# Effects of TDP-43 overexpression on neuron proteome and morphology *in vitro*

**Rachel AK Atkinson[1], Hannah L Fair[1], Richard Wilson[2], James C Vickers[1], Anna E King[1]**

[1]Wicking Dementia Research and Education Centre, University of Tasmania, Hobart, Tasmania, 7000, Australia

[2]Central Science Laboratory, University of Tasmania, Hobart, Tasmania, 7001, Australia

Corresponding Author: Rachel Atkinson, email Rachel.Atkinson@utas.edu.au

## Abstract

TDP-43 is pathologically and genetically associated ALS and FTD. These diseases are characterized by significant neurite defects, including cytoskeletal pathology. The involvement of TDP-43 in the degeneration of neurons in these diseases are not yet well understood, however accumulating evidence shows involvement in neurite outgrowth, remodelling and in regulation of many components of the neuronal cytoskeleton. In order to investigate how alterations to TDP-43 expression levels may exert effects on the neuronal cytoskeleton, primary cortical neurons from transgenic mice overexpressing one or two copies of human wildtype TDP-43 under the prion promoter were examined. Label-free quantitative proteomic analysis, followed by functional annotation clustering to identify protein families that clustered together within up- or down-regulated protein groups, revealed that actin-binding proteins were significantly more abundant in neurons overexpressing TDP-43 compared to wildtype neurons. Morphological analysis demonstrated that during early development neurons expressing one copy of human TDP-43 had an increased number of branches and alterations to growth cone morphology, while no changes were observed in neurons expressing two copies of TDP-43. These developmental processes require specific expression and organization of the cytoskeleton. The results from these studies provide further insight into the normal function of TDP-43 and how alterations in TDP-43 expression may impact the cytoskeleton.

**Keywords** TDP-43, neuron morphology, proteomics, ALS, FTD

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## Introduction

A prominent feature of ALS/FTD tissue is extensive neurite abnormalities, including neuromuscular dieback, axonal and dendritic swellings, swollen presynaptic terminals, reduced dendritic arborization, spine loss and transport deficits, indicated by accumulation of abnormal organelles (Ferrer et al., 1991, Sasaki and Iwata, 1996, Zhou et al., 1998, Vickers et al., 2009, King et al., 2011, Brettschneider et al., 2013, Brettschneider et al., 2014). Neurite abnormalities are linked to cytoskeletal disruption (Vickers et al., 2009).

TDP-43 pathology is present in 95% of ALS cases and 50% of FTD cases, usually presenting as mislocalization from the nucleus to the cytoplasm where it forms aggregates. TDP-43 pathology is also found within dystrophic neurites in ALS and FTD (Hatanpaa et al., 2008, Braak et al., 2010) and white matter tract degeneration is associated with FTLD-TDP (Armstrong, 2017). The links between TDP-43 pathology and neurite abnormalities are unclear, however there is accumulating evidence that TDP-43 plays a normal role in regulating the cytoskeleton, and that disruption to TDP-43 in disease conditions is sufficient for neurite abnormalities.

TDP-43 is essential for neuronal development (Sephton et al., 2012) and is upregulated during circuit formation (Liu et al., 2015). TDP-43 binds directly to *NEFL* mRNA to stabilize it, and regulates translocation of *NEFL* mRNA to the cytosol (Strong et al., 2007, Volkening et al., 2009). TDP-43 is a predominantly nuclear protein where it exerts the majority of its functions (reviewed in(Lee et al., 2012) but is also present in distal neuron compartments where it is important for transport of messenger ribonucleoprotein particles down axons to their distal targets (Alami et al., 2014). The localization of TDP-43 expression in neurites and its ability to directly regulate cytoskeletal elements suggest that alterations to TDP-43 expression levels may drive neurite abnormalities in disease. In fact, mutations to TDP-43 can cause cellular toxicity and abnormal translocation of TDP-43 to axons (Tripathi et al., 2014) as well as impair the transport of messenger ribonuclear proteins and reduce axonal outgrowth (Alami et al.,

2014, Fallini et al., 2012). These data suggest that alterations to TDP-43 could impair axonal function through a direct role of TDP-43 in the regulation of the integrity of the cytoskeleton.

This study sought to further investigate how alterations to TDP-43 expression levels, which occur in human disease (Mishra et al., 2007) may exert effects on the neuronal cytoskeleton. Primary neuronal cultures were prepared from transgenic mice overexpressing wildtype human TDP-43 under the control of the prion promoter (hTDP-$43_{Prp}$). The heterozygous and homozygous hTDP-$43_{Prp}$ mice express TDP-43 at 1.9 and 2.5 times the rates of endogenous TDP-43 expression, respectively (Xu et al., 2010). Postnatal heterozygous mice are viable and lack pathological changes, however homozygous mice develop a severe degenerative phenotype by 4 to 6 weeks of age, accompanied by TDP-43 aggregates and mislocalization to the cytoplasm in the brain and spinal cord (Xu et al., 2010). Other abnormalities include mitochondrial changes, gliosis and – importantly – axonal and myelin degeneration within the spinal cord (Xu et al., 2010).

Primary neurons cultured from these mice are an ideal model for detailed examination of the effect of TDP-43 overexpression on the expression of cytoskeletal proteins and the downstream effects of this on the neurite. Two main approaches were taken in this study. Firstly, proteomics, which has previously been used to identify interacting partners of TDP-43 (Freibaum et al., 2010), to allow analysis of global changes to proteins following alteration to TDP-43 expression. Secondly, neuron morphology was analyzed to determine the effect of TDP-43 alterations to developmental processes such as neurite outgrowth and branching, which require specific expression and organization of the cytoskeleton. The results from these studies provide further insight into how alterations in TDP-43 expression of may cause changes to neurites.

**Methods**

## Animals

TDP-43$_{Prp}$ mice (Xu et al., 2010) (C57BL/6-Tg(Prnp-TARDBP)3cPtrc/J, Jackson Laboratories, stock number 016608) were utilized in this study. Due to the phenotype developed by homozygous mice, colonies were maintained as heterozygotes for breeding stock. For culture studies, heterozygote mice were mated to obtain homozygous, heterozygous and wildtype embryos. All experiments involving animals were approved by the University of Tasmania Animal Ethics Committee (A15121) in accordance with the Australian Guidelines for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 2013) and followed ARRIVE guidelines.

