YMTHE, Volume *30*

Supplemental Information

Inhibition of Tau seeding by targeting Tau nucleation core within neurons with a single domain antibody fragment

Clément Danis, Elian Dupré, Orgeta Zejneli, Raphaëlle Caillierez, Alexis Arrial, Séverine Bégard, Justine Mortelecque, Sabiha Eddarkaoui, Anne Loyens, François-Xavier Cantrelle, Xavier Hanoulle, Jean-Christophe Rain, Morvane Colin, Luc Buée, and Isabelle Landrieu

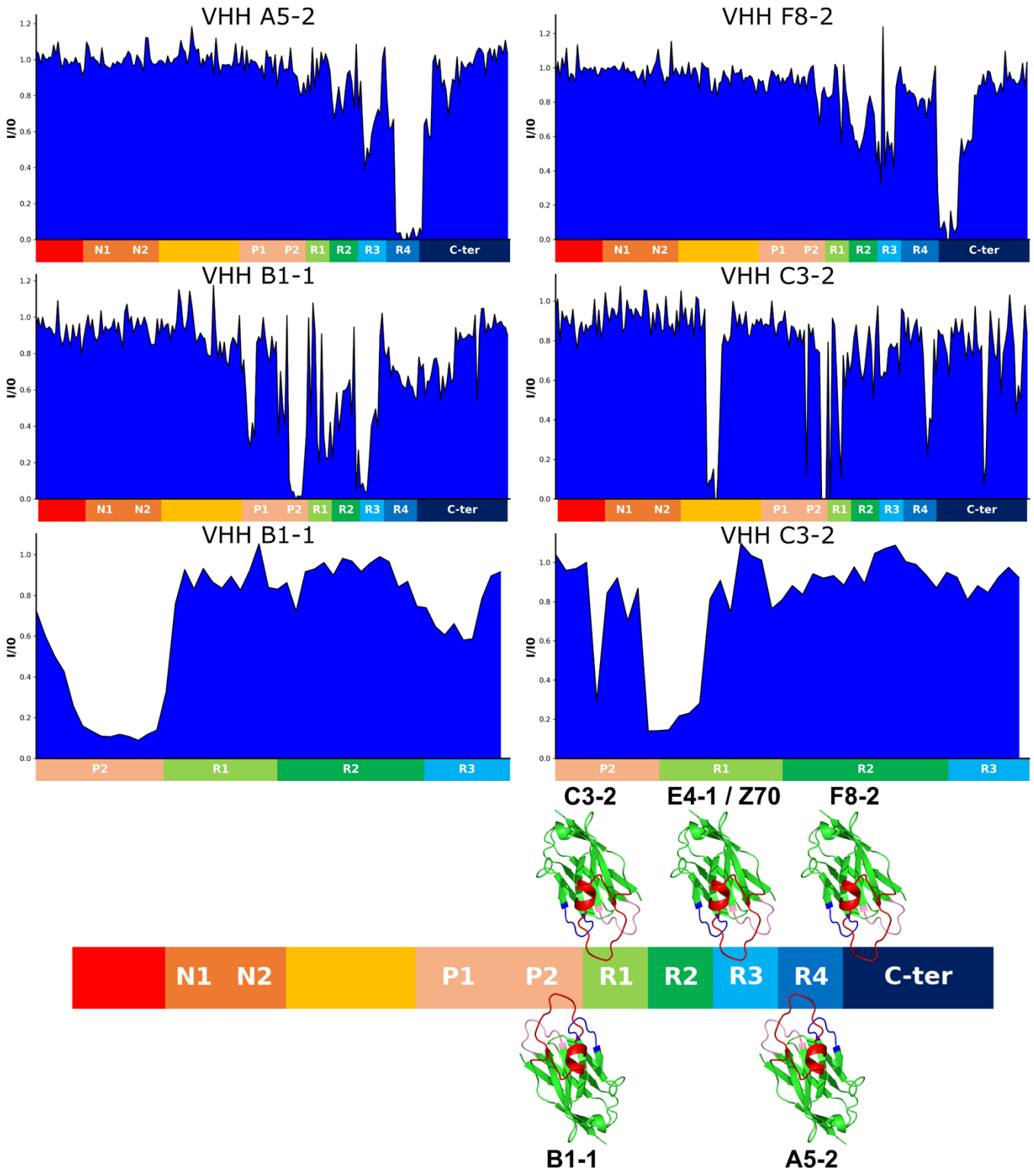


Figure S1 : Identification of synthetic VHHs directed against Tau microtubule-binding domain (MTBD) using 2D (two-dimensional) HSQC NMR experiment of Tau2N4R and Tau [208-324]. Intensity ratios I/I0 of corresponding resonances in the 2D spectra of Tau or Tau [208-324] with equimolar quantity of VHH (I) or free in solution (I0) for residues along the Tau or Tau [208-324] sequences. Binding regions are illustrated along the Tau sequence with VHH images (from PDB: 1MEL). CDR3 loop region is colored red.

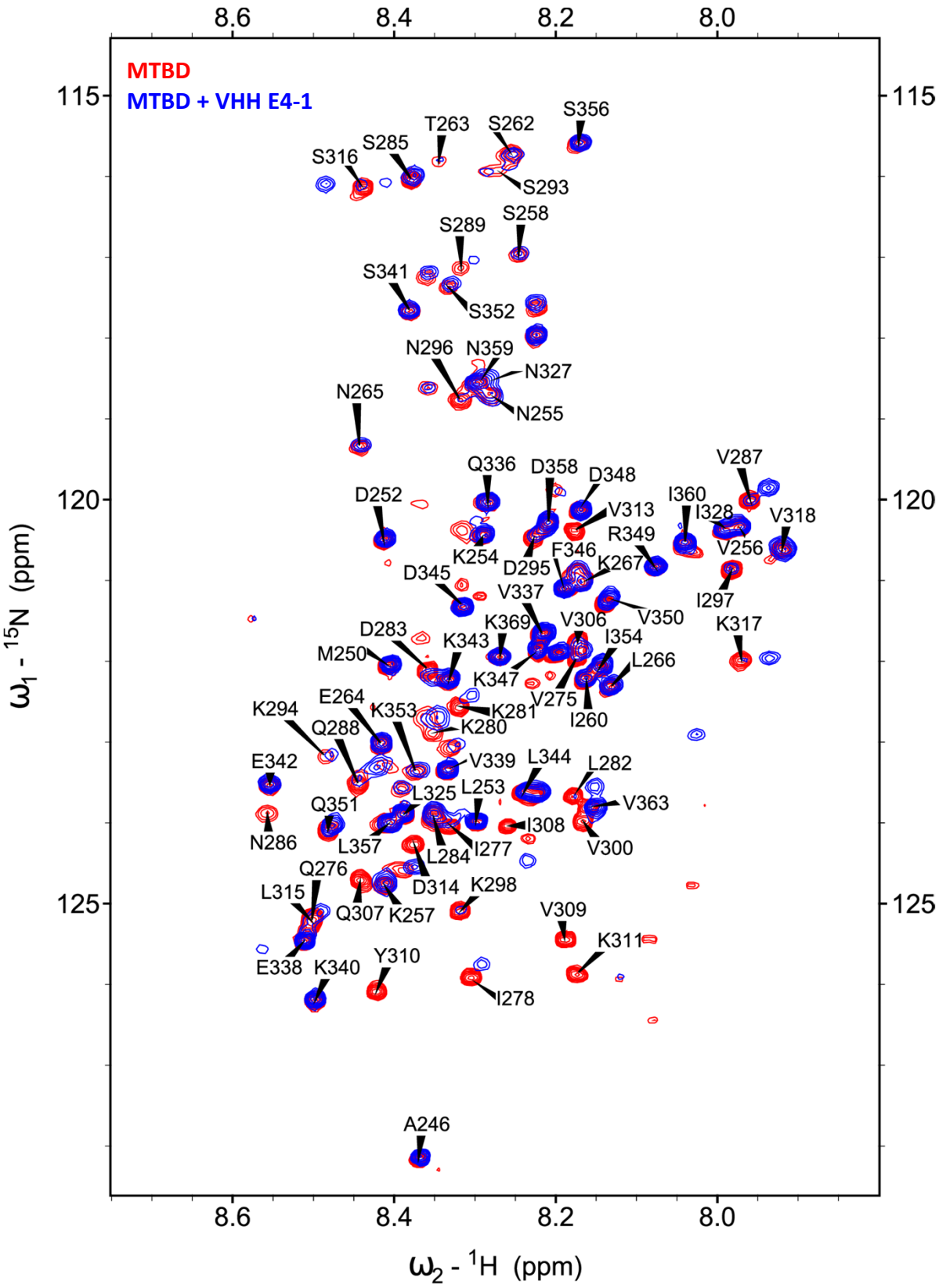


Figure S2 : Identification of VHH E4-1 binding region using 2D HSQC NMR experiment of Tau MTBD. Overlay of ^{1}H, ^{15}N, HSQC 2D spectra of ^{15}N-labelled Tau MTBD alone (red) or mixed with non-labelled VHH E4-1 spectra (blue). See enlarged regions of the spectra in **Figure S3 A**.

