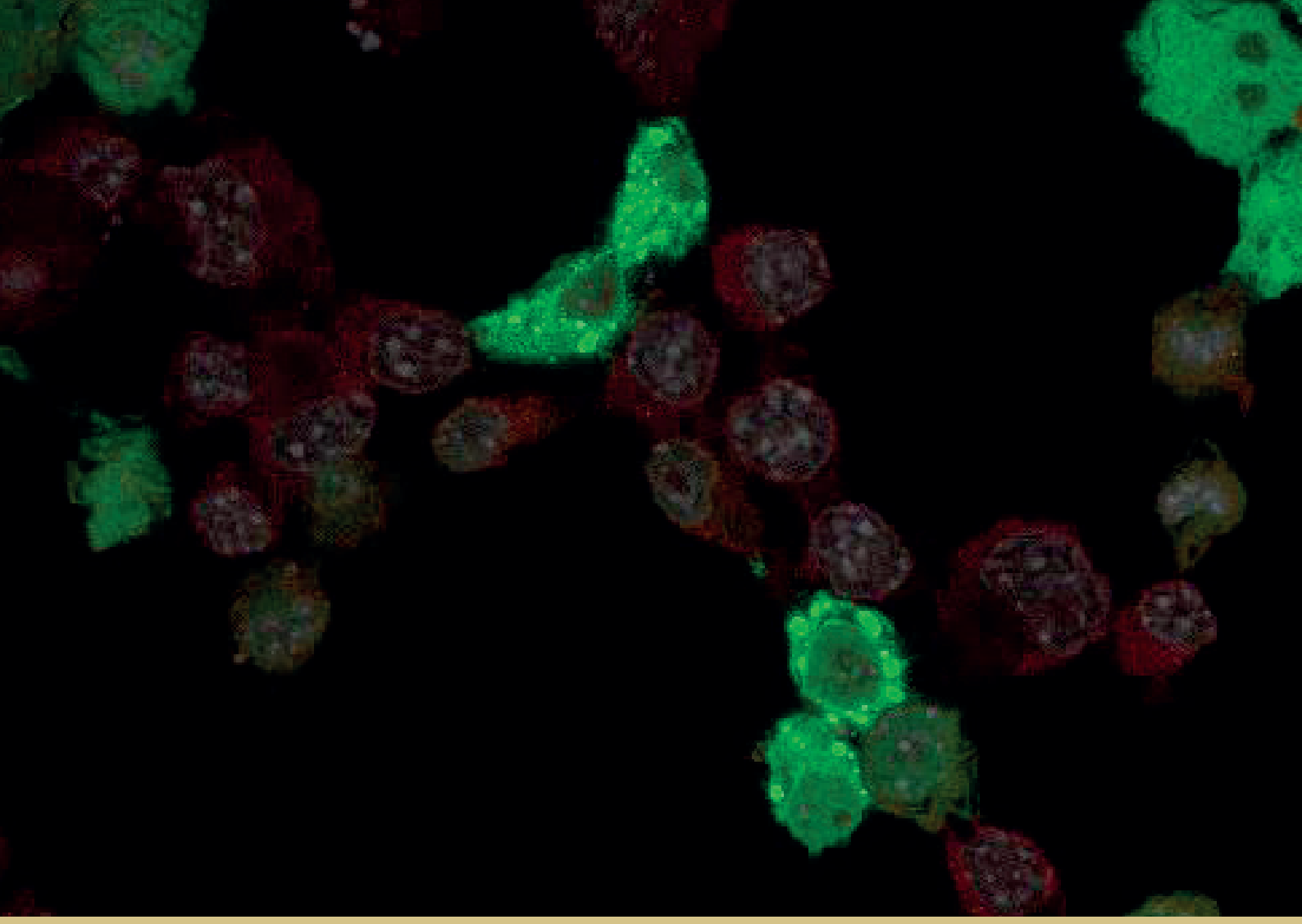

Sandra Marisa Oliveira Tomé

INSIGHTS INTO THE ROLE OF STRESS GRANULES IN SPINOCEREBELLAR ATAXIAS

Dissertação de Mestrado em Biologia Celular e Molecular, orientada pelo Doutor Clévio Nóbrega e apresentada ao Departamento Ciências da Vida, Faculdade de Ciências e Tecnologias da Universidade de Coimbra

Julho 2016

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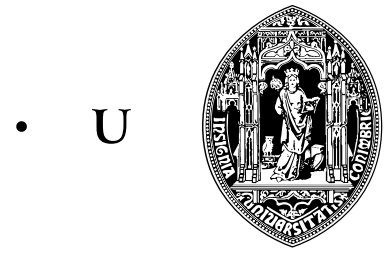
Universidade de Coimbra

Aos meus pais

Front cover composed of confocal microscopic image of Neuro2A cells transfected with GFP-MUT ataxin-3 and stained with DAPI and PABP, using 63x objective.



UNIVERSIDADE DE COIMBRA

Sandra Marisa Oliveira Tomé

Insights into the role of Stress Granules in Spinocerebellar ataxias

Dissertação apresentada à Faculdade de Ciências e Tecnologias da Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular com especialização em Neurobiologia, realizada sob a orientação científica do Doutor Clévio Nóbrega (Centro de Neurociências e Biologia Celular de Coimbra) e sob orientação interna da Professora Doutora Ana Luísa Carvalho (Universidade de Coimbra).

Julho 2016

The present work was performed in the Viral Vectors and Gene Therapy group of the Center for Neurosciences and Cell Biology (University of Coimbra, Portugal), leaded by Professor Luís Pereira de Almeida, and under supervision of Clévio Nóbrega PhD.

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Abbreviations

ALS – Amniotrophic Lateral Sclerosis
BSA - Bovine serum albumin
CAG - Cytosine-Adenine-Guanine
DMEM - Dulbecco's modified Eagle's medium supplemented
FBS - Fetal bovine sérum
G3BP1 - RasGTPase activating protein-binding 1
Htt - Huntingtin
MJD – Machado-Joseph's Disease
MUT - Mutant
Neuro2A – Mouse neuroblastoma cell line
PABP – Poly(A) Binding Protein
RBPs – RNA Binding Proteins
ROS – Reactive Oxygen Species
RT - Room temperature
SA – Sodium Arsenite
SGs – Stress Granules
SCA2 – Spinocerebellar ataxia type 2
SCA3 - Spinocerebellar ataxia type 3
TDP-43 – TAR DNA-binding protein 43
TIA-1 - TIA1 Cytotoxic Granule-Associated RNA Binding Protein 1
WT – Wild-type

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Abstract

Stress granules (SGs) are cytoplasmic messenger ribonuclear proteins (mRNPs) that occur in eukaryotic cells and assemble under several stress conditions, such as heat shock, UV irradiation, viral infection, and hypoxia. Its assembling is generally triggered by phosphorylation of the translation initiation factor 2 alfa (eIF2α) and culminates in translation arrest. SGs accumulate stalled untranslated mRNAs, and this process is regulated by several RNA-binding proteins (RBPs), such as TIA-1, G3BP1 and PABP, which are essential nucleators of SGs assembly. It is hypothesized that SGs appear during the course of several neurodegenerative disorders, as they could contribute to the increase in size as the RBPs consolidate by binding to each other through protein aggregation domain. On the other hand, it is also hypothesized that SGs could have a cytoprotective role, since the knockout of several SGs proteins render cells more vulnerable to stress and the inhibition of eIF2α dephosphorylation protects cells against some forms of stress. However, weather SGs induction may have beneficial effects in neurodegeneration is yet to be addressed.

Ataxin-2, the gene product of Spinocerebellar Ataxia Type 2 (SCA2) was proven to be recruited and to play a regulatory role in SGs, by assembling with polysomes and interacting with PABP, which is also a SGs component. Ataxin-2 also interacts with Ataxin-3, contributing significantly to Spinocerebellar Ataxia Type 3/Machado Joseph's Disease (SCA3/MJD) pathology, which could indicate a possible role of SGs in neurodegeneration.

The general purpose of this work was to provide an insight into the role that SGs might play in neurodegenerative disorders, namely SCA2 and SCA3/MJD, specifically in how the SGs induction could influence the causative proteins of these disorders: ataxin-2 and ataxin-3.

We observed that SGs induction is able to modulate ataxin-2 and/or ataxin-3 levels, whether by inducing oxidative stress with sodium arsenite (SA) or by overexpressing G3BP1. We also found that G3BP1 overexpression decreases significantly the aggregation of mutant ataxin-

3 in a cellular SCA3/MJD model and that oxidative stress is a prone environment to aggregation in a SCA2 cellular model. *In vivo*, we observed that G3BP1 overexpression reduces significantly the number of mutant ataxin-3 aggregates in mouse striatum in a SCA3/MJD lentiviral model, and also the loss of neuronal markers. Altogether, this study provides the first data about the role of the SGs in PolyQ disorders, specifically in SCA2 and SCA3/MJD. Nevertheless, future studies need to be performed in order to clarify the role of SGs in the neurodegeneration context.

Keywords: Stress Granules, Neurodegeneration, SCA2, Ataxin-2, SCA3/MJD, Ataxin-3, G3BP1, Sodium arsenite

Resumo

Os grânulos de stress (SGs) são estruturas citoplasmáticas, constituídas por RNA mensageiro de proteínas, sendo formados sob diversas condições de stress, tais como choque térmico, radiação UV, infeção viral e hipóxia. A sua formação é geralmente despoletada pela fosforilação do fator de iniciação da tradução 2 alfa (eIF2α) e culmina com a paragem da tradução. Os SGs acumulam mRNAs não traduzidos, num processo regulado por diversas proteínas que se ligam ao RNA (RBPs), tais como TIA-1, G3BP1 e PABP, sendo estas importantes na nucleação dos SGs. Nos últimos anos surgiu a hipótese de que os SGs podem ocorrer e contribuir para as doenças neurodegenerativas. Muitas destas doenças caracterizam-se pela acumulação de proteínas em agregados, sendo que os SGs podem contribuir para o aumento do tamanho dos agregados, por consolidação das RBPs, que se ligam entre si, através dos seus domínios de agregação proteica. Por outro lado, também está formulada a hipótese de que os SGs possam ter um papel cito-protetor, dado que o silenciamento de algumas proteínas dos SGs torna as células mais vulneráveis ao stress e que a inibição da fosforilação do eIF2α protege as células contra algumas formas de stress. Contudo, a hipótese de que os SGs possam ter um efeito benéfico no contexto degenerativo ainda está por ser clarificada.

Foi demonstrado que a ataxina-2, cuja forma mutada causa a Ataxia Espinocerebelosa Tipo 2 (SCA2) é recrutada e possui um papel regulatório nos SGs, pois interage com polissomas e com PABP, que também é um elemento dos SGs. A ataxina-2 também interage com a ataxina-3, contribuindo significativamente para a patologia da Ataxia Espinocerebelosa Tipo 3/Doença de Machado-Joseph (SCA3/MJD), o que aponta para um possível papel dos SGs na neurodegeneração.

O objetivo geral deste trabalho é fornecer uma compreensão do papel dos SGs em doenças neurodegenerativas, nomeadamente SCA2 e SCA3/MJD, especificamente o modo

como a indução dos SGs poderia influencia as proteínas causadoras destas patologias: ataxina-2 e ataxina-3.

Neste trabalho observámos que a indução dos SGs é capaz de modular os níveis de ataxina-2 e ataxina-3, quer induzindo stress oxidativo com arsenito de sódio (SA) quer sobre expressando G3BP1. Também observamos que a sobre expressão de G3BP1 reduz significativamente a agregação de ataxina-3 mutante num modelo celular de SCA3/MJD e que o stress oxidativo constitui um ambiente propício à agregação num modelo celular de SCA2. *In vivo,* observámos que a sobre expressão de G3BP1 reduz significativamente o número de agregados de ataxina-3 mutante no estriado de ratinho num modelo lentiviral de SCA3/MJD, bem como a perda de marcadores neuronais.

Em suma, este trabalho fornece os primeiros dados relativos ao papel dos SGs em doenças de poliglutaminas, especificamente SCA2 e SCA3/MJD. Não obstante, consideramos que futuramente serão necessários estudos adicionais para clarificar o papel dos SGs num contexto de degeneração.

Palavras-chave: Grânulos de Stress, Neurodegeneração, SCA2, Ataxina-2, SCA3/MJD, Ataxina-3, G3BP1, Arsenito de sódio

CHAPTER I - INTRODUCTION

1.1 Translation in Eukaryotes

Translation is a crucial and a complex process, responsible for processing mRNA into proteins, allowing the maintenance of cell and ultimately having a key role in the maintenance of life.

The first step in translation is the initiation step with the assembly of the eIF2-GTP-Met-tRNAi ternary complex. After its formation, the ternary complex binds to the small (40S) ribosomal subunit, which is facilitated by eIF 1, 1α, and 3. The resulting complex is called the 43S complex. The eIF4F complex assembles on the 5'-cap of the mRNA and unwinds structures found in the 5'-untranslated region (UTR). This complex, in conjunction with eIF3 and the poly(A) binding protein (PABP) bound to the 3'-poly(A) tail, and loads the mRNA onto the 43S complex. The 43S complex then begins scanning down the message in the 5' to 3' direction, looking for the initiation codon, which requires ATP hydrolysis. When encountering AUG codon, codon-anticodon base pairing takes place between the initiation codon and the initiator tRNA in the ternary complex. This then triggers GTP hydrolysis by eIF2, a reaction facilitated by the GTPase-activating protein (GAP) eIF5. After GTP hydrolysis by eIF2, eIF2-GDP releases the Met-tRNAi into the P site of the 40S subunit and then dissociates from the complex [1].

The second step in translation is elongation, which is a well-conserved mechanism between eukaryotes. Following translation initiation, an 80S ribosome is placed on a messenger RNA (mRNA) with the anticodon of Met-tRNAi in the P site base-paired with the start codon. The second codon of the open reading frame (ORF) is present in the acceptor site of the ribosome awaiting binding of the cognate aminoacyltRNA. The eukaryotic elongation factor eEF1A, binds aminoacyl-tRNA in a GTP-dependent manner and then directs the tRNA to the A site of the ribosome. Codon recognition by the tRNA triggers GTP hydrolysis by eEF1A, releasing the factor and enabling the aminoacyl-tRNA to be accommodated into the A site. Later on, peptide bond formation with the P-site peptidyl-tRNA occurs rapidly. Following peptide bond formation, tRNAs movement to the ribosomal subunits is triggered and this requires the elongation factor eEF2. Codon- anticodon base pairing induces three bases in the small ribosomal subunit's rRNA to swing out and interact with the resulting mRNA-tRNA duplex, which activates GTP hydrolysis and eEF1A releasing [1], [2]. The last step is termination, which takes place when the end of the coding sequence is reached by the ribosome and a stop codon (UAA, UGA, or UAG) enters the A site and is catalyzed by two protein factors, eRF1 and eRF3, that appear to collaborate in the process [3].

1.2 Integrated Stress Response (ISR)

The Integrated Stress Response (ISR) is a signalling pathway initiated upon phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2*α*) at serine 51 in response to different stress conditions, such as heat shock, hyperosmolarity, oxidative stress, viral infection, accumulation of unfolded or denatured proteins and the induction of apoptosis [4], [5]. These stresses can trigger translational arrest, the activation of signaling cascades responsible for reprogramming translation and ultimately lead to polysome disassembly. In these signaling cascades, different kinases like PKR, PERK/PEK, HRI and GCN2, act as "stress sensors" and phosphorylate eIF2α, which is a crucial component of the ternary complex necessary to initiate translation [6]. When eIF2α is phosphorylated, it will act as a dominant inhibitor of the guanine nucleotide exchange factor eIF2B, the GTP/GDP exchange factor that converts inactive ternary complex (GDP-associated) to active ternary complex. This will prevent the recycling of eIF2a between successive rounds of protein synthesis thereby reducing the levels of the eIF2–GTP–tRNAiMet and ultimately leading to translation inhibition and overall downregulation of protein synthesis (**Fig.1**).

Stress Granules (SGs) were discovered to appear in the cytoplasm of mammalian cells exposed to environmental stresses [7], such as those previously mentioned. These stresses, that ultimately lead to translation arrest and polysome disassembly, will activate a process of molecular "triage" in which mRNAs from disassembling polysomes are sorted and the fate of individual transcripts is determined. SGs are cytoplasmic mRNPs and form when translation initiation is impaired, suggesting that these granules contain mRNAs stalled in the process of translation initiation, which is consistent with their composition. They are, therefore, a consequence of this triage and selection process [8]. Furthermore, cells try to adapt to new and different conditions by reprograming their metabolism in order to repair stress-induced damage, for example, translation of mRNAs that code for molecular chaperones and enzymes involved in damage repair is enhanced, whereas mRNAs encoding *housekeeping* proteins is inhibited [9].