## Genotyping

Mice from the maintenance colony (heterozygous and wildtype mice), were genotyped using genomic DNA from tail clippings (Quanta Biosciences). Genotypes (heterozygote and wildtype) were then determined by conventional PCR using the MyTaq™ Red Mix (Bioline) using primers specific to hTDP-43 and Tcrd internal control (Table S1). The PCR conditions are outlined in Table S2. Products were electrophoresed with a 400bp band for mutant and 200bp band for wildtype. To determine zygosity of embryos cultured from two heterozygous parents, quantitative real time PCR (qPCR) was utilized. Genomic DNA was extracted from embryonic liver using the Isolate II Genomic DNA Extraction Kit (Bioline), with precaution taken to avoid contamination from the mother's blood. qPCR was carried out using GoTaq® Probe qPCR Master Mix with a maximum of 150ng of liver DNA and the following primer sets outlined in Table S3. qPCR conditions are outlined in Table S4.

## Primary Cell Culture

Primary dissociated cortical cultures were prepared as described previously (Atkinson et al., 2015) with slight modifications. Heterozygous mice were mated and resulting embryos were

harvested at embryonic day (E) 15.5. Each embryo was cultured individually and liver tissue was collected for genotyping as outlined in above. Following decapitation, heads were stored in Hibernate media (Gibco) at 4°C until time of culture (10 minutes to 1 hour), with samples blinded to the researcher until the end of experimental analysis. Cortical tissue (including both cortex and hippocampus) was collected into 1ml HBSS and enzymatically dissociated in 0.0125% trypsin for 4 minutes, prior to plating. Cells were plated onto a variety of surfaces, pre-coated with 10% poly L-lysine (Sigma Aldrich). For immunofluorescence and neurite outgrowth assays, cells were plated onto 12mm coverslips at a concentration of 30,000 viable cells per coverslip. For protein harvest, whole cells were plated into 12 well trays at a concentration of 200,000 viable cells per well.

**Western blotting**

Protein was harvested from whole cells plated in 12 well trays at 3 and 10 days in vitro (DIV; n=3 cultures/genotype/timepoint) in 100µL of RIPA buffer (Sigma) containing protease (Complete Mini Cocktail, Roche) and phosphatase inhibitors (AG Scientific). Protein quantification and Western blotting were carried out as described previously (Atkinson et al., 2015). Following Western blotting, membranes were incubated in human TDP-43 (Novus Biologicals, reference number H00023435, 1:1000; Zhang, 2008), total TDP-43 (binding to both mouse and human TDP-43; Proteintec Group; reference number 1078-2-AP, 1:1000), GAPDH (Millipore, reference number MAB374, 1:5000), Lamin-AC (Santa Cruz, reference number SC-376248, 1:250). For cellular fractionation, nuclear and cytoplasmic proteins were extracted from cells at 3 DIV (n=3 cultures/genotype) according to manufacturer's instructions (NE-PER Nuclear and Cytoplasmic Extraction Kit, Thermofisher Scientific).

**Proteomics analysis**

***Protein and peptide sample preparation***

Protein was harvested from wildtype and homozygous neurons (n=3 cultures/genotype) which had been plated in 12 well plates and grown to 10 DIV. One hundred microliters of lysis buffer (7M urea, 2M thiourea and 30mM tris) containing protease (Complete Mini Cocktail, Roche) and phosphatase inhibitors (AG Scientific) was used to extract protein. Cells were sonicated for 3 cycles of 15 seconds in the sonicator, 5 minutes out in ice, then kept at 4°C for 2 hours, centrifuged at 13,000 rpm for 15 minutes, and supernatant collected. Protein concentration was determined by performing a Bradford assay as per manufacturer's protocol (BioRad Protein Assay, BioRad) and approximately 90 ug of protein was trypsin-digested according to published methods (Wilson et al., 2010).

***Liquid chromatography separation and mass spectrometry***

The resulting tryptic peptides, equivalent to ~ 1µg digested protein, were analyzed using nano high performance liquid chromatography (HPLC) on an Ultimate 3000 RSLCnano system (Thermo Fisher Scientific, MA, USA). Firstly, peptides were concentrated on a 20mm x 75µm PepMap 100 trapping column (3µm C18) at a flowrate of 5µL/min, using 98% water, 2% acetonitrile and 0.05% trifluoroacetic acid (TFA). Peptides were then separated on a 250mm x 75µm PepMap 100 RSLC column (2µm C18) at a flowrate of 300nL/min, held at 40°C. Separation included a 240 minute gradient from 98% mobile phase A (0.1% formic acid in water) to 50% mobile phase B (0.08% formic acid in 80% acetonitrile and 20% water) and included the following steps: 3-10% B over 10 minutes, 10-40% B over 180 minutes, 40-50% B over 10 minutes, holding at 95% B for 10 minutes then re-equilibration in 2% B for 15 minutes. The HPLC system was coupled to an LTQ-Orbitrap mass spectrometer, controlled using Xcalibur 2.1 software in data-dependent mode. MS/MS spectra were acquired using a Top8 method and 30-second dynamic exclusion of fragmented peptides, as previously described (Wilson et al., 2016).

***Protein identification and analysis***

Data files were imported into MaxQuant version 1.5.1.2 (http://maxquant.org/) and MS/MS spectra were searched using the Andromeda engine against the complete *Mus musculus* (mouse) reference proteome (ID 000000589) comprising 44,455 protein entries. Default settings for protein identification by LTQ-Orbitrap MS/MS and label-free quantitation (LFQ) included a maximum of two missed cleavages, mass error tolerances of 20 ppm then 4.5 ppm for initial and main peptide searches, respectively, 0.5 Da tolerance for fragment ions, variable methionine oxidation and fixed carbamidomethylation. A false discovery rate (FDR) of 0.01 was used for peptide-spectrum matches and protein identification.

The MaxQuant algorithm MaxLFQ was used for peptide intensity determination and normalization (Cox et al., 2014), using pair-wise comparison of unique and razor peptide intensities and a minimum ratio count of 2. The MaxQuant proteinGroups output file was processed as follows: The normalized label-free quantification (LFQ) intensity values, MS/MS counts and the numbers of razor and unique peptides for each of the identified proteins were imported into Perseus software version 1.5.031 (http://perseus-framework.org/). Protein groups identified as potential contaminants and proteins only identified by site or by reverse database matching were removed and LFQ intensity values were log2–transformed. The proteins were filtered to include only those detected in all three replicates of at least one genotype. Missing values were replaced with random intensity values for low-abundance proteins based on a normal distribution of protein abundances using default MaxQuant parameters. This filter was applied to ensure that results were reproducible and that proteins detected at low abundance in one genotype but were below detection level in another genotype were still included. To determine proteins that were significantly altered in abundance between genotypes a two-sided t-test was used with significance set at $p < 0.05$, using 250 randomizations, and a minimum fold-change cut-off of

1.3. The data was exported from Perseus into Excel. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD022671. To extract functional information from the proteomic data, proteins that were significantly up- or down-regulated in homozygote samples compared to wildtype samples were imported into the online bioinformatics resource DAVID (version 6.8; https://david.ncifcrf.gov/; (Huang et al., 2009). Protein lists were analyzed using the mouse genome database, and annotation clusters were ranked using the Functional Annotation Clustering tool based on default parameters, with p values < 0.05 after adjustment using the Benjamini-Hochberg correction for multiple testing considered significant. The complete set of Functional Annotation Clusters is reported in the Supplementary Material (Table S5).

