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MMP-9 and MMP-2 Contribute to Neuronal Cell Death in iPSC Models of Frontotemporal Dementia with *MAPT* Mutations

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SUMMARY

How mutations in the microtubule-associated protein tau (*MAPT*) gene cause frontotemporal dementia (FTD) remains poorly understood. We generated and characterized multiple induced pluripotent stem cell (iPSC) lines from patients with *MAPT* IVS10+16 and tau-A152T mutations and a control subject. In cortical neurons differentiated from these and other published iPSC lines, we found that *MAPT* mutations do not affect neuronal differentiation but increase the 4R/3R tau ratio. Patient neurons had significantly higher levels of MMP-9 and MMP-2 and were more sensitive to stress-induced cell death. Inhibitors of MMP-9/MMP-2 protected patient neurons from stress-induced cell death and recombinant MMP-9/MMP-2 were sufficient to decrease neuronal survival. In tau-A152T neurons, inhibition of the ERK pathway decreased MMP-9 expression. Moreover, ectopic expression of 4R but not 3R tau-A152T in HEK293 cells increased MMP-9 expression and ERK phosphorylation. These findings provide insights into the molecular pathogenesis of FTD and suggest a potential therapeutic target for FTD with *MAPT* mutations.

INTRODUCTION

Frontotemporal dementia (FTD), the second most common form of presenile dementia, often causes changes in personality and behavior (Boxer and Miller, 2005). There is no effective treatment, and the molecular pathogenic mechanisms are poorly understood (Roberson, 2012; Irwin et al., 2015). Mutations in the gene encoding the microtubule-associated protein tau (*MAPT*) are responsible for a substantial fraction of FTD cases (Hutton et al., 1998; Loy et al., 2014). Tau pathology is also a hallmark of Alzheimer's disease (AD), and its pathogenic roles and utility as a potential drug target in AD have been extensively investigated (Wang and Mandelkow, 2016; Yoshiyama et al., 2013).

Tau helps assemble and stabilize microtubules. In the CNS, several isoforms of tau are produced from a single gene by alternative splicing (Goedert et al., 1989). More than 40 pathogenic *MAPT* mutations have been described in more than 100 families with FTD (Cruts et al., 2012; Alzheimer Disease & Frontotemporal Dementia Mutation Database, <http://www.molgen.vib-ua.be/FTDMutations>). Many *MAPT* mutations are either missense mutations or small deletions in the coding region or in introns, which can affect alternative splicing (e.g., of exon 10) (Hutton et al., 1998; Niblock and Gallo, 2012). Tau also modulates signaling cascades by acting as scaffolding proteins for

signaling complexes such as FYN, GRB2, and PLC γ , and may also affect other cellular functions (Ittner et al., 2010; Morris et al., 2011).

Many studies of tau toxicity have used cellular or animal models in which wild-type (WT) or mutant tau is ectopically overexpressed (Morris et al., 2011; Wittmann et al., 2001). Patient-specific induced pluripotent stem cells (iPSCs) are an exciting alternative approach to study disease genes in their native genetic context (Yamanaka, 2007) and uncover novel pathogenic mechanisms in AD, Parkinson's disease, FTD, and other neurodegenerative disorders (Israel et al., 2012; Soldner et al., 2009; Almeida et al., 2012). Here we generated and characterized multiple iPSC lines from an FTD patient with a *MAPT* IVS10+16 mutation, an FTD patient with the tau-A152T variant, and a control subject. The *MAPT* IVS10+16 mutation is relatively common and found in many families in different countries (Hutton et al., 1998; Janssen et al., 2002). The tau-A152T variant has been shown to significantly increase the risk for both FTD and AD (Coppola et al., 2012; Kara et al., 2012) and induce aggregation-independent toxicity (Pir et al., 2016). To study their pathogenic mechanisms, we differentiated these newly generated iPSC lines, as well as published control and tau-A152T lines (Almeida et al., 2012; Fong et al., 2013) into postmitotic cortical neurons and identified upregulation of matrix metalloproteinase 9



(MMP-9) through the ERK pathway as an important pathogenic mechanism in FTD with *MAPT* mutations.

