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(54) Title: METHOD FOR DETECTING A MODULATOR COMPOUND FOR CHEMICAL TARGETING OF PROTEIN-PROTEIN INTERACTIONS

Figure 1

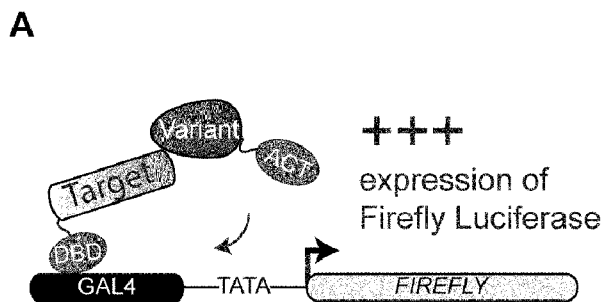
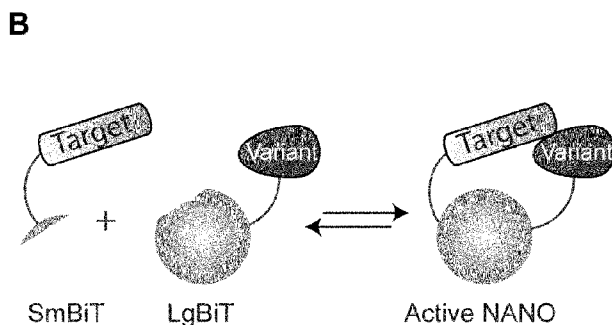


Figure 1 (cont.)



(57) Abstract: The present invention relates to a method for detecting a modulator compound of a protein-protein interaction between a first and a second protein, a modulator compound detected by any of the methods according to the present invention for use in the prophylaxis and/or treatment of a disease, an engineered high affinity variant used in the method of the present invention and a kit-of-parts comprising said engineered high affinity variant according to the present invention and a test system that is capable of detecting the extent of modulation of the modulator compound on said protein-protein interaction between the first and the second protein of the protein-protein interaction. Further, the present invention relates to the use of said methods and also relates to compositions comprising said engineered high affinity variant.

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METHOD FOR DETECTING A MODULATOR COMPOUND FOR CHEMICAL TARGETING OF PROTEIN-PROTEIN INTERACTIONS

TECHNICAL FIELD OF THE INVENTION

[001] The present invention relates to a method for detecting a modulator compound of a protein-protein interaction between a first and a second protein, a modulator compound detected by any of the methods described herein for use in the prophylaxis and/or treatment of a disease, an engineered high affinity variant used in the method of the present invention and a kit-of-parts comprising an engineered high affinity variant according to the present invention and a test system that is capable of detecting the extent of modulation of the modulator compound on said protein-protein interaction between the first and the second protein. Further, the present invention relates to the use of said methods and also relates to compositions comprising said modulator compound.

BACKGROUND ART

[002] Protein-Protein interactions (PPIs) are at the centre of virtually all life processes. On the cellular level, PPIs mediate cellular signal transduction, regulate transcription and translation and fulfil transport functions within and outside of cells. Therefore, modulation of PPIs in diseases with the intention to disturb a given PPI and inhibit the aberrant signalling pathway could result in potential new forms of therapy. Thus, pharmacological targeting of PPIs is of high importance.

[003] To target PPIs, researchers have predominantly focussed on small molecules because of their ease of administration and good bioavailability. Currently, a multitude of different technology platforms for primary high-throughput screening (HTS) of small molecule libraries are available. In principle, current small molecule screens can be divided into biochemical and cell-based screening approaches. Biochemical screens are *in vitro* assays that rely on the availability of the individual components in sufficient quality and quantity to ensure consistent and reproducible screening results. This poses a certain limitation in HTS, since availability of individual components for an *in vitro* assay in sufficient quantities requires high protein stability and/or expression levels.

[004] Cell-based screening platforms circumvent this problem since they rely on the intracellular expression of the assay components *in situ*. However, due to the complex topologies and weak affinities of protein interaction interfaces, it is notoriously difficult to develop efficient HTS platforms to interrogate large libraries of small molecules for compounds that

specifically bind a defined protein surface. Therefore, screening for inhibitors that target PPIs is difficult and often results in a poor Z-factor (a measure of statistical effect size and whether the effect is large enough to use in screening) due to the weak affinity of the targeted interaction. This results in a higher false positive rate, which significantly complicates downstream triage experiments and identification of true hits, increasing the total costs associated with HTS. Consequently, naturally weak PPIs, even if representing therapeutically relevant targets, are almost never considered for cell-based assay development because of the lack of robust HTS platforms.

[005] To overcome challenges in developing robust assays targeting PPIs that would be compatible with HTS, we utilised a different strategy, whereby mutations are introduced directly by protein engineering into one binding partner of an existing PPI, resulting in the generation of a variant of said binding partner that binds to the target protein of the existing PPI with high affinity. By doing so, the overall affinity of a protein complex, which is build between said variant and said target protein is significantly enhanced. Our approach allows maintenance of the existing interface of the given PPI while only minute molecular details of the interacting molecules are modified. This strategy is hardwired to target individual functionally relevant epitopes, therefore allowing targeting of pharmacological relevant protein interfaces in otherwise multifunctional, multi-domain full-length proteins.

[006] Applying high affinity variants generated by protein engineering approaches enables the development of robust cell-based assays for therapeutically relevant PPIs validated for applications in HTS. In proof-of-concept experiments, we used two existing assay systems – Dual Luciferase Reporter (DLR) and NanoBiT technologies - and demonstrated that several PPIs that play a role in diseases can now be chemically targeted.

[007] Dixon *et al.*¹ describes protein-fragment complementation assays, looking at protein-protein interactions (PPIs) in cells, without any identification of modulators or a compound, which influences the investigated protein-protein interaction.

[008] Rahighi *et al.*² reported on the binding capability of the UBAN (ubiquitin binding in ABIN and NEMO proteins) motif of NEMO to linear ubiquitin chains, without presenting any specific assay therefore or any assay, which allows the screening for compounds, which modulate or inhibit the respective protein-protein interaction.

[009] Instead, Wiechmann *et al.*³ investigated the small ubiquitin-like modifiers (SUMOs) and found that the E2-conjugated enzyme Ubc9 catalyzes the conjugation of SUMOs, meaning that the inhibition of the enzyme Ubc9 impairs SUMO chain formation.

[0010] Ernst *et al.*⁴ describes targeting enzymes that govern ubiquitination of protein substrates, looking specifically at inhibitors of four deubiquitinases and analyzed the inhibitor complexes by chrystallography.

[0011] Vincendeau *et al.*⁵ focusses on the inhibition of canonical NF- κ B (nuclear factor kappa B) signaling by a small molecule targeting NEMO-Ubiquitin interaction. The authors thereof describe an *in vitro* approach for targeting NEMO-Ubiquitin interaction.

[0012] However, the present invention is the first report of a method that provides proof-of-principle evidence for the application of protein engineering in the development of robust cell-based assays. Due to this method according to the present invention, one can envisage a wide application of this strategy in HTS, accelerating drug discovery programs in the future.

SUMMARY OF THE INVENTION

[0013] The present invention relates to a method for detecting a modulator compound of a protein-protein interaction between a first and a second protein, comprising the steps of:

- a) fusing the first protein of said protein-protein interaction with a reporter polypeptide or part thereof, and
fusing the second protein of said protein-protein interaction with a transcription activation domain or a part of the reporter polypeptide,
 - i) in the presence of a test modulator compound, and
 - ii) in the absence of said test modulator compound, wherein the first protein and the second protein of the protein-protein interaction are capable of binding to each other,
- b) measuring a signal of the reporter polypeptide in step a) i) and a signal of the reporter polypeptide in step a) ii), and
- c) comparing the measurements made in step (b), wherein an alteration of the signal of said reporter polypeptide in step a) ii) to the signal of said reporter polypeptide in step a) i) indicates the presence of a modulator compound of said protein-protein interaction, and
- d) quantifying the extent of modulation of the protein-protein interaction by the test modulator compound by step c).

[0014] In one embodiment of the method of the present invention, the alteration of the signal of said reporter polypeptide in step a) ii) to the signal in step a) i) is a reduction or deletion of the signal of the reporter polypeptide.

[0015] In a further embodiment of the method of the present invention, step a) of the method is within a cell, cell lysate, within a reaction mixture or outside a cell, preferably within a cell.

[0016] In an additional embodiment of the method of the present invention, the first protein of said protein-protein interaction is fused to a first part of the reporter polypeptide and the second protein of said protein-protein interaction is fused to a second part of the reporter polypeptide.

[0017] In a further embodiment of the method of the present invention, the modulator compound is a compound being capable of inhibiting said protein-protein interaction.

[0018] In an additional embodiment of the method of the present invention, the modulator compound is a compound being capable of inhibiting said protein-protein interaction, preferably a protein-protein interaction of the nuclear factor kappa B signaling pathway, more preferably the protein-protein interaction between NEMO and linear ubiquitin chains and most preferably the interaction between the UBAN domain of NEMO (SEQ ID NO: 2) and an Ubiquitin variant (SEQ ID NO: 6) as defined herein.

[0019] In one embodiment of the method of the present invention, fusing the first protein of said protein-protein interaction with the reporter polypeptide or part thereof is carried out by recruiting the first protein via an interaction between a DNA binding domain and an upstream activator sequence of the reporter polypeptide or part thereof to the reporter polypeptide or part thereof. This means, the first protein of said protein-protein interaction is recruited via an interaction between a DNA binding domain and an upstream activator sequence of the reporter polypeptide or part thereof to the reporter polypeptide or part thereof. Thereby, a first fusion is build for the method of the present invention or for the test system described herein.

[0020] In this embodiment, in the absence of said test modulator compound according to step a) ii) of the method of the present invention, the binding of the DNA binding domain to the upstream activator sequence of the reporter polypeptide and the binding of a transcription activation domain to a promoter sequence of the reporter polypeptide causes expression of the reporter polypeptide. A second fusion is build between the second protein of said protein-protein interaction and the transcription activation domain. The second fusion is build for the method of the present invention or for the test system described herein.

[0021] In another embodiment of the method of the present invention, the protein-protein interaction is the interaction between a protein of interest or a part thereof and an engineered high affinity variant, preferably the interaction between the UBAN domain of NEMO and an Ub variant, the interaction between UBC9 and a small ubiquitin-like modifier 2 (SUMO2) variant, the interaction between the ATG3 enzyme and a GATE16 variant, or the interaction between OPTN and a LC3B variant.

[0022] In another embodiment of the method of the present invention, the first protein of the protein-protein interaction is selected from the group consisting of UBAN domain of NEMO (SEQ ID NO: 2), UBC9 enzyme (SEQ ID NO: 3), ATG3 enzyme (SEQ ID NO: 4) and OPTN (SEQ ID NO: 5), preferably the UBAN domain of NEMO (SEQ ID NO: 2), and/or the second protein of the protein-protein interaction is selected from the group consisting of an Ub variant (SEQ ID NO: 6), a small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), a GATE16 variant (SEQ ID NO: 8) and a LC3B variant (SEQ ID NO: 9), preferably an Ub variant (SEQ ID NO: 6). In another embodiment of the method of the present invention, the first protein of the

protein-protein interaction is selected from the group consisting of UBAN domain of NEMO (SEQ ID NO: 18), UBC9 enzyme (SEQ ID NO: 3), ATG3 enzyme (SEQ ID NO: 4) and OPTN (SEQ ID NO: 5), preferably the UBAN domain of NEMO (SEQ ID NO: 18), and/or the second protein of the protein-protein interaction is selected from the group consisting of an Ub variant (SEQ ID NO: 6), a small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), a GATE16 variant (SEQ ID NO: 8) and a LC3B variant (SEQ ID NO: 9), preferably an Ub variant (SEQ ID NO: 6).

[0023] In a further embodiment of the method of the present invention, the reporter polypeptide according to the present invention is selected from the group consisting of a split ubiquitin, a fluorescent protein, preferably the green, red or yellow fluorescent protein, more preferably a split green fluorescent protein, a beta-lactamase, a beta-galactosidase, chloramphenicol acetyltransferase (CAT) and a luciferase, preferably Firefly luciferase, Renilla luciferase or split nanoluciferase.

[0024] In an additional embodiment of the method of the present invention, the method is a high-throughput screening assay, preferably a cell-based high-throughput screening assay, for detecting the modulator compound of the protein-protein interaction.

[0025] The present invention also relates to a modulator compound detected by any of the methods described herein for use in the prophylaxis and/or treatment of a disease selected from the group consisting of cancer, chronic inflammatory diseases, autoimmune diseases, neurodegenerative diseases, arthritides and infectious diseases.

[0026] The present invention also relates to a kit comprising a modulator compound as described herein.

[0027] The present invention is also directed to a protein or engineered high affinity variant selected from the group consisting of an Ub variant (SEQ ID NO: 6), a small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), a GATE16 variant (SEQ ID NO: 8) and a LC3B variant (SEQ ID NO: 9), preferably an Ub variant (SEQ ID NO: 6).

[0028] The present invention also relates to a kit comprising a protein or engineered high affinity variant selected from the group consisting of an Ub variant (SEQ ID NO: 6), a small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), a GATE16 variant (SEQ ID NO: 8) and a LC3B variant (SEQ ID NO: 9), preferably an Ub variant (SEQ ID NO: 6) as described herein.

[0029] In another embodiment, the present invention relates to a kit-of-parts comprising an engineered high affinity variant as described herein and a test system that is capable of detecting the extent of modulation of the modulator compound on a protein-protein interaction between a first protein and a second protein, wherein the second protein is the engineered high affinity variant. In this embodiment, it is preferred that the test system further comprises a reporter polypeptide, wherein the reporter polypeptide is selected from the group consisting of a split ubiquitin, a fluorescent protein, preferably the green, red or yellow fluorescent protein, more

preferably a split green fluorescent protein, beta-lactamase, beta-galactosidase, chloramphenicol acetyltransferase (CAT) and a luciferase, preferably Firefly luciferase or Renilla luciferase or split nanoluciferase.

[0030] Additionally, the inventors found for the first time the generation of a specific high affinity variant or engineered affinity variant that targets the interaction interface between the UBAN domain of NEMO and linear ubiquitin chains, an interaction that is critical for NF- κ B signalling pathway activation in response to TNF receptor stimulation². The UBAN domain of NEMO is usually engaged in binding to linear ubiquitin chains, wherein the mentioned variant specifically targets the molecular surface of the UBAN domain of NEMO that is responsible for this binding.

[0031] Furthermore, the inventors demonstrate that high affinity variants that were developed against NEMO, UBC9, ATG3 and OPTN protein targets significantly improve cell-based assay robustness and thus, enable chemical compound screening against PPIs of these proteins. These variants are used to illustrate the applicability of the method of the present invention against multiple functionally unrelated protein targets.

BRIEF DESCRIPTION OF THE FIGURES

[0032] **Figure 1** shows a theoretical description of the present invention. **Figure 1A** shows the schematic representation of a cellular assay based on the Dual Luciferase Reporter system (DLR) (Promega). Vectors modified to express a target protein (Target, relating to the first protein of the protein-protein interaction according to the present invention) fused to the DNA-binding domain (DBD) and an engineered high affinity variant (Variant, relating to the second protein of the protein-protein interaction according to the present invention) generated against the target protein fused to the VP16 activation (ACT) domain (SEQ ID NO: 13) are co-transfected into mammalian cells along with the firefly luciferase reporter vector (SEQ ID NO: 12), which contains five Gal4 binding sites upstream of the minimal TATA box. High affinity PPI between the given Target-Variant pair results in luciferase reporter expression and subsequent quantification of its activity. **Figure 1B** shows the schematic representation of a cellular assay based on the NanoBiT system (Promega). The system vectors are modified to express a target protein, relating to the first protein of the protein-protein interaction according to the present invention, and an engineered high affinity variant, relating to the second protein of the protein-protein interaction according to the present invention, as fusions to either small bit (SmBiT), relating to a first part of the reporter polypeptide of the present invention, or large bit (LgBiT), relating to a second part of the reporter polypeptide of the present invention, of split nanoluciferase (Active NANO), relating to the reporter polypeptide according to the present invention, are co-transfected into mammalian cells. High affinity PPI between the given Target-Variant pair results in functional complementation of nanoluciferase, which activity can be then quantified.

[0033] **Figure 2** shows the identification of the best high affinity Ub variant. **Figure 2A** shows a competition assay of generated Ub variants to the UBAN domain of NEMO (UBAN_{NEMO}, SEQ ID NO: 2). IC₅₀ values were determined in a competition ELISA assay as the concentration of mouse UBAN_{NEMO} (SEQ ID NO: 18) in solution that blocked 50% of Ub variant binding to immobilized mouse UBAN_{NEMO} (SEQ ID NO: 18). **Figure 2B** shows the sequence alignment of wild type (wt) Ub (SEQ ID NO: 14) and Ubv-A (SEQ ID NO: 6), corresponding to the best high affinity Ub variant according to the present invention. The amino acid sequence alignment shows only those positions that were diversified in the Ub variant library. Positions that were conserved as wt sequence are indicated by dashes. Affinity estimate is based on phage ELISA while competing with mUBAN_{NEMO} (SEQ ID NO: 18) in solution for binding to immobilized GST-mUBAN_{NEMO}.

[0034] **Figure 3** shows the specificity and selectivity validation of Ubv-A (SEQ ID NO: 6). **Figure 3A** shows that HEK293 cells were transiently co-transfected with control or vectors expressing HA-tagged Ubv-A (SEQ ID NO: 6) in combination with either wild type (wt) (SEQ ID NO: 2) or F312A human UBAN_{NEMO} (SEQ ID NO: 19). Ubv-A specifically (SEQ ID NO: 6) co-immunoprecipitated wt (SEQ ID NO: 2) and not F312A mutant UBAN_{NEMO} (SEQ ID NO: 19). **Figure 3B** indicates that GST fusions were immobilized on the ELISA plate, followed by incubation with the phage expressing Ubv-A (SEQ ID NO: 6) and detection of bound phages spectrophotometrically (optical density at 450 nm). Background binding to BSA was subtracted from the signal. **Figure 3C** shows surface plasmon resonance data of the interaction between UBAN of (i) NEMO (SEQ ID NO: 18), (ii) OPTN (SEQ ID NO: 22) and (iii) ABIN1 (SEQ ID NO: 25) with Ubv-A (SEQ ID NO: 6). GST-fused UBAN_{NEMO}, UBAN_{OPTN} and UBAN_{ABIN1} were immobilized on a GST antibody-coated CMD200m sensor chip. Ubv-A (SEQ ID NO: 6) molecules were loaded over the chip. Each measurement was performed at the indicated concentrations, and the fitted curves were used to calculate equilibrium dissociation constant (K_D) values.

[0035] **Figure 4** shows proof-of-concept using the assay technology according to Figure 1A (Dual Luciferase reporter system, DLR-system). **Figure 4A** shows that HEK293 cells were co-transfected with modified DLR assay vectors encoding the first fusion containing DBD-hUBAN_{NEMO} and the second fusion containing ACT-2xUbv-A of the present invention in combination with the Firefly reporter vector. Following 20-hour incubation cells were lysed and then assayed for Firefly and Renilla luciferase. Firefly/Renilla ratio served as normalization control for transfection efficiency between samples (as recommended by Promega assay protocols). The background level of the DLR assay is measured in the presence of DBD or ACT

empty vectors. Co-transfection of cells with combinations of wild type (wt) hUBAN_{NEMO} (SEQ ID NO: 2) and Ubv-A (SEQ ID NO: 6) constructs lead to a significant increase in DLR assay signal over the F312A mutant (SEQ ID NO: 19) control (n = 3, **NEMO assay p value= 0.0034). **Figure 4B** shows that HEK293 cells were co-transfected with modified DLR assay vectors encoding a fusion of DBD to either control SUMO2 (SEQ ID NO: 15) or high affinity SUMO2 variant (SEQ ID NO: 7) and a fusion containing ACT fused to UBC9 (SEQ ID NO: 3) in combination with the Firefly reporter vector. Firefly/Renilla ratio was determined 20 hours after transfection (n = 3, **UBC9 assay p value= 0.0012). **Figure 4C** shows that HEK293 cells were co-transfected with modified DLR assay vectors encoding DBD-ATG3 and the second fusion containing ACT fused to control GATE16 or generated high affinity GATE16 variant (SEQ ID NO: 8) in combination with the Firefly reporter vector. Similar to **Figure 4A** and **4B**, Firefly/Renilla ratio was determined 20 hours after transfection (n = 3, ***ATG3 assay p value= 0.0002).

