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The role of the Leucine-Rich (LeuR) domain of Rho Guanine Nucleotide Exchange Factor (RGNEF) in the regulation of amyotrophic lateral sclerosis (ALS) associated protein TAR DNA-binding protein of 43 KDa (TDP-43)

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Pathology and Laboratory Medicine

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Abstract

The presence of neuronal cytoplasmic inclusions (NCIs) composed of RNA-binding proteins (RBPs) and neurofilaments is considered to be ALS's neuropathological hallmark. RGNEF has been previously shown to interact with TDP-43 and to have a regulatory effect on the expression levels of *NEFL* mRNA and NFL protein *in vitro*. Here, I examined the mechanism of the RGNEF N-terminus, leucine-rich domain (LeuR) domain's interaction with TDP-43. I observed that the minimal domain required is 110 amino acids (LeuR¹¹⁰), that the Ankyrin domain adjacent to LeuR¹¹⁰ does not participate, and that LeuR¹¹⁰ forms of a high molecular weight complex with TDP-43 *in vitro* consistent with the co-aggregation of LeuR²⁴² and TDP-43 in double transgenic *drosophila melanogaster*. I also observed that RGNEF interacts directly with and destabilizes the *TARDBP* mRNA 3'UTR. These findings support that RGNEF interacts with TDP-43 through the formation of a high molecular weight complex and the down-regulation of its expression.

Keywords

Amyotrophic lateral sclerosis, RGNEF, TDP-43, Leucine Rich domain, protein-protein interaction, high molecular weight complex, down-regulation, *drosophila melanogaster*, co-aggregation.

Summary for Lay audience

Amyotrophic Lateral Sclerosis (ALS) is a progressive, adult-onset disease characterized by the degeneration of motor neurons. In the cytoplasm of neuronal cells, the buildup of RNA-binding proteins (RBPs), neurofilaments and other proteins are considered to be the disease's hallmark. Among these proteins, Rho guanine nucleotide exchange factor (RGNEF) has been identified as a key element in the development of ALS, where it accumulates in the cytoplasm of motor neurons. Previously, RGNEF has been shown to interact with another protein TDP-43 and to regulate a neurofilament protein NFL. Here, I focused on the mechanism of interaction between TDP-43 and a region of RGNEF, leucine-rich (LeuR). I observed that a minimal domain of LeuR interacts with TDP-43 by forming a complex of proteins, consistent with our observations in the brain and eye tissue of an ALS fly model. In addition to the interacting mechanism, I studied the role of RGNEF in the regulation of TDP-43. I observed that in the presence of the LeuR, there is a downregulation in TDP-43 levels. By defining the role of the interaction between two ALS-associated RBPs, RGNEF and TDP-43, we can get a step further into identifying a potential therapeutic target in ALS.

Dedication

I would like to dedicate my thesis to my parents, their unconditional love and support made it possible for me to be where I am today.

I also dedicate my work to my aunt and all the individuals who lived with ALS. Their strength and resilience are a real inspiration to all of us and may we one day live in a world free of disease.

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Contributions

The transgenic flies used in this project were previously generated and characterized by Dr Droppelmann and Ben Withers.

List of Abbreviations

3'UTR	3' untranslated region
ALS	Amyotrophic lateral sclerosis
<i>ANG</i>	Gene encoding angiogenin
ANK	Ankyrin repeat
<i>ARHGEF28</i>	Gene encoding Rho guanine nucleotide exchange factor
C9FTD/ALS	Frontotemporal dementia/amyotrophic lateral sclerosis linked to chromosome 9
<i>C9orf72</i>	Gene encoding Chromosome 9 open reading frame 72
CNS	Central nervous system
<i>DCTN1</i>	Gene encoding dynactin 1
DH-domain	Dbl homology domain
DLR	Dual luciferase reporter
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DRP	Dipeptide repeat proteins
DTSSP	Dithiobis(sulfosuccinimidylpropionate)
ECM	Extracellular matrix
<i>ERBB4</i>	Gene encoding erb-b2 receptor tyrosine kinase 4
<i>EWSR1</i>	Gene encoding Ewing sarcoma breakpoint region 1
FAK	Focal adhesion kinase
fALS	Familial amyotrophic lateral sclerosis
FTLD	Frontotemporal lobar degeneration
<i>FUS</i>	Gene encoding fused in sarcoma
Gal4	Yeast transcription activator protein Gal4
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GEF	Guanine exchange factor
HEK 293T	Human embryonic kidney 293 cells variant
<i>hnRNPa1</i>	Gene encoding Heterogeneous nuclear ribonucleoprotein A1
IF	Intermediate filaments

IHC	Immunohistochemistry
IP	Immunoprecipitation
LeuR	Amino terminal region of RGNEF containing the Leucine Rich domain
LMN	Lower motor neuron
LRR	Leucine-rich repeat
<i>MATR3</i>	Gene encoding Matrin 3
mRNA	messenger RNA
mtFUS-ALS	mutant FUS amyotrophic lateral sclerosis
mtTDP-43-ALS	mutant TDP-43 amyotrophic lateral sclerosis
mtSOD1	mutant SOD1 amyotrophic lateral sclerosis
NCI	Nuclear cytoplasmic inclusion
<i>NEFL</i>	Gene encoding low molecular weight neurofilament protein
NES	Nuclear export signal
NF	Neurofilament
NFL	Low molecular weight neurofilament protein
<i>NFH</i>	Gene encoding High molecular weight neurofilament protein
NLS	Nuclear localization signal
NP-40	Nonidet P-40
<i>OPTN</i>	Gene encoding Optineurin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PH-domain	Pleckstrin homology domain
PNS	Peripheral nervous system
<i>PRPH</i>	Gene encoding Peripherin
RAN	Repeat associated non-ATG
RGNEF	Rho guanine nucleotide exchange factor
RΔLeuR	RGNEF lacking the LeuR domain
RΔCOOH	RGNEF lacking the carboxy terminal region of RGNEF
RBD	RNA binding domain
RhoA	Rhas homolog family member A
RNA	Ribonucleic acid

RRM	RNA recognition motif
sALS	Sporadic amyotrophic lateral sclerosis
SD22-like	G-type lectin S-receptor-like serine/threonine-protein kinase
<i>SETX</i>	Gene encoding Senataxin
SG	Stress Granule
<i>SOD1</i>	Gene coding for Superoxide dismutase 1
SDS	Sodium dodecyl sulfate
<i>TAF15</i>	Gene encoding TATA-binding protein associated factor 2N
<i>TARDBP</i>	Gene encoding TAR DNA-binding protein of 43 kDa
TDP-43	TAR DNA-binding protein of 43 kDa
<i>UBQLN2</i>	Gene encoding Ubiquilin 2
<i>SETX</i>	Gene encoding Senataxin
UAS	Upstream activation sequence
UTR	Untranslated region
Znf	Zinc finger domain

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Chapter 1

1.1 Clinical aspects of ALS

1.1.1 History and description of ALS

Amyotrophic Lateral Sclerosis (ALS) is a progressive, adult-onset neurodegenerative disease first described by French neurologist Jean-Martin Charcot in 1869 (Charcot & Joffroy, 1869). Considered the founder of modern neurology, he revolutionized the field by coupling clinical observations and pathological findings associated with various neurological diseases that would allow for a more accurate classification. Through studies conducted on the first ALS cases from 1865 to 1869, alongside his pupil then colleague Alix Joffroy, Charcot observed that lesions of the anterior horn of the spinal cord resulted in paralysis accompanied by atrophy of muscle, whereas the lesions in the lateral column of the spinal cord resulted in progressive paralysis and no atrophy of muscles, thus establishing clinicopathological correlate of lower motor neuron (LMN) vs upper motor neuron (UMN) pathology (Charcot & Joffroy, 1869).

These observations supported Charcot's hypothesis that the various clinical outcomes of this motor neuron disease are a result of the location of the lesions. It wasn't until 1874 that the name amyotrophic lateral sclerosis was associated with motor neuron disease in Charcot's publication "Œuvres complètes" in which he described the unique features of ALS. Firstly, he noted that the paralysis of the upper limbs occurred without loss of sensation, shortly thereafter the legs became affected with atrophy of the muscles of the paralyzed limbs. With disease progression, these features worsened and were accompanied by the apparition of bulbar symptoms. The progress of the pathology through each phase was rapid and death occurred 2 to 3 years from the onset of the bulbar symptoms on average (Charcot, 1874).

Since then, neurologists around the world still rely on similar clinical features when describing patients with ALS (Rowland & Shneider, 2001) (Brown & Al-Chalabi, 2017) such that the progressive loss of upper (UMN) and lower (LMN) motor neurons in the brain and the spinal cord is a defining characteristic of the pathology.

1.1.2 Diagnosis and epidemiology

Over the past two decades, physicians have been using El Escorial Criteria and its revised version as a diagnostic tool (Brooks, 1994) (Brooks, Miller, Swash, & Munsat, 2000). These criteria are based on a combination of features and an algorithm for diagnostic certainty. Core features of the criteria include the history, physical and neurological examinations supported by electrophysiological studies to assess the degree of LMN involvement, and neuroimaging when appropriate to exclude other diseases and neuropathology. The concept of progression is also critical (Brooks, 1994). These criteria were subsequently modified to increase the sensitivity of ALS diagnosis (Carvalho, et al., 2008). These revised criteria (the Awaji criteria) increased the weighting applied to the neurophysiological data alongside the clinical observations as a single dataset to decrease the number of ALS false positives (Zarei, et al., 2015). This modification increased the sensitivity of probable or definite ALS diagnoses by over 20% (Costa, Swash, & Carvalho, 2012).

Because the initial symptoms of ALS are highly nonspecific and can mimic those of other muscle wasting diseases, misdiagnosis in early stages of the disease is very common and often leads to delays in ALS diagnosis. Since there are no valid diagnostic ALS biomarkers, the diagnosis remains based on clinical symptoms and electrophysiology that require spreading over time (Longinetti & Fang, 2019).

While the clinical presentation of ALS is heterogenous because of the various motor neuron populations involved, typically patients present with motor neuron lesions serving bulbar functions and limbs (Zarei, et al., 2015) (Brown & Al-Chalabi, 2017). Typically, these lesions result in weakness of the limbs and bulbar musculature. In general, most patients show an initial limb-onset (Brooks, 1996) with features including muscle stiffness and spasticity, fasciculation, weight loss, muscle cramps and as the degeneration progresses, muscle atrophy (Brown & Al-Chalabi, 2017) (Magnussen & Glass, 2017). The pathology later progresses toward the bulbar regions (Brooks, 1996). However, about a third of ALS cases have an early bulbar-onset, where the affected regions have a predominantly LMN involvement and the patients present with symptoms such as difficulty swallowing, chewing and speaking (Brown & Al-Chalabi, 2017) (Grad, Rouleau, Ravits, & Cashman, 2017). The disease progresses as the motor neurons of the brain and the spinal continue to degenerate affecting muscles of the limbs, oropharynx, tongue and eventually the diaphragm. In the later stages of ALS, the respiratory muscles are weakened,

which eventually leads to respiratory failures (Zarei, et al., 2015). However, ALS does not seem to affect the oculomotor neurons or those of Onuf's nucleus supplying bladder function (Brown & Al-Chalabi, 2017).

While the presence of motor dysfunction remains core to the diagnosis of ALS, there is an increasing awareness of nonmotor manifestations of the disease. The finding was first highlighted in the early 1980s by Arthur J. Hudson who noted that a proportion of ALS patients also demonstrated other neurological disorders including dementia, Parkinson's and other behavioral deficits (Hudson, 1981). Since then, the more contemporary literature has been defining ALS as a multisystem disorder with a subgroup of ALS patients showing symptoms of frontotemporal dementia (FTD) (Strong, et al., 2009) (Strong, et al., 2017). A prospective study conducted by the Miller group in 2002 show that 14% of patients diagnosed with FTD were later diagnosed with ALS whereas 36% presented with features of ALS and then developed ALS (Lomen-Hoerth, Anderson, & Miller, 2002). The pathological characterization of FTD in ALS patients shows frontal and temporal cortical degeneration with neuronal loss and spongiform degeneration (Hudson, 1981). The behavioral syndrome associated with FTD detected in ALS patients includes significant alterations of personality and social conduct while the memory is relatively spared (Strong, et al., 2009) (Strong, et al., 2017).

The average lifespan from disease onset is 3 to 5 years with respiratory failure as the most common cause of death (Yedavalli, Patil, & Shah, 2018). The incidence rate is estimated at 1.8/100,000 people per year with a mean prevalence of 3.4/100,000 in North America (Grad, Rouleau, Ravits, & Cashman, 2017). While over ninety percent of ALS cases are classified as sporadic (sALS), two other variants of ALS exist and are classified as follows: familial ALS (fALS) linked to specific genetic inheritance of multiple ALS-associated genes and a previously hyperendemic focus in the Kii Peninsula, Guam and Papua New Guinea known as the Western Pacific variant (Soraru, et al., 2009).

1.2 ALS-associated genes

While over 90% of ALS cases are classified as sporadic, approximately 10% are associated with a family history. Approximately half of these have an autosomal dominant transmission, while recessive (Andersen & Al-Chalabi, 2011) or X-linked (Kennedy, Alter, & Sung, 1968) (Harding, Thomas, Bradbury, Morgan-Hughes, & Ponsford, 1982) are less common. However, a clear

distinction between these two variants of ALS remains controversial if we follow the Mendelian inheritance definition. Indeed, the gene mutations identified in fALS cases have also been observed in sALS (Al-Chalabi, et al., 2013).

Although not all causative, mutations in over 25 genes have been associated with ALS (Andersen & Al-Chalabi, 2011). A genome-wide association study looking into the whole exome sequencing of a vast number of individuals allowed scientists to create a database of ALS genes with their corresponding clinical phenotype (Abel, Powell, Andersen, & Al-Chalabi, 2012). Amongst those genes known to be causative of ALS, a subset are more commonly observed, including *superoxidase dismutase 1 (SOD1)* (Rosen et al., 1993), *TAR DNA-binding protein 43 (TARDBP)* (Sreedharan, et al., 2008) (Davidson, et al., 2016), *fused in sarcoma (FUS)* (Deng, et al., 2010) and *Chromosome 9 open reading frame 72 (C9orf72)* (DeJesus-Hernandez, Mackenzie, Boeve, & al., 2011) (Renton, et al., 2011).

As indicated in table 1, there are also an increasing number of genetic mutations observed in association with ALS in which the mutation is considered to be rare or not causative, but rather disease modifying. Among those, mutations are observed in *optineurin (OPTN)* (Maruyama, et al., 2010), *TATA-binding protein-associated factor 15 (TAF-15)*, *Ewing sarcoma breakpoint region 1 (EWSR1)* (Couthouis J. , et al., 2012), *rho guanine nucleotide exchange factor (RGNEF)* (Keller, et al., 2012) (Droppelmann, et al., 2013) (Droppelmann, Keller, Campos-Melo, Volkening, & Strong, 2013), and intermediate filaments (*neurofilament NEFH* and *peripherin*) (Al-Chalabi, et al., 1999) (Wong, He, & Strong, 2000) (Gros-Louis, et al., 2004).

While the pathobiology of ALS is complex and heterogenous, the study of its genetic variants helped identify three molecular categories through which the disease is induced. These include alterations in protein homeostasis, cytoskeletal dynamics and RNA homeostasis (Brown & Al-Chalabi, 2017). In the next section, I will discuss the major ALS-associated proteins identified in the literature and their role in the pathobiology of ALS.

Table 1. ALS-associated genes

ALS pathology process	ALS-associated gene	Inheritance	Disease causative	Reference
RNA metabolism	<i>TARDBP</i>	Dominant	Yes	(Sreedharan, et al., 2008)
	<i>FUS</i>	Dominant and Recessive	Yes	(Kwiatkowski, et al., 2009) (Mackenzie, Rademakers, & Neumann, 2010)
	<i>TAF15</i>	Dominant and Recessive		(Ticozzi, et al., 2011)
	<i>EWSR1</i>	Inconclusive		(Couthouis J. , et al., 2012)
	<i>ANG</i>	Dominant		(Greenway, et al., 2004) (Greenway, et al., 2006)
	<i>SETX</i>	Dominant	Yes	(Chen, et al., 2004)
	<i>hnRNPa1</i>	Dominant	Yes	(Kim, et al., 2013)
	<i>MATR3</i>	Dominant		(Johnson, et al., 2014)
	<i>ARHGEF28</i>	Unknown		(Droppelmann, et al., 2013) (Ma, et al., 2014)
Proteinopathy	<i>C9orf72</i>	Dominant	Yes	(Renton, et al., 2011) (DeJesus-Hernandez, Mackenzie, Boeve, & al., 2011)
	<i>OPTN</i>	Dominant and Recessive	Yes	(Maruyama, et al., 2010)
	<i>UBQLN2</i>	Dominant	Yes	(Deng, et al., 2012)
Enzymatic activity	<i>SOD1</i>	Dominant and Recessive	Yes	(Rosen, et al., 1993)
Cytoskeletal	<i>NEFH</i>	Dominant		(Al-Chalabi, et al., 1999)

Dynamics	<i>PRPH</i>	Sporadic		(Gros-Louis, et al., 2004)
	<i>DCTN1</i>	Dominant		(Munch, et al., 2004) (Vilarino-Güell, et al., 2009)
Others	<i>ALS3</i>	Unknown		(Hand, et al., 2002)
	<i>ERBB4</i>	Dominant		(Takahashi, et al., 2013)
	<i>ALS7</i>	Dominant	Yes	(Sapp, et al., 2003)

Gene abbreviations: *ANG* = Angiogenin; *ARHGEF28* = Rho Guanine Nucleotide Exchange Factor; *C9orf72* = Chromosome 9 Open Reading Frame 72; *DCTN1* = Dynactin 1; *ERBB4* = erb-b2 receptor tyrosine kinase 4; *EWSR1* = Ewing Sarcoma Breakpoint Region 1; *FUS/TLS* = Fused in Sarcoma/Translocated in Liposarcoma; *hnRNPA1* = Heterogeneous Nuclear Ribonucleoprotein A1; *MATR3* = Matrin 3; *NFH* = High Molecular Weight Neurofilament; *OPTN* = Optineurin; *PRPH* = Peripherin; *SETX* = Senataxin; *SOD1* = Superoxide Dismutase 1; *TAF15* = TATA-Binding Protein Associated Factor 2N; *TARDBP* = TAR DNA-Binding Protein of 43 kDa; *UBQLN2* = Ubiquilin 2.

1.2.1 Protein homeostasis

Neuronal cytoplasmic inclusions (NCIs) identified in ALS post-mortem tissues are predominantly skein-like inclusions or Lewy-body like hyaline inclusions that are ubiquitinated (Haegawa, et al., 2008) (Robinson, et al., 2013). In fact, ubiquitinated inclusions are the most abundant form of inclusions detected in ALS motor neurons, in which ubiquitin binds and marks misfolded and abnormal proteins for proteasomal degradation. ALS motor neurons are unable to activate the proper pathways for protein degradation, resulting in the accumulation of ubiquitinated proteins in aggregates (Basso, et al., 2009) (Bendotti, et al., 2012). Researchers have identified various ALS-associated proteins linked to loss of protein homeostasis in ALS and they include SOD1, C9ORF72 and OPTN.

1.2.1.1 Cu/Zn Superoxide dismutase 1 (SOD1)

Cu/Zn Superoxide dismutase 1 (SOD1) is a 153 amino acid homodimer ubiquitously-expressed protein composed of two subunits with a stabilizing zinc and a catalytic copper ion in each subunit. SOD1 is an abundant cytoplasmic enzyme present in all cells in almost all organisms and is a highly conserved protein (Lui, et al., 2004). The primary function of SOD1 is to remove superoxide generated by the cells to reduce the generation of reactive oxygen species (Bendotti & Carri, 2004).

SOD1 was the first gene to be linked to ALS (Rosen et al., 1993). There are currently over 150 SOD1 mutations associated with fALS, accounting for 15 - 20% of fALS cases and approximately 3% of sALS cases (Anderson, 2006) (Ghasemi & Jr., 2018). Most mutations are missense mutations present across the coding sequence resulting in an exchange of amino acids without altering the size of the protein (Cleveland & Rothstein, 2001) (Andersen, et al., 2003) (Lui, et al., 2004). The rest of the mutations associated with ALS are nonsense mutations resulting in the alteration of the length of the final protein, silent mutations conserving both the length and the sequence of the protein, and nonpathogenic intronic mutations found in ALS cases and controls (Restagno, et al., 2005). In the case of many pathogenic SOD1 mutations, little to no effect has been observed on their enzymatic activity which excludes a loss of function mechanism. Indeed, the available evidence points to a gain of toxic function linked to the mutated SOD1 that results in motor neuron degeneration (Gurney, et al., 1994). Of relevance to

our understanding of ALS, neuropathological studies have demonstrated SOD1 immunoreactive neuronal cytoplasmic inclusions in cortical and spinal cord tissue of ALS patients with and without SOD1 mutations (Forsberg, et al., 2019).

In contrast, our group previously examined the expression of SOD1 using immunohistochemistry in three variants of ALS: familial, sporadic and mtSOD1 cases compared to healthy controls (Keller, et al., 2012). The results showed the specific expression of SOD1 in diffuse cytoplasmic inclusions in motor neurons of mtSOD1 cases only, suggesting that the mtSOD1-ALS variant possesses a unique pathological signature that distinguishes it from other variants of ALS. Among the possible mechanisms, cf mtSOD1 toxicity, our lab demonstrated the interaction of mtSOD1 with human *NEFL* mRNA and its effect on the RNA stability (Ge, Strong, Laystra-Lantz, & Strong, 2005) (Volkening, Leyster-Lantz, Yang, Jaffee, & Strong, 2009). By destabilizing *NEFL* mRNA, the stoichiometry of NF expression is altered in a manner that leads to the formation of NCIs (Ge, Leystra-Lantz, Wen, & Strong, 2003).

1.2.1.2 Chromosome 9 open reading frame 72 (C9orf72)

An autosomal dominant variant of ALS has been identified in association with genetic mutation on chromosome 9q21 encoding C9orf72 protein (Vance, et al., 2006) (Renton, et al., 2011) (DeJesus-Hernandez, Mackenzie, Boeve, & al., 2011). The number of hexanucleotide sequence repeats in *C9orf72* ranges from 0 to 22. In ALS patients, the mutation detected is a massive intronic hexanucleotide repeat expansion (GGGGCC) (DeJesus-Hernandez, Mackenzie, Boeve, & al., 2011) and the number of repeats ranges from 30 to several thousand (Renton, et al., 2011).

The pathological expansion of hexanucleotide repeats in C9orf72 is the most frequent genetic mutation causing ALS and frontotemporal dementia (FTD) in European and North American population, accounting for 11.7% of familial FTD cases and 23.5% of fALS cases (DeJesus-Hernandez, Mackenzie, Boeve, & al., 2011). It was also determined as a genetic cause in 5.1% of sporadic FTD patients and 5.9% of sporadic ALS patients (Blitterswijk, DeJesus-Hernandez, & Rademakers, 2012).

Previous findings related to non-coding repeat expansion disorders showed that by the formation of RNA foci in the nucleus and/or cytoplasm, RNA-binding proteins are sequestered which leads to the dysregulation of alternative mRNA splicing (Miller, et al., 2000) (Sofola, et al., 2007) (White, et al., 2010). Initial reports of RNA foci in patients with *C9orf72* repeat

expansions demonstrated their presence in approximately 25% of the cells in the frontal cortex and the spinal cord. Indeed, analyses of the brain tissue of patients with C9FTD/ALS showed the presence of sense and antisense RNA foci C9orf72 positive (DeJesus-Hernandez, Mackenzie, Boeve, & al., 2011). Previous findings showed that the most pathological feature of C9orf72 is the presence of RNA-binding protein TDP-43 positive inclusions in neurons of ALS cases with C9orf72 mutations (Davidson, et al., 2016).

Interestingly, despite their location in non-coding regions, *C9orf72* repeat expansions are capable of starting their own translation through a non-canonical mechanism known as repeat-associated non-ATG (RAN) translation (Zu, et al., 2011). This leads to the formation of neuronal inclusions containing dipeptide repeat proteins (DRPs) generated through RAN translation (Mori, et al., 2013).

As evidenced by the data from numerous studies on the role of *C9orf72* repeat expansions in ALS and other neurodegenerative diseases, three possible mechanisms seem to be involved in the pathogenesis of C9orf72-associated ALS and these include a loss of function of C9orf72 protein, the gain of toxic function caused by the sense and antisense *C9orf72* repeat expansions, or a direct toxicity of the DRPs (Balendra & Isaacs, 2018). Which of these proposed mechanisms is the dominant one remains to be determined.

1.2.1.3 Optineurin (OPTN)

Optineurin is a highly conserved protein that is involved in several cellular processes, including the inflammatory response, autophagy and vesicular transport. It is ubiquitously expressed in many tissues including the eye, kidney, heart, liver and brain (Rezaie & Sarfarazi, 2005) (Rezaie, Waitzman, Kaufman, & Sarfarazi, 2005).

OPTN is considered to be an ALS-associated gene for which greater than 20 mutations have been described (Li, et al., 2015). The identified mutations in a Japanese cohort of familial and sporadic ALS predicts that the recessive mutation causing ALS is a loss of function mechanism on the one hand and on the other hand, the upregulation and distribution of mutated *OPTN* causes a disruption in neuronal functions alongside the formation of inclusions in sporadic ALS (Ying & Yue, 2016). OPTN has been found in pathological structures in ALS (Maruyama, et al., 2010) where pathological skein-like inclusions immunoreactive for ubiquitin and TDP-43 are also immunoreactive for OPTN (Maruyama, et al., 2010) (Osawa, et al., 2011). A semi-

quantitative analysis performed on different variants of ALS spinal cord tissues in our lab showed that while OPTN immunoreactive NCIs are not present in healthy controls, they are observed in the spinal cord of a wide range of ALS variants including sALS, mtC9orf72-ALS, fALS, mtFUS-ALS and mtTDP-43 ALS patients (Keller, et al., 2012).

While specific mutations of *OPTN* have been determined to cause ALS in various studies (Maruyama, et al., 2010) (Fifita, et al., 2017), the specific role of OPTN in the pathogenesis of ALS pathology remains unclear. In fact, dysfunctions in various cellular mechanisms have been linked to the protein including endoplasmic reticulum stress (Vaibhava, et al., 2012), autophagy (Bansal, et al., 2018) and neuroinflammation (Sako, et al., 2012). The mechanism by which OPTN regulates neurodegeneration in ALS is however unknown.

