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Truncated tau disrupts autophagy and lysosomal biogenesis

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Abstract

The autophagy-lysosomal pathway plays a critical role in the clearance of tau protein aggregates that deposit in the brain in tauopathies, including Alzheimer's disease and defects in this system are associated with disease pathogenesis. Here, we report that expression of Tau35, a tauopathy-associated carboxy-terminal fragment of tau, reduces beclin-1 and microtubule-associated protein 1A/1B-light chain 3, indicating that Tau35 disrupts autophagy in cells. We demonstrate that Tau35 reduces autophagic flux by blocking activation of 5' AMP-activated protein kinase and activating mammalian target of rapamycin complex 1 (mTORC1), as seen by increased phosphorylation of S6 ribosomal protein and a reduction in phosphorylated raptor. Tau35 also induces neutral lipid accumulation in cells, indicating a block of autophagic clearance and a deficit in lysosomal degradative capacity. In support of this view, reductions in lysosomal-associated membrane protein 2 and cathepsin D in cells expressing Tau35 are accompanied by its increased colocalisation with lysosomes. These deleterious effects of Tau35 on autophagy are not apparent with full-length tau, indicating that sequences in the amino-terminal half of tau may be involved in the regulation of mTORC1 and autophagic activity. Notably, upon induction of autophagy by Torin 1, both Tau35 and full-length tau inhibited nuclear translocation of transcription factor EB (TFEB), a key regulator of lysosomal biogenesis. These findings implicate autophagic and lysosomal dysfunction as key pathological mechanisms through which abnormal tau could lead to the development and progression of tauopathy.

Keywords: Tau; dementia; Alzheimer's disease; autophagy; lysosomes; TFEB

Declarations

Not applicable.

Conflicts of interest

The Authors declare that they have no conflict of interest.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability

Not applicable.

Authors' Contributions

DD, TG and SP performed the experiments.

DPH, PGS and WN supervised the project.

GC provided expertise on quantitative microscopy.

DD, DPH and SP wrote the paper.

All authors read and approved the final manuscript.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Introduction

The tauopathies are a group of neurodegenerative diseases including Alzheimer's Disease (AD), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and some forms of frontotemporal dementia (FTD). Tauopathies are characterised by cognitive and motor dysfunction, combined with progressive deposition in the brain of pathological aggregates of abnormally phosphorylated and cleaved tau protein [1, 2]. The cause of this tau accumulation is unknown, but post-translational modifications [3] and/or impaired degradation of tau [4] have been suggested as potential mechanisms. Damage to these critical processes in neurons may result in both toxic gain-of-function acquired by aggregated tau, and loss of physiological tau function [5, 6], leading to neuronal damage.

Tau is degraded through the ubiquitin-proteasomal and autophagy-lysosomal pathways [7, 8]. Whilst the proteasome degrades soluble, monomeric proteins, the autophagy-lysosomal pathway is primarily responsible for the clearance of long-lived proteins, including aggregated tau species [9]. Autophagy is a highly conserved process, playing an essential role in regulating cell homeostasis and prolonging cell survival, through provision of energy and dietary components from recycling of intracellular proteins and organelles [10]. Autophagy is promoted by increased activity of 5' AMP-activated protein kinase (AMPK) via phosphorylation on Thr172 [11], whereas mammalian target of rapamycin complex 1 (mTORC1) negatively regulates autophagy through downstream effectors, including activation of ribosomal protein S6 kinase β-1 (S6K1) [12, 13]. mTORC1 also controls the nucleocytoplasmic shuttling of transcription factor EB (TFEB), which regulates expression of genes required for lysosomal biogenesis and autophagy [14, 15]. Inhibiting autophagy by overexpressing mTORC1 increases tau phosphorylation, enhances aggregation and cytotoxicity of mutant tau, and is detrimental to cell survival in the tauopathies [9, 16-18]. However, the effects of disease-associated tau fragments on these processes is unclear.

We previously identified a highly phosphorylated and aggregated carboxy-terminal fragment of tau (Tau35) in human tauopathy brain [19]. Minimal expression of Tau35 in transgenic mice induces key features of tauopathy, including deposition of highly phosphorylated and aggregated tau, progressive cognitive and motor deficits, autophagic/lysosomal dysfunction, and impaired synaptic plasticity [20, 21]. Recently, we showed that expression of Tau35 but not intact human tau, disrupts microtubule binding and insulin signalling in cells, and induces the unfolded protein response, all of which are features of

tauopathy [22]. However, the molecular mechanisms through which disease-associated tau species result in dysfunctional protein degradation in cells is unclear.

Here, we demonstrate that Tau35 expression disrupts basal autophagy and autophagic flux in cells, likely as a consequence of increased activation of mTORC1 and decreased AMPK activity. Furthermore, Tau35 expression reduces cellular protein degradative capacity and lysosomal protein expression. Notably, both Tau35 and intact tau disrupt nucleocytoplasmic shuttling of TFEB and blunt the cellular response to mTORC1 inhibition, suggesting new roles for tau in relation to lysosomal biogenesis and function.

Results

Tau35 impairs autophagy and leads to inefficient clearance of autophagosomes

We first confirmed expression of exogenous tau in stably transfected Chinese hamster ovary (CHO) cell lines. Western blots of lysates from CHO cells expressing 2N4R full-length tau (CHO-FL) or Tau35 (CHO-Tau35) were probed with antibodies to total and phosphorylated (PHF1) tau. The blots showed bands at the expected sizes of approximately 70 kDa and 35 kDa, corresponding to FL-tau and Tau35, respectively (Fig 1a), with both total and phosphorylated tau antibodies.

We next investigated the effect of Tau35 on initiation of autophagy and autophagosome formation in CHO cells. Initiation of autophagy is mediated by activation of the autophagy related 1 (Atg1)/Unc-51-like autophagy activating kinase (ULK1) complex and the beclin-1/phosphatidylinositol 3-kinase (PI3K) complex, which is required for autophagic vesicle formation [23]. Induction of autophagy also stimulates the conversion of microtubule-associated protein 1A/1B light chain 3B (LC3)-I to LC3-II, which localises to autophagosomal membranes and provides an estimate of the load of autophagosomes [24, 25]. Western blots of CHO cell lysates showed that the amount of beclin-1 relative to β-actin, was significantly reduced in CHO-Tau35 compared to CHO-FL and CHO cells (Fig 1b). Furthermore, Tau35 expression resulted in a significant reduction in LC3-II relative to β-actin, whereas LC3-II was increased by FL-tau expression (Fig 1c), suggesting that Tau35 might affect early autophagy and autophagosome formation. To confirm these changes in LC3, we quantified LC3 in CHO cells after standardising to cell area, which is important because CHO-Tau35 cells are significantly smaller than FL-tau and CHO cells [22]. In line

with the quantitation of LC3-II on western blots, Tau35 expression decreased LC3, whereas FL-tau increased LC3 fluorescence intensityin CHO cells (Fig 1d). However, antibody labelling of endogenous LC3 was somewhat diffuse and therefore, the CHO cells were transiently transfected with a plasmid expressing EGFP-LC3 to enable quantification of individual LC3-positive autophagosomes. There was a significant reduction in the number of GFP-positive autophagosomes in CHO-Tau35 cells, compared to untransfected CHO cells, whereas the number of autophagosomes was increased by FL-tau expression (Fig 1e). Taken together, this demonstrates that Tau35 reduces the autophagic markers LC3 and beclin-1, whereas FL-tau increases autophagosome formation.