[0036] **Figure 5** shows proof-of-concept being based on the NanoBiT assay technology. **Figure 5A** shows that HEK293 cells were reverse transfected with modified NanoBiT assay vectors encoding fusions of SmBiT to hUBAN_{NEMO} (SEQ ID NO: 2) and LgBiT to Ubv-A (SEQ ID NO: 6). Following 20-hour incubation cells were assayed for nanoluciferase (NANO) activity according to manufacturer's protocol (Promega). The background assay activation by LgBiT fusion was determined by transfection of cells with LgBiT-Ubv-A with SmBiT fusion to HaloTag. Co-transfection of cells with combinations of wild type (wt) hUBAN_{NEMO} (SEQ ID NO: 2) and Ubv-A (SEQ ID NO: 6) constructs lead to a significant increase in NanoBiT assay signal over the F312A mutant (SEQ ID NO: 19) control (n = 3, **NEMO assay p value = 0.0014). **Figure 5B** shows that HEK293 cells were reverse transfected with modified NanoBiT assay vectors encoding LgBiT-OPTN and the second construct expressing a fusion of SmBiT to either control LC3B or LC3B high affinity variant. NANO activity was determined 20 hours after transfection (n = 3, ***OPTN assay p value = 0.0002).

[0037] **Figure 6** shows a method validation in high-throughput screening. **Figure 6A** shows that HEK293, CHO or A549 cell lines were reverse transfected with modified NanoBiT constructs by either LgBiT-Ubv-A and SmBiT-wt UBAN_{NEMO} or LgBiT-Ubv-A and SmBiT-HaloTag construct combinations, denoted as assay or control, respectively. Transfected cells were then dispensed into 384-well plate and incubated for 20 hours. Nanoluciferase activity was assayed 30 minutes following substrate addition to the wells to determine relative luminescence units (RLU). Averages of RLU generated with assay or control transfections, signal-to-background ratios (S:B), standard deviations (StDEV) and Z' scores are indicated for each individual cell line. **Figure 6B** shows the concentration-dependent response of Aloe Emodin in A549 cells co-

transfected with NanoBiT NEMO assay constructs using a concentration range from 0.13 - 33.3 μM in two-fold dilution steps ($n = 3$). Aloe Emodin IC_{50} value is indicated as 7.844 μM within the context of this data. **Figure 6C** shows assay validation with a set of FDA approved drugs ($n = 774$) to determine data reproducibility. A549 cells were reverse transfected with NanoBiT constructs expressing LgBiT-Ubv-A and SmBiT-wt UBAN_{NEMO}, dispensed into 384-well plates on two separate occasions (shown as repeat 1 and repeat 2). Following 20-hour incubation, compounds were dispensed at 10 μM final concentration (f.c.) and nanoluciferase activity was determined two hours after compound addition. The response for each individual test compound was then quantified relative to the reference Aloe Emodin inhibitor (f.c. 50 μM) and depicted as percent inhibition. **Figure 6D** shows a subset of compounds with $\geq 50\%$ activity on both occasions classified based on the drug class. Calcium (Ca) antagonists ($n = 5$) are shown on the graph.

DETAILED DESCRIPTION OF THE INVENTION

[0038] The present invention relates to a method for detecting a modulator compound of a protein-protein interaction (PPI) between a first and a second protein, comprising the steps of:

- a) fusing the first protein of said protein-protein interaction with a reporter polypeptide or part thereof, and fusing the second protein of said protein-protein interaction with a transcription activation domain or a part of the reporter polypeptide,
 - i) in the presence of a test modulator compound, and
 - ii) in the absence of said test modulator compound, wherein the first protein and the second protein of the protein-protein interaction are capable of binding to each other;
- b) measuring a signal of the reporter polypeptide in step a) i) and a signal of the reporter polypeptide in step a) ii), and
- c) comparing the measurements made in step (b), wherein an alteration of the signal of said reporter polypeptide in step a) ii) to the signal of said reporter polypeptide in step a) i) indicates the presence of a modulator compound of said protein-protein interaction, and
- d) quantifying the extent of modulation of the protein-protein interaction by the test modulator compound by step c).

[0039] The method of the present invention provides means by which it is possible to identify whether or not a test modulator compound is a modulator compound of a protein-protein interaction (PPI) of interest. The method also allows quantification of the extent to which a test modulator compound is able to modulate PPIs, preferably the extent to which a modulator compound is able to inhibit a given PPI. This in turn allows a comparison to be made between

the ability of different test modulator compounds or different doses of the same test modulator compound, to modulate PPIs.

[0040] The method does not only enable the detection of a modulator compound of the protein-protein interaction between a first and a second protein, but also enables to identify or screen for such a modulator compound. This means, in the context of the present invention, the terms “detecting”, “identifying” and “screening” can be used interchangeably.

[0041] In the present invention, the term “protein” means a molecule in which two or more amino acids are linked by (a) peptide bond(s), and modified products thereof. Thus, the term is a concept including not only full-length proteins, but also so-called oligopeptides and polypeptides. Examples of the modification of the protein include phosphorylation, glycosylation, palmitoylation, prenylation (for example, geranylgeranylation), methylation, acetylation, ubiquitination, SUMOylation, hydroxylation, and amidation. Of course, the term “protein” also comprises parts of proteins as defined or described herein.

[0042] As the “first protein” and the “second protein” according to the present invention, it is possible to use desired proteins, intended for detection of interaction or which are able to build an interaction. In one specific embodiment of the present invention, the first protein of the protein-protein interaction is selected from the group consisting of the UBAN domain of NEMO (SEQ ID NO: 2), the UBC9 enzyme (SEQ ID NO: 3), the ATG3 enzyme (SEQ ID NO: 4) and OPTN (SEQ ID NO: 5). In a preferred embodiment of the present invention, the first protein of the protein-protein interaction is the UBAN domain of NEMO (SEQ ID NO: 2). In one specific embodiment of the present invention, the second protein of the protein-protein interaction is selected from the group consisting of an Ub variant (SEQ ID NO: 6), a small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), a GATE16 variant (SEQ ID NO: 8) and a LC3B variant (SEQ ID NO: 9). In a preferred embodiment of the present invention, the second protein of the protein-protein interaction is an Ub variant (SEQ ID NO: 6).

[0043] The “protein-protein interaction between a (the) first protein and a (the) second protein” according to the present invention includes not only direct interactions, but also indirect interactions such as an interaction for forming a complex. It might even be possible that in such a complex another molecule (protein, nucleic acid, sugar, lipid, low-molecular-weight compound, or the like) is interposed between the first protein and the second protein.

[0044] The present invention relates in one specific embodiment to a method for detecting a modulator compound of the protein-protein interaction (PPI) between the UBAN domain of NEMO according to SEQ ID NO: 2 and an Ub variant according to SEQ ID NO: 6, comprising the steps of:

- a) fusing the UBAN domain of NEMO according to SEQ ID NO: 2 with a reporter polypeptide or part thereof, and fusing the Ub variant according to SEQ ID NO: 6 with a transcription activation domain or a part of the reporter polypeptide,

- i) in the presence of a test modulator compound, and
- ii) in the absence of said test modulator compound, wherein the UBAN domain of NEMO according to SEQ ID NO: 2 and the Ub variant according to SEQ ID NO: 6 are capable of binding to each other;
- b) measuring a signal of the reporter polypeptide in step a) i) and a signal of the reporter polypeptide in step a) ii), and
- c) comparing the measurements made in step (b), wherein an alteration of the signal of said reporter polypeptide in step a) ii) to the signal of said reporter polypeptide in step a) i) indicates the presence of a modulator compound of said protein-protein interaction, and
- d) quantifying the extent of modulation of the protein-protein interaction by the test modulator compound by step c).

[0045] The present invention also relates in one specific embodiment to a method for detecting a modulator compound of the protein-protein interaction (PPI) between the UBC9 enzyme according to SEQ ID NO: 3 and a small ubiquitin-like modifier 2 (SUMO2) variant according to SEQ ID NO: 7, comprising the steps of:

- a) fusing the UBC9 enzyme according to SEQ ID NO: 3 with a reporter polypeptide or part thereof, and fusing the small ubiquitin-like modifier 2 (SUMO2) variant according to SEQ ID NO: 7 with a transcription activation domain or a part of the reporter polypeptide,
 - i) in the presence of a test modulator compound, and
 - ii) in the absence of said test modulator compound, wherein the UBC9 enzyme according to SEQ ID NO: 3 and the small ubiquitin-like modifier 2 (SUMO2) variant according to SEQ ID NO: 7 are capable of binding to each other;
- b) measuring a signal of the reporter polypeptide in step a) i) and a signal of the reporter polypeptide in step a) ii), and
- c) comparing the measurements made in step (b), wherein an alteration of the signal of said reporter polypeptide in step a) ii) to the signal of said reporter polypeptide in step a) i) indicates the presence of a modulator compound of said protein-protein interaction, and
- d) quantifying the extent of modulation of the protein-protein interaction by the test modulator compound by step c).

[0046] The present invention also relates in one specific embodiment to a method for detecting a modulator compound of the protein-protein interaction (PPI) between the ATG3 enzyme according to SEQ ID NO: 4 and a GATE16 variant according to SEQ ID NO: 8, comprising the steps of:

- a) fusing the ATG3 enzyme according to SEQ ID NO: 4 with a reporter polypeptide or part thereof, and fusing the GATE16 variant according to SEQ ID NO: 8 with a transcription activation domain or a part of the reporter polypeptide,
 - i) in the presence of a test modulator compound, and

- ii) in the absence of said test modulator compound, wherein the ATG3 enzyme according to SEQ ID NO: 4 and the GATE16 variant according to SEQ ID NO: 8 are capable of binding to each other;
- b) measuring a signal of the reporter polypeptide in step a) i) and a signal of the reporter polypeptide in step a) ii), and
- c) comparing the measurements made in step (b), wherein an alteration of the signal of said reporter polypeptide in step a) ii) to the signal of said reporter polypeptide in step a) i) indicates the presence of a modulator compound of said protein-protein interaction, and
- d) quantifying the extent of modulation of the protein-protein interaction by the test modulator compound by step c).

[0047] The present invention also relates in one specific embodiment to a method for detecting a modulator compound of the protein-protein interaction (PPI) between the OPTN protein according to SEQ ID NO: 5 and a LC3B variant according to SEQ ID NO: 9, comprising the steps of:

- a) fusing the OPTN enzyme according to SEQ ID NO: 5 with a reporter polypeptide or part thereof, and fusing the LC3B variant according to SEQ ID NO: 9 with a transcription activation domain or a part of the reporter polypeptide,
 - i) in the presence of a test modulator compound, and
 - ii) in the absence of said test modulator compound, the OPTN enzyme according to SEQ ID NO: 5 and the LC3B variant according to SEQ ID NO: 9 are capable of binding to each other;
- b) measuring a signal of the reporter polypeptide in step a) i) and a signal of the reporter polypeptide in step a) ii), and
- c) comparing the measurements made in step (b), wherein an alteration of the signal of said reporter polypeptide in step a) ii) to the signal of said reporter polypeptide in step a) i) indicates the presence of a modulator compound of said protein-protein interaction, and
- d) quantifying the extent of modulation of the protein-protein interaction by the test modulator compound by step c).

[0048] Fusing the first protein of said protein-protein interaction with a reporter polypeptide or part thereof can be called, in the context of the present invention, a “first fusion”. On the other hand, fusing the second protein of said protein-protein interaction with a transcription activation domain or a reporter polypeptide or part thereof can be called, in the context of the present invention, a “second fusion”.

[0049] It is also within the context of the present invention that the “first protein of the protein-protein interaction” or the “second protein of the protein-protein interaction” only comprises a part of a respective protein for building the first or second fusion, meaning that the part thereof is about 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%,

65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or even 99% of the protein.

[0050] The “reporter polypeptide” as used in the context of the present invention can be any protein, which is encoded by a reporter gene, wherein a reporter gene can be any genetic material, whose utilization by the transcriptional and/or translational apparatus derived from a cell (e.g., intact cell or cell-free extract) can be monitored, for example, any DNA sequence that is fused to a promoter sequence of interest so as to measure the relative activity of the promoter sequence. For example, the “reporter polypeptide” can be any luminescence-generating polypeptide, which is any polypeptide that is capable to generate bioluminescence. Preferably, the “reporter polypeptide” according to the present invention can be selected from the group consisting of a split ubiquitin, a fluorescent protein, more preferably the green, red or yellow fluorescent protein, most preferably a split green fluorescent protein, beta-lactamase, beta-galactosidase, chloramphenicol acetyltransferase (CAT) and a luciferase, more preferably Firefly luciferase, Renilla luciferase or split nanoluciferase. Luciferases catalyze light emission in the presence of their substrates, luciferins, and this property has made them attractive for various types of assay systems. There are different types of luciferases that occur in species including beetles, bacteria, worms, fungi, and squid with several of them cloned and tested for molecular biology research. Each of these luciferases has different characteristics, which makes them attractive for certain applications, but not optimal for others. For high-throughput applications and assays, an optimal luciferase would display the following characteristics: (1) enzyme stability over a variety of conditions; (2) highlight output for increased sensitivity; (3) non-invasive monitoring of enzymatic activity at different time points in real-time; and (4) the catalysis of stable light emission for minimal variability between thousands of screened wells.

[0051] In one embodiment of the method of the present invention, the alteration of the signal of said reporter polypeptide in step a) ii) to the signal of said reporter polypeptide in step a) i) is a reduction or deletion of the signal of the reporter polypeptide. In one specific embodiment of the method of the present invention, the signal of the reporter polypeptide is any signal of a reporter polypeptide as defined above. In a preferred embodiment of the present invention, the signal of the reporter polypeptide is a luminescence signal. The “alteration of the signal of the reporter polypeptide” in the context of the present invention can be any reduction of the signal received in step a) ii) of the methods as described above, meaning that the signal of the reporter polypeptide measured in step a) i) is only about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%,

75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or even 100% of the signal measured in step a) ii). When the signal of the reporter polypeptide in step a) i) is 100% of the signal of the reporter polypeptide measured in step a) ii) and vice versa, then the test modulator compound according to the present invention is not a modulator compound, meaning that the test modulator compound does not, in such a case, modulate the protein-protein interaction between the first and the second protein of said protein-protein interaction.

[0052] In a further embodiment of the method of the present invention, step a) of any method of the present invention as described herein is carried out within a cell, cell lysate, within a reaction mixture or outside a cell, preferably within a cell.

[0053] In an additional embodiment of the method of the present invention, the first protein of said protein-protein interaction is fused to a first part of the reporter polypeptide and the second protein of said protein-protein interaction is fused to a second part of the reporter polypeptide. For example, the first part of the reporter polypeptide can be 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the complete reporter polypeptide. For example, the second part of the reporter polypeptide can be 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the complete reporter polypeptide. For example, in such an embodiment of the method according to the present invention, the reporter polypeptide can be a split ubiquitin, a split luciferase or a split fluorescence protein, preferably a split luciferase. Such systems are for example known from WO 2016038750. Such a split luciferase is deactivated, when divided into an N-terminal and a C-terminal side fragment, being split in a particular position. Then both fragments show no luciferase activity. Luciferase activity can be measured, when both fragments build a functional complementation. The N-terminal fragment and C-terminal fragment of the split luciferase is also known as "split luciferase" collectively, wherein usually the smaller fragment thereof is called the "SmBiT" and the larger fragment thereof is called the "LgBiT". For example, the SmBiT of the split luciferase can be 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%,

13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the complete split luciferase. For example, the LgBiT of the split luciferase can be 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the complete split luciferase. When now a first protein of the protein-protein interaction according to the present invention is bound, fused or coupled to one fragment of the split luciferase, while the second protein of the protein-protein interaction according to the present invention is bound, fused or coupled to the other fragment of the split luciferase, the interaction between the first and the second protein will position both fragments of the split luciferase in close proximity within the cellular milieu and will result in functional complementation of the split nanoluciferase. The enzymatic activity of complemented luciferase is then quantified in live or lysed cells as relative luminescence units. However, when then a test modulator compound is able to bind to the first or the second protein of the protein-protein interaction, the protein-protein interaction cannot or in a reduced way take place so that the split luciferase fragments cannot get into close proximity with each other. A reduction or deletion of the luminescence units measured before, in the absence of a test modulator compound according to step a) ii) of the method according to the present invention, can be observed. In such an embodiment, the test modulator compound can then be named a modulator compound or can have the function of a modulator compound. In this embodiment, the term "coupled", "bound" or "fused" as used with respect to the present invention is not particularly limited, and a covalent bond, ionic bond, hydrogen bond, van der Waals forces, a hydrophobic bond or a non-covalent bond may also be encompassed by these terms.

[0054] The term "quantifying" or "quantifying the extent of the modulation of the protein-protein interaction" as used in the context of the present invention means measuring a reporter polypeptide activity, especially measuring a reduction in reporter polypeptide activity and determining an extent to which a modulator reduces reporter polypeptide activity. The term "measuring the/ a signal of the reporter polypeptide" includes "measuring reporter polypeptide activity", "measuring a reduction in reporter polypeptide activity" and can be used synonymously in the context of the present invention.

[0055] In a further embodiment of the method of the present invention, the modulator compound is a compound being capable to modulate the protein-protein interaction, preferably is capable of inhibiting said protein-protein interaction. In one specific embodiment of the method of the present invention, the modulator compound is a compound being capable of inhibiting a protein-protein interaction, preferably a protein-protein interaction of the nuclear factor kappa B signaling pathway, more preferably the protein-protein interaction between NEMO and linear ubiquitin chains and most preferably the interaction between the UBAN domain of NEMO (SEQ ID NO: 2) and an Ubiquitin variant (SEQ ID NO: 6).

[0056] This means that such a modulator compound according to the present invention can be an inhibitor. An "inhibitor" as used herein is defined as a compound/molecule reducing or blocking the activity of a target molecule and/or signaling pathway. The inhibitor may achieve this effect by reducing or blocking the transcription of the gene encoding the protein to be inhibited and/or reducing/blocking the translation of the mRNA encoding the protein to be inhibited. It can also be that the protein to be inhibited performs its biochemical function with decreased efficiency in the presence of the inhibitor or that the protein to be inhibited performs its cellular function with reduced efficiency in the presence of the inhibitor. Accordingly, the term "inhibitor" encompasses both molecules/compounds that have a directly reducing/blocking effect on the specific signaling pathway, but also molecules that are indirectly inhibiting, e.g. by interacting, for example, with molecules that positively regulate (e.g. activate) said pathway. The inhibitor can also be an antagonist of the pathway (receptor) to be inhibited. Methods for testing if a compound/molecule is capable to reduce or block the activity of a target molecule and/or signaling pathway are known to the skilled artisan. An "inhibitor" within the context of the present invention can also include a modulator compound that affects the activity of the reporter polypeptide and therefore reduces reporter polypeptide activation or activity. The term "affects" in this context of the present invention means that the activity of the reporter polypeptide is reduced for about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or even 100% compared to the activity of the reporter polypeptide without the presence of the inhibitor or modulator compound.

[0057] The modulator compound/molecule that can be used as an inhibitor can be any compound/molecule, which can reduce or block the respective pathway or which inhibits an activator of the signaling (pathway) to be inhibited. Exemplary inhibitors can include suitable

binding proteins as described herein, which are directed e.g. against activators of a certain pathway.

[0058] The inhibitor can also be a nucleic acid molecule, such as an RNA, siRNA, miRNA or a non-proteinaceous aptamer as described herein. Also the nucleic acid molecules may be used to suppress an activator of a pathway to be inhibited.

