



ADVANCING SCA2 RESEARCH

PROTEOMIC CHARACTERIZATION, AUTOPHAGY MODULATION,
AND CEREBELLAR ORGANOID MODEL DEVELOPMENT

PhD Thesis
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This PhD Thesis was submitted to the Graduate School of Health and Medical Sciences,
University of Copenhagen, June 12th, 2024

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PREFACE

This PhD project was carried out at the Danish Dementia Research Centre, Department of Neurology, Rigshospitalet, from January 2020 to June 2024. The experimental work took place at the Neurogenetics Clinic & Research Laboratory.

The proteomic analyses were performed in collaboration with Associate Professor Johan Palmfeldt, Head of Proteomics Core Facility at Research Unit for Molecular Medicine, Department of Clinical Medicine, HEALTH, Aarhus University (Study I).

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I would like to thank my principal supervisor, Jørgen Erik Nielsen, for admitting me as a PhD student into his laboratory. Jørgen fosters a trustful and relaxed environment, all while maintaining a strong focus on unraveling the mechanisms of neurodegenerative diseases. I consider myself fortunate and lucky to have completed both my master's thesis and PhD thesis at the Danish Dementia Research Centre and the Neurogenetics Research Laboratory.

I would also like to express my gratitude to my primary co-supervisor, Patrick Ejlerskov, who had the difficult task of taking on the daily supervision midway through this project, but he helped me navigate this academic challenge and maintain my trajectory.

I express my gratitude to Associate Professor Johan Palmfeldt at Aarhus University for generously allowing me to visit his laboratory and for helping me conduct the mass spectrometry work. His invaluable assistance was pivotal in deciphering the proteomics data and igniting my fascination with this field.

Together, their scientific prowess, support, encouragement, and occasional redirection propelled my progress. Throughout my PhD journey, I encountered several obstacles, and the culmination of this thesis owes much to Jørgen's understanding and patience, as well as Patrick's role as a catalyst and mentor. Thank you.

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I give my gratitude to everyone at the DDRC, especially to the people seated at the PhD office in the corner. I am particularly grateful to my fellow PhD colleague, Anders, whom I was lucky to have by my side throughout this battle. Our daily discussions ranged from statistics to Pokémon, providing encouragement, motivation, and camaraderie. Thank you for introducing me to the grimdark and dark fantasy genre, but more importantly, it was a delight to show off my useless knowledge of weird animals as well as share my "*Lord of the Rings*" obsession with you... for me at least, and therefore, it seems fitting to end my acknowledgments to you with a quote from J.R.R. Tolkien's *The Two Towers*

"... *Frodo wouldn't have got far without Sam, would he...?*".

In the final stages of my PhD project, I was blessed by Danai's collaboration. Without her extensive organizational intellect and kind help, I truly do not know what I would have done.

Amidst the intensive work on my thesis, it's easy to overlook the importance of taking a day off. Thankfully, my friends reminded me of the significance of maintaining a life outside academia. Without their support, the process of preparing this thesis would have been far less enjoyable. I am sincerely grateful to them.

Behind the scenes, we find the people who make the show tick and run smoothly. Therefore, I would like to thank Christina for always stepping up and helping when most needed, and to Michael, I am eternally grateful

for your unwavering support. I thank you wholeheartedly for enduring my long working hours, moments of self-doubt, and endless chatter about academia and ATXN2. Alongside my two sons, Ellio and Romeo, you bring me the greatest joy in life.

My family has been my rock throughout this PhD project. I cannot overstate the profound impact their support has on me. I can confidently say that this dissertation was only made possible by their unwavering support, help, and commitment to seeing me succeed.

My final remark and acknowledgment will go to the patients affected by this rare, hereditary form of ataxia. I truly hope this PhD thesis will help pave the way for future treatments, helping affected families combat this debilitating disorder.

LIST OF MANUSCRIPTS

1. Wardman JH, **Henriksen EE**, Marthaler AG, Nielsen JE, Nielsen TT. Enhancement of Autophagy and Solubilization of Ataxin-2 Alleviate Apoptosis in Spinocerebellar Ataxia Type 2 Patient Cells. *Cerebellum*. 2020 Apr;19(2):165-181. doi: 10.1007/s12311-019-01092-8. PMID: 31898278.

2. **Emil Elbæk Henriksen**, Anne Sigaard Bie, Jonathan Henry Wardman, Anders Toft, Cecilie Madsen, Jørgen Erik Nielsen, Johan Palmfeldt, Patrick Ejlerskov. PolyQ Length-Dependent ATXN2 Interaction with Chaperones and Ribosomal Proteins in Spinocerebellar Ataxia Type 2 Patient Fibroblasts. *Manuscript in Preparation*

ABSTRACT

Spinocerebellar ataxia type 2 (SCA2) is a progressive neurodegenerative disorder characterized by a CAG repeat expansion in the ATXN2 gene, resulting in a polyglutamine expansion in the ataxin-2 protein. Despite significant advances in elucidating the genetic underpinnings of SCA2, the precise cellular and molecular mechanisms driving its pathology remain inadequately understood, and effective disease-modifying treatments are currently unavailable. This PhD thesis aims to elucidate the complex pathomechanisms of SCA2 by applying advanced proteomic analyses, modulation of autophagy pathways, and developing hindbrain organoid models.

The first study examines the impact of polyglutamine expansion on the RNA binding profile, protein-protein interactions, and global proteome of SCA2 patient-derived cell lines. Immunoprecipitation coupled with RNA sequencing and mass spectrometry reveals significant alterations in the RNA binding profile and protein interaction network of ataxin-2 in SCA2 cells. The global proteomic analysis indicates dysregulation of pathways related to translation, ribosomal function, and cellular stress responses.

The second study investigates the roles of proteasome function and autophagy in SCA2 pathology. Pharmacological modulation of these pathways in patient-derived fibroblasts demonstrates that enhancing autophagy and solubilizing ataxin-2 can alleviate apoptosis and mitigate cellular phenotypes associated with SCA2.

The third study focuses on developing a novel protocol for differentiating induced pluripotent stem cells (iPSCs) into ventral hindbrain neurons, with the objective of establishing a cerebellar organoid model for SCA2. The protocol integrates strategies from recent studies and introduces a doxycycline-inducible *NGN2* expression system to enhance the efficiency and purity of the generated neurons.

Collectively, these studies provide novel insights into the molecular mechanisms underlying SCA2 pathology, underscore the therapeutic potential of targeting autophagy and protein aggregation, and establish a foundation for developing advanced cerebellar models to further investigate SCA2 and test therapeutic interventions. This thesis contributes to the understanding of SCA2 pathogenesis and paves the way for future research aimed at developing effective therapies for this debilitating disorder.

RESUMÉ

Spinocerebellar ataksi type 2 (SCA2) er en progressiv neurodegenerativ lidelse karakteriseret ved en CAG-repeat-ekspansion i *ATXN2*-genet, hvilket resulterer i en toksisk polyglutamin-ekspansion i ataxin-2-proteinet. På trods af betydelige fremskridt i forståelsen af de genetiske årsager til SCA2, er de præcise cellulære og molekylære mekanismer, der driver sygdommens patologi, stadig uklare, og effektive sygdomsmodificerende behandlinger er i øjeblikket ikke tilgængelige. Denne ph.d.-afhandling sigter mod at belyse de komplekse patomekanismer ved SCA2 gennem anvendelse af avancerede proteomiske analyser, modulering af autofagiveje og udvikling af baghjerne-organoidmodeller.

Det første studie undersøger påvirkningen af polyglutamin-ekspansion på ataxin-2 med fokus på RNA-bindingsprofilen, protein-protein-interaktioner samt hvordan det påvirker det globale proteom i celler afledt fra SCA2-patienter. Immunpræcipitation kombineret med RNA-sekventering og massespektrometri afslører betydelige ændringer i RNA-bindingsprofilen og proteininteraktionsnetværket af ataxin-2 i SCA2-celler, dertilhørende viser den globale proteomiske analyse en indikation på dysregulering af cellulære processer relateret til translation, ribosomfunktion og stressrespons.

Det andet studie undersøger rolle af proteasomet samt autofagi i SCA2-patologien. Farmakologisk modulering af disse cellulære processer i patientafledte fibroblaster demonstrerer, at aktivering af autofagi samt farmakologisk opløsning af ataxin-2 kan lindre apoptose og afbøde cellulære fænotyper forbundet med SCA2.

Det tredje studie fokuserer på at udvikle en ny protokol til differentiering af inducerede pluripotente stamceller (iPSCs) til ventrale baghjerne-neuroner med det formål at etablere en cerebellar organoidmodel for SCA2. Protokollen integrerer strategier fra nyere studier og introducerer et doxycyclin-inducerbart *NGN2*-ekspressionssystem for at forbedre effektiviteten og potentielt renheden af de genererede neuroner.

Samlet set giver disse studier nye indsigter i de molekylære mekanismer, der ligger til grund for SCA2-patologi, samt understreger det terapeutiske potentiale ved at modulere autofagi og proteinaggregering farmakologisk, og etablerer et fundament for udvikling af avancerede cerebellare modeller til yderligere undersøgelse af SCA2 samt test af terapeutiske interventioner. Denne afhandling bidrager til forståelsen af SCA2-patogenese og baner vejen for fremtidig forskning med henblik på at udvikle effektive behandlinger for denne invaliderende lidelse.

ABBREVIATIONS

5'-TOP mRNA: 5'-Terminal Oligopyrimidine Motif Containing mRNA

AD: Alzheimer's Disease

ADCA: Autosomal Dominant Cerebellar Ataxia

AFG3L2: ATPase Family Gene 3-Like 2

AIFM1: Apoptosis Inducing Factor Mitochondria Associated 1

AKT1/2: AKT Serine/Threonine Kinase 1/2

ALDH3A2: Aldehyde Dehydrogenase 3 Family Member A2

ALDOC: Aldolase C

ALS: Amyotrophic Lateral Sclerosis

ARCA: Autosomal Recessive Cerebellar Ataxia

ASO: Antisense Oligonucleotide

ATG8: Autophagy-Related Protein 8

ATOH1: Atonal BHLH Transcription Factor 1

ATP5J: ATP Synthase Membrane Subunit E

ATPase 6: Mitochondrially Encoded ATP Synthase Membrane Subunit 6

ATX-2: Caenorhabditis Elegans Ortholog Of ATXN2

Atx2: Drosophila ATXN2 Homolog

ATXN2: Ataxin-2

ATXN2L: Ataxin-2 Like

BAG3: BAG Cochaperone 3

BARHL1: Barh-Like Homeobox 1

BDNF: Brain-Derived Neurotrophic Factor

BMPs: Bone Morphogenetic Proteins

BSA: Bovine Serum Albumin

CAG: Cytosine-Adenine-Guanine

CALB1: Calbindin 1

CASA: Chaperone-Assisted Selective Autophagy

CHIR99021: Glycogen Synthase Kinase 3 Inhibitor

CKAP4: Cytoskeleton Associated Protein 4

CMA: Chaperone-Mediated Autophagy

CoQ10: Coenzyme Q10

CUL4B: Cullin 4B

CAA: Cytosine-Adenine-Adenine

DDX6: DEAD-Box Helicase 6

Dibutyryl-Camp: Dibutyryl Cyclic AMP

DISC: Death-Inducing Signaling Complex

DNAJC3: Dnaj Heat Shock Protein Family (Hsp40) Member C3

EBs: Embryoid Bodies

EEF1A1: Eukaryotic Translation Elongation Factor 1 Alpha 1

EEF2: Eukaryotic Translation Elongation Factor 2

EIF4A1: Eukaryotic Translation Initiation Factor 4A1

EN2: Engrailed Homeobox 2

ER: Endoplasmic Reticulum

ESCs: Embryonic Stem Cells

ETFA: Electron Transfer Flavoprotein Subunit Alpha

FGF: Fibroblast Growth Factor

FGF19: Fibroblast Growth Factor 19

FGF2/bFGF: Fibroblast Growth Factor 2/Basic FGF

FGF8: Fibroblast Growth Factor 8

FGF8b: Fibroblast Growth Factor 8b

FTD: Frontotemporal Dementia

GABARAP: GABA Type A Receptor-Associated Protein

GBX2: Gastrulation Brain Homeobox 2

GDNF: Glial Cell Line-Derived Neurotrophic Factor

GFAP: Glial Fibrillary Acidic Protein

GRID2: Glutamate Ionotropic Receptor Delta Type Subunit 2

GSK3: Glycogen Synthase Kinase 3

GSK3i: Glycogen Synthase Kinase 3 Inhibitor

H₂O₂: Hydrogen Peroxide

HD: Huntington's Disease

hESCs: Human Embryonic Stem Cells

HINT2: Histidine Triad Nucleotide Binding Protein 2

HSD17B10: Hydroxysteroid 17-Beta Dehydrogenase 10

HSP: Heat Shock Protein

HSP70: Heat Shock Protein 70

HSP90: Heat Shock Protein 90

HSP90AA1: Heat Shock Protein 90 Alpha Family Class A Member 1

HSPA1A: Heat Shock Protein Family A (Hsp70) Member 1A

HSPA5: Heat Shock Protein Family A (Hsp70) Member 5

HSPA8: Heat Shock Protein Family A (Hsp70) Member 8

HUWE1: HECT, UBA And WWE Domain Containing E3 Ubiquitin Protein Ligase 1

IDR: Intrinsically Disordered Region

IMM: Inner Mitochondrial Membrane

IP3R1: Inositol 1,4,5-Trisphosphate Receptor Type 1

IP-MS: Immunoprecipitation-Mass Spectrometry

IP-RNAseq: Immunoprecipitation-RNA Sequencing

iPSC: Induced Pluripotent Stem Cell

ITS: Insulin-Transferrin-Selenium

L7/PCP2: Purkinje Cell Protein 2

LAMP2: Lysosomal Associated Membrane Protein 2

LAMTOR1/2/3/5: Late Endosomal/Lysosomal Adaptor, MAPK And MTOR Activator 1/2/3/5

LC3: Microtubule-Associated Protein 1A/1B-Light Chain 3

LC3B/LC3-II: Microtubule-Associated Protein 1A/1B-Light Chain 3 Beta

LDN193189: Bone Morphogenetic Protein Inhibitor

LEPRE1: Leucine And Proline Enriched Proteoglycan 1

LHX1: LIM Homeobox 1

LHX5: LIM Homeobox 5

LRRFIP2: Leucine Rich Repeat Flightless-Interacting Protein 2

LSm: Like-Sm

LSM12: LSM12 Homolog

LSmAD: Like-Sm Associated Domain

MAP2: Microtubule Associated Protein 2

MG-132: Proteasome Inhibitor

MiSTR: Microfluidic-Controlled Stem Cell Regionalization

mPOS: Mitochondrial Precursor Over-Accumulation Stress

MRI: Magnetic Resonance Imaging

mRNP: Messenger Ribonucleoprotein

MS: Mass Spectrometry

mTOR: Mammalian/Mechanistic Target Of Rapamycin

mTORC2: Mammalian/Mechanistic Target Of Rapamycin Complex 1

NANOG: Nanog Homeobox

NDUFA13: NADH:Ubiquinone Oxidoreductase Subunit A13

NDUFA5: NADH:Ubiquinone Oxidoreductase Subunit A5

NDUFA6: NADH:Ubiquinone Oxidoreductase Subunit A6

NDUFAB1: NADH:Ubiquinone Oxidoreductase Subunit AB1

NDUFB10: NADH:Ubiquinone Oxidoreductase Subunit B10

NDUFV1: NADH:Ubiquinone Oxidoreductase Core Subunit V1

NEPH3: NEPHR0-3 Homolog

NGN1: Neurogenin 1

NGN2: Neurogenin 2

NIM: Neuronal Induction Medium

NMM: Neuronal Maturation Medium

OCT4: POU Class 5 Homeobox 1

OLIG2: Oligodendrocyte Transcription Factor 2

OTX2: Orthodenticle Homeobox 2

OXPHOS: Oxidative Phosphorylation

PABPC1: Poly(A) Binding Protein Cytoplasmic 1

PAM2: Poly(A) Binding Protein Interacting Motif 2

PAX2: Paired Box 2

Pbp1: Polyadenylate-Binding Protein 1

PBS: Phosphate-Buffered Saline

PCA: Principal Component Analysis

PI3K: Phosphatidylinositol 3-Kinase

PLA: Proximity Ligation Assay

PolyQ: Polyglutamine

PPI: Protein-Protein Interaction

PRDX3: Peroxiredoxin 3

PSCs: Pluripotent Stem Cells

PSMA4: Proteasome Subunit Alpha 4

PSMD4: Proteasome 26S Subunit, Non-Atpase 4

PSME3: Proteasome Activator Subunit 3

PTF1A: Pancreas Transcription Factor 1 Subunit Alpha

Purmorphamine: SHH Agonist

qPCR: Quantitative Polymerase Chain Reaction

RA: Retinoic Acid

RAB22A: RAB22A, Member RAS Oncogene Family

RBP: RNA Binding Protein

RHEB: Ras Homolog, Mtorc1 Binding

RNAseq: RNA Sequencing

ROS: Reactive Oxygen Species

RPL13A: Ribosomal Protein L13a

RPL8: Ribosomal Protein L8

RPS18: Ribosomal Protein S18

RPS26: Ribosomal Protein S26

RPS28: Ribosomal Protein S28

RPS3: Ribosomal Protein S3

RRAGD: Ras Related GTP Binding D

SAM: Sorting and Assembly Machinery

SB431542: TGF-Beta Inhibitor

SCA: Spinocerebellar Ataxia

SCA1: Spinocerebellar Ataxia Type 1

SCA2: Spinocerebellar Ataxia Type 2

SCA3: Spinocerebellar Ataxia Type 3

SCA6: Spinocerebellar Ataxia Type 6

SCA7: Spinocerebellar Ataxia Type 7

SDF1: Stromal Cell-Derived Factor 1

SDF1a: Stromal Cell-Derived Factor 1 Alpha

SG: Stress Granule

SHH: Sonic Hedgehog

SHH-C24II: Sonic Hedgehog C-Terminal Peptide

SLC25A6: Solute Carrier Family 25 Member 6

SNHG5: Small Nucleolar RNA Host Gene 5

SQSTM1/P62: Sequestosome 1

STAU1: Staufen Double-Stranded RNA Binding Protein 1

STUB1: STIP1 Homology And U-Box Containing Protein 1

SUCLA2: Succinate-CoA Ligase ADP-Forming Beta Subunit

SYNE2: Spectrin Repeat Containing Nuclear Envelope Protein 2

TDP-43: TAR DNA-Binding Protein 43

TGF-B: Transforming Growth Factor Beta

TIM: Translocase of Inner Mitochondrial Membrane

TIMM50: Translocase of Inner Mitochondrial Membrane 50

TOM: Translocase of Outer Mitochondrial Membrane

Torc1: Target of Rapamycin Complex 1

TSPO: Translocator Protein

TUBB3: Tubulin Beta 3 Class III

UBA5: Ubiquitin Like Modifier Activating Enzyme 5

UBA7: Ubiquitin Like Modifier Activating Enzyme 7

ULK1: Unc-51 Like Autophagy Activating Kinase 1

UPS: Ubiquitin-Proteasome System

VDAC1: Voltage-Dependent Anion Channel 1

VDAC2: Voltage-Dependent Anion Channel 2

VZ: Ventral Ventricular Zone

WDFY3: WD Repeat and FYVE Domain Containing 3

WNT: Wingless-Type MMTV Integration Site Family

WNT1: Wingless-Type MMTV Integration Site Family, Member 1

WNT3A: Wingless-Type MMTV Integration Site Family, Member 3A

Y-27632: ROCK Inhibitor

INTRODUCTION

Investigations into diseases often commence by outlining their personal and societal impacts, emphasizing the absence of effective cures or treatments. This approach is particularly pertinent for neurodegenerative disorders due to their relentless and progressive nature. These diseases are becoming more prevalent, especially in the aging populations of developed countries.

Neurodegenerative diseases such as Alzheimer's disease progressively impair critical aspects of the human experience, including memory and identity. Similarly, conditions like Amyotrophic Lateral Sclerosis (ALS) and Spinocerebellar Ataxia (SCA) effectively imprison a functioning mind within a deteriorating body. Despite extensive research efforts, there are no definitive cures available. Even the most advanced treatments, such as the 2023-approved Lecanemab for Alzheimer's disease (AD), only manage to slightly prolong life or maintain bodily functions.

This PhD thesis is dedicated to fundamental research into Spinocerebellar Ataxia type 2 (SCA2). It specifically investigates the cellular alterations induced by the causative protein, utilizing cells derived from patients. The aim is to unravel the cellular underpinnings of this disease, which could potentially lead to targeted therapies.

Moving from the general implications of neurodegenerative diseases, the thesis will next introduce the complexities of inherited ataxia. This section introduces the genetic and clinical diversity that characterizes these diseases, setting the stage for a detailed introduction to SCA2. This progression is designed to provide a comprehensive understanding of the broader category of ataxias, thereby enriching the focused investigation of SCA2.

Cerebellar Ataxias

Inherited ataxias constitute a heterogeneous group of cerebellar disorders characterized by distinct genetic foundations, inheritance modalities, clinical manifestations, and rates of progression. These disorders, which impair coordinated movement and balance, are systematically classified into four categories: autosomal dominant cerebellar ataxias, autosomal recessive cerebellar ataxias, X-linked ataxias, and mitochondrial ataxias.

Autosomal Cerebellar Ataxias: A Spectrum of Dominance and Recessivity

Autosomal Dominant Cerebellar Ataxia (ADCA) represents a spectrum of disorders where a single mutated allele is sufficient for disease manifestation, often presenting in adulthood but sometimes emerging in childhood or adolescence¹⁻⁴. The hallmark of these disorders is cerebellar dysfunction accompanied by a range of symptoms, including ophthalmoplegia, optic atrophy, and cognitive deficits¹⁻⁴. ADCA is further subclassified based on pathogenetic mechanisms^{1,2,4,5}: Type I involves CAG repeat expansions and presents with a mix of cerebellar, pyramidal, and neuropathic symptoms^{1,2,5}; Type II is associated with trinucleotide repeats outside protein-coding regions, often coupled with retinal degeneration^{1,2,5}; and Type III arises from specific gene mutations leading to pure cerebellar ataxia^{1,2,6}.

In contrast, Autosomal Recessive Cerebellar Ataxia (ARCA) requires two defective alleles for disease development, typically resulting in a more aggressive and rapidly progressing condition with an earlier onset^{4,7}. ARCA encompasses a variety of rare conditions, including developmental, metabolic, and degenerative ataxias, with

symptoms extending from cerebellar ataxia to cognitive impairment^{4,7}.

X-Linked and Mitochondrial Ataxias: Unique Inheritance and Varied Presentations

X-Linked Ataxias are characterized by cerebellar dysgenesis due to mutations on the X chromosome, leading to a range of neurological manifestations such as hypotonia, intellectual disability, and ataxia. Over 20 genes on the X chromosome have been linked to these conditions, which may present in isolation or alongside other brain malformations⁸.

Mitochondrial Ataxias, on the other hand, can follow either a maternally inherited or Mendelian pattern. Those following a Mendelian pattern are categorized within ADCAs or ARCAs. The maternally inherited mitochondrial ataxias, resulting from mutations in mitochondrial DNA like the ATPase 6 gene, can manifest as isolated ataxia or as part of a multisystem disorder, with pathological features that may include sensorineural deafness and myoclonus⁹.

The forthcoming sections will focus on Spinocerebellar Ataxias (SCAs) that follow an autosomal dominant inheritance pattern, isolating them from the broader ataxic landscape to underscore their global prevalence and distinct clinical features. This detailed examination will provide an understanding of the genetic and phenotypic intricacies that define these complex neurological conditions.

Spinocerebellar Ataxias

Spinocerebellar ataxias (SCAs) are a group of ADCAs characterized by complex genetics and variable phenotypic presentations. These disorders are primarily marked by progressive coordination difficulties due to cerebellar degeneration, accompanied by a range of other neurological symptoms. The incidence and prevalence of SCAs exhibit significant geographic and ethnic variations.

Epidemiological studies indicate that SCAs have a global prevalence estimated to range from 0 to 5.6 cases per 100,000 individuals^{2,10–13}. This figure, however, is likely an underestimation due to factors such as underdiagnosis and the lack of comprehensive genetic testing in many regions. In Europe, prevalence rates are heterogeneous, ranging from 0.9 to 3 cases per 100,000¹³, with regional hotspots identified in Italy, Poland, and Portugal¹⁰. The prevalence of specific SCA subtypes varies regionally; for example, SCA3 is the predominant subtype in the Americas and Europe¹¹, while SCA2 is more prevalent in India and South Korea, and SCA6 is predominant in Japan¹².

SCAs represent a genetically and clinically diverse group of cerebellar degenerative disorders. Over 40 genetically distinct SCAs have been identified, each associated with a specific genetic locus. The most common SCAs, including SCA1, SCA2, SCA3, SCA6, and SCA7, account for the majority of diagnosed cases¹³.

SCAs are unified by the hallmark symptom of progressive cerebellar ataxia, which manifests as unsteady and imprecise movements due to the deterioration of fine motor coordination. The clinical spectrum often extends beyond motor incoordination to include neurological symptoms such as oculomotor disturbances, dysarthria, and

cognitive impairment^{5,13}. The onset of symptoms and the rate of progression vary, typically beginning in adulthood⁵.

On a molecular level, most SCAs are characterized by genetic mutations that lead to the dysfunction of proteins critical for neuronal health. Many SCAs involve expansions of CAG trinucleotide repeats that encode polyglutamine (polyQ) tracts within the proteins^{4,5,14}. These expanded polyQ tracts are prone to misfolding and aggregation, leading to neuronal dysfunction and cell death. Cerebellar atrophy, a direct consequence of this neurodegeneration, is often detectable through neuroimaging techniques like MRI¹⁴. The degree of cerebellar atrophy correlates with the severity of ataxia and impairment of coordination.

These commonalities provide a framework for understanding the pathogenesis of SCAs and form the basis for ongoing research into potential therapeutic strategies. Despite their shared characteristics, the heterogeneity of SCAs necessitates an individualized approach to diagnosis and management, considering the specific genetic, clinical, and molecular context of each subtype.

Spinocerebellar Ataxia Type 2

SCA2 is among the more common forms of SCAs, second only to SCA3 in terms of prevalence¹⁵. However, the distribution of SCA2 is not uniform worldwide and exhibits significant geographic and ethnic variability. Certain populations show a markedly increased incidence, likely due to a founder effect or genetic drift. Notable clusters of SCA2 prevalence have been documented in specific regions:

- Cuba¹⁶: SCA2 is the most prevalent form of hereditary ataxia, particularly concentrated in the Holguín province.
- Mexico¹¹, South Africa¹⁷, India^{12,18}, South Korea¹², and Italy¹⁰: These regions have reported elevated frequencies of SCA2, often presenting with familial aggregations indicative of a higher disease burden.

The exact prevalence of SCA2 remains elusive in many countries, with challenges such as underdiagnosis and misdiagnosis obscuring the true global distribution. Comprehensive epidemiological studies are essential to accurately determine the prevalence and distribution of SCA2, which will aid in understanding the disease's impact and guiding research and healthcare strategies.

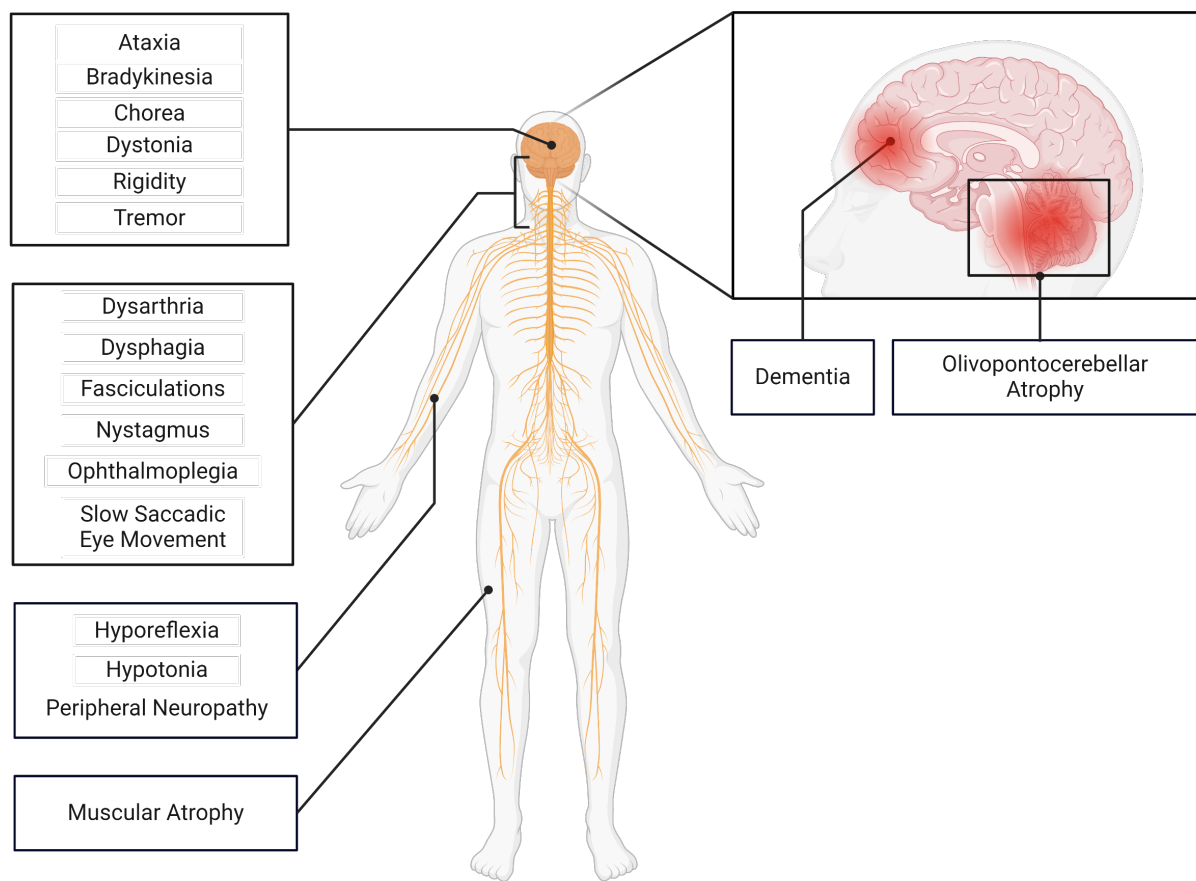
Clinical Features of SCA2

SCA2 is a progressive neurodegenerative disorder typically manifesting between the third and fourth decades of life, with an average onset around 30 years^{19,20}. Notably, some cases present earlier due to anticipation, a genetic phenomenon where successive generations experience earlier onset and more severe disease progression, particularly in younger individuals^{20,21}.

SCA2 is characterized by a spectrum of progressive symptoms that vary in onset and severity but generally converge on several key neuropathological phenomena:

The defining feature of SCA2 is progressive cerebellar ataxia, leading to a gradual decline in muscle coordination. This cerebellar dysfunction manifests through gait instability, dysarthria (motor speech difficulties), and limb incoordination, significantly impairing the quality of life^{19,22}.

Ophthalmoplegia, involving a range of ocular motor deficits, is another prominent feature. This includes nystagmus (involuntary oscillatory eye movements) and saccadic slowing (reduced speed of eye movements), which are critical diagnostic indicators and contribute to the disease's complexity^{19,22}.



INTRODUCTORY FIGURE 1 - Schematic illustration highlighting SCA2 features and the affected areas. Illustration created in Biorender.

Peripheral neuropathy in SCA2 presents as sensory loss, diminished reflexes, and progressive muscle weakness, potentially leading to severe muscle wasting and dystonia (involuntary muscle contractions), further deteriorating motor function^{19,22}.

Beyond primary ataxia symptoms, individuals with SCA2 may exhibit various movement disorders, prominently parkinsonism, characterized by bradykinesia (slowness of movement), tremor, and rigidity. These symptoms complicate the motor phenotype and management strategies²².

Cognitive impairment in SCA2 ranges from mild executive dysfunction to severe dementia, with deficits in short-term memory, executive function, and visuospatial skills. These cognitive challenges significantly affect daily living activities and contribute to the overall disease burden^{19,22}.

The clinical trajectory of SCA2 is marked by a progressive worsening of these symptoms, leading to increased

disability and dependence on care. Understanding these hallmark features is crucial for timely diagnosis and management, aiming to improve life quality and extend independence for affected individuals.

Genetics of SCA2

SCA2 is caused by a specific genetic mutation in the *ATXN2* gene. This gene, located on chromosome 12q24, plays a crucial role in the development of SCA2 through a pathogenic mutation characterized by the expansion of CAG trinucleotide repeats within exon 1^{23,24}.

The *ATXN2* gene spans approximately 130 kilobases and consists of 25 exons²⁴. Under normal conditions, it contains a tract of 22 or fewer CAG repeats²³. However, in the context of SCA2, this number is increased to 33 or more repeats²⁵, with a threshold of 37-39 CAG repeats being associated with complete pathology penetrance^{22,26}. This expansion is the hallmark mutation leading to the onset of SCA2 symptoms.

One of the intriguing aspects of the CAG repeat expansion mutation is its propensity for further expansion in successive generations, a phenomenon known as genetic anticipation. This results in affected offspring presenting with an earlier onset and more severe disease pathology compared to their predecessors. The presence of interruptions in the CAG repeat sequence by CAA codons has been observed to confer greater stability to the *ATXN2* gene, potentially mitigating the risk of further expansions²³.

Interestingly, alleles with 27 to 33 CAG repeats, while not always resulting in SCA2, have been linked to an elevated risk for other neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS)²⁷⁻²⁹ and frontotemporal dementia (FTD)³⁰. This overlap suggests a broader genetic interplay between these conditions, highlighting the significance of the *ATXN2* gene beyond SCA2 alone.

Research into the *ATXN2* gene has shown a high degree of conservation across various species, underscoring its importance in biological processes. However, studies, such as those conducted by Nechiporuk and colleagues, reveal that not all homologs of the *ATXN2* gene contain a CAG-repeat region. For instance, the mouse variant of *ATXN2*, despite its significant similarity to the human gene, contains only a single CAG triplet³¹. This finding suggests that the presence of a glutamine-rich segment, typically encoded by CAG repeats, may not be critical for the protein's functionality in some species.

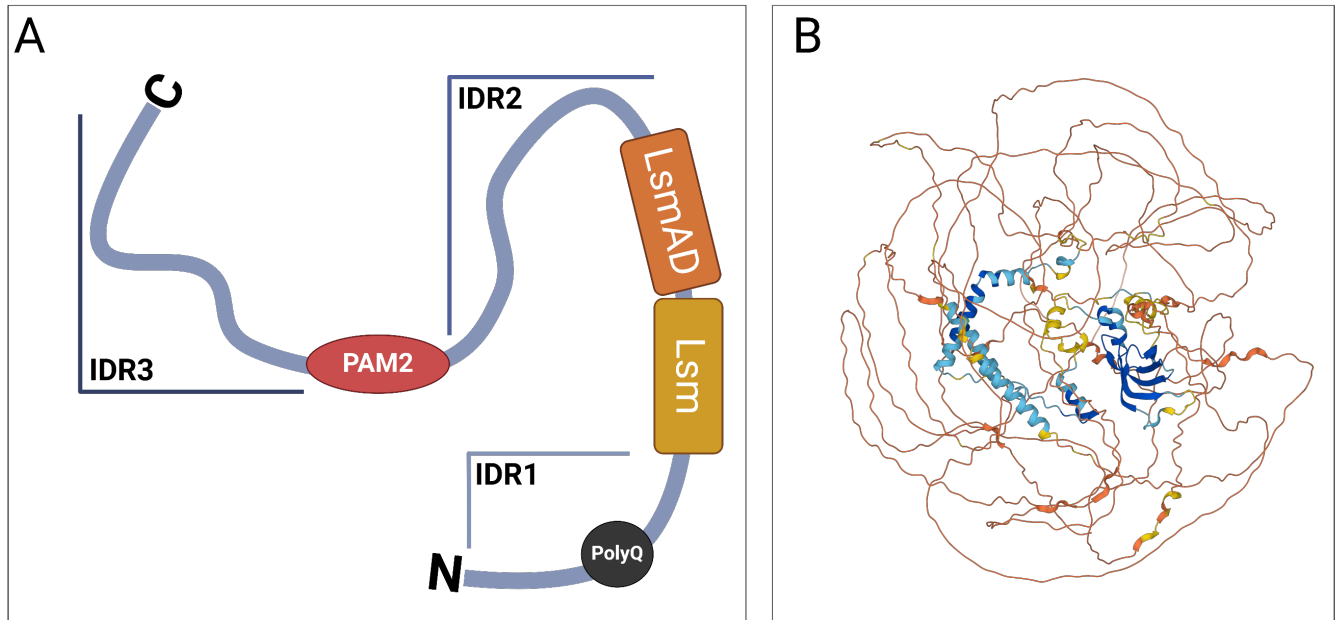
ATXN2 Protein: Structure, Domains, Protein-protein Interactors, and Function

The *ATXN2* gene encodes the ataxin-2 protein (referred to as ATXN2 in this thesis), also known in various species as Pbp1 in yeast and Atx2 in *Drosophila*³². This protein is characterized by its high affinity for RNA, playing a crucial role in RNA metabolism and cellular homeostasis. Human ATXN2 is predominantly a cytosolic protein with a molecular weight of approximately 140 kDa²⁴ and is ubiquitously expressed across various tissues. Notably, its expression is significantly elevated in neural tissues^{33,34}, with the cerebellum exhibiting one of the highest levels of ATXN2 expression³⁵.

ATXN2 is evolutionarily conserved across different species, and its homologs and paralogs share three structured domains: LSm, LSm-AD, and PAM2³⁶. The protein contains the Like-Sm (LSm) and LSm-associated (LSmAD)

domains near its N-terminus, which are conserved structures involved in RNA binding. Additionally, ATXN2 possesses a polyA-binding protein interacting motif (PAM2) near its C-terminus. This motif facilitates interaction with polyA-binding protein 1 (PABPC1), enhancing mRNA stability and indicating a role in translational activation

37–40



INTRODUCTORY FIGURE 2 - Protein structure of ATXN2

A: Schematic illustration of the ATXN2 protein structure. Illustration modified from Boeynants *et al.*, 2023 in Biorender. **B:** Predicted ATXN2 protein structure in 3D (AlphaFold structure prediction), <https://alphafold.ebi.ac.uk/entry/Q99700>

Functional Domains and Their Implications in ATXN2

The LSm, LSm-AD, and PAM2 domains within ATXN2 play critical roles in RNA binding and are essential for the protein's involvement in regulatory protein-protein interactions during RNA translational control^{40–43}. These domains underscore ATXN2's pivotal role in RNA metabolism, particularly in stabilizing mRNA within messenger ribonucleoprotein (mRNP) granules and modulating post-transcriptional gene regulation. ATXN2 acts as both a suppressor and an enhancer of translation for various mRNAs, highlighting its dual functionality in gene expression regulation^{39,44–46}.

Additionally, ATXN2 contains proline-rich regions and motifs that may facilitate various protein-protein interactions, contributing to processes such as receptor endocytosis and cellular growth signaling pathways. These interactions underscore the protein's versatility and its involvement in multiple cellular functions^{47,48}.

Intrinsically Disordered Regions and Their Biological Significance

ATXN2 is characterized as an intrinsically disordered protein, segmented into three intrinsically disordered regions (IDR1, 2, and 3)^{49,50}. These IDRs lack a fixed or ordered three-dimensional structure^{51,52}, which allows them to engage in weaker, more promiscuous interactions with various RNAs and protein partners^{53,54}. Despite their lack

of stable structure, IDRs are crucial for the protein's function, particularly in facilitating phase transitions or forming condensates^{50,52}. These are dynamic, membrane-less intracellular compartments that respond rapidly to changes in the cellular environment and stress, playing a vital role in cellular homeostasis^{51–53,55}.

PolyQ Region and Its Role in Neurodegeneration

The ATXN2 protein includes a polyQ region, which is critical in the context of SCA2. In SCA2 patients, expanded CAG repeats lead to an abnormally long polyQ tract³². This polyQ stretch, located near the N-terminal Lsm domain in humans and between the Lsm-AD and PAM2 domains in insects, varies in length across species and individuals^{32,36}. The length of the polyQ tract varies in the healthy population, with 22Q and 23Q being the most frequent variants. In the context of polyQ disorders, a pivotal threshold exists for the CAG repeat expansion necessary to precipitate SCA2^{32,36}.

Research suggests that the expanded polyQ tract may also influence ATXN2 stability, potentially increasing the protein's half-life through mechanisms that are yet to be fully understood²⁷.

Proteomic Insights into ATXN2 Interactions

Recent proteomic studies have significantly advanced our understanding of ATXN2's intracellular role and function. A notable study from 2016 identified ATXN2's interactions with a diverse array of proteins involved in translation regulation, ribosomal function, and RNA splicing events⁵⁶. These interactions underscore ATXN2's integral role in RNA metabolism and translation, consistent with its structural characteristics and its classification as a promiscuous, intrinsically disordered protein^{32,57,58}.

ATXN2 in mRNA Regulation and Translation

Further investigations have revealed that ATXN2 is actively involved in the assembly of mRNP complexes and polyribosomes, highlighting its direct engagement with the translational machinery and its potential role as a translational regulator^{49,59–61}. Additionally, ATXN2's interaction with the DEAD/H-Box RNA helicase (DDX6), a key player in mRNA control, reinforces its pivotal role in RNA metabolism⁴⁴. Additionally, the interaction and binding of PABPC1 to ATXN2 is also important for ATXN2's ability to regulate mRNA metabolism, mRNA stability, and translation efficiency^{50,58,60}. Moreover, a study reported that the ATXN2:PABPC1 interaction is critical for regulating the conformational changes of ATXN2's protein structure, enabling condensation and de-mixing into stress granules⁵⁰.

Role of ATXN2 in Stress Granule Dynamics

Under cellular stress, ATXN2 localizes to stress granules (SGs), which are transient cytoplasmic aggregates that form in response to environmental stressors^{50,62}. These membrane-less organelles are crucial for storing various biomolecules during unfavorable conditions, including ribosomal subunits, RNAs, translation factors, and RNA-

binding proteins, and exhibit dynamic, liquid-like properties^{62,63}. The presence of ATXN2 in SGs, although not essential for their assembly or disassembly, highlights its role as a client protein within these structures⁵⁰. The formation of SGs constitutes an essential cellular mechanism in response to various extracellular stressors, including mechanical or thermal stress, nutrient depletion, hypoxic conditions, and the presence of reactive oxygen species⁶³.

ATXN2 and Nutrient Stress Response

ATXN2 also plays a significant role in the regulation of nutrient stress responses, potentially through its interactions with the mTOR signaling pathway⁶¹. This suggests a broader function in metabolic regulation, further supported by the yeast ortholog of ATXN2, Pbp1, which acts as a negative Torc1 (mTORC1) regulator in response to heat or nutritional stress^{64–67}. This evidence indicates that Pbp1 modulates Torc1 signaling and serves as a stress-responsive modulator of mRNA translation at the pre-initiation complex, particularly under low cellular energy conditions⁶⁴. Additionally, ATX-2, the *Caenorhabditis elegans* ortholog of ATXN2, has been implicated in regulating TORC1-mediated nutrient responses via sequestration to stress granules⁶⁸.

In summary, ATXN2 is an intrinsically disordered multifunctional protein with significant roles in RNA binding, mRNA stability, translation, stress response, nutrient regulation, and potentially mTOR signaling. Its diverse functions highlight its importance in maintaining cellular homeostasis and responding to environmental changes.

Cellular Phenotype of SCA2

SCA2 manifests through distinct cellular pathological phenotypes, notably mitochondrial dysfunction⁶⁹, dysregulated calcium homeostasis^{70,71}, and autophagic dysregulation⁷², each contributing to the neurodegenerative process characteristic of the disorder. The following sections will delve into two cellular phenotypes that characterize SCA2 pathology: mitochondrial dysfunction and dysregulated autophagy.

Mitochondrial Dysfunction and Morphology

Mitochondria are essential for numerous cellular functions, including ATP production through oxidative phosphorylation (OXPHOS), phospholipid biosynthesis, fatty acid catabolism, calcium homeostasis, apoptosis regulation, and the synthesis of various metabolic intermediates and cofactors^{73,74}. The architecture of mitochondria, encapsulated by double membranes, is remarkably dynamic, influenced significantly by the processes of fusion and fission. These dynamics are crucial for maintaining the structural integrity and functional capacity of the mitochondrial network^{73,74}. Moreover, the balance between the removal of damaged mitochondria and the generation of new ones is vital for adapting the mitochondrial population to the cellular energy requirements^{75,76}. Consequently, mitochondrial dysfunction can disrupt these processes, leading to calcium dysregulation, energy deficits, excessive production of reactive oxygen species (ROS), and the activation of apoptotic pathways, all of which negatively impact cellular health and functionality. The implications of mitochondrial dysfunction extend to a wide range of disorders, encompassing neurodegenerative diseases and cardiac dysfunctions^{73,74}.

Studies underscore the pivotal role of oxidative stress and mitochondrial dysfunction in the progression of SCA2. Investigations into SCA2 patient-derived fibroblasts have revealed pronounced oxidative stress and notable alterations in mitochondrial morphology, highlighting the critical need to address these mitochondrial aspects for disease amelioration in SCA2⁶⁹. A 2019 study reported diminished oxygen consumption rates in SCA2-induced pluripotent stem cell (iPSC)-derived neurons compared to controls when cultured in a medium containing glutamate. Additionally, mitochondrial stress tests conducted under these conditions exhibited attenuated responses to stressor treatments, indicating a significant mitochondrial bioenergetic dysfunction in SCA2-iPSC-derived neurons⁷⁷.

Furthermore, the study on fibroblasts from SCA2 patients reported anomalies in the antioxidant system and reduced activity in mitochondrial electron transport complexes I and II/III relative to controls⁶⁹. These findings suggest that oxidative stress and mitochondrial dysfunction are integral to the pathophysiology of SCA2. The administration of the antioxidant coenzyme Q10 has demonstrated efficacy in mitigating oxidative stress and associated phenotypes in SCA2 patient fibroblasts⁶⁹, indicating that ROS and subsequent mitochondrial dysfunction are involved in the pathogenesis of SCA2. However, the causal relationship between mitochondrial dysfunction and SCA2 pathology remains to be fully elucidated, raising questions about whether mitochondrial dysfunction is a primary driver of SCA2 pathology or a secondary cofactor.

Dysregulation of Autophagy

Autophagy is an indispensable cellular process responsible for the degradation and recycling of cytoplasmic entities, such as damaged organelles and protein aggregates, which are essential for cellular homeostasis.

Autophagy can be subdivided into macroautophagy, microautophagy, and chaperone-mediated autophagy, each distinct in its mechanism despite converging at the lysosomal pathway. Microautophagy involves substrate engulfment via endolysosomal membrane invagination⁷⁸, whereas chaperone-mediated autophagy relies on chaperone-facilitated protein unfolding and translocation through a LAMP2A-constituted channel⁷⁹. Macroautophagy, often referred to as autophagy, encapsulates cargoes within autophagosomes, double-membraned vesicles⁸⁰. This self-cannibalization mechanism is orchestrated through a series of tightly regulated steps, each involving distinct molecular machinery components.

Autophagy initiation is triggered by various cellular signaling pathways, notably nutrient deprivation, which inhibits mTOR signaling and activates the Unc-51-like kinase 1 (ULK1) complex. This activation leads to the phosphorylation and activation of the class III phosphatidylinositol 3-kinase (PI3K) complex, essential for starting autophagosome formation⁸⁰.

