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The human *MAPT* locus generates circular RNAs

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ABSTRACT

The microtubule-associated protein Tau, generated by the *MAPT* gene is involved in dozens of neurodegenerative conditions ("tauopathies"), including Alzheimer's disease (AD) and frontotemporal lobar degeneration/frontotemporal dementia (FTLD/FTD). The pre-mRNA of *MAPT* is well studied and its aberrant pre-mRNA splicing is associated with frontotemporal dementia. Using a PCR screen of RNA from human brain tissues, we found that the *MAPT* locus generates circular RNAs through a backsplicing mechanism from exon 12 to either exon 10 or 7. *MAPT* circular RNAs are localized in the cytosol and contain open reading frames encoding Tau protein fragments. The *MAPT* exon 10 is alternatively spliced and proteins involved in its regulation, such as CLK2, SRSF7/9G8, PP1 (protein phosphatase 1) and NIPP1 (nuclear inhibitor of PP1) reduce the abundance of the circular MAPT exon 12→10 backsplice RNA after being transfected into cultured HEK293 cells. In summary, we report the identification of new bona fide human brain RNAs produced from the *MAPT* locus. These may be a component of normal human brain Tau regulation and, since the circular RNAs could generate high molecular weight proteins with multiple microtubule binding sites, they could contribute to taupathies.

1. Introduction

The human microtubule-associated protein Tau is highly expressed in the brain and promotes the assembly and stabilization of microtubules [1]. Tau protein can mis-fold into paired helical filaments and neurofibrillary tangles, which characterize a group of neurodegenerative diseases known as tauopathies, that include Alzheimer's disease (AD), frontotemporal lobar degeneration (FTLD-TAU), progressive supranuclear palsy (PSP), chronic traumatic encephalopathy (CTE), and primary age-related tauopathy (PART) [2]. The clearest connection between the *MAPT* gene and neurodegeneration is found in FTLD-MAPT, as the disease is caused by many different known mutations in the *MAPT* locus on chromosome 17 [3]. Studies in mice indicate that tau protein is necessary for amyloid-beta induced neuronal cell death [4], and thus plays a central role in AD.

The human *MAPT* gene contains 16 exons, with exons 2, 3, 4a, 6, 8 and 10 being alternatively spliced cassette exons (Fig. 1A). Alternative splicing of these exons in the normal adult human brain generates six major protein isoforms. These isoforms differ at the N-terminus due to alternative exons 2 and 3 in the tau projection domain and in the number of microtubule binding repeats due to alternative splicing of exon 10. Exon 10 encodes one of the four microtubule binding sites, and its alternative usage generates tau isoforms with either 3 or 4 binding sites (3R, 4R) that differ in their affinity to microtubules [5] and could thus 'fine tune' the interaction between the Tau protein and microtubules.

At least 19 mutations causing FTLD-MAPT have been identified in exon 10 and its 5′ splice site [3]. Pathological changes in exon 10 usage without mutations in exon 10 result in tauopathies, for example PSP and corticobasal degeneration (CBD) are characterized by 4R Tau, whereas Pick's disease is characterized by 3R Tau. Post mortem studies of AD brains indicated a slight increase of exon 10 usage [6–8], as well as deregulation of protein factors that regulate exon 10 splicing, namely the SR-like protein tra2-beta1 that promotes exon 10 usage and its kinase, CLK2, that inhibits exon 10 usage [6,9].

In addition to the well known linear RNAs, pre-mRNAs generate circular RNAs through a backsplicing mechanism, where a downstream 5′ splice site is joined with an upstream 3′ splice site [10]. In most cases, circRNAs are generated when the pre-mRNA forms a loop containing the exons undergoing backsplicing. This loop can be formed by either a large lariat or more commonly through intramolecular RNA base pairing, leading to double stranded RNA regions as short as 30–40 nt in length. Frequently, repeat elements that have regions of base complementarity provide the basis of loop formation. In humans, these elements are often Alu elements [11,12], comprising around 11% of the human genome [13]. Due to their self complementarity Alu elements form extensive double stranded RNA structures in pre-mRNA, which can influence alternative splicing [14] and promote the formation of

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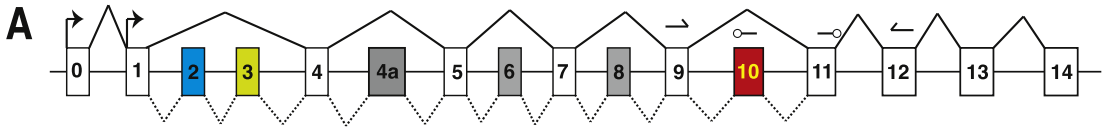