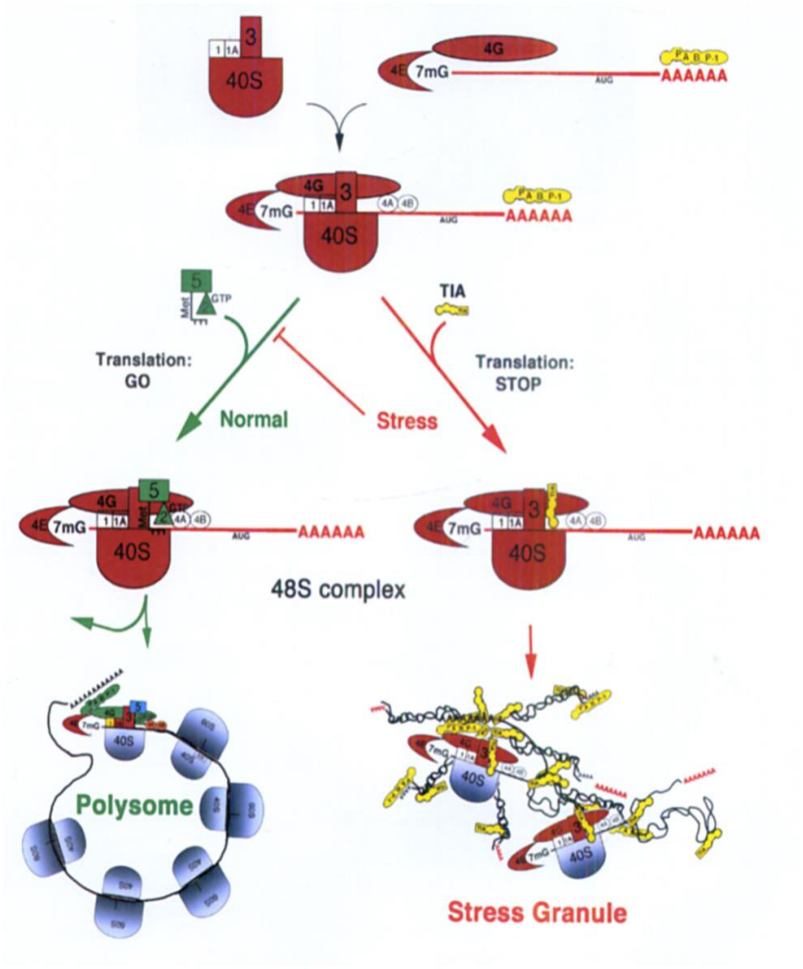

Figure 1 – **Normal translation versus stress condition translation.** In the absence of stress, the eIF2-GTP-tRNAMet ternary complex (green) is available to form a preinitiation complex at the 5' end of capped transcripts and scanning begins. Upon recognition by the anticodon of tRNAMet, early initiation factors are displaced by the 60 S ribosomal subunit and the mRNA is converted into a polysome. In stressed cells, phosphorylation of eIF2a depletes the stores of eIF2-GTP-tRNA, and the cytoplasmic amount of TIA- I (yellow) increases. Under these conditions, TIA- I is included in preinitiation complex that is translationally silent. TIA- I auto-aggregation then promotes the accumulation of these complexes at cytoplasmic foci known as SGs. Adapted from Kedersha N. and Anderson P., 2002.

Some models suggested that heat-shock SGs could act as a storage repository for untranslated mRNAs, however, mammalian SGs exhibit behavior inconsistent with that model [6]. Kedersha and colleagues (2002) observed that SG-associated mRNA is in a dynamic equilibrium with polysomes when observing the effect of some pharmacological inhibitors of protein translation on SG assembly [10]. The authors verified that the application of drugs that stabilize polysomes and inhibit RNA translation such as cycloheximide and emetine, would

inhibit the assembly of SGs and actively dissolve SGs by interfering with RNA translation at the step of protein elongation. In contrast, application of chemicals that destabilize polysomes (by releasing ribosomes from mRNA transcripts and consequently causing premature chain termination), such as puromycin, would promote the assembly of SGs. This observation suggested that there is a dynamic exchange between translating and SGs pools of mRNAs, supporting the idea that SGs are not independent or static structures.

SGs interact with Processing Bodies (PB) in mammalian stressed cells, which are defined as distinct cytoplasmic granules that contain components of the 5'-3' decay machinery, the nonsense-mediated decay pathway and the RNA-induced silencing complex. They are highly motile when compared to SGs and when encountering a SG, they are immobilized in a "docking" process. Furthermore, it was suggested that SGs act as intermediates between polysomes and PBs [8], [11], [12]. **(Fig.2).**

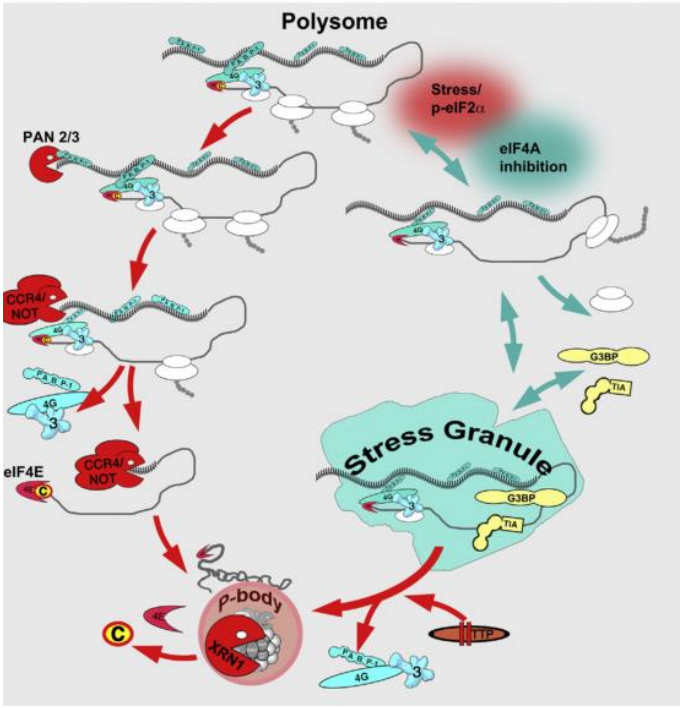

Figure 2 – Stress Granules and P-bodies interact in stressed cells. In response to cell-intrinsic and extracellular changes, mRNA translation is arrested and transcript can be routed from the polysomes to SGs (blue arrows) or PBs (red arrows). Transcripts directed for assembly into PBs are first deadenylated, which causes the release of PABP molecules from the mRNA poly(A) tail and ultimately leads to mRNA decay. In contrast, transcripts assembled into SGs retain their poly(A) tails and associated PABP. Stresses, that can trigger or not phosphorylation of eIF2α (red shading), disrupt translational initiation of the mRNA, resulting in accumulation of stalled mRNPs. Binding of mRNAs by aggregation-prone RNA binding proteins (such as TIA-1, G3BP1) subsequently promotes its assembly into SGs.

Some accumulating evidence indicate that some mRNAs shift from PBs to SGs: a) the fact that mammalian mRNAs within PBs can return to translation, suggests that they can transition from PBs to a state associated with translation factors [13]; b) the fact that mammalian PBs can assemble in an independent manner of SGs [9]; and c) the induction of SGs by arsenite treatment in HeLa cells leads to a sudden proliferation of SGs forming in association with PBs [14], which is consistent with SGs assembling from mRNAs exiting in PBs.

1.3 Stress Granules Dynamics and Composition

SGs include a diverse array of proteins and mRNAs in their composition **(Table 1)**, such as stalled initiation complexes that are bound to mRNAs and are recruited to SGs from disassembling polysomes (eg: eIF2, eIF3). SGs also harbor a large amount of RNA-binding proteins (RBPs), like PABP-1 (poly (A) binding protein 1), TIA-1 (T-cell intracellular antigen-1, and G3BP1 (Ras GTPase-activating SH3-domain-binding protein), which can shuttle in and out of SGs despite their large size. In response to environmental stress, TIA-1, a 3'UTR mRNA binding protein, accumulates in the cytoplasm, where it rapidly aggregate to form SGs [7]. Following removal of a non-lethal stress, the SGs increase in size owing to fusion of smaller SGs, and then rapidly disperse. [15] TIA-1 comprises three RNA-recognition motifs at their N-termini and a glutamine-rich domain at their C-termini. TIA mutants lacking the RNA-recognition motifs function as inhibitors of SG assembly in stressed cells [16], suggesting that TIA-1 is required for SG assembly. On the other hand, G3BP1 is recruited to SGs in cells exposed to arsenite and may determine the fate of mRNAs during cellular stresses. Tourrière et al. [17] demonstrated that SGs assembly can be induced by G3BP1 overexpression (wild type) or inhibited by expressing a phosphomimetic mutant, taking into account that arsenite dephosphorylates G3BP1. Both proteins, TIA-1 and G3BP1 are recruited to SGs after stress, and also are able to trigger SGs assembly and nucleation upon overexpression, therefore representing two very robust markers for SGs **(Fig. 2)**.

SGs can dissociate within minutes, when cells recover from stress [11], releasing the sequestered mRNAs and translation machinery to resume their normal functions.

In order to address this, Buchan and colleagues (2013) studied the mechanisms that control SG assembly, disassembly and clearance from the cell. Interestingly, they found that SGs clearance is affected by autophagy and by the function of Cdc48 or VCP (the human Cdc48 ortholog). Amongst many other cellular functions, VCP is involved in endoplasmic reticulum associated protein degradation [18]. They observed that endogenous VCP accumulates in SGs during stressful conditions and that it acts directly on SGs and it's required to promote their clearance. Moreover, they saw that inhibition of autophagy in mammalian cells leads to the accumulation of SGs and would reduce the rate at which they are cleared following removal of a stress. [19].

Despite these evidences, the full mechanism whereby SGs assemble and disassemble and the machinery driving this process still remains to be fully elucidated.

Table 1 – **Selected components of cytoplasmic stress granules.** Adapted from Anderson and Kedersha, 2007.

Protein	Relevant binding partners	Function
Ataxin-2	PABP-1	Translation
Caprin-1	G3BP	Cell growth
eIF3	40S, eIF4G (5)	Translation
eIF4E	CPEB, smaug, eIF4G, 4ET	Translation
eIF4G	eIF4E, eIF3, PABP-1	Translation
FMRP and FXR1	Ago2, RISC	Translation
G3BP1	Caprin	Ras signaling
PABP-1	eIF4G, eIF3, ataxin-2	Translation, stability
SRC3	TIA-1	Transcription
SMN	SMN complex	RNP assembly
TIA-1 and TIAR	FAST, SRC3, PMR1, FBP	mRNA silencing
TRAF2	eIF4G	Signaling
TTP and BRF-1	RCK (p54)	mRNA decay

1.4 Stress Granules Functions

SG assembly associates stalled initiation, polysome disassembly and mRNP aggregation in a series of reversible stages [11] and they sort their mRNP content to several cellular sites and fates. By sequestering untranslated mRNA from the degradation machinery, SGs could protect most cellular mRNAs, enabling translation re-initiation when environmental conditions improve. Moreover, some transcripts can be reinitiated and reconverted into polysomes. In addition to RNA- binding proteins, specific miRNAs might also facilitate the triage process in SGs, although this mechanism is not fully understood.

It is also important to highlight that the formation of SGs will lead to a higher local concentration of recruited mRNAs and associated proteins and a corresponding lower concentration in the cytosol, which could alter their interactions and rates of biochemical reactions, like for example, not letting those mRNAs interact with degradation enzymes. Furthermore, a high local concentration of components within a SG is likely to increase the rates of mRNP assembly or remodeling driven by these factors [20]. This helps to raise the possibility that SGs may form to promote assembly of translation initiation complexes, which can become very relevant in stress situations. It is also important to note that SGs represent pools of mRNAs primarily stalled to reenter translation, which is also supported by the observation that SGs disassemble faster than PBs [12].

Knowing that G3BP1 is a part of SGs and that it has been shown to regulate the stability and translation of several mRNAs (either by inhibiting the translation of the mitochondrial H^+-ATP synthase subunit, or by having an endoribonuclease activity to a subset of mRNAs), Takahashi and colleagues (2014) addressed SGs function in apoptosis. They observed that SGs suppress the elevation of reactive oxygen species (ROS), and this suppression is essential for inhibiting ROS-dependent apoptosis. This antioxidant activity of SGs is controlled by two SG components, G3BP1 and ubiquitin-specific protease 10 (USP10). G3BP1 elevates the steady-state ROS level by inhibiting the anti- oxidant activity of USP10. However, following exposure to sodium arsenite, G3BP1 and USP10 induce the formation of SGs, which promotes the antioxidant activity of USP10. Therefore, they concluded that SGs formation upon stresses like heat-shock, hypoxia or arsenite, inhibit apoptosis by reducing ROS production [5].

1.5 Stress Granules and Disease

Over the last years, several studies reported that SGs might be involved in the pathogenesis of some degenerative diseases. For example, conditions such as amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD), fragile X syndrome, inclusion body myopathy (IBM), and multisystem proteinopathy (MSP) can result from mutations in known SG proteins that often increase their tendency to aggregate. Additionally, a hallmark of ALS, FTLD, and some other degenerative diseases is the accumulation of cytoplasmic aggregates which often contain several SG factors and RNA. This leads to the hypothesis that inappropriate formation or persistence of SGs, might be related to the pathogenesis in these diseases or at least contributing to it[19].

SGs assemble when RBPs aggregate through their glycine rich domains and they act to recruit, silence and/or degrade RNA transcripts as part of a mechanism that adapts patterns of local RNA translation to facilitate the stress response [21]. Aggregation of RBPs is reversible and is tightly regulated through several pathways, such as phosphorylation of eIF2α, as explained above. RBPs are important in this context because of the large number of these proteins that are mutated in some forms of motor neuron diseases. Mutations in RBPs such as Tar DNA binding protein-43 (TDP-43), Fused in sarcoma (FUS), survival of motor neuron (SMN1) and ataxin-2 (ATX2) all cause neurodegenerative disorders, which might also indicate that SGs might be implicated in the pathogenesis

Moreover, SGs clearance in mammalian cells is reduced by inhibition of autophagy or by depletion or pathogenic mutations in valosin-containing protein VCP. Because mutations in VCP pre- dispose humans to ALS, frontotemporal lobar degeneration, inclusion body myopathy, and multisystem proteinopathy, it is possible that that autophagic clearance of SG-related and pathogenic RNP granules that arise in degenerative diseases may be important in reducing their pathology[19].

The importance of SGs for neurodegenerative disease becomes relevant because the process of SGs formation comprises a biological pathway that could be vulnerable to the protein aggregates that accumulate in neurodegenerative disorders. Previous studies shown that several of the neurodegenerative disease-causing RBPs co-localize with SGs markers (TIA-1, G3BP1) in cells undergoing stress [10], [22], [23].

McDonald *et al.* (2010) [22] demonstrated that TDP-43, which accumulates in ALS, co-localizes with SGs following heat-shock and oxidative stress and contributes to both the formation and maintenance of SGs in response to oxidative stress and differentially regulates

SGs dynamics by regulating G3BP mRNA levels. The authors also observed that the aggregation of TIA-1 is disrupted in the absence of TDP-43 resulting in slowed SG assembly.

SGs have been reported to be associated with age-related diseases. They represent one of the main pro-survival mechanisms whereby cells cope with environmental assaults by helping them reprogram mRNA metabolism and repair stress-induced damage. Some authors verified that a higher number of SGs form in aged fibroblasts that are exposed to heat shock or arsenite, than in growing cells, taking into account that senescent cells have a decreased ability to adapt to environmental stresses[24].

Upon stress removal, a large number of senescent cells were able to disassemble SGs and resume translation, although in a slower rate comparing to younger cells. This is important because addressing the functional relevance of SGs in senescent cells which are exposed to different extracellular drugs could be relevant to the treatment of age-related diseases such as cancer [25].

1.6 Stress Granules and PolyQ Disorders

Polyglutamine disorders are a group of neurodegenerative disorders caused by expanded cytosine-adenine-guanine (CAG) repeats, that encode a long PolyQ tract in the respective proteins. To date, a total of nine polyQ disorders have been identified and characterized: six spinocerebellar ataxias (SCA) types 1, 2, 6, 7, 17, Machado-Joseph Disease (MDJ/SCA3); Huntignton's Disease (HD); dentatorubral pallidoluysian atrophy (DRPLA) and spinal and bulbar muscular atrophy (SBMA) (**Table 2**). The translated PolyQ tend to aggregate in specific neuronal populations leading to their dysfunction and degeneration. Although animal models of these disorders have been created, there is neither a cure nor prevention for these pathologies [26].

