

The deubiquitinase OTULIN regulates tau expression and RNA metabolism in neurons

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The degradation of aggregation-prone tau is regulated by the ubiquitin-proteasome system and autophagy, which are impaired in Alzheimer's disease (AD) and related dementias (ADRD), causing tau aggregation. Protein ubiquitination, with its linkage specificity determines the fate of proteins, which can be either protein degradative or stabilizing signals. While the linear M1-linked ubiquitination on protein aggregates serves as a signaling hub that recruits various ubiquitin-binding proteins for the coordinated actions of protein aggregate turnover and inflammatory nuclear factor-kappa B (NF- κ B) activation, the deubiquitinase OTULIN counteracts the M1-linked ubiquitin signaling. However, the exact role of OTULIN in neurons and tau aggregates clearance in AD are unknown. Based on our quantitative bulk RNA sequencing analysis of human inducible pluripotent stem cell-derived neurons (iPSNs) from an individual with late-onset sporadic AD (sAD2.1), a downregulation of the ubiquitin ligase activating factors (MAGE-A2/A2B/H1) and OTULIN long noncoding RNA (OTULIN lncRNA) was observed compared to healthy control WTC11 iPSNs. The downregulated OTULIN lncRNA is concurrently associated with increased levels of OTULIN protein and phosphorylated tau at p-S202/p-T205 (AT8), p-T231 (AT180), and p-S396/p-S404 (PHF-1) in sAD2.1 iPSNs. Inhibiting the deubiquitinase activity of OTULIN with a small molecule, UC495 reduced the phosphorylated tau in iPSNs and SH-SY5Y cells, whereas the CRISPR-Cas9-mediated OTULIN gene knockout (KO) in sAD2.1 iPSNs decreased both the total and phosphorylated tau levels. CRISPR-Cas9-mediated OTULIN KO in SH-SY5Y resulted in a complete loss of tau at both mRNA and protein levels, and increased levels of polyubiquitinated proteins, which are being degraded by the proteasome as confirmed with an inhibitor, Lactacystin. In addition, SH-SY5Y OTULIN KO cells showed downregulation of various genes associated with inflammation, autophagy, ubiquitin-proteasome system, and the linear ubiquitin assembly complex that consequently may prevent development of an autoinflammation in the absence of OTULIN gene in neurons. Together, our results suggest, for the first time, a noncanonical role for OTULIN in regulating the gene expression and RNA metabolism, which may have a significant pathogenic role in exacerbating tau aggregation in neurons. Thus, OTULIN could be a novel potential therapeutic target for AD and ADRD.

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Introduction

The tau is encoded by the microtubule-associated protein tau or *MAPT* gene, which is located on chromosome 17q21 and expressed as six isoforms by alternative splicing in the human brain (1, 2). Tauopathy is a group of more than 20 neurodegenerative diseases, including Alzheimer's disease (AD), caused by intracellular aggregation of hyperphosphorylated tau as neurofibrillary tangles (NFTs) in neurons (3–5). Depending on the context in the neurons, tau can be a substrate of 20S proteasome, 26S proteasome, chaperone-mediated autophagy, microautophagy, macroautophagy, and aggrephagy, and their impaired degradative functions can lead to aggregation of tau as NFTs in tauopathy brains (reviewed elsewhere) (6, 7).

The linear ubiquitin assembly complex, LUBAC [Ring finger protein 31/HOIL-1-interacting protein (RNF31/HOIP), RanBP-type and C3HC4-type zinc finger-containing protein 1/Heme-oxidized IRP2 ubiquitin ligase 1 (RBCK1/HOIL-1) and SHANK-associated RH domain interactor (SHARPIN)] is the only known E3 ubiquitin ligase complex responsible for adding methionine1 (M1)-linked linear ubiquitin chains to protein aggregates (8). M1-linked ubiquitin is enriched around the nucleus and spread throughout the cell body of a neuron in various proteinopathies, including AD, frontotemporal dementia, Parkinson's disease (PD), polyglutamine (polyQ) diseases, and amyotrophic lateral sclerosis (8, 9). The ubiquitin chaperone, valosin-containing protein (VCP/p97 ATPase) recruits RNF31/HOIP to the sites of polyQ aggregates in Huntington's disease models for M1-linked polyubiquitination, which in turn recruits

autophagy receptors for efficient clearance of Huntingtin (Htt)-polyQ aggregates by autophagy (8). In another study, lysine48 (K48)-linked ubiquitination on tau aggregates is a prerequisite for recruiting VCP, which interacts with Heat Shock Protein (HSP70) to extract tau from aggregates for proteasomal degradation or extracellular secretion of seed-competent tau (10). Moreover, the tandem mass spectrometry analysis of tau-paired helical filaments (PHFs) isolated from the autopsy of human AD brains shows the presence of M1-linked polyubiquitin and various other ubiquitin-specific linkages, including K6-, K11-, K48-, and K63-linked ubiquitin chains (11–13).

In addition to recruiting autophagy receptors, the M1-linked ubiquitination on protein aggregates and host-invaded microbes is a signal for activation of nuclear factor-kappa B (NF- κ B), which regulates the transcription of DNA associated with immune response and cell survival under various stresses and infection (14–21). Most often, neuronally derived protein aggregates such as NFTs, α -synuclein, and Htt-polyQ become pro-inflammatory upon ubiquitination with M1-linked ubiquitin chains, which provide a binding platform to NF- κ B regulator/autophagy adaptor, NF- κ B essential modifier (NEMO), and autophagy receptors for NF- κ B activation and protein aggregate clearance, respectively (8, 9, 22, 23). Various other ubiquitin-specific linkages in NFTs recruit proteasome and aggrephagy machinery for tau clearance or extracellular secretion of seed competent tau upon impairment of proteostasis (6, 10).

The ovarian tumor (OTU) domain-containing deubiquitinase with linear linkage specificity (OTULIN) exclusively deubiquitinates interlinear

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M1-linked ubiquitin chains except the peptide bond between target proteins and the first ubiquitin (24), whereas cylindromatosis (CYLD) deubiquitinates both M1- and K63-linked ubiquitin chains (25–27). Both the deubiquitinases, OTULIN and CYLD bind to HOIP/RNF31 and decrease the LUBAC-mediated M1-linked ubiquitination on target proteins and consequently decrease NF- κ B activation under various inflammatory stimuli (28–32). Despite their role in negative regulation of NF- κ B activity, OTULIN knockout (KO) showed increased levels of M1-linked ubiquitination on LUBAC components, RBCK1/HOIL-1 and SHARPIN, but not with CYLD KO, suggesting that OTULIN directly regulates LUBAC activity in basal condition (27). Moreover, OTULIN tyrosine56 (Y56) is essential for bonding with LUBAC component, HOIP/RNF31 to prevent TNF α -induced NF- κ B activation, whereas phospho-OTULIN Y56 prevents interaction with HOIP and activates NF- κ B (29, 33). In another study, OTULIN deubiquitinates LUBAC itself polyubiquitinated M1-linked linear chains, and prevents LUBAC-mediated cell death and inflammation (34).

Mice KO of *HOIP* (35), *HOIL-1* (36), and *OTULIN* (34) genes are embryonic lethal, whereas *SHARPIN* deficiency causes chronic autoinflammatory disease in the skin of mice (37). Mutations in human HOIP L72P/Q399H (38, 39) and *HOIL-1* deficiency (40) cause immunodeficiency and autoinflammation due to a lack of LUBAC-mediated M1-linked ubiquitination. Whereas mutations in human OTULIN Y244C/L272P/G281R decrease deubiquitinase activity, resulting in increased levels of M1-linked polyubiquitination in patients with OTULIN-related autoinflammatory syndrome (41–43). The effect of *OTULIN* deficiency varies among cell types, such as in myeloid macrophages it causes elevated autoinflammation associated with increased M1-linked polyubiquitination and NF- κ B activity (42). *OTULIN* deficiency in lymphoid B or T cells causes no adverse inflammation due to complete loss of LUBAC components, HOIP, and SHARPIN at protein levels by proteasomal activity, but not their mRNAs levels (42). Despite OTULIN negatively regulates the M1-linked polyubiquitin and its associated autoinflammation in ORAS (41–43), OTULIN also functions as a negative and positive regulator of autophagy (44) and proteasomes (45), respectively. However, the role of OTULIN in neuron is largely unknown.

Here, we tested the hypothesis that stabilizing M1-linked polyubiquitination will reduce the levels of hyperphosphorylated tau by inhibiting M1-linked deubiquitinase activity of OTULIN and leading to the enhanced tau clearance by proteasome-, calpain- or autophagy-mediated degradation. However, results obtained significantly deviated from supporting this original hypothesis and suggests a novel noncanonical role of OTULIN in tau expression and RNA metabolism in neurons.

