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Selective Disruption of Drp1-Independent Mitophagy and Mitolysosome Trafficking by an Alzheimer's Disease Relevant Tau Modification in a Novel C. elegans Model.

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ABSTRACT

Background: Accumulation of inappropriately phosphorylated tau into neurofibrillary tangles (NFT) is a defining feature of Alzheimer’s Disease (AD), with specific epitopes such as Tau pT231 emerging early in the development of tau pathology. Previously, we demonstrated that a phosphomimetic mutant (T231E) of human tau drove the loss of neuronal function and structural integrity with age in a novel C. elegans single-copy gene insertion AD model. A critical finding was that T231E, unlike wild type tau, suppressed oxidative stress-induced mitochondrial autophagy, or mitophagy. Regulation of mitochondrial morphology by fission is important for mitophagy, which has been reported to be dysregulated by AD-relevant tau species. Dynamin Related Protein 1 (Drp1) is a GTPase that plays a central role in mediating mitochondrial fission, and its altered function may contribute to AD pathogenesis.

Methods: Genetically-encoded fluorescent biosensors and dynamic imaging approaches were combined with a genomic drp-1(-) loss-of-function and transgenic tau mutants to derive a comprehensive in vivo analysis of age-associated changes in mitochondria and mitolysosome (ML) morphology, abundance, neurite trafficking, and stress-induced mitophagy.

Results: Strain expressing disease-associated PTM mimetic Tau T231E demonstrated a surprisingly selective effect on ML development and trafficking, with no effect on lysosomes or autolysosomes, and a subtle effect on mitochondria that was apparent mainly in older animals. Unexpectedly, we found that drp-1(-) mutants mount a robust mitophagy response to oxidative stress, consistent with recent observations that adaptive mitophagy may occur independent of the canonical DRP1 pathway. Moreover, T231E continued to suppress oxidative stress-induced mitophagy in the drp-1(-) background.

Conclusions: Our C. elegans single-copy gene insertion model unveils multiple levels of selectivity – phenotypic selectivity for mutations that mimic pathologic tauopathy-associated PTM and physiologic selectivity for organelles that contain damaged mitochondria. In addition, our novel findings provide compelling support for DRP1-
independent mechanisms playing a pivotal role in regulating mitochondrial dynamics and function in the context of AD-relevant tau species and age-associated stress.

**Keywords.** Alzheimer’s disease, tau phosphorylation, *C. elegans*, mitochondria, Drp1, fission, mitophagy
BACKGROUND

Alzheimer’s disease (AD) is the most common progressive neurodegenerative disorder [1]. A prominent pathological hallmark of AD is the intraneuronal NFT, primarily comprised of fibrillar tau that is modified with a myriad of posttranslational modifications (PTMs) which accumulate stoichiometrically on tau as the disease progresses [2, 3]. Phosphorylation of tau at specific residues such as T231 occurs early in the disease process, leading to an attenuation in its normal function concurrent with an increased propensity to self-associate [4]. Indeed, the progressive phosphorylation of tau correlates with the evolution of cognitive impairment in AD, clearly indicating the importance of the phosphorylation of tau at key sites in the disease process [3, 5].

In the past decades, transgenic animal models of tau toxicity have been developed in organisms such as *C. elegans* [6-8], *D. melanogaster* [9-11], and mice [12, 13]. Toxicity in these model organisms has generally been achieved by over-expressing various tau species. Though these approaches have undoubtedly led to new insights, overexpression has the potential to mask subtle modifiers in a multifactorial, complex disease process.

Mitochondria play a complex role in the cell - they not only generate most of the energy needed to support the various neuronal functions [14], but also are mediators of homeostatic processes that are necessary for neuronal health [15]. Mitochondrial dysfunction is a prominent feature of many neurodegenerative diseases, including AD [16-19]. Pathological accumulation of tau in animal models is associated with mitochondrial reactive oxygen species (ROS) production and impacts oxidative phosphorylation (OXPHOS) [20-22], morphology and turnover [23, 24], and trafficking [25], though the precise mechanisms are still not well understood.
To better understand the role of gatekeeper tau PTMs in the context of AD relevant mitochondrial dysfunction, we developed transgenic, single-copy *C. elegans* models expressing wild type human tau or a phosphomimetic mutation of T231 (T231E) fused to a fluorescent reporter protein and expressed in a defined set of mechanosensory neurons. Because tau is not normally expressed in worms, these were by definition still overexpression models, albeit less so than previous models given the use of single-copy gene insertion techniques. A combination of behavioral assays and fluorescent biosensors clearly demonstrated a selective and age-dependent effect of T231E on sensory neuron function, morphology, and, perhaps most importantly, on mitochondrial autophagy (mitophagy) elicited by oxidative stress [26, 27]. This model confers the ability to discern between the pathological consequences of individual tau mutants and the age-dependence of mito-centric phenotypes with unprecedented precision.

In the context of AD, pathogenic forms of tau have been suggested to disrupt mitochondrial dynamics by selectively interacting with fission protein Drp1 [28, 29]. While mouse *Dnm1l* loss-of-function (lf) mutants in the gene coding for Drp1 are lethal, mutations in the *C. elegans* gene *drp-1*, which codes for the worm Drp1 ortholog DRP-1 [30], are viable [31, 32]. With this in mind, here we set out to examine the intersection of tau pT231, DRP-1 mediated fission, organelle trafficking, and mitochondrial turnover in promoting neurodegeneration using our single-copy transgenic tau mutant strains of *C. elegans*. The results of our study provide clear *in vivo* evidence that phosphomimetic T231E tau elicits profound and selective effects on stress-induced mitophagy and mitolysosomal trafficking starting in early adulthood. Moreover, these measures are in large part independent and additive to the surprisingly subtle defects associated with the
loss of *drp-1*. Our observations suggest that DRP-1 independent mitochondrial fission or adaptive mitophagy may be an unanticipated target of toxic tau phosphorylated at T231 (pT231), and may hence be worth exploring as a potential therapeutic target for tau pathology in AD.
METHODS

Propagation of *C. elegans*

Worms were maintained using standard techniques [33] at 20°C on Nematode Growth Media (NGM) plates made with Bacto Agar (BD Biosciences) and seeded with live *E. coli* OP50-1 bacterial strain (cultured overnight at 37°C in Luria Bertani (LB) Miller Broth). For experimental assays, 4th larval stage (L4) hermaphrodites (characterized by the appearance of a “Christmas tree vulva”) were generated by synchronization using standard alkaline-bleach treatment to isolate embryos. The synchronized animals were moved to plates containing 25 µM 2'-Deoxy-5-fluorouridine to suppress fertility and assayed as adults either 3 or 10 days later.

