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The hnRNPA1 D262V amyotrophic lateral sclerosis (ALS) mutation is linked to mitochondrial dysfunction

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Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder marked by motor neuron loss and has been genetically linked to mutations in RNA-binding proteins. A mutation D262V in the RNA-binding protein hnRNPA1, found in a subset of ALS patients, causes widespread splicing pattern changes. Cells expressing this hnRNPA1 mutation exhibit aggregation, reduced proliferation, altered stress granules and abnormal neuronal growth. To further elucidate how a single amino acid substitution in a splicing factor might impact cell growth, we employed ribosome profiling to study translational dynamics across the transcriptome in hnRNPA1 mutant cells. Differential ribosome occupancy was observed for a small number of transcripts linked to synaptic organization and GTPase functions, as well as disrupted codon usage and a global stalling of translation. This downregulation of translation coincided with suppression of the mTOR/AKT signaling pathway. RNA splicing changes in transcripts from genes linked to cilia/cell projections, GTPase cycles and glutamate signaling were also observed. Importantly, major mitochondrial dysfunction and mitochondrial fragmentation were found in hnRNPA1 D262V mutant cells. Overall, this study demonstrates how a single amino acid change in an RNA binding protein can contribute to disrupting cell growth and mitochondrial function related to defects linked to neuronal death in ALS.

[Keywords: amyotrophic lateral sclerosis (ALS); hnRNPA1; mitochondria]

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Amyotrophic lateral sclerosis (ALS) is the third most prevalent adult-onset neurodegenerative disease globally. It is characterized by progressive paralysis culminating in respiratory failure and death. Currently, no curative therapies exist, and available treatments offer limited disease-modifying effects. Human genetic studies have implicated mutations in RNA-binding proteins (RBPs), including FUS, TIA1, and TDP-43 (TARDBP) (Kwiatkowski et al. 2009; Vance et al. 2009; Kim et al. 2013; Johnson et al. 2014; Mackenzie et al. 2017), as well as heterogeneous nuclear ribonucleoproteins (hnRNPs) such as hnRNPA1 and hnRNPA2/B1, in the etiology of ALS (Kim et al. 2013; Liu et al. 2016; Beijer et al. 2021; Milicevic et al. 2022). Furthermore, hexanucleotide repeat expansions in the *C9orf72* gene, a major genetic cause of ALS, are associated with the formation of RNA foci that sequester low-solubility hnRNP proteins, suggesting a role for RNA toxicity through RBP sequestration in disease pathogenesis (Cooper-Knock et al. 2014; Conlon and Manley 2017; Balendra

and Isaacs 2018; Milicevic et al. 2022). Collectively, these findings underscore the critical role of genes encoding RBPs as key determinants in neurological disorders, particularly motor neuron diseases such as ALS (Milicevic et al. 2022).

The RNA-binding splicing factor hnRNPA1 is comprised of two N-terminal RNA recognition motifs (RRMs) and a C-terminal glycine-arginine-rich, prion-like low-complexity domain (LCD). Although predominantly localized to the nucleus at steady state, hnRNPA1 is a dynamic nucleocytoplasmic shuttling protein that continuously cycles between the nucleus and the cytoplasm (Pinol-Roma and Dreyfuss 1992). In fact, some mutations in hnRNPA1 result in its loss of nuclear localization and have been linked to multiple sclerosis (MS) (Salapa et al. 2024; Ansari et al. 2025). Beyond its canonical nuclear roles in pre-mRNA processing, cytoplasmic hnRNPA1 actively modulates mRNA translation, fundamentally acting as an internal ribosome entry site (IRES) *trans*-acting factor (ITAF) to regulate protein synthesis

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(Bonnal et al. 2005). Notably, single-point mutations within this C-terminal domain have been linked to ALS. Four such ALS-associated mutations in the hnRNPA1 LCD have been identified (Liu et al. 2016; Beijer et al. 2021). Among these, the aspartic acid to valine substitution at position 262 (D262V) represents the only disease-associated variant affecting an evolutionarily conserved residue in ALS patients (Kim et al. 2013). Our prior investigation (Lee and Rio 2024) revealed that a single amino acid substitution within the low-complexity domain of hnRNPA1 induced substantial alterations in global pre-mRNA splicing patterns and a broad spectrum of changes in *in vivo* RNA-protein interactions. The hnRNPA1 D262V mutation, which does not alter nuclear localization unlike the dramatic nuclear depletion typical of TDP-43 and FUS pathologies, uniquely manifests in slower growth, cell aggregation and neuronal cell differentiation defects, while also impacting splicing of thousands of RNA transcripts and potentially influencing mitochondrial integrity (Lee and Rio 2024). Quantitative proteomic experiments suggested that the hnRNPA1 D262V mutant protein interacted with ribosomal proteins to a greater extent than the wild-type protein (Lee and Rio 2024). This unexpected proteomic finding coupled with the known capacity of hnRNPA1 to modulate translation as an ITAF, suggested a possible connection between translational dysregulation and the observed D262V mutant phenotypes.

A critical gap remains in our understanding of how these primary, mutation-driven posttranscriptional regulatory defects manifest in complex macroscopic phenotypes, such as severely impaired cellular proliferation and progressive neurodegenerative phenotypes. To bridge these molecular-level alterations with cellular and phenotypic outcomes, an integrated multiomics approach is required. Here, we deploy high-resolution ribosome profiling (Ribo-seq) paired with deep transcriptomic sequencing and real-time metabolic profiling in cells carrying an ALS-linked hnRNPA1 D262V mutation. We found changes in translation of transcripts related to cell adhesion and nervous system development, show that there is a global alteration of translation, which parallels changes in the mTOR/AKT pathways and major mitochondrial dysfunction. We found that an hnRNPA1 mutation compromises homeostatic networks and leads to mitochondrial defects that could ultimately result in motor neuron death in ALS.

Results

Profiling ribosome occupancy to identify translationally affected genes in hnRNPA1 D262V mutant-expressing cells

The expression of the hnRNPA1 D262V mutant protein in SH-SY5Y neuronal cells resulted in several observable cellular phenotypes, including cell clumping and aggregation, dramatically reduced cell growth rates, and altered stress granule disassembly dynamics (Lee and Rio 2024). The slower growth rate was exhibited by both homozy-

gous and heterozygous hnRNPA1 D262V mutant cells compared to wild type suggesting a dominant negative effect. We also observed reduced levels of ribosomal proteins and many translation factors associated with the hnRNPA1 D262V mutant in cell extracts by quantitative proteomics (Lee and Rio 2024). Hence, we hypothesized that this growth defect might be due to changes in translational efficiency in the mutant cells.

To investigate a potential role for translational mechanisms driving these phenotypes, we employed ribosome profiling (Ribo-seq). Ribo-seq generates a precise, transcriptome-wide map of ribosome occupancy, revealing both the location and density of actively translating ribosomes (Ingolia et al. 2009, 2012). This approach provides a direct window into the process of protein synthesis, translation initiation sites and translational efficiency (Ingolia 2014). We utilized Ribo-seq to investigate translational dynamics in hnRNPA1 D262V mutant SH-SY5Y cells, hypothesized to contribute to the inhibited cell growth kinetics and observed proteomics data from our previous investigation (Lee and Rio 2024). By linking actively translated mRNAs to protein levels, we sought to determine whether the slower growth phenotype results from specific or global perturbations in the translato-

me. We generated Ribo-seq libraries from SH-SY5Y cells expressing either wild-type or homozygous mutant D262V hnRNPA1 to define and compare potential translational differences. A total of 497 differentially expressed genes were found in the ribosome protected footprint (RPF) libraries when comparing the hnRNPA1 D262V mutant to the wild type (Fig. 1A; Supplemental Table S1). Functional profiling of these differentially expressed RPF genes showed enrichment in developmental processes, anatomical structure development, cell adhesion and regulation of cell motility (Fig. 1B; Supplemental Table S2).

Gene-level ribosome occupancy (RO) serves as a quantitative measure of translational efficiency, calculated as the ratio of normalized ribosome-protected fragment (RPF) reads per million (RPM) to the normalized total RNA (RNA-seq) RPM for each gene. By normalizing RPF reads to the underlying mRNA pool, standard differential ribosome occupancy (DRO) analysis ensures that RO reflects the relative density of ribosomes actively translating a specific transcript. The accompanying table (Supplemental Table S3) presents both the raw and RPM-normalized read counts for each gene in both the RPF and RNA-seq libraries. Protein-coding and long non-coding RNA (lncRNA) genes that exhibit a minimum expression level of at least 10 reads in either the RPF or RNA-seq library were listed in Supplemental Tables 1 and 3. The comparative differential RO analysis revealed significant changes in only 26 gene transcripts (Fig. 1C; Supplemental Table S3). While this limited number of changes suggests that the growth deficits are not due to widespread shifts in translation, the affected genes, including *P2RX5*, *RBFOX1*, *TENM1*, and *NRG1*, are critical for nervous system development (Fig. 1D). Finding only a small number of genes with differential RO in hnRNPA1 D262V mutant cells was an unexpected result since we observed severe phenotypes, including slow growth,

