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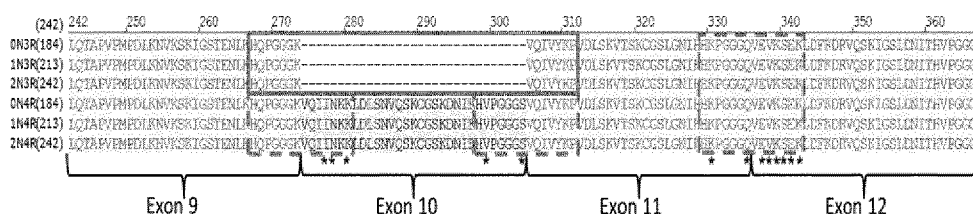


Figure 1

(57) Abstract: The present invention provides an antibody or antigen-binding fragment thereof that: (a) specifically binds 4R tau protein isoforms in a physiological sample; or (b) specifically binds 3R tau protein isoforms in a physiological sample. The present invention further provides nucleic acids and vectors encoding such an antibody or antigen-binding fragment, as well as host cells comprising such nucleic acids and vectors. The antibodies and fragments may be used in treating and diagnosing tauopathies.

ANTIBODIES

Field of Invention

The present invention relates to antibodies specific for particular tau proteins, and
5 in particular such antibodies that are specific for 4R or 3R tau protein isoforms. The
antibodies may be, for instance, used in detection, diagnostics, and therapeutics.

Background of Invention

10 Microtubule associated protein tau (tau) was discovered in 1975. It was initially
characterised as a microtubule binding protein essential for microtubule growth and
stabilisation within the axons of neurons. Binding to microtubules is achieved at the C-
terminal end of tubulin via the microtubule binding regions (MTBRs) of tau (encoded via
exons 9-12). Specifically it has been shown, by peptide competition NMR, that tau
15 interacts with the interface between α - and β -tubulin by residues 224-237, 245-253, 275-
284 and 300-317.

The tau gene (MAPT) comprises 16 exons located on chromosome 17q21.
Alternative splicing from a single tau pre-mRNA gives rise to six splice variant isoforms
of tau within the central nervous system ranging from 342-441 amino acids in length.
20 Tau isoforms are grouped into two categories based on inclusion or exclusion of exon 10
(within the MTBR) giving rise to four MTBRs (4 repeat (4R) tau) in the presence of exon
10 and three MTBRs (3 repeat (3R) tau) with the exclusion of exon 10. Within each of
these categories there are three N-terminal variants of tau based on the splicing of exons
2 and 3. This gives rise to 0N tau (with neither exon 2 or 3), 1N tau (with exon 2 only)
25 and 2N tau (containing both exons 2 and 3). Tau nomenclature is based upon amino acid
numbering from the largest (2N4R) isoform of tau and as such has four separate
functional domains; N-terminal projection (amino acids 1-165); proline rich domain
(amino acids 166-242); MTBR (amino acids 243-367); C-terminal domain (amino acids
368-441). The presence or absence of the sequence encoded by exon 10 in the MTBR
30 domain is what denotes 3R or 4R tau.

The tau MTBR is a repeating region of the protein which consists of three (3R
tau) or four (4R tau) repeat regions that are identical in sequence across the isoforms of
tau. Each repeat is a different tau exon; repeat 1 (exon 9) (amino acids 242-273); repeat 2
(exon 10) (amino acids 274-304); repeat 3 (exon 11) (amino acids 305-335); repeat 4

(exon 12) (336-367). Whilst there is only 34% complete identical sequence homology across all four repeat regions, the level of homology between amino acids of similar classes (polar, charged, non-charged) or between at least two of the repeat regions is 90%, with repeat region 1 and repeat 4 being the most divergent.

5 Tau is a largely unstructured protein, but FRET experiments have suggested that the N and C termini are oriented in such a way that they are in close proximity. Further studies using NMR have confirmed that the N and C terminal ends of tau are folded back in a “paperclip” like structure onto the central regions of the protein. It has also been demonstrated that tau has a propensity to form some more complex transient local
10 secondary structure, specifically, β – strands in the MTBR and polyproline helices in the proline rich domain.

 Tau is found expressed mainly in neurons and at low levels in oligodendrocytes and astrocytes. Tau was originally identified associated with axonal microtubules and to a lesser extent associated with the plasma membrane, nucleus and mitochondria. In
15 healthy adult neurons the distribution of tau to the axon cross links tubulin and allows interconnection with other cytoskeletal components such as neurofilaments and actin. These interactions stabilise tubulin assembly into microtubules and regulate their dynamically unstable nature allowing for reorganisation of the cytoskeleton as required for axonal growth. Tau has also been shown to directly regulate the microtubule binding
20 of motor transport proteins dynein and kinesin, which transport cargo in the retrograde and anterograde direction along axons respectively. This regulatory effect is thought to occur via direct competition with the aforementioned motor proteins for access to the microtubules. In tau overexpression systems it is observed a greater effect on kinesin resulting in net retrograde transport and an accumulation of cargo (such as mitochondria)
25 in cell bodies rather than spread out down axons. However, this regulatory effect is not completely understood and may have complementary mechanisms as it has been demonstrated in tau knock-out mice that there is no influence on axonal transport.

 Tau protein and its isoforms are involved in a number of diseases typically characterised by the deposition of tau fibrils, with those conditions being collectively
30 known as tauopathies. Although the majority of tauopathies occur sporadically in the population there are many tauopathies that are linked to a MAPT mutation that show the same disease phenotypes as the sporadic counterparts (Ghetti *et al.*, 2015, *Neuropathol Appl Neurobiol*, 41 (1), 24-46). Tauopathies can be further split into primary and secondary tauopathies. Primary tauopathies are a subgroup of the frontotemporal lobar

degeneration (FTLD), which are characterised by neuronal tau inclusions with predominant cell death in the frontal and temporal lobes of the brain. Within these lobes tau inclusions are believed to be the major driving factor in pathology. A well characterised example is progressive supranuclear palsy (PSP). In contrast, secondary tauopathies are diseases where tau pathology is observed in association with other brain pathologies. A well characterised example of a secondary tauopathy is Alzheimer's disease (AD), where disease pathology is characterised by both neuronal tau fibrils and extracellular amyloid plaques, as it was defined by Alois Alzheimer in 1907. It is worth noting that in AD there is still a link between tau load and cognitive decline, indicating the important role tau still plays in this multifactorial disease.

The ratio of tau isoforms appears to play an important role in maintaining tau in a non-pathogenic state. As such, tauopathies can exist as either 3R tauopathies or 4R tauopathies characterised by an excess of 3R or 4R tau respectively. Pick's disease is a tauopathy predominantly associated with an excess for 3R-forming filamentous 3R tau inclusions known as Pick bodies. The major pathology associated with Pick's disease are neuronal and glial loss in the frontal, temporal and parietal lobes of the brain. The much more common form of tauopathy associated with imbalance of tau isoforms are the 4R tauopathies. An excess of 4R tau and 4R tau inclusions has been observed in diseases such as: frontotemporal dementia and parkinsonism's linked to chromosome 17 (FTDP-17); progressive supranuclear palsy (PSP); and corticobasal degeneration (CBD).

Given the importance of tau in a large number of pathological conditions, there is an ongoing need for new diagnostic and therapeutic approaches that target tau.

Summary of the Invention

The invention provides an antibody or antigen-binding fragment thereof that:

- (a) specifically binds 4R tau protein isoforms in a physiological sample; or
- (b) specifically binds 3R tau protein isoforms in a physiological sample.

Where the antibody or antigen-binding fragment thereof specifically binds 4R tau protein, it may:

- (a) bind to the amino acid region encoded by exon 10 of the 4R tau protein when the 4R tau protein contains a post translational modification at one or more of amino acid positions 279, 280, 281, 285, and 289 of tau 4R;

(b) bind to a peptide comprising, or consisting of, amino acids 294 to 302 of 4R tau protein;

(c) bind to a peptide corresponding to a single epitope from the amino acid sequence encoded by exon 10 of tau; or

5 (d) cross-block or be cross-blocked by any of the antibodies or fragments of (a) to (c).

The antibody or antigen-binding fragment thereof may specifically bind 4R tau protein isoforms in cell lysates from cells expressing physiological 4R tau protein isoform(s), preferably in cell lysates from iPSC derived neuronal cells expressing 4R tau protein isoform(s). The antibody or antigen-binding fragment may detect 4R tau protein isoforms via immunofluorescence on, or in, cells expressing 4R tau protein isoform(s).

The antibody or antigen-binding fragment thereof to 4R tau protein may:

(a) comprise one or more of the light chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs 3, 5, and 7 and one or more of the heavy chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 11, 13, and 15, and preferably comprise the light chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs 3, 5, and 7 and heavy chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 11, 13, and 15;

(b) comprise the light and heavy chain variable regions of SEQ ID NOs 1 and 9;

20 (c) be an intrabody, preferably comprising the sequence of SEQ ID No: 17 or a sequence with at least 95% sequence identity thereto that is still able to specifically bind tau protein 4R isoforms; or

(d) cross-block or be cross-blocked by any of the antibodies or fragments of (a) to (c).

25 Where the antibody or antigen-binding fragment thereof specifically binds 3R tau protein isoforms, it may:

(a) bind to a peptide, such as a cyclic peptide, that comprises the amino acid sequence encoded by the region bridging exons 9 and 11 of tau; and/or

30 (b) bind to a peptide, such as a cyclic peptide, consisting of seven amino acids either side of the boundary of the sequence encoded by exons 9 and 11 of tau.

The antibody or antigen-binding fragment thereof to 3R tau protein may:

(a) comprise one or more of the light chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs 20, 22, and 24 and one or more of the heavy chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 28, 30, and 32, and preferably comprise

the light chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs 20, 22, and 24 and heavy chain CDR1, CDR2, and CDR 3 sequences of SEQ ID NOs: 28, 30, and 32;

(b) comprise the light and heavy chain variable regions of SEQ ID NOs 18 and 26;

5 (c) be an intrabody, preferably comprising the sequence of SEQ ID No: 34 or a sequence with at least 95% sequence identity thereto that is able to specifically bind tau protein 3R isoforms; or

(d) cross-block or be cross-blocked by any of the antibodies or fragments of (a) to (c).

10 The present invention also provides an antibody or antigen-binding fragment thereof that specifically binds 4R tau protein, wherein the antibody or antigen-binding fragment thereof binds an epitope of 4R tau protein comprising amino acids K294, D295, N296, and I297, optionally where the epitope further comprises K298 and V300 of 4R tau protein.

15 The antibody or antigen-binding fragment thereof may be an intrabody or a degrabody.

The invention also provides a nucleic acid or nucleic acids encoding an antibody or antigen-binding fragment of the invention; a vector or vectors comprising said nucleic acids; and a host cell comprising said nucleic acid or nucleic acids or said vector or

20 vectors.

The invention also provides a pharmaceutical composition comprising an antibody or antigen-binding fragment thereof of the invention, a nucleic acid or nucleic acids of the invention, or a vector or vectors of the invention; and a pharmaceutical carrier or excipient.

25 The invention also provides methods of detecting 4R or 3R tau protein isoforms comprising: (a) contacting a test sample with an antibody or antigen-binding fragment thereof of the invention; and (b) detecting binding of the antibody or antigen-binding fragment thereof. The invention also provides a method of determining the levels of 4R and 3R tau protein isoforms. The methods may be used to diagnose tauopathies, preferably PSP, CBD, or Pick's Disease.

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The antibody or antigen-binding fragment thereof may be used to treat a tauopathy, such as a tauopathy comprising an imbalance between 4R tau protein isoforms and 3R tau protein isoforms.

Brief Description of the Figures

Figure 1: Tau protein sequence exons 9-12 are shown. The TE9/11 peptide sequence is outlined in the solid lined box. Potentially cross-reactive epitopes at the exon 9-10, 10-11 and 11-12 boundaries are highlighted in the dashed box. The amino acid differences between TE9/11 are highlighted by asterisks.

Figure 2: Tau protein sequence exons 9-12 are shown. The TE10 peptide sequence is outlined in the solid lined box. Potentially cross-reactive epitopes within exons 9, 11 and 12 are highlighted in the dashed boxes. The amino acid differences between TE10 are highlighted via the asterisks. The documented post translational modifications at N279, K280/281 and S285/289 are highlighted in the dotted line boxes to the immediate left of the solid lined boxed region.

Figure 3: Primary B-cell culture supernatant screening homogenous fluorescence-based assay. Green points represent those that were selected for hit-picking and those in red were not selected.

Figure 4: TE10 v TE9/11 ELISA data displayed as fold change over background, selected wells for 3R specific marked in red in the upper left of the graph and for 4R specific marked in green in the lower right.

Figure 5: 0N3R v 0N4R ELISA data displayed as fold change over background, selected wells for 3R specific marked in red in the upper left and for 4R specific marked in green in the lower right

Figure 6: Neat TAP IgG expression products binding ELISA, one representative TAP expression per foci group. Values are expressed as fold change over background binding. 3R and 4R specific antibodies selected for cloning are highlighted in red in the upper left and green in the lower right respectively. Black spot represents a well containing an antibody not selected for cloning.

Figure 7: A ELISA optical density (OD) at 630nm for the cloned transients of each of the rabbit isoform-specific antibodies. This represents the signal at 10µg/ml for each of the clones. 4R-selective antibodies are shown in green whilst the 3R-selective antibodies are

highlighted in red. Clone 2 is not shown as it repeatedly failed to express. Circled in red and green respectively are clone 14 (VR7081) and clone 3 (VR7082) that were selected as the preferred isoform-specific antibodies for further study. **B** Heavy and light chain CDR3 sequencing of the cloned rabbit antibodies. Highlighted in green and red boxes respectively are the CDR3s of the antibodies selected to be 3R- and 4R-specific via ELSA.

Figure 8: Full titration of VR7081 (**A**) and VR7082 (**B**) against all tau isoforms directly coated onto ELISA plates. VR7081 is selective for the tau 3 isoforms and VR7082 for the tau 4 isoforms.

Figure 9: Flow cytometry assay, VR7081 (**A**) and VR7082 (**B**) titrated against intracellular expressed tau isoforms.

Figure 10: Western blot from single isoform overexpressing cell lysates probed with VR7081 (**A**) or VR7082 (**B**).

Figure 11: Western blot against 2N3R or 2N4R tau containing lysates blotted with non-tau reactive rabbit (**A**) or mouse (**A**) IgG.

Figure 12: Western blot from single isoform overexpressing cell lysates probed with VR7081 (**A**) or VR7082 (**B**) formatted as Mouse IgGs.

Figure 13: Immunofluorescent staining of CHOK1 cells expressing 0N3R or 0N4R tau co-stained with VR7081(AF647) and polyclonal anti-total tau antibody (AF488).

Figure 14: Immunofluorescent staining of CHOK1 cells expressing 0N3R or 0N4R tau co-stained with VR7082(AF647) and polyclonal anti-total tau antibody (AF488).

Figure 15: Immunofluorescent staining of iPSC derived neurons with VR7081.

Immunofluorescent staining of non-mutant (control) iPSC derived neurons and monoallelic 10+16 *MAPT* mutant neurons stained with VR7081 (3R-tau) AF647. In the merged image DAPI is shown in blue whilst tau staining is shown in red. Green arrows denote examples of axonal staining with VR7081

Figure 16: Immunofluorescent staining of iPSC derived neurons with VR7082

Immunofluorescent staining of non-mutant (control) iPSC derived neurons and monoallelic 10+16 *MAPT* mutant neurons stained with VR7082 (4R-tau) AF647. In the

merged image DAPI is shown in blue whilst tau staining is shown in red. Green arrows denote examples of axonal staining with VR7082

Figure 17: Simple Western immunoblots probed with either VR7081 (A) or VR7082 (B).

5 **Figure 18:** Western blot on human brain lysate and recombinant tau ladder probed with VR7081 (A) and VR7082 (B).

Figure 19: scFv mouse Fc conversion of VR7082 binding profile confirmation. Rabbit IgG (A) and scFv-Ms-Fc (B) format diagrams. VR7082 Rabbit IgG ELISA (C) flow cytometry (E) binding assays, and Western blot (G). VR7082 scFv-Ms-Fc ELISA (D),
10 Flow cytometry (F) binding assays and Western blot (H).

Figure 20: VR7082 scFv-GFP intrabody testing. A Flow cytometry fluorescence for VR7082-scFv-GFP transfected cells and mock transfected cells. B Western blot from four replicate transfections with VR7082-scFv-GFP. C Western blot of lysate pull down from four replicate VR7082-scFv-GFP transfected cells, pulled down with either TE10 or control peptides. D Western blot band densitometry from VR7082-scFv-GFP pull downs.
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Figure 21: Degradation screen with potential VR7082 degrabody constructs. A Representative Western blot for total tau and GAPDH following co-transfection of 0N4R tau with degrabody or control intrabody fusions. B Western blot band densitometry of N = 3 Western blots.

20 **Figure 22:** Flow cytometry assay of degradation with VR7082-degrabody. A Representative flow cytometry gating and histogram plots demonstrating levels of tau staining for a GFP control, non-degrading intrabody (A-1), a degrabody (A-2) and a degrabody in the presence of MG132 (A-3). B 0N4R and 0N3R tau staining, expressed as a percentage of the GFP, non-degrading, intrabody control for all degrabody
25 constructs in the presence and absence of MG132.

Figure 23: VR7082-XIAP degradation test in iPSC derived neurons. A Gating strategy for GFP based sorting A-1 Identification of live cells via ToPro-3 exclusion. A-2 Identification of cells from debris/cellular vesicles via FSC-A v SSC-A. A-3 GFP positive and GFP negative sort gates. A-4 Overlay plot of sort gating with VR7082-XIAP IRES GFP AAV treated cells (Green) and un-treated cells (Red). B GFP negative sorted cells Peggy Sue
30

Simple Western revealed with polyclonal anti-tau antibody. **C** GFP positive sorted cells
 Peggy Sue Simple Western revealed with polyclonal anti-tau antibody.

Figure 24: VR7082-XIAP degrabody mitochondrial membrane polarisation assay in
 iPSC derived neurons. **A** Representative gating strategy for iPSC derived neurone
 5 mitochondrial polarisation assay. **A-1** identification of nucleated cells via DAPI staining.
A-2 identification of intact cells via FSC-A v SSC-A. **A-3** Identification of neurons via
 β III tubulin staining. **A-4** Mitochondrial membrane polarisation assessment. **B**
 Representative mitochondrial membrane polarisation histograms derived from the ratio
 of mitochondrial polarisation/ total mitochondrial load. **B-1** WT cell iPSC derived
 10 neurons. **B-2** 10+16 monoallelic 10+16 MAPT mutant iPSC derived neurons (10+16
 mono). **B-3** A12 biallelic MAPT mutant iPSC derived neurons (10+16 biallelic) **C**
 Mitochondrial polarisation assay across each of the 3-iPSC derived neuronal lines and
 AAV treatment groups. ANOVA used to determine statistical significance between
 groups. **D** Normalised 3R tau staining across each of the 3-iPSC derived neuronal lines
 15 and AAV treatment groups. ANOVA used to determine statistical significance between
 groups.

Figure 25: Comparison of structures of the different tau isoforms. Shows the exons
 sequences present in different isoforms of Tau protein.

Figure 26: VR7082 antibody epitope analysis. The graph shows the relative binding of
 20 VR7082 antibody to various Tau mutants compared to binding to ON4R tau expressed as
 a percentage. The results demonstrate that the binding of VR7082 to tau is reliant on each
 of K294; D295; N296; and I297. When any of those residues are independently mutated
 to Alanine there is a complete ablation of antibody binding to ON4R tau. The results also
 show that binding is partially reliant on K298 and V300 as binding is decreased by
 25 around 50% upon mutation of those positions to alanine. The results show that H299,
 P301 and G302 are not involved in the binding of VR7082 to tau as when they are
 independently mutated to alanine there is no difference between the binding levels
 observed between the alanine mutants and ON4R tau. Finally, the results show that
 VR7082 is capable of binding both P301S and P301L forms of tau protein.

30

Detailed Description of the Invention

Tau proteins

5 The antibodies and antigen-binding fragments thereof provided by the present invention bind tau proteins. In one particularly preferred embodiment, the tau proteins referred to herein are human tau proteins. The convention of amino acid numbering based on the largest human isoform of tau protein, namely the 2N4R isoform, is adopted herein. The amino acid sequence of the 2N4R isoform is provided as SEQ ID No: 35. As
10 further discussed herein, the antibodies and fragments provided are specific for either 4R tau protein isoforms or 3R tau protein isoforms.

Antibodies specific for particular Tau proteins

 The present invention provides antibodies and antigen-binding fragments thereof that are specific for either 4R tau protein isoforms or are specific for 3R tau protein
15 isoforms. As discussed further below, typically the antibodies and antigen-binding fragments thereof that are specific for the 4R tau protein isoforms bind to a region of 4R tau protein isoforms that comprises at least part of the region encoded by exon 10 of the *tau* gene (the MAPT gene) as that region is unique to 4R tau protein isoforms. Conversely, typically antibodies or antigen-binding fragments of the present invention
20 that are specific for 3R tau protein isoforms recognise a portion of the protein that includes that bridging sequence encoded by the junction of exons 9 and 11 of the *tau* gene.

 In one embodiment, a particular advantage of the present invention is that the antibody or antigen-binding fragment thereof binds the 4R or 3R tau protein isoforms in
25 a physiological state, for example where the protein is not in denatured form. For instance, an antibody or an antigen-binding fragment thereof of the present invention may specifically bind 4R or 3R tau protein isoforms in intact cells, for example as determined by immunofluorescence. In one embodiment, it is able to detect the protein in flow cytometry analysis of cells. In another embodiment, it may be used to detect the
30 protein in a tissue sample, for example by immunofluorescence. In another, it may be able to detect the protein via immunohistochemistry. In a preferred embodiment, it may be possible to detect the tau protein via Western blot and in particular by Peggy Sue Simple Western.

In one embodiment, “specifically binds” as used herein means that the antibody or antigen-binding fragment thereof binds at least 10 times more strongly to whichever of 4R and 3R tau isoforms it is specific for. For example, it may bind more than 50, 100, 200, or 500 times more strongly to 4R tau than 3R tau protein isoforms. In another
5 embodiment, it may bind more than 50, 100, 200, or 500 times more strongly to 3R tau than 4R tau protein isoforms. In one embodiment, the binding is at least 1000 times stronger for whichever of 4R and 3R tau protein isoforms it is specific for. In one embodiment, the K_D value for binding is at least 10, 50, 100, 500 or 1000 times less for whichever of the 3R or 4R tau protein it is specific for. In another embodiment, the
10 binding may be 5,000, 10,000, or 50,000 times stronger for the form of tau protein for which it is specific. In one embodiment, the antibody or antigen-binding fragment thereof of the present invention does not bind the other form of tau protein at all or does not significantly do so. In another embodiment, the level of binding to non-tau proteins is at least as low as the level of binding to the form of tau for which the antibody is not
15 specific. In one embodiment, the antibody or antigen-binding fragment thereof does not bind non-tau proteins or does not significantly do so. Levels of binding may be measured by techniques well known in the art such as surface plasmon resonance or any of the other relevant techniques disclosed herein. In one embodiment, the level of specificities set out above are in relation to specificity for 4R tau protein isoforms over 3R tau protein
20 isoforms. In another embodiment, the level of specificities set out above are in relation to specificity for 3R tau protein isoforms over 4R tau protein isoforms.

As shown in Figure 25, 4R tau protein isoforms represent a group that all have the amino acid sequence encoded by exon 10 of the *tau* gene, whereas 3R tau protein
isoforms represent a group of isoforms that have the common feature of all lacking the
25 amino acid sequence encoded by exon 10. Those features denote how tau proteins are grouped into 4R and 3R tau protein isoforms, but within each of those designations there are isoforms that differ depending on whether or not the amino acid sequence encoded by exons 2 and 3 are present or absent. So, for instance, 4R tau protein isoforms are: 2N4R
which has the sequence encoded by exons 2 and 3; 1N4R which has the sequence
30 encoded by exon 2, but not that encoded by exon 3; and 0N4R which has neither the sequence encoded by exon 2 nor that encoded by exon 3. All three of 2N4R, 1N4R, and 0N4R though include the sequence encoded by exon 10 and so are designated 4R tau proteins. Hence, where reference is made herein to an antibody or antigen-binding
fragment thereof that specifically binds 4R tau protein isoforms it typically means that

the antibody or fragment binds all three of 2N4R, 1N4R, and 0N4R, but not any of the 3R tau protein isoforms 2N3R, 1N3R and 0N3R. In embodiments where the antibody or antigen-binding fragment is one specifically binding 3R tau protein isoforms, the converse will be the case and so the antibody will bind 2N3R, 1N3R and 0N3R, but not 2N4R, 1N4R, and 0N4R.

In one embodiment, the affinity of the antibody or antigen-binding fragment thereof for whichever of 4R or 3R tau protein isoforms that is specific is about 100 nM or stronger, such as about 50 nM, 20 nM, 10 nM, 1 nM, 500 pM, 250 pM, 200 pM, 100 pM or stronger. In one embodiment, the binding affinity is 50 pM or stronger. In one embodiment, the affinity of the antibody for whichever of 4R or 3R tau protein it is specific may be less than 1 μ M, less than 750 nM, less than 500 nM, less than 250 nM, less than 200 nM, less than 150 nM, less than 100 nM, less than 75 nM, less than 50 nM, less than 10 nM, less than 1 nM, less than 0.1 nM, less than 10 pM, less than 1 pM, or less than 0.1 pM. In some embodiments, the K_d is from about 0.1 pM to about 1 μ M.

Tau proteins include repeats with high levels of sequence identity in the MTBR region. In the case of 4R, four such repeats are present and, in the case of 3R, three such repeats are present. In one embodiment, an antibody or antigen-binding fragment thereof of the present invention binds only the repeat containing its epitope and not the other repeats. For example, in one embodiment, an antibody or antigen-binding fragment of the present invention binds to a repeat comprising the junction between exons 9 and 11, but does not bind to the other repeats and so is specific for 3R tau. In another embodiment, an antibody or antibody fragment of the present invention binds to a sequence within the repeat present in the sequence encoded by exon 10 of the *tau* gene, but not to the sequence of the other three repeats.

Tau 4R specific antibodies

In one particularly preferred embodiment, an antibody or antigen-binding fragment thereof of the present invention is specific for tau 4R protein isoforms. In one embodiment, an antibody or antigen-binding fragment thereof specifically binds tau 4R protein isoforms and not 3R tau protein isoforms. For instance, it may bind all isoforms of tau 4R protein for a given species or subject, but none of the isoforms of tau 3R protein for that species or subject. Hence, in one embodiment it may bind all of the 2N4R, 1N4R and 0N4R isoforms of tau 4R protein, but not bind any of the 2N3R, 1N3R and 0N3R isoforms of tau3R protein. In one embodiment, an antibody or antigen-binding

fragment thereof specifically binds tau 4R protein isoforms from human, rat, and mouse, but does not bind 3R tau protein isoforms from those species.

The common difference between 4R tau protein isoforms and those of 3R tau protein isoforms is that the latter lack the amino acid sequence encoded by exon 10 of the *tau* gene. In one embodiment, an antibody or antigen-binding fragment thereof that is specific for 4R tau protein isoforms therefore binds to a region that is unique to 4R tau protein isoforms and hence to that encoded by exon 10. In one preferred embodiment, the antibody or antigen-binding fragment thereof specific for 4R tau protein isoforms binds to an epitope that is wholly or partially in the sequence encoded by exon 10 of the *tau* gene. In one preferred embodiment, an antibody or antigen-binding fragment of the present invention binds to a region within the amino acid sequence encoded by exon 10 of the *tau* gene and in particular does not bind 3R tau protein isoforms.

In one embodiment, an antibody or antigen-binding fragment thereof of the present invention which is specific for 4R tau protein isoforms is able to bind a peptide that comprises at least part of the amino acid sequence encoded by exon 10 of the *tau* gene. In one embodiment, the peptide sequence is entirely within the region encoded by exon 10 of the *tau* gene. In one embodiment, the peptide is less than 15 amino acids in length. In one embodiment, it is less than 14, 13, 12, 11, or 10 amino acids in length. In one embodiment, the antibody or antigen-binding fragment thereof binds a peptide sequence which is nine amino acids in length. In one preferred embodiment, it is able to bind a peptide which consists of amino acids 294 to 302 of the 4R Tau protein isoforms. In one embodiment, the antibody or antigen-binding fragment thereof is able to bind such a peptide when the peptide is in linear form. In one embodiment, an antibody or antigen-binding fragment thereof of the present invention is able to bind a peptide corresponding to an amino acid sequence encoded by exon 10 of the *tau* gene which is of a length that only has one epitope present. In one embodiment, an antibody or antigen-binding fragment thereof is able to bind such peptides when conjugated to a carrier, for example KLH, ovalbumin, or BSA, particularly where the peptide is in linear form.