Results

sAD2.1 iPSCs downregulate *OTULIN* lncRNA and ubiquitin ligase activating factor *MAGE*

We performed bulk RNA sequencing analyses in sporadic Alzheimer's disease (sAD2.1) induced pluripotent stem cell line (iPSC)-derived neurons (iPSNs) and healthy control WTC11 iPSNs to identify the differentially expressed genes and transcripts. The complete details of the RNA sequencing data, including the mapped region, sequence coverage, read density, principal component analysis, Pearson correlation matrix, and significantly altered biological processes/pathways are shown in Supplementary Figures S1–S7 and Supplementary Tables S1–S2. The differentially expressed gene analyses obtained from the bulk RNA sequencing of sAD2.1 versus WTC11 iPSNs show that the expressions of 2390 and 2124 genes were upregulating and downregulated significantly in sAD2.1 iPSNs, respectively compared to WTC11 (Figure 1A and B). Similarly, 3828 and 1852 RNA transcripts were up and downregulated, respectively in sAD2.1 iPSNs (Figure 1C), which may likely indicate dysregulated RNA metabolism. Notably, the gene expression of *OTULIN* long non-coding RNA (lncRNA) derived from *OTULIN* divergent transcript (*OTULIN-DT*) was significantly downregulated (Figure 1D) that may influence the transcription of *OTULIN* gene. However, based on our quantitative bulk RNA sequencing analysis, the downregulated OTULIN-DT did not significantly upregulate the expression of *OTULIN* gene in sAD2.1 iPSNs (Figure 1D). Since pathological tau accumulate in sAD2.1 iPSNs (46), we analyzed whether there is any correlation between the gene expression of

MAPT and MAPT lncRNA or MAPT antisense RNA (MAPT-AS). Our quantitative bulk RNA analysis shows no significant difference between the MAPT and MAPT-AS1 gene expression levels in sAD2.1 iPSNs (Figure 1D). As expected, there was a positive correlation between sAD2.1 with AD and tauopathies in gene set enrichment analyses (GSEA) (Figure 1E and F). Gene Ontology (GO) mapped > 2000 genes significantly altered to be associated with “protein-binding” as a major molecular functional pathway (Supplementary Figure S5). The heatmap for gene expression analyses shows that the ubiquitin ligase activating factor of the melanoma antigen gene (MAGE) family members, including MAGE-A2/A2B/H1 were downregulated in sAD2.1 iPSNs (Figure 1G). However, we do not know whether there is any correlation between the downregulated ubiquitin ligase activator factor, MAGE and pathological tau accumulation in sAD2.1 iPSNs, which is currently beyond the scope of current study.

sAD2.1 iPSNs show increased levels of OTULIN and phosphorylated tau, whereas inhibition of OTULIN deubiquitinase or deletion of *OTULIN* gene decreases phosphorylated tau levels

The bulk RNA sequencing revealed that the downregulated OTULIN lncRNA did not significantly affect the expression of *OTULIN* gene at mRNA levels in sAD2.1 iPSNs (Figure 1D). However, it is plausible that increased stability of OTULIN at protein levels is most likely due to downregulation of the ubiquitin ligase activating factor MAGE and can negatively influence the pathological tau clearance in sAD2.1 iPSNs (Figure 1G). We therefore performed Western blotting experiments to identify if there is any correlation between the level of OTULIN protein and tau pathology in sAD2.1 iPSNs. Surprisingly, the OTULIN protein level was significantly elevated in sAD2.1 than in WTC11 iPSNs (Figure 2A and B). In addition, tau phosphorylation at p-S199/p-S202/p-T205 (AT8), p-T231 (AT180), and p-S396/p-S404 (PHF-1) was also significantly elevated in sAD2.1 iPSNs (Figure 2A and B). It is conceivable that the elevated OTULIN deubiquitinase counteracts with M1-linked polyubiquitinated proteins and consequently may increase tau pathology, and inhibiting the deubiquitinase activity of OTULIN may alleviate tau pathology in sAD2.1 iPSNs.

To identify a small molecule that can inhibit the catalytic activity of OTULIN deubiquitinase, compounds in a drug-like library were docked to the OTULIN deubiquitinase catalytic center (PDB: 3ZNV) using the Schrodinger Molecular Modeling Suite. In silico docking of small molecules with the deubiquitinase OTULIN catalytic center identifies UC495 as a potential OTULIN deubiquitinase inhibitor (Figure 2C). To test the hypothesis that the inhibition of OTULIN deubiquitinase might alleviate tau pathology, sAD2.1 iPSNs were treated with compound UC495 to inhibit OTULIN deubiquitinase activity and subsequently assessed hyperphosphorylation (Figure 2D). Inhibiting OTULIN deubiquitinase activity significantly reduced the level of phosphorylated tau at AT8 site, and modestly decreased PHF-1-positive tau levels compared to vehicle-treated sAD2.1 iPSNs (Figure 2D and E). However, neither OTULIN nor total tau levels were changed (Figure 2D and E).

Unlike pharmacological inhibition of OTULIN deubiquitinase activity with compound UC495, CRISPR-Cas9-mediated KO of *OTULIN* gene completely abolished its expression in sAD2.1 iPSNs (Figure 2F and G). As a result, both total and phosphorylated tau levels were significantly decreased in *OTULIN*-deficient sAD2.1 iPSNs (Figure 2F and G). The complete loss of tau in *OTULIN*-deficient sAD2.1 iPSNs intrigued us to investigate whether it is due to neuronal loss. We confirmed no adverse effect on neuronal differentiation despite the absence of both OTULIN and tau (Figure 2F and G). We also imaged the differentiated neurons using a bright field microscope, and found no significant morphological difference or alterations in neuronal marker, NeuN levels in *OTULIN*-deficient sAD2.1 iPSNs (Supplementary Figure S8). Together, these results suggest that CRISPR-Cas9-mediated *OTULIN* KO in sporadic sAD2.1 iPSNs causes complete loss of total tau and phosphorylated tau levels without affecting neuronal survivability.

Pharmacological inhibition of OTULIN deubiquitinase or deletion of *OTULIN* gene decreases tau in SH-SY5Y

In contrast to the effect of UC495 in reducing AT8 positive tau in sporadic sAD2.1 iPSNs (Figure 2F–G), inhibiting deubiquitinase activity of OTULIN with compound UC495 in human neuroblastoma cells, SH-SY5Y

