



The
University
Of
Sheffield.



Novel therapeutic strategies for Motor Neuron Disease and Frontotemporal Dementia: gene therapy approaches to target unconventional translation of C9ORF72 dipeptide repeat proteins

Submitted for the degree of Doctor of Philosophy (PhD) January 2023

Bridget Clare Benson 180154663

Supervisors: Dr Guillaume Hautbergue and Professor Mimoun Azzouz

Department of Neuroscience

Sheffield Institute for Translational Neuroscience, University of Sheffield

This thesis is dedicated to the patients, and to everyone who strives for
a world without neurodegenerative diseases

Acknowledgements

I would first like to thank my academic supervisors, Dr Guillaume Hautbergue and Professor Mimoun Azzouz. I would especially like to thank Dr Hautbergue for the opportunity to work in his lab for this PhD project, and for all the excellent guidance and mentorship I have received throughout my time at SiTraN. The chance to do a PhD has meant so much to me and I am grateful for everything I have learned.

Thank you to all the past and present members of the Hautbergue RNA Biology group for the friendship, support and the many times you gave me excellent advice in the lab. Special thanks must go to Dr. Lydia Castelli and Dr. Erica Lin, I could not have done this without you. I would also like to thank Dr. Robin Highley for all the time and attention spent helping with the pathology aspect of this project. Your advice, encouragement and expertise were invaluable. Thank you to Dr. Bridget Ashford for the double staining protocol, and for all the support and great conversations in histology. I am grateful to all the technical staff in the department, but especially to Dan Fillingham. Thank you for all the work you have done to help me with this project. Thank you to DiMeN, for making this opportunity possible for me, and especially to Emily for all your patience and support. And to all my friends at SiTraN, I can't thank you enough for helping me through.

I have met some wonderful people in Sheffield, who have helped shape my time here for the better. Special thanks to Claudia, you were always there to laugh, cry, celebrate and commiserate. You made my time in Sheffield so much better and your support got me through some difficult times, and I'll always be thankful for that. Thank you to all my friends, near and far, who have been so amazing during this PhD, I feel incredibly fortunate. To Alex, thank you for keeping me sane during the last few months of this PhD, and for always making things fun. And thank you to Stevie, who has made me feel better every day, and who sat beside me while I wrote this thesis.

Thank you to everyone who donated tissue that was used in this project. I hope they would feel proud to know their choice to donate their brain made my work possible, and I hope I have done justice to their decision.

Finally, I would like to thank my family. Thank you to Sim and Joe, I am so grateful for everything you have both done for me. But most of all, thank you to my parents Chris and Clare. You always encouraged me to be curious, and that is what has led me to this point. Thank you for your love, support and belief in me, it has made all the difference in the world.

Abstract

Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD) are rapidly progressing fatal neurodegenerative diseases, characterised by loss of upper and lower motor neurons, or degeneration of the frontal and temporal lobes. A polymorphic GGGGCC hexanucleotide repeat expansion mutation in intron one of the *C9ORF72* gene has been identified as the most common genetic cause of the ALS/FTD disease spectrum. SRSF1-dependent nuclear export of repeat *C9ORF72* transcripts from the nucleus enables Repeat Associated Non-AUG (RAN) translation of sense and antisense transcripts in all three frames, producing five neurotoxic dipeptide repeat proteins (DPRs). DPRs are the primary driver of pathogenesis in *C9ORF72*-ALS patient-derived neurons and animal models of disease. RNA helicases eIF4A1, eIF4A2, eIF4B, eIF4H and DHX36 resolve RNA secondary structures and are necessary for translation to initiate.

We hypothesised that partial depletion of RAN-associated RNA helicases will confer neuroprotection in preclinical models of C9-ALS/FTD, through disruption of RAN translation and preventing production of neurotoxic DPRs. Tools to target RAN-associated helicase expression were developed including shRNAs and dominant negative mutants. Partial depletion of individual RAN-associated RNA helicases reduced sense and antisense DPR production, alleviated DPR-induced toxicity and are non-toxic, while targeting multiple helicases is associated with toxicity. In post-mortem human tissue, RAN-associated RNA helicases have a predominantly cytoplasmic location. eIF4A1 forms a novel pathology in sALS and C9-ALS/FTD spinal cord motor neurons that is generally positive for p62, and can be either positive or negative for TDP-43 pathology. These findings have advanced our knowledge on helicases involvement in RAN translation, and provided evidence to support targeting RAN-associated helicases as a potential therapeutic strategy in C9-ALS/FTD.

Acknowledgements.....	3
Abstract.....	4
Abbreviations.....	11
List of Figures.....	16
List of Tables.....	22

Contents

1	Introduction.....	24
1.1	Epidemiology and Clinical Features of ALS	24
1.2	Genetic causes of ALS	25
1.3	<i>C9ORF72</i> G4C2 repeat expansions and the ALS/FTD disease spectrum	26
1.4	TDP-43 and <i>C9ORF72</i> pathologies in ALS/FTD	26
1.5	<i>C9ORF72</i> shares homology with DENN proteins and has functions relating to autophagy and energy homeostasis	29
1.6	Proposed pathogenic mechanisms in C9-ALS/FTD	33
1.6.1	Haploinsufficiency	33
1.6.2	RNA toxic gain-of-function mechanisms in C9-ALS/FTD	36
1.6.3	Dipeptide Repeat Protein toxic gain-of-function	40
1.6.4	Relative contributions of toxic gain-of-function mechanisms to C9-ALS/FTD	51
1.7	Mechanisms of Canonical translation.....	53
1.7.1	Key mechanisms in canonical translation initiation	53
1.7.2	eIF4A1 and eIF4A2 are DEAD-box helicases required for translation initiation.....	56
1.7.3	eIF4A1 and eIF4A2 helicase activity is stimulated by eIF4B and eIF4H.....	60
1.7.4	DHX36 is an RNA helicase that is a major resolver of G-quadruplexes.....	61
1.8	Mechanisms of DPR production by RAN translation	62
1.8.1	Introduction to RAN translation	62
1.8.2	Near-cognate start codons are used to initiate RAN translation and a frameshift produces DPRs in all three reading frames.....	63
1.8.3	RAN translation depends on the resolution of RNA secondary structures.....	64

1.8.4	The role of eIF4A1 and eIF4A2 in RAN translation initiation.....	65
1.8.5	eIF4A1 and eIF4A2 require co-factors eIF4B and eIF4H for initiation of RAN translation.....	66
1.8.6	RAN translation is enhanced by the ISR	69
1.8.7	Depletion of helicase DHX36 suppresses RAN translation.....	70
1.9	Treatment strategies for C9-ALS/FTD	72
1.9.1	Viral mediated gene therapy for C9-ALS/FTD	73
1.9.2	Other therapeutic strategies in C9-ALS/FTD include antisense oligonucleotides, small molecules, re-purposing of existing drugs, genome editing and anti-DPR antibodies among others.....	76
1.10	Introduction to the PhD project.....	79
1.10.1	Aims and Hypothesis	80
2	Materials and Methods	83
2.1	Materials	83
2.1.1	Cell Lines	83
2.1.2	Design of oligonucleotides for eIF4A1, eIF4A2, eIF4B and DHX36.....	83
2.1.3	shRNA design for insertion into pENTR/H1-TO-MCS, scAAV-KAS/H1 and LV-H1-TO vectors	89
2.1.4	Vectors.....	94
2.1.5	Antibiotics.....	106
2.1.6	Buffers and Solutions.....	106
2.1.7	Enzymes	109
2.1.8	Antibodies.....	110
2.1.9	Kits for Immunohistochemistry	111
2.1.10	Post-Mortem Human Tissue Donor Information	111
2.1.11	Mouse Spinal Cord.....	113
2.1.12	Imaging and Software.....	113
2.2	Methods	114
2.2.1	Cloning for pENTR/H1-TO-MCS and scAAV-KAS/H1 plasmids.....	114

2.2.2	Isolation of Plasmid DNA	116
2.2.3	Sub-Cloning.....	119
2.2.4	Western Blots	120
2.2.5	MTT Cell Proliferation Assays	124
2.2.6	Reverse Transcriptase qPCR	124
2.2.7	Immunohistochemistry	126
2.2.8	Immunofluorescence Staining of patient-derived neurons	128
2.2.9	Statistical Analysis	128
3	Identification and manipulation of RAN translation factors in human cell models of C9-ALS/FTD	129
3.1	Background, aims and rationale	129
3.2	Cloning of shRNAs into mammalian and viral vectors	130
3.2.1	pENRT/H1-T0-MCS-eIF4B shRNA.....	131
3.2.2	scAAV-KAS/H1 eIF4A1, eIF4A2, eIF4B and DHX36	134
3.2.3	Cloning of shRNAs targeting RAN-associated RNA helicases into a lentiviral plasmid.....	135
3.2.4	Testing Origene eIF4A1, eIF4A2, eIF4B and eIF4H shRNAs	138
3.3	Strategies to target RAN-associated RNA helicases and reduced DPR reduction in HEK293 cell models of C9-ALS/FTD.....	142
3.3.1	Transfection with RAN-associated RNA helicase shRNAs leads to a decrease in DPR production in HEK293T cell models of C9-ALS/FTD	143
3.3.2	Transfection with RAN-associated RNA helicase mutants leads to a decrease in DPR production in HEK293T cells models of C9-ALS/FTD	147
3.4	Chapter Summary and Discussion.....	153
3.4.1	Development of tools to target RAN-associated RNA helicases	153
3.4.2	Limitations associated with HEK293T cell models of C9-ALS/FTD	156
3.4.3	Strategy surrounding partial depletion of RAN-associated RNA helicases to reduce DPR production <i>in vitro</i>	157
3.4.4	Use of RAN-associated RNA helicase dominant negative mutants to reduce DPR production <i>in vitro</i>	160

