

THE ASSESSMENT OF UBIQUITIN E3 LIGASE ACTIVITY OF RNF4  
ON ALS-RELATED NEK1 PROTEIN

by

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## ABSTRACT

### THE ASSESSMENT OF UBIQUITIN E3 LIGASE ACTIVITY OF RNF4 ON ALS-RELATED NEK1 PROTEIN

Amyotrophic lateral sclerosis (ALS) is a serious and progressive motor-neuron disease in which neurons that control voluntary muscles gradually deteriorate. Patients lose the ability to speak, move and breathe. To date, 30 ALS associated genes have been discovered. The products of mutated genes generally follow the similar pattern which is protein aggregation. In 2014, the results of the global MinE project have announced that *NEK1* is a new ALS-related gene. NEK1 is a serine/threonine kinase functioning in cell cycle regulation, DNA damage response, cilia formation, etc. However, its contribution to disease phenotype is still not known. If NEK1 shares the same characteristic with other ALS-related proteins, it tends to aggregate. These aggregates can be cleared with the help of ubiquitin-proteasome system. In this study, we investigated whether NEK1 is a new target for RNF4 which is a special ubiquitin E3 ligase. If RNF4 is an E3 ligase for NEK1, it may affect the stability of NEK1 leading to its degradation by the proteasome. *In silico* analysis and co-immunoprecipitation experiments have suggested that NEK1 and RNF4 physically interact. RNF4 overexpression changes the stability of NEK1. In addition, the silencing of RNF4 with siRNA causes also the stabilization of NEK1. We have analyzed the ubiquitination of wild type NEK1 and one of its the disease-related versions, tNEK1. According to our preliminary results, NEK1 is ubiquitinated and tNEK is hyperubiquitinated. In conclusion, our study shows that NEK1 can be a new substrate of RNF4 and RNF4 can lead to further ubiquitination of NEK1 and tNEK1. These findings can contribute to improvements in therapeutic interventions for still incurable ALS.

## ÖZET

### RNF4 ENZİMİNİN ALS İLE İLİŞKİLİ NEK1 PROTEİNİ ÜZERİNDEKİ E3 LİGAZ AKTİVİTESİNİN DEĞERLENDİRİLMESİ

Amiyotropik lateral skleroz (ALS), istemli kasları kontrol eden nöronların giderek kötüleştiği ciddi ve ilerleyen bir motor nöron hastalığıdır. Hastalar zamanla konuşma, hareket etme ve nefes alma yeteneklerini kaybeder. Şimdiye kadar, 30 tane ALS ile ilişkili gen keşfedildi. Mutasyona uğramış genlerin ürünleri genellikle benzer bir modeli takip eder ve bu da proteinlerin çökmesidir. 2014 yılında, MinE projesinin sonuçlarına göre NEK1'in de ALS hastalarında mutasyona uğramış olan bir gen olduğunu keşfedildi. NEK1, hücre döngüsü düzenlemesinde, DNA hasarı tepkisinde, silia oluşumunda vs. çalışan bir serin / treonin kinazdır. Bununla birlikte, hastalık fenotipine olan katkısı hala bilinmemektedir. NEK1, diğer ALS ile ilişkili proteinlerle aynı özelliği paylaşıyorsa, çökme eğilimindedir. Bu agregatlar ubiquitin-proteozom sisteminin yardımı ile temizlenebilir. Bu çalışmada NEK1'in özel ubiquitin E3 ligaz olan RNF4 için yeni bir hedef olabileceğini araştırdık. Eğer RNF4, NEK1 için bir E3 ligaz ise, NEK1'in stabilitesini proteozoma bağlı olarak etkileyebilir. Bilgisayar ortamındaki analizler ve ko-immünopresipitasyon deneylerinde, NEK1 ve RNF4'ün fiziksel olarak etkileşime girdiği öne sürülmüştür. RNF4 aşırı ekspresyonunun, NEK1'in stabilitesini beklediğimizin tam tersi yönünde de değiştirdiğini gözlemledik. Ek olarak, RNF4'ün siRNA ile susturulması ayrıca NEK1'in stabilizasyonuna neden oldu. NEK1'in ve tNEK1 olan hastalıkla ilgili versiyonunun ubiquitinasyonunu kontrol ettik ve ön sonuçlarımıza göre NEK1'in ubiquitinlendiğini ve tNEK1'in de hiperubikitinlendiğini gözlemledik. Sonuç olarak, çalışmamız NEK1'in yeni bir RNF4 substratı olabileceğini ve RNF4'ün NEK1 ve tNEK1'in daha da ubiquitinlenmesine sebep olduğunu göstermektedir. Bu bulgular, henüz tedavisi olmayan ALS hastalığı için terapötik müdahalelerdeki gelişmelere katkıda bulunabilir.

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**LIST OF SYMBOLS**

bp	Basepair
g	Gram
L	Liter
ml	Milliliter
mm	Millimeter
mM	Millimolar
M	Molar
ng	Nanogram
rpm	Revolutions per Minute
v	Volume
w	Weight
$\mu$ M	Micromolar
$\mu$ l	Microliter
$^{\circ}$ C	Degree Celcius

**LIST OF ACRONYM/ABBREVIATIONS**

ALS	Amyotrophic Lateral Sclerosis
APL	Acute Promyelocytic Leukemia
ATL	Adult T-cell Leukemia/Lymphoma
ATP	Adenosine Triphosphate
ATR	Ataxia Telangiectasia
CC	Coiled-Coil
CHK1	Check Point Kinase 1
C9ORF72	Chromosome 9 Open Reading Frame 72
DNA	Deoxyribonucleic Acid
FDA	Food and Drug Administration
FUS1	Fused in Sarcoma 1
GST	Glutathione S-transferases
GWAS	Genome Wide Association Studies
HA	Hemagglutinin
HECT	Homologous to the E6-associated protein carboxyl terminus
HTLV-1	Human T-cell Lymphoma Virus 1
Lys	Lysine
mRNA	messenger RNA
NEK1	NIMA-related Kinase 1
NEM	N-Ethylmaleimide
NIMA	Never in Mitosis Gene A

PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PML	Promyelocytic Leukemia Protein
PML-RAR $\alpha$	Promyelocytic Leukemia Protein-Retinoic Acid Receptor Alpha
PRISM	Protein-protein Interaction Prediction by Structural Matching
PTM	Post-translational Modifications
RING	Really Interesting New Gene
RIPA	Radio Immunoprecipitation Assay Buffer
RNF4	Really Interesting New Gene Finger Protein 4
SDM	Side-Directed Mutagenesis
SIM	SUMO-Interacting Motif
siRNA	Small Interfering RNA
SOD1	Superoxide Dismutase
STUbLs	SUMO-targeted Ubiquitin E3 Ligase
SUMO	Small Ubiquitin-Like Modifier
tNEK1	truncated NEK1
UPS	Ubiquitin-Proteasome System
WCL	Whole Cell Lysate

# 1 INTRODUCTION

## 1.1 Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is a deadly motor neuron disease in which neurons that control muscles deteriorate, resulting in weakness in the legs and arms; and in later stages, difficulty in speaking, swallowing, and breathing. 2 out of 100,000 people are affected by ALS per year (O., L.H., & M.C., 2011). Most of the sufferers die because of respiratory failure after 3 years from disease onset (O. et al., 2011). The disease is diagnosed with definite symptoms, and unfortunately, there is no known cure for ALS. Some medications only help to slow down disease progression. The transition of the disease can be familial, meaning that it is inherited from parents, which covers 5% of all cases. 95% of the cases are sporadic, which means that the mutated gene or genes that are responsible for the acquisition of the disease are not inherited from parents (O. et al., 2011). It is known that certain genes are linked with ALS such as SOD1, FUS1, and C9ORF72 (Blokhuys, Groen, Koppers, Van Den Berg, & Pasterkamp, 2013). Mutations in these genes produce defective proteins that are found as aggregates in degenerated neurons. Although protein aggregation is the main pathological hallmark of ALS, there are still many questions which remain unanswered about the molecular and cellular mechanisms underlying disease phenotype.

With the help of Genome-Wide Association Studies (GWAS) and increased accessibility of DNA sequencing technology in recent years, new genes associated with ALS have been discovered. Currently, 30 ALS-related genes have been reported (Abel, Powell, Andersen, & Al-Chalabi, 2012). In 2014, public awareness of the disease had been increased with the help of the Ice Bucket Challenge. Thanks to the global MinE project, which initiated this campaign and donations, genomes of 15,000 ALS patients and 7,500 control subjects were analyzed. Based on the results from this project, the gene *NEK1* was discovered to be an ALS associated gene (Consortium et al., 2018).

## 1.2 NIMA-Related Kinase 1 (NEK1)

NIMA (Never in Mitosis Gene A)-related kinase 1 is a member of the NEK kinase cluster. It is a serine/threonine kinase and sometimes it harbors tyrosine kinase activity. The NEK family is composed 11 proteins whose functions vary from regulating cell cycle to DNA repair mechanisms (Quarmby, 2005). NEK1 plays a critical role in cell cycle regulation. Additionally, it has been shown that NEK1 is involved in the regulation of cilia formation and the modulation of DNA damage response (Fry, O'Regan, Sabir, & Bayliss, 2012; Meirelles et al., 2014). Upon DNA damage, the kinase activity of NEK1 increases. Owing to an extended C-terminal domain with several coiled-coil (CC) domains, it interacts with other proteins, and consequently phosphorylates its targets (Meirelles et al., 2014; Surpili, Delben, & Kobarg, 2003). It was previously stated that NEK1 influences the stabilization of the ATR complex and CHK1 phosphorylation (Liu, Ho, Ouyang, & Zou, 2013). The activation and stabilization of this complex is critical for proper DNA damage response, and the deficiency of NEK1 has been linked with genomic instability (Y. Chen, Chen, Riley, & Chen, 2011; Y. Chen, Chen, Chen, Jiang, & Riley, 2008). Apart from its role in DNA damage response, NEK1 is also associated with polycystic kidney disease (Upadhyya, Birkenmeier, Birkenmeier, & Barker, 2002). The murine version of *Nek1* is shown to be overexpressed in motor neurons in mice (Arama, Yanai, Kilfin, Bernstein, & Motro, 1998).

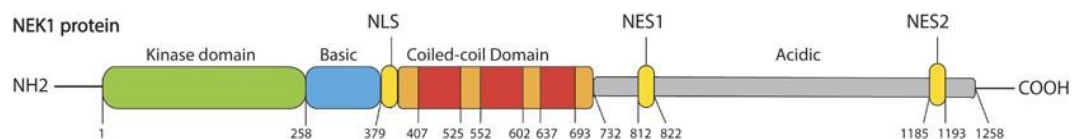


Figure 1.1: Domain architecture of ALS-linked NEK1 protein. (Monroe et al., 2016)

According to the findings of the MinE project and more recent studies, various mutations in the *NEK1* gene comprise 3% of all ALS cases (Kenna et al., 2016). However, the contribution of these mutations to the disease progression is not known. Until 2018, the only connection between ALS and NEK1 was the presence of point mutations of *NEK1*,

which were found in ALS patients. In 2018, one study showed that a truncation mutation in NEK1 (Arg812Ter) induces the accumulation of DNA damage in ALS patient-derived motor neurons (Higelin et al., 2018). Still, NEK1 has many different mutant versions which are related to ALS. Therefore, finding how NEK1 mutations contribute to disease pathology is intriguing.

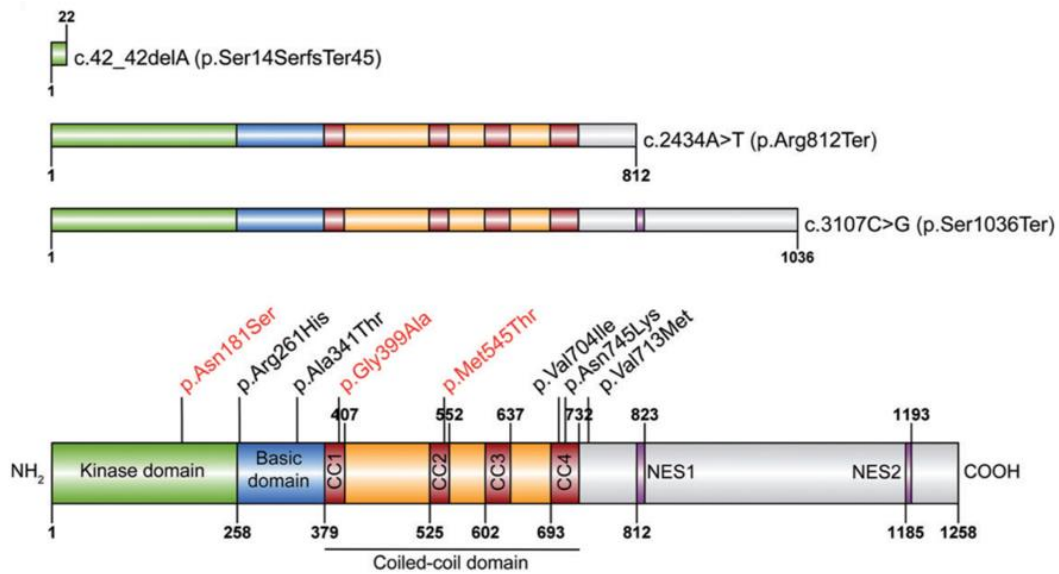


Figure 1.2: ALS-related mutations of NEK1 protein. (Oda, Izumi, & Kaji, 2011)

Our lab has recently discovered that NEK1 is conjugated by both SUMO1 and SUMO2/3. Apart from this exciting finding, we also showed that its ALS-mutant version, which leads to a truncated form of NEK1 protein (tNEK1) gets hyper-SUMOylated (Figure 1.3). SUMO, which stands for Small Ubiquitin-like Modifier, is a small peptide that is very similar to ubiquitin. SUMOylation is a posttranslational modification and it involves covalent addition of SUMO to target proteins. SUMO has three paralogues which are SUMO1, SUMO2, and SUMO3. SUMO2 and SUMO3 share 95% sequence similarity and antibodies cannot differentiate between these two proteins, thus they are called SUMO2/3. Once SUMO is conjugated to its target, it can change the function, stability, subcellular localization, solubility or the interaction partners of its target (Hay, 2005)(Swatek & Komander, 2016)(Swatek & Komander, 2016). It was shown previously that ALS-linked proteins can become modified by SUMO. SOD1 whose mutations are common in ALS patients, are shown to be modified by SUMO proteins (Fei et al., 2006). Apart from SOD1, other neurodegenerative disease-related proteins like Ataxin and Polyglutamine (PolyQ)

become modified by SUMO. In the context of neurodegeneration, sometimes SUMO can have a protective role by triggering the ubiquitylation and destruction of a SUMOylated, aggregation-prone protein, hence decreasing its stability. However, in other cases, SUMO may compete with the ubiquitin and leads to an increase in the protein's stability.

