

Threonine166 and serine189 of Rubicon RUN domain as LRRK2 kinase inhibition target sites.

CROSS REFERENCE TO RELATED APPLICATIONS

[001]. This application claims the priority to Singapore patent application No. 10201902046S, filed 7 March 2019, the contents of which are incorporated herein by reference.

[002]. FIELD

[003]. The present disclosure relates generally to methods antibodies, inhibitors and kits for diagnosing and/or providing treatments for diseases associated with Leucine-rich repeat kinase 2 (LRRK2) in particular diagnosing and/or providing treatments for Parkinson's disease (PD).

[004]. BACKGROUND:

[005]. The following discussion of the background to the invention is intended to facilitate an understanding of the present invention only. It should be appreciated that the discussion is not an acknowledgement or admission that any of the material referred to was published, known or part of the common general knowledge of the person skilled in the art in any jurisdiction as at the priority date of the invention.

[006]. Leucine-rich repeat kinase 2 (LRRK2) is a serine/threonine kinase. Mutations in LRRK2 are associated with PD, chronic inflammation, such a Crohn's disease and mycobacterial infections. Mutations in LRRK2 are the most common cause of autosomal-dominant and sporadic PD accounting for up to 40% of PD cases in some populations. G2019S is the most prevalent LRRK2 mutant and is reported to exhibit enhanced kinase activity compared to wild-type LRRK2. Asian LRRK2 variants like N551K, R1398H, R1628P and G2385R have been reported, though the functional impact of mutations are unknown as these variants have yet to be fully characterised. Familial LRRK2-linked PD has substantial overlap with idiopathic PD, suggesting that elucidating LRRK2 function may provide insights into both familial and idiopathic PD. Though LRRK2-linked toxicity has been associated with its kinase function, its physiological function remains unknown due to the lack of bona fide LRRK2 substrates. As a result, the quest for LRRK2 substrates and the development of LRRK2 kinase inhibitors has garnered interest as they may lead to therapeutic interventions for LRRK2-linked diseases like PD.

[007]. PD is a long-term degenerative disorder of the central nervous system and it primarily affects the motor system. PD is a global disease with no cure and treatment is mainly directed to improving symptoms. To date, there is no diagnostic assay to aid in the clinical diagnosis of PD. Current PD clinical diagnosis relies on motor symptoms and brain scans.

[008]. RUN domain protein as Beclin-1 interacting and cysteine-rich containing (Rubicon) is a protein known to be involved in autophagy, phagocytosis and immune responses.

[009]. There is a need to ameliorate at least some of the difficulties in diagnosing and/or providing treatments for Parkinson's disease.

[0010]. Summary

[0011]. It is envisioned that the methods, antibodies, inhibitors and kits described will be useful for diagnosing and/or providing treatments for diseases associated with Leucine-rich repeat kinase 2 (LRRK2), in particular diagnosing and/or providing treatments for Parkinson's disease (PD).

[0012]. Accordingly an aspect of the invention provides an *in vitro* method of detecting phosphorylation of a Rubicon protein comprising: isolating proteins from a sample; contacting the isolated proteins with an antibody that hybridises to a phosphorylated Threonine at 166 of a Rubicon protein to form a complex; detecting the complex wherein detection of the complex that varies from a predetermined value indicates that the sample comes from a subject having a disease associated with Leucine-rich repeat kinase 2 (LRRK2).

[0013]. Another aspect of the invention provides an antibody which is capable of binding to Rubicon phosphorylated at Threonine 166.

[0014]. Another aspect of the invention provides an antibody as described herein above for use in the diagnosis or treatment of a disease associated with Leucine-rich repeat kinase 2 (LRRK2).

[0015]. Another aspect of the invention provides an inhibitor of Rubicon interaction with Leucine-rich repeat kinase 2 (LRRK2) comprising a compound able to block interaction of LRRK2 with Threonine 166 of the Rubicon protein.

[0016]. Another aspect of the invention provides an inhibitor as described herein above for use in the treatment of a disease associated with Leucine-rich repeat kinase 2 (LRRK2).

[0017]. Another aspect of the invention provides use of an antibody as described herein above or an inhibitor as described herein above in the manufacture of a medicament for the treatment of a disease associated with Leucine-rich repeat kinase 2 (LRRK2).

[0018]. Another aspect of the invention provides a method of treating a subject in need having a disease associated with Leucine-rich repeat kinase 2 (LRRK2) comprising: administering a compound able to block interaction of LRRK2 with Threonine 166 and/or Serine 189 of the Rubicon protein.

[0019]. Another aspect of the invention provides a kit comprising an antibody described herein above or an inhibitor described herein above, and detection reagents for detecting a disease associated with Leucine-rich repeat kinase 2 (LRRK2).

[0020]. Other aspects and features of the present invention will become apparent to those of ordinary skill in the art upon review of the following description of specific embodiments of the invention in conjunction with the accompanying figures.

[0021]. BREIF DESCRIPTION OF THE DRAWINGS

[0022]. In the figures, which illustrate, by way of non-limiting examples only, embodiments of the present invention may include,

[0023]. **Figure 1. LRRK2 and Rubicon interaction.** (a) LRRK2 and Rubicon co-localisation in post-mortem human PD brain. Post-mortem human substantia nigra brain tissue was stained for endogenous LRRK2 and Rubicon expression. Confocal images were analysed by Imaris software to generate 2D histogram and co-localisation statistics (Table 1). (b) Co-immunoprecipitation of LRRK2 and Rubicon. Co-immunoprecipitation was carried out with HEK lysates over-expressing human LRRK2-GFP and/or Rubicon-Flag.

[0024]. **Figure 2. Identification of LRRK2-specific Rubicon phosphosite.** Wild-type or mutant LRRK2 kinase assay was carried out with wild-type or phosphor-null mutant Rubicon as substrate. Resultant phosphorylated Rubicon signal was tabulated as bar graphs and statistical significance was calculated using the Student's t-test against wild-type Rubicon. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

[0025]. **Figure 3. Validation of LRRK2-specific Rubicon phosphosite.** (a) LRRK2 ADP kinase assay carried out with wild-type or phosphor-null mutant Rubicon as substrate measured generated ADP from the kinase reaction. Statistical significance was calculated using the Student's t-test against wild-type Rubicon; * $p < 0.05$. (b) Human neuronal cells with endogenous LRRK2 knocked down or over-expressed LRRK2 were analysed for endogenous pT166 Rubicon expression using ELISA. Statistical significance was calculated using the Student's t-test against control and * $p < 0.05$, ** $p < 0.01$.

[0026]. **Figure 4. Drosophila climbing assay and lifespan studies.** Drosophila over-expressing human LRRK2 $\pm$ Rubicon were assessed for their climbing ability and their lifespan. Climbing assay was carried out every 10 days till Day 60 and the resultant climbing index was tabulated. Lifespan study was documented every seven days until all the flies have died. The last day for each drosophila line is labelled above the bar graph. Statistical analysis was carried out using multiple comparison with Tukey-Kramer post-hoc test. * $p < 0.05$, *** $p < 0.001$ (Tables 2 and 3).

[0027]. **Figure 5. Drosophila tyrosine hydroxylase (TH) expression.** Aged transgenic fly brains were stained for TH-positive neuronal clusters in five regions (PPL1, PPL2, PPM1/2, PPM3, PAL) and counted. Resultant count was tabulated and statistical significance was analysed using the Student's T-test after adjusting for multiple comparisons (Table 4).

[0028]. **Figure 6. Drosophila tyrosine hydroxylase (TH) expression.** Aged fly brain lysates were analysed by TH ELISA and statistical significance was carried out using the Student's t-test where * $p < 0.05$ and ** $p < 0.01$.

[0029]. **Figure 7. Validation of pT166 Rubicon sandwich ELISA using human serum samples.** Human serum from a local PD cohort harbouring LRRK2 variant mutations were analysed by pT166 Rubicon sandwich ELISA. 10 samples in each category represented as a dot plot with their respective median. Statistical significance was analysed using the Mann-Whitney test and significant numbers where $p < 0.05$ are depicted in **bold** (Table 5).

[0030]. **Figure 8. LRRK2 and Rubicon endogenous expression in microglia and macrophages.** (a) Endogenous protein expression of LRRK2 and Rubicon in human neuronal cells and microglia. (b) Rubicon was over-expressed in mouse macrophages and cell lysates were analysed by pT166 Rubicon sandwich ELISA. Statistical significance was analysed using multiple T-tests corrected for multiple comparisons using Holm-Sidak method. Statistical significance is achieved when $P < 0.05$ (Table 6).

[0031]. **Figure 9. Validation of pT166 Rubicon sandwich ELISA using human brain samples.** Co-localised LRRK2 and Rubicon staining in human post-mortem substantia nigra brain sections of an aged-match non-PD brain (left) and PD brain (right). Post-mortem human brain samples were analysed by pT166 Rubicon sandwich ELISA. Human brain tissues were homogenised and fractionated into soluble and membrane-associated proteins. There were four samples in each category and their respective mean is represented as bar graphs. Statistical significance was analysed using one-way ANOVA and statistical significance is achieved when $P < 0.05$ (Table 7).

