The stress granule protein G3BP1 reduces the levels of different polyglutamine proteins and alleviates spinocerebellar ataxia associated deficits

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Abstract

Polyglutamine (polyQ) diseases are a group of 9 rare neurodegenerative disorders caused by an abnormal expansion of the CAG trinucleotide in the codifying regions of the respective disease-associated gene. The trinucleotide abnormal expansion leads to the translation of a protein containing an overexpanded tract of glutamines. PolyQ mutant proteins undergo a gain of toxic function disrupting normal cellular pathways leading to neuronal death and, consequently, leading to selective neurodegeneration of specific brain regions. Spinocerebellar ataxia (SCA) 2 and SCA3 (also known as Machado-Joseph disease) are two different polyQ diseases in which the ataxin-2 and ataxin-3 proteins, respectively, bear abnormally long polyQ tracts. Until now, there is no treatment for these fatal diseases or therapies that could delay the normal pathologic progression. Stress granules (SGs) are important structures formed in response to cellular stress, having an important role in mRNA triage. A core component of SGs is the RNA-binding protein (RBP) Ras GTPase-activating protein-binding protein 1 (G3BP1), which is also implicated in the SGs assembly. Furthermore, G3BP1 is known to have endoribonuclease activity and an important role in modulating RNA metabolism. In this study, we showed that G3BP1 is decreased in context of SCA2 and SCA3 disease. For that, we assessed whether restoring the expression levels of G3BP1 might positively impact the SCA2 and SCA3 pathology. We showed that gene delivery of G3BP1 in two distinct lentiviral mouse model of SCA2 and SCA3 was able to i) reduce the number of aggregates and ii) reduce the loss of neuronal marker associated with the mutant toxic proteins. Importantly, in a polyQ transgenic mouse model, lentiviral delivery of G3BP1 in the cerebellum was able to i) preserve the number of Purkinje cells, ii) reduce the number of HA-ataxin-3 and, importantly iii) improve the motor performance, balance and coordination. Additionally, we identify the nuclear transport nuclear transport factor 2-like (NTF2-like) domain and the ser149 phosphorylation site of G3BP1 as a key players in the reduction of mutant ataxin-2 and ataxin-3 levels and aggregation.

Altogether these results showed that gene delivery of G3BP1 is able of mitigating the disease-associated phenotype in SCA2 and SCA3 disease, in three different disease mouse models. Therefore, this study suggests G3BP1 as a novel therapeutic target for SCA2 and SCA3 diseases.

Introduction

Polyglutamine (PolyQ) diseases are a group of hereditary neurodegenerative diseases including Huntington's disease (HD), Spinal bulbar muscular atrophy (SBMA), Dentatorubral-pallidoluysian atrophy (DRPLA), and several spinocerebellar ataxias (SCA1, 2, 3, 6, 7, and 17). These diseases are characterized by abnormal expansions of the trinucleotide CAG in coding regions of each disease-associated gene, which encode for an expanded polyglutamine tract in the respective proteins. A central feature of these diseases is the aggregation of the mutant protein, which promotes aberrant interactions with other proteins and mRNAs, leading to the impairment of several cellular pathways and organelles¹. Nevertheless, the complete picture of the molecular events leading to selective neurodegeneration of specific brain region, is yet to be fully understood. Moreover, until now there are no therapies able to stop or delay the disease progression that culminates in the premature death of patients with PolyQ diseases.

SCA2 and SCA3 (or Machado-Joseph disease – MJD) are two of the most prevalent spinocerebellar ataxias, being both characterized by a neurodegenerative profile that mainly affects the cerebellum and the brain stem. SCA2 is caused by an abnormal mutation in the *ATXN2* gene above 31-33 CAG repeats, resulting in an overexpanded ataxin-2 protein². SCA3 is caused by an abnormal mutation above 44-45 CAG in *ATXN3* gene, causing the ataxin-3 protein to be abnormally expanded^{3,4}. Both mutant ataxin-2 and ataxin-3, are prone to aggregate and form large inclusions capable of sequestering other proteins. Though large inclusions are often reported as hallmarks of the disease, whether they are directly leading to toxicity is still matter of debate⁵⁻⁸.

The pathological aggregation of polyQ proteins and the abnormal interactions in which they engage can result in significant changes in the cellular stress response pathways^{9,10}. To cope with stress, cells display several mechanisms that promote survival, including the assembly of stress granules (SGs).

These are transiently formed *foci* that act in the triage and regulation of RNA during stress periods¹¹. Recently, SGs dysregulation has been suggested to underlie the pathogenesis of several diseases, including neurodegenerative disorders¹². SGs are dynamic structures that can have different compositions depending on the type of stress and type of cell, although RNA binding proteins (RBPs) are their main components. One of these components, which is also a core nucleator and marker of SGs is the GTPase-activating protein-binding protein 1 (G3BP1)^{13,14}. G3BP1 has an important role in both mRNA stabilization and degradation¹⁵, and also in splicing modulation¹⁶. Additionally, it was shown to display endonuclease activity¹⁵, being able to degrade specific mRNAs through a mechanism that is regulated by phosphorylation of its Ser-149 residue¹⁷. Structurally, G3BP1 has at least two important domains, an RNA-recognition domain (RRM) and a nuclear transport factor 2-like domain (NTF2-like). The former is crucial for G3BP1 ability to bind mRNAs, whereas the latter is involved in the nuclear import of proteins through the pore complex¹⁵. While all these domains and catalytic site are important for the G3BP1 functions, the role of each one of them in specific steps of the RNA metabolism is yet to be elucidated^{13,14}.

Therefore, in this work we aimed at studying the involvement of SGs and more specifically of its core component G3BP1 in the SCA2 and SCA3 pathogenesis. We found that SGs assembly through oxidative stress did not change the aggregation profile or levels of mutant ataxin-2 and ataxin-3. On the other hand, the expression of G3BP1 led to a significant reduction in the number of cells with aggregates and in the levels of mutant ataxin-2 and mutant ataxin-3 proteins. We found that G3BP1 levels are reduced in SCA2 and SCA3 patients' samples and in models of both diseases. The re-establishment of G3BP1 levels in a lentiviral mouse model of SCA2 and of SCA3 reduced neuropathological anomalies associated with the expression of mutant ataxin-2 or mutant ataxin-3, respectively. In the same line, G3BP1 expression was able to significantly reduce behaviour and neuropathological deficits in a transgenic mouse model. Finally, we characterize the molecular mechanism of G3BP1 action, implicating both the NTF2-like domain and the phosphorylation of Ser149. Altogether, the results of this work identify G3BP1 as a relevant target for SCA2 and SCA3, whose modulation could be studied in the future as a therapeutic strategy for both diseases.

Materials And Methods

Plasmid vectors

Plasmids encoding for human ataxin-3 contain 28 glutamines (pEGFP-C1-Ataxin3Q28; #22122; Addgene) or 84 glutamines (pEGFP-C1-Ataxin3Q84; #22123; Addgene) were a gift from Henry Paulson and both are fused with a GFP protein at the N-terminal¹⁸. Plasmids encoding for human ataxin-2 containing 22 glutamines (pEGFP-Ataxin2Q22) or 104 glutamines (pEGFP-Ataxin2Q104) were kindly provided by Prof. Stefan Pulst¹⁹. The LacZ gene was cloned in our laboratory under the control of a phosphoglycerate kinase promoter (PGK)²⁰, and the GFP construct was cloned as previously described²¹. The plasmid encoding for human G3BP1 (GeneBank accession: DQ893058.2) purchased from Source Bioscience, was cloned into a lentiviral vector backbone using the GatewayTM LR ClonaseTM II Enzyme Mix, Invitrogen, according to the manufacturer instructions. The G3BP1- Δ NTF2 (G3BP1 deleted at the site 11-133) and G3BP1- Δ RRM (G3BP1 deleted at the site 340-415) constructs were synthesized from GeneScript and cloned into the vector pcDNA3.1+N-MYC.

