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# SYSTEMS-LEVEL INSIGHTS INTO MOLECULAR MECHANISMS OF AMYOTROPHIC LATERAL SCLEROSIS USING INTEGRATIVE NETWORK AND PATHWAY ENRICHMENT ANALYSIS

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**Abstract:**

*Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disorder marked by selective motor neuron degeneration and significant molecular heterogeneity. Understanding the complex interactions among ALS-associated genes is essential for elucidating disease mechanisms and identifying therapeutic targets. In this study, an integrative bioinformatics approach was applied to explore the functional organization of ALS-related genes. A curated gene set was analyzed using STRING (v11.5) to construct a high-confidence protein–protein interaction (PPI) network, followed by functional association analysis using GeneMANIA. Network topology parameters, including node degree and clustering coefficient, were evaluated to identify key hub genes and assess network connectivity. Functional and pathway enrichment analyses were conducted using STRING and Enrichr to determine significantly enriched biological processes and signaling pathways. The PPI network showed significant enrichment, indicating strong biological interconnectivity among ALS-associated genes. Hub genes were primarily involved in RNA metabolism, proteostasis, mitochondrial dynamics, and cytoskeletal regulation. Enrichment results highlighted pathways related to autophagy, MAPK signaling, neuroinflammation, vesicle trafficking, and cellular stress responses. GeneMANIA analysis further supported functional modules associated with axonal transport and mitochondrial quality control. Overall, this study provides a systems-level understanding of ALS pathogenesis and identifies potential targets for therapeutic intervention.*

**Keywords:** *Amyotrophic Lateral Sclerosis, Bioinformatics, Protein–Protein Interaction, Hub Genes, Pathway Enrichment, Neurodegeneration, Systems Biology.*

**Introduction**

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a neurological disorder that affects motor neurons and leads to muscle weakness, paralysis, and ultimately respiratory failure (1,2). ALS is diagnosed clinically through identifying both upper motor neuron symptoms such as hyperreflexia, impaired rapid movements, and increased muscle tone, and lower motor neuron manifestations including fasciculations and muscle atrophy (1). Despite extensive research, the molecular mechanisms underlying ALS are not completely understood (3,4). Historically, identifying genetic mutations involved in ALS development has been difficult because the disease is rare and only small numbers of patients have a documented family history (5). The disease exhibits marked clinical and genetic heterogeneity, with both sporadic and familial forms (6).

Genetically, ALS exhibits significant heterogeneity. Approximately 5–10% of cases are familial, while the majority are sporadic (5,6). Mutations in several genes, including SOD1, TARDBP, FUS, and C9orf72, have been implicated in ALS pathogenesis; however, these genes alone do not fully explain the variability observed in disease onset, progression, and severity (4,7).

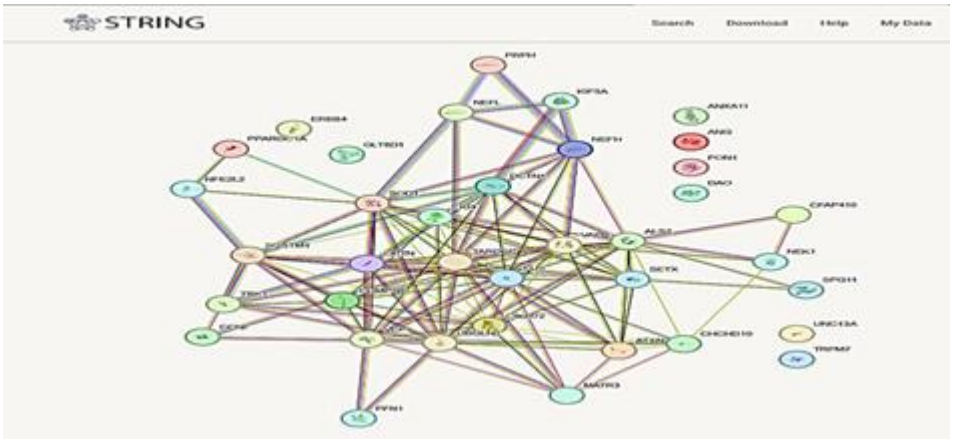
Recent findings indicate that ALS represents a convergence of multiple dysregulated biological processes, including protein homeostasis, defective autophagy, cytoskeletal instability, RNA metabolism, mitochondrial abnormalities, and neurotrophic signaling (3,8). Together, these processes contribute to the systems-level complexity of ALS pathology, which cannot be fully explained by traditional single-gene approaches (9). To explore the molecular mechanisms of amyotrophic lateral sclerosis (ALS) at a systems level, a variety of bioinformatics tools and databases were used in combination to provide information on gene–disease associations, protein interactions, functional associations, and pathway-level insights (10,11). Each database was selected to provide information on a specific aspect of gene analysis in ALS.

