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SMALL MOLECULE-MEDIATED UPREGULATION OF G3BP1 AS A THERAPY FOR ALS

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SMALL MOLECULE-MEDIATED UPREGULATION OF G3BP1

AS A THERAPY FOR ALS

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Résumé

Les troubles neurodégénératifs, tels que la sclérose latérale amyotrophique (SLA) et la démence frontotemporale (DFT), ont été associés aux protéines de liaison à l'ARN (RBP). Les principales caractéristiques de la SLA sont l'agrégation d'une protéine de liaison à l'ARN appelée protéine de liaison TAR (TDP-43). Il a été démontré que TDP-43 se lie à *G3BP1*, un facteur de nucléation pour l'assemblage des granules de stress, pour le stabiliser. Les granules de stress sont des structures séparées par phases qui se forment dans des conditions stressantes et favorisent la survie cellulaire. Une altération de l'assemblage des granules de stress et une réduction du *G3BP1* sont signalées dans la SLA. Cette réduction est due à un défaut dans les transcriptions codantes pour *G3BP1* stabilisant TDP-43. Par conséquent, une réponse défaillante des granules de stress pourrait jouer un rôle majeur dans la maladie. Ainsi, ce projet de recherche se concentre sur la restauration de G3BP1, dont la déplétion est liée à la perte de fonction de TDP-43. En utilisant des composés de petites molécules identifiés lors d'une campagne de dépistage de médicaments, nous cherchons à augmenter l'expression de G3BP1, rétablissant ainsi le mécanisme SG endogène et favorisant la survie neuronale. La découverte de candidats principaux (NPX-047, NPX-000-115 et NPX-001-280) qui sauvent efficacement l'expression et la fonction de G3BP1 est prometteuse pour des thérapies potentielles contre la SLA. Ces composés ont été testés sur des cellules SHSY5Y traitées avec du si-TDP, mais aucune récupération de l'ARNm de *G3BP1* n'a été observée malgré des niveaux plus élevés de signaux de luciférase. Ainsi, une enquête approfondie sur les divergences dans nos résultats constitue notre prochaine étape, ce qui n'a pas été possible pendant la durée limitée de cette mémoire. De plus, les cibles non ciblées de ces composés seront étudiées à l'aide du séquençage Bru Chase. Dans l'ensemble, cette étude explore de nouvelles stratégies pour restaurer l'expression de G3BP1, offrant ainsi une voie potentielle d'intervention thérapeutique dans la SLA.

Mots clés : ALS, FTD, TDP-43, G3BP1, SG, petite molécule, SH-SY5Y

Abstract

Neurodegenerative disorders, such as Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD), have been associated with RNA-binding proteins (RBPs). Major hallmark of ALS is aggregation of an RNA-binding protein called TAR binding protein (TDP-43). TDP-43 has shown to bind to *G3BP1*, a nucleating factor for stress granule assembly, to stabilize it. Stress granules (SGs) are phase separated structures that form under stressful conditions and promote cell survival. Impaired stress granules assembly and reduced G3BP1 is reported in ALS. This reduction is due to a defect in TDP-43 stabilizing *G3BP1* encoding transcripts; thus, a failed stress granule response could have a major role in the disease. Thus, this research focuses on restoring G3BP1, whose depletion is linked to TDP-43 loss of function. By utilizing small-molecule compounds identified through a drug screening campaign, we seek to increase G3BP1 expression, consequently reinstating the endogenous SG mechanism and promoting neuronal survival. The discovery of lead candidates (NPX-047, NPX-000-115, and NPX-001-280) that effectively rescue G3BP1 expression and function offers promise for potential ALS therapies. These compounds were tested on SH-SY5Y cells treated with si-TDP however no rescue of *G3BP1* mRNA was observed despite higher levels of luciferase signals. Thus, in-depth investigation of discrepancies in our results is our next step which was not possible during the limited timeline of this thesis. In addition, off-targets of these compounds will be investigated using BruChase-sequencing. Overall, this study explores novel strategies to restore G3BP1 expression, providing a potential avenue for therapeutic intervention in ALS.

Keywords: ALS, FTD, TDP-43, G3BP1, SGs, small-molecule, SH-SY5Y cells

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List of acronyms and abbreviations

3'UTR: 3' untranslated region – Région 3’ untranslated

5'UTR: 5' untranslated region – Région 5’untranslated

ALS: Amyotrophic lateral sclerosis

AD : Alzheimer’s disease

ASOs : Antisens Oligonucleotide

ATXN1: Ataxin-1

ATXN2: Ataxin-2

C.elegans: Caenorhabditis elegans

CAPRIN-1: Cytoplasmic activation/proliferation-associated protein-1

CBS: Syndrome corticobasal

DNA : Deoxyribonucleic acid

FTD: Frontotemporal dementia

FUS: Fused in sarcoma

G3BP1: Ras-GTPase-activating protein (GAP)-binding protein 1

GRN: Progranuline

hnRNP: Heterogeneous ribonuclear proteins

hnRNP A1: Heterogeneous ribonuclear protein A1

HuR: Human antigen R

IDR: Domaine intrinsèquement désordonné

JNK: c-Jun-N-terminal kinase

kDA: KiloDalton

LLPS: Séparation de phase liquide-liquide

MAGE-B2: Melanoma associated antigen B2

NES: Nuclear exportation signal - Signal d'exportation nucléaire

NFκb : Nuclear factor kappa B

NLS: Nuclear localization signal – Signal de localisation nucléaire

NTF2: Transport nucléaire 2

OPTN: Optineurine

PKR: Protein kinase R

PPA: Aphasie progressive primare

PxxP : Domaine intrinsèquement désordonné

RGG: Domaine riche en glycines et arginines

RIG-I: Retinoic acid-inducible gene I

RNA: Ribonucleic acid

RNP : Particule ribonucléoprotéique

RRM1: RNA recognition motif 1, motif de reconnaissance de l'ARN 1

RRM2: RNA recognition motif 2, motif de reconnaissance de l'ARN 2

SLA: Sclérose latérale amyotrophique

SOD1: Superoxyde dismutase 1

SP-10: Acrosomal protein

TAR: Trans-activation response element

TARDBP: Tar-DNA binding protein

TBK1: Tank-binding kinase 1

TDP-43: Tar-DNA binding protein 43

TIA-1: T-cell-restricted intracellular antigen-1

USP10: Ubiquitin specific peptidase 10

VCP: Valosin-containing protein

To my family

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Chapter 1– Introduction

1. Neurodegenerative diseases

Neurodegenerative disorders develop due to excessive apoptotic death of neurons, which probably occurs due to the accumulation of abnormal protein aggregates in the cytoplasm or nucleus of the cell, but also in the extracellular space (Mathis et al., 2017). In these diseases, there is increasing prevalence with age (Dugger & Dickson, 2017). Neurodegenerative diseases can be classified based on the anatomic distribution of neurodegeneration (e.g., frontotemporal degeneration, extrapyramidal disorders, or spinocerebellar degeneration), primary clinical features (e.g., dementia, parkinsonism, or motor neuron disease), or principal molecular abnormality (Dugger & Dickson, 2017). The protein abnormalities in these disorders have abnormal conformational properties (Dugger & Dickson, 2017). Several factors have been indicated to be involved in neurodegenerative diseases such as oxidative stress (Jaiswal, 2014), various gene polymorphisms, and pathogenic mutations which ultimately facilitate or accelerate the formation of protein aggregates (Hardiman et al., 2017). The combination of all the above-mentioned factors leads to neuronal damage as a result of the loss of functional proteins and consequently loss of function (Dugger & Dickson, 2017). The neuron is also damaged by the toxic effects of newly formed pathological protein aggregates (so-called gain-of-function) (Mathis et al., 2017). The most common neurodegenerative disorders are amyloidoses, tauopathies, α-synucleinopathies, and transactivation response DNA binding protein 43 (TDP-43) proteinopathies (Dugger & Dickson, 2017).

1.1 Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that affects upper and lower motor neurons leading to paralysis and ultimately death. According to macroscopic manifestations, the motor cortex in ALS patients may exhibit modest atrophy. In addition, the spinal cord is frequently atrophic and the anterior spinal nerve roots may also exhibit atrophy.

Lower motor neurons in the spinal cord and brain stem, as well as the upper motor neurons, exhibit neuronal loss and gliosis under a microscope (Brettschneider et al., 2013). The median survival of ALS patients is 30 months after symptoms onset (Talbot, 2009). Approximately 10% of ALS cases are familial (FALS) (Da Cruz & Cleveland, 2011) while the majority of ALS cases are sporadic meaning without a clear family history. Sporadic ALS is presumed to be a result of a combination of complex genetic predisposition, age-dependent alterations in cellular homeostasis, and environmental factors (Al-Chalabi et al., 2014). The heritability of ALS is high; in patients with sporadic ALS, it is believed to be between 30% and 60% (Al-Chalabi et al., 2010). In first- degree relatives of ALS patients, the risk of developing the disease doubles (Al-Chalabi et al., 2010). More than 20 genes have been associated with this condition to date. While monogenetic mutations with high effect sizes now account for 15% of ALS cases, both common and rare genetic variants with low and moderate effect sizes tend to increase the chance of developing ALS. The main pathological feature in ALS is that the TAR DNA-binding protein 43 (TDP-43), a common neuropathological marker in over 95% of cases of both sporadic and familial ALS, is redistributed from a predominantly nuclear localization to form insoluble ubiquitinated aggregates in the cytoplasm of affected motor neurons (Arai et al., 2006).

1.1.1 Clinical manifestation

Different clinical neurological disorders can be identified by the prevalence of upper motor neuron (UMN) degeneration (primary lateral sclerosis, PLS), lower motor neuron (LMN) impairment (progressive muscular atrophy, PMA), or both UMN and LMN degeneration (Masrori & Van Damme, 2020). ALS frequently starts with asymmetric weakness most frequently in the upper limb, which is accompanied by symptoms such as challenges in writing, difficulty swallowing, fasciculations in the arm, leg or tongue, and cramps. Weight loss is also observed which is usually caused by muscle atrophy as well as the disease itself. In the ALS variant linked with dementia (FTLD-ALS), executive and cognitive functions are also reduced (Masrori & Van Damme, 2020).

1.1.1.1 Amyotrophic lateral sclerosis phenotypes

Many different motor phenotypes of ALS exist, and they are mainly classified based on the relative UMN versus LMN involvement and the regional distribution of involvement (Van Es et al., 2017). Since the life expectancy differs between various phenotypes of ALS (Chiò et al., 2011), and various cognitive impairments can be present, thus it is critical to understand the different phenotypes of ALS. ALS is now more often acknowledged to be a clinically heterogeneous disease. The motor signs of the illness itself are quite heterogeneous, and the motor manifestations may be accompanied by varying degrees of frontotemporal impairment (Masrori & Van Damme, 2020). There are no established clinical criteria for the various ALS phenotypes thus there is an increasing demand for a new categorization scheme that takes into consideration the variety of the ALS disease (Al-Chalabi et al., 2016).

1.1.1.1.1 Subtypes of ALS related to relative UMN versus LMN involvement

The most prevalent phenotype of ALS is the classic form, which occurs in 65-70% of cases (Stetkarova & Ehler, 2021). Classic ALS is diagnosed if combined UMN and LMN loss is present in the patient (Masrori & Van Damme, 2020). Signs of LMN involvement include muscle weakness, fasciculations, and reduced muscle tone. Signs of UMN involvement include but are not limited to hyperreflexia and increased muscle tone (Masrori & Van Damme, 2020).

Primary lateral sclerosis (PLS) is characterized by increasing spasticity, slowing of movements, and only UMN symptoms. PLS occurs in 1-2% of people (Stetkarova & Ehler, 2021). Four years after the beginning of ALS symptoms, if electromyography (EMG) results do not show any denervation, noticeable fasciculations, or muscular atrophy, the disease could be considered as PLS (Pringle et al., 1992).

UMN-predominant ALS patients possess some characteristics of LMN involvement but much less than the UMN features (Masrori & Van Damme, 2020). A shorter survival in comparison to PLS is

reported however a slower disease progression is recognized in comparison to classic ALS (Masrori & Van Damme, 2020).

Patients with lower motor neurons predominate ALS have relatively few UMN symptoms and might proceed at varying speeds (Masrori & Van Damme, 2020). While up to 30% of individuals with progressive muscle atrophy will acquire UMN symptoms during follow-up, the disease is diagnosed by developing isolated LMN indications without clinical evidence of UMN dysfunction (Masrori & Van Damme, 2020).

1.1.1.1.2 Motor neuron disease subtypes based on the distribution of involvement by area

A catastrophic form of ALS known as bulbar ALS is defined by a fast deterioration and a median survival of two years from the beginning of the disease. Spastic dysarthria, which is recognized by distorted speech, is caused by bulbar UMN dysfunction. Tongue atrophy and fasciculation, together with flaccid dysarthria and dysphagia, are signs of bulbar LMN dysfunction. Although only around 30% of patients initially exhibit bulbar symptoms, speech, and swallowing problems ultimately affect the majority of ALS cases (Masrori & Van Damme, 2020). In later stages of the disease, axial muscular weakness should be seen along with poor posture and a drooping head (Stetkarova & Ehler, 2021). Pseudobulbar palsy lacks facial emotions, has spastic dysarthria, dysphagia, difficulties in eating, and protruding tongue but no fasciculation of the tongue nevertheless there is little agreement on this condition in the literature (Finegan et al., 2019). Approximately 3% of patients with respiratory ALS first report diaphragm weakness, such as orthopnea or dyspnea (Masrori & Van Damme, 2020). Patients who develop respiratory symptoms have a dismal prognosis (Masrori & Van Damme, 2020). In the axial variant of ALS, the onset of the disease is in paravertebral muscles, with stooped posture as a presenting symptom (Masrori & Van Damme, 2020). Flail arm ALS, also known as Vulpian-Bernhardt syndrome, is a progressive, primarily LMN pattern of weakness in the upper limbs that typically starts in proximal muscles and progresses to distal regions (Masrori & Van Damme, 2020). Up to 77% of these

patients suffer from bulbar symptoms (Masrori & Van Damme, 2020). Males predominate heavily (male-to-female ratio: 3:1) (Wijesekera et al., 2009).

1.1.2 Diagnostics

When the disease is still in its early stages, when it advances slowly, or when other neurological disorders are also present, making the diagnosis of ALS can be challenging (Masrori & Van Damme, 2020). According to reports, the likelihood of a misdiagnosis, including ALS-mimicking disorders, is between 7-8% (Traynor et al., 2000) As a result, it is critical to rule out certain ALS-mimicking diseases as soon as possible because waiting to start treatment might have a negative impact (Masrori & Van Damme, 2020). In order to receive a final diagnosis, it is crucial to transfer the patient to a reputable clinical facility with expertise in diagnosing ALS. This should be done on a frequent basis (Oskarsson et al., 2018). Three key tenets guide the determination of an ALS diagnosis. One or more of the following conditions must be present (De Carvalho, 2020):

(1) signs of functional impairment in a specific body part;

(2) symptoms of central and peripheral motor neuron involvement in one or more segmental anatomical locations; and

(3) Functional impairment progression.

Without satisfying these three criteria, a diagnosis of ALS is deemed suspect and necessitates more testing, however, it may not necessarily be incorrect (Stetkarova & Ehler, 2021).

1.1.3 Prognosis

There are various prognoses for different ALS subtypes. The average life expectancy is 2-4 years in general (Marin et al., 2016). Only approximately 5-10% of people survive more than ten years following the onset of the disease, around 50% pass away within three years, and 90% pass away within five years (Brown & Al-Chalabi, 2017). Patients who receive a diagnosis later in life or who are younger often live longer. Long-term survival is also better when UMN participation over LMN

injury predominates (Zoccolella et al., 2008). Given that hypermetabolic ALS patients exhibit higher levels of LMN involvement, a quicker pace of functional deterioration, and shorter life, the metabolic index appears to be significant for ALS prognosis (Steyn et al., 2018). Patients with an initial motor manifestation of ALS-FTLD proceed substantially more quickly than those with a cognitive manifestation (Ahmed et al., 2019), and frontotemporal syndrome in ALS is linked to a low prognosis (Govaarts et al., 2016). These results imply that alterations in physiology and motor function may be closely related to disease development in ALS-FTLD (Ahmed et al., 2020).

1.1.4 Incidence and Prevalence

Individuals of all races and ethnicities were thought to be affected by the disease (Masrori & Van Damme, 2020). Nonetheless, whites, non-Hispanics, men, people under the age of 60, and those with a family history of ALS were more prone to develop the disease (Mehta et al., 2018). The lowest incidence was measured to be 0.89 per 100,000 people in East Asia and 0.79 per 100,000 people in South Asia (Logroscino & Piccininni, 2019) while Japan has the highest prevalence of ALS (9.9/100,000 population) (Doi et al., 2014).

1.1.5 Etiology

The first ALS-related gene, *SOD1*, was identified in 1993 and accounts for 1%–2% of sporadic ALS (sALS) and 20% of familial ALS (fALS), respectively (Rosen et al., 1993). Mutations in this gene make the SOD1 protein more susceptible to aggregation, which interferes with a number of crucial physiological processes. Mutations in the *TARDBP* and *FUS* genes, which encode the RNA-binding proteins TDP-43 and FUS, were first identified in 2008 and 2009. 3%–5% of fALS and 1% of sALS are caused by these mutations (Kabashi et al., 2008; Kwiatkowski et al., 2009; Sreedharan et al., 2008; Vance et al., 2009). *C9orf72*, which accounts for 30%–50% of fALS and 7%–10% of sALS, was identified in 2011 (DeJesus-Hernandez et al., 2011). Patients with *C9orf72* hexanucleotide repeat expansions are more likely to develop cognitive impairment and bulbar

onset ALS. With up to 10% of individuals with ALS-FTD being affected, *TBK1* mutations are most likely the fifth most frequent cause of autosomal dominant ALS, accounting for roughly 1% of patients (Cirulli et al., 2015). The other genes indicated in ALS are known to have a reduced penetrance, which makes genetic counseling more challenging (Masrori & Van Damme, 2020). Patients with mutations in more than one of these genes are extremely rare, which suggests that ALS may have an oligogenic origin (van Blitterswijk et al., 2012).

