



Multiple motor proteins regulate TDP-43 anterograde axonal transport

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Abstract

TAR DNA-binding protein 43 (TDP-43) is an RNA-binding protein essential for RNA metabolism. Under physiological conditions, it predominantly resides in the nucleus but is also expressed in the cytoplasm, where it regulates mRNA trafficking and local translation. In nearly 97% of amyotrophic lateral sclerosis (ALS) cases, TDP-43 undergoes nuclear depletion and cytoplasmic aggregation. While its nuclear functions are well characterized, its axonal roles remain poorly understood, despite axonal degeneration being a hallmark of ALS pathology. To address this gap, we investigated TDP-43 localization and transport dynamics in axons of H9-derived human neurons. We compared fluorescently labeled TDP-43 with three well-characterized axonal cargoes, ras-related protein Rab5, synaptophysin, and amyloid precursor protein, and examined protein–protein interactions between TDP-43 and the axonal transport machinery. Our analyses revealed that TDP-43 exhibits active anterograde axonal transport and interacts with multiple kinesin motor proteins, including all three KIF5 isoforms, through the adaptor KLC1, and the synaptic vesicles motor KIF1A. This multi-motor engagement suggests a flexible transport system that ensures mRNA delivery to distal axons. In ALS, where TDP-43 accumulates abnormally in the cytoplasm, this flexibility may become compromised, with multiple transport mechanisms simultaneously affected. This could contribute to progressive accumulation of non-functional TDP-43 granules, disrupting mRNA trafficking and local translation. Our findings provide a foundation for understanding how physiological TDP-43 transport mechanisms may be impaired during disease, highlighting axonal TDP-43 transport pathways as potential therapeutic targets.

Keywords TDP-43 · Axonal transport · Amyotrophic lateral sclerosis · Motor proteins · Adaptors · Cargoes

Abbreviations

ALS	Amyotrophic lateral sclerosis	C9orf72	C9 open reading frame 72
APP	Amyloid precursor protein	DCTN1	Dynactin subunit 1
aCSF	Artificial cerebrospinal fluid	DIC1	Dynein intermediate chain 1
		FTLD	Frontotemporal lobar degeneration

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FUS	Fused in sarcoma
GFP	Green fluorescent protein
hNSCs	Human neural stem cells
hnRNPC	Heterogeneous nuclear ribonucleoprotein C
iPSC	Induced pluripotent stem cell
KHC	Kinesin heavy chain
KIF1A	Kinesin family member 1A
KIF5A/B/C	Kinesin family member 5A/B/C
KLC1	Kinesin light chain 1
LATE	Limbic-predominant age-related TDP-43 encephalopathy
LMN	Lower motor neuron
LV	Lentiviral
MLO	Membraneless organelle
mRNA	Messenger RNA
MyoV	Myosin V
NfL	Neurofilament light chain
NMJ	Neuromuscular junction
NOMc	Complete neuronal optimized medium
NT	Non-transduced
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PLA	Proximity ligation assay
pNfH	Phosphorylated neurofilament heavy chain
PVDF	Polyvinylidene difluoride
Rab5	Ras-related protein Rab5
RBP	RNA-binding protein
RNP	Ribonucleoprotein
shRNA	Short hairpin RNA
STMN2	Stathmin-2
SYP	Synaptophysin
TDP-43	TAR DNA-binding protein 43
tGFP	TurboGFP
UBQLN2	Ubiquilin-2
UMN	Upper motor neuron
UNC13A	Unc-13 homolog A

Introduction

TAR DNA-binding protein 43 (TDP-43) is a ubiquitously expressed and highly conserved RNA-binding protein (RBP) essential for RNA metabolism [5, 6]. TDP-43 shuttles between the nucleus and cytoplasm, but predominantly localizes to the nucleus under physiological conditions [19, 27]. Here, TDP-43 is an important regulator of transcription and splicing for a wide range of RNAs [92]. In the cytoplasm, TDP-43 supports trafficking and local translation of several messenger RNAs (mRNAs) in proximal and distal axonal and dendritic compartments, including its own [3, 14, 78].

TDP-43 pathology is a hallmark of several neurodegenerative diseases, most notably amyotrophic lateral

sclerosis (ALS) and Frontotemporal Lobar Degeneration with TDP-43 pathology (FTLD-TDP-43), and limbic-predominant age-related TDP-43 encephalopathy (LATE) [71]. It has also been observed in Alzheimer's disease [1], Huntington's disease [74], and several other neurodegenerative disorders. In approximately 97% of sporadic ALS (sALS) cases, TDP-43 is depleted from the nucleus and abnormally accumulates in the cytoplasm, forming aggregates that can contribute to neuronal dysfunction and death [6, 50, 80]. This dual pathology, characterized by nuclear depletion and cytoplasmic accumulation, disrupts TDP-43 function in both compartments. In the nucleus, TDP-43 normally regulates transcripts encoding stathmin-2 (STMN2), essential for axonal growth and microtubule dynamics, and UNC13 homolog A (UNC13A), which mediates synaptic vesicle priming at the neuromuscular junctions (NMJs) [16, 54, 69]. Loss of nuclear TDP-43 leads to cryptic exon splicing in these transcripts, resulting in non-functional proteins that compromise structural axonal stability and synaptic transmission [8, 14, 53, 76, 95]. Concurrently, cytoplasmic accumulation of TDP-43 impairs transport and local translation of mRNAs encoding cytoskeletal proteins such as neurofilament light chain (NfL) [2], as well as synaptic and nuclear-encoded mitochondrial proteins at NMJs [3, 15, 51], and ribosomal-associated protein mRNAs [70], among others. These combined deficits, loss of nuclear splicing regulation and impaired cytoplasmic mRNA transport and translation, may drive the axonal degeneration characteristic of ALS.

In the cytoplasm, TDP-43 associates with specific mRNA pools to form ribonucleoprotein (RNP) complexes, which assemble into what are currently defined as membraneless organelles (MLOs) [12, 17, 39, 65, 97]. These MLOs undergo long-distance transport along microtubules, facilitated by motor and adaptor proteins [84, 96]. Studies have revealed that some TDP-43-containing RNPs also 'hitchhike' on organelles to reach distal neuronal regions [29, 60]. Though, whether TDP-43-containing RNPs rely solely on this non-canonical transport mechanism, via organelle interaction, or can directly interact with adaptor and motor proteins remains unclear.

Despite extensive characterization of TDP-43's nuclear functions, its cytoplasmic role and axonal transport mechanisms remain poorly understood. It is unclear which motor proteins mediate TDP-43 trafficking in the axonal compartment, how this transport regulates mRNA delivery to distal subdomains, and at what level the disruption of these mechanisms contributes to ALS pathology [18, 26, 30]. Understanding TDP-43 dynamics within axons may provide crucial insight into early pathogenic events and either support or challenge the *dying-back* model of ALS, in which distal axonal dysfunction and synaptic impairment precede and potentially drive motor neuron degeneration [13, 32, 58].

To address these questions, we investigated the axonal transport dynamics of TDP-43 under physiological conditions. Using human neurons derived from the H9 embryonic stem cell line (H9-derived), we compared the movement of fluorescently labeled TDP-43 with three well-characterized axonal cargoes, ras-related protein Rab5 (Rab5), synaptophysin (SYP), and amyloid precursor protein (APP), each with distinct motor dependencies and directional bias linked to its role and localization in the axons. Through live-cell imaging, followed by segmental trajectory analysis, we demonstrated that TDP-43 exhibits processive anterograde transport with dynamics comparable to vesicular cargoes. Additionally, using both biochemical and proximity ligation approaches, we showed that TDP-43 engages with both anterograde and retrograde transport machinery, consistent with its bidirectional profile. Because anterograde transport is regulated by multiple kinesin isoforms [46], while retrograde transport is controlled by a single motor protein complex characterized by the dynein-dynactin complex [36, 68], we specifically examined which kinesin motors associate with TDP-43. We found that TDP-43 engages with multiple kinesin motor proteins, including all three kinesin-1 family isoforms, kinesin 5A, B, and C (KIF5A, B, and C), through the adaptor kinesin light chain 1 (KLC1), as well as the kinesin-3 family member kinesin 1A (KIF1A). In addition to previous finding describing alternative transport models [29, 33, 60], this multi-motor engagement suggests a flexible TDP-43-containing MLOs transport system that ensures robust mRNA delivery to diverse axonal compartments, and that due to changes in TDP-43 fitness might be selectively affected under pathological conditions.

Materials and methods

Differentiation of human neurons from an embryonic stem cell line

Human neural stem cells (hNSCs) derived from the NIH-approved H9 (WA09) human embryonic stem cell line were purchased from Merck (Germany). The hNSCs were plated on 100 mm Petri dishes precoated with Matrigel (Corning) for 1 h. Cells were then maintained in culture with NSCs expansion media (KO DMEM/F-12, 2% StemPro Neural Supplement, 1% Glutamax, 20 ng/ml β -FGF, 20 ng/ml EGF), which was exchanged every second day (day in vitro—DIV 0–3). Upon reaching confluency, we performed a complete media change to favor neural progenitors' induction (DMEM/F-12, 1% B27, 0.5% N2, 1% Glutamax) by feeding the cells every other day until DIV 9. Neural progenitors were then detached with accutase (Thermo Fisher), centrifuged at 300 $\times$ g for 5 min at room temperature (R.T.), and the pellet was resuspended in Neuronal Optimized Media

complete (NOMc) (DMEM/F-12, 2% B27, 1% N2, 1 μ g/ml laminin, 100 nM cAMP, 200 ng/ml ascorbic acid, 10 ng/ml BDNF, 10 ng/ml GDNF, 10 ng/ml IGF). Cells were counted and then seeded at different densities depending on the experimental needs. Neurons were cultured up to DIV 40 by feeding them with NOMc every 6 days.

Plasmid design and preparation for the over-expression of fluorescently labeled proteins for axonal transport studies

Plasmids overexpressing TDP-43, Rab5, SYP, and APP were designed in a pcDNA 3.1(+) backbone, under a cytomegalovirus (CMV) promoter and green fluorescent protein (GFP) tag placed for all the constructs in the C-terminal region, except for Rab5 where the fluorescent tag was placed at the N-terminal. The design was then transferred to GenScript (GenScript Biotech, New Jersey, US), which manufactured the stock plasmids. DH5 α bacterial competent cells (Thermo Fisher) were transformed by performing the following protocol: (i) bacterial cells were retrieved from -80°C and slowly thawed on ice, then the desired amount of stock DNA relative to each plasmid was incubated with the DH5 α competent cells; (ii) cells and DNA were incubated for 30 min on ice, then the mix exposed for 1 min at 42°C , and finally again on ice for 5 min; (iii) after that, Super Optimal broth with Catabolite repression (S.O.C.—Merck-Sigma—made of 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl_2 , 10 mM MgSO_4 , and 20 mM glucose) was added to the mix to reach a final volume of 500, and the mix was incubated for 1 h at 37°C shaking at 250 rpm; (iv) after the incubation cells were centrifuged a 5000 $\times$ g for 3 min and almost all the supernatant was discarded except for 50 μ l used for pellet resuspension; (v) mixed samples were plated independently on 100 mm Petri dishes completed with Luria Broth (LB) agar containing ampicillin (1 μ g/ μ l) to guarantee the selective growth of transformed bacteria. After 24 h of incubation up to 3 colonies per plasmid were selected and DNA was purified to be screened by gel electrophoresis. Gels were prepared with 2% agar to evaluate the correct size of the inserted plasmid. Next, in-gel DNA digestion was performed, and the purified DNA was sent for sequencing analysis of the open reading frames (ORFs) of interest. At last, the colonies selected per each plasmid were amplified to generate a long-term transformed bacteria stock stored at -80°C . The cells were grown into a liquid LB medium containing ampicillin (1 μ g/ μ l). On the second day, we performed a maxi prep DNA isolation according to the Qiafilter Plasmid Maxi kit (Qiagen) protocol with which DNA was purified. Plasmids were aliquoted for long term at -20°C , and transfections were performed always with the same mother DNA samples.