1.2.2 Cytoskeletal dynamics

Cortical and spinal motor neurons are polarized cells with a soma, dendrites and extended axons allowing them to participate in the directional flow of information from the central nervous system to the rest of the body systems. (Lambrechts, Robberecht, & Carmeliet, 2007) (Rolls & Jegla, 2015). To maintain its polarity and therefore function, the cytoskeletal organization of the motor neurons is critical. The main components of the cytoskeleton consist of a dynamic and complex network of protein filaments, including microtubules and intermediate filaments. The involvement of neuronal intermediate filaments in ALS has been determined in immunohistochemical studies identifying the presence of neurofilament subunits and peripherin in which defects in their metabolism resulted in the loss and degeneration of upper and lower motor neurons (Munoz, Green, Perl, & Selkoe, 1988) (Troost, Smith, Jong, & Swaab, 1992).

1.2.2.1 Intermediate filaments

Early studies of axonal transport identified three neuronal intermediate filament proteins of different molecular size 200kDa, 160kDa and 68kDa (Hoffman & Lasek, 1975) that further biochemical studies confirmed as the three subunits of the neurofilament (NF), the light (NFL), the medium (NFM) and the heavy (NFH) weight neurofilament (Liem, Yen, Salomon, & Shelanski, 1978). Together with the intermediate filament α -internexin, these make up for the primary neuronal intermediate filaments of the CNS (Pachter & Liem, 1985). In the peripheral nervous system (PNS), the fourth subunit constituting the NFs rather than internexin is peripherin (Portier, de Néchaud, & Gros, 1983).

In vivo studies on transgenic mice and *in vitro* studies in cells showed that in healthy neurons, neurofilaments are generated by the stoichiometric heteropolymerization of the NF subunits NFL and α -internexin/peripherin with NFM and/or NFH (Lee, Xu, Wong, & Cleveland, 1993) (Ching & Liem, 1993) (Yuan, et al., 2006) (Beaulieu, Robertson, & Julien, 1999). The stoichiometric co-assembly of these subunits into a filamentous structure is responsible for the axon caliber and cell integrity (Elder, et al., 1998).

Neurofilament accumulations are found in NCIs of both sALS and fALS and studies on *in vivo* models show that the overexpression of neurofilament proteins leads to alterations in the three subunits stoichiometry resulting in the disruption of axonal transport of NFs in motor neurons (Al-Chalabi & Miller, 2003). To better understand the correlation between the stoichiometric composition of the NFs and the ALS-related neuronopathy, the Julien group developed transgenic mice models expressing the human *NEFH* gene (Côté, Collard, & Julien, 1993). The results showed that, even with a modest overexpression of NFH subunit, the mice exhibited a progressive degeneration of their motor functions causing tremors and progressive signs of weakness like those observed in ALS patients. These features were accompanied by the presence of an abnormal accumulation of neurofilaments in the perikaryal and proximal axons of motor neurons of the anterior horn, similar to the neuronal swellings found in the motor neurons of ALS patients (Hirano, Donnemfeld, Sasaki, & Nakano, 1984).

Therefore, maintaining the NFs stoichiometry by controlling the expression levels of NF subunits is critical since alterations in the stoichiometry result in the formation of NF aggregates.

1.2.3 RNA homeostasis

The discovery of alterations in the metabolism of RNA-binding proteins (RBPs) in ALS, typically manifested as intracellular inclusions, provided a contemporary approach to the development of *in vivo* and *ex vivo* models of the disease process. Subsequent studies have supported the hypothesis that RNA dysmetabolism plays a critical role in the pathogenesis of ALS including impacts on transcription, alternative splicing, microRNA biogenesis, mRNA stability, mRNA compartmentalization and axonal transport of translationally quiescent mRNA (Strong, 2010).

1.2.3.1 TAR DNA-binding protein of 43kDa (TDP-43)

TDP-43 is a nucleic acid binding protein primarily localized in the nucleus with a small fraction localized in the cytoplasm. It has two highly conserved domains, RNA-recognition domains 1 and 2 (RRM1 and RRM2), allowing the protein to bind to highly (UG)_n enriched sequences (Lukavsky, et al., 2013). Its amino-terminal and carboxy-terminal ends are mainly responsible for protein-protein interactions involving TDP-43 (Budini, Baralle, & Buratti, 2014). In normal conditions, TDP-43 can shuttle between the nucleus and cytoplasm, mediated by a nuclear localization signal (NLS) located in the N-terminus region and the nuclear export signal (NES) in RRM2. It is involved in numerous RNA processes including transcription, splicing, RNA stability, transport, localization and stress granule (SG) formation (Buratti, 2015).

The observation of ubiquitin and TDP-43 immunoreactive neuronal cytoplasmic and glial inclusions in the spinal cord tissue of ALS patients, consisting of hyperphosphorylated TDP-43 provided the first evidence supporting the potential role of altered RNA homeostasis in the pathogenesis of ALS (Arai, et al., 2006). At the same time, Neumann et al (2006) observed similar TDP-43 immunoreactive inclusions in both frontotemporal lobar degeneration (FTLD) and ALS (Neumann, et al., 2006). Immunohistochemistry results from hippocampus, frontal and temporal cortex of ALS patients showed that the pathologic TDP-43 protein is ubiquitinated, hyperphosphorylated and cleaved resulting in the generation of toxic C-terminal fragments (Neumann, et al., 2006).

An additional breakthrough in understanding ALS pathogenesis was the discovery of dominant causative mutations on the encoding gene *TARDBP* in both fALS and sALS cases (Sreedharan, et al., 2008). The identified *TARDBP* mutations include missense, truncations and insertion/deletion, collectively accounting for about 5% of fALS cases (Buratti, 2015).

Under pathological conditions, TDP-43 accumulates in the cytoplasm in inclusions accompanied by TDP-43 clearing from the nucleus; the latter being proposed to cause a loss of the nuclear functions of TDP-43 (Ederle & Dormann, 2017). The formation of TDP-43 pathological inclusions is associated with extensive post-translational modifications including ubiquitination, phosphorylation, formation of C-terminal fragments and acetylation, that promotes the aggregation process and toxicity (Igaz, et al., 2009) (Buratti, 2018). The presence of TDP-43 protein inclusions in brain and spinal cord tissue are a signature of ALS pathology.

As previously demonstrated by our group, TDP-43 is an *NEFL* mRNA binding protein (Strong, et al., 2007) alongside mutant and wild type SOD1 (Ge, Strong, Laystra-Lantz, & Strong, 2005) and rho guanine nucleotide exchange factor (RGNEF) (Volkening, Leyster-Lantz, & Strong, 2010) (Droppelmann, Keller, Campos-Melo, Volkening, & Strong, 2013). *In vitro* data show that the presence of a unique UG motif at the 3'UTR of *NEFL* mRNA allows the RRM1 and RRM2 domains of TDP-43 to specifically interact with this mRNA (Volkening, Leyster-Lantz, Yang, Jaffee, & Strong, 2009). In ALS, TDP-43 co-localizes with an array of RNA granules including transport granules (Staufen-1 immunoreactive), stress granules (TIA 1 immunoreactive), processing granules (XRN1 immunoreactive). This suggest that TDP-43 may be sequestered in RNA granules in ALS, thus impacting their role in modulating RNA stability.

TDP-43 has also been determined to have many splicing targets including FUS/TLS and its own 3' untranslated region (UTR) (Polymenidou, et al., 2011), as well as playing a role in microRNA metabolism (Buratti, et al., 2010).

1.2.3.2 Fused in sarcoma/translocated in liposarcoma (FUS/TLS)

FUS/TLS (commonly abbreviated as FUS) is a DNA/RNA-binding protein mainly localized in the nucleus where it participates in various cellular processes including transcription and splicing (Ling, Polymenidou, & Cleveland, 2013). It is an hnRNP protein and belongs to the FET protein family that includes TAF15 and EWS (Morohoshi, Arai, Takahashi, Tanigami, & Ohki, 1996). The protein structure of FUS consists of an amino-terminal transcription activation domain - the Gln-Gly-Ser-Tyr (QGSY)-rich region, a highly conserved domain RNA recognition motif (RRM) and three Arg-Gly-Gly (RGG)-repeat regions, a zinc finger motif and a highly conserved carboxy-terminal region implicated in RNA binding (Iko, et al., 2004). FUS is heavily involved in transcription regulation, facilitated by the role of the amino-terminal region in FUS dimerization and binding to chromatin. Moreover, FUS is responsible for the phosphorylation of RNA polymerase II (RNA-polII) during transcription and therefore in regulating its activity.

About 5% of fALS cases occur in association with mutations in the FUS gene. The most abundant missense/deletion mutations identified in ALS occur in the carboxy-terminal region containing the nuclear localization signal where they have been shown to lead to an abnormal distribution of mutant FUS in the cytoplasm (Kwiatkowski, et al., 2009). In the presence of FUS ALS-associated mutations, the transcription activity of RNA-polII and FUS leads to a down-

regulation of the RNA species (Yang, Gal, Chen, & Zhu, 2014) (Masuda, et al., 2015). FUS is among the proteins identified in pathological NCIs in ALS motor neurons in both mutant FUS-linked fALS and sALS (Deng, Gao, & Jankovic, 2014).

Among the proposed mechanisms by which FUS causes the disease is a toxic gain of function. In fact, a homozygous *FUS* knockout mouse model demonstrated that the dysfunction of FUS led to a behavioral phenotype distinct from ALS suggesting that a loss of function alone is not enough for FUS to trigger ALS pathology (Kino, et al., 2015). On the other hand, results from transgenic mice overexpressing human *FUS* show an increase in the expression of cytoplasmic FUS present in globular and skein-like FUS-positive inclusions. Moreover, the homozygous animals developed an aggressive phenotype with pathological features like those seen in ALS patients (Mitchell, et al., 2013).

1.2.3.3 TATA box-binding protein-associated factor 15 (TAF15) and Ewing sarcoma b1 (EWS)

TATA box-binding protein (TBP)- associated factor 15 and Ewing sarcoma b1 (EWS) are RBPs that belong to the FET family of heterogeneous nuclear ribonucleoprotein proteins (hnRNPs). They are predominantly expressed in the nucleus. Both TAF15 and EWS are capable of shuttling from the nucleus to the cytoplasm and participate in transcription and alternative splicing (Morohoshi, Arai, Takahashi, Tanigami, & Ohki, 1996) (Couthouis J. , Hart, Shorter, & al, 2011). TAF15 has been shown to have unique intronic binding patterns in which it preferably binds to GGUA motifs in RNA (Kapeli, et al., 2016). However, TAF15 affects only a small number of alternative splicing events. It plays a more significant role in the stability of significant mRNA populations (Kapeli, et al., 2016) where TAF15 has been reported to recruit RNA polymerase II to sites of active transcription in complex with transcription factor II D (TFIID) (Bertolotti, Lutz, Heard, Chambon, & Tora, 1996) (Kwon, et al., 2013). In sALS patients, mislocalization of TAF15 to the cytoplasm in motor neurons is evident (Couthouis J. , Hart, Shorter, & al, 2011). Because of the presence of prion-like domains and its low-complexity feature it is prone to aggregation (Kwon, et al., 2013).

Of note, EWS mutations have only been detected in sALS patients, including three missense variants which were determined to be involved in the mislocalization of EWS in motor neurons (Couthouis J. , Hart, Shorter, & al, 2011) (Couthouis J. , et al., 2012). *In vivo* analysis of

the effects of EWS in transgenic flies expressing human EWS is associated with degeneration in the eye of the fly, a shortened life span and progressive loss of motor functions (Couthouis J. , et al., 2012). While these observations provided a new insight regarding EWS properties in ALS, the role of the protein in the pathogenesis of ALS awaits further investigation.

1.2.3.4 Rho Guanine Nucleotide Exchange Factor (RGNEF)

RGNEF is a 190 kDa RNA-binding protein, part of the diffuse B-cell lymphoma (Dbl) family of guanine exchange factors (GEFs). Its murine homologue was first cloned from the mouse brain cDNA library as p190rhoGEF (murine isoform). The activation of Rho GTPases leads to the activation of various cellular responses including cell adhesion, motility, transcription activation and cell cycle progression (Mackay & Hall, 1998). p190RhoGEF is a specific activator of RhoA that can interact with microtubules through its carboxy-terminal region (van Horck, Ahmadian, Haeusler, Moolenaar, & Kranenburg, 2001). In addition to its microtubule domain located within the carboxy-terminal end, RGNEF also has a focal adhesion kinase (FAK) domain. Located in a coil-coiled domain, it facilitates the direct interaction with FAK (Zhai, et al., 2003); which is in turn involved in cell adhesion to the extracellular matrix (ECM) (Kornberg, Earp, Parsons, Schaller, & Juliano, 1992). However, a dysregulation of these cell-matrix contact sites leads to a dysregulation in cell migration resulting in cancer cell growth and proliferation (Guan & Shalloway, 1992).

RGNEF has five important domains: a leucine-rich domain (LeuR) and a cysteine-rich Zn-binding domain in the amino terminal half of the protein; a Dbl homology domain (DH), a Pleckstrin homology domain (PH) and an RNA-binding domain in the carboxy-half of the protein (Cañete-Soler, Wu, Zhai, Shamim, & Schlaepfer, 2001) (Droppelmann, Campos-Melo, Volkening, & Strong, 2014) (Cheung, et al., 2017).

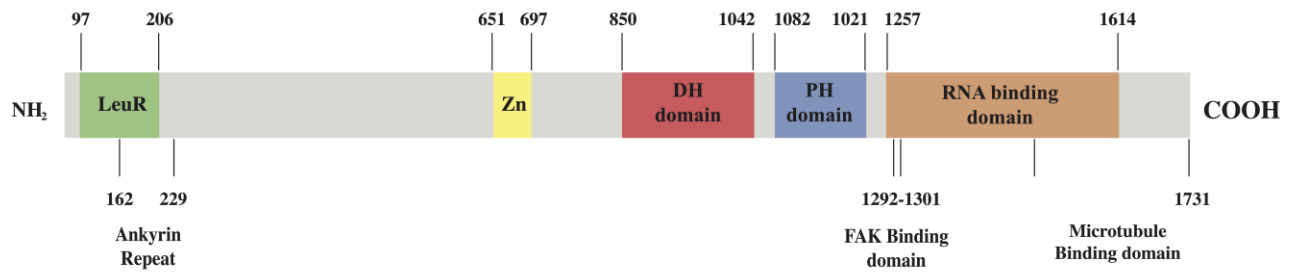


Figure 1. Structure of full length RGNEF. RGNEF is 1731 amino acid protein with eight putative domains: a leucine-rich domain (LeuR), an Ankyrin repeat domain overlapping with LeuR and a cysteine-rich Zn-binding domain in the amino terminal half of the protein ; a Dbl homology domain (DH), a Pleckstrin homology domain (PH), an RNA-binding domain, a FAK binding domain overlapping with the RBD and a microtubule binding domain overlapping with the RBD and extending to the amino acid 1731 in the carboxy-half of the protein (Droppelmann, Campos-Melo, Volkening, & Strong, 2014).

RGNEF is the only known RNA-binding protein to be also identified as a GEF protein with the capacity to activate RhoA. Our lab and others have observed mutations in the gene encoding RGNEF, *ARHGEF28*, in both sALS and fALS (Droppelmann, et al., 2013) (Song, et al., 2020). These mutations are predicted to generate either a frameshift mutation or a splicing mutation resulting in a truncated RGNEF of 319 or 259 amino acids (Fig. 2). The single nucleotide deletion detected results in the generation of a massively smaller protein compared to the normal 1731 amino acid RGNEF (Droppelmann, et al., 2013).

Schlaepfer and colleagues have demonstrated the critical role of RGNEF in the spread of colon carcinoma and fibroblast cell motility (Miller, Lawson, Chen, Ssang-Taek, & Schlaepfer, 2012). Their results suggest that RGNEF has a scaffolding role in the localization and activation of FAK (Miller, et al., 2013) in addition to its GEF activity regulating RhoA in control of cell migration (Miller, Lawson, Chen, Ssang-Taek, & Schlaepfer, 2012).

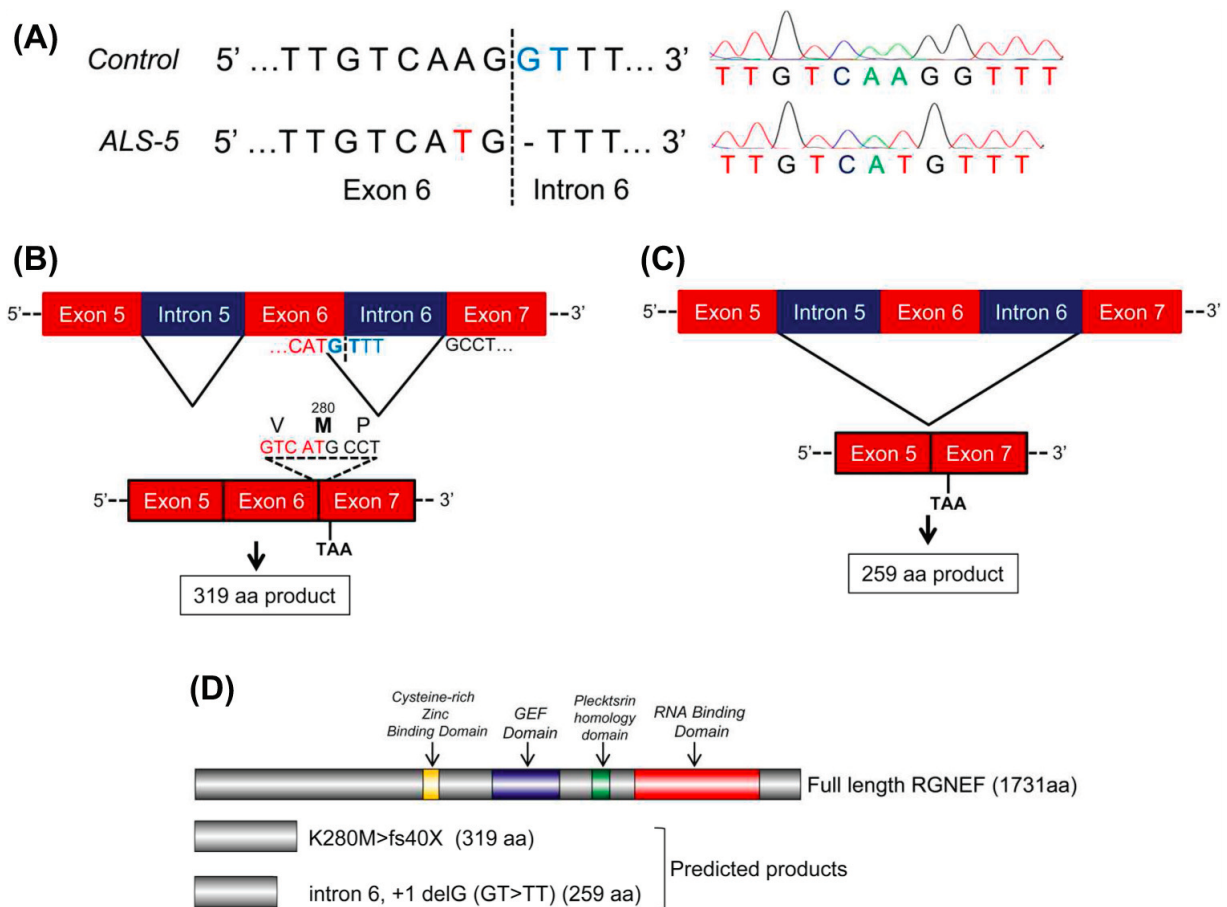


Figure 2. Frameshift mutation in RGNEF in a case of fALS. (A) Sequence profile showing the mutation and the single nucleotide deletion at the exon-intron 6 boundary found in ALS-5. These mutations are predicted to generate a frameshift mutation with premature truncation, namely K280M>fs40X (B) or a splicing mutation at the exon 6/intron 6 splice junction, namely intron 6, >1 delG (GT>TT) (C), which would cause exon 6 skipping. (D) Predicted protein products of frameshift mutation compared with full length RGNEF (Droppelmann, et al., 2013).

Under normal physiological conditions, RGNEF is mainly localized in the cytoplasm with moderate nuclear levels of expression (Droppelmann, Keller, Campos-Melo, Volkening, & Strong, 2013). RGNEF forms pathological NCIs in motor neurons in both sporadic and familial ALS forms (Keller, et al., 2012) (Droppelmann, Keller, Campos-Melo, Volkening, & Strong, 2013). Further RGNEF co-localizes with the RNA-binding proteins TDP-43 and FUS within motor neuron NCIs in ALS. We have also observed an interaction between full length RGNEF and TDP-43, further supporting the pathogenic role of RGNEF in ALS (Keller, et al., 2012).

In a murine model of neuronal stress (proximal sciatic axotomy), RGNEF is significantly upregulated, suggesting it acts as stress and survival factor (Cheung, et al., 2017). This was confirmed *in vitro* in HEK 293T cells in which RGNEF increased cell survival following exposure to oxidative or osmotic stress (Cheung, et al., 2017). In the same study, we observed the colocalization of RGNEF with Staufen-1 in SGs. The murine homologue of RGNEF, p190RhoGEF has been observed to interact with a destabilizing region in the 3' untranslated region (UTR) domain of murine *NEFL* mRNA in order to stabilize its transcript (Cañete-Soler, Wu, Zhai, Shamim, & Schlaepfer, 2001). Further studies looking into the aggregation process of NFL protein, showed that in an established cellular model, Neuro2a cells, p190RhoGEF co-localized with NFL protein aggregates and not with NFL subunit. Additionally, NFL protein aggregation was associated with the co-aggregation of p190RhoGEF in motor neurons in a transgenic mouse model of motor neuron disease (Lin, Zhai, & Schlaepfer, 2005). In our lab, we have shown that the RNA-binding domain of RGNEF, interacts with *NEFL* mRNA (Volkening, Leystra-Lantz, & Strong, 2010). However, in contrast to the stabilizing effect of p190RhoGEF on murine *NEFL* mRNA, RGNEF destabilizes human *NEFL* mRNA via its 3'UTR (Droppelmann et al. 2013)

Recently, we have examined the formation of micronuclei in ALS in response to stress (Droppelmann, Campos-Melo, Moszczynski, Amzil, & Strong, 2019). Despite the limited studies on micronuclei in neurodegeneration and ALS in particular, it has previously been noted that these structures are present in peripheral blood lymphocytes of Alzheimer's disease (AD) and Parkinson's disease (PD) patients (Migliore, Cappodè, Fenech, & Thomas, 2011). Using lactate stress-induced HEK 293T cells, we observed the formation of micronuclei, containing both TDP-43 and LeuR, the latter being a protein containing leucine repeats within the first 242 amino acids of RGNEF. In addition, we have observed that TDP-43 and LeuR co-immunoprecipitate in

a high molecular weight complex. These observations suggest a role for the LeuR in the co-aggregation of RGNEF and TDP-43 (Droppelmann, Campos-Melo, Moszczynski, Amzil, & Strong, 2019). The presence of micronuclei-like structures was also confirmed in the hippocampus and spinal cord of ALS patients with TDP-43 pathology.

1.3 Leucine-Rich Repeat protein family

Leucine Rich Repeats (LRR) are present in many proteins in humans and are one of the most conserved domain repeats across species (Matsushima, et al., 2007). Early studies looking into the structure of the porcine ribonuclease inhibitor enabled researchers to model other LRR proteins and their ligand complexes (Kobe & Deisenhofer, 1993). LRR are generally composed of 20 to 29 residues repeat in tandem containing a conserved 11-residue segment with a consensus sequence LxxLxLxxNxL (L= leucine; x= any amino acid; N= asparagine, cysteine, threonine or serine). The repeated tandems are grouped into highly conserved segments (HCS) and variable segments (VS) and are known to form a continuous superhelix (Kobe & Kajava, 2000) (Table 2). They have been shown to play a critical role in protein-protein interaction and other cellular processes such as neural development and modulation of different growth factor pathways (Ghiglione, et al., 1999).

Protein-protein interactions are involved in most biological processes of a cell in both normal and pathological conditions. Given the role of protein misfolding, aggregation and degradation in neurodegenerative disease, researchers are using interaction proteomics in order to establish the interacting partners of established neurodegenerative disorders-associated proteins, and by identifying binding partners, determine their potential functions (Hosp, et al., 2015).

The amino-terminal region of RGNEF contains an atypical leucine rich motif located between the amino acids 97 and 206 (Table 2). While the structure of full length RGNEF is still not known, a software analysis of the LeuR²⁴² sequence predicts the presence of alpha helices (Fig. 3) comparable to the structure of other leucine rich repeats in the literature (Dolan, et al., 2007).

Table 2. Identified subfamilies of Leucine Rich Repeat sequences. LRR conserved consensus sequences by Kobe and Kajava (Kobe & Deisenhofer, 1993) (Kobe & Kajava, 2000) and an identified atypical LeuR sequence of RGNEF.

Subfamily	Conserved consensus sequence
Ribonuclease inhibitor	LxxLxLxxN/CxL
SD22-like	LxxLxLxxNxL
Cys-containing	LxxLxLxxcx-x
Bacterial	LxxLxVxxNxL
Plant specific	LxxLxLxxNxL
Typical	LxxLxLxxNxL
TpLRR	LxxlxLx-xxLx
Atypical LeuR	LxxLLxxxxNxL

Abbreviations:SD22= G-type lectin S-receptor-like serine/threonine-protein kinase; Cys= cysteine; TpLRR= T. pallidum leucine-rich repeat; L= leucine; x= any residue; N= asparagine; - = possible insertion site.



Figure 3. Prediction of secondary structure of the LeuR domain of RGNEF. The model was created using I-Tasser (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>) based on the sequence of LeuR²⁴² amino acids 1 to 242. The modelling shows the presence of nine α -helices ranging from amino acids 95 through 220 in the middle of the leucine-rich domain of RGNEF. Pink indicates α -helix structure; yellow indicates β -unit structure.

Our group has previously demonstrated that when the full-length protein was truncated into various domains, the Leucine-Rich (LeuR) region proved to be critical for its cytoprotective effect under osmotic stress conditions (Cheung, et al., 2017). In addition, under stress, RGNEF co-localizes with Stauf-1 positive granules. Moreover, in unpublished observations, we have found that LeuR confers a protective effect *in vivo* against the toxicity of TDP-43 in a double transgenic LeuR;TDP-43 *drosophila melanogaster* model, improving the flies' lifespan, motor functions and the eye degeneration observed in single transgenic flies TDP-43 (Droppelmann, unpublished results).

