

André Filipe Vieira da Conceição

**Investigating the impact of stress granules in the
pathogenesis of spinocerebellar ataxia type 2**



Universidade do Algarve

Faculdade de Medicina e Ciências Biomédicas

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pathogenesis of spinocerebellar ataxia type 2**

PhD Program in Biomedical Sciences

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Para a Catarina, a minha maior inspiração, o meu maior amor

“What does it mean to live a finite, fragile life in an infinite, eternal universe?

We are definitely physically insignificant. The Earth is one planet, around one star, among 400 billion stars in one galaxy, and among two trillion galaxies in a small patch of the universe. We, humans, are just specks of dust.

If you think about what we are—everybody, me, you—we are just collections of atoms. Some of those atoms are as old as time; others were cooked in stars over billions of years. Our body is this collection of stardust, organized in such a pattern that it can now think.

This sounds unlikely—that you can have a few things that were cooked in the hearts of stars, you stick them together in a pattern, and suddenly it has some ideas and starts writing music, arts, and things. All of this is quite difficult to comprehend, but that happened here, on Earth. As far as we know, it did not happen anywhere else, or it is quite rare. We looked in every direction and found only nothingness.

While “meaning” is not a scientific concept, it might be a property that emerges from complex biological systems, like humans. If there's nothing beyond us, it might well be that Earth is the only place in the universe where meaning exists. This could be the only place in the universe where we mean something to someone, and that is enough for me.

Notwithstanding its insignificance, Earth is the only place where the universe created a meaning and has a way to understand and explore itself, which is us.”

— Brian Cox

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“It’s the job that’s never started
that takes the longest to finish”

— J. R. R. Tolkien

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Abbreviations

A2BP1	ATXN2-binding protein 1
aa	Amino acid
AAV1	Adeno-associated virus 1
AD	Alzheimer's disease (AD)
ALS	Amyotrophic lateral sclerosis (ALS)
AMPK	AMP-regulated kinase
APP	Amyloid-beta precursor protein
AraC	Cytosine-D-arabinofuranoside
BAC	Bacterial artificial chromosomes
CACNA1A	Calcium voltage-gated channel subunit alpha1 A
CAG	Cytosine-adenine-guanine
Calb1	Calbindin 1
CAT	Enzymatic catalyze
Cdk5	Cyclin-dependent kinase 5
cIDR	C-terminal intrinsically disordered region
CMV	Cytomegalovirus immediate early enhancer/promoter
CNS	Central nervous system
CRY1	Cryptochrome-1
D	Aspartate
DARPP-32	Dopamine- and cAMP-regulated neuronal phosphoprotein
DDX	DExD/H-box helicases
DIV"x"	Day in vitro "x"
DNA	Deoxyribonucleic acid
DRPLA	Dentatorubral pallidoluysian atrophy
E1	Ubiquitin-activating enzymes
E2	Ubiquitin-conjugating enzymes
E3	Ubiquitin ligases
EGFR	Epidermal growth factor receptor
eIF2 α	Eukaryotic initiation factor-2 α (eIF2 α)
eIF2 α kinase	EIF2AK
eIF2 α kinase 1	EIF2AK1
eIF2 α kinase 2	EIF2AK2
eIF2 α kinase 3	EIF2AK3
eIF2 α kinase 4	EIF2AK4
ER	Endoplasmic reticulum
FBXW8	Box/WD repeat domain containing 8
FTD	Frontotemporal-dementia
FUS	FUS RNA Binding Protein
GADD34	DNA damage-inducible protein

GCN2	General control nonderepressible
GFP	Green fluorescent protein sequence
GluN1	Glutamate Ionotropic Receptor NMDA Type Subunit 1
HA	Human influenza hemagglutinin
HD	Huntington's disease
HRI	Heme-regulated inhibitor
HRNBP1	RNA Binding Fox-1 Homolog 1
HSP	Heat shock protein
IDR	Intrinsically disordered region
IP3R	Inositol 1,4,5-trisphosphate receptor
ISR	Integrated stress response
kb	Kilobase
KO	Knock-out
LC3-II	Light chain 3 isoform II
LLPS	Liquid-liquid phase separation
Lsm	Like-Sm
LsmAD	Like-Sm-associated-domain
LV	Lentiviral
LVP	Lentiviral particle
MBNL1	Muscleblind-like splicing regulator
mGluN1	Metabotropic glutamate receptor 1
mIDR	Middle intrinsically disordered region
min	Minutes
mPGK	Mouse phosphoglycerate kinase 1 promoter
mRNA	Messenger RNA
mRNP	Messenger RNA protein
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex-1 (mTORC1)
NMDA	N-methyl D-aspartate
NMDR1	N-methyl-d-aspartate receptor
p-eIF2 α	Phosphorylated eIF2 α
P"x"	Postnatal "x"
PABP1	Poly(A)-binding protein 1
PABPC1	Poly(A) binding protein cytoplasmic 1
PAM2	Poly(A)-Binding Protein Interaction Motif 2
PARK2	the parkin ring E3 ubiquitin protein ligase
PASK	PAS domain containing serine/threonine kinase
Pcp2	Purkinje cell protein 2
PCR	Polymerase chain reaction
PD	Parkinson's disease (PD)
PER2	Period-2
PERK	(PKR)-like endoplasmic reticulum kinase
<i>Pgk-1</i> / PGK1	Phosphoglycerate kinase 1

PKR	Protein kinase double-stranded RNA-dependent
PolyQ	Polyglutamine
PP1	Protein phosphatase 1
pre-mRNA	Precursor mRNA (pre-mRNA)
PTM	Post-translational modifications
RACK1	Receptor for activated C kinase 1
Raptor	Regulatory-associated protein of mTOR
RBFOX1	RNA-binding protein fox-1 homolog 1
RBM9	RNA-binding motif protein 9
RBPM5	RNA-binding protein with multiple splicing
RER	Rough endoplasmic reticulum
RG	Arginine-glycine
RGS	Regulator of G protein signaling 8
RGS8	Regulator of G protein signaling 8
RNA	Ribonucleic acid
S	Serine
SA	Sodium arsenite
SCA	Spinocerebellar ataxia
SCA1	Spinocerebellar ataxia type 1
SCA17	Spinocerebellar ataxia type 17
SCA2	Spinocerebellar ataxia type 2
SCA3	Spinocerebellar ataxia type 3
SCA6	Spinocerebellar ataxia type 6
SCA7	Spinocerebellar ataxia type 7
SCH9	Serine/threonine protein kinase
SH3	Src homology 3
SMA1/SBMA	Spinal and bulbar muscular atrophy, X-linked
Snf1	Sucrose Non-fermenting protein 1
SOD2	Manganese superoxide dismutase
SQSTM1	Sequestosome 1
STAU1	Staufen double-stranded RNA binding protein 1
TAU	Microtubule Associated Protein Tau
TBP	TATA box binding protein
TDP43	TAR DNA-binding protein 43
TRIM21	Tripartite motif containing 21
TTFL	transcriptional-translational feedback loop
TYF	Twenty-four
Ub	Ubiquitin
UPS	Ubiquitin-proteome system
VCP	valosin-containing protein
WT	Wild-type

Abstract

Spinocerebellar ataxia type 2 (SCA2) is a polyglutamine disease caused by an abnormally long cytosine-adenine-guanine (CAG) expansion mutation within the coding region of the *ATXN2* gene. This mutation produces an ataxin-2 protein form, which bears an overexpanded glutamine tract with neurotoxic properties. Despite the efforts, the pathogenic mechanisms underlying neurodegeneration in SCA2 are still poorly understood, and no therapy is available to delay or stop disease progression. In this work, we investigated the impact of stress granules (SGs) in SCA2 pathogenesis.

SGs are dynamic membraneless organelles formed during the integrated stress response (ISR) to aid cells in coping with stress. Among other biological functions, these structures are thought to serve as local stores of untranslated mRNAs, allowing cells to prioritize the translation of stress-resolving factors while reducing the energetic burden of global translation during critical periods. Emerging evidence suggests that persistent SGs under chronic stress conditions may have deleterious effects, potentially contributing to cellular dysfunction and disease pathogenesis. Moreover, recent studies suggest that the ISR is overactivated in several neurodegenerative diseases, including Alzheimer's disease, amyotrophic lateral sclerosis, frontotemporal dementia, and Parkinson's disease, potentially promoting an exacerbated stress response and an aberrant formation of SGs. Nevertheless, the role of SGs in the context of chronic stress and neurodegenerative disorders, particularly in SCA2, is still poorly addressed.

Using both *in vitro* and *in vivo* models of SCA2, we observed that SGs assembly and disassembly differently modifies the pathological deficits associated with SCA2. Promoting the formation of SGs within a limited timeframe seems to have protective effects, while sustained induction results in an exacerbated SCA2-associated phenotype, supporting the hypothesis that chronic SGs persistence has deleterious effects. On the other hand, SGs sustained inhibition seemed to have discrete deleterious effects. These findings suggest that SGs are cellular structures requiring a concerted and tightly regulated control, and chronic

disturbances – either promoting or preventing their formation – negatively impact SCA2-associated pathology.

Keywords: spinocerebellar ataxia type 2, ataxin-2, neurodegeneration, stress response, stress granules

Resumo

A ataxia espinocerebelosa do tipo 2 (SCA2, do inglês *spinoocerebellar ataxia type 2*) é uma doença neurodegenerativa rara causada por uma expansão anormal do trinucleótido adenina-citosina-guanina (CAG) na região codificante do gene *ATXN2*. A expansão anormal do trinucleótido CAG está presente no primeiro exão do gene da *ATXN2*, e é responsável pela produção de uma proteína mutante, a ataxina-2, com um segmento de glutaminas anormalmente longo. A ataxina-2 mutante adquire funções tóxicas que comprometem o funcionamento normal das células, especialmente neurónios, resultando na sua morte. Indivíduos não afetados pela SCA2 também são portadores do gene *ATXN2*, o qual contém repetições CAG não patogénicas. Quando a expansão de CAG ultrapassa o limiar de 35 repetições, a SCA2 manifesta-se.

A SCA2 está inserida num grupo de mais oito doenças, igualmente caracterizadas por uma expansão anormal do trinucleótido CAG, nos respetivos genes causadores da doença, denominadas doenças de poliglutaminas. Neste grupo estão incluídas: a doença de Huntington, outras cinco ataxias espinocerebelosas (SCA1, SCA3, SCA6, SCA7 e SCA17), a atrofia dentato-rubro-palido-luisiana e atrofia muscular espinal e bulbar.

A SCA2 afeta maioritariamente o sistema nervoso central, mas é no cerebelo, e em particular nas células de Purkinje, onde se observa uma marcada neurodegeneração seletiva. Os indivíduos afetados manifestam um conjunto de sintomas que incluem a ataxia da marcha, movimento oculares lentos, dificuldades na fala (disartria), dificuldades na deglutição (disfagia), rigidez muscular e, em alguns casos, até parkinsonismo. Os mecanismos que conduzem à neurodegeneração, e consequentemente aos sintomas apresentados na SCA2, não estão ainda totalmente compreendidos; da mesma forma, a razão pela qual a expansão anormal de glutaminas é capaz de induzir toxicidade continua por esclarecer. Contudo, estão identificados diversos mecanismos celulares que contribuem para a SCA2 incluindo: agregação da proteína mutante ataxina-2, comprometimento da autofagia, toxicidade associada ao RNA expandido, disfunção mitocondrial, stress oxidativo exacerbado e desregulação na sinalização de cálcio.

De modo a minimizar os efeitos tóxicos dos mecanismos celulares desregulados, as células desenvolveram vias específicas capazes de mitigar os efeitos deletérios dessas disfunções, como é o caso da resposta integrada ao stress (ISR, do inglês *integrated stress response*). A ISR é um mecanismo capaz de detetar várias formas de stress celular (infecções virais, escassez de aminoácidos, stress oxidativo ou a presença de proteínas com conformações anormais), ativando respostas que visam restaurar a homeostasia celular. A ISR é iniciada quando, na presença de um estímulo de stress, uma de quatro cinases – PERK, PKR, GCN2, e HRI – fosforila o eIF2 α . Como consequência, a síntese global de proteínas é atenuada e formam-se os grânulos de stress (SGs, do inglês *stress granules*).

Os SGs são estruturas dinâmicas que se formam imediatamente após a deteção de um stress celular. A sua composição compreende mRNAs não traduzidos, moléculas envolvidas na tradução e proteínas ligantes a RNA, que coalescem na formação destes organelos. Pensa-se que os SGs tenham como principal função o sequestro de mRNAs não essenciais para combater o stress celular, de modo a que as células consigam concentrar os seus recursos energéticos na síntese de proteínas capazes de reestabelecer a homeostasia celular. Assim que o stress celular desaparece, os SGs são rapidamente dissociados. Esta dissociação dá-se após a desfosforilação do eIF2 α pelo complexo de proteínas PP1:GADD34.

Apesar do papel benéfico dos SGs em situações de stress celular, nos últimos anos novas evidências mostram que, em situações de stress celular crónico, os SGs deixam de ser dinâmicos, apresentando uma elevada persistência. Uma das hipóteses predominantes na área é que em condições de stress permanente, a persistência na formação e manutenção de SGs tem efeitos nocivos para as células, podendo promover a morte celular. Diversos estudos sugerem que, em algumas doenças neurodegenerativas, a ISR está ativa em níveis muito superiores aos normais, contribuindo para patologia associada. Contudo o impacto dos SGs nestas condições não está totalmente estabelecido. Assim sendo, neste trabalho colocamos a hipótese de no contexto de SCA2 se verificar uma ISR exacerbada e uma desregulação na formação de SGs, devido a uma persistente manutenção do stress celular, característico das doenças neurodegenerativas. Deste modo, o principal objetivo deste trabalho é tentar compreender o impacto da formação e dissociação dos SGs no contexto de SCA2.

Na primeira parte deste trabalho, desenvolvemos com sucesso ferramentas moleculares capazes de modular a dinâmicas dos SGs. Mais concretamente, desenvolvemos

uma estratégia genética capaz induzir a formação de SGs e uma estratégia genética capaz de inibir a sua formação. Para induzir a formação de SGs desenvolvemos um fosfomimético do eIF2 α (eIF2 α^{S52D}), enquanto que para inibir a formação de SGs desenvolvemos uma forma truncada do GADD34 Δ^{322} . Ambas as estratégias foram validadas *in vitro*.

Usando as estratégias para modular a dinâmica dos SGs observamos que, *in vitro*, através do uso de neurónios primários de estriado, a expressão de eIF2 α^{S52D} (que induz a formação de SGs) é capaz de reestabelecer o comprimento total das dendrites, que é reduzido pela expressão da proteína ataxina-2 mutante. Contudo a expressão de GADD34 Δ^{322} não impacta o comprimento das dendrites.

Posteriormente, através de estudos *in vivo*, usando um modelo estriatal de SCA2, verificamos que a expressão de eIF2 α^{S52D} exacerba as características neuropatológicas associadas à expressão da ataxina-2 mutante no estriado. Os resultados parecem indicar que a expressão de GADD34 Δ^{322} não impacta os achados neuropatológicos.

Na última parte deste trabalho, estudamos o impacto da expressão de eIF2 α^{S52D} (indução de SGs) e da expressão de GADD34 Δ^{322} (inibição dos SGs) num novo modelo transgénico de SCA2, desenvolvido anteriormente no nosso grupo e caracterizado mais detalhadamente neste trabalho. Utilizando este novo modelo transgénico de SCA2, verificamos que expressão de eIF2 α^{S52D} exacerba o fenótipo associado a SCA2, tanto a nível motor, como a nível neuropatológico. A expressão de GADD34 Δ^{322} parece ter também um impacto deletério na SCA2, embora mais discreto. Os resultados aparentemente discrepantes entres as experiências *in vivo* e *in vitro* dão consistência à hipótese de que a formação de SGs limitada no tempo têm um impacto benéfico, enquanto a permanente manutenção e persistência dos SGs no tempo tem um impacto deletério. Mais ainda, o facto de termos observado que a expressão de GADD34 Δ^{322} também tem um impacto negativo, ainda que discreto, na patologia da SCA2 reforça a ideia de que os SGs são estruturas que exigem uma regulação altamente coordenada. Os resultados sugerem que alterar de forma crónica a formação ou dissociação dos SGs afeta negativamente a patologia associada à SCA2, tendo a capacidade de alterar o curso normal da doença.

Palavras-chave: ataxia espinocerebelosa tipo 2, ataxina-2, neurodegeneração, stress celular, granules de stress.

Chapter 1 – Introduction

1.1 Neurodegenerative diseases

Neurodegeneration is a progressive process in which the nervous system, central and peripheral, suffers an irreversible loss of its function, structure, or both, within specific regions. Neurodegenerative diseases – which are characterized by neurodegeneration – are known to severely impact the patient's daily life and, in most cases, the life of their families. As the nervous system controls and regulates several body functions, neurodegenerative disorders can affect numerous processes that, among others, include sensory acquisition, cognition, hormone regulation, or movements. These impairments can occur alone or combined, leading to symptoms and signs typical of a specific disease. According to the World Health Organization (WHO), more than 50 million people are affected by a neurodegenerative disorder. Because most of these conditions are age-related, and due to the increase in life expectancy, more than 152 million people are expected to be affected in 2050 (Brown et al., 2005; Liao et al., 2021; Przedborski et al., 2003).

Currently, it is estimated to exist a few hundred of neurodegenerative diseases in which we can include for example: Alzheimer's disease, Parkinson's disease, Huntington's disease (HD), amyotrophic lateral sclerosis, and several spinocerebellar ataxias (Liao et al., 2021; Lieberman et al., 2019; Matos et al., 2019; Wilson et al., 2023). Some neurodegenerative diseases can lead to highly debilitating symptoms, leaving patients dependent on others, and ultimately leading to a premature death. To date, there is no cure for these devastating diseases, and people affected rely only on symptomatic and supportive treatment (Klockgether et al., 2019; Matos et al., 2019; Paulson, 2018). Despite extensive efforts to comprehend neurodegenerative diseases, our understanding of the pathological mechanisms that cause these disorders remains incomplete, even though several have been documented for decades. Some of them are known to have a genetic hereditary aetiology, others can arise from environmental factors combined with unknown genetic predispositions. Further studies on the pathogenic factors behind these diseases is crucial for understanding the cellular and molecular mechanisms involved in neurodegenerative diseases. This knowledge is essential for developing novel, personalized, and more effective treatments, which are able to have a meaningful impact on those affected by these debilitating conditions.

1.2 Polyglutamine diseases

General description

Expanded trinucleotide repeat diseases, despite being rare, are among the most common hereditary disorders caused by the expansion of DNA repetition sequences (Paulson, 2018). SCA2 is part of a group of disorders resulting from abnormal expansions of DNA triplet sequences, indicating that this genetic mutation is not exclusive to SCA2.

Six neurodegenerative diseases called spinocerebellar ataxia (SCA) types 1, 2, 3, 6, 7, and 17 are known to be caused by abnormal repeat expansions of the cytosine-adenine-guanine (CAG) trinucleotide within the coding regions of each respective disease-causing gene (**Table I1**). Additionally, three more diseases have been identified, known to be also caused by CAG trinucleotide expansions: spinal and bulbar muscular atrophy, X-linked (SMA1/SBMA), dentatorubral pallidoluysian atrophy (DRPLA), and Huntington's disease (HD). Due to the presence of an abnormal CAG expansion within the coding region, mutant genes will be translated into proteins bearing abnormally long tracts of the glutamine residue. For this reason, all these nine different disorders are categorized under the umbrella of polyglutamine diseases (polyQ) (Estevam et al., 2023; Fan et al., 2014; Lieberman et al., 2019).

PolyQ diseases are different neurodegenerative disorders, each of them with its own affected genes, specific neuronal populations affected, and, in some cases, with unique clinical features. However, they all share a common feature: an abnormally long CAG repetition within their causative genes that translates into a mutant protein bearing abnormal polyQ expansions, which are known to be the primary drivers of these diseases (Estevam et al., 2023; Fan et al., 2014; Klockgether et al., 2019; Lieberman et al., 2019; Paulson, 2018). Although the exact molecular mechanisms by which an abnormal polyQ tract leads to neurodegeneration are not fully understood, it is peculiar to observe that the same protein mutation, across different proteins (that have different functions), results in a similar outcome. For this reason, several researchers generally agree that polyQ diseases share pathological mechanisms.

Table I1 – Polyglutamine diseases features

Polyglutamine diseases

Disease	Gene	Protein	Normal CAG	Pathological CAG	Affected regions	Clinical phenotype
SCA1	<i>ATXN1</i>	Ataxin-1	9-39	41-91	Brainstem; cerebellum; cerebral cortex; dentate nucleus	Ataxia; bulbar palsy; dysarthria; muscular atrophy
SCA2	<i>ATXN2</i>	Ataxin-2	13-31	35-500	Brainstem; cerebellum; cerebral cortex	Ataxia; dysarthria; slow eye saccades; dementia; neuropathy
SCA3	<i>ATXN3</i>	Ataxin-3	11-44	60-87	Basal ganglia; brainstem; cerebellum; spinal cord	Ataxia; bulging eye; dystonia; dysphasia; parkinsonism; spasticity; fasciculations
SCA6	<i>CACNA1A</i>	Calcium voltage-gated channel subunit 1A	4-18	20-33	Cerebellar cortex	Ataxia; dysarthria; oculomotor disorders
SCA7	<i>ATXN7</i>	Ataxin-7	7-27	37-460	Brainstem; cerebellum; retina; visual cortex	Ataxia; dysphagia; dysarthria; retinal degeneration
SCA17	<i>TBP</i>	TATA box-binding protein	25-40	41-66	Cerebellum; cortex; substantia nigra; striatum	Ataxia; cognitive deficits; dystonia; epilepsy; psychiatric dysfunction
HD	<i>HTT</i>	Huntingtin	6-35	40-250	Cerebral cortex; striatum	Chorea; cognitive deficits; dystonia; psychiatric dysfunction
DRPLA	<i>ATN1</i>	Atrophin1	6-35	48-93	Basal ganglia; cerebellum; cerebral cortex; subthalamic nuclei	Ataxia; cognitive deficits; choreoathetosis; dementia; mioclinic epilepsy
SBMA	<i>AR</i>	Androgen receptor	5-34	38-70	Brainstem; spinal cord	Muscular atrophy; bulbar palsy; fasciculations; mild androgen insensitivity

Abbreviation: **SCA** – spinocerebellar ataxia; **HA** – Huntington’s disease; **DRPLA** - dentatorubral-pallidoluyian atrophy; **SBMA** – spinal and bulbar muscular atrophy. Adapted from: (Koppenol, 2024; Paulson et al., 2000; Tenchov et al., 2024)

Common features

While distinct in many aspects, polyQ diseases share several common clinical features and pathophysiological molecular pathways. At the core of these common features is the presence of the CAG expanded repeats. The convergence of features within clinically diverse polyQ diseases offers clues into the shared pathogenic mechanism that, while affecting different proteins, points to the influence of the polyglutamine expansion on cellular function and neurodegeneration across individual diseases (Conceição & Costa et al., 2024; Klockgether et al., 2019; Lieberman et al., 2019).

Genomic instability

A phenomenon common in polyQ diseases is genetic anticipation: successive generations are prone to develop symptoms earlier and more severe. This feature arises from the intrinsic instability of the expanded CAG repeat that tends to increase in length with every generation. This instability is much more pronounced across generations in the case of paternal transmissions (Bettencourt et al., 2008; Giuffrida et al., 1999; Giunti, 1998).

In SCA2, a pattern consistent with other polyQ disorders has been observed. While non-expanded alleles are usually stable during transmission, expanded alleles tend to undergo further expansions, especially when inherited from the father (Giunti, 1998; McMurray, 2010). This difference in instability between maternal and paternal transmissions is thought to relate to the different processes of gametogenesis in males and females. However, the exact mechanisms are not yet fully understood (Giuffrida et al., 1999; Giunti, 1998; McMurray, 2010).

CAG repeats and pathology

Another common feature of polyQ diseases is that the size of the CAG (glutamine) expansion directly correlates with the clinical manifestation of the disease. Several studies indicate that individuals with longer CAG repeat tracts tend to exhibit more severe symptoms and experience an earlier disease onset. The expanded glutamine tract seems to have a graded impact on neuronal dysfunction and degeneration, where larger expansions accelerate the pathogenic cascade. This positive correlation indicates that expanded CAG (and the number of glutamines) repeats are a key player in polyQ diseases. In SCA2, studies show that a higher number of CAG repetitions causes earlier disease onsets with more severe symptoms (Conceição & Costa et al., 2024; Giunti, 1998; Hayes, 2000; Klockgether et al., 2019; Lieberman et al., 2019; Pulst et al., 2005).

Aggregation

The primary hallmark that best characterizes polyQ diseases at the cellular level is the formation of mutant expanded protein aggregates within specific regions of the central nervous system (CNS) (Davies et al., 1997; DiFiglia et al., 1997). Depending on the specific polyQ disease, aggregates can be found in the cell nucleus, cytoplasm, or both. For instance, in HD, SBMA, SCA2, and SCA3, aggregates can be found both in the cell nucleus and cytoplasm. As for the other polyQ diseases (SCA1, 4, and 17; DRLPA), the aggregates are intranuclear. SCA6 displays aggregates exclusively in the cytoplasm (Conceição & Costa et al., 2024; Gatchel and Zoghbi, 2005; Schöls et al., 2004; Todd and Lim, 2013).

Aggregates are usually composed by the disease-causing mutant protein, ubiquitin, and components involved in protein quality control mechanisms. Elements of the ubiquitin-proteasome pathway and molecular chaperones present in the aggregates suggest a cellular response to the aggregation. This phenomenon might indicate that one of the polyQ pathogenic mechanisms is linked to protein aggregation. In this hypothesis, the abnormally expanded polyglutamine tracts initiate protein misfolding events, promoting aggregation. Consistent with this hypothesis, several studies have demonstrated that proteins with expanded polyglutamine sequences have a propensity to aggregate *in vitro* (Conceição & Costa et al., 2024; Jellinger, 2009; Klockgether et al., 2019; Paulson, 2018). Although some evidence might give clues about aggregates and their implications on polyQ diseases, their direct role in pathologic events is still a matter of debate (Huynh et al., 2000; Lieberman et al., 2019; Todd and Lim, 2013).

In SCA2, the mutant expanded ataxin-2 protein is known to be abnormally folded, forming aggregates. These aggregates are usually localized within the cytoplasm of neurons. However, the presence of ubiquitinated intranuclear aggregates of mutant ataxin-2 in pontine neurons of SCA2 patients, was also described. These observations suggest that, at least within a subset of neurons, the mutant ataxin-2 protein is capable of accumulating within the nucleus, where it may interfere with nuclear functions. Interestingly, aggregates present in Purkinje cells, the most affected neuronal population, are confined to the cytoplasm (Koyano et al., 1999; Matilla-Dueñas et al., 2010).

Mechanisms of pathology

The precise mechanisms by which proteins with expanded polyglutamine tracts cause neurodegeneration in polyQ disorders are still not fully understood. Despite extensive research, the whole landscape of pathogenic processes leading from the presence of these mutant proteins to the neurodegeneration is yet to be fully characterized. PolyQ proteins are known to undergo conformational changes and become prone to aggregate, particularly as the length of glutamine tracts increases (Klockgether et al., 2019; Lieberman et al., 2019; Williams and Paulson, 2008). Some studies suggest that the full-length, monomeric form of the pathogenic polyQ protein is responsible for the pathology. Other studies showed that oligomeric intermediates that arise from the aggregation process are responsible for disruptions. Even microaggregates, protofibrils, or truncated fragments of the mutant polyQ proteins might be involved in the pathogenesis of polyQ diseases. It is also possible that polyQ pathology arises not only from one of these entities but from their combined effect (Conceição & Costa et al., 2024; Klockgether et al., 2019; Todd and Lim, 2013; Williams and Paulson, 2008).

Nevertheless, some studies also showed that not only the mutant protein (and its different entities) but also messenger RNA (mRNA) transcripts, by themselves, with expanded CAG tracts, can exert toxicity in neurons. Both in SCA2 and SCA3, it was observed that mRNAs with expanded CAGs tracts induce toxicity. Interestingly, in a *Drosophila melanogaster* model of SCA2, expanded forms of *ATXN2* gene, with continuous CAG repeats, led to toxicity in the retina and neurons, while a form of *ATXN2* gene with CAA (that also codes for glutamines) interruptions within the CAG repeat did not (Stott et al., 1995). Similar findings were observed in SCA3, where mRNA transcripts from the *ATXN3* gene, containing pure CAG repeats, exhibited greater toxicity compared to those with a CAA interruption within the repeat.

Either through the presence of a mutant protein bearing abnormally long tracts of glutamines or through mRNA transcripts with overexpanded CAG repeats, several cellular pathways are known to be disrupted in polyQ diseases. For instance, mitochondrial dysfunction is known to be present in HD, SBMA, SCA1, SCA2, SCA3, SCA6 and SCA7 (Carmo et al., 2018; Cornelius et al., 2017; Leung et al., 2024; Lin et al., 2024; Ranganathan et al., 2009; Stucki et al., 2016; Ward et al., 2019). The dysregulation of cellular degradation systems,

such as the ubiquitin-proteasome system (UPS) or autophagy, was shown to be present in all polyglutamine diseases (Baron et al., 2017; Cortes et al., 2014; Cortes and La Spada, 2015, 2014; Marcelo et al., 2021a; Sathiyaseelan et al., 2019). Transcriptional dysregulation also seem to be affected in all polyQ diseases (Xiang et al., 2018). Calcium signaling was also described to be affected in HD, DRPLA, SBMA, SCA1, SCA2, SCA3, SCA6, and SCA17 (Bezprozvanny and Hayden, 2004; Kasumu and Bezprozvanny, 2012; Marchiorette et al., 2023; Nanclares et al., 2023; Weber et al., 2022). Neuroinflammation, neuronal loss, and defects in neuronal signal transmission are features that, at some point in the progression of polyQ disease pathogenesis, will eventually be present (Bhambri et al., 2020; Conceição & Costa et al., 2024; Cvetanovic and Gray, 2023; Lieberman et al., 2019). The nature of polyQ disease is highly complex, as multiple cell pathways are thought to be affected at the same time. PolyQ diseases are thought to result from the combined disruption of several cellular pathways rather than a single affected mechanism. Although common disrupted mechanisms have been identified across several polyQ disorders, their interplay may differ in each disease, as each polyQ protein exists within a unique cellular context. These differences can result in distinct clinical symptoms and patterns of disease progression among the different polyQ disorders.

Protein context and clues in selective neurodegeneration

All polyQ diseases result from the same type of genetic mutation in different associated genes. As discussed before, they also share several disease mechanisms and affected pathways. However, each disorder's unique characteristics imply that the players that converge in the same pathological pathway are different. While the CAG repeat threshold required for disease onset, the affected CNS regions, age of onset, and clinical symptoms may be similar across certain polyQ diseases, they never overlap entirely. Moreover, apart from the polyQ stretch, all nine polyQ proteins are unrelated and share no other homology (Conceição & Costa et al., 2024; Klockgether et al., 2019; Williams and Paulson, 2008). Additionally, it is well known that each polyQ protein has unique cellular functions and biochemical properties. Therefore, different polyQ proteins contribute in distinct ways to the disease-specific mechanisms, with each distinct pathological outcome being the result of

unique characteristics of each disease-causing protein (Estevam et al., 2023; Fan et al., 2014; Klockgether et al., 2019). This assumption gave rise to the hypothesis concerning the role of protein context: a particular context wherein the protein operates. As such, different proteins have different structures, unique protein-protein interactions and distinct post-translational modification.

Despite being expressed throughout the nervous system, polyQ-expanded proteins primarily induce neurodegeneration in specific brain regions. The selective vulnerability observed in polyQ diseases, where specific neuronal populations are preferentially affected, suggests that intrinsic properties of both the affected cells and the mutated proteins contribute to disease pathogenesis (Adegbiyiro et al., 2017; Moldovean-Cioroianu, 2024). The mechanism behind selective degeneration may result from a complex interplay of multiple factors. At the cellular level, susceptibility within specific neuronal populations can be mostly accounted by cell-intrinsic properties. For instance, neurons with low calcium buffering properties have higher susceptibility to neurodegeneration. This vulnerability can also be exacerbated by high energy demands, in particular those neurons with very extensive morphological features such as large cell bodies and complex axonal arborization (Beers et al., 2001; Foehring et al., 2009; Fu et al., 2018; Ho et al., 1996; Kampmann, 2024; Lewinski and Keller, 2005; Van Den Bosch et al., 2002). The unique demands and characteristics of each specific cell population also influence how cells cope with degeneration. Factors such as cell-specific expression, protein homeostasis, energy metabolism, calcium homeostasis, DNA damage responses, or even axonal transport are thought to impact each cell type differently (Fu et al., 2018; Kampmann, 2024).

Protein homeostasis is a crucial factor in selective degeneration, where the efficacy of both molecular chaperones and the protein degradation machinery, within specific cell populations, significantly influencing neuronal fate. How the cell's machinery can handle protein folding and clearance of aggregated proteins represents one of the key determinants of cellular vulnerability. For instance, it was shown that, depending on the neuronal cell type, polyQ tracts affect protein proteostasis differently (Fu et al., 2018; Kampmann, 2024; Samelson et al., 2023; Scheres et al., 2023; Tsvetkov et al., 2013). These studies suggest that different neuronal cell populations display different turnover rates regarding the polyQ

protein. Notably, cell populations that cleared the mutant protein more slowly died sooner (Fu et al., 2018; Kampmann, 2024; Tsvetkov et al., 2013).

Additionally, differential expression of disease risk-associated genes in selected neuronal populations also accounts for their susceptibility. Neuronal vulnerability largely depends on network context. Whole-genome expression studies and tissue-specific databases have revealed gene networks and pathways associated with vulnerable cell populations, as different cell populations exhibit different expression patterns (Bellenguez et al., 2022; Fu et al., 2018; Kampmann, 2024; J. J. Kim et al., 2024; Van Rheenen et al., 2021).

Taken together, these hypotheses illustrate that selective vulnerability in neurodegenerative diseases is the outcome of the complex interplay between intrinsic cellular properties and broader network dynamics.

The following sections will focus on the polyQ disease SCA2, focusing on its clinical features and current understanding of ataxin-2, as well as the effects of polyQ expansion on its functions.

1.3 Spinocerebellar ataxia type 2

Spinocerebellar ataxia type 2 (SCA2) is a hereditary, autosomal dominant, rare disorder caused by a mutation within the first exon of the *ATXN2* gene. The disease was first described by Wadia and Swami when they published their work describing patients with SCA2 in 1971. Their research began in 1962, focusing on nine Indian families experiencing unusual slow eye movements (slow saccades) along with other typical symptoms of spinocerebellar ataxias. At that time, those set of concomitant symptoms was called Wadia-Swami syndrome (Wadia and Swami, 1971). Several years later in 1989 and 1990, Orozco and colleagues described a high prevalence of similar symptoms in people with Spanish ancestry, in the Cuban province of Holguín (Orozco et al., 1990, 1989).

Epidemiology

SCA2 is considered a rare disorder, meaning that it affects fewer than 1 person per 2000. Nevertheless, SCA2 is the second most common dominantly inherited cerebellar ataxia, affecting 1.5 individuals per 150,000 worldwide (Ana et al., 2024). There are foci of high prevalence in specific regions, such as Cuba (6.57 affected individuals per 100,000 inhabitants), particularly in the Holguín province, where the prevalence of affected people ranges from 40.18 to 141.66 affected individuals per 100,000 inhabitants (Cruz-Mariño et al., 2015; Velázquez-Pérez et al., 2017). All the Cuban areas that show high prevalence rates belong to the eastern part of Cuba, a region where the Spanish explorers first settled during the maritime expansion of the 15th and 16th centuries. It is believed that a founder effect may be responsible for the high prevalence of SCA2 in specific Cuban regions, particularly in the Holguín province. In fact, most of the families affected have Spanish ancestry and share the same genetic haplotype near the SCA2-causative gene. Another hypothesis that might explain the high prevalence observed in Holguín is the occurrence of consanguineous marriage in the past (Magaña et al., 2014; Ragothaman et al., 2004; Velázquez-Pérez et al., 2017, 2009).

Among other inherited SCAs, SCA2 is the one of most common in Mexico where 43% of families with SCAs are affected with SCA2; 31% in Korea and 15-27% in India, Italy and Spain (Antenora et al., 2017; Klockgether et al., 2019).

Clinical features

SCA2 is a neurodegenerative disorder characterized by a myriad of motor symptoms that worsen during the normal course of the disease. Affected people start to experience the first symptoms during the third or fourth decade of life. SCA2 leads to a premature death of those affected within 15 years after the appearance of the first disease signs. According to the severity, the first symptoms may appear later or earlier in life. Extreme cases of early onset have been reported where infants started to manifest SCA2 symptoms at 10 months of age and at 2 years of age. On the opposite side, a case of late onset was also reported where the first symptoms appeared at the age of 86 (Auburger et al., 1990; Babovic-Vuksanovic et al., 1998; Fernandez et al., 2000; Orozco et al., 1990; Wadia and Swami, 1971).

Symptoms

Several motor and non-motor symptoms characterize SCA2 (**Figure I1**). The most common symptom of SCA2 and other SCAs is ataxia, which is characterized by a lack of coordination in the movement. Ataxia can result in uncoordinated gait, balance, or even speech. A distinctive symptom of SCA2 is slow saccades, characterized by difficulty performing horizontal eye movements. In severe cases, SCA2 patients with slow saccades often use head movements to scan texts while reading or to keep on moving objects. Other symptoms include slow reflexes of the upper limbs, dysphagia (difficulties swallowing), dysarthria (speech difficulties), ocular dysmetria, dystonia, and muscle spasticity (Mutesa et al., 2008; Orozco et al., 1989; Wadia and Swami, 1971). Postural tremors can also be sometimes present, and in

certain cases, tremors are also observed when patients attempt to reach for an object with their hands (intention tremors). SCA2 patients also suffer from involuntary muscle spasms characterized by non-controlled cycles of relaxation/contraction (myoclonic jerks) (De Rosa et al., 2006; Lastres-Becker et al., 2009). Reduced muscle tone is another reported symptom, and in some cases, early-stage hyperreflexia is observed, which eventually progresses to areflexia in later stages (Antenora et al., 2017; Orozco et al., 1989; Wadia and Swami, 1971). Like other ataxias, SCA2 patients often experience sleep disturbances, cognitive decline, dementia, and depression. Additionally, behavioral problems caused by frontal lobe neurodegeneration are sometimes observed (Burk et al., 1999; Lo et al., 2016; Velázquez-Pérez et al., 2017, 2011).

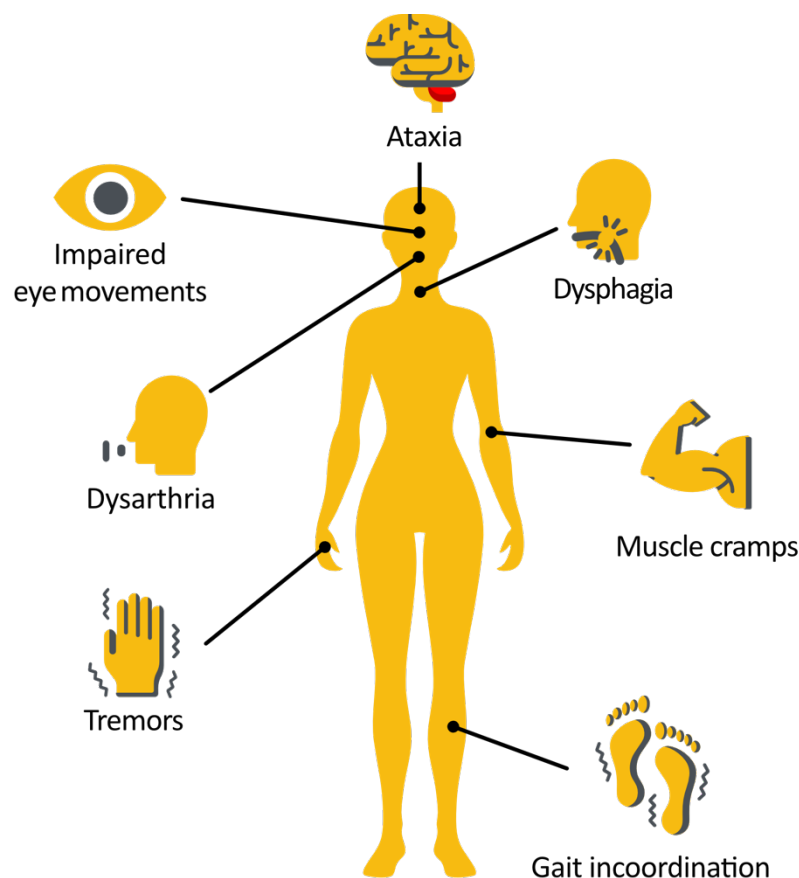


Figure I1 - Common spinocerebellar ataxia type 2 symptoms. Schematic representation of the symptoms commonly identified in patients affected with SCA2. The symptom that best characterizes SCA2 is ataxia. Slow saccades is one of the most distinctive SCA2 symptoms observed in many affected individuals. Symptoms, such as generalized movement incoordination, dysphagia, dysarthria, muscle cramps, and tremors, can also be observed in patients affected with SCA2.

Velázquez and co-workers also reported that, in preclinical stages of the disease, even before ataxia, SCA2 patient seems to develop several signs that include painful muscle

cramps, hypoesthesia (decreased touch sensation), paraesthesia (needle-like prickling skin sensations), hypopallesthesia (decreased vibration sensation), and hyperreflexia (increased reflexes). Other prodromal symptoms include slight slow horizontal saccades, pollakiuria (frequent, abnormal urination during the day), nocturia (frequent, abnormal urination during the night), constipation, and some cognitive dysfunctions. Additionally, brain MRI from non-ataxic SCA2 patients also show a slight cerebellar, pons, and frontal cortex atrophy (Rodríguez-Labrada et al., 2014; Velázquez-Pérez et al., 2014a, 2014b).

Neuropathological findings

Before the term SCA2 was coined, the first description named it “a new form of heredo-familial spinocerebellar degeneration” (Wadia and Swami, 1971). Due to the presence of cerebellar signs, even before any brain imageology or histology studies, it was clear that the symptoms observed emerged from a progressive degeneration of the cerebellum (Wadia and Swami, 1971). *In vivo* imageology confirmed the observations, where it was observed that in SCA2, the primary affected regions are the cerebellum and brainstem. The frontal and temporal lobes also showed some degree of degeneration. *Postmortem* analysis of SCA2 patient brains revealed reduced cerebellum and brainstem weights. These studies also showed an expansion of both cisterna magna and cisterna ambiens (Estrada et al., 1999; Orozco et al., 1989). More detailed observations revealed that cerebellar weight reduction could be explained by neurodegeneration of cerebellar folia and white matter. In fact, the number of cerebellar Purkinje cells is decreased, their arborization is less complex, and the axons are swollen, presenting a torpedo-like shape. Additionally, cerebellar parallel fibers and cerebellar nuclei are also affected by neurodegeneration, as they also are reduced (Estrada et al., 1999; Orozco et al., 1989; Scherzed et al., 2012). The brainstem weight reduction can be explained by a severe neurodegeneration of inferior olives along with a reduction of parallel fibres from the cerebellum-brainstem circuitry (Orozco et al., 1989; Scherzed et al., 2012).

It is also common to observe severe neuronal loss of the dopaminergic pars compacta region within the substantia nigra. This can explain why a few SCA2 patients develop signs of

atypical parkinsonism (Poewe et al., 2017). Nevertheless, not all patients who show severe degeneration of the pars compacta region develop signs of parkinsonism. This may be explained due to an anti-parkinsonian effect caused by neurodegeneration of the motor nuclei within the subthalamic region (also observed in SCA2 patients), inhibiting the parkinsonism tremors. This likely explains why less severe forms of SCA2 phenotypes are more prone to develop atypical parkinsonism: with less degeneration of the motor nuclei, within the subthalamic region, the anti-parkinsonian effect observed in severe cases is lost (Estrada et al., 1999; Park et al., 2015; Poewe et al., 2017; Schöls et al., 2015). SCA2 patients also show spinal cord neurodegeneration along with moderate reduction of the Clarke's nucleus, the motor fibers, and the dorsal ganglia root (Estrada et al., 1999; Hoche et al., 2011; Orozco et al., 1989).

Genetic aetiology

From its earlier descriptions, it was clear that SCA2 was a hereditary disease, meaning that a genetic cause underlies its manifestation. In 1993, Gispert and co-workers were the first to establish a link between SCA2 and its chromosomal *locus*, mapping the gene location within the 12q24.1 chromosome (Gispert et al., 1993; Hernández et al., 1995). A few years later, in 1996, three independent research groups identified and characterized the gene responsible for SCA2. This discovery enabled a more accurate disease diagnosis through genetic screening. Imbert, Pulst, Sanpei, and their respective collaborators determined that SCA2 is caused by a genetic mutation within the coding region of the human gene *ATXN2*, which codes for the mutant protein ataxin-2 (**Figure I2a-b**). Specifically, SCA2-causative alleles are characterized by an abnormal cytosine-guanine-adenine (CAG) trinucleotide expansion within the first exon of the human *ATXN2* gene (Imbert et al., 1996; Pulst et al., 1996; Sahba et al., 1998; Sanpei et al., 1996). In healthy individuals, the *ATXN2* gene presents different numbers of CAG expansions, with 22 being the most frequent repetition of the trinucleotide (Fernandez et al., 2000). On the other hand, CAG expansions above 35 repetitions are associated with SCA2, with the repetition of 37 CAGs being the most common allele associated with the disease.

Nevertheless, rare cases of CAG repeats ranging from 109 to 500 CAGs have been documented and associated with extremely severe and infantile-onset cases of SCA2 (Babovic-Vuksanovic et al., 1998; Mao et al., 2002; Spears et al., 2010; Tojima et al., 2018).

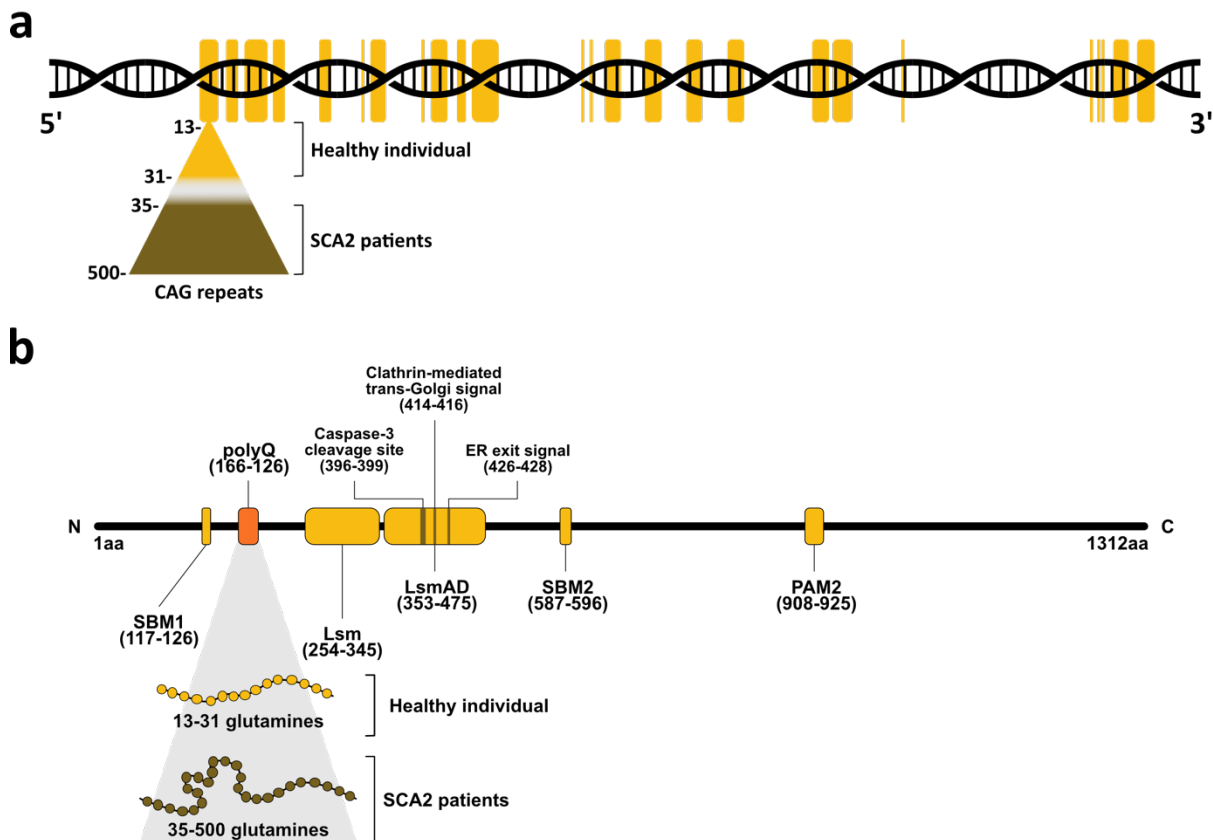


Figure 12 - ATXN2 and respective ataxin-2 protein. Schematic representation of the ataxin-2 gene and ataxin-2 proteins. **a)** Schematic representation of the *ATXN2* gene comprising 25 exons (yellow). Within the first exon, the *ATXN2* gene contains a CAG trinucleotide repetition, which is associated with SCA2. In healthy individuals, the number of CAG repeats ranges from 13 to 31. CAG repetitions ranging between 32-34 are associated with incomplete penetrance, whereas repeats above 35 CAGs are associated with the pathology of SCA2. **b)** Schematic representation of ataxin-2 protein domains. To date, several ataxin-2 domains have been described: polyQ, SBM1, Lsm, LsmAD, and PAM2. Within the LsmAD domain, there is a caspase-3 cleavage site, a clathrin-mediated trans-Golgi signal, and an endoplasmic reticulum exit signal. The mutant expanded form of the *ATXN2* gene with abnormally long CAG repeats gives rise to ataxin-2 protein forms with overexpanded polyQ tracts.

Alleles with intermediate sizes of CAG repeats, ranging between 32 to 34 trinucleotide expansions, have low penetrance or are associated with mild late-onset forms of SCA2. Moreover, individuals carrying intermediate CAG alleles may not exhibit the typical symptoms associated with SCA2. In these cases, when symptoms do appear, they often manifest much later in life. Curiously, a case of a late-onset form of SCA2 with *ATXN2* homozygous alleles

containing 31 trinucleotide CAG repeats was reported. These variations in the genetic manifestation of SCA2 are not yet understood. Still, they highlight the complexity of the disease, emphasizing the importance of understanding how genetic background and protein context may influence disease onset and severity.

The protein encoded by the *ATXN2* gene is present throughout the human body, particularly at high levels in the cerebellum (Human Protein Atlas [HPA] – www.proteinatlas.org). The abnormally long CAG expansion, within the coding region of the *ATXN2* gene, produces a mutant form of ataxin-2 that bears overexpanded tracts of the residue glutamine. While the exact mechanisms remain unclear, the mutant form of ataxin-2 is known to acquire toxic functions, disrupting normal cellular mechanisms and signaling pathways. These disruptions ultimately result in cell death and neurodegeneration, which are responsible for causing the symptoms previously described (Conceição & Costa et al., 2024; Klockgether et al., 2019; Sahba et al., 1998; Sanpei et al., 1996).

Ataxin-2

Ataxin-2 is a multifunctional protein encoded by the *ATXN2* gene, which plays crucial roles in several cellular processes and is implicated in several neurological disorders (Conceição & Costa et al., 2024; Laffita-Mesa et al., 2021). This protein is ubiquitously expressed across the human body, with high expression levels in the nervous system, particularly in the cerebellum, and is known to be involved in several cellular processes, such as RNA metabolism, translation regulation, stress response, and calcium homeostasis (Albrecht et al., 2004; Conceição & Costa et al., 2024; Laffita-Mesa et al., 2021; Ostrowski et al., 2017).

Ataxin-2 molecular features

Ataxin-2 is a large protein, with a molecular weight of 140 kDa, composed of 1312 amino acids (aa) in its full-length form (Imbert et al., 1996, 1996; Sahba et al., 1998). To date, several distinct ataxin-2 domains have been identified or predicted. The N-terminal region of ataxin-2 contains the polyQ tract, which, under normal conditions, consists of 22 glutamine residues (the most common repetition) (Fernandez et al., 2000). Two proline-rich Src Homology 3 (SH3) domain binding motifs – SBM1 and SBM2 – are located at amino acids 117-126 and 587-596, respectively. These motifs might mediate the interaction with SH3 domain-containing proteins, such as endophilins (Nonis et al., 2008). The central region of ataxin-2, from aa 254-475, is an acidic domain that includes two key RNA-binding domains: The Like-Sm (Lsm) domain, aa 254-345, and Like-Sm-associated-domain (LsmAD), from aa 357-475 (Albrecht et al., 2004; Huynh et al., 2000). The Lsm domain contains two important RNA splicing motifs, Sm1 (aa 230-233) and Sm2 (aa 283-286). These motifs are important for mRNA stabilization and regulation of protein abundance (Conceição & Costa et al., 2024; Petrauskas et al., 2024). The LsmAD serves to further enable the RNA interactions with ataxin-2. Within the LsmAD, many functional regions have been identified, including an endoplasmic reticulum (ER) exit signal, aa 426-428, a clathrin-mediated trans-Golgi signal, aa 414-416. Flanked upstream by the Lsm domain and downstream by the LsmAD domain (aa 396-399), ataxin-2 contains a caspase-3 cleavage site, named DDXX (Shibata, 2000). Furthermore, ataxin-2 protein has a poly(A)-binding protein interaction motif 2 (PAM2), located between aa 908-925. This domain allows ataxin-2 to interact with poly(A)-binding protein 1 (PABP1), a protein crucial for the circularization of mRNA during translation (Albrecht et al., 2004; Conceição & Costa et al., 2024; Ostrowski et al., 2017; Petrauskas et al., 2024; Roy et al., 2002). A feature of ataxin-2 structure is the presence of intrinsically disordered regions (IDRs) in its middle (mIDR) and C-terminal (cIDR) domains. These domains confer functional versatility, allowing ataxin-2 to participate in several cellular processes, due to the ability of IDRs to mediate protein-protein interactions, undergo post-translational modifications, and facilitate the formation of higher-order assemblies. Additionally, ataxin-2 also contains arginine-glycine (RG) peptide sequences, which, in other proteins, are known to bind RNA. At its N- and C-terminal, ataxin-2 has a proline-rich sequence, presumed to mediate interactions with protein containing Src homology 3 (SH3) domains (Li et al., 2024; Sahba et al., 1998; Singh et al., 2021).

Regarding ataxin-2 secondary structure, the Lsm and LsmAD domains adopt a similar structure, which is predicted to form five β -sheets packed and an N-terminal α -helix that forms an open barrel-like sheet (Lekontseva et al., 2021). The proline-rich domain is predicted to form a polyproline type II (PPII) helix (Adzhubei et al., 2013). Besides that, the polyQ domain tends to form β -sheets, but typically acquires an α -helix structure (Barbosa Pereira et al., 2023; Conceição & Costa et al., 2024; Nanajkar et al., 2024; Totzeck et al., 2017; Wen et al., 2017).

Ataxin-2 is mainly a cytoplasmic protein associated with the ER and polyribosomes. It can also be found in the Golgi apparatus and within stress granules (SGs) (Conceição & Costa et al., 2024; Nóbrega et al., 2015; Nonhoff et al., 2007; Nonis et al., 2008; Sundberg et al., 2022; Van De Loo et al., 2009). While predominantly cytoplasmic, evidence supports that ataxin-2 also has a nuclear localization, particularly under specific pathologic conditions, such as SCA2 (Huynh et al., 2003, 2000; Nóbrega et al., 2015). Ataxin-2 subcellular localization gives some insight into its biological functions and helps to understand the mechanisms impacted by this protein.

Ataxin-2 functions

Ataxin-2 is a protein involved in several cellular processes that range from circadian rhythm, translation, SGs formation, cellular growth, proliferation, endocytosis, cytoskeletal organization, calcium-mediated signaling or even cellular metabolism. The multifunctional feature of ataxin-2 shows its complexity and importance in maintaining cellular homeostasis.

Circadian rhythm

The circadian rhythm is an endogenous biological process common to all living organisms, from archaea to humans (Doherty and Kay, 2010). The basis of the circadian rhythm consists of gene expression oscillations of circadian clock components, acting like an

internal time-keeping mechanism (Patke et al., 2020). The core of the circadian system comprises a set of clock genes whose rhythmic expression forms the molecular basis of the circadian rhythm. This molecular clockwork is generally given by transcriptional-translational feedback loops (TTFL) that generate self-sustained oscillations at the cellular level. The TTFL mechanism is the most classic and relatively conservative circadian clock oscillation mechanism (Lee et al., 2017; Lim et al., 2011; Walker et al., 2020; Zhang et al., 2013, 2013; Zhuang et al., 2023). This feedback of gene expression is mainly regulated by the complex CLOCK–BMAL1 (Circadian Locomotor Output Cycles Kaput and BMAL1 Brain and Muscle ARNT-Like 1), which lead to transcriptional activation, and by the complex CRY–PER (Cryptochrome and Period), responsible for transcriptional repression, to regulate specific genes and biological function that are under circadian control (Luo et al., 2024; Ray et al., 2020). The CLOCK–BMAL1 complex binds to E-box elements (DNA sequences composed of 5′ – CANNTG – 3′ at the promoter region) present in several genes that are under circadian control. This interaction leads to activation of those specific genes (Ray et al., 2020). Two of those activated genes code for the proteins CRY and PER that, upon accumulation within the cytoplasm, form the complex CRY–PER. The complex translocates back to the nucleus and inhibits the activity of the CLOCK–BMAL1, thus repressing the activation of circadian genes, including CRY and PER (Langmesser et al., 2008; Luo et al., 2024; Ray et al., 2020). The eventual downregulation of CRY and PER proteins allows CLOCK–BMAL1 to start its activity again as a transcriptional activator. This constitutes an oscillating mechanism for activation/repression, which underlies the feedback loop of the circadian machinery, operating in cycles of approximately 24 hours (Langmesser et al., 2008; Luo et al., 2024; Patke et al., 2020; Ray et al., 2020).

Studies have suggested that ataxin-2 is a master regulator of the circadian rhythm by mediating the activity of the CLOCK–BMAL1/CRY–PER transcriptional activity feedback loop. These studies, using *Drosophila* models, showed that the twenty-four (TYF) protein interacts with ataxin-2 to activate the transcription of *per* genes. The LSM12 adaptor protein mediates the interactions between TYF and ATXN2 (in the Lsm/LsmAD domain), facilitating the interaction between TYF, the 5′cap-binding translation initiation factors complex, and PABP. Consequently, the translation of *per* mRNA is activated. Furthermore, data suggests that, in

the absence of ATXN2, TYF protein cannot activate *per* translation alone (Lee et al., 2017; Lim et al., 2011; Zhang et al., 2013; Zhuang et al., 2023).

Additionally, in mouse models, it was shown that ATXN2 and ATXN2L form phase-separated protein condensates that oscillate synchronously with the 24-hour cycle of the circadian rhythm. The condensates recruit ribosomes and both PER2 and CRY (the two major negative regulators in the TTFL) mRNAs to promote their translation. Data suggest that in the absence of ATXN2 and ATXN2L, condensates are no longer formed and translation of PER2 and CRY is downregulated. These disturbances were rescued upon ATXN2 reestablishment. Additionally, the absence of ATXN2 and ATXN2L disrupted the translation cycles of activation/repression and reduced the circadian rhythm (Zhuang et al., 2023).

Another mechanism by which ataxin-2 is proposed to regulate the circadian rhythm is through the formation of the ATXN2-ME31B-NOT1 (maternal expression at 31B and not1) complex. In *Drosophila*, the ME31B-NOT1 complex is known to mediate the silencing of genes known to be important for the circadian rhythm, contributing to the maintenance of high-amplitude behavioral rhythms. It was shown that NOT1-mediated gene silencing is supported by ATXN2 and that ME31B specifically promotes the assembly of the ATXN2-NOT1 complex. Moreover, data suggest that ATXN2 depletion reduces NOT1-mediated gene silencing activity, resulting in decreased amplitude rhythms in circadian behaviors. The effect associated with the gene silencing activity of NOT1 was shown to be independent of *per* activity (Lee et al., 2017).

Altogether, ataxin-2 appears to exert a multifaceted control over circadian rhythm depending on its protein-protein interaction partners. However, much of this function remains incompletely characterized since most studies were performed in *Drosophila* models. Nonetheless, emerging data support an important function for the ataxin-2 protein in mammalian circadian rhythm regulation (Lee et al., 2017; Lim et al., 2011; Zhang et al., 2013; Zhuang et al., 2023).

Calcium signaling

Calcium signaling is a universal, multifunctional cell communication process that regulates several cellular activities, including excitability, exocytosis, motility, apoptosis, and

transcription. This signaling system uses rapid and transient changes in intracellular calcium ions (Ca^{2+}) concentrations, where Ca^{2+} acts as a second messenger to trigger diverse cellular responses. Increase in cytoplasmic Ca^{2+} concentrations is generally either due to the release of Ca^{2+} from internal stores or due to the influx of Ca^{2+} from the extracellular space (Clapham, 2007; Dodd et al., 2010; Luan and Wang, 2021).

Ataxin-2 is suggested to have a role in Ca^{2+} signaling pathways. This role in calcium homeostasis is closely related to its interaction with the regulator of G protein signaling 8 (RGS8) mRNA and the Inositol 1,4,5-trisphosphate receptors (IP3Rs) (Dansithong et al., 2015; Liu et al., 2009). RGS8 is known to inhibit mGluR1-mediated signaling, which affects calcium levels in Purkinje cells. Previous studies suggest that ataxin-2 stabilizes RGS8 mRNA, preventing its degradation, consequently maintaining its protein levels and ability to inhibit mGluR1-mediated signaling (Dansithong et al., 2015).

Another mechanism by which ataxin-2 is suggested to influence calcium signaling is through the IP3R. However, this mechanism seems to be specific to the mutant expanded ataxin-2 and not its wild-type (WT) form (Liu et al., 2009). Thus, this subject will be covered in the following sections.

Cytoskeleton reorganization and endocytosis

The cytoskeleton is a dynamic network of protein filaments (microtubules, actin filaments, and intermediate filaments) extending throughout eukaryotic cells' cytoplasm. The cytoskeleton determines cell shape, organizes cellular contents, supports intracellular transport, supports cell motility, and provides mechanical stability. The cytoskeleton also plays an important role in cell division, signal transduction, and anchorage of cellular organelles (Fletcher and Mullins, 2010; Hohmann and Dehghani, 2019).

Additionally, the cytoskeleton is involved in the endocytic process as cells undergo cytoskeletal remodeling during endocytosis. Endocytosis is the cellular process by which cells internalize substances from the extracellular environment by invagination of the plasma

membrane, to form vesicles containing the ingested material, such as nutrients, receptors, membrane protein regulators, and cellular signaling molecules (Doherty and McMahon, 2009; Rennick et al., 2021; Sigismund et al., 2021).

Ataxin-2 has emerged as a central player in endocytosis and cytoskeleton reorganization, interacting with key components of the endocytic machinery. Indeed, several crucial binding partners have been identified for this protein, including endophilins A1 and A3, the E3 ubiquitin-ligase Cbl, the adaptor protein CIN85, and the protein kinase Src (Costa et al., 2024; Drost et al., 2013; Nonis et al., 2008; Ralser et al., 2005; Yang et al., 2018). Endophilins play an essential role in the assembly of endocytic vesicles and stimulate the internalization of several components from the extracellular space. They have also been shown to be associated with ataxin-2 (Doherty and McMahon, 2009; Rennick et al., 2021; Sigismund et al., 2021). This association is mediated between the SH3 domain of endophilins and the proline-rich domain of ataxin-2 (Nonis et al., 2008; Ralser et al., 2005b). It was shown that ataxin-2 regulates the internalization of the epidermal growth factor receptor (EGFR). Upon activation of EGFR, endophilins A1 and A3 combine in a complex with Cbl, CIN85, and Src to internalize the receptor. Ataxin-2 expression delays the internalization of EGFR, while its downregulation speeds the internalization of EGFR. These observations support the hypothesis that ataxin-2 acts as an endocytosis modifier (Nonis et al., 2008).

Ataxin-2 also might play an important role in the cytoskeletal remodeling during the dendritic spine morphogenesis. Studies showed that endophilin 1 regulates dendritic spine formation through the interaction with p140cap, a protein involved in cytoskeleton reorganization, facilitating actin polymerization. This mechanism has significant importance for synaptic plasticity and neuronal function, influencing dendritic morphogenesis, or even the formation and dynamics of dendritic spines, both crucial for the formation and maintenance of spine structure and synapses (Yang et al., 2015; Y. Yang et al., 2018). Considering that and the role of ataxin-2 in the cytoskeleton organization through the action of endophilins A1 and A3, there is also the possibility that ataxin-2 also plays a role in the remodeling of the cytoskeleton during spine formation via endophilin A1 interaction.

Although it was not empirically demonstrated, it is possible that ataxin-2 also plays a role in the ubiquitin-mediated activation of endocytosis. This process is mediated by the E3 ubiquitin-ligase Parkin. Parkin can ubiquitinate players of the endocytic machinery, such as

endophilins and their interacting partners, dynamin and synaptojanin-1 (Cao et al., 2014). Additionally, the growth factor receptor substrate 15, another protein that plays a role during endocytosis, was shown to be ubiquitinated by Parkin (Fallon et al., 2006). Notably, a physical interaction between ataxin-2 and Parkin has been reported (Huynh et al., 2007). Therefore, one can hypothesize a role of ataxin-2 in the ubiquitin-mediated activation of endocytosis through Parkin.

RNA metabolism and translation

RNA metabolism encompasses all processes involved in the life cycle of RNA molecules, such as their production, structural folding and unfolding, chemical modifications, processing, and eventual degradation. For instance, one mechanism of RNA metabolism is the splicing, an event by which non-coding regions (introns) from a precursor mRNA (pre-mRNA) are removed, and the coding sequences (exons) are joined together to create the mature mRNA. The RNA metabolism culminates in mRNA translation, a process by which the information coded in mature mRNA molecules is used to synthesize proteins.

Ataxin-2 is a known RNA-binding protein (RBP) that participates in several aspects of RNA metabolism. Besides direct interaction with RNA, ataxin-2 is also involved in RNA processing events through its interaction with splicing proteins (Conceição & Costa et al., 2024; Fittschen et al., 2015). These proteins include splicing regulators such as RNA Binding Fox-1 Homolog 1 (HRNBP1, also known as A2BP1), RNA-binding motif protein 9 (RBM9), and the RNA-binding protein with multiple splicing (RBPMS) (Conceição & Costa et al., 2024; Huttlin et al., 2021; Shibata, 2000). Notably, the putative role of ataxin-2 in splicing events carries a spatial inconsistency: splicing of RNA mainly happens in the nucleus, while the main location of the protein ataxin-2 is in the cytoplasm. This suggests that the regulatory putative role of ataxin-2 might occur by sequestering splicing factors in the cytoplasm and controlling their nuclear availability (Marasco and Kornblihtt, 2023; Neuwald and Koonin, 1997; Wilkinson et al., 2020).

Ataxin-2 is also thought to regulate protein translation. The Lsm, LsmAD, and PAM2 domains of ataxin-2 have been shown to interact with polyribosomes localized in the rough endoplasmic reticulum (RER) independently (Van De Loo et al., 2009). Furthermore, ataxin-2 is known to interact with PABP1. This interaction has been shown to negatively regulate mRNA translation, possibly by destabilizing the translation initiation complex (Conceição & Costa et al., 2024; Nóbrega et al., 2015; Satterfield and Pallanck, 2006). Conversely, through an Lsm domain-mediated interaction, ataxin-2 can stabilize mRNAs and upregulate protein levels by binding to uridine-rich elements within the 3' UTRs of specific mRNAs (Yokoshi et al., 2014).

Additionally, ataxin-2 has been shown to modulate mRNA-poly(A) tail length. Although less clear in the case of mammalian ataxin-2, studies in yeast models suggest that the ataxin-2 homolog – Pbp1 – cooperates with Pab-1 (PABP-1 homolog) to inhibit the activity of the deadenylation complex poly(A) nuclease. By inhibiting deadenylation, Pbp1 (ataxin-2 homolog) may promote translation since longer poly(A) tails are associated with increased translation efficiency (Mangus et al., 2004, 2003; Tuong Vi et al., 2021; Wakiyama et al., 2000; Yang et al., 2019).

Lastly, ataxin-2 might also have a role in regulating miRNA. Using *Drosophila* models, Atx2 (fly homolog of ATXN2) in cooperation with dFMR1 (*Drosophila* Fragile X messenger ribonucleoprotein 1 homolog) and other proteins involved in the miRNA pathway, such as Ago1 and Me31B (*Drosophila* homolog DEAD-Box Helicase 6), seem to be involved in long-term olfactory habituation. The role of Atx2 in the miRNA pathway is further supported by experiments showing that Atx2 knockdown increased the expression of a translational reporter carrying the 3'UTR of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) in olfactory neurons (Bellenguez et al., 2022; Sudhakaran et al., 2014). Additionally, it seems that protein synthesis-dependent long-term habituation, a crucial process in *Drosophila* olfactory memory, is modulated by Atx2, and this modulation is suggested to be dependent on SGs formation (Bakthavachalu et al., 2018).

Altogether, ataxin-2 seems to be a key regulator in gene expression, either by splicing regulation, impacting miRNA pathways or translation regulation. Nevertheless, some of these studies were conducted in non-mammal models, representing an important limitation when drawing conclusion to the human context.

Cellular metabolism and cellular growth

Cellular metabolism is broadly defined as a complex network of biochemical processes that occur in cells to maintain life. It includes the biochemical processes of energy production, synthesis of cellular components, and elimination of waste products (Kapnick et al., 2024). The mammalian target of rapamycin (mTOR) is the main player of cellular metabolism, growth, and survival, integrating various environmental signals. It senses nutrient availability to modulate essential cellular processes such as protein synthesis, autophagy, and cell proliferation (Saxton and Sabatini, 2017; Simcox and Lamming, 2022). In the presence of nutrients, the mTOR complex-1 (mTORC1) directly phosphorylates serine/threonine protein kinase (SCH9) and the autophagy-related protein 13 (ATG13), thereby driving cellular growth and inhibiting autophagy, respectively (Foltman and Sanchez-Diaz, 2023).

