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Supplemental Information

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

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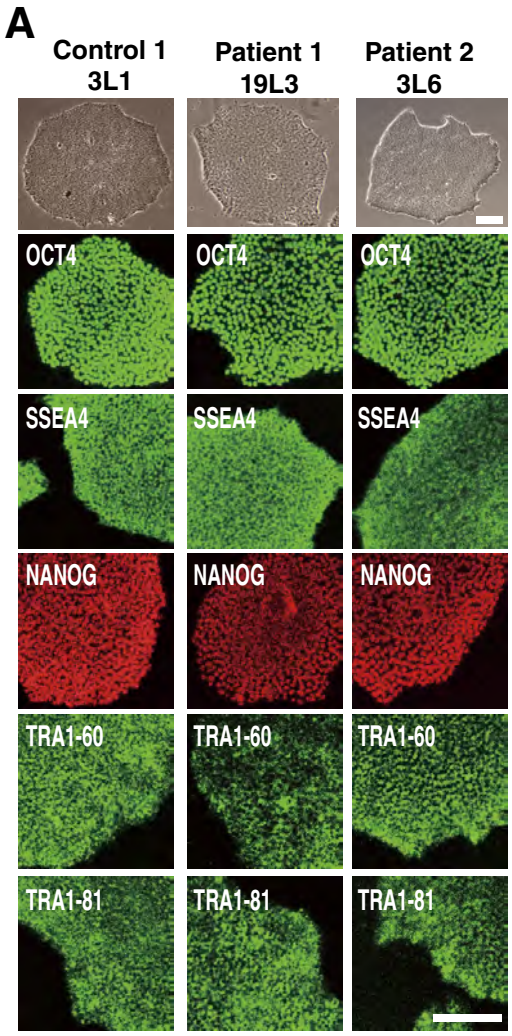
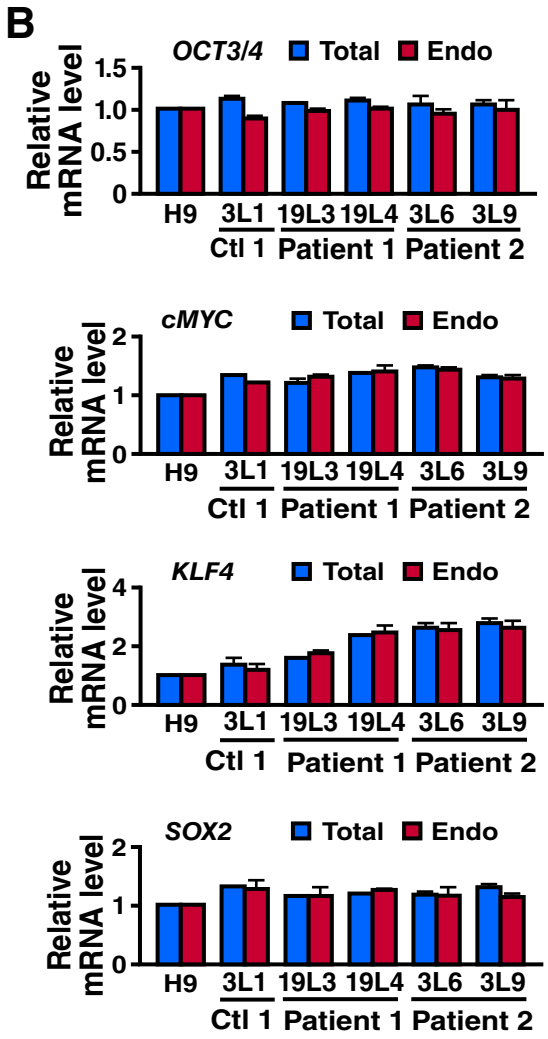