[0059] It is also encompassed by the present invention that the inhibitor is a small molecule or protein/polypeptide. As described herein, such a small molecule can have a low molecular weight of less than 900 daltons (da), less than 800 da, less than 700 da, less than 600 da or less than 500 da. The size of a small molecule can be determined by methods well known in the art, e.g., mass spectrometry. The inhibitor can also be an antagonist of the pathway/signaling pathway to be inhibited.

[0060] An inhibitor may reduce or decrease the pathway to be inhibited by 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90% or more (91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or even 100%) when compared to the activity of the pathway without the addition of the inhibitor. A block of the pathway to be inhibited is present when the pathway is inhibited by 100%, when compared to the activity of the pathway without the addition (or before the addition) of the inhibitor.

[0061] In a further embodiment of the present invention such a modulator compound according to the present invention can also be an activator. The term "activator", as used herein, is defined as a compound/molecule enhancing or achieving the activity of a target molecule and/or signaling pathway. The activator may achieve this effect by enhancing or inducing the transcription of the gene encoding the protein to be activated and/or enhancing the translation of the mRNA encoding the protein to be activated. It can also be that the protein to be activated performs its biochemical function with enhanced efficiency in the presence of the activator or that the protein to be activated performs its cellular function with enhanced efficiency in the presence of the activator. Accordingly, the term "activator" encompasses both molecules/compounds that have a directly activating effect on the specific signaling pathway but also molecules that are indirectly activating, e.g. by interacting, for example, with molecules that negatively regulate (e.g. suppress) said pathway. The activator can also be an agonist of the signaling pathway (receptor) to be activated. Methods for testing if a compound/molecule is capable to induce or enhance the activity of a target molecule and/or pathway are known to the skilled artisan. The compound/molecule that can be used as an activator can be any

compound/molecule, which can activate the respective pathway or which inhibits a suppressor of the pathway to be activated. Exemplary activators can include suitable binding proteins directed e.g. against suppressors of a certain pathway.

[0062] In another embodiment of the method of the present invention, fusing the first protein of said protein-protein interaction with the reporter polypeptide or part thereof is carried out by recruiting the first protein via an interaction between a DNA binding domain and an upstream activator sequence of the reporter polypeptide or part thereof to the reporter polypeptide or part thereof. Examples of such a DNA binding domain include, but are not limited to, Gal4 DNA-binding domain (DBD) (SEQ ID NO: 10). Examples of such an upstream activator sequence include, but are not limited to, Gal4 DBD binding site repeats (SEQ ID NO: 11). The term "recruiting" or "recruited" means in the context of the present invention and as used in this specific embodiment, aiming at bringing the interaction partners into contact, which includes any ways of direct and indirect contact, with each other or aiming at bringing the interaction partners into interaction. Thus, the terms "binding", "interacting" and "recruiting" can be used synonymously in the context of the present invention.

[0063] In this specific embodiment, in the absence of said test modulator compound according to step a) ii), the binding of the DNA binding domain to the upstream activator sequence and the binding of the transcription activation domain of the reporter polypeptide to a reporter sequence of the reporter polypeptide causes expression of the reporter polypeptide. Examples of a reporter sequence include, without limitation, Firefly luciferase (SEQ ID NO: 12). Examples of such a transcription activation domain include, but are not limited to, VP16 (SEQ ID NO: 13).

[0064] In another embodiment of the method of the present invention, the protein-protein interaction is the interaction between a protein of interest or a part thereof and an engineered high affinity variant, preferably the interaction between the UBAN domain of NEMO (SEQ ID NO: 2) and an Ub variant (SEQ ID NO: 6), the interaction between the UBC9 enzyme (SEQ ID NO: 3) and a small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), the interaction between the ATG3 enzyme (SEQ ID NO: 4) and a GATE16 variant (SEQ ID NO: 8), and the interaction between OPTN (SEQ ID NO: 5) and a LC3B variant (SEQ ID NO: 9). The term "protein of interest" is intended to mean a protein to be prepared, and includes physiologically active proteins such as growth hormone, proteins of signalling pathways, interferon, interleukin, granulocyte colony stimulating factor, erythropoietin, and membrane receptors. Additionally, the term "protein of interest", in the context of the present invention, means any protein desired for investigation, which is intended for detection of a protein-protein interaction and which is able to build a respective protein-protein interaction with a further protein or interaction partner. Unless specifically mentioned otherwise, the terms, "protein," "peptide," and "polypeptide" are used interchangeably.

[0065] In a specific embodiment of the method of the present invention, the engineered high affinity variant as used in the context of the present invention corresponds to the second protein of the protein-protein interaction according to the methods as described herein, wherein the engineered high affinity variant is then preferably selected from the group consisting of an Ub variant (SEQ ID NO: 6), a small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), a GATE16 variant (SEQ ID NO: 8) and a LC3B variant (SEQ ID NO: 9), more preferably an Ub variant (SEQ ID NO: 6).

[0066] "UBAN", as used herein, can mean the NEMO-like protein optineurin and is therefore termed UBD in ABIN proteins and NEMO (UBAN). For example, "UBAN" may comprise or have the sequence of SEQ ID NO: 2 or be a sequence, which is 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or even 100% identical to SEQ ID NO: 2. This amino acid sequence corresponds to amino acids 246 - 337 of human NEMO.

[0067] "NEMO", as used herein, can mean the regulatory subunit of the I κ B-kinase (IKK) in the NF- κ B activation. This highly conserved regulatory protein "NEMO" is also known as IKK γ or FIP-3. IKK activity relies on the interaction between the kinase and NEMO. For example, "NEMO" may comprise or have the sequence of SEQ ID NO: 1 according to UniProtKB Q9Y6K9 or be a sequence, which is 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or even 100% identical to SEQ ID NO: 1. However, for example, "NEMO" may also comprise or have the sequence of SEQ ID NO: 18 according to UniProtKB O88522 or be a sequence, which is 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or even 100% identical to SEQ ID NO: 18, which corresponds to mouse NEMO. "Ubiquitin", as used herein, can mean a small but extremely important protein that induces the "death" to other proteins or change the activity of other proteins. Ubiquitin consists of 76 amino acids. In the normal course of events, proteins are inactivated or their activity is changed by the attachment of ubiquitin to them, a process called ubiquitination. Ubiquitin acts as a tag by which the protein-transport machinery ferries a protein to the proteasome for degradation. Antagonizing this process are enzymes that remove ubiquitin from proteins. For example, ubiquitin may comprise or have the sequence of SEQ ID NO: 14 or be a sequence, which is 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 14.

[0068] "UBC9" as used herein can mean the E2-conjugating enzyme, which catalyzes the conjugation of small ubiquitin-like modifiers (SUMOs) to ϵ -amino groups of lysine residues in target proteins. For example, "UBC9" may comprise or have the sequence of SEQ ID NO: 3 according to UniProtKB P63279 or be a sequence, which is 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 3.

[0069] "SUMO" as used herein can mean a small ubiquitin-like modifier. Post-translational modifications by the small ubiquitin-like modifiers regulate a plethora of cellular processes, including genome integrity, gene expression and ribosome biogenesis. Complexity in

SUMOylation processes is introduced through the conjugation of at least three formally different proteins to substrates: SUMO1 and the highly similar SUMO2 and SUMO3. For example, "SUMO2" may comprise or have the sequence of SEQ ID NO: 15 according to UniProtKB P61956 or be a sequence, which is 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 15.

[0070] "ATG3" as used herein can mean an ubiquitin-like-conjugating enzyme. ATG3 is an E2 conjugating enzyme, which is required for the cytoplasm to vacuole transport (Cvt), autophagy and mitochondrial homeostasis. ATG3 is responsible for the E2-like covalent binding of phosphatidylethanolamine to the C-terminal Gly of ATG8-like proteins (GABARAP, GABARAPL1, GABARAPL2 or MAP1LC3A). For example, "ATG3" may comprise or have the sequence of SEQ ID NO: 4 according to UniProtKB Q9NT62 or be a sequence, which is 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or even 100% identical to SEQ ID NO: 4.

[0071] "GATE16" as used herein can mean Golgi-associated ATPase Enhancer of 16 kDa (GATE-16), also known as Apg8p2 and GABARAPL2. It is a 117 amino acid (aa) polypeptide and a member of the Autophagy-related 8 (Atg8) family of proteins. GATE-16/Apg8p2 has 100% amino acid sequence identity with its mouse and rat orthologs, and is orthologous to the yeast Atg8. GATE-16/Apg8p2 is best known for its role in autophagy. GATE-16/Apg8p2 covalently attaches to phosphatidylethanolamine the phagophore (autophagosome precursor) membrane using an Ubiquitin-like conjugation system that includes Ubiquitin-activating (E1)-, Ubiquitin-conjugating (E2)-, and Ubiquitin Ligase (E3)-like enzymes. It can be involved in the later stages of autophagosome formation. It may also be involved in cargo recruitment to autophagosomes. For example, "GATE16" may comprise or have the sequence of SEQ ID NO: 16 according to UniProtKB P60520 or be a sequence, which is 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or even 100% identical to SEQ ID NO: 16.

[0072] "OPTN" as used herein can mean optineurin and can play an important role in the maintenance of the Golgi complex, in membrane trafficking, in exocytosis, through its interaction with myosin VI and Rab8. OPTN links myosin VI to the Golgi complex and plays an important role in Golgi ribbon formation. It can play a role in the activation of innate immune response during viral infection. Mechanistically, it may recruit TBK1 at the Golgi apparatus, promoting its trans-phosphorylation after RLR or TLR3 stimulation. In turn, activated TBK1 phosphorylates its downstream partner IRF3 to produce IFN-beta. It may also act by regulating membrane trafficking and cellular morphogenesis via a complex that contains Rab8 and huntingtin (HD). Further, it may mediate the interaction of Rab8 with the probable GTPase-activating protein TBC1D17 during Rab8-mediated endocytic trafficking, such as of transferrin receptor (TFRC/TfR) and may regulate Rab8 recruitment to tubules emanating from the endocytic recycling compartment. For example, "OPTN" may comprise or have the sequence of SEQ ID

NO: 5 according to UniProtKB Q96CV9 or be a sequence, which is 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or even 100% identical to SEQ ID NO: 5.

[0073] "LC3B" as used herein can mean an ubiquitin-like protein that is a constituent of the ATG8-conjugation system, one of two evolutionarily conserved phosphatidylethanolamine conjugation systems necessary for the formation of the autophagosome. The human ATG8 system includes seven ubiquitin-like light chain proteins (LCPs) that are homologs of yeast LC3: MAP1LC3A, -B, -C, GABARAP, GABARAPL1, -2, and -3. Pro-LCPs are cleaved by ATG4B to expose a C-terminal glycine residue, the cytosolic LCP-I form. The exposed C-terminus is conjugated to the head group amine of phosphatidylethanolamine through an amide bond by a sequence of ubiquitination-like reactions that involves an E1 (ATG7), an E2 (ATG3), and an E3 (a complex including ATG5, ATG12, and ATG16L). For example, "LC3B" may comprise or have the sequence of SEQ ID NO: 17 according to UniProtKB Q9GZQ8 or be a sequence, which is 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 17.

[0074] In the context of the present invention, such an "engineered high affinity variant" means a modified protein variant (e.g. the "variant" shown in Figure 1A and B). The term "engineered" means in the context of the present invention and as it is used in "engineered high affinity variant" described herein to contain amino acid mutations that enhance the affinity of a specific target protein to an epitope. Amino acid mutations are selected from a combinatorial library of modified protein variants using phage display. However, other methods are also possible such as yeast display, ribosome display, mRNA display or computational methods. The term "variant" as used in the context of the invention means that any protein like ubiquitin variant (Ubv-A), LC3B or SUMO2 variants harbour affinity enhancing mutations with regard to the protein-protein interaction of a first and a second protein compared to the protein without these mutations. It is a variant of itself with altered properties, such as enhanced affinity, compared to the protein without these mutations. The term "modified", as used in the context of the invention, means the introduction of amino acid mutations into a protein so that the original protein is altered in its primary structure. The word "mutated" as being known to the skilled artisan can be used synonymously with the terms "modified" and "engineered" as used herein. The terms "variant", "engineered affinity variant", "engineered high affinity variant", "high affinity variant" and "specific high affinity variant" can be used synonymously here. In a specific embodiment, the engineered high affinity variant corresponds to the "second protein of the protein-protein interaction" as defined herein. "Affinity" and "epitope" are known to a skilled artisan and does not have to be defined in more concrete terms herein. The present invention is also directed to the engineered high affinity variant as described herein, wherein the high affinity variant is selected from the group consisting of the Ub variant (SEQ ID NO: 6), the small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), the GATE16 variant (SEQ ID NO: 8), and the LC3B variant (SEQ ID NO: 9). The present invention is also directed to the engineered high affinity variant as

described herein, wherein the engineered high affinity variant is selected from the group consisting of the small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), the GATE16 variant (SEQ ID NO: 8), and the LC3B variant (SEQ ID NO: 9). This means, the present invention is also directed to proteins selected from the group consisting of the Ub variant (SEQ ID NO: 6), the small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), the GATE16 variant (SEQ ID NO: 8), and the LC3B variant (SEQ ID NO: 9). This means, the present invention is also directed to proteins selected from the group consisting of the small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), the GATE16 variant (SEQ ID NO: 8), and the LC3B variant (SEQ ID NO: 9). Further, the present invention is directed to proteins selected from the group consisting of the Ub variant (SEQ ID NO: 6), the small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), the GATE16 variant (SEQ ID NO: 8), and the LC3B variant (SEQ ID NO: 9) for use in any of the methods as described herein. Further, the present invention is directed to proteins selected from the group consisting of the small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), the GATE16 variant (SEQ ID NO: 8), and the LC3B variant (SEQ ID NO: 9) for use in any of the methods as described herein.

[0075] In an additional embodiment of the method of the present invention, the method is a high-throughput screening assay, preferably a cell-based high-throughput screening assay, for the modulator compound of the protein-protein interaction. A person skilled in the art knows the term "high-throughput screening assay" and is aware thereof, when such an assay is given. No further definition is herein necessary for the person skilled in the art. The same applies for the term "cell-based high-throughput screening assay" or "cellular high-throughput screening assay".

[0076] The present invention also relates to a modulator compound detected by any of the methods described herein for use in the prophylaxis and/or treatment of a disease selected from the group consisting of cancer, chronic inflammatory diseases, autoimmune diseases, neurodegenerative diseases, arthritides and infectious diseases. Preferably, the disease is selected from the group consisting of cancer, chronic inflammatory diseases, autoimmune diseases, neurodegenerative diseases and arthritides, more preferably cancer, chronic inflammatory diseases, autoimmune diseases, and arthritides and most preferably chronic inflammatory diseases, autoimmune diseases, and arthritides. In a preferred embodiment, the modulator compound is a Ca antagonist.

[0077] The present invention also relates to a method as described herein for use in the prophylaxis and/or treatment of a disease selected from the group consisting of cancer, chronic inflammatory diseases, autoimmune diseases, neurodegenerative diseases, arthritides and infectious diseases. Preferably, the disease is selected from the group consisting of cancer, chronic inflammatory diseases, autoimmune diseases, neurodegenerative diseases and arthritides, more preferably cancer, chronic inflammatory diseases, autoimmune diseases, and

arthritis and most preferably chronic inflammatory diseases, autoimmune diseases, and arthritis.

[0078] The present invention also relates to a kit comprising an engineered high affinity variant as described herein. In a specific embodiment of the kit, the engineered high affinity variant corresponds to the second protein of the protein-protein interaction as described herein and vice versa. In a specific embodiment of the kit, the engineered high affinity variant is selected from the group consisting of the Ub variant (SEQ ID NO: 6), the small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), the GATE16 variant (SEQ ID NO: 8), and the LC3B variant (SEQ ID NO: 9). In a specific embodiment of the kit, the engineered high affinity variant is selected from the group consisting of the small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), the GATE16 variant (SEQ ID NO: 8), and the LC3B variant (SEQ ID NO: 9). This means, the present invention also provides a composition comprising a protein selected from the group consisting of the Ub variant (SEQ ID NO: 6), the small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), the GATE16 variant (SEQ ID NO: 8), and the LC3B variant (SEQ ID NO: 9). This means, the present invention also provides a composition comprising a protein selected from the group consisting of the small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), the GATE16 variant (SEQ ID NO: 8), and the LC3B variant (SEQ ID NO: 9). Thus, kits comprising one or more components (e.g., assay system components) described herein are also encompassed by the present invention. Such kits will typically contain printed instructions as to the use of the components contained therein.

[0079] In another embodiment, the present invention relates to a kit-of-parts comprising an engineered affinity variant as described herein and a test system that is capable of detecting the extent of modulation of a modulator compound on a protein-protein interaction between a first protein and a second protein as defined herein. In a specific embodiment thereof, the second protein is the engineered high affinity variant as defined herein. In one specific embodiment of the kit-of-parts, the engineered affinity variant is selected from the group consisting of the Ub variant (SEQ ID NO: 6), the small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), the GATE16 variant (SEQ ID NO: 8), and the LC3B variant (SEQ ID NO: 9). In a further embodiment thereof, the test system further comprises a reporter polypeptide, wherein the reporter polypeptide is selected from the group consisting of a split ubiquitin, a fluorescent protein, preferably the green, red or yellow fluorescent protein, more preferably a split green fluorescent protein, beta-lactamase, beta-galactosidase, chloramphenicol acetyltransferase (CAT) and a luciferase, preferably Firefly luciferase or Renilla luciferase or split nanoluciferase.

[0080] The present invention further provides a test system that is capable of detecting the extent of modulation of the modulator compound on a protein-protein interaction between a first and a second protein as described herein. In an embodiment of the test system, it is preferred

that the protein-protein interaction is the interaction between a protein of interest and an engineered high affinity variant, more preferably the interaction between the UBAN domain of NEMO (SEQ ID NO: 2) and a Ub variant (SEQ ID NO: 6), the interaction between UBC9 (SEQ ID NO: 3) and a small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), the interaction between the ATG3 enzyme (SEQ ID NO: 4) and a GATE16 variant (SEQ ID NO: 8), and the interaction between OPTN (SEQ ID NO: 5) and a LC3B variant (SEQ ID NO: 9). In a further embodiment of the test system, the first protein of the protein-protein interaction is selected from the group consisting of UBAN domain of NEMO (SEQ ID NO: 2), UBC9 enzyme (SEQ ID NO: 3), ATG3 enzyme (SEQ ID NO: 4) and OPTN (SEQ ID NO: 5), preferably the UBAN domain of NEMO (SEQ ID NO: 2), and/or the second protein of the protein-protein interaction is selected from the group consisting of an Ub variant (SEQ ID NO: 6), a small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), a GATE16 variant (SEQ ID NO: 8) and a LC3B variant (SEQ ID NO: 9), preferably an Ub variant (SEQ ID NO: 6), wherein the second protein of the protein-protein interaction is the engineered high affinity variant.

[0081] The present invention also relates to a composition comprising an engineered high affinity variant as described herein. This means, in a specific embodiment, the composition comprises a protein or an engineered high affinity variant selected from the group consisting of an Ub variant (SEQ ID NO: 6), a small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), a GATE16 variant (SEQ ID NO: 8) and a LC3B variant (SEQ ID NO: 9), preferably an Ub variant (SEQ ID NO: 6).

[0082] The present invention also comprises the use of an engineered high affinity variant as described herein in any of the methods as mentioned herein. In a specific embodiment, the present invention encompasses the use of a protein or an engineered high affinity variant selected from the group consisting of an Ub variant (SEQ ID NO: 6), a small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), a GATE16 variant (SEQ ID NO: 8) and a LC3B variant (SEQ ID NO: 9), preferably the use of an Ub variant (SEQ ID NO: 6), in any of the methods as described herein.

[0083] Further, the present invention comprises also the use of a method as described or defined herein. Such a use of a method as described herein can be for detecting a modulator compound of a protein-protein interaction between a first and a second protein as described or defined herein.

