Supporting information

RNA Stores Tau Reversibly in Concentrated Coacervates

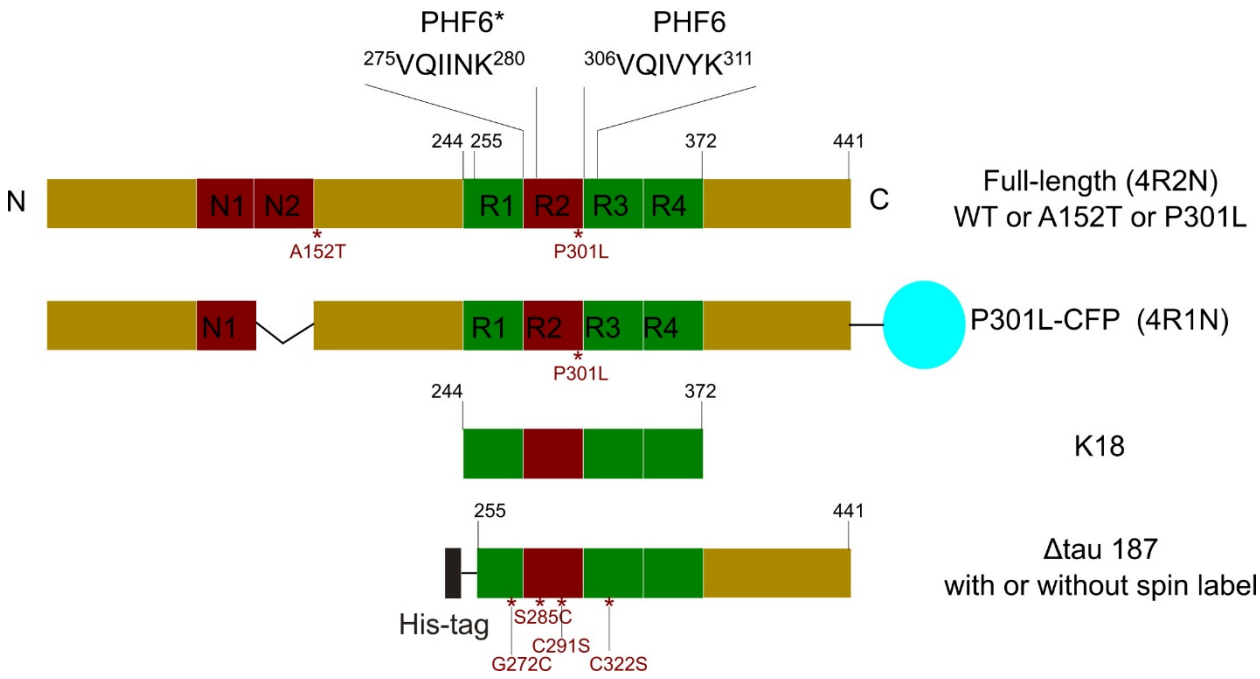


Fig. S1: Overview of Tau constructs used in this study.

Full length human Tau (the longest isoform, 4R2N) comprise the N-terminal projection domain (residues 1-243), the 4-repeat microtubule binding domain (residues 244-372) and the C-terminal region (residues 373-441). The inserts near the N-terminus−N1, N2−and the second repeat−R2−can be alternatively spliced, giving rise to six isoforms. Two hexapeptide motifs−PHF* and PHF−at the beginning of R2 and R3 repeats are known to promote paired helical filament (PHF) aggregation. Cells expressing 4R2N wild type, or mutant (A152T or P301L), or 4R1N P301L fused to CFP were used in PAR-iCLIP study, mutation sites marked here with asterisk. Full length 4R2N Tau, K18 Tau (residues 244-372) and Δtau187 (residues 255-441 with a His-tag at the N-terminus) were used for *in vitro* RNA binding and droplet formation studies. Two variants of Δtau187 were used for EPR line shape analysis and DEER study: Δtau187/322C contains a C291S mutation, leaving only one cysteine at site 322, and Δtau187G272C/S285C contains C291S, C322S, G272C and S285C mutations, leaving two

cysteines at sites 272 and 285.