The origin of sporadic ALS has not yet been determined. Glutamate- and homocysteine-mediated excitotoxicity, failure of proteostasis, mitochondrial malfunction, oligodendrocyte dysfunction, cytoskeletal disturbances, axonal transport defects, disturbed RNA metabolism, impaired DNA repair, oxidative stress, immune system dysfunction inducing chronic inflammation and nucleocytoplasmic transport deficits have all been taken into consideration, but these hypotheses have not been proven (Brown & Al-Chalabi, 2017; Longinetti & Fang, 2019; Talbott et al., 2016). Age, male sex, body mass index, blood lipid levels, and smoking are additional risk factors for the development of ALS (Longinetti & Fang, 2019; Zhan & Fang, 2019). Moreover, Low-density lipoprotein (LDL) is causally related to ALS, and both East Asian and European people are more likely to develop the disease when their LDL levels are greater (Zeng & Zhou, 2019). An epidemiological study have linked traumatic brain injury (TBI) to ALS (McKee et al., 2009) nonetheless another study have refuted this claim (Armon & Nelson, 2012).

1.1.6 Pathology

In genetic and pathological examinations of ALS patients, injury to the neuromuscular junction, dysfunction of motoneuron homeostasis, inadequate energy supply, abnormal axonal branching, and axonal transport have all been noted (Suzuki et al., 2020). The UMNs and LMNs lose their neuromuscular connections and experience subsequent cell death surrounded by astrogliosis and microgliosis, with ubiquitin-positive inclusions being seen in the remaining neurons (Masrori & Van Damme, 2020). This is the neuropathological characteristic of ALS. More than 95% of ALS patients have TDP-43 as the primary component of these inclusions (Neumann et al., 2006). TDP-43, is involved in some pathways, including RNA transport, transcription, microRNA maturation, splicing,

and the creation of stress granules (Neumann et al., 2006). TDP-43 shuttles back and forth between the nucleus and cytoplasm in accordance with its nuclear and cytoplasmic activities however it is mostly nuclear in location. A distinguishing feature of ALS is the mislocalization of TDP-43 to the cytoplasm, which results in nuclear TDP-43 depletion and cytoplasmic TDP-43 protein aggregation (Mackenzie et al., 2010). Excitotoxicity, proteostasis failure, mitochondrial dysfunction, neuroinflammation, oligodendrocyte dysfunction, oxidative stress, cytoskeletal abnormalities, disturbed RNA metabolism, axonal transport defects, impaired DNA repair, nucleocytoplasmic transport deficits are just a few of the molecular pathways that have been linked to the pathogenesis of ALS (Brown & Al-Chalabi, 2017; Taylor et al., 2016). It is interesting to note that many of the genes linked to ALS seem to congregate in important pathways, including RNA metabolism, cytoskeletal and axonal transport, protein quality control, and degradation (Masrori & Van Damme, 2020).

1.1.6.1 Failure of proteostasis

Cellular stress is brought on by protein aggregates or, more likely, the oligomeric complex progenitors of these aggregates (Webster et al., 2016). Molecular chaperones can help refold misfolded proteins and prevent protein aggregation however when the cell is overrun by misfolded proteins, the ubiquitin-proteasome system will target them for degradation. Alternatively, protein aggregates can bind to p62 (sequestosome 1) and then be degraded through the lysosomal route (autophagy pathway). Interestingly, Mutations in ALS-related genes, such as *C9orf72* (Webster et al., 2016), Optineurin (*OPTN*, functioning as a receptor for autophagy) (Maruyama et al., 2010), *TBK1* (activates OPTN by phosphorylation), *SQSTM1* (Fecto et al., 2011) and valosin-containing protein (*VCP*) (Johnson et al., 2010) cause protein aggregation and defective degradation. Thus, they indicate a significant role in the etiology of ALS.

1.1.6.2 Disturbed RNA metabolism

ALS etiology involves an important number of RNA-binding proteins. The process of deregulation of RNA metabolism has been linked to ALS as a result of the discovery of mutations in the genes of two similar RNA-binding proteins, FUS and TDP-43 (Buratti et al., 2010). Additional changes in RNA-binding proteins like matrin 3 (MATR3), angiogenin (ANG), ataxin 2 (ATXN2), senataxin (STX), heterogeneous nuclear ribonucleoproteins A1 (hnRNP A1) and A2/B1 (hnRNP A2/B1) support the hypothesis that disrupted RNA metabolism likely plays a significant role in ALS (Boeynaems et al., 2016). These proteins are mostly found in the nucleus of cells where they play a crucial role in the processes of non-coding RNA metabolism, miRNA synthesis, splicing, and transcription. Nuclear depletion can therefore be harmful and result in severe transcriptome abnormalities. Furthermore, aggregation and mislocalization to the cytoplasm may result in toxicity (Boeynaems et al., 2016).

1.1.6.3 Axonal transport abnormalities and cytoskeletal impairment

Profilin 1 (PFN1) and tubulin alpha 4A (TUBA4A) mutations, which only infrequently cause ALS, were found to destabilize the tubulin network and result in deficiencies in axonal transport (De Vos & Hafezparast, 2017). ALS or FTD may also result from point mutations in the gene encoding the dynactin1 (DCTN1), component of the dynactin complex (Bercier et al., 2019). The dynactin complex is a crucial dynein motor activator that modifies motor activity and stabilizes cargo binding. Additionally, the anterograde transport of cargo through the microtubules may be hampered by mutations in the C-terminus of kinesin-1, which is encoded by KIF5A (kinesin heavy chain isoform 5A) (Nicolas et al., 2018). All of the above-mentioned studies support the relevance of cytoskeletal integrity and axonal transport in ALS.

1.1.7 Treatments

In spite of massive efforts in recent years to find an effective treatment for ALS, the disease is still considered incurable nonetheless to date, some molecules have been approved by the FDA to treat the disease, such as Riluzole, Tofersen, and Edaravone. Riluzole, a glutamate-release inhibitor whose mechanism of action still needs further investigation, was approved in 1996 (Blyufer et al., 2021; Gordon, 2013). Initial research showed that the use of Riluzole extended the survival of patients by three to six months. However, new studies argue that it does not extend lifespan or improve motor functions in several animal models of ALS as previously reported (Andrews et al., 2020; Jaiswal, 2019). Human studies are also very variable. While some groups reported an increase in lifespan of 6 to 19 months, other groups reported no improvement (Andrews et al., 2020; Zoccolella et al., 2007). These observations have called into question the validity of the standards used for the study of potential therapies for ALS and the use of Riluzole as a treatment (Hogg et al., 2018). Edaravone, another FDA-approved drug and a low-specificity antioxidant that scavenges various free radicals, has been approved recently as a therapeutic agent for ALS in Japan (2015), the USA (2017), and Canada (2018) (Breiner et al., 2020; Hardiman & van den Berg, 2017; Kikuchi et al., 2017). Edaravone appears to be effective in patients meeting specific criteria, including early-stage disease and rapid progression (Abe et al., 2014; Okada et al., 2018). However, the therapeutic benefits of Edaravone on other patient subgroups remains unknown; therefore, further studies are yet to be performed. The mechanisms of action of Edaravone remain largely unknown. Despite all the efforts made, more than 40 clinical trials have proven inconclusive in recent years (Mitsumoto et al., 2014). Tofersen or BIIB067, was approved in April 2023 by the FDA to treat fALS associated with a mutation in the *SOD1* gene. Tofersen which is an antisense oligonucleotide targets the protein result of the mutation in *SOD1* (Miller et al., 2013). This drug slows down muscle degeneration in those eligible for treatment by targeting *SOD1* messenger RNA (mRNA) (Miller et al., 2013). Additionally, symptomatic treatment for ALS has advanced through the decades. Muscle cramps are a major source of discomfort for ALS patients. Although there is currently no effective treatment for muscle cramps, recent trials have shown that mexilitene significantly reduces cramps (Weiss et al., 2016). Furthermore, patients with pseudobulbar palsy can now be treated for pseudobulbar affect, which is

characterized by episodic emotional outbursts, using a combination of dextromethorphan and low-dosage quinidine, known as NUEDEXTA® (Brooks et al., 2004). The FDA authorized NUEDEXTA® after two controlled trials showed that it reduced emotional outbursts by more than 90% (Brooks et al., 2004; Pioro et al., 2010). Sialorrhoea is another symptom of patients that is frustrating and when oral agents become ineffective, botulinum toxin injection into salivary glands is usually effective (Jackson et al., 2009). Evidence suggests that radiotherapy of the salivary glands may be an effective alternative (Assouline et al., 2014). Effective treatment for respiratory insufficiency with non-invasive ventilation at night could alleviate dyspnoea, improve quality of life, and sleep, and extend survival. (Vrijsen et al., 2013). Thus, while we wait for more effective treatments we may make use of some of therapies to lessen the impact of this devastating disease (Miller & Appel, 2017).

1.2 Frontotemporal dementia (FTD)

Frontotemporal dementia (FTD) is the second most frequent cause of dementia in people under 65. FTD is marked by advancing alterations in behavior, personality, and/or language, with comparatively intact memory. The non-fluent and semantic variations of primary progressive aphasia (PPA), as well as the behavioral variant (bvFTD), are important clinical subgroups. Additionally, extrapyramidal movement disorders such as atypical parkinsonism or corticobasal syndrome (CBS) or motor neuron diseases like ALS are frequently linked to motor characteristics in FTD (Dugger & Dickson, 2017). TDP-43 is linked to the most frequent pathogenic form of frontotemporal dementia. There are three main kinds of TDP-43 inclusions: neuronal intranuclear inclusions, neuronal cytoplasmic inclusions, and dystrophic neurites (Fig.1 A-D) (Dugger & Dickson, 2017). Two teams, Mackenzie et al. (2006) and Sampathu et al. (2006) documented neuroanatomical and morphological subtype inclusions in FTD shortly after the identification of TDP-43. In order to create a unified categorization method, these two systems were integrated in 2011 (Mackenzie et al., 2011). According to this, patients with mutations in the progranulin gene (GRN) who are clinically diagnosed with either progressive nonfluent aphasia or behavioural variant FTD are more likely to have type A (Dugger & Dickson, 2017). TDP-43 is seen in more varied

neuronal cytoplasmic inclusions in the neocortex (Fig. 1B) and coarse granular inclusions in the hippocampus dentate fascia (Fig. 1A and Inset) (Dugger & Dickson, 2017). In addition, there are varying numbers of neuronal intranuclear inclusions (Fig. 1B's Inset) and short, thin dystrophic neurites in the cortex's outermost layers (Dugger & Dickson, 2017). In patients with hexanucleotide expansions in the *C9ORF72* gene, Type B is the most common (Dugger & Dickson, 2017). This type which can be related to motor neuron disease, is primarily characterised by neuronal cytoplasmic inclusions with few or no neurites and no neuronal intranuclear inclusions (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Patients who have Type C often exhibit a semantic variation of progressive aphasia (Dugger & Dickson, 2017). Long, thick dystrophic neurites randomly arranged in all cortical layers are what define this kind (Fig. 1D and Inset) (Dugger & Dickson, 2017). The hippocampus, amygdala, and basal ganglia all have well-circumscribed (Pick-body-like) neuronal cytoplasmic inclusions (Fig.1C and Inset) (Josephs et al., 2008). An uncommon fourth type, type D, is linked to mutations in the VCP, and it manifests as frontotemporal dementia, inclusion body myositis, and Paget's disease of the bones. Numerous neuronal intranuclear inclusions in the cortical and subcortical regions are a hallmark of type D (Dugger & Dickson, 2017).

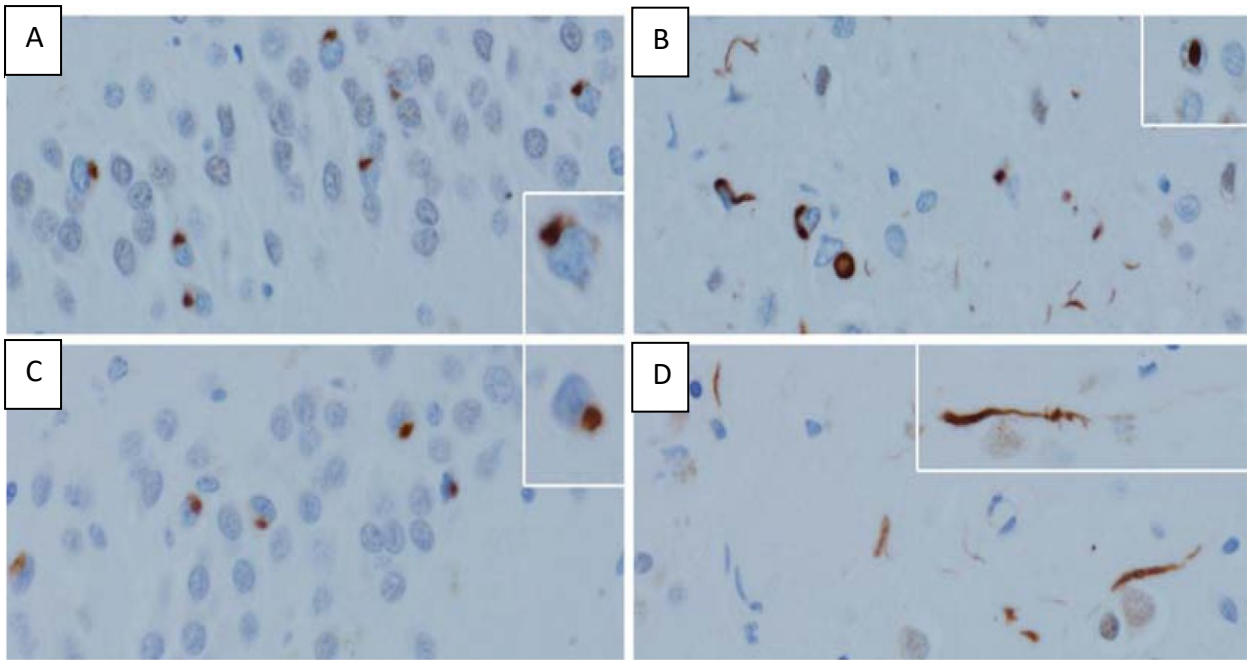

Figure 1. – Transactivation response DNA binding protein 43 (TDP-43) proteinopathies. Adapted from Dugger BN and Dickson DW, 2017. Neurons in the dentate fascia frequently exhibit granular neuronal cytoplasmic inclusions in FTLD-TDP Type A (A, inset), whereas the afflicted cortices have, short curved dystrophic neurites, more pleomorphic neuronal cytoplasmic inclusions and neuronal intranuclear inclusions (B, inset). Round, well-circumscribed (Pick-body-like) neuronal cytoplasmic inclusions are common in the hippocampus's dentate fascia (C), nevertheless, they are uncommon in the afflicted cortices, where lengthy, thick dystrophic neurites (D, inset) constitute the most distinguishing hallmark of FTLD-TDP Type C.

1.2.1 Spectral convergence with ALS

Due to the convergence of associated genes and the comorbidity of symptoms, it is now undeniable that ALS and FTD are part of the same pathological spectrum. About 50% of ALS patients may experience frontal and anterior temporal lobe degeneration, which may result in behavioral problems, language difficulties, and executive dysfunction (Masrori & Van Damme, 2020). At the same time, 30% of patients diagnosed with FTD have motor symptoms, and 13% are diagnosed with ALS (Wood et al., 2021). Roughly 50% of ALS patients will have normal cognition, but when the conditions for behavioral variant FTD or primary progressive aphasia are

met, the disease can be diagnosed as ALS-FTD in roughly 10% to 15% of patients (Table 1) (Masrori & Van Damme, 2020). Only two of the six criteria for behavioral variant FTD are necessary for ALS-related behavioral impairment (Masrori & Van Damme, 2020).

At first, the genetic convergence of the two diseases was suspected however it was with the discovery of TDP-43 as the main resident of pathological aggregates in post-mortem patients, and mutations in the *C9ORF72* gene as a major cause of ALS and FTD cases that the final piece of the puzzle was placed (Al-Chalabi & Hardiman, 2013; Neumann et al., 2006), and these two diseases were then recognized as being at the extremes of the same spectrum (DeJesus-Hernandez et al., 2011; Neumann et al., 2006; Renton et al., 2011). Histopathologically, the presence of TDP-43 or FUS inclusions in neurons and glial cells has also confirmed the similarity between these two diseases. The presence of these inclusions also suggests that there are common molecular mechanisms between these two diseases. Indeed, loss of RNA homeostasis (particularly of RNA granules), problems with nucleocytoplasmic transport and proteostasis are also observed in FTD (Balendra & Isaacs, 2018; Deleon & Miller, 2018; Wang et al., 2021). These findings further support that ALS and FTD are from the same spectrum.