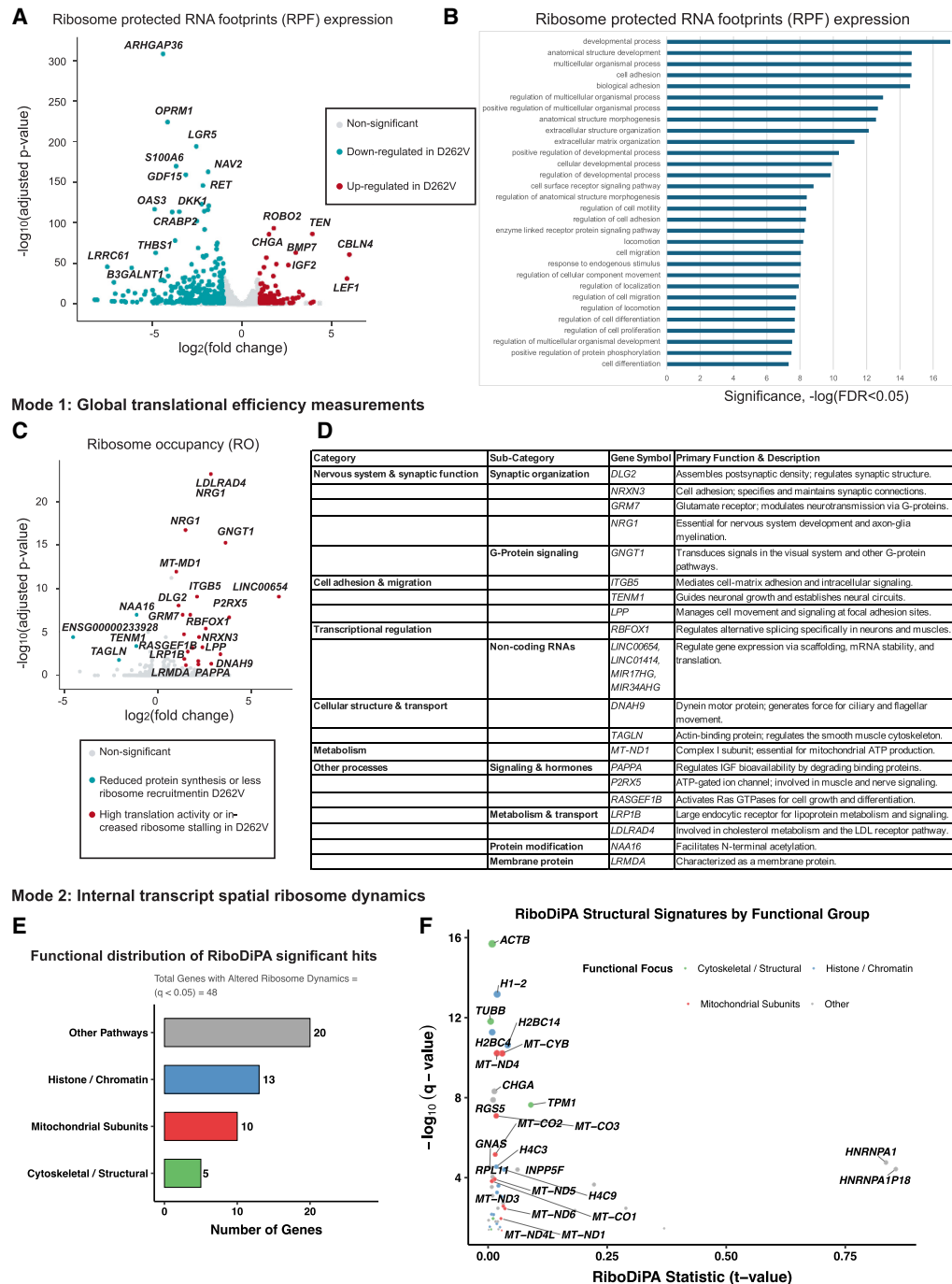


Figure 1. Ribosome-protected RNA footprint (RPF) expression and downstream translational dynamics in hnRNPA1 D262V mutant expressing cells. (A) Differential genes from the ribosome protected footprint (RPF) libraries, showing the analysis compares hnRNPA1 D262V mutant to the wild-type control. The x-axis shows the fold change [$\log_2(\text{fold change})$], and the y-axis shows significance [$-\log_{10}(\text{adjusted } P\text{-value})$]. Red (increased in mutant) and teal (decreased in mutant) dots represent genes that are significantly different between conditions. Gray dots represent genes that are not significant. (B) GO enrichment terminology analysis of 497 differentially expressed genes from RPF libraries that are involved in developmental process, anatomical structure development and multicellular organismal process. Downstream Ribo-seq analysis of translational dynamics. Mode 1: Global translational efficiency measurements. (C) Volcano plot displaying differential translational efficiency (TE), calculated as mRNA-normalized RPF abundance per gene to reflect changes in absolute ribosome occupancy between the hnRNPA1 D262V mutant and WT control. Red and teal points denote transcripts with significantly increased or decreased net translation in the mutant, respectively $q < 0.05$. (D) Tabulated summary outlining the primary biological functions and descriptions of the 26 genes exhibiting altered absolute translational efficiency. Mode 2: Internal transcript spatial ribosome dynamics. (E) Horizontal bar chart partitioning the 48 genes with significantly altered spatial ribosome distribution patterns (global $q < 0.05$, identified via RiboDiPA) into core functional categories. This analysis reveals that while net abundance changes are selective, the mutant explicitly targets the internal spatial patterning of mitochondrial and chromatin structural transcripts. (F) Volcano signature plot mapping the longitudinal RiboDiPA test statistic t -value against track significance [$-\log_{10}(q\text{-value})$]. Coordinated transcript cohorts are color-coded by functional focus.

reduced ribosomal protein interaction by quantitative MS, and pre-mRNA splicing defects (Lee and Rio 2024) in D262V mutant cells. Taken together these results suggest that the hnRNPA1 D262V mutation likely exerts its influence through a mechanism not involving changes in translational efficiency of very specific mRNAs across the transcriptome.

To identify localized variations in translation rather than global changes in translational efficiency (TE), we utilized RiboDiPA (Fig. 1E,F; Supplemental Table S4) to perform differential pattern analysis across conditions (Li et al. 2020). Unlike traditional computational methodologies (e.g., EclipseBio's eRibo Pro, deltaTE [Chothani et al. 2019], or riborex [Li et al. 2017]) that evaluate absolute alterations in total ribosome occupancy and necessitate paired RNA-seq data to normalize for baseline mRNA abundance, RiboDiPA evaluates shifts in the spatial trajectory and distribution of ribosomes along individual transcripts. By mapping ribosome protected footprint (RPF) data to binned P-site coordinates, this method uses a bin-based differential patterning analysis that utilizes a sliding-window framework to resolve site-specific kinetic alterations, such as stalling or initiation shifts, that are typically obscured by transcript-wide averaging. The RiboDiPA algorithm evaluates the relative distribution shape of footprints across a transcript rather than absolute abundance, localized read depletions are mathematically detected with the same sensitivity as localized accumulations. Computationally, these variations manifest in two distinct directional signatures: an increased regional dwell time yields a statistically enriched footprint peak in the mutant relative to the wild type at a specific bin coordinate, which may indicate a positional stall or pause. Conversely, a decreased regional dwell time produces a significant valley or drop relative to the wild type, indicating locally accelerated clearance or the resolution of a baseline translational pause. This bidirectional sensitivity allowed us to systematically identify both potential translation bottlenecks and alterations in baseline pause sites across the transcriptome.

While the RiboDiPA methodology tracked over 21,000 total transcripts, only a highly restricted cohort of 48 transcripts exhibited significant internal spatial dynamics (global $q < 0.05$) (Supplemental Table S4). We examined distinct instances of both induced ribosomal stalling and resolved baseline pauses within our target genes (*ACTB*, *MT-ND1*, and *MT-CO1*) (Supplemental Fig. S1). To investigate whether the mutation alters subtranscript translation dynamics on individual housekeeping genes, we examined the ribosome footprinting profile of β -actin mRNA (*ACTB*, ENSG00000075624.17) (Supplemental Fig. S1A), which emerged as one of the top statistically ranked targets in our RiboDiPA analysis. RiboDiPA revealed highly significant, localized variations in ribosome occupancy along the *ACTB* transcript between conditions (global $q = 2.03 \times 10^{-16}$) across 175 analyzed bins. Specifically, the framework identified distinct differential patterning (DP) spots indicating altered regional ribosomal dwell times. At the 5' region of the transcript (around bin 25), hnRNPA1 D262V mutant cells exhibited a sharp,

pronounced accumulation of ribosome footprint (RPF) counts relative to the wild type ($q = 2.10 \times 10^{-4}$), suggesting an induced positional ribosomal stall or pause. Conversely, toward the 3' region (around bin 145), a significant depletion of reads was observed in the mutant replicates compared to the occupancy peaks in the wild type ($q = 4.95 \times 10^{-2}$), consistent with locally accelerated clearance or the alteration of a baseline translational pause. Together, these bidirectional intragenic variations indicate that the mutation disrupts the spatial trajectory of ribosome distribution on highly expressed transcripts independent of total transcript abundance.

We next examined the translation profile of the mitochondrially encoded NADH dehydrogenase subunit 1 (*MT-ND1*, ENSG00000198888.2) (Supplemental Fig. S1B), which demonstrated significant subtranscript variation between conditions across 66 analyzed bins (global q -value = 1.11×10^{-2}). RiboDiPA identified two adjacent DP spots showcasing localized shifts in ribosome distribution. In the upstream region (q -value = 2.41×10^{-2}), mutant cells exhibited a depletion of ribosome footprints compared to the pronounced occupancy peaks present in the wild type, consistent with an alteration or clearance of a baseline translational pause. Conversely, directly downstream (q -value = 1.39×10^{-5}), the mutant profile displayed a sharp, localized accumulation of ribosome footprint counts, suggesting an induced positional ribosomal stall. This adjacent, bidirectional pattern highlights a complex regional rearrangement of ribosome dwell times on the *MT-ND1* transcript under mutant conditions. We additionally applied this framework to evaluate the mitochondrially encoded cytochrome c oxidase subunit I (*MT-CO1*, ENSG00000198804.2) (Supplemental Fig. S1C), which revealed a highly significant alteration in subtranscriptional ribosome profiling patterns across 67 bins (global q -value = 1.50×10^{-4}). RiboDiPA identified three localized DP spots. In the mid-upstream region (q -value = 2.62×10^{-7}), mutant cells displayed a prominent, sharp accumulation of ribosome footprint counts relative to the wild type, suggesting an induced positional ribosomal stall. Conversely, immediately downstream (q -value = 2.04×10^{-4}) and at a distal 3' site (q -value = 2.78×10^{-3}), the D262V mutant profile demonstrated a marked depletion of footprint counts compared to the occupancy peaks observed in the wild-type tracks. These coordinated, bidirectional shifts are consistent with local alterations in baseline pauses alongside the induction of distinct translation bottlenecks on the mitochondrial *MT-CO1* transcript.

