



Research Article

Open access

TDP-43 Knockdown In Neuronal Vs Astrocytic Cell Lines Reveals Similarities And Differences In the Biological Functions Between These Two Cellular Contexts

Alessandra Longo, Sara Cappelli, Demeke Geremew Debebe, Emanuele Buratti, Francesca Paron

International Centre for Genetic Engineering and Biotechnology, Trieste, Italy

DOI: 10.31383/ga.vol9iss2ga03

Abstract

TAR DNA-binding protein 43 (TDP-43) is a ubiquitously expressed RNA/DNA-binding protein of the heterogeneous nuclear ribonucleoprotein (hnRNP) family, involved in multiple stages of RNA metabolism. Since its identification in 2006 as the principal pathological component of cytoplasmic inclusions in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD), TDP-43 has become central to neurodegeneration research. While ALS was initially considered a motor neuron-specific disorder, mounting evidence implicates non-cell-autonomous mechanisms—particularly involving astrocytes—in disease onset and progression. These glial cells contribute to neurodegeneration via neuroinflammation, metabolic disruption, and excitotoxicity. To investigate the molecular consequences of TDP-43 loss of function, we performed RNA sequencing on human SH-SY5Y neuroblastoma and U87 glioma cell lines following TDP-43 knockdown, simulating neuronal and glial environments. Transcriptomic profiling revealed both shared and cell type-specific changes. Gene Ontology enrichment analysis highlighted dysregulation in pathways related to cell cycle control, DNA replication, and chromatin organization in both lines, suggesting convergent stress responses relevant to ALS pathology. Meanwhile, certain pathways—such as mitotic spindle assembly (SH-SY5Y) and DNA damage response (U87)—were uniquely affected. We identified several commonly deregulated genes with either concordant expression trends or divergent regulation between the two cell types, suggesting shared versus distinct adaptive responses. qRT-PCR validation confirmed the reliability of the transcriptomic data. Notably, many validated targets are involved in RNA processing, cytoskeletal dynamics, synaptic function, and stress responses. Altogether, our findings underscore the critical role of TDP-43 in maintaining transcriptomic stability and support a model where both neuronal and glial dysfunction contribute to ALS pathogenesis via overlapping yet distinct molecular mechanisms.

*Correspondence

E-mail: paron@icgeb.org

Received

August, 2025

Accepted

October, 2025

Published

November, 2025

Copyright: ©2025 Genetics & Applications, The Official Publication of the Institute for Genetic Engineering and Biotechnology, University of Sarajevo

Keywords

TDP-43, Neuronal cells, Glial cells, RNA sequencing, Neurodegeneration

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disorder marked by the selective degeneration of cortical and spinal motor neurons, resulting in muscular weakness, and paralysis failure. It is the third most common neurodegenerative disease in adults, following Alzheimer's disease and Parkinson's disease (Banks et al., 2008). ALS manifests in two main forms: most cases are sporadic (sALS), while only about 10% are familial (fALS), typically inherited in an autosomal dominant manner (Masrori and Van Damme, 2020). Central to ALS pathology is the aberrant behavior of RNA-binding proteins (RBPs), most notably the trans-activating response region (TAR) DNA-binding protein of 43 kDa (TDP-43), a member of the hnRNP family (Neumann et al., 2006).

Originally discovered in 1995 as a transcriptional repressor of HIV-1, TDP-43 was later recognized as a ubiquitous, highly conserved RNA-binding protein with broad functions in RNA metabolism including transcription, alternative splicing, microRNA biogenesis, mRNA transport, stability, and turnover (Buratti and Baralle, 2001; D'Ambrogio et al., 2009). Structurally, TDP-43 follows the typical architecture of hnRNP proteins, with an N-terminal domain (NTD) that mediates its nuclear localization, two RNA-recognition motifs (RRMs) involved in the RNA binding and recognition, and a C-terminal domain (CTD) which serves as a platform for protein-protein interaction, especially with other hnRNPs. This region is also critically involved in TDP-43 aggregation propensity, exhibiting features of a prion-like domain (Fuentelba et al., 2010).

Under pathological conditions, TDP-43 is mislocalized from the nucleus to the cytoplasm,

where it forms hyperphosphorylated and ubiquitinated inclusions (Arai et al., 2006; Neumann et al., 2006). This redistribution is responsible for both toxic gain-of-function and nuclear loss-of-function mechanisms. In the cytoplasm, TDP-43 aggregates may retain RNA-binding capacity, potentially interfering with the autoregulatory feedback, thus reducing the synthesis of newly translated TDP-43 and further exacerbating nuclear depletion (Lee et al., 2012). In parallel, the loss of nuclear TDP-43 disrupts its physiological roles in RNA processing, as cytoplasmic inclusions act as a functional sink, leading to widespread splicing and transcriptional deregulation (Appocher et al., 2017; Barmada and Finkbeiner, 2010; Igaz et al., 2011).

Since these toxic mechanisms are primarily affecting motor neurons, ALS has traditionally been viewed as a neuron-centric disease; however recent findings have significantly expanded our understanding of its pathogenesis, revealing the active contribution of non-neuronal cells—particularly astrocytes—in motor neuron degeneration (Boillée et al., 2006; Ilieva et al., 2009; Pehar et al., 2018). Indeed, astrocytic activation and neuroinflammation are present early and persist throughout disease progression, in both familial and sporadic ALS (Kushner et al., 1991; Nagy et al., 1994; O'Reilly et al., 1995).

Notably, astrocyte cell-autonomous toxicity has been demonstrated in vitro using differentiated induced pluripotent stem cells (iPSCs) derived from ALS patients, exhibiting hallmark pathological features such as cytoplasmic mislocalization and protein aggregation while expressing mutant TDP-43 (Rojas et al., 2023; Serio et al., 2013).

Finally, complementary *in vivo* studies in transgenic rodents have further demonstrated that expression of mutant TDP-43 in astrocytes alone is sufficient to induce motor neuron degeneration and muscle denervation (Tong et al., 2013). Importantly, astrocyte-mediated neurotoxicity is not restricted to familial ALS cases, as similar results have been obtained with astrocytes derived from iPSCs of sporadic ALS patients (Haidet-Phillips et al., 2013; Meyer et al., 2014).

Collectively, these findings clearly suggest that astrocytes are not just passively reacting to damage in degenerating neurons but actively contribute to the process of neurodegeneration (Huang et al., 2014; Yan et al., 2014; Yang et al., 2014).

From a molecular standpoint, astrocytes can contribute to neuronal loss through impaired glutamate uptake and defective alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor editing, leading to excitotoxicity (Rothstein et al., 1995). Additionally, disrupted neuron-astrocyte metabolic coupling, oxidative stress, mitochondrial dysfunction, and the release of proinflammatory mediators may further exacerbate neurodegeneration (Pehar et al., 2018). Abnormal crosstalk with other glial cells, including microglia and oligodendrocytes, can also amplify disease progression by promoting a dysfunctional glial environment (Peng et al., 2020).

Given the complexity of TDP-43 dysfunction across cell types, there is a pressing need to dissect its impact on gene expression in a cell-type-specific context. Next-generation sequencing (NGS) technologies have advanced our ability to capture transcriptomic alterations following RBP perturbation, including TDP-43 (Buratti et al., 2013; Cappelli et al., 2018). However, direct

comparative analyses of TDP-43 depletion in neuronal versus glial models remain limited.

In this study, we sought to explore the transcriptomic consequences of TDP-43 knockdown in two human cell models: SH-SY5Y neuroblastoma cells as a proxy for neuronal populations, and U87 glioma cells as an astrocyte-like glial model. By combining RNA-seq profiling with functional enrichment analysis, we aim to delineate shared and cell-specific transcriptional signatures following TDP-43 silencing, and contribute to a deeper understanding of the dual, neuronal and glial, landscape of TDP-43-linked ALS pathology.