[0032]. DETAILED DESCRIPTION

[0033]. Throughout this document, unless otherwise indicated to the contrary, the terms "comprising", "consisting of", "having" and the like, are to be construed as non-exhaustive, or in other words, as meaning "including, but not limited to".

[0034]. Furthermore, throughout the document, unless the context requires otherwise, the word "include" or variations such as "includes" or "including" will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

[0035]. Unless defined otherwise, all other technical and scientific terms used herein have the same meaning as is commonly understood by a skilled person to which the subject matter herein belongs.

[0036]. It was observed that LRRK2 kinase interacts with Rubicon RUN domain. LRRK2 is a serine/threonine kinase and Rubicon RUN domain contains 12 serine residues and three threonine residues. Rubicon phosphor-null mutants where a single serine/threonine residue

was substituted with alanine were cloned to delineate RUN domain residues that are critical to LRRK2 function. LRRK2 kinase assays utilising wild-type or mutant LRRK2 were used to methodically screen for LRRK2-specific phosphosites. The consensus Rubicon phosphosites identified were threonine 166 (T166) and serine 189 (S189). If Rubicon mediates LRRK2-induced toxicity via LRRK2 kinase activity, then Rubicon phosphor-null mutants T166A and/or S189A will rescue LRRK2-induced toxicity.

[0037]. Identifying LRRK2 substrates is key to elucidate the physiological function of LRRK2. Here, a novel LRRK2 substrate being Rubicon is reported and LRRK2-specific phosphosites are identified.

[0038]. Accordingly, in various embodiments, an *in vitro* method of detecting phosphorylation of a Rubicon protein comprising: isolating proteins from a sample; contacting the isolated proteins with an antibody that hybridises to a phosphorylated Threonine at 166 of a Rubicon protein to form a complex; detecting the complex wherein detection of the complex that varies from a predetermined value indicates that the sample comes from a subject having a disease associated with Leucine-rich repeat kinase 2 (LRRK2).

[0039]. The availability of a diagnostic assay that can be quantified and be easily carried out with minimal patient sample will enhance PD clinical diagnosis with added certainty. In various embodiments the use of sensitive ELISA detection is easily implemented in clinical settings. In various embodiments the use of blood samples such as serum samples or monocyte fraction samples further enhances the ease of implementation in clinical settings.

[0040]. As used herein the term "sample" refers to a sample obtained from a subject such as an animal or a patient. In various embodiments the sample is obtained from a patient, and may be e.g. a blood, fluid or tissue sample. In various embodiments the sample is a blood or blood-derived sample. That is, the sample may be whole blood obtained from the patient, or may be derived from a quantity of blood obtained from the patient. In some embodiments, a blood derived sample may be quantity of blood plasma or serum derived from the subject's blood. In some embodiments, a blood derived sample may be quantity of monocyte fractions or patient-derived macrophage cell lines derived from the subject's blood.

[0041]. In various embodiments the sample may be lung fluid. In various embodiments the sample may be tissue such as lung tissue, gastrointestinal tissue or brain tissue. In various embodiments the sample may be brain tissue.

[0042]. In various embodiments, a sample is obtained from the patient suspected of having a disease associated with Leucine-rich repeat kinase 2 (LRRK2). In various embodiments, diseases associated with Leucine-rich repeat kinase 2 (LRRK2) include but are not limited to Parkinson's disease (PD), chronic inflammation, such a Crohn's disease and mycobacterial infections such as tuberculosis.

[0043]. In various embodiments the methods comprise a step of obtaining a sample from the subject. In various embodiments the sample may be obtained and then stored, e.g. at -80°C. The stored sample can be thawed and analysed in accordance with the methods of the invention.

[0044]. In various embodiments, a sample is obtained from the patient at a pre-determined time point in relation to a proposed or contemporaneous course of treatment of the disease associated with Leucine-rich repeat kinase 2 (LRRK2). In various embodiments samples are obtained from the patient at more than one time point in relation to a proposed or contemporaneous course of treatment of the disease associated with Leucine-rich repeat kinase 2 (LRRK2).

[0045]. In various embodiments the subject having a disease associated with Leucine-rich repeat kinase 2 (LRRK2) is treated with a compound able to block interaction of LRRK2 with Threonine 166 and/or Serine 189 of the Rubicon protein. Blocking the interaction prevents phosphorylation of Threonine 166 and/or Serine 189 of the Rubicon protein slowing the deterioration of motor skills and/or extending life span. In various embodiments the subject having a disease associated with Leucine-rich repeat kinase 2 (LRRK2) is treated with a compound able to block interaction of LRRK2 with a phosphorylated Threonine 166 of the Rubicon protein. Blocking the interaction after phosphorylation of Threonine 166 and/or Serine 189 of the Rubicon protein will also have the effect of slowing the deterioration of motor skills and/or extending life span. In various embodiments the compound comprises a treatment antibody. In various embodiments the treatment antibody is a monoclonal antibody. In various embodiments the treatment antibody is a chimeric humanised antibody. In various embodiments the subject having a disease associated with Leucine-rich repeat kinase 2 (LRRK2) is treated with a compound able to block interaction of LRRK2 with a phosphorylated Threonine 166 of the Rubicon protein.

[0046]. In various embodiments the sample is or has been obtained from the patient up to 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 2 weeks, 3 weeks, 4 weeks, 2 months, 3 months, 4 months, 5 months, 6 months or 1 year prior to a therapeutic intervention to treat the disease.

[0047]. In various embodiments the sample is or has been obtained from the patient during the course of a therapeutic intervention to treat the disease. In various embodiments the sample is or has been obtained from the patient after commencement of a therapeutic intervention to treat the disease.

[0048]. In various embodiments the sample is or has been obtained from the patient up to 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 2 weeks, 3 weeks, 4 weeks, 2 months, 3 months, 4 months, 5 months, 6

months or 1 year after a therapeutic intervention to treat the disease (such as after commencement, i.e. first administration of, the therapeutic intervention). In various embodiments the sample is or has been obtained from the patient not more than 1 year, 6 months, 5 months, 4 months, 3 months, 2 months, 4 weeks, 3 weeks, 2 weeks, 13 days, 12 days, 11 days, 10 days, 9 days, 8 days, 7 days, 6 days, 5 days, 4 days, 3 days, 2 days or 1 day after a therapeutic intervention to treat the disease (such as after commencement, i.e. first administration of, the therapeutic intervention).

[0049]. In various embodiments the sample is or has been obtained from the patient on or after completion of the course of a therapeutic intervention to treat the disease. In various embodiments the sample is or has been obtained from the patient up to 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 2 weeks, 3 weeks, 4 weeks, 2 months, 3 months, 4 months, 5 months, 6 months or 1 year after completion of the course of a therapeutic intervention to treat the disease. In various embodiments the sample is or has been obtained from the patient not more than 1 year, 6 months, 5 months, 4 months, 3 months, 2 months, 4 weeks, 3 weeks, 2 weeks, 13 days, 12 days, 11 days, 10 days, 9 days, 8 days, 7 days, 6 days, 5 days, 4 days, 3 days, 2 days or 1 day after completion of the course of a therapeutic intervention to treat the disease. In various embodiments the sample is obtained from the patient post-mortem.

[0050]. As used herein the term "Rubicon protein" refers to RUN domain protein as Beclin-1 interacting and cysteine-rich containing. In various embodiments the Rubicon protein has an amino acid sequence set forth in SEQ ID NO. 1 or functional variants thereof that retains one or more of the known functions of Rubicon such as interact with Beclin-1, interact with Vps34, interact with UVRAG, interact with Rab7 or inhibition of autophagy and involvement in phagocytosis and immune responses. A functional variant may have one or more substitution, deletion or additional amino acid to SEQ ID NO. 1 that retains one or more of the known functions of Rubicon. In various embodiments a functional variant comprise a sequence sharing 60%, or 70% or 80% or 90% or 95% or 96% or 97% or 98% or 99% sequence homology with SEQ ID NO. 1. As used herein Threonine at 166 refers to a Threonine in the 166th amino acid of SEQ ID NO. 1 or a Threonine somewhere in the vicinity of the 166th amino acid of a functional variant. Serine at 189 refers to a Serine in the 189th amino acid of SEQ ID NO. 1 or a Serine somewhere in the vicinity of the 189th amino acid of a functional variant.

[0051]. As used herein the term "a disease associated with Leucine-rich repeat kinase 2 (LRRK2)" refers to any disease that has identified LRRK2 of SEQ ID NO. 3 as a risk factor. In various embodiments a disease associated with LRRK2 are selected from Parkinson's disease; chronic inflammation such as Crohn's disease or ulcerative colitis; and bacterial

infections such as mycobacterial infections. In various embodiments a disease associated with LRRK2 is Parkinson's disease. In various embodiments the disease associated with LRRK2 may comprise an intact LRRK2 represented as the amino acid sequence set forth in SEQ ID NO. 3. In various other embodiments the disease associated with LRRK2 may comprise mutations including additions, deletions or substitutions within the LRRK2 protein of SEQ ID NO. 3.

[0052]. In various embodiments the antibody is a monoclonal antibody. In various embodiments the antibody is a chimeric humanised antibody.

