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FTLD-MAPT mutations and short 5'UTR Tau mRNAs increase Tau translation

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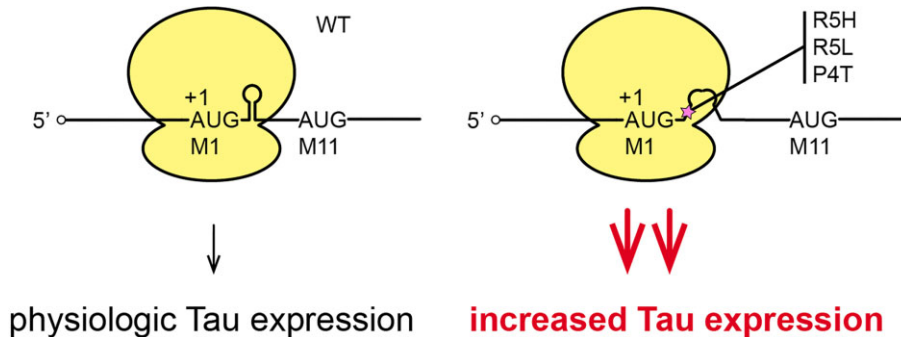
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Abstract

Tau protein plays a central role in Alzheimer's disease (AD) and other tauopathies. Tau proteins aggregate in degenerating neurons and their expression level is critical for disease development and progression. In addition, the generation of truncated Tau species is involved in the pathological process. In the human brain, several N-terminally truncated Tau species (including Met11- and Met127-Tau) have been detected. However, both the mechanisms of their synthesis and their relevance for the diseases remain largely unclear. Several Tau messenger RNA (mRNA) isoforms with distinct 5' untranslated regions (5'UTRs) were found in brains with tauopathies. In addition, single substitutions in the N-terminal coding sequence of Tau, namely R5H, R5L and R5C, have been associated with AD, progressive supranuclear palsy and Parkinson's disease, respectively. Here, we investigated how the 5' end of Tau mRNAs and the N-terminal mutations affect Tau protein synthesis. We show that both the length of the 5'UTR of Tau mRNAs and the N-terminal coding sequence largely modulate the expression level of Tau. Truncated Tau protein species are generated by an alternative translation initiation event. Thereafter, we show that the disease-associated mutations at codon #5 also increase the expression, mostly due to secondary structure modifications of the corresponding mRNAs.

Graphical abstract



Introduction

The microtubule-binding protein Tau (MAPT) is a component of the axonal cytoskeleton. Tau protein has been associated with microtubule dynamics, stability and regulation (1–3). Furthermore, Tau protein also plays a role in chromatin structure, signal transduction, synaptic plasticity, neuronal stress response and nucleic acid protection (4–8). Besides its physiological functions, Tau protein is known to form aggregates, alone or in association with other proteins, in several diseases termed tauopathies. Among tauopathies are disorders such as Alzheimer's disease (AD), Pick's disease, progressive supranuclear palsy (PSP) and frontotemporal lobar degener-

ation with mutations on MAPT (FTLD-MAPT) (9–11). Tau protein topology consists in four well-described domains: the N-terminal domain (also called projection domain), a proline-rich domain, a microtubule binding domain that contains microtubule binding repeats and a C-terminal domain (11,12). In the human adult brain, six major Tau isoforms are found. These isoforms are generated by the alternative splicing of exons 2, 3 and 10. Notably, the alternative splicing of exon 10 has been described to be an important feature in the onset of several neurodegenerative diseases (11,13,14). In fact, numerous mutations affecting exon 10 splicing were linked to Tau pathogenesis (10,11). To date, > 100 MAPT gene (coding for

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Tau) mutations have been described (<https://www.alzforum.org/mutations/mapt>, July 2024). Besides the mutations affecting the splicing, it was also reported that the total amount of Tau protein and its phosphorylation state are relevant for Tau-mediated pathologies (15–17). Noteworthy, some mutations were reported in the N-terminal part of Tau protein, hence not affecting the Tau exon 10 splicing. Interestingly, the fifth codon coding for an arginine residue (R5) located in exon 1 has been associated with Tau-mediated pathology (18,19). Indeed, the R5H, R5L and R5C mutations were associated with AD, PSP and Parkinson's disease (PD), respectively (20–22). Moreover, the R5H and R5L mutations were shown to affect Tau interactions with microtubules and promote Tau fibril formation (21,23,24). More recently, other features affecting the 5' end of Tau messenger RNAs (mRNAs) have been brought to light. Particularly, it has been reported that several 5' truncated Tau transcripts coexist in human brains (25). This report also showed that these 5' truncated mRNAs are overexpressed in AD and PSP brains. Of note, several of these 5' end truncated mRNAs were found exclusively in AD and/or PSP patient brains (−160, −54, −47, −35, −7, +90, +160 and +201) and were absent from healthy control individuals. In addition, Tau protein is known to undergo proteolysis, producing truncated proteins associated with neurodegenerative diseases in mice and humans (26–30). Thus, the existence of these 5' end truncated mRNAs in human brains can represent an overlooked source of N-terminally truncated Tau species. Besides that, N-terminally truncated Tau proteins were detected in human brain, two of them starting in the downstream methionine: Met11 and Met127 (31). Moreover, the N-terminally truncated Tau species displayed an impact on microtubule polymerization and showed stronger ability to bind microtubules. This gain-of-function property of Tau could be related to changes in its paperclip conformation or to a change in overall charges due to the loss of positively charged N-terminus residues, thereby affecting directly the binding to the negative surface of microtubules (31–34). Thus, changes in the N-terminal domain of Tau are relevant for Tau function and its ability to form neurofibrillary tangles. This makes us wonder whether these Tau species would arise from alternative translation initiation (on Met11 and Met127) rather than from proteolysis. Also, the impact of these 5' end truncated mRNAs with shorter 5' untranslated regions (5'UTRs) on Tau translation is yet to be established. However, the impact these 5' truncated mRNAs and the pathogenic mutation in the N-terminal coding sequence might have on the generation of different Tau protein species can be highly relevant. Moreover, the total amount of Tau protein present in a cell has been correlated with disease (15,16).

Here, we show that the size of the Tau 5'UTR mRNA affects the translation of Tau protein species. Mutations in the coding sequence of Tau N-terminal domain can also modify the translation efficiency of Tau. Our results show that the same mRNA can render different Tau species by alternative translation initiation. Additionally, we show that the mRNA (sequence and length) is the driving force to the translation changes. These changes in translation could be dependent on the secondary structure of Tau mRNA. Altogether, our work shows that 5' end changes of Tau mRNA can boost the translation of different Tau protein species. This underestimated source of Tau variants might contribute to the development of tauopathies.

Materials and methods

Plasmids

DNA templates

Coding sequence of human tau transcript variant 5, including 5'UTR sequence (exon 0), has been synthesized (GeneArt, Life Technologies SAS). The insert was cloned into BamHI–EcoRI sites of pcDNA3.1 vector (Invitrogen). The plasmid was then used to generate transcription templates containing different Tau sequences by polymerase chain reaction (PCR) (Supplementary Figure S1). The primers used in this work are listed in Table 1. The obtained DNA fragments were then used as templates for the *in vitro* transcriptions.