The autosomal-dominantly inherited spinocerebellar ataxia type 2 (SCA2) and Machado Joseph Disease (MJD) are caused by a trinucleotide expansion in the SCA2 and MJD1 genes, respectively. The mutated genes encode for a lengthened polyglutamine stretch in the gene product Ataxin-2 and Ataxin-3, for SCA2 and MJD/SCA3, respectively. The distinctive clinical characteristics of these disorders include progressive cerebellar syndrome, ataxic gait, cerebellar dysarthria, dysmetria, dysdiadochokinesia associated with slow saccadic movements, peripheral neuropathies, fasciculations, painful muscle contractures, sleep disorders, and

dysphagia [27], [28]. SCA3 is the most prevalent ataxia (21%), followed by SCA2, with 15% incidence of cases and both diseases constitute the prevalent ataxia among certain populations [26], [27].

MJD/SCA3

MJD/SCA3 has adult onset and is characterized by cerebellar ataxia and pyramidal signs and the clinical manifestations are progressive ataxia, dysfunction of motor coordination, ophthalmoplegia, dystonia, intention fasciculation-like movements of facial and lingual muscles, and also bulging eyes. The neuropathological features consist on a widespread of neuronal degeneration therefore affecting numerous neuronal systems, such as cerebellum, substancia nigra, brainstem, striatum, spinal cord, among others [29].

Table 2 - Polyglutamine Diseases and Their Causative Genes and Proteins. Adapted from Fan et al., 2014.

PolyQ Diseases	Locus	Protein	Normal CAG expansion repeats	Pathological CAG expansion repeats
SCA1	6p23	Ataxin-1	6-39	41-83
SCA2	12q24	Ataxin-2	14-32	34-77
SCA6	19p13	CACNAIA	4-18	21-30
SCA7	3p21-p12	Ataxin-7	7-18	38-200
SCA17	6q27	TBBP	25-43	45-63
MJD/SCA3	12q24-q31	Ataxin-3	12-40	62-86
HD	4p16.3	Huntingtin	6-35	36-121
DRPLA	12p13	Atrophin-1	3-38	49-88
SBMA	Xq11-q12	Androgen receptor	6-36	38-62

Ataxin-3 is a 42 kDa deubiquitinating protein and has shown to play a major role in the ubiquitin proteasomal system, contributing to proteasomal degradation by removing poly-ubiquitin chains from substrates before digestion[30]. It comprises a conserved N-terminal Josephin domain, followed by two ubiquitin-interaction motifs domains (UIMs) and a poly glutamine region [29] . Due to the several isoforms resulting from alternative splicing, it can contribute through different ways to the MJD/SCA3 neuropathology. Both the normal and

mutant ataxin-3 proteins are expressed ubiquitously throughout the brain, however in certain regions such as striatum, the expression is specific subset of neurons [30]. Strikingly, some authors discovered in flies that the wild-type ATXN3 could have neuro-protective roles, by suppressing degeneration by a process that requires ubiquitin-associated activities of the protein and is dependent upon proteasome function [31], [32].

SCA2

SCA2 is also a late-onset disease and the first symptoms usually appear after 30 years of age. However, when the number of CAG repetitions is higher than 130, the symptoms might have pediatric onset, implying that there's an inverse correlation between the number of CAG repeats and the onset of the disease, which also occurs in MJD/SCA3 [27], [33].

As for the neuropathological alterations, cerebellum of patient's post-mortem tissue shows a significant atrophy, weight decrease and loss of Purkinje cells on both hemispheres [34]; volume and weight diminution of the brain stem with a significant reduction of olivocerebellar fibers and also loss of pontine fibers, affecting also the locus coeruleus, cranialnuclei motor neurons, and cervical motor neurons [34], [35]; degeneration of thalamic nuclei with a corresponding significant demyelination of the spinal cord [36]; increase in astrocytes and microglia [37]. Substancia nigra, frontal lobe, medulla oblongata and cranial nerves are also affected [38].

Ataxin-2 is a 140 kD protein and comprises an acid region that corresponds to exons 2–7 of the gene, which forms the Like Sm domain (Lsm domain) and the Lsm-associated domain. It also presents two regions localized next to the C terminal region, which are denominated PABPC1-interacting motif-2 (PAM2) and ataxin-2 domain protein [33], [39]. Some evidence show that this protein might be involved in post-transcriptional and translational regulation: its Lsm domain is shared with proteins involved in RNA posttranscriptional modifications [33]; its PAM2 domain interacts with the PAB6 domain of PABP (polyadenylate-binding protein), which participates in RNA-messenger stability and translation regulation; it also interacts with A2BP1 (ataxin-2 binding protein) which is involved in RNA splicing [40]. Altogether, ataxin-2 appears to have a physiological role on mRNAs, possibly through direct RNA binding and through several protein binding partners, probably affecting their translation, but also their transport and stability [41], [42].

Since ataxin-2 associates with the endoplasmic reticulum, it is proposed that this protein may interact with the plasma membrane and therefore be involved in endocytosis and cytoskeleton

reorganization [27]. Ataxin-2 also seem to play a role in calcium-mediated cell signaling cascades, since the mutant ataxin-2 of a SCA2 transgenic mouse (Q58) interacts with a receptor of a calcium channel [38].

As previously mentioned, SGs harbor a wide variety or RBPs, which can be involved in disease, such as Ataxin-2, FUS and TDP-43, whose mutated forms cause SCA2 and Amyotrophic Lateral Sclerosis (ALS), respectively [27], [43], [44].

Mutations in RBPs increase their propensity to aggregate and to form SGs either by directly increasing the tendency of the protein to aggregate, or by preventing nuclear translocation [21]. These proteins have a strong tendency to form oligomers, and then fibrils, which are very stable structures. The consolidation of RBPs during SGs formation might promote oligomerization by creating cellular domains with higher concentrations of these proteins. Conversely, the increased stability of oligomers and fibrils might serve as a nidus for SG formation, leading to over-active SGs formation **(Fig. 3).**

Under stress, cells need to produce cytoprotective proteins and this is achieved by the assembly of SGs. Besides recruiting and silencing non-essential RNAs, SGs can interact with P-Bodies, routing RNAs for degradation and adapt local patterns of translation depending on the cell needs and conditions [21], as described above. Moreover, there are some accumulating evidence that suggest that SGs might play a cytoprotective role during stress situations: knock-out of SGs proteins, such as TIA-1, render cells more vulnerable to acute stress [45]; inhibiting eIF2α dephosphorylation protects against some forms of stress [46]; and the fact that SGs possess the ability to shift RNA translation towards cytoprotective proteins [10]. However, the mechanisms whereby SGs mediate cytoprotection are still poorly understood.

As previously explained, TIA-1 is one of the main markers of SGs and it is required for its assembly. Furokawa and colleagues (2009) saw that huntingtin (Htt) aggregation, which intracellular inclusions constitute a hallmark of the PolyQ Huntington's disease, functions as a template to promote TIA-1 fibrillation and that it could repress TIA-1 physiological function. Moreover, they shown that a cross-seeded aggregation of TIA-1 by Htt fibrils might lead to global suppression of translation by converging SGs and protein aggregates [47].

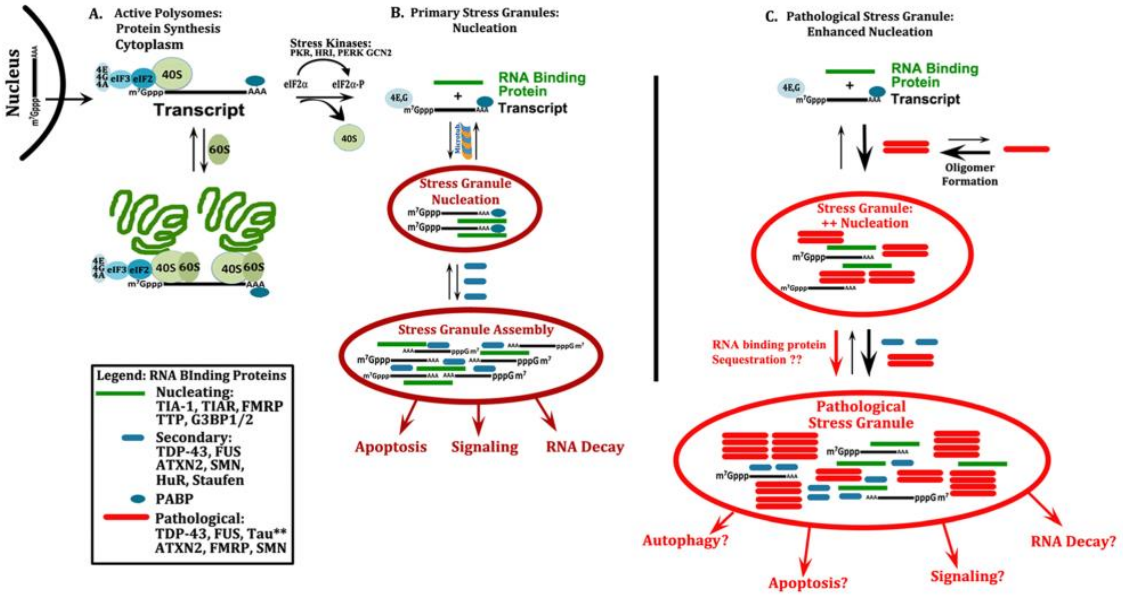

Figure 3 – **Mechanism of normal and pathological stress granule formation**. (A) Normal, physiological conditions and protein synthesis. (B) Primary Stress Granule: Nucleation. RNA binding proteins bind to the mRNA and to the nucleating RNA binding proteins to increase the size and complexity of SGs. These SGs are rapidly reversible upon removal of the stress. (C) Pathological Stress Granule: Enhanced Nucleation. Pathological proteins, such as TDP-43, FUS and tau, have a strong tendency to form fibrils, which will ultimately lead to enhanced SG nucleation and neurodegeneration. Adapted from Wolozin *et al*., 2012

Some studies previously shown that Ataxin-2 not only is recruited to SGs, but also plays a very important role there. Kaehler and colleagues [48] demonstrated that Ataxin-2 interacts with PABP1 to regulate mRNA polyadenylation and is required for SG assembly. Supporting this idea, Swisher *et al.,* [49] demonstrated that Pbp1, ataxin-2 homolog protein in yeast, plays a role in the formation of SGs by observing that the absence of Pbp1 would drastically reduce the levels of SGs. Thus, siRNA knockdown of ataxin-2 in mammalian cells leads to impairment and significant decreases in stress granule formation under arsenite treatment [50]. The same authors verified that mammalian cells with reduced ataxin-2 levels displayed a higher PABP level compared with control cells. Conversely, cells overexpressing ataxin-2 exhibited a lower PABP level. This finding could be important, because PABP is one of the key proteins regulating mRNA translation and stability. These results highlight the importance of this protein in regulation and formation of SGs and altogether, they help supporting the hypothesis that SGs are highly regulated and integrated into the biological response to stress and RNA translation.

Interestingly, neuronal intra-nuclear inclusions of ataxin-2 in SCA2 disease seems to recruit ataxin-3 and TATA box-binding protein (TBP), supporting the raising possibility that

nuclear aggregates could alter the transcriptional process [51]. The same authors also observed a co-localization of mutated ataxin-2 and mutated ataxin-3 in intra-nuclear inclusions of both SCA2 and SCA3 patients' post mortem tissue.

Moreover, it has been reported how TDP-43 and FUS, which mutated forms cause ALS, respond to stress stimulus, being recruited to SGs, and interacting with SGs components such as PABP and ataxin-2 [21], [22],[43], [52]. It was also shown that when the mutated forms of these proteins are present, SGs tend to be more difficult to disaggregate, and irreversible pathological alterations occur, which does not happen with the wild-type forms. We propose that the same might happen with ataxin-2 **(Fig.4).**

In a study in *Drosophila*, Lessing and Bonini [53] observed that normal activity of Atx2, the fly ortholog for human ATXN2, is critical for MJD/SCA3 degeneration and that Atx2 helps triggering the appearance of nuclear inclusions of Atxn3 (the fly ortholog for human ataxin-3), by interacting with PAPBP through a PAM2 motif of ataxin-2.

Moreover, Nóbrega and colleagues (2015) observed that ataxin-2 modulates in fact MJD/SCA3 neuropathology through interaction with PABP [42]. The authors observed that the number of aggregates in a MJD lentiviral mice model co-expressing PABP increased significantly, comparing to the hemisphere expressing only mutant ataxin-3. More importantly, they observed, in cells, that by blocking the interaction between ataxin-2 and PABP (by mutating PAM interaction motif), mutant ataxin-3 levels were not altered, in contrast to a significant reduction when upon normal ataxin-2 expression. This suggests that ataxin-2 may reduce the levels of mutant ataxin-3 through a regulation of translation, which could also lead to a reduction in overall protein synthesis. This data suggests that ataxin-2 might act as a negative regulator of translation reducing the neuropathology of MJD/SCA3 and that the regulation of translation of mutant ATXN3 could possibly constitute a therapeutic approach.

Overall, these findings indicate that SCA2 and SCA3 could be associated with one another and that the disease causing-proteins, ATXN2 and ATXN3, interact and affect each other's activity, making it relevant to address the effect of the modulation of SGs in both disorders.

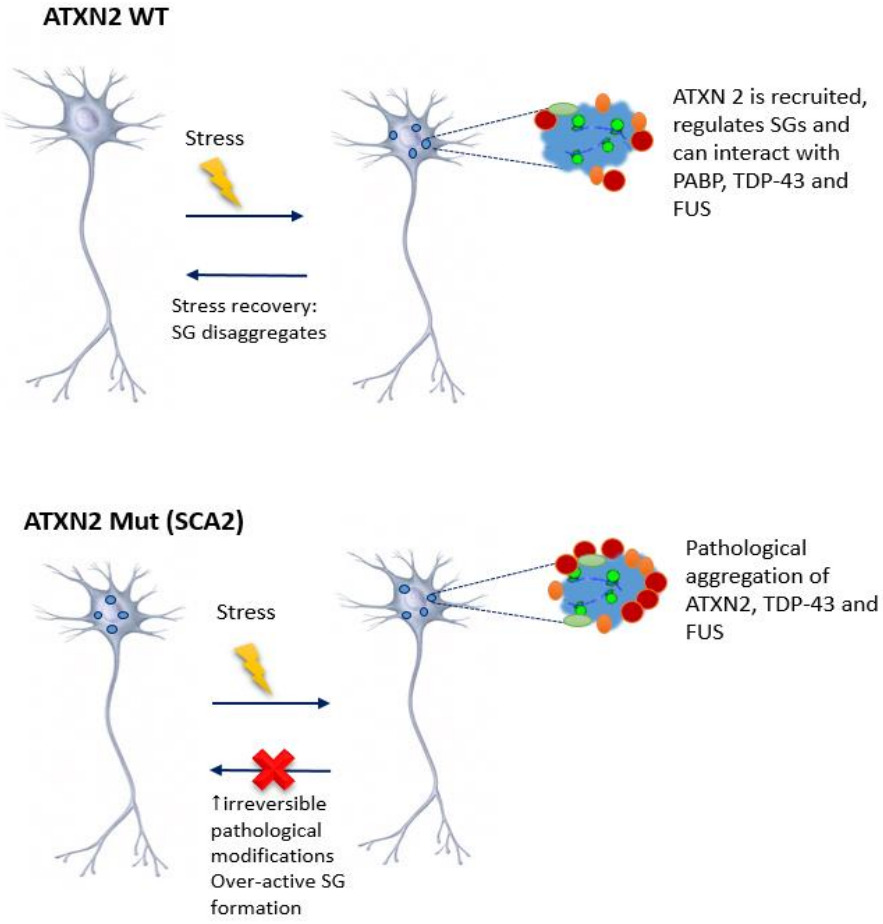

Figure 4 – **How mutant ataxin-2 could affect SGs in SCA2 pathology**. ATXN2 is a component of SGs and is required for their assembly and regulation. When pathologically expanded, it can underlie spinocerebellar ataxia type 2. (A) Upon stress and when ATXN2 comprises normal polyQ (Q22), SGs assemble and it can interact with PABP, FUS and TDP-43. When the stress is removed, SGs disaggregate and proteins return to the nucleus and their normal functions. (B) In the presence of the pathogenic expansion of ATXN2 (Q58), SGs are more difficult to disaggregate and there are irreversible pathological modifications, probably resulting of the increased stability of the ATXN2; The interactions with FUS and TDP-43 also increase significantly.

The general purpose of this work was to provide an insight into the role that SGs might play in neurodegenerative disorders, namely SCA2 and SCA3/MJD, specifically in how the SGs induction could influence the causative proteins of these disorders: ataxin-2 and ataxin-3.