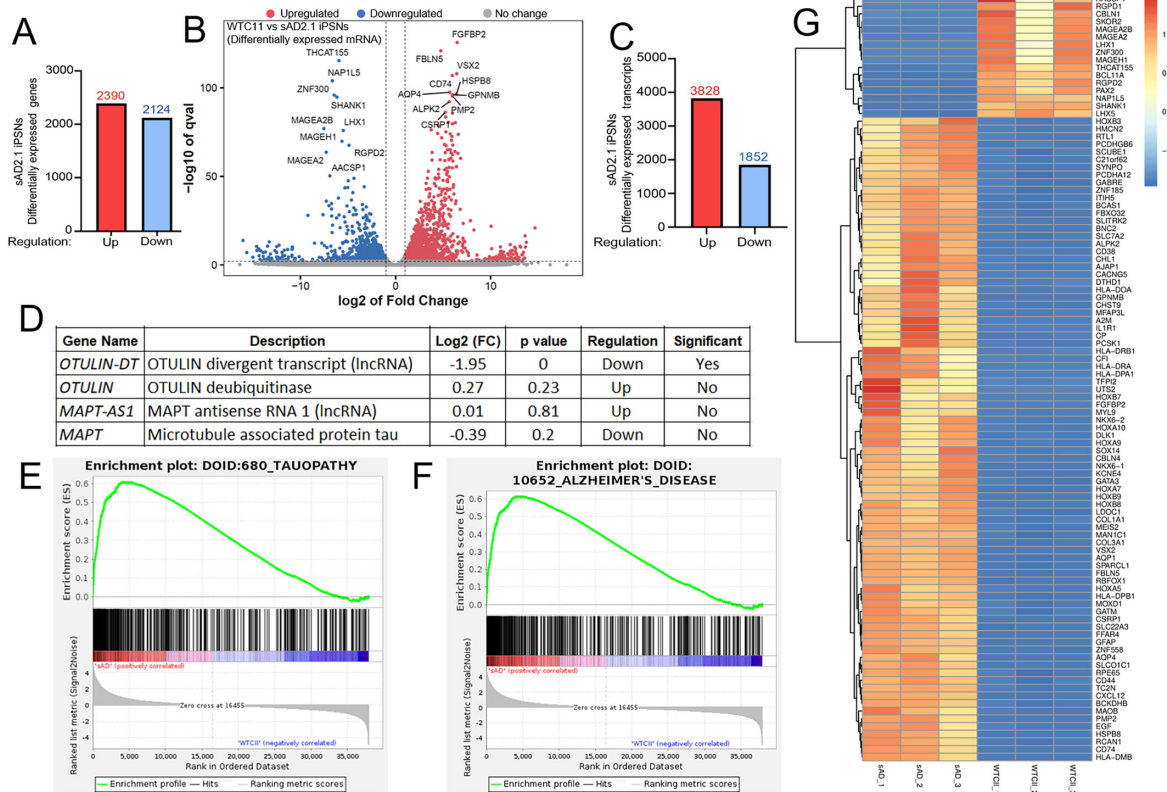


Figure 1. The sporadic Alzheimer's disease (SAD2.1) iPSC line-derived neurons (iPSNs) show significantly altered gene expression profile with increased *OTULIN* long noncoding RNA (*OTULIN* lncRNA) levels. (A) A comparative bulk RNA sequencing analysis of SAD2.1 vs WTC11 iPSNs shows upregulation of 2390 genes and downregulation of 2124 genes were differentially expressed in SAD2.1 iPSNs. (B) The volcano plot of differentially expressed genes in SAD2.1 iPSNs represents the log2 of fold change with a q value of < 0.05 as significant cutoff. On the plot, blue, red and gray dots indicate the levels of gene expression were downregulated, upregulated, and no changes, respectively. (C) SAD2.1 iPSNs shows altered RNA metabolism as reflected in the number of differentially expressed transcripts, with an upregulation of 3828 transcripts and downregulation of 1852 transcripts as compared to WTC11 iPSNs. (D) SAD2.1 iPSNs shows significant downregulation of *OTULIN* lncRNA (*OTULIN-DT*), which is inversely correlated with slightly upregulated *OTULIN* gene expression, which may exacerbate tau pathology. The *MAPT* gene expression may be slightly downregulated in contrast to either upregulated *MAPT-AS1* or downregulated *MAPT-IT1*, which may initially function as a defense mechanism to prevent tau pathology progression in SAD2.1 iPSNs. (E, F) The gene set enrichment analyses (GSEA) pathways showing enrichment of signaling pathways relevant to tauopathy and Alzheimer's disease. (G) Heatmap analysis of differentially expressed genes in SAD2.1 (sAD_1,2,3) versus WTC11 (WTC11_1,2,3) iPSNs with three numbers from each group of cells. Heatmap intensity in color codes indicates upregulated (orange to red) and downregulated (blue) gene expression, respectively. Notably, the melanoma antigen gene (MAGE) family members (MAGE-A2/A2B/H1), which are known to enhance ubiquitin ligase activity, are significantly downregulated in SAD2.1 compared to WTC11 iPSNs.

significantly reduced *OTULIN* and total/phosphorylated (AT8, AT180, and PHF-1) tau levels (Figure 3A and B). Despite the reduction in endogenous tau levels, we did not observe cell toxicity as observed by the relatively uniform level of actin between UC495-treated and control SH-SY5Y cells (Figure 3A).

Like the pharmacological inhibition of *OTULIN* deubiquitinase with compound UC495, the CRISPR-Cas9-mediated gene interfering with *OTULIN* sgRNA completely abolished the *OTULIN* protein expression in SH-SY5Y (Figure 3C and D). Interestingly, knocking out of *OTULIN* gene in SH-SY5Y caused a complete loss of tau (Figure 3D). Collectively, inhibiting deubiquitinase of *OTULIN* with small molecule UC495 or CRISPR-Cas9-mediated KO of *OTULIN* gene in SH-SY5Y cell line decreases *OTULIN* levels, which in turn significantly reduces total tau levels (Figure 3A–D).

We predicted that the absence of *OTULIN* protein expression in SH-SY5Y *OTULIN* KO might have enhanced the degradation of tau by proteostasis activity. To determine the proteostasis activity causing complete loss of tau, SH-SY5Y *OTULIN* KO cells were pretreated with protein translation inhibitor, cycloheximide (CHX) for 1 h and then incubated with various proteostasis inhibitors (Lactacystin for 20S proteasome, MG132 for 26S proteasome, Bafilomycin A1 or Baf A1 for autophagy, and PD 150606 for Calpains 1 and 2) individually or together for an additional 4 h. The absence of deubiquitinase *OTULIN* in SH-SY5Y increased the accumula-

tion of polyubiquitinated proteins, which are actively being degraded by the proteasome as confirmed with the 20S proteasome inhibitor, Lactacystin, but not with the 26S proteasome inhibitor, MG132 at 10 or 20 μ M concentrations (Figure 3D and E). Notably, the dynamics of polyubiquitinated proteins accumulation or degradation by the ubiquitin-proteasome system is enhanced in *OTULIN* KO cells compared to SH-SY5Y wild-type (WT) cells as confirmed with or without CHX under various proteostasis inhibitors (Figure 3C–E). Inhibiting the proteostasis activity did not increase the level of either *OTULIN* or Tau in SH-SY5Y WT cells except with 20 μ M MG132, which decreases tau levels by unknown mechanism (Figure 3C and E). Thus, we conclude that none of the proteostasis inhibitors restored tau level in *OTULIN* deficient SH-SY5Y cells (Figure 3D), which motivated us to investigate the presence of tau at *MAPT* mRNA level by real-time quantitative reverse transcription PCR (qRT-PCR) along with 18S ribosomal RNA (18S rRNA) as a housekeeping reference gene for normalizing qRT-PCR data. Surprisingly, the *MAPT* mRNA was undetectable in the *OTULIN*-deficient SH-SY5Y compared to the WT cell line (Figure 3F).

OTULIN may function as a master regulator of RNA metabolism and gene expression

To determine whether *OTULIN* deficiency has any global effects on the mRNAs of other genes or whether it is specific to *MAPT* mRNA, we isolated

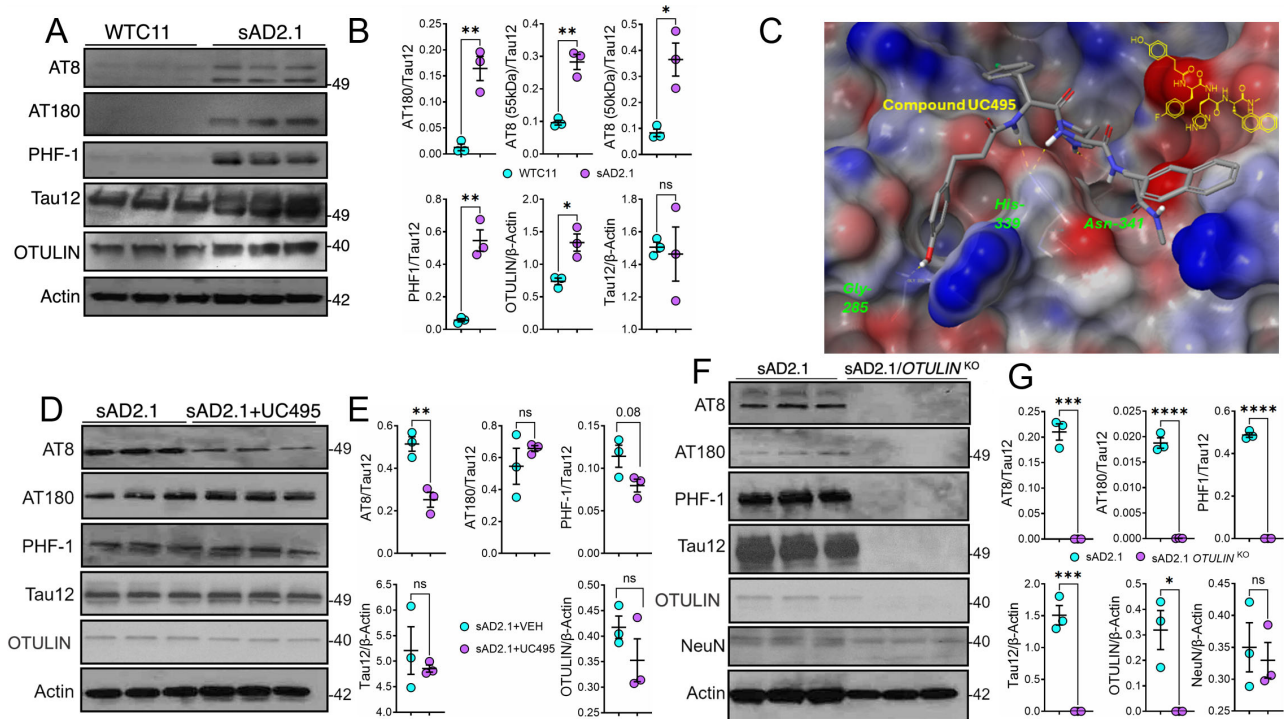


Figure 2. The sporadic sAD2.1 iPSNs increase the levels of OTULIN and phosphorylated tau, whereas inhibition of OTULIN deubiquitinase or deletion of *OTULIN* gene decreases phosphorylated tau levels. (A, B) OTULIN and phosphorylated tau levels were elevated in sporadic sAD2.1 iPSNs. Western blot analyses show significantly elevated phosphorylated tau at p-S199/p-S202/p-T205 (AT8), p-T231 (AT180), and p-S396/p-S404 (PHF-1) and OTULIN in sAD2.1 as compared to healthy control WTC11 iPSNs. (C) A Connolly surface representation of the major atoms of UC495 that interact with the OTULIN deubiquitinase binding pocket are shown in the figure. The OTULIN surface is shown with electrostatic potentials, with red color indicating regions of high negative potential (electron-rich), blue color indicating regions of high positive potential (electron-poor), and other colors representing intermediate potentials. (D, E) Pharmacological inhibition of OTULIN deubiquitinase with compound UC495 selectively decreases phosphorylated tau at AT8 site in sAD2.1 iPSNs. Western blot analyses showed significantly decreased AT8⁺ tau and a modest reduction of PHF-1⁺ tau upon inhibition of OTULIN deubiquitinase with compound UC495 in sAD2.1 iPSNs. (F, G) CRISPR-Cas9-mediated knockout (KO) of *OTULIN* causes a complete loss of total tau in sporadic sAD2.1 iPSNs. Western blot quantifications showing significantly reduced AT8, AT180, and PHF-1 positive as well as total tau in CRISPR-Cas9-mediated *OTULIN* KO of sporadic sAD2.1 as compared to sAD2.1 iPSNs. Note that the reduction is not due to toxicity to neurons, as no significant difference in the NeuN levels was noted. Data presented as mean + SEM; unpaired *t* test; **p* < 0.05; ***p* < 0.01; ****p* < 0.005; *****p* < 0.001; *n* = 3.