Nucleation, the initial phase, involves creating a phagophore that engulfs cellular components targeted for degradation. During membrane elongation, ATG8 family proteins (LC3 and GABARAP variants) undergo lipidation, integrating into the membrane and facilitating its expansion to enclose the cargo⁸⁰.

Maturation involves completing autophagosome formation and its fusion with a lysosome to form an autophagolysosome. This step is mediated by proteins that aid in the fusion process and lysosomal recognition⁷⁸.

Lysosomal hydrolases facilitate degradation within the autophagolysosome. They break down the engulfed content, allowing the breakdown products to be recycled back into the cytosol for reuse in various biosynthetic pathways. Selective autophagy targets specific cellular cargoes with high specificity, contrasting with non-selective autophagy, which randomly sequesters cytoplasm. The type of selective autophagy is named based on the cargo being degraded, such as mitophagy for mitochondria, ribophagy for ribosomes, and aggrephagy for protein aggregates⁸¹. In neurodegenerative disorders, aggrephagy is particularly significant due to the hallmark presence of intracellular protein/peptide aggregates in these diseases⁸². Aggrephagy is a selective autophagy pathway essential for removing protein aggregates, thereby maintaining cellular proteome functionality. It is regulated by the proteostasis network, encompassing chaperones, the ubiquitin-proteasome system, and autophagy⁸³. Aggresomal clearance can also occur via chaperone-assisted selective autophagy (CASA), which employs chaperones to target misfolded proteins for degradation through the autophagy-lysosome system. The CASA complex includes ATP-dependent HSPA/HSP70 chaperones, primarily HSPA1A and HSPA8, which bind misfolded proteins to promote refolding or degradation⁸³. HSPB8, a small heat shock protein, cooperates with HSP70 and binds strongly to the BAG3 scaffold protein. BAG3 links HSP70 to HSPB8 and client proteins, acting as a scaffold for the CASA complex^{83,84}. The E3 ubiquitin ligase STUB1/CHIP ubiquitinates client proteins, marking them for degradation^{83,84}. If refolding fails, ubiquitinated proteins are transported to the aggresome for autophagic degradation, facilitated by the autophagy receptor SQSTM1⁸⁴.

In SCA2, autophagy has gained attention due to its importance in neuronal health. Puorro *et al.* (2018) found increased expression of the autophagy scaffold protein WDFY3 mRNA in peripheral blood mononuclear cells of SCA2 patients, suggesting elevated peripheral autophagy markers as a potential compensatory mechanism⁸⁵. Marcelo *et al.* investigated autophagy's role in SCA2 pathogenesis and its therapeutic potential by analyzing autophagic markers in a new striatal lentiviral mouse model exhibiting SCA2 neuropathological hallmarks, as well as in a cellular model and human post-mortem brain samples⁷². The results showed altered levels of SQSTM1 and LC3B in cells and tissues expressing mutant ATXN2, and an abnormal accumulation of these markers was detected in SCA2 patients' striatum and cerebellum. Importantly, the molecular activation of autophagy mitigated the phenotypic alterations observed in the disease models. This study suggests that autophagy plays an important role in the context of SCA2 pathology and could be a potential target for treating SCA2 patients⁷².

Multiple studies have linked SCA2 to the aberrant interplay between ATXN2, STAU1, mTOR, and autophagy⁸⁶⁻⁹⁰. Staufen1 (STAU1), an RNA-binding protein and ATXN2 interactor, is elevated in SCA2 patient fibroblasts, ALS patients, and SCA2 mouse models due to abnormal autophagy and protein turnover rather than changes in STAU1 expression⁸⁶. STAU1 is recruited to polyQ-expanded ATXN2 aggregates in SCA2 patient and mouse model brain tissues. SCA2 cells exhibit impaired autophagy, SQSTM1 accumulation, and increased LC3-II levels⁸⁶. PolyQ-expanded ATXN2 expression leads to impaired autophagy, increased STAU1 levels, and STAU1-mediated mRNA decay, exacerbating SCA2 pathology, possibly through aberrant RNA processing.

In SCA2 patient fibroblasts and mouse cerebellar tissues, hyperactivated mTOR-signaling inhibits autophagy, as

evidenced by increased LC3-II levels. This is attributed to increased mTOR mRNA translation mediated by STAU1, which is highly elevated in SCA2 cells and models⁸⁷. Genetic reduction of STAU1 in SCA2 mice (*ATXN2^{Q127}*) improves autophagy markers, reduces mTOR activity and protein levels, and decreases caspase activity, suggesting STAU1 as a potential therapeutic target⁸⁸. Similar results were obtained in *ATXN2-Q58* knock-in HEK293 cells, where STAU1 reduction, mTOR inhibition by rapamycin⁸⁸, RNA interference⁸⁸, and AKT inhibition⁸⁹ decreased mTOR activity, protein levels, and autophagy markers⁸⁷.

Consequently, these phenotypes offer a window into the complex interplay of molecular events in SCA2 and provide potential targets for therapeutic intervention, underscoring the broader significance of these cellular dysfunctions within the disease's pathology.

Differentiation Protocols of Pluripotent Stem Cells Toward Cerebellar Neurons: A Chronological Overview

In the context of neurodegenerative diseases that cause ataxia, such as SCA2, understanding the development and function of the GABAergic neurons, Purkinje cells, is crucial. These cells are often the primary site of pathology in these conditions, and therefore, studying the development and function of Purkinje cells can provide important knowledge and contribute to new disease models to better mimic the pathogenesis and potentially guide the development of therapeutic strategies.

Induced pluripotent stem cells (iPSCs) have become a cornerstone in disease modeling as these cells have the unique ability to differentiate into various cell types, and significant strides have been made in the differentiation of iPSCs into cerebellar cell models, yielding cerebellar neurons, organoids, and progenitor populations with increased fidelity.

Early attempts to differentiate cerebellar neurons from pluripotent stem cells utilized mouse embryonic stem cells (ESCs). Su *et al.* (2006) generated cerebellar neuron precursors by treating ESCs with Wnt3a and BMP4, resulting in mitotically active neural cells expressing granule cell precursor markers. Purkinje cell progenitor induction was achieved under similar conditions but enhanced by FGF8 instead of WNT3a⁹¹. This study pioneered the *in vitro* recapitulation of early cerebellar neuron differentiation using ESCs, followed by Salero and Hatten (2007), who differentiated mouse ESCs into cerebellar neurons using WNT3a, WNT1, FGF8b, and retinoic acid for cerebellar patterning⁹².

In 2010 Muguruma *et al.* published a protocol that successfully generated Purkinje cells from mouse ESCs using the self-inductive signaling microenvironments of the isthmic organizer by treating the mouse ESCs with FGF2 and insulin, which promoted the expression of WNT1 and FGF8⁹³. To specify a dorsal hindbrain fate, they inhibited ventralization signals by adding the Smoothed inhibitor cyclopamine⁹³. The maturation of the Purkinje cells was achieved by co-culturing with granule cells derived from mice. That same year, another group published a differentiation protocol for generating mature cerebellar Purkinje cells from mouse ESCs using BMP4, FGF8, and

WNT3a as patterning factors⁹⁴. Also, in this study, a co-culture approach was used to mature the Purkinje cell progenitors. Additionally, in 2010 Erceg *et al.* (2010) published a protocol for differentiating human embryonic stem cells (hESCs) into cerebellar-like cells, including granule neurons, Purkinje cells, interneurons, and glial cells⁹⁵. In the protocol, they made use of various patterning factors such as heparin, FGF8, RA, insulin, BMP4, BMP6, BMP7, GDF7, and sonic hedgehog (SHH)⁹⁵. The neurons derived using this protocol expressed similar markers to the developed human cerebellum.

Early protocols for cerebellar differentiation shared similarities in their focus on caudalization, maturation, and the use of patterning factors like FGF8, RA, WNT3a, and WNT1. However, in 2012, Kirkeby *et al.* introduced a novel approach to pattern neural progenitors from telencephalic forebrain to posterior hindbrain fates, recapitulating human fetal brain development⁹⁶. The protocol utilized dual SMAD inhibition for neuroectodermal differentiation and a GSK3 inhibitor for dose-dependent WNT signaling activation, controlling the rostro-caudal positional specification. Combined with SHH-C24II, the researchers efficiently patterned cultures toward a ventral fate, controlling both rostro-caudal and dorsal-ventral axes⁹⁶. This study demonstrated that dose-dependent WNT signaling activation in hESCs efficiently generates authentic progenitors from the entire rostro-caudal neural axis, including hindbrain/cerebellar neurons.

In 2015 Muguruma *et al.* were able to generate neurons of the cerebellum by 3D culture of hESCs⁹⁷. They used a modified version of their previously published protocol⁹³ in which they introduced the addition of the transforming growth factor β (TGF- β)-receptor blocker SB431542 to promote neuroectodermal differentiation while adding FGF19 promoted the spontaneous generation of dorsoventrally polarized neural-tube-like structures⁹⁷. The addition of stromal cell-derived factor 1 (SDF1) promoted the generation of a continuous cerebellar plate neuroepithelium with a rhombic-lip-like structure and a three-layer cytoarchitecture similar to the embryonic cerebellum⁹⁷. However, the maturation of generated progenitor cells was still coculture-dependent.

Alsanie *et al.* (2017) developed several protocols for early neural fate restriction of murine pluripotent stem cells (PSCs), generating neural subtypes of the dorsal forebrain, ventral forebrain, ventral midbrain, and hindbrain without employing feeder layers or embryoid body formation⁹⁸. To derive hindbrain neural progenitors with a ventral identity, they directed differentiation of murine PSCs through the addition of LDN193189 (a BMP inhibitor promoting neuroectodermal differentiation), SHH, purmorphamine (SHH agonist), FGF8, and CHIR99021 (GSK3 inhibitor)⁹⁸.

The following year, Sundberg *et al.* published their protocol for human iPSC-derived Purkinje cell differentiation. While sharing similarities with the previous protocol, which utilized FGF8b, FGF2, dual SMAD inhibition, and CHIR99021, Sundberg *et al.* incorporated nicotinamide to enhance neural commitment during induction⁹⁹. A notable distinction was the temporal sequence of patterning factor application.

In 2020 Silva *et al.* presented a novel strategy for differentiating human pluripotent stem cells into cerebellar neurons without the need for coculture systems¹⁰⁰. This approach is significant as it reduces the variability introduced by feeder cells in co-culture systems, which can affect reproducibility and experimental outcomes. The differentiation protocol was an adaptation of the protocol published by Muguruma *et al.* in 2015⁹⁷, but the coculture-independent system was achieved by implementing advanced organoid strategies and using a defined medium for the maturation stages¹⁰⁰.

In the same year, the Kirkeby group published their study, which aimed to model early human neural tube development using human iPSCs cultured in a microfluidic device¹⁰¹. This approach, named microfluidic-controlled stem cell regionalization (MiSTR), exposed the iPSCs to signaling gradients that would mimic *in vivo* developmental patterning¹⁰¹. With MiSTR, they exposed the cells to a WNT-activating gradient, which generated a neural tissue exhibiting progressive caudalization from forebrain to midbrain to hindbrain, including the formation of isthmic organizer characteristics¹⁰¹. All within a single tissue. They also demonstrated that using RA was not able to induce a rostral-to-caudal neural axis¹⁰¹. The patterning factors used in this protocol were almost identical to those in the protocol published in 2012⁹⁶.

Over the past two decades, significant progress has been made in the development of protocols for differentiating iPSCs into cerebellar neurons. These advancements have enhanced our understanding of cerebellar development and function, providing valuable tools for investigating cerebellar diseases and developing potential novel therapies. As our knowledge of cerebellar neurons expands, it is anticipated that these protocols will undergo further refinement and optimization, opening new avenues for research and treatment in the field of neuroscience.

OBJECTIVES & AIMS

Neurodegenerative diseases, such as SCA2, present a complex interplay of genetic, molecular, and environmental factors that contribute to their pathogenesis. Despite the diversity in clinical manifestations and genetic underpinnings, these diseases share common mechanisms that drive their progression. Extensive research has yet to fully elucidate the precise mechanisms underlying SCA2 pathology, and disease-modifying treatments are still lacking. This PhD project aims to unravel the intricate cellular and molecular mechanisms underlying SCA2. The project is motivated by the urgent need for therapeutic strategies to combat this debilitating condition.

The overarching objective of this research is to elucidate the pathomechanisms of SCA2 by leveraging advanced proteomic analyses, modulating autophagy pathways, and developing sophisticated cerebellar organoid models. By investigating the proteomic landscape and autophagic processes in SCA2 patient-derived cells, this project seeks to identify key molecular players and pathways that could serve as potential therapeutic targets. Additionally, the development of hindbrain/cerebellar organoid models aims to provide a robust platform for studying disease mechanisms and testing therapeutic interventions in a controlled, three-dimensional environment.

STUDY I

Hypothesis: The elongated polyglutamine expansion in ATXN2 alters its protein-protein interactions and native RNA binding capacity, affecting the global proteome in SCA2 patient cell lines.

Aims:

- Assess the impact of polyglutamine expansion on the cellular proteome of SCA2 fibroblasts by mass spectrometry.
- Map the protein-protein interaction network of ATXN2 in SCA2 patient cell lines using immunoprecipitation followed by mass spectrometry.
- Examine the RNA binding profile of polyglutamine-expanded ATXN2 in SCA2 patient cell lines using immunoprecipitation and subsequent RNA sequencing (RNAseq).

STUDY II

Hypothesis: Proteasome function and/or autophagy are dysregulated, contributing to SCA2 pathology.

Aims:

- To evaluate the redox state in SCA2 patient-derived fibroblasts by applying caspase activity assays and measuring subsequent changes in the cellular redox process.
- To assess proteasome function and autophagic flux and measurement of protein levels of ATXN2.
- Determine if solubilization of ATXN2 protein and autophagic activation can alleviate the SCA2 pathology.

STUDY III

The objective was to develop a new differentiation protocol synthesizing the most effective strategies from Rifes *et al.*, 2020¹⁰²; Silva *et al.*, 2020¹⁰⁰; and Chen *et al.*, 2021¹⁰³, with adjustments for the initial neuronal induction and maturation phases of cerebellar neuron development.

Hypothesis: Integration of protocols from the aforementioned studies will enhance hindbrain patterning, leading to a refined protocol addressing both early hindbrain patterning and maturation of cerebellar neurons.

METHODS

STUDY I

see Methods and Materials section for Study I (page 43) and Manuscript I (page 115)

STUDY II

see Methods and Materials section for Study II (page 65) and Manuscript II (page 134)

STUDY III

see Methods and Materials section for Study III (page 74)

STUDY I

TITLE

PolyQ-Length-Dependent ATXN2 Interactome in Spinocerebellar Ataxia Type 2 Patient Cells

BRIEF INTRODUCTION

The RNA-binding protein ATXN2 is highly expressed in the brain, highlighting its critical role in neuronal function and health^{24,33,35}. ATXN2 is implicated in two distinct proteinopathies: polyglutamine (polyQ) disorders and TDP-43 proteinopathies. Spinocerebellar ataxia type 2 (SCA2), along with Huntington's disease (HD) and other spinocerebellar ataxias, belong to the polyQ disease group¹⁰⁴. These diseases are characterized by an expanded polyQ tract that induces a conformational change in the mutated protein, promoting the formation of oligomeric and fibrillar aggregates^{104–106}. However, the role of these aggregates in SCA2 pathogenesis is complex, as evidence suggests that intranuclear aggregates are not essential for SCA2 pathology, indicating that the toxicity of the polyQ expansion may involve mechanisms beyond intranuclear aggregate formation¹⁰⁷. For instance, cytoplasmic aggregations, mitochondrial dysfunction, and oxidative stress have been associated with SCA2 pathology without the formation of inclusion bodies^{69,107}.

Intermediate-length polyQ expansions in ATXN2 are associated with amyotrophic lateral sclerosis (ALS) and accumulation of intracellular TDP-43-positive aggregates^{27,108}. The formation of intracellular aggregates caused by RNA-binding proteins (RBP) is a known pathological feature in ALS and frontotemporal dementia (FTD)^{109–111}, which has given rise to the “two-hit” hypothesis of RBP-driven neurodegeneration¹¹². This model suggests that RBP aggregates induce toxicity through a dual mechanism: a toxic gain-of-function by the aggregating properties and a concurrent loss of the proteins' normal regulatory roles. The aggregation of RBPs is primarily driven by the presence of intrinsically disordered regions (IDRs) in their protein sequences, and mutations in these specific domains are linked to familial ALS/FTD^{113–115}.

Given the multifaceted roles of RBPs, which span transcription regulation, RNA splicing/export, translational regulation, RNA metabolism, and cellular stress responses^{63,116–121}, high-throughput methodologies are preferred for identifying potential alterations in cellular homeostasis, protein function, or interacting partners introduced by the given mutation. A proteomics study conducted in 2016 revealed that ATXN2 interacts with numerous proteins implicated in the regulation of translation, ribosomal proteins, and RNA splicing events⁵⁶. These protein-protein interactions underscore the involvement of ATXN2 in RNA metabolism and translation. The structural characteristics of ATXN2 and its reported interactions are further supported by previous findings demonstrating the physical assembly of ATXN2 with messenger ribonucleoprotein (mRNP) complexes and polyribosomes while also interacting with proteins involved in mRNA control and regulation^{32,44,49,50,57–61}. Moreover, ATXN2 has been shown to play a role in nutrient stress response regulation, suggesting a role in metabolic regulation. Research has shown that the expression of ATXN2 increases in response to nutrient and trophic stress, which is dependent on the mTOR signaling pathway⁶¹. Additionally, the yeast ortholog of ATXN2, Pbp1, has been reported to be implicated in the

stress-responsive translational regulation of precursor proteins that are targeted to the mitochondria¹²². The exact mechanisms through which ATXN2 mediates these responses are yet to be fully elucidated but are thought to involve its interactions with RNA and proteins involved in metabolic pathways¹²³. Given the involvement of ATXN2 in regulation of the mTOR pathway underscores its importance in the control of mRNA translation, lipid signaling, and autophagy¹²⁴.

OBJECTIVE

This explorative study aimed to investigate the alterations introduced by the polyQ expansion in ATXN2 on the protein-protein interaction and RNA binding and how this affects the cellular proteome to understand and elucidate the pathomechanisms of SCA2.

Hypothesis: The elongated polyglutamine expansion in ATXN2 alters its protein-protein interactions and native RNA binding capacity, affecting the global proteome in SCA2 patient cell lines.

AIMS

- Assess the impact of polyglutamine expansion on the cellular proteome of SCA2 fibroblasts by mass spectrometry.
- Map the protein-protein interaction network of ATXN2 in SCA2 patient cell lines using immunoprecipitation followed by mass spectrometry.
- Examine the RNA binding profile of polyglutamine-expanded ATXN2 in SCA2 patient cell lines using immunoprecipitation and subsequent RNA sequencing (RNAseq).

MATERIAL & METHODS

Cell Lines and Cell Line Generation

See Manuscript for Study I (Henriksen *et al. in preparation*)

General Culture Conditions

See Manuscript for Study I (Henriksen *et al. in preparation*)

iTRAQ Labeling, IEF Separation, and Purification of Peptides.

See Manuscript for Study I (Henriksen *et al. in preparation*)

Immunoprecipitation of ATXN2 with Protein-A Agarose Beads

See Manuscript for Study I (Henriksen *et al. in preparation*)

Nano-LC-MS/MS Analysis

See Manuscript for Study I (Henriksen *et al. in preparation*)

RNA Purification from ATXN2-IP

See Manuscript for Study I (Henriksen *et al. in preparation*)

RNA Sequencing

See Manuscript for Study I (Henriksen *et al. in preparation*)

Data Processing and Analyses

See Manuscript for Study I (Henriksen *et al. in preparation*)

Enrichment Annotation Analyses

See Manuscript for Study I (Henriksen *et al. in preparation*)

Proximity Ligation Assay (PLA)

See Manuscript for Study I (Henriksen *et al. in preparation*)

Statistical Analysis

See Manuscript for Study I (Henriksen *et al. in preparation*)

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (PFA) for 10-15 minutes at room temperature and washed twice with phosphate-buffered saline (PBS). Membrane permeabilization was performed using 0.2-0.5% TritonX-100 in PBS for 15 minutes. Cells were blocked with 5% bovine serum albumin (BSA) in PBS for a minimum of one hour at room temperature or overnight at 4°C. Primary antibodies were diluted in blocking buffer and incubated overnight at 4°C.

Following three PBS washes, cells were incubated with secondary antibodies (1:500-1:1000 diluted in blocking buffer) for one hour. Nuclei were stained with Hoechst (1:30,000 in PBS) for five minutes and washed thrice with PBS. Slides were mounted using ProLong Antifade Gold or a similar medium and allowed to solidify overnight at 4°C, protected from light.

For mitochondrial proteins, an optimized protocol adapted from Zorkau *et al.* (2021) was used. Cells were fixed in prewarmed 4% PFA in mitochondria-protective buffer (MPB; 10 mM Hepes/KOH, 10 mM NaCl, 5 mM MgCl₂, 300 mM sucrose, pH 7.5) for seven minutes at room temperature. Permeabilization was performed with 0.5% Triton X-100 in PBS, followed by blocking with 5% BSA in PBS for 10 minutes. Primary antibodies were diluted in 5%

BSA and incubated for one hour at room temperature, followed by three PBS washes. Secondary antibodies (1:1000 in 5% BSA) were incubated for 40 minutes. Nuclei were stained with Hoechst for five minutes, and cells were mounted with ProLong Glass Antifade or a similar medium.

Images were acquired using an EVOS 7000 fluorescent microscope (60x objective) and further processed for visualization and quantification using ImageJ software.

Subcellular Fractionation

Fibroblast patient cell lines were cultured in T75 flasks until 90% confluence. Upon reaching confluence, the medium was removed, and cells were washed with PBS. Cells were then scraped, transferred to Eppendorf tubes, centrifuged, and the supernatant discarded. The cell pellets were resuspended in 250 mM STM buffer and homogenized on ice for one minute using a Douncer with pestle B (30 strokes), followed by a 30-minute incubation on ice. The whole cell fraction was collected from this homogenate.

The remaining solution was vortexed and centrifuged at 600 x g for 10 minutes. The supernatant was designated as the mitochondrial and cytosolic fraction, and the pellet as the nuclear fraction.

The nuclear fractions were resuspended in 2 M STM buffer, vortexed, and centrifuged at 500 x g for 15 minutes. The supernatant was removed, and the pellet was washed, vortexed, and centrifuged again at 1000 x g for 15 minutes. The pellet was resuspended in NET buffer and lysed by sonication (22% amplitude, 0.5-second pulse on, 1-second pulse off) for five pulses, repeated three times with one-minute pauses on ice. The lysate was centrifuged at 9000 x g for 30 minutes at 4°C, and the supernatant was collected as the final nuclear fraction.

An initial centrifugation at 600 x g for 10 minutes was performed, and the supernatant was transferred to a new tube to purify the mitochondrial and cytosolic fractions. The mitochondrial fraction was pelleted by centrifugation at 11000 x g for 10 minutes. The supernatant (cytosolic fraction) was transferred to a new tube and precipitated in cold 100% acetone overnight at -20°C.

The mitochondrial pellet was washed in 250 mM STM buffer and centrifuged at 11000 x g for 10 minutes. The pellet was resuspended in SOL buffer and lysed by sonication (22% amplitude, 0.5-second pulse on, 1-second pulse off) for five pulses, repeated three times with one-minute pauses on ice. The cytosolic fraction was centrifuged at 12000 x g for five minutes, and the supernatant was discarded. The pellet was resuspended in 250 mM STM buffer. Protein concentration was measured using the Bradford assay and all subcellular protein fractions were stored at -20°C.

RESULTS

Characterization of the SCA2 Proteome Using Mass Spectrometry

A global mass spectrometry (MS) approach was employed to analyze the proteome of SCA2 patient cell lines. Initial MS data filtration identified 1,894 proteins, with 354 proteins exhibiting increased expression (fold change >1.25) and 337 proteins showing decreased expression (fold change <0.8) in SCA2 patient cell lines compared to

controls (**Figure 1C**, see manuscript of Study I). Enrichment analyses of differentially expressed proteins indicated that proteins with decreased expression were significantly enriched in pathways involved in the epigenetic regulation of gene expression, while proteins with increased expression were predominantly associated with pathways related to the cellular response to starvation, ribosomal function, and translation (**Figure 1D**, see manuscript of Study I). Notably, there was an increase in the abundance of proteins associated with translation, ribosomal components, and chaperone functions in SCA2 patient cells (**Figure 1E**, see manuscript of Study I).

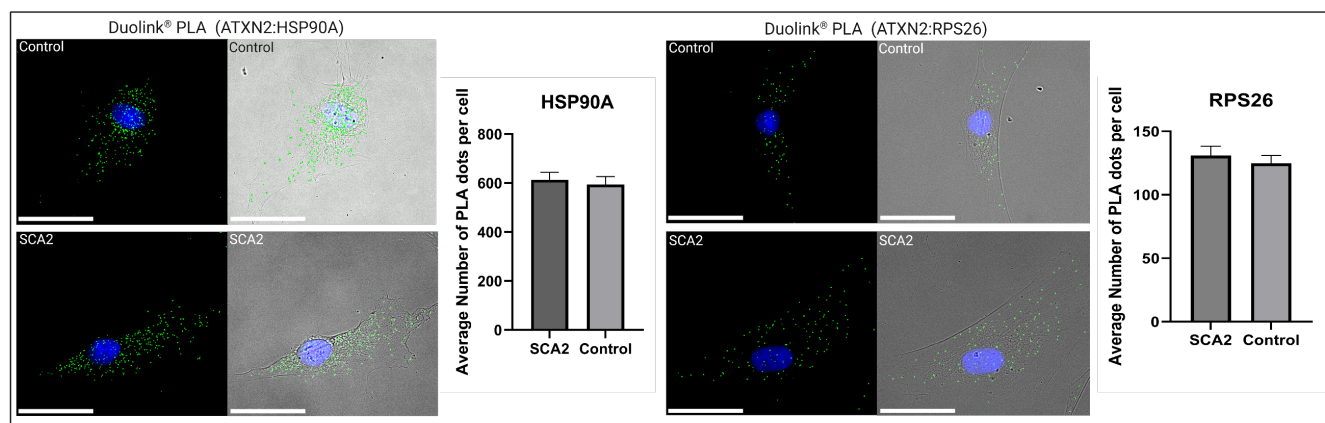
IP-MS Analysis of ATXN2 Protein Interactions in SCA2 Patient Cells

Immunoprecipitation coupled with mass spectrometry (IP-MS) was utilized to elucidate the protein-protein interactions (PPIs) of ATXN2 in fibroblasts derived from patients with SCA2. The analysis identified 232 proteins associated with ATXN2, implicated in translation, ribosome function, spliceosome activity, RNA metabolism, cytoskeletal structure, chaperone functions, mitochondrial processes, and RNA-binding activities (**Figure 2C, -E**, see manuscript of Study I). Principal component analysis (PCA) of the protein interaction profiles revealed significant overlap between patient and control cell lines, suggesting considerable variability in the ATXN2 interaction landscape across different cellular contexts (**Figure 2D**, see manuscript of Study I).

Notably, the PPI profile of ATXN2 in SCA2 patient cell lines revealed an increase in the abundance of proteins interacting with ATXN2 (**Figure 3A-B**, see manuscript of Study I). Specifically, 42 protein hits showed significantly enhanced interaction with ATXN2 across SCA2 patient cells compared to controls, while only one protein, NEK3, demonstrated a significantly reduced association. STRING analysis of the proteins with significantly increased interaction highlighted an enrichment of chaperone and ribosomal proteins, suggesting an enhanced association of ATXN2 with chaperone systems and translational machinery (**Figure 3C**, see manuscript of Study I). This was further supported by enrichment analysis of the proteins with increased PPI (threshold: >1.25-fold change), showing enrichment for proteins involved in translation, ribosomes, and rough endoplasmic reticulum membrane (**Figure 3D-E**, see manuscript of Study I). To verify these interactions, we selected candidates for proximity ligation assay (PLA), which showed a significant increase in ATXN2:RPS3 and ATXN2:RPS18 interactions in SCA2 patient cells compared to controls (**Figure 3F**, see manuscript of Study I). Although RPS26 and HSP90A had increased binding with ATXN2 in the MS analyses, we were not able to validate this with the proximity ligation assay (**Complementary Figure 1**).

Polyglutamine Length-Dependent Alterations in ATXN2 Protein-Protein Interactions

The study revealed a clear polyQ length-dependent increase in the number of proteins interacting with ATXN2 (**Figure 4A-B**, see manuscript of Study I). Cell lines with 36, 42, and 44 CAG repeat expansions in *ATXN2* showed a ≥ 2 -fold increase in binding, and the PPIs increased with the polyQ length to 46, 104, and 128 proteins, respectively (**Figure 4F**, see manuscript of Study I). Ribosomal and chaperone proteins were consistently present across different polyQ lengths and were among the 31 proteins present in the >2-fold fraction across all polyQ length groups (**Figure 4E**, see manuscript of Study I), suggesting their potential role in SCA2 pathology.



COMPLEMENTARY FIGURE 1 - The *in situ* proximity ligation assay (PLA) was used to detect interaction between ATXN2 and ribosomal proteins (RPS3 and RPS18) in control cells and SCA2 patient cells. Interaction events are shown as green dots (representative image, scale bar: 60 μ m). Quantification of interaction events is shown by bar plots. Unpaired Student's t-test; RPS26 (SCA2: n = 46, Control: 42), HSP90A (SCA2: n = 68, Control: 66).

Impact of PolyQ Expansion on ATXN2 RNA-Binding Capabilities in SCA2

RNA sequencing (RNAseq) of ATXN2 immunoprecipitations from both healthy control and SCA2 patient-derived cell lysates revealed a total of 8,794 RNAs associated with ATXN2. The RNAseq results displayed a significant alteration in the RNA-binding profile of ATXN2 in SCA2 patient cells compared to healthy controls, with a notable decrease in the abundance of RNAs bound to ATXN2 (**Figure 4C**, see manuscript of Study I). Specifically, there were increases in 723 RNAs (fold change >1.25) and decreases in 1,145 RNAs (fold change <0.8), suggesting that the polyQ expansion lowers the capacity of ATXN2 to bind RNA. This trend was further supported by the categorization of samples based on polyQ length, which revealed an inverse correlation between the length of the polyQ tract and the number of RNAs bound to ATXN2 (**Figure 4D, - G**, see manuscript of Study I).

COMPLEMENTARY RESULTS

Altered RNA Binding of ATXN2 - Analysis of IP-RNAseq Data

After data filtration, the total RNAs bound to or associated with ATXN2 amounted to 8794 RNAs, consisting of 13 antisense RNA species, 6 microRNA species, 13 non-coding RNA species, 1 PALM2-AKAP2 fusion RNA, 13, 8716 protein-coding RNAs, 21 pseudogene RNAs, 21 readthroughs, and 23 unknown RNA sequences.

The IP-RNAseq analysis displayed significant alterations in RNA binding to ATXN2 within patient cell lines, as evidenced in **Figure 4C-D, - G** (see manuscript of Study I).

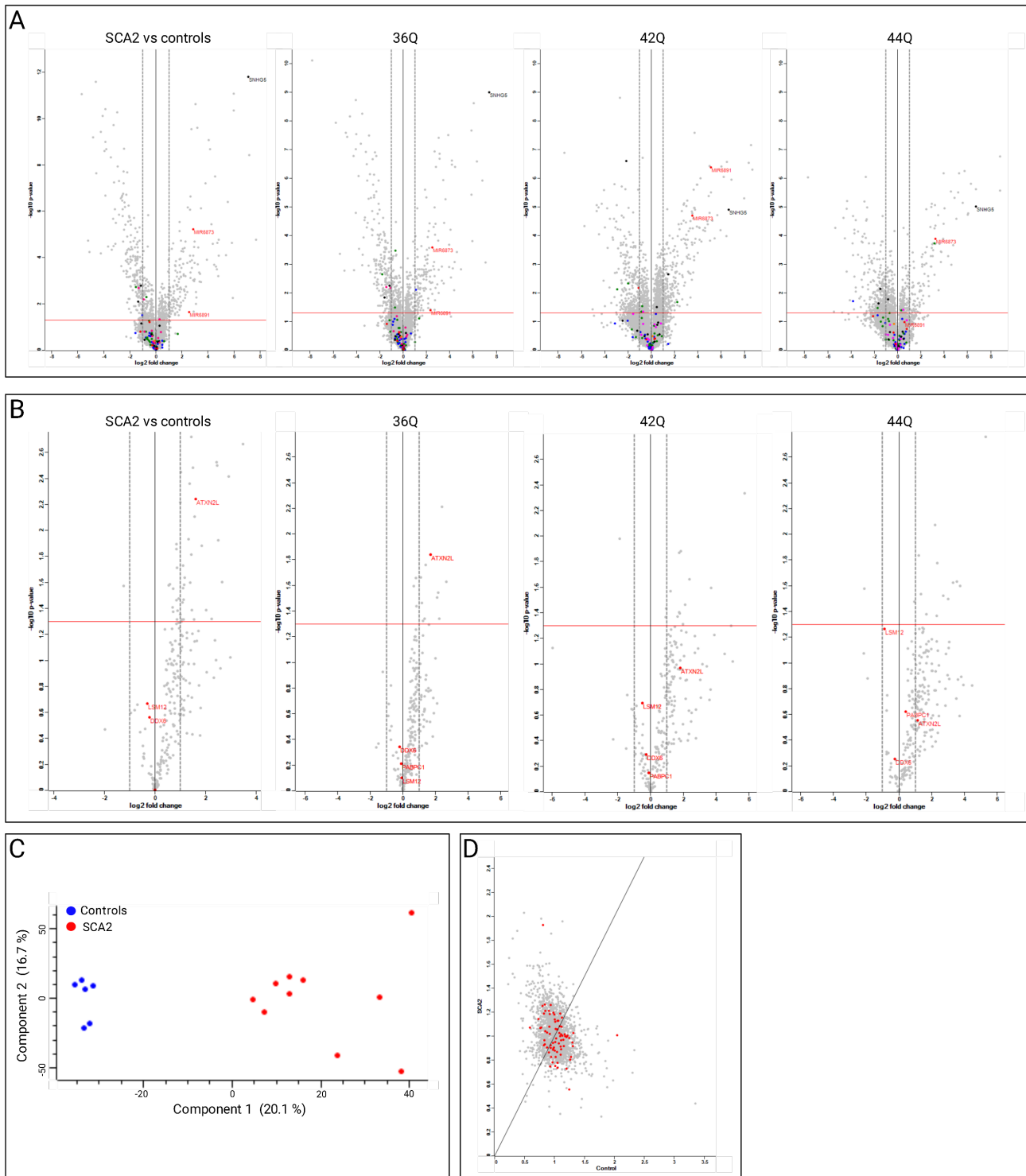
The PCA analysis further reinforces the significance of the altered RNA-binding profile observed in the IP-RNAseq data (**Complementary Figure 2C**). By reducing the dimensionality of the dataset and visualizing the variance between samples, PCA effectively distinguishes patient cell lines based on their RNA-binding profiles to ATXN2. The clustering of samples in the PCA plot (component 1, x-axis) indicates distinct groups characterized by their differential RNA-binding patterns to ATXN2, with patient samples exhibiting a decrease in the number of different RNA species bound to ATXN2 forming more dispersed clusters from control samples.

To further characterize the RNAs bound to ATXN2, we investigated the distribution of different RNA types

between control and patient samples. From the results visualized in **Complementary Figure 2A**, two types of RNAs are represented in the increased fraction of ATXN2 bound RNAs: the microRNA miR6873, the microRNA miR6891 (both labeled and colored red), and the long non-coding RNA SNHG5 (labeled and colored black), see **Complementary Figure 2A**. Predictions of functional targets of miR6873 using the online database TargetScanHuman (<https://www.targetscan.org/>) gave 984 predicted targets, while predictions of functional targets of miR6891 gave 774 predicted targets. However, the predicted targets did not correlate with the protein abundances of the global proteomics data (**Complementary Figure 2D**).

Known Protein-Protein Interactors of ATXN2

ATXN2 has been reported to bind to specific proteins, and the IP-MS assay found four of these reported binding partners, PABPC1 (Poly(A) Binding Protein Cytoplasmic 1), ATXN2L (Ataxin 2 Like), DDX6 (DEAD-Box Helicase 6), and LSM12 (LSM12 homolog). The cumulative IP-MS data display the two latter proteins, DDX6 and LSM12, to have a decreased, though not significant, interaction with ATXN2 in patient cell lines, see **Complementary Figure 2B**. while the interaction between ATXN2 and ATXN2L is significantly increased in the SCA2 patient cell lines, see **Complementary Figure 2A**. Regarding PABPC1, we see no difference in the interactions between the two proteins when comparing ATXN2-IP obtained from SCA2 patient cells and controls. However, by dividing the IP-MS data into groups corresponding to the length of the CAG repeats, we found a polyQ expansion length-dependent increase in the ATXN2:PABPC1 interaction as the SCA2 patient cells exhibiting 44 CAG repeats (Q44) display an increase in ATXN2 PPI compared to SCA2 patient cells exhibiting 36 (Q36) and 42 (Q42) CAG repeats (**Complementary Figure 2B**). Interestingly, the interaction with LSM12 gradually decreases with the increase in polyQ expansion length (**Complementary Figure 2B**).



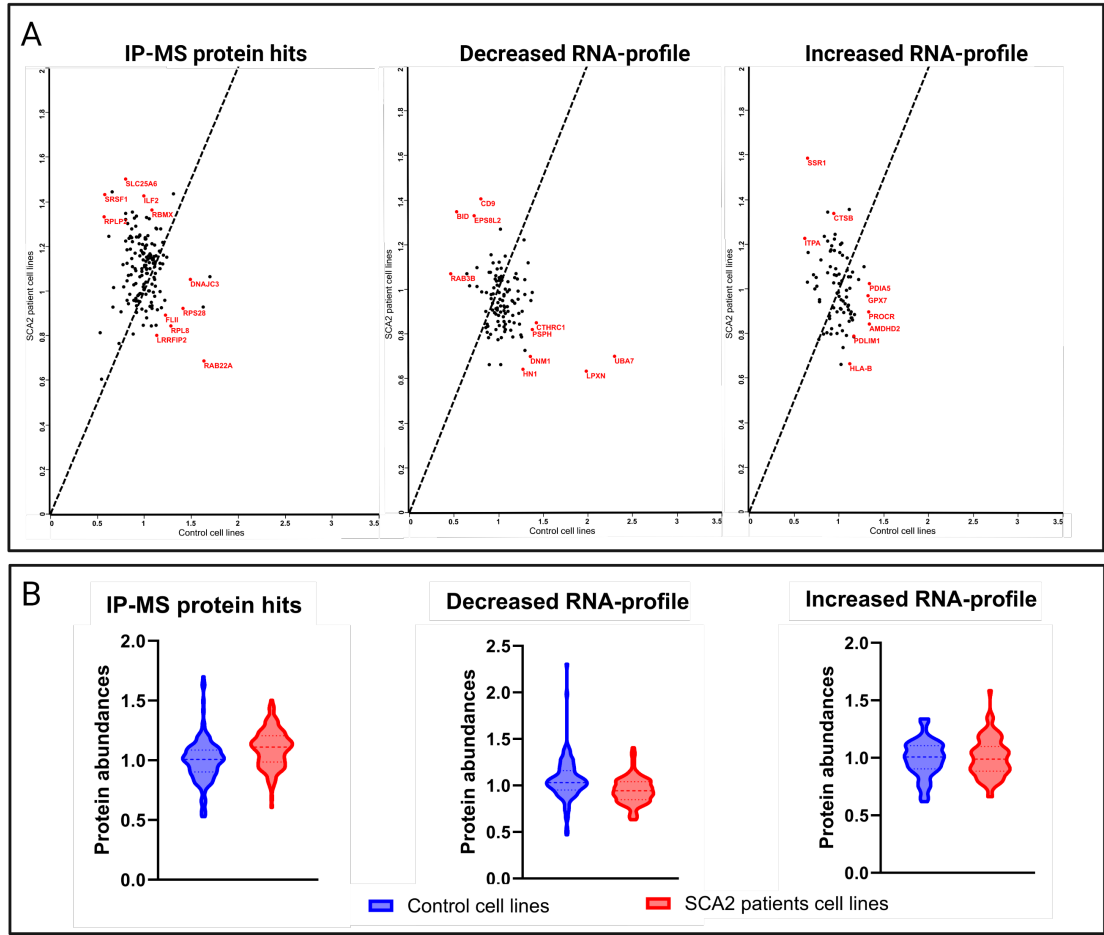
COMPLEMENTARY FIGURE 2 - PolyQ-length-dependent Alterations in ATXN2 Associated RNA Species and Interaction with Known Binding Partners

A: Volcano plot visualizing the statistical significance of RNA species associated with ATXN2 (red: microRNA, black: long non-coding RNA, gray: protein-coding mRNA, green: pseudogene, blue: readthrough, purple: antisense RNA, orange: PALM2-AKAP2 fusion RNA). The y-axis represents $-\log_{10}$ p-values, and on the x-axis is the $\log_2$ fold change. The vertical dashed lines indicate the 2-fold threshold, and RNAs with significant changes in ATXN2 association are above the red. The following three volcano plot depicts the distribution of values of known interaction partners of ATXN2 across groups with different CAG repeats: 36 CAG repeats (36Q), 42 CAG repeats (42Q), and 44 CAG repeats (44Q). **B:** Volcano plot of the proteomic data obtained from IP-MS of ATXN2 in SCA2 cells versus control cell lines. Known ATXN2 protein interactors are highlighted. The following three volcano plot depicts the distribution of values of known interaction partners of ATXN2 across groups with different CAG repeats: 36 CAG repeats (36Q), 42 CAG repeats (42Q), and 44 CAG repeats (44Q). The vertical dashed lines indicate the 2-fold threshold, while the red horizontal line depicts the p-value of 0.05. **C:** Principal component analysis (PCA) plot demonstrating the separation between SCA2 (red) and control (blue) cell lines based on the RNA profiles associated with ATXN2. The PCA plot was calculated using Perseus software. **F:** Scatter plot comparing the protein abundance between control (x-axis) and SCA2 patient cell lines (y-axis). Predicted targets of microRNA 6873 and microRNA 6891 are highlighted in red. Statistical assessment (student's t-test) of the expression data was done using Perseus software.

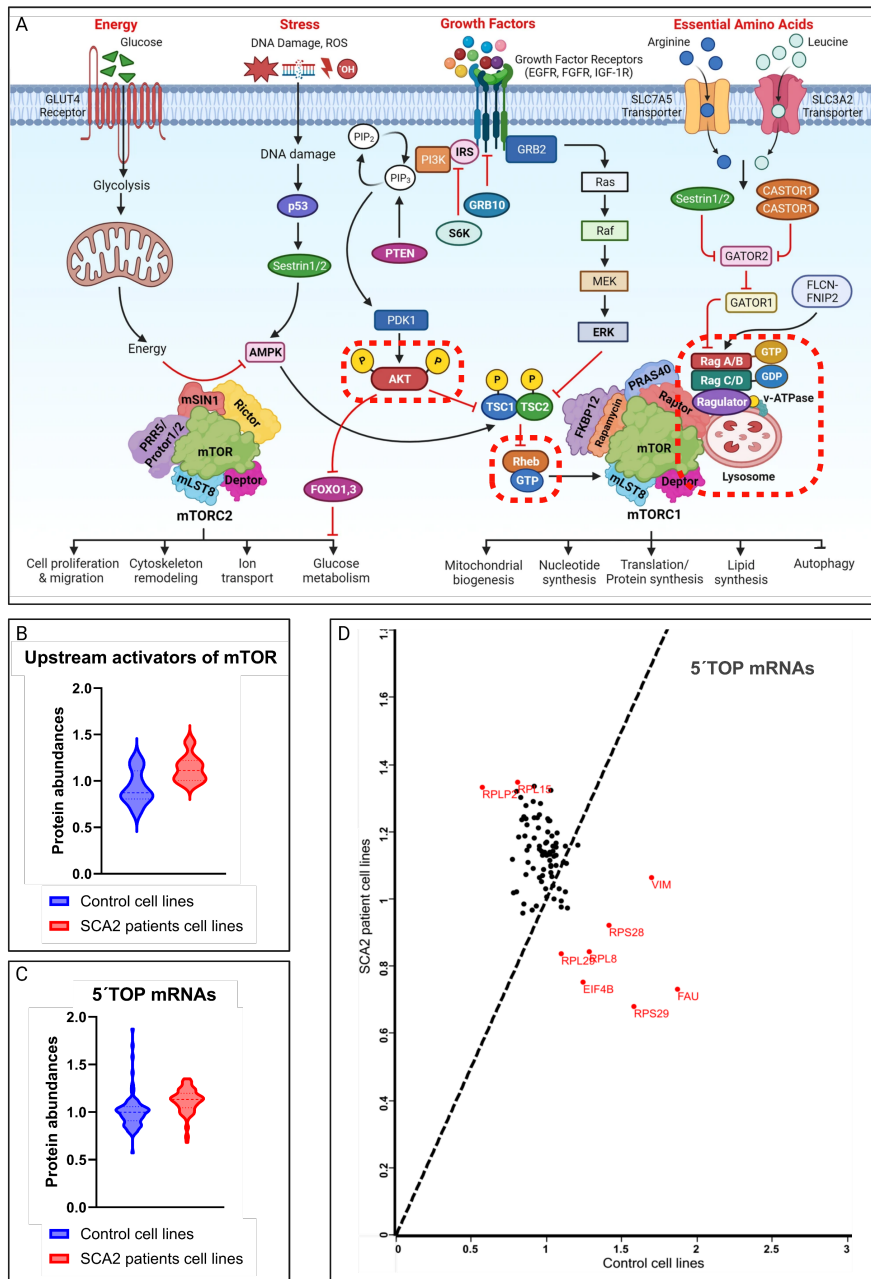
Comparisons of IP-RNAseq, ATXN2 IP-MS, and Global Proteomics of SCA2 Patient Cell Lines

The analysis aimed to elucidate the impact of the altered RNA-binding profile of ATXN2 on protein expression in SCA2 cells compared to control cell lines by comparing the most significant IP-RNAseq hits to the data obtained from the global proteomics. Our findings indicate no apparent correlation between the two data sets when comparing SCA2 and control cells (**Complementary Figure 3**). Although specific proteins exhibited upregulation or downregulation in the global proteomics, the analysis did not reveal any significant collective differences between the increased or decreased RNA profile of ATXN2 and the global proteomics. However, mRNAs with reduced binding to ATXN2 in SCA2 cells manifested a slight downregulation in protein expression across the total proteome in SCA2 cells (**Complementary Figure 3**).

We investigated whether these enhanced interactions were a result of increased availability and heightened protein expression of these specific proteins in SCA2 cells. The majority of ATXN2-associated proteins exhibited heightened expression in SCA2 cells, as evidenced by both the scatterplot and violin plot (**Complementary Figure 3B**) and **Table 1** (see manuscript for Study I). However, specific proteins, including RAB22A, DNAJC3, LRRFIP2, RPL8, and RPS28, displayed downregulations in SCA2 cells (**Complementary Figure 3A, Table 1** - see manuscript for Study I), indicating that the altered PPI of ATXN2 in SCA2 patient cells is not merely a consequence of increased availability.



COMPLEMENTARY FIGURE 3 - Comparative Analysis of Global Proteomics, IP-MS, and IP-RNAseq from SCA2 Patient and Control Cell Lines.
A: Scatter plots display the relative protein abundances identified from the IP-MS assay (**left**) and RNAs identified with decreased (**middle**) and increased (**right**) RNA profiles from the IP-RNAseq across control cell lines (x-axis) and SCA2 patient cell lines (y-axis). **B:** Violin plot of protein abundances from the IP-MS assay (**left**) and RNAs identified with decreased (**middle**) and increased (**right**) RNA profiles from the IP-RNAseq across control cell lines (x-axis) and SCA2 patient cell lines (y-axis). The color scheme distinguishes between SCA2 (**red**) and control (**blue**) samples.