3.4.5	Limitations associated with protein detection by Western Blotting.....	163
4	Characterising the expression pattern of RAN translation factors at tissue and cellular levels in human post-mortem CNS	166
4.1	Introduction	166
4.1.1	Background, aims and rationale	166
4.1.2	Post-mortem human tissue strategy	167
4.2	Antibody Optimisation	169
4.3	eIF4A1, eIF4A2, eIF4B, eIF4H, DHX36 and SRSF1 protein levels in human post-mortem tissue.....	174
4.3.1	Western blots in frozen human sporadic and C9-ALS/FTD spinal cord tissue	175
4.3.2	Western Blots of helicases in frozen human sporadic and C9-ALS motor cortex	177
4.4	eIF4A1 and eIF4A2 immunohistochemistry in human brain and spinal cord	180
4.4.1	eIF4A1 but not eIF4A2 forms neuronal cytoplasmic inclusions in spinal cord motor neurons of C9-ALS/FTD and sALS but not in control cases	180
4.4.2	Relationship between eIF4A1, phosphorylated TDP-43 and p62 pathology	187
4.5	Helicase immunohistochemistry in control and ALS brains.....	195
4.6	SRSF1 immunohistochemistry in control, sporadic ALS and C9-ALS/FTD post-mortem brain and spinal cord regions.....	197
4.7	eIF4A1 and eIF4A2 immunohistochemistry in C9-transgenic mouse spinal cord.....	206
4.8	Chapter Summary and Discussion.....	207
4.8.1	Investigations into novel eIF4A1-positive pathology in the ALS spinal cord.....	208
4.8.2	eIF4A1 double staining with phosphorylated TDP-43 and p62 in the ALS spinal cord.....	210
4.8.3	eIF4A1 and eIF4A2 immunohistochemistry in C9-transgenic mouse spinal cord.....	211
4.8.4	SRSF1 and RAN-associated RNA helicase immunohistochemistry in ALS spinal cord and CNS.....	212
4.8.5	Western blots of eIF4A1, eIF4A2 and eIF4H in frozen ALS spinal cord and motor cortex.....	214
4.8.6	Limitations associated with the use of post-mortem tissue	217

5	Manipulating RAN translation factors as potential gene therapeutic targets in C9-ALS/FTD	219
5.1	Background, aims and rationale	219
5.2	Exploring cytotoxicity associated with shRNA helicase depletion in HEK293T cell models of C9-ALS/FTD.....	220
5.2.1	shRNAs specifically targeting eIF4A1, eIF4A2, eIF4B and eIF4H are not associated with cytotoxicity in HEK293T cells	220
5.2.2	pENTR-shRNAs are associated with cytotoxicity in HEK293T cells only when multiple helicases are targeted simultaneously.	222
5.2.3	Partial depletion of helicases is associated with mild cellular stress	223
5.3	Investigating the effects of partial depletion of helicases on alleviation of DPR-induced toxicity.....	226
5.3.1	Partial depletion of helicases with shRNAs rescues cytotoxic effects of sense and antisense <i>C9ORF72</i> repeat expansion	226
5.3.2	Non-functional helicase mutants rescue cytotoxic effects of sense and antisense <i>C9ORF72</i> repeat expansion.....	228
5.3.3	Effects of partial depletion of helicases in mouse motor neurons and patient-derived astrocyte co-cultures	231
5.3.4	Effects of partial depletion of eIF4A1 and eIF4A2 in a <i>Drosophila</i> model of C9-ALS/FTD.....	233
5.4	Detection of eIF4A1 positive pathology in patient-derived neuronal lines	234
5.5	Chapter Summary and Discussion.....	246
5.5.1	Investigating cytotoxic effects linked to partial depletion of RAN-associated RNA helicases	246
5.5.2	Characterization of eIF4A1-positive inclusions in control and ALS patient-derived neuronal lines.....	253
5.5.3	Conclusions.....	254
6	Discussion and Conclusions.....	256
6.1	Overview of project aims and hypothesis.....	256
6.2	Targeting RNA helicases reduces DPR production in cell models of C9-ALS/FTD.....	257
6.3	Selective depletion of RNA helicase isoforms is non-toxic and rescues DPR-induced defects in cell proliferation and motor neuron survival	261

6.4	eIF4A1 forms cytoplasmic inclusions in sALS and C9-ALS/FTD spinal cord motor neurons.....	264
6.5	Immunohistochemistry of eIF4A1, eIF4A2, eIF4B and eIF4H shows predominantly cytoplasmic localisation	270
6.6	SRSF1 immunohistochemistry reveal positive glial cell staining in the C9-ALS/FTD motor cortex	271
6.7	Overall assessment of RAN-associated RNA helicases the most suitable to form a therapeutic strategy for C9-ALS/FTD	276
6.8	Future studies investigating SRSF1 and RAN-associated RNA helicases in C9-ALS/FTD.....	280
6.9	Summary and Conclusions	282
7	References.....	284
8	Appendix.....	314
	Appendix A: Immunohistochemistry Supplementary Images	314
	Appendix B: Copyright Statement.....	346

Abbreviations

AAV	Adeno-Associated Virus
ABS	Avidin-Biotin Complex
ALS	Amyotrophic Lateral Sclerosis
ALYREF	Aly/REF export factor
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	Alkaline Phosphatase
APS	Ammonium Persulfate
ASO	Antisense Oligonucleotide
ATP	Adenosine Triphosphate
ATXN8	Ataxin 8
BAC	Bacterial Artificial Chromosomes
BBB	Blood-Brain Barrier
bvFTD	Behavioural Variant Frontotemporal Dementia
BLF1	Burkholderia Lethal Factor 1
C9ORF72	Chromosome 9 Open Reading Frame 72
cDNA	Complementary DNA
CHOP	CCAAT-enhancer-binding protein homologous protein
CIP	Alkaline Phosphatase, Calf Intestinal
CNS	Central Nervous System
CRISPR	Clustered regularly interspaced short palindromic repeats
CSF	Cerebrospinal fluid
DAB	Diaminobenzidine
DAPI	4', 6-diamidino-2-phenylindole

DENN	Differentially expressed in normal and neoplastic cells
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DN	Dystrophic Neurites
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
DPRs	Dipeptide-repeat Proteins
DPX	Distyrene Plasticizer Xylene
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine Tetra acetic Acid
eIF	Eukaryotic initiation factor
EtOH	Ethanol
fALS	Familial Amyotrophic Lateral Sclerosis
FSB	First Strand Buffer
FTD	Frontotemporal Dementia
FTLD	Frontotemporal Lobar Degeneration
FUS	Fused in Sarcoma RNA-binding Protein
GA	Glycine-Alanine
GCI	Glial Cytoplasmic Inclusion
GDP	Guanosine diphosphate
GP	Glycine-Proline
GR	Glycine-Arginine
GTP	Guanosine triphosphate
HEK293T	Human Embryonic Kidney 293T Cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

hnRNP	Heterogeneous Nuclear Ribonucleoproteins
HSP	Heat Shock Protein
IgG	Immunoglobulin G
iPSC	Induced Pluripotent Stem Cell
IRES	Internal Ribosome Entry Site
ISR	Integrated Stress Response
ITRs	Inverted Terminal Repeats
IV	Intravenous
LB	Lysogeny Broth
LLPS	Liquid-Liquid Phase Separation
LV	Lentivirus
MAPT	Microtubule-associated protein tau
MICOS	Mitochondrial contact site and cristae organising system
miRNA	MicroRNA
MND	Motor Neuron Disease
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide
NCI	Neuronal Cytoplasmic Inclusion
NCT	Nucleocytoplasmic transport
NII	Neuronal Intranuclear Inclusion
NPM1	Nucleophosmin 1
ORFs	Open Reading Frames
PA	Proline-Alanine
PABP	Poly(A)-binding protein
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PEI	Polyethylenimine

PERK	protein kinase RNA-like endoplasmic reticulum kinase
PFA	Paraformaldehyde
PIC	Protease Inhibitor Cocktail
PMSF	phenylmethylsulfonyl fluoride
PNFA	Progressive Non-Fluent Aphasia
PNK	Polynucleotide Kinase
PPA	Primary Progressive Aphasia
PR	Proline-Arginine
qPCR	Quantitative polymerase chain reaction
RAB	Proteins Ras-associated binding Protein
RAN Translation	Repeat-associated non-AUG (RAN) translation
Ran Protein	Ras-related nuclear protein
RBP	RNA-Binding Protein
RHAU	RNA Helicase associated with AU-rich element
RNA	Ribonucleic Acid
RNAi	RNA interference
RNP	Ribonucleoprotein
ROS	Reactive Oxygen Species
RRM	RNA Recognition Motif
RT	Reverse Transcriptase
sALS	Sporadic Amyotrophic Lateral Sclerosis
SCA8	Spinocerebellar ataxia type 8
scAAV	Self-complementary adeno-associated virus
SD	Semantic Dementia
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SG	Stress Granule
shRNA	Short Hairpin RNA

siRNAs	Small interfering RNAs
SMCR8	Smith-Magenis Syndrome Chromosome Region, Candidate 8
snRNP	Small nuclear ribonucleoproteins
SOD1	Superoxide Dismutase 1
SRSF1	Serine/Arginine-Rich Splicing Factor
TAE	Tris-acetate-EDTA
TARDBP	TAR DNA-binding protein
TBS-T	Tris-Buffered Saline + 0.2% Tween 20
TEMED	N, N, N, N'-tetramethylethane-1,2-diamine
TIMMDC1	Translocase of inner mitochondrial membrane domain containing 1
TDP-43	Transactive Response (TAR) DNA-binding protein of 43
tRNA	Transfer RNA
TUDCA	Tauro-ursodeoxycholic acid
TUJ1	Class III beta-tubulin
ULK1	Unc-51-like kinase 1
Unc-119	Uncoordinated-119 Protein
UTR	Untranslated region
WDR41	WD Repeat domain 41