Plasmids transfection approach

All plasmids were transfected using the same protocol. 48 h before live-cell imaging of fluorescently labeled proteins, neurons were transfected with a 1:1 ratio of Lipofectamine 2000 (Thermo Fisher) and 1 µg of DNA of interest:

- pcDNA3.1(+)-TDP-43_GFP
- pcDNA3.1(+)-GFP_Rab5
- pcDNA3.1(+)-SYP_GFP
- pcDNA3.1(+)-APP_GFP

For the ibidi multichannel slide, we used 50 µl of transfection mix per channel, for a total of 300 µl of the mix for all 6 channels. The transfection mix was prepared according to the manufacturer's suggestion. In brief, 2 different tubes were prepared to incubate separately at R.T. for 5 min both Lipofectamine 2000 and the DNA of interest using NOMc. Then the two mixes were combined to form a final transfection mix, which was incubated for 20 min at R.T. prior to being added to the neuronal culture. The solution was kept in cell cultures for 2 h at 37 °C and 5% CO₂. After transfection, the Lipofectamine-containing solution was completely replaced with fresh NOMc, and cells were examined under a fluorescent microscope 24 h post-transfection.

Lentiviral vectors transduction for protein–protein interaction experiments

Lentiviral (LV) constructs expressing short hairpin RNA (shRNA) targeting KLC1 with the fluorescent reporter TurboGFP (LV-shKLC1-tGFP), or a scrambled shRNA control with tGFP (LV-scramble-tGFP), were designed for expression under the human synapsin 1 (hSyn1) promoter, and were cloned and packaged by OriGene (Rockville, MD, US). Human neurons (hNeurons) were transduced with LV particles at DIV 16. Particles were retrieved from −80 °C and slowly thawed on ice for 20 min. LV particles were then resuspended in NOMc media according to the validated multiplicity of infection (M.O.I.) and transduction unit (TU)/ml provided by the manufacturer. Transduction was performed by replacing the culture media with NOMc-containing LV particles for approximately 24 h. The following day, the lentivirus-containing media was completely replaced with fresh one. Transduction efficiency was monitored under a fluorescent microscope every 48 h until DIV 40.

Live imaging and tracking

Live-cell imaging recordings were performed at 4 frames per second (fps; total = 240 frames) using a confocal microscope equipped with a live module (Zeiss Confocal LSM780, Zeiss Live LSM7) and an immersion oil objective 63×/1.4 NA

Plan Apochromat. All the cell cultures were incubated with fresh artificial cerebrospinal fluid (aCSF—NaCl 121 mM, KCl 2.5 mM, CaCl₂ 2.2 mM, MgSO₄ 1 mM, NaHCO₃ 29 mM, NaH₂PO₄ 0.45 mM, Na₂HPO₄ 0.5 mM, Glucose 20 mM) at pH 7.4, before starting live imaging experiments.

Time-lapse movies were processed with ImageJ before the analysis in Imaris (version 9.2, Oxford Instruments). The latter calculates a variety of movement parameters relative to a specific reference point. During the preprocessing in ImageJ, we established the reference point. Here all the axon images were transformed such that the cell body oriented on the left and axon terminal on the right; hence, to track the anterograde movement from left to right and retrograde movement from right to left (Supplementary Movies 1, 2, 3, and 4). This preprocessing step is crucial for establishing the proportions of vesicles moving in anterograde or retrograde direction. Vesicles were segmented and tracked with the semi-automated spot tracking Imaris tool by applying an autoregressive motion algorithm. The algorithm required the input of the following parameters related mainly to the size of the vesicles and information about the continuity of movement over time: XY estimated diameter, max distance, and max gap size. The XY estimated diameter was chosen on an average empirical value for each specific cargo analyzed. For either max distance or max gap size, both spatial and temporal resolutions of the acquired movies were considered (temporal resolution = 4 fps; spatial resolution with the 63× objective described earlier in the paragraph = 0.104 µm/pixel). Among all the statistical values obtained, those used for analyses of axonal transport parameters were the spatial displacement ($\Delta D_x(t_1, t_0) = P_x(t_1) - P_x(t_0)$) of the particles in each frame and the track duration (t_d = total time during which a particle moves). For transport dynamics analysis, we first divided the tracks into stationary or moving. All the tracks moving at < 10 s were excluded. For the net axonal transport analysis, tracks with a total track displacement in $x < 250$ µm were considered completely stationary. All the tracks with ≥ 0.1 µm/s velocity were considered motile and classified as anterograde or retrograde based on the directionality of movement. In the segmental axonal transport analysis, we used the Δx displacement between frames to compute instantaneous changes in movement, which gave us more mechanistic information about cargoes features, like real-time movement ratios, reversal and pause frequencies, segmental velocities/run lengths. Finally, because the software computed the distance traveled by each particle along the x and y vector components, these values were reported as track lengths.

Immunocytochemistry (ICC)

Neurons differentiated either in ibidi chambers (6 channels or 8 multi well-8mw) were fixed in 4% paraformaldehyde

(PFA)/4% Sucrose for 40 min. Incubation with 0.1 M Glycine for 5 min was performed to quench the fixation buffer. After that, cells were permeabilized with 0.1% Triton X-100 for 10 min. Samples were blocked with 5% bovine serum albumin (BSA) for 30 min and then incubated overnight at 4 °C with primary antibodies in 3% BSA-0.1% Triton X-100. The second day, the cells were washed 3× 1× phosphate-buffer saline (PBS) for 5 min and then incubated with secondary antibodies in 3% BSA-1× PBS for 2 h. Finally, cells were stained with DAPI for 3 min, then washed with 1× PBS and mounted with 50% Glycerol in 0.01% Na azide/1× PBS. Samples were then imaged and stored at 4 °C.

Identification of axonal projections in a non-microfluidic chamber system

Studies relative to TDP-43, Rab5, SYP, and APP movement were carried in ibidi μ -Slides VI 0.4. Differently from the microfluidic chambers, the ibidi chamber lacks a physical division between the somato-dendritic and axonal compartments. For this reason, before seeding the neurons, the ibidi multichannel slides were marked with an arbitrary reference point to set an X; Y (0; 0). The reference point was then matched with the 0; 0 reference of the confocal microscope stage before time-lapse acquisition [38]. While performing live-cell imaging, the coordinates of selected neuronal projections were saved into a position list. Post-live imaging cells were then fixed, stained and imaged using the ICC protocol described above. The coordinates of each neuronal projection were retrieved to identify phosphorylated Neurofilament Heavy chain-positive (pNfH-(+)) neurites with overlapping GFP-(+) intensities, which were then included in the axonal transport analysis [38].

Image analysis

Images acquired following ICC were analyzed either using ImageJ or Imaris software.

- (i) *pNfH/GFP post-live imaging*: neurites were traced in ImageJ following GFP intensities for all the experiments that involved live-cell imaging of fluorescently labeled proteins. Intensity profiles of TDP-43-, Rab5-, SYP-, and APP-GFP tags were matched with those of pNfH to either include or exclude each analyzed neurite from further axonal transport quantifications [38].
- (ii) *Proximity Ligation Assay for protein–protein interaction*: Z-stacks were acquired using 40× or 63× objective lenses and analyzed to quantify PLA-(+) dots, representing close proximity between the two proteins of interest, thereby providing a measure of the frequency of these interactions. Images were

masked for axonal markers, either β III-tubulin and pNfH alone, or pNfH in combination with GFP, in the case of neuronal cell cultures transduced with LV-shKLC1-tGFP or LV-scramble-tGFP control. PLA dots along the axonal projections were measured using the surface/spots package of Imaris. On the other hand, the frequency of interaction was analyzed by tracing manually 10 axonal projections in each z-stack and then, with the Analyze particles tool from ImageJ the number of PLA(+) dots along axonal projections we counted.

Proximity ligation assay (PLA) for protein–protein interaction studies

To confirm protein–protein interactions with an alternative approach, besides the co-IPs experiments, we performed a series of PLAs, establishing a close interaction between two proteins that are less than 40 nm apart in their physiological environment.

In this study, we performed PLA to detect interactions between the following protein pairs:

- TDP-43: KLC1 (kinesin light chain 1)
- TDP-43: KIF5A (kinesin-5A)
- TDP-43: KIF5B (kinesin-5B)
- TDP-43: KIF5C (kinesin-5C)
- TDP-43: KIF1A (kinesin-1A)

In addition, we also performed PLAs for both technical and biological controls:

- Technical (–): one specific antibody incubated only with PLA buffers and enzymes
- Technical (+): 2 different antibodies recognizing different epitopes of the same protein.

For the biological controls we selected pairs of proteins known to be interacting/non-interacting with our protein of interest, in this case either KLC1 or TDP-43:

- KLC1:KHC (kinesin heavy chain—constitutively interacting)
- KLC1:MyoV (myosin V—not interacting)
- TDP-43:hnRNPC (heterogeneous ribonucleoprotein C—interacting in the nucleus)
- TDP-43:UBQLN2 (ubiquilin-2—interacting in the cytoplasm)

The first part of a PLA protocol follows the same steps of an ICC, up to the incubation step with primary antibodies (for further details see the previous paragraph on ICC). For the second part of the protocol, we followed the

manufacturer's instructions and used buffers, enzymes, and probes provided in the DuoLink PLA kit (Merck). Briefly, per each reaction performed in an 8 mw ibidi chamber, we used 10 µl of both PLUS (+) and MINUS (−) probes, each corresponding to the host species of the primary antibodies used in the first step (usually anti-rabbit PLUS, and either anti-mouse or anti-goat MINUS). The solution was prepared in antibody diluent in a final volume of 100 µl. After removing the primary antibodies mix, the probe solution was incubated with the sample for 1 h at 37 °C in a humid chamber. In the meantime, a ligation buffer and ligase enzyme were mixed. After removing the probes, the ligase mix was incubated for 30 min at 37 °C with the sample. During this step, the amplification mix was prepared by adding in the solution the amplification buffer and the polymerase (0.5 µl/PLA reaction) in a final 100 µl volume.

The ligase mix was then removed and exchanged with the polymerase solution, which was incubated for 100 min at 37 °C. After this step, additional secondary antibodies conjugated with fluorophores can be incubated to complete the PLA and the ICC of protein markers not involved directly in the assay. After the canonical final washes in PBS and the DAPI staining, samples were mounted in a 50% Glycerol in 0.01% Na azide/1× PBS solution, imaged, and then stored at 4 °C.

Protein extraction

For immunoprecipitations (IPs), proteins from 40 DIV differentiated hNeurons were collected using IP lysis buffer (1% Nonidet P-40, 25 mM Tris-buffer pH 7.4, 150 mM NaCl, 1 mM EDTA, 5% Glycerol). Cells were incubated for 30 min on ice and cell membranes were disrupted using an insulin syringe. Supernatants were collected into new tubes following centrifugation for 20 min at 14,000×g 4 °C. Total lysates were quantified using the BCA assay according to the manufacturer's protocol (Pierce™ BCA Protein Assay Kit, Thermo Fisher).

Immunoprecipitations of endogenous bait proteins.

