

RNA-binding deficient TDP-43 drives cognitive decline in a mouse model of TDP-43 proteinopathy

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Abstract

TDP-43 proteinopathies including frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS) are neurodegenerative disorders characterized by aggregation and mislocalization of the nucleic-acid binding protein TDP-43 and subsequent neuronal dysfunction. Here, we developed endogenous models of sporadic TDP-43 proteinopathy based on the principle that disease-associated TDP-43 acetylation at lysine 145 (K145) alters TDP-43 conformation, impairs RNA-binding capacity, and induces downstream mis-regulation of target genes. Expression of acetylation-mimic TDP-43^{K145Q} resulted in stress-induced nuclear TDP-43 foci and loss of TDP-43 function in primary mouse and human induced pluripotent stem cell (hiPSC)-derived cortical neurons. Mice harboring the TDP-43^{K145Q} mutation recapitulated key hallmarks of FTLD, including progressive TDP-43 phosphorylation and insolubility, TDP-43 mis-localization, transcriptomic and splicing alterations, and cognitive dysfunction. Our study supports a model in which TDP-43 acetylation drives neuronal dysfunction and cognitive decline through aberrant splicing and transcription of critical genes that regulate synaptic plasticity and stress response signaling. The neurodegenerative cascade initiated by TDP-43 acetylation recapitulates many aspects of human FTLD and provides a new paradigm to further interrogate TDP-43 proteinopathies.

eLife assessment

Necarsulmer et al describe an interesting new mouse model of TDP-43 proteinopathy in which gene editing was used to introduce a K145Q acetylation-mimic mutation previously shown to impair RNA-binding capacity and induce downstream misregulation of target genes. Mice homozygous for this mutation are **convincingly** shown to display cognitive/behavioral impairment, TDP-43 phosphorylation and insolubility, and changes in gene expression and splicing. This novel mouse model replicates some key hallmarks of human frontotemporal lobar degeneration and will be an **important** contribution to the field.

TDP-43 proteinopathies are characterized by the dysfunction and aggregation of Transactivation response element DNA-binding Protein of 43 kDa (TDP-43), with ~ 95% of all amyotrophic lateral sclerosis (ALS) and ~ 50-60% of all frontotemporal lobar dementia (FTLD-TDP) cases harboring TDP-43 pathology.^{1–4} There is significant neuropathologic and clinical overlap between FTLD and ALS with many individuals developing a mixed phenotype, providing strong evidence for a common FTLD/ALS spectrum of disorders.^{5–9} It is also notable that TDP-43 pathology is abundant in other sporadic neurodegenerative diseases including Alzheimer disease (AD),^{10,11} Limbic-Predominant Age-related TDP-43 Encephalopathy (LATE),^{12,13} and Parkinson's disease (PD).^{14,15} The clinical and neuropathological overlap suggests that common pathogenic mechanisms may link TDP-43 to neurodegeneration.^{16,17} However, modeling sporadic TDP-43 pathogenesis has been challenging since its expression levels are tightly regulated,^{18,19} which has precluded a clear separation of TDP-43 disease-related dysfunction from general toxicity resulting from TDP-43 over- or under-expression.^{20–23} Current knock-in models of TDP-43 disease-causing mutations^{24–28} provide valuable insights but may be limited in their application to sporadic disease.^{24–28}

Under normal physiological conditions, TDP-43 resides in the nucleus to control RNA processing (RNA splicing, transport, and stability) and gene transcription.^{29–31} Structurally, nuclear retention is primarily mediated by an N-terminal nuclear localization sequence (NLS) through interactions with α 1/ β -importins,^{32,33} and association with nucleic acids is mediated by two tandem RNA recognition motifs (RRM1/RRM2).^{34,35} However, there is interplay between nucleic acid binding and nuclear localization.^{36,37} The C-terminal glycine-rich domain (also termed the intrinsically disordered or low complexity domain) mediates protein-protein interactions.^{37–39} and harbors most, but not all, familial TARDBP mutations that are causative for FTLD/ALS.^{40,41} In sporadic and most familial TDP-43 proteinopathies, TDP-43 undergoes nuclear depletion and concomitant nuclear or cytoplasmic accumulation and aggregation.^{42–44} Both TDP-43 loss-of-function (e.g., aberrant cryptic splicing) and gain-of-function (e.g., aggregate-induced toxicity) mechanisms have been proposed as drivers of TDP-43 pathogenesis.^{45–48}

How TDP-43 becomes dysfunctional in sporadic disease remains unresolved, however, aberrant TDP-43 post-translational modifications (PTMs), such as phosphorylation, acetylation, and ubiquitination may play a role. PTMs modulate TDP-43's biochemical properties leading to conformational changes, modulation of nucleic acid binding affinity, regulation of liquid-liquid phase separation (LLPS), and propensity to form insoluble TDP-43 aggregates, all of which are disease-associated phenomena.^{49–51} Among the various PTMs, TDP-43 acetylation at lysine residue 145 (K145) within RRM1 has emerged as a critical regulator of both loss and gain of function toxicity.^{52,53} Acetylated TDP-43 is found within TDP-43 inclusions of sporadic ALS (sALS) spinal cord motor neurons but not age-matched control tissue.⁵² Inclusions in FTLD cortex are largely composed of C-terminal fragmented TDP-43 lacking the K145 residue,^{54,55} precluding an assessment of Ac-K145 in FTLD patients. However, *TARDBP* mutations that disrupt RNA binding, and thereby may act in a similar manner to TDP-43 acetylation, have been identified in FTLD-TDP patients (e.g. P112H and K181E),^{56,57} supporting a pathogenic role for altered nucleic acid binding in disease. Mimicking TDP-43 acetylation with a lysine-to-glutamine substitution (TDP-43^{K145Q}) is sufficient to neutralize the positive charge, disrupt RNA binding, and induce several hallmarks of TDP-43 pathology *in vitro*,^{52,53} supporting a model whereby TDP-43 acetylation drives both loss-of-function (e.g., RNA-binding deficiency) and gain-of-function (e.g., aggregation) toxicity.

Here, we used CRISPR/Cas9 genome editing to introduce a K145Q substitution into the endogenous mouse *Tardbp* gene, thereby generating acetylation-mimic TDP-43^{K145Q} knock-in mice, which enabled us to investigate the pathophysiological impacts of an aberrant TDP-43 PTM while leaving

native upstream and downstream genomic elements intact. Using mouse cortical neurons, human hiPSC-derived cortical neurons, and aged cohorts of TDP-43^{K145Q} homozygous mice, we found that acetylation-mimic TDP-43^{K145Q} induced nuclear TDP-43 foci and cytoplasmic TDP-43 accumulation, which coincided with several disease-associated and loss-of-function measures including widespread transcriptome and splicing alterations. Finally, we observed prominent FTL-like cognitive and behavioral deficits in acetylation-mimic TDP-43 mice that correlated with biochemical and splicing alterations in affected brain regions. Our study supports lysine acetylation of TDP-43 as a driver of dysfunction in sporadic TDP-43 proteinopathies.

Results

Mouse neurons expressing TDP-43^{K145Q} undergo stress-dependent formation of nuclear TDP-43 foci and loss of TDP-43 splicing function

We originally showed that TDP-43 acetylation can promote RNA-binding deficiency, aggregation, and pathology.^{52,53} We sought to expand these findings by exploring the behavior of acetylation-mimic TDP-43 variants in primary murine cortical neurons. We employed lentiviral vectors that encode either wild-type (TDP-43^{wt}), acetylation-deficient (TDP-43^{K145R}), and acetylation-mimic (TDP-43^{K145Q}) variants to overexpress TDP-43 species in neurons, and then examined their subcellular localization by immunofluorescence microscopy. In the absence of acute cellular stress, most neurons over-expressing TDP-43^{K145Q} showed distinct nuclear foci that were more prominent compared to TDP-43^{wt} or TDP-43^{K145R} constructs (**Figure 1A, B**). When neurons were exposed to an acute oxidative stressor (200 μ M sodium arsenite), a sensitizing agent commonly used to enhance TDP-43 dysfunction,^{58–61} there was a significant increase in TDP-43 foci formation with all variants (**Figure 1A, C**). The response in neurons expressing TDP-43^{K145Q} was very robust, resulting in the formation of numerous large, bright TDP-43-positive foci, as well as smaller TDP-43-positive nuclear foci. These aberrant TDP-43 structures were absent from cells expressing TDP-43^{wt} or acetylation-null TDP-43^{K145R}, indicating that acetylation-mimic TDP-43^{K145Q} alters TDP-43 conformation within the nucleus and sensitizes neurons to oxidative stress-induced foci formation. By coupling high content wide-field microscopy with quantitative image analysis, we observed a three-fold increase in TDP-43 foci formation in neurons expressing TDP-43^{K145Q} (**Figure 1C**).

TDP-43 overexpression can result in general toxicity and altered TDP-43 function, depending on the duration and the extent of over-expression.^{20,62,63} To avoid confounding non-specific toxicity, we transitioned to a more physiologically relevant model to further elucidate the impact of RNA-binding deficient acetylation-mimic TDP-43. We employed CRISPR-Cas9 mutagenesis to introduce a single amino acid substitution at position 145 (K145Q) into the endogenous mouse *Tardbp* locus, thereby generating TDP-43^{K145Q} knock-in mice (**Figure 2 - Figure supplement 1B**). By targeting the native mouse gene, we avoided both TDP-43 overexpression and disruption of the *Tardbp* untranslated regions (UTRs).¹⁸ A TDP-43^{K145Q} founder line was propagated as heterozygotes and continually re-sequenced to confirm retention and propagation of the K145Q substitution (**Figure 2 - Figure supplement 1C, D**). Both heterozygous and homozygous TDP-43^{K145Q} mice were born at normal mendelian frequencies and showed no obvious developmental defects.

We first investigated the effects of TDP-43^{K145Q} expression in neurons *in vitro* by isolating and culturing primary cortical neurons from homozygous TDP-43^{K145Q} mice, hereafter referred to as TDP-43^{KQ/KQ} mice and compared them to TDP-43^{wt}-derived neurons. Exposing neurons to acute oxidative stress induced more abundant TDP-43-positive nuclear foci in acetylation-mimic TDP-43^{KQ/KQ} neurons than in TDP-43^{wt} neurons (**Figure 2A, B**). Quantitative image analysis also

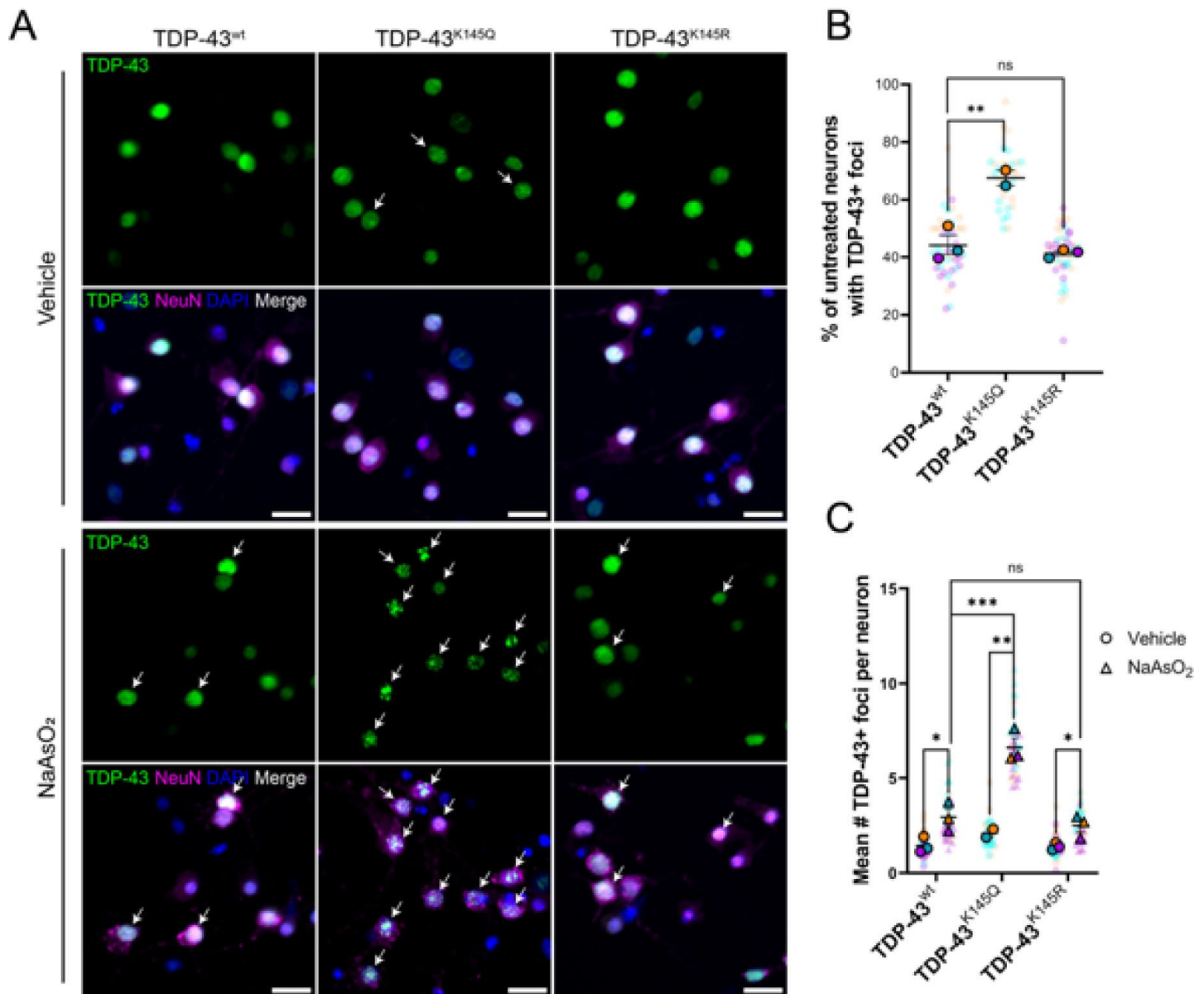


Figure 1

Ectopic expression of acetylation-mimic TDP-43^{K145Q}, an RNA-binding deficient mutant, shows accelerated formation of stress-induced nuclear TDP-43 foci

A Representative immunofluorescence images of TDP-43 in DIV14 mouse primary cortical neurons overexpressing TDP-43^{wt}, TDP-43^{K145Q}, or TDP-43^{K145R} after vehicle or 200μM NaAsO₂ treatment followed by labeling of TDP-43 (green), NeuN (magenta), and DAPI (blue). Arrows highlight nuclei with TDP-43+ foci. Scale bar = 20μm. **B** Quantification of percentage of neurons with TDP-43+ foci in vehicle-treated neurons. **C** Quantification of the average number of TDP-43+ foci per neuron. Data shown as Superplots⁸⁹; solid color bordered symbols and error bars indicate mean value of each biological replicate ± SEM; semi-transparent data points represent the average value per neuron in a single field of view, 10-110 neurons per field, 48 fields across n=2-4 biological replicates. Statistical analysis completed using a linear mixed-effect model. Statistical significance is represented by asterisks *p<0.05, **p<0.01, ***p<0.001. DIV= Day *in vitro*

revealed variable levels of nuclear clearing and cytoplasmic mislocalization of TDP-43 in TDP-43^{KQ/KQ} neurons, however, no statistically significant differences in TDP-43 localization was found between acetylation-mimic and TDP-43^{wt} neurons (**Figure 2A, C**). Because TDP-43 foci formation is associated with loss-of-function defects,^{64–66} we next investigated if TDP-43 function was impaired by evaluating endogenous targets of TDP-43 activity. We observed a trend toward increased *Tardbp* mRNA in TDP-43^{KQ/KQ} neurons at DIV 14 (**Fig 2D**), which was suggestive of TDP-43 loss of function and auto-regulation. We therefore investigated sortilin-1 (*Sort1*) mRNA splice variants, as a more sensitive endogenous indicator of loss of TDP-43-dependent splicing function. Functional nuclear TDP-43 results in the production of a mature spliced *Sort1* mRNA transcript, however, in the setting of TDP-43 depletion or loss of function, TDP-43 is unable to repress the inclusion of exon 17b, generating a longer *Sort1+ex17b* transcript variant.^{67,68} RT-qPCR analysis revealed a significant deficit in normal TDP-43 splicing function, with nearly a 10-fold increase in the ratio of *Sort1+ex17b* to *Sort1*-WT mRNA, without significant changes to total *Sort1* mRNA levels in TDP-43^{KQ/KQ} neurons compared to controls (**Fig 2E–F**). Overall, these results indicate that a single endogenously expressed acetylation-mimic TDP-43^{K145Q} mutation can sensitize TDP-43 to conformational changes that impair its normal splicing function in mouse neurons.

Acetylation-mimic TDP-43^{K145Q} alters TDP-43 function in human neurons

To assess this model's relevance to human neurons, we used CRISPR/Cas9 to generate a panel of human induced pluripotent stem cell (hiPSC) lines harboring homozygous acetylation-mimic TDP-43 (TDP-43^{K145Q.12} and TDP-43^{K145Q.18}), acetylation-deficient TDP-43 (TDP-43^{K145R.2} and TDP-43^{K145R.12}), or unmodified TDP-43 (isogenic control, TDP-43^{wt}) (**Figure 3 - Figure supplement 1**). We confirmed appropriate editing of the *TARDBP* gene in each line via Sanger sequencing (**Figure 3 - Figure supplement 1B**), verified the pluripotency of the hiPSC clones (**Figure 3 - Figure supplement 1C–E**), and then differentiated each of the lines into mature cortical neurons (**Figure 3 - Figure supplement 2**). We then used immunofluorescent labelling and confocal microscopy to qualitatively assess the distribution and morphology of TDP-43 protein within neurons, both with and without an acute exposure to sodium arsenite. Untreated hiPSC-derived TDP-43^{K145Q} cortical neurons were morphologically identical to TDP-43^{wt} and TDP-43^{K145R} neurons and showed similar patterns of TDP-43 localization (**Figure 3A**). All hiPSC-derived lines showed a granular nuclear TDP-43 localization pattern under normal conditions, consistent with physiologic de-mixing of nuclear TDP-43.⁶⁰ Following acute oxidative stress, however, TDP-43^{K145Q} neurons showed apparent TDP-43 nuclear clearing and the formation of large, intensely labeled TDP-43-positive foci (**Figure 3A**). In comparison, cortical neurons expressing TDP-43^{wt} or TDP-43^{K145R} maintained nuclear TDP-43 and formed small stippled TDP-43 puncta. We note that TDP-43 nuclear clearing and foci formation was more robust in hiPSC-derived neurons compared to mouse neurons, suggesting human neurons may be more sensitive to TDP-43 RNA-binding deficiency. Similar to mouse neurons, hiPSC-derived TDP-43^{K145R} neurons were indistinguishable from TDP-43^{wt} neurons, supporting acetylation-induced charge neutralization as a driver of TDP-43 loss of function rather than an inherent effect of mutation at the K145 locus.