In vitro transcription

The DNA templates generated by PCR amplification were transcribed by *in vitro* transcription with recombinant T7 RNA polymerase. The transcription was performed in the presence of m7GpppG cap analog (Jena Bioscience) to generate capped mRNAs as previously described (35). To check RNA integrity, an aliquot was loaded on 4% denaturing polyacrylamide gel. To eliminate unincorporated nucleotides, the RNA sample was further purified on a gel filtration Sephadex G25 column (Pharmacia Fine Chemicals). The concentration of purified RNA samples was determined by absorbance measurement at 260 nm.

In vitro translation with rabbit reticulocyte lysates

In vitro translation was performed with cell-free translation extracts [self-made untreated rabbit reticulocyte lysates (RRLs)], as previously described (35,36). All reactions were incubated at 30°C for 60 min. The translation reactions were performed using 100 nM of mRNA transcripts with amino acid mixture containing all the amino acids, except methionine (1 mM each), RNasin (Promega), 75 mM KCl, 0.5 mM MgCl₂ and 10.8 μCi [³⁵S]-methionine. Aliquots of translation mixture were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, 10%) and translation products were visualized by phosphor imaging or by western blot, using specific antibodies for Tau protein.

In vitro translation with HEK293T and SH-SY5Y cell extracts

The HEK293T or SH-SY5Y cell extracts were obtained as previously described (37). *In vitro* translation was performed using 200 nM of mRNA transcripts with amino acid mixture containing all the amino acids, except methionine (1 mM each), RNasin (Promega), 100 mM KAc, 1.5 mM MgAc₂ and 10.8 μCi [³⁵S]-methionine. These reactions were performed in a 20 mM HEPES–KOH (pH 7.6) buffer supplemented with 0.5 mM spermidine, 1 mM DTT, 0.8 mM ATP, 0.1 mM GTP, 8 mM creatine phosphokinase and 0.1 mg/ml phosphocreatine disodium salt hydrate. Aliquots of translation mixture were analyzed by SDS–PAGE (10%) and translation products were visualized by phosphor imaging or by western blot, using specific antibodies for Tau protein.

RNA transfection

HEK293T and SH-SY5Y cells were transfected with 3 μg of capped and polyadenylated RNA transcripts using Lipofectamine MessengerMAX according to the manufacturer's instructions. After 24 h, cells were lysed and translation products were visualized by western blot.

Table 1. Oligonucleotides used for PCR

Name	Sequence
Tau (−160)	CAACAAATATTAATACGACTCACTATAGGCGCGCCCTCGCAGTCACCGCC
Tau (−54)	CAACAAATATTAATACGACTCACTATAGGCTGTCCTCTCCCGTCTCGCCTCTG
Tau (−47)	CAACAAATATTAATACGACTCACTATAGTCTCCCGTCTCGCCTCTGTG
Tau (−35)	CAACAAATATTAATACGACTCACTATAGCCTCTGTGCGACTATCAGGTG
Tau (−7)	CAACAAATATTAATACGACTCACTATAGACCAGGATGGCTGAGCCCCGCCAGG
Tau (−5)	CAACAAATATTAATACGACTCACTATAGACCAGGATGGCTGAGCCCCGCCAGG
Tau (−1)	CAACAAATATTAATACGACTCACTATAGATGGCTGAGCCCCGCCAGG
Rev-Tau-FL	GAATTCTCACAACCCCTGCTTGGCCAGGGAGG
R5H (−7)	CAACAAATATTAATACGACTCACTATAGACCAGGATGGCTGAGCCCCACCAGAG
R5L (−7)	CAACAAATATTAATACGACTCACTATAGACCAGGATGGCTGAGCCCCCTCCAGGAGTTCC
R5H (−5)	CAACAAATATTAATACGACTCACTATAGACCAGGATGGCTGAGCCCCACCAGGAG
R5L (−5)	CAACAAATATTAATACGACTCACTATAGACCAGGATGGCTGAGCCCCCTCCAGGAGTTCC
R5H (−1)	CAACAAATATTAATACGACTCACTATAGATGGCTGAGCCCCACCAGGAGTTCC
R5L (−1)	CAACAAATATTAATACGACTCACTATAGATGGCTGAGCCCCCTCCAGGAGTTCC
P4T (−7)	CAACAAATATTAATACGACTCACTATAG ACCAGGATGGCTGAGACCCGCCAGGAG
R5C (−7)	CAACAAATATTAATACGACTCACTATAGACCAGGATGGCTGAGCCCCGCCAGGAGTTCC
R5G (−7)	CAACAAATATTAATACGACTCACTATAGACCAGGATGGCTGAGCCCCGCCAGGAGTTCC
R5R (−7)CGA	CAACAAATATTAATACGACTCACTATAGACCAGGATGGCTGAGCCCCGACAGGAGTTCC
R5R (−7)AGA	CAACAAATATTAATACGACTCACTATAGACCAGGATGGCTGAGCCCCAGACAGGAGTTCC
R5S (−7)AGC	CAACAAATATTAATACGACTCACTATAGACCAGGATGGCTGAGCCCCAGCCAGGAGTTCC
Rev-Tau (+200)	TTAGCATCAGAGGTTTCAGAGCCCGG

Western blot

The protein extracts were loaded on 10% SDS–PAGE. After migration, proteins were transferred at 10 V for 1 h in a semi-dry apparatus (Trans-Blot SD) on a polyvinylidene fluoride membrane (Immobilon[®]-P Transfer Membrane) previously activated with methanol for 1 min. After transfer, the membranes were immunoblotted with 9F6 (a homemade mouse monoclonal antibody against Tau amino acids 427–441, according to the longest human Tau isoform). After three washes by PBST (PBS 1×; 0.1% Tween 20), the membranes were incubated with secondary HRP-conjugated antibody (Bethyl #A90-116A) for 1 h at room temperature followed by washing steps. The signal was detected after adding ECL Kit (ECL Plus Western Blotting Detection System, GE Healthcare) with a Bio-Rad ChemiDoc (MP) apparatus.

Chemical probing

Probing with DMS

Modification by DMS was performed using 2 pmol of each RNA transcript. The RNAs were first incubated for 30 min at 37°C in DMS buffer (50 mM sodium cacodylate, pH 7.5, 100 mM KCl and 5 mM MgCl₂) and 1 μg of yeast total transfer RNA (tRNA; Sigma–Aldrich). Then, 1.25% DMS reagent diluted in 100% ethanol was added and incubated for 2 min at 37°C. The reaction was stopped on ice and modified transcripts were precipitated with 3 volumes of 100% ethanol and further precipitated with glycogen as previously described (38). Reactivity of nucleotides was detected by primer extension and quantification of the RT stops that were quantified.

Probing with CMCT

Similarly, modification by CMCT was performed using 2 pmol of each RNA transcript. Each RNA was first incubated for 30 min at 37°C in CMCT buffer (82.2 mM potassium borate, 100 mM KCl, 5 mM MgCl₂) and 1 μg of yeast total tRNA (Sigma–Aldrich). Then, modifications were performed with 10.5 g/l CMCT reagent with 20-min incubation at 20°C and stopped on ice. Modified transcripts were analyzed by primer extension as described in the previous paragraph.