Table1.- FTD criteria (Masrori & Van Damme, 2020)

Disorder	Variants	Clinical diagnosis	Imaging (^{18}F FDG PET/CT of the brain)
Primary progressive aphasia (PPA)	Non-fluent agrammatic variant primary progressive aphasia (naPPA)	At least one: • agrammatism errors and omissions, as well as samplification f grammatical forms • prosody (the rhythm or melody of speech), as well as speech sound errors (such as motor-based speech planning errors 'apraxia of speech') • at least two of the following criteria must be fulfilled:– 1) impaired comprehension of complex sentences 2) spared single-word comprehension 3) spared object knowledge	Atrophy of anterior perisylvian atrophy involving inferior, opercular and insular portions of the left frontal lobe
	Semantic variant of primary progressive aphasia (svPPA)	• Impaired confrontation naming • Impaired comprehension of single words • At least three of the following criteria must be fulfilled: 1) degraded object knowledge 2) surface dyslexia or dysgraphia, in which sight vocabulary words are pronounced as written 3) spared repetition 4) spared speech production	Atrophy of left anterior temporal atrophy affecting lateral and ventral surfaces as well as the anterior hippocampus and the amygdala
	Logopenic variant primary progressive aphasia (lv-PPA)	• Profound difficulty in word finding • Impaired repetition of phrases, partly as a result of limited auditory–verbal short-term memory • At least three of the following criteria must be fulfilled: 1) speech (phonologic) errors in spontaneous speech and naming 2) spared single-word comprehension and object knowledge 3) spared motor speech 4) absence of frank agrammatism	Atrophy of left posterior perisylvian or parietal lobe
Behavioural variant frontotemporal dementia (bvFTD)		At least three:• behavioural disinhibition • apathy or inertia • loss of sympathy or empathy • stereotypical, perseverative or compulsive behaviour • hyperorality or dietary changes • executive deficits with relative sparing of visuospatial skills and memory	Prefrontal or anterior temporal cortex loss, particularly in the right hemisphere

FTD, frontotemporal dementia; ^{18}F FDG PET/CT, ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography.

2. TDP-43

TDP-43 is a 43-kDa protein that is highly conserved throughout mammals and invertebrates and is widely expressed in almost all tissues. It has major roles regarding stress granules, RNA metabolism, gene splicing, and transcriptional repression (Ratti & Buratti, 2016). Normally a

nuclear protein, TDP-43 develops inclusion bodies throughout the cytoplasm, nucleus, and cell processes in neurodegenerative disorders. Nucleocytoplasmic transport and nuclear pore complexes can be directly disturbed by cytoplasmic clumps of TDP-43 (Chou et al., 2018). Moreover, Nucleoporins and transport factors mislocalize and aggregate as a result of TDP-43 aggregates (Chou et al., 2018). TDP-43 contains a nuclear localization signal (NLS) sequences that control its cellular localization in the cytoplasm and nucleus and TDP-43 mislocalization may be increased by certain missense variants, such as ALS-related A90V mutation in the NLS (Winton et al., 2008). TDP-43 was shown to be the main component of neuronal inclusions in ALS and frontotemporal lobar degeneration with ubiquitin inclusions (Neumann et al., 2006). Aside from ALS and FTD, aberrant TDP-43 may be seen in 25% to 50% of Alzheimer's disease patients, mostly in the limbic region (Josephs et al., 2014); therefore, TDP-43 pathology and its' mislocalization to the cytoplasm and subsequent aggregation are significant components in many neurodegenerative diseases which ultimately aids in neuronal dysfunction and toxicity.

2.1 TDP-43 structure

TDP-43 protein is made of the N-terminal domain (NTD), two RNA recognition motifs (RRM1 and RRM2; residues 104–200 and residues 191-262, respectively), and the C-terminal domain (CTD; residues 274–413) (Barmada et al., 2010). The NTD region contains a ubiquitin-like fold with one α-helix and six β-sheets and promotes TDP-43 self-oligomerization (Chang et al., 2012). It was discovered that the hydrophobic interface that surrounds the β7-strand is important for NTD dimerization (Jiang et al., 2017). TDP-43 undergoes disulfide formation to become a tetramer when protein concentration rises (Jiang et al., 2017). Reversible formation of TDP-43 polymers through the NTD has been demonstrated to be necessary for splicing activity (Afroz et al., 2017), and to contribute to phase separation via liquid-droplet (Afroz et al., 2017), and stress granule formation (Molliex et al., 2015). Full-length TDP-43 exhibits splicing activity in the nucleus in both the dimeric and tetrameric forms. The conserved phosphosite Ser 48 (A. Wang et al., 2018), one of three putative mitochondrial targeting sequences (Wang et al., 2016), and potential nucleotide binding site (A. Wang et al., 2018) are also located at the NTD. In order to participate in hydrogen

bonding interactions, phosphosite Ser 48 is moved towards the interface. According to research simulating phosphorylation at this location interferes with the NTD's ability to self-associate and alters splicing function (A. Wang et al., 2018). Additionally, TDP-43 appears to be shielded from aggregation and inclusion formation in the cytoplasm by the creation of dimers or tetramers (Jiang et al., 2017), and mutations of the nuclear localization sequence (NLS) in the NTD are the cause of cytoplasmic localization and aggregation of TDP-43 (Fig. 2). Two RRM domains in TDP-43, each with five β-strands and two α- helices, are present in order to carry out diverse tasks in translational control, transcriptional suppression, and pre-mRNA alternative splicing. These regions have been shown to bind UG/TG-rich single-stranded or double-stranded DNA/RNA (Kuo et al., 2009). The TDP-43 protein's C-terminus controls protein-protein interactions and is crucial for the protein's solubility and cellular localization (Ayala et al., 2008). Glycine, glutamine, and asparagine are all abundant in this domain. These sequences mimic the prion-like domain sequences (Patel et al., 2009). Prion-like domains are low-complexity sequences that are concentrated in glycine, asparagine, glutamine, and other uncharged polar amino acids, such as tyrosine. In controlling the solubility and folding of proteins, the prion-like domain is crucial. Proteins with prion-like domains phase separate into spherical, membraneless compartments, known as liquid-liquid phase separation (Shin & Brangwynne, 2017). It is proposed that through liquid-solid phase separation, the presence of mutations or abnormal posttranslational modifications causes the establishment of irreversible aggregation (Maharana et al., 2018). TDP-43 has been reported to contain several mutations that have been linked to ALS and FTLD, and the majority of these mutations are found in the C-terminal domain (Sreedharan et al., 2008). Some TDP-43 C-terminal mutations, including G376D, G294V, M337V, A315T and A382T encourage cytoplasmic mislocalization and aggregation through as-yet-unidentified mechanisms (Barmada et al., 2010; Mitsuzawa et al., 2018; Mutihac et al., 2015). TDP-43 mutations, such as N390S, G294A, M337V, N390D, Q331K, and Q343R when expressed in SH-SY5Y cells increase protein aggregation (Nonaka et al., 2009). Additionally, it has been discovered that several peptides with pathogenic TDP-43 mutations, including G295S, G294A, and G294V may twist into amyloid-like fibers (Sun et al., 2014). The most important part of TDP-43 that contributes to aggregation is therefore its CTD domain.

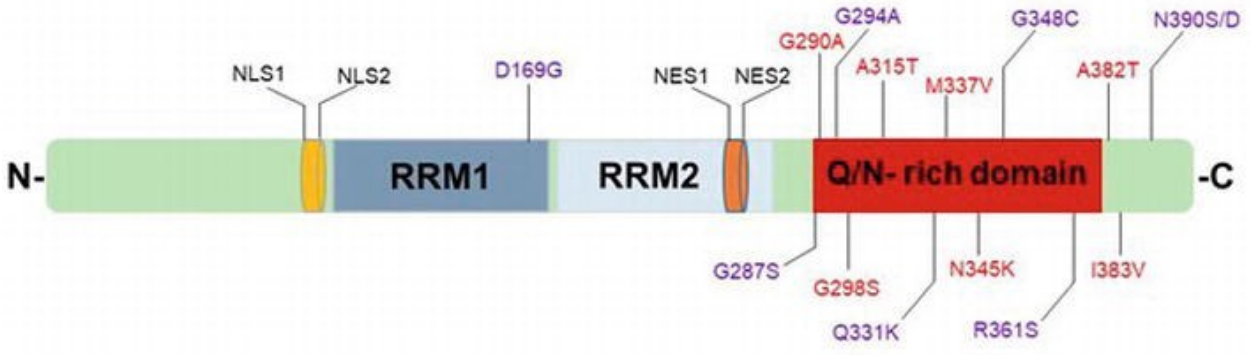

Figure 2. – TDP-43 structure, and ALS-related mutations. From (Joy & Muralidhar, 2020). Two RNA recognition motifs (RRMs), Nuclear localization sequence (NLS), nuclear export sequence (NES), and a glycine-rich domain make up the TDP-43 protein, which also comprises the bulk of the genetic alterations linked to ALS. Sporadic ALS-linked mutations are in blue color and familial ALS-related mutations are depicted in red.

2.2 TDP-43, splicing, and gene transcription

TDP-43 regulates mRNA stability, splicing, and translation as well as gene transcription by binding to both mRNA and DNA. TDP-43 was first identified as a transcriptional repressor in 1995. The Transactive Response Element (TAR) of the Human Immunodeficiency Virus (HIV) has a region rich in pyrimidines, and TDP-43 attaches to the TAR via its RNA binding domain (Ou et al., 1995). This binding prevents the virus's gene from being activated. The expression of the ACRV1 and SP-10 genes, is also suppressed by TDP-43, allowing spermatogenesis to proceed normally (Abhyankar et al., 2007). TDP-43 targets DNA regions like promoter sequences or regulates transcription by remodeling chromatin rather than interacting with the polymerase directly (Li et al., 2012). A significant alteration of the cellular transcriptional profile results after TDP-43 nuclear depletion. For instance, genes containing transposable elements are enriched when TDP-43 is lost from the nucleus (Morera et al., 2019). Additionally, TDP-43 colocalizes with active transcription sites in rat neurons, supporting its activity in transcription and the alternative splicing mechanism.

TDP-43 has been shown to have a more extensive function in the control of RNA expression and splicing in two studies. TDP-43, as demonstrated by research by Polymenidou et al., is essential for maintaining the mRNA levels of choline acetyltransferase, the disease-related proteins FUS/TLS, synaptic proteins, and progranulin, as well as others involved in different neurological diseases (Polymenidou et al., 2011). In the other study by Tollervey et al it was demonstrated RNAs that encode proteins that control neuronal growth and survival are bound by TDP-43 (Tollervey et al., 2011). They identified the noncoding RNAs, MALAT1 and NEAT1, as disease-related TDP-43 targets in FTLD. Future research may help to better understand how this fascinating family of noncoding RNAs regulates splicing in both healthy and diseased conditions (Cohen et al., 2011). These findings demonstrate that TDP-43 directly binds to a variety of essential target RNAs, It's interesting to note that the majority of these targets are involved in neurotransmitter modulation, synapse development and function, splicing, RNA maturation, as well as numerous genes linked to neurodegeneration, including ATXN1, ATNX2, and GRN suggesting that TDP-43 activity promotes and/or maintains healthy neuronal function and integrity (Cohen et al., 2011).

2.3 TDP-43 and stabilizing RNA

According to Fukushima et al., TDP-43 helps keep the cellular transcriptome stable (Fukushima et al., 2019). TDP-43 primarily binds RNAs via a GU dinucleotide repeat motif, according to early *in vitro* experiments (Buratti & Baralle, 2001), but a consensus TDP-43 binding site *in vivo* has yet to be conclusively identified (Cohen et al., 2011). The most significant RNA binding, nevertheless, occurred at either a GU-rich motif interrupted by a single adenine or unbroken GU repeats, according to previous assessments of global TDP-43 binding sites (Hallegger et al., 2021; Polymenidou et al., 2011; Tollervey et al., 2011). TDP-43 binds to UG-rich regions in order to control transcript stability. The degradation of the transcripts will be prevented or accelerated by the protein's binding to these sites. It has been proposed that cofactors that bind to a specific transcript and TDP-43 are the cause of the variation in how these transcripts are stabilized. To fully comprehend the underlying processes, further research is required. Studies demonstrate

that both TDP-43 and FUS/TLS bind and stabilize the mRNA encoding histone deacetylase 6 (HDAC6), a previously identified microtubule deacetylase (Lee et al., 2010). As HDAC6 has recently been linked to mitophagy and the removal of misfolded protein aggregates, TDP-43 and/or FUS/TLS may improve neuron survival or neuroprotection through increased HDAC6 expression (Kawaguchi et al., 2003). Recently, endogenous mouse TDP-43 was depleted using TDP-43 transgenic mice that expressed a TDP-43 mutant that is localized to the cytoplasm due to a faulty nuclear localization sequence (TDP-43-NLS). This provides a potential TDP-43 loss of function animal model (Igaz et al., 2011). Interestingly, a collection of genes whose mRNA expression profile was dramatically changed in the brain tissue of TDP-43-NLS mice included the expression of histones and histone regulatory proteins, which are normally expressed in mitotic cells (Cohen et al., 2011). In addition to transcriptional alterations (118 upregulated and 136 downregulated transcripts), Depletion of TDP-43 by siRNA in mouse embryonic motor neurons causes abnormalities in axonal development, reduced protein synthesis, and mitochondrial dysfunction in axonal compartments (Briese et al., 2020). Furthermore, Tank et al., discovered significant RNA transcript destabilization using patient-derived cells from sporadic ALS patients, which may have resulted in enhanced protein synthesis (Tank et al., 2018). Deletion of the mutant allele results in a 1.5-fold increase in endogenous wild-type protein production in mice models expressing human TDP-43 A382T or G348C heterozygously (Hasegawa et al., 2016). We now know that TDP-43's lack of nuclear function has the ability to raise the protein's levels, which might lead to further protein accumulation in the cytoplasm in ALS-FTD patients.

2.4 TDP-43 and stress granules (SGs)

2.4.1 SGs and neurodegenerative diseases

SGs are collections of membrane-free messenger ribonucleoprotein complexes (mRNPs) that have halted translation. RNA binding proteins (RBP) mediate eukaryotic cells' condensation to produce SGs in response to environmental stressors, such as oxidative conditions, heat,

hyperosmolarity, viral infections, and UV irradiation (Anderson & Kedersha, 2002; Buchan & Parker, 2009). The development of SGs controls signaling pathways that are strongly connected to nuclear function, cell death, and viral infection (Protter & Parker, 2016). Under stressful circumstances, SGs can potentially function as an oxidative regulator in cells (Takahashi et al., 2013). In addition, by isolating the invader and restricting the translation of viral mRNAs when host cells are attacked by viruses, SGs can fend off viral invasion however many viruses have developed ways to prevent the development of SGs and elude the host's antiviral response (Cohen et al., 2011). Neurodegenerative diseases have been associated with mutations in a variety of stress granule components (Wolozin & Ivanov, 2019). Studies on stress granule dynamics have been conducted in immortalized cell lines (such as HEK293 or HeLa) however to further understand how stress granules are impacted by neurodegenerative diseases, it is crucial to consider the role of stress granule dynamics in neurons and/or in vivo. According to three theories by Li et al., stress granules may be linked to neurodegeneration in three different ways (Li et al., 2013). First, improperly assembled stress granules that are not functioning might lead to increased neuronal susceptibility (Wolozin & Ivanov, 2019). Second, according to Baron et al., stress granules, which develop under stress but do not break down, gradually change from a liquid to a solid state, successfully seeding the cytosolic inclusions that are usually linked to neurodegenerative disorders (Baron et al., 2013). Last but not least, stress granules that do not disintegrate may sequester cellular chaperones or translation factors (Wolozin & Ivanov, 2019). Thus, understanding how stress granules act in neurons and the functional repercussions of their malfunction is crucial since it is probable that a combination of these mechanisms contributes to the development and progression of neurodegeneration.

2.4.2 TDP-43, pathogenic aggregates and SGs

As a part of stress granules, TDP-43 controls the formation and disassembly of stress granules (McDonald et al., 2011). According to some studies, loss of stress granule function results in loss of TDP-43 localization, whilst others contend that stress granules are probable antecedents of the

inclusions found in ALS and FTD (Colombrita et al., 2012). Additionally, RNA interference-mediated TDP-43 depletion decreased the size of stress granules and slowed the pace at which they were assembled, making cells more sensitive to stress (Khalfallah et al., 2018). Research also revealed that lymphoblastic cells from individuals expressing TDP-43R316S took longer to disassemble the Stress granules after being treated with sodium arsenite than lymphoblastic cells from patients with the wild-type allele (McDonald et al., 2011). Therefore, during stress exposure, TDP-43 is needed for both the assembling and disassembling of stress granules.

3. G3BP1

All cells express Ras-GTPase-activating protein (G3BP), a site-specific ribonucleic acid endonuclease (Kang et al., 2021). G3BP family consists of G3BP1, G3BP2a and G3BP2b. Different genes on human chromosomes 5 and 4 and mouse chromosomes 11 and 5 encode G3BP1 and G3BP2, respectively. While G3BP2 is substantially expressed in the small intestine and brain, G3BP1 is significantly expressed in the lungs and kidneys (Kennedy et al., 2001; Krapp et al., 2017). G3BP1 is critical for cell survival as well as a major participant in the fight against viral infections (Lloyd, 2016; White & Lloyd, 2011). The retinoic acid-inducible gene I (RIG-I) can be stimulated to recognize RNA viruses by G3BP1, which can operate as an antiviral factor (Onomoto et al., 2012) nevertheless by modifying G3BP, several viruses have developed new methods of action that let them replicate more easily and get through the host's cellular defense (Kang et al., 2021). Furthermore, G3BP1 is an essential piece in cancer invasion and metastasis. According to studies (Dou et al., 2016; Wang, Fu, et al., 2018; Zhang et al., 2015, 2019), it helps tumors and cancer cells resist chemotherapeutics and hostile environments (Wang et al., 2018; Zhang et al., 2019). Several studies point to G3BP targeting as a viable treatment approach for cancer (Kang et al., 2021) however G3BP1 regulation and precise functions are still mostly unknown. The development of *G3bp1*$^{-/-}$ mice, which show late embryonic lethality, highlights the significance of G3BP1 (Zekri et al., 2005). All organs were normal, with the only obvious anomaly being significant cell death in the brain, according to an examination of *G3bp1*$^{-/-}$ neonates (Zekri et al.,

2005). Internal capsule neurons, cortical neurons, and CA1 pyramidal neurons of the hippocampus all exhibited pyknosis and activated caspase-3 (Zekri et al., 2005). Importantly, the absence of this discovery in other organs suggests that the lethality is the result of CNS-based neuronal cell death. Given that G3BP1 is widely expressed it was unexpected to discover that genetic deletion of G3BP1 is crucial for physiological neuronal functioning (Sidibé et al., 2021).