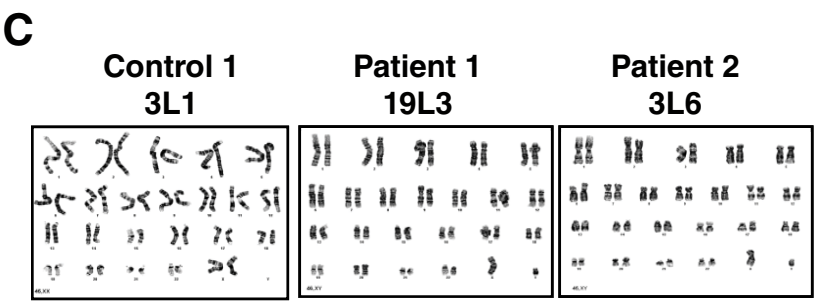
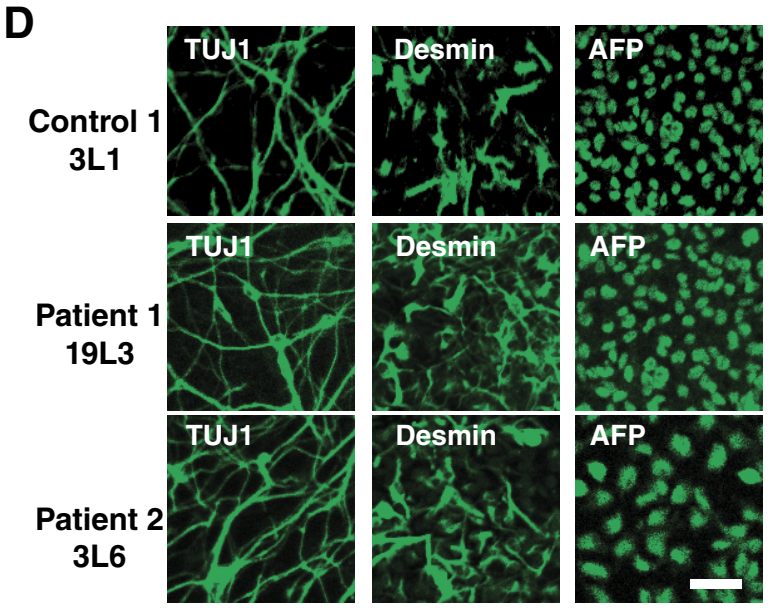
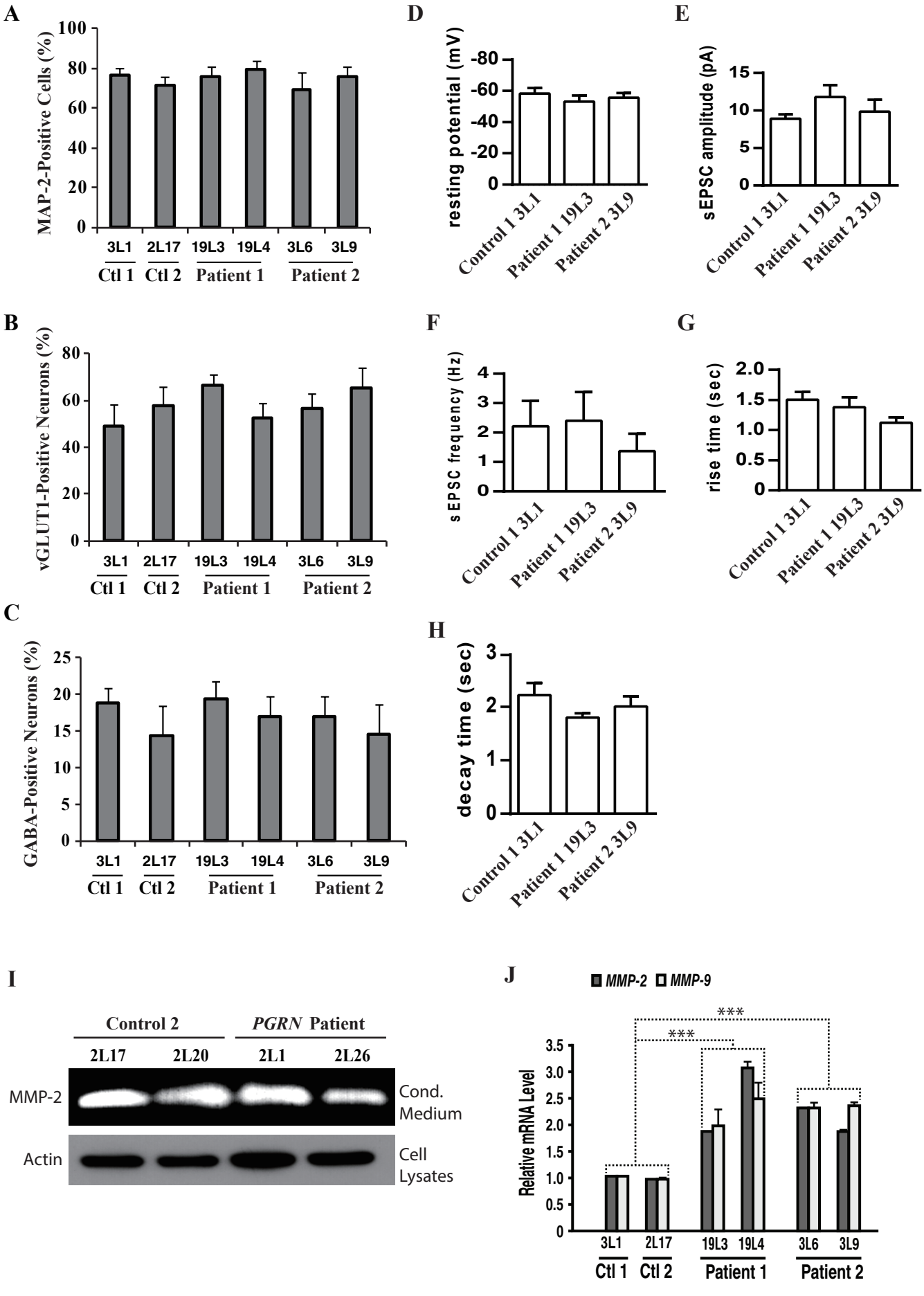
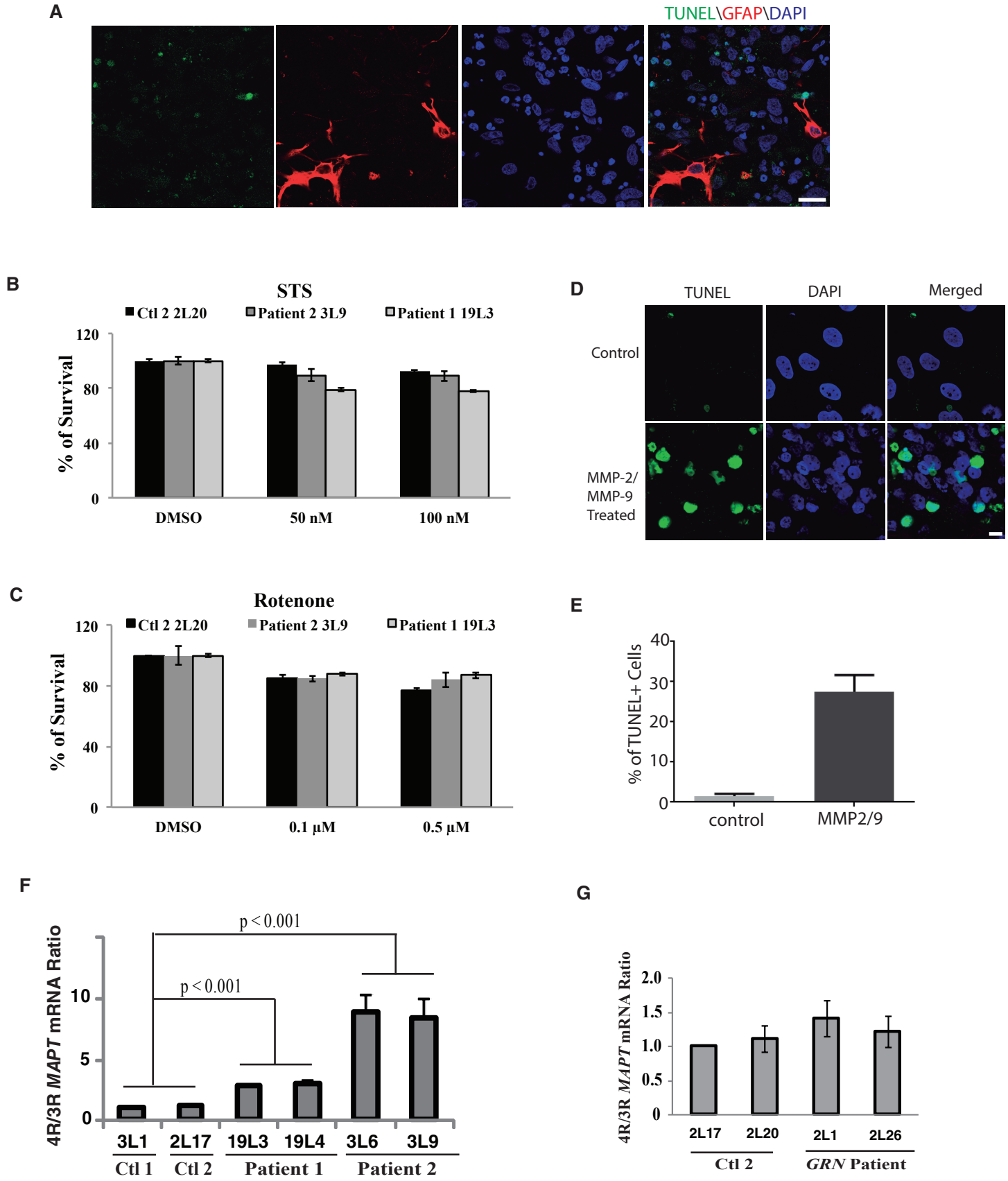