[0053]. In various embodiments the antibody-Rubicon complex may be detected and/or measured by various methods well known in the art, for example by western blot, immunohistochemistry, immunocytochemistry, flow cytometry, ELISA, ELISPOT, reporter-based methods, etc. In various embodiments the antibody-Rubicon complex is detected with an enzyme linked immunosorbent assay (ELISA). In various embodiments, the ELISA detection may be via direct ELISA that permits calculation of the absolute amount. In various embodiments, the ELISA detection may be via indirect ELISA. In various embodiments, the ELISA detection may be via sandwich ELISA which may provide more specific quantification. In various embodiments, the ELISA detection may be via any method that allows detection of antibody-Rubicon complex.

[0054]. In various embodiments, the predetermined value is the threshold for the normal range in a healthy individual that has no disease associated with Leucine-rich repeat kinase 2 (LRRK2) wherein variation from the predetermined value there is an indication that disease is present. In various embodiments, the predetermined value is a percentage, or a ratio of Rubicon protein phosphorylated at Threonine 166 of SEQ ID NO. 1 in relation to the total amount Rubicon protein in the sample. In various embodiments detection of the complex above a predetermined value indicates that the sample comes from a subject having a disease associated with Leucine-rich repeat kinase 2 (LRRK2). In various other embodiments, detection of the complex below a predetermined value indicates that the sample comes from a subject having a disease associated with Leucine-rich repeat kinase 2 (LRRK2).

[0055]. In various embodiments, the *in vitro* method further comprises removing blocking proteins prior to contacting the isolated proteins with the antibody. In various embodiments the blocking protein may comprise albumin. In various embodiments the blocking protein may be removed by filtration, centrifugation, tagged precipitation or any method known in the art to remove specific proteins. Advantageously, the method is sensitive and specific enough to detect Rubicon protein phosphorylated at Threonine 166 in all blood samples. Generally,

however, albumin removal from serum samples permits enhanced detection where serum samples are used.

[0056]. In various embodiments, an antibody which is capable of binding to Rubicon phosphorylated at Threonine 166 is provided.

[0057]. In various embodiments, the antibody comprises at least one variable region incorporating the CDR selected from amino acid sequences i) to vi):

i) LC-CDR1: RSSQSLVHSNGNTYLH (SEQ ID NO. 4);

ii) LC-CDR2: KLSNRFS (SEQ ID NO. 5);

iii) LC-CDR3: SQSTHVPLT (SEQ ID NO. 6);

iv) HC-CDR1: NYGVS (SEQ ID NO. 7);

v) HC-CDR2: TINSNGGSKYYPDSVKG (SEQ ID NO. 8);

vi) HC-CDR3: DVWLRRQWYFDV (SEQ ID NO. 9); and a functional variant with 99% amino acid sequence identity to any one of amino acid sequences i) to vi).

[0058]. In various embodiments, the antibody comprises amino acid sequences:

i) LC-CDR1: RSSQSLVHSNGNTYLH (SEQ ID NO. 4);

ii) LC-CDR2: KLSNRFS (SEQ ID NO. 5);

iii) LC-CDR3: SQSTHVPLT (SEQ ID NO. 6);

iv) HC-CDR1: NYGVS (SEQ ID NO. 7);

v) HC-CDR2: TINSNGGSKYYPDSVKG (SEQ ID NO. 8);

vi) HC-CDR3: DVWLRRQWYFDV (SEQ ID NO. 9); or a functional variant with 99% amino acid sequence identity to any one of amino acid sequences i) to vi).

[0059]. In various embodiments, the antibody comprises amino acid sequences set forth in SEQ ID NO. 12 and SEQ ID NO. 13 or a functional variant with 99% amino acid sequence identity to SEQ ID NO. 12 and SEQ ID NO. 13.

[0060]. In various embodiments, the functional variant comprises an antibody that is capable of binding to Rubicon phosphorylated at Threonine 166 at a supernatant dilution of at least 1:30. In various embodiments, the functional variant comprises an antibody that is capable of binding to Rubicon phosphorylated at Threonine 166 at a supernatant dilution of at least 1:90. In various embodiments, the functional variant comprises an antibody that is capable of binding to Rubicon phosphorylated at Threonine 166 at a supernatant dilution of at least

1:270. In various embodiments, the functional variant comprises an antibody that is capable of binding to Rubicon phosphorylated at Threonine 166 at a supernatant dilution of at least 1:810. In various embodiments, the functional variant comprises an antibody that is capable of binding to Rubicon phosphorylated at Threonine 166 at a supernatant dilution of at least 1:2,430. In various embodiments, the functional variant comprises an antibody that is capable of binding to Rubicon phosphorylated at Threonine 166 at a supernatant dilution of between 1:8 and 1:2,500. In various embodiments, the functional variant comprises an antibody that is capable of binding to Rubicon phosphorylated at Threonine 166 at a supernatant dilution of between 1:10 and 1:2,500. In various embodiments, the functional variant comprises an antibody that is capable of binding to Rubicon phosphorylated at Threonine 166 at a supernatant dilution of between 1:20 and 1:2,500. In various embodiments, the functional variant comprises an antibody that is capable of binding to Rubicon phosphorylated at Threonine 166 at a supernatant dilution of between 1:80 and 1:2,500. In various embodiments, the functional variant comprises an antibody that is capable of binding to Rubicon phosphorylated at Threonine 166 at a supernatant dilution of between 1:250 and 1:2,500. In various embodiments, the functional variant comprises an antibody that is capable of binding to Rubicon phosphorylated at Threonine 166 at a supernatant dilution of between 1:800 and 1:2,500.

[0061]. Polyclonal Antibodies

[0062]. In various embodiments the antibody may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant.

[0063]. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The intensity of the response is determined by several factors including the size of the immunogen molecule, its chemical characteristics, and how different it is from the animal's own proteins. Most natural immunogens are proteins with a molecular weight above 5 kDa that come from sources phylogenically far removed from the host animal (i.e., human proteins injected into rabbits or goats). It is desirable to use highly purified proteins as immunogens, since the animal will produce antibodies to even small amounts of impurities present as well as to the major component. The antibody response increases with repeated exposure to the immunogen, so a series of injections at regular intervals is needed to achieve both high levels of antibody production and antibodies of high affinity.

[0064]. In various embodiments the antibody engages, hybridizes to or binds the Rubicon protein phosphorylated at Threonine 166. In various embodiments the antibody engages,

hybridizes to or binds the Rubicon protein at Threonine 166. In various embodiments the antibody engages, hybridizes to or binds the Rubicon protein at phosphorylated Threonine 166. In various embodiments the amino acid sequence will be selected from the region of about 117 to 189 in the Rubicon protein. Sequences of at least 5, 6, 7, 8, 9, 10, 15, 20, 25, 30 amino acids from this region in SEQ ID NO. 1 will generally be used to generate those antibodies. In various embodiments the amino acid sequence is SEQ ID NO. 2. Desirably, the sequence selected will generate an antibody that specifically interferes with binding of Rubicon and LRRK2.

[0065]. Not all immunogenic molecules will however generate the level of antibody desired. To increase the intensity of the immune response immunogens are combined with complex mixtures called adjuvants. Adjuvants are a mixture of natural or synthetic compounds that, when administered with antigens, enhance the immune response. Adjuvants are used to (1) stimulate an immune response to an antigen that is not inherently immunogenic, (2) increase the intensity of the immune response, (3) preferentially stimulate either a cellular or a humoral response (i.e., protection from disease versus antibody production). Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate).

[0066]. If the immunogen is still unable to generate an acceptable response, it may be conjugated to a carrier protein that is more immunogenic. Small molecules such as drugs, organic compounds, and peptides and oligosaccharides with a molecular weight of less than 2-5 kDa like, for example, SEQ ID NO.: 2, are not usually immunogenic, even when administered in the presence of adjuvant. In order to generate an immune response to these compounds, it is necessary to attach them to a protein or other compound, termed a carrier that is immunogenic. When attached to a carrier protein the small molecule immunogen is called a hapten. Haptens are also conjugated to carrier proteins for use immunoassays. The carrier protein provides a means of attaching the hapten to a solid support such as a microtiter plate or nitrocellulose membrane. When attached to agarose they may be used for purification of the anti-hapten antibodies. They may also be used to create a multivalent antigen that will be able to form large antigen-antibody complexes. When choosing carrier proteins, remember that the animal will form antibodies to the carrier protein as well as to the attached hapten. It is therefore relevant to select a carrier protein for immunization that is unrelated to proteins that may be found in the assay sample. If haptens are being conjugated for both immunization and assay, the two carrier proteins should be as different as possible. This allows the antiserum to be used without having to isolate the anti-hapten antibodies from the anti-carrier antibodies.

[0067]. In various embodiments the immunizing agent is SEQ ID NO. 2 conjugated to a protein known to be immunogenic in the mammal being immunized.

[0068]. Examples of such immunogenic proteins include but are not limited to keyhole limpet hemocyanin (KLH), serum albumin, bovine thyroglobulin, soybean trypsin inhibitor, and a toxoid, for example tetanus toxoid. .

[0069]. KLH is a respiratory protein found in molluscs. Its large size makes it very immunogenic, and the large number of lysine residues available for conjugation make it very useful as a carrier for haptens. The phylogenic separation between mammals and molluscs increases the immunogenicity and reduces the risk of cross-reactivity between antibodies against the KLH carrier and naturally occurring proteins in mammalian samples.