List of Figures

Figure 1.1: Pathological, genetic and clinical correlations in FTLD.	29
Figure 1.2: C9ORF72 RNA transcript variants and associated protein isoforms.	30
Figure 1.3: Summary of Dipeptide Repeat Proteins produced from sense and antisense C9ORF72 transcripts	41
Figure 1.4: Summary of toxic gain-of-function mechanisms associated with RNA foci and DPR production in C9-ALS/FTD.	53
Figure 1.5: Summary of key mechanisms of canonical AUG-dependent translation.	55
Figure 1.6: Known structures involved in RAN translation initiation of sense G4C2 transcripts ..	67
Figure 1.7 Therapeutic Strategies currently in development for ALS/FTD.	73
Figure 2.1 schematic diagram of major features in oligonucleotides designed to target RAN- associated RNA helicases	84
Figure 2.2 schematic diagram of major features in shRNAs designed to target RNA helicases..	90
Figure 2.3 Plasmid map for pENTR eIF4A1 shRNA-3.....	95
Figure 2.4 Plasmid map for pENTR eIF4A2 shRNA-2.....	96
Figure 2.5 plasmid map for pENTR eIF4A1+2 shRNA-1	97
Figure 2.6 Plasmid map for pENTR eIF4B 29m shRNA.....	98
Figure 2.7 Plasmid map for pENTR DHX36 shRNA-1.....	99
Figure 2.8 Plasmid map for scAAV eIF4A1 shR-3.....	100
Figure 2.9 Plasmid map for scAAV eIF4A2 shR2	101
Figure 2.10 Plasmid map for scAAV DHX36 shR1	102
Figure 2.11 Plasmid map for LV-eIF4A1 shRNA3	103
Figure 2.12 Plasmid Map for LV-eIF4A2 shRNA2	104
Figure 2.13 : Plasmid Map for LV-eIF4A1+2 shRNA1	105
Figure 2.14 Plasmid Map for LV-DHX36 shRNA1	106
Figure 3.1 An overview of the cloning method and strategy.....	131
Figure 3.2 Agarose Gel Electrophoresis for cloning of eIF4B shRNA into a pENTR backbone.	132
Figure 3.3 Testing pENTR-eIF4A1, eIF4A2 and eIF4B shRNAs.....	133

Figure 3.4 Agarose gel electrophoresis to confirm cloning of eIF4A1, eIF4A2 and DHX36 shRNAs into an scAAV vector.....	135
Figure 3.5 Gel extraction of LV-GDNF vector and eIF4A1, eIF4A2, eIF4A1+2 and DHX36 shRNAs.	136
Figure 3.6 Agarose gel electrophoresis for cloning of eIF4A1, eIF4A2, eIF4A1+2 and DHX36 shRNA into an LV-GDNF vector..	136
Figure 3.7 Agarose gel electrophoresis to confirm cloning of eIF4A1, eIF4A2, eIF4A1+2 and DHX36 shRNAs into LV-GDNF.....	137
Figure 3.8 Western Blot to show knockdown of eIF4A1, eIF4A2, eIF4B or eIF4H by shRNA. ..	139
Figure 3.9 RT qPCR data showing the strength and specificity of eIF4A1, eIF4A2, eIF4B and eIF4H shRNA knockdown in HEK293T cells.	141
Figure 3.10 Western Blot to confirm consistent RAN-associated helicase knockdown using Origene LV-shRNAs.....	142
Figure 3.11 Western Blots for HEK293T cells co-transfected with pENTR shRNAs and Sense DPRs.....	143
Figure 3.12 Western Blots for HEK293T cells co-transfected with pENTR shRNAs and antisense DPRs.	144
Figure 3.13 Western Blot and quantified results for HEK293T cells co-transfected with RAN- associated RNA helicase shRNAs and Sense DPRs..	146
Figure 3.14 Western Blot and quantified results for HEK293T cells co-transfected with RAN- associated RNA helicase shRNAs and Antisense DPRs.	146
Figure 3.15 Schematic diagram of RAN-associated helicase mutants eIF4A1 Q339E/PRRVAA and eIF4A2 Q340E/PRRVAA.....	148
Figure 3.16 Expression of RAN-associated RNA Helicase mutants eIF4A1-Q339E and eIF4A2- Q340E leads to decreased production of sense DPRs in HEK293T cells.....	149
Figure 3.17 RAN-associated RNA Helicase mutants eIF4A1-Q339E and eIF4A2-Q340E result in decreased production of antisense DPRs in HEK293T cells.....	150
Figure 3.18 RAN-associated RNA Helicase mutants eIF4A1 PRRVAA and eIF4A2 PRRVAA result in decreased production of sense DPRs in HEK293T cells..	151

Figure 3.19 RAN-associated RNA Helicase mutants eIF4A1 PRRVAA and eIF4A2 PRRVAA result in decreased production of antisense DPRs in HEK293T cells. 1.	152
Figure 4.1 Summary of brain regions examined in post-mortem control, sporadic ALS and C9-ALS/FTD patients.....	169
Figure 4.2 eIF4A1 antibody optimisation for IHC.	171
Figure 4.3 eIF4A2 antibody optimisation for IHC.	171
Figure 4.4 eIF4B antibody optimisation for IHC..	172
Figure 4.5 eIF4H antibody optimisation for IHC.....	172
Figure 4.6 SRSF1 antibody optimisation for IHC.	173
Figure 4.7 Trials of alternative loading controls in frozen post-mortem human spinal cord..	175
Figure 4.8 Western Blot from frozen post-mortem human spinal cord probed for eIF4A1 and eIF4A2. 1.	176
Figure 4.9 Western Blot from frozen post-mortem human spinal cord probed for eIF4H.....	177
Figure 4.10 Western Blot from frozen post-mortem human motor cortex.....	179
Figure 4.11 eIF4A1 immunohistochemistry in control and ALS Spinal Cord.....	181
Figure 4.12 eIF4A2 immunohistochemistry in control and ALS Spinal Cord.....	182
Figure 4.13 Immunohistochemistry showing eIF4A1-positive inclusions in sALS Spinal Cord. .	183
Figure 4.14 Immunohistochemistry showing eIF4A1-positive inclusions in C9-ALS/FTD Spinal Cord.....	185
Figure 4.15 Immunohistochemistry in ALS Spinal Cord showing eIF4A1-positive ambiguous inclusions.....	186
Figure 4.16 Summary of eIF4A1-positive inclusion burden in spinal cord motor neurons..	187
Figure 4.17 Immunohistochemistry for alternative phosphorylated TDP-43 antibodies	188
Figure 4.18 Immunohistochemistry to test p62 antibody specificity and dilution	189
Figure 4.19 Double Immunohistochemistry of eIF4A1 and phosphorylated TDP-43..	190
Figure 4.20 Double Immunohistochemistry of eIF4A1 and phosphorylated TDP-43.	191
Figure 4.21 Double immunohistochemistry of eIF4A1 and phosphorylated TDP-43 positive pathologies in the same cell.....	192

Figure 4.22 Double immunohistochemistry of eIF4A1 and p62-positive pathologies in the same cell.....	194
Figure 4.23 SRSF1 immunohistochemistry in control and ALS spinal cord.	200
Figure 4.24 SRSF1-positive glial cell labelling in C9-ALS/FTD Motor Cortex.	201
Figure 4.25 SRSF1-positive glial cell labelling in motor cortex underlying white matter	202
Figure 4.26 SRSF1-positive glial labelling in Prefrontal Cortex underlying white matter.....	203
Figure 4.27 SRSF1-positive glial labelling in Temporal Lobe underlying white matter.....	204
Figure 4.28 SRSF1 immunohistochemistry in control and ALS Hippocampal neurons.....	205
Figure 4.29 eIF4A1 immunohistochemistry in mouse spinal cord..	206
Figure 4.30 eIF4A2 immunohistochemistry in mouse spinal cord..	207
Figure 5.1 MTT assay for LV-shRNAs targeting eIF4A1, eIF4A2, eIF4B or eIF4H.	221
Figure 5.2 MTT assay for pENTR-shRNAs targeting eIF4A1, eIF4A2, eIF4A1+4A2 and eIF4B.	223
Figure 5.3 Partial depletion of eIF4A1, eIF4A2 and eIF4B causes only mild stress granule formation..	225
Figure 5.4 MTT assays of HEK293T cells co-transfected with helicase shRNAs and sense or antisense DPRs..	227
Figure 5.5 MTT Assays of HEK293T cells co-transfected with helicase mutants and sense or antisense DPRs	229
Figure 5.6 MTT Assays of HEK293T cells co-transfected with RNA binding mutants and sense or antisense DPRs	230
Figure 5.7 Partial depletion of eIF4A1, eIF4A2, eIF4B and eIF4H on healthy mouse motor neurons and patient-derived astrocyte co-cultures.	232
Figure 5.8 Effects of partial depletion of eIF4A in Drosophila.....	234
Figure 5.9 Cellular distribution of TDP-43 in healthy controls, sALS and C9-ALS/FTD patient- derived neurons	235
Figure 5.10 Enlarged merged images of cellular distribution of C-terminal TDP-43 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons.	236

Figure 5.11 Cellular distribution of phosphorylated TDP-43 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons.	237
Figure 5.12 Enlarged merged images of cellular distribution of phosphorylated TDP-43 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons.	238
Figure 5.13 Cellular distribution of eIF4A1 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons.	239
Figure 5.14 Inclusion formation eIF4A1 in sALS and C9-ALS/FTD patient-derived neurons.....	240
Figure 5.15 Cellular distribution of eIF4A2 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons	241
Figure 5.16 Enlarged Merged images showing cellular distribution of eIF4A2 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons.	242
Figure 5.17 Cellular distribution of phosphorylated TDP-43 and eIF4A1 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons.....	243
Figure 5.18 Inclusion formation eIF4A1 in sALS and C9-ALS/FTD patient-derived neurons.....	244
Figure 5.19 Cellular distribution of phosphorylated TDP-43 and eIF4A2 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons.....	245
Figure 5.20 Cellular distribution of phosphorylated TDP-43 and eIF4A2 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons.....	246
Figure 8.1 eIF4A1 immunohistochemistry in control and ALS Motor Cortex	314
Figure 8.2 eIF4A1 immunohistochemistry in control and ALS Prefrontal Cortex.	315
Figure 8.3 eIF4A1 immunohistochemistry in control and ALS Temporal Lobe.	316
Figure 8.4 eIF4A1 immunohistochemistry in control and ALS Hippocampus.....	317
Figure 8.5 eIF4A1 immunohistochemistry in control and ALS Hippocampal neurons.....	318
Figure 8.6 eIF4A1 immunohistochemistry in control and ALS Cerebellum..	319
Figure 8.7 eIF4A2 immunohistochemistry in control and ALS Motor Cortex.	320
Figure 8.8 eIF4A2 immunohistochemistry in control and ALS Prefrontal Cortex.	321
Figure 8.9 eIF4A2 immunohistochemistry in control and ALS Temporal Lobe.	322
Figure 8.10 eIF4A2 immunohistochemistry in control and ALS Hippocampus.....	323
Figure 8.11 eIF4A2 immunohistochemistry in control and ALS Hippocampal neurons.....	324