RESULTS

Generation of Patient-Specific iPSC Lines with *MAPT* Mutations

To understand how endogenously expressed mutant tau contributes to eventual neurodegeneration, we obtained skin biopsies from two patients with *MAPT* mutations and a healthy control subject. Patient 1 was a male with a tau-A152T variant. The patient's clinical history and neurological examination were consistent with a diagnosis of progressive supranuclear palsy (PSP). Patient 2 was a male with an *MAPT* IVS10+16 mutation whose clinical diagnosis was largely normal at the time of skin biopsy but developed FTD later on. The control subject is a female family member of patient 2. Putative iPSC lines were derived from the fibroblasts as described previously (Yamanaka, 2007; Almeida et al., 2012), and 10–15 putative iPSC lines from each subject were extensively characterized. Immunocytochemistry analysis showed expression of stem cell makers such as OCT4, SSEA4, NANOG, TRA1-60, and TRA1-81 in all selected iPSC lines (Figure S1A). qRT-PCR showed that total and endogenous expression levels of the four reprogramming factors were the same in each line, confirming silencing of the transgene (Figure S1B). The iPSC lines had normal karyotypes (Figure S1C), and selected lines could differentiate into cell types of all three germ layers, confirming their pluripotency (Figure S1D). Based on these characterizations, we selected iPSC lines 3L1 of control 1, 19L3 and 19L4 of patient 1, and 3L6 and 3L9 of patient 2 for further study. In addition to the lines generated in our current study, we also used two published control iPSC lines, 2L17 and 2L20, derived from a male (Almeida et al., 2012), and one published iPSC line from another tau-A152T mutation male carrier (Fong et al., 2013).

MAPT Mutations Do Not Affect Early Neuronal Differentiation

At passages 25–30, all fully reprogrammed control and patient iPSC lines were differentiated into postmitotic cortical neurons as described (Figure 1A) (Almeida et al., 2012). Four weeks after terminal differentiation, 80% of cells were positive for the neuron-specific marker MAP2, and there was no significant difference between control and patient neurons (Figure S2A). The percentages of VGLUT1-positive excitatory and GABA-positive inhibitory neurons were similar in neuronal cultures differentiated from control and patient iPSCs (Figures S2B and S2C). Moreover, the total expression levels of tau and PSD-95 were the same in 1-month-old neurons derived from control and patient iPSCs (Figure 1B).

To further characterize differentiated human cortical neurons, we performed whole-cell voltage-clamp recordings. Tetrodotoxin-sensitive action potentials were induced in 64% of control neurons, 78% of tau-A152T neurons, and 78% of *MAPT* IVS10+16 neurons (9–11 cells were recorded for each group, Figure 1C). The resting membrane potential did not differ among neurons differentiated from all iPSC lines (Figure S2D). Functional synaptic connections formed in these cell lines, as shown by α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)-type glutamate receptor-mediated spontaneous excitatory postsynaptic currents (sEPSCs) that could be blocked with (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzof] quinoxaline-2,3-dione (NBQX) (Figure 1D). The mean amplitude, frequency, rise time, and decay time constant of sEPSCs were indistinguishable in all lines (Figures S2E–S2H). Thus, both molecular and electrophysiological analyses suggest *MAPT* mutations do not affect early neuronal differentiation. Therefore, any disease-relevant phenotypes would unlikely be due to changes in neuronal subtypes.

MAPT Mutations Increase the Level and Activity of Secreted MMP-9 and MMP-2

MMPs, a family of Zn-containing proteolytic enzymes, modulate both normal development and human pathophysiology. MMP-9 directly cleaves Huntingtin protein in Huntington's disease, a progressive neurodegenerative disorder (Miller et al., 2010). Moreover, dysregulation of MMP-9 in the CNS is a cause of neuronal developmental disorders (Reinhard et al., 2015). Thus, we set out to examine MMP-9 production in control and patient neurons with *MAPT* mutations. To this end, we performed zymography as described previously (Biswas et al., 2010), which measures the level and total activity of MMP-9. Tau-A152T neurons had >10-fold higher level and activity of secreted MMP-9 than control neurons (Figures 2A and 2B), as did *MAPT* IVS10+16 neurons to a lesser extent (Figures 2C and 2D). The total activity of secreted MMP-2 was more than 2-fold higher in patient-specific neurons than in controls (Figures 2A–2D). The level of secreted MMP-9 and MMP-2 was also increased in cortical neurons derived from a published iPSC line with the tau-A152T mutation (Fong et al., 2013) (Figures 2E and 2F). The effect on the total level and activity of secreted MMP-9/MMP-2 seems to be specific to mutant tau, since in progranulin-deficient FTD cortical neurons, there was no significant change in secreted MMP-2 level (Figure S2I) and the secreted MMP-9 level was below detection as in control neurons (not shown). This increase is likely in part due to transcriptional regulation, since the mRNA levels of *MMP-9* and *MMP-2* in tau-A152T and *MAPT* IVS10+16 neurons were higher than in control neurons (Figure S2J).