We next asked whether hiPSC-derived TDP-43^{K145Q} cortical neurons show more robust functional deficits compared to the mouse neuron data above. We again employed RT-qPCR to measure levels transcripts associated with TDP-43 loss of function, beginning with *TARDBP* as an indicator of impaired TDP-43 autoregulation. In contrast to mouse neurons, we observed an approximately 2-fold increase *TARDBP* mRNA in TDP-43^{K145Q.12} and TDP-43^{K145Q.18} neurons and a slight but significant reduction in *TARDBP* mRNA in TDP-43^{K145R.12} neurons (**Figure 3B**). We then assessed TDP-43-dependent splicing activity using primers specific for human *SORT1* splice variants, whose regulation by TDP-43 is conserved in mouse and humans.^{67,68} The aberrant

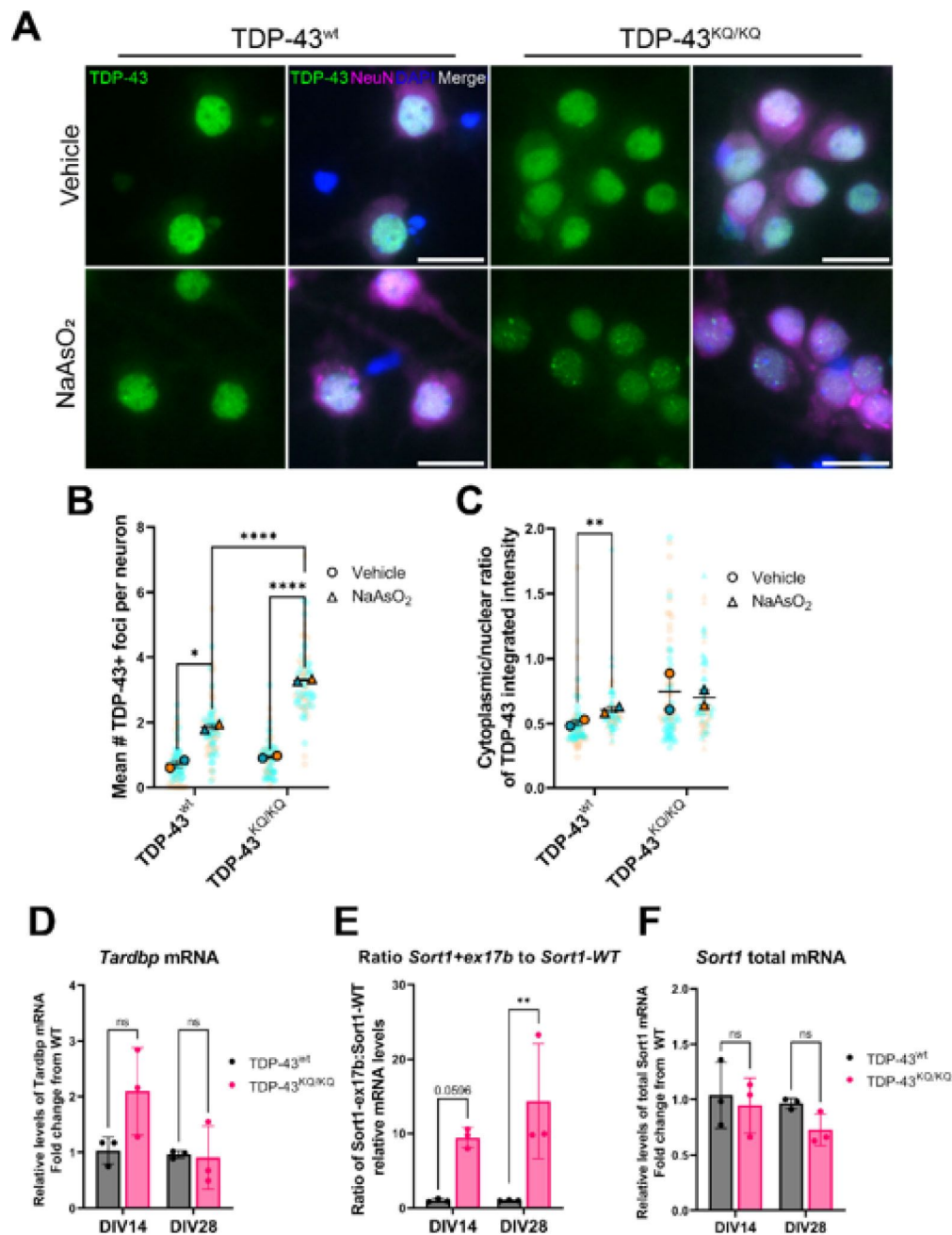


Figure 2

An endogenously encoded acetylation-mimic TDP-43^{K145Q} mutation causes altered TDP-43 localization and functional splicing deficits in mouse primary cortical neurons

A Representative images of primary cortical neurons derived from TDP-43^{wt} or TDP-43^{KQ/KQ} mice that were treated with vehicle or 200μM NaAsO₂ at DIV14 and immunolabeled for endogenous TDP-43 (green), NeuN (magenta), and DAPI (blue). **B-C** Quantification of the number of TDP-43+ foci (**B**) and the cytoplasmic:nuclear ratio of TDP-43 fluorescence intensity (**C**) in TDP-43^{KQ/KQ} compared to TDP-43^{wt} neurons. **D-G** RT-qPCR analysis of DIV14 and DIV28 untreated mouse primary cortical neurons using primers specific for mouse (**D**) *Tardbp* [F (1, 8) = 3.034, p=0.1197]; and *Sort1* splice variants: (**E**) ratio *Sort1+ex17b:Sort1-WT* [F (1, 8) = 23.02, p=0.0014] and (**F**) total *Sort1* mRNA [F (1, 8) = 5.086, p=0.0541]. **B-C** Data shown as Superplots⁸⁹; solid color bordered symbols and error bars indicate mean value of each biological replicate ± SEM; semi-transparent data points represent the average value per neuron in a single field of view, 10-110 neurons per field, 72 fields across n=2-4 biological replicates; one color represents one biological replicate. Statistical analysis performed using a linear mixed-effect model. **D-F** Analysis by two-way ANOVA followed by Šidák's multiple comparisons testing. F statistics represent main effect of genotype. Statistical significance is represented by asterisks, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns = not significant.

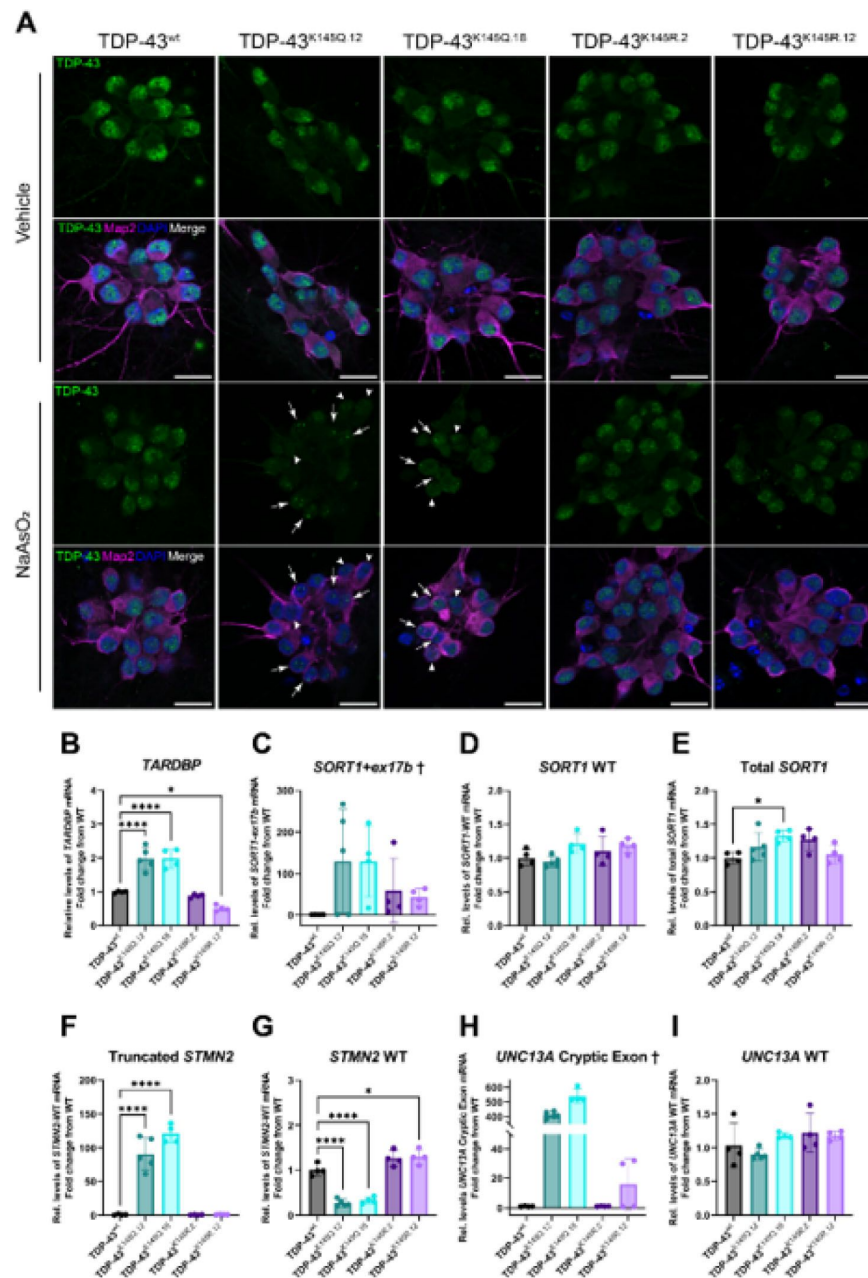


Figure 3

RNA-binding deficient TDP-43^{K145Q} in human iPSC-derived mature cortical neurons alters TDP-43 distribution and causes disease-relevant splicing dysregulation

(A) Representative confocal images of CRISPR-modified human iPSC-derived cortical neurons harboring homozygous TDP-43^{K145Q} (clonal lines 12, 18) or TDP-43^{K145R} (clonal lines 2, 12) knock-in mutations or an isogenic wild-type TDP-43^{wt} control lacking *TARDBP* modifications. Differentiated cortical neurons were treated with vehicle or 200μM NaAsO₂ and then immunolabeled for endogenous TDP-43 (green), Map2 (magenta), and DAPI (blue). Scale bars = 20μm. Arrows highlight nuclei with few, bright TDP-43+ foci. Arrowheads highlight nuclei with relative depletion of TDP-43 intensity. **B-I** RT-qPCR analysis of hiPSC-derived mature cortical neuron samples using primers specific for (B) human *Tardbp* [F=43.79, p<0.0001], (C) *SORT1+ex17b*, (D) *SORT1-WT* [F=2.975, p=0.0516], (E) total *SORT1* mRNA [F=3.461, p=0.0321], (F) truncated *STMN2* [F=79.06, p<0.0001], (G) *STMN2* WT [F=63.93, p<0.0001], (H) *UNC13A* cryptic exon; and (I) *UNC13A* WT mRNA [F=2.116, p=0.1262]. RT-qPCR results compared via ordinary one-way ANOVA (F statistics and p-values shown in brackets above) followed by Tukey's multiple comparisons testing. Statistical significance of multiple comparisons testing is represented by asterisks *p<0.05, ***p<0.001, ****p<0.0001. † Non-detectable levels of *UNC13A* cryptic exon- and *SORT1-ex17b* - containing transcripts in some or all control TDP-43^{wt} and TDP-43^{K145R} neurons prevented statistical comparisons among groups and therefore no statistical significance was reported and graphs are provided for visualization purposes only.

SORT1+ex17b splice variant was undetectable in TDP-43^{wt} neurons, but was present in 60% of TDP-43^{K145Q.12} and 100% of TDP-43^{K145Q.18}, TDP-43^{K145R.2}, and TDP-43^{K145R.12} neurons (**Figure 3C**). In samples confirmed to have detectable *SORT1+ex17b* transcript, levels were, on average, considerably higher levels in TDP-43^{K145Q} compared to TDP-43^{K145R} neurons. Appropriately spliced *SORT1*-WT and total *SORT1* transcript levels were comparable across all 5 lines, with only a slight increase in total *SORT1* transcript in TDP-43^{K145Q.12} neurons (**Figure 3D-E**). We conclude that *SORT1+ex17b* is a low-abundance transcript that accumulates in TDP-43^{K145Q} neurons.

We also examined neurons for the presence of truncated *STMN2* and *UNC13A* cryptic exon mRNA, which are implicated as ALS-FTLD biomarkers and/or risk genes.^{69–72} Strikingly, truncated *STMN2* mRNA was approximately 100-fold higher in both TDP-43^{K145Q.12} and TDP-43^{K145Q.18} neuron lines, respectively, compared to TDP-43^{wt} neurons, while no difference from WT was found in TDP-43^{K145R} neurons. (**Figure 3F**). The unperturbed *STMN2* splice variant (*STMN2* WT) was reduced about 50% in both acetylation-mimic TDP-43^{K145Q} lines but unchanged in TDP-43^{K145R.2} neurons (**Figure 3G**). We also noted a subtle increase in *STMN2* WT mRNA in TDP-43^{K145R} neurons, suggesting the fully deacetylated state, achieved by acetylation-deficient mimics, may augment *STMN2* levels. RT-qPCR analysis of cryptic exon-included *UNC13A* splice variant (*UNC13A* cryptic exon) failed to detect the abnormal splice product in TDP-43^{wt} and TDP-43^{K145R.2} lines, with only low levels observed in some TDP-43^{K145R.12} samples. In contrast, both TDP-43^{K145Q} lines dramatically accumulated the *UNC13A* cryptic exon containing mRNA (**Figure 3H**). The mRNA levels of the typical splice variant (*UNC13A* WT) were similar across all five lines (**Figure 3I**), indicating that the lack of detectable *UNC13A* cryptic exon mRNA in WT and acetylation-deficient neuron lines was not a result of reduced *UNC13A* expression, but rather is likely specific to a lack of cryptic splicing events. Taken together, these results demonstrate that endogenously expressed acetylation-mimic TDP-43^{K145Q} alters TDP-43 localization and conformation in response to stress and impairs splicing in a disease-relevant manner in human iPSC-derived neurons.

TDP-43 acetylation-mimic mice develop age-dependent cognitive and behavioral defects

To evaluate TDP-43 acetylation-mimic mice, we aged the animals and performed an extensive battery of behavioral analysis to assess cognitive and motor function, which reflect impairments that are commonly found in patients with TDP-43 proteinopathies.⁶ To be sufficiently powered to detect small differences in behavioral phenotypes, we initially focused on TDP-43^{wt} and homozygous TDP-43^{KQ/KQ} mice. Moreover, since there were no significant differences between males and females in any behavioral analyses described below, we pooled both sexes into either WT or TDP-43^{KQ/KQ} groups. At 12 months old, TDP-43^{KQ/KQ} mice showed significant reduction of body weight compared to WT littermates, and this difference was maintained until end point analysis at 18 months old (**Figure 4A**). Evaluation of exploratory activity and locomotion in an open field test demonstrated that TDP-43^{KQ/KQ} mice spend significantly more time in the center region (**Figure 4B**), with no differences in total distance traveled (**Figure 4 - Figure supplement 3A**), indicative of decreased anxiety-like behavior.^{73,74} Acoustic startle testing revealed impaired prepulse inhibition (PPI) in TDP-43^{KQ/KQ} mice at 12 months old (**Figure 4 - Figure supplement 2A**), indicative of deficits in sensorimotor gating, a form of inhibitory behavioral control,^{75,76} which is a phenomenon that can be observed in early dementia.⁷⁷ In 18-month-old animals, evaluation of acoustic startle response and PPI was confounded by hearing impairment (**Figure 4 - Figure supplement 2B**), however the altered activity reflected by increased time in the center region of an open field test was maintained at this age (**Figure 4B**). Thus, consistent patterns of behavioral disinhibition and reduced anxiety-like behavior were apparent in TDP-43^{KQ/KQ} mice over time.

We next performed contextual and cue-dependent fear conditioning as an index of hippocampal and cortical function.^{78–82} Context-dependent fear testing revealed reduced freezing times in TDP-43^{KQ/KQ} mice, with trends observed at 12 months old and significant deficits appearing at 18

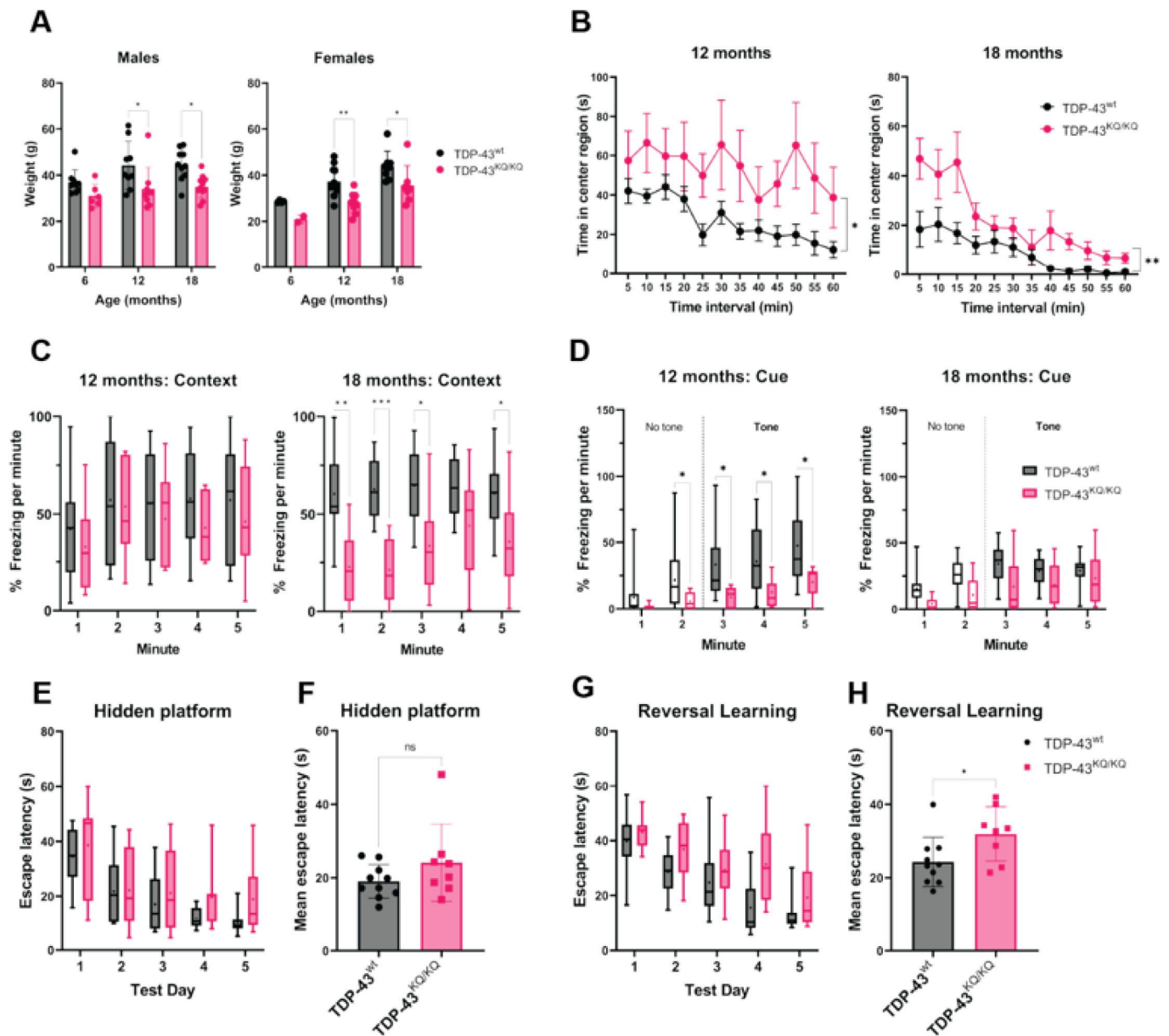


Figure 4

TDP-43^{KQ/KQ} mice develop age-dependent cognitive and behavioral defects.

