as second interaction protein and is indicated as “prey”. Preferably, said kinase domain is a mutant kinase domain. In one preferred embodiment, said mutant kinase domain is a constitutive mutant kinase domain. In another preferred embodiment, said mutant kinase domain is an inactive mutant kinase domain that is activated by addition of an exogenous small molecule. Several embodiments of the invention are represented in figure 1.

In one preferred embodiment said kinase is a constitutive kinase mutant derived from Tyk2, such as, but not limited to a constitutive Tyk2 deletion mutant or and/or a Tyk2 V678F mutant. Derived from Tyk2 as used here means that the kinase is a part of the human Tyk2 non-receptor tyrosine-protein kinase (Genbank accession number NP_003322; version NP_003322.3; SEQ ID N°1) or a mutant or variant thereof wherein said part shows constitutive kinase activity. A variant, as a non-limiting example, is a homologue, paralogue or orthologue. “Homologues” of a protein encompass peptides, oligopeptides, polypeptides, proteins and enzymes having amino acid substitutions, deletions and/or insertions relative to the unmodified protein in question and having similar biological and functional activity as the unmodified protein from which they are derived. “Orthologues” and “paralogues” encompass evolutionary concepts used to describe the ancestral relationships of genes. “Paralogues” are genes within the same species that have originated through duplication of an ancestral gene; “orthologues” are genes from different organisms that have originated through speciation, and are also derived from a common ancestral gene. Preferably, said homologue, “orthologue” or “paralogue” has a sequence identity at protein level of at least 50%, 51%, 52%, 53%, 54% or 55%, 56%, 57%, 58%, 59%, preferably 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, more preferably 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, even more preferably 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89% most preferably 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more as measured in a BLASTp (Altschul et al., 1997; Altschul et al., 2005). Variants and parts thereof according to the invention do show kinase activity. Preferably, said part is a part with constitutive kinase activity,

preferably fragment 589-1187 of SEQ ID N°1. Alternatively, said part is the part, corresponding to fragment 589-1187 of SEQ ID N° 1 in a homologue, paralogue or orthologue as defined above, wherein said part has constitutive kinase activity. In an alternative embodiment said constitutive kinase is a constitutive kinase derived from a Jak kinase, preferably from a Jak
5 kinase selected from the group consisting of Jak1 (Accession number P23458, version P23458.2), Jak2 (Accession number O60674, version O60674.2) and Jak3 (Accession number P52333, version P52333.2) or a mutant or variant thereof as defined above. Preferably, said constitutive kinase is a constitutive Jak2 deletion mutant. In still another alternative embodiment said constitutive kinase is a constitutive kinase derived from a Src kinase
10 (Accession number NP_005408, version NP_005408.1) or a mutant or variant thereof as defined above. Preferably said Src derived kinase is a kinase as depicted in SEQ ID N° 8.

In another preferred embodiment said mutant tyrosine kinase is an inactive mutant that is activated by addition of an exogenous small molecule. Such mutant kinase is known to the person skilled in the art, and has been described, as a non-limiting example, by Qiao et al
15 (2006) as a Src 388R/A mutant or a 391R/A mutation in the corresponding human Src protein (Accession number NP_938033, version NP_938033.1), or a mutant or variant thereof as defined above. Alternatively, it may be a similar mutation in the Jak kinase family, such as, but not limited to Tyk2 1027R/A, or a mutant or variant thereof.

A membrane span protein may be any membrane span protein known to the person skilled in
20 the art. Membranes include, but are not limited to the cellular membrane, the endoplasmatic reticulum and the mitochondrial membrane. A "membrane span" means that the protein crosses the membrane, while sticking out at both sides of the membrane. The "membrane span protein" as used here may contain a single membrane span, or multiple membrane spans. Preferably, said membrane span protein is a multiple membrane span protein,
25 comprising at least two membrane spans, even more preferably, said membrane span protein is a cellular membrane multispan membrane protein, most preferably said membrane span protein is a GPCR. A GPCR chain, as used here, means any polypeptide chain with 7 transmembrane spans that can function as a G-protein coupled receptor. In a preferred embodiment, it is a known GPCR; however, for the invention, the original GPCR may carry
30 mutations, insertions and/or deletions, and/or extension at the amino terminal and/or carboxyterminal end, as long as the capacity of binding with a ligand is not inhibited by the mutations or modifications.

Preferably, said kinase domain is fused at, or in the cytoplasmic part of the membrane span protein. In one preferred embodiment, said kinase domain is fused in a cytoplasmic loop of a
35 multispan membrane span protein, preferably in a cytoplasmic loop of a GPCR chain. In another preferred embodiment, said kinase domain is fused to the carboxyterminal end of the membrane span protein. The fusion may be direct, i.e. by direct coupling of the kinase domain

to the carboxyterminal end of the membrane span protein chain, or it may be indirect, using a linker sequence between the membrane span protein chain and the kinase domain. In case of a fusion within the membrane span protein chain, the linker may be situated at one side of the kinase domain, or at both sides. Preferably, said linker is shorter than 20 amino acids, more preferably shorter than 10 amino acids, even more preferably between 5 and 10 amino acids, most preferably 6 amino acids.

A reporter phosphorylation domain can be any domain that comprises a tyrosine, wherein said tyrosine can be phosphorylated by a tyrosine kinase. Preferably, said reporter phosphorylation domain is derived from or comprises a fragment of gp130, even more preferably said reporter phosphorylation domain consists of a fragment of gp130. Most preferably, said reporter phosphorylation domain consists of SEQ ID N° 2

Another aspect of the invention is the use of a recombinant membrane span protein complex according to the invention to detect compound-protein interaction, preferably protein-protein interactions. Detection of the compound-protein or protein-protein interaction may be direct or indirect. Direct detection of an interaction is the detection of the interaction of a fusion protein (fused to a reporter phosphorylation domain or a kinase domain), recruited to the membrane span protein chain (fused to a kinase domain or a reporter phosphorylation domain, complementary to the recruited fusion protein) wherein the membrane span protein or a domain thereof act as first interaction protein. In this case, the interaction of the first and second interaction protein brings the reporter phosphorylation domain close to the kinase domain and the interaction is detected by phosphorylation of the reporter phosphorylation domain. Indirect detection of an interaction is the detection of the phosphorylation of the reporter phosphorylation domain, wherein said reporter phosphorylation domain is brought in contact to the kinase domain by recruitment of a fusion protein to the receptor upon a compound-protein interaction that induces the recruitment of said fusion protein. Such compound-protein interaction may be, as a non-limiting example, the ligand-receptor binding, wherein ligand means every compound that can bind to the extracellular domain of a receptor and that is able to initiate the signaling pathway by binding to said extracellular domain. Initiating as used here means starting the events that normally directly follow the binding of the ligand to the extracellular domain of a receptor, e.g. multimerization for a multimerizing receptor, but it does not imply activation of the receptor and/or accomplishing of the signaling pathway. Compound means any chemical or biological compound, including simple or complex organic or inorganic molecules, peptides, peptido-mimetics, proteins, antibodies, carbohydrates, nucleic acids or derivatives thereof. In a special embodiment, the fusion protein that is recruited to the membrane span protein (fused to a reporter phosphorylation domain or a kinase domain) may be another membrane span protein fused to a kinase domain or a

reporter phosphorylation domain, complementary to that of the recruiting fusion protein, allowing the detection of homodimerization, homomultimerization, heterodimerization or heteromultimerization of membrane span proteins.

5 The detection of the phosphorylation of the reporter phosphorylation domain can be by any method known to the person skilled in the art. In one preferred embodiment, the reporter phosphorylation is inducing a signaling pathway, preferably a STAT3 dependent pathway, resulting in the activation of a reporter gene, such as a luciferase gene. Alternatively the phosphorylation of the reporter phosphorylation domain may be detected directly, e.g. by phosphorylation dependent binding of an antibody, or by detection of intermediates of the
10 signaling pathway such as STAT3 dimers. Still another alternative reporter system consist of a protein complementation assay, wherein one part of the protein is incorporated in or associated with the cytoplasmic protein complex according to the invention, and the second part of the protein is recruited to the phosphorylated reporter phosphorylation site, leading to a detectable activity of the reconstituted protein. Preferably, the readout of the receptor system
15 has a window of at least a factor 4, preferably at least a factor 5, even more preferably at least a factor 10. The readout window is defined as the ration of the signal to the noise (negative control).

Still another aspect of the invention is the use of a recombinant membrane span protein complex according to the invention to screen inhibitors of a compound-protein interaction, preferably a protein-protein interaction. Indeed, it is clear for the person skilled in the art that, if
20 the compound-protein interaction is giving a detectable signal, inhibitors of the compound-protein interactions can be screened by adding compounds to the test system and screening for those compounds that disturb the detectable signal.

The eukaryotic cell can be any eukaryotic cell capable of expressing a membrane span protein, including but not limited to yeast cells, fungal cells and mammalian cells. Preferably, said cell is a mammalian cell. In one preferred embodiment, the eukaryotic host cells comprising the recombinant membrane span protein chain fused to a kinase domain (or a reporter phosphorylation domain) are transformed with a library of polypeptides, all fused to the reporter phosphorylation domain (or a kinase domain, if the membrane span protein is
30 fused to a reporter phosphorylation domain). Cells, in which the reporter phosphorylation domain will be phosphorylated are comprising a prey-reporter phosphorylation domain construct that is capable of interacting with the membrane span protein chain. In another preferred embodiment the eukaryotic host cell comprises a recombinant GPCR chain fused to a kinase domain (or a reporter phosphorylation domain) and said cell is transformed with a
35 polypeptide, capable of interacting with the membrane span protein chain upon activation of the GPCR by ligand binding, wherein said polypeptide is fused to the reporter phosphorylation

domain (or a kinase domain, respectively), and the cell is contacted with compounds that may act as ligand. Binding of such compound to the GPCR will induce the recruitment of the polypeptide-phosphorylation domain fusion and result in the phosphorylation of the reporter phosphorylation domain. Polypeptide as used here
5 means any proteinaceous structure, independent of the length and includes molecules such as peptides, phosphorylated proteins and glycosylated proteins. Polypeptide as used herein is not necessarily indicating an independent compound but can also be used to indicate a part of a bigger compound, such as a domain of a protein.

Another aspect of the invention is a method to detect compound-protein interactions,
10 said method comprising (1) transforming a eukaryotic host cell with a first fusion protein, comprising a recombinant membrane span protein chain, fused to either a kinase domain or a reporter phosphorylation domain (2) transforming the same host cell with at least one second fusion protein, comprising a polypeptide, fused to either a reporter phosphorylation domain or a kinase domain, complementary to the first fusion protein
15 wherein said polypeptide is capable of interacting with the membrane span protein chain (3) adding the compound to be tested to the cell (4) optionally adding the ligand to the cell and (5) detecting the phosphorylation of the reporter phosphorylation domain. The sequence of the transformation steps may be inverted; a ligand is added in cases where the compound is not tested as a ligand; in this case, the steps of adding
20 compound and ligand may be interchanged.

Still another aspect of the invention is a method to screen inhibitors of a compound-protein interaction, said method comprising 1) transforming a eukaryotic host cell with first fusion protein, comprising a recombinant membrane span protein chain, fused to a either kinase domain or a reporter phosphorylation domain (2) transforming the same
25 host cell with at least one second fusion protein, comprising a polypeptide, fused to a either a reporter phosphorylation domain or a kinase domain, complementary to the first fusion protein, wherein said polypeptide is interacting with the membrane span protein chain (3) adding at least one possible inhibitor molecule (4) adding the ligand to the cell and (5) detecting the phosphorylation of the reporter phosphorylation domain.

Preferably, the same set up without inhibitor is used as positive control for the protein-protein interaction. The sequence of the transformation steps may be inverted; the steps of adding inhibitor and ligand may be interchanged.