[0070]. KLH is offered both in its native form, for conjugation via amines, and succinylated, for conjugation via carboxyl groups. Succinylated KLH may be conjugated to a hapten containing amine groups (such as a peptide) via cross-linking with carbodiimide between the newly introduced carboxyl groups of KLH and the amine groups of the hapten. Protocols for conjugation of haptens to carrier proteins are known in the art.

[0071]. The immunization protocol may be selected by one skilled in the art without undue experimentation. Protocols for preparing immunogens, immunization of animals, and collection of antiserum may be found in reference material available to a person skilled in the art.

[0072]. Monoclonal Antibodies

[0073]. In various embodiments the antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein (1975), *Nature*, 256:495. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized with an immunizing agent as described above to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized in vitro.

[0074]. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl

transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

[0075]. Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, Calif. and the American Type Culture Collection, Manassas, Va. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies.

[0076]. The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against Rubicon protein phosphorylated at Threonine 166.

[0077]. After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown in vivo as ascites in a mammal.

[0078]. The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

[0079]. The monoclonal antibodies may also be made by recombinant DNA methods known in the art.

[0080]. The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain cross-linking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent cross-linking.

[0081]. In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art.

[0082]. Human and Chimeric Humanized Antibodies

[0083]. The antibodies of the invention may further comprise chimeric humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab',

F(ab')₂ or other antigen-binding sub-sequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin.

[0084]. Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the methods known in the art, by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies, wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

[0085]. Human antibodies can also be produced using various techniques known in the art, including phage display. Similarly, human antibodies can be made by introducing of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire.

[0086]. Bispecific Antibodies

[0087]. In various embodiment bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for Rubicon protein phosphorylated at

Threonine 166, the other one is for another compound having Rubicon protein phosphorylated at Serine 189.

[0088]. Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities.

[0089]. In various embodiments, an antibody as described herein is provided for use in the treatment of a disease associated with Leucine-rich repeat kinase 2 (LRRK2).

[0090]. As mentioned above, in various embodiments the term “a disease associated with Leucine-rich repeat kinase 2 (LRRK2)” refers to any disease that has identified LRRK2 of SEQ ID NO. 3 as a risk factor. In various embodiments a disease associated with LRRK2 are selected from Parkinson's disease; chronic inflammation such as Crohn's disease or ulcerative colitis; and bacterial infections such as mycobacterial infections. In various embodiments a disease associated with LRRK2 is Parkinson's disease.

[0091]. In various embodiments the disease associated with Leucine-rich repeat kinase 2 (LRRK2) is Parkinson's disease.

[0092]. In various embodiments, an inhibitor of Rubicon interaction with Leucine-rich repeat kinase 2 (LRRK2) comprising a compound able to block interaction of LRRK2 with Threonine 166 of the Rubicon protein is provided.

[0093]. Current LRRK2 inhibitors directly targets LRRK2 kinase and cause adverse side effects when tested in *in vivo* models. Blocking or targeting specific phosphosites of LRRK2 substrate Rubicon protein has an indirect effect on the kinase. This exerts more control as the inhibition effects are confined to interactions between LRRK2 and Rubicon and pathways related to this interaction rather than affecting the generic function of LRRK2 alone.

[0094]. In various embodiments the inhibitor compound comprises an antibody. In various embodiments the compound comprises a chimeric humanised antibody. In various embodiments the compound comprises a monoclonal antibody.

[0095]. In various embodiments, an inhibitor as described herein above may be provided for use in the treatment of a disease associated with Leucine-rich repeat kinase 2 (LRRK2).

[0096]. As mentioned above, in various embodiments the term “a disease associated with Leucine-rich repeat kinase 2 (LRRK2)” refers to any disease that has identified LRRK2 of SEQ ID NO. 3 as a risk factor. In various embodiments a disease associated with LRRK2 are selected from Parkinson's disease; chronic inflammation such as Crohn's disease or ulcerative colitis; and bacterial infections such as mycobacterial infections. In various embodiments the inhibitor is for use in the treatment of the disease associated with Leucine-rich repeat kinase 2 (LRRK2) being Parkinson's disease.

[0097]. In various embodiments, use of an antibody as described herein above or an inhibitor as described herein above in the manufacture of a medicament for the treatment of a disease associated with Leucine-rich repeat kinase 2 (LRRK2).

[0098]. As mentioned above, in various embodiments the term “a disease associated with Leucine-rich repeat kinase 2 (LRRK2)” refers to any disease that has identified LRRK2 of SEQ ID NO. 3 as a risk factor. In various embodiments a disease associated with LRRK2 are selected from Parkinson's disease; chronic inflammation such as Crohn's disease or ulcerative colitis; and bacterial infections such as mycobacterial infections. In various embodiments the use of the antibody or the inhibitor in the manufacture of a medicament for the treatment of a disease associated with Leucine-rich repeat kinase 2 (LRRK2) is for Parkinson's disease.

[0099]. In various embodiments, a method of treating a subject in need having a disease associated with Leucine-rich repeat kinase 2 (LRRK2) comprising: administering a compound able to block interaction of LRRK2 with Threonine 166 and/or Serine 189 of the Rubicon protein.

[00100]. In various embodiments, the compound is able to block interaction of LRRK2 with phosphorylated Threonine 166 and/or Serine 189 of the Rubicon protein. Blocking the interaction either before or after phosphorylation of Threonine 166 and/or Serine 189 of the Rubicon protein will have the effect of slowing the deterioration of motor skills and/or extending life span.

[00101]. In various embodiments the compound used in the method comprises an antibody as described herein above. In various embodiments the antibody is a chimeric humanised antibody as described herein above. In various embodiments the antibody is a monoclonal antibody as described herein above.

[00102]. In various embodiments, the compound is administered any suitable way known in the art. In various embodiments, the compound is administered by injection. In various embodiments, the compound is administered by direct injection to the site of interaction of LRRK2 with Threonine 166 and/or Serine 189 of the Rubicon protein that is responsible for causing the disease associated with Leucine-rich repeat kinase 2 (LRRK2).

[00103]. In various embodiments, a kit comprising an antibody described herein above, and detection reagents for detecting a disease associated with Leucine-rich repeat kinase 2 (LRRK2) is provided.

[00104]. In various embodiments the detection reagents are those used for an enzyme linked immunosorbent assay (ELISA). In various embodiments, the detection reagents are those used for direct ELISA. In various embodiments, the detection reagents are those used for indirect ELISA. In various embodiments, the detection reagents are those used for

sandwich ELISA. In various embodiments, the detection reagents are those used for any method that allows detection of antibody-Rubicon complex. In various embodiments, depending on the method used the detection reagents may be selected from any one of: antigens to coat the microtiter plate; blocking reagents for unbound sites to prevent false positives; anti IgG conjugated enzymes; substrates that react with the enzyme to permit detection by colour change, fluorescence or any other means known in the art; additional reagents such as wash buffers, stop solutions, stabilizers; and any combination thereof.

[00105]. In various embodiments, the kit may comprise a microtiter plate precoated with the antibodies described herein above. This will facilitate more rapid detection and reduce the chance of contamination.

[00106]. As mentioned above, in various embodiments the term “a disease associated with Leucine-rich repeat kinase 2 (LRRK2)” refers to any disease that has identified LRRK2 of SEQ ID NO. 3 as a risk factor. In various embodiments a disease associated with LRRK2 are selected from Parkinson’s disease; chronic inflammation such as Crohn’s disease or ulcerative colitis; and bacterial infections such as mycobacterial infections.

[00107]. In various embodiments the disease associated with Leucine-rich repeat kinase 2 (LRRK2) is Parkinson’s disease.

[00108]. EXAMPLES

[00109]. Post-mortem human substantia nigra brain tissue was stained for endogenous LRRK2 (green fluorescence) and Rubicon (red fluorescence) expression. The extent of LRRK2 and Rubicon co-localisation (yellow fluorescence) was analysed by confocal microscopy and co-localisation statistics revealed a high degree of correlation between LRRK2 and Rubicon (Figure 1, Table 1). LRRK2 and Rubicon interaction was confirmed by co-immunoprecipitation.

[00110]. Table 1: Analysis of co-localization of LRRK2 and Rubicon

Co-localization statistics	Value	Interpretation
Pearson’s coefficient	0.7286	Strong correlation

[00111]. Two methods of LRRK2 kinase assay was used to screen for Rubicon phosphor-null mutants. The first method quantified the extent of Rubicon phosphorylation by LRRK2 kinase and determined T166 and S189 as the consensus LRRK2-specific phosphosites (Figure 2). The second method quantified the amount of generated ADP as a by-product of LRRK2 kinase activity and showed that Rubicon mutant’s 117-189 amino acids significantly decreased LRRK2 kinase activity (Figure 3a). Peptide analyses suggested that

LRRK2 prefers threonine residue as a phosphorylation site, therefore a customised monoclonal antibody targeting phosphor-T166 (pT166) Rubicon was generated to verify the identified phosphosite. Human neurons with endogenous LRRK2 knocked down had a 32.4% decrease in pT166-Rubicon compared to control; human neurons transiently over-expressing wild-type (WT) LRRK2 or mutant LRRK2-G2019S had a 12% and 26% increase in pT166-Rubicon respectively compared to control (Figure 3b).