Bait proteins were immunoprecipitated with Dynabeads Protein G (Thermo Fisher), which allows magnetic separation of immunocomplexes.

For IP of TDP-43, KLC1, dynactin 1 (DCTN1), KIF1A, and IgG isotype controls 50 µl of Dynabeads Protein G were used per each sample. Beads were vortexed for 30 s and then placed into DynaMag-2 to discard the storage solution. Then, the antibody conjugation step was performed by adding 200 µl of the Ab Binding Buffer (Thermo Fisher), and 5 µg of a primary antibody specific for each protein (Supplementary Table 1). The antibody mix was incubated for 1 h at 4 °C rotating end-over-end. After discarding the

Ab binding buffer 500 µg of total lysate was added to the beads, and samples were incubated for 2 h at 4 °C rotating end-over-end. Tubes were spun at 1000×g for 1 min and then placed into DynaMag-2 (Thermo Fisher) for separation of flow-through fractions. The beads were washed twice for 2 min with 200 µl of wash buffer (150 mM NaCl, 50 mM Tris/Cl pH 7.5). Protein complexes were eluted using 1× Lithium Dodecyl Sulfate (LDS) sample buffer, 1× NuPage dithiothreitol (DTT) reducing agent (Thermo Fisher) and heated at max 80 °C for 15 min. Eluates were transferred into new tubes to be used for SDS-PAGE and western blot analyses.

Quantification of bound fractions to the respective bait proteins was performed by analyzing the ratio of band intensities visualized in the blots of the co-IP complexes. The formula used for relative quantification was:

$$\text{Ratio of bound proteins} = \frac{\text{coIP}}{\text{IP}} = \frac{\text{intensity partner protein}}{\text{intensity bait protein}}.$$

SDS-PAGE and western blotting

Quantified protein samples were loaded into Bolt Bis-Tris Plus 4–12% precast gels (Invitrogen) and SDS-PAGE performed first for 10 min at 50 V and then at 100 V for 1 h 30 min. Proteins were then transferred onto PVDF 0.45 µm membranes (Thermo Fisher) using a Mini blot module (Invitrogen) for 1 h 50 min at 30 V. Membranes were washed 3× 5 min with 20 mM Tris-Buffer (TBS) and blocked in 5% non-fat dry milk (NFDM) in 20 mM TBS/0.2% Tween-20 (TBS-T) buffer for 1 h at R.T. After 3 washes in TBS, the membranes were probed overnight at 4 °C with primary antibodies (Supplementary Table 1) in 1% BSA/TBS-T 0.2%. The second day, HRP-conjugated antibodies were prepared in 1% NFDM TBS-T 0.2% and incubated for 2 h at 4 °C shaking. Last, the membranes were washed 3× 5 min with TBS-T 0.2% and proteins detected using Chemiluminescent Substrate (SuperSignal™ West Pico PLUS, Thermo Scientific) by acquiring images in a Chemidoc (BioRad).

Statistical analysis and data representation

All analyses were performed using Prism 10.0 (GraphPad Software). Data for axonal transport parameter determination were initially processed in Microsoft Excel tables, where multiple formulas were applied to calculate axonal transport parameters (e.g., real-time movement, reversal and pause frequency, segmental velocity). Statistical analyses and post hoc tests for multiple comparisons are described in detail in the figure legends, as well as the data representation for each graph shown in the corresponding figure. All experiments were performed at least three times using

independent biological samples. The statistical pooling of data was performed depending on the biological message that the graph wanted to show for that specific parameter, particularly in the case of axonal transport analyses (net or segmental approach). For axonal transport parameters the results are represented as SuperPlots [64] showing the individual data points in different colors, superimposed with the data points of either the biological replicate (net axonal transport analysis) or the grouped axons within that replicate. Regardless of grouping level, the mean of each experiment is shown as mean $\pm$ S.D.

The statistical analysis included unpaired *t*-test, unpaired *t*-test with Welch's correction, One-way ANOVA, and the Kruskal–Wallis test. Proportional parameters such as anterograde *vs* retrograde were compared within each protein using unpaired *t*-test. In the case of PLA experiments when comparing KIF5A-, KIF5B-, KIF5C-, and KIF1A-TDP-43 interactions *vs* control, unpaired *t*-test with Welch's correction was used. When comparing the means of three or more normally distributed samples such as for time in motion or track density and post shRNA-KLC1 PLA we performed a one-way ANOVA followed by Dunnett's and Tukey's multiple comparison tests, respectively, as specified in the figure legends. For data that did not follow a normal distribution such as reversal or pause frequency, track length, and frequency of segmental velocities, the Kruskal–Wallis test was performed followed by Dunn's post hoc test.

Correlations between segmental velocity and segment duration were assessed using Spearman's rank correlation coefficient (ρ). To evaluate whether the strength of the correlation differed between anterograde and retrograde transport, correlation coefficients were Fisher's *z*-transformed and compared using a two-tailed *z*-test.

No statistical methods were used to predetermine sample size; however, sample sizes are comparable to those reported in previous axonal transport publications [67, 73, 82]. As described earlier in the paragraph multiple independent experiments were performed using multiple sample replicates as detailed in the figure legends.

Results

Net TDP-43 axonal transport shows balanced proportions of anterograde and retrograde movement.

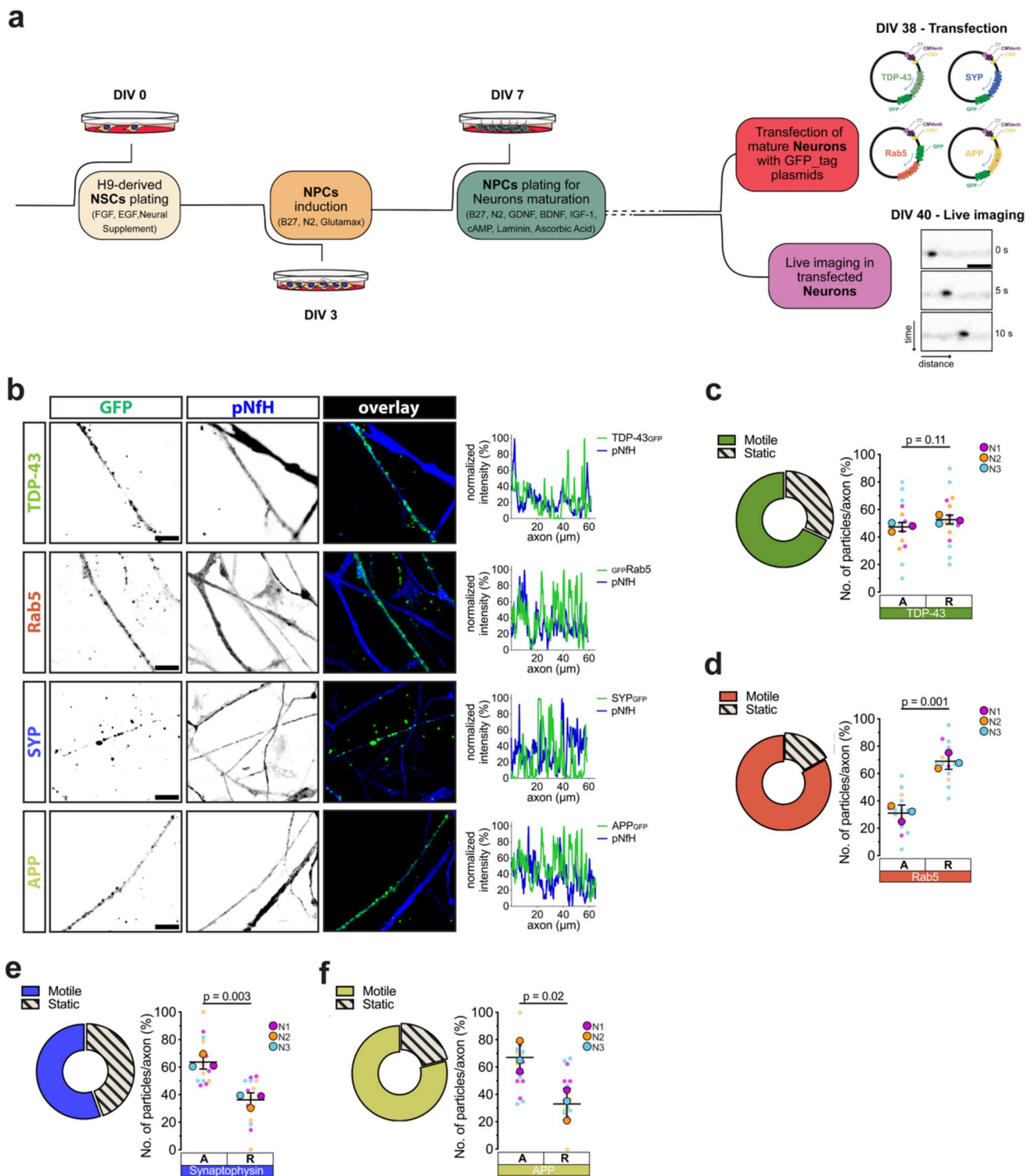
While TDP-43 has been primarily associated with nuclear functions, it is also present in axons under physiological conditions where it regulates mRNA trafficking and local translation [10, 51, 66, 96]. To investigate TDP-43 axonal transport mechanisms, we performed live imaging

experiments using previously characterized H9-derived human neurons for 40 DIV [79] (Fig. 1a). Neurons were transfected with Lipofectamine 2000 mixed with plasmids expressing GFP-labeled cargoes: TDP-43, Rab5, SYP, and APP 48 h prior to imaging (Supplementary Fig. 1a). We compared TDP-43 with three established axonal cargoes (Rab5, SYP, and APP) that exhibit distinct transport properties [22, 46, 49]. These cargoes were used as reference transport profiles for comparative analysis rather than as markers of co-transport with TDP-43. Movies were recorded at 4 frames per second (fps) for 60 s (s), and only axonal projections confirmed by post-imaging GFP/pNfH overlapping intensities were analyzed [38] (Supplementary Fig. 1b; Fig. 1b; Supplementary Movies 1, 2, 3, and 4).

Using semi-automated spot-tracking, we first confirmed that all four cargoes showed comparable GFP signal levels and track densities, validating the reliability of subsequent transport comparisons only in axonal projections (Supplementary Table 2, Fig. 1b—right, Supplementary Fig. 1c). We then assessed the proportion of motile versus static particles using a uniform movement threshold applied across all cargoes to enable direct comparison. This revealed that 50–80% of particles were motile while 20–50% of all four proteins remained static (pie charts—Fig. 1c–f). Because each protein has distinct cargo-specific transport dynamics, this uniform threshold necessarily captures particles with varying velocities, including slower-moving fractions and particles undergoing pausing or directional changes within the motile category.

Next, we assessed within the motile fractions the proportions of particles that for each cargo were moving toward the synaptic terminals (anterograde) or toward the soma (retrograde). TDP-43 exhibited balanced bidirectional transport with no significant directional preference (Fig. 1c—right), suggesting widespread axonal distribution similar to other mRNA-trafficking RNA-binding proteins (RBPs) [2, 42, 51]. In contrast, the other cargoes showed their canonical transport patterns: Rab5 displayed predominantly retrograde movement consistent with its endosomal recycling function [34, 44] (Fig. 1c—right). SYP showed strong anterograde bias reflecting kinesin-3/ kinesin-1A (KIF1A)-mediated transport to synapses [43, 55] (Fig. 1e—right). APP exhibited slightly anterograde-biased but relatively balanced bidirectional movement [25, 38, 57, 77] (Fig. 1f—right).