Results

Tau 5'UTR length is critical for Tau translation

Translation of Tau mRNAs was performed *in vitro* in RRLs using capped human Tau mRNAs. The Tau mRNA variants used in this study were previously found in AD and/or PSP patients' brains (25). These transcripts were tested to evaluate the role of 5'UTR length in the production of Tau protein species (starting at Met1, Met11 or Met127). The mRNA variants were named according to the number of nucleotides upstream the A of the start codon AUG (negative numbers) or downstream the A of the canonical start codon AUG (positive numbers), respectively. Indeed, we synthesized capped mRNA transcripts −160, −54, −47, −35, −7, −5, +90, +160 and +201 (Figure 1A) and tested the synthesis of Tau proteins in RRLs. We detected efficiently the Met1-Tau produced from the canonical AUG, as well as Met11-Tau and Met127-Tau species. Remarkably, the translation efficiency varied with the 5' end size of Tau mRNAs (Figure 1B). Indeed, Tau translation peaked for 35-nt-long 5'UTR Tau mRNA (−35). Noteworthy, all the variants with longer 5'UTRs (−160, −54, −47) showed very low translation efficiency compared to shorter Tau mRNAs (−35, −7, −5). The most efficient −35 mRNA generated five times or more Met1-Tau and Met11-Tau (Figure 1B and C) than all the longer variants (−160, −54 and −47). Tau mRNAs with shorter 5'UTRs (−7 and −5) generated ~70% of the Tau proteins produced with −35 Tau mRNA. Importantly, the Met1-Tau:Met11-Tau ratio remains identical with all the tested mRNAs (Supplementary Figure S2). As expected, further truncated variants that do not contain AUG#1 and AUG#11 (+90, +160 and +201) did not allow the detection of Met1-Tau or Met11-Tau but allowed the synthesis of Met127-Tau instead (Figure 1B and C). The amount of synthesized Met127-Tau also varied according to the size of the mRNA; the +201 mRNA that contains the shorter sequence upstream AUG#127 was more translated than the longer counterparts (+90, +108 and +160) (Figure 1C).

To further confirm these results, we performed *in vitro* translation in human HEK293T cell extracts (Figure 1D). The detection of Tau was performed by western blot with

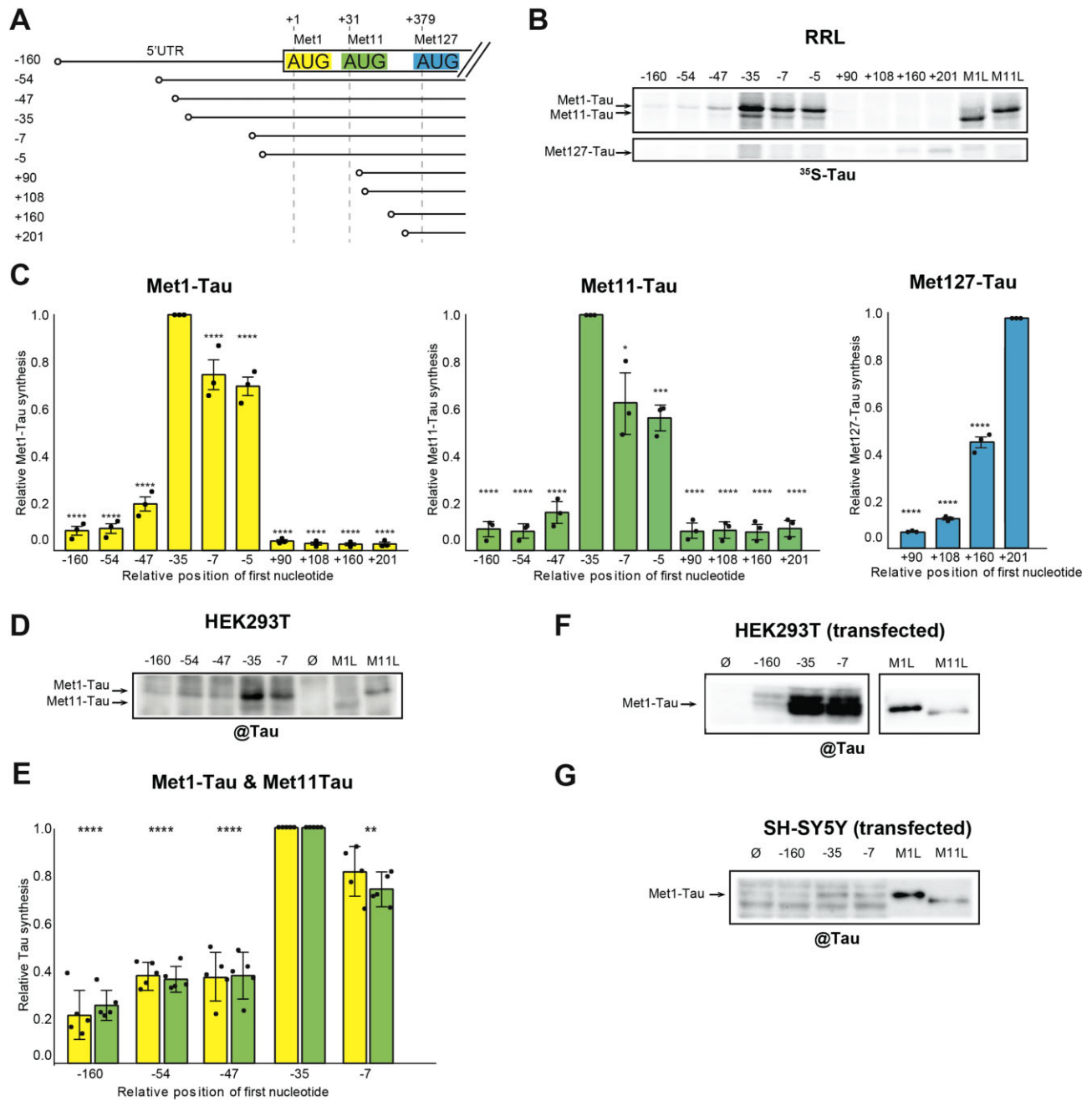


Figure 1. The 5' truncation of Tau mRNAs affects its translation efficiency. **(A)** Cartoon representing the sequential deletions in the 5' extremity of Tau mRNA constructs that were performed to assess their effect on Tau translation. The positions of the three N-terminal AUG codons are 1 (Met1), 31 (Met11) and 379 (Met127), shown in yellow, green and blue, respectively. **(B)** Representative SDS-PAGE of *in vitro* translation assays with RRLs. The translation products are labeled with [³⁵S]-methionine. **(C)** Histograms representing the translation efficiency fold changes of Met-Tau1 (yellow), Met11-Tau (green) and Met127-Tau (blue). The histograms represent relative expressions and statistical significance compared to -35 mRNA variant for Met1-Tau and Met11-Tau and +127 mRNA variant for Met127-Tau. **(D)** Representative SDS-PAGE of *in vitro* translation assays with HEK293T translation extracts. Tau proteins were detected by western blot with an antibody specific for Tau protein C-terminal domain (9F6 antibody). **(E)** Histogram representing the translation fold change of Met1-Tau and Met11-Tau in HEK293T extracts detected by western blot (9F6 antibody) compared to -35 Tau mRNA variant. **(F, G)** *In cellulo* translation assays. HEK293T cells (F) and SH-SY5Y cells (G) were transfected with no mRNA as negative control (∅) or with -160, -35 and -7 capped mRNA transcripts; Tau proteins are detected by western blot. M1L and M11L are Tau mRNAs bearing mutation in AUG1 and AUG31, respectively. All the histograms show mean ± standard error of the mean (SEM), *t*-test, *N* = 3 (C) and *N* = 5 (E), **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001.

9F6 antibody that specifically recognizes the Tau protein C-terminal domain. With this antibody, all N-terminal Tau variants were efficiently detected. The experiments performed with these cell extracts corroborated the results obtained in RRLs. Indeed, shorter variants (−35 and −7) generated more Met1-Tau and Met11-Tau than the longer variants (−160, −54 and −47) (Figure 1D). We found that −35 enabled the translation of ~5 times more Met1-Tau and Met11-Tau than the −160 mRNA (Figure 1E). The Met1-Tau:Met11-Tau ratio was similar among all variants as previously observed in RRLs (Supplementary Figure S2).