Figure 8.12 eIF4A2 immunohistochemistry in control and ALS Cerebellum..	325
Figure 8.13 eIF4B immunohistochemistry in control and ALS Spinal Cord.	326
Figure 8.14 eIF4B immunohistochemistry in control and ALS Motor Cortex..	327
Figure 8.15 eIF4B immunohistochemistry in control and ALS Prefrontal Cortex..	328
Figure 8.16 eIF4B immunohistochemistry in control and ALS Temporal Lobe..	329
Figure 8.17 eIF4B immunohistochemistry in control and ALS Hippocampus.....	331
Figure 8.18 eIF4B immunohistochemistry in control and ALS Hippocampal neurons.....	332
Figure 8.19 eIF4B immunohistochemistry in control and ALS Cerebellum.	333
Figure 8.20 eIF4H immunohistochemistry in control and ALS Spinal Cord.....	334
Figure 8.21 eIF4H immunohistochemistry in control and ALS Motor Cortex.....	335
Figure 8.22 eIF4H immunohistochemistry in control and ALS Prefrontal Cortex	336
Figure 8.23 eIF4H immunohistochemistry in control and ALS Temporal Lobe..	337
Figure 8.24 eIF4H immunohistochemistry in control and ALS Hippocampus.	338
Figure 8.25 eIF4H immunohistochemistry in control and ALS Hippocampal neurons. .	339
Figure 8.26 eIF4H immunohistochemistry in control and ALS Cerebellum..	340
Figure 8.27 SRSF1 immunohistochemistry in control and ALS Motor Cortex.....	341
Figure 8.28 SRSF1 immunohistochemistry in control and ALS Prefrontal Cortex.	342
Figure 8.29 SRSF1 immunohistochemistry in control and ALS Temporal Lobe..	343
Figure 8.30 SRSF1 immunohistochemistry in control and ALS Hippocampus.....	344
Figure 8.31 SRSF1 immunohistochemistry in control and ALS Cerebellum.	345

List of Tables

Table 2.1: Details of patient-derived neuronal cell lines used for immunofluorescence staining.	83
Table 2.2 Best miRNA targets for human eIF4A1.....	84
Table 2.3 Oligonucleotide design to target eIF4A1 and eIF4A1 + 2.....	85
Table 2.4 Best miRNA targets for human eIF4A2.....	86
Table 2.5 miR 1 and miR 2 to target eIF4A2	87
Table 2.6 Best miRNA targets for eIF4B.....	87
Table 2.7 Oligonucleotide to target eIF4B	88
Table 2.8 Best miRNA targets for human DHX36.....	88
Table 2.9 Oligonucleotide design to target DHX36.....	89
Table 2.10 pENTR/H1-TO-MCS/ eIF4A1 shRNA3	90
Table 2.11 scAAV-KAS/ H1-eIF4A1 shRNA_EF-1-eGFP	91
Table 2.12 pENTR/H1-TO-MCS/ eIF4A2 shRNA1	91
Table 2.13 pENTR/H1-TO-MCS/ eIF4A2 shRNA2	92
Table 2.14 scAAV-KAS/ H1-eIF4A2 shRNA_EF-1-eGFP.....	92
Table 2.15 pENTR/H1-TO-MCS/ eIF4AB 29mer-shRNA	93
Table 2.16 scAAV-KAS/ H1-eIF4B 29mer-shRNA_EF-1-eGFP	93
Table 2.17 scAAV-KAS/ H1-DHX36 shRNA1_EF-1-eGFP.....	94
Table 2.18 Antibiotics use for inoculations.....	106
Table 2.19 Restriction Enzymes	109
Table 2.20: Primary Antibodies	110

Table 2.21 Secondary Antibodies	110
Table 2.22 Details of Post-Mortem Human Tissue Donors	112
Table 2.23 Details of additional post-mortem human tissue used when main cohort was unavailable	113
Table 4.1 Summary of helicase immunohistochemistry across the spinal cord and CNS regions	196

1 Introduction

1.1 Epidemiology and Clinical Features of ALS

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease characterised by progressive degeneration of upper motor neurons of the motor cortex, and lower motor neurons of the brainstem and anterior horn of the spinal cord. Worldwide, the prevalence varies based on region and ranges between 4.1 and 8.4 people per 100,000. In one meta-analysis of 13,146 cases from 45 geographical regions, the average prevalence was found to be 1.75 people per 100,000 (Marin et al., 2017). Patients are typically in their 50s or 60s when diagnosed, with a subsequent decline in onset frequency from age 75 onwards (Longinetti & Fang, 2019). As no definitive test currently exists, diagnosis of ALS is based on clinical symptoms, family history, and ruling out other conditions. Clinically, approximately two-thirds of patients present with a limb-onset phenotype. Of the remaining third of patients, most experience a bulbar onset and present with difficulties in speech and swallowing (Hardiman et al., 2011). Less than 5% present with respiratory-onset ALS, which is associated with the shortest duration between diagnosis and patient death of all clinical presentations (Orrell & Guilloff, 2020). ALS progressive muscle paralysis eventually effects respiratory muscle function, and the majority of patients will die of respiratory failure within five years of diagnosis; fewer than 10% of patients survive longer than 10 years (Hobson et al., 2016). Currently, licenced treatments for ALS include Riluzole, an anti-glutamate agent (Miller et al., 2002) and the newer Edaravone, a neuroprotective free radical scavenger (Hardiman & van den Berg, 2017). However, access to these drugs can be limited, with Edaravone only licensed for use to treat ALS in the USA, Japan, South Korea and Switzerland (Breiner et al., 2020). The European Medicines Agency withdrew their application for use of Edaravone in 2019, due to the publication of several studies indicating limited clinical benefit (Witzel et al., 2022). It is currently not licenced for use for patients in the United Kingdom.

Overall, both these drugs have a very limited effect on disease progression, disability, and overall patient lifespan. Tofersen is a new antisense oligonucleotide that is currently undergoing trials in the USA, Canada and Europe for approval for treatment of SOD1-ALS (Miller et al., 2020) and while this is an encouraging step forward, SOD1-ALS patients represent a minority of total ALS patients and this drug is not suitable for other genetic subtypes of the disease.

1.2 Genetic causes of ALS

The majority of ALS cases are classed as sporadic (sALS) with only 10% associated with a known genetic cause (Talbot et al., 2016) and classed as familial (fALS). While over 30 ALS-linked gene mutations have now been identified, only four account for half of fALS cases (Zou et al., 2017). These are in *C9ORF72*, which accounts for 40-50% of fALS, equivalent to 4-5% of total cases; *TARDBP*, accounting for 4% of fALS cases and 1% sALS; *SOD1*, accounting for 20% of fALS and 2% sALS; and *FUS*, accounting for 5% fALS and <1% sALS (Chia et al., 2018). This thesis will focus on pathological G4C2 hexanucleotide repeat expansions in the *C9ORF72* gene, which in a seminal discovery for the field was found to represent the most common genetic cause of ALS (Renton et al., 2011); (DeJesus-Hernandez et al., 2011). In neurologically healthy patients, the G4C2 repeat is generally under 30 repeats, with over 30 repeats traditionally seen as the threshold for disease onset. These are polymorphic repeat expansions, and there is substantial variability in repeat length among patients, with many having an unambiguously pathogenic expansion of many hundreds, or thousands, of G4C2 repeats. Similarly to other repeat expansion disorders it had been hypothesised that repeat length influenced age of disease onset, but a recent study has found no significant effect on this or disease phenotype (Fournier et al., 2019). A number of environmental factors have been linked to ALS onset (Zufiría et al., 2016) and these may have a more influential role.

1.3 *C9ORF72* G4C2 repeat expansions and the ALS/FTD disease spectrum

For many years prior to the discovery of the *C9ORF72* G4C2 repeat expansion, ALS had been observed to exist on a single clinical spectrum with Frontotemporal Dementia (FTD). The discovery of the G4C2 repeat expansion explained the link between these two seemingly different forms of neurodegeneration, which have highly divergent sets of symptoms. FTD is characterised by progressive neurodegeneration of the frontal and temporal lobes. It has a prevalence of 15-22 people per 100,000 (Onyike & Diehl-Schmid, 2013), making it the second most common form of dementia in patients aged under 65 years (Rosso et al., 2003). Clinically, patients with FTD present with one of two variants primarily affecting behaviour (bvFTD) or language; the former is more commonly associated with the *C9ORF72* repeat expansion. Language variants are subdivided into semantic dementia (SD), also called primary progressive aphasia (PPA), or progressive non-fluent aphasia (PNFA) (Lashley et al., 2015). FTD has a larger heritable component compared to ALS, with familial cases accounting for up to 50% in FTD (Rohrer et al., 2009). There is substantial clinical overlap between ALS and FTD. Approximately 50% of patients originally diagnosed with ALS develop some cognitive or behavioural component to their illness with half of these meeting the diagnostic criteria for FTD (Couratier et al., 2017; Swinnen & Robberecht, 2014). Those first diagnosed with FTD can also then develop motor impairments, with 15% going on to receive an ALS diagnosis (Cruts et al., 2013). This is now known as the *C9ORF72* ALS/FTD disease spectrum, or C9-ALS/FTD.

1.4 TDP-43 and *C9ORF72* pathologies in ALS/FTD

The predominant pathology in all subtypes of ALS is of TDP-43 aggregates. TDP-43 is part of the heterogeneous nuclear RNA binding protein (hnRNP) family and has roles linked to RNA stability, regulation and processing (Buratti & Baralle, 2010). There is a mis-localisation of TDP-43 from the nucleus to the cytoplasm where it accumulates

into pathological aggregates, which are considered the hallmark pathology of ALS. Pathological forms of TDP-43 in ALS and FTD have a truncated N-terminal, and hyperphosphorylated, ubiquitylated C-terminal fragments (Feneberg et al., 2021). Cytoplasmic TDP-43 is prone to aggregation, with the 35kDa fragment acting as a seed and recruiting full-length TDP-43 to form further aggregates (Che et al., 2011). This hallmark pathology is seen in 97% of ALS patients and correlates well with neuronal loss (Neumann et al., 2006). A staging system for assessing burden of TDP-43 pathology was developed by Brettschneider and colleagues (Brettschneider et al., 2013). Mutations in *TARDBP*, the gene that codes for TDP-43, are associated with increased aggregation and formation of neurotoxic, insoluble TDP-43 species (Guo et al., 2011). C-terminal *TARDBP* mutations in particular are associated with increased TDP-43 cleavage (Sreedharan et al., 2008). Overall, both loss and gain of function mutations have been identified and linked to approximately 5% of fALS cases (Shatunov & Al-Chalabi, 2021).

