Nonmuscle myosin IIB regulates Parkin-mediated mitophagy associated with amyotrophic lateral sclerosis-linked TDP-43

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Abstract
C-terminal fragments of Tar DNA-binding protein 43 (TDP-43) have been identified as the major pathological protein in several neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). However, how they affect cellular toxicity and neurodegeneration, including the modulation process remains unknown. This study revealed that the C-terminal fragment of TDP-43 (TDP-25) was localized primarily to mitochondria and caused abnormal mitochondrial morphology, inducing Parkin-mediated mitophagy. Also, we discovered that the knockdown of selective autophagy receptors, such as TAX1BP, Optineurin, or NDP52 caused TDP-25 accumulation, indicating that TDP-25 was degraded by mitophagy. Interestingly, myosin IIB, a nonmuscle type of myosin and actin-based motor protein, is mostly colocalized to TDP-25 associated with abnormal mitochondria. In addition, myosin IIB inhibition by siRNA or blebbistatin induced mitochondrial accumulation of insoluble TDP-25 and Tom20, and reduced neuronal cell viability. Our results suggest a novel role of myosin IIB in mitochondrial degradation of toxic TDP-25. Therefore, we proposed that regulating myosin IIB activity might be a potential therapeutic target for neurodegenerative diseases associated with TDP-43 pathology.

Introduction
Transactive response DNA-Binding Protein 43 (TDP-43, 43 kDa) has been characterized as a major component of cytoplasmic protein inclusion and surprisingly detectable in approximately 97% of patients with sporadic ALS (sALS) and familial ALS (fALS) or approximately 50–55% of frontotemporal dementia patients, indicating the possible link of TDP-43 dysfunction to neurodegeneration1. Pathogenic TDP-43 extracted from patient brains and spinal cords cleaves into C-terminal fragments (CTFs) and is hyperphosphorylated or ubiquitinated1. Interestingly, more than 50 pathogenic mutations in 3% of fALS cases were identified in the aggregate-prone C-terminal region of TDP-43. Pathological TDP-43 is also found in Alzheimer’s disease (AD), corticobasal degeneration, Parkinson’s disease, Huntington’s disease, or progressive supranuclear palsy, supporting its contribution to neurodegeneration2. TDP-43 is a multifunctional protein containing several domains, including a multimeric N-terminal domain, RNA recognition domains (RRMs), and a glycine-rich C-terminal domain. Physiologically, TDP-43 is predominantly localized to the nucleus. In addition to its nuclear localization, TDP-43 also localizes to the cytoplasm and colocalizes with subcellular compartments such as the endoplasmic reticulum (ER), mitochondria, mitochondria associated membranes (MAMs), RNA granules, and stress granules. It is involved in RNA metabolism, including gene transcription, RNA processing, RNA stability, RNA transport, microRNA
biogenesis, and stress granule formation, regulation of ER-
mitochondrial tethering, or mitochondrial protein trans-
lation. In pathological conditions, TDP-43 is redis-
tributed to the cytoplasm and sequestered into inclusions,
where it is phosphorylated, ubiquitinated, and proteoly-
tically cleaved to generate 25-kDa and 35-kDa CTFs.
Accumulating evidence suggests that TDP-43 CTFs
recapitulate the pathological features of disease in vitro
and in vivo, including TDP-43 ubiquitination, hyperpho-
sphorylation, and cytoplasmic insoluble protein aggre-

gates. Notably, many postmortem studies have shown
that TDP-43 CTFs characterized disease pathology in the
frontal and temporal regions of the brain but are rarely
detected in the spinal cord, raising the possibility of its
toxicity in those brain regions. In silico analysis also
indicates that the C-terminal region of TDP-43 contains a
disordered peptide sequence called a prion-like domain,
which is aggregate-prone under some pathological con-
ditions. These pathogenic CTFs are toxic to neurons or

glial cells in vitro and in vivo, inducing protein aggregate
accumulation, sequestration of endogenous TDP-43,
neuronal loss, or motor or cognitive deficit.