[00112]. The *in vivo* effect of LRRK2 and Rubicon was consequently studied by co-expressing human LRRK2 and Rubicon in drosophila. Rubicon mutant lines based on previously identified phosphosites were generated: T166A, S189A, T166A+S189A drosophila Rubicon mutant (DM) and deletion 117-189 (del). T166A yielded no transformants after attempting three insertion sites on two chromosomes and S189A yielded transformants but lacked protein expression when verified by western blotting. As a result, all subsequent studies were carried out with WT, DM and del Rubicon lines after confirmed protein expression. Comparable to the human condition, the drosophila dopaminergic (DA) system is also involved in locomotor control. As distinct clusters of DA neurons identified by positive tyrosine hydroxylase (TH) staining are detectable in the adult fly brain, drosophila viability was assessed based on climbing ability, lifespan and TH-positive expression. First, the co-expression of Rubicon mutants significantly improved the ability of WT-LRRK2 and LRRK2-G2019S drosophila climbing until Day 50. After which, there was a sharp decline in performance that is characteristic of the drosophila model (Figure 4, Table 2). Next, the co-expression of Rubicon mutants significantly extended the lifespan of WT-LRRK2 drosophila by >25% and the co-expression of Rubicon significantly extended the lifespan of LRRK2-G2019S drosophila by >35% (Figure 4, Tables 3). Finally, five regions of the aged fly brains were stained for TH-positive neuronal clusters and counted (Figure 5). The co-expression of Rubicon mutants significantly increased TH count in >2 regions in WT-LRRK2 drosophila and in >3 regions in LRRK2-G2019S drosophila (Table 4).

[00113]. Drosophila climbing assay statistical analysis in Table 2 was carried out using multiple comparison with Tukey post-hoc test. Statistical significance is achieved when $p < 0.05$ (bold values).

[00114]. **Table 2a: LRRK2-WT climbing assay statistical analysis**

DAY		Control	LR-WT	LWT + RWT	LWT + RDM
30	Control	--	--	--	--
30	LR-WT	0.0005	--	--	--
30	LWT + RWT	0.0360	0.3559	--	--

30	LWT + RDM	0.9950	0.0002	0.0154	--
30	LWT + Rdel	0.9688	0.0009	0.0319	0.9977
40	Control	--	--	--	--
40	LR-WT	<.0001	--	--	--
40	LWT + RWT	<.0001	0.9736	--	--
40	LWT + RDM	0.0820	<.0001	<.0001	--
40	LWT + Rdel	0.0003	0.0339	0.0935	0.0500
50	Control	--	--	--	--
50	LR-WT	<.0001	--	--	--
50	LWT + RWT	<.0001	0.0739	--	--
50	LWT + RDM	0.0195	<.0001	<.0001	--
50	LWT + Rdel	<.0001	0.0227	0.0001	<.0001
60	Control	--	--	--	--
60	LR-WT	<.0001	--	--	--
60	LWT + RWT	<.0001	0.0061	--	--
60	LWT + RDM	<.0001	0.9728	0.0241	--
60	LWT + Rdel	0.0022	0.1005	<.0001	0.0364

[00115]. Table 2b: G2019S climbing assay statistical analysis

Day		Control	G2019S	GS + RWT	GS + RDM
30	Control	--	--	--	--
30	G2019S	0.0016	--	--	--
30	GS + RWT	<.0001	0.3543	--	--
30	GS + RDM	0.5960	0.0517	0.0006	--
30	GS + Rdel	0.9694	0.0003	<.0001	0.2568
40	Control	--	--	--	--
40	G2019S	<.0001	--	--	--
40	GS + RWT	<.0001	0.9897	--	--
40	GS + RDM	0.0023	<.0001	<.0001	--
40	GS + Rdel	0.0027	<.0001	<.0001	1.0000
50	Control	--	--	--	--

50	G2019S	<.0001	--	--	--
50	GS + RWT	<.0001	0.3031	--	--
50	GS + RDM	<.0001	<.0001	<.0001	--
50	GS + Rdel	<.0001	<.0001	<.0001	0.9860
60	Control	--	--	--	--
60	G2019S	0.0001	--	--	--
60	GS + RWT	0.0116	0.3811	--	--
60	GS + RDM	<.0001	0.9998	0.3015	--
60	GS + Rdel	0.0176	0.2933	0.9998	0.2265

[00116]. Drosophila lifespan statistical analysis was carried out using multiple comparison with Tukey-Kramer method. Statistical significance is achieved when $p < 0.05$ (bold values).

[00117]. **Table 3a: LRRK2-WT Lifespan statistical analysis**

Stratum1	Stratum2	Tukey
CTRL	LR-WT	<.0001
CTRL	LR-WTXR-DM	<.0001
CTRL	LR-WTXR-Del	0.0015
CTRL	LR-WTXR-WT	<.0001
LR-WT	LR-WTXR-DM	0.0364
LR-WT	LR-WTXR-Del	<.0001
LR-WT	LR-WTXR-WT	1.0000
LR-WTXR-DM	LR-WTXR-Del	0.0548
LR-WTXR-DM	LR-WTXR-WT	0.0497
LR-WTXR-Del	LR-WTXR-WT	<.0001

[00118]. **Table 3b: G2019S Lifespan statistical analysis**

Stratum1	Stratum2	Tukey
CTRL	G2019S	<.0001
CTRL	G2019S X RDM	0.0721
CTRL	G2019S X Rdel	<.0001

CTRL	G2019S X RWT	<.0001
G2019S	G2019S X RDM	<.0001
G2019S	G2019S X Rdel	0.0279
G2019S	G2019S X RWT	0.0036
G2019S X RDM	G2019S X Rdel	0.0004
G2019S X RDM	G2019S X RWT	0.0037
G2019S X Rdel	G2019S X RWT	0.9802

[00119]. **Table 4: Statistical significance of Drosophila tyrosine hydroxylase (TH) neuronal staining in aged transgenic flies.** Aged (Day 60) transgenic fly brains were stained for TH neuronal clusters in five regions (PPL1, PPL2, PPM1/2, PPM3, PAL) and counted. Statistical significance was analysed using the Student's T-test after adjusting for multiple comparisons.

	Control	LR-WT	LWT + RWT	LWT + RDM
Control	---	---	---	---
LR-WT	<u>PPL1, PPL2, PPM3, PPM1/2</u>	---	---	---
LWT + RWT	<u>PPL2, PPM3, PPM1/2</u>	<i>PPL1</i>	---	---
LWT + RDM	<u>PPM3</u>	<i>PPL2, PPM3, PPM1/2</i>	<i>PPL2, PPM1/2</i>	
LWT + Rdel	<u>PPL2, PPM3, PPM1/2</u>	<i>PPL1, PPM3</i>	---	<i>PPL1, PPL2, PPM1/2</i>
	Control	G2019S	GS + RWT	GS + RDM
Control	---	---	---	---
G2019S	<u>PPL2, PPM3, PPM1/2</u>	---	---	---
GS + RWT	<u>PPM3, PPM1/2</u>	<i>PPL2</i>	---	---
GS + RDM	<i>PPL2</i>	<i>PPL2, PPM3, PPM1/2</i>	<i>PPL1, PPL2, PPM3</i>	---
GS + Rdel	<i>PPL1, PPL2</i>	<i>PAL, PPL1, PPL2, PPM3, PPM1/2</i>	<i>PAL, PPL1, PPL2, PPM3, PPM1/2</i>	---

Unpaired T-test
Statistically significant decrease
Statistically significant increase

[00120]. After demonstrating that Rubicon T166 and S189 phosphor-null mutant is able to rescue LRRK2-induced toxicity *in vivo*, the significance of pT166 Rubicon in human PD samples using the customised pT166 Rubicon sandwich ELISA described herein was next investigated. Firstly, human serum from a local PD cohort harbouring Asian LRRK2 mutations was analysed. PD patient serum, regardless of wild-type or mutant LRRK2, had significantly higher pT166 Rubicon expression compared to healthy controls (Figure 7 and Table 5). Next, the expression of pT166 Rubicon was examined in macrophages using the customised pT166 Rubicon sandwich ELISA. Firstly, both LRRK2 and Rubicon had comparable endogenous expression in neurons and microglia, which are akin to brain macrophages (Figure 8a). Mouse macrophages with endogenous LRRK2 knocked out were analysed for pT166 Rubicon expression (Figure 8b, Table 6). Macrophages lacking LRRK2 had a 30% decrease in endogenous pT166 Rubicon and a 60% decrease in over-expressed pT166 Rubicon.

[00121]. Post-mortem human brain samples were fractionated into soluble and membrane-associated protein fractions and subsequently analysed for pT166 Rubicon expression. Post-mortem human brain sections stained for LRRK2 and Rubicon showed that PD brain had an overall lower expression compared to control brain (Figure 9). PD brains had significantly decreased pT166 Rubicon expression compared to controls in both the cytosolic and membrane-associated fractions (Table 7). Though differences exist between the peripheral and central nervous system samples examined i.e. serum vs brain, both systems had significantly altered pT166 Rubicon expression.

[00122]. **Table 5: Statistical significance of Human serum from a Singapore PD cohort harbouring LRRK2 variant mutations was analysed by pT166 Rubicon sandwich ELISA.** Statistical significance was analysed using the Mann-Whitney test.