Next, we further investigated the Tau translation efficiency *in cellulo* by transfecting these capped transcripts −160, −35 and −7 in human HEK293T and SH-SY5Y cells (Figure 1F and G, respectively). Using this experimental setup, we could efficiently detect Met1-Tau synthesis. Furthermore, the most efficient mRNA transcripts were the −35 and −7 Tau mRNAs, while the −160 did not produce any detectable Tau protein in both cell types.

Altogether, our results showed that the size of the 5'UTR is critical for Tau translation efficiency. Indeed, shorter Tau variants (−35 and −7) enhanced the translation of Tau when compared to longer variants (−160, −54 and −47). Furthermore, this boost in translation was observed for both Met1-Tau and Met11-Tau syntheses.

Pathogenic MAPT mutations in exon 1 (N-terminal coding sequence) affect Tau translation

Next, we tested the effect of disease-associated Tau N-terminal mutations (Table 2) on its translation in RRLs. We started to test the impact on translation of two mutations on the fifth codon of the proteins R5H (G14A) and R5L (G14U). Considering our previous results on 5'UTR truncations, we decided to test the effect of these mutations in efficiently translated Tau mRNA variants, meaning with short 5'UTRs (−35, −7 and −1) (Figure 2A). Interestingly, we found that the R5H and R5L mutations do not change the translation of the −35 Tau mRNA variants (Figure 2B and C). However, the shorter Tau mRNAs (−7 and −1) presented significantly increased translation of Tau proteins for both single mutations in R5H and R5L (Figure 2B and C). We then tested two other mutations with unclear pathogenicity that were previously reported in the same region of the mRNA, the P4T (C10A) and the R5C (C13U). We tested all the mutations with the −7 mRNA variants in RRLs (Figure 2B). Tau translation is boosted compared to the wild type (WT) with P4T, R5H and R5L (Figure 2B and C). In contrast, Tau translation with R5C (C13U) was almost unchanged. These results suggest that the changes in Tau expression are not related to the amino acid composition since the type of amino acid inserted at position 5 does not influence the Tau translation yield. However, the nucleotide sequence, here at positions 10 and 14, is likely to affect Tau translation efficiency. Next, we tested the P4T, R5H, R5L and R5C mRNAs in HEK293T cell extracts and found the same results as those obtained with RRLs, showing that P4T, R5H and R5L mutations also boosted Tau translation in HEK293T extracts, whereas R5C did not significantly change the Tau translation (Figure 2D and E). Altogether, all these experiments show that mutations in the N-terminal coding sequence can modify the Tau protein translation rate of Tau mRNAs with short 5'UTRs.

Table 2. N-terminal Tau mutations found in humans and their association with neurodegenerative diseases

Tau mutation	Nucleotide substitution	Disease association	Reference
R5H	G14A	PSP	(23)
		AD	(20)
		Familial FTD	(18)
		CT	(22)
		PPA and psychosis	(19)
R5L	G14U	PSP	(21)
R5C	C13U	PD + D	(22)
P4T	C10A	CT	(49)

PSP, progressive supranuclear palsy; AD, Alzheimer's disease; FTD, frontotemporal dementia; CT, control; PPA, primary progressive aphasia; PD, Parkinson's disease; D, dementia.

Tau translation is modulated by N-terminal coding sequence

To better understand the contribution of the N-terminal coding sequence to the translation of Tau mRNAs, we tested other point mutations at nucleotide positions 13, 14 and 15, encoding the original arginine amino acid at codon position #5 (Figure 3A). We tested mutations G14A, G14U, C13A, C13U and C13G that induce amino acid substitution and two silent mutations that do not affect the protein sequence (C15A and C13A–C15A) in RRLs (Figure 3B). Interestingly, all the tested mutations including the silent mutations increased Tau translation compared to the WT (Figure 3C). The biggest increases were found with G14A, G14U, C13A and the double mutant C13A–C15A. These mRNAs were also tested in HEK293T cell extracts (Figure 3D). Even though we also observed higher Tau translation upon G14A, G14U and C13A mutations, the biggest increase is found with the silent mutant C13A–C15A (Figure 3E). This indicates that the translation boost is due to the nucleotide sequence rather than changes in the amino acid sequence. Noteworthy, the C13U and C13G did not change Tau translation in HEK293T cell extracts. Our experiments confirmed that point mutations in the positions 13, 14 and 15 can change the Tau translation efficiency. Since some of the tested mutations are silent and influence translation efficiency, we speculate that the changes in the translation yield might be attributed to changes in the mRNA secondary structure. We then performed mRNA chemical probing on Tau mRNA bearing mutations at nucleotide positions 13, 14 and 15.

Tau mutations induced RNA secondary structure changes

We determined the impact of mutations G14A, G14U, C15A and C13A–C15A on the secondary structure of −7 transcripts by performing chemical probing by CMCT and DMS (Figure 4A). The average reactivity for each nucleotide to these chemical reagents from at least three replicates was determined for nucleotides 1–104 and compared to the reactivity of WT Tau mRNAs (Supplementary Figure S3). A high reactivity of a nucleotide indicated a high probability of this nucleotide being accessible and not base-paired in a secondary structure element. In contrast, low reactivity implied that this nucleotide was involved in base pairing. First, we found that in the WT Tau mRNA, residues from 9 to 16 are not reactive, indicating the presence of a secondary structure. By comparing with the mutant RNAs, the mutations at positions 13, 14 and 15 induce a significant secondary structure change in