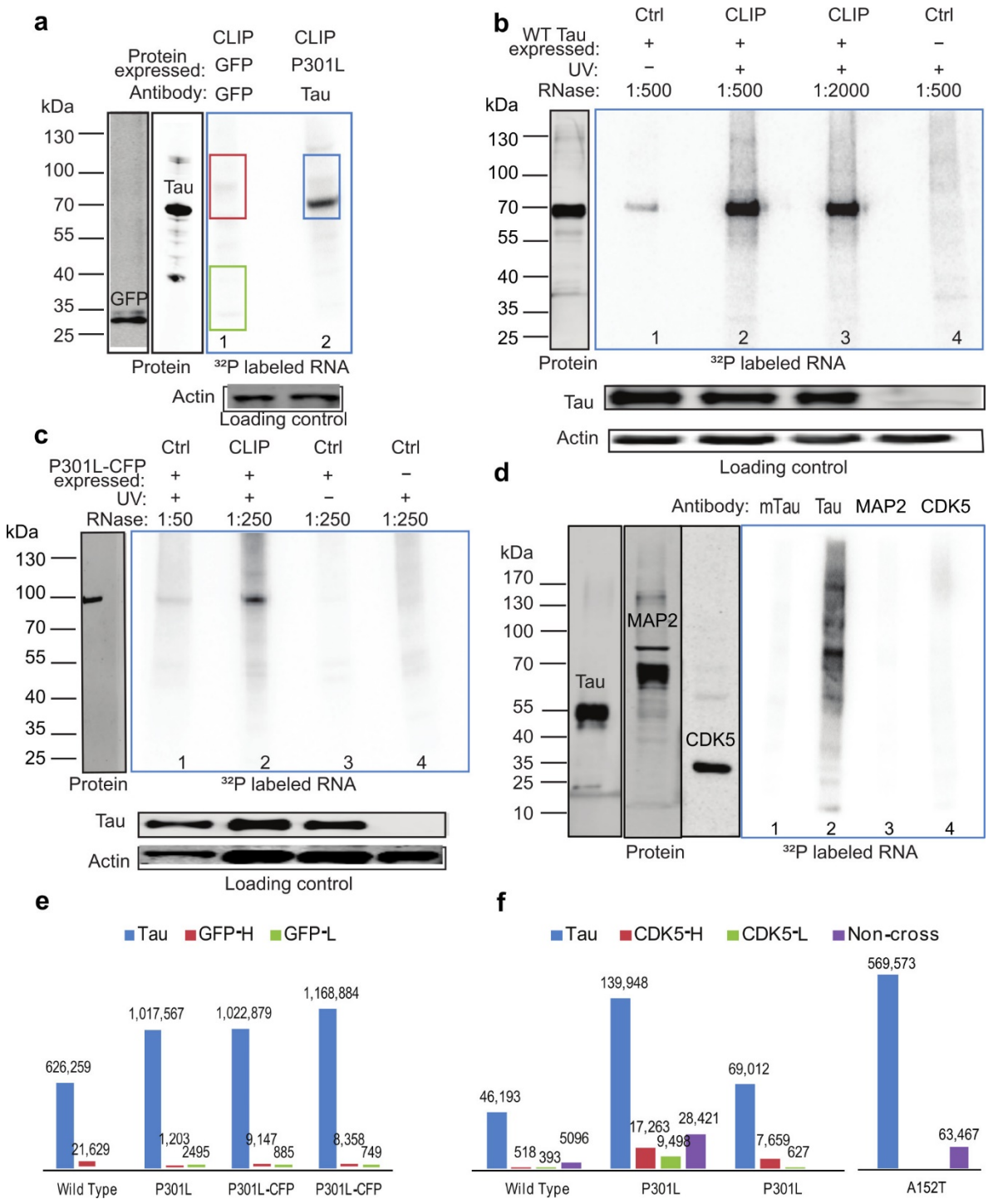
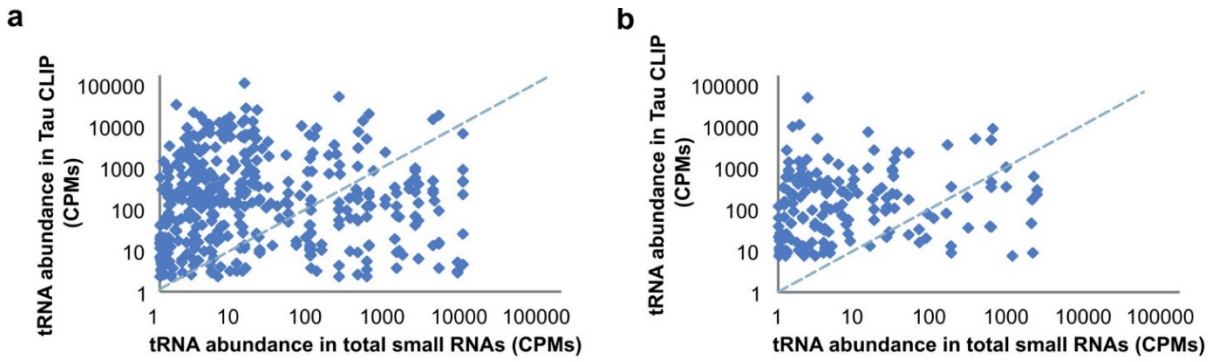


Fig. S2: Additional data of Tau PAR-iCLIP in Tau expressing HEK cells and hiPSC-derived neurons.