TDP-43 inclusions are classified based on localisation to the neuronal cytoplasm or nucleus as Neuronal Cytoplasmic Inclusions (NCIs) or Neuronal Intranuclear Inclusions (NIIs). Abnormal neuronal processes, including aberrant conformations, less complex morphology and shorter length, are classed pathologically as Dystrophic Neurites (DNs). Glial pathology in the form of Glial Cytoplasmic Inclusions (GCIs) are also seen (Arai et al., 2006; Brandmeir et al., 2008; Neumann et al., 2007). TDP-43-positive pathology is subdivided into four pathological subtypes: A, B, C and D. Type A displays frequent NCIs and DN with infrequent NIIs; type B displays frequent NCIs and infrequent DN in all cortical layers; type C displays frequent long, corkscrew-shaped DN; type D displays frequent NIIs and DN with infrequent NCIs. Type B is most commonly associated with the C9ORF72 repeat expansion, although in rare cases type A or C are also seen (Bigio et al., 2013; Lashley et al., 2015; Mahoney et al., 2012). C9-ALS/FTD has specific dipeptide repeat protein (DPR) pathology,

comprising star-shaped TDP-43 and tau-negative, ubiquitin and P62-positive inclusions in the cerebellar cortex, neocortex and hippocampus (Lashley et al., 2015).

Frontal Temporal Lobar Degeneration (FTLD) is the pathological term for the set of symptoms that comprise the clinical syndrome of FTD. Approximately 50% of FTD patients have TDP-43 pathology; other key proteins found in pathological aggregates in FTLD include tau and FUS, reviewed in full by Lashley and colleagues (Lashley et al., 2015). FTLD is a challenge for researchers in that it has substantial clinical, genetic and pathological heterogeneity. The lack of clear relationship between the clinical presentation of the disease and its underlying genetics and pathology is summarised in Figure 1.1. This may reflect differing disease mechanisms that converge on similar sets of symptoms and thus require different treatment strategies.

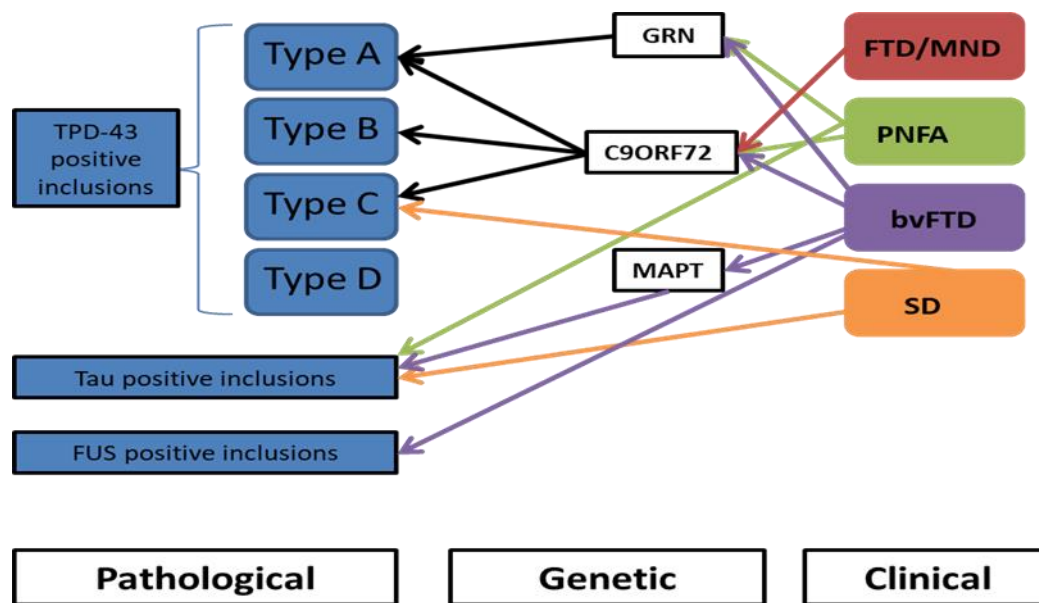


Figure 1.1: Pathological, genetic and clinical correlations in FTLD. Most common clinical presentations of FTLD (shown on right) are Frontotemporal Dementia with Motor Neuron Disease (FTD/MND), Progressive Non-Fluent Aphasia (PNFA), Behavioural Variant FTD (bvFTD) and Semantic Dementia (SD). Arrows indicate lack of clear relationship with genetics and pathology associated with each clinical presentation. Most common gene mutations associated with FTLD (shown centre) are progranulin (GRN), C9ORF72, and Microtubule Associated Protein Tau (MAPT). Patients with GRN mutations present with PNFA or bvFTD and have TDP-43 type A pathology; patients with C9ORF72 G4C2 repeat expansions present with FTD/MND, PNFA or bvFTD and have type B, and rarely type A or C pathology; patients with MAPT mutations present with bvFTD and have tau-positive TDP-43-negative pathology. SD rarely associates with a genetic cause and has TDP-43 type C or tau pathology. TDP-43 type D pathology is associated with valosin-containing protein (VCP) mutations, not shown here. (Bigio et al., 2013; Lashley et al., 2015; Mahoney et al., 2012)

1.5 C9ORF72 shares homology with DENN proteins and has functions relating to autophagy and energy homeostasis

Since the identification of C9-ALS/FTD, considerable research attention has focused on the normal function of C9ORF72. The *C9ORF72* gene is comprised of 11 exons, producing three transcript variants and two protein isoforms. These are summarised in Figure 1.2, including the location of the G4C2 expansion which varies depending on the transcript variant.

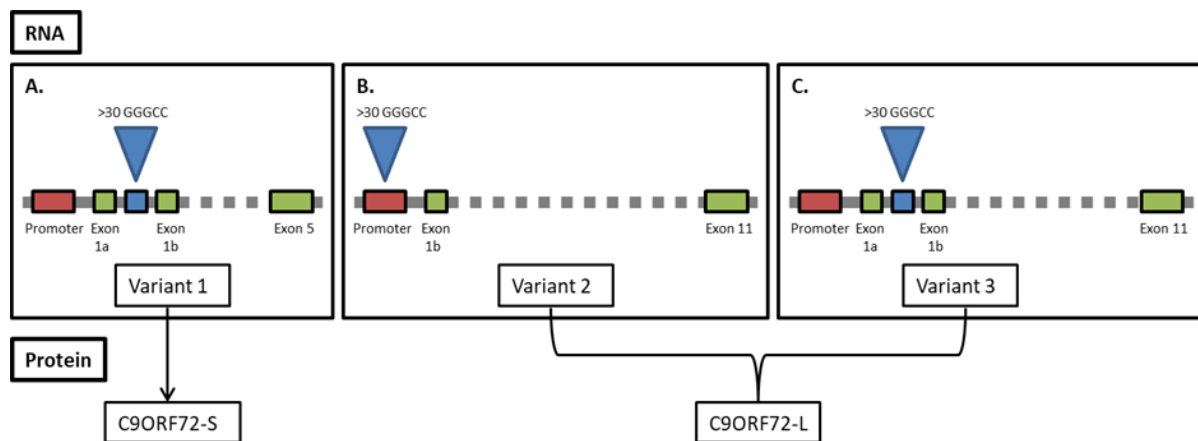


Figure 1.2: C9ORF72 RNA transcript variants and associated protein isoforms. (A) Shows variant 1, where the transcript contains a shortened first exon and terminates at exon 5 with a premature stop codon. The final six exons are excluded to encode a 222 amino acid short-form (C9ORF72-S) (Shangxi Xiao et al., 2016). The G4C2 repeat expansion is located in intron 1. (B) and (C) produce the 481 amino acid long-form (C9ORF72-L). (B) Shows variant 2, comprised of 11 exons commencing with exon 1b. The G4C2 repeat expansion is located in the promoter region. (C) shows variant 3, comprised of 11 exons and utilising full length exon 1a; the G4C2 repeat expansion is located in intro 1 (Douglas, 2018a)

The G4C2 repeat expansion is located in intron-1 in variants one and three, and the promoter region of variant two. Splicing of intron-1 was found to be unaffected in C9ORF72 ALS post-mortem human tissue and in patient-derived neurons. C9ORF72 pre-mRNAs retaining the intron-1 repeat expansion accumulate in the cytoplasm and are dependent on splicing factor SRSF1 for nuclear export (Hautbergue et al., 2017; Tran et al., 2015).

Some variation has been reported between the two isoforms C9ORF72-L and C9ORF72-S, which has been reviewed in full by Xiao and colleagues. Quantification of isoform levels indicates differential expression in C9-ALS/FTD cases with increased expression of C9ORF72-S and decreased C9ORF72-L especially in frontal and temporal cortices (Xiao et al., 2015; S. Xiao et al., 2016). Both isoforms show similar staining patterns in immortalised cell lines such as HEK293T cells, but variability in human brain tissue, indicating a level of functional divergence. Here, C9ORF72-L shows diffused labelling of the neuronal cytoplasm while C9ORF72-S is restricted to the nuclear membrane in healthy neurons and is re-localised to the plasma membrane

in ALS/FTD motor neurons. This appears to be the case even in non-C9 ALS cases indicating isoform variability may contribute to different forms of ALS (Xiao et al., 2015). Loss of C9ORF72-S from the nuclear membrane correlates with TDP-43 mislocalisation indicating this isoform may contribute to TDP-43 shuttling across the nuclear membrane (S. Xiao et al., 2016).

Looking to other species can provide additional information on *C9ORF72* function. It is a highly conserved gene, indicating its fundamental importance, and has orthologs in diverse species including *C.elegans*, zebrafish and mice. These show 23%, 76%, and 98% homology to the human counterpart, respectively (Therrien et al., 2013). The high homology between human and mouse *C9ORF72* suggests this species may be a particularly appropriate disease model for C9-ALS/FTD. However, the normal function of *C9ORF72* has not been fully defined. Functional divergence may exist between the two protein isoforms, C9ORF72-S and C9ORF72-L. In 2012, a bioinformatics analysis identified *C9ORF72* as encoding a differentially expressed in normal neoplastic cells (DENN) protein (Zhang et al., 2012). DENN proteins act as GTP-GDP exchange factors that activate Rab GTPases (Levine et al., 2013). These function as a molecular switch to regulate membrane trafficking (Mizuno-Yamasaki et al., 2012).