Evidence also suggests that as prominent early patho-
logical features, mitochondrial abnormalities are closely
associated with pathologically related TDP-43 in patients
with ALS and FTD, as well as experimental models. Recent-
ly, it has been reported that an increased expres-
sion of TDP-43 or TDP-43 CTFs induced mitochondrial
dysfunction, including calcium homeostasis alteration,
decreased mitochondrial membrane potential, and
increased production of reactive oxygen species (ROS),
suggesting a link between TDP-43 pathology and mito-
chondrial dysfunction or neurodegeneration.

Recent reports have described the role of actin-
cytoskeletal and actin-associated proteins in mitochon-
drial fission, transport, or the mitochondrial quality sys-
tem. Among actin-associated proteins, the actin-



Based motor protein nonmuscle myosin II is critically
involved in actin cytoskeleton organization and cellular
motility. A recent study has shown its new role in mito-
chondrial fission, suggesting its role in mitochondrial
regulation. Myosin II is abundantly expressed in the
adult nervous system and has three distinct isoforms (A,
B, and C), which are also present in isolated postsynaptic
densities of mature forebrain synapses. Disrupting myosin
II activity in cultured neurons alters the dendritic spine
development. Recently, a genetic mutation in Myh14
(Myosin IIC) was associated with impaired axonal trans-
port of nonfragmented mitochondria, thereby implicating
it in neurodegeneration. However, the role of myosin II
in neurodegeneration associated with TDP-43 and its link
to mitochondrial dysfunction remains unknown.

In this study, we used FTD/ALS linked CTFs of TDP-43
(25 kDa) to investigate the role of myosin IIB in
mitochondrial dysfunction and neurotoxicity associated
with TDP-43 in cultured cortical neurons. TDP-25 was
localized to abnormal mitochondria, causing their dys-
function. Furthermore, cytosolic Parkin was recruited to
TDP-25 associated with abnormal mitochondria. Selective
siRNA knockdown of autophagy receptors TAX1BP, Optineurin, or NDP52 induced the accumulation of
insoluble TDP-25 associated with damaged mitochondria,
thereby indicating their involvement in mitophagy. We
found that myosin IIB was localized to TDP-25 associated
with abnormal mitochondria, whereas its inhibition failed
to colocalize with TDP-25. Inhibition by myosin IIB
siRNA reduced mitophagy and induced the accumulation
of insoluble TDP-25 protein in the mitochondrial frac-
tion, thus inducing neuronal cell death. Therefore, we
proposed a novel role of myosin IIB in cellular degrada-
tion of toxic TDP-25 associated with mitophagy and
proposed that modulating the activity of myosin IIB may
be a potential strategy to alleviate TDP-43-induced
cytotoxicity in several neurodegenerative diseases with
TDP-43 pathology.

Materials and methods

Autophagic flux assay

To monitor autophagy flux, HEK293T cells were
with NH₄Cl (10 mM) or BafA1 (100 nM) and
incubated at 37 °C for 6 h before harvesting the cells
using a RIPA buffer (50 mM Tris-HCl (pH 7.5),
150 mM NaCl, 0.5% sodium deoxycholate, 0.5% SDS,
0.1% NP40, phosphatase and protease inhibitors). Cell
lysates were sonicated and centrifuged at 13,000 rpm
for 15 min at 4 °C. Protein concentrations were calcu-
lated using the BCA assay kit (Thermo Fisher Scien-
tific, #23227, USA).

Mitochondrial membrane potential measurement

Mitochondrial membrane potential was visualized
in cells with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetra-
ethylbenzimidazolcarbocyanine iodide (JC-1) (Invitro-
gen, #T3168, USA), which is a cationic, lipophilic
Fluoroprobe. In healthy mitochondria (high membrane
potential), JC-1 enters the mitochondria and forms red
fluorescent aggregates (J-aggregates). As membrane
potential decreases (depolarized mitochondria), JC-1
becomes green fluorescent monomers (JC-1 monomer).
Thus, the higher the ratio of red to green fluorescence,
the higher the mitochondrial membrane polarization.
The measurement of mitochondrial membrane potential
with JC-1 was performed according to the manu-
ufacturer’s instructions. Briefly, the cells were grown in a
confocal dish and treated with 2 mg/mL JC-1 dye in
DMEM medium for 20 min at 37 °C and 5% CO₂. Then,
the cells were observed using a confocal microscope
(Carl Zeiss, LSM880, Germany).
Results

The 25-kDa C-terminal fragment of TDP-43 is colocalized to Tom20-positive abnormal and damaged mitochondria

Despite the presence of TDP-43 CTFs in brain tissue, their cellular pathogenic effects are still controversial in different cellular and animal models. Furthermore, many postmortem studies have shown that TDP-43 CTFs characterized disease pathology in the frontal and temporal regions of the brain but are rarely detected in the spinal cord, thereby raising the possibility of its toxicity in those brain regions.