As in Fig. 1a-c, phosphor images in the blue frame (a-d) present the ^{32}P-labelled RNA crosslinked to the protein. Labels on top of the image indicate the experimental conditions in

each lane. Probed with the corresponding antibody, western blot to the left of the phosphor images shows the mobility of the control protein and Tau protein. Loading control below shows inputs. (a) PAR-iCLIP with Tau P301L (lane 2) or GFP (lane 1) expressed in HEK cell lysates. HJ 8.5 and anti-GFP were used for protein precipitation. The RNA-protein complexes marked within the rectangle were cut from the blot for library preparation. (b-d) Additional analysis of protein Tau-RNA complex isolated by PAR-CLIP in HEK cell under various conditions using anti-Tau antibody HJ 8.5. (b) PAR-CLIP experiments for wild type Tau expressed in HEK cell. Phosphor images demonstrates the ^{32}P-labelled RNA crosslinked to wild type Tau in experiments with different RNase dilutions (lane 2 and 3) and 2 other controls (lane 1, no UV-crosslinking, and lane 4 no expressed Tau nor GFP). (c) PAR-CLIP experiments as in b for mutant Tau P301L fused to CFP. CLIP experiment (lane 2) compared to 3 controls (lane 1, high RNase digestion; lane 3, no UV-crosslinking, and lane 4, no Tau). Note that the high RNase digested sample (lane 1) contains only 1/5 of the amount of protein in lanes 2-4. (d) PAR-CLIP of MAP2 (lane 3) in differentiated neuroblastoma cells using human Tau (lane 2), mouse Tau (lane 1) and CDK5 (lane 4) CLIP as positive and negative controls. MAP2 CLIP is similar to CDK5 CLIP with no apparent radio-labeled RNA signal. (e) PAR-iCLIP mapped reads in all four samples of HEK cells. (f) PAR-iCLIP mapped reads in all four samples of hiPSC-derived neurons.



c

Top 10 tRNAs bound by Tau in HEK cell and in hiPSC-derived neurons.

HEK cell	Bio-Complex	CLIP reads	tRNA abundance (control)	Log_2 (CLIP /control)
chr15.tRNA4-ArgTCG	4	80497	46	10.74
chr1.tRNA50-AsnGTT	4	3955	8	8.77
chr11.tRNA8-SerGCT	4	53213	125	8.72
chr1.tRNA25-AsnGTT	4	9007	24	8.49
chr1.tRNA26-AsnGTT	4	253216	796	8.31
chr17.tRNA19-ArgTCG	4	23692	91	8.00
chr2.tRNA5-IleTAT	4	42760	174	7.93
chr16.tRNA2-ArgCCT	4	33166	197	7.38
chr16.tRNA15-ThrCGT	4	13493	82	7.34
chr2.tRNA3-AlaAGC	4	15578	101	7.25

iPSC Neuron	Bio-Complex	CLIP reads	tRNA abundance (control)	Log_2 (CLIP /control)
chr15.tRNA4-ArgTCG	4	25774	257	6.64
chr17.tRNA19-ArgTCG	3	5510	98	5.80
chr2.tRNA5-IleTAT	4	6150	163	5.23
chr1.tRNA49-GluTTC	2	1583	52	4.90
chr6.tRNA73-ArgCCG	3	844	67	3.64
chr16.tRNA1-ArgCCG	3	554	67	3.03
chr6.tRNA4-ArgTCG	2	380	47	2.99
chr6.tRNA124-ArgTCG	3	519	67	2.93
chr6.tRNA63-IleTAT	2	218	29	2.87
chr1.tRNA58-LeuCAA	4	2923	402	2.86

Fig. S3: tRNA in PAR-iCLIP samples vs total small RNA controls.