In one preferred embodiment, an antibody or antigen-binding fragment thereof is able to specifically bind 4R tau protein isoforms irrespective of whether or not the protein has any post translational modifications at one or more of amino acid positions 279, 280, 281, 285, and 289. In one embodiment, it is able to specifically bind tau 4R protein isoforms irrespective of whether or not the protein is modified at any, or all, of amino acid positions 279, 280, 281, 285 and 289. Frequent post translational

modifications for these positions include glycosylation, phosphorylation, and acetylation. The most frequent posttranslational modification at position 279 is glycosylation, the most frequent post translational modification at positions 281 and 289 is acetylation, and the most frequent post translational modification at positions 285 and 289 is

5 phosphorylation. In one embodiment, an antibody or an antigen-binding fragment thereof of the present invention is able bind tau 4R protein isoforms irrespective of whether there is any glycosylation, acetylation, or phosphorylation at those positions. In one embodiment, an antibody or an antigen-binding fragment thereof of the present invention is able bind tau 4R protein isoforms irrespective of whether or not the protein is
10 glycosylated at position 279, acetylated at position 281, acetylated at position 289, phosphorylated at position 285, and/or phosphorylated at position 289. In other words, the antibody binds tau 4R protein both when glycosylated, acetylated, and/or phosphorylated at these positions and when not glycosylated, acetylated, and/or phosphorylated at these positions.

15 In one embodiment, an antibody or antigen-binding fragment specific for the 4R tau isoforms binds to repeat 2 within the 4R tau protein sequence, but not to the other three repeats present in the 4R tau protein isoforms. Each repeat corresponds to a different tau exon; repeat 1 (exon 9) (amino acids 242-273); repeat 2 (exon 10) (amino acids 274-304); repeat 3 (exon 11) (amino acids 305-335); repeat 4 (exon 12) (336-367).
20 Hence, in one embodiment, an antibody may bind to the sequence encoded by exon 10, but not by the sequences encoded by exons 9, 11, and 12 of the 4R tau protein isoforms.

In one embodiment, the antibody or antigen-binding fragment thereof specific for 4R tau proteins:

- (a) binds to the amino acid region encoded by exon 10 of tau when the tau 4R
25 protein contains a post translational modification at one or more of amino acid positions 279, 280, 281, 285, and 289 of tau 4R;
- (b) binds to a peptide comprising, or consisting of, amino acids 294 to 302 of tau 4R protein; and/or
- (c) binds to a peptide corresponding to a single epitope from the amino acid
30 sequence encoded by exon 10 of tau; or
- (d) cross-blocks or is cross-blocked by any of the antibodies or fragments of (a) to (c).

In one embodiment, an antibody or antigen-binding fragment of the present invention:

- (a) comprises one or more of the light chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs 3, 5, and 7 and one or more of the heavy chain CDR1, CDR2, and CDR 3 sequences of SEQ ID NOs: 11, 13, and 15, preferably comprising the light chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs 3, 5, and 7 and heavy chain CDR1, CDR2, and CDR 3 sequences of SEQ ID NOs: 11, 13, and 15; or
- (b) comprises the light and heavy chain variable regions of SEQ ID NOs 1 and 9;
- (c) is an intrabody, preferably comprising the sequence of SEQ ID No: 17 or a sequence with at least 95% sequence identity thereto that is still able to specifically bind tau protein 4R isoforms; or
- (d) cross-blocks or is cross-blocked by any of the antibodies or fragments of (a) to (c).

In one embodiment, an antibody or antigen-binding fragment of the present invention comprises a light chain CDR1, CDR2, and CDR3 corresponding to the sequences of SEQ ID NOs. 3, 5, and 7, or CDRs where each CDR has no more than two amino acid sequence changes compared to the sequence of SEQ ID NOs. 3, 5, and 7 and the antibody is still able to specifically bind 4R tau protein isoforms. In one embodiment, the sequence changes are conservative amino acid sequence changes. In another embodiment, an antibody or antigen-binding fragment of the present invention comprises heavy chain CDR1, CDR2, and CDR3 sequences corresponding to the sequences of SEQ ID NOs. 11, 13, and 15, or CDRs where each CDR has no more than two amino acid sequence changes compared to the sequence of SEQ ID NOs. 11, 13, and 15 and the antibody is still able to specifically bind 4R tau protein isoforms. In one embodiment, the antibody or antigen-binding fragment thereof comprises both such light and heavy chain CDRs and, in a preferred embodiment, comprises the light chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs. 3, 5, and 7 and the heavy chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs. 11, 13, and 15. In one embodiment, the light chain of the antibody may comprise one or more of the framework regions of the FW1, FW2, FW3, and FW4 of SEQ ID NOs 2, 4, 6, and 8. In one embodiment it may comprise all four of the FW1, FW2, FW3, and FW4 of SEQ ID NOs 2, 4, 6, and 8. In another embodiment, the heavy chain of the antibody may comprise one or more of the framework regions of the FW1, FW2, FW3, and FW4 of SEQ ID NOs 10, 12, 14, and 16. In one embodiment, it may comprise all four of those framework regions. In another embodiment, it may comprise both such heavy and light chain framework regions. In one

embodiment, an antibody or antigen-binding fragment thereof comprising the above sequences is humanized, for example so that all of the sequences of the antibody apart from the CDR sequences are human sequences, with the antibody or antigen-binding fragment thereof still specifically binds 4R tau protein isoforms. In another embodiment, all of the sequences of the antibody may be human apart from the variable regions.

In one embodiment, an antibody or antigen-binding fragment thereof comprises a light chain variable region of SEQ ID NO: 1 or a light chain variable region with at least 90% sequence identity thereto, where the antibody or antigen-binding fragment is still able to specifically bind 4R tau protein isoforms. In one embodiment, the light chain variable region has a least 95%, 98% or 99% sequence identity or has 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid sequence changes compared to SEQ ID NO: 1, whilst still being able to specifically bind 4R tau protein isoforms. In one embodiment, an antibody or antigen-binding fragment thereof comprises a heavy chain variable region of SEQ ID NO: 9 or a heavy chain variable region with at least 90% sequence identity thereto, where the antibody or antigen-binding fragment is still able to specifically bind 4R tau protein isoforms. In one embodiment, the light chain variable region has a least 95%, 98% or 99% sequence identity or has 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid sequence changes compared to SEQ ID NO: 9 and still specifically binds 4R tau isoforms. In one embodiment the sequence changes compared to the heavy and light chain sequences are solely in the framework regions of the variable regions. In one embodiment, an antibody or antigen-binding fragment thereof comprises both a light chain and heavy chain variable region as set out above. In one embodiment, an antibody or antigen-binding fragment of the present invention comprises any of the above light and/or heavy chain variable region sequences, but where they have been humanised so that all of the sequences apart from the CDR sequences are human.

As discussed further below, an antibody or antigen-binding fragment of the present invention may be provided in a variety of antibody and antibody fragment formats, and those which are specific for 4R tau protein isoforms may be provided in any of the antibody formats set out herein. They may also have any of the levels of sequence variation set out herein and any of the properties set out herein.

Tau 3R specific antibodies

In a further preferred embodiment, an antibody or antigen-binding fragment thereof of the present invention is specific for 3R tau protein isoforms. In one

embodiment an antibody or antigen-binding fragment thereof specifically binds 3R tau protein isoforms and not 4R tau protein isoforms. For instance, it may bind all isoforms of 3R tau protein for a given species or subject, but none of the isoforms of 4R tau protein for that species or subject. Hence, in one embodiment it may bind all of the
5 2N3R, 1N3R and 0N3R isoforms of 3R tau protein, but not bind any of the 2N4R, 1N4R and 0N4R isoforms of 4R tau protein.

The common difference between 3R tau proteins and those of 4R tau is that the former lack the amino acid sequence encoded by exon 10 of the *tau* gene. In one embodiment, an antibody or antigen-binding fragment thereof that is specific for 3R tau
10 protein isoforms therefore binds to a region that is unique to 3R tau protein isoforms and in particular to a region that includes the amino acid sequence corresponding to the junction between exons 9 and 11 of the *tau* gene. In one preferred embodiment, an antibody or antigen-binding fragment of the present invention binds to an epitope that comprises said junction between exons 9 and 11.

15 In one embodiment, an antibody or antigen-binding fragment thereof of the present invention which is specific for 3R tau protein isoforms is able to bind a peptide that comprises the sequence encoded by the junction between exons 9 and 11. In one embodiment, the peptide is less than 15 amino acids in length. In one embodiment, it is 14 amino acids in length. In one preferred embodiment, it is able to bind a peptide which
20 consists of 14 amino acids, with seven amino acids from each side of the junction between exons 9 and 11. In one embodiment, an antibody or antigen-binding fragment thereof of the present invention which is specific for 3R tau protein isoforms is able to bind a protein comprising amino acids 268 to 274 and 306 to 312 of SEQ ID NO: 35, but not amino acids 275 to 305 of SEQ ID NO:35. In one embodiment, an antibody or
25 antigen-binding fragment thereof of the present invention which is specific for 3R tau protein isoforms is able to bind a peptide that comprises the amino acid sequence of SEQ ID No: 36. In one embodiment, the antibody or antigen-binding fragment thereof is able to bind such a peptide when the peptide is in cyclic form and in particular when conjugated to a carrier, such as KLH, ovalbumin, or BSA.

30 In one embodiment, the antibody or antigen-binding fragment thereof specific for 3R tau protein isoforms:

(a) comprises one or more of the light chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs 20, 22, and 24 and one or more of the heavy chain CDR1, CDR2, and CDR 3 sequences of SEQ ID NOs: 28, 30, and 32, and preferably comprises

the light chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs 20, 22, and 24 and heavy chain CDR1, CDR2, and CDR 3 sequences of SEQ ID NOs: 28, 30, and 32; and/or

(b) comprises the light and heavy chain variable regions of SEQ ID NOs 18 and 26;

5 (c) is an intrabody, preferably comprising the sequence of SEQ ID No: 34 or a sequence with at least 95% sequence identity thereto that is still able to specifically bind tau protein 3R isoforms;

(d) cross-blocks or is cross-blocked by any of the antibodies or fragments of (a) to (c).

10 In one embodiment, an antibody or antigen-binding fragment of the present invention comprises a light chain CDR1, CDR2, and CDR3 corresponding to the sequences of SEQ ID NOs. 20, 22, and 24, or CDRs where each CDR has no more than two amino acid sequence changes compared to the sequence of SEQ ID NOs. 20, 22, and 24 and the antibody is still able to specifically bind 3R tau protein isoforms. In one
15 embodiment, the sequence changes are conservative amino acid sequence changes. In another embodiment, an antibody or antigen-binding fragment of the present invention comprises a heavy chain CDR1, CDR2, and CDR3 corresponding to the sequences of SEQ ID NOs. 28, 30, and 32, or CDRs where each CDR has no more than two amino acid sequence changes compared to the sequence of SEQ ID NOs. 28, 30, and 32 and the
20 antibody is still able to specifically bind 3R tau protein isoforms. In one embodiment, the antibody or antigen-binding fragment thereof comprises both such light and heavy chain CDRs and, in a preferred embodiment, comprises the light chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs. 20, 22, and 24 and the heavy chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs. 28, 30, and 32. In one embodiment, the light chain of
25 the antibody may comprise one or more of the framework regions of the FW1, FW2, FW3, and FW4 of SEQ ID NOs 19, 21, 23, and 25. In one embodiment it may comprise all four of the FW1, FW2, FW3, and FW4 of SEQ ID NOs 19, 21, 23, and 25. In another embodiment, the heavy chain of the antibody may comprise one or more of the framework regions of the FW1, FW2, FW3, and FW4 of SEQ ID NOs 27, 29, 31, and 33.
30 In one embodiment, it may comprise all four of those framework regions. In another embodiment, it may comprise both such heavy and light chain variable regions. In one embodiment, an antibody or antigen-binding fragment thereof comprising the above sequences is humanized, for example so that all of the sequences of the antibody apart

from the CDR sequences are human sequences, with the antibody or antigen-binding fragment thereof still being able to specifically bind 3R tau protein isoforms.

In one embodiment, an antibody or antigen-binding fragment thereof comprises a light chain variable region of SEQ ID NO: 18 or a light chain variable region with at least 90% sequence identity to SEQ ID No: 18, where the antibody or antigen-binding fragment is still able to specifically bind 3R tau protein isoforms. In one embodiment, the light chain variable region has a least 95%, 98% or 99% sequence identity or has 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid sequence changes compared to SEQ ID NO: 18, whilst still be able to specifically bind 3R tau protein isoforms. In one embodiment, an antibody or antigen-binding fragment thereof comprises a heavy chain variable region of SEQ ID NO: 26 or a heavy chain variable region with at least 90% sequence identity thereto, where the antibody or antigen-binding fragment is still able to specifically bind 3R tau protein isoforms. In one embodiment, the light chain variable region has a least 95%, 98% or 99% sequence identity or has 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid sequence changes compared to SEQ ID NO: 18 or 26 and is still able to specifically bind 3R tau protein isoforms. In one embodiment the sequence changes compared to the heavy and light chain sequences are solely in the framework regions of the variable regions. In one embodiment, an antibody or antigen-binding fragment thereof comprises both a light chain and heavy chain variable region as set out above. In one embodiment, an antibody or antigen-binding fragment of the present invention comprises any of the above light and/or heavy chain variable region sequences, but where they have been humanised so that all of the sequences apart from the CDR sequences are human.

As discussed further below, an antibody or antigen-binding fragment of the present invention may be provided in a variety of antibody and antibody fragment formats and those which are specific for 3R tau protein isoforms may be provided in any of the antibody formats set out herein. They may also have any of the levels of sequence variation set out herein.

Epitopes

In one embodiment, an antibody or antigen-binding fragment thereof of the present invention binds to the same epitope, or at least substantially the same epitope, as one of the antibodies or antigen-binding fragments disclosed herein. The specific region or epitope of Tau can be identified by any suitable epitope mapping method known in the

art in combination with any one of the antibodies provided by the present disclosure. Examples of such methods include screening peptides of varying lengths derived from tau protein for binding to the Tau-binding antibodies or binding fragments thereof of the present disclosure with the smallest fragment that can specifically bind to the antibody
5 containing the sequence of the epitope recognized by the Tau-binding antibodies or binding fragments thereof. In one particularly preferred embodiment, residues forming part of the epitope of the antibody may be identified using mutational analysis, for instance by swapping residues of the tau protein for alanine and determining the impact on binding of the antibody or antigen-binding fragment thereof to tau protein, particularly
10 4R tau protein.

In one embodiment at least one of K294, D295, N296, I297, K298, and V300 may be present in the epitope of the antibody, in particular where those amino acid positions are defined relative to that of SEQ ID NO: 35.

In one particularly preferred embodiment, an antibody or antigen binding
15 fragment is provided that binds to an epitope comprising at least one of the amino acid residues K294, D295, N296, and I297. Preferably, the antibody or antigen-binding antibody is provided that binds to an epitope that comprises at least two of the amino acid residues K294, D295, N296, and I297. More preferably, the antibody or antigen-binding fragment therefore binds to an epitope that comprises at least three of the amino acid
20 residues K294, D295, N296, and I297. In an even more preferred embodiment, the antibody or antigen-binding fragment thereof will bind to an epitope that comprises all four of the amino acid residues K294, D295, N296, and I297.

In a further preferred embodiment, the antibody or antigen-binding fragment thereof may bind to such an epitope that further comprises amino acid residues K298 and
25 V300 of tau protein. In one preferred embodiment, the antibody or antigen binding fragment thereof binds to an epitope comprising all of residues K294, D295, N296, I297, K298, and V300 of tau protein.

In any of the above embodiments, the antibody or antigen-binding fragment thereof may be preferably a 4R specific antibody. Hence, in one embodiment, the
30 antibody or antigen-binding fragment thereof is a 4R specific antibody that binds an epitope comprising K294, D295, N296, and I297 of 4R tau protein. In another preferred

embodiment, the antibody or antigen-binding fragment thereof is a 4R specific antibody that binds an epitope comprising K294, D296, N396, I297, K298 and V300 of 4R tau protein.

5 In one embodiment, the amino acids H299, P301, and G302 are not involved in the binding of the antibody or antigen-binding fragment thereof provided to tau protein. In one embodiment, the antibody or antigen binding fragment thereof is a 4R specific antibody that binds an epitope comprising K294, D296, N396, I297, K298 and V300 of 4R tau protein, but the H299, P301, and G302 residues are not involved in the binding of the antibody or antigen-binding fragment thereof to the tau protein.

10 In one embodiment, the overall epitope of the antibody or the antigen binding fragment thereof comprises amino acids in the region of residues K294 to V300 of tau 4R protein. In one embodiment, amino acids for the epitope of the antibody or antigen-binding fragment thereof are only in that region. In another embodiment, residues forming the epitope are at least in that region.

15 Any of the antibodies or antigen binding fragments thereof mentioned can bind such epitopes. In one embodiments such antibodies may be chimeric, humanized or fully human monoclonal antibodies or can be used to obtain chimeric, humanized or fully human monoclonal antibodies. In one particularly preferred embodiment, the antibody or antigen-binding fragment thereof binding the epitope is a degrabody.

20 *Antibody formats*

An antibody or antigen-binding fragment of the present invention may be provided in any suitable format. In one preferred embodiment, an antibody or antigen-binding fragment thereof of the present invention may be an IgG class antibody or fragment thereof. In one embodiment, it may be an IgG1, IgG2, IgG3, or IgG4 isotype antibody and in particular IgG1. In another embodiment, an antibody of antigen-binding fragment thereof of the invention may also be an IgA, IgE, IgD, or IgM class antibody.

The present disclosure refers to antibodies and antibody fragments. Wherever mention is made of an antibody an antibody fragment may be employed unless specifically stated otherwise. Hence, antibodies of the present invention may comprise a complete antibody having full length heavy and light chains or be an antigen-binding fragment, for instance, a Fab, modified Fab, Fab', modified Fab', F(ab')₂, Fv, single domain antibody (e.g. VH or VL or VHH), scFv, bi, tri or tetra-valent antibody, Bis-

scFv, diabody, triabody, tetrabody or epitope-binding fragments of any of the above (see for example Holliger and Hudson, 2005, Nature Biotech. 23(9):1126-1136; Adair and Lawson, 2005, Drug Design Reviews - Online 2(3), 209-217). The methods for creating and manufacturing antibody fragments are well known in the art (see for example Verma et al., 1998, Journal of Immunological Methods, 216, 165-181). Other antibody fragments for use in the present invention include the Fab and Fab' fragments described in International patent applications WO2005/003169, WO2005/003170 and WO2005/003171. Multi-valent antibodies may comprise multiple specificities, e.g. bispecific or may be monospecific (see for example WO 92/22853, WO05/113605, WO2009/040562 and WO2010/035012). Examples of possible antibody formats are known in the art, for example as disclosed in the review "The coming of Age of Engineered Multivalent Antibodies", Nunez-Prado *et al* Drug Discovery Today Vol 20 Number 5 Mar 2015, page 588-594, D. Holmes, Nature Rev Drug Disc Nov 2011:10; 798, Chan and Carter, Nature Reviews Immunology vol. 10, May 2010, 301 incorporated herein by reference.

In one embodiment, antibody formats include those known in the art and those described herein, such as wherein the antibody molecule format is, or comprises, one of those selected from the group comprising or consisting of: diabody, BYbe, scdiabody, triabody, tribody, tetrabodies, TrYbe, tandem scFv, FabFv, Fab'Fv, FabdsFv, Fab-scFv, Fab-dsscFv, Fab-(dsscFv)₂, diFab, diFab', tandem scFv-Fc, scFv-Fc-scFv, scdiabody-Fc, scdiabody-CH₃, Ig-scFv, scFv-Ig, V-Ig, Ig-V, Duobody and DVDIg.

In one particularly preferred embodiment an antibody of the invention is a scFv-Ms-Fc format antibody.

In one embodiment, an antibody or antigen-binding fragment thereof of the invention is small enough to pass through the blood brain barrier (BBB).

In one embodiment, an antibody or an antigen-binding fragment thereof is a bispecific or multi-specific antibody where at least one of the antigen-binding sites is specific for 4R tau protein isoforms or 3R tau protein isoforms. Hence, in one preferred embodiment, it is such a bispecific antibody.

In a particularly preferred embodiment, an antibody or antigen-binding fragment thereof is one that can be expressed inside a cell and in particular is an intrabody. Intrabodies are antibodies, or antibody fragments, that are capable of expression, correct folding and antigen binding intracellularly. Intrabodies are typically capable of folding correctly in the reducing environment of the cytoplasm typically due to a lack of inter-

and intra-chain disulphide bonds. Intrabodies are an alternative format for therapeutic antibodies of the present invention as in some instances they may be easier to target to cells than administration of the antibody itself.

In one embodiment, an antibody of the present invention is a targeted intrabody.
5 For example, in one embodiment ER-intrabodies are targeted to the ER lumen using a KDEL or SEKDEL sequence on their C-terminus (as set out in Wheeler, Chen, and Sane 2003 *Mol Ther*, 8: 355-66; Lewis and Pelham 1992 *Cell*, 68: 353-64, both of which are incorporated by reference in their entirety). In one embodiment, an intrabody of the present invention is targeted to the cytoplasm (such a specific class of intrabody may be
10 referred to as a cyto-intrabody) for example by the removal of a leader sequence. The intrabodies employed in the Examples of the present application are expressed in the cytoplasm of the cell. In another embodiment, an intrabody of the present invention is one targeted to the mitochondria or nucleus (for instance as set out in Biocca, Neuberger, and Cattaneo 1990 *EMBO J*, 9: 101-8, which is incorporated by reference in its entirety)
15 with the addition of a suitable targeting signal.

IgG-derived intrabodies may be produced using the variable region domains of IgGs linked via a Gly₄Ser peptide linker to make a scFv fragment (Bird et al. 1988 *Science*, 242: 423-6 which is incorporated by reference). These can then be directly used as intrabodies as they no longer require disulphide bond formation for correct folding. In
20 one embodiment, an intrabody of the present invention is a single domain antibody intrabody. For example, in one embodiment, an intrabody of the present invention is a heavy chain only antibody, for example *Camelidae* heavy chain only antibodies (HCabs) (Hamers-Casterman et al. 1993, *Nature*, 363: 446-8, which is incorporated by reference in its entirety). The single variable regions of HCabs (known as VHHs) can be used as
25 intrabodies.

In one embodiment, an antibody or antigen-binding fragment of the present invention is a non-disulphide stabilised scFv and so may be used as an intrabody. In the scFv format the heavy and light chain variable regions may be physically linked by a flexible linker. That ensures the heavy and light chains pair correctly inside the cell and
30 form a binding moiety without needing to form cysteine bridges between the heavy and light chains.

In one preferred embodiment, an antibody or antigen-binding fragment thereof provided may be one that comprises a degradation domain. Examples of possible degradation domains that may be employed include the C-terminal sequence of ornithine

decarboxylase (ODC), human FkBP-12 protein (FkBP), the C-terminus of Hsc70 interacting protein (CHIP), the X-linked inhibitor of apoptosis protein (XIAP), von Hippel-Lindau protein (VHL), and the N-terminal of Simb protein (NSImb). The degradation domain may be one that is dependent on ubiquitin proteasomes for degradation in one embodiment. In another embodiment it is not so dependent. In one embodiment, the degradation domain is present as an N-terminal fusion to the rest of the antibody, for example a C-terminal fusion to a scFv. In another embodiment, it is an N-terminal fusion, for example an N-terminal fusion to a scFv.

In one embodiment, an antibody or antigen-binding fragment of the present invention is an intracellular degrading antibody, also known as a target degrading intrabody, or degrabody. In the degrabody format the intrabody is linked to a degradation domain and so effectively a degrabody is a specific kind of intrabody. Hence, in one particularly preferred embodiment of the present invention the antibody or antigen-binding fragment thereof is a degrabody.

In one embodiment, an antibody or antigen-binding fragment of the present invention is a rabbit antibody or fragment thereof. In another embodiment, an antibody or antigen-binding fragment of the present invention is one that comprises rabbit variable regions and the rest of the sequences of the antibody are mouse sequences. In another embodiment, the CDRs of the antibody are rabbit CDRs and the rest of the antibody is mouse. In a further embodiment, the antibody is a fully human antibody. In a further embodiment, all of the sequence of the antibody apart from the CDR sequences are human sequences.

Variants

In one embodiment, rather than a specific sequence set out herein, an antibody or antibody fragment provided by the present invention may have a specific level of sequence identity or number of amino acid sequence changes compared to that specific sequence, so long as the antibody or fragment is still able to specifically bind whichever of 4R or 3R tau protein isoforms it is intended to be specific for. In another embodiment, a nucleic acid sequence may have a particular level of sequence identity compared to one of the specific sequences set out herein, provided that it still encodes an antibody or fragment thereof, or a constituent of those, which can still bind specifically to whichever of 4R or 3R tau protein isoforms it is intended to be specific for.

Degrees of identity and similarity can be readily calculated (Computational Molecular Biology, Lesk, A.M., ed., Oxford University Press, New York, 1988; Biocomputing. Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987, Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991, the BLAST™ software available from NCBI (Altschul, S.F. et al., 1990, J. Mol. Biol. 215:403-410; Gish, W. & States, D.J. 1993, Nature Genet. 3:266-272. Madden, T.L. et al., 1996, Meth. Enzymol. 266:131-141; Altschul, S.F. et al., 1997, Nucleic Acids Res. 25:3389-3402; Zhang, J. & Madden, T.L. 1997, Genome Res. 7:649-656,).

Hence, the present disclosure extends to novel polypeptide sequences disclosed herein and sequences at least 80% similar or identical thereto, for example 85% or greater, such 90% or greater, in particular 95%, 96%, 97%, 98% or 99% or greater in similarity or identity. "Identity", as used herein, indicates that, at any particular position in the aligned sequences, the amino acid residue is identical between the sequences. In one embodiment, a sequence may have one of those levels of sequence identity provided that the encoded antibody or fragment is still able to specifically bind 4R or 3R tau protein isoforms.

A particular amino acid sequence may differ from one of the specific amino acid sequences set out herein by up to 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid sequence changes, provided that it is still able to specifically bind 4R or 3R tau protein isoforms. In one embodiment, it may differ from the specific sequence by that number of sequence changes where the sequence changes are conservative ones.

As well as variant sequences defined by percentage identity or number of sequence changes, the present invention further provides an antibody or antigen-binding fragment defined by its ability to cross-block one of the specific antibodies or fragments set out herein. It may be that the antibody also has one of the recited levels of sequence identity or number of sequence changes as well. Cross-blocking antibodies can be identified using any suitable method in the art, for example by using competition ELISA or BIAcore assays where binding of the cross blocking antibody to antigen (the particular tau protein of interest, so for instance 4R or 3R, or one of the peptides from those peptides discussed herein) prevents the binding of an antibody or antibody fragment of the present invention or *vice versa*. In one embodiment, the antibody produces at least

50%, 60%, 70%, 80%, 90% or more reduction of binding of the specific antibody or antigen-binding fragment disclosed herein.

Peptides

The present invention also provides peptides that may be used to generate antibodies and antigen-binding fragments of such antibodies. In one embodiment, a peptide comprising the sequence bridging exons 9 and 10 of tau protein is provided. For example, in one embodiment, a peptide comprising, or consisting of, the sequence of SEQ ID No: 36 is provided, also known as the TE9/11 peptide. In another embodiment, the present invention provides a peptide sequence that corresponds to the amino acid sequence encoded by exon 10 of tau as that means the antibody is likely to recognise 4R tau protein isoforms specifically. In one embodiment, the peptide does not comprise an amino acid that represents a post-translational modification site in native 4R tau protein isoforms. In one embodiment, a peptide comprising, or consisting of, the amino acid sequence of SEQ ID No: 37 is provided, which is also referred to as the TE10 peptide.

In one embodiment, the peptide comprises further amino acid residues that do not originate from a tau protein, for example it may comprise a terminal cysteine to help conjugate the peptide to a carrier. In one embodiment, the peptide is conjugated to a carrier. For example, in one embodiment the carrier protein is KLH, ovalbumin, or BSA. In one embodiment, the peptide is conjugated as a linear peptide to a carrier and in particular where the portion of the peptide most likely to give rise to specific antibodies is further away from the carrier. In another embodiment, the peptide is a cyclic peptide, for example one joined to a carrier so that the amino acid residues most likely to give rise to an antibody specific for 4R or 3R tau protein isoforms are further away from the carrier.

In one embodiment, an antibody or antigen-binding fragment thereof which is obtainable by immunizing an animal with one of the above-mentioned peptides, in particular one conjugated to a carrier, is provided. In one embodiment, the antibody or antibody fragment is one obtained via such a method. In one embodiment, the immunized animal is a rabbit. Any suitable method may be used to identify the desired antibodies from such an immunized animal, for example screening via ELISA using tau peptides or proteins or any of the other methods discussed herein may be employed.

In one embodiment, an antibody or antigen-binding fragment of the present invention specific for 3R tau protein isoforms is not an antibody referred to in de Silva *et*

al (2003) *Neuropathology and Applied Neurobiology*, 29: 288-302. In one embodiment, the antibody or antigen-fragment is not the RD3 antibody disclosed in de Silva *et al*. In another embodiment, the antibody or antigen-binding fragment thereof is specific for 4R tau protein isoforms, but is not an antibody referred to in Croft *et al* (2018)

5 <https://doi.org/10.1371/journal.pone.0195211>. In one embodiment, the antibody or antigen-binding fragment thereof is specific for 4R tau protein isoforms but is not an antibody referred to in either of de Silva *et al* (2013) and Croft *et al* (2018).