1.4 Rationale and hypothesis

ALS is a progressive, adult-onset disease characterized by the degeneration of motor neurons. The presence of neuronal cytoplasmic inclusions (NCIs) composed of RNA-binding proteins, neurofilaments and other proteins, is considered to be the disease's neuropathological hallmark. Amongst ALS-associated proteins, RGNEF has been identified as a novel protein involved in the pathogenesis of ALS as it has been observed to co-aggregate with RNA-binding proteins TDP-43 and FUS within motor neuron NCIs in ALS. RGNEF is a 190 kDa RNA-binding protein with five important domains including a leucine-rich region (LeuR) domain in the amino terminal half of the protein. Given previous data demonstrating the interaction of RGNEF and the LeuR domain of RGNEF with TDP-43 and considering that RGNEF has been observed to regulate the levels of *NEFL* mRNA and NFL protein *in vitro*, **I hypothesize that there is a functional and biochemical interplay between RGNEF and TDP-43 in which RGNEF can regulate the levels of TDP-43 and where the interaction between these two proteins is regulated by a minimal domain contained within the LeuR region of RGNEF.**

1.5 Significance and impact of the research

This work will help elucidate the role of RGNEF in regulating the ALS-related protein TDP-43 and how a small domain contained in the LeuR region of RGNEF is critical for the physical interaction between RGNEF and TDP-43. Since the fly model used in this study shows a protective effect of LeuR over the toxic phenotype induced by TDP-43 overexpression, the data generated from immunostainings of brain and eye tissue from the flies will help us understand

how aggregates containing LeuR and TDP-43 could be a possible mechanism of protection against TDP-43 toxicity. By defining the role of the minimal domain of interaction between two ALS-associated RBPs, RGNEF and TDP-43, we can get a step further into identifying a potential therapeutic target in ALS.

Chapter 2

2.1 Specific aims

2.1.1 Aim 1: To determine the minimal domain of interaction between LeuR²⁴² and TDP-43

The first aim was to determine the minimal domain of interaction between the LeuR²⁴² region (Fig. 4) and TDP-43. The predictive structure of full length RGNEF suggests the presence of an atypical LeuR motif in the amino-terminal region of the protein. Given the role of the Leucine-Rich repeat proteins in protein-protein interaction, I hypothesized that within LeuR²⁴², a smaller fragment is required for the interaction with TDP-43. Previous results from a crosslink immunoprecipitation showed the presence of an interaction between LeuR²⁴² and TDP-43 in a high molecular weight complex (Droppelmann, Campos-Melo, Moszczynski, Amzil, & Strong, 2019). Since the LeuR²⁴² construct generated in our lab contains the leucine-rich motif, from amino acids 97 to 206, and sequences of RGNEF on both its amino and carboxy-terminal ends, we generated various constructs of LeuR²⁴² in order to determine the minimal domain critical for the protein-protein interaction observed.

2.1.2 Aim 2: To study the regulation of ALS-related protein TDP-43 by full length RGNEF and different RGNEF constructs (Δ LeuR and Δ COOH) designed to elucidate the role of LeuR²⁴²

The second aim was to study the regulation of ALS-related proteins by full length RGNEF and a variety of RGNEF constructs (Δ LeuR and Δ RBD) designed to elucidate the role of RGNEF. Given that RGNEF is an RNA-binding protein and given the role of these proteins on ALS-related proteins, such as TDP-43, I hypothesized that the amino-terminal region of RGNEF contains a domain responsible for the regulatory effect observed over TDP-43 *in vitro*. Considering that RGNEF has been observed to regulate the levels of *NEFL* mRNA and NFL protein, I investigated the regulatory effect of RGNEF over the mRNA stability and protein levels of TDP-43.

2.1.3 Aim 3: To determine the interaction between TDP-43 and LeuR²⁴² *in vivo* in a *drosophila melanogaster* model of TDP-43 toxicity.

The third aim was to determine how the LeuR²⁴² region modifies TDP-43 pathology *in vivo*. Preliminary experiments conducted in our lab have demonstrated that both RGNEF and LeuR²⁴² are critical modulators of the toxicity of wild-type TDP-43 *drosophila melanogaster* models with a significant protective effect on both survival and motor functions. To better understand the protective role of the LeuR²⁴² region against the TDP-43 toxicity in the flies, I analyzed the pathology of TDP-43 toxicity in the absence or presence of LeuR²⁴² using IHC on the brain and eye tissue of Elav-TDP-43 flies and Elav-LeuR²⁴²;TDP-43 flies.

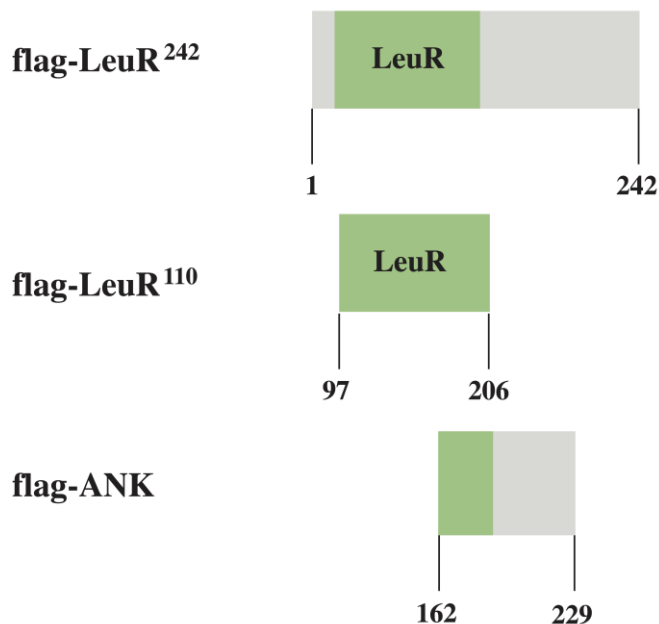


Figure 4. Schematic showing the LeuR region flag-tagged constructs used to study the minimal domain of interaction with TDP-43. flag-LeuR²⁴²: previously characterized in the interacting complex with TDP-43 and the LeuR region flag-tagged constructs used to study the minimal domain of interaction with TDP-43; flag-LeuR¹¹⁰: containing only the LRR (amino acids 97-206), flag-ANK: containing only the ANK repeat, including a small fragment of the C-terminal LRR (amino acids 162-229).

2.2 Methods

2.2.1 Constructs elaboration

The constructs, LeuR¹¹⁰ and ANK domains, were generated by PCR amplification using Phusion polymerase (Invitrogen, ThermoFisher Scientific) and primers specifically designed for each construct (Table 3). All the constructs created contain a flag-tag sequence on their amino-terminal region, which allows the detection of the proteins expressed using an anti-flag antibody. The PCR products used as insert were run on a 1% agarose gel at 90V for 60 minutes, purified by gel extraction using a NucleoSpin Gel Extraction Kit (Macharey-Nagel Inc) and quantified using a Nanodrop spectrophotometer (ThermoFisher Scientific). Each fragment insert was then inserted into a pcDNA3.0 vector using the restriction enzymes KpnI and XhoI, after a 15 minutes digestion at 37°C (Anza system, Invitrogen, ThermoFisher Scientific). The digested plasmids and inserts were then ligated overnight at room temperature using T4 DNA ligase (Promega) at 3:1 molar ratio of insert:vector, and transformed afterwards using chemically competent cells DH5α *E. Coli* per manufacturer's protocol (Invitrogen, ThermoFisher Scientific). The transformation was then plated onto a selective agar plate containing 100µg/ml ampicillin and incubated overnight at 37°C. The obtained colonies were picked from the plate and grown in LB broth containing 100µg/ml ampicillin overnight at 37°C with shaking at 225rpm. The next day, DNA plasmids of each construct were obtained using PureLink Quick Plasmid Miniprep Kit per manufacturer's protocol (Invitrogen, ThermoFisher Scientific). The purified plasmids were first screened through a 15minute enzymatic digestion at 37°C using Anza KpnI and XhoI, following which they were run on a 1% agarose gel for 60 minutes at 90V and compared to a control plasmid. 1Kb Plus molecular ruler (Bio-Rad) was used to determine the size of the plasmids on the agarose gel. Afterwards, the plasmids observed at the expected size were sent for sequencing (DNA Sequencing Facility - Robarts Research Institute) for confirmation before transiently transfecting HEK 293T cells.

Table 3. Primers used in PCR amplification to generate different constructs of LeuR

Name	Forward or Reverse primer	DNA sequence (5' - 3')
pcDNA3.0_flag-LeuR ¹¹⁰	Forward	<u>GTAGGTACCC</u> ACCATGGACTACAAGGACGAC GATGACAAGCTGGCTCGTCTGCTGGTGAC
pcDNA3.0_LeuR ¹¹⁰	Reverse	ACA <u>CTCGAG</u> TTATAAAGCTAAGTCTAATGGT GTGGCAC
pcDNA3.0_flag-ANK	Forward	<u>GTAGGTACCC</u> ACCATGGACTACAAGGACGAC GATGACAAGCACAGAGAATCTCTTCTACAC
pcDNA3.0_ANK	Reverse	ACA <u>CTCGAG</u> TTAGAAGCTTGGGGACCATCTG CCCTGAA

*Restriction site sequences are underlined: KpnI = GGTACC; XhoI = CTCGAG

2.2.2 Cell culture

HEK 293T cells were used for all *in vitro* experiments because of their amenability to transfection, their neuronal-like properties (Shaw, Morse, Arabat, & Graham, 2002) and their endogenous expression of TDP-43. They were maintained in Dulbecco's modified eagle's medium (Gibco, ThermoFisher Scientific) supplemented with 10% fetal bovine serum and kept in a water-jacketed incubator at 37°C, 5%CO₂ (ThermoFisher Scientific).

2.2.3 Transfections

For the *in vitro* experiments in aim 1 looking to determine the minimal domain of interaction of LeuR with TDP-43, the cells were transiently transfected with flag-tagged plasmid constructs of LeuR inserted in the pcDNA3.0 backbone vector or TDP-43 plasmid inserted in a pcDNA3.1(+)/myc-His, each validated through sequencing (DNA Sequencing Facility - Robarts Research Institute) and purified using the PureLink Midiprep kit according the manufacturer's protocol (Invitrogen, ThermoFisher Scientific). For the crosslink experiments of aim 1, the cells were seeded in 10cm dishes at 1,000,000 cells/dish and the transient transfections was performed using lipofectamine 2000 per the manufacturer's protocol (ThermoFisher Scientific). The cells were left to grow for 48h to attain a 70% confluency before transfection. Cells were transfected with 15µg of DNA at a 2.5µL lipofectamine 2000 per 1µg DNA ratio and per manufacturer's protocol, cells were incubated with the complex lipofectamine 2000 -DNA in OPTIMEM media (Invitrogen, ThermoFisher Scientific) for 3h. Afterwards, the cells were incubated in DMEM complete media for 36h for recovery before protein lysis.

For the *in vitro* experiments in aim 2 looking to study the regulation of endogenous TDP-43, HEK 293T cells were transiently transfected with RGNEF constructs previously generated in our lab by C. Droppelmann (Cheung, et al., 2017). The following constructs, inserted in a pcDNA3.1(+)/myc-His backbone vector, were used: full length RGNEF, RGNEF-ΔLeuR; and RGNEF-ΔCOOH (Fig. 5). HEK 293T cells were seeded in 6-well plates (200,000 cells per well) or 96-well plates (9,000 cells per well) and left to grow for 48 hours and 24 hours, respectively, until they reach 70% confluency. Afterwards, cells were transfected using lipofectamine 2000 with 2.5µg of plasmid DNA in 6-well plates or 120ng of plasmid DNA in 96-well plates, at a 2.5µL lipofectamine per 1µg DNA ratio and per manufacturer's protocol (Invitrogen, ThermoFisher Scientific). Figure 6 illustrates the steps followed for each transfection.

(A)

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RGNEF FL 1 MELSCSEAPLYGQMMIYAKFDKNVYLPEDAIFYTYDGSQHRVMIARIEDNVLQSSVPGHGLQETVTVSVCLCSEGYSPVTMGSGSVTVVDNMACRLARLLVTPQANRLTACSHQTLT
RΔLeuR 1 -----
RΔCOOH 1 MELSCSEAPLYGQMMIYAKFDKNVYLPEDAIFYTYDGSQHRVMIARIEDNVLQSSVPGHGLQETVTVSVCLCSEGYSPVTMGSGSVTVVDNMACRLARLLVTPQANRLTACSHQTLT

RGNEF FL 121 PFALTAGALPALDEELVLALTHLELPLEWTVLGGSSSLEVVSHRESLLHLAMRWGLAKLSQFFLCLPGVQALALPNEEGATPLDLALREGHSKLVEDVTNFQGRWSPSFSRVQLSEASL
RΔLeuR 121 -----
RΔCOOH 121 PFALTAGALPALDEELVLALTHLELPLEWTVLGGSSSLEVVSHRESLLHLAMRWGLAKLSQFFLCLPGVQALALPNEEGATPLDLALREGHSKLVEDVTNFQGRWSPSFSRVQLSEASL

RGNEF FL 241 HYIHSSETLTLTLNHTAEHLEADIKLFRKYFWDRAFLVKAFAQEARPEERTAMPSSGAETEEIKNVSSRSAAEKEDIKRVKSLVVQHNEHEDQHSLLDLDLDRSFDILKKSPPSTLLAA
RΔLeuR 241 --IHSETLTLTLNHTAEHLEADIKLFRKYFWDRAFLVKAFAQEARPEERTAMPSSGAETEEIKNVSSRSAAEKEDIKRVKSLVVQHNEHEDQHSLLDLDLDRSFDILKKSPPSTLLAA
RΔCOOH 241 HYIHSSETLTLTLNHTAEHLEADIKLFRKYFWDRAFLVKAFAQEARPEERTAMPSSGAETEEIKNVSSRSAAEKEDIKRVKSLVVQHNEHEDQHSLLDLDLDRSFDILKKSPPSTLLAA

RGNEF FL 361 GRLSMDLNGGDEVYANCVIDQVGLDLSYINIEGITATTSPESRGCTLWVQSSKHTLPTTETSPSVYPLSENVEGTAHTAQQSPMSPSSCASNLNLSFGWHGFEKEQSHLKKRSSSLD
RΔLeuR 361 GRLSMDLNGGDEVYANCVIDQVGLDLSYINIEGITATTSPESRGCTLWVQSSKHTLPTTETSPSVYPLSENVEGTAHTAQQSPMSPSSCASNLNLSFGWHGFEKEQSHLKKRSSSLD
RΔCOOH 361 GRLSMDLNGGDEVYANCVIDQVGLDLSYINIEGITATTSPESRGCTLWVQSSKHTLPTTETSPSVYPLSENVEGTAHTAQQSPMSPSSCASNLNLSFGWHGFEKEQSHLKKRSSSLD

RGNEF FL 481 ALDADSEGEHSEPHSHICYTPGSQSSSRTGIPSGDELDSFETNTEPDFNISRAESLPLSSNLQSKESLHSGVRSRSYSCSSPKISLGTKRLVRELTVCSSESSEQRAYSLSPEPPENRIQE
RΔLeuR 481 ALDADSEGEHSEPHSHICYTPGSQSSSRTGIPSGDELDSFETNTEPDFNISRAESLPLSSNLQSKESLHSGVRSRSYSCSSPKISLGTKRLVRELTVCSSESSEQRAYSLSPEPPENRIQE
RΔCOOH 481 ALDADSEGEHSEPHSHICYTPGSQSSSRTGIPSGDELDSFETNTEPDFNISRAESLPLSSNLQSKESLHSGVRSRSYSCSSPKISLGTKRLVRELTVCSSESSEQRAYSLSPEPPENRIQE

RGNEF FL 601 EEWKDYIIPAKSESEKYKVSRTFSLMNRMTSPRNKSKTKSKDAKDKEKLNRRHQFAPGTFSGVQLVCLVCKDTLLGKESLQCSNCNANVHGCKDAAPACTKFKFQEKYKNKPKQTLILGNSS
RΔLeuR 601 EEWKDYIIPAKSESEKYKVSRTFSLMNRMTSPRNKSKTKSKDAKDKEKLNRRHQFAPGTFSGVQLVCLVCKDTLLGKESLQCSNCNANVHGCKDAAPACTKFKFQEKYKNKPKQTLILGNSS
RΔCOOH 601 EEWKDYIIPAKSESEKYKVSRTFSLMNRMTSPRNKSKTKSKDAKDKEKLNRRHQFAPGTFSGVQLVCLVCKDTLLGKESLQCSNCNANVHGCKDAAPACTKFKFQEKYKNKPKQTLILGNSS

RGNEF FL 721 FRDIPQGLSLHPSSSVVGLPTGRRETGVQVHPLRSRVPGTTLSEFRRSATSLESESDHNSCRSRSHSDELLQSMGSSPSTESFIMEDVVDSSLWSLSDSDAQEFEAESWSLVVDPFSC
RΔLeuR 721 FRDIPQGLSLHPSSSVVGLPTGRRETGVQVHPLRSRVPGTTLSEFRRSATSLESESDHNSCRSRSHSDELLQSMGSSPSTESFIMEDVVDSSLWSLSDSDAQEFEAESWSLVVDPFSC
RΔCOOH 721 FRDIPQGLSLHPSSSVVGLPTGRRETGVQVHPLRSRVPGTTLSEFRRSATSLESESDHNSCRSRSHSDELLQSMGSSPSTESFIMEDVVDSSLWSLSDSDAQEFEAESWSLVVDPFSC

RGNEF FL 841 NRQEKDVIKRQDVI FELMQTEMHHIQTFLIMSEIFRKGMEELQLDHSITVDKIFPCLELLEIHRHFFYSMKERRQESCAGSDRNFVIDRIGDILVQQFSEENASKMKKIYGEFCCHHKE
RΔLeuR 841 NRQEKDVIKRQDVI FELMQTEMHHIQTFLIMSEIFRKGMEELQLDHSITVDKIFPCLELLEIHRHFFYSMKERRQESCAGSDRNFVIDRIGDILVQQFSEENASKMKKIYGEFCCHHKE
RΔCOOH 841 NRQEKDVIKRQDVI FELMQTEMHHIQTFLIMSEIFRKGMEELQLDHSITVDKIFPCLELLEIHRHFFYSMKERRQESCAGSDRNFVIDRIGDILVQQFSEENASKMKKIYGEFCCHHKE

RGNEF FL 961 AVNLFKELQQKKFQNFIKLRNSNLLARRRGIPECILLVTRITKYVPLVERILQYTKERTEEHKDLRKLALCLIKDMIATVDLKVNEYKNQKWLIEILNKIENKTYTKLNGHVFRKQAL
RΔLeuR 961 AVNLFKELQQKKFQNFIKLRNSNLLARRRGIPECILLVTRITKYVPLVERILQYTKERTEEHKDLRKLALCLIKDMIATVDLKVNEYKNQKWLIEILNKIENKTYTKLNGHVFRKQAL
RΔCOOH 961 AVNLFKELQQKKFQNFIKLRNSNLLARRRGIPECILLVTRITKYVPLVERILQYTKERTEEHKDLRKLALCLIKDMIATVDLKVNEYKNQKWLIEILNKIENKTYTKLNGHVFRKQAL

RGNEF FL 1081 MSEERTLLYDGLVYWKATGRFKDILALLLTDVLLFLQEKDQYIFAADVQKPSVISLQKLIAREVANEERGMFLISASSAGPEMYEIHNTNSKEERNWMMRRIQAVESCEPEKGGRTSE
RΔLeuR 1081 MSEERTLLYDGLVYWKATGRFKDILALLLTDVLLFLQEKDQYIFAADVQKPSVISLQKLIAREVANEERGMFLISASSAGPEMYEIHNTNSKEERNWMMRRIQAVESCEPEKGGRTSE
RΔCOOH 1081 MSEERTLLYDGLVYWKATGRFKDILALLLTDVLLFLQEKDQYIFAADVQKPSVISLQKLIAREVANEERGMFLISASSAGPEMYEIHNTNSKEERNWMMRRIQAVESCEPEKGGRTSE

RGNEF FL 1201 SDEDKRKAERVAKIQQCEILTNDQDQICAYLEEKLIHYAELGELSGFEDVHLEPHLLLIKPPDPEPPQAASLLAALKEAESLQVAVKASQMGAVSQSCEDSCGDSVLADTLSSHVDVPG
RΔLeuR 1201 SDEDKRKAERVAKIQQCEILTNDQDQICAYLEEKLIHYAELGELSGFEDVHLEPHLLLIKPPDPEPPQAASLLAALKEAESLQVAVKASQMGAVSQSCEDSCGDSVLADTLSSHVDVPG
RΔCOOH 1201 SDEDKRKAERVAKIQQCEILTNDQDQICAYLEEKLIHYAELGELSGFEDVHLEPHLLLIKPPDPEPPQAASLLAALKEAESLQVAVKASQMGAVSQSCEDSCGDSVLADTLSSHVDVPG

RGNEF FL 1321 SPTASLVTGGREGRCSDVDPGIQGVVTDLAVSDAGEKVECRNFGSSQSEIIQAIQNLRLLYSLQAALTIQDSHIEIHRVLVQQQEGLSLGHSLRGGPLQDQKSRDADRQHEELANV
RΔLeuR 1321 SPTASLVTGGREGRCSDVDPGIQGVVTDLAVSDAGEKVECRNFGSSQSEIIQAIQNLRLLYSLQAALTIQDSHIEIHRVLVQQQEGLSLGHSLRGGPLQDQKSRDADRQHEELANV
RΔCOOH 1321 -----

RGNEF FL 1441 HQLQHQLQOEQRWRRLRRCCEQQRAQATRESWLQERERECQSEELLLRSRGELDQLQEQYHSLERLREGQRLVEREQARMRAQQSLLGHWKHGRQRLSPAVLLPGGPEVMELNRSSELC
RΔLeuR 1441 HQLQHQLQOEQRWRRLRRCCEQQRAQATRESWLQERERECQSEELLLRSRGELDQLQEQYHSLERLREGQRLVEREQARMRAQQSLLGHWKHGRQRLSPAVLLPGGPEVMELNRSSELC
RΔCOOH 1441 -----

RGNEF FL 1561 HENSFFINEALVQMSFNTFNKLNPSVIHQDATYPTTQSHSDLVRTSEHQVDLKVDPSPQSNVSHKLWTAAGSGHQILPFHESKDKSCKNGSSMTKCSCTLTSPPGLWTGTTSTLKDLDTS
RΔLeuR 1561 HENSFFINEALVQMSFNTFNKLNPSVIHQDATYPTTQSHSDLVRTSEHQVDLKVDPSPQSNVSHKLWTAAGSGHQILPFHESKDKSCKNGSSMTKCSCTLTSPPGLWTGTTSTLKDLDTS
RΔCOOH 1561 -----