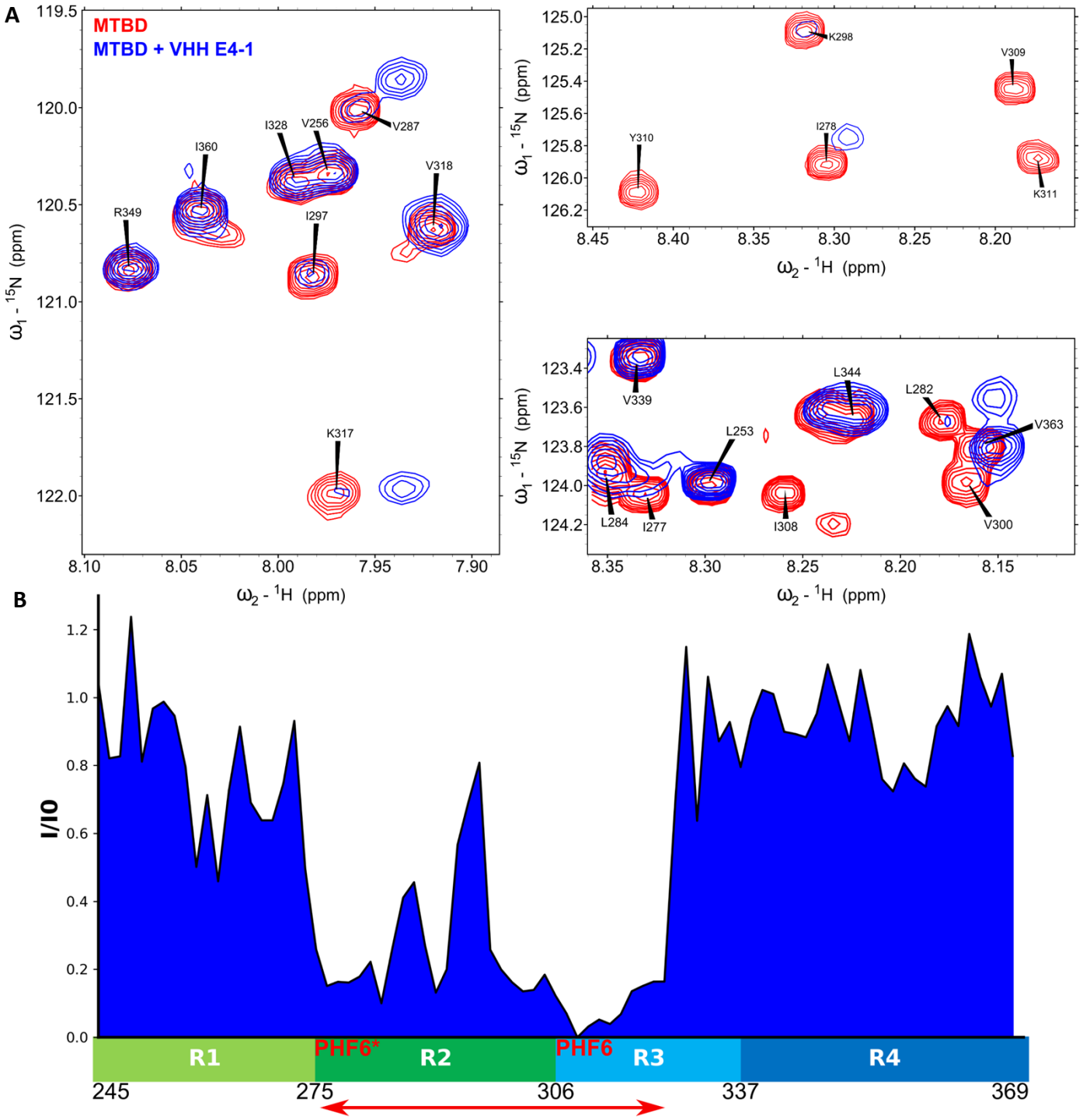


Figure S3 : Identification of VHH E4-1 epitope using 2D HSQC NMR experiment of Tau MTBD. A : Overlays of ^{1}H, ^{15}N, HSQC of 2D spectrum enlargements of ^{15}N-labelled Tau MTBD alone (red) or mixed with non-labelled VHH E4-1 (superimposed in blue). See full spectrum in **Figure S2. B :** Intensity ratios I/I0 of corresponding resonances in the 2D spectra of Tau MTBD with equimolar quantity of VHH E4-1 (I) or free in solution (I0) for residues along the Tau MTBD sequence. The red double-arrow indicates the region containing the corresponding major broadened resonances, which was mapped mostly on the R2-R3 repeats. Localization of the PHF6* and PHF6 peptide sequences is indicated.

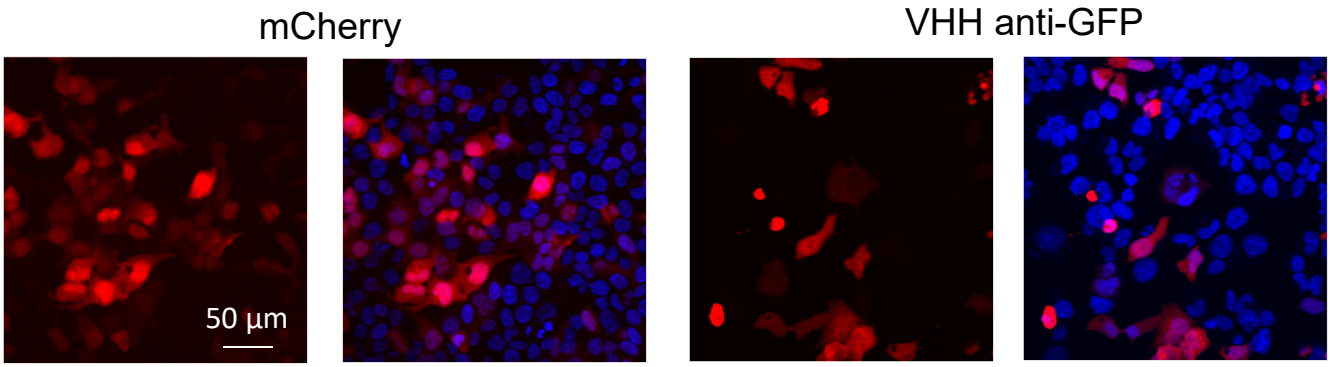


Figure S4 : VHH-70 is soluble inside cells - HEK293 cells were transfected with plasmid encoding either mCherry, mCherry-VHH E4.1 (**Figure 2C**), mCherry-VHH Z70 (**Figure 2D**) or mCherry-VHH anti-GFP. 48h later, cells were fixed and immunostained using a primary antibody against mCherry tag and visualized in red. Nuclei are vizualized in blue. The scale bar is indicated on the figure. See percentage of cells with puncta in **Figure 2E**.