3.1 Structure of G3BP

The *G3BP1* gene in humans, which is located on chromosome 5, encodes G3BP1. Only two of the 14 transcripts produced by the transcription of this gene code for the 466 amino acid protein; the rest are destroyed by a process known as non-sense mediated decay (GTEx) (Sidibé et al., 2021). The size of the two protein-coding transcripts' untranslated sections is what distinguishes them from one another (Kang et al., 2021). The larger transcript has a 132 nucleotide 5'UTR and an 8,694 nucleotide 3'UTR, whereas the shorter transcript has a 145 nucleotide 5'UTR and a 1,263 nucleotide 3'UTR (Figure 3a) (GTEx, UCSC genome browser) (Sidibé et al., 2021). These differences imply that the regulation and/or localization of the transcripts may differ (Sidibé et al., 2021). Although it has an apparent molecular weight of 68 kDa on a conventional Laemmli SDS-PAGE, the G3BP1 protein has a theoretical molecular weight of 55 kDa (Sidibé et al., 2021). G3BP1 has structural similarities with members of the heterogeneous nuclear ribonucleoprotein (hnRNP) group of proteins. Nuclear transport factor 2 (NTF2), a tiny homodimer that is critical for nuclear transport through RanGDP import, shares a significant degree of structural similarity with the protein's N-terminal region (amino acids 1–139) (Nehrbass & Blobel, 1996; Vognsen et al., 2013). Despite the fact that G3BP1 is generally found to be localized to the cytosol, this resemblance has led some researchers to hypothesize that the NTF2-like domain of G3BP1 has a similar role in nuclear shuttling (Vognsen et al., 2013). It was originally believed that this domain, which is the most conserved part of the G3BP1 protein sequence, acted as a RasGAP-binding site; however, this has now been called into doubt (Xu et al., 2013). G3BP1 dimerization and interactions with other proteins, such as cytoplasmic activation/proliferation-associated protein 1 (Caprin1),

require the NTF2-like domain. The area 139–340 comprises two intrinsically disordered regions (IDR1 and IDR2), the first half of which is enriched for proline residues (PxxP; amino acids 222–340), and the second half of which is mostly composed of acidic amino acids (139–221) (figure 3b). According to Booker et al. (1993), these areas are also connected to protein-protein interactions (Booker et al., 1993). The number of PxxP motifs can be used to distinguish G3BP1 and G3BP2 from one another. With just one PxxP motif, G3BP1 may be less able to interact with proteins than G3BP2a or G3BP2b, which contain four and five PxxP motifs, respectively (Figure 4b) (Krapp et al., 2017). G3BP1 has an RNA recognition motif (RRM) between amino acids 340-415, which indicates that it is an RNA-binding protein (RBP) (Sidibé et al., 2021). The binding platform created by beta sheets allows this RRM, which contains of two conserved amino acid sections of RNP1 and RNP2, to interact with RNA sequences of 2-8 nucleotides (Cléry et al., 2008). The change of Ile for Val in the RNP-2 common sequence is the primary distinction between the G3BP1 and G3BP2 RRMs (Kennedy et al., 2001). The arginine, glycine, and glycine repeat (RGG) domain of G3BP1 contains a third intrinsically disordered region (IDR3), which because of RGG clusters that promote non-specific RNA interactions, generates an exposed structure with no clear definition. The RGG domain's post-translational changes affect interactions with proteins and mRNAs (Sidibé et al., 2021).

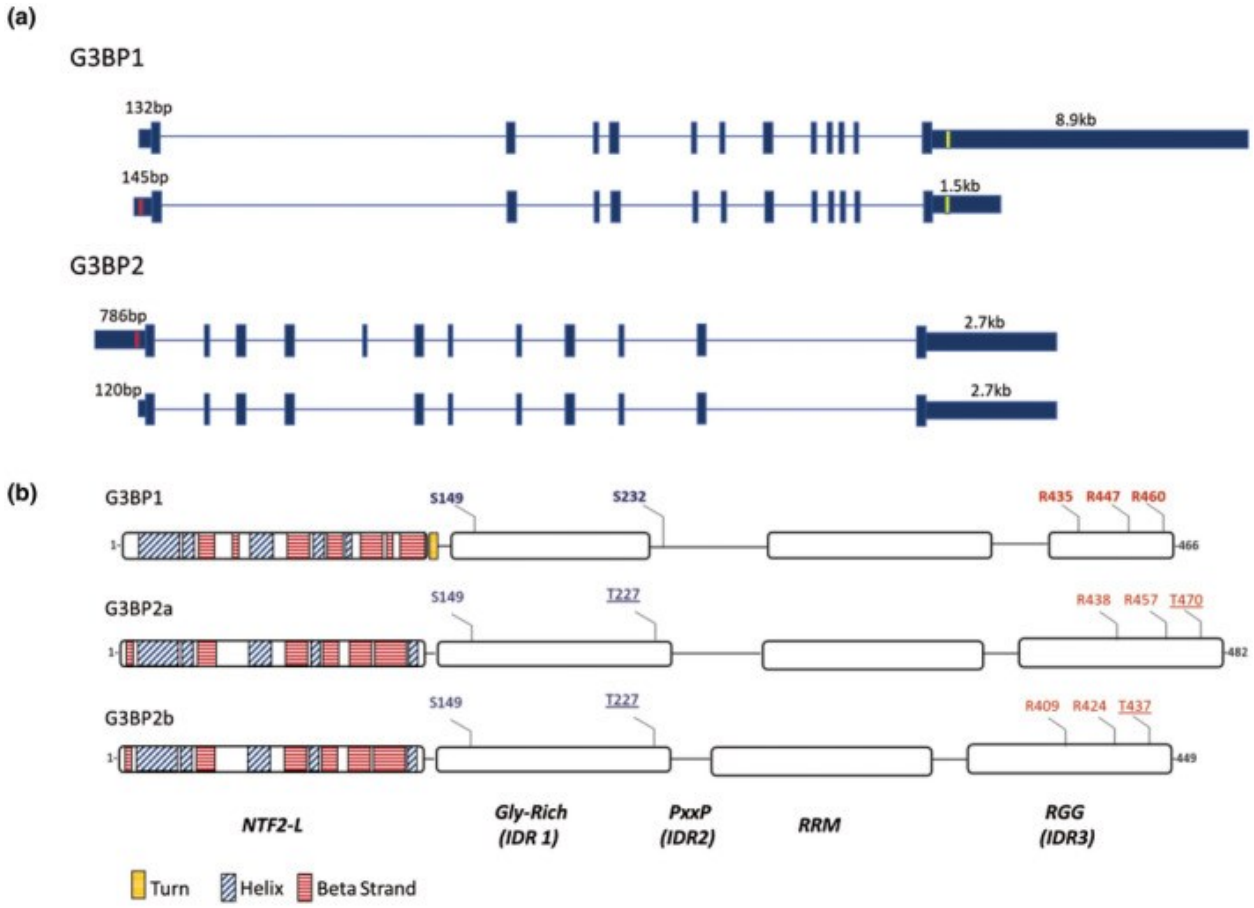

Figure 3. – G3BP sequence features. From Sidibé et al., 2021. (a) A schematic illustration of the transcripts for G3BP1 and G3BP2. (Yellow: TDP-43 binding site, Red: DDX5 binding site). (b) Schematic representation of the structural domains of G3BP1, G3BP2a, and G3BP2b: RNA recognition motif, Glycine rich domain (intrinsically disordered domain 1) nuclear transport factor 2 (NTF2) -like domain PxxP (intrinsically disordered domain 2), and RGG (Arginine, Glycine rich domain, intrinsically disordered domain 3). G3BP1 post-translational modification sites that are known are shown in bold (blue: phosphorylation; red: methylation). The locations that aligned with the changed G3BP1 amino acids are shown after the three proteins were aligned. Sites that are not preserved are marked with an underline.

3.2 G3BP and SGs

SGs are the cellular stress response's downstream players. G3BP1 and G3BP2 are nucleated in SGs in various human cells under stressful circumstances (Matsuki et al., 2013). In 2003, after cellular exposure to sodium arsenite G3BP1 was shown to co-localize with HuR and TIA1 in granular structures, demonstrating for the first time that it associates with stress granules (Tourrière et al., 2003). The creation of stress granules is triggered by a wide range of external insults, but the assembly, and dynamics of the granules differ depending on the cell type and degree of stress exposure (Aulas et al., 2017). G3BP1 has consistently been demonstrated to be a component of these cytoplasmic condensates, regardless of the kind of stress. Under stressful circumstances, G3BP1 generates structures rich in polyadenylated messenger RNAs which is brought on by stress-related eIF2 phosphorylation, together with other proteins including HuR and TIA1 (Kedersha & Anderson, 2002). Importantly, the phosphorylation of eIF2 caused translation to be stopped, resulting in the presence of polyadenylated mRNA in these puncta. For stress granules, several roles have been suggested over time including: providing an opportunity during which stress can be resolved or an apoptotic program will be launched, controling local concentrations of proteins and mRNAs, and allowing rapid resumption of protein synthesis when cells recover from stress (Eiermann et al., 2020; Kedersha & Anderson, 2002; McCormick & Khaperskyy, 2017). It is reported that the total number of early SGs is correlated with G3BP1 expression, and G3BP overexpression promotes SG formation (Kawaguchi et al., 2003). In addition, G3BP1 cellular depletion causes stress granule dynamics to malfunction, resulting in smaller, more numerous granules and an increase in cell mortality under stressful circumstances (McDonald et al., 2011).

The interactions between G3BP1's three separate intrinsically disordered regions (IDRs) control how the liquid-liquid phase separation of SGs occurs (Yang et al., 2020). The development of SGs is also influenced by G3BP's acetylation, phosphorylation, and methylation. For example, G3BP1 is dephosphorylated at Ser-149 by the pseudophosphatase MK-STYX (mitogen-activated protein

kinase phosphoserine/threonine/tyrosine-binding protein), which causes the Formation of SGs (Barr et al., 2013). Furthermore, by attaching to various proteins, G3BP controls how SGs are formed. For instance, G3BP's carboxyl (C)-terminus region can trigger eIF2's phosphorylation, which in turn can trigger the formation of SGs (Taniuchi et al., 2011). Caprin also interacts with G3BP, to facilitate the production of SGs (Kedersha et al., 2016). The nucleation of SGs requires ubiquitin-associated protein 2-like (UBAP2L). It functions upstream of G3BP and encourages the development of the G3BP1 core as well as the assembly and expansion of SGs (Cirillo et al., 2020). Despite G3BP1's role in SG assembly, G3BP1 is also a significant factor in SG disassembly. Histone deacetylase 6 (HDAC6) and CBP/p300 control the acetylation of G3BP1, which promotes the breakdown of SGs (Gal et al., 2019). By preventing G3BP from being translated, the testis-specific protein (melanoma-associated antigen gene B2) and DEAD-box decarboxylase 5 (DDX5) can prevent the development of SGs (Lee et al., 2020). In addition, protein arginine methyltransferase 1 (PRMT1), PRMT5, and low-density lipoprotein receptor-related protein 6 (LRP6) methylate G3BP1 to prevent the assembly of SGs (Ramachandran et al., 2018; Tsai et al., 2017). Mammalian ubiquitin-specific protease (USP10)'s FGDF motif also binds to G3BP, altering the way G3BP1/2 and Caprin1 interact and preventing the creation of SGs (Kedersha et al., 2016). Hence, according to all the above studies, stress granules are formed and disassembled in a manner that is closely Dependent on G3BP. Given that G3BP1 is the primary regulator of stress granules, studying it in the context of pathology may be able to shed light on the nature of the problems.

3.3 G3BP1 and G3BP2

G3BP1 and G3BP2 are paralogs that reside on different chromosomes rather than being isoforms of the same gene: While G3BP2 is located on chromosome 4, G3BP1 is found on chromosome 5. G3BP2 may be translated into 19 transcripts, three of which are responsible for the production of the isoforms G3BP2a and G3BP2b, proteins of 482 and 449 amino acids, respectively (Sidibé et al., 2021). According to DNA sequence analysis, the coding sequences of G3BP2a and G3BP2b are 60% and 55% comparable to those of G3BP1, respectively (Sidibé et al., 2021). G3BP1 and G3BP2

have frequently been investigated as a single unit. Findings from G3BP1 studies research routinely generalized to G3BP2 studies even when there is no supporting data (Sidibé et al., 2021) while both G3BP1 and G3BP2 have been heavily linked to cancer (Alam & Kennedy, 2019), work on stress granule formation and dynamics mostly concentrates on G3BP1. In addition, research on viral infection contend that viruses favor G3BP1 as a target (Zhang et al., 2019). These studies typically ignore the possibility that the activities of G3BP proteins may not entirely overlap despite the high degree of similarity among G3BP1 and G3BP2 (Sidibé et al., 2021). According to Matsuki et al., G3BP2 can also promote the creation of stress granules, while Kedersha et al., found that genetic deletion of both G3BP1 and G3BP2 is necessary to completely block the assembly of stress granules in transformed cell lines (Matsuki et al., 2013; Kedersha et al., 2016). G3BP2's phase separation characteristics, however, differ slightly from those of G3BP1 (Guillén-Boixet et al., 2020). It was shown that G3BP2 exhibits a greater tendency to phase separate with proteins and RNA compared to G3BP1 because a higher percentage of G3BP2 could form condensates with polyethylene glycol (PEG) or RNA at lower concentrations (Guillén-Boixet et al., 2020). This is due to G3BP2's intrinsically disordered sections and RNA-binding domain that are longer than those of G3BP1 (Figure 3b). Given these elements, it is likely that G3BP2 and G3BP1 play comparable roles in the generation and dynamics of stress granules (Sidibé et al., 2021).

3.4 Transcriptional regulation of G3BP1

Only a few studies have looked at how *G3BP1* mRNA is regulated. By binding to the 5'UTR (untranslated region) of G3BP1, Y-box binding protein 1 (YB-1), a highly conserved member of the cold shock protein family, controls translation (Somasekharan et al., 2020). Additionally controlling *G3BP1* transcripts, MAGE-B2 and DDX5 engage in antagonistic competition. While the Dead-box RNA helicase DDX5 (or p68) binds to the 5'UTR of G3BP1 to enhance translation, MAGE-B2, a member protein of the melanoma antigen family (MAGE), binds to the 5'UTR of G3BP1 to inhibit translation (Lee et al., 2020). Only the short G3BP1 transcript has the DDX5/MAGE-B2 binding site, suggesting differential regulation of transcripts. G3BP1 is also regulated by TDP-43.

3.4.1 Transcriptional regulation of G3BP1 by TDP-43

G3BP1 protein and transcript levels drop in response to TDP-43 knockdown (McDonald et al., 2011). In a recent study, it was indicated that TDP-43 stabilizes only the short *G3BP1* transcript through a UG-rich regulatory region in the 3'UTR while not affecting the long G3BP1 transcript (Sidibé et al., 2021). Additionally, this research showed that ALS/FTD neurons with TDP-43 inclusions and concurrent nuclear depletion had low levels of G3BP1 mRNA (Sidibé et al., 2021). It is yet to be proven whether this holds true in other conditions with TDP-43 pathology.