These distinct transport dynamics support the involvement of multiple motor proteins and adaptor complexes in cargo-specific axonal transport. In the case of TDP-43, the balanced bidirectional profile suggests that both anterograde and retrograde transport contribute to its distribution along the axon.



TDP-43 exhibits fast axonal transport comparable to vesicle-bound cargoes SYP and APP.

To characterize the transport mechanisms of individual cargo trajectories, we employed a ‘segmental analysis’ approach that divides each trajectory into vectorial

segments, allowing detailed examination of movement properties including direction, velocity, and pausing behavior by collecting real-time data from the Imaris software [38, 79] (Fig. 2a—top). This frame-by-frame analysis provides insight into the motor proteins or adaptor complexes that may mediate microtubule-directed transport of specific

Fig. 1 Axonal cargoes show distinct direction polarity. **a** Schematic representation of timeline differentiation of H9-derived neurons, and the two main time-points crucial for carrying axonal transport studies: DIV 38—transfection of 4 different plasmids; DIV 40 recording of axonal cargoes movement (scale bar = 3 μ m). Micrographs in the bottom right represent screenshot of a particle moving over time. **b** Representative micrographs showing signal from green fluorescent protein (GFP) tag over-expressed cargoes, TAR DNA binding protein 43 (TDP-43), ras-related protein Rab5 (Rab5), synaptophysin (SYP), and amyloid precursor protein (APP) (green), with axonal phosphorylated Neurofilament Heavy chain (pNFH) marker (blue). On the right of the panel normalized intensities graphs show the overlapping signals in the illustrated regions of interest (ROIs) (scale bar = 10 μ m). **c—left** Pie charts are showing the proportions of motile and completely static particles in the net axonal transport analysis of TDP-43; **c—right** superplots representing the proportions of motile TDP-43 particles moving anterograde and retrograde ($n = 15$ axons from $n = 3$ biological replicates). **d—left** Pie charts are showing the proportions of motile and completely static particles in the net axonal transport analysis of Rab5; **d—right** superplots representing the proportions of motile Rab5 particles moving anterograde and retrograde ($n = 15$ axons from $n = 3$ biological replicates). **e—left** Pie charts are showing the proportions of motile and completely static particles in the net axonal transport analysis of SYP; **e—right** superplots representing the proportions of motile SYP particles moving anterograde and retrograde ($n = 15$ axons from $n = 3$ biological replicates). **f—left** Pie charts are showing the proportions of motile and completely static particles in the net axonal transport analysis of APP; **f—right** superplots representing the proportions of motile APP particles moving anterograde and retrograde ($n = 15$ axons from $n = 3$ biological replicates). Data show normalized intensities along the GFP(+) and pNFH(+) ROIs (**b**), pie charts from $n = 3$ biological replicate means per each cargo (**c–f—left**), mean $\pm$ S.D. (**c–f—right**). Statistical analyses were performed using unpaired *t*-test, and comparisons result are shown with exact *p* values

cargoes, while also enabling categorization of trajectory types [72, 73] (Fig. 2a—bottom). To examine the regulatory aspects of distinct motor protein-cargo interactions, we chose to include only motile particles in the segmental analysis (Supplementary Table 3).

Segmental directionality analysis revealed that TDP-43 spent a significantly greater proportion of time in anterograde movement compared to Rab5 but showed similar anterograde proportions to SYP and APP (Fig. 2b—left). For retrograde movement, TDP-43 was comparable to Rab5, SYP and APP (Fig. 2b—center). Pausing time was significantly lower in TDP-43 compared to Rab5, with no differences observed relative to SYP or APP (Fig. 2b—right). These real-time motion patterns indicate that TDP-43 displays transport dynamics more similar to the fast-moving reference cargoes SYP and APP than to the retrograde-biased Rab5.

We next quantified directional reversals (frequency of direction changes during transport) and found that TDP-43 reversed direction significantly more frequently than Rab5, while showing similar reversal rates to SYP and APP (Fig. 2c). Similarly, pause frequency (number of pausing events per trajectory) was significantly lower for

TDP-43 compared to Rab5 and SYP, but comparable to APP (Fig. 2d). Finally, we measured total track length as an indicator of cargo processivity, which reflects the ability of a motor protein to step along microtubules in a constant way before detaching from the cytoskeletal track. TDP-43 particles traveled distances comparable to SYP and Rab5, and exhibited significantly shorter track lengths compared to APP, which displayed the widest range of distances (Fig. 2e). This suggests that TDP-43 transport may be characterized by both short- and long-distance movements, reflecting potential heterogeneity in cargo composition or motor protein engagement.

Collectively, these segmental analyses demonstrate that TDP-43-containing membraneless organelles (MLOs) can be considered as fast axonal cargoes with transport characteristics resembling the vesicle-associated proteins SYP and APP. Because these cargoes were imaged in separate experiments, these comparisons are correlative and do not demonstrate physical co-transport between TDP-43 and any individual reference cargo. Instead, they were used to guide subsequent biochemical and proximity ligation analyses of TDP-43 interactions with specific motor and adaptor proteins. Nevertheless, this comparison is particularly noteworthy because classical models of axonal transport have been predominantly developed from studies of membrane-bound vesicular cargoes, where transmembrane proteins serve as adaptor binding sites for motor protein recruitment and cargo sorting at axonal entry points [20, 81].

TDP-43 anterograde transport combines SYP-like processivity with APP-matching velocities

To determine whether longer uninterrupted transport events correlate with sustained velocity increase, we analyzed segmental velocities for all four cargoes during both anterograde and retrograde movement. First, relationship between segment duration and velocity was determined, which would indicate processive motor engagement. Correlation analysis using cubic spline fitting revealed cargo-specific patterns both in anterograde and retrograde direction.

For both TDP-43 and Rab5 segmental velocities correlated weakly but significantly with segment duration for both anterograde and retrograde transport (Fig. 3a, and b). The correlation strengths did not differ significantly between anterograde and retrograde, indicating that TDP-43 cargoes exhibit similar coupling of run length and speed in both directions (Supplementary Fig. 2a).

In the case of SYP, segmental velocity correlated positively with segment duration for both anterograde- and retrograde-biased transport, but the anterograde correlation was significantly stronger, indicating direction-specific coupling between segment duration and velocity for SYP-positive vesicles (Fig. 3c, and Supplementary Fig. 2a). Finally, we

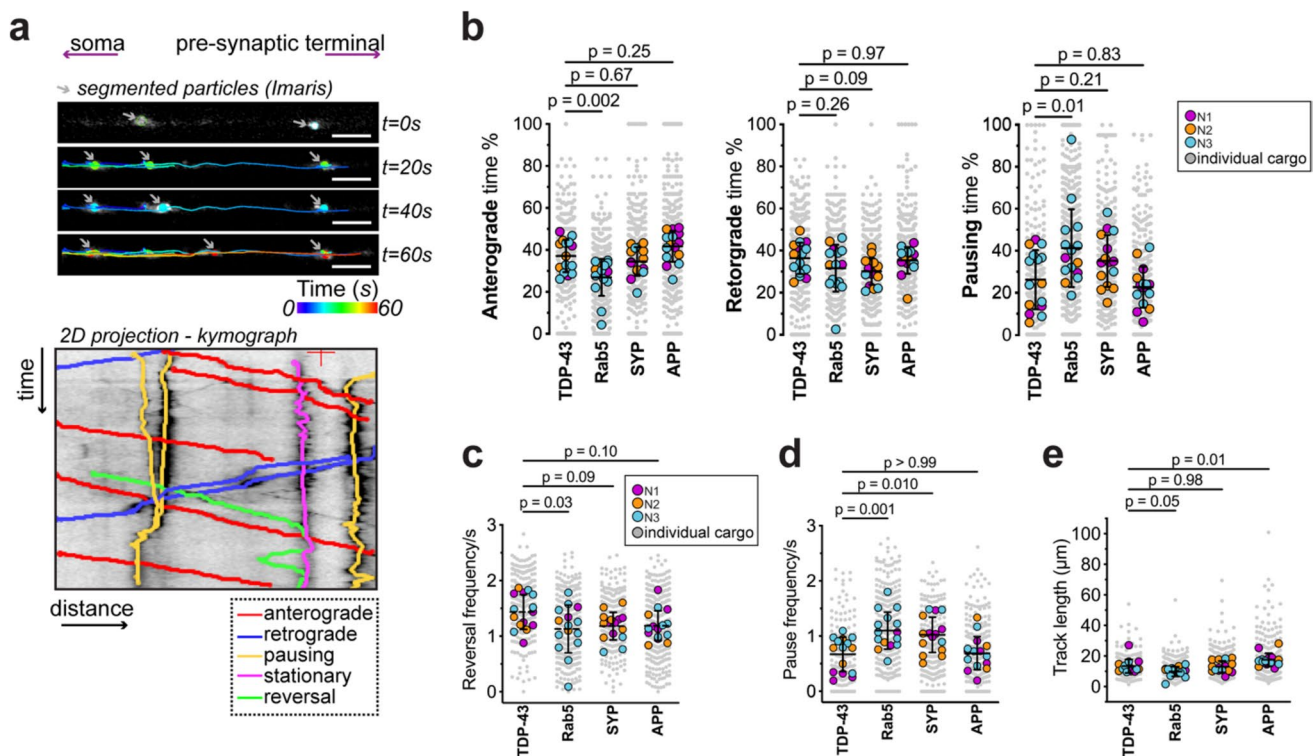


Fig. 2 TDP-43 exhibits active anterograde axonal transport similar to SYP and APP. **a—top** Representative micrographs of particles movement, segmentation and tracking determined with Imaris spots tracking algorithms. Gray arrows follow the particle movement over-time, as indicated by the time points illustrated on the right of the micrographs, while colored time scale bar indicates the length of movement as illustrated by the colored trajectories in the micrographs (scale bar = 5 μ m); **a—bottom** representative 2D projection (kymograph) of possible particles behavior (y =time, x =distance); trajectories can be traced to describe either motion towards the synaptic terminal (anterograde—red), to the soma (retrograde), no clear direction bias (pausing cargo—yellow), not moving cargo (stationary—magenta), or changing directionality (reversing cargo—green). **b** Quantification and comparisons of real-time movement analyzed as proportion of time spent by each TDP-43, Rab5, SYP, and APP particle in anterograde (left), retrograde (center), or pausing (right). SuperPlots represent single trajectories of particles (gray dots) superimposed by means per axon (solid-colored circles), with colors and

legends depicting the biological replicate to which every dot belongs ($n > 360$ tracks from $n = 15$ axons per each cargo analyzed from 3 different biological replicates). **c** Quantification and comparisons of reversal frequency normalized per second of motile TDP-43, Rab5, SYP, and APP particles ($n > 250$ tracks from $n = 15$ axons per each cargo analyzed from 3 different biological replicates). **d** Quantification and comparisons of pause frequency normalized per second of motile TDP-43, Rab5, SYP, and APP particles ($n > 250$ tracks from $n = 15$ axons per each cargo analyzed from 3 different biological replicates). **e** Quantification and comparisons of total track lengths of motile TDP-43, Rab5, SYP, and APP particles moving either anterograde or retrograde ($n > 250$ tracks from $n = 15$ axons per each cargo analyzed from 3 different biological replicates). Data show mean $\pm$ S.D. of superplot with axon means over the single tracks' dots (**b–e**). Statistical analyses were performed using One-way ANOVA followed by Dunnett's multiple comparisons test (**b**), or Kruskal Wallis test followed by Dunn's post hoc test (**c–e**), and the corresponding results shown with exact p values

examined APP segmental velocities and observed that they also correlated positively with segment duration for both anterograde and retrograde runs which was significantly stronger for anterograde transport, indicating that APP cargo exhibits a higher degree of coupling between run length and velocity during transport toward presynaptic terminals.