The EGFP-LC3 construct used in this study is a useful marker of autophagosomes, but not for autolysosomes, due to quenching of GFP fluorescence in the acidic environments of amphisomes and autolysosomes. In order to discern whether the changes observed in basal levels of LC3 were due to reduced autophagosome, amphisome or autolysosome formation, we used a plasmid expressing mCherry-GFP-LC3 [26]. Since mCherry is acid-stable, yellow ($mCherry^{+}GFP^{+}$) autophagosomes can be distinguished from red ($mCherry^{+}GFP^{-}$) amphisomes and autolysosomes, with an increasing ratio of red:yellow puncta indicating transition from autophagosomes to amphisomes/autolysosomes (Fig 2a). The numbers of both autophagosomes and autolysoosmes were significantly reduced in CHO-Tau35 cells, but were unaffected in CHO-FL cells, compared to naïve CHO cells (Fig 2a, b). All three CHO cell lines harboured a larger number of puncta corresponding to amphisomes and autolysosomes, compared to autophagosomes, suggesting that the transition to autophagosomes is not impaired by Tau35 (Fig 2b). Notably, tau expression in CHO-FL cells resulted in increased sizes of individual autophagosomes and autolysosomes (Fig 2c). This increased size was particularly marked for autophagosomes, which were approximately twice the size of those in CHO cells (Fig 2c). In contrast, Tau35 expression resulted in smaller autophagosomes and autolysosomes, with the area of autophagosomes in particular, being reduced to approximately half the size of those in CHO cells (Fig 2c). Together, these results show that Tau35 suppresses the number and area of autophagosomes and amphisomes/autolysosomes, whereas intact tau does not affect the number, but instead induces enlargement of these structures. These findings imply that intact and truncated tau have differing effects on components of the autophagy pathway, which may impact autophagic flux under basal conditions.

To further assess autophagic flux in cells, we monitored LC3-II turnover in the presence and absence of bafilomycin A1 to inhibit vacuolar H+-ATPase, preventing acidification of endosomes and lysosomes, blocking autophagosome maturation and leading to LC3-II accumulation [27]. Changes in the amount of LC3-II following bafilomycin A1 reflect LC3 delivery to lysosomes for degradation and thereby, provide an indirect assessment of autophagic flux [25, 28]. Exposing CHO cells to bafilomycin A1 (300 nM for 2 h), increased LC3-II in all three cell lines (Fig 3a). The increase in LC3-II was significantly lower in bafilomycin-treated CHO-Tau35, compared to CHO-FL and CHO cells (Fig 3a). These results suggest that under basal conditions, expression of Tau35 damages but does not completely block, autophagosome synthesis.

We next investigated the response of Tau35-expressing cells to rapamycin-mediated activation of autophagy. Rapamycin inhbits mTORC1 by binding to FKBP12 prolyl isomerase [29] and inducing autophagy, increasing the production and turnover of LC3-II [30]. Treratment with rapamycin (1 μM for 6 h) markedly increased LC3-II in all three cell lines (Fig 3b). However, whereas LC3-II increased to a similar extent in CHO-FL and naïve CHO cells, the effect of rapamycin on LC3-II was significantly blunted in CHO-Tau35 cells (Fig 3b). These findings demonstrate that Tau35 expression reduces both basal and rapamycin-induced autophagy.

To complement these findings, we investigated the effect of Tau35 on the degradation of lipid droplets, which constitute the primary store for intracellular neutral lipids and are targeted for lysosomal degradation via selective autophagy [31, 32]. Moreover, neutral lipid accumulation has been observed in Alzheimer's disease brain and apolipoprotein ε4, a risk factor for Alzheimer's disease, reduces fatty acid sequestration into lipid droplets and fatty acid oxidation, leading to lipid accumulation [33-35]. Autophagic dysfunction has also been implicated in lysosomal storage disorders, in which lipid accumulation is a neuropathological characteristic [31, 36]. Lipophagy was monitored using BODIPY 493/503 to probe for neutral lipids [37]. Whilst all three CHO cell lines contained cytoplasmic BODIPY 493/503-positive lipid droplets, the appearance of the BODIPY-labelled structures varied between the cell lines. CHO cells exhibited a homogeneous distribution of small lipid droplets, whereas both CHO-FL and CHO-Tau35 cells harboured larger clusters of lipid droplets, predominantly in perinuclear regions, particularly in CHO-Tau35 cells (Fig 4). Quantification of the lipid droplets in each cell line

revealed a significant increase in the number of BODIPY 493/503-positive puncta in CHO-Tau35 cells compared to CHO-FL and naive CHO cells (Fig 4). These findings suggest that Tau35 compromises the ability of cells to efficiently degrade and mediate the turnover of lipids, indicating a deficit in the degradative capacity of lysosomes.

Tau35 expression leads to lysosomal deficits

To investigate the apparent suppression of autophagy and lysosomal degradation caused by Tau35, we examined the effects of tau expression on lysosomal function. LysoTracker Red was used to label acific organelles, including amphisomes, lysosomes, late-stage endosomes and peroxisomes. Quantitation of LysoTracker-positive puncta showed that CHO-FL cells harboured a significantly increased number of acidic structures, compared to the other cell lines (Fig 5a), which was in line with the increased puncta area occupied by amphisomes/autolysosomes shown in Fig. 2. In contrast, Tau35 expression resulted in fewer LysoTracker-positive puncta compared to CHO cells.

We next examined the lysosomal proteins, lysosome-associated membrane protein 2 (LAMP2) and cathepsin D, in CHO cells. LAMP2 and cathepsin D puncta exhibited similarly diffuse distributions in all three CHO cell lines, suggesting that FL-tau and Tau35 do not affect lysosomal distribution (Fig 5b). Notably, however, we identified significant reductions of approximately three-fold, in the numbers of LAMP2 and cathepsin D puncta in CHO-Tau35 cells, but not in CHO-FL cells (Fig 5b). These findings suggest that Tau35 expression reduces the number of lysosomes, indicating a potential disruption in lysosomal biogenesis.