To induce oxidative stress, N, N'-dimethyl-4,4'-bipyridinium dichloride, commonly known as paraquat (PQT; Sigma-Aldrich, St. Louis, MO) was added to a final concentration of 8 mM in OP50-seeded NGM agar plates, and animals were maintained on these plates at 20°C overnight prior to analysis.

Strains and Reporters

A list of strains and reporter transgenes used in this study is provided in Supplementary Table 1. Reporters included MLS::GFP to measure mitochondrial morphology and dynamics [34], mito-mKeima to measure mitophagy and ML trafficking [35, 36], Atg8/LGG-1::mCherry::GFP to measure macroautophagy and autophagosome/autolysosome (AP/AL) trafficking [37], and LMP-1::mScarlet to measure lysosome trafficking (S. Swords and B. Grant, Rutgers University). All reporters were expressed under the control of a touch-cell specific mec promoter. Standard *C. elegans*
genetic techniques [38, 39] were used to cross reporters into single-copy tau strains and/or the *drp-1 (tm1108)* mutant. In all cases, “control” refers to reporters in an N2 background. Reporters were followed under a standard fluorescent dissecting microscope. Single worm genomic PCR was used to discern the presence of the tau expression cassettes in the Mos ttTi5605 locus on Chr II, as well as for *drp-1 (tm1108)* genotyping. Primers used for *drp-1* genotyping are as follows:

- **drp-1 geno F1**: ccagacctcgtgcgtgcga
- **drp-1 geno R1**: acgacacctccgcatagctca
- **drp-1 geno F2 (Int)**: cctgcactgaaagcccgtcgt

Primers for tau genotyping have been described [26]. T231A and T231E tau mutations were confirmed in all final transgenic strains by PCR sequencing, as described [26].

**Behavioral Assay**

The sensory response to light touch (using an eyelash) was assessed as described by [40] and modified according to [41]. Individual worms were visualized under a conventional dissecting scope on OP50-1 seeded NGM-agar plates and touched gently behind the terminal bulb of the pharynx a total of 10 times. Touches were separated by a minimum of 10 seconds to prevent habituation. Positive responses were scored if an omega turn or locomotory reversal was observed. Worms that crawled off the food were censored. The experimentalists used a *mec-4* mutant with a selective deficit in its response to light touch to calibrate their technique, thus ensuring that alternative harsh
touch response pathways that occur through separate genetically and physiologically distinguishable circuits were not inadvertently activated [42].

**Fluorescent Imaging**

*Mitochondrial morphology* - Animals expressing touch-cell MLS::GFP were mounted on 3% agarose pads on glass slides and anesthetized with 10 mM tetramisole hydrochloride. Imaging of the soma was performed using a Nikon Eclipse TE2000 inverted microscope coupled to a six channel LED light source (Intelligent Imaging Innovation, Denver, CO), an ORCA-Flash4.0 V2 Digital CMOS camera (Hamamatsu Photonics, Bridgewater Township, NJ) and Slidebook6 software (Intelligent Imaging Innovation, Denver, CO). The PLM cell body was brought into focus under visible light using DIC contrast, a 100x oil immersion objective, and a 1.5x magnifier. Once in focus, DIC was removed, and epifluorescence image acquisition was performed using 488-nm excitation, a 515-nm dichroic, and a 535/25 emission filter.

Freehand tracing of the mitochondria in ImageJ was used to calculate mitochondrial area and the proportion of somatic area occupied by mitochondria, which we termed density (1 pixel = 0.0019 µm²). Two experimentalists independently analyzed subsets of images and compared results to ensure the reproducibility of the analysis.

*Mitophagy* – Animals expressing touch-cell specific mito-mKeima were immobilized and imaged essentially as described above using dual excitation fluorescence ratiometry (ex. 550-nm/440-nm.; em. 600-nm+), with background subtraction, thresholding, and quantitation performed using Slidebook6 (Guha et al., 2020b).
**Autophagy** - Animals expressing touch-cell specific LGG-1::GFP::mCherry were immobilized as described above and imaged using a Nikon A1R HD scanning confocal microscope running NIS-Elements version 5.11 software. Images were acquired through a 60x oil objective using the resonance scanner mode with 488-nm or 561-nm laser excitation at 2.5% power, 2x zoom, 4x averaging, and a pixel size of 0.14 microns, and emissions were monitored through a 525/50 or 595/50 filter for the green and red channels, respectively. The PMT gain was HV=5 for the red channel and HV=10 for the green channel, and the z-interval was 0.6 µm. Maximum projection composite images were generated using ImageJ software and used to assess AP (green and red) and AL (red only) puncta.

**Motility assays** - Time-lapse recordings were acquired with a Nikon A1R HD Laser scanning confocal microscope (Eclipse Ti2) at 60X oil objective using NIS-Elements version 5.11 software with parameters similar to those describe above. Mitochondria were captured using 488-nm excitation and a 525/50 emission window, whereas lysosome, AL, and ML movements were captured using 561-nm excitation and a 595/50 emission window. The capture period was 5 minutes with 30 secs / frame interval. Particle dynamics were analyzed using the Imaris x6.2 version software. Run length was obtained as the distance between the starting and last stopping points of a motile particle. Other parameters considered were percent motility and respective speed as described (Butler et al., 2019; Morsci et al., 2016).

**Statistical Analysis**

All statistical analyses were conducted using Prism 8.0 (GraphPad Software), with alpha-error level of p < 0.05 considered to be significant. Data sets were first tested for
normalcy, and in most cases group differences were analyzed with either one-way or two-way ANOVA depending upon the variables. Data sets which did not fit normalcy were analyzed by a Kruskal-Wallis test. The sample sizes were based on those found previously in the laboratory to provide appropriate power for discerning phenotypic differences among genotypes. Graphs were plotted in Prism 8.0.
RESULTS

Phosphomimetic tau T231E, but not wild type tau, exacerbates an age-dependent reduction of mitochondrial area and density in PLM neurons and sensitizes them to oxidative stress.