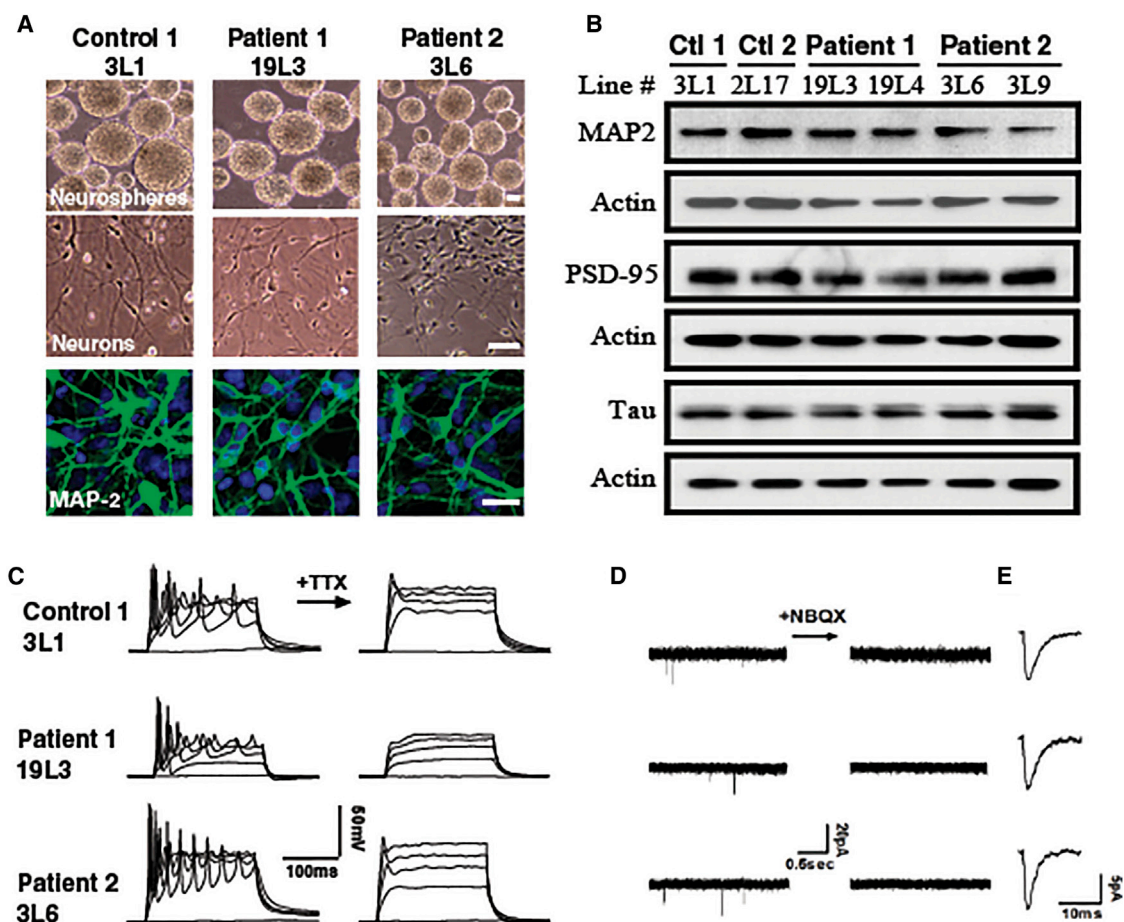


Figure 1. Differentiation and Characterization of Cortical Neurons Derived from iPSC Lines from FTD Patients and Control Subjects

(A) Phase-contrast images of neurospheres (upper), differentiated neurons (middle), and MAP-2 immunostaining of neurons (lower) from the iPSC lines. Scale bars, 50 μ m.

(B) Western blot analysis of MAP-2, PSD-95, and tau-expression levels in 4-week-old neurons differentiated from iPSC lines. Actin served as the loading control.

(C) Representative action potentials elicited by current injection (0–400 pA in 100-pA steps) and blocked by tetrodotoxin (TTX) of lines 3L1 of control 1, 19L3 of patient 1, and 3L6 of patient 2. Nine to eleven cells were recorded for each group.

(D) iPSC-derived neurons ($n = 7$ per subject) displayed synaptic responses and were sensitive to the AMPA receptor antagonist NBQX.

(E) Averaged sEPSC events from neurons of different subjects.

See also [Figures S1](#) and [S2](#).