Material and methods

Gene knockdown in SH-SY5Y and U87 MG cell lines

To achieve optimal knockdown efficiency, two rounds of siRNA-mediated silencing were performed on both cell lines using Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturers protocol. 50×10^4 U87 MG cells and 8×10^5 SH-Sy5Y cells were seeded in 35 mm Petri dishes in 1.5 mL of DMEM and DMEM:F12 medium, respectively. After 24 hours, silencing was performed using RNAiMAX reagent following manufacturer's instructions with 80nM of siRNA. A second round of silencing was carried out after 24 hours from the first knockdown, using the same protocol. 24 hours after the last silencing, cells were harvested and prepared for western blot and gene expression analysis.

The sequences of the siRNAs used are listed in Table 1.

Table 1. siRNA sequence from 5'-prime to 3'-prime

Target Gene	siRNA sequence	
Fire-fly luciferase	5'-uaaggcuaugaagagauac[dT][dT] -3'	Sense
(siLUC-control)	5'-guaucucucauagccuua[dT][dT] -3'	Antisense
TDP-43	5'-gcaaagccaagaugagccu[dT][dT]-3'	Sense
(siTDP-43)	5'-aggcucaucuuggcuuugc[dT][dT]-3'	Antisense

RNA-Sequencing and Differential Expression Analysis

Bulk RNA sequencing was performed on total RNA extracted from Fire-fly luciferase (used as negative control) and TDP-43 depleted SH-SY5Y and U87 MG cells. Three independent experiments for each condition. The sequencing was carried out by Novogene (Novogene Co., Ltd, Beijing, China). RNA-seq analysis was performed using Illumina HiSeq NovaSeq 600 instrument. CASAVA base recognition was used to transform the original raw data from Illumina to sequenced reads.

Standard quality control procedures were applied to remove low-quality reads and adapter contamination prior to downstream analysis.

Clean reads were mapped and quantified to the reference transcriptome (GRCh38) by using Salmon tool on the European Galaxy online platform (<https://usegalaxy.eu>).

Differential expression (DE) analysis was performed using 3D RNA-seq App (https://3drnaseq.hutton.ac.uk/app_direct/3DRNaseq/) which provides an interactive graphical user interface for RNA-seq data analysis using accurate quantification of RNA-seq reads based on Limma R package pipeline (Ritchie et al., 2015; Law et al., 2014). To determine differentially expressed

genes (DEGs), the $\log_2$ fold changes (Log_2FC) of gene abundance were calculated based on contrast groups, and significance of expression changes were determined using t-test. Furthermore, p-values of multiple testing were adjusted with Benjamini-Hochberg (BH) procedure to correct false discovery rate (FDR) (Benjamini and Yekutieli, 2001). A gene was significantly differentially expressed (DE) in a contrast group if it had adjusted p-value (padj) < 0.05 and $FC \geq 1.3$ for upregulation or $FC \leq 0.7$ for downregulation. Comparative analysis was performed on the results obtained from 3D RNA-seq App using R language through the interface RStudio (RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL <http://www.rstudio.com/>). Data visualization was performed using ggplot2 for volcano plots and clusterProfiler (v4.10.0) for Gene Set enrichment analysis (GSEA) and Gene Ontology (GO) enrichment analysis (Yu et al., 2012).

Western Blot Assay

For the protein analysis, cell pellets were resuspended with 1X RIPA buffer (Cell Signaling Technology, Danvers, Massachusetts, USA), supplemented with 1X of Protease Inhibitor (Roche, Basel, Switzerland). The mixture was incubated on ice for 30 minutes, followed by a centrifugation for 4 minutes at top speed to

Table 2. Primary and secondary antibodies for Western Blot assay

Primary Antibody	Host	Dilution	Media	Company
α-TDP-43	Rabbit	1:1000	PURITY Western Blot Solution (with α -rabbit-HRP diluted 1:2000)	Proteintech EU (Manchester, UK)
			Proteintech EU	
α-Tubulin	Mouse	1:10000	PURITY Western Blot Solution (with α -mouse-HRP diluted 1:2000)	Homemade

separate cellular debris, and leaving protein in the supernatant. The protein lysate concentration was measured using Protein assay Day Reagent Concentrated solution (Biorad, Hercules, California, USA) following manufacturer's instructions, and samples absorbance was read at 595 nm with D30 Eppendorf BioSpectrometre (Eppendorf, Hamburg, Germany). 20 g of whole-cell lysate was mixed with 4X NuPAGE LDS Sample Buffer (ThermoFisher Scientific, Waltham, Massachusetts, USA), and it was denaturated at 85°C for 5 minutes, cooled for 1 minute on ice and spin-downed before loading into SDS-PAGE gel. SDS-PAGE was performed using 10% NuPAGE Bis-Tris pre-cast Protein Gel (ThermoFisher Scientific, Waltham, Massachusetts, USA), and samples were separated with 1X NuPAGE MOPS SDS Running Buffer (20X) (ThermoFisher Scientific, Waltham, Massachusetts, USA) at 200V for 0.1A. Power Blotter-Semi-Dry Transfer System (ThermoFisher Scientific, Waltham, Massachusetts, USA). The Power Blotter Nitrocellulose Pre-cut Membranes and Filters, (ThermoFisher Scientific, Waltham, Massachusetts, USA) were used to transfer the protein from the gel to the membrane. A pre-programmed method of 15 minutes (25V, 1.3A)

for 1.5mm gels was chosen to blot the proteins. To check if the transfer was properly performed, the nitrocellulose membrane was treated with Pierce Reversible Protein Stain Kit for Nitrocellulose Membranes (ThermoFisher Scientific, Waltham, Massachusetts, USA) and the image was acquired with Alliance 9.7 Western Blot Imaging System (UVItect Limited, Cambridge, UK).

The membrane was then washed three times for 5 minutes in shaking with PURITY Washing Buffer (Vilber Lourmat Deutschland GmbH, Germany), successively it was incubated overnight at 4° C with primary antibody diluted in PURITY Western Blot Solution (Vilber Lourmat Deutschland GmbH, Germany). After antibody incubation, three washing steps were performed before protein detection with ECL Luminata Classico Western HRP substrate (Merck Millipore, Burlington, MA, USA) or SuperSignal West Femto, Trial Kit (Thermo Fisher Scientific).

Images were acquired using Alliance 9.7 Western Blot Imaging System (UVItect Limited, Cambridge, UK) and quantification was carried out with system's software. The antibodies used are reported in Table 2.

Real-Time quantitative PCR analysis

RNA extraction was performed by using the RNAeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. 500 nanograms of total RNA was transcribed into complementary DNA (cDNA) using random primers (Sigma-Aldrich, St. Louis, Missouri, USA) and Moloney Murine Leukemia Virus

Reverse Transcriptase (M-MLV RT) (Invitrogen, Carlsbad, California, USA). Reaction was performed at 37°C for 1-2 hours. The cDNA was subsequently diluted 1:5 for quantitative PCR analysis. For U87 MG cell line RPL32 and RPS23 were employed as the housekeeping genes while RPII and GAPDH were chosen for SH-SY5Y samples. Primer sequences are reported in Table 3.