**Immunocytochemistry**

At 3 and 10DIV coverslips were rinsed, fixed and ICC was carried out as described previously (Atkinson et al. 2015). Primary antibodies were diluted in 0.6% Triton-X-100 (Sigma) and included: TDP-43 (mouse and human, Proteintec Group, reference number 10782-2-AP, 1:1000); MAP2 (Millipore, reference number MAB3418, 1:1000); beta 3 tubulin (Promega, reference number G712A, 1:5000), SMI312 (Covance, reference number R500, 1:1000). Following overnight incubation at 4°C, coverslips were incubated in species-specific secondary antibodies for two hours. In some experiments, the f-actin stain phalloidin (1:200, Life Technologies) was applied to coverslips for 1 hour following secondary incubation. Nuclei were stained with DAPI (5µg/ml, Life Techologies) for 5 minutes. Coverslips were washed 3 x 10 minutes with PBS and mounted. Images were captured with a BX-50 Olympus microscope and a Photometrics Coolsnap HQ2 camera (3DIV) or a Cell Discoverer7 (Zeiss) microscope (10DIV).

**Nuclear and cytoplasmic distribution**

The distribution of total TDP-43 in the nucleus and cytoplasm was determined by immunocytochemistry and nuclear/cytoplasmic fractionation. For immunocytochemistry (n=3 cultures per genotype, minimum 10 images taken across 2 coverslips), careful attention was paid to ensure that coverslips were consistently fixed, incubated with antibody, washed and imaged with identical exposures. MAP2 and DAPI were used to delineate cytoplasm and nuclei, regions of interest (ROIs) were manually constructed around these cellular compartments and the integrated density of TDP-43 labeling in these areas was measured as previously described (Herzog et al., 2017).

**Growth cone analysis**

Growth cones were analyzed at 3DIV (n=3 cultures per genotype, minimum 5 images taken across 2 coverslips), and immunolabelled with beta 3 tubulin and f-actin. Growth cones were classified as filopodial, lamellipodial or blunt (Figure 4), based on the paper by Khazaei et al. (2014). For classification, 100 growth cones from each genotype were systematically examined across two coverslips per culture (three cultures per genotype) by a researcher blinded to the genotype groups. Beta 3 tubulin labeling and f-actin staining were used to examine length and number of filopodia; size of growth cone; and ratio of actin to tubulin. Approximately 30 images from the two coverslips for each culture were obtained, and ImageJ software was used for analysis as described previously (Khazaei et al., 2014).

**Neuron morphology analysis**

Phase contrast images of neurons at 3DIV were captured on a Nikon Live Cell microscope (Nikon Instruments Inc; NIS-Elements AR 4.00.12 Software, Nikon) by a researcher blinded to genotype groups. Approximately 60 individual neurons per genotype (n=3 cultures/genotype across 2 coverslips) were examined. Images were obtained systematically across each coverslip to capture neurons where the whole neurite tree could be visualized. Images were imported

into Neurolucida (MBF Bioscience) and cell bodies and neurites were traced. Figure 5A demonstrates the decision-making process for branch points. The longest neurite, normally considered to be the axon, was also traced separately. Traced images were imported into Neurolucida Explorer (MBF Bioscience) to determine cell body size; length of longest neurite; number of neurite trees; total length of neurite tree; number of branches in each order; and total number of branch points.

**Neurite density analysis**

Fluorescent images of neurons immunolabeled with SMI312 at 10DIV were captured by a researcher blinded to genotype groups. Two images containing 9 fields of view at 20x (n=3 cultures/genotype), equating to approximately 16% of the coverslip, were examined. Images were segmented using WEKA segmentation software to distinguish between neurites, cell bodies and background (Arganda-Carreras et al., 2017). The percentage area of neurites was expressed as a function of cell body area to give a measurement of neurite density, which took into account differences in plating density.

**Statistical analysis**

For all analyses, data from genotypes was grouped but genotypes were blinded until results were obtained. Unless otherwise stated, statistical analysis was carried out using a one-way ANOVA with Tukey's post hoc test. Neurolucida tracing data and growth cone morphology data were analyzed using mixed models with random intercepts to account for clustering within culture batches. The assumptions of normality of residuals and homogeneity of variance were checked using graphical methods (Q-Q and residual plots), and where the assumption of normality was violated, data were either log transformed or a generalized linear model with appropriate link function (*e.g.* Poisson link function for count data) was fitted to ensure that statistical conclusions were robust. Likelihood ratio tests were used to determine statistical

significance. To test differences in the distribution of growth cone morphology between genotypes, Pearson's Chi-square test of homogeneity was calculated. *Post-hoc* comparisons were corrected using the Bonferroni method. All statistical analysis was conducted in the R statistical language (R Core Team, 2016). Mixed models were fitted using the 'lme4' package in R (Bates, 2015). Data are presented ± standard error of the mean (SEM) and significance set at $p<0.05$.

## Results

### Expression of TDP-43 in development

Western blot analysis of protein harvested from cultured cortical neurons grown to 3 and 10 DIV confirmed the presence of human TDP-43 in cells from both homozygous and heterozygous embryos, and the absence from wild type cells, using a human-specific TDP-43 antibody (Zhang et al., 2008) (Figure 1 A and D). Human TDP-43 was significantly increased in homozygote cells compared to wildtype at 3 and 10DIV ($p<0.05$) (Figure 1 B and E), and also in heterozygote cells compared to wildtype cells at 10DIV. Although there was no statistical difference between heterozygote cells and wildtype cells at 3DIV, the blot (Figure 1 A) demonstrates the presence of the transgene in heterozygote cells. As TDP-43 is a self-regulating protein, the level of total TDP-43 expression was then determined using an antibody that recognizes both mouse and human (total) TDP-43. Total TDP-43 was significantly higher in both heterozygote and homozygote cells compared to wildtype cells at both 3 and 10DIV ($p<0.05$) (Figure 1 C and F).