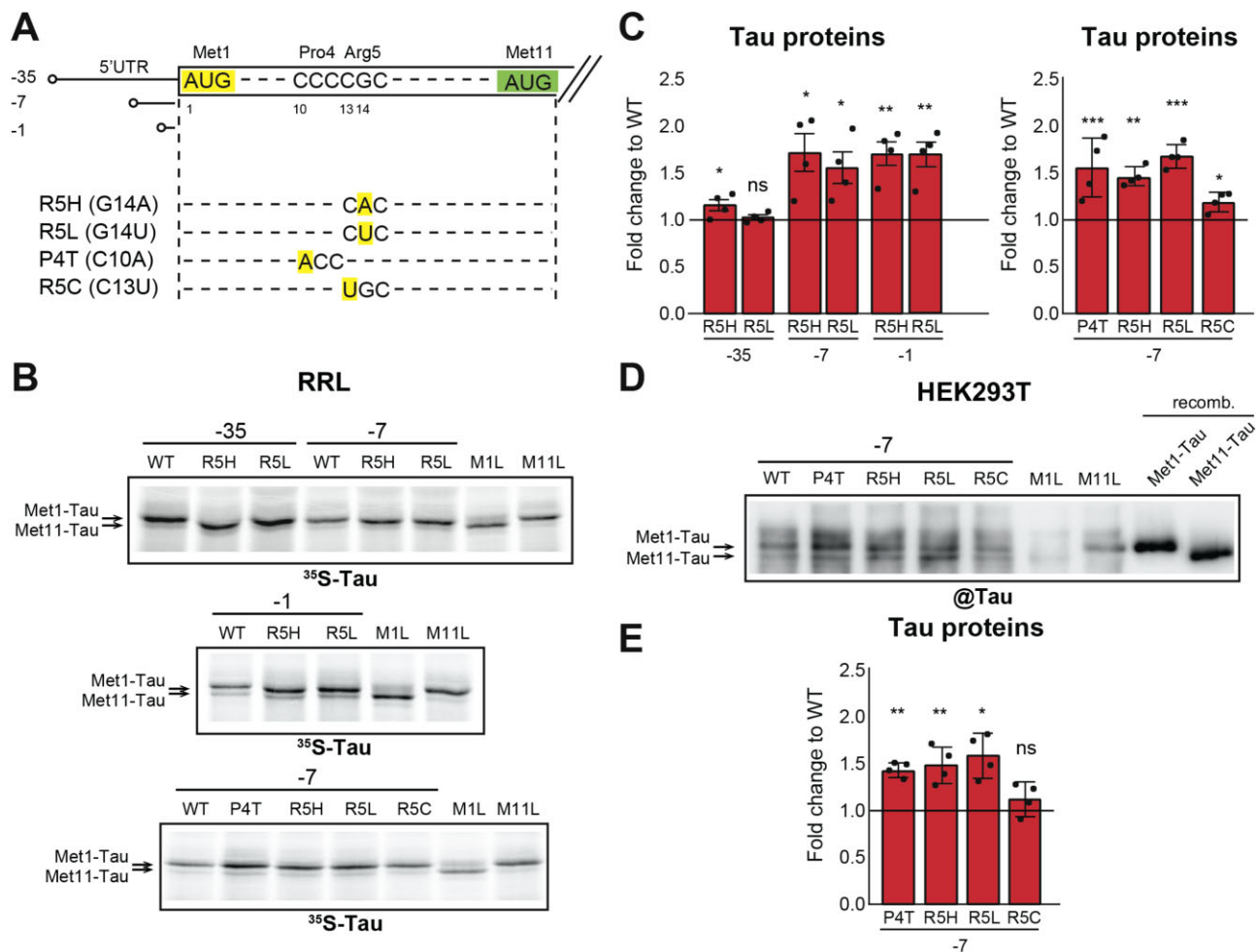


Figure 2. Disease-associated Tau N-terminal mutations affect translation of mRNA variants with short 5'UTRs. **(A)** Cartoon representing the deletions of the 5'UTR of Tau mRNA constructs (–35, –7, –1) and the positions of the mutations associated with diseases of proline codon #4 and arginine codon #5. The position of AUG codon #1 is shown in yellow and AUG codon #11 is shown in green. The nucleotides are numbered according to their distance to the A of AUG#1. The mutated nucleotides are highlighted in yellow. **(B)** Representative SDS–PAGE of *in vitro* translation assays with RRLs. The translation products are labeled with [³⁵S]–methionine. **(C)** Histograms representing the translation efficiency fold changes of all Met–Tau proteins synthesized (red) compared to WT –35 transcript with different 5'UTR lengths (–35, –7 and –1). **(D)** *In vitro* translation assay with HEK293T translation extracts of Tau transcripts with a 7-nt 5'UTR. Tau proteins are detected by western blot with C-ter Tau antibody (9F6 antibody). As control, recombinant Met1-Tau and Met11-Tau are loaded. **(E)** Histogram representing the translation fold change of Met–Tau proteins (red) synthesized in HEK293T extracts detected by western blot (9F6 antibody) compared to WT –7 Tau mRNA. M1L and M11L are Tau mRNAs bearing mutation in AUG1 and AUG31, respectively. All the histograms show mean ± SEM, *t*-test, *N* = 3 (C) and *N* = 5 (E), **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001.

nucleotides 13–16. In contrast, the rest of the reactivities are not changed, suggesting that the mutations induce only local changes in the secondary structure but do not affect the folding of the nucleotides downstream position 33 (Figure 4A). Based on our probing data, we can propose at least two models for secondary structure of the 5' proximal nucleotides of Tau mRNA (Figure 4B). In model A, the WT mRNA presents a stem-loop in close proximity to AUG#1. In model B, nucleotides 10–19 are involved in a long-distance interaction with nucleotides 373–380. When we focused on the changes induced by the mutations compared to the WT Tau mRNA in the region between codons #1 and #11, the overall impacts of the four tested mutations induced similar changes in the secondary structure pattern (Figure 5). More precisely, the mutations at positions 13, 14 and 15 induce an increase of the overall reactivity in this region of the mRNA, indicating that the mutations trigger the opening of secondary structures that are

present at these positions in the WT Tau mRNAs. In addition, the mutations induced a reduction of reactivity at positions 3, 8, 26 and 27, indicating that the introduction of the mutations allowed the formation of new base pairs that are not present in the WT Tau mRNAs. Interestingly, the most pronounced changes were observed with the C13A–C15A mutant, a mutant that was also the most efficiently translated in HEK extracts (Figure 3E). Consequently, this mRNA secondary structure change could explain differences in the translation levels of Tau mRNAs. In contrast, mutant C10A (P4T) does not induce significant structural changes in the region between codons #1 and #11 (Figure 5) or in nucleotides 30–104 (Supplementary Figures S4 and S5), suggesting that this point mutation does not interfere with mRNA folding. Altogether, these experiments corroborate our hypothesis that the N-terminal Tau mutations of codon #5 change the mRNA secondary structure.