In this study, we found that SGs induction is capable of modulating the levels of the disease-causing proteins ataxin-2 and ataxin-3. Upon sodium arsenite exposure, ataxin-2 levels are significantly reduced, whereas upon G3BP1 overexpression, ataxin-3 levels decrease significantly. We also investigated the role of SGs in aggregation using cellular disease models, and observed that G3BP1 overexpression reduces significantly the aggregation in a cellular model of SCA3/MJD and that SA-induced SGs increase significantly the aggregation in a SCA2

cellular model. Interestingly, we observed that Ataxin-2 co-localizes with SGs whereas ataxin-3 does not.

Further on, we addressed the effect of SGs induction in a SCA3/MJD lentiviral mouse model, by overexpression of G3BP1, and found that G3BP1 exerts a neuroprotective role, by diminishing the number of aggregates in the mouse brain, as well as decreasing SCA3/MJD neuropathology.

Altogether, this study provides an insight into the importance of SGs in PolyQ disorders. Nevertheless, further studies need to be performed in order to elucidate the mechanisms whereby SGs might contribute or influence these disorders.

CHAPTER II- OBJECTIVES

The general purpose of this work was to provide an insight into the role that SGs might play in neurodegenerative disorders, namely SCA2 and SCA3/MJD, specifically in how the SGs induction could influence the causative proteins of these disorders: ataxin-2 and ataxin-3. From this general objective several others were outlined:

- To evaluate how SGs induction (both pharmacologically or by molecular approaches) influences ataxin-2 and ataxin-3 levels, using a cellular SCA2 and SCA3 model.
- To investigate how SGs induction impacts the aggregation properties of ataxin-2 and ataxin-3.
- To investigate how the stress stimulus removal affects the levels of ataxin-2 and ataxin-3.
- To evaluate the neuroprotective effect of G3BP1 overexpression in a lentiviral MJD mouse model.

CHAPTER III- MATERIALS AND METHODS

3.1 IN VITRO Experiments

Neuroblastoma cell culture

Mouse neuroblastoma cell line (Neuro-2A cells) obtained from the American Type Culture Collection cell biology bank (CCL-131) were incubated in Dulbecco's modified Eagle's medium supplemented (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100mg/ml streptomycin (Gibco) (complete medium) at 37ºC in 5% CO2/ air atmosphere.

Neuroblastoma cell transfection

200.000 Neuro-2A cells were plated per well into 12 multi-well plates and transfected with plasmids of interest 24 hours after plating: 1ug of each plasmid DNA per well. PEI (Polyehtylenimine) was used to transfect the cells, that forms precipitates with the DNA. For this, PEI was pipetted into non-supplemented DMEM along with the DNAs of interest. After 10 minutes, PEI was inhibited with supplemented DMEM and this mixture was plated in the multi-wells. The plasmids expressing the following genes were used: GFP-ATXN2 (Q22), GFP-ATXN2 (Q58) [54]; EGFP-ATXN3 (Q24), EGFP-ATXN3 (Q84) [55], G3BP1-GFP [17] and G3BP1 (ID 100005688, Source BioScience). The multi-wells were incubated at 37ºC in 5% CO2/air for 48h.

Stress granules Induction

SGs were induced with Sodium Arsenite (SA, Sigma Aldrich 10µL/mL to a final 0,05 mM concentration) at 48h after transfection. After 1h hour of SA treatment (37ºC in 5% CO2/air), cells were collected and either fixed (for immunocytochemistry assay) or used for protein extraction (for Western blotting). For the reversion experiments, after 1h of treatment the medium was removed and fresh medium was applied and cells incubated for one additional hour at 37ºC in 5% CO2/air atmosphere.

Puromycin Translation Rate Assay

At 48h post-transfection, Neuro-2A cells were treated with SA to induce SGs formation. After 45min of the start this treatment, puromycin (10µg/ml) was added to all conditions. Ciclohexamide (CHX, 10µg) was used as a positive control and also non-treated cells as negative control. Cells were collected and the global protein translation levels were measured by Western Blot [56].

Protein extraction and Western blotting

Cells were lysed in RIPA-buffer solution (50mM Tris-HCl pH 8, 150nM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors (Roche diagnostics). Protein concentration was determined with the PierceTM BCA Protein Assay Kit (Thermo). Sixty micrograms of protein extract were resolved in SDS-polyacrylamide gels (10%). The proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (GE Healthcare) according to standard protocols. The membranes were blocked by incubation in 5% non-fat milk powder in 0.1% Tween 20 in Tris buffered saline (TBS-T) for 1 h at room temperature, and then incubated overnight at 4ºC with mouse monoclonal anti-ataxin-3 (Millipore, 1:5000), mouse anti-puromycin (Millipore, 1:20000), mouse anti-β-tubulin (Sigma Aldrich; 1:10000), mouse anti-ataxin-2 (BD Biosciences, 1:1000), and rabbit anti-G3BP1 (Millipore, 1:1000) antibodies. Blots were washed three times in TBS-T, for 10 min each, and incubated with the secondary antibody goat anti-mouse or anti-rabbit (1:10000; Vector Laboratories) for 2h at room temperature. Blots were washed three times in TBS-T, for 10min each and the bands were visualized with Enhanced Chemifluorescent substrate (ECF) (GE Healthcare) and chemifluorescence imaging (Versadoc Imaging System Model 3000, Bio-Rad). Semi-quantitative analysis was carried out based on the optical density of scanned membranes using ImageJ® 1.50i (Wayne Rasband) quantification software). The specific optical density was then normalized with respect to the amount of α-tubulin loaded in the corresponding lane of the same gel.

Immunocytochemistry procedure

The immunocytochemical procedure was initiated by fixating cells into glass coverslips with 4% paraformaldehyde fixative solution for 20 min, and after a washing with 0.1M phosphate buffer solution (PBS). Then samples were incubated for 10 min with PBS containing 0.25% Triton™ X-100. Blocking was made for 30 min in PBS with 1% bovine serum albumin (BSA,

Sigma). Primary antibody was incubated overnight in blocking solution in the proper dilution, and the secondary antibody (1:200) for 2 hours at room temperature. The secondary antibody used was coupled to a fluorophore (red 594 and green 488 Alexa Fluor®, Invitrogen) and followed by a nuclei staining reaction with 4',6-diamino-2-phenylindole (DAPI, 5 min, room temperature, 1:5000 dilution). The coverslips were then mounted in FluorSave™ Reagent (Calbiochem) on microscope slides.

Staining was visualized using Zeiss PALM Microbeam imaging microscope (Carl Zeiss Microimaging, Germany), equipped with AxioCam HR color digital cameras (Carl Zeiss Microimaging) using 5x, 20x, 40x and 63x Plan-Neofluar objectives and the AxioVision 4.7 software package (Carl Zeiss Microimaging). Quantitative analysis of both ataxin-2 and ataxin-3 aggregates was performed by counting the number of cells with aggregates within 100 transfected cells, using the 63x objective for each condition in each independent experiment.

3.2 IN VIVO Experiments

Lentiviral vectors

Viral vectors encoding for Atx3Q72 (Alves et al., 2008), as well as G3PB1 were produced in human embryonic kidney (HEK) 293T cells using a four-plasmid system described previously (de Almeida et al., 2001). The LV-G3BP1 was constructed using the cDNA from human G3BP1 (ID 100005688, Source BioScience) inserted into a lentiviral backbone using the gateway technology. The lentiviral particles were produced and resuspended in 0.1M PBS with 0.5% bovine serum albumin (BSA), and samples were matched for particle concentration by measuring HIV-1 p24 antigen content (RETROtek, Gentaur, Belgium). Viral stocks were stored at -80 C until use.

Animals

8-week-old C57/BL6 male mice (Charles River) were used in the *in vivo* experiments. The animals were housed in a temperature-controlled room maintained on a 12h light/ 12h dark cycle. Food and water were provided *ad libitum*. The experiments were carried out in accordance with the European Community directive (86/609/EEC) for the care and use of laboratory animals. The researchers received adequate training (FELASA-certified course).

Stereotaxic injection in the striatum

Concentrated viral stocks were thawed on ice and resuspended by repeated pipetting. Lentiviral vectors encoding for mutant Ataxin-3 and G3BP1 were stereotaxically co-injected into the striatum (or mutant ataxin-3) in the following coordinates: antero-posterior: +0.6mm; lateral; -1.8mm; ventral: -3.3mm; mouth bar: 0. Animals were anesthetized by administration of a mixture of ketamine (100mg/kg, Clorketam 1000, Vétaquinol) with xylazine (10mg/kg, Rompun®, Bayer) by intraperitoneal injection. Particle contents of the viral vectors were determined by p24 antigen ELISA (RETROtek, Gentaur, France) and a single injection of 400,000ng per construct was injected at a rate of 0.25 µl/min by means of an automatic injector (Stoelting Co., Wood Dale, IL, USA) into the mouse brain area through a 34-gauge blunt-tip needle linked to a Hamilton syringe (Hamilton, Reno, NV, USA). After injection, the syringe needle was left in place for an additional 5 min to allow the viral vector diffusion and to minimize backflow. Mice were kept in their home cages for 4 weeks after the injection before being sacrificed.

Immunohistochemical procedure

After an anaesthetic overdose the transcardial perfusion of the mice was performed by fixation with 4%paraphormaldehyde (PFA). The brains were removed and post-fixed in 4% PFA for 24h and cryoprotected by incubation in 25% sucrose/ phosphate buffer (PBS) for 48 h. The brains were frozen and stored at -80ºC. Subsequently 25µm coronal sections were cut using a cryostat (LEICA CM3050 S) at -21°C. Slices throughout the entire striatum were collected in anatomical series and stored in 48-well trays as free-floating sections in PBS supplemented with 0.05 µM sodium azide. The trays were stored at 4°C until immunohistochemical processing. The immunohistochemical procedure for light microscopy was initiated by incubating free-floating sections for 30 minutes at 37ºC in PBS containing 0.1% phenylhydrazine, to inhibit endogenous peroxidases. The sections were incubated at RT for 1h in blocking solution (0.1% TritonX™ 100 containing 10% Normal Goat Serum (NGS, Gibco) in PBS), and then with the appropriate antibodies: rabbit anti-ubiquitin (1:500; Enzo Life Sciences, O/N 4ºC), rabbit anti-DARP-32 (1:2000, Millipore; O/N 4ºC) diluted in the blocking solution. Three washings were performed and the sections were incubated with the corresponding biotinylated secondary antibody

(1:200; Vector Laboratories Inc., CA, USA) diluted in the blocking solution for 2h at RT. After three washes, bound antibodies were visualized by the ABC amplification system (Vectastain ABC kit, Vector Laboratories, West Grove, USA) and 3,3'- diaminobenzidine tetrahydrochloride (peroxidase substrate kit, DAB, Vector Laboratories, CA, USA) as the substrate. The sections were mounted, hydrated (with H2O mQ) and then dehydrated by passing through an increased degree of ethanol solutions (ETOH 75%, 96% and 100%) and xylene solution, and coverslipped with Eukitt (O. Kindler GmbH & CO, Freiburg, Germany). Staining was visualized using Zeiss open-field PALM Microbeam imaging microscope (Carl Zeiss Microimaging, Germany), equipped with AxioCam HR color digital cameras (Carl Zeiss Microimaging) using 5x and 20x Plan-Neofluar objectives and the AxioVision 4.7 software package (Carl Zeiss Microimaging). Quantitative analysis of DARP-23 loss and number of aggregates in the striatum was performed using ImageJ® 1.50i (Wayne Rasband) quantification software.

CHAPTER IV- RESULTS AND DISCUSSION

4.1 G3BP1 overexpression in Neuro2A cells leads to the formation of SGs

Firstly, we wanted to investigate if the human G3BP1 [17] gene was expressing correctly in the lentiviral vector backbone. So a western blot was performed using Neuro2A cells and transfected with the human G3BP1 gene (116 kD), which was detected along with the endogenous mouse G3BP1 (68kD) (**Fig 5A**).

G3BP1 has been reported to be one of the main markers, as well as main assembler and regulator of SGs, capable of inducing SGs when overexpressed [17], [19], [23], [57]. Aiming to observe the formation of SGs using G3BP1 as a marker and inducer, we transfected Neuro2A cells with eGFP-G3BP1 and also treated them with sodium arsenite (SA) for 1 hour to induce SGs. We verified that the overexpression of the eGFP-G3BP1 leads to the formation of cytoplasmic SGs (**Fig 5 B**). This result shows G3BP1 as a potent inducer of SGs.

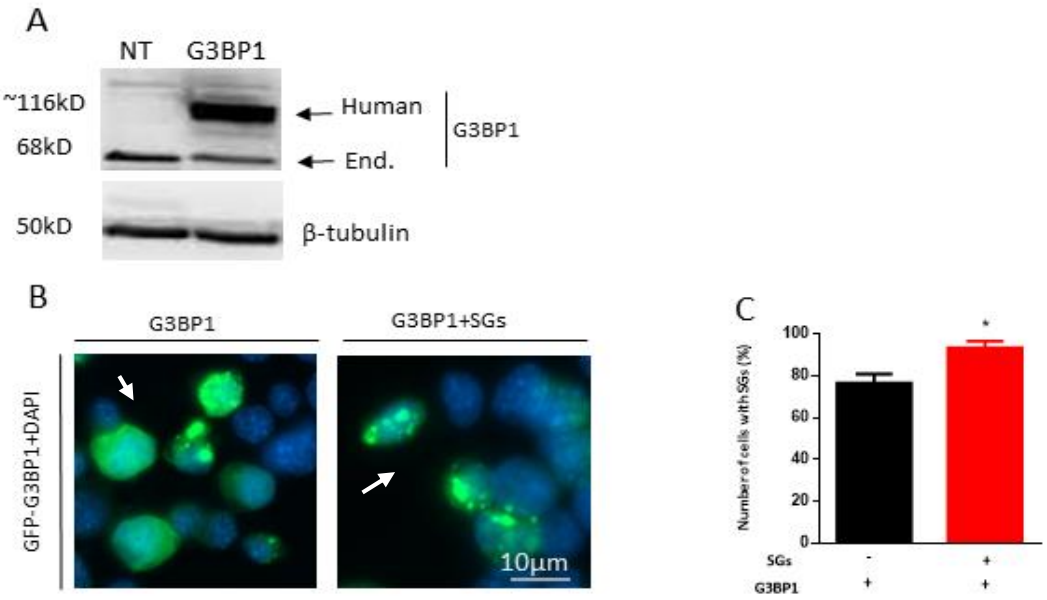

Figure 5 – **G3BP1 overexpression induces the formation of SGs**. (A) Human G3BP1 is being expressed. Western blotting analysis of lysates of N2a cells transfected with G3BP1 show human G3BP1 (116KD) band, as well as endogenous G3BP1 (68KD), this last in both transfected and non-transfected conditions. (B) G3BP1 induces the formation of SGs. Representative fluorescence microscopy images show GFP-G3BP1 as a robust marker and inducer for cytoplasmic SGs. Immunocytochemical analysis reveal that G3BP1 and sodium arsenite treatment increase significantly the formation of SGs (C). Quantification of the number of cells that contain SGs within 100 random transfected cells in a field. Values are expressed as mean ±SEM. *P<0,05, n=3 (Unpaired t-student test).

Previously, it has been shown that SA, a potent inducer of oxidative stress, causes most TIA-1 and G3BP1 to accumulate into SGs [7]. In fact, we observed that when adding SA to the cells transfected with G3BP1, the amount of cells containing SGs increased significantly **(Fig.5 C)**.

4.2 SGs induction reduces the global protein translation rate

Previous studies have shown that upon stress, the translation process might be arrested and the protein synthesis decreases significantly[11], [58]. Aiming to clarify if the induction of SGs with both SA and G3BP1 in fact contributes to translational arrest, we performed an assay to detect global protein synthesis levels. This puromycin-based assay described by Schmidt et al., 2009, is a useful and simple method to measure translation levels[56]. Puromycin is an aminonucleoside antibiotic produced by *Streptomyces alboniger*, and a structural analog of aminoacyl tRNAs, which is incorporated into the nascent polypeptide chain and prevents elongation [59]. When used in minimal amounts, puromycin incorporation in neosynthesized proteins reflects directly the rate of mRNA translation in vitro. Then a monoclonal antibody against puromycin is used to directly monitor translation levels. For that, N2a cells were transfected with G3BP1 or treated with SA 0,05mM for 1h, puromycin (10µg/ml) was added to all conditions and cycloheximide (CHX) was used as positive control (**Fig.6 A-B**).

We observed that SGs assembly (through G3BP1 overexpression or SA treatment) leads to a significant decrease in the levels of protein translation **(Fig.6 A-B**), which is in agreement with the previous studies reporting that SGs induction acts as an inhibitor of translation[8], [9]. Nevertheless, it is important to highlight that SA reduced protein translation in a more significant way, when comparing to G3BP1 overexpression.