[0084] The present invention also relates to a test system for detecting the extent of modulation of the modulator compound as described herein on a protein-protein interaction between a first protein and a second protein as described herein. The test system comprises the following components: a first fusion between a first protein of a protein-protein interaction as defined herein, a second fusion between a second protein of a protein-protein interaction as defined herein and a reporter polypeptide. The test modulator compound can then be applied to the test

system as described herein. In the test system according to the present invention, the first protein of the protein-protein interaction is selected from the group consisting of the UBAN domain of NEMO (SEQ ID NO: 2), the UBC9 enzyme (SEQ ID NO: 3), the ATG3 enzyme (SEQ ID NO: 4) and OPTN (SEQ ID NO: 5). In a preferred embodiment of the present invention, the first protein of the protein-protein interaction is the UBAN domain of NEMO (SEQ ID NO: 2). In one specific embodiment of the present invention, the second protein of the protein-protein interaction is selected from the group consisting of an Ub variant (SEQ ID NO: 6), a small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), a GATE16 variant (SEQ ID NO: 8) and a LC3B variant (SEQ ID NO: 9). In a preferred embodiment of the present invention, the second protein of the protein-protein interaction is an Ub variant (SEQ ID NO: 6). In one specific embodiment of the test system, the test system further comprises a reporter polypeptide, wherein the reporter polypeptide is selected from the group consisting of a split ubiquitin, a fluorescent protein, preferably the green, red or yellow fluorescent protein, more preferably a split green fluorescent protein, beta-lactamase, beta-galactosidase, chloramphenicol acetyltransferase (CAT) and a luciferase, preferably Firefly luciferase or Renilla luciferase or split nanoluciferase.

[0085] Although the present invention is described in detail herein, it is to be understood that this invention is not limited to the particular methodologies, protocols and reagents described herein as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which will be limited only by the appended claims. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art.

[0086] Herein, the elements of the present invention are described. Though, these elements are listed with specific embodiments, however, it should be understood that they may be combined in any manner and in any number to create additional embodiments. The variously described examples and preferred embodiments described throughout the specification should not be construed to limit the present invention to only the explicitly described embodiments. This description should be understood to support and encompass embodiments, which combine the explicitly described embodiments with any number of the disclosed and/or preferred elements. Furthermore, any permutations and combinations of all elements described herein should be considered disclosed by the description of the present application unless the context indicates otherwise.

[0087] Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated member, integer or step or group of members,

integers or steps, but not the exclusion of any other member, integer or step or group of members, integers or steps although in some embodiments such other member, integer or step or group of members, integers or steps may be excluded, i.e. the subject-matter consists in the inclusion of a stated member, integer or step or group of members, integers or steps. When used herein the term "comprising" can be substituted with the term "containing" or "including" or sometimes, when used herein, with the term "having". When used herein "consisting of" excludes any element, step, or ingredient not specified.

[0088] The terms "a" and "an" and "the" and similar reference used in the context of describing the invention (especially in the context of the claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein.

[0089] All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as"), provided herein is intended merely to better illustrate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

[0090] Unless otherwise indicated, the term "at least" preceding a series of elements is to be understood to refer to every element in the series. The term "at least one" refers to one or more such as two, three, four, five, six, seven, eight, nine, ten and more. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the present invention.

[0091] The term "and/or" wherever used herein includes the meaning of "and", "or" and "all or any other combination of the elements connected by said term".

[0092] When used herein "consisting of" excludes any element, step, or ingredient not specified in the claim element. When used herein, "consisting essentially of" does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claim.

[0093] The term "including" means "including but not limited to". "Including" and "including but not limited to" are used interchangeably.

[0094] The term "about" means plus or minus 10%, preferably plus or minus 5%, more preferably plus or minus 2%, most preferably plus or minus 1%.

[0095] Throughout the description and claims of this specification, the singular encompasses the plural, unless the context otherwise requires. In particular, where the indefinite article is

used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

[0096] It should be understood that this invention is not limited to the particular methodology, protocols, material, reagents, and substances, etc., described herein and as such can vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims.

[0097] Several documents are cited throughout the text of this specification. Each of the documents cited herein (including all patents, patent applications, scientific publications, manufacturer's specifications, instructions, etc.), whether *supra* or *infra*, are hereby incorporated by reference in their entirety. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention. To the extent the material incorporated by reference contradicts or is inconsistent with this specification, the specification will supersede any such material.

[0098] The content of all documents and patent documents cited herein is incorporated by reference in their entirety.

[0099] A better understanding of the present invention and of its advantages will be gained from the examples, offered for illustrative purposes only. The examples are not intended to limit the scope of the present invention in any way.

EXAMPLES OF THE INVENTION

[00100] The following Examples illustrate the invention, but are not to be construed as limiting the scope of the invention.

[00101] Proof-of-concept and method validation

[00102] Example 1: Generation and characterization of a Ub variant that binds to the UBAN domain of NEMO with high affinity

[00103] The inventors selected the interaction between the UBAN domain of NEMO (UBAN_{NEMO}) (SEQ ID NO: 2) with linear ubiquitin (Ub) chains (linear_{Ub}) as primary target protein-protein interaction (PPI) due to its crucial role in the activation of the NF- κ B signalling pathway. High affinity Ub variants that bind to the target protein, UBAN_{NEMO}, were developed using protein engineering, specifically phage display technology. The phage-displayed Ub variant (Ubv) library was generated using soft randomization strategy and mutations were introduced to the regions of Ub that are engaged in the UBAN_{NEMO} (SEQ ID NO: 2) and linear Ubiquitin interaction (named Regions 1, 2 and 3 in **Figure 2B**)⁴. Then the phage display library was screened against mouse UBAN_{NEMO} domain (mUBAN_{NEMO}) (SEQ ID NO: 18), which shares high conservation with the human UBAN_{NEMO} (hUBAN_{NEMO}) (SEQ ID NO: 2). After phage display selections, Ubv with improved affinity to the target were isolated by clonal enzyme-linked immunosorbent assay (ELISA) and twenty-three unique Ub variants were found by sequencing (**Figure 2A**). In order to identify Ub variants with the highest affinity, phage ELISA with surface immobilized mUBAN_{NEMO} (SEQ ID NO: 18) was performed, while competing with set concentrations of free mUBAN_{NEMO} (SEQ ID NO: 18) in solution. This assay allows an estimation of the IC₅₀ of the phage displayed Ubv. The Ubv with the best binding affinity to the UBAN_{NEMO} based on the estimated IC₅₀ values was named Ubv-A (SEQ ID NO: 6) and used for subsequent experiments (**Figure 2B**).

[00104] As reported in other studies, the F312A mutation in human UBAN_{NEMO} (hUBAN_{NEMO}) (SEQ ID NO: 19) abolishes NEMO binding to linear_{Ub}². To confirm the targeted epitope of the generated Ubv-A (SEQ ID NO: 6) in UBAN_{NEMO}, we performed immunoprecipitation experiment using the wild type (SEQ ID NO: 2) and F312A mutant (SEQ ID NO: 19) UBAN_{NEMO}. Only the wild type hUBAN_{NEMO} (SEQ ID NO: 2) and not F312A UBAN_{NEMO} (SEQ ID NO: 19) co-immunoprecipitated with Ubv-A (SEQ ID NO: 6), indicating that Ubv-A (SEQ ID NO: 6) indeed binds the UBAN_{NEMO} (SEQ ID NO: 2) and could potentially compete the interaction with linear_{Ub} in cells (**Figure 3A**).

[00105] The linear UBAN domain is highly conserved and can be also found in ABIN and optineurin (OPTN) proteins. Since the phage display strategy was designed to select high

affinity variants against UBAN_{NEMO}, Ubv-A (SEQ ID NO: 6) was then tested for selectivity in binding between UBAN domains of NEMO (SEQ ID NO: 2), ABIN1 (SEQ ID NO: 20) and OPTN (SEQ ID NO: 5). Ubv-A (SEQ ID NO: 6) preferentially interacted with UBAN_{NEMO} (SEQ ID NO: 2) with a dissociation constant of 0.29 μ M, which is ~ 7 and ~ 20 times smaller values of that of OPTN (SEQ ID NO: 5) and ABIN1 (SEQ ID NO: 20), respectively, indicating that Ubv-A (SEQ ID NO: 6) selectively binds the linear UBAN domain of NEMO (SEQ ID NO: 2) (**Figure 3B**). Additionally, the specificity of Ubv-A (SEQ ID NO: 6) was further validated by phage ELISA using wild type or mutant UBAN-domains of NEMO (aa 250-339, SEQ ID NO: 18), NEMO (aa 250-339, V293A Y301A K302A, SEQ ID NO: 21), OPTN (aa 454-514, SEQ ID NO: 22), OPTN (aa 454-577, SEQ ID NO: 23), OPTN (aa 454-514, D474N, SEQ ID NO: 24), ABIN1 (aa 465-525, SEQ ID NO: 25), ABIN1 (aa 465-525, D485A, SEQ ID NO: 26), ABIN1 (aa 465-525, D485N and F486A mutant, SEQ ID NO: 27). Furthermore, the Ub binding proteins HOIL (SEQ ID NO: 28, corresponding to UniProtKB Q9BYM8), HOIP (SEQ ID NO: 29, corresponding to UniProtKB Q96EP0), USP21 (SEQ ID NO: 30, corresponding to UniProtKB Q9UK80), USP7 (SEQ ID NO: 31, corresponding to UniProtKB Q93009), OTUD7A (SEQ ID NO: 32, corresponding to UniProtKB Q8TE49), USP14 (SEQ ID NO: 33, corresponding to UniProtKB P54578), USP5 (SEQ ID NO: 34, corresponding to UniProtKB P45974) were used as negative controls. In summary, these experiments demonstrate that Ubv-A (SEQ ID NO: 6) binds specifically to wt UBAN_{NEMO} (SEQ ID NO: 2) and recognizes an epitope on NEMO that is also involved in binding in linear_{Ub}, indicating that Ubv-A (SEQ ID NO: 6) binds the molecular surface on UBAN_{NEMO} occupied by linear_{Ub} (**Figure 3C**).

[00106] Example 2: Development of cell-based reporter assays using generated/engineered high affinity variants

[00107] The initial design of the assay system is based on the dual-luciferase reporter (DLR) technology (Promega), the theoretical model of which is presented in **Figure 1A**. The invention takes advantage of high affinity interaction between the target protein (e.g. UBAN_{NEMO}, SEQ ID NO: 2) and a variant (e.g. Ubv-A, SEQ ID NO: 6), coupling the expression of Firefly luciferase to the occurrence of a given interaction. In specific embodiments described herein, UBAN_{NEMO} (SEQ ID NO: 2) is expressed as a fusion to the Gal-4 DNA binding domain (DBD, SEQ ID NO: 10) that binds to the Gal4 promoter (SEQ ID NO: 11) upstream of the *FIREFLY* gene. The second component of the DLR system is the fusion of 2xUbv-A to a transcriptional activation module (ACT). Binding between UBAN_{NEMO} (SEQ ID NO: 2) and Ubv-A (SEQ ID NO: 6) at the *FIREFLY* promoter recruits the ACT domain (SEQ ID NO: 13) to the TATA element, which enhances Firefly expression. Importantly, the ACT fusion vector also contains the *RENILLA* luciferase gene that is constitutively expressed irrespective of the interaction between the fusion partners. Firefly and Renilla luciferases are evolutionary distinct and therefore have dissimilar

enzyme structures and substrate requirements. Therefore, activity determinations of both luciferases using subsequent addition of substrate reagents enable assay normalization to correct for variability associated with transient transfection.

[00108] In a proof-of-concept experiment, HEK293 cells were co-transfected with modified DLR vectors, incubated for 20 hours, lysed and then assayed for Firefly and Renilla luciferase activity by subsequent addition of assay reagents according to manufacturer's manual. Quantification of relative light units generated by Firefly and Renilla luciferases was recorded and Firefly/Renilla signal ratio served for normalization between samples. Since no activation of luciferase should occur in the absence of high affinity variant of the PPI under study, any signal indicative of activity of this reporter is considered to represent "background" for the DLR system. The background level of luciferase is measured in the presence of DBD and ACT empty vector controls. Co-transfection of cells with DLR assay vectors expressing the DBD-UBAN_{NEMO} first fusion protein and the ACT-2xUbv-A second fusion protein enabled the detection of the specific DLR assay activation. In contrast, the F312A UBAN_{NEMO} mutant (SEQ ID NO: 19) that does not interact with Ubv-A (SEQ ID NO: 6) did not produce any signal above the background activation (**Figure 4A**). Therefore, the binding between DBD-UBAN_{NEMO} and ACT-2xUbv-A fusions triggers the transcription of the reporter gene, thereby linking the PPI under study to production of the reporter.

[00109] Thus, the inventors present herein the first example of an approach for generation of cell-based assays that utilize high affinity variants to given PPI. To demonstrate a wide applicability of this approach on other PPIs, the inventors utilized the DLR system and exchanged the NEMO/Ubv-A binding pair with other engineered PPI pairs.

[00110] In a second example, the inventors tested a recently reported SUMO2 variant (SEQ ID NO: 7) that was engineered to bind UBC9 enzyme (SEQ ID NO: 3) with high affinity and block its enzymatic activity³. The DLR assay vectors were modified to express ACT-UBC9 and fusions of the DBD domain to either control SUMO2 or high affinity SUMO2 variant (SEQ ID NO: 7). As shown in **Figure 4B**, the interaction between UBC9 (SEQ ID NO: 3) and SUMO2 variant (SEQ ID NO: 7) results in a significant assay signal improvement over SUMO2 control, indicating that high affinity interaction between UBC9 (SEQ ID NO: 3) and SUMO2 variant (SEQ ID NO: 7) improves assay performance.

[00111] In a third example, the inventors use a GATE16 high affinity variant (SEQ ID NO: 8) developed to the ATG3 enzyme (SEQ ID NO: 4), which plays a key role in autophagy. The amino acid sequence of this engineered variant is given in SEQ ID NO: 8.

[00112] Similarly, the inventors of the present invention modified DLR assay vectors to express DBD-ATG3 and fusions of ACT to either control GATE16 or GATE16 variant (SEQ ID NO: 8). In contrast to the control GATE16 fusion, using the high affinity GATE16 variant (SEQ ID NO: 8) in the DLR assay results in significant assay activation (**Figure 4C**). Taken together, the examples

presented in **Figure 4** illustrate that specific high affinity variants significantly improve performance of the reporter assays for PPIs.

[00113] The NanoBiT assay system was then utilised as it is more advanced and enables HTS in live cells¹. The NanoBiT assay activation is independent of protein expression, which provides notable advantages in chemical screening as it limits the identification of compounds that affect protein expression machinery and therefore reduce false discovery rate (**Figure 1B**). The invention takes advantage of high affinity PPI between UBAN_{NEMO} (SEQ ID NO: 2) and Ubv-A (SEQ ID NO: 6) that are fused to the small bit (SmBiT) and the large bit (LgBiT) of split nanoluciferase, respectively. Following expression of fusion proteins in cells, high affinity PPI between UBAN_{NEMO} (SEQ ID NO: 2) and Ubv-A (SEQ ID NO: 6) leads to structural complementation of LgBiT with SmBiT, resulting in catalytically functional nanoluciferase. The activity of this complemented nanoluciferase can then be monitored by addition of the detection reagent containing cell-permeable furimazine substrate that upon binding to complemented nanoluciferase generates bright, luminescent signal that is quantified as relative light units (RLU). Therefore, PPIs of the fusion partners can be monitored in real-time inside living cells.

[00114] The assay background is determined by transfecting the LgBiT assay fusion together with a fusion of an unrelated protein (HaloTag) to SmBiT. The assay specificity is determined by the F312A point mutation in UBAN_{NEMO} (SEQ ID NO: 19) that abolishes the interaction between UBAN_{NEMO} (SEQ ID NO: 2) and high affinity Ubv-A (SEQ ID NO: 6) (**Figure 5A**).

[00115] The NanoBiT technology was then used for other target PPIs to demonstrate the flexibility of this novel approach. It was demonstrated that the current invention also enables targeting of a key selective autophagy receptor Optineurin (OPTN) with its partner protein LC3B. This PPI has a broad therapeutic application and a robust reporter assay for detecting this interaction is presented in **Figure 5B**. Taken together, the proof-of-concept experiments of the inventors demonstrate that application of phage display improved high affinity variants that target a specific PPI in assay development results in the generation of robust cell-based assays.

[00116] Example 3: Method validation in high-throughput screening

[00117] To test the application of the method in cell-based screening the NanoBiT NEMO assay was miniaturized and optimized for screening in 384-well plate format. A panel of available cell lines was first tested to identify a suitable cell line demonstrating a robust assay response. HEK293, CHO or A549 cells were co-transfected with constructs expressing LgBiT-Ubv-A in combination with either SmBiT-wt UBAN_{NEMO} or SmBiT-HaloTag fusion proteins and dispensed into 384-well plates. Following 20-hour incubation nanoluciferase activity was quantified as relative light units and Z' score for each cell line was determined. As shown in **Figure 6A**, co-transfection of A549 cells results in Z' score > 0.5 that is indicative of high assay quality.

[00118] The interaction between UBAN_{NEMO} and linear Ubiquitin chains was previously targeted

only *in vitro*, as this low affinity interaction is a roadblock towards developing a robust cell-based assay (data not shown). However, a previous study identified Aloe Emodin as a top hit emanating from a biochemical HTS targeting the interaction between UBAN_{NEMO} (SEQ ID NO: 2) and linear Ubiquitin chains. Therefore, we tested Aloe Emodin as a reference compound inhibitor for the developed cell-based NanoBiT NEMO assay. A549 cells were co-transfected with a combination of LgBiT-Ubv-A and SmBiT-wt UBAN_{NEMO} constructs, dispensed into 384-well plates and following 20-hour incubation the indicated concentrations of Aloe Emodin were dispensed into the wells and allowed to incubate for additional two hours. **Figure 6B** shows a concentration-dependent response of the assay to Aloe Emodin, validating its use as a reference inhibitor in HTS.

[00119] To examine the robustness of the NanoBiT NEMO assay as a pharmacological screening platform, the inventors used a compound library comprised of 774 FDA approved drugs. A549 cells were reverse transfected as before with a combination of LgBiT-Ubv-A and SmBiT-wt UBAN_{NEMO} constructs and dispensed into 384-well plates. Following 20-hour incubation, test compounds were dispensed at 10 μ M concentration and plates were further incubated for additional two hours. Assay response to test compounds was quantified relative to the maximum inhibition by Aloe Emodin reference inhibitor. Detected luminescence after incubation with compounds was normalized to luminescence values before compound addition to minimize the effects of transfection efficiency and variations in cell number. The assay demonstrates a suitable average Z' score of 0.42 and data correlation across assay repeats at $\geq$ 50% assay signal inhibition as threshold for minimal compound activity (**Figure 6C and 6D**). Taken together, the current embodiment validates the application of engineered high affinity variants in HTS in line with industry standards for cell-based assays.

[00120] An FDA approved drug library can also be used for drug repurposing screening. However, for many FDA approved medicines the detailed understanding of their molecular mechanism of action is currently unknown. Therefore, providing novel insights about drug mechanism of action can facilitate drug repurposing.

[00121] Top hits from this library identified within the context of these data constitute a subset of calcium antagonists (**Figure 6D**). These drugs are used for the treatment of hypertension, coronary heart diseases and as anti-hypertensive agents following acute brain injury with the protective effects through anti-inflammatory properties. Interestingly, the effect of calcium antagonists on inhibiting NF-kB transcription activation has also been described. Therefore, our data indicates a potentially new mechanism of calcium antagonists involving an inhibitory action on the interaction between NEMO and linear Ubiquitin, thereby attenuating NF-kB pathway activation, although further studies are necessary to test this possibility. It is also important to mention that some calcium antagonists are known nanoluciferase enzyme inhibitors, thus they may represent compounds that target complemented nanoluciferase enzymes.