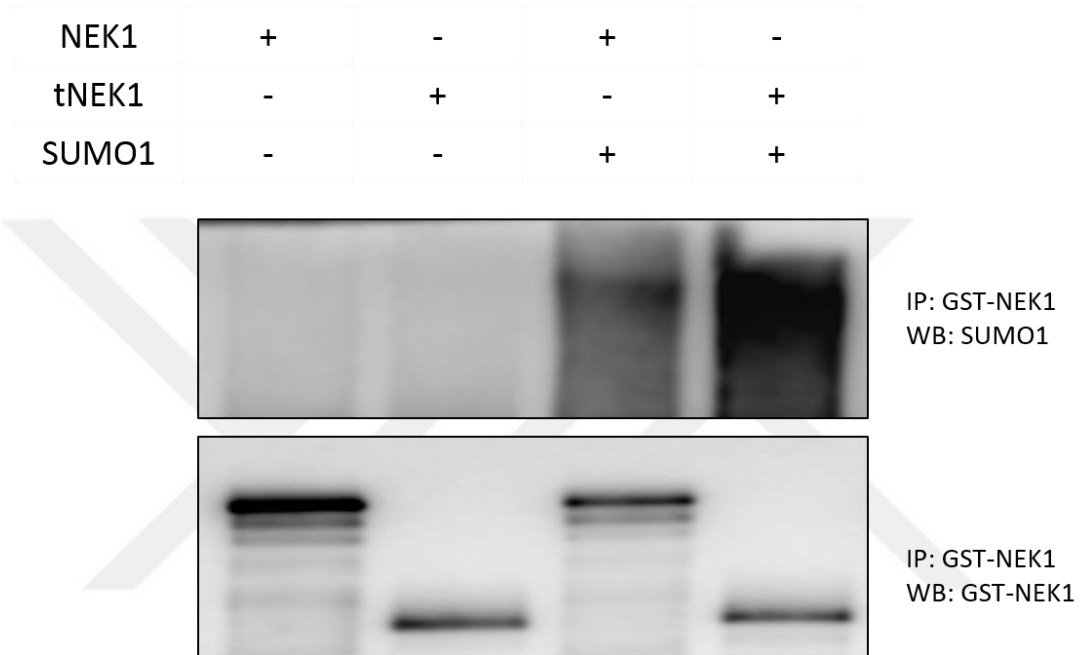


Figure 1.3: NEK1 and tNEK1 are modified by SUMO1 (Bahriye Erkaya, 2018, unpublished).

### 1.3 Protein Aggregation

Protein aggregation is a hallmark of neurodegenerative diseases. It is characterized by the accumulation of misfolded proteins either in the cytoplasm or in the extracellular matrix and it is implicated in a broad variety of diseases including ALS, prion disease, and Alzheimer's Disease (Aguzzi & O'Connor, 2010; Stefani & Dobson, 2003).

After translation, proteins fold into the most thermodynamically favorable three-dimensional conformation, which is called the 'native state' (Brüning, Jückstock, Khan, & Ruano, 2015). This folding is driven by hydrophobic effects, and protein structures are stabilized both by non-covalent interactions and disulfide bond formation between the

cysteine residues of the protein. In case of a change in amino acid sequence due to mutations, the above-mentioned covalent and non-covalent interactions may be affected, and the protein becomes susceptible to misfolding or completely unfolds. If the cell could not facilitate the refolding or the degradation of these abnormal proteins, the misfolded or unfolded proteins may form an aggregate by interacting with hydrophobic parts of other proteins leading to a disease condition (Gething & Sambrook, 1992; Roberts, 2007). Aggregated proteins can come together and create a structure called aggresome. Protein aggregates lead to the loss of function of the protein because aggregated proteins cannot carry out their function. Apart from this, these aggregates may interact with other parts of the cell and lead to full deterioration of neurons. Although it may seem that aggregates are harmful to cells, the formation of aggregates might as well be protective. If they were scattered in the cell, it may be more harmful to the cell.

Cells try different ways to retrieve their healthy state. Chaperone proteins assist the unfolded/misfolded proteins to unfold and then refold (Liberek, Lewandowska, & Ziętkiewicz, 2008). If they are not refolded correctly, they are immediately targeted to either the lysosome or the proteasome. The ubiquitin-proteasome system (UPS) is another system to eliminate misfolded proteins. With the help of a set of enzymes called E1, E2, and E3 in the ubiquitination pathway, the unfolded/misfolded proteins are labeled for degradation and get degraded by 26S subunit of the proteasome. Autophagy is another defense mechanism to clear protein aggregates. Cells try to remove toxic protein aggregates through macro-, micro- and chaperone-mediated autophagy.

#### **1.4 Ubiquitin Proteasome System**

The breaking down and the regulation of proteins is just as important as the synthesis of proteins. In the beginning of the 1980s, three scientists; Aaron Ciechanover, Avram Hershko, and Irwin Rose had focused on the breaking down of proteins and they discovered one of the most critical processes in the cell which is regulated protein degradation. The importance of this mechanism was realized with the Nobel Prize in Chemistry in 2004. A proteasome contains a set of domains which degrades the ubiquitin-tagged-proteins and proteases help the process. Generally, the ubiquitin serves as a

degradation signal, and the signal detection by the proteasome results in the degradation of ubiquitin-tagged-protein into peptide fragments. (Hershko & Ciechanover, 1998) UPS has many important roles in different cellular mechanisms such as apoptosis, turnover of proteins, cell cycle regulation, development, DNA repair mechanisms affecting the stability and the concentration of proteins (Swatek & Komander, 2016). Thus, the regulation of UPS and the amount of proteasome are linked to the pathology of serious diseases like neurodegenerative diseases, cancer, and aging.

### **1.5 Post-Translational Modifications**

Although the number of genes in the human genome is around 25,000, the total protein number in the human proteome is not the same. Several mechanisms enable a single gene to encode more than one protein. Alternative splicing and genomic recombination are mechanisms to generate different transcripts from a single gene. Along with these mechanisms, post-translational modifications (PTMs) can increase the complexity and the diversity of proteins and it is estimated to be over 1 million

PTMs have an important function in the modification of the end product of gene expression. (Muir, 2006). With the help of enzymes including kinases, phosphatases, and ligases which add or remove functional groups, sugars, and proteins, and proteases, the substrate is modified. Depending on the type of modification, some PTMs can be reversed with the help of special enzymes that remove functional groups or proteins from the substrate.

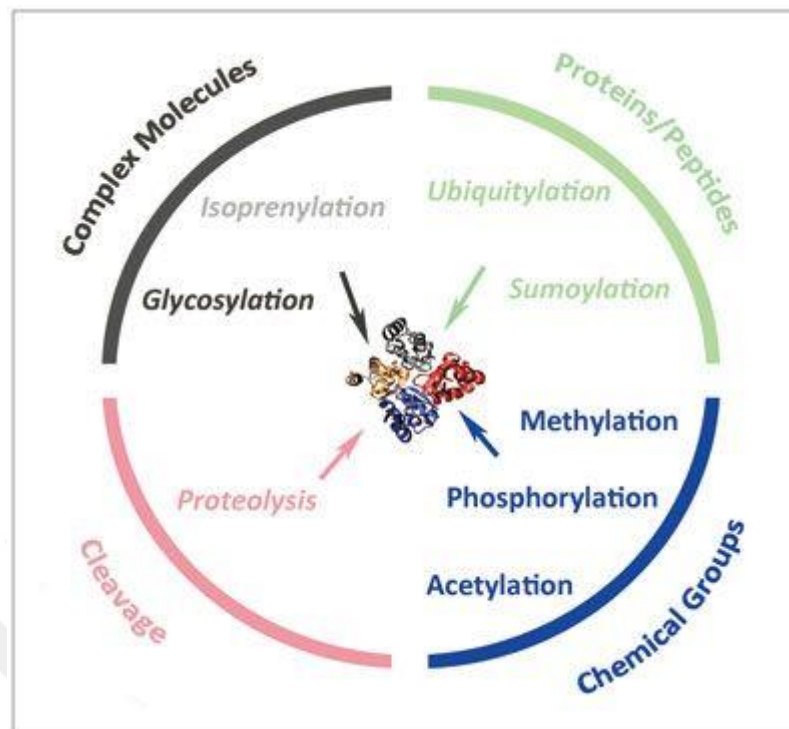


Figure 1.4: General scheme of post-translational modifications (Wang, Peterson, & Loring, 2014).

Phosphorylation, methylation, acetylation, and ubiquitination are the most well-known examples of PTMs. Localization, stability, activity, and the function of modified proteins can be affected by the modification. They can be involved in important mechanisms such as differentiation, signaling pathways, protein turnover, regulation of gene expression etc. (Geiss-Friedlander & Melchior, 2007). Any defects in the modification system might give rise to a disease condition, thus their regulation is quite critical to keep the system healthy.

## 1.6 Ubiquitylation

Ubiquitin is a small 76-amino acid regulatory protein, and is conserved through the evolutionary process in most eukaryotic organisms. Ubiquitylation is the addition of a ubiquitin molecule via an isopeptide bond to the target protein in between the C-terminal di glycine motif of the ubiquitin and a lysine residue on the target (Hershko & Ciechanover, 1998). Owing to seven lysine residues on the ubiquitin itself, this ubiquitin is subjected to

further modifications, leading to the formation of polyubiquitin and branched ubiquitin chains varying in length and composition. Due to the variation in the chain composition and length, the proper function of each different type of ubiquitination has not been fully understood. (Xu et al., 2009). In addition to this variation, other ubiquitin-like molecules such as SUMO or NEDD8 can also modify lysine residues on ubiquitin (Swatek & Komander, 2016).

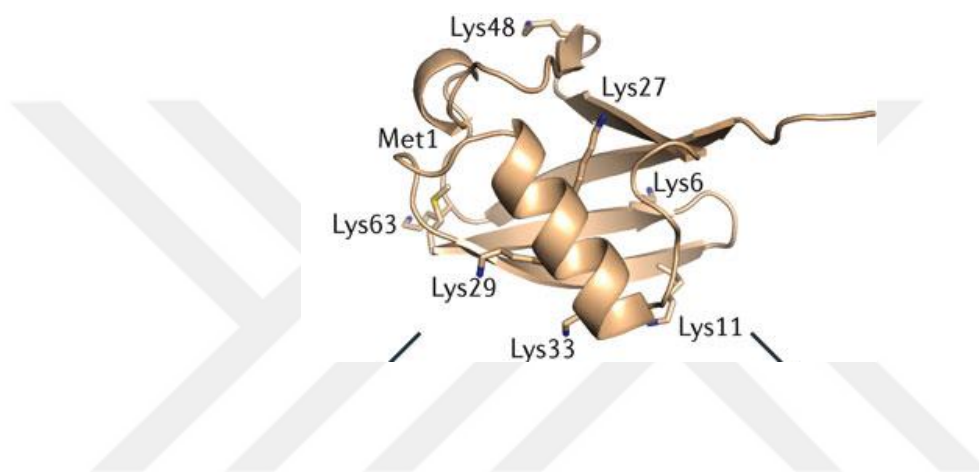


Figure 1.5: Structure of the ubiquitin protein. Lysine residues are shown on the ubiquitin. (Buetow & Huang, 2016)

Ubiquitination is a highly regulated ATP-dependent enzymatic cascade. Multiple enzymes act in this process. The cascade starts with the ATP-dependent attachment of C-terminal glycine of the ubiquitin through a thioester bond to the cysteine on the active-site of a Ubiquitin Activating Enzyme (E1). Then, the activated ubiquitin is passed onto another enzyme, which is a Ubiquitin Conjugating Enzyme (E2). In the last step, Ubiquitin E3 Ligase (E3) selects the substrate, recruits the ubiquitin loaded E2 enzyme into close proximity and correct alignment with the target protein to facilitate the transfer of the ubiquitin (Scaglione et al., 2013). Then, the isopeptide bond is formed between the substrate and the ubiquitin.

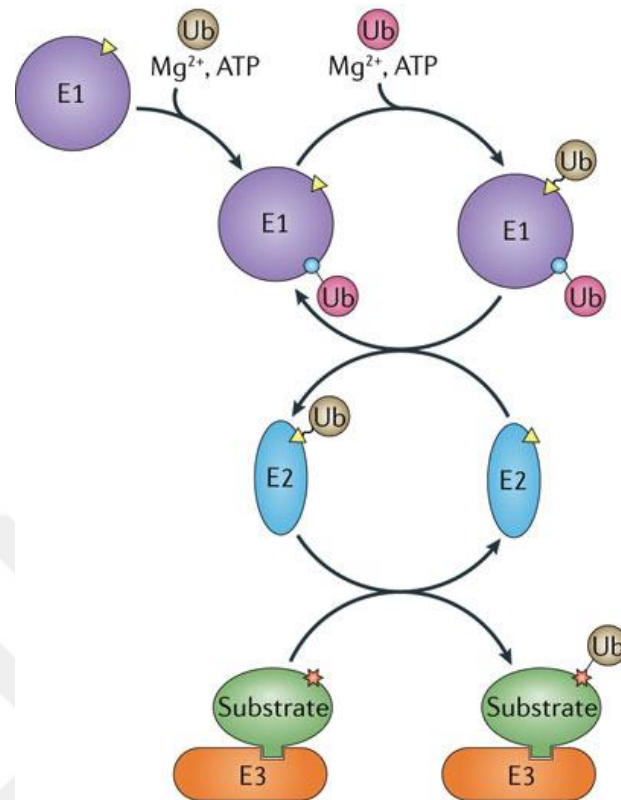


Figure 1.6: Enzymatic cascade of ubiquitination. After ATP-dependent activation of the ubiquitin, it is transferred to E2 Conjugating Enzyme and then E3 ligase takes place to transfer the ubiquitin from the E2 enzyme to the substrate. (Buetow & Huang, 2016)

A ubiquitinated protein can be charged with different roles after its modification, such as being marked for degradation, having its sub-cellular localization altered, or its activity may be affected. Depending on the lysine choice of the second ubiquitin, the fate of the substrate can be determined with different types of polyubiquitination. Lys48-linked polyubiquitin chains are the most dominant type in the cells (Hershko & Ciechanover, 1998). The main role of Lys48-linked polyubiquitination is tagging the proteins for proteasomal degradation. Lys63-linked polyubiquitination is the second common chain type and it has non-degradative roles (Z. J. Chen & Sun, 2009). It has a role in NF-Kb signaling by altering ubiquitin-dependent protein interactions (Hadian et al., 2011). Apart from Lys48 and Lys63, Lys11, Lys29, Lys33 are the other ubiquitination types that have distinct roles on proteins.

