TDP-43 loss of function increases TFEB activity and blocks autophagosome–lysosome fusion

Qin Xia1, Hongfeng Wang1, Zongbing Hao1, Cheng Fu2, Qingsong Hu1, Feng Gao1, Haigang Ren1, Dong Chen1, Junhai Han3, Zheng Ying1,4,** & Guanghui Wang1,2*

Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that is characterized by selective loss of motor neurons in brain and spinal cord. TAR DNA-binding protein 43 (TDP-43) was identified as a major component of disease pathogenesis in ALS, frontotemporal lobar degeneration (FTLD), and other neurodegenerative disease. Despite the fact that TDP-43 is a multi-functional protein involved in RNA processing and a large number of TDP-43 RNA targets have been discovered, the initial toxic effect and the overwhelmed ALP function. Importantly, inhibition of mTORC1 activation, leading to accumulation of immature autophagic vesicles and autophagic-lysosomal biogenesis and autophagy, through targeting the mTORC1 key component raptor. This regulation in turn enhanced global gene expressions in the autophagy–lysosome pathway (ALP) and increased autophagosomal and lysosomal biogenesis. However, loss of TDP-43 also impaired the fusion of autophagosomes with lysosomes through dynactin 1 downregulation, leading to accumulation of immature autophagic vesicles and overwhelmed ALP function. Importantly, inhibition of mTORC1 signaling by rapamycin treatment aggravated the neurodegenerative phenotype in a TDP-43-depleted Drosophila model, whereas activation of mTORC1 signaling by PA treatment ameliorated the neurodegenerative phenotype. Taken together, our data indicate that impaired mTORC1 signaling and influenced ALP may contribute to TDP-43-mediated neurodegeneration.

Keywords ALS; Autophagy; mTORC1; TDP-43; TFEB

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder that is characterized by degeneration of motor neurons, leading to progressive muscle weakness, atrophy, and eventually fatal paralysis and respiratory failure. Approximately 90% of ALS cases are sporadic and the remaining 10% are familial. SOD1 gene that encodes superoxide dismutase 1 (SOD1) was the first discovered causative gene of ALS, and a breakthrough of ALS research began with the discovery of TAR DNA-binding protein-43 (TDP-43) that is encoded by TARDBP gene (Ling et al., 2013). TDP-43 was identified as a key component of the ubiquitin-positive inclusions in both ALS and frontotemporal lobar degeneration (FTLD) patients (Neumann et al., 2006), and the clinical and pathological overlap between ALS and FTLD indicated a central role for TDP-43 in diverse disease pathogenesis. TDP-43 is a RNA-binding protein, which contains two RNA recognition motifs (RRM1 and RRM2) that allow TDP-43 to bind to nucleic acids and a C-terminal glycine-rich domain (GRD) that mediates protein–protein interactions. TDP-43 functions in multiple steps of RNA processing and homeostasis including transcription, splicing, and transport of target mRNAs, such as CFT, HDAC6, SNM, and Nefl (Bose et al., 2008; Volkening et al., 2009; Buratti & Baralle, 2010; Fiesel et al., 2010; Polymenidou et al., 2011; Alami et al., 2014). It is therefore conceivable that defects in RNA processing, due to loss of nuclear TDP-43, could be linked to neurodegeneration (Xu, 2012; Diaper et al., 2013; Vanden Broeck et al., 2013, 2014).

Autophagy–lysosome pathway (ALP) is a critical cellular quality control system that is tightly associated with various neurodegenerative diseases especially in ALS, since mutations in ALP-associated genes, such as p62/SQSTM1, OPTN, and FIG 4, are the genetic cause of ALS, indicating a critical linkage between ALP and ALS disease pathogenesis (Ling et al., 2013). Recently, the transcription factor EB (TFEB) was identified as a master regulator of ALP that controls ALP by driving the expressions of autophagic gene products such as ATG5, Beclin-1, and ATG9B, as well as lysosomal gene products such as LAMP1, cathepsins, and subunits of vacuolar ATPases.
The cellular localization and activity of TFEB are mainly controlled by Ragulator-Rag-mTORC1 (mTOR complex 1) (Pena-Llopis et al., 2011; Martina et al., 2012; Rocznia-Ferguson et al., 2012; Settembre et al., 2012; Martina & Puertollano, 2013), an important amino acid-sensing complex on the lysosomal surface that controls cell growth, proliferation, and autophagy (Zoncu et al., 2011b; Bar-Peled & Sabatini, 2014). It has been shown that ALP and TFEB functions are affected by proteins in association with neurodegenerative diseases. For examples, presenilins in Alzheimer’s disease, α-synuclein in Parkinson’s disease, and polyglutamine expanded androgen receptor in spinal and bulbar muscular atrophy could affect cargo recognition, autophagosome–lysosome fusion, and TFEB nuclear localization in ALP (Wong & Cuervo, 2010; Decressac et al., 2013; Chua et al., 2014; Cortes et al., 2014). In addition, autophagic vesicle accumulation is a common feature of neurodegenerative diseases including ALS (Iguchi et al., 2013; Ling et al., 2013), indicating that ALP is involved in neurodegeneration. However, it remains unclear whether the accumulation of autophagic vesicles reflects a compensatory neuroprotective response or results from a failure of autophagic degradation.

To investigate the molecular mechanism of ALP in association with loss of TDP-43 function, we comprehensively studied the effects of TDP-43 on ALP and TFEB in cellular and fly models with TDP-43 loss of function. We found that loss of TDP-43 strongly induced a nuclear translocation of TFEB by directly targeting raptor, a key component of mTORC1. TDP-43-mediated mTORC1 dysregulation could affect the expression of ALP genes and the neurotoxicity in cells and in vivo. The current study reveals a role of mTORC1 dysfunction, abnormal altered autophagy, and lysosomal biogenesis in TDP-43-mediated neurodegeneration.

**Results**

**TFEB nuclear translocation in TDP-43-depleted cells**

Given that ALP, the critical cellular quality control system, is involved in ALS (Ling et al., 2013) and that TFEB is a master regulator of ALP and is functionally associated with neurodegenerative diseases as recently reported (Sardiello et al., 2009; Settembre et al., 2011; Tsunemi et al., 2012; Decressac et al., 2013; Spamanpanato et al., 2013), we wonder whether TDP-43 is able to regulate TFEB. We knocked TDP-43 down in HeLa (non-neuronal) and SH-SY5Y (neuronal) cells and examined TFEB nuclear translocation, a process that is associated with autophagy activation and lysosomal biogenesis. We observed that knockdown of TDP-43 resulted in a dramatic increase of nuclear and a decrease of cytoplasmic GFP-tagged TFEB in those cells (Fig 1A and B). Similar results were obtained using non-GFP-tagged TFEB in other types of cells (Appendix Fig S1). Consistently, similar results were obtained using subcellular fractionation assays (Fig 1C).