Increased association of tau with LAMP2-labelled lysosomes in Tau35 cells

Highly phosphorylated and aggregated tau is degraded by the autophagy-lysosome pathway [9, 38, 39] and increased phosphorylation of tau provokes lysosomal dysfunction [40]. Given that Tau35 is highly phosphorylated in Tau35 transgenic mice [21] and in CHO cells (Fig. 1) [22] and that its expression results in reductions in LAMP2 and cathepsin D, we assessed the degree of colocalisation of LAMP2 with FL-tau and Tau35 in CHO cells (Fig 5c). We found that, despite LAMP2 being markedly reduced in CHO-Tau35 cells, it colocalised to a greater extent with Tau35 than with FL-tau (Fig 5c, Pearson's correlation coefficients of 0.64 +/- 0.01 and 0.35 +/- 0.03, respectively, $P<0.0001$). These results

demonstrate that Tau35 has a higher propensity to associate with lysosomes compared to FL-tau, which might be an attempt by the cells to degrade this more highly phosphorylated form of tau.

Tau induces deficits in TFEB expression at the transcriptional level

Given the reduction in lysosomes and autophagic structures observed in CHO-Tau35 cells, we investigated the possibility of a disruption in TFEB, since it controls the expression of genes regulating lysosomal and autophagosomal biogenesis. We performed RT-qPCR to determine the relative expression of TFEB and TFEB-regulated genes, including *Lamp1, Lamp2,* and cathepsin D (*Ctsd*) in the three CHO cell lines. Tau35 and FL-tau significantly reduced *Tfeb* expression (Fig 6a). Tau35 also induced a corresponding decrease in expression of *Lamp1* but the modest reductions in *Lamp2* and *Ctsd* did not reach significance. FL-tau expression did not significantly affect the expression of *Lamp1*, *Lamp2* or *Ctsd*. These results provide evidence of a reduction in *TFEB* transcription as a possible explanation for the decrease in lysosomes induced by Tau35.

We next investigated whether the tau-induced reduction in *Tfeb* translates to a decrease in TFEB protein. Endogenous TFEB migrated as a doublet of 60-75 kDa on western blots in all three CHO cell lines (Fig 6b, arrows) but the modest reductions apparent in total TFEB in the presence of either FL-tau or Tau35 did not reach significance. Immunofluorescence labelling of endogenous TFEB demonstrated a predominantly cytoplasmic localisation, with approximately 40% of cells harbouring nuclear TFEB, which unaffected by tau expression (Fig 6c). Dephosphorylated TFEB translocates to the nucleus to regulate the coordinated lysosomal expression and regulation (CLEAR) gene network [41, 42]. To investigate the effect of tau on TFEB localisation, nuclear localisation was quantified in the three CHO cell lines transfected with a plasmid expressing TFEB-EGFP. Under basal conditions, the proportion of transfected cells exhibiting exogenous nuclear TFEB-EGFP was approximately 75% in CHO cells and this was significantly reduced by Tau35, but not by FL-tau expression (Fig 6d, $P<0.01$).

TFEB translocation to the nucleus is, in part, mediated by inhibition of the mTOR pathway, and inhibitors of mTORC1, such as Torin 1, activate TFEB [41-43]. Therefore, we investigated nuclear-cytoplasmic TFEB translocation in fractions of the three cell lines treated with Torin 1 (1 μM for 2h) (Fig 6e). Western blots showed that endogenous TFEB is predominantly cytoplasmic under basal conditions and that Torin

1 induced translocation of TFEB to the nucleus in all cell lines (Fig 6d). However the Torin 1-induced increase in nuclear TFEB was markedly attenuated in the presence of tau (Fig 6e). Thus, Torin 1 induced an 8-fold increase in the nuclear-cytoplasmic ratio of TFEB in CHO cells, but only a 4-fold increase in CHO-FL and CHO-Tau35 cells. These findings demonstrate that, expression of either FL-tau or Tau35 represses the ability of Torin 1 to stimulate TFEB translocation to the nucleus upon induction of autophagy.

Tau35 expression disrupts mTOR signalling

Since the mTORC1 complex regulates TFEB subcellular localisation [12, 41] and we showed previously that Tau35 expression activates mTORC1 and its downstream effector S6K1 [22], we next investigated the effects of Tau35 on regulators of mTOR signalling. CHO-Tau35, but not FL-tau cells, exhibited a two-fold increase in Ser240/244 phosphorylation of S6 ribosomal protein, an S6K1 substrate (Fig 7a, $P<0.05$). The mTORC1 complex includes regulatory-associated protein of mTOR (raptor), which is an important scaffolding protein that regulates mTORC1 subcellular localisation and mTOR signalling [44]. Therefore, we determined the phosphorylation status of raptor at Ser792 and found that Tau35 expression significantly inhibited raptor phosphorylation, indicating a lack of repression of mTORC1 by raptor, and potentially inhibition of autophagy (Fig 7b). In contrast, expression of FL-tau did not affect raptor phosphorylation, which was in line with the lack of effect of FL-tau on S6 phosphorylation. Under nutrient-deficient conditions, AMPK, a key cellular energy sensor, phosphorylates raptor at Ser722 and Ser792 [45], resulting in functional inhibition of mTORC1 and activation of autophagy [46]. AMPK activation, assessed by its phosphorylation at Thr172 [47], was markedly reduced by Tau35 expression ($P<0.05$), whereas FL-tau expression did not affect AMPK phosphorylation (Fig 7c). Taken together, our findings indicate that Tau35 expression in CHO cells induces mTORC1 activity through a reduction in AMPK activity, which decreases raptor-mediated inhibition of mTORC1, and increases S6K1 activation. These effects were not apparent in CHO-FL cells, indicating that the N-terminal half of tau may have a regulatory role on mTORC1 activity.

Discussion

Dysfunction of the autophagy-lysosomal pathway has been implicated in the pathogenesis of AD and other neurodegenerative diseases [48, 49]. Autophagic-lysosomal abnormalities are apparent in post-

mortem tauopathy brain, tau transgenic mice and cultured cells expressing tau [40, 50-52]. However, the mechanisms by which disease-associated tau affects autophagy in AD and related tauopathies is unclear. We have previously shown in mouse and cell models of tauopathy that mTORC1 is abnormally activated, suggesting a disruption in autophagy [21, 22]. To elucidate the effects of this form of truncated, disease-associated tau on the autophagy pathway, we used a CHO cell model in which Tau35 is stably expressed. Our data show that whilst both FL-tau and Tau35 affect TFEB expression, only Tau35 disrupts autophagic-lysosomal degradation and mTORC1 signalling. A comparative summary of the differing effects of Tau35 and FL-tau on autophagy and mTORC1 signaling is shown in Table 2.