G3BP1 mutagenesis of serine149 residue

Sited-directed mutagenesis was performed using NZY Mutagenesis kit (NZYTech) according to manufacturer's indications. In the human variant of G3BP1 (GeneBank accession: DQ893058.2), a serine was changed by an alanine at the site 149 to generate a G3BP1 phospho-dead mutant (G3BP1_S149A). The pair of primers used to induce the substitution S149A were: 5'-CT GAG CCT CAG GAG GAG GCT GAA GAA GAA GTA GAG-3' and 5'-CT CTA CTT CTT CTT CAG CCT CCT CCT GAG GCT CAG - 3'. The mutation (S149A) was confirmed by DNA sequencing (Eurofins Genomics).

Neuroblastoma culture and transfection

Mouse neuroblastoma cell line (N2a cells) acquired from the American Type Culture Collection cell biology bank (CCL-131) were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% (v/v) foetal bovine serum (FBS), 100 U/mL penicillin and 100 μ g/mL streptomycin. Cells were seeded onto 12- or 6-multiwell plates. After 24h of growth, cells were transfected using polyethylenimine reagent (PEI; PEI MAX Polysciences, Inc.) following the manufacturer's instructions, with a concentration of 0.5-1 μ g of DNA per well. For SGs induction experiments, cells were treated with sodium arsenite (SA, Sigma Aldrich 10 μ g/mL) to a final concentration of 0.05 M, 1h before harvest.

Neuroblastoma culture with reduced levels of G3BP1

To create a cell line with reduced levels of G3bp1, N2a cells cultured in the same conditions described above were infected using the lentiviral vectors, encoding for CRISPR-Cas9 targeting G3bp1 mouse gene (custom CRISPR gRNA Plasmid DNA, LV05 vector, Sigma-Aldrich) and hexamethrine bromide (Sigma) at a concentration of 8 ng/ μ L. Cells were maintained in culture for 3 days post-infection and then were

selected by adding puromycin at a concentration of 10 µg/mL. To establish the cellular line, the cells undergone 4 cycles of stabilization/selection.

Cell viability assay

The cell viability under the different experimental conditions was assessed by alamar Blue assay. Prior to harvest, the cells were incubated for 40 min with Dulbecco's modified Eagle medium containing 10% (v/v) alamar Blue dye. Absorbance measurement and cellular viability was calculated as described previously²².

Human fibroblast culture

Human fibroblast cells from patients with spinocerebellar ataxia type 2 disease were kindly provided by Prof. Tetsuo Ashizawa. Human fibroblast cells from patients with spinocerebellar ataxia type 3 disease and control fibroblasts from healthy individuals were obtained from the Neurology and Pathology Services of University Hospital of Coimbra, Portugal. Fibroblast cells were kept in culture in Dulbecco's modified Eagle medium (DMEM), supplemented with 15% (v/v) foetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. All cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

Human brain tissue

Post-mortem striatum and cerebellum brain tissue from clinically and genetically confirmed SCA2 patients were obtained from the NIH NeuroBioBank (USA). Control striatum and cerebellum tissues from healthy individuals, without neurological conditions diagnosed were obtained from NIH NeuroBioBank (USA). Tissues preserved in 4% PFA solution, were dehydrated in a 30% sucrose/PBS for 48h, cryoprotected at -80°C degrees, dissected in 40µm slices using a cryostat (Cryostar NX50, ThermoFisher Scientific) and stored in free floating PBS/sodium azide solution at 4°C.

Animals

Adult C57BL/6 J wild-type animals, and transgenic polyQ mice²³, breed in in the animal facility of the Universidade do Algarve, were used. The animals were maintained in a temperature-controlled room on a 12h light-12 h dark cycle. Food and water were dispensed *ad libitum*. All experiments were carried out according to the European Community Council directive (86/609/EEC) for the care and use of laboratory animals. The researchers received certified training (FELASA course) and approval to perform the experiments from the Portuguese authorities (Direcção Geral de Alimentação e Veterinária) in the project Neuropath (421/2019).

Lentiviral vectors

The cDNA encoding for human G3BP1, GFP, Atx2MUT, and for Atx3MUT was cloned in a self-inactivating lentiviral vector under the control of PGK promoter, as described previously (Deglon et al., 2000). The

lentiviral vectors were produced in HEK (human embryonic kidney) 293T cells using a four-plasmid system described previously (de Almeida et al., 2002). The viral productions were quantified using a RetroTek HIV-1 p24 Antigen Enzyme-Linked Immunoabsorbent Assay (ELISA) (ZeptoMetrix), according to manufacturer's indications.

In vivo injection of lentiviral vectors

For the stereotaxic injection of lentiviral vectors, concentrated viral stocks were thawed on ice and homogenized. The animals were anesthetized through intraperitoneal injection (IP) of a mixture of ketamine (75mg/kg, Nimatek, Dechra) with medetomidine (0.75mg/kg, DOMTOR®, Esteve). For the SCA2 lentiviral mouse model, mice (10-12 weeks old) were injected with lentiviral particles encoding for human ATXN2MUT containing 82 glutamines or encoding for ATXN2MUT and G3BP1 at the left and right hemispheres of striatum, respectively, according to the following brain coordinates relative to bregma: Antero-Posterior (+0.6), Medial-Lateral (+/- 1.8), Dorsal-Ventral (-3.3)²⁴. A concentration of 400 ng p24/μl of lentivirus were injected at a rate of 0.20 μl/min. For the SCA3 lentiviral mice, viral particles encoding for human ATXN3MUT containing 72 glutamines or encoding for ATXN3MUT and G3BP1, were injected into mouse striatum (left hemisphere and right hemisphere, respectively) at 400 ng of p24/ml, using the same coordinates described above. To perform safety assays, wild-type C57/BL6 mice (10-12 weeks old) were injected into the striatum with lentiviral particles encoding for G3BP1 at a concentration of 400 ng p24/ul of lentivirus, using the same coordinates described above. For the transgenic polyQ animals, lentiviral particles encoding for G3BP1 or GFP, as respective control, were injected into mice cerebella (4-5 weeks old), at a concentration of 800 ng p24/μl of lentivirus at the coordinates: -1.6 mm rostral to lambda, 0.0 mm midline, and -1.0 mm ventral to the skull surface, with the mouth bar set at -3.3²¹. All stereotaxic injections were performed by means of an automatic injector (Stoelting Co.) using a 34-gauge blunt-tip needle linked to a Hamilton syringe. Mice were sacrificed for posterior analysis, a few weeks after surgery, according to the model, SCA2 lentiviral mice: 12 weeks; SCA3 lentiviral mice: 4 weeks; G3BP1 injected mice: 4 weeks; polyQ transgenic mice: 9 weeks.

Behavioural testing

The transgenic mice were subjected to several motor behaviour tests starting before the stereotaxic injection (4-5 weeks of age), every 3 weeks until 9 weeks post-injection. Motor and gait coordination were accessed by rotarod and footprint tests in a blind fashion way following the same procedure described before²¹. In the footprint test analysis, steps taken by mice at the beginning and at the end of the walking test are not included and not considered for the measures.

Swimming performance was assessed by placing mice at one end of a rectangular tank (100x10.5x20 cm), filled with water at room temperature. Mice freely swam a distance of 1 m until they reached a platform and the time taken to transverse the tank was recorded. Mice performed the trial three times, with an interval of 15-20 minutes per trial. The mean of the time taken to cross the tank in the tree trials was used for statistical analysis.

Histological processing

Animals were sacrificed by sodium pentobarbital overdose and either transcardially perfused with 0.1M phosphate buffer solution and a 4% paraformaldehyde fixative solution (Sigma Aldrich) for immunohistochemical assays or had cervical dislocation and striatal punches of the brains, using a Harris Core pen with 2.5 mm diameter (Ted Pella Inc.), for qPCR and western blot analysis. The brains and the striatal punches collected were post-fixed in 4% paraformaldehyde for 24h, dehydrated in a 30% sucrose/0.1M phosphate buffer solution (PBS) for 48h and cryoprotected at -80°C. Sagittal or coronal brain sections of 30 µm and 25 µm, respectively, were obtained using the cryostat-microtome model CryoStar NX50 (Thermofisher). For preservation, brain sections were stored at 4 °C, free-floating in 0.02% (w/v) sodium azide PBS.