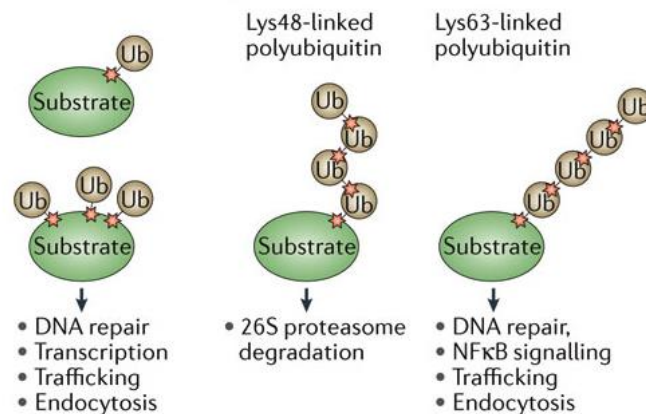


Figure 1.7: The types of ubiquitination and their effects on the target protein (Buetow & Huang, 2016). Monoubiquitination affect the pathways such as the transcription and DNA repair mechanisms. Lys48-linked and lys63-linked polyubiquitinations lead to the degradation of ubiquitinated proteins and endocytosis, respectively.

## 1.7 Ubiquitin E3 Ligases

Ligases have a crucial role to catalyze the formation of a new chemical bond between two molecules. Ubiquitin E3 ligases are vital enzymes in the ubiquitination process. More than 500 ubiquitin E3 ligases are encoded in the human genome because of their substrate specificities (Ardley & Robinson, 2005). E3 ligases take part in the final step of ubiquitination and are responsible for the addition of a ubiquitin molecule to the target. We can divide E3 ligases into two groups such as the HECT (Homologous to E6-AP Carboxyl Terminus)-type and the RING (Really Interesting New Gene)-type. The main role of the ubiquitin E3 ligase is that it brings the ubiquitin loaded E2 enzyme and the substrate together. It allows and helps the transfer of the ubiquitin from E2 to the target. One main feature that differentiates the HECT-type E3 ligases from the RING-type E3 ligases is that there is an intermediate step in the transfer of ubiquitin in the HECT-type. In the ubiquitination that involves HECTs, the ubiquitin is first transferred to an active cysteine on the E3 ligases and then it is passed to the protein that will be modified (Huibregtse, Scheffner, Beaudenon, & Howley, 2006; Lorick et al., 2002). However, in the RING-type, the E3 ligase creates a favorable environment by bringing the ubiquitin loaded

E2 and the substrate together. The ubiquitin is transferred directly from the E2 to the substrate without attaching to the E3 ligase.

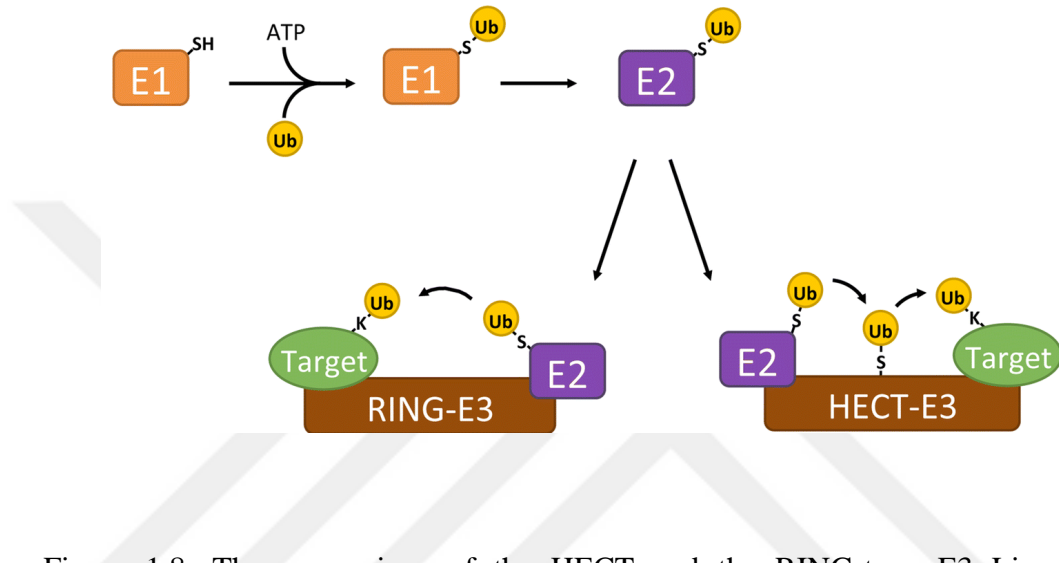


Figure 1.8: The comparison of the HECT and the RING-type E3 Ligase in the ubiquitination process. (Sluimer & Distel, 2018)

### 1.8 RING (Really Interesting New Gene) Finger Protein 4 (RNF4)

RNF4 (RING Finger Protein 4) is a special ubiquitin E3 ligase among 500 encoded in the human genome. It is extraordinary in terms of the substrate specificity. Although other ubiquitin E3 ligases have substrate specificity, RNF4 does not have this property. It can recognize SUMOylated proteins via its N-terminus. In the N-terminus, it contains special sequences that consist of 4 amino acids including isoleucine, valine, and leucine. This special motif is called SUMO-Interacting Motif (SIM). RNF4 can recognize poly-SUMOylated proteins with the help of these SIMs on its N-terminus. After the recognition of SUMOylated proteins by RNF4, it ubiquitinates the SUMOylated proteins to send them to the proteasome for their degradation. RNF4 acts neither as a pure HECT type nor as a pure RING-type E3 ligase. It possesses some similarities with the RING-type E3 ligases. Both have a RING domain which is important in binding to DNA.

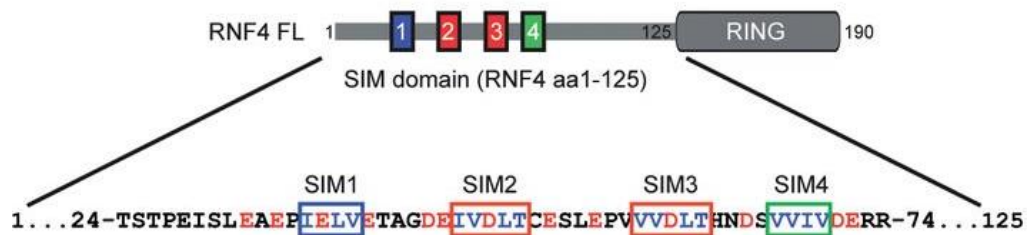


Figure 1.9: Representation of domains on RNF4: N-terminus end of it contains 4 SIMs and it is followed RING domain on C-terminus.

SUMO-dependent ubiquitination mechanism can be adapted to therapeutic inventions. For instance, SUMOylation of certain toxic proteins can be enhanced by FDA approved drugs such as arsenic, interferons or retinoic acid, resulting in accelerated degradation (Dassouki et al., 2015; Lallemand-Breitenbach et al., 2008). Viral oncoprotein TAX and the fusion oncoprotein PML-RARa are striking examples in this type of therapy (Dassouki et al., 2015; Lallemand-Breitenbach et al., 2008). TAX is a viral oncoprotein expressed by human T-cell lymphotropic virus type I (HTLV-1) that leads to Adult T-cell leukemia/lymphoma (ATL), which is a very aggressive form of leukemia. Upon infection, the virus leads to the transformation of CD4 + T cells (Matsuoka & Jeang, 2007). This transformation results in the production of viral oncoprotein TAX which plays a critical role in different cellular pathways including nuclear factor-KB (NFkB) and cell proliferation. Apart from that, TAX increases genomic instability and causes silencing of tumor suppressors, thus allowing the accumulation of genetic changes that drive transformation. TAX is a substrate of the ubiquitin-proteasome system and its degradation can be enhanced upon arsenic/interferon exposure (Dassouki et al., 2015). This degradation process relies on drug-induced hyper-SUMOylation of TAX, which is facilitated by Promyelocytic Leukemia Protein (PML) Nuclear Bodies (Dassouki et al., 2015) and SUMO-induced TAX poly-ubiquitination is accomplished by RNF4 (Dassouki et al., 2015). In this way, TAX-driven leukemic cells can be eliminated.

PML-RARa fusion oncoprotein is another important RNF4 target. It results from a chromosomal translocation between chromosomes 15 and 17, fusing the retinoic acid receptor alpha and the promyelocytic leukemia protein, eventually leading to Acute Promyelocytic Leukemia (APL). The fusion oncoprotein PML-RARa impairs retinoic acid signaling which is essential for myeloid cell differentiation resulting in tumorigenesis. In APL, arsenic trioxide in combination with retinoic acid induces the proteasome-dependent degradation of PML-RARa fusion oncoprotein via poly-SUMOylation and the subsequent Lys48-linked polyubiquitination through the recruitment of RNF4. Then, leukemic cells differentiate into the healthy stage. Now, this combination is used in clinics as a therapy for APL patients and it is one of the cornerstones of targeted therapies (De Thé & Chen, 2010).

## 2 HYPOTHESIS AND PURPOSES

Recent studies have shown that *NEK1* mutations have been found in 3% of ALS patients. However, how these NEK1 mutations cause neurodegeneration and the disease is not known. If NEK1 aggregates like other ALS-related proteins, the elimination of these toxic aggregates should diminish the disease phenotype. Based on the previous findings of our laboratory, we know that NEK1 is modified by both SUMO1 and SUMO2/3. Its toxic form, tNEK1, is hyperSUMOylated and colocalized in PML NBs, which also concentrate RNF4 (Geoffroy, Jaffray, Walker, & Hay, 2010). We thus hypothesized that NEK1 may be a substrate for ubiquitin E3 ligase.

### 3 MATERIALS

#### 3.1 Equipment

The following table contains the equipment used in this project.

Table 3.1: Equipment.

<b>Equipment</b>	<b>Supplier</b>
Agarose Gel Electrophoresis System	EASY-CAST, Thermo Fisher, USA
Autoclaves	Midas 55, Prior Clave, UK ASB260T, Astell, UK
Carbon dioxide Tank	Genc Karbon, Turkey
Cell Culture Dishes	TPP, Switzerland
Cell Culture Incubator	WTC, Binder, Germany
Cell Scraper	TPP, Switzerland
Centrifuges	Ultracentrifuge J2MC, Beckman, USA VWR CT15RE, Japan Allegra X-22, Beckman USA
Centrifuge Tubes	CAPP, Denmark
Cold room	Birikim Elektrik Soğutma, Turkey
Confocal Microscope	Leica SP8, USA
Cryovial Tubes (2ml)	CAPP, Denmark

Table 3.1. Equipment (cont.).

<b>Equipment</b>	<b>Supplier</b>
Deep freezers	(-20 °C) Ugur, UFR 370 SD, Turkey (-80 °C) ULT deep freezer, Thermo, UK (-150 °C) Sanyo MDF-1156, UK
Dish Washer	Mielabor G7783, Miele, Germany
Documentation System	Gel Doc XR System, Bio-Doc, ITALY Stella, Raytest, Germany G-BOX Chemi XX6, Syngene, UK
Electrophoresis Equipments	Mini-Protean III Cell, Bio-Rad, USA
Eppendorf Tubes (1,5 ml)	CAPP, Denmark
Eppendorf Tubes (2,0 ml)	CAPP, Denmark
Gloves	SemperCare, USA
Heat blocks	Block heater analog, VWR, USA
Ice Maker	Scotsman Inc. AF20, Italy
Inverted Microscope	Z1 Axio Observer, Zeiss, USA
Laboratory Bottles	VWR, USA
Laminal Flow Cabinet	Class II B, Tezsan, Turkey
Magnetic Stirrer	VMS-C7, VWR, USA
Microfuge Tubes	CAPP, Denmark
Micropipettes	Finnpipette, Thermo, USA

Table 3.1. Equipment (cont.).

<b>Equipment</b>	<b>Supplier</b>
Micropipette Tips	Axygen, USA
Microscopes	Inverted Microscope, Nikon, Eclipse TS100, Netherlands, Fluorescence Microscope, Observer.Z1, Zeiss, Germany
Microwave Oven	Arçelik, Turkey
Minifuge	VWR, USA
Multiwell Plates	TPP, Switzerland
Nitrocellulose Membrane	Amersham, GE Life Sciences, England
Oven	Gallenkamp 300, UK
PCR Tubes (0.2ml)	Axygen, USA
Petri Dishes	Fırat Plastik, Turkey
Ph Meter	Hanna Instrumentsi, USA
Pipette Tips (Bulk)	CAPP, Denmark
Pipette Tips (filtered)	BioPointe, USA
Power Supply	EC XL 300, Thermo Fisher, USA
Real-Time Quantitative PCR System	Bioneer Exicycler, Republic of Korea
Refrigerators	Ugur, USS 374 DTKY, Turkey
Rotator	Isolab, Germany
Serological Pipettes	CAPP, Denmark

Table 3.1. Equipments (cont.).

<b>Equipment</b>	<b>Supplier</b>
Shakers	Orbital shaker ANALOG, VWR, USA
Softwares	XStella 1.0, Stella, GERMANY
Syringes	Set Medikal, Turkey
Syringe Filter Units	EMD Millipore, USA
Thermal Cycler	Bio-Rad, USA
Vortex	VWR, USA
Water purification	WA-TECH UP, Germany
Water purification system	UTES, TURKEY

### 3.2 Cell Culture

HEK293 cells were kindly provided by Dr. Nurhan Özlü, Koc University.

### 3.3 Plasmids and Primers

Plasmids were purchased from Addgene and University of Dundee. Ubiquitin plasmid was gifted by Hugues de Thé, Collège de France. Primers and siRNAs were ordered from MacroGen (South Korea) and GenePharma (China), respectively.

Table 3.2: Plasmids.

Construct	Origin	Backbone
HA-RNF4	Addgene #59743	pRK5
pEBG NEK1 tv1	University of Dundee, DU41359	pEBG
His-Ubiquitin	Provided by Hugues de Thé, Collège de France	Unknown
pEGFP	Provided by GenReg, Bogazici University	pEGFP
pCMV-FLAG	Provided by CSL, Bogazici University	pCMV

Table 3.3: Sequences of Primers and siRNAs

Oligo ID	Sequence (5'-3')	Application
RNF4 DN F	AAAAGCTAGCTCCAAGTGCACCTCGGTTC	SDM
RNF4 DN R	AAAAAAGCTTATCGTCGTCCTTGTAGTCGAGT	SDM
siRNF4	CCCUGUUUCCUAAGAACGAAATT	Silencing
siCNTRL	UUCUCCGAACGUGUCACGUTT	Silencing

### 3.4 General Kits, Enzymes, and Chemicals

Following tables indicate kits, enzymes, and chemicals used in this study.

Table 3.4: Kits and Enzymes.