total RNAs from SH-SY5Y *OTULIN* KO and WT cell lines, respectively and performed bulk RNA sequencing for differentially expressed mRNA profiling (Supplementary Tables S3 and S4 and Supplementary Figures S9–S12). A significant upregulation of 774 genes and downregulation of 13,341 genes in SH-SY5Y *OTULIN* KO compared to WT cell line were observed (Figure 4A and C). To further confirm that OTULIN regulates RNA metabolism, we performed RNA transcript analyses and identified significant upregulation of 1113 transcripts and downregulation of 43,003 transcripts in the *OTULIN*-deficient SH-SY5Y line (Figure 4B and D). To classify the differentially expressed genes belonging to specific functional characteristics, we performed a GO enrichment analysis, which showed differentially expressed genes are mainly associated with protein, nucleotide and RNA-binding functions, ribonucleoprotein complex, transferase activity, DNA repair, and mitochondrial proteins (Figure 4F and Supplementary Figure S13). The Kyoto Encyclopedia of Genes and Genomes (KEGG) cellular pathway enrichment analysis revealed that most of the differentially expressed genes belongs to RNA degradation, RNA polymerase, neurodegeneration, lysosome, autophagy, oxidative phosphorylation, nucleotide excision repair, and N-Glycan biosynthesis pathways (Figure 4G). Heatmap comparative analysis showed a few highly significant up (yellow, orange, and red) and downregulated (blue) gene expression in SH-SY5Y *OTULIN* KO and WT (Figure 4E). Notably, the tau encoding *MAPT* gene transcript is downregulated along with OTULIN interaction partners predicted by STRING protein-protein interaction network (Supplementary Figure S14 and Supplementary Table S5). Since OTULIN is a deubiquiti-

nase and dysregulates RNA metabolism, we mainly focused on the expression level of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and RNA-binding ubiquitin ligase enzyme (E3), and RNA decay/degradation factors. OTULIN deficiency downregulated ubiquitin B/C and upregulated E1 enzyme UBA3, many E2 conjugating enzymes, and RNA-binding ubiquitin ligases, *RC3H2* and *MEX3C* (Supplementary Table S6). *OTULIN* KO also upregulated many RNA degradation/decay-associated proteins, heterogeneous ribonucleoproteins, and a few neurodegenerative disease-associated RNA-binding proteins (Supplementary Table S7). Details about mapped region, sequence coverage, ready density, principal component analysis, Pearson correlation matrix, and significantly altered biological processes/pathways are shown in Supplementary Figures S9–S13 and Supplementary Tables S3 and S4.

Discussion

The canonical function of OTULIN is deubiquitinate the linear M1-linked ubiquitin chains such as to counteract the LUBAC (RNF31/HOIP, RBCK1/HOIL-1, and SHARPIN) E3-ubiquitin ligase, which is destabilized by deubiquitinating the linear M1-linked polyubiquitin on HOIL-1 and SHARPIN. The deubiquitinase OTULIN functions as a negative regulator of LUBAC and M1-linked ubiquitin chains-associated NF-κB and autophagy. Since OTULIN is an M1-linked deubiquitinase, we anticipated that inhibiting its deubiquitinase activity in neurons would favorably stabilize the M1-linked ubiquitin chains on tau aggregates and clear the aggregates by proteasome- or autophagy-mediated degradation. However, we

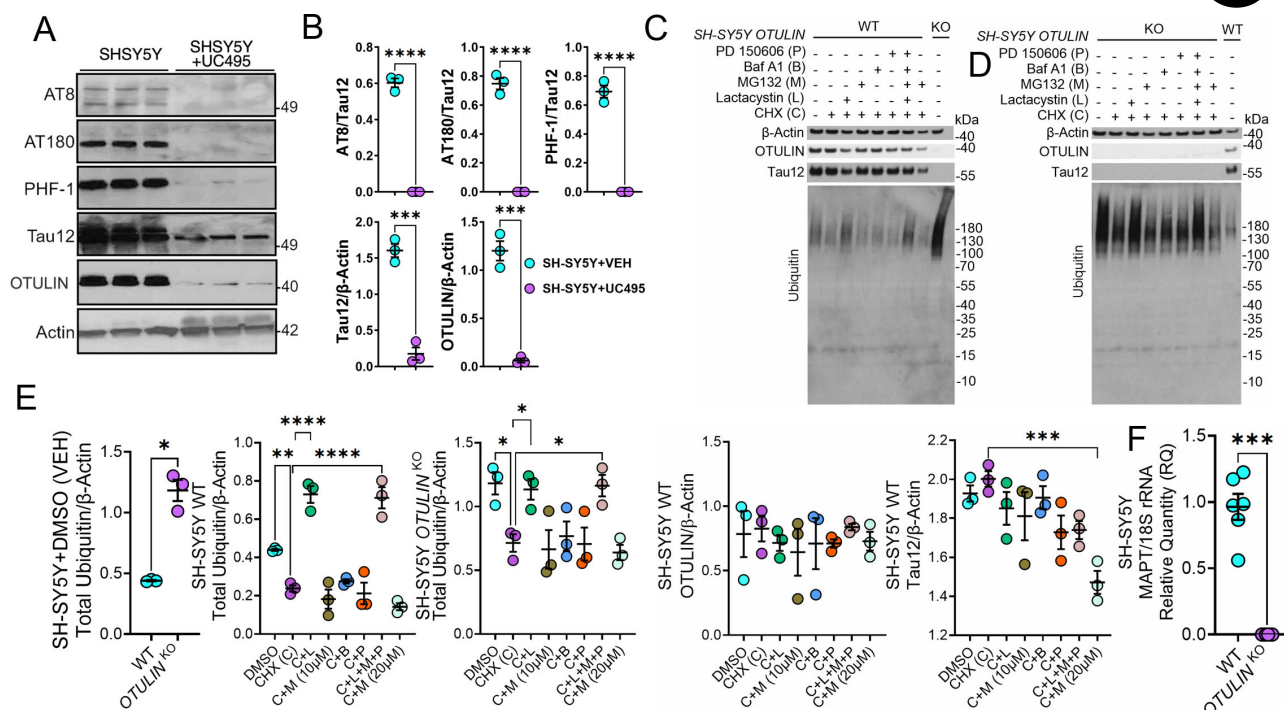


Figure 3. Pharmacological inhibition of OTULIN deubiquitinase or deletion of *OTULIN* gene decreases tau levels in human neuroblastoma cell line, SH-SY5Y. (A,B) Pharmacological inhibition of OTULIN with UC495 compound significantly decreases the levels of hyperphosphorylated and total tau, and OTULIN in SH-SY5Y cells. Western blot quantifications show significantly reduced AT8, AT180 and PHF-1 positive tau as well as total tau upon inhibition of OTULIN deubiquitinase with UC495 compound in SH-SY5Y cells as compared to DMSO-treated control. Inhibiting the deubiquitinase activity of OTULIN with UC495 also decreases the OTULIN levels. (C–E) CRISPR-Cas9-mediated *OTULIN* KO in SH-SY5Y causes a complete loss of tau and an accumulation of polyubiquitinated proteins. To study the dynamics of protein degradation in SH-SY5Y WT (C) and *OTULIN* KO (D) cells, protein translation was inhibited with 50 μ M cycloheximide (CHX) 1 h prior adding the proteostasis inhibitors, 10 or 20 μ M MG132 (26S proteasome), 10 μ M Lactacystin (20S proteasome), 100 nM Bafilomycin A1 (Baf A1, autophagy), 100 μ M PD 150606 (calpain 1/2) either individually or all four inhibitors together for 4 h. None of the inhibitors stabilizes tau in the absence of OTULIN (D) or change the OTULIN and Tau levels in wild-type cells except with 20 μ M MG132, which decreases tau levels (C, E). (F) *OTULIN* deficiency in SH-SY5Y downregulates the transcription of *MAPT* gene. The real-time quantitative reverse transcription PCR (qRT-PCR) analysis shows the absence of *MAPT* mRNA in *OTULIN* KO compared to wild type SH-SY5Y. 18S ribosomal RNA (18S rRNA) was used as a housekeeping reference gene for normalizing qRT-PCR data (F). Data presented as mean $\pm$ SEM; unpaired *t* test for comparing the two groups; Multiple comparisons were done by one-way ANOVA with Dunnett's multiple comparisons test; ***p* < 0.01; ****p* < 0.005; *****p* < 0.001; *n* = 3 to 6. Note that one of WT and *OTULIN* KO samples were loaded in blots in C and D (right most lanes) to control for samples run in two different gels.

surprisingly identified a complete loss of tau expression in *OTULIN* gene KO and explored an unappreciated noncanonical role of OTULIN in tau expression and RNA metabolism/gene expression in neurons.