(a-b) tRNA abundance in CLIP samples vs total small RNA controls in HEK cells (a) and hiPSC-derived neurons (b) indicate that tRNA distributions differ between the total tRNA pool and the CLIP tRNA pool. (c) Top 10 tRNAs bound by Tau in HEK cell and hiPSC-derived neurons.

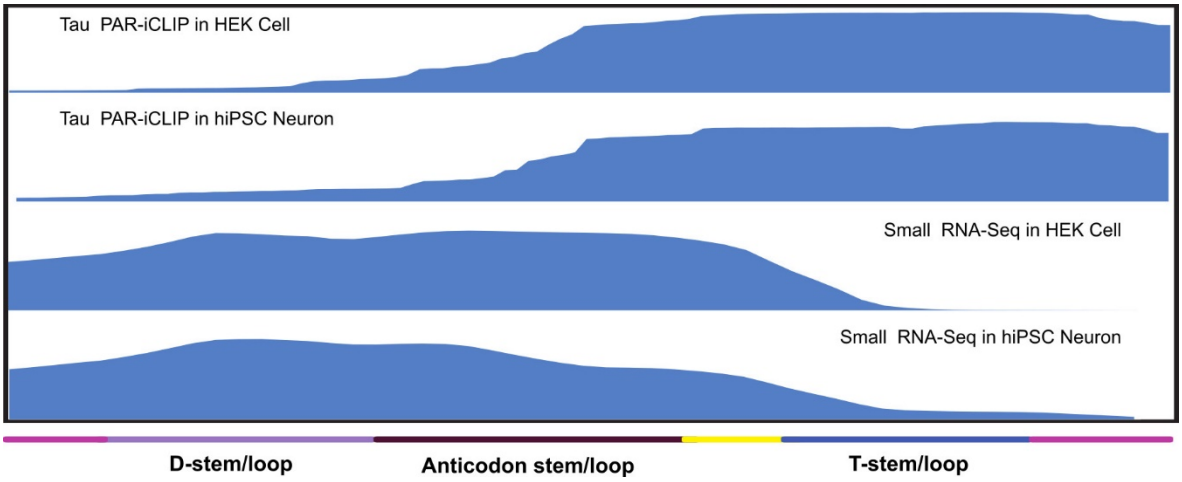
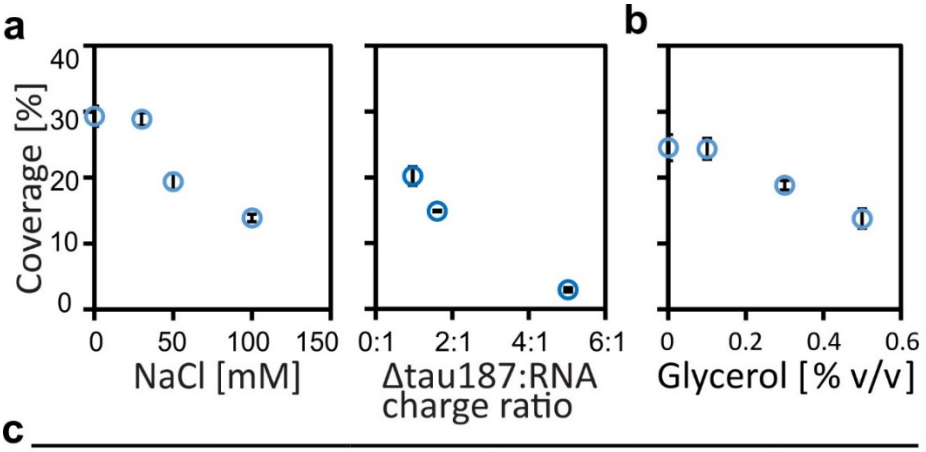


Fig. S4: Piled up reads from all tRNAs recovered in Tau PAR-iCLIP.

Piled up reads from all tRNAs recovered in Tau PAR-iCLIP of HEK cell and hiPSC-derived neurons (top two graphs) in comparison to those from total small RNA-Seq in HEK cells and hiPSC-derived neurons (bottom two graphs). Note the expected 5' bias of the total tRNAs in contrast to the sequencing block at the CLIP site.