Elevated Extracellular MMP-9 and MMP-2 Contribute to Neuronal Cell Death of iPSC-Derived Human Cortical Neurons with *MAPT* Mutations

The increased extracellular MMP-9 and MMP-2 activity in FTD patient-specific cortical neurons suggests that these molecular changes may contribute to disease phenotypes. To investigate this possibility, we first examined the relative survival of human cortical neurons derived from control subjects of patients with *MAPT* mutations without any inducers of cellular stress. We found that, indeed, TUJ1⁺ neurons showed reduced survival as measured by TUNEL analysis ([Figure 3A](#)). Interestingly, we did not observe

TUNEL labeling in more than 100 glial cells ([Figure S3A](#)), suggesting glial cells are less vulnerable to increased MMP-9 and MMP-2 levels. We then examined the sensitivity of iPSC-derived human cortical neurons to different inducers of cellular stress. We treated these neurons with staurosporine, a broad-spectrum kinase inhibitor that induces apoptosis ([Budd et al., 2000](#)); rotenone, a complex I inhibitor that induces mitochondrial dysfunction ([Sherer et al., 2003](#)); and rapamycin, an inhibitor of the mammalian target of the rapamycin (mTOR) signaling pathway with many downstream effects ([Li et al., 2014](#)). When measuring cell viability using the MTT assay, we found

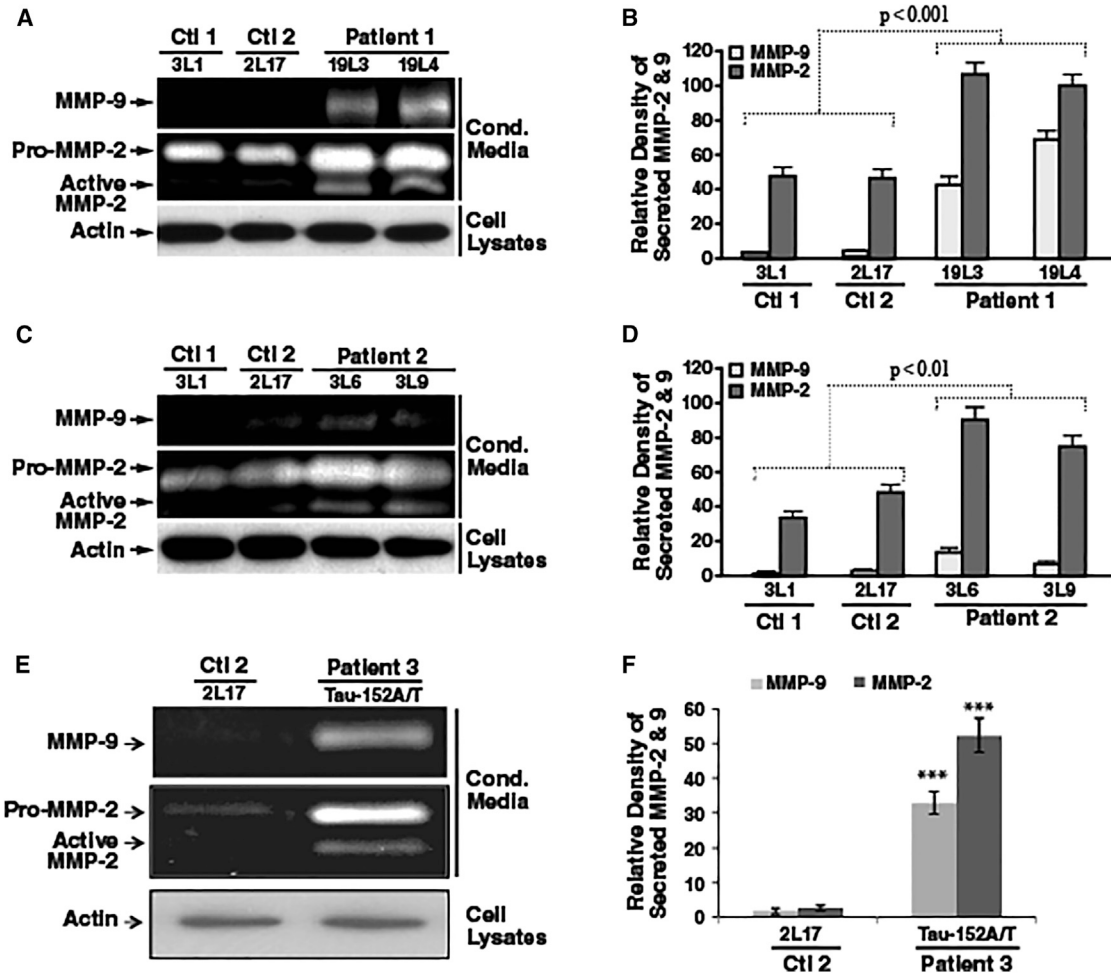


Figure 2. MMP-9 and MMP-2 Expression Is Increased in Human Neurons with *MAPT* Mutations

(A) Zymographic analysis of secreted MMP-9 and MMP-2 in conditioned media from tau-A152T human neurons (patient 1). Immunoblotting of cell lysates with actin served as the loading control.