<b>Name</b>	<b>Supplier</b>
Advansta Ecl	Advansta Inc., USA
Complete Mini Protease Inhibitor Cocktail	Roche, Switzerland
DNA Ladder (1 kb)	NEB, USA
DpnI restriction endonuclease	NEB, USA
ECL-Femto	Thermo, USA
ECL-Pico	Thermo, USA
PageRuler Prestained Protein Ladder	Thermo, USA
Q5 High-Fidelity DNA Polymerase	NEB, USA
Q5 10X Reaction Buffer	NEB, USA
Zymo Gel Extraction	Zymo Research, USA
ZymoPURE™ MaxiPrep Kits	Zymo Research, USA
ZymoPURE™ MidiPrep Kits	Zymo Research, USA
ZymoPURE™ Mini Prep Kits	Zymo Research, USA

Table 3.5: Chemicals.

<b>Chemical</b>	<b>Supplier</b>
Acetic Acid	Sigma-Aldrich, USA
Acrylamide	Bio-Rad, USA
Agar	Sigma-Aldrich, USA
Agarose	Peqlab, USA
Ammonium Persulfate (APS)	AppliChem, Germany
Ampicillin	Sigma-Aldrich, USA
$\beta$ -Mercaptoethanol	Merck, Germany
Bovine Serum Albumin (BSA)	Capricorn Scientific, Germany
Bromophenol Blue	Sigma-Aldrich, USA
Calcium chloride dehydrate	Sigma-Aldrich, USA
Disodium hydrogen phosphate	Merck, Germany
DMSO	Sigma-Aldrich, USA
Dulbecco's Modified Eagle Medium	Gibco, Fischer Scientific, USA
EDTA	Wisent Bioproducts, Canada
Ethanol	Merck, Germany
Ethidium Bromide	Sigma-Aldrich, USA
Fetal Bovine Serum (FBS)	Gibco, Fisher Scientific, USA
Glycerol	MP Biomedicals, USA
Glycine	NeoFROXX, Germany
HEPES Buffered Saline Solution (HBS)	Lonza, Switzerland
HiPerFect Transfection Reagent	Qiagen, Germany
Hydrochloric Acid	Sigma-Aldrich, USA
Kanamycin	Gold Biotechnology, USA

Table 3.5. Chemicals (cont.).

<b>Chemical</b>	<b>Supplier</b>
LB Broth	Caisson Laboratories, USA
Methanol	Merck, Germany
MG132	Calbiochem, Germany
Milk Powder	Havancızade, Istanbul
N-Ethylmaleimide	Sigma-Aldrich, USA
Penicillin/Streptomycin (100X)	Lonza, Switzerland
Potassium chloride	Sigma-Aldrich, USA
Potassium dihydrogen phosphate	Merck, Germany
Sodium Dodecyl Sulfate (SDS)	Merck, Germany
Sodium Hydroxide	Merck, Germany
TEMED	Sigma-Aldrich, USA
Tris-Base	Sigma-Aldrich, USA
Triton X-100	VWR, USA
Tween 20	Merck, Germany

### 3.5 Solutions, Buffers, and Antibodies

As an exception, the rabbit anti-RNF4 antibody was prepared in 5% BSA instead of skim milk in PBS-T. Unless documented in tables, all antibodies were used at indicated concentrations for western blotting.

Table 3.6: Antibodies.

<b>Antibody</b>	<b>Supplier</b>	<b>Source</b>	<b>Dilution</b>
Actin	Cell Signalling Technologies, USA	Rabbit	1:1000
GST	Cell Signalling Technologies, USA	Mouse	1:1000
HA	BioLegend, USA	Mouse	1:1000
Mouse IgG, HRP	Cell Signalling Technologies, USA	Horse	1:5000
NEK1	Santa Cruz Biotechnology, USA	Mouse	1:1000
Rabbit IgG, HRP	Cell Signalling Technologies, USA	Goat	1:5000
RNF4	Origene Technologies, USA #TA324594	Rabbit	1:1000
Tubulin	Santa Cruz Biotechnology, USA	Mouse	1:1000
Ubiquitin	Enzo Life Sciences, USA	Mouse	1:1000

Table 3.7: Solutions and Buffers.

Solution/Buffer	Content
4X Laemmli Buffer	200mM TrisHCl pH 6.8 8% (w/v) SDS 40% (w/v) 100% Glycerol 5% (w/v) $\beta$ -mercaptoethanol 50 mM EDTA 0.08% (w/v) Bromophenol Blue
8% Resolving Gel	375 mM TrisHCl pH 8.8 0.1% (w/v) SDS Acrylamide:Bisacrylamide (8% w/v) 0.05% (w/v) APS 0.005% (w/v) TEMED
10% Resolving Gel	375 mM TrisHCl pH 8.8 0.1% (w/v) SDS Acrylamide:Bisacrylamide (10% w/v) 0.05% (w/v) APS 0.005% (w/v) TEMED
12% Resolving Gel	375 mM TrisHCl pH 8.8 0.1% (w/v) SDS Acrylamide:Bisacrylamide (12% w/v) 0.05% (w/v) APS 0.005% (w/v) TEMED

Table 3.7. Solutions and Buffers (cont.).

Solution/Buffer	Content
4% Stacking Gel	0.125 mM TrisHCl pH 6.8 0.1% (w/v) SDS Acrylamide:Bisacrylamide 4% (w/v) 0.05% (w/v) APS 0.0075% (w/v) TEMED
10X SDS Running Buffer	1% (w/v) SDS 3.03% (w/v) Tris Base 14.41% (w/v) Glycine
10X Transfer Buffer	3.03% (w/v) Tris Base 14.41% (w/v) Glycine
1X Transfer Buffer	10% (w/v) 10X transfer buffer 20% (w/v) Methanol
1X Phosphate Buffered Saline with Tween-20 (PBS-T)	137 mM NaCl 2,7 mM KCl Na <sub>2</sub> HPO <sub>4</sub> 10 mM KH <sub>2</sub> PO <sub>4</sub> 1,8 mM %0.05 Tween-20
10% Sodium Dodecyl Sulfate	10% (w/v) SDS in ddH <sub>2</sub> O
10% Ammonium Persulfate	10% (w/v) ammonium persulfate in ddH <sub>2</sub> O Aliquoted and stored at -20 °C.

Table 3.7. Solutions and Buffers (cont.).

<b>Solution/Buffer</b>	<b>Content</b>
Ponceau S Solution	0.1% (w/w) Ponceau S dye 1% (v/v) Acetic acid
Mild Stripping Buffer pH=2.2	1.5% (w/v) Glycine 0.1% (w/v) SDS 1% (v/v) Tween 20
RIPA Buffer Ph=7.4	50 Mm Tris HCl pH 8.0 150-250 mM NaCl 2% NP-40 20% Glycerol
Western Blot Blocking Solution	5% (w/v) skim milk powder in TBS-T
1 <sup>st</sup> Antibody Solution	5% (w/v) skim milk in TBS-T
2 <sup>nd</sup> Antibody Solution	5% (w/v) skim milk in TBS-T
Immunoprecipitation Lysis Buffer	2% (w/v) SDS 0.05 M Tris-HCl (pH 8) 0.02 M NEM 1X Protease Inhibitor in ddH <sub>2</sub> O

Table 3.7. Solutions and Buffers (cont.).

<b>Solution/Buffer</b>	<b>Content</b>
0.5 M NEM	0.625-g NEM in 10 ml pure ethanol
NEM Working Solution (0.02 M)	0.5 M stock into 10 ml PBS.
Buffer A (His Pull-down)  pH 8.0	6 M guanidine-HCl  0.1 M Na <sub>2</sub> HPO <sub>4</sub> /NaH <sub>2</sub> PO <sub>4</sub>  10 mM imidazole  Adjust the Ph to 8.0.
Buffer TI (His Pull-down)  pH 6.8	25 mM Tris.Cl  20 mM imidazole
2 M CaCl <sub>2</sub>	2 M CaCl <sub>2</sub> was prepared in autoclaved ddH <sub>2</sub> O and filtered in cell culture.

## 4 METHODS

### 4.1 Competent Cell Preparation

50  $\mu$ l of competent cells from -80 °C was put on 5 ml of antibiotic-free LB and the culture was grown at 37 °C on the shaker for overnight. Next morning, 250  $\mu$ l from the overnight culture were inoculated into 25 ml LB and growth at 37 °C until OD 595 = 0.4. The culture was then centrifuged at 3000 rpm at 4 °C for 10 minutes. The pellet was gently resuspended in 12.5 ml ice-cold sterile 50 mM CaCl<sub>2</sub> with pipetting (no vortex) and the suspension was incubated on the ice for 30 minutes. Next, cells were centrifuged at 3000 rpm at 4 °C for 10 minutes and resuspended mildly in 2.5 ml of ice-cold sterile 50 mM CaCl<sub>2</sub> containing 10% glycerol. For storage, 50  $\mu$ l from the cell suspension were put into eppendorf tubes which were placed on ice or liquid nitrogen and cells were stored at -80 °C.

### 4.2 Site-directed Mutagenesis

In order to create a single amino acid change on proteins, site-directed mutagenesis was used. Primers used in the mutagenesis were ordered from Macrogen. We designed primers putting mismatched bases in the middle part of the primer and primer lengths were approximately 35 bases. PCR materials and reaction conditions were stated in Table 4.1 and Table 4.2. 100 ng DNA was used as a template for the amplification. After the PCR, the wild-type template was digested with *DpnI* (6x Cut Smart Buffer, 1x *DpnI* enzyme) overnight at 37 °C. Next day, samples were run on a 1% agarose gel for 1 hour but the duration may change depending on the length of the vector. If *DpnI* worked properly, possible mutated samples were transformed using *E. coli* DH5alpha strain as it was described in the Transformation section.

Table 4.1: PCR Components

Components	Volume
Q5Reaction Buffer	10 $\mu$ l
Forward primer (10 $\mu$ M)	1 $\mu$ l
Reverse primer (10 $\mu$ M)	1 $\mu$ l
DNA (100 ng stock template)	1 $\mu$ l
HighFidelity Polymerase (Q5)	1 $\mu$ l
DMSO	4 $\mu$ l
dNTP (10 mM)	1 $\mu$ l
ddH <sub>2</sub> O	31 $\mu$ l
Total	50 $\mu$ l

Table 4.2: PCR Conditions

Temperature	Time	
95 °C	3 minutes	
95 °C	30 seconds	x 25
64,6-65,6-68,8-70,5-72 °C	30 seconds	
72 °C	3 minutes	
72 °C	4 minutes	

### **4.3 Bacterial Transformation**

Competent cells were taken from -80 °C and waited for 5-10 minutes on ice. Then DNA (4 µl samples for SDM or ligation products, 100 ng for normal plasmid) was added to competent cells. They were mixed and incubated for 30 minutes on ice, followed by the heat shock at 42 °C for 45 seconds. Then, cells were incubated on ice for another 2 minutes. After that, 950 µl LB was added to cells which were incubated on a shaker at 37 °C for 1 hour. When the incubation was finished, cells were centrifuged at 4500 rpm and 850-900 µl LB from the supernatant was discarded. The pellet was resuspended in the remaining 100-150 µl LB-Broth and it was spread on a LB Agar plate that contains antibiotics compatible with the growing plasmid. After spreading cells on plates, the plates were left resting for 5 minutes. Then plates were inverted and incubated for overnight (16 hours) at 37 °C. Next day, single colonies were selected and incubated in 3-4 ml LB- Broth containing antibiotic against which the plasmid has the resistance gene, in 15 ml falcon tube for overnight at 37 °C. Then, plasmid DNA's were isolated using ZymoPURE Plasmid Mini-prep Kit according to the manufacturer's protocol. Finally, samples were sent to Macrogen for sequencing to validate the success of mutation.

### **4.4 Cell Maintenance & Seeding & Freezing**

The cell stock from -80/-150 °C was thawed and was added onto 8-10 ml full medium in 75 cm<sup>2</sup> cell culture flask. Next day, the medium was replaced with fresh medium. 1-2 days later, cells were passaged in the following manner. The medium was aspirated and 5 ml 1X PBS was slowly added from the wall of the flask. Then, PBS was removed, and 1.5 ml Trypsin was added over cells. It was incubated at 37 °C incubator for 2-3 minutes for cell detachment. Next, 5 ml full medium was added on the cells and the plate was washed with this suspension. The suspension was collected into 15 ml falcon tube and centrifuged at 2000 rpm for 3 minutes. The supernatant was removed, and the pellet was resuspended into 10 ml fresh medium. For the maintenance culture, 1 ml suspension was mixed with 9 ml full fresh medium into the flask and the flask was stored in the incubator.

For cell seeding for experiments, cells could be counted using a hemocytometer or cell counter machines. Instead of this, the amount can be approximated by naked eye. From 95-100% confluent plates, 1 ml cell suspension for the 10 cm<sup>2</sup> plates, 200 microliters for the 6-well plates, and 100 µl for the 12-well plates were seeded. The fresh full medium was added up to 10 ml for the 10 cm<sup>2</sup> plates, 2 ml for the 6-well plates and 1 ml for the 12-well plates.

Cells were frozen using the freezing buffer. All steps were the same as the cell passage except the resuspension of fresh medium. Instead of it, cells were resuspended in freezing buffer which contains 20% FBS, 10% DMSO in DMEM. Then, they were aliquoted in 1.5 ml eppendorfs or cryotubes for storage at -80 °C or -150 °C.

#### 4.5 Transfections

One day before transfection, cells were seeded on 6-well, 12-well or 10 cm<sup>2</sup> plates depending on the experiment type. The amount of solution mixtures for transfection can be seen in Table 4.3. First, ddH<sub>2</sub>O was put on 1.5 or 2 ml eppendorf tube and then DNA was added. 2 M CaCl<sub>2</sub> was added dropwise, and they were incubated 5 minutes. Next, 2X HBS was also added dropwise and make bubbles using a pipette. After the bubble formation, the mixture was incubated for 10 minutes to allow complex formation. Then, the transfection mixture was added slowly on cells.

Table 4.3: Transfection Mixture Ingredients and Amounts for well types

	12 well	6 well	10 cm <sup>2</sup> plate
ddH <sub>2</sub> O	109,5 µl	219 µl	438 µl
DNA	1 µl	1 µl	1 µl
2 M CaCl <sub>2</sub>	15.25 µl	30.5 µl	61 µl
2X HBS	125 µl	250 µl	500 µl

Total	250 $\mu$ l	500 $\mu$ l	1000 $\mu$ l
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#### 4.6 siRNA Reverse Transfection Protocol

Harun Öztürk had optimized this protocol following the manufacturer's protocol. 12-well plates and HEK293 cells were used. siRNA was dissolved in RNase free water, aliquoted in small amounts to prevent contamination and freeze-thaw and stored at -20 °C. Cells were seeded at the same time with siRNA transfection. For each 12 well, 100 microliter mixture including HiPerfect, culture medium without serum and antibiotics and siRNA was prepared according to Table 4.4.