A Body weight of TDP-43^{wt} and TDP-43^{KQ/KQ} mice in males [left panel; $F(1,48)=17.52$, $p=0.0001$] and females [right panel; $F(1,36)=14.17$, $p=0.0006$] at different ages. **B** Quantification of time in the center region of an open field in mice at 12 months [left panel; $F(1,20)=6.118$, $p=0.0225$] and 18 months [right panel; $F(1,17)=9.622$, $p=0.0065$]. **C** Quantification of time spent frozen (immobile) following context-dependent conditioned fear testing 18-months-old [right panel; $F(1,17)=5.402$, $p=0.0328$]. **D** Quantification of time spent frozen following cue-dependent conditioned fear testing at 12-months-old [left panel; $F(1,20)=6.136$, $p=0.0223$] and 18-months-old. Filled bars represent presence of auditory cue (tone) and period of statistical analysis. **E-F** Morris Water Maze (MWM) analysis displaying time to find a hidden platform (escape latency), quantified as daily trial means per animal (**E**) or average across all days (**F**). **G-H** Quantification of escape latencies during MWM reversal learning trials in daily trials (**G**) [$F(1,16)=5.273$, $p=0.0355$] and comparing averages per mouse across all days (**H**).

Bar and scatter plots shown as mean $\pm$ SD. Box and whiskers show line at median, '+' at mean, and whiskers run min to max. **a** Two-way ANOVA followed by Šidák's multiple comparisons test. **B-D,E,G** Two-way repeated measures ANOVA followed by Holm-Šidák's multiple comparisons tests; F statistics and p-values in legend represent main effect of genotype. **F, H** Unpaired student's t-test. Sample sizes as follows unless otherwise indicated: 12-month TDP-43^{wt} $n=15$; 12-month TDP-43^{KQ/KQ} $n=7$; 18-month TDP-43^{wt} $n=10$; 18-month TDP-43^{KQ/KQ} $n=9$; one 18-month TDP-43^{KQ/KQ} extreme outlier removed for MWM analysis (**E,F**). Statistical significance is represented by asterisks * $p<0.05$, ** $p<0.01$, *** $p<0.001$; ns=not significant. Further statistical information is located Figure 4 Source Data 1 file.

months (**Figure 4C**). Similarly, auditory cue-dependent fear testing revealed significant impairments in associative cue learning in TDP-43^{KQ/KQ} mice at 12 months of age (**Figure 4D**), a behavior thought to be mediated by the amygdala and higher-order cortical regions important in inhibitory control.⁸³ As mentioned above, general auditory defects in both genotypes at 18 months of age confounded interpretations of any cue-dependent learning deficits at this advanced age (**Figure 4D**). Morris Water Maze testing was used to evaluate swimming ability and spatial learning,⁸⁴ which showed equivalent swim speeds, suggesting no motor impairments in TDP-43^{KQ/KQ} mice at 18 months of age (**Figure 4 - Figure supplement 2D**). While assessment of spatial learning showed a trend towards impaired acquisition learning (**Figure 4E, F**), we observed more prominent defects in reversal learning after moving the location of the platform, as determined by significant delays in escape latency. Our findings support deficits in cognitive flexibility in TDP-43^{KQ/KQ} mice (**Figure 4G, H**).⁸⁵ We also analyzed cognitive impairments in heterozygous TDP-43^{wt/KQ} mice, which showed a mild intermediate phenotype (**Figure 4 - Figure supplement 1**).

To determine any ALS-like motor phenotypes, we assessed motor function and were surprised to find no overt signs of motor impairment in TDP-43^{KQ/KQ} mice at 18 months of age. TDP-43^{KQ/KQ} mice performed similarly to WT littermates as assessed by rotarod testing (**Figure 4 - Figure supplement 3C-D**) and in grip strength as measured using digital force meters (**Figure 4 - Figure supplement 3E**). Moreover, there were no differences in swim speed or distance traveled in an open field at any age tested (**Figure 4 - Figure supplement 3A, B**). We note that we cannot currently exclude the possibility of more subtle motor phenotypes or the emergence of motor defects in aged mice beyond 18 months old. The preferential deficits in learning and behavioral control support an age-dependent FTLT- or dementia-like phenotype in acetylation-mimic TDP-43^{KQ/KQ} mice.

TDP-43^{KQ/KQ} mice show hallmarks of TDP-43 dysfunction in the absence of neurodegeneration

Given the preferential cognitive phenotype observed in TDP-43^{KQ/KQ} mice (**Figure 4**), we focused on the neocortex and hippocampus of aged animals. We examined neuronal density in the neocortex of 12- and 18-month-old TDP-43^{wt} and TDP-43^{KQ/KQ} mice by immunofluorescence, widefield microscopy, and quantitative image analysis of the NeuN-positive neuronal cell density and area occupied by NeuN-positive cells in the neocortex. We did not observe any evidence of neuron loss in the neocortex of TDP-43^{KQ/KQ} mice at 12- or 18-months of age (**Figure 5A-C**). Similarly, we observed no astrogliosis (GFAP intensity and area occupied by GFAP+ cells) or microgliosis (Iba1 intensity and area occupied by Iba1+ cells) in the neocortex or hippocampus (**Figure 5 - Figure supplement 1**).

We next evaluated TDP-43 aggregation and cytoplasmic mislocalization in 18-month-old mice, a time at which cognitive deficits were most pronounced.^{17,43} We initially used confocal microscopy, and automated quantitative image analysis to assess TDP-43 morphology and localization in the neocortex and hippocampus, which did not reveal nuclear or cytoplasmic TDP-43-positive inclusions in any brain region or genotype (**Figure 5D, G**). In addition to searching for aggregate pathology, we also evaluated TDP-43 abundance, as measured by fluorescence intensity, in NeuN-positive neurons, as TDP-43 expression is altered in FTLT-TDP and other TDP-43 proteinopathies,^{18,86-88} and visualized the single-cell resolution data using SuperPlots.⁸⁹ We observed trends toward elevated TDP-43 protein levels in the neocortex and the CA3 region of the hippocampus (**Figure 5E-F, H-I**) and potential subtle increases in the cytoplasmic to nuclear TDP-43 ratio within CA3 neurons (**Figure 5G, J**), however these effects were variable and not statistically significant. Given the technical challenge of accurate image segmentation at the subcellular level within tissue samples, we sought more sensitive methods to assess TDP-43 pathology in TDP-43^{KQ/KQ} mice.

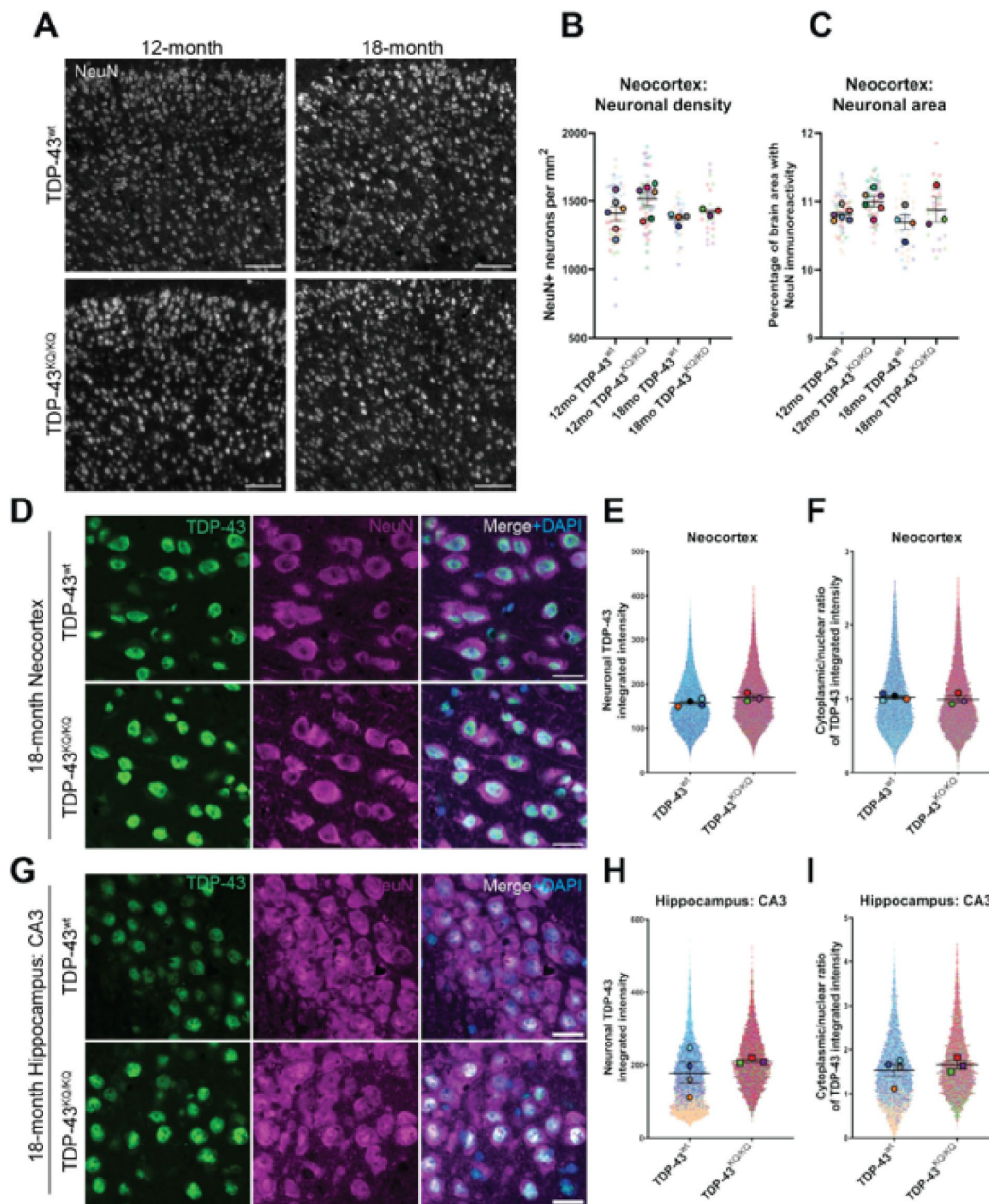


Figure 5

No evidence of neuronal loss in aged TDP-43^{KQ/KQ} mice with retention of predominantly nuclear TDP-43.

A Representative confocal immunofluorescence images of NeuN+ neurons in the neocortex of 12- and 18-month TDP-43^{wt} or TDP-43^{KQ/KQ} mice. **B** Quantification of NeuN+ neurons per square millimeter (mm) in TDP-43^{wt} and TDP-43^{KQ/KQ} mice neocortex. **C** Quantification of the percent of neocortex area that is occupied by cells with NeuN immunoreactivity. **D,G** Representative confocal images of neocortex (**D**) and hippocampus (**G**) sections from 18-month-old TDP-43^{wt} and TDP-43^{KQ/KQ} mice immunolabeled with TDP-43 (green), NeuN (magenta), and DAPI (blue). **F,H** Quantification of TDP-43 fluorescence intensity within NeuN+ neurons in the neocortex (**F**) or hippocampal CA3 region (**H**). **F,I** Quantification of the nuclear:cytoplasmic ratio of TDP-43 fluorescence intensity within NeuN+ neurons in the neocortex (**F**) and hippocampus (**I**). Scale bars = 100 μm (**A**), 20 μm (**D,G**). SuperPlots show average value per animal in solid color bordered symbol (as mean ± SEM) over top of semi-transparent individual values from each animal. One color represents one animal. Individual datapoints in (**B**) and (**C**) represent density in a single field of view, with 10-16 fields across 4 brain sections per animal, n = 3-4 mice per genotype of 18-month-old animals and n=6 mice per genotype of 12-month-old animals. Individual datapoints in **E-F** and **H-I** represent values from a single neuron within one field of view, with 1000-5000 neurons per animal across 12-16 fields from 4 (neocortex) or 2 (hippocampus) brain sections of n = 3-4 mice per genotype of 18-month-old animals. Statistical analyses performed using linear mixed-effect models. Omission of asterisks indicates no statistical significance.

We turned to an alternative biochemical approach of sequentially fractionating isolated hippocampus and neocortex tissue to generate soluble (RIPA-extracted) and insoluble (Urea-extracted) protein fractions. TDP-43^{KQ/KQ} mouse neocortex harbored insoluble phosphorylated TDP-43 at the disease-associated Ser409/410 locus⁹⁰ (p409/410) at 12 months of age, which was even more prominent at 18 months (**Figure 6A, C**), a timepoint at which TDP-43^{KQ/KQ} mice showed behavioral and cognitive defects (**Figure 4**). Notably, p409/410 abundance was minimal in the hippocampus of 12-month-old TDP-43^{KQ/KQ} mice but increased by 18 months (**Figure 6D, F**), coinciding with the onset of hippocampal-mediated learning deficits (**Figure 4C**). Though p409/410 was elevated in TDP-43^{KQ/KQ} mice, we were surprised to find that the total insoluble TDP-43 pool was not altered, suggesting that increased TDP-43 phosphorylation may precede overt conversion from soluble to insoluble TDP-43.

We also examined soluble TDP-43 levels, as acetylation-induced loss of function may result in autoregulatory feedback that increases the production of TDP-43.¹⁸ The soluble TDP-43 pool was significantly increased in TDP-43^{KQ/KQ} at 12 months in the neocortex and at 18 months in both neocortex (**Figure 6A, B**) and hippocampus (**Figure 6D, E**), an indicator of autoregulated TDP-43 protein levels. Biochemical analysis of TDP-43 solubility and phosphorylation in heterozygous TDP-43^{wt/KQ} animals revealed no significant differences in soluble TDP-43 or insoluble p409/410 levels compared to TDP-43^{wt} mice (**Figure 6 - Figure supplement 1A-C**, **Figure 6 - Figure supplement 2A-C**). To assess TDP-43 mislocalization using a biochemical approach, we performed subcellular fractionation to isolate nuclear and cytoplasmic proteins from the neocortex and hippocampus and found a striking increase in cytoplasmic TDP-43 in TDP-43^{KQ/KQ} mice at both 12 and 18 months of age (**Figure 6G-L**). Similar to the intermediate levels of cognitive decline in heterozygous TDP-43^{wt/KQ} mice, we observed trends toward increased cytoplasmic TDP-43 in TDP-43^{wt/KQ} mice, particularly at 18 months old (**Figure 6 - Figure supplement 1D-I**, **Figure 6 - Figure supplement 2D-I**).

Finally, we evaluated whether TDP-43^{KQ/KQ} mice show TDP-43 dysfunction in the spinal cord. Surprisingly, TDP-43^{KQ/KQ} mice harbored insoluble p(409/410)-TDP-43 (**Figure 6 - Figure supplement 3A,C**) and moderate increases in cytoplasmic TDP-43 mislocalization (**Figure 6 - Figure supplement 3D-F**) in the spinal cord at 18 months old, despite the lack of any overt motor deficits in TDP-43^{KQ/KQ} animals (**Figure 4 - Figure supplement 2**). We thus found that a similar biochemical signature of TDP-43 abnormalities is present in the spinal cord, yet does not correlate with motor deficits in 12- to 18-month-old mice. Our findings suggest TDP-43 phosphorylation and mislocalization occur as early events, prior to the formation of mature TDP-43 inclusion pathology and may drive the progressive FTLTDP-like phenotype in TDP-43^{KQ/KQ} mice.

Disease-linked transcriptomic and splicing defects

are prevalent in acetylation-mimic TDP-43^{KQ/KQ} mice

TDP-43 acetylation drives RNA dissociation and loss of TDP-43 function,⁵² implying that reduced RNA-binding capacity may impact transcriptional regulation and mRNA splicing.^{36,50,91} We performed total RNA sequencing to determine how mimicking TDP-43 acetylation affects RNA profiles *in vivo*. We examined the neocortex and hippocampus of 18-month-old TDP-43^{wt} or TDP-43^{KQ/KQ} mice, representing the regions and timepoints with the most striking biochemical and behavioral abnormalities and identified nearly 400 differentially expressed genes (DEGs) in each brain region in TDP-43^{KQ/KQ} mice compared to TDP-43^{wt}, after correcting for underlying batch effects. As expected by acetylation-induced loss of TDP-43 function and subsequent autoregulation, the *Tardbp* transcript was increased (**Supplementary File 1a-b**). Follow-up RT-qPCR analysis confirmed a 2- to 3-fold increase in *Tardbp* expression in the neocortex and hippocampus (**Figure 6M-N**), as well as the spinal cord (**Figure 7 - Figure supplement 1A**), which correlated with increased TDP-43 protein levels (**Figure 6B, E**; **Figure 6 - Figure supplement 3A-C**).