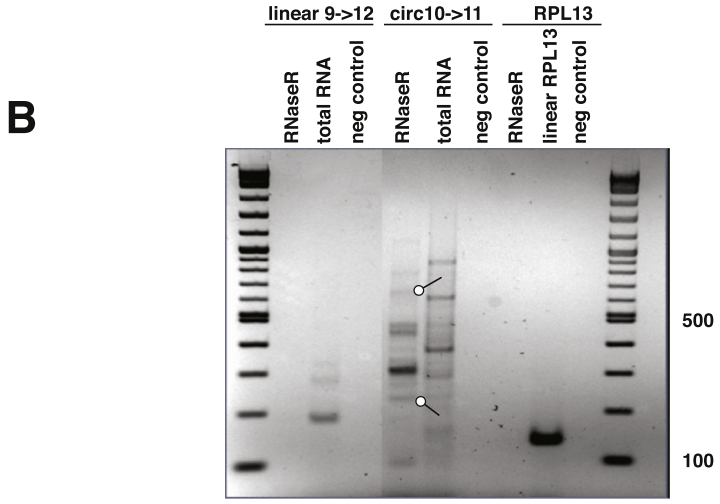

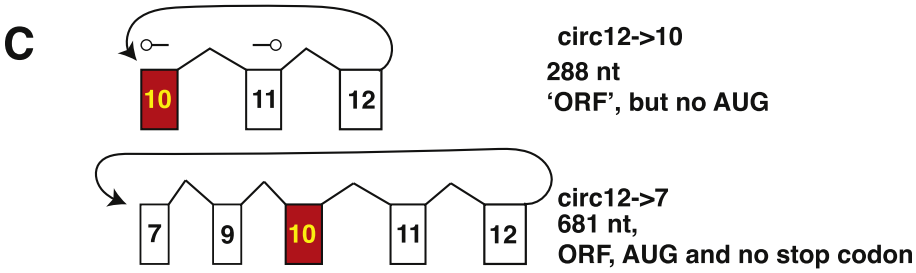

D

circ12->10:

GTGCAGATAATTAATAAGAAGCTGGATCTTAGCAACGTCCAGTCCAAGTGTGGCTCAAAGGATAATATCAAACACGTCCCGG
GAGGCGGCAGTGTGCAAATAGTCTACAAACCAGTTGACCTGAGCAAGGTGACCTCCAAGTGTGGCTCATTAGGCAACATCCA
TCATAAACCAG*GAGGTGGCCAGGTGGAAGTAAAATCTGAGAAGCTTGACTTCAAGGACAGAGTCCAGTCGAAGATTGGGTCC*
CTGGACAATATCACCCACGTCCCTGGCGGAGGAAATAAAAAG

E

circ 12->7: **GGGGCTGATGGTAAAACGAAGATCGCCACACCGCGGGGAGCAGCCCCTCCAGGCCAGAAGGGCCAGG**
CCAACGCCACCAGGATTCCAGCAAAAACCCCGCCCGCTCCAAAGACACCACCCAGCTCTG*GTGAACCTCCAAAATCAGGGGATC*
GCAGCGGCTACAGCAGCCCCGGCTCCCCAGGCACTCCCGGCAGCCGCTCCCGCACCCCGTCCCTTCCAACCCCACCCACCCGGG
AGCCCAAGAAGGTGGCAGTGGTCCGTACTCCACCCAAGTCGCCGTCTTCCGCCAAGAGCCGCCTGCAGACAGCCCCCGTGCCC
ATG*CCAGACCTGAAGAATGTCAAGTCCAAGATCGGCTCCACTGAGAACCTGAAGCACCAGCCGGGAGGCGGGAAG*GTGCAGATA
ATTAATAAGAAGCTGGATCTTAGCAACGTCCAGTCCAAGTGTGGCTCAAAGGATAATATCAAACACGTCCCGGGAGGCGGCAGT
GTGCAAATAGTCTACAAACCAGTTGACCTGAGCAAGGTGACCTCCAAGTGTGGCTCATTAGGCAACATCCATCATAAACCAG*GA*
GGTGGCCAGGTGGAAGTAAAATCTGAGAAGCTTGACTTCAAGGACAGAGTCCAGTCGAAGATTGGGTCCCTGGACAATATCACC
CACGTCCCTGGCGGAGGAAATAAAAAG

Fig. 1. The human *MAPT* gene generates circular RNAs through exon 12 backsplicing.

A. Schematic structure of the human *MAPT* gene.

Constitutive exons are in white, alternative exons are colored. Splicing patterns are indicated by lines. The two transcriptional start sites in exons 0 and 1 are indicated by arrows. The numbering of exons is from [29]. The arrows in exons 9 and 12 indicate the position of primers to amplify linear *MAPT* mRNA, arrows in exons 10 and 11 indicate primers to detect circular RNAs. The arrowheads indicate the 3′ ends.

B. Circular RNAs generated through backsplicing of exon 12.

10 μg of total human hippocampus RNA was amplified with primers pointing outwards in exon 10 and 11. Prior to RT-PCR, an equal part of the RNA was digested with RNase R. The negative control was performed without reverse transcription. The PCR-products were gel purified, subcloned and sequenced. Two bands indicated by arrows were circular RNAs generated through backsplicing of exon 12. The other bands were likely PCR artifacts, i.e. amplicons from different gene regions.

C. Structure of the RNAs generated through exon 12 backsplicing.

D. Sequence and predicted ORFs for circ12 → 10 The RNA part corresponding to exon 10 is underlined, exon 11 is in regular letters and exon 12 is underlined and italic.

E. Sequence and predicted ORFs for circ12 → 7. Exon 7 is bold, exon 9 italic, exon 10 is underlined, exon 11 is in regular letters and exon 12 is underlined and italic. The in-frame AUG is shown in bold.

circRNAs [11]. With about 50 highly expressed exceptions, circRNAs comprise only 1–5% of their linear counterparts. Since they lack a 3′ or 5′ end, they escape the exonucleic degradation of linear RNAs and are thus more stable. The analysis of a few circRNAs showed that they mainly reside in the cytosol, where they can function as microRNA sponges [12], and can undergo translation in the presence of an internal ribosomal entry site [15], possibly aided through methylation of adenine bases at the N6 position [16]. In addition, intron-containing circRNAs have been implicated in transcriptional control in the nucleus [17].

Here, we used a PCR approach to identify circular RNAs from the human tau locus that contain the alternatively spliced exon 10. These circular RNAs are low abundant, comprising < 1% of the linear tau RNA. The data shows that not all RNAs generated from the human tau locus have been identified.