Table 3. Quantitative Real Time PCR primers sequences

Name	Sequence 5'→3'	Housekeeping genes
RPL32 forward	5'-caccagtcagaccgatgtcaaaa-3'	
RPL32 reverse	5'-tgtgtgcaatgcctctgggtt-3'	
RPS23 forward	5'-ggcgcttctcatgcaaaagga-3'	
RPS23 reverse	5'-gcaaccgtcattgggtacaaa-3'	
RPII forward	5'-gcaccacgtccaatgacat-3'	
RPII reverse	5'-gtgcggctgctccataa-3'	
GAPDH forward	5'-cctgttcgacagtcagccg-3'	
GAPDH reverse	5'-cgaccaaaccgttgactcc-3'	
		Target genes
UBE2E3 forward	5'-tggagtgccgcttgactatt-3'	
UBE2E3 reverse	5'-atcctgtcgtgttctgctct-3'	
LIMS1 forward	5'-tggccgagttatcaagccat-3'	
LIMS1 reverse	5'-ctttctcacgattatgacagggg-3'	
CYP2E1 forward	5'-atgtctgccctcgagtgca-3'	
CYP2E1 reverse	5'-cgatgatgggaagcgggaaa-3'	
LAMC1 forward	5'-actgccactgacatcagagta-3'	
LAMC1 reverse	5'-gcttgctgtccattacattac-3'	
ADD2 forward	5'-ccatgatgacgctatcaatga-3'	
ADD2 reverse	5'-gtccaggagtcggtagacac-3'	
NRG2 forward	5'-acagcgggaagcagatgcac-3'	

NRG2 reverse	5'-gtttctctcctgatgacatggtc-3'
SERGAP3 forward	5'-tggattccccgaagtgacaagc-3'
SERGAP3 reverse	5'-ctggtgataacgcatgagcag-3'
FOXN3 forward	5'-ctgcctgacatccgattagaaga-3'
FOXN3 reverse	5'-atcgtagggcagtcagagt-3'
CDKL5 forward	5'-gtcggaggggaaagtgtact-3'
CDKL5 reverse	5'-atggcaccagtgaatagcct-3'
TDP-43 forward	5'-atctgggtgatgtgtcaactatcc -3'
TDP-43 reverse	5'-gaacttctccaaagtactaaaatactc-3'
KCNJ2 forward	5'-gtgcgaaccaaccgctaca-3'
KCNJ2 reverse	5'-ccagcgaatgtccacacac-3'
EGR2 forward	5'-tcttccaatgatcccagact-3'
EGR2 reverse	5'-ttacggattgtagagatggagt-3'
TLE2 forward	5'-gaaagcgacgaagacaagagtg-3'
TLE2 reverse	5'-aggatgagctccttggtct-3'
LHX9 forward	5'-tacttcaacggtacgggcac-3'
LHX9 reverse	5'-tcttctgcgagggtggataag-3'
NOVA2 forward	5'-aaggcgaatacttctgaaggt-3'
NOVA2 reverse	5'-tactaggcataccgctctgt-3'
EPHB3 forward	5'-atggaaaactgcgcctgg-3'
EPHB3 reverse	5'-gttgacaaggatgttcgagc-3'
HDAC9 forward	5'-agtagagaggcatcgagaga-3'
HDAC9 reverse	5'-gtgtaccagagctgggatgg-3'

Gene expression quantification was performed by quantitative Real-time PCR using PowerUp SYBR Green Master Mix (Applied biosystems, Foster City, California, USA) and QuantStudio5 instrument (Applied biosystems, Foster City, California, USA). Results were analysed using QuantStudio Design & Analysis software and the mean of relative expression levels $\pm$ standard error

of the mean (SEM) is reported in the corresponding figures ($n = 3$ independent experiments). For each sample, three technical qPCR replicates were considered. A nonparametric un-paired *t*-test was applied as statistical test (GraphPad Prism software, v10, La Jolla, California, USA). Statistical significance was displayed as $*P < 0.05$, $**P < 0.01$ and $****P < 0.0001$.

Results and Discussion

Differentially expression (DE) and differentially alternative splicing (DAS) analysis

We primarily verified the efficiency of TDP-43 knockdown in the samples used for RNA sequencing by assessing both protein and mRNA levels in SH-SY5Y (Figure 1A-B) and U87 MG cells (Figure 1C-D).

A general overview of RNA-seq data was obtained by performing a Gene Set Enrichment Analysis (GSEA), having an insight into the statistically

significant differences in SH-SY5Y and U87 microglia (MG) cells following TDP-43 knockdown (Figure 2A-B). Pathways related to DNA replication, chromosome segregation and cell cycle checkpoints were found to be enriched in both cell lines upon TDP-43 depletion. Looking at the suppressed categories, there were some differences: in U87 microglia, genes linked to Golgi apparatus compartmentalization as well as vesicular membranes (e.g. lysosomal and vacuolar) composition and transport were mostly affected (Figure 2A); while in SH-SY5Y, pre- and postsynaptic structural organization dedicated

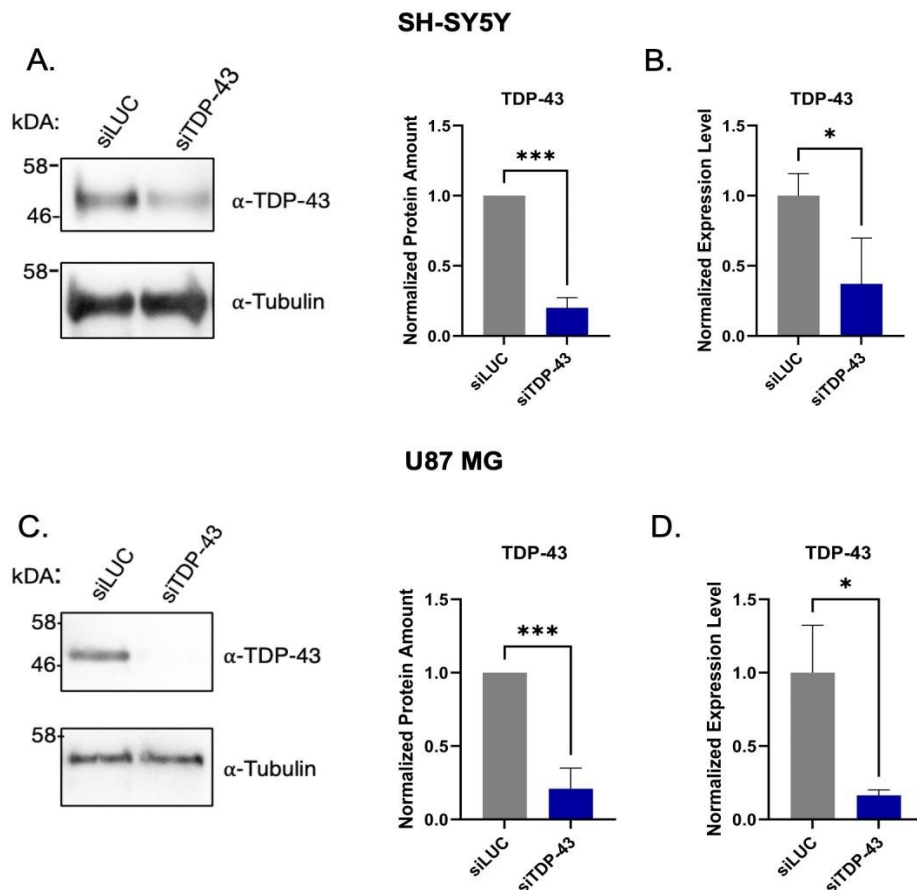


Figure 1. TDP-43 knockdown in SH-SY5Y and U87 MG cells. A-C) Western blot and densitometric quantification of TDP-43 protein levels in both cell lines (n=3). Tubulin was used as loading control. Statistical analysis was carried out by an unpaired t-test analysis using GraphPad software (GraphPad Software, La Jolla, California, USA).

pathways and, more in general, genes associated to proper synaptic signaling and modulation were significantly suppressed (Figure 2B).