Therefore, to investigate the cellular pathogenic effect of TDP-43 CTF (TDP-25) in cortical neurons, we examined its cellular localization in cultured cortical neurons. Since TDP-43 localizes to stress granules (SGs) upon cellular stress, we first examined whether Myc-TDP-25 is localized to SGs upon oxidative stress with sodium arsenite (SA, 0.5 mM, 1 h 30 min) using GFP-G3BP (an SG marker protein). Observably, Myc-TDP-43 (full-length) localized to G3BP-positive SGs upon oxidative stress, suggesting its role in stress response (Supplementary Fig. S1A, B). However, Myc-TDP-25 failed to colocalize with GFP-G3BP-positive SGs, thereby raising the possibility of impairment on its stress response in cortical neurons (Supplementary Fig. S1C, D). To further characterize its abnormal cellular localization, Myc-TDP-25 was transfected with an ER marker, GFP-Sec61, a Golgi apparatus marker, GFP-GaIT, or the mitochondrial marker GFP-Tom20 in cultured cortical neurons without stress conditions. As shown in Fig. 1a, b and Supplementary Fig. S2A, Myc-TDP-25 was mostly colocalized to GFP-Tom20-positive or Mitofusin2 (Mfn2)-positive mitochondrial structures but not to other ER or Golgi structures. As shown in Fig. 1a, b, we confirmed that Myc-TDP-25 colocalized to Mitotracker-positive mitochondria, indicating that Myc-TDP-25 was mislocalized to the mitochondria. Also, we confirmed that TDP-25 without a Myc-tag colocalized to Tom20-positive mitochondria (Supplementary Fig. S2B).

Next, to examine which domains within TDP-25 are required for mitochondria localization, we generated serial deletion mutants. Deleting the glycine-rich domain but not the NES domain impeded localization to the mitochondria (Fig. 1c–e), indicating that the TDP-25 glycine-rich domain is required for localization.

Furthermore, we examined the mitochondrial morphology in Myc vector or Myc-TDP-25 expressing neurons. From Supplementary Fig. S3A, compared with control neurons expressing Myc vector, neurons expressing Myc-TDP-25 showed fragmented mitochondria. Observably, the number of neurons with fragmented mitochondria was significantly increased compared with that of control neurons (Supplementary Fig. S3B).

To examine the mitochondrial structure at the ultrastructural level in Myc-TDP-25 expressing HEK293 cells, we performed electron microscopic analysis. As shown in Fig. 1f, observably, defective mitochondria showing swelling and enlargement were significant in Myc-TDP-25 expressing cells, while long and thin healthy mitochondria were abundant in control cells expressing Myc. Quantitative analysis of defective mitochondria with abnormal morphology in Myc-TDP-25 expressing cells and control cells showed that the Myc-TDP-25 expression significantly caused mitochondrial damage, thereby raising its possible link to mitochondrial dysfunction (Fig. 1g).

We used JC-1 dye (a mitochondrial membrane potential indicator), a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a
fluorescence emission shift from green (~525 nm) to red (~590 nm). Consequently, a decrease in the red/green fluorescence intensity ratio indicates mitochondrial depolarization. The potential-sensitive color shift is due to the concentration-dependent formation of red fluorescent J-aggregates. Indeed, CFP-expressing control neurons treated with carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (10 μM, 24 h) showed the potential-sensitive color shift (Fig. 1h, i). Interestingly, mitochondrial membrane potential detected using JC-1 dye was significantly altered in CFP-TDP-25 expressing cells without CCCP treatment compared with control cells expressing CFP (Fig. 1h, i). Collectively, our results demonstrate that TDP-25 is localized to mitochondria, induces both abnormal mitochondrial morphology and damage.