Table 4.4: siRNA Transfection Complex Components

Components	Amount
siRNA (20 nM final)	1,2 $\mu$ l
HiPerFect	4,5 $\mu$ l
DMEM -/-	94,3 $\mu$ l
Total	100 $\mu$ l

Empty medium, siRNA, and HiPerfect were put in the eppendorf tube and vortexed for 5 seconds. The tube was incubated at room temperature for 5-10 minutes. 1 ml full medium was put each well and approximately 100.000 cells were seeded on each well. After the incubation, the mixture was put on dropwise into the well. Cells were incubated at least 48 hours in 37°C and 5% CO<sub>2</sub>. If they need transfection, transfection was performed after 24 hours from the siRNA transfection.

## 4.7 Co-Immunoprecipitation

As the first step, solutions were prepared. For each group, 100  $\mu$ l beads were taken into a 1.5 ml eppendorf tube. (50  $\mu$ l for immunoprecipitation, 50  $\mu$ l for pre-clear). 750-1000  $\mu$ l RIPA buffer was added on the beads. Then, they were vortexed and centrifuged. The added amount of buffer was removed. This step was done for three times at least. After equilibration, beads were distributed into new 1.5 ml eppendorf tubes as 50  $\mu$ l each. They were stored at 4 °C.

After two days from the transfection, the media of them were removed. All steps should be done on ice when it is available. Cells were scraped with 5 ml 1X PBS and collected in the 15 ml falcon tubes. Then, they were centrifuged at 3000 g for 3 minutes and the supernatant was discarded, the pellet was resuspended in 5 ml 1X PBS. One more wash step was performed. The pellet was resuspended in 1 ml 1X PBS and the suspension was transferred to a 1.5 ml eppendorf. The centrifugation step was repeated, and the supernatant was removed. After that, the pellet was resuspended in 500  $\mu$ l lysis buffer which is RIPA buffer containing a protease inhibitor. Tubes were incubated on ice for 1 hour. During incubation, tubes were vortexed for 15 seconds at intervals of 15 minutes. After the completion of lysis, lysates were centrifuged at 13.000 rpm for 30 minutes at 4 °C. During the centrifugation, beads were equilibrated as described. After the centrifugation, the supernatant was transferred to a new 1.5 ml eppendorf. 50  $\mu$ l from the supernatant were taken as a whole-cell lysate sample and mixed with 16.66  $\mu$ l 4X Laemmli buffer. They were incubated at 95 °C for 10 minutes and store at 4 °C.

Lysates were added on the equilibrated beads and incubated on rotator-shaker for 30 minutes as a pre-clear. After the incubation, lysates were transferred to the equilibrated new beads. 2  $\mu$ l of antibodies were added on each lysate-bead mixture and they were incubated overnight at the 4 °C room on the rotator-shaker. Next day, tubes were centrifuged at 13.000 rpm for 30 seconds/1 minutes. The supernatant was transferred into a new tube for further control steps and tubes were stored at -20 °C. Beads were washed with 1 ml cold 0.2% Triton-X in PBS 3 times. In each step, 1 ml wash buffer was added, and tubes were shaken at least 1 minute. After that, beads were spun down and put on ice for 1

minute to ensure the proper precipitation of the beads. 100  $\mu$ l of the solution have remained in wash step in order to not lose beads until the last step. Before the elution of proteins on beads, 1 ml ice-cold 1X PBS was used for the final wash. Using a needle, all liquid part was removed. To elute proteins, 60  $\mu$ l 2X Laemmli buffer was added on the beads. They were incubated at 95 °C for 10 minutes and tubes can be vortexed 1 or 2 times for 5 seconds during boiling.

#### 4.8 Cell lysis

Two days after the transfection, media were aspirated. 2X Laemmli was added over the wells. Laemmli buffer amount was determined according to the cell confluency and well area and was stated in Table 4.5

Table 4.5: The amount of Laemmli Buffers for the Cell Lysis

Plate type	2X Laemmli Buffer Amount
12 well plate (per well)	150-200 $\mu$ l
6 well plate (per well)	300-400 $\mu$ l

Lysates were collected into the 1.5 ml eppendorf tubes and incubated at 95 °C for 10-15 minutes. After the incubation step, they were centrifuged at maximum speed for 5-10 minutes before loading on the SDS-PAGE.

#### 4.9 Treatments

MG132 powder was solved in dimethyl sulfoxide (DMSO) and the solution was stored at -20 °C. MG132 treatments were done according to Table 4.6. For control groups, the same volume of DMSO was used.

Table 4.6: MG132 Treatment Concentrations and Conditions

Treatment Duration	Final Concentration
Overnight	2 $\mu$ l
4-6 hours	10 $\mu$ l

#### 4.10 Immunoprecipitation

Cells were transfected two days before and treated with the proteasome inhibitor one day before the assay. Solutions were prepared according to the recipes in the material section. Media were removed from the plates and 1 ml working solution (NEM in PBS) was added carefully from the wall of plates and incubated for 1 minute. Then, working solution was removed and 1 ml 1X ice-cold PBS was added on cells. Then, the plates were scraped and collected into 1.5 ml eppendorf tubes. After the centrifugation at 3.000 g for 3 minutes, the supernatant was discarded, and 150  $\mu$ l SDS-containing lysis buffer was added to each group. Cutting the tip of the pipette tips, cells were resuspended into lysis buffer without introducing bubbles. Then, lysates were sonicated until they were clear. The lysate volume was completed to 1.5 ml with RIPA + PI solution. If they were not clear yet, the sonication was performed again. Next, samples were centrifuged at 13.000 rpm, 4°C for 30 minutes and the supernatant was transferred to a new tube. During this step, 60-100  $\mu$ l from the lysate were taken as whole-cell lysate (WCL) sample and mixed with 4X Laemmli buffer to have 1X laemmli concentration at the end. Before the lysate and the antibody incubation, the pre-clear step was performed. Beads were equilibrated as it was described in Co-IP protocol and equally distributed to eppendorf tubes. Lysates were added into the tubes and were incubated on rotator-mixer for 1 hour at room temperature. Then, beads were centrifuged using the minifuge and lysates were transferred to new eppendorf tubes and 2,5-4  $\mu$ l antibody was added for each tube. They were incubated at 4 °C for 4 hours and then the solution was incubated with equilibrated beads for 2 hours at 4 °C on the rotator-mixer. Before the elution step, beads were washed 5 times with 1 ml RIPA

buffer and 1 time with 1X PBS after discarding the lysate. Proteins on the beads were eluted with 2X Laemmli buffer and incubated at 95 °C for 10 minutes.

#### **4.11 His-Pull Down**

For each group, 50 µl of 50% Ni-NTA agarose beads were taken into a 1.5 ml eppendorf tube. Beads were washed with 1 ml Buffer A 3 times. First, 1 ml Buffer A was added on beads. Then, the beads were vortexed and spun down. 1 ml buffer from beads was discarded, and these were repeated 2 more times. They were distributed into eppendorf tubes as 50 µl beads each. The medium of cells was removed and 1 ml ice-cold PBS was added on each plate. Plates were scraped immediately and collected into eppendorf tubes. Then, they were centrifuged for 2 minutes at 3000 g. Next, the supernatant was removed, and the pellet was resuspended into 500 µl 1X PBS. 100 µl of each suspension was collected into new tubes as a whole-cell lysate sample and mixed with 33 µl 4X Laemmli buffer. They were mixed, incubated for 10 minutes at 95 degrees and stored at -20 °C for further use. Then, the rest of cell suspension was centrifuged at 3000 g for 2 minutes. The supernatant was discarded, and the pellet was dissolved in 1 ml Buffer A resulting viscous suspension. The suspension was sonicated until it becomes clear. After the sonication, lysates were centrifuged at 13.000 rpm for 30 minutes at room temperature. 800-850 µl of lysate was added on 50 µl equilibrated beads without disturbing the pellet. Lysate-bead mixture was rotated for 3 hours at room temperature on rotator-mixer. After 3 hours later, tubes were centrifuged for a short period for example 10-15 seconds at room temperature. The supernatant was carefully discarded but ~100 µl were remained. 1 ml Buffer A was added to each tube and tubes were incubated on rotator-mixer for 3-4 minutes at room temperature. 1 ml buffer from the beads was discarded, and this was the step was repeated with Buffer A one more time. Then, beads were washed with Buffer A/TI with 2 times. For last wash, 1 ml Buffer TI was added to beads and tubes were incubated for 3-4 minutes on rotator-mixer. Then, beads were centrifuged and most of the solution was discarded. The remaining liquid part was taken with a needle and the resin turned into white if all liquid part was taken. Beads were resuspended into 100 µl 2X Laemmli containing Imidazole and they were incubated for 10 minutes at 95 °C. During incubation, tubes were vortexed 2-3 times. Samples were run on the SDS-PAGE for Western Blot. For the WCL and Pulldown

control, 20  $\mu$ l each of the samples were loaded. To check the modified version of the protein of interest, 30-40  $\mu$ l were loaded from each group.

#### **4.12 Western Blot**

SDS-PAGE experiments were prepared according to the table in the material part. All solutions and chemicals except APS and TEMED were added. Then, APS and TEMED were added first to the separating gel mixture. It was poured and the top layer was leveled out with isopropanol. The separating gel mixture was left to fully polymerize. After separating gel was polymerized, APS and TEMED of the stacking gel were added and it was poured, then the comb was placed between the glass plates. When the stacking gel solution was polymerized, the gel was ready for sample loading.

Equal volumes from each group's lysates (10% of lysates for regular western blots) and 2-3  $\mu$ l ladder were loaded to the gel, and it was run for 30 minutes at 80 V for stacking. Then, the voltage was increased to 120 V and the running time was determined depending on the protein of interest. When the running was finished, proteins were transferred to the nitrocellulose membrane. The cassette, sponges, filter papers, gel, and membrane were established as it was shown in Figure 4.1. Then, the sandwich was placed into the transfer apparatus with an icebox and a magnetic fish, and it was run at 100 V for 3 hours at the 4 °C room on the magnetic stirrer. For siRNA experiments, the transfer step was performed for 2,5 hours.

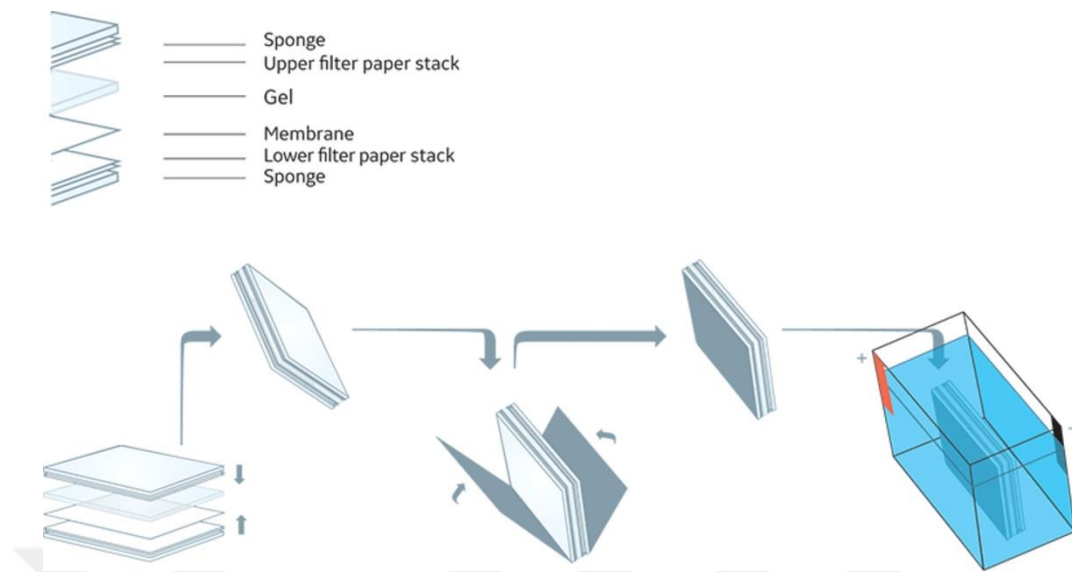


Figure 4.1: Schematic diagram of preparation of transfer cassette in wet transfer (Adopted from GE web page).

When the transfer of proteins was completed, the membrane was taken and incubated with the Ponceau solution to check efficiency of the transfer. 10 ml Ponceau S solution was poured on the membrane which was put on a small box. The membrane was incubated for 2-3 minutes. Then, it was washed with ddh<sub>2</sub>o to remove the excess Ponceau S dye until the background of the membrane turned to white. The image of the membrane was taken, and it was washed with 1X PBS-T until the remaining Ponceau S was removed. Then, it was blocked in 10 ml blocking buffer which contains 5% milk powder in PBS-T for 1 hour. After blocking, the membrane was incubated overnight with the antibody in blocking buffer (1:500-1.000) at the 4 °C. Next day, it was washed with 1X PBS-T for 3 times, 5 minutes each. Then, it was incubated with the HRP-linked secondary antibody (1:10.000) in blocking buffer for 1 hour at room temperature. After the secondary antibody incubation, the membrane was washed with 1X PBS-T 3 times, 10 minutes each. Then, the membrane was transferred to 1X PBS for storage and after this step it was ready for the visualization.

Depending on the size of the membrane, a sufficient amount of commercial chemiluminescence solutions were mixed with a 1:1 ratio. The surface of the membrane was covered with this mixture and put in the device for visualization.

## 5 RESULTS

### 5.1 *In silico* analysis of NEK1 and RNF4 interaction

Post-translational modification of a protein can pave the way for further modifications. For instance, phosphorylation of the protein leads not only to a change in its cellular localization but can sometimes trigger its subsequent ubiquitination. As mentioned above SUMOylation can induce further ubiquitination via RNF4. Our laboratory previously showed that NEK1 was SUMOylated and its ALS-linked form, tNEK1, was hyper-SUMOylated. Because RNF4 is a SUMO Targeted Ubiquitin E3 Ligase, we wondered whether there is any interaction between NEK1 and RNF4. To investigate the NEK1 and RNF4 interaction *in silico*, we used the PRISM database. PRISM (protein interactions by structural matching) uses a prediction algorithm to find new protein-protein interactions. This system merges the structure and the sequence conservation in the protein interfaces (Ogmen, Keskin, Aytuna, Nussinov, & Gursoy, 2005). This database uses possible interaction domains of proteins to predict a new possible interaction partner considering the previous interactor domains and the information from Protein Data Bank (PDB). After the analysis, it approximates an energy state and if it is smaller than -10, it indicates that the two analyzed proteins would have a good interaction. According to the results of our analysis, it gave us an approximate value of -30, which implies that NEK1 and RNF4 will possibly be interacting. In Figure 5.1, the red ribbon structure represents NEK1 which might be a substrate for RNF4, which is shown in blue. Thus, based on the *in silico* interaction analysis on PRISM, NEK1 and RNF4 probably interact with each other.