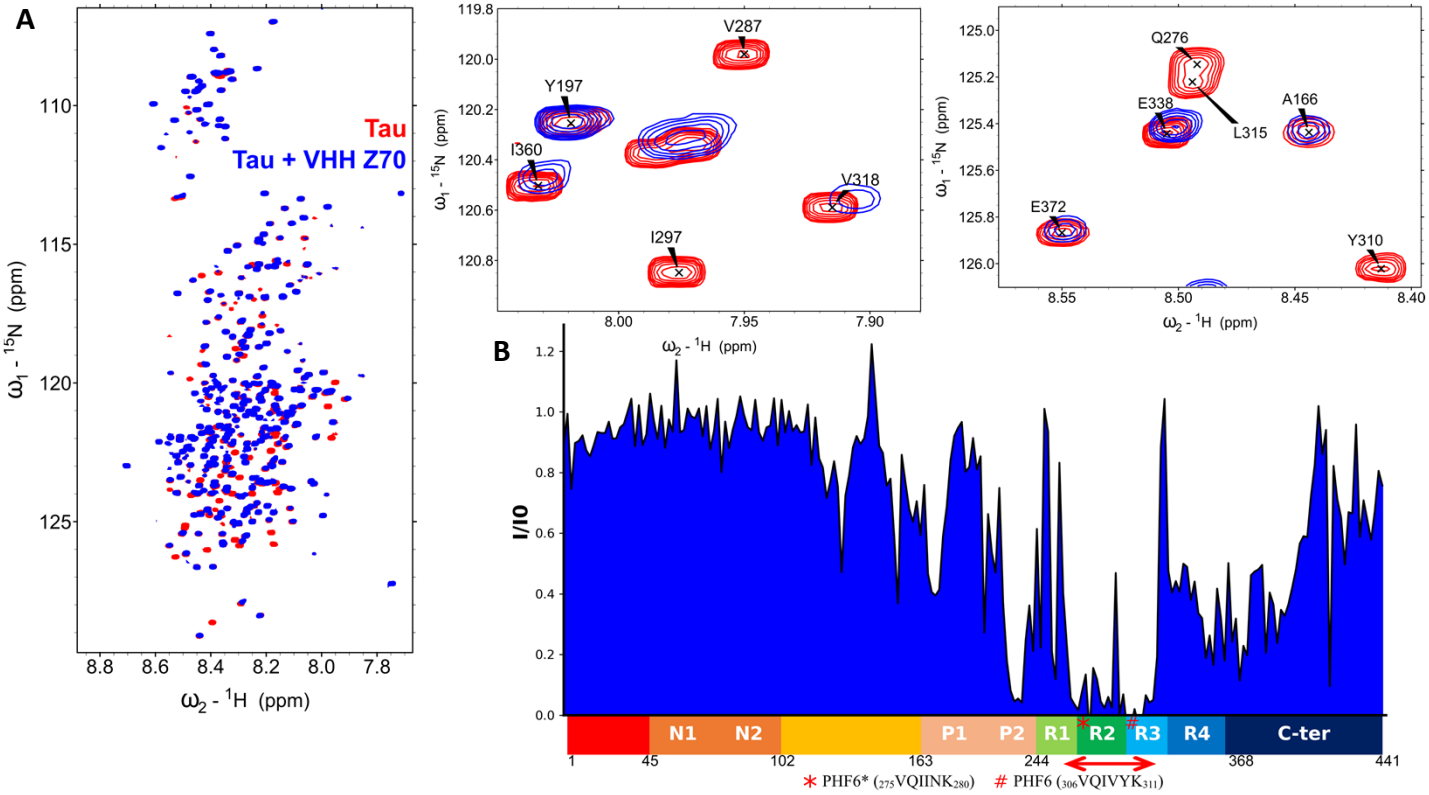


Figure S5 : Identification of VHH Z70 binding region using 2D HSQC NMR experiment of Tau2N4R. A : Overlays of ^{1}H, ^{15}N, HSQC full spectrum and enlargements of ^{15}N-labelled Tau, alone (red) or mixed with non-labelled VHH Z70 (in blue). Spectra enlargements show broadened resonances corresponding to residues implicated in the interaction **B :** Intensities ratio I/I0 of corresponding resonances in the 2D spectra of Tau with equimolar quantity of VHH Z70 (I) or free in solution (I0) for residues along the Tau sequence. The red double-arrow indicates the region containing the corresponding major broadened resonances, which was mapped mostly on the R2-R3 repeats. Localization of the PHF6* and PHF6 peptide sequences is indicated.

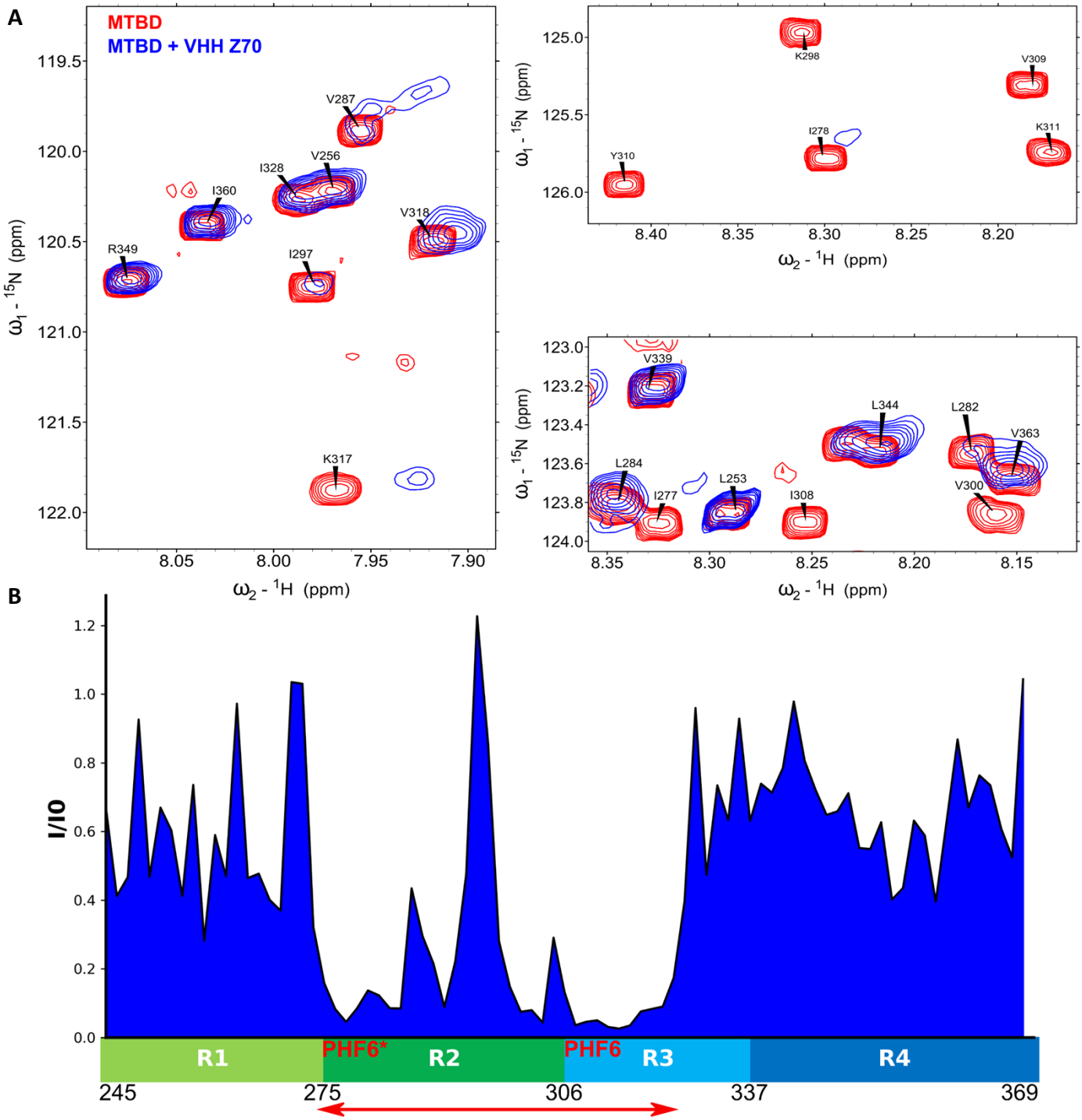


Figure S6 : Identification of VHH Z70 binding region using 2D HSQC NMR experiment of Tau MTBD. A : Overlays of ^{1}H, ^{15}N, HSQC enlargements of ^{15}N-labelled Tau MTBD alone (red) or mixed with non-labelled VHH Z70 (blue). **B :** Intensity ratios I/I0 of corresponding resonances in the 2D spectra of Tau MTBD with equimolar quantity of VHH Z70 (I) or free in solution (I0) for residues along the Tau MTBD sequence. The red double-arrow indicates the region containing the corresponding major broadened resonances, which was mapped mostly on the R2-R3 repeats. Localization of the PHF6* and PHF6 peptide sequences is indicated.