COMPLEMENTARY FIGURE 4 - mTOR Activity in SCA2 cells.

A: Illustration of the major upstream regulators of mTORC1 and mTORC2 (Adapted from Panwar *et al.*, 2023). **B-C:** violin plots displaying the protein abundances and distributions of upstream activators of mTOR (**B**) and 5' TOP mRNAs (**C**). The color scheme distinguishes between SCA2 (red) and control (blue) samples. **D:** Scatter plot displaying the relative protein abundances identified from the global proteomics across control cell lines (x-axis) and SCA2 patient cell lines (y-axis), differentially expressed proteins highlighted and labeled.

mTOR Activity in SCA2 Cells

Given the crucial role of the mTOR pathway in regulating ribosome biogenesis and translation initiation and that our preliminary analyses showed to be upregulated, we further explored involvement of this pathway in SCA2 cells.

The initial proteomics data filtration excluded mTOR protein due to insufficient peptide sequence identification. However, indications of increased mTOR expression were observed, with a 2.7-fold change in SCA2 cells compared to controls. Therefore, we focused on upstream and downstream targets of mTOR in the signaling pathway to

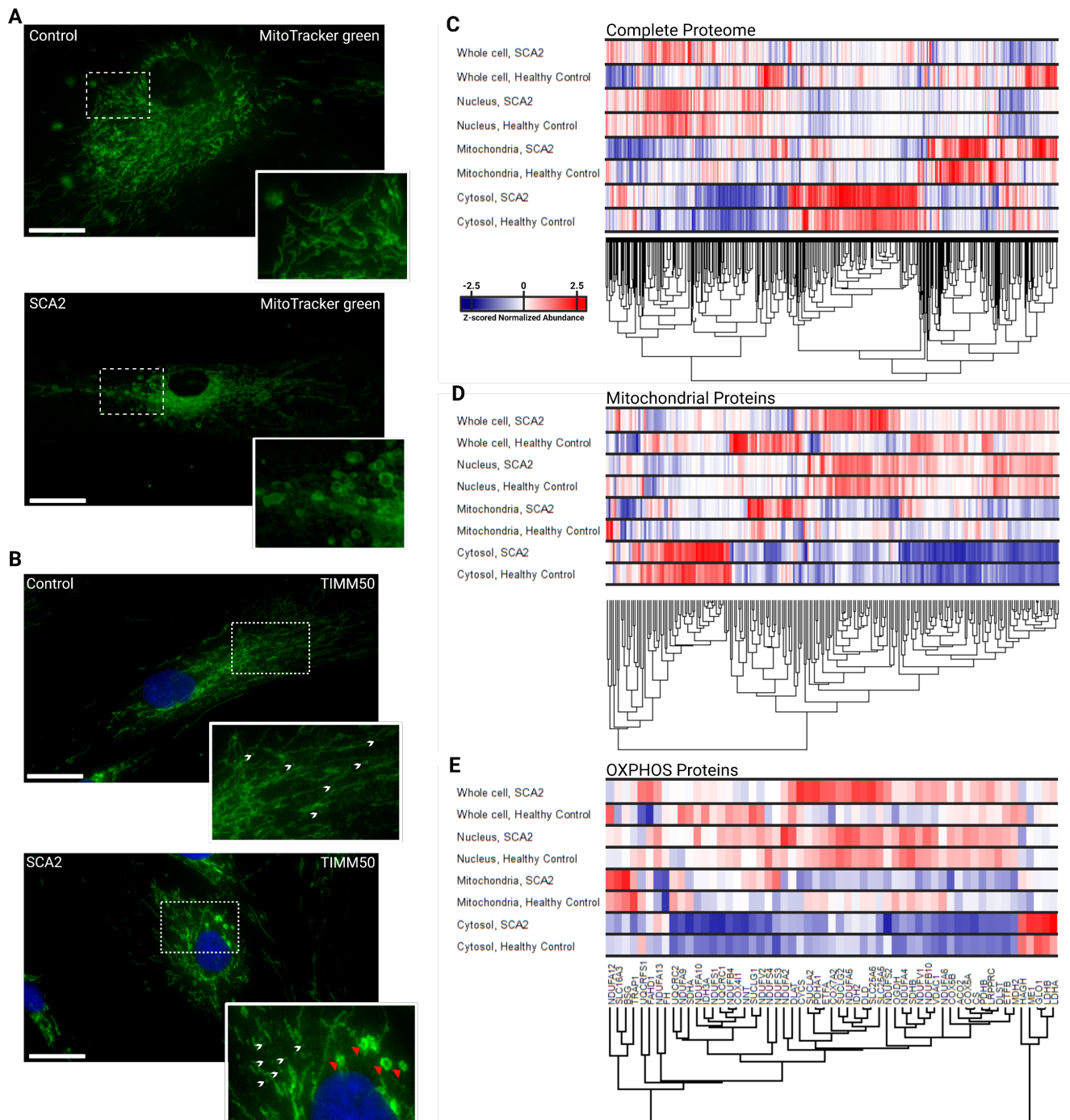
investigate alterations associated with SCA2 pathology. The upstream targets of mTOR investigated include AKT1/2, LAMTOR1/2/3/5, RHEB, and RRAGD. These proteins are crucial for mTORC1 regulation, either as components of the Ragulator complex that recruits mTORC1 to the lysosome (LAMTOR1, LAMTOR2, LAMTOR3, LAMTOR5), as mTORC1 activators (RHEB, RRAGD), or as upstream regulators modulating the pathway leading to mTORC1 activation (AKT1, AKT2). Their positions in the mTOR signaling pathway are shown in **Complementary Figure 3A** (highlighted with red and dashed squares). Analysis of the cumulative protein abundances of upstream mTOR activators in SCA2 patient cell lines compared to control cell lines reveals increased protein abundance in SCA2 cells (**Complementary Figure 4B**), suggesting dysregulation of the mTORC1 signaling pathway in SCA2.

To further investigate the mTOR signaling pathway, downstream targets were examined in the proteomics data set. As the proteomics data do not include protein modifications such as phosphorylation, a well-known post-translational modification regulated by mTORC1, we analyzed the abundances of proteins upregulated upon mTORC1 activation, particularly those containing a 5'-terminal oligopyrimidine (5'-TOP) motif at the beginning of their mRNAs. Many components of the translation machinery, including several ribosomal proteins and some translation factors, possess this 5'-TOP motif. These proteins were notably upregulated in SCA2 cells compared to controls, as shown in **Complementary Figure 4C-D**, indicating altered mTORC1 pathway activity in SCA2 cells.

Perturbed Expression Patterns of the Ubiquitin-Proteasome System and Autophagic System

The ubiquitin-proteasome system and autophagy are pivotal mechanisms for degrading cytotoxic proteins and maintaining cellular homeostasis. Previous studies have reported aberrant levels of autophagic markers, such as SQSTM1 and LC3B, in cells expressing polyQ-expanded ATXN2, suggesting a perturbation in at least the autophagic pathway^{72,85}.

The proteomic analysis of SCA2 cells reveals alterations in the expression profiles of proteins implicated in the proteasome system. Notably, there is a downregulation of UBA7, HUWE1, PSMD4, and UBA5 juxtaposed with an upregulation of PSME3, CUL4B, and PSMA4 in SCA2 cells (**Complementary Figure 5A**). The cumulative expression of proteasome system proteins demonstrates a modest increase in SCA2 cells compared to controls. In contrast, no such trend is observed for proteins associated with the autophagic system. The violin plot distributions for both the proteasome and autophagic systems manifest altered shapes, indicative of perturbed expression patterns within these protein cohorts (**Complementary Figure 5B**). Of particular significance is the upregulation (fold change: 4.14) of the lysosomal marker LAMP2 in SCA2 cells, suggestive of heightened lysosomal biogenesis or the selective autophagy activation termed chaperone-mediated autophagy.



COMPLEMENTARY FIGURE 6 - Subcellular Localization and Proteomic Profiling of Mitochondrial Proteins in SCA2 Fibroblasts

A: Images display the mitochondrial network within control and SCA2 fibroblasts, stained with MitoTracker Green. The dashed square highlights an area selected for magnification. Scale bar: 25 μ m. **B:** Similar to panel A, mitochondria are visualized using an antibody against TIMM50, a mitochondrial import receptor. Red triangles and white arrows identify small puncta morphology. Scale bar: 25 μ m. **C:** Hierarchical clustering heatmap of global proteomic distribution, Z-scores for protein abundances from global proteomics are displayed across four subcellular fractions: whole cell, cytosol, mitochondria, and nucleus. **D:** Hierarchical clustering heatmap of mitochondrial proteins: This heatmap focuses on the distribution of mitochondrial proteins across the same four subcellular fractions. It provides insights into mitochondrial proteins' localization and relative abundance, highlighting differences between SCA2 cells and controls. **E:** Hierarchical clustering heatmap of OXPHOS proteins: Specifically targeting proteins involved in oxidative phosphorylation (OXPHOS), this heatmap illustrates their abundance across the subcellular fractions. The color gradient emphasizes the differential expression and potential dysregulation of OXPHOS proteins in SCA2. Note: The heatmaps use a color gradient from blue (low abundance) to red (high abundance) to represent protein levels to identify subcellular localization patterns. All heatmaps were generated using Perseus software.

Dysregulation of Mitochondrial Proteins

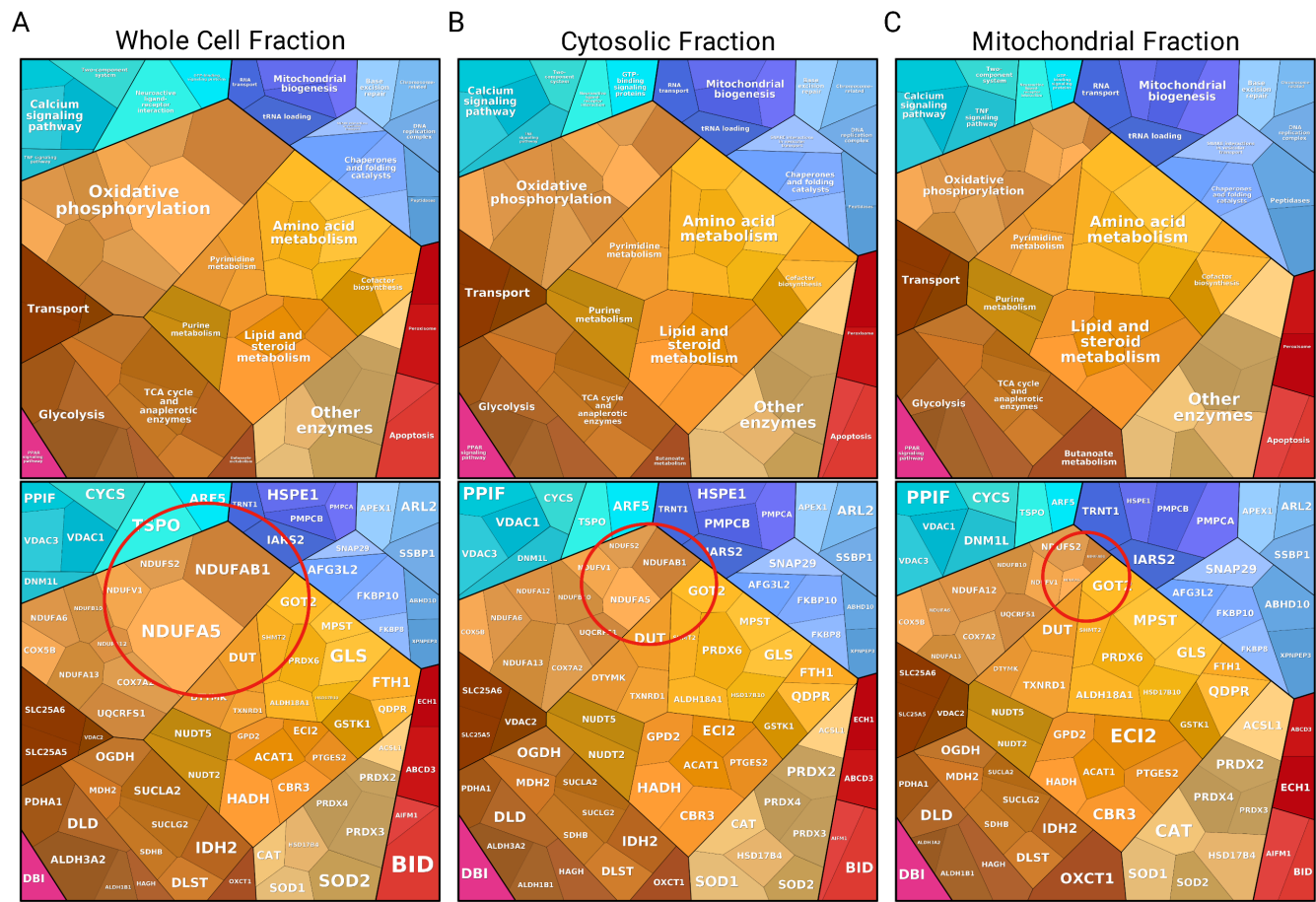
Analyses of mitochondrial protein abundance in SCA2 and control cells revealed changes in the expression of specific mitochondrial proteins. Upregulation was observed in proteins such as TSPO, NDUFA5, ALDH3A2, and SUCLA2, while downregulation was noted in VDAC2, HINT2, HSD17B10, and AIFM1, suggesting potential dysregulation of these particular mitochondrial proteins. However, the cumulative protein abundances shown in the violin plots did not indicate significant differences in mitochondrial proteins between the two groups (**Complementary Figure 4A-B**). Moreover, when examining the global MS data set for mitochondrial proteins identified in the IP-MS data set, a marked increase was observed for SLC25A6, which exhibited a 1.87-fold expression in SCA2 cells compared to control counterparts.

The initial experimental design aimed to evaluate the impact of polyQ-expanded ATXN2 on protein subcellular distribution. Subcellular fractionations of whole cells, cytosol, mitochondria, and nuclei were performed on control and SCA2 patient cell lines. Using iTRAQ methodology, which allows simultaneous identification and quantification of multiple peptides in complex protein samples, subcellular fractions from three controls and five SCA2 patient cell lines were pooled after labeling with distinct isobaric tags. MS analysis revealed significant differences in protein distribution within the mitochondrial fractions of SCA2 cells compared to control cells (**Complementary Figure 5C-E**). Specifically, certain mitochondrial proteins (AFG3L2, ALDH3A2, ETFA, NDUFA13, NDUFA5, NDUFA6, NDUFB10, NDUFV1, SLC25A6, SUCLA2, TIMM50, TSPO, and VDAC1) showed reduced protein abundances (fold change: <0.8) within the mitochondrial fraction in SCA2 cells. In contrast, these specific proteins exhibited increased protein abundances (fold change: >1.25) within the "whole cell" fraction in SCA2 cell lines compared to controls (**Complementary Figure 5D-E**).

The observed differences in protein abundances between the "whole cell" and mitochondrial fractions warranted further investigation, as the cause of the previously noted mitochondrial phenotype in SCA2 cells remains unclear. We hypothesized that impaired mitochondrial protein import or impaired trafficking of nucleus-encoded mitochondrial proteins to the mitochondria may lead to the mislocalization of these proteins. NDUFA5 and NDUFB10 were of particular interest, given their essential roles in oxidative phosphorylation and the notable differences in protein abundances between the "whole cell" and mitochondrial fractions in SCA2 cells (**Complementary Figure 6A, -C**). However, due to difficulties in obtaining reliable antibodies for these proteins, we instead focused on TIMM50.

SCA2 cells display a distinct mitochondrial phenotype with increased puncta morphology and enlarged, rounded mitochondria (**Complementary Figure 5A**), suggesting mitochondrial dysfunction or a cellular stress response. Fixed SCA2 and control cells were immunostained with TIMM50 antibody to further investigate the subcellular differences in protein localization. **Complementary Figure 5B** shows the cellular SCA2 phenotype for mitochondrial morphology (red triangle) visualized by the TIMM50 antibody. Small TIMM50-positive dots distinct from the surrounding mitochondria (white arrows) were also observed, potentially representing TIMM50 aggregates or fragmented mitochondria undergoing fission. However, verification of the subcellular fractionation protocol was unsuccessful, and thus, the findings of potential mislocalization of mitochondrial proteins as a phenotype of SCA2

pathology could not be confirmed.



COMPLEMENTARY FIGURE 7 - Distribution of Mitochondrial Proteins Between Subcellular Fractions
Proteomaps were used to visualize mitochondrial proteins and their ratios (SCA2 vs. control) of protein abundances in the “whole cell” fraction (**left**), cytosolic fraction (**middle**), and mitochondrial fraction (**right**). Each tile in the map represents a protein; size is proportional to its fraction in the total dataset. Tile colors represent different gene annotations. Proteins that are closer on the maps and share the same color are closer in function. Visualization was done using the Bionic Visualizations proteomaps (<https://bionic-vis.biologie.uni-greifswald.de/>).

DISCUSSION

This study presents a detailed investigation of the proteomic and RNA-binding modifications associated with polyQ expansions in the ATXN2 protein in SCA2. Our findings elucidate a complex interplay between augmented PPIs and altered RNA dynamics, modulated by the length of the polyQ tract within ATXN2. Notably, the results highlight a polyQ-length-dependent increase in the interaction of ATXN2 with ribosomal and chaperone proteins, suggesting a link between protein folding, altered translation, mRNA binding, but also indications of dysregulated mTOR signaling and an altered mitochondrial phenotype in SCA2. These alterations may play a multifaceted and intertwined role in the pathogenesis of SCA2.

PolyQ Expansion in ATXN2 Alters Protein-Protein Interactions in SCA2 Patient Cell Lines

The detection of known ATXN2 interactors, such as PABPC1, ATXN2L, DDX6, and LSM12, corroborates with previous studies and highlights the role of ATXN2 in mRNA regulation and stability. In particular, PABPC1 is

crucial for mRNA metabolism, including microRNA-mediated gene silencing and the regulation of mRNA stability and translation through binding to the poly(a) tail of mRNA¹²⁵. The link between PABPC1 and ATXN2 has been well studied in relation to SCA2^{50,58,60,126}. Our IP-MS analysis reveals that the ATXN2:PABPC1 interaction is dependent on the polyQ expansion length, as only a significant increase is observed in the group with 44 CAG repeats (**Complementary Figure 2B**) but not in groups with 36 or 42 CAG repeats. Damrath *et al.* reported sequestration of PABPC1 into an insoluble fraction in transgenic mice for ATXN2 with 42 CAG repeats¹²⁶. The absence of significant PABPC1 sequestration in heterozygous SCA2 patient-derived cells may be due to the presence of a normal ATXN2 allele, in contrast to homozygous transgenic disease models.

Interestingly, ATXN2 and ATXN2L exhibit an opposite interaction pattern, with a significant increase in the 36 CAG repeat group and a proportional decrease with increasing polyQ expansion length (**Complementary Figure 2B**).

Moreover, ATXN2:DDX6 and ATXN2:LSM12 interactions, though not significantly, decrease in patient cell lines (**Complementary Figure 2B**). DDX6 is involved in mRNA decay, translational repression, and the formation of P-bodies, while LSM12 has been shown to play a role in translational and mRNA regulation^{42–44,46}, and interacts with ATXN2 in active translating ribosome complexes¹²⁷.

Interestingly, ATXN2:DDX6 and ATXN2:LSM12 interactions occur directly through the LSm and LSmAD domains^{39,44,46}, which are harbored near the polyQ region^{44,128}. This could indicate that the polyQ expansion in ATXN2 imposes loss-of-function properties, impairing the binding of DDX6 and LSM12 to ATXN2.

These findings suggest a progressive decline in ATXN2:ATXNL2 and ATXN2:LSM12 interactions correlating with increasing polyQ expansion length (**Complementary Figure 2B**), suggesting a polyQ-expansion length-dependent impairment or loss of function. The altered interplay between ATXN2 and LSM12 could modulate posttranscriptional regulation, as LSM12 and DDX6 have been demonstrated to define distinct modes of posttranscriptional regulation by the Atx2 (*Drosophila* ATXN2 homolog) protein complex in circadian pacemaker neurons of *Drosophila*⁴⁶, and interestingly, sleep disturbances are reported as a common feature in SCA2 patients¹²⁹. The increased ATXN2 PPI may not solely result from polyQ-expansion-mediated aggregation but could also stem from deleterious properties affecting specific ATXN2 functions. Further research is needed to elucidate the impact of polyQ expansion on the LSm domain and the interaction profile between ATXN2 and LSM12, ATXN2L, DDX6, and PABPC1.

Increased Association of ATXN2 with Ribosomal Proteins in SCA2 Cells: Implications for Translational Dysregulation

Our investigations of the global proteomics of SCA2 cells revealed an increase in ribosomal proteins, and the increase in protein abundance of ribosomal proteins and other proteins involved in translation (**Table 1, Figure 1E, and Complementary Figure 5**) may reflect a compensatory mechanism as the cell attempts to restore normal ribosome function and protein synthesis capacity, potentially caused by increased association between ribosomal proteins and polyQ expanded ATXN2. It cannot be ruled out that the increase in ATXN2-associated ribosomal proteins is simply an artifact of the increased availability of these proteins. However, some of the most significantly increased ribosomal proteins associated with ATXN2 in the IP-MS assay, RPS28 and RPL8 (**Table 1**, see manuscript of Study

I), are, in fact, downregulated in SCA2 cells compared to controls, which contradicts the notion that the increased association of ATXN2 and ribosomal proteins is a matter of increased availability.

It is widely accepted that the expanded polyQ tract confers a toxic gain of function to the ATXN2 protein¹⁹ and is supported by ATXN2 knock-out studies in mice that do not show signs of cerebellar atrophy or symptoms of ataxia¹³⁰. Interestingly, a study on *Atxn2*^{-/-} mice found upregulations of ribosomal proteins similar to the results found in this study⁵⁸, indicating that the expanded polyQ region in ATXN2 may also exhibit deleterious properties to the native function of the protein. Interference with ribosomal biogenesis is often associated with cancer, aging, and age-related degenerative diseases¹³¹, and the fact that the onset of SCA2 also exhibits an age dependency further supports the potential involvement of reduced or altered translation through impaired ribosomal proteins in the progression of SCA2 pathology.

Previous studies have documented the association of ATXN2 with ribosomal proteins⁵⁹⁻⁶¹, and the current investigation corroborates these findings. Lastress-Becker *et al.* reported that ATXN2 assembles with polyribosomes⁶¹, while Terrence F. Satterfield and Leo J. Pallanck proposed a model in which this assembly interferes with polyribosome function, leading to translation stalling and arresting ribosomal movement along the mRNA⁶⁰. Interestingly, the IP-MS data from the current study display an enhanced association between ATXN2 and ribosomal proteins, which may contribute to the SCA2 pathology through impaired translation, as proposed by Satterfield and Pallanck, or by participating in the formation of aggregates that sequester key translation regulators such as Eukaryotic Translation Elongation Factor 1 Alpha 1 (EEF1A1), Eukaryotic Translation Initiation Factor 4A1 (EIF4A1), and Eukaryotic Translation Elongation Factor 2 (EEF2), see **Table 1** (see manuscript of Study I). EEF2 is indispensable for the elongation phase of protein synthesis by facilitating ribosomal translocation along the translated mRNA¹³², therefore the augmented PPI between EEF2 and ATXN2 in SCA2 cells potentially points towards an involvement of translational dysregulation in the pathogenesis of SCA2. Intriguingly, mutations in the EEF2 gene have been found implicated in neurodevelopmental disorders and neurodegenerative disorders, including spinocerebellar ataxia type 26 (SCA26)¹³²⁻¹³⁴, thereby accentuating the critical importance of translational mechanisms in the etiology of neurodegenerative diseases.

Based on our results, it is difficult to pin down the effect of the increased PPI between ATXN2 and ribosomal proteins in SCA2 patient cells; however, it is intriguing that the majority of proteins identified from the IP-MS are reported to be localized to messenger ribonucleoproteins (mRNPs). mRNPs play versatile roles in the regulation of translation, capable of both repressing and activating mRNA translation depending on the cellular context, the specific composition of proteins and RNA species, and the specific regulatory mechanisms at play¹³⁵.

Studies have reported the PAM2 domain and the intrinsically disordered regions (IDRs) of ATXN2 promote the formation of mRNP granules, while the LSm domain acts to inhibit this process^{49,59,136}. This antagonistic role is crucial because it helps to balance the formation and disassembly of mRNP granules, which are important for cellular stress responses and the regulation of mRNA stability and translation. The studies show that deletion of the LSm domain enhances the formation of mRNP granules and increases cytotoxicity, suggesting that the LSm domain normally acts to protect against neurodegeneration by preventing excessive mRNP granule formation^{49,136}. The

neighboring polyQ region, when expanded, may exacerbate the dysfunction of the LSm domain, thereby disrupting the equilibrium between mRNP granule assembly and disassembly. Together with the results from these previous studies, our data indicate that the increase in PPI of ATXN2 and proteins involved in translation could be a result of polyQ length-dependent impairment of the LSm domain promoting excessive mRNP assembly, potentially exhibiting aggregating properties.

Impact of Expanded Polyglutamine Region on RNA-Protein Interactions of ATXN2

The opposing roles of the LSm domain and the IDRs are also reported to be essential for ATXN2-mediated recruitment of mRNAs^{49,136} and our results obtained via immunoprecipitation coupled with RNAseq revealed significant alterations in the RNA binding profile of ATXN2 in patient cell lines compared to controls. Considering the possibility of expanded polyQ region impairment of the LSm domain and, thereby, the disruption of the interplay between these protein structures, the expanded polyglutamine region may very well directly affect the RNA-protein interactions of ATXN2. Notably, there was a decrease in the abundance of RNAs bound to ATXN2 in patient cells, suggesting an impaired RNA-protein interaction landscape. However, the IP-RNAseq results also revealed increased RNA binding to ATXN2 of specific RNAs, which contradicts this interpretation and instead might indicate a shift in RNA binding.

This altered RNA-binding profile of ATXN2 in SCA2 patient cells could affect the translation of these specific mRNAs. From the global proteomics and the IP-RNAseq results, we observed a subtle correlation between the mRNAs displaying decreased association to ATXN2 in the IP-RNAseq and a reduced abundance of the proteins encoded by these same mRNAs. This could suggest a dysregulation in the translation of specific mRNAs mediated by less RNA association to ATXN2 in SCA2 cells, as we do not see the same tendency in the protein abundances of the mRNAs with increased association to ATXN2 in SCA2 patient cell lines. However, one plausible explanation could be that the IP-RNAseq results reflect the global transcriptional differences between SCA2 cells and control cells, which exposes a weakness of this study. Quantifying the global transcriptome would have strengthened the results of both the IP-RNAseq and the global proteomics and paved the way for more comprehensive interpretations and conclusions. Therefore, more studies are needed to uncover the implications of ATXN2's RNA binding ability in the SCA2 pathology.

Potential Synergistic Role of ATXN2, microRNAs, and lncRNA SNHG5 in SCA2 Pathology

In addition to an altered RNA-binding profile of ATXN2 in SCA2 cells, we also found two types of RNAs, besides protein-coding mRNA, represented in the increased fraction of ATXN2 bound RNAs, namely the microRNAs miR6873 and miR6891 as well as the long non-coding RNA, SNHG5. Currently, there is limited research concerning the involvement of miRNAs in the pathology of SCA2, but McCann *et al.* demonstrated that ATXN2 is potentially necessary for miRNA activity in *Drosophila*⁴⁵.

The long non-coding RNA SNHG5 has mainly been studied in connection with cancer research, and SNHG5 has been reported to exert its functional activities by sponging miRNAs¹³⁷. A study reported SNHG5 to interact with

STAU1¹³⁸, an RNA binding protein studied intensively in connection with SCA2 pathology^{86–88,139}. The study by the Lund group at Biotech Research and Innovation Centre, University of Copenhagen, found SNHG5 to stabilize transcripts by blocking their degradation by STAU1¹³⁸. Our results showed an increased association between ATXN2 and SNHG5, which could impose an impaired function of SNHG5, resulting in decreased blocking of STAU1 activity. Interestingly, increased STAU1 activity has been reported and linked to SCA2 pathology⁸⁸. These findings could potentially link ATXN2, SNHG5, and STAU1 to synergize a possible role in SCA2 pathology, which future studies could investigate further.

Chaperone Proteins in SCA2 Pathology

Heat shock proteins (HSPs) are molecular chaperones that play a pivotal role in maintaining proteostasis by facilitating the proper folding of nascent polypeptides, preventing aberrant protein aggregation, and promoting the refolding or targeted degradation of misfolded proteins. In the context of various pathological conditions, HSPs exhibit a dichotomous nature: on one hand, they confer cytoprotection against stress-induced cellular damage; conversely, they may also contribute to the formation of protein aggregates, a hallmark of numerous neurodegenerative disorders¹⁴⁰. This sequestration phenomenon diminishes the availability of HSPs, consequently compromising their ability to execute their protective functions and maintain cellular proteostasis¹⁴⁰.

The observed increase in chaperone expression in SCA2 cells (**Figure 1E**, see manuscript of Study I) could be indicative of a cellular response to proteotoxic stress conditions induced by polyQ expansions in ATXN2, as the IP-MS data demonstrated an enhanced association of chaperone proteins with ATXN2 in SCA2 cells (**Figure 2C**, see manuscript of Study I), suggesting that ATXN2 may be recognized as misfolded or aggregated in SCA2 cells.

DNAJC3 abundance in SCA2 cells was decreased, while concurrently, ATXN2 in SCA2 cells exhibited an enhanced association with DNAJC3. DNAJC3, a co-chaperone, is known to translocate to the endoplasmic reticulum (ER) lumen under conditions of ER stress¹⁴¹. Previous studies have reported ATXN2 to be localized on the ER surface but not within the ER lumen³². This observation could be indicative of increased interaction between ATXN2 and the ER membrane, further corroborated by the elevated association of ATXN2 with ER-related proteins (HSPA5, CKAP4, LEPRE1, and SYNE2) and ribosomal proteins in SCA2 cells. The augmented association of DNAJC3 with ATXN2 in SCA2 cells, coupled with the reduced protein abundance of DNAJC3, could potentially result in a functional deficiency of this co-chaperone. Notably, a study implicated DNAJC3 deficiency as a causative factor in multisystemic neurodegeneration, including ataxia¹⁴². However, further investigations are warranted to elucidate the precise role of chaperone proteins and ER involvement in the pathogenesis of SCA2.

Chaperone-Mediated Autophagy in SCA2: The Association of HSPA8, HSP90AA1, and ATXN2

Heat shock proteins, such as HSPA8 and HSP90AA1, have been implicated in the selective lysosomal degradation pathway known as chaperone-mediated autophagy (CMA), and our findings revealed a significant association of these proteins with ATXN2 in SCA2 cells. The key molecular players orchestrating CMA include HSPA8, HSP90AA1, and the Lysosomal Associated Membrane Protein 2 (LAMP2A), which collectively facilitate the

recognition, unfolding, and translocation of substrate proteins across the lysosomal membrane for subsequent degradation^{79,84,143,144}, and the association between HSPA8, HSP90AA1, and ATXN2 could indicate that ATXN2 is being targeted for degradation via CMA. Additionally, LAMP2 (giving rise to three different splice variants: LAMP2A, LAMP2B, and LAMP2C)¹⁴⁵ is highly increased in SCA2 cells compared to controls. Together, these results could indicate an increase of CMA activity in SCA2 cells, perhaps as a compensatory mechanism of decreased/impaired macroautophagy or as a response to increased cellular stress such as oxidative stress, ER stress, or proteotoxic stress mediated by protein aggregation. However, subsequent research is needed to elucidate to what extent chaperone-mediated autophagy is involved in the pathogenesis of SCA2.

Mitochondrial Proteins and Mitochondrial Precursor Overaccumulation Stress (mPOS) in SCA2

Comparative analysis of mitochondrial protein levels between SCA2 and control cells reveals notable disparities in the global MS. Specifically, the mitochondrial proteins TSPO, NDUFA5, ALDH3A2, SUCLA2, PRDX3, SLC25A6, ATP5J, and NDUFA6 exhibit upregulation in SCA2 cells, while VDAC2, HINT2, HSD17B10, and AIFM1 were downregulated. These findings suggest a perturbation in the homeostatic regulation of specific mitochondrial proteins. The imbalance may be due to dysregulated translation, altered mRNA stability, or aberrant mTOR signaling^{146–148}.

The accumulation and aggregation of unimported mitochondrial proteins in the cytosol present significant proteostatic challenges that can be detrimental to cellular health. When the accumulation of these unimported proteins exceeds the cellular capacity for stabilization, sequestration, and degradation, a condition known as mitochondrial precursor overaccumulation stress (mPOS) ensues^{149–152}. The development of mPOS can be attributed to a perturbation in the delicate balance among mitochondrial precursor import efficiency, precursor handling and delivery, and proteasome activity^{149,150}. Additionally, mitochondrial health has been reported to be compromised in SCA2⁶⁹, leading to depolarization of the mitochondrial membrane, thereby disrupting the efficiency of mitochondrial precursor import^{151,153–155}. Additionally, mPOS can arise from the inability of cytosolic chaperones, such as HSP70, HSP90, and their co-chaperones, to effectively stabilize and deliver nascent precursors to the import machinery located on the outer mitochondrial membrane^{149,152}.

Interestingly, a study reported that the overexpression of the mitochondrial carrier protein SLC25A6 induces the formation of protein aggregates and increases cell death¹⁵⁶. Our findings reveal a 1.87-fold increase in SLC25A6 levels in SCA2 cells compared to controls, warranting further investigation, particularly in light of the dysfunctional mitochondria observed as a cellular hallmark phenotype of SCA2⁶⁹, and the potential impairment of chaperone capacity. Additional studies addressing these findings would be of high interest in relation to the SCA2 pathology.

mTOR Signaling in SCA2

The mTOR pathway plays a key role in regulating ribosome biogenesis and the initiation of translation, both of which are upregulated in our global MS analyses. Although the detection of the mTOR protein in the global proteomics data was suboptimal due to the limited number of identified peptide sequences, the preliminary

screening indicated a 2.7-fold increase in mTOR expression in SCA2 cells compared to control cells. Examination of the upstream and downstream targets of mTOR revealed elevated protein abundances, particularly for proteins encoded by mRNAs containing a 5' Terminal OligoPyrimidine (5' TOP) motif, which are known to be upregulated upon mTORC1 activation. Collectively, these findings suggest that mTORC1 activity is increased in SCA2 cells, corroborating the results of several studies demonstrating mTOR upregulation and activity in SCA2 disease models⁸⁷⁻⁹⁰. The IP-MS results revealed a significantly increased interaction between HSP90AA1 and ATXN2 in SCA2 cells. Interestingly, a study on HSP90AA1 suggested that this chaperone inhibits the PI3K/AKT/mTOR pathway by decreasing the phosphorylation of AKT and mTOR¹⁵⁷. It is plausible that polyQ-expanded ATXN2 may trap and impair HSP90AA1 function, leading to increased mTOR activity. However, a loss of function of ATXN2 could also potentially enhance mTOR activity in SCA2, as the *Caenorhabditis elegans* homolog of ATXN2 plays a crucial role in regulating the mTOR pathway by acting as a repressor of mTOR⁶⁸.

Moreover, a study investigating mTORC1 activity in cerebellar Purkinje cells found that mTORC1 hyperactivation significantly induced apoptosis, which was associated with abnormal mitochondrial activity and elevated cellular stress, disrupting both cellular and mitochondrial homeostasis¹⁵⁸. The researchers also reported enlarged mitochondrial morphology upon mTORC1 hyperactivation, reminiscent of the mitochondrial phenotype described in SCA2 cells (**Complementary Figure 5A**). Prolonged activation of mTORC1 has been shown to lead to dysregulated mitochondrial function. While short-term mTORC1 activation supports cellular growth and energy production, chronic activation can contribute to metabolic disorders and cellular stress. For example, in the context of hyperglycemia, mTORC1 activation induces impaired mitochondrial metabolism, resulting in decreased insulin content and altered glycolysis in pancreatic β -cells¹⁵⁹. This suggests that although mTORC1 activation is essential for normal cellular function and adaptation, sustained activation can have detrimental effects on mitochondrial function and overall cellular health. This could link the altered mitochondrial phenotype reported in SCA2 cells with the increased activation of mTOR observed in SCA2 pathology. However, further studies are necessary to elucidate the precise pathological link between the cellular SCA2 phenotypes, considering the interplay between mTOR activity and mitochondrial function.

STUDY II

TITLE

Enhancement of Autophagy and Solubilization of Ataxin-2 Alleviate Apoptosis in Spinocerebellar Ataxia Type 2 Patient Cells

BRIEF INTRODUCTION

Many neurodegenerative disorders are characterized by pathological accumulation of aggregated proteins, and they are known as proteinopathies. A subset of neurodegenerative diseases, which includes Huntington's disease (HD) and spinocerebellar ataxias, exhibit a common pathological feature: polyglutamine expansions within specific proteins¹⁶⁰. The clinical manifestations of these diseases vary, influenced by the specific brain regions and neuronal subtypes most adversely affected by the mutated proteins. Despite the varied and occasionally unknown functions of these proteins, they are pathophysiologically connected by their polyglutamine expansions. This shared polyglutamine motif not only disrupts the native function of the proteins but also imparts novel properties¹⁶¹. Clearance of misfolded or short-lived proteins involves chaperones, proteases, and, notably, the ubiquitin-proteasome system (UPS). Conversely, neurotoxic protein aggregates and damaged cellular organelles such as mitochondria are degraded by autophagy^{162,163}. Together, the proteasome and autophagy system maintain cellular homeostasis. However, larger protein aggregates can form when the degradation systems are impaired due to disease pathology or an excessive protein burden. These aggregates sequester vital cytosolic components, disrupting intracellular signaling and impairing the proteostatic system itself, leading to further protein accumulation and, eventually, cellular apoptosis^{164,165}. Disrupted autophagy is a prevalent aspect of the pathophysiology in many neurodegenerative diseases, affecting not only protein turnover and subsequent accumulation but also the degradation of damaged organelles, such as mitochondria¹⁶⁶⁻¹⁶⁸. Mitochondria are crucial for maintaining intracellular energy and regulating reactive oxygen species (ROS) levels, which are vital for cell signaling^{169,170}. However, excessive ROS production can lead to cellular damage, and dysfunctional mitochondria may initiate apoptosis¹⁷¹. The apoptotic machinery is activated by two distinct routes: the extrinsic pathway, primarily involving caspase-8, and the intrinsic pathway, primarily involving caspase-9. Caspase-8 is activated by signals from death receptors on the cell surface, leading to the formation of the death-inducing signaling complex (DISC) and subsequent activation of downstream effector caspases like caspase-3, -6, and -7^{172,173}. In contrast, caspase-9 is activated by intrinsic signals, such as intracellular stress signals, particularly those involving mitochondrial damage, which release cytochrome c, causing the formation of the apoptosome, subsequently resulting in apoptosis^{172,173}. Previous research reveals that fibroblasts from SCA2 patients exhibit elevated oxidative stress and display several abnormalities compared to controls⁶⁹. These include disruptions in the antioxidant system, alterations in the complexes that participate in oxidative phosphorylation, and changes in mitochondrial morphology, many of which are also observed in broader neurodegenerative disease contexts¹⁶⁸.

OBJECTIVE

Hypothesis: Proteasome function and autophagy are dysregulated, contributing to SCA2 pathology.

AIMS

- To evaluate the redox state in SCA2 patient-derived fibroblasts by applying caspase activity assays and measuring subsequent changes in the cellular redox.
- To assess proteasome function and autophagic flux in the context of caspase activity assays and measurement of protein levels of autophagy markers and ATXN2, utilizing chemical modulators of proteasome function and autophagic flux.
- Determine if solubilization of ATXN2 protein in SCA2 fibroblasts and autophagic activation can alleviate the SCA2 pathology.

MATERIAL & METHODS

Cell Lines and Cell Line Generation

See Manuscript for Study II (Wardman *et al.*, 2020)

General Culture Conditions

See Manuscript for Study II (Wardman *et al.*, 2020)

Pharmacological Treatments

See Manuscript for Study II (Wardman *et al.*, 2020)

Immunocytochemistry

See Manuscript for Study II (Wardman *et al.*, 2020)

Vitality Assay

See Manuscript for Study II (Wardman *et al.*, 2020)

Caspase Activation Assays

See Manuscript for Study II (Wardman *et al.*, 2020)

Protein Extraction

See Manuscript for Study II (Wardman *et al.*, 2020)

SDS/RIPA Extractions

See Manuscript for Study II (Wardman *et al.*, 2020)

Western Blot Analysis

See Manuscript for Study II (Wardman *et al.*, 2020)

Statistical Analysis

See Manuscript for Study II (Wardman *et al.*, 2020)

RESULTS

This investigation sought to delineate cellular pathologies of SCA2, with a particular emphasis on elucidating the roles of autophagy, protein aggregation, and oxidative stress in the disease's pathogenesis. The hypothesis posited that oligomerization of the polyglutamine-expanded ATXN2 protein impairs cellular function predominantly by disrupting autophagic processes and facilitating the production of ROS. To this end, five fibroblast cell lines derived from SCA2 patients, alongside three fibroblast cell lines from healthy controls, were subjected to a series of experiments that modulate autophagy, proteasome activity, and ATXN2 protein aggregation, and solubility dynamics.

Proteasomal Activity in SCA2 Cells

Fibroblasts from SCA2 patients displayed a modest yet statistically significant elevation in apoptotic activity via both caspase-9 (4% increase, $p=0.0410$) and caspase-8 (9% increase, $p=0.0276$) pathways compared to control fibroblasts. Subsequent exposure to the proteasome inhibitor MG-132 elicited comparable increases in caspase-9-mediated apoptosis across both control and SCA2 fibroblasts. In contrast, caspase-8-mediated apoptosis was notably more elevated in SCA2 fibroblasts (17.33% increase, $p=0.0208$) following MG-132 treatment, suggesting that proteasome inhibition exacerbates extrinsic apoptotic pathways in SCA2 pathology. Furthermore, SCA2 fibroblasts exhibited a decline in viable cells post-MG-132 treatment (23% greater decrease, $p<0.0001$), indicating an increased susceptibility to oxidative stress (**Figure 1**, Wardman *et al.*, 2020).

Western blot analysis revealed that ATXN2 protein levels remained unchanged upon proteasome inhibition, suggesting that the proteasome does not govern ATXN2 degradation. Evaluation of autophagy markers p62/SQSTM1

(p62) and LC3-II demonstrated stable p62 levels, but a significant upregulation of LC3-II (control: 5.63-fold increase, $p=0.0001$; SCA2: 3.44-fold increase, $p=0.0022$), which indicates either an increase or a blockage in the autophagy flux (**Figure 1**, Wardman *et al.*, 2020).

Autophagy in SCA2 Cells

To assess autophagic function, SCA2 fibroblasts were treated with chloroquine, a pharmacological inhibitor of autophagy that impedes lysosomal acidification and subsequent accumulation of autophagosomes. Chloroquine treatment caused a significant upregulation of caspase-9-mediated apoptosis in SCA2 fibroblasts relative to controls (19% greater increase, $p = 0.0006$), underscoring a heightened vulnerability of these cells to autophagic disruption (**Figure 2A**, Wardman *et al.*, 2020). In contrast, caspase-8-mediated apoptosis did not exhibit a comparable increase (**Figure 2B**, Wardman *et al.*, 2020). Additionally, chloroquine treatment generated oxidative stress in SCA2 cells, as indicated by a decline in the level of cytosolic reduced glutathione (14% greater decrease, $p = 0.0789$), see **Figure 2C**, Wardman *et al.*, 2020. This finding suggests that compromised autophagy in SCA2 cells exacerbates oxidative and mitochondrial-induced stress.

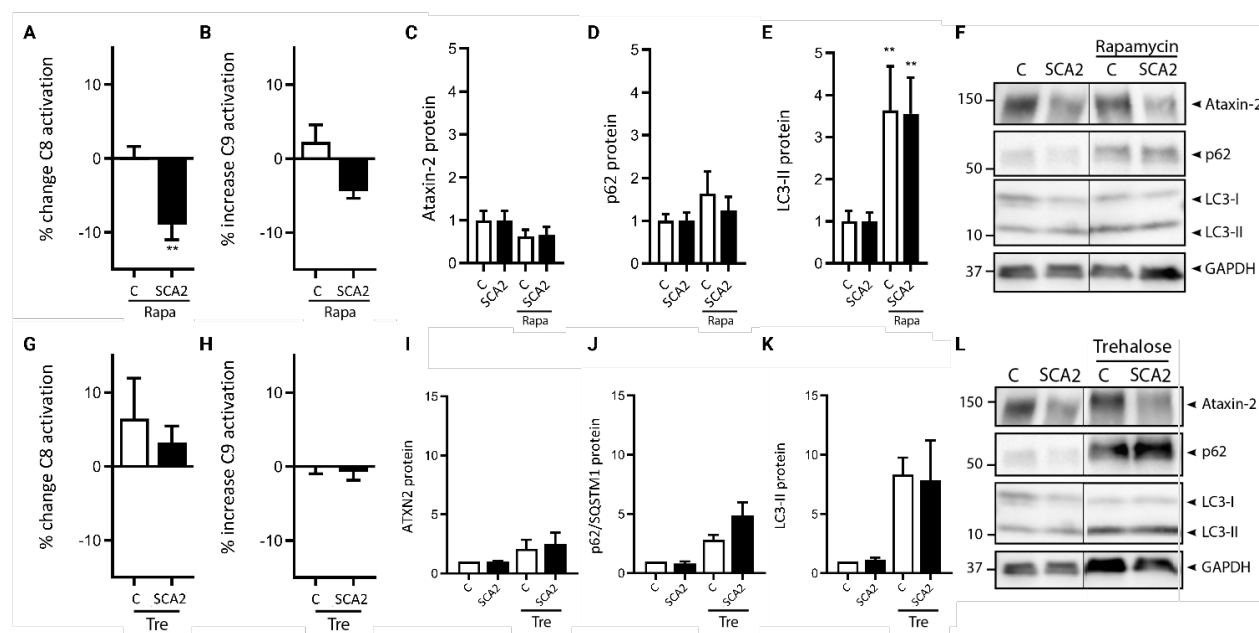
Western blot analysis revealed that chloroquine treatment led to elevated levels of ATXN2, p62, and LC3-II proteins in both control and SCA2 fibroblasts, suggesting that ATXN2 is degraded via autophagy (**Figure 2D-G**, Wardman *et al.*, 2020). This result also implies that the polyQ expansion in ATXN2 does not interfere with the formation of autophagosomes. However, a less pronounced accumulation of LC3-II protein levels was observed in SCA2 cells compared to controls under chloroquine treatment, indicating a reduced autophagic flux in SCA2 cells (**Figure 2G**, Wardman *et al.*, 2020).

Impact of Oxidative Stress and Autophagic Activation on SCA2 Pathology

Given the observed oxidative stress and altered mitochondrial morphology in SCA2 cells⁶⁹, we posited that autophagic clearance of dysfunctional mitochondria (mitophagy) was compromised and thereby contributed to increased caspase-9-mediated apoptosis. To investigate this hypothesis, cells were exposed to hydrogen peroxide (H_2O_2) to induce oxidative stress, and subsequently examined whether activation of autophagy could mitigate this stress. We evaluated the efficacy of two autophagy-inducing agents: rapamycin and trehalose. Rapamycin functions as an inhibitor of mammalian target of rapamycin (mTOR), a pivotal regulator of cellular growth, metabolism, and autophagy, while trehalose, a naturally occurring disaccharide, serves as a molecular chaperone and an mTOR-independent stimulator of autophagy.