RGNEF FL 1681 HTESPPTHSDNSHRPQLQAFITEAKLNLPTRTMTMQDGETGDGAKENIVYL
RΔLeuR 1681 HTESPPTHSDNSHRPQLQAFITEAKLNLPTRTMTMQDGETGDGAKENIVYL
RΔCOOH 1681 -----
```

(B)



Figure 5. RGNEF constructs used to study the effect of its putative regions on the endogenous expression of TDP-43 *in vitro*. (A) Aligned protein sequences of RGNEF constructs full length RGNEF, RΔLeuR and RΔCOOH: --- indicates deleted amino acids in RΔLeuR and RΔCOOH; green indicated the leucine rich domain; yellow indicates the cysteine-

rich Zn-binding domain; red indicates the Dbl homology domain (DH); blue indicates the Pleckstrin homology domain (PH) and orange indicates the RNA-binding domain. (B) Scheme of the RGNEF constructs used in transiently transfected HEK 293T cells to determine the regions of RGNEF involved in the regulation of endogenous TDP-43.

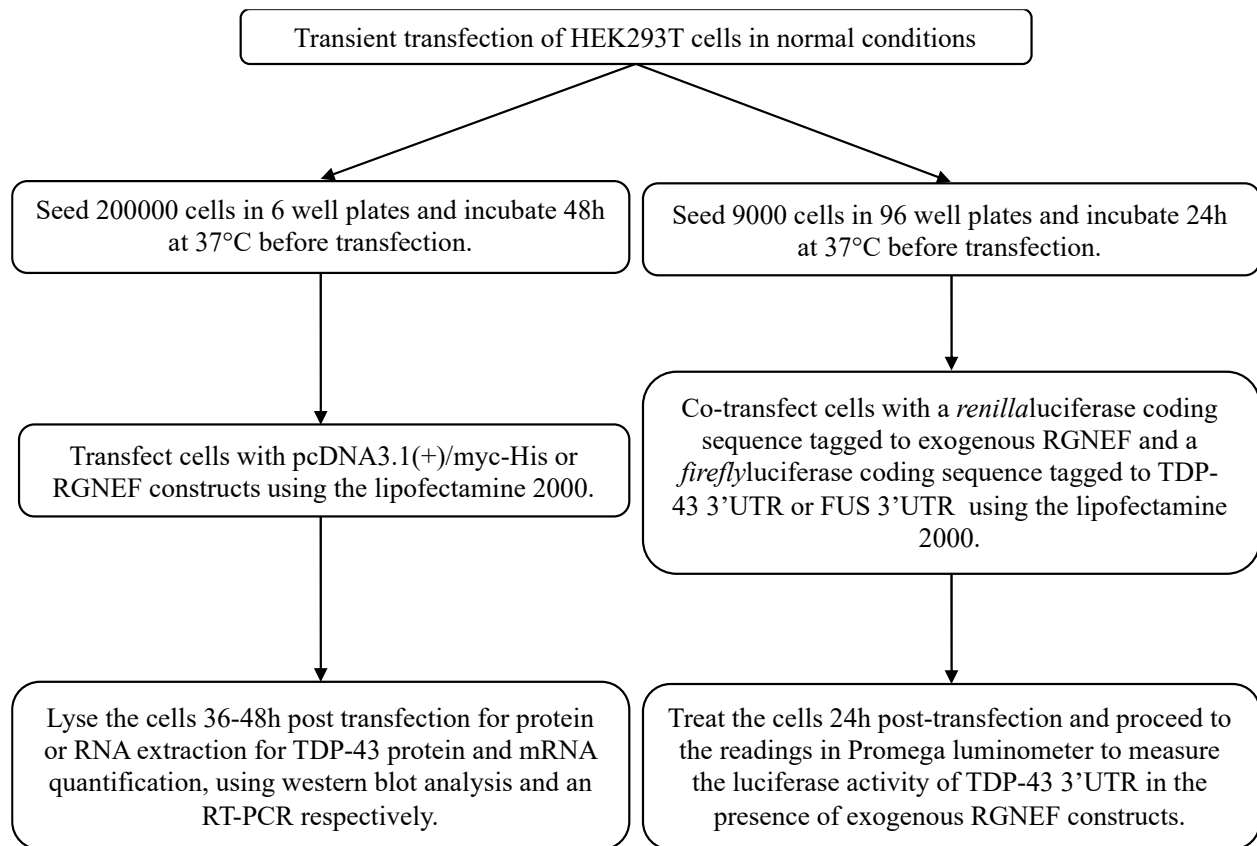


Figure 6. Flow chart describing the transfection conditions in HEK 293T cells used to study the regulation of TDP-43.

2.2.4 Dual-luciferase reporter assay

To determine the role of RGNEF in the regulation of *TARDP* 3'UTR mRNA, a dual-luciferase reporter assay was used on transiently co-transfected HEK 293T cells. The reporter plasmids, previously generated in our lab by D. Campos-Melo, were constructed by inserting *TARDBP* mRNA 3'UTR, or *FUS* mRNA 3'UTR in the pmirGLO vector (Promega) downstream of the *firefly* luciferase gene. HEK 293T cells were co-transfected with 2.83fmol of RGNEF-myc or pcDNA3.1 and 3.47fmol of pmirGLO-*TARDBP* 3'UTR; pmirGLO-*FUS* 3'UTR or pmirGLO using lipofectamine 2000 (Invitrogen, ThermoFisher Scientific) per the manufacturer's instructions. The total amount of DNA transfected was maintained at 120ng per well by adding an empty vector. Each condition was replicated in six wells and the mean luciferase activity was used. The experiment was performed in triplicate.

24 hours post-transfection, cells were treated with a luciferase assay buffer (Dual-Luciferase Reporter Assay, Promega) and the *firefly* luciferase activity was measured using a Luminometer (Turner Biosystems Luminometer, Promega) according to the manufacturer's protocol, following which the cells were treated with Stop & Glo reagent (Dual-Luciferase Reporter Assay, Promega) and the *Renilla* luciferase activity was measured using a Luminometer (Turner Biosystems Luminometer, Promega) according to the manufacturer's protocol. Using the normalization protocol previously reported from our lab (Campos-Melo, Droppelmann, Volkening, & Strong, 2014), the measured *firefly* luciferase activity was normalized to measured *Renilla* luciferase activity to account for the variations from transfection efficiency. The luciferase activity from RGNEF over *TARDBP* 3'UTR or *FUS* 3'UTR was normalized against the luciferase activity from RGNEF over mRNA without 3'UTR to determine the specific effect of RGNEF on the luciferase activity of *TARDBP* 3'UTR and *FUS* 3'UTR.

2.2.5 Protein lysates

Protein lysates from HEK 293T transfected cells were obtained after lysing cells on ice using the following lysis buffer: phosphate-buffered saline (PBS) pH 7.2 with 1% Nonidet P-40(NP-40) and cOmplete protease inhibitor cocktail (Roche). After the cells were washed with PBS pH 7.2, to each well and 10cm dish, 200µl and 1000µl of lysis buffer were added respectively and scraped using a cell scraper. The lysed cells were then sonicated to homogenize (Sonifier Cell Disruptor Vibracell, Sonics and Material Inc.), centrifuged at 10,000g, 4°C for 10 minutes to

pellet cell debris and the supernatant with the proteins was extracted and stored at -20°C. The quantification of the proteins in lysates were quantified using the Promega protein assay according to the manufacturer's protocol and the colorimetric analyses were measured using a Nanodrop (ThermoFisher Scientific) reading at 600nm.

2.2.6 Crosslinking experiment

To determine the minimal domain of interaction between LeuR²⁴² and TDP-43, the chemical crosslinker dithiobis(sulfosuccinimidylpropionate) (DTSSP) was used in the presence of protein lysates from HEK 293T transfected cells. A total of 500µg protein was used for the crosslinking, combining 250µg of protein lysate from TDP-43-myc HEK 293T transiently transfected cells and 250µg of protein lysate from flag-LeuR¹¹⁰ or flag-ANK HEK 293T transiently transfected cells. The lysates were incubated at room temperature for 15 minutes before adding 10mM of DTSSP for a final concentration of 1.2mM in a final volume of 500µl. The mixture was incubated at room temperature for 30 minutes and the crosslinking reaction was stopped afterward by adding 25µl of 1M Tris pH 7.5 for a final concentration of 50mM.

2.2.7 Immunoprecipitation

Immunoprecipitation was performed after the crosslinking using the Dynabeads Protein G IP Kit (Invitrogen, ThermoFisher Scientific). IP experiments were performed by incubating 50µl of protein G beads with 2µg of IP antibodies (Table 3) for 15 minutes on a rotator (Tube Revolver, ThermoFisher Scientific) at room temperature. Afterwards, the antibody-conjugated beads were washed and incubated with 500µg of previously crosslinked protein lysates for 15 minutes on a rotator (Tube Revolver, ThermoFisher Scientific) at room temperature. The magnetic beads-antigen-antibody complex were then washed according to the manufacturer's protocol (Dynabeads Protein G IP Kit, ThermoFisher Scientific), eluted in PBS pH 7.2 and loading buffer (0.32M Tris-HCl, 10% SDS, 50% glycerol, bromophenol blue). At this step, two different elution conditions were used: non-reducing conditions to detect the complex of interacting proteins and reducing conditions to detect the proteins after the complex was cleaved at the disulfide bond of DTSSP. In the case of non-reducing conditions, the beads were eluted in PBS with 5X loading buffer, while in reducing conditions the beads were eluted in PBS with 5X loading buffer and 0.68M β-mercaptoethanol. Samples were then heated at 94°C for 4 minutes and placed on a magnet to separate the beads-antigen-antibody complex.

2.2.8 Western blots

To complete the crosslink IP in aim 1, the supernatant containing the antigen-antibody complex was loaded onto a gradient sodium dodecyl(lauryl) sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4-20% Mini-PROTEAN TGX precast protein gels, Bio-Rad). To study the regulation of endogenous TDP-43 in the presence of various constructs of RGNEF, a total of 15µg of protein lysate in lysis buffer mixed with 5X loading buffer and 0.68M β-mercaptoethanol was loaded onto a 10% SDS-PAGE gel.

The gels were run on a constant of 100V for approximately 90 minutes, until the loading dye reached the bottom edge of the gel in a running buffer containing 50mM TRIS base, 200mM glycine, 2mM glycine in deionized water. Afterwards, the gels were transferred electrophoretically to a nitrocellulose membrane at a constant 300mA for 90 minutes. To ensure the transfer was successful, the nitrocellulose membranes were incubated with Ponceau-S red solution (0.1%(w/v) Ponceau S in 5%(v/v) acetic acid) for a few minutes after the transfer. Once confirmed, they were submerged in blotto solution (50mM TRIS base, 100mM NaCl, 1% Tween-20 pH 7.2), at 4°C overnight. The nitrocellulose membranes were first blocked using blocking solution (5% fat-free milk in blotto) for 60 minutes at room temperature on an orbital shaker (ThermoFisher Scientific), then primary antibodies were diluted in blocking solution afterwards, added to the membranes and incubated for 60 minutes at room temperature on an orbital shaker (ThermoFisher Scientific). Afterwards, the nitrocellulose membranes were washed three time in blocking solution 5 minutes each and then incubated with secondary antibodies diluted in blocking solution. The list and quantities of antibodies used are listed in Table 4. After secondary antibody incubation, the membranes were washed with blotto for 10 minutes each at room temperature on an orbital shaker (ThermoFisher Scientific). After the final wash, they were incubated in PBS pH 7.2 for 5 minutes at room temperature, and in Western Lightning ECL reagents (PerkinElmer) for 3 minutes at room temperature in order to generate a chemiluminescence signal. The signal was detected using ChemiDoc XRS+ system and visualized using Image Lab software (Bio-Rad).

Table 4. Antibodies used for western blot experiments

Primary antibody	Species	Dilution	Manufacturer	Secondary antibody
Anti-myc	Mouse	1:2500	Cederlane	Goat α -mouse, linked to horseradish peroxidase (titre: 1:3000) (Bio-Rad)
Anti-TDP-43	Rabbit	1:5000	ProteinTech	Goat α -rabbit, linked to horseradish peroxidase (titre: 1:5000) (Invitrogen, ThermoFisher Scientific)
Anti-flag	Rabbit	1:4000	ThermoFisher Scientific	Goat α -rabbit, linked to horseradish peroxidase (titre: 1:5000) (Invitrogen, ThermoFisher Scientific)
Anti-GAPDH	Rabbit	1:2500	Abcam	Goat α -rabbit, linked to horseradish peroxidase (titre: 1:5000) (Invitrogen, ThermoFisher Scientific)

2.2.9 RNA extraction

To study the regulation of *TARDBP* mRNA levels in the presence of the individual RGNEF constructs, RNA was extracted from transiently transfected HEK 293T cells in order to make cDNA and quantify *TARDBP* mRNA expression levels. After gently washing the cells with PBS, they were lysed by adding 400µl of Trizol (Ambion, ThermoFisher Scientific), homogenized and incubated for 5 minutes at room temperature in order to allow for complete disassociation of nucleoprotein complexes. Afterwards, 80µl of chloroform (ThermoFisher Scientific) was added to the homogenate, vigorously mixed, incubated for 2 minutes at room temperature and centrifuged for 15 minutes at 12,000g, 4°C. After separating the aqueous phase into a fresh tube, the RNA was precipitated using 200µl of isopropanol, incubated for 10 minutes at room temperature and centrifuged for 10 minutes at 12,000g, 4°C. The pelleted RNA was then washed with 400µl 75% ethanol and centrifuged for 5 minutes at 7,500g, 4°C. After discarding the supernatant, the pellet was dried at room temperature and resuspended in 20µl UltraPure RNase-free distilled water (Invitrogen, ThermoFisher Scientific). The resuspended RNA in the pellet was then incubated for 15 minutes at 60°C, quantified using a Nanodrop (Nanodrop 1000, ThermoFisher Scientific) and frozen at -80°C for storage.

2.2.10 Reverse transcription (RT)

The extracted RNA from HEK 293T transiently transfected cells was reverse transcribed using the SuperScript II Reverse Transcriptase system (Invitrogen, ThermoFisher Scientific). First, 2µg of total RNA was incubated for 15 minutes at room temperature with 2µl of DNase I (1U/µl) (Invitrogen, ThermoFisher Scientific) and 2µl of 10X DNase I Buffer in 20µl Ultrapure RNase-free distilled water (Invitrogen, ThermoFisher Scientific). 2µl of 25mM EDTA was then added to the DNase treated RNA and incubated for 10 minutes at 65°C. Afterwards, 1µl of random primers (3µg/µl) (Invitrogen, ThermoFisher Scientific) was added, incubated for 5 minutes at 70°C and immediately placed on ice. Subsequently, 8µl of 5X first strand buffer, 1µl of 25mM dNTPs, 1µl of RNaseOUT ribonuclease inhibitor (40U/µl) (Invitrogen, ThermoFisher Scientific), 0.5µl of 100mM DTT, 5.5µl of Ultrapure RNase-free distilled water and lastly 1µl of SuperScript Reverse Transcriptase (200U/µl) were added to the DNase treated RNA, incubated for 90 minutes at 42°C and for 15 minutes at 70°C afterwards. After the last incubation, the

cDNA was placed on ice until it was ready to use for the PCR reaction or kept at -80°C for storage.

2.2.11 Polymerase chain reaction (PCR)

The PCR reactions were performed using the Platinum Taq DNA polymerase (Invitrogen, ThermoFisher Scientific), *TARDBP* primers to quantify the expression of *TARDBP* mRNA and the 18S internal standard (QuantumRNA Universal 18S Internal Standard, Invitrogen, ThermoFisher Scientific) used as an internal control for quantitative RNA analysis. The PCR master mix used to amplify the cDNA and the incubation reactions performed on a thermal cycler (Bio-Rad) are listed in Table 5, while the list of primers used for the quantitative RNA analysis of *TARDBP* expression is detailed in Table 6. The PCR products were run on an agarose gel containing 1% of ultrapure agarose dissolved in 1X Tris-acetate-EDTA (TAE) buffer and RedSafe reactive (iNtRON Biotechnologies) at a 5µl:100ml ratio. The samples with 5X Orange G loading buffer were then loaded on the gel in a wide mini-sub cell GT system (Bio-Rad) and run at 100V for 60 minutes. The gel was observed under UV light and 1Kb plus molecular ruler (Bio-Rad) was used to detect the amplicons from the PCR reaction.

Table 5. PCR master mix components and incubation protocol in a thermal cycler.

PCR mix	Reactive	Volume
	10X PCR Buffer	5µl
	50mM MgCl ₂	1µl
	2.5mM dNTPs (each)	5µl
	10µM Primers	1.2µl
	18S Primer:Competimer (ratio 2:8)	0.2µl:0.8µl
	cDNA	2µl
	Taq Polymerase	0.2µl
	Ultrapure RNase-free distilled water	Up to 50µl
PCR incubation protocol in a thermal cycler	Stage 1	1 cycle
	94°C	3 minutes
	Stage 2	28 cycles
	94°C	30 seconds
	62°C	30 seconds
	72°C	30 seconds
	Stage 3	1 cycle
	72°C	5minutes
	Stage 4	1 cycle
	4°C	∞

* Stage 1= initial denaturation; Stage 2 = Denaturation, Annealing, Extension; Stage 3 = Final extension; Stage 4 = Hold

Table 6. Primers used in PCR to quantify *TARDBP* mRNA in transiently transfected HEK 293T cells.

Name	Forward or Reverse primers	DNA sequence (5' to 3')
TDP-43	Forward	5' CAAAGCCATTCAGGGCCTTTGCC 3'
	Reverse	5' GATGCTGATCCCCAACCAATTGCT 3'

2.2.12 Generation of transgenic flies

The transgenic flies used for the immunohistochemistry analysis were generated using the GAL4/UAS system (Drosophila Genomic Resource Center). The driver strain parent line contains transgenes that express the yeast GAL4 transcription factor in tissue-specific pattern *Elav*, pan-neuronal. The responder parent line express target genes, *TARDBP* and *LeuR*, sensitive to GAL4 transcription factor. The flies generated from crossing the responder and driver parent lines express target genes in a tissue specific pattern (Fig. 7). Single transgenic flies *Elav-TDP-43* and double transgenic flies *Elav-LeuR²⁴²;TDP-43* were previously characterized by C. Droppelmann and B. Withers using genomic analysis (Droppelmann et al., unpublished) (Withers, MSc Thesis). Fly stocks were maintained per standard procedures (Bloomington Drosophila Stock Center) and their food was provided by Dr Kramer's lab according to the standard fly food recipe (water, yeast, soy flour, yellow cornmeal, agar, light corn syrup, propionic acid). Flies were raised in 25°C, 70% humidity chambers.

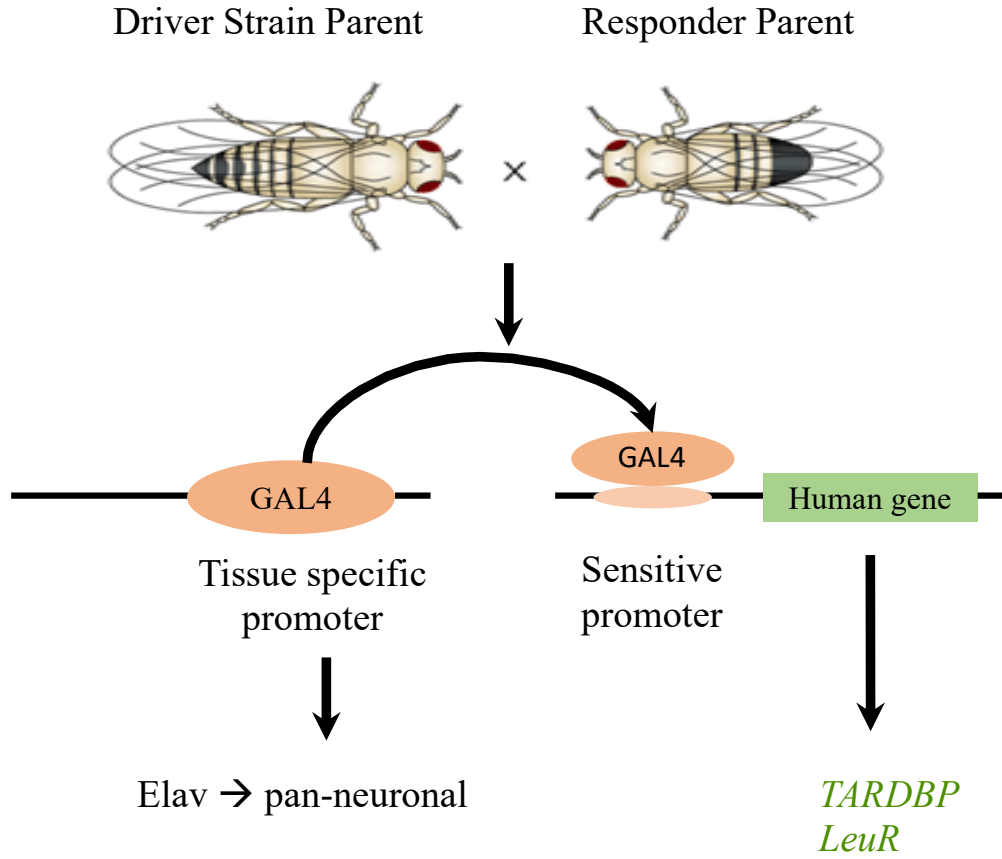


Figure 7. Scheme of the GAL4/UAS system used to generate transgenic *drosophila melanogaster* Elav-*LeuR*²⁴²;TDP-43 and Elav-TDP-43.

2.2.13 Fly fixation

To analyze the brain and eye tissue of transgenic *drosophila melanogaster* expressing TDP-43 and LeuR²⁴², immunohistochemistry (IHC) was performed on paraffin embedded sections of adult flies following the fixation protocol from the Shcherbata group (Kucherenko, et al., 2010). Whole flies in collars were first incubated in Carnoy's solution containing absolute ethanol, chloroform and glacial acetic acid 6:3:1 ratio, overnight at 4°C. The flies in Carnoy's solution were then dehydrated by incubating them in 40% ethanol for 20 minutes, 70% for 20 minutes and twice in 100% ethanol for 10 minutes each. Afterwards, they were incubated in methylbenzoate (MB) and MB with paraffin solution, 1:1 ratio, for 30 minutes each at 60°C following which they were incubated twice in paraffin solution for 60 minutes each at 60°C. The flies in paraffin were then allowed to solidify at room temperature overnight before proceeding to cutting the paraffin-embedded flies into 7µm sections in the Pathology Core facility (Robarts Research Institute).

2.2.14 Immunohistochemistry (IHC)

For deparaffinizing and rehydrating, the slide sections of the fly brain and eye tissue were first warmed on a slide warmer at 60°C for 30 minutes, then sequentially immersed in xylenes for 15 minutes, xylenes:ethanol (1:1 ratio) for 10 minutes, 100% ethanol, 95% ethanol, 70% ethanol, 50% ethanol and Millipore water for 5 minutes each, all at room temperature. Afterwards, the slides were incubated in citrate -EDTA buffer pH 6.2, containing 10mM citric acid, 2mM EDTA and 0.05% Tween-20, at 100°C for 30 minutes. This antigen retrieval buffer was designed to break the protein crosslinks and therefore expose the antigens and epitopes in the paraffin embedded tissue sections. Afterwards, the slides were incubated for 60 minutes at room temperature in PBS pH 7.2 blocking solution with 5% BSA and 0.3% Triton-X 100, following which they were incubated with primary antibodies at 4°C overnight in a humidifying chamber. After primary antibody incubation, they were washed three times with PBS pH 7.2 for 5 minutes each and then incubated with Alexa Fluor secondary antibodies for 60 minutes at room temperature in a humidifying chamber. The dilutions used for primary and secondary antibodies are indicated in table 7. The slides were then washed three times with PBS pH 7.2 and to visualize nuclei the slides were incubated with 1µg/ml DAPI for 3 minutes. This incubation was followed with three consecutive washes with Millipore water following which the slides were

incubated with an autofluorescence quenching kit (Vector TrueVIEW, Vector Labs) for 4 minutes at room temperature. The slides were then washed and left to dry overnight at room temperature. Once dry, coverslips were mounted to the slides using a fluorescent mounting media (Dako). All the slides were then examined using an SP8 Lightning Confocal microscopy system (Leica Microsystems Inc.) and visualized using the LAS X software (Leica Microsystems Inc.)

Table 7. Antibodies used for IHC experiments

Primary antibody	Species	Dilution	Manufacturer	Secondary antibody
Anti-TDP-43	Rabbit	1:500	ProteinTech	Donkey α -rabbit ALEXA fluor 555nm (titre: 1:200) (Invitrogen, ThermoFisher Scientific)
Anti-flag	Goat	1:400	ThermoFisher Scientific	Donkey α -rabbit, ALEXA fluor 488nm (titre: 1:200) (Invitrogen, ThermoFisher Scientific)

2.2.15 Statistical analysis

The statistical analysis in aim 2 was undertaken using GaphPad Prism 8.3 (GraphPad software Inc.). Student's t-test was used when comparing two groups: the control group transfected with the empty vector was compared to the experimental group transfected with the various constructs of RGNEF. Statistical analysis was generated and reported as the mean \pm (standard deviation) SD of the mean and they were considered to be statistically significant when $p < 0.05$. Experiments were carried out in triplicate.

Chapter 3

3. Experimental Results

3.1 Crosslink IPs in transiently transfected cells

Our lab had previously observed a potential interaction between RGNEF and TDP-43 *in vitro* HEK 293T and *in vivo* through observing co-aggregation of both proteins in spinal motor neurons of ALS patients (Keller, et al., 2012). Because of the presence of an atypical leucine rich motif (LRR motif) in the amino-terminal region of RGNEF and given the role of other LRR motifs in protein-protein interaction (Kobe & Deisenhofer, 1995), we postulated that the amino-terminal region of RGNEF is a critical domain mediating the interaction of RGNEF with TDP-43. This postulate was further supported by the observation that within the amino-terminal region of RGNEF, a 242 amino acid segment containing the LRR motif (LeuR²⁴²) (Fig. 8A) forms a high molecular weight complex of proteins with TDP-43 in HEK 293T cells (Droppelmann, Campos-Melo, Moszczynski, Amzil, & Strong, 2019).