**TDP-43 is required for mTOR lysosomal localization and mTORC1 activity**

Based on the observations that mTORC1 plays an important role in the regulation of TFEB (Pena-Llopis et al., 2011; Martina et al., 2012; Rocznia-Ferguson et al., 2012; Settembre et al., 2012; Martina & Puertollano, 2013), we hypothesized that TDP-43 may regulate TFEB nuclear translocation in an mTORC1-dependent manner. To test this possibility, we knocked TDP-43 down and checked the cellular localization of mTOR, which reflects the activity of mTORC1. TDP-43-depleted cells showed a dramatic reduction of the punctate mTOR distribution that could indicate its lysosomal localization according to the previous reports (Fig 2A). Furthermore, the activity of mTORC1 was suppressed after knockdown of TDP-43, indicated by the phosphorylation of mTORC1 substrates p70S6K (Fig 2B). Given that amino acid is a well-established regulator of mTORC1 and promotes mTOR translocation to the lysosomal surface, we next examined the intracellular localization of mTOR under starvation or re-addition of amino acids in cells after starvation with or without TDP-43 knockdown. Under normal conditions, mTOR was co-localized with a lysosomal-specific marker LAMP1, indicating that mTOR is indeed localized to the lysosomal surface (Fig 2C). The enrichment of mTOR on lysosomes was impaired once cells were starved, but was restored after amino acids re-feeding (Fig 2C, upper panel). Meanwhile, mTOR failed to localize to lysosomes in TDP-43-depleted cells regardless of the presence or absence of amino acids (Fig 2C, lower panel). Consistent with these data, biochemical assays showed that the level of phosphorylated p70S6K was relatively low after starvation. Re-treatment of amino acids failed to restore p70S6K phosphorylation in TDP-43-depleted cells, whereas it significantly induced p70S6K phosphorylation in control cells (Fig 2D).

**Raptor, the adaptor of mTORC1, is involved in TDP-43-mediated mTOR lysosomal localization**

Previous studies have shown that the subcellular localization and activity of mTOR are highly controlled by mTORC1 adaptor protein raptor, Rag GTPases, and Ragulator complex which is comprised of p18, p14, and MP1 proteins that reside directly on the lysosomal membrane surface (Sancak et al., 2008, 2010; Zoncu et al., 2011a; Bar-Peled et al., 2012). In Ragulator–Rag–mTORC1 complex, Ragulator anchors the Rag GTPases to lysosomes, which is essential for Rag GTPases activation through an amino acids-dependent manner. Meanwhile, raptor interacts with active Rag GTPase heterodimers in which GTP-bound RagA or RagB (very similar to RagA) associates with GDP-bound RagC or RagD (similar to RagC), and raptor targets mTOR to lysosome surface where mTOR could be activated by its activator Rheb on the lysosomes. We examined the cellular localization and expression level of components of Ragulator–Rag–mTORC1 complex in TDP-43-deficient cells. TDP-43-depleted cells showed a decrease of raptor, but not mTOR, p18, and RagB, compared with control cells (Fig 2B). Moreover, knockdown of TDP-43 did not affect the cellular localization of Rag GTPases (Fig 3D). To further investigate the effects of TDP-43 on the expression of raptor, we performed quantitative RT–PCR assays. Downregulation of raptor mRNA was observed in TDP-43-depleted cells, whereas mRNA levels of other known members of mTOR-associated machinery on the lysosomes, such as mTOR itself, RagB, p18, p14, HBXIP, C7orf59, RagA, RagC, RagD, and FLCN, were not changed (Figs 2E and 2F). The cells treated with siRNA against raptor had diminished mTOR localization to lysosomes, showing a similarity to the cells treated with siRNA against TDP-43 (Figs 2G and 2H). Similar effects were also observed in cells treated with
siRNAs against both TDP-43 and raptor (Figs 2F and G, and EV1A and B). Given that raptor is the key adaptor of mTORC1 and directly regulates mTOR lysosomal localization, we wondered whether TDP-43 regulates mTOR lysosomal localization in a raptor-dependent manner. In TDP-43-depleted cells, mTOR was recruited to lysosomes, indicated by a co-localization of lysosomal marker LAMP1.

Figure 1. Effect of TDP-43 on TFEB nuclear translocation.
A HeLa cells were transfected with the indicated siRNAs for 48 h, and then were re-transfected with TFEB-EGFP. Twenty-four hours after transfection, the cells were fixed. DAPI (blue) was used for nuclear staining. Cells were visualized using confocal microscopy. Scale bar, 5 μm. The quantification of TFEB localization in cytoplasm and nucleus is shown on the right side. Data are from three independent experiments, means ± S.E.M.; **, P < 0.01; one-way ANOVA.
B Similar experiments as in (A) were performed in SH-SY5Y cells. Scale bar, 5 μm. The quantification data are shown on the right side. Data from three independent experiments represented as means ± S.E.M.; **, P < 0.01; one-way ANOVA. White arrowheads indicate nuclear localization of TFEB.
C HEK 293 cells were similarly transfected as in (A). Lysates from the cells were separated into cytoplasmic and nuclear fractions and then subjected to immunoblot analysis using anti-GFP, -GAPDH, and -histone 2B antibodies. The relative densities are shown on the right side. The data from three independent experiments are presented as means ± S.E.M.; **, P < 0.01; one-way ANOVA.

Source data are available online for this figure.
Figure 2. Regulation of mTORC1–TFEB–autophagy–lysosome signaling by TDP-43

A

mTOR
si-Control
si-TDP-43

B

p-p70S6K
p70S6K
raptor
RagB
p18
mTOR
TDP-43
GAPDH

C

Control
Starved
Starved+AA
si-Control
si-TDP-43

D

si-Control
si-TDP-43
Starved
Starved+AA
p-p70S6K
p70S6K
TDP-43
GAPDH

E

si-Control
si-TDP-43

F

si-Control
si-TDP-43
si-raptor
si-TDP-43 + si-raptor
si-TDP-43 + HA-raptor

G

% cells with mTOR in lysosome

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Figure 2. TDP-43 regulates mTORC1 activity and localization.

A. HeLa cells were transfected with the indicated siRNAs. After 72 h, the cells were subjected to immunofluorescence assay using antibody against mTOR (red). DAPI (blue) was used for nuclear staining. The stained cells were visualized using confocal microscopy. Scale bar, 5 μm.

B. HeLa cells were transfected as in (A). After 72 h, cells lysates were subjected to immunoblot analysis using anti-TDP-43, -p70S6K, -phosphorylated p70S6K (T389), -mTOR, -RagB, -RagC, -raptor, and -GAPDH antibodies. The relative densities of phosphorylated/p70S6K are shown in the upper-right panel, and the relative densities of mTOR, RagB, P18, raptor, and TDP-43 to GAPDH are shown in the lower right panel. The data from three independent experiments are presented as means ± S.E.M.; ns, not significantly different; *P < 0.05; **P < 0.01; one-way ANOVA.

C. HeLa cells were transfected with the indicated siRNAs. After 48 h, the cells were re-transfected with LAMP1-RFP (red) for 24 h. The cells were then incubated with Earle’s balanced salt solution (Starvation) for 2 h or re-stimulated with amino acids (starvation + amino acids) for 30 min. The cells were stained with mTOR (green) and DAPI (blue) and then visualized using confocal microscopy. Regions within the dotted boxes are magnified in the insets. Scale bar, 5 μm.

D. Similar transfection and treatment as in (C) were performed, and the lysates from staned and re-feeding cells were subjected to immunoblot analysis using anti-TDP-43, -p70S6K, -phosphorylated p70S6K, and -GAPDH antibodies. The relative densities are shown on the right side. The data from three independent experiments are presented as means ± S.E.M.; ns, not significantly different; **P < 0.01; one-way ANOVA.

E. Similar transfection as in (B) was performed, and transfected cells were processed for qRT-PCR analysis. The level of TDP-43, mTOR, raptor, RagB, and P18 mRNA was quantified and normalized relative to GAPDH. The data from three independent experiments are presented as means ± S.E.M.; ns, not significantly different; **P < 0.01; one-way ANOVA.