The reductions in beclin-1 and LC3-II in the presence of Tau35 indicate a dysfunction in early autophagy/phagophore formation and in the number of autophagosomes. Several studies have implicated decreased beclin-1 in the pathogenesis of AD [53-55], possibly due to proteolysis [56]. Neuronal expression of beclin-1 gradually reduces during ageing but the rate of decline is enhanced in brains affected by AD [53, 55]. The decrease in beclin-1 caused by Tau35 is a likely consequence of reduced AMPK activity in CHO-Tau35 cells, because AMPK mediates its pro-autophagic effects through beclin-1 [57]. A deficit in autophagy caused by Tau35 would lead to a reduction in autophagosome formation, which is supported by the decrease in LC3-II. Since this deficit was not rescued by inhibition of late-phase autophagy using bafilomycin A1, this indicates that CHO-Tau35 cells have a reduced capacity to form autophagosomes. A disruption of autophagy by Tau35 is also supported by the accumulation of lipid droplets in CHO-Tau35 cells, suggesting inefficient lipid metabolism, similar to that found in Niemann-Pick Type C disease, in which tangles comprised of phosphorylated tau are a characteristic neuropathological feature [58]. Since we did not observe autophagosome accumulation with Tau35, autophagy could be disrupted by deficits in the numbers of autophagosomes, autolysosomes and/or lysosomes. Indeed, the reduced numbers of structures labelled by cathepsin D in CHO-Tau35 cells is consistent with the reduction in cathepsin D apparent in Tau35 mice [21]. Brains from frontotemporal lobar degeneration with abnormal tau pathology, caused by mutations in the tau-encoding *MAPT* gene, also exhibit reduced amounts of the lysosomal membrane protein LAMP1, suggesting a lysosomal deficit in this tauopathy [59]. Furthermore, complete loss of cathepsin D in mice results in the lysosomal neurodegenerative disorder, neuronal ceroid lipofuscinosis [60, 61], which is also characterised by impaired lipid metabolism [62]. We have shown previously that Tau35 expression

results in destabilisation of microtubules in CHO cells [22]. Given the importance of microtubule dynamics for autophagy [63] and that dissociation of tau from microtubules interferes with the retrograde transport of autophagosomes to lysosomes [64], it is possible that Tau35 might also impact the transport and delivery of dysfunctional proteins for degradation by this route.

We have previously demonstrated a build-up of phosphorylated tau at numerous disease-relevant epitopes in CHO-Tau35 cells [22]. The marked increase in colocalisation of LAMP2 puncta with Tau35 compared to FL-tau supports the observation that more highly phosphorylated forms of tau, such as Tau35 are substrates for autophagy, further differentiating the detrimental effects of this disease-associated tau fragment from intact human tau. These observations are not surprising because autophagy has been shown to be a primary route of clearance of phosphorylated tau [9, 51]. On the other hand, proteasomal activity appears to be reduced in AD brain, and insoluble tau isolated from human AD brain is not degraded by the proteasome, but instead has been demonstrated to inhibit the proteasome [65, 66].

It is possible that the reduction in lysosomal proteins observed in Tau35 cells is due to a reduced ability of TFEB to activate the co-ordinated lysosomal expression and regulation (CLEAR) network. TFEB is considered to be the master regulator of autophagy, as it influences expression of genes encoding proteins of the autophagy-lysosome pathway and controls lysosome biogenesis [14]. Furthermore, nuclear localisation of TFEB can be altered to match the cellular demand for lysosome biogenesis and autophagosome-lysosome function [42]. Interestingly, we found that expression of either FL-tau or Tau35 in CHO cells led to a reduction in *TFEB* mRNA but that only Tau35 reduced expression of the lysosomal gene, *Lamp1*. A reduction in TFEB has previously been observed in both aged [67] and AD brains, suggesting the possibility that TFEB activation might be a beneficial therapeutic approach [68]. In support of this view, accumulating evidence suggests that promoting autophagy through TFEB activation, enhances clearance of phosphorylated tau and rescues neurotoxicity in mouse models of tauopathy [17, 69, 70].

Tau expression also impeded Torin 1-induced nuclear translocation of TFEB, suggesting that tau species have a negative impact on nucleocytoplasmic transport of TFEB. However, the enhanced

activation of mTORC1 and decreased AMPK activity caused by Tau35 may exacerbate its effect on the autophagy-lysosomal pathway in comparison with FL-tau. Recently, AMPK has also been shown to be required for increased TFEB activity in mouse embryonic fibroblasts [71] and mouse embryonic stem cells (ESCs) [72]. AMPK-deficient ESCs also exhibit reduced levels of TFEB leading to reduced endolysosomal activity [72]. These frindings suggest that metabolic state and energy sensing via AMPK activity might be involved in the development or progression of tauopathy.

We found that Tau35 expression is sufficient to induce mTORC1 activity through reductions in AMPK activation and raptor inhibition, leading to increased activity of S6K1. Enhanced mTOR signalling has been implicated in AD pathogenesis and evidence from post-mortem human AD brain indicates that both phospho-mTOR and its downstream target p70S6K, are increased in AD compared to age-matched control cases, suggesting elevated mTOR activity in AD brain [73, 74]. Other studies have shown that mTOR activity is implicated in tau-mediated pathogenesis in AD and related tauopathies since mTORC1 induces tau expression, promotes glycogen synthase kinase (GSK)-3β-dependent tau phosphorylation [38] and enhances tau pathology [69, 75]. Therefore, the increased mTORC1 activity observed in this study is likely a consequence of increased GSK3β activity and increased tau phosphorylation, as we observed previously in CHO-Tau35 cells [22].

The direct effect of tau expression on TFEB has not been investigated previously, although TFEB has been shown to play a role in the selective lysosomal-mediated exocytosis of truncated mutant tau, without affecting secretion of endogenous wild-type tau [76]. There is increasing evidence for nucleocytoplasmic transport of proteins and RNA being disrupted in neurodegenerative disease, including for example, C9orf72-mediated amyotrophic lateral sclerosis and FTD [77]. Impaired nuclear transport has also been observed in cells positive for phosphorylated tau in AD brain, as well as in cell and mouse models of tauopathy, and the C-terminus of tau interacts with the nucleoporin, Nup98, *in vitro* [78]. Therefore, it is possible that the dysfunction in TFEB translocation we observed with Tau35 may be extended to the trafficking of other proteins and RNA between the cytoplasm and nucleus, although this remains to be investigated.