Cresyl violet staining

To stain brain sections with cresyl violet, they were mounted in gelatin-coated microscope slides. Brain sections were sequentially submersed in water, ethanol 96% (v/v), ethanol 100% (v/v), xylene, ethanol 75% (v/v) and the 0.1% (w/v) cresyl violet solution. To wash slices, brain sections were sequentially submersed in water, ethanol 75% (v/v), ethanol 96% (v/v), ethanol 100% (v/v) and xylene. Finally, brain sections were mounted with Eukitt (Sigma-Aldrich). Images were acquired with 10x objective in a Zeiss Axio Imager Z2.

Immunohistochemistry

The immunohistochemical procedure, for light imaging, started with the incubation of brain sections in phenylhydrazine diluted in phosphate buffer solution (1:1000; 15 min, 37 °C). For the human brain sections an additional step with a Tris-buffered saline pH 9 antigen retrieval method (30 min, 95°C) was performed. Brain sections went through blocking in 10% normal goat serum in 0.1% TritonTM X phosphate- buffered solution (1h, room temperature) and incubation with the respective primary (overnight at 4°C) and secondary biotinylated antibodies (2h at room-temperature) diluted in blocking solution, followed a reaction with the Vectastain elite avidin-biotin-peroxidase kit and by 3,3'-diaminobenzidine substrate (both from Vector Laboratories). Then, the sections were assembled over microscope slides, dehydrated in increasing degree ethanol solutions (75, 96 and 100%) and xylene, and finally cover slipped using mounting medium Eukitt (O. Kindler GmbH & CO). For fluorescence immunohistochemistry procedures, brain sections were incubated in the above-described blocking solution, followed by primary and secondary antibodies incubation. Brain sections were mounted in microscope slides with Fluoromount-G Mounting medium with 4', 6-Diamidino-2-Phenylindole, (DAPI) (Invitrogen). Images were acquired with 20x objective in a Zeiss Axio Imager Z2 and Axio Scan.Z1 Slide Scanner microscopes.

Immunocytochemistry

For the immunocytochemical procedure, cells were fixed using 4% paraformaldehyde (PFA) fixative solution for 20 min and washed with 0.1 M phosphate buffer solution (PBS). Samples were then incubated in PBS containing 0.1% TritonTM X-100 for 10 min. Blocking in PBS with 1% of bovine serum albumin (Sigma) was performed for 30 min. Samples were incubated with the primary antibody overnight in the proper dilution at 4°C and with the secondary antibody (1:200) for 2h at room temperature. The secondary antibody was coupled to a fluorophore (Alexa Fluor®, Invitrogen). Finally, the coverslips were mounted on microscope slides using Fluoromount-G mounting media with DAPI (Invitrogen). Images were acquired in a Zeiss Axio Imager Z2. Quantitative analysis of both of aggregates was blindly performed by counting the number of cells with aggregates within 100 transfected cells, using the 40x or 63x objective for each condition in each independent experiment.

Immunochemical antibodies

For immunochemical procedures, the following primary antibodies were used: mouse anti-ataxin-2 (1:1000, ref. 611378, BD Biosciences); mouse anti-ubiquitin (1:1000, ref. 3936S, Cell Signaling); rabbit anti-DARPP-32 (1:1000, ref. AB10518, Merck Millipore); rabbit anti-G3BP1 (1:1000, ref. 07-1801, Millipore); mouse anti-human G3BP1 (1:1000, ref. 611126, BD Biosciences); mouse anti-GFAP (1:1000, ref. 644702, BioLegend); rabbit anti-HA (1:1000, ref. Ab9110, Abcam); mouse anti-calbindin D-28K (1:1000, ref. C9848, Sigma Aldrich); mouse anti-PABP-1 (1:1000, ref. 04-1467, Millipore).

Western blot

Samples were either lysed in 10x RIPA solution (Merck Millipore) if cell extracts or homogenized in a urea/DTT solution if mouse striatal punches, both containing a cocktail of protease inhibitors (Roche), followed by an ultrasound sonication of 30 sec ON, 30 sec OFF, 5 cycles (Bioruptor Pico). Protein concentration levels were determined using PierceTM BCA Protein Assay Kit (Thermo Scientific) for cell lysates and NZYBradford reagent (Nzytech) for mouse samples. Protein extracts were resolved in SDS-polyacrylamide gels (7.5% and 12%), followed by protein transfer to PVDF membrane (Merck Millipore), membrane blocking in TBS-T with 3% of BSA or 5% of milk, and antibody probing overnight at 4 °C for primary and 2h at room temperature for secondary. The following antibodies were used: mouse anti-ataxin-2 (1:1000, ref. 611378, BD Biosciences); mouse anti-ataxin-3(1H9) (1:1000, ref. MAB5360, BD Biosciences); rabbit anti-G3BP1 (1:1000, ref. 07-1801, Merck Millipore); rabbit anti-phospho-G3BP1 (S149) (1:1000, ref. PA5-64827, Sigma); mouse anti-β-actin (1:5000, ref. A5316, Sigma Aldrich) mouse anti-B-tubulin (1:5000, ref. T7816, Sigma); mouse anti-puromycin (1:250, ref. MABE343, Millipore); mouse anti-GFP (1:1000, ref. 668205, BioLegend). Membranes were resolved using Enhanced Chemiluminescence (GE Healthcare) and scanned with a ChemiDocTM XRS+ (Bio-Rad). Optical densitometric analysis was carried out using Image J software.

RT-qPCR

Total RNA from mouse striatal punches started by Trizol (Invitrogen) tissue dissociation and RNA/DNA/protein chloroform separation. Then, both mouse and cell samples were extracted with NZY

Total RNA Isolation kit (Nzytech). RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). cDNA molecules of 1 µg of RNA were produced using iScript cDNA synthesis kit (Bio-Rad) according to manufacturer recommendations. Quantitative RT-qPCR was performed with the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), using home-made primers for gene of interest and for the human GAPDH housekeeping gene as a control and performed in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). mRNA expression levels relative to mRNA gene control were determined using amplification values. The following primers were used: human ATXN2 (QT01852480) and human ATXN3 (QT00094927) from QuantiTect Primer Assays, Qiagen. Human G3BP1 (Forward 5'– GAA ATC CAA GAG GAA AAG CC –3'; Reverse 5'– CCC AAG AAA ATG TCC TCA AG), human GAPDH (Forward 5'– ACA GTT GCC ATG TAG ACC –3'; Reverse 5'– TTG AGC ACA GGG TAC TTT A –3') and mouse Hprt (Forward 5'– AGG GAT TTG AAT CAC GTT TG –3'; Reverse 5'– TTT ACT GGC AAC ATC AAC AG –3') from KiCqStart Pre-designed Primers, Sigma-Aldrich. Mouse Pabpc1 (Forward 5'– GAA TAT GCC CGG TGC TAT CCG –3'; Reverse 5'– ACT CGT GGA ACC TGT GAG G –3'), mouse Tia-1 (Forward 5'– GCC CAA GAC TCT ATA CGT CGG –3'; Reverse 5'– GGT GCA AAC GCT GCT TTG ATG –3') and mouse Atxn2l (Forward 5'– CCT CAC CAT CTT GAC AAC AGC AG –3'; Reverse 5'– AAG GTC TTG GCA CCT CTC AGA G –3').

Statistical analysis

Statistical analysis was performed using either Student's *t*-test or one-way ANOVA complemented with Dunnett's multiple comparisons test, resorting to GraphPad software (La Jolla).