Differential expression analysis identified 1038 genes to be differentially expressed in TDP-43 depleted SH-SY5Y vs control, and 16 of them were also differentially alternatively spliced (Figure 3A). Concerning the U87 lineage (Figure 3B), 7168 genes were found to be differentially expressed in TDP-43 depleted U87 MG vs control, out of 15050 total analyzed genes. Among these DEGs, 564 being also differentially alternatively spliced.

Given the exploratory nature of this study, we applied a less stringent fold change threshold, considering genes with a fold change ≥ 1.3 or ≤ 0.7 as differentially expressed. This approach allowed us to capture a broader set of transcriptional changes, including modest but

potentially biologically relevant variations, enabling a more comprehensive comparison between the two cellular contexts and paving the way for subsequent refinement and experimental validation.

The analysis revealed a markedly higher number of affected genes in U87 MG cells compared to SH-SY5Y (7168 vs. 1038), a trend also observed for alternative splicing events. This striking difference may point to a greater transcriptional sensitivity of glial cells to TDP-43 loss. On one hand, glial cells act as active sensors and responders to cellular stress, often engaging broader compensatory programs. On the other hand, their homeostatic functions may render them particularly vulnerable to disruptions in RNA processing. These observations support the growing view that glial dysfunction can actively drive neurodegeneration through non-cell-autonomous mechanisms.

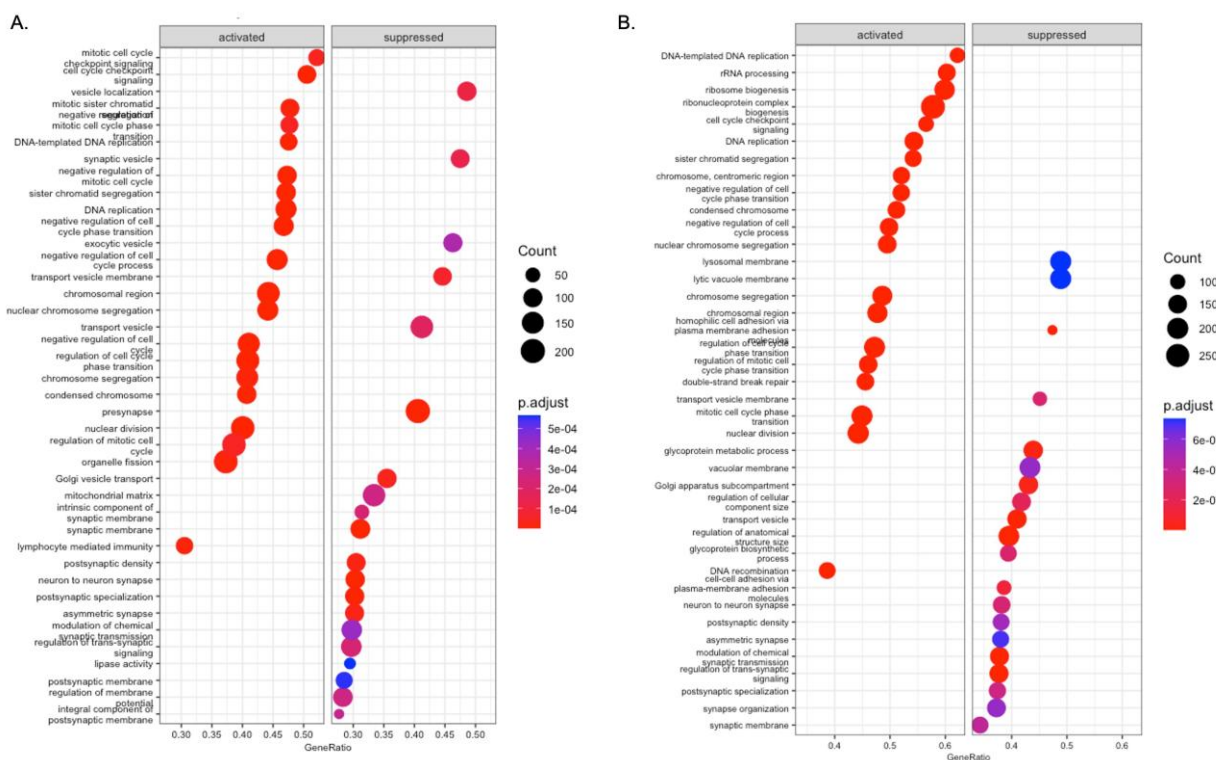


Figure 2. Gene Set Enrichment Analysis conducted on SH-SY5Y (A) and U87 MG (B) raw data

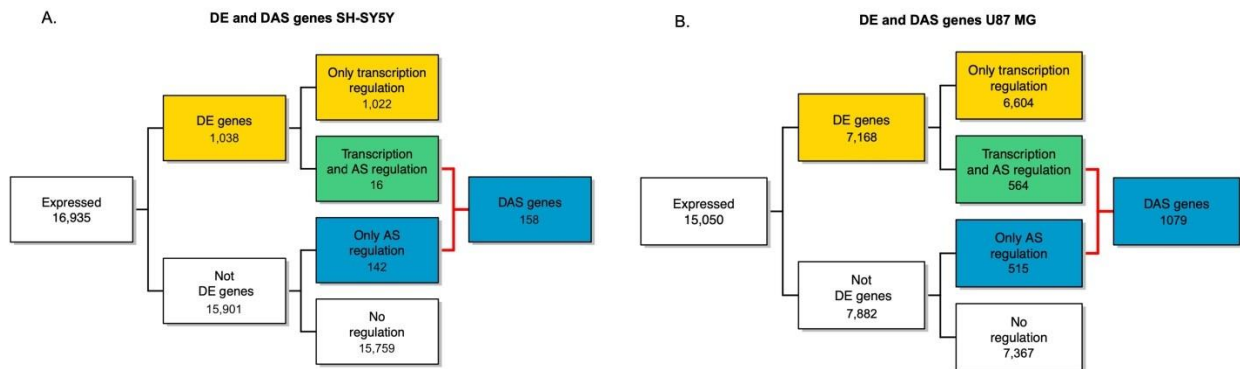


Figure 3. Summary diagrams: numbers of genes regulated only by transcription (DE), only by alternative splicing (DAS) and by both transcription and alternative splicing (DE+DAS) in SH-SY5Y (A) and U87 MG (B) cell lines

Comparative analysis of differentially expressed genes (DEGs) in SH-SY5Y and U87 cells

To identify shared and cell type-specific transcriptional responses to TDP-43 knockdown, we performed a comparative analysis of differentially expressed genes (DEGs) between SH-SY5Y and U87 MG cells. A total of 306 DEGs were uniquely detected in the neuronal-like SH-SY5Y cells (137 upregulated, 169 downregulated) (Figure 4A), while 6436 DEGs were specific to the glia-like U87 MG cells (3512 upregulated, 2924 downregulated) (Figure 4B). Cross-comparison revealed 732 DEGs commonly regulated in both cell lines, including 404 upregulated and 328 downregulated genes (Figure 4C). The DEG profiles were visualized using volcano plots, highlighting the most significantly up- and downregulated hits and these are reported in Figure 4.

We performed a comparative Gene Ontology (GO) enrichment analysis to have a look at the cellular pathways that were mostly affected by TDP-43 depletion. Over-representation analysis revealed both shared and cell type-specific functional

alterations (Figure 5). In particular, SH-SY5Y cells exhibited specific enrichment in processes related to mitotic spindle assembly and orientation, implicating alterations in cell division dynamics and polarity regulation (Figure 5A), while U87 MG cells showed enrichment in categories associated with DNA damage response, RNA localization, nuclear envelope organization, and protein kinase activity (Figure 5B). For what concerns the commonly enriched pathways, the most represented were related to DNA replication and cell cycle progression (Figure 5C).