Nucleic acids, vectors, and host cells

In a further aspect, there is provided a nucleotide sequence, for example a DNA
10 sequence encoding an antibody or antibody fragment of the present invention as described herein. In one embodiment, there is provided a nucleotide sequence, for example a DNA sequence encoding an antibody or fragment of the present invention as described herein. In one embodiment, the nucleotide sequence is collectively present on more than one polynucleotide but the nucleotide sequences together are able to encode an
15 antibody or antibody fragment of the present invention.

The invention herein also extends to a vector comprising a nucleotide sequence as defined above. The term “vector “ as used herein refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. An example of a vector is a “plasmid,” which is a circular double stranded DNA loop into which
20 additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the
25 genome of a host cell, where they are subsequently replicated along with the host genome. In the present specification, the terms “plasmid” and “vector” may be used interchangeably as a plasmid is the most commonly used form of vector. General methods by which the vectors may be constructed, transfection methods and culture methods are well known to those skilled in the art. In this respect, reference is made to
30 “Current Protocols in Molecular Biology”, 1999, F. M. Ausubel (ed), Wiley Interscience, New York and the Maniatis Manual produced by Cold Spring Harbor Publishing.

The term vector herein also includes, for example, particles comprising the vector, for example LNP (Lipid Nanoparticle) particles and in particular LNP-mRNA particles. It also includes viral particles used for transferring a vector of the present invention.

A vector of the present invention may include a selectable marker. The term
5 “selectable marker” as used herein refers to a protein whose expression allows one to identify cells that have been transformed or transfected with a vector containing the marker gene. A wide range of selection markers are known in the art. For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced.
10 The selectable marker can also be a visually identifiable marker such as a fluorescent marker for example. Examples of fluorescent markers include rhodamine, FITC, TRITC, Alexa Fluors and various conjugates thereof. In one embodiment, the selectable marker may be flanked by sequences that allow removal of the marker, for example loxP or frt.

Also provided is a host cell comprising one or more cloning or expression vectors
15 comprising one or more DNA sequences encoding an antibody or antigen-binding fragment thereof of the present invention. Any suitable host cell/vector system may be used for expression of the DNA sequences encoding the antibody or fragment of the present invention. Bacterial, for example *E. coli*, and other microbial systems may be used or eukaryotic, for example mammalian, host cell expression systems may also be
20 used. Suitable mammalian host cells include CHO, myeloma or hybridoma cells. A host cell comprising a nucleic acid molecule or vector of the present invention is also provided.

Detection of Tau and Diagnosis

25 The antibodies and antigen-binding fragments thereof of the present invention may be used in diagnosis/detection kits. In one embodiment, antibodies or antibody fragments of the present invention are fixed on a solid surface. The solid surface may for example be a chip, or an ELISA plate. The binding molecules, in particular antibodies, of the present invention may be for example conjugated to a fluorescent marker which
30 facilitates the detection of bound antibody-antigen complexes. They can be used for immunofluorescence microscopy. Alternatively, an antibody or antigen-binding fragment thereof, may also be used for western blotting or ELISA.

In one particularly preferred embodiment, the ability to bind 3R or 4R tau protein isoforms is measured where the tau protein is present in the same form that it is

physiologically, for example not in denatured form. In one preferred embodiment it is present in a cell. In one embodiment, the antibody or antigen-binding fragment thereof may bind the protein when it is present on a cell and hence be used to detect the protein when present on the cell. In one embodiment, the antibody or antigen-binding fragment thereof may bind the protein when it is present in the extracellular space, such as in the form of secreted fibrils.

Any suitable method may be employed to determine the binding of a given antibody or antigen-binding fragment thereof to 4R tau compared to 3R tau. In one embodiment, an ELISA is employed: for example, binding to immobilised tau 4R protein may be compared to that shown by immobilized 3R tau protein. Binding to the peptides discussed herein may also be measured, and compared, by ELISA.

In another embodiment, western blotting may be employed to detect binding of a given antibody or antigen-fragment thereof to 4R and 3R tau protein isoforms. Such western blots may be performed on any suitable material, for example on cell lysates of cells known to express 4R or 3R or both tau protein isoforms. In one embodiment, the cells employed are iPSc cells and in particular such cells which are known to express a particular tau protein. In another embodiment, they are neurones differentiated from iPSCs. In one embodiment, Western blots are performed on tissue samples, for example on brain tissue from a subject. In one preferred embodiment, a Peggy Sue Simple Western is employed.

In another embodiment, the ability of an antibody or antigen-binding fragment thereof to bind a particular form of tau protein is measured by immunofluorescence. For example, the antibody or antigen-binding fragment thereof may be conjugated to a fluorochrome itself and the present invention also provides such conjugated antibodies or antigen-binding fragments thereof. Alternatively, binding of an antibody or antigen-binding fragment thereof to a tau protein may be identified, and measured, using a secondary antibody, for example specific for the species of the primary antibody. In one embodiment such immunofluorescence is performed on cells expressing 4R or 3R tau protein, for example CHO cells, in particular CHOK1 cells over-expressing the desired tau. In another embodiment, immunohistochemistry is performed on iPSc cells. In another embodiment, binding may be measured on a tissue sample, for example via immunohistochemistry (IHC) by fluorescence or other means.

In one particularly preferred embodiment, iPSCs (Induced Pluripotent Stem cells) are used to assess an antibody or antigen-binding fragment of the present invention. In

another preferred embodiment, neurons are employed, for example neurons obtained by differentiating iPSCs. In one preferred embodiment, cells are isolated from a subject with a particular disorder, such as any of those mentioned herein and used to assess an antibody or antigen-binding fragment thereof of the present invention. In one
5 embodiment, fibroblasts are isolated from a subject with a tauopathy and used to produce iPSCs, then neurons. In one embodiment, the subject used to obtain the cells from is one with a mutation in the *tau* gene (MAPT) gene, for instance one of the mutations mentioned herein. For example, fibroblasts from such subjects may be used to generate iPSCs which will comprise the same mutation in the *tau* gene as the subject. In a
10 particularly preferred embodiment, such iPSCs are differentiated into neurons. In one embodiment, the neurons are cultured for at least five, six, seven, eight, or nine months before being used. In one embodiment, a comparison may be made with a control cell line that lacks any mutation associated with a tauopathy in the *tau* gene, for example cells isolated from a healthy subject lacking such mutations. In one embodiment, fibroblasts
15 are isolated from a healthy control subject, used to generate iPSCs, and then differentiated into neurons.

In another embodiment, rather than use cells from a subject with a specific disease mutation in the *tau* gene, mutations associated with a tauopathy are engineered into a chosen cell line, for example one which is, or is used to generate, iPSCs. In one
20 embodiment, the iPSCs are then differentiated into neurons comprising the mutation(s).

In one embodiment, an antibody or antigen-binding fragment of the present invention is used to detect 4R tau protein isoforms or 3R tau protein isoforms. For example, the present invention provides a method of detecting or measuring 4R tau protein isoforms comprising: (a) contacting a test sample with an antibody or antigen
25 binding fragment thereof of the present invention; and (b) detecting any binding of the antibody. Any of the detection means discussed herein may be employed, for example ELISA, immunofluorescence, flow cytometry analysis or immunohistochemistry may be used as the detection means. In one embodiment, the test sample comprises a cell lysate, or cells, or tissue, for example from a subject. In one embodiment, the test sample is one
30 comprising neurones or a lysate from them. In one embodiment, the test sample is brain tissue or lysate. In one embodiment, such a method may also comprise a positive control, for example one known to express 4R tau protein or which expresses it at normal levels. Such methods may also comprise performing the method separately with an antibody that

binds all tau protein isoforms of the subject to give an indication of the amount of 4R tau protein compared to that of the total amount of tau protein present.

In another embodiment, rather than an antibody or antigen-binding fragment thereof of the present invention specific for 4R tau protein isoforms, one specific for 3R tau protein isoforms is employed in a method as set out above for 4R tau protein isoforms, except the method is for detection or measurement of 3R tau protein isoforms.

The method may comprise analysing a test sample both for the level of 4R tau protein isoforms and for the level of 3R tau protein isoforms. For example, the method may comprise testing two portions of the same test material or staining simultaneously with both an antibody or fragment thereof specific for 4R tau protein isoforms and an antibody or fragment thereof specific for 3R tau protein isoforms. In one example, each is differently labelled, for example with different fluorochromes to allow a direct comparison of the relative amounts of 4R tau protein and 3R tau protein.

Such a method for detecting the relative amounts of 4R tau protein isoforms and 3R tau protein isoforms may be used to detect an imbalance of the two, for example compared to a sample from a healthy subject or a sample from the same subject before they developed a tauopathy. In one embodiment an antibody or fragment thereof of the present invention may be used to diagnose a tauopathy. In one preferred embodiment, an antibody or fragment thereof of the present invention is used to diagnose a condition involving an imbalance of 4R and 3R tau protein isoforms. For example, frontotemporal dementia and parkinsonism's linked to chromosome 17 (FTDP-17), progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD) are all thought to involve 4R tau protein isoforms predominating and so the invention may be used to detect such an imbalance as a way to diagnose the condition. Conversely, Pick's Disease is considered to be one where there is more 3R tau protein than 4R tau protein. Hence, again the present invention may be used to diagnosis that condition based on the identification of such an imbalance.

In one preferred embodiment, an antibody or antigen-binding fragment thereof is one which is able to specifically bind 4R tau protein isoforms but not 3R tau protein isoforms in a Peggy Sue Simple Western. In another it is able to show such specificity as measured by flow cytometry, in particular on cells over-expressing tau. In another embodiment, it shows such specificity when used to analyse a human brain sample, for instance by Western blot. In a particularly preferred embodiment, it will show such specificity when immunofluorescence is performed using the antibody or fragments on

neurones, particularly those obtained from iPSC expressing the relevant tau protein at physiological levels.

In one preferred embodiment, an antibody or antigen-binding fragment thereof is one which is able to specifically bind 3R tau protein isoforms but not 4R tau protein isoforms in a Peggy Sue Simple Western blot. In another it is able to show such specificity as measured by flow cytometry, in particular on cells over-expressing tau. In another embodiment, it shows such specificity when used to analyse a human brain sample, for instance by Western blot. In a particularly preferred embodiment, it will show such specificity when immunofluorescence is performed using the antibody or fragments on neurones, particularly those obtained from iPSC expressing the relevant tau protein at physiological levels.

Pathological conditions

In one embodiment, an antibody or antigen-binding fragment thereof may be used to treat or diagnose a tauopathy. In one embodiment, the condition to be treated is a primary tauopathy. In one embodiment, the condition may involve frontotemporal lobar degeneration (FTLD): for example, it may be characterised by neuronal tau inclusions with predominant cell death in the frontal and temporal lobes of the brain. In another embodiment, the condition may be a secondary tauopathy, so a condition where tau pathology is observed in association with other brain pathologies.

The present invention may be used to treat or diagnose tauopathies in general, for example, Alzheimer's Disease (AD) and a range of other conditions, including progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Pick's disease, or frontotemporal dementia (FTD).

In one preferred embodiment of the present invention, the condition to be treated or diagnosed is Alzheimer's Disease (AD), which is a secondary tauopathy.

In one embodiment, an antibody or antigen binding fragment thereof of the present invention is used to shift the balance of 3R and 4R tau protein in a subject with a tauopathy. For example, an antibody specific for 3R tau protein may be used to shift the balance more towards 4R tau protein. Alternatively, an antibody specific for 4R tau protein may be used to shift the balance more towards 3R tau protein.

In one preferred embodiment, the condition to be treated or diagnosed via the present invention may be Pick's Disease (PD). In one preferred embodiment, an antibody or antigen-fragment thereof of the present invention specific for 3R tau protein isoforms

may be used to treat Pick's Disease. In a preferred embodiment, the antibody or antigen-binding fragment thereof may be used to change the balance away from the 3R tau protein isoforms. For example, applying the present invention in that way may result in less or eliminate Pick bodies. Employing an antibody of the present invention specific for 3R tau protein isoforms may eliminate, reduce or stabilise neuronal and glial loss in the frontal, temporal and parietal lobes of the brain in a subject and in particular a subject with Pick's Disease. In one embodiment, treating a subject with an antibody or antigen-binding fragment thereof of the present invention specific for 3R tau protein isoforms may shift the ratio of 3R:4R tau proteins isoforms in the subject towards, or to, the level seen in individuals who does not have Pick's Disease.

In another embodiment, an antibody or antigen-binding fragment thereof of the present invention is used to treat a condition where there is more 4R tau protein than in a healthy subject and in particular one where the ratio of 4R:3R tau protein is shifted towards 4R tau protein. Hence, an antibody or antigen-binding fragment of the present invention specific for 4R tau protein may be used to treat a 4R tauopathy. Examples of such conditions that may be treated include: Frontotemporal dementia and parkinsonism's linked to chromosome 17 (FTDP-17); progressive supranuclear palsy (PSP); and corticobasal degeneration (CBD).

In one embodiment, the subject to be treated may have a MAPT mutation, i.e. a mutation in the *tau* gene. In one embodiment, the subject may have a splice mutation in or near intron 10 which results in overinclusion of exon10 (MTBR R3) and an increase in 4R tau. In one preferred embodiment the tauopathy involves a 10 + 16 mutation. In other words, in one embodiment the mutation is a IVS 10+16 C-T mutation. In one embodiment, the subject has a MAPT mutation that results in an increase in the mRNA for 4R tau protein. In one embodiment, an antibody or antigen-binding fragment thereof of the present invention specific for 4R tau protein may be used to treat such a condition. In one embodiment, the subject to be treated has FTDP-17 and in particular early onset FTDP-17.

The present invention may be applied to treat, prevent or diagnose tauopathies stemming from other mutations that can affect the inclusion of exon 10 and hence result in an increase in 4R tau mRNA. These can be within the stem loop structure such as S305N and S305I which in a similar way to the intronic mutations destabilise the stem loop causing increased inclusion of exon 10 (Hasegawa et al. 1998, *FEBS letters*, 443 (2), 93-96; Kovacs et al., 2008, *protein-based neuropathology and molecular*

classification of human neurodegenerative diseases: 251-272; Stanford et al., 2000, *Brain*, 123(5), 857-859).

Alternatively, mutations within regulatory elements of exon 10 have been demonstrated to increase exon 10 inclusion. The mutations N279K and L284L strengthen enhancer regions within exon 10 resulting in an increased inclusion and excess 4R tau expression (D'Souza and Schellenberg, 2002, *Journal of Biological Chemistry*, 277 (29), 26587-99; D'Souza et al., 1999, *Proceedings of the National Academy of Sciences*, 96 (10), 5598-603; Hasegawa et al., 1999, *supra*). Other mutations within exon 10 have been demonstrated to increase 4R tau expression include N296N and N296H which have been postulated to either disrupt silencer regions (D'Souza and Schellenberg 2002, *supra*) or create a new enhancer (Andrew Grover et al., 2002, *Neuroscience letters*, 323 (1), 33-36). Somewhat counterintuitively mutations within exons 12 and 13 (E342V and N410H respectively) have been reported to increase the expression of 4R tau within neurons (Lippa et al. 2000, *Annals of neurology*, 48 (6), 850-58). Again, the present invention may be employed to treat tauopathies involving such mutations.

Mitochondrial impairment can also be a feature of tauopathies. In one embodiment, treatment with an antibody or antigen-binding fragment thereof of the present invention may help restore mitochondrial function in a tauopathy. For example, in such tauopathies mitochondrial membrane potential may be affected, and treatment with an antibody or antigen-binding fragment thereof may help restore mitochondrial membrane potential to, or towards, normal levels. In one embodiment, the subject to be treated is one that displays mitochondrial dysfunction. In one embodiment, the subject may be one who displays altered mitochondrial membrane potential. In one embodiment, the subject displays lowered membrane potential. In a preferred embodiment, the subject displays raised membrane potential and in particular displays such raised membrane potential and has a 10+16 MAPT mutation. In one embodiment, such a subject may display excess 4R tau protein isoforms. In another embodiment, the subject has a P301L mutation and in particular may display hyperpolarised mitochondrial membranes. In one embodiment, employing the invention may mean that mitochondrial membrane potential may return, or at least be closer to, normal.

In one particularly preferred embodiment, antibodies or antigen-binding fragments thereof that are able to cross the blood brain barrier may be employed. In a further preferred embodiment, rather than administering an antibody or antigen-binding

fragment itself, a nucleic acid or nucleic acids or vector or vectors of the present invention may be administered, in one preferred embodiment ones encoding an intrabody or degrabody may be administered to a subject. In another embodiment, host cells of the present invention able to express an antibody or antigen-binding fragment of the present invention are employed.

Pharmaceutical compositions

In one aspect, there is provided a pharmaceutical composition comprising: (a) an antibody or antigen-binding fragment thereof, a nucleic acid molecule or molecules, or a vector or vectors of the present invention; and (b) a pharmaceutically acceptable carrier or diluent. In one preferred embodiment, the pharmaceutical composition comprises an antibody or antigen-binding fragment. The composition may be in solid, or liquid form and may be, *inter alia*, be in the form of a powder, a tablet, a solution or an aerosol.

Also provided is an antibody or antigen-binding fragment thereof, a nucleic acid molecule or molecules, a vector or vectors, or a pharmaceutical composition of the present invention for use in a method of treatment or diagnosis of the human or animal body. Further provided is the use of an antibody or antigen-binding fragment thereof, a nucleic acid molecule or molecules, a vector or vectors, or a pharmaceutical composition of the present invention for the manufacture of a medicament for the treatment of a pathological condition or disorder. In one embodiment where a therapeutic of the invention is administered to a subject who is also being given a second therapeutic agent, the two may be given, for example, simultaneously, sequentially or separately. In one embodiment, the two are given in the same pharmaceutical composition. In another embodiment, the two are given in separate pharmaceutical compositions.

A composition of the present invention will usually be supplied as a sterile, pharmaceutical composition. A pharmaceutical composition of the present invention may additionally comprise a pharmaceutically acceptable adjuvant. In another embodiment, no such adjuvant is present in a composition of the present invention. The present invention also provides a process for preparation of a pharmaceutical or diagnostic composition comprising adding and mixing the binding molecule, in particular antibody, of the present invention together with one or more of a pharmaceutically acceptable excipient, diluent or carrier.

The term “pharmaceutically acceptable excipient” as used herein refers to a pharmaceutically acceptable formulation carrier, solution or additive to enhance the

desired characteristics of the compositions of the present invention. Excipients are well known in the art and include buffers (e.g., citrate buffer, phosphate buffer, acetate buffer and bicarbonate buffer), amino acids, urea, alcohols, ascorbic acid, phospholipids, proteins (e.g., serum albumin), EDTA, sodium chloride, liposomes, mannitol, sorbitol, and glycerol. Solutions or suspensions can be encapsulated in liposomes or biodegradable microspheres. The formulation will generally be provided in a substantially sterile form employing sterile manufacture processes. This may include production and sterilization by filtration of the buffered solvent solution used for the formulation, aseptic suspension of the antibody in the sterile buffered solvent solution, and dispensing of the formulation into sterile receptacles by methods familiar to those of ordinary skill in the art.

The pharmaceutically acceptable carrier should not itself induce the production of antibodies harmful to the individual receiving the composition and should not be toxic. Suitable carriers may be large, slowly metabolised macromolecules such as proteins, polypeptides, liposomes, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers and inactive virus particles.

Pharmaceutically acceptable salts can be used, for example mineral acid salts, such as hydrochlorides, hydrobromides, phosphates and sulphates, or salts of organic acids, such as acetates, propionates, malonates and benzoates. Pharmaceutically acceptable carriers in therapeutic compositions may additionally contain liquids such as water, saline, glycerol and ethanol. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragées, capsules, liquids, gels, syrups, slurries and suspensions, for ingestion by the patient.

A thorough discussion of pharmaceutically acceptable carriers is available in Remington's Pharmaceutical Sciences (Mack Publishing Company, N.J. 1991).

The term "therapeutically effective amount" as used herein refers to an amount of a therapeutic agent needed to treat, ameliorate or prevent a targeted disease or condition, or to exhibit a detectable therapeutic or preventative effect. For any binding molecule, in particular antibody, the therapeutically effective amount can be estimated initially either in cell culture assays or in animal models, usually in rodents, rabbits, dogs, pigs or primates. The animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans. The precise therapeutically effective amount for a human subject will depend upon the severity of the disease state, the

general health of the subject, the age, weight and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities and tolerance/response to therapy. This amount can be determined by routine experimentation and is within the judgement of the clinician. Generally, a
5 therapeutically effective amount will be from 0.01 mg/kg to 50 mg/kg, for example 0.1 mg/kg to 20 mg/kg per day. Alternatively, the dose may be 1 to 500 mg per day, such as 10 to 100, 200, 300 or 400 mg per day. Pharmaceutical compositions may be conveniently presented in unit dose forms containing a predetermined amount of an active agent of the invention. In one embodiment, the amount in a given dose is at least
10 enough to bring about a particular function.

Any of a number of routes of administration may be employed to administer the present invention to a subject including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, transcutaneous (for example, see WO98/20734), subcutaneous, intraperitoneal,
15 intranasal, enteral, topical, sublingual, intravaginal or rectal routes. Hyposprays may also be used to administer the pharmaceutical compositions of the invention. Direct delivery of the compositions will generally be accomplished by injection, subcutaneously, intraperitoneally, intravenously or intramuscularly, or delivered to the interstitial space of a tissue. The compositions can also be administered into a specific tissue of interest. In
20 one embodiment, the administration is to the brain. Dosage treatment may be a single dose schedule or a multiple dose schedule. Where the product is for injection or infusion, it may take the form of a suspension, solution or emulsion in an oily or aqueous vehicle and it may contain formulatory agents, such as suspending, preservative, stabilising and/or dispersing agents. Alternatively, a pharmaceutical composition of the present
25 invention may be in dry form, for reconstitution before use with an appropriate sterile liquid. If the composition is to be administered by a route using the gastrointestinal tract, the composition may contain agents which protect the antibody from degradation but which release the antibody or antigen-binding fragment thereof once it has been absorbed from the gastrointestinal tract. A nebulisable formulation according to the present
30 invention may be provided, for example, as single dose units (e.g., sealed plastic containers or vials) packed in foil envelopes. Each vial contains a unit dose in a volume, e.g., 2 ml, of solvent/solution buffer.

In one embodiment, an antibody or antigen-binding fragment, nucleic acid, vector, host cell, or pharmaceutical composition of the present invention is administered to the brain or in such a way that it is able to gain access to the brain.

The present invention also provides a process for preparation of a pharmaceutical or diagnostic composition comprising adding and mixing an antibody or antigen-binding fragment thereof of the present invention, in particular an antibody, together with one or more of a pharmaceutically acceptable excipient, diluent or carrier. The antibody or antigen-binding molecule of the present invention may be the sole active ingredient in the pharmaceutical or diagnostic composition or may be accompanied by other active ingredients including antibody ingredients or non-antibody ingredients such as steroids or other drug molecules. The pharmaceutical compositions suitably comprise a therapeutically effective amount of the antibody or antigen-binding fragment thereof. The term “therapeutically effective amount” as used herein refers to an amount of a therapeutic agent needed to treat, ameliorate or prevent a targeted disease or condition, or to exhibit a detectable therapeutic or preventative effect.

Pharmaceutical compositions may be conveniently presented in unit dose forms containing a predetermined amount of an active agent of the invention per dose. A pharmaceutical composition of the present invention may be provided in a receptacle that provides means for administration to a subject. A pharmaceutical composition of the present invention may be provided in a prefilled syringe. The present invention therefore provides such a loaded syringe. It also provides an auto-injector loaded with a pharmaceutical composition of the present invention.

It is also envisaged that the binding molecule, in particular antibody, of the present invention may be administered by use of gene therapy. In order to achieve this, where the binding molecule is an antibody, DNA sequences encoding the heavy and light chains of the antibody molecule under the control of appropriate DNA components are introduced into a patient such that the antibody chains are expressed from the DNA sequences and assembled *in situ*. In another particular preferred embodiment, the sequences encoding an intrabody of the present invention may be administered to a subject.

The present invention also extends to a kit, comprising an antibody or antigen-binding fragment thereof of the invention, optionally with instructions for administration. In yet another embodiment, the kit further comprises one or more reagents for performing one or more assay or method, such as those discussed herein.

In one embodiment, molecules of the present invention including an antibody or antigen-binding fragment thereof of the invention is provided for use as a laboratory reagent.

“Purified form” as used supra is intended to refer to at least 90% purity, such as 91, 92, 93, 94, 95, 96, 97, 98, 99% w/w or more pure.

In the context of this specification "comprising" is to be interpreted as "including". Aspects of the invention comprising certain elements are also intended to extend to alternative embodiments "consisting" or "consisting essentially" of the relevant elements.

Positively recited embodiments may be employed herein as a basis for a disclaimer.

Where the singular is referred to herein, the plural is also encompassed unless otherwise stated or apparent. In particular, the singular forms “a,” “an,” “the” and the like include plural referents unless the context clearly dictates otherwise.

All references referred to herein are specifically incorporated by reference.

The sub-headings herein are employed to assist in structuring the specification and are not intended to be used to construct the meaning of technical terms herein.

Sequences of the invention are provided herein below.

EXAMPLE

Example 1 - Peptide immunogen design 3R tau

The amino acid sequence of the tau MTBR, exons 9-12 is shown in **Figure 1**. This splicing of exon 10 creates a unique exon boundary between exons 9 and 11 which only found in 3R tau. This epitope was used as a way to raise an antibody that would confer specificity for 3R tau over 4R tau. To drive an immune response to this epitope, a peptide was designed to cover amino acids 268-311 of tau (the peptide is named TE9/11), as denoted by the solid box in **Figure 1** and the TE9/11 sequences in **Table 1**.

Table 1: Sequence alignment of TE9/11 with potentially cross-reactive epitopes present on all tau isoforms

AA number	1	2	3	4	5	6	7	8	9	10	11	12	13	14
TE9/11	H	Q	P	G	G	G	K	V	Q	I	V	Y	K	P
Cross reactive - E10/11	H	V	P	G	G	G	S	V	Q	I	V	Y	K	P
Cross reactive - E9/10	H	Q	P	G	G	G	K	V	Q	I	I	N	K	K
Cross reactive - E11/12	H	K	P	G	G	G	Q	V	E	V	K	S	E	K

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TE9/11 shares homology to other regions of tau that exist at the exon boundaries between the all exons of the tau MTBR. These potentially cross-reactive epitopes are highlighted in the dashed boxes (**Figure 1**), with the asterisk highlighting sequence difference. For a clearer comparison Table 1 shows an exact peptide alignment of the potential cross-reactive epitope.

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The first of these is the exon boundary between exon 10 and 11 (tau residues 299-312 **Figure 1**)) which differs from TE9/11 by only two amino acids (peptide residues 2 and 7 (Table 1)). The second region of similarity exists at the boundary between exon 9 and 10 (tau residues 268-281 (**Figure 1**)), which differs by three amino acids (peptide residues 11, 12 and 14 (Table 1)). Finally, there is sequence homology at the site of the boundary between exons 11 and 12 (tau residues 330-343 (**Figure 1**)). However, there are 8 differences (peptide residues 2,7 and 9-14 (**Table 1**), representing 50% of the epitope space so this is less likely to drive non-specific antibody binding.

15

20 **Example 2 - Peptide immunogen design 4R tau**

As previously discussed, the unique feature of 4R tau over 3R tau is the presence of exon 10 in 4R tau. This represents an epitope for an antibody that would give specificity to 4R tau over 3R tau. It is worth noting that it might be possible to achieve 4R specificity by targeting the exon boundaries between Exons 9/10 and 10/11. In both cases there would only be one amino acid to target (I278 or S301 respectively). It is also important to consider there are several sites of known post translational moderation within exon 10, N279, K280/281 and S285/289) (Ercan et al. 2017, *Mol Neurodegener*, 12 (1), 87; Kontaxi et al. 2017, *Front Mol Biosci*, 4, 56; Mair et al. 2016, *Anal Chem*, 88 (7), 3704-14) (shown in **Figure 2** in the dashed box within exon 10). To ensure an antibody would be specific to 4R tau, and bind regardless of the post translational

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modification state of tau, peptide TE10 was designed to cover tau amino acids 294-302, (Figure 22), as this is within exon 10 and in a region of exon 10 and avoids the post translational modification sites. Figure 2 shows the location of TE10 in the solid box within the 4 exons of the tau MTBR. The TE10 line in Table 2 shows the exact peptide sequence.

Table 2: Sequence alignment of TE10 peptide with potential cross-reactive sites in Exons 9, 11 and 12 of tau.

AA number	1	2	3	4	5	6	7	8	9
TE10	K	D	N	I	K	H	V	P	G
Cross reactive-E12	L	D	N	I	T	H	V	P	G
Cross reactive-E11	L	G	N	I	H	H	K	P	G
Cross reactive-E9	T	E	N	L	K	H	Q	P	G

Given the repetitive nature of the MTBR, TE10 shares sequence homology to three other exons of the MTBR (highlighted in the dashed boxes in Figure 2 and aligned in Table 2). The first of these is within exon 12 (tau residues 357-365 (Figure 2)) which differs from the TE10 peptide by two amino acids (peptide residue 1 and 5 (Table 2)). The second region of similarity exists within exon 9 (tau residues 263-271 (Figure 2)) which differs by four amino acids (peptide residue 1, 2, 5 and 7 (Table 2)). Finally, there is sequence homology within exon 11 (tau residues 325-333 (Figure 2)) which differs by four amino acids (peptide residue 1, 2, 4 and 7 (Table 2)).