F, G. HeLa cells were transfected with the indicated siRNAs. After 48 h, the cells were transfected with LAMP1-RFP (red), with or without HA-raptor for 24 h. The cells were stained with anti-mTOR (green) antibody and DAPI (blue) and then visualized using confocal microscopy. Regions within the dotted boxes are magnified in the insets. Scale bar, 5 μm. The quantification of mTOR on lysosomes is shown in (G). Data from three independent experiments represented as means ± S.E.M.; **P < 0.01; one-way ANOVA.

Source data are available online for this figure.

Figure 3. TDP-43 regulates TFEB lysosomal localization.

A-C. HeLa cells were transfected with the indicated siRNAs. After 48 h, the cells were transfected with TFEB-EGFP (green) for 24 h. The cells were stained with anti-mTOR (red) antibody and DAPI (blue) and then visualized using confocal microscopy. Regions within the dotted boxes are magnified in the insets. Scale bar, 5 μm. The quantification data of TFEB puncta and nuclear localization are shown in (B and C), respectively. Data from three independent experiments represented as means ± S.E.M.; **P < 0.01; one-way ANOVA.

D. HEK 293 cells were transfected with the indicated siRNAs. After 48 h, the cells were re-transfected with LAMP1-RFP (red) and constitutively active HA-GST-tagged Rag GTPase mutants (RagAQ66L + RagC57S5L = RagAGTP + RagCGTP) for 24 h. The cells were stained with anti-HA (green) antibody and DAPI (blue). Cells were visualized using microscope IX71. Regions within the dotted boxes are magnified in the insets. Scale bar, 5 μm.

E. HEK 293 cells were transfected with siRNA targeting TDP-43. After 48 h, the cells were re-transfected with EGF-tagged TFEB-WT, TFEB-S211A, TFEB-Q10A/L11A, or TFEB-A30, along with LAMP1-RFP for 24 h. Cells were visualized using microscope IX71. Regions within the dotted boxes are magnified in the insets. Scale bar, 5 μm.

F. HEK 293 cells were transfected with the indicated siRNAs. After 48 h, the cells were re-transfected with EGF-tagged TFEB-WT or TFEB-R245-247A, along with LAMP1-RFP for 24 h. Then, the cells were incubated with Torin-1 (250 nM) for 2 h; Earle’s balanced salt solution (starvation) for 2 h or incubated with both. Cells were fixed and visualized using confocal microscopy. Regions within the dotted boxes are magnified in the insets. Scale bar, 5 μm.

TDP-43 regulates the localization of TFEB by targeting raptor, but not Rag GTPases

As we have shown that TDP-43 controls TFEB nuclear translocation and regulates the activity of mTORC1, we wonder how TDP-43 mediates TFEB localization. Recent studies showed that in fully fed cells, most TFEB appear to diffusely distributed in the cytoplasm and a little fraction of TFEB is recruited to activated Rag GTPases on the lysosomal surface, where TFEB is phosphorylated by mTORC1 on serine 211 (S211). Phosphorylated TFEB then interacts with cytosolic 14-3-3, resulting in the sequestration of TFEB in the cytosol. However, TFEB would transport into the nucleus once the interaction is abolished, resulting in an increased expression of TFEB targeting genes (Pena-Llopis et al, 2011; Martina et al, 2012; Rocznia-Ferguson et al, 2012; Settembre et al, 2012; Martina & Puertollano, 2013). In consistence with those findings, we observed that TFEB showed a diffuse cytoplasmic distribution in si-control cells, whereas mTOR was localized to lysosomes (Fig 3A–C). In contrast, in TDP-43 knockdown cells, TFEB and mTOR localization was dramatically changed, showing that TFEB was translocated from the cytosol to the nucleus and formed lysosomal puncta in cytoplasm, whereas mTOR had a diffusively cytoplasmic distribution (Figs 3A and EV2), suggesting an involvement of mTORC1 in TDP-43-mediated TFEB nuclear translocation. Moreover, similar results were obtained in raptor knockdown cells as well as in TDP-43 and raptor double-knockdown cells (Figs 3A and EV2). To further test whether TDP-43-induced redistribution of TFEB and mTOR depends on raptor, we performed raptor re-transfection assay in TDP-43-depleted cells and examined the subcellular localization of TFEB and mTOR. After a restoration of raptor, but not active Rag GTPases (RagAGTP/RagCGTP), TFEB shuttled back to the cytoplasm (Figs 3A, EV2, and EV3A and B), suggesting a critical role of raptor in TDP-43-mediated regulation of TFEB. Based on the studies that Rag GTPases functions in the recruitment of TFEB and mTOR to lysosomes (Martina & Puertollano, 2013), we next tested whether TDP-43 could influence the lysosomal localization of Rag GTPases. The enrichment of the active Rag GTPases (RagAGTP/RagCGTP) (Martina & Puertollano, 2013) on lysosomes was not disturbed after knockdown of TDP-43, but was strongly abolished after knockdown of p18, which anchors Rag GTPases to lysosomes (Fig 3D), indicating that loss of TDP-43 does not affect the localization of the Rag GTPases.
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B

% cells with TFEB

puncta

si-Control + - - - -
si-TDP-43 - + + + +
si-raptor - - - + +
HA-raptor - - - - +

C

% cells with TFEB

in nucleus

si-Control + - - - -
si-TDP-43 - + + + +
si-raptor - - - + +
HA-raptor - - - - +

TFEB-EGFP/ LAMP1-RFP

TFEB-R245-247A-EGFP / LAMP1-RFP

Figure 3.
The conversion of serine 211 to alanine in TFEB results in a loss of phosphorylation of TFEB by mTORC1, which in turn blocks the formation of the TFEB-14-3-3 complex in the cytosol, leading to a constitutive nuclear localization, as well as an increased lysosomal localization of TFEB (Martina et al., 2012; Rocznia-Ferguson et al., 2012; Martina & Puertollano, 2013). In contrast, TFEB-A30 mutant (deletion of the first 30 amino acids) and TFEB-Q10A/L11A mutant could not associate with Rag GTPases and lysosomes, leading to a failure of phosphorylation of Ser211 by mTORC1 and subsequently being transported easily into the nucleus (Martina et al., 2012; Rocznia-Ferguson et al., 2012). In our observations, knockdown of TDP-43 significantly increased wild-type (WT) TFEB, but not TFEB-A30 or TFEB-Q10A/L11A lysosomal localization (Figs 3E and EV3C and D), suggesting that Rag GTPases, but not TFEB-43, are critical for the association of TFEB with lysosomes. Meanwhile, knockdown of TDP-43 had no effects on TFEB-S211A localization (Figs 3E and EV3E), suggesting that TDP-43 may affect TFEB phosphorylation by mTORC1 to modulate TFEB lysosomal distribution, similarly as raptor does.