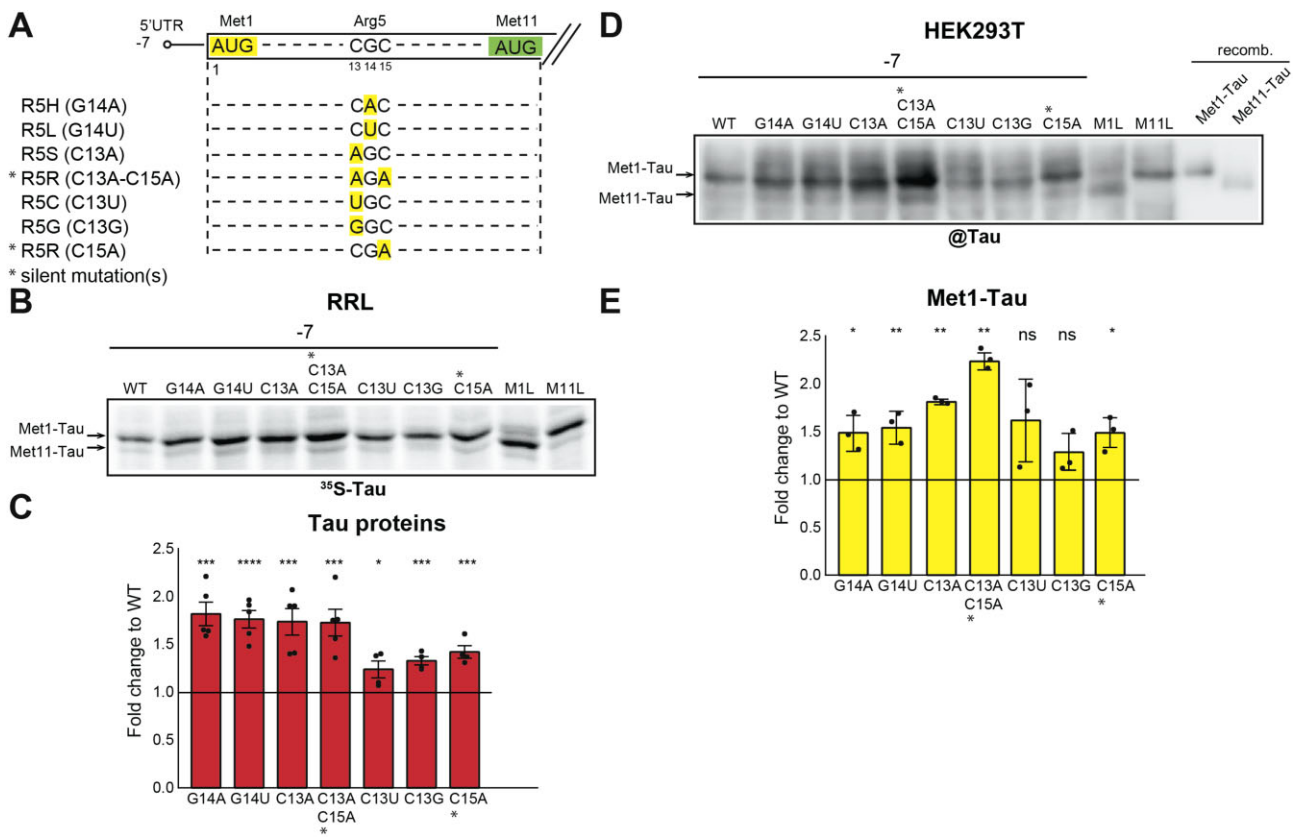


Figure 3. Tau translation efficiency is modulated by nucleotides in the N-terminal coding region. **(A)** Cartoon representing Tau mRNA transcripts with 7-nt 5'UTR. Mutations are highlighted in yellow. The transcripts are named according to the mutations (the mutated nucleotides are shown in parentheses). The silent mutations are marked by an asterisk. The AUG start codon is shown in yellow and the AUG#11 is shown in green. **(B)** Representative SDS-PAGE of *in vitro* translation assays with self-made RRLs. The translation products are labeled with [³⁵S]-methionine. **(C)** Histogram representing the translation efficiency fold changes of all Met-Tau proteins synthesized (red) compared to WT (–7) transcript. **(D)** *In vitro* translation assay with HEK293T translation extracts of Tau transcripts with a 7-nt 5'UTR. Tau proteins are detected by western blot (9F6 antibody). As control, recombinant Met1-Tau and Met11-Tau are loaded. **(E)** Histogram representing the translation fold change of Met1-Tau proteins (yellow) synthesized in HEK293T extracts detected by western blot (9F6 antibody) compared to WT –7 Tau mRNA. All the histograms show mean ± SEM, *t*-test, *N* = 5 (C) and *N* = 3 (E), **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001.

Discussion

Altogether, our results show, to our knowledge, a previously unknown role for the Tau mRNA structure in the regulation of Tau protein levels in cells. Previously, it has been reported that Tau protein levels are highly relevant for several neurodegenerative diseases (16,39–41). Here, we show that the relevance of the mRNA sequence and 5'UTR length has been overlooked. Since the discovery of novel shorter transcripts of Tau in human brains (25), the role of these mRNA species in Tau pathology or even their effect on regulation of Tau protein levels remained unexplored. Our results showed that 5' end truncated mRNAs, which render Tau mRNAs with shorter 5'UTRs, can induce higher Tau expression. Interestingly, the Tau mRNAs more efficiently translated (–35 and –7 variants) showed increased translation of both Met1- and Met11-Tau. These results indicate that Tau mRNAs have a previously unknown alternative translation initiation site. The lower translation efficiency of Tau longer isoforms can be due to high GC content of the 5'UTR (~75% for the full length). Thus, the long Tau mRNAs are expected to contain highly structured 5'UTRs. Such mRNAs, containing extensive secondary structured 5'UTRs, were shown to lower translation efficiency (42,43). In addition, cap-proximal structures can inhibit translation by hindering interaction between

the mRNA and the cap-binding translation initiation complex (eIF4F) (44). Throughout the years, many reports demonstrated the influence of 5'UTR structure on translational control (45,46). Besides the 5'UTR structure, the translation inhibition of longer isoforms can also be due to the presence of upstream open reading frames (uORFs). In fact, according to the uORF database (Ribo-uORF), seven active uORFs were found in Tau mRNAs. Therefore, we cannot exclude that the translation inhibition of longer isoforms might also be due to these uORFs (47). Moreover, further 5' end truncated Tau mRNAs that contain neither Met1 nor Met11 codons can initiate translation on Met127. Notably, Zilka *et al.* showed that a Tau species starting in Met127 promoted microtubule assembly ~4 times higher than the full-length Tau protein (29). Moreover, it was reported that a similarly truncated Gln124-Tau displayed stronger binding to the microtubules and protection against depolymerization (31). Thus, the production of Met127-Tau might also be relevant for the Tau pathology.

Previously, it has been reported that Tau protein levels have an essential role in neuronal loss, seeding capability and mouse survival (15). Besides that, reducing Tau levels in mice prevented/corrected behavioral deficits, substantially reversed transcriptional perturbations and rescued the phenotypes in tauopathy models (16,39-41,48). This suggests that lowering