A
Control 1
3L1
Patient 1
19L3
Patient 2
3L6
OCT4
SSEA4
NANOG
TRA1-60
TRA1-81


B
OCT3/4
cMYC
KLF4
SOX2
Total
Endo
Relative mRNA level
H9
3L1
19L3
19L4
3L6
3L9
Ctl 1
Patient 1
Patient 2


C
Control 1
3L1
Patient 1
19L3
Patient 2
3L6


D
Control 1
3L1
Patient 1
19L3
Patient 2
3L6
TUJ1
Desmin
AFP


A
MAP-2-Positive Cells (%)
100
80
60
40
20
0
3L1
2L17
19L3
19L4
3L6
3L9
Ctl 1
Ctl 2
Patient 1
Patient 2
B
vGLUT1-Positive Neurons (%)
80
60
40
20
0
C
GABA-Positive Neurons (%)
25
20
15
10
5
0
D
resting potential (mV)
-80
-60
-40
-20
0
Control 1 3L1
Patient 1 19L3
Patient 2 3L9
E
sEPSC amplitude (pA)
15
10
5
0
F
sEPSC frequency (Hz)
4
3
2
1
0
G
rise time (sec)
2.0
1.5
1.0
0.5
0
H
decay time (sec)
3
2
1
0
I
Control 2
PGRN Patient
2L17
2L20
2L1
2L26
MMP-2
Cond. Medium
Actin
Cell Lysates
J
MMP-2
MMP-9
***
***
Relative mRNA Level
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0


A
TUNEL\GFAP\DAPI
B
STS
Ctl 2 2L20
Patient 2 3L9
Patient 1 19L3
% of Survival
120
80
40
0
DMSO
50 nM
100 nM
C
Rotenone
Ctl 2 2L20
Patient 2 3L9
Patient 1 19L3
% of Survival
120
80
40
0
DMSO
0.1 µM
0.5 µM
D
TUNEL
DAPI
Merged
Control
MMP-2/
MMP-9
Treated
E
% of TUNEL+ Cells
40
30
20
10
0
control
MMP2/9
F
4R/3R MAPT mRNA Ratio
p < 0.001
p < 0.001
10
5
0
3L1
2L17
19L3
19L4
3L6
3L9
Ctl 1
Ctl 2
Patient 1
Patient 2
G
4R/3R MAPT mRNA Ratio
2.0
1.5
1.0
0.5
0
2L17
2L20
2L1
2L26
Ctl 2
GRN Patient