The present invention as claimed relates to:

- 5 - a recombinant membrane span protein complex, comprising (1) a first fusion protein, comprising a membrane span protein chain fused to either a tyrosine kinase domain or a reporter phosphorylation domain, and (2) a second fusion protein comprising a polypeptide, interacting with said membrane span protein chain, fused to either a reporter phosphorylation domain or a tyrosine kinase domain, wherein when the first fusion protein is
10 a fusion to a tyrosine kinase domain, the second fusion protein is a fusion to a reporter phosphorylation domain and vice versa, and wherein the tyrosine kinase phosphorylates the reporter phosphorylation domain;
- use of the recombinant membrane span protein complex of the invention, to detect a compound-protein interaction with the membrane span protein chain of the fusion
15 protein, or to screen for an inhibitor of a compound-protein interaction with the membrane span protein chain of the first fusion protein;
- a method to detect compound-protein interactions, said method comprising (1) transforming a eukaryotic host cell with a first fusion protein, comprising a recombinant membrane span protein chain, fused to either a tyrosine kinase domain or a reporter
20 phosphorylation domain; (2) transforming the same host cell with at least one second fusion protein, comprising a polypeptide, fused to a either a reporter phosphorylation domain or a tyrosine kinase domain, wherein when the first fusion protein is a fusion to a tyrosine kinase domain, the second fusion protein is a fusion to a reporter phosphorylation domain and vice versa, and wherein said polypeptide is interacting with
25 the membrane span protein chain; (3) adding the compound to be tested to the cell; (4) optionally adding a ligand to the cell; (5) detecting phosphorylation of the reporter phosphorylation domain; and (6) detecting a compound-protein interaction by detecting presence of a phosphorylated reporter phosphorylation domain in the cell; and

- a method to screen inhibitors of a compound-protein interaction, said method comprising 1) transforming a eukaryotic host cell with first fusion protein, comprising a recombinant membrane span protein chain, fused to a either tyrosine kinase domain or a reporter phosphorylation domain; (2) transforming the same host cell with at least one
- 5 second fusion protein, comprising a polypeptide, fused to a either a reporter phosphorylation domain or a tyrosine kinase domain, wherein when the first fusion protein is a fusion to a tyrosine kinase domain, the second fusion protein is a fusion to a reporter phosphorylation domain and vice versa, and wherein said polypeptide is interacting with the membrane span protein chain; (3) adding at least one possible
- 10 inhibitor molecule; (4) adding a ligand to the cell; (5) detecting phosphorylation of the reporter phosphorylation domain; and (6) detecting an inhibitor of compound-protein interaction by detecting absence of a phosphorylated reporter phosphorylation domain in the cell.

DEFINITIONS

- 15 The following definitions are set forth to illustrate and define the meaning and scope of various terms used to describe the invention herein.

Protein as used here means a chain composed of amino acids, independent of the length. The terms 'protein' and 'polypeptide' are interchangeable. The protein can be modified by modifications such as, but not limited to phosphorylation, glycosylation, ubiquitination and acetylation.

- 5 *Domain* as used here is a part of a polypeptide, wherein said part may carry a specific function, such as, but not limited to and enzymatic center or a phosphorylation site.

Protein complex as used here means a structure that comprises at least two, non-covalently linked, protein molecules. Protein complexes can consist of more than two proteins, and include other molecules that are not proteins. Some non-limiting examples of such molecules
10 are metal ions, ATP, or carbohydrate molecules.

A *kinase* as used here is a polypeptide that can transfer a phosphate group to an amino acid of the same or another polypeptide. Preferably, said amino acid is a serine, a threonine or a tyrosine. Even more preferably said amino acid is embedded in a phosphorylation site. A
15 *phosphorylation site* as used here is a pattern of several amino acids, preferably comprising a serine, threonine or a tyrosine, and determining the amino acid that will be phosphorylated by the kinase. Most kinases can occur in an inactive and in an active state, wherein the reporter phosphorylation site is only phosphorylated in the active state of the kinase. Kinases can be switched from the inactive from to the active form by phosphorylation, or by other modifications
20 such as proteolysis, or by mutation. The phosphorylation can be autophosphorylation, crossphosphorylation (by a protein complex of identical kinases) or by action of another kinase.

Constitutive as used here means that the kinase is continuously in the active state, normally as a consequence of a mutation, or by proteolytic cleavage removing an inhibitor. Constitutive
25 kinases are known to the person skilled in the art and comprise, but are not limited to truncated forms of Tyk2, truncated forms of Src kinase and point mutations such as Tyk2 (V678F), Jak1 (V658F) and Jak2(V617F) .

An *inactive* kinase mutant, means that that the mutant form shows a kinase activity that is significantly lower than the original non mutated form. Preferably, the remaining activity is lower than 50% of the original activity, even more preferably lower than 20%, more preferably
30 lower than 10%, most preferably lower than 5% of the original activity.

Activated by the addition an exogenous small compound as used here means that the activity of the inactive kinase is partly or totally restored by addition of a small compound to the cells, whereby said small compound, exogenous to the cell, is taken up by the cell and activates the kinase as an intracellular exogenous compound. "Activated by the addition an exogenous
35 small compound" is used to make a distinction with ligand-receptor induced activation, where a

ligand is binding to the extracellular part of a receptor, and induces activation of the kinase. "Exogenous" as used here means that the compound is normally not present in the cell.

Reporter phosphorylation site is the site that is phosphorylated in the protein complex upon interaction of the first and the second interaction polypeptide; it is distinct from a possible

5 phosphorylation site in the kinase domain that is autophosphorylated in the constitutive kinase. *First interaction polypeptide* as used here is a polypeptide of which one wants to study the interaction with one or more compounds. The first interaction polypeptide is normally referred to as a "bait" in the two hybrid terminology.

Second interaction polypeptide as used here is a polypeptide that is presented to study its

10 interaction with the first interaction polypeptide. The second interaction polypeptide is normally referred to as a "prey" in the two hybrid terminology. It is clear for the person skilled in the art that the first and the second interaction polypeptide are interchangeable in the invention, in this respect that either a "bait" or a "prey" may be fused to constitutive kinase according to the invention. Indeed, the resulting protein complex will have an identical overall composition,

15 composed of the four essential elements (first interaction polypeptide, second interaction polypeptide, constitutive kinase and reporter phosphorylation site), and independent whether the first interaction polypeptide is fused to the constitutive kinase or the reporter phosphorylation site (wherein the second interaction polypeptide is then fused to the reporter phosphorylation site, and the constitutive kinase, respectively), the interaction of the first with

20 the second interacting polypeptide will lead to the formation of a cytoplasmic protein complex according to the invention, and will result in the phosphorylation of the reporter phosphorylation site. In one preferred embodiment, the first and the second interaction protein are identical to study homodimerization or homomultimerization of a protein. In another preferred embodiment the first and the second protein are different, allowing to study protein-protein interactions of

25 heterodimers or heteromultimers.

Compound means any chemical or biological compound, including simple or complex organic or inorganic molecules, peptides, peptido-mimetics, proteins, antibodies, carbohydrates, nucleic acids or derivatives thereof.

Interaction means any interaction, be it direct or indirect. A direct interaction implies a contact

30 between the interaction partners. An indirect interaction means any interaction whereby the interaction partners interact in a complex of more than two compounds. This interaction can be completely indirect, with the help of one or more bridging compounds, or partly indirect, where there is still a direct contact that is stabilized by the interaction of one or more compounds.

35 BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Schematic representation of different embodiments of the recombinant membrane span protein complex according to the invention. "M" depicts a membrane.

- A. A membrane span protein (X) is fused to a constitutive kinase (K) and a polypeptide (Y) is fused to a reporter phosphorylation site (R). Interaction between the membrane span protein X and the polypeptide Y results in the reporter phosphorylation site being phosphorylated (P) by the constitutive kinase, leading to a detectable activity.
- 5 B. A membrane span protein (X) is fused to a reporter phosphorylation site (R) and a polypeptide (Y) is fused to a constitutive kinase (K). Interaction between the membrane span protein X and the polypeptide Y results in the reporter phosphorylation site being phosphorylated (P) by the constitutive kinase, leading to a detectable activity.
- 10 C. A membrane span protein (X) is fused to a constitutive kinase (K) and a second membrane span protein (Y) is fused to a reporter phosphorylation site (R). Interaction between the membrane span proteins X and Y results in the reporter phosphorylation site being phosphorylated (P) by the constitutive kinase, leading to a detectable activity.

Figure 2: Detection of the ligand-dependent interaction between human somatostatin receptor 2 (SSTR2) and human beta arrestin 2 (ARRB2) in an assay variant that comprises mutant Tyk2 kinase fusion proteins.

A. Schematic overview of the assay. The membrane span protein (X) is fused to the C-terminal region of Tyk2 comprising the kinase domain, whereas the polypeptide interacting with the membrane span protein (Y) is fused to a fragment of gp130 which contains phosphorylation sites. When membrane span protein X and the polypeptide Y interact, the Tyk2 kinase domain phosphorylates the phosphorylation sites of gp130. STAT3 transcription factors are recruited to these phosphorylated sites and are in turn phosphorylated by the Tyk2 kinase domain, which results in their activation. Dimers of activated STAT3 transcription factors are able to bind the specific rPAPI promoter which drives the expression of a firefly luciferase reporter gene. The activity of this reporter gene is measured as light production in a luciferase detection assay using a luminometer.

B. Application to the analysis of ligand-dependent interaction between SSTR2 and ARRB2. Cells were transfected with the indicated combination of plasmids, and either left untreated (NS) or treated with increasing doses (0,1-1-10 μ M) of somatostatin:

- 30 a) pMet7-HA-Tyk2(C) + pMG2-ARRB2 + pXP2d2-rPAPI-luciferase
 b) pMet7-SSTR2-Tyk2(C)-HA + pMG2-SVT + pXP2d2-rPAPI-luciferase
 c) pMet7-SSTR2-Tyk2(C)-HA + pMG2-ARRB2 + pXP2d2-rPAPI-luciferase

Luciferase activity is shown as fold induction relative to the luciferase activity measured in untreated cells. Error bars indicate standard deviation.

35 C. Detection of the ligand-dependent interaction between SSTR2 and ARRB2 using an alternative expression vector. Cells were transfected with the indicated combination of

plasmids, and either left untreated (NS) or treated with increasing doses (0,1-1-10 μ M) of somatostatin:

- a) pSVSport-HA-Tyk2(C) + pMG2-ARRB2 + pXP2d2-rPAPI-luciferase
- b) pSVSport-SSTR2-Tyk2(C)-HA + pMG2-SVT + pXP2d2-rPAPI-luciferase
- 5 c) pSVSport-SSTR2-Tyk2(C)-HA + pMG2-ARRB2 + pXP2d2-rPAPI-luciferase

Luciferase activity is shown as fold induction relative to the luciferase activity measured in untreated cells. Error bars indicate standard deviation.

- D. Dose-response curve of the ligand-dependent interaction between SSTR2 and ARRB2. Cells were transfected with a combination of the plasmids pMet7-SSTR2-Tyk2(C)-HA, pMG2-ARRB2 and pXP2d2-rPAPI-luciferase, and treated with increasing concentrations of somatostatin (SST-14). Luciferase activity is shown as relative light units (rlu). Error bars indicate standard deviation.

Figure 3: Analysis of the interaction between human angiotensin receptor 1 (AGTR1) and ARRB2.

- A. Detection of the ligand-dependent interaction between AGTR1 and ARRB2. Cells were transfected with the indicated combination of plasmids, and either left untreated (NS) or treated with increasing doses (0,1-1-10 μ M) of angiotensin II:

- a) pMet7-HA-Tyk2(C) + pMG2-ARRB2 + pXP2d2-rPAPI-luciferase
- 20 b) pMet7-AGTR1-Tyk2(C)-HA + pMG2-SVT + pXP2d2-rPAPI-luciferase
- c) pMet7-AGTR1-Tyk2(C)-HA + pMG2-ARRB2 + pXP2d2-rPAPI-luciferase

Luciferase activity is shown as fold induction relative to the luciferase activity measured in untreated cells. Error bars indicate standard deviation.

- B. Dose-response curve of the ligand-dependent interaction between AGTR1 and ARRB2. Cells were transfected with a combination of the plasmids pMet7-AGTR1-Tyk2(C)-HA, pMG2-ARRB2 and pXP2d2-rPAPI-luciferase, and treated with increasing concentrations of angiotensin II (AngII). Luciferase activity is shown as relative light units (rlu). Error bars indicate standard deviation.

Figure 4: Evaluation of the effect of GPCR antagonists on the interaction between GPCRs and ARRB2. Cells were transfected with the indicated combination of plasmids, and treated with the indicated combinations of GPCR ligand and antagonist (ligand: 1 μ M somatostatin for transfections a and b, 10 μ M angiotensin II for transfections c and d; antagonists: 0,05 or 0,5 μ M CYN154806; 0,1 or 1 μ M losartan or telmisartan):

- 35 a) pMet7-SSTR2-Tyk2(C)-HA + pMG1-EFHA1 + pXP2d2-rPAPI-luciferase
- b) pMet7-SSTR2-Tyk2(C)-HA + pMG2-ARRB2 + pXP2d2-rPAPI-luciferase
- c) pMet7-AGTR1-Tyk2(C)-HA + pMG1-EFHA1 + pXP2d2-rPAPI-luciferase

d) pMet7-AGTR1-Tyk2(C)-HA + pMG2-ARRB2 + pXP2d2-rPAPI-luciferase

Luciferase activity is shown as arbitrary light units. Error bars indicate standard deviation.