Experimental outcomes revealed that rapamycin treatment did lower the ATXN2 protein levels, though not significantly (**Complementary Figure 8C/Figure 3F**, Wardman *et al.*, 2020), but did not forestall H_2O_2 -induced apoptosis in SCA2 cells (**Figure 3C**, Wardman *et al.*, 2020). Conversely, trehalose treatment effectively inhibited the increase in caspase-9 activation triggered by oxidative stress from H_2O_2 exposure (**Figure 4B**, Wardman *et al.*, 2020). However, an increase in caspase-8 activity was observed in both control and SCA2 cells following trehalose treatment

(**Complementary Figure 8G**). This suggests that while trehalose may offer protective effects under stress conditions, its role under basal conditions could potentially exacerbate caspase-8-mediated apoptotic pathways.



COMPLEMENTARY FIGURE 8: Autophagic Activation Induced by Rapamycin and Trehalose in SCA2 Patient Cells.

A: Treatment with rapamycin in SCA2 patient fibroblasts resulted in a significant reduction of caspase-8 activation compared to control cells, with a 9.2% greater decrease ($p = 0.0019$). **B:** A 6.7% greater decrease in caspase-9 activation was observed in SCA2 patient fibroblasts following rapamycin treatment, indicating a significant reduction ($p = 0.0078$). **C:** Rapamycin treatment did not significantly alter ataxin-2 protein levels in either control or SCA2 fibroblasts. **D:** Levels of p62 protein remained unchanged in control and SCA2 fibroblasts after rapamycin treatment. **E:** Both control and SCA2 cells exhibited a significant increase in LC3-II protein upon rapamycin treatment, with a 2.63-fold increase in control cells ($p = 0.0059$) and a 2.56-fold increase in SCA2 cells ($p = 0.0025$). **F:** Western blot images display ATXN2, p62/SQSTM1, LC3-II, and GAPDH levels in vehicle and rapamycin-treated fibroblasts, serving as representative examples. **G:** Trehalose treatment led to increased caspase-8 activation in both SCA2 patient fibroblasts and controls. **H:** No significant change in caspase-9 activation was detected in SCA2 patient fibroblasts and controls after trehalose treatment. **I:** Trehalose treatment subtly increased ATXN2 protein levels in control fibroblasts, although not significantly. **J & K:** Trehalose treatment significantly elevated levels of p62/SQSTM1 and LC3-II proteins in both SCA2 and control cells, with SCA2 cells showing a greater increase in p62/SQSTM1 compared to controls. **L:** Western blot images illustrate ATXN2, p62/SQSTM1, LC3-II, and GAPDH levels in vehicle and trehalose-treated fibroblasts. Graphs represent mean and SEM from at least two independent experiments, utilizing three control and five SCA2 patient cell lines. Figure adapted from Wardman, J.H., Henriksen, E.E., Marthaler, A.G. et al. *Enhancement of Autophagy and Solubilization of Ataxin-2 Alleviate Apoptosis in Spinocerebellar Ataxia Type 2 Patient Cells*. *Cerebellum* 19, 165–181 (2020). <https://doi.org/10.1007/s12311-019-01092-8>.

Rapamycin exhibited positive effects on caspase activity under basal conditions in SCA2 patient cells, as demonstrated by the observed reductions in both caspase-8 and caspase-9 activities (**Complementary Figure 8A-B**). This outcome suggests that mTOR signaling may play a critical role in the pathophysiology of SCA2. Furthermore, our findings reveal a significant elevation in LC3-II protein levels (**Complementary Figure 8E/Figure 3G**, Wardman *et al.*, 2020) alongside only a modest increase in p62/SQSTM1 protein levels (**Complementary Figure 8D/Figure 3F**, Wardman *et al.*, 2020). This pattern indicates activation of autophagy, further substantiating the potential therapeutic efficacy of mTOR inhibition in mitigating the molecular abnormalities associated with SCA2.

Effects of Solubilization of ATXN2 in SCA2

Congo Red, recognized for its capacity to solubilize proteins containing expanded polyglutamine (polyQ) tracts, markedly reduced caspase-mediated apoptosis in SCA2 cells (**Figure 5A-B**, Wardman *et al.*, 2020). Treatment with

Congo Red mitigated the effects of chloroquine (**Figure 5C**, Wardman *et al.*, 2020), suggesting that Congo Red may play a protective role against apoptosis in SCA2 cells, potentially by solubilizing cytotoxic protein aggregates, including ATXN2 oligomers.

To further investigate this mechanism, we examined the differential solubility of ATXN2 under various extraction conditions. Utilizing RIPA buffer, known for its relatively mild solubilizing capability, and SDS buffer, which provides more stringent conditions, we observed a pronounced increase in ATXN2 levels in the insoluble fraction of SCA2 cells (**Figure 6B-C**, Wardman *et al.*, 2020). This finding indicates that ATXN2 with expanded polyQ tracts is more prone to oligomerize, thus reducing its solubility.

Intriguingly, Congo Red treatment normalized ATXN2 levels in the insoluble fractions between control and SCA2 cells and decreased the relative amount of ATXN2 in the soluble fraction (**Figure 6E-F**, Wardman *et al.*, 2020). This observation suggests that Congo Red may facilitate the solubilization and potential degradation of ATXN2 in SCA2 cells. These findings highlight that Congo Red dissolves ATXN2 aggregates, thereby mitigating the cellular stress associated with protein accumulation.

DISCUSSION

Autophagy is the primary pathway for clearing aggregated proteins in neurodegenerative disorders. Our study reveals elevated apoptotic activation and mitochondrial dysfunction in SCA2 cells, along with reduced LC3-II protein levels upon autophagic inhibition, suggesting hindered or defective autophagic flux in these cells. Similar findings have been reported in other SCA2 models⁷², highlighting a critical role for autophagy in SCA2 pathology. Conversely, inhibition of the proteasome did not affect ATXN2 levels or caspase-mediated apoptosis, indicating its limited involvement in the SCA2 disease mechanism.

Further investigations into enhancing autophagy via treatments with rapamycin and trehalose were conducted. Trehalose ameliorated apoptotic activation under oxidative stress in both SCA2 and control cells, suggesting its therapeutic potential. Despite the evidence supporting trehalose's role in autophagy activation, some studies have raised questions about its effectiveness in inducing autophagic flux, which is the complete process of autophagy from autophagosome formation to degradation in autolysosomes. A study using SH-SY5Y neuronal cells found that trehalose treatment increased the number of autophagosomes but not autolysosomes, suggesting that trehalose might inhibit autophagic flux rather than enhance it¹⁷⁴. This was further supported by the observation that trehalose treatment led to inefficient delivery of LC3 from autophagosomes to autolysosomes, and the levels of p62 did not decrease as would be expected if autophagic flux were completed¹⁷⁴.

Our study can support the notion that trehalose does not activate autophagy but might inhibit autophagic flux, as we see an increase in the protein levels of p62 in SCA2 cells upon treatment with trehalose (**Complementary Figure 8J/Figure 4E**, Wardman *et al.*, 2020). However, we still see a positive effect of trehalose on SCA2 cells during induced oxidative stress, which may stem from its chemical chaperone properties that may help stabilize misfolded proteins and prevent their aggregation^{175–178}.

In contrast, rapamycin diminished apoptosis in SCA2 fibroblasts but did not provide the same level of protection against oxidative stress as trehalose. Nonetheless, the observed reduction in caspase activity and signs of enhanced autophagic flux following rapamycin treatment position mTOR as a viable therapeutic target for SCA2. Supportive evidence suggests that rapamycin-induced inhibition of mTOR activity reduces caspase activity, reinforcing the potential utility of rapamycin-induced autophagy activation⁸⁸.

Our findings also indicate an increased presence of ATXN2 in the insoluble fraction of SCA2 cells, suggesting oligomerization or formation of insoluble protein aggregates that may contribute to the disease pathology. Treatment with Congo Red resulted in a decrease in insoluble ATXN2. Hence, preventing ATXN2 aggregation could be a therapeutic strategy.

The complexity of SCA2 pathology underlines the need for multifaceted therapeutic strategies. The differential impacts of compounds such as Congo Red, rapamycin, and trehalose on SCA2 pathology highlight the potential of targeting specific cellular pathways, such as autophagy, to mitigate disease progression.

STUDY III

TITLE

Development of a New iPSC-Derived Ventral Hindbrain Differentiation Protocol

BRIEF INTRODUCTION

The embryogenesis of cerebellar Purkinje cells represents a highly intricate and precisely coordinated sequence of events, encompassing their origination, migration, differentiation, and the formation of essential synaptic connections for cerebellar functionality. Purkinje cells are the primary efferent neurons in the cerebellum, and they emerge from the cerebellar primordium, encompassing two distinct progenitor zones: the dorsal rhombic lip and the ventral ventricular zone (VZ) (**Figure 1A**). These zones give rise to neuronal precursors destined for glutamatergic and GABAergic identities. The initial phase of cerebellar morphogenesis is critically dependent on the fibroblast growth factor 8b (FGF8b) secretion by the isthmus organizer located at the midbrain-hindbrain boundary (**Figure 1A**). This secretion triggers the activation of several region-specific transcription factors, notably EN2 (Engrailed Homeobox 2) and Gastrulation Brain Homeobox 2 (GBX2), alongside the induction of Wingless-Type MMTV Integration Site Family, Member 1 (WNT1) expression at the midbrain-hindbrain junction. A synergistic feedback loop between FGF8b and WNT1, involving EN2, plays a pivotal role in this process¹⁷⁹.

The cerebellar neuroepithelium undergoes regionalization under the influence of Atonal BHLH Transcription Factor 1 (ATOH1) and Pancreas Transcription Factor 1 Subunit Alpha (PTF1A), delineating two distinct domains: the VZ, characterized by PTF1A and Oligodendrocyte Transcription Factor 2 (OLIG2) expression, and the upper rhombic lip, marked by ATOH1 and BARHL1 expression. These domains generate GABAergic and glutamatergic neurons, respectively^{179–183}. Within the VZ, the expression of proneural genes Neurogenin 1 (NGN1) and Neurogenin 2 (NGN2) overlaps with that of PTF1A, suggesting a role in the final division cycle of progenitors before their transition to post-mitotic Purkinje cell precursors. This is further supported by the adjacent and partially overlapping expression of NGN1 and NGN2 with post-mitotic markers LIM Homeobox 1 (LHX1) and LIM Homeobox 5 (LHX5), which are implicated in Purkinje cell differentiation¹⁸⁴. Purkinje cells complete their terminal mitosis in the VZ, then migrate dorsally out of the VZ, often along radial glial processes, to form the developing cerebellar cortex (**Figure 1A**). The earliest-born Purkinje cells occupy the most dorsal positions within this structure^{179,185}. Notably, calbindin 1 (CALB1) expression, a principal calcium-binding protein that mitigates neuronal neurotoxicity, delineates three Purkinje cell clusters on either side of the midline around postnatal day 0. Calbindin is uniformly expressed across all Purkinje cells in the mature cerebellum, highlighting its significance in the adult cerebellar architecture^{186,187}.

MOTIVATION

The advent of three-dimensional (3D) cell culture models has revolutionized our ability to accurately recapitulate the complex multicellular interactions and 3D microenvironments inherent to human organs, transcending the limitations imposed by traditional two-dimensional (2D) systems. Recent research efforts have focused on the development of brain region-specific organoids, such as midbrain-specific organoids or hindbrain-specific organoids, which are specifically designed to facilitate the differentiation and maturation of cerebellar Purkinje cells. One of the primary objectives driving the development of these hindbrain organoids is the establishment of robust and physiologically relevant models for the study of cerebellar disorders.

In a study published in 2015, Muguruma *et al.* successfully generated cerebellar neurons using a 3D culture methodology employing human embryonic stem cells⁹⁷. Their approach has since served as the foundation for numerous differentiation protocols aimed at further optimizing the maturation of cerebellar. Building upon this groundwork, Silva *et al.* developed a novel protocol, published in 2020, which introduced a new strategy for the differentiation and maturation of cerebellar precursors without the need for co-culturing with feeder cells¹⁰⁰. This innovative approach established a long-term culture system that sustains the maturation of human cerebellar neurons in the absence of co-culturing, representing a significant advancement in the field of cerebellar research.

As an integral part of this PhD project, we sought to advance our investigations into SCA2 by transitioning from patient-derived fibroblast-based studies to a more physiologically relevant cell model, specifically cerebellar Purkinje cells, as this cell type is most prominently affected in the brains of SCA2 patients due to the pathogenically expanded polyglutamine region in ATXN2. Having generated induced pluripotent stem cells (iPSCs) from SCA2 patient-derived fibroblasts, we initiated efforts to explore the differentiation protocols published by Muguruma *et al.*⁹⁷ and Silva *et al.*¹⁰⁰. However, despite our efforts, we were unable to replicate their results, as we continuously experienced pronounced cell death or very limited growth of the organoids. Through a systematic troubleshooting process, we identified the primary obstacle as adherence to the basal differentiation medium employed, which was defined by the Muguruma group and termed growth-factor-free chemically defined medium (gfCDM). Our challenges in this regard prompted us to explore novel strategies for differentiating hindbrain/cerebellar organoids and to potentially enhance the differentiation process by optimizing the initial differentiation phases, but also if we could enhance the differentiation of Purkinje cells specifically.

OBJECTIVE

The primary objective of this study was to develop a novel differentiation protocol that synthesizes the most effective strategies from the seminal works of Rifes *et al.* (2020)¹⁰², Silva *et al.* (2020)¹⁰⁰, and Chen *et al.* (2020)¹⁰³, with a particular focus on optimizing the initial neuronal induction for ventral hindbrain patterning.

Hypothesis: The integration of key elements from the aforementioned protocols will enhance hindbrain patterning,

ultimately leading to the establishment of a comprehensive protocol that addresses ventral hindbrain patterning and the subsequent maturation of these progenitors into functional cerebellar neurons.

MATERIALS AND METHODS

Induced Pluripotent Stem Cells

Reprogramming human fibroblasts to induced pluripotent stem cells (iPSCs) using episomal vectors was adapted from Okita *et al.*, 2011¹⁸⁸. On the day of transfection, fibroblasts were transfected with Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific) and episomal plasmids: pCXLE-hOCT3/4-shp53-F (OCT3/4 and shRNA against p53, Addgene #27077), pCXLE-hSK (SOX2 and KLF4, Addgene #27078), and pCXLE-hMLN (L-MYC and LIN28, Addgene #27079). The cells were incubated overnight.

The culture medium, DMEM 1885 + NaCO₃ (Panum), 10% FBS, and 1% GlutaMax (Thermo Fisher Scientific), was supplemented with FGF2/bFGF. On days 3-4, the medium was switched to mTeSR1+ (StemCell Technologies) with Sodium Butyrate and SB431542 (StemCell Technologies).

Cells were monitored daily, and emerging iPSC colonies were manually transferred to new 24-well plates coated with laminin (Biolamina). Cells were passaged upon reaching confluency, using Revita (Thermo Fisher Scientific) during early sensitive stages. Freezing of established iPSCs commenced once homogenous colonies were obtained, with periodic batch freezing.

Characterization began around the fifth passage and included karyotyping, germ layer differentiation, embryoid body formation, and assessment of pluripotency gene expression (mRNA and protein levels).

Doxycycline Inducible *NGN2*-expression Model System

The Tet-On 3G Inducible Expression System, as described by Fernandopulle *et al.*¹⁸⁹, was utilized to create a doxycycline-inducible *NGN2* expression model. This system includes a regulatory vector expressing the mutant 2 reverse tetracycline-controlled transactivator (M2rtTA) and a response vector containing the *NGN2* gene under a tetracycline-responsive promoter (AAVS1-Neo-M2rtTA, Addgene #60843 and pUCM-AAVS1-TO-hNGN2, Addgene #105840). Both vectors contain antibiotic selection markers (Neomycin for AAVS1-Neo-M2rtTA and Puromycin for pUCM-AAVS1-TO-hNGN2), with the latter also expressing the fluorescent marker mCherry.

The plasmids were integrated into the AAVS1 "safe harbor" locus using the TALEN technique (AAVS1-Talen-L, Addgene #59025 and AAVS1-Talen-R, Addgene #59026). Transfection of the four plasmids was performed via nucleofection. iPSCs were cultured in a 6-well plate until 70-90% confluent. Two hours before nucleofection, wells were coated with laminin (Biolaminin 521 LN, Biolamina). Cells were dissociated with accutase (StemCell Technologies) for seven minutes, collected, and counted.

Approximately one million cells were centrifuged, resuspended in 82 μ l of solution and 18 μ l of supplement from the P3 Primary Cell 4D-Nucleofector™ X Kit (Lonza) and combined with 2 μ g of each plasmid. The mixture was transferred to a cuvette and nucleofected using program CA 167. Post-nucleofection, 500 μ l of mTeSR1+ medium

was added, and the cells were transferred to a laminin-coated well with 1:200 RevitaCell (Thermo Fisher Scientific) before incubation.

Cells recovered for 4-5 days post-nucleofection. Selection was performed with G418 (50 ng/ml) and Puromycin (100 ng/ml) for 5-7 days, with antibiotic concentrations and exposure times varying between cell lines. After selection, cell stocks were prepared and cryopreserved.

For further selection, mCherry-positive cells were sorted via FACS. The selected population was expanded and cryopreserved. The pUCM-AAVS1-TO-hNGN2 construct contains loxP sites for mCherry removal via the Cre-loxP system. iPSCs were transfected with a Cre recombinase plasmid (pCAG-Cre-IRES2-GFP, Addgene #26646) using Lipofectamine Stem Transfection Reagent and P3000™ enhancer reagent (Thermo Fisher Scientific), following the manufacturer's protocol.

After overnight incubation, GFP-positive and mCherry-negative cells were selected via FACS. Following these procedures, cell stocks were prepared and cryopreserved.

Ventral Hindbrain Differentiation of iPSCs

iPSCs were cultured in mTeSR+ medium (StemCell Technologies) in 6-well plates prior to differentiation. iPSCs were visually assessed for morphology, cell death, and spontaneous differentiation. An AggreWell 800 plate (24-well, StemCell Technologies) was coated with Anti-Adherence (StemCell Technologies) for at least 25 minutes at room temperature, followed by three PBS washes. When cultures reached 80-90% confluency, cells were detached with 1x accutase for five minutes at 37°C, collected, and centrifuged. The supernatant was removed, and cells were resuspended in a neuronal induction medium comprising 50/50 Advanced DMEM/F12 and Neurobasal (Thermo Fisher Scientific), 1% GlutaMax, 1% Insulin/Transferrin/Selenium-A, 1% N2 supplement, 2% B27 supplement minus vitamin A, 1% PenStrep, LDN193189 (100 nM), SB431542 (10 µM), CHIR99021 (2 µM), and Y-27632 (10 µM).

Approximately one million iPSCs in neuronal induction medium were transferred to each well of the prepared AggreWell 800 plate, centrifuged for one minute, and incubated at 37°C in a CO2 incubator. Embryoid bodies (EBs) were monitored, with 50% medium changes (excluding Y-27632) daily. On day two, FGF2/bFGF (50 ng/ml, PeproTech) was added to the medium.

On day four, EBs were transferred to a 6-well plate coated with Anti-Adherence and placed on an orbital shaker in a CO2 incubator at 37°C. Four ml of neuronal induction medium supplemented with LDN193189 (100 nM), SB431542 (10 µM), CHIR99021 (2 µM), and Purmorphamine (0.5 µM) were added to each well, with ¾ medium changes every other day. On day eight, FGF8b (100 ng/ml, PeproTech) was added to the medium. On day 14, the medium was completely replaced with fresh neural induction medium supplemented with FGF8b (100 ng/ml) and FGF19 (50 ng/ml, PeproTech), with ¾ medium changes every other day.

On day 22, neural spheres were transferred to either a T25 or T75 flask coated with Anti-Adherence, with a complete medium change to neuronal maturation medium consisting of BrainPhys (StemCell Technologies), 1% PenStrep, 2% B27, and supplemented with BDNF (20 ng/ml), GDNF (10 ng/ml), and Dibutyl-rl-cAMP (0.5 mM), with ¼

medium changes twice a week. From day 28 to day 35, SDF-1 α (50 ng/ml, PeproTech) was added to the neuronal maturation medium.

Doxycycline Treatment

To investigate if inducing *NGN2*-expression could enhance the ventral hindbrain/cerebellar differentiation of iPSCs, a doxycycline-inducible *NGN2*-expression iPSC model was used. The doxycycline was added to the neuronal induction medium on day 4 at a final concentration of 2 μ g/ml and throughout the following 10 days.

RNA Extraction and RNA Work

Cells, EBs, spheroids, or organoids were collected in an Eppendorf tube, and the medium was removed. TriZol reagent (Thermo Fisher Scientific) was added to the sample, and the protocol provided by the manufacturer was used. A minor modification was added to increase the yield of RNA; an increase in centrifugation from 7500 x g to 12000 x g in the final washing step.

RNA experiments were conducted after the workspace, pipettes, and essential equipment had been thoroughly cleaned using RNaseZap. All utilized reagents were guaranteed to be free from RNase and DNase contamination.

cDNA Synthesis and qPCR

Prior to cDNA synthesis, genomic DNA was removed. One microgram of RNA was combined with 1 μ l of 10X DNase I Reaction Buffer, 1 μ l of DNase I (Amp Grade, 1 U/ μ l), and RNase-free water to a final volume of 10 μ l in an RNase-free, 0.5 ml Eppendorf tube on ice. The mixture was incubated at room temperature for 15 minutes, followed by DNase I inactivation with 1 μ l of 25 mM EDTA solution, and then heated at 65°C for 10 minutes.

cDNA synthesis was performed using SuperScript II RT (Thermo Fisher Scientific) according to the manufacturer's protocol. Oligo (dT) primers and 200 ng of RNA were utilized. The synthesized cDNA was incubated at 37°C for 20 minutes with 1 μ l of RNase H to remove RNA complementary to the cDNA, and then diluted in 160 μ l of nuclease-free water.

Quantitative PCR (qPCR) was conducted by mixing approximately 100 ng of cDNA, 5 μ M of both forward and reverse primers, 5 μ l of SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix (Bio-Rad), and nuclease-free water to a final volume of 10 μ l. The solutions were maintained on ice throughout the preparation.

The qPCR was performed on a Bio-Rad CFX96 Real-Time PCR system with a Bio-Rad C1000 Thermal Cycler. The thermal cycling protocol included an initial polymerase activation and DNA denaturation step (30 seconds at 95°C), followed by 40 cycles of 10-second denaturation at 98°C and 60-second annealing/extension at 60°C. RPL13A served as the housekeeping gene.

2^(-ΔΔCt) Method

Each gene of interest and housekeeping gene was amplified in triplicates, and the average Cq value was calculated for these technical replicates. The ΔCt value for each sample was determined by subtracting the Cq value of the

housekeeping gene (RPL13A) from the C_q value of the gene of interest (Appendix C). The $\Delta\Delta C_t$ value was then calculated between the ΔC_t of the sample of interest and the ΔC_t of the iPSC sample. The expression level of the gene of interest relative to the reference sample was calculated using the formula $2^{(-\Delta\Delta C_t)}$. The housekeeping gene (RPL13A) exhibited stable expression across all samples.

2D Replating of Organoids

Organoids (day 35) were collected using a serological pipette and transferred to a 1.5 ml Eppendorf tube. After removing the medium and gently washing with prewarmed PBS, the organoids were resuspended in 1 ml of prewarmed accutase (StemCell Technologies). The tube was incubated at 37°C for 10 minutes, with resuspension using a pipette at 3 and 6 minutes to dissociate and dissolve the organoid. The tube was then centrifuged for 2 minutes at 300 x g, and the accutase was removed. The dissociated organoids were resuspended in neuronal maturation medium supplemented with BDNF (20 ng, StemCell Technologies), GDNF (10 ng/ml, StemCell Technologies), Dibutyryl-cAMP (0.5 mM, StemCell Technologies), Ascorbic acid (200 nM, Sigma-Aldrich), and Y-27632 (10 μ M, StemCell Technologies). The cell suspension was transferred to a Nunc™ Lab-Tek™ II CC2™ Chamber Slide System (8-well format, Thermo Fisher Scientific) coated with poly-L-ornithine (Sigma-Aldrich) and biolaminin 521 LN (Biolamina). A complete medium change was performed after one hour. The following day, 2/3 of the medium was changed without adding Y-27632. At day 52, the replated organoids were fixed in 4% PFA and further processed for immunofluorescence characterization.

Immunofluorescence of Organoids

Organoids were permeabilized by incubation with 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 15-30 minutes, depending on the size of the organoids. Subsequently, a blocking step was performed using 5% bovine serum albumin (BSA) in PBS to prevent non-specific binding. Primary antibodies, diluted in the blocking buffer, were added to the organoid sections and incubated overnight at 4°C. The following day, the organoids were washed three times with PBS and then incubated with secondary antibodies, diluted between 1:500 and 1:1000 in the blocking buffer. For nuclear staining, Hoechst dye was applied at a 1:30,000 dilution in PBS for 15 minutes, followed by three washes with PBS. Slides were mounted using ProLong Antifade Gold or a similar mounting medium and allowed to solidify at room temperature for 20-30 minutes. Imaging was conducted using an EVOS 7000 fluorescent microscope, and image processing was performed with ImageJ software.

RESULTS

Protocol Development and Theorization Framework

The initial phase of the new differentiation protocol is based on the work of the Kirkeby group (Rifes *et al.*, 2020)¹⁰², which identified the canonical WNT signaling pathway as crucial for brain regional specificity along the rostral-caudal axis. They simulated the WNT signaling gradient present during early neuronal embryonic

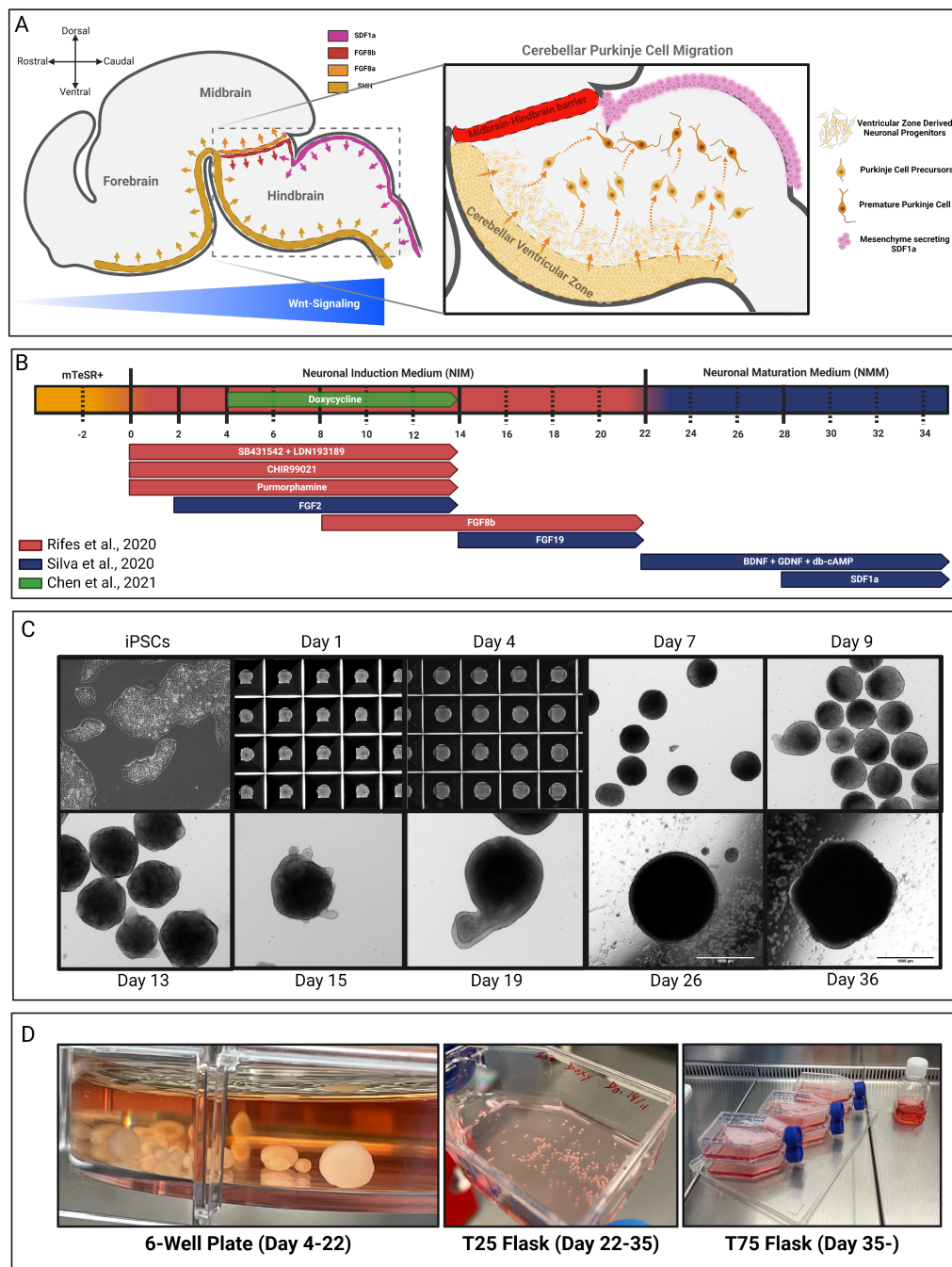


FIGURE 1 - Human Ventral Hindbrain Development and Differentiation Protocol

A: Schematic overview of the developing human hindbrain and cerebellar plate (sagittal view). **B:** Protocol schematic for generating ventral hind-brain/cerebellar organoids. Each color represents which previous differentiation protocol it originates from. **C:** Representative bright field images of cell aggregates taken at the indicated time points. **D:** Pictures showing brain organoids in the different cell culture formats used.

development by exposing iPSCs to a gradient concentration of the glycogen synthase kinase 3 inhibitor (GSK3i), CHIR99021, combined with dual SMAD inhibitors (SB431542 and LDN193189) to direct iPSCs into the ecto-derm layer. Additionally, they ventralized neuronal progenitor cells using Purmorphamine, an Shh agonist.

Our protocol employs the same initial patterning strategy, with the four patterning factors added from day 0 to day 14 (**Figure 1B**). On day 0, iPSCs were detached, and approximately one million cells were transferred to each well of

an AggreWell plate (24-well format with ~300 microwells per well, each 800 μm in size). For the first 24 hours, iPSCs were treated with ROCK inhibitor to maximize survival. EBs were maintained in the AggreWell for 4 days (**Figure 1C**) and cultured in neuronal induction medium (NIM) from day 0 to day 22. The medium consisted of a 1:1 mix of advanced DMEM/F12 and NeuroBasal, supplemented with N2, B27 without vitamin A, insulin/transferrin/selenium (ITS), and GlutaMax. On day 2, FGF2 was added to enhance the survival and proliferation of differentiating iPSCs, following the Muguruma and Silva protocols¹⁰⁰. From day 4 to day 22, the spheroids were transferred to a 6-well plate format to accommodate their increasing metabolic demands (**Figure 1D**). The 6-well plates were placed on an orbital shaker (100 rpm) to prevent further clustering of the spheroids. On day 8, FGF8b was added and maintained in the medium until day 22 to pattern the cerebellar morphogenesis of the neuronal progenitors, and Fibroblast Growth Factor 19 (FGF19) was supplemented from day 14 to day 22 to enhance the generation of Purkinje cells (**Figure 1B**).

Starting from day 22, the spheroids were transferred to a T25 or T75 flask (**Figure 1D**), depending on the experimental setup and batch size, and the medium was completely replaced with neuronal maturation medium (NMM) (**Figure 1B**). The NMM was supplemented with neurotrophins and dibutyryl-cAMP to promote neuronal survival and plasticity (**Figure 1B**). The addition of SDF1a, an enhancer of neural progenitor cell migration and Purkinje cell maturation, on day 28 marks the final stage of the differentiation protocol (**Figure 1B**). For long-term maturation, the spheroids were transferred to a T75 flask (**Figure 1D**). Representative bright field images of spheroids were taken at the indicated time points to monitor the EBs/spheroids growth (**Figure 1C**).

Ventral Hindbrain Differentiation – mRNA Expression Profile and Immunofluorescence

At day zero of differentiation, the iPSCs expressed the pluripotency genes POU Class 5 Homeobox 1 (OCT4) and Nanog Homeobox (NANOG) (**Figure 2A**). From day 7 and onwards, OCT4 and NANOG mRNA levels were significantly reduced, indicating that cells were committing to the differentiation state.

To characterize the brain-region specificity of differentiating spheroids, we quantified mRNA expression of Orthodenticle Homeobox 2 (OTX2), a rostral brain patterning marker, Paired Box 2 (PAX2), midbrain-hindbrain patterning marker, Engrailed Homeobox 2 (EN2), midbrain-hindbrain patterning marker, and Gastrulation Brain Homeobox 2 (GBX2), hindbrain patterning marker. On day 7, we observed elevated levels of midbrain-hindbrain patterning factors, particularly PAX2, indicating effective mid-hindbrain patterning. PAX2 expression peaked on day 7 and subsequently declined, whereas EN2 expression increased continuously until day 35. GBX2 exhibited a similar pattern to EN2, while OTX2 expression decreased throughout differentiation, favoring hindbrain over forebrain patterning (**Figure 2B**).

Regarding dorsal and ventral hindbrain markers, we anticipated low dorsal markers ATOH1 and BARHL1 expression compared to ventral markers OLIG2, PTF1A, and NEPH3. Contrary to expectations, ATOH1 expression increased, while BARHL1 remained low except in cell line 3, which showed a significant increase at day 21 (**Figure 2C**). Ventral markers PTF1A and NEPH3 exhibited rising expression until day 35, especially PTF1A. However, OLIG2 displayed consistently lower expression throughout the differentiation protocol. Notably, after day 21, the

transition from NIM to NMM enhanced PTF1A expression (**Figure 2D**).

With the addition of FGF19 on day 14, we see increases in the Purkinje cell markers CALB1, Purkinje Cell Protein 2 (L7/PCP2), and Aldolase C (ALDOC), reinforcing the importance of FGF19 in the differentiation of this cell type (**Figure 2E**). The three markers follow, to some extent, the same expression pattern after the addition of FGF19, especially L7/PCP2 and ALDOC, although in two batches cell line 2 and 3 showed no or low increase in CALB1. The Purkinje maturity marker Glutamate Ionotropic Receptor Delta Type Subunit 2 (GRID2) displayed a similar expression profile as L7/PCP2 and ALDOC, showing presence of Purkinje cell progenitor maturation (**Figure 2E**).

Glial Fibrillary Acidic Protein (GFAP) and Microtubule Associated Protein 2 (MAP2) were included as general markers for astrocytes and neurons, respectively. GFAP expression exhibited mixed transcription activation as early as day 7, with varying expression patterns across cell lines, potentially indicating batch differences (**Figure 2F**). In contrast, MAP2 expression significantly increased, following an exponential growth curve. From day 14 to day 35, we observed a significant rise in MAP2 expression compared to iPSCs (**Figure 2F**), suggesting enhanced neuronal differentiation from this point, which correlates with the increased expression of Purkinje cell markers.

To evaluate the mRNA result visually, the spheroids/organoids were dissociated with Accutase and replated onto poly-L-ornithine and laminin double-coated chamber slides. The dissociated spheroids/organoids were cultured in 2D for at least 17 days before immunofluorescence analysis. We performed immunofluorescence using antibodies against CALB1 (Purkinje cell marker), PTF1A, NEPH3 (both ventral hindbrain markers), and TUBB3 (a general neuronal marker) to identify neurons originating from the cerebellar ventricular zone. Immunofluorescence revealed multiple CALB1-positive neurons (**Figure 2G**, white arrows), indicating cerebellar Purkinje cell differentiation. Some neurons did not express CALB1 but were identified as distinct TUBB3-positive neurons overlapping with PTF1A and NEPH3 (**Figure 2G**), suggesting their origin from the cerebellar ventricular zone.

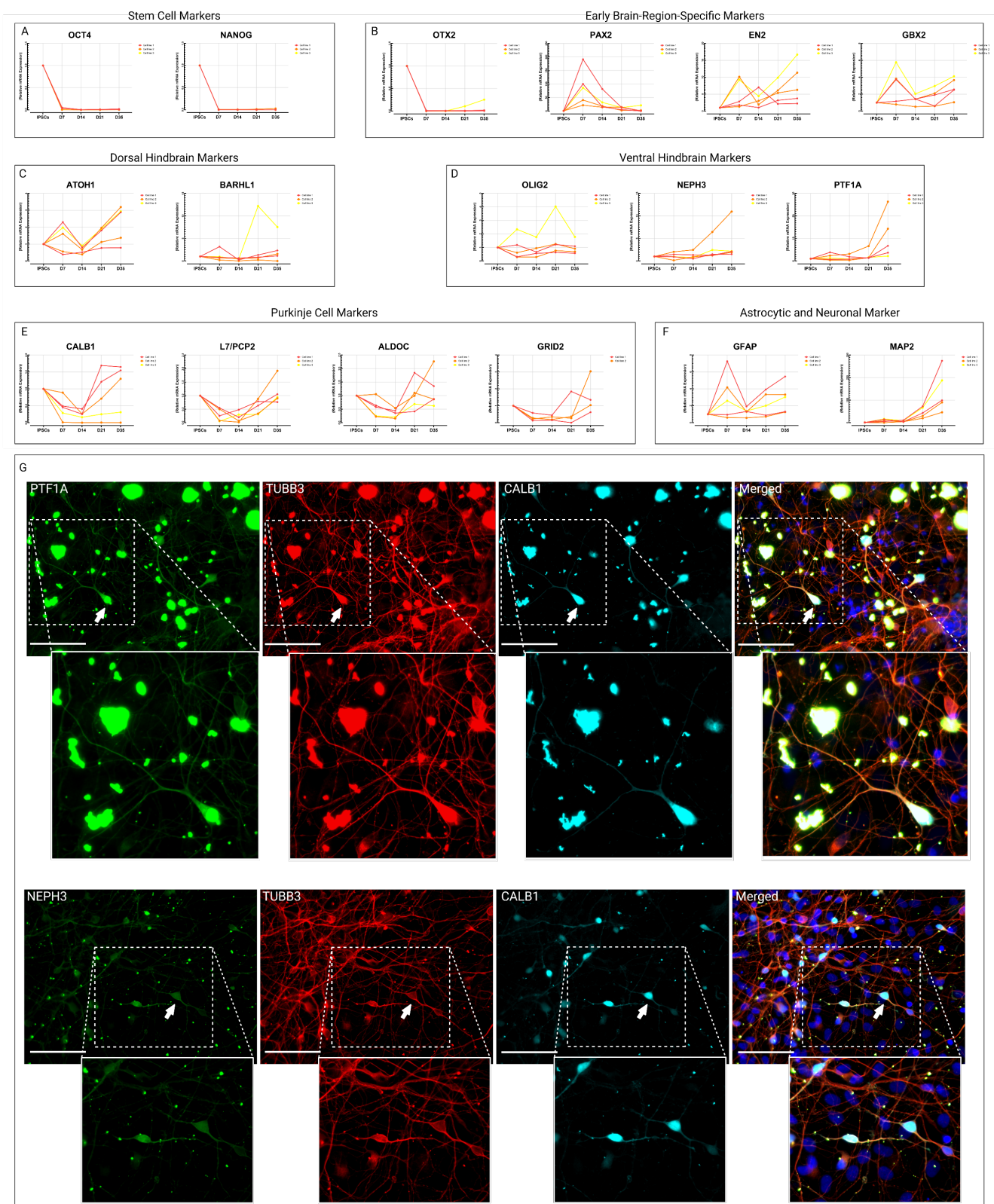


FIGURE 2 - Differentiation of Ventral Hindbrain Neurons.

A: qPCR analysis of differentiated 3D cultures derived from the H245 cell line for the indicated mRNA markers. The graphs depict mRNA expression levels ($2^{-\Delta\Delta C_t}$) relative to RPL13A (reference gene) and quantified mRNA levels for iPSCs (reference sample). Each color indicates a cell line. Red (H245), orange (H256), yellow (H250). H245 and H256 batches were duplicated (biological replicates). **B:** Immunofluorescence analysis on days 56 of differentiation of the H256 iPSC lines after being plated on day 35. The depicted neurons were stained with Hoechst and antibodies for either PTF1A, TUBB3, and CALB1 or NEPH3, TUBB3, and CALB1.

Enhanced Hindbrain Differentiation with Induced *NGN2* Expression - mRNA Expression Profile and Immunofluorescence

With the inclusion of an *NGN2*-inducible gene expression system, we aimed to streamline the generation of ventral hindbrain neurons from iPSCs and, at the same time, also ensure a high degree of purity and regional specificity, as seen in the protocol published by Chen *et al.*, in 2020¹⁰³. By introducing a more direct conversion system, we also aimed for a more rapid development of hindbrain neurons, bypassing the lengthy differentiation processes.

The differentiation protocol with induced *NGN2* expression is identical to our previous protocol, except for the addition of doxycycline to activate the Tet-On inducible expression system in transgenic iPSCs. According to Rifles *et al.*¹⁰², cells commit to their cell fate and regional specificity within 24-48 hours after exposure to dual SMAD inhibition and CHIR99021. Chen *et al.* demonstrate that pre-patterning before inducing *NGN2* expression is crucial for the regional specificity of differentiating neurons¹⁰³. Therefore, doxycycline was added to the NIM on day 4, allowing differentiating iPSCs to commit to a hindbrain identity and proliferate. The batches were run simultaneously, handled similarly, and given the same differentiation medium, with the only difference being the addition of doxycycline from day 4 to day 14. The starting material, iPSCs, originated from the same batch.

During differentiation, we observed distinct morphological and structural differences between batches that received doxycycline and those that did not. **Figure 3A** illustrates the development of spheroids over 19 days. In batches without doxycycline, the spheroids had axial elongations (marked by red dashed lines and arrows, **Figure 3A**). This axial elongation morphology began around day 9 and increased in prevalence until day 19. The axial elongation morphology was accompanied by developmental processes suggestive of self-organization in the forming spheroid. The cells positioned themselves in distinct populations, creating visually discernible morphological structures (**Figure 3A**; day 16 and day 19).

In batches exposed to doxycycline (doxy), we did not observe the same morphological development until days 16 to 19, and even then, it was not as pronounced as in the "no doxy" batches. The "no doxy" batches exhibited visual morphological compartmentalization, characterized by polarized darkening areas (**Figure 3A**; day 9 and day 13). In contrast, the "doxy" batches appeared uniform and rounded.

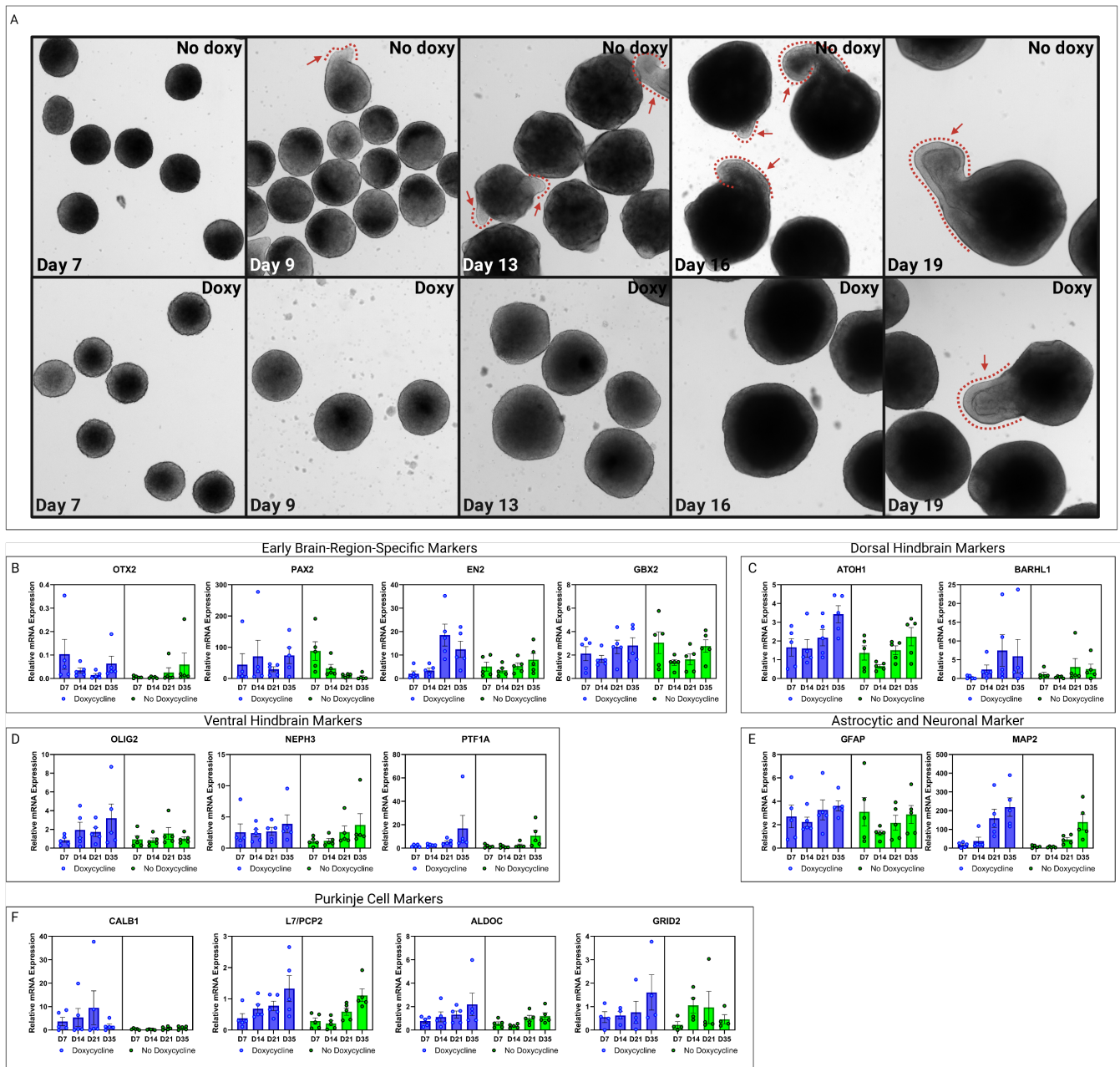


FIGURE 3 - Enhanced Hindbrain Differentiation with Induced NGN2 Expression

A: Brightfield images of EBs/spheroids at specific time points. “No doxy” or “doxy” indicates whether doxycycline was added to the differentiation protocol. Red arrows and dashed lines indicate morphological development. **B-F:** qPCR analysis of differentiated 3D cultures derived from the H245 cell line for the indicated mRNA markers. Doxycycline treatment (blue) and no doxycycline treatment (green). The graphs depict mRNA expression levels ($2^{-\Delta\Delta C_t}$) relative to RPL13A (reference gene) and quantified mRNA levels for iPSCs (reference sample).

Analysis of mRNA expression profiles also revealed differences imposed by doxycycline-induced *NGN2* expression. **Figure 3B-F** compares qPCR results between “no doxy” (green) and “doxy” (blue) batches. The midbrain-hind-brain markers, *EN2* and *PAX2*, displayed altered expression patterns upon *NGN2* induction (**Figure 3B**). *PAX2* exhibited continuous mRNA expression throughout the 35-day duration compared to “no doxy” batches. Interestingly, *EN2* expression significantly increased upon doxycycline withdrawal from day 14 (**Figure 3B**).