Biswas *et al*, Figure S4

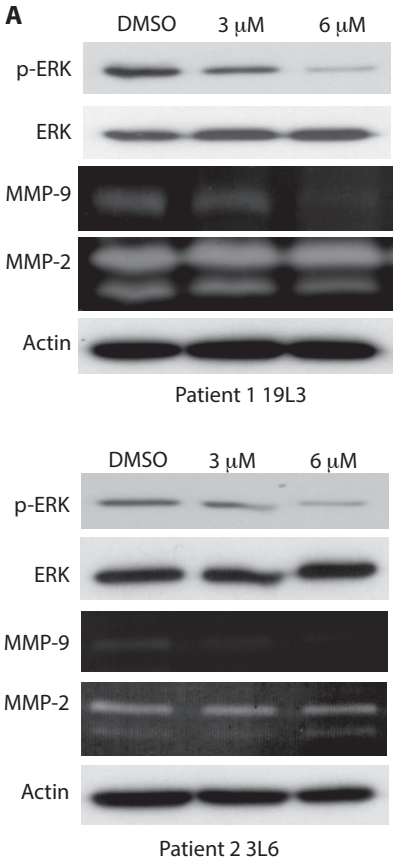


Figure S1. Generation and Characterization of iPSC Lines from Control and FTD Patients with *MAPT* Mutations (Related to Figure 1)

(A) Phase-contrast images (top panels, scale bar: 100 μm) and immunostaining of iPSC lines with different stem cell markers (scale bar: 50 μm).

(B) qRT-PCR analysis shows the expression levels of total and endogenous (Endo) reprogramming factors are similar, indicating silencing of the transgene. Values are mean ± S.D of three independent experiments.

(C) The karyotypes of newly generated iPSC lines are normal.

(D) *In vitro* spontaneous differentiation of different iPSC lines to demonstrate their pluripotency. Cells were immunostained with antibodies specific for markers of ectoderm (TUJ1), mesoderm (desmin), and endoderm (alpha-fetoprotein, AFP). Scale bar, 50 μm.

Figure S2. Characterization of Cortical Neurons Differentiated from Control and *MAPT* Mutant iPSCs (Related to Figure 1)

(A) Quantification of the percentage of MAP2-positive neurons in four-week-old neuron cultures Values are mean ± S.D of three experiments.

(B, C) Quantification of the percentage of VGLUT1-positive (glutametergic) neurons (B) and GABA-positive (GABAergic) neurons (C) among MAP2-positive neurons. No significant differences were found. Values are mean ± S.D of three independent experiments.

(D-H) Resting membrane potential (D), mean sEPSC amplitude (E), mean sEPSC frequency (F), rise time (G), and decay time constant (H) of human neurons differentiated from different iPSC lines. No significant differences were found. Values are mean ± SEM of two experiments and in each experiment, 7 neurons were recorded.

(I) The levels of secreted MMP-2 in 4-week-old control neurons and neurons with the progranulin S116X mutation are the same, as determined by Zymographic analysis. Cell lysates were analyzed by western blot with anti-actin antibody as a loading control.

(J) *MMP-2* and *MMP-9* mRNA levels in control and patient neurons measured by qRT-PCR. Values are mean ± SD of three experiments. ***: $p < 0.001$ by Student's t test.

Figure S3. Further Characterization of Human Cortical Neurons with *MAPT* Mutations (Related to Figure 2 and Figure 3)

(A) Lack of TUNEL labeling in GFAP-positive glial cells present in iPSC-derived cortical neuron cultures.

(B) Percentage of human neurons survival after treatment with DMSO or staurosporine (STS, 50 or 100 nM). Values are mean ± SD of three independent experiments.

(C) Percentage of human neurons survival after treatment with DMSO or rotenone (0.5 nM). Values are mean ± SD of three independent experiments.

(D) Neurotoxicity of MMP-2 and MMP-9 in iPSC-derived cortical neurons as assayed by TUNEL labeling. iPSC-derived cortical neurons were treated with 5 ug/ml MMP-2 and MMP-9 for 40 hours and then the extent of cell death was analyzed by TUNEL. Scale Bar: 10 μm.

(E) Quantification of the percentage of TUNEL-positive and MAP2-positive neurons from panel D. Values are mean ± SD of two independent experiments and in each experiment, 100 cells were counted for each condition.

(F) Quantification of 4R/3R *MAPT* mRNA ratios in control and patient cortical neurons as measured by RT-PCR. Values are mean ± SD from three experiments, by Student's t test.

(G) The 4R/3R *MAPT* mRNA ratio is not changed in *GRN* neurons compared with control neurons as measured by RT-PCR. Values are mean ± SD of three experiments, by Student's t test.