Figure 5: Dose-dependent effect of GPCR antagonists on the detection of the interaction between GPCRs and ARRB2.

A. Analysis of the effect of GPCR antagonists in an assay measuring the interaction between SSTR2 and ARRB2. Cells were transfected with the following combination of plasmids: pMet7-SSTR2-Tyk2(C)-HA + pMG2-ARRB2 + pXP2d2-rPAPI-luciferase. Cells were either left untreated, treated with 10 μ M somatostatin or treated with a combination of 10 μ M somatostatin and increasing doses (10^{-13} M up to 10^{-6} M) of either GPCR antagonist (CYN154806, losartan, telmisartan). Luciferase activity is shown as relative light units (rlu). Error bars indicate standard deviation.

B. Analysis of the effect of GPCR antagonists in an assay measuring the interaction between AGTR1 and ARRB2. Cells were transfected with the following combination of plasmids: pMet7-AGTR1-Tyk2(C)-HA + pMG2-ARRB2 + pXP2d2-rPAPI-luciferase. Cells were either left untreated, treated with 10 μ M angiotensin II or treated with a combination of 10 μ M angiotensin II and increasing doses (10^{-13} M up to 10^{-6} M) of either GPCR antagonist (CYN154806, losartan, telmisartan). Luciferase activity is shown as relative light units (rlu). Error bars indicate standard deviation.

Figure 6: Analysis of ERN1 dimerization.

A. Detection of ERN1 dimerization upon induction of ER (endoplasmic reticulum)-stress by treatment with tunicamycin. Cells were transfected with the following plasmids:

a) pcDNA5/FRT/TO-ERN1-Tyk2(C)-HA + pMG1 + pXP2d2-rPAPI-luciferase

b) pcDNA5/FRT/TO-ERN1-Tyk2(C)-HA + pMG2C-ERN1 + pXP2d2-rPAPI-luciferase

After transfection, cells were treated with 0-0.5-1-2 μ g/ml tunicamycin, final concentration. Error bars indicate standard deviation.

B. Detection of ERN1 dimerization upon induction of ER-stress by treatment with tunicamycin. Cells were transfected with the following plasmids:

a) pcDNA5/FRT/TO-ERN1-Tyk2(C)-HA + pMG1 + pXP2d2-rPAPI-luciferase

b) pcDNA5/FRT/TO-ERN1-Tyk2(C)-HA + pMG2C-ERN1 + pXP2d2-rPAPI-luciferase

c) pcDNA5/FRT/TO-ERN1-Tyk2(C)-HA + pMG2C-ERN1cyt + pXP2d2-rPAPI-luciferase

After transfection, cells were treated with increasing doses tunicamycin. Luciferase activity is shown as fold induction relative to the luciferase signal obtained in cells transfected with unfused gp130 (transfection a) and treated with the same concentration tunicamycin. Error bars indicate standard deviation. Expression of Tyk2(C) and gp130 fusion constructs was

evaluated through Western blot applying anti-HA and anti-gp130 antibodies, respectively. Beta-actin expression was stained as a control for equal loading.

C. Analysis of ERN1 structure-function relationship. Cells were transfected with the pXP2d2-rPAPI-luciferase plasmid combined with the indicated Tyk2(C) fusion constructs (pcDNA5/FRT/TO-ERN1-Tyk2(C)-HA, pcDNA5/FRT/TO-ERN1(K599A)-Tyk2(C)-HA or pcDNA5/FRT/TO-ERN1(D123P)-Tyk2(C)-HA) and gp130 fusion constructs (pMG1, encoding unfused gp130 or pMG2C-ERN1 encoding ERN1-gp130), and treated with either tunicamycin or vehicle control (DMSO). Luciferase activity is shown as fold induction relative to the luciferase signal obtained in cells transfected with unfused gp130. Error bars indicate standard deviation. Expression of Tyk2(C) fusion constructs was evaluated through Western blot applying an anti-HA antibody. Beta-actin expression was stained as a control for equal loading.

D. Detection of disruptors of ERN1 dimerization. Cells were transfected with the following plasmids:

- a) pcDNA5/FRT/TO-ERN1-Tyk2(C)-HA + pMG1 + pXP2d2-rPAPI-luciferase
- b) pcDNA5/FRT/TO-ERN1-Tyk2(C)-HA + pMG2C-ERN1 + pXP2d2-rPAPI-luciferase

After transfection, cells were treated with tunicamycin or vehicle control (DMSO) combined with increasing doses of Irestatin 9389. Luciferase activity of cells transfected with gp130-fused ERN1 (transfection b) is shown as fold induction relative to the luciferase signal obtained in cells transfected with unfused gp130 (transfection a) and treated with the same concentration of vehicle or tunicamycin with Irestatin 9389. Error bars indicate standard deviation.

Figure 7: Detection of the interaction between the serotonin transporter (SERT) and synaptobrevins 1 and 2 (VAMP1 and VAMP2). Cells were transfected with the pXP2d2-rPAPI-luciferase plasmid combined with the indicated Tyk2(C) and gp130 fusion constructs. Luciferase activity is shown as fold induction relative to the luciferase signal obtained in cells transfected with unfused gp130 (pMG2). Error bars indicate standard deviation.

EXAMPLES

Materials and methods to the invention

Plasmids used in the examples

A first type of plasmids express chimeric proteins consisting of an HA-tagged C-terminal portion of human Tyk2 fused at its N-terminus to the membrane span protein and are generated in the pMet7 vector, which contains a strong constitutive hybrid SR α promoter

(Takebe et al., 1988). A pMet7-dest-Tyk2(C)-HA Gateway destination vector was constructed by first amplifying the Gateway cassette from the pMG1 Gateway destination vector (Lievens et al., 2009) using primers 1 and 2 (see Table below). These primers contained an AgeI and PspOMI restriction enzyme recognition site, respectively, and these enzymes were used to digest the PCR amplicon. Next, the sequence encoding the C-terminal end of human Tyk2 comprising the kinase domain (starting from amino acids 589 and omitting the stop codon) was amplified by PCR on cDNA from HEK293 cells with primers 3 and 4 (see Table below). The former primer contained a NotI restriction site, whereas the latter contained an HA-tag coding sequence as well as an XbaI restriction enzyme recognition site. The PCR amplicon was digested with NotI and XbaI and, together with the AgeI and PspOMI cut fragment described above, ligated in the AgeI-XbaI cut pMet7 vector to generate the pMet7-dest-Tyk2(C)-HA Gateway destination vector. The pMet7-SSTR2-Tyk2(C)-HA and pMet7-AGTR1-Tyk2(C)-HA plasmids were produced by Gateway recombination mediated transfer of the full length sequence of human SSTR2 and AGTR1, respectively, from entry vectors of the hORFeome collection (Lamesch et al., 2007) into the pMet7-dest-Tyk2(C)-HA Gateway destination vector. Using the restriction enzymes EcoRI and MluI, the SSTR2-Tyk2(C)-HA insert (SEQ ID N° 3) of pMet7-SSTR2-Tyk2(C)-HA was subcloned into pSVSport (Invitrogen) to generate pSVSport-SSTR2-Tyk2(C)-HA. The AGTR1-Tyk2-HA construct is depicted in SEQ ID N° 4.

The control plasmids pMet7-HA-Tyk2(C) and pSVSport-HA-Tyk2(C), which are made of the same C-terminal Tyk2 fragment as described above, an HA-tag at the 5' end and a multiple cloning site at the 3' end were generated by PCR amplification of the Tyk2 sequence on the pMet7-dest-Tyk2(C)-HA template vector using primers 5 and 6 (see Table below). These primers contain an MfeI site and an HA-tag coding sequence together with an XbaI restriction site, respectively. The MfeI-XbaI digested amplicon was ligated both in the EcoRI-XbaI digested pMet7 vector to result in pMet7-HA-Tyk2(C), and in the EcoRI-XbaI digested pSVSport vector (Invitrogen) to generate pSVSport-HA-Tyk2(C).

pSVSport-HA-Tyk2(C)-RTp66 was produced by transfer of the RTp66 insert from pMG2-RTp66 (Pattyn et al., 2008) to pSVSport-HA-Tyk2(C) using the EcoRI and NotI restriction sites. The HA-Tyk2(C)-RTp66 construct is depicted in SEQ ID N° 28. To generate the pSVSport-HA-Tyk2(C)-SERT plasmid, human SERT was amplified on a SERT containing plasmid template using primers 18 and 19, containing EcoRV and NotI restriction sites, respectively. The amplicon was digested with EcoRV, rendered blunt end by the use of Pfu DNA polymerase and subsequently cut with NotI. This fragment was ligated in pSVSport-HA-Tyk2(C) that was cut with EcoRI, rendered blunt end through Pfu DNA Polymerase treatment and subsequently cut with NotI. The HA-Tyk2(C)-SERT construct is shown in SEQ ID N° 29.

To generate the pcDNA5/FRT/TO-ERN1-Tyk2(C)-HA plasmid, human ERN1 was amplified with primers 9 and 10, containing HindIII and NotI restriction enzyme recognition sites,

respectively, using an ERN1 entry clone from the hORFeome collection (Lamesch et al., 2007) as a template. The sequence encoding the C-terminal end of human Tyk2 comprising the kinase domain (starting from amino acids 589 and omitting the stop codon) was amplified by PCR on cDNA from HEK293 cells with primers 11 and 12. The former primer contained a NotI restriction site, whereas the latter contained an HA-tag coding sequence as well as an ApaI restriction enzyme recognition site. The PCR amplicon was digested with NotI and ApaI and, together with the HindIII and NotI cut ERN1 fragment described above, ligated into the HindIII-ApaI cut pcDNA5/FRT/TO vector (Invitrogen) to generate the pcDNA5/FRT/TO-ERN1-Tyk2(C)-HA expression plasmid. The ERN1-Tyk2-HA fusion is depicted in SEQ ID N° 5. The pcDNA5/FRT/TO-ERN1(K599A)-Tyk2(C)-HA plasmid was generated similarly, by amplifying ERN1 from a plasmid containing ERN1(K599A) instead of WT ERN1. The pcDNA5/FRT/TO-ERN1(D123P)-Tyk2(C)-HA plasmid was generated through site-directed mutagenesis of the pcDNA5/FRT/TO-ERN1-Tyk2(C)-HA plasmid using primers 16 and 17. The amino acid sequence of the ERN1(K599A)-Tyk2(C)-HA and ERN1(D123P)-Tyk2(K)-HA fusion proteins is depicted in SEQ ID N° 30 and 31, respectively.

The plasmids encoding the fusions with the second interacting polypeptide were of the type also used in MAPPIT, designated pMG2 (WO0190188, Eyckerman et al., 2001; Lemmens et al., 2003). These plasmids encode fusion proteins of the second interacting polypeptide coupled to a fragment of the human gp130 cytokine receptor chain, which contains multiple tyrosine residues that, upon phosphorylation, make up recruitment sites for STAT3. The SV40 large T containing control plasmid pMG2-SVT was generated by transfer of the SVT insert from the previously described pMG1-SVT plasmid (Eyckerman et al., 2001) into the pMG2 vector using EcoRI and NotI restriction enzymes. Human ARRB2 was PCR amplified on an ARRB2 entry clone from the hORFeome collection (Lamesch et al., 2007) using primers 7 and 8 (see Table below) and exchanged with the SVT insert of pMG2-SVT using EcoRI and NotI restriction sites to generate pMG2-ARRB2. pMG1-EFHA1, pMG1-VAMP1 and pMG1-VAMP2 were generated by Gateway recombination mediated transfer of the full length sequences of human EFHA1, VAMP1 and VAMP2, respectively, from entry vectors of the hORFeome collection (Lamesch et al., 2007) into a Gateway compatible version of the pMG1 vector as described earlier (Lievens et al., 2009). The flag tag-gp130-ARRB2, flag tag-gp130-VAMP1 and flag tag-gp130-VAMP2 fusion constructs are depicted in SEQ ID N° 6, 32 and 33, respectively.