### Cellular localization of TDP-43

In ALS/FTD, there is evidence that mislocalization of TDP-43 from the nucleus to the cytoplasm may cause both gain and loss of protein function (Lee et al., 2012). To determine

whether increased expression of human TDP-43 caused a change in localization of protein expression, the distribution of TDP-43 (mouse and human) was analyzed in the nucleus and cytoplasm at 3 DIV. Qualitative analysis of immunocytochemically labelled neurons suggested that TDP-43 was more abundant in the cytoplasm in homozygote cells (Figure 2 A). Integrated densitometry of TDP-43 fluorescence demonstrated higher levels in both the nucleus and cytoplasm in homozygote cells compared to wildtype cells ($p<0.05$) (Figure 2 B, C). These results qualitatively showed that both total TDP-43 and human TDP-43 were expressed at higher levels in the nuclear and cytoplasmic compartments in homozygote samples compared to heterozygote and wildtype samples.

**Global alterations to protein expression following TDP-43 overexpression**

TDP-43 interacts with over 30% of the genome (Sephton et al., 2011) and its effects are likely to be broad. A proteomics approach was used to determine global changes to protein expression in transgenic cells compared to wildtype. Protein was harvested at 10 DIV as there was insufficient protein for analysis at earlier timepoints. Principal component analysis of homozygote samples and wildtype samples demonstrated a relatively high degree of variability between the three homozygote replicates (Figure 3 A). In the 10 DIV samples, 85 proteins were downregulated and 94 proteins were upregulated in homozygote samples compared to wildtype samples (p-value $< 0.05$, FC $\pm$ 1.3) (Figure 3 B). The online bioinformatics database tool, DAVID, was used for functional annotation clustering to identify protein families that clustered together within up- or down-regulated protein groups (Figure 3 C). Functional terms associated with downregulated proteins included translation, rRNA binding, and mitochondria, whereas upregulated proteins were associated with the COP9 signalosome, proteasome complex and actin binding. These results demonstrated that proteins related to the normal function of TDP-43 were disrupted by increased expression, as well as proteins associated with functions known to be altered by abnormal TDP-43 (mitochondria and proteasome). Furthermore, increased

297 expression of TDP-43 upregulated proteins related to actin binding, including Actn1, Arpc4,
298 Capza1, Capza2, Cotl1, Marcksl1 and Myh9 (Figure 3 D). Many of these proteins are involved
299 in the regulation of growth cone dynamics and axon outgrowth.

300 **Effects of TDP-43 overexpression on growth cones**

301 Previous studies have suggested that altered TDP-43 affects neurite outgrowth (Tripathi et al.,
302 2014, Fallini et al., 2012). This is controlled by growth cones, found at the growing tip of
303 neurites, in which attractive or repulsive extracellular guidance cues cause reorganization of
304 microtubules and actin filaments. Since altered TDP-43 expression was found to affect actin-
305 dependent processes, the morphology and cytoskeletal composition of growth cones in
306 homozygous, heterozygous and WT TDP-43 cultured neurons were examined. Neuronal
307 growth cones were examined at 3 DIV, when cortical neurons are actively pathfinding in
308 culture before the majority of synapses form (Dotti et al., 1988). Immunolabelling with beta 3
309 tubulin and staining with the f-actin stain, phalloidin, were used to visualize growth cones.
310 Growth cones were classified as filopodial, lamellipodial or blunt (based on Khazaei et al.
311 (2014) (Figure 4 A). A chi-squared test for homogeneity demonstrated a significant difference
312 ($\chi^2_4$ =11, $p<0.05$) in the distribution of growth cone morphologies between genotypes;
313 specifically, heterozygote cultures had fewer lamellipodial growth cones, and more filopodial
314 growth cones (Figure 4 B). Filopodial growth cones across the genotypes were further analyzed
315 for changes to the number (Figure 4 C) or length (Figure 4 D) of growth cone filopodia,
316 however no differences were found. The area of phalloidin-stained f-actin (Figure 5 E) and
317 beta 3 tubulin (Figure 4 F) within filopodial growth cones was similar between genotypes, as
318 was the ratio of these areas (Figure 4 G).

319

320 **Effects of overexpression of TDP-43 on neurite morphology**

Initiation of neurites and branching is controlled by expression and reorganization of cytoskeletal proteins. To investigate whether changes to TDP-43 expression cause cytoskeletal alterations, neuron morphology across the three different genotypes was examined at an early developmental timepoint (3DIV) and a more mature timepoint (10DIV). Due to the differing complexities of neurons at these two timepoints, two separate approaches were taken for analysis. Neurons at 3 DIV were traced using Neurolucida and several morphological measures were assessed (branching schematic demonstrated in Figure 5A i). Representative images of neurons from the 3 genotypes are shown in Figure 5A ii. Axonal outgrowth, quantitated by measuring the longest neurite in each cell, and the total length of all neurites showed no significant differences between genotypes (Figure 5A iii, iv). Neuritogenesis was examined by looking at the number of neurite trees coming from the cell body (Figure 5 A v) and the total number of branch points (Figure 5 vi). From these analyses, the only parameter altered was the total number of branches in neurite trees, which was increased in heterozygous compared to wildtype cells ($p<0.05$) (Figure 5 vi).

Neurons at 10DIV were immunolabelled with SMI312, a marker of phosphorylated neurofilament highly expressed within axons. Neurite density was determined by determining the percentage area of neurites compared to cell density (Figure 5B). These results demonstrated there were no differences in the neurite density between the genotypes.

## Discussion

Following on from reports that TDP-43 plays a role in neurite development, this study focused on gaining a better insight into the relationship between TDP-43 and the neuronal cytoskeleton. Proteomics demonstrated that overexpression of wildtype TDP-43 lead to a range of proteins that were differentially regulated, of which actin-binding proteins were of particular interest.

Additionally, in cultured cells, increased branching and altered growth cone morphology were also observed.

A key pathological feature of FTD/ALS associated with TDP-43 is the mislocalization of TDP-43 from the nucleus to the cytoplasm. The current data shows that homozygous neurons have a modest increase in TDP-43 within both cytoplasm and nuclei, which may explain the differences in morphology phenotypes between the homozygous and heterozygous neurons. Increased expression of TDP-43 could alter its role in both compartments, including interactions with DNA and RNA targets, as well as transport in mRNA granules (Alami et al., 2014).