We then compared velocity distributions to gain insight into potential motor protein involvement. For anterograde movement, TDP-43 velocities were significantly higher than those of Rab5 and SYP, while they showed near-complete overlap with APP anterograde velocities (Fig. 3e, Supplementary Fig. 2b). The similarity between the anterograde velocity distributions of TDP-43 and APP suggests that

these cargoes may share features of kinesin-dependent plus-end-directed transport. However, because they were imaged in separate experiments, multicolor live-cell imaging will be required to determine whether individual TDP-43- and APP-positive particles undergo coordinated transport or physical co-transport within the same axon. In contrast, retrograde segmental velocities of TDP-43 were significantly different from all three reference cargoes (Fig. 3f, Supplementary Fig. 2c). This suggests that while the dynein-dynactin complex mediates retrograde transport for many cargoes, it exhibits cargo-specific processivity regulation [28, 36, 81]. The distinct retrograde velocity profile of TDP-43 MLOs may reflect unique adaptor proteins or post-translational

modifications that modulate dynein-dynactin activity on membraneless ribonucleoprotein (RNP) granules compared to vesicular cargoes [35].

Collectively, these velocity analyses suggest that TDP-43 anterograde transport shares kinetic features with kinesin-driven transport of APP and SYP, a conclusion further supported by the biochemical and proximity ligation evidence demonstrating TDP-43 interactions with kinesin family members. The similarities in anterograde transport characteristics of TDP-43, including processivity and velocity profiles, suggest that TDP-43-containing MLOs may engage kinesin-dependent transport mechanisms comparable to those used by APP and SYP to reach distal axonal compartments and synaptic regions. Such transport is likely to be functionally important, as TDP-43-mediated local mRNA translation at synapses is essential for maintaining synaptic plasticity and neuronal integrity [15, 51, 53, 56].

TDP-43 engages with KLC1 and DCTN1 adaptors for effective anterograde and retrograde transport

To identify the molecular machinery underlying TDP-43 axonal transport, we performed co-immunoprecipitation (co-IP) experiments in 40 DIV human neurons, using cytoplasmic protein lysates. Our experiments focused on kinesin light chain 1 (KLC1), a key adaptor for kinesin-5 (KIF5)-mediated anterograde transport [40, 41], and dynactin 1 (DCTN1), a core component of the retrograde transport dynein motor complex [7, 24] (Fig. 4a).

IP of endogenous TDP-43 revealed positive interactions with both KLC1 and DCTN1 (Fig. 4b, Supplementary Fig. 3a). Quantification of relative binding ratios to TDP-43 exhibited a more robust association with KLC1 rather than DCTN1 (Fig. 4c). This preferential binding to the anterograde adaptor is consistent with our live-imaging data showing that TDP-43 exhibits fast anterograde transport characteristics and spends considerable time moving toward distal axonal regions.

To confirm the specificity of these interactions, we performed reciprocal IPs. Pulldown of KLC1 successfully co-immunoprecipitated TDP-43, along with the expected positive control kinesin-5B (KIF5B), which forms the canonical KLC1-KIF5 motor complex (Fig. 4d, Supplementary Fig. 3b). Although KLC1 is an abundant neuronal protein involved in transporting multiple cargoes, quantification indicated that a fraction of TDP-43 associates with KLC1, suggesting this interaction is physiologically relevant (Fig. 4f). Similarly, IP of DCTN1 co-purified TDP-43 along with dynein intermediate chain 1 (DIC1), a core component of the dynein-dynactin complex [82, 91] (Fig. 4e, Supplementary Fig. 3c). Binding ratio analysis confirmed that TDP-43 associates with DCTN1-containing complexes,

supporting dynein-dynactin involvement in retrograde TDP-43 transport (Fig. 4g).

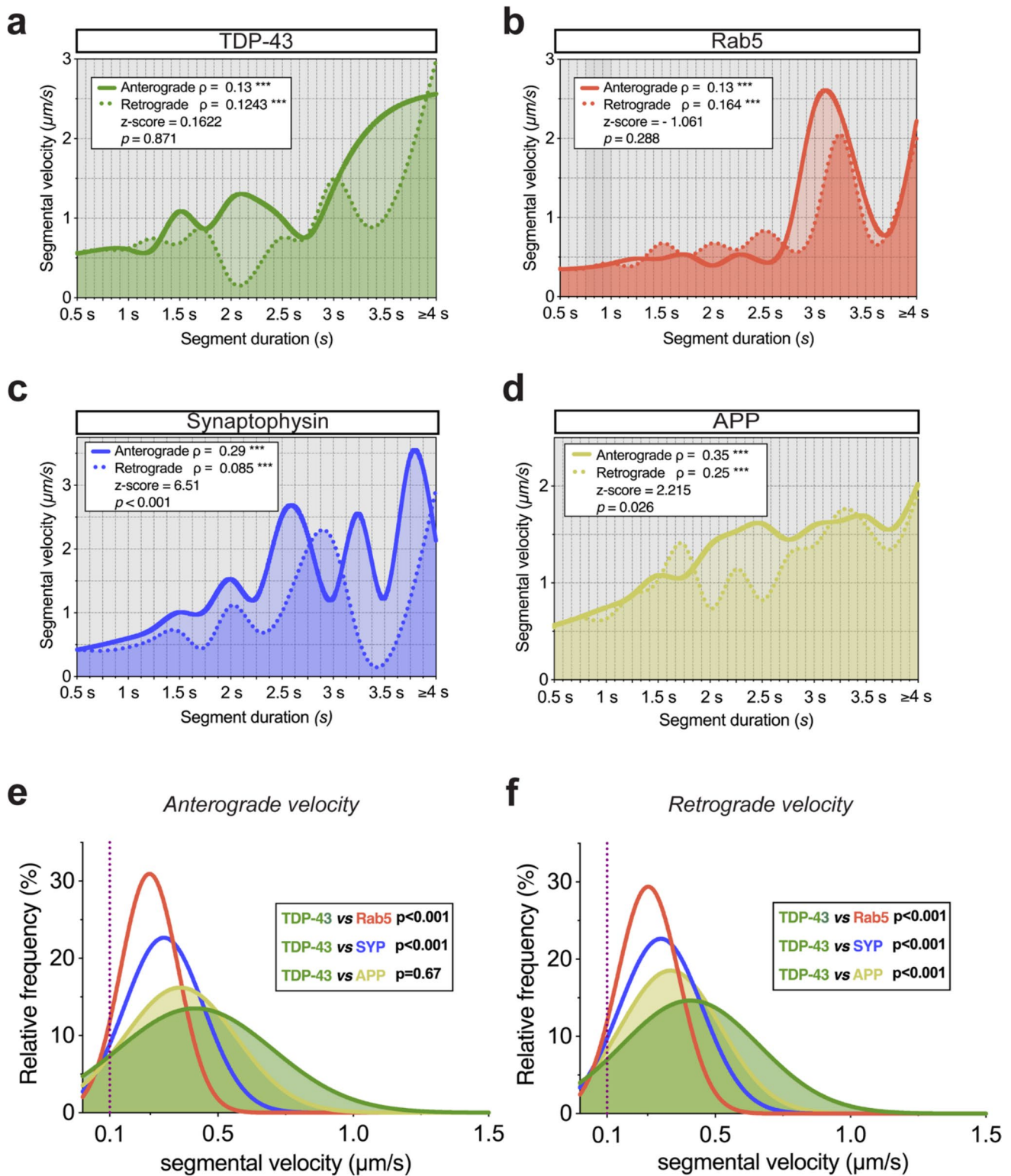
Collectively, these biochemical interactions, in addition to the axonal transport data, demonstrate that TDP-43 engages with both anterograde (KLC1/KIF5) and retrograde (DCTN1/dynein) motor complexes, with a strong association with the anterograde machinery. The presence of both interactions provides a molecular explanation for the bidirectional transport dynamics observed in our live-imaging analyses and implicates KIF5 as an important kinesin mediating TDP-43 anterograde movement.

TDP-43 associates with KLC1 and all three KIF5 isoforms in the axonal compartment

To validate the TDP-43-KLC1 interaction *in situ* and assess its functional specificity, we performed proximity ligation assays (PLAs) in our 40 DIV human neurons. PLA detects protein–protein interactions within a distance of ≤ 40 nm, allowing visualization of endogenous complexes in their native axonal environment.

We first established assay specificity using biological controls. For TDP-43, we used ubiquilin-2 (UBQLN2) as a positive control (known to bind TDP-43's C-terminus [23]) and hnRNPC as a negative control (known to bind TDP-43 predominantly in the nucleus [4]) (Supplementary Fig. 4a). For KLC1, we used kinesin heavy chain (KHC/KIF5) as a positive control [42] and myosin V (MyoV) (an actin-based motor protein [46]) as a negative control. These controls showed expected positive and negative overlapping intensity patterns, respectively, for both TDP-43 and KLC1 (Supplementary Fig. 4b). We then validated the overlapping signals for TDP-43 and KLC1 antibodies with the axonal pNfH to confirm their suitability for PLA (Supplementary Fig. 4c). Moreover, the KLC1 antibody alone showed no background binding in pilot PLA experiments and no overlap with pNfH (Supplementary Fig. 4d). Having validated assay performance and antibody specificity, we next examined the TDP-43-KLC1 interaction.

To test TDP-43-KLC1 binding we performed shRNA-mediated KLC1 knockdown (KD) from day 16 to day 40 *in vitro* human neurons, comparing three conditions: non-transduced (NT), scrambled control with TurboGFP (LV-scramble-tGFP), or with LV-shKLC1-tGFP (Fig. 5a, Supplementary Fig. 5e). KLC1 downregulation was induced with shRNAs of different efficiency, validated by western blot and densitometric analysis. First, we confirmed that β III-tubulin loading control levels were comparable across all samples, with no significant differences found, suggesting that the treatment was not affecting protein levels other than the KLC1 KD (Supplementary Fig. 5f). Next, we compared the levels of transduced scramble or different LV-shKLC1-tGFP constructs. By performing densitometric analysis



and normalizing KLC1 levels to β III-tubulin and tGFP, we observed that the most significant reduction was obtained with LV-shKLC1-tGFP A, which was selected for PLA analysis (Supplementary Fig. 5 g). We then performed PLAs, analyzing all conditions in pNfH-(+) axonal compartments.