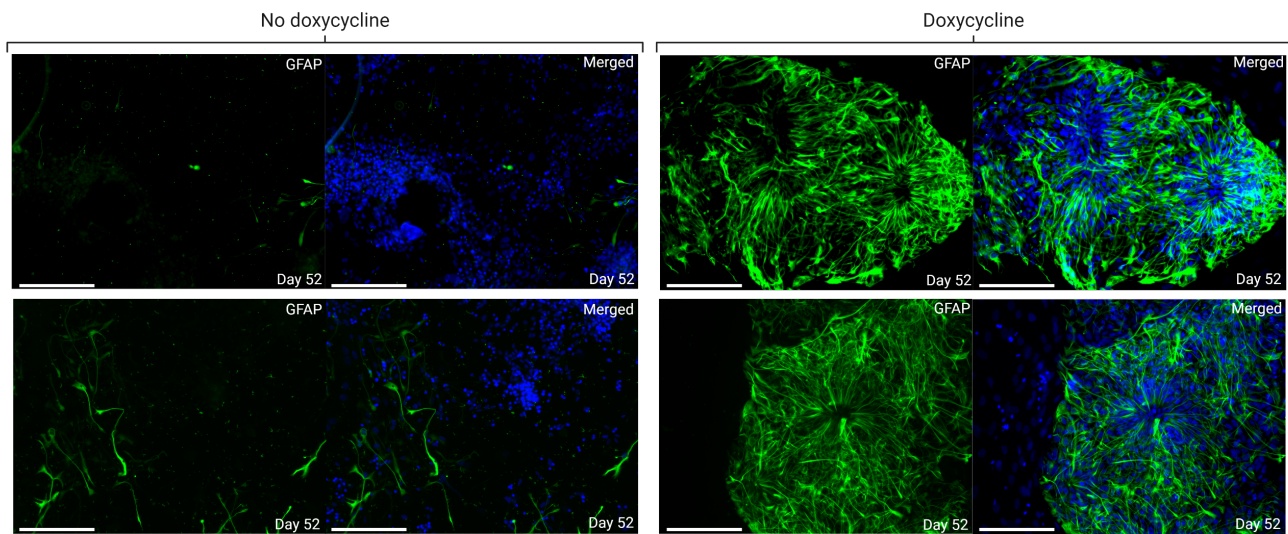


FIGURE 4 – GFAP⁺ Cells Indicate Increased Maturity in Doxycycline-Treated Spheroids.

Immunofluorescence analysis on days 52 of differentiation of the H245 iPSC lines. The depicted neurons were stained with Hoechst and GFAP, an astrocytic marker, after being dissolved and replated at day 35.

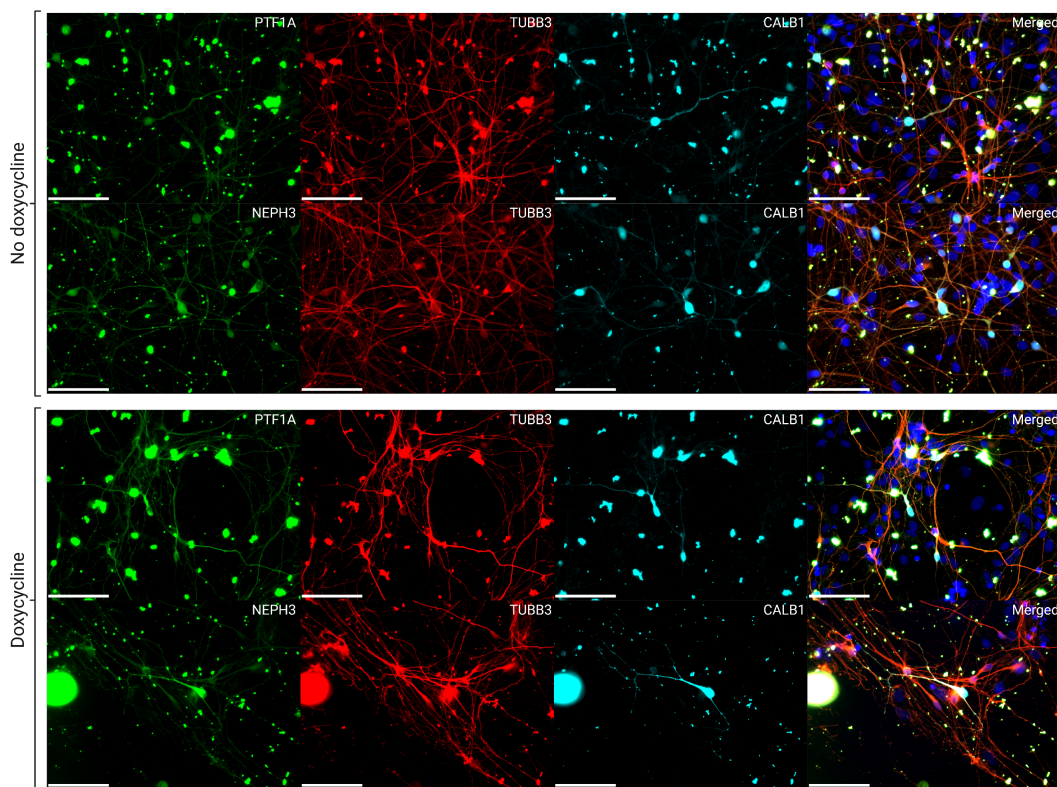


FIGURE 5 - Immunofluorescence Analysis Comparison of Ventral Hindbrain Markers with Doxycycline Treatment

Immunofluorescence analysis was performed on day 52 of the differentiation of the H256 iPSC line using the indicated antibodies. The depicted neurons were stained with antibodies for either PTF1A, TUBB3, and CALB1 or NEPH3, TUBB3, and CALB1. In merged, nuclei are stained with Hoechst.

The ventral hindbrain markers, PTF1A, NEPH3, and OLIG2, exhibited increased mRNA expression upon *NGN2* induction compared to "no doxy" batches, suggesting that forced *NGN2* expression enhances ventralization (**Figure 3D**). OLIG2 expression was particularly enhanced in "doxy" batches. However, a similar trend was observed for the dorsal hindbrain markers ATOH1 and BARHL1 (**Figure 3C**), indicating an overall enhancement of both dorsal and

ventral hindbrain identity. Interestingly, CALB1 mRNA expression was seemingly enhanced upon *NGN2* induction but decreased at day 35. In contrast, *L7/PCP2*, *ALDOC*, and *GRID2* showed early increased expression compared to "no doxy" batches (**Figure 3F**), suggesting that Purkinje cell differentiation and maturation phases are favored by doxycycline-induced *NGN2* expression.

The mRNA expression of GFAP, an astrocytic marker, showed a modest overall increase upon *NGN2* induction. In contrast, the neuronal marker MAP2 dramatically increased from day 7 onwards, with a more than 100-fold change in mRNA expression at day 35.

Immunofluorescence of replated 2D cultures revealed that "doxy" batches had more GFAP-positive cells than "no doxy" batches at day 52 (**Figure 4**), which does not reflect the qPCR results. Both "doxy" and "no doxy" cultures were positive for the markers *PTF1A*, *NEPH3*, *TUBB3*, and *CALB1* (**Figure 5**), but "doxy" *CALB1*⁺ neurons exhibited a more developed dendritic morphology, with dendritic spines and branching (**Figure 6**).

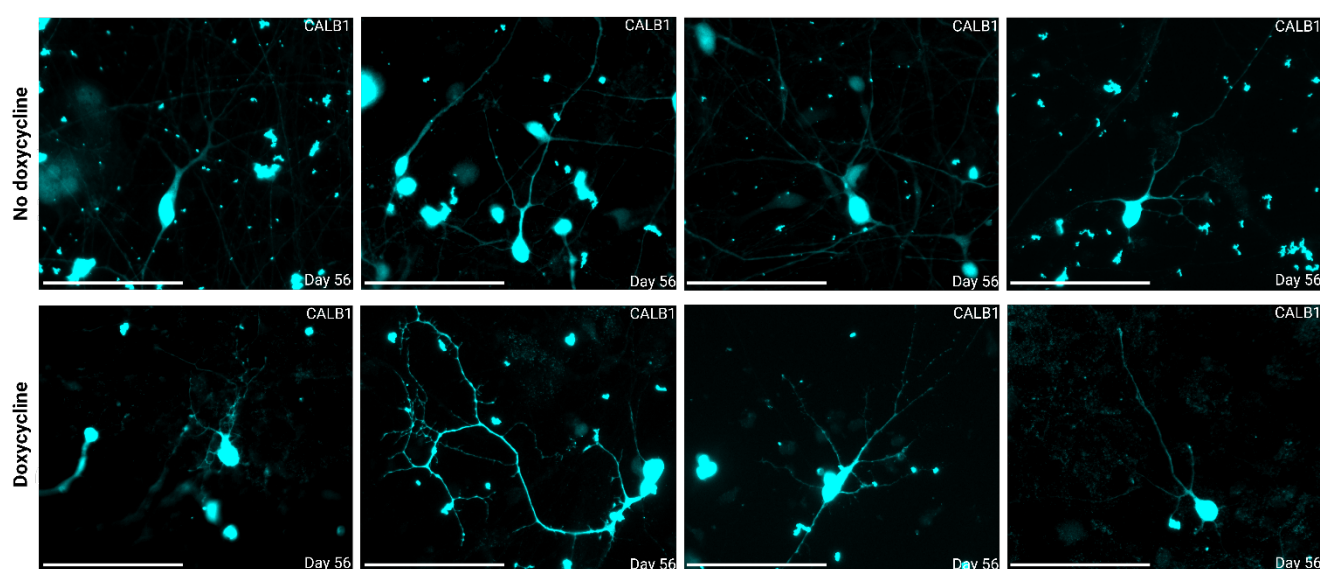


FIGURE 6 - Increased Maturity of Purkinje Cells with Forced *NGN2* Expression
Immunofluorescence analysis on days 52 of differentiation of H256 iPSC lines employing the anti-CALB1 antibody.

The process of dissolving and replating organoids had drawbacks, including pronounced cell death and detachment to the culture plates over time, limiting the maturation duration. Therefore, 3D culturing was preferred for long-term differentiation and maturation experiments. Hence, the organoids were also replated directly on laminin-coated

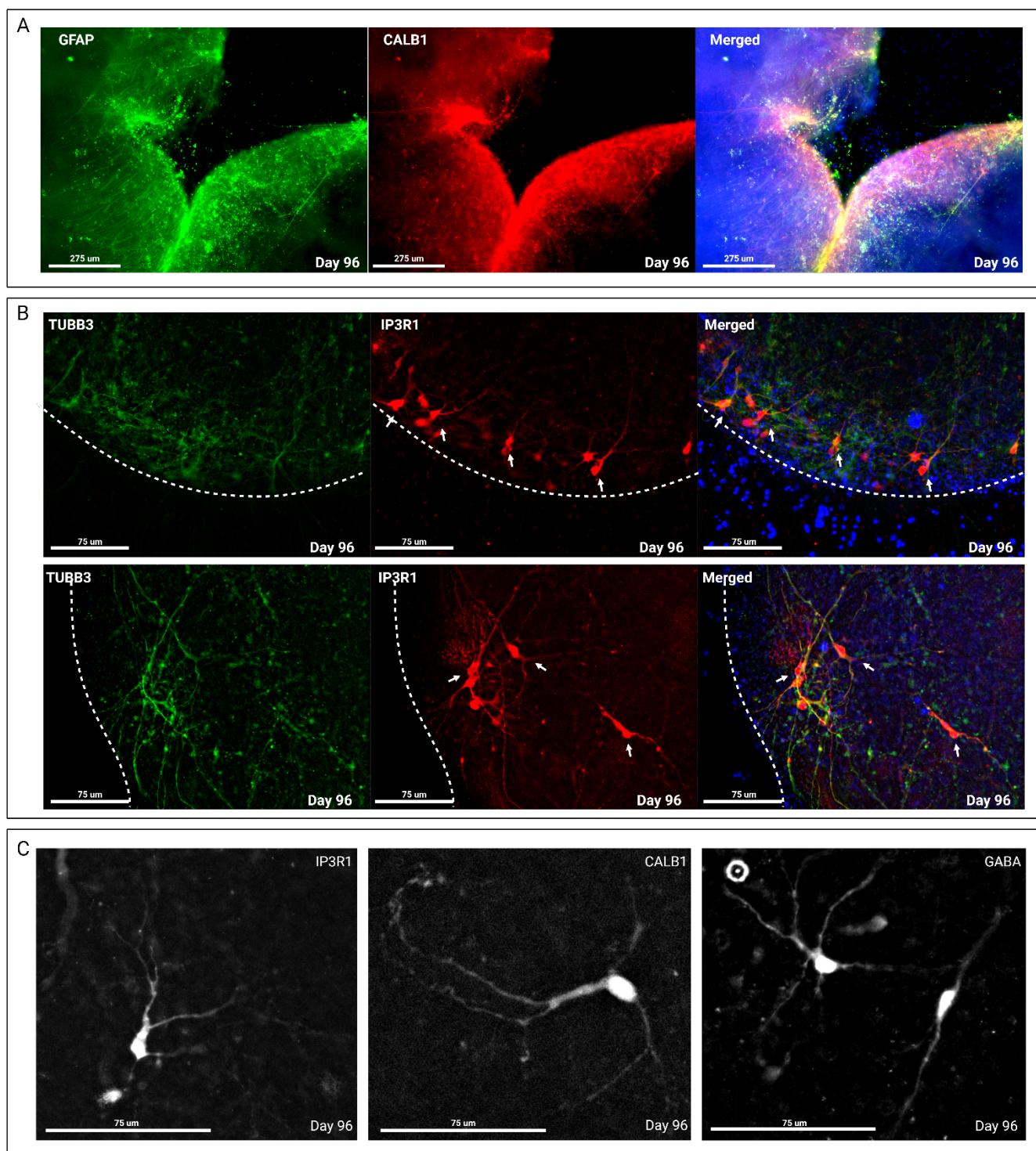


FIGURE 7 - Visualization of Cellular Structures in 96-Day-Old Organoids

A: Representative images of GFAP⁺ and CALB1⁺ cells within undissolved organoids. The images highlight the cellular structures, although background fluorescence presents a challenge in evaluating the detailed morphology of specific cellular subtypes. **B:** By subtracting background fluorescence and focusing on the edges of the organoids (indicated by the dashed line), we were able to distinguish the morphology of IP3R1⁺ cells. IP3R1 is highly expressed in Purkinje cells (white arrows). **C:** Detailed visualization of CALB⁺ and GABA⁺ cells in 96-day-old organoids, demonstrating the presence and distribution of these cell types.

plates with the aim of getting sprouting of cells from the rim of the. We visualized structures of GFAP⁺ cells and CALB1⁺ cells (**Figure 7A**). However, the intense fluorescence from the organoid limited the evaluation of cellular subtype morphology. By subtracting background fluorescence and focusing on the edges of the organoid (marked by the dashed line), we distinguished the morphology of Inositol 1,4,5-Trisphosphate Receptor Type 1 (IP3R1) positive cells (highly expressed in Purkinje cells) (**Figure 7B**, white arrows) and CALB⁺ and GABA⁺ cells in 96-day-old organoids (**Figure 7C**). Furthermore, we observed CALB1⁺ axonal and dendritic projections from 9-month-old organoids (**Figure 8**, white arrows).

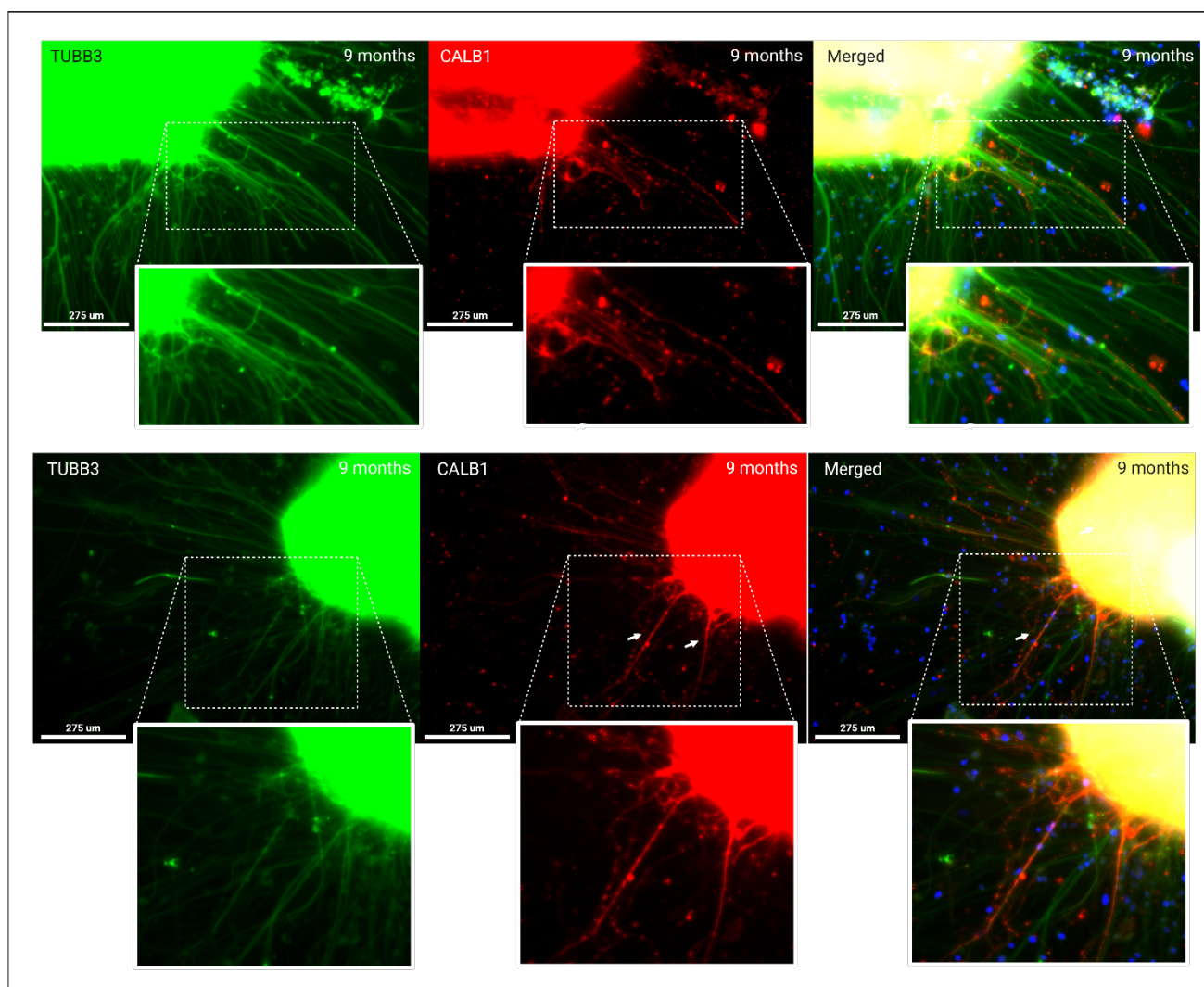


FIGURE 8 - Axonal and Dendritic Projections from 9-Month-Old Cultured Organoids
Visualization of CALB1⁺ axonal and dendritic projections in 9-month-old organoids (white arrows), indicating the advanced maturation and complex neuronal network formation achieved through long-term 3D culturing.

DISCUSSION

The development of a novel iPSC-derived ventral hindbrain differentiation protocol represents a significant advancement in neuroscience, particularly for studying cerebellar disorders such as SCA2. This study aimed to establish a new differentiation protocol that combines effective strategies from previous studies with specific adjustments to the initial neuronal induction of ventral hindbrain development. We hypothesized that integrating these protocols would enhance ventral hindbrain patterning, thereby facilitating more efficient generation of specific cerebellar neurons.

The differentiation protocol presented in this study merges key elements from three seminal works, employing a combination of WNT signaling pathway activation via a glycogen synthase kinase 3 inhibitor (GSK3i), dual SMAD-inhibition for ectoderm layer induction, and purmorphamine for ventralization of the cerebellar plate neuroepithelium. This approach successfully patterns iPSCs into neuronal progenitor cells with a hindbrain identity, while the addition of FGF19 further enhances the generation of Purkinje cells, a critical cell type in cerebellar function and development.

The mRNA expression profile and immunofluorescence analysis in this study provided valuable insights into the differentiation process, validating the efficacy of the novel protocol. A significant reduction in the mRNA from pluripotency genes, such as OCT4 and NANOG, from day 7 indicated successful differentiation of iPSCs towards a more specialized cell fate.

OTX2, a homeobox gene, plays a critical role in the early specification and patterning of the rostral brain, including the forebrain and midbrain^{190,191}. The absence of OTX2 expression, together with the expression patterns of midbrain-hindbrain markers, including PAX2^{192,193} and EN2¹⁹⁴, as well as the early hindbrain marker GBX2^{191,195}, suggest effective patterning of the hindbrain region.

The presence of the dorsal hindbrain markers ATOH1 and BARHL1, especially in the doxycycline-treated organoids, indicates that this protocol does not provide a clean ventralization of the spheroids/organoids. This could be attributed to the physical properties of the spheric spheroids/organoids, with the inner core being less exposed to the ventralizing factor, purmorphamine, a Shh receptor activator. The spherical morphology maintains physical properties, ensuring a gradient-dependent distribution of oxygen, nutrients, and likely also influencing the concentration of the patterning factors within the spheroid/organoid. Increasing the concentration of purmorphamine could potentially bypass this limitation. However, further studies are needed to clarify this hypothesis.

The addition of FGF8b at day 8 was intended to reinforce the patterning of ventralized caudal progenitors, as Kirkeby *et al.* demonstrated its importance in decreasing dorsal markers such as BARHL1¹⁹⁶. Our data (day 7 to day 14) suggest that midbrain-hindbrain markers (PAX2, EN2, and GBX2) are regulated by FGF8b, as we observed a drop in expression, particularly for PAX2. A similar decrease in expression was seen for the dorsal hindbrain

marker ATOH1. The withdrawal of initial patterning factors (CHIR99021, LDN193189, SB-431542, and purmorphamine) at day 14 increased the expression of ATOH1, EN2, and GBX2, indicating that FGF8b, combined with WNT signaling pathway activation, dual SMAD-inhibition, and purmorphamine for ventralization, are potent regulators favoring ventral hindbrain identity. To optimize the protocol further, it would be interesting to extend the exposure of the initial patterning factors to day 21 and analyze if ventralization would be enhanced. However, the withdrawal of initial patterning factors also increased the expression of ventral hindbrain markers, NEPH3 and PTF1A. Therefore, more studies are needed to optimize ventralization using FGF8b.

The addition of FGF19 on day 14 resulted in a notable increase in the mRNA expression patterns of Purkinje cell markers CALB1, L7/PCP2, and ALDOC, highlighting the crucial role of FGF19 in promoting Purkinje cell differentiation. However, the mRNA expression data clearly display variations in expression patterns between different cell lines and batches, revealing challenges in reproducibility when generating 3D brain organoids. Silva *et al.* also reported similar challenges¹⁰⁰.

The forced expression of *NGN2* via the inducible system significantly enhanced the differentiation of iPSCs, as evidenced by the overall increased expression of neuronal markers employed in this study and an accelerated maturation process (MAP2 and GRID2). Notably, the increased expression of the markers L7/PCP2, ALDOC, and GRID2 suggests a preferential differentiation towards Purkinje cells. This observation underscores the potential of enhanced *NGN2* expression in generating specific cerebellar neuronal subtypes for studying cerebellar disorders.

The three ventral hindbrain markers, particularly OLIG2, were upregulated in "doxy" batches. However, both dorsal hindbrain markers exhibited similar increased expression patterns, indicating a mixed ventral and dorsal cell population. Similar early neuronal markers OTX2, PAX2, and EN2 are also prolonged in "doxy" batches, suggesting a more immature neuronal expression profile.

The comparative analysis between doxycycline-treated ("doxy") and untreated ("no doxy") batches also revealed notable differences in cell type populations and neuronal network formation, with differences in compartmentalization and morphology visually observed between days 16 to 19 (**Figure 3A**), indicating aberrant compartmentalization processes potentially caused by a more pronounced neuronal monoculture, as the forced *NGN2* expression has been reported to favor neuronal differentiation.

Immunofluorescence analysis revealed differences between "doxy" and "no doxy" batches in 2D cultures. "Doxy" batches showed increased GFAP-positive cells (**Figure 4**) and more developed dendritic morphology in Purkinje cells (**Figure 5**), suggesting differences in cell type populations and possibly enhanced maturation of Purkinje cells. However, we observed that the survival rate of dissociated adherent neuronal cultures highly depends on the maturation state, as resuspending more mature neurons causes more cell death. This could explain the differences in the TUBB3⁺ network and neuronal network formation between "doxy" and "no doxy" batches in 2D cultures, as qPCR results indicate increased maturation in "doxy" batches. The increased GFAP-positive cells, an astrocytic marker, in "doxy" batches further support this, as astrocytes develop later in the embryonic development of the cerebellum,

indicating increased maturation^{197,198}. Therefore, future optimization could potentially benefit from replating "doxy" batches at an earlier time point to avoid disrupting and dissociating the neuronal network of more mature neurons. The transition from NIM to neural maturation medium (NMM) on day 21 marks the transition from dynamic (orbital shaker) to static cell culturing. This method relies heavily on the diffusion of nutrients and oxygen from the culture medium into the organoid, which can limit the growth and development of the core of the organoid due to insufficient nutrient and oxygen supply. Introducing movement or flow to the culture environment does improve nutrient distribution and waste removal. The decrease in several hindbrain markers could represent the effect of insufficient nutrient and oxygen supply. During the initial optimization of the protocol, dynamic culturing was favored due to its reported benefits in a follow-up study by Silva *et al*¹⁹⁹. However, dynamic culturing of organoids also introduces shear stress on the developing organoids. During protocol optimization, we observed increased cell debris in the medium, and the organoids started to dissociate from day 25 to day 35 when employing dynamic culturing (orbital shaker) for extended durations. Therefore, we decided to employ static cell culturing after day 21 to avoid the observed gradual dissociation of the organoids. Moreover, static cell culturing in T25 or T75 flasks allowed for upscaling of batch sizes, generating hundreds of organoids per flask. For future optimization, it is highly recommended to implement dynamic culturing in bioreactors, as reported by Silva *et al*¹⁹⁹. Nonetheless, immunofluorescence of 96-day-old and 9-month-old hindbrain organoids displayed both TUBB3⁺ neuronal populations and Purkinje cell populations (IP3R1⁺, CALB1⁺, and GABA⁺), indicating that it is possible to maintain these cell populations with a static cell culturing setup.

The protocol's efficiency in generating cerebellar neurons, especially Purkinje cells, holds significant promise for advancing the study of cerebellar disorders. However, this preliminary study leaves room for future work, particularly concerning further validation, repetitions (so far, the protocol has been tested on three control cell lines: H245, H250, and H256), and quantification. This includes maturation parameters such as dendritic spine classification and quantification, electrophysiology, live-cell calcium imaging, and additional maturation markers. Furthermore, additional techniques, such as cryo-sectioning of organoids followed by immunostaining, visualization of neuronal populations and compartmentalization of organoids by light-sheet fluorescence microscopy, quantification by flow cytometry, and characterization of neuronal subtypes by single-cell RNA sequencing, would elevate the study.

By integrating the proven strategies of Rifes *et al*.¹⁰², Silva *et al*.¹⁰⁰, and Chen *et al*.¹⁰³, we successfully differentiated iPSCs into ventral hindbrain neurons, including Purkinje cells. This novel approach provides a promising and valuable tool for modeling cerebellar disorders *in vitro*. The successful generation of Purkinje cells, which are particularly vulnerable in many cerebellar disorders, including SCA2, opens new avenues for investigating the molecular mechanisms of disease pathogenesis and identifying potential therapeutic targets.

OVERALL DISCUSSION

The findings from Study I and Study II provide valuable insights into the molecular mechanisms underlying the pathogenesis of SCA2. The convergence of evidence from these investigations highlights the pivotal roles played by the dysregulation of PPIs, impaired autophagy, and aberrant mTOR signaling in the disease pathology. Study III serves as a novel protocol development for iPSC-derived ventral hindbrain neurons, an area that is currently in its explorative phase.

Investigating SCA2 using *in vitro* models involves navigating a range of obstacles, from the disease's inherent complexity to the limitations of cellular models. Overcoming these challenges requires continuous refinement of model systems, validation against clinical data, and development of innovative techniques to better mimic the *in vivo* environment.

This is crucial for studying SCA2, which affects specific brain regions, particularly the cerebellum and brainstem¹⁰³. The ability of brain organoids to model these regions allows for a more accurate representation of the disease pathology compared to traditional 2D cultures, which often fail to capture the complexity of human brain structures, or animal models, which do not recapitulate the full clinical pathology in human.

Study III presents a novel iPSC-derived hindbrain differentiation protocol that integrates key elements from previous protocols by Rifes *et al.*¹⁰², Silva *et al.*¹⁰⁰, and Chen *et al.*¹⁰³. Our protocol effectively patterns iPSCs into neuronal progenitor cells with a hindbrain identity, enhancing the generation of Purkinje cells through the addition of FGF19. The mRNA expression-profiles and immunofluorescence analyses validated the efficacy of the protocol, showing significant reductions in pluripotency markers and increased expression of hindbrain and Purkinje cell markers. The inclusion of a doxycycline-inducible *NGN2* expression system further enhanced the differentiation and maturation of ventral hindbrain neurons, demonstrating accelerated neuronal development and increased expression of specific cerebellar markers. This protocol provides a promising tool for modeling cerebellar disorders, particularly SCA2, and offers an approach for investigating disease mechanisms and a biologically relevant model to test pharmacologic interventions.

ATXN2 is implicated in two distinct proteinopathies: polyQ disorders and TDP-43 proteinopathies. SCA2 belongs to the polyQ disease group, characterized by an expanded polyQ tract that induces a conformational change in the mutated protein, promoting the formation of oligomeric and fibrillar aggregates¹⁰⁴. Intermediate-length polyQ expansions in ATXN2 are also recognized as significant risk factors for amyotrophic lateral sclerosis (ALS), a TDP-43 proteinopathy^{27,108}. The formation of intracellular aggregates by RNA-binding proteins (RBPs) is a known pathological feature in ALS and frontotemporal dementia (FTD), giving rise to the “two-hit” hypothesis of RBP-driven neurodegeneration¹¹². This model posits that RBP aggregates induce toxicity through a dual mechanism: a toxic gain-of-function by the aggregating properties followed by or caused by a concurrent loss of the normal functions of the protein.

In Study I, we focused on elucidating the alterations in PPIs and RNA binding of ATXN2 caused by the polyQ expansion, while Study II investigated the roles of autophagy and protein aggregation in SCA2 pathogenesis. Together, these studies shed light on the complex molecular mechanisms underlying SCA2 and highlight potential targets for therapeutic intervention.

Study I highlights the potential impairment of the native functions of ATXN2 due to the polyQ expansion, as evidenced by the altered interactions with known binding partners such as PABPC1, ATXN2L, DDX6, and LSM12. The progressive decline in ATXN2:LSM12 interactions with increasing polyQ length suggests a loss-of-function mechanism as the expanded polyQ region is located near the LSm domain, which may impact the normal function of this domain. The LSm domain is important for binding LSM12 and regulating the formation and disassembly of mRNP granules^{46,49,136}. Dysfunction of the LSm domain could lead to excessive mRNP granule formation, disrupting the equilibrium and contributing to cytotoxicity^{49,136}. In other words, the polyQ expansion may impose a local loss-of-function on the LSm domain, which leads to a new gain-of-function enhancing mRNP granule formation. Interestingly, a central observation from Study I was the significant increase in the number of proteins interacting with ATXN2 in SCA2 patient-derived cell lines, exhibiting a clear polyQ length-dependent pattern. Notably, ribosomal proteins and molecular chaperones were consistently enriched among the proteins displaying enhanced association with ATXN2, suggesting potential implications for translational regulation and proteostasis maintenance. The increased association of ATXN2 with ribosomal proteins corroborates previous reports of ATXN2's involvement in mRNA metabolism and translation^{46,60,61}. This may contribute to SCA2 pathology by disrupting translation, either through direct interference with ribosomal function or by sequestering key translation regulators such as EEF1A1, EIF4A1, and EEF2 into insoluble aggregates.

These findings are complemented by the observations from Study II, which revealed a pronounced increase in ATXN2 levels in the insoluble fraction of SCA2 cells, indicative of oligomerization or the formation of insoluble protein aggregates. Notably, treatment with Congo Red, a compound known to disaggregate proteins, facilitated the solubilization of ATXN2 and normalized its levels in the insoluble fractions between control and SCA2 cells. This finding highlights the therapeutic potential of targeting protein solubility to mitigate SCA2 pathology.

Both studies provide evidence for the involvement of dysregulated mTOR signaling in SCA2 pathology. The *Caenorhabditis elegans* homolog of ATXN2 has been reported to repress the mTOR activity, thereby affecting a range of physiological responses to dietary restriction⁶⁸. In support, embryonic fibroblasts from ATXN2^{-/-} mice exhibit enhanced mTOR activity⁶¹. In accordance with these findings, Study I revealed an increase in the abundance of proteins associated with translation and ribosomal components in SCA2 patient cells, suggesting upregulated mTOR activity. This was further substantiated by the analysis of upstream and downstream targets of mTOR, which exhibited elevated protein abundances, particularly for proteins encoded by mRNAs containing the 5' Terminal OligoPyrimidine (5' TOP) motif, known to be upregulated upon mTORC1 activation. This could indicate yet another loss-of-function of ATXN2, mediated by the expanded polyQ tract. Potentially via an impaired function of SNHG5, as Study I showed an increased association between ATXN2 and SNHG5 in SCA2 patient cells. SNHG5

has been reported to block the activity of STAU1¹³⁸, and interestingly, increased STAU1 activity has been reported and linked to mTOR hyperactivation and AD, HD, FTD, ALS, and SCA2 pathology^{86–88,139}. These discoveries suggest a potential synergistic interplay between ATXN2, SNHG5, STAU1, and mTOR in the pathogenesis of SCA2, warranting further investigation to elucidate their precise roles and mechanisms in the context of this neurodegenerative disorder.

The potential link between mTOR dysregulation and SCA2 pathology was further explored in Study II, where treatment with rapamycin, an mTOR inhibitor, effectively reduced caspase-mediated apoptosis, possibly by inhibiting mTOR activity and enhancing autophagy in SCA2 patient cells. These findings position mTOR as a viable therapeutic target for SCA2, corroborating a previous study that reported beneficial effects of rapamycin-induced autophagy activation in SCA2 disease model⁸⁸.

STRENGTHS AND LIMITATIONS

The use of a multifaceted approach, including IP-MS, global proteomics, and IP-RNAseq, provides a comprehensive strategy for elucidating the pathological mechanisms underlying SCA2. IP-MS enables targeted insights into the ATXN2 interactome, while global proteomics identifies broader proteomic alterations in SCA2 cells. Simultaneously, IP-RNAseq provided insight into the alterations in the RNA-binding capabilities of ATXN2. Employing multiple techniques allows for cross-verification of findings, increasing confidence in the results. However, the inclusion of total transcriptome RNA sequencing would have further enhanced the comparative analyses and potentially aided in the cumulative interpretation of the results while also mitigating the risk of misinterpretation of the IP-RNAseq analysis.

Although IP-MS, IP-RNAseq, and total proteome MS are powerful techniques for uncovering the molecular landscape of cells, verifying findings using complementary techniques such as Western blot, qPCR, and immunofluorescence is crucial for data validation. These complementary methods can address issues related to specificity, functional relevance, and spatial localization of identified targets. The reliability of the findings in Study I would be improved by incorporating these complementary verification techniques. In the absence of such validation, the identified targets could potentially represent false positives, as each method possesses inherent limitations. Therefore, further validation through alternative methods is essential for building confidence in the obtained results.

Pooling SCA2 Patient Cell Samples

Pooling SCA2 patient cell samples with varying lengths of the expanded polyQ regions can introduce several limitations, as the length of the polyQ expansion in SCA2 correlates with disease severity and age of onset. Combining samples with different polyQ lengths, as in Study I and II, could potentially obscure specific genotype-phenotype correlations, making it challenging to draw precise conclusions about the cellular impact of the polyQ expansion. This is because distinct lengths of polyQ expansions may lead to distinct cellular pathologies, as evident in Study I, where distinct phenotypes in ATXN2 PPI and RNA-binding properties were found depending on the length of

the polyQ-expanded region. For instance, longer expansions caused a more pronounced increase in PPIs, while decreasing the amount of RNAs bound to ATXN2 in SCA2 patient cells.

However, it is important to note that pooling SCA2 patient cell samples with various lengths of the expanded polyQ regions also has significant strengths that can advance research, particularly in understanding common disease mechanisms and identifying common cellular and molecular pathways affected by the mutation, which might be relevant across different stages of the disease. By doing so, we could identify shared pathways and mechanisms that are perturbed across the spectrum of polyQ expansion lengths, providing insights into the core pathological processes underlying SCA2.

Statistical Analysis of the IP-MS Assay

The statistical analysis of the IP-MS data, including the adjustment for multiple testing, such as the False Discovery Rate (FDR), was not prioritized, as CALD1 would have been the only finding close to the statistical significance threshold. Instead, an exploratory approach to the analyses was favored to investigate the alterations introduced by the polyQ expansion in ATXN2. This exploratory strategy aimed to uncover potential alterations in the interactome and cellular pathways affected by the polyQ-expanded ATXN2 protein without being constrained by stringent statistical thresholds that could potentially overlook biologically relevant findings.

By adopting an exploratory approach, the identification of new as well as putative interactors and pathways that may be perturbed by the polyQ expansion in ATXN2 was prioritized, even if they did not meet strict statistical significance criteria. This approach acknowledges the inherent complexity of the disease mechanisms and the potential for subtle yet biologically relevant alterations in protein-protein interactions and cellular pathways. The exploratory analysis allows for generating hypotheses and identifying potential targets for further investigation, which can subsequently be validated and refined through additional experiments and statistical analyses.

Cell Model Relevance

Patient-derived fibroblast cell lines are valuable resources in SCA2 research, providing unique insights into the cellular and molecular mechanisms of the disease. However, their use is associated with specific limitations, particularly in recapitulating brain-specific features and challenges in disease modeling and phenotypic expression. Dividing fibroblasts, while easily accessible, differ significantly from postmitotic neurons and other brain cells in their native state, posing a challenge in modeling neurodegenerative diseases like SCA2. Neurons have specialized functions and gene expression profiles that differ from fibroblasts, especially regarding the expression of ATXN2, which is elevated in the cerebellum and Purkinje cells²⁰⁰.

Despite these limitations, patient-derived fibroblast cell lines remain valuable in SCA2 research, offering a patient-specific human model that is easily accessible and can be used to study disease mechanisms, identify potential therapeutic targets, and screen drugs. While SCA2 is a complex neurodegenerative disease affecting multiple cell types and systems, monocultures like patient-derived fibroblasts provide a simplified model to study specific aspects of the disease, which can be advantageous for dissecting the molecular basis of SCA2 and testing hypotheses about

disease mechanisms in a controlled environment.

Advances in 3D culture systems, brain organoids, and improved differentiation protocols will improve the gap between current models and the complex reality of SCA2 pathology in the brain. By employing a multi-faceted approach that combines various cell models and techniques, we can gain a more comprehensive understanding of the disease mechanisms and potentially identify more effective therapeutic strategies.

CONCLUDING REMARKS

This PhD thesis presents a comprehensive investigation into the cellular and molecular underpinnings of SCA2, a neurodegenerative disorder characterized by progressive cerebellar degeneration and a range of neurological symptoms. The studies encompassed in this work employed SCA2 patient-derived cells and a multifaceted approach, integrating proteomic analyses, RNA sequencing, and modulation of autophagy pathways to elucidate the pathogenic mechanisms of SCA2 and identify potential therapeutic targets, as well as contributing to the protocol for generating a hindbrain organoid model from iPSCs.

Study I delved into the proteomic landscape of SCA2 patient-derived cells, revealing significant alterations in the global proteome, protein-protein interactions, and RNA-protein interactions of ATXN2, the protein central to SCA2 pathology. The global proteomic analysis highlighted an upregulation of proteins involved in translation, ribosomal function, and chaperone activity in SCA2 cells, suggesting a compensatory response to the cellular stress induced by polyQ-expanded ATXN2. Notably, the study uncovered a polyQ length-dependent increase in ATXN2 interaction with chaperone and ribosomal proteins, implicating protein folding and translational dysregulation in SCA2 pathogenesis. Furthermore, the RNA-protein interaction profile of ATXN2 exhibited significant alterations in SCA2 cells, with a polyQ length-dependent decrease in the abundance of RNAs bound to ATXN2, indicating an impairment in its RNA-binding capacity.

Study II focused on the role of autophagy in SCA2 pathology and its potential as a therapeutic target. The findings revealed elevated apoptotic activation in SCA2 cells alongside evidence of impaired autophagy. Pharmacological induction of autophagy using rapamycin and trehalose demonstrated promising results, with trehalose ameliorating apoptotic activation under oxidative stress and rapamycin reducing caspase activity, positioning mTOR as a viable therapeutic target. Moreover, the study highlighted the increased presence of insoluble ATXN2 aggregates in SCA2 cells, which could be solubilized by treatment with Congo Red, presenting another potential therapeutic avenue.

Study III aimed to develop a differentiation protocol for generating ventral hindbrain neurons from iPSCs. By integrating strategies from recent studies, the protocol sought to enhance hindbrain patterning and maturation, providing a platform for modeling SCA2 pathology and testing therapeutic interventions in a physiologically relevant context.

Collectively, these studies provide novel insights into the complex pathogenic mechanisms underlying SCA2, highlighting the potential of an intricate interplay between dysregulated translation, proteostatic disturbances, and autophagic impairment. The identification of key molecular players and pathways, including polyQ length-dependent interactions of ATXN2 with chaperones, ribosomal proteins, and RNAs, as well as autophagy impairment, highlights the potential of autophagy induction and protein solubilization as therapeutic strategies for SCA2.

Furthermore, the establishment of a refined hindbrain differentiation protocol offers a powerful tool for future investigations, enabling the study of disease mechanisms and therapeutic efficacy in a more physiologically relevant

setting. This advancement holds promise for accelerating the translation of basic research findings into clinical applications.

In conclusion, this PhD thesis contributes to understanding SCA2 pathology by exploring the cellular and molecular mechanisms that drive this debilitating neurodegenerative disorder. The insights gained from these studies not only deepen our knowledge of SCA2 but may also lay the foundation for the development of novel therapeutic strategies, offering hope for improved management and treatment of this devastating condition.

PERSPECTIVES

The findings presented in this thesis underscore the multifaceted role of the polyQ expansion in ATXN2 in the pathogenesis of SCA2. The polyQ length-dependent alterations in the protein-protein interactions and the RNA-binding capacity of ATXN2 suggest a complex interplay between loss-of-function and gain-of-function mechanisms contributing to SCA2 pathology.

Further investigation into how the expanded polyQ tract affects the LSm domain is crucial, as this domain mediates ATXN2's interactions with key partners such as LSM12 and DDX6. The polyQ length-dependent decrease in these interactions points towards a loss-of-function mechanism, potentially impairing the native role of ATXN2 in RNA metabolism and translational regulation. Conversely, the polyQ length-dependent increase in the interaction between ATXN2 and PABPC1, as well as chaperone and ribosomal proteins, suggests a gain-of-function mechanism, possibly leading to translational dysregulation and proteostasis stress.

The dual nature of the polyQ expansion's impact on ATXN2 function presents a complex landscape for therapeutic intervention. Loss-of-function effects, such as impaired regulation of mTOR, impaired mRNA binding, and disrupted interactions mediated by the LSm domain, may not be fully addressed by strategies aimed at lowering the levels of ATXN2, such as antisense oligonucleotide (ASO) therapies^{201,202}. Conversely, gain-of-function effects, including increased interactions with translation factors, ribosomal proteins, chaperones, and the long non-coding RNA SNHG5, may require targeted approaches to mitigate their consequences.

To effectively address the multifaceted pathology of SCA2, a combination of therapeutic strategies may be necessary. Lowering ATXN2 levels could be combined with pharmacological regulation of mTOR activity to counteract the loss-of-function effects on mTOR regulation. Alternatively, more refined approaches, such as CRISPR-Cas9-mediated targeting of the polyQ-expanded ATXN2 or compounds that regulate the mRNP assembly properties of ATXN2, could be explored to selectively address the effects of the polyQ-expanded ATXN2.

Translating the findings of this project from fibroblasts to neurons, particularly to cerebellar neurons, is a critical next step in advancing SCA2 research. The unique cellular context of neurons, with their specialized morphology, connectivity, and metabolic demands, may reveal additional layers of complexity in the pathogenic mechanisms of SCA2. Investigating the polyQ length-dependent alterations in ATXN2's interactions and functions within the context of cerebellar neurons could provide more direct insights into the molecular basis of the selective neurodegeneration observed in SCA2.

Moreover, the development of relevant neuronal models, such as patient-derived iPSC-based models of cerebellar organoids, could enable the exploration of potential therapeutic strategies in a more disease-relevant context. These models could serve as platforms for testing the efficacy and safety of ASO therapies, mTOR modulators, or other targeted approaches while also allowing for the assessment of their impact on key disease phenotypes, such as neuronal survival, synaptic function, and electrophysiological properties.

Future investigations into the pathology of SCA2 should capitalize on novel approaches employing patient-specific iPSC-derived neuronal models and high-throughput methodologies. The availability of patient-derived material as a substrate for these techniques offers an unparalleled opportunity to elucidate the underlying mechanisms of SCA2 pathology. Moreover, personalized cellular models hold tremendous potential in cell-based therapy for future

treatment of neurodegenerative diseases. iPSC models, reprogrammed from SCA2 patient fibroblasts, accompanied by gene-corrected isogenic controls, have already been established in our lab, with current efforts focused on their utilization in neuronal cultures and cerebellar organoids.

Unraveling the precise impact of the polyQ expansions in ATXN2 in the context of iPSC-derived cerebellar neurons will improve the likelihood of generating viable therapeutic strategies.

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APPENDICES

APPENDIX A: Manuscript I (Henriksen *et al.* in preparation)

APPENDIX B: Manuscript II (Wardman *et al.* 2020)

APPENDIX C: Supplementary information for experimental procedures

APPENDIX A:

Manuscript I (Henriksen *et al.* in preparation)

PolyQ Length-Dependent ATXN2 Interaction with Chaperones and Ribosomal Proteins in Spinocerebellar Ataxia Type 2 Patient Fibroblasts

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Structured Abstract

Background: Spinocerebellar ataxia type 2 (SCA2) is an autosomal dominantly inherited neurodegenerative disease caused by a polyglutamine (polyQ) expansion in the *ATXN2* gene, leading to progressive motor coordination loss. The detailed molecular mechanisms of the disease, especially the impact of polyQ expansions on the function of ATXN2, are not fully understood.

Objective: To investigate how polyQ expansions influence protein-protein interactions (PPIs) and RNA-binding capabilities of ATXN2 in SCA2 patient-derived fibroblasts.

Methods: We employed immunoprecipitation and mass spectrometry (IP-MS) to map the PPIs of wild-type and polyQexpanded ATXN2. Concurrently, RNA sequencing was utilized to analyze changes in the RNA species bound by ATXN2.

Results: We found a polyQ-length-dependent increase in ATXN2 interactions, especially with ribosomal and chaperone proteins, indicating a toxic gain-of-function. A significant decrease in ATXN2-bound RNAs was also observed, suggesting compromised RNA binding.

Conclusion: This study provides insights into the complex interplay between altered protein and RNA dynamics and SCA2 pathology. Our results support a “two-hit” hypothesis of toxic gain of function ATXN2-related PPIs coupled with loss of RNA-binding capabilities. These findings underscore the multifaceted nature of SCA2 pathogenesis and potentially pave the way for targeted interventions.

Keywords

Spinocerebellar Ataxia Type 2, Ataxin-2, RNA-Binding Proteins, Polyglutamine disease, Neurodegenerative disease, Proteomics.