Interestingly, *MT-ND1* was the only mitochondrial subunit identified by the DRO model as having significantly altered transcript-wide occupancy ($P_{adj} = 1.2 \times 10^{-12}$) (Supplemental Table S3). However, immunoblot analysis revealed that MT-ND1 protein levels remained unchanged between wild-type and D262V mutant cells (Supplemental Fig. S2A). This discrepancy with increased ribosome density without a concomitant increase in protein output, strongly suggests a ribosome stalling phenotype, where higher RPF counts likely reflect a "traffic jam" of stalled ribosomes rather than productive

translation. In contrast, RiboDiPA analysis (Fig. 1E,F; Supplemental Table S4) revealed significant translational remodeling across a broad suite of mitochondrial transcripts, including subunits of complex I (*MT-ND1*, *MT-ND3*, *MT-ND4*, *MT-ND4L*, *MT-ND5*, and *MT-ND6*), complex III (*MT-CYB*), and the catalytic core of complex IV (*MT-CO1*, *MT-CO2*, and *MT-CO3*) (Supplemental Table S4). This included a highly significant dysregulation of *MT-CO1* (global q -value = 0.00015), which, unlike *MT-ND1*, was accompanied by a clear 1.49-fold increase in *MT-CO1* protein levels (Supplemental Fig. S2A). These results demonstrate that site-specific ribosome dynamics, rather than transcript-wide occupancy, are the primary drivers of proteomic imbalances in hnRNPA1 D262V mutant cells. This analysis highlights the necessity of subtranscriptomic analysis; while standard DRO analysis identified only *MT-ND1* (Fig. 1C), it failed to detect the broad translational remodeling of the mitochondrial transcriptome (Fig. 1E,F; Supplemental Table S4). Crucially, whereas the DRO-detected shift in *MT-ND1* did not alter protein levels suggesting a kinetic stall, RiboDiPA identified patterning shifts in *MT-CO1* (Supplemental Fig. S1C) directly manifested as increased protein output (Supplemental Fig. S2A), revealing the true site of proteomic impact. Our integrated Ribo-seq approach reveals that the hnRNPA1 D262V mutation induces site-specific stalling on complex I transcripts (*MT-ND1*) and altered ribosome dynamics on complex IV subunit transcripts (*MT-CO1*, *MT-CO2*, and *MT-CO3*). This decoupling should alter the stoichiometric balance required for normal levels of functional cellular respiration.

However, identifying such a highly restricted subset of altered transcripts, comprising only 26 genes by transcript-wide ribosome occupancy (DRO) analysis and 48 by differential pattern analysis presented an intriguing paradox given the severe slow-growth phenotype of the mutant cells. These remarkably selective signatures led us to hypothesize that the mutation might instead drive a uniform, global reduction in translation levels—a systemic defect that standard relative normalization procedures mathematically mask. To determine whether these seemingly uniform occupancy profiles across the remainder of the transcriptome mask a widespread kinetic bottleneck, we next proceeded to evaluate key components of the translational machinery.

Selective suppression of the mTORC1-S6K1, mTORC1-eEF2, and STAT3 signaling axes in hnRNPA1 D262V mutants

The absence of major changes in differential RO indicates that changes in translation of specific mRNAs are not the primary cause of the slower growth rate and reduced interaction of hnRNPA1 with ribosomal and translational proteins in hnRNPA1 D262V mutant cells. Instead, a global attenuation of translational capacity may explain the slower growth. Such a uniform reduction would yield lower total protein levels while maintaining a constant RO ratio, thereby preserving the relative proportions of the proteome despite the decrease in absolute output. To dis-

sect the subtranscriptomic translational architecture, riboWaltz (Lauria et al. 2018) was used to determine codon usage, stall sites, 5' UTR loading, and 3' read-through. This integrated analysis revealed that D262V mutant cells exhibit decreased general codon usage (Fig. 2A), a shift often associated with reduced global translation rates and compromised mTOR signaling (Ma and Blenis 2009; Quax et al. 2015; Thoreen 2017). A decrease in codon usage can slow down translation globally across the entire transcriptome (i.e., increases global ribosomal transit times and codon dwell times), yet the overall ribosome load or RO across most transcripts remains relatively constant.

The decrease in ribosomal protein levels is a frequent consequence of reduced mTOR activity (Mayer and Grummt 2006; Iadevaia et al. 2012). The mTORC1 pathway serves as the master switch for ribosome biogenesis; when mTORC1 is inhibited, the cell intentionally curtails its protein synthesis capacity to conserve energy. To evaluate whether a global reduction in translation is tightly correlated with compromised mTOR signaling, we investigated the phosphorylation status of key components within the PI3K/AKT/mTOR pathway to determine whether the pathway is compromised. The phosphorylation status of proteins downstream from mTORC1, for example, phospho-AKT (Fig. 2B) or phospho-p70 S6 Kinase (S6K1) (Fig. 2C) was examined by immunoblot analysis using phospho-specific antibodies. Indeed, we observed decreased levels of phosphorylation for phospho-AKT and phospho-S6K1 in hnRNPA1 D262V mutant cells compared to wild type. The total AKT or S6K1 protein levels remained relatively unchanged using control antibodies (Fig. 2B,C). Protein levels were quantitated after normalization to β -actin protein levels as a loading control.

To further investigate the molecular basis of the growth defects and altered codon usage in D262V mutant cells, we characterized the status of the PI3K/AKT/mTORC1 signaling axis (Fig. 2B). Immunoblot analysis revealed a 38% decrease in phospho-AKT (Ser473) in the mutant cells, signaling a significant reduction in upstream growth factor transduction. This was accompanied by a 50% reduction in p-S6K1 signaling (Fig. 2C). Since p-S6K1 is a requisite activator of translation elongation via the S6K1-eEF2 axis, we further assessed the phosphorylation status of eukaryotic elongation factor 2 (eEF2). Immunoblot analysis of hnRNPA1 wild-type and D262V mutant SH-SY5Y cells demonstrated that phospho-eEF2 (Thr56) expression is increased 1.91-fold ($P < 0.05$) in the mutant cells (Fig. 2D). The kinetic slowdown during translation elongation in hnRNPA1 D262V mutant cells is driven by the 1.91-fold upregulation of inactive phospho-eEF2 (Thr56, $P < 0.04$) (Fig. 2D). In line with this, SUNSET assays showed no statistically significant change in overall nascent protein synthesis between wild-type and D262V mutant cells (data not shown), mirroring our global ribosome profiling analysis where differential ribosome occupancy (DRO) was restricted to only a small set of 26 transcripts (Fig. 1C). Instead of a broad alteration in bulk translation, subtranscriptomic RiboDiPA profiling mapped site-specific ribosome traffic jams directly onto