Protein interactions currently identified for C9ORF72 include those related to the initiation of autophagy, nucleocytoplasmic transport of protein cargo, endosomal trafficking and vesicle release (Aoki et al., 2017; Behrends et al., 2010; Farg et al., 2014; Prpar Mihevc et al., 2017; Sellier et al., 2016; Webster et al., 2016). Further studies have made the link between C9ORF72 as a regulator of endosomal trafficking and lysosomal degradation (Farg et al., 2017). C9ORF72 regulates autophagy and membrane trafficking in a trimeric complex with SMCR8 and WDR41, which acts as a guanine exchange factor for RAB GTPases RAB8A and RAB39B. The complex interacts with ULK1 to regulate autophagy (Tang et al., 2020). A study looking at

several hundred missense mutations in *C9ORF72* revealed that such mutations affect protein stability and binding affinity of protein complexes. Binding between *C9ORF72* and *SMCR8* was destabilised which was critical for GTPase activity (Xue et al., 2022). In ALS/FTD patient-derived neurons carrying the *C9ORF72* repeat expansion, autophagy was impaired (Webster et al., 2016). P62, a substrate of the autophagy pathway, is found aggregated in FTD patient derived neurons from patients carrying a *C9ORF72* repeat expansion, but not a progranulin mutation (Almeida et al., 2013) further highlighting the link between *C9ORF72* and autophagy function.

C9ORF72 has also been functionally linked to ATP production and energy homeostasis through regulation of oxidative phosphorylation. This occurs via inhibition of protease-dependent degradation of essential component of electron transport chain complex 1, TIMMDC1. *C9ORF72* is imported into the mitochondria in a highly conserved manner, highlighting the importance of this mechanism for energy homeostasis and stabilisation of the electron transport chain. Approximately 6% of *C9ORF72* is imported into the mitochondrial inner membrane space where it assists with energy buffering capacity. Although a small proportion of overall *C9ORF72*, this is still highly concentrated in the inner membrane space (Wang et al., 2021). Mutations in *C9ORF72* have also been linked to reduction in Ca^{2+} buffering capacity, leading to reduced mitochondrial uptake of Ca^{2+} , thereby contributing to glutamate-induced excitotoxicity and eventually cell death (Dafinca et al., 2020). Additionally, *C9ORF72* has been linked to oxidative phosphorylation control through stabilisation of mitochondrial complex I. This is impaired in ALS/FTD patient-derived neurons, indicating not only that *C9ORF72* has a novel mitochondrial function, but also highlighting energy metabolism dysfunction as a possible contributor to neurodegeneration in C9-ALS/FTD (Wang et al., 2021).

1.6 Proposed pathogenic mechanisms in C9-ALS/FTD

Many investigations into the normal function of *C9ORF72* have looked at the impact of its depletion on biological systems. This has helped inform the first of three proposed disease mechanisms for the pathological *C9ORF72* G4C2 repeat expansion, that of haploinsufficiency. The other two proposed mechanisms are formation of RNA foci and translation of the repeat expansion to form toxic DPRs.

1.6.1 Haploinsufficiency

Several lines of evidence support *C9ORF72* haploinsufficiency as a contributing pathogenic mechanism in C9-ALS/FTD. In ALS/FTD patient-derived neurons with the G4C2 repeat expansion, deficits in autophagy can be alleviated by *C9ORF72* over expression, implicating haploinsufficiency of *C9ORF72* as underpinning dysfunction seen in ALS/FTD (Webster et al., 2016). In post-mortem brain tissue from C9-ALS/FTD patients, reduced levels of *C9ORF72* transcripts and protein are found compared to neurologically normal controls (Y. Liu et al., 2022; Waite et al., 2014). The *C9ORF72* repeat expansion is rich in CpG islands, increasing the likelihood of gene silencing via hypermethylation. Reduced *C9ORF72* expression is linked to trimethylation of lysine residues in histones H3 and H4 (Belzil et al., 2013). *C9ORF72*-L was found to be the predominant form expressed in the human brain, and levels were reduced by 80% in *C9ORF72* repeat expansion carriers compared to controls, although this did not correlate with any particular clinical phenotype (Frick et al., 2018).

In a study of 389 *C9ORF72* mutation carriers, there was a failure to identify any coding-region mutations that would lead to decreased production of functional *C9ORF72* protein (Harms et al., 2013). A significant piece of evidence that undermines the haploinsufficiency hypothesis is that homozygous and heterozygous *C9ORF72* repeat expansion carriers do not show any differences in clinical or pathological features to

their illness (Fratta et al., 2013). This is inconsistent with the hypothesis in that if haploinsufficiency was the main driving force in C9-ALS/FTD it would be expected that double mutation carriers would show a much worse disease phenotype.

Evidence from *C9ORF72* knock-outs results is mixed. The reasons for these inconsistencies are unclear but may relate to species-specific differences in *C9ORF72* function, or reflect issues in reproducing the human disease in the various models studied. In knock-outs of *C.elegans* and zebrafish *C9ORF72* orthologues, motor neuron degeneration occurs along with corresponding motor deficits (Therrien et al., 2013). Motor deficits can be rescued by injection of human *C9ORF72* mRNA thereby supporting a loss-of-function hypothesis for C9-ALS/FTD (Ciura et al., 2013). However, in mouse knock-outs of *C9ORF72*, behavioural and motor abnormalities, ALS/FTD pathology and neurodegeneration are absent (Koppers et al., 2015). Instead, a phenotype indicating immune dysfunction is seen (Atanasio et al., 2016). In other studies, mice expressing the G4C2 repeat expansion did show motor impairments, and these correlated with the extent of *C9ORF72* loss (Shao et al., 2019). *C9ORF72* deficiency has been linked to alterations in microglia transcriptional profile, promoting their alteration to an inflammatory state. Normally functioning *C9ORF72* appears crucial for maintaining microglia homeostasis (Hashimoto et al., 2022). Depletion in microglia leads to neuronal dysfunction and synapse loss. Crucially for the haploinsufficiency hypothesis, certain phenotypes such as synapse loss were only seen in homozygous *C9ORF72* knock-out mice (Lall et al., 2021). Dysfunctions in microglia and links with increased neuroinflammation have recently become of particular interest in the field of ALS, and it may be that *C9ORF72* haploinsufficiency is a contributor to this. If haploinsufficiency of *C9ORF72* alters microglia transcriptomics, this may lead to altered inflammatory response (Lall et al., 2021). This is supported by increased CD68 in ALS patient motor cortex. CD68 is a commonly used microglial marker in the CNS (Spiteri et al., 2022) and this increase is therefore suggestive of an enhanced inflammatory response (Brettschneider et al., 2012).

C9ORF72 haploinsufficiency has also been linked to failures in energy homeostasis through its interactions with electron transport chain complex I. Reduced C9ORF72 destabilised the electron transport chain and is associated with decreased complex I levels of up to 50% (Wang et al., 2021). C9ORF72 haploinsufficiency has been linked to deficits in autophagy, which C9ORF72 is responsible for regulating. This may in turn contribute to exacerbation of immune dysfunction and impaired proteostasis (Lai & Ichida, 2019). Haploinsufficiency of C9ORF72 in iPSCs led to reduced lysosomal expression and impaired vesicle trafficking (Shi et al., 2018). In turn this results in failure to clear DPR aggregates, therefore increasing neurodegeneration, while modulating vesicle trafficking rescued neurodegeneration. This suggests a co-operation, or mechanistic convergence, between C9ORF72 haploinsufficiency and gain-of-function mechanisms in C9-ALS/FTD.

Knocking out C9ORF72 in patient-derived motor neurons with the repeat expansion leads to deficits in axonal trafficking and eventually, apoptosis. Levels of HSP70 and HSP40 fell, further exacerbating the neurodegenerative phenotype. However, in healthy control cells knocking out C9ORF72 did not reproduce these deficits, suggesting a combination of loss-of-function and gain-of-function mechanism are in play in C9-ALS/FTD (Abo-Rady et al., 2020). Combined loss and gain of function models have been suggested by several groups (Shi et al., 2018) but lack of good disease models that incorporate both mechanisms holds back progress in this area. Failing to simultaneously consider loss and toxic gain-of-functions may be a reason why several animal models of C9-ALS/FTD have failed to show a disease phenotype. A recent study has shown that loss of C9ORF72 could enhance toxic gain-of-function effects resulting from the repeat expansion through autophagy suppression (Zhu et al., 2020). Haploinsufficiency may not be the immediate driving force of C9-ALS/FTD, but it likely remains a contributing factor to disease onset and progression.

1.6.2 RNA toxic gain-of-function mechanisms in C9-ALS/FTD

1.6.2.1 Sequestration of RNA binding proteins by C9ORF72 RNA foci leads to dysfunctional RNA metabolism

C9ORF72 repeat expansion sense and antisense RNA foci accumulate in the brain and spinal cord of C9-ALS/FTD patients (Gendron et al., 2013). Foci are formed in the nucleus from single mutant intronic RNA molecules with atypical secondary structures; in rare occasions cytoplasmic foci have also been detected (Liu et al., 2017) (McEachin et al., 2020). Foci are predominantly found in neurons although they can also appear in glia although much less frequently. Sense foci are thought to form more frequently than antisense foci although this may simply reflect that antisense foci can be harder to detect. In some cell types, such as cerebellar Purkinje cells and spinal motor neurons, antisense foci are thought to form more frequently (McEachin et al., 2020). Recently, foci formation was linked to paraspeckles, a type of ribonuclear body which are observed to form in the early stages of ALS (Bajc Česnik et al., 2019). RNA foci arising from the G4C2 repeat expansion share characteristics and co-localise with paraspeckles, and knockdown of essential paraspeckle protein SFPQ in fibroblasts harbouring the C4C2 repeat results in significant reductions in RNA foci formation (Bajc Česnik et al., 2019). There is some variation in frequency and size, but they are widespread and contribute to disease progression through sequestration of RNA binding proteins (RBPs). This alters their function in regulation of RNA metabolism, including transcription, processing, splicing and nuclear export of mRNA (Cooper-Knock et al., 2014b). It is possible that differences in proteins sequestered by RNA foci produce varying types of dysfunction that contributes to the onset of different clinical and pathological subtypes in C9-ALS/FTD.