It is speculated that ataxin-2 plays an important role in cellular metabolism, impacting the mTORC1 pathway. Using yeast models, it was shown that Pbp1 (ataxin-2 homolog) can function as a modulator of the mTOR pathway. In fact, overexpression of Pbp1 reduces the phosphorylation of mTORC1 downstream targets, especially Sch9 and Atg13. It was shown that Pbp1 exerts this effect by sequestering mTORC1 to stress granules upon heat-shock stress. These findings suggest that Pbp1 induces SGs formation in heat-shock stress situations, which in turn results in the sequestration of mTORC1 into SGs, inhibiting cellular growth. This mechanism was shown to play a key role in protecting cells from DNA damage that typically occurs during heat-shock stress situations (Takahara and Maeda, 2012). Additionally, under low-nutrient conditions, the AMP-regulated kinase (AMPK, Snf1 [Sucrose Non-fermenting protein 1] in yeast) directly phosphorylates and activates Psk1 (PASK [PAS domain containing serine/threonine kinase] homolog), which in turn phosphorylates Pbp1. This phosphorylation cascade results in the sequestration of mTORC1 into SGs, which might result in overall inhibition of cell growth due to the absence of mTORC1 downstream signal (DeMille et al., 2015). This mechanism seems to propose a role of ataxin-2 in hampering the anabolic activity of cells under low-nutrient-induced stress. Additionally, using *Caenorhabditis elegans*, it was suggested that ATX-2 (ataxin-2 homolog) inhibits Ras homolog enriched in brain (RHEB) protein, an activator of mTORC1. Consequently, as RHEB is inactivated, mTORC1 downstream signal is inhibited, resulting in decreased cellular growth (Bar et al., 2016).

The mTORC1 pathway is also known to regulate dendritic morphogenesis and spine growth (Bowling and Klann, 2014; Kumar et al., 2005; Tang et al., 2014). Considering the role of ataxin-2 homologs within the mTORC1 pathway, one might speculate that ataxin-2 might be involved in processes of dendritic morphogenesis and spine formation and dynamics, both crucial for maintaining neuronal function.

Altogether, ataxin-2 might regulate metabolism through the inactivation of the mTORC1 pathway, consequently leading to cell growth inhibition. Conversely, the absence of ataxin-2 might lead to cell growth activation (Bar et al., 2016; Takahara and Maeda, 2012). Converging with this notion is the fact that ataxin-2 is suspected to influence food intake and body weight, working as a nutritional and energy sensor (Carmo-Silva et al., 2017). Supporting this hypothesis, studies have shown that Atxn2-KO (knock-out) mice exhibit hepatic steatosis and abdominal obesity, along with insulin resistance. Additionally, it was demonstrated that hypothalamic-specific overexpression of ataxin-2 prevented obesity and insulin resistance caused by a high-fat diet. Moreover, restoring ATXN2 expression in Atxn2-KO mice seems to ameliorate metabolic dysfunction (Carmo-Silva et al., 2023, 2017; Lastres-Becker et al., 2008; Meierhofer et al., 2016).

Stress granules

Stress granules (SGs) are dynamic, membraneless cytoplasmic entities formed within cells as a response to a wide range of environmental stresses. They are composed of non-translated poly(A)-mRNA, RBPs, and factors from the translation initiation machinery (Bussi et al., 2023; Marcelo et al., 2021b; Nunes et al., 2019; Protter and Parker, 2016). SGs formation is a rapid cellular response to stress stimuli such as oxidative stress, heat shock, viral infections, or nutrient deprivation. SGs formation is initiated by the phosphorylation of eukaryotic initiation factor-2 α (eIF2 α), which leads to a global translational arrest (Anderson and Kedersha, 2002; Kedersha et al., 1999). Untranslated mRNAs then accumulate and interact with specific RNA-binding proteins to form SGs. The primary function of SGs is to protect cells under cellular stress by sequestering mRNAs and translation factors in order to save cellular

energy and resources during adverse conditions (Anderson and Kedersha, 2002; Marcelo et al., 2021b). This mechanism of storing mRNAs during stress allows the rapid reactivation of translation upon insult relief, hence aiding cellular survival and recovery (Anderson and Kedersha, 2002; Kedersha et al., 1999; Marcelo et al., 2021b; Protter and Parker, 2016). The specific mechanism of SGs biogenesis, their functions, as well as their implications in neurodegenerative diseases, will be discussed in detail in the following sections. This segment will focus on the specific role of ataxin-2 in regulating SGs dynamics.

The role of ataxin-2 in SGs has been investigated for several years. In fact, ataxin-2 was shown to be one of the RBPs present in SGs (Nonhoff et al., 2007; Ralser et al., 2005a). However, using cellular models, it was suggested that cells lacking ataxin-2 retain their ability to form SGs in the presence of cellular stress (Kaehler et al., 2012; Lastres-Becker et al., 2016). Additionally, also in cell models, a study also suggested that ataxin-2 overexpression does not induce the formation of SGs (Nonhoff et al., 2007).

Conversely, the first evidence suggesting that ataxin-2 may play a role in SGs dynamics came from its identification as a component of these structures (Nonhoff et al., 2007; Ralser et al., 2005a). Additionally, it was also observed that ataxin-2 interacts with SGs' key components, such as PABP1 and DEAD/H-Box RNA Helicase DDX6 (Nonhoff et al., 2007; Ralser et al., 2005a). From these studies, it is possible to hypothesize a role for ataxin-2 in SGs.

Using *Drosophila* cells, it was shown that Atx2 (ataxin-2 homolog) co-localizes in RNA-granules composed of proteins well known to be components of SGs (Me31B/DDX6, FMR1, Rox8, and Caprin) (Bakthavachalu et al., 2018; Farny et al., 2009). Additionally, the overexpression of Atx2 seems to promote the formation of RNA-granules. Moreover, the formation of these granules appears to be driven by the intrinsically disordered regions (IDRs) domains of atx2. It was shown that mIDR deletions have no apparent effect on the formation of the granules, but cIDR deletion decreases their formation, size, and morphology. Furthermore, the double deletion of mIDR and cIDR, almost completely abolished the ability of cells to form SGs (Bakthavachalu et al., 2018).

As discussed above, some studies state that ataxin-2 is not necessary for SGs to assemble, but other studies have opposite results. It seems that ataxin-2 downregulation leads to a significant decrease in both the number and size of SGs (Nonhoff et al., 2007).

Additionally, using yeast models, strains lacking Pbp1 (ataxin-2 homolog) were not able to form SGs upon stress stimuli (Buchan et al., 2008). Using mammal cells, the same result was observed – downregulation of ataxin-2 impairs SGs formation (Kaehler et al., 2012).

The contradictory results on the role of ataxin-2 regarding SGs formation might, in part, be explained by the presence of ataxin-2-like. Since both proteins are components of SGs and share multiple interactors, ataxin-2-like might have a redundant role and compensate for the disturbance in ataxin-2 (Kaehler et al., 2012; Lastres-Becker et al., 2016; Nonhoff et al., 2007; Ralser et al., 2005a). Convergent with this hypothesis is the fact that ataxin-2-like interacts with known partners of ataxin-2, involved in SGs dynamics, among them PABP, G3BP1, and DDXX6. Additionally, ataxin-2-like expression levels seem to modulate SGs dynamics. Using cellular models, it was shown that overexpression of ataxin-2-like leads to the formation of SGs, and its downregulation impairs SGs assembly (Kaehler et al., 2012; Nonhoff et al., 2007). Another hypothesis that can account for the contradictory results is the fact that distinct sources of cellular stress produce SGs granules with different components, which might slightly impact how SGs are formed (Aulas et al., 2017; Reineke and Neilson, 2019). Moreover, it was shown that depending on the source of stress and cellular type SGs composition is different. While the canonical pathways for SGs formation converge in the phosphorylation of eIF2 α , the transcriptomic and proteomic profile of each cell type, along with the stress stimuli, results in different players contributing to SGs formation and composition (Markmiller et al., 2018).

Ataxin-2 post-translational modifications

Post-translational modifications (PTMs) are a set of reversible and irreversible modifications that proteins undergo after their biosynthesis. To date, more than 650 types of translational modifications have been identified. These include the addition of small molecules to the amino acid chain, such as methyl groups, a sugar, ubiquitin, and phosphate, which alter the properties of proteins (Zhong et al., 2023). PTMs can also result in structural changes, which include proteolytic cleavage, where the protein backbone is cut. Overall, PTMs

are an essential mechanism by which proteins can alter their function, structure, or interactors in order to regulate and fulfill diverse molecular purposes (Rogers and Overall, 2013; Zhong et al., 2023). The PTMs are not very well studied in the context of ataxin-2. Nevertheless, some studies have already found evidence for their impact on the ataxin-2 functions.

Phosphorylation

Phosphorylation is a reversible post-translational modification characterized by adding a phosphate group to a specific protein's aa. This process is catalyzed by protein kinases that transfer a phosphate group from the adenosine triphosphate (ATP) and add it to particular aa – normally a serine, a tyrosine, or a threonine – on target proteins. Phosphorylation can alter proteins' subcellular localization, stability, and enzymatic activity. Phosphorylation has been linked to functions related to cell growth, apoptosis, DNA repair, and protein degradation (Ardito et al., 2017; Zhong et al., 2023).

Ataxin-2 comprises 47 regions composed of the aa serine or threonine followed by the aa proline (S/T)P. These sites are known to be preferential regions for the cyclin-dependent kinase 5 (Cdk5)-mediated phosphorylation (Asada et al., 2014; Dhavan and Tsai, 2001). In fact, Cdk5 has been found to phosphorylate ataxin-2, both N-terminal and in the central region of the protein, leading to its proteasomal degradation. This regulatory mechanism is crucial in controlling ataxin-2 abundance, especially in post-mitotic neurons, where Cdk5 exhibits its primary activity (Asada et al., 2014; Dhavan and Tsai, 2001).

Ubiquitination

Ubiquitination is a reversible PTM, characterized by the covalent binding of ubiquitin (Ub) to the target protein. Ubiquitin is a ubiquitous protein of 76 aa. Ubiquitination is mediated by a cascade process that involves ubiquitin-activating enzymes (E1), ubiquitin-

conjugating enzymes (E2), and ubiquitin ligases (E3), which ultimately link the Ub to a lysine aa of target proteins (Damgaard, 2021). Lysine residues can undergo mono-ubiquitination, where a single Ub is bound, or poly-ubiquitination, where multiple Ub molecules are linked end-to-end. Like phosphorylation, ubiquitination can regulate target proteins' subcellular localization, stability, or even enzymatic activity. Ubiquitination has been shown to have functions related to apoptosis, immune responses, DNA repair, and protein degradation (Ardito et al., 2017; Damgaard, 2021).

Parkin, a known E3 protein linked to Parkinson's disease, was shown to interact and to ubiquitinate ataxin-2. This process occurs at the N-terminal region of ataxin-2, targeting the protein for degradation via the ubiquitin-proteasome system (UPS) (Huynh et al., 2007). Parkin activity is regulated by the Cdk5 protein, which is also implicated in the degradation of ataxin-2 (Asada et al., 2014). However, it still remains unclear whether Cdk5-mediated regulation of Parkin is connected to ataxin-2 ubiquitination and degradation (Avraham et al., 2007; Huynh et al., 2007).

Proteolytic cleavage

Proteolytic cleavage is the process by which protein backbones are cleaved by the hydrolysis of peptide bonds. This process is considered to be an irreversible post-translational modification that can change the subcellular localization of proteins, their structure, interactions, and function (Klein et al., 2018).

Ataxin-2 contains a putative caspase-3 cleavage site at the LsmD domain, which leads to the formation of a 41.2 kDa fragment. In fact, two ataxin-2 fragments are detected within human cerebellar *post-mortem* samples of SCA2 patients – an N-terminal 42 kDa fragment and a C-terminal 70 kDa fragment. These observations suggest that ataxin-2 undergoes proteolytic cleavage as a result of caspase-3 activity (Huynh et al., 2000).

The 42 kDa fragment has been shown to react with the polyQ-specific 1C2 antibody, while the 70 kDa fragment was only detected by the ataxin-2 antibody, indicating that the

polyQ expansion is associated only with the smaller fragment (Huynh et al., 2000). Additionally, a study identified five different fragments of ataxin-2 – one corresponding to the 70 kDa and four minor others, corresponding to smaller fragments (Turnbull et al., 2004). While it was observed that the 70 kDa fragment is present both in healthy and SCA2 patients, the 42 kDa portion was enriched in the pathologic context (Huynh et al., 2000). Moreover, studies using cellular models showed that the presence of fragments, compatible with the molecular weight of 42 kDa, were only observed in cells transfected with a mutant polyQ-expanded form of ataxin-2. However, non-transfected cells and cells expressing non-expanded forms of ataxin-2 formed almost no fragments (Huynh et al., 2000).

These studies support the hypothesis that ataxin-2 cleavage is somehow contributing to SCA2 pathology, as it seems to be the case in other polyQ diseases. The formation of polyQ-containing fragments is exclusive of the disease context, as they are not found in healthy brains, and it seems that the mutant expansion within ataxin-2 drive the formation of these fragments (A. Matos et al., 2017; El-Daher et al., 2015; Haacke et al., 2006; Young et al., 2007). However, in transfected COS-1 cells, the expression of a polyQ-containing N-terminal truncated form of expanded ataxin-2 did not increase cytotoxicity as the number of glutamines increased, in contrast to the full-length ataxin-2, which progressively became more toxic as the polyQ expansions increased. In fact, with the same size expansion, truncated forms of ataxin-2 appeared to be less toxic than full-length forms (Ng et al., 2007). Strikingly, truncated forms of polyQ expanded ataxin-2 remain diffused throughout the cytoplasm without forming inclusions.

Overall, it seems that the formation of expanded polyQ-containing fragments does not directly exacerbate pathological mechanisms associated with the mutant ataxin-2, either by means of protein aggregation or cellular toxicity. Nevertheless, it is possible that the formation of ataxin-2 fragments, exclusively associated with the polyQ expansion, might affect its biological functions and molecular partners (A. Matos et al., 2017; Conceição & Costa et al., 2024; Huynh et al., 2000; Ng et al., 2007). These observations reinforce the idea that the mechanisms by which the mutant ataxin-2 with elongated polyQ expansions cause cellular toxicity are highly complex and likely arise from an intricate multifactorial context. In the following sections, we will explore some of the pathological mechanisms associated with the

polyQ expansion and how this abnormally long domain contributes to the cytotoxicity associated with the mutant protein ataxin-2.

PolyQ expansions, mutant ataxin-2 protein, and pathogenesis

Polyglutamine regions are one of the most frequent homopeptide repeats found in eukaryotes. Glutamine repeats are generally codified by CAG trinucleotides (also CAA) and can be found in at least 4800 different proteins. Interestingly, only nine of all predicted polyQ-containing proteins are associated with a pathological phenotype, which is less than 0.2% of proteins with glutamine repeats (Albà and Guigó, 2004; Faux et al., 2005; Lobanov and Galzitskaya, 2012; Mier et al., 2020; Mier and Andrade-Navarro, 2021).

The specific function of polyQ domains is yet to be determined. Still, due to their unstructured conformation, with propensity to form random coils, polyQ regions are thought to facilitate protein-protein interactions within macromolecular complexes (Almeida et al., 2013; Barbosa Pereira et al., 2023; Faux et al., 2005). PolyQ sequences exhibit high structural flexibility, enabling them to create versatile docking sites, either by promoting the approximation of distant domains within the protein itself or through direct interaction with the polyQ region, facilitating the contact with other molecules (Almeida et al., 2013; Huntley and Golding, 2002; Simon and Hancock, 2009). However, when engaging with different partners, such as nucleic acid and/or proteins, the polyQ unstructured domain may transition from random coils to adopt α -helices or extended loops, depending on: the specific interaction, flanking domains, or glutamine size (Barbosa Pereira et al., 2023; Faux et al., 2005; Totzeck et al., 2017). This structural flexibility is thought to provide a wider versatility to proteins-containing polyQ domains, allowing them to perform functions that require the assembly of large multiprotein or multiprotein/nucleic acid complexes where numerous protein interactions are needed. Such is the example of the polyQ protein ataxin-2, which is associated with molecular complexes in roles related to the circadian rhythm, RNA splicing, and protein translation (Conceição & Costa et al., 2024; Lee et al., 2017; Magaña et al., 2013; Neuwald and Koonin, 1997; Zhuang et al., 2023).

The fact that not all polyQ-containing proteins cause disease, along with the variability in polyQ length thresholds associated with each disorder, underscores the need to look at protein regions beyond the polyQ domain and to consider the role of context-specific neurodegeneration when investigating polyQ diseases.

Nevertheless, it is undeniable that, in polyQ diseases, abnormally long polyQ tracts are the driving force that leads to pathology (Estevam et al., 2023; Klockgether et al., 2019). Although the pathophysiology of abnormal polyQ expansions is not yet fully uncovered, growing evidence links these structures to several pathologic mechanisms.

Aggregation

Protein aggregation is the pathological process by which misfolded or mutant proteins accumulate within cells to form macromolecular entities called aggregates (Merlini et al., 2001). More than 30 different proteins linked to neurodegenerative disorders are known for their propensity to form intracellular aggregates under pathological conditions. The gradual accumulation of proteins is, in fact, regarded as a hallmark of many neurodegenerative diseases, which, among others, include Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), frontotemporal dementia, prion diseases, and polyQ diseases (Estevam et al., 2023; Fan et al., 2014; Klockgether et al., 2019; Wilson et al., 2023).

Proteins bearing abnormally long polyQ sequences undergo several structural changes, making them prone to misfold and aggregate. Overexpanded polyQ tracts can force proteins to predominantly transition towards β -sheet-rich structures, which are prone to form amyloid-like fibrils that consequently aggregate and accumulate in neurons as inclusions (Estevam et al., 2023; Fan et al., 2014; Todd and Lim, 2013; Wilson et al., 2023). The role of these aggregates and their implications in polyQ diseases remains controversial. While some studies depict aggregates as cytoprotective entities, others define them as toxic hubs. Current thoughts suggest that aggregates are formed to sequester disease-producing proteins and prevent them from carrying toxic functions. In fact, even before the formation of large macromolecular inclusions, misfolded monomers and small oligomers are thought to be the

actual toxic species causing cytotoxicity (Schaffar et al., 2004; Takahashi et al., 2010; Todd and Lim, 2013).

The presence of aggregates within neurons from the most affected brain regions of several polyQ diseases has prompted the speculation that macromolecular inclusions may directly contribute to cellular toxicity (Estevam et al., 2023; Fan et al., 2014; Klockgether et al., 2019). PolyQ-containing inclusions have been shown to sequester proteins other than the mutant protein itself. These sequestered proteins include, for instance, transcription factors, affecting gene transcription and contributing to pathogenesis (Chai et al., 2002; McCampbell, 2000; Yamanaka et al., 2008). It also includes the sequestration of UPS components, molecular chaperones, and ubiquitin-related proteins, disrupting protein homeostasis (Cummings et al., 1998; Jana et al., 2000; H. Yang et al., 2018; Yue et al., 2021). Additionally, polyQ-containing inclusions aberrantly interact with the ER, altering its membranes and dynamics (Bäuerlein et al., 2017; Van De Loo et al., 2009). Moreover, polyQ inclusions can also accumulate within axons, impairing axonal transport (Lee et al., 2004; Seidel et al., 2010; Wijegunawardana et al., 2025).

Overall, inclusions containing abnormally long polyQ disease-associated proteins are involved in cellular mechanisms that are known to be impaired. However, whether they are the primary drivers of polyQ pathogenicity remains unclear. It is possible that the role of aggregates may lie somewhere in between, as being protective and toxic (Todd and Lim, 2013; Wilson et al., 2023). There is the hypothesis that during the initial phases of polyQ pathogenesis, the sequestration of polyQ mutant proteins into inclusions aids in mitigating the deleterious effects of these proteins (Schaffar et al., 2004; Takahashi et al., 2010; Todd and Lim, 2013). However, in later stages, cells are unable to manage aggregation build-up, and inclusions start to abnormally sequester functional proteins and abnormally interact with cellular components (Chai et al., 2002; Cummings et al., 1998; Jana et al., 2000; McCampbell, 2000; Park et al., 2015; Yamanaka et al., 2008; H. Yang et al., 2018). At this point, it is likely that polyQ protein monomers, oligomers, and aggregates collectively exacerbate polyQ-associated pathophysiology.

Aggregation in spinocerebellar ataxia type 2

PolyQ-containing inclusions, marked by the accumulation of the disease-associated overexpanded protein, are a hallmark of all polyQ diseases (Conceição & Costa et al., 2024; Estevam et al., 2023). SCA2 disease is no exception, and abnormally long ataxin-2 undergoes aberrant structural changes that ultimately lead to the formation of intracellular inclusions (Huynh et al., 1999; Koyano et al., 1999). In SCA2 patients, the intracellular distribution of mutant ataxin-2 macromolecular aggregates is not restricted to a specific cellular compartment. While these inclusions are exclusively cytoplasmic in Purkinje cells of the cerebellum, they can also be intranuclear in the neurons of the pontine nuclei (Huynh et al., 1999; Koyano et al., 1999).

The mechanisms by which abnormally long tracts of glutamines lead to mutant ataxin-2 aggregation are not fully understood. For instance, the aa sequences flanking the polyQ expansion seem to affect the rate of ataxin-2 aggregation. Notably, studies have shown that, for the same polyQ expansion size, the aggregation rates differ depending on whether the polyQ tract is fused to the flanking polyQ regions of ataxin-2, ataxin-3, huntingtin, or atrophin proteins. Additionally, the substitutions of a few hydrophobic residues in the upstream flanking region of the ataxin-2 polyQ tract into charged hydrophilic aa dramatically decrease the aggregation rate. These observations not only support the hypothesis that flanking regions significantly impact the aggregation of ataxin-2, but also suggest that charged hydrophilic aa might increase protein solubility in the context of pathologic polyQ expansion (Nozaki et al., 2001). Moreover, charged hydrophobic aa (proline, lysine, arginine, glutamate, and aspartate), which are less prone to aggregate and display a high affinity to chaperones, are enriched upstream of the polyQ sequences, which are commonly prone to aggregate. The enrichment of these sequences near aggregation-prone regions could reflect an evolutionary pressure to minimize pathologic protein aggregation (Rousseau et al., 2006).

Expanded ataxin-2-positive cytoplasmic inclusions have been shown to sequester a variety of proteins involved in diverse cellular pathways (**Table I2**). Inclusions of mutant ataxin-2 were shown to sequester the regulatory-associated protein of mTOR (Raptor), known to be involved in the mTORC1 signaling (Liu et al., 2024; H. Yang et al., 2018). Studies also showed that cytoplasmic inclusions of mutant ataxin-2 are able to sequester ubiquitin protein ligases,

including the parkin ring E3 ubiquitin-protein ligase (PARK2) and the f-box/WD repeat domain containing 8 (FBXW8), both involved in the UPS pathway and degradation of ataxin-2 (Halbach et al., 2015). Additionally, the poly(A) binding protein cytoplasmic 1 (PABPC1) protein involved in mRNA translation is also sequestered into expanded ataxin-2-positive cytoplasmic inclusions (Damrath et al., 2012). The staufen double-stranded RNA binding protein 1 (STAU1), a protein involved in RNA metabolism, was shown to be recruited to inclusions of mutant ataxin-2 (Paul et al., 2018). Moreover, the TAR DNA-binding protein 43 (TDP-43) protein, which, among other functions, is involved with mechanisms of mRNA and a major player associated with hereditary and sporadic ALS, was shown to be sequestered by mutant ataxin-2 cytoplasmic inclusions (Wijegunawardana et al., 2025). Interestingly, and although only present within SCA2 neurons of the pontine nuclei, intranuclear inclusions positive for ataxin-2 were shown to recruit ataxin-1, ataxin-3, and TATA box binding protein (TBP). Since all these proteins are related to DNA transcription, it can be hypothesized that these intranuclear inclusions can interfere with gene expression (Estevam et al., 2023; Takahashi et al., 2010; Uchihara et al., 2001).

Table I2 – Proteins know to be sequestered into mutant ataxin-2 positive inclusions

Protein	Related pathway	Associated diseases
RAPTOR	mTOR signaling	Metabolic disorders
PARK2	UPS	Parkinson's disease
FBXW8	UPS	Cancer
PABPC1	RNA metabolism	ALS; ALS-FTD; Cancer
STAU1	RNA metabolism	ALS; ALS-FTD
TDP-43	RNA metabolism	ALS; ALS-FTD
Ataxin-1	Transcription	SCA1
Ataxin-3	Transcription	SCA3
TBP	Transcription	SCA17

Abbreviation: **SCA** – spinocerebellar ataxia; **UPS** – ubiquitin proteasome system; **ALS** – amyotrophic lateral sclerosis; **FTD** – frontotemporal dementia (Damrath et al., 2012; Estevam et al., 2023; Halbach et al., 2015; H. Yang et al., 2018; Liu et al., 2024; Uchihara et al., 2001; Takahashi et al., 2010; Wijegunawardana et al., 2025).

In the context of SCA2, pathways associated with all the proteins mentioned above were shown to be dysregulated. This strongly supports the idea that inclusions of mutant ataxin-2 play an important role in SCA2-associated pathophysiology (Damrath et al., 2012; Halbach et al., 2015; Liu et al., 2024; H. Yang et al., 2018). Nevertheless, other cellular

pathways, not directly associated with the mutant protein inclusions, were also described to be dysregulated in the context of the abnormally expanded ataxin-2 (Conceição & Costa et al., 2024; Estevam et al., 2023; Laffita-Mesa et al., 2021). In the following sections, several dysregulated cellular pathways are addressed to provide insight into how the mutant ataxin-2 affects normal cellular functions.

Autophagy impairment

Autophagy is a highly conserved physical process of lysosomal degradation that allows cells to turnover intracellular components. In this process, cells recycle proteins, organelles, or even parts of the cytoplasm, transforming them into nutrients and maintaining energy homeostasis, including in situations of cellular stress (Jimenez-Sanchez et al., 2012; Kroemer et al., 2010). Autophagy was shown to be a highly crucial process involved in the clearance of aggregates containing polyQ proteins (Jimenez-Sanchez et al., 2012; Paulino and Nóbrega, 2023).

Under normal physiological conditions, misfolded proteins are degraded by the UPS. Nevertheless, proteins bearing abnormally long sequences of glutamines seem to clog this system, ultimately hindering its capacity to degrade not only these mutant polyQ proteins but also other proteins (Ravikumar et al., 2002; Venkatraman et al., 2004). In such conditions, when the UPS fails, autophagy seems to be responsible for the degradation of proteins bearing abnormal tracts of glutamines, both in their soluble and aggregated forms (Ravikumar et al., 2002; Venkatraman et al., 2004).

Autophagy impairment seems to be a hallmark of polyQ diseases, as they were observed in HD, SBMA, SCA1, SCA2, SCA3, SCA7, SCA17, and possibly DRPLA (Cortes and La Spada, 2014; Marcelo et al., 2021a; Nisoli et al., 2010). Regarding SCA2, the degradation of proteins by the autophagic pathway seems to be impaired as proteins involved in the autophagic process are accumulated in the presence of mutant ataxin-2 (Paul et al., 2018). Autophagy impairments in SCA2-derived fibroblasts are evidenced by the accumulation of proteins involved in the autophagic process, including the light chain 3 isoform II (LC3-II),

which is essential for autophagosome maturation, as well as sequestosome 1 (SQSTM1/p62), which recognizes ubiquitinated proteins and targets them for autophagic degradation (Katsuragi et al., 2015; Lee and Lee, 2016; Paul et al., 2018). The difficulty in turnover of both LC3-II and SQSTM1/p62 strongly indicates defects in autophagy, suggesting this likely occurs in SCA2 (Ashkenazi et al., 2017; Paul et al., 2018). Additionally, previous work developed by our lab demonstrated that both in SCA2 mouse models and *post-mortem* brain tissue from SCA2 patients, LC3-II and SQSTM1/p62 expression levels are increased, further supporting the hypothesis that autophagy is impaired in SCA2 (Marcelo et al., 2021a).

To maintain their function, neurons rely on autophagy to prevent toxic proteins and damaged organelles from accumulating within cells. Loss-of-function studies demonstrated that autophagy suppression leads to neurodegeneration even in healthy contexts (Hara et al., 2006; Komatsu et al., 2006).

Considering that the UPS system is unable to efficiently degrade proteins with abnormal polyQ expansions, and that autophagy is also impaired in the context of expanded ataxin-2, it seems inevitable that the mechanisms of cellular clearance are compromised in the context of SCA2. With time, misfolded proteins and degraded organelles build up to a point that cells can no longer support and eventually die. In this panorama, the accumulation of non-degraded components in cells can be seen as a mechanism through which polyQ-expanded ataxin-2 exacerbates its cytotoxicity by severely compromising cellular proteostasis.

RNA-induced toxicity

RNA-mediated toxicity occurs when RNA molecules acquire cellular toxic function either by mechanisms of bidirectional transcription, formation of RNA foci or production of RNA species containing abnormally long repetitive sequences (Belzil et al., 2013; Sicot and Gomes-Pereira, 2013). RNA-induced toxicity is now a widely accepted mechanism of cytotoxicity commonly occurring in multiple neurodegenerative diseases, with particular relevance in the expansion repeat disorders (Conceição & Costa et al., 2024; Fiszler and Krzyzosiak, 2013; Swinnen et al., 2020).

In the context of PolyQ disorders, RNAs containing normal-size CAG repeat sequences undergo 3D structural changes, forming semi-stable hairpins. However, when the CAG repeats reach a certain threshold, formed hairpins become exceedingly stable, making it difficult to revert the acquired conformation (Fiszer and Krzyzosiak, 2013; Jasinska et al., 2003). These highly stable CAG-containing hairpins are believed to aberrantly interact with RBPs, preventing them from performing their normal functions and consequently contributing to cellular toxicity (Conceição & Costa et al., 2024; Fiszer and Krzyzosiak, 2013). RNA-mediated toxicity was observed in HD, SCA1, SCA2, and SCA7, but is likely to occur in most polyQ disorders. The exception could be SCA17, as studies showed that RNA-induced toxicity is not a feature. Studies suggest that the nucleotide sequences flanking mRNA CAG repeats modulate the toxic properties of these expanded RNAs. This indicates that RNA toxicity is not solely determined by the CAG expansion itself, but is also influenced by the specific surrounding sequences (Busan and Weeks, 2013, 2013; Jasinska et al., 2003; Nalavade et al., 2013; Nestor and Monckton, 2011). In fact, it was shown that particular nucleotide sequences, flanking the CAG repeats of mRNAs, can modulate the hairpin architecture/stability of polyQ protein transcripts from atrophin-1, ataxin-1, ataxin-3, and calcium voltage-gated channel subunit alpha1 A (CACNA1A). Moreover, the length of the CAG sequences is able to alter how hairpins are formed and how they are metabolized, affecting their biology (Jasinska et al., 2003; Michlewski and Krzyzosiak, 2004; Sobczak and Krzyzosiak, 2004).

Two different RNA-induced toxicity mechanisms are described to be associated with SCA2 pathology: the bidirectional transcription of the *ATNX2* gene and the formation of RNA foci enriched with ataxin-2 transcripts with abnormally long CAG tracts (Li et al., 2021, 2016; McGurk et al., 2021; Skariah and Albin, 2021). Evidence also suggests that ataxin-2-mediated toxicity depends not only on the length of the polyQ region but also on the specific RNA sequence that encodes for the glutamines. Studies using *Drosophila* models have shown that cytotoxicity is triggered only when the expanded form of ataxin-2 protein contains polyQ sequences exclusively encoded by the trinucleotide repeat CAG. Strikingly, when polyQ sequences within the ataxin-2 were encoded by CAA or CAA/G trinucleotide repeats (both also encoding for the glutamine aa), no toxic effect was detected, even though the polyQ sequence was abnormally elongated (McGurk et al., 2021).

Studies have shown that the *ATXN2* gene undergoes bidirectional transcription. This means that *ATXN2* transcription occurs on both the sense and antisense DNA strands. The sense transcriptions give rise to the *ATXN2* mRNA transcript, whereas the antisense transcriptions give rise to the antisense transcript termed *ATXN2-AS* (Li et al., 2016). *ATXN2-AS* transcripts, of the overexpanded *ATXN2* gene, display overexpanded CUG sequences known to further stabilize RNA hairpins (Li et al., 2016; Tian et al., 2000). These are only found in patient-derived fibroblasts and *post-mortem* cerebella from SCA2 patients, and not in healthy individuals. The *ATXN2-AS* transcript was also found in healthy individuals, but does not display abnormally elongated CUG sequences (Li et al., 2016). The polyQ-expanded *ATXN2-AS* transcripts containing abnormally long CUG sequences were shown to trigger cytotoxicity. This likely occurs due to the formation of CUG-rich *ATXN2-AS* transcript foci, able of sequestering the muscleblind-like splicing regulator 1 (MBNL1), a key protein involved in RNA splicing (Ho et al., 2004; Li et al., 2016; Masuda et al., 2012). It was shown that MBNL1 sequestration into CUG-rich transcript foci results in widespread RNA splicing abnormalities and cell degeneration (Miller et al., 2000). This mechanism aligns with the observations that amyloid-beta precursor protein (APP) and the N-methyl-d-aspartate receptor (NMDAR1) undergo abnormal splicing in SCA2, similar to what is observed in Alzheimer's disease (Hynd et al., 2001, 2001; Li et al., 2016). Further studies found that expanded *ATXN2-AS* transcripts abnormally interact with the transducing Beta-Like 3 (TBL3), a protein involved in the processing of ribosomal RNA (rRNA), consequently disrupting rRNA maturation, a crucial process for proper ribosomal assembly and protein synthesis, without which neurons would degenerate (Conceição & Costa et al., 2024; Li et al., 2021).

Overall, these studies provide evidence that SCA2 pathology arises from factors beyond the expanded amino acid sequence. Instead, it results from an intricate interplay of various pathophysiological mechanisms that appear to be the combined result of RNA-protein-mediated toxicity.

Enhanced oxidative stress and mitochondrial dysfunction

Oxidative stress is characterized by an imbalance between the accumulation of oxygen-reactive species (ROS; oxidants) and the ability to neutralize them with antioxidants

(Preiser, 2012; Valko et al., 2007). Enhanced oxidative stress is a feature of many neurodegenerative diseases, including polyQ diseases (Chen et al., 2019, 2012; Cornelius et al., 2017; Harmuth et al., 2022; Leung et al., 2024; Liu et al., 2022; Miyata et al., 2008; Ranganathan et al., 2009; Tichanek et al., 2020). Although often seen as harmful, in conditions of normal cell physiology, the controlled production of ROS plays an essential role in cell biology, including controlling cellular growth and division, regulating the immune system activity, and programmed cell death (apoptosis) (Preiser, 2012; Valko et al., 2007). However, when there is an excessive imbalance, the abnormal accumulation of ROS can aberrantly alter cellular structures such as lipids, proteins, and organelles (Chen et al., 2012). One of those affected organelles is mitochondria. They are the primary source of ROS, yet mitochondria are particularly sensitive to abnormal variations in ROS concentration (Palma et al., 2024).

To prevent abnormal ROS production caused by the accumulation of damaged mitochondria, the phosphatase and tensin homolog-induced kinase 1 (PINK1) phosphorylates several mitochondrial proteins, initiating mitophagy-mediated elimination of these dysfunctional organelles (Collier et al., 2023). The first pieces of evidence connecting mitochondrial dysfunction with SCA2 were obtained through deep transcriptome analysis of SCA2 patients' blood, revealing upregulated levels of PINK1 transcript. On the contrary, in an *ATXN2*-knockout mouse model, the levels of PINK1 were shown to be downregulated (Sen et al., 2016). Nevertheless, both studies suggest that *ATXN2* expression might modulate PINK1 levels. Increased levels of PINK1 should enhance mitochondrial quality control by clearing damaged mitochondria, consequently lowering ROS accumulation (Collier et al., 2023). Thus, the increased levels of PINK1 detected in patients might not be contributing directly to SCA2 pathology. However, Purkinje cells have high energy demands, which require a high number of functional mitochondria. In fact, Purkinje cells require more ATP than any other cerebellar cell subtype (Howarth et al., 2012). In this context, it is possible that PINK1 overexpression, observed in SCA2, "over-targets" mitochondria to degradation, collapsing the energetic balance of Purkinje cells.

Additionally, several studies suggested a direct link between SCA2-associated pathology and enhanced oxidative stress. SCA2 patient-derived fibroblasts have increased levels of superoxide compared to healthy controls, suggesting higher levels of mitochondrial oxidative stress. Moreover, the proteins and mRNA levels of the antioxidants manganese

superoxide dismutase (SOD2) and enzymatic catalyze (CAT) were dysregulated, clearly suggesting that cellular responses to oxidative stress are compromised (Cornelius et al., 2017). Stinkingly, these cells also displayed altered mitochondrial morphology, decreased enzymatic activity, and alterations in mitochondrial DNA transcription (Cornelius et al., 2017).

All these findings support that mitochondrial and oxidative stress imbalances are molecular mechanisms underlying SCA2 pathogenesis. The mitochondrial dysfunction, the putative dual role of PINK1, and the presence of dysregulated antioxidants in SCA2 only highlight the complexity of the pathological mechanisms surrounding this disease. Adding another layer of intricacy is the fact that mitochondria play a key role in calcium regulation through the uptake and buffering of Ca^{2+} (Choo et al., 2004; Sanz-Blasco et al., 2008). Mitochondrial dysfunction was shown, in other diseases, to provoke abnormal balances in concentrations of intracellular Ca^{2+} – a process well known to be highly toxic for neurons (Abeti and Abramov, 2015; Clapham, 2007; Marambaud et al., 2009).

Calcium dysregulation

Calcium homeostasis is the mechanism that allows cells to regulate the concentration of Ca^{2+} ions across the extracellular space, cytoplasm, and intracellular stores. As described in previous sections, Ca^{2+} is an important second messenger in cellular signaling, with critical roles in virtually every aspect of cell function (Clapham, 2007). For instance, Ca^{2+} has one of the most basal roles in neurons – synaptic transmission – the basis of communication between these neurons (Marambaud et al., 2009). The release of neurotransmitters from synaptic vesicles is not possible without Ca^{2+} (Südhof, 2012).

Calcium dysregulation is a feature observed across many neurodegenerative diseases, including polyQ disorders. These disruptions often result from abnormal cell mechanisms such as altered buffer capacity, dysfunctional channel activities, or excitotoxicity, typically leading to pathological alterations in Ca^{2+} concentrations within neurons (Marambaud et al., 2009). One of the most common mechanisms driving abnormal increases in intracellular calcium concentrations is the release of Ca^{2+} from the ER due to the overactivation of the IP3R (Kasumu

et al., 2012; Tada et al., 2016). Altered mitochondrial integrity also contributes to abnormal increases in intracellular calcium, as mitochondria actively buffer intracellular Ca^{2+} , for instance, upon signal transmission. Conversely, excessive Ca^{2+} uptake also induces abnormal production of ROS (Abeti and Abramov, 2015; Choo et al., 2004; Sanz-Blasco et al., 2008). Additionally, the proper function of calcium-binding and calcium-buffering proteins is essential to maintain balanced levels of intracellular Ca^{2+} (Clapham, 2007; Marambaud et al., 2009).

In SCA2, evidence suggests that several affected mechanisms have the ability to dysregulate calcium balance, further exacerbating pathology. The first evidence connecting SCA2 with excitotoxicity came from studies that showed a direct binding between ataxin-2 and ATXN2-binding protein 1 (A2BP1) – an RNA-splicing regulator that directly guarantees the presence of the exon 5 within the glutamate ionotropic receptor NMDA type subunit 1 (GluN1) transcript (Gehman et al., 2011; Shibata, 2000). GluN1 exon 5 ensures faster receptor deactivation, limiting Ca^{2+} intracellular influx. It was shown that, in SCA2 context, mutant ataxin-2 continues to interact with A2BP1, which might impair A2BP1 functions or directly sequester it to mutant polyQ-containing aggregates (Shibata, 2000). In either case, this disruption can prevent exon 5 from being included in the GluN1 transcripts, which leads to the formation of N-methyl D-aspartate (NMDA) receptors with slower deactivation, prolonging calcium influx to the cytosol and causing excitotoxicity (Lee et al., 2009). Under physiological conditions, upon neuronal depolarization, A2BP1 translocates into the nucleus, forcing GluN1 transcripts to include exon 5. This feedback works as a break against excitotoxicity by desensitizing the receptor (Lee et al., 2009). Hypothetically, the absence of a proper A2BP1 function, due to an aberrant interaction with the mutant ataxin-2, leads to excitotoxicity, as the inclusion of the GluN1 exon 5 is compromised.

Later, another study also supported excitotoxicity as a feature of SCA2 (Dansithong et al., 2015). The study showed that in SCA2, excitotoxicity results from an overactivation of the metabotropic glutamate receptor 1 (mGluN1) signaling pathway. Under physiological conditions, ataxin-2 is known to directly bind to the regulator of G protein signaling 8 (RGS8) transcripts to regulate their expression. RGS8 is almost exclusively expressed in cerebellar Purkinje cells, where it is responsible for the deactivation of the mGluR1 signaling by accelerating the conversion of GTP into GDP on its associated G protein (Itoh et al., 2001;

Larminie et al., 2004; Saitoh et al., 2002). However, the expanded ataxin-2 binds less efficiently to RGS8 transcripts, impairing their translation (Dansithong et al., 2015). Lower levels of RGS8 are likely to fail proper mGluR1 deactivation, prolonging receptor activity. As a downstream effect, IP3R is overactivated, which leads to excessive release of Ca^{2+} , into the cytosol, from internal calcium storage (Itoh et al., 2001; Larminie et al., 2004; Lee and Lee, 2016; Tada et al., 2016).

Multiple studies suggested that the GluR1/IP3R pathway is disrupted in SCA2 (Bezprozvanny and Hayden, 2004; Dansithong et al., 2015; Kasumu et al., 2012; Meera et al., 2017; Shibata, 2000). It is now known that in Purkinje cells, expanded ataxin-2, but not the normal form, binds to the IP3R, exacerbating its activity and enhancing Ca^{2+} released from the ER. Studies in SCA2 mouse models showed that increases in intracellular Ca^{2+} concentrations upon neuronal activation result in neurodegeneration and motor deficits. Conversely, blocking IP3R-mediated Ca^{2+} release mitigates neurodegeneration and improves mice's motor performance (Meera et al., 2017). Additionally, transcriptome analysis of SCA2 mouse models revealed a dysregulation in the expression of genes that encode for Ca^{2+} channels, Ca^{2+} transporters, and other elements from the mGluR/IP3R pathway, clearly suggesting severe disruptions in calcium homeostasis (Arsović et al., 2020). A noteworthy observation is that an abnormal increase in intracellular Ca^{2+} drives normal ataxin-2 to be displaced into SGs (Arsović et al., 2020). This could mean that under excessive Ca^{2+} increase, aberrant sequestration of WT ataxin-2 into SGs could exacerbate SCA2 pathology by depleting the normal protein of its functions.

The unique morphology and intrinsic characteristics of Purkinje cells make them especially vulnerable to calcium fluctuations. Their dendritic arborization, along with high expression levels of voltage-gated calcium channels, mGluR1 receptors, IP3Rs, and abundant intracellular calcium stores, enables Purkinje cells to efficiently communicate with the surrounding neurons (Gruol et al., 2012; L. H. Kim et al., 2024). While these features allow rapid input/output processing power and maintain Purkinje cells in a constant state of readiness for an accurate and adaptive motor coordination, these characteristics also make Purkinje cells highly susceptible to Ca^{2+} variations (Fernández Santoro et al., 2024; L. H. Kim et al., 2024; Payne et al., 2019; Popa et al., 2019). Considering that multiple mechanisms associated with Ca^{2+} are disrupted in SCA2 and given the high expression levels of ataxin-2 in

Purkinje cells, it is reasonable to position calcium dysregulation as a major contributor to SCA2 pathogenesis.

Alterations in stress granules dynamics

As described previously, SGs are organelles without membranes that form in response to situations of cellular stress. The direct role of ataxin-2 on SG dynamics is still not fully understood (Marcelo et al., 2021b). However, growing evidence position mutant ataxin-2 as a strong candidate able to disrupt SGs assembly/disassembly dynamics, either due to its presence within these entities or via abnormal structural changes that affect their dynamics (Nonhoff et al., 2007; Ralser et al., 2005a). Of the 312 estimated protein interactions involving ataxin-2, 200 (64.1%) are with proteins composing SGs. This accounts for 31.9% of the 627 proteins described to be present in SGs, highlighting the deleterious potential of the mutant ataxin-2 to disrupt these structures (Nunes et al., 2019; Oughtred et al., 2021; Stark et al., 2006).

In an indirect way, studies suggested that expanded forms of ataxin-2 can lead to the formation of persistent SGs and/or even lower the stress threshold by which they are able to form (Damrath et al., 2012; Huelsmeier et al., 2021; Paul et al., 2018; Petrauskas et al., 2024; Yamagishi et al., 2024). One of these mechanisms appears to occur through the upregulation of staufen double-stranded RNA binding protein 1 (STAU1). This protein is a known interactor of ataxin-2 within SGs. Studies have shown that mutant ataxin-2, but not the normal form, upregulates STAU1 (Paul et al., 2018). This correlates to the fact that STAU1 overexpression is able to induce SGs formation and simultaneously hinder autophagic degradation, a mechanism implicated in SGs clearance after stress release (Paul et al., 2021). Consistent with these observations is the fact that mutant ataxin-2 was shown to increase the time by which SGs disassemble upon stress release, possibly due to hindered autophagic clearance (Vieira de Sá et al., 2024). Altogether, these studies support a mechanism by which mutant ataxin-2 abnormally induces SGs or prevents their disassembling – upregulating STAU1 expression.

Another study proposed that mutant ataxin-2 can displace the content of SGs, affecting their composition. FUS and TDP-43, well-known SGs markers, were abnormally dispersed throughout the cytoplasm, upon their displacement from SGs-like mRNP granules, following mutant ataxin-2 expression (Nihei et al., 2012). Whether these specific displacements result in altered SGs dynamics or disrupt SGs functions is not known. However, it shows that mutant ataxin-2 has the ability to interfere with SGs components. Nevertheless, displacement of molecules involved in pathways that regulate SGs dynamics might, in fact, result in abnormal SGs formation. Through the combined action with the protein DExD/H-box helicase (DDX) 6 protein, studies have suggested that ataxin-2 might disrupt SGs dynamics (Buchan et al., 2008; Decker and Parker, 2012; Li et al., 2013; Nonhoff et al., 2007; Rao and Parker, 2017). It was proposed that DDX6 works as an RNA chaperone, restricting RNA-RNA interaction to prevent liquid-liquid phase separations caused by spatially confined accumulations of RNA, thus limiting the formation of SGs (Kim et al., 2022; Rajkowitsch et al., 2007; Ripin et al., 2024). In fact, it was shown that mutant ataxin-2 is able to displace DDX6 protein from its usual subcellular localization (Nonhoff et al., 2007). Taking in consideration studies showing that Lsm domain directly binds to DDX6, abnormal polyQ expansions likely result in ataxin-2 structural or affinity changes that might affect this interaction, ultimately leading to aberrant SGs formation (Lee et al., 2017; Majerciak et al., 2023; Nonhoff et al., 2007). In this sense, it is possible that the mutant ataxin-2 is affecting DDX6 functions of limiting SGs assembly. Either by decreasing its spatial availability to chaperone RNAs or by preventing it from interacting with RNAs. In both cases, eventually resulting in enhanced SGs formation. Another hypothesis is the possibility of the mutant ataxin-2 sequesters DDX6 into aggregates, impairing its activity and ability to suppress SGs nucleation. These ideas are consistent with the effects observed upon DDX6 depletion, which result in enhanced SGs formation (Ripin et al., 2024). Since DDX6 shares great structural similarities with the other DDXs, such as DDX1, DDX2A, DDX2B, DDX3, DDX6, DDX9, DDX15, and DDX17 – all of these known to interact with ataxin-2 and being described to be involved in responses of cellular stress – it is plausible to envision an analogous mechanism where the expanded polyQ proteins affects SGs homeostasis. Studies have shown that DDX3 and DDX1 mediate SGs formation, and its disruption affects SG dynamics. However, a role for ataxin-2 in this mechanism is mere speculation based only on the facts that these two proteins can alter SGs formation, are SGs components, and interact with ataxin-2 (Cui et al., 2020; Luo et al., 2023;

Valentin-Vega et al., 2016). Notably, as far as we could acknowledge, only DDX15 and DDX5 were not described as being found in SGs (Li et al., 2022; Nunes et al., 2019; Oughtred et al., 2021; Pattabhi et al., 2019; Powers et al., 2024; Shih and Lee, 2014; Valentin-Vega et al., 2016; Powers et al., 2024; Xu et al., 2022; Zhou et al., 2024; Tauber et al., 2020; Jongjitwimol et al., 2016). Nevertheless, the latter is known to interact with caprin-1, a key facilitator of SGs assembly (Solomon et al., 2007; Song et al., 2022; Vu et al., 2021).

Another mechanism by which ataxin-2 may drive aberrant formation of SGs is by pathologically condensing SGs-related components (Damrath et al., 2012; Petrauskas et al., 2024; Singh et al., 2021; Yamagishi et al., 2024). As mentioned in previous sections, ataxin-2 protein contains IDR regions, without which SGs do not assemble. In fact, IDRs and low-complexity regions, such as polyQ, are thought to be pivotal for SGs nucleation. These structures are found in most SGs nucleators and are necessary to trigger liquid-liquid phase separations in order to assemble SGs (Kedersha et al., 2013; Liu et al., 2023; Marcelo et al., 2021b; Protter et al., 2018; Wang et al., 2021). PolyQ regions, besides being low complexity structures, share multiple similarities with IDRs: structural flexibility, promiscuous binding to multiple partners via conformational adaptability, high glutamine content, and aggregation propensity (Mier and Andrade-Navarro, 2021; Totzeck et al., 2017; Trivedi and Nagarajaram, 2022; Wear et al., 2015). Moreover, polyQ sequences are prone to engage with proteins that contain IDRs, including SGs nucleators. An example is the fact that some SGs nucleators can usually be found in neuropathological protein aggregates of polyQ diseases (Marcelo et al., 2021b; Wolozin and Ivanov, 2019). In line with this, there is the hypothesis that ataxin-2 is able to pathologically induce/stabilize SGs by spatially condensing (liquid-liquid separation) their nucleators or components (Gandelman et al., 2020; Paul et al., 2021; Pulst et al., 2023; Vieira de Sá et al., 2024). In fact, it was shown that mutant ataxin-2 can sequester the protein PABPC1 into insoluble condensates, and its sole accumulation when bound to poly(A)-mRNAs leads to the formation of SGs (Damrath et al., 2012; Yamagishi et al., 2024). Note that one of the first steps of SGs formation starts with the accumulation of untranslated poly(A)-mRNAs (Anderson and Kedersha, 2002; Marcelo et al., 2021b). Additional work showed that compromising the interaction between ataxin-2 and PABPC1, either by expressing an ataxin-2 lacking the PAM motif or by mutating it, prevents SGs formation (Petrauskas et al., 2024;

Yamagishi et al., 2024). Together, these results suggest a role for the mutant ataxin-2 as a potentiator of SGs formation/stability by abnormally sequestering untranslated RNAs.

Gathering insights from different works also allows us to hypothesize whether the mutant ataxin-2 might be impacting SGs formation by dysregulating messenger RNA protein (mRNP) granules. These physiological granules, which are formed under normal stress-free conditions, serve as translationally “paused” storage composed of translation machinery and translationally stalled mRNAs ready to be translated. Some of the elements that compose mRNPs overlap with the SGs content, including proteins that aid in nucleating SGs and untranslated poly-(A) mRNA. In fact, the interplay between both structures has already been suggested (Adjibade and Mazroui, 2023; Buchan, 2014; Dalla Costa et al., 2021). Given these, one might hypothesize whether, in abnormal situations, the mRNP granule could “degenerate” and coalesce into SGs. This link can be established through an overlapping involvement of ataxin-2 in both SGs formation and mRNP biology (Lastres-Becker et al., 2016; Petrauskas et al., 2024; Singh et al., 2021; Yamagishi et al., 2024). Under physiological conditions, the ataxin-2 PAM motif binds to PABPC1-bound to poly-(A) mRNAs, while the Lsm domain interacts with translation machinery to regulate protein synthesis and prevent mRNP aggregation. Under specific translational needs (e.g., distant protein synthesis), ataxin-2 undergoes structural changes, exposing its IDRs regions and disconnecting the Lsm motif from the translational machinery, resulting in mRNP granule formation (**Figure I3**). Translation is stalled, and untranslated poly-(A) mRNAs accumulate (Ciosk et al., 2004; Lee et al., 2017; Petrauskas et al., 2024; Satterfield and Pallanck, 2006; Singh et al., 2021; Yamagishi et al., 2024). This process is closely related to SGs assembly, as in stress-induced translation arrest, one can observe the interaction of ataxin-2 with accumulated PABPC1-bound to poly-(A) mRNAs providing scaffold for SGs assembly (Anderson and Kedersha, 2002; Buchan, 2014; Protter and Parker, 2016; Singh et al., 2015). Studies have shown that not only is the ataxin-2 PAM-PABPC1 interaction needed to assemble SGs, but the absence of its Lsm domain also drives the formation of SGs (Petrauskas et al., 2024; Singh et al., 2021; Yamagishi et al., 2024). In these studies, under stress-induced conditions, the expression of ataxin-2 lacking the Lsm domain dramatically promotes SGs formation, whereas expressing an ataxin-2 with an additional Lsm resulted in the formation of many granules significantly smaller than typical SGs (Singh et al., 2021). Under physiological conditions, the Lsm domain normally promotes

disassembly of mRNP granules to aid translation. However, in induced cellular stress, ataxin-2 having two Lsm domains likely potentiate the disassembly of SGs, creating those small SGs-like structures. All considered, expanded polyQ sequences may structurally alter the ability of ataxin-2 Lsm domain to interact with the translation machinery, promoting abnormal granule formation, either by exposing its IDRs or by engaging in abnormal interactions at the sites of protein synthesis, such as the mRNPs. Since these sites are rich in poly-(A) mRNA and some SGs nucleators, the promiscuity of polyQ expanded sequences might lead to their sequestration, abnormally increasing their spatial concentration and laying the ground to start the nucleation of SGs. There is also the possibility that the abnormal mRNP accumulation *per se* could “degenerate” into SGs by spatially accumulating some of the SGs components. All together, it seems possible to mechanistically connect the ataxin-2 polyQ expansion with abnormal SGs formation (Buchan, 2014; Marcelo et al., 2021b; Petrauskas et al., 2024; Singh et al., 2021, 2015).

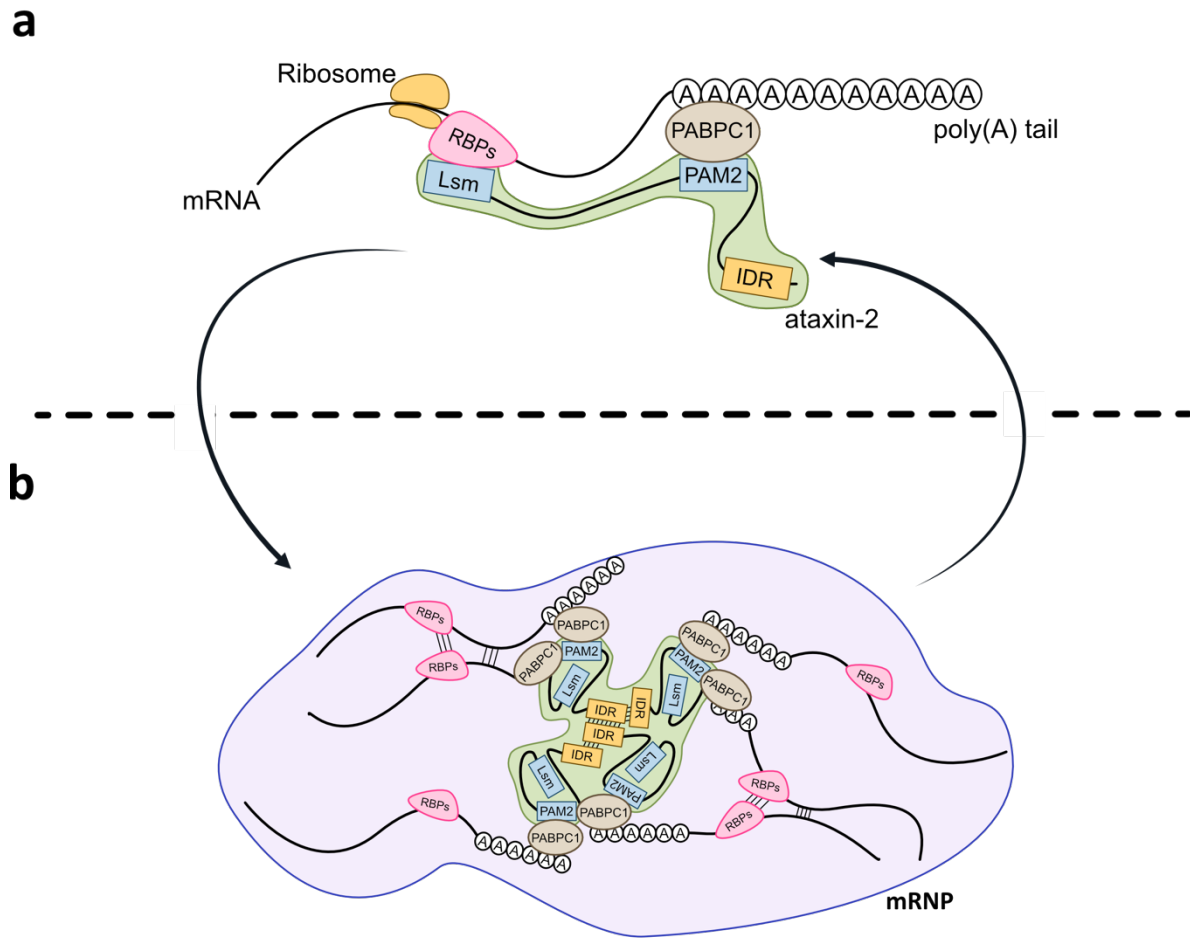


Figure I3 - Schematic representation of the proposed model for ataxin-2 on mRNP granules regulation. Proposed role of ataxin-2 in regulating mRNA translation and the formation of physiological mRNPs. **a)** The PAM2 of ataxin-2 binds to PABPC1, which is bound to poly-(A) mRNAs. Meanwhile, the Lsm domain interacts with RBPs and elements of the translation machinery. **b)** Under specific protein translation needs, ataxin-2 conformation is altered, and its IDRs domains are exposed, while the LSM domain disconnects from the translation machinery. Ataxin-2 exposed IDRs interact with other IDRs motifs, forcing the mRNAs bound to PABPC1 to coalesce into an mRNP granule, where RNA–RNA and protein-protein crosslinks are favored. The formation of these mRNPs is a reversible process that pauses translation depending on cellular demands for protein synthesis.

Overall, strong evidence points to the fact that mutant ataxin-2 might significantly impact SG dynamics, even though the specific mechanisms underlying these dysregulations are not fully understood. A complex interplay between ataxin-2, RBPs, SGs nucleators, and untranslated mRNAs challenges our understanding, suggesting multiple or even competing pathways that influence SGs dynamics in a context-specific manner.

Ataxin-2 as a modifier of other diseases

The role of ataxin-2 in the context of neurological disorders is not confined to SCA2. Several studies clearly positioned ataxin-2 as a key modulator in the pathology of several other neurodegenerative disorders. Ataxin-2 has been demonstrated to have connections with amyotrophic lateral sclerosis, Parkinson's disease, SCA3, and metabolic-related diseases.

a. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that mainly affects motor neurons. It is characterized by progressive muscle weakness, atrophy, and motor impairment (Masrori and Van Damme, 2020).

In ALS, it has been shown that intermediate CAG expansions within the *ATXN2* gene were associated with the disease. Individuals carrying *ATXN2* alleles with 27 to 33 CAG repetitions, typically not associated with SCA2, have an increased risk of developing ALS. This form of *ATXN2* is observed in a substantial number of ALS patients (Elden et al., 2010; Van Damme et al., 2011). Nevertheless, few reports have also connected the *ATXN2* CAG expansion, ranging from 34 to 39 (associated with full SCA2 penetration), with cases of ALS, although only 10 ALS patients with these expansions were reported so far (Tazen et al., 2013). The association between ALS and SCA2 was first described in 2010, and since then, several studies have strengthened this observation and described solid molecular mechanisms underlying this link (Conceição & Costa et al., 2024; Elden et al., 2010). Ataxin-2 was shown to abnormally interact with proteins that are directly associated with ALS, such as the TAR DNA-binding protein 43 (TDP43) – a protein typically found in ubiquitinated inclusions within neurons of ALS patients (Elden et al., 2010; Neumann et al., 2006). TDP-43 protein seems to be mislocalized in SCA2, whereas ataxin-2 appears mislocalized in ALS. This clearly shows an interplay between the two proteins in pathological contexts (Elden et al., 2010; Neumann et al., 2006).

Ataxin-2 with intermediate polyQ expansions seems to modulate ALS pathology by exacerbating the activity of several caspases. Under stress conditions, intermediate-length ataxin-2 induces caspase activity, driving excessive cleavage of TDP-43, resulting in the excessive production of C-terminal fragments, which are known to form highly phosphorylated/ubiquitinated inclusions in ALS (Elden et al., 2010). This effect, observed only in conditions of induced cellular stress, aligns with reports linking TDP-43 and ataxin-2 to SGs dynamics. It also aligns with studies showing that in ALS models, SGs display abnormal dynamics. Interestingly, it was shown that, by preventing ataxin-2 from forming granules, ALS-associated phenotype is mitigated (Bakthavachalu et al., 2018; Elden et al., 2010; Vieira de Sá et al., 2024).

An interesting note is that the number of CAG expansions within the *ATXN2* does not correlate with the severity of ALS, as it is observed in SCA2. This implicates a mechanism by which the simple fact of carrying a particular *ATXN2* CAG length is sufficient to trigger ataxin-2-ALS-associated pathology (Ross et al., 2011; Sorarù et al., 2011; van Blitterswijk et al., 2014). An interesting case was reported where, in a familiar case of SCA2/ALS, an individual carrying an *ATXN2* allele with 39 developed ALS, but a direct descendant carrying 40 CAGs developed SCA2 (Tazen et al., 2013).

The clearest piece of evidence positioning ataxin-2 as a modifier of ALS pathology comes from studies using the TDP-34 mice, which mimics TDP-43 inclusion formation and motor deficits (Wils et al., 2010). In this model, downregulating the normal-length ataxin-2 reduces the formation of TDP-43 toxic inclusions, reduces neurodegeneration, improves motor performance, and lowers the rate of disease progression (Becker et al., 2017).

Another link connecting ALS and ataxin-2 is through the formation of SGs. A study established that ataxin-2-mediated SGs formation contributes to the ALS-associated pathology in *Drosophila* models. Preventing ataxin-2 from forming SGs mitigated the ALS phenotype. Here, ataxin-2 poses as an ALS disease modifier via SGs dynamics (Bakthavachalu et al., 2018).

b. Frontotemporal dementia

Frontotemporal dementia is a spectrum of neurodegenerative disorders that are characterized by the degeneration of frontal lobe neurons. Clinically, these diseases present progressive intellectual function loss and behavioral changes. Frontotemporal dementia-ALS (FTD-ALS) is a particular condition characterized by the manifestation of FTD signs that overlap with ALS (Cividini et al., 2022).

Ataxin-2 has also been associated with the FTD-ALS spectrum. Some studies showed that intermediate-length *ATXN2* alleles ranging from 27 to 32 CAG repeats are a risk factor for developing FTD-ALS. However, the number of CAGs seems to not correlate with the severity of the symptoms (Lattante et al., 2014; Narain et al., 2017; van Blitterswijk et al., 2014). The (GGGGCC)*n* hexanucleotide abnormal repeat expansion within the *C9ORF72* gene is the most common cause of familial ALS, FTD, or FTD-ALS. *C9ORF72* mutations result in protein loss-of-function, generation of RNA foci, and production of toxic dipeptide repeat proteins, which aggregate (Balendra and Isaacs, 2018; Smeyers et al., 2021). A proposed mechanism for pathogenesis suggests that *C9orf72* loss-of-function disrupts autophagy, affecting the turnover of several proteins, which eventually accumulate. Among these proteins, normal-length ataxin-2 and TDP-43 were identified. This protein accumulation leads to TDP-43 toxic aggregates, which solely drive the ALS-associated phenotype. However, when an intermediate-length form of ataxin-2 is expressed, this process is exacerbated, further potentiating autophagy dysregulation and the accumulation of toxic TDP-43. Although the exact interplay between these factors and how they collectively lead to ALS-FTD, is not clear, it poses intermediate-length ataxin-2 as a modulator that drives *C9orf72*-associated pathology (Sellier et al., 2016).

c. Parkinson's disease

Parkinson's disease (PD) is the most common neurodegenerative disorder affecting the dopaminergic neurons. It is characterized by tremors and other debilitating motor signs (Balestrino and Schapira, 2020).

Ataxin-2 has been suggested to be linked with atypical forms of PD. In such rare cases, PD patients carry *ATXN2* alleles with CAG repeats spanning from 33 to 43 CAGs repeats (above the threshold to develop SCA2). However, a key nuance in these patients is the presence of redundant CAA trinucleotide interruptions within the *ATXN2* CAG repeat tract (Casse et al., 2023; Charles et al., 2007; Kim et al., 2007). It is curious to observe that interrupting the CAG sequence with a redundant CAA sequence is able to shift the SCA2 phenotype into a PD-like phenotype. Since both trinucleotide (CAG and CAA) sequences encode for glutamine, it suggests a mechanism operating beyond the amino acid sequence of the polyQ expansion. The phenotype of these patients is generally characterized by mild to non-existing cerebellar signs accompanied by parkinsonian features that respond to L-DOPA treatment. Similar to the other diseases associated with *ATXN2*, and contrary to SCA2, the length of CAG repeats does not affect the symptoms of these patients (Casse et al., 2023; Charles et al., 2007; Gwinn-Hardy et al., 2000; Infante et al., 2004; Lu et al., 2002).

Parkin protein is associated with most of the cases of early-onset forms of PD. Parkin was shown to directly bind to the ataxin-2 C-terminal region of both normal and expanded forms, but with a preference for the latter. Parkin aids ataxin-2 degradation by ubiquitinating it and targeting it to the proteasome. In fact, it was shown that parkin overexpression reduces the levels of ataxin-2 and mitigates some expanded polyQ-associated disruptions. Additionally, Purkinje neurons from SCA2 patients showed that parkin is less immunoreactive, suggesting a reduced expression (Huynh et al., 2007).

Studies using patient-derived cells and comparing PD, SCA2, and PD-associated *ATXN2* showed that DDX6 and PABPC1 proteins displayed altered expression patterns. In PD and PD-associated *ATXN2*, DDX6 was observed to be increased, while in SCA2 patients, it was reduced. On the contrary, PABPC1 also exhibits a similar but inverse pattern (Nkiliza et al., 2016). This expression profile aligns with the previously described mechanisms for ataxin-2-related SGs dysregulation, in which it was observed that DDX6 downregulation or PABPC1-bound to poly-

(A) mRNA upregulation could potentiate SGs assembly (Ripin et al., 2024; Yamagishi et al., 2024). This link raises a speculative question: could SGs homeostasis explain why the same expansion, ranging 33–43 CAG repeats, cause two different phenotypes, SCA2 or PD-like? As such, this could mean that SGs upregulation would act as a disease modifier, potentially shifting the pathology towards a pure SCA2 phenotype, in the cases where the mutant ataxin-2 presents 33-43 glutamine expansions.

d. Spinocerebellar ataxia type 3

Spinocerebellar ataxia type 3 (SCA3) arises from abnormal expansion of the CAG trinucleotide within the human *ATXN3* gene. As a result, a polyQ expanded ataxin-3 protein is produced (Estevam et al., 2023). It was observed that in SCA3 patients, ataxin-2 was both downregulated and mislocalized (Nóbrega et al., 2015). The re-establishment of ataxin-2 levels mitigated SCA3-associated phenotype, including motor deficits in a SCA3 transgenic mouse model. Additional experiments showed that overexpression of ataxin-2 resulted in mutant ataxin-3 downregulation. Since PABPC1 binds ataxin-2 to regulate protein synthesis, these observations could be linked to the regulation of ataxin-3 translation. Mechanistically, this would mean that the mutant ataxin-3 reduces the levels of ataxin-2, leaving PABPC1 free to elevate general protein synthesis, including the mutant protein (Ciosk et al., 2004; Nóbrega et al., 2015; Satterfield and Pallanck, 2006).

e. Metabolic-related disorders

A metabolic disorder is any condition characterized by the inability of the body to synthesize, process and distribute macronutrients such as lipids, carbohydrates and proteins (DeBerardinis and Thompson, 2012).

Studies using two different *ATXN2*-knockout mouse models observed a markedly obese phenotype (Kiehl et al., 2006; Lastres-Becker et al., 2008). Additionally, in one of those models, it was shown that mice displayed altered insulin metabolism with downregulation of the insulin receptor, both in the liver and brain. Furthermore, similar to what is observed in humans with insulin resistance syndrome, the insulin levels were increased in the pancreas and blood serum of *ATXN2*-knockout mice (Lastres-Becker et al., 2008).

Ataxin-2 has been shown to play a role in mechanisms involving metabolic pathways, such as the mTORC1. This pathway is closely connected to diabetes, as mTORC1 overactivation is associated with enhanced insulin resistance. In line with this, mice lacking ataxin-2 fail to regulate this pathway, resulting in excessive mTORC1 activity and, consequently, insulin resistance (Amin et al., 2024; Lastres-Becker et al., 2016). The obese phenotype can also be associated with overactivation of the mTORC1, as its activation is associated with lipid deposition and adipose tissue expansion (Gong et al., 2023; Magdalon and Festuccia, 2017). However, SCA2 patients typically show marked weight loss. Although never demonstrated, it is possible that the mutant ataxin-2 might excessively limit the mTORC1 pathway, which could result in overactivation of the autophagic processes, reduced protein synthesis, and ultimately in weight loss (Rodriguez-Graña et al., 2022).

The dysfunctions described above are somewhat related to ataxin-2 involvement in SGs dynamics. Overexpression of ataxin-2, in unstressed cells, sequesters mTORC1 into SGs, limiting its activity. Moreover, under stress conditions, mTORC1 activity is only reactivated following SGs disassembly (Lastres-Becker et al., 2016; Takahara and Maeda, 2012). In conditions of cellular stress, when protein synthesis is needed to be repurposed and/or attenuated, mTORC1 sequestration might be a mechanism to limit a specific type of protein synthesis (Lastres-Becker et al., 2016; Liu and Sabatini, 2020; Panwar et al., 2023; Takahara and Maeda, 2012).

The observations of obesity in *ATXN2*-knockout mouse models raise the question of whether this phenotype could result from the inability of SGs to assemble due to the absence of ataxin-2. Without ataxin-2 and SGs, mTORC1 would not be sequestered to these structures, leaving mTORC1 free to overactivate its pathway, leading to the metabolic alterations observed (Amin et al., 2024; Lastres-Becker et al., 2016; Liu and Sabatini, 2020; Panwar et al., 2023; Takahara and Maeda, 2012). Additionally, the observations related to marked weight

loss in SCA2 patients could result from an excessive constraint of mTORC1 signaling due to its sequestration by the mutant ataxin-2 into SGs (Lastres-Becker et al., 2016; Takahara and Maeda, 2012).