[00122] The invention is further characterized by the following items:

1. Method for detecting a modulator compound of a protein-protein interaction between a first and a second protein, comprising the steps of:
 - a) fusing the first protein of said protein-protein interaction with a reporter polypeptide or part thereof, and fusing the second protein of said protein-protein interaction with a transcription activation domain or a part of the reporter polypeptide,
 - i) in the presence of a test modulator compound, and
 - ii) in the absence of said test modulator compound, wherein the first protein and the second protein of the protein-protein interaction are capable of binding to each other;
 - b) measuring a signal of the reporter polypeptide in step a) i) and a signal of the reporter polypeptide in step a) ii), and
 - c) comparing the measurements made in step (b), wherein an alteration of the signal of said reporter polypeptide in step a) ii) to the signal of said reporter polypeptide in step a) i) indicates the presence of a modulator compound of said protein-protein interaction, and
 - d) quantifying the extent of modulation of the protein-protein interaction by the test modulator compound by step c).
2. Method according to item 1, wherein the alteration of the signal of said reporter polypeptide in step a) ii) to the signal in step a) i) is a reduction or deletion of the signal of the reporter polypeptide.
3. Method according to item 1 or item 2, wherein step a) is within a cell, cell lysate, within a reaction mixture or outside a cell, preferably within a cell.
4. Method according to any one of the preceding items, wherein the first protein of said protein-protein interaction is fused to a first part of the reporter polypeptide and wherein the second protein of said protein-protein interaction is fused to a second part of the reporter polypeptide.
5. Method according to any one of the preceding items, wherein the modulator compound is a compound being capable of inhibiting said protein-protein interaction, preferably a protein-protein interaction of the nuclear factor kappa B signaling pathway, more preferably the

protein-protein interaction between NEMO and linear ubiquitin chains and most preferably the interaction between the UBAN domain of NEMO (SEQ ID NO: 2) and an Ubiquitin variant (SEQ ID NO: 6).

6. Method according to any one of the preceding items, wherein fusing the first protein of said protein-protein interaction with the reporter polypeptide or part thereof is carried out by recruiting the first protein via an interaction between a DNA binding domain and an upstream activator sequence of the reporter polypeptide or part thereof to the reporter polypeptide or part thereof.
7. Method according to item 6, wherein, in the absence of said test modulator compound according to step a) ii), the binding of the DNA binding domain to the upstream activator sequence and the binding of the transcription activation domain to a reporter sequence of the reporter polypeptide causes expression of the reporter polypeptide.
8. Method according to any one of the preceding items, wherein the protein-protein interaction is the interaction between a protein of interest or a part thereof and an engineered high affinity variant, preferably the interaction between an UBAN domain of NEMO (SEQ ID NO: 2) and an Ub variant (SEQ ID NO: 6), the interaction between UBC9 (SEQ ID NO: 3) and a small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), the interaction between the ATG3 enzyme (SEQ ID NO: 4) and a GATE16 variant (SEQ ID NO: 8), or the interaction between OPTN (SEQ ID NO: 5) and a LC3B variant (SEQ ID NO: 9).
9. Method according to any one of the preceding items, wherein the first protein of the protein-protein interaction is selected from the group consisting of UBAN domain of NEMO (SEQ ID NO: 2), UBC9 enzyme (SEQ ID NO: 3), ATG3 enzyme (SEQ ID NO: 4) and OPTN (SEQ ID NO: 5), preferably the UBAN domain of NEMO (SEQ ID NO: 2), and/or wherein the second protein of the protein-protein interaction is selected from the group consisting of an Ub variant (SEQ ID NO: 6), a small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), a GATE16 variant (SEQ ID NO: 8) and a LC3B variant (SEQ ID NO: 9), preferably an Ub variant (SEQ ID NO: 6).
10. Method according to any one of the preceding items, wherein the reporter polypeptide is selected from the group consisting of a split ubiquitin, a fluorescent protein, preferably the green, red or yellow fluorescent protein, more preferably a split green fluorescent protein, beta-lactamase, beta-galactosidase, chloramphenicol acetyltransferase (CAT) and a luciferase, preferably Firefly luciferase, Renilla luciferase or split nanoluciferase.

11. Method according to any one of the preceding items, wherein the method is a high-throughput screening assay, preferably a cell-based high-throughput screening assay, for detecting the modulator compound of the protein-protein interaction.
12. Modulator compound detected by any of the methods according to items 1 to 11 for use in the prophylaxis and/or treatment of a disease selected from the group consisting of cancer, chronic inflammatory diseases, autoimmune diseases, neurodegenerative diseases, arthritides and infectious diseases.
13. Engineered high affinity variant selected from the group consisting of an Ub variant (SEQ ID NO: 6), a small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), a GATE16 variant (SEQ ID NO: 8) and a LC3B variant (SEQ ID NO: 9), preferably an Ub variant (SEQ ID NO: 6).
14. A kit-of-parts comprising an engineered high affinity variant according to item 13 and a test system that is capable of detecting the extent of modulation of the modulator compound on a protein-protein interaction between a first protein and a second protein, wherein the second protein is the engineered high affinity variant.
15. Kit-of-parts according to item 14, wherein the test system further comprises a reporter polypeptide, wherein the reporter polypeptide is selected from the group consisting of a split ubiquitin, a fluorescent protein, preferably the green, red or yellow fluorescent protein, more preferably a split green fluorescent protein, beta-lactamase, beta-galactosidase, chloramphenicol acetyltransferase (CAT) and a luciferase, preferably Firefly luciferase or Renilla luciferase or split nanoluciferase.

References:

1. Dixon, A. S. *et al.* NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. *ACS Chem. Biol.***11**, 400–408 (2016).
2. Rahighi, S. *et al.* Specific Recognition of Linear Ubiquitin Chains by NEMO Is Important for NF- κ B Activation. *Cell***136**, 1098–1109 (2009).
3. Wiechmann, S. *et al.* Site-specific inhibition of the small ubiquitin-like modifier (SUMO)-conjugating enzyme Ubc9 selectively impairs SUMO chain formation. *J Biol Chem***292**, 15340–15351 (2017).
4. Ernst, A. *et al.* A strategy for modulation of enzymes in the ubiquitin system. *Science (New York, NY)***339**, 590–595 (2013).
5. Vincendeau, M. *et al.* Inhibition of Canonical NF- κ B Signaling by a Small Molecule Targeting NEMO-Ubiquitin Interaction. *Sci. Rep.***6**, 18934 (2016).

CLAIMS

1. Method for detecting a modulator compound of a protein-protein interaction between a first and a second protein, comprising the steps of:
 - a) fusing the first protein of said protein-protein interaction with a reporter polypeptide or part thereof, and
fusing the second protein of said protein-protein interaction with a transcription activation domain or a part of the reporter polypeptide,
 - i) in the presence of a test modulator compound, and
 - ii) in the absence of said test modulator compound, wherein the first protein and the second protein of the protein-protein interaction are capable of binding to each other;
 - b) measuring a signal of the reporter polypeptide in step a) i) and a signal of the reporter polypeptide in step a) ii), and
 - c) comparing the measurements made in step (b), wherein an alteration of the signal of said reporter polypeptide in step a) ii) to the signal of said reporter polypeptide in step a) i) indicates the presence of a modulator compound of said protein-protein interaction, and
 - d) quantifying the extent of modulation of the protein-protein interaction by the test modulator compound by step c).
2. Method according to claim 1, wherein the alteration of the signal of said reporter polypeptide in step a) ii) to the signal in step a) i) is a reduction or deletion of the signal of the reporter polypeptide.
3. Method according to claim 1 or claim 2, wherein step a) is within a cell, cell lysate, within a reaction mixture or outside a cell, preferably within a cell.
4. Method according to any one of the preceding claims, wherein the first protein of said protein-protein interaction is fused to a first part of the reporter polypeptide and wherein the second protein of said protein-protein interaction is fused to a second part of the reporter polypeptide.
5. Method according to any one of the preceding claims, wherein the modulator compound is a compound being capable of inhibiting said protein-protein interaction, preferably a protein-protein interaction of the nuclear factor kappa B signaling pathway, more preferably the

protein-protein interaction between NEMO and linear ubiquitin chains and most preferably the interaction between the UBAN domain of NEMO (SEQ ID NO: 2) and an Ubiquitin variant (SEQ ID NO: 6).

6. Method according to any one of the preceding claims, wherein fusing the first protein of said protein-protein interaction with the reporter polypeptide or part thereof is carried out by recruiting the first protein via an interaction between a DNA binding domain and an upstream activator sequence of the reporter polypeptide or part thereof to the reporter polypeptide or part thereof.
7. Method according to claim 6, wherein, in the absence of said test modulator compound according to step a) ii), the binding of the DNA binding domain to the upstream activator sequence and the binding of the transcription activation domain to a reporter sequence of the reporter polypeptide causes expression of the reporter polypeptide.
8. Method according to any one of the preceding claims, wherein the protein-protein interaction is the interaction between a protein of interest or a part thereof and an engineered high affinity variant, preferably the interaction between an UBAN domain of NEMO (SEQ ID NO: 2) and an Ub variant (SEQ ID NO: 6), the interaction between UBC9 (SEQ ID NO: 3) and a small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), the interaction between the ATG3 enzyme (SEQ ID NO: 4) and a GATE16 variant (SEQ ID NO: 8), or the interaction between OPTN (SEQ ID NO: 5) and a LC3B variant (SEQ ID NO: 9).
9. Method according to any one of the preceding claims, wherein the first protein of the protein-protein interaction is selected from the group consisting of UBAN domain of NEMO (SEQ ID NO: 2), UBC9 enzyme (SEQ ID NO: 3), ATG3 enzyme (SEQ ID NO: 4) and OPTN (SEQ ID NO: 5), preferably the UBAN domain of NEMO (SEQ ID NO: 2), and/or wherein the second protein of the protein-protein interaction is selected from the group consisting of an Ub variant (SEQ ID NO: 6), a small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), a GATE16 variant (SEQ ID NO: 8) and a LC3B variant (SEQ ID NO: 9), preferably an Ub variant (SEQ ID NO: 6).
10. Method according to any one of the preceding claims, wherein the reporter polypeptide is selected from the group consisting of a split ubiquitin, a fluorescent protein, preferably the green, red or yellow fluorescent protein, more preferably a split green fluorescent protein, beta-lactamase, beta-galactosidase, chloramphenicol acetyltransferase (CAT) and a luciferase, preferably Firefly luciferase, Renilla luciferase or split nanoluciferase.

11. Method according to any one of the preceding claims, wherein the method is a high-throughput screening assay, preferably a cell-based high-throughput screening assay, for detecting the modulator compound of the protein-protein interaction.
12. Modulator compound detected by any of the methods according to claims 1 to 11 for use in the prophylaxis and/or treatment of a disease selected from the group consisting of cancer, chronic inflammatory diseases, autoimmune diseases, neurodegenerative diseases, arthritides and infectious diseases.
13. Engineered high affinity variant selected from the group consisting of an Ub variant (SEQ ID NO: 6), a small ubiquitin-like modifier 2 (SUMO2) variant (SEQ ID NO: 7), a GATE16 variant (SEQ ID NO: 8) and a LC3B variant (SEQ ID NO: 9), preferably an Ub variant (SEQ ID NO: 6).
14. A kit-of-parts comprising an engineered high affinity variant according to claim 13 and a test system that is capable of detecting the extent of modulation of the modulator compound on a protein-protein interaction between a first protein and a second protein, wherein the second protein is the engineered high affinity variant.
15. Kit-of-parts according to claim 14, wherein the test system further comprises a reporter polypeptide, wherein the reporter polypeptide is selected from the group consisting of a split ubiquitin, a fluorescent protein, preferably the green, red or yellow fluorescent protein, more preferably a split green fluorescent protein, beta-lactamase, beta-galactosidase, chloramphenicol acetyltransferase (CAT) and a luciferase, preferably Firefly luciferase or Renilla luciferase or split nanoluciferase.

Figure 1

A

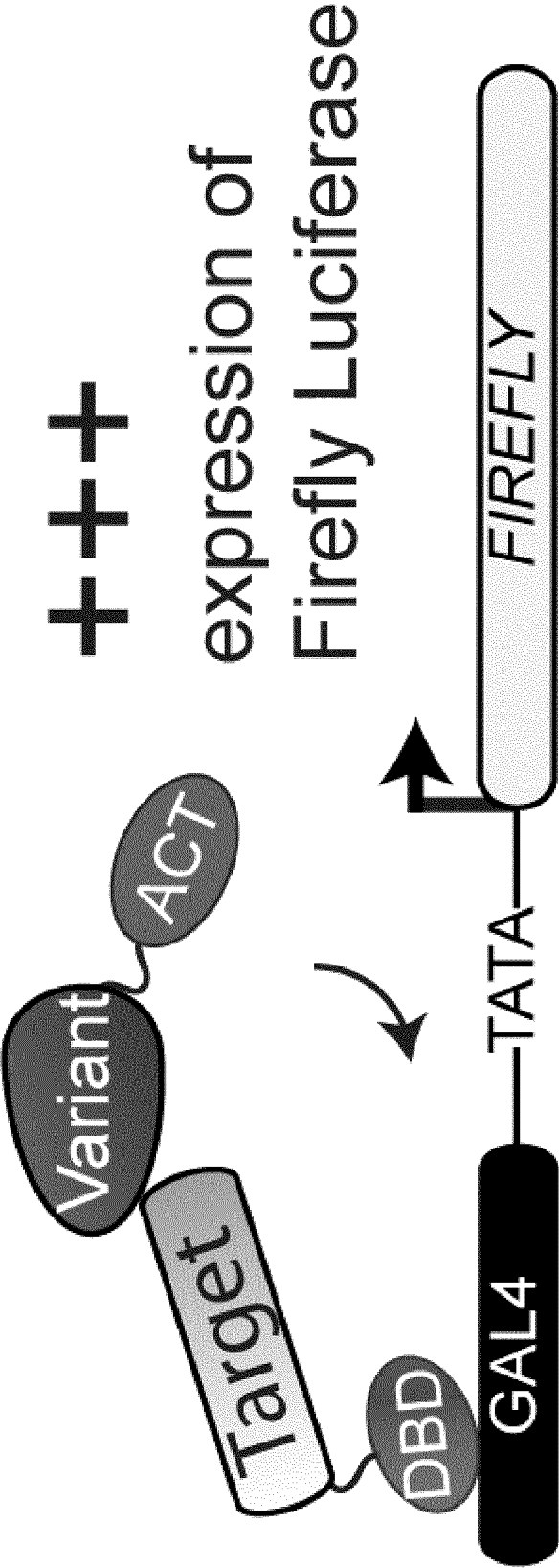


Figure 1 (cont.)

B

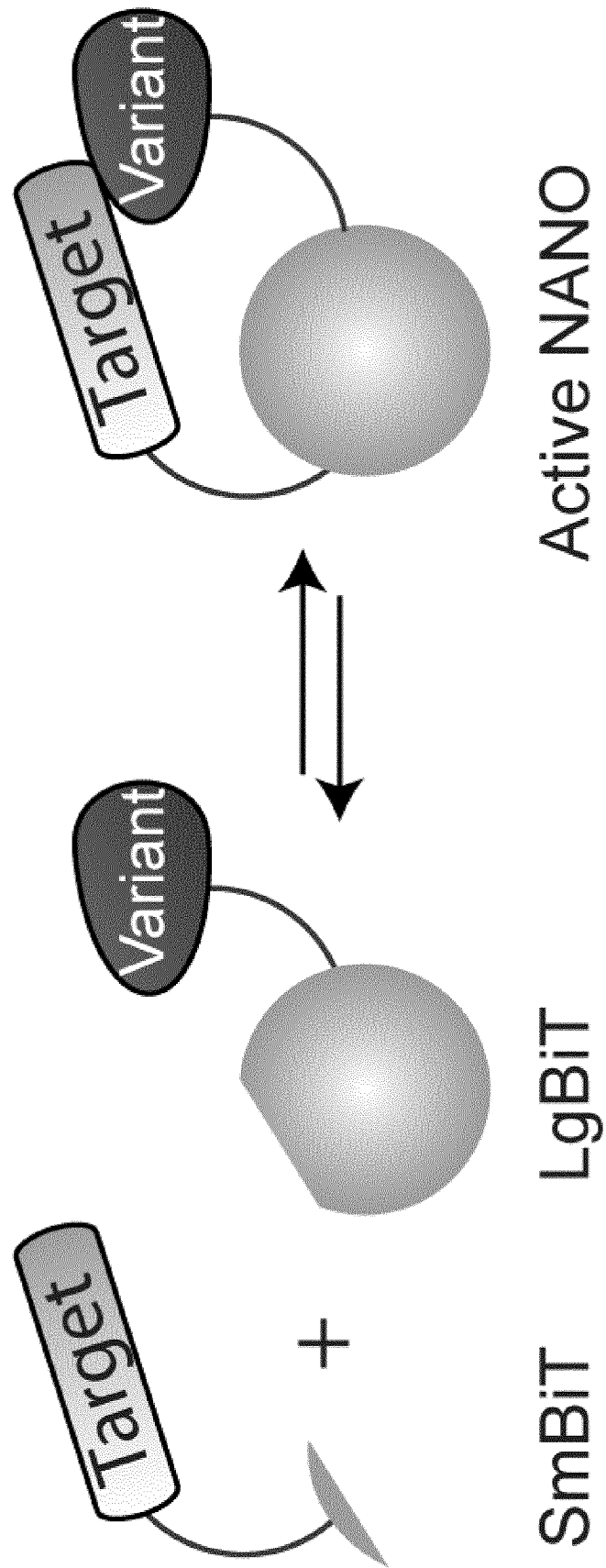
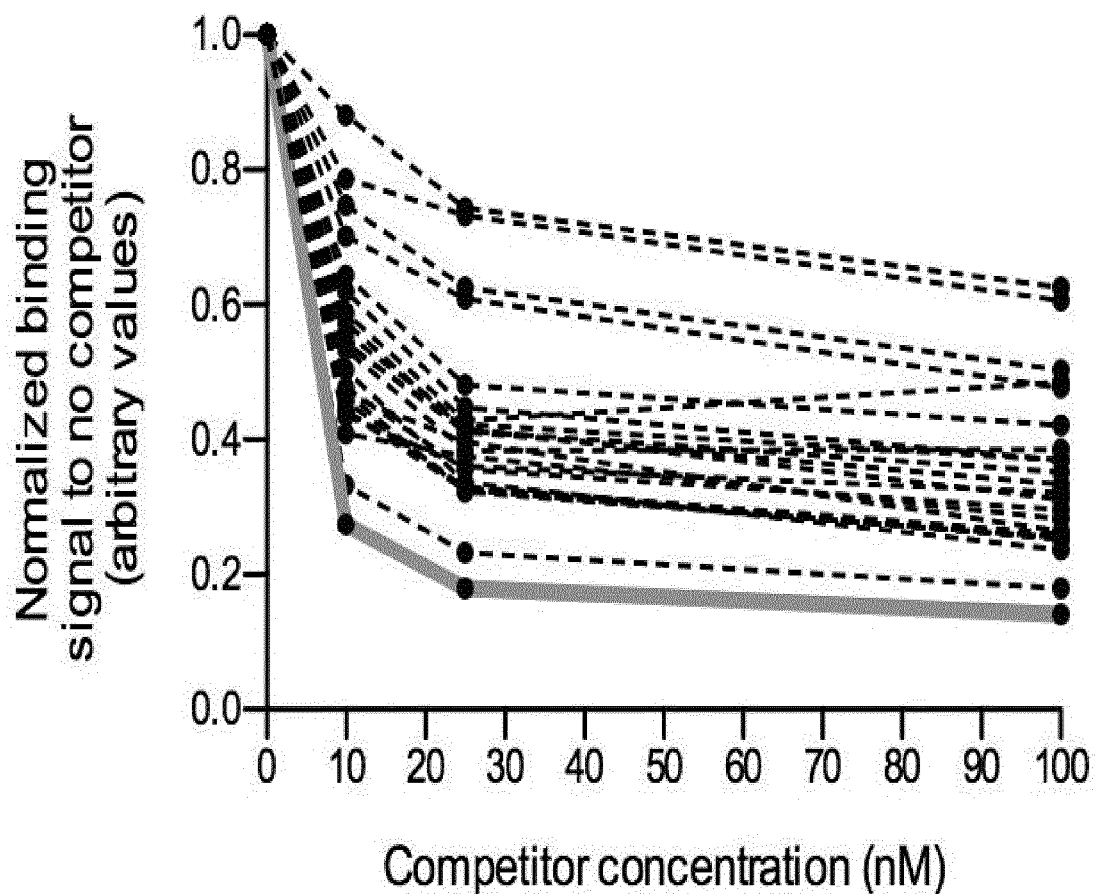


Figure 2

A

--●--	Ubv-1	--●--	Ubv-7	--●--	Ubv-13	—●—	Ubv-A
--●--	Ubv-2	--●--	Ubv-8	--●--	Ubv-14	--●--	Ubv-20
--●--	Ubv-3	--●--	Ubv-9	--●--	Ubv-15	--●--	Ubv-21
--●--	Ubv-4	--●--	Ubv-10	--●--	Ubv-16	--●--	Ubv-22
--●--	Ubv-5	--●--	Ubv-11	--●--	Ubv-17	--●--	Ubv-23
--●--	Ubv-6	--●--	Ubv-12	--●--	Ubv-18		

Figure 2 (cont.)