Introduction

Spinocerebellar ataxia type 2 (SCA2) is an autosomal dominantly inherited neurodegenerative ataxia caused by an expanded CAG trinucleotide repeat region within the *ATXN2* gene. Due to its toxic gain-of-function, this expanded CAG repeat is central to SCA2 pathogenesis, causing progressive ataxia and cerebellar dysfunction, reflecting the degeneration of cerebellar Purkinje cells and other brain regions.

The *ATXN2* gene encodes the 140 kDa RNA-binding protein (RBP), ataxin-2 (ATXN2), that is highly expressed in the brain, underscoring its significance in neuronal function and health¹⁻³. ATXN2 is evolutionarily conserved and harbors three structured domains: LSm, LSm-AD, and PAM2⁴. The N-terminal region harbors the Like-Sm (Lsm) and Lsm-associated (LsmAD) domains, while the C-terminal region encompasses a polyA-binding protein interacting motif (PAM2), which is vital for mRNA stability and may contribute to translational activation⁵⁻⁸. Like other RBPs, an aspect of ATXN2 is the presence of intrinsically disordered regions (IDRs)⁹, which are protein regions lacking a fixed or ordered three-dimensional structure under physiological conditions.

Interestingly, ATXN2 is associated with two distinct proteinopathies: polyglutamine (polyQ) disorders and TAR DNA-binding protein 43 (TDP-43) proteinopathies. SCA2, along with Huntington's disease and several spinocerebellar ataxias (SCA1-3, SCA6-7, and SCA17), is part of the group of polyQ diseases¹⁰. PolyQ diseases are characterized by an expanded polyQ tract that leads to a structural shift of the mutated protein, which is believed to favor the formation of oligomeric and fibrillar aggregates. Despite this commonality, each disease presents a unique pathological profile, with the implicated proteins being structurally and functionally unrelated, sharing no homology besides the polyQ tract¹⁰⁻¹². This

suggests that the pathology of each disease is influenced by the function of the protein rather than the PolyQ region per se^{13–15}. Furthermore, the role of protein aggregates in the SCA2 pathogenesis is intricate, and evidence indicates that intranuclear aggregates are not crucial for SCA2 pathology, suggesting that the toxicity of the polyQ expansion may involve mechanisms beyond intranuclear aggregate formation¹⁶. For example, cytoplasmic aggregations have been linked to SCA2 pathology in mouse models¹⁶, and studies have demonstrated that the polyQ expansion can lead to cellular dysfunctions, such as mitochondrial dysfunction and oxidative stress¹⁷, without necessarily forming protein aggregates.

As mentioned, ATXN2 is also associated with TDP-43 proteinopathies as intermediate-length polyQ expansions in ATXN2 are recognized as significant risk factors for amyotrophic lateral sclerosis (ALS)^{18,19}. The formation of intracellular aggregates by RBPs is a known pathological feature in ALS and frontotemporal dementia (FTD). The identification of cytoplasmic TDP-43²⁰ and FUS^{21,22} as central elements of ubiquitin-positive aggregates in motor neurons and glial cells have given rise to the "two-hit" hypothesis of RBP-driven neurodegeneration²³. This model posits that RBP aggregates induce toxicity through a dual mechanism: a toxic gain-of-function by the aggregated proteins and a concurrent loss of the normal regulatory roles of the protein. This hypothesis has gained substantial empirical support, with many studies illustrating that modulating RBP expression levels can trigger neuronal death in various experimental models^{24,25}. The aggregation of RBPs is primarily driven by the presence of IDRs in their protein sequences. This is particularly evident in RBPs associated with ALS, such as TDP-43, FUS, and TAF15. The RBPs are known to form complexes with other proteins and are prone to aggregate due to the IDRs in their protein sequences^{26,27}. Mutations in these specific domains are linked to familial ALS/FTD, and they are characterized by pathological protein aggregation and sequestration of secondary proteins²⁸.

Given the multifaceted roles of RBPs, which span transcription regulation, RNA splicing/export, translational regulation, RNA metabolism, and cellular stress responses^{29–35}, high-throughput methodologies are preferred for identifying potential alterations in cellular homeostasis, protein function, or interacting partners introduced by the given mutation. Immunoprecipitation followed by mass spectrometry (IP-MS) is a powerful technique for elucidating extensive networks of RBP protein-protein interactions (PPIs) and the biological pathways they influence. Understanding these PPIs is crucial for comprehending the roles of RBPs and how the mutational alterations may disrupt these by aggregation.

To explore the intracellular alterations introduced by the polyQ expansion in ATXN2, we applied a battery of explorative bottom-up high-throughput methodologies, including global proteomics and IP-MS together with IP-RNAseq of ATXN2 on cellular lysates from SCA2 patient-derived fibroblast cells and healthy control cell lines. We identified increased levels of ribosomal proteins and protein chaperones in SCA2 patient cells as well as novel and altered PPIs of ATXN2 in SCA2 patient cells. Our findings link SCA2 pathology to biological processes predominantly associated with translation and mRNA processing in SCA2 patient cells. Additionally, our IP-MS data suggests that in SCA2 patient cells, ATXN2 has a polyQ length-dependent interaction with chaperones and ribosomal proteins, which is correlated with lower binding of RNA. These results highlight an altered PPI landscape for ATXN2 in SCA2 contributing to the cellular pathology.

Methods and materials

Clinical and Genetic Assessment

The study was approved by the Ethics Committee of the Capital Region of Denmark (H-4-2011-157). Patients were recruited through the Neurogenetics Clinic, Danish Dementia Research Centre, Rigshospitalet, and written informed consent was obtained from all participating patients and control individuals. Each patient diagnosed with SCA2 underwent a thorough clinical evaluation to document the presence and severity of characteristic SCA2 symptoms and signs, including ataxic gait, slowed saccades, and dysarthria. Genetic counselling and testing were independently performed before enrollment in the study. The elongated CAG repeat alleles were within a range of 36 to 44 repeats.

Cell Culture Preparation and Maintenance

Fibroblast cell cultures were established from forearm skin biopsies obtained from eight participants, divided into two groups: three control individuals without any reported health issues and five patients genetically diagnosed with SCA2. Fibroblast cells, derived from patients with SCA2 and healthy controls, were cryopreserved and stored in liquid nitrogen at the Danish Dementia Research Centre. Upon thawing, cells were cultured in T25 flasks containing 5 ml of Dulbecco's Modified Eagle Medium (DMEM) 1885 supplemented with NaCO₃, 10% fetal bovine serum (FBS), 1% GlutaMax, and 1% penicillin-streptomycin (all from Thermo Fisher Scientific). To reduce the residual dimethyl sulfoxide (DMSO) from the cryopreservation medium, the initial culture medium was replaced with fresh complete culture medium the day following initial plating. Cell growth was monitored daily using light microscopy. Upon reaching over 90% confluency, cells were dissociated using trypsin, transferred to T75 flasks, and maintained at 37°C in a humidified incubator with 5 % CO₂. The medium was refreshed every second days. Once confluency exceeded 90%, the cells were passaged into a new stock T75 flask.

Immunoprecipitation of ATXN2

Immunoprecipitations were carried out as previously described with a mild detergent to maintain RNA integrity (Thermo Fisher Scientific, 87787)³⁶. ATXN2 were immunoprecipitated with Protein-A agarose beads (Cell Signaling Technology, #9863). Briefly, on day 1, the beads were blocked by incubating in 5 % BSA (bovine serum albumin) in lysis buffer for two hours at 4 °C. The beads were washed three times for five minutes in lysis buffer. The cells were harvested with cell scrapers and incubated in 0.5 % formaldehyde in PBS for seven minutes. The cells were washed in PBS, and lysis buffer was added, followed by three cycles of freeze/thaw and sonification, and subsequently centrifuged at 13,000 x g for 30 minutes at 4 °C. The cell lysates were precleared on Protein A-Agarose beads for 1 hour at 4 °C while shaking. Following, the beads were pelleted by centrifugation at 1000 x g for five minutes at 4 °C, and the supernatants were transferred to a fresh tube on ice. A lysate aliquot (40 μ l) was kept for input reference. Primary ATXN2 antibody (1 μ g, Novus Biologicals, NBP1-90063) was added to total cellular protein (500 μ g) and incubated for one hour at 4 °C. Subsequently, prewashed Protein A-Agarose was added and incubated overnight at 4°C rotating. The following day, the Protein A-Agarose pellet was centrifuged at 1000 x g for five minutes at 4 °C, and the supernatant was saved as flow through. The Protein A-agarose pellets were washed four times in lysis buffer, once with PBS-T (0.5 %), each time repeating the centrifugation step above. The immunoprecipitates destined for mass spectrometry were eluted by adding 1x LDS sample buffer containing reducing agent to the Protein A-agarose pellets and incubated at 96 °C for three minutes, followed by centrifugation at 1000 x g, for five minutes at 4 °C, and supernatant were collected for downstream analyses. Immunoprecipitation was performed using an anti-ATXN2 antibody from Novus Biologicals (NBP1-90063) and an anti-IgG from SigmaAldrich (I5006-10MG).

iTRAQ labeling.

For iTRAQ labeling, iTRAQ® Reagent - 8PLEX Multiplex Kit (Sciex iChemistry, 4390812) was applied according to the manufacturer's instructions. From each cell line, 90 μ g protein was pooled into a healthy control group (n = 3) and a SCA2 group (n = 5). Proteins were precipitated with 10 volumes of ice-cold acetone and incubated overnight at -20°C. Before iTRAQ labeling, the protein samples were incubated with a dissolution buffer, a denaturant, a reducing reagent, and a cysteine-blocking reagent to reduce the proteins and block cysteine and, followed by trypsin digestion (2 μ g) incubated overnight at 37°C. The protein digests from each pooled group were labeled with specific amine-modifying labeling reagents to distinguish the samples in the following mass spectrometry analysis. After the peptides had been labeled, the contents of each sample were combined in a new tube.

Proteome sample preparation and nano-LC-MS/MS analysis

Proteomics analyses were carried out as previously described³⁷. Briefly, A SCX (strong cation exchange) cartridge purification was performed of the peptides and subsequently vacuum-dried. Following, the peptides were rehydrated using isoelectric focusing (IEF), fractionated, and extracted. The digests were purified using PepClean C-18 Spin Columns and vacuum-dried. Proteins were identified and quantified using nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS). Initially, protein samples were prepared by slicing each gel lane into pieces measuring 1 $\times$ 1 mm. The cysteine residues were then protected through reduction and alkylation, employing tris(2-carboxyethyl)phosphine and iodoacetamide, respectively. Trypsin was used for in-gel digestion, and the peptides produced were extracted from the gel fragments with acetonitrile and trifluoroacetic acid. These peptides were subsequently purified using PepClean C-18 Spin columns. The analysis was conducted on an EASY nanoLC system linked to a Q Exactive Plus Hybrid QuadrupoleOrbitrap Mass Spectrometer. Peptides were separated on a PepMap C-18 reverse phase column, which has a dimension of 25 cm in length, a 75 μ m internal diameter, and two μ m particle size. The peptides were eluted over a 90-minute linear acetonitrile gradient (4% to 40%) mixed with 0.1% formic acid. The mass spectrometer operated in a data-dependent acquisition mode, alternating between full scans (MS1) and fragment scans (MS2), with mass resolutions set at 70,000 for MS scans and 17,500 for MS2 scans. The instrument selected up to ten of the most intense ions for fragmentation by higher-energy collisional dissociation in each full MS scan. A dynamic exclusion period of 10 seconds was implemented to prevent repetitive selection of ions, and ions with a single charge or those with unassigned charge states were excluded from fragmentation. MaxQuant software (version 1.5.2.8) was utilized for protein identification and quantification based on the peptide peak areas' analysis. The raw MS data were cross-referenced against a database containing 20,197 human protein sequences sourced from UniProt. To validate the reliability of the identified proteins and peptides, the false discovery rate (FDR) was determined by contrasting the results against a reverse decoy database, with both protein and peptide identification FDR thresholds set at 0.01.

RNA purification from ATXN2-IP

The Protein-A agarose immunoprecipitates were centrifuged at 1000 x g for 5 minutes at 4°C, and the supernatant was removed, with 800 μ l of Lysis-Urea buffer added to the beads. The beads were washed with 500 μ l of Lysis-Urea buffer and subsequently with 500 μ l of Wash buffer not containing UREA. Next, 130 μ l of lysis buffer, 1.5 μ l of Proteinase K, and 150 μ l of 0.1% SDS solution were added and incubated at 37°C for 30 minutes. This was followed by the addition of 30 μ l of 3M sodium acetate and 350 μ l of phenol – chloroform – isoamyl alcohol mixture (Sigma-Aldrich, 77618), after which the samples were vortexed and centrifuged at 16,000 x g for five minutes at room temperature. The aqueous layer was transferred to a new tube with 900 μ l of 99% ethanol and left to precipitate overnight at -20°C. On Day 3, the samples were centrifuged

at 16,000 x g for 20 minutes at 4°C, washed in 70% ethanol, and spun again under the same conditions. The ethanol was removed, and the pellets were left to air-dry. The pellet was then resuspended in 10 µl of DNase solution (Invitrogen) and incubated for 15 minutes at room temperature. The DNase was quenched by adding 1 µl of 25 mM EDTA and heated for 10 minutes at 65°C. Subsequently, 20 µl of 3M Sodium Acetate, 170 µl of RNase-free water, and 200 µl of PCA were added, vortexed, and centrifuged at 16,000 x g at room temperature. The aqueous layer was transferred to a fresh tube, mixed with 500 µl of 100% ethanol, and left to precipitate overnight. On Day 4, the procedure for RNA purification was repeated as on Day 3. Finally, the pellet was resuspended in RNase-free water, rendering it ready for downstream application.

RNA sequencing

RNA obtained from the ATXN2-IP samples was sent to BGI Genomics for general transcriptome sequencing using the Illumina HiSeq platform and the human genome as a reference genome. The ATXN2-IP samples were run in duplicates with an IgG control for unspecific binding. Upon completion of the sequencing process, a collection of raw data reads was obtained. To obtain a 'clean' data set, the data was rigorously filtrated for reads of substandard quality, contamination with adaptor sequences, or a high proportion of unidentified bases (denoted as 'N'). Novel transcripts were integrated with the existing coding transcripts from the reference to formulate an augmented reference transcriptome. Gene expression analysis was conducted utilizing the enhanced references, which enables the identification and characterization of differentially expressed genes.

Data processing and analyses

For iTRAQ proteomics, the data was processed and normalized using Excel and Perseus (software version 2.0.11.0). Protein abundances from both groups (healthy controls and SCA2 patients) were normalized by centering the data to the median of the distribution of each sample. This was done to minimize any unwanted batch variabilities. The detected proteins were filtrated by Protein FDR (thresholds set at 0.01) and peptide-spectrum matches, PSMs (threshold: ≥ 3). Downstream analysis was performed using Excel and Perseus software to identify differentially abundant proteins between the two groups. Perseus software was used for data visualization.

For IP-MS, the data was processed and normalized using Excel and Perseus software (version 2.0.11.0). Raw iBAQ protein abundances of each sample were normalized to the abundance of the corresponding immunoprecipitated protein (ATXN2 or IgG). Proteins were filtered out if they were only identified by a modification site or if the protein matched the reversed part of the decoy database, and potential contaminants were left out, such as keratins and trypsin. Unspecific binding of the IgG control was evaluated on statistical differences (unpaired Student's t-test) and fold change between the averaged values from ATXN2 and IgG within each group (healthy controls and SCA2 patients). Filter criteria were set to a p-value below 0.05 and a fold change above 2 (ATXN2 vs IgG). To address missing data, the dataset was filtered to retain only variables with at least 70% presence in either the controls or SCA2 patient group. Subsequently, the data underwent a log2 transformation. An imputation strategy was implemented in instances of remaining missing values by drawing values from a normal distribution for each sample individually.

For the IP-RNAseq data, any unspecific binding of the IgG control was evaluated on statistical differences (unpaired Student's t-test) and fold change between the values from ATXN2 and IgG within each cell line sample. Filter criteria were set to a p-value below 0.05 and a fold change above 2 (ATXN2 vs IgG), which resulted in 2214 (healthy controls) and 2353 (SCA2 patients) RNAseq reads being filtered out. The CPM (Counts Per Million) method was used to normalize the RNA sequencing data ($\text{CPM} = \frac{\text{Total reads in the sample}}{1,000,000 \text{ number of reads for a gene}}$). The data was filtered for missing values, and a threshold of a minimum of 70% of valid values in either the healthy control or SCA2 patient group was set, followed by log2-transformation. Data imputation from normal distribution was performed separately for each sample. Data processing and analyses were done with the Excel and Perseus software.

Enrichment annotation analyses

The enrichment annotation analyses were performed using DAVID (Database for Annotation, Visualization, and Integrated Discovery) version 6.8, Enrichr, and STRING version 12.0. The differentially expressed gene lists were submitted to DAVID for functional annotation clustering using custom settings (high classification stringency and annotation categories: KEGG, Reactome, Gene Ontology Biological Process, Gene Ontology Molecular Function, Gene Ontology Cellular Component). Only annotation clusters with an enrichment score above 1.3 were considered reliable. Similarly, the gene lists were analyzed using Enrichr against the KEGG, Reactome, Gene Ontology Biological Process, Gene Ontology Molecular Function, and Gene Ontology Cellular Component. In STRING, functional association networks and functional enrichment analyses were generated for the differentially expressed proteins (settings: network type: full STRING network, network edges: confidence, active interaction sources: experiments and databases, minimum required interaction score: high confidence 0.700). Clustering options for STRING network: MCL clustering with an inflation parameter of 3 with no edges between clusters. Overlapping enriched terms identified by DAVID, Enrichr, and STRING were examined to derive biological insight from the gene expression changes.

Proximity ligation assay (PLA)

The protein-protein interaction studies were performed with PLA. A Duolink® In Situ Detection Reagents (SigmaAldrich, DUO92014, DUO92001, DUO92005) and used according to the manufacturer's instructions. PFA (4%) fixed cells were permeabilized (0.5% Triton X-100) and blocked with PBS containing 5% BSA (bovine serum albumin) for one hour at room temperature. The cells were incubated with primary antibodies overnight at 4°C in PBS with 5% BSA, followed by incubation with Duolink PLA anti-Mouse PLUS and PLA anti-Rabbit PLUS proximity probes. For nuclei staining, Hoechst staining was added, after which the cells were washed three times in PBS. The slides were mounted using ProLong Antifade Gold mounting medium (Thermo Fisher Scientific, P36934). The slides were visualized using a fluorescence microscope (EVOS™ M7000 Imaging System). Images were analyzed with a ComDet plugin (v0.5.5) in ImageJ software.

Statistical analysis

The statistical differences between control and SCA2 patient cell lines were calculated using an unpaired Student's t-test. The statistical calculations were carried out using Perseus Software and GraphPad Prism (v10.2.3).

Results

Characterization of the SCA2 Proteome Using Mass Spectrometry

We employed a global mass spectrometry (MS) approach to analyze the proteome of SCA2 patient cell lines, aiming to identify alterations linked to the polyQ expansion in the ATXN2 protein. Initial MS data filtration revealed the presence of 1,894 proteins. Of these, 354 proteins exhibited increased expression (fold change >1.25), and 337 proteins showed decreased expression (fold change <0.8) in SCA2 patient cell lines compared to controls. The protein abundance distributions were similar between the SCA2 and control cell lines, although the control group displayed more dispersed protein abundances (Figure 1A-C).

Enrichment analyses of the differentially expressed proteins (fold change >1.25 for increased and <0.8 for decreased expression) revealed significant enrichment pathways involved in the epigenetic regulation of gene expression, including arginine methylation, histone deacetylation and acetylation (Figure 1D). Conversely, proteins with increased expression were predominantly associated with pathways related to the cellular response to starvation, ribosomal function, and translation (Figure 1D).

Further detailed analysis of translation-related proteins demonstrated an increased abundance of these proteins in SCA2 patient cells compared to controls (Figure 1E). Notably, there was a marked enhancement in the abundance of proteins associated with translation, ribosomal components, and chaperone functions, which relates to the polyQ-expanded ATXN2-mediated SCA2 pathology (Figure 1E). The upregulations of ribosomal proteins were verified via Western Blot (Figure 1F, not done yet).

IP-MS Analysis of ATXN2 Protein Interactions in SCA2 Patient Cells

To elucidate the PPIs of ATXN2 in cells derived from patients with SCA2, we employed IP-MS. Figure 2A provides a schematic representation of the IP-MS procedure and workflow. We performed immunoprecipitation of ATXN2 from whole cell lysates from healthy control and SCA2 patient-derived cells. IgG antibodies were used as a control to assess non-specific binding. A mild detergent was used during the immunoprecipitation process to preserve RNA-mediated interactions, allowing for the identification of both strong and transient ATXN2-associated proteins. Immunoblot analysis confirmed the enrichment of ATXN2 in the immunoprecipitated fractions (Figure 2B), while no ATXN2 was detectable in the IgG control pulldowns, indicating specific immunoprecipitation of ATXN2 from the cell extracts.

The immunoprecipitated proteins were analyzed with nano-LC-MS/MS. The analysis identified 232 proteins that are associated with ATXN2 (Figure 2C). These proteins are implicated in various cellular processes, e.g., translation and ribosome function, spliceosome activity, RNA metabolism, cytoskeletal structure, chaperone functions, mitochondrial processes, and RNA-binding activities (Figure 2E). Notably, these candidate proteins were consistently detected in at least 70% of either the healthy control or SCA2 patient samples and exhibited at least a 2-fold increase in abundance compared to the IgG controls, underscoring their specificity to ATXN2.

Principal component analysis (PCA) of the protein interaction profiles showed a significant overlap between the patient and control cell lines (Figure 2D), which suggests considerable variability in the ATXN2 interaction landscape across different cellular contexts.

Alterations in ATXN2 Protein-Protein Interactions in SCA2 Patient Cell Lines

Immunoprecipitation coupled with IP-MS analysis revealed significant alterations in the protein binding profile of ATXN2 in cell lines derived from patients with SCA2. There was a notable increase in the abundance of proteins interacting with ATXN2, as evidenced by the identification of 42 protein hits showing significantly enhanced interaction with ATXN2 in

SCA2 patient cells compared to controls (Figure 3A-B). This change suggests that the expanded polyQ tract in ATXN2 may mediate alterations in its PPIs. Conversely, only one protein, NEK3, demonstrated a significantly reduced association with ATXN2. Further exploration using STRING analysis of the proteins with increased interaction revealed a significant enrichment of ribosomal proteins, including both 40S and 60S ribosomal subunits (Figure 3C). This enrichment indicates a potential shift toward increased ribosome-associated processes or translation regulation in the pathology of SCA2. The analysis also highlighted a subset of chaperone proteins (HSPA6, HSP90AA1, and DNAJC3) and endoplasmic reticulum (ER)-associated proteins (CALD1, CKAP4, and LEPRE1) among the proteins with increased interactions with ATXN2. This finding suggests an enhanced association of ATXN2 with chaperone systems and the ER, which may reflect cellular stress responses or alterations in protein folding and processing pathways in SCA2 patient cells. Enrichment analyses of the proteins with increased interaction with ATXN2 highlighted their involvement in critical cellular pathways, including translation, ribosomal subunit integration, and amino acid metabolism and sensing (Figure 3E). Moreover, PLA assays showed a significant increase in ATXN2:RPS3 and ATXN2:RPS18 interactions in SCA2 patient cells compared to controls (Figure 3F).

Polyglutamine Length-Dependent Alterations in ATXN2 Protein-Protein Interactions

Previous studies have established a strong inverse correlation between the length of the CAG repeat in the ATXN2 gene and the age of onset of SCA2, with longer expansions typically leading to an earlier onset of the disease^{38–40}. Based on this correlation, we hypothesized that variations in the length of the expanded polyQ tract in ATXN2 might influence its PPIs, potentially elucidating mechanisms driving SCA2 pathology. To investigate this hypothesis, we categorized our samples into four groups based on the identified polyQ lengths: 22Q (control), 36Q, 42Q, and 44Q. This stratification allowed us to assess the impact of increasing polyQ lengths on the interaction profile of ATXN2. The results demonstrated a clear polyQ length-dependent increase in the number of proteins interacting with ATXN2: 36Q cell lines exhibited a ≥ 2 -fold increase in binding or association with ATXN2 for 46 proteins (Figure 4B, F). 42Q cell lines with a 42 CAG repeat expansion showed a ≥ 2 -fold increase for 104 proteins (Figure 4B, F). 44Q cell lines displayed a ≥ 2 -fold increase for 128 proteins (Figure 4B, F). These findings indicate that as the polyQ length increases, so does the number of ATXN2 PPIs, which correlates with the known association between CAG repeat length and the severity and onset of disease. The consistent presence of ribosomal and chaperone proteins across varying polyQ tract lengths, coupled with their inclusion among the 31 proteins exhibiting a greater than two-fold enrichment in all polyQ length cohorts (Figure 4E) strongly implies their putative involvement in the pathogenesis of SCA2.

Impact of PolyQ Expansion on ATXN2 RNA-Binding Capabilities

Building on our findings of polyQ length-dependent increases in ATXN2 PPIs, we explored the potential impact on the RNA-binding capabilities of ATXN2. Given the observed increase in PPIs, particularly with ribosomal and chaperone proteins, we hypothesized that the sequestration of ATXN2 might compromise its canonical RNA-binding functions. This hypothesis was predicated on the notion that polyQ expansion could induce structural and functional alterations in ATXN2, particularly affecting its RNA-binding domains, LSm and LSmAD.

To investigate this hypothesis, we performed RNA sequencing on ATXN2 immunoprecipitations from both healthy control and SCA2 patient-derived cell lysates. The immunoprecipitation procedure was optimized to preserve RNA integrity, using IgG as a control to assess nonspecific binding. Post-immunoprecipitation, the RNA molecules associated with ATXN2 were sequenced to identify and quantify the bound RNA species. The specificity of ATXN2-RNA interactions was evaluated using an unpaired Student's t-test, with significance set at a p-value below 0.05 and a fold change greater than 2 compared to the IgG control.

The sequencing results revealed a total of 8794 RNAs associated with ATXN2, encompassing various RNA species, including antisense RNA species, microRNA, non-coding RNA, a PALM2-AKAP2 fusion RNA, protein-coding RNAs, pseudogene RNAs, readthroughs, and unknown RNA sequences. Further volcano plot analysis highlighted significant alterations in RNA bound or associated with ATXN2 in SCA2 patient cell lines (Figure 4C). The plot demonstrated a notable decrease in the abundance of RNAs bound to ATXN2, indicating an altered RNA-binding profile in the context of polyQ expansion. Specifically, we observed increases in 723 RNAs (fold change >1.25) and decreases in 1145 RNAs (fold change <0.8), with an overall trend showing a reduction in the number of RNAs bound to ATXN2 across patient cell lines.

To further elucidate the impact of polyQ length on RNA-binding, samples were categorized as previously based on polyQ length into four groups: 22Q (control), 36Q, 42Q, and 44Q. Analysis of these groups revealed a correlation between the length of the polyQ tract and a decrease in RNAs bound to ATXN2 (Figure 4D, G). Hence, these results show an inverse correlation between protein binding and RNA binding of ATXN2, which is determined by the polyQ length.

Discussion

IP-MS was employed to explore the PPIs of ATXN2 in SCA2 patient-derived cells. The analysis displayed significant alterations in ATXN2 PPIs in SCA2 patient cell lines, with a notable increase in the abundance of proteins associated with ATXN2 compared to controls. This highlighted ribosomal proteins and chaperones as being enriched in the interactome of ATXN2 in SCA2 patient cells.

Heat shock proteins (HSPs) function primarily as molecular chaperones, assisting in the proper folding of proteins, preventing aggregation, and facilitating the refolding or degradation of misfolded proteins. HSPs play a dual role in diseases: they protect cells from stress-induced damage but may also participate in the formation of protein aggregates¹³². On the one hand, they act as chaperones to prevent aggregation but can be sequestered into aggregates themselves, reducing their availability to perform protective functions¹³². Interestingly, our data displays an increased interaction between ATXN2 and chaperones, especially DNAJC3, HSPA6, and HSP90AA1, in SCA2 patient-derived cells, which indicate that ATXN2 in SCA2 is recognized as misfolded, aggregated, or marked for degradation.

Notably, we found the co-chaperone DNAJC3 to be decreased in the global MS of SCA2 cells compared to controls, while ATXN2 in SCA2 cells had increased association with DNAJC3. This could reflect that polyQ-expanded ATXN2 scavenges DNAJC3 and reduces the availability of DNAJC3. DNAJC3, also known as P58IPK, is a co-chaperone that translocates to the ER lumen upon ER stress¹³³, and the co-chaperone is involved in the unfolded protein response (UPR), a control system that manages ER stress caused by the accumulation of misfolded proteins. Interestingly, studies have reported DNAJC3 deficiency to cause mitochondrial dysfunction⁴¹ and the cause of multisystemic neurodegeneration, including ataxia¹³⁴.

In addition, ATXN2 significantly increased the interaction with Heat Shock Protein 90 kDa Alpha (Cytosolic) Member A1 (HSP90AA1) in SCA2 cells. HSP90AA1 is the stress-inducible isoform of the HSP90 chaperones, while HSP90AB1 is expressed constitutively⁴². Xiao *et al.* suggest that HSP90AA1 activates autophagy by inhibiting the PI3K/AKT/mTOR pathway by decreasing the phosphorylation of AKT and mTOR⁴³. Hyperactivation of mTOR has been reported in SCA2 disease models^{44,45}, hence, the binding of polyQ-expanded ATXN2 to HSP90AA1 could reduce autophagy activation, which is known to cause neurodegenerative disease⁴⁶. However, treatment with an HSP90 inhibitor, 17-DMAG, in ATXN2-Q58 HEK293 cells reduced ATXN2 levels while upregulating HSP70. Collective, this restored autophagy flux and reduced the ER-stress⁴⁷. This could indicate that HSP90 proteins are involved in disease progression by potentially stabilizing polyQ-expanded ATXN2 and facilitating a pathogenic aggregation.

Through our global proteomics data, we found a slight increase in the abundance of proteins exhibiting chaperone functions (Figure 1E), possibly reflecting a response to cellular proteotoxic stress caused by the polyQ expansion in ATXN2, as we also found an increase in chaperones associated with ATXN2 in SCA2 cells (Figure 3C). Further research is necessary to ascertain the importance of these interesting chaperone proteins in the cellular pathology of SCA2.

Our global proteomics data also revealed alterations in ribosomal proteins and translation-related processes. The increased abundance of ribosomal proteins in SCA2 cells and their enhanced association with ATXN2 warrant further investigation into ribosome function and protein synthesis. As the global proteomics of SCA2 cells revealed a general increase in ribosomal proteins (Figure 1D, -E), we cannot exclude that the increase in ATXN2-associated ribosomal proteins is caused by the increased availability of these proteins. However, some of the significantly increased ribosomal proteins associated with ATXN2 in the IP-MS assay, RPS28 and RPL8 (Table 1), are, in fact, downregulated in SCA2 cells compared to controls. Alternatively, this could reflect a compensatory response aimed at restoring normal ribosomal function and overall protein synthesis capacity. This could be attributed to the activation of the mTORC1. Notably, mTORC1 can regulate the translation of mRNAs containing the 5' terminal oligopyrimidine (TOP) motif sequences predominantly found at the 5' ends of mRNAs encoding components of the protein synthesis machinery, including ribosomal proteins and translation factors. The translation of these 5' TOP mRNAs is tightly controlled by mTORC1, which adjusts their expression in response to cellular growth signals and nutrient availability. Our global MS analysis further supports an increase in ribosome biogenesis and the initiation of translation, processes critically governed by mTORC1. Additionally, hyperactivation of mTOR has been documented in disease models of SCA2^{44,45}.

Our study extends these observations by showing a pronounced association between ATXN2 and ribosomal proteins in SCA2 patient cells. This enhanced association could be a contributing factor to the SCA2 pathomechanism, potentially through disrupted translation processes or the formation of aggregates that sequester key translation regulators like EEF2 (Table 1). EEF2, encoded by the EEF2 gene, is essential for the elongation phase of protein synthesis, facilitating ribosomal translocation along mRNA⁴⁸. Its increased protein-protein interaction (PPI) with ATXN2 in SCA2 cells underscores the significance of translational dysregulation in SCA2's pathogenesis. Moreover, mutations in the EEF2 gene have been linked to neurodevelopmental disorders, including spinocerebellar ataxia 26 (SCA26)⁴⁸⁻⁵⁰, further highlighting the critical role of translational mechanisms in neurodegenerative diseases.

The development of SCA2 is closely associated with the expansion of the polyQ tract within the ATXN2 protein. Our IPMS data shows a polyQ-length-dependent increase in PPIs to mutant ATXN2, suggesting a toxic gain-of-function mechanism. Among these, the frequent association with ribosomal and chaperone proteins across samples with expanded polyQ tracts points to a significant role for these proteins in the molecular underpinnings of SCA2. This polyQ-length-dependent increase in ATXN2-associated PPIs is consistent with the established inverse relationship between the length of the CAG repeat expansion and the age at which the disease manifests. This correlation emphasizes the critical influence of the polyQ tract on the network of interactions involving ATXN2, shedding light on its integral role in the advancement of SCA2 pathology.

Conversely, the expansion of the polyQ tract reduces the RNA-binding capacities of ATXN2 in a length-dependent manner. The canonical role of ATXN2 in RNA dynamics, mediated through its LSm and LSmAD domains, is essential for various aspects of RNA metabolism^{51,52}. Our results suggest that the polyQ expansion induces structural perturbations that extend to these RNA-binding domains, thereby altering the RNA-binding profile of ATXN2. The RNA sequencing of ATXN2 immunoprecipitates from SCA2 patient cells revealed a significant shift in the RNA-binding profile compared to healthy controls. Principal component analysis (PCA) effectively segregated the patient-derived cell lines based on their ATXN2 RNA-binding patterns, indicating a disease-specific alteration in RNA-protein interactions (Figure 4E). The altered RNA-binding profile may contribute to dysregulation of gene expression and disruption of cellular homeostasis.

Based on our findings, the impact of enhanced PPI between ATXN2 and ribosomal proteins in SCA2 patient cells remains elusive. However, it is noteworthy that the majority of proteins identified via IP-MS localizes to messenger ribonucleoproteins (mRNPs). mRNPs are crucial in translation regulation, capable of both repressing and activating mRNA translation depending on the cellular context, specific protein and RNA composition, and regulatory mechanisms⁵³.

Previous studies have shown that the PAM2 domain and IDRs of ATXN2 facilitate mRNP granule formation, while the LSm domain inhibits this process⁵⁴⁻⁵⁶. The neighboring polyQ region, when expanded, may exacerbate LSm domain dysfunction, disrupting the balance between mRNP granule assembly and disassembly and potentially interfering with translational regulation. In conjunction with these previous studies, our data suggest that the increased PPI of ATXN2 and translation-related proteins may result from polyQ length-dependent impairment of the LSm domain, promoting excessive mRNP assembly with potential aggregating properties. However, Singh et al. also reported an increase in ATXN2:mRNA interactions upon deletion of the LSm domain, while the IP-RNAseq data show a decrease in RNAs bound to ATXN2 in SCA2 cells.

The inverse correlation between the increased interaction of ribosomal proteins and the decreased abundance of RNAs bound to ATXN2 in SCA2 patient cells could manifest as disrupted protein synthesis, resulting from ribosome stalling and collision, and impaired RNA metabolism, both of which are critical for neuronal health. Interestingly, ribosome stalling and suppression of protein synthesis have also been proposed as a pathomechanism of HD⁵⁷, while mutations in proteins that also regulate protein synthesis (SMN, FUS, TDP-43) are known to cause neurodegenerative disorders⁵⁸⁻⁶¹. The expanded polyQ tract in ATXN2 is widely recognized for conferring a toxic gain-of-function⁶², as evidenced by our findings of a polyQ-length-dependent increase in ATXN2 PPIs. In support, ATXN2 knockout mice (ATXN2^{-/-}) do not exhibit cerebellar atrophy or ataxia symptoms, suggesting that loss of ATXN2 function does not recapitulate SCA2 pathology^{16,63}. Intriguingly, research on ATXN2^{-/-} mice has shown upregulations of ribosomal proteins⁶⁴, similar to those observed in our study. This aligns with the "two-hit" hypothesis of RBP-driven neurodegeneration, proposing that SCA2 pathogenesis may require both loss of normal function and a toxic gain-of-function.

Conclusion

This study provides a comprehensive analysis of the proteomic and RNA-binding alterations associated with polyQ expansions in the ATXN2 protein in SCA2. Our findings underscore a complex interplay between increased PPIs and altered RNA dynamics, which are influenced by the length of the polyQ tract within ATXN2. Notably, the results highlight a polyQ-length-dependent increase in the association of ATXN2 with ribosomal and chaperone proteins, suggesting a shift towards disrupted protein synthesis and folding processes that may contribute to the pathogenesis of SCA2.

Patient-derived fibroblast cell lines may not fully replicate the neuronal-specific pathology of SCA2. To address this gap, future research should focus on neuron-specific ATXN2 PPIs. Investigating these interactions in neuronal models will be essential to understanding the precise mechanisms by which polyQ-expanded ATXN2 contributes to disease progression and to identifying potential therapeutic targets within these pathways. This neuron-focused approach will provide deeper insights into the cellular dysfunctions that underlie SCA2, potentially leading to more effective treatments.

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Tables:

Table 1 - Proteins involved in translation identified in IP-MS and global MS

Table of identified translational proteins from the ATXN2 IP-MS listing the protein names, gene symbols, significance compared to control cell lines (p-value >0.05, marked with +), and fold change compared to control.

Protein	Gene	ATXN2 interaction (p-value < 0.05)	ATXN2 interaction (fold change)	Global MS (fold change)
ATP-dependent RNA helicase DDX1	DDX1		1,15	1,10
ATP-dependent RNA helicase DDX3X	DDX3X		1,01	1,23
Probable ATP-dependent RNA helicase DDX5	DDX5		1,45	1,03
Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	DHX15		1,81	0,78
Elongation factor 1-alpha 1	EEF1A1		1,83	1,45
Elongation factor 2	EEF2	+	2,76	1,07
Eukaryotic initiation factor 4A-I	EIF4A1		2,23	1,15
Fragile X mental retardation syndrome-related protein 1	FXR1		1,73	0,86
Nucleolin	NCL		2,03	1,30
Polyadenylate-binding protein 1	PABPC1		1,00	1,09
Polyadenylate-binding protein 4	PABPC4		1,03	0,96
60S ribosomal protein L10	RPL10		3,27	0,95
60S ribosomal protein L10a	RPL10A		1,89	1,28
60S ribosomal protein L11	RPL11		1,62	1,48
60S ribosomal protein L12	RPL12		2,41	1,00
60S ribosomal protein L13	RPL13		1,65	1,11
60S ribosomal protein L13a	RPL13A	+	1,91	1,22
60S ribosomal protein L14	RPL14		1,68	1,03
60S ribosomal protein L15	RPL15		2,49	1,68
60S ribosomal protein L17	RPL17		1,72	1,11
60S ribosomal protein L18	RPL18		1,66	1,39
60S ribosomal protein L18a	RPL18A		1,67	0,90
60S ribosomal protein L19	RPL19	+	2,08	1,20
60S ribosomal protein L21	RPL21		1,63	1,00
60S ribosomal protein L22	RPL22	+	2,82	1,03
60S ribosomal protein L23	RPL23	+	2,49	1,12
60S ribosomal protein L23a	RPL23A		1,69	1,07
60S ribosomal protein L24	RPL24		2,94	1,10
60S ribosomal protein L26	RPL26	+	2,19	1,04
60S ribosomal protein L27	RPL27		2,05	1,58
60S ribosomal protein L27a	RPL27A		1,40	1,11
60S ribosomal protein L28	RPL28	+	5,45	1,05
60S ribosomal protein L3	RPL3		1,79	1,32
60S ribosomal protein L30	RPL30		1,86	ND
60S ribosomal protein L31	RPL31	+	1,99	1,10
60S ribosomal protein L32	RPL32	+	5,69	0,95
60S ribosomal protein L34	RPL34	+	4,06	1,08
60S ribosomal protein L35	RPL35		1,75	1,26
60S ribosomal protein L35a	RPL35A	+	2,63	ND
60S ribosomal protein L36	RPL36		2,14	1,16
60S ribosomal protein L36a	RPL36A		2,74	ND
60S ribosomal protein L37a	RPL37A		2,97	0,96
60S ribosomal protein L4	RPL4	+	1,84	1,45
60S ribosomal protein L6	RPL6		1,64	1,15
60S ribosomal protein L7	RPL7	+	2,13	1,17
60S ribosomal protein L7a	RPL7A	+	2,26	1,05
60S ribosomal protein L8	RPL8	+	2,98	0,66

60S acidic ribosomal protein P0	RPLP0		2,10	1,48
60S acidic ribosomal protein P1	RPLP1		3,16	1,21
60S acidic ribosomal protein P2	RPLP2	+	2,16	2,34
40S ribosomal protein S11	RPS11		2,02	1,24
40S ribosomal protein S13	RPS13		1,72	1,22
40S ribosomal protein S14	RPS14		2,06	1,46
40S ribosomal protein S15a	RPS15A	+	2,00	1,07
40S ribosomal protein S16	RPS16		1,95	1,42
40S ribosomal protein S17	RPS17		1,91	ND
40S ribosomal protein S18	RPS18	+	2,72	1,19
40S ribosomal protein S19	RPS19		2,41	1,31
40S ribosomal protein S2	RPS2		1,50	1,45
40S ribosomal protein S20	RPS20		2,40	0,91
40S ribosomal protein S23	RPS23	+	1,84	1,07
40S ribosomal protein S24	RPS24		1,25	1,10
40S ribosomal protein S25	RPS25	+	1,66	1,22
40S ribosomal protein S26	RPS26	+	2,15	ND
40S ribosomal protein S28	RPS28	+	7,57	0,65
40S ribosomal protein S3	RPS3	+	2,04	1,43
40S ribosomal protein S3a	RPS3A	+	2,71	1,35
40S ribosomal protein S4, X isoform	RPS4X		1,57	1,32
40S ribosomal protein S6	RPS6	+	1,80	1,18
40S ribosomal protein S8	RPS8		1,51	1,40
40S ribosomal protein S9	RPS9		1,59	1,65
Ribosome-binding protein 1	RRBP1		1,43	1,12
Nuclease-sensitive element-binding protein 1	YBX1		1,37	1,22

Figures:

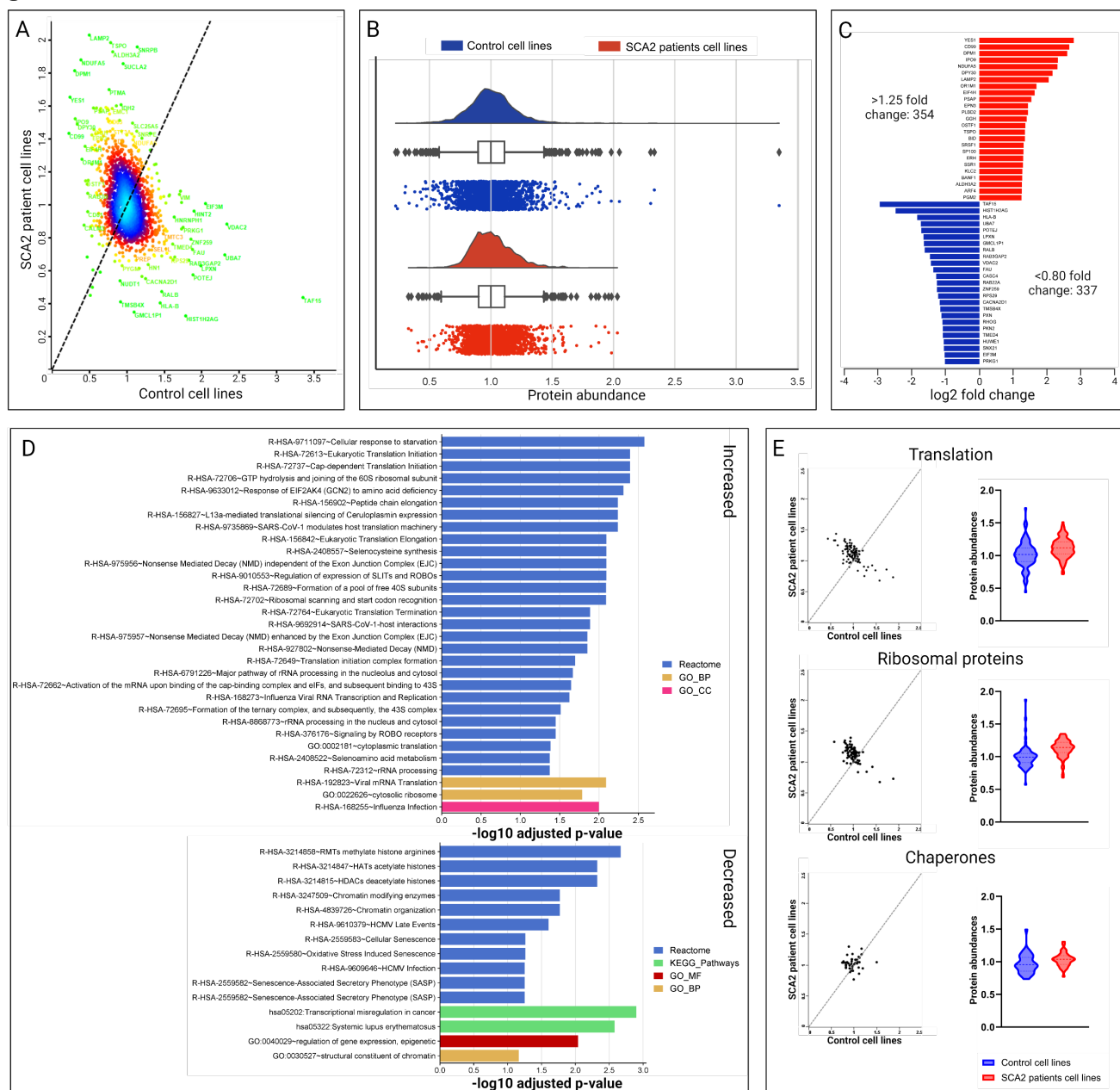


Figure 1 - Global Mass Spectrometry Analysis of SCA2 Patient Cell Lines and Controls

A: Density plot comparing the overall protein abundance distributions between control (x-axis) and SCA2 patient cell lines (y-axis). Differentially expressed proteins are highlighted and labeled. **B:** Raincloud plot for data distribution of protein abundances. SCA2 patient cell line data are shown in red, and control cell line data are in blue. **C:** Bidirectional bar plot showcases the top 25 proteins with increased expression and the bottom 25 proteins with decreased expression in SCA2 cells compared to controls. The x-axis indicates the log2 fold change, emphasizing the proteins with the most significant changes in expression. **D:** Top - Functional enrichment analysis of increased protein expression in SCA2 cells (>1.25-fold change) using DAVID for Biological Process, Cellular Compartments, and REACTOME pathways are presented in a bar graph. The x-axis indicates the -log10 of the adjusted p-values, identifying the most significantly enriched GO terms and REACTOME pathways. Bottom - Functional enrichment analysis of decreased protein expression in SCA2 cells (>0.8-fold change) using DAVID for REACTOME and KEGG pathways and Biological Process and Molecular Functions. The x-axis indicates the -log10 of the adjusted p-values, highlighting the pathways most significantly associated with ATXN2-interacting proteins in SCA2 cells. **E:** Scatter plots and violin plots display the relative protein abundances and distributions of proteins from distinct functional protein groups: translation (top), ribosomes (middle), chaperones (bottom) across control cell lines (scatterplot, x-axis) and SCA2 patient cell lines (scatterplot, y-axis). The color scheme distinguishes between SCA2 (red) and control (blue) samples.