**Toxic TDP-25 recruits Parkin into damaged mitochondria and is degraded by selective mitophagy**

Based on our cellular analysis, TDP-25 was associated with MFN2 and accumulated in damaged mitochondria. Thus, cells expressing TDP-25 might induce mitophagy as a quality control system to remove damaged mitochondria. Next, we investigated whether Parkin-mediated mitophagy is involved in cells expressing Myc-TDP-25.

First, we examined the cellular localization of parkin, which is a well-known protein localized to mitochondria in response to mitochondrial malfunction in Parkin-dependent mitophagy in cultured cortical neurons expressing Myc-TDP-25. As shown in Fig. 2a–c, GFP-Parkin was mostly recruited into Tom20-positive mitochondria associated with Myc-TDP-25 and was mostly diffused in the cytoplasm of cells expressing Myc, suggesting that Myc-TDP-25 expression induced Parkin-dependent mitophagy. Indeed, as shown in Fig. 2d, e, endogenous LC3A/B, GABARAP-L1 as mammalian autophagosome markers, or phosphorylated p62 as an autophagy adapter is localized to TDP-25-positive mitochondria.

To determine whether mitophagy is responsible for degrading insoluble TDP-25 with damaged mitochondria, we examined the protein level of insoluble TDP-25 by...
knockdown of mitophagy receptors such as Tax1BP, Optineurin, or NDP52 by siRNA targeting in Myc-TDP-25 expressing cells. As shown in Fig. 2f, g, knockdown of mitophagy receptors such as TAX1BP, Optineurin, or NDP52 by siRNA increased insoluble TDP-25 protein, suggesting that Parkin-mediated mitophagy is responsible for reducing insoluble TDP-25 associated with damaged mitochondria. Therefore, our results indicate that Myc-TDP-25 activates Parkin-mediated autophagy and is degraded by selective mitophagy.

**Myosin IIB, an actin-based motor protein, is localized to TDP-25-positive abnormal mitochondria**

Recent studies have raised the possible role of myosin II in mitochondrial fission. Nonmuscle myosin II is found adjacent to mitochondria but is not specifically enriched at the constriction sites, thereby supporting its involvement in mitochondrial regulation. However, its role in regulating damaged mitochondria associated with neurodegeneration and mitophagy is barely known. Myosin II has three distinct isoforms, myosin IIA, IIB, and IIC. We first examined the mRNA expression level of each myosin II in induced pluripotent stem cell-derived postmitotic human neurons. From Supplementary Fig. S4, myosin IIB is highly expressed in neurons compared with myosin IIA or myosin IIC. Therefore, in this study, to identify myosin IIB contribution in regulating damaged mitochondria or insoluble TDP-25, we examined its cellular localization. Interestingly, cytoplasmic TDP-43 is partially localized to the GFP-myosin IIB. However, GFP-myosin IIB is mostly colocalized to TDP-25 and insoluble TDP-25 expressed in myc-TDP-25. As shown in Fig. 3a, b, Myc-TDP-43 is mostly localized to the nucleus with some localization in the cytoplasm. Therefore, our results indicate that TDP-25 activates Parkin-mediated autophagy and is degraded by selective mitophagy.
compared with Myc-TDP-43, supporting their colocalization data (Fig. 3c).

Next, to determine its role in damaged mitochondria associated with TDP-25, we inhibited myosin IIB with blebbistatin, which can inhibit its ATPase activity in neurons expressing Myc-TDP-25. Interestingly, myosin IIB inhibition reduced the colocalization of myosin IIB with TDP-25 and mitochondria significantly accumulated (Fig. 3d–f, i, j). These data suggest that the myosin IIB ATPase activity is required for myosin IIB association with TDP-25 in mitochondria.