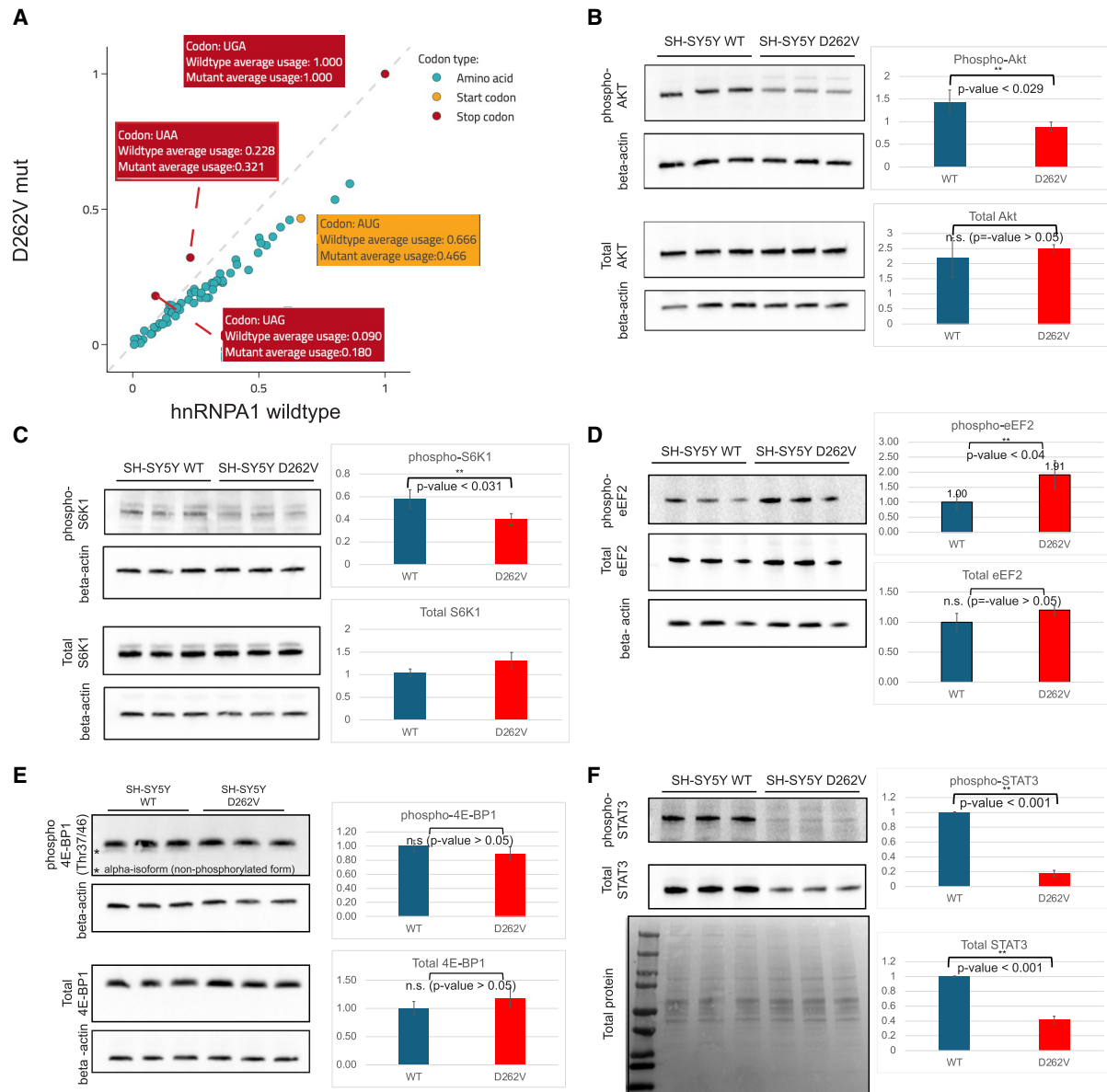


Figure 2. hnRNPA1 D262V mutation impairs translational elongation kinetics and selectively suppresses downstream AKT, STAT3, and eEF2 signaling. (A) Comparison of global ribosome P-site codon occupancy. Scatter plot illustrating average codon usage in cells expressing hnRNPA1 wild-type (WT) or the D262V mutant. Codon usage is defined as the scaled, normalized frequency of P-site enrichment. The mutant (y-axis) exhibits a shallower global slope (<1) relative to the wild type (x-axis), reflecting a systematic reduction in translation rate and altered codon dwell times. Off-diagonal codons highlight specific synonymous triplets with differential translational efficiency between conditions. (B) Impaired AKT phosphorylation in hnRNPA1 D262V mutant cells. Representative western blot analysis of total and phosphorylated AKT (p-AKT) levels. While total AKT expression remains constant, a significant reduction in p-AKT is observed in D262V mutant-expressing cells compared to wild type, indicating a defect in the PI3K/AKT signaling pathway. β -Actin levels serve as the loading control. (C) Attenuated S6K1 activation in hnRNPA1 D262V mutant cells. A marked decrease in the levels of phosphorylated S6K1 (p-S6K1 and Thr389) was observed in the D262V mutant compared to wild type, while total S6K1 protein levels remained unchanged, indicating a specific defect in kinase activation rather than protein expression. The β -actin protein was used as a loading control to ensure accurate protein quantitation across lanes. (D) Immunoblot analysis of phospho-eEF2 and total eEF2 in hnRNPA1 WT and D262V mutant SH-SY5Y cells. Phospho-eEF2 expression is increased 1.91-fold ($P < 0.04$) in hnRNPA1 D262V mutant SH-SY5Y cells, indicating a defect in translational elongation. (E) Immunoblot analysis of phospho-4E-BP1 and total 4E-BP1 in hnRNPA1 WT and D262V mutant SH-SY5Y cells. Quantification revealed no significant difference in the levels of phospho-4E-BP1 ($0.89 \times$ of WT; $P > 0.05$) or total 4E-BP1 ($1.18 \times$ of WT; $P > 0.05$) between the two groups. Significance was determined using a two-tailed Student's *t*-test. β -Actin or total protein levels were used as a loading control. Data are representative of three independent biological replicates. (F) Suppression of STAT3 expression and phosphorylation in hnRNPA1 D262V mutant cells. The D262V mutant exhibits a marked reduction in both phosphorylated STAT3 (p-STAT3, Tyr705; 18% of wild type) and total STAT3 protein levels (41% of wild type). Total protein staining served as a loading control to ensure accurate quantification. Data are representative of three independent biological replicates.

specific structural and metabolic mRNAs (Fig. 1E,F; Supplemental Tables 3, 4). Altogether, these findings indicate that the D262V mutation induces targeted elongation bottlenecks rather than a global shutdown in translational output. Because eEF2 phosphorylation serves to inhibit its activity, this elevation, likely resulting from the loss of S6K1-mediated inhibition of eEF2 kinase, suggests a restriction on the rate of ribosomal translocation during translation elongation.

Paradoxically, despite this suppression of the AKT/mTORC1 pathway, total levels of phosphorylated 4E-BP1 (p-4E-BP1) remained relatively stable, suggesting that the molecular scaffolding for cap-dependent initiation was nominally preserved. Notably, we observed an absence of the α -isoform—the rapidly migrating, hypophosphorylated state of 4E-BP1—in mutant lysates compared to wild type (Fig. 2E). This indicates that the mutant 4E-BP1 pool is effectively sequestered in a phosphorylated state (specifically at Thr37/46), which licenses the initiation machinery by preventing 4E-BP1 from sequestering eIF4E. These data point toward a specific decoupling of translation stages, where initiation signaling is maintained but downstream elongation signaling is markedly attenuated.

This phenomenon was revealed by our gene-level differential ribosome occupancy (DRO) and subtranscriptomic RiboDiPA profiling, which mapped site-specific ribosome traffic jams directly onto mRNAs encoding components of structural, synaptic, and signaling networks (*DNAH9*, *TENM1*, *NRXN*, *RBFOX1*, *DLG2*, and *LRP1B*), as well as abundant cytoskeletal elements (*ACTB*, *TUBB*, and *TPM1*) and core mitochondrial-encoded respiratory machinery (*MT-ND1* and *MT-CO1*) (Fig. 1C,F; Supplemental Tables 3, 4). Together with the observed reduction in specific codon usage metrics, these target profiles suggest that while active cytoplasmic translation elongation is heavily constrained by p-eEF2, a large pool of translation complexes becomes structurally stalled. While our metabolic signaling immunoblots utilized a mild, native lysis buffer to capture the active, soluble cytoplasmic protein pool, total protein denaturation with SDS solubilized cell lysates uncovered this ribosome-associated macromolecular fraction of stalled translation complexes anchored to structural frameworks, altered stress granules, or organellar frameworks, which are otherwise discarded in native lysate supernatants (Simsek et al. 2017).

Beyond the mTORC1 axis, we observed a profound and selective collapse in STAT3 signaling (Fig. 2F), a critical pathway for cellular proliferation and survival. Immunoblot analysis of hnRNPA1 wild-type and D262V mutant SH-SY5Y cell lysates revealed a marked reduction in both phosphorylated STAT3 (p-STAT3 and Tyr705), which was reduced to 18% of wild-type levels, and total STAT3 protein levels, which fell to 41% of wild type (Fig. 2F). Notably, this downregulation appears specific to the STAT3 pathway, as we observed no significant changes in either the phosphorylation or total protein levels of STAT5 in D262V mutant cells (Supplemental Fig. S3). These observations, represented by three independent biological replicates, suggest that the D262V mutation in-

duces a targeted rather than global suppression of key anabolic signaling pathways, likely underpinning the observed phenotypic growth defects by specifically altering STAT3-mediated survival and mTOR-driven translation.

Collectively, these biochemical findings provide a mechanistic explanation for the global attenuation of translational capacity in D262V mutant cells. The selective impairment of the S6K1–eEF2 axis (Fig. 2), despite the maintenance of 4E-BP1-mediated initiation licensing, suggests that the mutant cells suffer from a “bottleneck” at the translation elongation stage. This specific decoupling, combined with the drastic reduction in STAT3-mediated survival signaling (Fig. 2F), reconciles our ribosome profiling observations: the stable RO ratios across the transcriptome do not reflect healthy translation, but rather a uniform, nonselective slowing of the protein synthesis machinery. These results suggest that the D262V mutation does not selectively reshape the translationalome. Rather, it imposes a global kinetic bottleneck on protein synthesis that diminishes absolute cellular protein output, which parallels the observed growth impairment of the mutant cells.

RNA-seq analysis of gene expression and pre-mRNA splicing patterns of undifferentiated hnRNPA1 D262V mutant vs. wild-type cells

While the Ribo-seq and immunoblotting data (Figs. 1, 2) highlight a significant global translational slowdown without substantial changes in ribosome occupancy, these translational defects must be contextualized within the broader transcriptional and RNA processing environment of the cell. The D262V mutation, located within the C-terminal low-complexity domain (LCD) of hnRNPA1, is critically linked to the pathogenesis of neurodegenerative diseases, particularly amyotrophic lateral sclerosis (ALS) (Kim et al. 2013). Given that the LCD is essential for mediating protein–protein interactions, mutations in this region often disrupt an important role of the protein in RNA metabolism. To determine whether the translational perturbations we observed are secondary to broader defects in transcript stability, abundance or alternative splicing, we performed a comparative RNA-seq analysis. This much deeper level of RNA profiling allows us to delineate which aspects of the D262V phenotype might arise from altered mRNA levels or RNA splicing changes versus those driven specifically by the translational bottlenecks identified in our ribosome profiling experiments.

To ensure that the observed molecular perturbations were driven specifically by the hnRNPA1 D262V mutation rather than the cellular differentiation process characterized in our previous investigation (Lee and Rio 2024), we performed RNA-seq on the same undifferentiated SH-SY5Y cell populations used for the Ribo-seq experiment. This approach allowed us to discriminate between constitutive RBP-driven defects and those emerging during neuronal differentiation. To characterize the transcriptional landscape in the D262V mutant cells, we performed differential gene expression and alternative

pre-mRNA splicing analyses on these deeply sequenced RNA-seq data sets. We identified approximately 6329 significant alternative splicing event changes in D262V mutant cells compared to wild-type controls (Fig. 3A; Supplemental Table S5) using the JUM software (Wang and Rio 2018). Interestingly, the GO term enrichment analysis revealed regulation of the glutamate receptor signaling pathway and the positive regulation of excitatory postsynaptic potential categories, as well as the regulation of dendritic spine morphogenesis, and cell projection morphogenesis (Fig. 3B; Supplemental Table S6). Furthermore, the simultaneous enrichment of terms associated with the regulation of RNA biosynthetic processes and DNA duplex unwinding mirrors the broad impact of hnRNPA1 on RNA metabolism (Kim et al. 2013; Guerousov et al. 2017; Lee and Rio 2024), suggesting widespread disruption of cellular processes spanning from synaptic architecture to genomic stability.

To delineate how these altered splicing patterns impact motor neuron biology, we focused on functional profiling of the 4162 genes exhibiting altered splicing patterns that was performed against the Reactome database (Fig. 3C). Notably, we observed a pronounced enrichment of genes involved in the cilium assembly, RHO GTPase cycle, and glutamate binding and activation pathways ($P < 0.05$ using g:SCS threshold) (Fig. 3C). These results indicate that alternative splicing extensively targets structural and signaling modules essential for neuronal homeostasis. In amyotrophic lateral sclerosis (ALS), the convergence of excitotoxic glutamate signaling and dysregulated Rho GTPase cycling forms a central mechanism driving motor neuron degeneration (Foran and Trotti 2009; Arnold et al. 2024). As an essential member of the Ras superfamily, the Rho GTPase cycle translates synaptic stimuli into the cytoskeletal changes necessary for axonal stability (Hall and Lalli 2010; Stankiewicz and Linseman 2014; Arnold et al. 2024). Furthermore, aberrant glutamate receptor activity triggers calcium-dependent hyperactivation of the Ras/MAPK and PI3K/Akt signaling cascades, which converge on the mTOR pathway to suppress autophagic flux and exacerbate proteotoxicity (Heras-Sandoval et al. 2014; Sahana and Zhang 2021). Notably, the dysregulation of the glutamate signaling axis observed in our data mirrors the pathology of the *C9ORF72* gene, a primary genetic driver of ALS. Loss of *C9ORF72* function has been shown to reduce the expression of the excitatory amino acid transporter EAAT2 (Fomin et al. 2018). This resulting proteostatic imbalance disrupts the cell's ability to maintain homeostasis, ultimately facilitating the structural and functional death of the motor neurons. The perturbation of these pathways, even in the undifferentiated cell state, suggests that the D262V mutation fundamentally alters multiple cellular signaling networks linked to cell growth and function. These early molecular defects likely prime the cell for the axonal growth failure and genomic stress that ultimately drive motor neuron degeneration in ALS (Kim et al. 2013; Butti and Patten 2019). Functional enrichment analysis was also performed using g:Profiler against the KEGG database, with the identified signaling pathways shown in Supplemental Figure S4, with the

most prominent enrichment observed is in axon guidance (KEGG:04360), the neurotrophin signaling pathway (KEGG:04722), and ubiquitin-mediated proteolysis (KEGG:04120). In addition to this synaptic vulnerability, our analysis highlighted significant splicing perturbations within structural and architectural modules, specifically the RHO GTPase cycle, which is essential for axonal stability, growth cone dynamic and cilium assembly. Furthermore, complementary KEGG analysis identified extensive alterations in the axon guidance and neurotrophin signaling pathways (Supplemental Fig. S5). Because these synaptic, structural and neurotrophic modules are strictly required for motor neuron homeostasis, their early breakdown in the undifferentiated state strongly indicates that the hnRNPA1 D262V mutation fundamentally primes the mutant cells for a coordinated collapse of synaptic, structural, and survival machinery.