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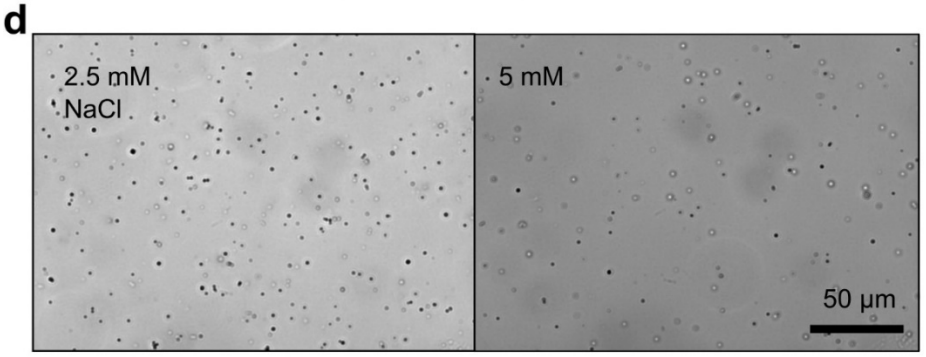
Property	tRNA	poly(A)	poly(U)	ΔTau187	4R2N
MW (kDa)	25	66~660	800~1000	22	45.8
Charge (pH7)	77	194~1906	2473~3090	11.4	-
Charge (pH6)	77	194~1906	2473~3090	-	9
Charge / Mass	3.08	2.94	3.09	0.52	0.2

Exp.	pH	Tau	Tau Conc. (μM)	RNA	RNA Conc. (μg/ml)	RNA Conc. (μM)	Molar ratio	Mass ratio	Charge ratio
1	7	ΔTau187	160	tRNA	480	19.2	8	7	1.23
2			400	poly(A)	800	1.21~12.12	33~330	11	1.97
3			80	poly(A)	240	0.36~3.63	22~220	7	1.29
4			160	poly(U)	480	0.48~0.60	267~333	7	1.23
5	6	4R2N	80	poly(A)	174	0.26~2.63	30~303	21	1.43
6			80	poly(A)	349	0.53~5.29	15~151	10	0.7



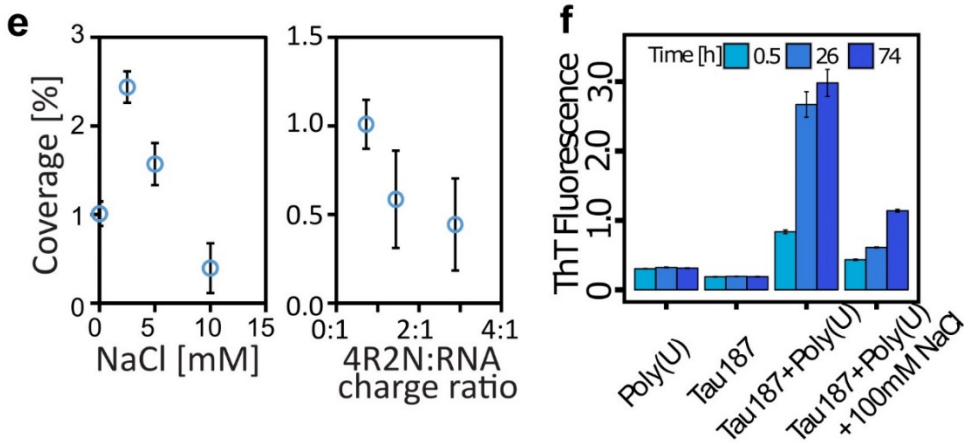


Fig. S5: Additional data about Tau and RNA forms droplet *in vitro*.

(a) Mixing Δtau187 with poly(U) RNA lead to droplet formation *in vitro*. Left: droplet coverage varying NaCl concentration, while a constant charge ratio of Δtau187:poly(U) RNA of 1.2:1 is maintained. Right: droplet coverage varying Δtau187:poly(U) charge ratios, no NaCl was added. A total mass concentration of Δtau187 and poly(U) was kept at ~4 mg/ml. (b) Droplet coverage of Δtau187 and poly(A) RNA varying glycerol concentration. Experimental condition was maintained with tauΔ187:poly(U) charge ratio 2.4 : 1, total mass concentration ~2 mg/ml and no NaCl. (c) Calculations on Tau-RNA molar or charge ratio, based on their chemical properties (top table), for various experimental conditions (bottom table). The charge of a protein at specific pH was estimated (see the method). (d-e) Mixing full length Tau 4R2N with poly(A) RNA lead to droplet formation *in vitro*. (d) Representative bright-field images of Tau-RNA droplet at two NaCl concentrations. (e) Left: droplet coverage varying NaCl concentration, a charge ratio of Tau 4R2N and RNA at 0.7:1. Right: droplet coverage varying Tau 4R2N:RNA charge ratios, no NaCl was added. A total polymer mass concentration ~3.6 mg/ml was maintained in the experiments in d-e. All data and images in a-e were acquired 10 minutes after mixing of Tau, RNA and 19% glycerol in 20 mM ammonium acetate at pH 7. (f) Poly(U) induced fibrilization of Δtau187 measured by Thioflavin T assay. Error bars show standard deviation of three images across one sample (a-b and d-e) or 4 parallel samples (f).