**Methodology**

***1) DisGeNet for gene set***

ALS-associated genes were retrieved from the DisGeNET database using the search term “Amyotrophic Lateral Sclerosis”. Only protein-coding human genes were included, while non-coding elements, non-human entries, and duplicates were excluded (12). A stringent Gene–Disease Association score ≥ 0.9 was applied to retain high-confidence genes (10,11). This filtering produced 37 ALS-associated genes for downstream network and enrichment analyses (13).

***2) Protein–protein interaction network construction and functional enrichment analysis***



The curated ALS-associated gene set was subjected to protein–protein interaction (PPI) network analysis using the STRING database (v11.5). Interactions were restricted to *Homo sapiens*, and a high-confidence interaction score threshold (≥ 0.7) was applied to ensure robust and biologically meaningful associations. Only experimentally validated and curated database interactions were prioritized to minimize false-positive connections (13).

Network topology parameters, including node degree, clustering coefficient, and PPI enrichment p-value, were assessed to evaluate the biological connectivity of the gene set relative to random expectation (10). To further characterize the biological relevance of the network, functional enrichment analyses were performed within STRING. Gene Ontology (GO) enrichment analysis was conducted across three categories: Biological Process (BP), Molecular and Function (MF).

***3) Functional association network analysis using GeneMANIA***

To further investigate functional relationships among the curated ALS-associated genes, network analysis was performed using the GeneMANIA web server. The gene list was submitted with *Homo sapiens* selected as the organism of interest. Default parameters were applied, including automatic network weighting and the addition of up to 20 related genes to enhance functional context (14).

GeneMANIA integrates multiple data sources, including physical interactions, co-expression, genetic interactions, pathway associations, co-localization, and shared protein domains (14). The resulting composite network was examined to determine the relative contribution of each interaction category and to identify central hub genes (10,11).

***4) Reactome pathway mapping analysis***

To obtain mechanistic insights beyond enrichment-based tools, pathway over-representation analysis was performed using the Reactome Pathway Browser (15). The curated ALS-associated gene set was submitted to the Reactome Analyze Data module with *Homo sapiens* selected as the reference organism (15). Statistical significance was assessed using false discovery rate (FDR)-corrected p-values. Pathways were ranked according to FDR, and biologically relevant parent pathway categories were examined to identify major functional modules (4,8).

***5) HUB genes analysis using Enrichr***

Enrichr was used to identify key molecular pathways enriched within the selected gene set and to determine which genes exhibit functional prominence in ALS pathogenesis (16). Enrichment significance was assessed using adjusted p-values and combined scores (which integrate the p-value and Z-score), thereby improving the reliability and biological interpretability of the results. Clustergram visualizations were generated to evaluate gene-term associations across enriched pathways. Particular emphasis was placed on identifying genes demonstrating functional recurrence defined as genes appearing across multiple significantly enriched pathways and across independent databases (15,16). The frequency of gene occurrence across these non-redundant pathway libraries was recorded as a metric of functional centrality. Genes exhibiting repeated enrichment across pathways related to autophagy, mitophagy, oxidative stress response, protein aggregation, and neurodegeneration were classified as functional hub genes (4,8,17). This recurrence-based approach enabled the identification of biologically central regulators potentially contributing to the complex and multifactorial molecular mechanisms underlying ALS pathogenesis (3,9).

***6) ALS mutation validation using ALSoD***

To validate the clinical relevance of the identified hub genes, mutation data were retrieved from the ALS Online Database (ALSoD) (18). Each high-confidence gene identified through network and pathway analyses was queried individually to assess reported ALS-associated mutations, inheritance patterns, and mutation types. Genes with documented familial or sporadic ALS mutations were considered clinically validated (ALSoD, n.d.). This step ensured that the computationally identified genes corresponded to experimentally and clinically reported ALS-associated variants (7,18).

**Results**

***1. STRING***

***1.1. Protein–Protein Interaction (PPI) network analysis***

A protein–protein interaction (PPI) network was constructed using the STRING database (v11.5) with *Homo sapiens* selected and a high-confidence interaction score threshold (≥ 0.7) (13). The network comprised 37 nodes and 133 edges, substantially exceeding the 4 edges expected for a random gene set of similar size. The PPI enrichment p-value ($< 1.0 \times 10^{-16}$) confirmed that the observed interactions were significantly greater than chance, consistent with established principles of network enrichment analysis (10,11). The average node degree was 7.19, and the average local clustering coefficient was 0.573, indicating strong interconnectivity and modular organization (11).

Topological analysis revealed a densely connected core cluster. Based on degree centrality (≥ 8), seventeen genes were identified as hub genes. TARDBP showed the highest connectivity, followed by ALS2, FUS, SOD1, UBQLN2, C9orf72, VAPB, VCP, OPTN, and SQSTM1. These hubs represent central interaction nodes within the ALS-associated network and are well-established contributors to ALS molecular pathology (4,6,7).

Functional clustering highlighted biologically coherent modules involving RNA metabolism (e.g., TARDBP and FUS) (8), autophagy and proteostasis (e.g., TBK1, SQSTM1, OPTN, VCP, UBQLN2) (19), axonal transport and cytoskeletal integrity (e.g., DCTN1, NEFL, NEFH, KIF5A) (20), and oxidative stress pathways (e.g., SOD1, NFE2L2) (21,22). Peripheral genes displayed limited connectivity, suggesting more specialized roles within the broader disease network (10).

Overall, the high edge density, strong enrichment significance, and presence of highly connected hubs indicate that ALS pathogenesis involves disruption of an integrated molecular network rather than isolated gene effects (10,11).

***1.2 Functional enrichment analysis***

Gene Ontology – Biological Process (GO-BP)

Biological Process (Gene Ontology)

| GO-term | description | count in network | strength | ▼ signal | false discovery rate |
|---|---|---|---|---|---|
| GO:0050808 | Synapse organization | 9 of 298 | 1.21 | 1.3 | 1.05e-05 |
| GO:0060052 | Neurofilament cytoskeleton organization | 3 of 9 | 2.25 | 0.98 | 0.0026 |
| GO:0007528 | Neuromuscular junction development | 4 of 42 | 1.71 | 0.93 | 0.0026 |
| GO:0006996 | Organelle organization | 27 of 3470 | 0.62 | 0.78 | 4.00e-09 |
| GO:0006914 | Autophagy | 7 of 303 | 1.09 | 0.75 | 0.0026 |

(more ...)

The enriched biological processes primarily reflect neuronal structural organization and protein homeostasis mechanisms. Strong enrichment of synapse organization and neuromuscular junction development indicates that the gene set is highly involved in maintaining motor neuron connectivity and synaptic integrity (20,23).

Enrichment of neurofilament cytoskeleton organization highlights the importance of intermediate filament stability in ALS pathogenesis (4,8).

Notably, autophagy and organelle organization were also significantly enriched, suggesting disruption of intracellular trafficking, mitochondrial regulation, and protein degradation pathways (17,19). These findings collectively support the concept that ALS involves coordinated impairment of synaptic maintenance, cytoskeletal stability, and proteostatic control (3,9).

***1.3 Gene Ontology – Molecular Function (GO-MF)***

| Molecular Function (Gene Ontology) | | | | | |
|---|---|---|---|---|---|
| GO-term | description | count in network | strength | signal | false discovery rate |
| GO:0031593 | Polyubiquitin modification-dependent protein binding | 4 of 54 | 1.6 | 0.77 | 0.0068 |
| GO:0042802 | Identical protein binding | 17 of 2144 | 0.63 | 0.6 | 0.00020 |
| GO:0099184 | Structural constituent of postsynaptic intermediate filament cytosk... | 2 of 3 | 2.55 | 0.57 | 0.0336 |
| GO:0005515 | Protein binding | 31 of 7242 | 0.36 | 0.42 | 2.86e-05 |
| GO:0019899 | Enzyme binding | 14 of 2084 | 0.55 | 0.39 | 0.0159 |

Molecular function analysis demonstrated significant enrichment of polyubiquitin modification-dependent protein binding and general protein binding activities. These results indicate that many genes within the network participate in ubiquitin-mediated signaling and protein–protein interaction complexes (8,19).

The enrichment of structural constituents of the postsynaptic intermediate filament cytoskeleton further supports cytoskeletal destabilization as a key feature of ALS (20). Together, these findings emphasize the central role of protein interaction networks and ubiquitin-dependent regulatory mechanisms in disease progression (9,10).

***1.4 KEGG pathway enrichment***

| KEGG Pathways | | | | | |
|---|---|---|---|---|---|
| pathway | description | count in network | strength | signal | false discovery rate |
| hsa05014 | Amyotrophic lateral sclerosis | 25 of 350 | 1.58 | 5.31 | 2.11e-32 |
| hsa04137 | Mitophagy - animal | 3 of 64 | 1.4 | 0.48 | 0.0449 |

Pathway analysis demonstrated highly significant enrichment of the Amyotrophic lateral sclerosis (hsa05014) pathway as defined by the KEGG database. The magnitude of enrichment confirms that the constructed interaction network captures established ALS-associated molecular mechanisms (4,9).

Additionally, enrichment of the Mitophagy – animal (hsa04137) pathway implicates mitochondrial quality control processes in the disease network (15,17). This supports growing evidence that mitochondrial dysfunction and impaired mitophagy contribute to motor neuron degeneration (3,8).

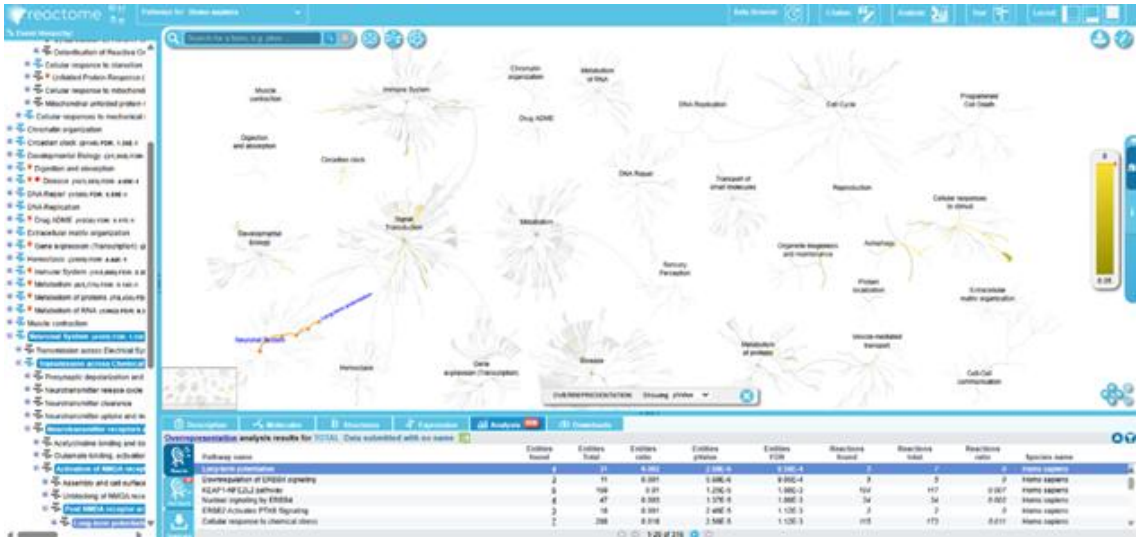
***2. GeneMANIA***

GeneMANIA analysis revealed a densely interconnected functional association network dominated by physical interactions (58.13%), followed by co-expression (17.35%) and pathway-based interactions (11.14%) (10,14). This indicates that the ALS-associated genes form tightly connected molecular complexes and participate in shared biological pathways.

Several well-established ALS-related hub genes, including TBK1, TARDBP, SOD1, FUS, VCP, C9orf72, and KIF5A, occupied central positions within the network (6,7). The connectivity pattern highlighted modules related to autophagy regulation, axonal transport, RNA processing, and cytoskeletal organization (17,20).

The strong predominance of physical interaction evidence supports the existence of functional protein complexes underlying ALS pathology. Integration of predicted and pathway-based associations further reinforced the involvement of mitochondrial quality control and neuroinflammatory signaling mechanisms (4,8,23).

***3. Reactome***



Reactome analysis identified significant enrichment of pathways associated with autophagy and mitochondrial quality control, including Macroautophagy (FDR = $2.51 \times 10^{-3}$), Autophagy (FDR = $2.84 \times 10^{-3}$), and PINK1-PRKN mediated mitophagy (FDR = $2.84 \times 10^{-3}$) (15,17). These findings reinforce the central role of impaired autophagic flux and defective mitochondrial clearance in ALS pathogenesis (3,8). Pathways related to oxidative stress regulation were also significantly enriched, including the KEAP1–NFE2L2 pathway (FDR = $1.08 \times 10^{-3}$) and Nuclear events mediated by NFE2L2 (FDR = $1.66 \times 10^{-3}$), indicating disruption of antioxidant defense mechanisms (21,22). Additionally, receptor tyrosine kinase signaling pathways involving ERBB family members were enriched, suggesting alterations in neuronal survival and signaling cascades (4,9).

Collectively, Reactome mapping supports a mechanistic framework in which ALS-associated genes converge on autophagy dysfunction, oxidative stress response impairment, and altered neuronal signaling pathways (24).

***4. Enrichr***

Functional enrichment analysis using Enrichr mapped the prioritized ALS-associated genes to statistically significant biological modules primarily involving autophagy dysregulation, mitophagy impairment, oxidative stress response, and proteostasis failure (8,16).

Cross-database validation across KEGG 2026, Reactome 2024, and WikiPathways 2024 Human consistently identified TBK1, SQSTM1, OPTN, SOD1, VCP, and NFE2L2 as the most recurrent and biologically robust hub genes. Their repeated occurrence across independent pathway repositories indicates a central regulatory role within the molecular framework of Amyotrophic Lateral Sclerosis (ALS) (4,9).

***Mechanistic insights from enrichment clusters***

- Autophagy and Selective Cargo Recognition Failure: Significant enrichment of selective autophagy and macroautophagy pathways highlights TBK1, SQSTM1, and OPTN as key mediators of impaired autophagic flux. Their recurrence across multiple enriched terms suggests defective clearance of protein aggregates and damaged organelles as a core pathogenic mechanism in ALS (8,17).
- Mitophagy and Mitochondrial Dysfunction: Enrichment of mitophagy-associated pathways implicates TBK1, OPTN, and VCP in disrupted mitochondrial quality control. Given the high metabolic demand of

motor neurons, impairment of mitochondrial turnover likely contributes directly to neuronal vulnerability and degeneration (3,17).

- Oxidative Stress and Redox Imbalance: Pathways associated with reactive oxygen species detoxification prominently featured SOD1 and NFE2L2, reinforcing the role of oxidative damage and compromised antioxidant defense in ALS progression (21,22). RNA Metabolism and Protein Aggregation: The enrichment of neurodegeneration-related pathways involving FUS and TARDBP further supports the contribution of RNA-binding protein dysfunction and cytoplasmic aggregation to motor neuron pathology (4,7). Integrated Molecular Interpretation: Collectively, the enrichment profile indicates that ALS pathogenesis is characterized by a coordinated breakdown of cellular quality-control systems, encompassing autophagy, mitochondrial surveillance, redox regulation, and proteostasis (8,9).

The consistent recurrence of TBK1, SQSTM1, OPTN, SOD1, VCP, and NFE2L2 across multiple independent enrichment databases suggests that these genes function as regulatory bottlenecks within the ALS molecular network (4,11). Importantly, this designation reflects functional centrality based on enrichment recurrence, rather than topological centrality derived from protein–protein interaction degree (10). These findings prioritize key molecular nodes that may serve as strategic targets for therapeutic interventions aimed at restoring cellular homeostasis in ALS (25).

***5. ALSoD***

| Gene | Mutation Type | Inheritance | Evidence Type |
|---|---|---|---|
| SOD1 | Missense | Autosomal Dominant | Familial ALS |
| TARDBP | Missense | Autosomal Dominant | Familial + Sporadic |
| FUS | Frameshift / Missense | Autosomal Dominant | Familial |
| TBK1 | Loss-of-function | Autosomal Dominant | Familial |
| C9orf72 | Hexanucleotide repeat expansion | Autosomal Dominant | Familial |

The data showed computationally identified genes corresponding to experimentally and clinically reported ALS-associated variants (7,18)

**Discussion**

Amyotrophic Lateral Sclerosis (ALS) is increasingly recognized as a complex neurodegenerative disorder driven by the interaction of multiple dysregulated molecular pathways rather than a single causative mechanism (3,4). In this study, an integrative bioinformatics strategy combining gene–disease association analysis, protein–protein interaction (PPI) network construction, functional enrichment, pathway mapping, and mutation validation was applied to investigate the molecular architecture underlying ALS (10,11).

The STRING-based PPI network demonstrated significant enrichment ($p = 6.21 \times 10^{-11}$), indicating that ALS-associated genes form a biologically interconnected network rather than random associations (10,13). Several hub genes, including SOD1, TARDBP, TBK1, FUS, and C9orf72, showed high connectivity within the network, suggesting their central regulatory roles in maintaining neuronal homeostasis (6,7). These genes are involved in key cellular processes such as RNA metabolism, protein quality control, cytoskeletal organization, and mitochondrial regulation, all of which are known to be disrupted in ALS (4,8).

Functional enrichment analyses revealed significant overrepresentation of pathways associated with organelle organization, autophagy, axonal transport, and cellular stress responses (9). The enrichment of autophagy and lysosomal transport pathways across STRING, Enrichr, and Reactome analyses highlights impaired proteostasis

as a major contributor to motor neuron degeneration (16,17). Dysfunctional autophagy can lead to the accumulation of misfolded proteins such as mutant SOD1 and TDP-43 aggregates, which are characteristic pathological features of ALS (7,8).

Mitochondrial dysfunction also emerged as a key pathogenic mechanism. Reactome pathway analysis identified enrichment of mitophagy and PINK1–PRKN mediated mitochondrial clearance pathways, suggesting defective removal of damaged mitochondria (15,17). Since motor neurons have high metabolic demands, impaired mitochondrial quality control may increase oxidative stress and promote neuronal degeneration (3,4).

Additionally, oxidative stress pathways involving the KEAP1–NFE2L2 antioxidant response system were significantly enriched, indicating disruption of cellular defense against reactive oxygen species (21,22). Enrichment of axonal transport pathways involving genes such as KIF5A, DCTN1, NEFL, and NEFH further suggests that impaired intracellular trafficking contributes to synaptic dysfunction and neuromuscular junction degeneration (20).

Although the study relies on computational datasets and requires experimental validation, the integrative approach provides a comprehensive systems-level perspective of ALS (26). Overall, the findings highlight interconnected mechanisms involving proteostasis failure, mitochondrial dysfunction, oxidative stress imbalance, RNA metabolism defects, and disrupted axonal transport, offering potential targets for future therapeutic research (3,24).

**Conclusion**

This research utilized a comprehensive bioinformatics framework to examine the molecular mechanisms associated with Amyotrophic Lateral Sclerosis (ALS) (27). The STRING database was used to construct protein–protein interaction networks, while GeneMANIA and Enrichr were used to perform functional association and pathway enrichment analyses, respectively (13,14,16). The STRING-based network exhibited significant interaction enrichment, suggesting that ALS-associated genes form a highly interconnected molecular system rather than random associations (10). Network topology analysis identified highly connected hub proteins that may act as important regulators of disease progression (11). Functional enrichment analysis revealed overrepresentation of pathways related to RNA metabolism, protein homeostasis, mitochondrial dysfunction, autophagy, and cellular stress responses (8,9). GeneMANIA results further highlighted the importance of RNA-binding proteins, axonal transport mechanisms, and mitochondrial quality control processes in ALS pathogenesis (17,20). These integrated analyses highlight interconnected molecular pathways driving ALS pathology and suggest potential therapeutic targets (24).

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