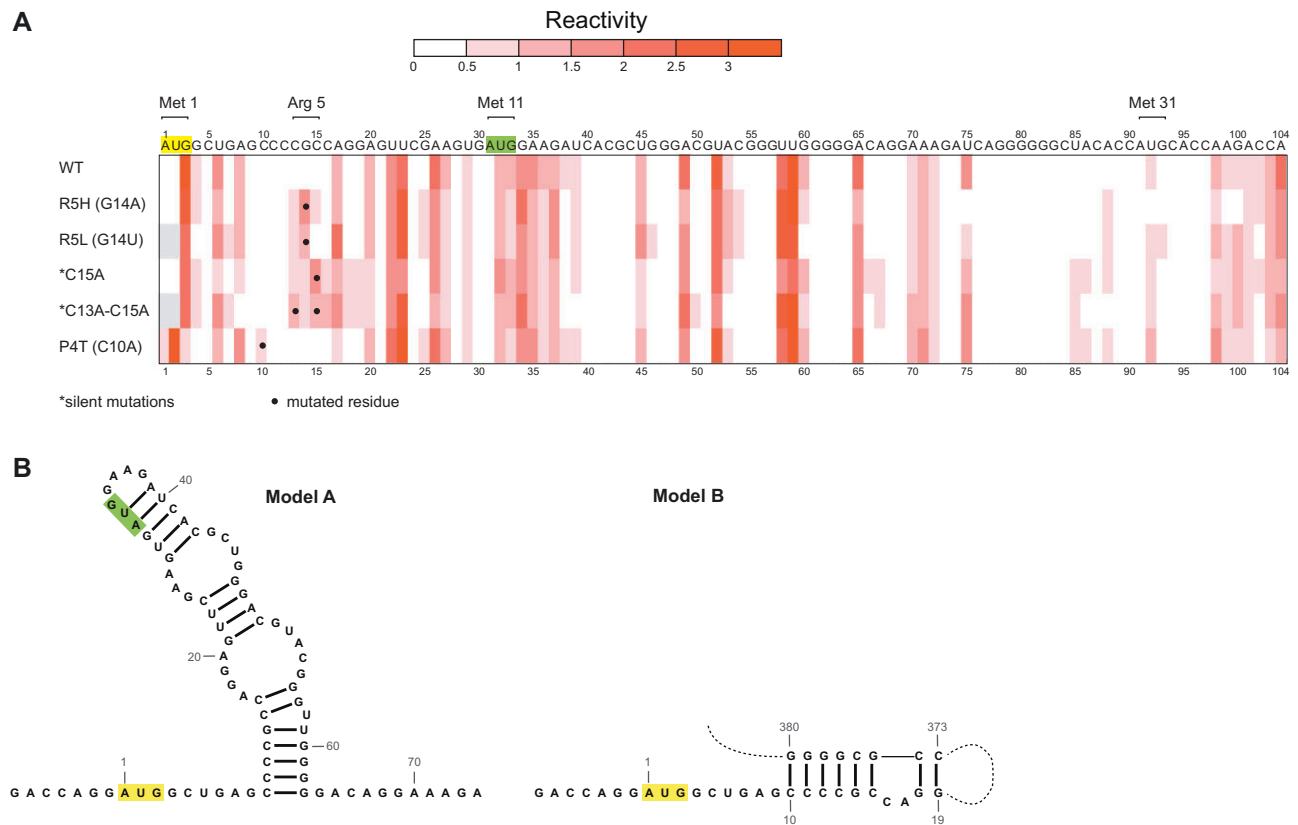


Figure 4. Tau mutations in the coding region induce structural changes on Tau mRNA. **(A)** The N-terminal (1–104) sequence of WT Tau mRNA is shown. The nucleotides are numbered from 1 (A from AUG start codon) to 104. The AUG start codon is highlighted in yellow and AUG codon #11 is highlighted in green. The amino acid sequence is shown on top of the codon sequences. The heatmap shows the DMS (A/C) and CMCT (G/U) reactivities of the WT and G14A, G14U, C15A, C13A–C15A and C10A mutant Tau mRNAs. The average reactivities calculated from three replicates are represented for residues from nucleotides 1 to 10 using the reactivity scale from white (no reactivity) to red (>3). The positions of the mutations G14A, G14U, C15A, C13A–C15A and C10A are represented by a black dot and overlaid on the heatmap. **(B)** Models of secondary structure of the 5' proximal nucleotides of Tau mRNA.

Tau levels might be beneficial to tauopathies such as AD. Our results show that Tau 5'UTR length plays a role in Tau translation, and subsequently in Tau protein accumulation. Thus, the ratio of Tau mRNAs with different 5'UTR lengths may have a previously unknown role in tauopathy development.

Regarding the disease-associated mutations R5H and R5L, we showed that these mutations when associated with short 5'UTRs (7, 5 and 1 nt) can boost Tau translation up to ~2-fold (Figure 2B). These results showed that Tau mutations can impact beyond the well-described post-translational level (phosphorylation and truncation) [reviewed in (11)]. Here, we demonstrate that mutations in the coding sequence of protein N-terminus can contribute to Tau accumulation *in vitro*.

Afterward, we decided to test other Tau N-terminal mutations found in humans, such as P4T and R5C (<https://www.alzforum.org/mutations/mapt>). Although neither P4T nor R5C has proven pathogenicity, R5C was reported in a patient with PD + D (22,49). On the other hand, the P4T mutation was found in a person without known Tau-associated pathology (49). To test the impact of P4T, R5H, R5L and R5C mutations on Tau translation, we synthesized all these mRNA variants with 7-nt 5'UTR as a template for the *in vitro* translation in RRLs. We chose the 7-nt 5'UTR background since it was found only in AD patient brains and was highly translated compared to the longer 5'UTR variants. Once more our results showed that the reported mutations can affect Tau translation. Indeed, the P4T, R5H and R5L displayed increased

translation compared to the WT mRNA (Figure 2B and C). These results were further confirmed in HEK293T extracts (Figure 2D and E). Regarding the R5C mutation, we observed a slight increase in RRLs that was not observed in HEK extracts. This might be due to the R5C impact on Tau translation that is less prominent than for the other mutations. We cannot exclude that there is a very modest effect, but this suggests that this effect is likely to be due to technical issues in our replicates. Previously, the R5 mutation's association with disease was attributed to the arginine substitution. Since this would eliminate the positive charge of the arginine, it is predicted to increase aggregation propensity (50,51). Moreover, the R5H and R5L mutations were both associated with impaired ability to promote microtubule assembly without altering the ratio 3R/4R of Tau isoforms (21,23). Furthermore, the R5L mutation showed increased fibrillation and nucleation *in vitro* (21,52). Also, R5L modification disrupts axonal transport and promoted the polymerization process (53,54). More recently, Mutreja *et al.* stated that R5L mutation showed subtle differences on various parameters of polymerization kinetics. Indeed, R5L did not have a significant effect on the kinetics of polymerization for 1N4R isoforms (the one used in our study) (24). Interestingly, the amount of soluble Tau in the brain bearing the R5L mutation was 1.5- to 2-fold higher compared with sporadic PSP and healthy controls (21). This increase in Tau accumulation is similar to the one observed in our study. Thus, our results indicate that the

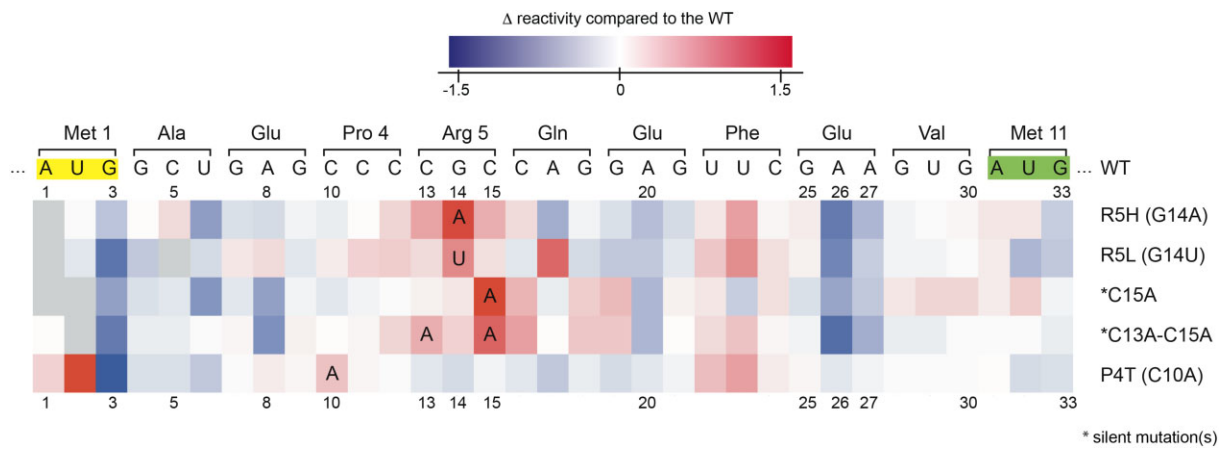


Figure 5. Tau mutations in the coding region induce structural changes on Tau mRNA. The N-terminal sequence of WT Tau mRNA is shown. The nucleotides are numbered from 1 (A from AUG start codon) to 33 (G from AUG#11). The AUG start codon is highlighted in yellow and AUG codon #11 is highlighted in green. The amino acid sequence is shown on top of the nucleotide sequence. The heatmap shows the DMS (A/C) and CMCT (G/U) reactivity fold changes of G14A, G14U, C15A, C13A–C15A and C10A mutant Tau mRNAs compared to WT Tau mRNA. The average reactivity differences from three replicates are represented for residues from nucleotides 1 to 33 using a blue–white–red color scheme. The reactivity fold changes span from blue (–1.5) to red (+1.5); no change (0) is indicated by white. The positions where no information could be obtained are shown in gray. The mutations G14A, G14U, C15A, C13A–C15A and C10A are overlaid on the heatmap.

translation of Tau mRNAs alone can be the source of this accumulation.