Protein ubiquitination is a three-step posttranslational modification that requires the sequential action of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase enzyme (E3) (47) (Figure 5). However, protein ubiquitination becomes complex when decoding the functions of differential ubiquitination on the same protein with various linkages, such as M1-, K6-, K11-, K27-, K29-, K33-, K48-, and K63-linked ubiquitin chains. It also adds further complexity to understand the roles of homotypic, heterotypic, linear, and branched ubiquitination with various linkages on the same protein (6, 48). Such a complex ubiquitination occurs on the pathological tau aggregates in the brains of patients with tauopathies. Notably, prior studies have suggested that PHFs from human postmortem AD brains show M1-, K6-, K11-, K48-, and K63-linked ubiquitin chains via mass spectrometry (6, 11–13). Tau has been shown as a substrate of 20S proteasome, 26S proteasome, chaperone-mediated autophagy, microautophagy, macroautophagy, and aggrephagy depending on the posttranslational modifications, suggesting that ubiquitin can modify tau function and its half-life depends on the cellular contexts (6). However, it is unclear why tau requires differential ubiquitination to undergo degradation. Most likely, the sequential failure of various proteostasis machinery adds differential linkages of ubiquitin chains, such as K63- and M1-linked polyubiquitin, which

recruit ubiquitin-binding adaptors or receptors to activate the transcription factors. These factors, in turn, trigger gene expression for cell survival, immune responses, and the replenishment of proteostasis machinery for efficient clearance of protein aggregates (6, 8, 9, 22, 23).

The LUBAC is composed of RNF31/HOIP, RBCK1/HOIL-1, and SHARPIN, which function as an E3-ubiquitin ligase for M1-linked polyubiquitination on protein aggregates associated with various neurodegenerative diseases (8). The deubiquitinase OTULIN specifically deubiquitinates M1-linked ubiquitin chains, such as those found in HOIL-1 and SHARPIN, whereas CYLD deubiquitinates both M1- and K63-linked ubiquitin chains but not the LUBAC (24–27). OTULIN functions as a negative regulator of LUBAC, NF- κ B, and autophagy (8, 9, 22, 23). We, therefore, aimed to understand the role of OTULIN in clearance of tau aggregates in AD. We utilized healthy control WTC11 and sporadic AD2.1 human iPSC line-derived neurons (sAD2.1 iPSCs), and human neuroblastoma SH-SY5Y WT/*OTULIN* KO lines as model systems to understand the role of OTULIN in tau clearance and differential gene expressions. Our first RNA sequencing analyses of WTC11 and sAD2.1 iPSCs indicated that over 4000 genes and transcripts are differentially expressed in sporadic sAD2.1 iPSCs compared to healthy control WTC11 iPSCs (Figure 1A–C). The most notable hits are the melanoma antigen gene (*MAGE*) family members, including *MAGEA2/A2B/H1*, which are known to positively regulate the ubiquitin ligase activity of RING domain proteins (49). The *MAGE* family members, *MAGEA2/A2B/H1* are significantly downregulated in sAD2.1 iPSCs

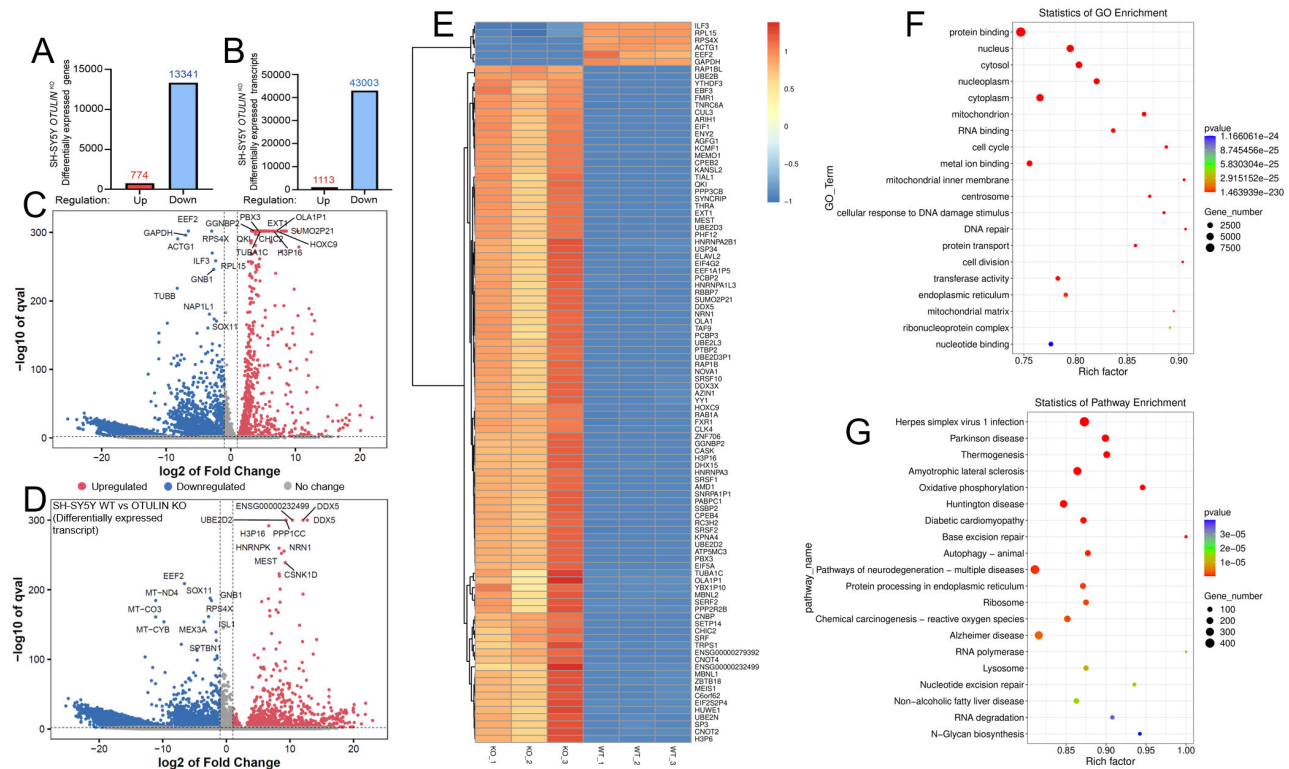


Figure 4. Dysregulated gene expression and RNA metabolism in SH-SY5Y *OTULIN* KO reveals a potential non-canonical role of *OTULIN*, which may serve as a master regulator of gene expression and RNA metabolism in neurons. (A) Bulk RNA sequencing analysis of SH-SY5Y *OTULIN* KO shows upregulation of 774 genes and a downregulation of 13,341 genes compared to wild type (WT), with three replicates from each group. (B) *OTULIN* gene deficiency dysregulates RNA metabolism in SH-SY5Y as reflected in the number of differentially expressed transcripts, with a downregulation of 43,003 transcripts and an upregulation of 1,113 transcripts compared to WT. (C) The volcano plot of differentially expressed genes in SH-SY5Y *OTULIN* KO represents the log₂ of the fold change with a q value of < 0.05 as the significant cutoff. On the plot of log₂ of fold changes, red dots at positive and blue dots at negative zones represent upregulated and downregulated gene expression, respectively. Whereas gray dots at the neutral zone denote no significant change of gene expression in both WT and KO lines. (D) The volcano plot of differentially expressed transcripts represents the log₂ of fold change with a q value of < 0.05 as the significant cutoff for upregulated (red dots) or downregulated (blue dots) and no change (gray) of transcript levels in *OTULIN* KO as compared to WT. (E) Heatmap analysis of differentially expressed genes in *OTULIN* KO (KO_1,2,3) and wild type (WT_1,2,3) SH-SY5Y lines. Heatmap intensity in color codes indicates upregulated (orange to red) and downregulated (blue) gene expression, respectively. Most of the upregulated gene expressions in *OTULIN* KO are linked with mRNA degradation/decay and splicing pathways. (F) A Gene Ontology (GO) scatter plot represents a set of genes that are associated with protein-to-nucleotide binding and were significantly differentially expressed in the SH-SY5Y *OTULIN* KO line. (G) The cellular pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) scatter plot describes the enrichment of differentially expressed genes in *OTULIN* KO. RNA metabolism and neurodegenerative disease-associated pathway genes were enriched among the differentially expressed genes. The size and color of dots on the scattered plots (F, G) denote the number of genes and levels of significantly enriched differentially expressed genes.