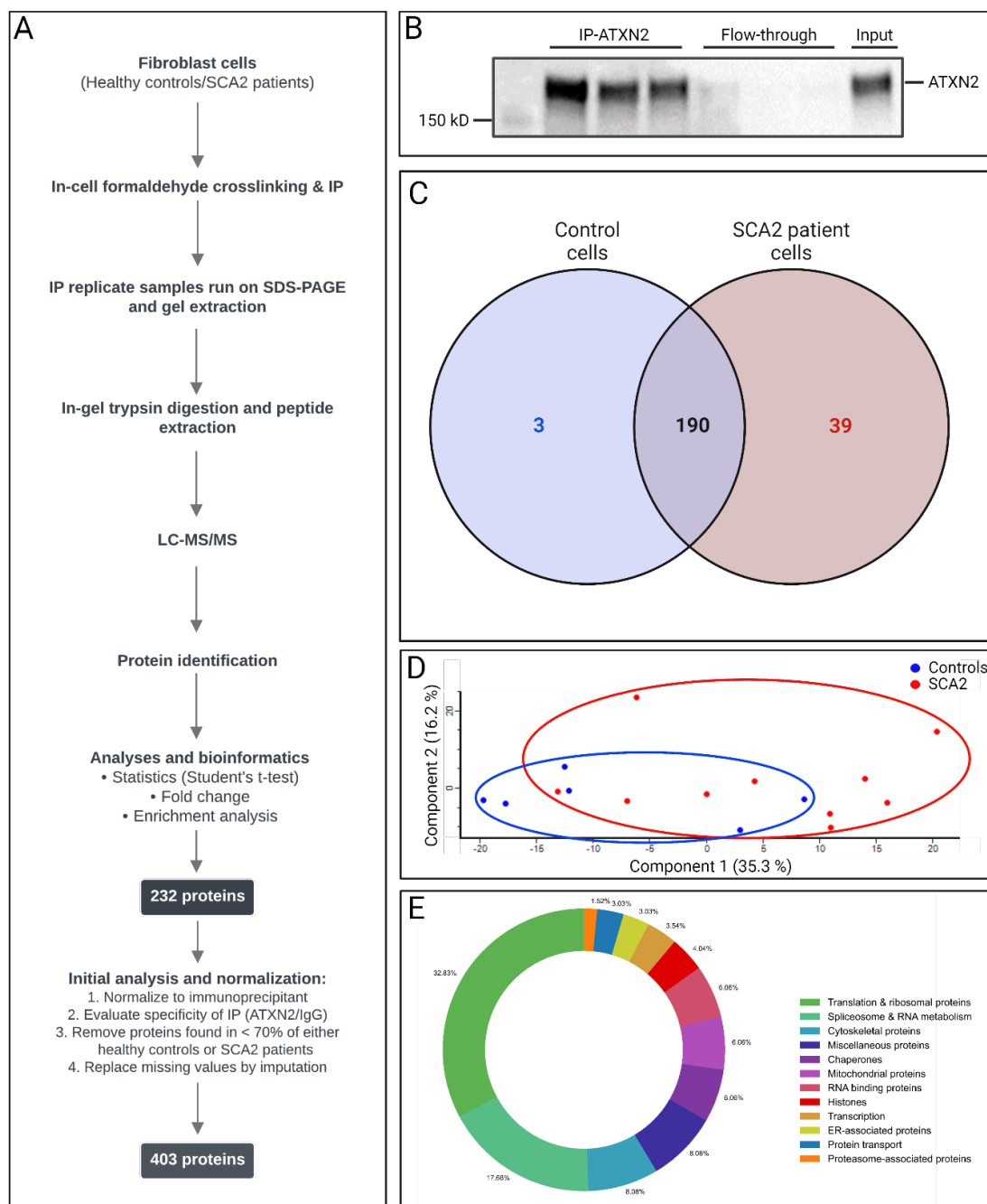
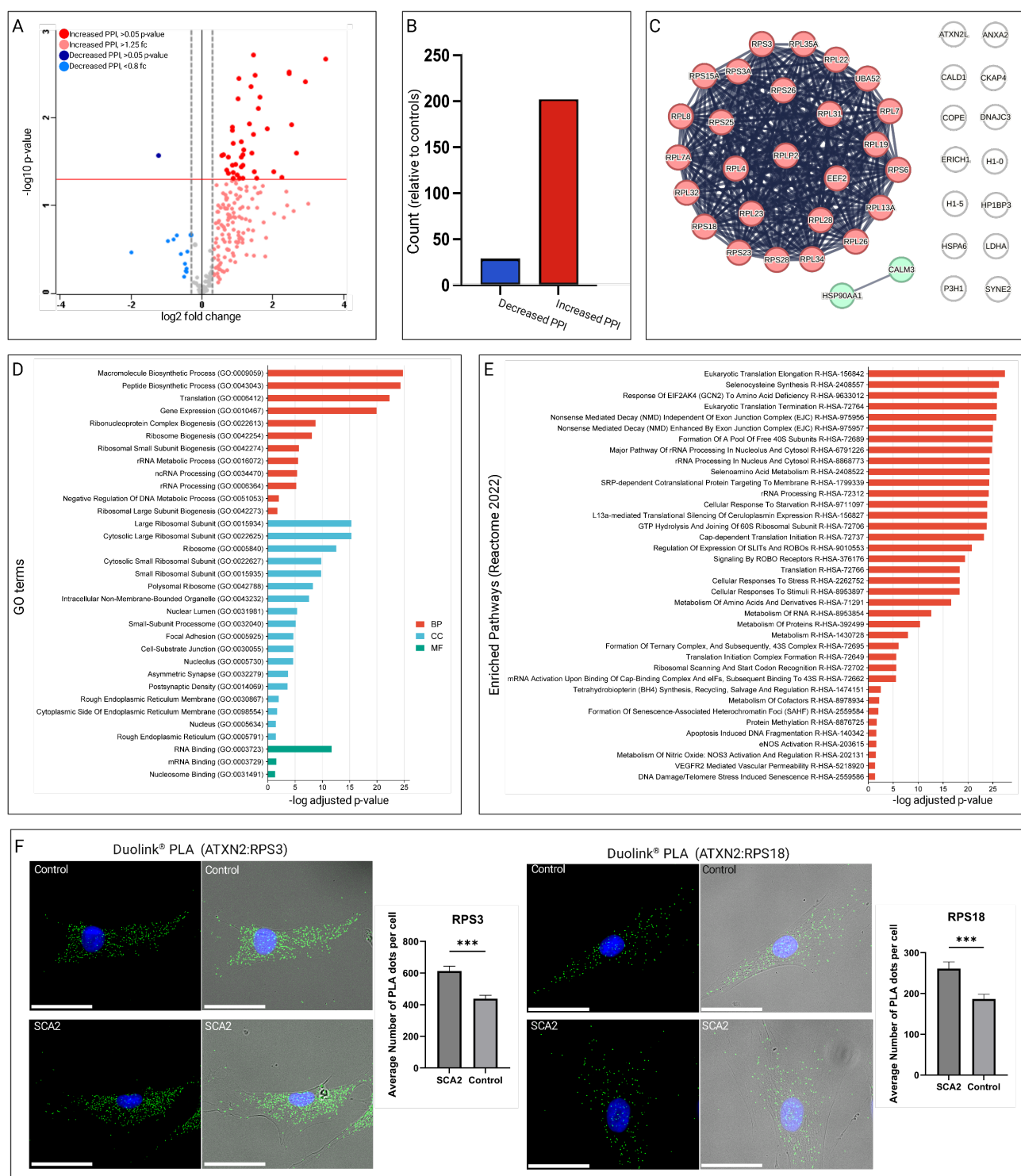


Figure 2 - Proteomic Profiling of ATXN2 Interactions in SCA2 and Control Cell Lines

A: Diagram of the IP-MS approach to identify the ATXN2 interacting proteins. **B:** Western blot image confirms the specificity of the ATXN2 immunoprecipitation, showing bands for the immunoprecipitated ATXN2 (triplicates), indicating the presence of ATXN2. The flow-through lanes show proteins not bound by the antibody, and the input lane confirms the presence of ATXN2 in the starting material. **C:** Venn diagram displaying the distribution of the 232 proteins identified from the IP-MS of ATXN2 in control cells and SCA2 patient cell lines. After filtration and analysis, 3 proteins were found specifically (in 70% of the samples within the group) in IP-MS from the control cell lines, while 39 proteins were specific for SCA2 patient cell lines. The remaining 190 proteins were found in both groups. **D:** The PCA plot visually represents the overall proteomic differences between SCA2 and control cell line samples based on the data from IP-MS. The plot shows the clustering of samples based on either component 1 (x-axis) or component 2 (y-axis). **E:** Pie chart illustrating the categorization of proteins identified from IP-MS assay of ATXN2 from SCA2 patient cell lines and control cell lines based on their primary biological functions. Percentages are stated for each category.



patient cells. Interaction events are shown as green dots (representative image, scale bar: 60 μ m). Quantification of interaction events are shown by bar plots. *** $P < 0.0005$ vs. control; unpaired Student's t-test; RPS3 (SCA2: $n = 82$, Control: 46), RPS18 (SCA2: $n = 84$, Control: 70).

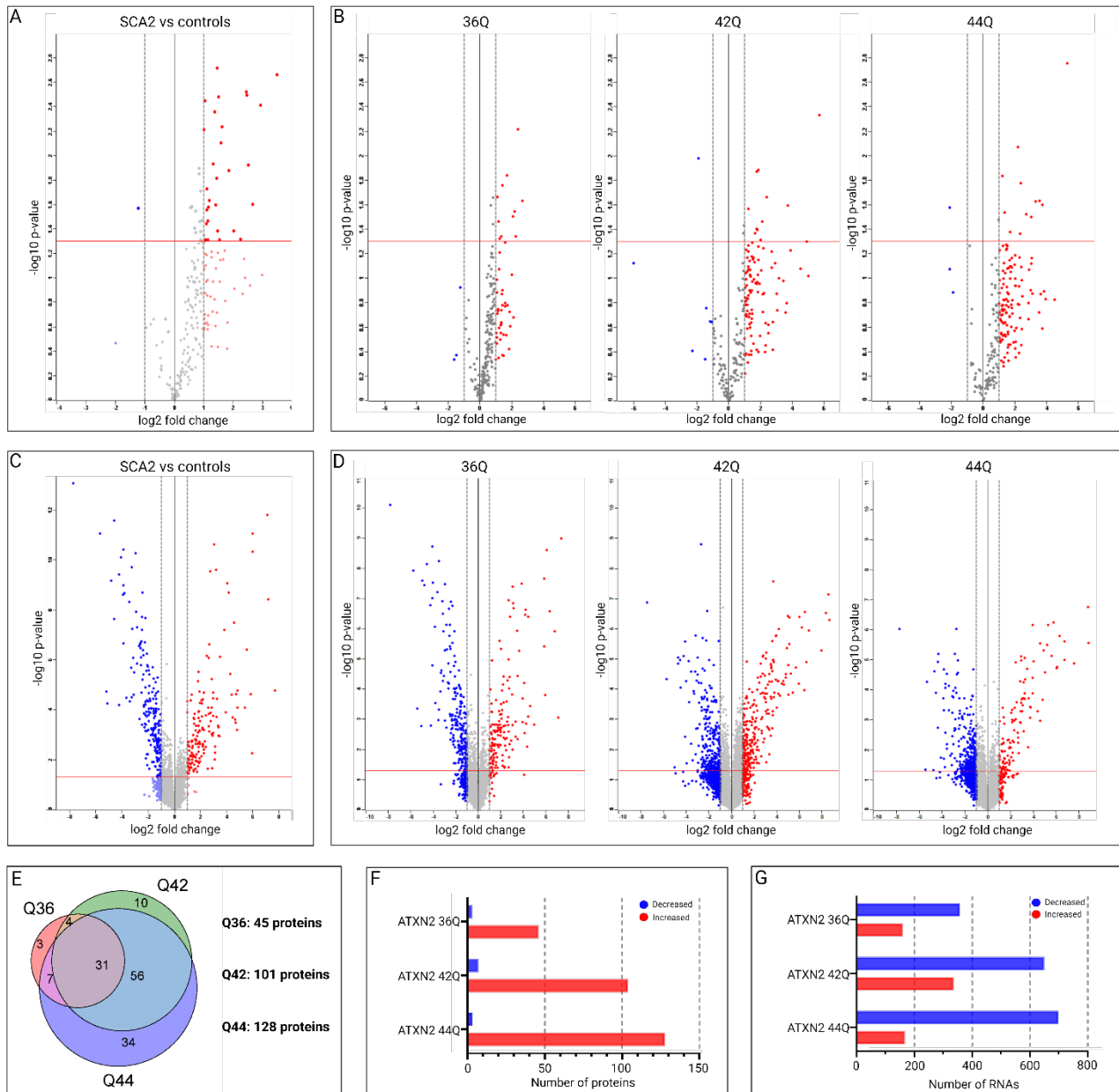


Figure 4 - PolyQ-length-dependent alterations in ATXN2 PPIs and RNA-protein binding

A: Volcano plot of the proteomic data obtained from IP-MS of ATXN2 in SCA2 cells versus control cell lines. The vertical dashed lines indicate the 2-fold threshold and proteins beyond this threshold are depicted in blue (decrease) or red (increase). Proteins with significant changes in ATXN2 association are highlighted above the red line (bright blue: significant decrease, bright red: significant increase). **B:** Proteomics IP-MS data divided into groups of different CAG repeats: 36 CAG repeats (36Q), 42 CAG repeats (42Q), and 44 CAG repeats (44Q). Each point represents a protein, with the x-axis displaying the log₂ fold change in protein levels and the y-axis showing the -log₁₀ p-value. **C:** Volcano plot visualizing the statistical significance of RNAs associated with ATXN2. The y-axis represents -log₁₀ p-values, and on the x-axis is the log₂ fold change. The vertical dashed lines indicate the 2-fold threshold, and RNAs beyond this threshold are depicted in blue (decrease) or red (increase). RNAs with significant changes in ATXN2 association are highlighted above the red line (bright blue: significant decrease, bright red: significant increase). **D:** IP-RNAseq data divided into groups of different CAG repeats: 36 CAG repeats (36Q), 42 CAG repeats (42Q), and 44 CAG repeats (44Q). Each point represents a specific identified RNA, with the x-axis displaying the log₂ fold change in protein levels and the y-axis showing the -log₁₀ p-value. **E:** Principal component analysis (PCA) plot demonstrating the separation between SCA2 (red) and control (blue) cell lines based on the RNA profiles associated with ATXN2. The PCA plot was calculated using Perseus software. **F:** Horizontal bar plot illustrating the number of proteins within the 2-fold threshold in the different CAG repeat groups. The blue bars represent proteins/RNAs in the ≥ 2 -fold decrease fraction, while the red bars represent proteins/RNAs in the ≥ 2 -fold increase fraction. Statistical assessment of the expression data was done using Perseus software.

APPENDIX B:

Manuscript II (Wardman *et al.* 2020)



Enhancement of Autophagy and Solubilization of Ataxin-2 Alleviate Apoptosis in Spinocerebellar Ataxia Type 2 Patient Cells

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Abstract

Spinocerebellar ataxia type 2 (SCA2), a rare polyglutamine neurodegenerative disorder caused by a CAG repeat expansion in the ataxin-2 gene, exhibits common cellular phenotypes with other neurodegenerative disorders, including oxidative stress and mitochondrial dysfunction. Here, we show that SCA2 patient cells exhibit higher levels of caspase-8- and caspase-9-mediated apoptotic activation than control cells, cellular phenotypes that we find to be exacerbated by reactive oxygen species (ROS) and inhibition of autophagy. We also suggest that oligomerization of mutant ataxin-2 protein is likely to be the cause of the observed cellular phenotypes by causing inhibition of autophagy and by inducing ROS generation. Finally, we show that removal of ataxin-2 oligomers, either by increasing autophagic clearance or by oligomer dissolution, appears to alleviate the cellular phenotypes. Our results suggest that oligomerized ataxin-2 and oxidative stress affect autophagic clearance in SCA2 cells, contributing to the pathophysiology, and that activation of autophagy or clearance of oligomers may prove to be effective therapeutic strategies.

Keywords Autophagy . Ataxia . Polyglutaminatedisease . Neurodegenerativedisease

Introduction

Many of the most common neurodegenerative diseases are characterized by aggregation or accumulation of cytosolic or extracellular proteins, accumulation that is often caused by mutations in the native protein [1, 2]. This aspect of pathophysiology links the various neurodegenerative diseases despite diverse and sometimes poorly characterized native function of the aggregated proteins in each disease. Among the neurodegenerative diseases exist a symptomatically diverse group of syndromes, including Huntington's disease and the spinocerebellar ataxias, which manifest polyglutamine expansions in specific proteins [3]. The different symptoms of each of these diseases are dictated by the brain areas and the neuronal subtypes most affected by the respective mutated proteins. These proteins, of diverse and sometimes unknown

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function, are nonetheless pathophysiologically linked by the dysfunctions caused by their polyglutamine expansions. This common polyglutamine motif contributes not only to disruption of the native protein function but also confers new aggregative properties regardless of the protein's original role [4]. Non-functioning or damaged proteins are typically cleared by cellular chaperones and proteases, especially the ubiquitin-proteasome system (UPS), whereas accumulated, aggregated, or oligomerized proteins are cleared by autophagy [5, 6]. Together, these pathways comprise the cellular proteostatic system. However, when clearance is inefficient, either due to the pathology of the disease or due to excess load on the system, the accumulated protein can form larger structures that bind essential cytosolic components and these, in turn, impair proper cellular function, both by interrupting the normal function of sequestered proteins and by impairing the proteostatic system itself [7, 8]. This results in accumulation of protein waste, which can impair cellular function and cause apoptosis [9]. Disrupted autophagy is a common feature of the pathophysiology of many neurodegenerative diseases, in which mechanisms of autophagic dysfunction are diverse [10]. This dysfunction, which affects not only protein accumulation, also impacts other autophagic roles, including the

Cerebellum

removal of damaged organelles [11]. This could lead to accumulation of old and damaged organelles, especially mitochondria, a phenotype displayed in several neurodegenerative diseases [12–14]. As a major source of reactive oxygen species (ROS) in the cell, the mitochondria maintain appropriate ROS levels for cell signaling, but overproduction can damage cellular function, and dysfunctional mitochondria can trigger cell death via a specific apoptotic pathway (caspase-9) [15–17].

Previous studies in our laboratory have shown that spinocerebellar ataxia type 2 (SCA2) fibroblasts display many of the abovementioned mitochondrial phenotypes [13], and other studies have shown that SCA2 shares some of the dysfunctions common among the well-characterized neurodegenerative diseases, including protein aggregation [3, 13, 18, 19].

SCA2 is a rare, progressive, late-onset neurodegenerative disease, characterized clinically by cerebellar ataxia, lower limb weakness, decreased muscle tone and vibration sense, postural tremor, dystonia, nystagmus, slow saccades, and cognitive decline [20]. Late-stage SCA2 is characterized by widespread neuronal loss in the thalamus, brainstem, and cerebellum [2]. Cerebellar Purkinje cells and several pontine, mesencephalic, and thalamic neurons are particularly affected by progressive neurodegeneration [21]. SCA2 is genetically characterized by a meiotically unstable expansion of a trinucleotide repeat (CAG)

in exon 1 of the ataxin-2 gene (*ATXN2*) on chromosome 12q24.12, which codes for ataxin-2. Ataxin-2 appears to have roles in regulating many cellular processes, including stress granule formation, starvation and stress response, translation, RNA processing, metabolism, mitochondrial function, and calcium signaling [18, 22–31], and like in several other CAG repeat disorders, the pathology is believed to be caused at least in part by a toxic gain of function, giving the mutant protein new

deleterious functions in the cell [32]. The CAG repeat expansion results in an elongated polyglutamine domain in the gene product, ataxin-2, which can lead to protein aggregation and to the sequestration of other proteins, a common feature of polyglutamine diseases [33, 34]. Late stages of SCA2 are neuropathologically characterized by intracellular protein aggregates comprised of expanded ataxin-2 with many other associated proteins, including components of the ubiquitin-proteasome system among others [21]. Consequently, loss of function is also believed to contribute to the disease-causing mechanism either by disruption of ataxin-2's native function or by sequestration of proteins unable to exert their normal function. This is supported by studies showing that knockout

of ataxin-2 in mice causes multiple disrupted cellular pathways,

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including branched-chain amino acid pathways, effects on lipid metabolism, circadian behaviors, and protein translation [22, 30, 35, 36]. Considering that diverse proteins containing polyglutamine expansions in several neurodegenerative diseases display similar properties, and can lead to common pathologies, including autophagic and mitochondrial dysfunction, it is important to investigate what role these processes play in

SCA2. If disruption of these processes is a common feature of this diverse set of diseases, then targeting of these pathways could prove therapeutically valuable in the treatment of neurodegeneration in general. Our previous investigations in SCA2 patient cells revealed mitochondrial dysfunction and oxidative stress similar to that observed in other neurodegenerative diseases [13]. These experiments, as well as multiple other studies, also display the relevance and utility of using a fibroblast-based cellular model derived from a genetically diverse patient population (differing in age, age-of-onset, CAG repeat length, and Scale for Assessment and Rating of Ataxia (SARA) score) to study the mechanistic aspects of SCA2 or other neurodegenerative diseases [19, 37, 38]. In this study, we investigated the redox state changes and the apoptotic activation elicited by various compounds modulating autophagy, proteasome function, and ataxin-2 oligomerization, as well as the effect of these compounds on ataxin-2 protein and autophagic activation. We find that ataxin-2 is increased in the insoluble protein fraction of SCA2 cells and that re-solubilization of ataxin-2 leads to reduced caspase activation in these cells. We also find that the SCA2 cells are particularly susceptible to autophagic inhibition, leading to increased oxidative stress and apoptotic activation in these cells upon chloroquine treatment, a phenotype which can be alleviated by preventing oligomerization of ataxin-2. Finally, we find that enhancing autophagy via multiple pathways leads to alleviation of apoptotic activation in SCA2 cells.

Materials and Methods

Subjects and Samples

Patients were recruited through the Neurogenetics Clinic, Danish Dementia Research Centre, Rigshospitalet, and written informed consent was obtained from all participating patients and control individuals. Skin biopsies were obtained from the forearm of five patients and three age-matched controls of Danish origin and representative of the general Danish population. The biopsies were cut into small pieces and left untouched for 10 days in DMEM 1885 supplemented with 10% fetal bovine serum (FBS), 1% GlutaMAX (Gibco, Life Technologies), and 1% penicillin/streptomycin (BioSera, In Vitro) at 37 °C, 5% CO₂, and 95% relative humidity. Subsequently, media was changed every 2–3 days and the fibroblasts were expanded for further experiments. Clinical assessment: A complete neurological examination was conducted and the clinical diagnosis of SCA2 was based on positive family history and disease manifestation such as ataxic gait, dysarthria, dysmetria, dysdiadochokinesia, and slow saccades. The age of onset was defined as the onset of motor impairment as stated by the patient, and disease severity was assessed according to the SARA [39].

CAG Repeat Size Determination

Genomic DNA was isolated from peripheral blood leukocytes or cultured fibroblasts, and the *ATXN2* CAG repeat length was assessed as previously described [40]. The age of the patients ranged from 24 to 68 years, and the CAG repeat length ranged from 36 to 44 CAG repeats. Specific descriptions of patients and their respective CAG repeat size has previously been published [13].

Cell Culture

The fibroblasts were cultured in DMEM 1885 medium, supplemented with 1% GlutaMAX, 10% FBS (in vitro), and 1% penicillin/streptomycin at 37 °C, 5% CO₂ 95% relative humidity. The fibroblasts were plated in 6-well plates and allowed to grow for a minimum of 2 days before being treated with either various compounds or their appropriate vehicles. Single-compound treatments consisted of overnight (24 h) treatment of the cells with either MG-132 (50 µM), chloroquine (50 µM), or hydrogen peroxide (H₂O₂) (200 µM), dissolved in serum-free media, using an appropriate vehicle (DMSO or H₂O), while an equal number of wells were treated with the appropriate vehicle only. Experiments were also carried out using other cytotoxic compounds, including tigecycline (100 µM), etoposide (100 µM), and cycloheximide (100 µg/ml) and vehicles, showing no differential effects in SCA2 cells. The combined Congo red/chloroquine treatments consisted of either 24 h of Congo red treatment (25 µM), 24 h of chloroquine treatment (50 µM), or 24 h of Congo red treatment (25 µM), followed by 24 h of chloroquine treatment (50 µM). The combined rapamycin/H₂O₂ treatments consisted of either 24 h of rapamycin treatment (0.2 mg/ml), 24 h of H₂O₂ treatment (200 µM), or 24 h of rapamycin treatment (0.2 mg/ml), followed by 24 h of H₂O₂ treatment (200 µM). The combined trehalose/H₂O₂ treatments consisted of either 24 h of trehalose treatment (100 mM), 24 h of H₂O₂ treatment (200 µM), or 24 h of trehalose treatment (100 mM), followed by 24 h of H₂O₂ treatment (200 µM). Concentrations used for all drugs were determined experimentally using dose-response curve experiments in control and patient fibroblasts.

Immunocytochemistry

Control and SCA2 fibroblasts were first plated on polylysine-coated coverslips in 12-well dishes, allowed to grow to low confluence, then washed with PBS and fixed in 4% paraformaldehyde in PBS. The cells were then rinsed twice with PBS and permeabilized with 0.1% Triton X-100 detergent in PBS. Cells were then blocked using 4% bovine serum albumin (BSA) in PBS for 1 h. Primary antibody to ataxin-2 (1:500, BD Transduction Laboratories, 611,378, monoclonal, host:

mouse) was then incubated with cells in 1% BSA for 1 h. Cells were rinsed with PBS and incubated for 1 h with anti-mouse CF488A-conjugated secondary antibodies (Sigma-Aldrich). The cells were then rinsed thoroughly with PBS and affixed to microscope slides using Prolong Gold reagent anti-fade reagent containing 4,6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA, USA). Microscopic evaluation of the cells was conducted on an Olympus IX73 microscope using the × 40 objective.

Vitality Assay

Cell vitality was measured via image cytometry by analysis of reduced glutathione using Vitabright-48 (VB-48) reagent (Chemometec) according to the manufacturer's instructions. Briefly, the cells were washed with PBS, trypsinised and resuspended in PBS containing 1× (manufacturer's dilution) VB-48-propidium iodide-acridine orange solution. The stained cells were measured for fluorescence using a NucleoCounter NC-3000 image cytometer (Chemometec), and subsequent analyses of the proportion of healthy and low-vitality cells were performed using NucleoView 2.1 software (Chemometec). The acridine orange staining was used as masking channel to define the cells to include into the analyses, and the low-vitality cells were defined as cells displaying above-threshold VB-48 intensity levels compared with the healthy control cell populations, while above-threshold propidium iodide staining was used to exclude the dead (necrotic) cells. The experiments were conducted in biological triplicates, and the results were replicated in independent experiments for confirmation.

Caspase Activation Assays

Activation of either caspase-8- or caspase-9-mediated apoptosis was measured using the FAM-FLICA caspase assay kit (Immunochemistry) according to the manufacturer's instructions. Briefly, the cells were washed, trypsinised and resuspended in PBS, and incubated at 37 °C for 60 min in PBS containing 1× (manufacturers dilution) FAM-LETD-FMK (caspase-8-targeted FLICA reagent) or FAM-LEHD-FMK (caspase-9-targeted FLICA reagent) and Hoechst 33342 (10 µg/ml). Subsequently, the cells were washed twice with apoptosis wash buffer (immunochemistry) and re-suspended in apoptosis wash buffer containing propidium iodide (10 µg/ml). The measurements of cellular fluorescence were performed on a NucleoCounter NC-3000 image cytometer (Chemometec), and the subsequent analyses of proportion of healthy and apoptotic cells were performed using NucleoView 2.1 software (Chemometec). The Hoechst staining was used as masking channel to define which cells to include into the analyses, and the apoptotic cells were defined as cells displaying above-threshold caspase-FAM intensity levels

compared with healthy control cell populations, while above-threshold propidium iodide staining was used to exclude the dead (necrotic) cells. The experiments were conducted in biological triplicates, and the results were replicated in separate experiments for confirmation.

Cell Collection

When the cells were collected for RNA or protein extraction, culture medium was poured off and thereafter the cells were rinsed in PBS. PBS was added, and cells were scraped off the surface of the flasks using a plastic cell scraper. The content of the flask was transferred with a pipette to an Eppendorf tube and spun down for 5 min at 1700×g. The supernatant was carefully removed, ensuring not to disturb the pellet. The cell samples were then stored at − 80 °C.

Protein Extraction from Cell Cultures

The cell samples stored at − 80 °C were thawed on ice and CellLytic M (Sigma-Aldrich), including 2% protease inhibitor cocktail, was added to the samples and incubated on ice for 15 min. After incubation, the cell samples were centrifuged at 13,000×g for 30 min at 4 °C. The supernatant was removed and the protein was quantified using Bradford reagent and a Multiskan FC (Thermo Scientific) as described [13].

SDS-PAGE and Western Blot Analysis

SDS-PAGE and western blot was used to quantify the levels of various proteins in samples from control and SCA2 fibroblasts. Briefly, 7 µg protein was prepared with sample buffer mix containing sample buffer and reducing agent (Invitrogen), then heated at 95 °C for 7 min, followed by brief centrifugation. Protein samples were loaded into NuPAGE polyacrylamide gels or Criterion TGX (BioRad) gels, using appropriate running buffer (Invitrogen/BioRad), and run at 150 V for roughly 90 min on either an Xcell SureLock (Novex) electrophoresis system or a Criterion cell system (BioRad). The proteins were transferred to a nitrocellulose membrane using either the Xcell SureLock or the BioRad system at appropriate voltage.

After the transfer, the membrane was blocked in TBST containing 5% non-fat dry milk (NFDM) at room temperature for an hour while shaking. The membranes were then incubated at 4 °C overnight on a shaker with primary antibodies prepared in 5% NFDM against ataxin-2 (1:1000, BD Transduction Laboratories, 611,378, monoclonal, host: mouse), GAPDH (1:3000, Abcam ab9485, host: rabbit), p62 (1:1000 Abcam, ab56416, monoclonal, host: mouse), and LC3 (1:1000 Novus Biologicals, NB600-1384, polyclonal, host: rabbit). The following day, the membranes were washed three times with TBST. Afterwards, a horseradish-peroxidase-

coupled secondary antibody solution (Agilent/Dako) prepared in 5% NFDM in TBST was added to the membrane and incubated on a shaker for an hour at room temperature, and then washed in TBST. The blots were visualized using Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare Life Sciences) using a chemiluminescent imaging system from Syngene. The quantification of the protein bands was carried out by measuring the optical density of the emission from the bands using Genetools software (Syngene). The proteins were quantified by normalizing to respective loading control bands, and the individual sample results were normalized to their respective control sample depending on the experimental setup.

SDS/RIPA Extractions

To prepare RIPA fraction, proteins for SDS-polyacrylamide gel electrophoresis (PAGE), immunoblotting, and quantitative densitometry were extracted from primary skin fibroblasts in RIPA buffer ((Sigma Aldrich) with 1 mM EDTA; 1 mM EGTA; 1 mM PMSF and Complete Protease Inhibitor Cocktail (Roche) added) and incubated on ice for 15 min. The lysates were centrifuged at 16,000×g for 20 min at 4 °C, and supernatant was separated and kept on ice until further processing. To prepare the SDS fraction: The remaining pellet was dissolved in 2× SDS buffer (137 mM Tris-HCl (pH 6.8); 4% SDS; 20% glycerol; Complete Protease Inhibitor Cocktail (Roche)) by sonification. After 10 min of centrifugation at 16,000×g, the pellet was removed, and the supernatant represented the SDS fraction. For SDS-PAGE, the samples were processed as above, using the Xcell Surelock system (dual extraction protocol adapted from Halbach et al. [18]). The membranes were blocked with 5% NFDM in TBST and incubated with primary antibodies in 5% NFDM in TBST against ataxin-2 (1:400, BD Transduction Laboratories, 611,378, monoclonal, host: mouse), GAPDH (1:3000 Abcam ab9485, host: rabbit) or β-ACTIN (1:10,000, Abcam, ab6276, monoclonal, host: mouse). After normalization of ataxin-2 protein values versus either GAPDH (RIPA fraction) or β-ACTIN (SDS fraction), changes in protein expression were calculated and statistics were evaluated and plotted using GraphPad Prism software.

Statistical Analysis

The comparison of the effects of the individual drug treatments on control and patient cells (caspase activation, change in caspase activation elicited by drug treatment, and change in redox state upon drug treatment) and the comparison of ataxin-2 levels in the dual-extraction experiments (all experiments in which only two data sets were compared with one another) were analyzed using a Student's *t* test. The experiments in which more than two data sets were compared,

including the caspase activation effect with multiple drug treatments, and the protein levels after drug treatment, were analyzed using ANOVA with multiple comparisons and corrected with Dunnett's multiple comparison test to distinguish changes relative to both, the control and patient vehicle-treated groups. All statistical analysis was performed using GraphPad Prism software.

Results

Activation of Caspases in SCA2 Cells

Apoptosis is an important route to cell death in neurodegenerative disease [17], and it has been shown previously to be activated in SCA2 pathology [41], for example, in response to cellular stress [42]. However, the previous work describing ataxin-2's involvement in apoptosis has reported activation of the effector caspase, caspase-3, in immortalized cell lines exogenously expressing expanded ataxin-2. Additionally, studies have shown that SCA2 cells displayed higher levels of oxidative stress and dysfunctional mitochondria, which can also cause activation of specific apoptotic pathways [13, 16, 42–44]. To explore apoptotic activation in our cell model, we measured activation of the initiator caspases of the mitochondrially mediated (caspase-9) and the extrinsic (caspase-8) apoptotic pathways. We found that untreated SCA2 cells showed significantly enhanced activation of both, caspase-9 and caspase-8, relative to control cells (Fig. 1a, b). In addition to aberrant calcium signaling and mitochondrial dysregulation, this increased basal activation of apoptotic pathways could also be due to possible proteostatic dysfunction [45].

To assess the roles of the various arms of the proteostatic pathway, as well as the involvement of ataxin-2 in these pathways, the cells were treated with various drugs acting upon the autophagic machinery, proteasome, aggregated or oligomerized polyglutamine, and mitochondrial function, and these treatments led to differential effects in the SCA2 cells relative to control cells (discussed in detail below); whereas general cytotoxic compounds like tigecycline, cycloheximide, and etoposide, did not cause significantly different effects between control and SCA2 cells, indicating that SCA2 cells are not generally more vulnerable to cytotoxic insult (results not shown).

Effects of Proteasomal Inhibition on SCA2 Cells

The polyglutamine expansion in ataxin-2 may cause the protein to misfold, to aggregate or oligomerize, and may also disrupt the primary function of the protein. Misfolded and dysfunctional proteins can be eliminated by either of the main proteostatic pathways of the cell,

autophagy or the ubiquitin-proteasome system (UPS). To investigate the role of the UPS in SCA2 fibroblasts, we treated cells with the proteasome inhibitor MG-132. These treatments did not cause significantly differential increases in caspase-9-mediated apoptosis in SCA2 cells (Fig. 1c), indicating that proteasome inhibition does not specifically contribute to mitochondrially mediated apoptosis in SCA2 cells. Proteasomal inhibition, however, caused a significantly higher degree of caspase-8 activation in SCA2 cells relative to control cells (Fig. 1d). Previous studies have shown that proteasomal inhibition is able to activate caspase-8-mediated apoptosis via an autophagy-related mechanism, so our results showing an increase in caspase-8 activation, combined with an increase in autophagic activation, might be expected in this case [45, 46]. Although MG-132 did not cause activation of mitochondrially mediated apoptotic pathway, the treatment was paralleled by increased oxidative stress in SCA2 cells, as indicated by a lower number of "healthy" (based on measurements of reduced-glutathione) SCA2 cells upon treatment (Fig. 1e). A recent study suggests that polymorphisms in glutathione-S-transferase might be associated with age of onset in SCA2, and, therefore, we cannot entirely exclude the possibility that polymorphisms in glutathione-S-transferase might influence the measurements of healthy cells, although such association was not established in the study [47].

To investigate the pathway by which ataxin-2 is degraded, we investigated the change in ataxin-2 levels upon proteasome inhibition using western blot. There appeared to be no significant change in the level of ataxin-2 protein in either control or SCA2 fibroblasts upon treatment with MG-132, indicating that the proteasome is not the major pathway of ataxin-2 elimination in either control or SCA2 cells (Fig. 1f, g). Considering that the proteasome does not seem to have a crucial role in eliminating ataxin-2, we assessed autophagy activation in these cells via two protein markers, p62 and LC3-II. p62 is an autophagosome cargo marker protein that targets and directs its binding partners for autophagy [48], while LC3-II acts as an integral part of the autophagic machinery, helping to select the autophagic cargo and to form autophagosomes [46]. There was no change in p62 protein levels with proteasomal inhibition (Fig. 1f, h). In contrast, we observed a significant increase in LC3-II levels (and presumably autophagic activity) in both control and SCA2 fibroblasts, likely a compensatory effect for the inhibition of another arm of the proteostatic system (Fig. 1f, i). Thus, proteasomal disruption does not appear to have specific effects on either accumulation or elimination of ataxin-2, although it does lead to general oxidative stress and autophagic activation, perhaps as secondary effects.

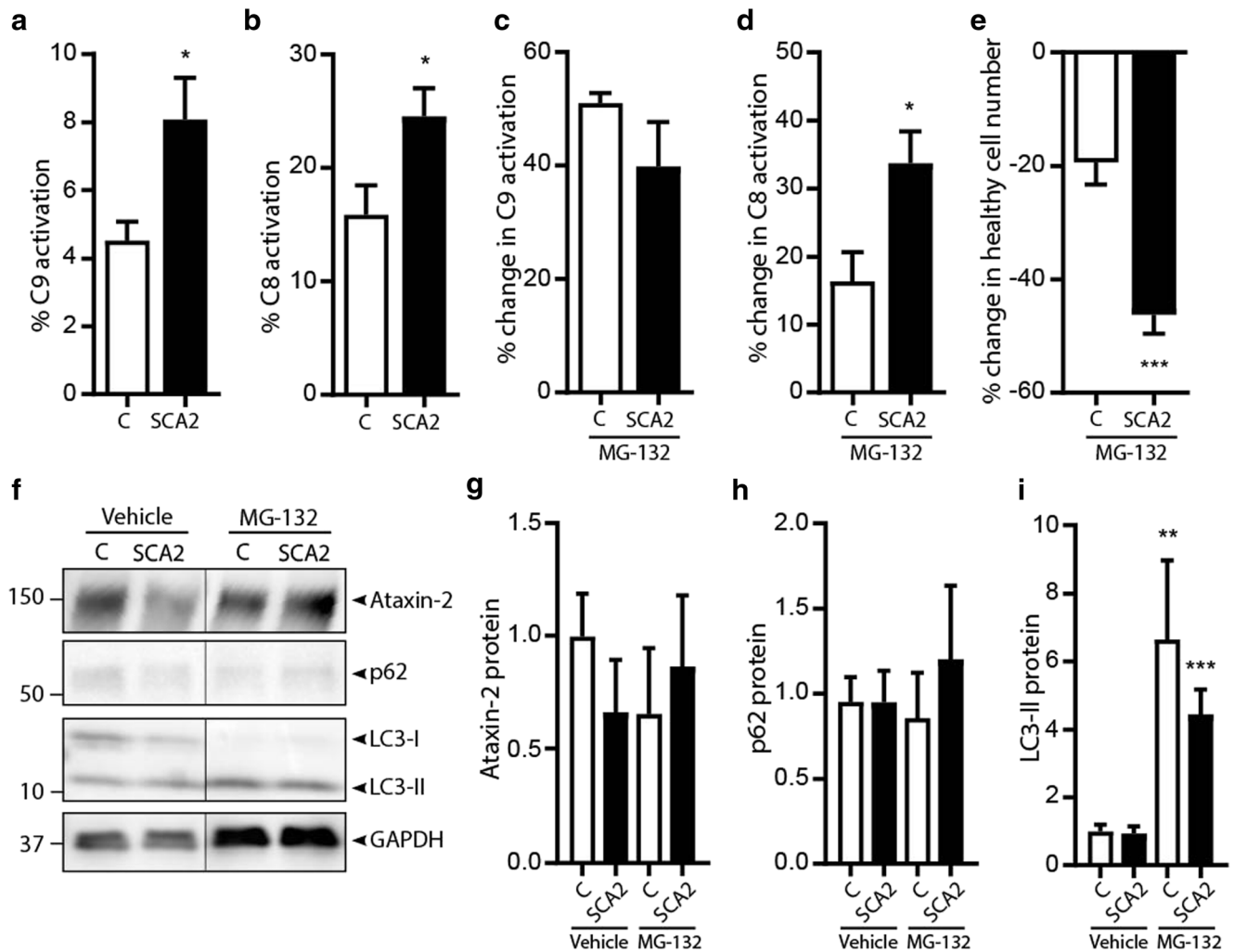


Fig. 1 Proteasome inhibition by MG-132: **a** Untreated SCA2 patient fibroblasts showed higher levels of caspase-9-mediated (4% increase, $p = 0.0410$) and **b** caspase-8-mediated (9% increase, $p = 0.0276$) apoptotic activation relative to controls. **c** Caspase-9-mediated apoptosis was increased in control and SCA2 fibroblasts to a similar degree when treated with proteasome inhibitor MG-132. **d** Caspase-8-mediated apoptosis is significantly increased in SCA2 cells relative to control cells with MG-132 treatment (17.33% increase, $p = 0.0208$). **e** SCA2 patient fibroblasts showed greater decrease in oxidatively healthy cells relative to controls when treated with MG-132 (23% greater decrease, $p < 0.0001$)

in a vitality assay, measuring cellular reduced glutathione. **f** Representative western blot images of ataxin-2, p62, LC3-II, and GAPDH levels in vehicle and MG-132-treated fibroblasts. **g–h** MG-132 treatment did not cause significant changes in the levels of either ataxin-2 or p62 protein in control or SCA2 fibroblasts. **i** MG-132 caused significant increase in LC3-II protein in both control and SCA2 cells compared with untreated counterparts (control 5.63-fold increase, $p = 0.0001$, SCA2 3.44-fold increase, $p = 0.0022$). Graphs represent mean and SEM from at least two independent replicate experiments, using three control and five SCA2 patient cell lines

Effects of Autophagic Inhibition on SCA2 Cells

Our results indicate that the UPS does not appear to have a significant role in the elimination of extended ataxin-2, a finding that is supported by other studies examining this phenomenon in SCA2 [19]. Therefore, it seems likely that autophagy, the other major arm of the proteostatic system, is playing an important role in elimination of the protein. To explore the functionality of this pathway in SCA2 fibroblasts, we first treated the cells with the autophagy inhibitor chloroquine, which prevents the acidification of autophagosomes once they have engulfed cargo. This, in turn, prevents cargo elimination and causes accumulation of autophagosomes, preventing

autophagic function [49]. Chloroquine treatment caused activation of caspase-9-mediated apoptosis in a significantly higher proportion of the SCA2 fibroblasts than in controls, indicating that the SCA2 cells are more susceptible to interruption of this pathway (Fig. 2a). Conversely, caspase-8-mediated apoptotic activation was not increased by chloroquine treatment in the SCA2 cells compared with controls (Fig. 2b). Chloroquine treatment also increased oxidative stress significantly more in SCA2 cells than in controls, as shown by measuring cytosolic reduced glutathione (indicated in Fig. 2c by a significantly greater reduction in “healthy” cells). This result, combined with the observed increase in mitochondrially mediated apoptosis, could indicate that

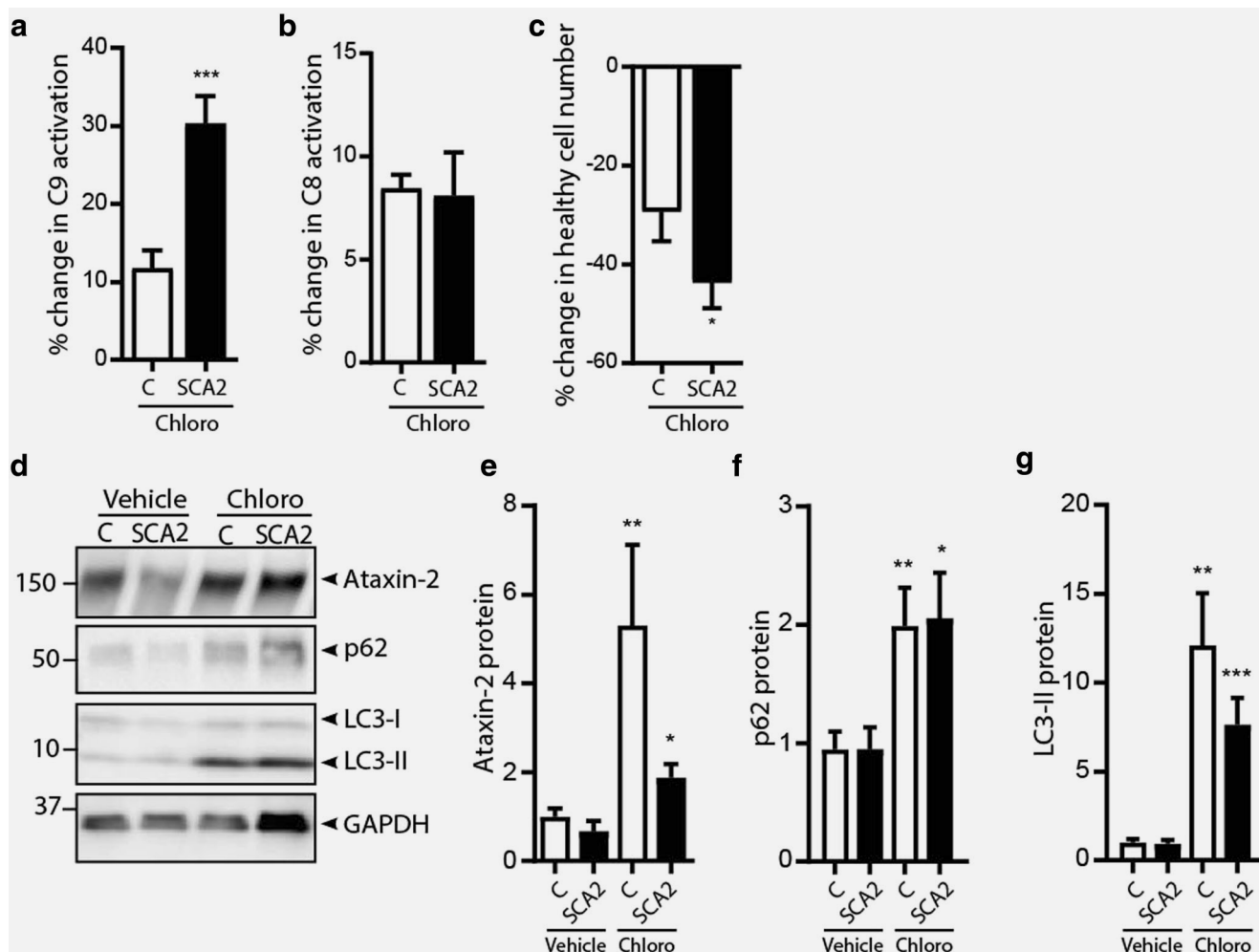


Fig. 2 Autophagy inhibition with chloroquine: **a** Caspase-9-mediated apoptosis (mitochondrial pathway) was increased significantly more in SCA2 fibroblasts when treated with the autophagy inhibitor chloroquine (19% greater increase, $p = 0.0006$) relative to control cells. **b** Caspase-8-mediated apoptosis (extrinsic pathway) was also increased but not to a significant degree. **c** SCA2 patient fibroblasts also showed greater decrease in oxidatively healthy cells relative to controls when treated with chloroquine (14% greater decrease, $p = 0.0789$). **d** Representative western blot images of ataxin-2, p62, LC3-II, and GAPDH levels in vehicle

and chloroquine-treated fibroblasts. **e** Chloroquine treatment caused a significant increase in levels of ataxin-2 protein (control, 4.32-fold, $p = 0.0002$). **f**, **g** Chloroquine treatment caused significant increase in p62 (control 2.04-fold increase, $p = 0.0406$, SCA2 2.10-fold increase, $p = 0.0108$) and LC3-II (control 11.10-fold increase, $p = 0.0001$, SCA2 6.55-fold increase, $p = 0.0006$) protein in control cells and SCA2 cells. Graphs represent mean and SEM from at least two independent replicate experiments, using three control and five SCA2 patient cell lines

hampered elimination of damaged organelles, specifically the mitochondria, is the source of increased ROS, previously observed in the SCA2 cells [13]. Mutant ataxin-2 could be contributing to this apparent autophagic disruption in two ways: ataxin-2 and its yeast orthologue have previously been shown to inhibit mTORC1 activity under stress condition, thereby inducing autophagy [23, 50, 51]. The polyglutamine expansion in mutant ataxin-2 could possibly prevent such induction. Alternatively, the elimination of excess mutant protein, present as insoluble aggregates or oligomers (that is not otherwise eliminated by the UPS), could add pressure to an already-taxed autophagic system.