B

	Region 1					Region 2				Region 3								< Φ IC ₅₀
	4	6	11	12	14	42	44	46	48		66	68	71	72	76	77	78	
Ub wt	F	K	K	T	T	R	I	A	K		T	H	L	R	G	-	-	N/A
Ubv-A	N	-	T	-	G	I	F	S	M		R	Y	F	S	R	L	G	10nM

Figure 3

A

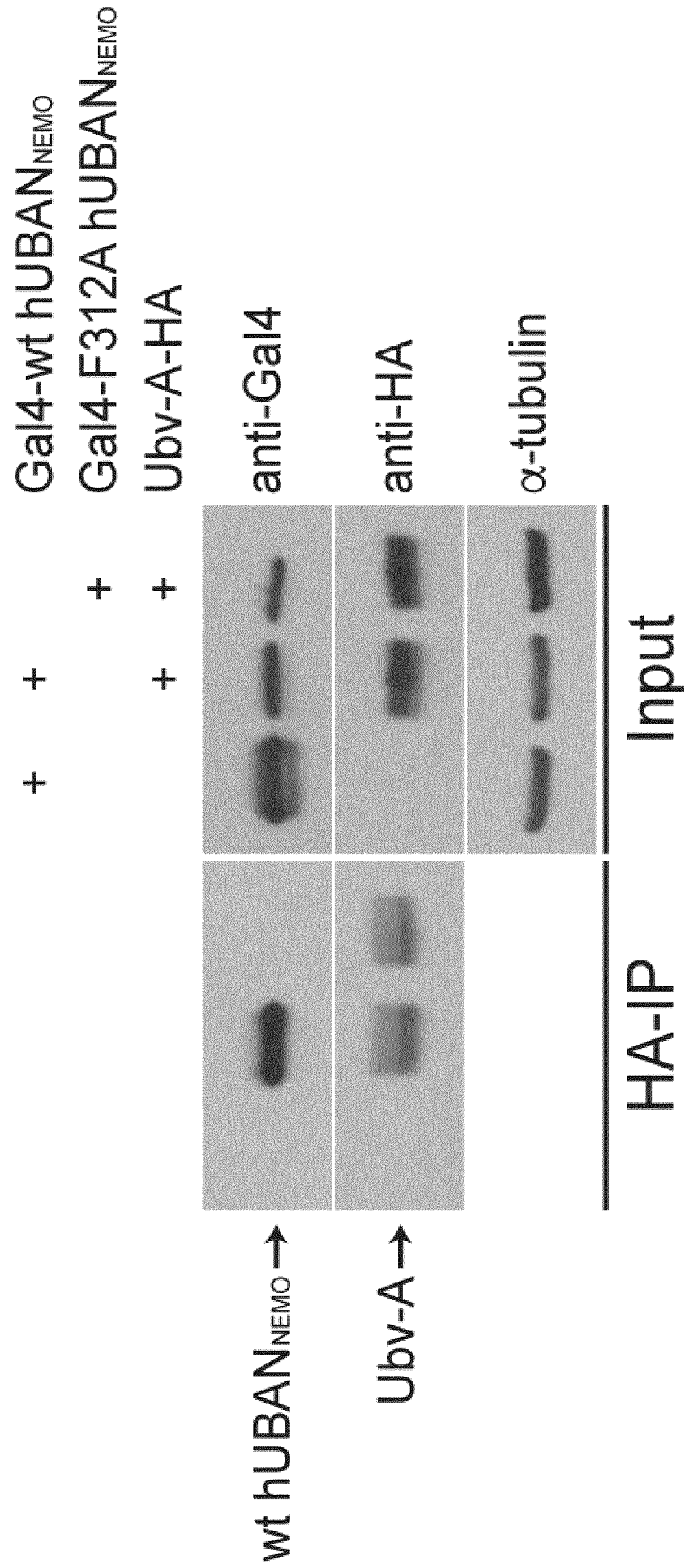


Figure 3 (cont.)

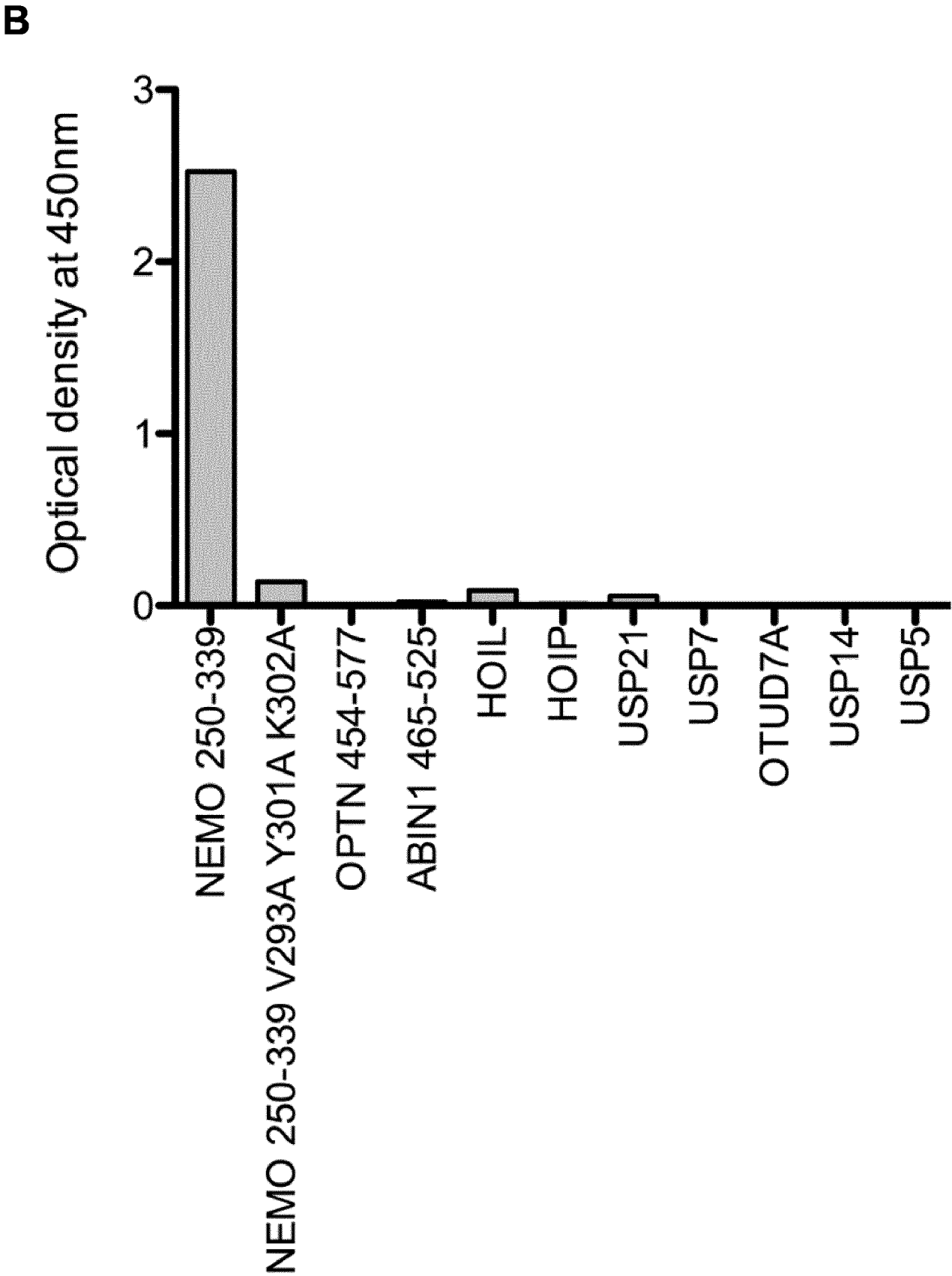


Figure 3 (cont.)

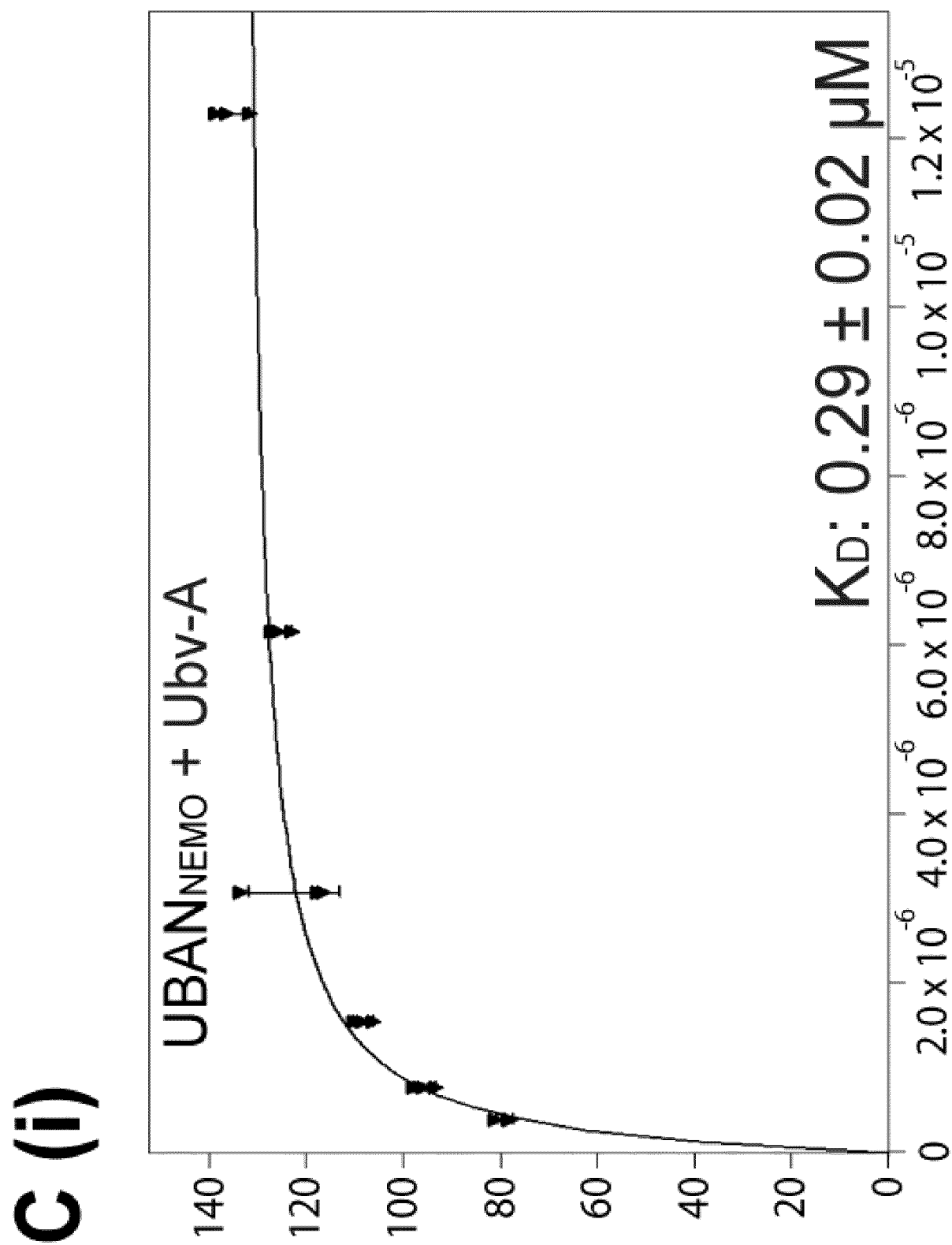


Figure 3 (cont.)

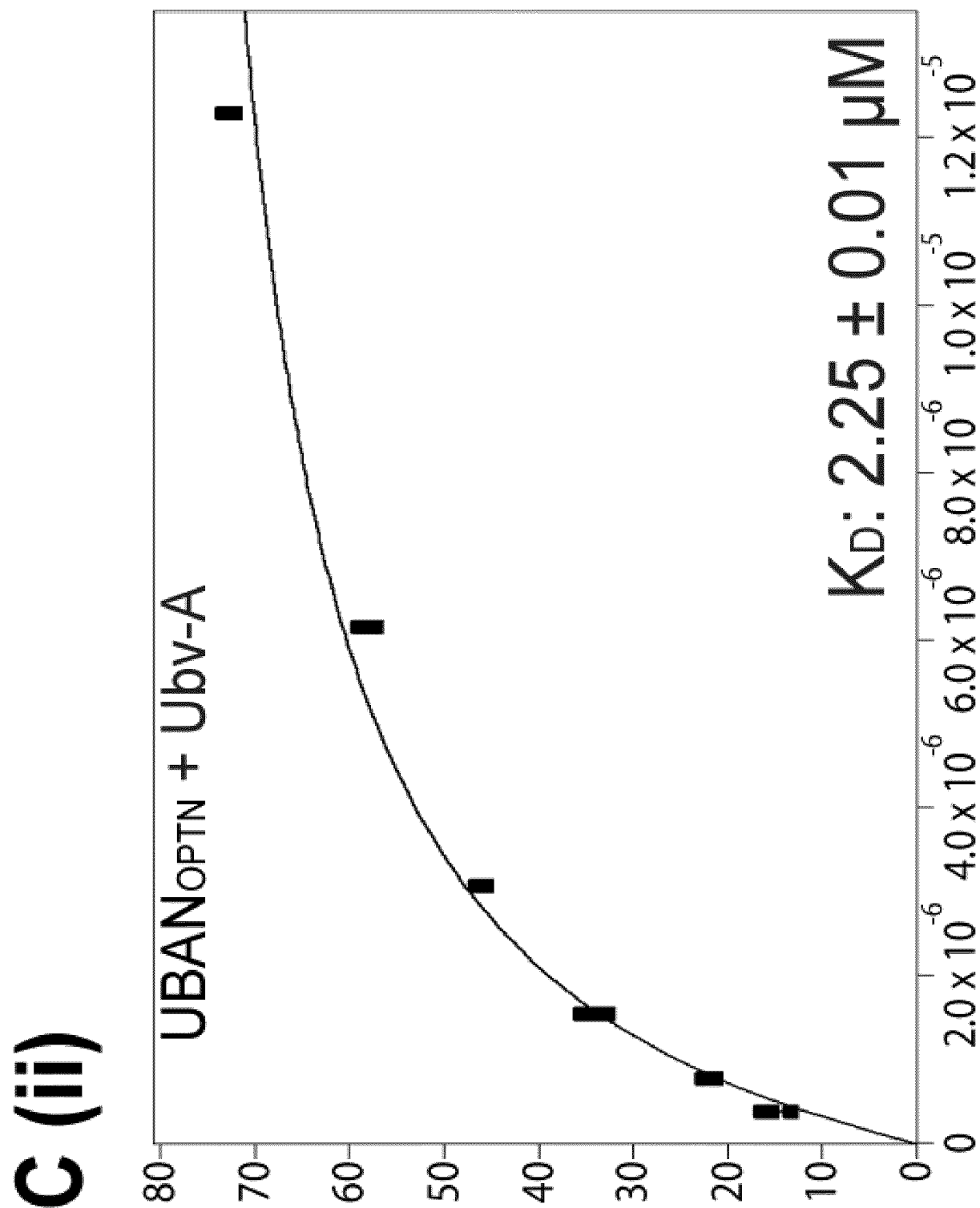


Figure 3 (cont.)