***Global changes to protein expression***

This study examined whether altered TDP-43 levels could result in changes to cytoskeletal proteins. Due to the vast number of interaction pathways affecting cytoskeletal and associated proteins, a proteomic approach was taken to give a global view. Bioinformatic analysis of the proteomes of wildtype and homozygous mice demonstrated changes across a range of functional domains, including several linked to established TDP-43 roles. For example, proteins related to RNA binding and translation were highly down-regulated, consistent with the role of TDP-43 as an RNA binding protein (Sephton and Yu, 2015). Previous proteomic studies examining interacting partners of TDP-43 demonstrate strong associations with translation machinery (Freibaum et al., 2010). Alterations to the localization, and therefore function, of TDP-43 in homozygote cells would be consistent with alterations to these protein families. Other observed changes included the ubiquitin-proteosome system (UPS), which is highly associated with ALS/FTD. TDP-43 is ubiquitinated in protein aggregates in diseased tissue (Neumann et al., 2006). Levels of TDP-43 protein are regulated by the UPS through degradation of monomeric TDP-43, preventing accumulation and aggregation (Scotter et al.,

2014), emphasizing the importance of regulation to this system. Overexpression of a protein is also consistent with an upregulation of the protein degradation system to try and normalise protein levels within the cell, and may be consistent with pathological TDP-43 aggregation.

Down regulation of mitochondrial proteins was also observed, in accordance with previous research, which demonstrated clustering of mitochondria within axons and dendrites in the same mouse model (Xu et al., 2010). Mitochondrial dysfunction is a common theme in TDP-43 mouse models of ALS and FTD, causing aggregation, fragmentation or vacuolation of mitochondria (Magrane et al., 2014, Wang et al., 2013). Abnormal mitochondria are also a feature of human disease with swollen and vacuolated mitochondria observed in neurofilamentous axon swellings (Sasaki and Iwata, 2007).

***The effect of TDP-43 alterations on the cytoskeleton***

Of particular interest to the current study was the finding that actin-binding proteins were among the most up-regulated proteins in the proteomic analysis. Actin-binding proteins are important for controlling the cytoskeletal network through actions such as filament nucleation, severing, crosslinking, end capping and monomer sequestering. For cortical neurons during development, these processes affect pathfinding, neurite outgrowth and branching. A number of actin binding proteins were altered, including non-muscular myosin heavy chain 11B (NMHC11-B), also known as myosin-9 (MYH9), which was upregulated in homozygote cells. This protein has previously been associated with TDP-43 alterations; SH-SY5Y cells with a TDP-43 knockdown resulted in a cytoplasmic increase and a nuclear decrease of MYH9 levels compared to controls (Stalekar et al., 2015), and immunoprecipitation of HEK293T cells transfected with FLAG-TDP-43 demonstrated that MYH9 interacts with TDP-43 (Freibaum et al., 2010). In neurons, MYH9 is important for driving neurite outgrowth, is involved in growth cone motility (Wylie, 1998) and for NMDA receptor trafficking (Amparan et al., 2005).

Alterations to NMHC11-B/MYH9 protein expression have been found in both ALS patient brains containing TDP-43 aggregates and in transgenic pigs overexpressing the TDP-43 M337V mutation (Wang et al., 2015). In more developed neurons, the actin cytoskeleton and actin binding proteins become important for supporting synaptic transmission and synaptic plasticity, as the main structural component of synapses (Dillon, 2005). Arp2/3, highlighted by the proteomic data, aids the formation of F-actin networks (Dos Remedios, 2003). Its putative interaction with TDP-43 and the identified actin-binding proteins is yet to be determined.

***Effects of altered cytoskeleton on neuronal phenotype***

In the current study, morphology was examined at 3DIV and 10DIV. At 3DIV cultured neurons have become polarized and are undergoing dynamic branching and pathfinding (Dotti et al., 1988). At 10DIV neurons are mature but continue to increase in size and elongate neurites to form a network of axons and dendrites (Dotti et al., 1988). Filopodial growth cones, important for sampling the extracellular environment (Omotade et al., 2017), were increased in heterozygous cultures at 3DIV. The formation of filopodia and lamellipodia is thought to be controlled by rapid polymerization of the actin cytoskeleton at the leading edge of the growth cone, followed by depolymerization within the growth cone. TDP-43 interacts with members of the guanosine triphosphate hydrolase enzyme family (GTPase) involved in these processes. Rac1 is important for the formation of lamellipodia while Cdc42 is important for forming filopodia (Nobes, 2017). Knockdown of TDP-43 has previously been shown to inactivate both Rac1 and Cdc42 (Iguchi et al., 2009), plausibly explaining alterations to growth cone morphology following TDP-43 overexpression.

In the current study, total branch number was also increased in heterozygous neurons at 3DIV, although the length of the neurite trees was similar across genotypes, implying that neurons became more ramified without a change in neurite length. Branch formation requires

coordinated changes in actin and microtubules, and differs in axons and dendrites. A general feature of neurite branching appears to be the protrusion of actin filaments from filopodia and/or lamellipodia on the neurite, followed by invasion of microtubules as the branch matures and continues extending (Armijo-Weingart and Gallo, 2017). In line with these findings, Lu and colleagues (Lu et al., 2009) found that overexpression of *Drosophila* TDP-43, or human TDP-43 in *Drosophila*, increased dendritic branching of sensory neurons. Additionally, Schwenk and colleagues (2016) found that knockdown of TDP-43 in cultured hippocampal neurons reduced the complexity of dendrites at both 10 and 19DIV, highlighting the important role for TDP-43 in maintaining neuron connectivity. In contrast to these findings, at 10DIV in the current study, neurite density appeared similar between genotypes, however there was a high degree of variability, likely due to the analysis method.

Results from the current study may indicate that there is a differential effect of increased TDP-43 in heterozygotes and homozygotes, demonstrated by changes to growth cone morphology and branching observed only in heterozygote mice. One possible explanation is that the increased cytoplasmic expression of TDP-43 in homozygous neurons that was observed may become toxic (for example undergoing early protein oligomerization), negating the results observed in heterozygous neurons.

During FTD/ALS cells are known to undergo loss of connectivity, and alterations to dendritic arborization and dendrite length have been observed in cases of FTD/ALS (Ferrer et al., 1991). During disease states, cells may increase their amount of TDP-43 to try and reestablish connectivity. However, concomitant stresses on the UPS system may alter the ability to degrade excess TDP-43, leading to aggregation and mislocalization. The results of this study

highlight the importance of tight regulation of TDP-43 levels, and suggest future studies examining TDP-43's role in regulation of the cytoskeleton.