Recently, we developed an AD model where single-copy transgenes coding for tau fused to the fluorescent protein Dendra2 were expressed specifically in *C. elegans* mechanosensory (mec) touch neurons; strains expressing phosphomimetic mutant human tau T231E (T231E hereafter), but not wild type tau (0N4R or TauT4 hereafter) or a phosphoablation mutant T231A (T231A hereafter) exhibited mild fragmentation of the PLM cell mitochondrial network and progressive morphologic and sensory defects [26]. To more fully explore this effect in a quantitative manner, we measured mitochondrial area and the proportion of mitochondrial area in total somatic area, which we termed density, as surrogates for changes in mitochondrial distribution through PLM – similar to approaches taken to define morphologic remodeling with age [43]. The mitochondria of touch neurons were visualized using transgenic matrix-targeted GFP driven by a touch neuron specific *mec-7* promoter. In order to assess the impact of age, we performed identical analyses in young adults and old adults at Days 3 and 10 following the onset of reproductive maturity – with the normal worm adult lifespan being ~ two weeks.

Representative images from the upper and lower quartiles of our combined datasets clearly indicate modest effects, with most neurons exhibiting relatively normal tubuloretibular networks (Fig 1 A, B). Correspondingly, there is no significant difference between control, TauT4, T231A and T231E at Day 3 of adulthood (Fig. 1C, F). However, by Day 10 of adulthood, T231E exhibited a slight but significant reduction in both
mitochondrial area and density as compared to either TauT4 or T231A (Fig. 1D, G), reminiscent of age-dependent morphologic decline shown to occur in extremely old animals [43].

Age is associated with the accumulation of oxidative damage [44, 45] and chronic mitochondrial stress is likely to be a factor in neurodegenerative diseases including AD [46, 47]. Here, we used paraquat (PQT), a redox cycler that stimulates ROS production in mitochondria [48], to address the idea that T231E exacerbates ROS sensitivity. Following a 24h exposure to PQT at d2 of adulthood, all strains exhibited reduced mitochondrial area compared to untreated controls (Fig 1E). However, the magnitude of the reduction exhibited by T231E was greater than that exhibited by TauT4 or T231A. Moreover, mitochondrial density was not significantly impacted by PQT in either the TauT4 or T231A strains, but only in the T231E strain (Fig 1H). These results suggest that the mitochondria of animals that express T231E are susceptible to ROS-induced remodeling, even in early adulthood.

**Phosphomimetic tau T231E, but not WT tau, reduces mitochondrial density and motility in neuronal processes.**

Mitochondria are dynamic organelles, both with respect to morphology as well as to movement through neuronal processes [16]. Abnormal mitochondrial morphology, distribution, and axonal transport have been observed in AD postmortem brains and in AD mouse models [49, 50], while pathologic tau reduces mitochondrial mobility in cortical neurons treated with Aβ [51, 52] as well as in worm neurites expressing pro-aggregate forms of tau [53].
GFP-labeled mitochondria in PLM neuronal processes appear as sphere and rod-shaped organelles of varying size distributed at specific intervals, similar to axonal mitochondria in mammalian neurons [54, 55] (Fig 2A). Given the established role of tau in facilitating axonal transport, we investigated the impact of TauT4, T231A and T231E on the distribution of mitochondria with respect to abundance, spacing, and active trafficking dynamics such as motile run length and speed in the distal segment of PLM processes ranging over 300 µm.

As observed for somatic mitochondria, tau genotype had no significant impact on these measures at Day 3 (Fig 2B-E), but T231E became distinguishable from control for all four metrics by Day 10 (Fig 2F-I). Importantly, zero values were triaged by limiting trafficking metrics to motile mitochondria, which represented ~1/2 of the pool at Day 10 (Fig 2J). Hence, while both total and motile mitochondria were reduced in T231E compared to control, overall fractional motility remained similar (Fig 2J). These results demonstrate that our model can detect subtle differences in mitochondrial number, distribution, and transport induced by toxic tau species associated with “disease” progression, where mitochondrial dysfunction may contribute to neurite deficits (Fig 2K). These results motivated us to query the selectivity of T231E for mitochondria by examining the movement of other dynamic organelles.

Neurite transport of mitolysosomes is suppressed early and selectively by pathologic Tau T231E

Mitophagy is a type of cargo-selective autophagy [56, 57], where damaged mitochondria are engulfed by autophagosomes (AP) and subsequently degraded by fusion with lysosomes to form a distinct type of autolysosome (AL) termed mitolysosome.
(ML). We previously demonstrated a failure in oxidative-stress induced mitophagy in T231E but not TauT4 or T231A [26].

Here, we used the mitophagy reporter mito-mKeima to follow the movement of ML along PLM neurites (Fig 3A). Mito-mKeima is a matrix-targeted variant of the genetically-encoded pH biosensor Keima [58]. Upon fusion of the engulfed mitochondria with the acidic lysosome, mito-mKeima – which is resistant to degradation by acid proteases – exhibits a spectral shift in the excitation maxima from 440 to 550 nm. Mito-mKeima ratios have been widely used as a metric to assess mitophagy [37, 59] but this property also permits focusing on ML movement through the neurite by selectively imaging the red channel.

Unlike mitochondria, the fractional motility of ML in T231E was lower than either TauT4 or T231A, even at Day 3 of adulthood (Fig 3D; Additional Movies S1 and S2). This was particularly apparent when comparing kymographs depicting movement of individual ML over-time (Fig 3G). Moreover, even though the total number of ML in the neurites remained constant across the genotypes (Fig 3D), the run length and the average speed of motile ML were also significantly lower at both Day 3 (Fig 3B, C) and Day 10 in T231E (Fig 3E, F).

In order to address the selectivity of this transport deficit for ML, we used two approaches. First, we asked whether it applies generally to lysosomes by measuring their trafficking with a lysosomal-associated membrane protein 1 (LMP-1)::mScarlet fusion transgene, generated using miniMos single-copy insertion [60]. However, neither their motile run length nor their speed was impacted by T231E at either Day 3 or Day 10 of adulthood (Fig S1A, B). We then narrowed our query by using a tandem mCherry-GFP-
Atg8/LGG-1 reporter that labels touch neuron autophagic vesicles in both red and green, but is non-selective for cargo [37]. Once again, our results suggested a lack of effect on autophagic vesicle trafficking with respect to tau genotype at either Day 3 or Day 10 (Fig S1C-F). To summarize, pathological tau mutation T231E impacts ML movement in young and old adults, whereas its effects on mitochondria motility are limited to older animals, and neither lysosome nor autolysosome (AL) motility are affected (Fig S1G).