2. Material and methods

2.1. Primers

9 → 11 Forward: TGT CAA GTC CAA GAT CGG CT
9 → 11 Reverse: CTG GCC ACC TCC TGG TTT
11 → 10 Forward: GAC CTC CAA GTG TGG CTC AT
11 → 10 Reverse: TGG ACT GGA CGT TGC TAA GA
RPL13 Forward: GCC ATC GTG GCT AAA CAG GTA
RPL13 Reverse: GTT GGT GTT CAT CCG CTT GC
Tau 12 → 10 reverse: cag ctt ctt att aat tat ctg cac ctt tt
Tau 10 → 11 forward: gag gcg gca gtg tgc aa
HIPK3f: tcg gcc agt cat gta tca aa
HIPK3r: tgc ttg gct cta ctt tga gtt tc
Tau exon12 Rev.: ccc aat ctt cga ctg gac tc
Tau exon 9 Forw: tgt caa gtc caa gat cgg ct

2.2. Minigene generation

The tau exon 9–12 minigene was generated using Gibson cloning by assembling exons 9, 10, 11 and 12 in pcDNA3.1 using Q5 DNA polymerase (NEB) and these primers:

Vector1 AAGCTTAAGTTTAAACGCTAGCCAGCTTG
Vector 2 CTCGAGTCTAGAGGGCCCGTTTAAACC
Exon 9f ctggctagcgtttaaacttaagcttACGCTGGCCGCAGGGATT
Exon 9r cctgtggatttttGGCCCACGAGTGGAGATGC
Exon 10f ccactcgtgggccAAAAATCCACAGGTGATTCTGATGCC
Exon 10r gagtggggtatctGCGGCAGCCCAGTCTCAG
Exon 11f actgggctgccgcAGATACCCCACTCCTGCCTTTCCA
Exon 11r aacatcctgtaaaccatgaccccacAGTAGCTGGGACTACAGGCG
Exon 12f GTGGGGTCATGGTTTACAG
Exon 12r tttaaacgggccctctagactcgagAAACTGCAGTGACTTAGGCC

2.3. RNA isolation

Samples were derived from short-postmortem interval (PMI) autopsies. All methods conformed with a University of Kentucky IRB protocol. Premortem clinical evaluations and pathological assessments were as described previously [18]. The inclusion criteria that were applied: generally PMI < 4 h; no evidence of frontotemporal dementia; no cancer in the brain parenchyma; and no large infarctions in the brain, or microinfarcts found within 3 cm of the brain tissue samples. We also obtained information on agonal events for each subject, and additional criteria for exclusion from the study were an extended interval of premortem hypoxia, any medical ventilator use, brain edema, or large infarct. The characterization of the samples is shown in Supplemental Fig. 1. The RNA isolation was performed using Trizol and the PureLink RNA mini kit from Ambion (Life Technologies).

2.4. Cell fractionation

Cells were harvested through trypsination, washed once with PBS and then resupendend in Buffer A (10 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) and incubated on ice for 10 min. After swelling in buffer A, cells were ruptured in a 0.5 ml Dounce homogenizer using 10 strokes of the tight pestle “B”. Cytosol was recovered by centrifugation at 2000*g* for 10 min. Nuclei were resuspended in Buffer S1 (0.25 M sucrose, 10 mM $MgCl_2$) and layered over 4 ml of Buffer S2 (0.35 M sucrose, 10 mM $MgCl_2$). Nuclei were recovered through 20 min of centrifugation at 3500*g*.

2.5. Transfection assays

Transfection assays were performed as described [19]. Briefly, DNA was incubated with PEI (Polyethylenimine, 1 mg/ml, (Sigma), final concentration 3 μg/μg PEI/DNA, per 300,000 cells) for 10 min and then added to HEK293 cells that were 60% confluent in six-well plates. A GFP construct was added to visualize transfection efficiency (> 80%). The RNA was isolated after 24 h using the PureLink RNA Mini Kit (Ambion, Life Technologies).

2.6. RT-PCR

RT-PCR was performed as described [20] using an H^- MMLV reverse transcriptase (100 U per reaction, Invitrogen) 500 μM, dNTPs, 0.5–1 μg RNA, 500 nM gene-specific primers. PCR was performed using Platinum Taq Polymerase (Invitrogen) (200 μM dNTPs, 500 nM primer). PCR was performed on a Mastercycler Nexus2 (Eppendorf). Denaturation was at 94° for 30 s, extension at 68° for 30 s. Annealing temperatures and cycles used were: 46°, 20×, (SNORD2), 55°, 20× (HIPK3), 55°, 30× (linear tau). Circular tau was amplified using touchdown, starting at 65° lowered per cycle by 0.5° to 58° (14 cycles), followed by 16 cycles at 58°.

2.7. RNase protection

RNase protection was performed as described [21] using the RPAIII kit (Ambion/Invitrogen) and one million cpm of a ^{32}P uniformly labeled probe. Hybridization was overnight, the digestion used RNaseA and T1 for one hour.

3. Results

3.1. Tau generates circular RNAs through exon 12 backsplicing

No human circular *MAPT* RNAs have been reported in databases. Given the extensive alternative pre-mRNA processing of the human tau gene (Fig. 1A), we used a PCR approach to search for possible human circular *MAPT* RNAs. We concentrated on exon 10, since this exon is alternatively spliced and deregulated in both Alzheimer's disease and FTLD-MAPT [6,7]. To amplify circular RNAs, we used a reverse primer upstream of the forward primer, i.e. a reverse primer in exon 10 and a forward primer in exon 11, which amplifies circular, but not linear RNAs (Fig. 1A, C). To further enrich for circular RNAs, we digested the RNA with RNase R, a bacterial RNA exonuclease that removes linear RNAs [8].