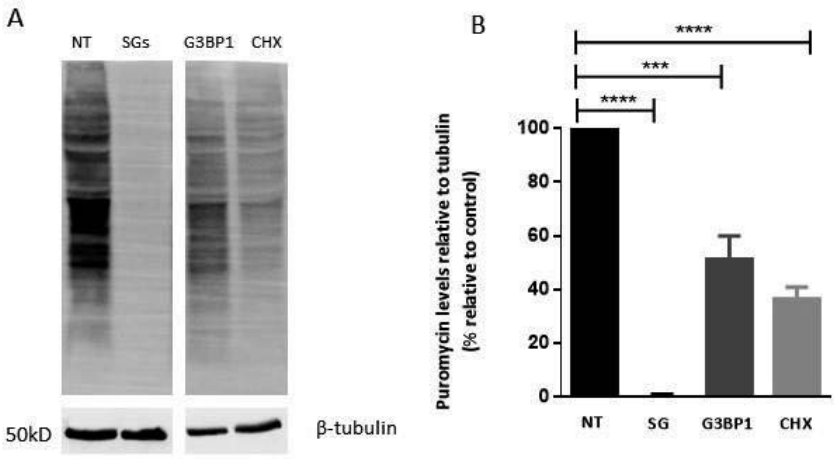

Figure 6 – SGs induction decreases the global protein translation levels in N2a cells. (A) Western blotting analysis of lysates of N2a cells treated 0,05mM of sodium arsenite for 1h or transfected with G3BP1, treated with CHX (10µg), and N2a non-transfected cells (NT) as control. (B)Optical densitometry analysis for puromycin levels. A significant decrease of puromycin incorporation in the cells treated with SA is observed, and also in the G3BP1 transfected cells. Each puromycin line was normalized according to the tubulin loading control band. Results were expressed as puromycin/tubulin ratio. Values are expressed as mean ± SEM, n=4. ***P<0,001; ****P<0.0001 (Unpaired Student's t-test).

4.3 Ataxin-2 co-localizes with SGs whereas Ataxin-3 does not

Previous studies have reported that Ataxin-2, besides causing Spinocerebellar Ataxia Type 2 (SCA2) in its mutated form, is actively recruited to SGs, acting as a regulator and affecting its formation[52], [50]. Furthermore, it has been shown that Ataxin-2 interacts with Ataxin-3, the protein causing Machado Joseph's Disease, affecting MJD's neuropathology [42], [53]. Thus, it is important to investigate the sub-cellular localization of both proteins upon SGs assembly.

We observed that GFP-Ataxin-2 co-localizes with SA-induced SGs, whereas GFP-Ataxin-3 does not. In **Figure 7A** we can clearly observe the mutant ataxin-2 aggregates overlapping with the SGs marker, meaning that this protein is in fact recruited and translocated to the SGs, when cells are exposed to SA. The same does not happen with mutant ataxin-3 protein, as is observed in **Figure 7B**. These observations are similar regardless of the protein form (mutant or wild-type).

This result was somehow expected as despite the fact that ataxin-2 interacts with Ataxin-3 and affect its function and pathology upon being recruited to mutant ataxin-3 aggregates in MJD [42], it might not be recruited along with Ataxin-2 to the cytoplasmic granules. However, the mechanism whereby Ataxin-2 is translocated to SGs still remains to be fully elucidated.

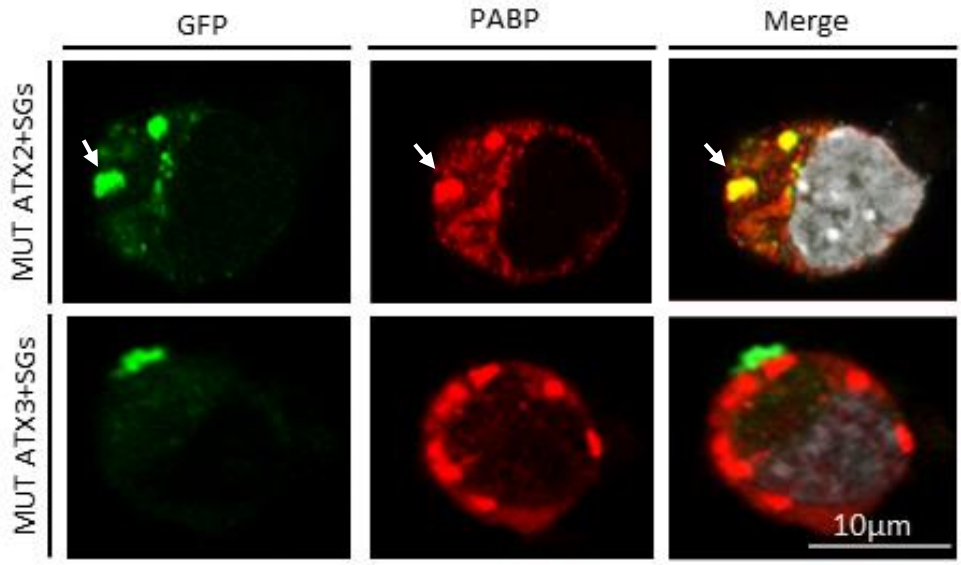

Figure 7 – **Ataxin-2 is recruited to SGs but not Ataxin-3.** Neuro2A cells were transfected with either GFP-Ataxin-2 (both wild-type and mutant forms) or GFP-Ataxin-3 (both wild-type and mutant forms) and exposed to SA to induce SGs formation. An immunostaining assay was performed, with anti-PABP antibody (which is a SGs marker) and nuclei were stained with DAPI. Co-localization of SGs with GFP-Ataxin-2 is observed, whereas GFP-Atx3 does not co-localize with PABP. Confocal representative images from two independent experiments.

4.4 Sodium arsenite induction of SGs modulates the levels of Ataxin-2 and Ataxin-3

As previously shown, upon SGs induction using SA, the translation is stalled and thus we next aimed to evaluate how this assembly correlates with the levels of both mutant and wild-type ataxin-2 and ataxin-3.

We observed that SGs induction upon SA exposure affects the expression of ataxin-2 and ataxin-3, probably due to translation inhibition upon SGs assembly. When cells are exposed to SA, SGs are induced and some RBPs are recruited to these cytoplasmic granules, such as TIA-1, PABP and ataxin-2. We observed a significant decrease in the levels of wild-type ataxin-2 but not in the mutated form (**Fig. 8 A-D**). This could result of the association of ataxin-2 with PABP, that are translocated to the SGs [48], and it might be no longer available in the soluble form in the cytoplasm, and for that, the detected levels are significantly reduced. As for the mouse endogenous levels of both ataxin-2 and ataxin-3 no alterations were observed (data not shown).

As for ataxin-3, there is an overall reduction of both wild and mutant forms of this protein when upon SGs assembly, although it is not significant, as observed in **Figure 8 E-H**.

This general reduction in both ataxin-2 and ataxin-3 levels could be the result of the reduction in the translation rate, which results from SGs assembly.

Overall, these results show that the SA induction of SGs is able to modulate and alter the levels of ataxin-2 and ataxin-3, which could suggest that this pathway might be important in the context of disease caused by these two proteins.

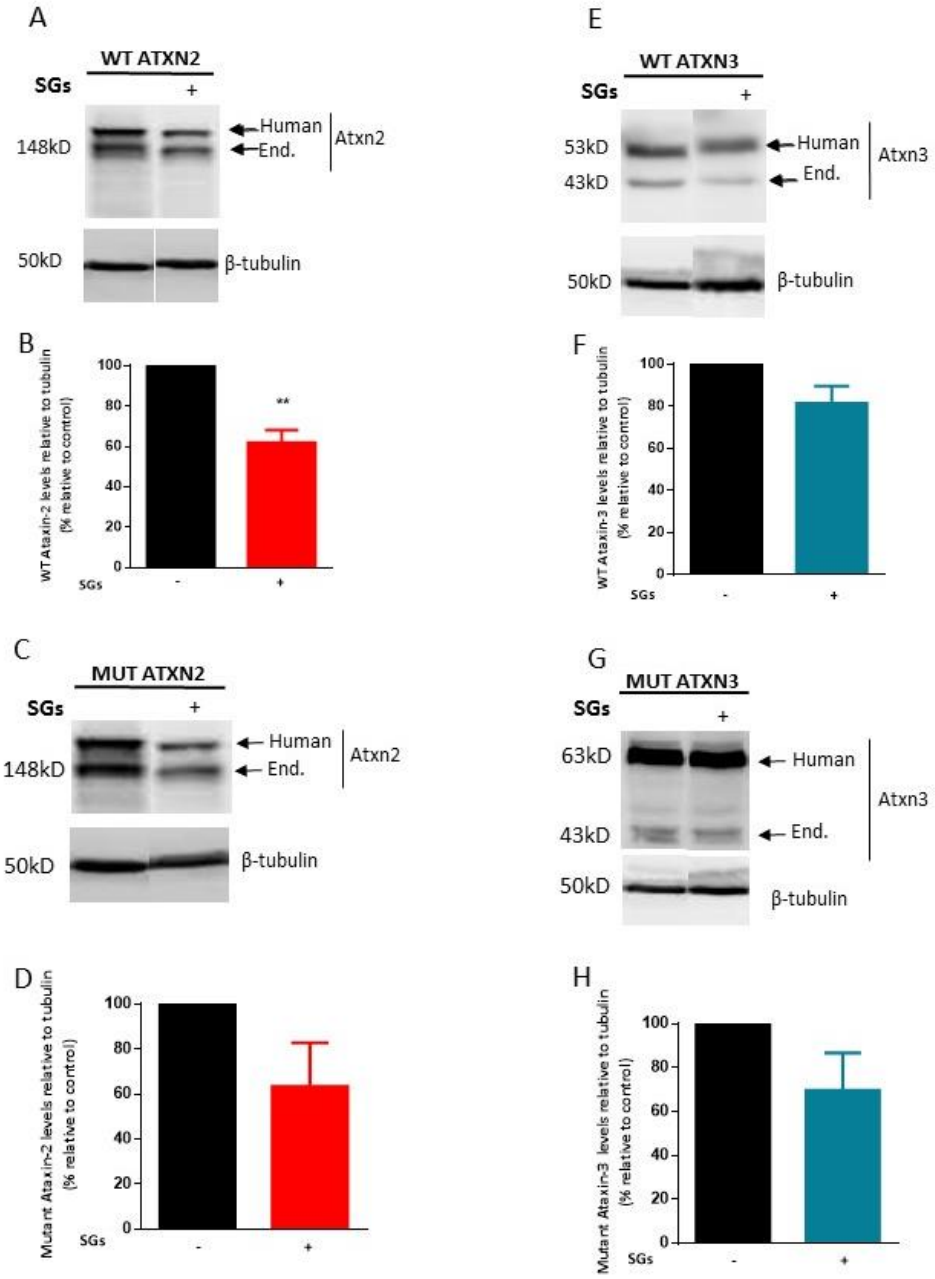

Figure 8 – **Induction of SGs with sodium arsenite modulates levels of Ataxin-2 (A-D) and Ataxin-3 (E-H) in Neuro2A cells.** Western Blot analysis and optical densitometry quantification of WT Ataxin-2 (A-B), MUT Ataxin-2 (C-D), WT Ataxin-3 (E-F) and MUT Ataxin-3 (G-H). WT Ataxin-2 is significantly reduced upon SG induction, whereas no significant reductions are observed in the remaining conditions. Neuro2A Cells were transfected with WT Ataxin-2 (Q22), MUT Ataxin-2 (Q58), WT Ataxin-3 (Q24) or MUT Ataxin-3 (Q84) and sodium arsenite 0,05mM was applied for 1h, 48h post-transfection. Representative western blots are displayed, probed for anti-atx2 (A, C) or anti-atx3 (E,G), and anti-tubulin (A-H). Values are expressed as mean ± SEM, n=5. **P<0.01 (One-way ANOVA)

4.5 G3BP1 over-expression significantly reduces the levels of Ataxin-2 and Ataxin-3

G3BP1 is a phosphorylation-dependent endoribonuclease that associates with RasGAP in dividing cells and is tightly associated with a subset of poly(A) chains[57]. As previously mentioned, G3BP1 is an important SGs constituent and marker and also a potent inducer of SGs assembly [17]. In this set of experiments, we aimed to address the effect of G3BP1 overexpression in ataxin-2 and ataxin-3 levels in Neuro2A cells.

We observed that G3BP1 overexpression leads to a significant reduction in both ataxin-2 and ataxin-3 protein levels, and this effect is particularly pronounced in wild type and mutant ataxin-3 (**Fig.9 F, H**). This interesting result is somehow expected for ataxin-2 (**Fig.9D**), considering that this protein is recruited to SGs, and this fact could decrease the soluble protein levels in the cytoplasm. However, both wild-type and mutant ataxin-3 levels are also significantly reduced upon G3BP1 overexpression, which is unexpected as they are not recruited to SGs. Also interesting was the fact that there were no differences between wild-type and mutant forms within ataxin-2 and ataxin-3 levels upon G3BP1 overexpression (**Fig. 9 B, C** and **F, H**, respectively). The levels of mouse endogenous Ataxin-2 and Ataxin-3 were not altered (data not shown).

These results indicate that ataxin-3 is somehow being affected upon assembly of SGs, but only in a more pronounced effect upon G3BP1 overexpression. This fact could occur possibly by an action of ataxin-2, G3BP1 and ataxin-3 not yet described. Furthermore, we have shown that unlike ataxin-2, ataxin-3 is not sequestered to SGs, which makes us hypothesize that other SGs-independent mechanism may be implicated in the reduction of ataxin-3 levels upon G3BP1 overexpression. G3BP1 has been reported as a SGs inductor that promotes protein interaction to preserve polyadenylated mRNAs[61]. These interactions are crucial in the context of disease, and G3BP1 was reported to be involved in several pathologies as a SGs nucleator, such as in SCA2 and ALS, by being involved in the interaction and sequestration of RBPs like mutant TDP-43, FUS, TIA1, PABP and Ataxin-2[50], [61],[21]. This makes us suggest that G3BP1 might somehow interact with both wild-type and mutant forms of ataxin-3 or its mRNA, decreasing its levels in a significant manner. Alternatively, this reduction in protein levels could result from the translation repression which is promoted upon SGs assembly. Nevertheless, the effect of G3BP1 is far more pronounced in the reduction of ataxin-3 levels, than the observed upon SA exposure

and also the global reduction in the global protein synthesis. This might suggest that G3BP1 reduces the levels of this protein independently of the SGs pathway.