Figure S4. Regulation of MMP-9 Expression (Related to Figure 4)

(A) The level and activity of secreted MMP-9 in iPSC-derived cortical neurons of Patient 1 and Patient 2 directly correlates with the extent of ERK phosphorylation. Cortical neurons differentiated from patient iPSCs were treated with DMSO or different concentrations of the MEK inhibitor PD98059.

(B) Western blot analysis of human 3R WT or 3R tau-A152T in HEK293 cells.

(C) Zymographic analysis of secreted MMP-2 and MMP-9 in conditioned media from HEK293 cells expressing 3R WT and 3R tau-A152T, and western blot analysis of actin in the respective cell lysates.

(D) Western blot analysis of p-ERK and ERK in HEK293 cells expressing 3R WT or 3R tau-A152T.

EXPERIMENTAL PROCEDURES

Karyotyping Analysis

Standard G-banding analysis was performed by Cytogenetics Laboratory, University of Massachusetts Memorial Medical Center, Worcester, MA.

Neuronal Differentiation of iPSCs

IPSCs were differentiated into neurons as described (Almeida et al., 2012). Briefly, iPSC colonies were detached and grown in suspension for 6–8 days in iPSC medium without basic FGF to form embryoid bodies (EBs), which were seeded on poly-L-ornithine/laminin coated dish and maintained in neural induction medium to form rosettes. After 10 days, neuroepithelial cells in the rosettes were isolated from the surrounding cells with 0.2 mg/ml dispase. After 3–4 weeks, neurospheres were dissociated with accutase (Millipore), placed on glass coverslips coated with poly-D-lysine and laminin (BD), and cultured in neuron medium for 4 weeks. For spontaneous differentiation, EBs were obtained as described above, seeded on poly-L-ornithine/laminin-coated coverslips and cultured for 8 additional days. Cells migrating out of the EBs were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, immunostained for pluripotency markers, and analyzed by confocal microscopy.

Materials

The use of human skin biopsies was approved by the Institutional Review Board and Ethics Committee at the University of California, San Francisco (UCSF). Written informed consent was obtained in all cases. Experimental procedures are described in detail in Supplemental Methods.

Rapamycin, rotenone, and staurosporine were from Sigma. Horseradish peroxidase–conjugated secondary antibody was from Jackson ImmunoResearch Laboratories. PD98059, an MEK inhibitor, MMP-2 and MMP-9 inhibitors as well as recombinant MMP-2 and MMP-9 were from Calbiochem.

MTT Assay

Neurons used in the MTT assays were cultured in 24-well plates for 4 weeks. The MTT assays were done as recommended by the manufacturer (Promega).

Gene Expression Analysis

For mRNA expression analysis, total RNA was isolated with RNeasy kits (Qiagen), and 500 ng of RNA was reverse transcribed into cDNA with a Taqman reverse transcription reagent kit (Applied Biosystems). Quantitative RT-PCR was done with Taqman Gene Expression Master Mix and Taqman primers (for *OCT3/4*, *cMYC*, *SOX2, KLF4* and *GAPDH*) or SYBR Green PCR Master Mix (Applied Biosystems). The sequences of the primers used with SYBER Green are as following. 4R *MAPT*: forward - GAAGCTGGATCTTAGCAACG and reverse – GACGTGTTTGATATTATCCT. 3R *MAPT*: forward - AGGCGGGAAGGTGCAAATAG and reverse – TCCTGGTTTATGATGGATGTT. *GAPDH*: forward –TGCACCACCACCTGCTTAGC and reverse – GGCATGGACTGTGGTCATGAG. *MMP-2*: forward – ATGACAGCTGCACCACTGAG and reverse – TGATGTCATCCTGGGACAGA. *MMP-9*: forward - ATAAGGACGACGTGAATGGC and reverse – TCAAAGACCGAGTCCAGCTT. Ct values for each target gene were normalized to that of the *GAPDH*. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative expression of each gene.