The pMG2C-ERN1 plasmid encoding a fusion protein of the human ERN1 protein N-terminally coupled to a fragment of the human gp130 cytokine receptor chain was generated by PCR amplification of the ERN1 encoding sequence on an ERN1 entry clone from the hORFeome collection (Lamesch et al., 2007) using primers 13 and 14 and cloning this into a MAPPIT vector containing a gp130 encoding sequence at the 3' end of a Flag-tag encoding sequence

and a multi-cloningsite (Pattyn et al., 2008) using EcoRI and XhoI restriction enzymes. The flag tag-ERN1-gp130 fusion construct is depicted in SEQ ID N°7. The pMG2C-ERN1cyt plasmid encoding a fusion protein of the cytoplasmic portion of the human ERN1 protein fused N-terminally to the gp130 fragment was produced by amplifying the ERN1 cytoplasmic domain on an ERN1 entry clone (see higher) using primers 15 and 14 and cloning this into a MAPPIT vector containing a gp130 encoding sequence using EcoRI and XhoI restriction enzymes, similarly to described above. The flag-tag-ERN1cyt-gp130 fusion construct is depicted in SEQ ID N° 34.

pMG2-RTp51 has been described elsewhere (Pattyn et al., 2008). The flag tag-gp130-RTp51 fusion construct sequence is shown in SEQ ID N° 35. The pMG1 and pMG2 plasmids encoding an unfused gp130 receptor fragment were obtained by cutting out the MAPPIT prey insert of a pMG1 vector using EcoRI and XhoI or of a pMG2 vector using EcoRI and Sall, respectively, blunting the vector backbone through Pfu DNA Polymerase and self-ligation. The amino acid sequence of the polypeptide encoded by pMG1 and pMG2 is depicted in SEQ ID N° 36 and 37, respectively.

The reporter plasmid pXP2d2-rPAPI-luciferase used in the examples contains the STAT3-dependent rPAPI (rat Pancreatitis-Associated Protein I) promoter driving a firefly luciferase reporter gene as described previously (WO0190188, Eyckerman et al., 2001).

Transfection procedure

Transfections were carried out using a standard calcium phosphate method. HEK293-T cells were seeded in black tissue-culture treated 96-well plates at 10.000 cells/well in 100µl culture medium (DMEM supplemented with 10% FCS). Twenty-four hours later, plasmid DNA mixes were prepared that contained plasmids encoding fusion proteins with the first and second interacting proteins and reporter plasmids. The DNA was supplemented with 5µl 2.5M CaCl₂ and double distilled water to a final volume of 50µl. This mixture was added drop wise to 50µl 2xHeBS buffer (280mM NaCl, 1.5mM Na₂HPO₄, 50mM Hepes; pH 7.05) while vigorously vortexing. After incubation at room temperature for 15 min. to allow DNA precipitates to form, the solution was added to the cells at 10µl/well. Cells were incubated at 37°C, 8% CO₂. Twenty-four hours after transfection, cells were treated with the indicated amounts of ligand, either alone or combined with the indicated amount of antagonist. In the case of Irestatin 9389, cells were pre-treated with the antagonist before adding vehicle (DMSO) or tunicamycin. Another twenty-four hours later, luciferase activity was measured using the Luciferase Assay System kit (Promega) on a TopCount luminometer (Perkin-Elmer). Each transfection was done in triplicate and the average of the luciferase activity readings was used in the calculations.

Induction of dimerization

Tunicamycin (Sigma T7765; 2 mg/ml stock in DMSO) was diluted in culture medium and added to the cells 24h prior to luciferase signal read-out.

(Ant)agonists applied in the examples

- 5 Somatostatin (Sigma S1763) and angiotensin II (Sigma A9525) were solubilized in PBS to make stock concentrations of 500µM and 10mM, respectively. CYN154806 trifluoroacetate salt (Sigma C2490) and losartan potassium (Fluka 61188) were dissolved in PBS at a final concentration of 500µM and 10mM, respectively. Telmisartan (Sigma T8949) was dissolved in DMSO at a concentration of 10mM. Irestatin 9389 (Axxon Medchem) was dissolved in DMSO
- 10 at a concentration of 50mM.

Western blotting

- Cells were lysed in 1xCCLR buffer (25mM Tris-phosphate (pH 7.8), 2mM DTT, 2mM CDTA (trans-1,2-diaminocyclo- hexane-N,N,N,N-tetra acetic acid), 10% glycerol, 1% Triton X-100). Lysates were centrifuged and supernatants were separated by SDS-PAGE. Proteins were
- 15 detected by immunoblotting using rat anti-HA (Roche), rabbit anti-gp130 (Santa Cruz Biotechnology) or mouse anti-beta-actin (Sigma) antibodies.

Oligonucleotide primer	Sequence (5' > 3')
1	CCCACCGGTCCGGAATTGACAAGTTTGTACAAAAAAGC (SEQ ID N° 9)
2	GGGGGGCCCCAACCACTTTGTACAAGAAAGC (SEQ ID N° 10)
3	CCCGCGGCCGCTGGCGGTTTCGATCACCCAGCTGTCCCACTTG G (SEQ ID N° 11)
4	TCTAGACTAAGCATAATCTGGAACATCATATGGATACTCGAGG CACACGCTGAACACTGA AGG (SEQ ID N° 12)
5	CCCCAATTGACCATGTATCCATATGATGTTCCAGATTATGCTTT AATTAAAATCACCCAGCTGTCCCACTTGG (SEQ ID N° 13)
6	GGGTCTAGAGCGGCCGCACCGGTCTTAATTAAGTCGACGAAT TCGCACACGCTGAACACT GAAG (SEQ ID N° 14)
7	CCCAAGCTTGAATTCACCATGGGGGAGAAACCCGGGAC (SEQ ID N° 15)
8	GGGGCGGCCGCCTAGCAGAGTTGATCATCATAG (SEQ ID N° 16)
9	CCCAAGCTTGGTACCACCATGCCGGCCCCGGCGGCTGCTG (SEQ ID N° 17)

10	CCCGCGGCCGCGCTAGCGAGGGCGTCTGGAGTCACTGG (SEQ ID N° 18)
11	CCCGCGGCCGCTGGCGGTTGATCACCCAGCTGTCCCACTTG G (SEQ ID N° 19)
12	GGGCCCCTAAGCATAATCTGGAACATCATATGGATACTCGAG GCACACGCTGAACACTGA AGG (SEQ ID N° 20)
13	CCCGAATTCATGCCGGCCCGGCGGCTGCTG (SEQ ID N° 21)
14	CCCCTCGAGGGGAGGGCGTCTGGAGTCACTGG (SEQ ID N° 22)
15	CCCGAATTCTTCTGTCCCAAGGATGTCCTG (SEQ ID N°23)
16	GGGTAAAAAGCAGCCCATCTGGTATGTTATTGACC (SEQ ID N°24)
17	GGTCAATAACATACCAGATGGGCTGCTTTTTACCC (SEQ ID N°25)
18	CCCGATATCTATGGAGACGACGCCCTTGAA (SEQ ID N°26)
19	GGGGCGGCCGCTTACACAGCATTCAAGCGGA (SEQ ID N°27)

Example 1: Detection of the ligand-dependent interaction between SSTR2 and ARRB2

G-protein coupled receptors (GPCRs) are integral membrane proteins that contain 7 transmembrane domains. Upon binding of the appropriate ligand GPCRs are activated, leading to the recruitment of cytoplasmic beta arrestin proteins. In order to determine whether the assay can detect the somatostatin-dependent interaction between the GPCR SSTR2 and ARRB2, the following combinations of plasmids were transfected (Figure 2A; 250ng of the Tyk2(C) fusion construct, 250ng of the gp130 fusion construct and 50ng of the luciferase reporter construct) according to the methods described above:

- a) pMet7-HA-Tyk2(C) + pMG2-ARRB2 + pXP2d2-rPAPI-luciferase
- b) pMet7-SSTR2-Tyk2(C)-HA + pMG2-SVT + pXP2d2-rPAPI-luciferase
- c) pMet7-SSTR2-Tyk2(C)-HA + pMG2-ARRB2 + pXP2d2-rPAPI-luciferase

Transfected cells were either left untreated (NS) or treated with increasing doses (0,1-1-10µM) of the SSTR2 agonist somatostatin. The fold induction for each sample was calculated as the ratio of the measured luciferase activity relative to the luciferase activity for the untreated sample of the same transfection. The results (Figure 2B) show a clear ligand dose-dependent signal specifically in the cells co-transfected with both the SSTR2-Tyk2(C) and gp130-ARRB2 fusion constructs (transfection c). No signal was observed when either of the fusion constructs was transfected in combination with a negative control fusion construct (gp130-ARRB2 fusion

construct combined with an unfused Tyk2(C) construct in transfection a, or SSTR2-Tyk2(C) fusion construct together with a fusion of gp130 to a fragment of the SV40 large T protein in b). The assay was further optimized by transferring the Tyk2(C) fusion construct into another vector system (pSVSport) and testing the resulting constructs in a similar experiment as described above. The following combinations of plasmids were transfected (500ng of the Tyk2(C) fusion construct, 250ng of the gp130 fusion construct and 50ng of the luciferase reporter construct) according to the methods described above:

a) pSVSport-HA-Tyk2(C) + pMG2-ARRB2 + pXP2d2-rPAPI-luciferase

b) pSVSport-SSTR2-Tyk2(C)-HA + pMG2-SVT + pXP2d2-rPAPI-luciferase

c) pSVSport-SSTR2-Tyk2(C)-HA + pMG2-ARRB2 + pXP2d2-rPAPI-luciferase

Transfected cells were either left untreated (NS) or treated with increasing doses (0,1-1-10 μ M) of the SSTR2 agonist somatostatin, and signals were calculated as indicated above. The resulting graph (Figure 2C) shows strong and specific ligand dose-dependent signals up to 30-fold stronger compared to untreated samples.

In another experiment, cells were transfected with 31ng of the pMet7-SSTR2-Tyk2(C)-HA plasmid, 250ng of the pMG2-ARRB2 plasmid and 50ng of the pXP2d2-rPAPI-luciferase plasmid, and transfected cells were treated with a concentration gradient of somatostatin (a 1/3 serial dilution series down from 10 μ M). The resulting dose-response curve is shown in Figure 2D.

Together, these data illustrate that the method is able to detect ARRB2 recruitment to the SSTR2 integral membrane GPCR induced by treatment with the SSTR2 agonist somatostatin.

Example 2: Detection of the ligand-dependent interaction between AGTR1 and ARRB2

Likewise as in example 1, the ligand-induced recruitment of ARRB2 to another GPCR family member, AGTR1, was tested by transfecting the following combinations of plasmids (250ng of the Tyk2(C) fusion construct, 250ng of the gp130 fusion construct and 50ng of the luciferase reporter construct) according to the methods described above:

a) pMet7-HA-Tyk2(C) + pMG2-ARRB2 + pXP2d2-rPAPI-luciferase

b) pMet7-AGTR1-Tyk2(C)-HA + pMG2-SVT + pXP2d2-rPAPI-luciferase

c) pMet7-AGTR1-Tyk2(C)-HA + pMG2-ARRB2 + pXP2d2-rPAPI-luciferase

Transfected cells were either left untreated (NS) or treated with increasing doses (0,1-1-10 μ M) of angiotensin II, an AGTR1 agonist. The fold induction for each sample was calculated as the ratio of the measured luciferase activity relative to the luciferase activity for the untreated sample of the same transfection. The results (Figure 3 A) show a clear ligand dose-dependent signal specifically in the cells cotransfected with both the AGTR1-Tyk2(C) and gp130-ARRB2 fusion constructs (transfection c). No signal was observed when either of the fusion constructs

was transfected in combination with a negative control fusion construct (gp130-ARRB2 fusion construct combined with an unfused Tyk2(C) construct in transfection a, or AGTR1-Tyk2(C) fusion construct together with a fusion of gp130 to a fragment of the SV40 large T protein in b).

- 5 In another experiment, cells were transfected with 62ng of the pMet7-AGTR1-Tyk2(C)-HA plasmid, 250ng of the pMG2-ARRB2 plasmid and 50ng of the pXP2d2-rPAPI-luciferase plasmid, and transfected cells were treated with a concentration gradient of angiotensin II (a 1/3 serial dilution series down from 10μM). The resulting dose-response curve is shown in Figure 3B.
- 10 These results confirm the method's ability to detect the interaction between the AGTR1 integral membrane protein and ARRB2, in a ligand-dependent manner.