(Figure 1G), suggesting a likely loss of a few unknown ubiquitin ligases activity, which may explain the elevated pathological tau levels in sAD2.1 iPSNs. However, the level of M1-linked LUBAC E3-ligase components, including *HOIP*, *HOIL-1*, and *SHARPIN*, were not significantly altered in sAD2.1 iPSNs. Since MAGEs interact with more than 50 RING-type E3 ubiquitin ligases (50), a link between MAGEs and impaired tau clearance in AD needs further investigation.

Interestingly, a significant downregulation of *OTULIN* lncRNA is detected in sAD2.1 iPSNs (Figure 1D), which negatively correlated with elevated *OTULIN* protein levels. Since lncRNAs are intronic (51), overlapping, or antisense to protein-coding mRNAs (exons) like microRNAs, the downregulated *OTULIN* lncRNA may likely increase the level of *OTULIN* expression in sAD2.1 iPSNs. In a previous study, we observed an inverse correlation between elevated mRNA and reduced lncRNA or circular RNA (52). Notably, increased *OTULIN* is positively correlating with elevated levels of total tau and phosphorylated tau at AT8 (p-S202/p-T205), AT180 (p-T231), and PHF-1 (p-S396/p-S404) epitopes (Figure 2A and B), suggesting that *OTULIN* may regulate tau pathology.

To understand the role of the M1-linked deubiquitinase activity of *OTULIN* in tau pathology, we treated the sporadic sAD2.1 iPSNs with compound UC495. Inhibiting the *OTULIN* deubiquitinase with UC495

decreases phosphorylated tau at the AT8 epitope, but not at the AT180 and PHF-1 epitopes or total tau levels. Notably, inhibiting the deubiquitinase activity of *OTULIN* does not affect the level of *OTULIN* in sAD2.1 iPSNs, suggesting that inhibiting *OTULIN* deubiquitinase alone is sufficient to prevent tau pathology moderately (Figure 2D and E). However, the exact mechanism by which phosphorylated tau at the AT8 epitope is decreased upon inhibiting the *OTULIN* deubiquitinase with UC495 is unknown. It is possible that the compound UC495 may promote partial clearance of pathological tau by restoring proteostasis activity, such as by enhancing the E3-ubiquitin ligase activity of LUBAC or by stabilizing M1-linked ubiquitin chains on ATG13 (44), a key regulator of autophagosome initiation. Alternatively, *OTULIN* inhibition may also likely downregulate the activation of certain tau kinases, such as CDK5, GSK-3 β , and MAPK, or any protein kinases that phosphorylate tau at S202 and T205 (AT8). In contrast to the effect of compound UC495 in sAD2.1 iPSNs, *OTULIN* inhibition with UC495 in SH-SY5Y WT cells significantly decreases *OTULIN* and total tau; consequently, phosphorylated tau was not detectable with AT8, AT180, and PHF-1 antibodies (Figure 3A and B). Although the effect of compound UC495 is inconsistent with cell types such as sAD2.1 iPSNs (1 μ M) and SH-SY5Y (2 μ M) most likely is due to concentration and cell type dependent, UC495 could be a potential therapeutic molecule (based on

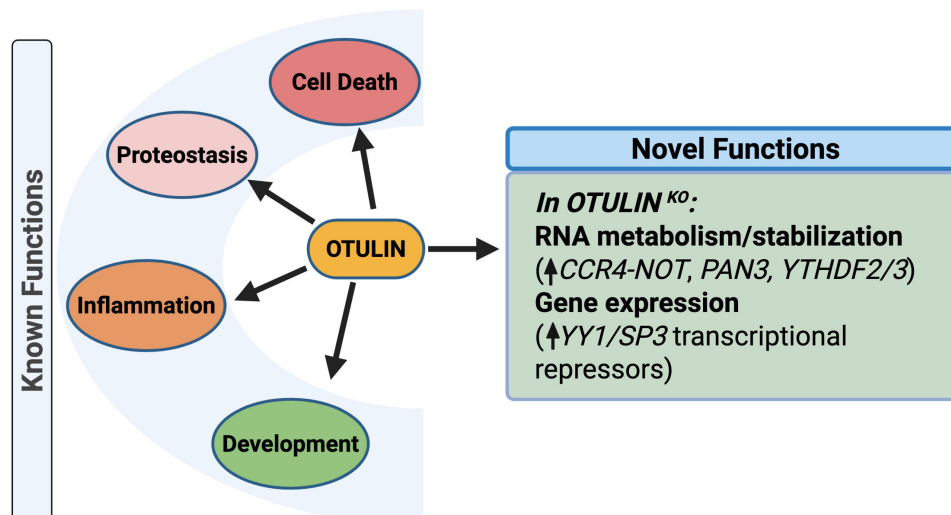


Figure 5. The working model shows the known and novel functions of OTULIN. OTULIN regulates proteostasis, cell death, inflammation, and cell survival/development. The novel non-canonical function identified in this study demonstrates OTULIN role as a regulator of RNA metabolism/stability and gene expression, including tau.

rather modulatory role than complete inhibition) that may alleviate tau pathology in tauopathy brains for future study.

To better understand the role of OTULIN in tau pathology, the *OTULIN* gene was knocked out in sAD2.1 iPSNs or the SH-SY5Y neuroblastoma line. We observed that the tau protein has completely blunted in its expression in both cell types with *OTULIN* KO (Figure 2F and G and Figure 3C and D). The overall morphology of sAD2.1 iPSNs was not affected in the absence of *OTULIN* (Supplementary Figure S8). However, it does exhibit morphological defects, including a lack of neurite-like projections, in the majority of SH-SY5Y *OTULIN* KO cells (Supplementary Figure S15). However, after 7–10 days in culture, the SH-SY5Y *OTULIN* KO cells were spontaneously differentiated into neuron-like cells without expressing tau (Supplementary Figures S15 and S16). We assumed that the loss of tau in SH-SY5Y *OTULIN* KO is due to enhanced proteostasis activity. We, therefore, used various proteostasis inhibitors with protein translation inhibitor, CHX, to understand the dynamics of tau degradation, including Lactacystin (20S proteasome), MG132 (26S proteasome), Bafilomycin-A1 (Baf-A1; autophagy), and PD 150606 (Calpains 1 and 2). Surprisingly, none of the proteostasis inhibitors stabilized tau (Figure 3D), which eventually motivated us to analyze *MAPT* mRNA levels by qRT-PCR, which suggests that *MAPT* mRNA has completely disappeared in the absence of *OTULIN* in SH-SY5Y line (Figure 3F). This finding further motivated us to perform bulk RNA sequencing to analyze whether the effects of *OTULIN* KO are specific to *MAPT* mRNA or dysregulated RNA metabolism globally.

Bulk RNA sequencing analysis revealed that SH-SY5Y *OTULIN* KO dysregulates RNA metabolism by downregulating the expression of 13,341 genes and 43,003 transcripts and upregulating 774 genes and 1113 transcripts, respectively, compared to SH-SY5Y WT (Figure 4A and B). It confirms that the effect of *OTULIN* KO is not specific to the downregulation of the *MAPT* gene. Still, it does globally and most likely function as a master regulator of RNA metabolism. However, the mechanism of *OTULIN* deficiency-associated dysregulated RNA metabolism is unknown. We, therefore, searched the possible candidates from our RNA sequencing data relevant to OTULIN-binding proteins, RNA metabolism associated with mRNA decay/degradation, mRNA stability, DNA transcription repressors, ubiquitination, and RNA-binding proteins. Primarily, we searched the differential expression of OTULIN-binding proteins obtained from the STRING protein-protein interactions database and identified that none of them were detectable at mRNA levels in our bulk RNA sequencing analysis, including the ubiquitin pathway-associated LUBAC components (*HOIP*, *HOIL-1*, *SHARPIN*), *VCP*, Ubiquitin B (*UBB*), and Ubiquitin C (*UBC*), and Neural Precursor Cell Expressed, Developmentally Down-Regulated 8 (*NEDD8*) (Supplementary Figure S14 and Supplementary Table S5).