Non-transduced neurons and those transduced with scramble-tGFP displayed robust PLA signals along pNfH-(+) axons, confirming TDP-43-KLC1 association in the axonal compartment (Fig. 5b-left, center). In contrast, LV-shKLC1-tGFP-treated neurons had dramatically reduced PLA signals

Fig. 3 Positive correlations between velocity and run lengths represent processive anterograde and retrograde TDP-43 movement. Cubic spline fitting of correlation analyses for both anterograde and retrograde segmental velocities vs run length (segment duration) for TDP-43 (a), Rab5 (b), SYP (c), and APP (d). Graph shows fitting of all analyzed segments for anterograde (solid line) and retrograde (dashed line) moving TDP-43 particles, with Spearman's correlation (ρ) values and z-scores testing the correlation strength in either anterograde or retrograde ($n > 1500$ segments analyzed from 15 axons from 3 biological replicates). e Frequency distribution of anterograde segmental velocity compared across the 4 cargoes TDP-43, Rab5, SYP, and APP, with colored area under the curve (AUC) of APP and TDP-43 frequencies representing the overlap between TDP-43 and APP velocities ($n > 1500$ segments analyzed from 15 axons from 3 biological replicates). f Frequency distribution of retrograde segmental velocity compared across the 4 cargoes TDP-43, Rab5, SYP, and APP, with colored AUC of APP and TDP-43 frequencies representing the overlap between TDP-43 and APP velocities ($n > 1500$ segments analyzed from 15 axons from 3 biological replicates). Data are shown as cubic spline fitting models, where each point is crossed by fitting line curve (a–d), and frequency distributions in percentage of segmental velocity values (e and f). Statistical analyses were performed by using Spearman's correlation and Fisher's z-transformed then compared with a two-tailed z-test (a–d) Spearman's ρ values and z-scores are stated in the inlet tables, as well as corresponding significance (*** $p < 0.001$). Kruskal–Wallis followed by Dunn's multiple comparisons test was used for comparison between frequency distributions (e and f)

in tGFP(+) axons (Fig. 5b–right). Quantification revealed significantly higher PLA puncta density in both NT and scramble neurons compared to shRNA KLC1-treated neurons, with no significant difference between NT and scramble conditions (Fig. 5c). These results demonstrate that reduced KLC1 levels specifically decrease TDP-43–KLC1 interaction, validating the specificity of this association.

Having confirmed TDP-43 interacts with KLC1, we next investigated which kinesin-1 heavy chain isoform associates with TDP-43. The mammalian kinesin-1 family includes three isoforms, kinesin-5A, B, and C (KIF5A, B, and C), that are differentially expressed in different tissues and can exhibit cargo specificity [47]. KIF5B is ubiquitously expressed, while KIF5A and KIF5C are neuron-specific, making all three relevant candidates for neuronal TDP-43 transport. We performed PLA in 40 DIV neurons using isoform-specific antibodies against each KIF5 variant and TDP-43, with a β III-tubulin-GFP cytoskeletal tracker marking neurite compartments (Fig. 5d, Supplementary Fig. 5a). Remarkably, confocal imaging revealed positive PLA puncta for all three isoforms with TDP-43 (Fig. 5e), and quantification confirmed significantly elevated PLA puncta for TDP-43 with KIF5A, KIF5B, and KIF5C compared to negative controls (Fig. 5f). This suggests that TDP-43 granules can engage with multiple kinesin-1 isoforms, which may contribute to the robust and heterogeneous anterograde transport observed in our live-imaging analyses.

Collectively, these PLA experiments, combined with KLC1 knockdown, validate the biochemical interaction

data and demonstrate that TDP-43 specifically associates with the KLC1/KIF5 anterograde transport machinery in axons. The engagement with all three KIF5 isoforms is consistent with TDP-43's robust anterograde transport characteristics. However, whether TDP-43 exhibits preferential binding to specific isoforms for delivery of specific mRNAs to distinct axonal subdomains remains to be determined.

TDP-43 is alternatively recruited by the synaptic vesicle motor KIF1A

Our comparative transport analyses showed that TDP-43 and SYP have similar processive anterograde transport characteristics. Although these comparisons were made using separately imaged cargoes and therefore do not demonstrate physical co-transport, SYP is primarily transported by KIF1A, a kinesin-3 family member specialized for the rapid anterograde delivery of synaptic vesicle precursors to presynaptic terminals. The similarity in transport dynamics raised the possibility that TDP-43 may also engage KIF1A-dependent transport mechanisms. This hypothesis was subsequently supported by our biochemical and proximity ligation analyses [48, 59], suggesting that KIF1A contributes to TDP-43 anterograde trafficking, and may provide an additional or complementary transport pathway to the KIF5/KLC1 motor complex mechanism.

To test this hypothesis, we performed co-IPs and PLAs in DIV 40 human neurons (Fig. 6a). IP of TDP-43 successfully co-purified KIF1A (Fig. 6b, and Supplementary Fig. 6a), with relative binding ratios indicating that a fraction of KIF1A associates with TDP-43 (Fig. 6d). Reciprocal IP of KIF1A confirmed this interaction, co-purifying TDP-43 (Fig. 6e, Supplementary Fig. 6b). The observed binding ratios likely reflect the relative abundance of these proteins and the stoichiometry of motor-cargo complexes.

On the other hand, PLA confirmed close spatial association between TDP-43 and KIF1A in neuronal projections. Using β III-tubulin-GFP cytoskeletal tracker to mark neurites, confocal imaging revealed positive PLA puncta densities along neuronal projections (Fig. 6f, Supplementary Fig. 6c). Quantification showed significantly higher PLA puncta density for TDP-43–KIF1A compared to negative controls (Fig. 6g). This validates the biochemical interaction data and demonstrates that TDP-43 and KIF1A associate within the axonal compartment.

Collectively, these findings reveal that TDP-43 engages with multiple anterograde motor systems, both kinesin-1 (KIF5A/B/C via KLC1) and kinesin-3 (KIF1A). This dual motor engagement may provide functional flexibility for TDP-43-containing MLOs transport regulation.

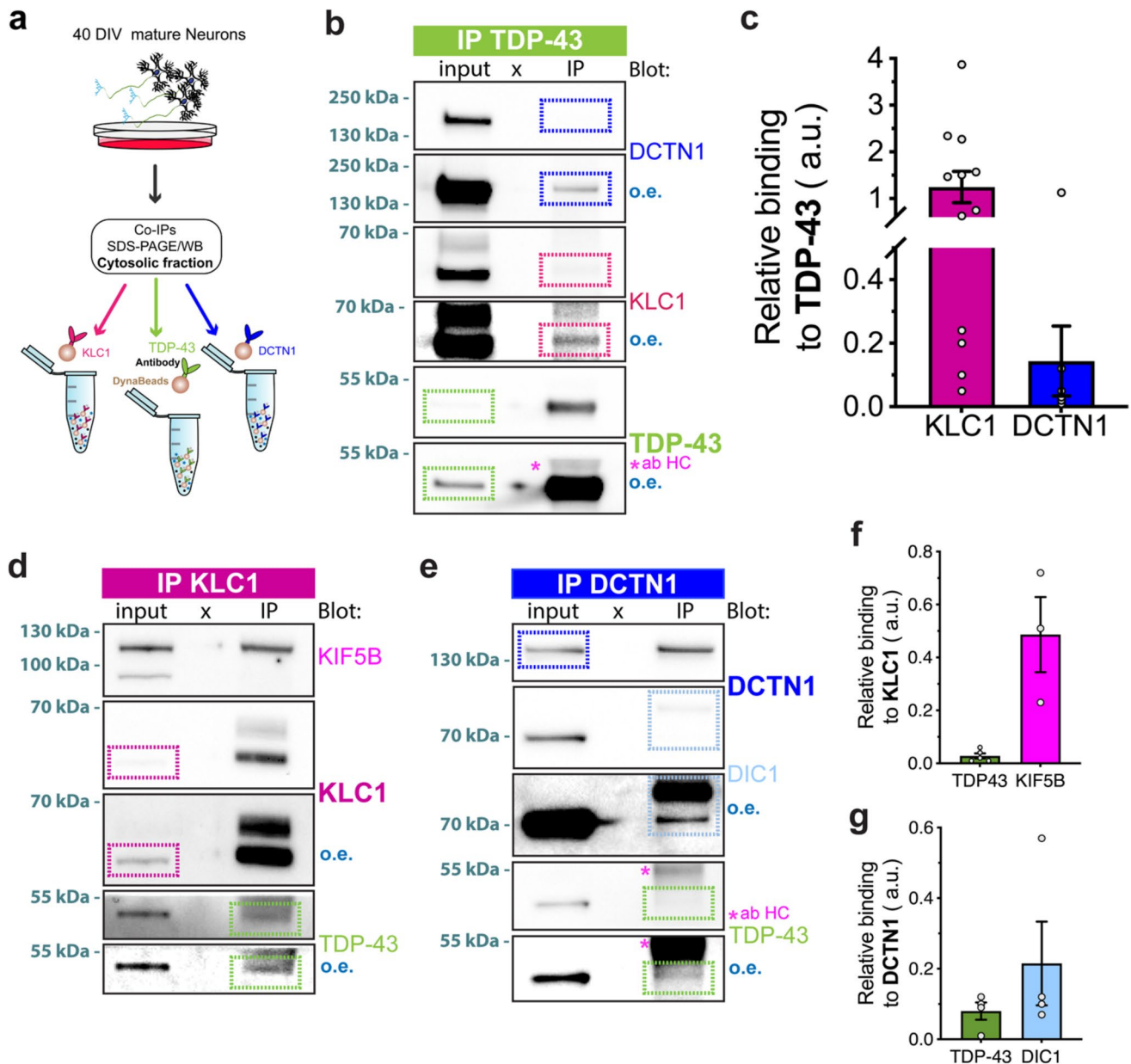


Fig. 4 TDP-43 axonal transport is sustained by interaction with anterograde KLC1 and retrograde DCTN1 motor protein adaptors. **a** Schematic representation of co-immunoprecipitations (co-IP) experiments performed in 40 DIV human neurons to validate interactions between TDP-43, kinesin light chain 1 (KLC1), and dynactin 1 (DCTN1). **b** Representative immunoblots of TDP-43 IP bands (green box) and DCTN1 (blue box) and KLC1 (dark magenta box) co-IP bands. Each protein is represented by two images to show both unsaturated and overexposed images (o.e.). **c** Co-IPs ratios expressed as KLC1 and DCTN1 intensity relative to TDP-43 IP (IP TDP-43 $n=12$ biological replicates with KLC1 co-IP analyzed in $n=12$ and DCTN1 co-IP intensities analyzed in $n=10$ biological replicates). **d** Representative immunoblots of KLC1 IP bands (dark magenta box) and kinesin 5B

(KIF5B) (magenta box) and TDP-43 (green box) co-IP bands. Each protein is represented by two images to show both unsaturated and overexposed images (o.e.). **e** Co-IPs ratios expressed as TDP-43 and KIF5B intensity relative to KLC1 IP (IP KLC1 $n=5$ biological replicates with TDP-43 co-IP analyzed in $n=5$ and KIF5B co-IP intensities analyzed in $n=3$ biological replicates). **f** Representative immunoblots of DCTN1 IP bands (blue box) and dynein intermediate chain 1 (DIC1) (light blue box) and TDP-43 (green box) co-IP bands. Each protein is represented by two images to show both unsaturated and overexposed images (o.e.). **g** Co-IPs ratios expressed as TDP-43 and DIC1 intensity relative to DCTN1 IP ($n=5$ biological replicates). Data are shown as mean $\pm$ S.E.M. with individual dots indicating single biological replicates

Discussion

TDP-43 was initially defined as a predominantly nuclear protein, with its presence in the cytoplasm considered primarily pathological. However, accumulating evidence indicates that TDP-43 physiologically localizes to the cytoplasm at lower concentrations than in the nucleus, including axonal and dendritic compartments where it regulates mRNA transport and local translation [37, 85]. TDP-43 axonal transport has been demonstrated in vivo in spinal motor neurons and other neuronal types using live imaging in *Drosophila*, zebrafish, and mouse models [2, 89]. The present study was designed to define the physiological axonal transport machinery engaged by TDP-43 in human neurons. This distinction is important because ALS-associated mutations, post-translational modifications, aggregation state and patient-specific disease background may alter TDP-43 transport behavior.