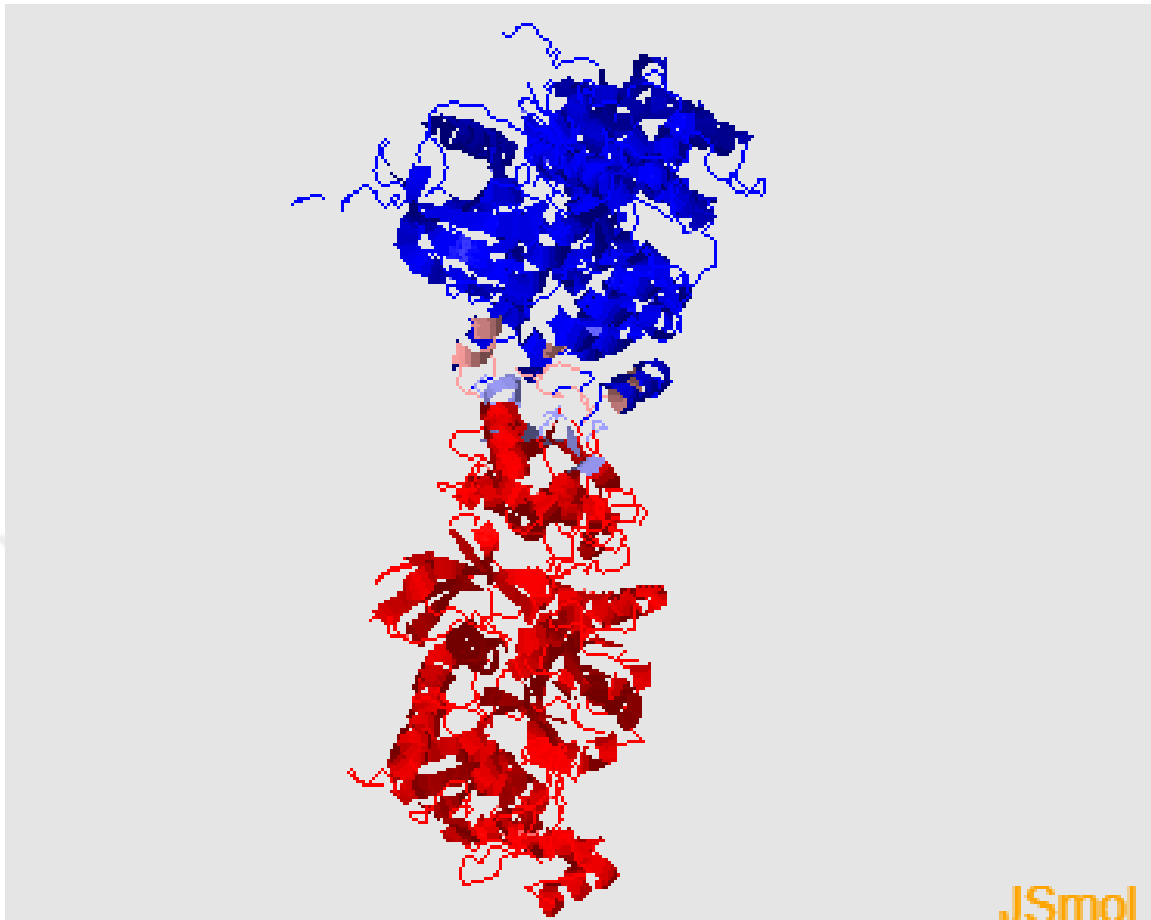


Figure 5.1: *In silico* analysis of the interaction of E2 and Ubiquitin loaded RNF4 (blue) and our protein of interest (red) using the PRISM database.

## 5.2 Confirmation of the Physical Interaction between NEK1 and RNF4

We know that NEK1 is SUMOylated, and NEK1-RNF4 might have a strong interaction according to the *in silico* analysis of PRISM. In the light of this information, we speculate that NEK1 may be a new target of RNF4. To investigate whether there is a real physical interaction between NEK1 and RNF4, we performed co-immunoprecipitation experiments in HEK293 cells. We over-expressed GST-NEK1 and HA-RNF4 in HEK293 cells, and the cells were treated overnight with proteasome inhibitor after 24 hours from the transfection. Next day, we lysed the cells with NP-40 containing mild lysis buffer (RIPA) in order not to disrupt the interaction between proteins. Then, we pulled down HA-RNF4 using anti-HA antibodies and A/G Agarose Beads eluted proteins and broke their interaction using Laemmli buffer.

Eluted proteins were analyzed with SDS-PAGE followed by a Western Blot using anti-GST antibody to detect the co-immunoprecipitated GST-NEK1. Our Western Blot results show that NEK1 co-immunoprecipitated with RNF4. This suggested that NEK1 and RNF4 physically interact (Figure 5.2).

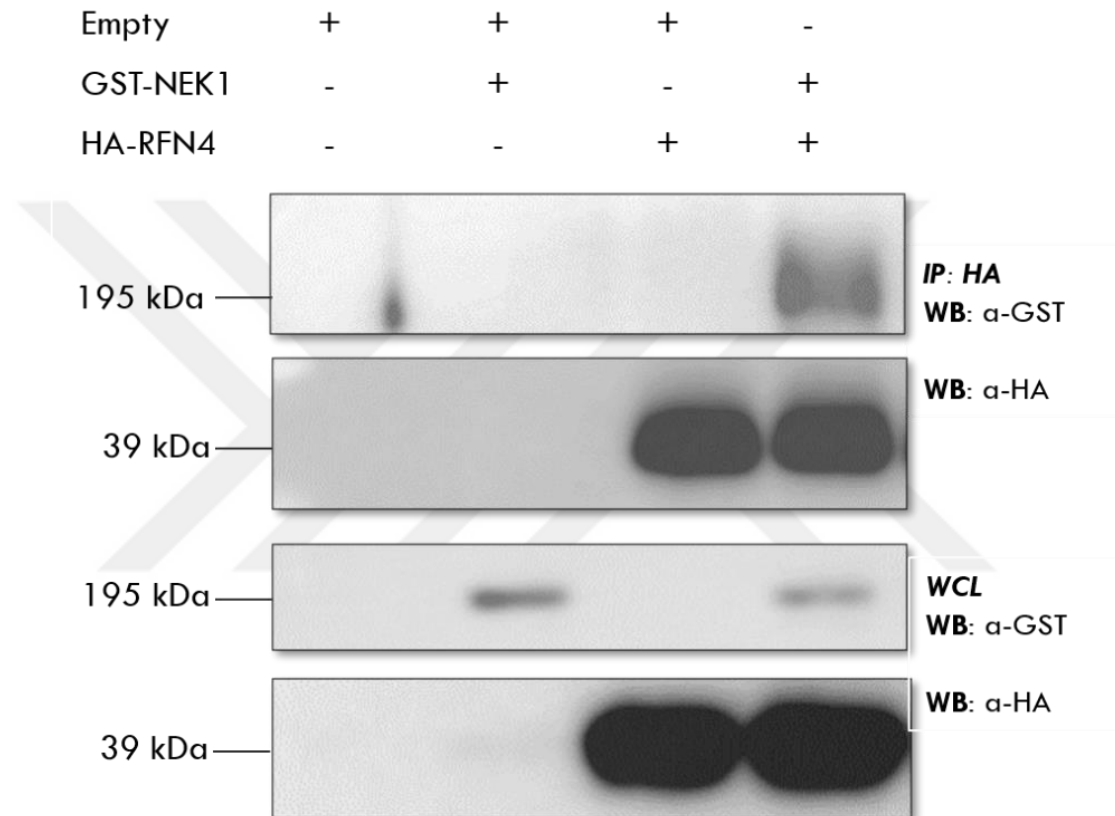


Figure 5.2: Co-immunoprecipitation experiments confirm physical interaction between NEK1 and RNF4. HA-RNF4 was pulled with HA antibodies and GST-NEK1 was co-immunoprecipitated with RNF4.

### 5.3 The Effect of RNF4 on NEK1 and tNEK1 Stability

If NEK1 is a target of RNF4, the stability of NEK1 might be affected because ubiquitinated forms of NEK1 will be sent to the proteasome for degradation. To investigate the effect of RNF4 on NEK1 stability; we co-expressed GST-NEK1 either with HA-RNF4, or with a dominant negative version of RNF4 (HA-RNF4 DN) in HEK293 cells. The dominant-negative version of RNF4 has a mutation in its catalytic site and prevents the

transfer of ubiquitin to the target. One day after transfection, one group of cells was treated with 2  $\mu$ M MG132, which is a proteasome inhibitor, to test whether this degradation can be reversed by proteasome inhibition or not. Two days after transfection, we lysed the cells with 2X Laemmli buffer and ran the samples on SDS-PAGE for Western Blot analysis. In the presence of RNF4 overexpression, we observed in some cases the destabilization (Figure 5.3) and in some cases the stabilization of NEK1 (Figure 5.4) in RNF4 overexpression conditions.

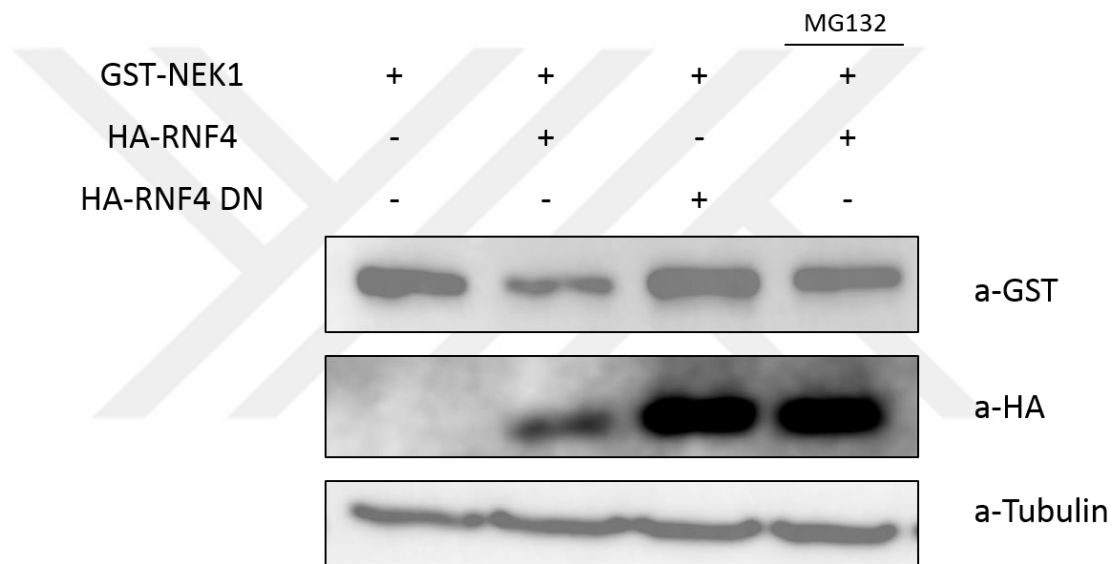


Figure 5.3: Destabilization of NEK1 in the RNF4 overexpression system. The dominant-negative version has no effect on the degradation of NEK1 and MG132 can reverse the destabilization of NEK1 although wild type RNF4 is there.

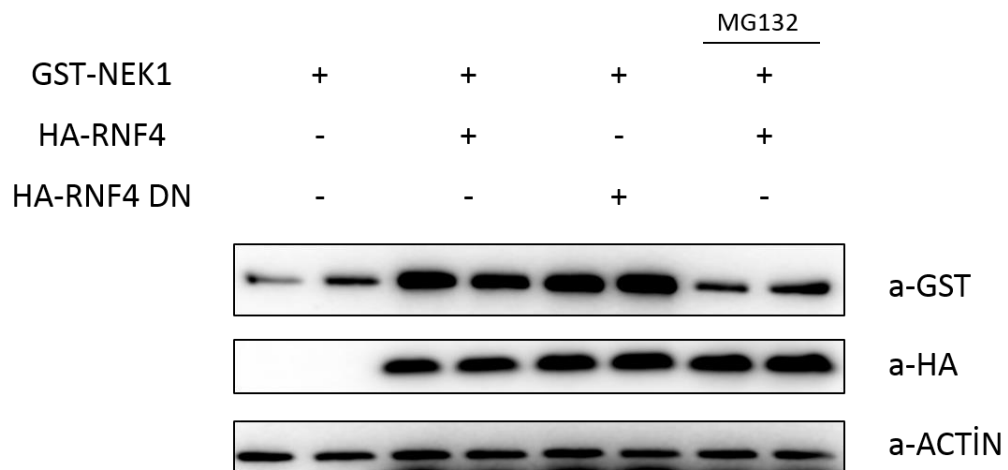


Figure 5.4: RNF4 overexpression leads to the stabilization of NEK1 in some experiments contrary to what we observed in other experiments.

The performed co-transfection experiments did not provide us a with consistent result. To find an effective concentration of RNF4, we transfected our cells with increasing concentration of RNF4 while keeping the NEK1 concentration stable. We observed an increase of NEK1 stabilization with increased concentration of RNF4 concentration, contrary to our expectations.

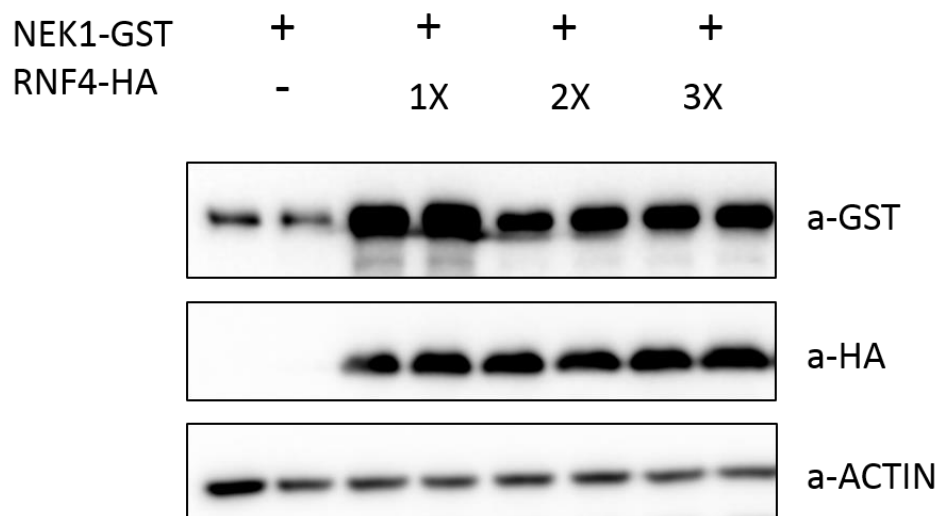


Figure 5.5: Increasing concentration of RNF4 leads to the stabilization of NEK1 in some experiments.