Pipeline of PAR-iCLIP in identifying RNA-Tau interaction in intact cells.

	Steps	Procedure and Purpose
1	Cross-linking	Introduce 4-thiouridine to cell, UV 365 nm, cross-linking RNA to protein according to PAR-CLIP protocol.
2	Nuclease digestion	Add DNase, and RNase at various concentration
3	Immuno-precipitation	Pull down protein Tau and RNA crosslinked to it, high stringency wash
4	Ligate 3' adapter	Dephosphorylation of the 3'-end and incorporate 3'DNA linker
5	Label RNA with p32 ATP at 5' end	By PNK on beads, tracking RNA in following step
6	Separate on Gel	Run on Nupage Bis-tris gel to eliminate IgG non-specific binding, transfer to WB, and expose on phosphoimager to detect RNA on membrane.
7	Degrade the protein and extract RNA	Cut band from gel, add proteinase K, extract with Phenol/Chloroform
8	Reverse transcription	Prepare cDNA: using iCLIP method, the primer contains cleavable adapter region and barcode
9	Size selection of cDNA on TBU gel	Clear off RT Primer, purify cDNA.
10	Circlize and re-linearize	Self-ligation, digesting with BAMHi at the cleavable site of the two adapter region, to re-linearize, cDNA from RT will have one adapter each side
11	PCR library	PCR amplification with primer used for sequencing
12	Quantify library and sequencing	Quantify and analyze the size of oligonucleotides on fragment analyzer, high-throughput sequencing on Ion Torrent Proton
13	Data analysis	Trim the primer, separate by barcode, align to human genome

Fig. S6: Key steps of PAR-iCLIP.

Pipeline of PAR-iCLIP in identifying RNA-Tau interaction in intact cells. The protocol of PAR-iCLIP can be found in the materials and methods and the supplementary file.

Supplementary text

Expression and purification of recombinant Tau

The gene of Δtau187, a truncated version of full length Tau which consists of residues 255-441, was cloned into the pET28a vector [1,2]. The plasmid was then transformed and expressed using *E. coli* BL21 (DE3). Cell pellets were harvested and the resulting pellets were resuspended in lysis buffer (Tris-HCl pH 7.4, 100 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA, 1mM PMSF) with 1 Pierce protease inhibitor tablet (Thermo Scientific). Lysis was initiated by the addition of lysozyme (2 mg/ml), DNase (20 µg/ml), and $MgCl_2$ (10 mM) and incubated for 30 min on ice. Lysate was then sonicated for 10 min, boiled for 12 min. Samples were cooled on ice for 20 min and then centrifuged to remove the precipitant. The supernatent was loaded onto a Ni-NTA agarose column equilibrated in wash buffer A (20 mM sodium phosphate pH 7.0, 500 mM NaCl, 10 mM imidazole, 100 µM EDTA). The column was then washed with 20 ml of buffer A, 15 ml buffer B (20 mM sodium phosphate pH 7.0, 1 M NaCl, 20 mM imidazole, 0.5 mM DTT, 100 µM EDTA), and then 10 ml of buffer A again. Purified Δtau187 was eluted with buffer C (20 mM sodium phosphate pH 7.0, 0.5 mM DTT, 100 mM NaCl) supplemented with varying amounts of imidazole increasing from 100 mM to 300 mM. The protein was then precipitated using an equal volume of methanol, in the presence of 10 mM DTT, at -20^{o}C. The protein was then collected by centrifugation at 5,000 rpm at 4^{o}C for 45 min. The final protein concentration was determined by UV-Vis absorption at 274 nm using an extinction coefficient of 2.8 $cm^{-1}mM^{-1}$, calculated from absorption of Tyrosine [3].