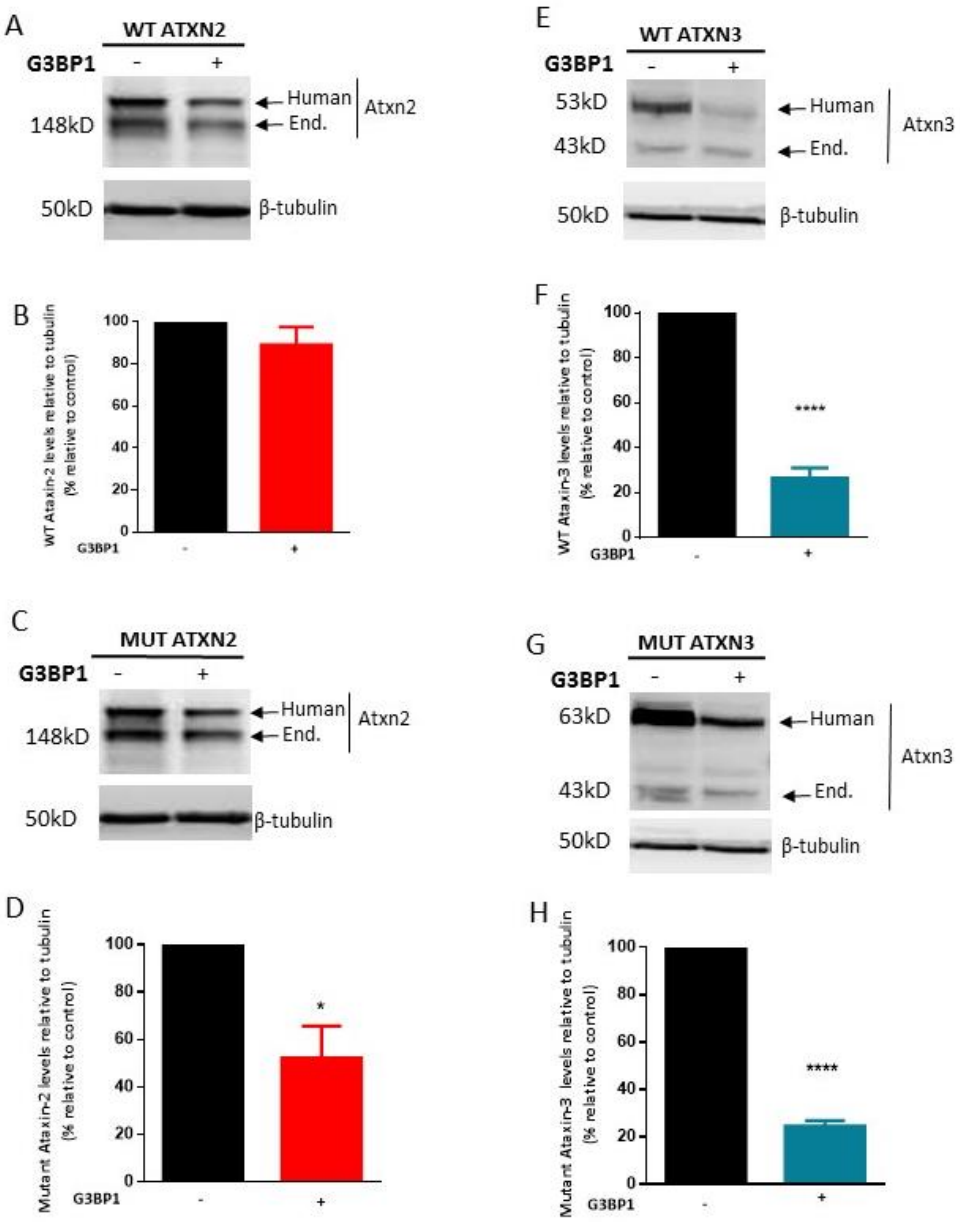

Figure 9 – **Overexpression of G3BP1 significantly reduces the levels of Ataxin-2 and Ataxin-3 in Neuro2A cells (A-H).** Western Blot analysis and optical densitometry quantification of WT Ataxin-2 (A-B), MUT Ataxin-2 (C-D), WT Ataxin-3 (E-F) and MUT Ataxin-3 (G-H). MUT Ataxin-2 is significantly reduced upon G3BP1 overexpression (C-D), whereas the decrease of WT Ataxin-2 is not significant (A-B). WT and MUT Ataxin-3 are significantly reduced upon G3BP1 overexpression (E-H). Neuro2A Cells were co-transfected with G3BP1 and WT Ataxin-2 (Q22), MUT Ataxin-2 (Q58), WT Ataxin-3 (Q24) or MUT Ataxin-3 (Q84). Representative western blots are displayed, probed for anti-atx2 (A,C) or anti-atx3 (E,G), and anti-tubulin (A-H). Values are expressed as mean ± SEM, n=5. *P<0.05, ****p<0,0001 (One-way ANOVA).

4.6 Combined effect of G3BP1 and sodium arsenite reduces the levels of Ataxin-2 and Ataxin-3

In this next set of experiments, we aimed to investigate the combined effect of the induction of SGs using both SA and G3BP1 overexpression. These experiments showed that the levels of both forms of Ataxin-2 and Ataxin-3 were strongly reduced upon the combined treatment.

As shown before, G3BP1 had a significant effect in reducing ataxin-3 levels, whereas SA treatment reduced ataxin-2 levels more significantly. This makes sense because oxidative stress was shown to recapitulate some key aspects of SCA2, being a co-factor or this disorder[62].

It is clear that SA is not enough to provoke a significant reduction in Ataxin-3 levels, both WT and MUT forms, however, when G3BP1 overexpression is present together with SA, the levels of this protein are reduced (**Fig.10 F, H**). This tells us that G3BP1 is modulating ataxin-3 levels in a SGs-independent manner and that SA-induced stress is not relevant for this protein's levels. Overall, the mouse endogenous levels of both forms of ataxin-2 and ataxin-3 were not altered (data not shown).

These observations suggest that G3BP1 affects ataxin-3 levels, but not the SA treatment. As previously explained, SA-induced stress causes oxidative stress, whether G3BP1 induces SGs assembly and promotes interaction and recruitment of several disease-causing proteins. Unlike to what we observed with ataxin-2, ataxin-3 may not respond to oxidative stress in the same. This corroborates previous studies that indicate that oxidative stress is a key factor in SCA2, promoting its pathology, which tells us that it may not be as relevant in a SCA3/MJD context[62]. In turn, G3BP1, which highly interacts with SGs in a SCA2 pathology, may somehow be interacting with ataxin-3.

Furthermore, it is assumed that the common toxic gain of function mechanisms for the polyglutamine-containing protein, is aggregation and deposition of misfolded proteins in SGs or its permanent presence in different tissues including membranes, that may lead to neuronal dysfunction and eventually to cell death[43], [50], [62],[21]. This becomes very relevant in PolyQ disorders such as SCA2 and SCA3/MJD, and could mean that these misfolded proteins can accumulate in SGs, decreasing its levels and becoming less detectable. However, ataxin-3 was not yet shown to be recruited to SGs.

In sum, it is evident that SGs assembly is affecting these disease-causing proteins, ataxin-2 and ataxin-3, whether by overexpressing G3BP1 or by treatment with SA, which might be relevant to both SCA2 and SCA3/MJD pathologies.

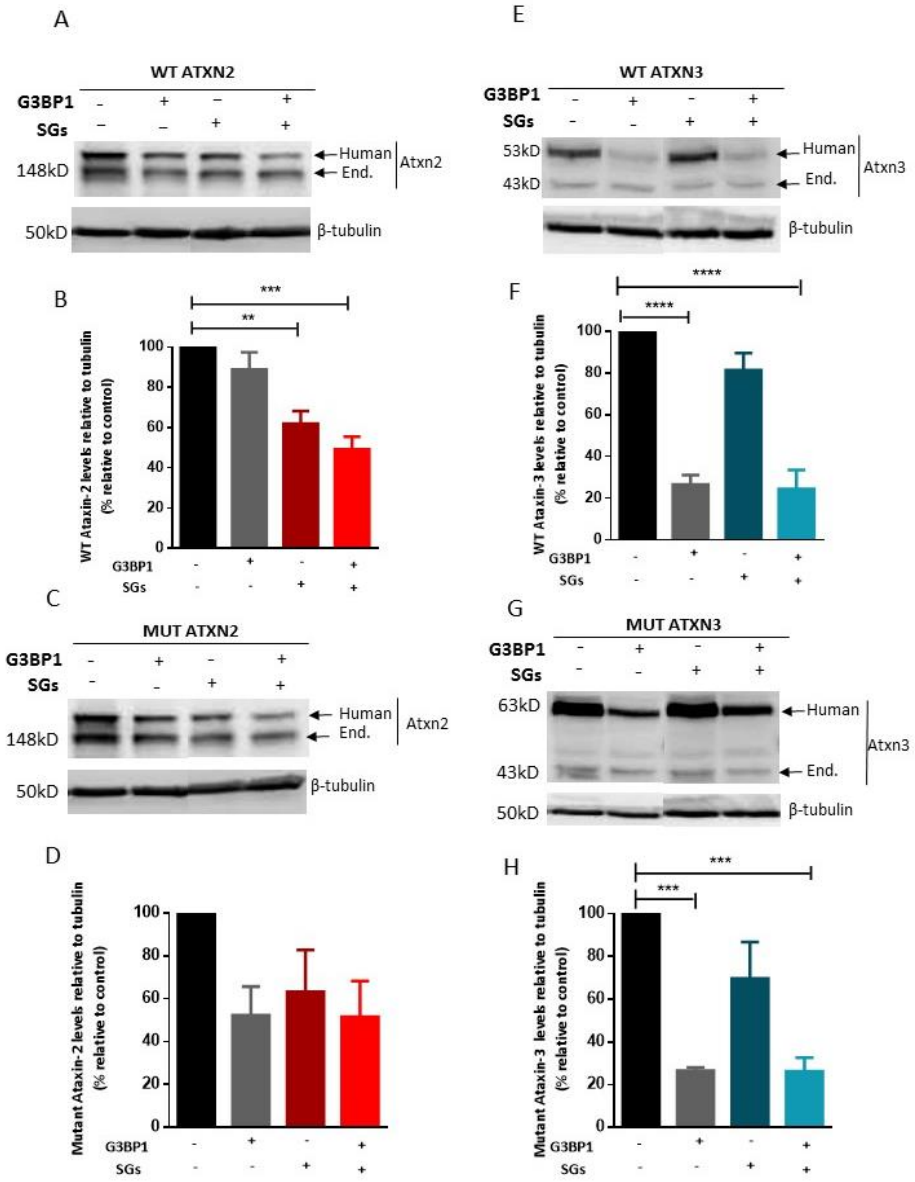

Figure 10 – **Combined effect of G3BP1 and SA reduces the levels of Ataxin-2 and Ataxin-3 (A-H).** Western Blot analysis and densitometry analysis quantification of WT Ataxin-2 (A-B), MUT Ataxin-2 (C-D), WT Ataxin-3 (E-F) and MUT Ataxin-3 (G-H). SA and G3BP1 combined effect reduce significantly the levels of WT Ataxin-2 (A-B), whereas this effect is not significant in MUT ataxin-2 (D-E), relatively to control. SA is not enough to reduce significantly the levels of WT ataxin-3 (E-F) or MUT ataxin-3 (G-H), but when combined with G3BP1 overexpression, the levels of both proteins are significantly reduced. Neuro2A Cells were co-transfected with G3BP1 and WT Ataxin-2 (Q22), MUT Ataxin-2 (Q58), WT Ataxin-3 (Q24) or MUT Ataxin-3 (Q84). Representative western blots are displayed, probed for anti-atx2 (A, C) or anti-atx3 (E, G), and anti-tubulin (A-H). Values are expressed as mean ± SEM, n=5. **p<0,01, ***p<0,001, ****p<0,0001 (One-way ANOVA).

4.7 SGs assembly induction alters aggregation properties of Ataxin-2 and Ataxin-3

It has been extensively studied how mutant forms of ataxin-2 and ataxin-3 aggregate and cause neuropathology which ultimately leads to cell death in SCA2 and SCA3/MJD, respectively [28], [34], [35], [63]. Thus, we next wanted to address the effect of SGs induction in ataxin-2 and ataxin-3 aggregation.

We first observed that in this cellular model, both wild-type and mutant forms of the proteins ataxin-2 and ataxin-3, have propensity to aggregate [55] (**Fig.11 B, C**), nevertheless and as expected, this aggregation is much stronger upon transfection with the mutant forms.

We observed that SGs formation is capable of modulating aggregation in cellular models of SCA2 and SCA3.

In ataxin-2, SA-induced SGs formation seems to play a key role in aggregation, which occurs in both forms of the protein, wild-type and mutant. We can observe a significant increase in the aggregation of ataxin-2 when cells are exposed to SA, whereas G3BP1 does not seem to affect ataxin-2 aggregates significantly (**Fig.11 B, C**).

As previously explained, as SGs are assembled, disease-causing proteins and RBPs (such as Ataxin-2, PABP, TDP-43 and FUS), are sequestered to SGs, where they interact significantly (see proposed model in **Fig.4**), which will lead to a more stable aggregation. Upon stress, (such as SA induced stress) and when pathologically expanded, these proteins form fibrils and irreversible pathological modifications occur. Ultimately, this impacts aggregation, which increases significantly in stress environments, which helps us understanding why SA has a more significant impact in aggregation, when comparing to G3BP1.

When G3BP1 is overexpressed with the wild type ataxin-2 and ataxin-3, the number of cells with aggregates increase (**Fig.11 B, D**), being significant in the case of ataxin-3. Interestingly, G3BP1 overexpression significantly decreases the number of cells with aggregates in mutant form of ataxin-3, in comparison to control, in SCA3/MJD model (**Fig.11 E**), which was not observed in the case of mutant ataxin-2 (**Fig.11 C**). As suggested before, G3BP1 might interact differently with ataxin-3 when comparing to ataxin-2, reducing its aggregates in a significant way.

In all cases, there is no significant difference in aggregation when cells are exposed to SA alone when comparing to the combined effect of SA and G3BP1.

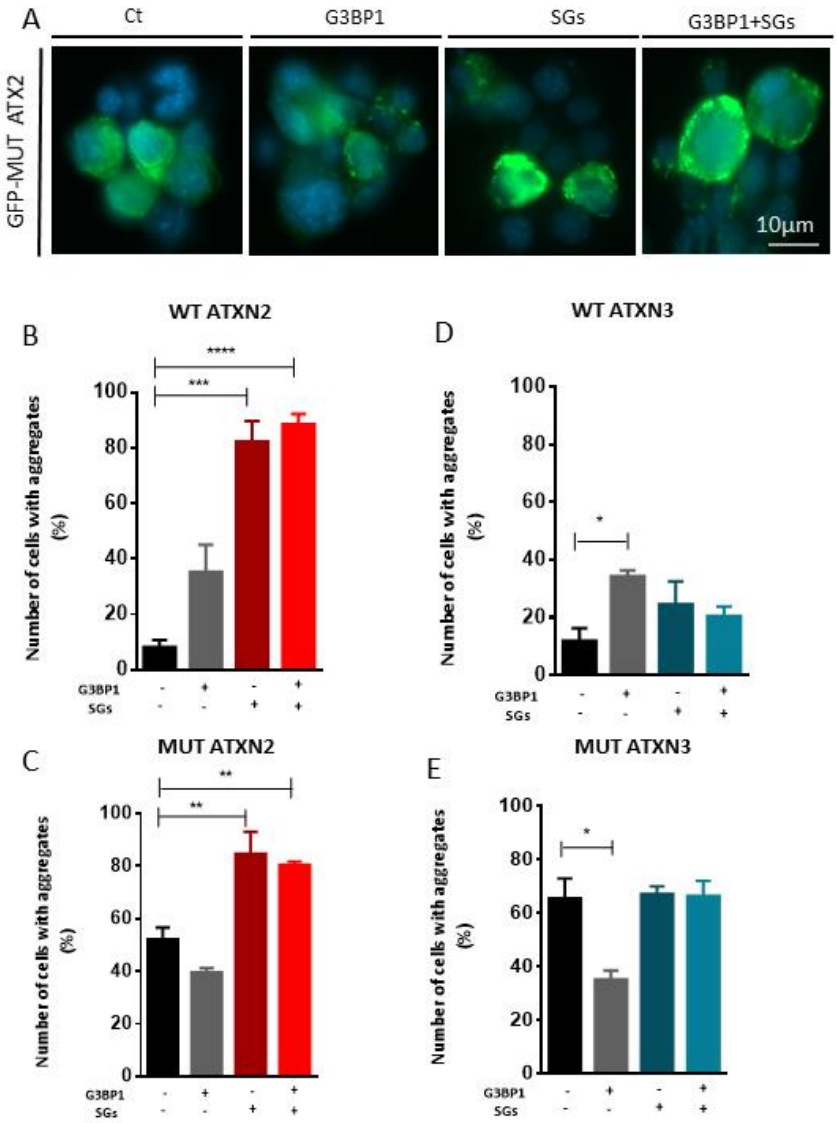

Figure 11 –SGs assembly affects ataxin-2 and ataxin-3 aggregation. SA-inducted SGs assembly increase significantly the number of cells with aggregates in both forms of ataxin-3, whereas G3BP1 has no relevant effect (B, C). In contrast, G3BP1 impacts ataxin-3 aggregates, and reducing significantly the aggregation of mutant ataxin-3 (D, E). Neuro2A cells were transfected with the following DNAs: GFP-WT ataxin-2, GFP-MUT ataxin-2, GFP-WT ataxin-3, GFP-MUT ataxin-3 and GFP- G3BP1, to induce SGs. SA was also used to induce SG in certain conditions. Cells were then stained with DAPI and the number of cells with aggregates were quantified directly in the microscope (63x), in a field within 100 random transfected cells. Representative images are shown. Values are expressed as mean ± SEM, n=4. *p<0,05** p<0,01, ***p<0,001, ****p<0,0001 (One-way ANOVA).

Altogether, these results suggest that SGs induction by SA exerts the main role in aggregation in these cellular disease models. In the same line, it was shown that oxidative stress

plays an important role in the pathogenesis of neurodegenerative disorders, including ataxias[62]. Furthermore, oxidative stress acts as co-factor, namely in SCA2. In aggregation, activation of apoptosis/caspases, autophagy, calcium and dopaminergic signaling, endoplasmic reticulum signaling, gene transcription, heat shock pathway, mitochondrial dysfunction, and synaptic neurotransmission deficits are involved [62],[64],[65]. Some of these molecular mechanisms generate reactive species that in turn are messengers or mediators of the related process, which will ultimately lead to oxidative stress. Thus, we can postulate that an oxidative stress environment is prone to aggregation in a SCA2 cellular model but not in a SCA3 cellular model, where G3BP1 seems to have a key role in aggregation.