We further comprehensively analyzed the localization of TFEB under various conditions. In agreement with other studies (Martina et al., 2012; Rocznia-Ferguson et al., 2012; Settembre et al., 2012; Martina & Puertollano, 2013), TFEB was dramatically translocated to the nuclei and lysosomes after the treatment of Torin-1, a specific mTORC1 inhibitor. In contrast, starvation or depletion of RagB led to an increase of nuclear, but not lysosomal translocation of TFEB, even if the cells were subsequently incubated with Torin-1 (Fig 3F). Meanwhile, the localization of TFEB in TDP-43- or raptor-depleted cells was similar to that in Torin-1-treated cells, but not in starved or RagB-depleted cells (Fig 3F). Similar results were obtained using cells that were transfected with the TFEB-R245-247A mutant, which lacks a nuclear localization signal but still has the lysosomal localization signal by targeting Rag GTPases (Fig 3F). In those cells, raptor- or TDP-43 depletion, or Torin-1 treatment still induced an increased translocation of TFEB-R245-247A to lysosomes (Fig 3F). All together, our data reveal that TDP-43 regulates the localization of TFEB by affecting raptor and mTORC1, but not Rag GTPases.

TDP-43 directly binds to raptor mRNA through its RRM domains and regulates raptor mRNA stability

To confirm that the decreased raptor level following TDP-43 depletion was a specific effect, we re-expressed wild-type TDP-43 after the cells were treated with siRNA targeting the 3’UTR of TARDBP, which does not affect the re-transfected TARDBP with coding sequences. The mRNA and protein levels of raptor were restored upon re-expression with wild-type TDP-43 in TDP-43-deficient cells, indicating a role of TDP-43 in the regulation of raptor gene expression level (Fig 4A and B). TDP-43 is a DNA/RNA-binding protein containing two RNA recognition motifs (RRM1 and RRM2) and a C-terminal glycine-rich domain (GRD) that allow its binding to nucleic acids and mediate protein–protein interactions. To further verify which domains could affect the regulation of raptor by TDP-43, we constructed TDP-43 deletion mutants that lack the domains of RRM1 (ARRM1), RRM2 (ARRM2), or both RRM1 and RRM2 (ARRM1+ARRM2), respectively. In TDP-43-depletion cells, both mRNA and protein levels of raptor failed to be restored by these TDP-43 depletion mutants (Fig 4C and D). Similarly, in TDP-43-depleted cells, raptor levels and phosphorylated p70S6K levels were not restored by a 25-kDa C-terminal fragment of TDP-43 (named TDP-C25) lacking the RRM4s, which could form cytoplasmic aggregates in cells (Appendix Fig S2A–C), suggesting that the cytoplasmic TDP-43 aggregates themselves cannot affect raptor level or mTORC1 activity. Therefore, our results indicate that the raptor level and mTORC1 activity are regulated by the functions of endogenous TDP-43 in cells. In addition, we found that GRD deletion also failed to restore the raptor levels in TDP-43 depletion cells (Appendix Fig S2D and E). Since the GRD domain of TDP-43 could interact with other key regulators (such as hnRNPs) that are critical for the TDP-43 function, these factors could cooperate together with TDP-43 to regulate TDP-43 RNA targets such as raptor.

Given that the mutation sites of above TDP-43 deletion mutants were RNA-binding domains, we suspected that TDP-43 could bind to raptor mRNA directly. In agreement with this, RNA immunoprecipitation (RIP) analysis showed that wild-type TDP-43, but not the RRM1 and RRM2 domain deletion mutant, could bind to raptor mRNA (Fig 4E). Therefore, our data suggest that RRM domains of TDP-43 specifically bind to raptor mRNA and mediate the regulation of raptor mRNA level.

To further explore how TDP-43 mediates the regulation of raptor mRNA, we examined the transcription and the stability of raptor in TDP-43-deficient cells. We used Click-iT technology to label the newly transcribed RNA in TDP-43-depleted cells and control cells, and then, the labeled RNA was selected and subjected to qRT–PCR. Results showed that the level of newly synthesized raptor was not affected in TDP-43-deficient cells (Fig 4F). To test whether TDP-43...
Figure 4.

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can affect the stability of raptor mRNA, we analyzed half-lives of raptor mRNA by inhibiting transcription with the treatment of actinomycin D (ActD). Importantly, we observed that the mRNA stability of raptor, but not RagB, was decreased in TDP-43-deficient cells (Fig 4G and Appendix Fig S3). Taken together, our results suggest that TDP-43 can bind to raptor mRNA and regulate raptor mRNA stability at the post-transcriptional level.

**TDP-43 regulates lysosomal and autophagosomal biogenesis in a TFEB-dependent manner**

As nuclear translocation of TFEB is associated with TFEB transcriptional activity, which is tightly involved in the regulation of lysosomal biogenesis and autophagic level, we wonder whether TDP-43 could regulate ALP. To address this, we first employed Lysotracker (a specific lysosomal fluorescent probe) as a marker to monitor lysosomal biogenesis. In TDP-43-depleted cells, the fluorescent intensity of Lysotracker was markedly increased (Fig 5A and Appendix Fig S4A and B). We next broadly assessed the expression levels of lysosomal and autophagic genes in TDP-43-depleted cells. TDP-43-depleted cells showed a global increase of both mRNA and protein expression levels of a set of TFEB target genes, including lysosomal and autophagic genes, such as genes involved in lysosomal biogenesis and function (LAMP1, cathepsins, and genes encoding subunits of vacuolar ATPases), and genes involved in autophagy (ATG5, Beclin-1, and ATG9B; Fig 5B and C). Similar results were obtained in various cell lines, such as SH-SY5Y (Fig 5D and E). However, the relative genes of ALP were no longer upregulated in TDP-43-depleted cells if TFPB was silenced (Fig 5F and G).

As the protein level of LC3-II and the numbers of LC3-labeled vesicles reflect the autophagosomal numbers, we used biochemical and immunofluorescent assays to determine whether autophagy is induced by TDP-43 depletion. Indeed, we observed a significant enhancement of LC3-II levels in 8 various types of TDP-43-depleted cell lines, including HeLa (human cervical), A549 (human epithelial), HEK 293 (human kidney), HT22 (mouse neuronal), PC12 (rat neuronal), and Atg5 wild-type (autophagy-competent) mouse embryonic fibroblasts (ATG5-WT MEFs), but not in Atg5 knockout (autophagy-deficient) mouse embryonic fibroblasts (ATG5-KO MEFS) (Fig EV4A), suggesting that TDP-43 could strikingly affect autophagosome formation across cell types of diverse species and tissues with functional autophagy. Consistent with these results, immunofluorescent assay showed a dramatic increased punctuate LC3 in the absence of TDP-43 (Fig EV4B and C). Knockdown of TDP-43 also increased punctuate p62 (an autophagic substrate and marker protein) and LC3 that were co-localized in cells (Fig EV4D and E). Moreover, immunoblot and electron microscopy (EM) analyses demonstrated that loss of TFEB could abolish the increases of levels of LC3-II, autophagosomal, and lysosomal numbers in TDP-43-depleted cells (Fig 6A and B). Taken together, our data suggest that TDP-43 regulates lysosomal and autophagosomal biogenesis in a TFEB-dependent manner.