Example 3 - Peptide conjugation for immunisation

To elicit an immune response, the peptides were conjugated to the carrier proteins keyhole limpet hemocyanin (KLH), ovalbumin (OVA) and bovine serum albumin (BSA). All peptides were also conjugated to biotin to allow for screening via streptavidin capture to beads or plates (carried out by CRO Peptide Synthetics).

Throughout an immunisation the immunodominant position of a peptide immunogen is located furthest from the carrier protein, as this is most exposed to the immune system. Therefore TE9/11 was conjugated as cyclic a peptide to ensure the predicted key residues 2, 7, 11 and 12 (**Table 2**), were presented in the most immunodominant positions. TE10 (designed to confer a 4R tau specific immune response) was designed as linear peptide coupled to the carrier protein via its C-terminus. This was to ensure the lysine residues at positions 1 and 5 of the peptide, the only 2 amino acids that are unique to TE10 (**Table 2**), were in the immunodominant position. For both peptides, a single cysteine was added to the peptide for linkage via the thiol group to the maleimide linker and carrier protein or biotin-PEG-maleimide. Cyclisation of the peptides was achieved via N to C-terminal amide bond formation to generate a cyclic peptide loop.

Example 4 - 3R and 4R specific antibody identification from rabbits

The conjugated peptides were used to immunize rabbits. B-cells from rabbit 6170 and 6171 were activated into antibody secreting B-cells in a 400 x 96 well plate culture. Culture supernatants were primary screened for binding. This homogenous fluorescence assay was to a mix of the biotinylated TE9/11 and TE10 peptides captured onto streptavidin beads. **Figure 3** shows the primary screening data from the 50 x 384 well assay screening plates. Highlighted in green are the wells where bead associated fluorescence was observed, indicating antibodies with specificity to either TE10 or TE9/11. Effectively, the colonies highlighted green are roughly above a bead associated fluorescence of 1000 in the graph. These hits were selected based on a binding value threshold of 1000 bead associated fluorescent units. This threshold gives high confidence in the binding profile and allowed for consolidation of samples into 12 x 80-well master plates.

Example 5 - Rabbit B-cell culture secondary screening

Following the identification of supernatants that showed reactivity to either TE10 or TE9/11 (**Figure 3**) and cryopreservation of the activated B-cells, secondary supernatant screening was carried out to identify those wells that had antibodies demonstrating isoform specificity. This was achieved by ELISA using each of the peptide antigens (**Figure 4**) and to recombinant 0N3R and 0N4R tau (**Figure 5**)

Importantly, the remaining four tau isoforms are all identical within the MTBR and there are no other potential cross-reactive epitopes present within the rest of the protein.

The data obtained is shown in **Figure 4** shows the results of the peptide ELISA and **Figure 5** shows the results of the protein ELISA. All ELISA data is plotted as fold-change over background for each data point. Supernatants from wells which showed binding to single peptides and exhibited at least a 4-fold higher binding to the desired tau ON isoform were selected for B-cell isolation and variable region gene recovery. In each case, the wells selected to be 3R-specific are highlighted in red and the 4R-specific wells are highlighted in green. It was interesting to note that some wells appeared to show cross-reactivity to both peptides. These are likely recognising a common epitope on the peptides. The lack of sequence homology between the two tau peptides suggests the cross reactivity was likely due to the linker that conjugates the peptide to the biotin molecule or carrier protein or polyreactive antibodies within the developing immune repertoire of the rabbits.

Example 6 - B-cell isolation, RT-PCR and transcriptionally active PCR recombinant transient screening

B-cell isolation, via the fluorescent method, was performed on all of the selected wells highlighted red or green in **Figure 4** and **Figure 5**. Reverse transcription and three rounds of PCR were carried out, to recover the variable region genes and create transfection ready linear expression cassettes. **Table 3** shows the recovery levels from B-cell isolations, the number of PCRs and success of those PCRs from each well along with the specificity to either 3R or 4R tau for that well.

Table 3: B-cell isolation and PCR recoveries for selected wells

Well reference	Tau isoform specificity	Number of foci picked	Number PCR attempted	Heavy chain PCR recovered	Kappa chain PCR recovered
SSUP0011921834	4R	8	5	5/5	5/5
SSUP0011921958	4R	8	5	4/5	5/5
SSUP0011924815	4R	0	0	n/a	n/a
SSUP0011917532	4R	4	4	4/4	4/4
SSUP0011934401	4R	8	5	5/5	5/5
SSUP0011918493	4R	8	5	5/5	5/5
SSUP0011919075	4R	7	5	5/5	5/5
SSUP0011934305	4R	8	5	5/5	5/5
SSUP0011929468	4R	8	5	4/5	4/5
SSUP0011926345	4R	8	5	5/5	5/5
SSUP0011933382	4R	8	5	5/5	5/5
SSUP0011919104	4R	8	5	5/5	5/5
SSUP0011923507	3R	0	0	n/a	n/a
SSUP0011924325	3R	0	0	n/a	n/a
SSUP0011921782	3R	0	0	n/a	n/a
SSUP0011928511	3R	8	4	4/4	4/4
SSUP0011924609	3R	8	4	4/4	4/4
SSUP0011931102	3R	8	4	4/4	4/4
SSUP0011928754	3R	8	4	3/4	4/4
SSUP0011929699	3R	8	4	4/4	4/4
SSUP0011927754	3R	8	4	4/4	4/4
SSUP0011929686	3R	8	4	4/4	4/4
SSUP0011917961	3R	8	4	4/4	4/4
SSUP0011917987	3R	8	4	4/4	4/4

In general, the results obtained demonstrated good B-cell isolation efficiencies, generating fluorescent foci from 83% of wells. Variable region recovery was observed from 97% of vH PCRs and 99% of vK PCRs. This represented an overall paired V-region recovery rate of 96%.

5 Linear expression cassettes, generated via the tertiary TAP PCR, were directly transfected into Expi293F cells. Following expression, the antibody containing supernatants were assayed for IgG expression via ELISA. Expression of IgG was observed in all cases where paired V-regions were recovered in the PCR. In total there were 77 samples containing IgG from 90 wells. All wells were subsequently assayed for
10 binding to either 0N3R or 0N4R tau via ELISA. The ELISA data with one representative TAP expression per foci group is shown in **Figure 6**. Wells selected for cloning and further testing are highlighted in green and red for the 4R and 3R specific antibodies respectively, in **Figure 6**.

 It is interesting to note that all except one of the foci wells picked retained their
15 binding specificity to 3R or 4R tau following the TAP expressions. For the single antibody highlighted in black, that no longer appears to bind either 3R or 4R tau, it is likely that this was a false positive in the B-cell supernatant screening ELISA on protein (**Figure 5**) and bound to a non-tau representative epitope on the TE10 peptide. This would explain how it was able to produce fluorescent foci, as these were performed using
20 peptide, then subsequently not show binding to tau protein as a TAP transient.

Example 7 - Cloned transient screening and sequencing of rabbit IgGs

 The variable region fragments, generated via PCR, were cloned as full-length rabbit IgGs. All cloned antibodies were sequenced, expressed as 30ml transients in
25 Expi293F cells and the resulting culture supernatants were assayed to determine the concentration of IgG they contained. All the supernatants were assayed via ELISA at 10ug/ml IgG against 0N3R and 0N4R tau, to determine if they had retained their specificity profiles following cloning. **Figure 7A** displays the ELISA data for all the cloned antibodies, whilst **Figure 7B** shows the CDR3 sequences of these antibodies.

30 As can be seen, groups of antibodies with similar or the same antibody sequence clustered very closely together in their ELISA values, **Figure 7**. There was a clear split between the 3R and 4R specific antibodies, with the 4R specifics generally demonstrating more cross reactivity. Two antibody sequences, clone 3 (VR7082) (4R-specific) and

clone 14 (VR7081) (3R-specific), that were considered to possess the suitable level of isoform-selectivity (without any measurable unwanted cross-reactivity) to justify larger scale expression/purification and more detailed characterisation.. The sequences for the two antibodies and those of the subsequently generated scFv intrabodies generated are given in the Table below, which also includes other relevant sequences.

Table 4 - Amino acid sequences including those of the VR7082 and VR7081 antibodies

SEQ ID No.	Sequence
1	VR7082 - Light chain variable region AVVLTQTPAS VEAAVGGTVS INCQASEDIE SYLAWYQQKP GQRP KLLIYR ASTLASGVSSRFKSGSGSTE YTLTISGVEC ADAATYYCQ E GYSIHVDNVF GGGTEVVVK
2	VR7082 - FW1 - AVVLTQTPAS VEAAVGGTVS INC
3	VR7082 - CDR1 - QASEDIESYL A
4	VR7082 - FW2 - WYQQKPGQRP KLLIY
5	VR7082 - CDR2 - RASTLAS
6	VR7082 - FW3 - GVSSRFKSGS SGTEYTLTIS GVECADAATY YC
7	VR7082 - CDR3 - QEGYSIHVDN V
8	VR7082 - FW4 - FGGGTEVVVK
9	VR7082 - Heavy chain variable region QSVEESGGRL VTPGTPLTLT CTVSGIDLSN YAMIWVRQAP GEGLE YIGII SSTGNTFYAT WAKGRFTISK TSTTVDLKIT SPTAEDTATY F CARDTNSDS HYGWEAFNIW GPGTLVTVSS
10	VR7082- FW1 - QSVEESGGRL VTPGTPLTLT CTVS
11	VR7082 - CDR1 - GIDLSNYAMI
12	VR7082 - FW2 - WVRQAPGEGL EYIG
13	VR7082 - CDR2 - IISSTGNTFY ATWAKG
14	VR7082 - FW3 - RFTISKSTTT VDLKITSPTA EDTATYFCAR
15	VR7082 - CDR3 - DTNSDSHYGW EAFNI
16	VR7082 - FW4 - WGPGTLVTVS S

17	VR7082 - derived scFv intrabody MAQSVEESGGRLVTPGTPLTLTCTVSGIDLSNYAMIWVRQAPGEGLE EYIGIISSTGNTFYATWAKGRFTISKSTTTVDLKITSPTAEDTATYFC ARDTNSDSHYGWEAFNIWGPGLTVTVSSGGGGSGGGGGSGGGGGSG GGGSAVVLTPQTPASVEAAVGGTVSINCQASEDIESYLAWYQQKPG QRPKLLIYRASTLASGVSSRFKSGSGTEYTLTISGVECADEAATYYC QEGYSIHVDNVFGGGTEVVVK
18	VR7081 - Light chain variable region AQVLTQTPAS VSAAVGGTVS ISCQSSPSVL NTNDLSWFQQ KPGQ PPKVLI YGASTLASGV PSRFKSGSG TQFTLTISDV QCDDAATYY C AGGYDVNIDI YGFGGGTEVV VK
19	VR7081 - FW1 - AQVLTQTPAS VSAAVGGTVS ISC
20	VR7081 - CDR1 - QSSPSVLNTN DLS
21	VR7081 - FW2 - WFQQKPGQPP KVLIIY
22	VR7081 - CDR2 - GASTLAS
23	VR7081 - FW3 - VR7081 - GVPSRFKSGSG SGTQFTLTIS DVQCDDAATY YC
24	VR7081 - CDR3 - AGGYDVNIDI YG
25	VR7081 - FW4 - FGGGTEVVVK
26	VR7081 - Heavy Chain variable region QSLEESGGRL VTPGTPLTLT CTVSGFSLSN YAMIWVRQAP GGGLE WIGTM GTNGRITYYAS WAKGRFTISK TSTMDLSMT SLTAEDTAT Y FCARDAGITY STYYFNIWGP GTLTVTVSS
27	VR7081 - FW1 - QSLEESGGRL VTPGTPLTLT CTVS
28	VR7081 - CDR1 - GFSLSNYAMI
29	VR7081 - FW2 - WVRQAPGGGL EWIG
30	VR7081 - CDR2 - TMGTNGRITYY ASWAKG
31	VR7081 - FW3 - RFTISKSTT MDLSMTSLTA EDTATYFCAR
32	VR7081 - CDR3 - DAGITYSTYY FNI
33	VR7081 - FW4 - WGPGLTVTVS S
34	VR7081 derived scFv intrabody MAQSLEESGGRLVTPGTPLTLTCTVSGFSLSNYAMIWVRQAPGGGL EWIGTMGTNGRITYYASWAKGRFTISKSTTMDLSMTSLTAEDTAT YFCARDAGITYSTYYFNIWGPGLTVTVSSGGGGSGGGGGSGGGGGSG GGGSAQVLTQTPAS VSAAVGGTVS ISCQSSPSVL NTNDLSWFQQ

	KPGQPPKVLI YGASTLASGV PSRFKSGSG TQFTLTISDV QCDDAATYYC AGGYDVNIDI YGFGGGTEVV VK
35	Amino acid sequence of the 2N4R isoform of tau MAEPRQEFVMDHAGTYGLGDRKDQGGYTMHQDQEGD TDAGLKESPLQTPTE DGSEEPGSETSDAKSTPTAEDVTAPLVDEGAPGKQAAAQPHTEIPEGTTAEEAGIG DTPSLEDEAAAGHVTQARMVSKSKDGTGSDDKKAKGADGKTKIATPRGAAPPGQ KGQANATRIPAKTPPAPKTPPSSGEPKSGDRSGYSSPGSPGTPGSRSRTPSLPTPPT REPKKVAVVRTPPKSPSSAKSRLQTAPVPMPLKLVKSKIGSTENLKHQPGGGKV QIINKKLDLSNVQSKCGSKDNIKHVPGGGSVQIVYKPVDSLKVTSKCGSLGNIHHK PGGGQVEVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKT DHGAEIVYKSPVVSGDTSRHLNSVSTGSIDMVDSPQLATLADEV SASLAKQGL
36	TE9/11 peptide sequence – HQPGGGKVQIVYKP
37	TE10 peptide sequence - KDNIKHCPG

Example 8 - 3R and 4R specific antibody characterisation

Following the identification of 3R and 4R tau specific antibodies, VR7081 and VR7082, their suitability for all further studies was assessed. That was done by characterisation in a range of assays against recombinant and natively expressed tau.

(a) *Recombinant tau binding and characterisation assays*

As described previously, VR7081 and VR7082 demonstrated exquisite specificity to 0N3R and 0N4R tau at 10µg/ml via ELISA (**Figure 7**). It was important to ensure this specificity had been retained following purification, therefore VR7081 and VR7082 were therefore tested against all six recombinantly expressed isoforms of tau via ELISA (**Figure 8A and B**).

Following confirmation of the selectivity profiles of VR7081 and VR7082 to 3R and 4R via ELISA, **Figure 8**, it was important to check the specificity of these antibodies against tau in a cellular context. The data for this flow cytometry assay can be seen in **Figure 9**. **Figure 9** demonstrates that both VR7081 (**A**) and VR7082 (**B**) show absolute specificity to tau in a cellular context via flow cytometry. To ensure this specificity was retained in a Western blot, lysates were generated from cells overexpressing all the isoforms of tau and validated using the polyclonal anti-total tau antibody. The Western blots with VR7081 and VR7082 can be seen in **Figure 10 A and B** respectively. Peptide TE9/11 and peptide TE10 represent linear epitopes within across the tau exon 9/11 boundary, unique to 3R tau, and within exon 10, unique to 4R tau. These epitopes were therefore unlikely to be affected via the sample reduction performed for Western blot.

Figure 10 shows that both VR7081, **Figure 10 A** and VR7082, **Figure 10 B** retain their specificity profiles via Western blot to 3R and 4R tau respectively.

Both antibodies also appeared to show reactivity with a non-tau band of approximately 49kDa (VR7081) or 60kDa (VR7082). It is unclear what is driving this extra band, it could be a result of the Rabbit IgGs, the secondary HRP conjugated antibody or driven through off target variable region binding.

To address this issue, a Western blot was performed with 2N3R and 2N4R tau containing lysate as well as the recombinant tau ladder and mock lysate and probed with a non-tau reactive rabbit or mouse IgG with the results shown in **Figure 11**. The Western blots in **Figure 11 A** show a similar nonspecific banding pattern to VR7081 and VR7082 with non-specific bands around 60 kDa and 42 kDa with the non-tau reactive rabbit IgG. However, in **Figure 11 B**, with non-tau reactive mouse IgG these bands are not observed. The results clearly indicate that any non-tau bands observed in **Figure 10** are because of what the rabbit IgG constant regions or HRP reveal rather than the tau specific variable regions.

Further experiments were then performed to ensure that the antibodies were of more general utility. To ensure this was possible the rabbit IgGs were converted to mouse IgGs with rabbit variable regions. This was performed as the non-tau reactive mouse IgG showed no banding in tau over expression lysates, **Figure 11 B**. The resulting chimeric antibodies were then assayed via Western blot against individual tau isoform overexpressing cell lysates. The results of these blots can be seen in **Figure 12**. It is clear to see, from **Figure 12**, that the conversion to a mouse chimeric antibody with both VR7081 and VR7082 has removed the non-tau band from the blots. This data, along with the banding seen with a non-tau antibody in **Figure 11 A**, demonstrates that this non-tau reactive band was a result of the rabbit IgG constant regions or the secondary HRP conjugated antibody used for Western blotting. It is important to note that this is a Western blot artefact, likely due to the presentation of linear epitopes of other cellular proteins, as this non-tau reactivity is not observed in the same cells in a flow cytometry assay.

The conversion to a mouse IgG proved very successful in helping the specificity of these antibodies via Western blot, however as discussed previously this non-tau reactive band is not a problem for the experiments performed so, unless otherwise stated all data is generated with the rabbit IgG versions of these antibodies.

Another key area it was important to assess of these antibodies in was immunofluorescence. To assess these antibodies 0N3R and 0N4R tau was overexpressed in adherent CHOK1 cell before being fixed, permeabilised and co-stained with either VR7081/VR7082 and the polyclonal anti-total tau antibody. The resulting immunofluorescent data can be seen in **Figure 13** for VR7081 and **Figure 14** for VR7082. In each case with VR7081 and VR7082 staining with that antibody was only seen for cells expressing the tau protein that the antibody is specific for. The data shown in **Figure 13** and **Figure 14** clearly demonstrates both VR7081 and VR7082 retain exquisite specificity for 3R and 4R tau respectively via immunofluorescence. Polyclonal anti-total tau antibody staining enables visualisation of all the cells expressing tau within these populations. It is therefore important to note that in both cases the staining with either VR7081 or VR7082 overlay with all polyclonal anti-total tau antibody staining for the appropriate tau isoform.

The data obtained therefore clearly shows that VR7081 and VR7082 are highly specific for 3R and 4R tau respectively. In all assays that were performed, both antibodies showed no off-target binding, to the non-desired tau isoforms.

(b) Native tau binding and characterisation assays

Binding to tau expressed in a native system of iPSC derived neurones or from human brain samples was next demonstrated.

Binding of VR7081 and VR7082 was initially assayed via immunofluorescence on iPSC-derived neurons. Both control neurons with no tau mutation (express only 0N3R tau) or mono allelic 10+16 *MAPT* mutant neurons (that express both 0N3R tau and 0N4R tau) were used. The resulting images can be seen in **Figure 15** for VR7081 and **Figure 16** for VR7082.

As expected VR7081 binds to 3R tau in both the non-mutant control cells and in the 10+16 *MAPT* mutant neurones, **Figure 15**. VR7082 demonstrates binding to 4R tau within the 10+16 mutant neurones, **Figure 16**, and as expected shows no binding to the non-mutant control neurones, as these express no 4R tau.

Specificity was also ensured in immunoblot using lysates from control, 10+16 monoallelic and biallelic *MAPT* mutation neurons via Peggy Sue Simple Western together with lysates from ExpHEK-293F cells over expressing 0N3R or 0N4R tau as well as tau negative cell lysates and a recombinant tau ladder containing all tau isoforms

(**Figure 17**). It can be seen from the blots in **Figure 17A** and **B** that both VR7081 and VR7082 retain their specificity in the over expression lysates to both 3R and 4R respectively. VR7081 can be seen to bind to a 0N3R tau sized band in all three of the iPSC lysates as expected. As previously discussed at this age the non-mutant neurones do not express 4R tau. Therefore, as expected, VR7082 demonstrates binding to 0N4R tau sized band in only the 10+16 mutant iPSC derived neurones.

It is interesting to note with both VR7081 and VR7082 the non-tau bands observed in **Figure 10** are no longer seen via this method. This blot uses a different secondary antibody to a standard Western blot and it appears that this has solved the non-tau banding. This gives a further indication that this non-tau band observed with both VR7081/VR7082 and the non-tau rabbit antibody, **Figure 10** and **Figure 11**, were a result of the secondary HRP conjugated antibody.

Tau is able to undergo significant post translational modification (Ercan et al., 2017, *supra*; Kontaxi et al., 2017, *supra*; Mair et al., 2016, *supra*). It was therefore important to confirm that both VR7081 and VR7082 were able to bind 3R and 4R tau, respectively, regardless of the post translational modification state of tau. Aged brain lysate contains a range of tau species with many post translational modifications and tau truncations. It therefore, represented a good source of material to test in Western blot and confirm that both VR7081 and VR7082 were able to bind to many forms of tau (**Figure 18**).

Multiple bands are observed in both blots with either VR7081 or VR7082 **Figure 18**, importantly however three distinct and different bands are present in the tau ladder for VR7081 and VR7082. The multiple bandings with the brain lysate are likely due to multiple phosphorylation states of tau, higher molecular weight aggregates of tau and or truncated tau variants. This data is consistent with other data using these lysates. This data gives a high degree of confidence that both VR7081 and VR7083 are capable of binding to all post translational modification states of 3R and 4R tau.

The data shown in the section on “Native tau binding and characterisation assays” demonstrates that VR7081 and VR7082 are both able to bind to natively expressed tau in a range of assays. It has also been demonstrated in **Figure 17** that the non-tau banding observed in standard Western blot was due to the secondary HRP conjugated antibody. Finally, **Figure 18** demonstrates that both VR7081 and VR7082 are capable of binding tau in a range of post translational modification states.

Example 9 - Generation of 4R degrading tau degrabody

IgGs or derivatives of such as Fab fragments, cannot be used as intracellular antibodies as their disulphide bonds do not form correctly in the reducing environment of the cytoplasm. This ensures that the heavy and light chains do not associate and hence no binding moiety is formed. However, IgGs can be re-formatted to non-disulphide stabilised scFvs allowing for use as intrabodies. In the scFv format the heavy and light chain variable regions are physically linked by a flexible linker. This ensures the heavy and light chains pair correctly inside the cell and form a binding moiety. To ensure that VR7082 reformatted in that way retained its binding properties, the IgG was initially re-formatted into an scFv-msFc. This is a screening construct where the scFv is fused to a mouse Fc to allow for detection with anti-mouse secondary antibodies, a diagrammatic representation of IgG and scFv-Fc can be seen in **Figure 19A** and **Figure 19B** respectively. VR7082 scFv-Ms-Fc was tested. The results of the VR7082 scFv-Ms-Fc conversions can be seen in **Figure 19**. The conversion was tested via ELISA (**Figure 19D**), flow cytometry (**Figure 19F**) and Western blot (**Figure 19H**). For comparative purposes the data seen in **Figure 19C** and **Figure 19E** is ELISA and flow cytometry data respectively. **Figure 19G** is Western blot data utilising VR7082 IgG for comparison with the scFv format of VR7082.

To allow for intracellular cytoplasmic expression of an antibody fragment, or any other protein, expression vectors are designed devoid of any specific leader sequence. Tau exists predominantly within the cytoplasm of the cell, so for the intrabody work it was important that the intrabodies were also expressed in the cytoplasm. Expression constructs were therefore designed without any leader or localisation signals. Despite the successful conversion of VR7082 to an scFv format (**Figure 19**), it was important to ensure that the scFv fragment was capable of cytoplasmic expression and specific binding to the immunising peptide. To allow for testing of VR7082 as a scFv intrabody a fusion construct with GFP was designed, by fusing GFP to VR7082-scFv with a flexible linker. GFP was chosen as it could be used as a surrogate for future fusions and it allows for detection of expression in live cells, via fluorescence, and detection of intrabody via Western blot, via anti-GFP antibodies.

The subsequent expression construct was transfected (n = 4) into HEK-293F cells. These were then assessed for GFP fluorescence via flow cytometry (**Figure 20A**)

and the cells were lysed. The lysates were then assayed via Western blot (**Figure 20B**), to ensure the intrabody GFP fusion was intact and the correct molecular weight. Finally, these lysates were used in a pull-down experiment where TE10, the 4R tau specific peptide, was used to pull down the intrabody before subsequent Western blot this was compared to a pull down with an irrelevant mix of peptide controls (**Figure 20C** and **Figure 20D**).

It can be seen from this data that the intrabody-GFP fusion was expressed in HEK-293F cells, and the GFP folded correctly as indicated by GFP fluorescence (**Figure 20A**). To demonstrate that VR7082 is correctly fused to GFP a Western blot of transfected HEK-293F cells with a GFP antibody was performed. If intact, scFv-GFP fusion intrabody should be observed at a molecular weight of approx. 53 kDa (scFv 26kDa + linker 1kDa + GFP 26kDa). Western blotting of the cell lysates (**Figure 20B**) indicates that this construct was expressing intact fusion protein, with a single band present at 53kDa. Finally, the intrabody was assessed for binding to its immunising peptide. The Western blot (**Figure 20C**) shows that where the VR7082-scFv-GFP lysates were pulled down with TE10 coated beads (lanes 1-4) a band the size of VR7082-scFv-GFP is observed. However, when the VR7082-scFv-GFP lysates are pulled down with irrelevant peptide coated beads (lanes 7-10) or when mock transfected lysate is pulled down with TE10 coated beads (lane 5-6) no band is observed (**Figure 20C**). Band densitometry from this blot, (**Figure 20D**), indicated that the scFv-GFP intrabody construct is still capable of specifically binding the TE10 peptide.

This phase of work showed the successful conversion of VR7082, a 4R tau specific rabbit IgG, to an scFv-Ms-Fc and an scFv-GFP intrabody both of which still demonstrated specific binding to 4R tau or 4R tau peptide. The next phase of work was to focus on the conversion of this intrabody into a target degrading intrabody, namely a degrabody.

Example 10 - Generation of a degrabody from VR7082-scFv intrabody

To convert VR7082-scFv intrabody into a degrabody capable of degrading 4R tau, the GFP domain was replaced with one of six degradation domains that were selected from the literature. These domains were fused as an N or C terminal fusion to VR7082-scFv, dependent on the orientation suggested by the literature (Table 5).

Table 5: Degradation domains selected for fusion to VR7082-scFv intrabody to form a degrabody.

Degradation domain	Acronym	N or C terminal fusion to scFv	Method of degradation	Literature reference
C-terminal sequence of Ornithine decarboxylase	ODC	C terminal	Ubiquitin proteasome independent	Zhao, Pferdehirt, and Segatori 2018
Human FkBP-12 protein	FkBP	C-terminal	Ubiquitin proteasome dependent	Banaszynski et al. 2006
C-terminus of Hsc70 interacting protein	CHIP	C-terminal	Ubiquitin proteasome dependent	Portnoff et al. 2014
X-linked inhibitor of apoptosis protein	XIAP	C-terminal	Ubiquitin proteasome dependent	Gross et al. 2016
von Hippel–Lindau protein	VHL	N-terminal	Ubiquitin proteasome dependent	Fulcher et al. 2017
N-terminus of Slmb protien	NSlmb	N-terminal	Ubiquitin proteasome dependent	Caussinus, Kanca, and Affolter 2011

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All degrabody fusion constructs as well as GFP N and C terminal fusions (as non-degrading intrabody controls), were co-transfected into HEK-293F cells with either 0N3R or 0N4R tau. 48h post-transfection, the cells were lysed and analysed via Western blot utilising the polyclonal total tau antibody (Dako). Band densitometry was then carried out and values were expressed as a percentage of the GFP fusion control. A representative Western blot (**Figure 21A**) and the data for the n=3 densitometry from the tau Western Blots (**Figure 21B**) can be seen.

To allow for accurate comparison of degradation each construct is expressed as % tau compared with the non-degrading VR7082-GFP control (fused to the respective N or C termini) (**Figure 21B**). This was important as it shows the effect of the degrabody over and above a binding, but non-degrading intrabody.

The data presented in **Figure 21** demonstrates that the co-transfection of VR7082 degrabody with 0N4R tau induces the degradation of 0N4R compared with the non-degrading VR7082-GFP intrabody control. It is also possible to see that this is not the case when the degrabody constructs are co-transfected with 0N3R tau.

Following this work, it was interesting to determine if the presence of MG132 (a proteasome inhibitor) would stop the degradation of 4R tau. This should be the case in all degrabodies except for ODC, which has been previously demonstrated to be less dependent on the ubiquitin proteasome (Erales and Coffino, 2014, *Biochim Biophys Acta*, 1843 (1), 216-21). Degrabody constructs were co-transfected with 0N4R or 0N3R tau in

HEK-293F cells, following a 6-hour incubation, MG132 was added to half of the cultures. All cultures were then incubated for a further 18 hours before analysis via flow. Flow cytometry was used rather than western blotting in these experiments as it allowed for higher throughput screening of all the degrabody constructs against 0N3R and 0N4R tau with and without MG132 treatment. Whilst technically possible by Western Blotting the numbers of blots required meant this technique was much less practical when testing many conditions and constructs. It was also important to ensure that any degradation was not a Western blotting artefact and that it could be confirmed by multiple techniques. Representative flow cytometry plots and comparative assay data can be seen in **Figure 22A** and the geomean of all single cell populations co-transfected with VR7082 degrabodies normalised to VR7082-GFP levels can be seen in **Figure 22B**.