Full length Tau protein (4R2N,1-441) or the microtubule binding domain (K18, 244-372) was purified with slight modification to previously published protocols [4]. Briefly, 4R2N Tau in the pRK172 plasmid were expressed in E. coli BL21 (DE3). Cell pellets were harvested and resuspended in cell lysis buffer (50 mM MES pH 6.5, 5 mM DTT, 1mM PMSF, 1 mM EGTA) + cOmplete protease inhibitor tablets (Roche). Lysate was sonicated, boiled for 10 min, and then centrifuged at 50,000 x g for 30 min at 4^{o}C. The supernatant was then precipitated with ammonium sulfate (20% w/v) and centrifuged at 20,000 x g for 30 min at 4^{o}C. The pellet was resuspended into 4ml of MonoS Buffer A (50 mM MES pH 6.5, 50 mM NaCl, 2mM DTT, 1 mM PMSF, 1mM EGTA) and dialyzed overnight against the same buffer. The protein was loaded on

to a MonoS column (GE Healthcare) and eluted with a linear gradient of NaCl using MonoS Buffer B (Buffer A + 1 M NaCl). Fractions containing 4R2N Tau were pooled, concentrated and dialyzed overnight into PBS pH 7.4. Protein concentration was determined using a BCA assay (Thermo Scientific).

Detailed PAR-iCLIP protocol

UV crosslinking

Cells were treated with 4-thiouridine (4SU) (Sigma-Aldrich) for 1h at a final concentration of 500 µM at 37°C, rinsed with ice-cold 1x PBS and irradiated one time with 400 mJ/cm^2 of 365 nm UV light on ice. The cells were centrifuged and the pellet stored at -80°C.

Bead preparation

Protein A/G plus beads (Thermo Scientific) (40 µl) were added to a spin column (Thermo Scientific) and washed 4 times with 500 µl conjugation buffer (1x PSB made from 10x buffer using RNase-free water). Anti-Tau antibody (HJ 8.5, 20 µl, 4.5 mg/ml) or anti-GFP (abcam, 1.5 µl) or anti-CDK5 (Santa Cruz , 40 µl, 0.2 mg/ml) was added to the beads in 800 µl of conjugation buffer and rotated for 1 hour at room temperature. The supernatant was filtered and the beads were washed twice with 800 µl conjugation buffer. The antibody was crosslinked to the beads using 800 µl of 450 µM DSS (Thermo Scientific) in conjugation buffer and rotated for 1 hour at room temperature. The supernatant was discarded and the reaction was quenched with 800 µl 0.2 M Tris buffer for 15 minutes at room temperature. *The beads were then washed 3 times with 600 µl IP buffer (*50 mM Tris pH 7.4, 150 mM NaCl, 1 % NP40*) and resuspended in 0.8 ml lysis buffer* (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP40 and 1x protease inhibitor from Roche).

Cell lysis and partial RNA digestion

Lysis buffer (500 µl) was added to each cell pellet and incubated for 10 minutes on ice. For experiments using RNase, RNase I (Thermo Fisher) in lysis buffer were prepared, a 1:50 dilution for high RNase and a 1:250 or 1:500 or 1:2000 dilution for low RNase. 10 µl of diluted RNase was added to 500 µl lysate. To save material 2 µl of high RNase was added to 100 µl lysate (1/5 total lysate) and used as reference for high RNase digestion. Turbo DNase (2 µl, Thermos Fisher)

was added to 500 µl lysate and to 0.4 µl Turbo DNase was added to 100 µl lysate. The samples were immediately placed at 37°C for exactly 3 minutes. Afterwards the samples were centrifuged at 20,000 x g for 10 minutes at 4°C. The supernatant was collected without touching the pellet. The protein concentrations of the supernatants were determined using the BCA protein assay kit (Thermo Scientific). The final concentration of protein was adjusted with lysis buffer to 0.4 -1 mg in 500 µl. Input of the control was kept at the same amount for direct comparison.

Immunoprecipitation

Lysate (500 µl) was added to the beads and rotated overnight at 4°C. The next day the supernatant was discarded and the beads were washed twice with 600 µl cold high salt buffer (50 mM Tris pH 7.4, 1 M NaCl, 1% NP-40, 1 mM EDTA), each for 5 minutes. Then was washed twice with 600 µl wash buffer (20 mM Tris pH 7.4, 10 mM $MgCl_2$, 0.2% Tween-20) and the beads were left in the last wash.

RNA 3' end dephosphorylation

The supernatant was discarded and the beads were resuspended in 40 µl of the following mixture:

8 µl, 5x PNK pH 6.5 buffer (350 mM Tris, pH 6.5, 50 mM $MgCl_2$, 5 mM DTT);
1 µl, PNK (NEB);
1 µl, RNasin (Promega);
30 µl, ddH_2O.