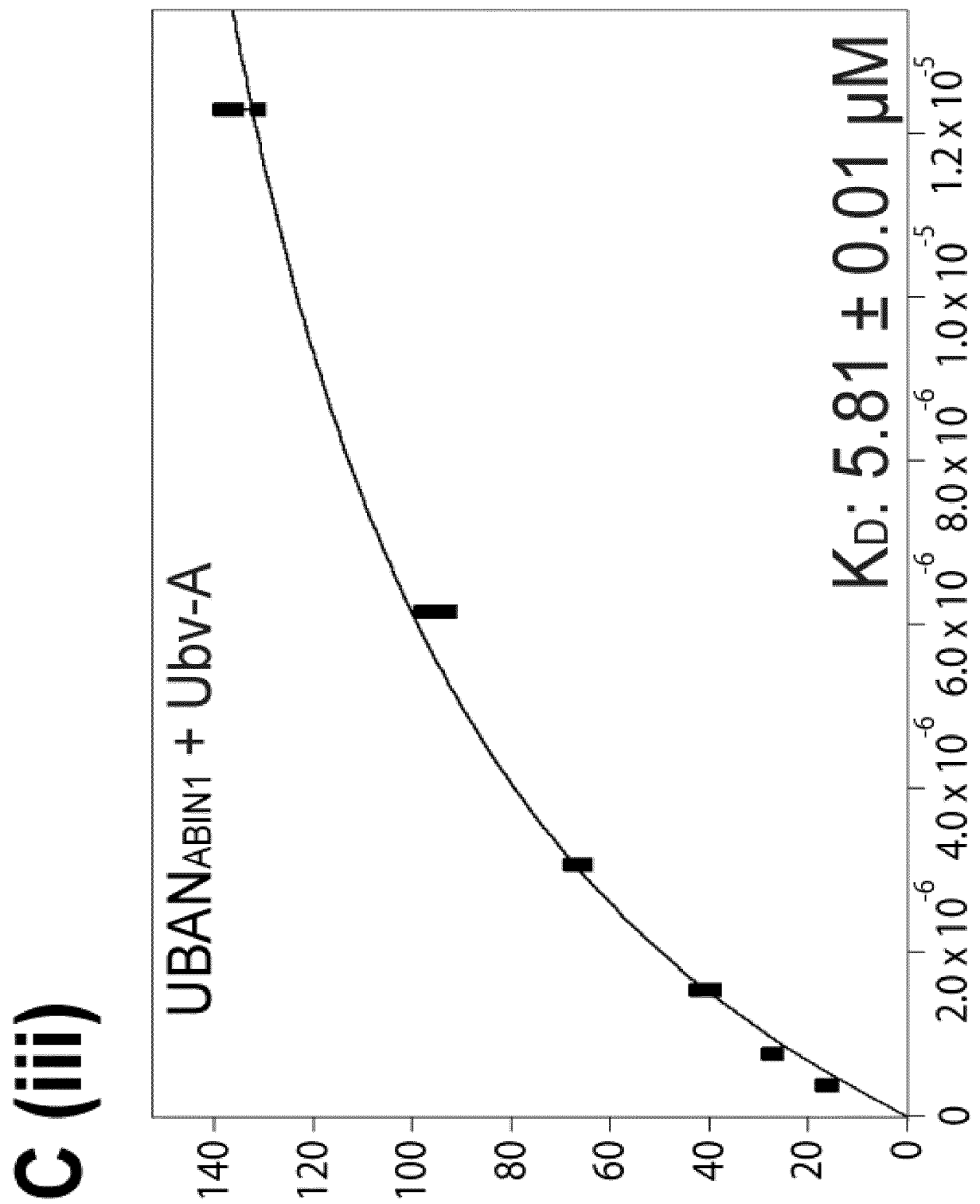


Figure 4

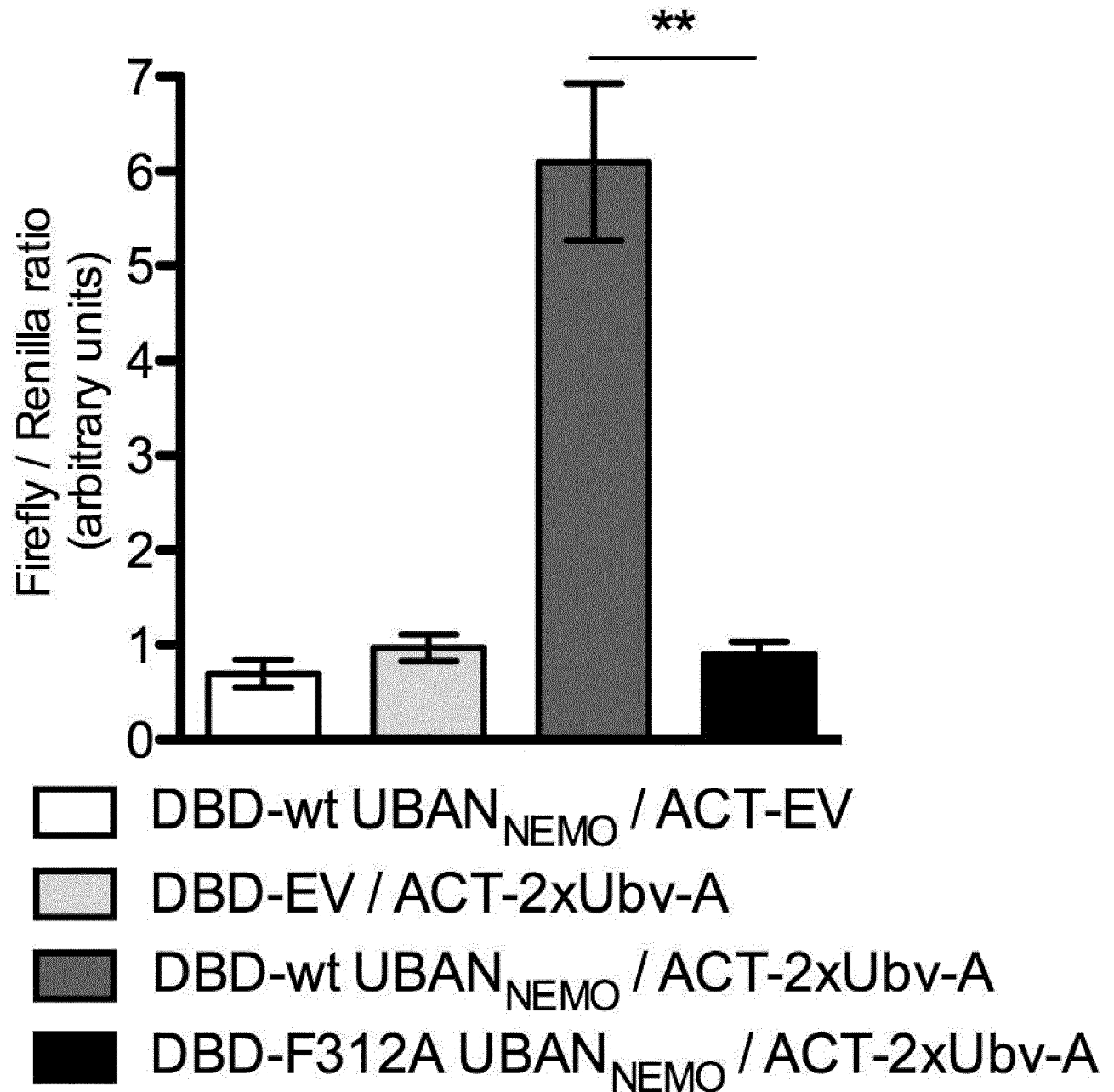
A**DLR NEMO assay**

Figure 4 (cont.)

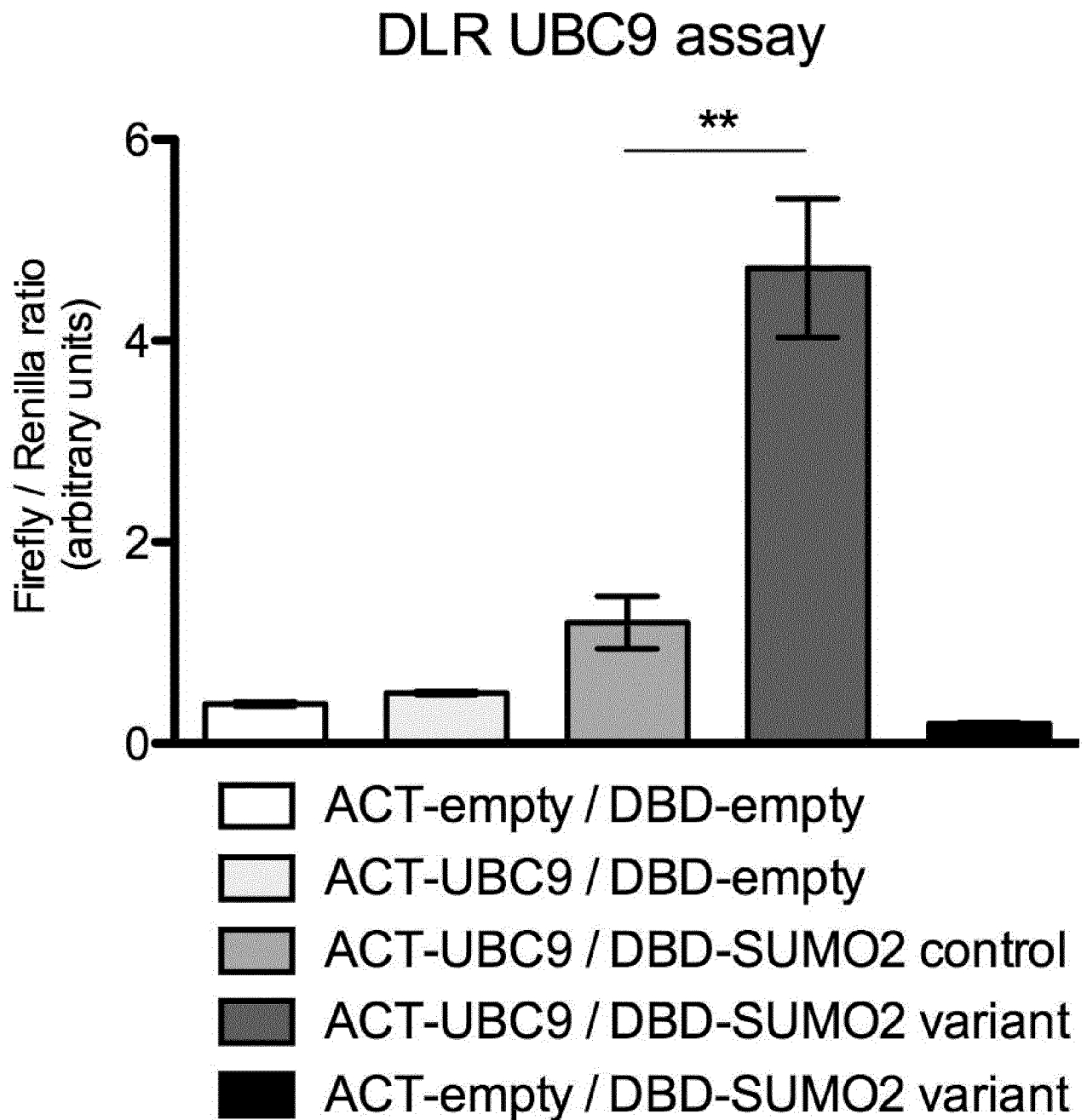
B

Figure 4 (cont.)

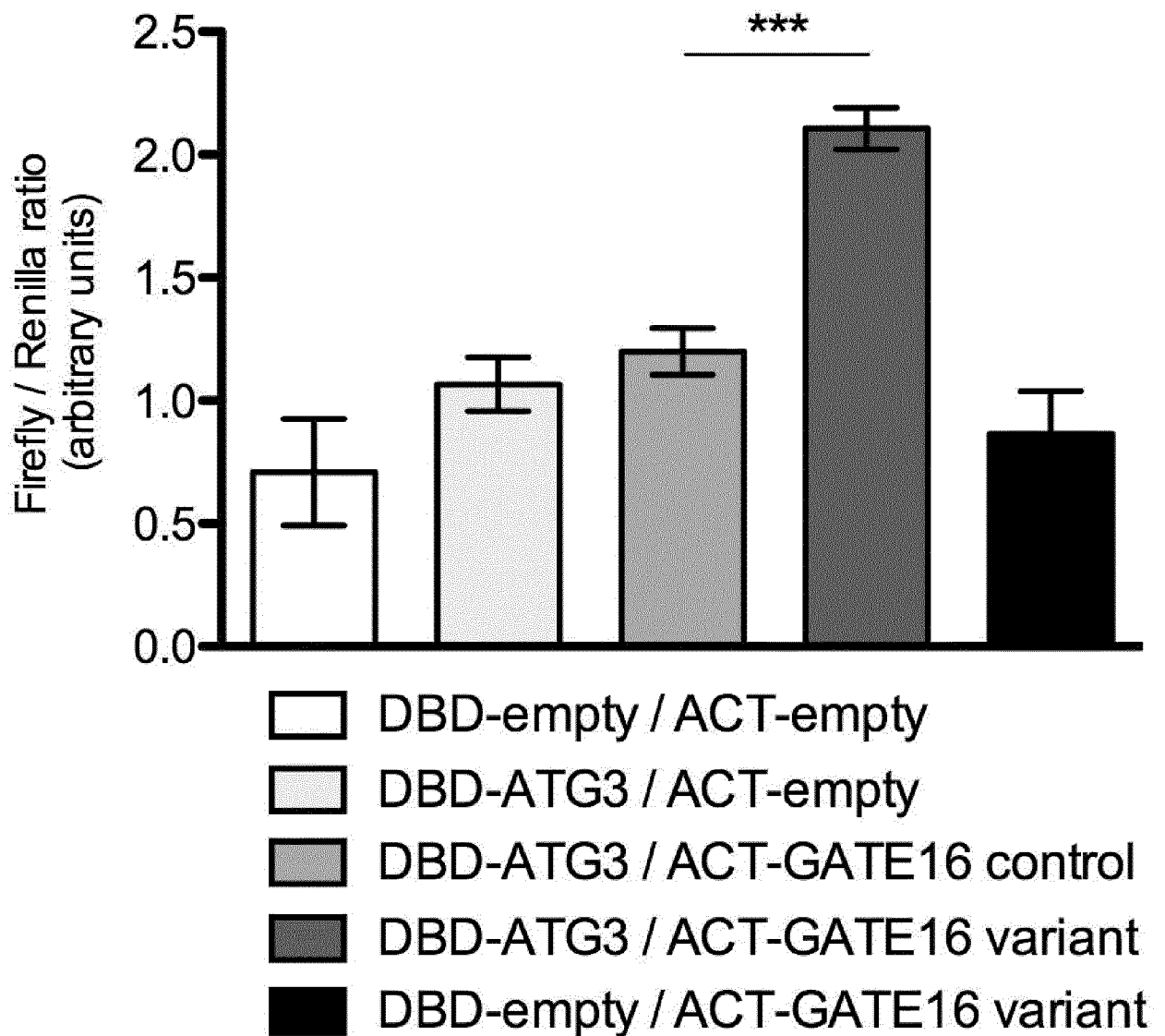
C**DLR ATG3 assay**

Figure 5

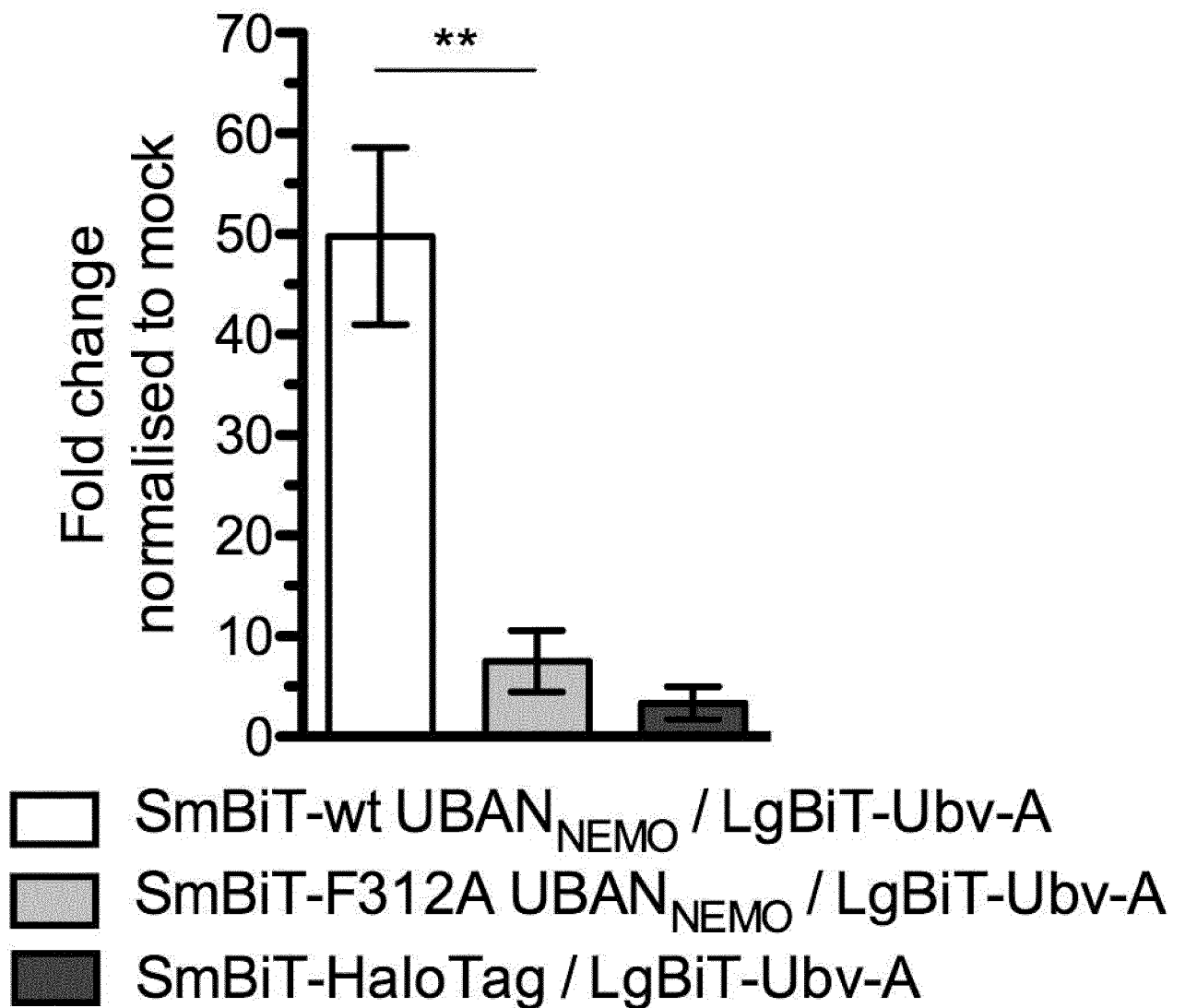
A**NanoBiT NEMO assay**

Figure 5 (cont.)

B

NanoBiT OPTN assay

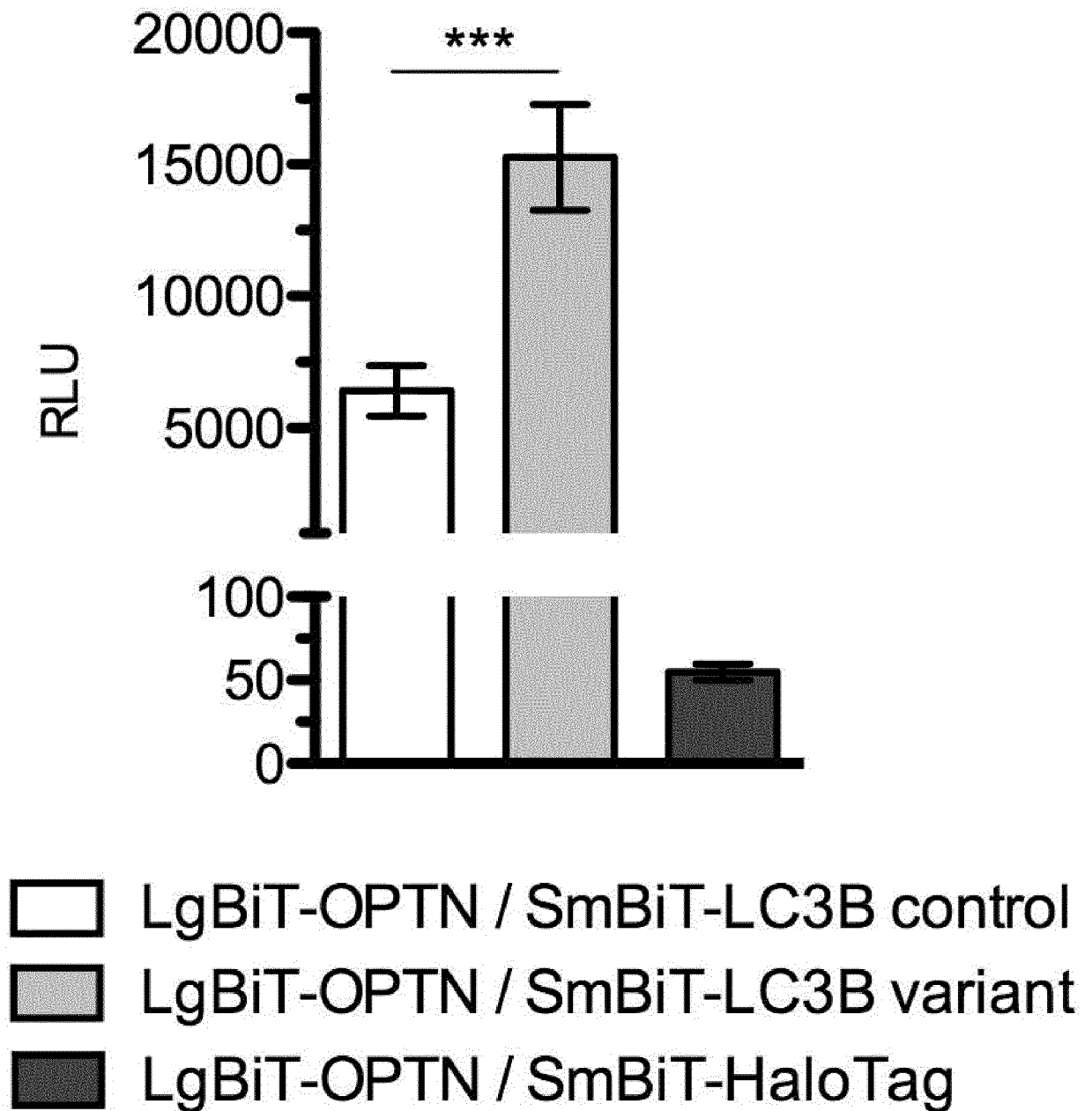
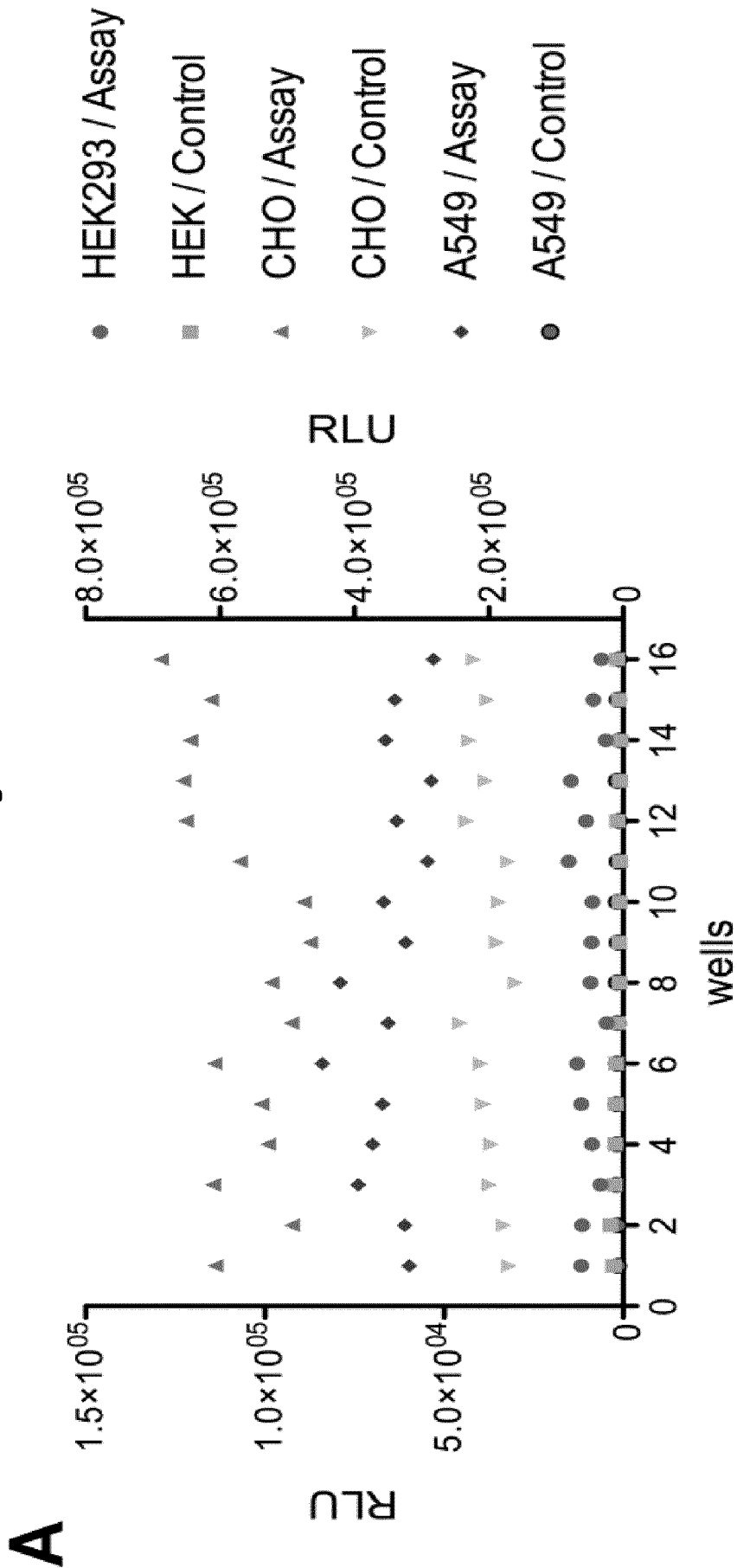