The findings from this study indicate that expression of Tau35 in CHO cells leads to dysfunction of the autophagy and lysosomal pathways. Increased mTORC1 signalling, reduced AMPK activity and increased cytoplasmic retention of endogenous TFEB are possible underlying mechanisms leading to the disruption in autophagy caused by this form of truncated tau. Together, these findings provide important molecular links between protein degradation and disease pathogenesis in thetauopathies.

Experimental Procedures

Generation and culture of stable CHO cell lines

Chinese hamster ovary (CHO) cells stably expressing either FL-tau (FL-CHO, expressing 2N4R tau isoform, 441 amino acids) or Tau35 (Tau35-CHO, expressing truncated 4R tau isoform, residues 187-441), and untransfected CHO cells were generated as described previously [22]. Cells were maintained as monolayer cultures in Ham's F-12 medium (Gibco™) containing foetal bovine serum (PAA Laboratories Ltd, UK) at 37°C in an atmosphere of 5% CO_2 and passaged at 80-90% confluence. For biochemical analyses, CHO cells were plated at 3 x 10^5 cells per well of a 6-well plate or 2 x 10^6 cells per 100mm dish for 24 h. For immunofluorescence, CHO cells were plated on 18 mm coverslips at 1 x 10^5 cells per well of a 12-well plate for 24 h.

Plasmids and CHO cell transfection

The mammalian expression plasmid encoding human transcription factor EB (TFEB), pEGFP-N1-TFEB, was a gift from Shawn Ferguson (Addgene plasmid #38119; http://n2t.net/addgene:38119; RRID:Addgene_38119) [42]. CHO cells were transfected using JetPEI® (Polyplus Transfection, Illkirch-Graffenstaden, France) with 1 μg DNA/well of a 12-well plate, according to the manufacturer's instructions.

The EGFP-LC3 plasmid was a gift from Karla Kirkegaard (Addgene plasmid #11546; http://n2t.net/addgene:11546; RRID:Addgene_11546) [79]. CHO cells were transfected using Lipofectamine™ 2000 (Thermo Fisher Scientific, Waltham, MA, USA) with 0.5 μg DNA/well of a 12-well plate, according to the manufacturer's instructions.

mCherry-GFP-LC3 lentivirus production and transduction

The FUW-mCherry-GFP-LC3 plasmid was a gift from Anne Brunet (Addgene plasmid #110060; http://n2t.net/addgene:110060; RRID:Addgene_110060) [26]. FUW-mCherry-GFP-LC3 lentivirus was produced by transfection of the plasmid (21 μg DNA) in HEK293T cells with 30 μl TransIT® lentivirus packaging mix (Mirius, Cambridge Bioscience) using Lipofectamine™ 2000 (Thermo Fisher Scientific, Waltham, MA, USA) in a T-175 flask. Supernatant was collected 48 h post-transfection, filtered (45 μm), concentrated using a Lenti-X™ Concentrator (Takara Bio Inc, Kusatsu, Shiga, Japan), and strored at -80°C. For lentiviral infection, 24h after plating, CHO cells (0.05 x 10^6 cells per well of a 24-well plate) were transduced with lentiviral particles at an MOI of 5, for 72 h. After replacing the medium, cells were fixed with 4% (w/v) paraformaldehyde (PFA) in PBS for 15 min. Nuclei were counterstained using 2.5 ng/ml Hoechst 33342 (Sigma Aldrich, St. Louis, MO, USA) in PBS and coverslips were mounted onto slides using fluorescence mounting medium (DAKO).

CHO cell treatments

24 h after seeding, CHO cells were treated with either 1 μM rapamycin (Sigma-Aldrich, St. Louis, MO, USA) for 6 h, to activate mTOR-dependent autophagy, or 300 nM bafilomycin A1 (Sigma-Aldrich, St. Louis, MO, USA) for 2 h, to inhibit v-ATPase and block autophagy. For TFEB localisation, CHO cells were treated with 1 μM Torin 1 (Tocris Bioscience, Bristol, UK) to activate mTOR-dependent autophagy and nuclear TFEB translocation or vehicle (0.1% [v/v] dimethyl sulfoxide [DMSO]) in culture medium for 2 h.

CHO cell lysis and fractionation

Cells were washed three times with ice-cold phosphate-buffered saline (PBS) and scraped into 2x or 4x Laemmli sample buffer containing PhosSTOP™ (Sigma-Aldrich, St. Louis, MO, USA) and cOmplete™ ethylenediamine tetraacetic acid (EDTA)-free protease inhibitor cocktail (Roche, Basel, Switzerland). For analysis of total TFEB, cells were lysed in 100 μl radio-immunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) (25 mM Tris-HCl, pH7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors, for 15 min on ice. Cell lysates were centrifuged at 16,000g for 15 min at 4°C. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and 20 μg protein

was loaded onto western blots. Nuclear and cytoplasmic fractions were prepared as described [80]. Briefly, following two washes in ice-cold PBS, cells were lysed in 500 µl lysis buffer per 100mm dish (50 mM Tris-HCl, pH7.5, containing 0.5% (w/v) Triton X-100, 137.5 mM NaCl, 10% (v/v) glycerol, 5mM EDTA, PhosSTOP™ (Sigma-Aldrich, St. Louis, MO, USA) and cOmplete™ EDTA-free protease inhibitor (Roche, Basel, Switzerland) for 15 min on ice. Cell lysates were centrifuged at 2500g for 15 min at 4°C and 250 µl supernatant was retained (cytosolic and membrane fraction). The pellet was washed with 500 µl lysis buffer and resuspended in 100 µl lysis buffer containing 0.5% (w/v) SDS, then sonicated for 3x3 s at 10% amplitude (VC 130 Vibra-Cell™ Ultrasonic Processor) at 4°C. The sonicated lysate was centrifuged at 16,000g for 15 min at 4°C and the supernatant was retained (nuclear fraction) and analysed on western blots.