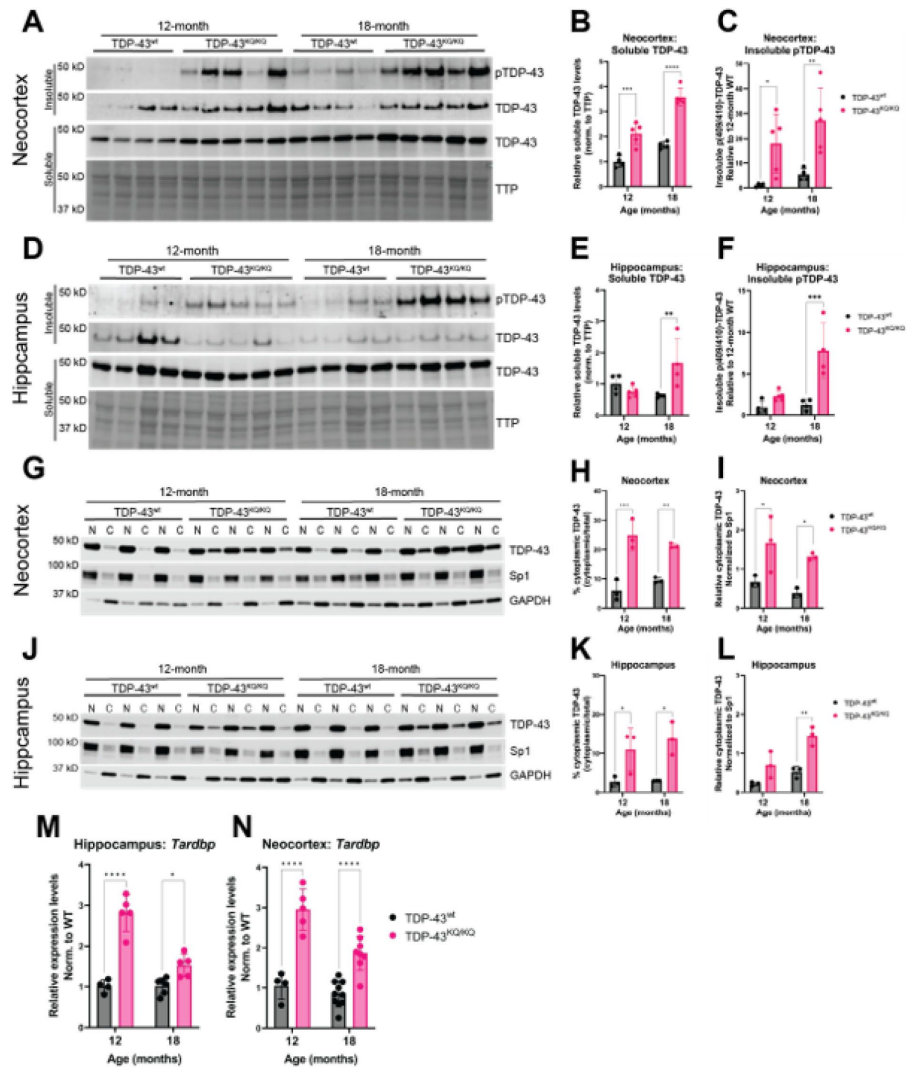


Figure 6

Hyperphosphorylated and mislocalized TDP-43 protein and autoregulated induction of the *Tardbp* transcript is found in the neocortex and hippocampus of aged TDP-43^{KQ/KQ} mice

A-F Western blot images comparing soluble and insoluble protein fractions in neocortex (**A**) and hippocampus (**D**) from TDP-43^{wt} and TDP-43^{KQ/KQ} mice at 12- and 18-months of age. Quantification of soluble TDP-43 protein levels in neocortex and hippocampus tissue relative to total transferred protein (TTP) [neocortex (**B**) F (1, 14) = 97.67, $p < 0.0001$; hippocampus (**E**) main effect of genotype F (1, 13) = 3.699, $p = 0.0766$ and main effect of genotype*age interaction F (1, 13) = 10.21, $p = 0.0070$]. Quantification of insoluble phosphorylated p(409/410)-TDP-43 levels in neocortex and hippocampus tissue [neocortex (**C**) F (1, 14) = 18.81, $p = 0.0007$; hippocampus (**F**) F (1, 13) = 23.07, $p = 0.0003$]. **G-I** Western blot images following isolation of soluble nuclear and cytoplasmic protein fractions from neocortex (**G**) and hippocampus (**J**) of TDP-43^{wt} and TDP-43^{KQ/KQ} mice at 12- and 18-months of age. **J-L** Quantification of cytoplasmic TDP-43 measured as percent of total TDP-43 in neocortex and hippocampus [neocortex (**H**) F (1, 8) = 69.38, $p < 0.0001$; hippocampus (**K**) F (1, 8) = 22.40, $p = 0.0015$] and relative cytoplasmic mislocalization of TDP-43 after normalizing to cytoplasmic/nuclear ratio of the nuclear Sp1 protein in neocortex and hippocampus [neocortex (**I**) F (1, 8) = 19.79, $p = 0.0021$; hippocampus (**L**) F (1, 8) = 29.94, $p = 0.0006$]. **M-N** *Tardbp* expression was analyzed by qPCR in the (**M**) hippocampus [F (1, 16) = 76.86; $p < 0.0001$] and (**N**) neocortex [F (1, 23) = 80.74; $p < 0.0001$] of TDP-43^{KQ/KQ} and TDP-43^{wt} mice at 12- and 18-months-old. Relative expression (RQ) values in each sample were determined using the Plaff method and *B-actin* and *Pgk1* as housekeeping genes. **A-F** n=4 TDP-43^{wt} and n=5 TDP-43^{KQ/KQ} at all ages and regions except 18-month hippocampus n=4 TDP-43^{KQ/KQ}. **F-L** n=4 all ages and regions. **M** Neocortex n=4 12-month TDP-43^{wt}, n=5 12-month TDP-43^{KQ/KQ}, n=10 18-month TDP-43^{wt}, n=9 18-month TDP-43^{KQ/KQ}. **N** Hippocampus n=4 12-month TDP-43^{wt}, n=5 12-month TDP-43^{KQ/KQ}, n=6 18-month TDP-43^{wt}, n=6 18-month TDP-43^{KQ/KQ}. Data are presented as mean $\pm$ SD. Two-way ANOVA followed by Šidák's multiple comparisons test. F statistics represent the main effect of genotype unless otherwise stated. Statistical significance represented by asterisks, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Further statistical information is available in the Figure 6 Source Data 1 file. Figures showing uncropped western blot images are available in Figure 6 Source Data 2 files. Full images of western blots are available in Figure 6 Source Data 3 files.

We then clustered the DEGs based on their up- or down-regulation and the brain region affected (**Figure 6**), which revealed largely similar patterns of transcriptional alterations, with some distinct differences (**Figure 7**, **Figure 7 - Figure supplement 2**, **Supplementary File 1a-b**). To investigate the potential biological implications of the altered transcriptome, we performed Gene Ontology (GO) term enrichment analyses on DEGs identified in each of the six clusters (**Supplementary File 1c**). In both brain regions, the most highly downregulated genes were involved in developmental processes, including many related to CNS development and maintenance, such as neurogenesis (e.g., *Sema5b*, *Rnd2*, *Brinp1*), gliogenesis (e.g., *Tlr2*, *Olig2*, *Sox10*), and myelination (e.g., *Nkx2-2*, *Nkx6-2*, *Sox10*). *Sema5b* was the most dramatically reduced transcript in the hippocampus, and the third most highly reduced in the cortex, (**Supplementary File 1a-b**) with a two-fold reduction in expression in TDP-43^{KQ/KQ} mice. Downregulated genes in both the hippocampus and neocortex were also enriched for terms related to synapse homeostasis and transmembrane signaling, however the dysregulated pathways were distinct. Genes related to GABAergic synapses (e.g., *Gad1*, *Gad2*, *Abat*, *Gnb5*) were selectively downregulated in the neocortex, while trans-synaptic signaling and ion transport mechanisms (e.g., *Homer3*, *Camk4a*, *Nsmf*, *Cnih2*) were decreased in the hippocampus.

In contrast, there was significant upregulation of cellular stress response genes (*Sesn1*, *Nrros*, *Plat*, *Klf15*) and many apoptotic regulators (e.g., *Trp53inp1*, *Pmaip1*, *Bcl2l1*, *Plekhf1*) in both the hippocampus and neocortex, along with an over-representation of GO terms related to metabolism, localization, cell adhesion (**Figure 7**, **Supplementary File 1c**). Several pathways were uniquely altered including coagulation and complement cascades, which were only upregulated in the hippocampus (e.g., *F3*, *Plat*, *Cd59a*). Interestingly, while genes associated with trans-synaptic signaling were decreased in the hippocampus, another set of genes involved in this same pathway was upregulated in the cortex (e.g., *Syt7*, *Synpo*, *Nptx1*, *Spg11*) (**Supplementary File 1c**).

Given the cognitive defects in TDP-43^{KQ/KQ} mice, we sought to draw parallels between the TDP-43^{KQ/KQ} mouse and the human FTLTDP transcriptome. Comparison of the DEGs in TDP-43^{KQ/KQ} mice to the mouse orthologs of those found in FTLTDP frontal or temporal cortex tissue⁹² revealed marked overlap between our mouse and their human datasets (**Figure 7**, see “FTLTDP Cortex” bar), particularly in the hippocampus, as measured by statistical enrichment analyses that found over-representation of FTLTDP DEGs in our TDP-43^{KQ/KQ} dataset (**Supplementary File 1d**; $p = 0.0003$ “Hippocampus Down” vs Downregulated in FTLTDP frontal cortex; $p = 0.0014$ “Hippocampus Down” vs Downregulated in FTLTDP temporal cortex; $p = 0.0048$ “Hippocampus Up” vs Upregulated in FTLTDP frontal cortex). A similar alignment comparing DEGs from TDP-43^{KQ/KQ} mice to those following TDP-43 knockdown in mouse striatum also identified commonly altered genes, particularly in the TDP-43^{KQ/KQ} downregulated gene sets (**Figure 7**, see “TDP-43 knockdown” bar; **Supplementary File 1d**; $p = 0.019$ “Hippocampus Down” vs Downregulated in TDP-43-KD; $p = 0.046$ “Cortex Down + Hippocampus Down” vs Upregulated in TDP-43-KD). Taken together, we identified distinct FTLTDP signatures including altered synaptic gene expression and stress response signaling that reflect acetylation-induced TDP-43 dysfunction.

Alternative splicing defects, particularly impaired repression of cryptic exons, due to TDP-43 dysregulation are strongly implicated in FTLTDP and ALS pathogenesis.^{71,72,93–96} In line with our findings above that TDP-43^{KQ/KQ} mouse primary cortical neurons *in vitro* show splicing deficits (**Figure 2E-F**), we identified widespread splicing alterations *in vivo*. Analysis of TDP-43^{KQ/KQ} neocortex identified 289 differentially spliced genes (DSGs), with 81.7% of loci containing at least one cryptic splice junction, and 29.8% containing two cryptic splice junctions (**Supplementary File 1e**). In the hippocampus, we found 126 DSGs, 77.0% of which contain a cryptic splice junction and 41.3% that are formed by two cryptic splice sites (**Supplementary File 1f**). The alternative splicing events were relatively consistent between brain regions, as over 70% of the DSGs identified in the hippocampus were also present in the cortex. Among the most

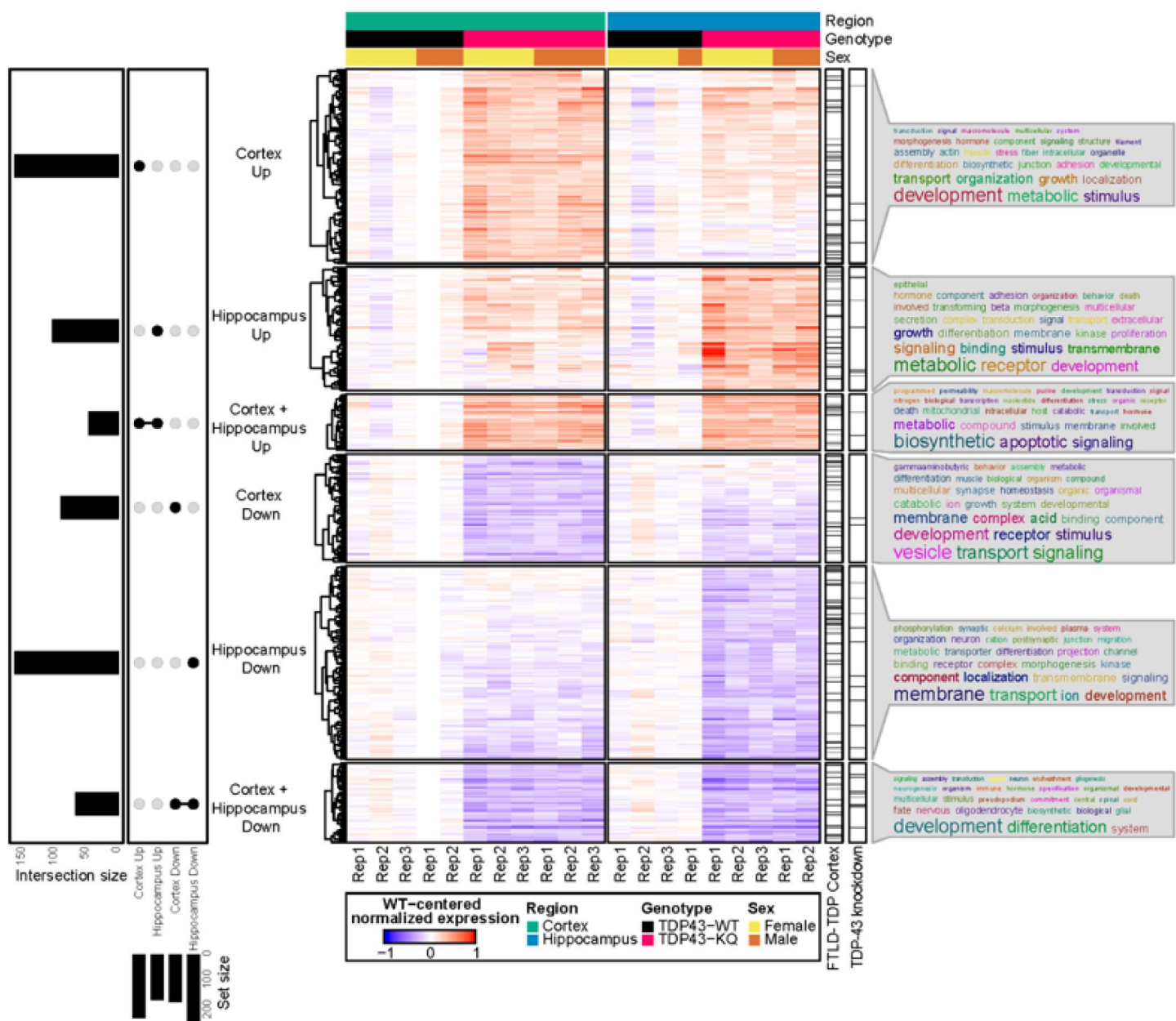


Figure 7

RNA sequencing of aged TDP-43^{KQ/KQ} mouse brain reveals dysregulation of neurodegeneration and FTLD-TDP-associated pathways

Genes differentially expressed between TDP-43^{KQ/KQ} (TDP43-KQ) and TDP-43^{WT} (TDP43-WT) cortex and hippocampus were compared and their intersections were defined as 6 gene groups, as represented by an UpSet plot (left). Normalized gene expression values, centered around the mean of TDP-43^{WT} samples for each brain region, were then hierarchically clustered and plotted as a heatmap (center, red indicates expression higher than that of WT, blue indicates expression lower than WT). Genes that were significantly altered in human FTLD-TDP temporal and/or frontal cortex (Hasan et al.⁹², "FTLD-TDP Cortex" bar), or striatal mouse brain TDP-43-knockdown (Polymenidou et al.⁹⁷, "TDP-43 knockdown" bar) were demarcated with tick marks. Each gene group was assessed for over-enrichment of Gene Ontology terms and these results were summarized as word clouds (right). Neocortex n=5 TDP-43^{WT}, n=6 TDP-43^{KQ/KQ}; hippocampus n=4 TDP-43^{WT}, n=5 TDP-43^{KQ/KQ}.

significant DSGs identified were known TDP-43 splicing targets (e.g., *Kcnp2*, *Pdp1*, *Poldipp3*, *Ppfibp1*, *Dnajc5*, *Tmem2*, *Sort1*),^{24,94,97} transcripts associated with particular neurodegenerative diseases (e.g., *Mapt*, *Atxn1*, *Lrrk2*),^{98–104} and also many robustly altered transcripts that are poorly characterized but linked to neurodegeneration (e.g., *Nrxn3*, *Nos1*, *Arfge2*, *Arhgap10*, *Lrp8*, *Smarca4*, *Rims2*). Of all identified DSGs, the most substantially altered transcript was *Sort1*, encoding the Sortilin-1 (Sort1) protein. We noticed that exclusion of a *Sort1* 3' exon was reduced by 55.9% in TDP-43^{KQ/KQ} cortex and by 57.0% in the hippocampus (**Figure 8A**), such that the aberrantly spliced *Sort1* transcript was the predominant variant detected, comprising nearly 80% of all *Sort1* transcripts, aligning with our findings from TDP-43^{KQ/KQ} primary mouse cortical neurons.

SORT1, the human homolog of mouse *Sort1*, is a highly expressed neurotrophic factor receptor that binds progranulin (PGRN) and regulates endosomal/lysosomal function through a pathway that is genetically linked to FTLTDP.^{105–109} Because TDP-43 depletion results in *SORT1* exon 17b inclusion, this leads to the production of a soluble and putatively toxic SORT1 variant that is increased in FTLTDP patients.^{67,68,97} To confirm that the altered 3' splicing in our sequencing data was indeed *Sort1* exon 17b inclusion, we performed qPCR using primers specific for the mouse *Sort1+ex17b* transcript or the appropriately spliced variant (*Sort1-WT*), and a primer pair that recognizes all *Sort1* variants (*Sort1* total) on tissues isolated from TDP-43^{KQ/KQ} or WT mice.⁶⁷ We found the ratio of aberrant *Sort1-ex17b* to appropriately spliced *Sort1-WT* was increased approximately 10-fold in the neocortex (**Figure 8B**) and hippocampus (**Fig 8C**) of TDP-43^{KQ/KQ} mice at both 12- and 18-months of age, which corroborates our RNA sequencing analysis showing that the exon-included *Sort1+ex17b* variant predominates in these tissues. The total level of *Sort1* transcript *in vivo* varied with age, with slightly increased levels at 12 months and reduced levels at 18 months of age, suggestive of a negative feedback mechanism regulating *Sort1* expression. Spinal cord tissue revealed similar levels of abnormal *Sort1* splicing in TDP-43^{KQ/KQ} animals at 12- and 18-months of age, however total *Sort1* mRNA expression did not significantly change with age (**Figure 7 - Figure supplement 1B-C**).

To determine whether the *Sort1+ex17b* transcript in TDP-43^{KQ/KQ} mice leads to the generation of a distinct Sort1 protein product, we immunoblotted hippocampus and cortex homogenates and identified a higher molecular weight Sort1 variant in TDP-43^{KQ/KQ} compared to WT mice (**Figure 8D, E**), consistent with *Sort1* exon 17b inclusion. The abnormal Sort1 protein showed decreased steady-state stability in TDP-43^{KQ/KQ} mice, as suggested by reduced protein levels in acetylation-mimic animals, compared to controls (**Figure 8F, G**). These data are strongly suggestive of failure to repress exon inclusion in TDP-43^{KQ/KQ} mice, leading to an altered Sort1-progranulin axis and supporting an aberrant splicing profile that resembles alterations seen in human FTLTDP.

Discussion

Here, we developed novel neuronal and mouse models of RNA binding-deficient acetylation-mimic TDP-43 (TDP-43^{K145Q}). Using a combination of molecular, cellular, and transcriptomic readouts combined with *in vivo* behavioral assays, we provide evidence that aberrant TDP-43^{K145Q} recapitulates key hallmarks of TDP-43 proteinopathies, including altered TDP-43 solubility and localization, impaired autoregulation, and abnormal splicing function. Taken together, our findings suggest that TDP-43 acetylation-induced dysfunction likely contributes to disease progression.