VHH	kon (1/M.s)	koff (1/s)	Kd (nM)
E4-1	4982	0.0017	345
Z70	18100	0.0026	147

Tau peptide	sequence	kon (1/M.s)	koff (1/s)	Kd (nM)
Tau[273-318]	$_{273}$GKVQIINKKLDLSNVQSKCGSKDNIKHVPGGGSV QIVYKPVDLSKV$_{318}$	106158	0.00214	21
K18	Tau[245-368]	23523	0.00343	146
K18 PHF6x2	Tau[245-368] with [274-282] mutated as SVQIVYKPV	29426	0.00099	34
K18 PHF6*x2	Tau[245-368] with [301-309] mutated as KVQIINKKL	16534	0.00659	398

Figure S7 : Thermokinetic parameters of the interaction of VHH Z70 and VHH E4-1 with Tau, Tau MTBD and the PHF6/PHF6* sequences. Tables corresponding to k_{on}, k_{off} and resulting Kd obtained from SPR experiments with biotinylated Tau (*upper table*) or VHH-Z70 biotinylated on a C-terminal Cys residue (*lower table*) immobilized on the chips. Sequence of the Tau[273-318] peptide (containing both PHF6* and PHF6 sequences) is included (*lower table*). See also **Figure S10.**

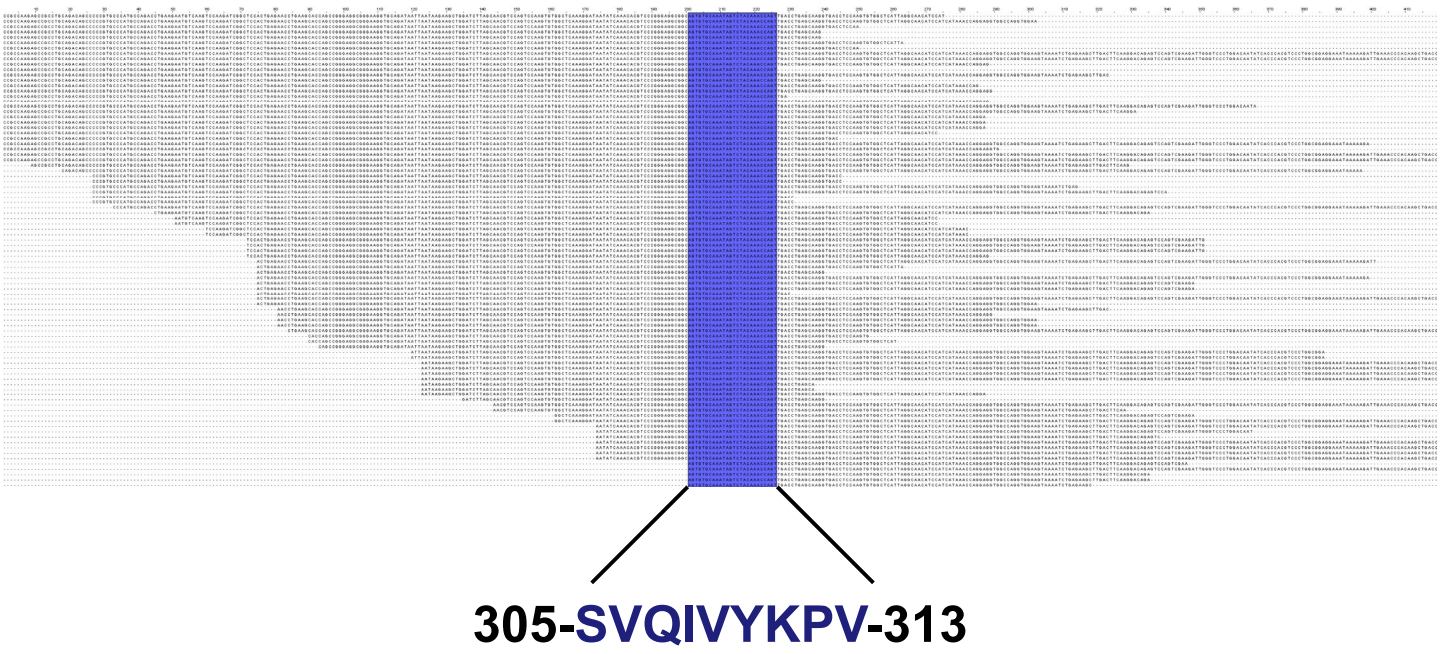


Figure S8 : Identification of the minimal epitope recognized by VHH Z70 using Tau fragment library and yeast two hybrid. Sequence alignment of the 90 Tau fragments corresponding to the 90 positive colonies picked on selective growth conditions and thus binding VHH Z70. The minimal common sequence is highlighted. Sequences are not meant to be read.

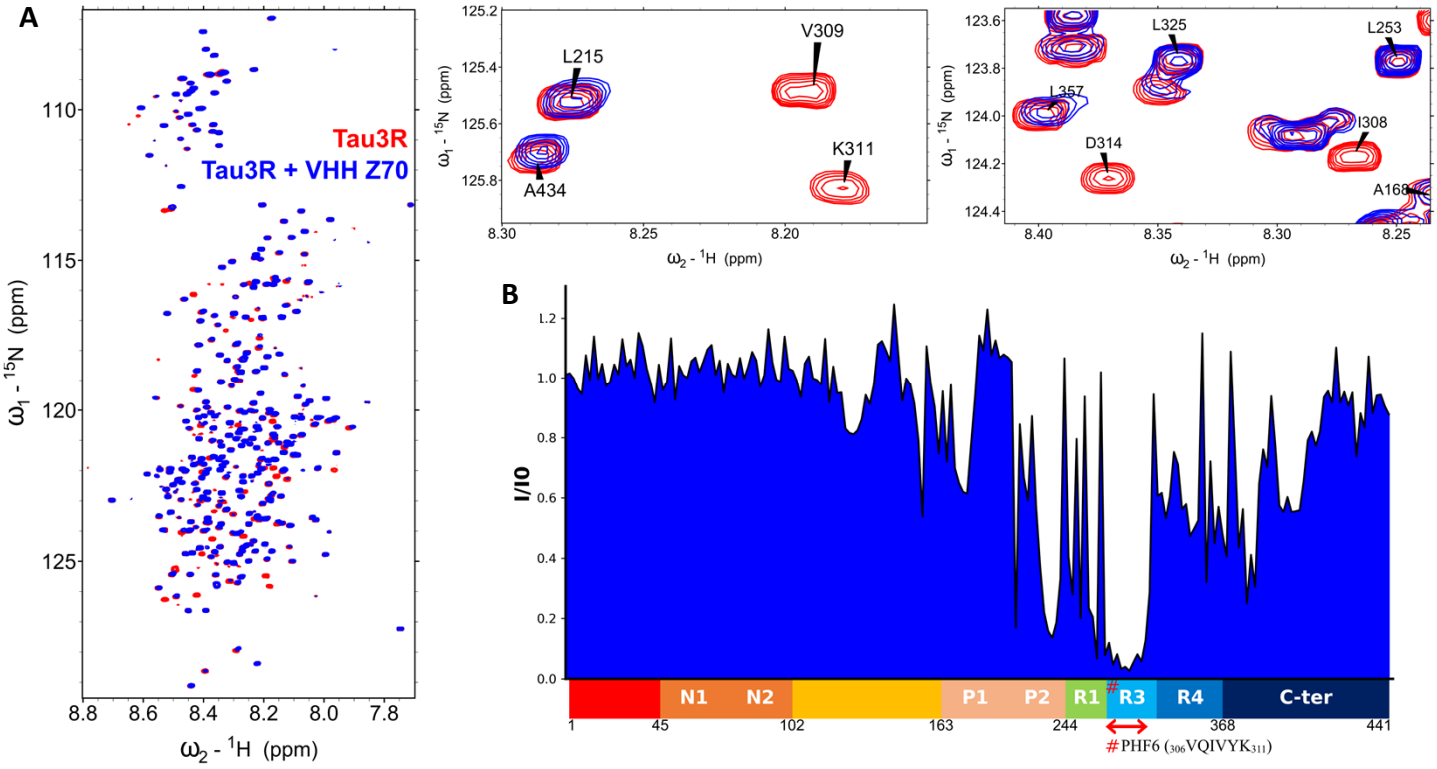


Figure S9 : Identification of VHH Z70 epitope using 2D HSQC NMR experiment of Tau2N3R. A : Overlays of ^{1}H, ^{15}N, full spectrum and enlargements of ^{15}N-labelled Tau 2N3R alone (red) or mixed with non-labelled VHH Z70 (blue). Spectra enlargements show broadened resonances corresponding to residues implicated in the interaction. **B :** Intensities ratio I/I0 of corresponding resonances in the 2D spectra of Tau 2N3R with equimolar quantity of VHH (I) or free in solution (I0) for residues along the Tau 2N3R sequence. Tau 2N3R lacks the R2 repeats. Tau 2N3R residue numbering corresponds to the Tau 2N4R sequence, for clarity. The red double-arrow indicates the region containing the corresponding major broadened resonances, which was mapped mostly on the PHF6 motif, showing PHF6 is sufficient for VHH Z70-Tau binding. Localization of the PHF6 peptide sequence is indicated.