Noteworthy, by default accumulation/aggregation tests performed with Tau mutations used the same starting amount of Tau protein. This disregards the impact of the translation itself on Tau accumulation, function and aggregation propensity. All of them are characteristics relevant to tauopathies' onset. Here, we show that mutations such as R5H, R5L and even P4T can have an impact on Tau accumulation in the cells by directly affecting its translation. Although P4T was found in a control individual, information such as age of the mutation carrier and details regarding cognitive health were not reported. Thus, we should not ignore the possible onset of a Tau pathology *a posteriori* (49).

Afterward, we decided to further investigate the role of the mRNA sequence in Tau translation. To this purpose, we generated several mutations in the fifth codon (nucleotide positions 13, 14 and 15) of Tau mRNA. More precisely, we added to the R5H (G14A), R5L (G14U) and R5C (C13U) mutations, two more nonsynonymous mutations R5S (C13A) and R5G (C13G), and two synonymous mutations (C13A/C15A and C15A). By including two synonymous mutations, we can exclude the contribution of the amino acid chain and subsequent charge or structural changes to the translation efficiency. Our results show that the Tau translation depends on the mRNA sequence rather than the amino acid sequence of the nascent chain of Tau protein (Figure 3C and E). In fact, the two synonymous mutations in Arg5 (C13A/C15A and C15A) resulted in a significant increase in Tau protein translation in both RRL and HEK293T extracts. These results make us wonder whether these mutations were affecting the secondary structure of the mRNAs. Then, we performed chemical probing of the RNAs (WT, G14A, G14U, C13A/C15A and C15A) with CMCT/DMS (Figure 4A). For both modified/unmodified RNAs, we compared the intensity of the RT arrests for each nucleotide. By performing this, we were able to determine the mRNA structural changes induced by these mutations. Our results showed that mutations that boost Tau translation displayed conformational changes in

this region. When compared to the WT RNA, the mutations increased the intensity of the RT stops. This means that this region becomes less structured and most probably these mutations disrupt a double-stranded RNA region as depicted in the models in Figure 4B. Through these structural changes, the mutations might grant a more accessible and easier to translate mRNA, thus contributing to enhance Tau translation *in vitro*. Based on these results, we propose the following model to explain the increased translation for mutated Tau mRNAs (Figure 6). In the WT Tau mRNA, nucleotides between 9 and 16 are embedded in a secondary structure. An initiating ribosome that is assembled on the AUG start codons covers ~16 nucleotides downstream the start codon. Therefore, the assembly of the ribosomal subunits on the start codon requires the opening of any structure located in the immediate vicinity of the start codon (37). Since this structure is close to the AUG start codon (+9 to +16), efficient initiation requires prior unfolding of the structure for efficient translation initiation. This is achieved by an ATP-dependent helicase activity that occurs during the 5'–3' scanning step (55). In the Tau mRNA containing mutations at codon #5, the structure between nucleotides 9 and 16 is partially or totally unfolded by the mutations. Therefore, translation initiation can occur without the need of helicase activity and is hence more efficient, resulting in a higher expression of Tau protein. The fact that we observe a higher expression of the mutants of arginine 5 only with short 5'UTR (–7) further corroborates this explanation since short 5'UTR mRNAs are translated without any scanning step like leaderless mRNAs. This suggests that the Tau mRNAs with a short 5'UTR are more sensitive to the presence of structures in the vicinity of the start codon. Concerning mutant P4T, we did not find structural perturbations induced by the mutation. However, we also observed an increased Tau expression level with P4T. In this case, the explanation might be different. The position of the mutation (C10A) is closer to the AUG start codon. During the initiation process, the mRNA is in close contact with the ribosome at different key positions. For instance, the +4 residue interacts with eIF1A and the ribosomal protein uS19. The +10 residue is also in contact with ribo-

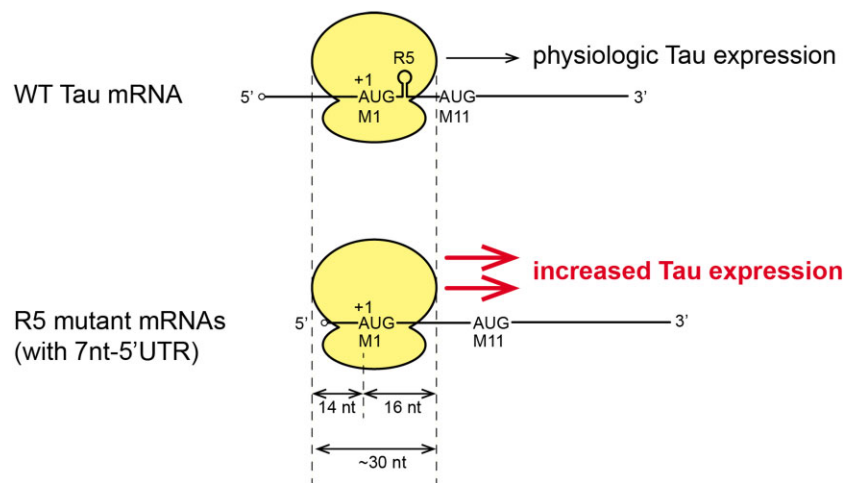


Figure 6. Model of Tau mRNA translation. In the WT Tau mRNA, nucleotides 9–16 between codon AUG#1 and AUG#11 are structured. The structure needs to be unwinded for efficient translation. The mutations at codon CGC#5 coding for an arginine residue impair base pairing; therefore, nucleotides 9–16 become single stranded. The absence of structure in these mutant RNAs increases the translation efficiency and therefore induces a higher expression of Tau proteins.

somal protein uS3 (56). The mutation C10A in Tau mRNA might improve the interaction with uS3 during the translation initiation process and thereby increase the translation efficiency of Tau protein synthesis.

Our data show that translation of Tau protein can be influenced by various factors, more precisely the 5'UTR length and N-terminal missense mutations. Beyond the results presented here, there are several reports stating the importance of translation regulation in neurodegenerative diseases. Aberrant mRNA translation was already implicated in several neurodegenerative diseases, such as FTD, PD, amyotrophic lateral sclerosis, spinal muscular atrophy and Charcot–Marie–Tooth disease. The aberrant translation in neurodegenerative diseases can arise from global inhibition of translation, increased translation of a subset of mRNAs and/or mutations in translation-related proteins [reviewed in (57)]. Thus, our work establishes a new link between translation regulation and neurodegenerative diseases.

Altogether, we demonstrate that Tau 5'UTR length is critical for its translation efficiency. Similarly, our results show that Tau N-terminal disease-associated mutations can boost Tau translation. Furthermore, we show that Tau translation is dependent on its mRNA sequence, particularly in the fifth codon. Also, we show that these mutations induced RNA secondary structure changes. Our results show that the presence of a variety of mRNAs in human brains regulates Tau protein levels of different species. Once these mRNAs affect the translation itself, these variants could play a neglected role in Tau accumulation and the tauopathies' onset.

Data availability

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Supplementary data

[Supplementary Data](#) are available at NARMME Online.

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Author contributions: L.B. conceived the original idea for this study on MAPT mutations. F.M. and L.B. vision guided this research. P.J.d.C. performed most of the experimental work described in this manuscript, interpreted the results and participated in the writing of the manuscript, A.P. also performed experimental work, L.B. and M.H. interpreted the results and participated in the critical reading and writing of the manuscript, and F.M. designed the experimental strategy, interpreted the results, made the figures and wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of interest statement

None declared.

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