**Supplementary methods:**

**Table S1 Conventional PCR primer information**

| Primer | Sequence 5'-3' |
|---|---|
| hTDP-43 Forward(20µM) | GGATGAGCTGCGGGAGTTCT |
| hTDP-43 Reverse (20µM) | TGCCCATCATACCCCAACTG |
| Tcrd internal control forward (20µM) | CAAATGTTGCTTGTCTGGTG |
| Tcrd internal control reverse(20µM) | GTCAGTCGAGTGCACAGTTT |

**Table S2 Conditions for conventional PCR**

| Cycle step | Incubation times |
|---|---|
| Initial denaturation | 94°C 2 min |
| 10 cycles | Step 1: 94°C for 20 sec<br>Step 2: 64°C (-0.5°C/cycle) for 15 sec<br>Step 3: 68°C for 10 sec |
| Amplification (28 cycles) | Step 1: 94°C for 15 sec<br>Step 2: 60°C for 15 sec<br>Step 3: 72°C for 10 sec |
| Final extension | 72°C for 2 min |
| Hold | 11°C infinite |

**Table S3 qPCR primer information**

| Primer | 5’ label | Sequence 5’-3’ | 3’ label |
|---|---|---|---|
| Tg Forward(40µM) | | GTACGGGGATGTGATGGATG | |
| Tg Reverse (40µM) | | CGCAATCTGATCATCTGCAA | |
| Tg probe (40µM) | 6-FAM | CCAAGCCATTCAGGGCCTTTGC | Black Hole Quencher 1 |
| Apob internal control forward (100µM) | | CACGTGGGCTCCAGCATT | |
| Apob internal control reverse(40µM) | | TCACCAGTCATTTCTGCCTTTG | |
| Internal control probe (5µM) | Cy5 | CCAATGGTCGGGCACTGCTCAA | Black Hole Quencher 2 |

453

454 **Table S4 Conditions for qPCR**

| Cycle step | Incubation times |
|---|---|
| Initial denaturation | 94°C 2 min |
| Hot start (10 cycles) | Step 1: 94°C for 30 sec<br>Step 2: 64°C (-1°C/2 cycles) for 45 sec<br>Step 3: 68°C for 30 sec |
| Amplification (28 cycles) | Step 1: 94°C for 30 sec<br>Step 2: 60°C for 45 sec<br>Step 3: 72°C for 30 sec (aquisition) |
| Final extension | 72°C for 2 min |

455

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459

460 **References:**

Alami, N. H., Smith, R. B., Carrasco, M. A., Williams, L. A., Winborn, C. S., Han, S. S. W., Kiskinis, E., Winborn, B., Freibaum, B. D., Kanagaraj, A., et al. 2014. Axonal Transport of TDP-43 mRNA Granules Is Impaired by ALS-Causing Mutations. *Neuron,* 81**,** 536-543.

Arganda-Carreras, I., Kaynig, V., Rueden, C., Eliceiri, K. W., Schindelin, J., Cardona, A. & Sebastian Seung, H. 2017. Trainable Weka Segmentation: a machine learning tool for microscopy pixel classification. *Bioinformatics,* 33**,** 2424-2426.

Armijo-Weingart, L. & Gallo, G. 2017. It takes a village to raise a branch: Cellular mechanisms of the initiation of axon collateral branches. *Molecular and Cellular Neuroscience***,** 1-12.

Armstrong, R. A. 2017. White matter pathology in sporadic frontotemporal lobar degeneration with TDP-43 pathology. *Clinical Neuropathology,* 36**,** 66-72.

Atkinson, R. a. K., Fernandez-Martos, C. M., Atkin, J. D., Vickers, J. C. & King, A. E. 2015. C9ORF72 expression and cellular localization over mouse development. *Acta Neuropathologica Communications,* 3.

Bates, D. M., M.; Bolker, B.; Walker, S. 2015. Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software,* 67**,** 1-48.

Braak, H., Ludolph, A., Thal, D. R. & Del Tredici, K. 2010. Amyotrophic lateral sclerosis: dash-like accumulation of phosphorylated TDP-43 in somatodendritic and axonal compartments of somatomotor neurons of the lower brainstem and spinal cord. *Acta neuropathologica,* 120**,** 67-74.

Brettschneider, J., Arai, K., Del Tredici, K., Toledo, J. B., Robinson, J. L., Lee, E. B., Kuwabara, S., Shibuya, K., Irwin, D. J., Fang, L., et al. 2014. TDP-43 pathology and neuronal loss in amyotrophic lateral sclerosis spinal cord. *Acta neuropathologica,* 128**,** 423-437.

Brettschneider, J., Tredici, K. D., Toledo, J. B., Robinson, J. L., Irwin, D. J., Grossman, M., Suh, E., Van Deerlin, V. M., Wood, E. M., Baek, Y., et al. 2013. Stages of pTDP-43 pathology in amyotrophic lateral sclerosis. *Annals of neurology,* 74**,** 20-38.

Cox, J., Hein, M. Y., Luber, C. A., Paron, I., Nagaraj, N. & Mann, M. 2014. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Molecular & cellular proteomics : MCP,* 13**,** 2513-2526.

Dillon, C. G., Y. 2005. The actin cytoskeleton: integrating form and function at the synapse. *Annu Rev Neurosci,* 28**,** 25-55.

Dos Remedios, C. G. C., D.; Kekic, M.; Dedova, L. V.; Tsubakihara, M.; Berry, D. A.; Nosworthy, N. J. 2003. Actin binding proteins: regulation of cytoskeletal microfilaments. *Physiological reviews,* 83.

Dotti, C. G., Sullivan, C. A. & Banker, G. A. 1988. The establishment of polarity by hippocampal neurons in culture. *The Journal of Neuroscience,* 8**,** 1454-1468.

Fallini, C., Bassell, G. J. & Rossoll, W. 2012. The ALS disease protein TDP-43 is actively transported in motor neuron axons and regulates axon outgrowth. *Human molecular genetics,* 21**,** 3703-3718.

Ferrer, I., Roig, C., Espino, A., Peiro, G. & Matias Guiu, X. 1991. Dementia of frontal lobe type and motor neuron disease. A Golgi study of the frontal cortex. *Journal of neurology, neurosurgery, and psychiatry,* 54**,** 932-934.

Freibaum, B. D., Chitta, R. K., High, A. A. & Taylor, J. P. 2010. Global analysis of TDP-43 interacting proteins reveals strong association with RNA splicing and translation machinery. *Journal of proteome research,* 9**,** 1104-1120.

Hatanpaa, K. J., Bigio, E. H., Cairns, N. J., Womack, K. B., Weintraub, S., Morris, J. C., Foong, C., Xiao, G., Hladik, C., Mantanona, T. Y., et al. 2008. TAR DNA-binding protein 43 immunohistochemistry reveals extensive neuritic pathology in FTLD-U: a midwest-southwest consortium for FTLD study. *Journal of neuropathology and experimental neurology,* 67**,** 271-279.