Common target characterization

From the bioinformatic analysis, we then proceeded with the experimental validation of the main hits. We focused our attention on specific genes selected among the ones found to be misregulated in both SH-SY5Y and U87 MG cells because they presented the highest fold difference detected in our analyses. From the list of DEGs shared between the two cell lines, we prioritized candidate genes based on the following criteria: (i) fold change $FC > 1.3$ for upregulated genes, (ii) $FC < 0.7$ for downregulated genes, (iii) consistent

direction of regulation across both cell lines, (iv) established association with brain function or neurodegenerative processes, and (v) presence of predicted TDP-43 binding sites. The latter was evaluated based on publicly available CLIP-seq data (Tollervey et al., 2011, data not shown). This filtering led to the selection of nine candidate genes, four upregulated and five downregulated, summarized in Figure 6. Additionally, we identified seven DEGs that displayed opposite regulation trend between the two cell lines, suggesting divergent, cell-type-specific effects of TDP-43 depletion (Figure 6). Quantitative real-time PCR analysis showed consistent expression changes for the majority of the selected target genes, thus validating the RNA-seq results. Specifically, upregulation of UBE2E3, LIMS1, and LAMC1 mRNAs following TDP-43 knockdown was confirmed in both SH-SY5Y and U87 MG cell lines. Although CYP2E1 expression

in U87 MG cells did not reach statistical significance ($p = 0.05$), it still displayed the same upregulation trend observed in the transcriptomic data. Similarly, the downregulation of ADD2, NRG2, SRGAP3, FOXN3, and CDKL5 was consistently validated in both models (Figure 7). In line with these results, we also investigated the subset of seven genes that showed opposite dysregulation patterns between the two cell lines. In SH-SY5Y cells, a significant difference was confirmed for all identified targets, except for TLE2 and KCNJ2, which, despite not reaching statistical significance, followed the expected trend (i.e., KCNJ2 upregulated and TLE2 downregulated). In U87 MG cells, TLE2 expression showed a non-significant increase, together with EPHB3, while EGR2 exhibited only a minimal downregulation (Figure 8). Taken together, these findings support the robustness of our RNA-seq data and underscore the existence of

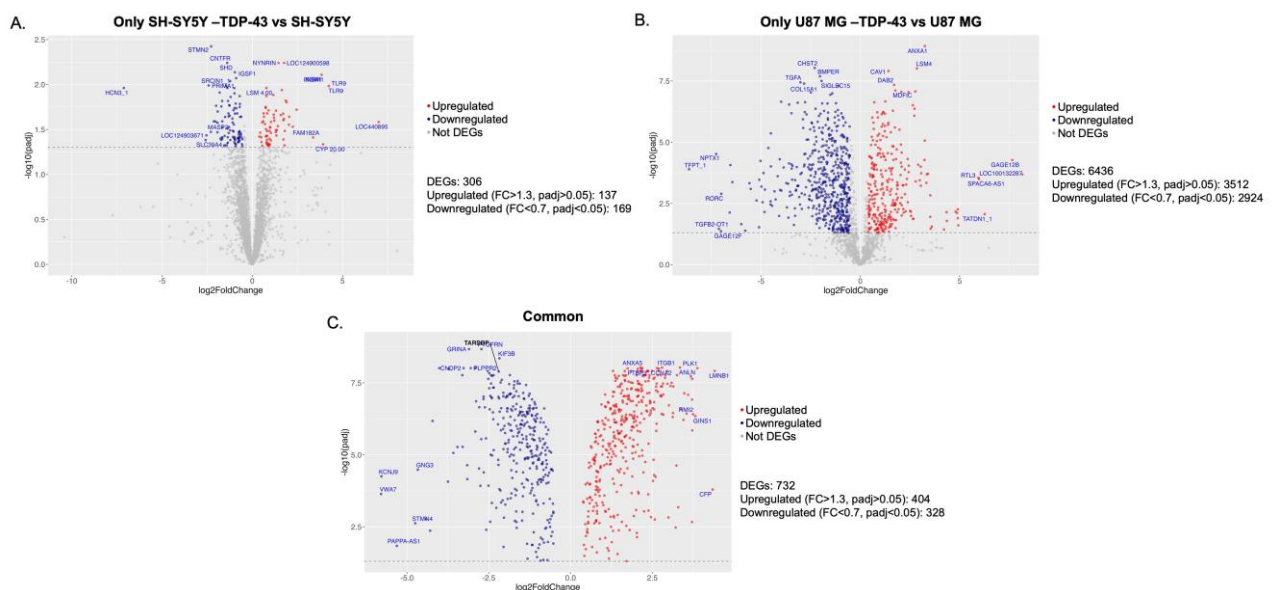


Figure 4. Volcano plots of differentially expressed genes (DEGs) exclusively found in SH-SY5Y (A), U87 MG (B), and shared between the two cell lines (C). Volcano plot reports in x-axis the magnitude (Log2FoldChange) and in y-axis the significance ($-\log_{10}(padj)$) of siLUC versus siTDP-43 treated cells. Upregulated and downregulated genes are highlighted in red and blue, respectively. The horizontal dashed line signals statistical significance threshold ($P = 0.05$). Not DEGs are represented as grey dots. In light blue are reported the items that change the most

distinct, cell-type-specific transcriptional responses to TDP-43 depletion. Starting from a global perspective, Gene Ontology and Gene Set Enrichment Analyses revealed that TDP-43 depletion consistently impacts key biological pathways in both contexts, particularly those related to cell cycle regulation, DNA replication, and chromatin organization. This suggests a common molecular vulnerability that may be relevant to neurodegenerative processes. From a neurodegenerative standpoint, cell cycle dysregulation could be particularly relevant, as neurons undergoing degeneration have been reported to re-enter the cell cycle, ultimately triggering apoptotic pathways rather than successful cell division, as recently reviewed by Campos-Melo and colleagues (Koseoglu et al., 2019). In astrocytes, aberrant cell cycle progression in response to TDP-43 depletion could likewise disrupt their normal supportive functions and alter neuron–glia homeostasis (Sofroniew and Vinters, 2010).

Consistent with the enrichment of pathways related to cell cycle control, several differentially expressed genes — including CDK2, CDC6, CDC25C, and HDAC1 — emerged as key contributors to these signatures (data not shown). Although not examined in depth here, these findings align with previous reports showing that the loss TDP-43 or mutation at its phosphorylation sites lead to replication stress and DNA damage accumulation, ultimately impairing genome maintenance and activating cell cycle checkpoints (Wood et al., 2020, Paron et al., 2022).

Moving from pathway-level analysis to individual genes, we identified a subset of differentially expressed genes commonly deregulated in both cell lines. This group includes genes with roles in extracellular matrix composition, cytoskeletal dynamics, oxidative stress response, and transcriptional control. For instance, LAMC1, encoding the laminin γ 1 chain, is a critical component of the astrocyte-derived basement