**Accumulation of immature autophagic vesicles and overwhelmed autophagic flux in TDP-43-depleted cells**

Given that the protein level of LC3-II reflects both autophagosome synthesis and degradation, we employed bafilomycin A1, a V-ATPase inhibitor which blocks the fusion of autophagosome with lysosome and ultimately leads to the inhibition of autophagosome degradation, to monitor autophagosome synthesis in TDP-43-depleted cells. The level of LC3-II was also increased in TDP-43-depleted cells treated with bafilomycin A1 compared with control cells treated with bafilomycin A1 (Fig 6C), indicating that loss of TDP-43 enhanced autophagosome synthesis. To better study the effect of TDP-43 on autophagic flux and function, we tested mCherry-EGFP-LC3 as a reporter of autophagic flux. An significant increase of autophagosomes (yellow dots with both mCherry and EGFP signals) was observed in TDP-43-depleted cells compared with control cells, but the formation of autolysosomes (red dots with only mCherry signal since quenching of the EGFP signal in the acidic lysosomes) was not enhanced to a same extent as autophagosomes (Fig 6D and E). In addition, formation of autophagosomes (yellow) was also enhanced in TDP-43-depleted cells treated with bafilomycin A1 (Fig 6D and E). Notably, under starvation condition, we observed an increase of autophagosomes and a decrease of autolysosomes in TDP-43-depleted cells compared with control cells, suggesting that knockdown of TDP-43 could impair autophagosome–lysosome fusion in a mTORC1-independent manner, thereby inhibiting autophagic flux under starvation condition (Fig 6D and E). In supporting with this notion, the protein level of autophagic substrate p62 was upregulated in TDP-43-deficient cells under basal and starvation conditions (Fig 6G), whereas the mRNA level of p62 was not changed in those cells (Fig EV4F).

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**Figure 5. TDP-43 regulates global gene expression levels in ALP through TFEB.**

A HeLa cells were transfected with the indicated siRNAs. After 72 h, the cells were stained with Lysotracker (red). Then, the cells were harvested and processed for flow cytometry analysis.

B Similar transfection as in (A) was performed, and transfected cells were processed for qRT–PCR analysis. The mRNA levels of lysosomal and autophagic genes were quantified and normalized relative to GAPDH. The data from three independent experiments are presented as means ± S.E.M.; *, P < 0.05; **, P < 0.01; one-way ANOVA.

C HeLa cells were similarly transfected as in (A), and the cell lysates were subjected to immunoblot analysis using anti-TFEB, -LAMP1, -LAMP2, -ATG5, -Beclin-1, -cathepsin L, and -GAPDH antibodies.

D Similar transfection as in (A) and processed as in (B). Data from three independent experiments represented as means ± S.E.M.; *, P < 0.05; one-way ANOVA.

E Similar experiments as in (C) were performed in SH-SY5Y cells.

F HeLa cells were transfected with the indicated siRNAs. Cell lysates were subjected to immunoblot analysis using anti-TFEB, -LAMP1, and -GAPDH antibodies.

G Similar transfection as in (F) was performed, and transfected cells were processed for qRT–PCR analysis as in (B). The data from three independent experiments are presented as means ± S.E.M.; ns, not significantly different; *, P < 0.05; **, P < 0.01; one-way ANOVA.

Source data are available online for this figure.
Regulation of mTORC1–TFEB–autophagy–lysosome signaling by TDP-43

Figure 5.
Figure 6.

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To further explore the role of altered ALP in TDP-43-mediated cytotoxicity, we examined the cell viability in TDP-43-depleted cells that were transfected with EGFP-tagged Nhtt-150Q, a N-terminal truncated huntingtin known as a autophagy substrate that could damage ALP function and increase the sensitivity of cell death induced by autophagic dysfunction (Wong & Cuervo, 2010). Knockdown of TDP-43 induced a significant cell death in ATG5-WT MEFs that were transfected with Nhtt-150Q, but not in ATG5-KO MEFs (Fig 6F). Taken together, our data indicate that abnormal accumulation of autophagic cargoes and overwhelmed ALP function could trigger cytotoxicity in TDP-43-depleted cells.

To study how TDP-43 affects autophagosome–lysosome fusion, we examined a set of genes involved in autophagosome–lysosome fusion, including syntaxin-17 (Itakura et al., 2012), SNAP-29 (Diao et al., 2015), VAMP8 (Jean et al., 2015), VPS33A (Jiang et al., 2014), VPS16 (Jiang et al., 2014), Rab7, DCTN1, MYO1C (Brandstaetter et al., 2014), DYNCH1 (Jahreiss et al., 2008), DNA11/DIC, DYNLL1, DYNCL1L2, ARL8B, myrin, SERCA (Mauvezin et al., 2015), PLEKHM1 (McEwan et al., 2015). Our results showed that the gene expression level of DCTN1, which encodes dynactin 1, was significantly decreased in TDP-43-depleted cells (Fig EV5A and B). Furthermore, protein expression level of dynactin 1, but not dynein heavy and intermediate chains (DHC and DIC), was decreased in those cells (Fig 7A). Dynactin 1 is a motor protein component of the dynein–dynactin complex which mediates the retrograde transport of lysosomes, and dysfunction of dynein–dynactin complex results in abnormality of lysosomal positioning and autophagosome–lysosome fusion as reported (Jahreiss et al., 2008; Korolchuk et al., 2011). Under starvation condition, we could observe a dramatic increase of perinuclear lysosomes in si-controls cells (Fig EV5C) as reported (Korolchuk et al., 2011), and we found more dispersed distributed lysosomes in si-TDP-43 cells, as well as si-dynactin 1 or si-DHC cells, but not si-KIF5B (KIF5B mediates the anterograde transport) cells (Figs 7B and C, and EV5D–H). Importantly, we observed significant decreased LC3/LAMP1 co-localization (autophagosome–lysosome fusion) in dynactin 1-deficient cells, and loss of TDP-43 was not able to decrease LC3/LAMP1 co-localization in dynactin 1-deficient cells (Fig 7D and E). Meanwhile, re-transfection of dynactin 1 could increase LC3/LAMP1 co-localization in TDP-43-depleted cells (Fig 7F and G), indicating that dynactin 1 deficiency contributes to the impairment of autophagosome–lysosome fusion in TDP-43-depleted cells. Taken together, these data suggest that TDP-43 could regulate lysosomal positioning and autophagosome–lysosome fusion by targeting dynactin 1.
Figure 7. Regulation of mTORC1–TFEB–autophagy–lysosome signaling by TDP-43.
**Discussion**

Since cytoplasmic TDP-43 aggregation is accompanied by a loss of TDP-43 function in cells, it is presently unclear whether the TDP-43-mediated neuropathological changes are caused by a toxic gain of function or by a loss of normal function (Lee et al., 2012). However, based on a series of recent studies, a loss-of-function mechanism, rather than a gain-of-function mechanism, may be the major factor involved in TDP-43-related neuropathies (Xu, 2012; Diaper et al., 2013; Vanden Broeck et al., 2013, 2014). In supporting with this notion, numerous animal models with TDP-43 loss of function, such as TDP-43 knockdown or knockout mice, flies, and worms, have been demonstrated to have a neurodegenerative phenotype (Feiguin et al., 2009; Fiesel et al., 2010; Lin et al., 2011; Vaccaro et al., 2012; Wu et al., 2012; Zhang et al., 2012; Iguchi et al., 2013; Yang et al., 2014). Loss of TDP-43 function results in a dysregulation of RNA pathways, such as RNA splicing, transport, and stability, leading to motor neuron neurodegeneration (Lee et al., 2012). Although a lot of TDP-43 RNA targets have been identified according to the previous reports (Polymenidou et al., 2011; Ling et al., 2013), the molecular mechanism by which loss of TDP-43 causes neurotoxicity remains elusive. Here, our findings reveal that raptor, a key component of mTORC1, as a new TDP-43-binding target (Fig 4). In TDP-43-depleted cells, raptor deficiency-mediated mTORC1 dysfunction induces TFE3 nuclear translocation (Fig 3) and enhances gene expressions in ALP (Fig 5), but not the autophagosome–lysosome fusion, which results in accumulated immature autophagosome vesicles and overwhelmed overall ALP function (Fig 6). Thus, our findings provide evidence that TDP-43-linked neurotoxicity is triggered by a loss of normal TDP-43 function and that raptor is the molecular target in TDP-43-mediated mTORC1–TFEB pathway (Figs 2–4).