Overall, the observations linking ataxin-2 with other diseases suggest that this protein is a potential disease modifier. Its multifaceted functions and numerous interactions confer ataxin-2 with a great potential to abnormally engage with cellular components, disturbing mechanisms beyond those typically associated with SCA.

1.4 Models of disease

According to the Cambridge Dictionary, a model is “something that a copy can be based on because it is an extremely good example of its type.” In research, models should accurately copy the mechanisms under study and poses as good examples of their type. Models are essential tools in biomedical research for understanding complex physiological processes and disease mechanisms. Biomedical research models allow researchers to perform extensive studies by manipulating different biological systems under controlled conditions. By recapitulating aspects of human disorders, disease models are the cornerstone of developing novel or refining preexisting therapies (Dawson et al., 2018; Egorova and Bezprozvanny, 2019; Loewa et al., 2023).

In the preclinical stage of research and development, models can be divided into three main groups: *in silico*, *in vitro*, and *in vivo*. *In silico* models use computer simulations or tools to mimic biological systems, whereas *in vitro* models rely on cells or tissues to study biological processes at a cellular and molecular level outside a living organism. *In vivo* models use live organisms to study biological mechanisms, considering the influence of an interconnected system (Benam et al., 2015; Dawson et al., 2018; Shaker et al., 2021). While *in silico* and *in vitro* are often associated with simplicity and controlled environments, making them so appealing to use, they lack the intricate physiological interactions in living organisms. *In vivo* models try to represent an organism as a whole, providing results under the complex interactions between cells, tissues, and organs (Benam et al., 2015; Dawson et al., 2018; Egorova and Bezprozvanny, 2019; Shaker et al., 2021).

Whether *in silico*, *in vitro*, or *in vivo*, it is crucial to choose the model that best suits the specific context and purpose of the study. Researchers should consider aspects such as cost and time efficiency, biological relevance, translational potential, and, above all, ethics.

Mouse models for SCA2

The brain, the primary organ affected in SCA2, poses several challenges to its study, including complexity and access. In the past, researchers were limited to studying *post-mortem* brain tissue, which provides a picture that only represents the late phase of the disease. Therefore, animal models of SCA2 are key tools that allow researchers to understand the processes via which a polyQ protein with an elongated glutamine tract induces the observed pathology, as well as associated pathways with the pathogenesis and their consequences. Animal models are among the most reliable platforms for studying human diseases. They have the potential to recapitulate a human disorder in a complex, multi-systemic organism, in which inter-tissue communications are preserved, making them invaluable for studying pathological mechanisms and developing disease therapies (Dawson et al., 2018; Tello et al., 2022).

Several different animals have already been used to unveil the mechanism underlying the SCA2 pathology, including *Caenorhabditis elegans* (nematode), *Drosophila melanogaster* (fruit fly), *Danio rerio* (zebrafish), and *Mus musculus* (mouse), all of which present advantages and disadvantages. However, mice are considered the gold standard of neurodegenerative disease studies associated with motor signs (Cendelin et al., 2022; Sellier et al., 2016). To date, nine different mouse models have been developed to help researchers understand the biology of ataxin-2 and SCA2. They included seven mice expressing expanded forms of ataxin-2 and two *ATXN2*-knockout mice lacking ataxin-2.

Knockout mouse models

Knockout mouse models are genetically engineered animals that lack the expression of a specific gene due to its inactivation or removal. Two different knockout mice for *ATXN2* have been developed, representing powerful platforms for ataxin-2 loss-of-function studies (Kiehl et al., 2006; Lastres-Becker et al., 2008).

The first *ATXN2*-knockout mouse (*ATXN2*-KO-2006), developed in 2006, was able to produce viable offspring but showed reduced fertility rates compared to WT mice. This model did not develop any motor deficits. At birth, the *ATXN2*-KO-2006 model did not display any weight alterations. Still, after the first year of age, they started to exhibit signs of obesity with abnormal accumulation of abdominal and liver fat. At the histological level, no alterations were observed in the nervous system (Kiehl et al., 2006).

The second *ATXN2*-knockout mice (*ATXN2*-KO-2008), developed two years later in 2008, displayed several similarities with the previous model. They produced viable offspring with reduced fertility, there were no visible motor impairments besides traits of hyperactivity, and they were obese. At 6 months of age, animals displayed some metabolic alterations, namely increased blood insulin and increased insulin production, accompanied by enlargement of pancreatic islets. Also, mice displayed altered lipid metabolism, as observed by dyslipidemia and alteration in lipid-related brain proteins, especially in the cerebellum. At 6 months of age, lipid-related proteins, including the acid sphingomyelinase, neutral sphingomyelinase, and the peroxisome proliferator-activated receptor δ , were shown to be downregulated, while the brain lipid-binding protein was upregulated (Lastres-Becker et al., 2008). A later study using the *ATXN2*-KO-2008 mice performed a transcriptional analysis and showed that the absence of ataxin-2 globally upregulates transcription of genes associated with translation signals and machinery. Interestingly, the global rate of protein synthesis was reduced in mouse embryonal fibroblasts from this model (Fittschen et al., 2015).

Striatal lentiviral mouse model

Although the striatum is not the most affected brain region in SCA2, growing evidence suggests that other brain regions contribute to non-ataxic symptoms (Seidel et al., 2012). In line with this, our laboratory developed a novel SCA2 striatal lentiviral (LV) mouse model (LV-SCA2) (Marcelo et al., 2021a). This model was generated by delivering lentiviral vectors (LV) encoding the mutant *ATXN2* gene (80 or 104 CAGs), under the control of the mouse phosphoglycerate kinase 1 (*Pgk-1*) promoter, directly into one of the striatal hemispheres of

mice through stereotaxic injection. Additionally, as a control, the contralateral hemisphere was injected with LV coding for the WT form of the *ATXN2* gene (Marcelo et al., 2021a).

The striatal LV mouse model is not suitable for motor assessment, as the only behavioral alteration observed is hyperactivity. However, at the histological level, striatal hemispheres expressing expanded ataxin-2 displayed a progressive accumulation of the mutant protein aggregates and, at the same time, a gradual loss of the dopaminergic neuronal markers. These pathological findings were observed at 4 weeks of age and worsened over time until 12 weeks of age. Moreover, neuroinflammation markers, such as astrogliosis and microgliosis, were also shown to appear progressively. Overall, the striatal LV mouse model presents a rapid pathological progression while preserving key neuropathological hallmarks of SCA2, closely approaching those observed in the human context (Marcelo et al., 2021a).

Knock-in mouse models

Knock-in mouse models refer to genetically engineered mice in which an exogenous gene was inserted, via homologous recombination, at a specific locus. One of the main advantages of these models is the fact that the inserted gene is under the control of the endogenous promotor, thereby closely replicating the endogenous context of genetic regulation (Damrath et al., 2012; Sen et al., 2019).

a. Atxn2-CAG42-KIN

The first *ATXN2* knock-in mouse was generated in 2012 using homologous recombination to replace the normal mouse exon 1 of *Atxn2* with an exon containing 42 CAG repeats (above the threshold associated with full penetrance for SCA2). As such, the mutant allele is under the control of the endogenous mouse *Atxn2* (Conceição & Costa et al., 2024; Damrath et al., 2012).

Molecular analysis demonstrated positive expression for both protein and mRNA within the cerebellum and cortex. The *Atxn2*-CAG42-KIN mice exhibited weight loss from 1 week of age that remained for at least 21 months, a similar feature also present in SCA2 patients (Damrath et al., 2012; Rodriguez-Graña et al., 2022).

At the behavior level, these mice displayed very mild symptoms, with only the homozygous showing some significant alterations at 72 weeks of age. At the histological level, there were no signs of cerebellar degeneration. However, ataxin-2 aggregates were observed in Purkinje cells at 56 weeks of age and later, at 96 weeks of age in higher amounts (Damrath et al., 2012).

The *Atxn2*-CAG42-KIN model exhibits a mild SCA2-associated phenotype with late onset; however, it is crucial to acknowledge that neurodegenerative diseases are generally progressive and frequently present later in life. This feature makes the model particularly valuable for studying the long-term development and progression of SCA2, as it accurately reflects how the disease progresses in humans (Conceição & Costa et al., 2024; Damrath et al., 2012).

b. *Atxn2*-CAG100-KIN

In 2019, another *ATXN2* knock-in was generated, employing the same strategies as the *Atxn2*-CAG42-KIN mouse model previously described. Genetically, they only differ in the number of CAG repetitions, as the new model bears 100 repetitions within the exon 1 of the mouse *Atxn2* gene, instead of 42 (Sen et al., 2019). Since the *Atxn2*-CAG42-KIN models exhibited mild symptoms, the rationale behind the development of the new *Atxn2*-CAG100-KIN is related to the fact that a greater number of CAG repetitions is associated with more severe phenotypes and earlier onsets (Conceição & Costa et al., 2024; Sen et al., 2019). Like the human context, the *Atxn2*-CAG100-KIN exhibited genomic instability, with later generations presenting larger CAG tracts (128 repetitions) than their predecessors (100 repetitions). Homozygous mice had to be sacrificed at 14 months of age due to severe locomotor deficits, and even before this advanced stage, mice exhibited increased rates of death (Damrath et al., 2012; Sen et al., 2019).

As previously described for the other knock-in model, weight loss was also a late-stage feature. Consistent with other models, hyperactivity was also present in the *Atxn2*-CAG100-KIN model but was reduced as the disease progressed (Damrath et al., 2012; Lastres-Becker et al., 2008; Marcelo et al., 2021a). Several behavioral tests were used to assess motor deficits and revealed a progressive impairment throughout the disease. The first symptoms were observed at 3 months of age using the grip strength test. The rotarod and footprint tests revealed patterns of motor incoordination that started to manifest at 20 weeks and 12 months of age, respectively.

At the histological level, ataxin-2 aggregates were observed in several regions, including in cerebellar Purkinje neurons, inferior olivary neurons, and pontine nuclei neurons (Sen et al., 2019)

Overall, the *Atxn2*-CAG100-KIN is characterized by a more pronounced phenotype compared to the *Atxn2*-42-KIN, confirming that the number of CAG repetitions is associated with the severity and onset of symptoms. The development of this model allows researchers to study the pathology associated with the mutant ataxin-2 in a faster timeframe, while preserving the endogenous context of genetic regulations.

Transgenic mouse models

Transgenic mouse models refer to genetically engineered mice in which an exogenous gene, via pronuclear injection, was inserted at a random locus. Unlike knock-in mice, the gene of interest is under the control of a specific promoter of choice. Because researchers can virtually choose any promoter they desire, transgenic models are normally associated with robust and directed expression of the gene of interest (Alves-Cruzeiro et al., 2016; Cendelin et al., 2022). Several transgenic mouse models were developed for SCA2.

a. TG-SCA2-Q58

The first SCA2 transgenic mouse model was generated in 2000. Through the pronuclei injection of the exogenous gene, the TG-SCA2-Q58 transgenic mouse expresses a full-length version of the human *ATXN2* cDNA with 58 CAGs repeats. The transgene is under the control of the L7 promoter, which directs the expression to the Purkinje cells. To study this model, as a control, researchers developed transgenic mice expressing a normal-length form of the *ATXN2* gene with 22 CAGs. Several TG-SCA2-Q58 mouse lines were studied, and the Q58–11 was the one better characterized. Cerebellar extracts from transgenic mice showed robust transgene expression at protein and mRNA levels (Huynh et al., 2000).

At the histological level, the TG-SCA2-Q58 exhibits decreased immunoreactivity for Calbindin-28K, a marker typically used to assess the loss of Purkinje cells. This decrease was observed at 4 weeks of age and progressively worsened throughout disease progression. At a later stage, 24-27 weeks of age, mice also displayed less Purkinje cells than control animals. Additionally, electrophysiological studies revealed a significantly reduced firing frequency of Purkinje cells, which worsened progressively (Huynh et al., 2000; Kasumu et al., 2012).

The rotarod, clasping, and footprint tests showed that TG-SCA2-Q58 mice exhibited a progressive motor decline that started at 16 weeks of age. At 26 weeks of age, transgenic mice were severely affected by motor deficits. Comparisons between transgenic controls with 22 CAGs and WT animals revealed no differences (Huynh et al., 2000).

Overall, the TG-SCA2-Q58 transgenic mouse recapitulates some key features related to SCA2: progressive motor dysfunction and neurodegeneration. At the same time, the TG-SCA2-Q58 also displays a relatively fast disease onset, which enables a quick assessment of pathological mechanisms associated with the mutant ataxin-2

b. TG-BAC-Q72

In 2015, a transgenic mouse model for SCA2 was developed using a bacterial artificial chromosome (BAC). BACs are large DNA constructs derived from bacterial chromosomes, capable of accommodating large DNA fragments, up to 500 kilobases (kb). The BAC used to generate the SCA2 model contained the entire human *ATXN2* gene with 72 CAG repetitions, plus 16 kb of the 5' flanking genomic sequence and 3 kb of the 3' flanking genomic sequence. This way, the transgene includes all exons, introns, and regulatory regions, thereby reflecting the regulation of gene expression present in the context of SCA2. The whole DNA construct was injected into fertilized eggs of mice to generate the SCA2 transgenic model. The transgene is under the control of the human *ATXN2* gene promoter; thus, its expression is expected to be ubiquitous (Dansithong et al., 2015; Mogi et al., 2025; Van Keuren et al., 2009).

The TG-BAC-Q72 exhibited a robust transgene expression, both at the protein and mRNA levels, throughout the CNS and in other organs, including the liver and the heart (Dansithong et al., 2015).

Beginning at 9 weeks of age and maintained for at least 24 weeks, the TG-BAC-Q72 displayed decreased transcript levels from key markers of the cerebellum, which is suggestive of cerebellar degeneration. These observations were accompanied by reduced protein levels of Purkinje cell markers, calbindin 1 (Calb1), and Purkinje cell protein 2 (Pcp2). This reduction correlated with abnormalities in Purkinje cell morphology and shrunken arborization, observed at 24 weeks of age (Dansithong et al., 2015).

At the behavioral level, some alterations were observed at 8 weeks of age, where transgenic mice exhibited a smaller body size than their WT littermates. In terms of motor performance, these mice began to show motor impairments at 16 weeks of age, as assessed by the accelerated rotarod test (Dansithong et al., 2015).

Altogether, the TG-BAC-Q72 transgenic model exhibits certain features that facilitate translational research into the human context. However, it lacks pronounced neurodegeneration and marked motor deficits. Nevertheless, the greatest advantage of this model is that it preserves the human *ATXN2* genetic context, making it an excellent tool for studying mechanisms related to genetic regulation and expression.

c. TG-SCA2-Q75

In 2006, a SCA2 transgenic mouse was developed using the pronuclei injection strategy to generate a model with a full-length human version of the *ATXN2* gene. The particularity of this model lies in its use of *ATXN2* cDNA with 75 CAG repeats (Aguilar et al., 2006).

The *ATXN2* transgene is under the control of its own promoter, which drives ubiquitous expression. At the protein level, expression was detected throughout the CNS, including the cerebellum, and in the liver, skeletal muscle, lung, and kidney. Behavioural tests revealed that heterozygous TG-SCA2-Q75 mice began exhibiting motor deficits at 12 weeks, while homozygous mice started performing poorly at 6 weeks. Some neuropathological features were also observed in the transgenic model, as Purkinje cells exhibited both reduced dendrites and shrinkage of the cell body (Aguilar et al., 2006).

Altogether, the TG-SCA2-Q75 model exhibits few pathological features associated with the SCA2 phenotype. Nevertheless, unlike other transgenic models that typically employ the specific Purkinje promoters, the TG-SCA2-Q75 used the *ATXN2* promoter. This approach made *ATXN2* expression closer to its natural context while ensuring efficient ubiquitous expression and the presence of motor deficits.

d. TG-SCA2-Q127

In 2013, a SCA2 transgenic mouse model was developed using the full-length version of the human *ATXN2* cDNA with 127 CAG repetitions. The transgene is under the control of the L7 promoter, which directs its expression to the Purkinje cells (Hansen et al., 2013).

Like the TG-BAC-Q72, the TG-SCA2-Q127 displayed decreased transcript levels from markers of several cerebellar layers, suggesting cerebellar degeneration. Behavioral tests using the rotarod demonstrated that the TG-SCA2-Q127 mice were progressively affected by robust motor deficits, which started manifesting at 8 weeks of age. At 36 weeks of age, the differences between WT mice and transgenic mice were marked, with the TG-SCA2-Q127

exhibiting a motor performance that was 50% worse than control animals (Hansen et al., 2013).

At a functional level, electrophysiology studies demonstrated a progressive decline of Purkinje cells' activity, as they showed slower firing frequency starting at 6 weeks of age. Strikingly, the regularity with which the Purkinje cell fired was not affected (Hansen et al., 2013).

Neuropathological experiments with the TG-SCA2-Q127 model showed a progressive reduction in the thickness of the cerebellar molecular layer, starting at 12 weeks of age. This was followed by a significant loss of Purkinje cells at 40 weeks of age. Additionally, ataxin-2 protein aggregates were also detected in Purkinje cells as early as 4 weeks of age (Hansen et al., 2013).

The TG-SCA2-Q127 transgenic mouse model recapitulates many pathological features observed in the context of SCA2. It presents a marked motor phenotype, accompanied by pronounced electrophysiological alterations. Aggregation is also a feature, as well as cerebellar neurodegeneration. Importantly, its early onset enables faster and more efficient research. However, considering that SCA2 is typically a late-onset disorder, there is a chance that eventual pathological mechanisms, which might depend on a gradual disease progression, could be underestimated.

Overall, the mouse models generated to study the mechanisms underlying ataxin-2 biology, whether in its expanded or WT form, provide important clues about its physiological roles, the cellular processes it impacts, and its involvement in disease pathogenesis (**Table I3**). Doing research using mice provides researchers with tools that have a great potential to, at the same time, recapitulate both neurodegeneration and motor impairments, as observed in SCA2.

Table I3 – Key traits of SCA2 mouse models

Mouse model	Behavioral observations	Age of onset (weeks)	Associated Pathology	Age of onset (weeks)	Reference
<i>ATXN2-KO-2006</i>	Increased body weight	13	—	—	(Kiehl et al., 2006)
<i>ATXN2-KO-2008</i>	Reduced fertility	—	Metabolic alterations	12	(Lastres-Becker et al., 2008)
	Progressive obesity	12	Insulin receptor protein levels reduction in the liver and brain	24	
<i>LV-SCA2</i>	Hyperactive behavior	8 (post injections)	Ataxin-2 aggregates DARPP-32 marker depletion volume	12 (post injections)	(Marcelo et al., 2021a)
<i>Atxn2-CAG42-KIN</i>	Reduce body weight	1	Cytoplasmic insoluble aggregates	56	(Damrath et al., 2012).
	Impairments in rotarod	72 (only in homozygous)	No signs of neuronal loss	—	
<i>Atxn2-CAG100-KIN</i>	Reduce body weight	20 (M) 40 (F)	Progressively larger cytoplasmic aggregates	12-56	(Sen et al., 2019)
	Impairments in rotarod	20			
	Irregular footprint	48			
	Reduced forelimb strength	12	Reduced brain weight	56	
TG-SCA2-Q58	Impairments in rotarod	16	Loss of calbindin-28K immunoreactivity	4	(Huynh et al., 2000)
	Irregular footprint	16	Decreased PC number	24	
	Limb claspings	32	Cytoplasmic microaggregates	—	
	Beam walk test imbalance	32	Decrease in the firing PC number and firing frequency	24	
TG-BAC-Q72	Reduced body weight	8	Loss of calbindin-28K immunoreactivity	24	(Dansithong et al., 2015)
	Impairments in rotarod	16	Shrinkage of dendritic trees in PCs	—	
			No signs of neuronal loss	—	
TG-SCA2-Q75	Impairments in rotarod	6	Loss of calbindin-28K immunoreactivity	52	(Aguilar et al., 2006)
			Loss of dendrites and shrinkage of cell bodies in PCs	52	
TG-SCA2-Q127	Impairments in rotarod	8	Decreased PC number	40	(Hansen et al., 2013)
			Perinuclear aggregates	4	
			Loss of cerebellar molecular layer thickness	12	
			Slower firing frequency of PCs	6	

Abbreviation: **SCA** – spinocerebellar ataxia; **TG** – transgenic; **BAC** – bacterial artificial chromosome Adapted from: (Koppenol, 2024)

1.5 Integrated stress response

Across evolution, cells developed a plethora of mechanisms to cope with both external and internal stress stimuli, ensuring their adaptation and survival. One of those mechanisms is the integrated stress response (ISR) – a highly conserved molecular pathway that plays a vital role in response to a wide variety of cellular stressors. The conservation of ISR across eukaryotes highlights its critical importance as a key mechanism for stress adaptation and homeostasis restoration (Marcelo et al., 2021b; Pakos-Zebrucka et al., 2016). Among others, stress stimuli known to activate ISR include amino acid deprivation, viral infection, heme deprivation, ER stress, oxidative stress, or the presence of unfolded proteins (Harding et al., 2003; Heather P Harding et al., 2000; Lu et al., 2001; Marcelo et al., 2021b; Srivastava et al., 1998; Wek et al., 1995).

The ISR is initiated by a variety of stress signals that converge on the phosphorylation of the α -subunit of eukaryotic translation initiation factor 2 (eIF2 α) on serine 52. To phosphorylate eIF2 α , four eIF2 α kinases (EIF2AKs) are activated in response to different stresses. They include: EIF2AK1 (or heme-regulated inhibitor – HRI); EIF2AK2 (or protein kinase double-stranded RNA-dependent – PKR); EIF2AK3 (or protein kinase R (PKR)-like endoplasmic reticulum kinase – PERK); EIF2AK4 (or general control nonderepressible – GCN2) (Harding et al., 2003; McEwen et al., 2005; Srivastava et al., 1998; Wek et al., 1995). Each of these EIF2AKs shares a conserved kinase domain. However, each of them also contains a distinct regulatory domain responsible for recognizing a specific group of stress stimuli (Donnelly et al., 2013). For example, HRI is associated with cellular stress caused by heme deprivation, PKR with viral infection (dsRNA), PERK with ER stress/accumulation of misfolded proteins, and GCN2 with amino acid deprivation (Harding et al., 2003; McEwen et al., 2005; Srivastava et al., 1998; Wek et al., 1995).

The ISR manages stress essentially by reprogramming the translation activity, which enables cells to become more efficient in dealing with stress. The ISR maximizes the translation efficiency towards the production of specific stress proteins that are crucial for tackling stressful events (Marcelo et al., 2021b; Pakos-Zebrucka et al., 2016; Riggs et al., 2020). At the same time, this also allows cells to conserve energy and resources, facilitating a proper

stress response. As an example, the ISR is able to upregulate anti-apoptotic factors to suppress apoptosis, thereby promoting cell survival during acute stress (Arimoto et al., 2008; Fujikawa et al., 2023; Tian et al., 2021).

One of the most notorious downstream effects of attenuating the global protein synthesis during ISR is the accumulation of stalled translation machinery, which, in turn, results in the formation of membraneless organelles named stress granules (SGs) (**Figure I4**) (Kedersha et al., 2000, 1999). SGs are cellular structures typically composed of translation initiation components, untranslated poly-(A)-mRNAs, and RNA-binding proteins that rapidly coalesce during stress conditions (Estevam et al., 2023; Marcelo et al., 2021b; Wolozin and Ivanov, 2019). In the following sections, we will cover some aspects regarding SGs biology, focusing on their functions, dynamics, and importance in regulating neurodegenerative disorders.

Integrated stress response

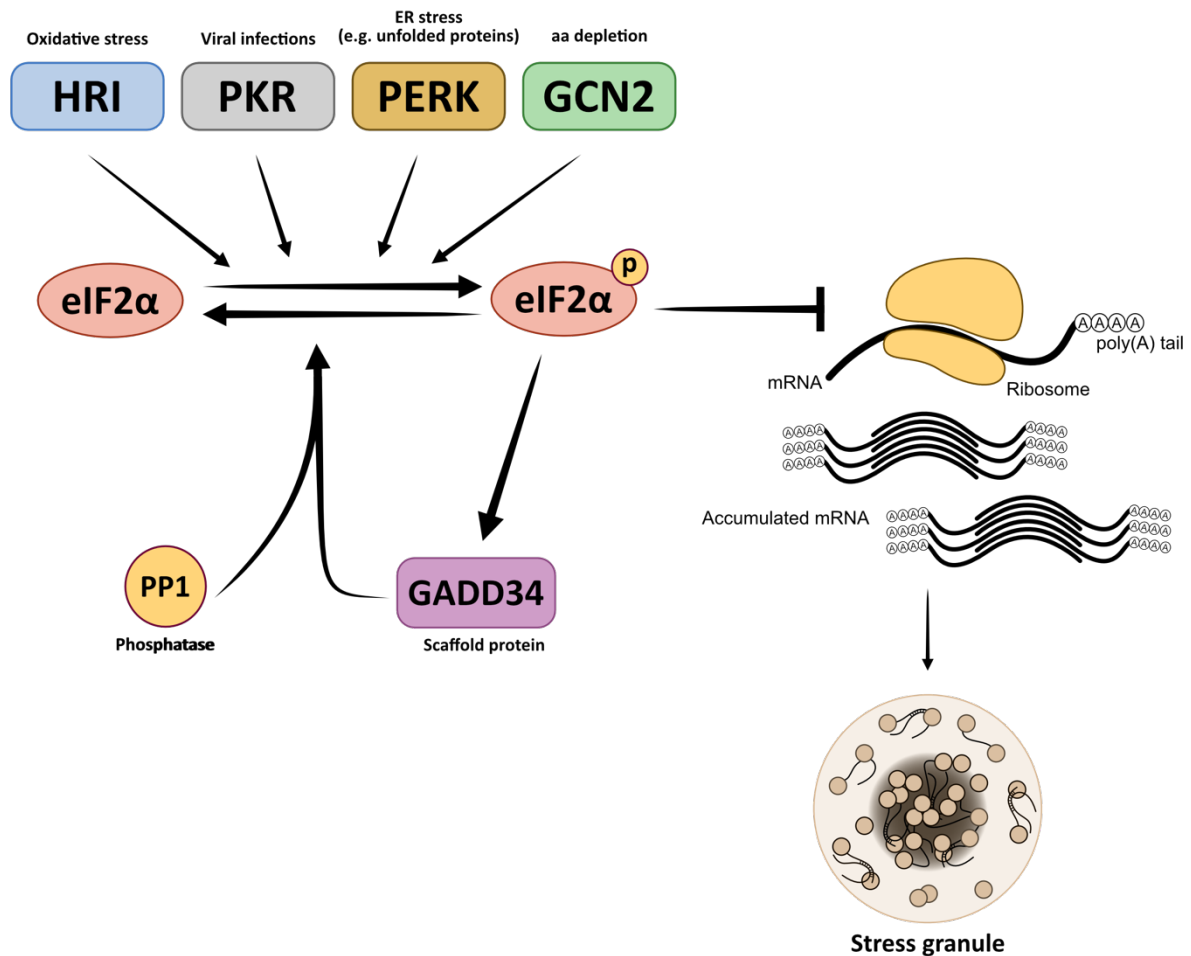


Figure I4 - Schematic representation of the integrated stress response (ISR) pathway. Under conditions of cellular stress, including oxidative stress, viral infections, ER stress, or aa depletion, the ISR is activated. Four stress kinases – HRI, PKR, PERK, or GCN2 – are able to detect different sources of stress. Upon sensing a stress stimulus, one of the stress kinases phosphorylates eIF2α. Consequently, global protein synthesis is arrested, resulting in the accumulation of untranslated poly(A)-mRNAs – a process that precedes stress granule formation. Simultaneously, GADD34 is upregulated and, upon stress relief, serves as a scaffold to mediate the dephosphorylation of p-eIF2α.

Stress Granules

SGs are dynamic membraneless organelles that form within the cytoplasm of eukaryotic cells in response to stress stimuli. They are biomolecular condensates essentially

composed of translationally stalled mRNAs and translation machinery. SGs are formed downstream of the ISR upon accumulating untranslated poly-(A) mRNA. As part of the ISR, SGs are among the most critical elements that help cells manage and recover from several stress conditions.

Stress granules assembly

During translation, the genetic information encoded in the form of mRNA is used to synthesize proteins. To initiate translation, eIF2 α is responsible for delivering the initiator methionyl-tRNA (Met-tRNAⁱ) to ribosomes through its GTP-bound ternary complex (eIF2 α - GTP-Met-tRNAⁱ). The ternary complex aids the Met-tRNAⁱ in scanning the mRNA until it finds the start codon to initiate translation (Donnelly et al., 2013; Merrick and Pavitt, 2018). Since proteins are virtually involved in every aspect of cellular functions, their synthesis emerges as a fundamental biological mechanism essential to all known life forms. As such, a proper regulation of mRNA translation is crucial in maintaining an adequate cellular homeostasis (Pakos-Zebrucka et al., 2016; Riggs et al., 2020).

The formation of SGs is highly dependent on translation alterations that occur in response to stress signals. Under specific stress conditions, translation needs to be repurposed to aid cells to cope with insults. At the beginning of the ISR, eIF2 α is phosphorylated (p-eIF2 α) at serine 52 by one of the four stress sensor kinases – HRI, PKR, PERK, and GCN2 – which were activated upon detecting a specific cellular stress signal (Harding et al., 2003; Kedersha et al., 2000, 1999; Marcelo et al., 2021b; McEwen et al., 2005; Srivastava et al., 1998; Wek et al., 1995). Consequently, the phosphorylation of eIF2 α prevents the conversion of GDP to GTP, locking eIF2 α into a permanent eIF2 α -GDP-bounded state (Pakos-Zebrucka et al., 2016; Riggs et al., 2020). As a result, the availability of eIF2-GTP-tRNA^{Met} ternary complexes is decreased, resulting in polysome disassembly and the release of poly(A)-mRNAs from ribosomes into the cytoplasm. Consequently, global translation is stalled, and poly-(A) mRNAs accumulate (Kedersha et al., 2002). The local and confined increase in concentration of untranslated poly-(A) mRNAs, translation machinery, and other locally

recruited factors results in the formation of SGs via a process called liquid-liquid phase separation (LLPS) (Protter and Parker, 2016; Riggs et al., 2020; Van Treeck et al., 2018; Yamagishi et al., 2024).

In the context of SGs formation, LLPS occurs when untranslated poly-(A) mRNAs coalesce with other proteins, and both accumulate beyond a certain concentration threshold, forming visible foci. In this context, LLPS can be viewed as a process through which cells establish liquid-like segregated compartments within the cytoplasm composed of RNA and proteins (Protter and Parker, 2016; Riggs et al., 2020; Van Treeck et al., 2018; Yamagishi et al., 2024). During the formation of SGs, and for LLPS to occur, accumulated poly-(A) mRNAs must recruit and interact with proteins enriched with IDRs and/or low complexity regions in a way that favors their condensation (Bakthavachalu et al., 2018; Gilks et al., 2004; Protter et al., 2018). The importance of LLPS as an essential process of SGs formation emerged from studies with proteins enriched with low complexity whose overexpression triggered SGs assembly. These proteins are SGs nucleators and, among others, include TIA1, caprin-1, or G3BP1. Additionally, *in vitro* studies revealed that high concentrations of IDRs-containing proteins were able to trigger LLPS spontaneously (Kedersha et al., 2016, 2000, 1999; Riggs et al., 2020; Yang et al., 2020).

Recently, it was shown that ataxin-2 also plays a key role in promoting SGs formation upon poly(A)-mRNA accumulation. Under stress, PABPC1-bound to translationally silenced poly(A)-mRNA recruits ataxin-2, forming a PABPC1-mRNA-ataxin-2 complex (Yamagishi et al., 2024). The increasing formation of PABPC1-mRNA-ataxin-2 complexes, due to the accumulation of PABPC1-bound to translationally silenced poly(A)-mRNA, acts as a scaffold for SGs formation (Yamagishi et al., 2024). Moreover, it was also shown that preventing either the PABPC1 from binding poly(A)-mRNAs or disrupting the interaction between ataxin-2 and PABPC1 downregulates SGs assembly (Damrath et al., 2012; Petrauskas et al., 2024; Yamagishi et al., 2024). These findings position PABPC1 and ataxin-2 as key players in the formation of SGs. This study also showed results suggesting that SGs formation relies on the ability of ataxin-2 to self-aggregate into multimers through its C-terminal region (Yamagishi et al., 2024). A proposed model suggests a sequential assembly mechanism that takes place after stress detection and follows the formation of the PABPC1-mRNA-ataxin-2 complex. Through its c-terminal region, ataxin-2 interacts with multiple other PABPC1-mRNA-ataxin-2 complexes (via

c-terminal—c-terminal interactions), forming multimer aggregates. This aggregation mechanism locally increases the concentration of poly(A)-mRNA, PABPC1, ataxin-2, and other SGs-related proteins beyond critical thresholds, triggering LLPS and consequently SGs assembly (**Figure I5**) (Kedersha et al., 2002; Marcelo et al., 2021b; Petruskas et al., 2024; Protter and Parker, 2016; Yamagishi et al., 2024).

Overall, the process by which SGs assemble is complex and still not fully understood. However, the concerted interaction between RNA-RNA, RNA-protein, and protein-protein, along with LLPS, seems to be at the core of SGs formation.

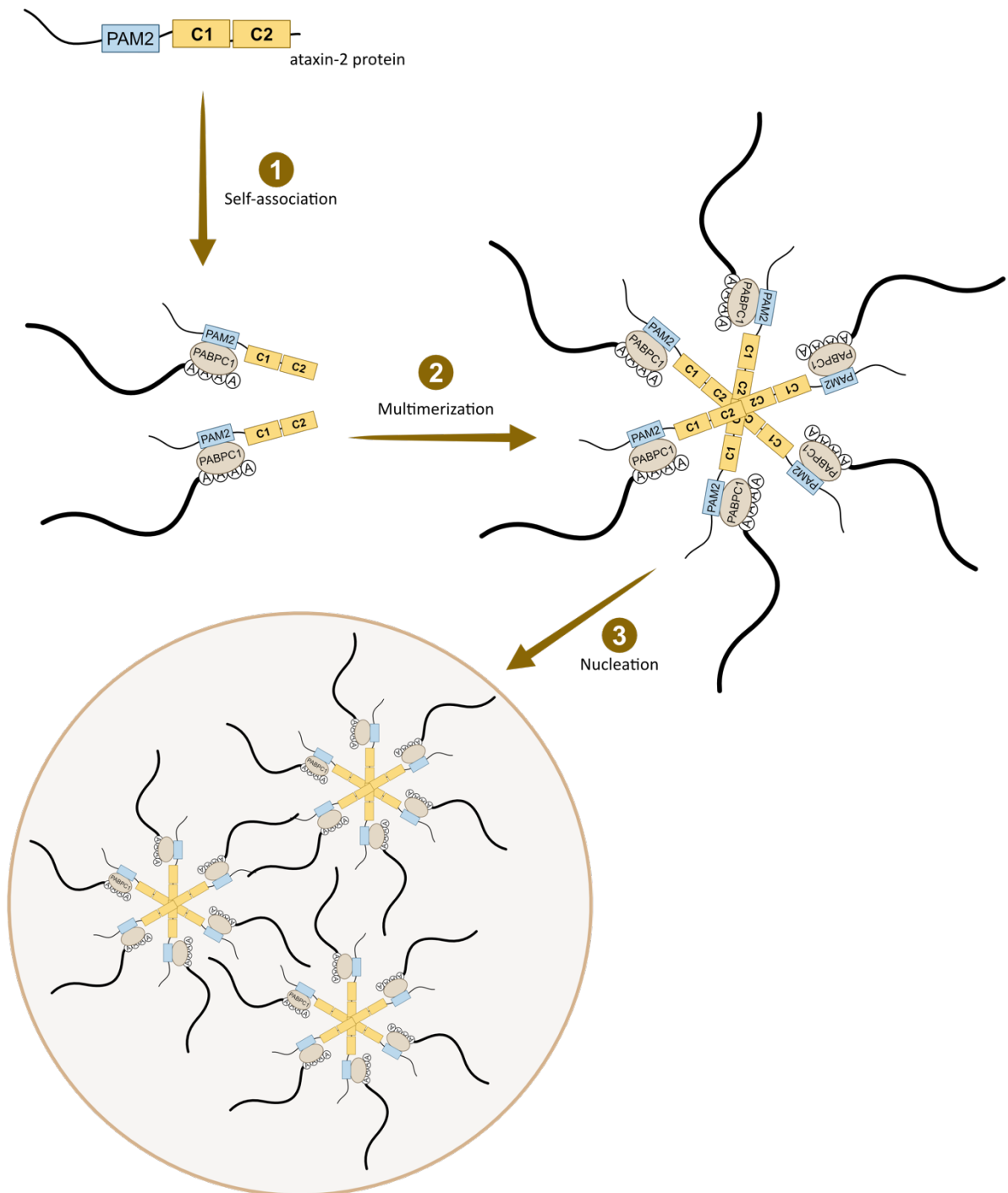


Figure I5 - Schematic representation of the proposed model for ataxin-2 on SGs nucleation. After eIF2 α , the global protein synthesis is arrested, and poly(A)-mRNAs bound to PABPC1 begin to accumulate. Ataxin-2 recruitment gives rise to the poly(A)-mRNAs-PABPC1-ataxin-2 complex that progressively accumulates as translation remains arrested. Due to the increased concentration of this complex, ataxin-2 starts to self-associate through its C-terminal region, until it starts to multimerize and form a multimer aggregate. The increased concentration of poly(A)-mRNAs-PABPC1-ataxin-2 multimers starts to nucleate SGs and eventually recruits other SGs-related proteins, triggering the LLPS that precedes the formation of SGs

Stress granules composition and structure

In response to a cellular stress, downstream the phosphorylation of eIF2 α , SGs start to form in response to the accumulation of translationally silenced poly(A)-mRNA, which, in turn, starts the LLPS process. As mRNAs accumulate, alongside with the PABPC1-mRNA-ataxin-2 complexes, several other proteins are recruited into the liquid-like segregated compartment, ultimately forming a non-membranous aggregate composed of many RNA-protein-bound assemblies (Marcelo et al., 2021b; Protter and Parker, 2016; Riggs et al., 2020).

Apart from variable components, and as mentioned before, SGs are almost entirely composed of translation-associated factors and mRNAs. Pre-initiation translation complexes are among the most enriched components of SGs, which include 40S ribosomal subunits, translation initiation factors (eIF4A, eIF4B, eIF4E, and eIF4G), PABPC1, and poly(A)-mRNAs. Other components, such as RNA helicases, constituents of ribosomes, and cytoskeletal proteins, are also present at SGs (Marcelo et al., 2021b; Nunes et al., 2019; Protter and Parker, 2016; Riggs et al., 2020). Nevertheless, the precise composition of SGs in terms of proteome and transcriptome components is not yet fully known.

The exact composition and structure of SGs are highly complex. SGs' composition can vary depending on the specific stress stimuli, the cell type involved, and the cellular needs during the stress (Aulas et al., 2017; Markmiller et al., 2018). Nevertheless, in the past few years, studies have shown that SGs have a dynamic structure composed of many different proteins and mRNAs. To date, at least 627 proteins have been identified as SGs components (Jain et al., 2016; Khong et al., 2017; Nunes et al., 2019; Van Treeck et al., 2018). Additionally, more than 78% of the SGs components are mRNAs (Khong et al., 2017). In fact, transcriptomic analysis estimates that, to some extent, virtually every mRNA being expressed at the time of the stress stimuli can be found within SGs (Khong et al., 2017). However, a group of 185 specific gene transcripts are significantly enriched, making up more than 50% of the total SGs transcriptome (Khong et al., 2017). These mainly include mRNAs that are either unnecessary for an immediate stress response or are being protected until the normal translation program can resume, this way ensuring rapid restoration of cellular homeostasis once the stress is resolved (Khong et al., 2017; Namkoong et al., 2018; Protter and Parker, 2016). Recent studies have suggested that some proteins that compose SGs are, in fact, ubiquitinated, including

TDP-43, FUS, and G3BP1. The ubiquitination of these specific proteins is suspected to be involved in the disassembly of SGs (Gwon et al., 2021; Krause et al., 2022; Shang et al., 2024; Tolay and Buchberger, 2022; Yang et al., 2023).

Regarding SGs structure, studies suggest that they have a biphasic architecture, consisting of a stable core surrounded by a dynamic shell. The core consists of proteins and mRNAs at higher concentrations, while the shell is formed by a less concentrated layer of proteins and mRNAs (Glauninger et al., 2022; Jain et al., 2016; Khong et al., 2017; Wheeler et al., 2016). Studies have suggested that SGs have a quasi-solid immobile interior region, which rarely alters its content. On the other hand, the outer region seems more fluid and can exchange its content with the cytoplasm rapidly (Jain et al., 2016; Protter and Parker, 2016; Wheeler et al., 2016). At the core of SGs, it is possible to find some of the components that initiate SGs nucleation, including G3BP1, caprin-1, and TIA1, along with poly-(A)-mRNAs and, according to recent studies, ataxin-2 (Wolozin and Ivanov, 2019). Recent evidence also suggests that SGs are only fully mature when several of these cores coalesce and fuse together into a stable assembly (Gilks et al., 2004; Jain et al., 2016; Kedersha et al., 2016; Protter and Parker, 2016; Wheeler et al., 2016).

The composition of the SGs' outer shell remains unclear. Nevertheless, its higher fluidity character has been speculated to be essential to stabilize the core while permitting the bidirectional exchange of components with the surrounding cytosol, depending on cellular needs during the stress response (Jain et al., 2016; Wheeler et al., 2016). Besides the exchanges with the cytosol, the shell also allows the bidirectional exchange with its own core components. For instance, it has been demonstrated that while G3BP1 is a component within the core of SGs, it can quickly move to the shell region (Jain et al., 2016). Studies have suggested that the dynamic fluidity of the shell is maintained by chaperones and other RNA-protein remodeling complexes, to promote weak interactions between the components of the outer SGs region, thereby facilitating the exchange of components, either with the cytosol or with the core (Jain et al., 2016; Wheeler et al., 2016).

Overall, understanding the composition, structure, and dynamics of SGs is challenging. Not only due to their heterogeneity between different cell types or distinct sources of stress but also due to their highly dynamic character

Stress granules functions

The most well-studied and fundamental purpose of an SGs is to serve as a hub where non-essential mRNAs are halted in translation while cells prioritize the synthesis of proteins needed to cope with stress. Attenuating global translation not only alleviates cells' energetic needs but also allows them to employ an efficient expression program that is able of dealing with stress conditions. However, in recent years, SGs have also been associated with other biological functions that extend beyond their role of temporarily storing mRNAs during stress conditions (Reineke and Neilson, 2019; Riggs et al., 2020; Wolozin and Ivanov, 2019).

SGs have been implicated in antiviral response upon viral infection as a mechanism of innate immune response. It was shown that, upon infection, SGs are assembled via G3BP1. Either preventing SGs assembly or downregulating G3BP1 (also decreases SGs assembly) impairs the stress response against viruses (Onomoto et al., 2012; Reineke et al., 2015; Reineke and Lloyd, 2014). In fact, it seems that many viruses developed mechanisms to cleave G3BP1, preventing it from nucleating SGs and impairing the stress response (Long et al., 2024; Visser et al., 2019; Yang et al., 2024). The mechanisms behind G3BP1-dependent SG assembly in response to viral infection are not fully understood. Nevertheless, it has been speculated that G3BP1-dependent SGs formation might be responsible for the sequestration of both translation machinery and viral genome into SGs, this way limiting the synthesis of viral proteins (Marcelo et al., 2021b; Markmiller et al., 2018; Nikolic et al., 2016; Reineke and Lloyd, 2014; White and Lloyd, 2011). Additionally, PKR (one of the four ISR-sensing kinases) is known to phosphorylate eIF2 α after being activated in the presence of viral RNA. At the same time, PKR activates a transcriptional program (e.g., proinflammatory cytokines and interferons) that triggers the innate immune responses (Balachandran et al., 2000; Cesaro and Michiels, 2021). However, it has also been suggested that PRK is recruited to SGs to further enhance an antiviral response. Once PKR is recruited to SGs, it engages with viral RNA accumulated/sequestered within SGs, further amplifying the PKR signaling pathway and intensifying the immune responses (Reineke and Lloyd, 2014).

SGs also play an essential role in regulating apoptosis-related pathways, in particular, SGs seem to have an anti-apoptotic function. It was shown that SGs can sequester the receptor for activated C kinase 1 (RACK1), a known activator of the p38/c-Jun N-terminal kinases (JNK;

p38/JNK) signaling pathway, which induces apoptosis. Once sequestered into SGs, RACK1 is unable to bind mitogen-activated protein kinase (MTK1). Without the RACK1-MTK1, the p38/JNK pathway is no longer activated (Arimoto et al., 2008). Some studies have connected the anti-apoptotic effect of SGs with enhanced cancer proliferation and worse cancer prognosis. It has been suggested that, in the context of cancer, SGs formation is exacerbated, which over-inhibits cellular apoptosis, resulting in a worsened prognosis. In fact, some cancer drugs that target SGs formation were shown to improve cancer treatment and prognosis (Mateju and Chao, 2022; Zhou et al., 2023).

Previous studies suggested that SGs modulate the mTORC1 pathway (Lastres-Becker et al., 2016; Takahara and Maeda, 2012). In a high-nutrient state, mTORC1 is known to induce protein and lipid synthesis, while in conditions of nutrient deprivation, mTORC1 is associated with catabolic events to produce energy, such as autophagy (Heberle et al., 2015). It was shown that SGs can sequester mTORC1 and attenuate its signal pathways in conditions of nutrient deprivation. In fact, as described in previous sections, ataxin-2 seems to be responsible for the mTORC1 inhibition via SGs (Carmo-Silva et al., 2017; Lastres-Becker et al., 2016; Takahara and Maeda, 2012). In this context, SGs seem to play a key role in regulating the metabolic status of cells via mTORC1 signaling.

Recently, SGs granules were also suggested to be required for long-term memory. Although the mechanism was not fully established, it was proposed that ataxin-2-dependent SGs formation is required to consolidate olfactory memory in *Drosophila*. It was suggested that impairing the capacity of ataxin-2 to induce SGs formation impaired long-term memory (Bakthavachalu et al., 2018).

The degree of influence that SGs exert on cells is poorly known, and their functions are yet to be fully characterized. Nevertheless, SGs have been implicated in a distinct array of cellular mechanisms, positioning SGs as multifaceted structures with key functions throughout distinct pathways. One should also consider that SGs have the potential to alter the cellular pathways of those proteins recruited and sequestered within the organelle.

Stress granules disassembly

An effective cellular stress response also encompasses an appropriate transition from a stress-activated state to a non-stress mode. This transition involves the coordinated action between the deactivation of the stress signaling, the disassembly of SGs, and the restoration of the pre-stress translation program. In fact, some pathological mechanisms have been associated with poor SGs disassembly and their chronic persistence (Reineke and Neilson, 2019). The mechanisms by which SGs disassembly are way less studied and known than the mechanisms that lead to their assembly. Nevertheless, some studies suggest that, upon stress release, SGs can be disassembled by chaperones or, alternatively, cleared via autophagy (Marcelo et al., 2021b). Additionally, other studies have shown that the ubiquitination of specific proteins is crucial to promoting the disassembly of SGs (Gwon et al., 2021; Maxwell et al., 2021).

Chaperones from the heat shock protein (HSP) family, such as HSP27, HSP60, HSP70, HSP90, and HSP104, were identified as SGs components and demonstrated to be involved in facilitating SGs disassembly. While the exact mechanism by which HSPs facilitate the disassembly of SGs remains unclear, some studies have demonstrated that disrupting HSP activity hinders SGs disassembly (Akaree et al., 2025; Bard and Drummond, 2024; Mateju and Chao, 2022; Siwach and Kaganovich, 2017).

Currently, it is believed that SGs are resolved from the cytoplasm by two distinct processes: i) disassembly, which is typically associated with transient stress situations, and characterized LLPS loss and dispersion SGs components; or ii) undergo clearance by an autophagic process termed granulophagy, typically associated with chronic stress situations and persistent SGs, and involves the complete engulfment and subsequent degradation of SGs (Buchan et al., 2013; Chitiprolu et al., 2018; Gwon et al., 2021; Maxwell et al., 2021; Ryan and Rubinsztein, 2024; Ryu et al., 2014).

Recent studies have suggested that SGs disassembly (not to be confused with clearance by granulophagy) is mediated by the valosin-containing protein (VCP). VCP is a chaperone with the ability to interact with ubiquitinated proteins to extract them from macromolecular complexes, thereby facilitating their degradation (Meyer and Wehl, 2014; Protter and Parker, 2016). It is suggested that the VCP role in SGs disassembly relates to G3BP1

ubiquitination. In fact, it has been demonstrated that VCP can bind to poly-ubiquitinated G3BP1 within SGs. This interaction leads to the extraction of G3BP1 from SGs, weakening the overall stability of interactions within the granule. As a result, the LLPS falls below the threshold, ultimately disassembling the SGs (Gwon et al., 2021; Maxwell et al., 2021). In the past few years, proteins from the ubiquitination machinery were identified as SGs components. These findings suggest that ubiquitination-related events might have a role in SGs dynamics. In fact, it was shown that the E3 ubiquitin ligase tripartite motif containing 21 (TRIM21) ubiquitinates and co-localizes with G3BP1 within SGs. The study also demonstrated that TRIM21 could directly promote SGs disassembly via G3BP1 ubiquitination (Yang et al., 2023). In line with this observation, a proposed mechanism suggested that TRIM21 is recruited to SGs to prepare their disassembly by ubiquitinating G3BP1 (Gwon et al., 2021; Maxwell et al., 2021; Yang et al., 2023).

Interestingly, VCP also plays an essential role in directing SGs to the granulophagy pathway, but only in situations of persistent stress or chronic stress (Maxwell et al., 2021). First studies on the role of VCP-mediated clearance of SGs by granulophagy showed that by inhibiting VCP activity, SGs accumulate within the cytosol and, simultaneously, reduce the number of SGs within autophagic vesicles (Buchan et al., 2013). Moreover, autophagy inhibition results in increased formation of SGs, whereas promoting autophagy results in SGs clearance (Buchan et al., 2013; Ryu et al., 2014). Moreover, downregulating key proteins in the autophagic pathway, such as ATG3, ATG5, or ATG7, resulted in the persistence of SGs, even in the absence of stress (Ryan and Rubinsztein, 2024). Another essential player in autophagy, the SQSTM1/p62, was shown to co-localize within SGs periphery while binding LC3, suggesting that SGs could be targeted for autophagy. In fact, SQSTM1/p62 downregulation resulted in SGs persistence even after cells were released from stress (Chitiprolu et al., 2018).

Another mechanism that seems to be at the core of SGs disassembly is the active dephosphorylation of eIF2 α . Upon stress sensing, eIF2 α becomes phosphorylated, which in turn upregulates the DNA damage-inducible protein (GADD34). GADD34 serves as a scaffold to promote the interaction between protein phosphatase 1 (PP1) and p-eIF2 α (GADD34-PP1-p-eIF2 α complex), ultimately resulting in the dephosphorylation of p-eIF2 α , thus resuming translation (Connor et al., 2001; Magg et al., 2024; Novoa et al., 2001). Essentially, GADD34-mediated dephosphorylation of eIF2 α works as a negative feedback loop to re-initiate

translation. This is considered the last step of the ISR (Dalet et al., 2017; Goh et al., 2018; Kojima et al., 2003; Lu et al., 2004; Magg et al., 2024).

Overall, it seems that SGs can either be disassembled by extracting key nucleators to prevent LLPS or be cleared by granulophagy. Interestingly, it appears that cells also developed mechanisms to deal with persistent SGs maintenance by targeting granules to the autophagic pathway in prolonged stress situations. The fact that there is a complementary mechanism to resolve SGs, in situations of chronic stress, seems to suggest that the SGs assembly and disassembly is a process that requires tight regulation for proper cell functions. Moreover, it may indicate that the persistence of stress (and consequently SGs) is a deleterious process for cellular homeostasis.

Chronic stress – is there a link with neurodegenerative diseases?

SGs are dynamically active structures that assemble in response to cellular stress and disassemble when the stress is no longer active. SGs are even able to bi-directionally exchange some components with the cytosol. These observations support the notion that SGs are highly dynamic structures (Jain et al., 2016; Protter and Parker, 2016; Wheeler et al., 2016).

Under chronic stress conditions, such as those observed during the epithelial to mesenchymal transition in cancer or in neurodegenerative diseases like ALS and FTD, SGs' dynamics are thought to be disrupted. In these situations of chronic stress, it was shown that eIF2 α phosphorylation is upregulated, which leads to persistent activation of the ISR and inappropriate SGs formation (Kedersha et al., 1999; Pakos-Zebrucka et al., 2016). However, the link between chronic phosphorylation of p-eIF2 α , chronic stress formation, and how it impacts cellular fate is yet to be determined. A study showed that homozygous cells for an inactive form of eIF2 α , which cannot be phosphorylated, were unable to assemble SGs and, at the same time, survived longer in a context of chronic nutrient deprivation. These observations suggest that overactivation of ISR might be deleterious for cells under chronic stress conditions (Reineke et al., 2018; Reineke and Neilson, 2019).

Although the link between chronic SGs and neurodegenerative disease has not yet been established, this matter has been subject to great interest. Indeed, many studies have been trying to elucidate how ISR and SGs might impact the progression of neurodegenerative diseases, especially those characterized by the accumulation of misfolded proteins within the cytoplasm.

During the formation of misfolded proteins, eIF2 α is phosphorylated by PERK in order to stop translation globally and further prevent the cytosolic accumulation of proteins with aberrant structures (H. P. Harding et al., 2000; Kondratyev et al., 2007; Smith and Mallucci, 2016). At the same time, elements involved in the clearance of misfolded proteins are activated (e.g. chaperones, reticulum-associated protein degradation, autophagy). Once cells are cleared from misfolded proteins, the GADD34-PP1 complex, which was upregulated during ISR, dephosphorylates eIF2 α , and protein synthesis is resumed (Connor et al., 2001; Magg et al., 2024; Novoa et al., 2001). Essentially, this mechanism aims to prevent the endoplasmic reticulum (ER) from being overwhelmed. By arresting translation globally, ER's ability to fold protein is increased, and its capacity to send misfolded proteins for degradation is improved (Smith and Mallucci, 2016; Walter and Ron, 2011).

However, a mechanism that is supposed to prevent the deleterious effects of misfolded protein accumulation seems to have the contrary effect in prolonged stress conditions. Studies have demonstrated that inhibition of p-eIF2 α signaling, which restores protein synthesis, improved survival of prion-diseased mouse models even though it increased prion replication. These findings indicate that, over time, a prolonged stress response aimed at addressing misfolded proteins may produce more harmful effects than allowing those aberrant proteins to be synthesized (Moreno et al., 2013). Similar results were also observed in mouse models of FTD. The Tg4510 mouse, used as a model of FTD and characterized by the overexpression of the human tau mutation P301L, exhibits upregulated p-eIF2 α levels and displays a decrease in global protein synthesis. Although not directly shown, this closely correlates with the formation and functions of SGs (Radford et al., 2015). Interestingly, the onset of the disease was shown to be associated with the increase of p-eIF2 α and global protein arrest. In this model, and mirroring the results observed in the prion disease model, inhibition of the p-eIF2 α signaling resulted in restoration of protein synthesis and decreased neuronal death (Radford et al., 2015). Additionally, it was observed that p-

eIF2 α levels are increased in *post-mortem* brains from patients affected with AD, ALS, FTD, and PD (Hoozemans et al., 2009, 2007; Walter and Ron, 2011).

Another observation that correlates exacerbated SGs formation and increased neuropathology in neurodegenerative diseases emerges from findings that downregulating GADD34 negatively impacts ALS pathology related to TDP-43 (Kim et al., 2014). Previous studies have shown that GADD34 downregulation aberrantly increases the SGs formation, while p-eIF2 α remains upregulated (Ruggieri et al., 2012). In ALS *Drosophila* models, it was demonstrated that *Gadd34* reduction exacerbates TDP-43-associated pathology by impairing the locomotion of flies (Kim et al., 2014).

Altogether, it seems that during situations of chronic stress, the ISR is overactivated through the upregulation of p-eIF2 α . Additionally, low levels of GADD34 also seem to be deleterious in the context of neurodegeneration. Given the observation that modulating ISR via eIF2 α or GADD34 has an impact on some neurodegenerative diseases, it is compelling to hypothesize that SGs dynamics could underlie these observations. Additionally, proteins associated with neurodegenerative disease have already been shown to co-localize with SGs, which, among others, include TDP-43, FUS RNA Binding Protein (FUS), microtubule-associated protein tau (TAU), and ataxin-2. These observations further support the hypothesis that SGs might be dysregulated in these neurodegenerative disorders (Wolozin and Ivanov, 2019).

Stress granules and polyglutamine diseases

The interplay between SGs and polyQ diseases remains to be elucidated. The impact of SGs on the pathogenesis of polyQ proteins is not known. Similarly, whether polyQ proteins can alter SGs dynamics is also unclear. However, some properties of polyQ proteins prompt the hypothesis that they might influence SGs functions.

A key similarity between SGs and polyQ proteins lies in their aggregation, which is driven by IDR domains in both cases (Protter et al., 2018; Protter and Parker, 2016; Trivedi and Nagarajaram, 2022). Interestingly, it is known that IDR-containing proteins, such as those that

typically nucleate (or are present within SGs), are prone to be recruited by proteins containing polyQ expansion (Protter et al., 2018; Wear et al., 2015). In fact, some SGs components have been found within aggregates of pathological proteins linked to polyQ diseases. In the case of HD, using mouse models, the SGs protein TIA-1 was observed to co-localize with aggregates of mutant huntingtin. Similarly, in patients with SCA2, TDP-43 (another SGs component) was detected within aggregates of mutant ataxin-2 (Elden et al., 2010; Furukawa et al., 2009; Marcelo et al., 2021b). These observations suggest that abnormal polyQ expansion might disrupt SGs normal function. Additionally, and as mentioned, chronic activation of the ISR, via p-eIF2 α , due to the presence of misfolded proteins seems to contribute to the pathology of some neurodegenerative disorders. Since mutant polyQ proteins are well known for their propensity to misfold, a similar mechanism might be in play, where an overactivation of ISR mediates pathology by driving SGs persistence (Smith and Mallucci, 2016).

A well-recognized hallmark of polyQ diseases is the impairment of the autophagy pathway (Cortes and La Spada, 2014; Marcelo et al., 2021a; Nisoli et al., 2010). As mentioned above, autophagy is responsible for clearing SGs when stress conditions are chronic (Gwon et al., 2021; Maxwell et al., 2021). The autophagy impairments seen in polyQ disorders, combined with the continued activation of the ISR (due to the buildup of misfolded proteins), may drive the accumulation of persistent SGs by both enhancing their formation and hindering their clearance (Cortes and La Spada, 2014; Gwon et al., 2021; Marcelo et al., 2021a, 2021a; Maxwell et al., 2021; Nisoli et al., 2010). Additionally, the sequestration of chaperones by mutant polyQ aggregates, to counteract aggregation, might also result in abnormal SGs dynamics. Chaperones are known for their role in modulating SGs' fluidity and facilitating their disassembly. In a scenario where mutant polyQ proteins sequester chaperones, they become less available to disassemble SGs (Ganassi et al., 2016; Marcelo et al., 2021a; Sakahira et al., 2002; Yoo et al., 2022). Interestingly, misfolded proteins have been shown to accumulate and form aggregates within SGs. These mutant aggregates are able to transform SGs into rigid, quasi-solid structures, making them more resistant to disassembly (Mateju et al., 2017; Siwach and Kaganovich, 2017).

The function of the polyQ protein ataxin-2, regarding the formation of SGs, has already been detailed in a previous section. Given the fact that ataxin-2 has the propensity to misfold when mutated, it also has the potential to over-activate ISR, which was suggested to be

pathologic in other neurodegenerative diseases (Moreno et al., 2013; Radford et al., 2015). Moreover, as described before, misfolded proteins were shown to accumulate and aggregate within SGs, hindering their disassembly. Given that ataxin-2 facilitates SGs nucleation, recruiting its mutant form, which is prone to misfold and aggregate, can plausibly generate SGs that are resilient to disassembly.

Overall, the link between SGs and polyQ diseases, particularly SCA2, has not yet been established. However, considering the evidence gathered from previous studies and shared mechanisms between some neurodegenerative diseases, there is a strong possibility that inadequate stress responses might impact how SCA2 progresses.

Chapter 2 – Objectives

Objectives

Currently, the role of stress granules (SGs) in the context of neurodegenerative diseases is not known. Several studies have demonstrated that the integrated stress response (ISR) is overactivated in several neurodegenerative diseases, which correlates with impaired SGs homeostasis. However, in the context of spinocerebellar ataxia type 2 (SCA2), the role of the ISR, and in particular SGs, was never explored.

Thus, the main objective of this work is to contribute to the understanding of the impact of SGs in the context of SCA2. This work investigated whether SGs contribute to the pathogenesis of SCA2 and might constitute a potential therapeutic target.

We first aimed to develop genetic tools capable of either inducing SGs formation or inhibiting their assembly. To achieve this goal, we looked to the ISR pathway and key SGs components, and designed genetic tools that could induce SG assembly or, conversely, prevent their formation. Then, we used primary cultures of mouse striatal neurons to understand the impact of manipulating SGs dynamics in the context of the mutant ataxin-2, and studied how it affected cells' morphology.

Next, to understand the impact of manipulating SGs dynamics in a more disease-relevant context, we used an SCA2 lentiviral mouse model previously developed by our lab. Using this model, we induced and inhibited the formation of SGs *in vivo* and studied how it impacted neuropathology.

Since most existing SCA2 mouse models are either unavailable or exhibit mild pathological features, such as mild or late-onset phenotypes, which limit their reliability in research, we aimed to develop a novel SCA2 mouse model with more robust and relevant disease characteristics, which could better translatable to the human context. To achieve this, we used mice expressing a transgene that comprises a mutant form of the human *ATXN2* gene bearing 129 CAG repeats (codes for 129 glutamines). Transgenic mice were behaviorally and neuropathologically characterized.

Finally, we aimed to understand how SGs impacted the behavioral traits and neuropathology associated with the newly developed SCA2 mouse model. For that, we manipulated the assembly and disassembly of SGs *in vivo* and submitted animals to behavioral tests and histopathological studies.

Chapter 3 – Materials and methods

Plasmid design and validation

All plasmids used were designed by the authors, and their production was outsourced to VectorBuilder (<https://www.vectorbuilder.com/>).

eIF2 α ^{S52D}–mCherry (SGs induction)

The plasmid used to induce SGs formation is named eIF2 α ^{S52D}–mCherry (or abbreviated eIF2 α ^{S52D}). It comprises a phosphomimetic form of the *eIF2 α* gene, in which the triplet that codes for the amino acid serine at position 52 (TCC) was replaced by a triplet that codes for the amino acid aspartate (GAC). eIF2 α ^{S52D} is under the control of the promoter PGK1. The plasmid also comprises a sequence encoding for the fluorescent protein mCherry, which is under the control of the promoter CMV. Both sequences are inserted within the AAV1 backbone.

TIA1–mCherry (SGs induction)

The plasmid used to alternatively induce SGs formation is named TIA1–mCherry. It comprises a gene sequence encoding for *TIA1*. The plasmid also comprises a sequence encoding for the fluorescent protein mCherry, which is under the control of the promoter CMV. Both sequences are inserted within the AAV1 backbone.

GADD34 Δ ³²²–mCherry (SGs inhibition)

The plasmid used to induce SGs formation is named GADD34 Δ ³²²–mCherry (or abbreviated GADD34 Δ ³²²). It comprises a truncated form of the *GADD34* gene, in which the nucleotide sequence that encodes for the first 322 amino acids was removed. GADD34 Δ ³²² is under the control of the promoter PGK1. The plasmid also comprises a sequence encoding for the fluorescent protein mCherry, which is under the control of the promoter CMV. Both sequences are inserted within the AAV1 backbone.

CT-mCherry

The plasmid used as control is named CT-mCherry. It comprises the luciferase gene sequence. The plasmid also comprises a sequence encoding for the fluorescent protein mCherry, which is under the control of the promoter CMV. Both sequences are inserted within the AAV1 backbone.

shRNA targeting TIA1

The plasmids used to express the shRNAs targeting TIA1 named shTIA1#1 or shTIA1#2 are termed shTIA1#1-mCherry or shTIA1#2-mCherry, respectively. shTIA1#1 sequence is under the control of the U6 promoter. shTIA1#2 sequence is under the control of the U6 promoter. Both plasmids also comprise a sequence coding for the fluorescent protein mCherry, which is under the control of the promoter CMV. Constructs are inserted into AAV1 backbone.

The plasmid used as control for the shRNAs is named shCT-mCherry. Regarding its design, shCT-mCherry is similar to shTIA1#1-mCherry and shTIA1#2-mCherry, except the shRNA sequence. The shCT, does not have any known human or mice target.

shTIA1#1 sequence:

CGATGGTGGATGTTTGCCAATCTCGAGATTGGCAAACATCCACCATCG

shTIA1#2 sequence:

CATGCGATTGTTTCTGTTAATCTCGAGATTAACAGAAACAATCGCATG

CT sequence:

CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG

ataxin-2^{WT(23Q)}–GFP

The plasmid used to express the WT, no-expanded form of the human *ATXN2* gene, is named ataxin-2^{WT(23Q)}–GFP (or abbreviated WT(23Q)). It comprises a human *ATXN2* sequence bearing a CAG tract of 23 CAG repetitions (encodes for 23 glutamines (Q)), within the first exon. At the end of the ataxin-2^{WT(23Q)} sequence, an HA-tag was inserted. Ataxin-2^{WT(23Q)} sequence is under the control of the promoter PGK1. The plasmid also comprises a sequence coding for the fluorescent protein GFP, which is under the control of the promoter CMV. Both sequences are inserted within a lentiviral backbone.

ataxin-2^{MUT(80Q)}–GFP

The plasmid used to express the mutant, expanded form of the human *ATXN2* gene is named ataxin-2^{MUT(80Q)}–GFP (or abbreviated MUT(80Q)). It comprises a human *ATXN2* sequence with a CAG tract of 80 CAG repetitions (encodes for 80 glutamines (Q)), within the first exon. At the end of the ataxin-2^{MUT(80Q)} sequence an HA-tag was inserted. Ataxin-2^{MUT(80Q)} sequence is under the control of the promoter PGK1. The plasmid also comprises a sequence encoding for the fluorescent protein GFP, which is under the control of the promoter CMV. Both sequences are inserted within a lentiviral backbone.

Plasmid amplification

For all plasmids, transformed bacteria were ordered from VectorBuilder. The cloning host used was “VB ultrastable” *Escherichia coli*. Transformed bacterial stocks were picked, plated and incubated overnight at 37°C in dishes containing lysogeny broth agar (LB agar) – 10% (w/v) tryptone, 5% (w/v) yeast extract, 15% (w/v) agar – (NZYTech, Portugal). Next day, colonies were picked and grown in LB at 37°C with rotation overnight. Plasmids were purified using NZYMaxiprep kit (NZYTech, Portugal), following the manufacturer’s recommendations. DNA concentration and purity were assessed using NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, USA).

Enzymatic restriction of plasmids

Purified plasmids were subjected to enzymatic restriction to verify whether generated DNA fragments matched the predicted sizes. The plasmids: eIF2 α ^{S52D}–mCherry, TIA1–mCherry, GADD34^{A322}–mCherry CT–mCherry, shTIA1#1–mCherry and shTIA1#2–mCherry, were digested using the enzymes FastDigest *Xba*I and FastDigest *Pvu*I (ThermoFisher, USA). The plasmids ataxin-2^{WT(23Q)}–GFP, and ataxin-2^{MUT(80Q)}–GFP were digested using the FastDigest *Kpn*I and the FastDigest *Nhe*I enzymes (ThermoFisher, USA). Plasmid digestion was performed following the manufacturer's instructions. Digested plasmids were submitted to a 2% (w/v) agarose gel electrophoresis during 45 minutes at 90 V. After electrophoresis, gels were scanned using the iBright™ CL750 Imaging System (ThermoFisher, USA).

Cell culture and transfections

Cell lines

Mouse neuroblastoma (N2a) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% (v/v) fetal calf serum (FCS), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 44 mM of NaCO₃ and 1% (v/v) penicillin-streptomycin at a pH 7.2. Cells were incubated in 75 cm² flasks at 37°C, with a humidified atmosphere containing 5% CO₂. Media was changed every 3/4 days and cells were split upon reaching a confluence of 80%-90%.

The day prior to transfections, N2a cells were seeded and incubated for 24h in 12-multiwell plates containing 0.1% (w/v) porcine gelatin-coated glass coverslips. Next day, cells were transfected with 500 ng of plasmid DNA using 3 μ l of polyethylenimine (PEI, 1 mg/ml, Polysciences Inc., USA). After 48h, cells were 3x rinsed with cold phosphate buffered saline (PBS) and fixed with fixed with 4% (w/v) paraformaldehyde in PBS (PFA/PBS) for 15 min. When direct fluoresce was assessed, without immunochemistry, cells were rinsed 3x with PBS, 10 minute each, and the coverslips were mounted on glass microscope slides with Fluoromount-G™ Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, USA).

Primary cultures of mice striatal neurons

C57BL/6 mice at P0 were decapitated, and the striatum was isolated and cleaned in complete dissociation media (DM) – 81.8 mM of Na₂SO₄, 30 mM of K₂SO₄, 5.85 mM of MgCl₂, 5.85 mM of MgCl₂, 250 µM of CaCl₂, 1 mM of HEPES, 0.2 % (v/v) of phenol red, 20 mM of glucose, 10.48 mM of kynurenic acid and 100 mM of MgCl₂ in ddH₂O. Isolated striata were incubated at 37°C, 2x for 20 minutes in 3 ml of enzymatic solution (EZ) – 10 U/ml of papain latex (Cell Systems GmbH, Germany) and 450 µg/ml of L-cysteine in complete DM with pH adjusted to 7.5 – 8.0 using NaOH. Every 5 minutes, striata were carefully swirled. Enzymatic digestion was stopped with 3 washes, 1 minute each, with 10 mg/ml of trypsin inhibitor (Sigma-Aldrich, USA) in DM at 37°C with the pH adjusted to 7.5 – 8.0 using NaOH. Then, striata were washed 3x, 1 minute each, with 2 ml of growth media (GM) – 97% (v/v) of Neurobasal™-A Medium (Gibco Scientific- Thermofisher, USA), 2% (v/v) of Rat Serum – S2150 (Biowest, USA), 0.5 mM of L-Glutamine and 0.5% (v/v) of penicillin-streptomycin. Next, to dissociate cells, striata were triturated 50x using 1000 µl pipette (up&down), in 15 ml conic tube with 2 ml of GM. The dissociation process was repeated 3x. After each dissociation, 2 ml of GM were added to the tube and the mixture was let to rest for 5 minutes before collecting 2 ml GM's superior portion containing the cells in suspension. Collected cells were counted by using a Neubauer chamber loaded 9 µl of the cell suspension diluted in 1 µl of trypan blue. Counted cells were diluted in GM to a final concentration of 0.25 million cells/ml and plated on 12-multiwell plates containing poly-D-lysine and laminin-coated glass coverslips. Cells were maintained 37°C, with a humidified atmosphere containing 5% CO₂.