We also performed a global differential analysis of gene expression using DESeq2 to assess shifts in steady-state mRNA abundance caused by the D262V mutation (Supplemental Table S7). Consistent with our previous observations in neuronally-differentiated SH-SY5Y cells (Lee and Rio 2024), the D262V mutation in the undifferentiated cell state induced a significantly higher number of alternative splicing events compared to the total number of differentially expressed genes (DEGs). Specifically, we identified 919 DEGs between wild-type and mutant cells (Fig. 4A; Supplemental Table S7), a relatively modest figure when contrasted with the 6329 significant splicing alterations (Fig. 3A; Supplemental Table S8). This disproportionate ratio suggests that the primary transcriptomic impact of the hnRNPA1 D262V mutation is the disruption of pre-mRNA processing patterns and isoform expression, rather than changes in transcript abundances. The gene ontology (GO) analysis of these 919 DEGs further identified significant enrichment in terms typically associated with developmental morphogenesis, including cell differentiation in the spinal cord, metanephros development, and regulation of viral budding via the host ESCRT (endosomal sorting complex required for transport) (Fig. 4B; Supplemental Table S7). To better understand how these transcriptomic shifts relate to ALS pathology, we examined specific DEGs critical to motor neuron identity and survival signaling pathways (Fig. 4D). Among these genes, the homeobox transcription factor *HOXD8* and the G-protein signaling subunit *GNGT1*, both established markers used to define differential motor neuron vulnerability between resistant and vulnerable pools, were significantly altered. Furthermore, we observed pronounced dysregulation of *IGFBP5* (a key negative regulator that sequesters neuroprotective insulin-like growth factor-1) and *CRABP2* (a critical vehicle for retinoic acid-mediated cellular stress responses). The differential expression of these structural and survival determinants indicates that the D262V mutation disrupts the homeostatic networks required to maintain motor neuron identity and survive environmental stress.

To distinguish whether these transcriptional shifts represent constitutive, mutation-driven effects or are dependent on specific cellular contexts, we compared the

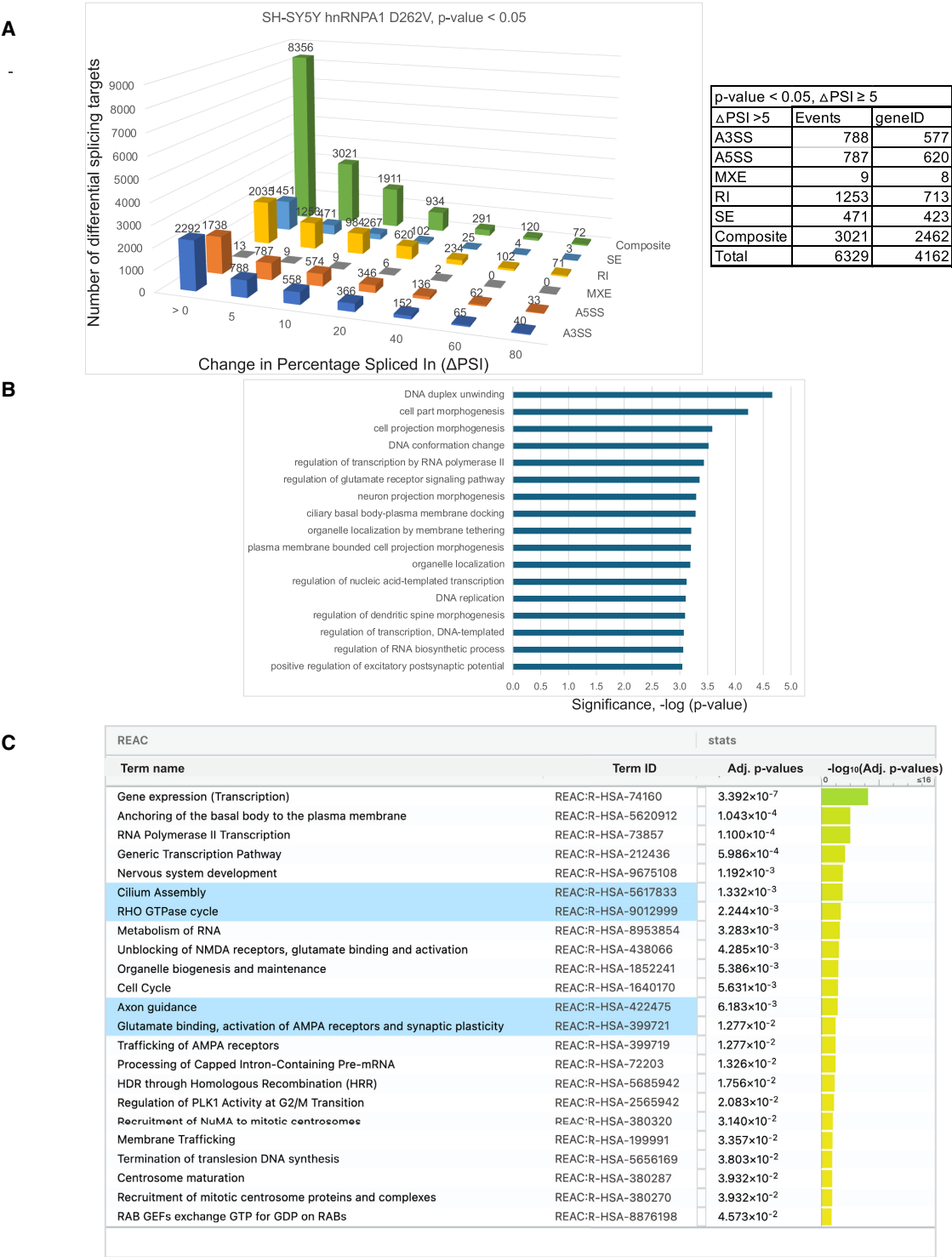


Figure 3. Differential splicing events in hnRNPA1 D262V mutant SH-SY5Y neuroblastoma cells. (A) Differential alternative splicing (AS) events detected in undifferentiated SH-SY5Y neuroblastoma cells expressing the ALS-associated hnRNPA1 D262V mutation, compared to wild-type control cells. A total of 6329 splicing events in 4162 genes were significantly altered ($P < 0.05$, $|\Delta\text{PSI}| > 5$). The y-axis represents the number of differential splicing events, while the x-axis indicates the difference in percentage spliced in (ΔPSI). Splicing pattern types are indicated at the right. (B) Gene ontology (GO) term enrichment analysis of differentially spliced transcripts in hnRNPA1 D262V mutant SH-SY5Y cells. (C) Functional profiling was performed using g:Profiler to analyze the 4162 differentially spliced genes against the Reactome database. The bar length represents the $-\log_{10}$ of the adjusted P -value, with the significance threshold set at $P < 0.05$ using g:SCS threshold (set counts and sizes). Significant enrichment is observed in the cilium assembly, RHO GTPase cycle, and glutamate receptor signaling pathways.