Example 3: Effect of GPCR antagonists on the detection of the interaction between GPCRs and ARRB2

- 15 In order to test whether the assay allows evaluating the effect of GPCR antagonists, GPCR ligands were combined with specific antagonists of SSTR2 and AGTR1 in the assay for detection of their interaction with ARRB2. A peptide antagonist that specifically inhibits SSTR2 activation was tested (CYN154806), together with two small molecule AGTR1-selective antagonists (losartan and telmisartan).
- 20 Cells were transfected with the following combinations of plasmids (250ng of the Tyk2(C) fusion construct, 250ng of the gp130 fusion construct and 50ng of the luciferase reporter construct) according to the methods described above:
 - a) pMet7-SSTR2-Tyk2(C)-HA + pMG1-EFHA1 + pXP2d2-rPAPI-luciferase
 - b) pMet7-SSTR2-Tyk2(C)-HA + pMG2-ARRB2 + pXP2d2-rPAPI-luciferase
 - 25 c) pMet7-AGTR1-Tyk2(C)-HA + pMG1-EFHA1 + pXP2d2-rPAPI-luciferase
 - d) pMet7-AGTR1-Tyk2(C)-HA + pMG2-ARRB2 + pXP2d2-rPAPI-luciferase

One day after transfection, cells were treated with combinations of GPCR ligand and antagonist (ligand: 1μM somatostatin for transfections a and b, 10μM angiotensin II for transfections c and d; antagonists: 0,05 or 0,5μM CYN154806; 0,1 or 1μM losartan or telmisartan), and luciferase was measured one day after treatment. The results are shown in Figure 4 and clearly indicate the specific inhibition by the corresponding antagonist of the GPCR-ARRB2 interactions. The interaction between SSTR2 and ARRB2 (transfection b) can be specifically inhibited by the SSTR2-selective antagonist CYN154806, whereas the AGTR1-specific antagonists losartan and telmisartan have no effect. Conversely, AGTR1-ARRB2 interaction as detected by the assay (transfection d) can be selectively inhibited by the AGTR1-specific antagonists losartan and telmisartan, whereas the SSTR2-selective antagonist CYN154806 has no effect. In both cases, the inhibition through application of the antagonists

goes down to background levels observed for cells that had not been treated with GPCR ligand (NS). The inhibitory effect is specific for the GPCR-ARRB2 interaction, as the signal obtained for control interactions of the GPCR-Tyk2(C) fusion construct with a positive control gp130 fusion construct containing EFHA1 (which binds to Tyk2(C) itself), are not affected by the GPCR antagonists.

In a second experiment (shown in Figure 5), a dose-response curve was generated for the different GPCR antagonists. Cells were transfected with 125ng of the pMet7-SSTR2-Tyk2(C)-HA or pMet7-AGTR1-Tyk2(C)-HA fusion construct, 250ng of the pMG2-ARRB2 gp130 fusion construct and 50ng of the pXP2d2-rPAP1-luciferase reporter plasmid, according to the methods described above. Cells were either left untreated, treated with 10 μ M of the appropriate ligand (somatostatin in the case of SSTR2 and angiotensin II in the case of AGTR1) or treated with a combination of the cognate ligand and increasing doses (10⁻¹³ M up to 10⁻⁶ M) of either GPCR antagonist (CYN154806, losartan, telmisartan). The results are presented in Figure 5A (for the interaction between SSTR2 and ARRB2) and Figure 5B (for the interaction between AGTR1 and ARRB2). Again, these data clearly indicate the specific inhibition by the corresponding antagonist of the GPCR-ARRB2 interactions. The interaction between SSTR2 and ARRB2 can be specifically and completely inhibited by the SSTR2-selective antagonist CYN154806, whereas the AGTR1-specific antagonists losartan and telmisartan have no effect. Conversely, AGTR1-ARRB2 interaction as detected by the assay can be selectively and completely inhibited by the AGTR1-specific antagonists losartan and telmisartan, whereas the SSTR2-selective antagonist CYN154806 has no effect. It is of note that the observed stronger effect of telmisartan compared to losartan in this assay corresponds with the reported higher binding affinity of telmisartan versus losartan towards AGTR1 (Kakuta *et al.*, 2005).

Together, these results confirm the specificity of the GPCR-ARRB2 interactions as detected by the assay and indicate that the assay can be applied to identify inhibitors of these interactions.

Example 4: Detection of context-dependent dimerization of a transmembrane protein

To support the ability of the method to detect protein-protein interactions under physiological conditions, we studied dimerization of ERN1. ERN1 is a single-span transmembrane protein involved in the cellular response to ER-stress. The ERN1 protein is able to sense unfolded proteins in the ER through its N-terminal domain which is exposed to the ER lumen. This leads to its dimerization and activation of the kinase and endoribonuclease enzymatic domains in its C-terminal moiety exposed towards the cytoplasm. To mimic ER-stress, tunicamycin was applied to the cells, an inhibitor of protein glycosylation which is generally used to induce ER-stress.

In a first experiment, cells were transfected with the following combinations of plasmids (500ng of the kinase fusion construct, 100ng of the gp130 fusion construct and 50ng of the luciferase reporter construct) according to the methods described above:

- a) pcDNA5/FRT/TO-ERN1-Tyk2(C)-HA + pMG1 + pXP2d2-rPAPI-luciferase
- 5 b) pcDNA5/FRT/TO-ERN1-Tyk2(C)-HA + pMG2C-ERN1 + pXP2d2-rPAPI-luciferase

After transfection, cells were treated with 0-0.5-1-2µg/ml tunicamycin, final concentration. The results shown in Figure 6A show a dose-dependent signal upon addition of tunicamycin, only in cells expressing both ERN1-Tyk2(C) and ERN1-gp130 fusion constructs (transfection b). No signal was observed when the ERN1-Tyk2(C) fusion construct was combined with an unfused gp130 fragment (transfection a).

In a second experiment (Figure 6B), cells were transfected with the following combinations of plasmids (62,5ng of the kinase fusion construct, 125ng of the gp130 fusion construct and 50ng of the luciferase reporter construct) according to the methods described above:

- a) pcDNA5/FRT/TO-ERN1-Tyk2(C)-HA + pMG1 + pXP2d2-rPAPI-luciferase
- 15 b) pcDNA5/FRT/TO-ERN1-Tyk2(C)-HA + pMG2C-ERN1 + pXP2d2-rPAPI-luciferase
- c) pcDNA5/FRT/TO-ERN1-Tyk2(C)-HA + pMG2C-ERN1cyt + pXP2d2-rPAPI-luciferase

After transfection, cells were treated with 0-0.04-0.2-1-5µg/ml tunicamycin, final concentration. The luciferase data are presented as fold induction relative to the signal obtained in cells transfected with unfused gp130 (empty prey; transfection a) and treated with the same concentration tunicamycin. Expression of the different fusion proteins was confirmed using Western blot. These data show that in accordance with the requirement of the ERN1 luminal domain to sense ER stress, no signal is produced upon overexpression of full length ERN1 kinase fusion and a gp130 fusion containing only the cytoplasmic portion of ERN1 (transfection c).

25 In a next experiment (Figure 6C), cells were transfected with combinations of the pXP2d2-rPAPI-luciferase construct (50ng), a WT or mutant ERN1 kinase fusion construct (62,5ng) and either unfused or ERN1-fused gp130 construct (125ng). After transfection, cells were either vehicle (DMSO) treated or treated with 1µg/ml tunicamycin (final concentration). The mutant ERN1 kinase fusions have mutations in either the luminal domain (D123P) or cytoplasmic ATP-binding pocket (K599A). Both mutations are expected to block ERN1 oligomerization. As evident from Figure 6C we indeed find that both mutations block the interaction with full length ERN1 gp130 fusion, despite equal expression and similar (aspecific) interaction signals with unfused gp130 constructs.

35 In another experiment (Figure 6D), cells were transfected with combinations of the pXP2d2-rPAPI-luciferase construct (50ng), the ERN1 kinase fusion construct (62,5ng) and either unfused or ERN1-fused gp130 construct (125ng). After transfection, cells were treated with tunicamycin (1µg/ml tunicamycin final concentration) or vehicle (DMSO) combined with

increasing doses of Irestatin 9389. This molecule was recently reported to inhibit ERN1 endonuclease activity (Feldman and Koong, 2007). Although the molecular mode of action of Irestatin 9389 was not reported, the molecule induced a dose-dependent disruption of ERN1 dimerization in the assay described here.

- 5 Together, these data indicate that the method is able to specifically detect the ER-stress-induced dimerization of the ERN1 protein and to analyze the structure-function relationship of this protein and pharmacological interference with dimerization of the protein in more detail.

Example 5: Detection of heterologous interactions among transmembrane proteins

- 10 To further corroborate the ability of the assay to analyse protein-protein interactions involving integral membrane proteins, heterologous interactions between transmembrane proteins were analyzed. Serotonin transporter (SERT) is a multispan integral membrane protein that transports serotonin from the synaptic spaces into presynaptic neurons, this way terminating the action of serotonin and recycling it. In this example, we show its interaction with the
15 synaptobrevins VAMP1 and VAMP2, which are transmembrane proteins involved in fusion of synaptic vesicles with the presynaptic membrane.

Cells were transfected with combinations of the pXP2d2-rPAPI-luciferase construct (50ng), a SERT or RTp66 kinase fusion construct (1000ng) and either unfused (pMG2) or one of the indicated gp130 fusion constructs (pMG2-RTp51, pMG1-VAMP1 or pMG2-VAMP2; 500ng).

- 20 Luciferase activity is shown as fold induction relative to the luciferase signal obtained in cells transfected with unfused gp130 (pMG2).

The results (Figure 7) show a clear signal when VAMP1 and VAMP2 gp130 fusion constructs were transfected in combination with the SERT kinase fusion construct, and not when combined with the HIV-1 RTp66 (reverse transcriptase subunit p66) fusion construct. The
25 strong signal obtained for the co-transfection of the RTp66 kinase and the RTp51 gp130 fusion constructs, which has been previously described (WO2012117031), is included as a control for proper expression and functioning of the RTp66 kinase fusion.

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CLAIMS:

1. A recombinant membrane span protein complex, comprising (1) a first fusion protein, comprising a membrane span protein chain fused to either a tyrosine kinase domain or a reporter phosphorylation domain, and (2) a second fusion protein comprising
5 a polypeptide, interacting with said membrane span protein chain, fused to either a reporter phosphorylation domain or a tyrosine kinase domain, wherein when the first fusion protein is a fusion to a tyrosine kinase domain, the second fusion protein is a fusion to a reporter phosphorylation domain and vice versa, and wherein the tyrosine kinase phosphorylates the reporter phosphorylation domain.
- 10 2. The recombinant membrane span protein complex according to claim 1, wherein said tyrosine kinase domain is a constitutive mutant tyrosine kinase domain.
3. The recombinant membrane span protein complex according to claim 1, wherein said tyrosine kinase domain is an inactive mutant tyrosine kinase domain that is activated by addition of an exogenous small molecule.
- 15 4. The recombinant membrane span protein complex according to any one of claims 1 to 3, wherein said tyrosine kinase domain is derived from TYK2, from a Jak kinase or from a Src kinase.
5. The recombinant membrane span protein complex according to any one of claims 1 to 4, wherein the domain fused to the membrane span protein chain of the first
20 fusion protein is fused to the carboxyterminal end of the membrane span protein chain.
6. The recombinant membrane span protein complex according to any one of claims 1 to 5, wherein said membrane span protein chain is a multispan membrane span protein chain.
7. The recombinant membrane span protein complex according to claim 6,
25 wherein said multispan membrane span protein chain is a G protein coupled receptor.
8. The recombinant membrane span protein complex according to any one of claims 1 to 7 wherein the first fusion protein consists of a membrane spanning domain

fused to a tyrosine kinase domain, wherein the tyrosine kinase domain has at least 95% sequence identity to amino acids 589-1187 of SEQ ID NO: 1 and has tyrosine kinase activity.

9. The recombinant membrane span protein complex according to any one of
5 claims 1 to 7 wherein the first fusion protein consists of a membrane spanning domain fused to a tyrosine kinase domain, wherein the tyrosine kinase domain has at least 95% sequence identity to the Tyk domain of any one of SEQ ID NOs:3, 4, 5 or 29 and has tyrosine kinase activity.

10. The recombinant membrane span protein complex according to any one of
10 claims 1 to 9 wherein the second fusion protein consists of an interaction domain fused to a reporter phosphorylation domain, wherein the phosphorylation domain has an amino acid sequence of SEQ ID NO: 2.

11. Use of the recombinant membrane span protein complex according to any
one of claims 1 to 10, to detect a compound-protein interaction with the membrane span
15 protein chain of the fusion protein.