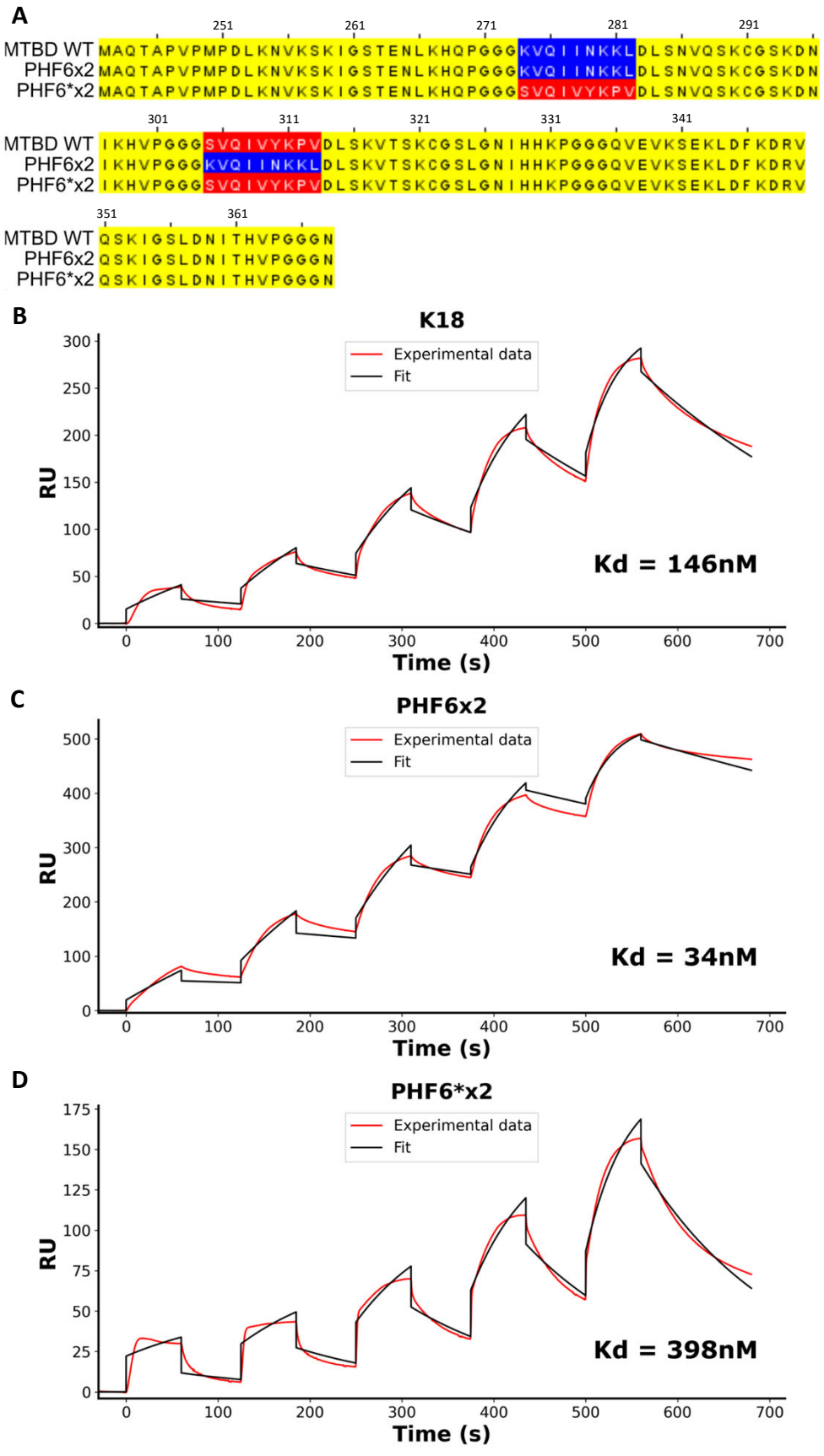
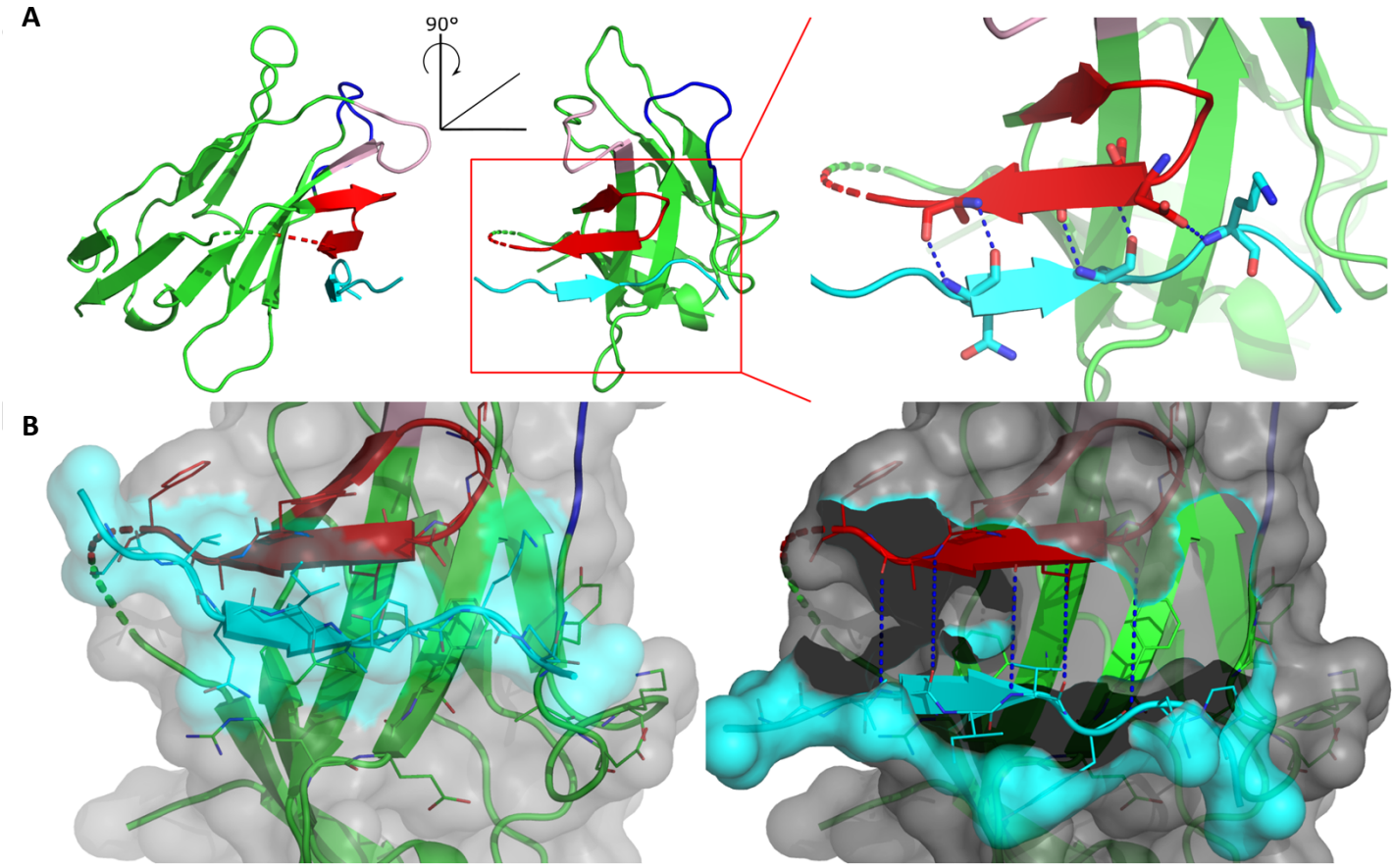


Figure S10 : The PHF6 peptide sequence is the main binding site for VHH Z70

A : Sequence alignment of Tau MTBD (starting at Q244) and chimeric MTBD sequences. PHF6 peptide is highlighted in blue, PHF6* in red. **B-D :** Sensorgrams (reference subtracted data) of single cycle kinetics analysis performed on immobilized biotinylated VHH Z70, with five injections of **B :** MTBD or K18 or **C :** chimeric MTBD with two PHF6/PHF6x2 or **D :** chimeric MTBD with two PHF6*/ PHF6*x2, at 0.125 µM, 0.25 µM, 0.5 µM, 1 µM, and 2 µM (n=1). Dissociation equilibrium constant Kd were calculated from the ratio of off-rate and on-rate kinetic constants koff/kon (**Figure S7**). Black lines correspond to the fitted curves, red lines to the measurements.