Our prior mass spectrometry analysis originally identified acetylated lysines K145 and K192,⁵² though a more recent study identified residues K84 (within the NLS) and K136 (within RRM1) as additional lysine residues of interest.⁶⁴ Any normal physiological or cellular role for TDP-43 acetylation remains unclear. However, given its impact on RNA binding, one can imagine TDP-43 acetylation may fine-tune TDP-43's normal nuclear and cytoplasmic functions, but becomes

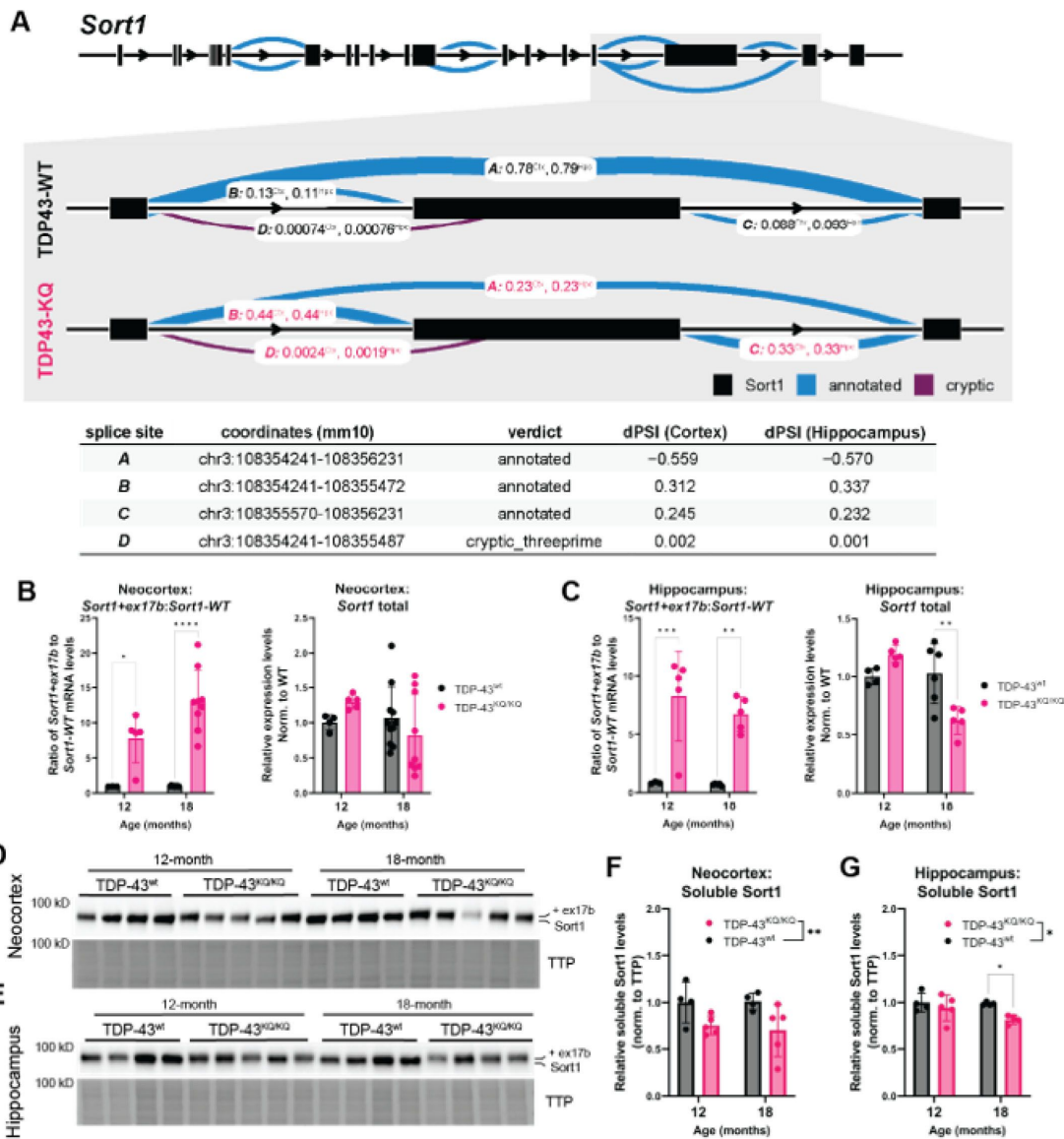


Figure 8

Dysfunctional splicing regulation of the *Sort1* transcript in TDP-43^{KQ/KQ} mice

A Differential splicing analysis of TDP-43^{wt} and TDP-43^{KQ/KQ} neocortex ("Ctx") and hippocampus ("Hpc") using LeafCutter¹⁷⁷ (visualized with LeafViz¹⁷⁸) demonstrates reduction in exclusion of a 3' exon within the *Sort1* transcript. Diagram in **(A)** shows full-length *Sort1* gene in upper panel and highlights the differentially spliced region in gray. Chromosomal location, intron start and end points, annotation status, and Δ percent spliced in (dPSI) of the intronic region is listed in the lower table. **B-C** qPCR analysis of additional WT and TDP-43^{KQ/KQ} neocortex **(B)** and hippocampus **(C)** samples using primers specific for *Sort1* splice variants to show the ratio of *Sort1-ex17b:Sort1-WT* mRNA levels and total *Sort1* mRNA levels [**B** neocortex; *Sort1-ex17b:Sort1-WT* ratio, $F(1, 22) = 51.85$, $p < 0.0001$; *Sort1* total, $F(1, 25) = 0.01856$, $p = 0.8927$; **C** hippocampus; *Sort1-ex17b:Sort1-WT* ratio, $F(1, 14) = 39.54$, $p < 0.0001$, *Sort1* total $F(1, 16) = 2.086$, $p = 0.1679$]. **D-G** Images of western blots probed for Sort1 protein from neocortex **(D)** and hippocampus **(E)** lysates from 12- and 18-month-old mice. The Sort1 protein band intensity is plotted relative to total transferred protein (TTP) and quantified in **F-G** [neocortex, $F(1, 14) = 9.308$, $p = 0.0086$; hippocampus, $F(1, 13) = 6.117$, $p = 0.0280$]. **A** Neocortex $n = 5$ TDP-43^{wt}, $n = 6$ TDP-43^{KQ/KQ}; hippocampus $n = 4$ TDP-43^{wt}, $n = 5$ TDP-43^{KQ/KQ}. **B** Neocortex $n = 3$ 12-month TDP-43^{wt}, $n = 5$ 12-month TDP-43^{KQ/KQ}, $n = 9$ 18-month TDP-43^{wt}, $n = 9$ 18-month TDP-43^{KQ/KQ}. **C** Hippocampus $n = 3$ 12-month TDP-43^{wt}, $n = 5$ 12-month TDP-43^{KQ/KQ}, $n = 5$ 18-month TDP-43^{wt}, $n = 9$ 18-month TDP-43^{KQ/KQ}. **D-G** $n = 4$ 12-month TDP-43^{wt}, $n = 5$ 12-month TDP-43^{KQ/KQ}, $n = 4$ 18-month TDP-43^{wt}, $n = 4$ (hippocampus) or $n = 5$ (neocortex) 18-month TDP-43^{KQ/KQ}. Data are presented as mean $\pm$ SD. Two-way ANOVA followed by Šidák's multiple comparisons test. F statistics represent main effect of genotype, unless otherwise stated. Statistical significance is represented by asterisks, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Further statistical information is located Figure 8 Source Data 1 file. Figures showing uncropped western blot images are available in Figure 8 Source Data 2 files. Full images of western blots are available in Figure 8 Source Data 3 files.

corrupted due to aging and/or stress exposure.¹¹⁰ It is likely that acetylation within the RRM generally impairs TDP-43 RNA-binding function, as multiple studies have shown that acetylation mimic TDP-43^{K136Q} or TDP-43^{K145Q} variants phenocopy one another, with both showing nuclear foci formation and reduced affinity for target RNAs. Therefore, deficiency in RNA binding may explain why acetylation, or acetylation-mimic mutations within RRM1, alter TDP-43 dynamics and lead to pathology. Indeed, RNA-binding deficient TDP-43, including RRM mutations or deletions, showed increased TDP-43 phase separation, aggregation, nuclear egress, and neurotoxicity.^{34,64,66,111} Conversely, enhancing RNA binding was suggested to mitigate these phenotypes,^{65,112,113} in part due to TDP-43 retention within the nucleus.³⁶ Thus, impaired RNA binding achieved by excessive lysine acetylation, or other RRM modifications, likely promotes TDP-43 pathogenesis in the form of mislocalization, aggregation, RNA dysregulation, and impaired autoregulation, as we observe with the TDP-43^{K145Q} models described in this study.

Primary TDP-43^{KQ/KQ} knock-in mouse and human iPSC-derived cortical neurons formed nuclear TDP-43+ foci that were exacerbated by acute oxidative stress. The acetylation-induced loss of RNA binding likely destabilized TDP-43 thus creating a more aggregate-prone conformation that can be further modulated by stressors (e.g. sodium arsenite) and likely other factors. Based on our original description of these TDP-43 foci, and several recent studies of acetyl-mimic TDP-43 variants,^{64,66,114} the nuclear TDP-43+ foci observed in our neuronal models likely represent a spectrum of phase-separated structures, including anisosomes,⁶⁶ and more immobile gel-like or solid aggregates. Future studies are needed to determine the molecular composition of these TDP-43+ foci, which could lead to a better understanding of their biogenesis, progression, and perhaps their dissolution. For example, it is conceivable that re-establishing TDP-43/RNA interactions could alleviate TDP-43 foci formation, suppress aggregation, and provide potential therapeutic avenues to consider.¹¹⁵

Homozygous TDP-43^{KQ/KQ} knock-in mice showed impaired cognitive function, as well as behavioral disinhibition in the absence of overt motor deficits up to 18-months of age, supporting a FTL-like phenotype in this model. The progressive behavioral defects and parallel increases in insoluble phosphorylated TDP-43 in the neocortex and hippocampus are also consistent with age as a driver of the FTL-like TDP phenotype. Even heterozygous TDP-43^{wt/KQ} mice showed intermediate levels of both cognitive impairment and biochemical perturbations when compared to wild-type and homozygous littermates, suggesting a dose-dependent effect of TDP-43 acetylation. We were surprised to find no overt motor deficits at 18-months-old despite the presence of mislocalized and phosphorylated TDP-43 in the spinal cord of TDP-43^{KQ/KQ} mice. We speculate this could be due to any of the following non-mutually exclusive possibilities. 1) The levels of insoluble phosphorylated TDP-43 in the spinal cord have not crossed a critical threshold required for functional decline. 2) There is a time-dependent delay in the accumulation of TDP-43 and the onset of motor symptoms that could emerge beyond 18 months, which will require additional studies in aged cohorts of mice. 3) Spinal motor neurons in mice may possess some intrinsic resilience that suppresses functional decline. 4) It is possible that TDP-43^{KQ/KQ} mice do indeed show subtle motor deficits that could be detected by more sensitive measures, such as electromyography-based techniques,^{116,117} for which future studies are warranted. 5) Finally, it remains plausible that phosphorylated TDP-43 is not inherently toxic or associated with functional decline in the spinal cord or other tissues.^{118,119}

TDP-43^{KQ/KQ} mice show striking similarities to other TDP-43 depletion models. For example, the RNA profiles in TDP-43^{KQ/KQ} brain, particularly the hippocampus, were similar to a mouse model of striatal TDP-43 knock down (**Figure 7**), including upregulation of immune response genes (*Serpine1*, *Serping1*) and altered splicing of TDP-43 targets (*Kcnp2*, *Sort1*, *Dnajc5*, *Pdp1*, *Poldip3*).⁹⁷ Similar splicing deficits were found in conditional CAMKIIa-driven *Tardbp* knockout mice, accompanied by an FTL-like phenotype with disinhibitory behavior and spatial learning deficits resembling those seen in TDP-43^{KQ/KQ} mice.^{120,121} Hippocampal knockdown of TDP-43 in adult mice impaired learning and memory, likely due to synaptic loss.¹²² This aligns with

our finding of cognitive decline without overt cortical neuron loss or neurodegeneration. We suspect that TDP-43 loss of function causes synaptic dysfunction that may eventually lead to neurodegeneration beyond 18 months. In contrast to the lethality associated with complete TDP-43 depletion²³, the K145Q substitution creates a powerful yet viable partial loss of function model, which lacks developmental defects and ALS-like motor phenotypes. In addition, transcript profiles in TDP-43^{KQ/KQ} mice do not entirely align with striatal TDP-43 knockdown.⁹⁷ This is likely in part because the K145Q mutation retains some level of RNA binding capacity, leaving some TDP-43-dependent processes unperturbed. Acetylated TDP-43 could also lead to gain-of-function toxicity due to altered RNA binding patterns and conformational instability, leading to nuclear export, enhanced phosphorylation and aggregation, and a dysregulated transcriptome, as we observe in TDP-43^{KQ/KQ} mice. Both loss and gain of function effects have been proposed for familial disease-causing *TARDBP* mutations,^{45,46,94,123} and TDP-43 acetylation may act in a similar manner.

RNA sequencing revealed profound transcriptomic alterations within aged TDP-43^{KQ/KQ} mouse brains. Many up- and down-regulated transcripts identified in acetylation-mimic mice were also differentially expressed in FTLD-TDP human tissue (**Figure 7**), particularly genes associated with cellular stress response, synaptic regulation, apoptotic signaling, and cellular adhesion.^{92,124} Disease-related alternative splicing events were also common in TDP-43^{KQ/KQ} neurons and mouse brain. For example, the altered splicing of *Sort1* and subsequent generation of *Sort1+ex17b* observed in TDP-43 acetylation-mimic mice (**Figure 8**) are also observed in FTLD-TDP brain.^{67,106} Moreover, SORT1 facilitates the endocytosis and lysosomal degradation of PGRN,^{106,108} a protein whose deficiency is causative of 10-30% familial FTLD cases.^{125–127} In humans, SORT1+ex17b contains an additional proteolytic site, resulting in the production of a soluble toxic variant that impairs the function of SORT1, prevents binding to PGRN and impacts neuronal survival.^{67,105} A recent study showed that TDP-43 depletion increased SORT1+ex17b, impaired brain derived neurotrophic factor (BDNF) signaling, and reduced synaptic plasticity in mice.⁶⁸ Importantly, hiPSC-derived cortical neurons expressing acetylation-mimic TDP-43^{K145Q} showed impaired splicing of putative FTLD-ALS spectrum disease biomarkers *UNC13A* and *STMN2*.^{69–72} We speculate that acetylated TDP-43 alters SORT1/PGRN signaling, as well as other pathways critical to neurotransmission, contributing to the synaptic dysregulation that is so evident in our transcriptome data. Future mechanistic studies are aimed at interrogating SORT1-PGRN, UNC13A, and STMN2-related signaling pathways in TDP-43^{KQ/KQ} mice and hiPSC-derived neurons.

Other aberrantly spliced transcripts in TDP-43^{KQ/KQ} mice provide additional insight into pathogenic mechanisms. The synaptic regulators *Arfgef2*¹²⁸ and *Arhgap10*^{129,130} were among the most affected transcripts. Concurrently, *Sema5b* which regulates synaptic connections and axon guidance,^{131–134} was strongly downregulated in TDP-43^{KQ/KQ} hippocampus and cortex, as were other genes involved in synapse homeostasis and signaling. Furthermore, splicing of LRP8 (low-density lipoprotein receptor-related protein 8) was significantly altered in TDP-43^{KQ/KQ} mice, and abnormalities in LRP8 can cause learning and memory defects, likely by disrupting Reelin-mediated synaptic plasticity and long-term potentiation (LTP).^{135–138} Similarly, *Nos1*, encoding the neuronal nitric oxide synthase (nNOS) protein that regulates LTP and synaptic plasticity via the production of nitric oxide,^{139,140} showed aberrant splicing patterns in TDP-43^{KQ/KQ} mice. Importantly, all of these synaptic regulators (*Nos1*, *Lrp8*, *Argef2*, *Sema5b*) and other significantly altered splice variants (e.g., *Adipor2*, *Mapk14*, *Smarca4*, *Sort1*, *Mapt*) have been linked to AD and other neurodegenerative diseases.^{99,138,140–148} Our transcriptome data strongly suggests that splicing and transcriptional abnormalities due to TDP-43 acetylation impacts synaptic plasticity, neurotransmission, and neuronal survival pathways in the TDP-43^{KQ/KQ} model, which overlaps with abnormalities found in human FTLD-TDP patients. We suspect that this prominent RNA signature may underlie the cognitive decline in TDP-43^{KQ/KQ} neocortex and hippocampus.

Given the correlation between TDP-43 acetylation and FTL-D-TDP presented in this study, it is perhaps surprising that acetylated TDP-43 (detected with an acetylation-specific ac-K145 antibody) is found within ALS spinal cord inclusions but has not yet been detected in FTL-D-TDP cortex.⁵² However, TDP-43 pathology in FTL-D-TDP cases is dominated by the presence of C-terminal fragments lacking RRM1 thereby precluding detection of the acetylated K145 site.^{54,149} It remains possible that TDP-43 acetylation still occurs in FTL-D-TDP at one or more additional lysine residues, and thus future mass spectrometry studies will be needed to determine whether a TDP-43 acetylation profile occurs earlier in the progression of FTL-D-TDP.

In conclusion, disrupting RNA-binding with an acetylation mimic TDP-43^{K145Q} mutation results in an age-dependent, dementia-like phenotype characterized by many signatures of FTL-D, including progressive cognitive deficits, TDP-43 mislocalization and phosphorylation, impaired autoregulation, and prominent RNA dysregulation. Interrogation of the affected genes linked TDP-43 acetylation to many known FTL-D-associated pathways, including STMN2, SORT1, and stress response signaling, while also pinpointing new putative factors as mediators of disease pathogenesis. By developing new human and mouse models of TDP-43 acetylation, we expand our understanding of sporadic TDP-43 proteinopathies and provide valuable approaches to identify and test new avenues for therapeutics that target pathogenic mechanisms underlying TDP-43 proteinopathy.

Methods

Animal Husbandry

Mice were housed in ventilated microbarrier cages on racks providing HEPA filtered air supply to each cage. Animals were kept on a 12hr light-dark cycle with access to food and water *ad libitum*. All animal husbandry, experiments, and procedures were performed in strict compliance with animal protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Chapel Hill (Protocol #21.257).

Primary neuron cultures

Murine primary cortical neurons were performed using wild-type C57Bl/6 mice (Charles River) or TDP-43^{wt/KQ} breeding pairs. Timed pregnant females at embryonic day 15-16 were lethally anesthetized with isoflurane. The abdominal cavity was opened, and the uterus incised to remove the placentas and embryonic sacs, which were washed briefly in ice-cold 70% ethanol and then placed in cold HEPES-buffered Bank's balanced salt solution (HBSS). The embryos were transferred into a 10cm dish containing cold HBSS, the embryos removed from the amniotic sac, and the brains were extracted from the cranium. For TDP-43^{wt/KQ} dissections, process was paused for approximately 2 hours to permit genotyping of the fetuses, during which the brains were stored at 4° C protected from light in a Hibernate-E (BrainBits NC9063748) solution supplemented with B27 (Gibco 17504044) and GlutaMAX (ThermoFisher 35050061). After genotyping, if applicable, the cerebral cortices from each brain were isolated under a stereomicroscope, pooled by genotype, minced with forceps, and digested for 30min at 37°C in a filter-sterilized HBSS solution containing 20U/mL papain (Worthington Biochemical LS003126), 1mM EDTA, 0.2mg/mL L-cysteine, and 5U/mL DNase (Promega M6101). The enzyme solution was removed, and the digested tissue was washed twice with sterile HBSS. Warm plating media [BrainPhys media (Stemcell 05790), 5% fetal bovine serum, 1x penicillin/streptomycin (Gibco 15140122), 1x B27, 1x GlutaMAX] containing 5U/mL DNase, was added and the tissue was dissociated mechanically using a P1000 pipette. The resulting cell suspension was spun down for 5min at 1.5rcf to pellet the cells, resuspended in plating media, and filtered through a 40 mm cell strainer. Cells were counted using a hemocytometer and plated onto poly-D-lysine (PDL)-coated 12-well tissue culture plates (Corning 356470) at 300K cells/well (for RNA extraction) or 96-well glass bottom black wall plates (Cellvis P96-1.5H-N) at 30K/well (for immunofluorescence and microscopy). 16-24hours after

plating, all plating media was removed and replaced with neuronal cell media (BrainPhys, 1x GlutaMAX, 1x B27, 1x penicillin/streptomycin). Cultures were incubated at 37°C, 5% CO₂ and 95% humidity with half-media exchanges every 3 days for the duration of all experiments.