4. Rational to the project

A growing number of RNA-binding Proteins (RBPs) have been related to myopathies and motor neuron disorders in a stream of genetic investigations. For instance, RBP gene mutations have been linked to disorders like spinocerebellar atrophy and spinomuscular atrophy for more than 20 years (Lefebvre et al., 1995). After TDP43 was identified as the main pathogenic protein aggregate that accumulates in ALS and certain FTDs, interest turned to RBP aggregation pathways (Lefebvre et al., 1995). Other RBP genes with ALS-linked mutations, such as FUS were discovered shortly after TDP43 was identified as the main pathogenic protein in ALS (Kwiatkowski et al., 2009; Vance et al., 2009). These findings supported the emerging theory that RBP biology might contribute to neurodegenerative disorders (Wolozin & Ivanov, 2019). The discoveries that TDP43 and FUS lead to the formation of SGs, that disease-linked mutations in the genes encoding these RBPs cause an increased accumulation of SGs in cells under stress, and that the aberrant accumulation of these proteins in the human brain co-localizes with SG markers represent one view on our understanding of the mechanisms underlying diseases exhibiting TDP43 and ALS pathology (Dormann & Haass, 2011; Liu-Yesucevitz et al., 2010). A definitive proof-of-concept, however, will only come from research showing that targeted inhibition of the SG pathway stops disease development in animal models. Another view which concentrates on TDP-43 loss of function leading to stress granule dysfunction as a contributor to stress granules has also gained attention. According to the first view SGs act as a crucible for triggering pathogenic protein aggregation and this was developed as a result of linking the SG pathway to neurodegenerative

diseases (Liu-Yesucevitz et al., 2010). Since the coalescence of RBPs into SGs draws aggregation-prone proteins together into local domains that concentrate RBPs 100–400-fold, this route was suggested to be susceptible to disorders of aggregation (Maharana et al., 2018). At the moment, RBP biological models are the main focus of research on SGs and neurodegenerative pathologies. RBPs direct the translation, transport, and destruction of intracellular RNA, which controls and regulates the production of proteins. Due to this theory that stress granule formation results in persistent granules that change from a liquid phase to a solid structure and may serve as seeds for the TDP-43 inclusions seen in patients with ALS, stress granules have drawn more attention in this disease (Jeon & Lee, 2021; Wolozin & Ivanov, 2019). This is supported by the findings that many RBPs have been noted to aggregate in ALS post-mortem tissue which localize to stress granules in certain in vitro situations (Bakkar et al., 2018; Liu-Yesucevitz et al., 2010; Vu et al., 2021). Liu et al., demonstrated that TDP-43 disease-linked mutations enhanced the production of TDP-43 inclusions in response to stressful stimuli. In this study, they discovered that therapy with translational inhibitors that prevent or reverse SG formation can reduce inclusion development caused by WT or mutant TDP-43. Finally, they showed that TDP-43 positive inclusions co-localize with a number of stress granule protein markers, including TIA-1 and eIF3, in pathological CNS tissue. However, TDP-43 inclusions in ALS/FTD patients were not found to contain mRNA, an obligate component of stress granules (Mann et al., 2019). TDP-43 aggregates and stress granules appear to develop independently, according to investigations in cell models (Aulas et al., 2012; Mann et al., 2019). Therefore, another theory is that defective stress granule production may enhance neuronal susceptibility in the context of disease. *In vitro* data from cells exposed to sodium arsenite show that TDP-43 knockdown with a silencing RNA result in sub-optimal stress granule development, with granules having smaller size (Aulas et al., 2012; McDonald et al., 2011). The production of stress granules is likewise defective in patient fibroblasts carrying the TDP-43^{A382T} mutation and neural cells expressing solely cytoplasmic TDP-43 (TDP-$43^{\Delta NLS}$) in response to arsenite (Besnard-Guérin, 2020; Orrù et al., 2016). Additionally, it has been demonstrated that primary mouse motor neurons or motor neurons generated from ESCs with the TDP-43^{M337V} mutation exhibit impaired stress granule assembly (Gordon et al., 2019). Depletion of nuclear TDP-43 is linked to the loss of G3BP1, a key stress granule protein, which

might be one explanation for these reported deficiencies in stress granule formation (Sidibé et al., 2021). Together, these results show that TDP-43 represents loss of function with regard to stress granule formation.

A vast range of post-translational modifications, microtubule networks, and protein remodeling complexes control how briefly SGs exist under particular stress situations. One important strategy for managing cell fate or treating disease may be to regulate SG assembly or disassembly. Therefore, targeting SG proteins in relevant disorders is a promising avenue for the development of possible treatment methods. Currently, a variety of compounds have been shown to have an impact on how SGs are maintained, assembled, and disassembled via various mechanisms. SGs have been targeted by blocking and sequestering signaling molecules including TRAF2 (NF- κb. signaling), RACK1 (p38/JNK signaling), RhoA/ROCK1 (Wnt signaling), and Raptor (mTOR signaling) (Cao et al., 2020; McCormick & Khaperskyy, 2017; Wolozin & Ivanov, 2019). Many small-molecule inhibitors, such as those aiming for the eIF4 pathway, which involves mTOR, the eIF2α pathway, which includes the PERK and PKR pathways, heat shock protein 104, valosin containing protein (VCP), importin, and transportin, are among the many known small-molecules that target SG pathways and are easily accessible (Wang et al., 2020). In models of neurodegenerative disorders, inhibitors of several of these SG pathway inhibitors have already demonstrated neuroprotection (Wang et al., 2020). Additionally, increasing stress granule disassembly by targeting the VCP/FAF2 axis may help in the removal of persistent stress granules: nonetheless, Gwon et al. have noted that this process may be highly context-specific, which may limit its therapeutic application (Gwon et al., 2021). Numerous groups have investigated the PERK pathway in various contexts (Smith & Mallucci, 2016). In transgenic mice models where the disease is triggered by the production of mutant TDP43 or tau, or transmission of the prion protein, inhibiting the PERK pathway slows disease development (Kim et al., 2014; Radford et al., 2015). According to research on TDP43 and the prion protein, PERK inhibition decreases eIF2 phosphorylation, which suggests that it has an impact on the translational regulation and SG pathway (Wolozin & Ivanov, 2019). However, PERK inhibition has pleiotropic effects that control the endoplasmic reticulum's chaperone pathways, raising the likelihood that the advantages of PERK inhibition result from mechanisms other than the SG pathway (Wolozin & Ivanov, 2019). In a model of AD based on the overproduction of Aβ,

inhibition of GCN2, a different kinase that controls eIF2α, also offered neuroprotection (Ma et al., 2013). Another neuroprotective agent, rapamycin, is likely to block an SG route mediated by eIF4 since it suppresses mTOR (Jackson et al., 2010). Various animal models of neurodegeneration, including those caused by tau or TDP43, have successfully been treated with rapamycin (Khurana et al., 2006; Wang et al., 2012).The development of SGs, including TDP43-positive granules, in cells is inhibited by inhibitors of cyclin-dependent kinase inhibitors that disrupt the SG response (Moujalled et al., 2015). Lastly, high-throughput drug screening can be used for better understanding of SGs. Motor neuron-differentiated induced pluripotent stem cells (iPSCs) may now be employed for pharmacological screens targeting TDP43, FUS, or hnRNPA2B1 (Martinez et al., 2016).

Assessment in a neuronal context and using *in vivo* paradigms will be necessary to determine the true impact of stress granule dynamics on neurodegenerative disease, whether as a participant in disease initiation or progression or impairments in their assembly or disassembly (Dubinski & Vande Velde, 2021). TDP-43 simultaneously loses its nuclear localization in conjunction with this cytoplasmic localization. Splicing, transcription, miRNA and lncRNA processing, and RNA stability maintenance are only a few of the mRNA-related tasks that TDP-43 carries out in the nucleus (Prasad et al., 2019). Nuclear depletion causes TDP-43 to stop performing these activities. One of the most significant impacts of TDP-43 depletion is its effects on G3BP1. Recent research has shown that TDP-43 stabilizes G3BP1-encoding mRNA and that G3BP1 mRNA is reduced in ALS/FTD patient neurons exhibiting TDP-43 pathology (Sidibé et al., 2021). Consequently, G3BP1 may be a desirable druggable target. The disruption of stress granule dynamics caused by this TDP-43-induced G3BP1 depletion can be corrected by reintroducing G3BP1 (Aulas et al., 2012). This generally points to a TDP-43 loss of function mechanism and downstream impairment of stress granule production (Sidibé et al., 2021). It should follow that methods to support their survival should be a cellular priority if one takes into account that motor neurons are terminally differentiated cells (Sidibé et al., 2021). Normalizing this process would be a possible therapy due to the known function of stress granules to support cell survival (Sidibé et al., 2021).

Trans-acting factors (miRNAs and RNA binding proteins) specifically target cis-acting elements (sequence-specific control elements) to modulate mRNA stability (Moore, 2005). It was demonstrated that the cis-acting regulatory sequence motifs found in mRNAs may be used by small molecules to control the translation and stability of mRNA (Kastelic et al., 1996; Mak et al., 2007). These studies suggest RNA as a target for therapeutic intervention. The ability of small compounds to directly attach to RNA has been demonstrated by several studies (Warner et al., 2018; Childs-Disney & Disney, 2016). Based on these discoveries, Novation (a pharmaceutical company) has created a platform-technology to uncover new small molecules that can influence gene expression. This novel small technology has now been applied to G3BP1 and is based on work in our lab. In human neuronal-like cells, a variety of chemicals that upregulate G3BP1 mRNA and protein levels when TDP-43 is suppressed is identified by screening using different drug-like compounds from libraries that are available commercially. Through this screen three promising lead candidates (NPX-047, NPX-000-115, and NPX-001-280) that restore endogenous G3BP1 expression and function after TDP-43 loss of function were identified. According to the study that the regulatory element is conserved in *C. elegans* and *gtbp-1* mRNA, the worm orthologue of G3BP1, has lower levels in *tdp-1-/-* (ok803) (Sidibé et al., 2021), this model was used in order to investigate the lead candidates at the organismal level. These compounds rescued G3BP1 expression as well as age-related paralysis in TDP-43 (*tdp-1*) knockout worms. Also, all of these compounds depicted low EC_{50} in SH-SY5Y cells as well as high brain permeability according to Novation.

5. Hypothesis and objective

Recently, it was indicated that TDP-43 stabilizes and directly binds one of the two transcripts that code for G3BP1 by way of a highly conserved, UG-rich regulatory region in the 3'UTR (Sidibé et al., 2021). Additionally, it was indicated that there is a strong connection TDP-43 pathology and the loss of *G3BP1* transcripts in ALS/FTD (Sidibé et al., 2021). Our hypothesis is that restoring

physiological G3BP1 expression will enhance neuronal survival by restoring the endogenous stress granule mechanism, resulting in a positive therapeutic impact for ALS/FTD patients. We hypothesize that restoration of physiological G3BP1 expression by governing mRNA stability within the G3BP1 transcript will protect neuronal survival in challenging circumstances. In the end, this will have a positive therapeutic impact on ALS patients. Therefore, as a potential ALS therapy, our therapeutic approach and objective is to identify and functionally verify small molecular weight compounds that increase G3BP1 expression as screened by novation, restore the stress granule response, and sustain neuronal health. We also aim for finding structural analogs of potential compounds to optimize restoration of G3BP1 expression and function.

Chapter 2- materials and methods

Cell culture and transfection

SH-SY5Y cells were cultured in Dulbecco's high glucose modified Eagle medium/Nutrient Mixture F-12 Ham (DMEM-F12, ThermoFisher Scientific) supplemented with 10% FBS (Corning), 2 mM L-glutamine and 1% MEM non-essential amino acids (ThermoFisher Scientific). Cells were collected 48h or 72 h after transfection with 125 pmol Stealth siRNA using Lipofectamine® 2000 (Invitrogen). Sequences of si-RNAs were: TDP-43 (siTDP-43): 5' AAGCAAAGCCAAGAUGAGCCUUUGA-3'; Control (siCTL): #12935-200 (Invitrogen).

Cell lines qRT-PCR

Using RNAeasy Minikit (Qiagen) RNA was extracted and treated with DNAse I (Qiagen) according to the manufacturer's instructions. Using the QuantiTect Reverse Transcription kit (Qiagen), 500ng of RNA were used for reverse transcribtion. The QuantStudio 7 Flex Real-Time PCR System (Life Technologies) was used for qPCR. PrimeTime Standard qPCR assays (IDT) were: *TARDBP*: Hs.PT.58.26912658; *G3BP1* total (targeting exon 6 and 7) Hs.PT.58.20396264; *GAPDH* (targeting exon 1-2) Hs.PT.39a.22214836; *G3BP1* long forward: 5'-TCTTACTGGACACTCAACCTTG-3'; *G3BP1* long reverse: 5'-TGCCATAACTTTTGTGACTTCATG-3'; *18S:* Hs.PT.39a.22214856. g. Data was analyzed using the 2-ΔΔCt method. The genes of interest were normalized to the geometric mean of two housekeeping genes (*GAPDH* and *18S*).

Cellular viability

400000 of SH-SY5Y cells were incubated at 37°C for 48 or 72 hours and treated with the compounds from Novation and then were stained with trypan blue (Multicell) and were counted immediately using a hemocytometer.

Animals

The use of animals and all procedures were performed according to guidelines of the Canadian Council on Animal Care and were approved by the CRCHUM animal care committee (CIPA). The non-transgenic group of mice were a combination of non-transgenic littermates from TDP-43^{M337V} and B6/N mice (Charles River). Mice of both sexes were used in this study at 4 and 18 months of ages.

Mice qRT-PCR

Spinal cords from previously stored tissues at -80˚C were used. RNA was extracted using TRIzol® (Invitrogen) and chloroform (Fisher Scientific). RT was performed with QIAGEN kit. PrimeTime Standard qPCR assays (IDT) were: *hTARDBP*: Hs.PT.58.26912658; *mTARDBP*: Mm.PT.58.30210473; G*3BP2:* Mm.PT.58.9030133. *G3BP1* total Mm.PT.58.6655287; *G3BP1* long forward: 5'-TGTAATCGAGGCTGgTTTTGG-3'; *G3BP1* long reverse: 5'-TGGCGAATGTTTTAGTCCTGG-3'; *Rplp0:* Mm.PT.58.43894205; *Actin*: Mm.PT. 39a.22214843.g

Statistics

Data were graphed and analyzed using Prism version 9.00 (GraphPad Software). Data were compared via two-way ANOVA with statistical significance at $p<0.05$.

Chapter 3- Results

3.1 Cell viability assay using different doses of compounds

In order to assess the toxicity of the compounds, Trypan blue test of cell viability was performed. This test determines the number of viable cells based on the fact that live cells exclude Trypan blue because of their intact membrane, however dead cells do not. In this method, the dead cells will have a blue cytoplasm whereas the alive cells will contain a clear cytoplasm. After seeding 400,000 SH-SY5Y cells, the next day the cells were transfected with either si-CTL or si-TDP. 5h later, compounds were given to the cells. For the next 48h, cells were treated with the compounds at 20uM every 24h. On day 5 the cells were harvested. This is called the 72h protocol (Fig.4A). DMSO, NPX 000-280 and NPX 000-115 all caused toxicity less than 20% however this was around 60% for NPX-047 (Fig.4B). Next, to reduce the exposure time of cells to the compounds a 48h protocol where cells were harvested on day 4 instead of day 5 was used. DMSO, NPX 000-280 and NPX 000-115 all indicated less than 20% cell death. Whereas this was ∼45% for NPX-047 (Fig.4C). Next, various concentrations of NPX-047 for toxicity were tested. Less than 20% of toxicity for all the concentrations except for 20uM was observed (Fig.4D). Then, Because of the low toxicity observed with the compounds NPX 000-280 and NPX 000-115, the concentration of these compounds was increased to 40uM and the experiments were performed according to 48h protocol. NPX 000-280 showed slightly higher than 20% toxicity while NPX 000-115 indicated about 36% toxicity (Fig.4E). Old batches and new batches of the compounds were tested to investigate whether there was any difference in terms of toxicity. In addition, the effects of the compounds on cell toxicity without si-treatment was investigated. In this experiment, on day 1 400,000 cells were seeded. For the next 48h cells were treated with the old and new batches of the compounds every 24h. For NPX-047 only new batch was available. All the compounds were used at 20uM. No significant differences were detected between old and new batches of the compounds (Fig.4F). NPX-047 indicated less toxicity without si-treatment.

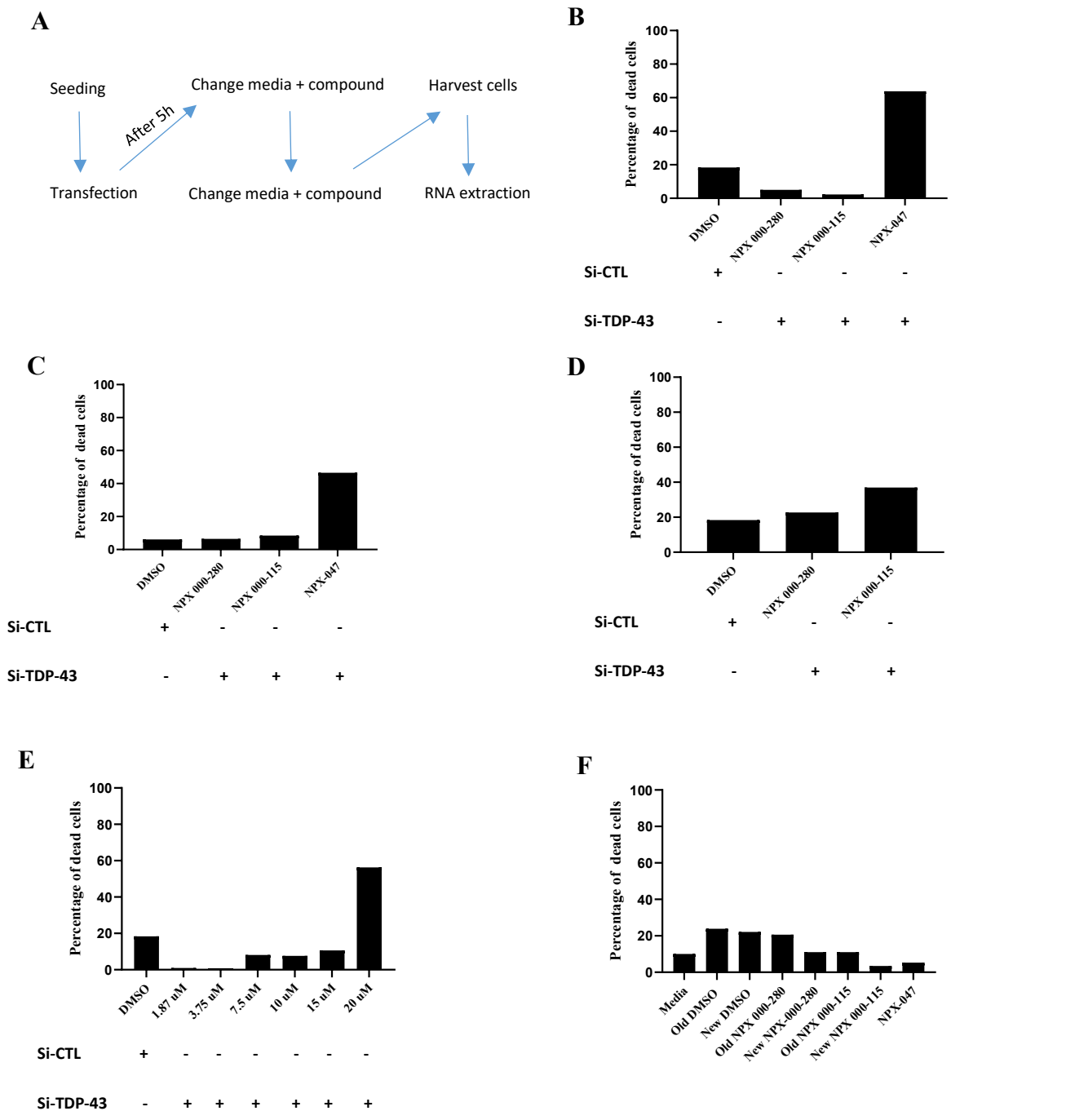

Figure 4. – Cell viability assay using different concentrations of the compounds. (A) Schematic figure for 72h protocol. (B) SH-SY5Y cells were seeded on day 1, the next day they were treated with either si-CTL or si-TDP. After 5 hours of transfection the compounds were given to cells. The next 48h they cells were treated with the compounds every 24h. On day 5 cells were harvested. All the compounds had 20uM concentration. N=1 (C) SH-SY5Y cells were seeded on day 1, the next day they were treated with either si-CTL or si-TDP. After 5 hours of transfection the compounds were given to the cells. After 24h, the compounds cells

were treated with the compounds following half-media change. After 24h, cells were harvested and cell viability was measured (48h protocol). 20uM of all compounds was used. N=1 (D) SH-SY5Y cells were transfected and treated with DMSO, NPX 000-280 and NPX 000-115 according to the 48h protocol. The concentration of the compounds was 40uM. N=1. (E) Cells were treated with DMSO or different concentrations of NPX-047 after si-CTL or si-TDP treatment according to the 72h protocol. N=1. (F) SH-SY5Y cells were seeded on day one, the next two days they were treated with old and new batches of DMSO, NPX 000-280, NPX 000-115 and new batch of NPX-047. All the compounds were used at 20uM. On day fourth cells were harvested and cell viability was assessed using Trypan blue. N=1