Results

Stress granules assembly does not alter the levels of ATXN2MUT and ATXN3MUT proteins

SGs are cellular *foci* formed in response to stress in which mRNAs, translation factors and RNA-binding proteins (RBPs) coalesce together to prevent cellular damage^{25,26}. Therefore, we aimed at investigating the impact of SGs assembly in mutant ataxin-2 (ATXN2MUT) and mutant ataxin-3 (ATXN3MUT), which are proteins involved in SCA2 and SCA3 diseases, respectively. For that, in Neuro2A cells expressing ATXN2MUT (pEGFP-ATXN2-Q104) or ATXN3MUT (pEGFP-ATXN3-Q84), SGs assembly was pharmacologically induced using sodium arsenite. As previously reported, ataxin-2 is recruited to SGs²⁷, however, ataxin-3 is not. The expression of both mutant ataxin-2 and mutant ataxin-3 in Neuro2A cells leads to the formation of aggregates (Fig. 1a). However, the assembly of SGs did not alter the number of cells with ATXN2MUT (0.67 ± 0.1818 , $n=3$) or ATXN3MUT (0.67 ± 0.0306 , $n=3$) aggregates, compared to the control conditions (ATX2MUT: 0.52 ± 0.0458 , $n=3$; ATXN3MUT: 0.65 ± 0.0767 , $n=3$) in which stress stimulus was not induced (Fig. 1b, 1c). SGs assembly is accompanied by a translation inhibition²⁸, leading to a reduction in the overall protein synthesis (0.06 ± 0.0475 , $n=4$, $P < 0.0001$) (Fig. S1a, S1b). Thus, we next rationalized that ATXN2MUT and ATXN3MUT soluble proteins levels could be impacted upon SGs assembly and therefore we analyzed their levels by western blot (Fig. 1d–1g). However, no alterations were observed in the levels of these proteins among the different experimental conditions

(ATXN2MUT+SG: 0.075 ± 0.2052 , $n=4$; ATXN3MUT+SGs: 0.96 ± 0.2538 , $n=6$). Altogether, these results show that although SGs assembly reduces the overall translation, it does not seem to interfere with mutant ataxin-2 and mutant ataxin-3 aggregation neither with its protein levels.

G3BP1 overexpression reduces the number of cells with aggregates and the levels of ATXN2MUT and ATXN3MUT.

It was previously shown that SGs assembly can also be induced by overexpression of its core components^{13,29}. One of those SGs components is the G3BP1 protein, which is an RBP able of both mRNA stabilization and degradation¹⁵. In Neuro2a cells, we observed that G3BP1 overexpression alone is less effective in inducing SGs formation than when combining it with a sodium arsenite stimulus (Supplementary Fig. 2). Additionally, in SCA2 patient-derived fibroblasts, G3BP1 has a diffuse expression, contrarily to what happens upon sodium arsenite treatment, where G3BP1 self-assembles in SGs resembling structures (Supplementary Fig. 3). Moreover, upon G3BP1 overexpression there is an inhibition of protein synthesis (0.52 ± 0.0618 , $n=4$, $P=0.0048$), nevertheless, in lower levels than the inhibition observed in SGs induction with sodium arsenite (Supplementary Fig. 1a, 1b and 1c). Taking this in consideration, we next aimed to investigate the impact of G3BP1 overexpression in ATXN2MUT and ATXN3MUT proteins and assess whether the results were in line with those observed upon stress granules induction by sodium arsenite. To achieve this goal, we co-transfected Neuro2A cells either with ATXN2MUT and G3BP1 or ATXN3MUT and G3BP1 (Fig. 2a, 2e). We found that G3BP1 overexpression was able to significantly reduce the number of cells with aggregates of both ATXN2MUT (ATXN2MUT+G3BP1: 0.39 ± 0.0153 , $n=3$, *versus* ATXN2MUT: 0.53 ± 0.0361 , $n=3$, $P=0.0233$) and ATXN3MUT (ATXN3MUT+G3BP1: 0.35 ± 0.0361 , $n=3$, *versus* ATXN3MUT: 0.66 ± 0.0736 , $n=3$, $P=0.0201$), compared to control conditions (Fig. 2b, 2f). Next, we used Neuro2A cells with reduced levels of mouse endogenous G3bp1 to further investigate the specificity of G3BP1 overexpression (Supplementary Fig. 4a). These cells had a significant reduction in cell viability compared to non-transduced cells (74.22 ± 5.767 $n=3$, $P=0.0111$) (Supplementary Fig. 4b). No difference in the number of pathological aggregates was observed in the Neuro2A cells with reduced levels of G3bp1, compared to control Neuro2a cells. However, in line with the results obtained above, the overexpression of G3BP1 in these Neuro2A cells with low G3bp1 levels, significantly reduced the number of cells with aggregates of ATXN2MUT (0.50 ± 0.0683 , $n=4$) and ATXN3MUT (0.50 ± 0.1594 , $n=4$), compared to control conditions (1.02 ± 0.0991 , $n=4$, $P=0.0052$; 1.00 ± 0.0417 , $n=4$, $P=0.0217$, respectively) (Supplementary Fig. 4c–S4e). Next, we investigated whether the observed reduction in aggregation upon G3BP1 overexpression was related with a reduction in the protein levels of ATXN2MUT and ATXN3MUT. Through western blot (Fig. 2c and 2g) we found that G3BP1 overexpression was able to significantly reduce the expression levels of both ATXN2MUT (0.52 ± 0.1343 , $n=5$, $P=0.0075$) and ATXN3MUT (0.25 ± 0.0223 , $n=5$, $P<0.0001$), when compared with control conditions (Fig. 2d, 2h). However, no alteration was observed in mouse endogenous levels of Ataxin-2 (0.85 ± 0.1176 , $n=5$) and Ataxin-3 (0.86 ± 0.0742 , $n=4$) upon G3BP1 overexpression (Supplementary Fig. 5). Moreover, in an additional control condition, GFP levels were not

altered when G3BP1 is overexpressed (0.92 ± 0.0762 , $n=4$) (Supplementary Fig. 6). These results suggest that G3BP1 expression might be impacting mutant ataxin-2 and mutant ataxin-3 proteins in a SGs-independent manner.

The NTF2-like domain is important for G3BP1 action on mutant ataxin-2 and mutant ataxin-3 proteins.

Besides being involved in the regulation of SGs assembly¹³, G3BP1 is an RBP with several molecular and biological functions, including mRNA binding, DNA binding³⁰, helicase and endonuclease activity³¹, and has important functions in immune response³². Overall, RBPs, including G3BP1, interact with mRNAs through a very specific and restrict set of RNA-binding domains^{33,34}. The G3BP1 RRM motif is known for interacting with target RNA sequences³⁵. Moreover, G3BP1 harbors a NTF2-like domain that is involved in the nuclear shuttling of proteins through the nuclear pore complex³⁶. This domain also facilitates protein-protein interactions³⁷, mediates G3BP1 dimerization and interferes in SGs formation¹³. Therefore, to better understand G3BP1 mechanism of action, we developed two different forms of the protein, one with the deletion of the NTF2-like domain and the other with the deletion of the RRM domain (Fig. 3a, 3b). Next, we co-transfected Neuro2a cells with the ATXN2MUT and either with NTF2-like deleted G3BP1 (G3BP1- Δ NTF2) or with RRM deleted G3BP1 (G3BP1- Δ RRM) (Fig. 3c). We observed that when the cells were transfected with G3BP1- Δ NTF2 the number of cells with mutant ataxin-2 aggregates increased (70.5 ± 1.893 , $n=4$), comparing with the control mutant condition (62 ± 0.8165 , $n=4$; $P=0.0062$). However, when the cells were expressing G3BP1- Δ RRM there was a significant decrease in the number of cells with ataxin-2 aggregates (55 ± 0.8165 , $n=4$) when compared to the control condition (62 ± 0.8165 , $n=4$, $P=0.0009$) (Fig. 3d). Subsequently, we analyzed the mutant protein levels through western blot and found that the G3BP1- Δ RRM condition led to a significant reduction of ATXN2MUT levels (0.49 ± 0.0352 , $n=3$, $P<0.0001$) compared to control condition, while no difference was observed in the cells expressing the G3BP1- Δ NTF2 (Fig. 3e, 3f). We performed a similar experiment using the ATXN3MUT (Fig. 4a). Likewise, we observed an increase of cells with aggregates upon transfection with G3BP1- Δ NTF2 (ATXN3MUT+ Δ NTF2: 183.67 ± 0.3333 , $n=3$; ATXN3MUT+LacZ: 78.25 ± 1.315 , $n=4$, $P=0.0187$), and a reduction in the number of cells with aggregates upon G3BP1- Δ RRM expression (66.5 ± 2.327 , $n=4$, $P=0.0046$) (Fig. 4b). Moreover, protein levels of mutant ataxin-3 were also reduced upon G3BP1- Δ RRM expression (0.52 ± 0.0366 , $n=4$, $P<0.0001$) and unchanged in the G3BP1- Δ NTF2 condition (0.91 ± 0.0389 , $n=4$, $P=0.0594$) (Fig. 4c, 4d).