Prior studies have shown that TDP-43-containing mRNA granules undergo bidirectional movement and that ALS-causing *TARDBP* mutations impair this transport [2]. More recent studies using mutant *TARDBP* models further support the concept that pathological forms of TDP-43 exhibit transport characteristics distinct from those of physiological wild-type TDP-43 [21, 88]. Accordingly, our findings establish a physiological framework for TDP-43 axonal transport against which *TARDBP*, *chromosome 9 open reading frame 72* (*C9orf72*), *superoxide dismutase 1* (*SOD1*), *Fused in sarcoma* (*FUS*), or sALS-associated pathology can be compared in future studies.

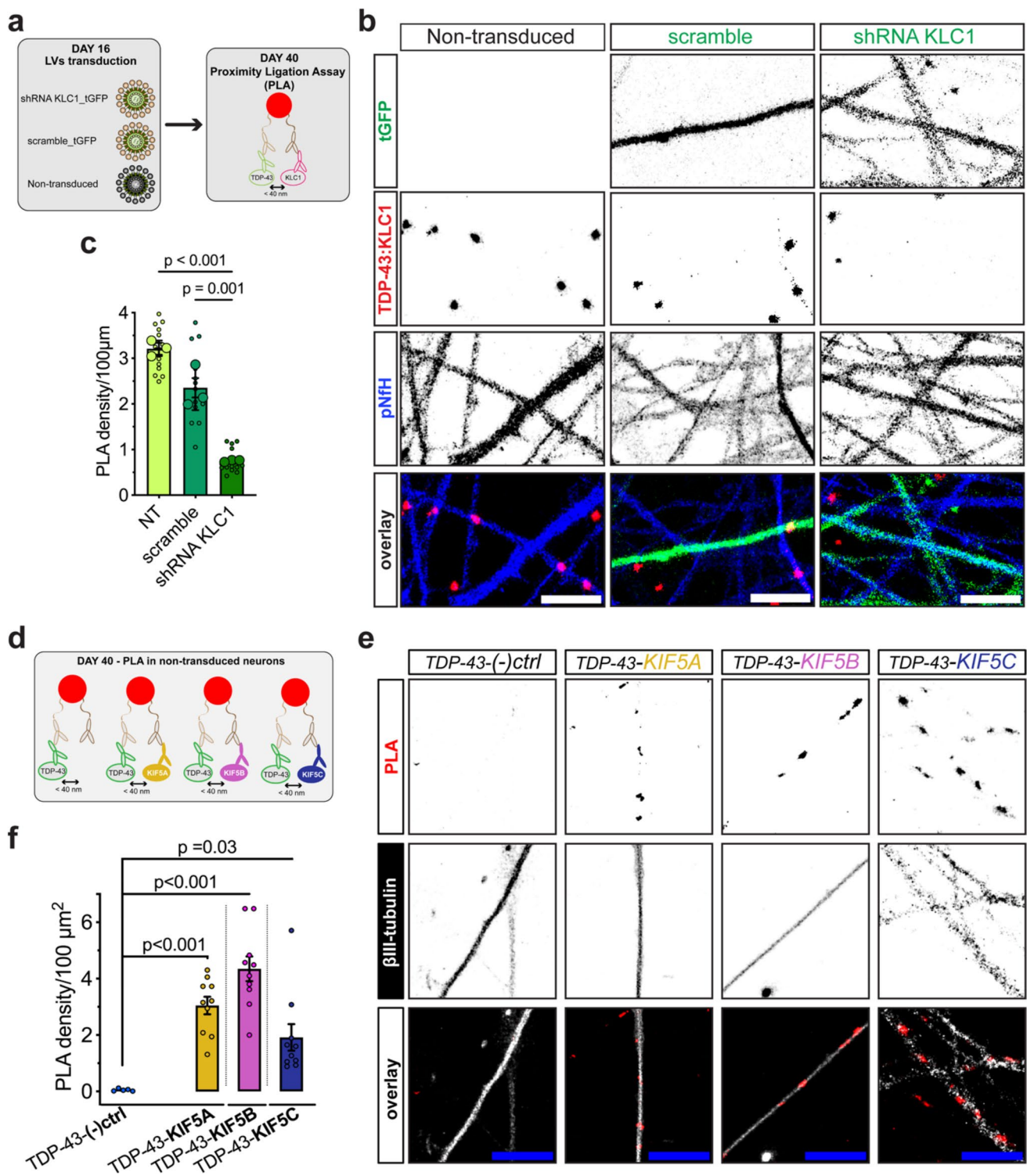
Published in vivo studies have also shown that pathological TDP-43 can propagate through neural connections and produce circuit-specific neurodegeneration. Tsuboguchi et al. demonstrated that mutant TDP-43 spreads along neuro-glial connections within the corticospinal pathway, resulting in distinct patterns of degeneration in spinal motor circuits [93]. Using adeno-associated virus (AAV)-mediated expression of mutant TDP-43 in mice, the authors showed that TDP-43 expressed in upper motor neurons (UMNs) underwent anterograde transport along corticospinal tract axons and was transferred to oligodendrocytes, leading to mild axonal degeneration. In contrast, mutant TDP-43 expressed in lower motor neurons (LMNs) did not undergo retrograde propagation but instead induced severe motor neuron loss and degeneration of neighboring spinal neurons, resulting in muscle atrophy. These findings demonstrate that pathological TDP-43 can affect neural circuits in a direction- and cell-type-dependent manner. However, they should not be interpreted as evidence that physiological axonal transport of wild-type TDP-43 characterized in the present study is equivalent to pathological TDP-43 propagation in human ALS.

Given the marked clinical and neuropathological heterogeneity of ALS, it is unlikely that a single mechanism of TDP-43 propagation accounts for all disease phenotypes. Classical ALS, bulbar-onset ALS, primary lateral sclerosis, progressive muscular atrophy, and genetically defined forms of ALS differ in the relative involvement of upper and lower motor neurons, patterns of TDP-43 pathology, and disease progression. Accordingly, the bidirectional physiological transport of TDP-43 demonstrated here should not be viewed as supporting an exclusively anterograde or retrograde model of disease spread. Rather, it provides a mechanistic framework within which multiple modes of pathological propagation may operate depending on disease subtype and stage.

To understand the physiological mechanisms that may become disrupted in disease, we characterized TDP-43 axonal transport by comparing its dynamics to those of well-established cargoes with defined motor dependencies: Rab5 (predominantly retrograde, dynein-mediated) [44], SYP (predominantly anterograde, KIF1A-mediated) [55], and APP (bidirectional with slight anterograde bias, kinesin-1/dynein) [25]. Because these cargoes were imaged in separate cargo conditions rather than by simultaneous multicolor live-cell imaging, these comparisons identify shared and distinct transport characteristics but do not demonstrate physical co-transport between TDP-43 and the reference cargoes. Importantly, all the movies relative to each protein were acquired under identical experimental conditions and analyzed using the same tracking pipeline. This comparative framework therefore served to guide subsequent biochemical and proximity ligation experiments testing direct association between TDP-43 and motor/adaptor proteins.

Our net transport analysis revealed that TDP-43 MLOs exhibit balanced bidirectional movement, with nearly equal proportions of anterograde and retrograde transport. This feature, also observed for other mRNA-localizing RBPs [42, 63], distinguishes TDP-43 from membrane-bound vesicular cargoes, which generally display a stronger directional bias. Such balanced dynamics suggest that TDP-43 granules deliver mRNA cargoes to multiple axonal regions rather than a single subdomain, consistent with TDP-43's role in maintaining diverse aspects of RNA axonal homeostasis [11, 62, 69].

Despite this bidirectional behavior, TDP-43 displayed fast and processive dynamics resembling those of APP and SYP rather than the slower, pause-prone Rab5. TDP-43 exhibited higher reversals, but fewer pauses, and traveled distances comparable to vesicular cargoes. Although total TDP-43 track lengths were similar to those of SYP and Rab5, they were significantly shorter than those of APP, which exhibited the greatest range of traveled distances. Together, these findings indicate that TDP-43 transport supports both short- and long-range movements, consistent with the requirement



for local mRNA delivery and translation throughout the axon.

Velocity distribution analysis provided further mechanistic insight. TDP-43 anterograde velocities almost completely overlapped with those of APP, supporting the possibility that TDP-43-containing MLOs use kinesin-dependent

plus-end-directed mechanisms with properties comparable to APP transport. In contrast, TDP-43 retrograde velocities differed from those of all three reference cargoes, suggesting that although dynein-dynactin broadly mediates retrograde transport, its processivity is likely regulated in a cargo-specific manner through differential adaptor engagement [7, 20,

Fig. 5 KLC1 and the three KIF5 isoforms associate with TDP-43 sustaining anterograde axonal transport. **a** Schematic representation of LV transduction strategy for KLC1 downregulation experiments, followed by proximity ligation assay (PLA) at DIV 40. **b** Representative micrographs of non-transduced (NT), scrambled control with TurboGFP (LV-Scramble-tGFP), and shRNA targeting KLC1 with the fluorescent reporter tGFP (LV-shKLC1-tGFP) transduced neurons showing tGFP signals (green), in scramble and shRNA, PLA dots representing positive/negative interaction between TAR DNA-binding protein 43 (TDP-43):KLC1 (red), axonal marker pNfH (blue) and overlay images (scale bar=10 μ m). **c** Quantification of PLA densities analyzed along axonal projections (pNfH) performed in NT, scramble, and LV-shKLC1-tGFP neurons ($n=14$ images for NT and scramble and $n=16$ images for shRNA KLC1 from 3 different biological replicates; mean values per image were obtained from $7 \leq n \leq 24$ traced axons per image). **d** Schematic representation of PLA experiments conducted at DIV 40 in human neurons using specific kinesin-5A, B, and C (KIF5A, B, and C) antibodies to detect interactions with TDP-43. **e** Representative micrographs of the PLA experiments performed to validate the interaction of TDP-43 with the three different isoforms of motor protein family kinesin-1 including KIF5A, KIF5B, and KIF5C. Images show (+) PLA signal (red), and the axonal marker β III-tubulin (white), along which is possible to follow the red spots. Pictures show negative control with TDP-43 antibody only (left), TDP-43-KIF5A (center left), TDP-43-KIF5B (center right), and TDP-43-KIF5C (right); last row represents overlay images of PLA signal along neuronal projections (scale bar=10 μ m). **f** Quantification of PLA densities in TDP-43:KIF5A, TDP-43:KIF5B, and TDP-43:KIF5C vs negative control ($n=5$ images and $n=10$ images from 3 different biological replicates for TDP-43(-) ctrl and TDP-43:KIF5A/B/C PLAs, respectively). Data show density of PLA dots over 100 μ m axonal distance as mean $\pm$ S.D (e), and density of PLA dots over 100 μ m² as mean $\pm$ S.D (f). Statistical comparison was performed using a One-way ANOVA followed by Tukey's multiple comparisons test (e) and unpaired *t*-test with Welch's correction for independent comparisons since different antibodies cannot be compared between each other, though TDP-43(-) ctrl was run in parallel (f). Approximate or exact *p* values are shown in the figure

35]. This distinct retrograde profile may reflect specialized adaptor engagement or unique biophysical properties of TDP-43-containing RNP granules.

Co-immunoprecipitation and proximity ligation assays provided independent evidence that TDP-43 associates with both anterograde and retrograde transport machinery. TDP-43 interacted with KLC1 and DCTN1, consistent with its bidirectional transport profile, and KLC1 knockdown markedly reduced the TDP-43–KLC1 proximity ligation signal, confirming specificity of this interaction. Remarkably, TDP-43 also interacted with all three mammalian kinesin-1 isoforms, KIF5A, KIF5B, and KIF5C [52, 90]. This broad engagement may provide functional flexibility, enabling TDP-43 to use distinct transport pathways depending on neuronal compartment, mRNA cargo composition, or local axonal demand. This model gained further support when we observed that TDP-43 also associates with KIF1A, a kinesin-3 family motor primarily responsible for transporting synaptic vesicle precursors [45, 55].