Whole table statistical analysis using 1 way-ANOVA (Kruskal-Wallis test):

P = 0.0168

Mann-Whitney Test	Control – LR WT	Control – LR mutants	PD – LR WT	PD – LR mutants
Control – LR WT	---	0.6917	0.0433	0.0172
Control – LR mutants	---	---	0.0717	0.0127
PD – LR WT	---	---	---	---
PD – LR mutants	---	---	0.4285	---

[00123]. **Table 6: Statistical analysis of LRRK2 macrophage ELISA.**

	Over-expressed Rubicon	Endogenous Rubicon
Multiple T-test	P = 0.0154	P = 0.0227

[00124]. **Table 7: Statistical significance of Human brain samples from post-mortem Human brains samples were analysed by pT166 Rubicon sandwich ELISA.**

Statistical significance was analysed using one-way ANOVA and statistical significance is achieved when $P < 0.05$.

	Cytosolic pT166 Rubicon	Membrane pT166 Rubicon
One-way ANOVA	P = 0.022	P = 0.0004

[00125]. The altered expression of pT166 Rubicon in PD serum and post-mortem PD brain compared to healthy controls displayed its potential as a PD diagnostic biomarker.

[00126]. *In vivo*, the co-expression of Rubicon phosphor-null mutants was able to rescue LRRK2-induced toxicity. This suggests that Rubicon phosphosites, T166 and S189, are possible drug target sites for LRRK2-linked diseases. Many drugs have off-target effects, therefore an allosteric inhibitor to LRRK2-specific substrate sites will enhance precision and minimise adverse off-target effects.

[00127]. An antibody was formed using the synthesized peptide DAHV{pThr}AMLQCLEAVE (SEQ ID NO. 2) conjugated to Keyhole limpet hemocyanin (KLH) and the resultant immunogen peptide was used to generate phosphor-specific antibody in a mammal.

[00128]. Few bona fide LRRK2 substrates have been identified to date. Among them, not many were methodically screened for LRRK2-specific phosphosites.

[00129]. Polyclonal antibodies were developed in 3 Balb/c mice and 3 C57 mice using the synthesized peptide DAHV{pThr}AMLQCLEAVE (SEQ ID NO. 2) conjugated to Keyhole limpet hemocyanin (KLH). Test bleeds were taken after the 3rd and 5th immunisation with the peptide conjugate. To test titer and specificity of the antibodies 96 well plates were coated with either SEQ ID NO. 2: DAHV{pThr}AMLQCLEAVE or SEQ ID NO. 14: DAHVTAMLQCLEAVE epitopes, whereby the sequence surrounding T166 of the Rubicon protein of SEQ ID NO. 1 was either phosphorylated or not phosphorylated. Samples of pre-immunised serum and antiserum collected after the 3rd and 5th immunisation were then used in an indirect ELISA assay in phosphate buffered saline, pH 7.4 to detect the phosphorylated

antigen SEQ ID NO. 2 or the un-phosphorylated antigen SEQ ID NO. 14 across a series of supernatant dilutions. The secondary antibody was a peroxidase-AffiniPure Goat Anti-Mouse IgG, Fcγ Fragment Specific (min X Hu, Bov Hrs Sr Prot). The results are listed in table 8. The dilution extended to 1: 512,000, however the results for the full range of dilution is not listed.

[00130]. **Table 8: ELISA results for polyclonal antibodies.**

Serum type	sample	Supernatant dilution						Blank	Titer	SEQ ID NO. coating
		1:1,000	1:2,000	1:4,000	1:8,000	1:16,000	1:32,000	PBS		
Pre-immunisation serum	1	0.08	--	--	--	--	--	0.06	<1,000	2
		0.09	--	--	--	--	--	0.08	<1,000	14
	2	0.07	--	--	--	--	--	0.06	<1,000	2
		0.06	--	--	--	--	--	0.08	<1,000	14
antiserum collected after the 3 rd immunisation	1	1.62	0.96	0.62	0.40	0.25	0.16	0.06	32,000	2
		0.09	0.08	0.07	0.07	0.06	0.06	0.08	<1,000	14
	2	2.31	1.27	0.74	0.45	0.31	0.19	0.06	64,000	2
		0.1	0.8	0.08	0.06	0.07	0.06	0.08	<1,000	14
antiserum collected after the 4 th immunisation	1	2.62	2.06	1.84	1.29	0.81	0.44	0.06	512,000	2
		1.13	0.71	0.35	0.21	0.14	0.10	0.08	16,000	14
	2	2.64	2.17	1.78	1.19	0.75	0.42	0.06	512,000	2
		1.72	1.15	0.77	0.50	0.34	0.19	0.08	64,000	14
antiserum collected after the 5 th immunisation	1	2.89	2.60	1.98	1.88	1.07	0.71	0.06	64,000	2
		1.99	1.57	1.00	0.75	0.42	0.25	0.08	16,000	14
	2	2.59	2.41	1.88	1.41	0.96	0.51	0.06	64,000	2
		2.00	1.77	1.00	0.94	0.57	0.51	0.08	64,000	14

[00131]. Monoclonal antibodies were developed against the target site and 5 primary clones were selected with 2 monoclonal cell lines being established for each clone. To test titer and specificity of the antibodies 96 well plates were coated with either SEQ ID NO. 2: DAHV{pThr}AMLQCLEAVE or SEQ ID NO. 14: DAHVTAMLQCLEAVE epitopes, whereby the sequence surrounding T166 of the Rubicon protein of SEQ ID NO. 1 was either phosphorylated or not phosphorylated. All of the 10 antibody cell lines were then used in an indirect ELISA assay in phosphate buffered saline, pH 7.4 to detect the phosphorylated antigen SEQ ID NO. 2 or the un-phosphorylated antigen SEQ ID NO. 14 across a series of

supernatant dilutions. The secondary antibody was a peroxidase-AffiniPure Goat Anti-Mouse IgG, Fcy Fragment Specific (min X Hu, Bov Hrs Sr Prot). The results are listed in table 9.

[00132]. Table 9: ELISA results of hybridoma culture supernatant

Primary clone No.	Cell lines	Supernatant dilution						Blank	Titer	SEQ ID NO. coating	isotype
		1:10	1:30	1:90	1:270	1:810	1:2,430	PBS			
1	4B9-1	2.60	2.56	2.54	2.33	1.83	0.98	0.08	>2,430	2	IgG2a,K
		0.07	0.07	0.07	0.06	0.06	0.06	0.08	<10	14	
	4B9-2	2.57	2.55	2.36	2.11	1.32	0.72	0.08	>2,430	2	IgG2a,K
		0.09	0.08	0.07	0.06	0.06	0.06	0.08	<10	14	
2	6A1-1	2.60	2.31	1.95	1.31	0.59	0.24	0.08	>2,430	2	IgG2a,K
		0.07	0.06	0.06	0.06	0.06	0.05	0.08	<10	14	
	6A1-2	2.59	2.30	2.12	1.45	0.88	0.49	0.08	>2,430	2	IgG2a,K
		0.06	0.06	0.06	0.06	0.06	0.06	0.08	<10	14	
3	9B7-1	2.65	2.64	2.44	2.05	1.48	1.05	0.08	>2,430	2	IgG2,K
		0.07	0.06	0.06	0.06	0.05	0.05	0.08	<10	14	
	9B7-2	2.75	2.63	2.59	1.99	1.32	0.80	0.08	>2,430	2	IgG2,K
		0.07	0.07	0.07	0.06	0.06	0.06	0.08	<10	14	
4	9D1-1	2.46	2.27	2.18	2.14	1.60	1.02	0.08	>2,430	2	IgG2a,K
		0.09	0.07	0.07	0.07	0.06	0.06	0.08	<10	14	
	9D1-2	2.51	2.46	2.42	2.36	1.93	1.36	0.08	>2,430	2	IgG2a,K
		0.15	0.15	0.06	0.06	0.06	0.06	0.08	<10	14	
5	12H1-1	2.39	2.34	2.30	1.97	1.51	0.99	0.08	>2,430	2	IgG2a,K
		0.07	0.07	0.06	0.06	0.06	0.05	0.08	<10	14	
	12H2-2	2.45	2.43	2.38	2.12	1.60	0.95	0.08	>2,430	2	IgG2a,K
		0.08	0.07	0.07	0.06	0.05	0.05	0.08	<10	14	

[00133]. A specific monoclonal antibody against the identified target site was customised and a sandwich ELISA using the customised pT166 Rubicon antibody was developed. The pT166 Rubicon sandwich ELISA was validated with human PD serum and brain samples. The developed pT166 Rubicon sandwich ELISA may be used as a diagnostic assay for PD.

[00134]. The produced monoclonal antibody was sequenced. Total RNA was isolated from the hybridoma cells following the technical manual of TRIzol® Reagent. Total RNA was then reverse-transcribed into cDNA using either isotype-specific anti-sense primers or universal primers following the technical manual of PrimeScript™ 1st Strand cDNA

Synthesis Kit. Antibody fragments of heavy chain and light chain were amplified according to the standard operating procedure (SOP) of rapid amplification of cDNA ends (RACE) of GenScript. Amplified antibody fragments were cloned into a standard cloning vector separately. Colony PCR was performed to screen for clones with inserts of correct sizes. The consensus sequence is provided below. All 5 clones screened had at least 99% amino acid sequence identity. Similarly, analysis found at least 96% sequence identity in both the heavy chain (96.53% nucleotide identity) and the light chain (98.64% nucleotide identity).