Next, we thought that RNF4 may not affect the stability of wild-type NEK1. Then, we analyzed the stability of ALS-linked form of NEK1 (tNEK1) with increasing RNF4 concentrations. Two days after transfection, cells were lysed, and lysates were run on a SDS-PAGE. In the anti-GST blot against GST-NEK1, we observed the stabilization of both WT and the mutant version of NEK1.

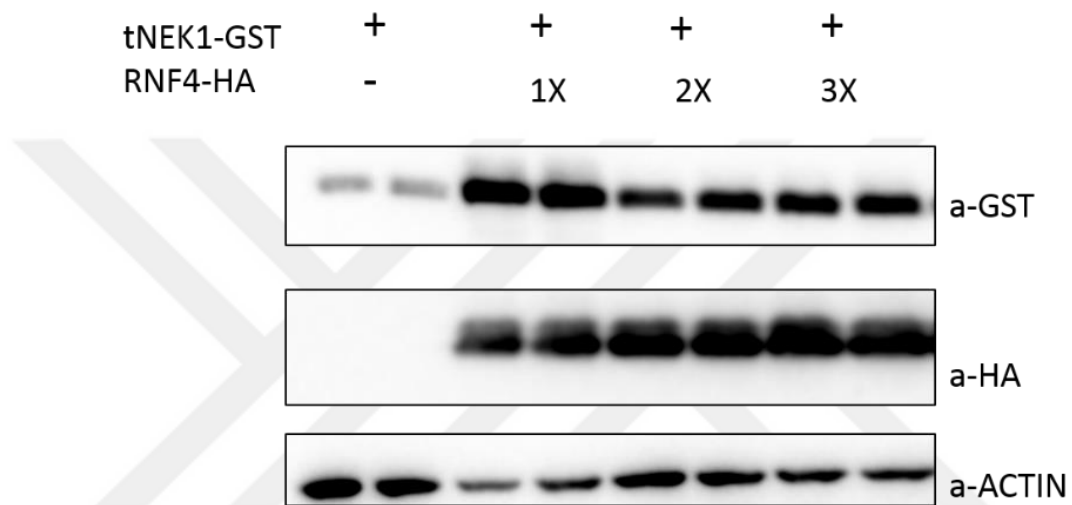


Figure 5.6: Stabilization of tNEK1 in the overexpression of increasing RNF4 concentrations.

#### 5.4 Silencing of RNF4 by siRNA affects the stability of NEK1

After receiving inconsistent results in the over-expression system, we decided to work on the endogenous system. Instead of over-expressing RNF4, we knocked down RNF4 using siRNA. siRNA for RNF4 and non-targeting control were transfected during cell seeding. 48 hours after transfection, cells were lysed with 2X Laemmli buffer before SDS-PAGE and Western blot analysis. After confirming the silencing of RNF4 at the protein level, we blotted the membrane with anti-NEK1 antibody to determine NEK1 levels and observed that the silencing of RNF4 with siRNA increased the stability of NEK1.

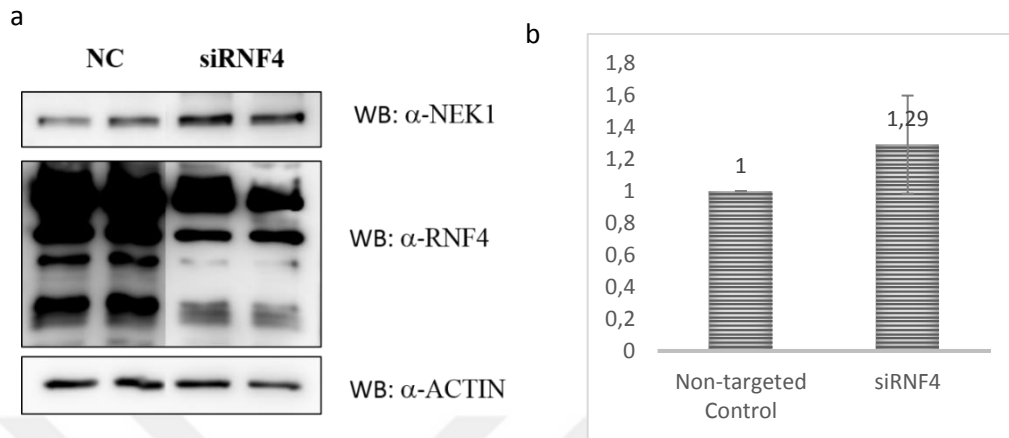


Figure 5.7: The effect of RNF4 silencing on NEK1 level. a) Representative image of the western blot analysis of silencing of RNF4 and the stabilization of NEK1. b) Quantitative analysis of five technical replicates and at least two biological replicates of each technical replica.

### 5.5 His Pull-Down Assays to Probe NEK1-Ubiquitination

So far, we showed that NEK1 and RNF4 physically interact and that the depletion of RNF4 affects endogenous NEK1 levels in the cell. In the light of these findings, we performed His pull-down experiments to investigate the ubiquitination of NEK1 in the presence of RNF4 overexpression. To do this, we transfected HEK293 cells with different combinations of GST-NEK1, GST-tNEK1, HIS-UB, and HA-RNF4 plasmids. 24 hours after the transfection, cells were treated with 2  $\mu$ M of the proteasome inhibitor MG132. Next day, cells were lysed with 6 M Guanidine-containing harsh lysis buffer to disrupt the interaction between proteins. Equilibrated beads and lysates were incubated for 3 hours at room temperature. During the incubation, all proteins modified by His-UB were first bound to nickel beads using the affinity of histidine to the nickel. Then, proteins on nickel beads were eluted with imidazole-containing Laemmli buffer. Imidazole has an affinity to nickel and thus, depending on the concentration, it either prevents the non-specific binding, or it elutes the bound proteins.

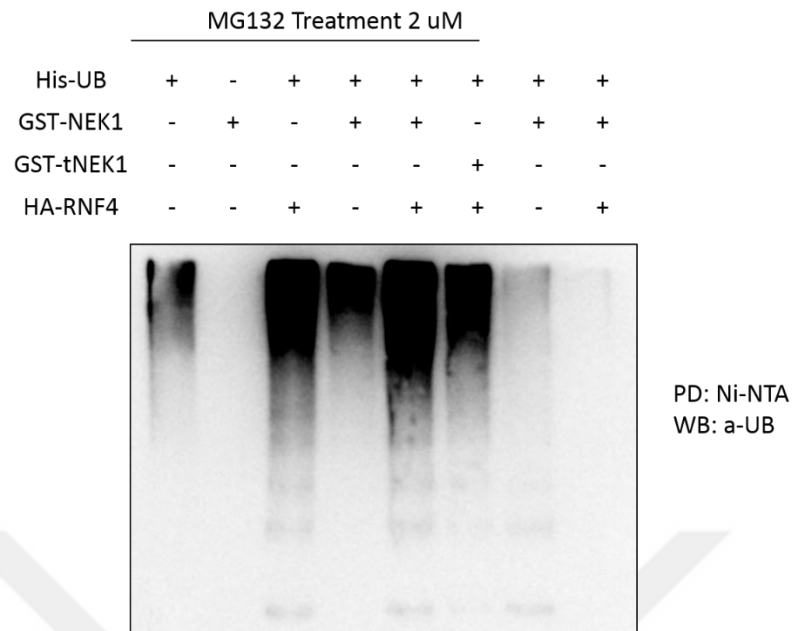


Figure 5.8: Pull-down of all His-Ubiquitin modified proteins via Ni-NTA beads. In this blot, all ubiquitinated proteins which were pulled with Nickel beads were seen.

In his pull-down experiments, we pulled down all ubiquitinated proteins including modified NEK1 and tNEK1 because of the His-tag of our ubiquitin plasmid. (Figure 5.8). Afterwards, we performed the western blot using GST antibody to detect the ubiquitinated NEK1 and tNEK1 in the pool of all ubiquitinated proteins. According to our blots, we observed an increase in the ubiquitination of NEK1 in the presence of Ubiquitin and RNF4 overexpression (Figure 5.9). If we compare the ubiquitination of wild type NEK1 and disease-related tNEK1; although the pull-down of ubiquitinated proteins of tNEK1 containing group (lane 6) is less than the wild type NEK1 group (lane 5), the ubiquitination of tNEK1 was already equal with wild type. In the equal pull-down condition, we easily see the hyperubiquitination of the truncated version. If we compare lane 4 and 5 in Figure 5.8, pull-down of lane 5 had more protein than that of lane 4. Since RNF4 increases the ubiquitination of SUMOylated proteins in cells, the amount of the ubiquitinated proteins is much more in RNF4 containing groups in the presence of proteasome inhibitor. So, we pulled down more proteins in the RNF4 containing groups. This problem might be misleading us in determining the effect of RNF4 on NEK1 ubiquitination. To deeply investigate this finding and have a more accurate result, we decided to perform immunoprecipitation experiments.

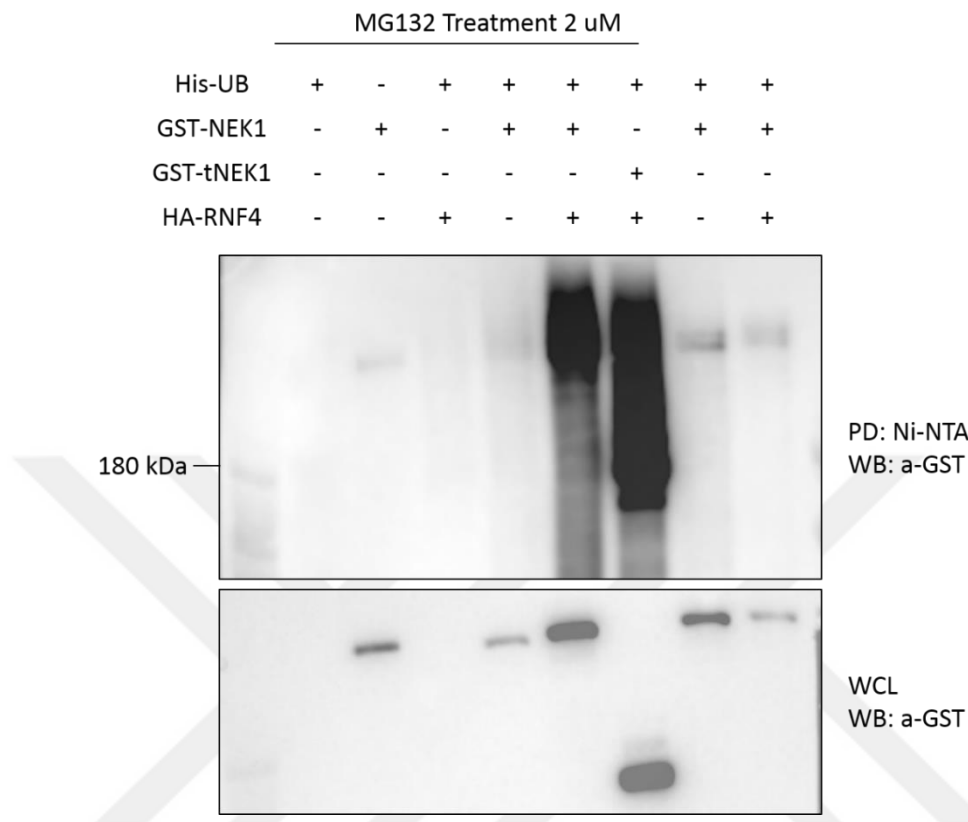


Figure 5.9: Ubiquitination of NEK and tNEK1 levels in His pull-down experiment. Upper panel shows GST blot of pull-down samples and it is seen that NEK1 and tNEK1 were ubiquitinated. Lower panel belongs to whole cell lysates samples and expression levels of GST-NEK1 in HEK293 cell can be seen.

## 5.6 Immunoprecipitation to detect NEK1 ubiquitination

To further investigate the results of His Pull-down experiments and to acquire a more precise result about the NEK1 ubiquitination, we performed immunoprecipitation assays. We over-expressed GST-NEK1, HIS-Ubiquitin, and HA-RNF4 plasmids in different combinations one day after cell seeding. One day after transfection, a proteasome inhibitor treatment (MG132) was performed for all groups except one control group. The next day, cells were lysed with SDS containing lysis buffer after NEM treatment. Cells were sonicated for proper lysis, SDS was diluted with RIPA from 2% to 0.2%, and lysates were incubated with equilibrated beads for pre-clear step for 30-45 minutes at room temperature.

Then, lysates were collected and incubated with GST antibody for 4 hours at 4 °C on the rotator-mixer. Next, the lysate-antibody mixture was incubated to catch the antibody-lysate conjugation on the beads. After a few washing steps, proteins on beads were eluted with Laemmli buffer. Then, they were loaded and run on the SDS-PAGE for Western Blot analysis to detect ubiquitinating NEK1.

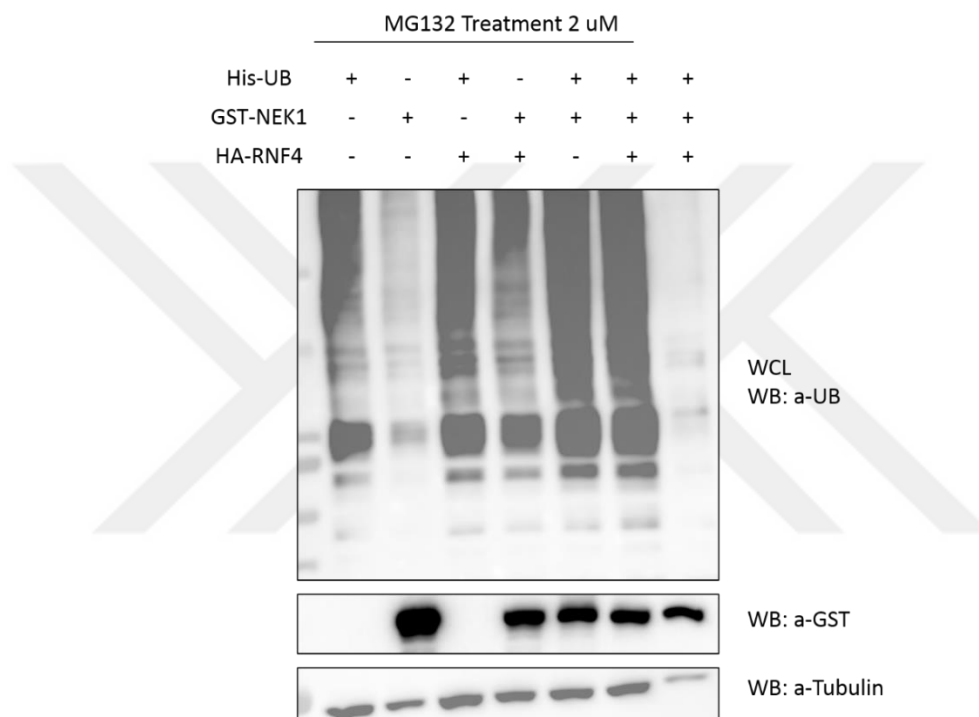


Figure 5.10: The expression of ubiquitin and NEK1 in whole-cell lysates of immunoprecipitation samples. The membranes were blotted with a-UB, a-GST and a-Tubulin respectively.