To block proliferation of glia cells, plated cells were treated at 3 days *in vitro* (DIV3) with 2.8 µM of cytosine-D-arabino-furanoside (Ara-C) (Sigma-Aldrich, USA). At DIV8, cells were transfected with 1.3 µg of plasmid DNA using 2.5 µl of Lipofectamine 2000 (Invitrogen, USA). Separately, using polystyrene tubes, DNA and lipofectamine were diluted in 50 µl of transfection media (TM) – 7.2 µM of insulin, 7.2 µM of transferrin, 2.9 µM of sodium selenite, 10% (v/v) of minimum essential medium (MEM) with Earle's salts, without L-glutamine (Gibco Scientific-Thermofisher, USA), 0.5% (v/v) of penicillin-streptomycin, 114 mM of NaCl, 26.08 mM of NaHCO₃, 5.292 mM of KCl, 10 mM of HEPES, 1 mM of glycine, 30 mM of D-glucose, 0.5 mM of Na-pyruvate, 0.001% (v/v) of phenol red, 1 mM of MgCl₂, 2 mM of CaCl₂. Both the

diluted DNA and diluted Lipofectamine were incubated for 30 minutes before mixed them into a single polystyrene tube and add 400 μ l of TM. After removing 50% of media from plated cells, the transfection mixture was applied to cells dropwise and cells were incubated for 2.5h. After incubation all media was replaced by TM and 48h post transfection, all media was removed, and cells were fixed with 4% (w/v) paraformaldehyde plus 4% (w/v) sucrose in PBS for 20 min. When direct fluorescence, without immunochemistry, was assessed, cells were rinsed 3x with PBS for 10 minutes each. Coverslips were mounted on glass microscope slides with previously prepared MOWIOL® 4-88 Reagent mounting media. MOWIOL® 4-88 Reagent was prepared according to manufacturer's recommendations.

Pharmacological induction of stress granules

Pharmacological stress granules induction was carried using 0.5 μ M of sodium arsenite diluted in cell's media. N2a cells were treated with sodium arsenite 48h after transfection for 1h. Then, cells were immediately fixed with 4% (w/v) paraformaldehyde for 10 minutes, and then rinsed 3x with PBS, for 10 minutes each. Cells were then immunoassayed.

Immunocytochemistry

Cell lines

After fixation, cells were rinsed 3x with cold PBS and permeabilized with 0.2% (v/v) of Triton X-100™ (Thermo Fisher, USA) in PBS for 5 min at RT. Permeabilized cells were rinsed 3x with PBS and blocked with 3% (w/v) of BSA in PBS for 1h RT. Next, cells were incubated with the primary antibody (Table M1), at 4°C overnight. The next day, cells were rinsed 3x with PBS and incubated with the respective secondary antibody (Table M1) for 2h at RT. After incubation, cells were rinsed 3x with PBS, 10 minutes each, and the coverslips were mounted on glass microscope slides with Fluoromount-G™ Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, USA).

Primary cultures of mice striatal

After fixation, cells were rinsed 3x with PBS at RT and incubated with the primary antibodies (Table M1) overnight at 4°C. Next day, cells were rinsed 3x with PBS for 10 minutes each, and incubated with the secondary antibody (Table M1) for 1h at RT. After incubation cells were rinsed 3x with PBS for 10 minutes each, and incubated with 2 µg/ml of Hoechst for 5 minutes at RT. Then, cells were rinsed 3x with PBS for 10 minutes each. Coverslips were mounted on glass microscope slides with previously prepared MOWIOL® 4-88 Reagent mounting media. MOWIOL® 4-88 Reagent was prepared according to the manufacturer's recommendations.

Table M1 - Antibodies used in immunocytochemistry

Primary antibodies – Cell lines			
Marker	Name	Dilution	Reference
Stress granules	Rabbit anti-G3BP1	1:1000	Proteintech 13057-2-AP
HA-tag (ataxin-2)	Rabbit anti-HA	1:1000	Abcam #ab9110
Secondary antibodies – Cell lines			
	Name	Dilution	Reference
	AlexaFluor® 488 anti-rabbit	1:400	Invitrogen #A11008
	AlexaFluor® 594 anti-rabbit	1:400	Invitrogen #A11012
Primary antibodies – Primary cultures of mice striatal			
Marker	Name	Dilution	Reference
Striatal Neurons	Mouse anti-DARPP32	1:500	Abcam #ab40801
Neurons	Rabbit anti-NeuN	1:500	Sigma-Aldrich #ABN78
Secondary antibodies — Primary cultures of mice striatal			
	Name	Dilution	Reference
	AlexaFluor® 488 anti-mouse	1:200	Invitrogen #A11001
	AlexaFluor® 594 anti-rabbit	1:200	Invitrogen #A11005

Animal experiments

Care, maintenance and well-being

All animals used in this work were housed in our Animal Facility (ABC-RI Animal Facility). The 3Rs principles were applied, and none of the mice were submitted to unnecessary manipulations. When mice were manipulated, it was ensured that the techniques used exerted the minimum stress and minimum discomfort as possible. Mice were housed in a room with controlled temperature with a cycle of 12h of light and 12h of darkness. Food and water were provided *ad libitum*. All the experiments were conducted according to the directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for experimental and other scientific purposes. The researchers who manipulated the animals have a valid certifier training (FELASA), and the experiments were approved by the Portuguese authority for animal health and protection – Direção Geral de Alimentação e Veterinária – under the Project Neuropath (421/2019).

Transgenic mice generation

A novel transgenic SCA2 mouse model was developed and named hATXN2^{MUT(129Q)} or abbreviated as TG^{MUT(129Q)}. The transgene cassette was custom-designed following the recommendations for creating transgene constructs, and it was synthesized by GeneScript (Haruyama et al., 2009). The transgene cassette encodes a codon-optimized version of the human *ATXN2* gene bearing 129 repetitions of the trinucleotide CAG (codes for 129 glutamines). The transgene is under the control of the L7-6 promoter (Nitta et al., 2017; Tomomura et al., 2001). It also includes an SV40 intron, a Kozak consensus sequence, and an SV40 poly(A) signal intron. The plasmid containing the transgene cassette was linearized and 1-2 ng/μl introduced into fertilized oocytes from B6:129 mixed-background mice via pronuclear microinjection, the embryos were then implanted into Swiss/CD1 foster mothers.

Apart from the transgene cassette design, all the described procedures were outsourced to the i3S Institute. After weaning, the resulting founder (F0) mice were transferred to the ABC-RI Animal Facility, where they were housed and bred. F0 animals were backcrosses with WT C57/BL6 mice to produce the F1 offspring. The transgene integration was then confirmed by PCR and agarose gel resolving. To phenotypically characterize the transgenic mouse lines, we congenic crosses F1 vs. F1 heterozygous transgenic mice, which originated the F2 generation.

Transgenic mice genotyping

Genotyping of transgenic mice was carried using 2 mm of ear tissue punches, which were digested, the DNA extracted and purified using the GeneJet Genomic DNA Purification Kit (#K0722 ThermoFisher, USA), according to the manufacturer's instructions. DNA concentration and purity were assessed using NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, USA). Transgene and genomic DNA amplification was performed by polymerase chain reaction (PCR) assay, using specific primers (Table M2). The PCR was performed using the DreamTaq DNA Polymerase (ThermoFisher, USA) in a C1000 Touch Thermal Cycler (BioRad, USA) according to the protocol in Table M2.

Table M2 – Primers, mix and the protocol used to genotype transgenic mice

Primers			
Target	Name	Sequence	
Transgene	Primer F	AAATTCTGCAGACCCGTGAG	
	Primer R	TCTGTCTGCTTGGTGGAGGT	
Endogenous Genomic DNA	Primer CF	TGACTTGGGCAGAAATGGGA	
	Primer CR	TGCCACTGAAACACGTCAGGTA	
PCR reaction mix			
Reagent		Quantity	
10X Taq Buffer		2.5 µl	
dNTP Mix, 2 mM each		0.5 µl	
Primer F 10 µM		1 µL	
Primer R 10 µM		1 µL	
Primer CF 10 µM		1 µL	
Primer CR 10 µM		1 µL	
Template DNA		100 ng	
Taq DNA Polymerase 1.25 U		0.25 µl	
Water, nuclease-free		up 25 µl	
PCR cycle			
Step	Temperature (°C)	Time	Cycles
Initial denaturation	95	3 minutes	1x
Denaturation	95	30 seconds	30x
Annealing	68.5	30 seconds	
Extension	72	45 seconds	
Final extension	72	5 minutes	1x

Stereotaxic injection

a. SCA2 lentiviral mouse model

For the experiments using the SCA2 lentiviral mouse model, C57BL/6J mice were first anesthetized by intraperitoneal injection using a pharmacologic mixture of 0.67 mg/kg of medetomidine, 3.3 mg/kg of midazolam and 33 µg/kg of fentanyl. Mice were injected at 8 weeks of age, into the striatum according to the coordinates relative to bregma: antero-posterior (+0.6 mm), medial-lateral (-/+1.8 mm), and dorsal-ventral (-3.3 mm) (Marcelo et al., 2021a). Lentivirus packaging and AAV1 packaging were outsourced to VectorBuilder. In the

left striatal hemisphere, mice were injected with a mixture of 2 μ l 1×10^{13} GC/ml of AAV1 particles encoding for the CT-mCherry plus 2 μ l 10^9 TU/ml of lentiviral particles encoding for the ataxin-2^{MUT(80Q)}-GFP. In the right striatal hemisphere mice were injected with a mixture of 2 μ l 10^9 TU/ml of lentiviral particles encoding for the ataxin-2^{MUT(80Q)}-GFP plus, either with 2 μ l 1×10^{13} GC/ml of AAV1 particles encoding for the eIF2 α ^{S52D}-mCherry or 2 μ l 1×10^{13} GC/ml of AAV1 particles encoding for the GADD34 ^{Δ 322}-mCherry. Mice were injected at a rate of 0.20 μ l/minute with an automatic injector (Ugo Basile, Italy) using a 34-gauge blunt-tip needle linked to a Hamilton syringe. Mice were sacrificed 12 weeks post-injections.

b. SCA2 transgenic mouse model

For the experiments using the novel SCA2 transgenic mouse model, animals were first anesthetized by intraperitoneal injection using a pharmacologic mixture of 0.67 mg/kg of medetomidine, 3.3 mg/kg of midazolam and 33 μ g/kg of fentanyl. Mice were injected at 4 weeks of age, into the cerebellum according to the coordinates relative to lambda: antero-posterior (-1.6 mm), medial-lateral (0 mm), and dorsal-ventral (-2.9 mm and -2.4 mm). AAV1 packaging was outsourced to VectorBuilder. Mice were injected either with CT-mCherry, or eIF2 α ^{S52D}-mCherry or GADD34 ^{Δ 322}-mCherry. All mice were injected with 6 μ l of the respective AAV1 particles (3 μ l at dorsal-ventral (-2.9 mm) + 3 μ l at dorsal-ventral (-2.4)) at the concentration of 1×10^{13} GC/ml. Mice were injected at a rate of 0.25 μ l/minute, with an automatic injector (Ugo Basile.) using a 34-gauge blunt-tip needle linked to a Hamilton syringe. Mice were sacrificed 17 weeks post injection.

Behavioral testing

a. Motor performance of the transgenic hATXN2^{MUT(129Q)} mouse model

To assess the motor performance of the transgenic hATXN2^{MUT(129Q)} mouse model, mice were submitted to battery of behavioral tests at 4 weeks of age. Test were repeated every four weeks until mice were 44 weeks old (Line 36) or 24 weeks of (Line 40).

Mice weight was monitored and registered every 4 weeks.

Mice were submitted to the grip strength test using the 47200 GSM Grip Strength Meter (Ugo Basile, Italy). The grip strength was measured by gently holding the tail of the mice and pulling the animals while they gripped a ring. The peak force exerted on the ring was then measured. Three trials were performed with 3-5 minutes interval. The mean peak force of the three trials was normalized for the respective mice weight.

Mice were submitted to the accelerated rotarod test using the 47650 Rota-Rod NG (Ugo Basile, Italy). Mice were placed on the rotating rod at a speed of 4 rotation per minute (rpm) that were accelerated up to 40 rpm in 5 minutes. The time that mice remained in the apparatus without falling (latency to fall) was recorded. Three trials were performed with 10-15 minutes interval between each trial. The mean of latency to fall of the three trials was used for statistical.

Mice were submitted to the beam walking test using a 50 cm long horizontal squared beam with 1.8 cm or 1 cm width and a cylindrical beam with 1 cm diameter. Mice performed 3 trials per beam with 10-15 minutes interval between each trial. The means of the time taken by the animals to traverse the beams was used for statistically analysis.

Mice were submitted to the swimming test using a rectangular water-filled tank with 10 cm width and 1.5 m long. The water used was at room temperature. Mice were placed at one end of the water-filled tank and swam freely until reaching the other end where a water-level platform was placed. Mice performed 3 trials with 10-15 minutes interval between each trial. The means of the time taken by the animals to traverse the water-filled tank to reach the platform was used for statistically analysis.

b. Motor performance of transgenic hATXN2^{MUT(129Q)} animals subjected to *in vivo* SGs modulation

At 5 weeks of age (1 week post injection) transgenic mice were submitted to battery of behavioral tests to access their motor performance. Tests were repeated every four weeks until mice were 21 weeks old.

Mice weight was monitored and registered every 4 weeks.

Mice were submitted to the grip strength test using the 47200 GSM Grip Strength Meter (Ugo Basile, Italy). The grip strength was measured by gently holding the tail of mice and pulling the animals while they gripped a ring. The peak force exerted on the ring was then measured. Three trials were performed with 3-5 minutes interval. The mean peak force of the three trials was normalized for the respective mice weight.

Mice were submitted to the accelerated rotarod test using the 47650 Rota-Rod NG (Ugo Basile, Italy). Mice were placed on the rotating rod at a speed of 4 rotation per minute (rpm) that were accelerated up to 40 rpm in 5 minutes. The time that mice remained in the apparatus without falling (latency to fall) was recorded. Three trials were performed with 10-15 minutes interval between each trial. The mean of latency to fall of the three trials was used for statistical analysis.

Mice were submitted to the walking test, beam walking test and swimming test using the MotoRater system. The MotoRater system allows the characterization of mice's kinetic profile while performing the tests. This system is composed of a set of mirrors and a camera that records movement while mice traverse the test lane (1.7 meters). The mirror systems enable the recording of mice's left, right, and bottom sides. For the walking test, mice are placed on the test lane and freely traverse it. For the beam walking test, mice were placed on cylindrical beam with 0.5 cm diameter and freely traverse it. For the swimming test mice were placed in the water (testing lane filled with water) and freely traverse it. For each test, mice's movement was recorded at 100–200 frames per second, using the Simi Motion software 10.3.0.250 and the kinetic profile was analyzed using MotoRater TSE Automator software 1.0. Movement is analyzed and measured in each frame while mice are moving. One trial was performed in the walking test, three trials were performed for the beam walking test, and three trials were performed for the swimming test, with 10-15 minutes interval between each trial. For statistical analysis, for each kinetic parameter, it was used the measurements recorded at each frame divided by the total number of frames. We measured 35 kinetic parameters for each test, including: time to traverse, step width (hind), step width (front), step length (left), step length (right), toe clearance forepaw (right), toe clearance hindpaw (right), toe clearance forepaw (left), toe clearance hindpaw (left), iliac crest height (right), iliac crest height (left), hip height (right), hip height (left), tail base height (right), tail base height (left),

protraction/retraction (right), protraction/retraction (left), nose height, tail tip height, head rotation, hip angle (right), knee angle (right), ankle angle (right), hip angle (left), knee angle (left), ankle angle (left), tail angle (side view), front right paw velocity, front right paw acceleration, front left paw velocity, front left paw acceleration, hind right paw velocity, hind right paw acceleration, hind left paw velocity, hind left paw acceleration. For simplicity we only presented measurements with statistical or near statistical significance.

Brain tissue collection

For histological analysis mice were euthanized by an intraperitoneal injection of a pharmacological mixture of 75 mg/kg of Ketamine (Nimatek, Dechra, UK) and 0.75 mg/kg of medetomidine (DOMTOR®, Orion, Finland). Mice were then transcardiacally perfused with 4% (w/v) paraformaldehyde in PBS, pH 7. Whole brains were removed incubated for 24h in 4% (w/v) PFA in PBS and then 48h in 20% (w/v) sucrose in PBS. Tissue was then frozen at -80 °C.

For RNA extraction and purification mice were sacrificed by cervical displacement and the cerebellum was removed and directly frozen at -80 °C.

RNA sequencing

Differently expression analysis was performed by comparing: (TG^{MUT(129Q)}+CT / TG^{MUT(129Q)}+Non-injected); (TG^{MUT(129Q)}+CT / TG^{MUT(129Q)}+eIF2^{αS52D}) and (TG^{MUT(129Q)}+CT / TG^{MUT(129Q)}+GADD34^{Δ322}). Transgenic hATXN2^{MUT(129Q)} mice were injected (according to previous description) at 4 weeks of age and sacrificed at 14 weeks of age (10 weeks expression). To consider differentially expressed genes, we established a cutoff of $|\geq 2|$ -fold change (for upregulation and downregulation) with $p < 0.05$.

RNA from mice cerebella was extracted and purified using the RNeasy® Plus Mini kit (Qiagen, Germany) according to the manufacturer's recommendations. RNA concentration and purity were assessed using NanoDrop One Microvolume UV-Vis Spectrophotometer

(Thermo Scientific, USA). RNA integrity was measured using the Qubit™ 4 Fluorometer (Invitrogen, USA) with the Qubit RNA Integrity and Quality (IQ) Assay Kit (Thermo Scientific, USA) according to manufacturer's recommendations.

The RNA sequence and all data analysis was outsourced to MacroGen Inc. (www.macrogen.com). Before analysis, quality control of the samples was carried out using the Illumina TruSeq Stranded mRNA kit. After, the RNA samples were sequenced with the Illumina NovaSeqX instrument using 2x150 cycles (paired end reads). The raw-sequencing data underwent quality check with FastQC v0.11.7, followed by read trimming with Trimmomatic 0.38. Trimmed data was then mapped to the reference genome GRCm39 with HISAT2 v2.1.0. Finally, known transcripts were assembled with StringTie v2.1.3b. The transcripts quantity was calculated using the read counts normalized values as Fragments Per Kilobase of transcript per Million mapped reads (FPKM) and Transcripts Per Kilobase Million (TPM).

Curated RNA sequencing data was analyzed using RStudio 2024.04.1 Build 748. Differential expression analysis was performed using the DESeq2 R package, which models normalized gene count data with a negative binomial generalized linear model to account for overdispersion commonly observed in RNA-seq experiments (Love et al., 2014). The variability within each group is modeled by a gene-specific dispersion parameter, which is estimated using Bayesian shrinkage methods to improve accuracy. Log2 fold changes (LFCs) are also modeled through Bayesian shrinkage, applying a zero-centered normal prior, to reduce the likelihood of low-count genes exhibiting overestimated LFCs. The shrunken LFCs are then subjected to Wald significance testing, and the resulting p-values are adjusted for multiple comparisons using the Benjamini-Hochberg procedure, with a default target false discovery rate (FDR) of 0.1. DESeq2 automatically determines an optimal filtering threshold to maximize the number of differentially expressed genes identified at the specified FDR.

Gene ontology (GO) enrichment analysis was carried using the GOstar R package (Falcon and Gentleman, 2007). The analysis assesses whether a specific GO term (representing biological processes, molecular function or cellular components) are statistically overrepresented in the list of differentially expressed genes. This approach enables the identification of biologically meaningful patterns and insights from transcriptome-wide gene

expression data. All analyses were applied to the set of genes found to be differentially expressed in the comparisons made.

Histological processing and staining

SCA2 lentiviral mouse model

For the experiments using the lentiviral mouse model, frozen fixed brains were sectioned in a coronal orientation with a thickness of 25 μ M using the Cryostar NX50 (Thermofisher, USA). Brain sections were collected and stored free-floating at 4°C in plates containing 0.05% sodium azide in PBS.

For each mouse, six central coronal sections were used for immunostaining. Mice sections were rinsed 3x in PBS, 10 minutes each. Then, sections were incubated in 0.1% (v/v) of diphenylhydrazine in PBS for 30 min at 37°C. Next, sections were rinsed 3x in PBS, 10 minutes each, and then blocked in 10% (v/v) of normal goat serum (Gibco, USA), 0.1% (v/v) of Triton X in PBS, for 1h at RT. Then, sections were incubated in with the primary antibody (Table M3) overnight at 4°C. Next day, sections were incubated with the respective secondary antibody (Table M3) for 2h at RT. Signal detection was performed using Vectastain ABC peroxidase kit (Vector Laboratories, USA) for 40 min, at RT, followed by incubation with 3,3'-diaminobenzidine (DAB) substrate kit (Vector Laboratories, USA). Next, sections were mounted on porcine gelatin-coated microscope slides and dehydrated using ethanol solutions with increasing concentrations of 75% (v/v), 96% (v/v) and 100% (v/v), and treated with xylene substitute (Sigma-Aldrich, USA), each for 3 minutes. Mounted slices were covered with a coverslip containing Eukitt® (Kindler, Germany).

Transgenic hATXN2^{MUT(129Q)} mouse model

For the experiments using the transgenic hATXN2^{MUT(129Q)} animals frozen fixed brains were sectioned in a sagittal orientation with a thickness of 30 μ M using the Cryostar NX50 (ThermoFisher, USA). Brain sections were collected and stored free-floating at 4°C in plates containing 0.05% sodium azide in PBS.

For cresyl staining eight mid-sagittal sections from each mouse were mounted in gelatin-coated microscope slides and left drying overnight. Then, sections were stained with cresyl violet, passing them sequentially through water, ethanol 96% (v/v), ethanol 100% (v/v), xylene, ethanol 75% (v/v) 5 minutes each and the 0.5% (w/v) cresyl violet solution, 5 minutes each. Sections were then washed in water and sequentially passed through ethanol 75% (v/v), ethanol 96% (v/v), ethanol 100% (v/v) and xylene 3 minutes each. Slides containing dyed sections were then mounted with Eukitt (Sigma-Aldrich, USA).

For immunohistochemistry staining, eight mid-sagittal sections from each mouse were used. Mice sections were rinsed 3x in PBS, 10 minutes each, and then blocked in 10% (v/v) of normal goat serum (Gibco, USA), 0.1% (v/v) of Triton X in PBS, for 1h at RT. Then, sections were incubated in with the primary antibody (Table M3) overnight at 4°C. Next day, sections were incubated with the respective secondary antibody (Table M3) for 2h at RT. The next day, sections were rinsed 3x with PBS, a then incubated with the respective secondary antibody for 2h at RT. Finally, sections were mounted on porcine gelatin-coated microscope slides and covered with a coverslip containing Fluoromount-G™ Mounting Medium with DAPI (Invitrogeb, USA).

Table M3 - Antibodies used in immunohistochemistry

Primary antibodies – Lentiviral model			
Marker	Name	Dilution	Reference
Striatal Neurons	Rabbit anti-DARPP32	1:1000	Merk Milipore #AB10518
HA-tag (ataxin-2)	Rabbit anti-HA	1:1000	Abcam #ab9110
Secondary antibodies – Lentiviral model			
	Name	Dilution	Reference
	Biotinylated anti-rabbit	1:200	Vector Laboratories #BA-1000
Primary antibodies – Transgenic hATXN2 ^{MUT(129Q)}			
Marker	Name	Dilution	Reference
Purkinje cells	Mouse anti-Calbindin-D-28K	1:1000	Sigma #C9848
Secondary antibodies – Transgenic hATXN2 ^{MUT(129Q)}			
	Name	Dilution	Reference
	AlexaFluor® 488 anti-mouse	1:500	Invitrogen #A11001

Microscopy and data analysis

To quantify the stress granules, 5 fields of view were randomly and blindly acquired on the mCherry channel to avoid bias. Then the number of transfected cells forming aggregates was counted. The percentage of cells forming aggregates was calculated using the following formula: $\frac{n^{\circ} \text{ of transfected cells with SGs}}{\text{total } n^{\circ} \text{ of transfected cells}} \times 100$, and the mean was used for statistical analysis. The surface area of stress granules was measured only in cells that formed stress granules, and the mean was used for statistical analysis. The number of stress granules per cell was measured only in cells that formed stress granules, and the mean was used for statistical analysis. Images were acquired in Zeiss LSM710 confocal microscope (Carl Zeiss, Germany).

To quantify the total dendritic length using Sholl analysis, a minimum of 4 neurons from 3 independent preparations, were randomly and blindly acquired on the mCherry and GFP channels. Images were acquired 48h after transfection, using a Leica TCS SP2 or SP8 laser-scanning confocal microscope with a resolution of 1024x1024 pixels with 0.172 μm /pixel. For each neuron, a z-stack including the entire cell was acquired. The morphometric analysis was performed by importing z-stack to FIJI software 2.16.0. Z-projections were used to manually

trace dendrites using the FIJI software 2.16.0 and the FIJI SNT plugin software 4.3.0 (Longair et al., 2011). The mean of total dendritic sum was used for statistical analysis. For the Sholl analysis, the central ring was placed at the center of the soma and subsequent rings were spaced 5 μm using the FIJI SNT plugin software 4.3.0 (Longair et al., 2011; Sholl, 1953). Then the number of intersections was measured by the sum of all intersections between the traced neurons and the rings at a specific distance from the soma. The mean of the total number of intersections at a specific distance was used for statistical analysis.

To quantify the number HA aggregates (ataxin-2) or the DARPP-32 marker depletion volume, six central coronal sections images were acquired using the Axio Scan.Z1 Slide Scanner microscope (Carl Zeiss, Germany). For all animals, images were analyzed using the ZEISS ZEN lite software 3.4. HA aggregates (ataxin-2) were counted within the striatum and multiplied the total distance between sections of 150 μm . The total DARPP-32 marker depletion volume was measured within the striatum and multiplied the total distance between sections of 150 μm according to the formula *Depletion volume* = $(a1 + a2 + a3 + a4 + a5 + a6) \times 150$, where a=depletion area of section.

To quantify the total number of Purkinje cells eight-mid sagittal sections were used covering a central distance of 240 μm , and images were acquired using the Axio Scan.Z1 Slide Scanner microscope (Carl Zeiss, Germany). The total number of Purkinje cells was counted within the mCherry expression area of each experimental group for each section, using the ZEISS ZEN lite software 3.4. The mean total number of Purkinje cells was used for statistical analysis.

To quantify the thickness of each cerebellar lobule, we used eight-mid sagittal sections covering a central distance of 240 μm , and images acquired using the Axio Scan.Z1 Slide Scanner microscope (Carl Zeiss, Germany). For each section both molecular and granular layers of each cerebellar lobule were measured using the ZEISS ZEN lite software 3.4. All cerebellar lobules were measured, but for simplification only statistical or near statistically significant alterations were presented. The mean thickness of each layer of each lobule was used for statistical analysis.

Representative images and schematic representation

All microcopy representative images are original and acquired during this work. All graphics were obtained from original data and drawn using GraphPad 10 software (GraphPad Software, USA).

All schematic representations were envisioned and drawn by André Conceição using Affinity designer 2 software 2.6.2. Exceptions: Figure R14 was adapted from (Koppenol, 2024) using open access elements; Figure R16 schematic representation of the behavioral test was drawn using BioRender (www.biorender.com); Figure R21 **a**) MotoRater photos were obtained from the website manufacturer (www.tse-systems.com), **b**) was adapted from (Preisig et al., 2016). Figure R22 **a**) was adapted from (Preisig et al., 2016). Schematic representations of mice's anatomic points measured, next to the behavioral test graphics, were adapted from (Preisig et al., 2016).

Statistical analysis

Statistical analysis was performed using the GraphPad 10 software (GraphPad Software, USA). In general, comparisons between two groups were carried using unpaired Student's t-test. In general, multiple comparisons between three groups were performed using one-way analysis (ANOVA).

Statistical analysis of mice performance overtime was carried using two-way analysis (ANOVA) with mixed-effect analysis followed by Bonferroni correction for multiple comparisons. The statistical significance considered was of the differences between groups relative to controls ("column factor").

All results are expressed as mean $\pm$ standard error of the mean (SEM) and significance was set at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$.

Chapter 4 – Results

4.1 Manipulation of SGs dynamics

Introduction

The ISR begins with the phosphorylation of eIF2 α (p-eIF2 α) by a group of four different kinases (HRI, PKR, PERK, and GCN2), which are considered stress sensors. Upon phosphorylation of eIF2 α , global translation is arrested, culminating in the accumulation of stalled translation machinery and translationally paused poly-(A) mRNA. Thus, a pro-survival protein synthesis program is activated, enabling cells to become more resilient in adverse conditions (Harding et al., 2003; McEwen et al., 2005; Srivastava et al., 1998; Wek et al., 1995). Simultaneously, to facilitate the dephosphorylation of p-eIF2 α , the expression of the scaffold GADD34 is upregulated, allowing the interaction between PP1 and p-eIF2 α . This way, the standard translation program is re-initiated faster as soon as the stress stimulus is no longer present (Dalet et al., 2017; Goh et al., 2018; Kojima et al., 2003; Lu et al., 2004; Magg et al., 2024).

Another crucial element involved in the ISR is stress granules (SGs). SGs are membraneless organelles mainly composed of stalled translation machinery and the translationally paused poly-(A) mRNA. SGs form in response to the accumulation of stalled translation machinery and the translationally paused poly-(A) mRNA upon eIF2 α phosphorylation (Kedersha et al., 2002, 1999; Riggs et al., 2020). SGs can prevent the translation and trap mRNAs: i) that need to be protected during stress conditions, ii) are unnecessary to address stress, or iii) whose translation could harm cells. Thus, SGs play a crucial role in maintaining a protein synthesis program that allows cells to counteract situations of cellular stress (Marcelo et al., 2021b; Riggs et al., 2020; Wolozin and Ivanov, 2019).

Although the formation of SGs is essential for cells to cope with stress, current hypotheses point out that their persistence within cells under chronic stress conditions, such as in the context of neurodegenerative disorders, might be harmful. Studies related to the ISR have revealed that p-eIF2 α is upregulated in several neurodegenerative disorders and that preventing its phosphorylation mitigates disease-associated features (Feng et al., 2014;

Reineke and Neilson, 2019; Smith and Mallucci, 2016, 2016). Other studies have shown that SGs formation can directly impact how neurodegenerative diseases progress (Bakthavachalu et al., 2018; Gutiérrez-García et al., 2023; Reineke and Neilson, 2019). However, the precise role of SGs assembly and disassembly dynamics in modulating the progression of neurodegenerative diseases via the activation and deactivation of the ISR was never addressed.

To understand how the ISR, particularly the SGs granules, impact the phenotype associated with neurodegenerative disorders, specifically SCA2, the following experiments aimed to develop tools that allowed us to manipulate the formation of SGs.

Tools to manipulate SGs assembly and disassembly

SGs assembly tools

Previous results have shown that eIF2 α phosphorylation at its serine 52 is the first step of ISR, which then culminates in SGs formation (Harding et al., 2003; Kedersha et al., 1999). Thus, we aimed to develop an eIF2 α phosphomimetic construct by substituting the amino acid serine (S) 52 with the amino acid aspartate (D), from now on termed eIF2 α^{S52D} . By performing the S $\rightarrow$ D modification, cells will process this change as if eIF2 α is permanently phosphorylated. The construct also expresses a mCherry fluorescent protein, allowing us to track expression levels. To minimize any interference between the eIF2 α^{S52D} and mCherry proteins, even though they are in the same construct, they are under the control of different promoters. Respectively, the eIF2 α^{S52D} is under the control of the mouse phosphoglycerate kinase 1 promoter (PGK1), and mCherry is under the control of the human cytomegalovirus immediate early enhancer/promoter (CMV). Additionally, the construct is inserted into an adeno-associated virus 1 (AAV1) backbone. From now on, the whole construct is termed eIF2 α^{S52D} -mCherry. As a control, we developed a similar construct, identical in every respect to the one

previously described, except that it expresses the protein luciferase. From now on, the control construct will be termed CT-mCherry (control). Both constructs are schematically represented in **Figure R1a**. First, we started by validating both constructs' design through enzymatic restriction using *PvuI* and *XbaI* enzymes. After digestion, samples were then resolved in an agarose gel electrophoresis. For the undigested eIF2 α^{S52D} -mCherry construct we expected a 6844 bp band; and for the digested, we expected a 5440 bp band as well as a 1404 bp band. For the undigested CT-mCherry construct, we expected a 7549 bp band; and for the digested, we expected a 6145 bp band as well as a 1404 bp band (**Figure R1b**). The DNA bands of both constructs had the expected weights (**Figure R1b**). Since identifying the cells that were specifically expressing our constructs was a crucial step in this work, we first confirmed the presence of the mCherry fluorescent protein in transfected mouse neuroblastoma (N2a) cells. As expected, in both cases, cells transfected either with eIF2 α^{S52D} -mCherry or the CT-mCherry positively expressed the fluorescent protein (**Figure R1c**).

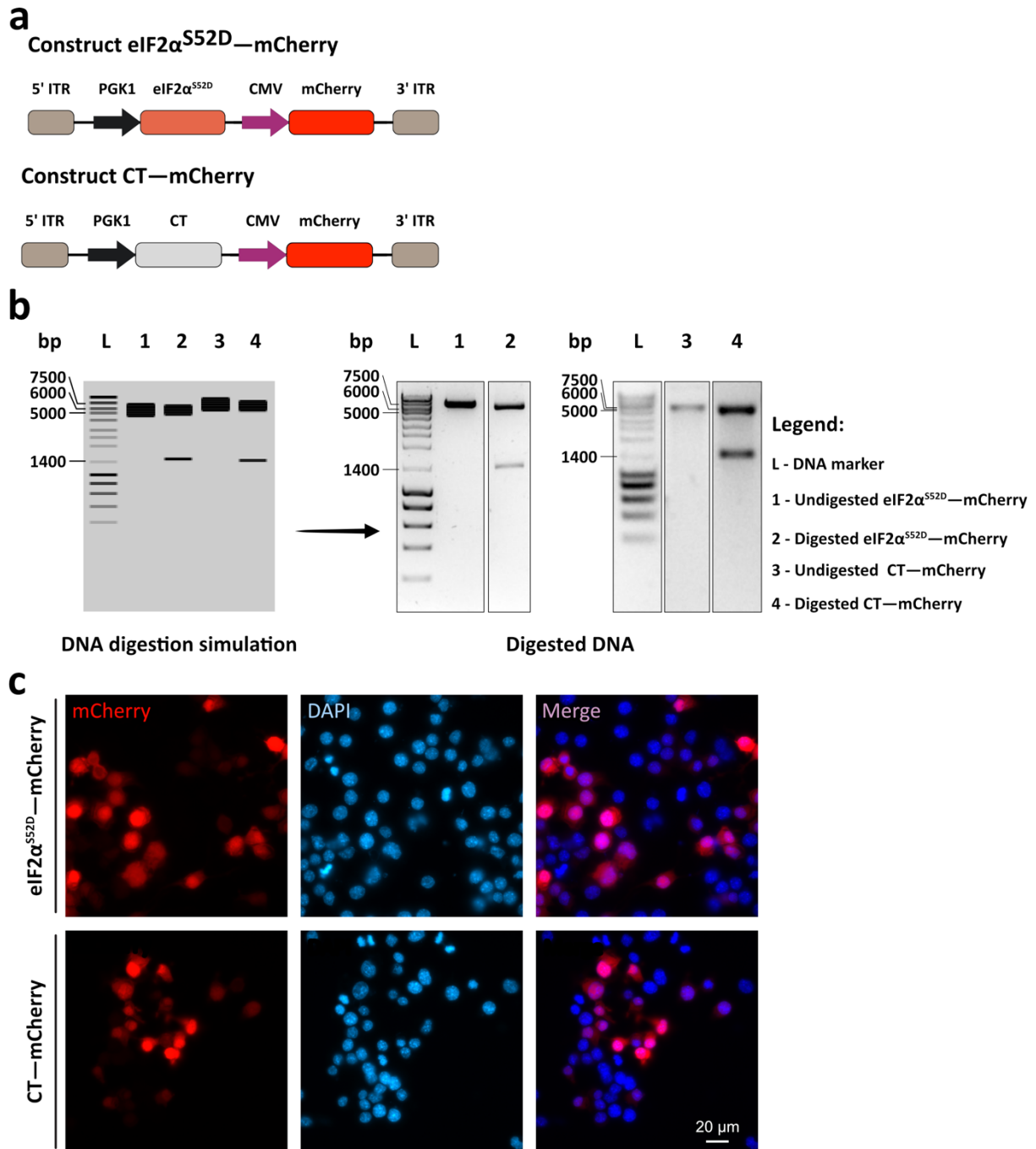


Figure R1 - Validation of genetic strategies to induce SGs assembly. We developed an eIF2 α^{S52D} phosphomimetic (eIF2 α^{S52D}) form in order to activate ISR and induce the formation of SGs. **a)** Schematic representation of the plasmid used. **a)** eIF2 α^{S52D} —mCherry construct [a)upper]; **a)** CT—mCherry [a) down]. Both plasmids are inserted within an AAV backbone; eIF2 α^{S52D} and the CT genes are under the control of the PGK1 promotor, while mCherry is under the control of the CMV promotor. **b)** Schematic representation of the DNA electrophoresis after PvuI and XbaI enzymatic digestion. **b)** *In silico* schematic simulation of the digestion eIF2 α^{S52D} —mCherry and CT—mCherry constructs with the predicted DNA fragment weights [b)left]. For the undigested eIF2 α^{S52D} —mCherry, it was expected a 6844 bp band (lane 1), and for the digested, we expected a 5440 bp band and a 1404 bp band (lane 2). For the undigested CT—mCherry, it was expected a 7549 bp band (lane 3), and for the digested, we expected a 6145 bp band and a 1404 bp band (lane 4). **b)** Representative images of the electrophoresis agarose gel showing the digested constructs matching the predicted DNA weights [b)right]. **c)** Representative microscopy images showing the expression of the mCherry fluorescent protein. N2a cells were transfected either with the eIF2 α^{S52D} —mCherry [c) upper]; or with the CT—mCherry [c) down].

Next, we aimed to evaluate whether the expression of eIF2 α^{S52D} -mCherry was able to induce the formation of SGs in unstressed cells. To achieve that, we transfected N2a cells either with eIF2 α^{S52D} -mCherry or, as control, with CT-mCherry. Cells were then immunocytochemically stained for the well-established SGs marker G3BP1 in order to observe SGs (**Figure R2a-b**) (Kedersha et al., 2005; Marcelo et al., 2021b). Then, we characterized the formation of SGs only in cells that were expressing mCherry (**Figure R2b-c**). While the presence of SGs was virtually absent in cells expressing CT-mCherry (**Figure R2b upper row; Figure R2c**), the expression of eIF2 α^{S52D} -mCherry was able to induce the formation of SGs, even though cells were not subjected to other cellular stress (**Figure R2 b bottom row; Figure R2c**). We then decided to characterize the properties of SGs formation. Among the transfected cells exhibiting SGs, we observed an average of three SGs per cell (**Figure R2d**), each with an area of approximately 5 μm^2 (**Figure R2e**).

Overall, our results show that the phosphomimetic form of eIF2 α can activate ISR, as seen by the presence of SGs in unstressed cells transfected with eIF2 α^{S52D} -mCherry. Thus, we successfully developed a genetic strategy to study the impact of SGs formation.

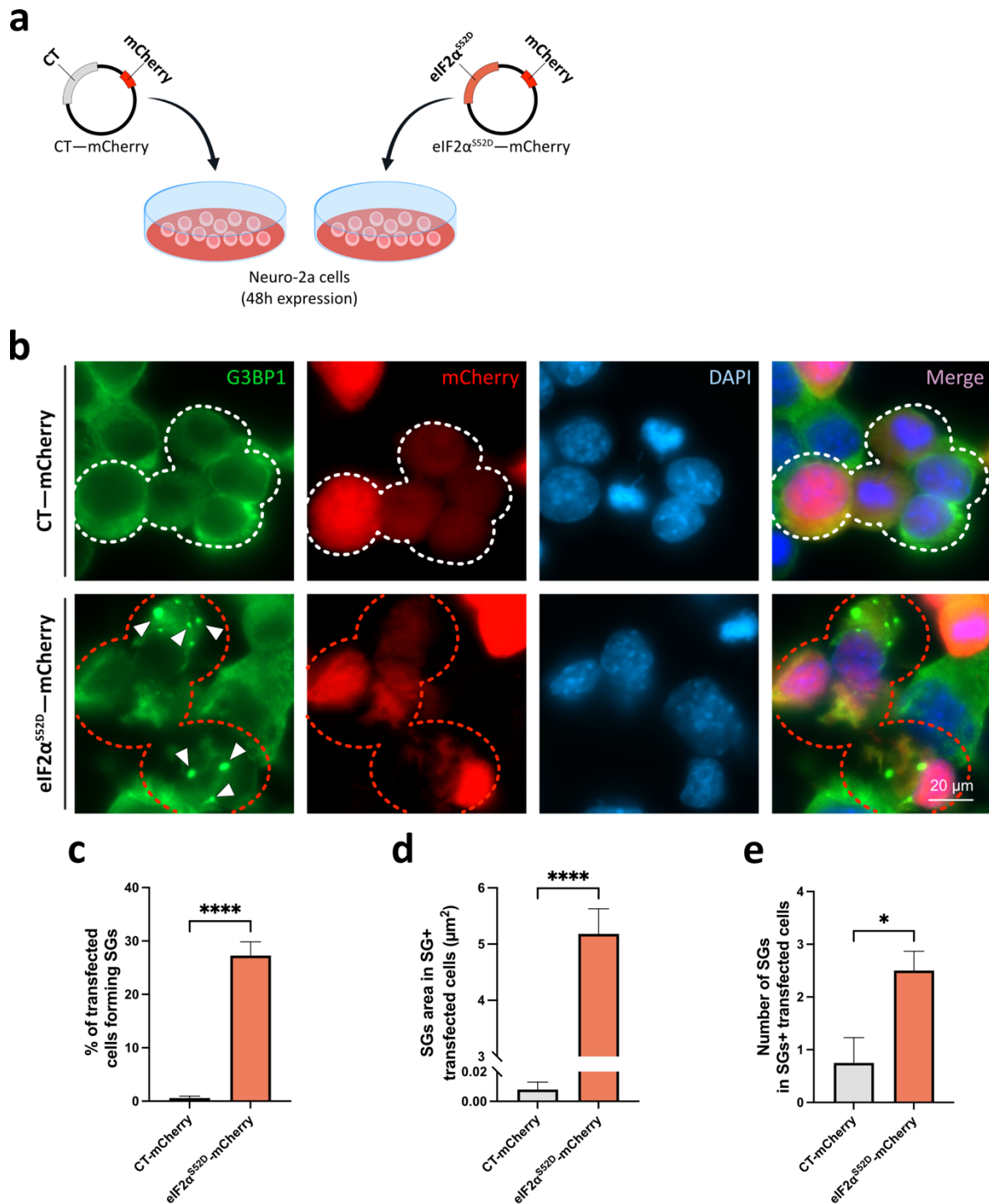


Figure R2 - EIF2 α phosphomimetic induces the formation of SGs in unstressed cells. The developed eIF2 α^{S52D} -mCherry construct, which contains a phosphomimetic form of eIF2 α , induces SG formation in cells not subjected to cellular stress. **a)** Schematic representation of the experimental design used to evaluate SGs assembly. Neuro-2a cells were either transfected with eIF2 α^{S52D} -mCherry or, as control, with CT-mCherry; 48h after transfection, SGs formation was characterized. **b)** Representative microscopy images from Neuro-2a cells stained with SGs marker G3BP1 (green) and expressing either CT-mCherry [b) upper row] or eIF2 α^{S52D} -mCherry [b) bottom row] (red). The white dashed line highlights that CT-mCherry transfected cells do not form SGs. The red dashed line highlights that eIF2 α^{S52D} -mCherry transfected cells display SGs. Arrowheads are highlighting the SGs formed in cells transfected with eIF2 α^{S52D} -mCherry. **c) - e)** The expression of the eIF2 α phosphomimetic successfully induced the formation of SGs in unstressed Neuro-2a cells (arrowhead). **c)** Nearly 30% of unstressed transfected cells expressing eIF2 α phosphomimetic were able to form SGs. **d)** Formed SGs in eIF2 α^{S52D} -mCherry transfected cells displayed approximately 5 μ m². **e)** Transfected cells with eIF2 α^{S52D} -mCherry showed approximately three SGs per cell. (n = 4 independent experiments; *P<0.05; ****P<0.0001, Student's t-test; values are expressed as mean $\pm$ SEM).

SGs disassembly tools

The ISR begins with the phosphorylation of eIF2 α , which culminates in the formation of SGs. At the same time, GADD34 is upregulated and scaffolds the interaction between the phosphatase PP1 and p-eIF2 α , promoting the dephosphorylation of eIF2 α and SGs disassembly through a negative feedback loop (Connor et al., 2001; Magg et al., 2024; Novoa et al., 2001). Thus, we aimed to evaluate whether the expression of GADD34 was able to prevent the formation of SGs in cells subjected to cellular stress.

Previous studies have shown that the PEST domains (peptide sequence rich in proline, glutamate, serine, and threonine) within the GADD34 protein were responsible for GADD34-PP1 complex recruitment to p-eIF2 α . It was also shown that removing the GADD34 amino acid sequence, located upstream of the PEST domains, enhanced the ability of the GADD34-PP1 complex to target p-eIF2 α and dephosphorylate it (Choy et al., 2015). Thus, we decided to use a truncated form of GADD34, lacking the first 323 aa (from now on termed GADD34 ^{Δ 322}; **Figure R3a**). Like the previously described constructs, GADD34 ^{Δ 322} is under the control of the PGK1 promoter and contains a mCherry sequence under the control of the CVM promoter, both inserted within an AAV backbone (**Figure R3b**). From now on, the construct is termed GADD34 ^{Δ 322}-mCherry. Again, we started by validating the construct design through enzymatic restriction using *PvuI* and *XbaI* enzymes. After digestion, samples were then resolved in an agarose gel electrophoresis. For the undigested GADD34 ^{Δ 322}-mCherry construct, we expected a 6958 bp band; and for the digested, we expected a 5554 bp band as well as a 1404 bp band (**Figure R3c**). According to what was expected, DNA bands matched the predicted molecular weights (**Figure R3c**). To evaluate the expression of mCherry fluorescent protein, N2a cells were transfected. Cells transfected with GADD34 ^{Δ 322}-mCherry positively expressed the fluorescent protein (**Figure R3d**).

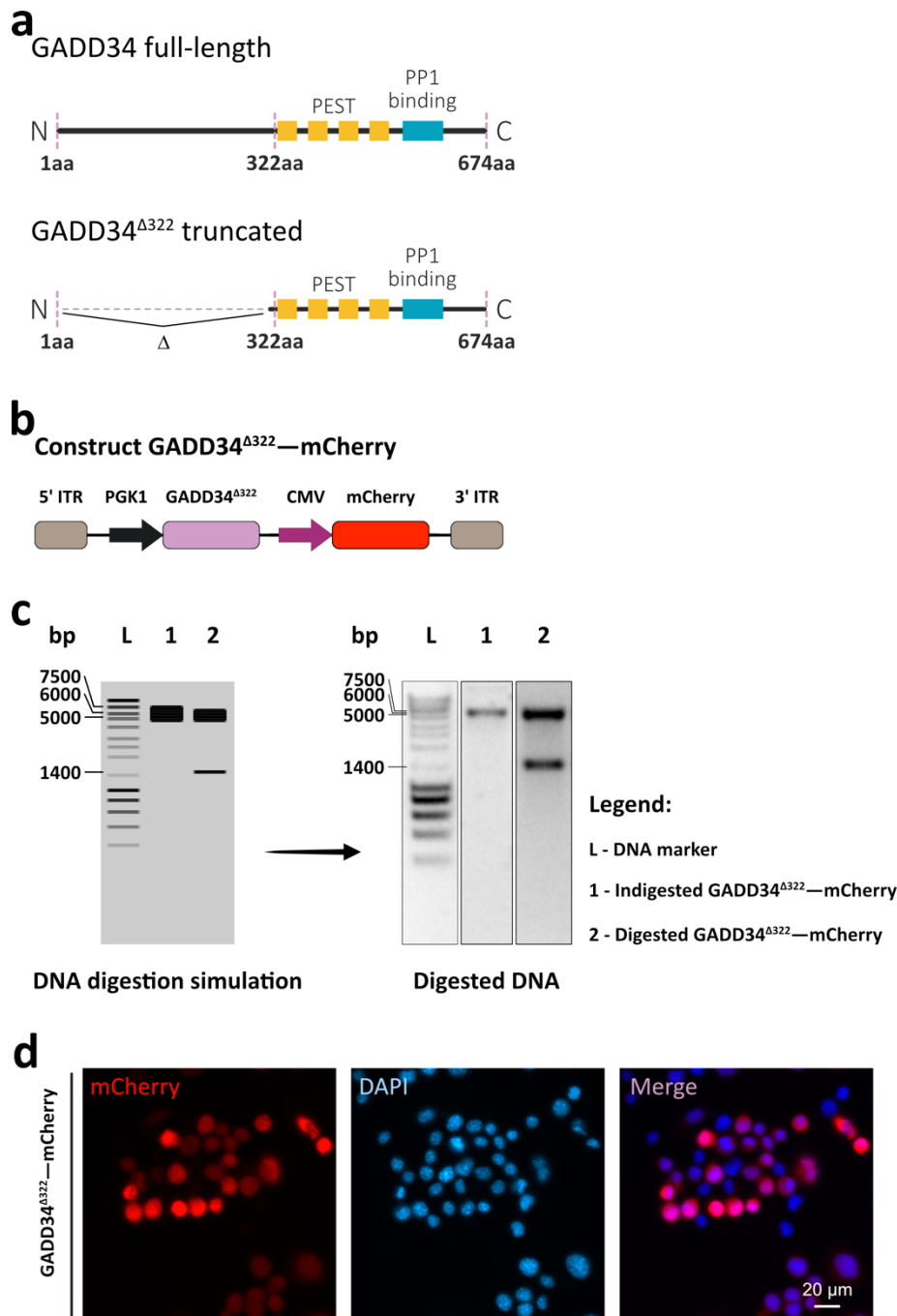


Figure R3 - Validation of genetic strategies to inhibit SGs assembly. In order to inhibit the formation of SGs, we developed a GADD34 truncated form (GADD34^{Δ322}) lacking 322 aa upstream of the PEST domains. **a)** Schematic representation of the GADD34 protein used. **a)** Schematic representation of the full-length GADD34 protein, with the respective PEST and PP1 binding domains [a] upper]. Schematic representation of the truncated GADD34 protein lacking the 322aa upstream of the PEST motifs [a] bottom]. **b)** GADD34^{Δ322}—mCherry construct. The plasmid is inserted within an AAV backbone; GADD34^{Δ322} is under the control of the PGK1 promoter, while mCherry is under the control of the CMV promoter. **c)** *In silico* schematic representation of the DNA electrophoresis after PvuI and XbaI enzymatic digestion. **c)** *In silico* schematic representation of the digestion GADD34^{Δ322}—mCherry construct with the predicted DNA fragment weights [c]left]. For the undigested GADD34^{Δ322}—mCherry, it was expected a 6958 bp band (lane 1); for the digested, we expected a 5554 bp band and a 1404 bp band (lane 2). **c)** Representative images of the electrophoresis agarose gel showing the digested constructs matching the predicted DNA weights [b]right]. **d)** Representative microscopy images showing the expression of the mCherry fluorescent protein. Neuro-2a cells were transfected with the GADD34^{Δ322}—mCherry. Legend: Δ - deletion.

To evaluate the ability of GADD34^{Δ322}-mCherry to prevent the formation of SGs, we used a well-established cellular stress paradigm in which cells are subjected to a treatment with sodium arsenite (SA). SA is known to trigger the unfolded protein response, activating PERK (one of the four stress kinase sensors), which then phosphorylates eIF2α (Sun et al., 2017). Since GADD34 facilitates the dephosphorylation of p-eIF2α, the expression of GADD34^{Δ322} is expected to prevent the assembly of SGs. Therefore, we transfected N2a cells either with GADD34^{Δ322}-mCherry or, as control, with CT-mCherry. Two days after transfection (48h), transfected cells were treated with SA for 60 minutes (**Figure R4a**). We stained and characterized SGs as previously described. It is expected that virtually all cells exposed to SA consistently form SGs (Streit et al., 2022). While SGs were present in more than 80% of SA-treated cells expressing CT-mCherry (**Figure R4b upper row; Figure R4c**), the expression of GADD34^{Δ322}-mCherry almost resulted in the complete ablation of SGs assembly (**Figure R4b bottom row; c**). In the few transfected cells still displaying SGs, both GADD34^{Δ322}-mCherry and CT-mCherry SGs surface area remained unchanged (**Figure R4d**). However, the number of SGs per cell was lower in GADD34^{Δ322}-mCherry transfected cells when compared to cells transfected with CT-mCherry (**Figure R4e**).

Overall, our results show that truncated GADD34^{Δ322} can inhibit ISR, as seen by the absence of SGs in cells treated with SA in GADD34^{Δ322}-mCherry transfected cells. Thus, we successfully developed a genetic strategy to study the impact of SGs.

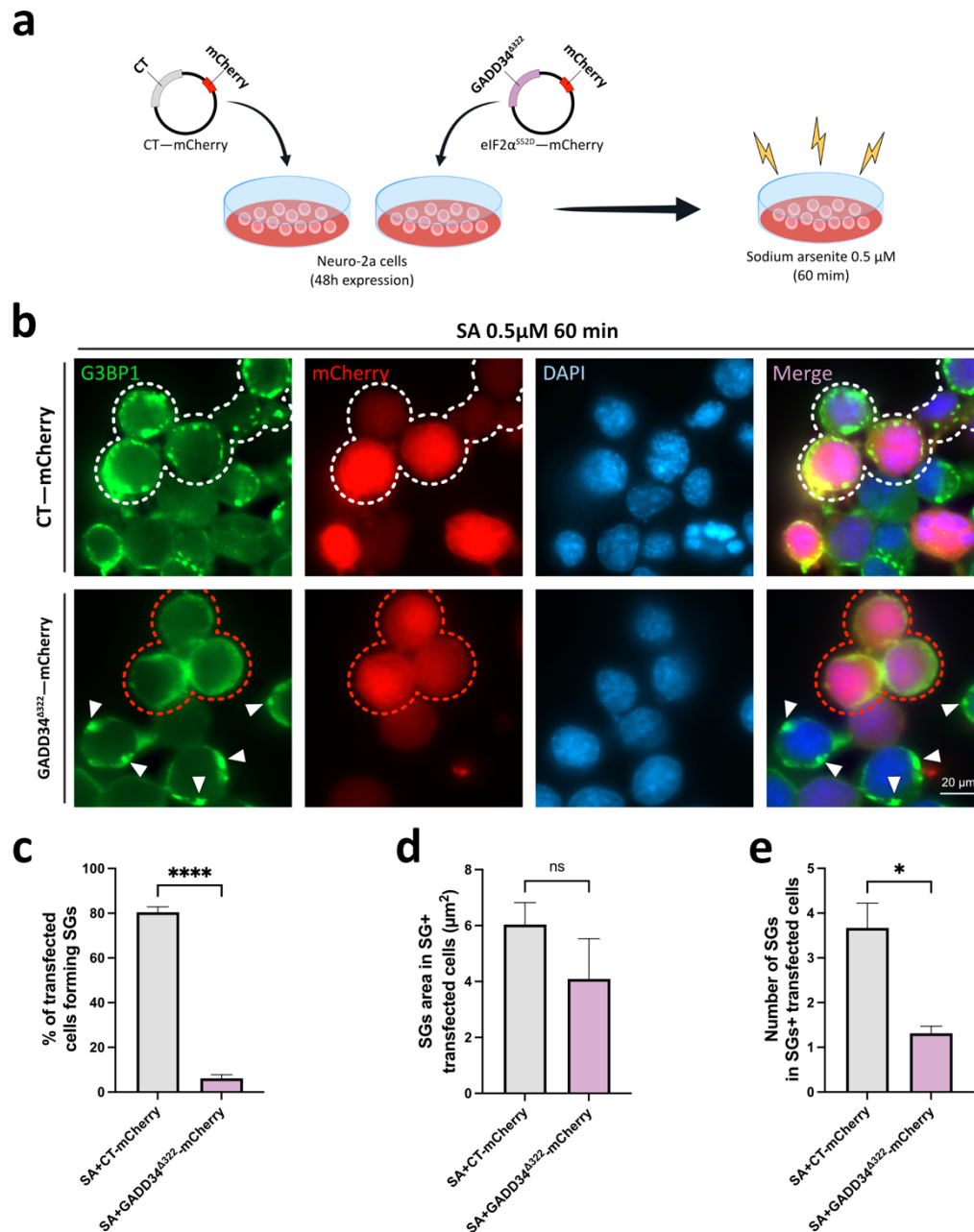


Figure R4 - Truncated GADD34^{Δ322} prevents the formation of SGs in cells subjected to cellular stress. The developed GADD34^{Δ322}-mCherry construct, which contains a truncated GADD34 sequence, prevented the formation of SG in cells treated with SA. **a)** Schematic representation of the experimental design used to evaluate SGs disassembly. Neuro-2a cells were either transfected with GADD34^{Δ322}-mCherry or, as control, with CT-mCherry; 48h after transfection, cells were treated with sodium arsenite for 60 minutes; then SGs formation was characterized. **b)** Representative microscopy images from Neuro-2a cells stained with SGs marker G3BP1 (green) and expressing either CT-mCherry [b) upper row] or GADD34^{Δ322}-mCherry [b) bottom row] (red). The white dashed line highlights that CT-mCherry transfected cells display SGs. The red dashed line highlights that GADD34^{Δ322}-mCherry transfected cells do not exhibit SGs. Arrowheads are highlighting that in the conditions GADD34^{Δ322}-mCherry, if cells are not transfected, SGs still form. **c) - e)** The expression of the truncated GADD34^{Δ322} successfully prevented the formation of SGs in SA-treated Neuro-2a cells. **c)** Only 6% of SA-treated cells expressing GADD34^{Δ322} were able to form SGs, opposing the 80% of cells transfected CT-mCherry. **d)** Formed SGs in both GADD34^{Δ322}-mCherry and CT-mCherry transfected cells displayed approximately the same area. **f)** Transfected cells with GADD34^{Δ322}-mCherry showed approximately one SG per cell, while CT-mCherry cells displayed more than three SGs per cell. (n = 4 independent experiments; *P<0.05; ****P<0.0001, ns - no significance; Student's t-test; values are expressed as mean $\pm$ SEM).

Alternative strategies to manipulate SGs dynamics

We also evaluated alternative genetic strategies to manipulate SGs assembly and disassembly dynamics that do not directly impact the ISR. Those strategies relied on expressing or downregulating SGs core components (Kedersha et al., 2002, 1999; Pakos-Zebrucka et al., 2016). Thus, we aimed to evaluate how the expression and downregulation of TIA1, a SGs core component, impacted the formation of SGs. To achieve that goal, we used a similar approach as described before. To overexpress TIA1, we developed a TIA1–mCherry construct and used the CT-mCherry as control (**Figure R5a**). Conversely, to downregulate TIA1, we evaluated two short hairpin RNAs (shRNA) designed to target *TIA1* mRNA (from now on termed shTIA1#1–mCherry and shTIA1#2–mCherry) (**Figure R5b**). These shRNA-expressing constructs feature the sequence targeting TIA1 under the control of the U6 promoter and, similarly to the other constructs, a mCherry sequence under the control of the CMV promoter. As a control for the shRNAs, we used a sequence without any known target (from now on termed shCT–mCherry). We started by validating the construct using the previously described approaches (**Figure R5c-d**).

Regarding the DNA agarose electrophoresis, for the undigested constructs, we expected a 7024 bp band for the TIA1-mCherry construct, while for all the shRNAs, we expected a 5450 bp band. Regarding the digested constructs, for TIA1-mCherry, it was expected a 5620 bp band, as well as a 1404 bp band. For the digested shRNAs constructs, it was expected a 4036 bp band and a 1404 bp band (**Figure R5c left**). According to what was expected, all DNA bands matched the predicted molecular weights. Additionally, all N2a cells transfected with the constructs were expressing the fluorescent protein mCherry (**Figure R5c right**).

To assess how TIA1 expression impacts the formation of SGs, we transfected unstressed N2a cells with either TIA1–mCherry or CT-mCherry (**Figure R6a**). We observed that TIA1 expression triggered significant SGs formation compared to the control condition (**Figure R6b-c**). Moreover, in transfected cells that formed SGs, the area of these granules also increased (**Figure R6d**). Furthermore, in the rare cases where CT-mCherry transfected cells form SGs, their number was smaller when compared to those formed in TIA1-transfected cells (**Figure R6c**). Together, these results show a robust tool to induce the formation of SGs, which is not directly involved in the ISR initiation pathway.

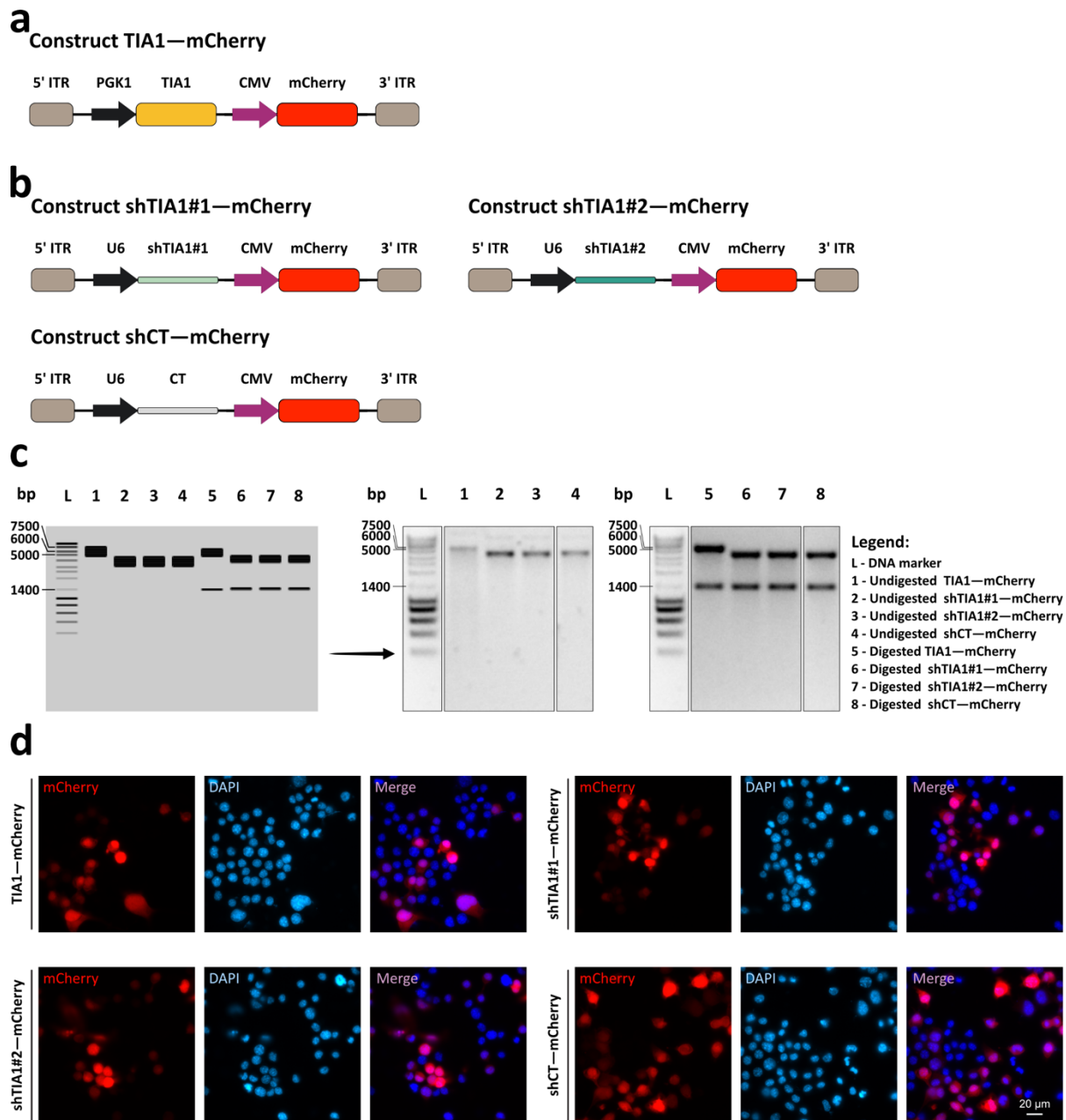
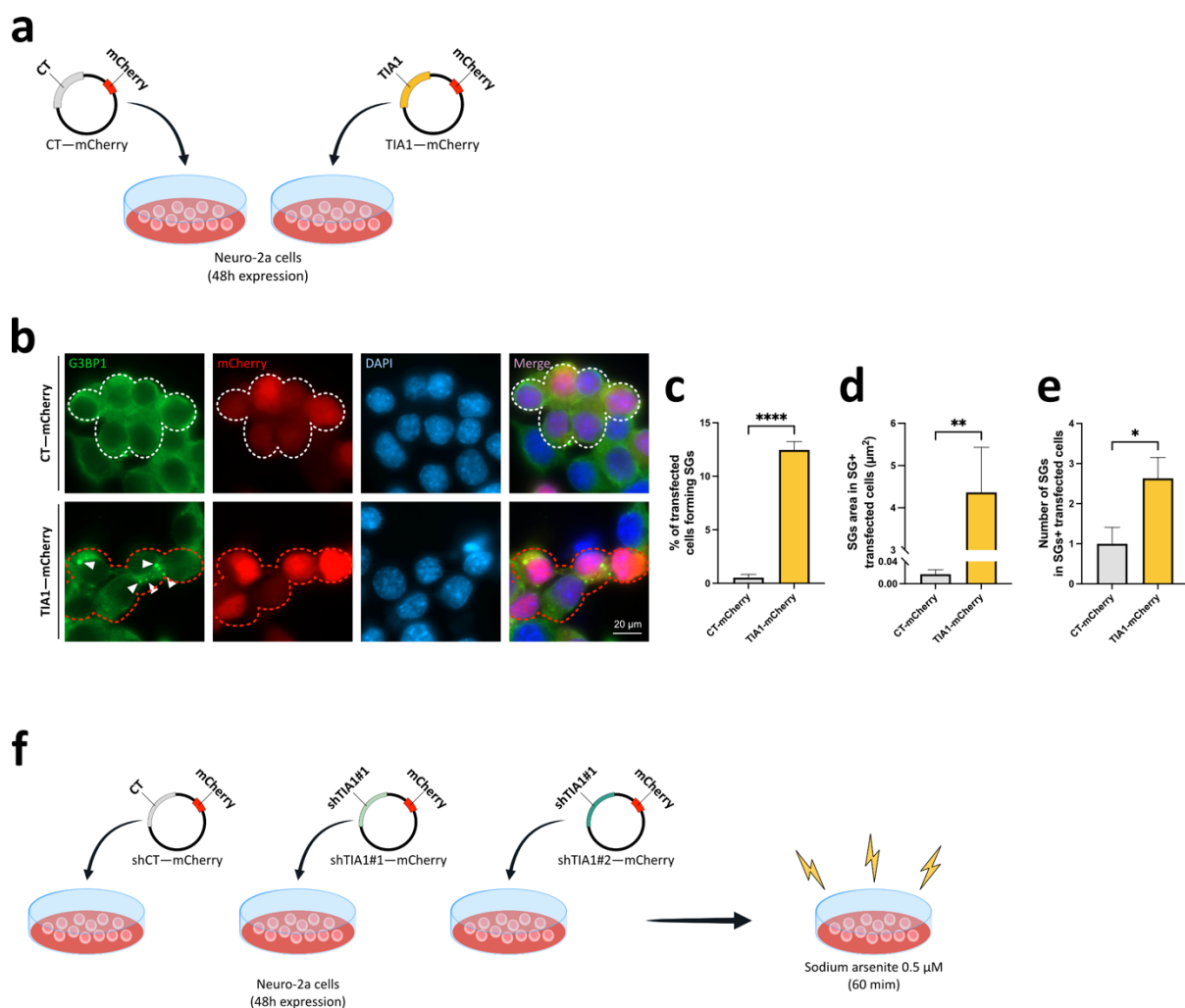


Figure R5 - Validation of genetic strategies to manipulated SGs assembly and disassembly. In order to induce or inhibit the formation of SGs without directly acting on the ISR activation or deactivation, we developed a construct to overexpress TIA1 and two shRNAs constructs targeting TIA1. **a)** Schematic representation of the TIA1-mCherry construct used to overexpress TIA1. **b)** Schematic representation of the shRNAs constructs used to target TIA1: shTIA1#1-mCherry [b) upper left], shTIA1#2-mCherry [b) upper right] and shCT-mCherry [b) bottom]. **a-b)** All constructs are inserted within an AAV backbone; while TIA1 is under the control of the PGK1 promoter, the shRNAs are under the control of the U6 promoter. The mCherry sequence is under the control of the CMV promoter. **c)** *In silico* schematic simulation of the TIA1-mCherry, shTIA1#1-mCherry, shTIA1#2-mCherry and shCT-mCherry constructs digestions, with the predicted DNA fragment weights [c)left]. For the undigested TIA1-mCherry and for the shRNAs, it was expected, respectively, a 7024 bp band (lane 1) and a 5450 bp band (lanes 2-4). For the digested TIA1-mCherry construct, we expected a 2620 bp and a 1404 bp band (lane 5). For the digested shRNA constructs, we expected a 4036 bp and a 1404 bp band (lanes 6-8). **c)** Representative images of the electrophoresis agarose gel showing the digested constructs matching the predicted DNA weights [c)right]. **d)** Representative microscopy images showing the expression of the mCherry fluorescent protein. N2a cells were transfected either with the TIA1-mCherry [c) upper left]; or shTIA1#1-mCherry [c) upper right]; shTIA1#2-mCherry [c) bottom left]; or shCT-mCherry [c) bottom right].

Regarding our strategies to inhibit the formation of SGs using shRNAs targeting *TIA1* mRNA, N2a cells were either transfected with the shCT-mCherry, as control, or with shTIA1#1-mCherry or shTIA1#2-mCherry. As previously mentioned, 48h after transfection, cells were treated with SA (**Figure R6f**). While none of the shRNA strategies robustly affected SGs assembly in cells subjected to stress (**Figure R6g-i**), it seems that shTIA1#2-mCherry reduced SGs area (**Figure R6j**).