Lentivirus preparation and neuron transduction

Lentiviral vectors were constructed in-house using the pUltra vector as a backbone for cloning via restriction enzyme digestion followed by ligation. The pUltra construct was acquired from Addgene (gift from Malcolm Moore; Addgene plasmid # 24129 ; <http://n2t.net/addgene:24129> ; RRID:Addgene_24129)¹⁵⁰. To generate TDP-43 constructs, TDP-43 variant gene fragments were PCR amplified using TDP-43 F' and R' primers (Supplementary File 3) and a pcDNA5/TO- myc-TDP-43^{wt}, -myc-TDP-43^{K145Q}, or -myc-TDP-43^{K145R} plasmid⁵² as the template. The pUltra construct was digested with AgeI and BamHI, and then incubated with the appropriate TDP-43 PCR product in the presence of T4 DNA ligase and T4 Polynucleotide Kinase for 1 hour at room temperature. The resulting ligation product was transformed into NEB stable competent cells using standard protocols.

Lentiviral production was performed by co-transfecting (CalPhos™ Mammalian Transfection Kit, TakaRa 631312) 37.5 µg lenti-plasmid with 25 µg psPAX2, 12.5 µg VSVG, and 6.25 µg REV for each 15 cm dish of lenti-X 293T cells (Takara 632180) and 3 dishes of cells were used for each lentiviral production. 3 days after transfection, culture media were collected and centrifuged at 2000g for 10 min. Lentiviral particles were purified using a double-sucrose gradient method. Briefly, the supernatants were loaded onto a 70%-60%-30%-20% sucrose gradient and centrifuged at 70,000g for 2 hr at 17°C (Beckman Optima LE-80K Ultracentrifuge, SW 32 Ti Swinging-Bucket Rotor). The 30%-60% fraction containing the viral particles was retrieved, resuspended in PBS, filtered with a 0.45µm filter flask before loaded onto a 20% sucrose cushion, and centrifuged a second time at 70,000g for 2 hr at 17°C. The supernatants were carefully discarded, and the viral particles present in the pellet were resuspended in PBS, aliquoted and stored at -80°C.

Lentivirus aliquots were evaluated for neuronal transduction ability prior to each experiment and diluted to approximately equal concentrations of effective virus. Lentiviral transductions were performed on DIV10 for a DIV14 harvest or DIV24 for a DIV28 harvest. In brief, lentiviruses were diluted to a 2x concentration in neuronal media, and then added to plates via half-media exchange. 24 hours after viral transduction, all virus-containing media was removed and replaced with 50% fresh neuronal media and 50% conditioned media from plates containing WT non-transduced neurons. Neurons were then cultured to DIV14 or DIV28 as the experiment required.

hiPSC-derived cortical neurons

iPSC maintenance: The HPC26 iPSCs were maintained on Matrigel-coated dishes (Corning 354480) in StemFlex Medium (Thermo Fisher Scientific A3349401) and passaged every 3-4 days with 0.5 mM EDTA dissociation solution as previously described¹⁵¹.

CRISPR/Cas9 genome editing: We used a control wildtype HPC26 cell line¹⁵¹ to generate two separate point mutations in the TARDBP gene that encodes the TDP-43 protein. Both mutations created single amino acid substitutions to change lysine 145 to glycine or arginine (K145Q and K145R). Benchling was used to design two guide RNAs and the corresponding single-stranded DNA donor oligos. The guide RNAs were purchased from Synthego, and the donor oligos from IDT. Genome editing was performed as described in Battaglia et. al.¹⁵². Briefly, 3x10⁵ iPSCs were electroporated on the Neon electroporation system (Thermo Fisher Scientific) with recombinant protein complexes made of Cas9 v2 protein (Thermo Fisher Scientific A36498), 900 ng sgRNA-TARDBP and 2700 ng of single-stranded donor oligonucleotide-K145Q or donor oligonucleotide-K145R (Supplementary File 3). Seventy-two hours after electroporation, cells were plated for single cell screening on a 96-well plate format using the limited dilution method. After two weeks,

single cells were expanded, genome DNA collected and the exon 4 of TARDBP amplified using the specific primers Ex4Fr and Ex4Rv (Supplementary File 3 [3](#)). Screening for single or double allele gene edits was performed by Sanger sequencing (Figure 3 - Figure supplement 1a-c [3](#)).

Characterization of edited iPSC clones: Stemness of edited iPSCs was assessed by immunofluorescent staining of the pluripotency factors OCT4, SOX2, SSEA4 and Tra-1-60 using specific antibodies (Supplementary File 4 [4](#)) as described¹⁵¹ (Figure 3 - Figure supplement 1d-e [3](#)). To confirm stemness and differentiation capabilities of the edited iPSCs we used the Taqman hPSC Scorecard (ThermoFisher A15871) as described in Battaglia et al.¹⁵² Briefly, iPSCs were differentiated into all three germ layers using STEMdiff Trilineage Differentiation Kit (StemCell Technologies), a mono-layer based protocol to directly differentiate hES cells in parallel into the three germ layers. Non-differentiated and differentiated cells were lysed and total RNA purified using the RNeasy kit (QIAGEN 74004). RNA reverse transcription was performed with the high-capacity cDNA Reverse Transcription kit (ThermoFisher 4368813) following the Taqman Scorecard's manufacture guidelines. qRT-PCR was carried out using the QuantStudio 7 Flex Real-Time PCR system. The TaqMan PCR assay combines DNA methylation mapping, gene expression profiling, and transcript counting of lineage marker genes¹⁵³.

iPSC-derived cortical neurons: We adapted, modified and standardized a protocol to generate mature cortical neurons from iPSCs using a dual SMAD inhibition protocol^{154,155}. First, undifferentiated iPSCs were collected with Accutase, counted and 3×10^5 cells cultured in StemScale PSC medium (A4965001) supplemented with 10 μ M Y27632 (PeproTech 1293823) and cultured in suspension on an orbital shaker at 37 C and 5% CO₂. After 48 hours, cells were dissociated with Accutase and 3×10^5 cells differentiated into NPCs as neurosphere in StemScale PSC medium supplemented with 10 μ M Y27632 (PeproTech 1293823) and 1.5 μ M CHIR99021 (PeproTech 2520691), 10 μ M SB431542 (PeproTech 3014193) and 50 nM LDN-193189 (Sigma SML0755). CHIR99021 was removed after 24 hours and cells were cultured for 10 days with daily medium changes and neurospheres dissociated at 1:3 ratio twice a week. Then NPCs were expanded for 7 days in the presence of 20 ng/ml FGF (Peprotech 100-18B) in Neuronal Expansion Medium [1:1 Advanced DMEM/F12 (12634028) and Neural Induction Supplement (A1647801)]. NPCs were plated for maturation on PDL/Laminin coated plates or coverslips in cortical neuron maturation medium [1:1 Advanced DMEM/F12 and Neurobasal; 1X GlutaMAX, 100mM B-mercaptoethanol, 1x B27, 0.5x N2, 1x NEAA and 2.5 mg/ml insulin (Sigma-Aldrich I9278)] supplemented with 10 ng/ml BDNF, 10 ng/ml GDNF, and 10 μ M DAPT. Seventy-two hours after plating, cells were treated with 1 μ g/ml Mitomycin C for (Sigma M5353) for 1 hour. Cellular identity was assessed specific markers of neuronal progenitor (PAX6, SOX2, and SOX1), and mature neurons (MAP2, TUJ1, CTIP2 and SATB2) (Figure 3 - Figure supplement 2 [3](#)) (Supplementary File 4 [4](#)). All reagents were purchased from ThermoFisher unless otherwise noted.

Primary mouse neuron and hiPSC-derived cortical neuron arsenite treatments and immunocytochemistry

On DIV14 (mouse primary neurons) or mature hiPSC-derived cortical neurons (~DIV50), neurons were treated for 2 hours with 200 μ M sodium arsenite (NaAsO₂) or vehicle (molecular biology grade water). Following treatment, neurons were fixed with 4% paraformaldehyde (PFA) in 1x phosphate buffered saline (PBS) and washed with 1xPBS. Neurons were permeabilized with 0.3% Triton X-100 in 1xPBS for 15min at RT, blocked in an 8% normal goat serum (NGS) 0.2% Triton X-100 in 1xPBS solution for 1-2 hours at RT, and incubated with primary antibodies (Supplementary File 4 [4](#)) diluted in 4% NGS, 0.2% Triton X-100, 1xPBS solution overnight at 4°C. The next day, the neurons were washed with 1xPBS, followed by application of fluorescent secondary antibodies (Supplementary File 4 [4](#)) diluted in 4% NGS, 0.2% Triton X-100, 1xPBS solution for 2 hours at RT, covered. Cells were washed four times with 1xPBS, with the third wash containing 1 μ g/mL 4',6-Diamidino-2'-phenylindole dihydrochloride (DAPI). 96-well plates were preserved in an 85%

glycerol in 1xPBS solution containing 0.4% sodium azide for microbial prevention. Coverslips were mounted onto pre-cleaned glass slides using ProLongTM Diamond Antifade Mountant (Invitrogen P36961).

Cultured neuron fluorescence microscopy and image analysis

Mouse primary cortical neurons in 96-well plates were visualized via automated fluorescence microscopy using EVOS M7000 Imaging System (ThermoFisher AMF7000) equipped with Olympus 20x/0.75 NA UPlanSApo and Olympus 40x/0.95 NA UPlanSApo objectives, and the following filter cubes: DAPI (357/44 nm Excitation; 447/60 nm Emission), GFP (482/25 nm Excitation; 524/24 nm Emission), Texas Red (585/29 nm Excitation; 628/32 nm Emission), and Cy5.5 (655/40 nm Excitation; 794/160 nm Emission). Images were acquired in an automated fashion, using DAPI fluorescence as the autofocus substrate, taking 16-32 images per well of 2-3 wells per experimental condition. Automated quantitative image analysis was performed using CellProfiler 4.0¹⁵⁶[157](#) to measure TDP-43 fluorescence intensity within neuronal compartments and to count TDP-43-positive foci within neurons. To identify neurons and subcellular compartments, DAPI was used to delineate nuclei and NeuN was used to label neuronal soma. hiPSC-derived cortical neurons were imaged on a Leica SP8X Falcon confocal microscope equipped with a 63x/1.40 NA Plan Apochromatic (oil) objective and hybrid GaAsP detectors, Leica Application Suite X Life Sciences software (Leica, Wetzlar, Germany).

Mouse model generation and genotyping

CRISPR/Cas9 Reagents

Cas9 guide RNAs targeting the mouse *Tardbp* K145 codon were identified using Benchling software (Benchling, San Francisco, CA, USA). Three guide RNAs were selected for activity testing. Guide RNAs were cloned into a T7 promoter vector followed by in vitro transcription and spin column purification (RNeasy, Qiagen). Guide RNAs were tested for cleavage activity by in vitro cleavage assay. Each guide RNA was incubated with Cas9 protein (UNC Protein Expression and Purification Core Facility) and PCR-amplified guide RNA target site. The products were run on an agarose gel for analysis of target site cleavage. Based on this assay, the guide RNA selected for genome editing in embryos was *Tardbp*-g79T (Supplementary File 3¹⁵⁸[159](#)). The donor oligonucleotide for insertion of the K145Q and silent genotyping mutations was *Tardbp*-K145Q-T (Supplementary File 3¹⁵⁸[161](#)). Two silent mutations were induced to create a unique HinfI restriction enzyme digestion site and permit genotyping by PCR and gel electrophoresis.

C57BL/6J zygotes were microinjected with microinjection buffer (5 mM Tris-HCl pH 7.5, 0.1 mM EDTA) containing 1) 20 ng/ul Cas9 mRNA, 10 ng/ul g79T guide RNA and 50 ng/ul donor oligonucleotide (founder #9) or 2) 20 ng/ul Cas9 mRNA, 400 nM Cas9 protein, 10 ng/ul g79T guide RNA and 50 ng/ul donor oligonucleotide (founders #23, 26, 39, 44, 53). Injected embryos were implanted in recipient pseudopregnant females and resulting pups were screened by PCR and sequencing for the presence of the desired mutant allele. Founder lines were propagated as heterozygotes and regularly sequenced to confirm retention of the K145Q and silent mutations.

We assessed the likelihood of off-target mutations using prediction algorithms to ensure low likelihood of off-target effects. The Benchling MIT off-target score for *Tardbp*-g79T is 72¹⁵⁸[159](#), and the CRISPOR-generated MIT off-target score is 86. CRISPOR also gave a Cutting frequency determination score of 86¹⁵⁹[161](#). Moreover, all of the predicted exonic off-target sites from CRISPOR have 4 mismatches, making them unlikely to be mutated. Sequencing of the founder lines and their progeny did not detect any insertions or deletions within a 550bp region surrounding the *Tardbp* locus.

TDP-43^{K145Q} mouse line genotyping

Each mouse was genotyped prior to any experimental use. DNA was extracted from ear punch or toe clip tissue using the HotSHOT method¹⁶². PCR amplification of the modified Tardbp locus was performed with ApexRed master mix (Genesee Scientific 42-138B) and *TDPKQ* F' and *TDPKQ* R' primers (Supplementary File 3) using the following cycling parameters: 95°C denaturation for 3min; 15 cycles of 95°C for 30sec, 72°C for 30sec then -1°C per cycle, 72°C 60sec; 25 cycles of 95°C for 30sec, 58°C for 30sec, 72°C for 60sec; 72°C extension for 5min. The PCR product was then incubated with HinfI enzyme (New England Biosystems R0155S) in CutSmart buffer (New England Biosystems B7204) for 1hr at 37°C. Digested PCR products were separated on a 2% agarose Tris-acetate-EDTA gel containing SybrSafe stain (Invitrogen S33102) and visualized on an ImageQuant LAS4000 machine.

Mouse Behavior

All testing was performed by experimenters blinded to mouse genotype.

Open field. Exploratory activity in a novel environment was assessed by a one-hour trial in an open field chamber (41 cm x 41 cm x 30 cm) crossed by a grid of photobeams (VersaMax system, AccuScan Instruments). Counts were taken of the number of photobeams broken during the trial in five-minute intervals, with separate measures for locomotor activity (total distance traveled) and vertical rearing movements. Time spent in the center region was used as an index of anxiety-like behavior.

Conditioned fear. Mice were evaluated for conditioned fear using the Near-Infrared image tracking system (MED Associates, Burlington, VT). The procedure had the following phases: training on Day 1, a test for context-dependent learning on Day 2, and a test for cue-dependent learning on Day 3.

Training. On Day 1, mice were placed in the test chambers, contained in sound-attenuating boxes. The mice were allowed to explore the novel chambers for 2 minutes before presentation of a 30-sec tone (80 dB), which co-terminated with a 2-sec scrambled foot shock (0.4 mA). Mice received 2 additional shock-tone pairings, with 80 sec between each pairing, and were removed from the test chambers 80 sec following the third shock.

Context- and cue- dependent learning. On Day 2, mice were placed back into the original conditioning chambers for a test of contextual learning. Levels of freezing (immobility) were determined across a 5-min session. On Day 3, mice were evaluated for associative learning to the auditory cue in another 5-min session. The conditioning chambers were modified using a Plexiglas insert to change the wall and floor surface, and a novel odor (dilute vanilla flavoring) was added to the sound-attenuating box. Mice were placed in the modified chamber and allowed to explore. After 2 min, the acoustic stimulus (an 80 dB tone) was presented for a 3-min period. Levels of freezing before and during the stimulus were obtained by the image tracking system.

Grip strength. Grip strength was evaluated using precision force gauges. Measures were based on paw grasp of a metal grid by a mouse gently pulled by the tail. Digital force meters (Chatillon DFIS-10; Largo, FL) were mounted on an acrylic platform (San Diego Instruments), with two different grids: a left-hand grid for the front paws, and a right-hand grid for all-four paws. Each grid connected to a force transducer, which provided measures of peak force (newtons). Each mouse was given 3 trials, with at least one minute between each trial. Each trial had 2 components: front-paw measures from the left-hand grid, immediately followed by all-four-paw measures from the right-hand grid.

Rotarod. Subjects were tested for motor coordination and motor learning on an accelerating rotarod (Ugo Basile, Stoelting Co., Wood Dale, IL). For the first test, mice were given three trials, with 45 seconds between each trial. Two additional trials were given 48 hours later. Rpm (revolutions per minute) was set at an initial value of 3, with a progressive increase to a maximum of 30 rpm. across five minutes (the maximum trial length). Measures were taken for latency to fall from the top of the rotating barrel.

Morris water maze. The water maze was used to assess spatial and reversal learning, swimming ability, and vision. The water maze consisted of a large circular pool (diameter = 122 cm) partially filled with water (45 cm deep, 24–26°C), located in a room with numerous visual cues. The procedure involved three separate phases: a visible platform test, acquisition in the hidden platform task, and a test for reversal learning (an index of cognitive flexibility).

Visible platform test. Each mouse was given 4 trials per day, across 2 days, to swim to an escape platform cued by a patterned cylinder extending above the surface of the water. For each trial, the mouse was placed in the pool at 1 of 4 possible locations (randomly ordered), and then given 60 sec to find the visible platform. If the mouse found the platform, the trial ended, and the animal was allowed to remain 10 sec on the platform before the next trial began. If the platform was not found, the mouse was placed on the platform for 10 sec, and then given the next trial. Measures were taken of latency to find the platform and swimming speed via an automated tracking system (Noldus Ethovision).

Acquisition and reversal learning in a hidden platform task. Following the visible platform task, mice were tested for their ability to find a submerged, hidden escape platform (diameter = 12 cm). Each mouse was given 4 trials per day, with 1 min per trial, to swim to the hidden platform. The criterion for learning was an average group latency of 15 sec or less to locate the platform. Mice were tested until the group reached criterion, with a maximum of 9 days of testing. When the group reached criterion (on day 5 in the present study), mice were given a 1-min probe trial in the pool with the platform removed. Selective quadrant search was evaluated by measuring the number of crosses over the location where the platform (the target) had been placed during training, versus the corresponding area in the opposite quadrant. Following the acquisition phase, mice were tested for reversal learning, using the same procedure as described above. In this phase, the hidden platform was re-located to the opposite quadrant in the pool.