The present study highlights the role of mTORC1–TFEB signaling in TDP-43-linked neurodegeneration (Fig 9D). TDP-43 regulates mTORC1 activity by specifically targeting mTORC1 component raptor, but not other factors associated with mTORC1 activity, such as Ragulator or Rag GTPases (Figs 2–4). Given that Ragulator-Rag complex can target mTOR to lysosomes and is necessary for its activation by amino acids (Fig 9D), we thought to examine the effect of TDP-43 on Ragulator and Rag GTPases. Our data indicate that TDP-43 does not affect the expression or cellular localization of p18, the key component of Ragulator complex, or Rag GTPases (Figs 2B and E, and 3D). Our results further suggest that TDP-43 acts on raptor (Figs 2B and E, and 4), a component and an adaptor of mTORC1, to dissociate mTOR from lysosomes.

Raptor interacts with Rag GTPases and targets mTOR to lysosomes where mTOR can phosphorylate TFE3 on serine 211, and then, phosphorylated TFE3 would stay in the cytosol by interacting with 14-3-3 (Roczniak-Ferguson et al., 2012; Martina & Puertollano, 2013). We note that knockdown of TDP-43 not only leads to a nuclear transport of TFE3, but also leads to an increase of TFE3 lysosomal localization (Fig 3A–C). In raptor-deficient cells, an inactivation of mTOR inhibits the interactions between TFE3 and 14-3-3, leading to an enrichment of TFE3 in both nuclei and lysosomes (Martina & Puertollano, 2013). An explanation of this lysosomal enrichment is that dissociation of the cytosolic TFE3-14-3-3 complex will cause a more stable interaction between TFE3 and Rag GTPases on lysosome membrane (Martina et al., 2012; Roczniak-Ferguson et al., 2012; Martina & Puertollano, 2013). Thus, Rag GTPases are the direct basis for TFE3 lysosomal localization, and raptor indirectly modulates TFE3 lysosomal localization with mTOR and 14-3-3. Based on this, we conclude that TDP-43 regulates TFE3 lysosomal localization by specifically targeting raptor, but not Rag GTPases. In support of this notion, the effects of TDP-43 on TFE3 lysosomal localization are diminished in raptor re-transfected cells (Fig 3A).

As neurons are not capable of self-renewal and cell division, they are quite susceptible to various cellular stress and stimuli. Thus, the protein quality control system such as autophagy is
Figure 8. mTORC1 activity was strikingly impaired in TBPH-knockout flies.

A W1118 and TBPH−/− larvae were blotted using antibodies against phosphorylated p70S6K (T389), phosphorylated 4E-BP1 (T37/46), and tubulin.

B Total RNA was prepared from W1118 and TBPH−/− larvae and subjected to RT-PCR analysis. Ribosomal protein L32 (RPL32) was used as a loading control.

C Total RNA was prepared from W1118 and TBPH−/− larvae and subjected to qRT-PCR analysis. The level of TBPH and raptor was quantified and normalized relative to RPL32. Data from three independent experiments represented as means ± S.E.M.; **, P < 0.01; one-way ANOVA.

D The heads of W1118 and TBPH−/− larvae were cut and blotted using antibodies against phosphorylated p70S6K (T389), phosphorylated 4E-BP1 (T37/46), and tubulin.

E The first-, second- and third-instar larvae of W1118 and TBPH−/− were blotted using antibodies against phosphorylated p70S6K (T389), phosphorylated 4E-BP1 (T37/46), and tubulin.

F–H Similar experiments as in (A–C) were performed in W1118 and TBPH−/− adult flies. Data from three independent experiments, means ± S.E.M.; **, P < 0.01; one-way ANOVA.

I Similar experiments as in (C) were performed in W1118 and TBPH−/− adult flies. The mRNA levels of lysosomal and autophagic genes were quantified and normalized relative to GAPDH. The data from three independent experiments are presented as means ± S.E.M.; ns, not significantly different; **, P < 0.01; one-way ANOVA.

Source data are available online for this figure.
essential for neuronal cells to maintain cellular homeostasis to clear abnormally accumulated proteins (Feiguin et al., 2009; Wong & Cuervo, 2010; Chua et al., 2014). With this in mind, it is reasonable that neurons can exhibit a higher level of ALP-associated autophagosome turnover, which is particular important in disease conditions (Boland et al., 2008). Although the current
view of autophagy in neurons is that an induction of autophagy helps to remove cytoplasmic aggregate-prone proteins in neurodegenerative diseases, which is considered to exhibit therapeutic benefit (Harris & Rubinsztein, 2012), the manner by which autophagy affects ALS is controversial. Recent reports showed that an induction of defective autophagy by blocking mTOR activity could actually exacerbate neurotoxicity and worsen disease in ALS (Zhang et al., 2011; Ching et al., 2013), whereas a reduction of autophagy had a rescue effect to the disease animals (Nassif et al., 2014). In accordance with these studies, our findings show that an inhibition of mTOR by rapamycin impairs larval movement and developmental viability of TBPH–/– flies, whereas mTOR activation with PA treatment benefits TBPH–/– flies (Fig 9), suggesting that a rescue of impaired mTOR activity which in turn suppresses the enhanced autophagy would have benefit for TDP-43-mediated neurodegeneration.

In our observations, immature autophagic vesicles strikingly accumulated in TDP-43-depleted cells and flies (Fig 6B and D, and Appendix Fig S5), similar to the phenomena in other neurodegenerative disease and ALS models (Wong & Cuervo, 2010; Iguchi et al., 2013; Chua et al., 2014), suggesting that an impairment of ALP in those models. Relevant to this, we observed that a depletion of TDP-43 has both mTORC1-dependent and mTORC1-independent effects on ALP (Fig 9D). Our data demonstrate that the autophagosomal and lysosomal biogenesis is increased through mTORC1 inhibition and TFEB activation in TDP-43-deficient cells, whereas the autophagosome–lysosome fusion, which is the later step of ALP, is impaired through a mTORC1-independent manner (a dynactin 1–associated manner) in those cells (Fig 7). Regarding the mTORC1-independent manner, our data showed that re-transfection of dynactin 1 could rescue the impaired autophagosome–lysosome fusion in TDP-43-deficient cells (Fig 7F and G), suggesting that TDP-43 deficiency could impair autophagosome–lysosome fusion by affecting dynactin 1. Our data indicate that impaired autophagosome–lysosome fusion might be a cause of defective autophagic degradation in TDP-43-depleted cells (Fig 6G). Importantly, we reason that as autophagosome synthesis is faster than the degradation in TDP-43-depleted cells (Fig 6G). Importantly, we reason that as autophagosome synthesis is faster than the degradation in TDP-43-depleted cells, whereas mTOR activation with PA treatment benefits TBPH–/– flies (Fig 9), suggesting that a rescue of impaired mTOR activity which in turn suppresses the enhanced autophagy would have benefit for TDP-43-mediated neurodegeneration.