4.8 Stress removal does not re-establish Ataxin-2 and Ataxin-3 levels

Previous studies have reported that when a stress is removed from cells, SGs are capable of disassemble [20], [58]. Next, we wanted to investigate if, by removing the stress stimuli, we could re-establish ataxin-2 and ataxin-3 protein levels. We co-transfected Neuro2A cells with G3BP1, Ataxin-2 (WT and MUT) and Ataxin-3 (WT and MUT). After 48 hours, we induced SGs with SA for 1 hour. After that, we removed the medium from our condition of interest, applied new medium and cells incubated for one more hour.

Altogether, the stress removal does not impact the levels of ataxin-2 and ataxin-3, either wild-type or the mutant forms. As already described, we observed an overall reduction in the protein levels when the SA is applied although it is not significant (except in the case of MUT Ataxin-2). However, when re-establishing the normal conditions, the protein levels did not recover to its previous state. This might have occurred because the time cells had to recover from the stress was not enough for the protein translation to be re-established.

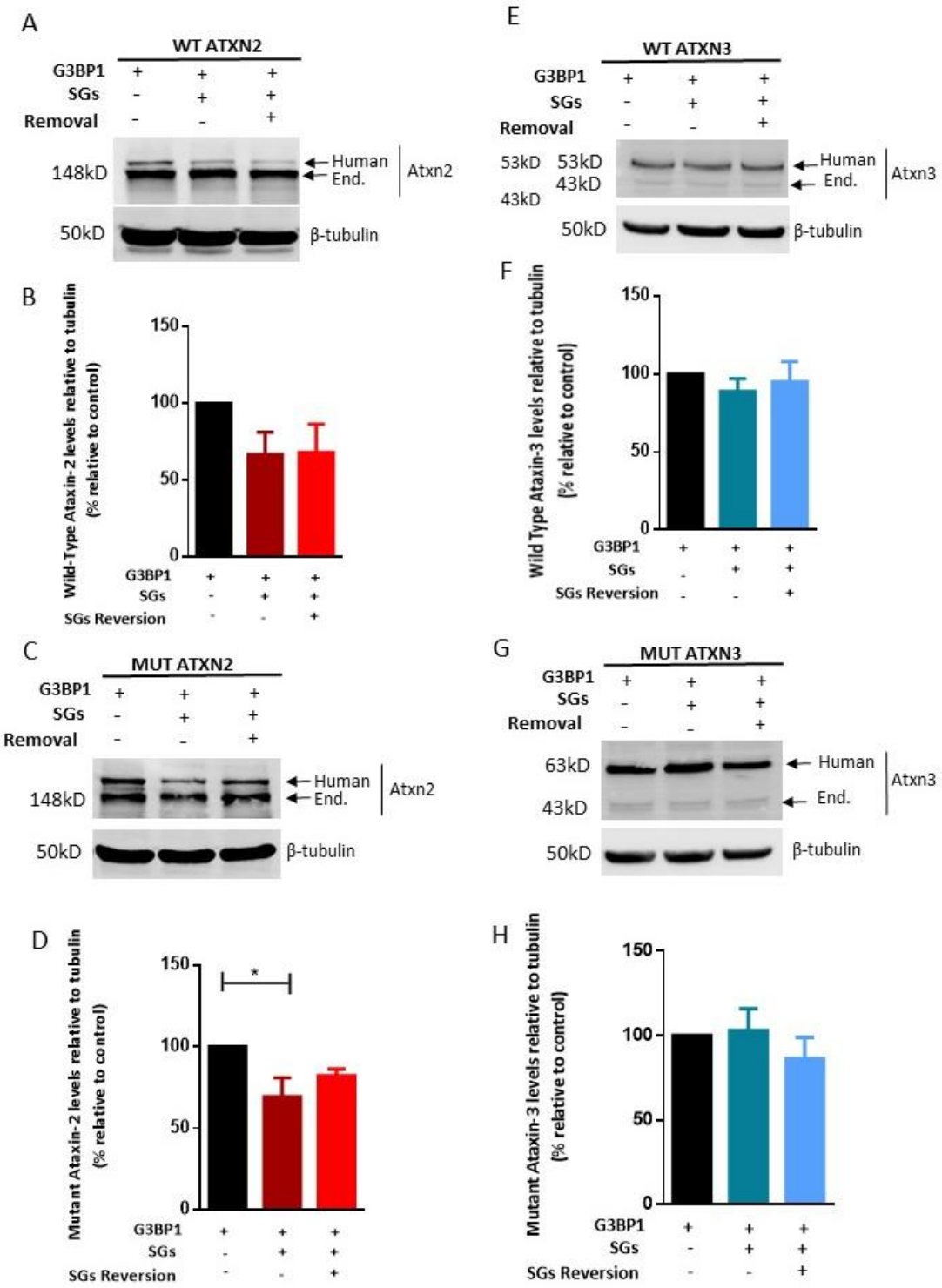

Figure 12 – **Stress stimulus removal does not impact ataxin-2 and ataxin-3 levels (A-H).** Western Blot analysis and densitometry quantification of WT Ataxin-2 (A-B), MUT Ataxin-2 (C-D), WT Ataxin-3 (E-F) and MUT Ataxin-3 (G-H). In all cases except for MUT ataxin-3 (H), there is a slight reduction in protein levels when SA is applied, which is significant in the case of MUT Ataxin-2 (D). When SA is removed from cells, proteins are not re-established to previous basal levels. Neuro2A Cells were co-transfected with G3BP1 and WT Ataxin-2 (Q22), MUT Ataxin-2 (Q58), WT Ataxin-3 (Q24) or MUT Ataxin-3 (Q84). Representative western blots are displayed, probed for anti-atx2 (A, C) or anti-atx3 (E,G), and anti-tubulin (A-H). Values are expressed as mean ± SEM, n=4. *p<0,05 (One-way ANOVA).

4.9 G3BP1 overexpression mitigates the neuropathology in a MJD Lentiviral Mouse Model

Last, and taking into account the previous results, we aimed to address the effect of the G3BP1 overexpression in a MJD lentiviral mouse model[66]. First, we injected the lentiviral vectors encoding for G3BP1 in the striatum of wild-type mice. No toxicity was detected upon viral transduction at 4 weeks post-injection, as no loss of DARPP-32 neuronal marker was detected (data not shown).

As previously reported, mutant ataxin-3 is able to form ubiquinated aggregates when expressed in the mouse striatum [60]. In order to investigate the effects of SGs in neuropathological features in vivo, we used a MJD lentiviral based mouse model [66], and overexpressed mutant ataxin-3 in one hemisphere and mutant ataxin-3 with G3BP1 in the other hemisphere. Then, we performed an immunohistological staining with Ubiquitin to evaluate the mutant ataxin-3 aggregates. We observed a significant reduction in the number of ubiquitin aggregates, comparing to the control hemisphere, at 4 weeks post-injection (**Fig. 13B**). These results are in agreement with the reduction of aggregates that we already observed in Neuro2A cells, upon G3BP1 overexpression. According to these results we then stained the histological sections with DARP-32, a marker for neuronal degeneration, to investigate if G3BP1 overexpression was capable of reducing neuronal degeneration. In fact, G3BP1 overexpression led to a robust reduction of neuronal loss, thus revealing a neuroprotective effect.

As previously mentioned, G3BP1, which is a RasGAP-binding protein, possess a diverse array of functions, namely recruiting and interacting with mRNAs upon inducing SGs assembly. Taking into consideration the previous results regarding G3BP1 effect in aggregation in a cellular SCA3/MJD model, it is clear that G3BP1 is specifically interacting with ataxin-3 somehow specifically, affecting its function. In this case, mutant ataxin-3 aggregates are significantly reduced when G3BP1 is being overexpressed. It has been reported that, when expressed, G3BP1 induces SGs and recruits several RNAs, in order to preserve them in a stress situation [61]. Furthermore, it has been shown its interaction with several proteins as well, in order to modulate SGs assembly [57], [61],[67]and it is also able to control and restrict mRNA translation in a stressful environment [68]. Taking this into consideration, it is possible that G3BP1 might be interacting with mutant ataxin-3 protein, and thus reducing the number of aggregates. Taking

into account these results, it seems that G3BP1 might play neuroprotective role in a SCA3/MJD lentiviral mouse model, reducing significantly the neuropathology.

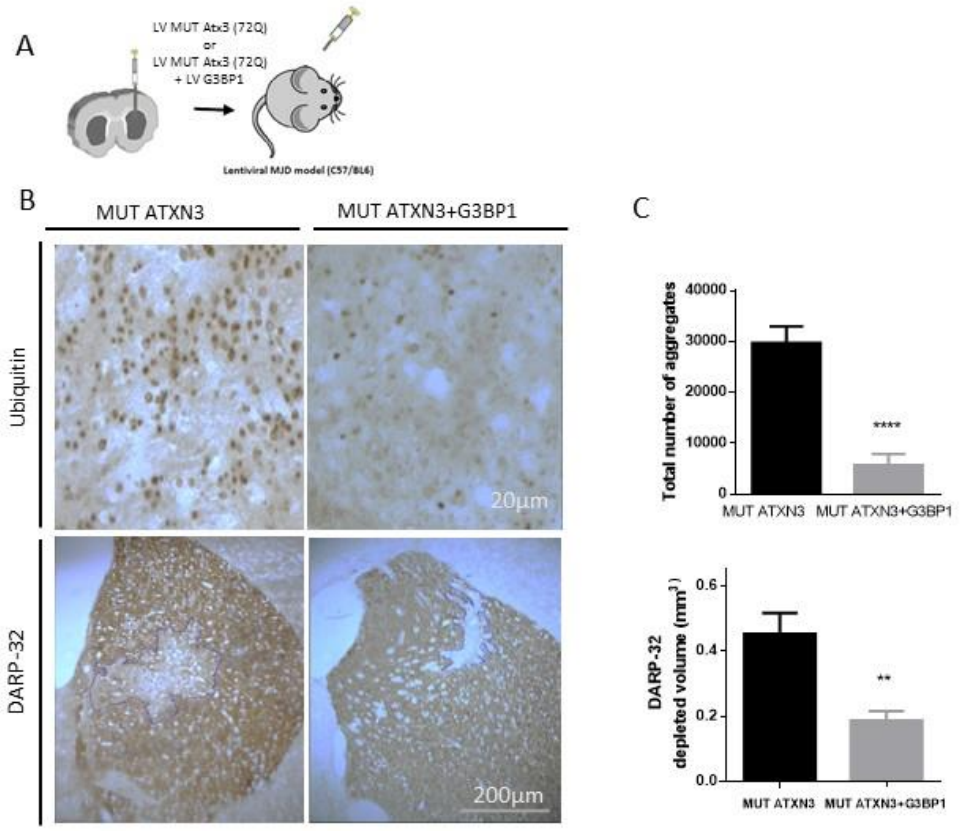

Figure 13 – *In vivo* G3BP1 overexpression reduces neuropathology of SCA3/MJD LV mouse model. (A) Schematic representation of stereotaxic unilateral injection of lentiviral vectors in mice striatum. Lentiviral vectors encoding human mutant ataxin-3 with 72 glutamines (Atx3 MUT) and G3BP1 were co-injected in the striatum of 8 weeks old C57/Bl6 mice. (B) Upper panel, Immunohistochemical staining for ataxin-3-positive inclusions (Ubiquitin). Lower panel, DARP-32 loss. (C) Upper graphic, quantification of absolute number of mutant ataxin-3-positive inclusions upon 4 weeks of injection. Values are expressed as mean ±SEM. (Unpaired Student's t-test) ****P<0.0001. N=6 for controls and N=8 for G3BP1 conditions. Lower graphic, quantification of DARP-32 depleted volume (mm3) 4 weeks after co-injection. Values are expressed as mean ±SEM. (paired Student's t-test). **P<0.01.

CHAPTER V- FINAL REMARKS AND FUTURE PERSPECTIVES

In this study we addressed the effect of the induction of SGs in a cellular model of SCA2 and SCA3/MJD and in a lentiviral mouse model of SCA3/MJD. Considering that the SCA2-causing protein ataxin-2 is recruited to SGs [43], [50], [69] and that it has been reported that this protein interacts with ataxin-3, affecting MJD's pathology[42], [53], it became important to study the effect of SGs induction in the levels of these diseases, as well as investigating if SGs could have a beneficial role in a neurodegeneration context.

We observed that by modulating SGs, either by inducing cellular stress (with SA) or by overexpressing a SGs inductor – G3BP1 – the levels of ataxin-2 and ataxin-3 were altered. We observed that SA-induced SGs decrease significantly the levels of ataxin-3, having no significant effects in aatxin-3 levels, whereas G3BP1 overexpression reduces significantly the levels of ataxin-3, having no significant effects in ataxin-2 levels. We can conclude that G3BP1 might be interacting somehow specifically with ataxin-3, despite the fact that ataxin-3 is not recruited to SGs. As for ataxin-2, it makes sense that oxidative stress impacts more the levels of this protein, given the fact that ataxin-2 is sequestered to SGs (acting as a regulator) and that it may become less available for detection.

We also observed that SA increases significantly the aggregation in a cellular model of SCA2, and that G3BP1 reduces significantly aggregation in a cellular model of SCA3/MJD. This is important because it shows once again that G3BP1 may be having some effect on ataxin-3, affecting its function, and ultimately being relevant in the reduction of aggregation in disease context. Thus, it is clear that an oxidative stress environment is prone to aggregation in a SCA2 cellular model.

We also verified that by removing the stress stimuli, the levels of ataxin-2 and ataxin-3 were not re-established to control levels, but further experiments need to be performed, namely without G3BP1 being expressed, as a control.

As a future study, we consider that it might be interesting to silence G3BP1 in a cellular model, to understand the impact of SGs induction impairment in ataxin-2 and ataxin-3 levels, as well as in aggregation.

Finally, we observed that by overexpressing G3BP1 in a lentiviral mouse model, the total number of mutant ataxin-3 aggregates were significantly reduced, and that the neuronal loss is also significantly decreased. This tells us that SGs induction might be having a neuroprotective role in the mouse brain, which is very important in a neurodegenerative disorder context, such as SCA3/MJD. We consider that overexpressing G3BP1 in a SCA2 disease model would be relevant to address if the effect is similar to what we observed in cells.

CHAPTER VI - REFERENCES

[1] L. D. Kapp and J. R. Lorsch, “E UKARYOTIC T RANSLATION,” pp. 657–704, 2004.

[2] T. E. Dever and R. Green, “Phases of Translation in Eukaryotes,” 2012.

[3] E. Z. Alkalaeva, A. V Pisarev, L. Y. Frolova, L. L. Kisselev, and T. V Pestova, “In Vitro Reconstitution of Eukaryotic Translation Reveals Cooperativity between Release Factors eRF1 and eRF3,” pp. 1125–1136, 2006.

[4] B. Levine, “Review Autophagy and the Integrated Stress Response,” 2010.

[5] M. Takahashi, M. Higuchi, H. Matsuki, M. Yoshita, T. Ohsawa, M. Oie, and M. Fujii, “Stress Granules Inhibit Apoptosis by Reducing Reactive Oxygen,” vol. 33, no. 4, pp. 815–829, 2013.

[6] N. Kedersha and P. Anderson, “Stress granules: sites of mRNA triage that regulate mRNA stability and translatability.,” *Biochem. Soc. Trans.*, vol. 30, no. Pt 6, pp. 963–969, 2002.

[7] N. L. Kedersha, M. Gupta, W. Li, I. Miller, and P. Anderson, “eIF-2 ⌴ to the Assembly of Mammalian Stress Granules,” *J. Cell Biol.*, vol. 147, no. 7, pp. 1431–1441, 1999.

[8] N. Kedersha and P. Anderson, “Mammalian Stress Granules and Processing Bodies,” *Methods Enzymol.*, vol. 431, no. 07, pp. 61–81, 2007.

[9] P. Anderson and N. Kedersha, “RNA granules,” *J. Cell Biol.*, vol. 172, no. 6, pp. 803–808, 2006.

[10] N. Kedersha, M. R. Cho, W. Li, P. W. Yacono, S. Chen, N. Gilks, D. E. Golan, and P. Anderson, “Dynamic shuttling of TIA-1 accompanies the recruitment of mRNA to mammalian stress granules,” *J. Cell Biol.*, vol. 151, no. 6, pp. 1257–1268, 2000.

[11] P. Anderson and N. Kedersha, “Stress granules: the Tao of RNA triage,” *Trends Biochem. Sci.*, vol. 33, no. 3, pp. 141–150, 2008.

[12] J. R. Buchan, D. Muhlrad, and R. Parker, “P bodies promote stress granule assembly in Saccharomyces cerevisiae,” *J. Cell Biol.*, vol. 183, no. 3, pp. 441–455, 2008.