As before, measures were taken of latency to find the platform. On day 5 of testing, the platform was removed from the pool, and the group was given a probe trial to evaluate reversal learning.

Acoustic startle test. This procedure was used to assess auditory function, reactivity to environmental stimuli, and sensorimotor gating. The test was based on the reflexive whole-body flinch, or startle response, that follows exposure to a sudden noise. Measures were taken of startle magnitude and prepulse inhibition, which occurs when a weak prestimulus leads to a reduced startle in response to a subsequent louder noise.

Mice were placed into individual small Plexiglas cylinders within larger, sound-attenuating chambers. Each cylinder was seated upon a piezoelectric transducer, which allowed vibrations to be quantified and displayed on a computer (San Diego Instruments SR-Lab system). The chambers included a ceiling light, fan, and a loudspeaker for the acoustic stimuli. Background sound levels (70 dB) and calibration of the acoustic stimuli were confirmed with a digital sound level meter (San Diego Instruments). Each session began with a 5-min habituation period, followed by 42 trials of 7 different types: no-stimulus (NoS) trials, trials with the acoustic startle stimulus (AS; 40 msec, 120 dB) alone, and trials in which a prepulse stimulus (20 msec; either 74, 78, 82, 86, or 90 dB) occurred 100 msec before the onset of the startle stimulus. Measures were taken of the startle amplitude for each trial across a 65-msec sampling window, and an overall analysis was

performed for each subject's data for levels of prepulse inhibition at each prepulse sound level (calculated as $100 - [(response\ amplitude\ for\ prepulse\ stimulus\ and\ startle\ stimulus\ together / response\ amplitude\ for\ startle\ stimulus\ alone) \times 100]$).

Tissue harvest and preparation

At each end point, mice were anesthetized deeply with isoflurane and euthanized via rapid decapitation. The hippocampus and neocortex were dissected out of the brain on a cold surface using clean surgical tools, placed into cryo-safe nuclease-free microcentrifuge tubes, flash frozen in liquid nitrogen (LN2), and stored at -80°C until processing. Frozen tissue was then pulverized in LN2-cooled stainless steel Cryo-Cups using cold stainless-steel pestles and transferred into cold nuclease-free microcentrifuge tubes and immediately stored at -80°C until use.

Solubility fractionation, nucleocytoplasmic fractionation, and immunoblotting

All steps of protein fractionation were performed on ice unless otherwise indicated. For solubility fractionation, pulverized tissue was suspended in $5\mu\text{L}/\text{mg}$ of ice-cold 1xRIPA buffer (50mM Tris pH 8.0, 150mM NaCl, 1%NP-40, 5mM EDTA, 0.5% sodium deoxycholate, 0.1%SDS) containing a mix of protease, phosphatase, and deacetylase inhibitors [1 $\mu\text{g}/\text{mL}$ Peptstatin A (Sigma P4265), 1 $\mu\text{g}/\text{mL}$ Leupeptin (Sigma L2023), 1 $\mu\text{g}/\text{mL}$ N_{α} -Tosyl-L-lysine chloromethyl ketone hydrochloride (TPCK) (Sigma T7254), 1 $\mu\text{g}/\text{mL}$ Trypsin inhibitor (Sigma T9003), 1 $\mu\text{g}/\text{mL}$ N-p-Tosyl-L-phenylalanine chloromethyl ketone (TLCK) (Sigma T4376), 0.67 $\mu\text{g}/\text{mL}$ trichostatin A, 10mM nicotinamide, 1mM phenylthiohydantoin fluoride, 1mM phenylmethylsulfonyl fluoride]. The solution was homogenized by sonication and centrifuged at 4°C for 45min at 18000xrcf. The supernatant was removed and saved as the RIPA-soluble ("soluble") protein fraction. The pellet was resuspended in RIPA buffer with inhibitor mixture, sonicated, and centrifuged as described above, and the supernatant discarded. The resulting pellet of RIPA-insoluble material was resuspended in approximately 1 $\mu\text{L}/\mu\text{g}$ of Urea buffer (7M urea, 2M Thiourea, 4%CHAPS, 30mM Tris, pH 8.5) with the inhibitor mixture (as above), sonicated to homogenize, and centrifuged at room temperature (RT) for 45min at 21000xrcf. The resulting supernatant of RIPA-insoluble, urea-soluble protein fraction was saved as the "insoluble" protein fraction.

Nuclear and cytoplasmic soluble proteins were isolated using the ThermoFisher NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific 78835) per the manufacturer's instructions, with the addition of the protease, phosphatase, and deacetylase inhibitors (as above) to each buffer.

All soluble protein fractions were analyzed by BCA assay (Thermo Scientific 23225) to determine protein concentration. Equal quantities of protein per sample were run onto 4-20% Tris-Glycine SDS-PAGE gels (Biorad 5671095) under reducing conditions and then transferred onto nitrocellulose membranes. Total transferred protein (TTP) was assessed using PonceauS (Research Products International Corp P56200) protein stain on nitrocellulose membranes per manufacturer's instructions. Membranes were washed three times in TBST, once in TBS, and blocked in 2% nonfat milk in 1xTBS for 1-2 hours at RT. Membranes were incubated with primary antibodies ([Supplementary File 4](#)) diluted in 2% milk overnight at 4°C . The primary antibody solution was removed and membranes washed three times in TBST, once in TBS, and incubated with cross adsorbed HRP-conjugated goat secondary antibodies ([Supplementary File 4](#)) diluted in 2% milk at 1:10000 or 1:2000 for soluble or insoluble protein immunoblots, respectively, for 1-2 hours at RT. Blots were then visualized by chemiluminescent imaging using an ImageQuant LAS4000 machine. Densitometry analysis to quantify western blot images was performed in LI-COR Image Studio Lite (Lincoln, NE, USA).

Mouse brain RNA isolation, RNA sequencing, and data analysis

Mouse brain tissue was isolated, flash frozen, and pulverized as described above. Approximately 20mg of pulverized brain tissue per sample was used to isolate RNA. 1mL of TRIzol (Invitrogen 15596018) was added to each nuclease-free eppendorf tube containing pulverized tissue, and tissues were lysed via trituration with a P1000 pipette tip, followed by trituration with a 21G and then a 25G needle on a 1mL syringe. Samples were centrifuged at 4°C for 5min at 10,000rcf to remove tissue debris. The supernatant was removed and added to a new tube containing 200uL of chloroform, which were mixed by inversion and cooled on ice for 5min. Samples were centrifuged at 4°C for 15min at 10,000rcf, and the upper aqueous phase containing RNA was transferred into a new tube, followed by the addition of 100uL of isopropanol to precipitate RNA and overnight incubation at -20°C. The next day, samples were centrifuged at top speed (18,000rcf) for 20min at 4°C to pellet the RNA. The pellets were washed twice with 1mL of ice-cold 70% molecular biology grade ethanol and then air dried for 15min at RT. The RNA pellets were resuspended in 20uL of nuclease-free water. On-column DNase digestion and RNA clean-up was then performed using the Qiagen RNeasy mini kit (Qiagen, Inc. 74106) per manufacturer's instructions, followed by elution in nuclease-free water.

RNA concentration was assessed using Qubit® RNA BR Assay Kit (Q10210) and a Qubit® 3.0 Fluorometer. RNA integrity was assessed using an Agilent 4150 TapeStation system and associated RNA screen tape reagents (Agilent 5067-5576). Only samples with an estimated RNA integrity number (RIN) ≥ 7.0 were sent to the New York Genome Center (NYGC) for bulk total RNA sequencing. Upon receipt at NYGC, RNA samples were re-evaluated for quantification and integrity, using Ribogreen and Fragment Analyzer 5300, respectively. Total RNA libraries were prepped using Kapa Total library prep with Ribo-Erase, in accordance with manufacturer recommendations. Briefly, 500ng of total RNA was used for ribosomal depletion and fragmentation of total RNA. Depleted RNA underwent first and second strand cDNA synthesis. cDNA was then adenylated, ligated to Illumina sequencing adapters, and amplified by PCR (using 9 cycles). The cDNA libraries were quantified using Fragment Analyzer 5300 (Advanced Analytical) kit FA-NGS-HS (Agilent DNF-474-1000) and Spectramax M2 (Molecular Devices) kit Picogreen (Life Technologies P7589). Libraries were sequenced on an Illumina NovaSeq sequencer, using paired end sequencing (2 x 100 bp cycles) to a depth of $>75M$ read pairs per sample.

Raw reads were then trimmed and filtered of adapter sequencing using cutadapt¹⁶³ and filtered such that at least 90% of bases had a quality score of at least 20. Reads were then aligned to the reference mouse genome (mm10, RefSeq gene annotations) using STAR v2.5.2b¹⁶⁴, and transcript abundance was estimated using salmon¹⁶⁵. Differential expression between TDP43-KQ and TDP43-WT cortex and hippocampus was then detected using DESeq2 v1.34.0¹⁶⁶ in R v4.1.0¹⁶⁷, using a design that corrects for both mouse sex and litter effects. These batch effects were also removed from the VST-normalized expression values using limma¹⁶⁸. The correlation between Log2 fold change values between differentially expressed genes in the cortex and hippocampus was determined and Pearson correlation coefficient and two-sided p-value were computed by ggpubr stat_cor^{169,170}, and the smoothed linear model was fit using geom_smooth with method="lm".

Differentially expressed genes ($\text{padj} < 0.05$) were then separated into 6 groups based on their intersections between the two brain regions using UpSetR v1.4.0¹⁷¹ and plotted with ComplexUpset¹⁷². Normalized expression values were then centered around the mean of TDP43-WT for each respective brain region and plotted with ComplexHeatmap¹⁷³. Gene Ontology enrichments were then assessed using gprofiler2 v0.2.1^{174,175} and summarized using simplifyEnrichment v1.7.2¹⁷⁶. Previously published differentially expressed genes from Hasan et al.⁹² and Polymenidou et al.⁹⁷ were retrieved from the respective publications; significant over-enrichments as well as human gene symbol mappings to mouse orthologs were performed using gprofiler2 (g:Orth). Differential splicing analyses were performed on splice

junctions extracted from genome-aligned BAM files using regtools and LeafCutter¹⁷⁷, where tests compared TDP43-KQ to TDP43-WT correcting for sex for each brain region. Results were then summarized and visualized using LeafViz¹⁷⁸.

Quantitative RT-PCR

RNA was isolated from mouse brain tissue as described above. For RT-PCR experiments involving mouse primary cortical neurons or human iPSC-derived cortical neurons, cells were washed with 1xPBS, harvested from the culture dish, and RNA was extracted using the Qiagen RNeasy mini kit (Qiagen, Inc. 74106) with on-column DNase digestion per manufacturer's instructions. RNA concentration was determined using a NanoDrop 2.0 spectrophotometer, 250ng-1μg of RNA was used to generate cDNA using Applied Biosystems High-capacity RNA-to-cDNA kit (#4387406) per manufacturer's instructions. cDNA was generated from 250-500ng of RNA using Applied Biosystems High-capacity RNA-to-cDNA kit (#4387406) per manufacturer's instructions. Quantitative PCR was performed on a QuantStudio 6 Real Time PCR system (with Thermo Fisher Design & Analysis Software version 2.6.0) using PowerUp SYBR Green master mix (Applied Biosystems A25776). The PCR phase consisted of 40 cycles of 15s at 95°C and 1min at 60°C. Forward and reverse primer sequences for *Tardbp*, *Sort1-WT*, *Sort1-total*, *Sort1-ex17b*, *TARDBP*, *SORT1-WT*, *SORT1-ex17b*, *SORT1-total*, *UNC13A*, *UNC13A* cryptic exon, *STMN2*, truncated *STMN2* and the reference genes *β-Actin*, *Pgk1*, and *RPLP0* are listed in Supplementary File 3. Relative quantification of transcripts was performed using the Pfaffl method¹⁷⁹ with both *β-Actin* and *Pgk1* as reference genes for mouse tissue and mouse primary neurons, and *RPLP0* used as the reference gene for hiPSC-derived neurons. Primers generated for this paper were designed using PrimerBank¹⁸⁰ and synthesized by Integrated DNA Technologies (IDT).

Tissue Collection, Staining, and Immunofluorescence

Mice were transcardially perfused with 1xPBS followed by 4% PFA, and brains were removed and post-fixed for 24 hours in 4% PFA. Brains were cryoprotected in 15% sucrose in 1xPBS for 24hrs followed then 30% sucrose in 1xPBS for 48hrs, and then embedded in Fisher Tissue-Plus OCT compound (4585). Forebrain (~Bregma +1.78 mm), midbrain (~Bregma +0.50 mm), and hippocampal (~Bregma -1.94 mm) 10 mm cryosections were collected onto Superfrost Plus charged slides using a Leica CM1950 cryostat.

Luxol Fast Blue and Cresyl Violet staining. Slides were acclimated to room temperature and rinsed in distilled water. They were then incubated in 70% ethanol for ~60 hours followed by 95% ethanol for 30 minutes. Tissues were stained with 0.1% Luxol Fast Blue Solution overnight at 60°C, rinsed in distilled water, and differentiated as needed by 0.05% Lithium Carbonate followed by 70% ethanol. They were then counterstained with 0.1% Cresyl Echt Violet, rinsed in distilled water, and differentiated again by 95% ethanol. Finally, slides were dehydrated in 100% ethanol, cleared in Xylene, and coverslipped with DPX mountant (Millipore-Sigma 44581).

Immunofluorescence. Slides were acclimated to room temperature and rinsed in distilled water. Heat-induced epitope retrieval was performed at 120°C using pH 6.0 buffer (Epredia, TA-135-HBL). The tissues were then blocked in 10% normal goat serum for 1 hour. Primary antibodies were applied overnight at 4°C: Mouse Anti-NeuN (clone A60) conjugated to Alexa Fluor 555 (Millipore, MAB377A5), and Rabbit Anti-TDP-43 (Cell Signaling, 89789) or Rabbit anti-Iba1 (Wako, 019-19741) and mouse anti-GFAP conjugated to Alexa Fluor 488 (Cell Signaling, 3655) (Supplementary File 4). After rinsing, secondary antibodies were applied for 2 hours at room temperature: Alexa Fluor 488 Goat Anti-Rabbit IgG (Invitrogen, A32731) or Alexa Fluor 680 Goat anti-Rabbit IgG (Invitrogen 32734). All antibodies were diluted using Da Vinci Green Diluent (Biocare Medical, PD900L). Finally, slides were coverslipped using Fluorogel II with DAPI (Electron Microscopy Sciences, 17985-50).

Tissue microscopy and Image Analysis

Brain sections stained with Cresyl Violet (CV) and brain sections labelled with Iba1 and GFAP antibodies were imaged on a Nikon Eclipse Ti2 widefield microscope equipped with a Nikon DS-Fi3 CMOS color camera (for CV imaging) and a pco.edge 4.2Q High QE sCMOS camera (PCO, Kelheim, Germany) using a 20x/0.5 NA Plan Fluor objective and NIS-Elements software (Nikon, Minato City, Tokyo, Japan). Immunofluorescently labeled brain sections were imaged on a Leica SP8X Falcon confocal microscope equipped with hybrid GaAsP detectors using a 40x/1.30 NA Plan Apochromatic (oil) objective and Leica Application Suite X Life Sciences software (Leica, Wetzlar, Germany). Cell counting (density of CV-positive and NeuN-positive cells), as well as TDP-43 fluorescence intensity, TDP-43 localization measurements, GFAP fluorescence intensity and area, and Iba1 fluorescence intensity and area were performed using CellProfiler^{156,157}. Prior to quantifying TDP-43 immunofluorescence intensity and prior to confocal image segmentation, immunofluorescent images were denoised using NIS-Elements Batch Denoise.ai under default conditions. Prior to quantifying Iba1 and GFAP fluorescence characteristics, images of the neocortex and hippocampus were cropped to exclude fluorescent signal from the corpus callosum. Within CellProfiler, the Cellpose 2.0 plugin^{181,182} was used to perform neuron identification and the subcellular segmentation of nucleus and cytoplasm within neurons. DAPI fluorescence was used to identify nuclei, and NeuN immunoreactivity was used to identify neuronal nuclei and the surrounding soma. Images were pseudocolored and formatted for publication using Fiji ImageJ¹⁸³.

Statistical Analysis

Statistical analysis of RNA sequencing data was performed as described above. For data shown in **figures 1B-C**, **2B-C**, **4B-C**, **4E-F**, and **4H-I**, we used Linear Mixed Effects (LME) Models with random intercept to allow for an animal or a well specific effect. Restricted Maximum likelihood (REML) approach was used for parameter estimation. For **Fig 1B-C** and **2B-C**, wells were used as a “by-subject” random effect. For **Fig 4B-C**, animals were used as a “by-subject” random effect. For 4E-F and 4H-I, animal ID and image ID were included as nested random effects. Either genotype or treatment group were included as fixed effects in each LME model. Results are presented as fixed effect estimates, standard errors and 95% confidence intervals (CI) (**See Supplementary File 2**). All statistical analyses were performed in R version 4.2.1 (R Core Team, 2022). Complete case analysis was considered, with $P < .05$ determining statistical significance. All other data was analyzed in GraphPad Prism Version 9.4.1 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com (<http://www.graphpad.com/>). For data sets with $n > 10$, outliers were identified and removed (if any) using the ROUT method¹⁸⁴ at $Q = 1\%$. Details regarding the statistical test performed, sample sizes, and what the data points and error bars represent can be found in the appropriate figure legends. All statistical tests were two-sided. Statistical significance was determined as $p < 0.05$. Supplementary statistical information about data presented in main and supplemental figures can be found in the figure’s accompanying source data files.

Declarations

Data Availability

The TDP-43^{K145Q} mouse line is now available at the Mutant Mouse Resource and Research Center (MMRRC) at University of North Carolina at Chapel Hill, an NIH-funded strain repository, with the following identifiers (RRID:MMRRC_068119-UNC). Raw and processed RNA-seq data have been deposited to the Gene Expression Omnibus (GEO) under accession GSE216294. All data generated in this work are included in the manuscript and the supporting files. Any plasmids or lentiviruses generated in this study are available upon request.

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Reviewer #1 (Public Review):

With this work, the authors address a central question regarding the potential consequences of post-translational modifications for the pathogenesis of neurodegenerative diseases. Phosphorylation and mislocalization of the RNA binding protein TDP43 are characteristic of ~50% of frontotemporal lobar degeneration (FTLD), as well as >95% of amyotrophic lateral sclerosis (ALS). To determine if acetylation is a primary, disease-driving event, they generated a TDP-43 mutant harboring an acetylation-mimicking mutation (K145Q). Animals carrying the acetylation-mimic mutation (K145Q) displayed key pathological features of disease, including more cytoplasmic TDP43 and impaired TDP43 splicing activity, together with behavioral phenotypes reminiscent of FTLD.

This is a well-written and well-illustrated manuscript, with clear and convincing findings. The observations are significant and emphasize the importance of post-translational modifications to TDP-43 function and disease phenotypes. In addition, the TDP43(K145Q) mice may prove to be a valuable model for studying TDP-43-related mechanisms of neurodegeneration and therapeutic strategies.