Dysregulation of autophagy has been linked with neurodegenerative diseases, including AD [61-64]. Given the selective effect of T231E on ML motility, but not bulk AL motility, we asked whether there was a similarly selective impact on mitophagy over autophagy. Autophagy was triggered using food deprivation [65] and measured as a function of tau genotype, with acidic quenching of GFP in the mCherry-GFP-Atg8/LGG-1 reporter as a means of distinguishing between AP and AL (Fig S1H-N). In all cases, fasting induced an aging and genotype-independent increase in AL number (Fig S1I-N). In addition, AL were more abundant than AP, particularly after fasting, suggesting that they turn over more slowly than AP. This validated the autophagy reporter, but also confirmed the selectivity of T231E in impairing mitophagy over bulk autophagy given that overall lysosome and autolysosome number were unchanged by tau mutant expression.

Finally, we explored whether PQT stimulation, which fails to induce mitophagy in the PLM soma of the T231E mutant [26], has a similar phenotype with respect to ML formation in the neuronal processes, and whether this in turn affects trafficking of this very specific cargo containing vesicle. Interestingly, while PQT treatment stimulates increased ML abundance in both control and TauT4 but not T231E (Fig S2A), it also suppresses ML speed and motility such that both TauT4 and T231A phenocopied T231E (Fig S2B, C).
We conclude that the effect of T231E is selective for ML formation and trafficking, though perhaps through different mechanisms.

**Loss of Drp-1 obfuscates morphological mitochondrial defects caused by phosphomimetic Tau T231E in the soma.**

Alterations in mitochondrial dynamics are increasingly being recognized as early-stage pathological events in neurodegenerative diseases, including AD, Huntington's disease, and Parkinson's disease [50, 66, 67]. Regulation of mitochondrial morphology by fission is important for mitophagy [68, 69]. The canonical fission mediator, Drp1, is a monomeric GTPase that oligomerizes at the mitochondrial outer membrane upon activation [70]. Previous studies indicate that hyper-phosphorylated tau interacts directly with Drp1, likely increasing its activity and inducing mitochondrial fragmentation [28]. This led us to ask whether Drp1 contributes to the changes in mitochondrial morphology, trafficking or oxidative stress induced clearance that we observe in our model. Unlike the embryonic lethality observed in in Dnm1l knockout mice, loss of drp-1 is tolerated in C. elegans, and a mutant allele (tm1108) has been well studied [32, 71].

The drp-1(tm1108) mutant exhibits swollen mitochondrial clusters that are predominantly localized to opposite poles of the soma (Fig 4A), irrespective of the tau background. In general, both the qualitative and quantitative effect of drp-1 mutation on mitochondrial area and density was dominant to that of the tau genotype at both Day 3 and Day 10 (Fig 4B-E). We also note that de facto statistical significance is observed at Day 3 when comparing mitochondrial area and density between the drp-1(tm1108) background and the drp-1(tm1108); TauT4 or drp-1(tm1108); T231E strains (Fig 4B, C).
However, given the relatively limited magnitude of these differences, we suspect that they may not be biologically relevant.

We also examined the distribution and movement of GFP-labelled mitochondria in the PLM cell process (Fig 4F, G) via time-lapse fluorescent imaging (Fig 4H-P). Our analysis focused on addressing how T231E impacts mitochondrial abundance and motility in the *drp-1(tm1108)* mutant. As in the soma, the effects of *drp-1(tm1108)* were dominant over T231E. The *drp-1(tm1108)* mutant exhibited reduced abundance (Fig 4H, M), increased separation (Fig 4I, N), and a reduction in both motile run length (Fig 4J, O) and speed (Fig 4K, P) of mitochondria, regardless of T231E’s presence or absence. In short, the *drp-1(tm1108)* mitochondria were smaller, spaced further apart, and did not move appreciably, and the independent effect of T231E on these parameters paled in comparison. This motivated us to question the extent to which mitochondrial morphology per se and specifically motility in the cell processes affects neuronal function.

We addressed this by measuring touch sensitivity in the *drp-1(tm1108)* mutant and comparing it to the deficit previously reported for T231E [26]. Mec neurons such as PLM, where our imaging results were obtained, mediate the locomotion response to light touch [41, 42]. At Day 3, *drp-1(tm1108)* by itself displayed a subtle, but not statistically significant touch defect (Fig 4L); however, this was exacerbated by age, and by Day 10 had achieved significance (Fig 4Q). As reported previously, T231E also exhibited an age-dependent functional decline in touch responsiveness (Fig 4Q). Most interestingly, however, the T231E phenotype was additive to *drp-1(tm1108)* at both Day 3 and Day 10 (Fig 4Q), suggesting that they contribute to neuronal dysfunction through independent mechanisms. Combined with the non-additivity of mitochondrial transport and
moldology, these results suggest that some other target of T231E negatively influences neuronal function. Thus, we investigated the other notable output of T231E - oxidative stress induced mitophagy - using a similar genetic approach with \textit{drp-1(tm1108)}.

**T231E suppresses Drp-1 independent mitophagy following oxidative stress.**

Mito-mKeima was used to assess baseline mitophagy in PLM neurons as a function of \textit{drp-1} and T231E. As reported previously [26], T231E had no impact on baseline mitophagy at either Day 3 or Day 10 (Fig. 5A, B). In contrast, \textit{drp-1(tm1108)} exhibited a significant increase in mitophagy in both young and old animals, independent of T231E (Fig. 5A, B). Given that DRP-1 is thought to participate in mitophagy, this finding was surprising and led us to examine ML abundance and motility in the neuronal processes of \textit{drp-1(tm1108)}.

Unlike mitochondria (Fig 4), \textit{drp-1(tm1108)} ML in neurites were indistinguishable from control at both Days 3 and 10, and \textit{drp-1(tm1108)} did not impact the ML motility and speed defect observed in T231E (Fig 5C, D, E, F). Hence, \textit{drp-1(tm1108)} and T231E appear to act through separable pathways with respect to their effect on ML motility. The combined results of \textit{drp-1} loss-of-function on mitochondria, mitophagy, and ML transport are schematized in Fig 5G.