Overall, our results indicate that TIA1 expression can induce the formation of SGs as seen by their presence in unstressed cells transfected with TIA1-mCherry. In contrast, targeting TIA1 with a shRNA (shTIA1#2-mCherry) appears to alter SGs assembly, reducing their size. Thus, we successfully developed two opposing genetic strategies that affect SGs assembly but do not directly act on either the activation or deactivation of the ISR.



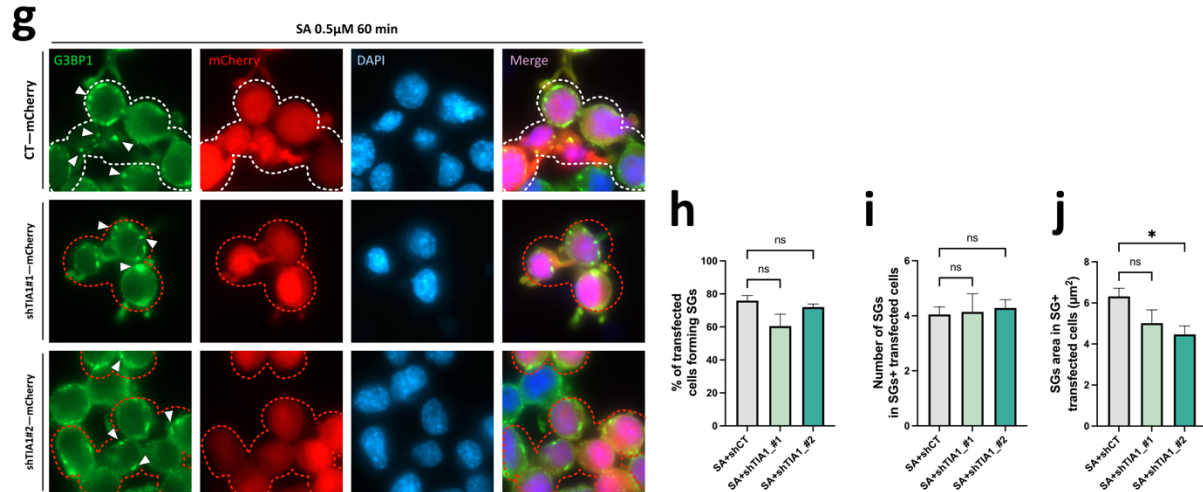


Figure R6 - TIA1 expression affects SGs dynamics. The developed TIA1—mCherry construct was able to induce SGs formation in cells not subjected to cellular stress. Contrarily, the expression of a shRNA targeting TIA1 slightly perturbed SGs assembly dynamics. **a)** Schematic representation of the experimental design used to evaluate SGs assembly. Neuro-2a cells were either transfected with TIA1—mCherry or, as control, with CT—mCherry; 48h after transfection, SGs formation was characterized. **b)** Representative microscopy images from Neuro-2a cells stained with SGs marker G3BP1 (green) and expressing either CT—mCherry [b] upper row] or TIA—mCherry [b] bottom row] (red). **c) - e)** The expression of the TIA1 successfully induced the formation of SGs in unstressed Neuro-2a cells (arrowhead). **c)** Nearly 13% of unstressed transfected cells expressing TIA1 were able to form SGs. **d)** Formed SGs in TIA1—mCherry transfected cells displayed approximately 4 μm^2 . **e)** Transfected cells with TIA1—mCherry showed approximately three SGs per cell. **f)** Schematic representation of the experimental design used to evaluate SGs disassembly. Neuro-2a cells were either transfected with shTIA1#1—mCherry or shTIA1#1—mCherry. As control, cells were transfected with shCT—mCherry. After 48h, cells were treated with sodium arsenite for 60 minutes; then SGs formation was characterized. **g)** Representative microscopy images from Neuro-2a cells stained with SGs marker G3BP1 (green) and expressing either shCT—mCherry [b] upper row]; or shTIA1#1—mCherry [b] middle row]; or shTIA1#2—mCherry (red). **h)-j)** the expression of a shRNA targeting TIA1 modestly affected SGs formation. **i)** Targeting TIA1 with shTIA1#2—mCherry seems to affect SGs assembly by reducing their area. (n = 4 independent experiments; *P<0.05; **P<0.01; ****P<0.0001, ns - no significance; *Student's t*-test (two conditions); One-way Anova followed by Bonferroni correction; values are expressed as mean (three conditions); values are expressed as mean $\pm$ SEM).

Altogether, we were able to develop different genetic strategies able to manipulate the assembly/disassembly dynamics of SGs (**Table R1**). These strategies included expressing factors that were directly involved in the ISR and upregulating/downregulating an SGs component.

Table R1 –SGs manipulations results summary

	% cells with SGs	SGs area	SGs number per cells
eIF2 α ^{S52D} –mCherry	↑	↑	↑
GADD34 ^{Δ322} –mCherry	↓	—	↓
TIA1-mCherry	↑	↑	↑
shTIA1#1–mCherry	—	—	—
shTIA1#2–mCherry	—	↓	—

Abbreviation: ↑ – increases; ↓ – decreases; — – unaltered.

4.2 Inducing SGs in the context of SCA2

Introduction

The ISR is a crucial mechanism that aids cells in tackling situations of cellular stress. In fact, some evidence has pointed out that an altered stress response affects a proper cellular homeostasis (Pakos-Zebrucka et al., 2016). Some studies have suggested that alterations in the ISR can contribute to the pathology associated with several neurodegenerative disorders (Hoozemans et al., 2009, 2007; Moreno et al., 2013; Radford et al., 2015; Walter and Ron, 2011). However, the same studies fail to map which ISR pathways and players are directly involved in those deleterious effects observed in neurodegenerative diseases. In the context of SCA2, the role of the IRS, and particularly SGs, in modifying the associated pathology was never explored.

Given the fact that ataxin-2 is suggested to play a central role in the formation of SGs, it can be hypothesized that its mutant form might be impacting a proper IRS response (Yamagishi et al., 2024). Moreover, in SCA2 cells are chronically exposed to internal stress stimuli due to the presence of a mutant protein known to disrupt multiple cellular pathways (Conceição & Costa et al., 2024). In situations of prolonged chronic stress, the ISR and the formation of SGs are hypothesized to become detrimental rather than beneficial to cells (Pakos-Zebrucka et al., 2016; Reineke and Neilson, 2019). Taking it all together, we aimed to evaluate the effects of inducing the formation of SGs in the context of SCA2 to assess their impact on the disease. To achieve this goal, we used different *in vitro* and *in vivo* models of SCA2 and made use of the eIF2 α^{S52D} -mCherry construct, which we previously showed to induce the formation of SGs.

Inducing SGs in vitro in the context of mutant ataxin-2 expression

We have previously shown that expressing a phosphomimetic form of eIF2 α (eIF2 α^{S52D} –mCherry) was able to induce the formation of SGs. To understand the impact of inducing the formation of SGs in the context of SCA2, we first relied on an *in vitro* approach to mimic the mutant ataxin-2 expression context. To achieve that goal, we used primary cultures of mouse striatal neurons expressing either a WT form or a mutant form of ataxin-2 (**Figure R7**). These constructs contain a WT or a mutant ataxin-2 sequence under the control of the PGK1 promoter and a green fluorescent protein sequence under the control of the CMV promoter. Both constructs were inserted within an LV backbone. The WT ataxin-2 construct bears 23 CAG repetitions (23 glutamines) (**Figure R7a**), and the mutant ataxin-2 construct bears 80 CAG repetitions (80 glutamines) (**Figure R7a**). Additionally, to help us distinguish the endogenous ataxin-2 from these forms, the constructs have an additional human influenza hemagglutinin (HA) sequence tag at the end of both ataxin-2 sequences. From now on, the WT ataxin-2 construct will be termed ataxin-2^{WT(23Q)}–GFP (or abbreviated WT(23Q)) and the mutant ataxin-2 construct will be termed ataxin-2^{MUT(80Q)}–GFP (or abbreviated MUT(80Q)). Like the constructs developed in previous sections, we first validated the plasmid design through a DNA electrophoresis agarose gel (**Figure R7b**). To digest the DNA, we used the *KpnI* and *NheI* enzymes. For the undigested ataxin-2^{WT(23Q)}–GFP and the ataxin-2^{MUT(80Q)}–GFP, we expected a 11535 bp and 11712 bp DNA band, respectively. For the digested ataxin-2^{WT(23Q)}–GFP we expected a 6192 bp and a 5343 bp bands, while for the ataxin-2^{MUT(80Q)}–GFP, it was expected a 6369 bp and a 5343 bp bands (**Figure R7b left**). According to what was expected, bands matched the predicted DNA weights (**Figure R7b right**). Next, to evaluate the expression of GFP and the presence of the HA-tag within the constructs, we transfected N2a cells (**Figure R7 c**). As expected, in both conditions, cells transfected with ataxin-2^{WT(23Q)}–GFP or ataxin-2^{MUT(80Q)}–GFP (**Figure R7c 1st column**) displayed green fluorescence and immunostained positively for the HA-tag (**Figure R7c 2nd column**).

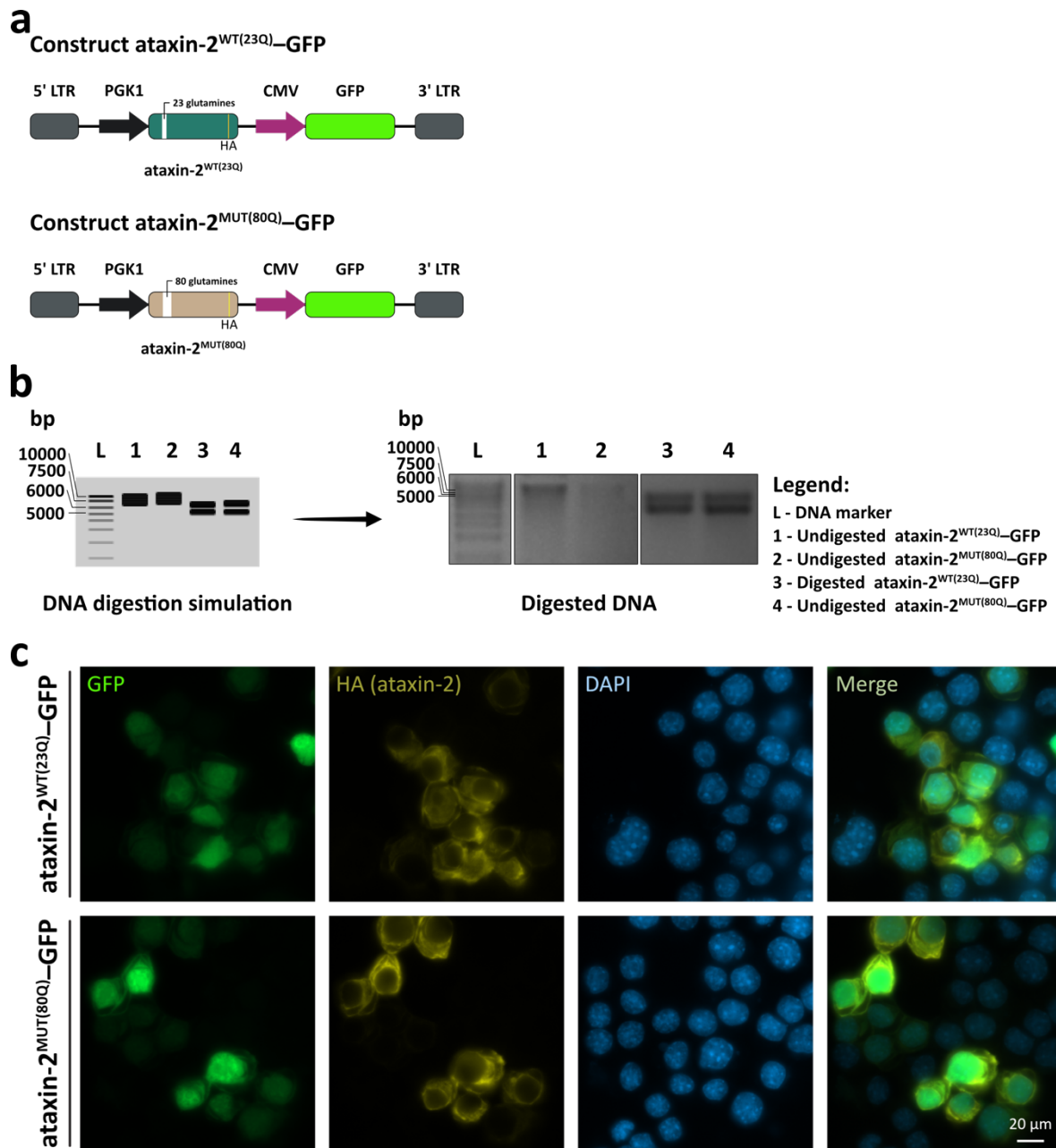


Figure R7 - Validation of the WT and mutant ataxin-2 constructs. We developed a WT (ataxin-2^{WT(23Q)}) and a mutant (ataxin-2^{MUT(80Q)}) ataxin-2 constructs in order to study the impact of manipulating the dynamics of SGs in the context of SCA2. **a)** Schematic representation of the plasmid used. **a)** ataxin-2^{WT(23Q)}—GFP construct [a)upper]; **a)** ataxin-2^{MUT(80Q)}—GFP [a) bottom]. Both plasmids are inserted within an LV backbone; ataxin-2^{WT(23Q)} and ataxin-2^{MUT(80Q)} genes are under the control of the PGK1 promotor, while GFP is under the control of the CMV promoter. An HA-tag sequence was added at the end of both ataxin-2 sequences (WT and mutant). **b)** Schematic representation of the DNA electrophoresis after KpnI and NheI enzymatic digestion. *In silico* schematic simulation of the digestion ataxin-2^{WT(23Q)}—GFP and ataxin-2^{MUT(80Q)}—GFP constructs with the predicted DNA fragment weights [b)left]. For the undigested ataxin-2^{WT(23Q)}—GFP, it was expected a 11535 bp band (lane 1). For the undigested ataxin-2^{MUT(80Q)}—GFP, it was expected a 11712 bp band (lane 2). For the digested ataxin-2^{WT(23Q)}—GFP, a 6192 bp and a 5343 bp bands were expected (lane 3). For the digested ataxin-2^{MUT(80Q)}—GFP, it was expected a 6369 bp and a 5343 bp bands (lane 4). Representative images of the electrophoresis agarose gel showing the digested constructs matching the predicted DNA weights [b) right]. **c)** Representative microscopy images showing the expression of the GFP fluorescent protein (red) and the presence of the HA-tag (yellow). N2a cells were transfected either with ataxin-2^{WT(23Q)}—GFP [c)upper]; or with ataxin-2^{MUT(80Q)}—GFP [c)bottom].

To evaluate the impact of inducing the formation of SGs in the context of the mutant ataxin-2, we used primary cultures of mouse striatal neurons (**Figure R8a-b**). Primary neurons were obtained from neonatal mice on the day of birth – postnatal zero (P0) – and plated on the same day (**Figure R8a**). The protocol used yielded cultures with approximately 70% of striatal neurons (**Figure R8b**). Neurons were maintained in culture and transfected at day *in vitro* 10 (DIV10), either with ataxin-2^{WT(23Q)}–GFP and CT–mCherry (**Figure R8c upper**); or ataxin-2^{MUT(80Q)}–GFP and CT–mCherry (**Figure R8c bottom left**); or ataxin-2^{MUT(80Q)}–GFP and eIF2 α ^{S52D}–mCherry (**Figure R8 bottom right**). Then, 48h after transfection, we did a morphometric analysis of the neurons expressing both mCherry and GFP to assess dendritic morphology (**Figure R9b**).

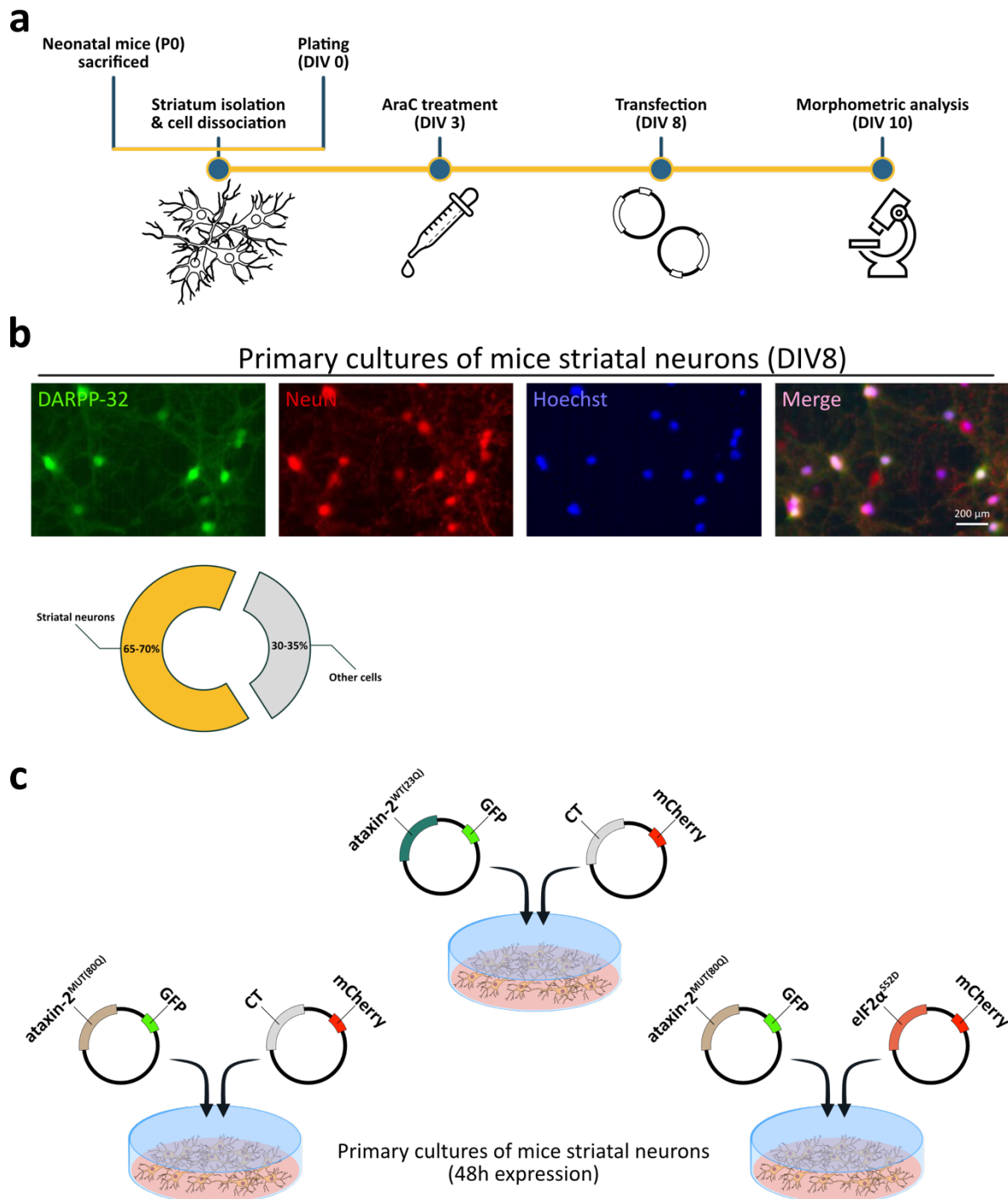


Figure R8 - Experimental design used to study the impact of inducing the formation of SGs in the context of the mutant ataxin-2 expression. We used primary cultures from mice striatal neurons to assess the effects of manipulating the assembly of SGs. **a)** Schematic representation of the timeline used to study the impact of inducing SGs assembly in the context of the mutant ataxin-2 expression. Briefly, mice were sacrificed at P0. The brains were dissected, the striatum isolated, and cells were dissociated. Finally, cells were plated, which represented zero days *in vitro* (DIV0). At DIV3, cells were treated with cytosine-D-arabino-furanoside (AraC) to block the proliferation of glial cells. At DIV8, cells were transfected, and at DIV10, cells were processed for analysis. **b)** Representative microscopy images of primary cultures of mice striatal neurons. To identify striatal neurons, we used the DARPP-32 marker (green). To identify all neurons without discrimination, we used the NeuN marker (red). Striatal neurons represented approximately 65-70% of all neurons present with the cultures at DIV8. **c)** Schematic representation of the experimental conditions used primary cells transfected with ataxin-2^{WT(23Q)}—GFP and CT—mCherry [c)upper]; or with ataxin-2^{MUT(80Q)}—GFP and CT—mCherry [c)bottom left]; or with ataxin-2^{MUT(80Q)}—GFP and eIF2 α ^{S52D}—mCherry [c)bottom right].

To perform the morphometric analysis, we traced the dendrites from the striatal neurons 48h after transfection (**Figure R9a**). The tracing consists of drawing a line that represents the dendritic arborization (**Figure R9b left column**). Then, we calculated the total dendritic length in the experimental conditions (**Figure R9c**). We observed that the expression of ataxin-2^{MUT(80Q)}-GFP + CT-mCherry was able to reduce by 13% the total dendritic length of striatal neurons when compared to neurons transfected with ataxin-2^{WT(23Q)}-GFP + CT-mCherry. Furthermore, the expression of eIF2 α ^{S52D}-mCherry + CT-mCherry mitigated the reduction of dendritic length (**Figure R9c**).

Then, we counted how many dendrites are connected from a specific branch, referred to as dendritic order. This means that dendrites directly connected to the soma are 1st order; those extending from 1st order are of 2nd order; those linked to 2nd order are of 3rd order; and those branching from 3rd order are of 4th order (**Figure R9c upper**). Counting the dendritic order allows us to understand how many dendrites are branching from a specific branch. However, we were not able to detect differences regarding the number of dendrites per order in the different experimental conditions (**Figure R9c bottom**).

We also performed a Sholl analysis, which, in our experimental design, consisted of counting the number of intersections between dendrites and a diagram with predefined spaced rings (5 μ m) centered on the cell soma (**Figure R9b right; Figure Rd**). This measurement allows us to count how many branches cross a specific ring at a specific distance from the soma, which is indicative of the arborization complexity at that distance (Mauceri et al., 2015; Sholl, 1953). Despite the differences observed in the total dendritic length, we were not able to detect differences between experimental conditions regarding the neuronal morphology using the Sholl the analysis (**Figure R9e**).

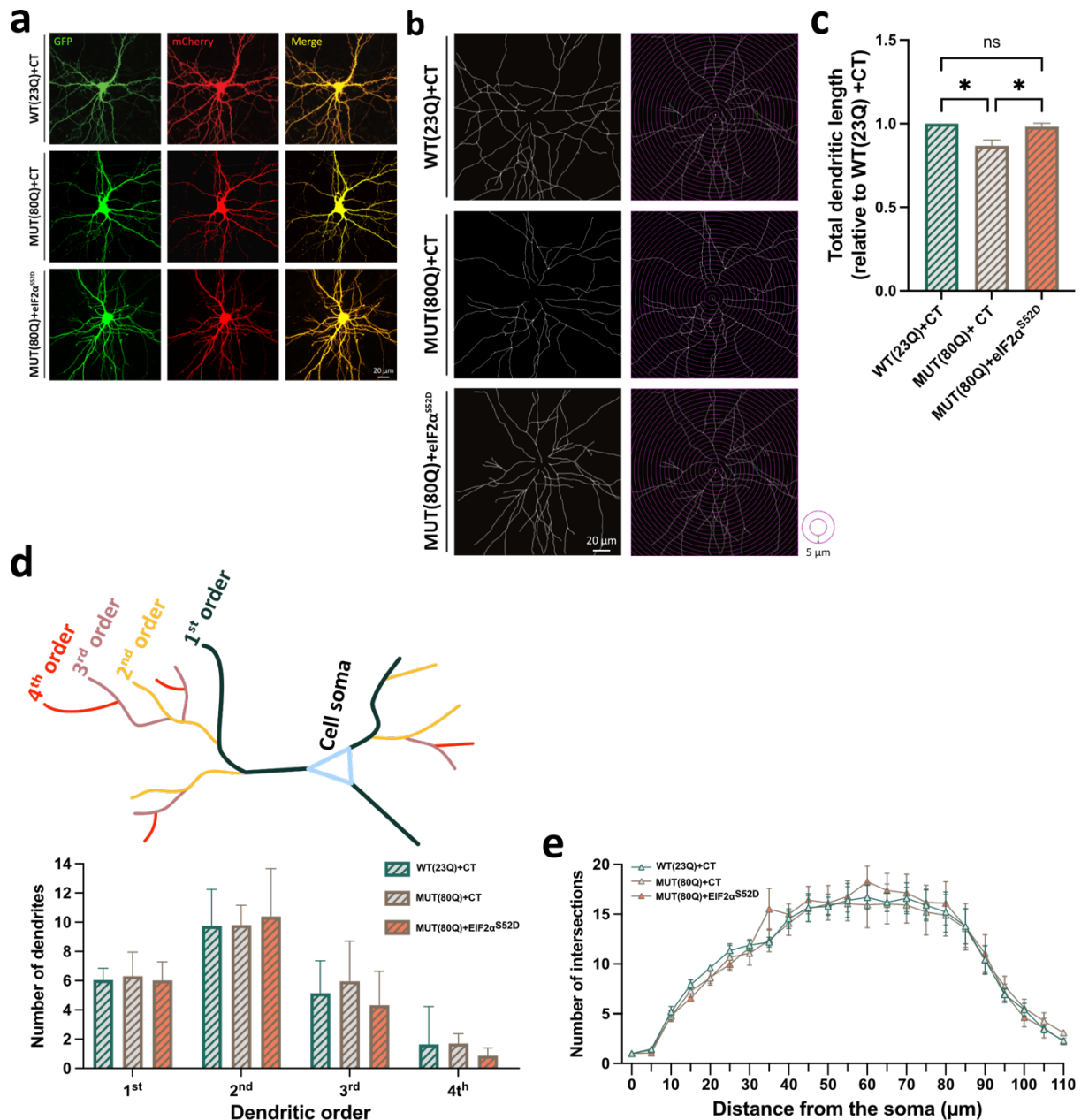


Figure R9 - eIF2α phosphomimetic expression reverts deficits associated with the expression of mutant ataxin-2. Expression of eIF2α^{S52D}—mCherry in primary cultures of mice striatal neurons was able to mitigate the phenotype associated with the expression of ataxin-2^{MUT(80Q)}—GFP (ataxin-2 mutant form). **a**) Representative microscopy images of primary cultures of mice striatal neurons expressing ataxin-2^{WT(23Q)}—GFP or ataxin-2^{MUT(80Q)}—GFP (green) [b]left column]; and CT—mCherry or eIF2α^{S52D}—mCherry (red) [b]middle column]. **b**)—**d**) Expression of eIF2α^{S52D}—mCherry was able to mitigate the phenotype associated with the expression of ataxin-2^{MUT(80Q)}—GFP. Dendrites from striatal cultures were traced to evaluate the morphometric parameters. **b**) Representative image depicting the dendritic skeleton after the tracing (white lines) [b]left column]. Representative images depicting the diagram of the Sholl analysis with the rings spaced 5 μm (purple) [b]right column]. **c**) The expression of ataxin-2^{MUT(80Q)}—GFP resulted in the reduction of the total dendritic length of striatal neurons (≈13%), compared to neurons expressing ataxin-2^{WT(23Q)}—GFP. The expression of eIF2α^{S52D}—mCherry resulted in the reestablishment of the total dendritic length in striatal neurons expressing ataxin-2^{MUT(80Q)}—GFP. **d**) No differences were detected in the number of dendrites order across the different experimental conditions. **e**) No differences were detected with the Sholl analysis regarding the number of intersections between dendrites and the rings across the different experimental conditions. (n = 4 independent experiments; *P<0.05; ns - non-significance; One-way Anova followed by Bonferroni correction; values are expressed as mean.

Inducing SGs in vivo in an SCA2 lentiviral mice model

SCA2 is characterized by a progressive impairment of motor functions accompanied by a neuropathological profile marked by neurodegeneration and the formation of mutant ataxin-2 aggregates within the affected brain regions (Conceição & Costa et al., 2024). Due to the limitation of studying neuropathology hallmarks in cellular models, we used a more robust model to study the impact of inducing the formation of SGs *in vivo* in the context of SCA2. Therefore, to assess the effects of expressing the phosphomimetic form of eIF2 α , we used an LV mouse model of SCA2 previously developed by our lab (Marcelo et al., 2021a). Briefly, the LV model of SCA2 is characterized by the expression of the mutant ataxin-2 using LV particles stereotactically injected directly into the striatum. This mouse model offers a fast and convenient method for studying SCA2-associated neuropathology *in vivo* (Marcelo et al., 2021a).

To achieve our goal, in the right striatal hemisphere, mice were stereotactically co-injected with LV particles encoding for ataxin-2^{MUT(80Q)}—GFP and AAV particles encoding for CT—mCherry, as control. Additionally, in the left striatal hemisphere, mice were co-injected with LV particles encoding for ataxin-2^{MUT(80Q)}—GFP and AAV particles encoding for eIF2 α ^{S52D}—mCherry. Mice were injected at 8 weeks of age and sacrificed 12 weeks later for tissue processing and analysis (**Figure R10 a-b**). The co-expression of ataxin-2^{MUT(80Q)}—GFP and eIF2 α ^{S52D}—mCherry led to a significant loss of DARPP-32 neuronal marker staining volume when compared with the contralateral control hemisphere, which was co-injected with ataxin-2^{MUT(80Q)}—GFP and CT—mCherry (**Figure R10c upper row; Figure R10d**). However, regarding the number of ataxin-2^{MUT(80Q)} aggregates formed, assessed by HA staining, we were not able to detect differences between the two injected hemispheres (**Figure R10c bottom row; R10e**).

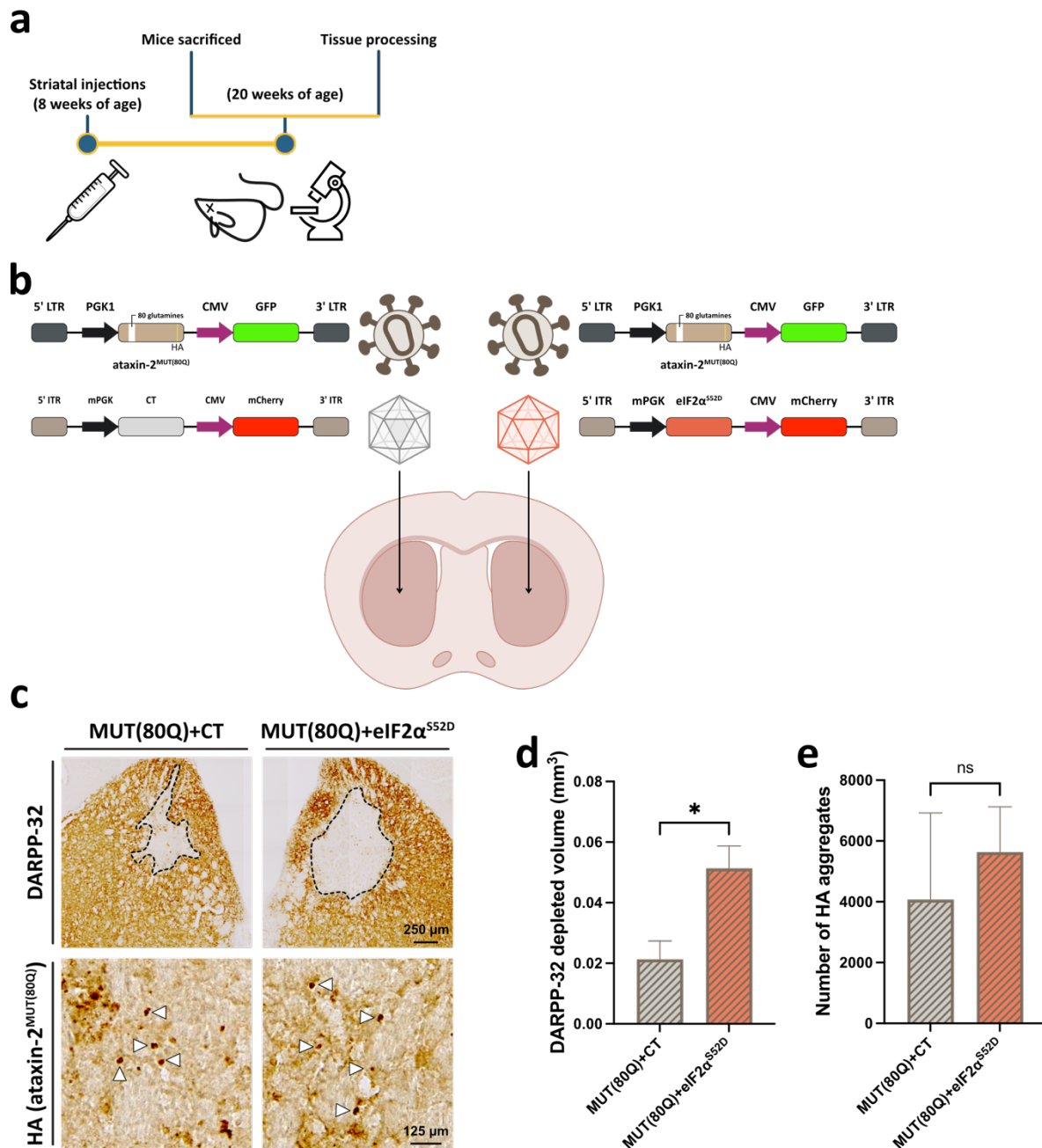


Figure R10 - *In vivo* expression of the eIF2α phosphomimetic induces the loss of the neuronal dopaminergic marker DARPP-32. To assess the impact of expressing eIF2α phosphomimetic in the context of SCA2, we used a LV mouse model of SCA2. **a)** Schematic representation of the timeline used in the study. Mice were sacrificed stereotactically injected at 8 weeks of age. Then, 12 weeks post-injection, mice were sacrificed, and brains were processed for posterior analysis. **b)** Mice were stereotactically injected into the striatum. Into the left striatal hemisphere, mice were stereotactically injected with LV particles coding for ataxin-2^{MUT(80Q)}—GFP and AAV particles coding for CT—mCherry. Into the right striatal hemisphere, mice were stereotactically injected with LV particles coding for ataxin-2^{MUT(80Q)}—GFP and AAV particles coding for eIF2α^{S52D}—mCherry. **c)** Representative microscopy images of mice brain sections immunostained for DARPP-32 or HA-tag. We measured the loss of the neuronal marker DARPP-32 [c]upper row; dashed line]. We counted the number of ataxin-2^{MUT(80Q)} aggregates [c]bottom row; arrowheads]. **d)** LV-mediated expression of eIF2α^{S52D}—mCherry resulted in an increased volume loss of the DARPP-32 marker. **e)** No differences were found between both injected hemispheres regarding the number of ataxin-2^{MUT(80Q)} aggregates formed. (n = 5 animals; *P<0.05; Student's *t*-test; values are expressed as mean ± SEM).

4.3 Inhibiting SGs in the context of SCA2

Introduction

The role of ISR and the importance of SGs in the context of neurodegenerative disease are still poorly understood. Overall, it is known that ISR is critical for cells to deal with multiple stress stimuli, but the exact mechanisms of how this pathway impacts diseases remain unclear (Pakos-Zebrucka et al., 2016). In the past few years some studies begun trying to establish a connection between ISR, SGs, and neurodegenerative diseases (Hoozemans et al., 2009, 2007; Moreno et al., 2013; Radford et al., 2015; Walter and Ron, 2011). Nevertheless, it is still not clear whether SGs mitigate or contribute to the pathology. The current mainstream hypothesis speculates that in the first stages of stress, SGs play an essential role in trying to mitigate the stress impact (Marcelo et al., 2021a; Riggs et al., 2020). However, when the stress stimulus shifts to a permanent chronic state, cells become susceptible to the stress response, and in that case, SGs' permanent assembly could promotes cell death (Pakos-Zebrucka et al., 2016).

In the first part of this work, we started by studying the impact of inducing the formation of SGs in the context of the mutant ataxin-2 expression. In this part, we are interested in understanding the effects of inhibiting the formation of SGs. To achieve this goal, we used the genetic tool GADD34^{Δ322}–mCherry, which has previously been developed and shown to ablate the assembly of SGs. The next experiments, which follow up on the previous findings reported here, are expected to contribute not only to the understanding of SG's role in the context of SCA2 but also help map within the ISR pathway which key players are impacting the disease.

Inhibiting SGs in vitro in the context of mutant ataxin-2 expression

We have previously shown that expressing a truncated form of GADD34 (GADD34^{Δ322}–mCherry) was able to prevent the formation of SGs. To understand the impact of inhibiting SGs assembly in the context of the mutant ataxin-2 expression, we used primary cultures of mouse striatal cells, previously described (**Figure 11a**). Primary neurons were handled in the same way as described before and were transfected at DIV10 either with ataxin-2^{WT(23Q)}–GFP and CT–mCherry (**Figure R11c upper**); or ataxin-2^{MUT(80Q)}–GFP and CT–mCherry (**Figure R11c bottom left**); or ataxin-2^{MUT(80Q)}–GFP and eIF2α^{S52D}–mCherry (**Figure R11c bottom right**). Then, 48h after transfection, we performed a morphometric analysis of the neurons expressing both mCherry and GFP to assess dendritic morphology (**Figure R12**).

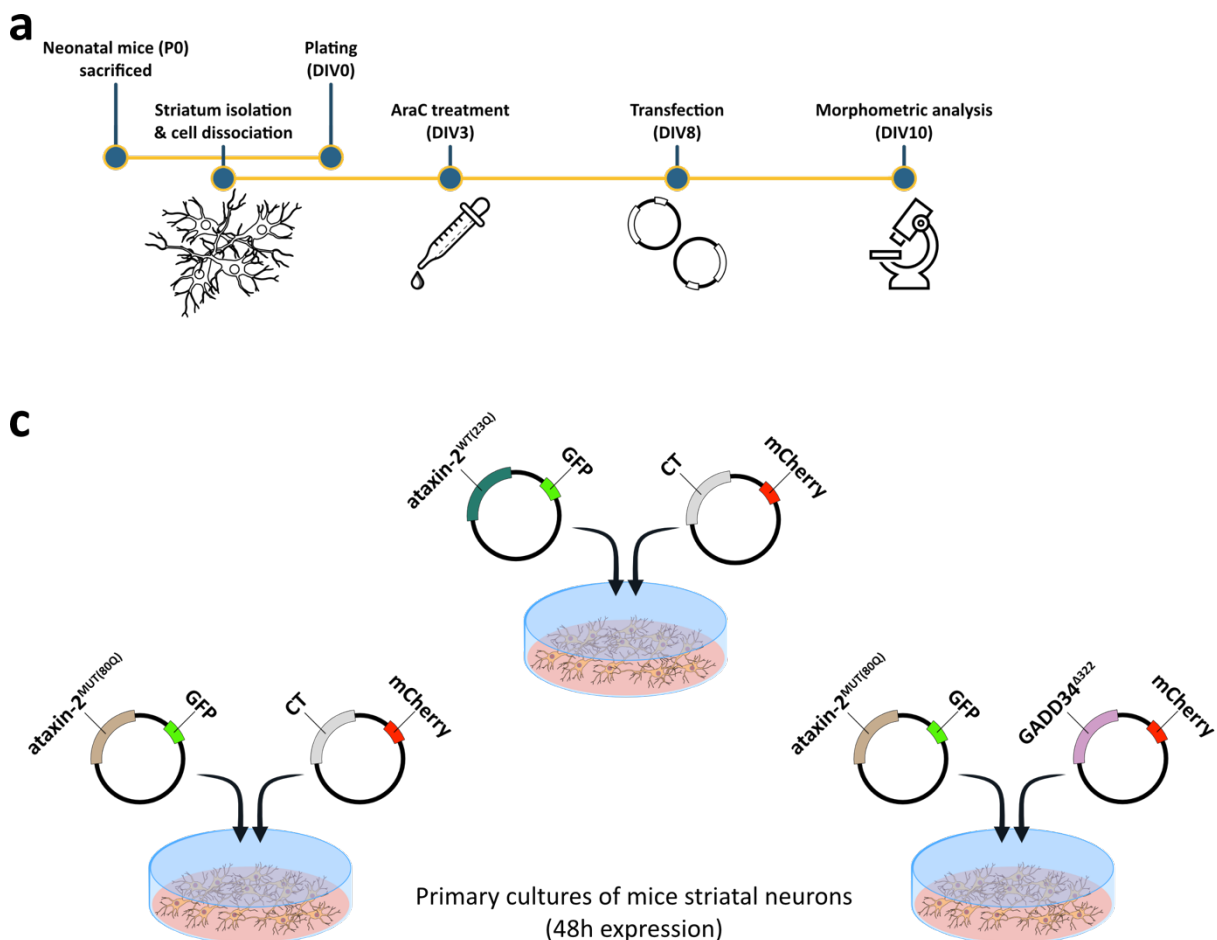


Figure R11 - Experimental design used to study the impact of inhibit SGs formation in the context of the mutant ataxin-2. We used primary cultures from mice striatal neurons to assess the effects of preventing the assembly of SG in the context of the mutant ataxin-2. **a)** Schematic representation of the timeline used to study the impact of inhibit SGs assembly in the context of the mutant ataxin-2. Mice were sacrificed at P0. Mice brains were dissected, the striatum isolated, and cells were dissociated. Finally, cells were plated, which represented zero days in vitro (DIV0). At DIV3, cells were treated with cytosine-D-arabofuranoside (AraC) to block the proliferation of glial cells. At DIV8, cells were transfected, and at DIV10, cells were processed for analysis. **b)** Schematic representation of the experimental conditions used to study the impact of inhibit the formation of SGs in the context of the mutant ataxin-2. We had three different experimental conditions: primary cells transfected with ataxin-2^{WT(23Q)}—GFP and CT—mCherry [c)upper]; or with ataxin-2^{MUT(80Q)}—GFP and CT—mCherry [c)bottom left]; or with ataxin-2^{MUT(80Q)}—GFP and + GADD34^{Δ322}—mCherry [c)bottom right].

We observed that the expression of ataxin-2^{MUT(80Q)}—GFP + CT—mCherry expression reduced the total dendritic length of striatal neurons when compared to neurons transfected with ataxin-2^{WT(23Q)}—GFP + CT—mCherry (**Figure R12a-c**). The co-expression of ataxin-2^{MUT(80Q)}—GFP + GADD34^{Δ322}—mCherry also showed a significant reduction regarding the total dendritic length when compared to primary neurons co-transfected with ataxin-2^{WT(23Q)}—GFP + CT—mCherry (**Figure R12a-c**). We were not able to detect differences between the experimental conditions ataxin-2^{MUT(80Q)}—GFP + CT and ataxin-2^{MUT(80Q)}—GFP + GADD34^{Δ322}—mCherry regarding the total dendritic length of the primary striatal neurons (**Figure R12a-c**).

After, we counted the number of dendrites by order, as described (**Figure R11d upper**). Using this morphometric analysis, we were not able to detect differences regarding the number of dendrites per each order between ataxin-2^{WT(23Q)}—GFP + CT—mCherry, ataxin-2^{MUT(80Q)}—GFP + GADD34^{Δ322}—mCherry, and ataxin-2^{MUT(80Q)}—GFP + GADD34^{Δ322}—mCherry conditions (**Figure R12d bottom**).

Finally, we performed a Sholl analysis and counted the number of intersections between neuronal branches of primary neurons and the predefined ringed diagram (**Figure R12b right column**). We were not able to detect differences in this analysis between ataxin-2^{WT(23Q)}—GFP + CT—mCherry, ataxin-2^{MUT(80Q)}—GFP + GADD34^{Δ322}—mCherry, and ataxin-2^{MUT(80Q)}—GFP + GADD34^{Δ322}—mCherry conditions (**Figure R12d**).

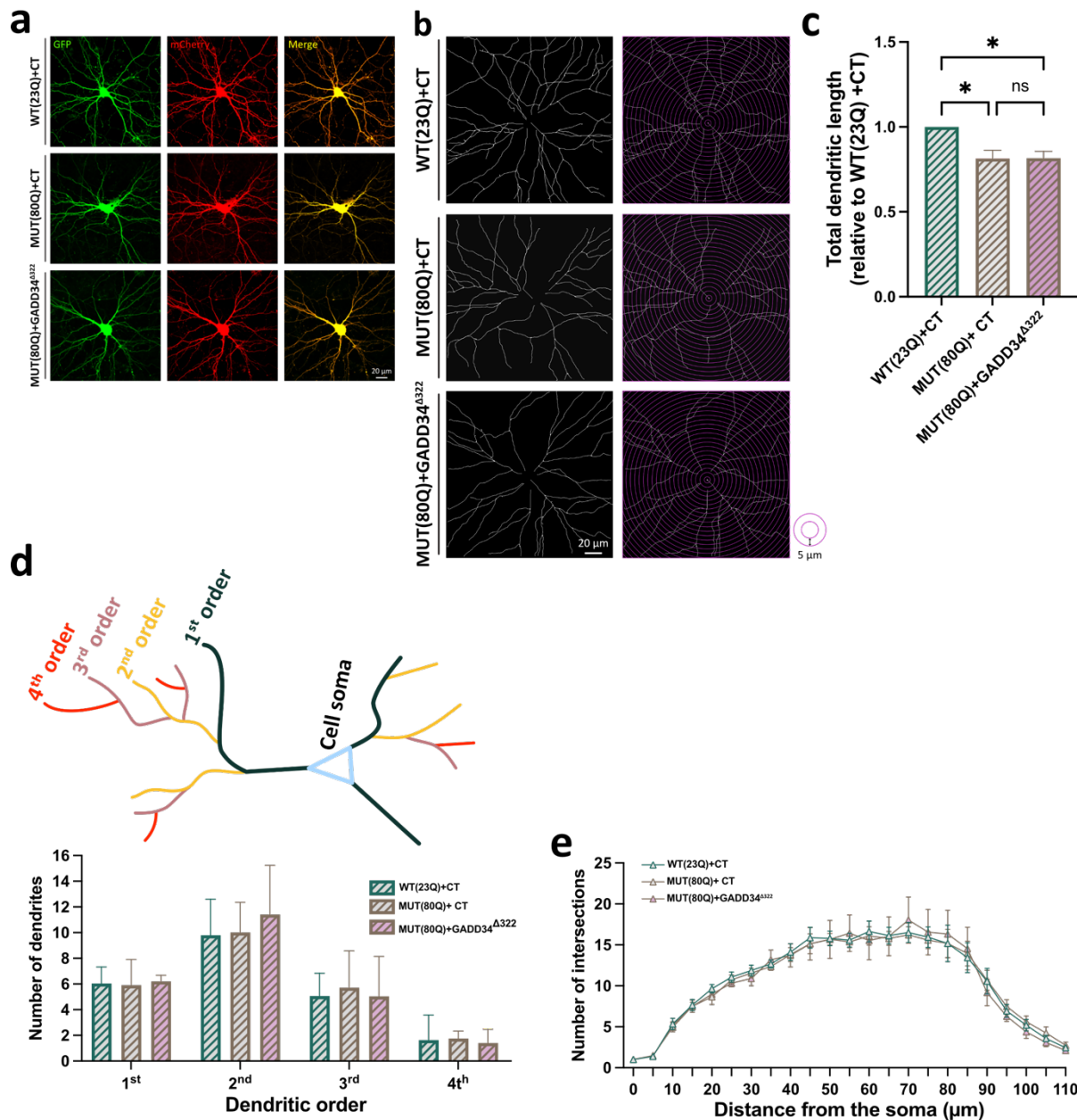


Figure R12 - Truncated GADD34 expression does not impact the phenotype associated with mutant ataxin-2 expression. The expression of GADD34^{Δ322}—mCherry in primary cultures of mice striatal neurons did not impact the phenotype associated with the expression of ataxin-2^{MUT(80Q)}—GFP (ataxin-2 mutant form). **a**) Representative microscopy images of primary cultures of mice striatal neurons expressing ataxin-2^{WT(23Q)}—GFP or ataxin-2^{MUT(80Q)}—GFP (green) [b]left column]; and CT—mCherry or GADD34^{Δ322}—mCherry (red) [b]middle column]. **b)-d**) Expression of GADD34^{Δ322}—mCherry did not impact the phenotype associated with the expression of ataxin-2^{MUT(80Q)}—GFP. Dendrites from striatal cultures were traced to evaluate the morphometric parameters. **b**) Representative images depicting the dendritic skeleton after the tracing (white lines) [b]left column]. Representative images depicting the diagram of the Sholl analysis with the rings spaced 5 μm (purple) [b]right column]. **c**) The expression of ataxin-2^{MUT(80Q)}—GFP resulted in the reduction of the total dendritic length of striatal neurons, compared to neurons expressing ataxin-2^{WT(23Q)}—GFP + CT—mCherry; or expressing ataxin-2^{MUT(80Q)}—GFP + GADD34^{Δ322}—mCherry. **d**) We did not detect differences in the number of dendrites of each order across our experimental conditions. **e**) We did not detect differences with the Sholl analysis regarding the number of intersections between dendrites and the rings across our experimental conditions. (n = 4 independent experiments; *P<0.05; ns - non-significance; One-way Anova followed by Bonferroni correction; values are expressed as mean (three conditions); values are expressed as mean ± SEM).

Inhibiting SGs in vivo within the context of an SCA2 lentiviral mouse model

SCA2 is a neurodegenerative disease featuring a neuropathological profile characterized by the formation of mutant ataxin-2 aggregates followed by neuronal death (Conceição & Costa et al., 2024). To assess whether expressing the truncated form of GADD34, which we have shown to inhibit the formation of SGs, has the potential to modify the pathology associated with SCA2, we decided to investigate its impact *in vivo*. To achieve that goal, we used the previously described LV mouse model of SCA2 (Marcelo et al., 2021a) (**Figure R13a**). Briefly, in the right striatal hemisphere, mice were stereotactically co-injected with LV particles encoding for ataxin-2^{MUT(80Q)}–GFP and AAV particles encoding for CT–mCherry, as control. In the left striatal hemisphere, mice were co-injected with LV particles coding for ataxin-2^{MUT(80Q)}–GFP and AAV particles coding for GADD34^{Δ322}–mCherry (**Figure R13b**). Mice were injected at 8 weeks of age and sacrificed 12 weeks later for tissue processing and analysis (**Figure R13a**). The co-expression of ataxin-2^{MUT(80Q)}–GFP + CT–mCherry showed no differences regarding the loss of DARPP-32 neuronal marker staining volume when compared with the contralateral hemisphere, which was co-injected with ataxin-2^{MUT(80Q)}–GFP + GADD34^{Δ322}–mCherry (**Figure R13c upper row; Figure R13d**). After, we counted the number of mutant ataxin-2 aggregates (HA-tagged) formed within the two injected hemispheres. We did not detect differences regarding the number of mutant ataxin-2 aggregates formed between the two injected hemispheres (**Figure R13c bottom row; Figure R13e**).

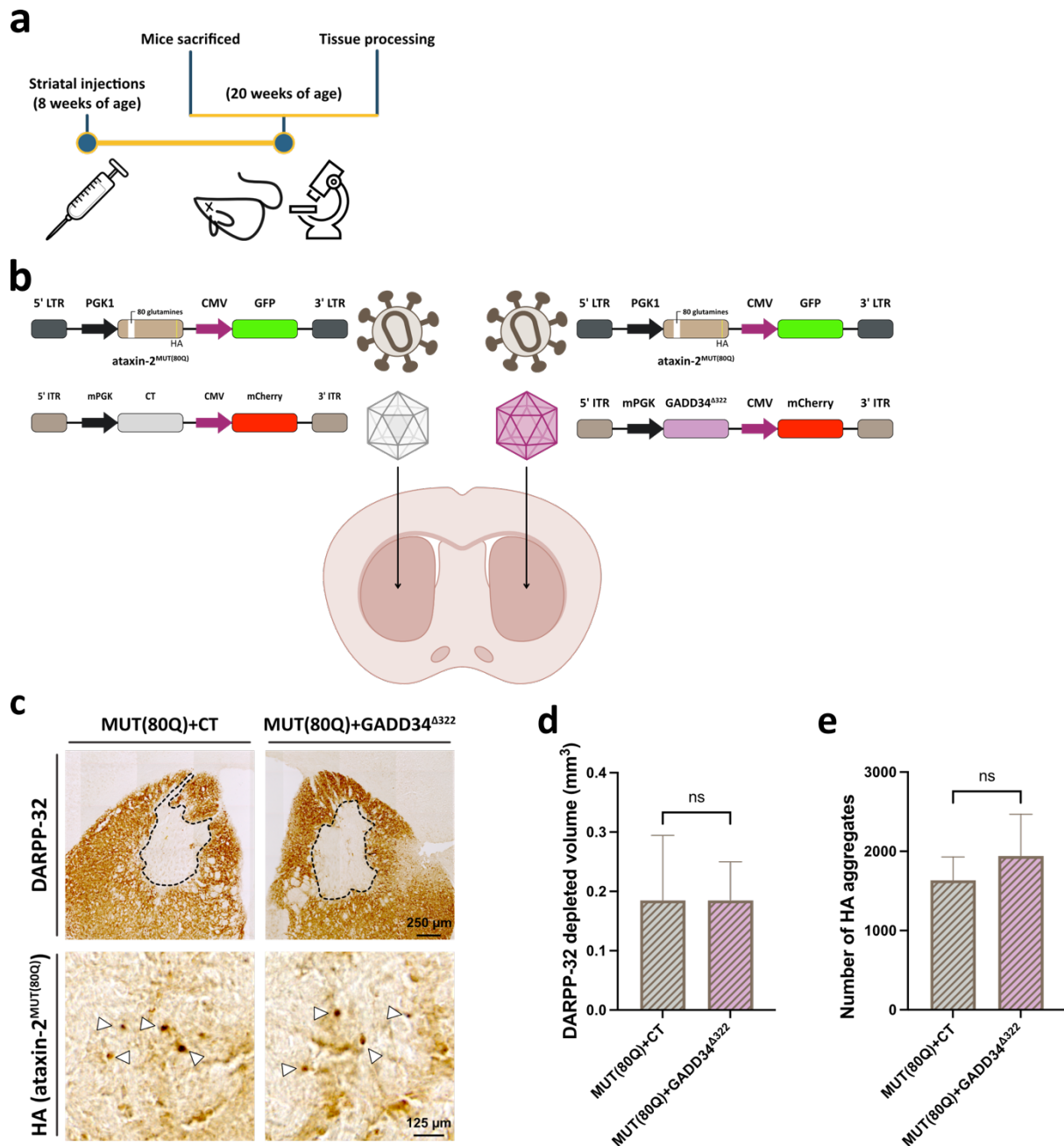


Figure R13 - *In vivo* expression of GADD34 truncated form does not modify the pathology associated to SCA2 disease. To assess the impact of expressing truncated GADD34 in the context of SCA2, we used an LV mouse model of SCA2. **a)** Schematic representation of the timeline. Mice were stereotactically injected at 8 weeks of age. Then, 12 weeks post-injection, mice were sacrificed, and brains collected for posterior analysis. **b)** We used lentiviral particles to transduce striatal neurons with ataxin-2^{MUT(80Q)}—GFP and AAV particles to express either CT—mCherry or GADD34^{Δ322}—mCherry. In the left striatal hemisphere, mice were stereotactically injected with LV particles encoding for ataxin-2^{MUT(80Q)}—GFP and AAV particles coding for CT—mCherry. In the right striatal hemisphere, mice were stereotactically injected with LV particles encoding for ataxin-2^{MUT(80Q)}—GFP and AAV particles encoding for GADD34^{Δ322}—mCherry. **c)** Representative microscopy images of mice brain sections immunostained for DARPP-32 or HA-tag. We measured the loss of the neuronal marker DARPP-32 [c]upper row; dashed line]. We counted the number of ataxin-2^{MUT(80Q)} aggregates [c]bottom row; arrowheads]. **d)** LV-mediated expression of GADD34^{Δ322}—mCherry did not change the volume loss of the DARPP-32 marker. **e)** We did not detect differences between injected hemispheres regarding the number of ataxin-2^{MUT(80Q)} aggregates formed. (n = 3 animals; t-test; values are expressed as mean ± SEM).

4.4 Development of a novel SCA2 transgenic mouse model

Introduction

In biomedicine, it is important to use proper models to withdraw significant conclusions. While *in silico* or *in vitro* approaches may be sufficient in the initial stages of preclinical studies, at more advanced stages, relying on animal models becomes essential to mimic specific disease contexts more accurately. For instance, translating experimental outcomes obtained from cellular models to aspects of motor performance in humans would be impossible. Despite their limitations, animal models remain one of the best available options for studying neurodegenerative diseases, especially those characterized by motor impairments (Alves-Cruzeiro et al., 2016; Dawson et al., 2018). To help us predict a hypothetical human outcome in the context of a genetic disease, animal models (as any other model) should recapitulate the human genetic context of the disease, exhibit relevant phenotypes, and mimic the disease progression (Alves-Cruzeiro et al., 2016; Dawson et al., 2018; Tello et al., 2022).

Mice have been used in research since at least the 17th century, but their use significantly increased in the 1900s following Mendel's studies on inheritance (Phifer-Rixey and Nachman, 2015). As in any other model, mice display limitations when studying human diseases, and findings may not always fully replicate human pathophysiology or treatment responses. For example, mice have a significantly shorter lifespan, and while their genetic context and anatomy are relatively similar to humans (which can be seen as an advantage), they are not identical (Jucker, 2010). Genetically and anatomically, mice also exhibit enough similarities that make them relevant for translational studies in a human context (Bryda, 2013). Nevertheless, the main advantages of using mice in biomedical research include their small size, ease of housing, easy breeding, and relatively low associated costs compared to other mammals.

SCA2 is characterized by a progressive decline in motor performance. In fact, motor deficits, particularly ataxia, are main clinical hallmarks of SCA2. Despite efforts to understand

its pathophysiology, much remains unknown, and SCA2 remains an incurable and devastating disease. Affected individuals rely solely on symptomatic and supportive treatments, which neither cure nor delay the disease, meaning that affected individuals die prematurely (Conceição & Costa et al., 2024; Pulst, 2025).

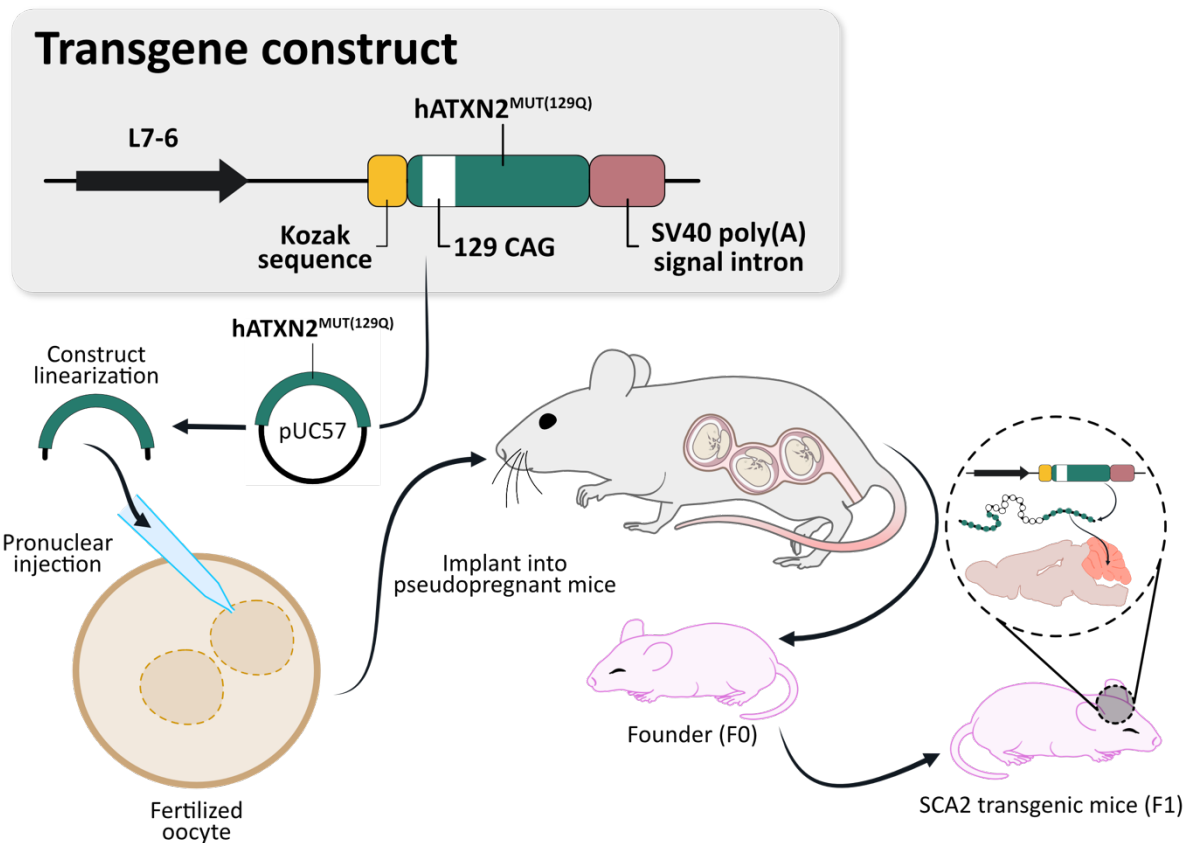
To better understand the mechanisms underlying SCA2, there is a need to develop proper disease models. To date, nine different mouse models have been developed, contributing to the understanding of both ataxin-2 functions and SCA2 pathophysiology. However, these models have certain features that make their use in some applications limited. While some SCA2 mouse models are unavailable to the scientific community, others display mild phenotypes with a disease late onset. Therefore, aiming to address these limitations, we developed a novel mouse model of SCA2 expressing a human form of the *ATXN2* gene bearing 129 CAG repeats. This new SCA2 mouse model exhibits the highest number of CAG repeats found in any currently existing SCA2 mouse model. Therefore, considering that a greater number of CAG repeats correlates to earlier onset and more severe phenotypes, we expect this novel model to exhibit more pronounced motor deficits that begin to manifest earlier when compared to other models (Conceição & Costa et al., 2024; Giunti, 1998; Hayes, 2000; Klockgether et al., 2019; Lieberman et al., 2019; Pulst et al., 2005).

Development of a novel SCA2 mouse model

We aimed to develop a novel transgenic mouse model of SCA2, expressing a human form of the mutant expanded *ATXN2* gene with 129 glutamines. From now on, the transgenic mice will be termed hATXN2^{MUT(129Q)} mice or abbreviated as TG^{MUT(129Q)} mice. In order to develop the hATXN2^{MUT(129Q)} mice, a transgene construct containing a human form of *ATXN2* was developed. This construct includes an L7-6 Purkinje cell-specific promoter to regulate and direct the expression of the hATXN2^{MUT(129Q)} to the cerebellum (Torashima et al., 2008). Additionally, the hATXN2^{MUT(129Q)} sequence is flanked upstream by a Kozak sequence and downstream by an SV40 poly(A) signal intron sequence (**Figure R14a**). Briefly, the Kozak sequence is a set of nucleotides that serves as a site to initiate translation, and SV40 poly(A) signal intron improves transcription and enhances gene expression (Kozak, 1984; Yew et al.,

1997). First, the transgene plasmid was linearized and injected into the pronucleus of a previously fertilized mouse oocyte. Subsequently, injected cells were implanted into pseudo pregnant recipient mice, giving rise to the first litter (F0). Then, the F0 generation was crossed with WT C57/BL6 (WT/B6), originating the F1 generation. Finally, the F1 generation was crossed in an inbred manner, originating the F2 generation (**Figure R14b**). To characterize the novel transgenic hATXN2^{MUT(129Q)} mice, we used the F2 generation and subsequent generations of lines 36 and 40 (**Figure R14b; yellow box**). Line 41 and Line 53 were previously characterized in other study (Koppenol, 2024).

a



b

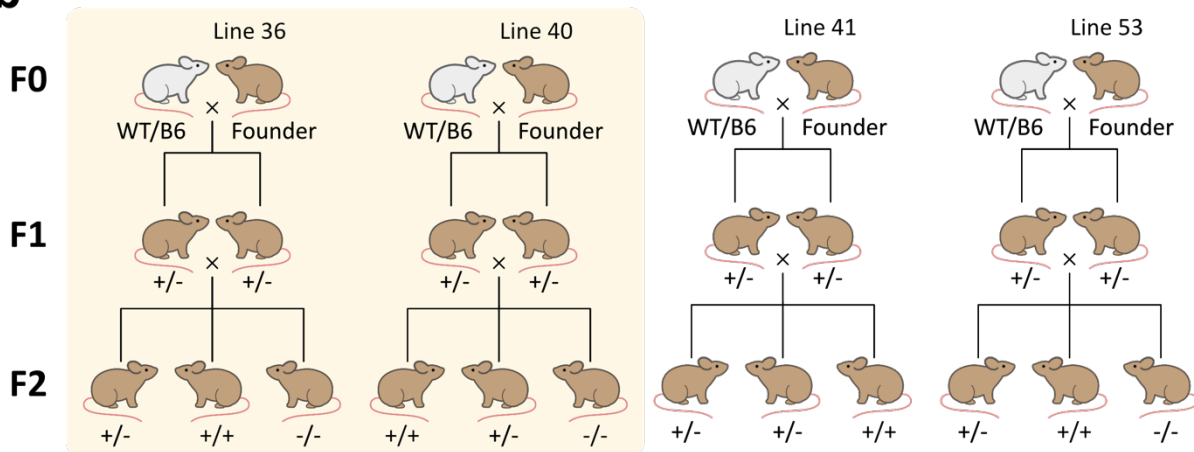


Figure R14 - Generation of a novel SCA2 transgenic mouse model. We developed a new transgenic mouse model for SCA2, designed to express a human form of ataxin-2 with 129 glutamines – hATXN2^{MUT(129Q)}. **a)** Schematic representation of the methodology used to generate the SCA2 transgenic mouse model. We used a human form of the ATXN2 gene, which is expressed under the control of the L7-6 Purkinje cell-specific promoter. The construct was linearized and injected into a pronucleus of fertilized oocytes, which were then implanted into pseudopregnant recipient mice. The recipient mice gave origin to the founder generation (F0), which subsequently produced the F1 generation. **b)** Representation of the breeding scheme used to develop the SCA2 transgenic mice. Mice from the F0 generation were crossed with WT C57/BL6 mice, originating the F1 generation. Subsequently, F1 transgenic mice were congenic crossed to originate the F2 generation. Mice from the F2 generation used in this study are either WT (-/-); or heterozygous for hATXN2^{MUT(129Q)} (+/-); or homozygous for hATXN2^{MUT(129Q)} (+/+). The yellow background box represents the lineages characterized in this study – Line 36 and Line 40. Other lineages – Line 41 and Line 53 – were previously characterized by our lab in a different study.

Characterization of the novel transgenic mouse model – Line 36

To characterize the novel transgenic mouse model of SCA2, we started to study Line 36. These mice were genotyped by PCR to discriminate WT mice from transgenic hATXN2^{MUT(129Q)} mice (**Figure R15a**). The genotyping method involved the amplification of a DNA fragment from the inserted transgene and, as a control, the amplification of a DNA fragment from the mouse endogenous DNA (**Figure R15b**). To generate an amplicon corresponding to the transgene construct, we used Primer F and Primer R (see Methods) to target the inserted sequence. Additionally, to amplify endogenous DNA, we used Primer CF and Primer CR to target the mouse genome. The two pairs of primers (F-R and CF-CR) were used simultaneously to genotype mice. As such, when in the presence of a WT animal, the genotyping PCR will generate a single 828 bp DNA band (**Figure R15b left**). However, when in the presence of transgenic hATXN2^{MUT(129Q)} mice, the genotyping PCR will generate the control DNA band (828 bp), and a 336 bp DNA band corresponding to the inserted transgene fragment (**Figure R15b left**). The control amplicon is always present independently of the transgene insertion. What differentiates WT mice from transgenic mice is the 336 bp DNA band that corresponds to the amplification of the inserted transgene (**Figure R15b left**). As expected, after crossing F1 mice, we were able to detect the presence of a 336 bp DNA amplicon corresponding to the amplification of the inserted transgene (**Figure R15b right**).

As previously detailed, to develop the transgenic mice, we inbred F1 heterozygous mice (for hATXN2^{MUT(129Q)}) with other F1 heterozygous mice. According to the Mendelian law of inheritance, the generated progeny was expected to be composed of a population of 75% transgenic mice (25% homozygous and 50% heterozygous) and 25% WT. In fact, the generated progenies were closely balanced according to Mendelian laws. Furthermore, both males and females generated were closely balanced (**Figure R15b**). Using this genotyping methodology, we were not able to distinguish homozygous from heterozygous hATXN2^{MUT(129Q)} mice. Additionally, the average litter size ranged between 6 and 9 pups, remaining stable for at least three generations (**Figure R15c**).