Altogether, these results show that the deletion of the NTF2-like domain impacts the G3BP1 molecular mechanism of action. On the contrary, the deletion of the RRM domain did not alter the levels of mutant ataxin-2 and mutant ataxin-3 proteins.

G3BP1 reduces mutant ataxin-2 and mutant ataxin-3 mRNAs levels, which could be mediated through its endoribonuclease activity

In G3BP1 protein, the NTF2-like domain is closely located to a phosphorylation site (ser-149), which provides its endoribonuclease activity^{17,31,34}. Therefore, we next aimed to investigate the contribution of this phosphorylation site to the observed results. First, in Neuro2A cells we analyzed the mRNA levels of mutant *ATXN2* and mutant *ATXN3*. We found that both mRNAs are significantly reduced upon G3BP1 overexpression (*ATXN2MUT*+G3BP1: 61.89 ± 13 , $n=3$, $P=0.0427$; *ATXN3MUT*+G3BP1: 8.32 ± 2.543 , $n=4$, $P<0.0001$), compared to control condition (Fig. 5a, 5b), which suggest that G3BP1 could be degrading these mRNAs. Through the analysis of *TIA-1*, *PABP1* and *ATXN2L* mRNA levels, which interact with G3BP1³⁸, we observed no alteration of their mRNA levels, upon expression of G3BP1, both in Neuro 2A cells expressing *ATXN2MUT* (Fig. 5c) and *ATXN3MUT* (Fig 5d) conditions ($n=4$). To further investigate the importance of this phosphorylation site, we performed a mutagenesis site-directed for ser-149 in G3BP1, changing this residue to alanine (S149A) (Figs. 5e and Supplementary Fig. 7a). The expression of this construct in Neuro2A cells results in a non-significant reduction of phosphorylated G3BP1 ratio relatively to the total G3BP1 expression, comparing to cells expressing normal G3BP1 and non-transfected cells (Supplementary Fig. 7b, 7c). Next, in Neuro2A cells we co-transfected the G3BP1-S149A with *ATXN2MUT* and *ATXN3MUT* (Fig. 5f). In opposition to the previous observations of G3BP1-mediated reduction of aggregation, here no differences were found in the number of cells with aggregates of both mutant ataxin-2 (*ATXN2MUT*+G3BP1(S149A): 20.25 ± 2.496 , *ATXN2MUT*: 24.5 ± 6.665 , $n=4$) and mutant ataxin-3 (*ATXN3MUT*+G3BP1(S149A): 15.5 ± 2.723 , *ATXN3MUT*, 17.5 ± 2.217 , $n=4$) upon the expression of the G3BP1-S149A (Fig. 5g, 5H). Altogether, these results suggest that Ser-149 phosphorylation site is critical for G3BP1 molecular activity and could be modulating the levels of mutant ataxin-2 and mutant ataxin-3 mRNAs.

G3BP1 expression is reduced in SCA2 and SCA3 patients and disease models

Previous studies report that mutant polyQ proteins, as *ATXN2MUT* and *ATXN3MUT*, can dysregulate the expression of several genes^{1,39}. In fact, it was previously shown that the expression of mutant ataxin-3 drives an abnormal reduction of wild-type ataxin-2 levels²². In this line, we next analyzed the levels of G3BP1 in samples from SCA2 and SCA3 patients and also in disease models. In post-mortem brain samples of SCA2 patients we detected a reduction in the immunodetection of G3BP1, comparing with healthy individuals, both in striatum and cerebellum (Supplementary Fig. 8). Furthermore, in fibroblasts from SCA2 and SCA3 patients we detected a significant reduction in the mRNA levels of G3BP1 (SCA2: 0.46 ± 0.0477 , $n=4$, versus Control: 0.82 ± 0.0945 , $n=8$, $P=0.0267$; SCA3: 0.67 ± 0.0552 , $n=10$, versus Control: 1 ± 0.0643 , $n=5$, $P=0.0032$), compared to fibroblasts from healthy controls (Fig. 6a, 6b). Next, we analyzed the G3BP1 protein levels in fibroblasts from SCA2 by western blot, which we found to be significantly reduced compared to healthy controls (0.49 ± 0.0153 $n=2$, $P=0.0394$) (Fig. 6c, 6d). Consistently, the G3BP1 immunoreactivity in SCA3 patient's fibroblasts was also significantly reduced when compared to healthy

controls (SCA3: 4.41 ± 0.2511 , $n=7$, versus Control: 5.84 ± 0.5151 , $n=6$, $P=0.0239$) (Fig. 6e, 6f). Finally, we also observed that the G3BP1 protein levels are significantly reduced in a transgenic mouse model of SCA3 (Tg_SCA3: 0.45 ± 0.1686 , $n=4$, versus WT: 1.19 ± 0.2585 , $n=4$, $P=0.0267$) (Fig. 6g, 6h). Altogether, these results show that G3BP1 levels are decreased in SCA2 and SCA3 diseases.

Restoring G3BP1 levels alleviates neuropathology in SCA2 and SCA3 lentiviral mouse models

The expression of ATXN2MUT and ATXN3MUT, mediated by lentiviral vectors leads to formation of intraneuronal aggregates and to the loss of neuronal markers⁴⁰, which are neuropathological signs also found in post-mortem human tissue^{41–43}. Thus, we next aimed to investigate whether restoring G3BP1 levels restoring could improve neuropathological abnormalities induced by ATXN2MUT and ATXN3MUT *in vivo*. For that we co-expressed lentiviral vectors encoding ATXN2MUT (or ATXN3MUT) and human G3BP1 in one hemisphere of the striatum and, as control, in the contralateral hemisphere we injected lentiviral vectors encoding ATXN2MUT (or ATXN3MUT) (Fig. 7a, 7e). At 12 weeks post-injection for SCA2 lentiviral mouse model, and at 4 weeks post-injection for SCA3 lentiviral mouse model, the animals were sacrificed, and the striatum histologically analyzed. In both models, the expression of G3BP1 was able to significantly reduce the number of ATXN2MUT (ATXN2MUT+G3BP1: 1466 ± 31.13 , $n=5$, versus ATXN2MUT: 2131 ± 71.04 , $n=5$, $P=0.0002$) and ATXN3MUT (ATXN3MUT+G3BP1: 6066 ± 1958 , $n=8$, versus ATXN3MUT: 30076 ± 2717 , $n=6$, $P<0.0001$) aggregates (Fig. 7b, 7c, 7f and 7g). Accordingly, G3BP1 expression led to a preservation of neuronal marker DARPP-32 in both models, as compared to the control hemispheres (ATXN2MUT+G3BP1: 0.02 ± 0.0078 , $n=5$, versus ATXN2MUT: 0.08 ± 0.0078 , $n=5$, *Student's t-test*, $P=0.001$; ATXN3MUT+G3BP1: 0.19 ± 0.0291 , $n=8$, versus ATXN3MUT: 0.45 ± 0.0647 , $n=6$, *Student's t-test*, $P=0.0072$) (Fig. 7b, 7d, 7f and 7h).

Altogether these results show that G3BP1 expression mediates neuroprotection, reducing the neurodegeneration hallmarks associated with the expression of mutant ataxin-2 and mutant ataxin-3.

Overexpression of G3BP1 in the brain of wild-type mice did not produce neuronal loss nor inflammation

Based on the previous results, we next aimed to evaluate the long-term impact of G3BP1 expression. For that, lentiviral particles coding for human G3BP1 were injected in one hemisphere of the striatum of wild-type C57BL/6 mice, while in the contralateral side was injected with PBS, as control (Fig. 8a). At 4 weeks post-injection the loss of the neuronal marker DARPP-32 in the hemisphere injected with G3BP1 was significantly smaller comparing to the control hemisphere injected with PBS (LV-G3BP1: 0.003 ± 0.0014 , $n=10$, versus PBS: 0.01 ± 0.0011 , $n=3$ *Student's t-test*, $P=0.035$) (Fig. 8b, 8c). In fact, in the G3BP1-injected animals the lesion area was restricted to the injection site. Moreover, no differences in astrocytes activation, was observed upon G3BP1 overexpression, as compared to control hemisphere injected with PBS (Fig. 8d). Altogether these results point to a safety profile of G3BP1 overexpression in the brain.