Immunofluorescence

Cells cultured on cover slips were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.5% Triton X-100 for 5 min. After incubation with 7% FBS in PBS for 1 h, cells were incubated with the primary and secondary antibodies in 7% FBS in PBS, and mounted on slides with Slow Fade Antifade reagent (containing DAPI). The primary antibodies used were mouse anti-OCT4 (1:100, Santa Cruz Biotechnology, sc-5279), mouse anti-SSEA4 (1:100, Abcam, ab16287), goat anti-NANOG (1:100, R&D Systems, AF1997), mouse anti-TRA-1-60 (1:100, Millipore, MAB4360), mouse anti-TRA-1-81 (1:100, Millipore, MAB4381), mouse anti-TUJ1 (1:200, Promega, G7121), rabbit anti-desmin (1:100, Thermo Scientific, RB-9014-PO), mouse anti-alpha-fetoprotein (1:200, R&D Systems, MAB1368), mouse anti-MAP2 (1:500, Sigma, M9942), rabbit anti-GABA (1:100, Sigma, A2052), and rabbit anti-VGLUT1 (1:500, Synaptic Systems, 135303). Slides were analyzed by confocal microscopy.

Electrophoresis and Immunoblotting

Electrophoresis and immunoblotting were done as described (Biswas et al., 2010). Briefly, proteins were subjected to SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked and incubated with respective primary antibody at 4°C, overnight and with the secondary antibody at room temperature for 1 h. The primary antibodies used were mouse antibodies against MAP-2 (1:1000, Sigma, M9942), actin (1:2000, Sigma, A2228), and PSD95 (1:1000, Antibodies Inc., 75-028) and rabbit antibodies against tau (1:1000, Dako, A0024), ERK (1:1000, Cell Signaling, 4695), and p-ERK (1:1000, Cell Signaling, 9101). Proteins were visualized by enhanced chemiluminescence (Thermo Scientific).

Electrophysiology

Electrophysiological recordings were obtained from 4–5-week-old iPSC-derived neurons as described (Almeida et al., 2012). Action potentials were evoked with 200-msec depolarizing currents of 0–400 pA in 100-pA steps. AMPAR-mediated spontaneous excitatory postsynaptic currents (sEPSCs) were measured in whole-cell voltage-clamp mode with bath application of picrotoxin (0.1 mM, Sigma) and analyzed with Mini Analysis software (Synaptosoft). Results are shown as mean ± SEM. Statistical significance of changes in sEPSCs was determined by one-way ANOVA; $p < 0.05$ was considered statistically significant.

Measurement of MMP-2 and MMP-9 Activity by Zymography

MMP-2 and MMP-9 activities in conditioned medium were assayed by zymography as described (Biswas et al., 2010). Conditioned medium were subjected to SDS-PAGE with 0.3% Type-A gelatin (Sigma). The gels were washed, incubated for 18 h at 37°C in the reaction buffer (50 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$), stained with 0.1% Coomassie Brilliant Blue (CBB) for 1 hour, and destained in 50% H_2O, 40% methanol, and 10% acetic acid by volume. Finally, gel was sandwiched in two sheets of cellophane and air dried overnight. Activity of MMP-2 and MMP-9 was visualized as a transparent band against CBB background and quantified using ImageJ software (Biswas et al., 2010).

TUNEL assay

We performed TUNEL assay in one-month-old iPSC-derived cortical neurons from control and *MAPT* mutation subjects. In addition, we treated iPSC-derived cortical neurons with a combination of 5 μg/ml MMP-2/MMP-9 for 48 hours. We fix the neurons

from the two experiments with 4% PFA for 15 minutes and then we performed TUNEL assay with the Fluorescein in situ cell death detection kit (Roche). After we performed the TUNEL assay we did immunostaining with the mouse anti-MAP2 primary antibody (Sigma, 1:1000, M9942) followed by Alexa 568 anti-mouse secondary antibody (1:500, Thermo Scientific, A10037).

REFERENCES

Almeida, S., Zhang, Z., Coppola, G., Mao, W., Futai, K., Karydas, A., Geschwind, M.D., Tartaglia, M.C., Gao, F., Gianni, D. et al. (2012). Induced pluripotent stem cell models of progranulin-deficient frontotemporal dementia uncover specific reversible neuronal defects, *Cell Rep. 2*, 789–798.

Biswas, M.H., Du, C., Zhang, C., Straubhaar, J., Languino, L.R., and Balaji, K.C. (2010). Protein kinase D1 inhibits cell proliferation through matrix metalloproteinase-2 and matrix metalloproteinase-9 secretion in prostate cancer. *Cancer Res. 70*, 2095–2104.