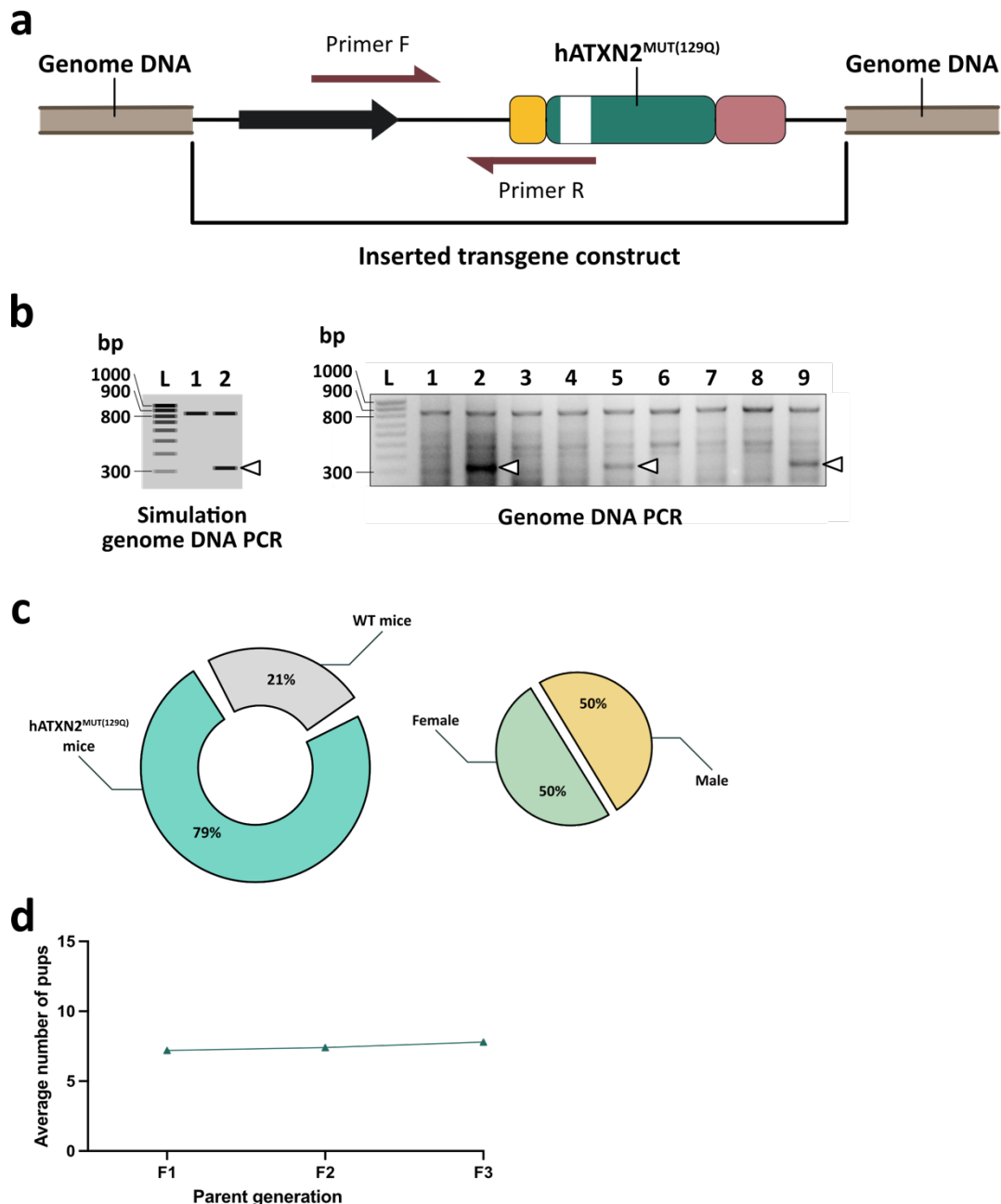


Figure R15 - SCA2 transgenic mouse Line 36 birth characteristics. Transgenic hATXN2^{MUT(129Q)} mice from Line 36 were viable and presented the transgene integrated into the genome. **a)** Schematic representation of the transgene construct inserted into the mice genome. A specific pair of primers were used for genotyping and discriminating transgenic hATXN2^{MUT(129Q)} mice from WT mice. It was also used a pair of control primers that amplifies a fragment of mouse endogenous DNA. **b)** Schematic representation of the DNA electrophoresis after PCR amplification of mice genome. **b)** *In silico* schematic simulation of the DNA bands after DNA genome amplification, using the control primers and primers F and R [b]left]. For WT animals, it was expected a single 828 bp DNA band (lanes 1; 3; 4; 6; 7 and 8). For transgenic hATXN2^{MUT(129Q)} mice it was expected a 828 bp band and a 336 bp band (lanes 2; 5; and 9). The 828 bp band represents the fragment generated from the amplification of the genomic DNA, while the 336 bp band represents the amplicon generated from the amplification of the inserted transgene (arrowheads). **b)** Representative images of the electrophoresis agarose gel showing the DNA fragments matching the predicted DNA weights [b]right]. **c)** The offspring generated from Line 36 comprised approximately 21% WT mice and 79% transgenic hATXN2^{MUT(129Q)} mice [c]left]. The offspring originated from Line 36 comprised approximately 50% females and 50% males [c]right]. **d)** The originated offspring averaged between 9-6 pups per litter across the different generations. Legend: L – DNA marker.

Since SCA2 is a highly debilitating disease characterized by progressive motor impairment, we then submitted WT mice and hATXN2^{MUT(129Q)} mice to a battery of behavioral tests to understand whether the transgenic population displayed performance deficits (**Figure R16**). Mice started to be tested at 4 weeks of age and then tested every 4 weeks until 44 weeks of age (**Figure R16a**). Mice performed the beam walk test, the swimming test, the rotarod test, and the grip strength test; mice were also weighted at every time point (**Figure R16b-h**).

Since several reports associate ataxin-2 with metabolic perturbations, including weight disturbances, we also weighed mice at every time point (Carmo-Silva et al., 2017). However, no differences regarding mice weight were observed throughout the study (**Figure R16b**).

The grip strength test consisted of holding the tail of the mouse and gently pulling the animal while it gripped a ring. The force exerted on the ring was recorded. According to the grip strength test, we were not able to detect differences in mice strength when comparing WT with transgenic hATXN2^{MUT(129Q)} animals (**Figure R16c**).

The rotarod test consisted of placing mice on an apparatus with a rotating rod that accelerated overtime. The latency to fall from the rod was measured to evaluate the performance of both groups. WT and transgenic hATXN2^{MUT(129Q)} mice exhibited similar latencies to fall across the different time points (**Figure R16d**).

The beam walking test consisted of placing mice on an elevated beam and measuring the time it took them to cross it. In our experimental setup, we used three different beams: a square 1.8 cm with beam (large beam); a square 1 cm with beam (small beam); and a cylindrical 1 cm diameter beam. Comparing WT mice with transgenic hATXN2^{MUT(129Q)} mice, we were not able to detect motor impairments using the beam walking test. Both groups performed similarly in the large beam (**Figure R16e**), in the small beam (**Figure R16f**), and in the cylindrical beam (**Figure R16g**).

Finally, the swimming test consisted of placing mice in a water-filled tank and measuring the time it took them to cross it. Using this test, we were not able to detect motor performance differences between WT and transgenic hATXN2^{MUT(129Q)} mice (**Figure R16h**).

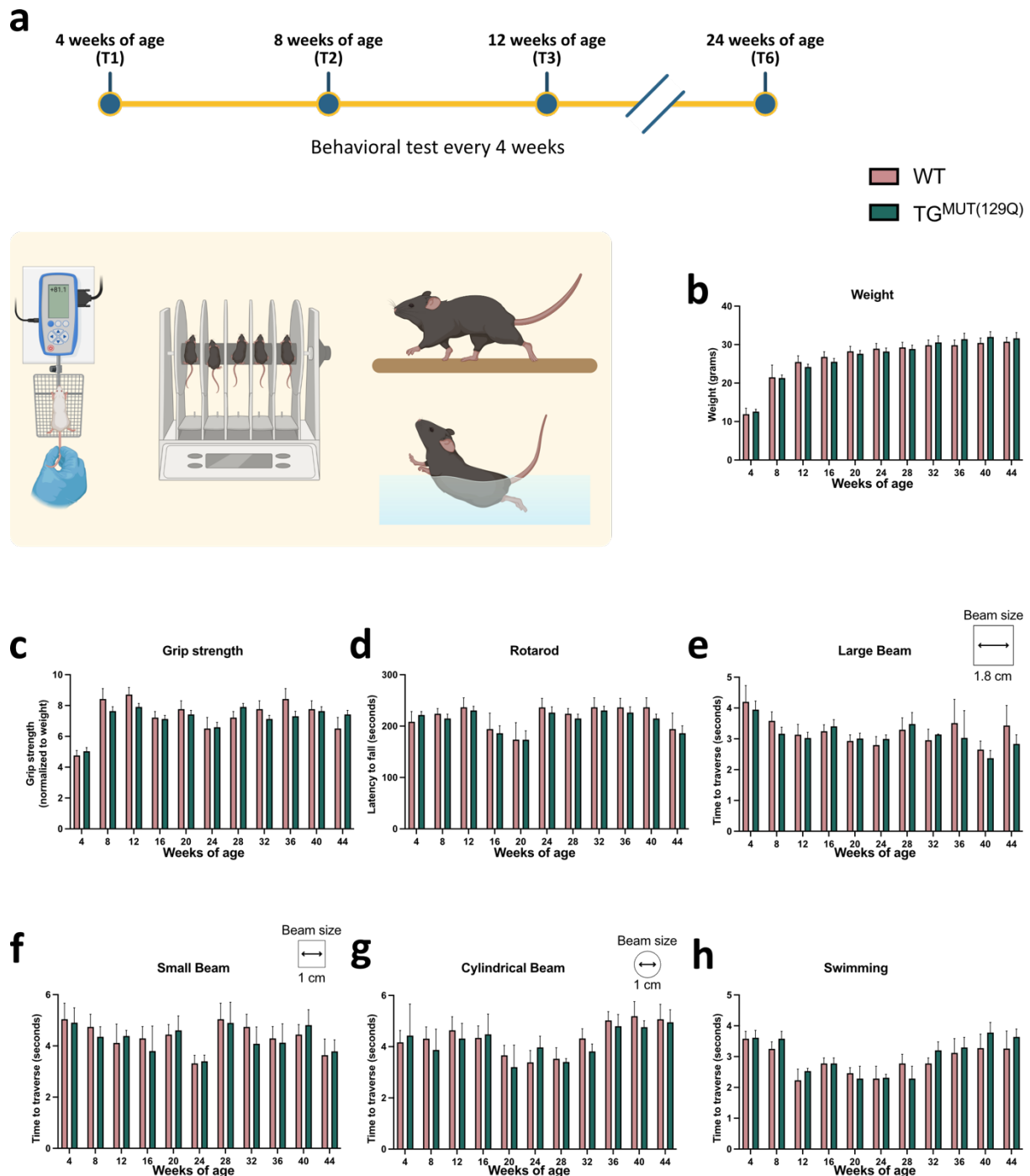


Figure R16 - SCA2 transgenic mouse Line 36 was analyzed through a battery of behavioral tests. To characterize the novel transgenic model, mice were submitted to a battery of behavioral tests. **a)** Schematic representation of the timeline used to test transgenic hATXN2^{MUT(129Q)} mice motor performance. Mice started being tested at 4 weeks of age, every 4 weeks until 44 weeks of age (11 time points). Mice were submitted to the following tests: grip strength test, rotarod, beam walking test and swimming test (yellow box). **b)** Transgenic hATXN2^{MUT(129Q)} mice and WT mice displayed similar weights. **c)** Both group tested did not display performance differences regarding the grip strength test. **d)** Transgenic hATXN2^{MUT(129Q)} and WT mice performed similarly in the rotarod test. **e)** Transgenic hATXN2^{MUT(129Q)} mice did not display differences in motor performance regarding the large beam when compared to WT mice. **f)** Transgenic hATXN2^{MUT(129Q)} and WT mice performed similarly in the medium beam. **g)** There were no significant differences in motor performance when comparing transgenic hATXN2^{MUT(129Q)} and WT mice in the cylindrical beam. **h)** We could not detect any behavioral difference in the swimming test, comparing hATXN2^{MUT(129Q)} with WT mice. n= 10 WT mice and n= 32 hATXN2^{MUT(129Q)} transgenic mice; Two-Way Anova with mixed-effect analysis followed by Bonferroni correction; values are expressed as mean $\pm$ SEM).

SCA2 disease is characterized by progressive neurodegeneration, which is translated into the loss of cerebellar Purkinje cells among other neuronal populations (Conceição & Costa et al., 2024). Therefore, and since the hATXN2^{MUT(129Q)} is Purkinje cell-specific, we proceeded with a qualitative histological analysis of Purkinje cells as an indication of neuronal loss. To do that, we immunostained cerebellar sections of WT animals and hATXN2^{MUT(129Q)} transgenic mice using the calbindin cell-specific marker of Purkinje neurons. The representative image obtained from WT and transgenic animals, at 44 weeks of age, seem to indicate that loss of Purkinje cells is not a feature present in the transgenic mice.

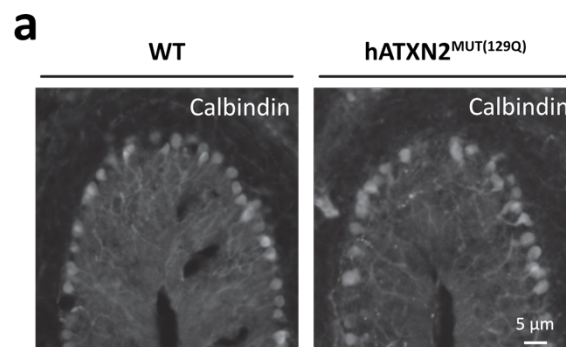


Figure R17 - SCA2 transgenic mouse Line 36 cerebellar histology. Cerebellar sections from mouse Line 36 were stained with the with the Purkinje cell-specific marker calbindin. **a)** Staining of cerebellar sections from mouse Line 36 suggest no evidence of Purkinje cell loss, as evaluated through the calbindin marker. Cerebellar sections were processed from mice at 48 weeks of age.

Overall, using this methodology to characterize the hATXN2^{MUT(129Q)} transgenic mouse Line 36, and although the inserted transgene containing a mutant expanded *ATXN2* bearing 129 CAG repeats was detected by PCR, the mice do not to exhibit a phenotype characteristic of SCA2.

Characterization of the novel transgenic mouse model – Line 40

According to the methodology used previously to characterize the transgenic mouse Line 36, we analyzed the transgenic mouse Line 40 phenotype. The genotyping method, as well as the behavioral setup used, were performed as described for the previous Line.

Considering the characterization of the mouse Line 40, when genotyping animals, we detected by PCR the presence of a single DNA band of 828 bp, which corresponded to the WT animal. Conversely, the PCR generated using transgenic hATXN2^{MUT(129Q)} mice DNA, originated an 828 bp DNA band and a 336 bp DNA band (**Figure R18a**). According to the Mendelian law of inheritance, the generated progeny was expected to be composed of a population of 75% transgenic mice (25% homozygous and 50% heterozygous) and 25% WT. However, the generated progenies seemed to be slightly unbalanced, in favor of WT mice, according to Mendel's law of inheritance (37% WT mice and 63% transgenic hATXN2^{MUT(129Q)} mice) (**Figure R18b**). Additionally, regarding the distribution of male and female mice generated, we expected a population close to 50%/50%. However, the transgenic mouse Line 40 appears to produce more females than males (**Figure R18b**). Additionally, the average litter size seems to decline over generations, from approximately 6–9 pups to approximately 3–1 pups (**Figure R18c**).

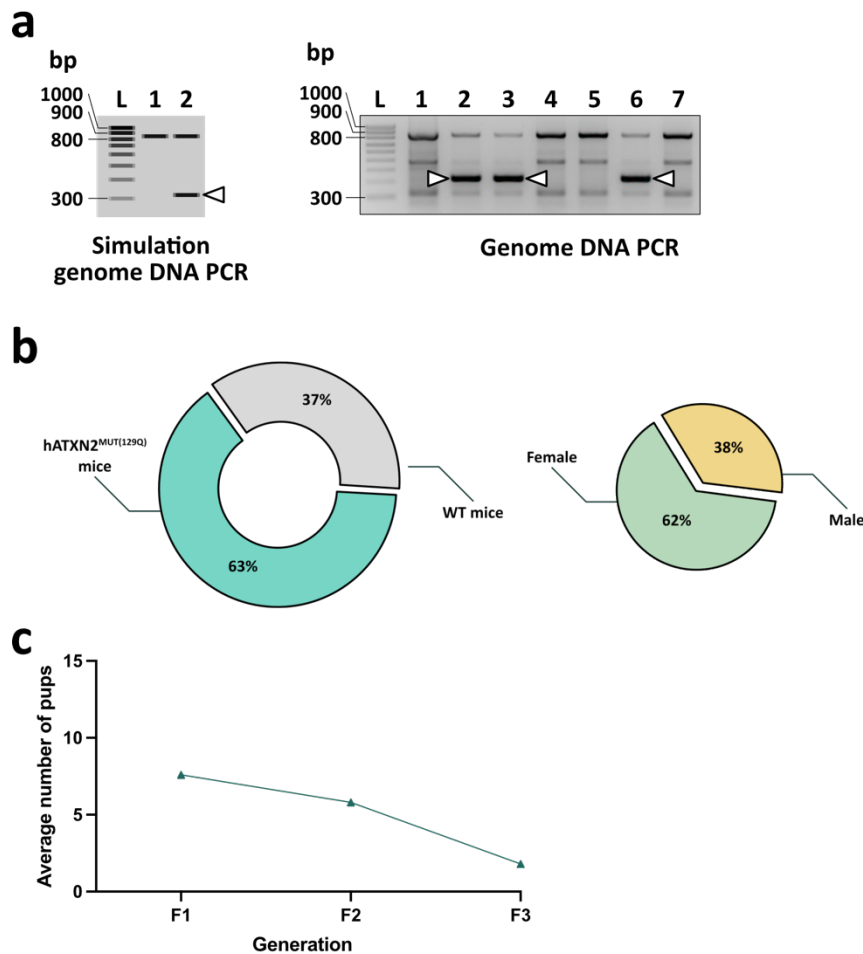


Figure R18 - SCA2 transgenic mouse Line 40 birth characteristics. Transgenic hATXN2^{MUT(129Q)} mice from Line 40 were viable and presented the transgene construct integrated into the genome. **a)** Schematic representation of the DNA electrophoresis after PCR amplification of mice genome. **a)** *In silico* schematic simulation of the DNA bands after DNA genome amplification, using the control primers and primers F and R [a]left]. For WT animals, it was expected a single 828 bp DNA band (lanes 1; 3; 4; 5; and 7). For transgenic hATXN2^{MUT(129Q)} mice it was expected a 828 bp band and a 336 bp band (lanes 2; 3; and 6). The 828 bp band represents the amplicon generated from the amplification of the genomic DNA, while the 336 bp band represents the amplicon generated from the amplification of the inserted transgene (arrowheads). **b)** Representative images of the electrophoresis agarose gel showing the DNA amplicons matching the predicted DNA weights [a]right]. **b)** The offspring generated from Line 40 comprised approximately 31% WT mice and 63% transgenic hATXN2^{MUT(129Q)} mice [c]left]. Additionally, the offspring originated from Line 40 comprised approximately 62% females and 38% males [c]right]. **d)** The average litter size declined over the generations from approximately 9-6 pups to 3-1 pups.

We then moved to the behavioral characterization of the transgenic mouse Line 40 (**Figure 19**). Due to the decline of pups generated by the transgenic hATXN2^{MUT(129Q)} mice over a generation, we could not apply the same timeline used to characterize Line 36. However, the behavioral tests and the methodology to perform them remained unchanged. The transgenic Line 40 started to be tested at 4 weeks of age, every 4 weeks until 24 weeks of age (**Figure R19a**).

First, we did not observe weight differences between WT and hATXN2^{MUT(129Q)} mice **(Figure R19b)**.

However, in the grip strength test, hATXN2^{MUT(129Q)} mice seem to perform poorer at early stages compared to WT mice . These differences were statistically significant at 4 weeks of age and 8 weeks of age **(Figure R19c)**.

Likewise, through the analysis of the rotarod test, results suggest that hATXN2^{MUT(129Q)} mice display motor deficits when compared to WT mice. These deficits are especially evident at 8 weeks of age, 20 weeks of age, and 24 weeks of age **(Figure R19d)**.

Using the beam walking test, we were not able to detect differences between WT and transgenic hATXN2^{MUT(129Q)} mice, as they performed similarly in the large beam **(Figure R19e)**, saml beam **(Figure R19f)**, and cylindrical beam **(Figure R19g)**.

Finally, we were not able to detect differences in motor performance between WT and transgenic hATXN2^{MUT(129Q)} mice using the swimming test **(Figure R19h)**.

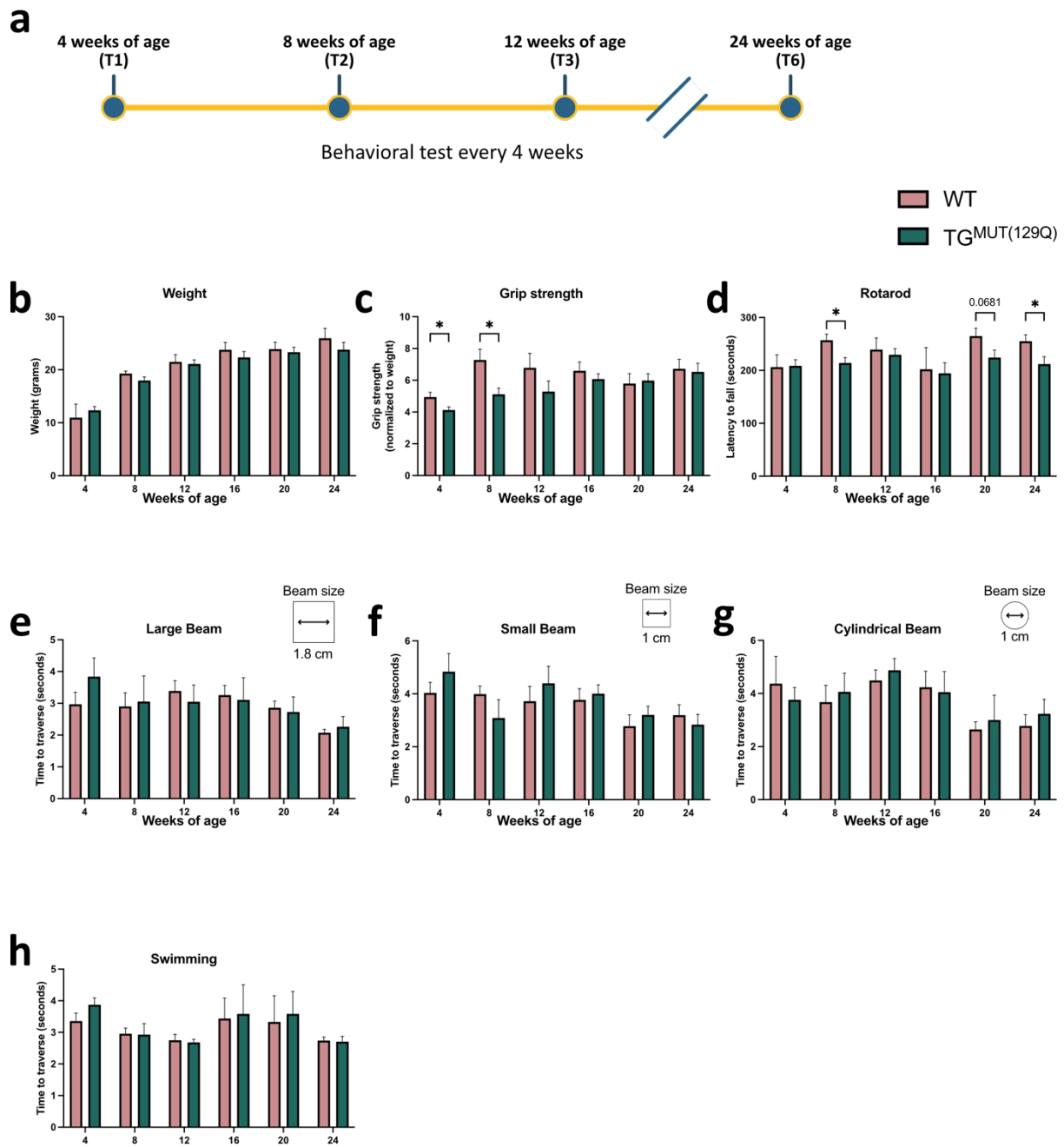


Figure R19 - SCA2 transgenic mouse Line 40 displays phenotypic traits associated with SCA2 disease. To characterize the novel transgenic mouse model, mice were submitted to a battery of behavioral tests. **a)** Schematic representation of the timeline used to test transgenic hATXN2^{MUT(129Q)} mice motor performance. Mice started being tested at 4 weeks of age, every 4 weeks until 24 weeks of age (6 time points). **b)-i)** Mice were submitted to the following tests: grip strength test, rotarod, beam walking test and swimming test. **b)** Transgenic hATXN2^{MUT(129Q)} and WT mice displayed similar weights. **c)** At 4 weeks of age and 8 weeks of age, transgenic hATXN2^{MUT(129Q)} mice exhibited reduced grip strength comparing to WT mice. **d)** At 8 weeks of age, 20 weeks and 24 weeks of age, transgenic hATXN2^{MUT(129Q)} mice performed worst in the rotarod test comparing to WT mice. **e)** Transgenic hATXN2^{MUT(129Q)} mice did not display differences in motor performance regarding the large beam when compared to WT mice. **f)** Transgenic hATXN2^{MUT(129Q)} mice and WT mice performed similarly in the medium beam. **g)** There were no significant differences in motor performance when comparing transgenic hATXN2^{MUT(129Q)} and WT mice in the cylindrical beam. **h)** We could not detect any behavioral difference in the swimming test, comparing hATXN2^{MUT(129Q)} with WT mice. n= 7 WT mice and n= 12 hATXN2^{MUT(129Q)} mice; *p<0.05; **p<0.01; Two-Way Anova with mixed-effect analysis followed by Bonferroni correction; values are expressed as mean ± SEM).

As previously described, we also did a histological qualitative analysis as an indication of neuronal loss. To do that, we immunostained cerebellar sections of WT animals and hATXN2^{MUT(129Q)} transgenic mice using the calbindin cell-specific marker of Purkinje neurons. The representative image obtained from WT and transgenic animals, at 44 weeks of age, seem to indicate that loss of Purkinje cells is a characteristic of the transgenic hATXN2^{MUT(129Q)} mice from Line 40 (**Figure R20**).

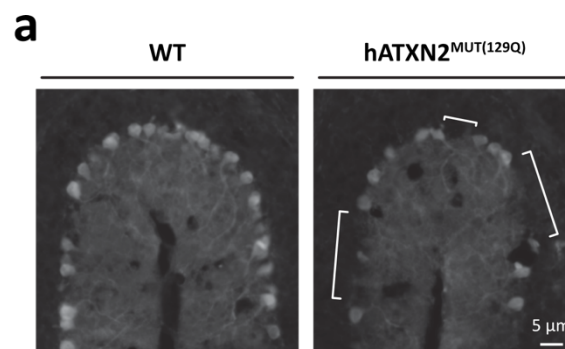


Figure R20 - SCA2 transgenic mouse Line 40 cerebellar histology. Cerebellar sections from mouse Line 40 were stained with the with the Purkinje cell-specific marker calbindin. **a)** Staining of cerebellar sections from mouse Line 40 suggest the loss of Purkinje cells, as evaluated through the calbindin marker. Cerebellar sections were processed from mice at 24 weeks of age (white brackets).

Overall, using the methodology described here to characterize the hATXN2^{MUT(129Q)} transgenic mouse Line 40, we successfully detected the transgene insertion. Additionally, we detected phenotypic traits relevant to the SCA2 context, including motor deficits at specific time points. However, the low birth rates observed make this Line unreliable and not useful for use in a laboratory setting. Thus, the following experiments were performed using the hATXN2^{MUT(129Q)} transgenic mouse Line 41, previously characterized by our lab using a similar methodology as described here (Koppenol, 2024). This model starts to exhibit motor impairments at 12 weeks of age and signs of cerebellar degeneration starting 4 weeks of age (Koppenol, 2024).

4.5 *In vivo* SGs manipulation

Introduction

SCA2 is an incapacitating and fatal disease. Affected people rely on symptomatic and supportive treatments that only alleviate symptoms without the ability to either delay or cure the disease. SCA2 symptoms usually appear between the ages of 30 and 40 and progress over approximately 10 years, ultimately leading to incapacitation and premature death. This represents not only a healthcare burden but also a significant challenge for the families of SCA2 patients (Conceição & Costa et al., 2024; Klockgether et al., 2019). All considered, there is a clear unmet medical need to develop therapies to modify SCA2 progression.

To better understand the pathological mechanism underlying SCA2 we aimed to create a novel mouse model for this disease. However, the transgenic lines previously described in this document did not exhibit suitable characteristics for reliable use in a laboratory context. Line 36 did not present a phenotype that could be associated with SCA2, and Line 40, while it presented some motor impairments, its birth rates decreased overtime. Nevertheless, our lab previously characterized a SCA2 transgenic line that proved viable and exhibited phenotypic traits that are relevant in a disease context (Koppenol, 2024).

The novel transgenic model was characterized using the traditional behavioral test to assess the motor performance of mice, which includes the rotarod test, the footprint test, and the beam walking test (Koppenol, 2024). For many years, these tests have been the gold standard for studying diseases associated with motor impairments and assessing how potential therapies impact this motor performance. While these tests often offer measurements that can be translated into a human context, they disregard the kinetic motion measurements that would help to better understand the mice's movement under a specific experimental context (Alves-Cruzeiro et al., 2016; Conceição & Koppenol et al., 2023). A kinesiology study would produce finer and more precise metrics that characterize motion, thus allowing us to better understand traits of mice's movement. In that line of thought, we aimed to further characterize the previously developed hATXN2^{MUT(129Q)} transgenic mice and conduct a thorough kinetic analysis to understand how WT mice compare with the novel

transgenic SCA2 model. To achieve that goal, we used the MotoRater systems that enable the characterization of mice's movement kinetic profile, while animals perform several behavioral tests.

Kinetic profiling of the novel SCA2 mouse model

In general, SCA2, as well as other rare diseases, face many challenges that hinder the clinical research of new medicines, such as the low prevalence of the disease, the genetic and clinical heterogeneity among patients, and the lack of objective disease scales. Additionally, the lack of suitable SCA2 models, as well as the scarcity of well-validated pre-clinical data, hinders the translation to the human context, further delaying the development of therapies (Brooker et al., 2021). To address some of the challenges faced in preclinical research in understanding SCA2 pathological mechanisms and, thus, developing novel therapies, we proceeded with the kinetic profiling of the transgenic hATXN2^{MUT(129Q)} mice Line 40. This would allow the generation of additional pre-clinical data outside the conventional behavioral tests, which is fundamental to validate potential therapeutic therapies.

The kinetic profiling was performed using an apparatus called MotoRater from TSE systems (**Figure R21a**). This device comprises a set of mirrors and a camera that records movement while mice traverse the test lane (**Figure R21b**). The mirror systems enable the recording of mice's left, right, and bottom sides (**Figure R21c**). Using the MotoRater, we characterized the kinetic profile of mice while they performed the walking test, beam walking test, and swimming test (**Figure R21c**).

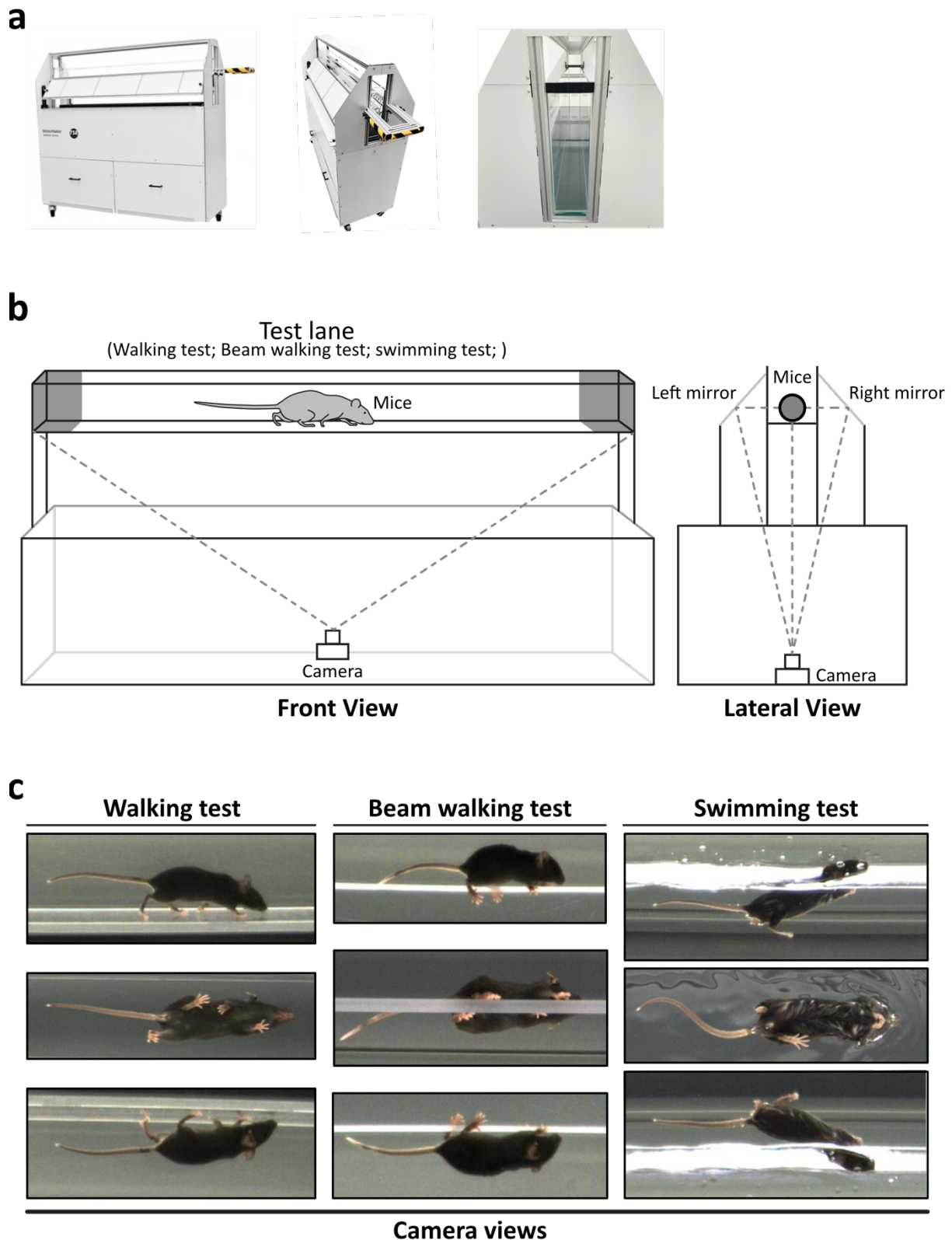


Figure R21 – The MotoRater system is a powerful tool for assessing the kinetic profile of mice's movement. The MotoRater is a system that enables the characterization of mice's movement, allowing the elaboration of a kinetic profile. **a)** Representative photographs of the MotoRater apparatus as viewed from different perspectives. **b)** Schematic representation of the MotoRater system. The MotoRater system comprises a camera that records, directly below, the movement of mice that traverse the test lane. A system of mirrors allows the recording of mice's left and right sides. **c)** The MotoRater system allows the walking test [c]left column], beam walking test [c]middle column], and swimming test [c]right column] to be performed on the same device. In every test, the left [c]left row], bottom [c]middle row], and right sides [c]right row] of the moving mice are recorded.

To the best of our knowledge, this type of kinetic profiling has never been performed on models of other SCA disorders. The main advantage of using the MotoRater system is that it can automatically detect and monitor anatomical points of interest to elaborate a kinetic map (**Figure R22a-b**). To elaborate on the kinetic profile of WT and transgenic hATXN2^{MUT(129Q)} mice, we analyzed more than 100 kinetic parameters on the tests performed (35 parameters for each test). Nevertheless, we will only present the kinetic measurements that showed differences with statistical relevance between genotypes, due to the large volume of data produced and to simplify findings. Mice were tested similarly to the previously described methodology. Tests started at 5 weeks of age and were repeated every 4 weeks until 21 weeks of age. Finally, mice were sacrificed between 1 and 3 days after finishing the last timepoint (**Figure R22c**).

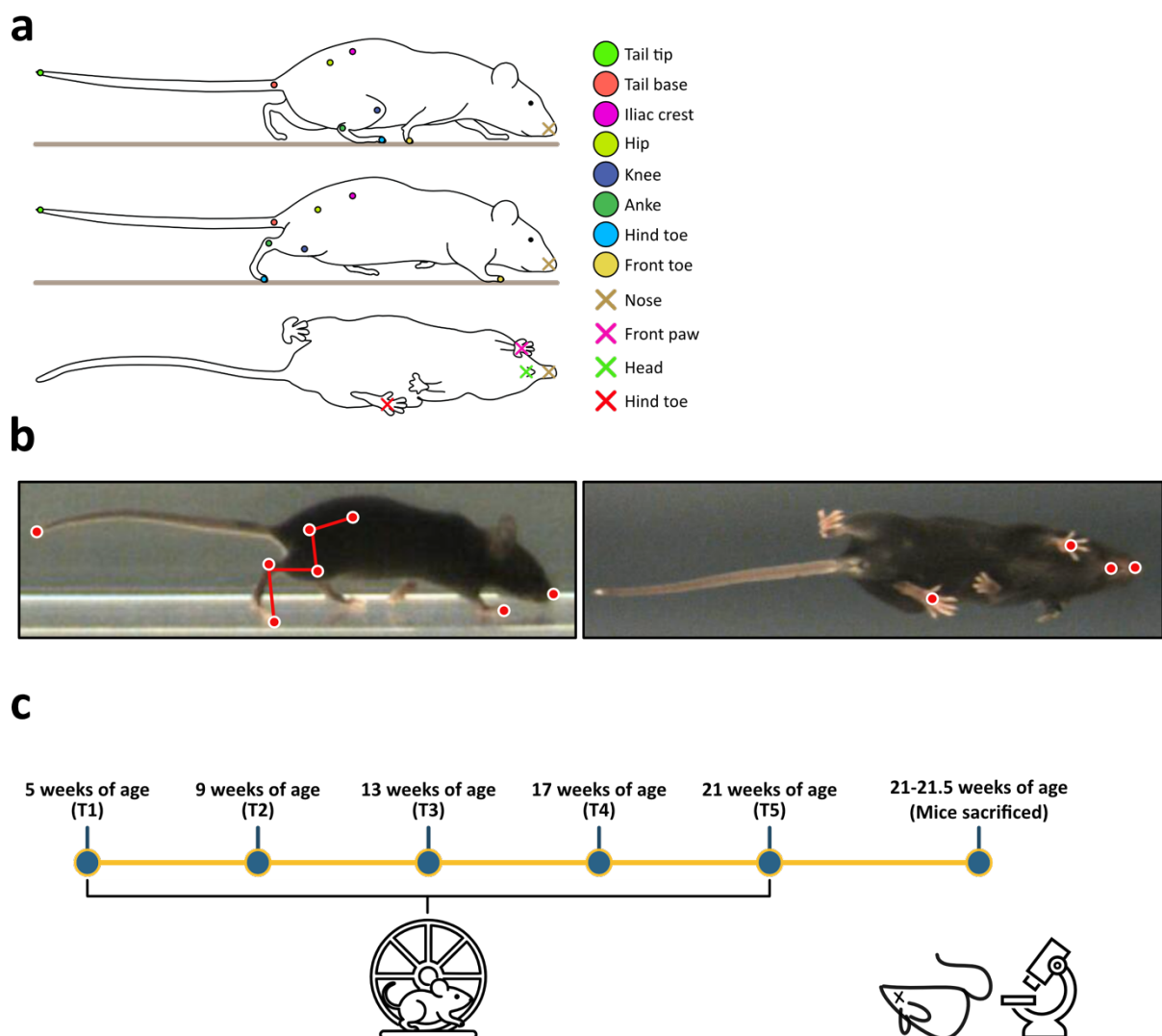
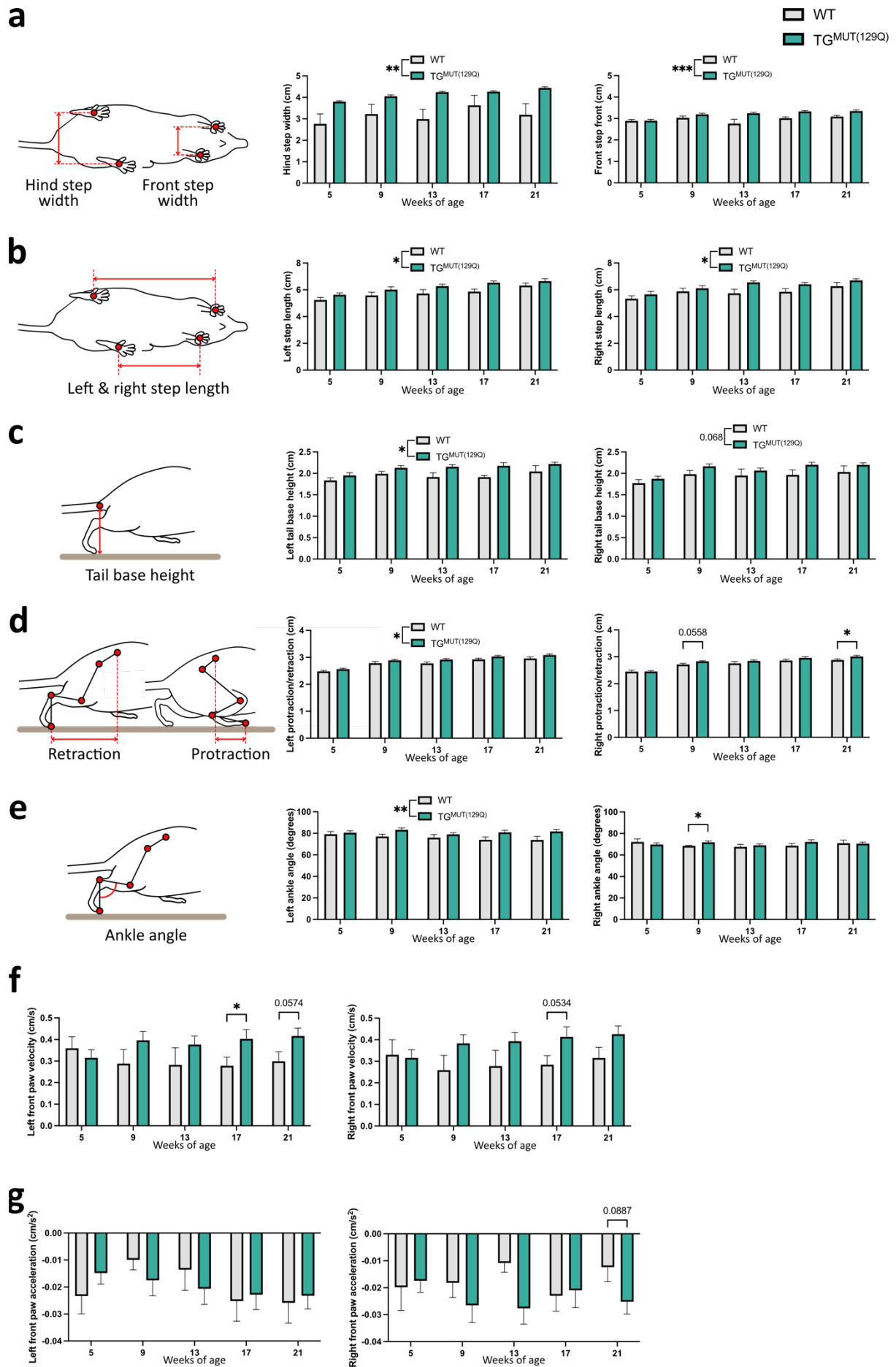


Figure R22 – The MotoRater system automatically detect defined anatomical points in the mice to elaborate a detailed kinetic profile. The MotoRater system detects multiple anatomical points of choice at the same time to map and profile the kinetic metrics that characterize mice's movements. **a)** Schematic representation of the anatomical points used to characterize the kinetic profile of mice tested in this work. **b)** Schematic photographs representing the anatomical points detected by the MotoRater system (red circles). **c)** Schematic representation of the timeline used to characterize the kinetic profile of WT and transgenic hATXN2^{MUT(129Q)} animals. Mice started to be tested at 5 weeks of age. Tests were repeated every 4 weeks until 21 weeks of age.

First, we characterized the kinetic profile of mice performing the walking test (**Figure R23**). We were able to detect differences in the width of steps both in the hind- and frontpaws (**Figure R23a**). The length of the mice's steps also presented differences on the left and right sides (**Figure R23b**). The base tail height of mice shows differences on the left side, with near statistical differences also on the right side ($p=0.068$) (**Figure R23c**). The relation between protraction and retraction movement was altered on the mice's left side. There are near statistical differences on the right side at 9 weeks of age ($p=0.0558$) and significant differences at 21 weeks of age (**Figure R23d**). Regarding the ankle angle, we detected differences on the mice's left side. The ankle angle on the right side exhibited differences only at 9 weeks of age (**Figure R23e**). The velocity of the left frontpaw exhibited significant alteration at 17 weeks of age and near statistical significance at 21 weeks of age ($p=0.057$). The velocity of the right frontpaw exhibited near statistical significance at 17 weeks of age (**Figure R23f**). The acceleration of the left frontpaw did not show differences, but the right frontpaw acceleration displayed a near statistical significance at 21 weeks of age (**Figure R23g**). Both left and right hindpaw velocities showed near statistical significance at 21 weeks of age ($p=0.0867$ and $p=0.0844$, respectively) (**Figure R23h**). The acceleration of the left hindpaw did not exhibit differences, while the right hindpaw acceleration displayed differences (**Figure R23i**). Overall, the MotoRater system enabled us to detect alterations between WT and transgenic hATXN2^{MUT(129Q)} mice regarding the kinetic profile of animals performing the walking test.



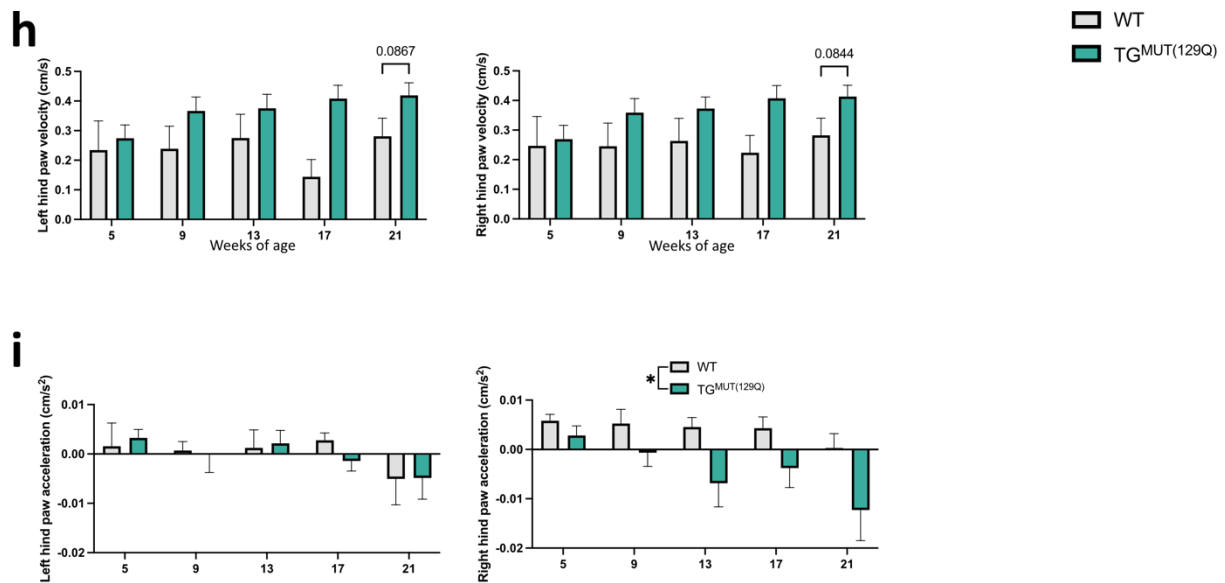


Figure R23 – WT and transgenic hATXN2^{MUT(129Q)} animals exhibit different kinetic profiles on the walking test. Mice were submitted to the walking test to characterize their kinetic profile using the MotoRater system. **a)-i)** To evaluate mice's movement, we evaluated several kinetic parameters: speed width, step length, tail base height, protraction/retraction relation, paw velocity, and paw acceleration. All comparisons were made between WT and transgenic hATXN2^{MUT(129Q)} mice **a)** Transgenic mice show differences regarding the step width. **b)** Differences in the step length were also observed. **c)** The tail-based height was also altered in transgenic mice. **d)** In the relation between protraction and retraction, it was possible to observe significant differences between the two groups. **e)** The ankle angle exhibited differences between the two groups. **g)** Front paws acceleration exhibited a near statistical difference between both groups ($p=0.0887$). **h)** When comparing WT and transgenic mice, the acceleration of hindpaw exhibited a near statistical significance both on left and right sides ($p=0.0867$ and $p=0.044$). **i)** Hindpaws acceleration displayed differences between WT and transgenic mice. (n: WT = 8 and hATXN2^{MUT(129Q)} = 17; * $P<0.05$; ** $P<0.01$; *** $P<0.001$; Two-Way Anova with mixed-effect analysis followed by Bonferroni correction; values are expressed as mean $\pm$ SEM).

Next, we characterized the kinetic profile of mice performing the beam walking test (**Figure R24**). We found differences on the left forepaw clearance at 5 and 13 weeks of age. However, no differences were detected on the opposite side (**Figure R24a**). The hindpaw clearance on the left side also exhibited differences at 13 weeks of age. Additionally, the right hindpaw clearance displayed differences between the two groups (**Figure R24b**). The iliac crest height did not show differences on the left side, but the right side exhibited a near statistical difference ($p=0.053$) (**Figure R24c**). The hip height did not exhibit differences on the left side. However, on the right side, we were able to detect significant alterations between experimental groups (**Figure R24d**). The left knee angle displayed near statistical significance at 17 weeks of age, with significant differences observed at 21 weeks of age. We could not observe differences in the right knee angle measurements (**Figure R24e**). Additionally, the ankle angle on the left side exhibited differences at 17 weeks of age and showed near

statistical significance at 21 weeks of age ($p=0.0759$). The opposite side did not exhibit differences (**Figure R24f**). Finally, we were able to detect differences regarding the nose height (**Figure R24g**). Overall, the MotoRater system enabled us to detect alteration between WT and transgenic hATXN2^{MUT(129Q)} mice regarding the kinetic profile of animals performing the beam walking test.

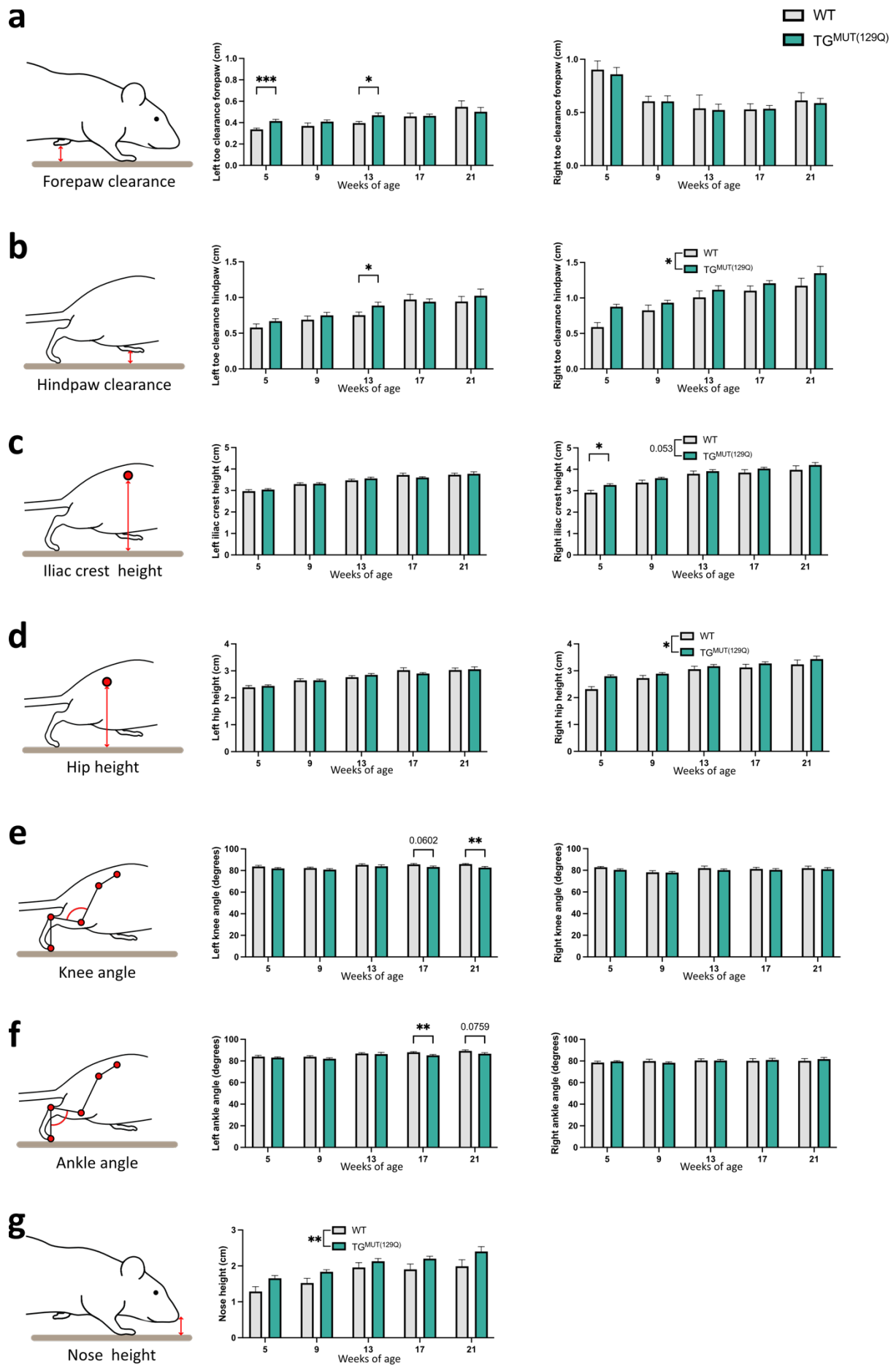


Figure R24 – WT and transgenic hATXN2^{MUT(129Q)} mice exhibit different kinetic profiles on the beam walking test. Mice were submitted to the beam walking test in the MotoRater system to characterize their kinetic profile. **a)-g)** To evaluate mice's movement, we evaluated several kinetic parameters: paw clearance, iliac crest height, hip height, knee angle, ankle angle, and nose height. All comparisons were made between WT and transgenic hATXN2^{MUT(129Q)} mice **a)** Transgenic show differences regarding the forepaw clearance at 5 and 13 weeks of age. **b)** Transgenic mice exhibited differences in the left hindpaw clearance at 13 weeks. They also displayed alterations regarding the right hindpaw clearance. **c)** The iliac crest height displayed near statistical significance, with significant alterations observed at 5 weeks of age. **d)** Transgenic mice exhibited differences regarding hip height. **e)** It was possible to observe near statistical differences regarding the knee angle at 17 weeks of age and significant alterations at 21 weeks of age. **f)** Transgenic mice displayed alteration regarding the ankle angle at 17 weeks of age, with near statistical significance at 21 weeks of age. **g)** comparing WT with transgenic mice, we observed differences regarding the nose height. (n: WT = 8 and hATXN2^{MUT(129Q)} = 17; *P<0.05; **P<0.01; Two-Way Anova with mixed-effect analysis followed by Bonferroni correction; values are expressed as mean ± SEM).

Finally, mice were also submitted to a swimming test to characterize their kinetic profile. Regarding the forepaw clearance, we could only detect a near statistical significance on the left side at 9 weeks of age (0.0995). Conversely, we were able to detect differences on the forepaw clearance in the right side (**Figure R25a**). The hindpaw clearance exhibited differences on the left side, while no differences were detected on the opposite side (**Figure R25b**). The iliac crest height displayed a near statistical difference on the left side (p=0.0995), while no differences were observed on the right side (**Figure R25c**). We detected a near statistical difference regarding the hip height on the left side (p=0.0794). However, no differences were detected in the hip height on the right side (**Figure R25d**). The base tail height exhibited near statistical differences (p=0.0856), while the opposite side did not show differences (**Figure R25e**). The relation between protraction and retraction was not altered on the left side. However, on the right side, we were able to detect differences at 9 weeks of age (**Figure R25f**). Finally, we also observed differences regarding the nose height between WT and transgenic hATXN2^{MUT(129Q)} mice. Overall, the MotoRater system enabled us to detect alterations regarding the kinetic profiling of mice performing the swimming test when comparing WT and transgenic hATXN2^{MUT(129Q)} animals.

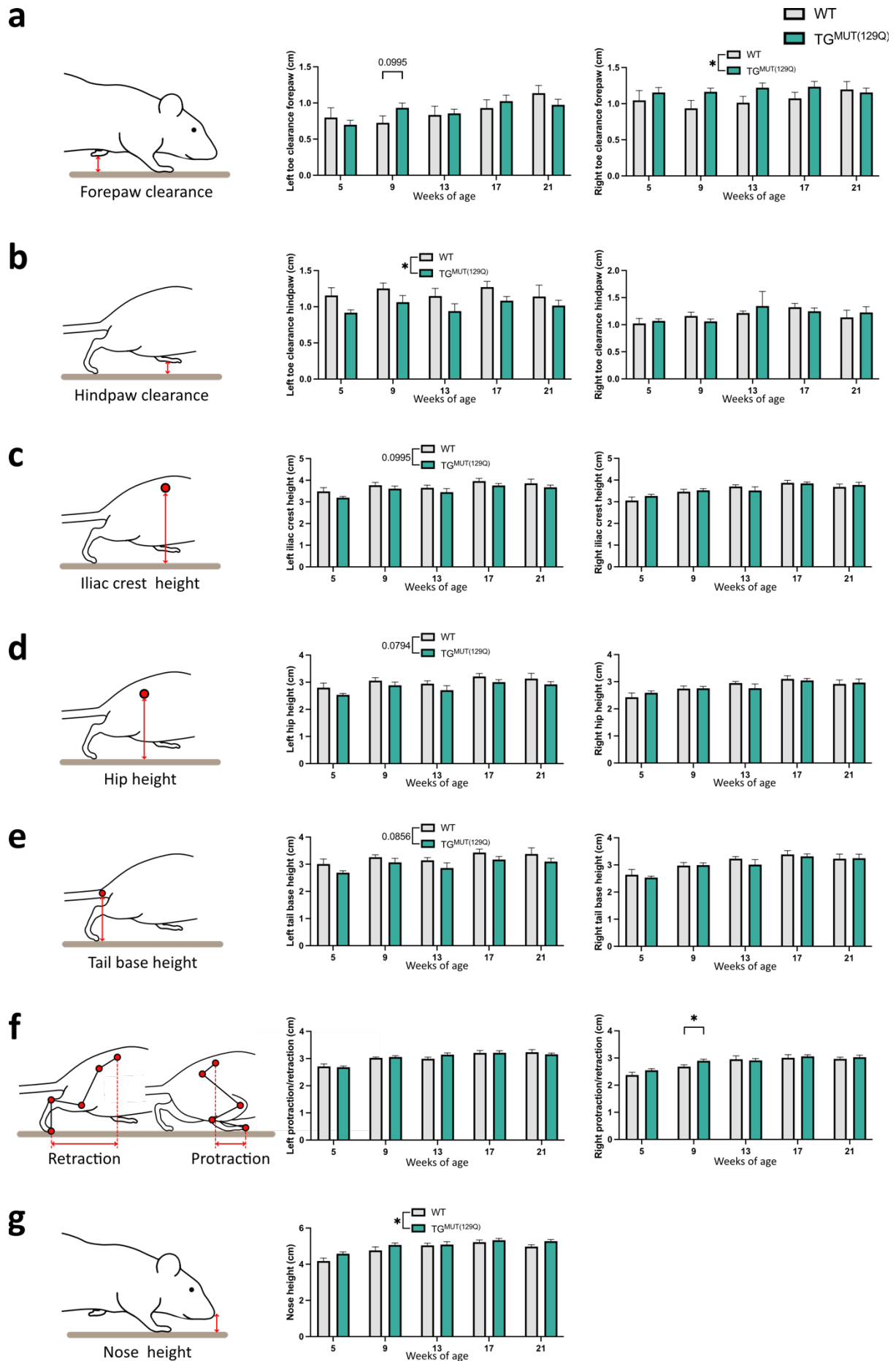


Figure R25 – WT and transgenic hATXN2^{MUT(129Q)} mice exhibit different kinetic profiles on the swimming test. Mice were submitted to the swimming test in the MotoRater system to characterize their kinetic profile. **a)-g)** To evaluate the mice's movement, we evaluated several kinetic parameters: paw clearance, iliac crest height, hip height, tail base height, protraction/retraction relation, and nose height. All comparisons were made between WT and transgenic hATXN2^{MUT(129Q)} mice **a)** Transgenic mice showed near statistically significant differences regarding the left forepaw clearance at 9 weeks of age ($p=0.0995$). Additionally, transgenic mice exhibited right forepaw clearance differences compared to WT. **b)** Transgenic mice, exhibited differences regarding the hindpaw clearance **c)** The differences regarding iliac crest height were near statistical significance ($p=0.0995$). **d)** The differences regarding hip height were near statistical significance ($p=0.0794$). **e)** The differences regarding the tail base height were near statistical significance ($p=0.0856$). **f)** We observed alterations regarding the relation between protraction and retraction at 9 weeks of age. **g)** Transgenic mice displayed alterations regarding nose height. (n: WT = 8 and hATXN2^{MUT(129Q)} = 17; * $P<0.05$; Two-Way Anova with mixed-effect analysis followed by Bonferroni correction; values are expressed as mean $\pm$ SEM).

Taking all together, the MotoRater system allowed us to elaborate a kinetic profile of both WT and transgenic hATXN2^{MUT(129Q)} mice using different tests, namely the walking, beam, and swimming tests. Due to its ability to detect fine kinetic metrics, the MotoRater is a powerful tool for detailed mouse movement analysis. The generation of detailed, precise, and fine pre-clinical data is crucial for studying the pathology mechanism underlying SCA2, to identify motor disease biomarkers and for developing novel therapies.

SGs modulation in the context of the novel SCA2 transgenic mouse model

The pathogenesis of SCA2, namely the molecular mechanisms that contribute to the disease are still poorly understood. The fact that ataxin-2 is a multifaceted protein with diverse molecular functions not only challenges the understanding of its biological roles but also adds an additional layer of complexity when studying it in the context of SCA2 (Conceição & Costa et al., 2024). One of the proposed roles of ataxin-2 is its involvement in the biogenesis of SGs, especially due its presence in these foci (Damrath et al., 2012; Petrauskas et al., 2024; Yamagishi et al., 2024).

SGs are membraneless structures that are assembled when cells are under stress conditions. SGs are part of the ISR and play a crucial role in maintaining cellular homeostasis

during stress. Upon sensing a stress stimulus, the ISR is initiated by the phosphorylation of eIF2 α , which leads to the SGs assembly. After the stress stimulus disappears or is resolved by the ISR, GADD34 acts as a scaffold protein to promote the interaction between the phosphatase PP1 and p-eIF2 α , leading to p-eIF2 α dephosphorylation. Ultimately, the increased presence of non-phosphorylated eIF2 α leads to SGs disassembly. Both p-eIF2 α and GADD34 are crucial players in SGs dynamics. While p-eIF2 α acts as the main driver that initiates SGs assembly, GADD34 plays a fundamental role in triggering SG disassembly (Choy et al., 2015; Kedersha et al., 1999; Marcelo et al., 2021b; Pakos-Zebrucka et al., 2016).

Currently, it is established that the ISR and SGs are fundamental mechanisms to promote cellular homeostasis in situations of cellular stress. However, evidence has suggested that prolonged maintenance of SGs assembly in conditions of chronic stress might lead to cellular death (Reineke and Neilson, 2019). In fact, in the context of neurodegenerative disorders, it has been proposed that an exacerbated ISR can further lead to neurodegeneration (Reineke and Neilson, 2019).

However, the role of SGs in the context of neurodegenerative disorders remains unclear. Whether SGs are beneficial or further contribute to neurodegeneration is still a matter of debate. Therefore, to elucidate the role of SGs in the context of neurodegeneration, particularly in SCA2, we sought to modulate SGs dynamics *in vivo*. To achieve that goal, we used the previously developed tools to modulate SGs assembly, as well as the previously characterized SCA2 transgenic hATXN2^{MUT(129Q)} mice.

The following sections describe the impact of expressing the tools designed to modulate SGs, assessing their impact at the behavioral, histological, and transcriptomic levels in the context of the SCA2 transgenic hATXN2^{MUT(129Q)} mice.

Behavioral testing – conventional

To understand how SGs dynamics impact SCA2 pathogenesis, we used the novel transgenic hATXN2^{MUT(129Q)} mice, previously characterized by our lab and kinetic profiled in

this work. To manipulate the ISR, namely the assembly and disassembly of SGs, we used the strategies that were developed and described in the previous sections. Briefly, to induce the formation of SGs it was developed a construct expressing a phosphomimetic form of eIF2 α (eIF2 α^{S52D}) and, to inhibit the formation of SGs, it was developed a construct expressing a truncated form of GADD34 (GADD34 $\Delta 322$).

The transgenic hATXN2^{MUT(129Q)} mice were stereotactically injected into the cerebellum at 4 weeks of age with AAV particles encoding for the genes that modulate SGs dynamics. One week after the injection (5 weeks of age), mice were submitted to a battery of behavioral tests, which were repeated every 4 weeks until 21 weeks of age. At that timepoint, mice were sacrificed, and the cerebella was processed for posterior histologic analysis (**Figure R26a**). Our study was composed of three different experimental groups: i) transgenic hATXN2^{MUT(129Q)} mice injected with AAV particles encoding for the control (CT; TG^{MUT(129Q)}+CT) (**Figure R26b**); ii) transgenic hATXN2^{MUT(129Q)} mice injected with AAV particles encoding for eIF2 α^{S52D} (TG^{MUT(129Q)}+eIF2 α^{S52D}) (**Figure R26c**); and iii) transgenic hATXN2^{MUT(129Q)} mice injected with AAV particles encoding for GADD34 $\Delta 322$ (TG^{MUT(129Q)}+GADD34 $\Delta 322$) (**Figure R26d**). All comparisons were performed with controls.

None of the experimental groups exhibited any alterations regarding weight (**Figure R26e**). Using the conventional behavioral tests to assess motor performance, mice injected with AAV particles encoding for the eIF2 α phosphomimetic (TG^{MUT(129Q)}+eIF2 α^{S52D}) displayed significantly worse performance when compared to controls (TG^{MUT(129Q)}+CT), as they showed less strength in the grip test at 5, 17, and 21 weeks of age when compared to transgenic mice injected with the control, with an overall worse performance near statistical significance ($p=0.0598$) (**Figure R26f**). Likewise, mice injected with AAV particles encoding for the eIF2 α phosphomimetic, displayed less latency to fall from the rotarod apparatus (**Figure R26g**). No alterations were observed in the described tests regarding transgenic mice injected with AAV particles encoding for GADD34 $\Delta 322$ (TG^{MUT(129Q)}+ GADD34 $\Delta 322$), compared with control animals.

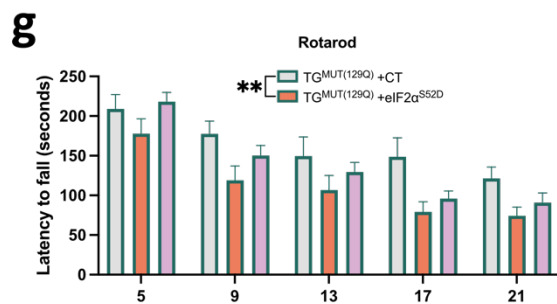
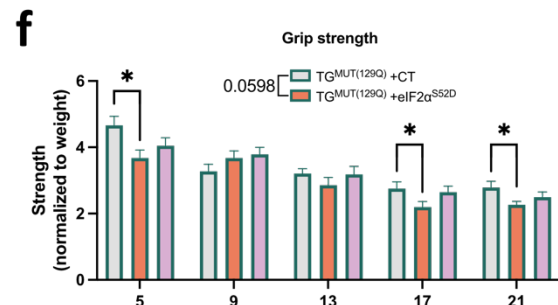
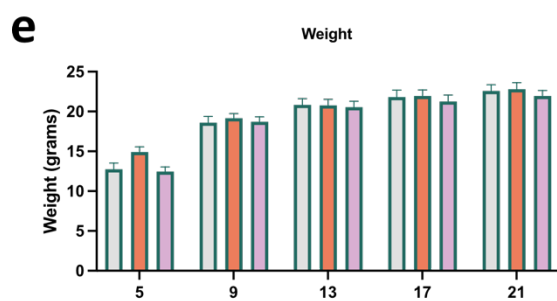
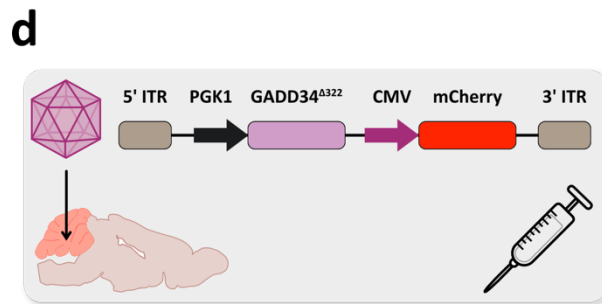
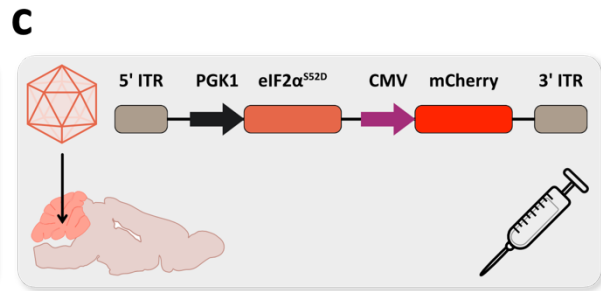
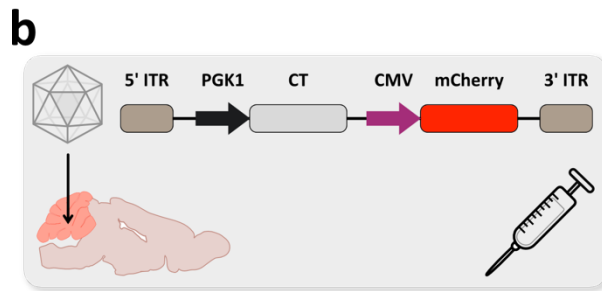
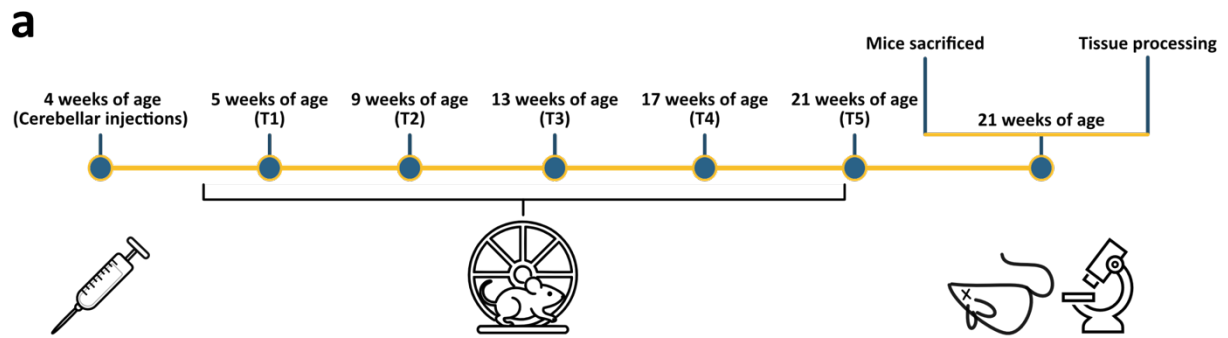


Figure R26 - AAV-mediated expression of eIF2 α^{S52D} worsens the phenotype of transgenic hATXN2^{MUT(129Q)} mice. To understand the impact of SGs dynamics in the context of SCA2, mice were stereotactically injected into the cerebellum with AAV particles encoding for the strategies to modulate SGs assembly. **a)** Mice were injected at 4 weeks of age. One week after injection, mice were submitted to a battery of behavioral tests every 4 weeks until 21 weeks of age. Mice were sacrificed and cerebella was processed for histological analysis. **b)** Transgenic mice were stereotactically injected into the cerebellum with AAV particles encoding for the luciferase protein, as a control. **c)** Transgenic mice were stereotactically injected into the cerebellum with AAV particles encoding for eIF2 α^{S52D} (induces SGs assembly). **d)** Transgenic mice were stereotactically injected into the cerebellum with AAV particles encoding for GADD34 ^{$\Delta 322$} . As previously described, all expressing vectors contain a mCherry fluorescent protein sequence. **e)** No alterations regarding mice weight were observed. **f)** Transgenic mice expressing eIF2 α^{S52D} showed less grip strength, when comparing to injected controls, at 5, 17 and 21 weeks of age. **g)** Transgenic mice expressing eIF2 α^{S52D} exhibited a worse motor performance compared with mice injected with the control. (n: TG^{MUT(129Q)}+CT= 14, TG^{MUT(129Q)}+eIF2 α^{S52D} = 14, TG^{MUT(129Q)}+GADD34 ^{$\Delta 322$} = 14; *P<0.05, **P<0.01; ****P<0.001, ***P<0.001; Two-Way Anova with mixed-effect analysis followed by Bonferroni correction; values are expressed as mean $\pm$ SEM).