In summary, our study demonstrates that TFEB nuclear translocation and sustained activation are caused by a decreased raptor level and an impaired mTORC1 signaling, leading to abnormal enhanced autophagy and lysosomal biogenesis in TDP-43-depleted cells. Moreover, more autophagosomes and lysosomes are accumulated in the cells since the fusion between autophagosomes and lysosomes is blocked due to dynactin 1 deficiency in those cells. This stressful situation in turn impairs cell homeostasis and induces neurological damage (Fig 9D). It is helpful for both understanding the basic mechanism in TDP-43-associated neurotoxicity and developing therapeutic treatments of related neurodegenerative disorders.

Materials and Methods

Plasmid constructs

The 3×FLAG-TFEB plasmid (Sardiello et al., 2009) was kindly provided by Dr. Andrea Ballabio (Telethon Institute of Genetics and Medicine, Italy). EGFP-N3-tagged TFEB was generated by excising full-length TFEF CDNA from 3×FLAG-TFEB and inserting it into the pEGFP-N3 (Clontech) vector at HindIII/BamHI sites. The point mutations and deletion mutants of 3×FLAG-TFEB were generated by the site directed mutagenesis using MutanBEST kit (Takara) with following primers: 5′-GACCCCAAGGCGAGTCACAGAT-3′ and 5′-AGGTCGCAAGGCAGCCTGCTTGTA-3′ for 3×FLAG-TFEB S211A in which the serine 211 was converted to alanine; 5′-GCGATCCGGGACACGCCC-3′ and 5′-CGCCATGCGAAAACCCTAT-3′ for 3×FLAG-TFEB Q10A/L11A in which the glutamine 10 and leucine 11 were converted to alanines; 5′-GCGCAAGATGTCACGCA-3′ and 5′-GCGCAAGATGTCACGCA-3′ for 3×FLAG-TFEB R245-247A in which the arginine 245-247 were converted to alanines; 5′-ATGACATATCGACGACGAGCCG-3′ and 5′-CCGCGCGCAAGCG-3′ for 3×FLAG-TFEB lacking 1–30 amino acids (A30). The point mutations and deletion mutants of EGFP-N3-TFEB were generated by excising TFEB mutants from above-mentioned 3×FLAG-TFEB constructs and inserting them into the pEGFP-N3 vector at HindIII/BamHI sites. HA-TDP-43 was created by subcloning TDP-43 PCR product from pGEX-5x-1-TDP-43 (Wang et al., 2010) into pHK3-HA vector at HindIII/XbaI sites. Plasmid-tagged TDP-43 was generated by excising TDP-43 cDNA from HA-TDP-43 and inserting it into the p3×FLAG-CMV24 vector at HindIII/XbaI sites. The deletion mutants of TDP-43-FX were generated by using the following primers: 5′-GTCGCAATTAATCAGCCAT-3′ and 5′-ACTTCTTTTATACTTGCTATT-3′ for FLAG-TDP-43 Δ274-314 (ΔGRD); 5′-GGACGATGTGCTGACTGCAA-3′ and 5′-GGATGTGTGACTGCAA-3′ and 5′-GGATGTGTGACTGCAA-3′ and 5′-GGATGTGTGACTGCAA-3′ and 5′-GGATGTGTGACTGCAA-3′ for FLAG-TDP-43 Δ194–257 (ΔGRM2); 5′-GGACGATGTGCTGACTGCAA-3′ and 5′-GGATGTGTGACTGCAA-3′ and 5′-GGATGTGTGACTGCAA-3′ and 5′-GGATGTGTGACTGCAA-3′ and 5′-GGATGTGTGACTGCAA-3′ for FLAG-TDP-43 Δ105–169 (ΔARRM1); 5′-GACCCCAAGGCGAGTCACGACG-3′ and 5′-GTCGCAATTAATCAGCCAT-3′ for FLAG-TDP-43 Δ194–257 (ΔARRM2); 5′-GTCGCAATTAATCAGCCAT-3′ and 5′-GTCGCAATTAATCAGCCAT-3′ for FLAG-TDP-43 Δ105–169 (ΔARRM1 + ΔARRM2). The following plasmids were obtained from Addgene: plasmid 1817, LAMP1-RFP; plasmid 8513, HA-raptor; plasmid 19300, pRK5-HA GST RagAGTP (RagA Q66L); plasmid 19305, pRK5-HA GST RagCGDP (RagC S75L); plasmid 22418, mCherry-EGFP-LC3. The CD63-EGFP plasmid was a kind gift from Dr. Shumin Duan (Zhejiang University, China), and the myc-tagged dynactin 1 (myc-dynactin 1) plasmid was a kind gift from Dr. Jia-Jia Liu (Institute of Genetics and Developmental Biology, CAS, China). FLAG-TDP-25 was created by subcloning PCR products using primers previously described (Wang et al., 2010). The EGFP-LC3, FLAG-p62, and Nhf150Q-EGFP expression vectors.
were described previously (Ying et al., 2009; Li et al., 2010; Zhou et al., 2013).

All constructs were confirmed by sequencing.

Antibodies

The following primary antibodies were used: anti-FLAG antibody (Sigma), anti-GFP antibody (Santa Cruz), anti-GAPDH antibody (Millipore), anti-tubulin antibody (Santa Cruz), anti-TDP-43 antibody described previously (Wang et al., 2010), anti-TDP-43 antibody (Proteintech), anti-HA (Santa Cruz), anti-histone 2B antibody (Epitomics), anti-phospho-p70S6K antibody (T389) (Cell Signaling Technology), anti-p70S6K antibody (Epitomics), anti-mTOR antibody (Cell Signaling Technology), anti-raptor antibody (Cell Signaling Technology), anti-RagB antibody (Cell Signaling Technology), anti-p18 antibody (Cell Signaling Technology), anti-TFPB antibody (Cell Signaling Technology), anti-LAMP1 antibody (Abcam), anti-cathepsin L antibody (Abcam), anti-LC3 antibody (Novus Biologicals), anti-Beclin-1 antibody (Abcam), anti-LAMP2 antibody (Abcam), anti-phospho-4E-BP1 antibody (T37/46) (Cell Signaling Technology), anti-phospho-p70S6K antibody (T389) (Cell Signaling Technology), anti-phospho-LC3 antibody (Cell Signaling Technology), and anti-p62 antibody (Enzo life science).

The following secondary antibodies were used: horseradish peroxidase-conjugated sheep anti-mouse and anti-rabbit antibodies (Amersham Pharmacia Biotech). The proteins were visualized with an ECL detection kit (Amersham Biosciences).

The following fluorescent secondary antibodies were used: anti-rabbit Rhodamine (Santa Cruz Biotechnology), Alexa Fluor 488 donkey anti-mouse IgG (Invitrogen), Alexa Fluor 488 donkey anti-rabbit IgG (Invitrogen), Alexa Fluor 594-conjugated AffiniPure Donkey Anti-mouse IgG (Invitrogen), and Alexa Fluor 594-conjugated AffiniPure Donkey Anti-rabbit IgG (Invitrogen).