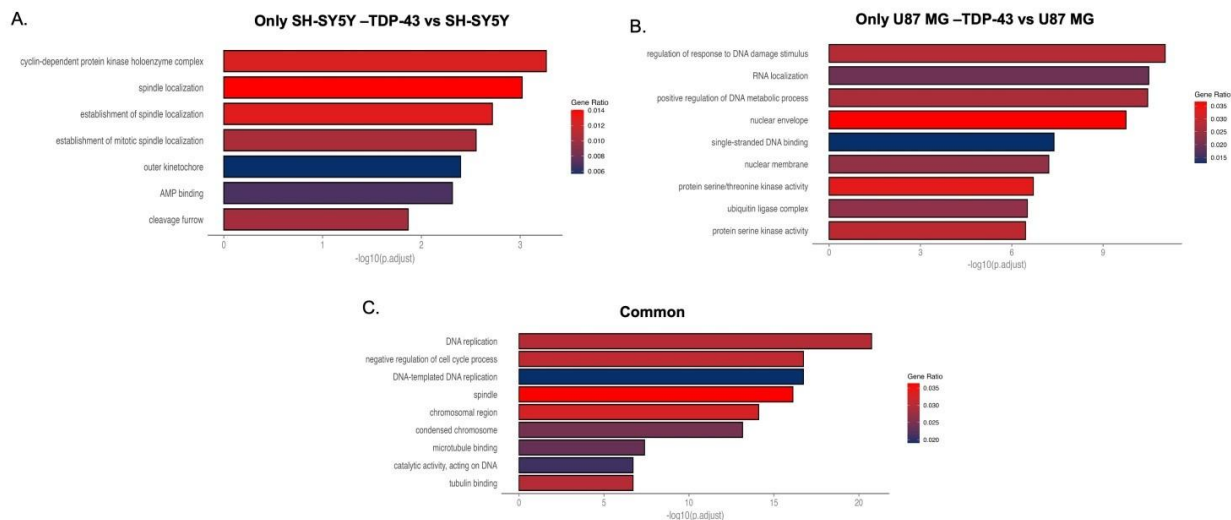


Figure 5. GO Enrichment Analysis of exclusive differentially expressed genes found in SH-SY5Y (A), U87 MG (B) and shared between the two cell lines (C). Top categories significantly enriched ($p_{adj} < 0.05$) are considered

membrane and has been associated with blood-brain barrier (BBB) disruption in ALS models (Nguyen et al., 2021). Its upregulation in both systems may reflect astrocytic reactivity or maladaptive extracellular matrix remodeling linked to neuroinflammation.

UBE2E3, an E2 ubiquitin-conjugating enzyme, has been previously implicated in regulating the solubility and turnover of TDP-43 itself (Hans et al., 2014; Plafker and Plafker, 2015). Its dysregulation may contribute to a feedback disruption in the ubiquitin-proteasome system, further compromising protein quality control and possibly promoting TDP-43 aggregation.

Among the downregulated genes, ADD2, a membrane protein highly expressed at synaptic sites and known to be stabilized by TDP-43, could reflect impaired maintenance of synaptic integrity

(Costessi et al., 2014). Similarly, the repression of NRG2, a glia-secreted neuregulin supporting neuronal survival and plasticity, could point toward diminished neuroglial support mechanisms in response to TDP-43 loss (Ou et al., 2021).

Of particular interest are also those genes that exhibited an opposite expression trend between the two cell lines, potentially reflecting cell-type-specific regulatory mechanisms in response to TDP-43 loss. Among them, KCNJ2, encoding an inward-rectifying potassium channel, also showed an opposite regulation. Recent studies using human cortical organoids and TBI models identified KCNJ2 as a key modulator of TDP-43-associated neurodegeneration, with its inhibition shown to mitigate injury-induced neuronal death, likely through modulation of cytosolic calcium levels and downstream stress responses (Lai et al.,

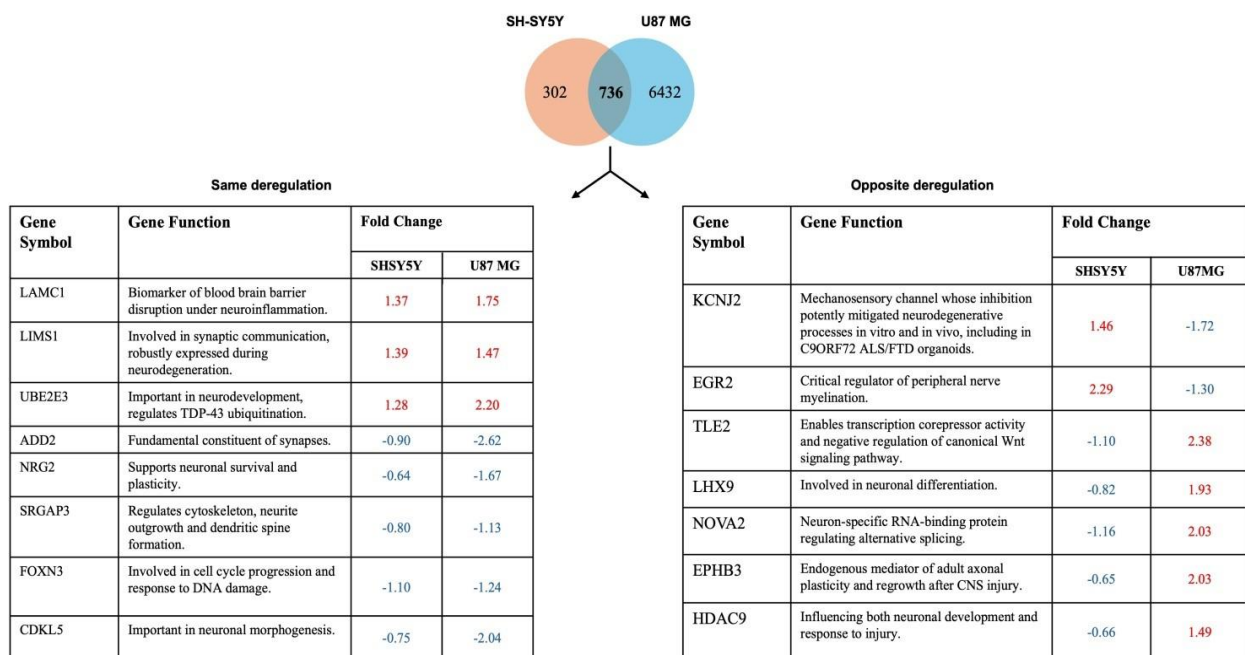


Figure 6. Common differentially expressed genes following TDP-43 knockdown in neuronal and glial cells. Venn diagram showing the overlap of differentially expressed genes (DEGs) identified in SH-SY5Y and U87 MG cell lines upon TDP-43 depletion, revealing 736 genes commonly affected. The tables highlight selected hits among these shared DEGs, categorized based on consistent (left) or opposite (right) expression trends between the two cell types. Gene functions are annotated based on established roles in neurodevelopment, synaptic function, and transcriptional regulation. Fold change values are reported for each cell line