Behavioral testing – MotoRater: walking test

The kinetic profile of transgenic mice injected with the strategies to modulate SGs assembly was assessed using the MotoRater system. All comparisons were performed vs. controls.

Using the walking test, at 5 and 9 weeks of age, the TG^{MUT(129Q)}+eIF2 α^{S52D} animals showed a near statistical significance (p=0.0888 and 0.0878, respectively) regarding the hind step width measure, as compared to controls. Additionally, at 17 weeks of age, TG^{MUT(129Q)}+GADD34 ^{$\Delta 322$} mice exhibited a decrease in the front step width (**Figure R27a**).

The left step length of mice injected with eIF2 α^{S52D} showed an overall near statistical significance (p=0.0888), with a significant increase in length at 5 and 9 weeks of age, as compared to controls. Additionally, the right step length of mice injected with eIF2 α^{S52D} showed an overall near statistical significance (p=0.0779) with a significant increase at 9 weeks of age (**Figure R27b**).

Regarding the right iliac crest height, TG^{MUT(129Q)}+eIF2 α^{S52D} mice exhibited an overall alteration near statistical significance (p=0.0921). Moreover, GADD34 ^{$\Delta 322$} -injected mice showed a significant reduction in both left and right iliac crest heights (**Figure R27c**).

Concerning left hip height, mice injected with eIF2 α^{S52D} showed an increase at 5 weeks of age. Additionally, mice injected with GADD34 ^{$\Delta 322$} displayed an overall decrease in the left hip height (**Figure R27d**).

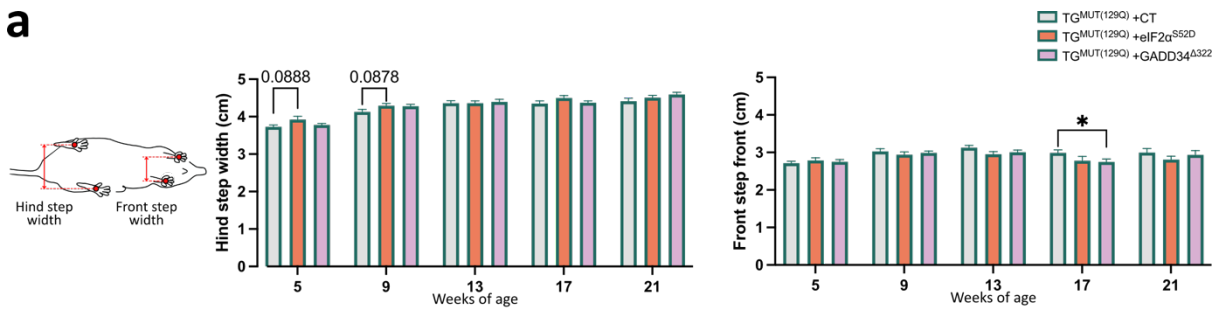
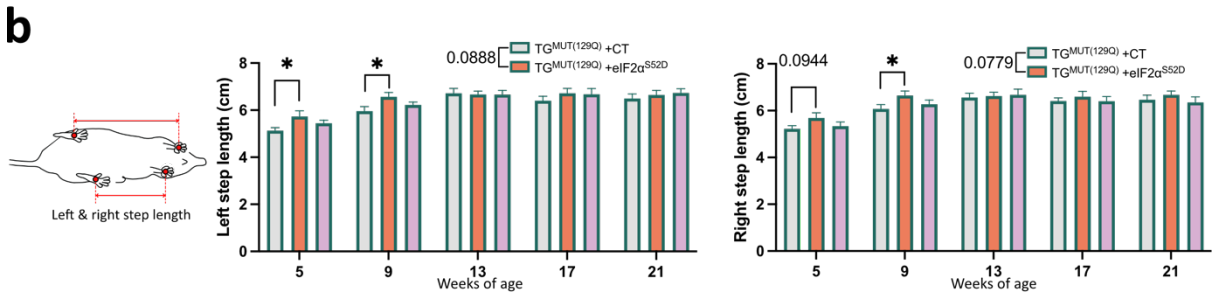
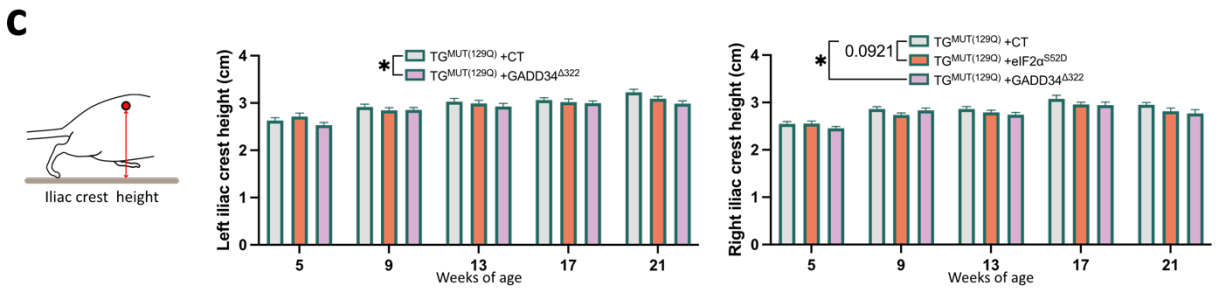
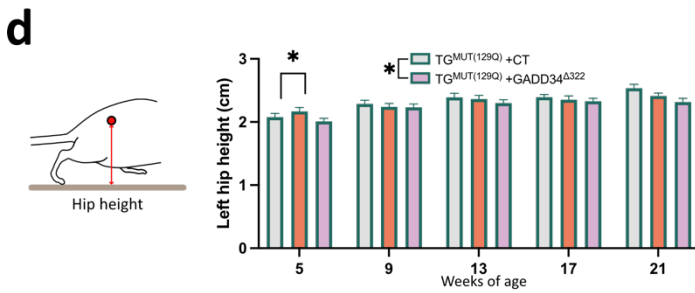
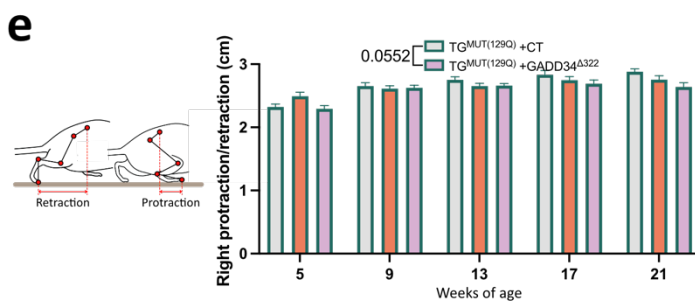
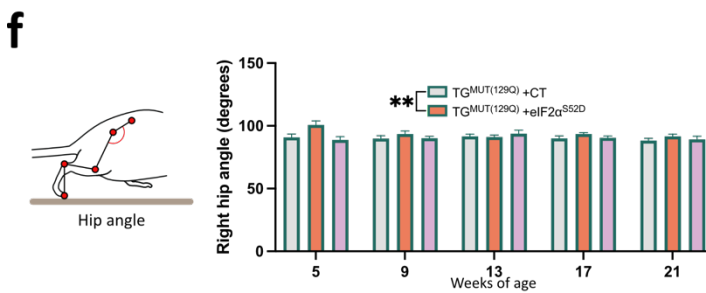
TG^{MUT(129Q)}+GADD34^{Δ322} mice displayed an overall near statistical significance (p=0.0552) regarding the relation between protraction/retraction on the left side, with a tendency to decrease, as compared to controls (**Figure R27e**).

Another kinetic parameter that showed an overall significant alteration was the right hip angle, with eIF2α^{S52D}-injected mice displaying an increase in this measure compared with controls (**Figure R27f**).

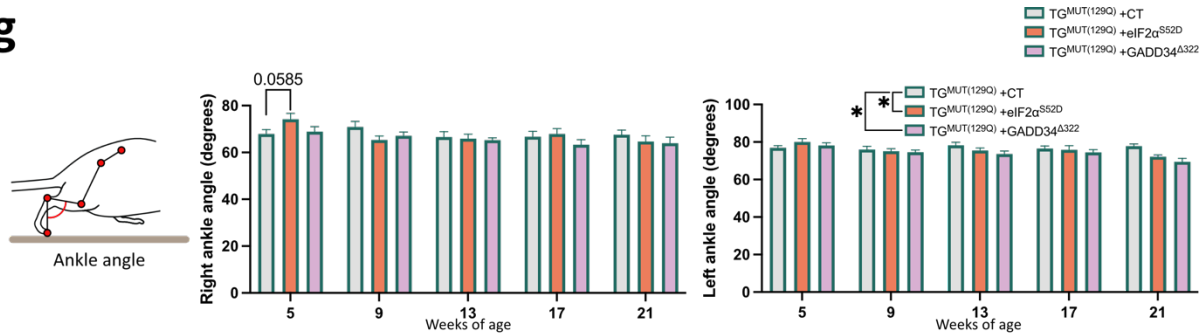
We also observed alterations in injected mice regarding the ankle angle. Both TG^{MUT(129Q)}+eIF2α^{S52D} and TG^{MUT(129Q)}+GADD34^{Δ322} mice displayed an overall decrease in the left ankle angle. Additionally, TG^{MUT(129Q)}+eIF2α^{S52D} mice also showed a near statistically significant increase at 5 weeks of age on the right ankle angle **Figure R27g**).

Finally, the left knee angle of both TG^{MUT(129Q)}+eIF2α^{S52D} and TG^{MUT(129Q)}+GADD34^{Δ322} mice was reduced, compared to controls (**Figure R27h**).

Overall, we observed that expressing the tools to manipulate SGs assembly/disassembly dynamics were able to alter the kinetic profile of mice's movement while performing in the walking test

a**b****c****d****e****f**

g



h

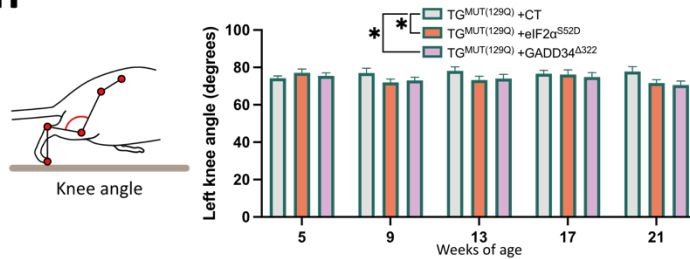


Figure R27 - AAV-mediated expression of eIF2α^{S52D} and AAV-mediated expression of GADD34^{Δ322} impact the kinetic profile of transgenic hATXN2^{MUT(129Q)} mice in the walking test. To understand the impact of SGs dynamics on the kinetic profile of mice transgenic mice expressing eIF2α^{S52D} or GADD34^{Δ322}, animals movement was analyzed while performing the walking test. **a)** Transgenic mice expressing GADD34^{Δ322}, showed alterations regarding the front step length at 17 weeks of age. **b)** Transgenic mice expressing eIF2α^{S52D} exhibited step length alterations on the left side at 5 and 9 weeks of age and on the right side at 9 weeks of age. **c)** Transgenic mice expressing GADD34^{Δ322} showed an overall alteration regarding the iliac crest height. **d)** Transgenic mice expressing eIF2α^{S52D} exhibited alterations in the hip height at 5 weeks of age. Additionally, transgenic mice expressing GADD34^{Δ322} displayed an overall alteration in the hip height. **e)** Transgenic mice expressing GADD34^{Δ322} showed an overall near statistical significance regarding the relation between protraction and retraction at the right side. **f)** Transgenic mice expressing eIF2α^{S52D} exhibited an overall alteration in the hip angle. **g)** Transgenic mice expressing eIF2α^{S52D} showed a statistical significance at 5 weeks of age regarding the angle of the right ankle. Additionally, both transgenic mice expressing eIF2α^{S52D} and transgenic mice expressing GADD34^{Δ322} showed an overall alteration in the left ankle angle. **h)** Both transgenic mice expressing eIF2α^{S52D} and transgenic mice expressing GADD34^{Δ322} showed an overall alteration in the left knee angle. (n: TG^{MUT(129Q)}+CT= 14, TG^{MUT(129Q)}+eIF2α^{S52D}= 14, TG^{MUT(129Q)}+GADD34^{Δ322}= 14; *P<0.05, **P<0.01; ****P<0.001, ***P<0.001; Two-Way Anova with mixed-effect analysis followed by Bonferroni correction; values are expressed as mean ± SEM).

Behavioral testing – MotoRater: beam walking test

The kinetic profile of injected transgenic mice during the beam walking test was also evaluated. All comparisons were performed vs. controls.

Transgenic mice injected with AAV particles encoding for GADD34^{Δ322} (TG^{MUT(129Q)}+GADD34^{Δ322}) showed an overall tendency for reduction regarding the hind step with near statistical significance (p=0.0850) (**Figure R28a**).

The forepaw clearance on both the left and right sides were increased in mice injected with $eIF2^{\alpha S52D}$. Moreover, $TG^{MUT(129Q)}$ -injected mice also displayed alterations, with an overall tendency for an increase in the forepaw clearance on the right side ($p=0.0921$) and an overall significant increase on the left side (**Figure R28b**).

The iliac crest height was increased at 5 weeks in mice injected with $eIF2^{\alpha S52D}$, both on the right and left sides (**Figure R28c**).

The hip height on the left side of $TG^{MUT(129Q)}+eIF2^{\alpha S52D}$ exhibited an overall significant increase (**Figure R28d**).

The hip angle on the left side of $TG^{MUT(129Q)}+eIF2^{\alpha S52D}$ also exhibited an overall significant increase (**Figure R28e**).

Finally, $TG^{MUT(129Q)}+eIF2^{\alpha S52D}$ mice took more time to traverse the beam when compared to the control group (**Figure R28f**).

Overall, we observed that expressing the tools to manipulate SGs assembly/disassembly dynamics were able to alter the kinetic profile of mice's movement while performing in the beam walking test.

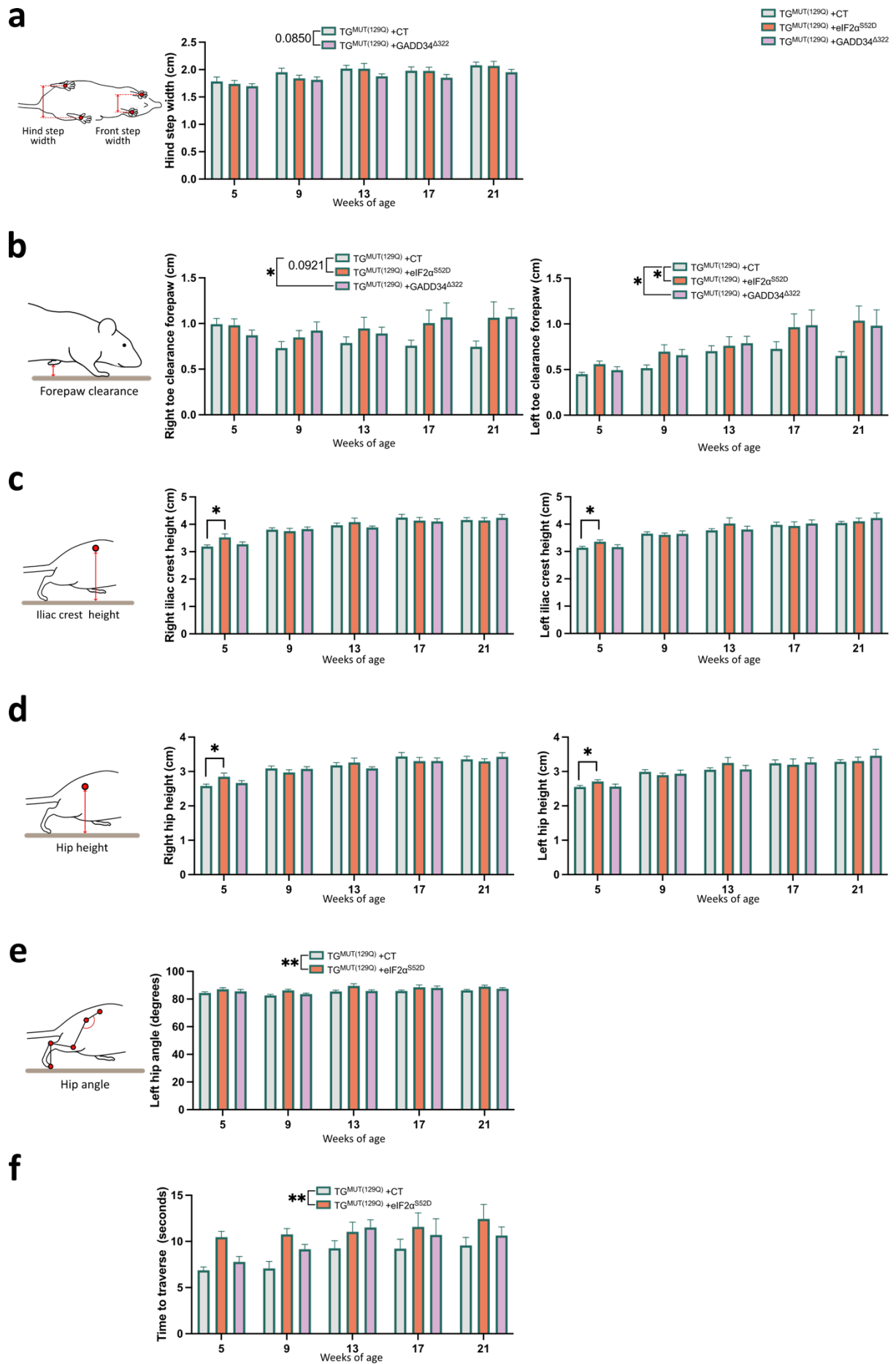


Figure R28 - AAV-mediate expression of eIF2 α^{S52D} and AAV-mediated expression of GADD34 Δ^{322} impact the kinetic profile of transgenic hATXN2^{MUT(129Q)} mice in the beam walking test. To understand the impact of SGs dynamics on the kinetic profile of transgenic mice expressing eIF2 α^{S52D} or GADD34 Δ^{322} , the animals' movement was analyzed while performing the beam walking test. **a)** Transgenic mice expressing GADD34 Δ^{322} displayed an overall near statistical significance regarding alterations in the hind step width. **b)** Transgenic mice expressing eIF2 α^{S52D} exhibited an overall alteration regarding the forepaw clearance on the left side. Additionally, transgenic mice expressing GADD34 Δ^{322} showed an overall alteration regarding the forepaw clearance both on the right and left sides. **c)** Transgenic mice expressing eIF2 α^{S52D} exhibited alterations regarding the iliac crest height at 5 weeks of age. **d)** Transgenic mice expressing eIF2 α^{S52D} exhibited alterations regarding the hip height at 5 weeks of age. **e)** Transgenic mice expressing eIF2 α^{S52D} displayed overall alteration in the hip angle. **f)** Transgenic mice expressing eIF2 α^{S52D} took more time to traverse the beam. (n: TG^{MUT(129Q)}+CT= 14, TG^{MUT(129Q)}+eIF2 α^{S52D} = 14, TG^{MUT(129Q)}+GADD34 Δ^{322} = 14; *P<0.05, **P<0.01; ****P<0.001, ***P<0.001; Two-Way Anova with mixed-effect analysis followed by Bonferroni correction; values are expressed as mean $\pm$ SEM).

Behavioral testing – MotoRater: swimming test

The kinetic profile of injected transgenic mice during the swimming test was also evaluated. All comparisons were performed versus controls.

Transgenic mice injected with AAV particles encoding for GADD34 Δ^{322} (TG^{MUT(129Q)}+GADD34 Δ^{322}) showed an overall reduction regarding both left and right step length (**Figure R29a**).

The left hindpaw clearance of mice injected with GADD34 Δ^{322} showed an overall near significant statistical decrease (p=0.0756) (**Figure R29b**).

The left iliac crest height of mice injected with eIF2 α^{S52D} was decreased at 9 weeks of age. Additionally, mice injected with GADD34 Δ^{322} showed an overall decrease in the left hip height (**Figure R29c**).

Regarding the relation protraction/retraction on the left side, both TG^{MUT(129Q)}+eIF2 α^{S52D} and TG^{MUT(129Q)}+GADD34 Δ^{322} animals showed an overall decrease (**Figure R29d**).

The right ankle angle of both eIF2 α^{S52D} and GADD34 Δ^{322} -injected mice showed an overall decrease. Additionally, the left ankle angle of mice injected with GADD34 Δ^{322} showed an overall near statistically significant increase (**Figure R29e**).

Finally, both TG^{MUT(129Q)}+eIF2 α^{S52D} and TG^{MUT(129Q)}+GADD34 Δ^{322} animals showed an overall decrease regarding the left ankle angle (**Figure R29f**).

Overall, we observed that expressing the tools to manipulate SGs assembly/disassembly dynamics were able to alter the kinetic profile of mice's movement while performing in the swimming test

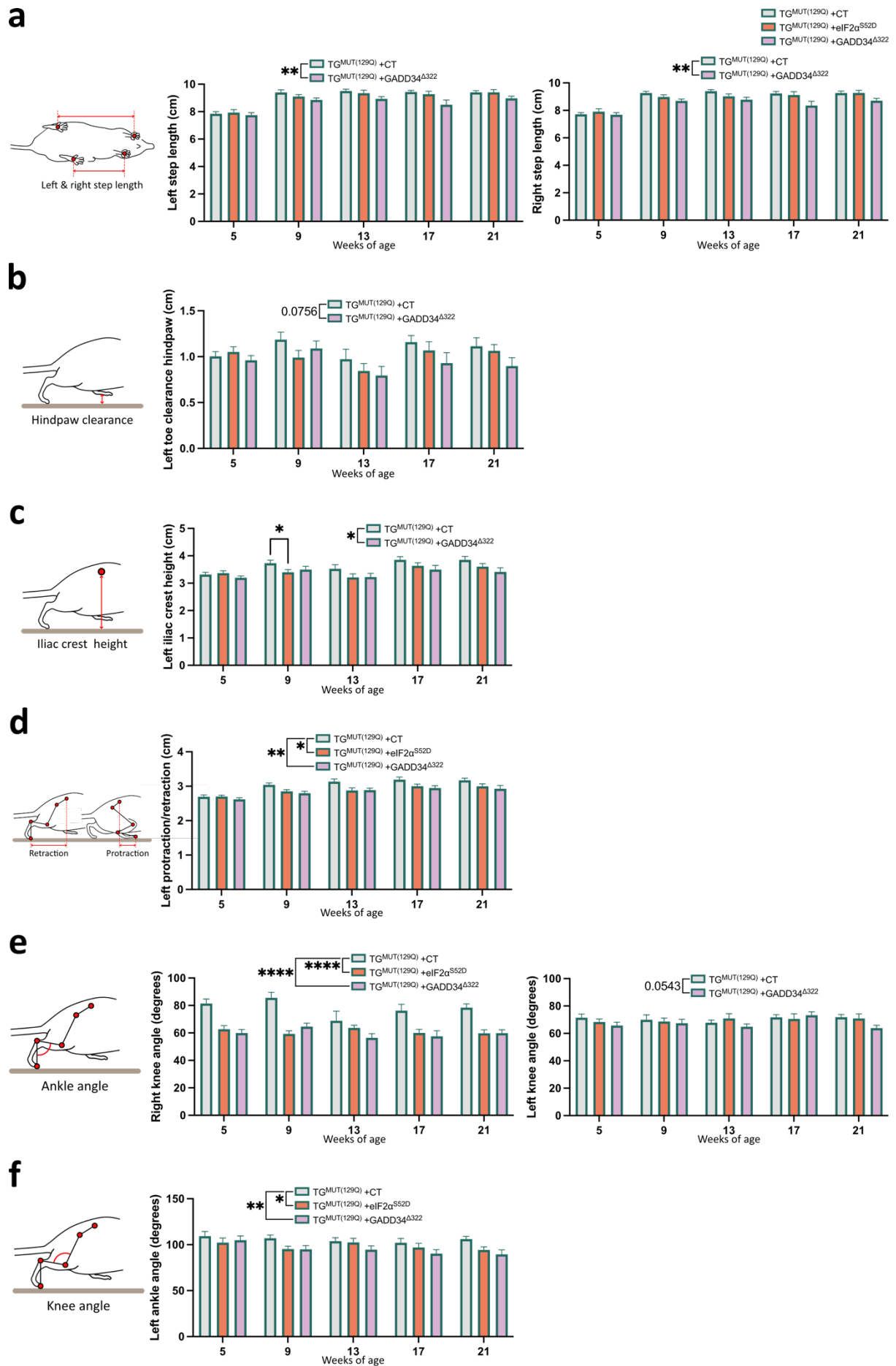


Figure R29 - AAV-mediated expression of eIF2 α^{S52D} and AAV-mediated expression of GADD34 Δ^{322} impact the kinetic profile of transgenic hATXN2^{MUT(129Q)} mice in the swimming test. To understand the impact of SGs dynamics on the kinetic profile of transgenic mice expressing eIF2 α^{S52D} or GADD34 Δ^{322} , the animals' movement was analyzed while performing the swimming walking test. **a)** Transgenic mice expressing GADD34 Δ^{322} displayed an overall alteration regarding the left and right step length. **b)** Transgenic mice expressing GADD34 Δ^{322} showed an overall near statistical significance regarding alterations in hindpaw clearance. **c)** Transgenic mice expressing eIF2 α^{S52D} exhibited alterations regarding the left iliac crest height at 9 weeks of age. Additionally, transgenic mice expressing GADD34 Δ^{322} displayed an overall alteration in the height of the left iliac crest. **d)** Both transgenic mice expressing eIF2 α^{S52D} and transgenic mice expressing GADD34 Δ^{322} exhibited an overall alteration regarding the left protraction/retraction relation. **e)** Both transgenic mice expressing eIF2 α^{S52D} and transgenic mice expressing GADD34 Δ^{322} exhibited an overall alteration regarding the right ankle angle. **f)** Both transgenic mice expressing eIF2 α^{S52D} and transgenic mice expressing GADD34 Δ^{322} exhibited an overall alteration regarding the left knee angle. (n: TG^{MUT(129Q)}+CT= 14, TG^{MUT(129Q)}+eIF2 α^{S52D} = 14, TG^{MUT(129Q)}+GADD34 Δ^{322} = 14; *P<0.05, **P<0.01, ****P<0.001, ***P<0.001; Two-Way Anova with mixed-effect analysis followed by Bonferroni correction; values are expressed as mean $\pm$ SEM).

Neuropathological analysis

To better understand the impact of AAV-mediated expression of eIF2 α^{S52D} and GADD34 Δ^{322} at the neuronal level, mice's cerebella were processed and analyzed.

As previously described, the constructs developed to express the control (CT), eIF2 α^{S52D} , and GADD34 Δ^{322} contain a mCherry reporter. First, we qualitatively observed cerebellar sections from injected mice to assess the distribution of AAV-mediated expression throughout the cerebellum. Overall, the expression distribution was high on lobules I/II, lobule III, and lobules X; high/moderate on lobules IV/V; low on lobules VIII and IX; and low/absent on lobules VI and VII (**Figure R30a**).

In SCA2, the most affected brain region is the cerebellum, particularly Purkinje cells (Conceição & Costa et al., 2024). Our model expresses a mutant form of the hATXN2 under the control of the L6-7 promoter that specifically directs the transgene expression to the Purkinje cells (Torashima et al., 2008). Given that, we started our neuropathological analysis by quantifying the total number of Purkinje cells within the mCherry expression area in each experimental group (**Figure R30b**). Cerebellar sections were immunostained with the Purkinje specific cell marker calbindin to count the number of Purkinje cells. We found that AAV-mediated expression of eIF2 α^{S52D} (TG^{MUT(129Q)}+eIF2 α^{S52D}) led to a significant reduction in the total number of Purkinje cells within the mCherry expression area compared to mice injected with the control (TG^{MUT(129Q)}+CT). No alterations regarding the total number of Purkinje cells were observed when comparing mice expressing GADD34 Δ^{322} with controls (**Figure R30c**).

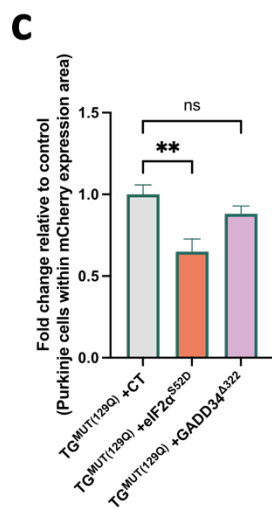
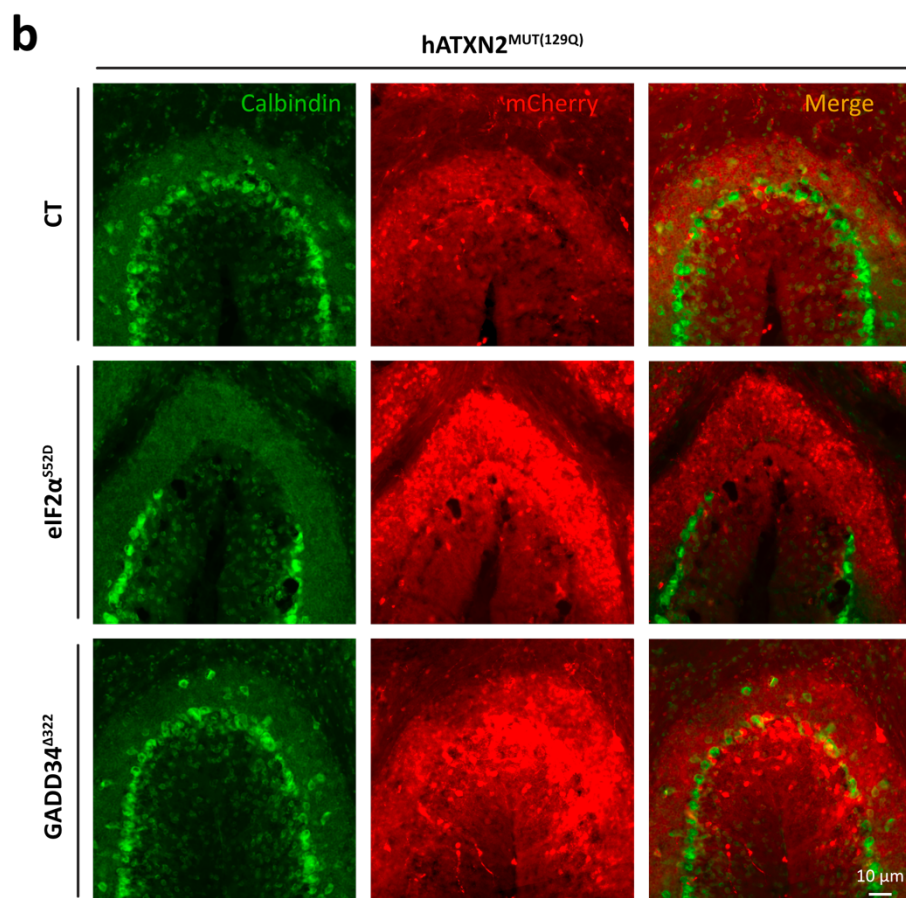
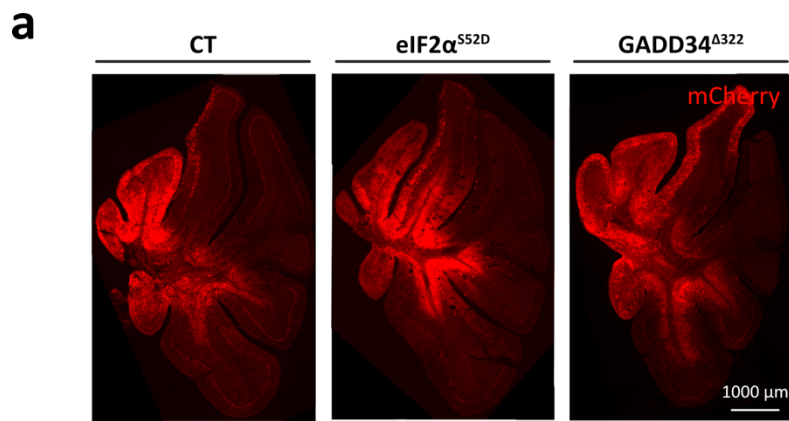
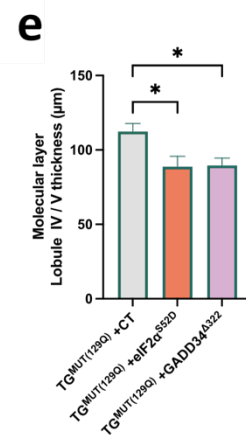
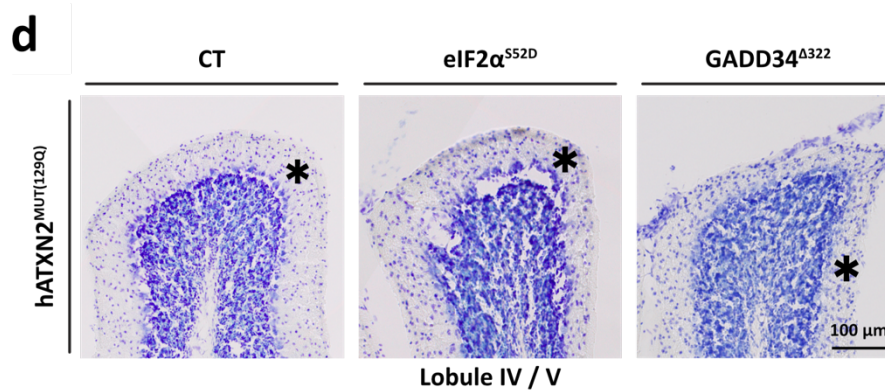
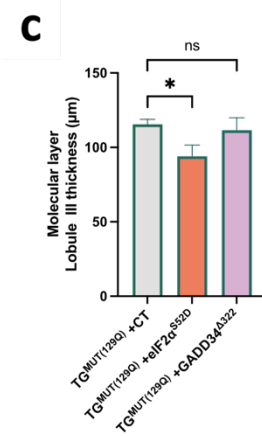
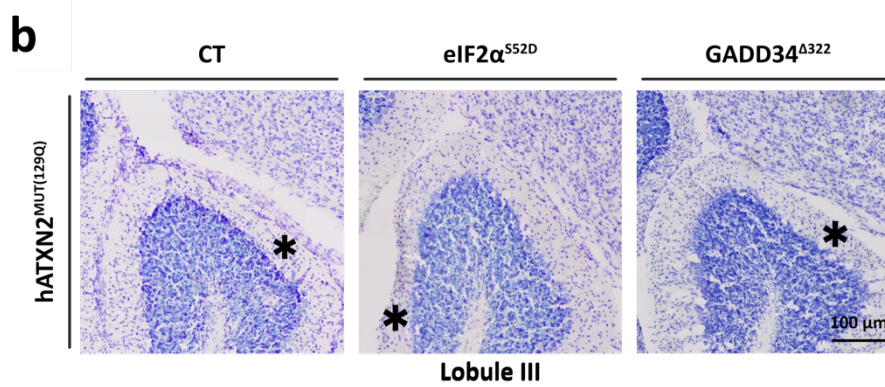
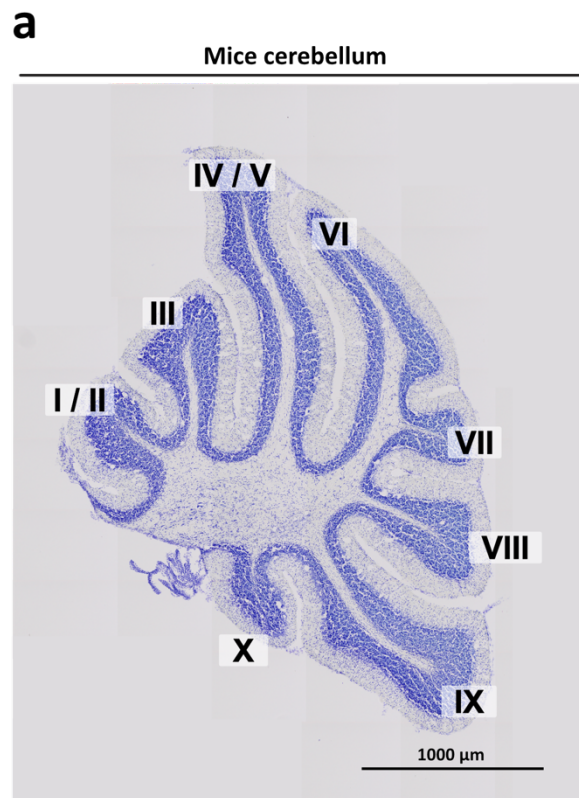
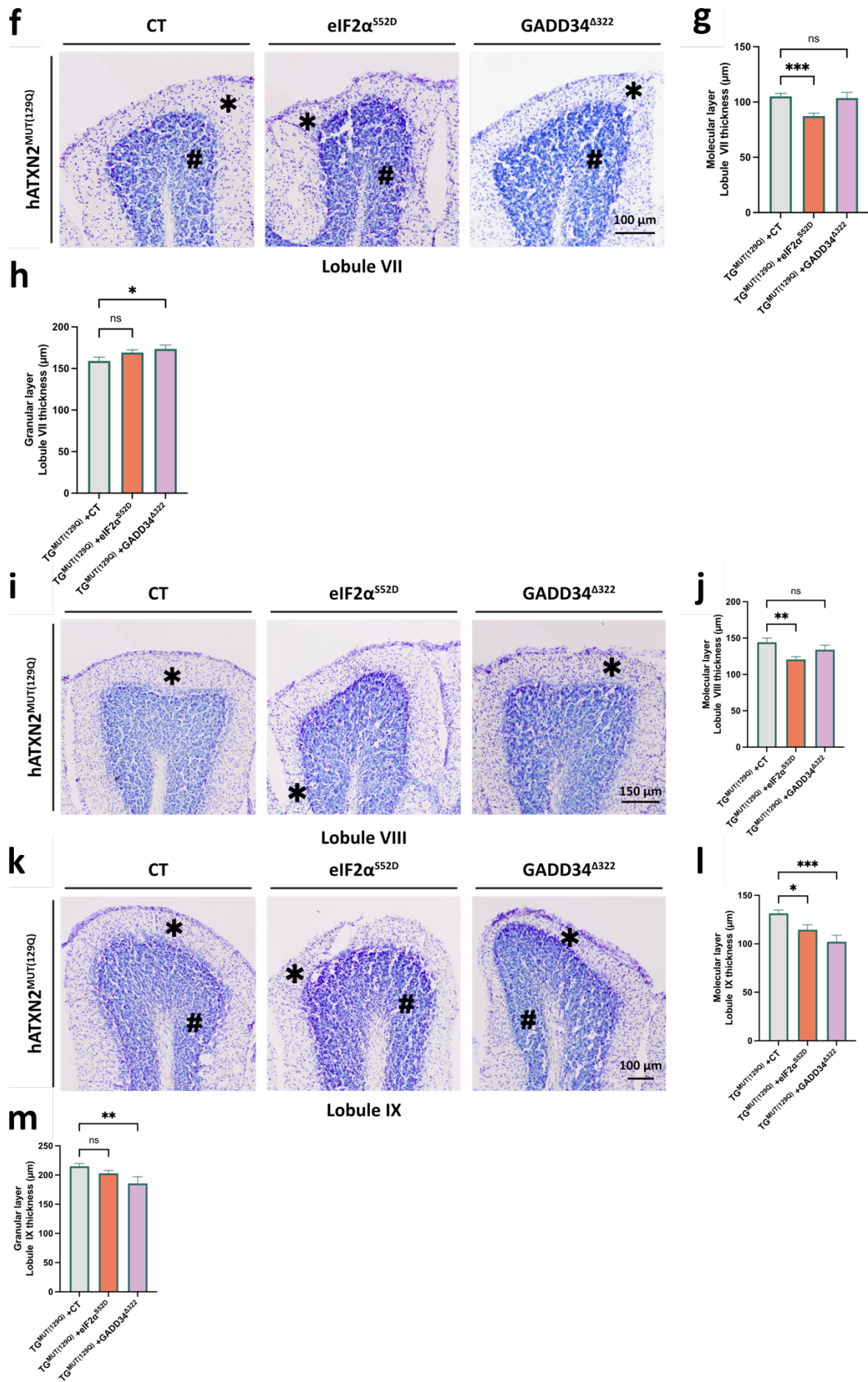


Figure R30 - AAV-mediated expression of eIF2 α^{S52D} promotes the loss of cerebral Purkinje cells in transgenic mice. Cerebella from transgenic mice expressing eIF2 α^{S52D} or GADD34 Δ^{322} were processed for histological analysis to understand the impact of SGs modulation at the neuronal level. **a)** Representative microscopy images showing the overall AAV-mediated expression pattern in cerebellar sections of transgenic mice, as shown by the presence of the mCherry fluorescent protein. Mice were injected with AAV particles encoding either the control, eIF2 α^{S52D} , or GADD34 Δ^{322} . **b)** Cerebellar sections were immunostained for the Purkinje cell marker calbindin. Representative microscopy images showing the Purkinje cells (green) and the expression of the fluorescent protein mCherry (red). **c)** The total number of Purkinje cells within the area of mCherry expression was quantified. Transgenic mice expressing eIF2 α^{S52D} showed a reduction in the total number of Purkinje cells when compared with transgenic mice expressing the control. We could not detect alterations regarding the total number of Purkinje cells in transgenic mice expressing GADD34 Δ^{322} . (n: TG^{MUT(129Q)}+CT= 6, TG^{MUT(129Q)}+eIF2 α^{S52D} = 6, TG^{MUT(129Q)}+GADD34 Δ^{322} = 6; *P<0.05, **P<0.01; ****P<0.001, ***P<0.001; One-Way Anova followed by Bonferroni correction; values are expressed as mean $\pm$ SEM).

Next, to complement our neuropathological analysis, cerebellar sections were stained with cresyl violet, and the thickness of the molecular and granular layers across the cerebellar lobules were measured (**Figure R31**). All comparisons were performed versus control animals. Regarding the cerebellar lobule III, transgenic mice expressing eIF2 α^{S52D} showed a significant reduction in the thickness of the molecular layer (**Figure R31 b-c**). In the same line, the lobule IV/V exhibited a decrease in the thickness at the molecular layer, both in TG^{MUT(129Q)}+eIF2 α^{S52D} and TG^{MUT(129Q)}+GADD34 Δ^{322} mice (**Figure R31 d-e**). Additionally, in lobule VII, expression of eIF2 α^{S52D} resulted in a significant decrease in the thickness of the molecular layer, while GADD34 Δ^{322} expression increased the granular layer thickness (**Figure R31 f-h**). The molecular layer thickness of the cerebellar lobule VIII was decreased in transgenic mice expressing eIF2 α^{S52D} (**Figure R31 i-j**). In transgenic mice expressing eIF2 α^{S52D} , the molecular layer thickness of lobule IX was reduced, while in transgenic mice expressing GADD34 Δ^{322} , both molecular and granular layers were reduced (**Figure R31 k-m**). Finally, the lobule X showed a reduction in the thickness of the granular and molecular layers in the transgenic mice expressing eIF2 α^{S52D} (**Figure R31 n-p**).





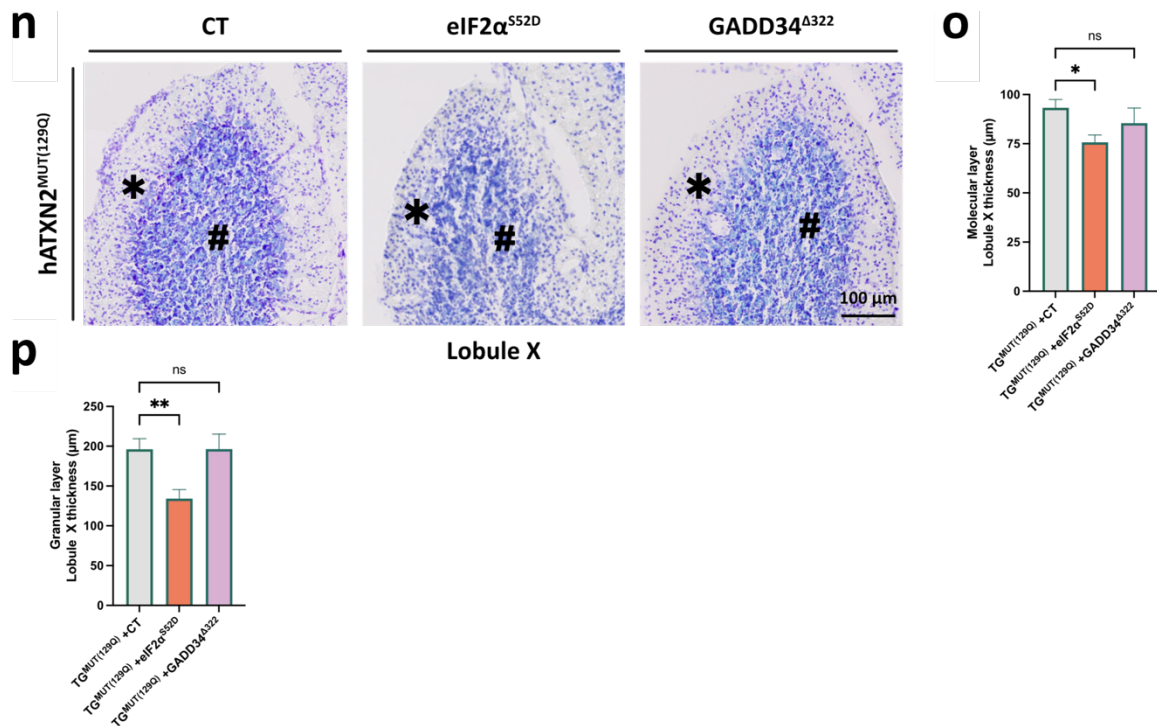


Figure R31 - AAV-mediated expression of eIF2α^{S52D} and AAV-mediated expression of GADD34^{Δ322} alters the thickness of molecular and granular layers of the cerebellum. Cerebella from transgenic mice expressing eIF2α^{S52D} or GADD34^{Δ322} were stained with cresyl violet. **a)** Representative microscopy image of a whole mice cerebellum, at 21 weeks of age, depicting each lobule. **b-p)** The thickness of the molecular and granular layers of the different cerebellar lobules was measured. The asterisk symbol (*) represents the molecular layer, and the number sign (#) represents the granular layer. **b)** Representative microscopy images of the cerebellar lobule III. **c)** Transgenic mice expressing eIF2α^{S52D} exhibited a reduced molecular layer thickness. **d)** Representative microscopy images of the cerebellar lobule IV/V. **e)** Both transgenic mice expressing eIF2α^{S52D} and transgenic mice expressing GADD34^{Δ322} showed a reduction in the molecular layer. **f)** Representative microscopy images of the cerebellar lobule VII. **g)** Transgenic mice expressing eIF2α^{S52D} exhibited a reduced molecular layer thickness. **h)** Transgenic mice expressing GADD34^{Δ322} showed an increase in the granular layer thickness. **i)** Representative microscopy images of the cerebellar lobule VIII. **j)** Transgenic mice expressing eIF2α^{S52D} exhibited a reduced molecular layer thickness. **k)** Representative microscopy images of the cerebellar lobule IX. **l)** Both transgenic mice expressing eIF2α^{S52D} and transgenic mice expressing GADD34^{Δ322} showed a reduction in the molecular layer. **m)** Transgenic mice expressing GADD34^{Δ322} showed a reduction in the granular layer thickness. **n)** Representative microscopy images of the cerebellar lobule X. **o)** Transgenic mice expressing eIF2α^{S52D} exhibited a reduced molecular layer thickness. **p)** Transgenic mice expressing eIF2α^{S52D} showed a reduction in the granular layer thickness. (n: TG^{MUT(129Q)}+CT= 6, TG^{MUT(129Q)}+eIF2α^{S52D}= 6, TG^{MUT(129Q)}+GADD34^{Δ322}= 6; *P<0.05, **P<0.01; ****P<0.001, ***P<0.001; One-Way Anova with mixed-effect analysis followed by Bonferroni correction; values are expressed as mean ± SEM).

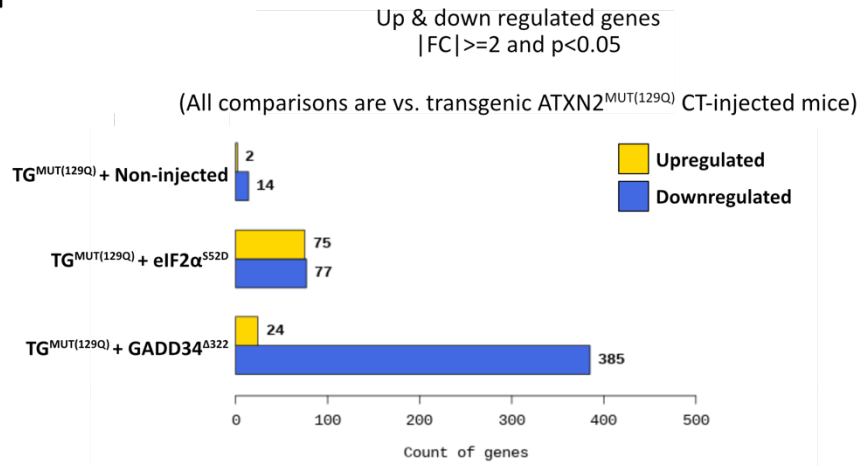
Overall, we observed that expressing the tools to manipulate SGs assembly/disassembly dynamics were able to alter the neuropathology profile associated with the transgenic mouse model of SCA2.

Transcriptomic analysis across the different experimental groups

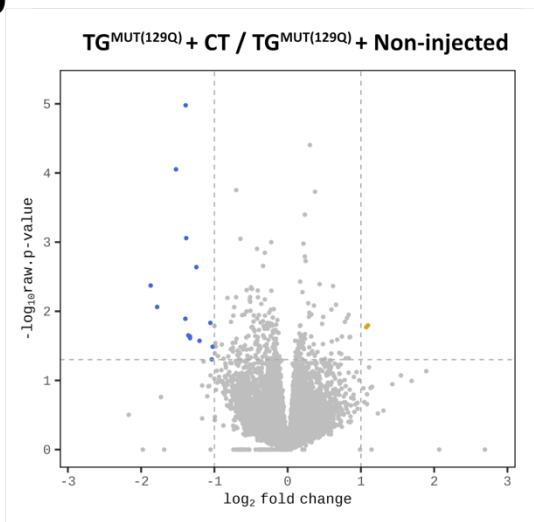
To understand the impact of AAV-mediated expression of eIF2 α^{S52D} and GADD34 $\Delta 322$ at the transcriptomic level, cerebella from 14 weeks of age mice, with 10 weeks of expression, were processed for RNA sequencing analysis. The differentially expressed genes were assessed in the following comparisons: i) (TG^{MUT(129Q)}+CT / TG^{MUT(129Q)}+Non-injected); ii) (TG^{MUT(129Q)}+CT / TG^{MUT(129Q)}+eIF2 α^{S52D}); and iii) (TG^{MUT(129Q)}+CT / TG^{MUT(129Q)}+GADD34 $\Delta 322$). To consider differentially expressed genes, we established a cutoff of $|\geq 2|$ -fold change (for upregulation and downregulation) with $p < 0.05$.

First, we analyzed how many genes were upregulated and downregulated in the different comparisons. Comparing TG^{MUT(129Q)}+CT / TG^{MUT(129Q)}+Non-injected, only 16 genes were differentially expressed (2 upregulated and 14 downregulated). Comparing TG^{MUT(129Q)}+CT / TG^{MUT(129Q)}+eIF2 α^{S52D} mice, 152 genes were differentially expressed (75 upregulated and 77 downregulated). Finally, comparing TG^{MUT(129Q)}+CT / TG^{MUT(129Q)}+GADD34 $\Delta 322$, we observed 409 differently expressed genes (24 upregulated and 385 downregulated) (**Figure R32a**). Then, we plotted the differentially expressed genes considering the cutoffs applied to visualize their distribution, with statistical significance as a function of fold change (**Figure R32b-d**).

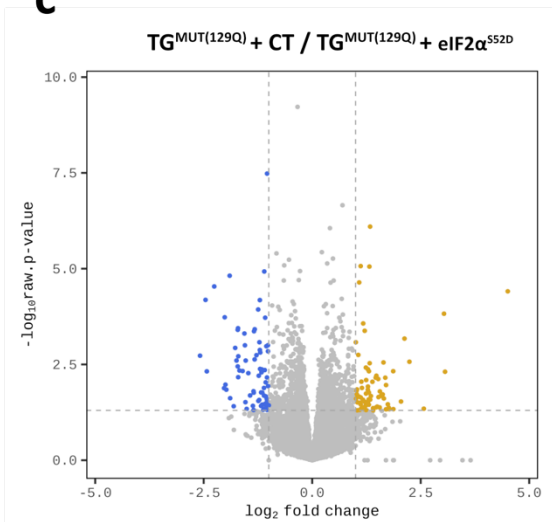
a



b



c



d

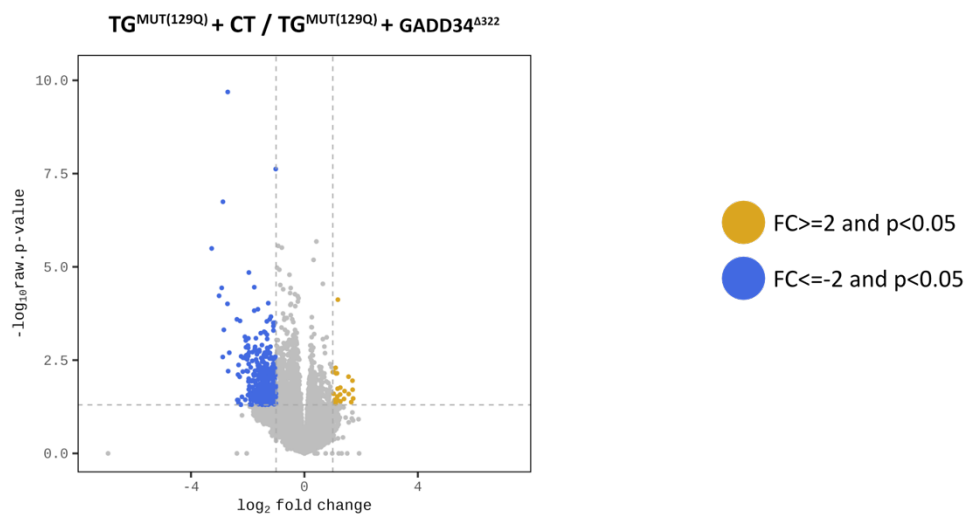


Figure R32 - Transcriptomic analysis of cerebellar samples from mice expressing the control, eIF2 α^{S52D} or GADD34 Δ^{322} . Cerebella from mice stereotactically injected with AAV particles encoding for the control, eIF2 α^{S52D} , or GADD34 Δ^{322} , were processed for transcriptomic analysis. Mice were injected at 4 weeks of age and sacrificed at 14 weeks of age. All expression comparisons were performed with mice expressing the control (TG^{MUT(129Q)} + CT). The cutoff for differentially expressed genes was set at a $\geq \pm 2$ -fold change with a $p < 0.04$. **a)** In non-injected transgenic mice, only 16 genes were differentially expressed. Transgenic mice expressing eIF2 α^{S52D} exhibited 152 differentially expressed, and transgenic mice expressing GADD34 Δ^{322} showed a total of 408 differentially expressed genes. **b)** Volcano plot representation of the differentially expressed genes (TG^{MUT(129Q)} + CT / TG^{MUT(129Q)} + Non-injected), showing the genes with statistical significance as a function of fold change. **c)** Volcano plot representation of the differentially expressed genes (TG^{MUT(129Q)} + CT / TG^{MUT(129Q)} + eIF2 α^{S52D}) showing the genes with statistical significance as a function of fold change. **d)** Volcano plot representation of the differentially expressed genes (TG^{MUT(129Q)} + CT / TG^{MUT(129Q)} + GADD34 Δ^{322}) showing the genes with statistical significance as a function of fold change. (n: TG^{MUT(129Q)}+CT= 4, TG^{MUT(129Q)}+Non-injected= 6, TG^{MUT(129Q)}+eIF2 α^{S52D} = 4, TG^{MUT(129Q)}+GADD34 Δ^{322} = 4).

We also analyzed the overall gene expression patterns by hierarchical clustering the different conditions and compared them with the TG^{MUT(129Q)}+CT mice (**Figure R33**). Overall, the TG^{MUT(129Q)}+Non-injected animals exhibited distinct expression signatures when compared with the TG^{MUT(129Q)}+CT (**Figure R33a**). Additionally, the TG^{MUT(129Q)}+eIF2 α^{S52D} mice exhibited completely distinct expression signatures when compared with TG^{MUT(129Q)}+CT (**Figure R33b**). Finally, the TG^{MUT(129Q)}+GADD34 Δ^{322} animals showed an overall distinct expression signature compared with TG^{MUT(129Q)}+CT (**Figure R33c**). Overall, the different experimental groups exhibit expression signatures that are distinctly different from TG^{MUT(129Q)}+CT.

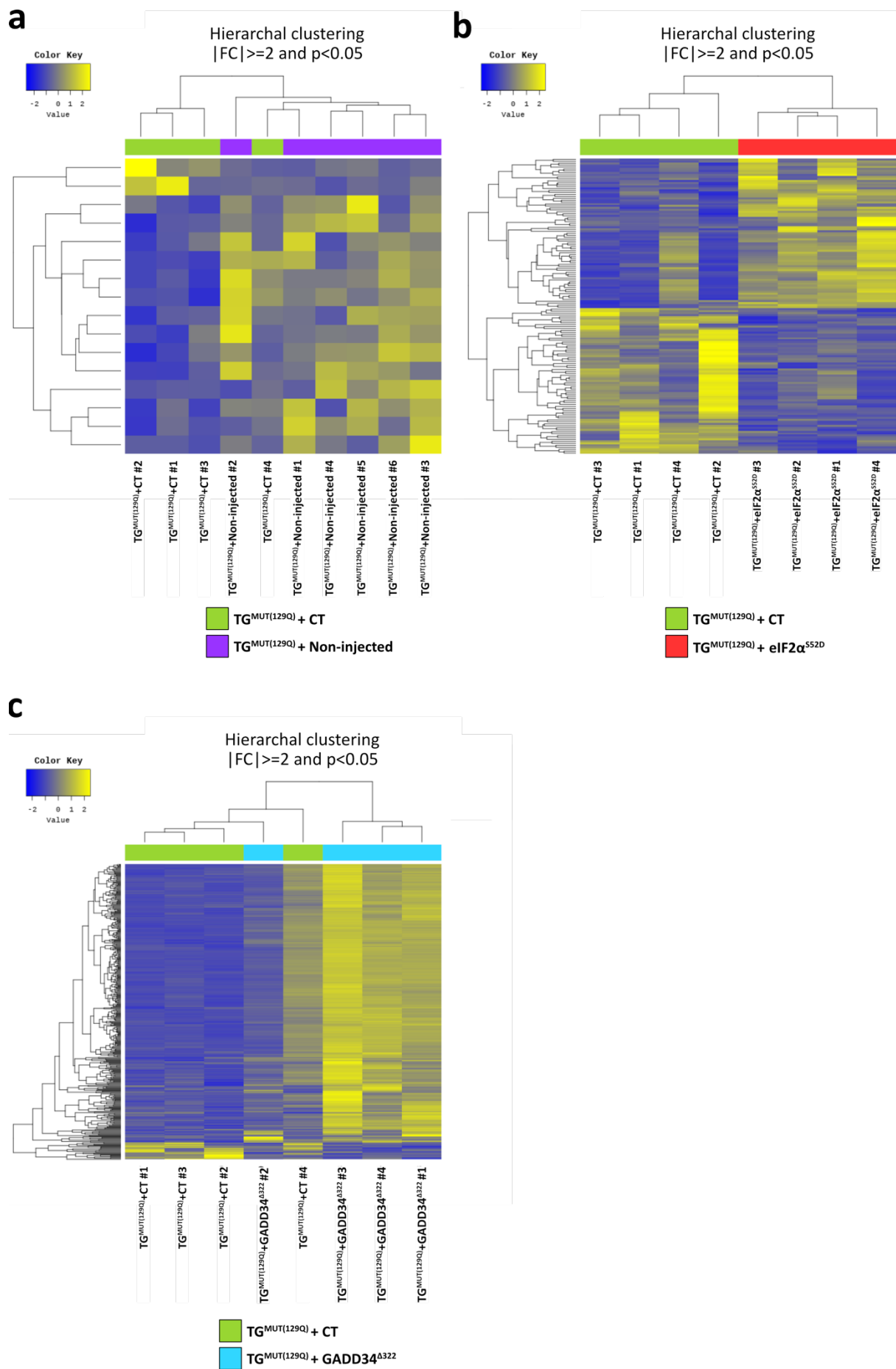


Figure R33 - Hierarchical clustering and transcriptomic pattern of cerebellar samples. Samples from mice cerebella were hierarchically clustered based on the transcriptomic signature. Hierarchical clustering was performed with TG^{MUT(129Q)} + CT samples. **a)** TG^{MUT(129Q)} + Non-injected mice displayed an overall homogenous expression pattern, distinct from TG^{MUT(129Q)} + CT. **b)** TG^{MUT(129Q)} + eIF2 α ^{S52D} mice displayed an homogenous expression pattern, distinct from TG^{MUT(129Q)} + CT. **c)** TG^{MUT(129Q)} + GADD34 Δ ³²² mice displayed an overall homogenous expression pattern, distinct from TG^{MUT(129Q)} + CT (n: TG^{MUT(129Q)}+CT= 4, TG^{MUT(129Q)}+Non-injected= 6, TG^{MUT(129Q)}+eIF2 α ^{S52D}= 4, TG^{MUT(129Q)}+GADD34 Δ ³²²= 4).

Finally, to understand the overall biological significance of the gene expression analysis we performed a gene ontology analysis. Overall, the alterations observed in the comparison TG^{MUT(129Q)}+CT / TG^{MUT(129Q)}+Non-injected animals, were related mainly related to immune responses involving immunoglobulins (**Figure R34**). The overall observed alterations in the comparison between TG^{MUT(129Q)}+CT / TG^{MUT(129Q)}+eIF2 α ^{S52D} animals were mainly related with lipid metabolism, immune-related processes, transmembrane transport activities and neuronal-related functions (**Figure R35**). Finally, the alterations observed between TG^{MUT(129Q)}+CT / TG^{MUT(129Q)}+GADD34 Δ ³²² animals were mainly related to processes associated with immune responses (**Figure R36**).

TG^{MUT(129Q)}+CT / TG^{MUT(129Q)} + Non-injected

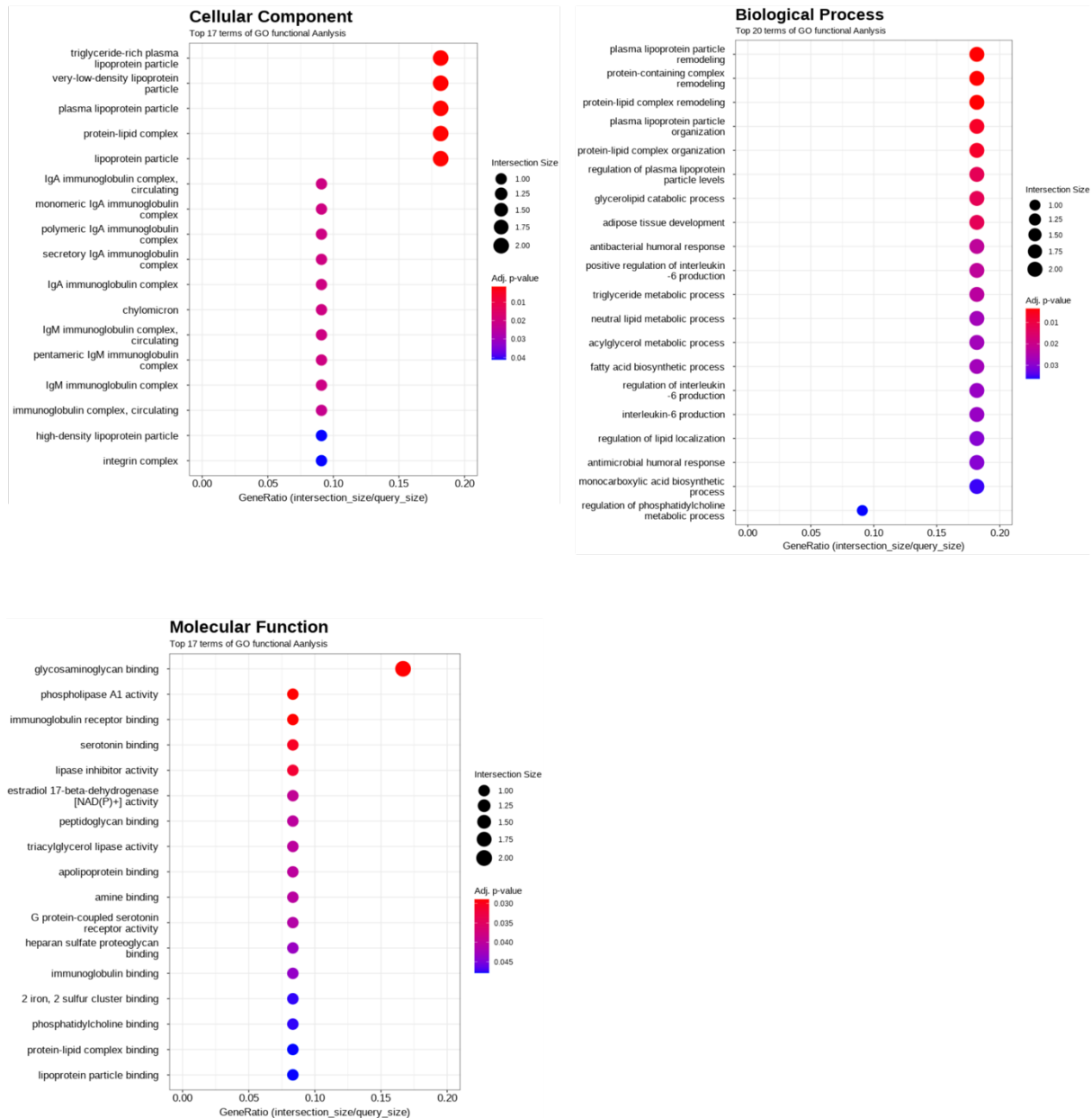


Figure R34 - Gene ontology analysis of differentially expressed genes (TG^{MUT(129Q)} + CT / TG^{MUT(129Q)} + Non-injected). Differentially expressed genes were categorized into Cellular Components, Biological Processes, and Molecular functions. Dot plot to show the top most significant gene ontology terms, whose term size is more than 10 and less than 500, in each category and their adjusted p-value < 0.05 (n: TG^{MUT(129Q)}+CT= 4, TG^{MUT(129Q)}+Non-injected= 6, TG^{MUT(129Q)}+eIF2 α ^{S52D}= 4, TG^{MUT(129Q)}+GADD34 Δ ³²²= 4).

TG^{MUT(129Q)}+CT / TG^{MUT(129Q)} + eIF2 α ^{S52D}

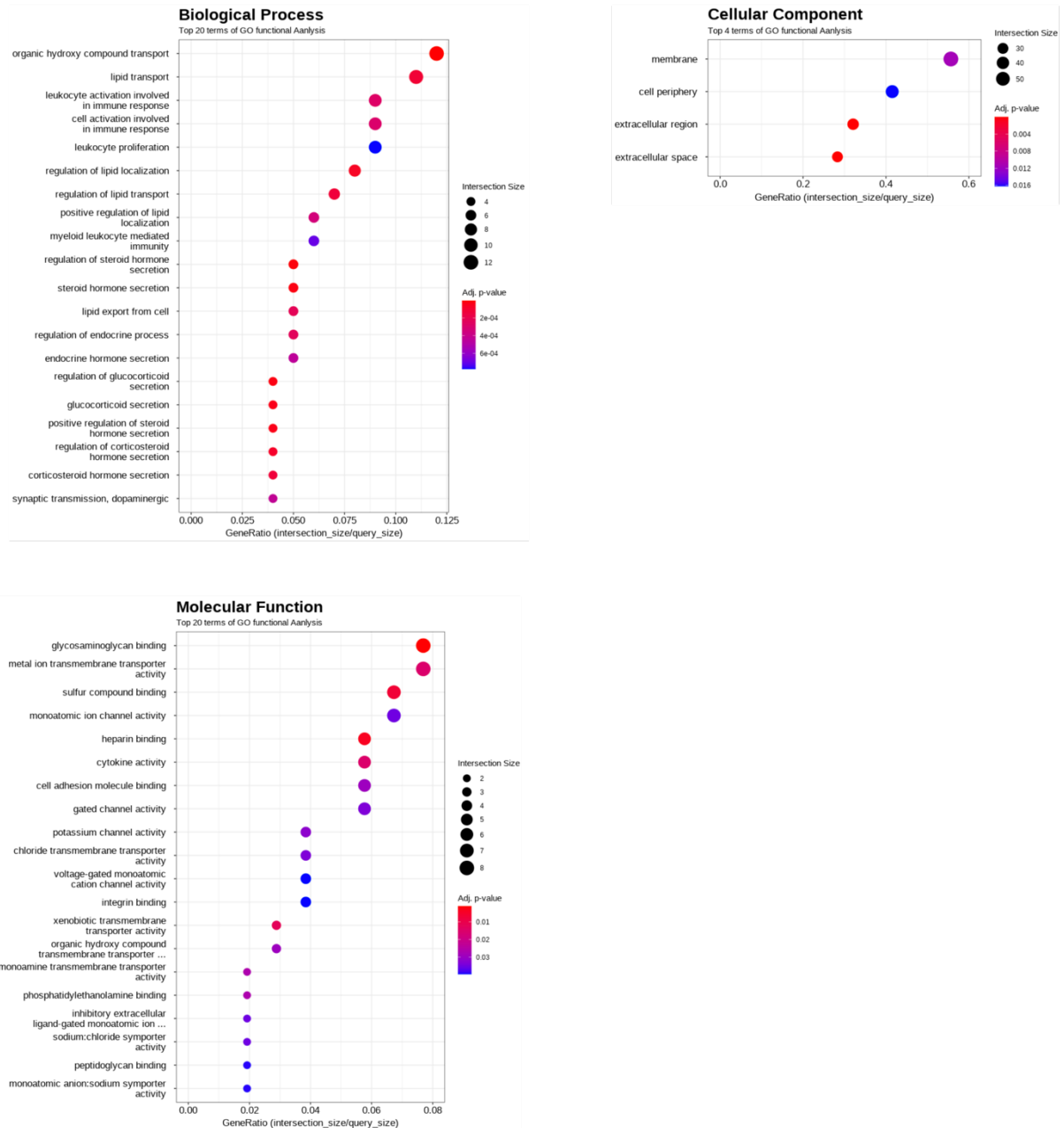


Figure R35 - Gene ontology analysis of differentially expressed genes (TG^{MUT(129Q)} + CT / TG^{MUT(129Q)} + eIF2 α ^{S52D}). Differentially expressed genes were categorized into Cellular Components, Biological Processes, and Molecular functions. Dot plot to show the top most significant gene ontology terms, whose term size is more than 10 and less than 500, in each category and their adjusted p-value <0.05 (n: TG^{MUT(129Q)}+CT= 4, TG^{MUT(129Q)}+Non-injected= 6, TG^{MUT(129Q)}+eIF2 α ^{S52D}= 4, TG^{MUT(129Q)}+GADD34^{Δ322}= 4).