As illustrated in figure 8, LeuR²⁴² is predicted to contain an Ankyrin (ANK) repeat domain between the amino-acids 162 and 229 in the amino-terminal region (Fig. 8A). The ANK repeats are one of the most abundant protein classes in protein-protein interaction and are involved in numerous physiological processes including transcriptional regulation (Li, Mahajan, & Tsai, 2006). The role of ANK in protein-protein interaction and its localization as part of the LeuR²⁴² suggests that the ANK domain could be a good candidate for the minimal region involved in the interaction between RGNEF and TDP-43. To determine if this was the case, we examined using cross-linking studies the ability of various flag-tagged constructs containing either LeuR²⁴² (and thus containing both the LRR and ANK domain), LeuR¹¹⁰ (amino acids 97 through 206 containing only LRR), or ANK (amino acids 162 – 229 and thus containing only ANK as a small C-terminus fragment of LRR) (Fig. 8A) to interact with myc-tagged TDP-43. The structure prediction of LeuR¹¹⁰ and ANK with the flag-tag was conserved which would not affect the co-aggregation of each sequence with TDP-43.

To determine the presence of protein-protein interaction between TDP-43 and LeuR *in vitro*, protein lysates from transiently transfected HEK 293T cells with myc-tagged TDP-43 and flag-

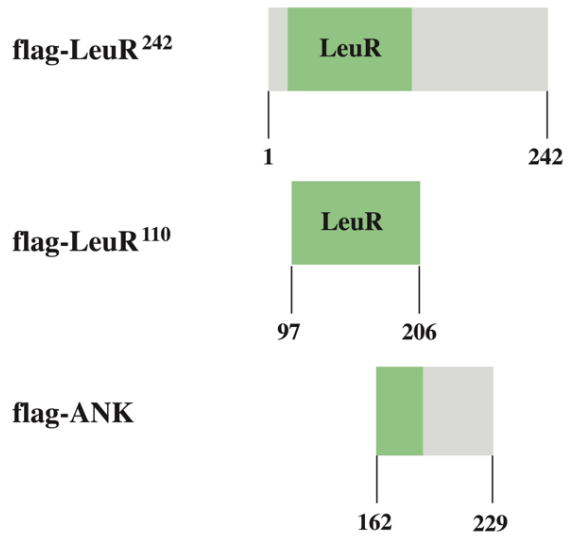
tagged LeuR¹¹⁰ were used in a chemical DTSSP crosslinking experiment. This water soluble crosslinker is characterized by central disulfide bond sensitive to reducing conditions and its amine-reactive *N*-hydroxysulfosuccinimide esters at each end of a 12Å spacer arm that react to primary amines and form stable bonds at pH 7-9. While the use of this crosslinker might non-specifically pull-down other proteins through their amine-reactive-NHS ester ends around the 12Å spacer arm, the following immunoprecipitation experiment using a monoclonal antibody increases the specificity to our protein of interest TDP-43-myc. After running the immunoprecipitation pulling down myc-tagged proteins and the western blot detecting flag-tagged proteins, we observed a high molecular weight complex (>250 KDa) containing myc-tagged TDP-43 in the presence of flag-tagged LeuR¹¹⁰ in non-reducing conditions. However, in the presence of β-mercaptoethanol, causing the di-sulfide bond of DTSSP to be cleaved, we detected the presence of a low molecular weight protein (50kDa) (Fig. 8B). These results suggest that the cleavage of the disulfide bond resulted in breaking the complex of proteins in which only the flag-tagged protein was detected in the western blot. While the expected size of flag-LeuR¹¹⁰ is 12kDa, the detected band size in reducing conditions is 40kDa suggesting that while the disulfide bond of DTSSP was cleaved, the flag-LeuR¹¹⁰ pulled down was still in a complex with other proteins which would explain the difference between the expected and detected protein band size.

To determine if the ANK domain participates in the formation of this high molecular weight complex, we conducted the same crosslink IP using protein lysates from transiently transfected cells with myc-tagged TDP-43 and flag-tagged ANK. Given the role of the ANK domain in protein-protein interaction, we expected the presence of an interaction between ANK and TDP-43. However, we observed no interaction between flag-tagged ANK and myc-tagged TDP-43 suggesting that the ANK binding domain does not play a role in the interaction between RGNEF and TDP-43.

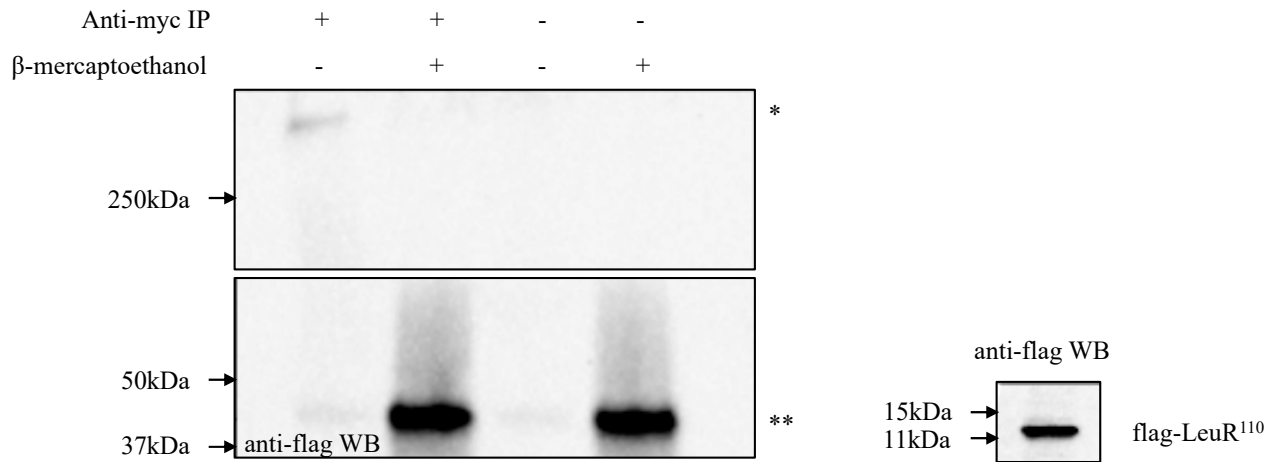
This series of experiments indicated that the 110 amino-acids fragment contained in the LeuR region is sufficient for the interaction with TDP-43. In addition, given that the observed molecular weight of the immunoprecipitant was > 250 kDa, significantly greater than that which would have been predicted if LeuR¹¹⁰ interacted alone with TDP-43 (Fig. 8B), we postulated that this interaction is within a larger complex of proteins. These results suggest that the LeuR¹¹⁰

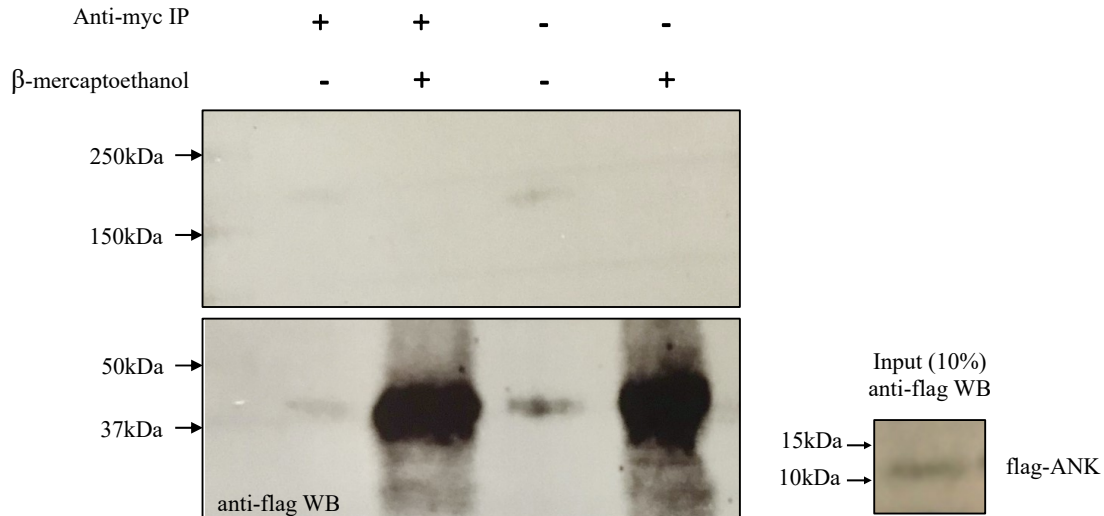
sequence contained in the amino-terminal region of RGNEF is the minimal domain of the protein required for its indirect interaction with TDP-43.

(A)



(B)





(C)

Figure 8. flag-LeuR¹¹⁰ and TDP-43-myc form a complex after DTSSP crosslinking treatment while flag-ANK and TDP-43-myc do not form a complex. (A) Scheme showing LeuR²⁴² previously characterized in the interacting complex with TDP-43 and the LeuR region flag-tagged constructs used to study the minimal domain of interaction with TDP-43: LeuR¹¹⁰ containing only the LRR (amino acids 97-207), ANK containing only the ANK repeat, including a small fragment of the C-terminal LRR (amino acids 162-229). (B) Using DTSSP as the crosslinking agent, we observed a high molecular weight complex between myc-tagged TDP-43 and flag-tagged LeuR¹¹⁰. This complex could be dissociated with β -mercaptoethanol. The immunoprecipitants were run on a gradient (4-20%) SDS-PAGE gel and detected using immunoblotting probed with anti-flag antibody (1:4000). (C) In contrast, we failed to observe any interaction between flag-tagged ANK and myc-tagged TDP-43.

3.2 Endogenous expression of TDP-43 in HEK 293T cells in the presence of RGNEF constructs

To determine the critical domains of RGNEF involved in the regulation of TDP-43, the endogenous expression of TDP-43 was measured in transiently transfected HEK 293T cells. HEK 293T cell line was chosen because of its amenability to transfection, its neuronal-like properties (Shaw, Morse, Arabat, & Graham, 2002) and its endogenous expression of TDP-43. The plasmids used were myc-tagged: full length RGNEF, R Δ LeuR or R Δ COOH. The control plasmid used was a pcDNA3.1 empty vector. The first experiment conducted was to determine the effect of RGNEF on the endogenous expression levels of TDP-43 using a western blot. The goal was to determine the endogenous levels of expression of TDP-43 in the presence of various constructs of RGNEF. In addition to protein levels, the expression of *TARDBP* mRNA expression levels was also analyzed in the presence of different RGNEF constructs. Finally, given the role of RGNEF as a destabilizing factor of *NEFL* through its 3' untranslated region (UTR) (Droppelmann, Keller, Campos-Melo, Volkening, & Strong, 2013), the role of this region on the *TARDBP* mRNA stability was analyzed using luciferase reporter assay in the presence of full length RGNEF. The 3'UTR of the ALS-associated gene, *FUS* was used as control.

3.2.1 Effect of various RGNEF constructs on the endogenous expression of TDP-43 in HEK 293T

Previously our lab has examined the effect of RGNEF on the expression levels of endogenous NFL in HEK 293T cells (Droppelmann, Keller, Campos-Melo, Volkening, & Strong, 2013). We observed that in the presence of full length RGNEF, the endogenous levels of NFL decreased significantly. The effect of RGNEF on NFL expression levels could be normalized in the presence of RGNEF knockdown.

In order to determine whether a similar regulatory effect of RGNEF is present in relationship to TDP-43 and to determine if there is a minimal domain involved in its regulation, HEK 293T cells were transiently transfected with a pcDNA3.1 empty vector and three different myc-tagged constructs full length RGNEF-myc, R Δ LeuR-myc and R Δ COOH-myc (Fig. 9A). To do so, protein extracts from cell lysates were generated and anti-TDP-43, anti-myc, antibodies for

western blotting were used to quantify the endogenous expression of TDP-43. The expression levels of TDP-43 were normalized to the housekeeping protein GAPDH and each group from cell lysates transfected with RGNEF constructs, was compared to the control transfection containing an empty vector. All studies were done in triplicate.

We observed that the expression levels of TDP-43 were significantly decreased in the presence of full length RGNEF and R Δ COOH (fold change: -0.184 ± 0.042 ; -0.236 ± 0.039 ; both $p < 0.05$; respectively, Fig. 9C). However, in the absence of the amino-terminal region, R Δ LeuR, there was no significant change in the levels of expression of endogenous TDP-43 in the cells (Fig. 9B-C). These results suggest that LeuR²⁴² of RGNEF, is involved in the down-regulation of endogenous TDP-43 expression levels of the protein in HEK 293T cells in normal conditions.

(A)

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RGNEF FL 1 MELSCSEAPLYGQMMIYAKFDKNVYLPEDAIFYFTYDGSQRHVMI AERIEDNVLQSSVPGHGLQETVTVSVCLCSEGYSPVTMGSGSVTVYVDMACRLARLLVTFQANRLTACSHQTLT
RΔLeuR 1
RΔCOOH 1 MELSCSEAPLYGQMMIYAKFDKNVYLPEDAIFYFTYDGSQRHVMI AERIEDNVLQSSVPGHGLQETVTVSVCLCSEGYSPVTMGSGSVTVYVDMACRLARLLVTFQANRLTACSHQTLT

RGNEF FL 121 PFALTAGALPALDEELVLA LTHLELPLEWTVLGSSSLEVSSHRESLLHLAMRWGLAKLSQFFLCLPGGVQALALPNEEGATPLDLALREGHSKLVEDVTNFQGRWSPSFSRVQLSEEASL
RΔLeuR 121
RΔCOOH 121 PFALTAGALPALDEELVLA LTHLELPLEWTVLGSSSLEVSSHRESLLHLAMRWGLAKLSQFFLCLPGGVQALALPNEEGATPLDLALREGHSKLVEDVTNFQGRWSPSFSRVQLSEEASL

RGNEF FL 241 HYIHSETLTLTLNHTAEHLEADIKLFKRYFDRAFLVKA FEQEARPEERTAMPSSGAETEEIKNVSSRSAAEKEDIKRVKSLVVQHNEHEDQHS LLDLDRSFDILKKS KPPSTLLAA
RΔLeuR 241 --IHSETLTLTLNHTAEHLEADIKLFKRYFDRAFLVKA FEQEARPEERTAMPSSGAETEEIKNVSSRSAAEKEDIKRVKSLVVQHNEHEDQHS LLDLDRSFDILKKS KPPSTLLAA
RΔCOOH 241 HYIHSETLTLTLNHTAEHLEADIKLFKRYFDRAFLVKA FEQEARPEERTAMPSSGAETEEIKNVSSRSAAEKEDIKRVKSLVVQHNEHEDQHS LLDLDRSFDILKKS KPPSTLLAA

RGNEF FL 361 GRLSDMLNGGDEVYANCMVIDQVGDLDISYINIEG IATTSPE SRGCTLWPQSSKHTLP TETS PSVYPLSENV ECTAHTEAQQSFMSPSSSCASN LNSFGWHGFEKEQSHLKKRSSSLD
RΔLeuR 361 GRLSDMLNGGDEVYANCMVIDQVGDLDISYINIEG IATTSPE SRGCTLWPQSSKHTLP TETS PSVYPLSENV ECTAHTEAQQSFMSPSSSCASN LNSFGWHGFEKEQSHLKKRSSSLD
RΔCOOH 361 GRLSDMLNGGDEVYANCMVIDQVGDLDISYINIEG IATTSPE SRGCTLWPQSSKHTLP TETS PSVYPLSENV ECTAHTEAQQSFMSPSSSCASN LNSFGWHGFEKEQSHLKKRSSSLD

RGNEF FL 481 ALDADSEGEHSEPSHICYTPGSQSSRTGIPSGDELDFETNTEPDFNISRAESLPLSSNLQSKESL LSGVRSRSYSCSSPKISLGKTRLVRELTVCSSSEEQRAYSLSEPPRENRIQE
RΔLeuR 481 ALDADSEGEHSEPSHICYTPGSQSSRTGIPSGDELDFETNTEPDFNISRAESLPLSSNLQSKESL LSGVRSRSYSCSSPKISLGKTRLVRELTVCSSSEEQRAYSLSEPPRENRIQE
RΔCOOH 481 ALDADSEGEHSEPSHICYTPGSQSSRTGIPSGDELDFETNTEPDFNISRAESLPLSSNLQSKESL LSGVRSRSYSCSSPKISLGKTRLVRELTVCSSSEEQRAYSLSEPPRENRIQE

RGNEF FL 601 EEWDKYIIPAKSESEKYKVSRTFSFLMNRMTSPRNKSKTKSKDAKDKELNRHOFAPGTFSGVLQCLVCDKTL LKGESLQCSNCNANVHKGCCKDAAPA TKKFQEKYNNKPKQ TILGNSS
RΔLeuR 601 EEWDKYIIPAKSESEKYKVSRTFSFLMNRMTSPRNKSKTKSKDAKDKELNRHOFAPGTFSGVLQCLVCDKTL LKGESLQCSNCNANVHKGCCKDAAPA TKKFQEKYNNKPKQ TILGNSS
RΔCOOH 601 EEWDKYIIPAKSESEKYKVSRTFSFLMNRMTSPRNKSKTKSKDAKDKELNRHOFAPGTFSGVLQCLVCDKTL LKGESLQCSNCNANVHKGCCKDAAPA TKKFQEKYNNKPKQ TILGNSS

RGNEF FL 721 FRDIPQPLSLHPSSSVVGLPTGRRET VGVHPLSRSPVGT TLESFRRSATSLESESDHNSCRSRSHSDELLQSMGSSPSTESFIMEDVVDSSLWSDLS SDAQFEAEWSLVVDPSFC
RΔLeuR 721 FRDIPQPLSLHPSSSVVGLPTGRRET VGVHPLSRSPVGT TLESFRRSATSLESESDHNSCRSRSHSDELLQSMGSSPSTESFIMEDVVDSSLWSDLS SDAQFEAEWSLVVDPSFC
RΔCOOH 721 FRDIPQPLSLHPSSSVVGLPTGRRET VGVHPLSRSPVGT TLESFRRSATSLESESDHNSCRSRSHSDELLQSMGSSPSTESFIMEDVVDSSLWSDLS SDAQFEAEWSLVVDPSFC

RGNEF FL 841 NRQEKDVIKRQDVIFELMQTEMHHIQTLFIMSEIFRKG MKEELQDHS TVDKI FPCLELLEIHRHFFYSMKERRQES CAGSDRN FVIDRIGDILVQQFSEENASKMKKIYGEFCCHHKE
RΔLeuR 841 NRQEKDVIKRQDVIFELMQTEMHHIQTLFIMSEIFRKG MKEELQDHS TVDKI FPCLELLEIHRHFFYSMKERRQES CAGSDRN FVIDRIGDILVQQFSEENASKMKKIYGEFCCHHKE
RΔCOOH 841 NRQEKDVIKRQDVIFELMQTEMHHIQTLFIMSEIFRKG MKEELQDHS TVDKI FPCLELLEIHRHFFYSMKERRQES CAGSDRN FVIDRIGDILVQQFSEENASKMKKIYGEFCCHHKE

RGNEF FL 961 AVNLFKELQONKFFQNF IKLRSNLLARRRGIPEC ILLVQRITKYPV LVERILQYTKERTEEHKDLRKALCLIKDMIATVDLKVNEYEKQK WLEILNKIENKTYTKLKNGHVFRKQAL
RΔLeuR 961 AVNLFKELQONKFFQNF IKLRSNLLARRRGIPEC ILLVQRITKYPV LVERILQYTKERTEEHKDLRKALCLIKDMIATVDLKVNEYEKQK WLEILNKIENKTYTKLKNGHVFRKQAL
RΔCOOH 961 AVNLFKELQONKFFQNF IKLRSNLLARRRGIPEC ILLVQRITKYPV LVERILQYTKERTEEHKDLRKALCLIKDMIATVDLKVNEYEKQK WLEILNKIENKTYTKLKNGHVFRKQAL

RGNEF FL 1081 MSEERTLLYDGLVYWKATGRFKDILALLLTDVLLFLQEKDQKYIFA AVDQKPSV ISLQKLIAREVANEERGMFLISASSAGPEMYE IHTNSKEERNW MRRIQAVESCP ECKGGRTSE
RΔLeuR 1081 MSEERTLLYDGLVYWKATGRFKDILALLLTDVLLFLQEKDQKYIFA AVDQKPSV ISLQKLIAREVANEERGMFLISASSAGPEMYE IHTNSKEERNW MRRIQAVESCP ECKGGRTSE
RΔCOOH 1081 MSEERTLLYDGLVYWKATGRFKDILALLLTDVLLFLQEKDQKYIFA AVDQKPSV ISLQKLIAREVANEERGMFLISASSAGPEMYE IHTNSKEERNW MRRIQAVESCP ECKGGRTSE

RGNEF FL 1201 SDEDKRKAERAVAKIQQCQEILTNQDQICAYLEEKLHIYAE LGELSGFEDVHLEP HLLIKPDGPEPPQAASLLAALKEAESLQVAVKASQMGAVSQSCEDSCGDSVLADT LSSHDPVG
RΔLeuR 1201 SDEDKRKAERAVAKIQQCQEILTNQDQICAYLEEKLHIYAE LGELSGFEDVHLEP HLLIKPDGPEPPQAASLLAALKEAESLQVAVKASQMGAVSQSCEDSCGDSVLADT LSSHDPVG
RΔCOOH 1201 SDEDKRKAERAVAKIQQCQEILTNQDQICAYLEEKLHIYAE LGELSGFEDVHLEP -----

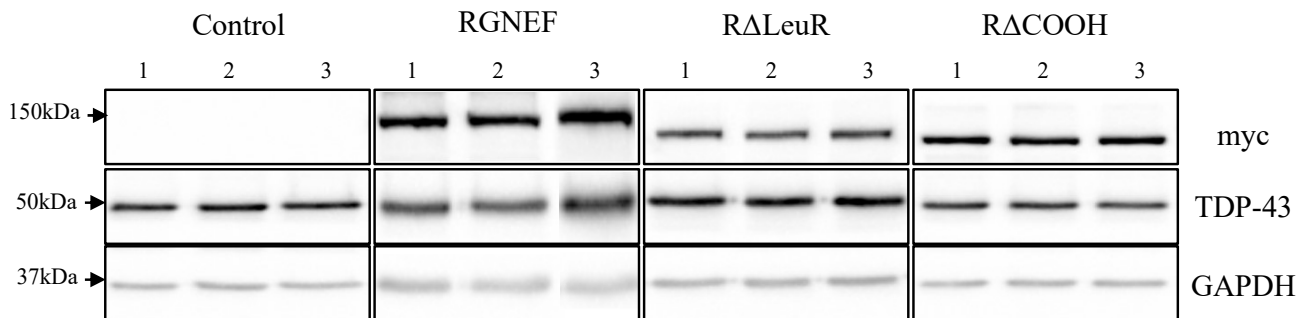
RGNEF FL 1321 SPTASLVTTGREGRCSDVDPGIQGVVTDLAVSDAGEKVECRNFP GSSQSEI IQAIQNLTRLLYSLQAALTIQDSHIEIHRVLVQQEGLSLGHSILRGGPLQDQKSRDADRQHEELANV
RΔLeuR 1321 SPTASLVTTGREGRCSDVDPGIQGVVTDLAVSDAGEKVECRNFP GSSQSEI IQAIQNLTRLLYSLQAALTIQDSHIEIHRVLVQQEGLSLGHSILRGGPLQDQKSRDADRQHEELANV
RΔCOOH 1321 -----

RGNEF FL 1441 HQLOHQLOQEQRRWLRRC EQQRAQATRESWLQERERECQS QEBELLLRSRGELDLQLQEQYHSLERLREGQLVREVEQARMRAQSSLGHGHWKGRQSLPAVLLPGGP EVMELNRSSELC
RΔLeuR 1441 HQLOHQLOQEQRRWLRRC EQQRAQATRESWLQERERECQS QEBELLLRSRGELDLQLQEQYHSLERLREGQLVREVEQARMRAQSSLGHGHWKGRQSLPAVLLPGGP EVMELNRSSELC
RΔCOOH 1441 -----

RGNEF FL 1561 HENSFFINEALVQMSFNTFNKLNPSVIHQDATYPTTQSHS DLVRTSEHQVDLKV DPSQPSNVSHKLWTAAGSGHQILPFHESKDSCKNGSSMTKCSCTLTSP PGLWTGTSTLTKDLDT S
RΔLeuR 1561 HENSFFINEALVQMSFNTFNKLNPSVIHQDATYPTTQSHS DLVRTSEHQVDLKV DPSQPSNVSHKLWTAAGSGHQILPFHESKDSCKNGSSMTKCSCTLTSP PGLWTGTSTLTKDLDT S
RΔCOOH 1561 -----

RGNEF FL 1681 HTESPTPHDSNSHRPQLQAFITEAKLNLPTRTMTRODGETGDGAKENIVYL
RΔLeuR 1681 HTESPTPHDSNSHRPQLQAFITEAKLNLPTRTMTRODGETGDGAKENIVYL
RΔCOOH 1681 -----
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(B)



(C)

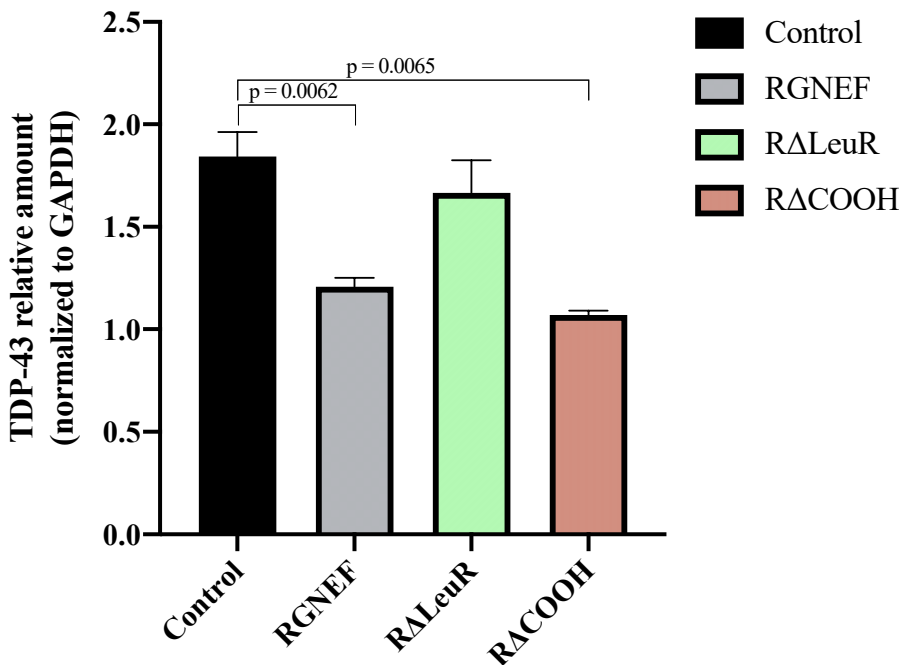


Figure 9. TDP-43 protein expression levels are decreased in HEK 293T transfected cells expressing full length RGNEF. (A) Aligned protein sequences of RGNEF constructs (RGNEF full length, RΔLeuR and RΔCOOH) used to study the effect of putative regions of RGNEF on the endogenous expression of TDP-43 *in vitro*. --- indicates deleted amino acids in RΔLeuR and RΔCOOH. Green indicated the leucine rich domain, yellow indicates the cysteine-rich Zn-binding domain red indicates the Dbl homology domain (DH), blue indicates the Pleckstrin homology domain (PH) and orange indicates the RNA-binding domain. (B) The expression level of TDP-43 protein was analyzed by western blotting in HEK 293T cells transfected with either full length RGNEF, RΔLeuR or RΔCOOH, using the endogenous levels of TDP-43 as the control. (C) Expression levels of TDP-43 protein were quantified comparing the TDP-43 protein levels between the control and HEK 293T RGNEF-full length, RΔLeuR and RΔCOOH cells. A significant reduction in TDP-43 protein levels was only observed in RGNEF-full length and RΔCOOH transfected cells. (n=3 independent experiments, the results are presented as mean, standard deviation of the mean. A student *t*-test was performed; p-value significant: $p < 0.05$).

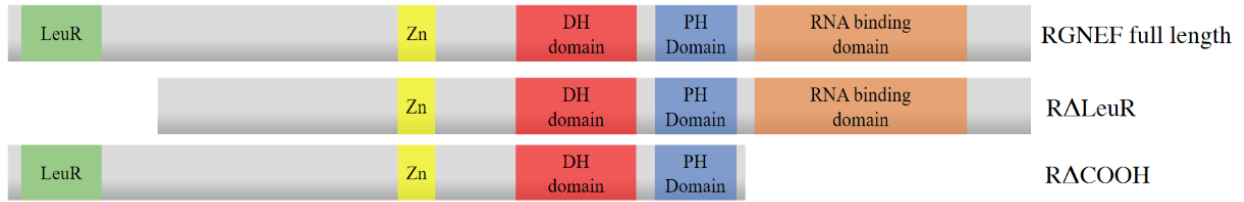
3.2.2 Effect of RGNEF constructs on the endogenous expression of *TARDBP* mRNA in HEK 293T cells

It has been previously reported that the murine homolog of RGNEF (p190RhoGEF) enhances the stability of murine *NEFL* mRNA through a direct interaction within the hinge domain of *NEFL* mRNA 3'UTR (Cañete-Soler, Wu, Zhai, Shamim, & Schlaepfer, 2001). In contrast, our lab observed that RGNEF destabilizes human *NEFL* mRNA in HEK 293T and that this effect is critically dependent on the presence of the N-terminus domain of RGNEF (Droppelmann, Keller, Campos-Melo, Volkening, & Strong, 2013).

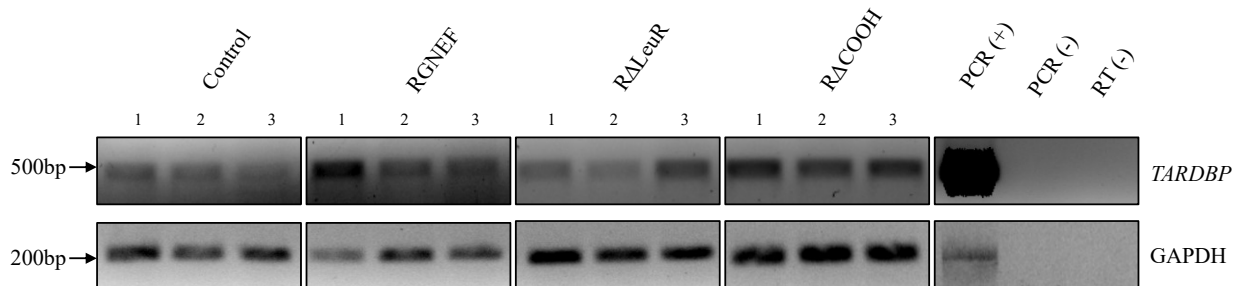
Given this and my previously described observation that RGNEF down-regulates the expression of TDP-43 *in vitro*, we further examined the effect of RGNEF on the endogenous levels of *TARDBP* mRNA. To do so, we undertook transient transfections in HEK 293T cells using full length RGNEF or its other constructs (Fig. 10A).

The endogenous expression of *TARDBP* mRNA in transiently transfected HEK 293T cells was normalized to the housekeeping gene *GAPDH*. Each group of transfected cells with full length RGNEF and its constructs was compared to the control group transfected with an empty vector. We observed that in the presence of full length RGNEF and its other constructs there was no significant effect on the expression level of endogenous *TARDBP* mRNA (Fig. 10C). Contrary to the significant reduction of endogenous TDP-43 protein levels observed in the presence of full length RGNEF and R Δ COOH construct, there was no effect on the endogenous expression of *TARDBP* transcript, which suggests that the down-regulation effect observed at the protein level is not a result of a down-regulation of its transcript levels.

(A)



(B)



(C)

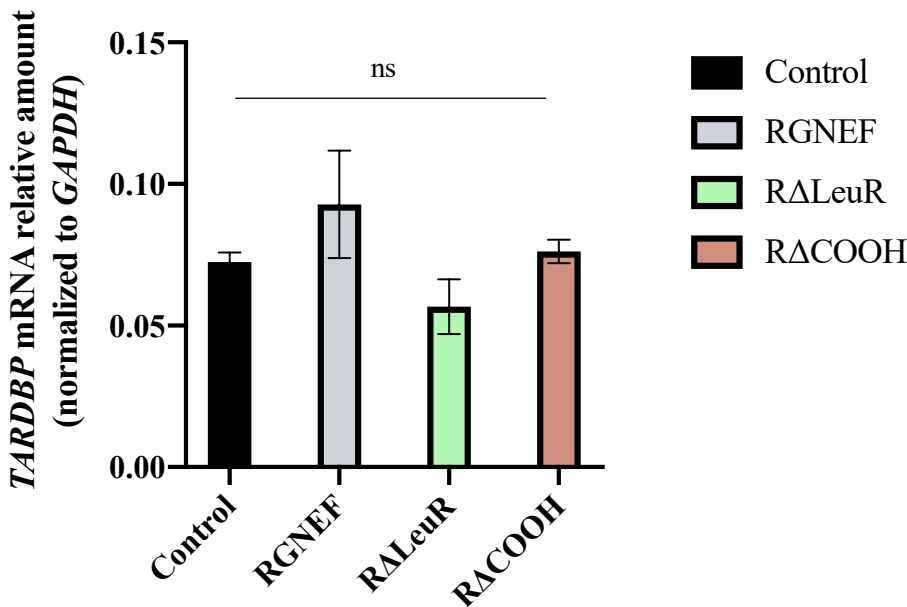


Figure 10. *TARDBP* mRNA endogenous expression in the presence of RGNEF constructs.

(A) Schematic of the RGNEF constructs used in transiently transfected cells to evaluate the effect of putative regions of RGNEF on the expression levels of endogenous *TARDBP* mRNA. (B) The expression of *TARDBP* mRNA in HEK 293T RGNEF-full length, RΔLeuR and RΔCOOH cells was analyzed by relative quantitative polymerase chain reaction (RT-PCR) and compared with the amount of *TARDBP* mRNA in control cells. No significant difference was

observed between the groups expressing RGNEF constructs and the control group. (C)
Expression levels of *TARDBP* mRNA were quantified comparing the *TARDBP* transcript levels between the control and HEK 293T RGNEF-full length, R Δ LeuR and R Δ COOH cells. No significant effect was observed when comparing *TARDBP* mRNA of transfected cells with RGNEF constructs to control cells (n=3 independent experiments, the results are presented as mean, standard deviation of the mean. A student *t-test* was performed; ns: not significant, p-value $p>0.05$).

3.2.3 Regulation of *TARDBP* 3'UTR

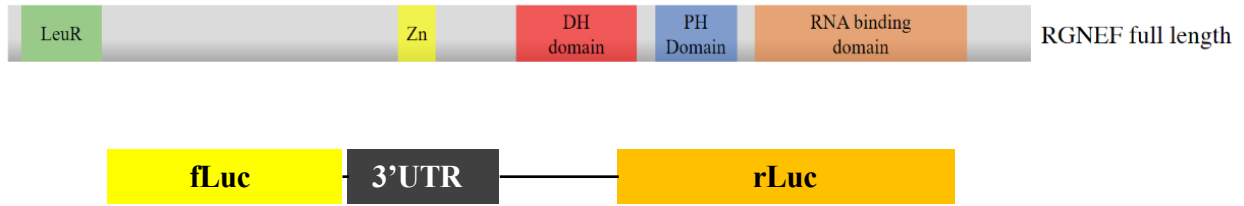
As previously reported, RGNEF interacts directly with *NEFL* transcript through its 3'UTR (Droppelmann, Keller, Campos-Melo, Volkening, & Strong, 2013). We had also observed that RGNEF could interact with *NEFL* mRNA in human lumbar spinal cord homogenates (using RNA-IP), but only in homogenates derived from ALS patients (Volkening, Leystra-Lantz, & Strong, 2010).

In order to further explore the genesis of the reduction in TDP-43 protein levels in the presence of RGNEF, having failed to observe a reduction in *TARDBP* mRNA levels in the previous experiment, we next examined whether RGNEF has a destabilizing effect on *TARDBP* mRNA. The 3'UTR of the ALS-associated gene, *FUS* was used as control. To do this, we used a dual luciferase reporter assay measuring the effect of exogenous RGNEF-full length on the luciferase activity when linked to the 3'UTR of either *FUS* or *TARDBP*. HEK 293T cells were transiently transfected with a *firefly* luciferase pmirGLO linked to the 3'UTR of either *FUS*, *TARDBP* mRNA or full length RGNEF (Fig. 11A). The controls used for normalizing the assay were the pmirGLO and the pcDNA3.1 empty vector and the normalization of the assay was calculated as previously described by our group in a comprehensive study using the luciferase-based reporter gene assay (Campos-Melo, Droppelmann, Volkening, & Strong, 2014).

In these experiments, we observed a significant down-regulation (-0.047 ± 0.0036 , $p < 0.05$) (Fig. 11B) in the relative luciferase activity of *TARDBP* 3'UTR in the presence of full length RGNEF. However, we did not observe an effect on the relative luciferase activity of *FUS* 3'UTR when exposed to RGNEF.