Herzog, J. J., Deshpande, M., Shapiro, L., Rodal, A. A. & Paradis, S. 2017. TDP-43 misexpression causes defects in dendritic growth. *Sci Rep,* 7**,** 15656.

Huang, D. W., Sherman, B. T. & Lempicki, R. A. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols,* 4**,** 44-57.

Iguchi, Y., Katsuno, M., Niwa, J.-I., Yamada, S.-I., Sone, J., Waza, M., Adachi, H., Tanaka, F., Nagata, K.-I., Arimura, N., et al. 2009. TDP-43 depletion induces neuronal cell damage through dysregulation of Rho family GTPases. *The Journal of biological chemistry,* 284**,** 22059-22066.

Khazaei, M. R., Girouard, M.-P., Alchini, R., Ong Tone, S., Shimada, T., Bechstedt, S., Cowan, M., Guillet, D., Wiseman, P. W., Brouhard, G., et al. 2014. Collapsin Response Mediator Protein 4 regulates growth cone dynamics through the actin and microtubule cytoskeleton. *Journal of Biological Chemistry*.

King, A., Maekawa, S., Bodi, I., Troakes, C. & Al-Sarraj, S. 2011. Ubiquitinated, p62 immunopositive cerebellar cortical neuronal inclusions are evident across the spectrum of TDP-43 proteinopathies but are only rarely additionally immunopositive for phosphorylation-dependent TDP-43. *Neuropathology : official journal of the Japanese Society of Neuropathology,* 31**,** 239-249.

Lee, E. B., Lee, V. M. Y. & Trojanowski, J. Q. 2012. Gains or losses: molecular mechanisms of TDP43-mediated neurodegeneration. *Nature reviews. Neuroscience,* 13**,** 38-50.

Liu, Y., Atkinson, R. a. K., Fernandez-Martos, C. M., Kirkcaldie, M. T. K., Cui, H., Vickers, J. C. & King, A. E. 2015. Changes in TDP-43 expression in development, aging, and in the neurofilament light protein knockout mouse. *Neurobiology of aging,* 36**,** 1151-1159.

Lu, Y., Ferris, J. & Gao, F.-B. 2009. Frontotemporal dementia and amyotrophic lateral sclerosis-associated disease protein TDP-43 promotes dendritic branching. *Molecular brain,* 2**,** 30.

Magrane, J., Cortez, C., Gan, W. B. & Manfredi, G. 2014. Abnormal mitochondrial transport and morphology are common pathological denominators in SOD1 and TDP43 ALS mouse models. *Human molecular genetics,* 23**,** 1413-1424.

National Health and Medical Research Council 2013. Australian code for the care and use of animals for scientific purposes, 8th edition. *In:* COUNCIL, C. N. H. A. M. R. (ed.).

Neumann, M., Sampathu, D. M., Kwong, L. K., Truax, A. C., Micsenyi, M. C., Chou, T. T., Bruce, J., Schuck, T., Grossman, M., Clark, C. M., et al. 2006. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science (New York, N.Y.),* 314**,** 130-133.

Nobes, C. D. H., A. 2017. Rho, Rac, and Cdc42 GTPases Regulate the Assembly of Multimolecular Focal Complexes Associated with Actin Stress Fibers, Lamellipodia, and Filopodia. *Cell* 81**,** 53-62.

Omotade, O. F., Pollitt, S. L. & Zheng, J. Q. 2017. Actin-based growth cone motility and guidance. *Molecular and Cellular Neuroscience***,** 1-7.

Perez-Riverol, Y., Csordas, A., Bai, J., Bernal-Llinares, M., Hewapathirana, S., Kundu, D. J., Inuganti, A., Griss, J., Mayer, G., Eisenacher, M., et al. 2019. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Research,* 47**,** D442-D450.

559 R Core Team 2016. R: A language and environment for statistical computing. *R Foundation*
560 *for Statistical Computing, Viena, Austria*.
561 Sasaki, S. & Iwata, M. 1996. Ultrastructural study of synapses in the anterior horn neurons of
562 patients with amyotrophic lateral sclerosis. *Neuroscience letters,* 204**,** 53-56.
563 Sasaki, S. & Iwata, M. 2007. Mitochondrial Alterations in the Spinal Cord of Patients With
564 Sporadic Amyotrophic Lateral Sclerosis. *J Neuropathol Exp Neurol,* 66.
565 Schwenk, B. M., Hartmann, H., Serdaroglu, A., Schludi, M. H., Hornburg, D., Meissner, F.,
566 Orozco, D., Colombo, A., Tahirovic, S., Michaelsen, M., et al. 2016. TDP-43 loss of
567 function inhibits endosomal trafficking and alters trophic signaling in neurons. *The*
568 *EMBO journal,* 35**,** 2350-2370.
569 Scotter, E. L., Vance, C., Nishimura, A. L., Lee, Y. B., Chen, H. J., Urwin, H., Sardone, V.,
570 Mitchell, J. C., Rogelj, B., Rubinsztein, D. C., et al. 2014. Differential roles of the
571 ubiquitin proteasome system and autophagy in the clearance of soluble and aggregated
572 TDP-43 species. *Journal of cell science,* 127**,** 1263-1278.
573 Sephton, C. F., Cenik, B., Cenik, B. K., Herz, J. & Yu, G. 2012. TDP-43 in central nervous
574 system development and function: clues to TDP-43-associated neurodegeneration.
575 *Biological chemistry,* 393**,** 589-594.
576 Sephton, C. F. & Yu, G. 2015. The function of RNA-binding proteins at the synapse:
577 implications for neurodegeneration. *Cellular and molecular life sciences : CMLS*.
578 Strong, M. J., Volkening, K., Hammond, R., Yang, W., Strong, W., Leystra-Lantz, C. &
579 Shoesmith, C. 2007. TDP43 is a human low molecular weight neurofilament (hNFL)
580 mRNA-binding protein. *Molecular and cellular neurosciences,* 35**,** 320-327.
581 Tripathi, V. B., Baskaran, P., Shaw, C. E. & Guthrie, S. 2014. Tar DNA-binding protein-43
582 (TDP-43) regulates axon growth in vitro and in vivo. 65**,** 25-34.
583 Vickers, J. C., King, A. E., Woodhouse, A., Kirkcaldie, M. T., Staal, J. A., Mccormack, G. H.,
584 Blizzard, C. A., Musgrove, R. E. J., Mitew, S., Liu, Y., et al. 2009. Axonopathy and
585 cytoskeletal disruption in degenerative diseases of the central nervous system. *Brain*
586 *research bulletin,* 80**,** 217-223.
587 Volkening, K., Leystra-Lantz, C., Yang, W., Jaffee, H. & Strong, M. J. 2009. Tar DNA binding
588 protein of 43 kDa (TDP-43), 14-3-3 proteins and copper/zinc superoxide dismutase
589 (SOD1) interact to modulate NFL mRNA stability. Implications for altered RNA
590 processing in amyotrophic lateral sclerosis (ALS). *Brain research,* 1305**,** 168-182.
591 Wang, G., Yang, H., Yan, S., Wang, C.-E., Liu, X., Zhao, B., Ouyang, Z., Yin, P., Liu, Z.,
592 Zhao, Y., et al. 2015. Cytoplasmic mislocalization of RNA splicing factors and aberrant
593 neuronal gene splicing in TDP-43 transgenic pig brain. *Molecular Neurodegeneration,*
594 10**,** 42.
595 Wang, W., Li, L., Lin, W. L., Dickson, D. W., Petrucelli, L., Zhang, T. & Wang, X. 2013. The
596 ALS disease-associated mutant TDP-43 impairs mitochondrial dynamics and function
597 in motor neurons. *Human molecular genetics,* 22**,** 4706-4719.
598 Wilson, R., Diseberg, A. F., Gordon, L., Zivkovic, S., Tatarczuch, L., Mackie, E. J., Gorman,
599 J. J. & Bateman, J. F. 2010. Comprehensive profiling of cartilage extracellular matrix
600 formation and maturation using sequential extraction and label-free quantitative
601 proteomics. *Molecular & cellular proteomics : MCP,* 9**,** 1296-1313.
602 Wilson, R., Golub, S. B., Rowley, L., Angelucci, C., Karpievitch, Y. V., Bateman, J. F. &
603 Fosang, A. J. 2016. Novel Elements of the Chondrocyte Stress Response Identified
604 Using an in Vitro Model of Mouse Cartilage Degradation. *Journal of proteome*
605 *research,* 15**,** 1033-1050.
606 Wylie, S. R. W., P.-J.; Patel, H.; Chantler, P. D. 1998. A conventional myosin motor drives
607 neurite outgrowth. *Proceedings of the National Academy of Sciences,* 95**,** 12967-12972.