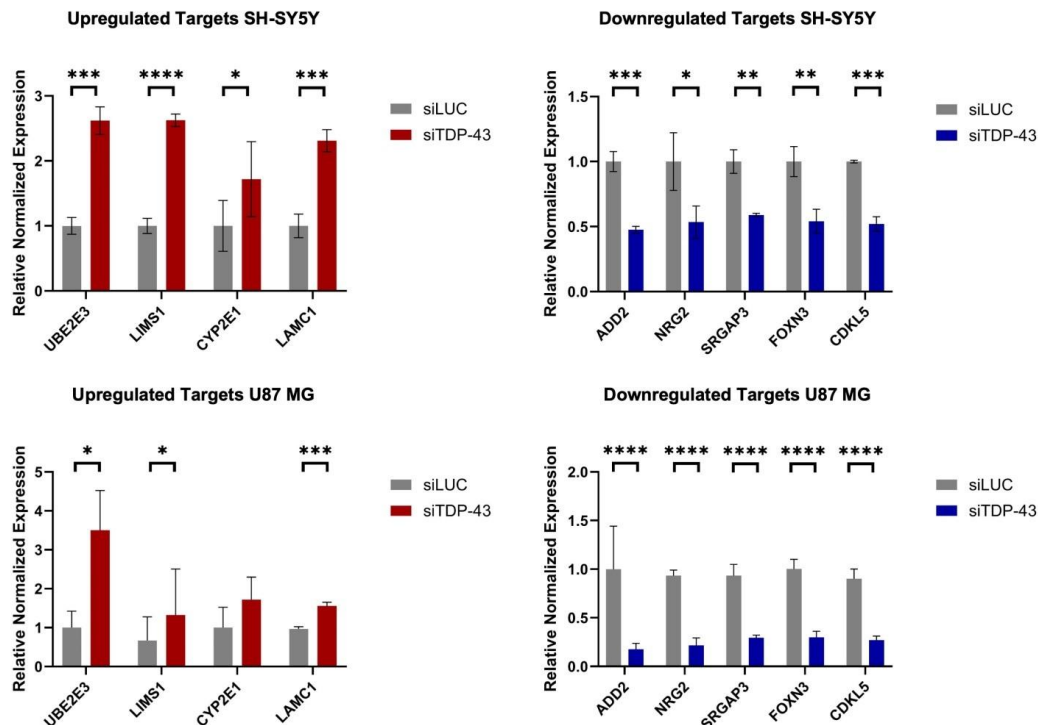


Figure 7. RNA sequencing validation qPCR on selected genes in SH-SY5Y and U87 MG cell lines upon TDP-43 depletion. Validation analysis was performed on commonly downregulated and upregulated genes (n=3). Statistical analysis was carried out by using an unpaired t-test with the GraphPad software (GraphPad Software, La Jolla California, USA)

2024). NOVA2, a neuron-specific RNA-binding protein and key regulator of alternative splicing, also showed discordant regulation, which is particularly intriguing given its overlapping function with TDP-43 in RNA processing (Saito et al., 2016). Its altered expression could amplify splicing disturbances in a cell-type-dependent manner. HDAC9 emerged with divergent regulation, adding to the growing evidence implicating histone deacetylases in ALS pathology. The HDAC family has already been linked to neurodegenerative processes: HDAC6 and HDAC1 regulate TDP-43 deacetylation while HDAC4 deficiency in neurons leads to motor coordination deficits (Boutillier et al., 2019; Sanna et al., 2020). Given this background, HDAC9 could represent a compelling candidate for further

investigation, both as a potential disease modifier and as a therapeutic target.

Of note, while SH-SY5Y and U87 MG cell lines are widely adopted as neuronal- and glial-like models, it is important to acknowledge their inherent limitations. Both are tumor-derived and only partially reproduce the properties of mature neurons and astrocytes. Nonetheless, both models have been extensively used to study TDP-43 biology and successfully recapitulate key disease-associated phenotypes — such as cytoplasmic mislocalization, aggregation, DNA damage, and cellular stress responses — supporting their suitability for exploratory molecular studies (Lee et al., 2021; Nonaka et al., 2009). Future work should aim to validate these findings in more physiologically relevant systems, such as iPSC-

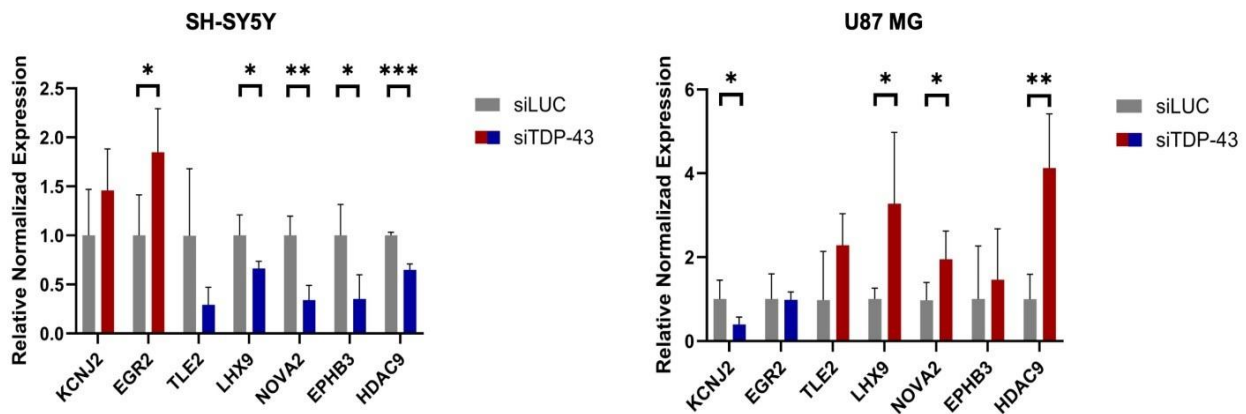


Figure 8. RNA sequencing validation qPCR on selected genes in SH-SY5Y and U87 MG cell lines upon TDP-43 depletion. Validation analysis was performed on commonly downregulated and upregulated genes (n=3). Statistical analysis was carried out by using an unpaired t-test with the GraphPad software (GraphPad Software, La Jolla California, USA)

derived motor neurons and astrocytes, to better dissect the cell-autonomous and non-cell-autonomous effects of TDP-43 dysfunction.

Conclusion

In this study, we performed transcriptomic profiling and functional enrichment analysis to investigate the effects of TDP-43 knockdown in human neuronal- (SH-SY5Y) and glial- (U87 MG) like cell lines.

Our findings highlight the complexity of TDP-43-mediated gene expression changes and identify a set of candidate targets potentially involved in ALS-related mechanisms. Looking forward, an important step will be to directly confirm the interaction of TDP-43 with the identified transcripts through RNA immunoprecipitation (RIP) assays, to distinguish direct from indirect targets and validate the functional binding. In parallel, it will be crucial to explore the cell-type-specific transcriptional signatures more deeply, as they may reveal distinct pathogenic pathways or compensatory responses activated in neurons versus glia. Finally, given the well-established role

of TDP-43 in RNA splicing regulation, a focused analysis of differential splicing events — particularly cryptic exon inclusion — could provide additional mechanistic insight into how TDP-43 loss shapes cellular vulnerability. By integrating these approaches, it may be possible to elucidate not only the neuronal consequences of TDP-43 dysfunction, but also the contribution of glial cells to ALS pathogenesis — a dimension still underexplored but potentially key to fully understanding disease progression and heterogeneity.

Authors' contributions

A. Longo: Investigation; Methodology; Formal Analysis; Writing-original draft; Writing-editing.

S. Cappelli: Conceptualization; Methodology; Data-curation.

D. Geremew Debebe: Resources.

E. Buratti: Supervision; Conceptualization.

F. Paron: Investigation; Writing review & editing.

Conflict of interest

The authors have no conflicts of interest to disclose.