Our previous result from quantifying the endogenous expression of *TARDBP* mRNA showed no significant effect on the endogenous expression of *TARDBP* mRNA in the presence of full length RGNEF. However, the quantification of the endogenous expression of TDP-43 protein in the presence of full length RGNEF demonstrated a significant down-regulation when compared to the control group which suggests that the loss of TDP-43 protein in the presence of full length RGNEF is due to a direct destabilizing effect of RGNEF on the *TARDBP* mRNA 3'UTR.

(A)



(B)

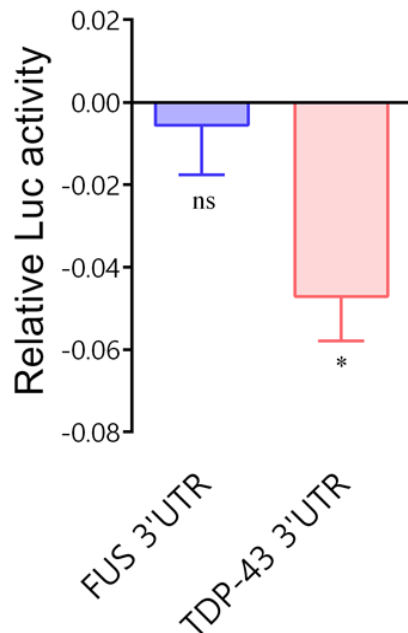


Figure 11. Full length RGNEF destabilizes the luciferase activity of *TARDBP* 3'UTR. (A) Schematic showing the constructs used in the assay: *FUS* 3'UTR or *TARDBP* 3'UTR linked *firefly* luciferase (*fLuc*) upstream and to *renilla* luciferase downstream; exogenous full length RGNEF or pcDNA3.1 empty vector. (B) Reporter gene assay showing decreased normalized luciferase activity in HEK 293T cells co-transfected with pcDNA-RGNEF-myc and pmirGLO linked to the 3' UTR of *TARDBP* or *FUS* mRNA. We observed a significant reduction of the luciferase activity of *TARDBP* 3' UTR in the presence of RGNEF-full length compared to the luciferase activity of *FUS* 3'UTR. A *t-test* was performed to compare the effect of RGNEF on *FUS* and *TARDBP* 3'UTRs. p-value (*) = $p < 0.05$; ns = not significant.

3.3 The expression of hTDP-43 and flag-LeuR²⁴² in transgenic *drosophila melanogaster* models.

To study the relationship between TDP-43 and LeuR of RGNEF, our lab in collaboration with Dr Kramer's lab developed *drosophila melanogaster in vivo* models expressing these particular proteins. Preliminary results characterizing the lifespan, motor functions and eye degeneration in double transgenic flies Elav-LeuR²⁴²;TDP-43 showed a significant improvement when compared to single transgenic flies Elav-TDP-43 (Withers, MSc Thesis). While the expression of human wild-type TDP-43 showed a dramatic reduction in survival and motor functions, in addition to eye degeneration, the co-expression of human wild-type TDP-43 and LeuR²⁴² showed a significant improvement in the flies' lifespan and, motor functions. Similar to our *in vitro* data demonstrating the cytoprotective effect of the amino-terminal region of RGNEF against osmotic stress in cells (Cheung, et al., 2017), the *in vivo* data suggests that the LeuR²⁴² offers a protective effect against TDP-43 toxicity in our *drosophila melanogaster* model (Withers, MSc Thesis).

We next directed our attention to exploring this effect of RGNEF and LeuR²⁴² in reducing the toxicity of TDP-43 *in vivo*. As has been described, we have observed that LeuR¹¹⁰ is able to interact with TDP-43 through the formation of a high molecular weight complex. We have shown that RGNEF induces a reduction in TDP-43 protein levels and that this effect is dependent on the LRR contained within the N-terminus domain of RGNEF. And we have shown that full length RGNEF interacts with the 3'UTR of TDP-43 to induce destabilization of the firefly luciferase transcript. These *in vitro* data are strongly supportive of a direct effect of RGNEF at both the protein and RNA level of TDP-43.

Given this, and to better understand the protective role of the LeuR²⁴² region in mitigating the TDP-43 toxicity observed in the flies, we studied the pathology of TDP-43 toxicity in the absence or presence of LeuR²⁴² using fluorescent immunohistochemistry (IHC) of the brain and eye tissue of single and double transgenic flies. In the following experiments, the double transgenic flies Elav-LeuR²⁴²;TDP-43 were generated using the LeuR²⁴² construct only because the fly study in the lab started before we determined the LeuR¹¹⁰ as the minimal domain of interaction with TDP-43. Since the results from our *in vitro* study confirmed that the interaction with TDP-43 is independent of the ANK domain contained in LeuR²⁴² we used LeuR²⁴² as a surrogate for LeuR¹¹⁰ in the *in vivo* study. All the transgenic flies were generated using the

GAL4/UAS system, in which human TDP-43 and flag-tagged LeuR²⁴² were expressed in a tissue specific manner. Both Elav-TDP-43 and Elav-LeuR²⁴²;TDP-43 fly models express their transgenes in neurons.

In the images of the brain and eye tissue of transgenic flies, we observed the expression of TDP-43 in aggregates. In the case of single transgenic flies Elav-TDP-43, these aggregates were more condensed, and their size was bigger (Fig. 12C) when compared to TDP-43 aggregates in double transgenic flies (Fig. 12F). In the Elav-LeuR²⁴²;TDP-43 flies we detected the presence of higher number of dispersed punctate aggregates expressing TDP-43 in both the brain and eye tissue (Fig. 12G-H), absent in the brain and eye tissue of single transgenic flies Elav-TDP-43 (Fig. 12D-E). The images from the tissue of Elav-LeuR²⁴²;TDP-43, looking into the expression of flag-tagged LeuR²⁴² and human TDP-43, showed that both proteins are expressed in aggregates (Fig. 13E-F; I-J) where they colocalize (Fig. 13G-L).

This suggests that the co-aggregation of TDP-43 and the LeuR²⁴² alongside the distribution and shape of these aggregates are possible mechanisms of protection against TDP-43 toxicity in these fly models.

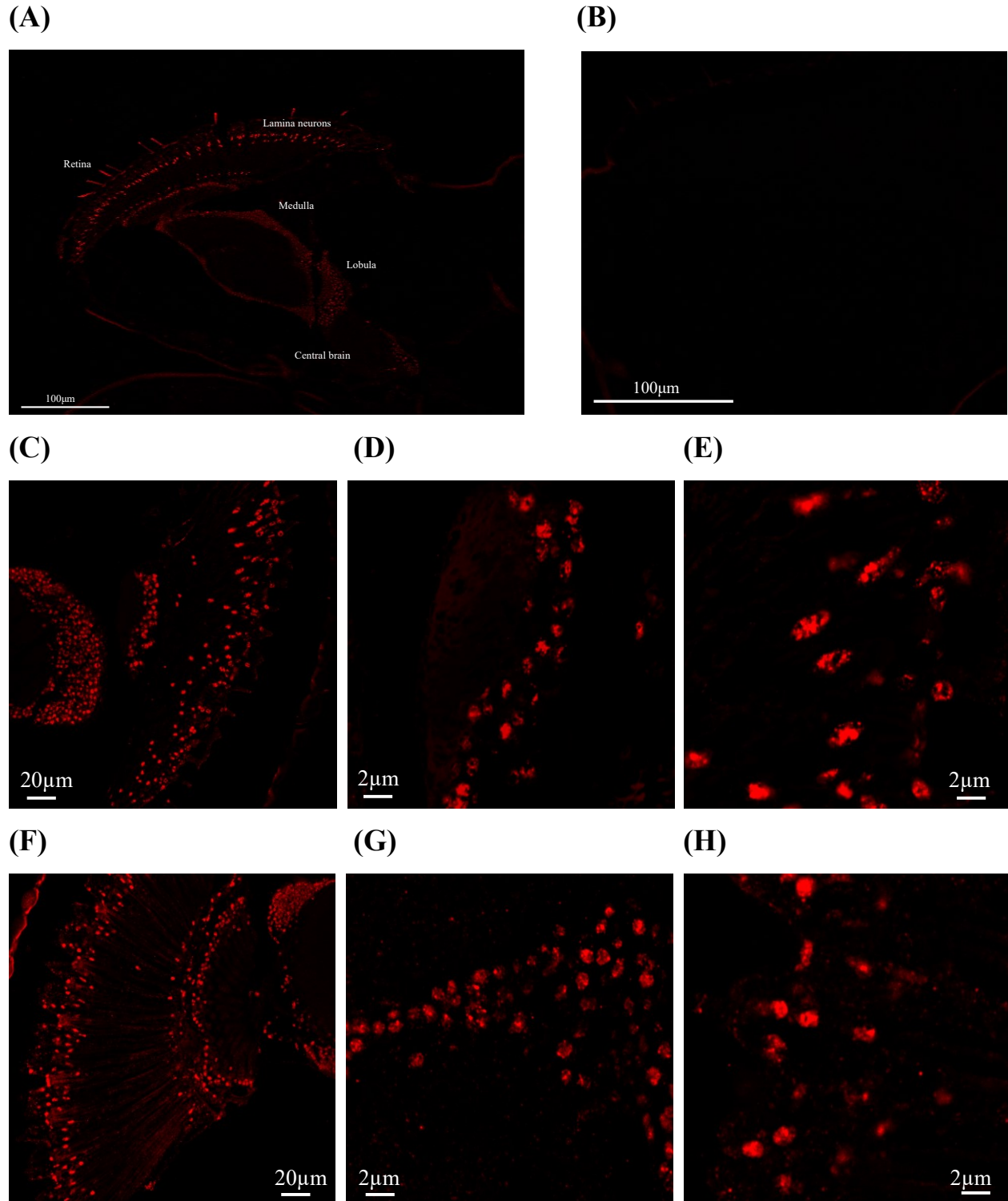


Figure 12. Confocal images show the localization of TDP-43 in the brain and eye tissue of single and double transgenic flies. Immunofluorescence using anti-TDP-43 antibody, visualized in red, shows the localization of TDP-43 in aggregates in single transgenic Elav-TDP-43 and double transgenic Elav-LeuR²⁴²;TDP43 *drosophila melanogaster*. Panel (A) shows an

overview of the brain and eye anatomy of Elav-LeuR²⁴²;TDP43 *drosophila melanogaster*. Scale bar = 100µm. Panel (B) shows a negative control of TDP-43 staining of Elav-LeuR²⁴²;TDP-43 flies. Scale bar = 100µm. Panel (C) shows an overview of the brain and eye tissue of single transgenic flies. Scale bar = 20µm. Panels (C and D) show the aggregation of TDP-43 in the brain and eye tissue, respectively, of single transgenic flies. Scale bar = 2µm. Panel (E) shows an overview of the brain and eye tissue of Elav-LeuR²⁴²;TDP43 transgenic flies. Scale bar = 20µm. Panels (F and G) show the aggregation of TDP-43 in the brain and eye tissue, respectively, of Elav-LeuR²⁴²;TDP43 transgenic flies. Scale bar = 2µm.

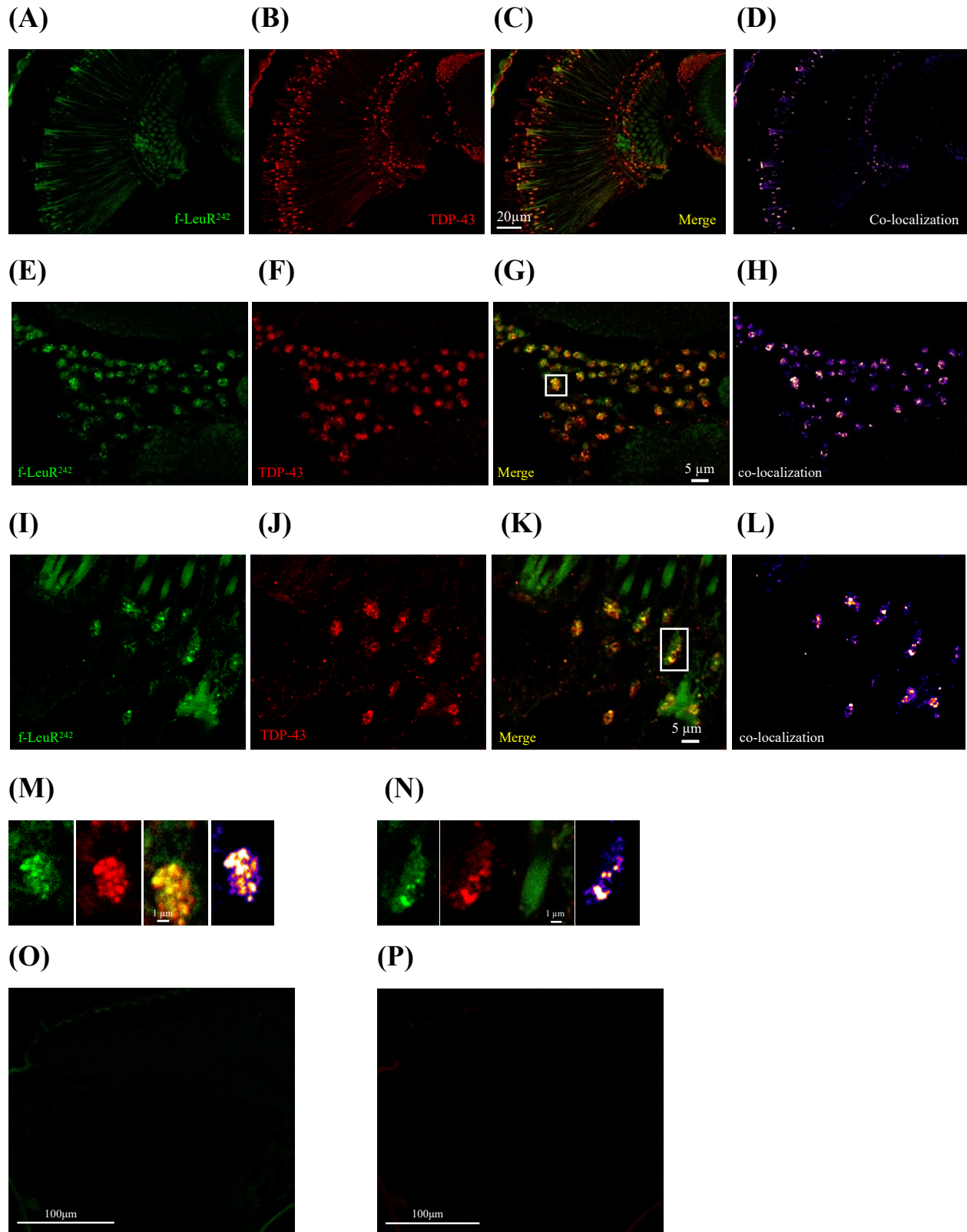


Figure 13. flag-LeuR²⁴² co-aggregates with TDP-43. Immunofluorescence using anti-flag antibody visualized in green, and anti-TDP-43 antibody visualized in red, in the brain and eye

tissue of *Elav-LeuR²⁴²;TDP-43 drosophila melanogaster*. Panels (A and B) show an overview of the brain and eye tissue of double transgenic flies with *LeuR²⁴²* visualized in green, *TDP-43* visualized in red and the panels (C and D) show the merge of *TDP-43* and *LeuR²⁴²* with their co-aggregation. Scale bar = 20 μ m. Panels (E and F; I and J) show the co-aggregation (G and H; K and L) of *TDP-43* and *LeuR²⁴²* in the brain and eye tissue, respectively, of double transgenic flies. Scale bar = 5 μ m. Panels (M and N) are a magnification of inset boxes in panels (G and K) respectively, and they show the co-aggregation of *TDP-43* and *LeuR²⁴²*. Both *TDP-43* and *LeuR²⁴²* co-aggregate in the brain and eye tissue of double transgenic flies with a distinctive phenotype consisting of dispersed punctate aggregates. Scale bar = 1 μ m. Panels (O and P) are negative controls for flag and *TDP-43* staining respectively in *Elav-LeuR²⁴²;TDP-43* flies. Scale = 100 μ m.

Chapter 4

4. Discussion

4.1 LeuR¹¹⁰ of RGNEF is critical for the interaction with TDP-43

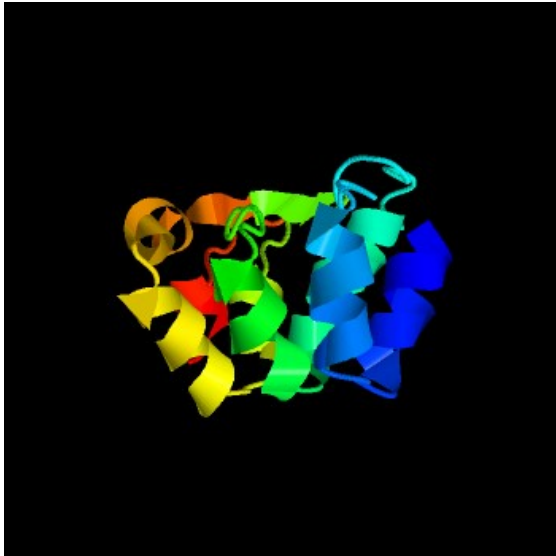
While previous data demonstrated the co-aggregation of TDP-43 and RGNEF in motor neurons of ALS patients and the interaction between the two proteins *in vitro* (Keller, et al., 2012), the question remains what domain of RGNEF is critical for the interaction. More recent *in vitro* data from our group showed the presence of an interaction between TDP-43 and LeuR²⁴² contained in the amino-terminal end of RGNEF (Droppelmann, Campos-Melo, Moszczynski, Amzil, & Strong, 2019). The presence of LeuR¹¹⁰ and ANK (Fig. 14A-B) in the LeuR²⁴² sequence and their role in protein-protein interaction led us to the hypothesis that there is a functional and biochemical interplay between RGNEF and TDP-43 where the interaction between these two proteins is regulated by a minimal domain contained within the LeuR region of RGNEF.

Here we report the presence of high molecular weight complex composed of flag-LeuR¹¹⁰ and TDP-43-myc through a crosslink experiment using a chemical crosslinker, DTSSP, sensitive to reducing conditions. In non-reducing conditions, the size of the detected complex (>250kDa) suggests that in addition to TDP-43 and LeuR¹¹⁰, other proteins are present in the complex. In reducing conditions, by breaking the disulfide bond of DTSSP, the high molecular weight complex was dissociated and only a smaller band was detected at approximately 40kDa which suggests that the interaction between LeuR¹¹⁰ and TDP-43 in the complex has been broken.

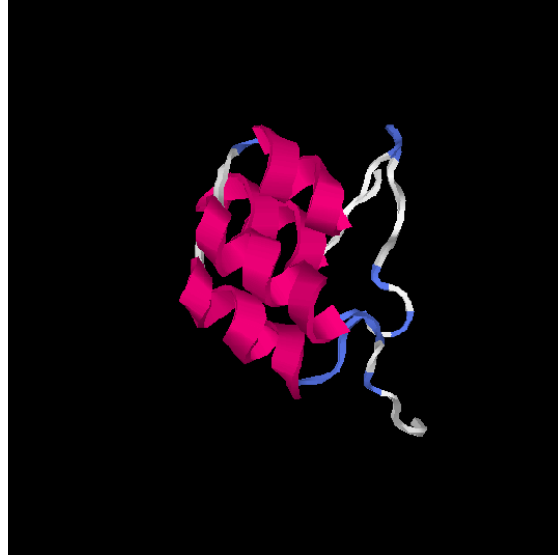
A software analysis (Fig. 14B-C) of the full length RGNEF predicts the presence of an ANK repeat between the amino-acids 162 and 229 contained in the LeuR²⁴² sequence. Given the role of the ANK in protein-protein interaction (Li, Mahajan, & Tsai, 2006), we also investigated the role of ANK in the interaction with TDP-43. However, under the same experimental conditions, we were unable to immunoprecipitate the complex containing ANK and TDP-43. These findings suggest that within the LeuR²⁴² sequence, LeuR¹¹⁰ (amino acids 97 through 206) is the minimal domain of RGNEF required for the interaction with TDP-43.

The leucine-rich repeats are evolutionary conserved in proteins and play a central role in protein-protein interaction (Kobe & Deisenhofer, 1995). Their secondary structure consisting of repeated structural units, such as α -helix, 3_{10} -helix and β -turns, is critical for their role in protein-protein interaction (Kobe & Kajava, 2000). The unique structure of LeuR¹¹⁰ consisting of eight consecutive α -helices (amino acids 97 through 206) (Fig. 14A), is consistent with the role of LeuR¹¹⁰ as the minimal domain of RGNEF involved in the interaction with TDP-43. However, whether the interaction between TDP-43 and RGNEF is direct or indirect remains unknown. While our data demonstrate that within RGNEF, LeuR¹¹⁰ is the minimal domain required for the interaction with TDP-43, in which the proteins are interacting in a complex involving at least another protein, the nature of the interaction is not clear.

(A)



(B)



(C)

LeuR ¹¹⁰	97	LARLLVTQANRLTACSHQTLTLPFALTAGALPALDEELVLA LTHLELPLEWTVLGSSSLEVSSHRESLLHLAM
ANK	97	-----
LeuR ¹¹⁰	171	RWGLAKLSQFFLCLPGGVQALALPNEEGATPLDLAL-----
ANK	171	-----GATPLDLALREGHSKLVEDVTNFGRWSPSF

Figure 14. Prediction of secondary structure of LeuR¹¹⁰ and ANK domains of RGNEF. (A) Secondary structure of LeuR¹¹⁰ showing the presence of eight α -helices (amino acids 97 through 206). (B) Secondary structure ANK showing the presence of five α -helices (amino acids 166 through 221). The model was created using I-Tasser (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>). (C) Aligned protein sequences of LeuR¹¹⁰ (amino acids 97 through 206) and ANK (amino acids 162 – 229 a small C-terminus fragment of LRR). -- indicates deleted amino acids in ANK; green indicates the leucine rich domain (amino acids 97 through 206).

4.2 The co-aggregation of LeuR and TDP-43 in transgenic *drosophila melanogaster* models

In human tissue, our lab has previously characterized the co-aggregation of RGNEF and TDP-43 in motor neurons of ALS patients (Keller, et al., 2012). *In vitro* data showed that LeuR²⁴² interacts with TDP-43 (Droppelmann, Campos-Melo, Moszczynski, Amzil, & Strong, 2019). To investigate the functional and biochemical interplay between TDP-43 and RGNEF, our lab developed *drosophila melanogaster* transgenic models co-expressing human TDP-43 and flag-LeuR²⁴² in neuronal cells. Here, by comparing the pathology of TDP-43 in the presence or absence of LeuR²⁴² in neuronal cells, we noted that TDP-43 is expressed in neuronal aggregates both in the eye and brain tissue. By comparing the brain and eye tissue of Elav-TDP43 flies to Elav-flag-LeuR²⁴², we observed a difference in the aggregates' phenotype containing TDP-43. In the presence of LeuR²⁴² there is co-aggregation of TDP-43 with LeuR²⁴² in neurons of the brain and eye tissue where the two proteins co-aggregate in small aggregates. These results suggest that LeuR²⁴² offers a cytoprotective effect against TDP-43 pathogenicity by sequestering toxic TDP-43 in aggregates. In addition to the qualitative data from our histological analysis, comparing the aggregates' phenotype in the absence or presence of LeuR²⁴², a quantitative study comparing the size of the aggregates containing TDP-43 in the brain and eye tissue of single transgenic versus double transgenic flies would help elucidate the formation of these inclusions act as a protective mechanism against TDP-43 toxicity. A crosslink IP experiment using DTSP crosslinker and protein lysates from Elav-LeuR²⁴²;TDP-43 double transgenic flies should be used to correlate our *in vivo* observations to the interaction of LeuR with TDP-43 *in vitro*.

Previous data from the literature demonstrated the toxic effect of TDP-43, resulting in a significant loss of motor functions and a decrease in the flies' lifespan (Voigt, et al., 2010) (Hanson, Wassarman, & Tibbetts, 2010) (Langellotti, et al., 2016) which led to the possibility of investigating genetic modifiers of TDP-43 and therefore possible therapeutic targets. RGNEF has previously been described to form intracellular inclusions in motor neurons of both familial and sporadic ALS patients (Droppelmann, Keller, Campos-Melo, Volkening, & Strong, 2013). The LeuR²⁴² has been shown to have a cytoprotective effect under osmotic stress conditions (Cheung, et al., 2017).

Previously, RGNEF has been identified as an ALS-protein where frameshift mutations in *ARHGEF28* encoding RGNEF are predicted to generate truncated proteins containing the LeuR region (Droppelmann, et al., 2013). However, our *in vivo* model suggests that the expression of the LeuR²⁴² offers a protective effect against TDP-43 toxicity (Withers, MSc Thesis). The data from a study comparing Elav-TDP-43 flies and D42-TDP-43 to Elav-flag-LeuR²⁴²;TDP-43 flies and D42-flag-LeuR²⁴²;TDP-43 expressing their transgenes in neuronal cells and motor neurons respectively illustrate the role of LeuR²⁴² as a modifier of TDP-43 toxicity *in vivo*. The co-expression of LeuR²⁴² and TDP-43 resulted in a significant increase in the lifespan and motor functions of double transgenic flies compared to the flies expressing TDP-43 alone. These observations alongside the pathology of TDP-43 in the presence LeuR²⁴² in this study reinforces the role of LeuR²⁴² as a modifier of TDP-43 toxicity, providing a protective effect in neurons and motor neurons. Additionally, the recent data, demonstrating the co-aggregation of LeuR with TDP-43 in a high molecular weight complex suggest that the protective effect of LeuR against TDP-43 toxicity involves at least an additional protein. However, the mechanism underlying the protective role LeuR²⁴² against TDP-43 toxicity remains unclear.

4.3 RGNEF plays a role in the regulation of ALS-associated protein TDP-43 *in vitro*

For the *in vitro* study, HEK 293T cell line was chosen because of its neuronal-like properties and amenability to transfection. Unlike the human neuroblastoma cell line SH-SY5Y in which the efficiency of transient transfection is very low, HEK 293T cells offer a high transfection efficiency and transiently maintain a high copy number of transfected plasmid DNA.

Additionally, given the endogenous expression of RGNEF's murine homolog p190rhoGEF in murine cell line neuro-2a and its stabilizing effect on murine *NEFL* mRNA (Lin, Zhai, & Schlaepfer, 2005), only HEK 293T cells were used to study the role of RGNEF in the regulation of endogenous TDP-43.

RGNEF has previously been reported as destabilizing *NEFL* mRNA through the interaction of RGNEF with *NEFL* mRNA 3'UTR (Droppelmann, Keller, Campos-Melo, Volkening, & Strong, 2013). Here we report the effect of RGNEF on the endogenous expression of TDP-43 in HEK 293T cells.

While the endogenous expression of *TARDBP* transcript in cells is not affected by the expression of full length RGNEF, the luciferase assay results demonstrate the destabilizing effect of full length RGNEF on *TARDBP* mRNA through its 3'UTR. For my thesis, the endogenous *TARDBP* mRNA expression levels were measured using RT-PCR only. While a more sensitive experiment was being set using real-time qRT-PCR, the lab and the university went under lockdown for a few months because of the pandemic and consequently only the RT-PCR experiment was completed. At the protein level, we note the down-regulatory effect of full length RGNEF and R Δ COOH on the expression levels of TDP-43 protein in cells. However, in the absence of LeuR²⁴² demonstrated by the expression levels of TDP-43 in HEK 293T cells transfected with R Δ LeuR, there was no significant effect on the regulation of TDP-43 when compared to controls. Through a dual reporter luciferase assay we measured the effect of full length RGNEF only on the luciferase activity of *TARDBP* mRNA 3'UTR, however our conclusions remain incomplete. In addition to full length RGNEF, we need to account for the effect of R Δ LeuR and R Δ COOH on *TARDBP* mRNA 3'UTR stability under the same experimental conditions.

Early studies focusing on the role of RGNEF's murine homologue p190RhoGEF *in vivo*, identified the protein's interactive properties and its role in post-transcriptional regulation of *NEFL* mRNA (Cañete-Soler, Wu, Zhai, Shamim, & Schlaepfer, 2001). Using transgenic mice expressing human *NEFL* mRNA, Schlaepfer and colleagues showed that the interaction between the C-terminal domain of p190RhoGEF and the 3'UTR region of *NEFL* mRNA resulted in the alteration of *NEFL* mRNA stability which in turn led to the post-transcriptional regulation of murine NFL protein.

With the available data confirming the interaction of TDP-43 with LeuR¹¹⁰ and LeuR²⁴², these findings suggest a regulatory mechanism by which the interaction of TDP-43 with RGNEF through the LeuR region results in the destabilization of *TARDBP* mRNA 3'UTR and consequently in the down-regulation of endogenous expression of TDP-43.

The C-terminal region of TDP-43 has been implicated in numerous protein-protein interactions (Buratti, et al., 2005) in addition to its role in the autoregulation of its protein levels through *TARDBP* mRNA 3'UTR (Ayala, et al., 2011) (Bhardwaj, Myers, Buratti, & Baralle, 2013). *In vitro* data showed that the interaction of C-terminal region of TDP-43 protein with *TARDBP* mRNA 3'UTR resulted in a decreased level of expression of TDP-43. Given our data

demonstrating the interaction between LeuR¹¹⁰ and TDP-43 alongside the destabilizing effect of RGNEF over *TARDBP* mRNA 3'UTR and the down-regulation of endogenous levels of TDP-43 in the presence of the LeuR region of RGNEF, it is possible that by binding the 3'UTR of *TARDBP* mRNA, the LeuR region of RGNEF activates the TDP-43 negative feedback loop which in turn leads to the down-regulation of its protein levels.