3.2 NPX 000-280 has no effect on total G3BP1 and long G3BP1 transcripts at 20uM

The compounds were able to increase G3BP1 luciferase levels in luciferase assay as reported by Novation; however, this does not indicate endogenous levels of *G3BP1* mRNA. In our experiments it is necessary to indicate the impact of the compounds on endogenous *G3BP1* levels to confirm our hypothesis. Thus, the effect of NPX 000-280 at 20uM on *G3BP1* mRNA levels was assessed. According to cell viability results, 20uM was an appropriate concentration for our experiments. First, whether there was a suitable knockdown of *TDP-43* and that NPX 000-280 had any effect on TDP-43 was investigated. Around 70% knock down of *TDP-43* following si-TDP treatment was observed. Also, no effect of NPX 000-280 on *TDP-43* mRNA levels was detected (Fig.5A). Then, the *G3BP1* mRNA levels following si-treatment was assessed to investigate whether NPX 000-280 can rescue the *G3BP1* levels. There was no rescue of *G3BP1* levels (Fig 5B). NPX 000-280 had also no effect on *Long G3BP1* mRNA levels (Fig 5C). Next, the 72h protocol was used to assess *G3BP1* mRNA following treatment with NPX 000-280. Again, we about 80% of *TDP-43* knock down was detected(n=1) (Fig.5D) however, there was no rescue of *G3BP1* mRNA levels (n=1) (Fig.5E). In addition, no effect on long *G3BP1* mRNA was detected, as expected (n=1) (Fig.5F).

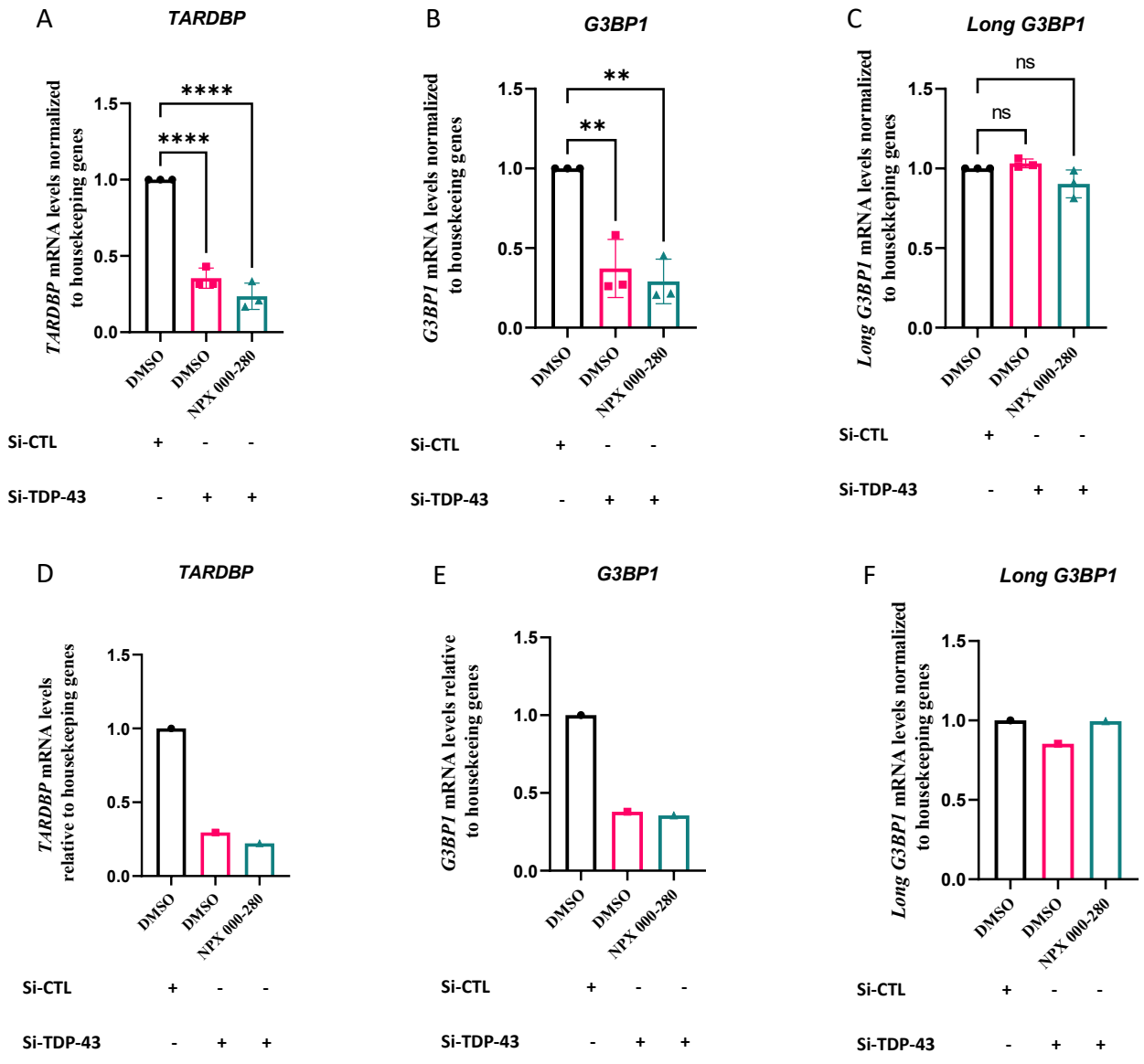

Figure 5. – NPX 000-280 has no effect on total G3BP1 and long G3BP1 transcripts at 20uM.

Relative mRNA expression levels of (A) TARDBP, (B) G3BP1 and (C) Long G3BP1 in SHSY5Y cells transfected with si-CTL and si-TDP followed by treating with DMSO and NPX 000-280 according to 48h protocol. Data are shown as mean +s.d. of n = 3 independent experiments after normalization to GAPDH and 18S. Statistical analysis using one-way ANOVA with ****p <0.001, **p <0.01, ns=not significant. Relative mRNA expression levels of (D) TARDBP, (E) G3BP1 and (F) Long G3BP1 in SHSY5Y cells transfected with si-CTL and si-TDP followed by

treating with DMSO and NPX 000-280 according to 72h protocol. Data are shown as mean +s.d. of n = 1 experiment after normalization to GAPDH and 18s.

3.3 NPX 000-115 has no effect on total G3BP1 and long G3BP1 transcripts at 20uM

As Novation implicated that NPX 000-115 was able to increase G3BP1 luciferase signals, it was important to assess this compound's effect on G3BP1 mRNA levels in our SH-SY5Y cells to assess our hypothesis. Based on the cell viability findings, 20uM was a suitable concentration for this compound for our experiments. Same as NPX 000-280, NPX 000-115 had no influence on *TDP-43* and approximately a 70% reduction in TDP-43 levels after si-TDP treatment was detected. (Fig. 6A). Subsequently, when G3BP1 mRNA levels were quantified following si-treatment to explore the potential of NPX 000-115 in rescuing G3BP1 levels, no restoration of G3BP1 levels was observed. (Fig. 6B). Furthermore, NPX 000-115 exhibited no impact on Long G3BP1 mRNA levels, as anticipated (Fig. 6C). In our continued investigation using the 72-hour protocol again an approximately 80% reduction in TDP-43 levels was observed following si-treatment (n=1) (Fig. 6D). Nevertheless, there was no evidence of G3BP1 mRNA level rescue (n=1) (Fig. 6E), and as expected, no effect on Long G3BP1 mRNA was detected (n=1) (Fig. 6F).

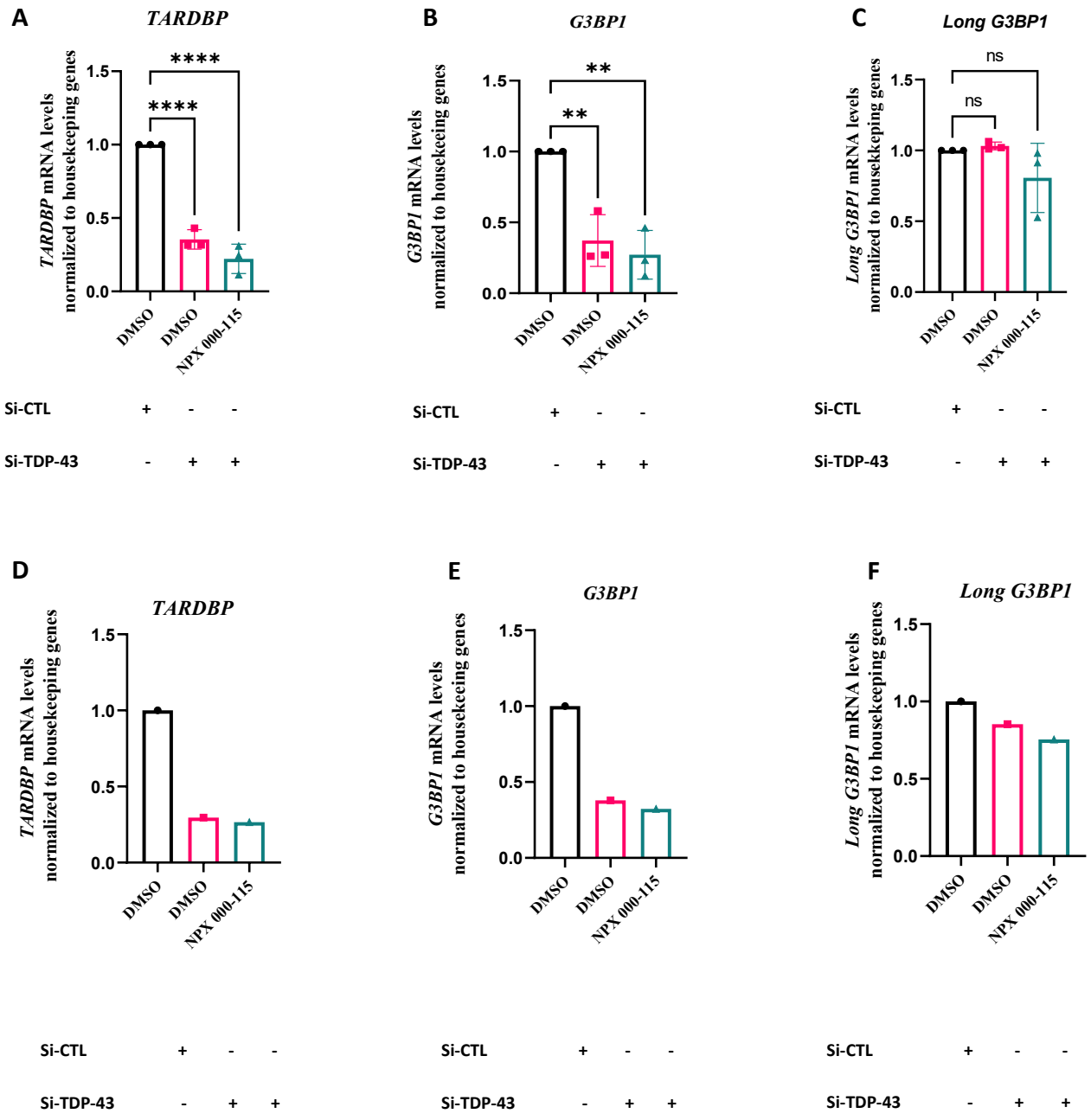

Figure 6. – NPX 000-115 has no effect on total G3BP1 and long G3BP1 transcripts at 20uM.

Relative mRNA expression levels of (A) TARDBP, (B) G3BP1 and (C) Long G3BP1 in SHSY5Y cells transfected with si-CTL and si-TDP followed by treating with DMSO and NPX 000-115 according to 48h protocol. Data are shown as mean +s.d. of n = 3 independent experiments after normalization to GAPDH and 18S. Statistical analysis using one-way ANOVA with ****p <0.001, **p <0.01, ns=not significant. Relative mRNA expression levels of (D) TARDBP, (E) G3BP1 and (F) Long G3BP1 in SHSY5Y cells transfected with si-CTL and si-TDP followed by

treating with DMSO and NPX 000-115 according to 72h protocol. Data are shown as mean +s.d. of n = 1 experiment after normalization to GAPDH and 18s.

3.4 NPX 000-280 and NPX 000-115 do not rescue G3BP1 mRNA levels at 40uM

Since our earlier observation that 20uM failed to restore G3BP1 mRNA levels following a 48h and 72h protocol, the impact of NPX 000-280 and NPX 000-115, both administered at a concentration of 40uM with the aim of rescuing *G3BP1* mRNA levels was investigated subsequently. Initially, the efficacy of TDP-43 knockdown was assessed and remarkably, an approximate 70% reduction in TDP-43 levels following si-TDP treatment was observed (Fig. 7A and 7E) and no discernible effect of either of the two compounds on TDP-43 mRNA levels was detected (Fig. 7A and 7D). Next, our findings revealed no restoration of G3BP1 levels (Fig. 7B and 7E) and exhibited no impact on Long G3BP1 mRNA levels (Fig. 7C and 7F).

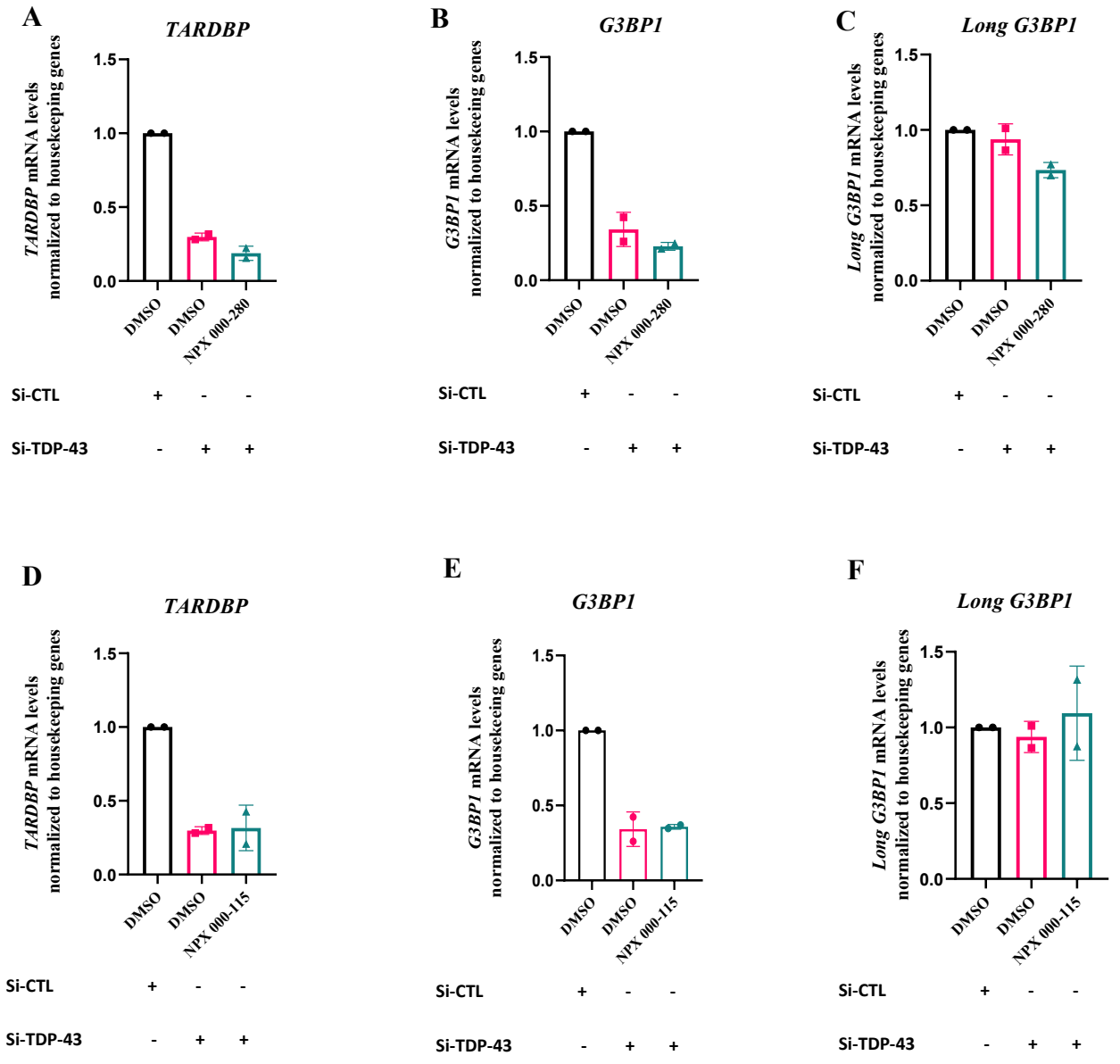

Figure 7. – NPX000-280 and NPX 000-115 have no effect on total G3BP1 and long G3BP1 transcripts at 40uM. Relative mRNA expression levels of (A) TARDBP, (B) G3BP1 and (C) Long G3BP1 in SHSY5Y cells transfected with si-CTL and si-TDP followed by treating with DMSO and NPX 000-*280* according to 48h protocol. Data are shown as mean +s.d. of n = *2* independent experiments after normalization to GAPDH and 18S. Statistical analysis using one-way ANOVA with ****p <0.001, **p <0.01, ns=not significant. Relative mRNA expression levels of (D) TARDBP, (E) G3BP1 and (F) Long G3BP1 in SHSY5Y cells transfected with si-CTL and si-TDP followed by treating with DMSO and NPX 000-115 according to *48*h protocol. Data are shown as mean +s.d. of n = *2* experiments after normalization to GAPDH and 18s.