Restoring G3BP1 levels in the cerebellum mitigates behavior deficits and neuropathological abnormalities in a polyQ transgenic mouse model.

PolyQ SCAs diseases are characterized by a progressive neuronal loss and motor dysfunctionality. Thus, to mimic this phenotype, we used a transgenic mouse model expressing a truncated form of mutant ataxin-3 with 69 glutamines and characterized by a severe motor dysfunctions, neurodegeneration and early onset²³. This can also be a relevant polyQ model, considering that only contains a small region of the ataxin-3 protein, and a significant tract of glutamines, causing pathology, as observed in other polyQ diseases^{23,44}. Therefore, we next aimed to investigate the impact of G3BP1 overexpression in this polyQ transgenic mouse model. For that, at 4 weeks of age, lentiviral vectors encoding for G3BP1 were stereotactically injected into the cerebellum⁴⁵. The control animals were injected with lentiviral vectors encoding for GFP. The animals were subjected to several behavior tests every 3 weeks until 9 weeks post-injection (Fig. 9a). At the final timepoint, G3BP1-injected animals stayed for longer times in the rotating rotarod, comparing to the control animals (G3BP1: 1.45 ± 0.0124 , $n=7$, versus NI: 0.84 ± 0.1082 , $n=7$, $P=0.0254$) (Fig. 9b). In the same line, in the swimming test, at 9 weeks post-injection, G3BP1-injected animals took less time to reach the safe platform (G3BP1: 0.55 ± 0.0974 , $n=7$, versus NI: 0.99 ± 0.173 , $n=7$, $P=0.0476$) (Fig. 9c). Finally, in the footprint patterns test, the animals injected with G3BP1 had a smaller footprint overlap, as compared to control animals (G3BP1: 1.06 ± 0.1081 , $n=7$, versus NI: 1.62 ± 0.1997 , $n=7$, $P=0.0297$) (Fig. 9d). Similarly, these results show that G3BP1 expression in the cerebellum is able to mitigate motor deficits. This mouse model is characterized by the formation of aggregates in the Purkinje cells of the cerebellum, showing a high reduction in the number of these cells and a strong disarrangement of cerebellar layers architecture^{23,46}. Thus, we next aimed to evaluate the impact of G3BP1 in the neuropathological abnormalities (Fig. 9e). We found that animals injected with lentiviral particles encoding for G3BP1 exhibited significantly reduced the number of pathological aggregates, compared to controls (G3BP1: 63.12 ± 10.17 , $n=2$, versus NI: 101.2 ± 15.29 , $n=4$, $P=0.0397$) (Fig. 9f). In the same line, G3BP1-injected animals showed a preservation in the number of Purkinje cells as compared to controls (G3BP1: 1.62 ± 0.2405 , $n=2$, versus NI: 0.91 ± 0.1904 , $n=4$, $P=0.0437$) (Fig. 9g). Importantly, in non-transduced lobes no differences were found between the experimental groups regarding HA ataxin-3 aggregates (G3BP1: 44.45 ± 7.169 , $n=2$, versus NI: 49.7 ± 9.385 , $n=4$) or the number of cerebellar Purkinje cells (G3BP1: 1 ± 0.1457 , $n=2$, versus NI: 0.97 ± 0.1988 , $n=4$) (Supplementary Fig. 9). Additionally, the cerebellar molecular layer thickness of transduced lobules (II/III) was significantly wider compared to non-injected controls (G3BP1: 64.99 ± 3.189 , $n=2$, versus NI: 56.03 ± 1.824 , $n=4$, $P<0.0118$) (Supplementary Fig. 10a and 10b). Moreover, in non-transduced lobules no differences were observed regarding thickness of the cerebellar molecular layer (G3BP1: 67.65 ± 2.301 , $n=2$, versus NI: 69.56 ± 1.642 , $n=4$) (Supplementary Fig. 10a and 10c). Altogether, G3BP1 overexpression in the cerebellum significantly reduced motor behavior impairments while it reduces the neuropathological abnormalities.

Discussion

Proteins containing abnormally expanded polyQ tracts have been implicated with the impairment of several cellular pathways, which ultimately lead to cellular death. The high propensity of the mutant polyQ proteins to aberrantly aggregate are either directly involved or at least contribute to aggravate particular toxic outcomes, acting decisively in the polyQ pathogenesis. In the last decade it has been hypothesized that the abnormal protein aggregation, characteristic of several neurodegenerative disorders, not only subjects cells to stress, but can also impair cellular stress-response pathways⁴⁷. The formation of stress granules is one important player in stress response, as they play an important role as mediator of protein synthesis. During SGs assembly, several key players, such as RBPs and mRNAs are sequestered into the granule preventing these components from integrating the translational machinery^{12,48}. Previous evidence has also shown that SGs co-localize with several protein aggregates, which are characteristic of different neurodegenerative diseases⁴⁹. Therefore, we hypothesized that an activation of the stress response through the formation of SGs could either sequester mutant polyQ proteins, or promote a translational arrest, decreasing its expression. We found that chemically-inducing SGs assembly, in Neuro2a cells expressing either mutant ataxin-2 or mutant ataxin-3, leads to a significant decrease in global translation levels, however it seems not to interfere with the expression levels of both ATXN2MUT and ATXN3MUT, nor with the aggregation of those proteins.

G3BP1 is an RBP, a core component of SGs and in its dephosphorylated state can induce SGs formation¹³. It has been reported that cellular stress induction by sodium arsenite, reduces the constitutive phosphorylation state of G3BP1^{13,50}. However, in recent years, this hypothesis was challenged⁵⁰, and it is not clear if there is a correlation between cellular stress induction through sodium arsenite and phosphorylation/dephosphorylation status of G3BP1. To clarify this possible link, we overexpressed G3BP1 in SCA2 patients-derived fibroblasts. We found that G3BP1 shows a diffuse expression within the cell, contrasting to what happens when we treat the cells with sodium arsenite. Upon sodium arsenite treatment, G3BP1 self-assembles in structures resembling SGs. As G3BP1 functions vary depending on its phosphorylation/dephosphorylation state, our next aim was to study the impact of G3BP1 overexpression in Neuro2a cells expressing ATXN2MUT or ATXN3MUT. Upon overexpression of G3BP1 we observed a reduction in the number of cells with mutant protein aggregates and in the expression levels of mutant polyQ proteins. We hypothesized that, while the phosphorylated G3BP1, diffusely spread in the cells, performs its catalytic activity on the mutant polyQ proteins, the non-phosphorylated G3BP1 assembles in SGs-like structures, switching its functions.

To clarify the specificity of G3BP1 action on ATXN2MUT and ATXN3MUT levels and aggregates, we next used Neuro2A cells with low levels of mouse endogenous G3bp1. We observed that when ATXN2MUT and ATXN3MUT are expressed in this cell line, the number of cells with aggregates is maintained, comparing to a normal Neuro2a cell line. However, when we co-express G3BP1 and the mutant proteins, the results are in line to what is observed in the Neuro2a cells, i.e., a decrease in the number of cells with aggregates. These observations lead us to suggest that is G3BP1 expression the responsible for the decrease of the levels and number of aggregates of mutant polyQ proteins.

Next, we wanted to investigate which domains of G3BP1 could be implicated in its molecular mechanisms of action. Thus, we focused our study in the NTF2-like domain, which is involved in the nuclear transport via nuclear pores and has been shown to facilitate protein-protein interactions⁵¹. Additionally, we also investigated the contribution of the RRM domain, which interacts with target RNA sequences and can also bind with other proteins⁵². By expressing truncated constructs of G3BP1 with deletions of NTF2 or RRM domains, we observed a reduction in the number of cells with aggregates of both ATXN2MUT and ATXN3MUT and their expression levels, when the RRM domain is deleted. Oppositely, no differences were found among the experimental conditions when NTF2 is deleted. This led us to suggest that NTF2 domain could be essential for G3BP1 action. We next went to analyze the impact of G3BP1 expression on the mRNA levels of ATXN2MUT and ATXN3MUT. We found that those levels were significantly decreased upon G3BP1 expression. G3BP1 protein was found to interact with ATXN3 RNA³⁸, which could be the cause for the more robust results found in ATXN3 mRNA, comparing with ATXN2. Previous studies demonstrated that phosphorylated G3BP1 translocates to the cellular nucleus, probably to perform its endoribonuclease activity^{17,31}. As mentioned before, the NTF2-like domain of G3BP1 is very close to an important phosphorylation site, serine 149. This phosphorylation site is also believed to be connected to the endonuclease activity of G3BP1³¹. To evaluate the impact of G3BP1 phosphorylation we performed a sited-directed mutagenesis in G3BP1, switching the serine149 for an alanine, therefore generating a phospho-dead protein at the 149 aa site. Using this phospho-dead construct we found that the expression of G3BP1 lost its impact in the number of cells with aggregates of ATXN2MUT and ATXN3MUT, leading us to suggest that G3BP1 phosphorylation is crucial for its molecular functions.