(B) Quantification of secreted MMP-9 and MMP-2 in (A). Values are mean \pm SD of three independent experiments. $p < 0.001$ by Student's t test.

(C) Zymographic analysis of the level of secreted MMP-9 and MMP-2 in conditioned media from *MAPT* IVS10+16 human neurons (patient 2). Immunoblotting of cell lysates with actin served as the loading control.

(D) Quantification of secreted MMP-9 and MMP-2 in C. Values are mean \pm SD of three independent experiments. $p < 0.01$ by Student's t test.

(E) Zymographic analysis for MMP-9 and MMP-2 in conditioned media from tau-A152T human neurons (patient 3; Fong et al., 2013). Immunoblotting of cell lysates with actin served as the loading control.

(F) Quantification of MMP-2 and MMP-9 levels in (E) based on three experiments. Values are mean \pm SD of three experiments. $***p < 0.001$ by Student's t test.

See also Figure S3.

that patient-specific neurons are more sensitive than control neurons to cell death induced by rapamycin (Figure 3B) but not by staurosporine or rotenone (Figures S3B and S3C), suggesting compromised function in the mTOR pathway or related molecular pathways. For this experiment, we compared relative survival of neurons derived from the same iPSC line with or without the treatment with inducers of cellular stress; thus, the value from the

MTT assay without stressors for each line was always taken as 100%.

To examine the contribution of MMP-9 and MMP-2 to reduced survival of iPSC-derived cortical neurons with *MAPT* mutations, we quantified their mRNA levels in iPSC-derived 4-week-old human neurons treated with rapamycin for an additional 3 days. *MMP-9/MMP-2* mRNA levels in tau-A152T and *MAPT* IVS10+16 neurons were