**Myosin IIB Inhibition impairs mitophagic degradation of TDP-25 or Tom20 and reduces viability in cells expressing Myc-TDP-25**

To determine the myosin IIB effect on Parkin-mediated mitophagy caused by TDP-25, we examined the Tom20 protein level, which is a mitochondrial protein and Myc-TDP-25 in cells expressing Myc-TDP-25 after myosin IIB inhibition. To do this, myosin IIB or myosin VI was knocked down by its specific siRNA, and Western blotting was performed using the mitochondrial fraction. Myosin VI, as the other myosin protein family, was also used since a recent study showed that myosin VI is involved in CCCP-induced mitophagy. Surprisingly, Myc-TDP-25 and Tom20 accumulated 48 h after siRNA transfection in the presence of NH$_4$Cl. The bar graphs indicate the percentage of normalized protein levels of Myosin IIB, Myosin VI, Myc-TDP-25, or Tom20 with β-actin. Cellular images showing mitotracker-positive mitochondria expressing Myc-TDP-25 in the presence or absence of blebbistatin in cultured cortical neurons. Scale bar; 20 μm. The bar graph indicates the ratio of mitotracker-positive mitochondrial area per cell expressing Myc-TDP-25 in the presence or absence of blebbistatin. *p < 0.05. Values represent mean ± SEM (n ≥ 13).

Furthermore, when we inhibited lysosomal degradation with NH$_4$Cl in cells expressing Myc-TDP-25 in the presence of myosin IIB or myosin VI siRNAs, no changes in the protein level of Myc-TDP-25 or Tom20 were observed, indicating that the degradation of Myc-TDP-25 or Tom20 by mitophagy was blocked by inhibition of myosin IIB or myosin VI. We examined the mitochondrial level by myosin IIB inhibition with blebbistatin to determine whether myosin could control mitophagy associated with TDP-25. Observably, blebbistatin caused mitochondrial accumulation in neurons expressing Myc-TDP-25 (Fig. 3i, j). Therefore, our data indicate that these myosin IIB motor proteins regulate mitophagy associated with ALS linked to TDP-43.
myosin IIB inhibition can affect neuronal cell viability in neurons expressing Myc-TDP-25 treated with blebbistatin, we quantified cells with DNA fragmentation with DAPI in neurons expressing Myc-TDP-25 in the presence or absence of blebbistatin. Neuronal cell viability was significantly reduced by myosin IIB inhibition with blebbistatin in neurons expressing Myc-TDP-25 (Fig. 3k). However, no significant difference in cell survival was observed between Myc expressing control neurons treated with DMSO or blebbistatin (Supplementary Fig. S5A, B). Our results suggest a novel regulatory role of myosin IIB in TDP-43 associated with Parkin-mediated mitophagy and represent a potential new therapeutic target in several neurodegenerative diseases linked to TDP-43 pathology.

Discussions

Accumulating evidence indicates that multiple mitochondrial pathways are perturbed by pathological TDP-43, such as its C-terminal fragment, including mitochondrial dynamics, trafficking, bioenergetics, and mitochondrial quality control, suggesting mitochondria as likely targets of TDP-43 proteinopathy. However, how pathogenic TDP-43 affects mitochondrial abnormality and modulation of mitochondrial damage/dysfunction associated with TDP-43 is barely known. Despite the evidence that mitochondrial dysfunction underlies the pathogenesis of TDP-43–related ALS and the potential role of myosin II in mitochondrial dynamics, the roles of myosin II in mitochondrial dysfunction in TDP-43 pathology are unknown.

In this study, we characterized a novel role of nonmuscle myosin IIB in regulating pathogenic TDP-25 associated with damaged mitochondria and neurodegeneration. We found that TDP-25 was localized to mitochondria with an abnormal morphology inducing an altered mitochondrial membrane potential, strongly supporting the causative link between TDP-43 pathology and mitochondrial dysfunction. Our deletion study (Fig. 1c–e) showed that the glycine-rich domain is required for TDP-25 cellular localization into mitochondria. It has been reported that the glycine-rich domain is involved in protein–protein interaction. Mitochondrial proteins probably associate with TDP-25 for mitochondrial targeting of TDP-25. Among mitochondrial proteins, MFN2 represents a key player in these mitochondrial activities (fusion, trafficking, turnover, contacts with other organelles), the balance of which results in the appropriate mitochondrial shape, function, and distribution within the cell.