The gating strategy for this assay was to first enable identification of HEK-293F cells (**Figure 22A-1**) followed by single cell identification via forward scatter area compared with height (**Figure 22A-2**). The single cells were then assessed for intracellular tau staining via polyclonal total tau antibody (Dako) in either 0N4R- (**Figure 22A-3**) or 0N3R- (**Figure 22A-4**) transfected cells.

As described above for **Figure 21B** in **Figure 22B**, all tau levels are expressed as a % of the VR7082-GFP (binding but non-degrading intrabody) control. The data in **Figure 22** clearly demonstrates in a separate assay system that all of the degrabody constructs are capable of degrading 0N4R tau whilst retaining levels of 0N3R tau within HEK-293F cells. With a statistically significant reduction of 4R tau levels compared to 3R tau levels (via T-test) for all constructs (**Figure 22 B**). All fusions except for ODC were proteasome-dependent as MG132 treatment blocked the degradation of 4R tau (**Figure 22 B**). With a statistically significant difference (via T-test) for all 0N4R co-transfections with an without the addition of MG132 (**Figure 22 B**). Once again this is not the case with 0N3R tau transfected cells which show no statistical difference between the MG132 treated and untreated groups (**Figure 22 B**) indicating that treatment with MG132 is effecting only the proteasome degradation and not the general tau proteostasis within the cell.

The data presented in **Figure 21** and **Figure 22** clearly demonstrate that VR7082-scFv intrabody has been successfully converted into an scFv degrabody capable of the specific degradation of 0N4R tau.

Example 11 - Degradation of 4R tau using VR7082 degrabody in iPSC derived neurones

In transfection of neurons via lipid-based transfection methodologies only a small percentage of cells receive transfected DNA (Karra and Dahm 2010). For this reason, viral transfection using Adeno-associated virus (AAV) was selected as the method of delivery for degrabodies and controls for this project. Three AAV constructs were acquired from GeneCopeia, two of which contained VR7082-scFv fused to either the XIAP degradation domain or Halo tag (non-degrading control) as well as an AAV vector negative control with a construct lacking an. The VR7082-XIAP construct was also acquired with and without IRES expression of a GFP transfection marker.

The VR7082-XIAP + IRES GFP AAV was initially used to transfect the 10+16 biallelic *MAPT* mutant, neurons at day 90 post induction. Cells were analysed seven days post-transfection to allow for expression of the degrabody and GFP transfection marker. Two populations of cells were then identified and sorted using flow cytometry based on GFP positive or negative signal (**Figure 23A-3**). These two populations were lysed and tau protein levels were assessed using Peggy Sue simple Western (**Figure 23B** and **Figure 23C**). The full gating strategy can be seen in **Figure 23A**.

Live cells were initially identified via lack of ToPro3 staining (**Figure 23A-1**). Intact cells were identified via FCS-A v SSC-A (**Figure 23A-1-2**). It is interesting to note that there are lots of events that are ToPro3 negative but are very small and in the debris area (bottom left of the plot) of the FSC-A v SSC-A plot. It is possible they are cellular debris that has formed a small lipid micelle. Such debris would in theory have an intact lipid bilayer membrane and as such would exclude the ToPro3 stain (in the way a live cell does). Finally, cells were sorted two ways based on GFP expression (**Figure 23A-3**).

Following sorting, the two cell populations were lysed and then run on a Peggy Sue Simple Western. The resulting traces can be seen from the sorted GFP-negative cells (**Figure 23B**) and the sorted GFP-positive cells (**Figure 23C**). The trace for the GFP-negative cells displays two peaks, a large peak representative of 0N3R, and a small peak corresponding to 0N4R tau (**Figure 23B**). In contrast, the GFP-positive cells had a peak representing 0N3R tau, but no peak representing 0N4R tau was detected (**Figure 23B**). This suggests that 0N4R has been degraded in cells transduced with XIAP.

Taken together, the data presented in **Figure 23** demonstrates that it is possible to deliver a degrabody to iPSC derived neurons and degrade 4R tau protein from these cells.

Example 12 - Phenotypic effect of 4R tau removal on iPSC derived neurons

Previous work has shown that GFP can be toxic to neuronal cells (Detrait et al. 2002, *Mol Ther*, 5 (6), 723-30). As the effect of GFP on the mitochondrial polarisation assay is unknown, for this work an AAV vector was used without GFP. For the same reason a VR7082-Halo tag intrabody as a non-degrading control rather than the previously used VR7082-GFP intrabody.

Neurons were differentiated from WT, 10+16 mono and 10+16 biallelic *MAPT* mutant iPSC lines cell lines. At day 80-90 the lines were treated with AAV containing VR7082-XIAP, VR7082-Halo or a negative AAV construct and were incubated for a further 7 days. Following incubation the cells were run in a mitochondrial membrane polarisation assay. In which mitochondrial membrane polarisation is assessed via staining with MitoTracker Deep Red CMX ROS and normalised to total mitochondrial load via staining with Mito Tracker deep red. The resultant data can be seen in **Figure 24**.

The gating strategy for this assay is to identify intact neuronal cells which can be assayed for mitochondrial membrane polarisation and total mitochondrial load (**Figure 24A**). A representative example of each treatment group on each iPSC derived neuronal cell line (**Figure 24B**) demonstrates that in WT control neurons, (**Figure 24B-1**) there is no effect of either the control AAV, VR7082-Halo or VR7082-XIAP treatment groups.

However, when these treatments are applied to 10+16 mono neurons, (**Figure 24B-2**) or 10+16 biallelic neurons, (**Figure 24B-3**) treatment with VR7082-XIAP degrabody reduced the level of mitochondrial membrane polarisation. This was observed in n=5 neuronal inductions for each genotype and treatment group (**Figure 24C**). As expected, no statistically significant difference in mitochondrial polarisation was observed in

10+16 *MAPT* mutant neurons between the control AAV particle treatment and VR7082-Halo intrabody for the 10+16 mono or 10+16 biallelic lines ($p=0.145 + p=0.922$, ANOVA). This is because although the VR7082-Halo intrabody will bind to 4R tau within these cells, it will not induce degradation of 4R tau. However, when cells were transfected with VR7082-XIAP degrabody a statistically significant decrease of mitochondrial polarisation is observed in both the 10+16 mono and 10+16 biallelic mutant cell lines from the control AAV ($p<0.0001 + p=0.0052$, ANOVA) and from the VR7082-Halo treated groups ($p<0.0001 + p=0.0018$, ANOVA) (**Figure 24C**). It is interesting to note that whilst only a modest decrease of the mutant phenotype is

observed in the 10+16 biallelic neurons, in the 10+16 mono neurons the level of mitochondrial polarisation is reduced to levels that are not significantly different from the WT neurons ($p=0.567$, ANOVA) (**Figure 24C**).

Because the VR7082-RbIgG 4R specific antibody has the same epitope as the VR7082-scFv degrabody, it was not possible to assess 4R tau levels in these experiments. However, it has been previously demonstrated (**Figure 21**, **Figure 22** and **Figure 23**) that VR7082-XIAP specifically induced 4R tau degradation and not 3R degradation. It was confirmed that there was no change in 3R tau levels between any of the treatment groups, suggesting no off target degradation effect in these experiments (**Figure 24D**).

Here have we successfully demonstrated that the VR7082 degrabody can be applied to 10+16 *MAPT* mutant iPSC-derived neurons to restore non- mutant levels of mitochondrial membrane polarisation.

Example 13 - Epitope characterisation of VR7082 antibody

Intracellular tau expression constructs were designed to make single alanine point mutations across the peptide epitope TE10 as well as the P301S and L mutations common in disease models of tauopathy. The constructs were synthesised by TWIST bioscience into a vector suitable for the intracellular expression of tau.

Upon receipt of the constructs, single constructs were transiently transfected into Expi293 cells (Thermo-Fisher) according to the instructions from the manufacturer. The cells were incubated at 38°C, shaking at 220RPM, in a humidified 5% CO₂ environment. Following a 48 hour incubation, the cells were harvested via centrifugation at 350G_(av) for 10 minutes. The cells were fixed and permeabilized with Invitrogens Fix and Perm kit, according to the instructions from the manufacturers. The cells were then stained with a non-VR7082 cross blocking antibody HT7 labelled with alexa-488, for 30 minutes on ice before washing in PBS+1% BSA, and then seeded at 20,000 cells/well. Each well was subsequently stained with 10 µg/ml VR7082 for 30 minutes on ice and the cells were washed once in PBS+1%BSA and a secondary anti-rabbit-Fc -Alexa647 (Jackson Laboratories) reveal antibody was added at 1:1000 dilution. The cells were incubated on ice for 30 minutes before being washed in PBS+1% BSA and resuspended in 50µl/well. The cells were run on an IQue3 and tau containing cells were gated via the alexa-488 fluorescence from HT7 and tau binding assessed via alexa-647 staining. The data was

normalised and expressed as a percentage of the binding signal observed for not-mutated tau.

The results obtained are presented in **Figure 26**. The data in **Figure 26** demonstrates the knockdown of tau binding signal in the presence of a single specific alanine mutation across the peptide-binding site (2nd to 10th bars going from left to right), the binding to P301S tau (11th bar going from left to right), P301L tau (12th bar going from left to right) and mock-transfected cells (final bar going from left to right). All values are expressed as a percentage of binding to unmutated 0N4R tau (the 1st bar going from left to right). The results show that mutation of any of K294; D295, N296, and I297 to alanine almost completely ablates binding of VR7082 antibody to tau. Mutation of either of K298 and V300 to alanine roughly halves binding of VR7082 antibody to tau. Mutation of H299, P301, and G302 did not affect binding of VR7082 to tau. The results also demonstrated that VR7082 is capable of binding both P301S and P301L indicating that it may be employed in animal or cellular models of tauopathies containing those mutations.

CLAIMS

1. An antibody or antigen-binding fragment thereof that:
 - (a) specifically binds 4R tau protein isoforms in a physiological sample; or
 - 5 (b) specifically binds 3R tau protein isoforms in a physiological sample.
2. The antibody or antigen-binding fragment thereof of claim 1, wherein the antibody or antigen-binding fragment thereof specifically binds 4R tau protein isoforms.
3. The antibody or antigen-binding fragment thereof of claim 2, wherein the antibody or antigen-binding fragment thereof:
 - 10 (a) binds to the amino acid region encoded by exon 10 of tau when the 4R tau protein contains a post translational modification at one or more of amino acid positions 279, 280, 281, 285, and 289 of tau 4R;
 - (b) binds to a peptide comprising, or consisting of, amino acids 294 to 302 of 4R tau protein;
 - 15 (c) binds to a peptide corresponding to a single epitope from the amino acid sequence encoded by exon 10 of tau; or
 - (d) cross-blocks or is cross-blocked by any of the antibodies or fragments of (a) to (c).
- 20 4. The antibody or antigen-binding fragment thereof of claim 2 or 3, wherein:
 - (a) the antibody or antigen-binding fragment specifically binds 4R tau protein isoforms in cell lysates from cells expressing physiological 4R tau protein isoform(s), preferably in cell lysates from iPSC derived neuronal cells expressing 4R tau protein isoform(s); and/or
 - 25 (b) the antibody or antigen-binding fragment is able to detect 4R tau protein isoforms via immunofluorescence on, or in, cells expressing 4R tau protein isoform(s).
5. The antibody or antigen-binding fragment thereof of any one of claims 2 to 4, wherein the antibody or antigen binding fragment thereof:
 - 30 (a) comprises one or more of the light chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs 3, 5, and 7 and one or more of the heavy chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 11, 13, and 15, and preferably comprises the light

chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs 3, 5, and 7 and heavy chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 11, 13, and 15;

(b) comprises the light and heavy chain variable regions of SEQ ID NOs 1 and 9;

5 (c) is an intrabody, preferably comprising the sequence of SEQ ID No: 17 or a sequence with at least 95% sequence identity thereto that is still able to specifically bind tau protein 4R isoforms; or

(d) cross-blocks or is cross-blocked by any of the antibodies or fragments of (a) to (c).

10 6. The antibody or antigen-binding fragment thereof of any one of claims 2 to 5, wherein the antibody or antigen-binding fragment thereof binding an epitope of 4R tau protein comprising amino acids K294, D295, N296, and I297.

7. The antibody or antigen-binding fragment thereof of any one of claims 2 to 5, wherein the epitope further comprises K298 and V300 of 4R tau protein.

15 8. The antibody or antigen-binding fragment thereof of claim 1, wherein the antibody or antigen-binding fragment thereof specifically binds 3R tau protein isoforms.

9. The antibody or antigen-binding fragment thereof of claim 8, wherein the antibody or antigen-binding fragment thereof:

20 (a) binds to a peptide that comprises the amino acid sequence encoded by the region bridging exons 9 and 11 of tau; and/or

(b) binds to a peptide consisting of seven amino acids either side of the boundary of the sequence encoded by exons 9 and 11 of tau.

10. The antibody or antigen-binding fragment thereof of claim 9, wherein the peptide is a cyclic peptide.

25 11. The antibody or antigen-binding fragment thereof of any one of claims 8 to 10, wherein the antibody or antigen binding fragment thereof:

(a) comprises one or more of the light chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs 20, 22, and 24 and one or more of the heavy chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 28, 30, and 32, and preferably comprises

- the light chain CDR1, CDR2, and CDR3 sequences of SEQ ID NOs 20, 22, and 24 and heavy chain CDR1, CDR2, and CDR 3 sequences of SEQ ID NOs: 28, 30, and 32;
- (b) comprises the light and heavy chain variable regions of SEQ ID NOs 18 and 26;
- 5 (c) is an intrabody, preferably comprising the sequence of SEQ ID No: 34 or a sequence with at least 95% sequence identity thereto that is able to specifically bind tau protein 3R isoforms; or
- (d) cross-blocks or is cross-blocked by any of the antibodies or fragments of (a) to (c).
- 10 12. An antibody or antigen-binding fragment thereof that specifically binds 4R tau protein, wherein the antibody or antigen-binding fragment thereof binds an epitope of 4R tau protein comprising amino acids K294, D295, N296, and I297, optionally where the epitope further comprises K298 and V300 of 4R tau protein.
13. An antibody or antigen-binding fragment thereof according to any one of the
- 15 preceding claims wherein the antibody is an intrabody or a degrabody.
14. A nucleic acid or nucleic acids encoding an antibody or antigen binding fragment according to any one of claims 1 to 13.
15. A vector or vectors comprising a nucleic acid or nucleic acids according to claim 14.
- 20 16. A host cell comprising a nucleic acid or nucleic acids according to claim 14 or a vector or vectors according to claim 15.
17. A pharmaceutical composition comprising:
- (a) an antibody or antigen-binding fragment thereof according to any one of claims 1 to 13, a nucleic acid or nucleic acids according to claim 14, or a vector or
- 25 vectors according to claim 15; and
- (b) a pharmaceutical carrier or excipient.
18. A method of detecting 4R tau protein isoforms comprising:
- (a) contacting a test sample with an antibody or antigen-binding fragment thereof according to any one of claims 2 to 7 or 12; and

(b) detecting binding of the antibody or antigen-binding fragment thereof.

19. A method of detecting 3R tau protein isoforms comprising:

(a) contacting a test sample with an antibody according or antigen-binding
5 fragment thereof to any one of claims 8 to 11; and

(b) detecting binding of the antibody or antigen-binding fragment thereof.

20. A method of determining the levels of 4R and 3R tau protein isoforms comprising
performing the methods of both claims 17 and 18.

10

21. The method of claim 19 which further comprises determining whether the relative
amount of 4R and 3R tau protein isoforms is different from that expected for a subject,
optionally by comparing to the relative amount of 4R and 3R in a sample from a healthy
subject.

15

22. The method of claim 19 or 20, wherein the method is used to diagnose
tauopathies, preferably PSP, CBD, or Pick's Disease.

23. The method of any one of claims 18 to 22 wherein:

20

- (a) the detection is via immunofluorescence;
- (b) the detection is via a Peggy Sue Simple Western blot;
- (c) the detection is via ELISA; or
- (c) the test sample comprises a cell lysate, cells, or tissue.

25

24. An antibody or antigen-binding fragment thereof according to any one of claims 1
to 13, a nucleic acid or nucleic acids according to claim 14, a vector or vectors according
to claim 15, or a pharmaceutical composition according to claim 16, for use in a method
of treating a tauopathy.

30

25. The antibody, antigen-binding fragment thereof, nucleic acid, nucleic acids,
vector or vectors, or pharmaceutical composition for use in a method according to claim
24, wherein the tauopathy comprises an imbalance between 4R tau protein isoforms and
3R tau protein isoforms.

26. A method of treating a tauopathy comprising administering an antibody or antigen-binding fragment thereof according to any one of claims 1 to 13, a nucleic acid or nucleic acids according to claim 14, a vector or vectors according to claim 15, or a pharmaceutical composition according to claim 15, to a subject with the tauopathy.

5

27. The method of claim 26, wherein the tauopathy comprises an imbalance between 4R tau protein isoforms and 3R tau protein isoforms.

28. Use of an antibody or antigen-binding fragment thereof according to any one of claims 1 to 113, a nucleic acid or nucleic acids according to claim 14, a vector or vectors according to claim 15, or a pharmaceutical composition according to claim 16, in the manufacture of a medicament for use in treating a tauopathy.

29. Use according to claim 28, wherein the tauopathy comprises an imbalance between 4R tau protein isoforms and 3R tau protein isoforms.