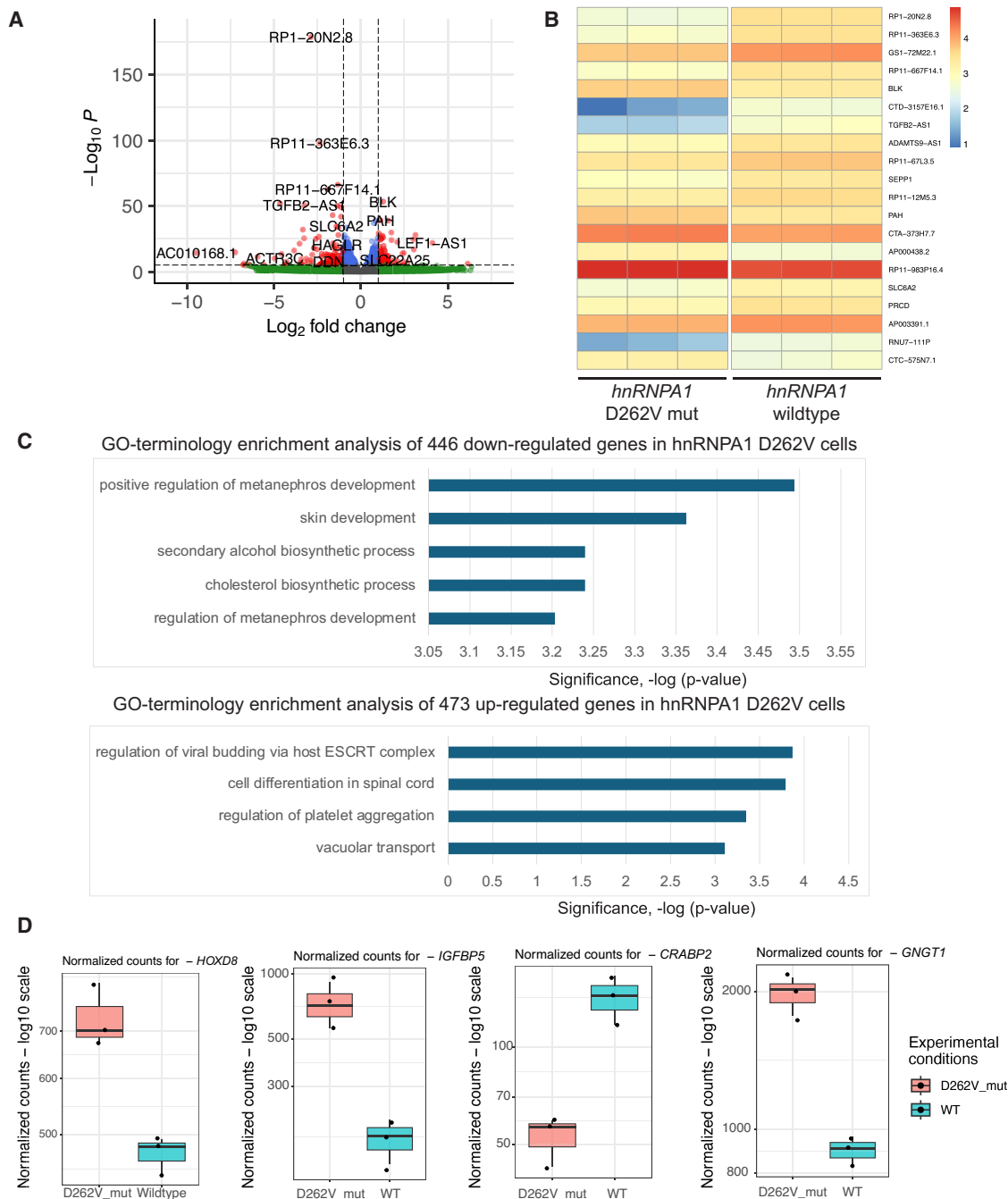


Figure 4. DESeq2 gene expression level analysis reveals 919 genes are differentially expressed in the *hnRNPA1* D262V mutant compared to wild-type cells. (A) A volcano plot is shown with some upregulated and downregulated gene names shown. DESeq2 expression analysis of the *hnRNPA1* D262V mutant compared to the wild-type control cells reveals 919 differentially expressed genes (adjusted P -values < 0.05 , $FC > 1.5$). (B) Heat map for the top 20 differentially expressed genes with an absolute $\log_2$ fold change ≥ 0.58 in the *hnRNPA1* D262V mutant cells. (C) A graph of gene ontology (GO) term enrichment of either 446 downregulated genes or 473 upregulated genes in the *hnRNPA1* D262V mutant protein-expressing neuronal cells compared to the wild-type control cells. (D) Box plots showing differential expression of selected genes (*HOXD8*, *IGFBP5*, *CRABP2*, and *GNGT1*) in the *hnRNPA1* mutant cells compared to the wild-type control cell samples.

profiles of differentially spliced genes (DSGs) and differentially expressed genes (DEGs) across different differentiation states (Supplemental Fig. S5A,D; Supplemental Tables 8, 9; Lee and Rio 2024). While both processes ex-

hibited context dependency, we observed a significantly higher degree of conservation in splicing patterns than in transcript abundance. Specifically, 30% of DSGs were common to both undifferentiated and differentiated

D262V mutant cells, whereas only 16% of DEGs were shared between the two differentiation states (Supplemental Fig. S5A,D). We identified a core signature of 785 DSGs common to both states (Supplemental Fig. S5A; Supplemental Table S9), representing the fundamental impact of the D262V mutation on RNA processing regardless of differentiation status. Interestingly, the significant nonoverlap between the two states suggests that the D262V mutation interacts with the cell's transcriptome and differentiation state to trigger distinct splicing vulnerabilities as neurons mature. Gene ontology (GO) analysis of the shared 785 DSGs revealed a high enrichment in pathways critical for neuronal integrity, including cell projection morphogenesis and the regulation of axonogenesis (Supplemental Fig. S5B). This analysis indicates that the state-independent effect of the mutation is specifically tuned to disrupt the structural and functional modules necessary for long-term neuronal health. Notably, this core 785-gene signature contains several prominent DSGs with established roles in ALS pathogenesis and neurodegenerative biology, including *NEFL*, *DCTN1*, *ANK3*, *PICALM*, *MAP2*, *APP*, and *SHANK2* (Supplemental Fig. S5C). As a validation of several splicing pattern changes between hnRNPA1 wild-type and D262V mutant cells detected in our data set, we show sashimi plots for several genes with the relevant junction read numbers and splicing patterns shown (Supplemental Fig. S6). Together, this comparative analysis reinforces our conclusion that the primary, state-independent effect of the hnRNPA1 D262V mutation is a profound disruption of pre-mRNA processing rather than a uniform reshaping of steady-state transcript abundance.

Importantly, while alternative splicing is the dominant driver of the phenotype, the specific subset of genes altered at the steady-state expression level points toward a distinct transcriptional effect. Notably, our GO analysis of the 919 primary DEGs highlighted a significant enrichment in the regulation of viral budding via the host ESCRT (endosomal sorting complex required for transport) (Fig. 4B). The ESCRT machinery is the primary cellular component for membrane remodeling, vesicle budding, and autophagy (Zhen et al. 2020; Jun et al. 2023). In fact, recent research confirms that the ESCRT machinery is crucial for mitochondrial health (Horbay et al. 2025) by facilitating the piecemeal removal of damaged inner mitochondrial membrane (IMM) sections and maintaining cristae organization (Prashar et al. 2024). Based on this finding and because of the slow growth of the mutant cells, we sought to employ confocal microscopy with organelle-specific fluorescent staining (lysosome marker antibodies and MitoTracker) to assess lysosomal and mitochondrial morphology and to evaluate mitochondrial respiratory function through oxygen consumption rate (OCR) measurements.

Mitochondrial dysfunction in hnRNPA1 D262V mutant cells

Our ribosome profiling analysis identified several mitochondrially-encoded subunit mRNAs with significant

translational dysregulation in D262V mutant cells. While standard DRO analysis highlighted a significant increase in transcript-wide occupancy for *MT-ND1* ($P_{adj} = 1.2 \times 10^{-12}$), the RiboDiPA pipeline revealed a highly significant shift in the positional patterning of *MT-CO1* ($P_{adj} = 0.00015$) (Fig. 1C,F; Supplemental Fig. S1B,C; Supplemental Tables S3 [for DRO], S4 [for RiboDiPA]). While the altered pattern of *MT-ND1* suggests potential ribosome stalling during the assembly of the complex I foundational scaffold, *MT-CO1* exhibited a translational dysregulation by a 1.49-fold increase in protein expression (Supplemental Fig. S2). Given that these subunits are essential for the assembly and function of complex I (NADH-ubiquinone oxidoreductase) and complex IV (cytochrome c oxidase), respectively, we hypothesized that their collective translational dysregulation leads to downstream structural and functional catastrophic failures within the electron transport chain (ETC).

To evaluate the morphological integrity of the mitochondrial and lysosomal networks, we performed high-resolution confocal fluorescence microscopy in SH-SY5Y cells stably expressing either wild-type hnRNPA1 or the D262V mutant. Cells were seeded 24 h prior to imaging and costained with either MitoTracker or antibodies to lysosomal markers (LAMP1/LAMP2) to assess organelle spatial distribution and interorganelle cross talk. While wild-type cells exhibited an elongated, interconnected mitochondrial reticulum, the D262V mutant cells displayed marked mitochondrial fragmentation and a shift toward a punctate morphology (Fig. 5A). These observations provide visual evidence that the D262V mutation compromises mitochondrial homeostasis and disrupts the organellar network architecture. Parallel experiments utilizing LAMP2 immunostaining revealed only a marginal decrease in lysosomal content within D262V-expressing cells. However, this trend did not reach statistical significance (data not shown).

The Mito stress test (Roy-Choudhury and Daadi 2019; Parmar et al. 2024) using the Seahorse XF analyzer is a well-established assay for assessing mitochondrial function in cells by measuring oxygen consumption. This test quantifies different aspects of cellular respiration, including basal, ATP-linked and maximal respiration, as well as spare respiratory capacity and nonmitochondrial respiration. By employing specific inhibitors and uncouplers of the electron transport chain, the assay yields significant insights into mitochondrial health and dysfunction. To quantitatively assess bioenergetic capacity, we employed the Seahorse XF cell mito stress test to monitor real-time oxygen consumption rates (OCR). The hnRNPA1 D262V mutant cells exhibited profound reductions in basal respiration and maximal respiratory capacity compared to wild-type controls (Fig. 5B). Mitochondrial fragmentation is a well-documented hallmark of mitochondrial distress and often serves as a morphological precursor to the loss of mitochondrial membrane potential and subsequent respiratory failure (Sheridan and Martin 2010; Youle and van der Bliek 2012). This functional impairment was further substantiated by high-resolution confocal microscopy, which revealed significant

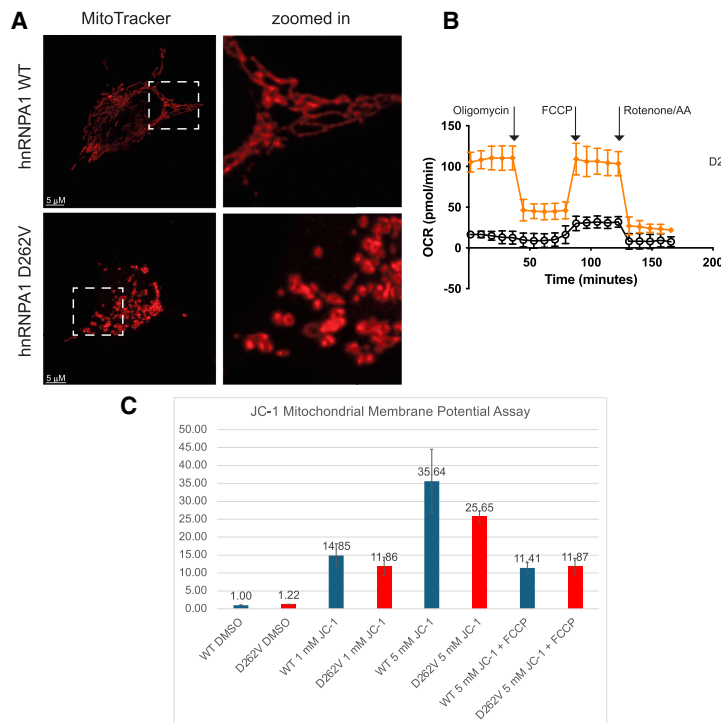


Figure 5. Mitochondrial dysfunction in hnRNPA1 D262V mutant-expressing cells. (A) SH-SY5Y cells expressing either hnRNPA1 wild-type or mutant D262V genes were stained for mitochondria and confocal microscopy was used to examine mitochondria morphology using MitoTracker Deep Red dye. (B) Oxygen consumption rates (OCR) were measured following overnight cell proliferation. OCR directly reflects the rate at which cells consume oxygen, which is the final electron acceptor in the electron transport chain (ETC) within the mitochondria. The difference between maximal and basal respiration is shown, indicating the cell's ability to meet increased energy demands. (C) Ratiometric JC-1 analysis of mitochondrial membrane potential ($\Delta\Psi_m$) in wild-type and D262V mutant SH-SY5Y cells. Bars represent the ratio of red J-aggregates (Ex 530 nm/Em 595 nm) to green monomers (Ex 480 nm/Em 530 nm) under basal conditions and following a 4 h treatment with the uncoupler FCCP at 100 μ M. FCCP serves as a positive control for maximal membrane depolarization.

mitochondrial fragmentation and an increased presence of punctate organelles (Fig. 5A), indicating a clear correlation between disrupted mitochondrial structure and a respiratory deficit. This defect in mitochondrial function might contribute to the death of motor neurons that is characteristic of ALS. Disruption of pre-mRNA splicing patterns may contribute to slow growth of the cells and the disruption of mitochondrial function.