A major family of RNA binding proteins sequestered by RNA foci in C9-ALS/FTD are the hnRNPs. These are a large, abundant and diverse family of RBPs including hallmark pathological proteins TDP-43 and FUS, with functions including DNA

replication and repair, transcriptional and splicing regulation, mRNA transcript export from the nucleus, and localization and initiation of protein synthesis (He & Smith, 2009). Depletion of TDP-43 from the nucleus results in its abnormal interactions with hnRNPA1, promoting inclusion of elongated prion-like exon 7B thereby producing the hnRNPA1B isoform. This increases the propensity for aggregation and is found co-localised with TDP-43 pathology. TDP-43 and hnRNPA1 together modulate over 30% of the transcriptome, so this one example demonstrates how altered TDP-43 splicing and mis-localisation results in a neurotoxic phenotype that has the potential for a very widespread impact on many RNA transcripts (Deshaies et al., 2018). In C9-ALS/FTD, hnRNP A1 and hnRNP H/F bind to sense RNA foci (Cooper-Knock, Higginbottom, et al., 2015; Davidson et al., 2017); sequestration results in abnormal splicing and correlates with DNA damage, telomere instability and overall severity of the disease phenotype (Conlon et al., 2016; Douglas, 2018b; Mohagheghi et al., 2016).

Pur- α , an RNA processing regulator, is also sequestered by RNA foci, along with some of its numerous binding partners including splicing factor SRSF1 (Reddy et al., 2013; Xu et al., 2013). Splicing abnormalities, accompanied by widespread transcriptome alterations, have been reported in the brains of patients with C9-ALS/FTD (Prudencio et al., 2015). RNA found in the brain undergoes more splicing than RNA found in other tissues (Yeo et al., 2004), and therefore the brain is very sensitive to this aspect of RNA metabolism and any associated dysfunctions therein. Almost all genes require splicing, and correct splicing is required to maintain proper neuronal function and relies on complex interactions between a number of RBPs and RNAs. Splicing is regulated by spliceosomes, which are large molecular complexes that include small nuclear RNAs and associated regulatory proteins (Rino & Carmo-Fonseca, 2009). Splicing deficits including splicing dysregulation and altered spliceosome dynamics are widespread in ALS motor neurons. RNA processing genes are preferentially affected by splicing errors, leading to altered expression of genes with splicing functions, thereby creating a toxic feedback loop (Highley et al., 2014).

Sequestration of SRSF1 and other nuclear export regulators such as ALYREF contribute to nuclear export of repeat-containing C9ORF72 mRNA transcripts to the cytoplasm for translation into neurotoxic DPRs (Cooper-Knock et al., 2014b; Hautbergue et al., 2017). SRSF1 is of particular interest as it has been shown to be required for the export of repeat-containing C9ORF72 mRNA transcripts to the cytoplasm, and inhibition of this function alleviates degeneration and associated motor deficits (Hautbergue et al., 2017). SRSF1 sequestration is also linked to increased formation of R-loops, thereby increasing genomic instability (Li & Manley, 2005). Nucleolin sequestration results in increased nucleolar stress and deficits in RNA processing and ribosome maturation. This causes downstream dysfunctions in proteostasis (Haeusler et al., 2014). This implicates sequestration of RBPs as an event starting a cascade of knock-on dysfunctions and linking this mechanism to the third proposed mechanism of DPR production as responsible for C9-ALS/FTD neurodegeneration.

Abnormal intron retention, reflecting splicing abnormalities, is seen in familial and sporadic ALS subtypes, and could be considered a unifying feature of the disease (Luisier et al., 2018). However splicing errors do not correlate well with ALS pathology, but there is a strong correlation with protein solubility states. It may be the case that neuronal vulnerability to splicing errors depends on other factors, namely the ratio of soluble to aggregated RBPs (Conlon & Manley, 2017). Shifts in solubility may reflect a wide range of underlying biochemical changes affecting RBPs, and splicing errors are simply one of many responses to this.

1.6.2.2 Promotion of abnormal phase separation through dysfunctional RNA metabolism

In C9-ALS/FTD, longer G4C2 repeats are associated with increased recruitment of proteins and RNAs to RNP granules. Dysfunction in RNA metabolism has been

implicated as a key mechanism through which RNP granules convert to a more aggregate prone state, and the recruitment seen in C9-ALS/FTD results in further downstream detrimental effects on RNA metabolism (Fay et al., 2017). It has previously been believed that in any membrane-less compartment solutes are evenly distributed. In fact, phase separation is a crucial biological mechanism whereby biochemical reactions can be organized into separate cellular compartments not bound by a membrane. Abnormal phase transitions are linked to dysfunctional RNA metabolism and appear to play an important role in neurodegeneration (Alberti & Hyman, 2016). TDP-43 and other RBPs have low complexity prion-like domains which are highly interactive with a propensity for aggregation. They initiate liquid-liquid phase separation (LLPS), the first step of formation of insoluble aggregates of certain low-complexity proteins (Harrison & Shorter, 2017). Failures in autophagy and protein degradation systems more broadly contribute to a shift towards aggregation and a failure to prevent these aberrant transitions. Aggregates that form are not cleared, and instead are able seed further inclusions. RNP granules recruiting further proteins overwhelm cellular degradation systems, exacerbating the abnormal conversions from liquid to solid states (Hyman et al., 2014). Other factors linked to the *C9ORF72* repeat expansion that influence abnormal phase transition include altered gene expression profiles, genomic instability, and DNA damage. This has been hypothesized to further alter the balance that maintains normal RNP granule dynamics to push them towards an insoluble state (Jain & Vale, 2017).

RNA transcripts act as a buffer to inhibit phase separation of nuclear RBPs. Depleted or dysfunctional RNA metabolism promotes aggregated, pathological TDP-43 which lacks an RNA recognition motif (RRM) (Kitamura et al., 2016; Maharana et al., 2018). This lack of an RRM alters the TDP-43 protein: transcript ratio, which is linked to increased aggregate formation. Without the ability to bind RNA, TDP-43 loses its dynamism, and is excluded from stress granules and transitions to an aggregate prone state (Mann et al., 2019). Therefore, correct RNA metabolism is of fundamental

importance for the cell to resist this aberrant transition: stress granule interactions between RBPs and their target RNAs helps maintain cellular liquidity. Under prolonged stress conditions, stress granules disintegrate resulting in release of pathological, aggregate prone TDP-43 (McGurk et al., 2018). While contrary to the original hypothesis that aggregation would be linked to increased stress granule recruitment (Portz et al., 2021), it is possible that stress granules play multiple roles. Some may prevent aggregate formation while others seed inclusions, possibly via a shift in composition and dynamics that promotes abnormal phase separation (Mann et al., 2019). The role of stress granules in ALS has been explored by a number of groups and reviewed thoroughly (Baradaran-Heravi et al., 2020; Hu et al., 2022). Stress granule dynamics are mediated by a number of ALS/FTD-linked RBPs including TDP-43. These have been shown to contribute to aberrant stress granule dynamics through their low-complexity domains thereby contributing to stress granule-mediated protein aggregation (Baradaran-Heravi et al., 2020). Stress granules undergo several assembly and disassembly stages throughout their lifecycles, with impairment in disassembly particularly linked to aberrant dynamics which lead to the accumulation of pathological stress granules in cells and contribute to ALS progression. Several groups have now begun to consider targeting the lifecycle of stress granules as a potential therapeutic approach in ALS (Hu et al., 2022).

1.6.3 Dipeptide Repeat Protein toxic gain-of-function

The third proposed mechanism for C9-ALS/FTD is the translation of the G4C2 repeat expansion into five dipeptide repeat proteins (DPRs). These are aggregate-prone proteins and form the hallmark pathology uniquely associated with the *C9ORF72* repeat expansion (Mori et al., 2013). Despite being located in an intron and lacking an AUG start codon, the G4C2 repeats are transcribed and translated in both sense and antisense direction through an unconventional mechanism called Repeat Associated Non-AUG (RAN) translation. This is seen in a number of microsatellite repeat

disorders in addition to C9-ALS/FTD (Zu et al., 2011). The DPRs produced are glycine-alanine (GA) and glycine-arginine (GR) from sense transcripts, proline-arginine (PR) and proline-alanine (PA) from antisense transcripts, and glycine-proline (GP) from both directions (Ash et al., 2013). A summary of DPR production is seen in Figure 1.3.

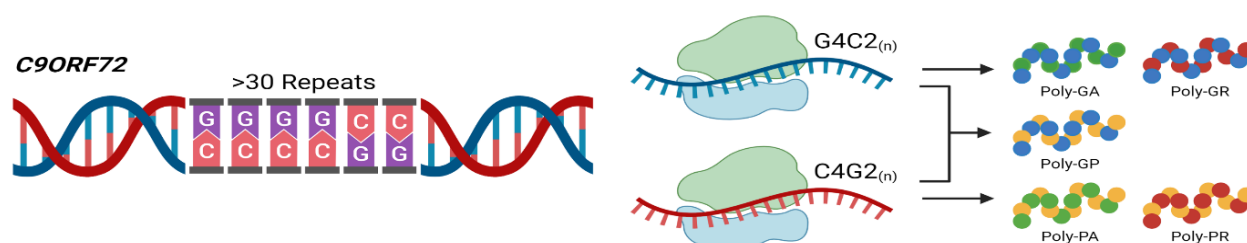


Figure 1.3: Summary of Dipeptide Repeat Proteins produced from sense and antisense C9ORF72 transcripts. Over 30 C9ORF72 repeats are associated with C9-ALS/FTD. The repeat undergoes bi-directional RAN translation, producing poly-GA and poly-GR from sense repeat-containing mRNA transcripts, poly-PA and poly-PR from repeat-containing antisense mRNA transcripts, and poly-GP in both sense and antisense directions. Figure made with BioRender.

DPRs vary in abundance and aberrant affects. There has been a lot of research interest surrounding DPRs and their respective toxicities; it is likely different species of DPRs exert different toxic effects. Poly-GP has been reported as showing minimal toxicity. Of alanine-containing DPRs, poly-GA was found to be less toxic than poly-PA (Jovičić et al., 2015). Arginine-containing DPRs have been reported to be the most toxic species, with consistent reports to support this from *Drosophila*, mouse studies, and post-mortem human tissue (Boeynaems et al., 2016; Kwon et al., 2014; S. Mizielinska et al., 2014; Wen et al., 2014). Toxicity of arginine-containing DPRs may be attributed to their localisation, aggregation state, size, or some organelle or cell-specific vulnerability. There may be mechanisms specifically associated with arginine and its specific properties. It is likely some combination of these factors that produces such toxicity in these DPRs.