As expected, RNAse R treatment removed the signal for linear *MAPT*, detected by primers in exons 9 and 12 and RPL13 mRNA, but enriched the signal for circular RNAs (Fig. 1B). The bands from RNAse R treated RNA generated by the circular RNA primers were subcloned and sequenced. Two of the bands corresponded to circular RNAs made from exon 12 back splicing, either to exon 10 or 7 (Fig. 1C). The exon sequences present in the linear *MAPT* mRNA were completely present in the circular RNAs, indicating usage of the linear splice sites. Both circRNAs were divisible by 3 (288, and 681 nt, respectively) and circ12 → 7 contained an in frame AUG start codon. No AUG start codon

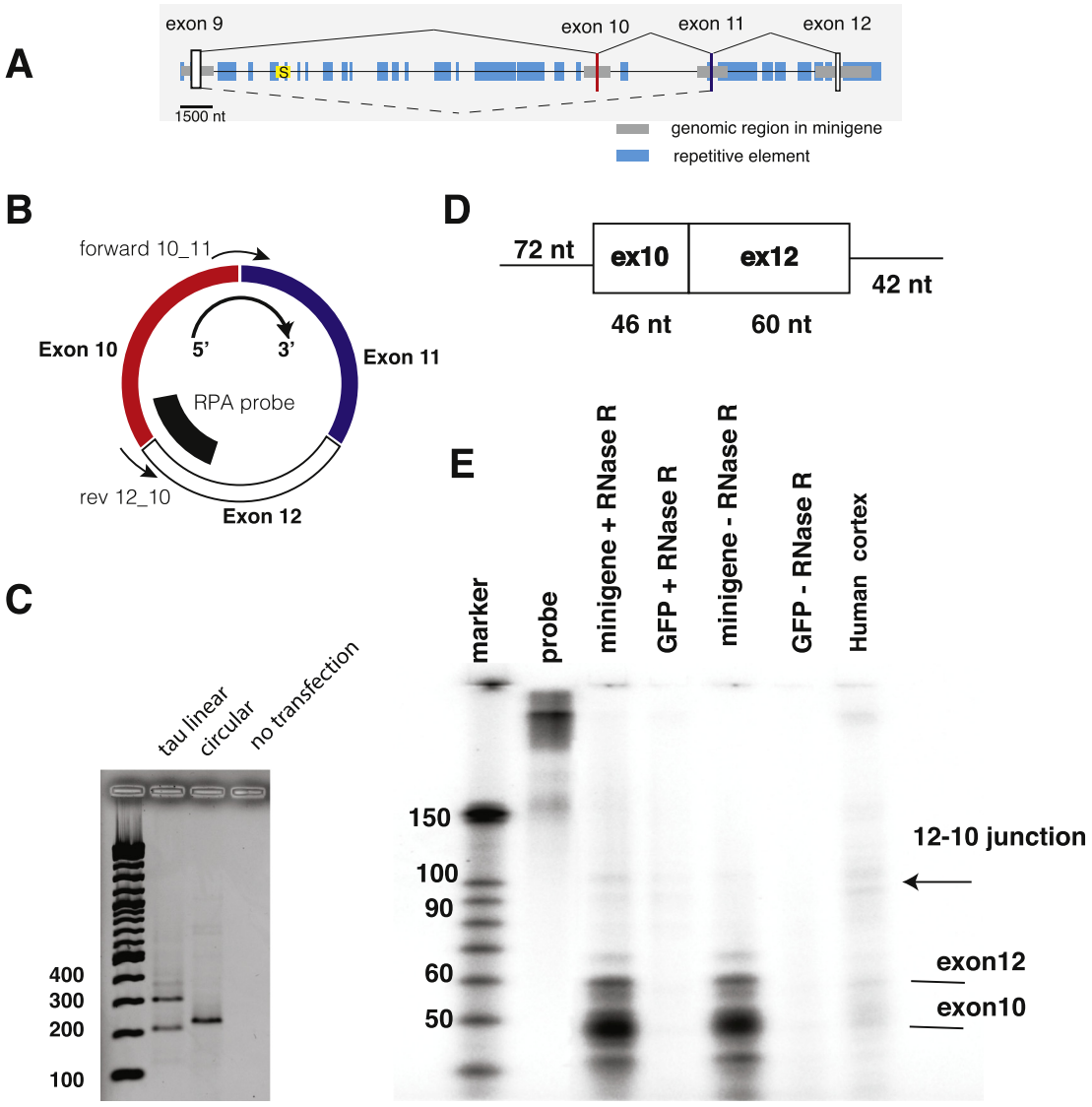

Fig. 2. A minigene containing exons 9–12 generates circ12 → 10.

A. Structure of the minigene. Exons are indicated as vertical boxes, horizontal gray boxes indicate the genomic regions used for cloning the minigene. Blue boxes indicate repetitive elements defined by the UCSC genome browser repeat masker. The yellow box with an 'S' depicts the intronless Saitohin reading frame. The drawing is to scale, as indicated.

B. Detection of circ12 → 10. The orientation of the circular RNA is clockwise 5′→3′. The location of the detection primer for10_11 and rev12_10 is indicated. The probe used for RPA is shown as a bold line.