Because respiratory chain impairment is tightly coupled with changes in the electrochemical gradient, we next assessed whether this respiratory deficit was accompanied by a loss of mitochondrial membrane potential ($\Delta\Psi_m$) using ratiometric JC-1 staining (Fig. 5C). Under basal conditions, D262V mutant cells exhibited a significant decrease in the red-to-green (J-aggregate to monomer) fluorescence ratio, indicative of basal mitochondrial depolarization. Dissipation of the membrane potential using the uncoupler FCCP abolished these differences, reducing the ratiometric signal to equivalent minimums across both genotypes and confirming the specificity of the assay. Together, these findings demonstrate that structural fragmentation and bioenergetic exhaustion are deeply intertwined in the mutant cells, a defect that may ultimately contribute to the motor neuron death characteristic of ALS.

While these data establish a profound state of bioenergetic failure, the upstream molecular triggers driving this mitochondrial dysfunction remained unclear. Disruption of pre-mRNA splicing patterns represents one potential pathway and might be related to reduced ATP production by defective mitochondria. However, our earlier observations also pointed toward localized translational defects. Having confirmed that the translational dysregulation of MT-CO1 results in protein accumulation

while MT-ND1 levels remain stable (Supplemental Fig. S2A), we investigated whether this increase stemmed from a general induction of mitochondrial biogenesis. To evaluate this, we examined levels of the mitochondrial poly(A) polymerase PAPOLG, a key regulator of mitochondrial gene expression and transcript stability. Immunoblotting revealed that PAPOLG levels were unchanged in the D262V mutant cells (Supplemental Fig. S2B, top). These results suggest that MT-CO1 accumulation is independent of the mitochondrial polyadenylation machinery and does not reflect an overall increase in mitochondrial mass, but rather a transcript-specific translational effect.

This directed our attention toward a potential failure in organelle clearance. Consistent with the hypothesis of a proteostatic collapse, immunoblot analysis of the autophagic marker LC3B showed a significant 25% decrease in hnRNPA1 D262V mutant cells (Supplemental Fig. S2B, bottom). This reduction suggests that the accumulation of specific mitochondrial subunits is exacerbated by impaired LC3B-mediated turnover, preventing the effective clearance of dysfunctional mitochondrial components via mitophagy. Collectively, these findings indicate that the hnRNPA1 D262V mutation induces a systemic failure, possibly linking altered translation kinetics with a downstream defect in autophagic flux, ultimately leading to the fragmentation and metabolic collapse of the mitochondrial network.

Discussion

Amyotrophic lateral sclerosis (ALS), a prevalent adult-onset neurodegenerative disorder affecting motor neurons,

leads to progressive paralysis and respiratory failure. Genetic investigations have identified mutations in RNA binding proteins (RBPs), including FUS (Kwiatkowski et al. 2009; Vance et al. 2009), TIA1 (Mackenzie et al. 2017), TARDBP (TDP-43) (Johnson et al. 2014), hnRNPA1 (Kim et al. 2013; Liu et al. 2016; Beijer et al. 2021; Milicevic et al. 2022), and hnRNPA2/B1 (Liu et al. 2016; Beijer et al. 2021; Milicevic et al. 2022), as well as hexanucleotide repeat expansions in the *C9orf72* gene associated with RNA toxicity through RBP sequestration and aggregation (Cooper-Knock et al. 2014; Conlon and Manley 2017; Balendra and Isaacs 2018; Milicevic et al. 2022), underscoring the pivotal role of RBP-encoding genes in ALS etiology. It is clear from these studies that changes in RNA metabolism and activity are linked to the neuronal death observed in ALS. hnRNPA1 mutations have also been linked to multiple sclerosis (MS) (Salapa et al. 2024; Ansari et al. 2025).

Our focus has been on hnRNPA1, known RNA splicing factor carrying mutations linked to ALS (Liu et al. 2016; Beijer et al. 2021). Previous research from our laboratory demonstrated that human cells harboring the hnRNPA1 D262V mutation exhibited several growth defects and macroscopic cell clumping, dysregulation of stress granule dynamics, and impaired neuronal differentiation. hnRNPA1 is known to shuttle between the nucleus and cytoplasm and quantitative proteomics suggested changes in ribosomal protein interactions of the D262V protein in mutant cells (Lee and Rio 2024). We hypothesized that the slow growth observed in the hnRNPA1 D262V SH-SY5Y mutant cells might be due to changes in mRNA translation. We employed ribosome profiling (Ribo-seq) to examine whether changes in translation might be linked to the slow growth of the hnRNPA1 D262V mutant cells. While there were only limited effects on global ribosome occupancy and bulk translation rates assayed by puromycin incorporation (data not shown), our results indicated that the D262V mutation exerts its primary influence on eEF2-mediated elongation bottlenecks, supported by decreased codon usage and localized ribosome stalling. This translational-wide reduction in translation appears to be linked to the PI3K/Akt/mTOR pathway, which serves as a master sensor for cellular energy and nutrient status. We observed a significant reduction in the phosphorylation of AKT, S6K1, and 4E-BP1, key components in this signaling pathway. Importantly, the suppression of the S6K1 axis was linked to an increase in the inhibitory phosphorylation of eukaryotic elongation factor 2 (p-eEF2; Thr56), driving this kinetic elongation bottleneck. Since eEF2 is the primary GTPase driving ribosomal translocation, its inactivation provides an explanation for the global reduction in translation observed by ribosome profiling. We also observed a profound collapse in STAT3 signaling and a significant decrease in the LC3B autophagy marker protein levels. Because STAT3 and mTORC1 coordinate the autophagic machinery, their joint suppression in D262V mutant cells appears to cripple mitophagy. This global alteration of translation levels provides a plausible mechanistic explanation for the severe proliferative defects observed here and in our previous study (Lee and Rio 2024).

Because hnRNPA1 is known to be RNA binding splicing factor, we performed deep RNA-seq analysis on undifferentiated hnRNPA1 D262V SH-SY5Y cells, which were used in the ribosome profiling experiments. We identified 6329 significantly different alternative splicing events in the mutant cells. Functional enrichment analysis of the differentially spliced gene transcripts identified disruptions in synaptic homeostasis, specifically through the dysregulation of the glutamate receptor signaling pathway and excitatory postsynaptic potentials, as well as RNA biosynthetic processes and polymerase II activity, cilium assembly, the rho GTPase cycle and axon guidance indicating a broad impact of hnRNPA1 affecting many pathways (Kim et al. 2013; Gueroussov et al. 2017; Lee and Rio 2024). These results indicate that alternative splicing extensively targets pathways essential for neuronal homeostasis. We also analyzed differential gene expression, which revealed that the D262V mutation causes many fewer transcript abundance changes than alternative splicing events. This disproportionate ratio, consistent with our findings in differentiated neurons (Lee and Rio 2024), suggests that the D262V mutation has its primary impact on the alteration of pre-mRNA processing patterns rather than on steady-state mRNA levels. However, interestingly, among the DEGs, we observed changes in the elevated levels of ESCRT-related genes, which provide a molecular link to mitochondrial dysfunction. The ESCRT plays a major role in autophagy (Zhen et al. 2020) but also in mitochondrial health (Horbay et al. 2025) by repairing the inner mitochondrial membrane and maintaining cristae organization (Prashar et al. 2024).

Our ribosome profiling analysis revealed divergent translational signatures among some mitochondrially-encoded transcripts. The slow growth of the mutant cells might be linked to energy production. Using confocal microscopy, we found that while wild-type SH-SY5Y cells maintained elongated, interconnected mitochondrial cristae, the hnRNPA1 D262V mutant cells exhibited marked mitochondrial fragmentation and a punctate morphology. Assays for mitochondrial function showed respiratory, oxygen consumption and membrane potential defects in the D262V mutant cells compared to the wild-type cells. This respiratory failure correlates directly with the observed mitochondrial fragmentation, a hallmark of cellular distress (Sheridan and Martin 2010). These results suggest that hnRNPA1 D262V-driven disruptions culminate in a catastrophic loss of mitochondrial function, likely contributing to the motor neuron death characteristic of ALS.

By combining high-resolution Ribo-seq with deep transcriptomic profiling in an undifferentiated human cell line, our study addresses two critical knowledge gaps in the fields of RNA-binding protein (RBP) pathology and neurodegeneration. While prior studies have typically evaluated RBP mutations in mature or actively differentiating neurons, those systems inherently confound primary, mutation-driven defects with the complex gene expression programs required for cellular maturation. By analyzing the hnRNPA1 D262V mutation in undifferentiated cells, we uncovered fundamental alterations of RNA

processing, translational and metabolic networks that occur independently of neuronal differentiation. Most importantly, our experiments establish a novel link between the hnRNPA1 D262V mutation and mitochondrial dysfunction. While these insights enhance our understanding of hnRNPA1 pathology, several limitations of the current study should be noted. The SH-SY5Y cell model provides a highly tractable, human cell culture system to uncouple cell-autonomous RNA-based effects due to mutations in hnRNPA1. However, this system lacks the multicellular architecture, non-cell-autonomous glial cell interactions and aging effects characteristic of the human spinal cord or patient-derived primary motor neurons. Nonetheless, this study captures the early molecular homeostasis changes and organelle vulnerabilities linked to hnRNPA1 inherited mutations found in some ALS patients.