C

VHH residue	Atom	Tau residue	Atom	Distance (Å)
Asp 106	OD1	Lys 311	N	2.91
Leu 107	N	Vas 309	O	2.89
Leu 107	O	Val 309	N	2.80
Ala 109	N	Gln 307	0	2.77
Ala 109	O	Gln 307	N	2.88

Figure S11 : Crystal structure of the PHF6 peptide sequence bound to VHH Z70
A : Cartoon representation of VHH Z70 in complex with Tau[301-312] peptide. The two views correspond to a 90° rotation around the *y* axis. A 3D rotating view is provided as a supplemental **Video S1**. Framework regions of the VHH are represented in green and the PHF6 Tau peptide in cyan. VHH CDR1 is represented in pink, CDR2 in dark blue and CDR3 in red. The region boxed in red is enlarged. Dashed blue lines correspond to intermolecular H-bonds as detail in **C.** Red/green dashed cartoon line is undefined in the structure. See supplemental **Video S1**. **B :** Cartoon and transparent surface representation. Color code as in **A.** Buried residues are represented as lines. Right panel is a tearing through the interface. Intermolecular H-bonds are represented as blue dashes and the residues involved in the interaction are represented as sticks. **C :** Table of the intermolecular H-bonds

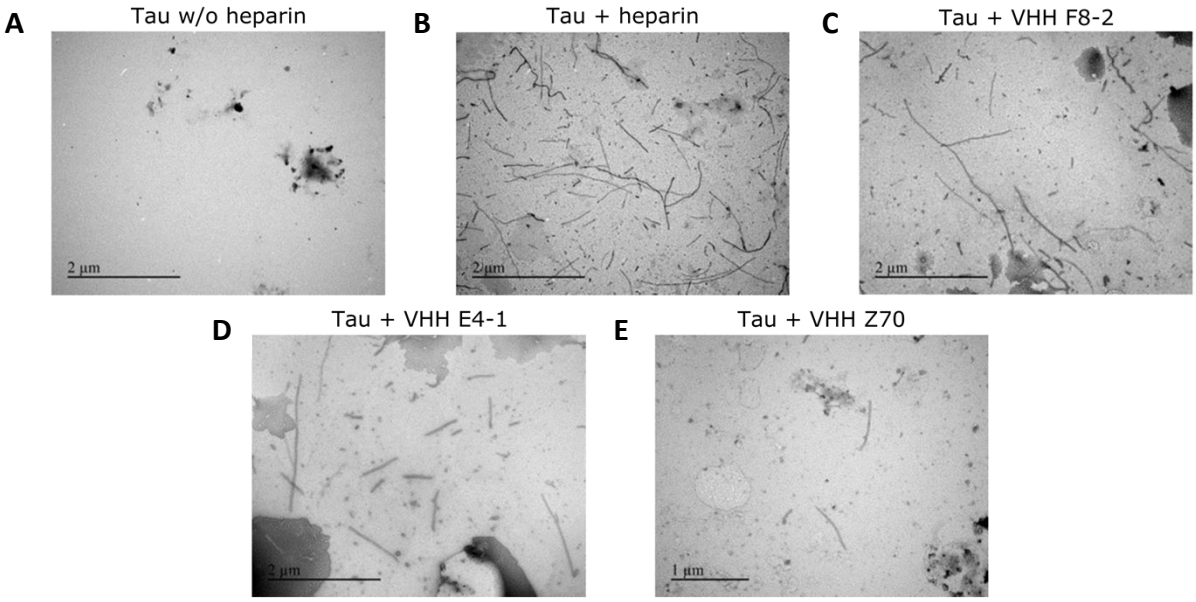


Figure S12 VHH E4-1 and VHH Z70 inhibit *in vitro* Tau aggregation Transmission electron microscopy images at the end point of the aggregation assays (**Figure 4**) **A :** in the absence of heparin or **B :** in the presence of heparin and **C-E** in the presence of heparin and the additional presence of **C :** VHH F8-2 **D :** VHH E4-1 **E :** VHH Z70 (for Tau/VHH molar ratio of 1 : 1) (n=2).

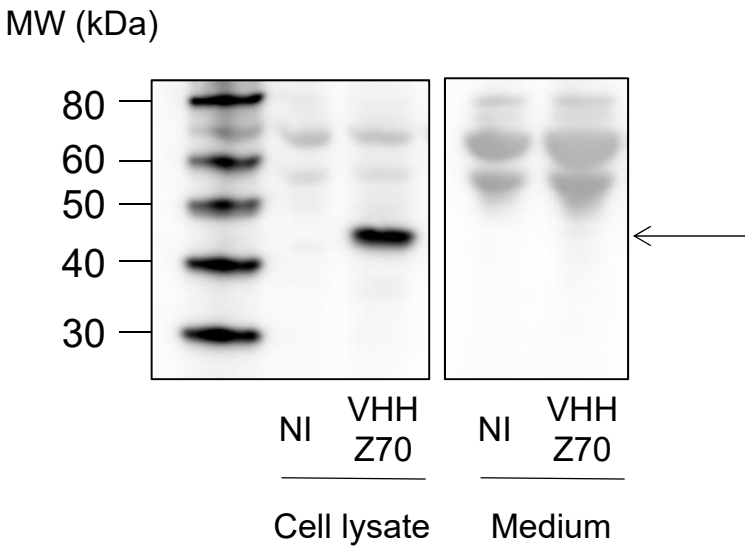


Figure S13 : Intracellular expression of VHH-Z70: HEK293 cells were infected with LVs encoding VHH-Z70 N-terminally fused to mCherry or non infected (NI). Forty-eight hours later, the cell lysate and the medium were recovered and analysed by western-blotting. VHH 70 expression (black arrow) was revealed thanks to the mCherry tag using primary antibody against mCherry.

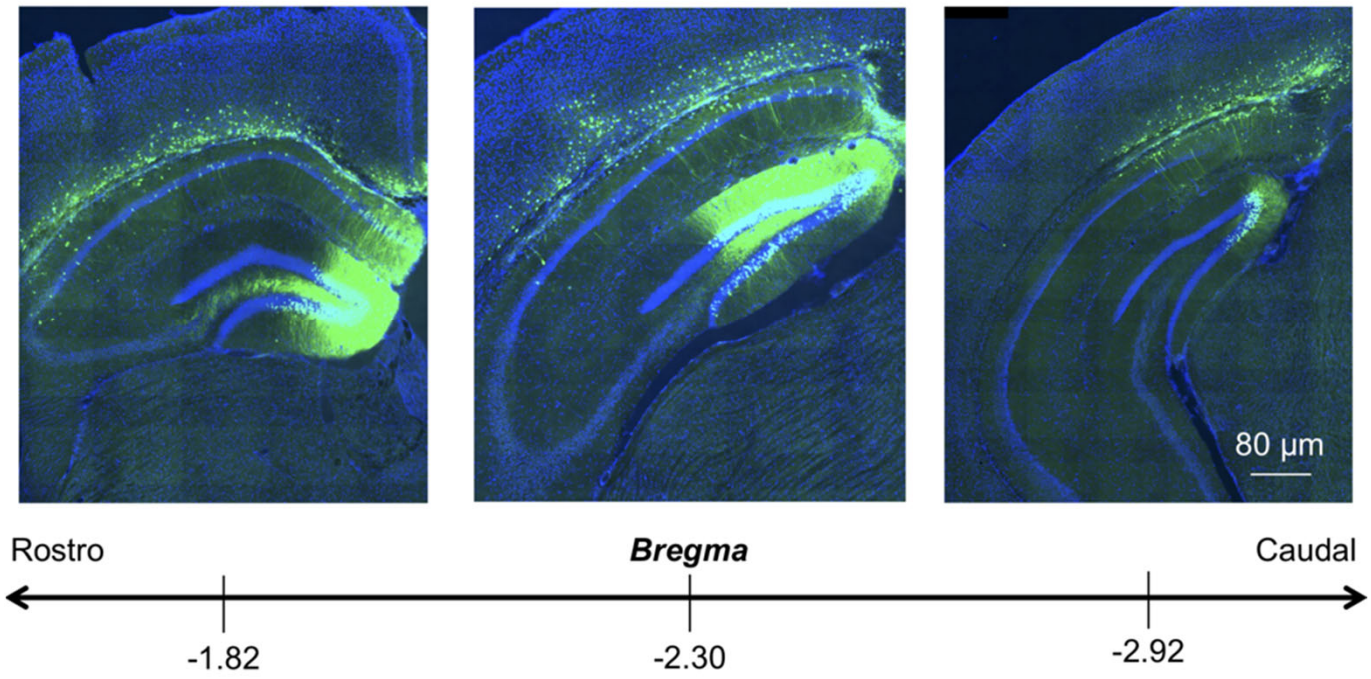


Figure S14 : VHH Z70 expression in the hippocampus. One month-old THY-tau30 mice were treated with bilateral intracranial injections of LVs encoding VHH Z70. Two weeks later mice received stereotaxic injection of AD brain lysate. Mice were sacrificed 4 weeks later and the whole brains were processed for immunohistochemical analyses. VHH Z70 was detected using a primary antibody against mCherry tag (visualised in green). VHH Z70-immunoreactivity is detected in all regions covering the bregma where Tau pathology has been quantified.