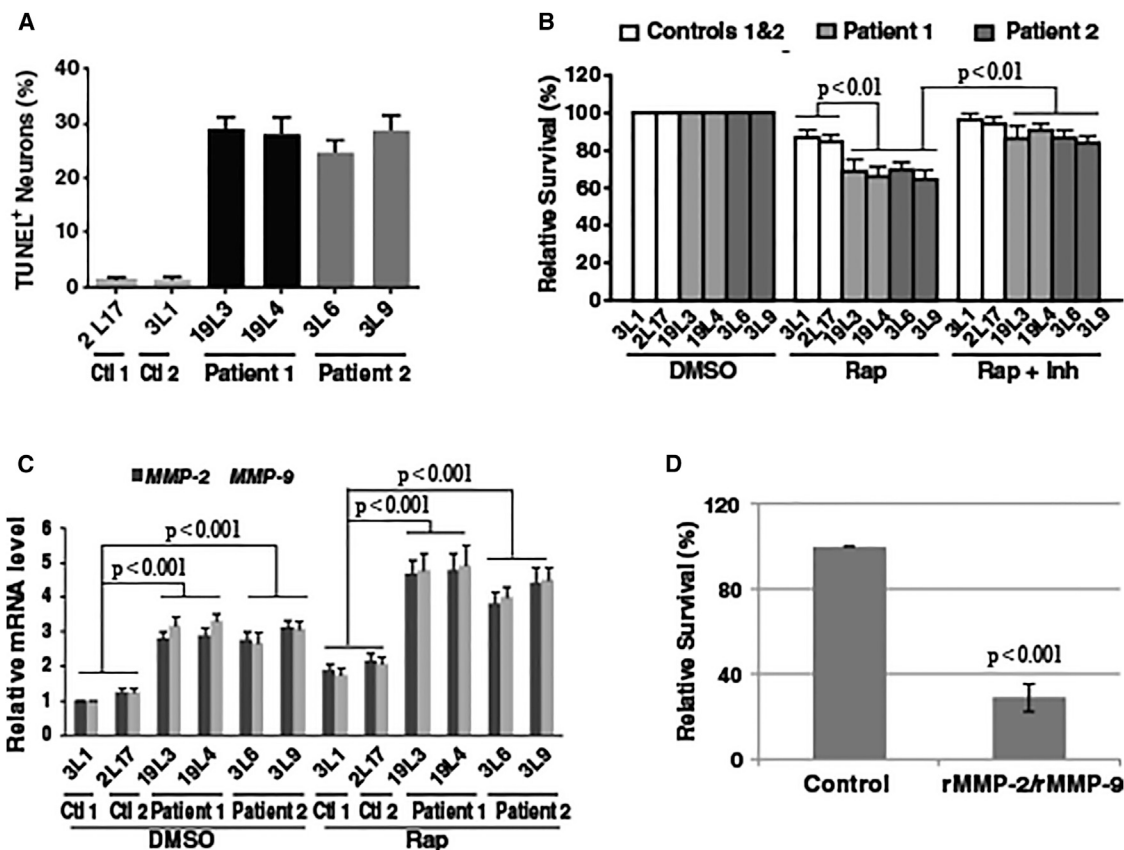


Figure 3. Increased MMP-9 and MMP-2 Expression Contributes to Neurodegeneration of Patient Neurons under Stress

(A) TUNEL analysis of TUJ1⁺ 4-week-old cortical neurons from controls and tau patients under basal conditions. Values are mean ± SD of two independent experiments and in each experiment, 100 cells were counted for each iPSC line.

(B) Percentage of human neuron survival after 48 hr of treatment with DMSO, 40 nM rapamycin, or 40 nM rapamycin and inhibitors (Inh) of MMP-2 (5 μM) and MMP-9 (200 nM). Values are mean ± SD from three independent experiments.

(C) qRT-PCR analysis of *MMP-2* and *MMP-9* mRNA levels in human neurons after treatment with DMSO or 40 nM rapamycin (Rap). Values are mean ± SD from three independent experiments.

(D) Survival of 4-week-old control human neurons 48 hr after treatment with recombinant MMP-2 (5 μg/mL) and MMP-9 (5 μg/mL). Values are mean ± SD of three independent experiments.

Statistical significance is indicated by p values and was determined by Student's t test. See also Figure S3.

significantly higher than that in control neurons (Figure S2J), and rapamycin increased these levels ($p < 0.01$) (Figure 3C). Human neurons differentiated from iPSCs are difficult to transfect at high efficiency. To examine the contributions of increased MMP-9 and MMP-2 to neuronal cell death, we used two compounds that are known to inhibit MMP-9 and MMP-2 activity (catalog #444278 and #444244, Calbiochem). We found that this treatment partially rescued the increased sensitivity to rapamycin and improved survival (Figure 3B). Furthermore, treatment with recombinant MMP-9/MMP-2 induced neuronal cell death in 4-week-old postmitotic cortical neurons differentiated from iPSCs of a healthy control subject, as measured by MTT assay (Figure 3D). MMP-2 and MMP-9 seem to induce cell death directly, since this

treatment also increased the TUNEL signal in iPSC-derived neurons (Figures S3D and S3E). These results suggest that elevated extracellular MMP-9 and MMP-2 are sufficient to cause neuronal cell death in human neurons. Taken together, our data suggest that elevated level and activity of extracellular MMP-9 and MMP-2 are in part responsible for the observed loss of patient neurons under rapamycin-induced stress.

4R Tau-A152T Activates the ERK Pathway to Increase MMP-9 Expression

It is known that MMP-9 expression is regulated by ERK (Wang et al., 2006). Therefore, to explore the mechanism by which mutant tau increases MMP-9 production in iPSC-derived patient neurons with *MAPT* mutations, we