12. The use according to claim 11, wherein said compound-protein interaction is an interaction between 2 or more membrane span protein chains.

13. The use according to claim 11, wherein said membrane span protein chain is a G protein coupled receptor and said compound-protein interaction is an interaction
20 between the G protein coupled receptor and a compound that acts as a ligand.

14. Use of the recombinant membrane span protein complex according to any one of claims 1 to 10, to screen for an inhibitor of a compound-protein interaction with the membrane span protein chain of the first fusion protein.

15. The use according to claim 14, wherein said inhibitor of the compound-
25 protein interaction is an inhibitor of membrane span protein chain dimerization or multimerization.

16. The use according to claim 14, wherein said membrane span protein chain is a G protein coupled receptor and said inhibitor of the compound-protein interaction is an inhibitor of the binding of a ligand to the G protein coupled receptor.

17. A method to detect compound-protein interactions, said method comprising
5 (1) transforming a eukaryotic host cell with a first fusion protein, comprising a recombinant membrane span protein chain, fused to either a tyrosine kinase domain or a reporter phosphorylation domain; (2) transforming the same host cell with at least one second fusion protein, comprising a polypeptide, fused to a either a reporter phosphorylation domain or a tyrosine kinase domain, wherein when the first fusion protein is a fusion to a
10 tyrosine kinase domain, the second fusion protein is a fusion to a reporter phosphorylation domain and vice versa, and wherein said polypeptide is interacting with the membrane span protein chain; (3) adding the compound to be tested to the cell; (4) optionally adding a ligand to the cell; (5) detecting phosphorylation of the reporter phosphorylation domain; and (6) detecting a compound-protein interaction by detecting presence of a
15 phosphorylated reporter phosphorylation domain in the cell.

18. A method to screen inhibitors of a compound-protein interaction, said method comprising 1) transforming a eukaryotic host cell with first fusion protein, comprising a recombinant membrane span protein chain, fused to a either tyrosine kinase domain or a reporter phosphorylation domain; (2) transforming the same host cell with at
20 least one second fusion protein, comprising a polypeptide, fused to a either a reporter phosphorylation domain or a tyrosine kinase domain, wherein when the first fusion protein is a fusion to a tyrosine kinase domain, the second fusion protein is a fusion to a reporter phosphorylation domain and vice versa, and wherein said polypeptide is interacting with the membrane span protein chain; (3) adding at least one possible inhibitor molecule; (4)
25 adding a ligand to the cell; (5) detecting phosphorylation of the reporter phosphorylation domain; and (6) detecting an inhibitor of compound-protein interaction by detecting absence of a phosphorylated reporter phosphorylation domain in the cell.

19. The recombinant membrane span protein complex of claim 1, wherein the tyrosine kinase domain is the Tyk2 domain comprised in SEQ ID NO: 3, SEQ ID NO: 4,
30 SEQ ID NO:5 or SEQ ID NO:29.

Figure 1

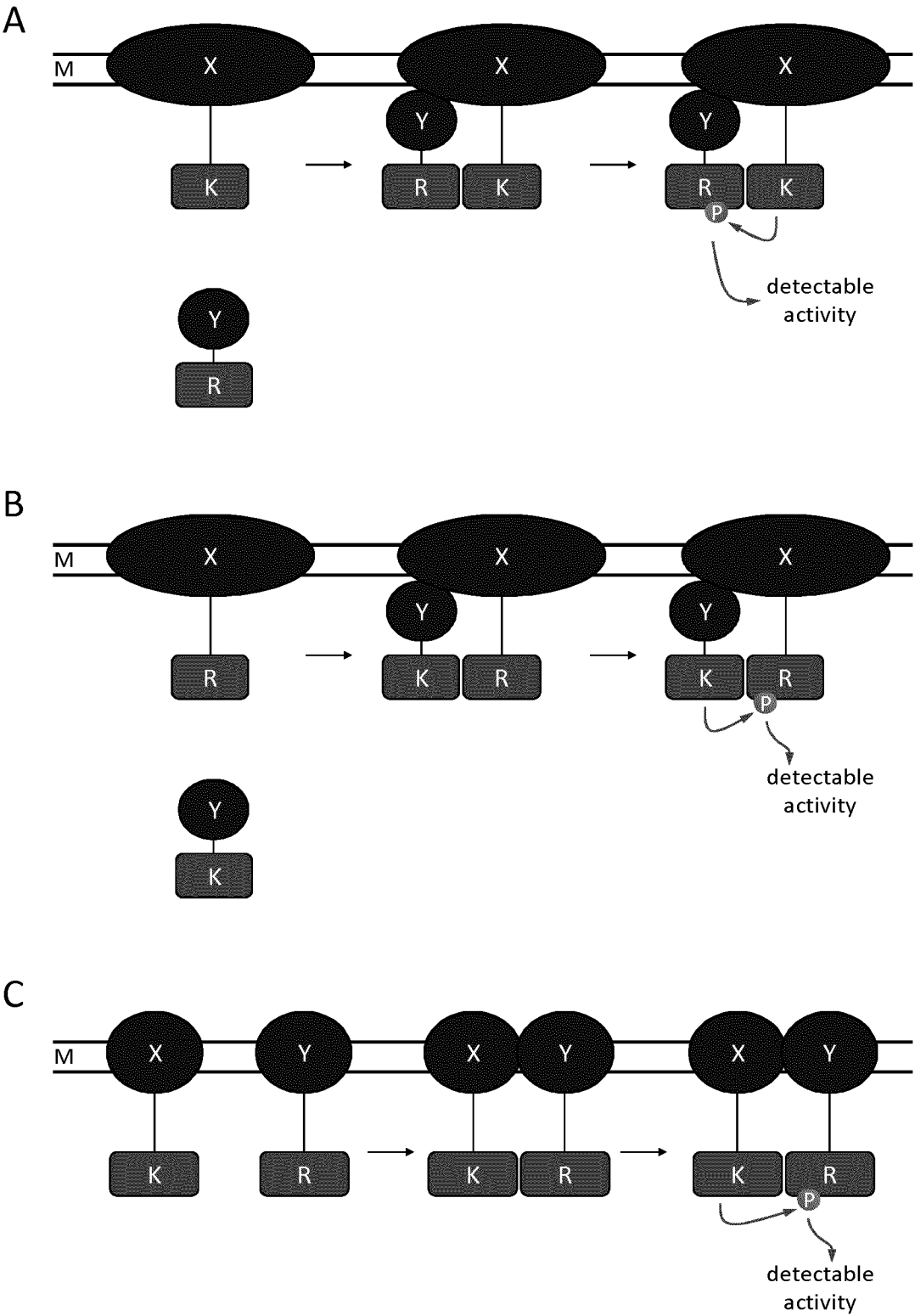
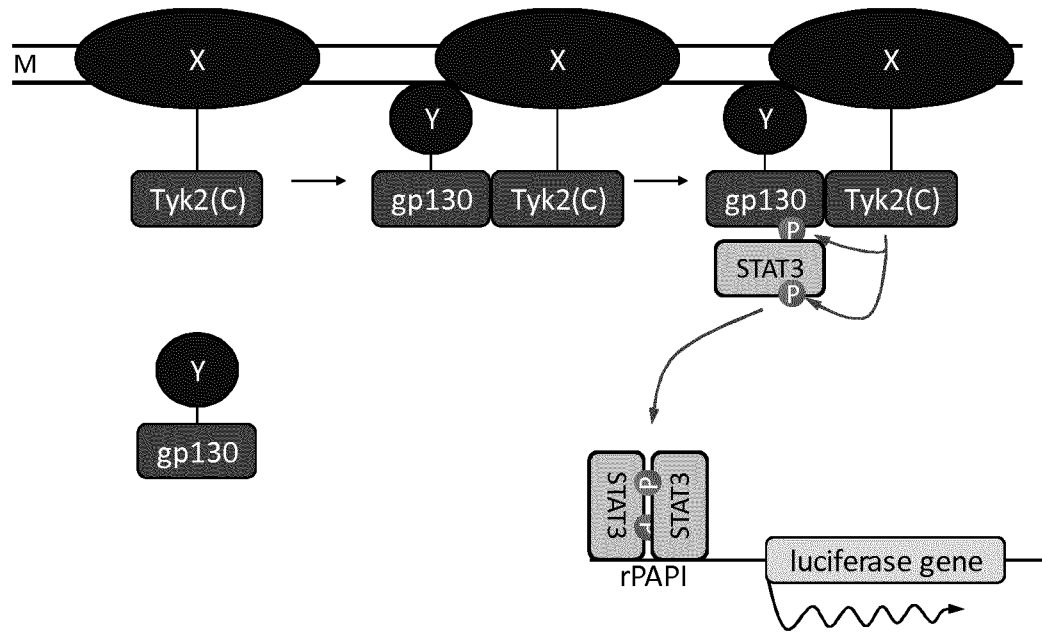
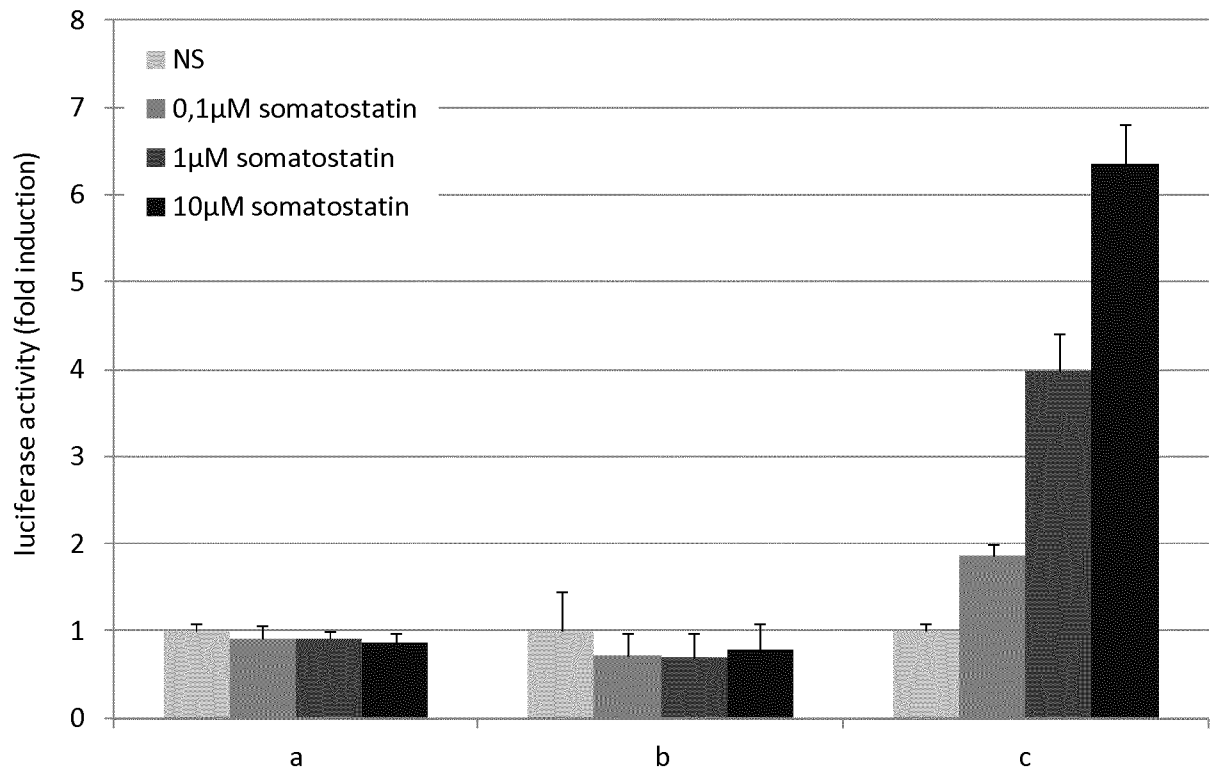