Figure 6



Cell line	HEK293 / Assay	HEK293 / Control	CHO / Assay	CHO / Control	A549 / Assay	A549 / Control
Average	9670	1730	573565	200635	65240	1630
S:B	6		3		40	
StDEV	3225	801	71197	24245	8637	259
Z' score	-0,52		0,23		0,58	

Figure 6 (cont.)

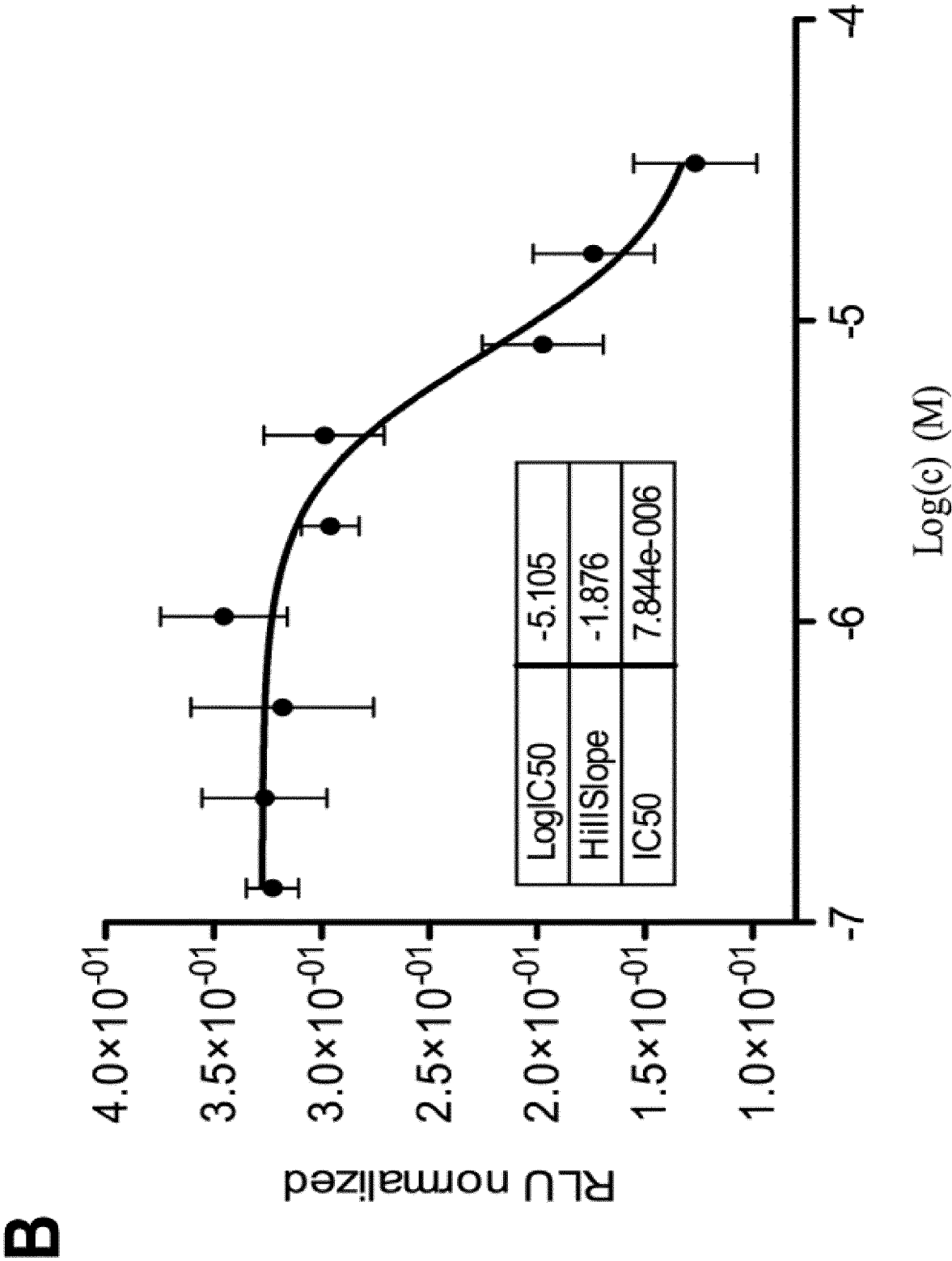


Figure 6 (cont.)

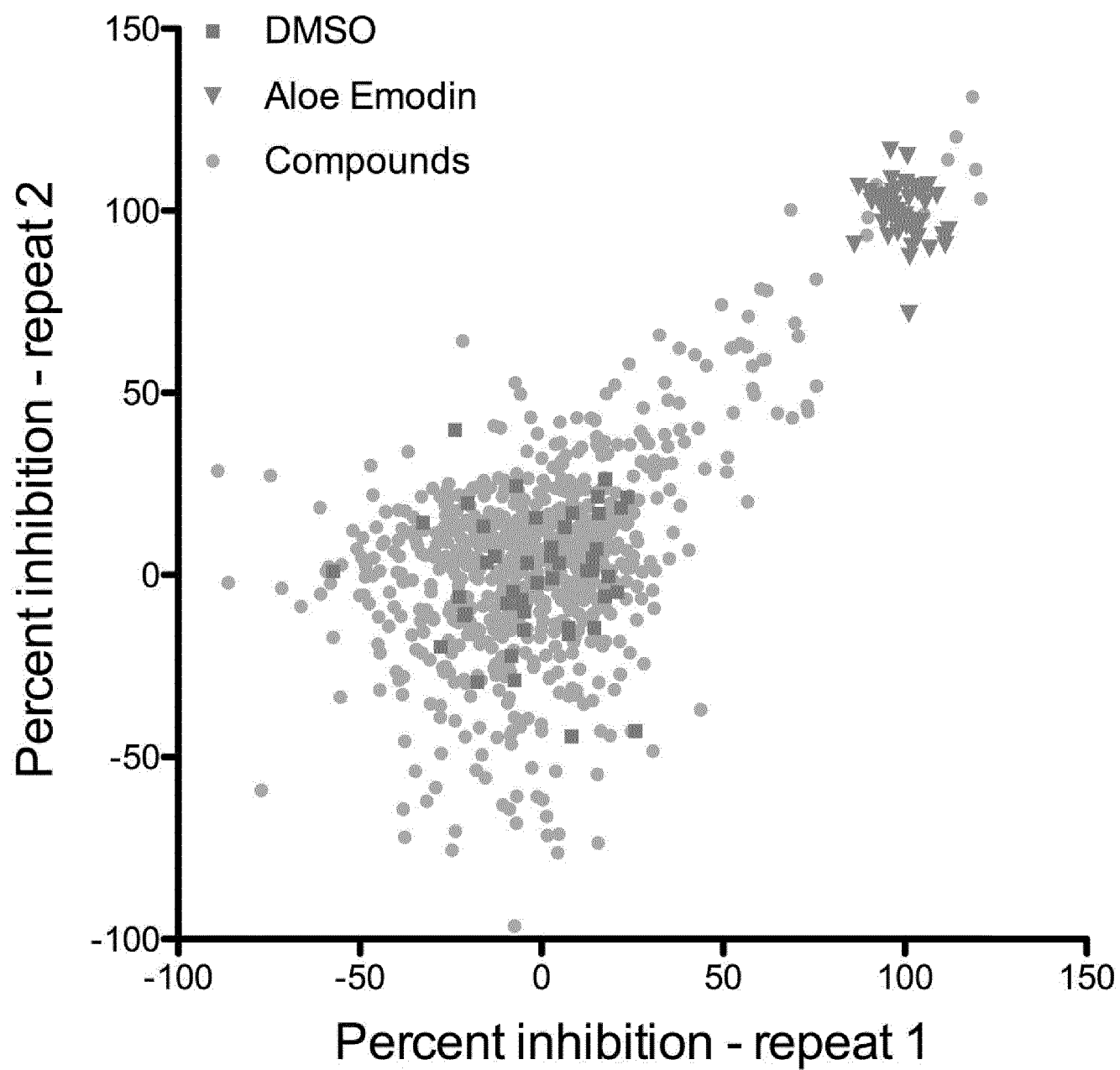
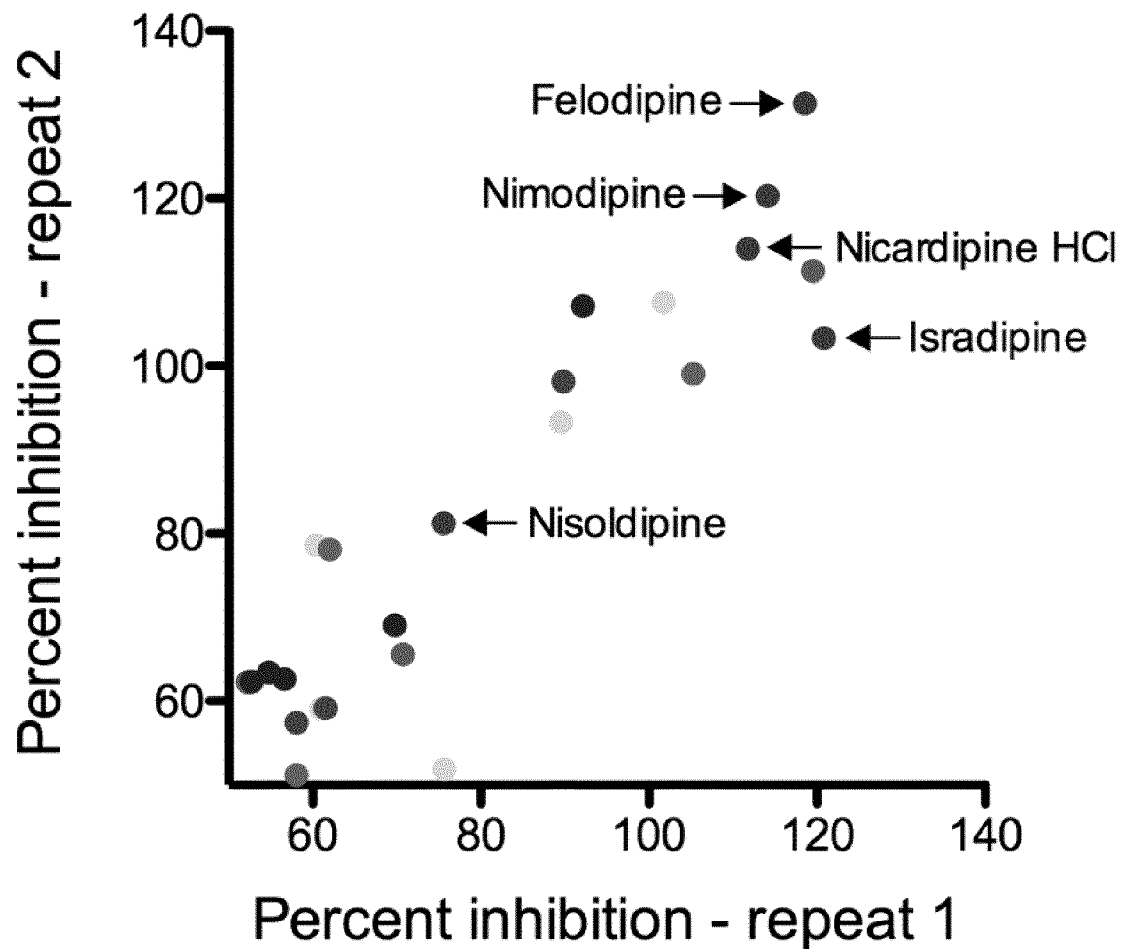
C

Figure 6 (cont.)

D

- Ca antagonists (n=5)
- ER antagonist (n=3)
- Conazole (n=5)
- Cytotoxic/cytostatic (n=3)
- Frequent hitters (n=3)
- Others (n=5)

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2019/073058

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N33/542 C07K14/435
ADD.

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)

G01N C07K A61K

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)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/127538 A1 (INVITROGEN CORP [US]; LEE K J; POLLOK B A; ZHONG Z) 8 November 2007 (2007-11-08) the whole document, in particular paragraphs [0003], [0021], [0064], [0065], [00113], [00114], [00119], [00120], [00143], [00157]; figure 12B; claim 10 ----- -/--	1-8,10, 11



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

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

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 November 2019

Date of mailing of the international search report

28/01/2020

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Authorized officer

Weber, Peter

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2019/073058

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	S O'NEILL ET AL: "Quantitative interaction analysis permits molecular insights into functional NOX4 NADPH oxidase heterodimer assembly", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 293, no. 23, 19 April 2018 (2018-04-19), pages 8750-8760, XP055526258, DOI: 10.1074/jbc.RA117.001045 the whole document, in particular figure 2A; paragraph spanning pages 8753 and 8754; paragraph spanning pages 8755 and 8756 -----	1-5,10, 11
X	S J DUELLMAN ET AL: "Cell-based, bioluminescent assay for monitoring the interaction between PCSK9 and the LDL receptor", JOURNAL OF LIPID RESEARCH, vol. 58, no. 8, 13 June 2017 (2017-06-13), pages 1722-1729, XP055534190, DOI: 10.1194/jlr.D074658 the whole document, in particular abstract; figure 1A, 1C -----	1-5,10, 11
X	J LI ET AL: "A Cell-Based High-Throughput Assay for the Screening of Small-Molecule Inhibitors of p53-MDM2 Interaction", JOURNAL OF BIOMOLECULAR SCREENING, vol. 16, no. 4, 1 April 2011 (2011-04-01), pages 450-456, XP055533983, DOI: 10.1177/1087057111399191 the whole document, in particular abstract; figure 1 -----	1-3,5-7, 10,11
X	E H Han ET AL: "A small-molecule inhibitor targeting the AURKC-I[kappa]B[alpha] interaction decreases transformed growth of MDA-MB-231 breast cancer cells", Oncotarget, 29 June 2017 (2017-06-29), pages 69691-69708, XP055534166, DOI: 10.18632/oncotarget.18883 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5642509/pdf/oncotarget-08-69691.pdf [retrieved on 2018-12-13] the whole document, in particular abstract; figure 6C; page 69704, section "mammalian two-hybrid assay" ----- -/--	1-3,5-7, 10

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2019/073058

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	M VINCEDEAU ET AL: "Inhibition of Canonical NF-[kappa]B Signaling by a Small Molecule Targeting NEMO-Ubiquitin Interaction", SCIENTIFIC REPORTS, vol. 6, no. 1, 7 January 2016 (2016-01-07), XP055534020, DOI: 10.1038/srep18934 the whole document, in particular paragraph "Identification of NEMO-Ub binding inhibitors from a natural compound library" spanning pages 3 and 4 -& Michelle Vincendeau ET AL: "Inhibition of Canonical NF-[kappa]B Signaling by a Small Molecule Targeting NEMO-Ubiquitin Interaction - Supplementary Information", Scientific Reports, vol. 6, no. 1 1 May 2016 (2016-05-01), pages 1-16, XP055538326, Retrieved from the Internet: URL:https://media.nature.com/original/nature-assets/srep/2016/160107/srep18934/extra/srep18934-s1.pdf [retrieved on 2019-01-04] supplementary figure 3 -----	1-11, 13-15
A	B STYNEN ET AL: "Diversity in Genetic In Vivo Methods for Protein-Protein Interaction Studies: from the Yeast Two-Hybrid System to the Mammalian Split-Luciferase System", MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, vol. 76, no. 2, 1 June 2012 (2012-06-01), pages 331-382, XP055533931, DOI: 10.1128/MMBR.05021-11 the whole document -----	1-11, 13-15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2019/073058

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

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

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

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

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

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

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

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-11, 13-15(all partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-11, 13-15(all partially)

Method for detecting a modulator compound of an interaction between the UBAN domain of NEMO (SEQ ID NO: 2) and an engineered high affinity variant of ubiquitin (SEQ ID NO: 6). Engineered high affinity variant as such. Kit-of-parts comprising the engineered high affinity variant.

2. claims: 1-11, 13-15(all partially)

Method for detecting a modulator compound of an interaction between UBC9 (SEQ ID NO: 3) and an engineered high affinity variant of SUMO2 (SEQ ID NO: 7). Engineered high affinity variant as such. Kit-of-parts comprising the engineered high affinity variant.

3. claims: 1-11, 13-15(all partially)

Method for detecting a modulator compound of an interaction between ATG3 (SEQ ID NO: 4) and an engineered high affinity variant of GATE16 (SEQ ID NO: 8). Engineered high affinity variant as such. Kit-of-parts comprising the engineered high affinity variant.

4. claims: 1-11, 13-15(all partially)

Method for detecting a modulator compound of an interaction between OPTN (SEQ ID NO: 5) and an engineered high affinity variant of LC3B (SEQ ID NO: 9). Engineered high affinity variant as such. Kit-of-parts comprising the engineered high affinity variant.

5. claim: 12

Medical uses of PPI modulator compounds.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2019/073058

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