C. Detection of RNAs made from the exon 9–12 minigene. 1 μg of the minigene was transfected into HEK293 cells and after 24 h, RNAs were detected by RT-PCR. The negative control are untransfected HEK293 cells, using circRNA primers.

D. RNase protection probe to detect circ12 → 10 RNA. The T7 antisense RNA is shown with the nucleotide lengths indicated.

E. RNAse protection using RNA from transfected cells as well as human cortex. 50 μg total RNA was used, which was digested with RNaseR, as indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was present in circ12 → 10 (Fig. 1D, E). The other bands contained non-canonical splice sites and thus their mechanism of generation is unclear and they could be PCR artifacts.

3.2. A minigene spanning exons 9 to 12 generates circ12 → 10

We concentrated on circ12 → 10, as it was the most abundant RNA. To determine the sequences necessary for its formation, we created a minigene consisting of exons 9, 10, 11 and 12, each flanked by 1–2 kb of intronic sequence (Fig. 2A). This minigene was transfected into HEK293 cells and circular RNAs were detected using PCR exon junction primers selective for the 12 → 10 backsplice, consisting of a 10→12 reverse primer and a forward primer in exon 10 (Fig. 2B). In addition, we amplified linear RNA, using primers in exons 9 and 12 (Fig. 1A). The minigene contained the repetitive elements surrounding exon 12 and an intronic repetitive element upstream of exon 9. After minigene transfection, we could detect both the exon 9 to 12 linear RNA containing the alternative exon 10, as well as the circ12→10 backsplice RNA (Fig. 2C). Next we tested the expression of circRNAs using a different method. We chose RNase protection analysis that has a linear readout, but is less sensitive than RT-PCR. Using a uniformly radioactively labeled probe exhibiting sequence complementarity towards the exon12 → 10 backsplice junction (Fig. 2B, D), we could detect a faint signal in RNA from transfected HEK293 cells, which was enriched by RNAse R treatment (Fig. 2E). In addition, the probe detected parts of exon 10 and 12, which derived from linear RNA indicating that RNAse

R treatment does not remove all linear RNAs. A signal of the same length could also be detected from 50 µg of total human cortex RNA. The circular RNA signal was < 1% of the linear RNA signal in transfected cells, indicating that despite our ability to detect circ12 → 10 using RT-PCR, this RNA is very weakly expressed (Fig. 2E). Since our minigene can produce circular RNA, we conclude that all cis-acting sequence elements to generate the tau circ12 → 10 RNA are present in exons 9 to 12 and their immediate intronic vicinity.

3.3. Tau circ12 → 10 is regulated by clk2 and sensitive to mutations in exon 10

Tau circ12 → 10 contains exon 10, which is alternatively spliced in linear *MAPT*. Several exonic mutations causing FTLD/FTD have been identified that change exon 10 usage [3]. In addition, exon 10 is regulated by several trans-acting factors, most notable tra2-beta1/*TRA2B* [9], promoting its inclusion as well as the cdc2-like kinase CLK2 [6,9], the SR-protein SRSF7/9G8, protein phosphatase 1 (PP1) and its nuclear inhibiter NIPP1 promoting exon 10 skipping [22–24].

To test a possible regulation, we transfected the tau 9–12 minigene with plasmids expressing EGFP, CLK2, the inactive CLK2 variant CLK2KR, tra2-beta1, DYRK, 9G8, SRPK1, NIPP1 and PP1. The SR-protein kinase SRPK1 [25], was tested as it phosphorylates tra2-beta1. To investigate the effect of an FTLD/FTD mutation on the formation of the circular RNA, we introduced the N279K mutation promoting exon 10 inclusion into the minigene [3].

First, we tested the effect of the trans-acting factors in *MAPT* linear RNA using PCR primers in exon 9 and 12. CLK2, 9G8, NIPP1 and PP1 caused exon 10 skipping, as expected from previous data using exon 9–12 reporter genes (Fig. 3A). This experiment was repeated with a minigene harboring the N279 K mutation. This exon 10 mutation (ATAA to AGAA) creates a stronger tra2-beta1 binding site, as tra2-beta1 binds to NGAA sequences [26]. The generation of this tra2-beta1 binding site promotes inclusion of exon 10, by promoting the formation of tra2-beta1 dependent splicing enhancer complexes. Importantly, the N279K mutation causes FTLD/FTD [3]. Testing the trans-acting factors on this minigene revealed that the effect on exon 10 skipping was strongly reduced for CLK2, 9G8, NIPP1 and PP1.

RNA from these experiments was tested next for the expression of the tau12 → 10 circRNA. CLK2 and 9G8, NIPP1 and PP1 strongly reduced the expression of this circRNA. A similar effect was observed when we used the N279 K minigene. The trans-acting factors had no detectable effect on the HIPK3 circular RNA (Fig. 3C), and we thus quantified our data by calculating the ratio between circtau12 → 10 and circHIPK3. The analysis of three independent experiments showed a statistically significant reduction of circtau12 → 10 caused by 9G8, NIPP1 and PP1 in both minigenes and by CLK2 in the wild-type minigene context (Fig. 3D).

Finally, we investigated possible synergistic effects between the SR-proteins and their kinases and cotransfected CLK2 and SRPK1 together with tra2-beta1 and 9G8 expression clones. SRPK1 slightly increased the effect of tra2-beta1 and 9G8 on tau12 → 10 circRNA formation in the wild-type exon 10 context and had no detectable effect on the N279 K mutations. In contrast, clk2 reduced the effect of tra2-beta1 in both wild-type and N279 K background, whereas it had no effect on 9G8 (Fig. 3E).