Finally, we measured oxidative-stress induced mitophagy. As we have reported previously [26], PQT treatment increased mitophagy in the control at both Day 3 and Day 10 of adulthood (Fig. 6A, B, C), and T231E suppressed this effect (Fig. 6D, E, F). Mitophagy was likewise increased by PQT treatment in \textit{drp-1(tm1108)}, and this increase was also suppressed by T231E (Fig. 6D, E, F). However, consistent with the idea that \textit{drp-1} loss-of-function and T231E act through separable mechanisms, the \textit{T231E; drp-}
double mutant exhibited more mitophagy than the T231E mutant itself (Fig. 6E, F). This suggests that suppression is selective for oxidative-stress induced mitophagy and not for the increased mitophagy observed upon loss of drp-1. To conclude, the drp-1 mutant mounts a robust mitophagy response to oxidative stress, consistent with mitochondrial fission occurring independent of the canonical DRP1 pathway (Fig. 7A). In addition, the T231E phosphomimetic mutant tau continues to suppress oxidative stress-induced mitophagy in the drp-1(lf) background (Fig. 7B).
DISCUSSION

Tau accumulation is a cardinal feature of AD brain, with increasing PTMs as the disease progresses [3, 72-74]. While it is clear that tau is a necessary component of the pathologic pathways in AD [75], the primary toxic entity is likely not the insoluble NFTs [12, 76]. Instead, toxicity is likely due to soluble or oligomeric forms of tau [4, 12, 77, 78] that possess increased, disease-associated PTM such as phosphorylation at specific residues that alter tau turnover and function [74, 79]. One such disease relevant residue is T231, where phosphorylation occurs early in the evolution of tau pathology [72, 80, 81]. Additionally, phosphorylation at this specific site precedes the formation of tau oligomers [4], which likely contribute to tau toxicity [82], suggesting that this PTM may be, at least in part, causative for AD pathology. However, the precise cellular mechanism of how increased phosphorylation at this site negatively impacts neuronal health remains unclear.

We demonstrated previously that *C. elegans* touch neurons expressing a single-copy tau transgene with a T231E phosphomimetic mutant are unable to elicit mitophagy in the face of oxidative stress [26]. Here, we have gone on to show that neurite transport of MLs is also selectively impacted by T231E (Fig. 3). While many studies have linked impaired mitochondrial transport with overexpression of tau [52, 83], both in vitro and in vivo (for a review, see [84], to our knowledge this is the first demonstration of a selective impairment of ML movement. Moreover, this effect is limited to a subtle gatekeeper mutation mimicking early pathologic modifications to tau in a single-copy gene insertion model, at a time when other phenotypic deficits have not yet manifested.
The dynamic distribution of mitochondria via bidirectional trafficking in the neurite processes helps to maintain local energy demands in axons and dendrites [85]. Likewise, the turnover of damaged and dysfunctional mitochondria via mitophagy contributes to mitochondrial quality control, and supports neuronal health [86]. These processes are facilitated by changes in mitochondrial size and shape, which are dynamically regulated through fusion and fission [87, 88]. Indeed, a major role of fission is to facilitate the clearance of dysfunctional mitochondria by mitophagy, and defective mitophagy is a prominent dysfunction in age-related diseases [89]. Defective mitophagy likely occurs early in AD [90], and contributes to premature aging and neurodegeneration such as that observed in Werner’s syndrome patients [91].

Another well-established role of fission is to create mitochondrial fragments that can be transported more easily along neuronal processes, and of course, tau is a mediator of microtubule dynamics [92]. In fact, it has been shown that overexpression in hippocampal neurons of a tau construct that mimics a caspase cleaved form significantly impairs mitochondrial transport in the neurites [93]. However, many studies of tau, microtubules and trafficking have utilized tau overexpression models, and there is data that challenges the conclusion that tau disrupts neuronal transport mechanisms [94-96]. Moreover, our results suggest a relatively subtle effect on mitochondrial trafficking, and none at all on AP or lysosome trafficking (Figs. 2 and S1). Hence, it is unclear that the exquisitely selective defect observed here in ML transport is comparable to these more wide-spread defects reported in the literature. It is interesting to speculate that defective mitochondria may prime adjacent lysosomes in a manner that allows them to be
selectively recognized by transport machinery, as there is ample evidence supporting
mitochondria-lysosome communication axes (for review, see [97]).

More surprising to us was the observation that neither oxidative stress-induced
mitophagy nor the deficit in ML trafficking was impacted by the loss of the fission mediator
Drp1. Studies have shown that in AD patient brain samples, phospho-tau interacts with
Drp1 [28], which may lead to increased mitochondrial fragmentation and possibly
contributing to cognitive decline [98, 99]. In C. elegans, drp-1(If) is able to suppress
certain forms of genetically-induced mitochondrial fragmentation [100], and hence
seemed to be a reasonable target to test in our model, given the significant, albeit subtle,
morphology phenotype in T231E.

Interestingly, we saw an upregulation of baseline mitophagy in drp-1(tm1108)
mutants, independent of tau presence or genotype (Fig. 5). While initially counter to our
expectations, this corresponds well with very recent work suggesting a reduction in
mammalian Drp1 elevates mitophagy and may be beneficial in a symptomatic tau
transgenic mice [29], perhaps through a mechanism where APs remove larger-than-
normal mitochondrial segments [101]. We also noted that oxidative stress-induced
mitophagy was additive to the increased baseline mitophagy observed in the drp-
1(tm1108) mutant, and that T231E suppressed mitophagy equally well in the drp-
1(tm1108) and wild type backgrounds (Fig. 6). We interpret this to mean that mitophagy
can be induced through at least three separate mechanisms: a canonical drp-1 dependent
pathway, a second that is drp-1 independent yet is unaffected by T231E (such as that
observed in the drp-1(tm1108) mutant at baseline), and a third that is drp-1-independent,
upregulated in response to oxidative stress, and suppressed by T231E.
There is some precedent for alternative pathways compensating for the loss of canonical mitophagy. Recent work suggests the importance of mitochondrial-derived vesicles (MDVs) [102], which can deliver cargo to the endolysosomal system through a Pink1/Parkin/Syntaxin 17 dependent pathway [103], and may compensate for the loss of LC3-mediated mitophagy [104]. Interestingly, MDV formation has been associated with oxidative stress [105, 106].