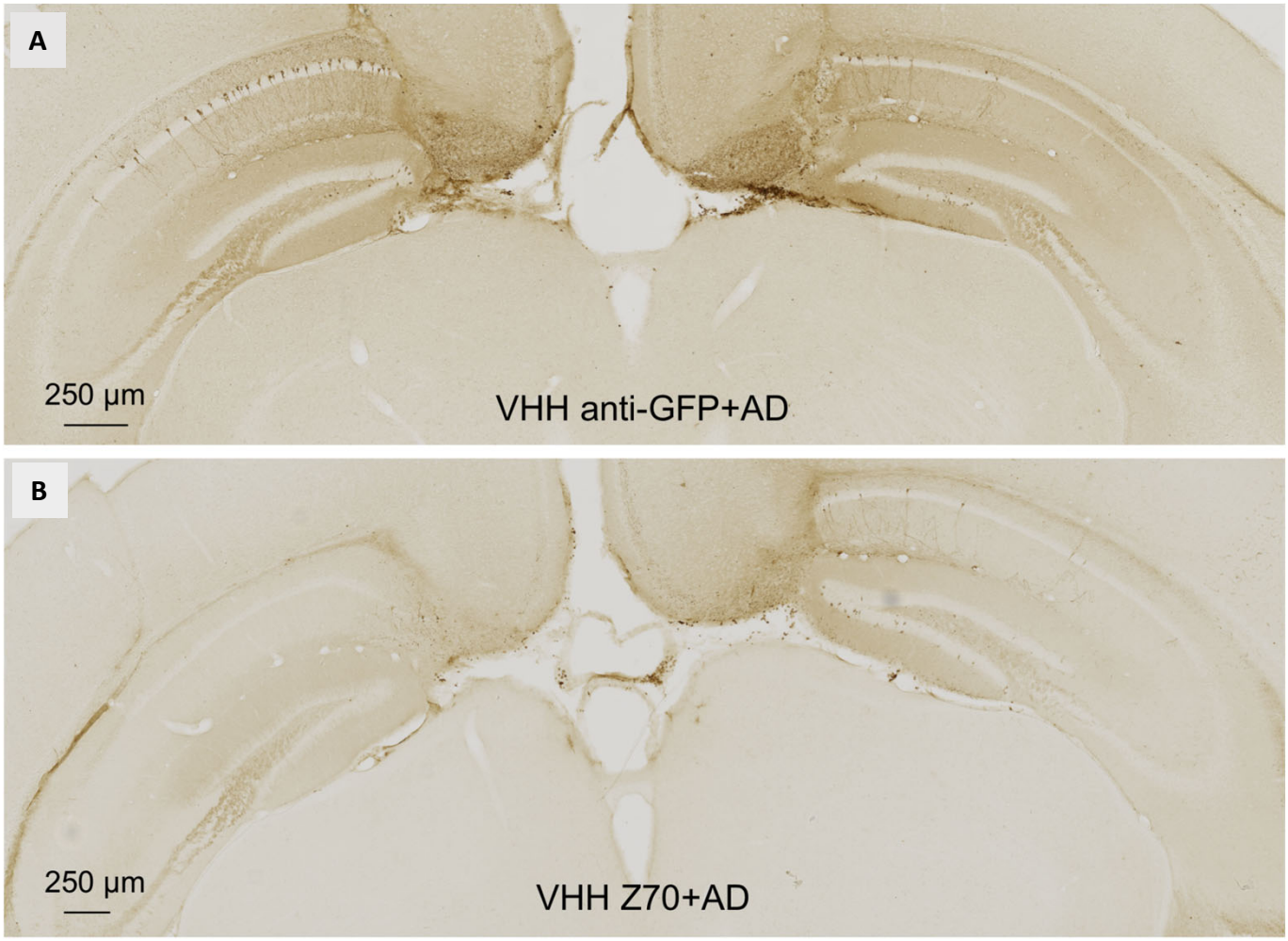


Figure S15 : VHH Z70 reduces human Tau seeding induced by extracellular human pathological Tau species: One month-old THY-tau30 mice were treated with bilateral injections of LVs **A :** encoding VHH-anti GFP or **B :** VHH Z70. Two weeks later mice received stereotaxic injection of AD brain lysate. Mice were sacrificed 4 weeks later and the whole brains were processed for immunohistochemical analysis using AT8. Sections from the hippocampus (injection site) are shown. Scale bars are indicated on the figure.

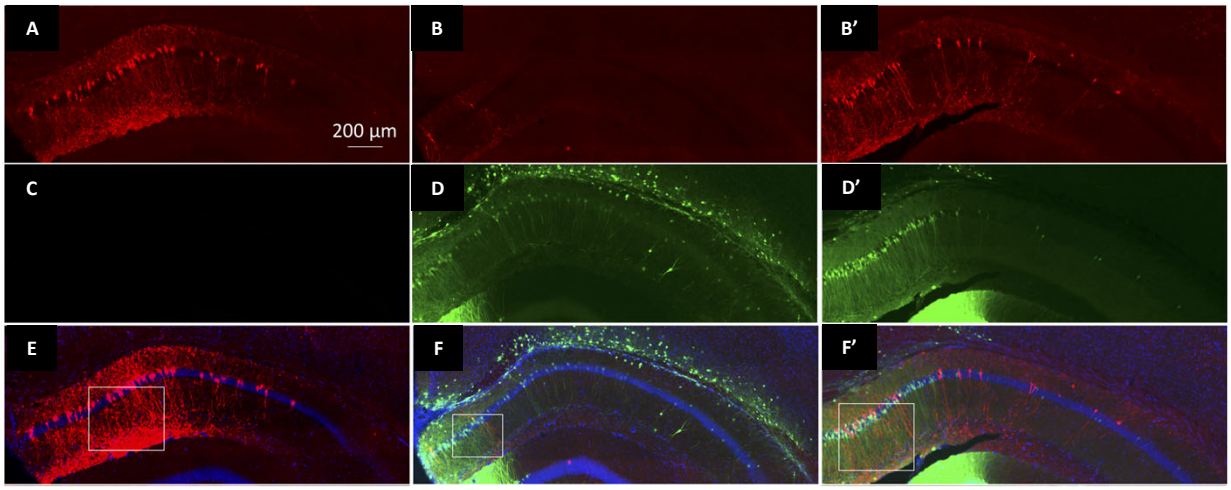


Figure S16 : No Tau lesion in VHH 70-positive neurons- One month-old Tg30tau mice were treated with bilateral intracranial injections of LVs encoding GFP-specific VHH (**A**-**C**-**E**, one mouse) or VHH Z70 (**B**-**D**-**F** and **B'**-**D'**-**F'**, two different mice). Two weeks later mice received stereotaxic injection of AD brain lysate. Mice were sacrificed 4 weeks later and the whole brains were processed for AT8 (red) and mcherry (green) immunoreactivities (VHH Z70 was detected using a primary antibody against mCherry fusion domain). Nuclei are vizualized in blue. Enlargements (white rectangles) of E, F, F' are shown in **Figure 6D**. The scale bar is indicated on the figure.