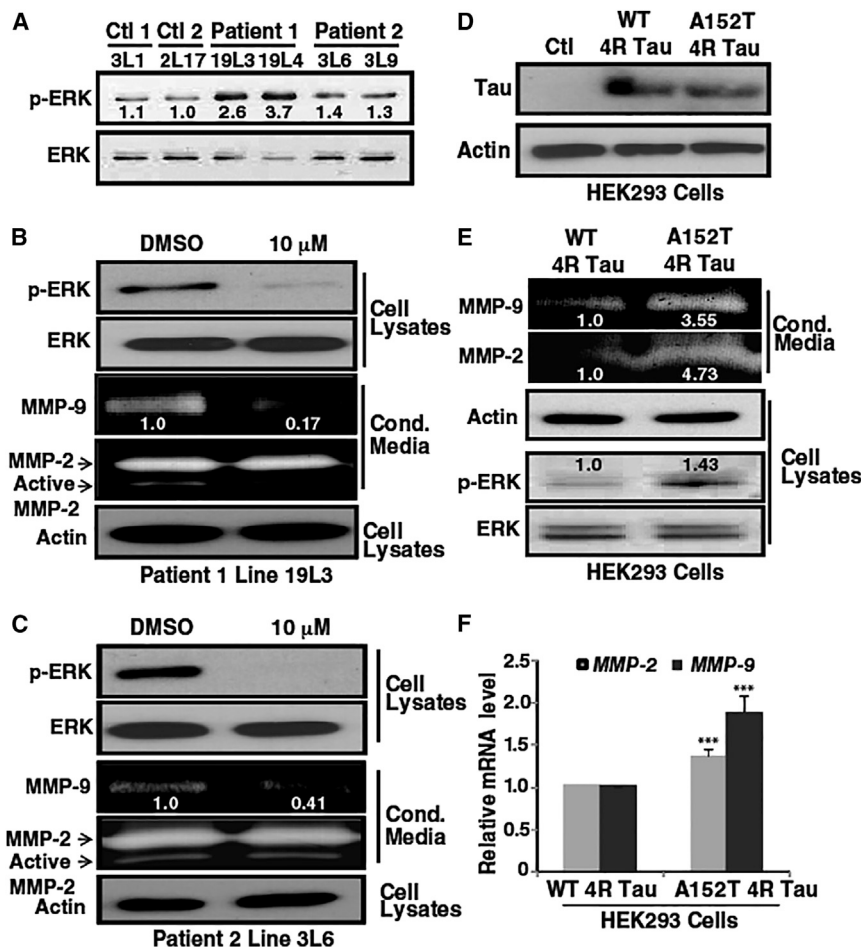


Figure 4. The tau-A152T Mutation Increases MMP-9 Expression through Effects on ERK

(A) Western blot analysis of ERK and p-ERK in 4-week-old iPSC-derived human cortical neurons.

(B and C) Neurons from line 3 of patient 1 (B) and line 6 of patient 2 (C) were treated with DMSO or the MEK inhibitor PD98059. Cell lysates were analyzed by western blot with antibodies against p-ERK, ERK, and actin, and conditioned media were analyzed by zymography for MMP-9 and MMP-2. The numbers indicate the levels of MMP-9 relative to actin.

(D) Western blot analysis of tau levels in HEK293 cells transfected with human 4R WT or 4R tau-A152T.

(E) Zymographic analysis of secreted MMP-9 and MMP-2 in conditioned media, and western blot analysis of actin, p-ERK, and ERK levels in respective lysates from HEK293 cells expressing 4R WT or 4R tau-A152T.

(F) qRT-PCR analysis of the expression levels of *MMP-2* and *MMP-9* mRNA in HEK293 cells expressing 4R WT or 4R tau-A152T. Values are mean \pm SD from three independent experiments. *** $p < 0.001$ by Student's *t* test.

first examined ERK-pathway activity. The level of phosphorylated ERK (p-ERK) was significantly increased in tau-A152T neurons and to a lesser extent in *MAPT* IVS10+16 patient neurons (Figure 4A). It is worth noting that the level and activity of secreted MMP-9 in tau-A152T neurons is also substantially higher than that in *MAPT* IVS10+16 neurons (Figure 2). ERK phosphorylation in *MAPT* mutant neurons was blocked by treatment with PD98059, a specific inhibitor of MEK-mediated activation of ERK (Li et al., 2001) (Figures 4B and 4C). Correspondingly, the level and activity of secreted MMP-9 was greatly suppressed in both tau-A152T and *MAPT* IVS10+16 patient neurons (Figures 4B and 4C), suggesting that MMP-9 expression is regulated by the ERK signaling pathway in cortical neurons. To further confirm the regulation of MMP-9 by ERK activation, we treated cortical neurons of both patient 1 and patient 2 with lower concentrations of PD98059. We found the level and activity of secreted MMP-9 to correlate well with the extent of ERK phosphorylation (Figure S4A). Interestingly, the level and activity of MMP-2 was not affected after the PD98059 treatment in

tau-A152T neurons (Figure 4B) and *MAPT* IVS10+16 neurons (Figure 4C). These results suggest that FTD-causing *MAPT* mutations increase MMP-9 through ERK activation, while the molecular regulation of MMP-2 induction in patient neurons remains to be identified.

To further confirm the role of mutant tau in MMP-9 production, we first examined the 4R/3R tau ratio in iPSC-derived patient neurons. As expected, the 4R/3R tau ratio is greatly increased in *MAPT* IVS10+16 neurons (Figure S3F) but not in *GRN* mutant neurons (Figure S3G). Interestingly, this ratio is also increased, but to a lesser extent, in tau-A152T neurons (Figure S3F). We then transfected HEK293 cells, which do not normally express tau, with either 3R or 4R WT tau or tau-A152T. 4R WT and 4R tau-A152T were expressed at comparable levels, as shown by western blot (Figure 4D). However, the secreted MMP-9 level and activity in conditioned medium was significantly higher in cultures of cells expressing tau-A152T than WT tau (Figure 4E). The *MMP-9* mRNA level was also higher in cells expressing tau-A152T (Figure 4F). Correspondingly, the level of p-ERK was also elevated in HEK293 cells expressing