The effect of DRP-1 loss on trafficking is similarly intriguing. Mitochondria are effectively stalled in the PLM neurites in the \textit{drp-1\textit{(tm1108)}} mutant, yet only a very subtle touch response defect can be detected and is limited to older adults (Fig 4). This suggests that mitochondrial trafficking (or lack thereof) does not impact the behavioral surrogate of neurodegeneration in our model, albeit this in an organism with very short neuronal processes – which admittedly may not translate well to vertebrates. Yet, somewhat counterintuitively, ML motility is normal in the \textit{drp-1\textit{(tm1108)}} mutant, as is the reduced motility observed in T231E (Fig 5). Collectively, these data suggest an unexpected functional independence between DRP-1 and T231E for both defects, and we suspect that both trafficking and mitophagy may contribute to neurodegeneration in our model in a manner that does not require DRP-1.

We must acknowledge the caveat that severe mitochondrial dysfunction can impact lysosomal activity [107], and under such circumstances lysosomes may not be properly acidified, hence suppressing the spectral shift in mito-mKeima even in the face of functional mitophagy. However, unlike most models of severe mitochondrial dysfunction, T231E has a modest, subtle phenotype, and baseline mitophagy appears normal; it is only following oxidative stress that we observe a suppression in oxidative-
stress induced mitophagy. Moreover, the trafficking defect that we report here assesses ML motility by virtue of their containing mitochondrial in acidified vesicles, and by definition focuses on functional organelles. However, we appreciate the potential interplay between mitochondrial dysfunction and lysosomal activity in regulating metabolism, and new tools are being developed to interrogate those connections [108].

Moving forward, it will be important to identify the molecular mechanism(s) through which oxidative stress-induced mitophagy occurs. Mitophagy signaling can involve multiple diverse recognition cues on the outer membrane, the most well-studied being the ubiquitin-dependent Pink1/Parkin pathway [109], as well as ubiquitin-independent mitophagy receptors including BNIP, NIX, and FUNDC1 [110], with the later implicated in hypoxic mitophagy in both mammals and worms [111, 112]. There is also emerging interest in what’s been termed “adaptive mitophagy” [113, 114], where proteins other than Drp1 have been shown to mediate mitochondrial removal. For example, Fis1 can regulate mitochondrial morphology independently of Drp1 [115] and has been implicated in both homeostatic and adaptive signaling regiments that contribute to mitochondrial quality control through mitophagy [116-119]. Additionally, femtosecond laser wounding triggers mitochondrial fragmentation in C. elegans and has also been shown to occur independent of DRP-1 (as well as other known regulators of mitochondrial morphology), and it instead requires the mitochondrial Rho GTPase Miro-1 and cytosolic calcium signaling [120].

Finally, the mechanism by which tau T231E selectively suppresses mitophagy in a phosphomimetic-specific manner also remains to be established. One possible mechanism could be that phospho-tau inserts directly into the mitochondrial membrane, disrupting Pink1/Parkin-mediated mitophagy [24, 121]. Another possibility involves tau
targeting one of the alternative receptors that function in stress-induced mitophagy. Whether the mechanism that impacts mitophagy is related to the ML motility deficit is unknown, but it is intriguing to speculate that these may be conjoined at the level of mitochondrial-lysosomal communication. Moving forward, we anticipate being able to use our single-copy model to provide a genetic perspective on how tau modification impacts the molecular mechanisms through which mitochondria respond to stress in the context of aging and to further decipher how these processes contribute to neurodegeneration in AD.
CONCLUSIONS

It is apparent that tau toxicity in our single-copy gene insertion model is selective for the phosphomimetic T231E mutation. It is likewise apparent that there is additional selectivity for acidic organelles that contain damaged mitochondria, and that these organelles are derived from mitochondria by non-canonical processing that does not involve typical DRP1 mediated fission. Moving forward, we anticipate being able to use our model to provide a genetic perspective on how tau modification impacts the specific molecular mechanisms through which mitochondria respond to stress in the context of aging and to further decipher how these processes contribute to neurodegeneration in AD.

Abbreviations. AD – Alzheimer’s disease; AL – Autolysosome; AP – Autophagosome; CRISPR – clustered regularly interspaced short palindromic repeats; Cas9 – CRISPR associated protein 9; Drp1 – Dynamin Related Protein 1; ETC – electron transport chain; GFP – Green Fluorescent Protein; LMP-1 - Lysosomal-associated Membrane Protein 1; Mec – Mechansensory; ML – Mitolysosome; MLS – Mitochondrial Localization Sequence; MQC – Mitochondrial quality control; MosSCI – Mos-mediated single-copy insertion; NGM – nematode growth media; NFT – neurofibrillary tangle; PQT – paraquat; PTM – post translational modification; ROS – reactive oxygen species
DECLARATIONS

Availability of Data and Materials. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests. None declared.

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Author contributions. Conceived and designed the experiments: SG GJ KN. Generated reagents or performed the experiments: SG AC TC DK SK SS. Analyzed the data: SG AC TC DK SK. Wrote the paper: SG TC GJ KN. All authors read, edited, and approved the final manuscript.