Figure 2

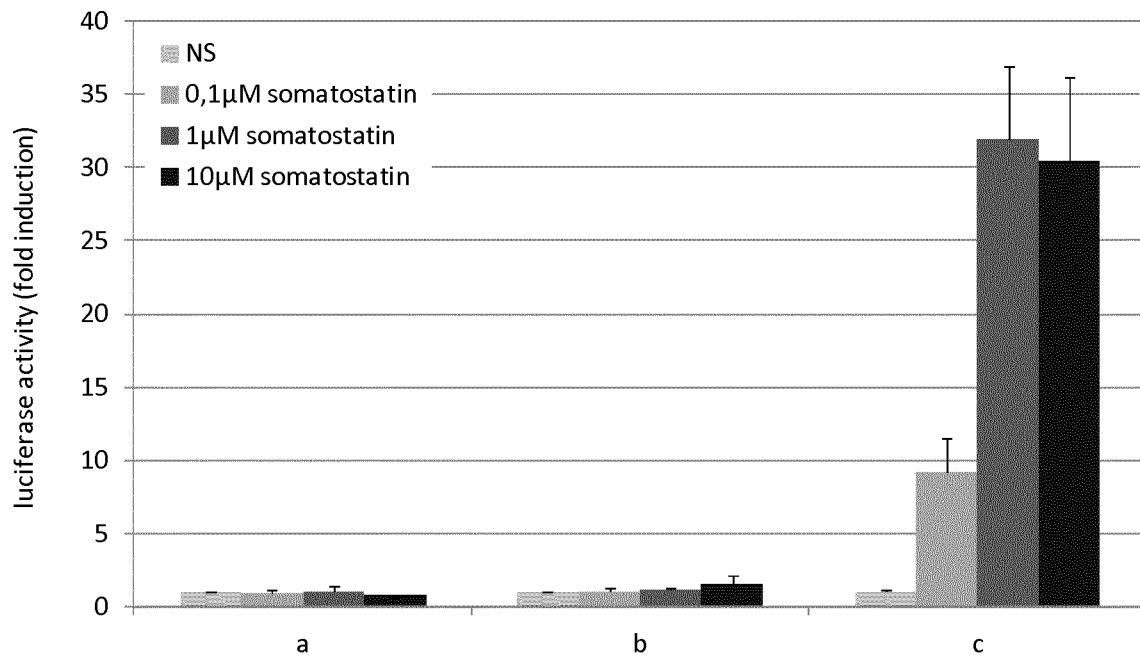
A



B



C



D

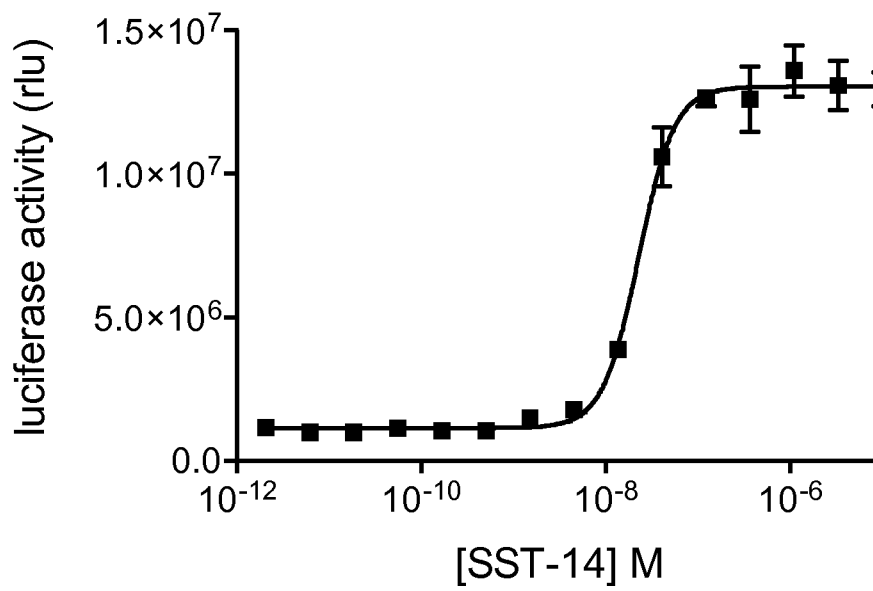
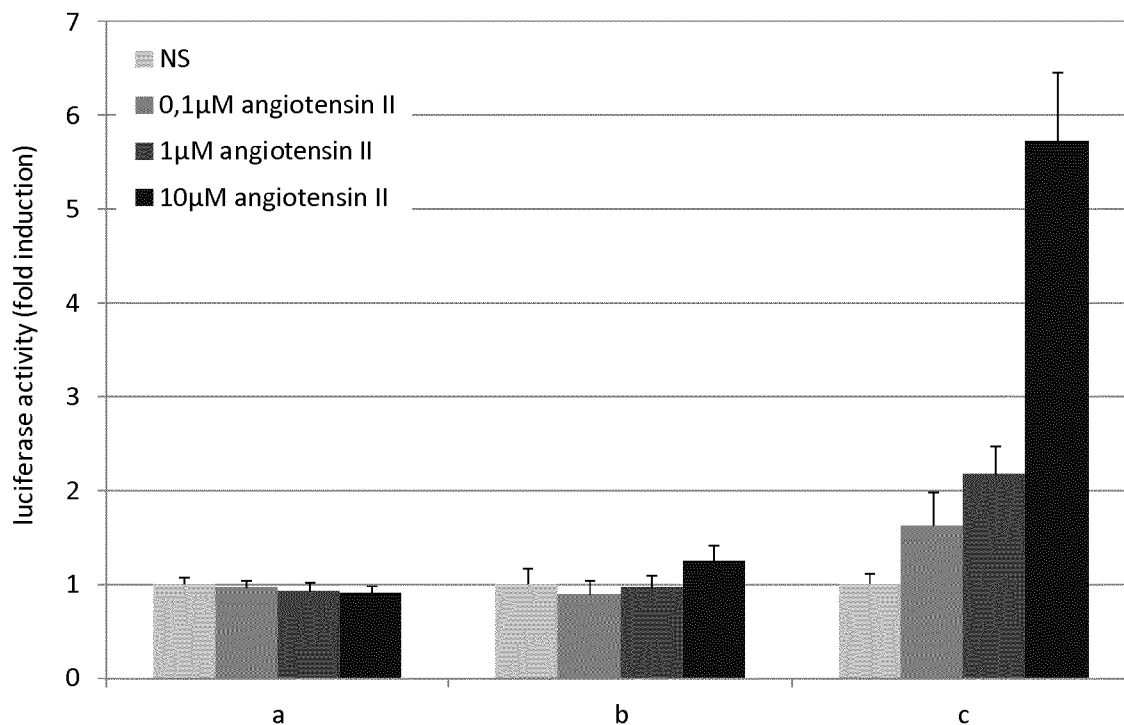


Figure 3

A



B

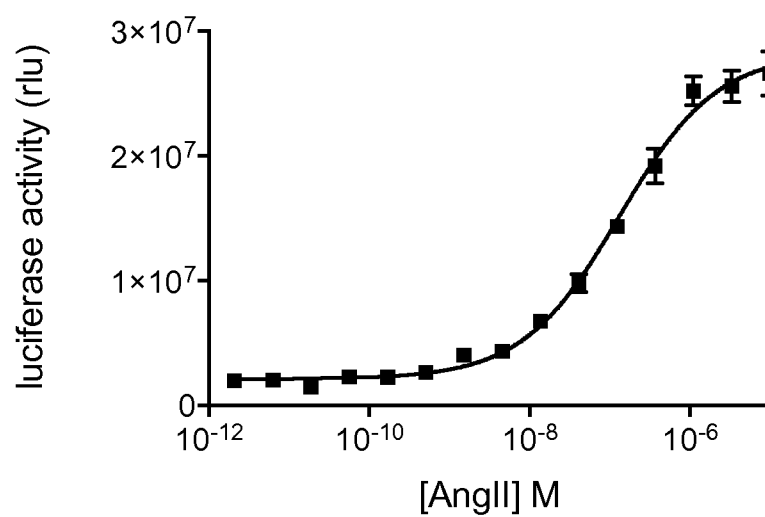


Figure 4

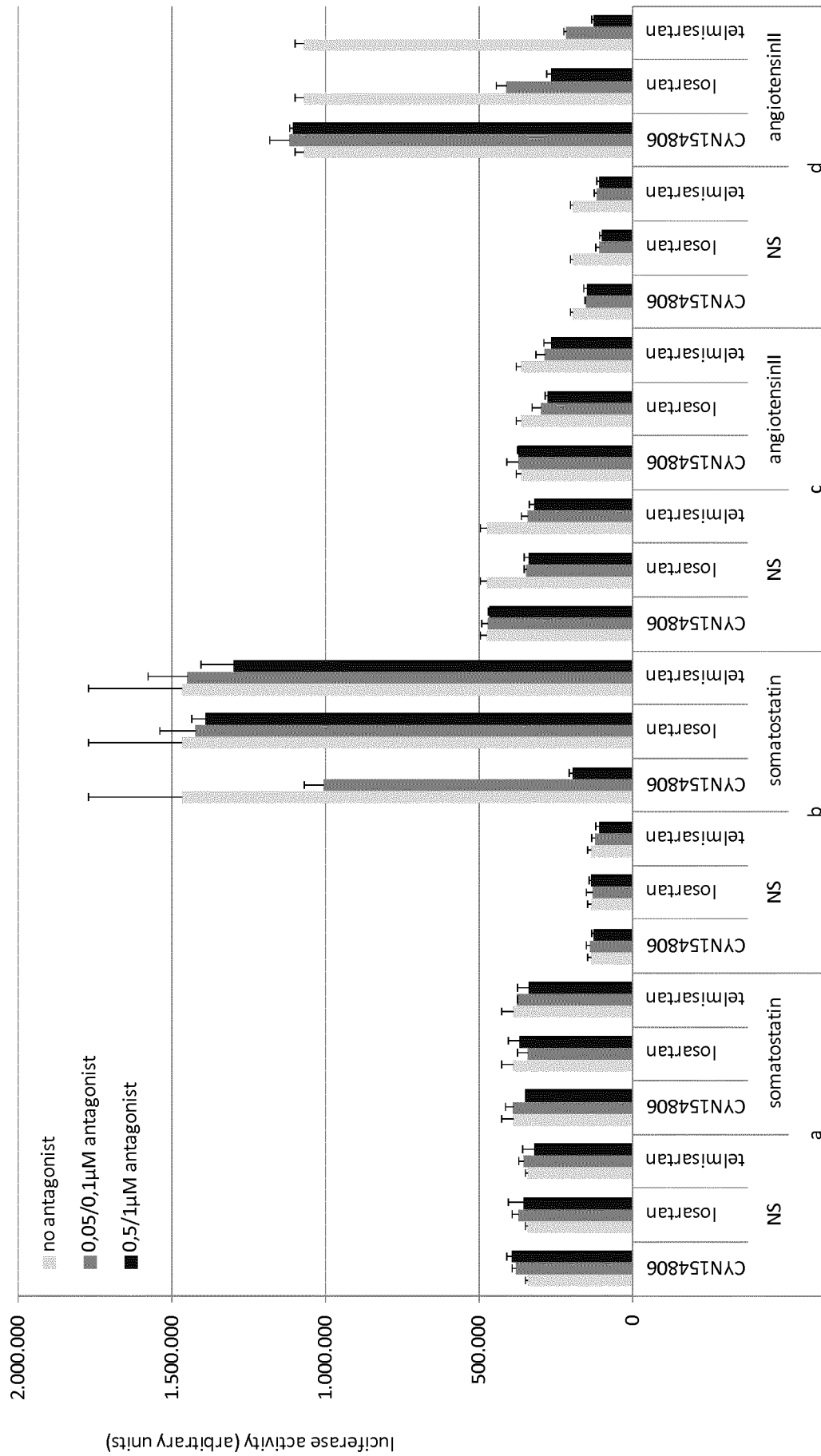
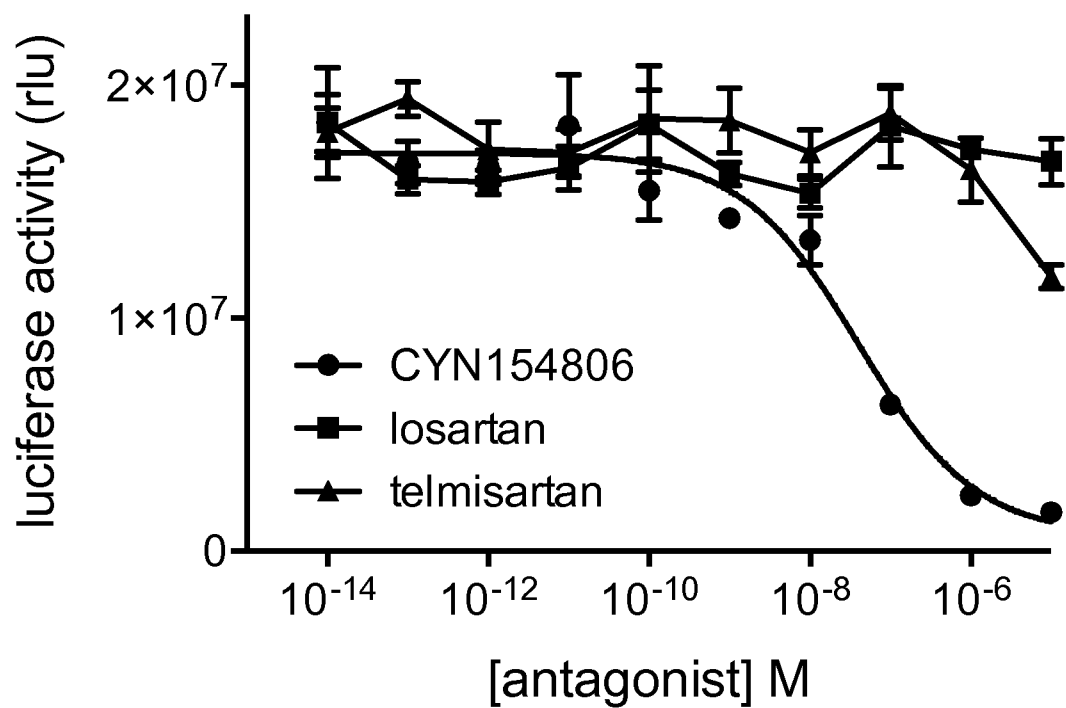