Our RNA sequencing analysis showed that *OTULIN* KO in SH-SY5Y upregulated the expression of 774 genes and 1113 transcripts (Figure 4A and B), most likely required for cell survival under autoinflammation by suppressing gene transcription and accelerating RNA catabolism. We identified upregulation of ubiquitin-like modifier enzyme (*UBA3*), which is a component of NEDD8-activating enzyme 1 (NAE1), and its associated neddylation has been shown to be a regulator of neuronal aging and neurodegeneration in AD (53). Many ubiquitin-conjugating enzymes (UBE2s), including *UBE2B/D2/D3/E3/H/K/L3/L5/M/N/Q1/V2/W* were upregulated (Supplementary Table S6) among the approximately 40 E2 enzymes. However, many proteasome subunit genes were downregulated except proteasome activator subunit 3 (*PSME3*), proteasome 20S subunit beta 7 (*PSMB7*), proteasome 26S subunit, ATPase 6 (*PSMC6*), proteasome 26S subunit ubiquitin receptor, non-ATPase 2 (*PSMD2*), and proteasome 26S subunit, non-ATPase 14 (*PSMD14*) genes that were upregulated. Since more than 600 ubiquitin ligases (E3s) are involved in the ubiquitin-proteasome pathway, we searched for RNA-binding ubiquitin ligases (54). We identified that most of them are downregulated except Ring finger and CCH-type domains 2 (*RC3H2*) and Mex-3 RNA-binding family member C (*MEX3C*) (Supplementary Table S6), which are known to bind to 3 prime untranslated region (3'-UTR) of mRNAs, leading to deadenylation and degradation of mRNAs such as MHC-I mRNA (55). Interestingly, we identified that many mRNA stability regulator proteins encoding genes such as carbon catabolite repression 4-negative on TATA-less (*CCR4-NOT*) complex (*CNOT*) (56, 57), YTH N6-Methyladenosine RNA-binding protein F (*YTHDF1/2/3*), Poly(A) specific ribonuclease subunit 2 (*PAN2*), and *PAN3* were either upregulated or downregulated in *OTULIN* KO cells (Figure 5 and Supplementary Table S7) that might have regulated mRNA stability or degradation depending on the context. In addition to accelerating mRNA degradation/decay, *OTULIN* KO may suppress DNA transcription globally by upregulating the expression of transcription factors such as Yin Yang 1 (*YY1*) and Specificity protein 3 (*SP3*), which are known to function as suppressors of gene expression (Figure 5 and Supplementary Table S7). It is conceivable from this interpretation that either the gene expression suppressors or mRNA stability regulators might have played an important role in the loss of tau expression in SH-SY5Y *OTULIN* KO cells. *OTULIN* KO also upregulates many heterogeneous ribonucleoproteins (*HNRNPs*) and neurodegenerative disease-associated RNA-binding proteins such as Fragile X messenger ribonucleoprotein 1 (*FMR1*), TAR DNA-binding protein (*TARDBP/TDP-43*), Ataxin 2 (*ATXN2*), and Musashi RNA-binding protein 1 (*MSI1*) (Supplementary Table S7). Since pathological tau is an inflammatory protein, it is plausible that knocking out of *OTULIN* gene in SH-SY5Y may hyperactivate LUBAC and stabilize



M1-linked linear ubiquitin chains causing autoinflammation, which in turn may negatively regulate LUBAC activity by down-regulating its components in addition to many thousands of genes, including tau to prevent developing an autoinflammation in neurons for cell survival.

In conclusion, sAD2.1 iPSCs showed elevated OTULIN and hyperphosphorylated tau. Inhibiting OTULIN deubiquitinase activity with UC495 has moderately decreased tau levels, whereas *OTULIN* KO has completely abolished tau expression at mRNA levels which indicates a possible novel, noncanonical role for OTULIN in RNA metabolism or gene expression, including those of tau. Notably, inhibiting the deubiquitinase OTULIN with UC495 in SH-SY5Y also decreased the level of OTULIN along with total tau which implies loss of deubiquitinase activity might have promoted OTULIN degradation, and eventually reduction in tau levels. Currently, we do not know whether it is regulated at the transcriptional level or at the translational level (RNA metabolism). Our quantitative bulk RNA sequencing analysis of SH-SY5Y *OTULIN* KO revealed a widespread change in the transcriptome and promoted neurite-like outgrowth spontaneously. The newly identified noncanonical role of OTULIN in neurons might function as a master regulator of RNA metabolism and gene expression, besides its canonical regulatory role of M1-linked deubiquitination of its target proteins. Therefore, while OTULIN could serve a potential drug target for AD/DRD (Alzheimer's disease and related dementias), caution is warranted because of its pleiotropic role in the CNS.

Methods

Cell culture

The WTC11 iPSCs (58) were a kind gift from Dr. Li Gan and sAD2.1 iPSCs (46) were obtained from Coriell. Neural progenitor cells (NPCs) of WTC11 and sAD2.1 were initially cultured in STEMdiff forebrain neuron differentiation kit (STEMCELL Technologies # 08600) and then matured in BrainPhys neuronal medium with SM1 supplement (STEMCELL Technologies # 05792). HEK293T cells were cultured in DMEM (Thermo Fisher Scientific # 11965092) and supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific # 16140071) and 2 mM L-Glutamine (Thermo Fisher Scientific # 25030081). All the cell cultures were maintained at 37°C with 5% CO₂ and moisture. SH-SY5Y cells were grown in DMEM-F12 (1:1) (Thermo Fisher Scientific # 11320033) culture medium supplemented with 10% FBS and 1X concentration of penicillin-streptomycin (10,000 U/mL stock) (Thermo Fisher Scientific # 15140122) for UC495 treatment and *OTULIN* KO.

CRISPR-Cas9-*OTULIN* single-guide RNA cloning

Targeting *OTULIN* single-guide RNA (sgRNA) sequences were obtained from the Genome-scale CRISPR Knock-OUT v2 libraries released by Dr. Feng Zhang Laboratory (59). The oligo nucleotide sequence of the target gene for Homo sapiens *OTULIN* gene is 5'-CGAGCGACCGCATGAGTCGG-3' and its reverse complement oligo nucleotide pair was cloned into the LentiCRISPRv2 as described here briefly. Each pair of Oligos (100 μM) was mixed with T4 ligation buffer (NEB # B0202S; New England Biolabs) and nuclease-free ddH₂O at 37°C for 30 min followed by 95°C for 5 min and cooled down to room temperature naturally to form double-stranded DNA fragments. A total of 5 μg of LentiCRISPRv2 (Addgene # 52961) was digested with restriction enzyme BsmBI in FastDigest buffer at 37°C for 30 min. Agarose gel-purified vectors were ligated with the above annealed oligonucleotide duplexes using ligase (NEB # M2200S). The resulting lenti-sgOTULIN plasmids were transformed into competent DH5α cells, which were selected by ampicillin to collect the positive clones for purifying the plasmid DNA carrying CRISPR-Cas9-*OTULIN* sgRNA.

Lentivirus production

The day prior to lentiviral transduction of sgRNA of *OTULIN*, HEK293T cells were plated onto a 6-well plate at a 70% confluency. A total of 60 μL (6 μg) of the sgRNA to *OTULIN* (sgOTULIN) plasmid (32) was added to 30 μL (3 μg) of psPAX packaging plasmid DNA and 15 μL (1.5 μg) of pVSV-G envelope plasmid DNA in 345 μL of nuclease-free water with 50 μL of 2M CaCl₂. The DNA mix was then added dropwise to 500 μL of 2x HBS buffer with slow vortexing and incubated at room temperature for 30 min. Then, the DNA mix solution was added to HEK293T cells and incubated at 37°C for overnight in a cell culture incubator. The culture medium was

changed 24 h later. The conditioned medium was harvested 48 h later and passed through a 0.2-μm syringe filter. The lentivirus was then aliquoted and kept frozen at -80°C.

Lentivirus transduction of NPCs

Thawed Lentivirus (100 μL) was added to WTC11, and sAD2.1 NPCs were cultured in STEMdiff media onto a 6-well plate precoated with 200 μL Gel-trex (Thermo Fisher Scientific # A1569601). After overnight incubation, the lentivirus was aspirated and replaced with fresh growth medium. A 0.5 μg/mL of puromycin (Thermo Fisher Scientific # J67236.XF) was added 48 h later for selection. After reaching a 70% confluency with puromycin, cells were transferred to a 6-well plate precoated with 10 μg Poly-L-Ornithine (Sigma # P4957-50ML) and 15 μg Laminin (Sigma # L6274-5MG) and fed with BrainPhys neuronal medium (STEMCELL Technologies # 05790) for 20 days. Cells were then harvested and lysed in cold RIPA buffer (Thermo Fisher Scientific # 89901) with 1% Phenylmethylsulfonyl Fluoride (PMSF) (Sigma # 93482-50ML-F), 1X concentration of protease (Sigma # P8340-1ML) and phosphatase (Sigma # P2850-1ML) inhibitor cocktails. Lysates were spun down at 14,000 × g for 20 min at 4°C. Lysates were transferred to new collection tubes and stored at -80°C for further analysis.

Inhibition of deubiquitinase OTULIN with compound UC495

The compound UC495 was originally identified through a virtual screening effort to identify small molecules of OTULIN deubiquitinase inhibitor and subsequently synthesized UC495. The compound UC495 was reconstituted in DMSO to make stock solution and kept at -20°C freezer until further use. Differentiated and fully matured sAD2.1 iPSCs were treated with 1 μM of UC495 overnight in a cell culture incubator. SH-SY5Y cells were treated with 2 μM of UC495 overnight, and DMSO (vehicle) alone treated cells as a control experiment. After overnight treatment with UC495, cells were washed with 1X PBS (1 mL/washing, three times) and harvested for Western blotting.