Western blots

Cell lysates were separated by SDS-polyacrylamide gel electrophoresis using 4-12% pre-cast polyacrylamide gels (Invitrogen, Carlsbad, CA, USA), to detect S6, raptor and AMPK proteins, or 10% (w/v) acrylamide gels, to detect beclin-1 and TFEB, or 12.5% (w/v) acrylamide gels to detect LC3. Separated proteins were transferred onto 0.45 µm nitrocellulose membranes (GE Healthcare, Chicago, IL, USA), blocked in Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE, USA) or 3% (w/v) dried skimmed milk in Tris-buffered saline containing 0.2% (v/v) Tween 20 for 1 h at ambient temperature, then incubated with primary antibody (Table 1) overnight at 4°C. After washing, membranes were incubated with the appropriate secondary antibody (Table 1) for 1 h at ambient temperature. Antigens were visualised using an Odyssey® infrared imaging system (Li-Cor Biosciences, Lincoln, NE, USA) and analyzed using using Fiji-ImageJ (https://imagej.net/Fiji) [81]. Unless otherwise stated, quantification of blots included 3 technical replicates from 3 independent experiments.

RNA extraction and reverse transcription quantitative PCR

For RT-qPCR, cells were seeded at 100,000 cells/well in 6-well plates and incubated for 48 h. Total RNA was extracted using TRIzol™ reagent (Sigma Aldrich, St. Louis, MO, USA), following the manufacturer's protocol. Isolated RNA quantity and quality was assessed from 260/280nm and 260/230 absorbance (NanoDrop 2000,Thermo Fisher Scientific, Waltham, MA, USA) and 1 µg RNA was reverse transcribed using the Maxima H Minus First Strand cDNA Synthesis Kit, with dsDNase (Thermo Fisher

Scientific). RT-qPCR was carried out using the QuantStudio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific) using 10x diluted cDNA and 500nM primers (Table 2) in SYBR™ Green PCR Master Mix (Thermo Fisher Scientific) in a 96-well plate. Samples were heated for 2 min at 95°C and amplified using 40 cycles of 95°C for 15 s, 60°C for 15 s, and 70°C for 1 min. Each sample was run in triplicate and the threshold cycle (Ct) value for each gene was used to calculate the difference between the Ct of the target gene and the Ct of the housekeeping genes *Actb* and *Gapdh* using ΔΔCt. The relative mRNA expression in FL-tau and Tau35 cells was normalised to untransfected CHO cells.

Immunofluorescence labelling of CHO cells

For LC3 antibody labelling, CHO cells were fixed and permeabilised in ice-cold methanol for 5 min at -20°C, then blocked in 2% (w/v) bovine serum albumin (BSA) in PBS overnight at 4°C. Cells were incubated with LC3 antibody diluted in BSA blocking solution for 1 hour at ambient temperature, washed and incubated with secondary antibody Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) for 30 min at ambient temperature. For immunofluorescence labelling with other antibodies, CHO cells were fixed in 4% (w/v) paraformaldehyde (PFA) in PBS for 20 min at ambient temperature, then permeabilised in 0.5% (w/v) Triton X-100 in PBS (3 x 5 min) and blocked in 10% (v/v) goat serum (Sigma Aldrich, St. Louis, MO, USA) in 0.5% (w/v) Triton-X100 in PBS for 30-60 min at ambient temperature. Cells were incubated in primary antibodies diluted in blocking buffer overnight at 4°C. After washing with 0.5% (w/v) Triton X-100 in PBS, cells were incubated with the appropriate secondary antibody diluted in 0.5% (w/v) Triton X-100 in PBS for 1 h at ambient temperature. Nuclei were counterstained using 2.5 ng/ml Hoechst 33342 (Sigma Aldrich, St. Louis, MO, USA) in PBS and coverslips were mounted onto slides using fluorescence mounting medium (DAKO).

Staining of neutral lipids in CHO cells

To label intracellular lipids, CHO cells were incubated with 2 µM boron-dipyrromethene 493/503 (BODIPY, Invitrogen, Carlsbad, CA, USA) for 15 min at 37°C, washed in PBS and fixed with 4% (w/v) PFA in PBS for 30 min at ambient temperature. Nuclei were stained with 2.5 ng/ml Hoechst 33342 and cells were mounted as above.

Staining of acidic organelles in CHO cells

To label acidic organelles, CHO cells were incubated with 100 nM LysoTracker™ Red DND-99 (Invitrogen™, Thermo Fisher Scientific) diluted in Ham's F-12 medium for 1 h. Cells were fixed with 4% (w/v) PFA in PBS for 15 min at ambient temperature and nuclei were stained with 2.5 ng/ml Hoechst 33342 before mounting on glass slides.

Acquisition of confocal and super-resolution fluorescent images

Images were acquired using a Leica TCS-SP5 confocal microscope with a 63X HCX PL APO lambda blue CS 1.4 oil UV objective (Leica Microsystems). Images were collected using single excitation for each wavelength sequentially using a 405 nm laser and a 425–475 nm emission band pass for Hoechst; 488 nm Argon laser line and a 500–550 nm emission for BODIPY (Fig. 4A) and LAMP2 (Fig. 5B); 561 nm laser line and a 575–650 nm emission band pass for LC3 (Fig. 1D) and Tau (Fig. 5C).

Images in Fig 1D and E, Fig. 2 and Fig. 5A were captured using a Nikon Inverted A1R confocal microscope with a 60X oil objective. Images were collected at each wavelength sequentially, using a 405 nm laser for Hoechst; 488 nm Argon Laser line for EGFP-LC3 (Fig. 1E) and FUW-GFP-LC3 (Fig. 2); 561 nm Laser line for LC3 (Fig. 1D), FUW-mCherry-LC3 and LysoTracker (Fig. 5).

Images in Fig. 5B were captured using a Nikon Spinning disk confocal system with a CSU-X1 scanning head (Yokogawa - Japan) and a Nikon Eclipse Ti-E inverted microscope coupled with a 60xCFI Apo/1.4NA objective and a Du 897 iXon Ultra EMCCD camera (Andor - Oxford Instruments). Laser Illumination was supplied from a Nikon LU-NV emitting 405nm, 488nm and 561nm wavelengths. Light was collected through emission filters for DAPI (Chroma ET460/50m), LAMP2 (Chroma AT535/40m) and cathepsin D (Chroma ET645/75m). Acquisition was controlled and data stored using NIS-Elements v5.0 (Nikon).

Fluorescent images in Fig. 6C were collected using a Nikon Eclipse Ti-E microscope equipped with Intenslight C-HGFI light source (Nikon), CFI Apo Lambda S 60x/1.40 objective (Nikon) and an Andor Neo scientific complementary metal-oxide-semiconductor camera (Andor – Oxford Instruments). Acquisition was controlled and data stored using NIS-Elements v5.0 (Nikon). Excitation and emission

filter sets were used for DAPI (Chroma Technology - 49000) and DsRed (Chroma Technology - 49005) filter sets for Hoecsht and TFEB fluorescence collection, respectively.