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Figure 1. Mitochondrial morphology in PLM soma as a function of single-copy Tau mutants. Fluorescent imaging of an MLS::GFP reporter was used to measure mitochondrial morphology in the soma of PLM neurons. (A-B) Green images are the
original fluorescent micrographs obtained from animals with mitochondrial networks that differ qualitatively within the scope of our observations, whereas black and white images represent a binary mask used to derive area and density metrics, shown below each image. These images are representative, falling in ~the upper and lower quartile of the metrics we use to assess morphology, and illustrate the range of morphologies we observe. Scale bar: 5 µm. (C, D, F, G) Quantitative analysis of mitochondrial area or density at Day 3 and Day 10 as a function of single-copy tau genotype. (E, H) Area and density as a function of 24 h treatment with 8 mM PQT starting at d2 of adulthood. Data are the mean ± SD from three independent technical replicates performed on different days by two independent researchers. Individual data points indicate values from single PLM cells from separate animals (N = 55 ± 5). Statistical analysis within Day 3 and Day 10 datasets was by one-way ANOVA with Tukey’s multiple comparison test, with *** P< 0.001, **P<0.01, *P<0.05 when comparing bracketed samples. Comparisons between treated and untreated samples with PQT at Day 3 was by two-way ANOVA followed by Tukey’s post hoc test, with *** P< 0.001, **P<0.01, *P<0.05 when comparing bracketed samples.
Figure 2. Mitochondrial distribution and transport is reduced by pathological phosphomimetic Tau T231E. Mitochondria were visualized through dynamic fluorescent imaging of an MLS::GFP reporter to track their movement in the PLM neuronal process of live worms. (A) Representative confocal image of a PLM neurite expressing MLS::GFP. Scale bar: 20 µm. (B, F) Mitochondrial abundance (number of mitochondria observed within 300 µm neurite length), (C, G) inter-mitochondrial distance (distance between two consecutive mitochondria), (D, H) run length (distance traversed on average by a healthy mitochondrion), and (E, I) speed, as a function of age and single-copy tau genotype. Motility metrics were limited to mitochondria that exhibited movement over the course of 5 min imaging. (J) Total and motile mitochondria at Day 10 as a function of single-copy tau genotype. (K) Schematic of how dysfunction of different mitochondrial health parameters might contribute to neurodegeneration in worms expressing phosphomimetic tau (T231E). The PLM neurite shown in red portrays three key features of damage: beading, aberrant branching, and wavy processes. Data are the mean ± SD from two independent technical replicates performed on different days. Individual data points demarcate values from single PLM cells from separate animals (N = 20 ± 5). Statistical analysis within Day 3 and Day 10 datasets was by one-way ANOVA with Tukey’s multiple comparison test, with *** P< 0.001, **P<0.01, *P<0.05 when comparing bracketed samples.
Figure 3. Pathological phosphomimetic tau T231E reduces mitolysosome motility. Mitolysosomes (ML)s were visualized through dynamic fluorescent imaging of mito-mKeima (550-nm excitation) to track their movement in the PLM neuronal process of live worms. (A) Representative confocal image of a PLM neurite expressing mito-mKeima
Scale bar: 20 µm. (B, E) ML run length and (C, F) speed, as a function of age and single-copy tau genotype. Motility metrics were limited to MLs that exhibited movement over the course of 5 min imaging. (D) Total and motile MLs at Day 3 as a function of single-copy tau genotype. (G) Diagram showing the C. elegans touch-sensitive mechanosensory neurons, including the PLM neuronal process (demarcated by dotted lines), with representative kymographs of ML movement in TauT4 and T231E, as labeled. Data are the mean ± SD from two independent technical replicates performed on different days. Individual data points demarcate values from single PLM neurites from separate animals (N = 20 ± 5). Statistical analysis within Day 3 and Day 10 datasets was by one-way ANOVA with Tukey’s multiple comparison test, with ***P< 0.001, **P<0.01, *P<0.05 when comparing bracketed samples.
**Figure 4.** Drp-1 effects on mitochondrial morphology are dominant to T231E, but additive with respect to touch sensitivity. Mitochondria were visualized through time-lapse fluorescent imaging of a MLS::GFP reporter to measure mitochondrial morphology in the soma and to track their movement in the PLM neuronal process of live worms. (A) Representative binary images used to quantitate morphology in the genotypes indicated. Note that the mitochondria are localized toward polar ends of the soma in the *drp-1(tm1108)* mutant, irrespective of the tau genotype and age. Scale bar: 5 µm. (B-E) Quantitative analysis of mitochondrial area and density in the distal PLM cell bodies at Day 3 and Day 10 as a function of *drp-1* and tau genotype. Control indicates data points from MLS::GFP reporter. Data are the mean ± SEM from three independent technical replicates performed on different days by two independent researchers. Individual data points demarcate values from single PLM cells from separate animals (N = 55 ± 5). (F, G) Representative confocal images of the PLM neurite from control and *drp-1(tm1108)* expressing MLS::GFP. Scale bar: 20 µm. Quantitative analysis of mitochondria (H, M) abundance, (I, N) distribution, (J, O) run length, and (K, P) speed in distal PLM processes at Day 3 or Day 10 as a function of *drp-1* or T231E, as indicated. Individual data points indicate values from single PLM neurites from separate animals (N = 20 ± 5). (L, Q) Responsiveness to touch was plotted as a function of *drp-1* and T231E at Day 3 and Day 10 (N = 100 animals, from three independent biological replicates, by two researchers). Statistical analysis for all datasets was by one-way ANOVA with Tukey’s multiple comparison test, with *** P< 0.001, **P<0.01, *P<0.05 when comparing bracketed samples. Some of the data has been reproduced from earlier figures for the sake of comparison with the addition of new data points.
Figure 5. Drp-1 and phosphomimetic Tau T231E are independent effectors of mitophagy and mitolysosome motility. Mito-mKeima was used to measure mitophagy in the PLM soma and to visualize PLM neurite ML trafficking through dynamic fluorescent imaging. Drp-1 refers to the drp-1(tm1108) allele. (A, B) Dual excitation ratio imaging was used to derive a mitophagy index as a function of age, drp-1 genotype, and T231E, as indicated. Individual data points demarcate values from single PLM cells from separate animals (N = 50 ± 5) from two independent researchers. (C-F) Quantification of ML trafficking parameters (run length and speed) in the distal PLM cell neurites at Day 3 and Day 10 as a function of age, drp-1 genotype, and T231E, as indicated. Individual data points demarcate average values from single PLM cells from separate animals (N = 20 ±
5). (G) Cartoon illustrating the location of mitochondria in green and MLs in red in a neurite process. Drp1 loss of function severely affects mitochondrial morphology and affects mitochondrial transport. The effects of tau mutant T231E are independent and additive, leading to further loss of neuronal touch sensitivity. Data are the mean ± SD from two independent technical replicates performed on different days, by two researchers. Statistical analysis within Day 3 and Day 10 datasets was by one-way ANOVA with Tukey’s multiple comparison test, with *** $P<0.001$, ** $P<0.01$, * $P<0.05$. 
Figure 6. Oxidative stress stimulated mitophagy and suppression by phosphomimetic Tau T231E are *drp-1* independent. Mitophagy was measured using dual excitation ratio imaging of mito-mKeima expressed in *C. elegans* PLM neurons. *Drp-1* refers to the *drp-1(tm1108)* allele. (A, B) Representative merged images where 440-nm excitation was used to detect mitochondria (green) and 550-nm excitation was used to detect mitolysosomes (red) as a function of *drp-1* genotype, T231E or 24h PQT treatment, as indicated. Scale bar: 2 µm. (C-F) Quantitative analysis of mitophagy as a function of age, *drp-1* genotype, T231E or PQT. Data are the mean ± SD from two independent technical replicates performed by two researchers (N = 50 ± 5 cells from...
separate animals). Statistical analysis was by two-way ANOVA followed by Tukey’s post hoc test, with ***$P < 0.001$ denoting significance when comparing bracketed samples.
Figure 7. Relationship of Drp1 to mitophagy under stress and in presence of T231E.