15

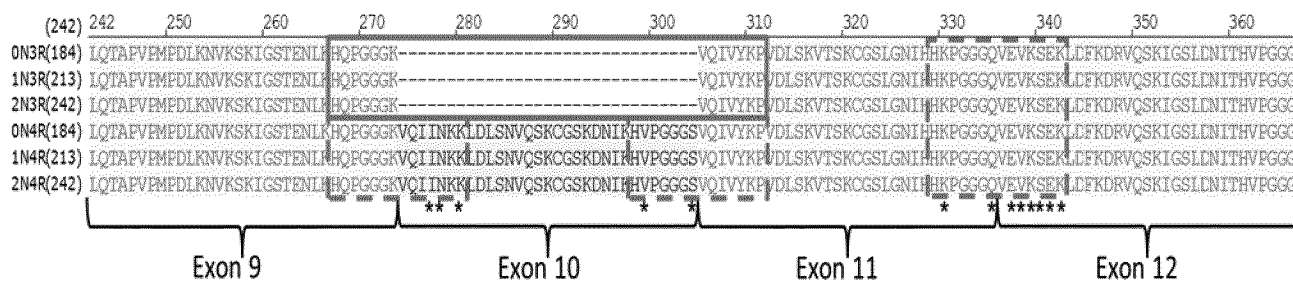


Figure 1

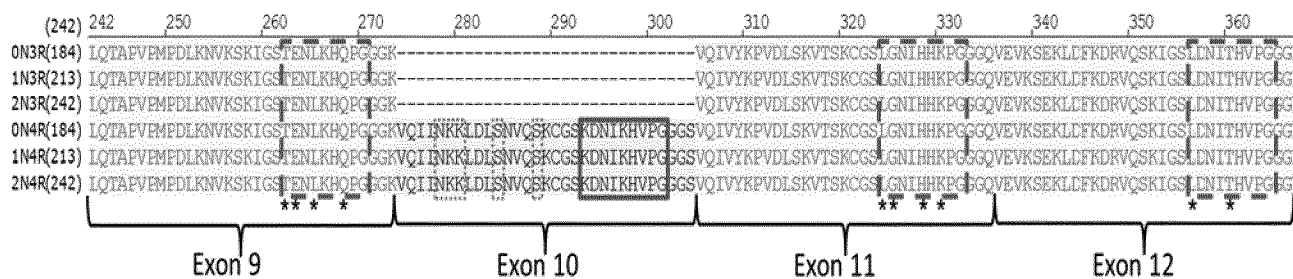


Figure 2

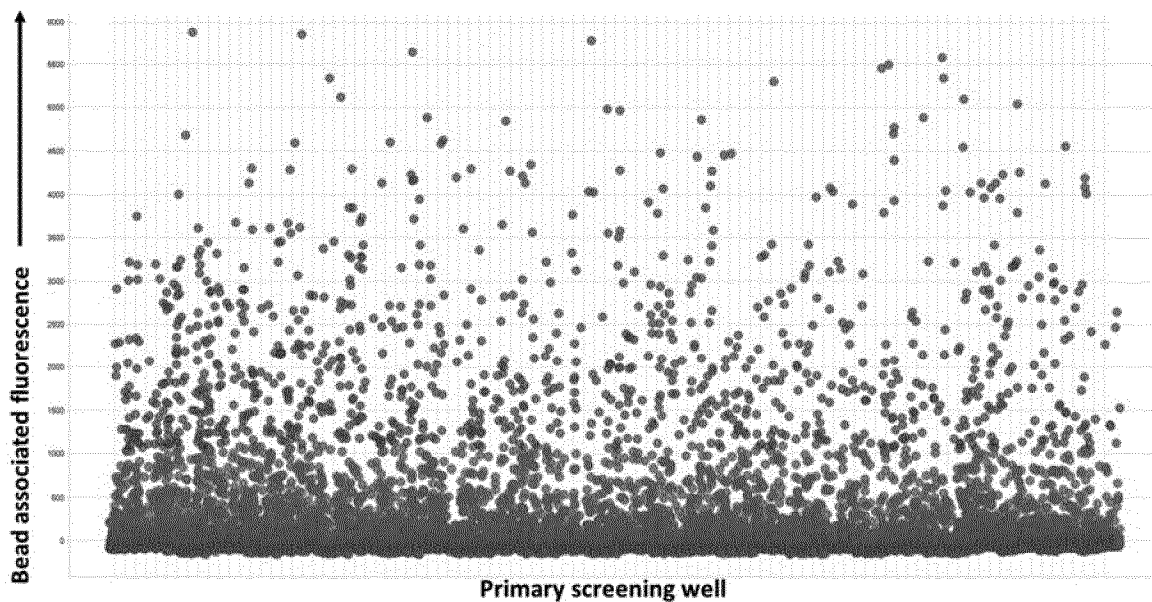


Figure 3

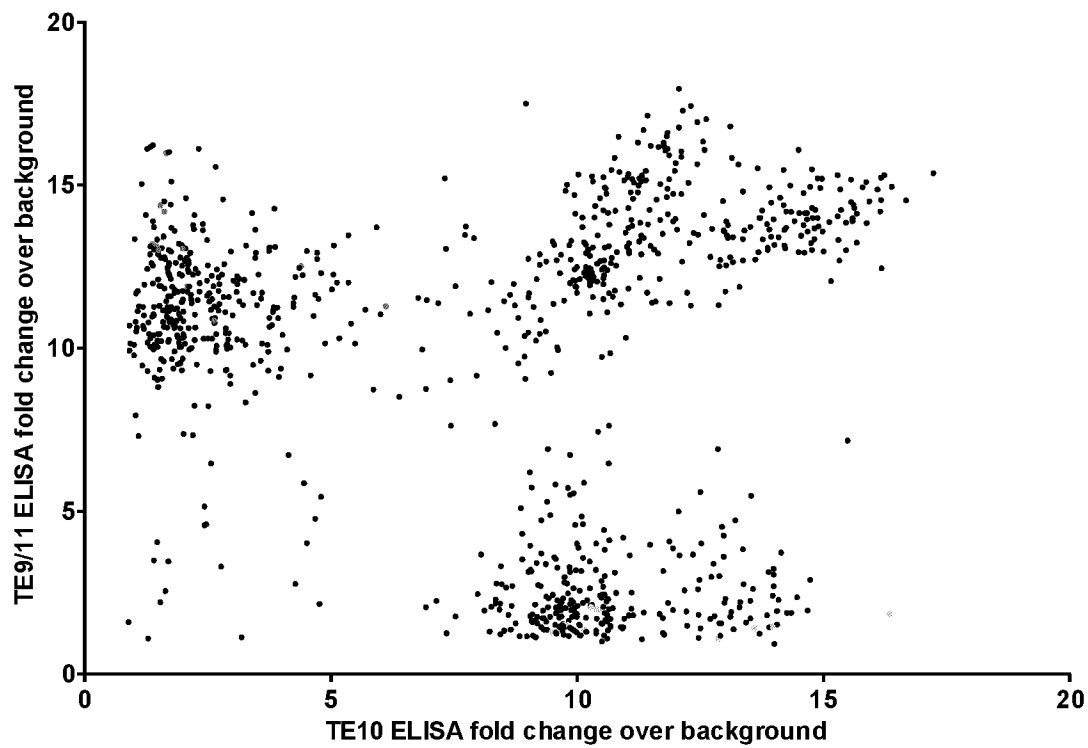


Figure 4

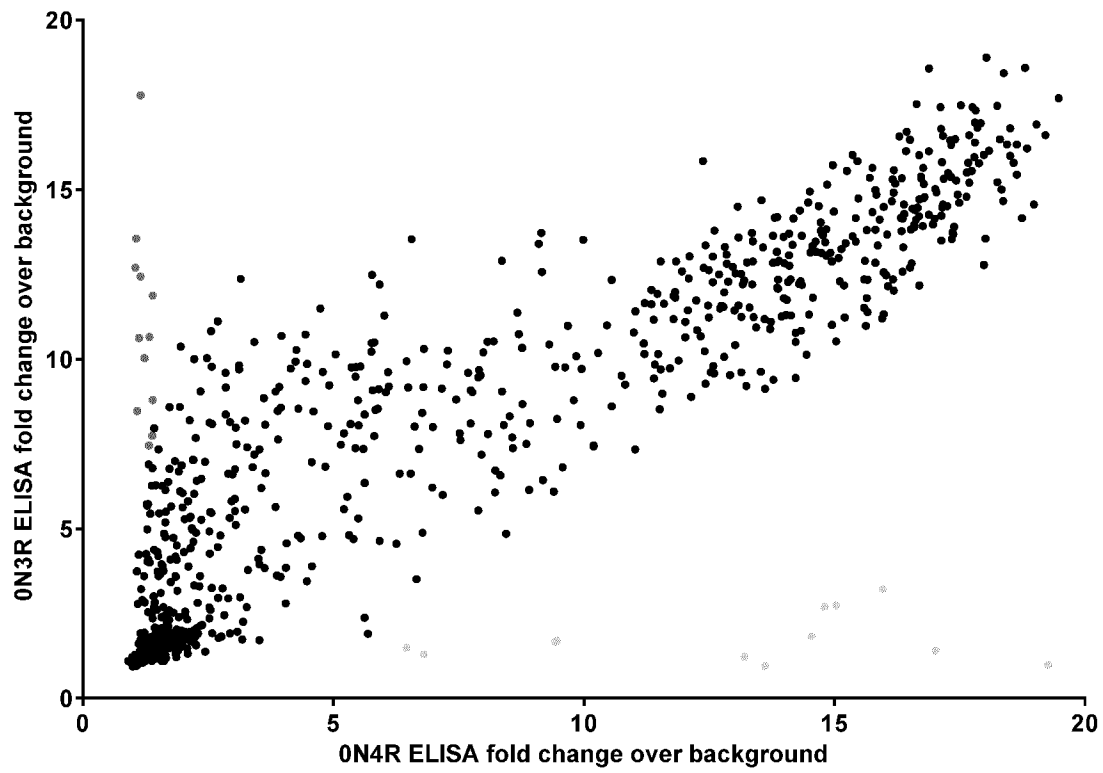


Figure 5

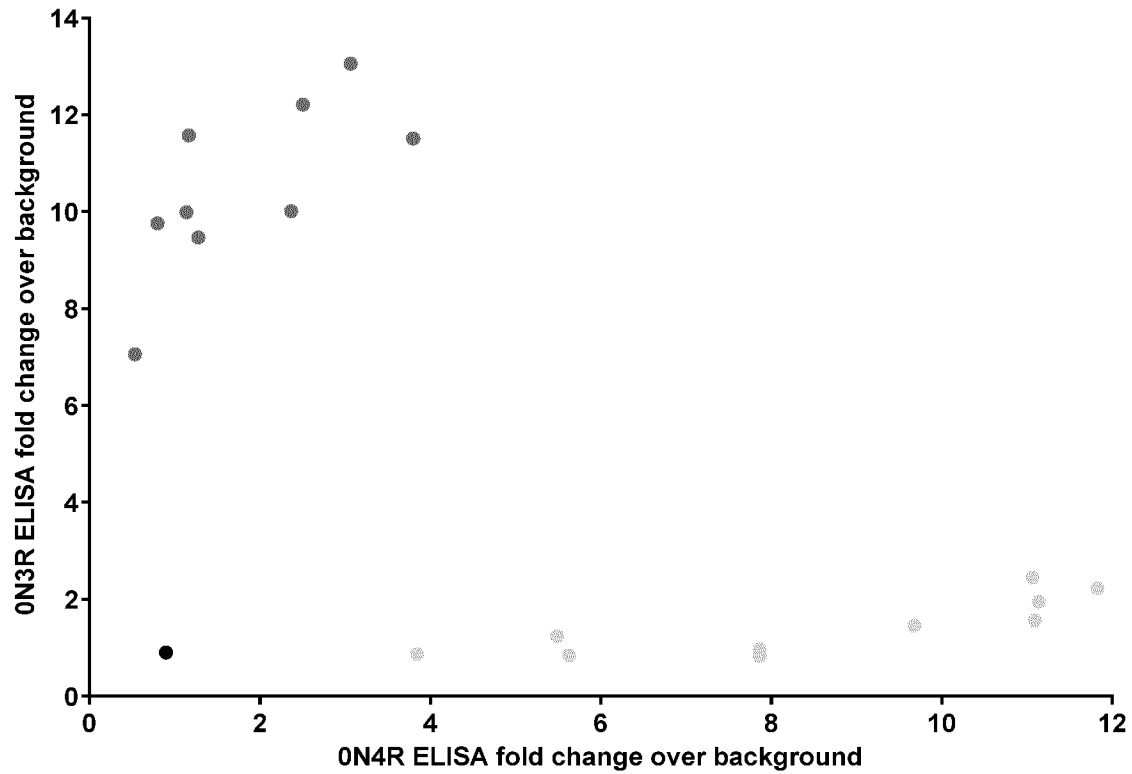


Figure 6

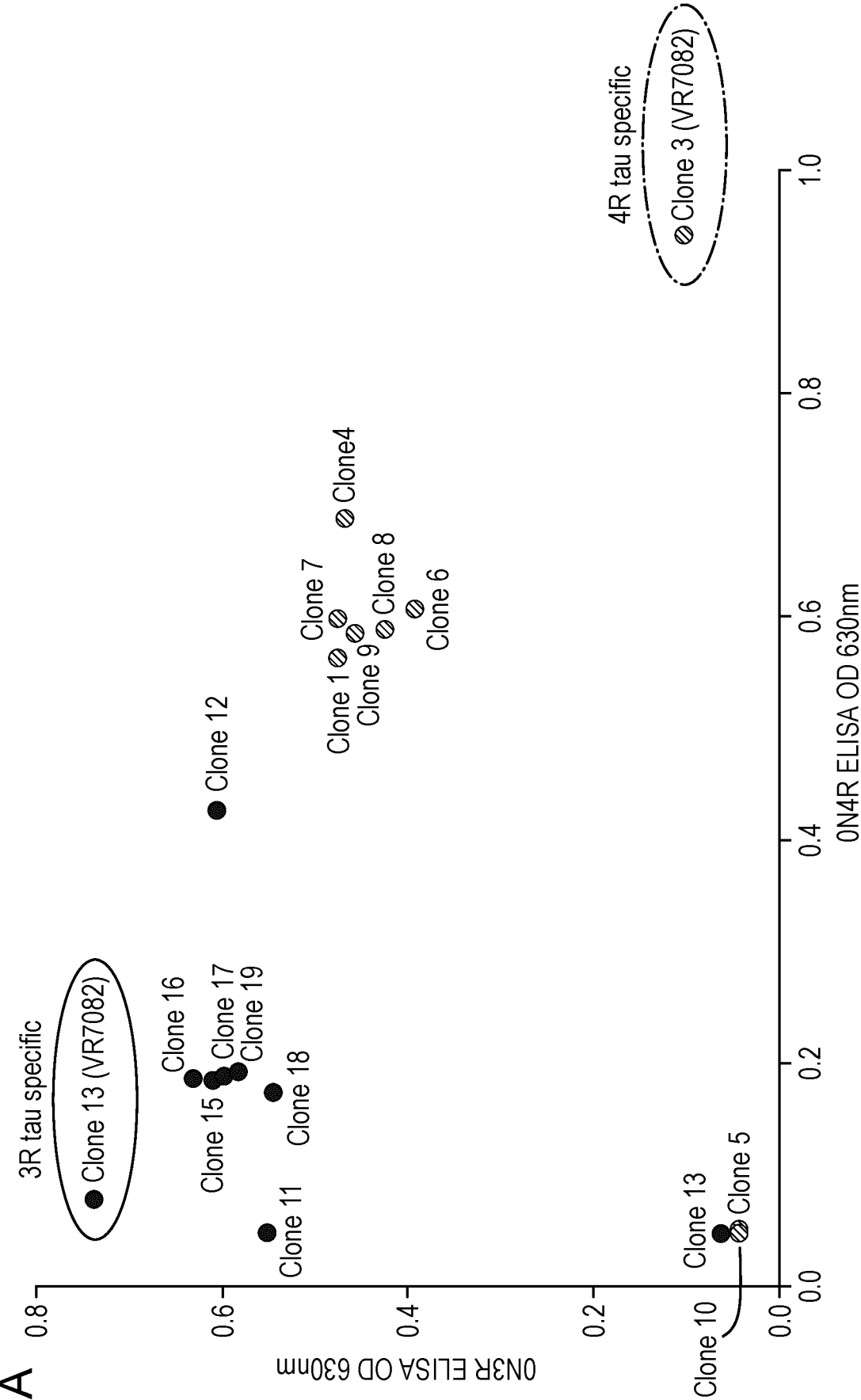


Figure 7

B

	vH CDR3										vK CDR3																		
	1	10	20	31	1	10	29	4R Tau specific antibodies																					
(VR7082) Clone 3	YFCARDTNSD	SHYGWEAFNI	WGPGTL	MTVSS	YYQ	QEG---	YSIHVDNV	FGGTE	VVV	KRT																			
Clone 2	YFCARDTNSD	SHYGWEAFNI	WGPGTL	MTVSS	YYQ	QEG---	YSIHVDNV	FGGTE	VVV	KRT																			
Clone 10	YFCARAADT	-----	ATSGYDIWRP	GTLDTVSS	YYQ	QC--	TDYGS	TYIGAF	GGGTE	VVV	ART																		
Clone 8	YFCARVRGNS	-----	RYFFMDLWGQ	TLMTVSS	YYCE	SNYWSFNT	NYGAP	FGGTE	VVV	KRT																			
Clone 5	YFCARWTTKY	-----	AEDGYDIWSP	GTLMTVSS	YYCAG	--CKST	ITID	SNTE	FGGTE	VVV	KRT																		
Clone 4	YFCARWTTKY	-----	AEDGYDIWRP	GTLMTVSS	YYQ	QSNYWSFNT	NYGAP	FGGTE	VVV	KRT																			
Clone 9	YFCARWTTKY	-----	AEDGYDIWSP	GTLMTVSS	YYQ	QSNYWSFNT	NYGAP	FGGTE	VVV	KRT																			
Clone 1	YFCARGTTKY	-----	AEDGYDIWGP	GTLMTVSS	YYQ	QSNYWSFNT	NYGAP	FGGTE	VVV	RR																			
Clone 7	YFCARGTTKY	-----	AEDGYDIWGP	GTLMTVSS	YYQ	QSNYWSFNT	NYGAP	FGGTE	VVV	KRT																			
Clone 6	YFCARGTTKY	-----	AEDGYDIWGP	GTLMTVSS	YYQ	QSNYGSFNT	NYGAP	FGGTE	VVV	KRT																			
	1	10	29	1	10	27																							
(VR7081) Clone 14	YFCARDAGIT	YSTYYFNI	QGP	GTLTVSS	YYCAG	GYDVNI	DIYGF	GGTE	VVV	KRT																			
Clone 11	YFCARDAGIT	YSTYYFNI	QGP	GTLTVSS	YYCAG	DYDVNI	DIYGF	GGTE	VVV	KRT																			
Clone 13	YFCARG	-----	LEMWGP	GTLTVSS	YYQ	VGD	FSG--	WIFAF	GGTE	VVV	KRT																		
Clone 12	YFCARG	-----	LEMWGP	GTLTVSS	YYCL	GGYIG	--HI	YVFF	GGTE	VVV	KRT																		
Clone 15	YFCARG	-----	LEIWGP	GTLTVSS	YYCL	GGYID	--HI	YVFF	GGTE	VVV	KRT																		
Clone 18	YFCARG	-----	LEIWGP	GTLTVSS	YYCL	GGYSG	--HI	YVFF	GGTE	VVV	KRT																		
Clone 19	YFCARD	-----	LEIWGP	GTLTVSS	YYCL	GGYSG	--HI	YVFF	GGTE	VVV	KRT																		
Clone 17	YFCARG	-----	LEIWGP	GTLTVSS	YYCL	GGYSG	--HI	YVFF	GGTE	VVV	KRT																		
Clone 16	YFCARG	-----	LEIWGP	GTLTVSS	YYCL	GGYLG	--HV	YVFF	GGTE	VVV	KRT																		

4R Tau specific
antibodies3R Tau specific
antibodies

Figure 7

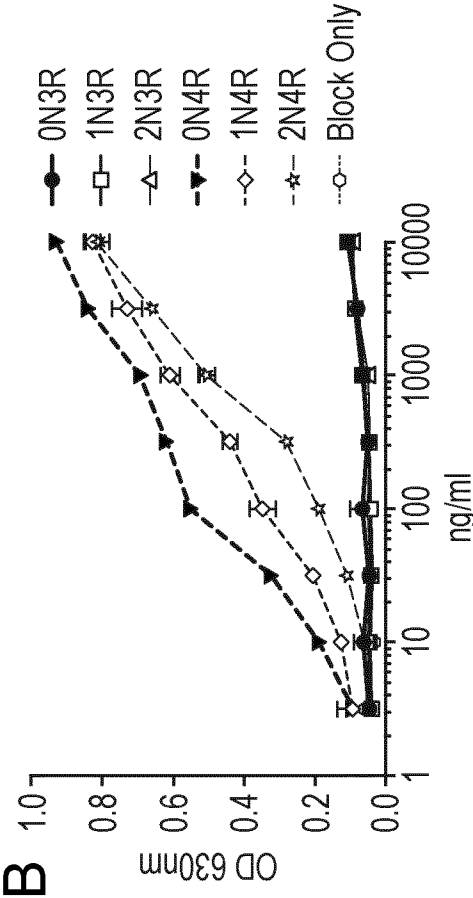


Figure 8

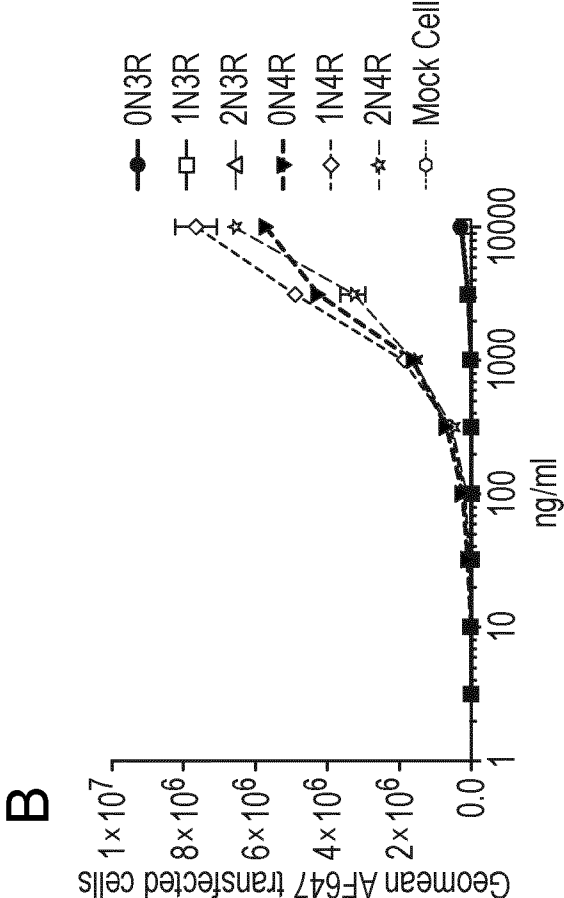
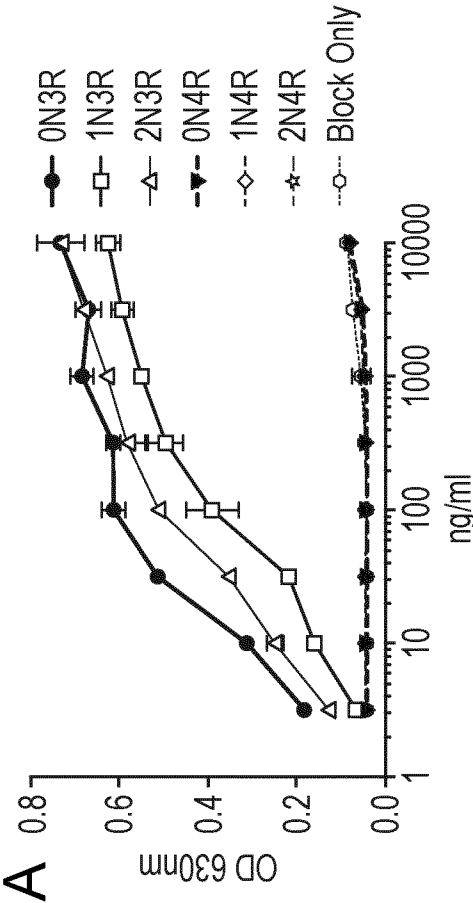
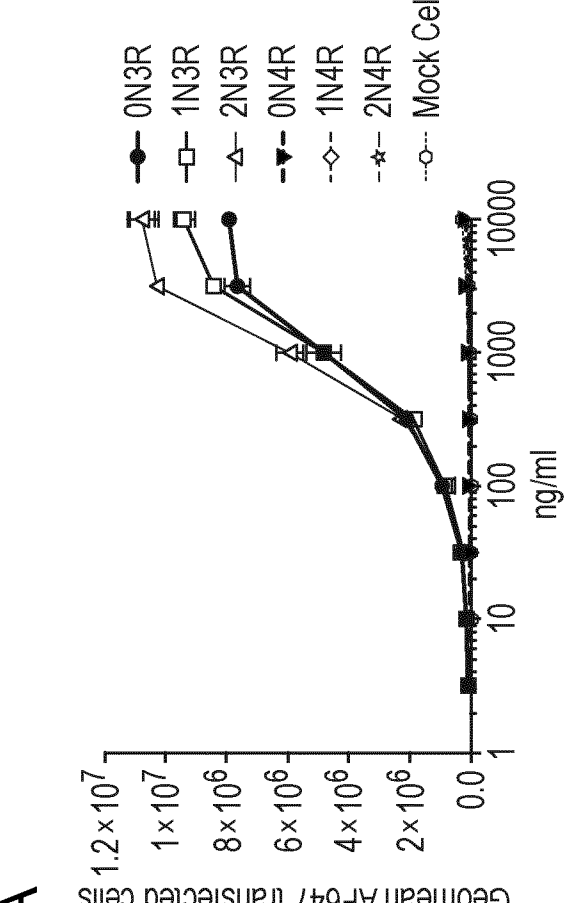
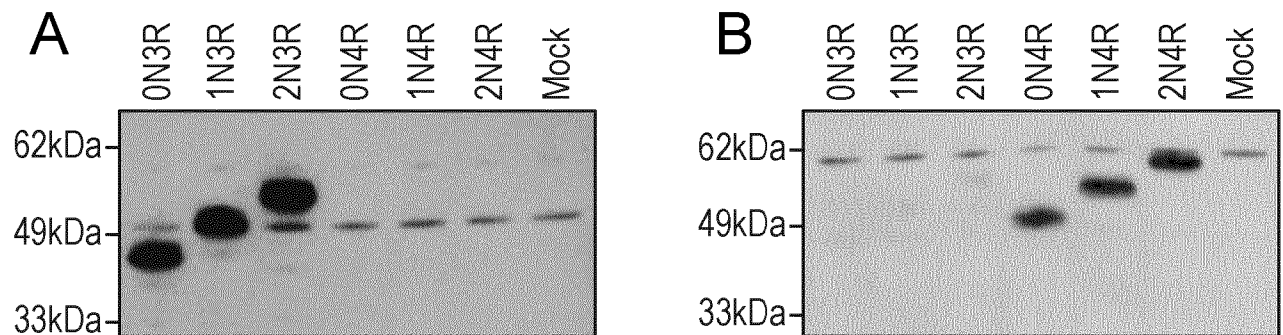
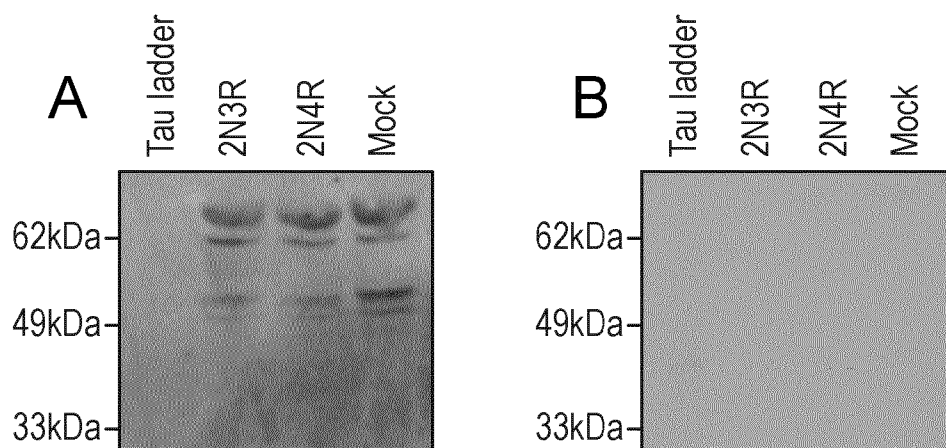
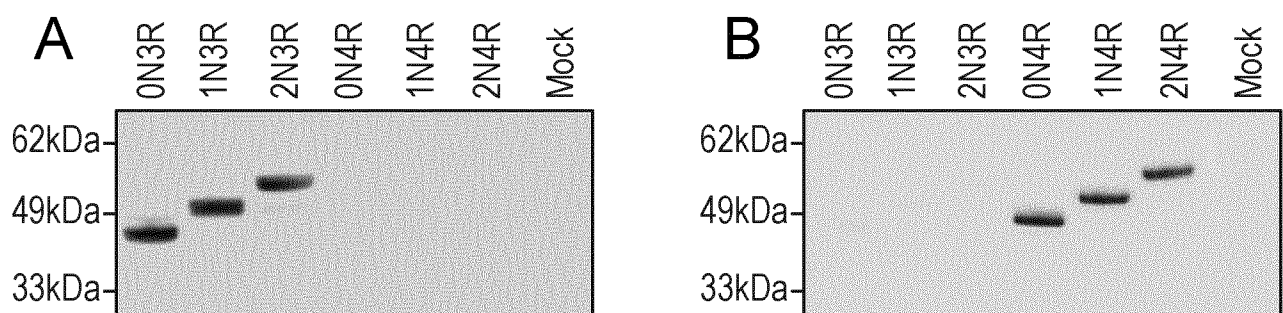


Figure 9



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**Figure 10****Figure 11****Figure 12**

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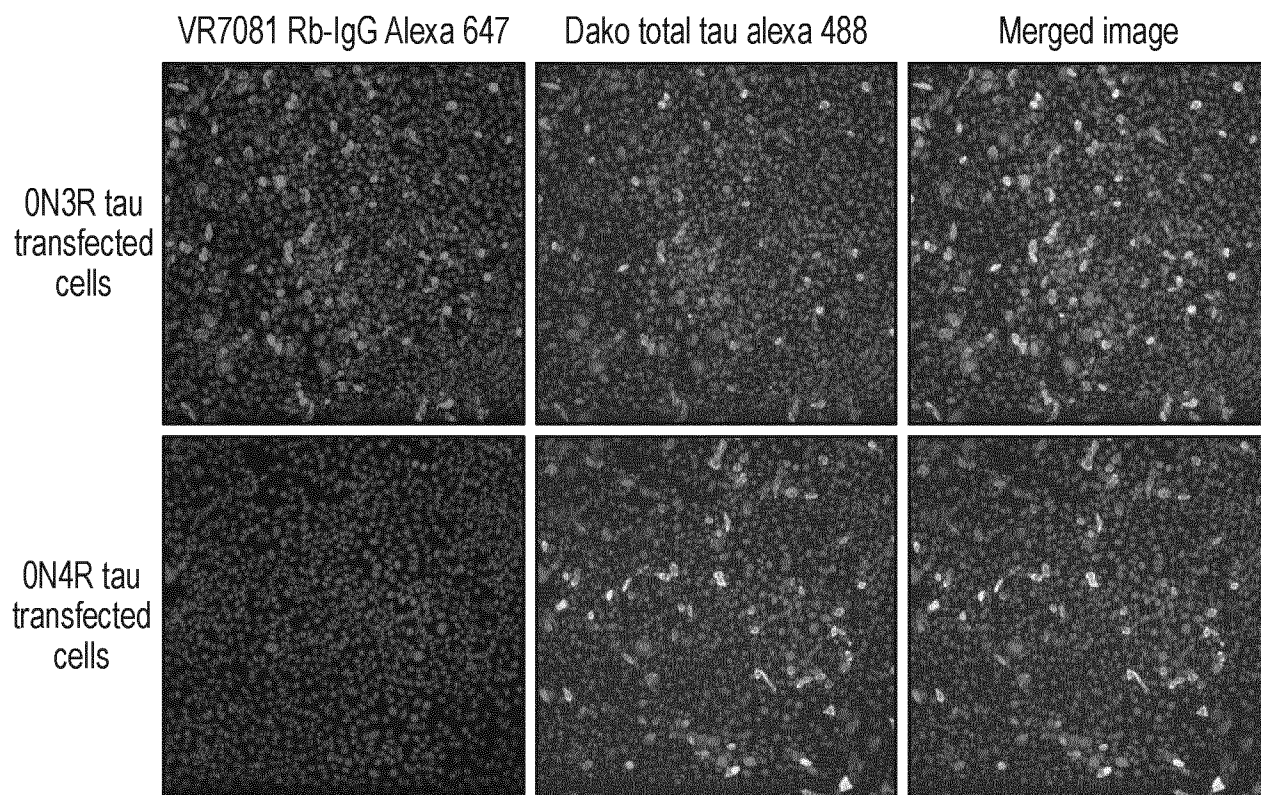