tau-A152T (Figure 4E). Interestingly, the level and activity of secreted MMP-9 in HEK293 cells expressing WT 3R tau and 3R tau-A152T are similar (Figures S4B and S4C). ERK phosphorylation is also not affected by A152T mutation in 3R tau (Figure S4D). Taken together, these findings strongly suggest that 4R tau-A152T activates the ERK pathway more strongly, which in turn increases the level and activity of secreted MMP-9. 4R tau-A152T expression (Figures 4E and 4F) but not inhibition of ERK activity (Figures 4B and 4C) also increases the total activity of secreted MMP-2, suggesting that tau-A152T increases the level and activity of secreted MMP-2, likely through an ERK-independent pathway.

DISCUSSION

In this study, we found a significant increase in the level and activity of secreted MMP-9 and MMP-2 in cortical neurons derived from iPSCs of three subjects with *MAPT* mutations. More importantly, this increase contributed to neuronal cell loss induced by cellular stress, and inhibition of MMP-9/MMP-2 activity was neuroprotective. Our results are consistent with a recent paper published during our study in which the authors showed that MMP-9 is specifically expressed in fast motor neurons and contributes to selective neuronal vulnerability in amyotrophic lateral sclerosis (ALS) (Kaplan et al., 2014). Moreover, reduction of MMP-9 through genetic or pharmacological approaches delayed muscle denervation in vivo (Kaplan et al., 2014). Thus, MMP-9 is a potential therapeutic target for both FTD and ALS, although how MMP-9 induces cell death in iPSC-derived cortical neurons remains to be determined at a mechanistic level.

Our studies also provide mechanistic insight into the increased level and activity of secreted MMP-9 in cortical neurons derived from FTD patient-specific iPSCs with *MAPT* mutations. The increase seems to be mediated by ERK-pathway activation. Indeed, ectopic expression of mutant 4R but not 3R tau in HEK293 cells resulted in ERK phosphorylation and increased MMP-9 production. However, the molecular pathways of MMP-2 induction remain to be further delineated. These results further support the notion that tau is not only a key substrate for phosphorylation but also functions upstream of several kinase signaling pathways (Ittner et al., 2010; Morris et al., 2011; Vossel et al., 2015). Our iPSC lines with different *MAPT* mutations will be useful for further investigations of the roles of endogenous tau in cell signaling and neuronal vulnerability, such as in an accompanying paper by Silva et al. (2016). In the future, it will be useful to generate isogenic iPSC lines with different *MAPT* mutations and confirm the effect of endogenous mutant tau

on the ERK-MMP-9 pathway. Moreover, since different tau strains with prion-like properties cause different tauopathies in vitro and in vivo (Sanders et al., 2014), it will be also interesting to determine which tau strains are present in iPSC-derived human neurons and activate the ERK pathway.

EXPERIMENTAL PROCEDURES

Isolation of Primary Human Skin Fibroblasts and Generation of iPSCs

Skin biopsies were obtained from two patients with *MAPT* mutation. Patient 1 was a 68-year-old man with a 6-year history of personality changes and cognitive and speech deterioration. He was diagnosed with PSP and has a tau-A152T mutation. Patient 2 was presymptomatic at the time of skin biopsy but had an extensive family history of FTD and has a *MAPT* IVS10+16 mutation.

iPSCs were generated as described by Almeida et al. (2012). In brief, fibroblasts were transduced for 7 days with equal volumes of supernatant from retroviruses expressing human *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC* and seeded onto mitomycin C-treated SNL feeder cells. Five weeks after viral transduction, individual colonies were picked and transferred to 12-well plates containing feeder cells for expansion. Well-established iPSC lines were adapted to feeder-free conditions and maintained with mTeSR1 medium (STEMCELL Technologies) on Matrigel (BD Biosciences).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2016.08.006>.

AUTHOR CONTRIBUTIONS

B.L.M. and F.B.G. initiated the project. M.H.U.B., S.A., and F.B.G. designed the studies, analyzed the data, and wrote the manuscript. M.H.U.B. did most of the experiments. Z.Z. helped S.A. with iPSC generation. R.L.-G. differentiated some iPSC lines and performed TUNEL assays. W.M. and K.F. conducted the electrophysiological analysis. A.K., M.D.G., and B.L.M. provided patient skin biopsies. J.B. and E.-M.M. generated tau-expression vectors. F.B.G. supervised the project.

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