In conclusion, our findings redefine the hnRNPA1 D262V mutation as a multimodal driver of cellular failure that extends far beyond changes in RNA splicing. We document a vast collection of over 6000 alternative splicing events, including critical disruptions to cilium assembly, RHO GTPase signaling and the glutamate receptor pathway, with few changes in steady-state levels of gene expression. This unique production of aberrant mRNA isoforms coupled with a systemic, mTOR/eEF2-mediated reduction in translational flux, creates a state of profound proteomic stress. When combined with localized translational bottlenecks in essential mitochondrial transcripts this coordinated regulatory failure results in the catastrophic fragmentation and functional mitochondrial collapse. By linking the hnRNPA1 LCD mutation to the convergence of altered RNA processing defects, kinetic translational bottlenecks and a systemic failure of mitochondrial function, our findings provide a more comprehensive view of how these molecular perturbations drive the progressive neurodegeneration characteristic of ALS.

Materials and methods

Cells and generation of hnRNPA1 D262V cell lines

Human SH-SY5Y cells (American Type Culture Collection) were maintained at 37°C in DMEM/F-12 (Gibco 11320082) medium supplemented with 10% FBS and 1% penicillin/streptomycin. Generation of genome-edited hnRNPA1 D262V mutant SH-SY5Y cell line was described previously (Lee and Rio 2024).

Ribosome-associated RNA (Ribo)-seq

SH-SY5Y hnRNPA1 wild-type or homozygous mutant D262V cells (1.5×10^6 cells) were plated 48 h prior to harvest. Ribosome-associated RNA (Ribo)-seq was performed by Eclipsebio (eclipsebio.com). Cells were washed twice with $1 \times$ PBS and pelleted. For each sample, total RNA was isolated and split: 1 μ g was heat-fragmented for RNA-seq, and 9 μ g was RNase I-digested to obtain ribosome-protected fragments (RPFs). Both underwent rRNA

depletion and adapter ligation, followed by reverse transcription, PCR library preparation, and sequencing on a NextSeq 2000. Sequencing data were processed using Eclipsebio's pipeline (v1). UMIs were pruned (UMI-tools v1.1.1), and 3' adapters were trimmed (Cutadapt v3.2). Reads were mapped to repeat/rRNA databases and then to hg38 (STAR v2.7.7a), retaining only uniquely mapping reads. PCR duplicates were removed (UMI-tools v1.1.1). Gene coverage and ribosome occupancy (RPF coverage/RNA-seq coverage) were calculated using Eclipsebio's algorithms. Differential expression and occupancy were assessed using DESeq2 (Love et al. 2014). Transcriptome alignment (mudskipper v0.1.0) enabled codon usage, stall site, UTR loading, and read-through analyses (riboWaltz [Lauria et al. 2018] and custom scripts). ORF detection was performed on genome alignments (ribotricer v1.3.3 [Lauria et al. 2018]). GOrilla (Eden et al. 2009) was used for identifying and visualizing enriched GO terms for the differentially expressed genes.

To resolve site-specific changes in ribosome distribution, we employed the RiboDiPA pipeline (v1.16.0 [Li et al. 2020]), which utilizes a sliding-window (bin-based) approach to detect shifts in the positional distribution of ribosome protected fragments (RPFs). The identical adapter-trimmed libraries utilized for the DRO efficiency analysis were subjected to differential pattern analysis using the RiboDiPA R package (v1.16.0) (Li et al. 2020). Positional analysis across transcript bins was executed via the master wrapper function `ribodipa()` under default parameters. The spatial distribution of RPFs (represented by P-site counts) across these bins was evaluated across biological replicates under wild-type and mutant conditions. Transcript annotations were derived from GENCODE release 41 (GRCh38) and default package thresholds to ensure sufficient read coverage for robust spatial binning. P-site offsets were calculated per RPF length, and differential patterning was assessed using a generalized linear model (GLM) with a permutation-based significance test. *P*-values were corrected for multiple testing using the Benjamini-Hochberg procedure, with a global *q*-values, with *q*-value < 0.05 defined as the threshold for significant translational dysregulation.

Immunoblot analyses

Cells were incubated in lysis buffer (1% Triton X-100, 10 mM β -glycerol phosphate, 10 mM sodium pyrophosphate, 4 mM EDTA, 40 mM HEPES pH 7.4, supplemented with Pierce protease inhibitor tablets) for 10 min at 4°C with rocking to ensure complete lysis. Lysates were harvested and cleared by centrifuging at 17,000g for 10 min at 4°C. Protein concentrations in the supernatants were measured by Bradford assay. Equivalent amounts of each sample (10 μ g protein) were subjected to immunoblotting with the following antibodies: phospho-p70 S6 kinase (Thr389; 108D2) rabbit mAb (1:1000 dilution; Cell Signaling Technologies 9234T), p70 S6 kinase (49D7) rabbit mAb (1:1000 dilution; Cell Signaling Technologies 2708T), phospho-Akt (Ser473; D9E) XP rabbit mAb (1:1000 dilution; Cell Signaling Technologies 4060T),

Akt (pan) 40D4 antimouse mAb (1:1000 dilution; Cell Signaling Technologies 2920T), Stat3 (D3Z2G) rabbit mAb (1:1000 dilution; Cell Signaling 12640), phospho-Stat3 (Tyr705; D3A7) rabbit mAb (1:1000 dilution; Cell Signaling Technologies 9145), Stat5 (D2O6Y) rabbit mAb (1:1000 dilution; Cell Signaling Technologies 94205), phospho-Stat5 (Tyr694; D47E7) rabbit mAb (1:1000 dilution; Cell Signaling Technologies 4322), phospho-eEF2 (Thr56) rabbit (1:1000 dilution; Cell Signaling Technologies 2331), anti-eEF2 rabbit (1:1000 dilution; Abcam ab33523), phospho-4E-BP1 (Thr37/46) rabbit mAb (1:1000 dilution; Cell Signaling Technologies 2855), 4E-BP1 (53H11) rabbit mAb (1:1000 dilution; Cell Signaling Technologies 9644), and β -actin (1:3000 dilution; Sigma A2228). For phosphorylation site-specific antibodies, antibodies were diluted in 5% BSA in PBS-Tween with overnight incubation with primary antibodies.

Alternative splicing RNA-seq library preparation and sequencing

RNA isolation was performed using the RNeasy minikit (Qiagen 74104) followed by 30 min of DNase treatment (Ambion AM2238) at 37°C. Poly(A)⁺ RNA transcripts were isolated [NEBNext poly(A) mRNA magnetic isolation module; New England Biolabs E7490] from 1 μ g of total RNA for RNA library preparation and sequencing using the NEBNext Ultra directional RNA library preparation kit for Illumina (New England Biolabs E7420S) according to the manufacturer's instructions. The samples were sequenced on an Illumina HiSeq 4000 NovaSeq SP with 100 bp paired-end reads at the Vincent J. Coates Genomics Sequencing Laboratory at the University of California, Berkeley.

Analysis of pre-mRNA splicing patterns using JUM and differential expression analysis using DESeq2

Pre-mRNA splicing analysis using JUM 2.0.2 software to detect pre-mRNA splicing patterns was performed as described before (Lee et al. 2018; Lee and Rio 2024). A *P*-value of 0.05 was used as the statistical cutoff for differentially spliced alternative splicing events. To remove low-expressed genes prior to differential expression analysis, genes were retained if they reached a minimum threshold of 10 raw reads per sample (Chen et al. 2016). For differential expression analysis, uniquely mapped reads were mapped to hg38 using STAR_2.5.3a_modified version using --outFilterMultimapNmax 1. Read counts were calculated using the HTSeq script htseq-count with the following specifications: -s yes -r pos -f. Differential gene expression analysis was performed using DESeq2 (Love et al. 2014), only genes with at least 10 reads across all samples were considered for further analysis.

Confocal microscopy

SH-SY5Y cells (3×10^5 cells) expressing wild-type hnRNAP1 or the D262V mutant were seeded onto glass coverslips in 24 well plates and allowed to adhere for

24 h. Cells were incubated with 250 nM MitoTracker Deep Red for mitochondrial labeling (Deep Red, Invitrogen M22426) in prewarmed complete growth medium for 45 min at 37°C in a 5% CO₂ incubator. Following incubation, the staining medium was removed, and cells were washed twice with phosphate-buffered saline (PBS). Cells were fixed in 4% paraformaldehyde solution and incubated for 10 min at 37°C, followed by methanol fixation for 10 min. The cells were blocked using 6% BSA for 1 h at room temperature and mounted with DAPI-containing VectaShield mounting medium (Vector Laboratories). The images were obtained at the College of Natural Resources Biological Imaging Facility, University of California, Berkeley.

Oxygen consumption rates assay

Approximately 50,000 SH-SY5Y cells expressing either wild-type or the D262V mutant cells were seeded onto Agilent Technologies Seahorse XFp cell culture miniplates. Oxygen consumption rates (OCR) were measured following an overnight period of cell proliferation to ensure adherence. The Mito stress test was performed according to the manufacturer's instructions using an Agilent Seahorse XF analyzer. OCR serves as a direct measurement of the rate at which cells consume oxygen, the terminal electron acceptor in the mitochondrial electron transport chain (ETC). We specifically evaluated the spare respiratory capacity, defined as the difference between maximal and basal respiration, which indicates the ability of the cells to meet increased metabolic energy demands.

Data deposition

The data reported here have been deposited in the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE342413>).

Competing interest statement

The authors declare no competing interests.

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Author contributions: D.C.R. supervised the study. Y.J.L. established the SH-SY5Y hnRNPA1 D262V mutant cell lines and prepared the cells used for ribosome profiling experiments. Y.J.L. performed the GO enrichment and RiboDiPA analysis of the ribosome profiling data, confocal microscopy imaging, and immunoblot analysis. Additionally, Y.J.L. conducted the bulk RNA-seq library preparation and subsequent bioinformatics analysis, including alternative splicing and differential expression profiling, generating all figures and tables, including all **Supplemental Material**, with the sole exception of Figure 5B. K.S. performed the OCR assays using the Agilent Seahorse platform and generated Figure 5B. Y.J.L. and D.C.R. wrote the initial draft of the manuscript. All authors contributed to and edited the final version of the manuscript.

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