Next, we aimed to analyze G3BP1 expression levels in the context of SCA2 and SCA3 patients and animal models. We found that in postmortem samples of human brain tissue from SCA2, G3BP1 staining was substantially decreased, suggesting low levels of expression. Accordingly, we also observed that SCA2 and SCA3 patient-derived fibroblasts have reduced levels of G3BP1 mRNA and protein. The results led us to hypothesize that molecular pathologic phenotype observed in SCA2 and SCA3 might be exacerbated due to the joint effect of the polyQ mutant proteins toxicity and the lower expression levels of G3BP1. Therefore, we next investigate the potential of G3BP1 re-establishment in disease mitigation, using different mice models of these two diseases. Using SCA2 and SCA3 lentiviral mouse models, we observed that the injection into the striatum of lentiviral particles encoding for G3BP1 lead to the preservation of brain tissue (DARPP-32 staining) and to a decrease in the number of aggregates. Moreover, in a transgenic mouse model, characterized by severe neurodegeneration and motor deficits, we found that the injection in the cerebellum of lentiviral particles encoding for G3BP1, reduces the number of aggregates and preserves the number of Purkinje cells. Importantly, G3BP1 expression in mice cerebella significantly improved the overall motor performance, in particular, balance and coordination.

With this work we found that G3BP1 expression levels are decreased in both patient-derived fibroblast and brain sample of SCA2 and SCA3 affected individuals. Additionally, we show that G3BP1 expression is able to decrease the expression of mutant ataxin-2 and ataxin-3. These results strongly suggest that, in

SCA2 and SCA3 disease, the ability of G3BP1 to downregulate the mutant ataxin-2 and ataxin-3 might be impaired, due to G3BP1 decreased expression levels, leading to an exacerbation of the phenotype. Additionally, we also show that the G3BP1 NTF2-like domain and the ser 149 phosphorylation site, are essential to mitigate mutant ataxin-2 and mutant ataxin-3 aggregation.

Despite the extensive efforts developed over the last years, polyQ diseases pathogenesis is not completely understood, neither exist therapeutic options exist to delay or stop the disease progression. Therefore, it is essential to identify new molecular targets, implicated in the disease pathogenesis and that could be targetable in future therapeutic strategies. The implication of G3BP1 in neurodegenerative diseases has only recently being proposed, and therefore more studies are needed to unveil this link. Nevertheless, our results strongly support that gene delivery of G3BP1 is efficient and safe in the mitigation SCA2 and SCA3 pathology, suggesting that G3BP1 might be a novel therapeutic target, not only for SCA2 and SCA3, but possible to other polyQ diseases.

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Figures

Figure 1

Stress granules assembly does not alter the number of aggregates nor protein levels of ATXN2MUT and ATXN3MUT. Stress granules assembly was induced in Neuro2A cells expressing mutant forms of ATXN2 or ATXN3 (which are prone to aggregate) by adding sodium arsenite to the cell media to a final concentration of 0,05 M. The stress stimulus had a duration of 1 hour. A Representative confocal images of immunocytochemistry from Neuro2A cells showing aggregates of ATXN2MUT or ATXN3MUT (green).

Stress granules marker PABP is labelled in red. Scale: 10 μ m. Stress granules induction did not alter aggregates of B ATXN2MUT or (c) ATXN3MUT (n=3; Student's t-test), compared to the control conditions. D and F Representative blots of protein lysates from Neuro2A cells, transfected with mutant forms of D ATXN2 or F ATXN3 in which, stress granules formation was induced. Stress granules induction did not alter protein levels of E ATXN2MUT or G ATXN3MUT (n=4; Student's t-test). Values are expressed as mean $\pm$ SEM.

Figure 2

Stress granules core protein, G3BP1, reduces the levels of ATXN2MUT and ATXN3MUT proteins and the number of cells with aggregates. Neuro2a cells were either transfected with mutant forms of ATXN2 or ATXN3, or co-transfected with the mutant forms and G3BP1. (A) Representative images of immunocytochemistry from Neuro2A cells showing aggregates of ATXN2MUT (green). (B) G3BP1 expression significantly reduces the number of cells with ATXN2MUT aggregates when compared with the control conditions (n=3; *p<0.05; Student's t-test). (C) Representative blot of protein lysates from Neuro2A cells transfected either with a ATXN2MUT or co-transfected with G3BP1 and ATXN2MUT. (D) G3BP1 protein was able to significantly reduce the levels of ATXN2MUT when compared with the control condition (n=5; **p<0.001; Student's t-test). (E) Representative images of immunocytochemistry from Neuro2A cells showing aggregates of ATXN3MUT (green). (F) G3BP1 protein significantly reduces the number of cells with ATXN3MUT when compared with the control conditions (n=3; *p<0.05; Student's t-test). (G) Representative blot of protein lysates from Neuro2A cells transfected either with a ATXN3MUT or co-transfected with G3BP1 and ATXN3MUT. (H) G3BP1 protein was able to significantly reduce the levels of ATXN3MUT when compared with the control condition (n=5; ****p<0.0001; Student's t-test). Values are expressed as mean $\pm$ SEM.

Figure 3

The NTF2-like domain is important for G3BP1 modulation of mutant ATXN2 protein. Neuro2a cells were co-transfected with a mutant form of ATXN2, and either the G3BP1-ΔNTF2 or G3BP1-ΔRRM. A co-transfection with LacZ was used as control. (A) Schematic representation of G3BP1 structural domains and the respective constructs with deleted NTF2 domain and deleted RRM domain. Δ: deletion. NTF2: nuclear transport factor 2 domain. Ser: serine. PxxP: proline-rich region. RRM: RNA recognition motif. RGG box: arginine and glycine rich box. (B) Representative blot of protein lysates from Neuro2A cells transfected either with G3BP1-ΔNTF2 or G3BP1-ΔRRM depicting the expression of G3BP1 truncated forms. (C) Representative images of immunocytochemistry from Neuro2A cells showing aggregates (arrowheads) of ATXN2MUT (green). Scale: 10 μm. (D) G3BP1-ΔNTF2 expression increases the number of cells with ataxin-2 aggregates. On the other hand, G3BP1-ΔRRM significantly reduces the number of

cells with ATXN2MUT when compared with the control conditions (n=4; **p<0.01; Student's t-test). (E) Representative blot of protein lysates from Neuro2A cells co-transfected with ATXN2MUT and each of the G3BP1 deleted constructs. (F) At protein levels, G3BP1-ΔNTF2 does not alter the levels of mutant ataxin-2, but consistently, G3BP1-ΔRRM significantly reduces the levels of the mutant protein, when compared to the pathologic control (n=4; ***p<0.001; Student's t-test). Values are expressed as mean ± SEM.

Figure 4

The NFT2-like domain is important for G3BP1 modulation of mutant ATXN3 protein. Neuro2a cells were co-transfected with a mutant form of ATXN3, and either the G3BP1-ΔNTF2 or G3BP1-ΔRRM. A co-transfection with LacZ was used as control. (A) Representative images of immunocytochemistry from Neuro2A cells showing aggregates (arrowheads) of ATXN3MUT (green). Scale: 10 μm. (B) Similarly, G3BP1-ΔNTF2 expression increases the number of cells with ataxin-3 aggregates observed by fluorescence microscopy. However, G3BP1-ΔRRM significantly reduces the number of cells with ATXN3MUT when compared with the control conditions (n=4; **p<0.01; Student's t-test). (C) Representative blot of protein lysates from Neuro2A cells co-transfected with ATXN2MUT and each of the

G3BP1 deleted constructs. (D) At protein levels, G3BP1-ΔNTF2 does not alter the levels of mutant ataxin-3, however, G3BP1-ΔRRM shows a significative decrease of the ATX3MUT protein levels (n=4; ****p<0.0001; Student's t-test). Values are expressed as mean ± SEM.