The whole-cell lysates were run and blotted to check the transfection efficiency and the expression level. All groups were successfully transfected and proteasome inhibitor MG132 worked well when we compare the sixth and last lane (Figure 5.10). Pull down samples were run to determine the pull-down efficiency and detect NEK1 ubiquitination. In the ubiquitin blot of pull-down samples, we could not detect any ubiquitination although we used a proteasome inhibitor to prevent the degradation of ubiquitinated proteins and the pull-down was successful. Therefore, we speculate that there was a technical problem and

that the protocol should be further optimized to investigate the ubiquitination profile of this protein.

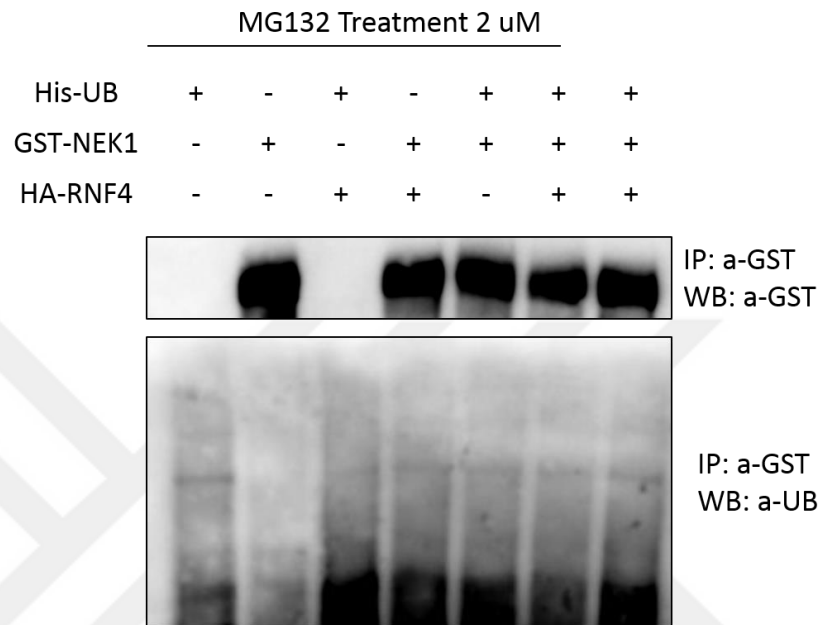


Figure 5.11: Pull down control of GST-NEK1 (top) and ubiquitin blot (bottom) of pull-down samples. Ubiquitinated forms of NEK1 have not been observed in Ubiquitin blot.

## 6 DISCUSSION

### 6.1 NEK1 is a new substrate for RNF4

Studies on ALS and neurodegeneration show that the main cause of ALS is the aggregation of misfolded proteins in motor neurons resulting in neurodegeneration. FUS, TDP-43, UBQLN2, and the gene product of mutant C9ORF72 are predominantly found in ALS-linked cellular aggregates. Self-aggregation capacities of proteins and malfunctions in the protein quality control system also contribute to these aggregate formations (Blokhuys et al., 2013). Thanks to the MinE project, in recent years some novel ALS-linked genes like *NEK1* have been discovered (Kenna et al., 2016). Although it is known that *NEK1* mutations are found in some ALS patients, how mutant NEK1 leads to the disease phenotype is still not known. There are around 30 genes related to ALS and mutations in these 30 different genes result in the same condition: degeneration of motor neurons. What all the mutations of these genes may have in common as a consequence is the misfolding or aggregation of the mutant proteins. In recent years, it has been discovered that different *NEK1* mutations were observed in ALS patients. If NEK1 also follows the same path as other proteins in ALS, it or its disease-related versions may tend to aggregate. In our laboratory, we have chosen one of the common mutants which has a single amino acid change leading to a termination codon in position 812 and we named it truncated NEK1 (tNEK1). One of our lab members showed that tNEK1 has a tendency to lose solubility and aggregate, having been trapped in the nucleus. These results showed that tNEK1 shares some common characteristics with other ALS-related proteins. Apart from that, it has been shown by our laboratory that NEK1 undergoes SUMO modification. Furthermore, the ALS-linked mutant version tNEK1 is hyperSUMOylated which might be a sign of its toxicity or aggregation. The stability of some proteins like Tax or PML-RAR $\alpha$  is affected by SUMOylation. Their SUMOylation can cause their subsequent ubiquitination and degradation. In this step, an important enzyme takes to the stage. It is a special ubiquitin E3 ligase which differs from the others because of its substrate specificity. All SUMOylated proteins can be a target for RNF4. Because NEK1 and tNEK1 are SUMOylated, they might be a substrate for RNF4. For this purpose, we performed co-immunoprecipitation experiments involving RNF4 and NEK1. These experiments showed

that RNF4 and NEK1 physically interact. This suggests that, RNF4 possibly ubiquitinates NEK1 and causes its degradation by the proteasome. As a next step, tNEK1 and RNF4 interaction should be further investigated. In addition to co-immunoprecipitation experiments, this interaction can be investigated with proximity ligation assay (PLA) (Bagchi, Fredriksson, & Wallén-Mackenzie, 2015). PLA is a sensitive method to detect closely interacting proteins (in proximity smaller than 16 nm). Furthermore, with this method, the localization of this interaction can be observed and it can be determined whether it occurs in the cytoplasm or the nucleus.

## **6.2 The effect of RNF4 on the stability of NEK1**

Since we have shown that NEK1 and RNF4 physically interact, RNF4 could affect the stability of NEK1 via ubiquitination. If RNF4 is a ubiquitin E3 ligase for NEK1, NEK1 will be ubiquitinated after its SUMOylation and degraded in the proteasome. To observe the effect of RNF4 on the stability of NEK1, we co-expressed NEK1 along with RNF4 or DN (dominant negative) RNF4, which is catalytically inactive. In Western blot, we did not observe convincing destabilization of NEK1. Variability of NEK1 levels may result from different circumstances. Since NEK1 has a role in the cell cycle, the level of NEK1 can vary in different stages of the cell cycle. If ubiquitination of NEK1 occurs in specific conditions, we may not be able to capture the ubiquitination and further degradation. NEK1 is a serine/threonine kinase which phosphorylates its substrates after interacting with them via its coiled-coil domain. Depending on our previous experiments such as co-immunoprecipitation of NEK1 and RNF4, SUMOylation of NEK1 and degradation of ALS-linked version of NEK1, we speculated that NEK1 might be a new substrate of RNF4. However, the co-immunoprecipitation experiment did not give an idea about the interaction domains. It is known that the coiled-coil domain of NEK1 is important for interaction with its partners. Generation of a coiled-coil deleted version of NEK1 would give an idea about its involvement and significance in protein-protein interactions. Contrary to our hypothesis, NEK1 may be regulating the activity of RNF4. In some experiments, we observed the stabilization of NEK1 in the presence of RNF4. If RNF4 is phosphorylated by NEK1 in specific times, it might change the E3 ligase activity of RNF4. In the literature, it was shown that phosphorylation affects the activity of SUMO E3 ligases

(Roscic et al., 2006). To clarify whether NEK1 is phosphorylating RNF4, the kinase domains of NEK1 can be mutated and inactivated. Another issue is that ubiquitination occurs after SUMOylation. If only a small percentage of NEK1 can be SUMOylated with the limited endogenous SUMO machinery in the cells, the non-SUMOylated NEK1 would far outnumber the modified proportion. In that case, even if RNF4 indeed affects NEK1 ubiquitination and degradation, it would be very hard to detect this effect. This could also explain our results.

We finally investigated the steady-state levels of endogenous NEK1 when RNF4 is silenced. For this purpose, we used siRNA to knockdown RNF4 in HEK293 cells. When we knocked down RNF4 with siRNA, we did observe some stabilization of NEK1, suggesting that RNF4 may indeed destabilize NEK1.

### **6.3 Ubiquitination Level of NEK1 in the Presence of RNF4**

After confirming the interaction between NEK1 and RNF4, we investigated the effect of RNF4 on NEK1 ubiquitination via two approaches: His-pull down and immunoprecipitation assays. For both assays, we transfected HEK293 cells with GST-NEK1 along with HA-RNF4 and His-tagged ubiquitin (His-Ub). To prevent the degradation of ubiquitinated proteins in the proteasome, we treated cells with a proteasome inhibitor (MG132). In the His-pull down experiment, we used Ni-NTA beads in order to employ the affinity of nickel to histidine. In this way, all ubiquitinated proteins were pulled down. Then, the level of ubiquitinated NEK1 in RNF4 + and RNF4 – groups was analyzed in order to understand the effect of RNF4 on the ubiquitination levels of NEK1. When we compared the ubiquitination profile of NEK1/UB and NEK1/UB/RNF4 groups, the latter had more ubiquitinated proteins than the former one. Although we observed an increase in ubiquitinated NEK1 in NEK1/UB/RNF4 groups, we were not sure if this is a real increase or if it comes from the inequality between the pull down of the whole ubiquitinated proteins. Apart from that, GST-NEK1 nonspecifically binds the Ni-NTA beads even without His-Ub modification. In the GST-NEK1 only group, we observed a small number of precipitated proteins although this group did not have a His-tag and was not modified by any His-tagged protein. To overcome these problems, we switched from His pull-down

assays to immunoprecipitation assays. Since we used GST antibody to pull down GST tagged NEK1, we eliminated the inequality in the pull-down groups. Using GST antibody, we isolated both the modified and non-modified version of NEK1. By blotting with a ubiquitin antibody, we expected to detect ubiquitinated NEK1 and to observe the effect of RNF4. However, we could not see any ubiquitinated NEK1 in a number experiments we performed, thus we cannot ascertain that RNF4 is a ubiquitin E3 ligase for NEK1 and leads to its ubiquitination. After optimization of the immunoprecipitation protocol for ubiquitination, the effect of RNF4 on NEK1 ubiquitination can be determined by comparing the presence and absence of RNF4. As an alternative approach for ubiquitination, PLA (Proximity Ligation Assay) can also detect the ubiquitination of NEK1 when RNF4 overexpressed case or in the case when RNF4 is silenced. In conditions with MG132 treatment, we can compare the number of PLA signals in different groups. In this way, the effect of RNF4 can easily be determined.

#### **6.4 Therapeutic usage of RNF4 dependent degradation of NEK1**

Our co-immunoprecipitation results indicate that NEK1 and RNF4 interact. This result leads to the following question: Is NEK1 a target for RNF4? Previous experiments from our lab showed that the disease-related version of NEK1 (tNEK1) is basally degraded in PML nuclear bodies, possibly via hyper-SUMOylation. PML NB-dependent tNEK1 degradation can be induced by Interferon alpha (Harun Öztürk, unpublished results). There is a mechanism in the cell in which PML NB, SUMO, and RNF4 work together. Thus, we speculated that the degradation in PML NBs might be RNF4 dependent. Aggregation of NEK1 leads to the degeneration of motor neurons as has been observed for other ALS-related proteins. Therefore, devising a method for the elimination of the toxic proteins contributes to the research of targeted therapies against ALS. In this study, we revealed the importance of post-translational modifications and suggest a possible mechanism to regulate the stability of ALS-related protein NEK1. If the hypothesized/proposed mechanisms are further confirmed, these speculations and findings may help to develop a cure for ALS patients.

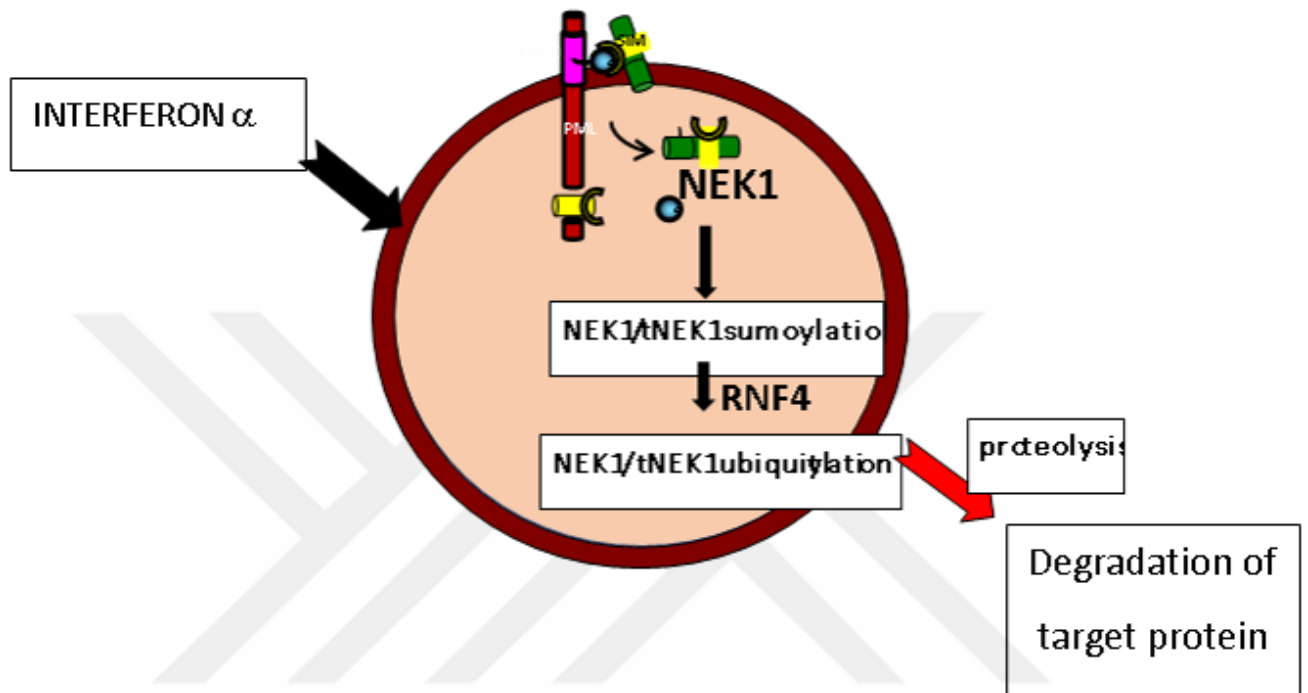


Figure 6.1: Proposed model for our hypothesis. NEK1/tNEK1 is recruited into PML NBs and is SUMOylated. STUbL RNF4 can facilitate the ubiquitination of SUMO-modified NEK1/tNEK1 and send them to the proteasome for degradation. Via this mechanism, the clearance of toxic proteins can be achieved. ( Adapted from Sahin Umut et al., 2014)

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