Comments on the latest version:

The authors have addressed most concerns. The additional analysis demonstrating a lack of neuron loss is quite different from the original study -- it is good that the authors pursued this question. In addition, new data focusing on native TDP-43 splice targets, rather than the splicing reporter, are excellent.

Reviewer #2 (Public Review):

This paper extends prior work demonstrating the importance of K145 acetylation of TDP-43 as a post-translational modification that impacts its RNA-binding capacity and may contribute to pathology in FTLD-ALS. The main strengths of this paper are the generation of a novel mouse model, using CRISPR gene editing, in which an acetylation-mimetic mutation (K to Q) is introduced at position 145. Behavioral, biochemical, and genetic analyses indicate that these mice display phenotypes relevant to TDP-43-associated disease and will be a valuable contribution to the field.

Reviewer #3 (Public Review):

Numerous experimental models are phenotyped in this manuscript including mouse neurons, iPSC-derived human neurons, knock-in mice, and knock-in iPSCs. Expression of

acetylation-mimic or acetylation-null TDP-43 protein is achieved either with overexpression or CRISPR-Cas9-based knock-in. A complex phenotype is observed including loss of TDP-43 function (reduced autoregulation, increased cryptic splicing) and a gain of TDP-43 (increased insoluble TDP-43 protein). These correlate with downstream neurobehavioral changes which are most consistent with a cortical/hippocampal phenotype without a motor phenotype. Post-translational modifications of disease-associated proteins are thought to contribute to neurodegenerative disease pathogenesis, and this study succeeds in demonstrating that TDP-43 acetylation results in downstream molecular and behavioral phenotypes.

TDP-43 acetylation is a post-translational modification that is known to be associated with TDP-43 inclusions that are characteristic of human diseases. An important strength is the rigorous use of multiple different experimental models (rodent cells, iPSC-derived neurons, mice, overexpression, knock-in) with overall consistent results. Moreover, multiple orthogonal endpoints are presented including histology/cytology/immunostaining, biochemistry, molecular biology, and neurobehavioral assays. As TDP-43 acetylation is known to block RNA binding, these novel cellular and mouse models represent interesting albeit complex tools to study the functional consequences of a partial loss of function. As TDP-43 regulates its own expression (i.e. autoregulation), the complexity lies at least in part due to the loss of RNA binding leading to a functional loss of TDP-43 function which includes the increased expression of the TARDBP transcript and TDP-43 protein.

Conceptually, there is a disconnect in that the mouse model exhibits primarily a cortical/hippocampal phenotype more akin to frontotemporal lobar degeneration with TDP-43 inclusions (FTLD-TDP), while TDP-43 acetylation is only seen in ALS tissues and not in FTLD-TDP tissues because most of the pathologic protein in the latter is N-terminally truncated (i.e. the acetylation site is not present). That being said, there is no mouse model which completely and faithfully recapitulates the human disease, and this mouse model avoids overt overexpression (increased TDP-43 protein expression stemming from altered autoregulation) and avoids the use of synthetic/artificial mutation (such as mutation of the TDP-43 nuclear localization signal).

This revision addresses most of my prior comments including documenting the lack of neurodegeneration in this model, the use of more appropriate statistical methods, and the use of more robust/quantitative aberrant splicing measures. The one thing which would still be helpful is sequencing the top predicted off target genomic loci for their various CRISPR'd models irrespective of whether these loci are exonic or noncoding. Having actual sequencing verification of the lack of mutations at these loci is preferable over relying only on computational likelihood estimates.

Author Response

The following is the authors' response to the original reviews.

Reviewer #1

1.1 Fig. 1: A good control for these studies would be a TDP-43 variant with an RRM1 mutation that impairs RNA binding, but not an acetylation mimic (i.e. mutations affecting W113, R151, F147, or F149)

In our original paper (Cohen et al, Nat Commun. 2015 Jan 5; 6:5845), we already characterized TDP-43 acetylation and employed a complete RNA-deficient mutant (F147/149L), as the reviewer suggested. In that original study, this mutant showed maximal RNA binding-

deficiency, and therefore we proposed that acetylation mimic mutations represent a comparable RNA-binding deficient variant.

1.2 Fig. 1: time and expression level can influence nuclear TDP-43 puncta formation. It is important that the authors take these into account when measuring puncta number/frequency.

All expression levels and transfection/transduction times were identical across samples. We chose the optimal times to express TDP-43 constructs yet minimize toxicity and found that neuronal transduction at DIV10 and arsenite exposure on DIV14 in mature neurons was optimal.

1.3 Fig. 2: to accurately refer to the nuclear foci as anisosomes, the authors will need to conduct higher-resolution imaging.

We agree with the reviewer and since anisosomes are not well characterized in terms of their relationship to TDP-43 nuclear foci (and may represent only a subset of foci), we have now omitted any mention of anisosomes but instead refer to them in the discussion, where we suggest that TDP-43 K145Q foci may partially represent anisosomes.

1.4 Fig. 2D: it seems as though the splicing reporter should have a fluorescence-based readout (red/green ratio, for instance). Is this the case, and is the ratio informative?

We have now removed the splicing reporter data and replaced this with much more robust data showing RT-qPCR of downstream TDP-43 targets including Sortilin-1 (see the new revised Figure 2E and 3B-I).

1.5 Line 145: "Overall, these results indicate that a single endogenously expressed acetylation-mimic TDP-43(K145Q) mutation is sufficient to alter TDP-43 localization, induce TDP-43 phase separation, and impair splicing in a murine primary neuron culture model." The authors did not assess phase separation in this study. Moreover, it would be more convincing to assess native splice targets of TDP-43 in K145Q primary neurons, rather than an exogenous splicing reporter.

See comment 1.1 above. We have now avoided mentioning phase separation in the main text but mention this as a potential mechanism in the discussion. In addition, we have now evaluated native TDP-43 splice targeted in primary neurons.

1.6 Fig. 4A: is the loss of neurons selective for a specific layer or region of the cortex?

Since we did not observe any gliosis, we have gone back and completely re-evaluated neuronal loss since the concept of neurodegeneration is a critical question in the TDP-43KQ/KQ mice. We do not find any significant neuronal loss in the homozygous TDP-43KQ/KQ mice (see Figure 5).

1.7 Fig. 6: The authors suggest that the large majority of splicing changes are direct results of the TDP43(K145Q) mutation and impaired RNA binding by TDP-43. However, without a direct assessment of TDP43(K145Q) target RNAs in comparison to those of TDP-43(WT), this is only an assumption. Moreover, given the fact that RNA-seq was performed in aged animals, the potential for indirect gene expression changes is very high.

In our original study (Cohen et al, Nat Commun. 2015 Jan 5;6:5845), we showed that the K145Q is severely deficient in RNA binding. In this study, we now show strong evidence that many known targets of direct TDP-43 binding are dysregulated, supporting the expected loss

of function if TDP-43 K145Q mutation abrogated RNA binding. Although we have not performed direct RNA binding studies to the Sort1 transcript, for example, other studies have clearly indicated that wild-type TDP-43 binds these targets. We infer that loss of function mutations (i.e., K145Q) impact direct targets of TDP-43. Future studies employing RNA-immunoprecipitation followed by RNA sequencing (RIP-seq) could be useful in this regard and will be required to mechanistically address this point.

1.8 Sup Fig. 8 is very interesting and suggests that any TDP-43 variant that is unable to bind RNA may lead to upregulation of TDP43 RNA and phenotypes similar to those observed in K145Q animals. This is alluded to in the discussion but never specifically tested.

Yes, we agree with this reviewer's comment. Loss of RNA binding, whether due to acetylation (e.g., K145Q) or otherwise is expected to cause autoregulatory up-regulation of the TARDBP transcript and impact other targets, potentially yielding phenotypes similar to the TDP-43KQ/KQ mice. However, new in vivo models would be needed to prove this point. For example, in the future, we will consider this possibility by characterizing recently identified RNA-binding deficient familial TARDBP mutants (e.g., P112H or K181E).

1.9 The authors should also provide some comment or potential explanation for why TDP43(K145Q) animals show no signs of motor neuron disease.

We now show a moderate level of TDP-43 aggregation and hyper-phosphorylation in spinal cord of mutant mice in Figure 6 – Figure Supplement 3. We also speculate in the discussion why we observe aspects of TDP-43 dysfunction in spinal cord without overt motor phenotypes up until 18 months old.

1.10 Line 79: "However, TARDBP mutations that disrupt RNA binding, and thereby may act in a similar manner to TDP-43 acetylation, have been identified in FTLD-TDP patients." Evidence suggests that the D169G mutation does not interfere with RNA binding. See Furukawa et al., 2016.

We thank the reviewer for pointing this out. We have now removed the D169G mutation from the discussion.

1.11 It is unclear why the authors focused solely on homozygous K145Q animals, rather than heterozygous mice.

We focused initially on homozygous mutant mice to provide better statistical power to detect small effect sizes. However, we have now included a thorough analysis of heterozygous mice including molecular analysis of brain tissue and mouse behavior, as shown in Figure 4 – Figure Supplements 1-2 and Figure 6 – Figure Supplements 1-3.

Reviewer #2

2.1 A strength of this paper is the generation of a new mouse model to study the effects of K145 acetylation in TDP-43 proteinopathy. While the authors note an absence of a behavioral phenotype on neuromuscular testing in aged animals, it would be appropriate to include some analysis of spinal cord and skeletal muscle in this initial description of their model. At a minimum, I wonder if there is pathology in the cord (neuron loss, gliosis) or muscle (fiber atrophy) if insoluble p-TDP-43 is detectable in these tissues, and whether dysregulated splicing of TDP-43 target genes (such as shown in Fig 7) occurs at these sites.

See comment 1.9 above. We analyzed TDP-43 aggregation, localization, and splicing in the spinal cord of TDP-43KQ/KQ mice and found mild loss of TDP-43 function that was comparable, though not to same extent, as that seen in hippocampus and cortex. We discussed these findings in the discussion and provide several possibilities for why there are no overt motor phenotypes in these mice. We note that TDP-43 Q331K knock-in mice also have cognitive but no motor deficits, suggesting TDP-43 dysfunction may preferentially (or at least initially) impact cognitive function (White et al, Nat Neurosci. 2018 Apr;21(4):552-563).

2.2 Fig 2: Differences in the splicing reporter are hard to appreciate from the images shown in panel E. Is the quantification shown in panel F corroborated by an analysis of green vs yellow fluorescence or by another method? Quantification of results shown in panel 2G (from 3 biological replicates) should be included.

We have now removed the splicing reporter data in lieu of the more robust RT-qPCR data shown in Figure 2E and 3B-I. We have also now included more biological replicates from our iPSC neuron imaging, as shown in Figure 3A. Due to time and resource constraints, we were not able to quantify the images shown in figure 3A, and reinforce in the text that our statements are qualitative. However, we were able to add quantitative analysis of TDP-43 dysfunction, by detecting genotype-dependent splicing changes in hiPSC neurons, as mentioned above, which strengthens our claim that TDP-43 dysfunction is prominent in this culture mode.

2.3 Fig 4: Differences in NeuN quantification without changes in cresyl violet staining or gliosis are surprising and a bit difficult to understand. Is there confirmation of neuron loss through another metric? Is it possible that NeuN expression is lower in mutants without frank neuron loss? Also, although no significant differences were seen by IF for TDP-43 staining, did IF for phospho TDP-43 show differences? One might expect this to be the case given the biochemical findings in Fig 5.

See comment 1.6 above. After a much more in-depth and rigorous assessment, we find little evidence for neurodegeneration. Given the transcriptome data showing that TDP-43 regulates a subset of synaptic genes, we suggest that synaptic deficits underlie the behavioral phenotype rather than neuronal loss.

Regarding phospho-TDP-43 pathology by immunofluorescence (IF) staining, after much effort, we have not been able to detect phospho-TDP-43 pathology by IF in TDP-43KQ/KQ mice. Currently available phospho-TDP-43 antibodies (including those acquired from collaborators) do not work well to detect endogenous mouse TDP-43 by histology or IF staining, and therefore we are somewhat limited technically. Nonetheless, given the increase in phospho-TDP-43 in the insoluble fractions by western blotting combined with the increase in cytoplasmic TDP-43 via biochemical fractionation, our data suggest that phospho-TDP-43 is the relevant species accumulating in the cytoplasm of TDP-43KQ/KQ mice.

2.4 Fig 5: Probing the NC fractions for phospho TDP-43 would be an interesting addition to support the conclusion that increased cytoplasmic localization of the KQ mutant occurs prior to its phosphorylation.

We agree that this would be an excellent addition to our data. Unfortunately, after rigorous antibody validation experiments, we were not able to find a phospho-TDP-43 antibody that specifically detected phosphorylated TDP-43 and did not cross-react with unphosphorylated TDP-43 in the buffers used for N-C fractionations. We tested phospho-TDP antibodies in RIPA (soluble), Urea (detergent-insoluble), and the N-C fractionation buffers, using samples treated or untreated with lambda phosphatase (to de-phosphorylate TDP-43). Only one antibody reliably detected the phosphorylated TDP-43 and not the lambda phosphatase-treated TDP-43

samples, and only did so in the Urea buffer, which is shown by straight westerns in our manuscript. Because of these technical difficulties with the phospho-TDP-43 antibodies, this was a challenging point to address at the moment. As better phospho-TDP antibodies become available, we hope to be able to address this. We therefore cannot definitively conclude that cytoplasmic phospho-TDP-43 pathology is present in these mice, but nonetheless the total phospho-TDP-43 levels are significantly elevated in urea (insoluble) fractions.

2.5 Fig 1: What quantitative criteria were used to distinguish between puncta and foci, as highlighted in panel A? What is the biological significance of this distinction? From the images in panel A, it is difficult to see the TDP-43 foci in wt and K145R expressing cells.

Although the size of nuclear TDP-43 foci can be quite variable, and we are certainly interested in the biological significance of this parameter, we did not focus this study on size profiles of K145Q-induced foci, only their accelerated formation and abundance. Therefore, in the revised manuscript we chose not to explicitly state any differences in “foci” vs. “puncta” and now refer to all nuclear TDP-43 structures as “foci” (removed the word “puncta” throughout).

2.6 Fig 3: In describing the results of context-dependent fear testing, it is more appropriate to state that significant deficits appeared at 18 months, deleting the word “more” on line 186.

We have deleted the word “more”.

Reviewer #3

3.1 Multiple figures (1b, 1c, 2b, 2c, 4b, 4d, 4f, 4g, 4i, 4j) include data with multiple measurements per field of view and multiple fields of view per condition. It appears that each measurement was considered an “n” for ANOVA or t-tests, but the data structure violates the requirement that data points are independent. More rigorous statistical methods such as mixed effect models should be considered (see DOI: 10.1016/j.neuron.2021.10.030) which in many cases provide more statistical power. Mixed effects models are the more appropriate statistical method for much of their data. Should the authors want to reanalyze their data with this method, they can reach out to me for an introduction to this statistical model.

We have now re-evaluated the figures mentioned using linear mixed effects models, similar to what the reviewer has mentioned. The new statistical measurements have been incorporated into the revised Figures 1, 2, and 5 (formerly Figure 4). A description of the statistical methods used is now provided in the revised methods section.

3.2 In the introduction, the authors write “we avoid both TDP-43 overexpression and disruption of autoregulatory genomic elements of the endogenous Tardbp transcript” but they show that autoregulation is altered. So shouldn't the acetylation sites be considered a genomic element that regulates autoregulation?

We agree and have now stated that our knock-in approach avoids disrupting surrounding genomic elements (as could occur with transgenic or gene replacement strategies, for example) in order to retain the native Tardbp gene in its unaltered form.

3.3 Suggest editing the language regarding potential neurodegeneration/neuron loss as the same results could be obtained with tissue volume and/or developmental effects independent of progressive neurodegeneration.

See comments 1.6 and 2.3 above. The language has been edited to reflect no apparent neurodegeneration.

3.4 Sequencing the top predicted off-target loci in CRISPR'd mice and iPSC cell lines would help show the absence of off-target mutations.

We described in the methods how potential off-target effects were avoided. We assessed the likelihood of off-target mutations using prediction algorithms to ensure low likelihood. All of the predicted exonic off-target sites have 4 mismatches, making them extremely unlikely to be mutated.

3.5 The authors describe a subtle shift in electrophoretic mobility of the SORT1 protein band in figure 7d/e, but it is unclear why the entire SORT1 band should be shifted up in mutant mice given that the RNA analysis suggests that WT species (not the cryptically spliced +ex17b) is still the major RNA that is expressed. In addition, others have shown that the WT versus +ex17b bands can be resolved (see DOI: 10.1073/pnas.1211577110). Perhaps knockout/knockdown cells can facilitate by providing a positive control for sizing/separation of Sort1 by immunoblotting.

Please refer to our RNA-seq data shown in Figure 8A. In WT mice, nearly 80% of Sort1 transcripts lack exon17b, while this number drops to 23% in the TDP-43KQ/KQ mice. Therefore, the abnormally spliced +ex17b becomes the dominant transcript in TDP-43KQ/KQ mice. Given the prominent +ex17b inclusion that we are observing at the transcript level, it is not surprising that we mostly observe the up-shifted ex17b-containing Sort1 protein band. We have been unable to resolve two distinct bands by immunoblotting in mouse tissues using multiple Sort1 antibodies, including those used in Prudencio et al Proc Natl Acad Sci U S A. 2012 Dec 26;109(52):21510-5. Nonetheless, the up-shifted Sort1 protein is clearly the abnormal variant, as it becomes destabilized in our mice. Another possibility is that partial loss of TDP-43 function, as we suspect occurs in the TDP-43KQ/KQ mice, may magnify (or enhance) the effects on Sort1 such that the dominant Sort1 variant observed is the +ex17b containing variant. We suspect this to be true since this phenomenon was also observed in the Prudencio et al study (see Figures 1-2 in that study).

3.6 The authors may try to corroborate their CFTR splicing results by examining fluorescence as it appears that the construct allows for analysis of splicing differences using GFP vs mCherry expression. This is a minor point as RNA-seq analysis demonstrates abundant splicing changes in acetylation-mimic expression models.

We have now removed the CFTR splicing data entirely and replaced it with more robust readouts of endogenous TDP-43 splicing targets both in vitro (Figure 2E, 3B-I) and in vivo (Figure 8B-C).

3.7 Should the bars in figure 3d for 1 and 2 min be colored in grey/pink? It is unclear why they are clear and only outlined in color.

This point is clarified in the revised Figure 4D legend. In our cue-dependent conditioned fear testing, the filled bars beyond 2 min represents the presence of the auditory cue (tone) and the period of statistical analysis.

3.8 The statistical test used (Fisher's exact test?) for determining overlap between transcriptome datasets should be stated.

We clarified our comment in the results section to reflect the use of over-enrichment analysis. In the methods section, it reads “Previously published differentially expressed genes from Hasan et al⁹⁵ and Polymenidou et al⁹⁶ were retrieved from the respective publications; significant over-enrichments as well as human gene symbol mappings to mouse orthologs were performed using gprofiler2 (g:Orth).”