[13] S. N. Bhattacharyya, R. Habermacher, U. Martine, E. I. Closs, and W. Filipowicz, “Stress-induced Reversal of microRNA repression and mRNA P-body localization in human cells,” *Cold Spring Harb. Symp. Quant. Biol.*, vol. 71, pp. 513–521, 2006.

[14] S. Mollet, N. Cougot, A. Wilczynska, F. Dautry, M. Kress, E. Bertrand, and D. Weil, “Translationally repressed mRNA transiently cycles through stress granules during stress.,” *Mol. Biol. Cell*, vol. 19, no. 10, pp. 4469–79, Oct. 2008.

[15] Q. Tian, M. Streuli, H. Saito, S. F. Schlossman, and P. Anderson, “A polyadenylate binding protein localized to the granules of cytolytic lymphocytes induces DNA fragmentation in target cells.,” *Cell*, vol. 67, no. 3, pp. 629–639, 1991.

[16] N. L. Kedersha, M. Gupta, W. Li, I. Miller, and P. Anderson, “RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2?? to the assembly of mammalian stress granules,” *J. Cell Biol.*, vol. 147, no. 7, pp. 1431–1441, 1999.

[17] H. Tourrière, K. Chebli, L. Zekri, B. Courselaud, J. M. Blanchard, E. Bertrand, and J. Tazi, “The RasGAP-associated endoribonuclease G3BP assembles stress granules,” *J. Cell Biol.*, vol. 160, no. 6, pp. 823–831, 2003.

[18] D. H. Wolf and A. Stolz, “Biochimica et Biophysica Acta The Cdc48 machine in endoplasmic reticulum associated protein degradation ☆,” *BBA - Mol. Cell Res.*, vol. 1823, no. 1, pp. 117–124, 2012.

[19] J. R. Buchan, R. Kolaitis, J. P. Taylor, and R. Parker, “Eukaryotic Stress Granules Are Cleared by Autophagy and Cdc48 / VCP Function,” *Cell*, vol. 153, no. 7, pp. 1461–1474, 2013.

[20] J. R. Buchan and R. Parker, “Eukaryotic Stress Granules: The Ins and Outs of Translation,” *Mol. Cell*, vol. 36, no. 6, pp. 932–941, 2009.

[21] B. Wolozin, “Regulated protein aggregation: stress granules and neurodegeneration,” *Mol Neurodegener*, vol. 7, no. 1, p. 56, 2012.

[22] K. K. McDonald, A. Aulas, L. Destroismaisons, S. Pickles, E. Beleac, W. Camu, G. a. Rouleau, and C. Vande Velde, “TAR DNA-binding protein 43 (TDP-43) regulates stress granule dynamics via differential regulation of G3BP and TIA-1,” *Hum. Mol. Genet.*, vol. 20, no. 7, pp. 1400–1410, 2011.

[23] J. P. White, A. M. Cardenas, W. E. Marissen, and R. E. Lloyd, “Inhibition of Cytoplasmic mRNA Stress Granule Formation by a Viral Proteinase,” *Cell Host Microbe*, vol. 2, no. 5, pp. 295–305, 2007.

[24] X. J. Lian and I. E. Gallouzi, “Oxidative stress increases the number of stress granules in senescent cells and triggers a rapid decrease in p21waf1/cip1 translation,” *J. Biol. Chem.*, vol. 284, no. 13, pp. 8877–8887, 2009.

[25] I. E. Gallouzi, “Could stress granules be involved in age-related diseases?,” *Aging (Albany. NY).*, vol. 1, no. 9, pp. 753–757, 2009.

[26] H. Fan, L. Ho, C. Chi, S. Chen, G. Peng, T. Chan, S. Lin, and H. Harn, “Review Polyglutamine (PolyQ) Diseases : Genetics to Treatments,” vol. 23, no. 235, pp. 441–458, 2014.

[27] J. J. Magaña, L. Velázquez-Pérez, and B. Cisneros, “Spinocerebellar ataxia type 2: Clinical presentation, molecular mechanisms, and therapeutic perspectives,” *Mol. Neurobiol.*, vol. 47, no. 1, pp. 90–104, 2013.

[28] M.-U. Manto, “The wide spectrum of spinocerebellar ataxias (SCAs).,” *Cerebellum*, vol. 4, no. 1, pp. 2–6, 2005.

[29] S. A. Type, C. Nóbrega, and L. P. De Almeida, “Machado-Joseph Disease /.”

[30] G. Wang, N. Sawai, S. Kotliarova, I. Kanazawa, and N. Nukina, “Ataxin-3, the MJD1 gene product, interacts with the two human homologs of yeast DNA repair protein RAD23, HHR23A and HHR23B.,” *Hum. Mol. Genet.*, vol. 9, no. 12, pp. 1795–1803, 2000.

[31] W. L. Tsou, A. a. Burr, M. Ouyang, J. R. Blount, K. M. Scaglione, and S. V. Todi, “Ubiquitination regulates the neuroprotective function of the deubiquitinase ataxin-3 in vivo,” *J. Biol. Chem.*, vol. 288, no. 48, pp. 34460–34469, 2013.

[32] J. M. Warrick, L. M. Morabito, J. Bilen, B. Gordesky-Gold, L. Z. Faust, H. L. Paulson, and N. M. Bonini, “Ataxin-3 suppresses polyglutamine neurodegeneration in Drosophila by a ubiquitin-associated mechanism,” *Mol. Cell*, vol. 18, no. 1, pp. 37–48, 2005.

[33] M. Albrecht, M. Golatta, U. Wüllner, and T. Lengauer, “Structural and functional analysis of ataxin-2 and ataxin-3,” *Eur. J. Biochem.*, vol. 271, no. 15, pp. 3155–3170, 2004.

[34] R. Estrada, J. Galarraga, G. Orozco, A. Nodarse, and G. Auburger, “Spinocerebellar ataxia 2 (SCA2): Morphometric analyses in 11 autopsies,” *Acta Neuropathol.*, vol. 97, no. 3, pp. 306–310, 1999.

[35] J. T. Pang, P. Giunti, S. Chamberlain, S. F. An, R. Vitaliani, T. Scaravilli, L. Martinian, N. W. Wood, F. Scaravilli, and O. Ansorge, “Neuronal intranuclear inclusions in SCA2: a genetic, morphological and immunohistochemical study of two cases.,” *Brain*, vol. 125, no. Pt 3, pp. 656–663, 2002.

[36] U. Rüb, D. Del Turco, K. Bürk, G. Orozco Diaz, G. Auburger, M. Mittelbronn, K. Gierga, E. Ghebremedhin, C. Schultz, L. Schöls, J. Bohl, H. Braak, and T. Deller, “Extended pathoanatomical studies point to a consistent affection of the thalamus in spinocerebellar ataxia type 2,” *Neuropathol. Appl. Neurobiol.*, vol. 31, no. 2, pp. 127–140,

2005.

[37] A. C. Elden, H.-J. Kim, M. P. Hart, A. S. Chen-Plotkin, B. S. Johnson, X. Fang, M. Armakola, F. Geser, R. Greene, M. M. Lu, A. Padmanabhan, D. Clay-Falcone, L. McCluskey, L. Elman, D. Juhr, P. J. Gruber, U. Rüb, G. Auburger, J. Q. Trojanowski, V. M.-Y. Lee, V. M. Van Deerlin, N. M. Bonini, and A. D. Gitler, "Ataxin-2 intermediate-length polyglutamine expansions are associated with increased risk for ALS.," *Nature*, vol. 466, no. 7310, pp. 1069–1075, 2010.

[38] D. P. Huynh, K. Figueroa, N. Hoang, and S. M. Pulst, "Nuclear localization or inclusion body formation of ataxin-2 are not necessary for SCA2 pathogenesis in mouse or human.," *Nat. Genet.*, vol. 26, no. 1, pp. 44–50, 2000.

[39] I. Nascimento-Ferreira, C. Nóbrega, A. Vasconcelos-Ferreira, I. Onofre, D. Albuquerque, C. Aveleira, H. Hirai, N. Déglon, and L. Pereira De Almeida, "Beclin 1 mitigates motor and neuropathological deficits in genetic mouse models of Machado-Joseph disease," *Brain*, vol. 136, no. 7, pp. 2173–2188, 2013.

[40] H. Shibata, D. P. Huynh, and S. M. Pulst, "A novel protein with RNA-binding motifs interacts with ataxin-2.," *Hum. Mol. Genet.*, vol. 9, no. 9, pp. 1303–1313, 2000.

[41] D. Camilli, M. H-m, and I. Region, "Spinocerebellar ataxia 2," vol. 2, no. 1993, pp. 972–975, 1994.

[42] C. Nóbrega, S. Carmo-Silva, D. Albuquerque, A. Vasconcelos-Ferreira, U. G. Vijayakumar, L. Mendonça, H. Hirai, and L. Pereira De Almeida, "Re-establishing ataxin-2 downregulates translation of mutant ataxin-3 and alleviates Machado-Joseph disease," *Brain*, vol. 138, no. 12, pp. 3537–3554, 2015.

[43] C. M. Dewey, B. Cenik, C. F. Sephton, B. a. Johnson, J. Herz, and G. Yu, "TDP-43 aggregation in neurodegeneration: Are stress granules the key?," *Brain Res.*, vol. 1462, pp. 16–25, 2012.

[44] Y. R. Li, O. D. King, J. Shorter, and A. D. Gitler, "Stress granules as crucibles of ALS pathogenesis," *J. Cell Biol.*, vol. 201, no. 3, pp. 361–372, 2013.

[45] K. Phillips, N. Kedersha, L. Shen, P. J. Blackshear, and P. Anderson, "Arthritis suppressor genes TIA-1 and TTP dampen the expression of tumor necrosis factor alpha, cyclooxygenase 2, and inflammatory arthritis.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 101, no. 7, pp. 2011–2016, 2004.

[46] M. Boyce, K. F. Bryant, C. Jousse, K. Long, H. P. Harding, D. Scheuner, R. J. Kaufman, D. Ma, D. M. Coen, D. Ron, and J. Yuan, "A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress.," *Science*, vol. 307, no. 5711, pp. 935–939, 2005.

[47] Y. Furukawa, K. Kaneko, G. Matsumoto, M. Kurosawa, and N. Nukina, "Cross-Seeding Fibrillation of Q/N-Rich Proteins Offers New Pathomechanism of Polyglutamine Diseases," *J. Neurosci.*, vol. 29, no. 16, pp. 5153–5162, 2009.

[48] C. Kaehler, J. Isensee, U. Nonhoff, M. Terrey, T. Hucho, H. Lehrach, and S. Krobitsch, "Ataxin-2-Like Is a Regulator of Stress Granules and Processing Bodies," *PLoS One*, vol. 7, no. 11, pp. 1–12, 2012.

[49] K. D. Swisher and R. Parker, "Localization to, and effects of Pbp1, Pbp4, Lsm12, Dhh1, and Pab1 on stress granules in Saccharomyces cerevisiae," *PLoS One*, vol. 5, no. 4, 2010.

[50] U. Nonhoff, M. Ralser, F. Welzel, I. Piccini, D. Balzereit, M.-L. Yaspo, H. Lehrach, and S. Krobitsch, "Ataxin-2 interacts with the DEAD/H-box RNA helicase DDX6 and interferes with P-bodies and stress granules.," *Mol. Biol. Cell*, vol. 18, no. 4, pp. 1385–96, Apr. 2007.

[51] T. Uchihara, H. Fujigasaki, S. Koyano, a Nakamura, S. Yagishita, and K. Iwabuchi, "Non-expanded polyglutamine proteins in intranuclear inclusions of hereditary ataxias--triple-labeling immunofluorescence study.," *Acta Neuropathol.*, vol. 102, no. 2, pp. 149–152, 2001.

[52] D. a. Bosco, N. Lemay, H. K. Ko, H. Zhou, C. Burke, T. J. Kwiatkowski, P. Sapp, D. Mckenna-Yasek, R. H. Brown, and L. J. Hayward, "Mutant FUS proteins that cause amyotrophic lateral sclerosis incorporate into stress granules," *Hum. Mol. Genet.*, vol. 19, no. 21, pp. 4160–4175, 2010.

[53] D. Lessing and N. M. Bonini, "Polyglutamine genes interact to modulate the severity and progression of neurodegeneration in Drosophila," *PLoS Biol.*, vol. 6, no. 2, pp. 0266–0274, 2008.

[54] D. P. Huynh, H. Yang, H. Vakharia, D. Nguyen, and S. M. Pulst, "Expansion of the polyQ repeat in ataxin-2 alters its Golgi localization , disrupts the Golgi complex and causes cell death," vol. 12, no. 13, pp. 1485–1496, 2003.

[55] C. A. Matos, C. Nóbrega, S. R. Louros, B. Almeida, E. Ferreiro, J. Valero, L. P. De Almeida, S. M. Ribeiro, and A. L. Carvalho, "Ataxin-3 phosphorylation decreases neuronal defects in spinocerebellar ataxia type 3 models," vol. 212, no. 4, pp. 465–480, 2016.

[56] E. K. Schmidt, G. Clavarino, M. Ceppi, and P. Pierre, "SUnSET , a nonradioactive method to monitor protein synthesis," vol. 6, no. 4, pp. 275–277, 2009.

[57] J. Mouaikel, P. V. A. N. D. E. R. Geer, and J. Tazi, "RasGAP-Associated Endoribonuclease G3BP : Selective RNA Degradation and Phosphorylation-Dependent Localization," vol. 21, no. 22, pp. 7747–7760, 2001.

[58] P. Anderson and N. Kedersha, "Stressful initiations.," *J. Cell Sci.*, vol. 115, no. Pt 16, pp. 3227–3234, 2002.

[59] I. Of, P. Into, and P. Chains, "INCORPORATION OF PUROMYCIN INTO locyte," vol. 51, no. 1963, 1964.

[60] S. Alves, I. Nascimento-Ferreira, G. Auregan, R. Hassig, N. Dufour, E. Brouillet, M. C. Pedroso de Lima, P. Hantraye, L. P. de Almeida, and N. Déglon, "Allele-specific RNA silencing of mutant ataxin-3 mediates neuroprotection in a rat model of Machado-Joseph disease," *PLoS One*, vol. 3, no. 10, 2008.

[61] A. Aulas, G. Caron, C. G. Gkogkas, N. Mohamed, L. Destroismaisons, N. Sonenberg, N. Leclerc, J. A. Parker, and C. Vande Velde, "G3BP1 promotes stress-induced RNA granule interactions to preserve polyadenylated mRNA," vol. 209, no. 1, pp. 73–84, 2015.

[62] M. Guevara-garcía, L. G. Valle, L. Velásquez-pérez, and J. C. García-rodríguez, "Oxidative stress as a cofactor in spinocerebellar ataxia type 2," vol. 17, no. 2, 2012.

[63] A. Durr, "Autosomal dominant cerebellar ataxias: Polyglutamine expansions and beyond," *Lancet Neurol.*, vol. 9, no. 9, pp. 885–894, 2010.

[64] I. Casetta, V. Govoni, and E. Granieri, "Oxidative Stress , Antioxidants and Neurodegenerative Diseases," pp. 2033–2052, 2005.

[65] M. A. Liebert, J. Reichenbach, R. Schubert, D. Schindler, K. Müller, H. Böhles, and S. Zielen, "Forum Rapid Letter," vol. 4, no. 3, pp. 465–469, 2002.

[66] S. Alves, E. Régulier, I. Nascimento-Ferreira, R. Hassig, N. Dufour, A. Koeppen, A. L. Carvalho, S. Simões, M. C. P. De Lima, E. Brouillet, V. C. Gould, N. Déglon, and L. P. De Almeida, "Striatal and nigral pathology in a lentiviral rat model of Machado-Joseph disease," *Hum. Mol. Genet.*, vol. 17, no. 14, pp. 2071–2083, 2008.

[67] L. Melnychuk, K. Vyboh, L. Ajamian, I. Gallouzi, F. Valiente-echeverrı, N. Bernard, and A.

J. Mouland, “during HIV-1 infection,” 2014.

[68] V. Cobos, F. O. Martinez, T. Booiman, K. A. Van Dort, M. A. A. Van De Klundert, S. Gordon, T. B. H. Geijtenbeek, and N. A. Kootstra, “G3BP1 restricts HIV-1 replication in macrophages and T-cells by sequestering viral RNA,” *Virology*, vol. 486, pp. 94–104, 2015.

[69] C. Kaehler, J. Isensee, U. Nonhoff, M. Terrey, T. Hucho, H. Lehrach, and S. Krobitsch, “Ataxin-2-like is a regulator of stress granules and processing bodies.,” *PLoS One*, vol. 7, no. 11, p. e50134, Jan. 2012.