Our finding also suggests a possible mechanism by which LeuR acts as a modifier of pathogenic TDP-43 in our *in vivo* model. The co-aggregation of LeuR²⁴² with cytoplasmic TDP-43 in neuronal cells leads to the down-regulation its expression levels which in turn results in reducing the toxic effect of TDP-43 observed in single transgenic flies Elav-TDP-43.

4.4 Future directions

While our *in vitro* results identify the LeuR¹¹⁰ as the minimal domain of RGNEF required for the interaction with TDP-43, the mechanism by which this interaction occurs remains unclear. Here we report that the interaction of TDP-43 and LeuR¹¹⁰ is within a high molecular weight complex which suggests the presence of at least another protein in the complex. By using mass spectrometry on the purified IP complex, it will be possible to identify the proteins contained within this interactive complex therefore elucidating the mechanism by which TDP-43 and LeuR¹¹⁰ are brought into the complex. Still the question remains, is the interaction between TDP-43 and LeuR¹¹⁰ direct or indirect? To answer the question, a crystal structural analysis of this protein-protein interaction is necessary. By elucidating the atomic structure of the protein-protein interaction through X-ray crystallography and by identifying the proteins contained in the complex, we can determine the nature of the interaction between TDP-43 and LeuR¹¹⁰.

Our *in vivo* data focusing on the pathology of TDP-43 in the presence of LeuR²⁴², provides us with additional evidence regarding the protective effect of LeuR²⁴² where it co-aggregates with TDP-43. Additional experiments, analyzing the pathology of TDP-43 in the absence of LeuR alongside a biochemical study of the co-aggregation of TDP-43 and LeuR²⁴² are necessary to determine if other putative regions of RGNEF act as a modifier of TDP-43 pathogenicity.

While our findings help elucidate the effect of LeuR²⁴² over the endogenous expression of TDP-43, our experiments were limited to normal conditions. Given the cytoprotective role of LeuR²⁴² in cells under osmotic stress and its protective effect against TDP-43 toxicity *in vivo*, it

is necessary to investigate the effect of the putative regions of RGNEF beyond LeuR²⁴² under osmotic, metabolic and lactic stress conditions. Additionally, while in normal conditions, TDP-43 is mainly localized in the nucleus where it regulates numerous RNA processing pathways (Polymenidou, et al., 2011), under stress conditions TDP-43 aggregates in the cytoplasm resulting in cellular toxicity. Therefore, by analyzing the localization of RGNEF and TDP-43 in the cells, in addition to evaluating the effect putative regions of RGNEF on *TARDBP* mRNA and TDP-43, we could better understand how RGNEF acts as modifier of TDP-43.

5. Bibliography

- Abel, O., Powell, J. F., Andersen, P. M., & Al-Chalabi, A. (2012). ALSod: A user-friendly online bioinformatics tool for amyotrophic lateral sclerosis. *Human Mutation*, *33*, 1345-1351.
- Al-Chalabi, A., & Miller, C. C. (2003). Neurofilaments and neurological disease. *BioEssays*, *25*, 346-355.
- Al-Chalabi, A., Andersen, P. M., Nilsson, P., Chioza, B., Andersson, J. L., Russ, C., . . . Leigh, P. N. (1999). Deletions of the heavy neurofilament subunit tail in amyotrophic lateral sclerosis. *Human Molecular Genetics*, *8*, 157-164.
- Al-Chalabi, A., Kwak, S., Mehler, M., Rouleau, G., Siddique, T., Strong, M., & Leigh, P. N. (2013). Genetics and epigenetics of amyotrophic lateral sclerosis. *Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration*, *14*, 44-52.
- Andersen, P. M., & Al-Chalabi, A. (2011). Clinical genetics of amyotrophic lateral sclerosis. *Nature reviews. Neurology*, *7*, 603-615.
- Andersen, P. M., Sims, K. B., Xin, W. W., Kiely, R., O'Neill, G., Ravits, J., . . . Brown, R. H. (2003). Sixteen novel mutations in the Cu/Zn superoxide dismutase gene in amyotrophic lateral sclerosis: a decade of discoveries, defects and disputes. *Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders: Official Publication of the World Federation of Neurology, Research Group on Motor Neuron Diseases*, *4*, 62-73.
- Anderson, P. M. (2006). Amyotrophic lateral sclerosis associated with mutations in the CuZn superoxide dismutase gene. *Current Neurology and Neuroscience Reports*, *6*, 37-46.
- Arai, T., Hasegawa, M., Akiyami, H., Ikeda, K., Nonaka, T., Mori, H., . . . Oda, T. (2006). TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochemical and Biophysical Research Communications*, *351*, 602-611.
- Ayala, Y. M., Conti, L. D., Avendaño-Vásquez, S. E., Dhir, A., Romano, M., D'Amborgio, A., . . . Baralle, F. E. (2011). TDP-43 regulates its mRNA levels through a negative feedback loop. *The EMBO Journal*, *30*, 277-288.
- Balendra, R., & Isaacs, A. M. (2018). C9orf72-mediated ALS and FTD: multiple pathways to disease. *Nature Review: Neurology*, *14*, 544-558.

- Bansal, M., Moharir, S. C., Sailasree, S. P., Sirohi, K., Sudhakar, C., Sarathi, D. P., . . . Swarup, G. (2018). Optineurin promotes autophagosome formation by recruiting autophagy-related Atg12-5-16L1 complex to phagophores containing the Wipi2 protein. *The Journal of Biological Chemistry*, *293*, 132-147.
- Basso, M., Samengo, G., Nardo, G., Massignan, T., D'Alessandro, G., Tartari, S., . . . Bonetto, V. (2009). Characterization of detergent-insoluble proteins in ALS indicate a causal link between oxidative stress and aggregation in pathogenesis. *PLoS One*.
- Beaulieu, J. M., Robertson, J., & Julien, J. P. (1999). Interactions between peripherin and neurofilaments in cultured cells: disruption of peripherin assembly by the NF-M and NF-H subunits. *Biochemistry and Cell Biology*, *77*, 41-45.
- Bendotti, C., & Carri, M. T. (2004). Lessons from models of SOD1-linked familial ALS. *Trends in Molecular Medicine*, *10*, 393-400.
- Bendotti, C., Marino, M., Cheroni, C., Fontana, E., Crippa, V., Poletti, A., & Biasi, S. D. (2012). Dysfunction of constitutive and inducible ubiquitin-proteasome system in amyotrophic lateral sclerosis: Implication for protein aggregation and immune response. *Progress in Neurobiology*, 101-126.
- Bertolotti, A., Lutz, Y., Heard, D. J., Chambon, P., & Tora, L. (1996). hTAF(II)68, a novel RNA/ssDNA-binding protein with homology to the pro-oncoproteins TLS/FUS and EWS is associated with both TFIID and RNA polymerase II. *The EMBO Journal*, *15*, 5022-5031.
- Bhardwaj, A., Myers, M. P., Buratti, E., & Baralle, F. E. (2013). Characterizing TDP-43 interaction with its RNA targets. *Nucleic Acids Research*, *41*, 5062-5074.
- Blitterswijk, M. v., DeJesus-Hernandez, M., & Rademakers, R. (2012). How do C9ORF72 repeat expansions cause ALS and FTD: can we learn from other non-coding repeat expansion disorders? *Current Opinion in Neurology*, *25*, 689-700.
- Brooks, B. R. (1994). El Escorial World Federation of Neurology criteria for the diagnosis of amyotrophic lateral sclerosis. *Journal of the Neurological Sciences*, *124*, 96-107.
- Brooks, B. R. (1996). Natural history of ALS: Symptoms, strength, pulmonary function, and disability. *Neurology*, *47*, S71-S82.

- Brooks, B. R., Miller, R. G., Swash, M., & Munsat, T. L. (2000). El Escorial revisited: Revised criteria for the diagnosis of amyotrophic lateral sclerosis. *Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders*, 1, 293-299.
- Brown, R. H., & Al-Chalabi, A. (2017). Amyotrophic lateral sclerosis. *The New England Journal of Medicine*, 377, 162-172.
- Budini, M., Baralle, F. E., & Buratti, E. (2014). Targeting TDP-43 in neurodegenerative diseases. *Expert Opinion on Therapeutic Targets*, 18, 617-632.
- Buratti, E. (2015). Functional significance of TDP-43 mutations in disease. *Advances in Genetics*, 91, 1-53.
- Buratti, E. (2018). TDP-43 post-translational modifications in health and disease. *Expert Opinion on Therapeutic Targets*, 22, 279-293.
- Buratti, E., Brindisi, A., Giombi, M., Tisminetzky, S., Ayala, Y. M., & Baralle, F. E. (2005). TDP-43 binds heterogeneous nuclear ribonucleoprotein A/B through its C-terminal tail: An important region for the inhibition of cystic fibrosis transmembrane conductance regulator exon 9 splicing. *Journal of Biological Chemistry*, 280, 37572-37584.
- Buratti, E., Conti, L. D., Stuani, C., Romano, M., Baralle, M., & Baralle, F. (2010). Nuclear factor TDP-43 can affect selected microRNA levels. *The FEBS Journal*, 277, 2668-2681.
- Campos-Melo, D., Droppelmann, C. A., Volkening, K., & Strong, M. J. (2014). Comprehensive Luciferase-Based Reporter Gene Assay Reveals Previously Masked Up-Regulatory Effects of miRNAs. *International Journal of Molecular Sciences*, 15592-15602.
- Cañete-Soler, R., Wu, J., Zhai, J., Shamim, M., & Schlaepfer, W. W. (2001). p190RhoGEF binds to a destabilizing element in the 3' untranslated region of light neurofilament subunit mRNA and alters the stability of the transcript. *Journal of Biological Chemistry*, 276, 32046-32050.
- Carvalho, M. d., Dengler, R., Eisen, A., England, J. D., Kaji, R., Kimura, J., . . . Swash, M. (2008). Electrodiagnostic criteria for diagnosis of ALS. *Clinical Neurophysiology: Official Journal of the International Federations of Clinical Neurophysiology*, 119, 497-503.
- Charcot, J. M. (1874). De la sclérose latéral amyotrophique. *Prog. Med.*, 2, 325-327; 341-342; 453-455.

- Charcot, J. M., & Joffroy, A. (1869). Deux cas d'atrophie musculaire progressive avec lésions de la substance grise et des faisceaux antéro-latéraux de la moelle épinière. *Arch. Physiol. Neurol. Pathol.*, 2, 744-754.
- Chen, Y. Z., Bennett, C. L., Huynh, H. M., Blair, I. P., Puls, I., Irobi, J., . . . Chance, P. F. (2004). DNA/RNA helicase gene mutations in a form of juvenile amyotrophic lateral sclerosis (ALS4). *American Journal of Human Genetics*, 74, 1128-1135.
- Cheung, K., Droppelmann, C. A., MacLellan, A., Cameron, I., Withers, B., Campos-Melo, D., . . . Strong, M. J. (2017). Rho guanine nucleotide exchange factor (RGNEF) is a prosurvival factor under stress conditions. *Molecular and Cellular Neuroscience*, 82, 88-95.
- Ching, G. Y., & Liem, R. K. (1993). Assembly of type IV neuronal intermediate filaments in nonneuronal cells in the absence of preexisting cytoplasmic intermediate filaments. *Journal of Cell Biology*, 122, 1323-1335.
- Cleveland, D. W., & Rothstein, J. D. (2001). From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. *Nature Reviews Neuroscience*, 2, 806-819.
- Costa, J., Swash, M., & Carvalho, M. .. (2012). Awaji Criteria for the diagnosis of amyotrophic lateral sclerosis. *Neurological Review*, 69, 1410-1416.
- Couthouis, J., Hart, M. P., Erion, R., King, O. D., Diaz, Z., Nakaya, T., . . . Green, R. (2012). Evaluating the role of the FUS/TLS-related gene EWSR1 in amyotrophic lateral sclerosis. *Human Molecular Genetics*, 21, 2899-2911.
- Couthouis, J., Hart, M., Shorter, J., & al, e. (2011). A yeast functional screen predicts new candidate ALS disease genes. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 20881-20890.
- Côté, F., Collard, J.-F., & Julien, J.-P. (1993). Progressive neuronopathy in transgenic mice expressing the human neurofilament heavy gene: a mouse model of amyotrophic lateral sclerosis. *Cell*, 73, 35-46.
- Davidson, Y., Robinson, A. C., Liu, X., Wu, D., Troakes, C., Rollinson, S., . . . Mann, D. M. (2016). Neurodegeneration in frontotemporal lobar degeneration and motor neuron disease associated with expansions in C9orf72 is linked to TDP-43 pathology and not associated with aggregated forms of dipeptide repeat proteins. *Neuropathology and Applied Neurobiology*, 42, 242-254.

- DeJesus-Hernandez, M., Mackenzie, I. R., Boeve, B. F., & al., e. (2011). Expanded GGGGCC hexanucleotide repeat in noncoding regions of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron*, 72, 245-256.
- Deng, H., Gao, K., & Jankovic, J. (2014). The role of FUS gene variants in neurodegenerative diseases. *Nature reviews. Neurology*, 10, 337-348.
- Deng, H.-X., Chen, W., Hong, S.-T., Boycott, K. M., Gorrie, G. H., Siddique, N., . . . Siddi. (2012). Mutations in UBQLN2 cause dominant X-linked juvenile and adult onset ALS and ALS/dementia. *Nature*, 477, 211-215.
- Deng, H.-X., Zhai, H., Bigio, E. H., Yan, J., Fecto, F., Arjoud, K., . . . Siddique, T. (2010). FUS-immunoreactive inclusions are common features in sporadic and non-SOD1 familial amyotrophic lateral sclerosis. *Annals of Neurology*, 67, 739-748.
- Dolan, J., Walshe, K., Alsbury, S., Hokamp, K., Okeeffe, S., Okafuji, T., . . . Mitchell, K. J. (2007). The extracellular leucine-rich repeat superfamily; A comparative survey and analysis of evolutionary relationships and expression patterns. *BMC Genomics*, 8, 320.
- Droppelmann, C. A., Campos-Melo, D., Moszczynski, A. J., Amzil, H., & Strong, M. J. (2019). TDP-43 aggregation inside micronuclei reveals a potential mechanism for protein inclusion formation in ALS. *Scientific Reports*, 9, 19928.
- Droppelmann, C. A., Campos-Melo, D., Volkening, K., & Strong, M. J. (2014). The emerging role of guanine nucleotide exchange factors in ALS and other neurodegenerative diseases. *Frontiers in Cellular Neuroscience*, 8, 1-14.
- Droppelmann, C. A., Keller, B. A., Campos-Melo, D., Volkening, K., & Strong, M. J. (2013). Rho guanine nucleotide exchange factor is an NFL mRNA destabilizing factor that forms cytoplasmic inclusions in amyotrophic lateral sclerosis. *Neurobiology of Aging*, 34, 248–262.
- Droppelmann, C. A., Wang, J., Campos-Melo, D., Keller, B., Volkening, K., Hegele, R. A., & Strong, M. J. (2013). Detection of a novel frameshift mutations and regions with homozygosity within ARHGAP28 gene in familial amyotrophic lateral sclerosis. *Amyotrophic Lateral Sclerosis & Frontotemporal Degeneration*, 14, 444-451.
- Ederle, H., & Dormann, D. (2017). TDP-43 and FUS en route from the nucleus to the cytoplasm. *FEBS letters*, 591, 1489-1507.

- Elder, G. A., Jr, V. L., Kang, C., Bosco, P., Gourav, A., Tu, P. H., . . . Lazzarini, R. A. (1998). Requirement of heavy neurofilament subunit in the development of axons with large calibers. *The Journal of Cell Biology*, *143*, 195-205.
- Fifita, J. A., Williams, K. L., Sundaramoorthy, V., Mccan, E. P., Nicholson, G. A., Atkin, J. D., & Blair, I. P. (2017). A novel amyotrophic lateral sclerosis mutation in OPTN induces ER stress and golgi fragmentation in vitro. *Amyotrophic Lateral Sclerosis and Frontotemporal degeneration*, *18*, 126-133.
- Forsberg, K., Graffmo, K., Pakkenberg, B., Weber, M., Neilsen, M., Marklund, S., . . . Andersen, P. M. (2019). Misfolded SOD1 inclusions in patients with mutations on C9orf72 and other ALS/FTD-associated genes. *Journal of Neurology, Neurosurgery & Psychiatry*, *90*, 861-869.
- Ge, W. W., Strong, W., Laystra-Lantz, C., & Strong, M. J. (2005). Mutant copper-zinc superoxide dismutase binds to and destabilizes human low molecular weight neurofilament mRNA. *The Journal of Biological Chemistry*, *280*, 118-124.
- Ge, W.-W., Leystra-Lantz, C., Wen, W., & Strong, M. J. (2003). Selective loss of trans-acting instability determinants of neurofilament mRNA in amyotrophic lateral sclerosis spinal cord. *The Journal of biological chemistry*, *278*, 26558-26563.
- Ghasemi, M., & Jr., R. H. (2018). Genetics of amyotrophic lateral sclerosis. *Cold Spring Harbor Perspectives in Medicine*, *8*, a024125.
- Ghiglion, C., III, K. L., Amundadottir, L. T., Boswell, R. E., Perrimon, N., & Duffy, J. B. (1999). The transmembrane molecule Kekk1 acts in a feedback loop to negatively regulate the activity of the Drosophila EGF receptor during oogenesis. *Cell*, *96*, 847-856.
- Grad, L. I., Rouleau, G. A., Ravits, J., & Cashman, N. R. (2017). Clinical spectrum of amyotrophic lateral sclerosis (ALS). *Cold Spring Perspectives in Medicine*, *7*, a024117.
- Greenway, M. J., Alexander, M. D., Ennis, S., Traynor, B. J., Corr, B., Frost, E., . . . Hardiman, O. (2004). A novel candidate region for ALS chromosome 14q11.2. *Neurology*, *63*, 1936-1938.
- Greenway, M. J., Andersen, P. M., Russ, C., Ennis, S., Cashman, S., Donaghy, C., . . . Hardiman, O. (2006). ANG mutations segregate with familial and 'sporadic' amyotrophic lateral sclerosis. *Nature Genetics*, *38*, 411-413.

- Gros-Louis, F., Larivière, R., Gowing, G., Laurent, S., Camu, W., Bouchard, J.-P., . . . Julien, J.-P. (2004). A frameshift deletion in peripherin gene associated with amyotrophic lateral sclerosis. *The Journal of Biological Chemistry*, *279*, 45951-45956.
- Guan, J., & Shalloway, D. (1992). Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature*, *358*, 690-692.
- Gurney, M. E., Pu, H., Chiu, A. Y., Dal Canto, M. C., Polchow, C. Y., Alexander, D. D., . . . Deng, H. X. (1994). Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science*, *264*, 1772-1775.
- Haegawa, M., Arai, T., Nonaka, T., Kametani, F., Yoshida, M., Hashizume, Y., . . . Akiyama, H. (2008). Phosphorylated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Annals of Neurology*, *60*-70.
- Hand, C. K., Khoris, J., Salachas, F., Gros-Louis, F., Lopes, A. A., Mayeux-Portas, V., . . . Rouleau, G. A. (2002). A novel locus for familial amyotrophic lateral sclerosis on chromosome 18q. *American Journal of Human Genetics*, *70*, 251-256.
- Hanson, K. A., Wassarman, S. H., & Tibbetts, R. A. (2010). Ubiquitin modifies TDP-43 toxicity in a drosophila model of amyotrophic lateral sclerosis (ALS). *Journal of Biological Chemistry*, *285*, 11068-11072.
- Harding, A. E., Thomas, P. K., Bradbury, P. G., Morgan-Hughes, J. A., & Ponsford, J. R. (1982). X-linked recessive bulbospinal neuropathy: a report of 10 cases. *Neurology, Neurosurgery & Psychiatry*, *45*, 1012-1019.
- Hirano, A., Donnenfeld, H., Sasaki, S., & Nakano, I. (1984). Fine structural observations of neurofilamentous changes in amyotrophic lateral sclerosis. *Journal of Neuropathology and Experimental Neurology*, *43*, 461-470.
- Hoffman, P. N., & Lasek, R. J. (1975). The slow component of axonal transport. Identifications of the major structural polypeptides of the axon and their generality among mammalian neurons. *Journal of Cell Biology*, *66*, 351-366.
- Hosp, F. V., Arumughan, A., Wyler, E., Consortium, G. a., Landthaler, M., Hubner, N., . . . Selbach, M. (2015). Quantitative interaction proteomics of neurodegenerative disease proteins. *Cell Reports*, *11*, 1134-1146.
- Hudson, A. J. (1981). Amyotrophic lateral sclerosis and its association with dementia, parkinsonism and other neurological disorders: A review. *Brain*, *104*, 217-247.

- Igaz, L. M., Kwong, L. K., Chen-Plotkin, A., Unger, M. J., Xu, Y., Neumann, M., . . . Lee, V. M. (2009). Expression of TDP-43 C-terminal fragments in vitro recapitulates pathological features of TDP-43 proteinopathies. *The Journal of biological chemistry*, *284*, 8516-8524.
- Iko, Y., Kodama, T. S., Kasai, N., Oyama, T., Morita, E. H., Muto, T., . . . Morikawa, K. (2004). Domain architectures and characterization of an RNA-binding protein, TLS. *The Journal of Biological Chemistry*, *279*, 44834-44840.
- Johnson, J. O., Pioro, E. P., Boehringer, A., Chia, R., Feit, H., Renton, A. E., . . . Traynor, B. J. (2014). Mutations in Matrin 3 gene cause familial amyotrophic lateral sclerosis. *Nature Neuroscience*, *17*, 664-666.
- Kapeli, K., Pratt, G., Vu, A. Q., Hutt, K. R., Martinez, F. J., Sundararaman, B., . . . Hoon, S. (2016). Distinct and shared functions of ALS-associated proteins TDP-43, FUS and TAF15 revealed by multisystem analyses. *Nature Communications*, *7*, 12143.