[00135]. Heavy chain: cDNA sequence (420 bp)

[00136]. Signal sequence-*FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4*, SEQ ID NO. 10:
 ATGAACTTAGGGCTCAGCTTCATTTTCCTTGCCCTTATTTTAAAAGGTGTCCAGTGTGAG
 GTGCAGCTGGTGGAGTCTGGGGGAGGCTTAGTGCAGCCTGGAGGGTCCCTGAAACTC
 TCCTGTGCAGCCTCTGGATTCACCTTCACTAATTATGGCGTGTCTTGGGTTCCGCCAGAC
 TCCAGACAAGAGGCTGGAGTTGGTCGCAACCATTAATAGTAATGGTGGTAGTAAATAT
TATCCAGACAGTGTGAAGGGCCGATTCACCATTTCCAGAGACACTGCCAAGAACACCC
 TGTACCTGCATATGAGCAGTCTGAAGTCTGAGGACACAGCCATGTATTACTGTGCAAGA
GATGTATGGTTACGACGTCAGTGGTACTTCGATGTCTGGGGCGCAGGGACCACGGTC
 ACCGTCTCCTCA.

[00137]. Light chain: DNA sequence (393 bp)

[00138]. Signal sequence-*FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4*, SEQ ID NO. 11:
 ATGAAGTTGCCTGTTAGGCTGTTGGTGCTGATGTTCTGGATTCCTGCTTCCAGCAGTGA
 TGTTGTGATGACCCAACTCCTCTCTCCCTGCCTGTCAGTCTTGGAGATCCAGCCTCCA
 TCTCTTGCAGATCTAGTCAGAGCCTTGTACACAGTAATGGAAACACCTATTTACATTG
 GTACCTGCAGAAGACAGGCCAGTCTCCAAAGCTCCTGATCTACAAACTTTCCAACCGA
TTTTCTGGGGTCCCAGACAGGTTCACTGGCAGTGGATCAGGGACAGATTTCACTCA
 AGATCAGCAGAGTGGAGGCTGAGGATCTGGGAGTTTATTTCTGCTTCTCAAAGTACACA
TGTTCTCTCACGTTCCGGTGCTGGGACCAAGCTGGAGCTGAAA.

[00139]. Light chain: Amino acid sequence (131 aa)

[00140]. Signal peptide-*FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4*, SEQ ID NO. 12:
 MKLPVRLVLVLMFWIPASSSDVVMQTPLSLPVSLGDPASISCRSSQSLVHSNGNTYLHWYL
 QKTGQSPKLLIYKLSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHVPLTF
 GAGTKLELK.

[00141]. Heavy chain: Amino acid sequence (140 aa)

[00142]. Signal peptide-*FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4*, SEQ ID NO. 13:
 MNLGLSFIFLALILKGVQCEVQLVESGGGLVQPGGSLKLSCAASGFTFTNYGVSWVRQTPD
 KRLELVATINSNGGSKYYPDSVKGRRFTISRDTAKNTLYLHMSSLKSEDTAMYCARDDVWLR
RQWYFDVWGAGTTVTVSS.

[00143]. Identified LRRK2-specific Rubicon phosphosites, T166 and S189, LRRK2-linked toxicity was able to be rescued *in vivo*, highlighting the phosphosites potential as drug target sites.

[00144]. Current work aims to build on pT166 Rubicon as a diagnostic biomarker for PD. This is achieved by assaying a small amount of patient serum protein (2 µg) with the developed pT166 Rubicon sandwich ELISA.

[00145]. The quantified pT166 Rubicon expression in patient serum will determine if the assayed sample belongs to healthy control or PD group based on pre-set thresholds. Ideally, if pT166 Rubicon expression varies between short disease duration (<5 years) and long disease duration (>5 years), the quantified pT166 Rubicon expression will be able to reveal if PD is early stage or late stage.

[00146]. In various embodiments Rubicon is an amino acid sequence represented by SEQ ID NO.1 :

MRPEGAGMELGGGEERLPEESRREHWQLLGNLKTTEGLVSTNSPNVWSKYGGGLERLCR
DMQSILYHGLIRDQACRRQTDYWQFVKDIRWLSPHSALHVEKFISVHENDQSSADGASERA
VAELWLQHSLQYHCLSAQLRPLLGDQRQYIRKFYTDAAFLSDAHV**T**AMLQCLEAVEQNNPR
LLAQIDA**S**MFARKHESPLLVTKSQSLTALPSSTYTPPNSYAHQHSYFGSFSSLHQSVPNNGS
ERRSTSFPLSGPPRKPQESRGHVSPAEDQTIQAPPVSVSALARDSPLTPNEMSSSTLTSPIE
ASWVSSQNDS PGDASEGPEYLAIGNLDPRGRTASCQSHSSNAESSSSNLFSSSSSQKPDS
AASSLGDQEGGGESQLSSVLRRSSFSEGQTLTVTSGAKKSHIRSHSDTSIASRGAPESCND
KAKLRGPLPYSGQSSEVSTPSSLYMEYEGGRYLCSGEGMFRRPSEGQSLISYLSEQDFGS
CADLEKENAHFSISESLIAAIELMKCNMMSQCLEEEEEEEDSDREIQELKQKIRLRRQIRT
KNLLPMYQEAHGSFRVTSSSSQFSSRDSAQLSDSGSADEVDEFEIQDADIRRNATSSSKS
FVSSQSFSHCFLHSTSAEAVAMGLLKQFEGMQLPAASELEWLVPEDAPQKLLPIPDSLPI
PDDGQHADIYKLIRVRGNLEWAPPRPQIIFNVHPAPTRKIAVAKQNYRCAGCGIRTPDYIK
RLRYCEYLGKYFCQCCHENAQMAIPSRVLRKWDFSKYYVSNFSKDLLIKIWNDFLNVQDI
NSALYRKVKLLNQVRLLRVQLCHMKNMFKTCRLAKELLDSDFTVPGHLTEDLHLYSLNDLT
ATRKGEGLPRLAELTRAGATHVERCMLCQAKGFICEFCQNEDDIIFPFELHKCRTCEECKAC
YHKACFKSGSCPRCERLQARREALARQSLESYLSDYEEEPAEALALEAAVLEAT

[00147]. Whereby the phosphosites T166 and S189 are represented as the bold underlined amino acids.

[00148]. SEQ ID NO. 2: DAHV{pThr}AMLQCLEAVE.

[00149]. SEQ ID NO. 3:

MASGSCQGCEEDEETLKKLIVRLNNVQEGKQIETLVQILEDLLVFTYSERASKLFQGKNIHVP
LLIVLDSYMRVASVQQVGWSLLCKLIEVCPGTMQSLMGPQDVGNDWEVLGVHQLILKMLTV
HNASVNLVSVIGLKTLDLLTSGKITLLILDEESDIFMLIFDAMHSFPANDEVQKLGCKALHVLFE