The data indicate that the abundance of circtau12 → 10 RNA can be regulated by the cell through trans-acting factor expression, whose actions is in turn regulated through their kinases. In addition, an FTLD/FTD mutation that promotes exon 10 inclusion also influences tau circ12 → 10 RNA formation.

3.4. Tau circ12 → 10 RNA is predominantly localized in the cytosol

We detected tau12 → 10 circRNA in the human neuroblastoma cells SH-SY5Y allowing to analyze the cellular localization of the endogenous RNA. We employed cell fractionation using different lysis of plasma and nuclear membranes followed by RT-PCR. The tau circ12 → 10 RNA was localized in the cytosol (Fig. 4), similar to other circRNAs analyzed [11]. circHIPK3 RNA showed a similar cytosolic localization, whereas the C/D box snoRNA SNORD2 was exclusively nuclear.

3.5. No significant correlation between circ12 → 10 expression and Braak stages

Tau exon 10 and CLK2 splicing is deregulated in temporal cortext in sporadic Alzheimer's disease [6,9]. We therefore tested brain samples from 15 subjects for a correlation between tau circ12 → 10 circRNA and Braak stages (Supplemental Data 1, Fig. 5A–C). RNA from the gray matter of superior and middle temporal gyri was used. The amount of tau circ12 → 10 was normalized to circular HIPK3. Although we found expression of tau circ12 → 10 in all subjects, there was no statistical significant correlation with Braak stages, when the circ12 → 10 RNA was normalized to circHIPK3 RNA (Fig. 5D). For the statistical analysis, only RNAs from samples with RIN numbers larger than seven were used. In addition, we tested these samples for expression of the kinase clk2 using primers spanning its alternative exon 4. Skipping of this exon generates an inactive kinase isoform [27]. Again, there was no correlation between the amount of full-length clk2 and tau12 → 10 circRNA (Supplemental Fig. 2).

4. Discussion

4.1. MAPT as a contributor to human disease

The *MAPT* gene and its polypeptide products are acutely relevant to human diseases. A deregulation of *MAPT* is involved in Alzheimer's Disease, Chronic Traumatic Encephalopathy, Frontotemporal Lobar Degeneration-tau, and many other 'tauopathies' [28].

4.2. Circular RNAs from the MAPT locus could be species specific

Here we present evidence that the human *MAPT* locus generates circular RNAs through backsplicing from exon 12 to either exon 10 or 7. Our data show that the regulation of *MAPT* gene expression and its molecular biology are still incompletely understood, despite numerous studies focusing on alternative splicing of MAPT [29].

We concentrated on circ12 → 10, as it is the most predominant circular RNA. Currently, human circular tau RNAs have not been reported in databases. For mouse, a backsplice from exon six to four has been identified [30,31]. We could not amplify the human orthologue for this mouse circ6 → 4 RNA, nor could we identify mouse orthologues for our described 12 → 10 and 12 → 7 circRNAs. It is thus possible that *MAPT* generates species-specific circRNAs, similar to exon 10 usage that is alternatively spliced in adult humans, whereas it is constitutively used in adult mouse [29].

CircRNA formation through backsplicing is facilitated by repetitive elements that form regions of complementarity in the pre-mRNA, allowing to position exons for backsplicing [11]. Human *MAPT* contains at least 83 Alu elements, 56 on the sense strand and 27 on the antisense strand [32]. Since Alu elements are primate-specific, they could cause a difference in circular RNA formation between mouse and humans.

We generated a minigene consisting of exons 9 to 12 with about 2 kb flanking intron each. We found that this construct forms tau circ12 → 10 RNA when transfected into HEK293 cells, indicating that these regions are sufficient to generate the circular RNA. Importantly, in this construct, exon 10 is not surrounded by repetitive elements, suggesting that far-distance interactions, for example between repeats near exon 12 and 9 present in our minigene could form the basis for circRNA formation.