The mixture was incubated and shaken at 1100 rpm for 20 minutes at 37°C. Then washed once with 600 µl wash buffer and for at least 5 minute with 600 µl high salt buffer at 4°C. At the end it was washed twice with 600 µl wash buffer.

L3 adapter ligation

The supernatant was discarded and the beads were resuspended in 40 µl of the following mixture:

4 µl, ddH_2O;
10 µl, 4x ligation buffer (*200 mM Tris-HCl PH7.8; 40 mM $MgCl_2$, 40mM DTT)*;
2 µl, T4 RNA ligase (NEB);
1 µl, RNasin (Promega);
3 µl, pre-adenylated adapter L3-App (20 µM)

(IDT rAppAGATCGGAAGAGCGGTTCAG/ddC/);

20 µl, PEG8000 50%.

The mixture was incubated at 16°C at 1100 rpm overnight. The next day 600 µl wash buffer was added, then washed twice with 600 µl high salt buffer, each wash was rotated at 4°C for 5 minutes and then washed twice with 600 µl wash buffer.

5' end labeling

Remove the supernatant and 36 µl of hot PNK mix was added to the beads*:*

1.8 µl, PNK (NEB);

3.6 µl, PNK 10x buffer (NEB);

29.4 µl, H_2O;

1.2 µl, ATP [γ-^{32}P].

The mixture was incubated at 37°C for 5 minutes. The radioactive solution was removed and discarded in the radioactive waste container.

Run 4-12% NuPAGE gel and transfer to nitrocellµlose membrane

2x NuPAGE gel loading buffer with ddH_2O was prepared and 25 µl was added to the beads. The samples were heated for 5 minutes at 70°C.

The NuPAGE gel was run at 180 volts for about 35-50 minutes with 20x MOPS-SDS running buffer (Thermo Fisher) and transferred for 90 minutes at 30 volt with 20x Transfer buffer (Thermo Fisher) to a nitrocellulose membrane (Protran *BA85*, VWR). *T*he membrane was rinsed twice with 1x PBS and it was blotted briefly with Kimwipe at the back side. Then the membrane was wrapped with parafilm and placed on the top of the paper board, which has been unsymmetrically labeled with radioactive dye marker (prepared from blue marker dye and old ATP [γ-^{32}P]) and wrapped in parafilm, for later positioning the radioactive signal. The packet of membrane and paper board was screened under phosphor imaging for 1 hour. The phosphor imager was scanned and analyzed using Quantity One software (Bio-Rad) and the packet was also scanned for image alignment.

RNA isolation

An area of the membrane that contained the target protein and up to an additional 45 kDa was cut out. The same region was cut out in the control sample as well as the region corresponding to the irrelevant protein GFP or CDK5. The membranes were transferred to a 1.5 ml tube and was added the mixture of 40 µL proteinase K (20 mg/ml, Thermo Fisher) in 160 µl PK buffer (100 mM Tris–HCl, pH 7.4, 50 mM NaCl, 10 mM EDTA), shaken at 1100 rpm at 37°C for 30 min. Another solution containing 160 µl PK and 40 µl 20% SDS (final concentration of SDS is 2%) was then added to the samples, shaken at 1100 rpm at 55°C for 30 min. The supernatant together with 450 µl of saturated phenol: chloroform (1:1) mixture was added to a 2 ml Phase Lock Gel Heavy tube (VWR), incubated for 5 min at 30°C, followed by spinning for 5 min at full speed room temperature.

The aqueous layer was transfered into a new tube, precipitated by adding 0.75 µL Glycoblue (Thermo Fisher), 0.1 volume of 3M sodium acetate pH 5.5 (Thermo Fisher), vortexed and added 3x volume of 100% ethanol, mixed again and placed at -20°C overnight.

The mixture was centrifuged at 14,000 rpm at 4°C for 20 min. The radioactivity was monitored to make sure the precipitation of RNA. The pellet was washed with 0.9 ml of 80% ethanol and spinned again for 5 min, then resuspended in 5 µl of H_2O and transferred to a PCR tube.

The further protocol was assumed from published procedures [5], except that for the PCR amplification the following specific primers for one directional sequencing the primer mix of A-P5Solexa/ trP1-P3Solexa was used:

A **Target Forward P5Solexa**

5’-CCATCTCATCCCTGCGTGTCTCCGACTCAG|CACGACGCTCTTCCGATCT-3’ 49 nt

trP1 **Target Reverse P3Solexa**

5’-CCTCTCTATGGGCAGTCGGTGAT|CTGAACCGCTCTTCCGATCT-3’ 43 nt

References:

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