Figure 13

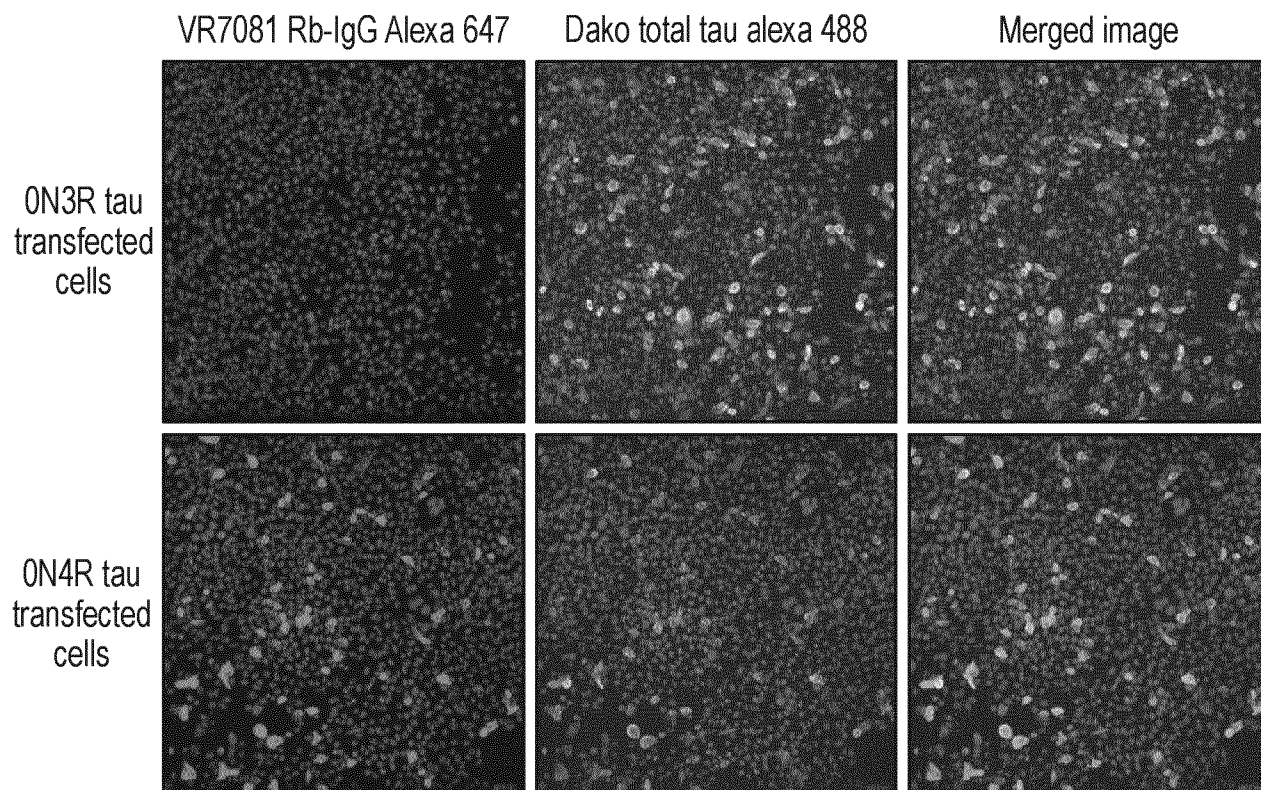


Figure 14

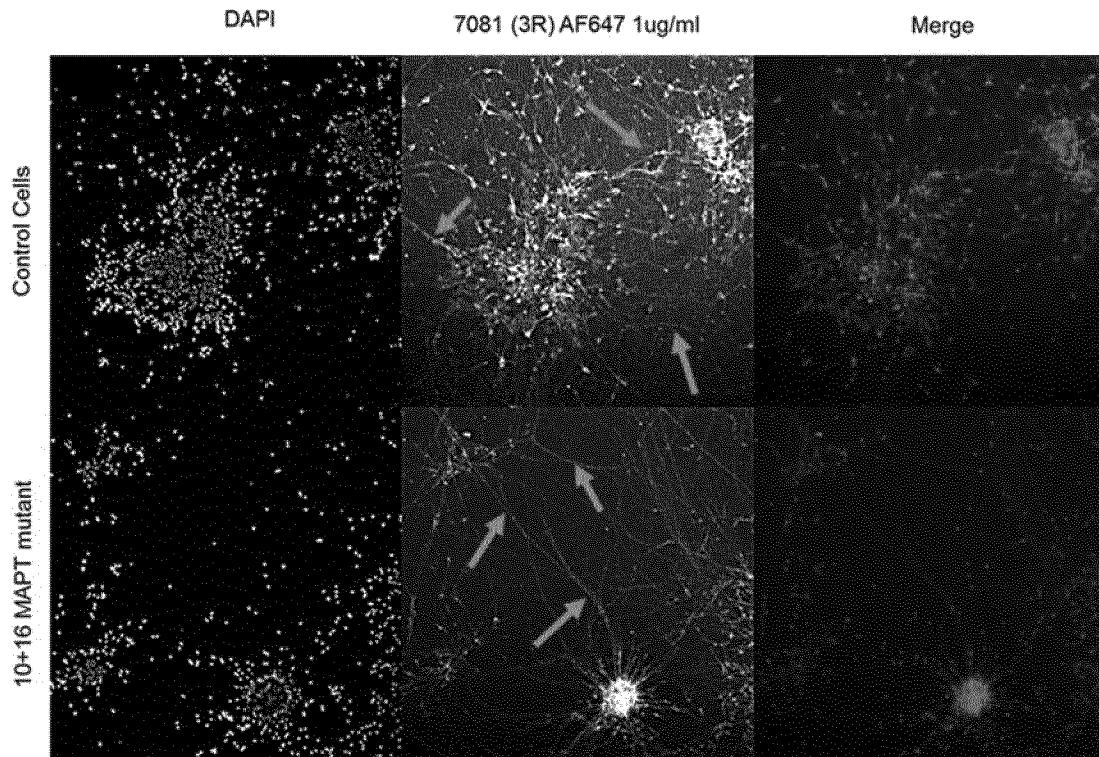


Figure 15

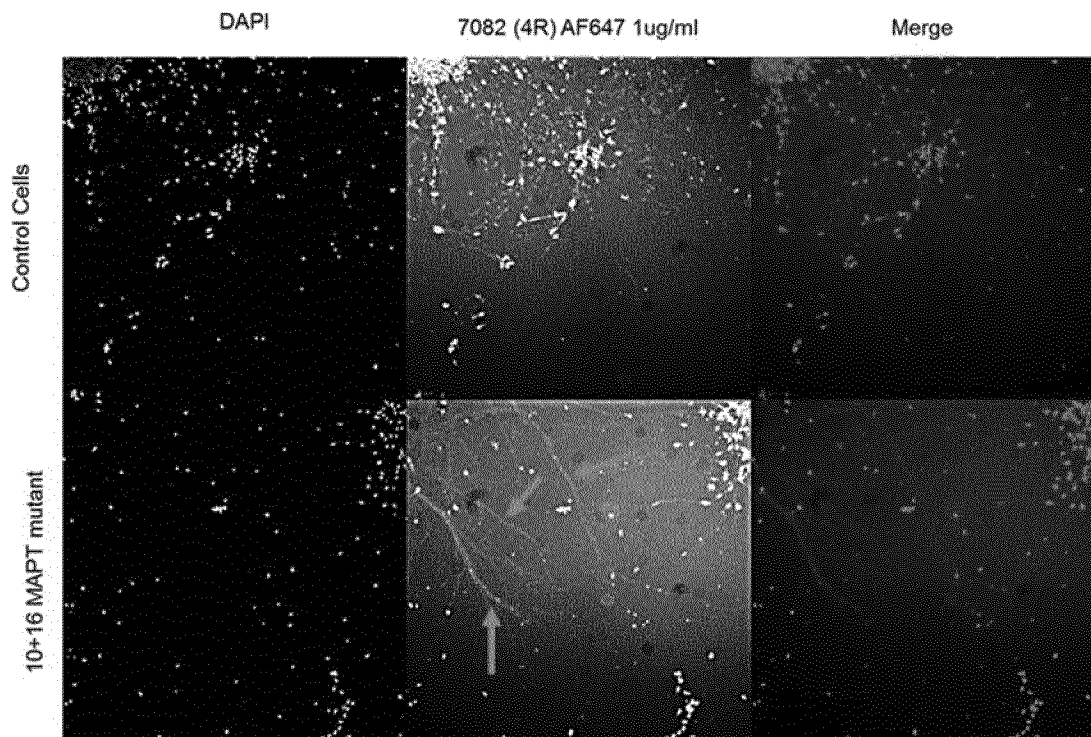


Figure 16

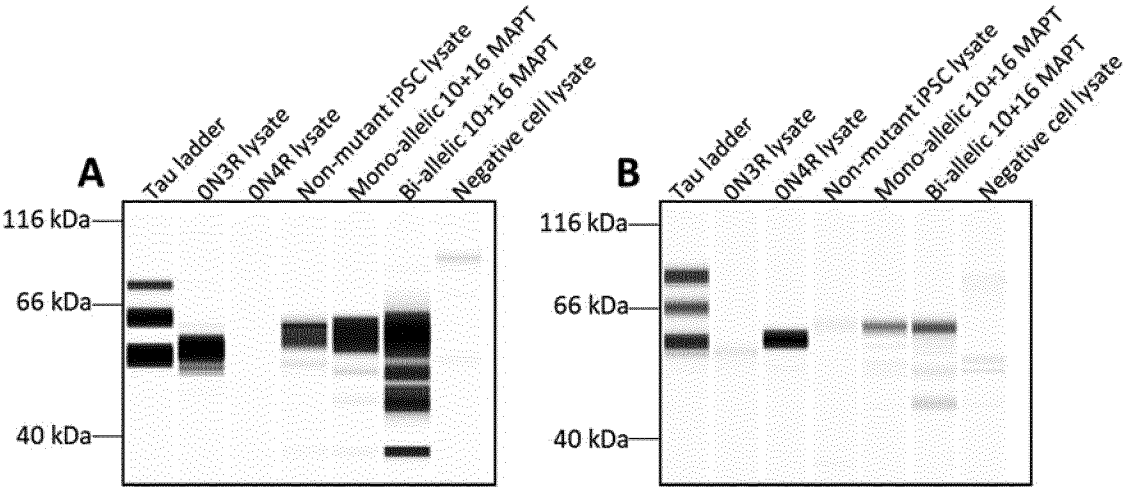


Figure 17

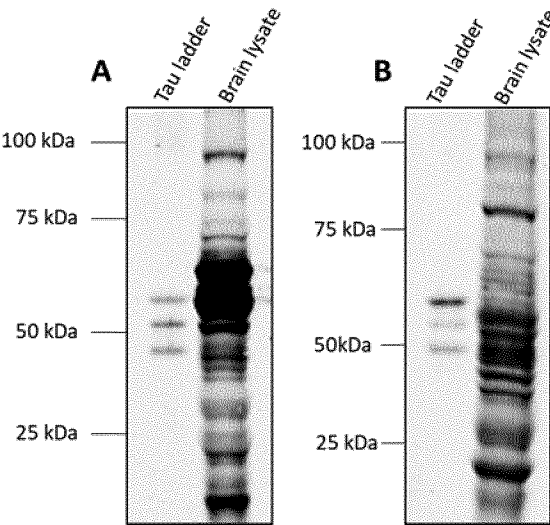


Figure 18

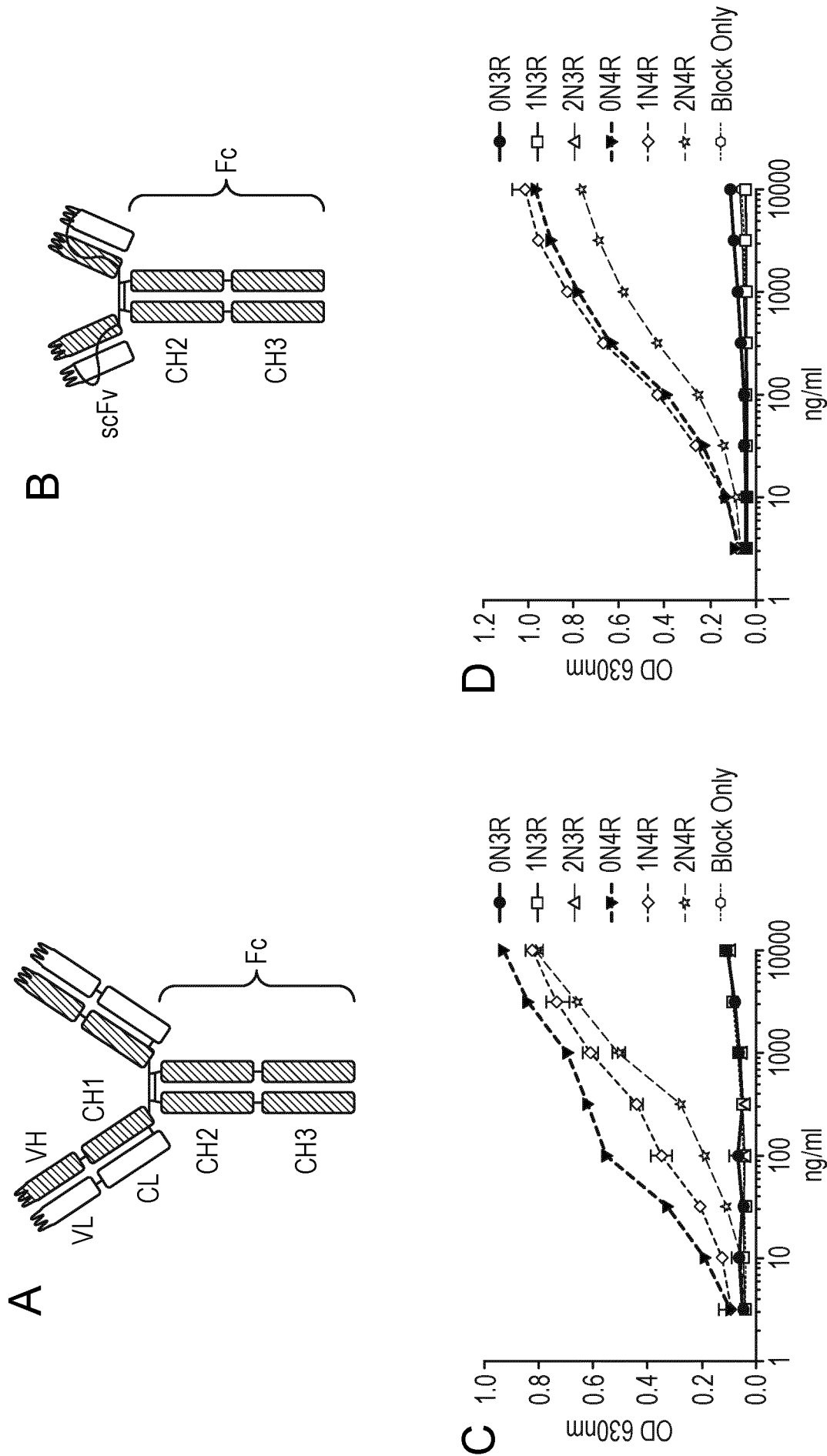


Figure 19

12/23

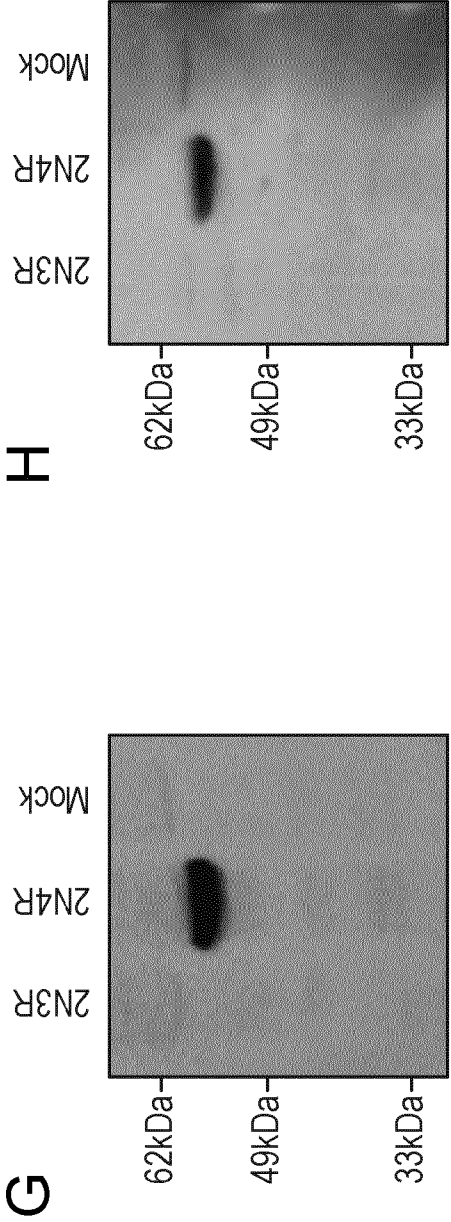
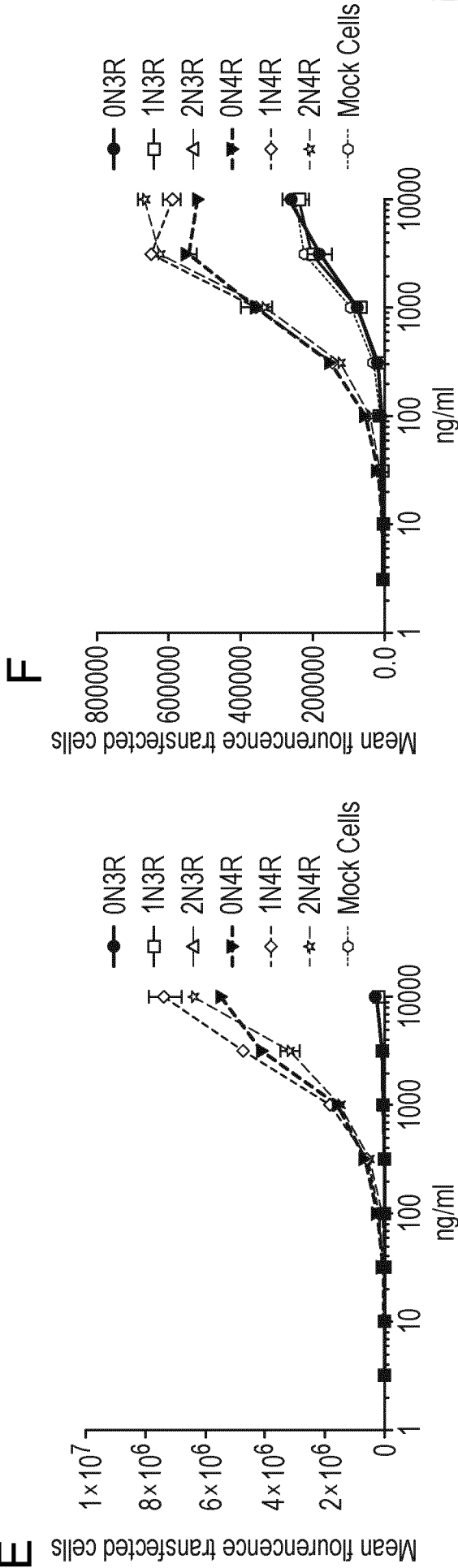


Figure 19

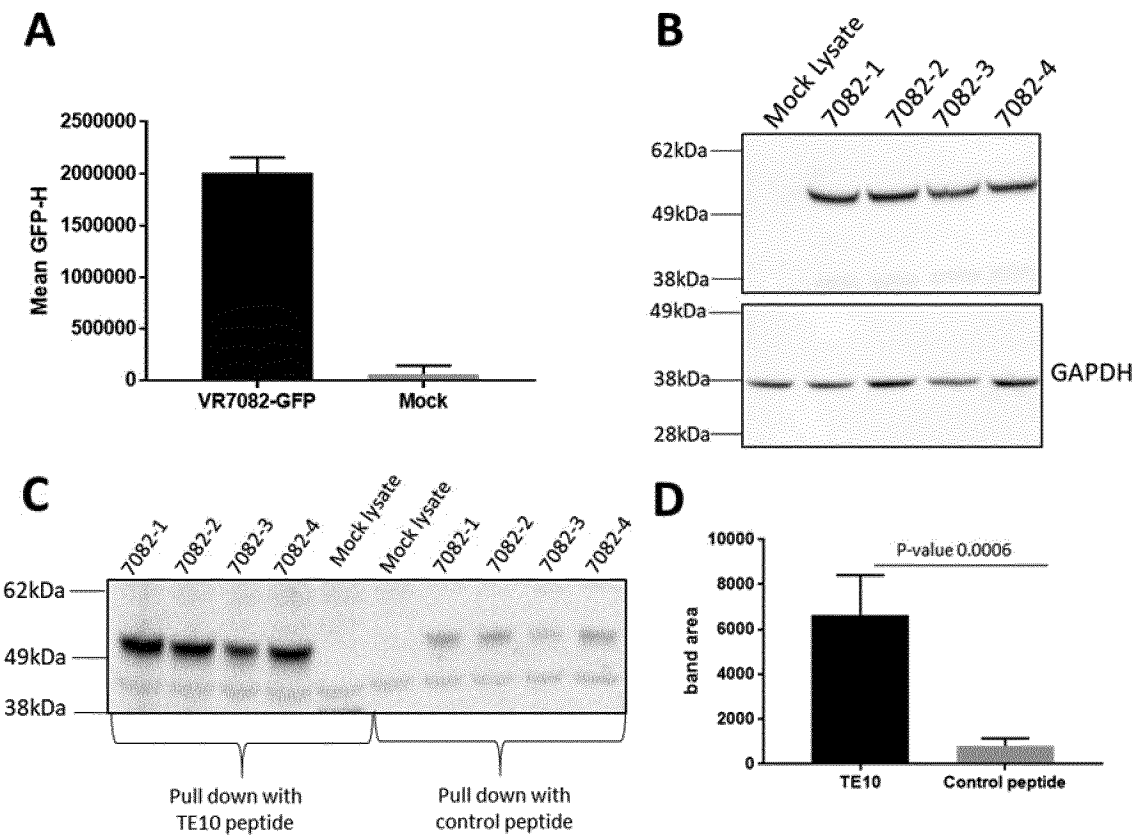
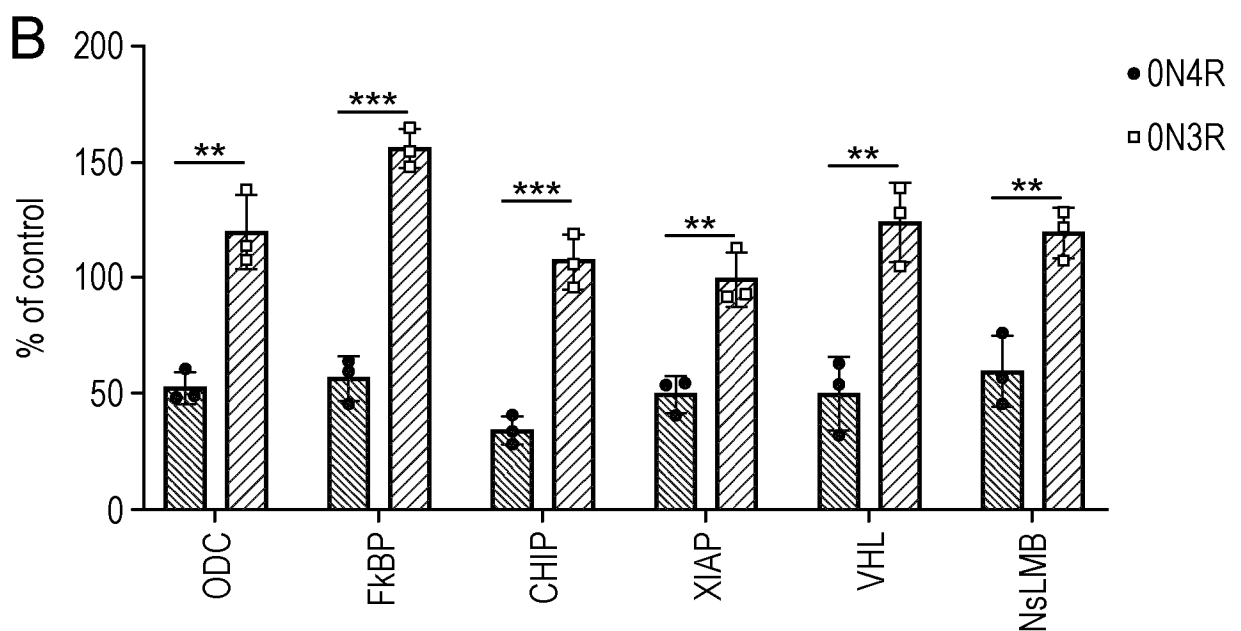
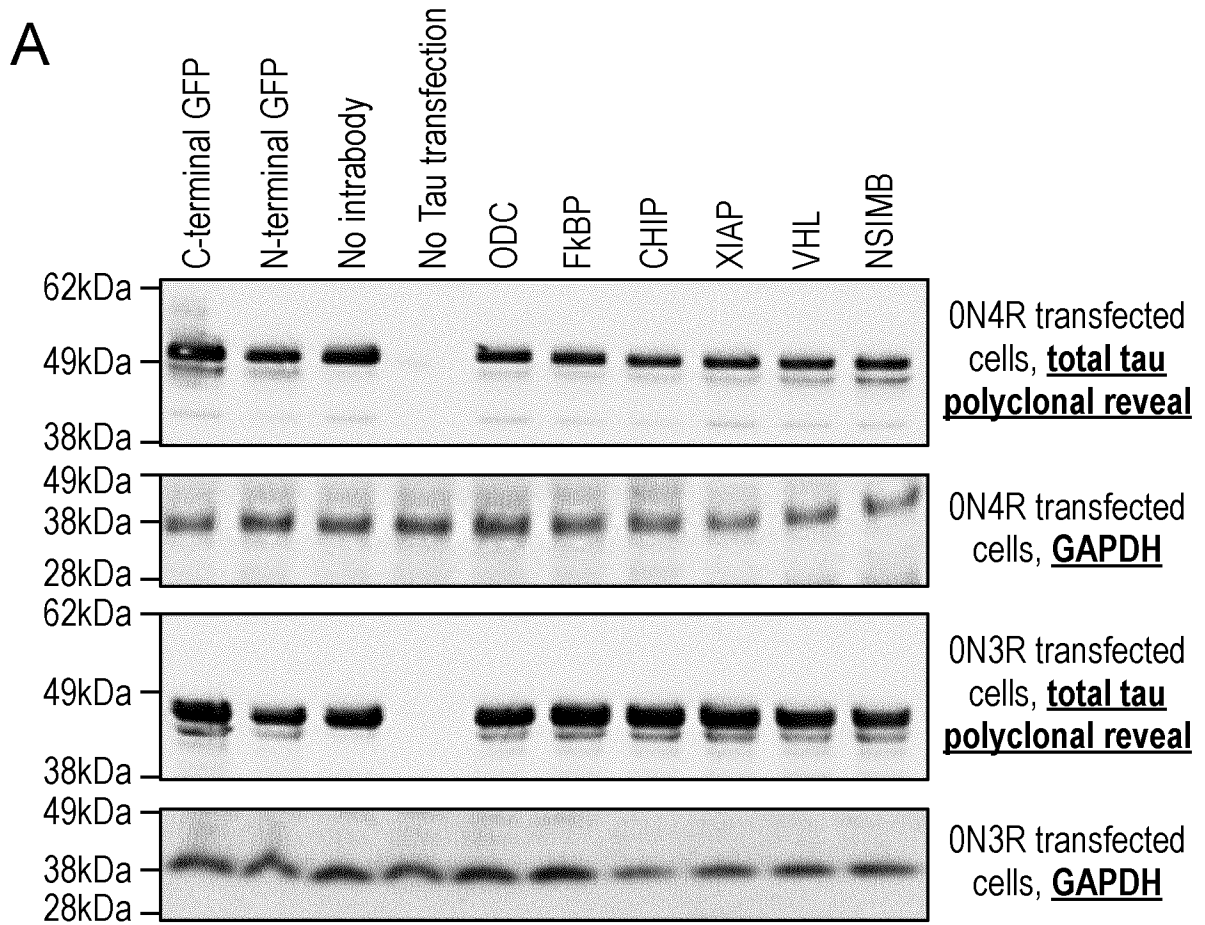