Quantitative analysis of fluorescent images

Intracellular puncta (EGFP-LC3, BODIPY 493/503, LAMP2 and cathepsin D) were analysed using the GFP-LC3 macro (NIH) in Fiji-ImageJ, which records puncta number, area and size based on manually thresholding. The numbers of puncta were standardised to the area of each cell . Signal intensities were determined using Fiji-ImageJ (https://imagej.nih.gov/ij/). Intracellular mCherry-GFP-LC3 puncta were analysed using the General Analysis function on NIS-Elements software (Nikon, RRID:SCR_014329). Briefly, $mCherry^+$ and GFP^+ puncta were thresholded and quantified using the spot detection algorithm. The overlap of mCherry mask with GFP mask was determined to calculate the number and size of $mCherry^+GFP^+$ puncta and $mCherry^+GFP^-$ puncta as autophagosomes and autolysosomes, respectively. LAMP2/tau colocalisation was performed using NIS-Elements software to obtain Pearson's correlation coefficient.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Prior to conducting statistical analyses, the assumptions of normality and homogeneity of variance were assessed using the Shapiro-Wilk test and the Brown-Forsythe test, respectively. If the data were not normally distributed, a Kruskal-Wallis test was performed. If the variances were significantly different, a Welch ANOVA test was performed.

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Table 1: Antibodies used in this study

Primary antibodies			
Antibody	**Species (clone)**	**Dilution**	**Source (Catalogue #)**
β-Actin	Mouse monoclonal (AC74)	WB: 1/5,000	Sigma Aldrich (A2228)
AMPK	Rabbit polyclonal	WB: 1/1,000	Cell Signaling Technology (2532)
Phospho-AMPKα (Thr172)	Rabbit monoclonal (40H9)	WB: 1/1,000	Cell Signaling Technology (2535)
Beclin-1	Rabbit polyclonal (D40C5)	WB: 1/500	Cell Signaling Technology (3495)
Cathepsin D	Goat polyclonal (C-20)	ICC: 1/100	Santa Cruz Biotechnology (sc-6486)
GAPDH	Mouse monoclonal (6C5)	WB: 1/5,000	Santa Cruz Biotechnology (sc-32233)
Histone H3	Rabbit polyclonal	WB: 1/5,000	Cell Signaling Technology (9715)
LAMP2	Mouse monoclonal (H4B4)	ICC: 1/50	Santa Cruz Biotechnology (sc-18822)
LC3B	Rabbit polyclonal	WB: 1/1,000 ICC: 1/200	Sigma Aldrich (L7543)
Raptor	Rabbit polyclonal (24C12)	WB: 1/1,000	Cell Signaling Technology (2280)
Phospho-raptor (Ser792)	Rabbit polyclonal	WB: 1/1,000	Cell Signaling Technology (2083)
S6	Mouse monoclonal (54D2)	WB: 1/1,000	Cell Signaling Technology (2317)
Phospho-S6 (Ser240/244)	Rabbit polyclonal	WB: 1/500	Cell Signaling Technology (2215)
Tau	Rabbit polyclonal (K9JA)	ICC: 1/1,000	Agilent (DAKO, A0024)

PHF-1 (Tau pSer396/404)	Mouse monoclonal	WB: 1/1,000	Gift from Professor Peter Davies
TFEB	Rabbit polyclonal	WB: 1/2,000 ICC: 1/400	Bethyl Laboratories (A303-673A)
Secondary antibodies			
Antibody label	**Species**	**Dilution**	**Source (Catalogue #)**
Alexa Fluor® 488	Donkey, anti-goat	ICC: 1/1,000	Invitrogen (A-11055)
Alexa Fluor® 488	Goat, anti-mouse	ICC: 1/1,000	Invitrogen (A-11001)
Alexa Fluor® 568	Goat, anti-rabbit	ICC: 1/1,000	Invitrogen (A-11011)
Alexa Fluor® 680	Goat, anti-mouse	WB: 1/5,000	Invitrogen (A-21057)
IRDye® 800CW	Goat, anti-rabbit	WB: 1/5,000	Li-Cor Biosciences (926-32211)
IRDye® 680RD	Goat, anti-mouse	WB: 1/5000	Li-Cor Biosciences (926-68070)

ICC: immunocytochemistry

WB: western blot

Table 2: List of primers used for RT-qPCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Reference
Tfeb	ATGTACTGTCCACCTCAGCCG	CTCGGGGTTGATGTAGCCCA	XM_035451172.1
Lamp1	GCGTTCAGGTCCAGGCTTTC	CCTGTCCACCGGCTAGATG	NM_001246830.1
Lamp2	TGCTACCTGTCTGCTGGCTAC	TGACAGCTGCCGGTGAAGTTA	NM_001246749.1
Ctsd	TGCAGACCCTCGGCATCTTG	GAGCCGCCCACTTCTGTCAT	XM_003510234.5
Gapdh	CTCTCTGCTCCTCCCTGTTCTA	TGAAGGGGTCATTGATGGCA	NM_001244854.2
Actb	CCCCAAGGCCAACCGTGAA	GCATGAGGGAGAGCGTAGC	NM_001244575.1

Table 3: Comparative summary of the effects of Tau35 and FL-tau on mTORC1 signaling and autophagy compared to untransfected CHO cells

Factor	FL-tau	Tau35
Beclin-1	No change	Decreased
LC3-II	Increased	Decreased
LC3-labelled autophagosomes	Increased	Decreased
LC3-labelled autolysosomes	Increased	Decreased
LC3-II increase following autophagy induction	No change	Attenuated
Lipid accumulation	Increased lipid area	Increased lipid area and puncta/cell
Acidic structures	Increased	Decreased
LAMP2 +ve structures	No change	Decreased
Cathepsin D +ve structures	No change	Decreased
LAMP2/tau colocalization	-	Increased compared to FL-tau
TFEB mRNA expression	Decreased	Decreased
Nuclear TFEB increase following mTORC1 inhibition	Attenuated	Attenuated
S6 phosphorylation	No change	Increased
Raptor phosphorylation	No change	Decreased
AMPK phosphorylation	No change	Decreased

Figure Legends

Fig 1. Tau35 reduces the autophagy markers LC3 and beclin-1

Western blots of cell lysates prepared from CHO-FL, CHO-Tau35 and CHO cells probed with antibodies recognising (a) total and phosphorylated (PHF1) tau, (b) beclin-1 (n=3), and (c) LC3-I/II (n=4), with β-actin as loading control. Molecular weights (kDa) are shown on the left. Graphs show quantitation of proteins relative to β-actin (mean ± SEM). One-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P<0.01, ****P<0.0001. (d) Immunofluorescence of methanol-fixed CHO-FL, CHO-Tau35 and CHO cells labelled with antibody to LC3 (red) and Hoescht 33342 (blue). Scale bar=10 µm. Graph shows LC3 intensity per μm^2 cell area (mean ± SEM, n=30 cells from 3 independent experiments for each cell line). Welch ANOVA with Dunnett's T3 multiple comparisons test, *P<0.05, ****P<0.0001. (e) Paraformaldehyde-fixed CHO-FL, CHO-Tau35 and CHO cells transfected with 1 µg EGFP-LC3 plasmid (green). Scale bar=10 µm. Graph shows the number of EGFP-LC3 puncta per μm^2 cell area (mean ± SEM, n=70-100 cells from 3 independent experiments for each cell line). Kruskal-Wallis with Dunn's multiple comparisons test, **P<0.01, ****P<0.0001.