TG^{MUT(129Q)}+CT / TG^{MUT(129Q)}+ GADD34^{Δ322}

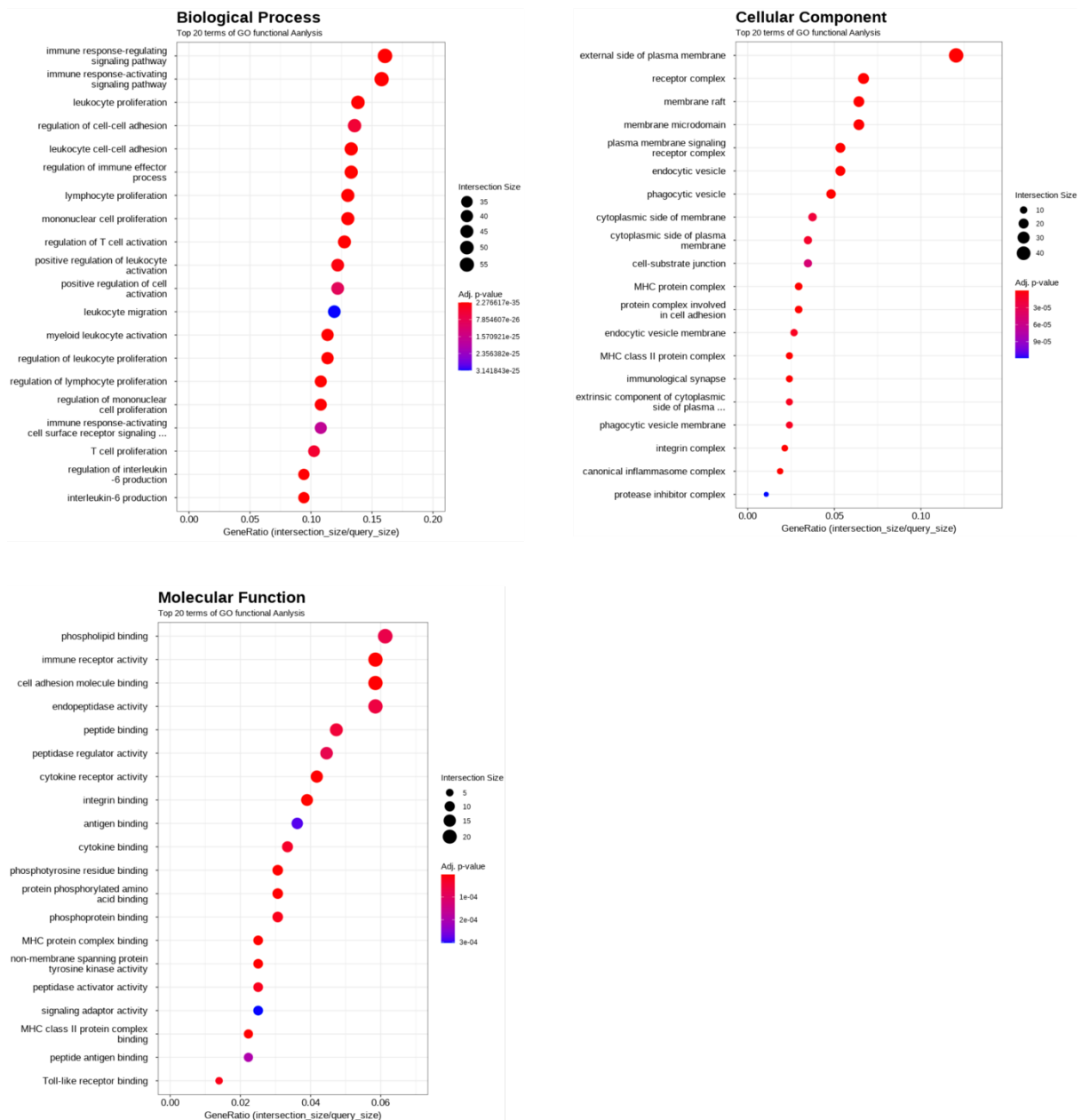


Figure R36 - Gene ontology analysis of differentially expressed genes (TG^{MUT(129Q)} + CT / TG^{MUT(129Q)} + GADD34^{Δ322}). Differentially expressed genes were categorized into Cellular Components, Biological Processes, and Molecular Functions. Dot plot to show the top most significant gene ontology terms, whose term size is more than 10 and less than 500, in each category and their adjusted p-value <0.05 (n: TG^{MUT(129Q)}+CT= 4, TG^{MUT(129Q)}+Non-injected= 6, TG^{MUT(129Q)}+eIF2α^{SS2D}= 4, TG^{MUT(129Q)}+GADD34^{Δ322}= 4).

Since we were studying the impact of SGs in the context of the neurodegenerative disease SCA2, we decided to elaborate a list of differentially expressed genes according to the following categories: stress-related genes and neuronal-related genes. Moreover, as the

mutant ataxin-2 toxicity is connected to alterations in proteostasis, we also included the category proteostasis-related genes. Since the gene ontology analysis revealed several alterations related to immune responses, we also decided to include an immune-related genes category (**Table R2, Table R3 and Table R4**). To compile this list, we used the GeneALaCart platform (www.genealacart.genecards.org), which allows batch querying of gene symbols, enabling us to retrieve gene functions based on previously published data. Note that we only used protein-coding genes to elaborate this list.

According to our categorization, differentially expressed genes $TG^{MUT(129Q)+CT}$ / $TG^{MUT(129Q)+Non-injected}$, few protein-coding genes were altered, and most of them were related to the immune system (**Table R2**). Additionally, comparing $TG^{MUT(129Q)+CT}$ / $TG^{MUT(129Q)+eIF2\alpha^{S52D}}$ most of the genes related to the stress response, proteostasis and immune responses were upregulated, whereas genes related to neuronal function were all upregulated (**Table R3**). Finally, comparing $TG^{MUT(129Q)+CT}$ / $TG^{MUT(129Q)+GADD34^{\Delta 322}}$ almost all differentially expressed genes were downregulated (**Table R4**).

Overall, we observed that expressing the tools to manipulate SGs assembly/disassembly dynamics were able to alter the expression profile associated with the transgenic mouse model of SCA2.

Table R2 – Differentially expressed genes $TG^{MUT(129Q)+CT}$ / $TG^{MUT(129Q)+Non-injected}$

Cellular stress-related genes			
Gene ID	Fold-change	pvalue	Description
Slpi	2.6115635	0.00087493	Protease inhibitor stress/immune modulation
Neuronal-related genes			
Gene ID	Fold-change	pvalue	Description
Htr1f	2.13756124	0.01600525	Serotonin receptor modulates neuronal signaling
Immune response-related genes			
Gene ID	Fold-change	pvalue	Description
Itgax	-2.0495421	0.04962942	CD11c dendritic cell marker antigen presentation
Jchain	-2.8765624	8.8691E-05	Joins immunoglobulin subunits antibody secretion
Pou2af1	-2.5193788	0.02315974	Transcription factor B-cell development
Cd3001b	-2.3021747	0.02672457	Immune receptor modulates inflammation

Table R3 – Differentially expressed genes TG^{MUT(129Q)}+CT / TG^{MUT(129Q)}+eIF2^{αS52D}

Cellular stress Related Genes			
Gene ID	Fold-change	pvalue	Description
Atf3	-2.3236646	0.00083185	Transcription factor induced by stress signals
Atf5	-2.0559888	3.3021E-08	Part of unfolded protein response (UPR) in ER stress
Gdf15	-4.044944	0.00018588	Induced by stress and inflammation
Trib3	-2.292643	0.00154313	Stress signaling and apoptosis
Spp1	-2.0707681	0.03566064	Osteopontin upregulated in stress/inflammation
Slpi	-3.9967044	0.01063698	Protease inhibitor stress/immune modulation
Adm2	-2.4359958	0.00181005	Peptide stress-related homeostasis
Ccl6	-2.9521206	0.00049246	Chemokine inflammatory stress responses
Proteostasis related genes			
Gene ID	Fold-change	pvalue	Description
Npc1l1	2.22549834	0.04188448	Linked to lipid/membrane protein homeostasis
Timp1	-2.8931231	0.00250475	Metalloproteinase inhibitor regulates proteolysis
Plau	-2.7735645	0.00533171	Urokinase extracellular protein degradation
Pappa	-2.1507147	1.1841E-05	Protease cleaving IGF-binding proteins
Mmp19	-2.1363788	0.04520126	Matrix metalloproteinase extracellular matrix remodeling
Neuronal-related genes			
Gene ID	Fold-change	pvalue	Description
Crh	3.65308596	0.04633347	Corticotropin-releasing hormone key in neuronal stress
Ecel1	4.13044515	0.02932954	Neuronal differentiation and axonal growth
Gabre	2.82361297	0.03979166	GABA receptor subunit inhibitory neurotransmission
Glr1	5.94470478	0.04516543	Glycine receptor subunit neuronal inhibition
Npas1	3.33681374	0.04591581	Transcription factor neuronal development
Slc6a2	22.7207467	3.9024E-05	Norepinephrine transporter neurotransmitter reuptake
Slc18a1	2.31312952	0.02990437	Vesicular monoamine transporter neurotransmitter storage
Th	2.49341703	8.8171E-06	Tyrosine hydroxylase catecholamine synthesis
Nrg1	2.08828755	0.00178281	Neuronal development synaptic plasticity
Rab3b	2.1688223	8.5602E-06	GTPase synaptic vesicle release
Immune response-related genes			
Gene ID	Fold-change	pvalue	Description
Cd68	-2.2470555	0.00507894	Marker of macrophages immune surveillance
Cd84	-2.0545826	0.02767506	Immune cell receptor regulates lymphocyte activation
Fcgr3	-2.5038915	0.0003812	Low-affinity Fc receptor immune cell activation
Itgax	-3.1952895	0.00455667	CD11c dendritic cell marker antigen presentation
Itgb2	-2.0527479	0.02812415	CD18 integrin complex in leukocyte adhesion
Ly86	-2.1823576	0.04790287	Co-receptor with TLR4 enhances immune signaling
Slc11a1	-2.0787568	0.04405296	Regulates macrophage pathogen resistance
Pou2af1	-3.9411095	0.01439648	Transcription factor B-cell development
Tyrobp	-2.299699	0.00414085	Adapter protein immune cell signaling (NK cells macrophages)
Trem2	-2.3072276	0.00132568	Receptor on microglia/macrophages immune modulation
Cd3001b	-3.4215889	0.0011704	Immune receptor modulates inflammation
Gpr183	-2.1016314	0.00927256	G-protein-coupled receptor immune cell migration

Table R4 – Differentially expressed genes TG^{MUT(129Q)}+CT / TG^{MUT(129Q)}+GADD34^{Δ322}

Stress response-related genes			
Gene ID	Fold-change	pvalue	Description
Alox5	-2.6767757	0.01744464	Lipoxygenase oxidative stress mediator
Atf3	-2.1992956	0.02166906	Transcription factor induced by stress signals
Birc5	-2.8544384	0.00665283	Survivin inhibits apoptosis
Casp1	-2.0860763	0.01604508	Caspase-1 inflammasome activation
Casp8	-2.0633687	0.01025842	Caspase-8 apoptosis signaling
Cryab	-2.0169116	2.3855E-08	Heat shock protein stress chaperone
Cyba	-2.6452117	0.00550176	NADPH oxidase subunit oxidative stress
Gsdmd	-2.5340814	0.02260845	Gasdermin D pyroptosis mediator
Ncf1	-2.5116708	0.00441498	NADPH oxidase subunit ROS production
Ncf2	-2.4840061	0.00513103	NADPH oxidase subunit ROS production
Ncf4	-3.3469552	0.00608146	NADPH oxidase subunit ROS production
Nlrp3	-3.0263758	0.00894025	Inflammasome component stress response
Pycard	-2.636015	0.00803049	ASC inflammasome adaptor
Sl00a4	-2.4557963	0.02221052	Calcium-binding protein stress signaling
Slpi	-9.6501263	3.2075E-06	Protease inhibitor stress/immune modulation
Timp1	-3.4303537	0.00822828	Metalloproteinase inhibitor stress response
Top2a	-3.5760013	0.00331351	Topoisomerase DNA stress response
Ube2c	-3.5663851	0.01668208	Ubiquitin-conjugating enzyme stress regulation
Proteostasis-related genes			
Gene ID	Fold-change	pvalue	Description
Capn6	-2.3306303	0.017529	Calpain-6 protease
Ctsc	-2.5188003	0.03991259	Cathepsin C lysosomal protease
Ctsd	-2.3337479	0.000254	Cathepsin D lysosomal protease
Ctsh	-2.2905847	0.02661977	Cathepsin H lysosomal protease
Ctss	-2.8292136	0.04065895	Cathepsin S lysosomal protease
Ctsw	-3.975461	0.00128247	Cathepsin W lysosomal protease
Ctsz	-2.2922878	0.01548767	Cathepsin Z lysosomal protease
Derl3	-3.4054378	3.5073E-05	Derlin-3 ER protein degradation
Hexb	-2.01148	0.03072767	Hexosaminidase B lysosomal enzyme
Hpse	-3.7336569	0.03671465	Heparanase extracellular matrix degradation
Plau	-3.9375766	0.00082458	Urokinase extracellular protein degradation
Mmp19	-2.497927	0.04768095	Matrix metalloproteinase extracellular matrix remodeling
Neuronal-related genes			
Gene ID	Fold-change	pvalue	Description
Cnr2	-3.4718739	0.00762069	Cannabinoid receptor 2 neuronal signaling
Htr1f	2.93894883	0.00878632	Serotonin receptor neuronal signaling
Lck	-3.0597557	0.02056604	Tyrosine kinase neuronal signaling
Nts	2.03919343	0.00656046	Neurotensin neuropeptide signaling
Immune-related genes*			
Gene ID	Fold-change	pvalue	Description
Ighm	-8.054129	5.9639E-05	Immunoglobulin heavy chain B-cell immunity
Igkc	-7.5494793	3.6619E-05	Immunoglobulin kappa chain B-cell immunity
Jchain	-7.3288477	1.7998E-07	Joins immunoglobulin subunits antibody secretion
Pou2af1	-7.16825	0.00048725	B-cell transcription factor immunity
H2-DMb2	-6.2739911	0.0020013	MHC class II chaperone antigen presentation
H2-Eb1	-5.1547828	0.0370808	MHC class II antigen presentation
GpnmB	-5.0656282	0.00775219	Glycoprotein immune modulation
Ciita	-5.044725	0.03739018	MHC class II transactivator antigen presentation
H2-Aa	-5.0049431	0.04477017	MHC class II antigen presentation
H2-Oa	-4.997689	0.00425948	MHC class II accessory antigen presentation
Ltb	-4.8690886	0.00883269	Lymphotoxin beta immune signaling
H2-Ab1	-4.7866735	0.0479045	MHC class II antigen presentation

* More than 300 immune-related genes were differentially expressed. For simplification, we considered the top 13 based on fold change.

Chapter 5 – Discussion

Discussion

The ISR plays a crucial fundamental role when cells are exposed to stress conditions. When cells detect a stressor, the ISR starts with the phosphorylating eIF2 α , which leads to the global arrest of translation, accumulation of untranslated poly-(A) mRNA, and, consequently, the formation of SGs (Harding et al., 2003; Pakos-Zebrucka et al., 2016; Riggs et al., 2020). SGs, the main players of the ISR, are central in aiding cells to cope and recover from various sources of stress (Buchan and Parker, 2009; Marcelo et al., 2021a; Riggs et al., 2020).

The exact biological functions of SGs are not fully understood. Nevertheless, the most accepted view is that SGs serve as hubs to store mRNAs that are not essential to be translated during a specific time under stressful conditions (Kedersha et al., 2002, 1999; Pakos-Zebrucka et al., 2016). Therefore, cells can activate a new translation program that is more efficient and prioritizes the synthesis of proteins that help cells resolve the stress. Simultaneously, this response reduces the energetic burden of global translation while promoting the production of factors more suitable to cope with stress (Marcelo et al., 2021a; Protter and Parker, 2016; Williams and Rousseau, 2024). SGs are known to be formed in response to cellular stresses such as oxidative stress, viral infections, amino acid starvation, and ER stress, as well as during the unfolded protein response (Harding et al., 2003; Kedersha et al., 2000, 1999; Marcelo et al., 2021b; McEwen et al., 2005; Srivastava et al., 1998; Wek et al., 1995).

In the context of pathological conditions, recent studies have shown that several proteins associated with neurodegenerative diseases (TDP-43 – ALS; TAU – Alzheimer's disease; ataxin-2 – SCA2) are components of SGs. Moreover, in the context of polyQ disorders, it has been suggested that aggregates of the mutant proteins can sequester SGs components (Marcelo et al., 2021b; Wolozin and Ivanov, 2019).

Recently, ataxin-2 has also been identified as a central player in the formation of SGs, acting as a scaffold for their assembly (Yamagishi et al., 2024). Given the role of ataxin-2 in SGs nucleation, its mutant form has the potential to disrupt the homeostasis of SGs, and its assembly and disassembly dynamics. It can be hypothesized that due to the increased rigidity of polyQ-expanded proteins, mutant ataxin-2 may promote the formation of SGs, which are

more resilient to disassembly. In fact, several studies have shown that in the context of different neurodegenerative diseases (ALS, FTD, prion-disease), the ISR is overactivated, contributing to the exacerbation of disease-associated phenotypes (Hoozemans et al., 2009, 2007; Walter and Ron, 2011). This may result from chronic stress stimuli, which are characteristic of neurodegenerative conditions, supporting the hypothesis that sustained formation and/or maintenance of SGs can be deleterious.

The dynamics of SGs in SCA2 are not known. Given the role of ataxin-2 in SGs formation, its potential to disrupt these structures when the protein is expanded, and the chronic nature of SCA2, we hypothesized that SGs might influence SCA2 pathogenesis. Since the impact of SGs on the disease progression—whether beneficial or detrimental—remains unknown, this work aimed to investigate how SGs assembly and disassembly dynamics influence SCA2-associated deficits.

The ISR starts when one (or more, depending on the stress stimuli) of the four stress kinases (HRI, PKR, PERK, and GCN2) detects a stress stimulus and phosphorylates eIF2 α (Harding et al., 2003; McEwen et al., 2005; Srivastava et al., 1998; Wek et al., 1995). The downstream effect of the increased accumulation of p-eIF2 α is the assembly of SGs (Kedersha et al., 2002). Thus, to induce the formation of SGs, we envisioned two different approaches: i) expressing a key factor that is directly involved in the ISR activation pathway, and ii) expressing an SGs component that is not directly involved in the ISR activation pathway. In line with that, we first developed a phosphomimetic form of eIF2 α , in which the serine 52 was substituted by an aspartate (eIF2 α^{S52D}). Previous studies have demonstrated that, in order for the stress response to be triggered, eIF2 α is phosphorylated at the serine 52 (Kedersha et al., 2002; Marcelo et al., 2021b; Pakos-Zebrucka et al., 2016). The work presented here showed that the *in vitro* expression of eIF2 α^{S52D} , which mimics the phosphorylation state of eIF2 α , successfully induced the formation of SGs in cells that were not subjected to an exogenous stress stimulus. This observation is in line with the previous observations that the phosphorylation of eIF2 α is not only at the center of the ISR activation but also plays a role in the formation of SGs (Kedersha et al., 2002, 1999; Marcelo et al., 2021b; Pakos-Zebrucka et al., 2016; Riggs et al., 2020).

We next induced the formation of SGs without directly acting at the activation/deactivation pathway of the ISR. We used a core component of the SGs. For that,

we expressed, *in vitro*, TIA1, a known component of SGs and a widely used SGs marker (Kedersha et al., 2000, 1999; Marcelo et al., 2021b). We observed that the expression of a core component of SGs successfully induced the formation of SGs in cells that were not submitted to an exogenous stress stimulus. Other authors have already shown that the sole expression of the core component G3BP1 or TIAR induced the formation of SGs (Kedersha et al., 1999; Tourrière et al., 2023).

When comparing the two strategies to induce the formation of SGs, we observed that eIF2 α ^{S52D} (phosphomimetic mutant) induced SGs assembly in approximately 30% of transfected cells, whereas TIA1 was only able to induce in 13%. The differences in the efficiency of both strategies to induce the formation of SGs might be explained by the cascade that precedes the phosphorylation of eIF2 α . When eIF2 α is phosphorylated, several factors accumulate within cells, such as multiple untranslated RNAs, ribosomes, and Met-tRNAi, which might contribute to a better signal amplification to nucleate SGs. In fact, the local and confined increased concentration of these factors is the basis for SGs formation by triggering the LLPS (Protter and Parker, 2016; Riggs et al., 2020; Van Treeck et al., 2018; Yamagishi et al., 2024). However, TIA1 overexpression alone may be less efficient at achieving sufficient concentrations of untranslated mRNAs and co-factors to trigger LLPS, likely because it must recruit these components actively rather than benefiting from their stress-induced accumulation.

Then, to inhibit the formation of SGs, we envisioned a similar rationale. This means that i) we expressed a key element directly involved in the deactivation of the ISR and ii) downregulated the expression of an SGs component that is not directly involved in the ISR deactivation pathway. One of the mechanisms contributing to SGs disassembly is the dephosphorylation of p-eIF2 α . During the initiation of the ISR, while eIF2 α is phosphorylated, GADD34 expression is increased. GADD34 is a scaffold protein that mediates the interaction between p-eIF2 α and the phosphatase PP1, leading to the dephosphorylation of eIF2 α (Connor et al., 2001; Magg et al., 2024; Novoa et al., 2001). Furthermore, studies had suggested that overexpression of GADD34 increases the dephosphorylation of p-eIF2 α (Brush et al., 2003; Kojima et al., 2003; Novoa et al., 2001; Zhu et al., 2021). In line with that, we developed a truncated form of GADD34, lacking the nucleotide sequence that codes for the first 322 amino acids (GADD34 Δ ³²²). We chose to use a truncated form based on studies

showing that the deletion of the $\Delta 322$ amino acids in GADD34 increases its binding affinity for both PP1 and phosphorylated eIF2 α , suggesting enhanced dephosphorylation activity (Brush et al., 2003; Choy et al., 2015). In the experiments presented here, we showed that expressing GADD34 $\Delta 322$ nearly led to the ablation of SGs assembly in cells treated with sodium arsenite. Under the sodium arsenite treatment, virtually all treated cells form SGs, meaning that GADD34 $\Delta 322$ drastically prevented the SGs assembly. Our observations are in line with previous results showing that the expression of GADD34 facilitates the dephosphorylation of p-eIF2 α . Additionally, other authors have demonstrated that GADD34 knockdown not only increases the levels of p-eIF2 α but also hinders SGs disassembly (Ruggieri et al., 2012; Zhu et al., 2021). Moreover, it was already shown that cell lines overexpressing GADD34 prevented the formation of SGs upon viral infection, a stimulus that typically causes the formation of SGs (Ruggieri et al., 2012).

Next, we tried to limit the formation of SGs in cells under cellular stress without directly acting on the ISR pathway. Since *in vitro* expression of TIA was able to induce a modest formation of SGs, we tried to knockdown this SGs component. For that, we used shRNAs targeting TIA1. We could not succeed in manipulating the formation of SGs, as the shRNAs used were not able to prevent or reduce their formation. Contrary to our observation, several other studies have suggested that TIA downregulation impairs the formation of SGs (Gilks et al., 2004; Kedersha et al., 2000, 1999). Thus, our results could reflect a technical difficulty rather than actual biological meaning, possibly explained by an insufficient TIA1 levels knockdown.

After developing the genetic tools that enable us to modulate the formation of SGs, we decided to test their impact on relevant models for SCA2. To do that, we used primary cultures of mouse striatal neurons either expressing a non-expanded form of ataxin-2 with 23 glutamines or expressing a mutant form of ataxin-2 with 80 glutamines. First, we observed that neurons expressing a mutant form of ataxin-2 exhibited a reduction in the total dendritic length compared to those expressing the non-expanded form, possibly reflecting the toxic effects associated with polyQ-expanded proteins. In fact, recently, our lab showed that SCA2 patients' iPSC-derived neurons also displayed reduced dendritic length compared to neurons derived from healthy controls (Koppenol, 2024). Then, we observed that the expression of

eIF2 α^{S52D} prevented the deleterious effects caused by the mutant ataxin-2, restoring the total dendritic length to levels close to those observed in neurons expressing the non-expanded form of ataxin-2. The expression of GADD34 Δ^{322} had no impact on the total dendritic length of neurons expressing mutant ataxin-2.

The fact that eIF2 α^{S52D} mitigated the deleterious effects of mutant ataxin-2 suggests that SGs may play a protective role in counteracting the toxicity of the expanded polyQ protein. This aligns with the proposed role of SGs as structures that are assembled in order to aid cells in coping with cellular stress, which, in this case, is the presence of a protein with toxic properties (Marcelo et al., 2021b; Pakos-Zebrucka et al., 2016; Riggs et al., 2020). Moreover, compromised proteostasis is one of the stressors that can induce SGs formation in order to prevent the accumulation of defective proteins (H. P. Harding et al., 2000; Kondratyev et al., 2007; Smith and Mallucci, 2016). Given that, by inducing SGs formation, we may be promoting a more robust cellular response to the accumulation of defective proteins. In this way, boosting cellular proteostasis could reduce the cytotoxic impact of these proteins and support cellular homeostasis under stress conditions.

Considering the results obtained by the expression of eIF2 α^{S52D} – which promotes SGs formation – we would hypothesize an opposite effect when preventing SGs formation by expressing GADD34 Δ^{322} . Recent studies have indicated that inhibiting SGs formation in primary neurons from rat models of ALS, and FTD exacerbates the associated pathology (Glineburg et al., 2024). However, we did not observe any impact in cells related to the inhibition of SGs, which could indicate that the toxicity of mutant ataxin-2 has reached a 'floor effect. Meaning that the damage caused by the expression of the mutant protein may have already reached a threshold beyond which additional SGs inhibition can no longer exacerbate the toxicity associated with the expression of the mutant ataxin-2. Nevertheless, to gain a clearer understanding, we could complement the morphometric analysis with cellular viability and metabolic assays to evaluate whether neuronal survival is impacted by the expression of GADD34 Δ^{322} .

After testing our strategies to induce and inhibit the formation of SGs *in vitro*, we wanted to understand whether the expression of eIF2 α ^{S52D} or GADD34 ^{Δ 322} would impact traits associated with SCA2 rodent models. Thus, we used an LV striatal mouse model of SCA2 previously developed by our lab (Marcelo et al., 2021a). This model is characterized by the formation of mutant ataxin-2 aggregates and by the loss of dopaminergic DARPP-32 marker staining. AAV-mediated expression of eIF2 α ^{S52D} – which promotes SGs formation – exacerbated the loss of the dopaminergic DARPP-32 marker staining, a trait associated with the LV mouse model. However, eIF2 α ^{S52D} seemed not to impact the total number of mutant ataxin-2 aggregates formed. Expressing eIF2 α ^{S52D} in this model seems to have the opposite effect compared with the *in vitro* experiments. Several studies have proposed that the chronic stress characteristic of neurodegenerative diseases may result in the prolonged presence SGs (Hoozemans et al., 2009, 2007; Moreno et al., 2013; Walter and Ron, 2011). It is hypothesized that under such sustained cellular stress conditions, persistent SGs may start to lose their protective role and instead contribute to cellular dysfunction, ultimately becoming deleterious to neurons. In fact, it was shown that p-eIF2 α levels are increased in patients affected with AD, ALS, FTD, and PD. Moreover, it was described that downregulating eIF2 α , which reduces its availability to be phosphorylated, mitigated disease-associated traits in some models of these diseases (Hoozemans et al., 2009, 2007; Moreno et al., 2013; Walter and Ron, 2011). We interpret the differences observed between the *in vitro* and *in vivo* experiments because of the duration of SGs induction. *In vitro*, the expression of eIF2 α ^{S52D} promotes SG formation over a period of several hours, likely mimicking a transient stress response that does not reach the threshold for chronic, deleterious effects. In contrast, in the SCA2 LV mouse model, sustained expression of eIF2 α ^{S52D} over several weeks leads to the maintained formation of SGs, which are thought to contribute to cellular toxicity and disease progression.

The AAV-mediate expression GADD34 ^{Δ 322} in the LV SCA2 mouse model did not impact the loss of the DARPP-32 marker staining nor the number of ataxin-2 aggregates. Based on previous studies, where downregulating GADD34 was linked to higher p-eIF2 α levels, uncontrolled SGs formation, and worsened ALS-associated phenotype, we hypothesized that promoting p-eIF2 α dephosphorylation would mitigate the deficits associated with mutant ataxin-2 in the striatum. Moreover, considering the role of GADD34 on p-eIF2 α

dephosphorylation, we would expect an opposite impact compared with the expression of eIF2 α^{S52D} (Kim et al., 2014; Ruggieri et al., 2012). All considered, it is possible that the lack of phenotypic changes following AAV-mediated expression of GADD34 Δ^{322} likely reflect an already established ISR overactivation, present in the LV SCA2 model, which GADD34 Δ^{322} is no longer able to counteract. Although GADD34 plays a crucial role in dephosphorylating p-eIF2 α within the ISR pathway, the stress stimulus that trigger eIF2 α phosphorylation might exert a stronger signal, potentially fading GADD34 activity. The formation of SGs is triggered by the phosphorylation of eIF2 α , an event mediated by one of four distinct stress kinases that respond to various stress stimuli (Harding et al., 2003; Kedersha et al., 2000, 1999; Marcelo et al., 2021b; McEwen et al., 2005; Srivastava et al., 1998; Wek et al., 1995). In SCA2, where multiple cellular and molecular mechanisms are disrupted and may contribute to several kinds of stress stimuli, the simultaneous activation of the four stress kinases (due to the detection of multiple stressors) may further amplify eIF2 α phosphorylation, thereby diminishing the efficacy of GADD34 activity (Conceição & Costa et al., 2024; Estevam et al., 2023).

Our final aim was to understand the impact of expressing eIF2 α^{S52D} and GADD34 Δ^{322} in a more disease-relevant model. However, SCA2 mouse models that exhibit phenotypic translatable traits are currently limited. Additionally, some of the models presently developed present late disease onsets that hinder their use in a laboratory context (Alves-Cruzeiro et al., 2016). This issue presents a significant challenge in advancing our understanding of SCA2, as disease research requires models that not only replicate the clinical context but also present disease-associated traits within a timeframe suitable for experimental investigations. Taking all into consideration, we aimed to develop a model that has a disease onset within a convenient research timeframe; a model that displays disease-associated traits, such as motor impairments and neuropathology; and a model that is focused on the expression of an expanded ataxin-2 in the cerebellar Purkinje cells. To achieve that goal, we developed a new transgenic mouse model of SCA2 expressing a human form of ataxin-2 with 129 glutamines. This polyglutamine repeat length not only exceeds the disease-associated threshold (>35 glutamines) but also represents the highest number of repeats reported in a mouse model to date (Alves-Cruzeiro et al., 2016). Thus, given the positive correlation between polyglutamine

expansion length and disease severity, where longer expansions are associated with earlier onset and more pronounced motor symptoms, we expected this model would exhibit a more robust and easily detectable phenotype (Conceição & Costa et al., 2024; Giunti, 1998; Hayes, 2000; Klockgether et al., 2019; Lieberman et al., 2019; Pulst et al., 2005).

To characterize the novel SCA2 mouse model, we used four different lines: Line 36, Line 40, Line 41, and Line 53. In this work, we characterized Lines 36 and 40, while our lab has previously characterized the other two lines (Koppenol, 2024). Regarding Line 36, even though we could detect the transgene integration within mice's genome, we were not able to observe, by the methodology used, any trait that could be translated to the human context of SCA2. Our transgenic mice from Line 36 did not display motor impairments or neuropathology that could be associated with SCA2. Mice from Line 40 started to manifest motor impairments at 4 weeks of age on the grip strength test and at 8 weeks of age on the rotarod test. Additionally, mice from Line 40, at 44 weeks of age showed a loss of cerebellar Purkinje cells. However, the number of pups generated by mice started to decline rapidly over the generations.

The fact that Line 36 did not exhibit any SCA2-associated phenotype may be related to the integration site of the transgene. Although the PCR could detect the human *ATXN2* transgene during genotyping, it is possible that it was inserted into a transcriptionally inactive genomic region, thereby reducing or even silencing its expression. Thus, compromising the manifestation of a phenotype associated with SCA2. Additionally, the copy number integration, when low, can also affect the levels of transgene expression (Day et al., 2000; Kooter, 1999; Matzke and Matzke, 1998). The phenotypic traits of the animals from the transgenic Line 40 are in line with the observations previously described by our lab for Lines 41 and 53 (Koppenol, 2024).

However, Line 40 displayed the first motor impairments at 4 weeks of age, while the onset of Lines characterized by Koppenol was at 12 weeks of age (Koppenol, 2024). Although Line 40 seems to exhibit a more pronounced phenotype, compared to other Lines, the number of pups born progressively declined over time, making it difficult to breed them. As a result, this line became unviable to use in the context of research.

A limitation of the novel SCA2 mouse model characterization is that we have not yet been able to determine which animals, produced through heterozygous crossing, are

homozygous or heterozygous. Since we are characterizing the entire group of mice, it is possible that alterations might be masked or underestimated due to the variability introduced by a population with mixed zygosity. Having mice separated by heterozygous and homozygous groups would refine our characterization both at the behavioral level and neuropathological level.

Due to the low fertility observed in Line 40, we chose to use the SCA2 transgenic mouse Line 41, which was previously characterized by Koppenol, to test the expression of eIF2 α ^{S52D} and GADD34 ^{Δ 322}. However, we did a further motor characterization of this line by analyzing the kinetic motion of transgenic mice, using the MotoRater system while the mice performed the walking, beam, and swimming tests. This system enables the characterization of movement by automatically detecting anatomic points of interest to draw a kinetic profile throughout the performance of the tests. It is a powerful tool with high sensitivity that enables a fine characterization of mice's movement, allowing objective and precise phenotyping. We analyzed over 35 kinetic parameters, but due to the large volume of data generated, we decided to only present the measurements that were statistically significant or reached a near statistical significance

Using the MotoRater system, we were able to detect phenotypic differences between WT animals and SCA2 transgenic mice from Line 41 while performing the walking, beam walking, and swimming tests. These differences were observed as early as 5 weeks of age, whereas Koppenol only detected behavioral differences at 12 weeks of age, using traditional behavior tests. This further supports the sensitivity and power of the MotoRater system in detecting fine phenotypic alterations in rodent models of diseases associated with movement.

While some kinetic parameters are difficult to translate into a human context, some alterations detected in the MotoRater have already been described by other authors as characteristic traits of mouse models of ataxia (Nascimento-Ferreira et al., 2013; Nóbrega et al., 2013; Santana et al., 2020). We showed that in the walking test, transgenic mice exhibited an increased front step width as well as an increased hind step width. Previous studies using the footprint test—which, to some extent, is similar to the MotoRater walking test—have also

reported increased step width in ataxia mouse models, consistent with our observations (Nascimento-Ferreira et al., 2013; Nóbrega et al., 2013; Santana et al., 2020). Thus, the MotoRater is a powerful tool that detects fine movement alterations and, at the same time, it generates data that aligns with well-established conventional behavioral tests.

In the last part of this work, using the novel transgenic mouse model developed by our lab and further characterized here, we evaluated the impact of *in vivo* AAV-mediated expression of eIF2 α^{S52D} and GADD34 Δ^{322} . To do that, we injected transgenic mice directly into the cerebellum with the strategies to either induce or inhibit the formation of SGs. Overall, we observed a strong impact of AAV-mediated expression of eIF2 α^{S52D} in exacerbating the pathological phenotype. Transgenic mice expressing eIF2 α^{S52D} showed a reduced latency to fall from the accelerated rotarod and, at specific time points, a reduced grip strength. These observations strongly suggest that eIF2 α^{S52D} – which promotes the formation of SGs – exacerbates the motor deficits of the transgenic mouse model. We could not detect any phenotypic alteration in transgenic mice expressing GADD34 Δ^{322} using conventional behavioral tests.

Using the MotoRater system, we were able to verify that the strategy to induce SGs (eIF2 α^{S52D}) and the strategy to prevent their assembly (GADD34 Δ^{322}) were able to modify the natural course of the disease by altering the characteristic kinetic profile of the SCA2 transgenic mice. To infer whether some kinetic parameters improved or worsened, we looked at the direction of the change of the measurement when the kinetic phenotype of hATXN2^{MUT(129Q)} mice was characterized by comparing it with wild-type (WT) animals. To infer whether certain kinetic parameters had worsened or improved, we examined in which direction the measurement changed (increased or reduced) when the kinetic profile of hATXN2^{MUT(129Q)} mice vs WT animals was determined. For instance, in the walking test, both the step width and step length of transgenic mice expressing eIF2 α^{S52D} followed the same pattern of change as hATXN2^{MUT(129Q)} mice, suggesting a phenotype worsening. In the beam walking test, regarding the iliac crest height and hip height, mice expressing eIF2 α^{S52D} (at 5 weeks of age) also followed the same pattern of change as hATXN2^{MUT(129Q)} mice, again suggesting a phenotype worsening. On the swimming test at 9 weeks of age, transgenic mice exhibited a decreased iliac crest height, following the same pattern of change as hATXN2^{MUT(129Q)} mice. Furthermore, transgenic mice expression eIF2 α^{S52D} took more time to

traverse the beam, a clear evidence of phenotype worsening. Moreover, several other kinetic parameters appeared altered, which were not previously changed when we first did the hATXN2^{MUT(129Q)} mice vs WT movement characterization. Thus, by integrating the previous results, the data suggest that eIF2 α ^{S52D} expression induces defects in the kinetic profile of transgenic mice.

Furthermore, the expression of GADD34 ^{Δ 322} in the transgenic mouse model seems to follow the same pattern of change as hATXN2^{MUT(129Q)} mice as the expression of eIF2 α ^{S52D} in many kinetic parameters. Thus, our data seem to suggest that GADD34 ^{Δ 322} might as well negatively impact the kinetic profile of transgenic mice. For instance, on the walking test, both eIF2 α ^{S52D} and GADD34 ^{Δ 322} expression reduced the ankle angle on the mice's left side. In the beam walking test, the forepaw clearance of transgenic mice expressing GADD34 ^{Δ 322} and eIF2 α ^{S52D} also displayed the same increasing trend. Moreover, both the ankle and knee angles of transgenic mice expressing eIF2 α ^{S52D} or GADD34 ^{Δ 322} follow the same reduction trend. All considered, AAV-mediated expression of GADD34 ^{Δ 322} might as well have a deleterious effect on the SCA2 pathology.

From a neuropathological point of view, our histopathological analysis revealed a marked loss of Purkinje cells in transgenic mice expressing eIF2 α ^{S52D}. This was accompanied by a reduction in the thickness of the molecular and/or granular layers in cerebellar lobules III, IV/V, VII, VIII, IX, and X. Notably, only lobules I/II and VI did not exhibit alterations in the thickness of these layers. This further supports the results observed in behavior and the hypothesis that inducing the formation of SGs by expressing eIF2 α ^{S52D}, exacerbates the SCA2 pathology.

Although GADD34 ^{Δ 322} expression in transgenic mice does not appear to affect the number of cerebellar Purkinje cells, a reduction in the thickness of the molecular and/or granular layers is observed in lobules IV/V and IX. Despite the absence of a detectable motor phenotype in the conventional rotarod test, GADD34 ^{Δ 322} expression seems to slightly compromise the cerebellar structure. This is an interesting observation, as Koppenol, when characterizing this model, similarly reported a reduced thickness in lobules IV/V and IX at 4 weeks of age – prior to the presence of any detectable motor deficits (Koppenol, 2024). This is aligned with the observations that SCA2 patients, at near symptomatic stages, already exhibited atrophy of lobules V and VI (Jacobi et al., 2013). In this case, a similar parallel can be

drawn, in which GADD34^{Δ322} further impairs the cerebellar structure of the transgenic mice, but at a level, not yet detected at the behavioral levels. Altogether, these results suggest that GADD34^{Δ322} may exacerbate the cerebellar phenotype associated with SCA2, but its expression levels or the duration of expression may not yet be sufficient to elicit more pronounced motor deficits, a similar state as observed in near symptomatic patients (Jacobi et al., 2013).

Finally, we performed a transcriptomic analysis to assess the impact of eIF2α^{S52D} or GADD34^{Δ322} expression on the global gene expression profile of transgenic mice. For that, we used cerebella injected with eIF2α^{S52D} or GADD34^{Δ322}. The expression of eIF2α^{S52D} resulted in a distinct transcriptomic signature comparing to mice expressing the control. Similar results were also observed in transgenic mice expressing GADD34^{Δ322}, with samples displaying distinct expression profiles compared with control animals.

Gene ontology analysis reveals that mice expressing eIF2α^{S52D} and GADD34^{Δ322} exhibited several altered genes related to lipid metabolism compared to controls. It was previously reported that the ATXN2 KO mouse model (ATXN2-KO-2008, previously mentioned in the Introduction) displays changes in the expression of genes involved in brain lipid metabolism (Lastres-Becker et al., 2008). Since ATXN2-KO-2008 lacks the expression of ataxin-2, our observation could reflect a shared pathway involving ataxin-2 dysfunction, where manipulating SGs exacerbates a loss of function caused by the abnormal polyQ expansions within the ataxin-2 protein. Moreover, some neuronal-related genes were also differentially expressed in transgenic mice expressing eIF2α^{S52D}, compared to controls.

In both transgenic mouse models expressing eIF2α^{S52D} and GADD34^{Δ322}, most genes associated with the stress response were downregulated. This observation may reflect a compensatory mechanism aimed at limiting excessive stress signaling under conditions of chronic stress, possibly by attenuating the activation of stress response pathways.

Moreover, of the 152 differently expressed transcripts in mice expressing eIF2α^{S52D}, 110 encoded for proteins, of which approximately 9% were related to neuronal functions, all of them upregulated. Moreover, gene ontology analysis from transgenic mice samples expressing eIF2α^{S52D}, revealed expression alterations on transcripts related to monoatomic ion channel activity, voltage-gated monoatomic cation channel activity, ligand-gated

monoatomic ion channel activity, sodium:chloride symporter activity and potassium channel activity. These terms suggest a connection to neuronal activity through ion transport and channel functions, which are fundamental for signal transmission. Alterations in the expression of genes related to neuronal functions can profoundly impact signal transmission, potentially leading to impaired synaptic plasticity, disrupted neuronal communication, and ultimately contributing to neurodegenerative processes and motor impairments (Alexander et al., 2017; Lai and Jan, 2006; Tipton and Russek, 2022; Urrutia et al., 2024; Zhang et al., 2018).

Considering all the results obtained in this work, we propose a model in which a temporally limited induction of SGs exerts a protective effect against the toxic properties of mutant ataxin-2, as observed in *in vitro* experiments. However, when the induction of SGs is maintained for extended periods, SGs themselves become toxic, exacerbating the phenotype associated with SCA2 (*in vivo experiments*). Furthermore, considering that GADD34 – which inhibits the formation of SGs – might as well present discrete toxic properties, we hypothesize that SGs assembly and disassembly dynamics need to be tightly regulated. Excessive or insufficient SGs formation might lead to detrimental effects, as an optimal balance of SGs dynamics is crucial for maintaining cellular homeostasis and preserving neuronal function. A better understanding of the mechanisms that govern ISR and SGs dynamics in the context of neurodegenerative diseases might uncover key insights into novel pathways driving SCA2 pathogenesis. Gaining insight into new disease-associated mechanisms creates the opportunity to discover novel therapeutic targets, ultimately opening the way to developing innovative and curative treatments for SCA2.

Chapter 6 – Conclusions and future perspectives

Conclusions and future perspectives

PolyQ diseases are a group of nine dominantly inherited neurodegenerative disorders caused by an abnormal expansion of the trinucleotide CAG within the coding regions of the respective disease-associated gene. The gene product of the CAG expansion is the translation of a protein with an abnormally long tract of glutamines (Conceição & Costa et al., 2024; Estevam et al., 2023). These proteins, with abnormal polyQ expansions, are known to gain toxic functions that dysregulate cellular mechanisms, ultimately leading to cell death and neurodegeneration (Adegbuyiro et al., 2017; Conceição & Costa et al., 2024; Estevam et al., 2023; Fan et al., 2014).

One of the nine polyglutamine diseases is SCA2, which is caused by a CAG expansion within the first exon of the *ATXN2* gene. SCA2 is characterized by several motor dysfunctions, among which the most common are the ataxia and slow eye movements (slow saccades). The motor performance of people affected by SCA2 progressively declines until patients experience severe disabilities and die prematurely (Conceição & Costa et al., 2024; Klockgether et al., 2019). Unfortunately, to this date, there is no therapy or cure for SCA2, and affected people rely only on symptomatic and supportive treatments, which are unable to change the natural course of the disease (Conceição & Costa et al., 2024). This represents an unmet medical need with a great impact not only on patients but also on their families, underscoring the necessity to continue the research to better understand SCA2.

It is well established that a mutant *ATXN2* gene, with an expanded CAG sequence within the first exon 1, underlies the etiology of SCA2 (Imbert et al., 1996; Pulst et al., 1996; Sanpei et al., 1996). The mutant ataxin-2 protein, with an abnormally long tract of glutamines, has been associated with the disruption of several cellular mechanisms, including abnormal cellular homeostasis, disrupted proteostasis, disrupted calcium signaling, enhanced oxidative stress, metabolic dysfunctions, and autophagy impairments. All these disrupted mechanisms ultimately culminate in the selective neurodegeneration of the cerebellum and, in less extended, other brain regions (Conceição & Costa et al., 2024; Estevam et al., 2023; Klockgether et al., 2019; Laffita-Mesa et al., 2021).

To cope with situations of cellular stress, such as enhanced oxidative stress, ER stress, or even the accumulation of unfolded proteins, cells rely on a mechanism called ISR. Under stress conditions, the ISR alters the cellular translation program to enhance efficiency and help cells cope with stressors (Pakos-Zebrucka et al., 2016; Riggs et al., 2020). The ISR starts with the phosphorylation of eIF2 α upon the detection of cellular stress by cells. The phosphorylation of eIF2 α results in the global translation arrest and in the formation of SGs, which are membraneless organelles mainly composed of untranslated mRNAs and RBPs (Kedersha et al., 2002, 1999). The proposed function of SGs is to sequester mRNAs that are not immediately required to cope with cellular stress. This allows cells to efficiently translate mRNAs that are crucial both for tackling the stressor and to promote cell survival (Buchan and Parker, 2009; Marcelo et al., 2021b; Reineke and Neilson, 2019). Ultimately, when the stress is no longer present, p-eIF2 α is dephosphorylated by PP1, which requires the scaffolding action of GADD34. A negative feedback loop regulates SGs assembly-disassembly, as the accumulation of p-eIF2 α leads to the upregulation of GADD43 (Choy et al., 2015). In a simplistic point of view, the first step of SGs assembly is the eIF2 α phosphorylation and terminates with its dephosphorylation aided by GADD43.

The formation of SGs emerges as a key mechanism that helps cells cope with and survive conditions of cellular stress. However, the precise mechanism through which SGs exert their functions remains unclear. It is also hypothesized that SGs have toxic functions as their persistent formation in conditions of chronic stress is associated with deleterious effects (Reineke and Neilson, 2019). Additionally, SGs have been suggested to exacerbate the pathology associated with several neurodegenerative disorders (Feng et al., 2014; Koromilas, 2015; Smith and Mallucci, 2016). Notably, several proteins associated with neurodegenerative disorders have been identified as components of SGs (Wolozin and Ivanov, 2019). Moreover, in the particular case of polyQ disorders, components of SGs were found in aggregates of polyQ proteins, strengthening the connection between SGs and these neurodegenerative diseases (Marcelo et al., 2021b; Wolozin and Ivanov, 2019). However, whether this connection establishes a deleterious or a beneficial effect on cells is unknown.

Taking all together, the main goal of this work was to understand how the assembly and disassembly of SGs impact SCA2 pathology.

We started our study by developing genetic strategies to manipulate the assembly and disassembly of SGs, which are directly involved in the ISR. To induce the formation of SGs, we developed a construct to express a phosphomimetic form of the eIF2 α . Thus, in the eIF2 α nucleotide sequence, the triplet that codes for the serine 52 was substituted by a triplet that codes for an aspartate – eIF2 α^{S52D} . We then transfected cells with the construct expressing the eIF2 α^{S52D} and saw that nearly 30% of cells that were not submitted to exogenous cellular stress were able to form SGs.

To prevent the formation of stress granules, we developed a construct to express a truncated form of GADD34 lacking the first 322 amino acids – GADD34 Δ^{322} . Previous studies showed that this truncated form increases the binding to PP1 and dephosphorylation of p-eIF2 α (Choy et al., 2015). We observed that expressing GADD34 Δ^{322} prevented the formation of SGs in transfected N2a cells treated with sodium arsenite – a stress stimulus that induces the formation of SGs virtually in all cells treated.

Overall, we successfully developed two strategies to modulate SGs dynamics. These strategies act in opposite directions within the ISR pathway, with eIF2 α^{S52D} functioning at the onset of the stress response and GADD34 Δ^{322} acting during its resolution.

Our next approach aimed to understand the impact of SGs in the context of the mutant ataxin-2 expression. To study this, we used primary cultures of mouse striatal neurons transfected either with a non-expanded form of ataxin-2 or an expanded form of ataxin-2 with 80 glutamines. Additionally, cells were either transfected with eIF2 α^{S52D} or GADD34 Δ^{322} . First, we observed that the expression of a mutant form of ataxin-2 in primary cultures of mouse striatal neurons reduced the total dendritic length when compared with cells transfected with the non-expanded form. Additionally, the expression of eIF2 α^{S52D} reverted the effects on dendritic length associated with the expression of the mutant ataxin-2, suggesting that SGs formation had a protective effect. We did not detect alterations regarding the dendritic length or complexity when co-expressing GADD34 Δ^{322} with the non-expanded or expanded ataxin-2.

Overall, the results show that SGs induction, in the context of mutant ataxin-2 expression, has a protective effect by reverting the reduction in total dendritic length to values like those observed in neurons expressing non-expanded ataxin-2.

Next, to understand the impact of manipulating SG dynamics in a more disease-relevant context, we used an *in vivo* approach, resorting to a SCA2 lentiviral mouse model. This model was previously developed and characterized by our group (Marcelo et al., 2021a). To generate the SCA2 lentiviral mouse model, mice are stereotactically injected into the striatum with LV particles encoding for the mutant form of ataxin-2. To understand the impact of manipulating SGs, in this context, animals were either stereotactically co-injected into the striatum with LV encoding for mutant ataxin-2 and AAV encoding for eIF2 α ^{S52D} or GADD34 ^{Δ 322}.

The AAV-mediated expression of eIF2 α ^{S52D} led to a more pronounced loss of DARPP-32 marker staining – dopaminergic neurons marker – when compared with mice expressing the control. We also counted the number of ataxin-2 aggregates but no differences were observed.

We assessed the impact of expressing GADD34 ^{Δ 322} in the context of the SCA2 LV mice, but we could not detect differences either in the loss of the DARPP-32 marker staining nor in the number of aggregates formed.

Altogether, in the context of the SCA LV model, our results show that expression of eIF2 α ^{S52D} exacerbates SCA2-associated deficits.

Given the lack of available SCA2 transgenic mouse models for research purposes, we developed a novel SCA2 mouse model. This model expressed a human form of the mutant ataxin-2 with 129 glutamines. The expression of the transgene was directed to the Purkinje cells of the cerebellum – the most affected brain region in SCA2 (Conceição & Costa et al., 2024). The transgenic Lines 36 in this work, although expressing a mutant form of ataxin-2

with a polyglutamine tract far beyond the disease threshold, did not exhibit a motor phenotype, as assessed by behavioral test that could be associated with SCA2. Nevertheless, Line 40 exhibited motor impairments and seemed to display a loss of cerebellar Purkinje cells. However, the decline of pups that were born overtime hindered its use in the research context.

Our lab previously characterized another transgenic line (Line 41), which exhibited both motor phenotype and neurodegeneration features. In this work, we further characterized this line to study the kinetic profile of mice's movement while performing different behavioral tests. To do that, we used the MotoRater system, which enabled us to track the mice's movement and draw a kinetic profile. Using this system, we profiled the movement of mice while performing the walking test, beam walking test, and swimming test. We were able to detect alterations in the kinetic profile of transgenic mice from Line 41 on the walking, beam a swimming test, when compared with WT animals.

Altogether, our lab developed a novel transgenic mouse model of SCA2, with motor phenotype and neuronal loss, which was further validated and characterized in this work.

Finally, we studied the impact of modulating SGs dynamics in the context of the novel SCA2 transgenic mouse model. To study the impact of modulating SGs, transgenic mice were injected into the cerebellum either with AAV particles encoding for eIF2 α ^{S52D} or AAV particles encoding for GADD34^{A322}. Using the traditional motor behavior tests, we observed that AAV-mediated expression of eIF2 α ^{S52D} worsens the phenotype of transgenic mice in the rotarod test. Additionally, transgenic mice expressing eIF2 α ^{S52D} also showed less grip strength compared with control transgenic mice. No alteration in the mice's weight was observed. The AAV-mediated expression of GADD34^{A322} did not affect mice's performance in the rotarod or the grip strength test. Similarly to mice expressing eIF2 α ^{S52D}, AAV-mediated expression of GADD34^{A322} did not affect the mice's weight. Using the MotoRater system, we were able to observe that both AAV-mediated expression of eIF2 α ^{S52D} and AAV-mediated expression of GADD34^{A322}, altered the kinetic profile of transgenic mice compared with control transgenic mice.

Neuropathologically, the AAV-mediated expression of eIF2 α^{S52D} led to the loss of Purkinje cells from the cerebellum compared with control animals. However, AAV-mediated expression of GADD34 Δ^{322} did not alter the number of Purkinje cells. Moreover, we observed a reduction in the thickness of both the molecular and granular layers in many lobules of the cerebellum of mice expressing eIF2 α^{S52D} . GADD34 Δ^{322} also appears to affect these cerebellar layers, with some lobules showing reduced thickness of both the molecular and granular layers.

Altogether, the AAV-mediated expression of eIF2 α^{S52D} seems to have a clear impact on SCA2-associated pathology, by worsening the phenotype of transgenic mice. This highlights that promoting SGs assembly exacerbates the SCA2-associated phenotype. The impact of AAV-mediated expression of GADD34 Δ^{322} on the phenotype of transgenic mice was not as clear as on mice expressing eIF2 α^{S52D} , and we only detected alterations in the kinetic profile of mice and in the layers of some cerebellar lobules.

Our final set of experiments aimed to analyze the transcriptomic profiles of transgenic mice expressing the strategies to either induce or inhibit SGs formation. Compared to transgenic mice expressing a control, both AAV-mediated expression of eIF2 α^{S52D} and GADD34 Δ^{322} led to distinct gene expression signatures. The expression of eIF2 α^{S52D} in transgenic mice affected the expression of genes involved in lipid metabolism, immune responses, and neuronal activity. Additionally, the expression of GADD34 Δ^{322} in transgenic mice also influenced genes related to lipid metabolism but primarily impacted those associated with the immune system.

Taken together, these findings indicate that modulating SGs in the context of SCA2 impacts the progression of the disease. Moreover, SGs formation appears to have a clear effect in exacerbating the pathology associated with SCA2 as seen by the AAV-mediated expression of eIF2 α^{S52D} in different mouse models.

Despite the insights gained in this study regarding the impact of SGs in the context of SCA2, several questions remain unanswered, and further studies are needed to better

understand how these structures influence SCA2 pathology. For instance, SGs induction seems to have a dual role *in vitro* and *in vivo*. While inducing SGs *in vitro* seems to be protective, *in vivo* SGs induction appears to exacerbate pathology. This difference raises important questions regarding the context-dependent functions of SGs. It highlights the need to investigate whether their role depends on the cellular environment, disease stage, or duration of stress exposure. Furthermore, investigating the molecular mechanisms behind SGs assembly and disassembly dynamics and their direct interaction with mutant ataxin-2 could reveal insights into their precise role in disease pathology.

Additionally, it remains to be determined whether the timing of SGs modulation differently affects the progression of the disease. A study in which SGs modulation is conducted at various stages of the disease may result in different outcomes, revealing therapeutic windows where inducing or inhibiting the formation of SGs could be beneficial. We cannot also exclude that the outcomes observed could be dependent on a dosage effect, where different expression levels of the SGs-modulating strategies may have a different impact. Future experiments should consider varying the dose and timing of vector administration.

Since we could not identify a clear impact of GADD34^{Δ322} expression regarding the phenotype associated with SCA2, an alternative approach to inhibit SGs formation should clarify the impact of preventing the assembly of these structures. Finally, since chronic SGs induction through the expression of the eIF2α phosphomimetic seems to exacerbate the phenotype associated with SCA2, strategies that directly modulate the expression or phosphorylation of eIF2α could be explored as potential therapeutic approaches for SCA2.

Appendix

Brain Journal cover image by André Conceição



Cover image from brain journal: The Helix and the Cerebellum: a journey into spinocerebellar ataxias, **by André Conceição**. Based on the article 'The stress granule protein G3BP1 alleviates spinocerebellar ataxia-associated deficits'. Pp. 2346–63.

REVIEW ARTICLE

OPEN



The polyglutamine protein ATXN2: from its molecular functions to its involvement in disease

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A CAG repeat sequence in the *ATXN2* gene encodes a polyglutamine (polyQ) tract within the ataxin-2 (ATXN2) protein, showcasing a complex landscape of functions that have been progressively unveiled over recent decades. Despite significant progresses in the field, a comprehensive overview of the mechanisms governed by ATXN2 remains elusive. This multifaceted protein emerges as a key player in RNA metabolism, stress granules dynamics, endocytosis, calcium signaling, and the regulation of the circadian rhythm. The CAG overexpansion within the *ATXN2* gene produces a protein with an extended poly(Q) tract, inducing consequential alterations in conformational dynamics which confer a toxic gain and/or partial loss of function. Although overexpanded ATXN2 is predominantly linked to spinocerebellar ataxia type 2 (SCA2), intermediate expansions are also implicated in amyotrophic lateral sclerosis (ALS) and parkinsonism. While the molecular intricacies await full elucidation, SCA2 presents ATXN2-associated pathological features, encompassing autophagy impairment, RNA-mediated toxicity, heightened oxidative stress, and disruption of calcium homeostasis. Presently, SCA2 remains incurable, with patients reliant on symptomatic and supportive treatments. In the pursuit of therapeutic solutions, various studies have explored avenues ranging from pharmacological drugs to advanced therapies, including cell or gene-based approaches. These endeavours aim to address the root causes or counteract distinct pathological features of SCA2. This review is intended to provide an updated compendium of ATXN2 functions, delineate the associated pathological mechanisms, and present current perspectives on the development of innovative therapeutic strategies.

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FACTS

- ATXN2 is a highly abundant protein in the nervous system that is important for the maintenance of neuronal homeostasis by participating in RNA metabolism and translation, stress response, and calcium regulation.
- The *ATXN2* gene is associated with certain neurodegenerative diseases and conditions, including spinocerebellar ataxia type 2 (SCA2), amyotrophic lateral sclerosis (ALS), parkinsonism, and Machado-Joseph disease (MJD/SCA3).
- Despite the ubiquitous expression of expanded ATXN2, neurodegeneration in SCA2 occurs in a region-selective manner.
- Neuronal inclusions can be found in spared brain areas of SCA2 patients while neuronal death still occurs in their absence.
- Expanded ATXN2 is associated with SCA2 that is an incurable disease, thus patient care relies on symptomatic and supportive treatments.

OPEN QUESTIONS

- How is the *ATXN2* gene involved in different diseases despite the number of CAG repeats overlap among them?

- What mechanisms can explain the region-selective neurodegeneration in SCA2?
- What is the role of neuronal inclusions in these diseases? Are they harmful or cytoprotective?
- How do the various pathways and mechanisms intricately implicated in the pathogenesis of SCA2 contribute to both the onset and progression of the disease?

INTRODUCTION

Ataxin-2 (ATXN2) is a versatile protein with key roles in several molecular, cellular, and physiological functions. For example, it binds RNA but also plays a crucial role in regulating the overall body metabolism [1–4] and the circadian rhythm [5–7]. On the other hand, the malfunctioning of ATXN2 has been associated with many diseases, especially spinocerebellar ataxia type 2 (SCA2) [8–10] and amyotrophic lateral sclerosis (ALS), but also parkinsonism, SCA1, SCA3, obesity, and diabetes [3, 11–19].

ATXN2 is a polyglutamine (polyQ) protein that typically contains a 22-glutamine tract [20]. Nevertheless, genetic instability can originate mutations that produce abnormally expanded polyQ tracts that alter the protein's conformational dynamics, which

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The polyglutamine protein ATXN2: from its molecular functions to its involvement in disease

On the role of RNA binding proteins in polyglutamine diseases: from pathogenesis to therapeutics

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Polyglutamine (polyQ) diseases are a group of different neurodegenerative disorders characterized by an abnormal expansion of the trinucleotide cytosine-adenine-guanine (CAG) within coding regions of each disease-associated gene. The abnormal expansion translates into a protein bearing an abnormally long tract of glutamines. The expanded proteins are prone to aggregate, promote aberrant interaction with other proteins and mRNAs and contribute to cellular pathway disruption (Matos et al., 2019). To date, nine different polyQ diseases are described, including among others, Huntington's disease, and six different spinocerebellar ataxias (SCA). Patients affected by polyQ diseases, suffer a myriad of motor symptoms that include ataxia, dysphagia, tremors, dysarthria, and even dementia. Unfortunately, there is no cure nor treatment able to delay the disease and patients rely only on symptomatic and supportive treatments culminating in premature death (Takahashi et al., 2010).

SCA2 and SCA3 (also known as Machado-Joseph disease [MJD]) are two polyQ diseases characterized by selective neurodegeneration within specific brain regions that include the cerebellum and brain stem. SCA2 patients have an abnormal CAG expansion above 33 glutamines within the *ATXN2* gene, whereas SCA3/MJD patients harbor CAG expansions above 61 glutamines in the *ATXN3* gene (Klockgether et al., 2019; Matos et al., 2019).

The molecular mechanisms by which a protein bearing abnormally expanded polyQ tracts leads to neurodegeneration are still not understood. Nevertheless, several mechanisms and pathways are involved, including impaired autophagy, RNA toxicity, enhanced oxidative stress, aberrant mRNA processing, and disrupted cellular calcium release (Lieberman et al., 2019; Marcelo et al., 2021b). In recent years, several studies suggest that stress granules (SGs) might play an important role in neurodegenerative diseases and their disruption might contribute to the pathology (Wolozin and Ivanov, 2019; Marcelo et al., 2021a). SGs are dynamic structures formed in response to cellular stress, being mainly formed by messenger RNAs, RNA-binding proteins (RBPs), and protein translation machinery components (Marcelo et al., 2021a). Additionally, it is also widely accepted that disruption of RBPs and widespread RNA processing defects are critical determinants of neurodegenerative diseases (Nussbacher et al., 2019). In fact, we showed that the RBP ataxin-2

is downregulated in the context of SCA3/MJD, and restoring its levels ameliorates the disease phenotype, due to its interaction with poly(A)-binding protein, which is another RBP (Nóbrega et al., 2015). It is noteworthy to mention that both ataxin-2 and poly(A)-binding protein are proteins recruited to SGs in response to cellular stress and disruption of cellular stress responses might contribute to disease pathology (Nunes et al., 2019; Marcelo et al., 2021a).

In this line, we sought to understand how the SGs nucleator Ras GTPase-activating protein-binding protein 1 (G3BP1), which is an RBP could impact SCA2 and SCA3/MJD disease phenotype. In cellular models, we showed that G3BP1 expression was able to reduce the levels and the number of aggregates of expanded ataxin-2 and ataxin-3 proteins (Koppenol et al., 2022). However, this mechanism seems to be independent of SGs assembly. To elucidate the mechanism of action of G3BP1 we focused on two important domains, an RNA-recognition domain that bind RNA and a nuclear transport factor 2-like domain, responsible to import proteins through the nuclear pore (Winslow et al., 2013). Using truncated forms of G3BP1 lacking specifically each of these domains, we found that nuclear transport factor 2-like domain seems important to the action of G3BP1 in the reduction of polyQ proteins levels and aggregates. We further explored the role of serine 149 phosphorylation site, described to have an important functional role in G3BP1 functions (Tourrière et al., 2003). Using a phospho-dead and a phosphomimetic form of G3BP1 at this site, we found that it plays a key role in the modulation of polyQ proteins levels and aggregation.

To evaluate the functional relevance of G3BP1 in SCA2 and SCA3/MJD molecular pathogenesis, we accessed the levels of G3BP1 mRNA and protein in SCA2 and SCA3/MJD patients and models. Importantly, we found that G3BP1 levels are reduced in the patient's fibroblasts and in a SCA3/MJD transgenic mouse model. Additionally, we observed that reducing endogenous G3BP1 levels in lentiviral mouse models of SCA2 and SCA3/MJD increases the number of ataxin-2 and ataxin-3 aggregates, respectively (Koppenol et al., 2022). Altogether, these results suggest that G3BP1 levels are reduced in SCA2 and SCA3/MJD and this contributes to disease pathogenesis.

Next, we evaluated the impact of G3BP1 levels reestablishment in a lentiviral mouse model of SCA2 (Marcelo et al., 2021b) and SCA3/MJD (Alves et al., 2008). We found that lentiviral-mediated

G3BP1 expression mitigates mutant ataxin-2 and ataxin-3 disease-associated neuropathological abnormalities, reducing the number of aggregates and preserving the loss of the neuronal marker DARPP-32. Furthermore, we observed that lentiviral-mediated G3BP1 expression did not produce neuronal loss or neuroinflammation.

Aiming to translate these results to a more clinically relevant model, we investigated the impact of G3BP1 levels reestablishment in a symptomatic transgenic mouse model of SCA3/MJD, which is characterized by severe motor deficits and marked Purkinje cell loss (Torashima et al., 2008). G3BP1 expression in the cerebellum mediated by lentiviral vectors led to a significant improvement in motor performance and to a significant reduction of aggregates and preservation of Purkinje cells, compared to control animals injected with green fluorescent protein.

PolyQ diseases enclose sets of disrupted cellular and molecular mechanisms caused by abnormal glutamine expansions in 9 different proteins, all leading to neuronal death and selective degeneration within specific brain regions. Our latest study shows, using *in vitro* and *in vivo* disease models for SCA2 and SCA3/MJD that the expression of G3BP1, an RBP, can ameliorate neuropathologic and motor features (Figure 1). The shared pathologic features of polyQ diseases, based on the same type of genetic mutation allow one to envision common therapeutic strategies to counteract the progressive pathologic cascade. In this line, our study suggests that viral delivery of G3BP1 might constitute a viable therapeutic target for SCA2 and SCA3/MJD, and even for the other PolyQ diseases.

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On the role of RNA binding proteins in polyglutamine diseases: from pathogenesis to therapeutics



The stress granule protein G3BP1 alleviates spinocerebellar ataxia-associated deficits

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Polyglutamine diseases are a group of neurodegenerative disorders caused by an abnormal expansion of CAG repeat tracts in the coding regions of nine, otherwise unrelated, genes. While the protein products of these genes are suggested to play diverse cellular roles, the pathogenic mutant proteins bearing an expanded polyglutamine sequence share a tendency to self-assemble, aggregate and engage in abnormal molecular interactions. Understanding the shared paths that link polyglutamine protein expansion to the nervous system dysfunction and the degeneration that takes place in these disorders is instrumental to the identification of targets for therapeutic intervention. Among polyglutamine diseases, spinocerebellar ataxias (SCAs) share many common aspects, including the fact that they involve dysfunction of the cerebellum, resulting in ataxia. Our work aimed at exploring a putative new therapeutic target for the two forms of SCA with higher worldwide prevalence, SCA type 2 (SCA2) and type 3 (SCA3), which are caused by expanded forms of ataxin-2 (ATXN2) and ataxin-3 (ATXN3), respectively. The pathophysiology of polyglutamine diseases has been described to involve an inability to properly respond to cell stress. We evaluated the ability of GTPase-activating protein-binding protein 1 (G3BP1), an RNA-binding protein involved in RNA metabolism regulation and stress responses, to counteract SCA2 and SCA3 pathology, using both *in vitro* and *in vivo* disease models. Our results indicate that G3BP1 overexpression in cell models leads to a reduction of ATXN2 and ATXN3 aggregation, associated with a decrease in protein expression. This protective effect of G3BP1 against polyglutamine protein aggregation was reinforced by the fact that silencing *G3bp1* in the mouse brain increases human expanded ATXN2 and ATXN3 aggregation. Moreover, a decrease of G3BP1 levels was detected in cells derived from patients with SCA2 and SCA3, suggesting that G3BP1 function is compromised in the context of these diseases. In lentiviral mouse models of SCA2 and SCA3, G3BP1 overexpression not only decreased protein aggregation but also contributed to the preservation of neuronal cells. Finally, in an SCA3 transgenic mouse model with a severe ataxic phenotype, G3BP1 lentiviral delivery to the cerebellum led to amelioration of several motor behavioural deficits. Overall, our results indicate that a decrease in G3BP1 levels may be a contributing factor to SCA2 and SCA3 pathophysiology, and that administration of this protein through viral vector-mediated delivery may constitute a putative approach to therapy for these diseases, and possibly other polyglutamine disorders.

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The stress granule protein G3BP1 alleviates spinocerebellar ataxia-associated deficits

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