Figure 20

**Figure 21**

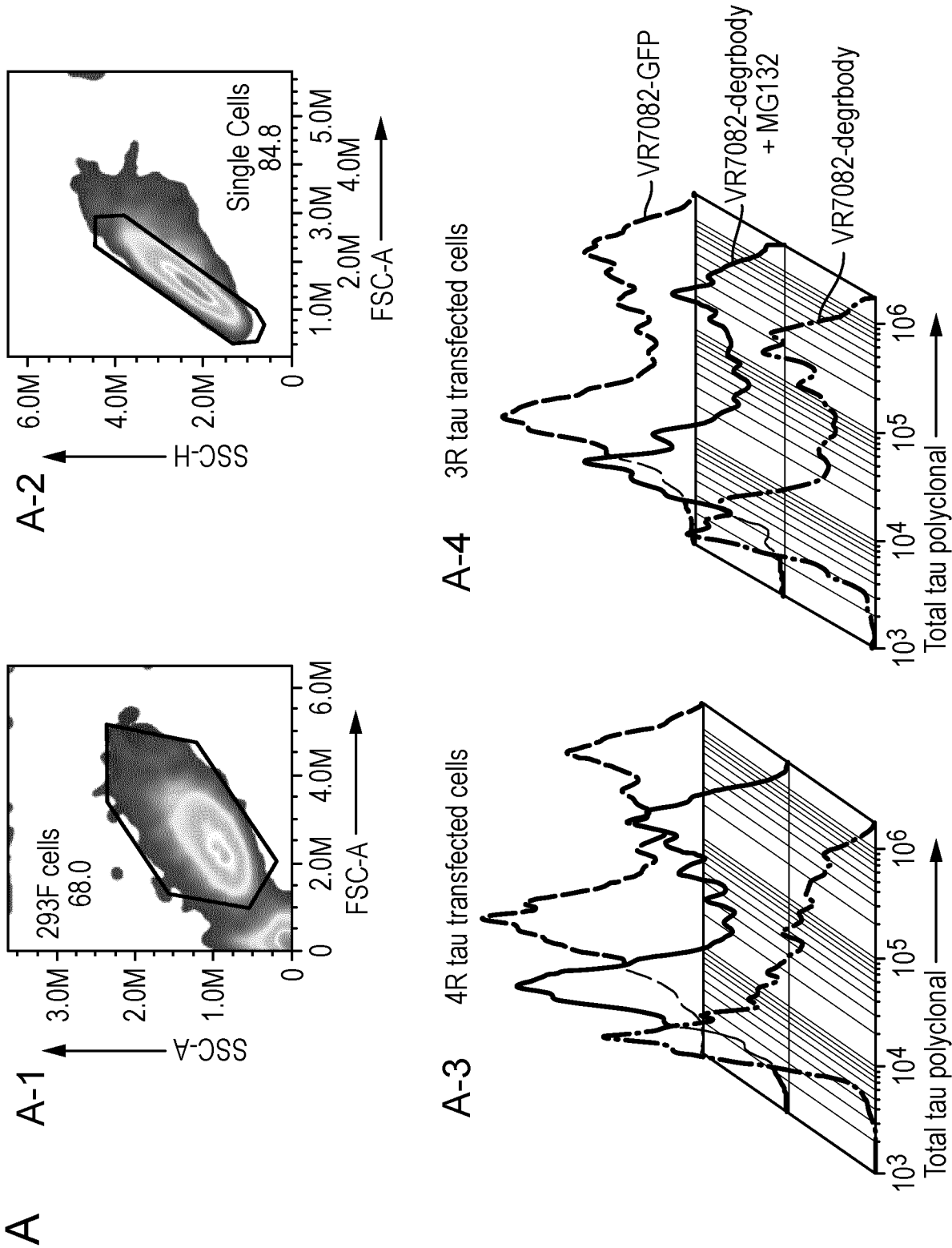


Figure 22

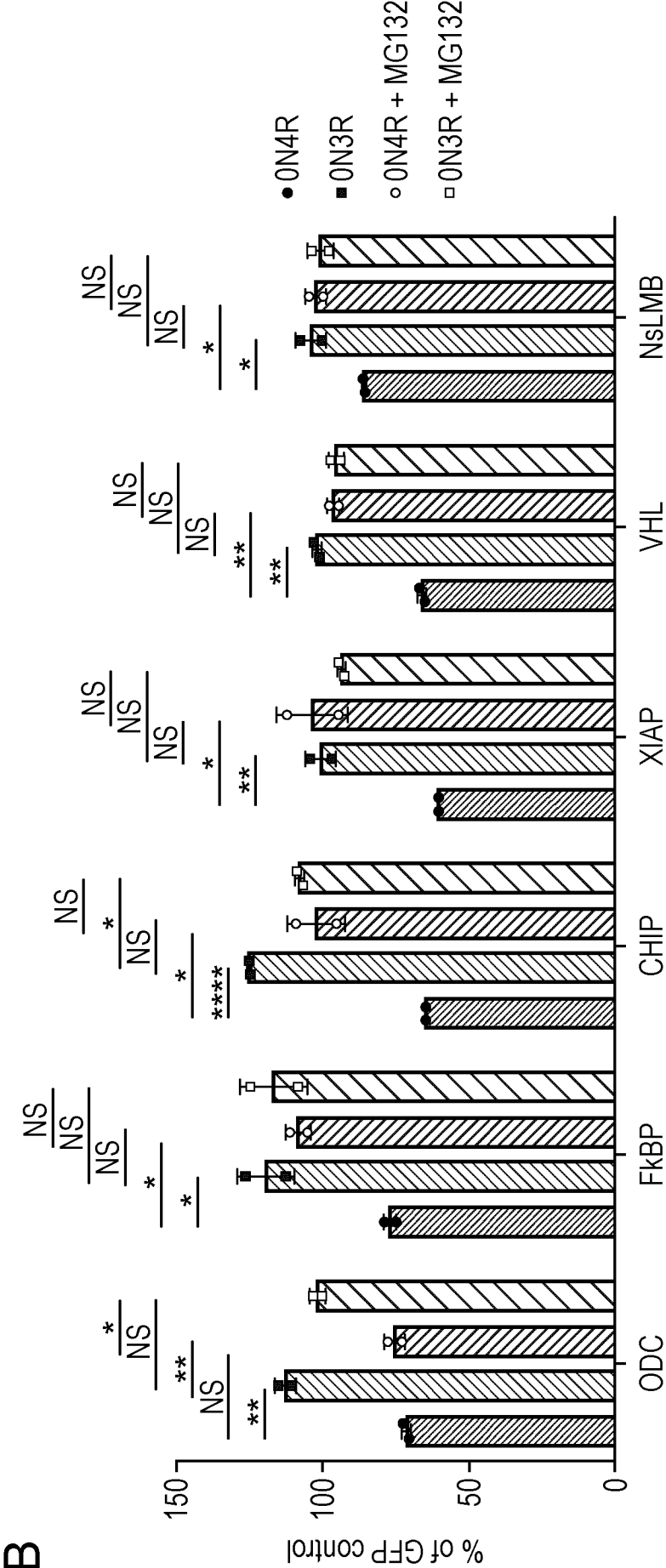
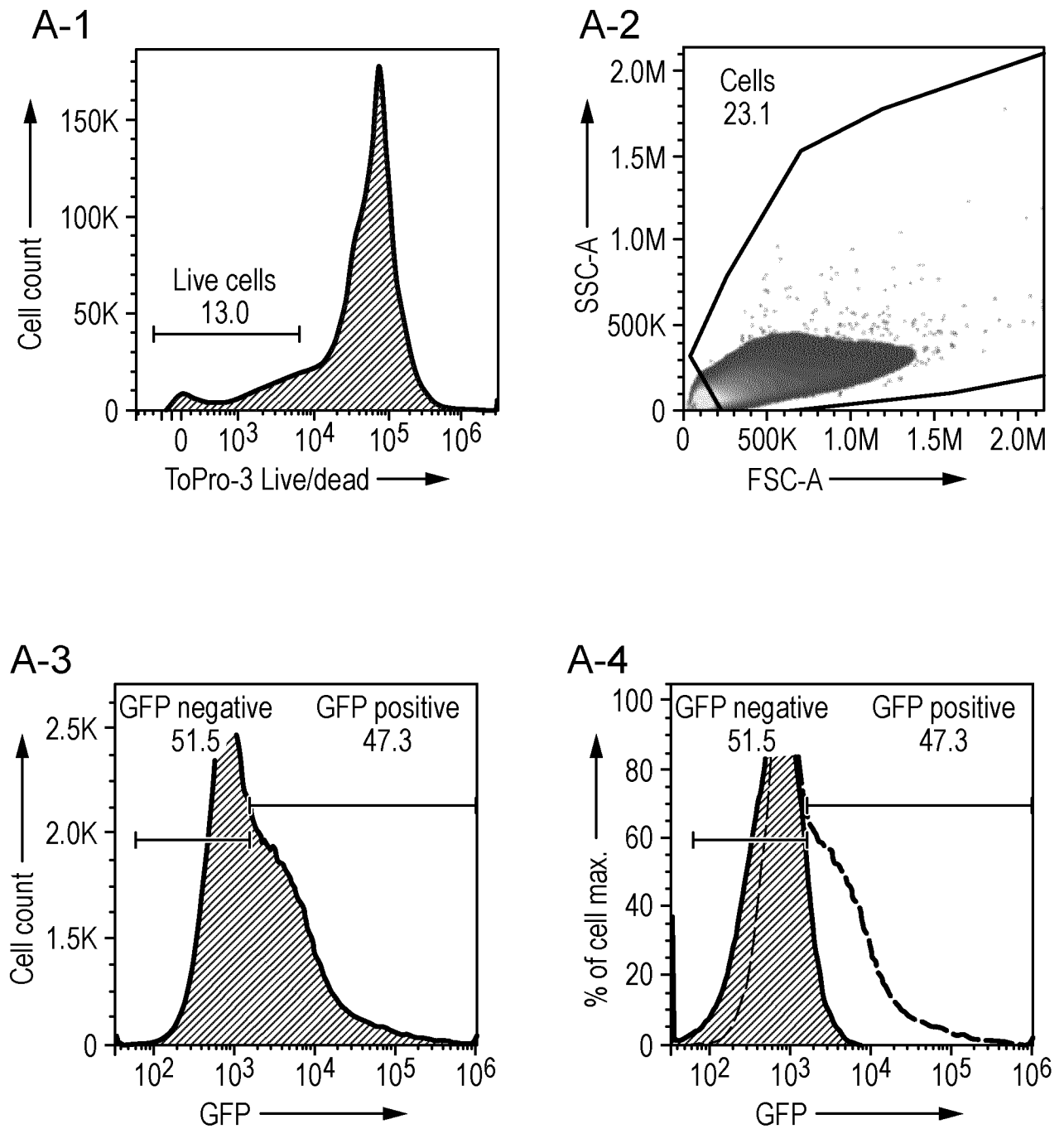
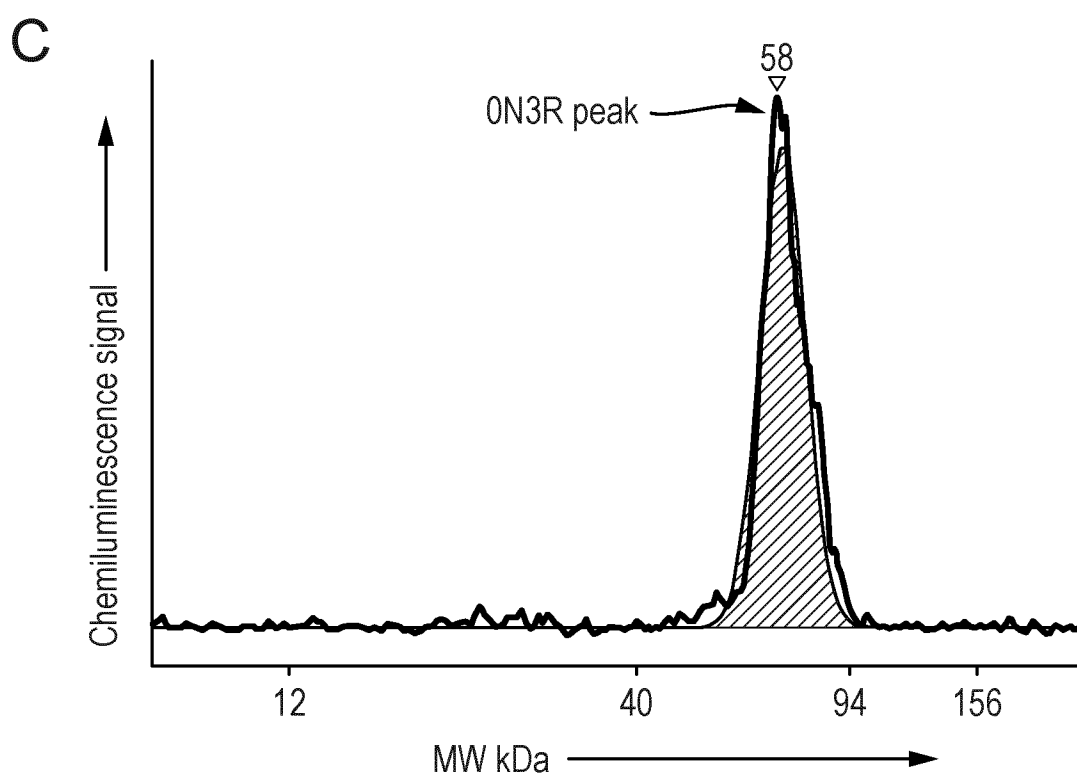
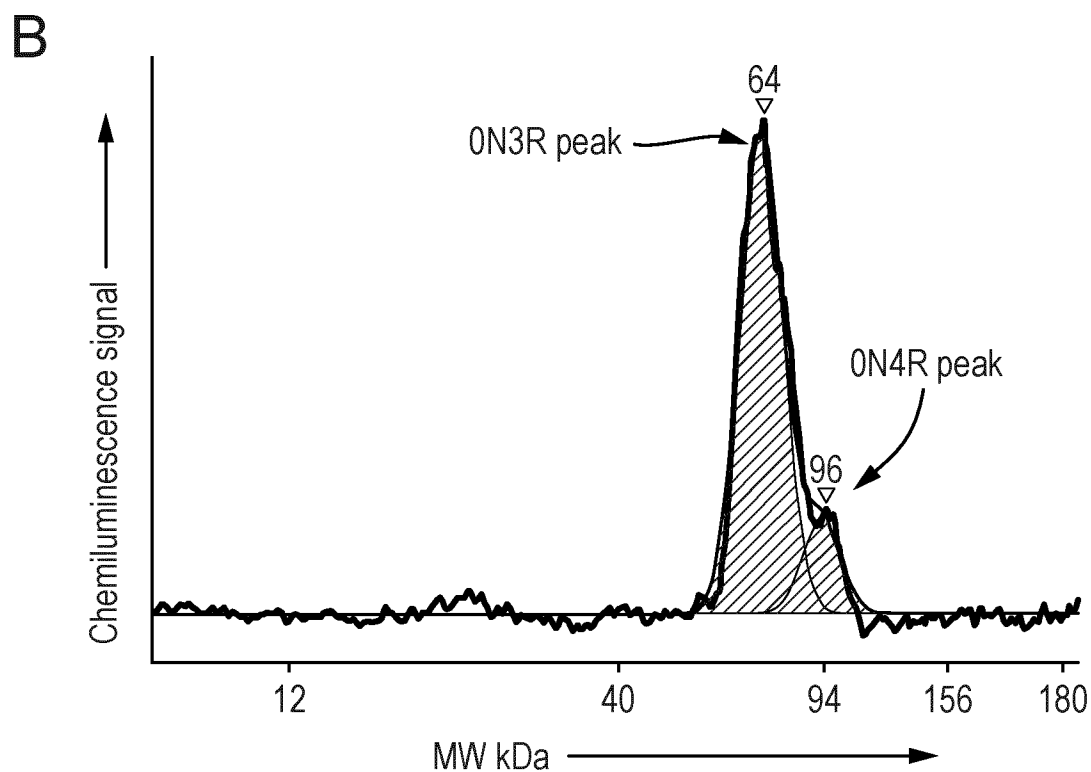
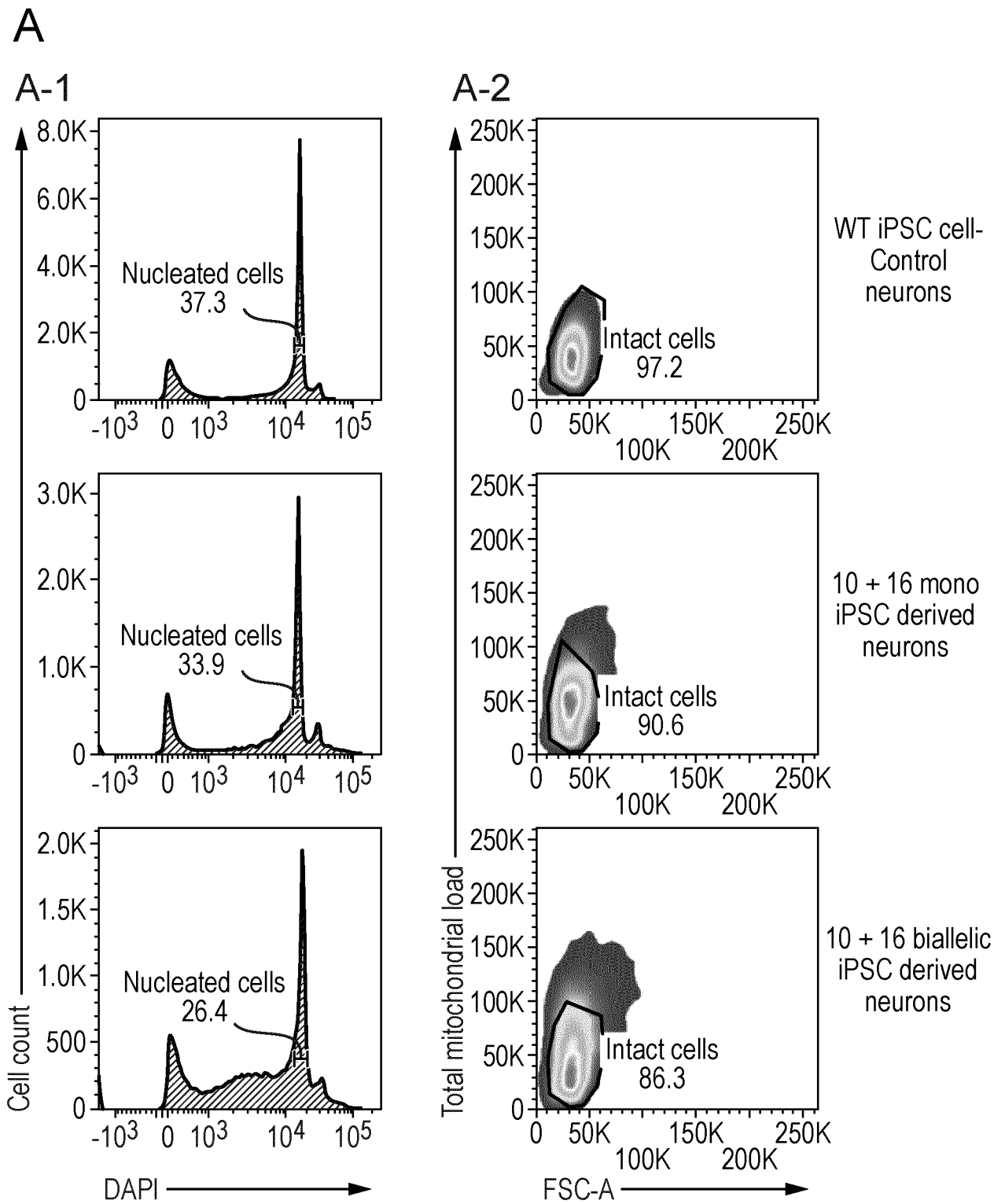


Figure 22

A**Figure 23**

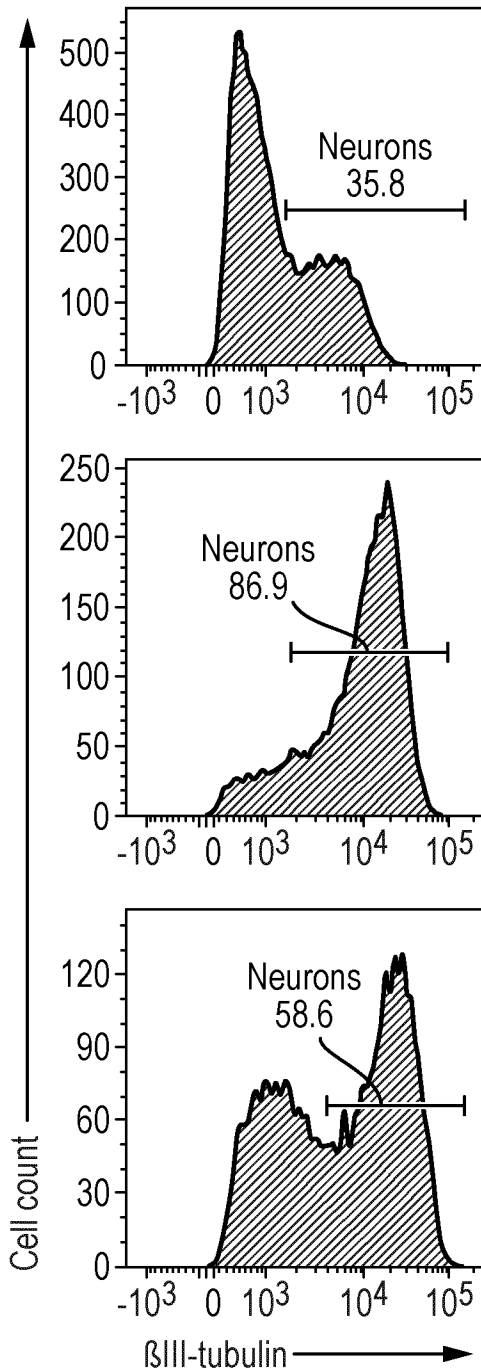
**Figure 23**

19/23

**Figure 24**

A

A-3



A-4

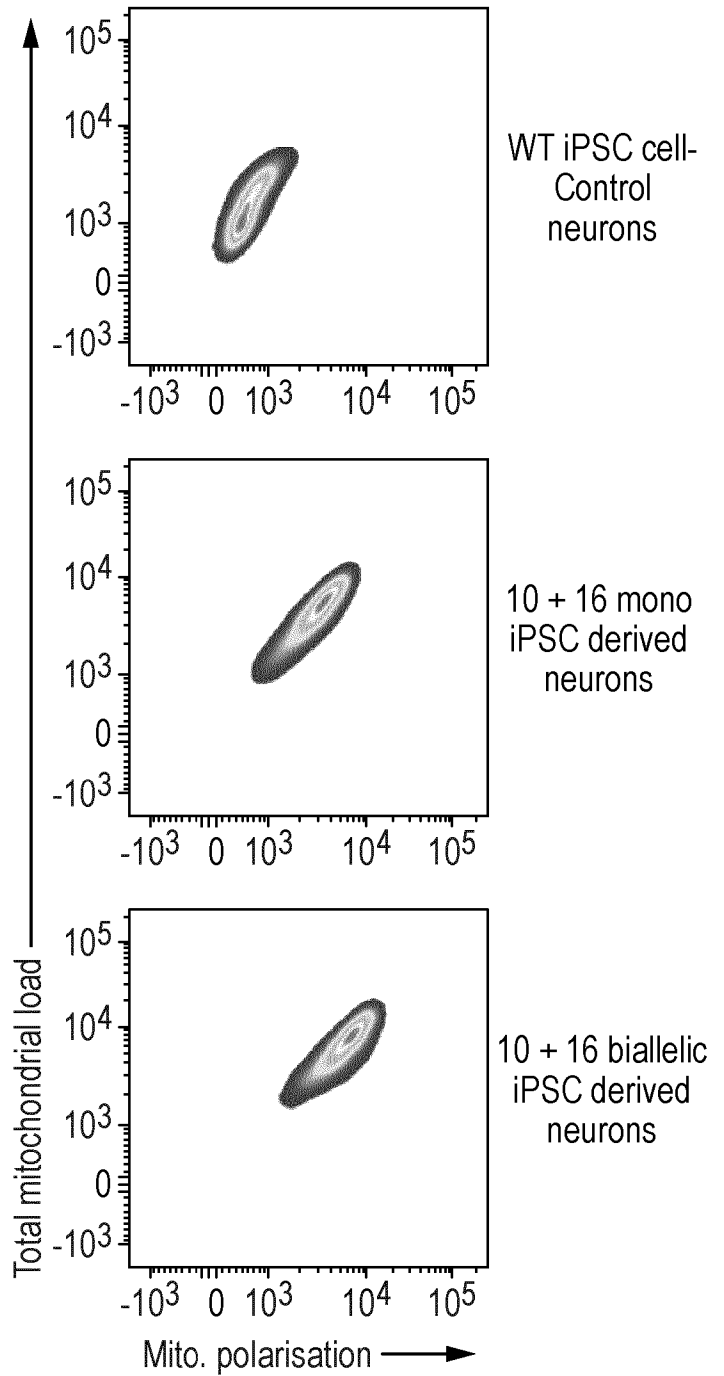


Figure 24

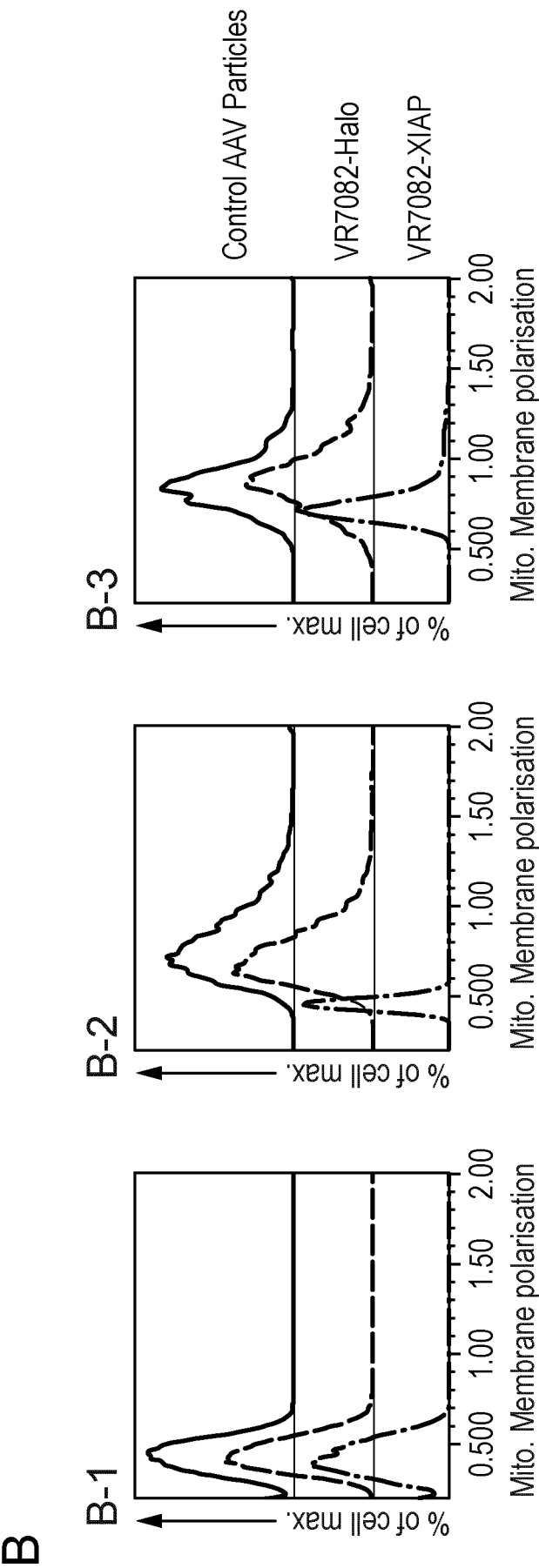
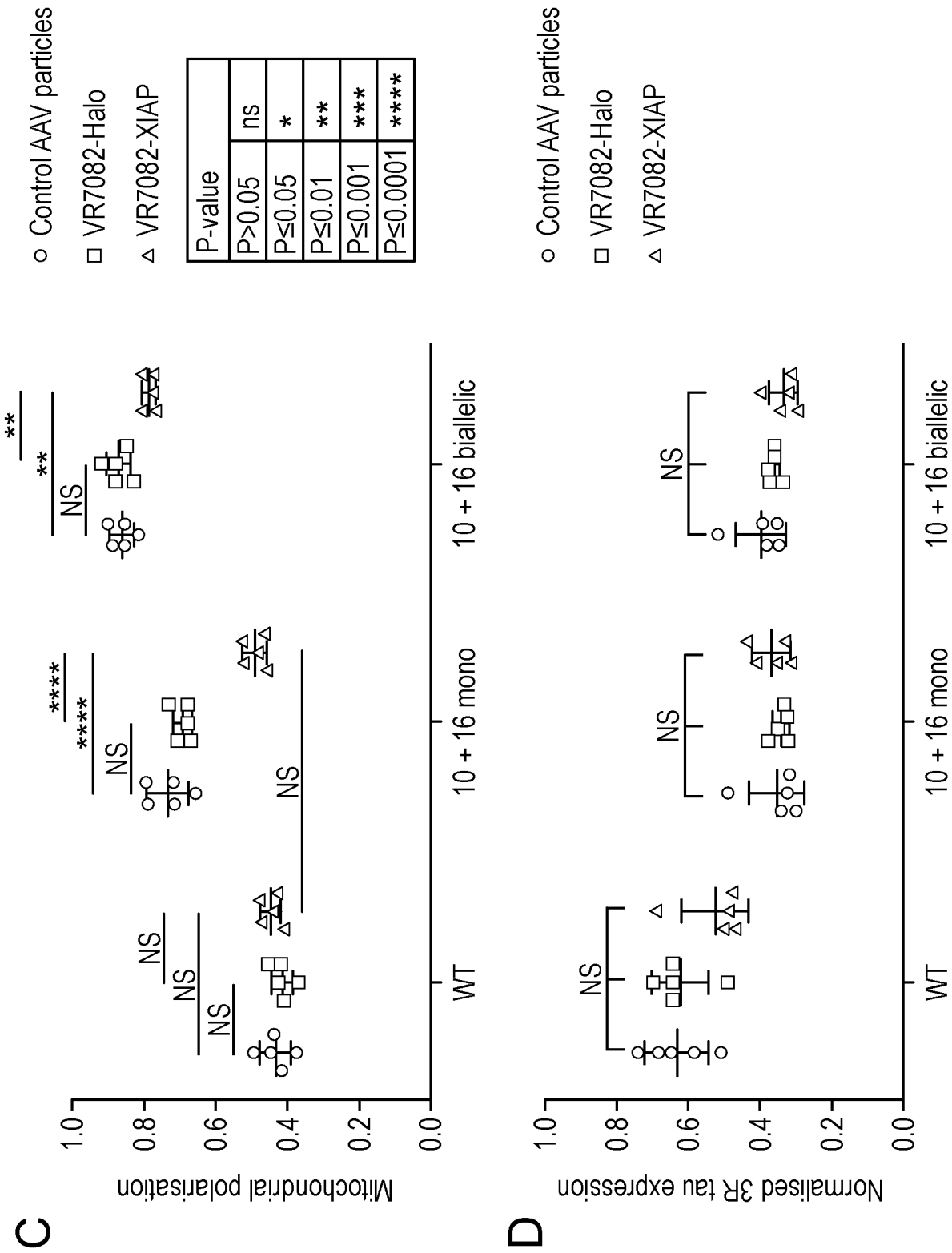


Figure 24



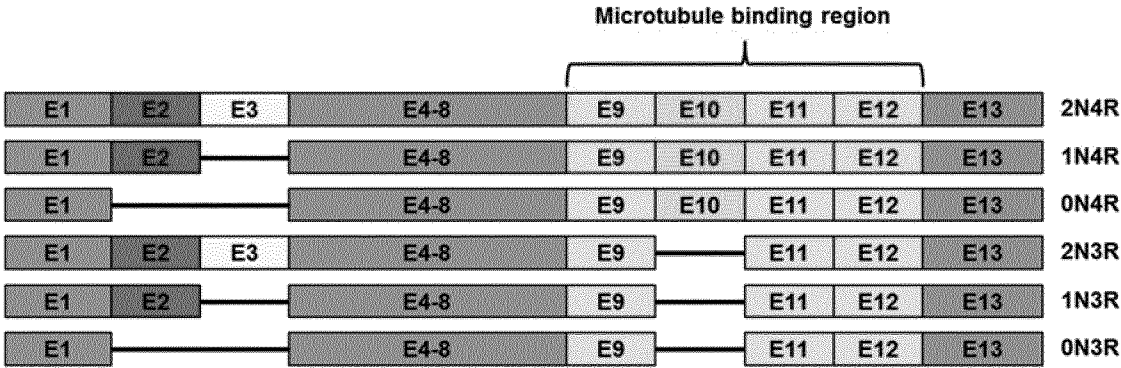


Figure 25

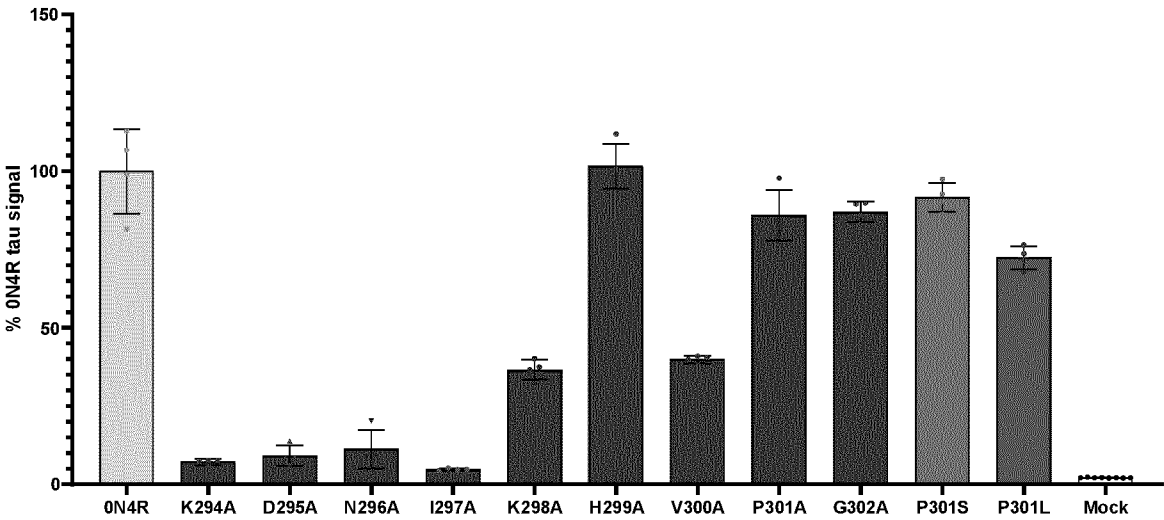


Figure 26

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2022/053694

A. CLASSIFICATION OF SUBJECT MATTER INV. C07K16/18 A61P25/28 G01N33/533 G01N33/68 A61K39/00 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 A61P A61K C07K		
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, Sequence Search, 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	ROHAN DE SILVA ET AL: "An immunohistochemical study of cases of sporadic and inherited frontotemporal lobar degeneration using 3R and 4R-specific tau monoclonal antibodies", ACTA NEUROPATHOLOGICA, SPRINGER, BERLIN, DE, vol. 111, no. 4, 1 April 2006 (2006-04-01), pages 329-340, XP019340887, ISSN: 1432-0533, DOI: 10.1007/S00401-006-0048-X page 331, column 2, paragraph 3 - page 332, column 1, paragraph 1 -----	1-4, 8
X	WO 2018/085653 A1 (UNIV CALIFORNIA [US]) 11 May 2018 (2018-05-11) claims 1-11 ----- ----- -/--	1-4, 8
☒ 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		Date of mailing of the international search report
30 May 2022		10/06/2022
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Klee, Barbara

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2022/053694

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ESPINOZA ET AL: "Differential Incorporation of Tau Isoforms in Alzheimer's Disease", JOURNAL ALZHEIMER'S DISEASE, vol. 14, no. 1, 1 May 2008 (2008-05-01), pages 1-16, XP055904175, Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2882247/> abstract; page 2, paragraph 4; page 4, first paragraph "monoclonal antibodies"; page 4, paragraph 2 page 7, paragraph 1 - page 8, paragraph 1 -----	1-4, 8-11, 14-23
X	WO 2019/133799 A1 (UNIV FLORIDA [US]) 4 July 2019 (2019-07-04) page 1, last paragraph to page 3, line 15; page 2, paragraph 4; claims 1-25 -----	1-8, 11-29
A	GROVER ANDREW ET AL: "Effects on splicing and protein function of three mutations in codon N296 of tau in vitro", NEUROSCIENCE LETTERS, vol. 323, no. 1, 1 April 2002 (2002-04-01), pages 33-36, XP055922912, AMSTERDAM, NL ISSN: 0304-3940, DOI: 10.1016/S0304-3940(02)00124-6 page 35, column 2, paragraph 2; figure 3 -----	1-29

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2022/053694

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2018085653 A1	11-05-2018	US 2020181245 A1 WO 2018085653 A1	11-06-2020 11-05-2018
WO 2019133799 A1	04-07-2019	US 2021070847 A1 WO 2019133799 A1	11-03-2021 04-07-2019

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2022/053694

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments: