

**T.C.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL**

**REGULATORY NETWORKS OF ETS TRANSCRIPTION
FACTORS AND THEIR POTENTIAL ALS-RELATED TARGET
GENES IN AMYOTROPHIC LATERAL SCLEROSIS**

AYŞENUR KOCAMAN

**A THESIS OF MASTER OF SCIENCE
DEPARTMENT MOLECULAR BIOLOGY AND GENETICS**

ADVISOR: PROF. DR. İŞİL KURNAZ

JANUARY 2026

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LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

ETS TRANSKRİPSİYON FAKTÖRLERİNİN
REGÜLASYON AĞLARI VE AMİYOTROFİK
LATERAL SKLEROZDA ALS İLE İLGİLİ
POTANSİYEL HEDEF GENLERİ

AYŞENUR KOCAMAN

YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI: PROF. DR. IŞIL KURNAZ

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ABSTRACT

The E26 transformation-specific (ETS) transcription factor family plays a fundamental role in the regulation of various cellular functions, including differentiation, proliferation, inflammation, and apoptosis. Studies on neurons have shown that ELK1, a member of the ETS transcription factor family, plays a role in neuronal growth and survival and has a neuroprotective effect. In our laboratory's previous microarray data, it was noted that ELK1 overexpression in SH-SY5Y cells and significantly changed genes are associated with neurodegenerative diseases. ELK1 has been studied in our laboratory before in relation to Huntington and Parkinson's disease, and its neuroprotective effect has been demonstrated. Within the scope of this thesis, the effect of ELK1 in Amyotrophic Lateral Sclerosis was examined.

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that affects both upper and lower motor neurons, which control voluntary muscle movements. As with many neurodegenerative diseases characterized by neuronal dysfunction and subsequent cell death. ALS is also characterized by the accumulation of protein aggregates in cells throughout the nervous system. As ALS progresses, proteins begin to accumulate even more in the spinal motor neurons. These cytoplasmic protein aggregates are commonly seen in both sporadic and familial ALS patients. In both cases, gene mutations also play a role in the onset of the disease. Mutations in the most common genes, Cu/Zn Superoxide dismutase (SOD1), Fused in sarcoma (FUS), and TAR DNA binding protein-43 (TDP-43), result in protein accumulations in the cytoplasm. In this thesis, public transcriptomic data were used for identifying potential gene regulatory networks (GRNs) of ETS transcription factors and this regulatory network was validated in cell culture by *in vitro* ALS disease modelling. The results obtained from *in silico* and *in vitro* experiments revealed findings related to the potential regulation of ELK1 with genes associated with ALS. These findings are expected to pave the way for potential therapeutic and/or neuroprotective studies targeting ALS disease that may be developed in the future.

Key Words: Neurodegenerative Diseases, Amyotrophic Lateral Sclerosis, ETS Transcription Factors, ETS Like-1 Protein ELK1.

ÖZET

E26 dönüşümüne özgü (ETS) transkripsiyon faktörü ailesi, farklılaşma, proliferasyon, inflamasyon ve apoptoz dahil olmak üzere çeşitli hücre fonksiyonların düzenlenmesinde temel bir rol oynar. Nöronlar üzerinde yapılan çalışmalar, ETS transkripsiyon faktörü ailesinin bir üyesi olan ELK1'in nöronal büyüme ve hayatta kalmada rol oynadığını ve nöroprotektif bir etkiye sahip olduğunu göstermiştir. Laboratuvarımızın önceki mikroyarray verilerinde, SH-SY5Y hücrelerinde ELK1'in aşırı ekspresyonu ve önemli ölçüde değişen genlerin nörodejeneratif hastalıklarla ilişkili olduğu belirtilmiştir. ELK1, laboratuvarımızda daha önce Huntington ve Parkinson hastalığı ile ilişkili olarak incelenmiş ve nöroprotektif etkisi gösterilmiştir. Bu tez kapsamında, ELK1'in Amyotrofik Lateral Skleroz üzerindeki etkisi incelenmiştir.

Amyotrofik lateral skleroz (ALS), istemli kas hareketlerini kontrol eden üst ve alt motor nöronları etkileyen ilerleyici bir nörodejeneratif hastalıktır. Nöronal disfonksiyon ve ardından hücre ölümü ile karakterize edilen birçok nörodejeneratif hastalıkta olduğu gibi, ALS de sinir sistemi boyunca hücrelerde protein agregatlarının birikmesi ile karakterizedir. ALS ilerledikçe, proteinler omurilik motor nöronlarında daha da fazla birikmeye başlar. Bu sitoplazmik protein agregatları, hem sporadik hem de ailesel ALS hastalarında yaygın olarak görülür. Her iki durumda da, gen mutasyonları da hastalığın başlangıcında rol oynar. En yaygın genlerdeki mutasyonlar, Cu/Zn süperoksit dismutaz (SOD1), sarkomda füzyon (FUS) ve TAR DNA bağlayıcı protein-43 (TDP-43), sitoplazmada protein birikimlerine neden olur. Bu tezde, ETS transkripsiyon faktörlerinin potansiyel gen düzenleyici ağlarını (GRN) tanımlamak için kamuya açık transkriptomik veriler kullanılmış ve bu düzenleyici ağ, *in vitro* ALS hastalığı modellenmesi ile hücre kültüründe doğrulanmıştır. *In silico* ve *in vitro* deneylerden elde edilen sonuçlar, ELK1'in ALS ile ilişkili genlerle potansiyel düzenlemesi ile ilgili bulguları ortaya koymuştur. Bu bulguların, gelecekte geliştirilebilecek ALS hastalığını hedef alan potansiyel terapötik ve/veya nöroprotektif çalışmaların önünü açması beklenmektedir.

Anahtar Kelimeler: Nörodejeneratif Hastalıklar, Amyotrofik Lateral Skleroz, ETS Transkripsiyon Faktörleri, ETS Like-1 Proteini ELK1.

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LIST OF SYMBOL AND ABBREVIATIONS

ALS	: Amyotrophic Lateral Sclerosis
BAM	: Binary Alignment Map
DEGs	: Differentially Expressed Genes
ELK1	: ETS-like Transcription Factor-1
ETS	: E26 Transformation Specific
FBS	: Fetal Bovine Serum
FUS	: Fused in Sarcoma
GEO	: Gene Expression Omnibus
GRN	: Gene Regulatory Network
GSEA	: Gene Set Enrichment Analysis
GO	: Gene Ontology
LB	: Lysis Buffer
LMN	: Lower Motor Neuron
MND	: Motor Neuron Disease
NB	: Neutralization Buffer
PCA	: Principle Component Analysis
PI	: Propidium iodide
RB	: Resuspend Buffer
SOD1	: Cu/Zn Superoxide dismutase
SNP	: Single Nucleotide Polymorphism
SRA	: Short Read Archive
SREs	: Serum Response Elements
SRF	: Serum Response Factor
TCF	: Ternary Complex Factors
TDP-43	: TAR DNA binding protein - 43
TF	: Transcription Factor
TSS	: Transcription Start Site
UMN	: Upper Motor Neuron
vst	: Variance Stabilizing Transformation
WB	: Washing Buffer

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1. INTRODUCTION

1.1. ETS Transcription Factors

Transcription factors are protein molecules that bind to specific DNA sequences in the genome to induce or inhibit gene transcription. The ETS domain family is one of the transcription factor families. All members contain a conserved ETS DNA-binding domain that recognizes the 5'-GGAA/T-3' sequence [1]. The ETS family plays a role in various functions such as the regulation of cellular differentiation, cell cycle control, cell proliferation, apoptosis, and angiogenesis [2,3]. They are divided into 11 subfamilies based on the homology of the sequences in the DNA-binding domain, the conservation of other domains, and the location of the ETS domains. Dysregulation of ETS factors has been linked to oncogenesis, developmental disorders, and neuronal function. ETS superfamily is subclassified into the 12 subfamilies based on the sequence and position of the ETS domain.

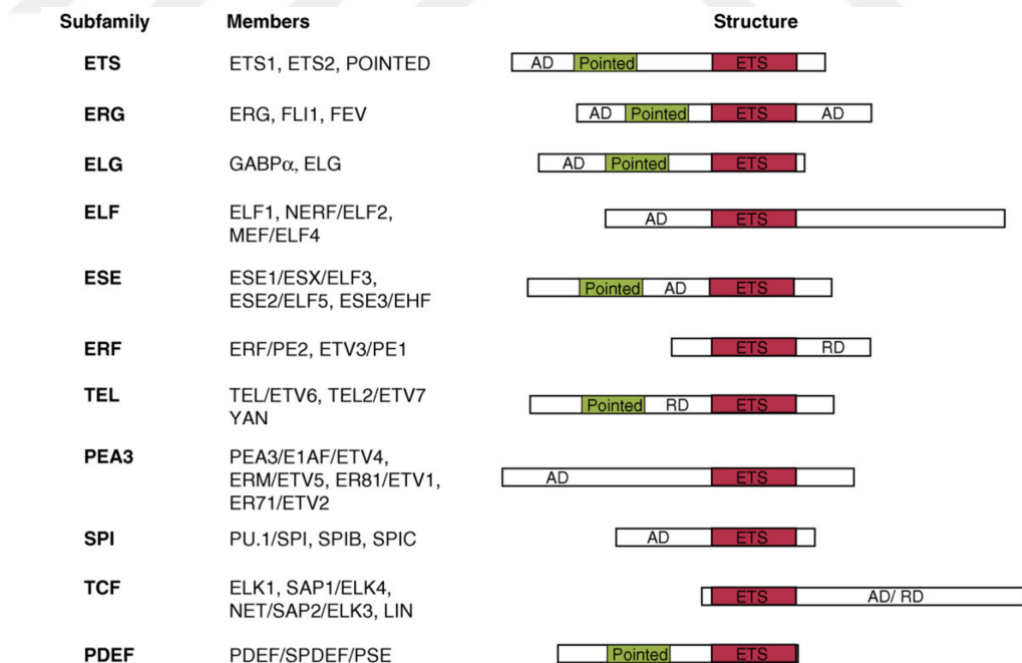


Figure 1.1: The ETS transcription factors. ETS transcription factors are subclassified into the 12 subfamilies based on the amino acid sequence and position of the ETS domain [2].

1.1.1. ETS-like Transcription Factor-1

Ternary Complex Factors (TCFs) form a subgroup of the ETS transcription factor family and primarily include the ELK1, ELK3 (NET), and ELK4 (SAP1) proteins. TCFs, along with Serum Response Factor (SRF), regulate the transcription of immediate early genes such as c-fos by binding to promoters containing Serum Response Element (SRE) [4]. These proteins are activated particularly through phosphorylation via the MAPK/ERK signaling pathway and play a critical role in initiating rapid transcriptional responses in processes such as cell cycle progression, cellular adaptation, and neuronal plasticity [2, 5].

ELK1, a member of this subfamily, has different roles in various biological processes such as differentiation, cell survival, apoptosis, inflammation, wound healing, cytoskeletal dynamics, and cancer [6]. Studies on neurons have shown that it plays a role in neuronal growth and neuronal survival.

ELK3 (NET) is a TCF member structurally similar to ELK1, but differs in that it functions as a transcriptional repressor under basal conditions. Following activation of MAPK/ERK signaling, the repressive effect of ELK3 decreases, and the expression of target genes can be stimulated, making it a regulator sensitive to environmental signals [7, 8].

ELK4 (SAP1) is another TCF member that forms complexes with SRF to regulate the transcription of serum and growth factor-responsive genes. ELK4 activity is also dependent on MAPK-mediated phosphorylation and plays a role in controlling gene programs that respond specifically to cellular stress and proliferative signals [2, 9].

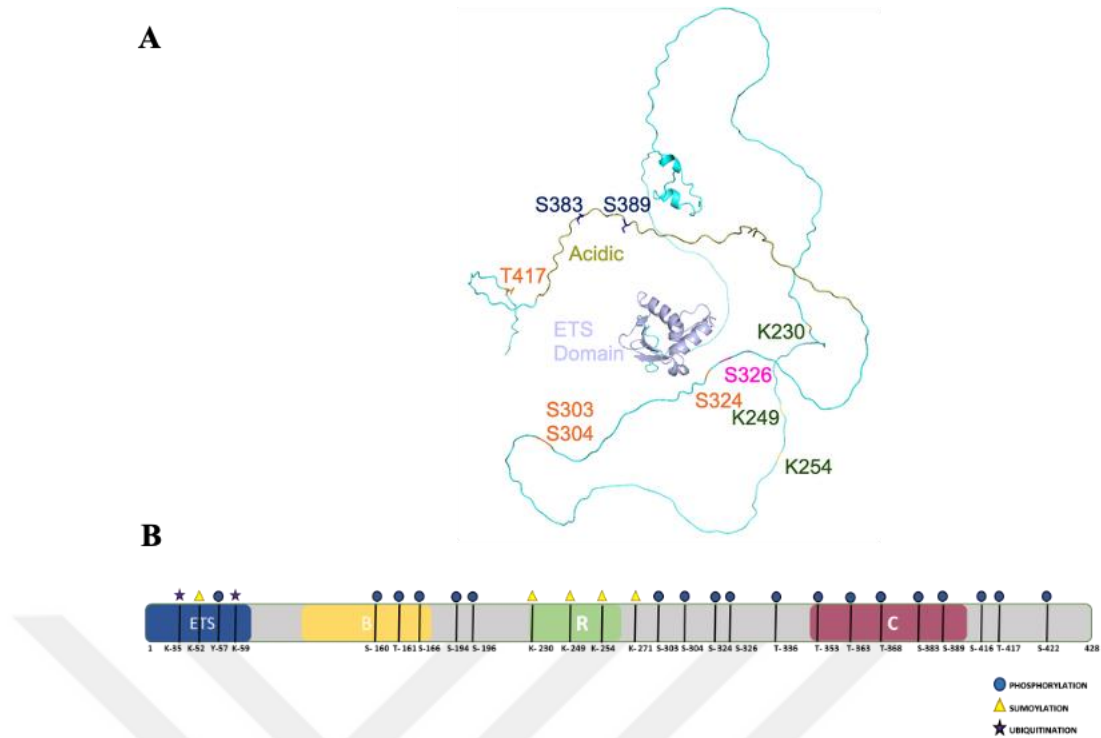


Figure 1.2: Representative schematics of ELK1 domains and putative posttranslational modification sites. (A) The representative 3D structure of ELK1. This figure is created by PyMOL by using AlphaFold prediction AF-P19419-F1-v4. (B) Domain structure of ELK-1 highlighting the N-terminal ETS DNA-binding domain (blue); B, SRF-binding domain (yellow); R, lysine-rich domain (green); and C, activation domain (purple). PTM sites were curated from PhosphoSitePlus, iPTMnet, and UniProt.

ELK1 is a member of the ETS (E-twenty six) transcription factor family and belongs to a specific subgroup within this family known as ternary complex factors (TCFs). TCFs are transcriptional regulators that play a critical role in regulating gene expression in response to serum and growth factors. These proteins bind to serum response element (SRE) sequences located on target gene promoters together with the serum response factor (SRF), forming a ternary protein-DNA complex [4]. This complex formation is one of the fundamental mechanisms that directly links signal transduction to the transcriptional response. TCF–SRF interaction enables the precise control of gene expression, particularly in rapid cellular processes such as cell proliferation, differentiation, and stress response. Although SRE sequences can be recognized by SRF alone, the recruitment of TCF proteins such as ELK1 to the promoter region is necessary for transcriptional activity. This demonstrates that ELK1

is not a passive factor that binds to DNA, but also a key regulator of signal-dependent transcriptional activation [4, 5].

ELK1, one of the best characterized members of the TCF family, has been identified as an important integrator protein that converts extracellular signals into a gene expression response at the nuclear level. ELK1 undergoes phosphorylation, particularly upon activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway, thereby initiating the transcription of early response genes [10]. Among these early response genes, *c-fos* encodes a transcription factor that plays a central role in processes such as cell cycle progression, cellular adaptation, and neuronal plasticity [5]. Therefore, ELK1 activation contributes to triggering not just a single gene but a broader gene regulation network.

Structurally, ELK1 consists of three main regions that are functionally distinct from each other. The ETS DNA-binding domain located in the N-terminal region enables ELK1 to recognize specific DNA sequences and bind to target promoters. This domain contains an ETS family-specific helix-turn-helix-like structure and interacts with consensus ETS binding sequences on DNA [11]. The SRF interaction domain in the middle region allows ELK1 to establish protein-protein interaction with SRF, thereby increasing the stability of the ternary complex. The transactivation domain located at the C-terminus contains critical serine residues that are phosphorylated by MAPKs.

The transcriptional activity of ELK1 is largely dependent on the phosphorylation of specific serine residues in the C-terminal region. Specifically, Ser383 and Ser389 residues have been identified as the primary regulatory sites phosphorylated by ERK1/2 [12]. These phosphorylation events induce conformational changes in ELK1, facilitating the binding of co-activator proteins (e.g., CBP/p300). Consequently, the chromatin structure loosens and transcription of target genes is initiated. When phosphorylation is inhibited, ELK1 remains transcriptionally inactive or may assume an inhibitory role [13, 14].

In this context, ELK1 acts as a bidirectional regulator depending on cellular binding and signaling status. It can suppress the expression of target genes under basal conditions or in the absence of appropriate signals, while acting as a potent transcriptional activator in the presence of mitogenic or stress-related signals. This feature positions ELK1 as a functional “transcription switch” between intracellular signaling networks and gene expression pathways. ELK1's binding-dependent

regulatory role is particularly important in tissues requiring dynamic gene regulation, such as the nervous system, for both physiological and pathological processes.

In the nervous system, ELK1 is a transcription factor that plays multifaceted roles in regulating neuronal development and homeostasis. The literature has demonstrated that ELK1 is involved in regulating neuronal differentiation, synaptic plasticity, and cellular responses to oxidative or excitotoxic stress conditions [11]. These processes are critically important, particularly for the survival of long-lived neurons with high metabolic activity. In this context, ELK1 is considered an important transcriptional control point that regulates the balance between neuronal survival and apoptosis, depending on the cellular context and signaling state.

In microarray analyses previously conducted in our laboratory, it was determined that ELK1 and significantly changed genes as a result of overexpression of ELK1 in the SH-SY5Y cell line were associated with neurodegenerative diseases [6]. These findings suggest that ELK1 plays a role in regulating not only individual genes but also broader transcriptional networks associated with neurodegeneration. Indeed, ELK1 has previously been studied in our laboratory in the context of Huntington's and Parkinson's diseases and has been shown to exhibit neuroprotective effects that support neuronal survival under cellular stress conditions. These results indicate that ELK1 may regulate common protective mechanisms in different neurodegenerative diseases.

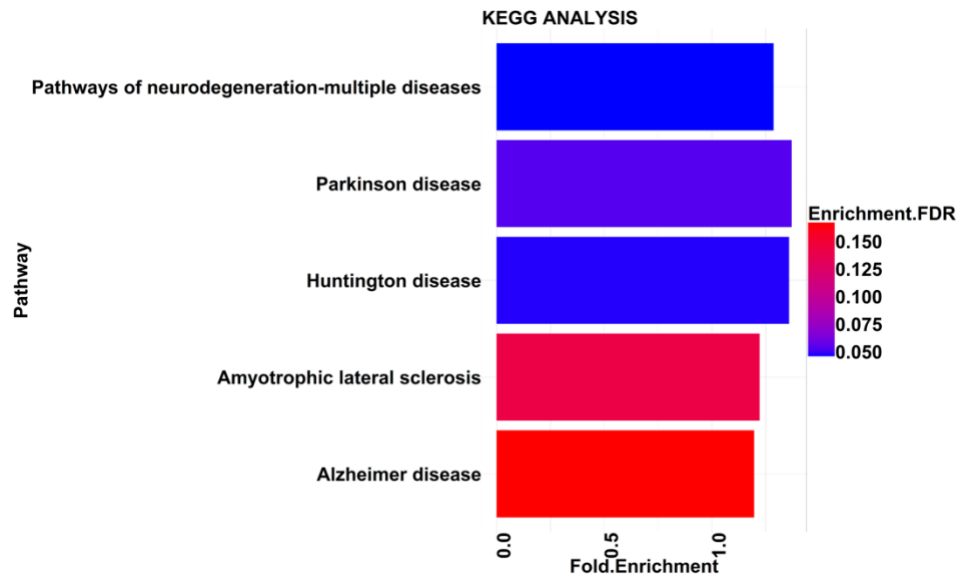


Figure 1.3: KEGG pathway enrichment analysis of ELK1-regulated genes in SH-SY5Y cells. KEGG pathway enrichment analysis was performed on genes showing significant expression changes following ELK1 overexpression in the SH-SY5Y neuroblastoma cell line. Enriched pathways were predominantly associated with neurodegenerative disease-related pathways, including amyotrophic lateral sclerosis, Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and pathways of neurodegeneration. Bars represent fold enrichment, while color intensity indicates the false discovery rate (FDR) of enrichment (in preparation).

In this context, it is predicted that ELK1 signaling pathway may affect transcriptional regulatory networks critical for motor neuron survival. Particularly in diseases characterized by motor neuron degeneration, such as amyotrophic lateral sclerosis (ALS), the delicate balance of the stress response, oxidative damage, and apoptotic signaling are among the key factors determining disease progression. The direct involvement of ELK1 in these processes makes it meaningful to investigate its potential role in ALS pathogenesis.

Studies in the literature have demonstrated that ELK1 plays a transactivated regulatory role in gene expression by binding to the promoter region of SOD1, one of the genes directly associated with ALS [15, 16]. SOD1 is of central importance in ALS pathogenesis, and its mutations are responsible for a significant proportion of familial ALS cases. The transcriptional regulation of SOD1 expression by ELK1 suggests that this factor may play an indirect but critical role in protecting motor neurons against oxidative stress.

In parallel, a study using a Huntington's disease mouse model showed that the absence of ELK1 was associated with increased apoptosis in the disease model and that increasing ELK1 levels provided a significant neuroprotective effect by activating survival mechanisms [17]. This finding supports the notion that ELK1 plays a fundamental role in regulating cellular stress and death mechanisms that are common to different neurodegenerative processes, not just disease-specific ones.

When all these data are considered together, it is thought that ELK1 may be an important component of transcriptional networks affecting motor neuron survival in ALS pathogenesis, and that alterations in ELK1 signaling may play a decisive role in disease progression. Therefore, detailed investigation of ELK1 and its potential target genes in the context of ALS may contribute to elucidating the molecular mechanisms of the disease and identifying new therapeutic targets.

1.2. Amyotrophic Lateral Sclerosis

Rare diseases are defined as disorders that affect a small proportion of the population and may arise from hereditary or sporadic origins. Although environmental factors can contribute in certain cases, the majority of rare diseases are caused by mutations in a single gene or by complex genetic alterations [18]. These diseases are typically chronic and progressive, with clinical manifestations that may appear at early or late stages of life and significantly impair daily functioning. Due to their genetic heterogeneity and highly variable clinical presentation among patients, rare diseases often pose substantial challenges in diagnosis and are associated with limited therapeutic options [19].

Within this broad group of rare disorders, motor neuron diseases (MNDs) represent a distinct class of neurodegenerative conditions characterized by the progressive degeneration of motor neurons. Motor neuron diseases can be either inherited or acquired and result from the selective vulnerability and eventual loss of motor neurons [20]. The motor neuron system consists of upper motor neurons (UMNs), located in the primary motor cortex and projecting to the brainstem and spinal cord, and lower motor neurons (LMNs), which innervate skeletal muscles of the limbs, trunk, and bulbar region. Damage to this system leads to progressive loss of voluntary muscle

control, with clinical manifestations varying according to the anatomical level and extent of neuronal involvement [21].

Amyotrophic lateral sclerosis (ALS) is the most common form of motor neuron disease and is also classified as a rare disease. Epidemiological data indicate that ALS has a global prevalence of approximately 4–6 cases per 100,000 individuals, with an annual incidence of 1–2 cases per 100,000 persons [22]. The disease typically manifests in mid-to-late adulthood and shows a slightly higher incidence in males. Approximately 90–95% of ALS cases are sporadic, whereas 5–10% are familial, often associated with mutations in genes such as SOD1, TDP-43 and FUS [23, 24]. These epidemiological characteristics underscore both the rarity and the substantial clinical burden of the disease.

Clinically, ALS is defined by the combined involvement of upper and lower motor neurons, which distinguishes it from other motor neuron disorders. Upper motor neuron degeneration leads to symptoms such as spasticity, hyperreflexia, increased muscle tone, and pathological reflexes, whereas lower motor neuron degeneration results in muscle weakness, atrophy, fasciculations, and reduced or absent reflexes [25]. The coexistence of UMN and LMN signs within the same patient reflects the multisystem nature of ALS and contributes to the heterogeneity of disease presentation and progression.

At the molecular level, ALS pathogenesis is driven by interconnected and mutually reinforcing mechanisms, rather than independent pathological events. Mitochondrial dysfunction, impaired RNA metabolism, and oxidative stress form a tightly linked pathogenic network [26]. Defective mitochondrial function leads to impaired energy production and increased generation of reactive oxygen species, thereby exacerbating oxidative stress. In parallel, disruptions in RNA processing, transport, and stability—particularly involving RNA-binding proteins such as TDP-43 and FUS—further compromise cellular homeostasis [27, 28]. These alterations collectively promote protein aggregation, synaptic dysfunction, and activation of apoptotic pathways, ultimately resulting in selective motor neuron degeneration.

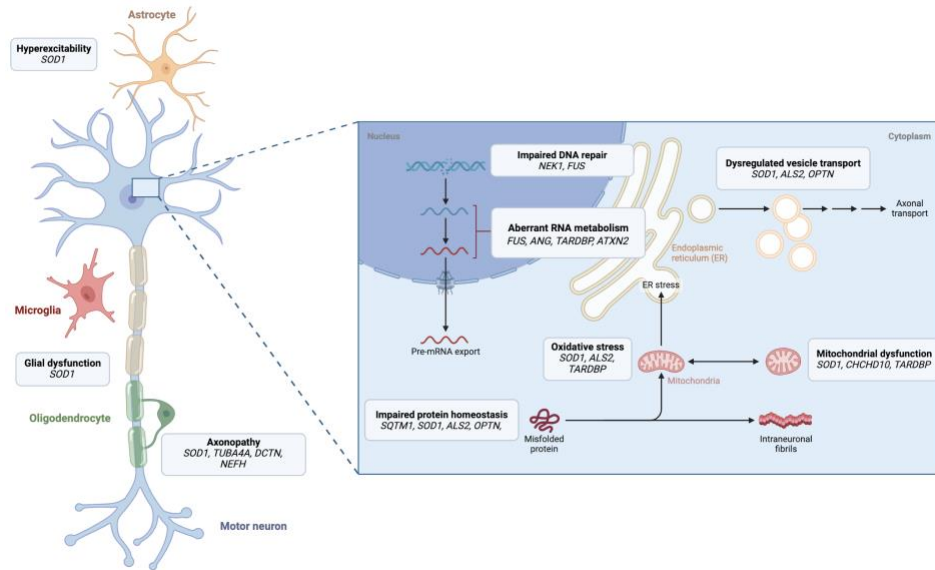


Figure 1.4: Cellular and molecular mechanisms of ALS. Amyotrophic lateral sclerosis (ALS) is characterized by progressive motor neuron degeneration triggered by multiple interconnected pathological mechanisms. Motor neuron hyperexcitability, glial cell dysfunction, and axonal degeneration contribute to neuronal dysfunction. At the subcellular level, impaired RNA metabolism, impaired DNA repair, mitochondrial dysfunction, oxidative stress, and defective vesicle trafficking lead to protein misfolding and cellular stress. These overlapping processes threaten the survival of motor neurons and accelerate the progression of the disease [29].

The earliest sign of ALS development is the appearance of abnormal proteins in motor neurons and glial cells. SOD1, copper zinc superoxide dismutase, is responsible for converting the harmful superoxide anion into the less harmful hydrogen peroxide. Mutations in this gene result in disruptions in mitochondrial function and axonal transport, leading to neuronal damage and loss [30].

A second sign is the disruption or impairment of RNA metabolism. This occurs as a result of mutations in RNA-binding proteins such as TDP-43 and FUS, or their mislocalization. TDP-43, a DNA- and RNA-binding protein, plays a role in the regulation of transcription, transport, and mRNA splicing. Under normal conditions, this protein is located in the nucleus but begins to accumulate in the cytoplasm of damaged motor neurons in ALS [31]. The FUS protein plays a role in regulating DNA and RNA binding and mRNA splicing. Most FUS mutations that cause ALS localize in the nuclear region via the C-terminal nuclear localization signal. This leads to dysfunctional nuclear import and cytoplasmic mislocalization [32, 33].

Mitochondrial dysfunction and oxidative stress are important markers in the development of ALS. In the disease state, morphological abnormalities and impaired ATP production mechanisms are observed in nerve cells. Mutant SOD1 and TDP-43 have been found to be associated with impaired calcium homeostasis in mitochondrial membranes [34]. Excess extracellular glutamate and impaired glutamate utilization by astrocytes are important processes in the development of the disease [35]. In this situation, NMDA receptors become excessively activated. This activation causes excessive calcium loading. Excessive calcium loading leads to damage and even death in motor neurons.

1.3. Purpose of Thesis

ALS research has primarily focused on protein aggregation, RNA metabolism, and mitochondrial dysfunction, relatively little is known about the transcription regulators that regulate these processes. ETS transcription factors, particularly ELK1, may regulate ALS-associated genes and play a critical role in disease progression by initiating cellular differentiation, proliferation, and apoptosis.

Therefore, it has been hypothesized that ELK1 may potentially play a role in disease progression through its neuroprotective effects on the potential regulatory network of ALS-associated genes. In this study, we address the role of changes in the expression of ETS transcription factors and their potential target genes in the pathogenesis of ALS.

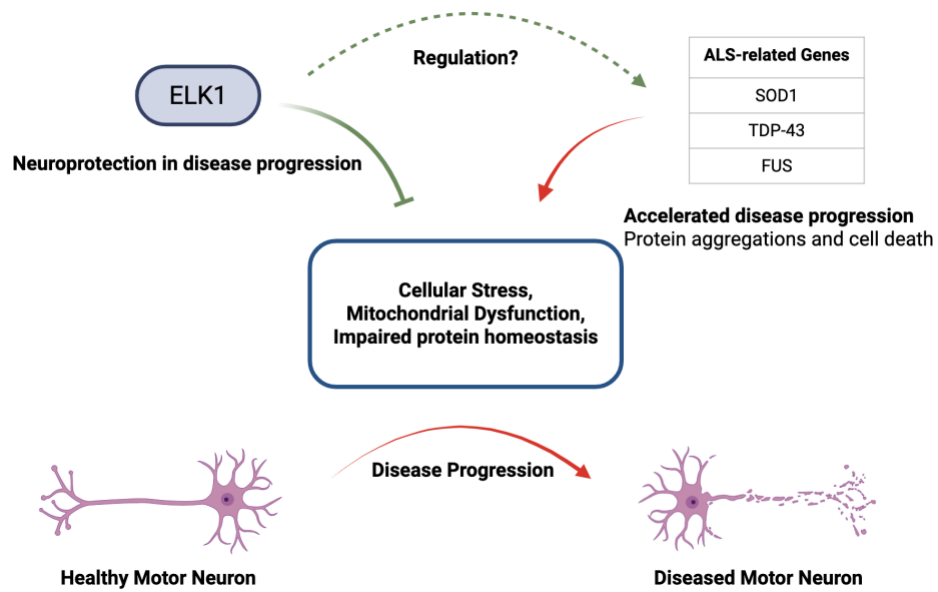


Figure 1.5: Schematic representation of the study hypothesis. This figure illustrates the proposed role of the ELK1 in ALS. It is hypothesized that ELK1 may play a neuroprotective role by regulating the network of ALS-associated genes. Changes in ELK1-mediated transcriptional regulation may influence cellular stress responses, mitochondrial dysfunction, and protein homeostasis. This schematic summarizes the central biological question of the study: how changes in the expression of ELK1 and its potential target genes contribute to ALS pathogenesis and motor neuron degeneration.

To answer these questions, previously published public ALS-related bulk transcriptomic data were analyzed, shown in Table 2.1 Potential regulatory networks were determined and numerous ALS-related genes were identified among the potential target genes in the ELK1 gene regulatory network. The in vitro validation of transcriptomic analysis was conducted to determine whether these genes were indeed regulated by ELK1 in cell culture using an in vitro ALS model.

2. MATERIALS AND METHODS

2.1. Transcriptomic Analysis

2.1.1. GALAXY and Data Pre-Processing

Publicly available gene expression datasets were obtained from the Gene Expression Omnibus (GEO) database. Datasets were selected based on relevance to amyotrophic lateral sclerosis (ALS), species (human or mouse), and the presence of ALS-associated genetic mutations. The final dataset set included multiple independent GEO series (GSE accession codes listed in Figure 2.1), comprising both human samples and established mouse models of ALS. These datasets represent different genetic backgrounds, including mutations in ALS-related genes, and were chosen to capture transcriptional changes of ELK1 and ALS related genes with disease pathogenesis across species.

Table 2.1: Publicly available bulk RNAseq datasets used in this study. This table summarizes the Gene Expression Omnibus (GEO) datasets included in the analysis. Each dataset is identified by its GSE accession code and categorized according to species (human or mouse) and the presence of ALS-associated genetic mutations.

GSE CODE	Human	Mouse	Mutation
GSE192755	X		SOD1
GSE203168	X		FUS
GSE98289	X		TDP-43
GSE157713		X	FUS
GSE146141		X	SOD1
GSE38820		X	SOD1
GSE54409	X		SOD1

The Galaxy platform was used for pre-analysis of these raw data (<https://usegalaxy.org/>). The FASTQ format from NCBI SRA, FastQC Read Quality reports, Trim Galore!, HISAT2, and featureCounts tools were used for raw RNA-seq data pre-analysis. Reads obtained from raw data were uploaded to the GALAXY platform using the Fast Download and Extract Reads tool (Galaxy Version 3.1.0+galaxy0). Read quality was checked with the FASTQC tool (Galaxy Version 0.74+galaxy1). Raw sequencing reads may contain adapter sequences and low-quality regions. These parts must be removed. The TrimGalore! tool was used to eliminate low-quality reads and adapter sequences with a quality cut-off value of 28 (Galaxy Version 0.6.10+galaxy0). After trimming, quality control was repeated using FASTQC to confirm that all samples had acceptable read quality. High-quality reads were then aligned to the reference genome to determine their genomic locations by using the HISAT2 tool (Galaxy Version 2.2.1+galaxy1). The hg19 genome for humans and the mm10 genome for mice were used for alignment based on the source of the bulk RNA-seq data. The alignment output was generated in BAM (Binary Alignment Map) format. The resulting BAM files were used as input for featureCounts to quantify the number of reads mapped to each gene (Galaxy Version 2.2.1+galaxy0). Finally, count data from all samples were combined to generate a gene expression matrix. The flowchart of our study methodology is shown in Figure 2.1.

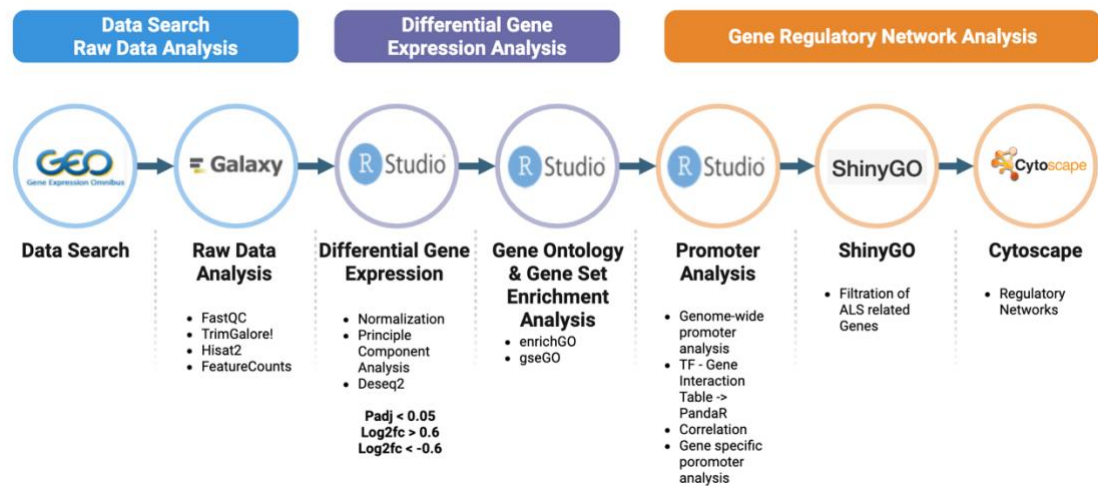


Figure 2.1: Methodology of transcriptome analysis. Publicly available gene expression data were retrieved from the Gene Expression Omnibus (GEO) and processed using the Galaxy platform for raw data quality control, trimming, read alignment, and read counting. Differential gene expression analysis was performed in RStudio, followed by gene ontology and gene

set enrichment analyses. Promoter and transcription factor–gene interaction analyses were subsequently conducted to identify regulatory relationships. Finally, ALS-related genes were filtered using ShinyGO, and regulatory networks were constructed and visualized using Cytoscape.

2.1.2. Differential Gene Expression Analysis

Differential Gene Expression Analysis was performed using the R programming language (3.6 and 4.2 versions). The count data matrix generated by FeatureCounts on the Galaxy platform was imported into R. Since raw count values show a wide range, data normalization was required before further analysis. First, genes with lower counts, specifically those with a row sum below the 10 threshold, were pre-filtered. Normalization was carried out using variance stabilizing transformation (VST), which reduces the dependence of variance on mean expression levels and allows more consistent comparisons between samples [36]. The normalized data were used for principal component analysis (PCA) to identify outliers and visualize the distributions between samples. Differential gene expression analysis was then conducted using the DESeq2 R package, which uses raw count data as input [36]. Differentially expressed genes (DEGs) were determined based on a Benjamini & Hochberg adjusted p-value < 0.05 as the significance threshold. Upregulated DEGs were defined by a log2 fold change ($\log_2\text{FC}$) > 0.6 , whereas downregulated DEGs were defined by a $\log_2\text{FC} < -0.6$. Visualization of DEGs for each comparison (healthy vs diseased) was performed using volcano plots generated with the ggplot R package [37]. These significantly changed genes were used for subsequent functional analyses. Gene Ontology (GO) analysis was performed to investigate the biological processes, signaling pathways, and cellular components associated with these significantly changed genes. GO enrichment analysis was conducted using the clusterProfiler and enrichGO R package [38, 39]. The top ten enriched GO terms were further used for Gene Set Enrichment Analysis (GSEA) to group differentially expressed genes into functional categories. GSEA was conducted using the gseGO R package. The results were visualized using the ggplot2 R package [37].

2.1.3. Gene Regulatory Network Analysis

Gene regulatory networks (GRNs) were constructed to investigate the regulatory relationships between ETS transcription factors (TFs) and significantly changed genes. In these networks, nodes represent transcription factors and target genes, while edges represent regulatory interactions between TFs and genes.

To examine transcription factor binding sites within the selected promoter regions, a genome-wide promoter analysis was conducted using the FindM online tool (<https://ccg.epfl.ch/ssa/findm.php>). Letter probability matrices corresponding to transcription factors were obtained from the CIS-BP database for the appropriate organism and used as input for the FindM analysis (<https://cisbp.ccbr.utoronto.ca/index.php>).

Promoter regions spanning from -100 bp upstream to +5000 bp downstream of the transcription start site (TSS) were analyzed. Transcription factor binding motifs were identified using a p-value cutoff of 0.001. The resulting output file (fps format) was subsequently processed in R to generate a readable transcription factor–gene interaction table.

Following the generation of the TF–gene interaction dataset, this interaction file, together with the corresponding gene list and expression data, was used as input for the PANDA package. PANDA was employed to infer and construct the estimated gene regulatory network based on transcription factor binding information and gene expression profiles [40, 41].

Correlation analysis was performed using the `corr()` function to assess the relationship between the expression levels of genes and the expression of ETS transcription factors across different experimental groups. Interactions were filtered using a correlation threshold of $R > 0.75$ indicating strong positive correlations and $R < -0.75$ indicating strong negative correlations.

Promoter sequences of candidate target genes were retrieved from the UCSC Genome Browser (<https://genome.ucsc.edu/>). The TxDb annotation and BSgenome packages were utilized for mouse (mm10) and human (hg19) genomes [42, 43]. Full transcript coordinates were obtained using the `transcriptsBy()` function, and promoter regions were extracted using the `getPromoterSeq()` function. Sequences spanning from 5000

bp upstream to 100 bp downstream of the transcription start site (TSS) were selected. For motif analysis, transcription factor binding motifs of ETS family members were obtained from the JASPAR database and analyzed using the MotifDB R package [44, 45]. For transcription factor binding site prediction, the false positive probability (α) threshold was set to 0.001.

As a result, edge-weighted gene regulatory networks were generated for each ETS transcription factor. To identify ALS-related genes among these potential target genes, the analysis was performed using the ShinyGO web tool (<http://bioinformatics.sdstate.edu/go76/>). Potential target genes and potential regulatory networks involving these potential genes and ETS transcription factors were identified for each ETS transcription factor and their networks were visualized in Cytoscape [46].

2.2. Validation Studies

2.2.1. Establishing Disease Modelling

2.2.1.1. Plasmid Isolation

Addgene Inc. plasmids SOD1^{WT} (pSOD1WTAcGFP1, #26407) and SOD1^{G93A} (pSOD1G93AAcGFP1, #26411) were obtained for the generation of an in vitro disease model. As an initial step, plasmid isolation and validation were performed prior to experimental use.

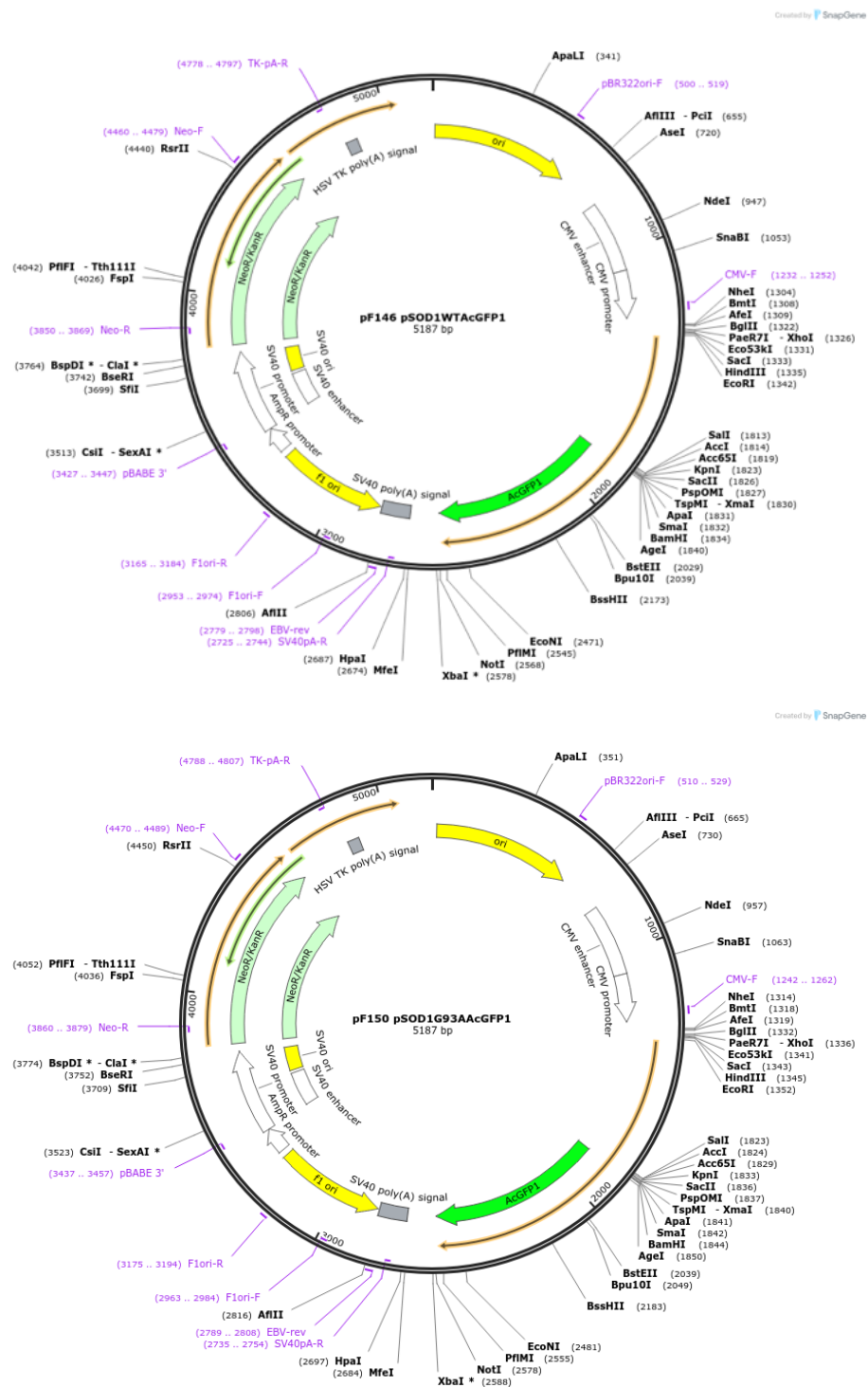


Figure 2.2: Plasmid maps of SOD1^{WT} and SOD1^{G93A} expression constructs. This figure shows the plasmid maps of SOD1^{WT} (pSOD1WTAcGFP1, #26407) and SOD1^{G93A} (pSOD1G93AAcGFP1, #26411). Both constructs are 5187 bp in length and were designed for the expression of the human SOD1 gene fused to the AcGFP1 reporter under the control of the cytomegalovirus (CMV) promoter. The AcGFP1 fusion enables visualization and tracking of SOD1 expression in transfected cells. Plasmid maps were generated using SnapGene software and arrows indicate the direction of transcription.

Bacterial cultures containing the SOD1^{WT} and SOD1^{G93A} plasmids obtained from Addgene Inc. were inoculated onto kanamycin-containing agar plates and incubated overnight at 37°C. The next day, bacterial colony growth was observed on the plate. One of these growing colonies was taken and inoculated with kanamycin at a concentration of 0.1 ng/mL in 5 mL of LB for miniprep and left overnight at 37°C. The next day, growth (turbidity) was observed in the inoculum compared to the control that has no antibiotics.

The miniprep protocol was performed according to the manufacturer's instructions (EasyPure Plasmid MiniPrep Kit (Lot#R50923-V2)). The bacterial inoculum was centrifuged at 10,000g for 1 minute. The supernatant was discarded, the pellet was resuspended in 250 µL RB (Resuspend Buffer) and the sample was transferred to a 1.5 mL eppendorf tube. 250 µL LB (Lysis Buffer) was added and the tube was inverted 6 times. 350 µL NB (Neutralization Buffer) was added, the tube was inverted 6 times, and it was left to stand at RT for 2 min. The tube was centrifuged at 12,000g for 5 min. The supernatant was added to the column. Centrifuged for 1 min at 12,000g and the supernatant was discarded. Added 650µL WB (Washing Buffer), centrifuged for 1 min at 10,000g, and the supernatant was discarded. The tube was centrifuged empty for 3 min at 10,000g and incubated for 2 min at 65°C. The column was transferred to a new Eppendorf tube and 40 µL of water incubated at 65°C was added. The tube was incubated at RT for 3 min and centrifuged at 10,000g for 1 min. Then the supernatant was measured at NanoDrop and its concentration was found.

To determine whether the plasmid obtained after miniprep was the desired plasmid, plasmid digestion was performed. The cut was performed with EcoR1 according to the plasmid map to yield 500 ng. Mixes were prepared according to the measurements in the table for the cut and incubated in a water bath (37 degrees) for two hours. The cut plasmids were run on a 1% agarose gel, calculated to yield 300 ng.

After the correctness of the plasmids was confirmed, transformation was performed to scale up the plasmids (midiprep). 2 µL of the plasmid to be amplified was added to a 1.5 mL Eppendorf tube containing 50 µL of DH5α competent cells. The tubes were first placed on ice for 15 minutes, then in a 37°C water bath for 90 seconds, and finally on ice again for 2 minutes. 200 µL of LB Broth was added to the tubes and shaken at 37°C at 200 rpm for 1 hour (in a warm room). 100 µL of the growing culture was

spread onto Kanamycin-containing agar using a glass pipette and incubated overnight at 37°C.

The following day, bacterial colonies were observed growing on the agar plates. One of these colonies was taken and inoculated into 100 mL of liquid medium containing kanamycin at a concentration of 0.1 ng/mL, and incubated overnight at 37°C. The next day, turbidity (bacterial growth) was observed in the inoculum compared to the antibiotic-free control culture.

The Midiprep protocol was performed according to the manufacturer's instructions (DNA-midi GT Plasmid DNA Purification Kit). From the 100 mL LB culture prepared the previous day, 50 mL was transferred to culture falcon tubes and centrifuged at 10,000 g for 10 minutes. The supernatant was discarded, the remaining LB culture in each tube was added to the pellet, and the tubes were centrifuged again at 10,000g for 10 minutes under the same conditions. After removing the supernatant, 8 mL of M1 Buffer stored at +4°C, provided with the kit, was added to the pellet. Then, 8 mL of M2 Buffer was added, the tubes were mixed by inverting them 15 times, and incubated for 3 minutes. Subsequently, 8 mL of M3 Buffer was added, the tubes were mixed by inverting them 10 times, and the samples were centrifuged at 10,304 g for 30 minutes. While the centrifugation was ongoing, the columns were activated. 5 mL of CPE Buffer was added to each column, and the filtered CPE Buffer was discarded. The supernatant obtained after centrifugation was poured onto the activated columns and allowed to filter. Next, 12 mL of Washing Buffer was added to the columns and allowed to filter. The columns were transferred to new falcon tubes, and 8 mL of Elution Buffer was added. After filtration was complete, 6 mL of isopropanol was added to the eluate, and the tubes were inverted 10 times to mix. The samples were centrifuged at 10,304g for 60 minutes. The supernatant was discarded, and 5 mL of 70% ethanol was added to the pellet. The samples were centrifuged at 10,304g for 45 minutes. After removing the supernatant, the pellet was dried at 70°C for 10–15 minutes. The dried pellet was dissolved in 300 µL of deionized water (dH₂O). The DNA concentration was measured using a NanoDrop device.

2.2.1.2. Cell Culture Conditions

The NSC-34 mouse motor neuron-like hybrid cell line (CLU140) was used in all experiments. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; 4.5 g/L glutamine, without pyruvate) supplemented with 10% Fetal Bovine serum (FBS) and 1% penicillin–streptomycin, and cultured in a humidified incubator at 37 °C with 5% CO₂.

For cell seeding, frozen NSC-34 vials were removed from liquid nitrogen and quickly thawed in a 37 °C water bath for approximately 1 minute. The cell suspension was then transferred into a 40-mm culture dish containing 2 mL of complete growth medium. After allowing the cells to attach to the culture surface, the medium was replaced with fresh growth medium. For NSC-34 cells, cell attachment was observed within approximately 4 hours.

2.2.1.3. Transient Transfection of Cells

300,000 NSC-34 cells were seeded into each 40 mm culture dish and 6-well plate well. Transfection was performed 24 hours after cell seeding. For transfection, 3 and 6 µg of DNA (eGFP, SOD1^{WT} and SOD1^{G93A} plasmids) per well and transfection reagent proportional to the DNA amount (1:1 PeiPRO (Polyplus), 1:3.5 Pei, 1:4 Pei, 1:5 Pei (Polysciences, Inc.)) were mixed and added to 250 µl serum-free medium. After transfection, images of eGFP, SOD1^{WT} and SOD1^{G93A} transfected cells were taken every 24 hours.

2.2.1.4. Cell Viability Assays

2.2.1.4.1. MTT Assays

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity [47]. This colorimetric test relies on the reduction of a yellow tetrazolium salt into purple formazan crystals by metabolically active cells. This conversion can only occur in living cells, therefore the amount of purple formazan produced is directly proportional to the number of living cells in the sample. Living

cells contain NAD(P)H-dependent oxidoreductase enzymes that reduce MTT to formazan. Insoluble formazan crystals will be dissolved using a dissolution solution, and the resulting colored solution will be quantified by absorbance measurement at 570 nanometers using a multi-well spectrophotometer. The darker the solution, the greater the number of living, metabolically active cells.

For MTT Assay, NSC-34 cells were seeded into a 96-well plate, with 5,000 cells per well. Transfection was prepared in 10 μ L of serum-free medium using 0.03 μ g DNA and 0.105 μ L Pei per well. The DNA-Pei mixture (10 μ L) was added to the respective wells (columns 2, 3, and 4). 10 μ L of DMSO was added to column 5 as a control. The medium was removed from the 0-48-72 hour wells, and 10 μ L of MTT reagent (BioFroxx) was added to each well. After 3 hours of incubation, the formed formazan crystals were dissolved with DMSO, and absorbance measurements were taken. Statistical analysis was performed on percentage-based experimental data between different groups (eGFP, SOD1^{WT}, and SOD1^{G93A}) and different time points (0 hours, 48 hours, 72 hours) in the experimental set-up. Two-way analysis of variance (two-way ANOVA) was used to evaluate the effects of the experimental factors, followed by Tukey's post hoc test for multiple comparisons. Differences were considered statistically significant at $p < 0.05$.

2.2.1.4.2. PI / Hoechst Staining

The double-staining apoptosis test (PI/Hoechst) was used to see viability of diseased cells. Propidium iodide (PI) is used as a red dye that cannot penetrate the cell membrane; that is, while no staining occurs in living cells performing vital functions, staining occurs in the damaged membrane structure of dead cells, and the dead cells are stained [48]. Hoechst dyes are a type of fluorescent dye cell-permeable dyes that emit blue fluorescence and stain all cells [49]. The staining pattern resulting from the simultaneous use of these dyes makes it possible to distinguish normal, apoptotic, and dead cell populations.

300,000 NSC-34 cells were seeded into each 40 mm culture dish. Transfection was performed 24 hours after cell seeding. For transfection, 3 μ g of DNA (eGFP, SOD1^{WT}, and SOD1^{G93A} plasmids) and transfection reagent (1:3.5 Pei) in proportion to the DNA amount were mixed in 250 μ L of serum-free medium for each culture dish. Images

were taken at 0, 48, and 72 hours after transfection using PI-Hoescht staining. PI-Hoescht staining was performed by removing the medium from the cells, incubating for 30 minutes with 10 μ L of PI (1:100) (Thermo Fisher Scientific) in 1 mL of PBS, then adding 0.2 μ L of Hoescht (1:5000) (Thermo Fisher Scientific) and incubating for 10 minutes at room temperature with a shaker.

2.2.2. Target Gene Validation

2.2.2.1. RNA Isolation and cDNA Synthesis

At 0, 48, and 72-hour time points, 2,000,000 NSC-34 cells were seeded into each 100 mm culture dish. Transfection was performed 24 hours after cell seeding. For transfection, 12 μ g of DNA (SOD1^{WT} and SOD1^{G93A} plasmids) and transfection reagent (1:3.5 Pei) in proportion to the DNA amount were mixed in 1 mL of serum-free medium per well. At 0, 48, and 72-hour time points, the media in the culture dishes were removed, the cells were washed with 1 ml of PBS, and transferred to -80°C for RNA isolation.

RNA isolation was performed according to the manufacturer's instructions (iNNUPREP RNA MINI KIT, Lot 006-23). Culture dishes were scraped with 500 μ L of PBS using a cleaned scraper that had been rinsed through a sequential dH₂O–ethanol–dH₂O–ethanol–dH₂O–ethanol–dH₂O–dH₂O washing cycle. After collecting the sample into a 1.5 mL Eppendorf tube, the culture dish was cleaned again with 500 μ L of PBS, and the samples were collected into the previous 1.5 mL Eppendorf tube. Centrifuge at 1500 rpm for 10 minutes at room temperature. PBS was removed from the samples, and 400 μ L of Lysis Solution RL was added to the pellet and incubated for 2 minutes at room temperature. The pellet was dissolved with this solution and incubated again for 2 minutes at room temperature. Spin Filter D was placed in the collector tube, samples were added, and it was centrifuged at 11,000g for 2 minutes. Spin Filter R was placed in the collector tube. 400 μ L of 70% ethanol was added to the collector tube from the previous step and pipetted. Samples were added to Spin Filter R and it was centrifuged at 11,000g for 2 minutes. Spin Filter R was transferred to another collector tube, 500 μ L of Washing Solution HS was added, and it was centrifuged at 11,000g for 1 minute. Spin Filter R was transferred to another collector tube, 500 μ L of Washing Solution LS was added, and it was centrifuged at 11,000g

for 1 minute. Spin Filter R was transferred to yet another collector tube and centrifuged at 11,000g for 3 minutes. Spin Filter R was transferred to an elution tube, 35 μ L of RNase-free water was added, and it was centrifuged at 11,000 g for 1 minute. Spin Filter R was discarded, and the elution tube was labeled. RNA samples were measured using NanoDrop. Each RNA sample was diluted to 100 ng/ μ L (volume 100 μ L).

Table 2.2. Components used for cDNA synthesis and the volumes of each in the reaction mixture.

Component	Volume
Enzyme	1ul
Mix	4ul
RNA (100ng/ul)	10ul
dH2O	5ul

Table 2.3. Thermal cycles applied in the cDNA synthesis reaction.

Temperature	Duration
25°C	5 minutes
46°C	20 minutes
95°C	1 minutes
4°C	forever

2.2.2.2. Quantitative PCR (qPCR)

In accordance with the manufacturer's instructions for qPCR (Luna Universal qPCR Master Mix), the amounts listed in the table above were added to 96-well culture plates. Each prepared 96-well culture plate was processed using an Agilent qPCR device under the conditions specified in the table below. The reaction was prepared in a total volume of 10 μ L by combining Syber, primers, and cDNA in appropriate ratios. The reaction was run at 95 °C for 30 seconds, 95 °C for 15 seconds, and 60 °C for 30 seconds, respectively.

Table 2.4. Primer list used in qPCR

Gene	Forward primer	Reverse primer
<i>Sod1</i>	GTCGGCTTCTCGTCTTGCTC	CTGGTTCACCGCTTGCCTTC
<i>Tdp43</i>	GCAGTGGCCTAGCGGAGAT	CCCCTGGAAACTGGGCTGT
<i>Fus</i>	TCAATAAATTTGGTGGTCCTCGG	CTGTTGCCTCACCCCTTCAAC
<i>Grin2a</i>	CTGGGAAGTTGGACGCTTTC	AGCTCTTCCATCTCACCGTCA
<i>Grin2d</i>	CTGAGTGAGTGTGCGGGAAC	CTCCTCTTCTTGGTCCTGCC
<i>Grin2b</i>	GCCTTTCTATCCCCGGCATC	CAAAGCGTCCCCTTCCGGT
<i>Nrg1</i>	GCGCTCCAGGTGATCGAG	GGAGGCAATGCTGGGCT
<i>Gria1</i>	CTGAACGAGCAGGGGCTTTT	AGCACTGGTCTTGTCTTGG
<i>Hap1</i>	GATGCTGGCCTCAGAGAAGG	GTGAGTGTCACGTCTCTCCA
<i>Kif5a</i>	CCAACATGGACAATGGAAATGCC	GGAGGAGACATCAAACAGTCCA
<i>Rab39b</i>	TCGAGCCAGGAAAACGCATC	GGCGCGAGTGATGGATCTGAA
<i>Nrg3</i>	TGCAAGGAAGGCTACCAAGG	CCCCAAGTGGTCTGTTGGAT
<i>Tubb3</i>	CCCAGCGCAACTATGTAGG	GCACATACTTGTGAGAGGAGGC
<i>Ppp3cc</i>	CCTTCAAACAGGAATGTCGGA	ACTGCTGGTTTAAGAGGGCA
<i>Tuba1a</i>	GCAGCAACCATGCGTGA	TCCTTGCCTGTGATGAGCTG
<i>Tubb2b</i>	CAGCAAGAAGCTAACCAGGC	CACTGATGACCTCCCAAACTTG
<i>Nefl</i>	GCCTGCTCAAGGCTAAGACC	CTTCGCCTTCCAAGAGTTTTCTGT
<i>Pink1</i>	GATATGCTGCCCCCACAATA	AGGGACAGCCATCTGAGTCC
<i>Ppp3cb</i>	GCTCCAATTACAGTGTGTGGT	AGCAGGTGATCCTCCTACTTC
<i>Bcl2</i>	CGTCGTGACTTCGCAGAGAT	TAGTTCCACAAAGGCATCCCAG
<i>Nefh</i>	GGTCAGATCCAGGGCTGC	CCTCCAACCTCTGTGGTCCTG
<i>Ppp3r1</i>	CCCTGTTCCGTGCCTTGAG	CTCGCCTCATTTCCTCATCTTG
<i>Elk1</i>	GCTTCCTACGCATACATTGACC	ACTGGATGGAACTGGAAGG
<i>Gapdh</i>	CATGACCACAGTCCATGCCATC	ACGGACACATTGGGGGTAGG
<i>Actnb</i>	CTTGGGTATGGAATCCTGTGG	TGGCATAGAGGTCTTTACGG

Table 2.5. Components of the mixture prepared for the qPCR reaction and the volume of each component in the reaction.

Component	Volume
Syber	5ul
dH ₂ O	1.8ul
Forward Primer	0.6ul
Reverse Primer	0.6ul
cDNA (5ng/ul)	2ul

Table 2.6. Thermal cycles applied in the qPCR reaction.

Temperature	Duration	Cycle
95°C	30 seconds	1
95°C	15 seconds	45
60°C	30 seconds	
Melting	6 seconds	1

After instrument reading, mRNA expression was normalized according to the expression of the internal control genes β -actin and GAPDH (housekeeping). The difference in gene expression levels was calculated using the following formulas:

$$\Delta Ct = Ct_{target\ gene} - Ct_{housekeeping\ gene} \quad (2.1)$$

$$\Delta\Delta Ct = \Delta Ct_{transfected} - \Delta Ct_{non-transfected} \quad (2.2)$$

$$Relative\ Expression = Fold\ Change = 2^{-\Delta\Delta Ct} \quad (2.3)$$

In statistical analyses, the F-test was applied to assess the homogeneity of variances between the control and experimental groups. To determine the significance of the difference between the means of the two groups, a two-tailed (2-tail) and type 2 independent samples t-test was performed, based on the assumption that variances are not equal. A threshold value of $p < 0.05$ was accepted for the statistical significance level.

3. RESULTS

Based on the hypothesis that the ETS transcription factor ELK1 may play a neuroprotective role in ALS by regulating ALS-related genes, this study aimed to determine whether changes in ELK1 expression and its potential ALS-related target gene network. To address the proposed hypothesis and biological question, transcriptomic and regulatory network-based analysis was performed, followed by in vitro validation experiments. Using this approach, differentially expressed genes, enriched biological processes, and ETS transcription factor-centered regulatory networks were identified. In particular, ELK1-associated gene regulatory network was examined in vitro ALS disease model. The results of these analyses, including differential gene expression profiles, functional enrichment outcomes, predicted regulatory interactions, and experimental validation findings, are presented in the following section.

3.1. Transcriptomic Analysis

To address the central question of whether ETS transcription factors, particularly ELK1, contribute to ALS pathogenesis through transcriptional regulation, transcriptomic analysis was selected to assess gene expression changes with identifying transcription factors and potential targets involved in disease-related molecular changes. For this reason, multiple publicly available ALS-related transcriptomic datasets were systematically screened and analyzed.

When bulk RNAseq data from different types and different ALS mutation types were analyzed. Differential expression, enrichment, and regulatory analyses were performed; however, only one dataset showed ELK1 to be significantly changed and potential target genes were found to be associated with ALS. Focusing on the dataset in which ELK1 showed significant change, differentially expressed genes, enriched biological processes and potential gene regulatory network were identified. Data sets in which ELK1 was not significantly altered were excluded from further analysis, and the detailed results of these preliminary analyses are presented in the Appendix.

Table 3.1: Results of transcriptomic datasets analyzed for ELK1 relevance in ALS. This table summarizes the publicly available GEO transcriptomic datasets analyzed in this study. Each dataset is classified according to species (human or mouse), the presence of ALS-associated mutations, and ELK1-related findings. ELK1 columns indicate whether ELK1 showed significant differential expression or how it change in each dataset. Only datasets in which ELK1 demonstrated meaningful transcriptional significance were selected for downstream analyses.

GSE CODE	Human	Mouse	Mutation	ELK1	ELK1
GSE192755	X		SOD1	Significantly Changed	Up-regulated
GSE203168	X		FUS	Not Significantly Changed	Down-regulated
GSE98289	X		TDP-43	Not Significantly Changed	Up-regulated
GSE157713		X	FUS	Not Significantly Changed	Up-regulated
GSE146141		X	SOD1	Not Significantly Changed	Down-regulated
GSE38820		X	SOD1	Not Significantly Changed	Up-regulated
GSE54409	X		SOD1	Not Significantly Changed	Up-regulated

The GSE192755 dataset consists of transcriptome data from motor neurons derived from induced pluripotent stem cells (iPSCs) obtained from twins with SOD1 mutations, some of whom exhibit ALS symptoms and others who do not.

This dataset was analyzed to identify gene expression profiles of healthy vs diseased samples. Principal Component Analysis (PCA) was performed to confirm clear separation of healthy and diseased samples (Fig.3.1.A). Differential gene expression analysis of this dataset identified DEGs that changed significantly between diseased and healthy samples (Fig.3.1.B). Volcano plot shows statistical significance (P_{adj}) versus magnitude of change (fold change), where the most upregulated genes are towards the right, the most downregulated genes are towards the left, and the most statistically significant genes are towards the top. In the diseased group of this study,

a total of 3,454 genes were upregulated ($\log_2FC > 0.6$) and 3,408 genes were downregulated ($\log_2FC < 0.6$) compared to healthy controls (adjusted p-value < 0.05) (Fig.3.1.B).

Gene Ontology (GO) of differentially expressed genes revealed a significant enrichment of biological processes related to the nervous system and synaptic function (Fig. 3.1.C). The most enriched GO terms included modulation of chemical synaptic transmission and regulation of trans-synaptic signaling, processes that are documented to be disrupted in amyotrophic lateral sclerosis (ALS) and are associated with early synaptic dysfunction and motor neuron hyperexcitability [50, 51]. Synaptic alterations and excitatory–inhibitory imbalance are considered early pathogenic events in ALS, often preceding overt motor neuron degeneration. In addition, enrichment of axonogenesis and regulation of nervous system development terms is consistent with previous studies describing ALS as a distal axonopathy, in which axonal degeneration and impaired axonal maintenance occur prior to neuronal cell body loss [52, 53]. Disruption of axonal growth and guidance pathways has been shown to compromise motor neuron connectivity and resilience, contributing to disease progression. The identification of vesicle-mediated transport in synapse as a significantly enriched term further supports the relevance of the transcriptomic findings, as defects in vesicle trafficking and synaptic transport have been widely reported in ALS and linked to mutations in ALS-associated genes such as SOD1, TDP-43 and FUS [54, 55]. Impaired vesicle transport contributes to synaptic failure and altered neuronal communication, reinforcing the pathogenic significance of these pathways. Overall, the enrichment of synaptic, axonal, and vesicle transport–related GO terms observed in this study is highly consistent with established ALS disease mechanisms [56, 57], indicating that the identified differentially expressed genes capture key molecular features of ALS-related neurodegeneration.

Among the differentially expressed genes, ELK1 was identified as a significantly upregulated gene, indicating increased transcriptional activity in the analyzed ALS-related condition. This upregulation suggests that ELK1 may be actively involved in regulating downstream gene expression associated with neuronal stress responses and disease-related molecular pathway.

Genome-wide promoter analysis was performed in order to identify the potential gene regulation network between these significantly changed genes and ELK1 (Fig.3.1.D).

The potential gene regulatory network was generated through a defined multi-layer procedure that integrates (i) predicted promoter binding and (ii) expression-based co-variation. Briefly, a potential target gene list was first defined from the significantly changed genes identified in the differential expression analysis. For each potential gene, the promoter region was extracted from the reference genome (mouse mm10 or human hg19) using UCSC coordinates, and a fixed window surrounding the transcription start site (TSS) was used (−5000 bp upstream to +100 bp downstream). ETS transcription factor binding motifs were obtained from JASPAR and scanned across these promoter regions using MotifDB to identify genes that carry putative ETS-binding sites, resulting in an initial TF–gene interaction potential list. This promoter-derived TF–gene interaction table was then combined with the normalized gene expression matrix to infer a condition-specific regulatory network using the PANDA package. PANDA assigns an edge weight to each TF–gene pair by integrating the TF–gene interactions with gene expression co-variation patterns across samples, thereby prioritizing interactions that are both motif-supported and expression-consistent. In parallel, a correlation matrix between ETS transcription factors and candidate target genes was computed in R using the `corr()` function, and only strong associations were retained ($R > 0.75$ or $R < -0.75$). The promoter-based PANDA output and the correlation-supported TF–gene associations were then used to define the final edge list, yielding an edge-weighted ELK1 regulatory network for downstream interpretation. 381 genes were initially identified within the potential regulatory network; subsequent correlation filtering reduced this set to 343 genes.

Finally, to focus the analysis on ALS-relevant biology, predicted target genes were filtered using ShinyGO to retain ALS-related genes. Following this filtering step, the network was further refined to 19 ALS-related genes, which were retained as potential target genes. The resulting networks were visualized in Cytoscape for interpretation of relation between ELK1 and its potential ALS-related target genes.

ELK1 is directly linked to the identified potential target genes. The resulting network was a potential regulation network where ELK1 interacts with multiple genes that which are significantly downregulated; genes these genes were mainly found to be related to ‘axonal transport and intracellular traffic’, ‘cellular stress response and survival’, ‘axonal integrity and microtubule dynamics’ and ‘synaptic transmission and glutamatergic response’ (Fig.3.1.D). Genes related to ‘axonal transport and

intracellular traffic' were also identified in the network ; these include HAP1, RAB39B and KIF5A [19, 58- 65]. The presence of these genes suggests that ELK1 may be linked to processes regulating intracellular transport along axons. Genes related to 'cellular stress response and survival' were also identified in the network; these include BCL2, PINK1, NRG1 and NRG3[66- 75]. These genes play a role in apoptotic regulation and mitochondrial quality control under cellular stress conditions. In addition, Several protein phosphatase regulatory subunits (PPP3R1, PPP3CB, PPP3CC) that are associated with ELK1, demonstrating potential involvement in calcium-dependent signaling pathways [76- 79]. The inclusion of these phosphatase-related genes suggests that ELK1-associated regulatory activity may interfere with intracellular signaling cascades in modulating neuronal function. Genes related to 'axonal integrity and microtubule dynamics' including TUBA1A, TUBB2B, TUBB3, NEFL and NEFH were identified [80- 89]. These genes are essential for the maintenance of neuronal architecture. And finally, Another group consists of genes related to 'synaptic transmission and glutamatergic response' that were identified as part of ELK1 GRN, such as GRIN2A, GRIN2B, GRIN2D and GRIA1 [90- 96]. These genes encode glutamate receptor subunits that play critical roles in stimulating synaptic transmission and neuronal communication.

The presence of ALS-related genes involved in axonal transport, intracellular trafficking, stress response mechanisms, mitochondrial regulation, cytoskeletal stability, and glutamatergic synaptic signaling supports the hypothesis that ELK1 functions as a transcription regulator in disease-related molecular pathways. The observation that a large proportion of potential ALS-related target genes are downregulated further suggests that dysregulation of ELK1-dependent transcriptional control may contribute to impaired neuronal maintenance and survival. This study, which combines promoter-based motif identification and network inference with differential expression analysis, provides evidence that transcription networks associated with ELK1 are consistent with the fundamental pathogenic features of ALS. Although these results were obtained from computational analyses, they provide evidence for hypothesis-driven experimental studies. Accordingly, subsequent studies focused on experimentally validating direct ELK1-target gene interactions and functionally evaluating ELK1 activity under ALS-related conditions. Such approaches will be critical for determining whether modulation of ELK1 expression or activity

affects disease-related cellular phenotypes and may reveal ELK1 as a potential therapeutic target in ALS.

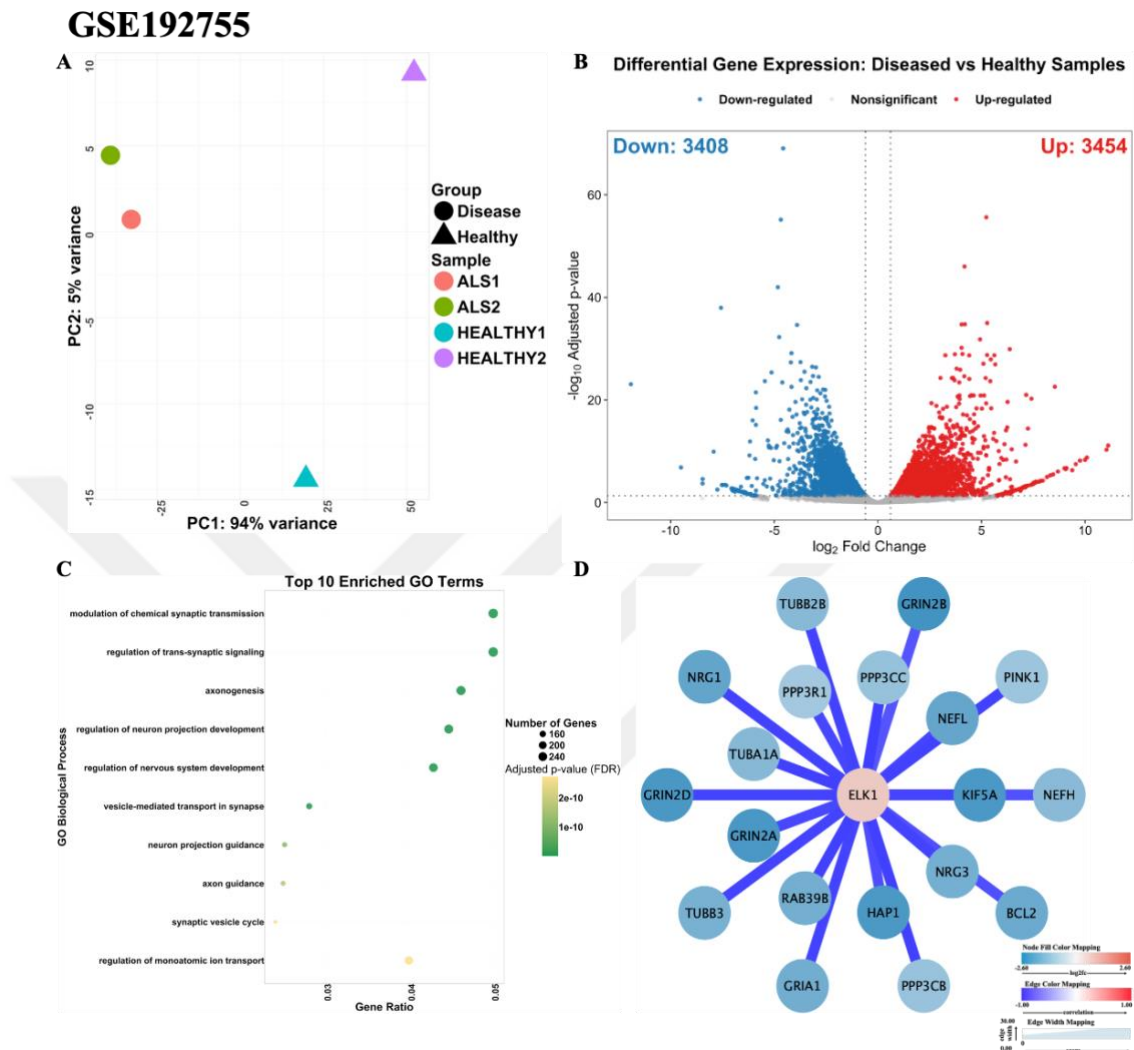


Figure 3.1: Transcriptome analysis of the GSE192755. (A) Principal Component Analysis. It defines samples depending on the diseased or healthy group. (B) Volcano plot. In volcano plot, the most upregulated genes are towards the right, the most downregulated genes are towards the left. (C) Gene Ontology Analysis. It shows Top 10 related GO Term that were used for further analysis and these terms are related with ALS disease conditions. The size of each circle corresponds to the number of associated counts, while adjusted p-values are encoded by the color scale. (D) Gene Regulatory Network of ELK1. It shows potential regulation network between ELK1 and its potential target ALS-related genes. All potential target genes are downregulated, ELK1 is upregulated. Edge width represents the strength of regulatory evidence between each transcription factor and its target gene. Edge color indicates the correlation between transcription factors and target genes, while node color reflects the corresponding $\log_2$ fold change ($\log_2$ FC).

3.2. Validation Studies

In order to confirm the genes in the ELK1 GRN, NSC-34 mouse motor neuron-like hybrid cell line was used that overexpresses wildtype or mutant SOD-1 expression plasmid. To maximize gene transfer efficiency and minimize toxicity in NSC-34 cells, both transfection reagent ratios (1:1 PeiPRO; 1:3.5, 1:4, and 1:5 Pei) and DNA amounts (3 μ g and 6 μ g eGFP, SOD1^{WT}, and SOD1^{G93A} plasmids) were initially optimized. This optimization, performed using 300,000 cells in each 6-well dish or 40 mm dish, involved microscopic monitoring of transfection success and cell viability.

When the transfection reagent ratios were evaluated (Figure 3.2.), it was determined that 1:1 PeiPRO caused high cell death, while the 1:4 Pei ratio provided insufficient yield, and although the 1:5 Pei ratio offered similar yield to 1:3.5, more cell death was observed. Thus, the 1:3.5 Pei ratio was determined to provide the optimum transfection/survival ratio best balance among the reagent conditions ratios. However, the result was obtained from the comparison of DNA quantity: in all groups using 6 μ g DNA, overall transfection efficiency was found to be lower and cell health was negatively affected compared to the groups using 3 μ g DNA. In contrast, using 3 μ g DNA at a 1:3.5 Pei ratio resulted in both satisfactory eGFP expression and the lowest level of cell death. Therefore, it was decided that using 3 μ g DNA at a 1:3.5 Pei ratio is the most suitable protocol for the SOD1^{WT} and SOD1^{G93A} plasmids to be used in subsequent stages in NSC-34 cells.

The ALS disease model was then created by transfecting NSC-34 cells with eGFP, SOD1^{WT} and SOD1^{G93A} plasmids using these optimized transfection conditions (3 μ g DNA; 1:3.5 Pei) (Fig. 3.3.). eGFP signaling, starting 24 hours after transfection, reached its maximum level between approximately 72 and 96 hours and was maintained at a high level for up to 120 hours. SOD1^{WT} and SOD1^{G93A} plasmids exhibited the same signaling duration as the eGFP control. During the 120-hour period, there was a significant difference in cell morphology and viability between the eGFP, SOD1^{WT} and SOD1^{G93A} groups. Bright-field imaging showed that cells in all groups were healthier in the eGFP and SOD1^{WT} groups compared to the SOD1^{G93A} group for 5 days. In the SOD1^{G93A} group, cell health was significantly negatively affected over time, and therefore, overall transfection efficiency was found to be lower compared to other groups.

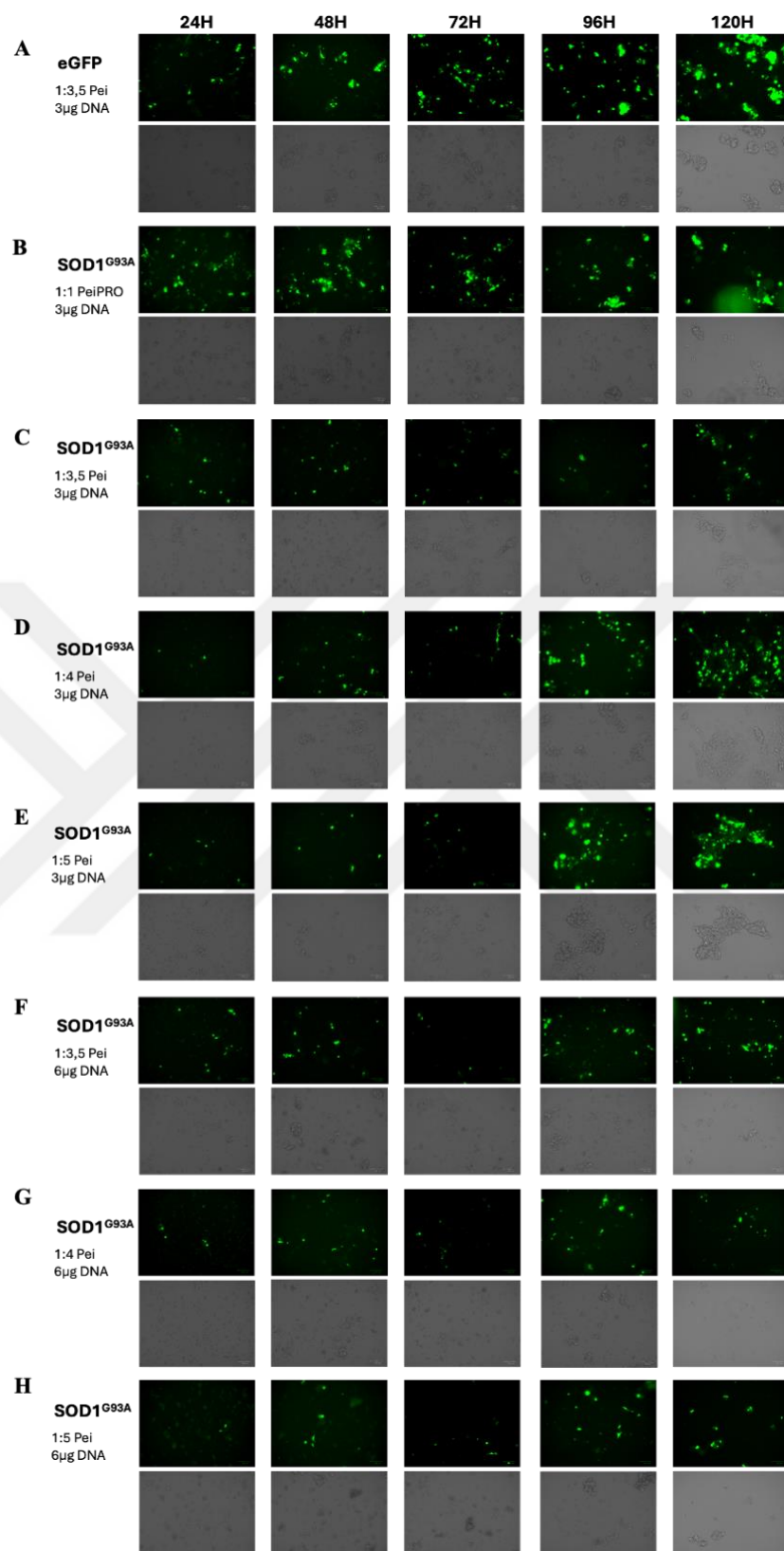


Figure 3.2: Time-Dependent (Up to 120 Hours) Morphological Analysis of eGFP Control and SOD1^{G93A} in NSC-34 Cells Under Different Transfection Conditions. The expression of the eGFP plasmid under the optimized condition (1:3.5 Pei) was compared with the expression of the SOD1G93A plasmid under different reagents (1:1 PeiPRO; 1:3.5, 1:4 and 1:5 Pei) and

DNA amounts (3 μ g and 6 μ g SOD1-Mutant plasmid). At each time point, the upper panel represents fluorescence microscopy images and the lower panel represents brightfield images showing cell health and density. (A) Control cells transfected with eGFP with 3 μ g DNA in 1:3.5 Pei. (B) Cells transfected with SOD1^{G93A} with 3 μ g DNA in 1:1 PeiPRO. (C) Cells transfected with SOD1^{G93A} with 3 μ g DNA in 1:3.5 Pei. (D) Cells transfected with SOD1^{G93A} with 3 μ g DNA in 1:4 Pei. (E) Cells transfected with SOD1^{G93A} with 3 μ g DNA in 1:5 Pei. (F) Cells transfected with SOD1^{G93A} with 6 μ g DNA in 1:3.5 Pei. (G) Cells transfected with SOD1^{G93A} with 6 μ g DNA in 1:4 Pei. (H) Cells transfected with SOD1^{G93A} with 6 μ g DNA in 1:5 Pei.

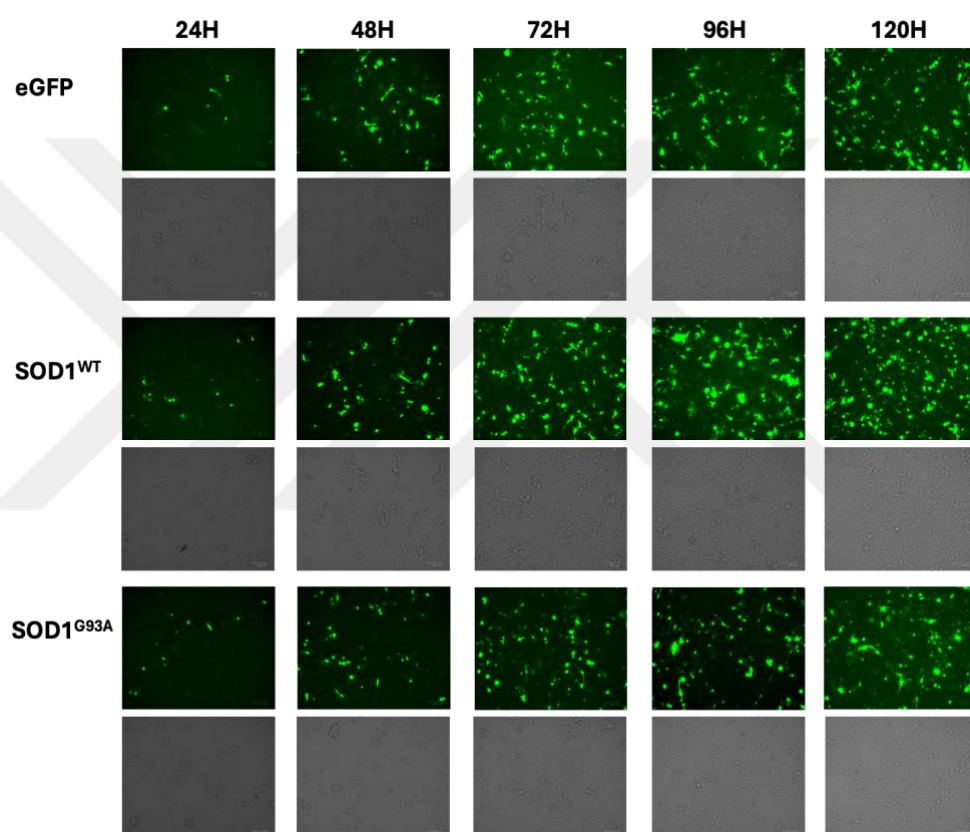


Figure 3.3: Expression of eGFP, SOD1^{WT} and SOD1^{G93A} Plasmids in NSC-34 Cells Under Optimized Transfection Conditions. Cells were transfected using previously optimized 3 μ g DNA and a 1:3.5 Pei ratio. Cell morphologies were monitored at 24, 48, 72, 96, and 120-hour time points. For each plasmid, the upper panel shows fluorescence, and the lower panel shows bright-field (cell morphology) images.

Cell viability and death analyses can generally be assessed by changes in metabolic activity or in cell membrane integrity. The MTT assay test indirectly measures the number of functionally viable cells by reliance on the reduction of a yellow salt into

purple formazan crystals by metabolic enzymes in the mitochondria of living cells. In contrast, PI/Hoechst staining directly assesses the physical state of the cell membrane: while Hoechst stains the nuclei of all cells, Propidium Iodide (PI) enters only the nuclei of dead cells with disrupted membrane integrity in the absence of membrane permeabilization, giving a red signal. When these two methods are used together, strong and complementary quantitative data on both the metabolic health and physical integrity of cells can be obtained.

These assays are used in this study to determine whether the SOD1^{G93A}, a common mutation in familial ALS pathology, causes acute metabolic dysfunction and cellular death in motor neuron-like cells. The viability of transfected NSC-34 cells under optimized transfection conditions was measured at 0, 48, and 72 hours using the MTT test and results were normalized to those obtained from non-transfected cells (NTC) in parallel time period (Fig. 3.4.). The SOD1^{WT} group showed a slight decrease in metabolic activity levels compared to NTC. This slight decrease is consistent with the basal level of cell death caused by the transfection procedure. However, a significant decrease was observed in the SOD1^{G93A} group: cell viability decreased at 48 hours. This decrease represents the most pronounced loss of metabolic activity among all groups. Although the viability rate increased to approximately 80% at 72 hours, this sharp decrease at 48 hours clearly demonstrated that the mutant protein caused acute metabolic dysfunction.

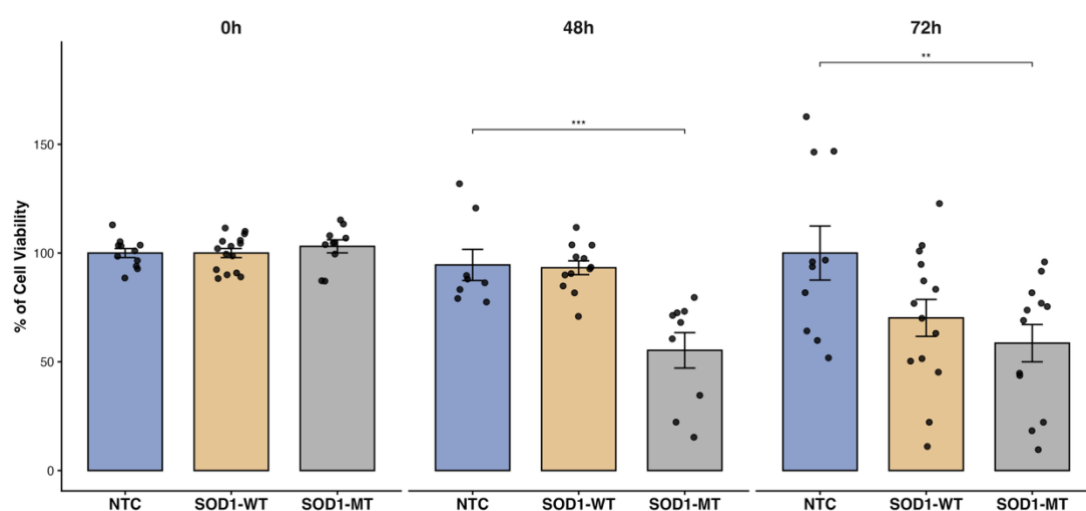


Figure 3.4: Analysis of the Effect of SOD1^{WT} and SOD1^{G93A} Expression on Cell Metabolic Activity in NSC-34 Cells by MTT Test. Cell viability rates were normalized relative to the non-transfected control group (NTC). Statistical

significance was analyzed by two-way ANOVA followed by Tukey's post hoc test. Differences were considered statistically significant at $p < 0.05$.

Complementary to the MTT assay, PI-Hoechst staining was performed on non-membrane-permeabilized cells. Fluorescence microscopy images were obtained at 0, 48, and 72 hours from NSC-34 cells transfected with eGFP, SOD1^{WT} and SOD1^{G93A} using optimized transfection conditions (3 μ g DNA; 1:3.5 Pei) (Fig. 3.5). The Hoechst signal (Blue) indicated the total number of cells in NTC and all transfected groups, while the PI signal (Red) stained only the nuclei of dead cells with compromised cell membrane integrity. When the 48-hour images were examined, it was observed that the number of PI-positive nuclei remained at a similar level to the number of Hoechst-positive nuclei in the NTC, eGFP, and SOD1^{WT} groups. In contrast, the SOD1^{G93A} mutant group contains a much higher percentage and intensity of red (PI) signal compared to all other groups (Fig. 3.5). This visual evidence demonstrates that the transfected SOD1^{G93A} mutant protein has a strong harmful effect as early as 48 hours.

Under conditions optimized for transfection efficiency and cell death analysis (3 μ g DNA; 1:3.5 Pei), NSC-34 cells were transfected with eGFP, SOD1^{WT}, and SOD1^{G93A} plasmids, and cell viability was quantitatively examined over time using PI-Hoeschst staining (Fig. 3.6). At the 0-hour time point, all groups had a similar viability rate to NTC, while at the 48-hour time interval, a certain amount of cell loss was observed in all transfected groups. The eGFP and SOD1^{WT} groups maintained similar viability rates, at 48 hours. This decrease was assumed to be primarily due to the stress caused by the transfection procedure. However, a significant and dramatic decrease was observed in the SOD1^{G93A} group at 48 hours. This data indicated that mutant protein expression caused acute cellular death. At the 72-hour time point, the viability rate of the SOD1^{G93A} group was found to have increased. This may be due to the division and proliferation of healthy cells during this time interval. However, it should be noted that even transfection of empty eGFP plasmid resulted in elevated PI-positive cells (Fig. 3.5), therefore these results can only be interpreted in combination with the above-mentioned MTT Assay (Fig. 3.4)

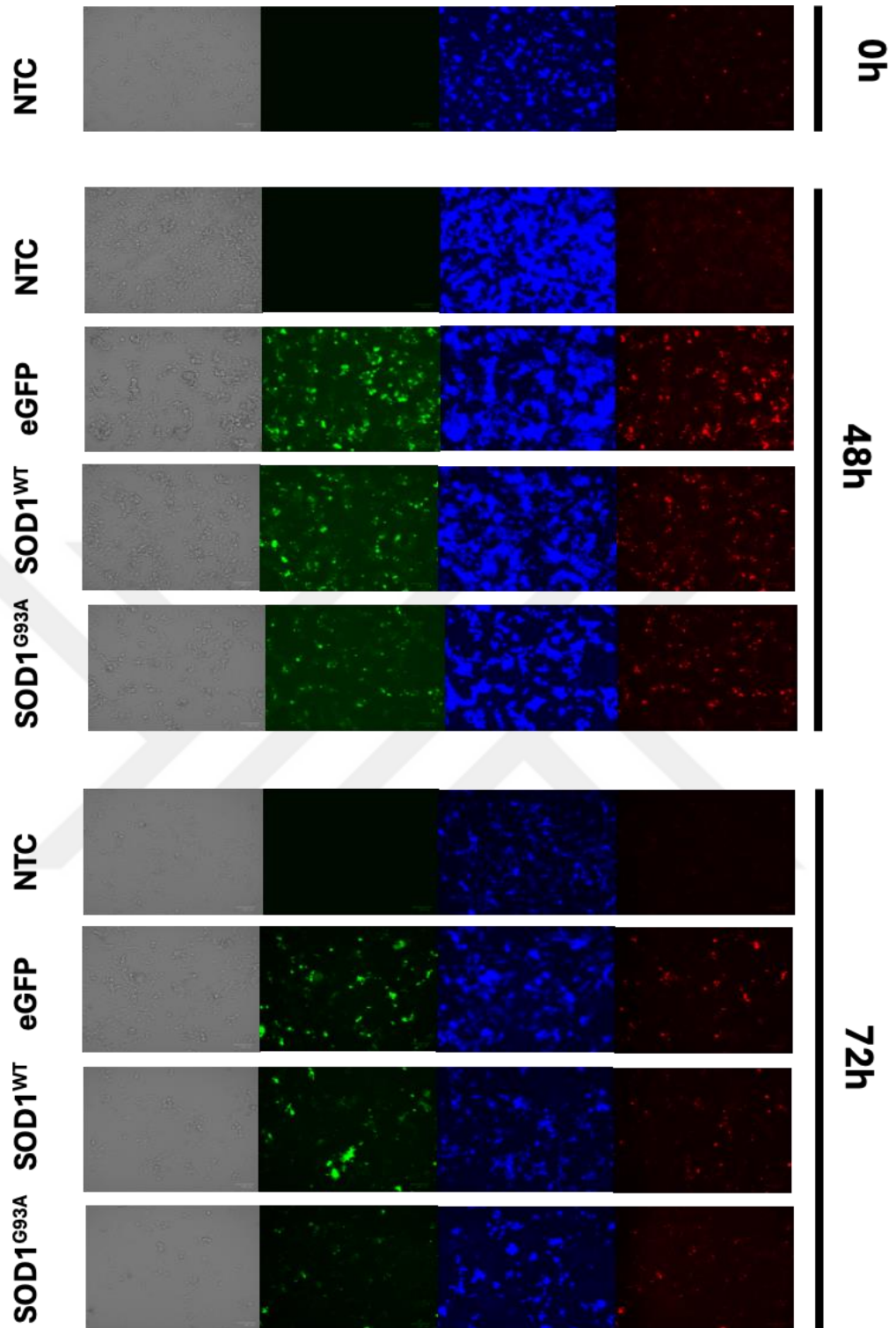


Figure 3.5: Visual Analysis of eGFP, SOD1^{WT}, and SOD1^{G93A} Groups in NSC-34 Cells by Time-Dependent PI-Hoechst Staining. Cells were transfected using optimized 3 μ g DNA and a 1:3.5 Pei ratio and examined with PI and Hoechst stains at time points of 0, 48, and 72 hours. From left to right: Bright Field (Morphology), eGFP/SOD1 Expression (Green), Hoechst Staining (Blue - All Nuclei), PI Staining (Red - Dead Cells).

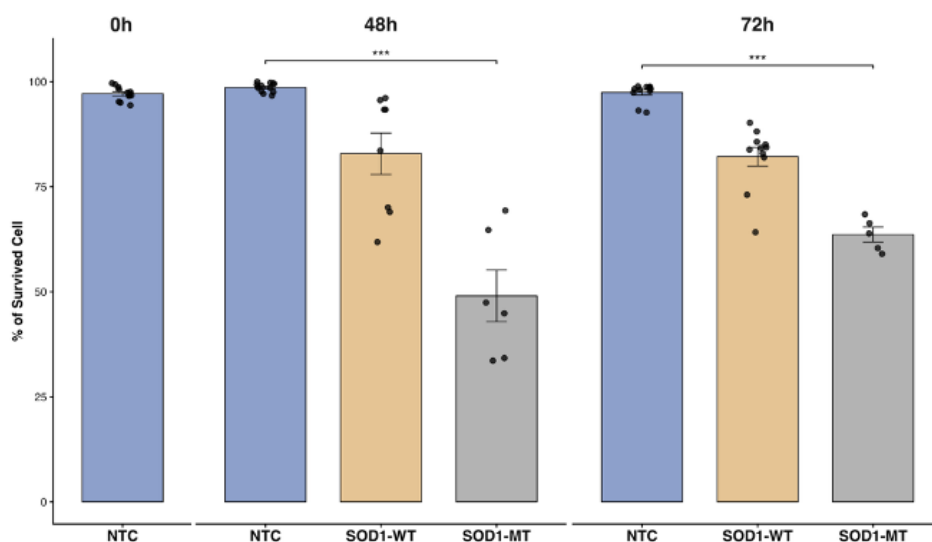


Figure 3.6: Quantitative Cell Viability Analysis of eGFP, SOD1^{WT} and SOD1^{G93A} Groups in NSC-34 Cells by Time-Dependent PI-Hoechst Staining. Cell viability rates were determined by cell counting at 0, 48, and 72-hour time points using the PI-Hoechst staining method under optimized transfection conditions (3 μ g DNA, 1:3.5 Pei). Results were normalized to the non-transfected control group (NTC). Statistical significance was analyzed by two-way ANOVA followed by Tukey's post hoc test. Differences were considered statistically significant at $p < 0.05$.

Next, quantitative Real-Time PCR (qPCR) analysis was performed to test the potential gene regulation network of ELK1 and to elucidate the effects of the SOD1^{G93A} mutant on intracellular gene regulation. Data obtained by the $\Delta\Delta$ Ct method using internal control genes such as β -actin and GAPDH quantitatively. Gene expression levels were determined by qPCR using cDNA templates (SOD1^{WT} and SOD1^{G93A} transfected at 48 and 72 hours) (Figure 3.7.).

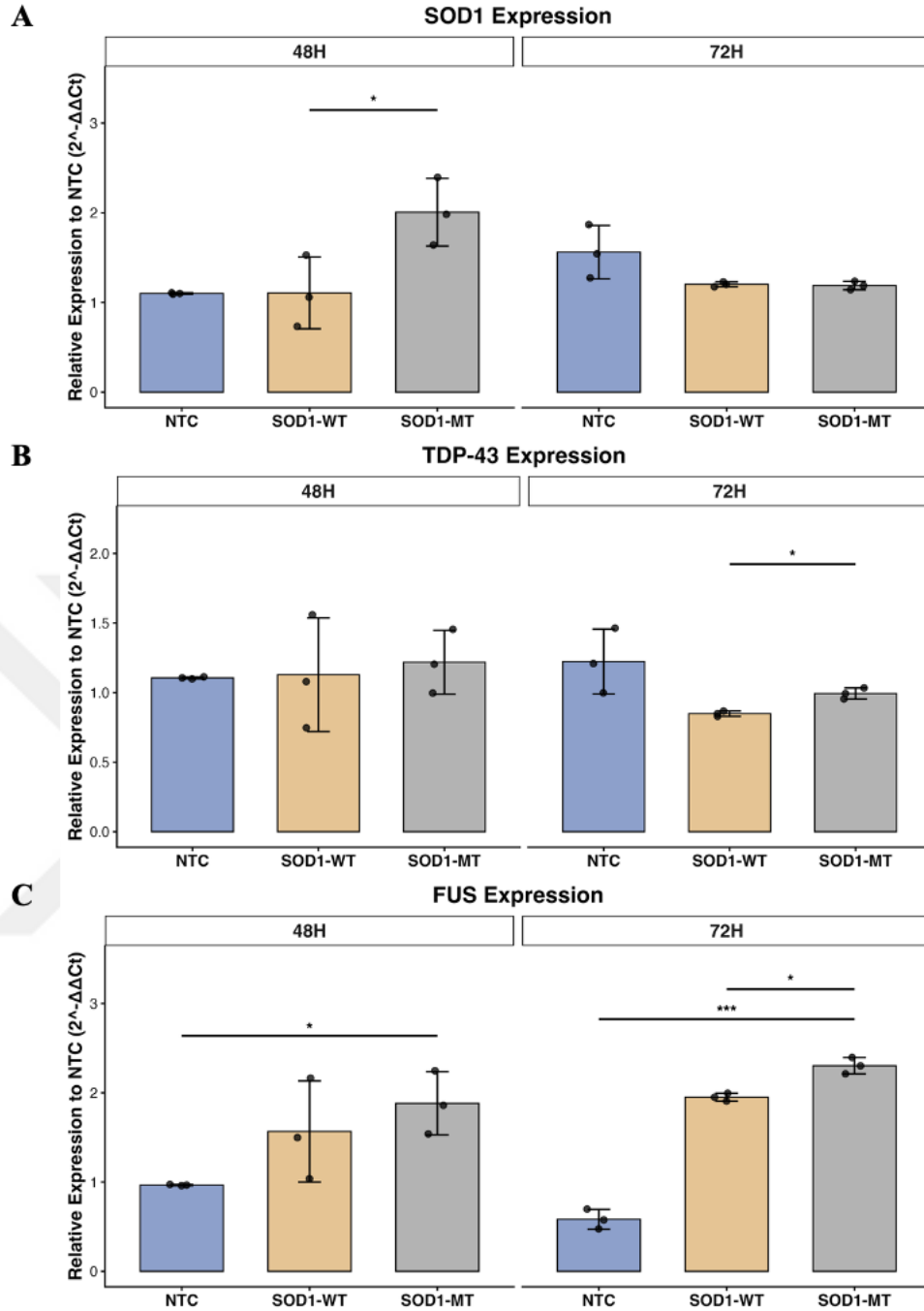


Figure 3.7: Relative Expression Levels of SOD1, TDP-43, and FUS Genes in NTC, SOD1^{WT} and SOD1^{G93A} Groups in NSC-34 Cells. The effect of NTC, SOD1^{WT} and SOD1^{G93A} expression on the relative expression of SOD1 (A), TDP-43 (B) and FUS (C) genes at 48 and 72 hours. Expression levels obtained by the $\Delta\Delta C_t$ method using internal control genes such as β -actin and GAPDH quantitatively. Expression levels were normalized according to the NTC group. In statistical analyses, the F-test and t-test were performed. Differences were considered statistically significant at $p < 0.05$.

At 48 hours, SOD1 expression showed a significant increase in the SOD1^{G93A} group compared to both NTC and SOD1^{WT}, indicating a mutation-dependent upregulation of SOD1 transcript levels ($p < 0.05$). In contrast, no significant difference was observed between the NTC and SOD1^{WT} groups. At 72 hours, SOD1 expression levels were comparable among groups, indicating that the early mutation-driven transcriptional effect observed at 48 hours is attenuated over time. For TDP-43, no statistically significant change was detected among groups at 48 hours, suggesting that short-term SOD1 overexpression does not immediately alter TDP-43 transcription. Analysis of FUS expression revealed a significant increase in both SOD1^{WT} and SOD1^{G93A} groups compared to NTC at 48 hours ($p < 0.05$), with a more pronounced elevation in the mutant condition. At 72 hours, FUS expression remained significantly elevated in SOD1-transfected cells, particularly in the SOD1^{G93A} group ($p < 0.001$), indicating sustained transcriptional dysregulation.

Therefore, since the aim was to examine the expression levels of potential target genes in the potential regulatory network of ELK1 with ALS-related genes in the disease state (SOD1^{G93A} transfected 48-hour period), it was decided to perform qPCR with cDNA templates in the 48-hour period.

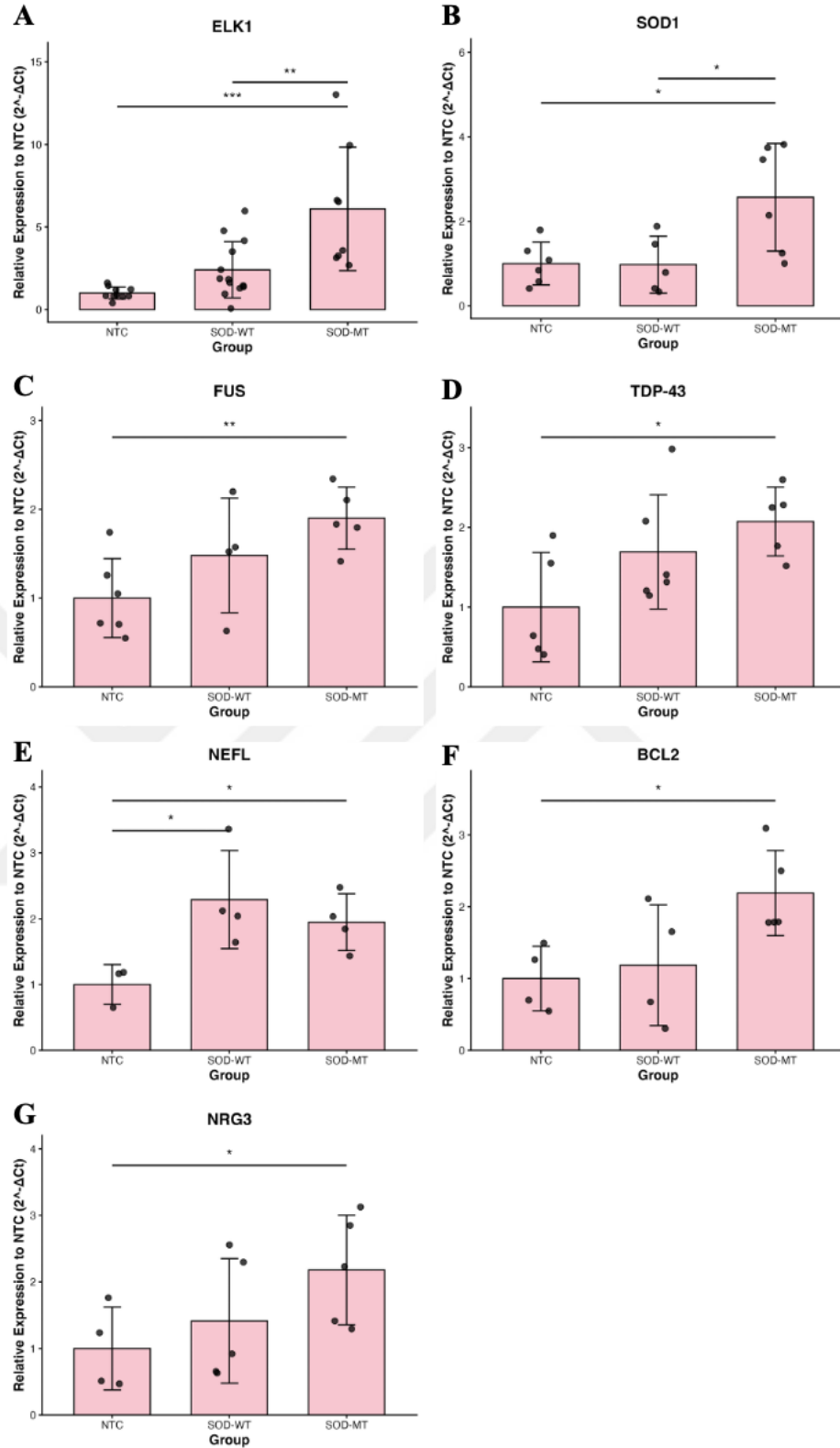


Figure 3.8: Analysis of the Effect of SOD1^{G93A} Mutant Expression on Potential Target Genes in NSC-34 Cells by qPCR (significantly changed genes). The genes in the figure are the genes that showed significant changes in qPCR among the target genes in the potential regulatory network, and their expression levels were normalized according to the NTC group. (A) Relative ELK1 mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups

normalized to NTC. (B) Relative SOD1 mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC. (C) Relative FUS mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC. (D) Relative TDP-43 mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC. (E) Relative NEFL mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC. (F) Relative BCL2 mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC. (G) Relative NRG3 mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC.

SOD1 expression was significantly increased ($p < 0.05$) in the SOD1^{G93A} group compared to both the NTC and SOD1^{WT} groups. Similarly, TDP-43 and FUS showed significantly higher expression ($p < 0.05$ and $p < 0.01$) levels in SOD1^{G93A} cells, suggesting that mutant SOD1 expression is associated with changes in the regulation of key genes associated with ALS. ELK1 expression showed statistically significant increase ($p < 0.001$) in the SOD1^{G93A} group compared to both the NTC and SOD1^{WT} groups. Consistent with the potential regulatory network of ELK1, significant expression changes were observed in several ALS-related target genes. NEFL, a marker of neuronal integrity and cytoskeletal organization, was significantly upregulated ($p < 0.05$) in SOD1^{WT} cells compared to NTC, but its expression remained unchanged in the SOD1^{G93A} group. BCL2, a key regulator of apoptotic and cell survival, showed a significant increase in expression ($p < 0.05$) in SOD1^{G93A} cells, suggesting increased activation of survival-related pathways. Additionally, NRG3 is a marker of synaptic communication and its expression was significantly upregulated ($p < 0.05$) in the SOD1^{G93A} group compared to NTC controls.

To investigate the effect of mutant SOD1 expression on genes associated with axonal transport and intracellular transport, the expression levels of HAP1, RAB39B, and KIF5A in NSC-34 cells were analyzed by qPCR. HAP1 expression was significantly increased in the SOD1^{WT} group compared to the NTC group ($p < 0.05$), while a significant decrease was observed in the SOD1^{G93A} group compared to SOD1^{WT} ($p < 0.01$). This indicates that mutant SOD1 expression alters the regulation of HAP1 compared to the wild-type group. Similarly, RAB39B expression showed a significant increase ($p < 0.05$) in SOD1^{WT} cells compared to the NTC group, while a significant decrease ($p < 0.01$) was observed in the SOD1^{G93A} group compared to SOD1^{WT}. The contrasting expression pattern between wild-type and mutant SOD1 conditions

suggests that mutant SOD1 disrupts the transcriptional regulation of RAB39B. Unlike HAP1 and RAB39B, KIF5A expression did not show statistically significant differences between the NTC, SOD1^{WT}, and SOD1^{G93A} groups, although an increased trend was observed in the SOD1^{G93A} condition. This result indicates that KIF5A expression is less sensitive to transcriptional changes caused by SOD1 mutation under the tested conditions.

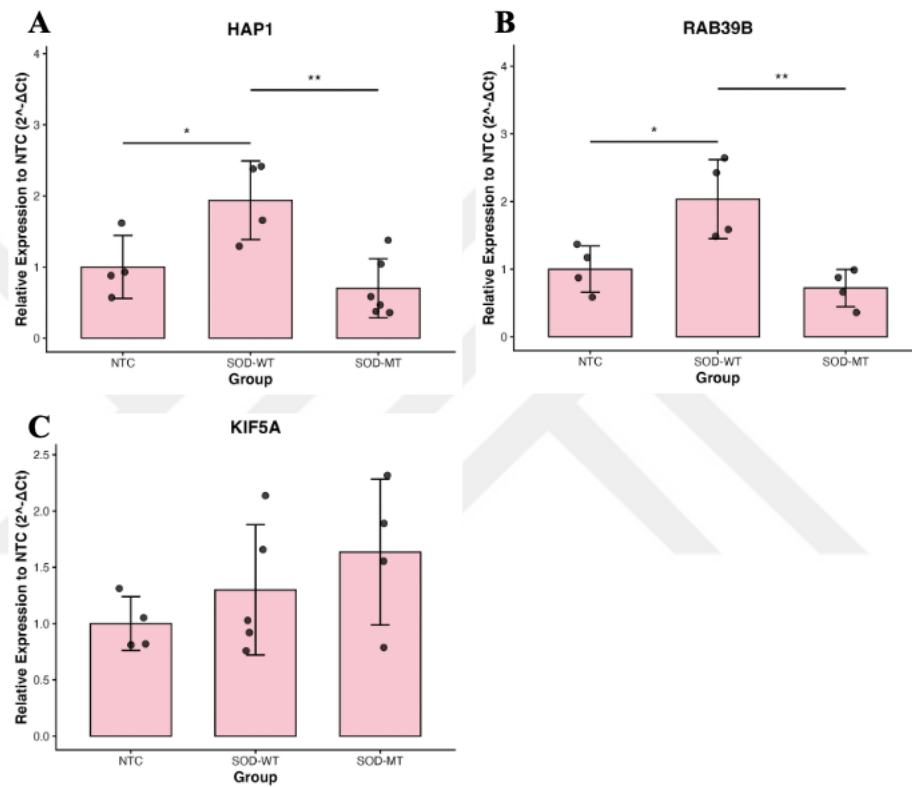


Figure 3.9: Analysis of the Effect of SOD1^{G93A} Mutant Expression on Potential Target Genes in NSC-34 Cells by qPCR (genes related to axonal transport - intracellular traffic). The genes in the figure are the genes that showed significant changes in qPCR among the target genes in the potential regulatory network, and their expression levels were normalized according to the NTC group. (A) Relative HAP1 mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC. (B) Relative RAB39B mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC. (C) Relative KIF5A mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC.

To examine the effect of mutant SOD1 expression on genes associated with cellular stress response and neuronal survival, the expression levels of PINK1, NRG1, PPP3R1, PPP3CB, and PPP3CC were analyzed by qPCR in NSC-34 cells expressing

wild-type or mutant SOD1. PINK1 expression showed an increase in the SOD1^{WT} group compared to NTC. However, no statistically significant differences were observed among the experimental groups. In contrast, NRG1 expression was significantly reduced in the SOD1^{G93A} group compared to SOD1^{WT} ($p < 0.05$), indicating changed regulation of this survival-related gene in response to mutant SOD1 expression. The expression of PPP3R1 was significantly decreased in the SOD1^{G93A} group compared to SOD1^{WT} ($p < 0.05$), while no significant difference was observed between NTC and SOD1^{WT} groups. Similarly, PPP3CB expression was significantly increased in SOD1^{WT} cells compared to NTC ($p < 0.01$), followed by a reduction in the SOD1^{G93A} group. These results indicate that mutant SOD1 expression disrupts the expression pattern of calcineurin-associated genes. In contrast, PPP3CC expression did not show statistically significant differences among NTC, SOD1^{WT}, and SOD1^{G93A} groups, suggesting relative stable expression of this gene under the experimental conditions.

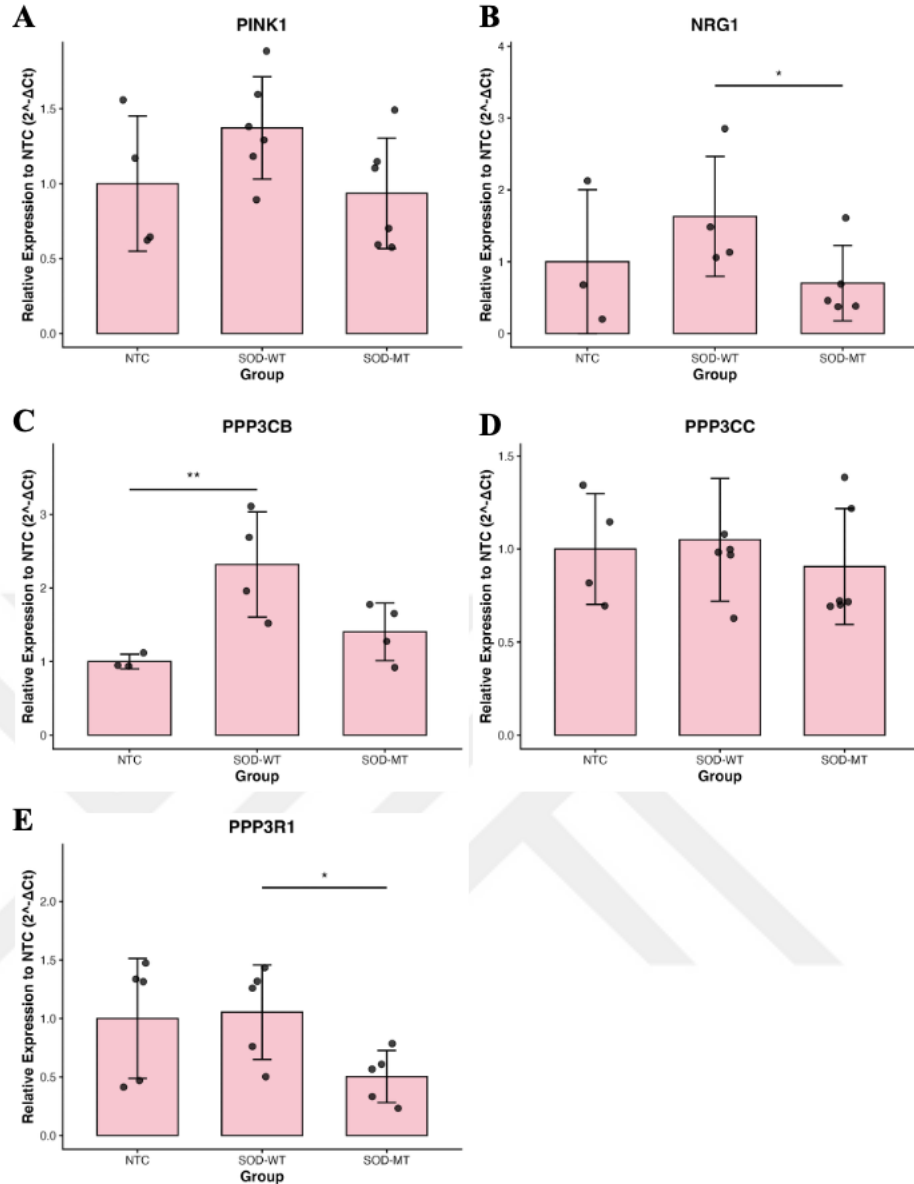


Figure 3.10: Analysis of the Effect of SOD1^{G93A} Mutant Expression on Potential Target Genes in NSC-34 Cells by qPCR (genes related to cellular stress response - survival). The genes in the figure are the genes that showed significant changes in qPCR among the target genes in the potential regulatory network, and their expression levels were normalized according to the NTC group. (A) Relative PINK1 mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC. (B) Relative NRG1 mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC. (C) Relative PPP3CB mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC. (D) Relative PPP3CC mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC. (E) Relative PPP3R1 mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC.

To evaluate the effect of mutant SOD1 expression on genes involved in axon integrity and microtubule dynamics, the expression levels of TUBA1A, TUBB2B, TUBB3, and NEFH genes were analyzed by qPCR in NSC-34 cells expressing wild-type or mutant SOD1. TUBA1A expression was significantly decreased in the SOD1^{WT} group compared to the NTC group ($p < 0.05$). In the SOD1^{G93A} group, TUBA1A expression showed a partial recovery compared to SOD1^{WT} but remained lower than NTC levels. In contrast, TUBB2B expression did not show statistically significant differences between the NTC, SOD1^{WT}, and SOD1^{G93A} groups, although increased variability was observed, particularly under the SOD1^{G93A} condition. Similarly, TUBB3 expression remained relatively constant across all experimental groups, and no significant difference was detected between NTC, SOD1^{WT}, and SOD1^{G93A}. The expression level of NEFH, a marker associated with axon structure and neuronal integrity, did not show a statistically significant difference between the experimental groups. However, a trend towards increased expression was observed in the SOD1^{G93A} group compared to the NTC and SOD1^{WT} conditions.

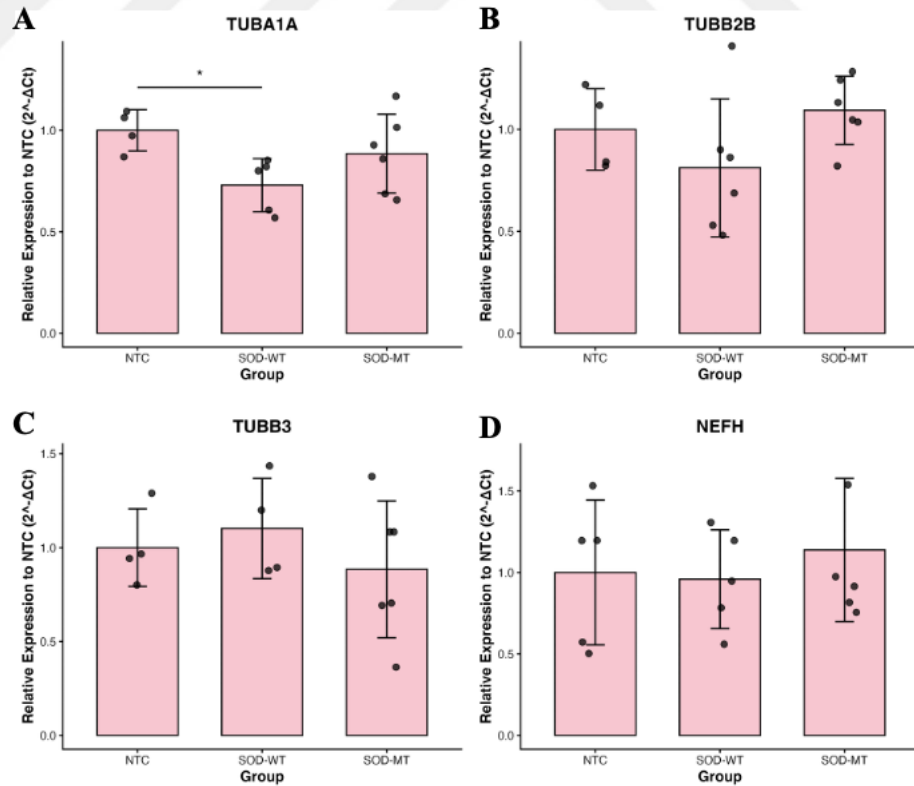


Figure 3.11: Analysis of the Effect of SOD1^{G93A} Mutant Expression on Potential Target Genes in NSC-34 Cells by qPCR (genes related to axonal integrity – microtubule dynamics). The genes in the figure are the genes that showed

significant changes in qPCR among the target genes in the potential regulatory network, and their expression levels were normalized according to the NTC group. (A) Relative TUBA1A mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC. (B) Relative TUBB2B mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC. (C) Relative TUBB3 mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC. (D) Relative NEFH mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC.

To evaluate the impact of mutant SOD1 expression on genes involved in synaptic transmission and glutamatergic signaling, the expression levels of GRIA1, GRIN2A, GRIN2B, and GRIN2D were analyzed by qPCR in NSC-34 cells expressing wild-type or mutant SOD1. GRIA1 expression did not show statistically significant differences among NTC, SOD1^{WT} and SOD1^{G93A} groups, although variability was observed across replicates. This indicates that GRIA1 transcription is not markedly affected by either wild-type or mutant SOD1 expression under the experimental conditions. In contrast, GRIN2A expression was significantly reduced in the SOD1^{WT} group compared to NTC ($p < 0.05$). In the SOD1^{G93A} group, GRIN2A expression showed partial recovery relative to SOD1^{WT}, but no significant difference was detected when compared to NTC. GRIN2B expression did not exhibit statistically significant differences among the experimental groups, although a tendency toward reduced expression was observed in both SOD1^{WT} and SOD1^{G93A} conditions compared to NTC. Similarly, GRIN2D expression did not show significant differences between NTC, SOD1^{WT} and SOD1^{G93A} groups. However, increased variability and a trend toward higher expression were observed in the SOD1^{G93A} condition.

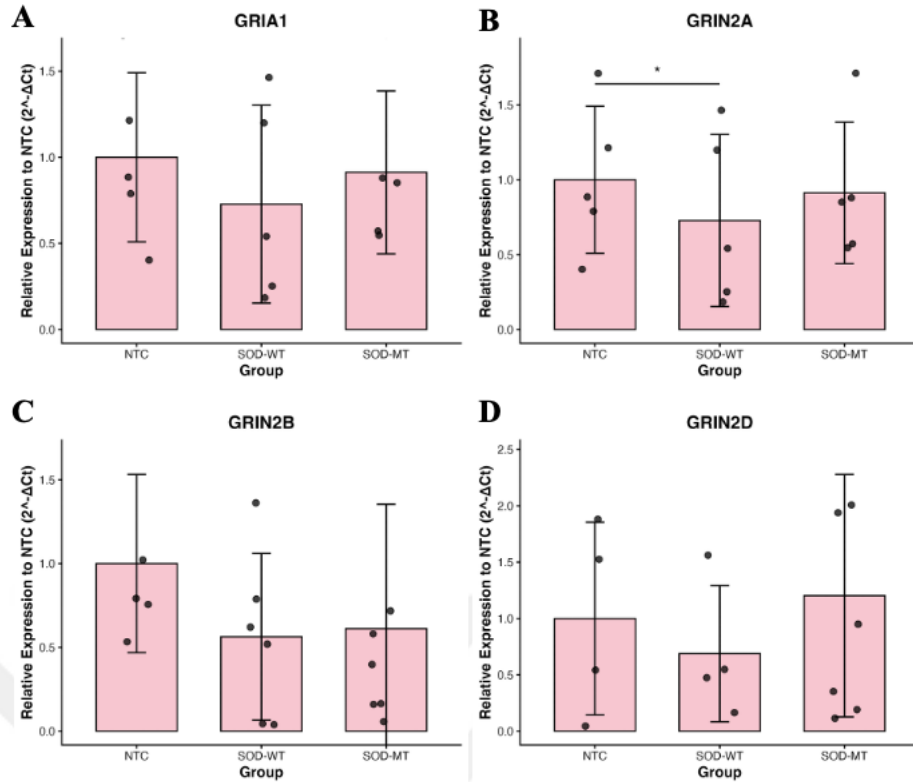


Figure 3.12: Analysis of the Effect of SOD1^{G93A} Mutant Expression on Potential Target Genes in NSC-34 Cells by qPCR (genes related to synaptic transmission– glutamatergic response). The genes in the figure are the genes that showed significant changes in qPCR among the target genes in the potential regulatory network, and their expression levels were normalized according to the NTC group. (A) Relative GRIA1 mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC. (B) Relative GRIN2A mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC. (C) Relative GRIN2B mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC. (D) Relative GRIN2D mRNA expression levels in NTC, SOD1^{WT}, and SOD1^{G93A} groups normalized to NTC.

4. DISCUSSION AND CONCLUSION

During the progression of ALS, protein aggregation, impaired RNA metabolism, and mitochondrial dysfunction are usually observed in motor neurons, leading to neuronal dysfunction and ultimately death [19]. Many genes were reported to be associated with or causative to familial or sporadic forms of ALS, however there are still many aspects of ALS that are unclear. In this study, we mainly focused on a transcription factor of the ETS superfamily, namely ELK1, that our laboratory hypothesizes to cause a neuroprotective effect by initiating cellular differentiation, proliferation and apoptosis. Therefore, ELK1 may regulate ALS-related genes and play a critical role in the progression of the disease.

This study sought to determine whether ELK1 plays a role in ALS pathogenesis by regulating the transcription of disease-related genes through an integrated approach combining transcriptomic analysis and in vitro experimental validation. By analyzing numerous publicly available ALS-related transcriptomic datasets, we identified changes in the activity of ELK1 and its potential ALS-related target genes. Specifically, among the analyzed datasets, only in the GSE192755 dataset was ELK1 expression found to be significantly changed along with potential ALS-related target genes.

The GSE192755 dataset is a bulk RNAseq data whose samples are derived from iPSC-derived motor neurons from ALS-discordant twins with SOD1 mutation. Clear separation of healthy and diseased samples in PCA analysis, together with the identification of thousands of differentially expressed genes, confirmed the validity of this dataset. Importantly, functional enrichment analyses revealed strong overrepresentation of pathways related to synaptic transmission, axonal development, vesicle-mediated transport, and nervous system development. These processes are well-established hallmarks of ALS pathology, supporting the biological relevance of the transcriptomic changes observed.

Within this disease-associated transcriptional context, ELK1 has been found as significantly upregulated. Given the known role of ELK1 as a signal-responsive regulator involved in neuronal activity, stress responses, and survival pathways, its upregulation suggests that ELK1 may play an active role in disease-related

mechanisms. Rather than representing a passive change, increased ELK1 expression may reflect a compensatory response to neuronal stress or an attempt to maintain cellular homeostasis under pathological conditions.

To further explore this possibility, a genome-wide promoter-based gene regulatory network was constructed. Integration of ETS-binding motif predictions with expression-based co-variation identified an ELK1 regulatory network with potential ALS-related genes. Importantly, potential ALS-related target genes were significantly downregulated in the disease condition and clustered into functional groups directly relevant to ALS pathology, including axonal transport and intracellular trafficking, cellular stress response and survival, cytoskeletal organization, calcium-dependent signaling, and glutamatergic synaptic transmission.

Experimental validation using the NSC-34 motor neuron-like cell line further confirmed the validity of the computational analysis. The expression of the ALS-associated SOD1^{G93A} mutant caused significant morphological changes, decreased cell viability, impaired metabolic activity, and increased loss of membrane integrity compared to wild-type SOD1 and control conditions. These findings confirm that the in vitro model reflects the key features of ALS-related cellular stress and neuronal dysfunction.

Consistent with transcriptomic analysis, mutant SOD1 expression was associated with significant upregulation of ELK1, as well as changed expression of multiple ALS-related target genes. Genes involved in axonal transport and intracellular trafficking, such as HAP1 and RAB39B, showed opposite expression patterns between wild-type and mutant SOD1 conditions, suggesting that mutant SOD1 disrupts normal transcriptional regulation of transport-related pathways. Similarly, genes associated with cellular stress responses and survival, including BCL2 and NRG3, were altered under mutant conditions, indicating activation of compensatory or stress-induced transcriptional programs. Changes observed in calcineurin-associated genes (PPP3R1, PPP3CB) further suggest that mutant SOD1 affects calcium-dependent signaling cascades, which are critical for neuronal function and survival. In contrast, several genes related to cytoskeletal organization and glutamatergic signaling displayed more subtle or variable transcriptional changes. This may reflect pathway-specific sensitivity to mutant SOD1 expression, temporal regulation differences, or post-transcriptional mechanisms not captured by gene expression analysis alone. The partial

recovery or increased variability observed for certain genes in the mutant condition suggests that transcriptional dysregulation in ALS is complex and may involve both compensatory and degenerative components.

Overall, this study provides converging computational and experimental evidence that ELK1 is involved in transcriptional networks associated with ALS-related cellular dysfunction. The integration of transcriptomic screening, regulatory network inference, and *in vitro* validation supports the hypothesis that ELK1 contributes to disease-associated molecular pathways, potentially acting as a compensatory or stress-responsive regulator. Future studies should focus on direct validation of ELK1–target gene interactions, analysis of ELK1 activity during disease progression, and functional manipulation of ELK1 to determine whether modulation of its activity can influence ALS-related cellular phenotypes.

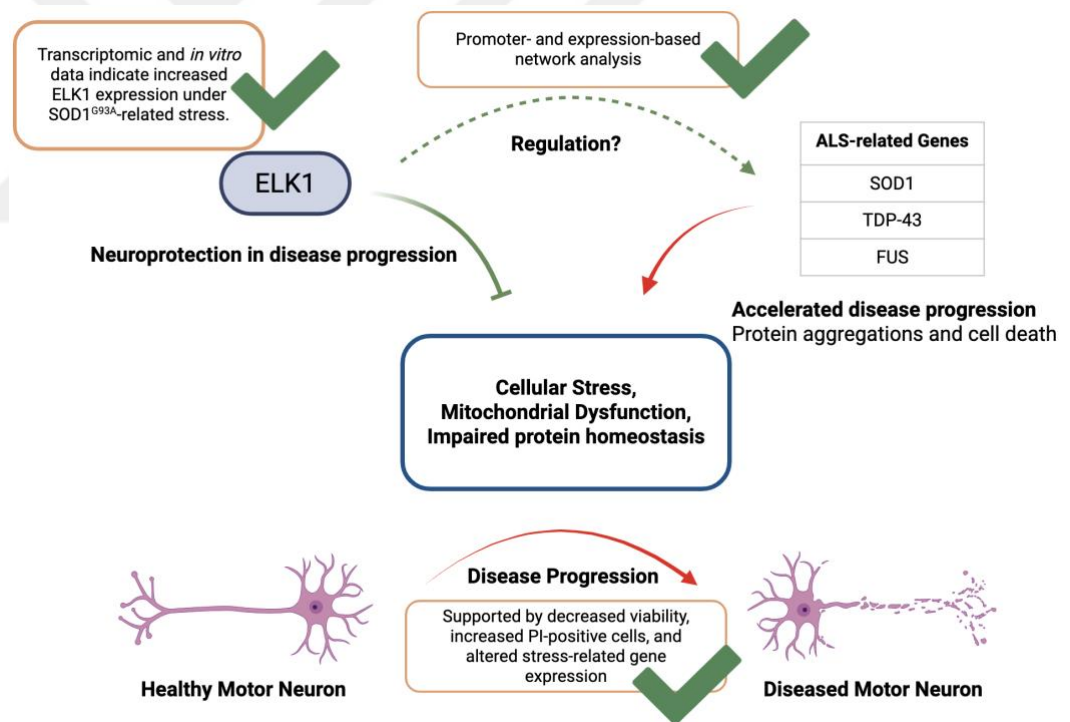


Figure 4.1: Summary of the findings illustrating the potential role of ELK1 in ALS. This schematic summarizes the integration of transcriptomic and *in vitro* findings indicating increased ELK1 expression under SOD1^{G93A}-related cellular stress. Promoter- and expression-based network analysis suggests a potential regulatory relationship between ELK1 and ALS-related genes. The model illustrates how altered transcriptional regulation may contribute to cellular stress, mitochondrial dysfunction, and impaired protein homeostasis, ultimately accelerating disease progression and motor neuron degeneration. Orange boxes represent experimentally and computationally

supported results obtained in this study, including ELK1 upregulation, decreased cell viability, increased PI-positive cells, and altered expression of stress-related genes. Green arrows indicate potential compensatory or neuroprotective responses, whereas red arrows represent disease-promoting processes leading to motor neuron degeneration.

As summarized in Figure 4.1, the integrated transcriptomic and in vitro analyses suggest that ELK1 is involved in transcriptional networks associated with cellular stress, mitochondrial dysfunction, and neuronal degeneration under ALS-related conditions. In conclusion, this study demonstrates that ELK1 is associated with ALS-related transcriptional changes and is positioned within a gene regulatory network linked to key pathogenic processes, including synaptic dysfunction, axonal transport impairment, cellular stress responses, and neuronal survival. Transcriptomic analyses identified ELK1 as a significantly altered transcription factor in a disease-relevant dataset, and in vitro validation using an SOD1^{G93A} motor neuron model confirmed ELK1 upregulation together with altered expression of several ALS-related target genes. Collectively, these findings suggest that ELK1 may act as an important transcriptional regulator in ALS-related molecular pathways and provide a basis for future studies aimed at defining its functional role in ALS pathogenesis.

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BIOGRAPHY

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She also contributed to poster presentations titled “Regulatory Network of ELK1 in Amyotrophic Lateral Sclerosis Based on Distinct Mutation Types” at the 23rd Turkish Neuroscience Congress, “ELK1 and Mitochondrial Gene Regulatory Networks in Neurodegenerative Diseases” at the 29th FEBS Congress and “Integrated Cell Models for Neurodegenerative Diseases and Senescence” at Biotech4Sus where the study received the Poster Third Prize.

PUBLICATIONS AND PRESENTATIONS FROM THE THESIS

[1] Regulatory Network of ETS Genes in Amyotrophic Lateral Sclerosis. 21. Turkish Neuroscience Congress, Bolu Abant İzzet Baysal University, Turkey. (Poster 1st Prize)

[2] Regulatory Network of ELK1 in Amyotrophic Lateral Sclerosis Based on Distinct Mutation Types. 23. Turkish Neuroscience Congress, Dokuz Eylül University İzmir, Turkey.

[3] Elk-1 and Mitochondrial Gene Regulatory Networks in Neurodegenerative Diseases. 29. FEBS Congress, İstanbul, Turkey.

[4] Integrated Cell Models for Neurodegenerative Diseases and Senescence. Biotech4Sus, Gebze Technical University Kocaeli, Turkey. October 2025. (Poster 3rd Prize)

APPENDICES

APPENDICIES-A: ALS Transcriptomic Datasets

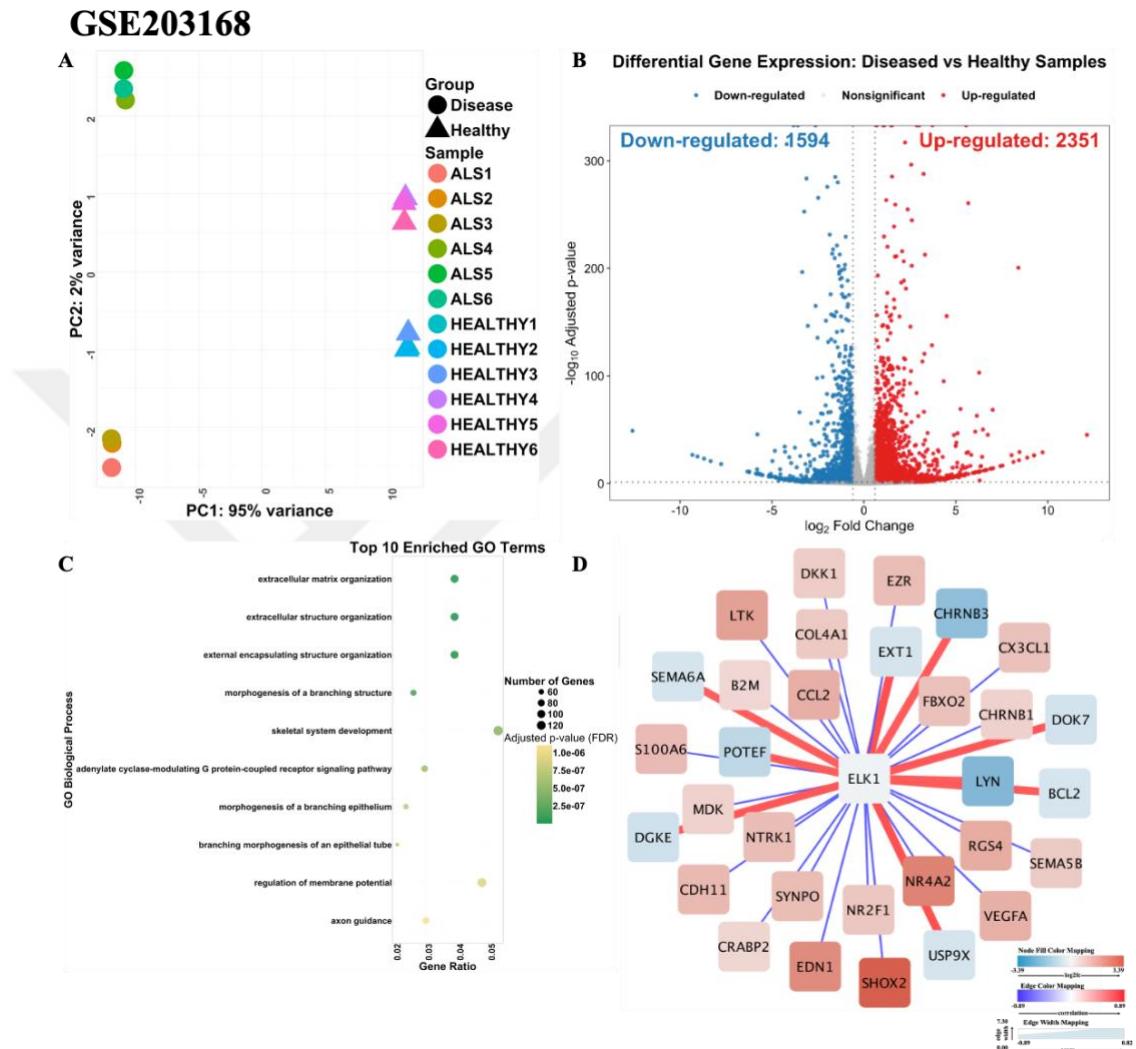


Figure A.1.1: Transcriptome analysis of the GSE203168. (A) Principal Component Analysis. It defines samples depending on the diseased or healthy group. (B) Volcano plot. In volcano plot, the most upregulated genes are towards the right, the most downregulated genes are towards the left. (C) Gene Ontology Analysis. The size of each circle corresponds to the number of associated counts, while adjusted p-values are encoded by the color scale. (D) Gene Regulatory Network of ELK1. It shows potential regulation network between ELK1 and its potential target genes. Some potential target genes are downregulated, some potential target genes are upregulated and ELK1 is downregulated. Edge width represents the strength of regulatory evidence between each transcription factor and its target gene. Edge color indicates the

correlation between transcription factors and target genes, while node color reflects the corresponding log2 fold change (Log2FC).

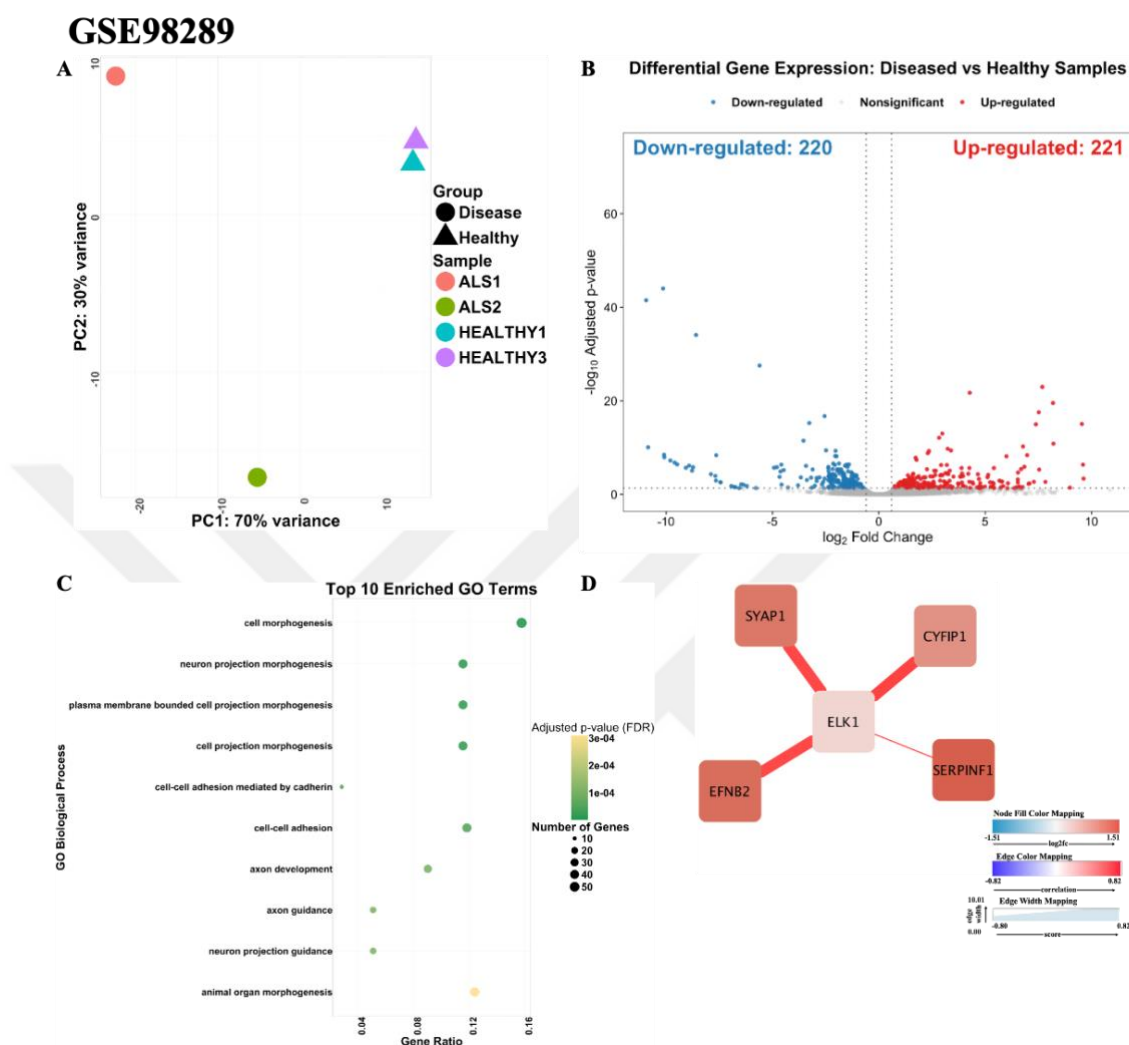


Figure A.1.2: Transcriptome analysis of the GSE98289. (A) Principal Component Analysis. It defines samples depending on the diseased or healthy group. (B) Volcano plot. In volcano plot, the most upregulated genes are towards the right, the most downregulated genes are towards the left. (C) Gene Ontology Analysis. The size of each circle corresponds to the number of associated counts, while adjusted p-values are encoded by the color scale. (D) Gene Regulatory Network of ELK1. It shows potential regulation network between ELK1 and its potential target genes. All potential target genes are upregulated and ELK1 is upregulated. Edge width represents the strength of regulatory evidence between each transcription factor and its target gene. Edge color indicates the correlation between transcription factors and target genes, while node color reflects the corresponding log2 fold change (Log2FC).

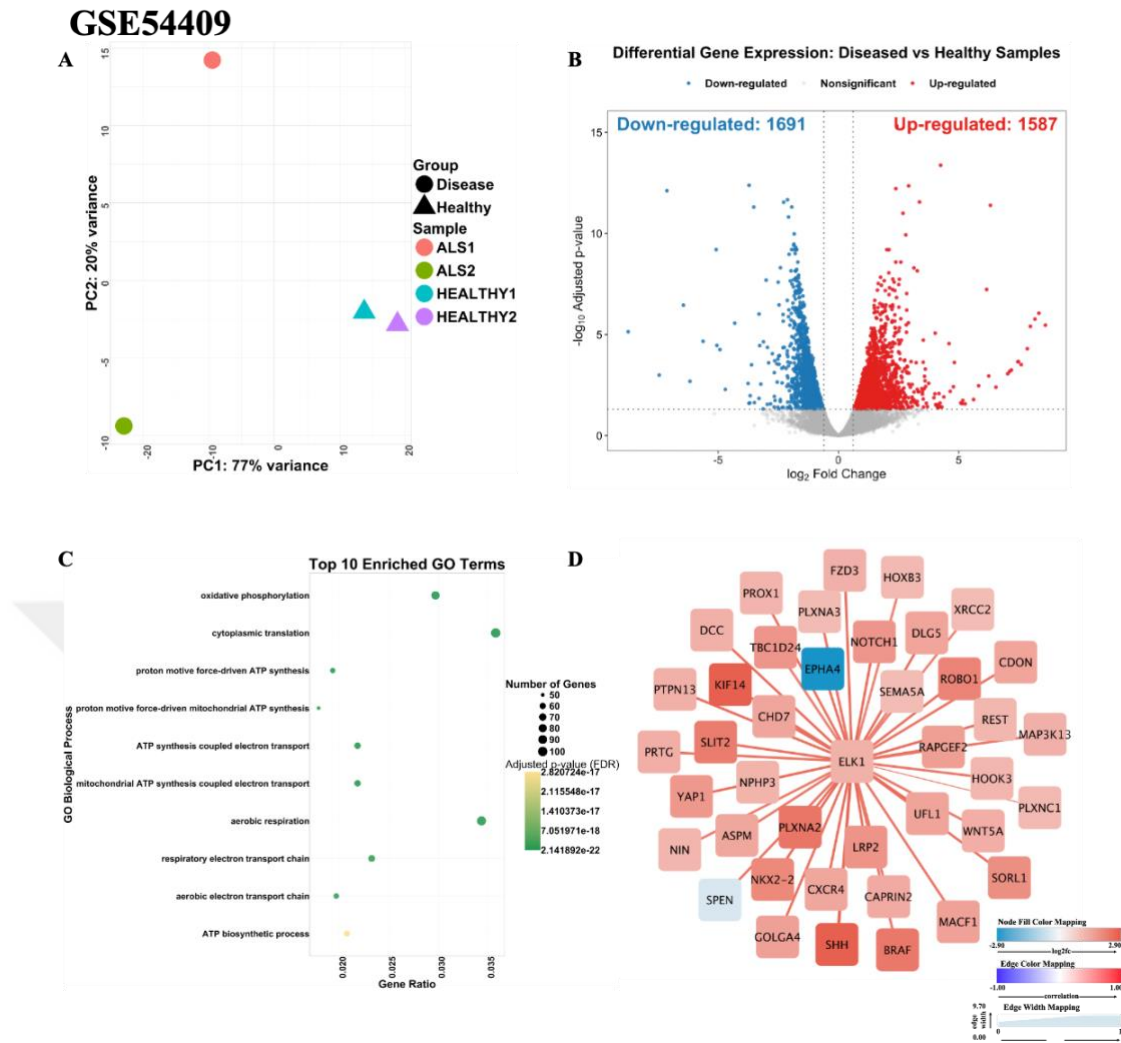


Figure A.1.3: Transcriptome analysis of the GSE54409. (A) Principal Component Analysis. It defines samples depending on the diseased or healthy group. (B) Volcano plot. In volcano plot, the most upregulated genes are towards the right, the most downregulated genes are towards the left. (C) Gene Ontology Analysis. The size of each circle corresponds to the number of associated counts, while adjusted p-values are encoded by the color scale. (D) Gene Regulatory Network of ELK1. It shows potential regulation network between ELK1 and its potential target genes. Some potential target genes are downregulated, some potential target genes are upregulated and ELK1 is upregulated. Edge width represents the strength of regulatory evidence between each transcription factor and its target gene. Edge color indicates the correlation between transcription factors and target genes, while node color reflects the corresponding $\log_2$ fold change ($\log_2FC$).

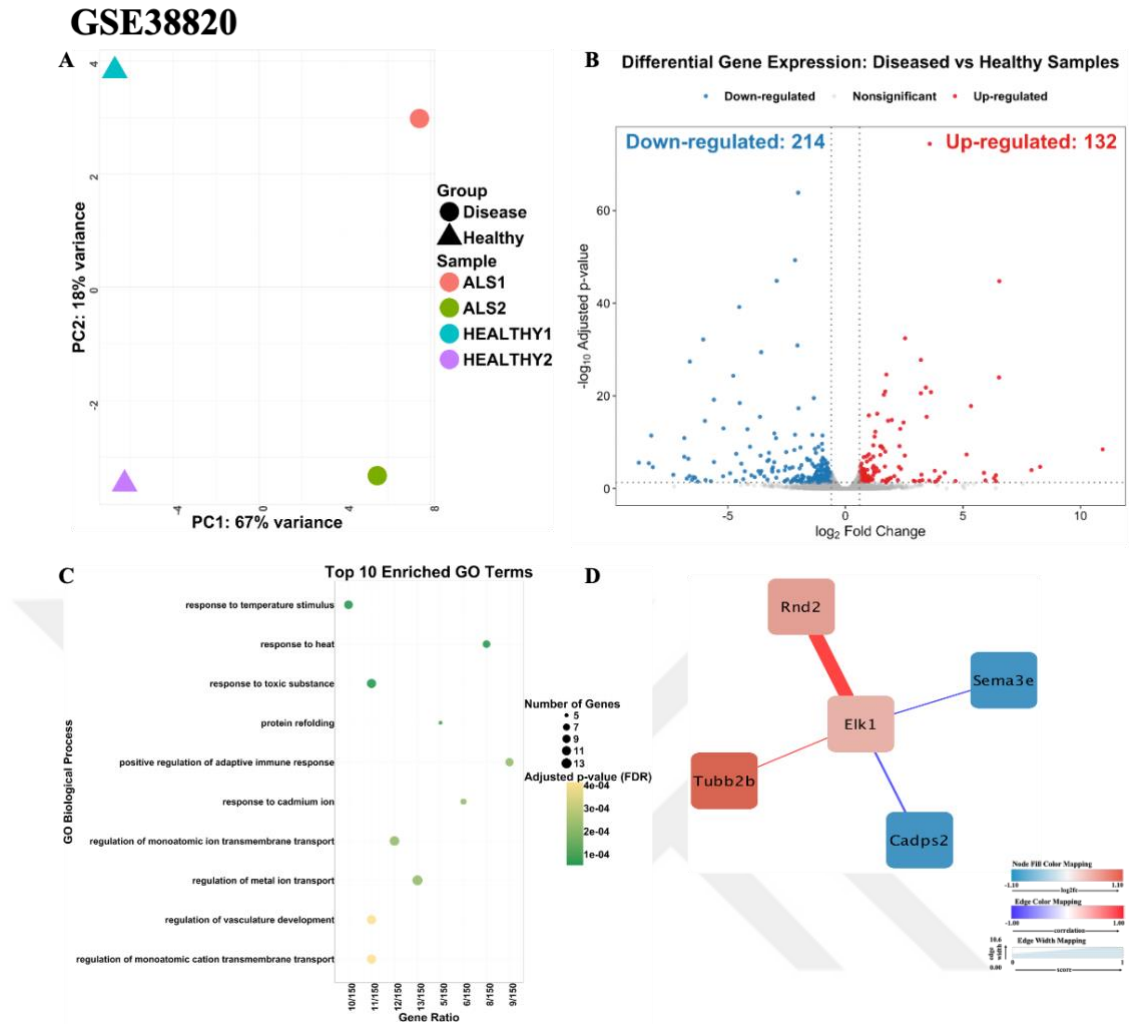


Figure A.1.4: Transcriptome analysis of the GSE38820. (A) Principal Component Analysis. It defines samples depending on the diseased or healthy group. (B) Volcano plot. In volcano plot, the most upregulated genes are towards the right, the most downregulated genes are towards the left. (C) Gene Ontology Analysis. The size of each circle corresponds to the number of associated counts, while adjusted p-values are encoded by the color scale. (D) Gene Regulatory Network of ELK1. It shows potential regulation network between ELK1 and its potential target genes. Some potential target genes are downregulated, some potential target genes are upregulated and ELK1 is upregulated. Edge width represents the strength of regulatory evidence between each transcription factor and its target gene. Edge color indicates the correlation between transcription factors and target genes, while node color reflects the corresponding log₂ fold change (Log₂FC).

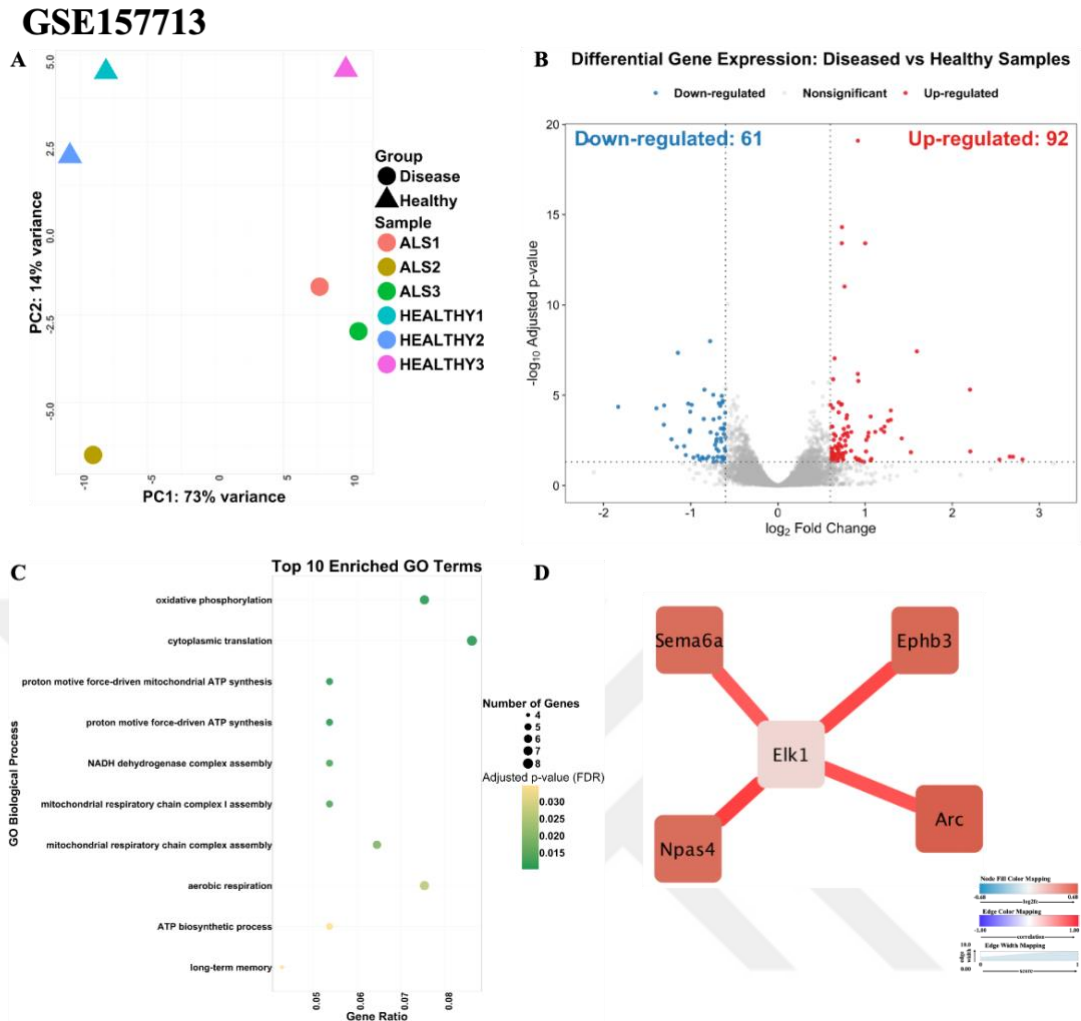


Figure A.1.5: Transcriptome analysis of the GSE157713. (A) Principal Component Analysis. It defines samples depending on the diseased or healthy group. (B) Volcano plot. In volcano plot, the most upregulated genes are towards the right, the most downregulated genes are towards the left. (C) Gene Ontology Analysis. The size of each circle corresponds to the number of associated counts, while adjusted p-values are encoded by the color scale. (D) Gene Regulatory Network of ELK1. It shows potential regulation network between ELK1 and its potential target genes. All potential target genes are upregulated and ELK1 is upregulated. Edge width represents the strength of regulatory evidence between each transcription factor and its target gene. Edge color indicates the correlation between transcription factors and target genes, while node color reflects the corresponding log2 fold change (Log2FC).

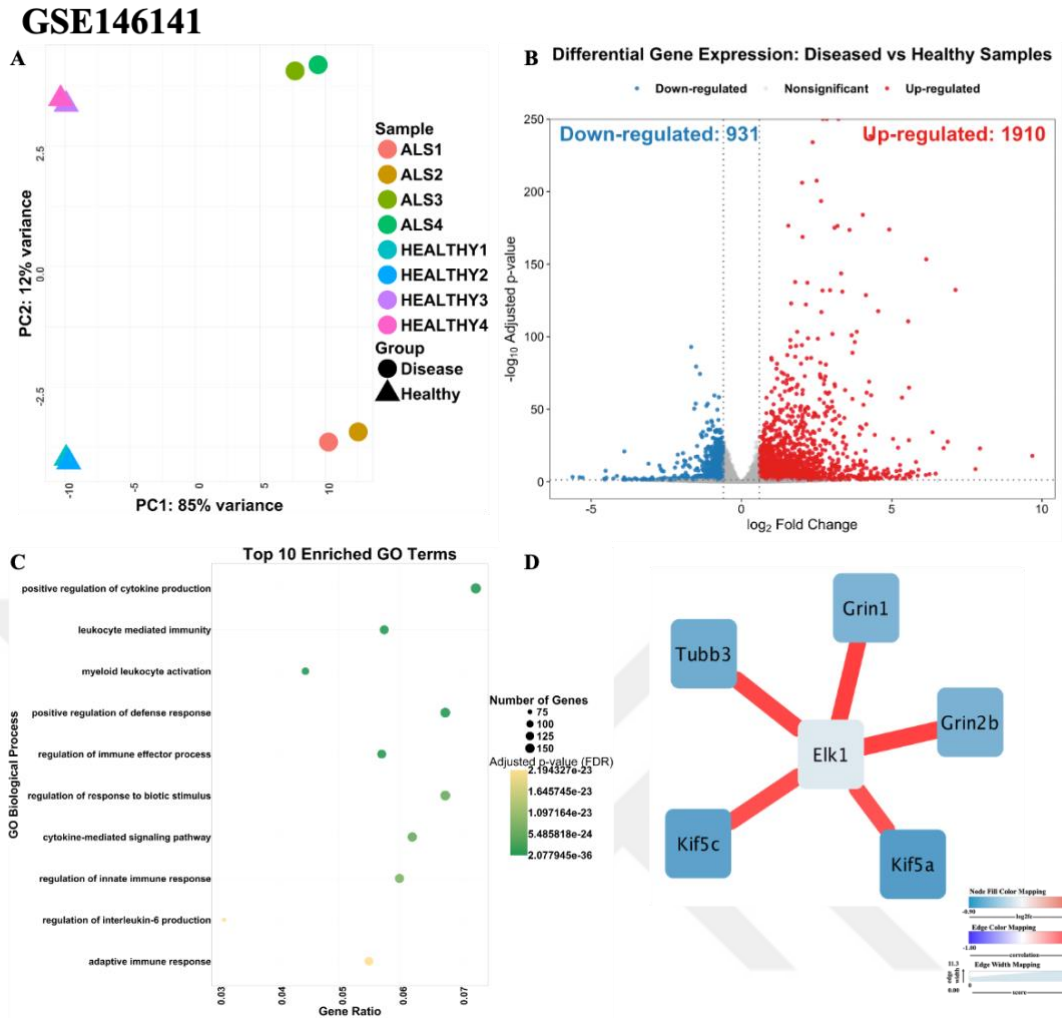


Figure A.1.6: Transcriptome analysis of the GSE146141. (A) Principal Component Analysis. It defines samples depending on the diseased or healthy group. (B) Volcano plot. In volcano plot, the most upregulated genes are towards the right, the most downregulated genes are towards the left. (C) Gene Ontology Analysis. The size of each circle corresponds to the number of associated counts, while adjusted p-values are encoded by the color scale. (D) Gene Regulatory Network of ELK1. It shows potential regulation network between ELK1 and its potential target genes. All potential target genes are downregulated and ELK1 is downregulated. Edge width represents the strength of regulatory evidence between each transcription factor and its target gene. Edge color indicates the correlation between transcription factors and target genes, while node color reflects the corresponding log2 fold change (Log2FC).

APPENDICES-B: MTT Test

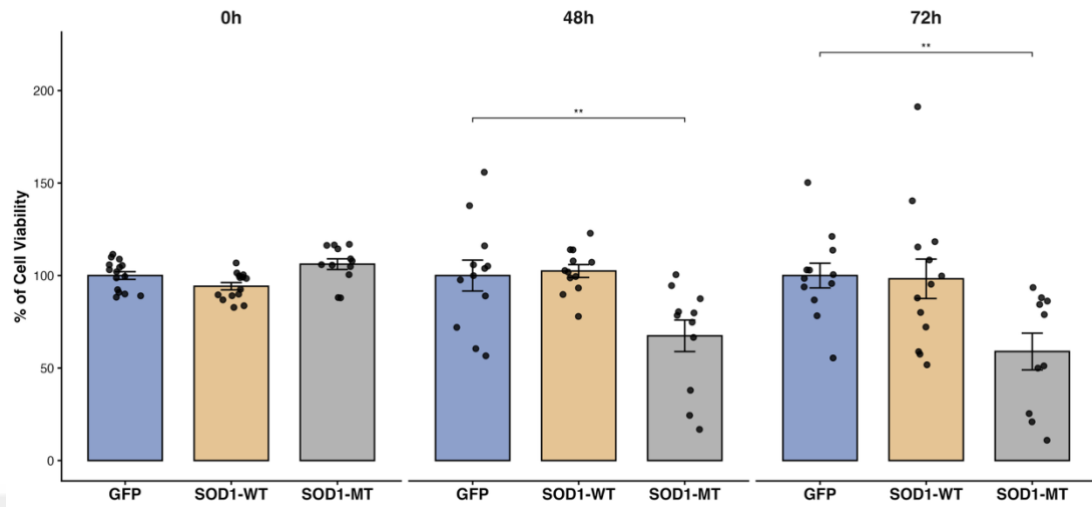


Figure B.1: Analysis of the Effect of SOD1^{WT} and SOD1^{G93A} Expression on Cell Metabolic Activity in NSC-34 Cells by MTT Test. Cell viability rates were normalized relative to the eGFP transfected control group (GFP). Statistical significance was analyzed by two-way ANOVA followed by Tukey's post hoc test. Differences were considered statistically significant at $p < 0.05$.