Figure 5

A



B

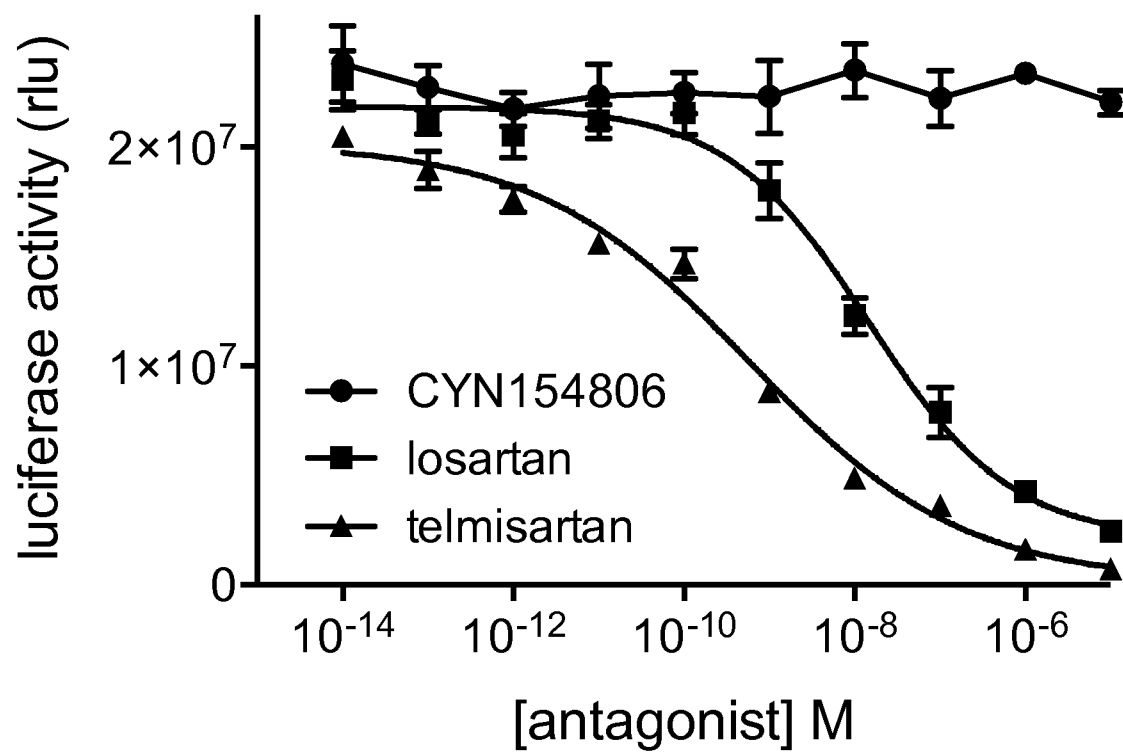
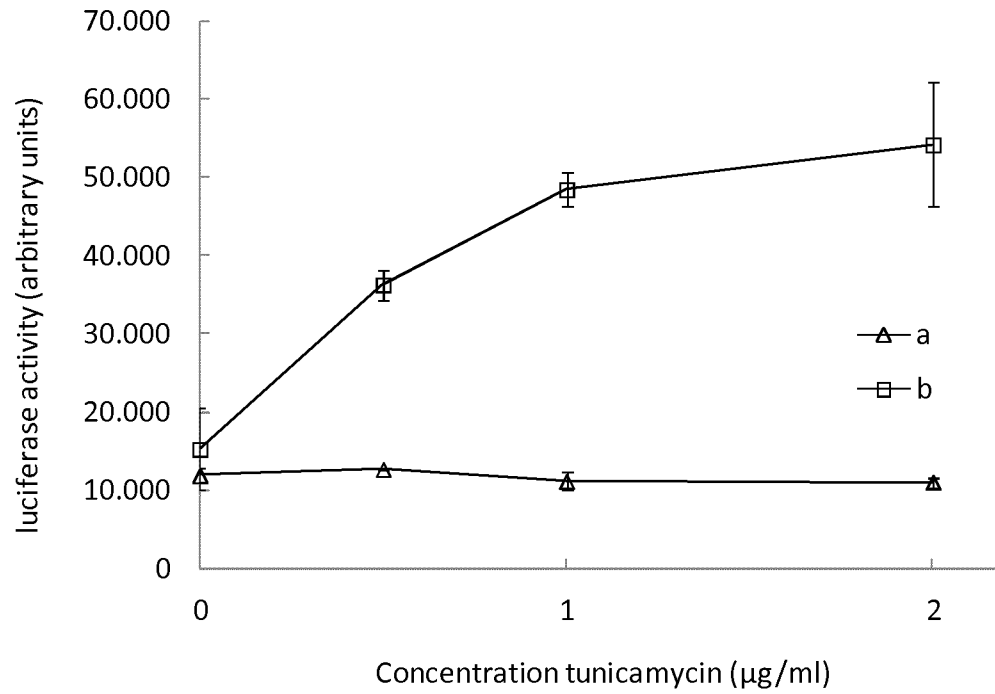
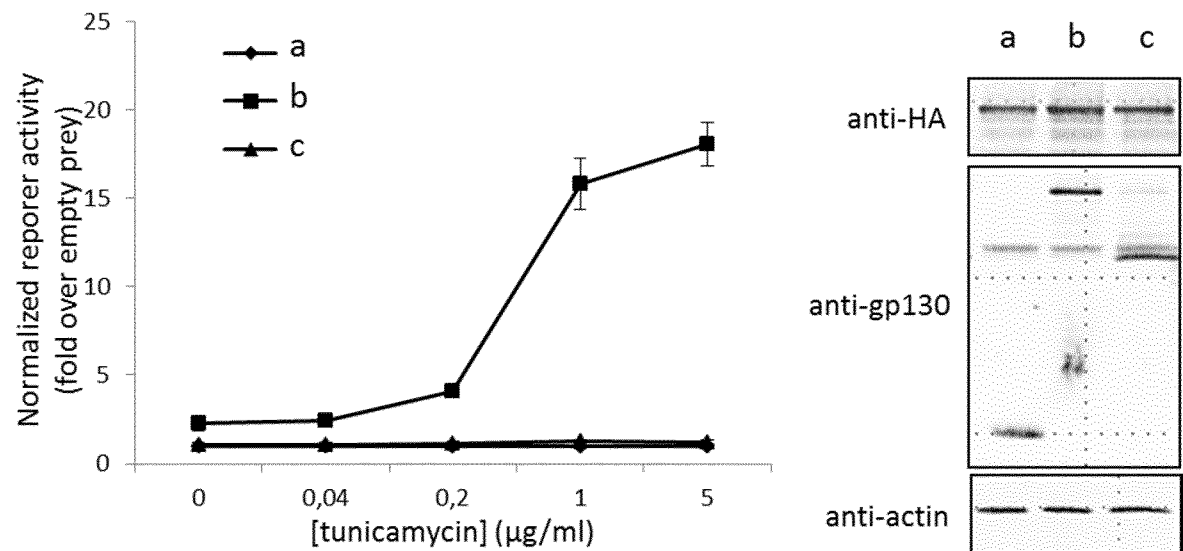
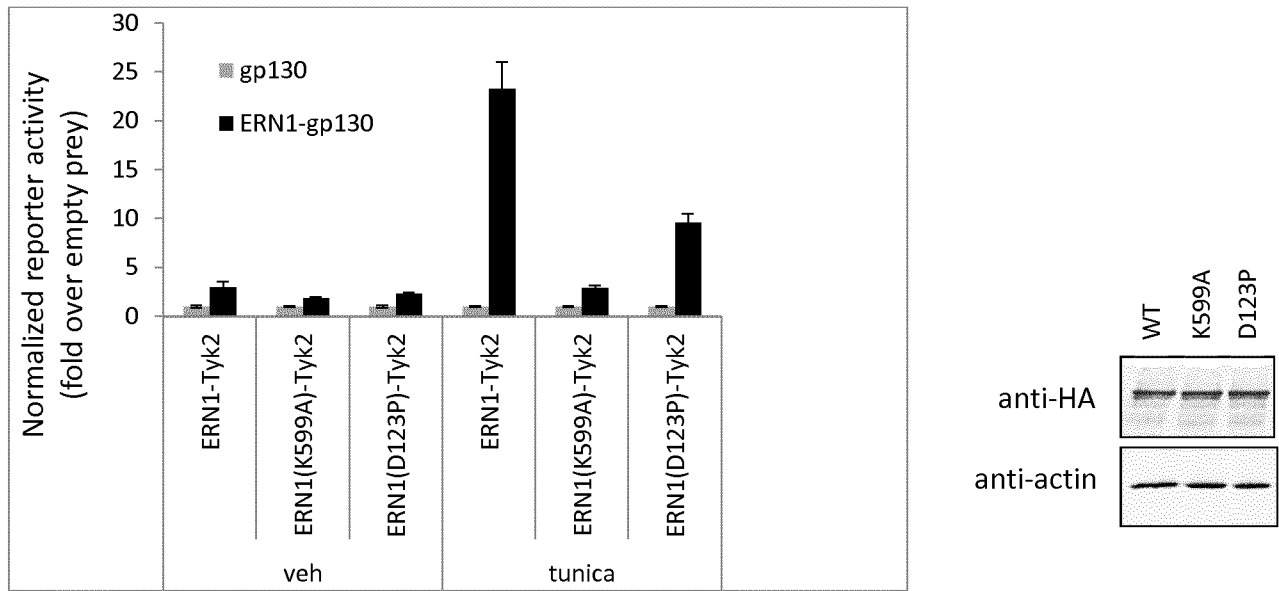


Figure 6**A****B**

C



D

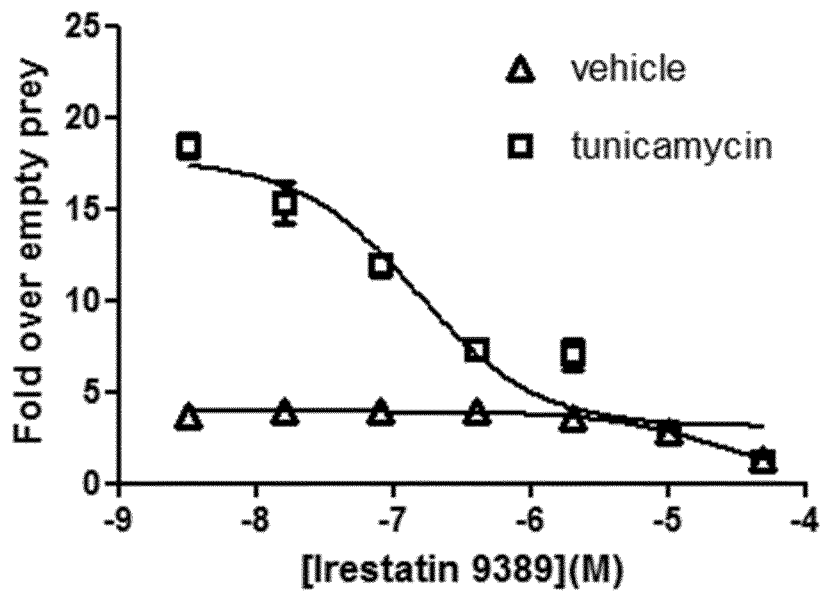


Figure 7