In order to investigate if aggregation could be involved, we performed immunocytochemistry to visualize ataxin-2 on our

patient fibroblasts. However, we did not find any evidence of actual aggregates (results not shown), indicating that elongated ataxin-2 does not aggregate in fibroblasts. However, we cannot exclude the possibility that ataxin-2 forms oligomers that cannot be detected by immune fluorescence and fluorescence microscopy. The role of autophagy in the elimination of ataxin-2 is further supported by western blot, indicating a significant increase in ataxin-2 protein upon chloroquine treatment in both control and SCA2 cells (Fig. 2d, e). Interestingly, ataxin-2 levels were lower in SCA2 cells than in controls. A possible explanation could be that oligomerization of ataxin-2 causes it to locate into a fraction insoluble under normal protein extraction conditions, thus making it unavailable. This is in line with previous reports, showing that extended

polyglutamine sequences can cause aggregation of affected proteins in neurodegenerative disease [1]. The accumulation of autophagosomes after chloroquine treatment led to increased levels of both, p62 and LC3-II proteins, in control as well as in SCA2 fibroblasts as expected (Fig. 2f, g), indicating that autophagosome formation can proceed normally even in SCA2 cells, as there is no significant difference in levels of either of these proteins between control and SCA2 cells. The fact that these cells can still efficiently activate autophagy indicates that there is no significant primary role of the polyglutamine expansion in this process.

Effects of Oxidative Stress and Autophagic Activation Using Rapamycin and Trehalose

Previous studies on mitochondrial function in SCA2 cells [13] indicated that these cells are under increased oxidative stress and are unable to efficiently dispose of dysfunctional mitochondria. Presumably, this increased oxidative stress would contribute to an increase in damaged mitochondria, stressing the autophagic system, and leading to increased caspase-9-mediated apoptosis. The susceptibility of the cells to caspase-9-mediated apoptosis (mitochondrial pathway) also supports this aspect of the SCA2 pathological mechanism. To investigate this, cells were treated with hydrogen peroxide (H_2O_2), and the patient cells were found to display a significantly increased response in both caspase-9- and caspase-8-mediated apoptosis (Fig. 3a, b). Eliciting oxidative stress in this manner is likely to cause an increased amount of damaged proteins and organelles that would lead to increased pressure on an autophagic system already tasked with eliminating mutant ataxin-2. SCA2 cells are predictably sensitive to disruptions of the autophagic pathway and, therefore, may be amenable to treatment with enhancers of autophagy. To this end, the cells were treated with the autophagic enhancer rapamycin, which specifically targets mTOR to activate autophagy [52]. As expected, activation of the mitochondrially mediated apoptotic pathway (caspase-9) and activation of the extrinsic apoptotic pathway (caspase-8) decreased significantly in SCA2 cells relative to controls upon rapamycin treatment (Fig. 3c, d), suggesting that rapamycin had a positive effect on cell survival via both apoptotic pathways. Moreover, rapamycin caused a tendency in decreasing the ataxin-2 protein levels in both control and SCA2 cells lines, as would be expected by enhancing autophagy, but these changes did not meet the threshold of significance (Fig. 3e, f). This is, however, interesting, given the recent study by Lastres-Becker et al., showing that ataxin-2 expression increases in response to nutrient and trophic stress in a mTOR-dependent manner [23], which could counteract the expected decrease in ataxin-2 levels upon autophagy induction. We also assessed the p62 protein levels, which appeared to be increased by rapamycin treatment in control and SCA2 cells, although this result did

not reach significance either (Fig. 3e, g). However, rapamycin seemed to be able to activate autophagy in both the control and SCA2 fibroblasts, as indicated by the significant increase in LC3-II protein in both cell types (Fig. 3e, h). Taken together, these data suggest that rapamycin, while having positive effects on apoptosis in SCA2 cells, does not activate autophagy in a manner which significantly depletes ataxin-2 or prevents ROS-elicited apoptosis. This is consistent with the fact that enhancement of autophagy with rapamycin was unable to significantly depress H_2O_2 -mediated apoptotic activation in patient cells (Fig. 3c).

Promoting autophagy using rapamycin appears to both activate autophagy effectively and decrease apoptotic activation in SCA2 cells, but it is unable to alleviate apoptotic activation elicited by ROS insult. In order to enhance autophagy via another pathway, the cells were treated with trehalose, a sugar molecule, proposed to be a chemical chaperone, which has been shown in previous studies to activate autophagy via a non-mTOR-mediated mechanism [53–55]. Unlike rapamycin, trehalose treatment alone caused no change in caspase-9 activation in either the control or patient fibroblasts, indicating that autophagic activation via this pathway does not alleviate the basal mitochondrially mediated apoptosis seen previously in SCA2 cells (Fig. 4a, b). As expected, H_2O_2 treatment caused an increase in caspase-9 activation in both the control and SCA2 cells, but with a significantly greater effect in the SCA2 fibroblasts (Fig. 4a). However, when the cells were pre-treated with trehalose, the effects of peroxide on caspase-9 activation were completely prevented, indicating that the activation of autophagy via this pathway alleviates oxidative-insult-mediated apoptosis. The same result occurred for caspase-8 activation by peroxide treatment, which was also increased in the SCA2 cells, and this effect was also alleviated by trehalose treatment (Fig. 4b). This alleviation of caspase-8 and caspase-9 activation was accompanied by increases in p62 and LC3-II proteins, increases which were significant in the SCA2 patient fibroblasts, indicating that such treatments do indeed activate autophagy (Fig. 4c, e, f). Neither trehalose, nor H_2O_2 alone appeared to increase levels of either p62 or LC3-II protein significantly (Fig. 4c, e, f). Furthermore, treatment with peroxide and/or trehalose had no significant effect on ataxin-2 levels in either cell line (Fig. 4c, d). Considering that enhancing autophagy via multiple pathways has positive effects on the basal apoptotic activation and on apoptosis elicited by oxidative insult, it is important to determine how the polyglutamine extension in ataxin-2 causes the cytotoxic stress eliciting this apoptosis. As we have established that SCA2 cells activate autophagy efficiently, it is likely that mutant ataxin-2 contributes to secondary autophagic pressure, perhaps via its own elimination.

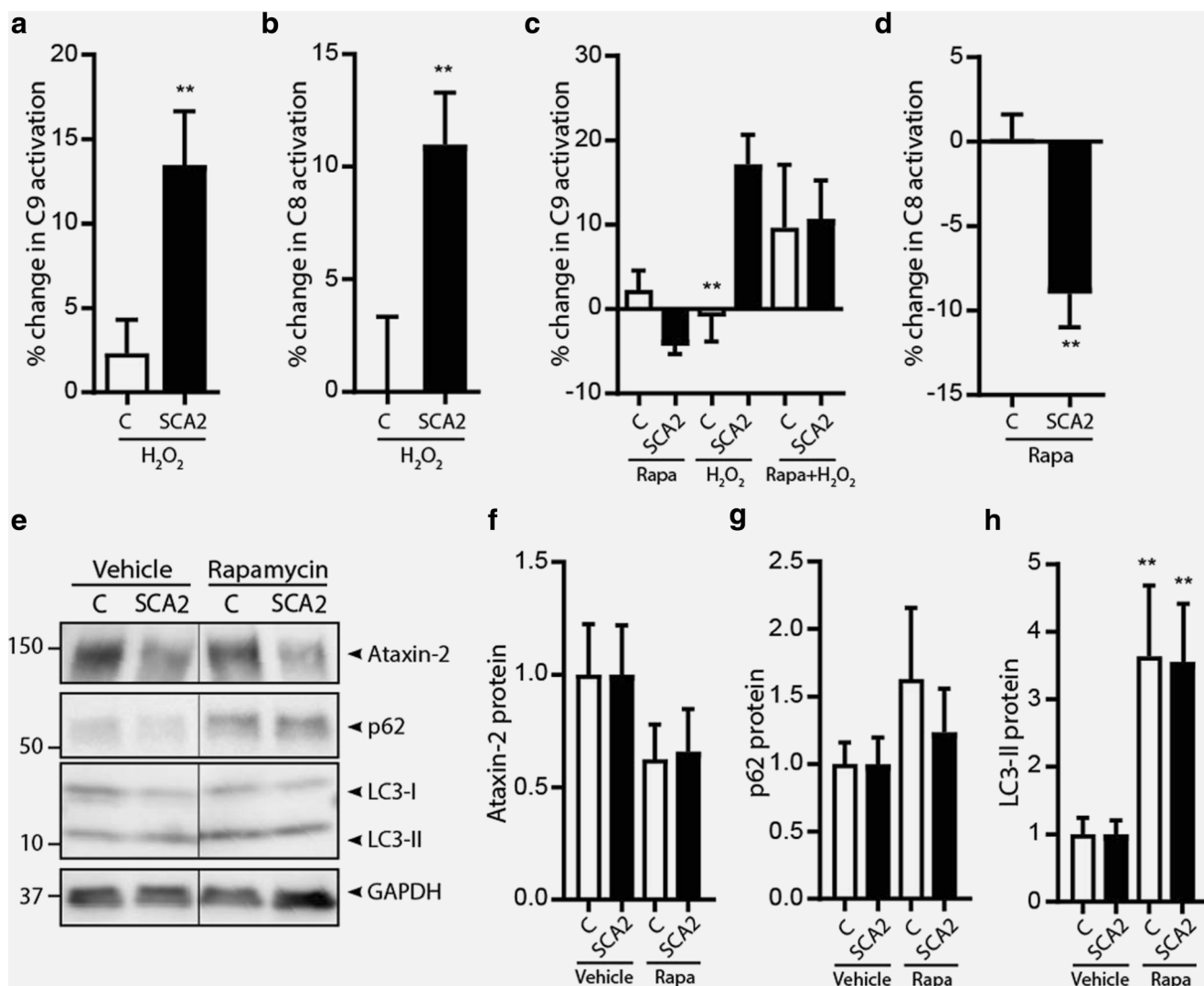


Fig. 3 Oxidative insult and autophagy enhancement by rapamycin: **a**, **b** SCA2 patient fibroblasts showed significantly increased levels of both caspase-9 (11.17% increase, $p = 0.0096$) and caspase-8 (10.98% increase, $p = 0.0078$) activation when treated with hydrogen peroxide (H_2O_2). **c** SCA2 patient fibroblasts showed significantly decreased levels of caspase-9 activation (6.7% greater decrease, $p = 0.0078$) upon rapamycin treatment, but the treatment exerted no significant effect on caspase-9 activation in either control or SCA2 fibroblasts, elicited by H_2O_2 treatment. **d** SCA2 patient fibroblasts showed significantly decreased levels of caspase-8 activation (9.2% greater decrease, $p = 0.0019$) upon treatment

with rapamycin, relative to control cells. **e** Representative western blot images of ataxin-2, p62, LC3-II, and GAPDH levels in vehicle and rapamycin treated fibroblasts. **f** Rapamycin treatment did not cause significant changes in the level of ataxin-2 protein in control or SCA2 fibroblasts. **g** Rapamycin treatment did not cause significant changes in the level of p62 protein in control or SCA2 fibroblasts. **h** Rapamycin caused significant increase in LC3-II protein in both control and SCA2 cells (control 2.63-fold increase, $p = 0.0059$, SCA2 2.56-fold increase, $p = 0.0025$). Graphs represent mean and SEM from at least two independent replicate experiments, using three control and five SCA2 patient cell lines

Effects of Congo Red in SCA2

Oligomerization or aggregation of mutant protein is a common characteristic of the polyglutamine neurodegenerative disorders and has been noted in SCA2 neurons as well as in multiple SCA2 disease models [1, 56]. Aggregation occurs due to polyglutamine expansions in the mutant proteins, causing complex structures consisting of many ataxin-2 proteins as well as other cytosolic proteins, and these aggregates can be eliminated via autophagy [4, 5]. The combined pressure of the aggregates and the

dysfunctional mitochondria in SCA2 cells, and sequestration of autophagic proteins can disrupt autophagy, leading to cell death [57]. To explore the role of possible oligomerization of ataxin-2 in the SCA2 disease mechanism, we treated cells with the chemical chaperone Congo red, which has previously been shown in other neurodegenerative diseases to efficiently solubilize proteins with expanded polyglutamine tracts, making the individual proteins available to degradation [2, 58]. If polyglutamine-expanded ataxin-2 oligomerization is one of the causes of apoptotic activation in these cells, then Congo red treatment should alleviate the

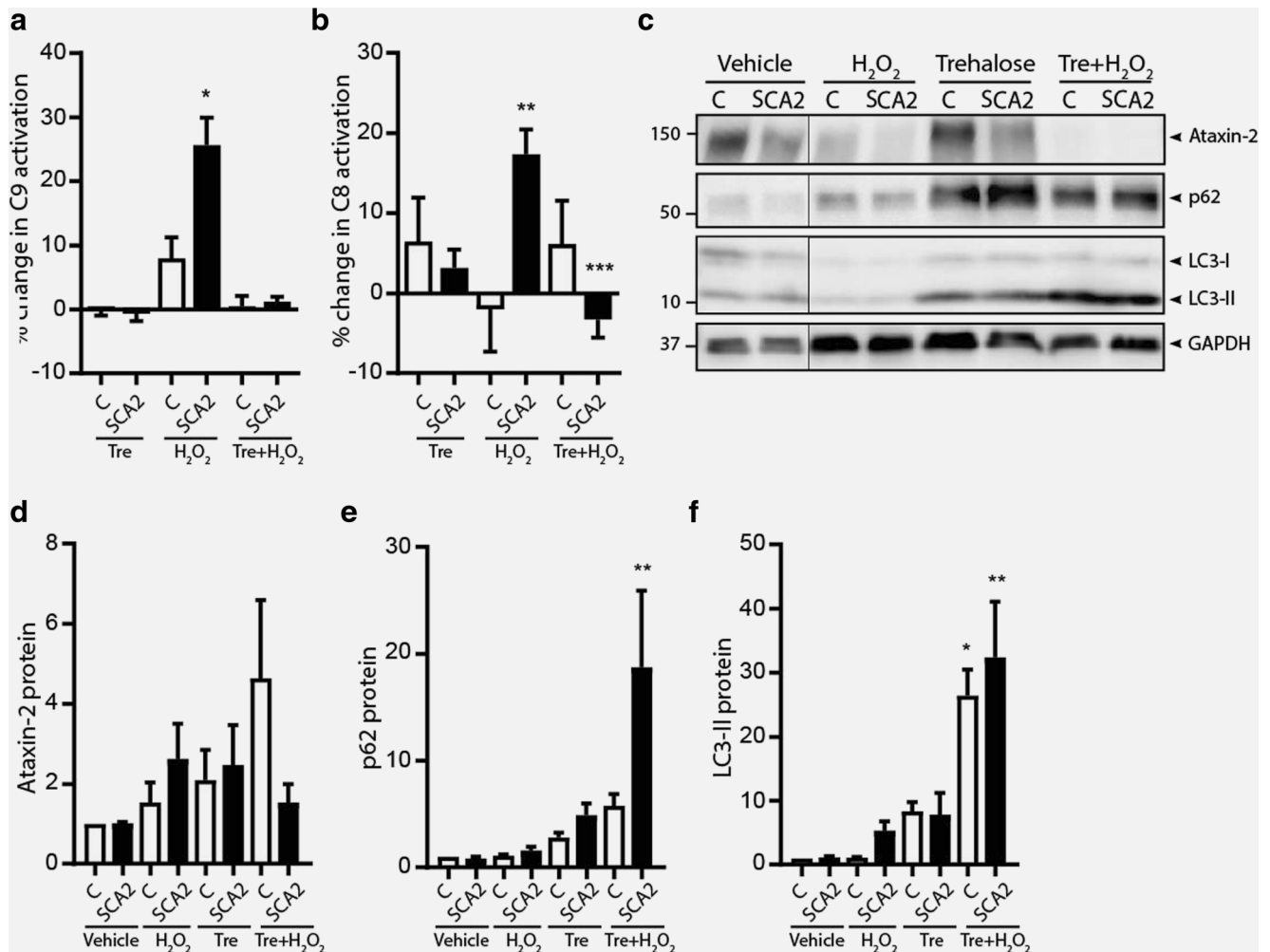


Fig. 4 Oxidative insult and autophagy enhancement by trehalose: **a** SCA2 patient fibroblasts showed significantly increased levels of caspase-9 activation (17.7% greater increase, $p = 0.0063$) upon treatment with hydrogen peroxide relative to control cells. Despite this increased response to hydrogen peroxide in SCA2 patient cells, treatment with molecular chaperone/autophagy activator trehalose was able to reverse caspase-9 activation to untreated control levels in both control and SCA2 patient cells. **b** The same result was observed for caspase-8 activation, with SCA2 cells showing significantly greater degree of activation upon hydrogen peroxide treatment (19.38% greater increase, $p = 0.0030$), and this activation was completely reversed when hydrogen peroxide-treated

SCA2 cells were treated with trehalose. **c** Representative western blot images of ataxin-2, p62, LC3, and GAPDH levels in vehicle, H₂O₂, trehalose, and trehalose-H₂O₂-treated fibroblasts. **d** Hydrogen peroxide, trehalose, and trehalose-H₂O₂ treatments had no significant effects on ataxin-2 protein levels relative to untreated control fibroblasts. **e, f** Levels of p62 and LC3-II proteins were significantly increased only in SCA2 cells treated with trehalose followed by H₂O₂ (p62, 17.7-fold increase, $p = 0.0011$; LC3-II, 34.7-fold increase, $p = 0.0099$). Graphs represent mean and SEM from at least two independent replicate experiments, using three control and five SCA2 patient cell lines

observed apoptosis. It should be noted that, when we immunocytochemically investigated cells for ataxin-2 aggregates, no punctae were detected by the ataxin-2 antibody (data not shown). This could be due to the small size of oligomers of ataxin-2 or it could be due to epitope sequestration in oligomers. The lack of visible punctae could also indicate that aggregates are not present, and that Congo red could exert effects independent of deligomerization of ataxin-2. We found that Congo red treatment caused significantly greater decreases in both the caspase-9- and caspase-8-mediated apoptosis in the SCA2 cells relative to controls (Fig. 5a, b). Considering these results, it is important to determine how the proteostatic system, responsible for the

elimination of dysfunctional and oligomerized proteins, plays a role in apoptotic cell death in these SCA2 cells. If autophagy is responsible for elimination of oligomerized ataxin-2, as it is responsible for eliminating polyglutamine aggregates in other neurodegenerative diseases [8], and Congo red can deplete ataxin-2 oligomers or aggregates, then Congo red treatment should help to alleviate apoptosis caused by autophagy inhibition. To test this, we pre-treated cells with Congo red before chloroquine treatment, and found that addition of Congo red alleviated apoptotic activation, elicited by chloroquine, almost completely (Fig. 5c). We also examined the effect of Congo red on ataxin-2 protein levels via western blot and found that ataxin-2 levels were not

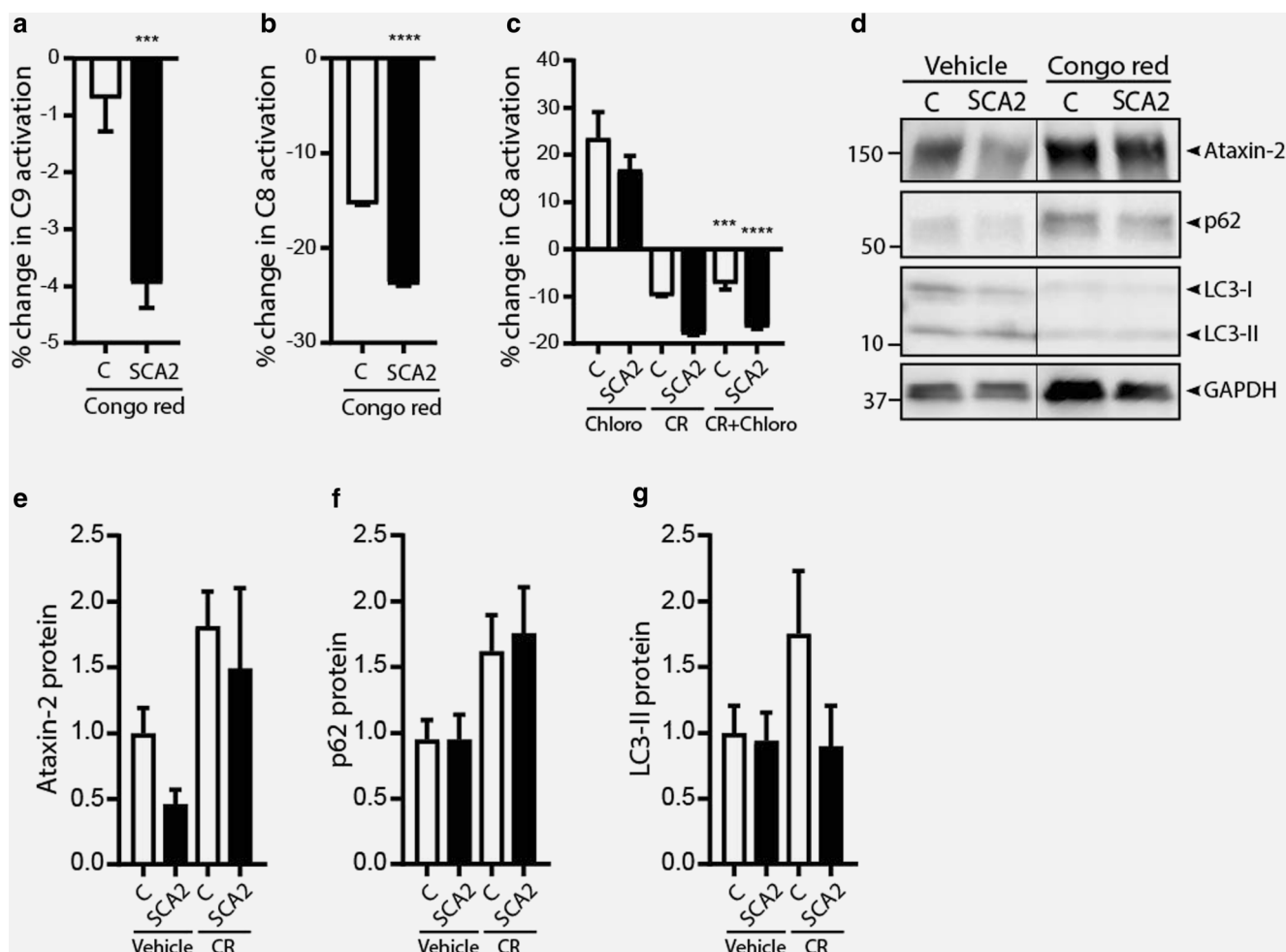


Fig. 5 Congo red treatment: **a** Treatment with the chemical chaperone Congo red caused a significantly greater decrease in apoptotic cells versus vehicle-treated controls as measured by caspase-9 activation (3.25% greater decrease, $p = 0.0005$) and **b** caspase-8 activation (8.51% greater decrease, $p < 0.0001$). **c** Pretreatment with Congo red significantly reversed the increases in caspase-8 activation, elicited by chloroquine treatment in both, control cells (7.57% decrease, $p = 0.0011$) and SCA2

patient cells (7.92% decrease, $p = 0.0001$). **d** Representative western blot images of ataxin-2, p62, LC3, and GAPDH levels in vehicle and Congo red-treated fibroblasts. **e–g** Congo red treatment caused no significant changes in ataxin-2, p62, or LC3-II protein levels in either control or SCA2 fibroblasts. Graphs represent mean and SEM from at least two independent replicate experiments, using three control and five SCA2 patient cell lines

significantly different, although there seemed to be a tendency of lower ataxin-2 levels in the untreated SCA2 cells relative to controls; a surprising result, as the polyglutamine extension should prevent elimination of ataxin-2, leading to its accumulation. Interestingly, this tendency disappeared when the cells were treated with Congo red (Fig. 5d, e), which also increased the overall levels of ataxin-2 protein in both, the control and SCA2 cells. Though these results were not statistically significant, they could indicate the extraction of ataxin-2 is insufficient under our normal extraction conditions for western blot, leading to the lack of availability of ataxin-2 in the SCA2 cells, which is then reversed once cells are treated with Congo red. Treatment with Congo red caused no significant increase in either of the autophagy proteins p62 or LC3-II (Fig. 5d, f, g). It should be noted that untreated SCA2 cells show no basal elevation of p62 or LC3-II protein, indicating that there is no increase in autophagic

activation or targeting of cargo to autophagosomes in the disease state. These data could indicate that SCA2 cells do not efficiently compensate for increased pressure on the autophagic system exerted by expanded ataxin-2, or that autophagy is not responsible for elimination of expanded ataxin-2.

Previous studies have shown that expanded polyglutamine-containing proteins can oligomerize to form aggregate bodies, which are unavailable to weak protein extraction buffers, and, therefore, require more stringent extraction conditions to reveal the levels of these proteins via western blot [18]. To test whether mutant ataxin-2 aggregates and accumulates in an insoluble fraction, we applied a dual-extraction protocol to separate and extract both, the soluble cytosolic proteins and insoluble protein aggregates and oligomers from the SCA2 patient cells. This revealed that, while there was no difference in ataxin-2 protein levels in the control and SCA2 cells under

typical “weak” extraction conditions (RIPA buffer), a more stringent extraction (SDS buffer) resulted in significantly higher levels of ataxin-2 in the SCA2 fibroblasts, indicating that more ataxin-2 sequesters to the insoluble fraction in SCA2 fibroblasts, possibly due to a greater degree of polyglutamine-mediated oligomerization (Fig. 6a–c). To test if expanded-polyglutamine-mediated oligomerization was causing oligomerization in our cells, they were treated with Congo red to solubilize ataxin-2 in the insoluble fraction. This revealed that Congo red treatment eliminated the difference in ataxin-2 levels in the insoluble fraction between control and SCA2 cells (Fig. 6d–f), and, interestingly, this treatment also caused a decrease in the relative amount of ataxin-2 in the soluble fraction, possibly by making more of the mutant, expanded ataxin-2 available for degradation (Fig. 6d, e). These data could indicate that ataxin-2, with an expanded polyglutamine tract, oligomerizes to insolubility and can be solubilized by Congo red, suggesting that the positive effects of Congo red on cell health are likely due to dissolution of these oligomers. However, Congo red has been shown to exert other effects, in diseases characterized by protein toxicity, including in Alzheimer’s disease models of β -amyloid toxicity, or via effects on blockade of channel formation, interference with immune functions, and by modulation of gene expression [59]. Therefore, it is important to consider these alternate effects, especially in light of the lack of microscopically observed aggregates in these cells. Nevertheless, the combined data presented here, showing that chloroquine treatment causes accumulation of ataxin-2 and caspase activation, and that treatment with Congo red counteracts the effect of chloroquine, suggests that in this context, Congo red functions, at least in part, through solubilization of oligomers.

Discussion

Solubilization of Ataxin-2

While aggregation is a hallmark of many of the polyglutamine neurodegenerative diseases, previous studies have shown that it is not necessary for SCA2 pathogenesis [60]. Although we were unable to detect ataxin-2 aggregates in the SCA2 cells, we found that they show a significant increase in ataxin-2 in the insoluble fraction of cytosolic protein (Fig. 6b, c), which could be due to oligomerization of ataxin-2; alternatively, it could be a result of stress granule formation, in which ataxin-2 has also been implicated [27]. In addition, the fact that the polyglutamine expansion in ataxin-2 might prevent appropriate disintegration of oligomers or stress granules could contribute to SCA2 pathology. To alleviate potential secondary ataxin-2 toxicity, the cells were treated with Congo red to dissociate the insoluble polyglutamine-containing proteins. Congo red treatment depressed the relative preponderance of

ataxin-2 protein in the insoluble fraction of the SCA2 fibroblast proteins (Fig. 6e, f). In neurodegenerative diseases, the main route of disposal of aggregated proteins is via autophagy, and the role of aggregated protein in the various pathological mechanisms of these diseases is not definitive [11, 19, 52, 61]. Our SCA2 fibroblast model shows higher levels of apoptotic activation versus controls (Fig. 1a, b) and depleting insoluble or oligomerized ataxin-2 appears to have positive effects on these phenotypes (Fig. 5a–c). Particularly interesting among these results is the effect on mitochondrially mediated (caspase-9) apoptosis. Excess oxidative stress and mitochondrial dysfunction are thought to be part of the disease mechanism of many neurodegenerative disorders [62], including the polyglutamine disorders [56]. Previous studies in our laboratory have exposed an increase in oxidative species in SCA2 patient cells, changes in antioxidant enzyme expression, a decrease in mitochondrial metabolism, and distinct morphological changes in the mitochondria [13]. These cells display increased proportions of punctate mitochondria, indicating increased mitochondrial fission, a hallmark of mitochondrial damage. Punctate mitochondria appear to accumulate in these cells, indicating problems with their disposal, a process also carried out by the autophagic pathway. It is possible that the bivalent increases in pressure upon the autophagic system, from protein oligomers and damaged mitochondria, lead to reduced autophagic efficiency and a consequent accumulation of both species. This could result in cell death due to the aggregates themselves [63] and increased ROS from un-degraded, damaged mitochondria [64].

Proteasomal Elimination of Ataxin-2

Autophagy is not the only route of elimination for unfolded, misfolded, or mutated proteins. Before forming aggregates or oligomers, these proteins can be disposed of by the UPS, which degrades the proteins individually. If ataxin-2 is a substrate of this system, then its dysfunction could harm SCA2 fibroblasts. Though MG-132 treatment shows significant differential effects on oxidative stress and caspase-8 activation in SCA2 fibroblasts relative to controls, we did not observe significant changes in caspase-9-mediated apoptosis (Fig. 1c, d). Therefore, the resultant oxidative stress is likely not related to mitochondria, but rather a result of an increase in ROS, secondary to other aspects of proteostatic dysfunction, a connection previously established in other studies of the proteasome [65]. The fact that no change in ataxin-2 levels is observed with proteasome inhibition in either control or SCA2 cells could indicate that the proteasome is not the major route of either normal or expanded ataxin-2 elimination. It is also possible that expanded ataxin-2 that is not processed properly by the proteasome, finds its way into oligomers, which are in turn eliminated by autophagy, as supported by the significant increase in LC3-II protein levels in this case (Fig. 1f, i). This is

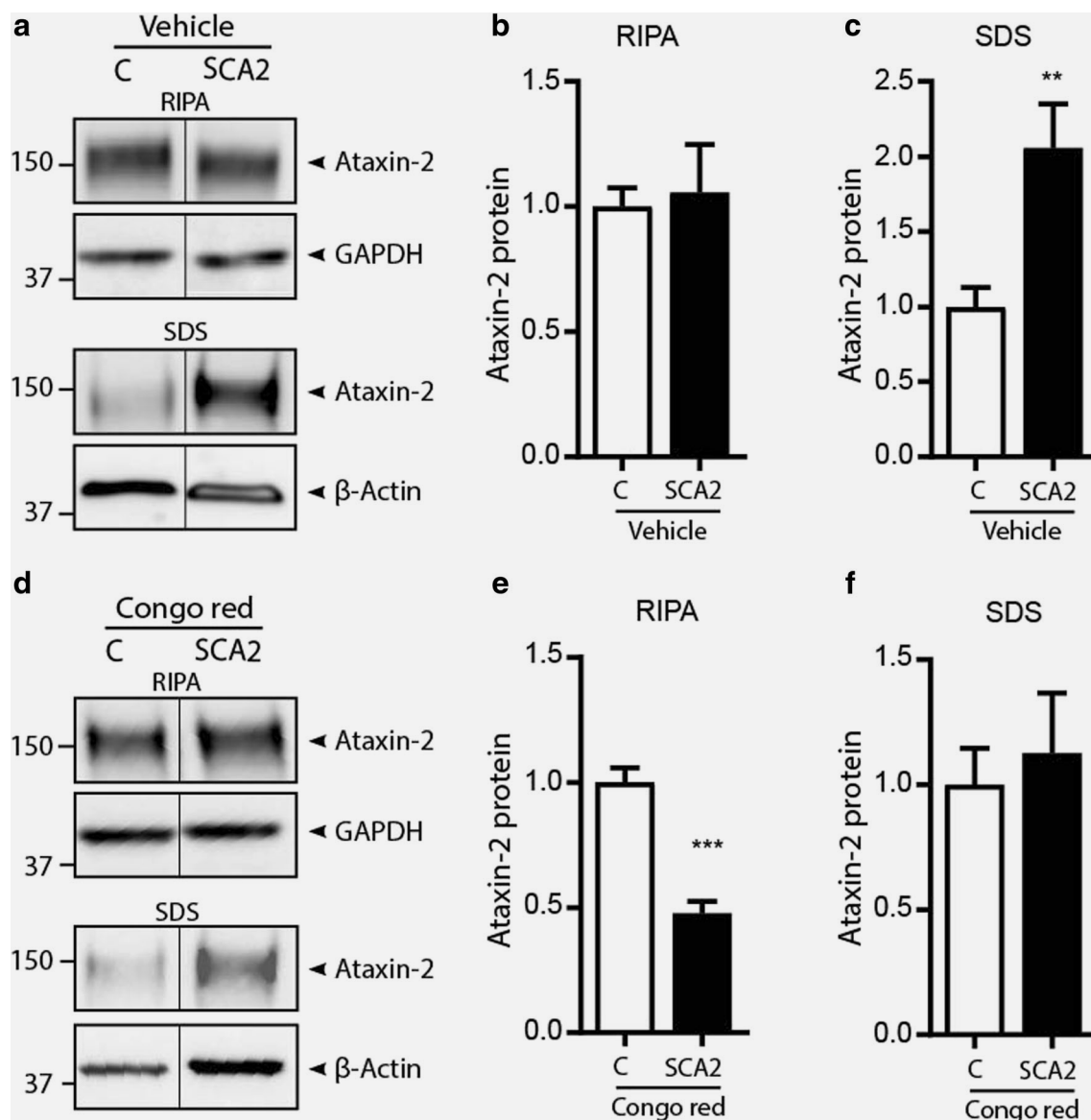


Fig. 6 Solubilization of polyglutamine by Congo red: Densitometry quantification of ataxin-2 from western blots of control and SCA2 patient cell extracts: **a** Representative western blot images of ataxin-2, GAPDH, and β -actin levels in vehicle treated fibroblasts under differential extraction conditions. **b** Under weak extraction conditions for soluble cytoplasmic proteins (RIPA buffer), there was no significant difference in ataxin-2 levels between control and SCA2 fibroblast extracts. **c** Under strong extraction conditions for insoluble proteins (SDS buffer), SCA2 patient cells exhibited significantly higher levels of ataxin-2 (2.06-fold increase,

$p = 0.044$). **d** Representative western blot images of ataxin-2, GAPDH, and β -actin levels in vehicle Congo red-treated fibroblasts under differential extraction conditions. **e** Treatment with Congo red decreased ataxin-2 in the soluble (RIPA) fraction in SCA2 patient cells to below control levels (0.52-fold decrease, $p < 0.0001$). **f** No significant difference was observed in ataxin-2 levels in the insoluble (SDS) fraction. Graphs represent mean and SEM from at least two independent replicate experiments, using three control and five SCA2 patient cell lines

interesting in the context of MG-132-mediated caspase-8 activation, as these data recapitulate previous findings on proteasome inhibition and autophagy-dependent activation of caspase-8, with LC3-II protein increases after MG-132 treatment [45]. This increase in autophagy could also be due to the observed increase in oxidative stress, causing more damaged organelles, which eventually will also be degraded by autophagy.

Alleviating Autophagic Pressure

Since the UPS does not appear to play a significant role in the elimination of either normal or expanded ataxin-2, as inhibition of this system caused no changes in ataxin-2 protein levels (Fig. 1f, g), the role of autophagy was more specifically targeted. Inhibiting autophagic clearance caused a significantly higher increase in caspase-9-mediated apoptosis in SCA2

cells versus control fibroblasts (Fig. 2a), though this could be due to the inhibition of autophagic clearance of the previously mentioned damaged mitochondria. This could also explain the decreased numbers of oxidatively healthy cells. However, if aggregated ataxin-2 is indeed contributing to autophagic pressure and, in turn, apoptosis, then depleting ataxin-2 aggregates with Congo red should decrease apoptosis in these cells. Predictably, Congo red treatment was able to alleviate caspase-8-mediated apoptosis elicited by chloroquine (Fig. 5c). Caspase-8-mediated apoptosis was measured in this case to avoid confounding effects of mitochondrial dysfunction on caspase-9-mediated apoptosis. Another explanation for the susceptibility of SCA2 cells to autophagic disruption could be that ataxin-2 has a primary functional role in autophagic activation, as suggested by its interaction with stress granules in other studies [19]. In our fibroblast model, we found SCA2 cells to be equally capable of activating autophagy as control cells, as indicated by increases of p62 and LC3-II proteins after chloroquine treatment (Fig. 2f, g). This is also true for treatments that increase autophagy, including rapamycin and trehalose, indicating that mutant ataxin-2 interrupting normal autophagic activation does not play a role in the deleterious phenotypes we observe in SCA2 fibroblasts (Figs. 3 and 4).

With previous studies showing increased ROS and mitochondrial dysfunction in SCA2 fibroblasts [13], and indications of oxidative stress in the above studies, it is unsurprising that hydrogen peroxide treatment causes acutely detrimental consequences in SCA2 cells (Fig. 4a–c). However, this could be the result of ROS causing cytotoxic stress itself, or it could be due to increased numbers of damaged mitochondria, in turn leading to more stress on the autophagic system. Considering what appears to be a proteostatic system doubly stressed by both, damaged mitochondria as well as protein aggregates, but with a functioning autophagic system, enhancement of autophagy might alleviate some of the apoptosis observed in these cells. Previous studies on other polyglutamine-expansion neurodegenerative diseases have shown that rapamycin was able to prevent mutant protein accumulation and cell death [5]. Treatment of cells with rapamycin caused a decrease in caspase-9- and caspase-8-mediated apoptosis in SCA2 patient fibroblasts but not in control fibroblasts (Fig. 3c). Despite this positive effect on mitochondrially mediated apoptosis, rapamycin did not affect apoptotic activation in the context of oxidative insult, indicating that autophagic activation with rapamycin is enough to overcome basal differences in apoptosis but is not protective (Fig. 3d). Other studies have shown that rapamycin can efficiently activate both, autophagy and mitophagy [66], but this activation is apparently not sufficient to overcome the dual pressure of ROS and aggregate pressure in these cells. This is despite efficient activation of autophagy in these cells, with increased levels of LC3-II protein in both cell types, indicating that there are no mechanistic problems with the mTOR-mediated autophagic pathway causing autophagosome formation and maturation (Fig. 3h).

Interestingly, there appears to be a slight (though non-significant) decrease in ataxin-2 protein levels upon rapamycin treatment, indicating that autophagic activation can potentially cause a decrease in ataxin-2, possibly in insoluble form (Fig. 3f). Though, as previously mentioned, this could be confounded by increases in ataxin-2 upon mTOR activation [23].

To explore the effect of other pathways of autophagic activation, we treated cells with the disaccharide trehalose, which has been implicated as both, a molecular chaperone and as an mTOR-independent activator of autophagy that can alleviate protein accumulation and degeneration in various neurodegenerative diseases [53, 54, 61]. Interestingly, upon treatment with trehalose, there was no significant decrease in caspase-9- or caspase-8-mediated apoptotic activation in either control or SCA2 cells. However, in the context of H₂O₂ treatment, which activates both, caspase-9 and caspase-8, significantly more in SCA2 patient cells than in control cells, trehalose can completely alleviate oxidative stress effects on caspase activation in both cell lines and decreases caspase-8 activation in SCA2 cells relative to vehicle treated cells (Fig. 4a, b). This is accompanied by significant increases in both p62 and LC3-II protein levels in SCA2 cells treated with trehalose and H₂O₂ together, indicating marked activation of autophagy in this context (Fig. 4e, f). These data suggest that trehalose, possibly by activating autophagy via an alternative pathway, can alleviate apoptotic activation elicited by ROS. This could be due to activation of autophagy or to alleviation of protein accumulation, both of which have been shown to occur with trehalose treatment in other studies [52, 54, 55]. These results suggest that treatment with a drug like trehalose, or activating the specific pathway affected by trehalose using another drug, could be a potential therapy for not only SCA2 but also other polyglutamine neurodegenerative diseases or those characterized by protein accumulation and mitochondrial dysfunction. The strategy of enhancing autophagic activity in these diseases certainly requires more investigation, especially the various autophagic pathways and their specific roles in eliminating the toxic species in these diseases.

Conclusions

To conclude, it appears that both oligomerization of ataxin-2 and oxidative stress contribute to the dysfunction and apoptotic activation observed in our SCA2 fibroblast model. It is likely that both of these stressors prevent proper disposal of damaged organelles and proteins, which in turn leads to cell death. Changes in levels of autophagic proteins upon activation by well-characterized autophagy-promoting compounds indicate that in control and SCA2 cells, autophagy is efficiently initiated and is presumably still an important route for elimination of proteins and organelles, even in SCA2 cells. However, accumulation of ataxin-2 in the insoluble fraction in

SCA2 cells, as well as the higher level of apoptotic activation in these cells upon inhibition of autophagy indicate that the autophagic pathway may be more susceptible to disruption, and may, therefore, be vulnerable in SCA2. As either enhancement of autophagy or solubilization of ataxin-2 alleviates some of the apoptotic effects in SCA2 cells, it appears that autophagy plays an important role in the elimination of ataxin-2, especially in its expanded form. Moving forward, it will be important to study the specifics of these mechanisms further and to investigate them in different models, including pluripotent stem cell-derived neurons and animal models. Furthermore, this study indicates that diverse treatments, including drugs that deplete ROS, remove oligomerized or aggregated proteins, or activate autophagy, could serve as potential therapies for neurodegenerative diseases sharing these cellular phenotypes.

Author Contributions JHW, EEH, AGM, JEN, and TTN designed the experiments. JHW and EEH performed the experiments and analyzed the corresponding results. JHW wrote the paper with editing and consultation from EEH, AGM, JEN, and TTN.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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APPENDIX C:

Supplementary information for experimental procedures

Primer list:

Gene	Forward Primer	Reverse Primer
<i>ATOH1</i>	TCCTTTCCGCCCACCTTCT	AGCGTATTGGCAGCAGGGAA
<i>BARHL1</i>	CAGCGGCTGAGCTGTACATACC	GGCACAGGCCACAGACTCACTA
<i>CALB1</i>	GCTCCATTTTCGACGCTGACG	CAGGTGATAACTCCAATCCAGCC
<i>EN2</i>	GTCCATCTGTGGCTGGGACA	GCGAAGTTCACCCTCAGTCCTC
<i>GBX2</i>	GTTCCGCCGTCGCTGATGAT	GCCGGTGTAGACGAAATGGCCG
<i>GFAP</i>	CAAGATGAAACCAACCTGAGGCT	GGCTTGGCCACATCCATCT
<i>ALDOC</i>	ACTCCATACCACAGCCCTTG	GCAATTTCTTCTGCCCTCAG
<i>PAX2</i>	AACGACAGAACCCGACTATGT	GAGCGAGGAATCCCCAGG A
<i>OTX2</i>	AGAGGACGACGTTCACTCG	TCGGGCAAGTTGATTTTCAGT
<i>L7/PCP2</i>	ACCAGGAGGGCTTCTTCAAT	CTGTCACACGTTGGTCATCC
<i>GRID2</i>	AGCTCTTCCTCTCTTGGTTTCC	GCCCCACGTTGCCTAGAAAT
<i>MAP2</i>	GGATTCCGAGGTTCCAACACA	CATGAATAACTACAACCAGCCATTGA
<i>NANOG</i>	GAGGTCTCGTATTTGCTGCATCG	TCCTTCTCCACCCCAACCAAA
<i>NEPH3</i>	AGACGCCTCGTTCCTGACTGA	AACAGCACATCCAGCGCAGT
<i>OCT4</i>	CCCCAGGGCCCCATTTTGGTACC	ACCTCAGTTTGAATGCATGGGAGAGC
<i>OLIG2</i>	GACAAGCTAGGAGGCAGTGG	CGGCTCTGTCATTTGCTTCTTG
<i>PTF1A</i>	AGCAGGACACTCTCTCTCATGGACT	GTCCAGACTTTGGCTGTTCG
<i>RPL13A</i>	TTCCAAGCGGCTGCCGAAGA	TTCCGGCCCCAGCAGTACCTGT

Antibody list:

Antibody	Host Species	Company
Anti- β -ACTIN	Mouse	Abcam, ab6276
Anti-ATXN2	Rabbit	Novus Biologicals, NBP1-90063
Anti-ATXN2	Mouse	BD Biosciences, 611,378
Anti-CALB1	Rabbit	Sigma-Aldrich, HPA023099
Anti-GABA	Rabbit	Sigma-Aldrich, A2052
Anti-GAPDH	Rabbit	Abcam ab9485
Anti-GFAP	Mouse	Abcam, ab4648
Anti-HSP90	Rabbit	Abcam, ab203126
Anti-IP3R1	Rabbit	Invitrogen, PA1-901
Anti-LC3	Rabbit	Novus Biologicals, NB600-1384
Anti-MAP2	Chicken	Novus Biologicals, NB-300-213
Anti-NEPH3	Goat	Santa Cruz Biotechnology, sc70195
Anti-PTF1A	Goat	RD Systems, AF6119
Anti-RPS18	Rabbit	Atlas Antibodies, HPA055007
Anti-RPS26	Rabbit	Atlas Antibodies, HPA043961
Anti-RPS3	Rabbit	Atlas Antibodies, HPA063339
Anti-SQSTM1 / p62	Mouse	Abcam, ab56416
Anti-TIMM50	Mouse	Santa Cruz Biotechnology, sc-393678
Anti-TOMM20	Rat	Abcam, ab289670
Anti-Tubulin, beta III isoform	Mouse	Merck/Millipore, MAB1637

Secondary Antibody	Congugation	Company
Anti-chicken IgG	568	Life Technologies, A11041
Anti-goat IgG	488	Novus Biologicals, NBP1-90063
Anti-mouse IgG	488	Sigma-Aldrich, SAB4600388
Anti-mouse IgG	568	Sigma-Aldrich, SAB4600066
Anti-mouse IgG	633	Sigma-Aldrich, SAB4600138
Anti-rabbit IgG	488	Thermo Fisher Scientific, A55053
Anti-rabbit IgG	568	Sigma-Aldrich, SAB4600068
Anti-rabbit IgG	633	Sigma-Aldrich, SAB4600140
Anti-rat IgG	488	Sigma-Aldrich, SAB4600037
Anti-rat IgG	633	Sigma-Aldrich, SAB4600133