- Keller, B. A., Volkening, K., Droppelmann, C. A., Ang, L. C., Rademakers, R., & Strong, M. J. (2012). Co-aggregation of RNA binding proteins in ALS spinal motor neurons: evidence of a common pathogenic mechanism. *Acta Neuropathologica*, *124*, 733–747.
- Kennedy, W. R., Alter, M., & Sung, J. H. (1968). Progressive proximal spinal and bulbar muscular atrophy of late onset. *Neurology*, *18*, 671-680.
- Kim, H. J., Kim, N. C., Wang, Y. D., Scarborough, E. A., Moore, J., Diaz, Z., . . . Trojanowki, J. Q. (2013). Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS. *Nature*, *495*, 467-473.
- Kino, Y., Washizu, C., Kurosawa, M., Yamada, M., Miyazaki, H., Akagi, T., . . . Nukina, N. (2015). FUS/TLS deficiency causes behavioural and pathological abnormalities distinct from amyotrophic lateral sclerosis. *Acta Neuropathologica Communications*, *3*, 24.
- Kobe, B., & Deisenhofer, J. (1993). Crystal structure of porcine ribonuclease inhibitor, a protein with leucine-rich repeats. *Nature*, *366*, 751-756.
- Kobe, B., & Deisenhofer, J. (1995). A structural basis of the interactions between leucine-rich repeats and protein ligands. *Nature*, 183-186.
- Kobe, B., & Kajava, A. V. (2000). When protein folding is simplified to protein coiling: the continuum of solenoid protein structures. *Trends in Biochemical Sciences*, *25*, 509-515.

- Kornberg, L., Earp, H. S., Parsons, J. T., Schaller, M., & Juliano, R. L. (1992). Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. *The Journal of Biological Chemistry*, 23439-23442.
- Kucherenko, M. M., Marrone, A. K., Rishko, V. M., Yatsenko, A. S., Klepzig, A., & Shcherbata, H. R. (2010). Paraffin-embedded and frozen sections of Drosophila adult muscles. *Journal of Visualized Experiments*, 1-3.
- Kwiatkowski, T. J., Bosco, D. A., Leclerc, A. L., Tamrazian, E., Vanderburg, C. R., Russ, C., . . . Jr, R. (2009). Mutations in the FUS/TLS gene in chromosome 16 cause familial amyotrophic lateral sclerosis. *Science*, 323, 1205-1208.
- Kwon, I., Kato, M., Xiang, S., Wu, L., Theodoropoulos, P., Mirzaei, H., . . . McKnight, S. L. (2013). Phosphorylation-regulated binding of RNA polymerase II to fibrous polymers of low-complexity domains. *Cell*, 155, 1049-1060.
- Lambrechts, D., Robberecht, W., & Carmeliet, P. (2007). Heterogeneity in motoneuron disease. *Trends in Neurosciences*, 30, 536-544.
- Langellotti, S., Romano, V., Romano, G., Klima, R., Feiguin, F., Cragnaz, L., . . . Baralle, F. E. (2016). A novel Drosophila model of TDP-43 proteinopathies: N-terminal sequences combined with the Q/N domain induce protein functional loss and locomotion defects. *Disease Models & Mechanisms*, 9, 659-669.
- Lee, M. K., Xu, Z., Wong, P. C., & Cleveland, D. W. (1993). Neurofilaments are obligate heteropolymers in vivo. *Journal of Cell Biology*, 122, 1337-1350.
- Li, C., Ji, Y., Tang, L., Zhang, N., He, J., Ye, S., . . . Fan, D. (2015). Optineurin mutations in patients with sporadic amyotrophic lateral sclerosis in China. *Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration*, 16, 485-489.
- Li, J., Mahajan, A., & Tsai, M.-D. (2006). Ankyrin Repeat: A Unique Motif Mediating Protein-Protein Interactions. *Biochemistry*, 15168-15178.
- Liem, R. K., Yen, S.-H., Salomon, G. D., & Shelanski, M. L. (1978). Intermediate filaments in nervous tissues. *Journal of Cell Biology*, 79, 637-645.
- Lin, H., Zhai, J., & Schlaepfer, W. W. (2005). RNA-binding protein is involved in aggregation of light neurofilament protein and is implicated in the pathogenesis of motor neuron degeneration. *Human Molecular Genetics*, 14, 3643-3659.

- Ling, S. C., Polymenidou, M., & Cleveland, D. W. (2013). Converging mechanisms in ALS and FTD: Disrupted RNA and protein homeostasis. *Neuron*, *79*, 416-438.
- Lomen-Hoerth, C., Anderson, T., & Miller, B. (2002). The overlap of amyotrophic lateral sclerosis and frontotemporal dementia. *Neurology*, *59*, 1077-1079.
- Longinetti, E., & Fang, F. (2019). Epidemiology of amyotrophic lateral sclerosis: an update of recent literature. *Current Opinion in Neurology*, *32*, 771-776.
- Lui, J., Lillo, C., Jonsson, P. A., Valde, C. V., Ward, C., Miller, T. M., . . . Cleveland, D. W. (2004). Toxicity of familial ALS-linked SOD1 mutants from selective recruitment of spinal mitochondria. *Neuron*, *43*, 5-17.
- Lukavsky, P. J., Daujotyte, D., Tollervey, J. R., Ule, J., Stuani, C., Buratti, E., . . . Allain, F. H.-T. (2013). Molecular basis of UG-rich RNA recognition by the human splicing factor TDP-43. *Nature Structural & Molecular Biology*, *20*, 1443-1449.
- Ma, Y., Tang, L., Chen, L., Zhang, B., Deng, P., Wang, J., . . . Fan, D. (2014). ARHGEF28 gene exon 6/intron 6 junction mutations in chinese amyotrophic lateral sclerosis cohort. *Amyotrophic Lateral Sclerosis & Frontotemporal Degeneration*, *15*, 309-311.
- Mackay, D. J., & Hall, A. (1998). Rho GTPases. *The Journal of Biological Chemistry*, *273*, 20685-20688.
- Mackenzie, I. R., Rademakers, R., & Neumann, M. (2010). TDP-43 and FUS in amyotrophic lateral sclerosis and frontotemporal dementia. *The Lancet Neurology*, *9*, 995-1007.
- Magnussen, M. J., & Glass, J. D. (2017). Chapter 2 - Natural history of amyotrophic lateral sclerosis. In N. Boulis, D. O'Connor, & A. Donsante, *Molecular and Cellular Therapies for Motor Neuron Diseases* (pp. 25-41). Atlanta: Academic Press.
- Maruyama, H., Morino, H., Ito, H., Izumi, Y., Kato, H., Watanabe, Y., . . . Kawakami, H. (2010). Mutations of optineurin in amyotrophic lateral sclerosis. *Nature*, *465*, 223-226.
- Masuda, A., Takeda, J., Okuno, T., Ohkawara, B., Ito, M., Ishigaki, S., . . . Ohno, K. (2015). Position-specific binding of FUS to nascent RNA regulates mRNA length. *Genes & Development*, *29*, 1045-57.
- Matsushima, N., Tanaka, T., Enkhbayar, P., Mikami, T., Taga, M., Yamada, K., & Kuroki, Y. (2007). Comparative sequence analysis of Leucine-rich-repeats (LRRs) within vertebrate toll-like receptors. *BMC Genomics*, *8*, 124.

- Migliore, L., Cappodè, F., Fenech, M., & Thomas, P. (2011). Association of micronucleus frequency with neurodegenerative diseases. *Mutagenesis*, *26*, 85-92.
- Miller, J. W., Urbinati, C. R., Teng-Umuay, P., Stenberg, M. G., Byrne, B. J., Thornton, C. A., & Swanson, M. S. (2000). Recruitment of human muscleblind proteins to (CUG)(n) expansions associated with myotonic dystrophy. *The EMBO Journal*, *19*, 4439-4448.
- Miller, N. L., Lawson, C., Chen, X. L., Ssang-Taek, L., & Schlaepfer, D. D. (2012). Rgnef (p190RhoGEF) knockout inhibits RhoA activity, focal adhesion establishment, and cell motility downstream of integrins. *PLoS ONE*, *7*, e37830.
- Miller, N. L., Lawson, C., Kleinschmidt, E. G., Tancioni, I., Uryu, S., & Schlaepfer, D. D. (2013). A non-canonical role for Rgnef in promoting integrin-stimulated focal adhesion kinase activation. *Journal of Cell Science*, *126*, 5074-5085.
- Mitchell, J. C., McGoldrick, P., Vance, C., Hortobagyi, T., Sreedharan, J., Rogelj, B., . . . Shaw, C. E. (2013). Overexpression of human wild-type FUS causes progressive motor neuron degenerations in an age- and dose-dependant fashion. *Acta Neuropathologica*, *125*, 273-288.
- Mori, K., Weng, S. M., Arzberger, T., May, S., Rentzsch, K., Kremmer, E., . . . Edbauer, D. (2013). The C9orf72 GGGGCC repeat is translated into aggregating dipeptide-repeat proteins in FTL/ALS. *Science*, *339*, 1335-1338.
- Morohoshi, F., Arai, K., Takahashi, E., Tanigami, A., & Ohki, M. (1996). Cloning and mapping of a human RBP56 Gene encoding a putative RNA binding protein similar to FUS/TLS and EWS proteins. *Genomics*, *38*, 51-57.
- Munch, C., Sedlmeier, R., Meyer, T., Homberg, V., Sperfeld, A. D., Kurt, A., . . . Ludolph, A. C. (2004). Point mutations of the p150 subunit of dynactin (DCTN1) gene in ALS. *Neurology*, *63*, 724-726.
- Munoz, D. G., Green, C., Perl, D. P., & Selkoe, D. J. (1988). Accumulation of phosphorylated neurofilaments in the anterior horn motor neurons of amyotrophic lateral sclerosis patients. *Journal of Neuropathology and experimental Neurology*, *47*, 9-18.
- Neumann, M., Sampathu, D. M., Kwong, L. K., Truax, A. C., Micsenyi, M. C., Chou, T. T., . . . Le. (2006). Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science*, *314*, 130-133.

- Osawa, T., Mizuno, Y., Fujita, Y., Takatama, M., Nakazato, Y., & Okamoto, K. (2011). Optineurin in neurodegenerative diseases. *Neuropathology*, *31*, 569-574.
- Pachter, J. S., & Liem, R. K. (1985). alpha-internexin, a 66-kD intermediate filament-binding protein from mammalian central nervous system. *Journal of Cell Biology*, *101*, 1316-1322.
- Polymenidou, M., Lagier-Tourenne, C., Hutt, K. R., Huelga, S. C., Moran, J., Liang, T. Y., . . . Cleveland, D. W. (2011). Long pre-mRNA depletion and RNA missplicing contribute to neuronal vulnerability from loss of TDP-43. *Nature Neuroscience*, *14*, 459-468.
- Portier, M. M., de Néchaud, B., & Gros, F. (1983). Peripherin, a new member of the intermediate filament protein family. *Developmental Neuroscience*, *6*, 335-344.
- Renton, A. E., Majounie, E., Waite, A., Simón-Sánchez, J., Rollinson, S., Gibbs, J. R., . . . Traynor, B. J. (2011). A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. *Neuron*, *72*, 257-268.
- Restagno, G., Gomez, A., Lombardo, F., Cocco, E., Calvo, A., Ghiglione, P., . . . Chiò, A. (2005). The IVS1 +319 t>a of SOD1 gene is not an ALS causing mutation. *Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders: Official Publication of the World Federation of Neurology, Research Group on Motor Neuron Diseases*, *6*, 45-49.
- Rezaie, T., & Sarfarazi, M. (2005). Molecular cloning, genomic structure, and protein characterization of mouse optineurin. *Genomics*, *85*, 131-138.
- Rezaie, T., Waitzman, D. M., Kaufman, J. L., & Sarfarazi, M. (2005). Molecular cloning and expression profiling of optineurin in the rhesus monkey. *Investigative Ophthalmology & Visual Science*, *46*, 2404-2410.
- Robinson, J. L., Geser, F., Steiber, A., Umoh, M., Kwong, L. K., Deerlin, V. M., . . . Trojanowski, J. Q. (2013). TDP-43 Skeins show properties of amyloid in a subset of ALS cases. *Acta Neuropathologica*, 121-131.
- Rolls, M. M., & Jegla, T. J. (2015). Neuronal polarity: an evolutionary perspective. *The Journal of Experimental Biology*, *218*, 572-580.
- Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., . . . (1993). Mutations in Cu/Zn superoxide dismutase gene are associates with familial amyotrophic lateral sclerosis. *Nature*, *362*, 59-62.

- Rowland, L., & Shneider, N. A. (2001). Amyotrophic lateral sclerosis. *The New England Journal of Medicine*, *344*, 1688-1700.
- Sako, W., Ito, H., Yoshida, M., Koizumi, H., Kamada, M., Fujita, K., . . . Kaji, R. (2012). Nuclear factor κB expression in patients with sporadic amyotrophic lateral sclerosis and hereditary amyotrophic lateral sclerosis with optineurin mutations. *Clinical Neuropathology*, *31*, 418-423.
- Sapp, P. C., Hosler, B. A., McKenna-Yasek, D., Chin, W., Gann, A., Genise, H., . . . Brown, R. H. (2003). Identification of two novel loci for dominantly inherited familial amyotrophic lateral sclerosis. *American Journal of Human Genetics*, *73*, 397-403.
- Shaw, G., Morse, S., Arabat, M., & Graham, F. L. (2002). Preferential transformation of human neuronal cells by human adenoviruses and the origin of HEK 293 cells. *The FASEB Journal*, *16*, 869-871.
- Sofola, O. A., Jin, P., Qin, Y., Duan, R., Liu, H., Haro, M. d., . . . Botas, J. (2007). RNA-Binding proteins hnRNP A2/B1 and CUGBP1 suppress fragile X CGG premutation repeat-induced neurodegeneration in a Drosophila model of FXTAS. *Neuron*, *55*, 565-571.
- Song, Y., Lin, F., Ye, C.-h., Huang, H., Li, X., Yao, X., . . . Wang, C. (2020). Rare, low-frequency and common coding variants of ARHGEF28 gene and their association with sporadic amyotrophic lateral sclerosis. *Neurobiology of Aging*, *87*, 138.e1-138.e6.
- Sorarù, G., Ermani, M., Logroscino, G., D'Ascenzo, C., Orsetti, V., Volpe, M., . . . Angelini, C. (2009). Natural history of upper motor neuron-dominant ALS. *Amyotrophic Lateral Sclerosis*, *11*, 424-429.
- Sreedharan, J., Blair, I. P., Tripathi, V. B., Hu, X., Vance, C., Rogelj, B., . . . Shaw, C. E. (2008). TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science*, *319*, 1668-1672.
- Strong, M. J. (2010). The evidence for altered RNA metabolism in amyotrophic lateral sclerosis (ALS). *Journal of the Neurological Sciences*, *288*, 1-12.
- Strong, M. J., Abrahams, A., Goldstein, L. H., Woolley, S., McLaughlin, P., Snowden, J., . . . Turner, M. R. (2017). Amyotrophic lateral sclerosis - frontotemporal spectrum disorder (ALS-FTSD): Revised diagnostic criteria. *Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration*, *18*, 153-173.

- Strong, M. J., Grace, G. M., Freedman, M., Lomen-Hoerth, C., Woolley, S., Goldstein, L. H., . . . Figlewicz, D. (2009). Consensus criteria for the diagnosis of frontotemporal cognitive and behavioural syndromes in amyotrophic lateral sclerosis. *Amyotrophic Lateral Sclerosis*, *10*, 131-146.
- Strong, M. J., Volkening, K., Hammond, R., Yang, W., Strong, W., Leystra-Lantz, C., & Shoesmith, C. (2007). TDP-43 is a human low molecular weight neurofilament (hNFL) mRNA-binding protein. *Molecular and Cellular Neuroscience*, *35*, 320-327.
- Takahashi, Y., Fukuda, Y., Yoshimura, J., Toyoda, A., Kurppa, K., Moritoyo, H., . . . Tsuji, S. (2013). ERBB4 mutations that disrupt the neuregulin-ErbB4 pathway cause amyotrophic lateral sclerosis type 19. *American Journal of Human Genetics*, *93*, 900-905.
- Ticozzi, N., Vance, C., LeClerc, A. L., Keagle, P., Glass, J. D., McKenna-Yasek, D., . . . Landers, J. E. (2011). Mutational analysis reveals the FUS homolog TAF15 as a candidate gene for familial amyotrophic lateral sclerosis. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics: The Official Publication of the International Society of Psychiatric Genetics*, *156B*, 285-290.
- Troost, D., Smith, P. A., Jong, J. M., & Swaab, D. F. (1992). Neurofilament and glial alterations in the cerebral cortex in amyotrophic lateral sclerosis. *Acta Neuropathologica*, *84*, 664-673.
- Vaibhava, V., Nagabhushana, A., Chalasani, M. L., Sudhakar, C., Kumari, A., & Swarup, G. (2012). Optineurin mediates a negative regulation of Rab8 by the GTPase-activating protein TBC1D17. *Journal of Cell Science*, *125*, 5026-5039.
- van Horck, F. P., Ahmadian, M. R., Haeusler, L. C., Moolenaar, W. H., & Kranenburg, O. (2001). Characterizations of p190RhoGEF, a RhoA-specific guanine nucleotide exchange factor that interacts with microtubules. *The Journal of Biological Chemistry*, *276*, 4948-4956.
- Vance, C., Al-Chalabi, A., Rudy, D., Smith, B. N., Hu, X., Sreedharan, J., . . . Shaw, C. E. (2006). Familial amyotrophic lateral sclerosis with frontotemporal dementia is linked to a locus on chromosome 9p13.2-21.3. *Brain*, *129*, 868-876.
- Vilariño-Güell, C., Wider, C., Soto-Ortolaza, A. I., Cobb, S. A., Kachergus, J. M., Keeling, B. H., . . . Farrer, M. J. (2009). Characterization of DCTN1 genetic variability in neurodegeneration. *Neurology*, *72*, 2024-2028.

- Voigt, A., Heholz, D., Fiesel, F. C., Kaur, K., Müller, D., Kersten, P., . . . Schulz, J. B. (2010). TDP-43-mediated neuron loss in vivo requires RNA-binding activity. *PLoS One*, *5*, e12247.
- Volkening, K., Leyster-Lantz, C., Yang, W., Jaffee, H., & Strong, M. J. (2009). Tar DNA binding protein of 43 kDa (TDP-43), 14-3-3 proteins and copper/zinc superoxide dismutase (SOD1) interact to modulate NFL mRNA stability. Implications for altered RNA processing in amyotrophic lateral sclerosis (ALS). *Brain Research*, *1305*, 168-182.
- Volkening, K., Leystra-Lantz, C., & Strong, M. J. (2010). Human low molecular weight neurofilament (NFL) mRNA interacts with a predicted p190RhoGEF homologue (RGNEF) in humans. *Amyotrophic Lateral Sclerosis*, *11*, 97-103.
- White, M. C., Gao, R., Xu, W., Mandal, S. M., Lim, J. G., Hazra, T. K., . . . Ashizawa, T. (2010). Inactivation of hnRNP K by expanded intronic AUUCU repeat induces apoptosis via translocation of PKCdelta to mitochondria in spinocerebellar ataxia 10. *PLoS Genetics*, *6*, e1000984.
- Wong, N. K., He, B. P., & Strong, M. J. (2000). Characterization of neuronal intermediate filament protein expression in cervical spinal neurons in sporadic amyotrophic lateral sclerosis. *Journal of Neuropathology and Experimental Neurology*, *59*, 972-982.
- Yang, L., Gal, J., Chen, J., & Zhu, H. (2014). Self-assembled FUS binds active chromatin and regulates gene transcription. *Proceedings of the National Academy of Sciences of the United States of America*, *111*, 17809-17814.
- Yedavalli, V. S., Patil, A., & Shah, P. (2018). Amyotrophic Lateral Sclerosis and its Mimics/Variants: A Comprehensive Review. *Journal of Clinical Imaging Science*, *8*, 53.
- Ying, H., & Yue, B. Y. (2016). Optineurin: the autophagy connection. *Experimental Eye Research*, *144*, 73-80.
- Yuan, A., Rao, M. V., Sasaki, T., Chen, Y., Kumar, A., Veeranna, . . . Nixon, R. A. (2006). α -internexin is structurally and functionally associated with neurofilament triplet proteins in the mature CNS. *The Journal of Neuroscience*, *26*, 10006-10019.
- Zarei, S., Carr, K., Reiley, L., Diaz, K., Guerra, O., Altamirano, P. F., . . . Chinea, A. (2015). A comprehensive review of amyotrophic lateral sclerosis. *Surgical Neurology International*, *6*, 171.

Zhai, J., Lin, H., Nie, Z., Wu, J., Cañete-Soler, R., Schlaepfer, W. W., & Schlaepfer, D. D.

(2003). Direct interaction of Focal Adhesion Kinase with p190RhoGEF. *The Journal of Biological Chemistry*, 278, 24865-24873.

Zu, T., Gibbens, B., Doty, N. S., Gomes-Pereira, M., Huguet, A., Stone, M. D., Ranum, L.

P. (2011). Non-ATG-initiated translation directed by microsatellite expansions. *PNAS*, 108, 260-265.

Curriculum Vitae

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2009/9 - 2013/7 Bachelor's, Biochemistry Molecular Medicine, Université de Montréal

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2007/9 Diploma, Medicine, Catholic University of Louvain (French)

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2017/2 - 2017/6 Tutor

Tutor Doctor

2015/8 - 2016/12 Tutor

Succès Scolaire

2013/8 - 2015/7 Research Assistant
Cancer Research, McGill, Lady Davis Institute for Medical Research

2011/8 - 2011/5 Research Apprentice
Neuroscience research, NYU, Langone Health Neuroscience Institute

Affiliations

2018/9 M.Sc Candidate, University of Western Ontario

Poster Presentations

Pathology Research Day - March 28, 2019:

Determination of the Leucine rich (LeuR) domain of Rho Guanine Nucleotide Exchange Factor's (RGNEF) role in the regulation of ALS related protein TDP-43.

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Keystone Symposia RNA-Protein interaction - February 24-28, 2019:

Determination of the Leucine rich (LeuR) domain of Rho Guanine Nucleotide Exchange Factor's (RGNEF) role in the regulation of ALS related protein TDP-43.

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ALS Canada - April 28-30, 2018:

Determination of interaction regions between Rho guanine nucleotide exchange factor (RGNEF) and TDP-43 using *in vitro* and *in vivo* analyses.

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Publications

Droppelmann CA, Campos-Melo D, Moszczynski AJ, Amzil H, Strong MJ. *TDP-43 aggregation inside micronuclei reveals a potential mechanism for protein inclusion formation in ALS*. Sci Rep. 2019 Dec 27;9(1):19928. doi: 10.1038/s41598-019-56483-y.

Krikor B., Dominik W., Su J., Amzil H., Nicolas M., Alaoui-Jamali M. *Novel Aurora A and Protein kinase C (α , $\beta 1$, $\beta 2$, Θ) multi-target inhibitors: Impact of selenium atoms on the potency and selectivity*. Submitted.