Figure 5

G3BP1 has endoribonuclease activity in a phosphorylated-dependent manner. In Neuro2a cells transfected either with ATX2MUT or ATX3MUT or co-transfected with the mutant polyQ proteins and G3BP1, an analysis of the mutant proteins' mRNA levels was performed by RT-qPCR. The quantification of (A) ATXN2 and (B) ATXN3 mRNA levels when G3BP1 is expressed, comparing to control conditions, shows a significant reduction. (C and D) The quantification of TIA-1, PABP1 and ATXN2L mRNA levels when G3BP1 is expressed both in ATXN2MUT and ATXN3MUT, comparing to control conditions, shows no difference between conditions. (E) Schematic representation of G3BP1 gene structure with the mutagenesis site where a serine was changed for an alanine, creating a phospho-dead construct at the 149 aa site (G3BP1-S149A). f Representative images of immunocytochemistry from Neuro2a cells with low levels of mouse endogenous G3bp1 transfected with ATX2MUT or ATX3MUT and co-transfected with

the mutant polyQ proteins and G3BP1-S149A. Scale: 10 μ m. (G and H) Co-transfection of G3BP1-S149A with either ATXN2MUT or ATXN3MUT, did not altered the number of cells with aggregates when comparing with the respective controls (n=4; Student's t-test). Values are expressed as mean $\pm$ SEM.

Figure 6

G3BP1 expression is decreased in SCA2 and SCA3 patients and models of disease. To analyse the basal expression levels of G3BP1 we used SCA2 and SCA3 patient-derived fibroblast. Fibroblasts from a healthy individual were used as control. The mRNA levels of G3BP1, in both (A) SCA2 (n=4) and (B) SCA3 (n=10) patient-derived fibroblasts were reduced when compared to the basal G3BP1 levels from healthy-derived (n=13) fibroblast (*p<0.05; **p<0.01; Student's t-test). (C) Representative images of western blot, from SCA2 patient-derived fibroblast lysates. (D) G3BP1 protein levels from SCA2 patient's fibroblasts were significantly reduced when compared with the control condition (n=2; *p<0.05; Student's t-test). (E) G3BP1 immunofluorescence staining (red) of SCA3 patient-derived fibroblasts. Scale 20 μ m. (F) In SCA3 fibroblasts there is a decrease in the G3BP1 immunoreactivity when compared to healthy fibroblasts (n=7; *p<0.05; Student's t-test). (G) We also analysed, through western blot, protein lysates from cerebellar tissue of a transgenic mouse model of SCA3. (H) G3BP1 protein levels are significantly decreases in the transgenic animals expressing mutant ATXN3 compared with wild-type animals (n=4; *p<0.05; Student's t-test). Values are expressed as mean $\pm$ SEM.

Figure 7

G3BP1 reduces the number of aggregates and mitigates neurodegeneration in lentiviral mouse models of SCA2 and SCA3. Mice were stereotactically injected into the striatum either with lentiviral particles encoding for mutant forms of ATXN2 or ATXN3, or co-injected with lentiviral particles encoding for the mutant form and G3BP1. (A) Schematic representation of the injection site and the constructs used to treat mouse models of SCA2. Animals were bilaterally injected at 8-12 weeks of age and euthanized 12

weeks after lentiviral vectors injection, for tissue collection. (B) Representative images of immunohistochemistry brain sections, from the lentiviral mouse model of SCA2. Images show aggregate of ATXN2MUT (black arrowheads; Scale: 20 μ m) and the neuronal marker (DARPP-32; Scale: 200 μ m) loss volume (black line). (C) The hemisphere expressing G3BP1 significantly presented a reduced number of ATXN2MUT aggregates, compared to control hemisphere (n=5; ***p<0.0001; Student's t-test). (D) G3BP1 expression rescues neuronal marker loss, compared to the contralateral hemisphere only expressing mutant ATXN2 (n=5; ***p<0.0001; Student's t-test). (E) Schematic representation of the injection site and the constructs used to treat mouse models of SCA3. Animals were unilaterally injected at 8 weeks of age and euthanized 4 weeks after surgery, for tissue collection. (F) Representative images of immunohistochemistry brain sections, from the lentiviral mouse model of SCA3. The figures show ubiquitinated ATXN3MUT aggregates (dark dots; Scale: 20 μ m) and the neuronal marker (DARPP-32; Scale: 200 μ m) loss volume (black line). (G) The expression of G3BP1 led to a significant reduction in the number of ubiquitinated ATXN3MUT aggregates, compared to control condition (n=6 for ATXN3MUT injected; n=8 for ATXN3MUT and G3BP1); ***p<0.0001; Student's t-test). (H) G3BP1 expression rescues neuronal marker loss, as compared to controls (n=6 for ATXN3MUT injected; n=8 for ATXN3MUT and G3BP1; ***p<0.0001; Student's t-test). Values are expressed as mean $\pm$ SEM.

Figure 8

Overexpression of G3BP1 in the brain of normal mice did not produce neuronal loss or inflammation. Mice at 8-12 weeks of age were stereotactically injected into the striatum (bilaterally) either with PBS or with lentiviral particles encoding for human G3BP1 and euthanized for tissues collection 4 weeks after injection. (A) Schematic representation of the injection site and the constructs used. (B) Immunohistochemistry images analysis of DARPP-32 depletion volume (dashed black line; upper panel; Scale: 200 μ m) and G3BP1 (bellow panel; Scale: 50 μ m) labelling in brain sections from mice injected with PBS and in the contralateral hemisphere injected with lentiviral particles encoding for G3BP1. (C)

The total area of DARPP-32 depletion, in mice brain sections, was reduced upon injection of lentiviral particles encoding for G3BP1 when compared to the injection with PBS (n=5; *p<0.05; Student's t-test). (D) Representative images of immunohistochemistry labelling of GFAP in brain sections from mice injected with PBS and in the contralateral hemisphere injected with lentiviral vectors encoding G3BP1. Scale: 50 μ m. Values are expressed as mean $\pm$ SEM.

Figure 9

G3BP1 expression is able to mitigate motor deficits and neuropathological abnormalities in a transgenic mouse model. SCA3 transgenic mice animals expressing a truncated form of the ataxin-3 protein containing 69 glutamines were stereotactically injected into the cerebellum with lentiviral particles encoding for GFP (control group) or injected with lentiviral particles encoding for G3BP1 (treated group). (A) Schematic representation of the timeline that was used to perform behavioural test. Mice, at 4-5 weeks of age, were first tested 1-2 days prior to injection and then repeatedly tested every three weeks until 9 weeks post-injection, to be euthanized at 10 weeks post-surgery. (B–D) Representative plots of mice motor performance 9 weeks post injection. (B) Mice injected with G3BP1 significantly improved motor performance (assessed by the rotarod test) as they remain more time at the rotating rod comparing to control mice treated with GFP or non-injected mice. (C) Mice injected with G3BP1 significantly reduced the time needed to cross the water-filled tank and reach the platform comparing to control mice treated with GFP or non-injected animals. (D) Footprint analysis showed that mice injected with G3BP1 improved overlap measures comparing to controls treated with GFP. (E) Representative images of immunohistochemistry brain sections from mice cerebella either injected with lentiviral particle encoding for GFP (control) or injected with lentiviral particles encoding for G3BP1. upper panel: ataxin-3 aggregates assessed by HA-tag immunoreactivity (arrowhead; Scale: 50 μ m and 200 μ m); bellow panel: Purkinje cells assessed by calbindin immunoreactivity (red; Scales: 100 μ m and 200 μ m). (F) G3BP1 injection significantly reduced the number of HA-ataxin-3 aggregates (arrowhead) in mice cerebella when compared to control mice injected with GFP or non-injected. (G) G3BP1 injection significantly preserved the number of Purkinje cells (red) in mice cerebella, within lobe IX, when compared to non-injected and GFP injected controls. (n=2; *p<0.05; Student's t-test). Values are expressed as mean $\pm$ SEM.

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