Fig 2. Tau35 and FL tau affect the formation of autophagosomes and autolysosomes

(a) Paraformaldhyde-fixed CHO-FL, CHO-Tau35 and CHO cells transduced with mCherry-GFP-LC3 lentivirus for 72 h. Insets show zoomed in regions in the white dashed box. Scale bar=10 µm. Graphs show (b) the number of $mCherry^+GFP^+$ (autophagosomes) puncta and $mCherry^+GFP^-$ (autolysosomes) puncta per μm^2 cell area and (c) the sizes of autophagosomes and autolysosomes. Values shown are mean ± SEM, n=37-40 cells from a single transduction for each cell line. Kruskal-Wallis with Dunn's multiple comparisons test, **P<0.01, ***P<0.001, ****P<0.0001.

Fig 3. Tau35 impairs autophagy flux

Western blots of CHO-FL, CHO-Tau35 and CHO cell lysates probed with antibodies to LC3-I/II and β-actin. Cells were treated with (a) bafilomycin A1 (300 nM, 2 h) or (b) rapamycin (1 µM, 6 h). Molecular weights (kDa) are shown on the left. Graphs show quantitation of LC3-II relative to β-actin under each condition. Values shown are mean ± SEM (n=3). Two-way ANOVA with Sidak's multiple comparisons test, *P<0.05, ***P<0.001, ****P<0.0001.

Fig 4. Tau35 inhibits the clearance of neutral lipid droplets

Labelling of paraformaldehyde-fixed CHO-FL, CHO-Tau35 and CHO cells with BODIPY 493/503 (green) and Hoescht 33342 (blue). Scale bar=10 µm. Graphs show the number of BODIPY puncta per μm^2 cell area. Values shown are mean ± SEM, n=30 cells from 3 independent experiments for each cell line. Kruskal-Wallis with Dunn's multiple comparisons test, ***P<0.001.

Fig 5. Tau35 leads to lysosomal deficits

(a) Labelling of acidic structures using LysoTracker Red in CHO-FL, CHO-Tau35 and CHO cells. Scale bar=10 µm. Graph shows the number of LysoTracker positive puncta per μm^2 cell area. Values are shown as mean ± SEM, n=30-40 cells from 3 independent experiments for each cell line. Welch ANOVA with Dunnett's T3 multiple comparisons test, ****P<0.0001, *P<0.05. (b) Labelling of paraformaldehyde-fixed CHO-FL, CHO-Tau35 and CHO cells with antibodies to LAMP2 (green), cathepsin D (red), and Hoescht 33342 (blue). Scale bar=10 µm. Graphs show the number of LAMP2 and cathepsin D puncta per cell, standardised to cell area. Values shown are mean ± SEM, n=30 cells from 3 independent experiments for each cell line. Welch ANOVA with Dunnett's T3 multiple comparisons test, ****P<0.0001. (c) Immunofluorescence of paraformaldehyde-fixed CHO-FL and CHO-Tau35 cells labelled with antibodies to LAMP2 (green), tau (red), and Hoescht 33342 (blue). Scale bar=10 µm. Graph shows the Pearson correlation coefficient for LAMP2 and tau in CHO-FL and CHO-Tau35 cells. Values shown are mean ± SEM, n=30 cells from 3 independent experiments for each cell line. Unpaired t-test with Welch's correction, ****P<0.0001.

Fig 6. Tau35 leads to defects in the expression of TFEB-regulated genes

(a) Graphs of RT-qPCR analyses showing the relative quantification (RQ) of mRNA expression, using CHO cells as control. Data show mean ± SEM from 3 independent experiments for each cell line. RT-qPCR was performed on *Tfeb*, *Lamp1*, *Lamp2* and *Ctsd* and normalised to *Gapdh* and *Actb*. One-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P<0.01. (b) Western blots of CHO-FL, CHO-Tau35 and CHO cell lysates probed with antibodies to TFEB and β-actin. The lanes were re-ordered from the same blot. Molecular weights (kDa) are shown on the left. Arrows indicate the TFEB doublet. The graph show quantitation of total TFEB relative to β-actin (n=3). (c) Immunofluorescence of paraformaldehyde-fixed CHO-FL, CHO-Tau35 and CHO cells labelled with antibody to TFEB (red) and

Hoescht 33342 (blue). Scale bar=10 μm. Graph shows the percentage of cells with nuclear TFEB (n=240-300 cells from each condition, 3 independent experiments). (d) Paraformaldehyde-fixed CHO-FL, CHO-Tau35 and CHO cells expressing EGFP-TFEB (green) labelled with Hoechst 33342 (blue). Scale bar=10 μm. Graph shows the percentage of cells exhibiting nuclear TFEB (n=330-540 cells/5-10 fields of view of each cell type, 3 independent experiments). One-way ANOVA with Tukey's multiple comparisons test, **P<0.01. (e) CHO-FL, CHO-Tau35 and CHO cells treated with 1 μM Torin 1 (T1) for 2 h or vehicle (V) were separated into nuclear and cytoplasmic fractions and probed on western blots with antibodies to TFEB, GAPDH and histone H3. C: cytoplasmic fraction, N: nuclear fraction. Graphs show quantification of the normalised nuclear/cytoplasmic TFEB ratio (n=3). Two-way ANOVA with Sidak's multiple comparisons test, *P<0.05, **P<0.01, ****P<0.0001.

Fig 7. Tau35 expression activates mTORC1 and reduces AMPK activity

Western blots of CHO-FL, CHO-Tau35 and CHO cell lysates probed with antibodies to (a) phosphorylated and total S6 (n=6), (b) phosphorylated and total raptor (n=6), and (c) phosphorylated and total AMPK (n=3). Molecular weights (kDa) are shown on the left. Graphs show quantitation of the phosphorylated/total proteins (mean ± SEM). One-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P<0.01.

Figures

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