Bulk RNA sequencing and bioinformatics analysis

RNA was extracted from healthy control WTC11 iPSC line- and sporadic sAD2.1 iPSC line-derived neurons, and human neuroblastoma SH-SY5Y WT as control and SH-SY5Y *OTULIN* KO, respectively. RNA was extracted with TRIzol reagent (Thermo Fisher Scientific # 15596026) and quantified by NanoDrop. A total of 2.0 mg of RNA was sent to LC Sciences and performed poly(A) RNA sequencing—sample quality control, library preparation, sequencing (150 bp PE, 6 GB data per sample), and analysis. *De Novo* assembly, alignment of RNA sequencing reads to reference genome, identification and construction of splice-junctions, report of known and novel transcripts with annotation and abundance, identification of alternate splicing and report of isoform abundance, test for differential expression at gene level and transcript level, GO and KEGG annotation and enrichment analysis GSEA were done by the LC Sciences. All raw RNA sequencing analysis data were deposited to NCBI Gene Expression Omnibus (GEO) with accession # GSE294290 (WTC11/sAD2.1 iPSCs) and # GSE294134 (SH-SY5Y WT/*OTULIN* KO).

Library construction and sequencing Poly(A) RNA sequencing library was prepared following Illumina's TruSeq-stranded-mRNA sample preparation protocol. RNA integrity was checked with Agilent Technologies 2100 Bioanalyzer. Poly(A) tail-containing mRNAs were purified using oligo-(dT) magnetic beads with two rounds of purification. After purification, poly(A) RNA was fragmented using divalent cation buffer in elevated temperature. The DNA library construction is shown in the following workflow. Quality control analysis and quantification of the sequencing library were performed using Agilent Technologies 2100 Bioanalyzer High Sensitivity DNA Chip. Paired-ended sequencing was performed on Illumina's NovaSeq 6000 sequencing system.

Transcripts assembly First, Cutadapt (60) and perl scripts in house were used to remove the reads that contained adaptor contamination, low quality bases and undetermined bases. Then sequence quality was verified using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). We used HISAT2 (61) to map reads to the genome of ftp://ftp.ensembl.org/pub/release-112/fasta/homo_sapiens/dna/. The mapped



reads of each sample were assembled using StringTie (62). Then, all transcriptomes were merged to reconstruct a comprehensive transcriptome using perl scripts and gffcompare. After the final transcriptome was generated, StringTie (63) and ballgown (<http://www.bioconductor.org/packages/release/bioc/html/ballgown.html>) was used to estimate the expression levels of all transcripts.

Different expression analysis of mRNAs: StringTie (63) was used to perform expression level for mRNAs by calculating FPKM (fragments per kilobase of transcript per million mapped fragments). The mRNAs differential expression analysis was performed by R package DESeq2 (63) between two different groups [and by R package edgeR (64) between two samples]. The mRNAs with the parameter of false discovery rate below 0.05 and absolute fold change ≥ 2 were considered differentially expressed mRNAs. The following bioinformatics software was used to analyze the bulk RNA sequences: FastQC version 0.10.1 (Quality control), Cutadapt version 1.10 (Adapter remove), HISAT version 2.0 (Mapping), StringTie version 1.3.4 (Transcripts assembly), DESeq2/edgeR (Differential expression analysis), Perl scripts in house (GO and KEGG enrichment analysis), samtools version 0.1.19 (SNP/Indel analysis), ANNOVAR version 2017.09 (SNP/Indel annotation), and rMATS version 4.1.1 (Alternative splicing).

Real-time quantitative reverse transcription PCR

A total of 1.0 μ g of purified RNA was diluted in RNase free water to 25 μ L as template for synthesizing complementary deoxyribonucleic acid (cDNA). The high capacity cDNA reverse transcription kit (Thermo Fisher Scientific # 4374966) was used to synthesize cDNA. A total of 25 μ L (1 μ g) of RNA was mixed with 25 μ L of reverse transcription components, including 10X reverse transcriptase (RT) buffer (5 μ L), 100 mM dNTP mix (2 μ L), 10X RT random primers (5 μ L), and MultiScribe RT (2.5 μ L). The reaction mixture was incubated in a PCR machine by programming the temperature condition at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. The resulting cDNA was used as a template to quantify the levels of target gene expression by the real-time qRT-PCR using Applied Biosystems StepOnePlus Real-Time PCR System with the software 2.3 version. A 25 μ L of real-time qRT-PCR mixture was prepared with 12.5 μ L TaqMan universal PCR mix, 1.25 μ L gene target primer, 2.5 μ L cDNA, and 8.75 μ L RNase free water. The reaction mixture in a 96-well plate was kept in the machine and the program was set to amplify and quantify the target gene at the specified temperature of 50°C for 2 min and 95°C for 10 min as predenaturation and followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The following TaqMan gene expression assay primers were used from Thermo Fisher Scientific: Homo sapiens *MAPT* (TaqMan assay ID: Hs009021944_m1) as target gene, and eukaryotic 18S rRNA as housekeeping gene (Catalog no. 4319413E; TaqMan assay ID: Hs99999901_s1).

Proteostasis inhibitors treatment and Western blotting

Human neuroblastoma SH-SY5Y WT/OTULIN KO cells (0.1×10^6) were seeded in a 12-well plate. After overnight culturing of cells, protein translation was inhibited with 50 μ M of CHX 1 h prior to adding the proteostasis inhibitors, 10 μ M MG132 (26S proteasome), 10 μ M Lactacystin (20S proteasome), 100 nM Bafilomycin A1 (Baf A1, autophagy), 100 μ M PD 150606 (calpain 1/2) either individually or all four inhibitors together for 4 h. All the inhibitors were reconstituted with DMSO to prepare stock solutions and stored at -20°C until further use. After treatment, cells were washed with 1X PBS three times and lysed with ice-cold RIPA buffer with PMSF, protease, and phosphatase inhibitor cocktails and incubated on a rocker for 30 min at 4°C. Then the cell lysates were briefly sonicated on ice 20 kHz frequency for 30 s and centrifuged at $14,000 \times g$ for 10 min at 4°C. The protein concentration was quantified with the bicinchoninic acid assay kit (Thermo Fisher Scientific 23227) and measured the resulting purple-color intensity at 562 nm wavelength using the TECAN multi-mode microplate reader. The resulting supernatant was stored at -80°C or heat-denatured with 1X LDS-sample buffer (Thermo Fisher Scientific # NP0007) at 95°C for 10 min. Prior to loading the denatured samples onto a gel for protein separation, samples were centrifuged to collect the supernatant at $14,000 \times g$ for 5 min at 4°C. Western blots band intensities were quantified using ImageJ software. The level of β -Actin was used as

a loading control to normalize the resulting Western blots of protein of interest. The ratio of protein interest/ β -Actin levels were analyzed statistically using the GraphPad Prism software.

Proteostasis inhibitors and antibodies

The following proteostasis inhibitors were procured from Cayman Chemical. Cycloheximide (Cayman # 26924), Lactacystin (Cayman # 70980), MG132 (Cayman # 13697), Baf A1 (Cayman # 11038), and PD 150606 (Cayman # 13859). The following antibodies were procured from various vendors and their dilution mentioned here. β -Actin, Rb mAb (1:1000; Cell Signaling Technology # 4970S), OTULIN, Rb pAb (1:1000; Cell Signaling Technology # 14127S), Ubiquitin, Mo mAb (1:1000; Enzo Life Sciences # ENZ-ABS840), Tau12, Mo mAb (1:2000; Sigma # MAB2241), AT8, Mo mAb (1:8000; Thermo Fisher Scientific # MN1020), AT180, Mo mAb (1:8000; Thermo Fisher Scientific # MN1040), PHF-1 (1:10,000, a kind gift from Dr. Peter Davies), and NeuN (1:1500; GeneTex # GTX638922). Abbreviations: Mo – mouse; Rb – rabbit; mAb – monoclonal antibody; pAb – polyclonal antibody.

Data availability

The RNA sequencing data are available at NCBI GEO, with accession # GSE294290 (WTC11/sAD2.1 iPSCs) and # GSE294134 (SH-SY5Y WT/OTULIN KO). All other data are available from the corresponding author and shared upon a reasonable request.

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Author contributions

KB and FFL developed the project, acquired funding, and designed the experiment as well as oversaw the entire study. KB supervised KT and VB while FFL supervised ML. KT designed and conducted most of the experiments, analyzed the data, and wrote the manuscript. VB standardized and performed CRISPR-Cas9 KO experiments on iPSCs and performed Western blot analyses. ML and WL generated the CRISPR-Cas9 against *OTULIN* and designed/characterized the UC495 compound. All authors take full responsibility for all data, figures, and text and approve the study's content and submission. No related work is under consideration elsewhere. All authors state that all unprocessed data are available, and all figures accurately present the original data. Corresponding authors: Professor KB for any aspect of the work except for UC495 and the CRISPR-Cas9 construct. Professor FFL for CRISPR-Cas9 construct and OTULIN antibodies. Professor WL for UC495. These corresponding authors take full responsibility for the submission process.

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Author disclosures

The author(s) have confirmed that no conflict of interest exists.

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