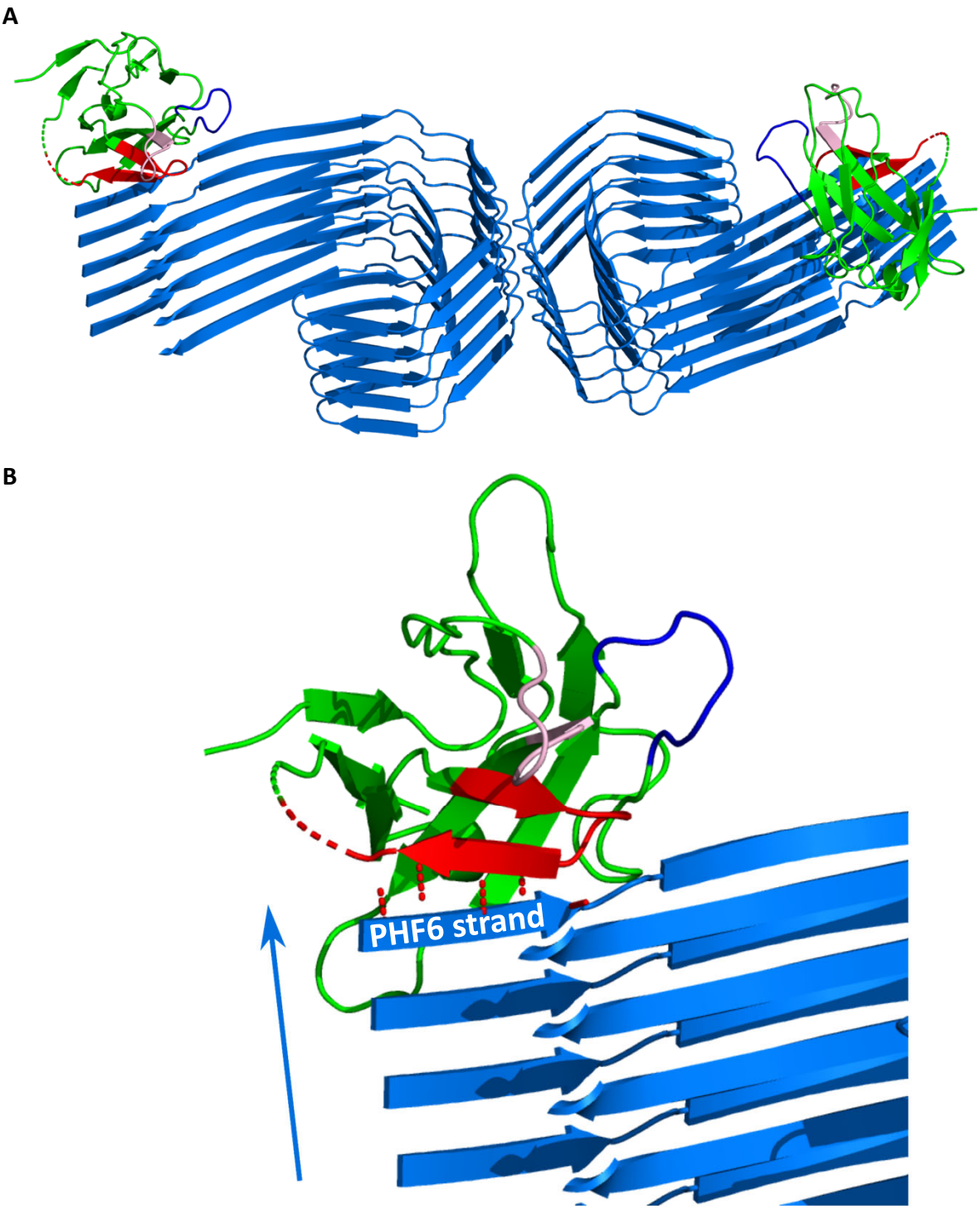


Figure S17 : Structure-based representation of VHH Z70 interaction with AD Tau fiber

A-B : Structure of VHH Z70 (in green) was solved in this study. Cryo-EM structure of Tau fiber (in blue) was obtained from PDB: 5O3L. Color code of the loops as in **Figure S11**. Dashed cartoon line at CDR3 / FR4 junction illustrates the unresolved segment due to high flexibility. Structures were positioned by hand using superimposition of the PHF6 ß-strand respectively in complex with VHH Z70 and within the fiber (with Pymol). **B :** Enlargement of the red-boxed zone of **A**. Intermolecular H-bonds are illustrated by dashed red lines. The fiber elongation axis is illustrated by an arrow. The only accessible PHF6 strand at the fiber extremity is annotated.

This representation of the complex shows the complementation of the fiber ß-sheet by the VHH Z70 ß-hairpin in the CDR3 upon interaction with the PHF6 ß-strand within the fiber. This complex representation supports the hypothesis that the interaction is sterically only feasible at the free extremity of the fiber or oligomer of Tau. Such interaction would be expected to disrupt the seeding and fiber elongation.

3390
5pTCTGCACAATATTTCAAGCTATACCAAGCATACAATCAACTCCAAGCTAGAACCATGGCGGAAGTGCAGCTGCAGGCTC
3880
3pTCTTCTTTTTTGGAGGCTCGGGAATTAATTCCGCTTTATCCATCTTTGCGGCGGCCGCGCTACTCACAGTTACCTG

6690
5pCAGGGCAATAAAGTCGAACT,
6972
5pGACCTACAGGAAAGAGTTACTC

10829
5pCTATTCGATGATGAAGATACCCCACCAAACCCAAAAAAAGAGATCCTAGAACTAGACACTCTTTCCCTACACGACGCTCTTCC
10830
5pCCGGGCCTCTAGACACTAGCTACTCGAGGGGCCCCAGTGGCCCTATCTATGCGGCCGCTCAGACTGGAGTTCAGACGTGTGCTC

Figure S18 Oligonucleotide sequences (Material and Methods)

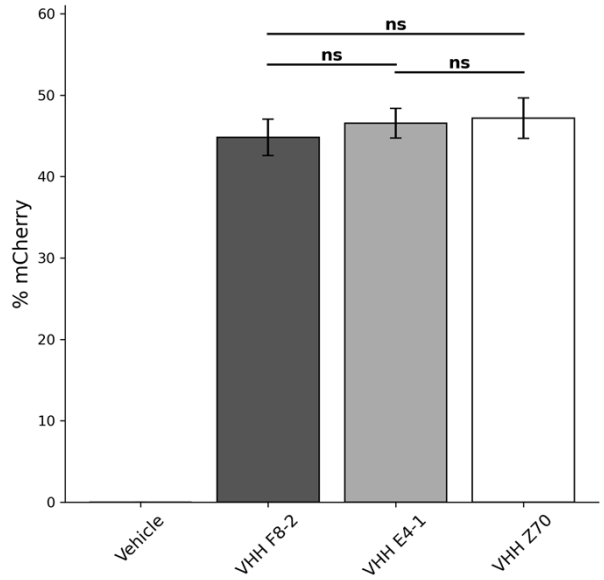


Figure S19 : VHH expression in the biosensor seeding reporter cells. HEK293 Tau RD P301S cells were transfected with plasmids encoding the different mCherry-VHH. mCherry fluorescence was evaluated by flow cytometry showing that transfection efficiency is equivalent : 44.8 % (± 2.2%) for VHH-F8-2, 46.6 % (± 1.8%) for VHH VE4-1 and 47.2 % (± 2;5%) for VHH Z70.

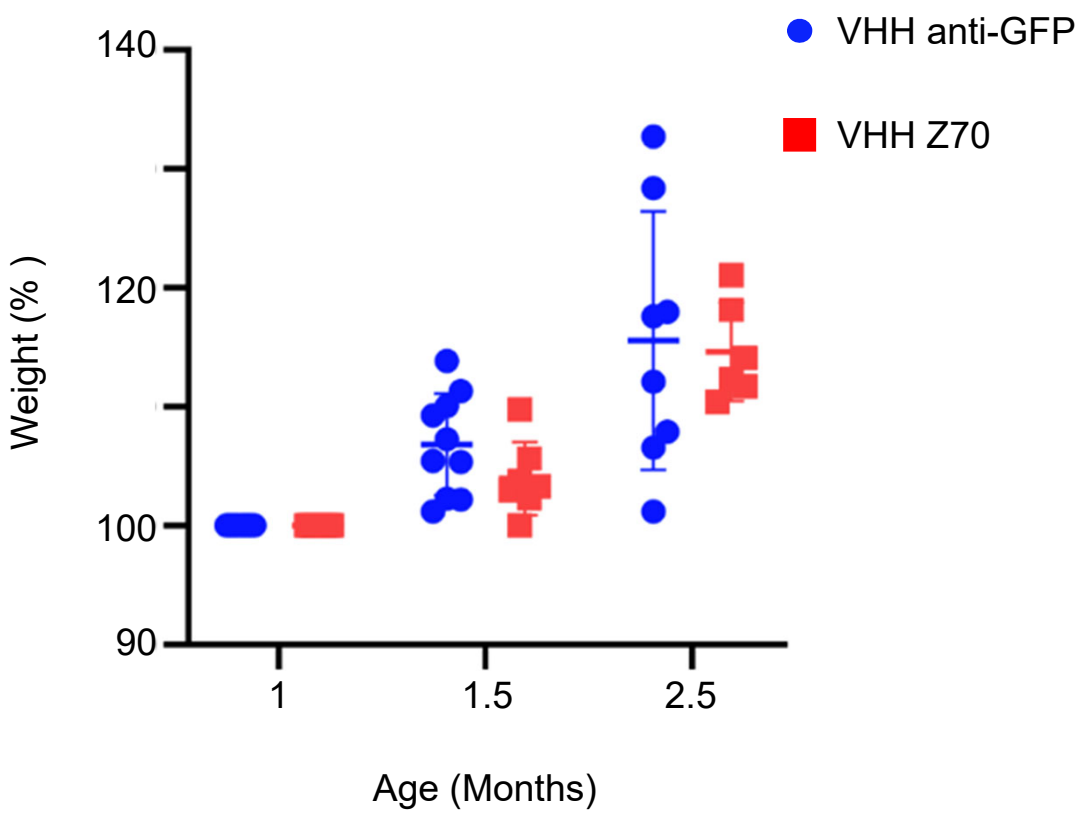


Figure S20 : No VHH toxicity *in vivo*- Mice were weighted three times: 1 Month : before injection of LVs, 1.5 Month : before injection of human brain lysate and 2.5 Month : at sacrifice. Weights were normalized to the first weighting (100%). VHH 70 and VHH anti-GFP correspond to mice injected with one of these VHHs and AD human brain lysate.