RVSEEQLTEFVENKDYMILLSALTNFKDEEEIVLHVLHCLHSLAIPCNNVEVLMSGNVRCYNI
VVEAMKAFFPMSERIQEVSCCLLHRLTLGNFFNILVLNEVHEFVVKAVQQYPENAAALQISALS
CLALLTETIFLNQDLEEKNNENQENDDEGEEDKFLWLEACYKALTWHRKNKHVQEAACWAL
NNLLMYQNSLHEKIGDEDGHFPAHREVMLSMLMHSSSKEVFQASANALSTLLEQNVNFRKI
LLSKGIHLNVLELMQKHIHSPEVAESGCKMLNHLFEGSNTSLDIMAADVVPKILTMKRHETS
PVQLEALRAILHFIVPGMPESREDTEFHKLNMVKKQCFKNDIHKLVLAALNRFIGNPGIQK
CGLKVISSIVHFPDALEMLSLEGAMDSVLHTLQMYPPDDQEIQCGLSLIGYLITKKNVFIGTGH
LLAKILVSSLYRFKDVAEIQTKGFQTLAILKLSASFSLLVHHSFDLVIFHQSSNIMEQKDQ
QFLNLCKCKFAKVAMDDYLKNVMLERACDQNNSIMVECLLLLGAADANQAKEGSSLICQVCE
KESSPKLVELLLNSGSREQDVRKALTISIGKGDSSQIISLLLRRLALDVANNISICLGGFCIGKVE
PSWLGPLFPDKTSNLRKQTNIASTLARMVIRYQMKSAVEEGTASGSDGNFSEDVLSKFDE
WTFIPDSSMDSVFAQSDDDLDEGESEGSFLVKKKSNSISVGEFYRDAVLQRCSPNLQRHSN
SLGPIFDHEDLLKRKRKILSSDDSLRSSKLQSHMRHSDSISSLASEREYITSLDLSANELRDID
ALSQKCCISVHLEHLEKLELHQNALTSFPQQLCETLKSLEHLHSLNKFTSFPSYLLKMSCIA
NLDVSRNDIGPSVVLDPVTVCPTLKQFNLSYNQLSFVPENLTDVVEKLEQLILEGNKISGICS
PLRLKELKILNLSKNHISLSENFLACPKVESFSARMNFLAAMPFLPPSMTILKLSQNKFS
PEAILNLPHLRSLDMSSNDIQYLPGAHWKSLNLRRELLFSHNQISILDSEKAYLWSRVEKLH
LSHNKLKEIPPEIGCLENLTSLDVSYNLELRSFPNEMGKLSKIWDLPDELHLNFDKFKHIGCK
AKDIIRFLQQRLKKAVPYNRMKLMIVGNTGSGKTTLLQQLMKTCKSDLGMSATVIGIDVKD
WPIQIRDKRKRDVLNVWDFAGREEFYSTHPHMTQRALYLAVYDLKSGQAEVDAMKPWL
FNIKARASSSPVILVGTHLDVSDEKQRKACMSKITKELLNKRGFPAIRDYHFVNATEESDALA
KLRKTIINESLNFKIRDQLVVGQLIPDCYVELEKIILSERKNVPIEFPVIDRKRLQLVRENQLQL
DENELPHAVHFLNESGVLLHFQDPALQLSDLYFVEPKWLCKIMAQILTVKVEGCPKHPKGIIS
RRDVEKFLSKKRKFPKNYMSQYFKLLEKFQIALPIGEEYLLVPSSLSDHRPVIELPHCENSEIII
RLYEMPYFPMGFWSRLINRLLEISPYMLSGRERALRPNRMVWRQGIYLNWSPEAYCLVGS
EVLNHPESFLKITVPSCRKGCILLGQVVDHIDSLMEEWFPGLLEIDICGEGETLLKKWALYS
FNDGEEHQKILLDDLKKAEEGDLLVNPDPRLTIPISQIAPDLILADLPNIMLNDELEFEQ
APEFLLGDGSGFSVYRAAYEGEEVAVKIFNKHTSLRLLRQELVVLCHLHHPSLISLLAAGIRP
RMLVMELASKGSLDRLLQQDKASLTRTLQHRIALHVADGLRYLHSAMIIYRDLKPHNVLLFTL
YPNAAIIAKIADYGIAQYCCRMGIKTSEGTGPFRAPEVARGNVIYNQQADVVSFGLLLYDILT
GGRIVEGLKFPNEFDELEIQGKLPDPVKEYGCAPWPMVEKLIKQCLKENPQERPTSAQVFDI
LNSAELVCLTRRILLPKNVIVECMVATHHNSRNASIWLGCIGHTDRGQLSFLDLNTEGYTSEE
VADSRILCLALVHLPVEKESWIVSGTQSGTLLVINTEDGKKRHTLEKMTDSVTCLYCNSFSK
QSKQKNFLLVGTADGKLAIFEDKTVKLKGAAPLKILNIGNVSTPLMCLSESTNSTERNVMWG
GCGTKIFSFSNDFTIQKLIETRTSQLFSYAAFSDSNIITVVVDALYIAKQNSPVVEVWDKTE
KLCGLIDCVHFLREVMVKENKESKHMSYSGRVKTLCLQKNTALWIGTGGGHILLDLSTRR

LIRVIYNFCNSVRVMMTAQLGSLKNVMLVLGYNRKNTEGTQKQKEIQSCLTVWDINLPHEV
QNLEKHIEVRKELAEKMRRTSVE

[00150]. It should be further appreciated by the person skilled in the art that variations and combinations of features described above, not being alternatives or substitutes, may be combined to form yet further embodiments falling within the intended scope of the invention.

[00151]. As would be understood by a person skilled in the art, each embodiment, may be used in combination with other embodiment or several embodiments.

Claims

1. An *in vitro* method of detecting phosphorylation of a Rubicon protein comprising:
isolating proteins from a sample;
contacting the isolated proteins with an antibody that hybridises to a phosphorylated Threonine at 166 of a Rubicon protein to form a complex;
detecting the complex wherein detection of the complex that varies from a predetermined value indicates that the sample comes from a subject having a disease associated with Leucine-rich repeat kinase 2 (LRRK2).
2. The *in vitro* method according to claim 1, wherein the disease associated with Leucine-rich repeat kinase 2 (LRRK2) is Parkinson's disease.
3. The *in vitro* method according to claim 3, wherein the antibody is a chimeric humanised antibody.
4. The *in vitro* method according to claim 3, wherein the antibody is a monoclonal antibody.
5. The *in vitro* method according to any one of the preceding claims, wherein the complex is detected with an enzyme linked immunosorbent assay.
6. The *in vitro* method according to any one of the preceding claims, wherein the subject having a disease associated with Leucine-rich repeat kinase 2 (LRRK2) is treated with a compound able to block interaction of LRRK2 with Threonine 166 and/or Serine 189 of the Rubicon protein.
7. The *in vitro* method according to any one of the preceding statements, wherein the compound comprises a treatment antibody.
8. The *in vitro* method according to claim 7, wherein the treatment antibody is a chimeric humanised antibody.
9. The *in vitro* method according to claim 7, wherein the treatment antibody is a monoclonal antibody.
10. The *in vitro* method according any one of the preceding claims, further comprising removing blocking proteins prior to contacting the isolated proteins with the antibody.
11. The *in vitro* method according to claim 10, wherein the blocking proteins comprise albumin.
12. An antibody which is capable of binding to Rubicon phosphorylated at Threonine 166 and/or phosphorylated at Serine 189.
13. The antibody according to claim 12, comprising at least one variable region incorporating a CDR selected from amino acid sequences i) to vi):
 - i) LC-CDR1: RSSQSLVHSNGNTYLH (SEQ ID NO. 4);

- ii) LC-CDR2: KLSNRFS (SEQ ID NO. 5);
- iii) LC-CDR3: SQSTHVPLT (SEQ ID NO. 6);
- iv) HC-CDR1: NYGVS (SEQ ID NO. 7);
- v) HC-CDR2: TINSNGGSKYYPDSVKG (SEQ ID NO. 8);
- vi) HC-CDR3: DVWLRRQWYFDV (SEQ ID NO. 9); and

a functional variant with 99% amino acid sequence identity to any one of amino acid sequences i) to vi).

14. The antibody according to claim 12, comprising amino acid sequences:

- i) LC-CDR1: RSSQSLVHSNGNTYLH (SEQ ID NO. 4);
- ii) LC-CDR2: KLSNRFS (SEQ ID NO. 5);
- iii) LC-CDR3: SQSTHVPLT (SEQ ID NO. 6);
- iv) HC-CDR1: NYGVS (SEQ ID NO. 7);
- v) HC-CDR2: TINSNGGSKYYPDSVKG (SEQ ID NO. 8);
- vi) HC-CDR3: DVWLRRQWYFDV (SEQ ID NO. 9); or

a functional variant with 99% amino acid sequence identity to any one of amino acid sequences i) to vi).

15. The antibody according to any one of claims 12 to 14, wherein the antibody is a chimeric humanised antibody.

16. The antibody according to any one of claims 12 to 14, wherein the antibody is a monoclonal antibody.

17. The antibody according to any one of claims 12 to 16, for use in the detection or treatment of a disease associated with Leucine-rich repeat kinase 2 (LRRK2).

18. The antibody for use according to claim 17, wherein the disease associated with Leucine-rich repeat kinase 2 (LRRK2) is Parkinson's disease.

19. An inhibitor of Rubicon interaction with Leucine-rich repeat kinase 2 (LRRK2) comprising a compound able to block interaction of LRRK2 with Threonine 166 and/or Serine 189 of the Rubicon protein.

20. The inhibitor according to claim 19, wherein the compound comprises an antibody.

21. The inhibitor according to claim 20, wherein the antibody is a monoclonal antibody.

22. The inhibitor according to claim 20, wherein the antibody is a chimeric humanised antibody.
23. The inhibitor according to any one of claims 19 to 22, for use in the treatment of a disease associated with Leucine-rich repeat kinase 2 (LRRK2).
24. The inhibitor for use according to claim 23, wherein the disease associated with Leucine-rich repeat kinase 2 (LRRK2) is Parkinson's disease.
25. Use of an antibody according to any one of claims 11 to 15 or an inhibitor according to any one of claims 19 to 22, in the manufacture of a medicament for the treatment of a disease associated with Leucine-rich repeat kinase 2 (LRRK2).
26. Use according to the claim 25, wherein the disease associated with Leucine-rich repeat kinase 2 (LRRK2) is Parkinson's disease.
27. A method of treating a subject in need having a disease associated with Leucine-rich repeat kinase 2 (LRRK2) comprising:
administering a compound able to block interaction of LRRK2 with Threonine 166 and/or Serine 189 of the Rubicon protein.
28. The method according to claim 27, wherein the compound comprises an antibody according to claim 12.
29. The method according to claim 28, wherein the antibody is a chimeric humanised antibody.
30. The method according to claim 28, wherein the antibody is a monoclonal antibody.
31. A kit comprising an antibody according to any one of claims 12 to 16, and detection reagents for detecting a disease associated with Leucine-rich repeat kinase 2 (LRRK2).
32. The Kit according to claim 31, wherein the disease associated with Leucine-rich repeat kinase 2 (LRRK2) is Parkinson's disease.