(A) Schematic view illustrating how Drp1 plays a key role in regulating mitochondrial fission by driving scission of mitochondria, so two unequal daughters are born. The damaged mitochondrion is purged by mitophagy. (B) A model figure illustrating how the
rate of mitophagy changes under various conditions, be it oxidative stress (via PQT), loss of \( drp-1 \), or pathological tau T231E mutation. Although both loss of \( drp-1 \) and oxidative stress result in a subtle-to-moderate increase of mitophagy, suppression by T231E is apparently limited to the later form of adaptive mitophagy.
Additional Figure S1. Selectivity of phosphomimetic T231E in terms of cellular organelle trafficking. Lysosomes and autophagosomes (AP) / autolysosomes (AL) were visualized through dynamic fluorescent imaging of LMP-1::mScarlet (lysosomes) or LGG-1::GFP::mCherry (AP/AL) to quantify their movement in the PLM neuronal process of live worms. (A, B) Lysosome run length and speed in control and T231E at Day 3 and Day 10, as indicated. (C-F) AP/AL run length and speed as a function of age and tau genotype. Both of these were run on the red channel. Control refers to biosensor strains that lack tau entirely. Statistical analysis within Day 3 and Day 10 lysosome datasets was by one-way ANOVA with Tukey’s multiple comparison test, while AP/AL datasets were analyzed using a Kruskal-Wallis test (N = 20 ± 5). (G-M) Starvation induced autophagy occurs normally in T231E mutant. (G) Table summarizing the selectivity of organelle trafficking defects in T231E as a function of age. (H) Representative merged fluorescent images of a LGG-1::GFP::mCherry reporter expressed in the PLM cell body at baseline and fasted conditions. Because the intensity of the red channel (AP and AL) is relatively higher than the green channel (AP only), the acquisition settings were scaled accordingly (see Methods). (I-N) Quantitative analysis of APs and ALs at baseline or fasted for 16 hours as a function of tau genotype. Data are the mean ± SD of ≥ 30 animals combined from two independent biological replicates. Data for the parental LGG-1 reporter strain lacking Tau transgenes, which is very similar to control Tau strain, is not shown. Statistical analysis was by two-way ANOVA followed by Tukey’s post hoc test. *** P< 0.001, **P<0.01, *P<0.05 denoting significance when comparing bracketed samples.
Additional Figure S2. PQT treatment phenocopies the effect of T231E on mitolysosome motility. Dynamic fluorescent imaging of mito-mKeima (control) was used to determine the effect of PQT on ML movement in single-copy tau mutants. ML (A) abundance, (B) run length, and (C) speed in the distal PLM cell neurites at Day 3 animals, as a function of 24h PQT treatment and tau genotype. Comparisons between treated and untreated samples with PQT at Day 3 was by two-way ANOVA followed by Tukey’s post hoc test, with *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ when comparing bracketed samples.
| Strain   | Transgene                                      | Purpose                              |
|----------|------------------------------------------------|--------------------------------------|
| KWN167   | rnySi24 (Pmec-7::Dendra2::TauT4)              | Tau in touch neurons                |
| KWN788   | rnySi51 (Pmec-7::Dendra2::TauT4(T231A))      | Phosphoablation mutant              |
| KWN789   | rnySi52 (Pmec-7::Dendra2::TauT4(T231E))      | Phosphomimetic mutant               |
| ZB4587   | jsIs609 [Pmec-7::MLS::GFP]                   | Touch neuron mitochondria            |
| KWN840   | jsIs609; rnySi24                              |                                      |
| KWN842   | jsIs609; rnySi51                              |                                      |
| KWN844   | jsIs609; rnySi52                              |                                      |
| KWN796   | rnyEx336 [pSKG1(Pmec-4::mito-mKeima)]        | Touch neuron mitophagy              |
| KWN803   | rnyEx336; rnySi24                             |                                      |
| KWN806   | rnyEx336; rnySi51                             |                                      |
| KWN804   | rnyEx336; rnySi52                             |                                      |
| KWN820   | rnyEx337 [pSKG7(Pmec-4::LGG1::GFP::mCherry)] | Touch neuron autophagy              |
| KWN822   | rnyEx337; rnySi24                             |                                      |
| KWN824   | rnyEx337; rnySi51                             |                                      |
| KWN823   | rnyEx337; rnySi52                             |                                      |
| RT4056   | pwSi222 [pcf910 pmec7::LMP-1::mscarlet let858] | Touch neuron lysosomes              |
| KWN852   | pwSi222; rnySi52                              |                                      |
| CU6372   | drp-1 (tm1108)                                |                                      |
| KWN850   | jsIs609; drp-1 (tm1108)                       |                                      |
| KWN854   | jsIs609; drp-1 (tm1108); rnySi51              |                                      |
| KWN851   | jsIs609; drp-1 (tm1108); rnySi52              |                                      |
| KWN855   | rnyEx336; drp-1 (tm1108)                      |                                      |
| KWN853   | rnyEx336; drp-1 (tm1108); rnySi52             |                                      |

**Supplementary Table 1. List of strains used in the study.**
Tau PTM-mimetic display reduced mitolysosomal trafficking

Additional Movie S1 and S2. mKeima expression in the PLM neuron demonstrating ML trafficking in phosphoablation mutant (S1) (A) and in phosphomimetic mutant (S2)(B) (this video corresponds to Fig. 3).
**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- MovieS1.mp4
- MovieS2.mp4