This finding is consistent with our transport analyses showing that TDP-43 shares processive anterograde

features with SYP, while the biochemical and proximity ligation result provide direct evidence for TDP-43–KIF1A association. The engagement of TDP-43 with both kinesin-1 and kinesin-3 families suggests a multi-motor transport mechanism serving distinct functional roles. KIF1A may facilitate rapid long-distance delivery of selected TDP-43 granule populations to distal synaptic terminals, whereas kinesin-1 may support more regulated transport or delivery to intermediate axonal regions. Alternatively, distinct TDP-43-containing MLO populations, defined by their mRNA cargoes or phase-separation properties, may preferentially recruit different motor systems.

An important implication of our findings is that TDP-43 MLOs display transport dynamics comparable to classical vesicular cargoes. Current models of axonal transport have largely been developed from studies of membrane-bound vesicles, in which transmembrane proteins provide recognition sites for motor-adaptor complexes [39, 60]. The observation that TDP-43-containing MLOs engage similar motor systems raises several important mechanistic questions: How do motors recognize and bind to these condensates in the absence of membranes? Are specific RNA sequences or RNA binding proteins (RBPs) motifs serving as adaptor recruitment sites? Does the biophysical state of TDP-43 condensates regulate motor engagement? Previous studies have proposed that RNA granules can ‘hitchhike’ on motile organelles such as lysosomes or mitochondria [29, 60, 86]. Our results demonstrate that TDP-43 can closely associate with molecular motor proteins, suggesting that TDP-43-containing complexes possess an intrinsic capacity to engage transport machinery independently of membrane-bound organelles, similar to other RBPs [42], and regulatory microtubule-associated binding proteins involved in mRNA transport [10]. Whether direct motor engagement and organelle hitchhiking operate in parallel to transport distinct TDP-43-bound mRNA populations remains an important question for future investigation.

Although the present study was performed in human neurons expressing physiological TDP-43 rather than ALS patient-derived iPSC motor neurons, it defines the physiological transport mechanisms that may become vulnerable during disease. In TDP-43 proteinopathies, disruption of anterograde transport could impair the delivery of mRNA required for local translation, synaptic function, and axonal maintenance [8, 15, 51, 53], whereas disruption of retrograde transport could alter the redistribution and homeostasis of TDP-43-containing ribonucleoprotein (RNP) granules within axons. Consistent with this concept, *TARDBP* mutations and *C9orf72* repeat expansions have been associated with impaired mRNA trafficking and local translation [3, 31, 56]. In addition, mutations affecting components of the dynein-dynactin complex have been linked to ALS and other motor neuron disorders [35, 87, 88]. Pathological TDP-43

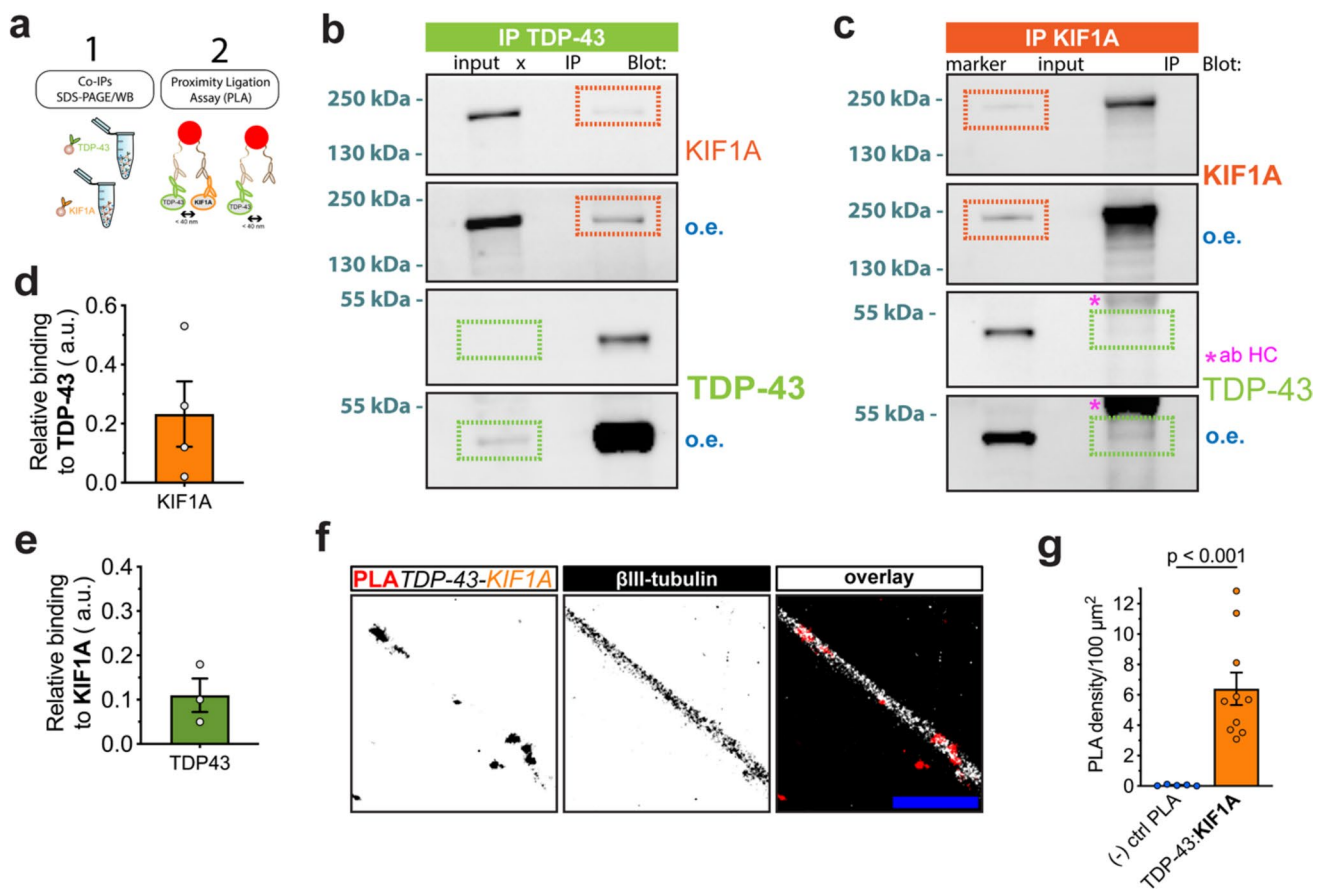


Fig. 6 TDP-43 alternatively associates with KIF1A synaptic vesicle motor protein. **a** Schematic representation of co-IP and PLA experiments conducted at DIV 40 in human neurons using TDP-43 and KIF1A antibodies to test their interaction. **b** Representative immunoblots of TDP-43 IP bands (green box) and kinesin 1A (KIF1A) co-IP bands (orange box). Each protein is represented by two images to show both unsaturated and overexposed images (o.e.). **c** Representative immunoblots of KIF1A IP bands (orange box) and TDP-43 co-IP bands (green box). Each protein is represented by two images to show both unsaturated and overexposed images (o.e.). **d** Co-IPs ratios expressed as KIF1A intensity relative to TDP-43 IP ($n=4$ biological replicates). **e** Co-IPs ratios expressed as TDP-43 intensity relative to

KIF1A IP ($n=3$ biological replicates). **f** Representative micrographs of the PLA experiments performed to validate the interaction of TDP-43 with KIF1A. Images show (+) PLA signal (red), and the axonal marker β III-tubulin (white), and the overlay of the two channels highlighting the red spots distribution (scale bar = 10 μm). **g** Quantification of PLA densities in TDP-43:KIF1A vs TDP-43:(-)ctrl ($n=5$ images and $n=10$ images from 3 different biological replicates for TDP-43:(-)ctrl PLAs and TDP-43:KIF1A, respectively). Data show mean $\pm$ S.D. (**d**, **e**, and **g**). Statistical comparison was performed using unpaired *t*-test with Welch's correction. Approximate *p* values are shown in the figure

aggregates may further compromise axonal transport by sequestering motor proteins or adaptor molecules [50, 83, 94]. The engagement of multiple kinesin motor proteins observed in the present study may provide physiological redundancy, allowing partial compensation when individual transport pathways become impaired, for example in the context of KIF5A dysfunction, a recognized genetic cause of ALS [9, 75]. Likewise, rare *KIF1A* variants associated with ALS further support the broader relevance of kinesin-dependent anterograde transport for motor neuron integrity [59]. However, combined impairments of cytoskeletal integrity, mitochondrial function, RNA granule homeostasis, and proteostasis may ultimately overwhelm this coordinated transport system.

In non-TDP-43 ALS forms, such as *SOD1*- or *FUS*-associated ALS, canonical TDP-43 pathology is generally absent, making disruption of TDP-43 transport unlikely to represent the initiating pathogenic event. Because *SOD1* and *FUS* mutations represent two different genetic backgrounds of ALS, driven by distinct molecular mechanisms, any effects on TDP-43 transport are likely to be mutation- and cell-type-specific. Nevertheless, because RNA metabolism, axonal transport, cytoskeletal organization, and mitochondrial function are commonly disrupted across diverse forms of ALS, physiological TDP-43 RNP transport may still become secondarily affected. Future studies using patient-derived iPSC motor neurons should determine whether TDP-43 transport is selectively altered in *TARDBP*- and

C9orf72-ALS mutations or more broadly perturbed across other genetic and sporadic ALS subtypes.

These considerations are particularly important in light of the marked clinical and pathological heterogeneity of ALS. Our findings should therefore not be interpreted as supporting a single directional model of disease propagation or a uniform corticofugal mechanism. Instead, they demonstrate that physiological TDP-43 undergoes bidirectional axonal transport and engages both anterograde and retrograde transport machinery, although anterograde transport exhibits a prominent association with kinesin-dependent pathways. Consequently, disruption of TDP-43 trafficking in ALS could involve impaired delivery of RNP granules to distal axons, altered retrograde redistribution, or both. The relative contribution of these mechanisms is likely to vary across ALS subtypes, neuronal populations, genetic backgrounds, and disease stages.

Together, our findings demonstrate that physiological TDP-43 utilizes a multi-motor transport system within axons. Rather than supporting a single model of disease propagation, our results establish a mechanistic framework for understanding how different components of physiological TDP-43 transport may become disrupted in disease and highlight the importance of identifying cortical neurons, which model upper motor neurons, from spinal motor neurons, which model lower motor neurons, in studying axon biology in ALS pathogenesis [61]. This framework provides a foundation for future studies in ALS-relevant models to determine whether TDP-43 trafficking contributes to impaired mRNA delivery, disrupted local translation, abnormal RNP granule dynamics, and ultimately axonal degeneration.

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Author contributions M.F. and G.B.S. conceived the study, developed the methodology, and wrote the original draft. M.F. and V.M.P.D. performed the investigation, software development, formal analysis, and data curation. M.F., V.M.P.D., N.D., M.Č., and K.K. conducted experimental investigations, including co-immunoprecipitation assays and plasmid design and preparation. C.L.-S. and K.L.M. supervised the study and contributed to manuscript editing. G.F., R.A.S., and C.N.S. provided supervision and funding acquisition and contributed to manuscript editing. G.B.S. managed project administration and funding acquisition. All authors reviewed the manuscript.

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Data availability All relevant data associated with the current study are available in the manuscript or the supplementary material. Additional information about data and protocols is available from the authors upon request.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval Not applicable.

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