References

- Appocher C, Mohagheghi F, Cappelli S, Stuani C, Romano M, Feiguin F, Buratti E (2017) Major hnRNP proteins act as general TDP-43 functional modifiers both in *Drosophila* and human neuronal cells. *Nucleic Acids Research*, 45: 8026–8045.
- Arai T, Hasegawa M, Akiyama H, Ikeda K, Nonaka T, Mori H, Mann D, Tsuchiya K, Yoshida M, Hashizume Y, Oda T (2006) TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochemical and Biophysical Research Communications*, 351: 602–611.
- Banks GT, Kuta A, Isaacs AM, Fisher EMC (2008) TDP-43 is a culprit in human neurodegeneration, and not just an innocent bystander. *Mammalian Genome*, 19: 299–305.
- Barmada SJ, Finkbeiner S (2010) Pathogenic TARDBP mutations in amyotrophic lateral sclerosis and frontotemporal dementia: Disease-associated pathways. *Reviews in the Neurosciences*, 21: 251–272.
- Boillée S, Yamanaka K, Lobsiger CS, Copeland NG, Jenkins NA, Kassiotis G, Kollias G, Cleveland DW (2006) Onset and progression in inherited ALS determined by motor neurons and microglia. *Science*, 312: 1389–1392.
- Buratti E, Baralle FE (2001) Characterization and functional implications of the RNA binding properties of nuclear factor TDP-43, a novel splicing regulator of CFTR exon 9. *Journal of Biological Chemistry*, 276: 36337–36343.
- Buratti E, Romano M, Baralle FE (2013) TDP-43 high throughput screening analyses in neurodegeneration: Advantages and pitfalls. *Molecular and Cellular Neuroscience*, 56: 465–474.
- Cappelli S, Romano M, Buratti E (2018) Systematic analysis of gene expression profiles controlled by hnRNP Q and hnRNP R, two closely related human RNA binding proteins implicated in mRNA processing mechanisms. *Frontiers in Molecular Biosciences*, 5: 79.
- D'Ambrogio A, Buratti E, Stuani C, Guarnaccia C, Romano M, Ayala YM, Baralle FE (2009) Functional mapping of the interaction between TDP-43 and hnRNP A2 in vivo. *Nucleic Acids Research*, 37: 4116–4126.
- Fuentealba RA, Udan M, Bell S, Wegorzewska I, Shao J, Diamond MI, Weihl CC, Baloh RH (2010) Interaction with polyglutamine aggregates reveals a Q/N-rich domain in TDP-43. *Journal of Biological Chemistry*, 285: 26304–26314.
- Haidet-Phillips AM, Gross SK, Williams T, Tuteja A, Sherman A, Ko M, Jeong YH, Wong PC, Maragakis NJ (2013) Altered astrocytic expression of TDP-43 does not influence motor neuron survival. *Experimental Neurology*, 250: 250–259.
- Huang C, Huang B, Bi F, Yan LH, Tong J, Huang J, Xia XG, Zhou H (2014) Profiling the genes affected by pathogenic TDP-43 in astrocytes. *Journal of Neurochemistry*, 129: 932–939.
- Igaz LM, Kwong LK, Lee EB, Chen-Plotkin A, Swanson E, Unger T, Malunda J, Xu Y, Winton MJ, Trojanowski JQ, Lee VM-Y (2011) Dysregulation of the ALS-associated gene TDP-43 leads to neuronal death and degeneration in mice. *Journal of Clinical Investigation*, 121: 726–738.
- Ilieva H, Polymenidou M, Cleveland DW (2009) Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. *Journal of Cell Biology*, 187: 761–772.
- Kushner PD, Stephenson DT, Wright S (1991) Reactive astrogliosis is widespread in the subcortical white matter of amyotrophic lateral sclerosis brain. *Journal of Neuropathology and Experimental Neurology*, 50: 263–277.
- Lee EB, Lee VM-Y, Trojanowski JQ (2012) Gains or losses: molecular mechanisms of TDP-43-mediated neurodegeneration. *Nature Reviews Neuroscience*, 13: 38–50.

- Lee Y-B, Scotter EL, Lee D-Y, Troakes C, Mitchell J, Rogelj B, Gallo J-M, Shaw CE (2021) Cytoplasmic TDP-43 is involved in cell fate during stress recovery. *Human Molecular Genetics*, 31: 166–175.
- Masrori P, Van Damme P (2020) Amyotrophic lateral sclerosis: a clinical review. *European Journal of Neurology*, 27: 1918–1929.
- Meyer K, Ferraiuolo L, Miranda CJ, Likhite S, McElroy S, Renusch S, Ditsworth D, Lagier-Tourenne C, Smith RA, Ravits J, Burghes AH, Shaw PJ, Cleveland DW, Kolb SJ, Kaspar BK (2014) Direct conversion of patient fibroblasts demonstrates non-cell autonomous toxicity of astrocytes to motor neurons in familial and sporadic ALS. *Proceedings of the National Academy of Sciences of the United States of America*, 111: 829–832.
- Nagy D, Kato T, Kushner PD (1994) Reactive astrocytes are widespread in the cortical gray matter of amyotrophic lateral sclerosis. *Journal of Neuroscience Research*, 38: 336–347.
- Neumann M, Sampathu DM, Kwong LK, Truax AC, Micsenyi MC, Chou TT, Bruce J, Schuck T, Grossman M, Clark CM, McCluskey LF, Miller BL, Masliah E, Mackenzie IR, Feldman H, Feiden W, Kretzschmar HA, Trojanowski JQ, Lee VM-Y (2006) Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science*, 314: 130–133.
- Nonaka T, Arai T, Buratti E, Baralle FE, Akiyama H, Hasegawa M (2009) Phosphorylated and ubiquitinated TDP-43 pathological inclusions in ALS and FTLD-U are recapitulated in SH-SY5Y cells. *FEBS Letters*, 583: 394–400.
- O'Reilly SA, Roedica J, Nagy D, Hallelwell RA, Alderson K, Marklund SL, Kuby J, Kushner PD (1995) Motor neuron-astrocyte interactions and levels of Cu,Zn superoxide dismutase in sporadic amyotrophic lateral sclerosis. *Experimental Neurology*, 131: 203–210.
- Pehar M, Harlan BA, Killoy KM, Vargas MR (2018) Role and therapeutic potential of astrocytes in amyotrophic lateral sclerosis. *Current Pharmaceutical Design*, 23: 1–10.
- Peng AYT, Agrawal I, Ho WY, Yen Y-C, Pinter AJ, Liu J, Phua QXC, Koh KB, Chang J-C, Sanford E, Man JHK, Wong P, Gutmann DH, Tucker-Kellogg G, Ling S-C (2020) Loss of TDP-43 in astrocytes leads to motor deficits by triggering A1-like reactive phenotype and triglial dysfunction. *Proceedings of the National Academy of Sciences*, 117: 29101–29112.
- Rojas F, Aguilar R, Almeida S, Fritz E, Corvalán D, Ampuero E, Abarzúa S, Garcés P, Amaro A, Diaz I, Arredondo C, Cortes N, Sanchez M, Mercado C, Varela-Nallar L, Gao F-B, Montecino M, van Zundert B (2023) Mature iPSC-derived astrocytes of an ALS/FTD patient carrying the TDP43A90V mutation display a mild reactive state and release polyP toxic to motoneurons. *Frontiers in Cell and Developmental Biology*, 11: 1226604.
- Rothstein JD, Van Kammen M, Levey AI, Martin LJ, Kuncel RW (1995) Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. *Annals of Neurology*, 38: 73–84.
- Serio A, Bilican B, Barnada SJ, Ando DM, Zhao C, Siller R, Burr K, Haghi G, Story D, Nishimura AL, Carrasco MA, Phatnani HP, Shum C, Wilmut I, Maniatis T, Shaw CE, Finkbeiner S, Chandran S (2013) Astrocyte pathology and the absence of non-cell autonomy in an induced pluripotent stem cell model of TDP-43 proteinopathy. *Proceedings of the National Academy of Sciences of the United States of America*, 110: 4697–4702.
- Tong J, Huang C, Bi F, Wu Q, Huang B, Liu X, Li F, Zhou H, Xia X-G (2013) Expression of ALS-linked TDP-43 mutant in astrocytes causes non-cell-autonomous motor neuron death in rats. *EMBO Journal*, 32: 1917–1926.
- Yan S, Wang C-E, Wei W, Gaertig MA, Lai L, Li S, Li X-J (2014) TDP-43 causes differential pathology in neuronal versus glial cells in the mouse brain. *Human Molecular Genetics*, 23: 2678–2693.
- Yang C, Wang H, Qiao T, Yang B, Aliaga L, Qiu L, Tan W, Salameh J, McKenna-Yasek DM, Smith T, Peng L, Moore MJ, Brown RH, Cai H, Xu Z (2014) Partial loss of TDP-43 function causes phenotypes of amyotrophic lateral sclerosis. *Proceedings of the National Academy of Sciences*, 111: E1121–E1129.