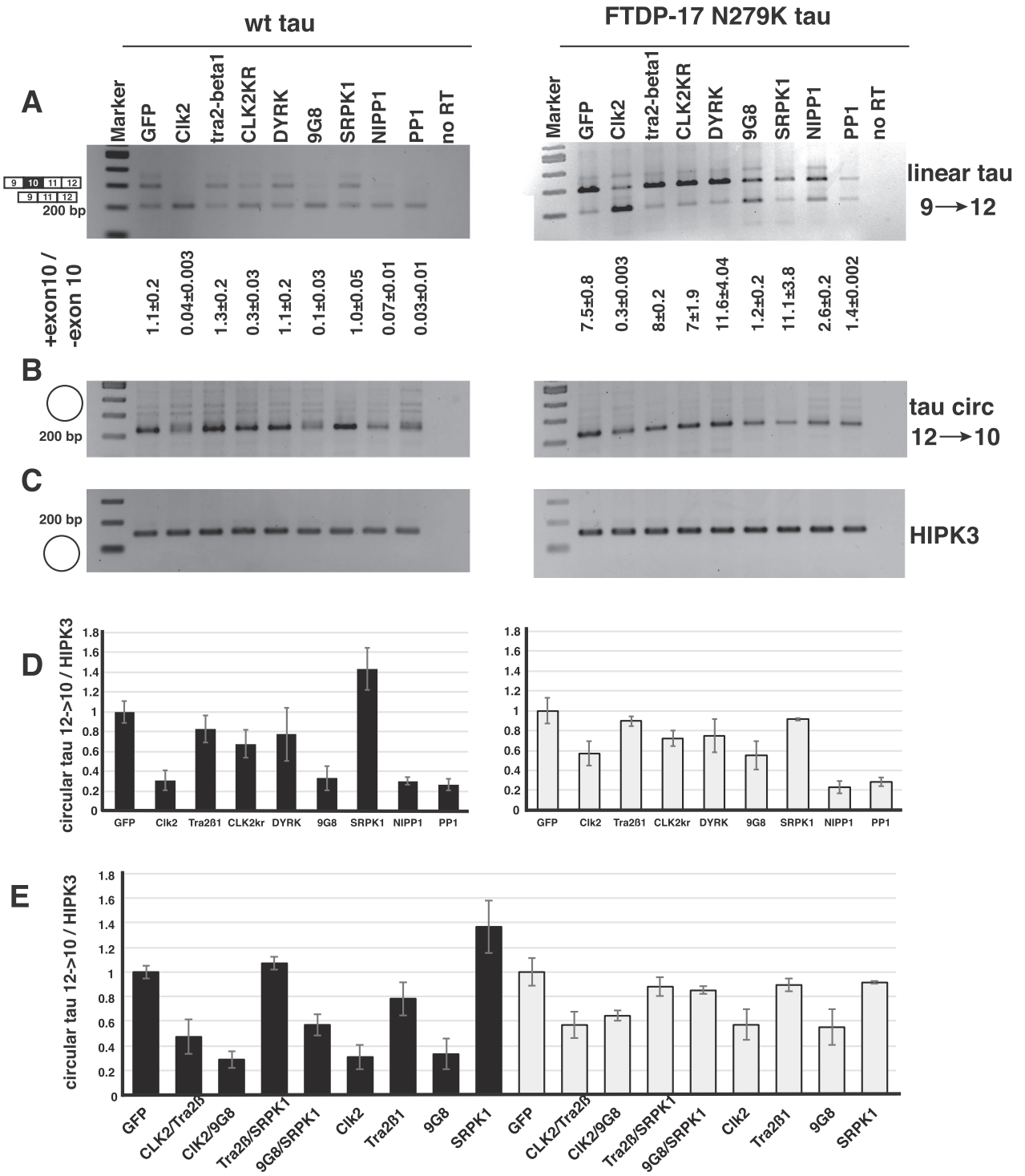

Fig. 3. The abundance of exon 10 containing circ RNA is regulated by the kinase clk2.
One μg of the tau exon 9–12 minigene was transfected into HEK293 cells together with 1 μg of the plasmids expressing the trans-acting factors indicated.
A. Linear RNA was amplified using primers in exon 9 and 12. The ratio of the isoform with exon 10 inclusion to the isoform with exon 10 skipping from three experiments is indicated below.
B. Circular RNA was amplified using primers rev12–10 and forward_exon 10.
C. The circular HIPK3 RNA was amplified as a loading control.
D. Quantification of the circtau to circHIPK3 ratio.
E. Quantification of a cotransfection of the SR-proteins tra2-beta1 and 9G8 in the presence of their kinases CLK2 and SRPK1. 500 ng of each expressing vectors were cotransfected and three independent experiments were analyzed.

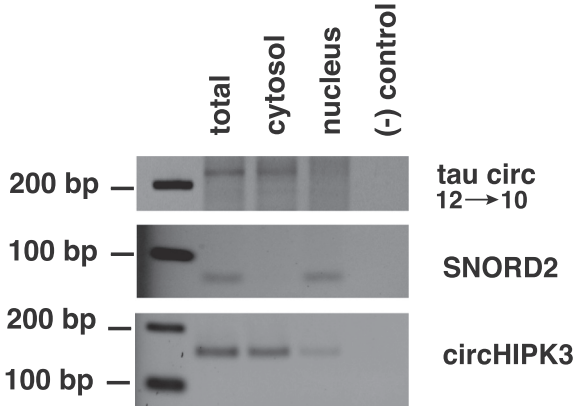

Fig. 4. tau circ12 → 10 is cytosolic.
SH-SY5Y cells were separated into cytosol and nucleus and RNA isolated from each fraction.
A. Detection of circ12 → 10.
B. Detection of SNORD2, a C/D box snoRNA with nuclear localization.
C. Detection of circHIPK3, a circular RNA known to be cytosolic.

4.3. Possible functions of tau circRNAs

To gain insight into the possible function of tau circ12 → 10, we first determined its cellular localization in human SH-SY5Y neuroblastoma cells. Similar to other circular RNAs [11], tau circ 12 → 10 is almost exclusively cytosolic. Inspection of the sequence of tau circ 12 → 10 and tau circ12 → 7 showed that both RNAs contain a number of nucleotides divisible by three. Tau circ12 → 10 contains a reading frame without a stop or start codon. This reading frame is identical to a portion of the tau protein containing the microtubule binding site encoded by exon 10. Despite the absence of an AUG codon, it is conceptually possible that translation could occur, for example one of the two in frame AUA triplets could be edited by ADAR1 or 2 to AUI, where the inosine (I) is read as a G by the initiator tRNA, leading to protein synthesis. Further, repeat-associated non-ATG (RAN) translation [33] from CAGs that are also present in tau circ 12 → 10 are possible and finally a translation of the RNA could theoretically occur in mitochondria, that recognize AUA start codons [34]. Initiation at any of these non-AUG start codons could generate a tau protein fragment containing a microtubule binding site. Since the tau circ12 → 10 RNA is divisible by three, it could be translated through a rolling circle mechanism that has been shown for model