Xu, Y.-F., Gendron, T. F., Zhang, Y.-J., Lin, W.-L., D&Apos;Alton, S., Sheng, H., Casey, M. C., Tong, J., Knight, J., Yu, X., et al. 2010. Wild-type human TDP-43 expression causes TDP-43 phosphorylation, mitochondrial aggregation, motor deficits, and early mortality in transgenic mice. *Journal of Neuroscience*, 30**,** 10851-10859.

Zhang, H.-X., Tanji, K., Mori, F. & Wakabayashi, K. 2008. Epitope mapping of 2E2-D3, a monoclonal antibody directed against human TDP-43. *Neuroscience letters*, 434**,** 170-174.

Zhou, L., Miller, B. L., Mcdaniel, C. H., Kelly, L., Kim, O. J. & Miller, C. A. 1998. Frontotemporal dementia: neuropil spheroids and presynaptic terminal degeneration. *Annals of neurology*, 44**,** 99-109.

Please use the following details to access the mass spectrometry proteomics data via the Proteome Exchange http://www.ebi.ac.uk/pride
**Username:** reviewer_pxd022671@ebi.ac.uk
**Password:** XbKZM8YP

## Figure legends

**Figure 1 Characterization of total and human TDP-43 expression.** (A, D) Expression of total and human TDP-43 were analyzed by Western blot from cortical neuron protein from the three genotypes at 3 and 10 DIV (n=3 cultures per genotype, per timepoint). Quantification was carried out relative to GAPDH for human TDP-43 (B, E) and total TDP-43 (C, F). Results are mean and standard error. Statistical significance is defined as $*p<0.05$.

**Figure 2 Cellular localization of TDP-43.** (A) Cortical neurons from the three genotypes at 3DIV (n= 3 cultures per genotype, n= >20 neurons per culture) immunolabelled for TDP-43 (red), MAP2 (green) and DAPI (blue). MAP2 and DAPI were used to delineate the nucleus (B) and cytoplasm (C) within which the integrated density of TDP-43 was determined. Results are mean and standard error. Statistical significance is defined as $*p<0.05$. Scale bar 10µm.

**Figure 3 Proteomic analysis and protein families enriched in homozygote samples compared to wildtype samples.** (A) Principal component analysis of LFQ proteomic data for proteins extracted at 10 DIV from homozygote and wildtype cortical neurons (n= 3 cultures per genotype). (B) Results of t-test analysis displayed as a volcano plot showing $-\log_{10}$ transformed p-values versus $\log_2$ transformed fold change between wildtype and homozygote samples. A p-value threshold of 0.05 is signified by the solid line and proteins significantly more abundant in homozygous and wildtype samples are displayed as red and green data points, respectively. (C) Significant functional terms, based on Fisher's exact test, with the enrichment scores higher than 2.0. Terms associated with the proteins that were significantly more abundant in wildtype and homozygous samples are shown in green and red, respectively.

(D) Heat map of actin binding proteins showing the z-scored protein LFQ values for each sample replicate. Abbreviations: gene ontology (GO), biological processes (BP), cellular component (CC), UniProt (UP), molecular function (MF) constitutive photomorphogenesis 9 (COP9), ribosomal ribonucleic acid (rRNA).

**Figure 4 Alterations to growth cones.** Cortical neurons from the three genotypes at 3DIV (n= 3 cultures per genotype) were immunolabelled for B3 tubulin (green) and stained with phalloidin (red). Growth cones were classified as filopodial, lamellipodial or blunt (A) and counted for each genotype (n= 100 growth cones per culture) (B). Filopodial growth cones were analyzed further (C-G) (n= >15 filopodial growth cones per culture). Values are mean and standard error. Statistical significance is defined as $*p<0.05$, scale bar 5µm.

**Figure 5 Alterations of neuron morphology.** (A) Cortical neurons were imaged at 3DIV from the 3 genotypes (n=5 cultures per genotype, n= >20 neurons per culture). Cell bodies, neurites, and the longest neurites were traced using Neurolucida and branch points defined (i). (ii) Example phase contrast images of neurons showing branch morphology. Analysis yielded measures of morphological features (iii-vi). (B) Cortical neurons were imaged at 10DIV from the 3 genotypes (n=3 cultures per genotype, n=2 images containing 9 fields of view at 20x) and neurite density was calculated based on the percentage area of neurites compared to the percentage area of cell bodies. Values are mean and standard error. Statistical significance is defined as $*p<0.05$. Scale bar 50µm.