Furthermore, during mitophagy, MFN2 recruits Parkin into damaged mitochondria and it has been recently reported that MFN2 interacts with TDP-43. Therefore, we investigated whether TDP-25 is colocalized to MFN2 in postmitotic neurons. Myc-TDP-25 was mostly colocalized to cytosolic MFN2 (Supplementary Fig. S2).

Our electron microscopy analysis showed that TDP-25 expression induced abnormal mitochondrial morphology with swelling and enlarged without cristae. In a recent study, electron microscopy of patient samples revealed considerable mitochondrial impairment, including abnormal cristae and cristae loss; these ultrastructural changes were consistently observed in both our and other cellular or animal models of TDP-43 proteinopathy. In these previous studies, increased TDP-43 expression induced mitochondrial dysfunction, suppressed mitochondrial complex I activity, and reduced mitochondrial ATP synthesis.

The maintenance of a healthy and functional mitochondrial network is essential for the development, as well as diverse biological processes in response to physiological adaptations and stress conditions. Mitophagy is a major mitochondrial quality system that eliminates damaged mitochondrial proteins or parts of the mitochondrial network. In this and other studies, TDP-25 aggregates seem to be associated with Parkin-dependent mitophagy by recruiting mitophagy components such as parkin, p62, or mitophagy receptors. Also, CTF of TDP-43 activates mitophagy to rescue mitochondrial dysfunction. Other studies have shown that TDP-43 interacts with and regulates MFN2 and Prohibitin 2, thus affecting mitochondrial dynamics and mitophagy.

Although considerable progress has been made in elucidating the actin cytoskeleton involvement in regulating mitochondrial network dynamics and fission/fusion events, cellular cytoskeletal components to regulate mitophagy associated with TDP-43 pathology are unknown. Surprisingly, from our results, myosin IIB inhibition induced a significant accumulation of TDP-25 and Tom20 by inhibiting selective mitophagy. Indeed, this inhibition also aggravated neuronal cell death by accumulating damaged mitochondria associated with toxic TDP-25.

Myosins as molecular motors can regulate the dynamics of actin filaments and cellular transport/trafficking of cellular components. Myosin IIB superfamily comprises 40 myosin genes classified into 12 classes. In mammals, three isoforms of nonmuscle myosin II are expressed (nonmuscle myosin IIA, IIB, and IIC). Nonmuscle myosin II regulates cell migration and protrusion, cell adhesion, and cytokinesis. Recent studies have shown that myosin IIB regulates mitochondrial fission. In our study, myosin IIB associates with TDP-25 and is redistributed to TDP-25–positive mitochondria. How does the myosin IIB regulate selective mitophagy in cells expressing Myc-TDP-25? Redistributed myosin IIB might facilitate mitochondrial fission, which can accelerate selective mitophagy.
associated with toxic TDP-25. Therefore, our study showed that its inhibition caused TDP-25 accumulation in damaged mitochondria. However, further study is required to determine myosin IIB importance in the spatial and temporal regulation of mitophagy and mitochondrial quality control in pathogenic conditions associated with TDP-43.

Our study has simultaneously uncovered a previously unknown role of myosin II in regulating mitophagy associated with TDP-43 pathology and advanced our understanding of the pathogenic mechanisms for TDP-43 proteinopathy regarding selective mitophagy. Therefore, our study suggests that regulating myosin II activity provides a therapeutic approach to several neurodegenerative diseases shown in TDP-43 pathology.

Acknowledgements
J.-A.L. was supported by the Basic Research Program of the National Research Foundation (NRF-2020R1A2C2005021), the Neurological Disorder Research Program (NRF-2020M3E5D9079908), the Bio and Medical Technology Development Program of the NRF funded by the Ministry of Science and ICT (2017M3A9G7073521), and Science Research Center Program of the NRF funded by the Ministry of Science and ICT (2020R1A5A1019023). J.K. was supported by KBI basic research program through Korea Brain Research Institute funded by the Ministry of Science and ICT (20-DR-02-05).

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Conflict of interest
The authors declare that they have no conflict of interest.

Publisher’s note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary Information accompanies this paper at (https://doi.org/10.1038/s41419-020-03165-7).

Received: 21 July 2020 Revised: 22 October 2020 Accepted: 23 October 2020 Published online: 05 November 2020

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