3.5 NPX-047 at 15 and 10uM do not rescue G3BP1 mRNA levels

According to Novation, NPX-047 was able to increase G3BP1 luciferase signals at 10 uM and because based on the cell viability results, it was determined that 10 uM and 15 uM were suitable for our experiments, these concentrations were chosen to proceed. As previous experiments, knock down efficiency was first assessed which was around 70% (Fig. 8A). Furthermore, no impact of NPX-047 on *TDP-43* mRNA levels was detected (Fig. 8B). Moving forward, *G3BP1* mRNA levels indicated no recue (Fig. 8B). Additionally, NPX-047 showed no effect on *Long G3BP1* mRNA levels (Fig. 8C). Next, a 72-hour protocol to assess *G3BP1* mRNA levels following NPX-047 treatment was tested. Once again, an approximately 80% reduction in *TDP-43* levels (n=1) (Fig. 8D) and no recue of *G3BP1* mRNA levels was detected (n=1) (Fig. 8E). In addition, as expected, no impact on *Long G3BP1* mRNA was observed (n=1) (Fig. 8F).

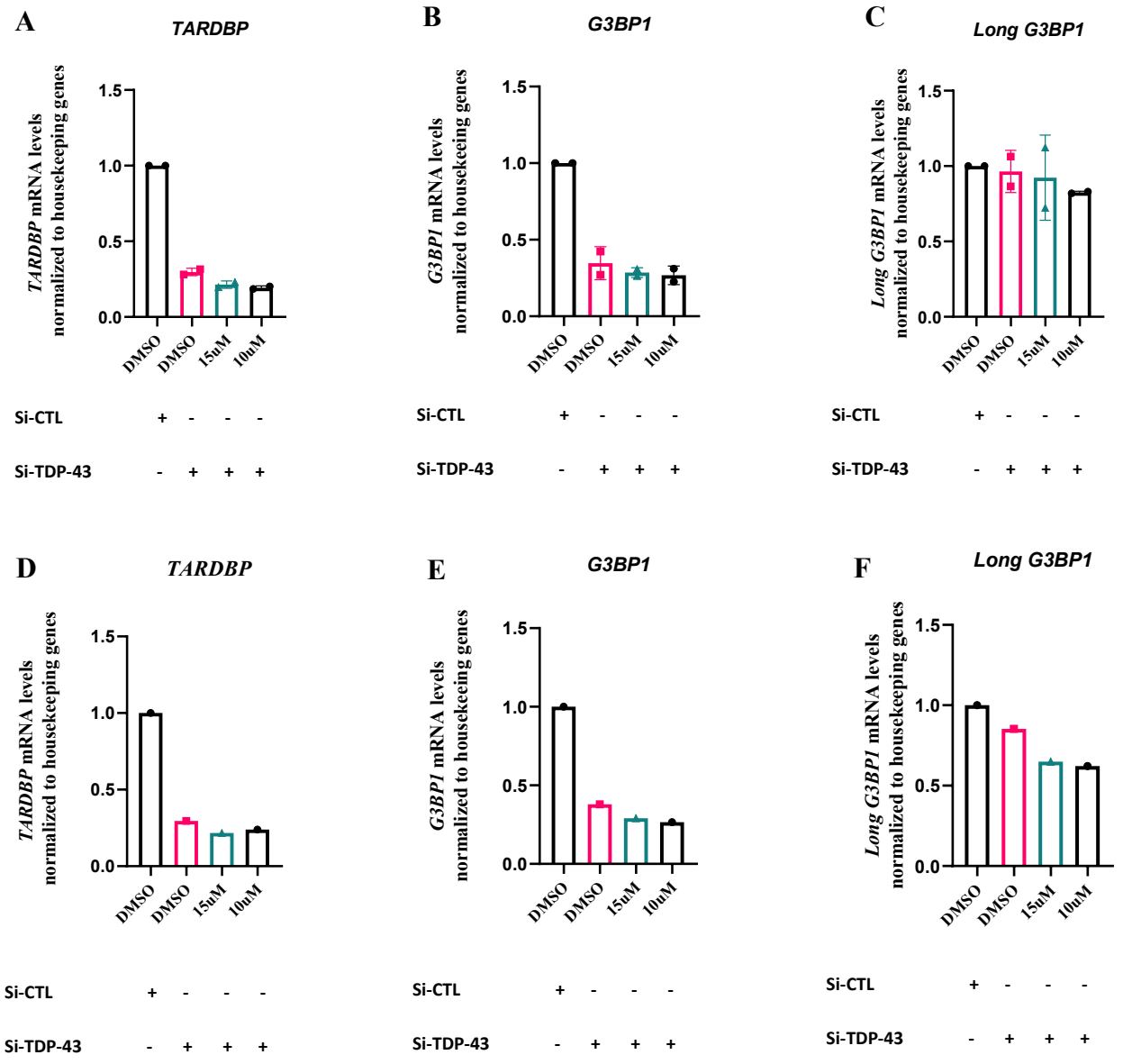

Figure 8. – NPX-047 has no effect on total G3BP1 and long G3BP1 transcripts at 15uM and 10uM. Relative mRNA expression levels of (A) TARDBP, (B) G3BP1 and (C) Long G3BP1 in SHSY5Y cells transfected with si-CTL and si-TDP followed by treating with DMSO and NPX-047 according to 48h protocol. Data are shown as mean +s.d. of n = 2 independent experiments after normalization to GAPDH and 18S. Relative mRNA expression levels of (D) TARDBP, (E) G3BP1 and (F) Long G3BP1 in SHSY5Y cells transfected with si-CTL and si-TDP followed by treating with DMSO and NPX-047 according to 72h protocol. Data are shown as mean +s.d. of n = 1 experiment after normalization to GAPDH and 18S.

3.6 Edaravone did not have any effect on G3BP1 mRNA levels at 20uM

According to our previous results in worms, Edaravone at 20uM could serve as a positive control because it enhanced *G3BP1* mRNA levels in tdp-l-. Hence, the effect of Edaravone as a positive control at 20uM on *G3BP1* mRNA levels in our SH-SY5Y cells was determined. First, around 70% knock down of *TDP-43* following si-TDP treatment was detected. There was also not any effect of Edaravone on *TDP-43* mRNA levels. N=1(Fig.9A). Then, the *G3BP1* mRNA levels was investigated following si-treatment and no rescue of *G3BP1* levels was observed N=1(Fig 9B). Edaravone had also no effect on *Long G3BP1* mRNA levels N=1 (Fig 9C).

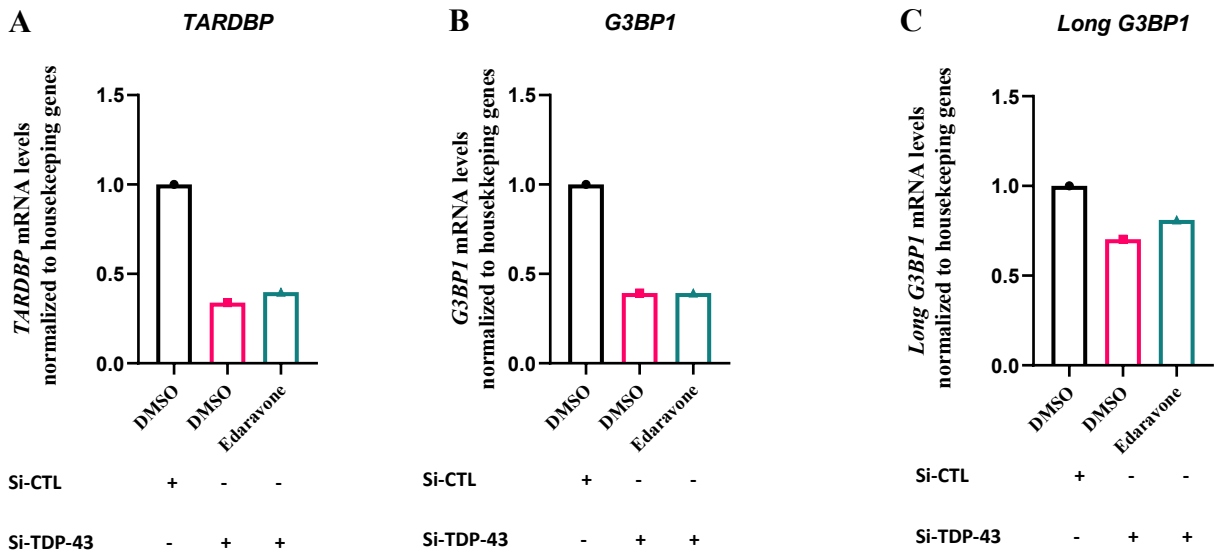

Figure 9. – Edaravone has no effect on total G3BP1 and long G3BP1 transcripts at 20uM.

Relative mRNA expression levels of (A) TARDBP, (B) G3BP1 and (C) Long G3BP1 in SHSY5Y cells transfected with si-CTL and si-TDP followed by treating with DMSO and Edaravone according to 72h protocol. Data are shown as mean +s.d. of n = 1 experiment after normalization to GAPDH and 18S.

3.7 Edaravone at 20nm and various doses of NPX-047 at nM concentration do not rescue G3BP1 levels

Since some drugs like some antidepressants show a bell-shaped dose response curve, meaning that enhancing dose leads to more efficiency but only up to a certain point whereas further increasing of the drug concentration leads to less efficiency. Hence, the effects of the compounds at various nM concentrations were tested. A70% knock down of *TDP-43* following si-TDP treatment was detected about 70% and no effect of the compounds on *TDP-43* mRNA levels was observed N=1 (Fig.10A). Additionally, *G3BP1* mRNA levels indicated no rescue (Fig.10B) and no change of on *Long G3BP1* mRNA levels was detected (Fig.10C).

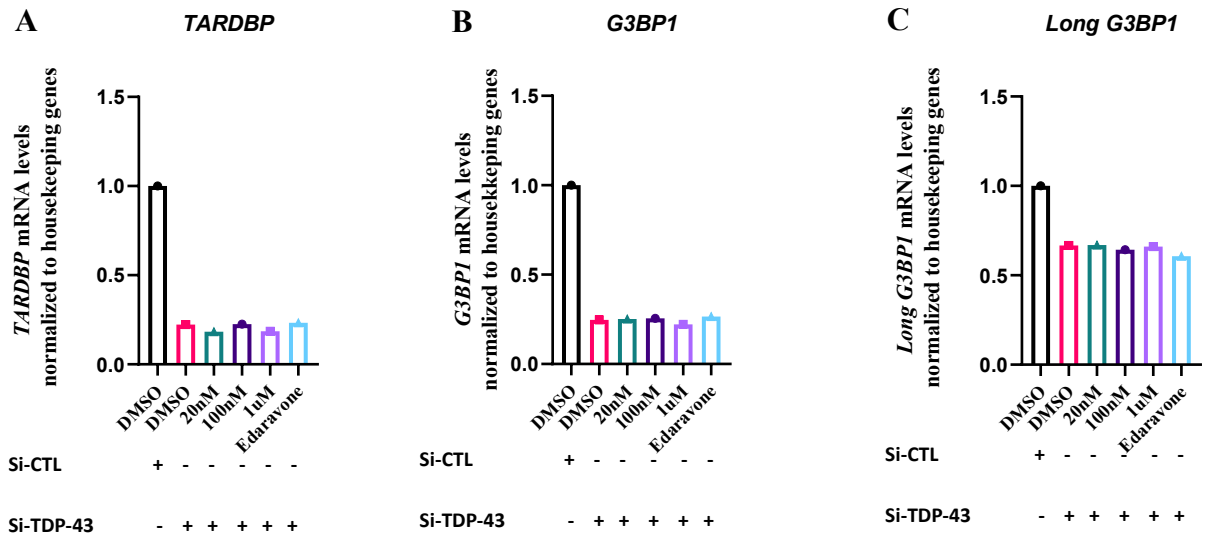

Figure 10. – NPX-047 and Edaravone has no effect on total G3BP1 and long G3BP1 transcripts at different nM concentrations. Relative mRNA expression levels of (A) TARDBP, (B) G3BP1 and (C) Long G3BP1 in SHSY5Y cells transfected with si-CTL and si-TDP followed by treating with DMSO, NPX-047 and Edaravone according to 72h protocol. Data are shown as mean +s.d. of n = 1 independent experiments after normalization to GAPDH and 18S.

3.8 TARDBP, G3BP1, Long G3BP1 and G3BP2 mRNA levels do not change in 4 months old TDP-43 M337V mice.

Considering for a future strategy involving one of the primary compounds for ALS therapy, our subsequent objective was to assess these compounds *in vivo*. Initially the levels of mouse *TARDBP* and *human TARDBP (hTARDBP)* mRNA in 4-month-old TDP-43 ^{M337V} mice was assessed using real-time qPCR. Notany significant change in *TARDBP* mRNA levels in TDP-43 homo (homozygotes) versus non transgenic mice was detected however, the level of *hTARDBP* was significant in homo mice (Fig 11A and 11B). Next, *G3BP1* mRNA levels were assessed. No change between TDP-43 ^{M337V} homo and NTg mice was observed (Fig.11C). Furthermore, *long G3BP1* mRNA levels were insignificant between TDP-43 ^{M337V} homo and NTg mice (Fig.11D). G3BP2 is similar to G3BP1 in structure and function. Thus, levels of *G3BP2* as also investigated and no significant changes were observed between 4 months old TDP-43 ^{M337V} mice and their NTg counterparts (Fig.11E).

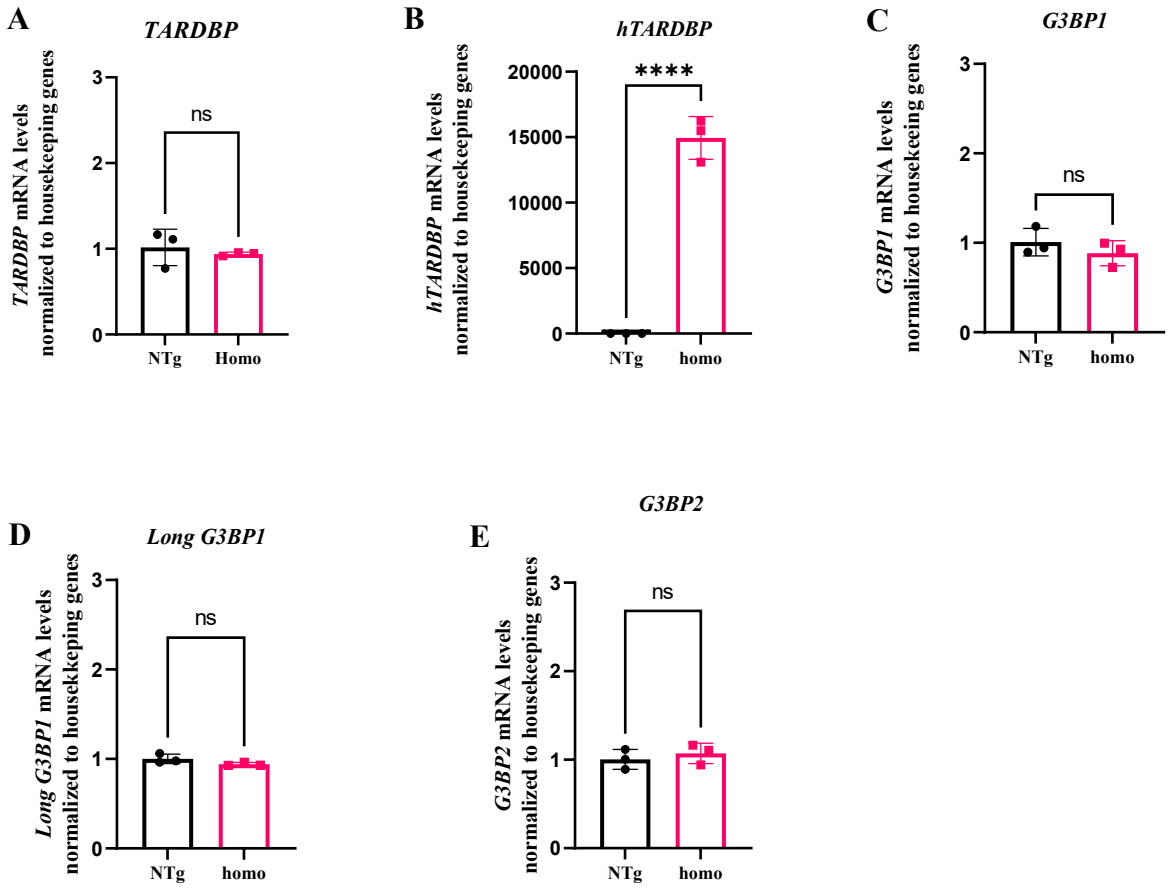

Figure 11. – TARDBP, hTARDBP, G3BP1, Long G3BP1 and G3BP2 mRNA levels in 4months old TDP-43 ^{M337V} mice. Relative mRNA expression levels of (A) TARDBP, (B) hTARDBP, (C) G3BP1, (D) Long G3BP1 and (E) G3BP2 in 4 months old NTg and homo TDP-43 ^{M337V} mice Data are shown as mean +s.d. of n = 3 per group after normalization to Rplp0 and Actin. Statistical analysis using unpaired t-test with ****p <0.001, ns=not significant. NTg= non transgenic. Homo=homozygotes.