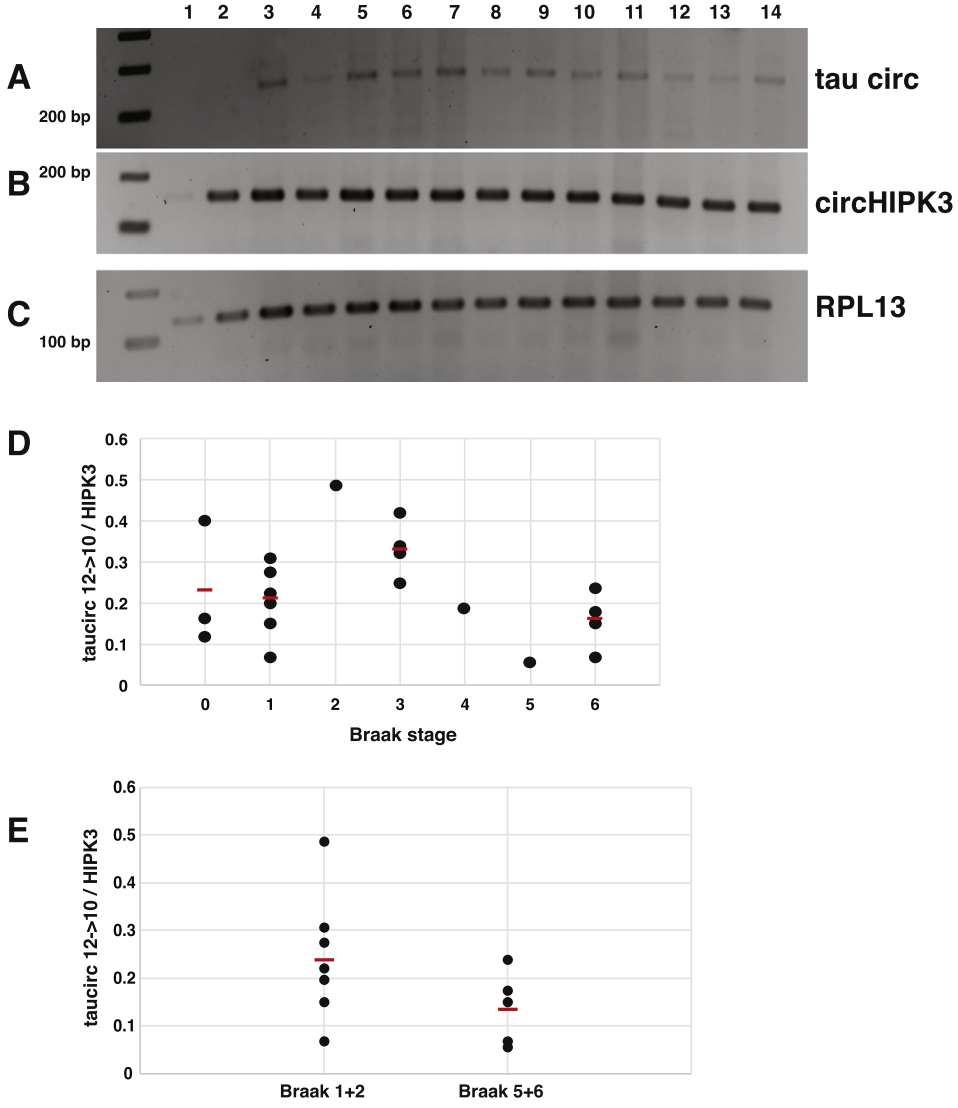

Fig. 5. The ratio between tau circ12→10 and circHIPK3 differs between individuals.
Temporal cortex from 14 patients with Braak stages 0 to 6 were analyzed by RT-PCR amplifying (A) circTau12 → 10 and (B) the abundant circHIPK3 RNA, as well as (C) linear RPL13 mRNA. D shows the quantification of tau circ12 → 10 normalized to HIPK3. There are no statistical significant changes between the Braak stages. E. Summary of tau circ12 → 10 abundance in Braak stages 1 and 2, compared with Braak stages 5 and 6. Red lines indicate the averages.

circular RNAs [35,36]. A similar translational mechanism is possible for tau circ12→7 that contains an in frame start codon, but no stop codon. However, both circular Tau transcripts are very weakly expressed. Thus, their translation will be a rare event. If translation occurs, the expected product would be a high molecular weight tau multimer containing several microtubule binding sites.

4.4. Relation to disease

The alternative splicing regulation of tau exon 10 has been extensively studied, due to exon 10's involvement in human disease. Mutations that interfere with the inclusion ratio of tau exon 10 cause frontotemporal dementia [3] and exon 10 usage as well as CLK2 splicing isoforms are changed in Alzheimer's disease [6]. We thus tested the expression of tau circ12→10 in AD brains of various Braak stages, using gray matter of the superior and middle temporal gyrus (SMTG), but did not find a statistically significant correlation between tau circ12→10 expression, normalized to circHIPK3. However, the translation of circular RNAs depends on the creation of an internal ribosomal entry site likely caused by RNA methylation [16] and thus the biological effect of the tau circRNAs might be correlated with modifications and not abundance. In addition, our sample number was low and we thus cannot rule out a connection with AD.

In a minigene, we re-created one FTDP-17 mutation that strengthens exon 10 usage and found that it generates an amount of taucirc12→10 RNA similar to the wild-type in transfection assays, indication that mutations causing frontotemporal dementia can also form circular tau RNAs.

In summary, our data show that the *MAPT* locus can generate circRNAs that could play a role in neurodegenerative diseases, which warrants further investigation.

Transparency document

The http://dx.doi.org/10.1016/j.bbadis.2018.04.023 associated this article can be found, in online version.

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Appendix A. Supplementary data

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