Cell culture, transfection, and drug treatment

Human cervical cancer cells (HeLa), HeLa cells stably expressing EGFP-LC3 (Wang et al., 2010), human alveolar epithelial cells (A549), human embryonic kidney 293 cells (HEK 293), human embryonic kidney 293FT cells (HEK 293FT), mouse hippocampal neuronal cells (HT22), rat adrenal medulla cells (PC12), human neuroblastoma cells (SH-SY5Y), ATG5 WT, and KO MEF cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) containing 10% fetal bovine serum (FBS) (Gibco) with penicillin (100 mg/ml) and streptomycin (100 mg/ml). Cells were transfected with siRNAs using the RNAiMAX transfection reagent upon splitting and transfected with plasmids using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. Cells were starved with Earle’s balanced salt solution (Gibco) for 2 h and re-feeding with the RPMI-1640 medium (Gibco) supplemented with 10% FBS. Cells were treated with DMSO (Sangon Biotech), Torin-1 (250 nM) (Tocris Bioscience) for 1 h, bafilomycin A1 (100 nM) (Sigma) for 24 h or actinomycin D (1 µg/ml) (Sigma) for 1–4 h.

siRNAs

The following primers were used: human TDP-43: 5′-CAATGCTGAACCTAAGCA-3′, human TFEB: 5′-CAGAGGAGGTTTCAACATCA-3′, human raptor: 5′-TGGGCTAGCTGTTTTCGAA-3′, human raptor: 5′-GGAGGAAGCTTGGACATT-3′, human p18: 5′-CGAGAGACCCAGGCUU-3′, human RagA: 5′-CCAAUCUCGCCGUUCCAU-3′, human RagA: 5′-CCCGGACCAUCUUGAGAA-3′, human RagB: 5′-CCAGAGACCAUCUUGAGAA-3′, human RagB: 5′-GCCGUUGGACCAUCUUGUUTT-3′, human si-KIF5B: 5′-GCCGUUGGACCAUCUUGUUTT-3′, human si-DHC: 5′-GCCGUUGGACCAUCUUGUUTT-3′, human si-dynactin 1: 5′-CGAGGCTCAGTACCTAAGCA-3′, rat TDP-43: 5′-GAGGCTTGGACAGTTCAACATCA-3′, human TDP-43: 5′-TTCCCGGACCAUCUUGAGAA-3′, human TFEB: 5′-GGGAGAAGGTTTCAACATCA-3′, human raptor: 5′-TGGGCTAGCTGTTTTCGAA-3′, human raptor: 5′-GGAGGAAGCTTGGACATT-3′, human p18: 5′-CGAGAGACCCAGGCUU-3′, human RagA: 5′-CCAAUCUCGCCGUUCCAU-3′, human RagA: 5′-CCCGGACCAUCUUGAGAA-3′, human RagB: 5′-CCAGAGACCAUCUUGAGAA-3′, human RagB: 5′-GCCGUUGGACCAUCUUGUUTT-3′, human si-KIF5B: 5′-GCCGUUGGACCAUCUUGUUTT-3′, human si-DHC: 5′-GCCGUUGGACCAUCUUGUUTT-3′, human si-dynactin 1: 5′-CGAGGCTCAGTACCTAAGCA-3′, rat TDP-43: 5′-CAATGCTGAACCTAAGCA-3′, human TFEB: 5′-CAGAGGAGGTTTCAACATCA-3′, human raptor: 5′-TGGGCTAGCTGTTTTCGAA-3′, human raptor: 5′-GGAGGAAGCTTGGACATT-3′, human p18: 5′-CGAGAGACCCAGGCUU-3′, human RagA: 5′-CCAAUCUCGCCGUUCCAU-3′, human RagA: 5′-CCCGGACCAUCUUGAGAA-3′, human RagB: 5′-CCAGAGACCAUCUUGAGAA-3′, human RagB: 5′-GCCGUUGGACCAUCUUGUUTT-3′, human si-KIF5B: 5′-GCCGUUGGACCAUCUUGUUTT-3′, human si-DHC: 5′-GCCGUUGGACCAUCUUGUUTT-3′, human si-dynactin 1: 5′-CGAGGCTCAGTACCTAAGCA-3′.
Cells were washed with PBS and incubated with MTT (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) for 1 h. Then, the formazan crystals were dissolved in DMSO. Subsequently, the optical density (OD) was measured by a photometer at 570 nm, and background at 630 nm was subtracted.

**Quantitative real-time PCR (qRT–PCR) and RT–PCR**

Total RNA from Drosophila larvae, adult flies, HeLa, and SH-SY5Y cells were extracted with TRIzol reagent (Invitrogen) and then were reverse-transcribed into cDNA using PrimeScript RT Master Mix (Takara). The analysis of real-time PCR was used for SYBR Green Real-Time PCR Master Mix (Takara) with relevant primers using a CFX96 Real-Time System (Bio-Rad).

The following primers were used: human GAPDH: 5'-AGAGGAGCTG GAG-3' and 5'-GTCACTCTCGTAC-3', human RB1: 5'-GAGAG-3' and 5'-CCTCTTCACTC-3', human AKT: 5'-TGGATCTGTTGCTTGGATA-3' and 5'-TCTCTGTGATAGACCGTTCG-3', human Beclin-1: 5'-CTGTGGACCATCA-3' and 5'-CTGTAAGGATATAATG-3', human TDP-43: 5'-GGTG-3' and 5'-AGAATGATGAGC-3', human CTSD: 5'-TCTCTGTGATAGACCGTTCG-3', human ARSB: 5'-GTGTCCGTT-3', human ATG12: 5'-GTTCAGTATGTGGAACAG-3', human ATG16L1: 5'-CAGCAGCTTGAAGTTGAAGACCTG-3' and 5'-TGTGGACCATCA-3'.

**RNA immunoprecipitation**

HeLa cells were lysed in the polysome lysis buffer containing 100 mM KCl, 5 mM MgCl2, 10 mM HEPES (pH 7.0), 0.5% NP-40, 100 mM KCl, 5 mM MgCl2, and then were re-suspended with 100 mM KCl, 5 mM MgCl2, 10 mM HEPES (pH 7.0), 0.5% NP-40.

**Flow cytometry**

HeLa cells were digested with 0.05% trypsin for 2 min. The cells were harvested and washed with 1× PBS three times, and then the cells (10,000) were analyzed at 575 nm on a flow cytometer (Beckman Coulter, FC500).
Click-iT technology

Newly transcribed RNA was prepared and isolated according to the manual of Click-iT Nascent RNA Capture Kit (Life Technologies). Briefly, newly transcribed RNA in cells was metabolically labeled by the uridine analog 5-ethynyl uridine (EU) for 1 h, and then, EU was attached to biotin. The labeled RNA was collected on streptavidin beads and subjected to qRT–PCR analysis.

Fly stocks and culture

W1118 flies were obtained from Institute of Biochemistry and Cell Biology, SIBS, CAS. TBPH−/− flies were kindly provided by Dr. Francisco Baralle (International Centre for Genetic Engineering and Biotechnology, Italy). All fly stocks were kept on standard food (yeast, cornmeal, and molasses) according to standard procedures at 25°C.

Drosophila drug treatment

Rapamycin (50 µM, 100 µM or 200 µM) (Selleck) in DMSO or PA (phosphatidic acid) (5 mM or 10 mM) (Guidechem) in chloroform was added to Drosophila food. DMSO or chloroform alone was used as a control.

Larval movement

Thirty third-instar larvae per genotype were washed with distilled water and then placed on a LB agar plate. Larvae were allowed to recover for 30 s. Subsequently, the number of peristaltic waves was counted over two minutes. The average movement distance of the larvae per genotype was analyzed using ImageJ software.

Expanded View for this article is available online.

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Author contributions

QX, JH, ZY, and GW designed research; QX, HW, ZH, CF, QH, FG, HR, DC, and ZY performed experiments; QX, HW, ZY, and GW analyzed data; QX, HW, ZY, and GW wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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