3.9 TARDBP, G3BP1, Long G3BP1 and G3BP2 mRNA levels do not change in 18 months old TDP-43 M337V mice.

We have shown previously that stress granule response in mice worsens with age (Dubinski et al., 2023). As a result, the mRNA levels of *G3BP1 and G3BP2* were investigated to see the effects of age on their expression *in vivo*. Initially, the *TARDBP* mRNA levels in NTg, homo and het(heterozygote) group was assessed and no significant changes of *TARDBP* mRNA was detected among these groups (Fig.12A). Next, no significant changes were observed in the *G3BP1*, *long G3BP1* and *G3BP2* mRNA levels among NTg, homo and het groups however a tendency towards lower levels of these mRNAs was indicated in homo group (Fig.12B, 12C and 12D).

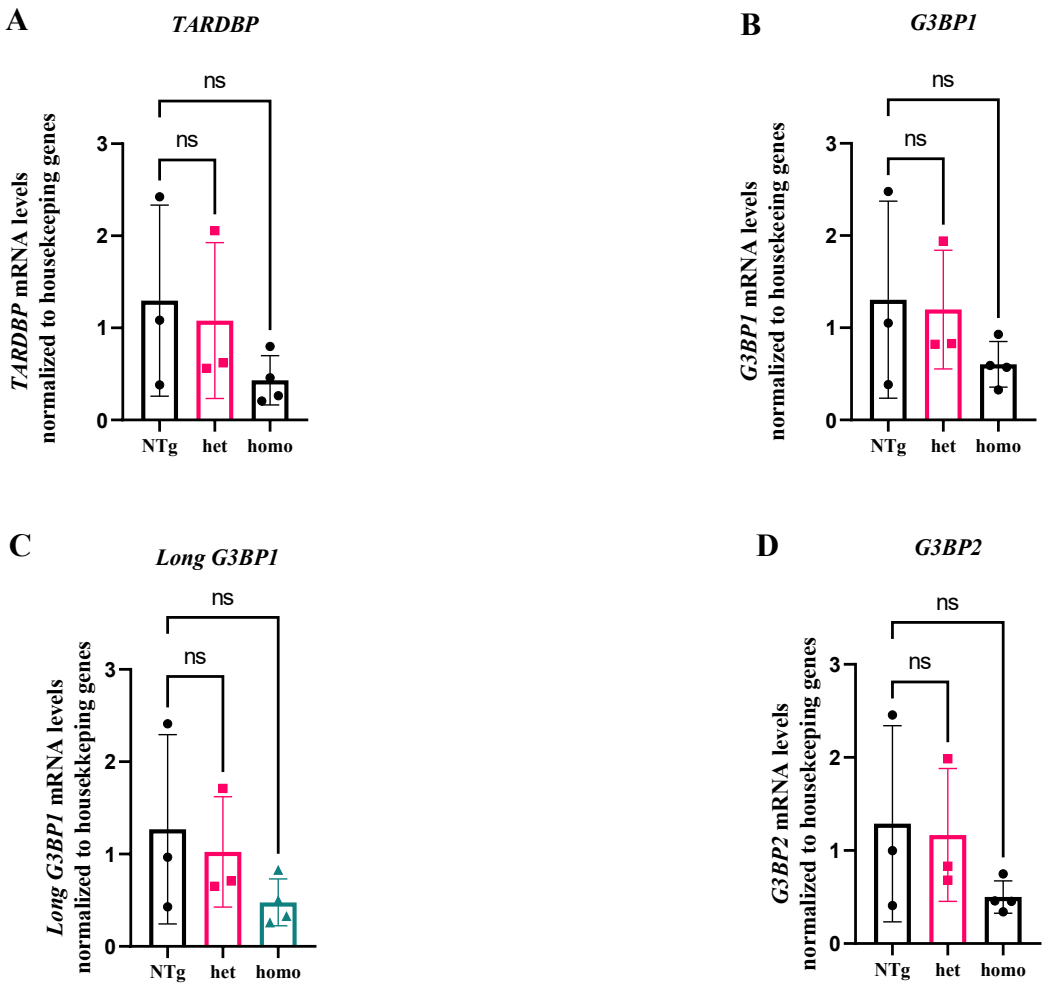

Figure 12. – TARDBP, G3BP1, Long G3BP1 and G3BP2 mRNA levels in 18 months old TDP-43 M337V mice. Relative mRNA expression levels of (A) TARDBP, (B) hTARDBP, (C) Long G3BP1 and (D) G3BP2 in 18 months old NTg, het and homo TDP-43 ^{M337V} mice Data are shown as mean +s.d. of n = 3-4 per group after normalization to Rplp0 and Actin. Statistical analysis using one-way ANOVA. ns=not significant. NTg= non transgenic. Homo=homozygotes.

Chapter 4- Discussion

Based on our hypothesis, Novation's compounds were tested on SH-SY5Y cells. First, the toxicity of these compounds was assessed using Trypan blue assay. When cells were treated for 72h with 20 µM of the compounds, NPX 000-047 caused around 60% toxicity while the other two compounds had low toxicity even compared to DMSO. A 72h protocol was an established protocol due to previous similar experiments in our lab. 20 µM was the preferred dose (except for NPX 000-047) according to Novation's screening of the compounds. Less than 20% of toxicity is optimal for these experiments, thus in order to reduce the toxicity, the 72h protocol was replaced by the 48h protocol. With the 48h protocol, the toxicity of the compound NPX 000-047 was reduced to around 40%; therefore, lower doses of NPX-047 had to be used for further experiments. Next, NPX 000-280 and NPX 000-115 were used at 40 µM according to the 48h protocol. This experiment was necessary to validate the maximum concentration of the compounds to be used. This experiment was conducted based on the 48h protocol to limit toxicity. At 40 µM NPX 000-280 indicated around 20% while around 36% of toxicity was observed with NPX 000-115, which indicated quite toxicity. For NPX 000-047 different concentrations were assessed and less than 20% toxicity was detected with all of the concentrations except for 20 µM. Since different batches of the compounds were present, the level of toxicity among them without si-treatment had to be compared. There was not a significant difference between old batches and new batches in terms of toxicity however new batches slightly indicated less toxicity. As a result, new batches were used for further experiments, also previous experiments were conducted using new batches. Next, NPX 000-280 and NPX 000-115 were used at 20 µM based on the 48h protocol. 20 µM concentration was selected for these experiments since higher levels of these compounds caused more than 20% toxicity. Despite lower levels of *G3BP1* levels due to TDP-43 knockdown, no rescue of *G3BP1* mRNA levels was observed when compounds were given to the cells. This was quite surprising as these compounds were screened before by Novation to rescue *G3BP1* mRNA levels. Although NPX 000-280 and NPX 000-115 at 40 µM were quite toxic to the cells, their effect on the rescue of G3BP1 was tested. This was due to the fact that 20 µM of the compounds did not rescue *G3BP1* mRNA levels. No rescue of *G3BP1* mRNA was observed. Also, the levels of *long G3BP1* did not

change however this was expected. Additionally, NPX 000-047 was tested at 10 and 15 μM. These experiments were conducted according to 48h protocol. 10 and 15 μM concentrations were chosen according to cell viability results. Surprisingly, both concentrations did not rescue *G3BP1* mRNA levels. No effect on *long G3BP1* was also observed. Nevertheless, the same experiments were conducted according to 72h and the results remained the same with no rescue of *G3BP1* mRNA levels. In previous experiments on worms, Edaravone indicated a rise in *G3BP1* mRNA levels; therefore, it was used as a positive control in the next experiments however, interestingly, no upregulation of G3BP1 was demonstrated in SH-SY5Y cells at 20 μM. Then, since many drugs like antidepressants have a bell curve response (Terao et al., 2020), it was speculated the optimum dose for the functions of these compounds might be at much lower concentrations, such as nM. Furthermore, since most of these compounds were used at 20uM on worms, we speculated that concentrations at nM may be more appropriate on cells due to the fact that cells cannot metabolize drugs as organisms, such as worms. However when Edaravone at 20nM, and NPX 000-047 at various nM concentrations were tested, no effect on *G3BP1* mRNA levels was detected.

Despite our results, in terms of G3BP1 mRNA levels when using the compounds at 20μM, Novation reported that they observed a higher luciferase level of *G3BP1* when using NPX 000-280, NPX 000-115 at 20μM and NPX 000-047 at lower concentrations than 20μM. These discrepancies between Novation and our experiments might be because of the following reasons, first of all, Novation's results were based on luciferase assay which measures transfected gene expression and not endogenous *G3BP1*, thus these experiments have to be repeated by Novation assessing endogenous *G3BP1*. Second, Novation's protocol for treating cells with the compound was different. They treated SH-SY5Y cells with si-TDP for 48h, then compounds were added only once and 48h later luciferase assay was performed whereas in our experiments cells were treated with si-RNAs for 5 hours, then media with the compounds were added to the cells. Depending on whether a 48h protocol or 72h protocol was used, cells were treated with the compounds for another 24 or 48h, respectively. Another contributing factor to the different results could be the different sources of cells, media, and FBS used. Novation used MEM/EBSS: F12 in a 1:1 ratio while we used DMEM F12 in a 1:1 ratio with sodium bicarbonate. After precise examination of the components of these media, more than a 3-fold difference was noted in glycine, serine,

niacinamide, pyridoxal HCL, pyridoxine HCL, and riboflavin however we do not speculate the difference in these components of the media contributed significantly to the different results. Despite this, the difference in FBS used may contribute to different results. While FBS is essential for cell cultures, small molecules within FBS are poorly understood and how they affect cultured cells are yet unknown. To increase the reproducibility of experimental results in scientific articles, metabolites in FBS should be taken into account when performing cell studies (Liu et al., 2023). Different FBS were used in our experiments versus those performed by Novation; therefore, this might play a role in the observed opposite results and needs to be further investigated.

A compound's ability to be developed and ultimately the dose and route of delivery depend on how efficiently it is absorbed, distributed, and metabolized. Therefore, it is crucial to assess the characteristics of compounds when exposed to an organism. In an attempt to select an appropriate model for this purpose, we examined the TDP-43^{M337V} transgenic mice. These mice express the full-length mutant human TDP-43. In these transgenic a single copy BAC construct that contains human TDP-43, either with an ALS-associated TDP-43 mutation (M337V) or wild-type (Edupuganti et al.) has been inserted into the mouse Rosa26 (Gt (ROSA26) Sor locus. Despite human TDP-43^{WT}, expression of TDP-43^{M337V} causes motor dysfunction and loss of neuromuscular integrity from 6 months, and reduced survival. In our prior research, it was discovered that homo hTDP-43^{M337V} transgenic mice at 4 months of age have lower amounts of G3BP1 protein than NTg animals (Dubinski et al., 2023). As a precursor to evaluating our compounds in mice, first assess the levels of *G3BP1* mRNA in this model was investigated. Surprisingly, no reduction of *G3BP1* mRNA levels was detected in these mice despite the reduction in G3BP1 protein that was recently reported in our lab in these mice. Reduced protein levels with steady mRNA levels observed can indicate different matters. One is that a ribosomal malfunction, or a defect in the translation of mRNA to protein, might be indicated by a decreased protein level. This could be one explanation for these results according to the fact that one of the functions of TDP-43 is ribosome stabilization. However, it may also indicate that the protein is degrading more quickly due to an accelerated turnover rate. Another explanation is that there may be a problem with the protein's post-translational modifications, if any, which may render it less stable.

G3BP1 and its paralog G3BP2 have been often investigated as a single protein despite the lack of supporting data (H. Sidibé et al., 2021). These studies typically ignore the possibility that, despite the great degree of similarity between the proteins, the activities of G3BP proteins may not entirely overlap. For example, G3BP2's phase separation characteristics differ slightly from those of G3BP1's (Guillén-Boixet et al., 2020). It was shown that G3BP2 exhibits a greater tendency to phase separate with proteins and RNA than G3BP1 because a higher percentage of G3BP2 could form condensates with RNA at lower concentrations (Guillén-Boixet et al., 2020). Also, Depletion of G3BP1 in the axon results in the loss of these structures which cannot be compensated by G3BP2. (Sahoo et al., 2018). Furthermore, G3BP1 specifically binds TARDBP mRNA and G3BP2 specifically binds HNRNPA1 mRNA; both of these genes have been associated with ALS and FTD (Edupuganti et al., 2017). The differences between the mRNAs that G3BP1 and G3BP2 regulate are probably crucial for identifying the specific roles that each protein plays (H. Sidibé et al., 2021). These observations support the notion that functions of G3BP1 and G3BP2 could be different in various contexts despite their similarity (H. Sidibé et al., 2021). Due to this matter, levels of *G3BP2* were also assessed in 4 months old homo TDP-43_{M337V} mice and no change was observed in *G3BP2* mRNA levels. Next, since aging is the main risk factor for ALS and that senescent cells are unable to produce stress granules (Omer et al., 2020), hTDP-43_{M337V} transgenic mice at 18 months of age were also assessed for *G3BP1*, *G3BP2* and *long G3BP1* levels. Surprisingly, no reduction of *G3BP1* was observed in het and homo hTDP-43_{M337V} transgenic mice however there was a trend towards lower levels in these mice. Interestingly, this was also true for *G3BP2*, and *long G3BP1* mRNAs. This might suggest a different regulatory mechanism of G3BP1 in mice compared to humans which requires further investigation. Another area requiring further research is the effect of sex differences on these results. In our experiments a combination of female and male mice was used; nevertheless, future assessment is required to investigate these results in female vs. male mice.

we have identified small-molecule compounds that were able to have functional impacts at the organismal level with the potential to restore stress granule response in neuronal context although it was not confirmed with our recent results. With the goal of solving inconsistency observed between our and results of Novation and identification of a lead chemical compound,

forthcoming investigations within our laboratory will involve chemical modifications of the initial compounds to amplify their efficacy for restoring G3BP1 expression and function and mitigate toxicity. Within neuronal cells and nematodes for restoring *G3BP1* expression and function. The preliminary screening will involve utilizing a high-throughput screening (HTS) assay that targets *G3BP1* mRNA. This method was used in our initial screening. The chosen compounds will then undergo additional mRNA-targeted assays to measure their selectivity, EC50 values, and cytotoxicity within SH-SY5Y cells. Compounds that demonstrate low EC50 values will be chosen for further functional assessment within the C. elegans model. Additionally, we will investigate gtbp-1 mRNA levels through quantitative real-time polymerase chain reaction (qRT-PCR) to gain deeper insights into the efficacy of the compounds after TDP-43 knock down. This validation process will be carried out in SH-SY5Y cells and human induced pluripotent stem cell (iPSC)-derived cortical neurons. Importantly, our project's main focus is to screen compounds that can elevate G3BP1 expression specifically to physiologically relevant levels since higher levels of G3BP1 is associated with cancer (Li et al., 2020).

Besides assessing the selectivity of these compounds through counter-screening, their influence on the transcriptome also needs to be examined. This examination is crucial not only to unveil potential off-target effects but also to explore the pleiotropic impacts in the context of ALS/FTD, where the expression of a multitude of genes that are regulated by TDP-43 is altered. In order to achieve this goal, BruChase-sequencing in SH-SY5Y cells will be used in both TDP-43-depleted and non-depleted cells, followed by treatment with the top candidate compounds. With the use of BruChase-sequencing, both nascent RNA synthesis and RNA stability can be measured. To validate our findings, qRT-PCR, incorporating +/- actinomycin D will be used to halt transcription and transcripts that display the most significant alterations in mRNA stability following treatment with lead candidates or analogs will be selected. This aim offers broader insights of the intricate effects of our candidate compounds on RNA stability. Furthermore, by analyzing control si-RNA-treated samples changes unrelated to TDP-43 will be detected and will provide valuable insights into the potential implications of these compounds for neurons characterized by normal nuclear TDP-43 levels. These upcoming aims will serve as a critical foundation for subsequent stages of this

project which includes assessing the candidate compounds in the mouse models of ALS contribute to the broader quest for innovative treatments for ALS, FTD, and related disorders.

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