In terms of abundance, poly-GA and poly-GP have been reported as the most frequent DPRs produced, while poly-PA and poly-PR are less common (Mackenzie et al., 2015). Poly-GP appears at stable levels with no alterations seen that are linked to clinical

progression (Gendron et al., 2017). Therefore, it appears there is an inverse relationship between the toxicity of a DPR and its abundance, although it may be that this simply reflects failures in detecting all species of DPRs to the same sensitivity. Specific antibodies have now been developed for DPRs, meaning they can be detected in immunoassays or through immunostaining techniques (Gendron et al., 2015). However, as DPRs are inherently of low complexity there are still challenges in developing antibodies that are highly specific and do not show cross-reactivity with other DPR species. It is possible that poly-PR is still toxic at levels undetectable by these studies. It may also be the case that these studies are showing a survivorship bias favouring less toxic DPRs, whereby the most toxic DPRs quickly lead to the death of cells. This could lead to them appearing less abundant in subsequent post-mortem pathology studies. It has also been hypothesized that the low toxicity, but high abundance of poly-GP pathology is a response to upstream events rather than responsible for disease progression in and of itself. This is consistent with the observation that poly-GP inclusions do not correlate well with neurodegeneration indicating the neurotoxicity is not coming from these inclusions but from some other source (Mackenzie et al., 2015). It is only poly-GR that shows any correlation with brain regions relevant to C9-ALS/FTD (Saber et al., 2018).

Accumulating DPRs result in a number of toxic effects, including disruption of major cellular signalling pathways such as Notch. In *Drosophila*, poly-GR production leads to suppressed Notch signalling and corresponding wing degeneration, while in iPSCs Notch genes are downregulated (Yang et al., 2015). Interestingly in this study, poly-GA recruited poly-GR into inclusions and this was accompanied by a restoration of Notch signalling. This suggests different DPRs likely have different roles and very different contributions to C9-ALS/FTD progression. While inclusion formation is a clear sign of failure of essential cellular processes, they may be a response to toxic events rather than the toxic species themselves. However, in the case of C9-ALS/FTD, there

are many examples of DPR inclusions incorporating other proteins, leading to toxic effects from their loss-of-function.

1.6.3.1 DPR production leads to ribosome stalling, increases ER stress and impairs proteostasis in C9-ALS/FTD

Poly-PR and poly-GR have been shown to be able to stall ribosomes, thereby blocking protein synthesis and impairing proteostasis. This is DPR species and length-dependent, with only arginine-rich DPRs longer than 10 repeats (i.e., 20 amino acids) able to stall ribosomes. This contrasts with less toxic poly-GP and poly-GA which showed sparse binding in comparison. Arginine-rich DPR interactions with the proteome are related to translation, ribosome biogenesis, RNA splicing and mitochondrial function. (Radwan et al., 2020). The stalling of ribosomes by arginine-rich DPRs may be an important pathogenic mechanism in C9-ALS/FTD. Poly-GR required at least 10 DPR repeats and poly-PR at least 40 DPR repeats for up to 25% stalling; over 40 poly-GR and 50 poly-PR repeats are required for severe translational arrest of more than 50% (Kriachkov et al., 2022). It has been hypothesised that long DPR lengths are needed to stall the ribosome through an electrostatic jamming mechanism by way of filling up the ribosome exit tunnel (Loveland et al., 2022; Radwan et al., 2020). Importantly, the presence of arginine-rich DPRs appears to alter the ribosome quality control mechanism, indicating limited ability to resolve stalled ribosomes induced by arginine rich DPRs and therefore contributing to increasing, unresolvable levels of cellular stress (Kriachkov et al., 2022).

Arginine-containing DPRs have also been linked to proteostasis disruption through widespread hypomethylation of the proteome. The brains of individuals with the *C9ORF72* repeat expansion show patterns of methylated inclusions (Boeynaems et al., 2016). It has been suggested that arginine-rich motifs lead to alterations in methylation patterns. Arginine methylases are recruited to induce hypomethylation of

ribosomal and splicing proteins. This could have widespread effects on many aspects of proteostasis, splicing, and ribosome function (Radwan et al., 2020).

Using proteomics to identify partners of poly-PR shows co-localisation with RNA binding protein hnRNPA1. Both poly-PR and poly-GR form insoluble complexes with mRNAs and ribosomal proteins, restricting translation factor access thereby blocking translation. In this way, poly-PR and poly-GR induce toxicity and neurodegeneration through global impairment of proteostasis (Kanekura et al., 2016). The ability of the cell to maintain proteostasis is heavily dependent on cellular degradation systems. Failures in these systems leads to accumulations of damaged, misfolded proteins, increases in ER stress, and eventual cell death (Claessen et al., 2012). In C9-ALS/FTD primary neuronal cultures, cells expressing poly-GA show increased levels of ER stress sensor PERK. This activates the PERK-CHOP pathway, reduces protein synthesis and eventually leads to cell death (Hetz, 2012). Administration of TUDCA, an inhibitor of ER stress and blocker of PERK-CHOP activation, is neuroprotective against poly-GA. Poly-GA itself is not reduced, indicating its toxic effects are not direct and are via proteasome inhibition increasing ER stress (Zhang et al., 2014). A vicious cycle commences, as increasing oxidative stress triggers phosphorylation of eIF2 α via the integrated stress response, which promotes eIF2 α -dependent RAN translation of poly-GA (Cheng et al., 2018). Therefore, in this way DPR toxicity leads to further aggregation and increased cellular stress, which induces further DPR production (Sonobe et al., 2018). Sonobe and colleagues have shown that the presence of G4C2 repeat constructs induced the ISR, and ISR-mediated poly-GA translation is accompanied by decreased conventional translation and that poly-GA translation itself further triggers the ISR. This was shown both *in vitro* and *in vivo* and reveals a continuous pathogenic cycle where DPR translation triggers the ISR and the ISR triggers further DPR production (Sonobe et al., 2018).

Further evidence supports DPR-induced translational repression as a mechanism of *C9ORF72* neurodegeneration. Poly-GR and poly-PR directly interact with ribosomal proteins to inhibit translation in iPSC-derived motor neurons and *Drosophila*. Translational arrest could be restored through expression of eIF1A, a translation initiation factor (Moens et al., 2019). Moens and colleagues performed a purification of DPRs from the brain of *Drosophila* and identified their protein interaction partners through mass spectrometry, followed by a screen of 81 translation-associated proteins. This led to identification of ribosomal proteins that provide a mechanistic link between poly-GR, poly-PR, and toxicity induced by translational arrest in the context of the G4C2 repeat expansion. Additionally, eIF1A was identified as a novel modifier of this toxicity and a potential therapeutic target in C9-ALS/FTD, highlighting the important role of translational repression by arginine rich DPRs as a contributor to C9-ALS/FTD (Moens et al., 2019).

Poly-GA inclusions have been shown to sequester proteins such as HR23 which are known to be involved in proteasomal degradation; this is consistent with links between poly-GA expression in primary neurons and their impaired proteasome activity and increased endoplasmic reticulum stress (Zhang et al., 2016). Poly-GA incorporates numerous proteins into aggregates, notably Unc119, a neuromuscular axonal protein (May et al., 2014). Aggregated Unc119 is associated with decreased dendritic complexity and is neurotoxic. This was rescued by Unc119 overexpression, suggesting it is poly-GA that drives this depletion-induced toxicity by recruiting the protein into inclusions. Unc119 does not co-aggregate with TDP-43 or any other DPR, suggesting DPR species have specific toxic effects based on their interactions inducing loss-of-function in other proteins. Among five DPRs, there are many opportunities for inducing toxicity via this mechanism, which may contribute to the clinical heterogeneity associated with the *C9ORF72* repeat expansion.

1.6.3.2 DPRs induce dysfunction in nucleocytoplasmic transport and intracellular trafficking in C9-ALS/FTD

Poly-GA has also been mechanistically linked to toxicity in ALS through dysfunctional nucleocytoplasmic transport. Cytoplasmic poly-GA aggregates induce mislocalisation of TDP-43 into cytoplasmic granules while nuclear poly-GA prevented this mislocalisation. It has been suggested that poly-GA impairs the importin- α/β dependent pathway thus providing a link between formation of the main pathologies seen in C9-ALS/FTD (Khosravi et al., 2017). Dysfunctional nucleocytoplasmic transport is central to C9-ALS/FTD toxicity.

C9ORF72 directly interacts with Ran GTPase activating protein 1 (RanGAP1), a key regulator of nucleocytoplasmic transport that, along with HR23, is co-recruited into poly-GA aggregates (Zhang et al., 2016). Of gene knock-outs screened in yeast to identify those that could suppress or enhance DPR-induced toxicity, the strongest modifiers were found to be members of the karyopherin family of nuclear importers. Others included NDC1, a key component of the nuclear pore complex (Jovičić et al., 2015). In a genetic screening of a *Drosophila* model expressing the G4C2 repeat expansion, components of the nuclear pore complex and regulators of RNA export and protein import were identified as key genetic disease modifiers. This corresponds to morphological abnormalities of the nuclear envelope and dysfunctional RNA export leading to retained nuclear RNA. This study makes a clear link between the C9ORF72 repeat expansion, DPR production, and compromised nucleocytoplasmic transport leading to increased neurodegeneration (Freibaum et al., 2015).

1.6.3.3 DPRs cause widespread splicing abnormalities that contribute to toxicity in C9-ALS/FTD

Poly-PR and poly-GR have been implicated as contributing to toxicity in C9-ALS/FTD through a mechanism of blocking spliceosome assembly through aberrant interactions with U2 snRNP. This may account for as many as 44% of the mis-spliced exons that are widespread in C9-ALS/FTD (Yin et al., 2017). Some 5000 splicing errors have been observed in astrocytes in culture with poly-PR providing strong evidence for the role of DPRs in mis-spliced RNA transcripts (Kwon et al., 2014). Formation of RNA foci also contribute to splicing errors through sequestration of essential RBPs; one key example is hnRNP-H which was found to co-localise with up to 70% of G4C2 RNA foci in the brains of C9-ALS/FTD patients (Lee et al., 2013). This is exacerbated by the additional mis-splicing attributed to DPRs.

A major class of these mis-spliced genes dependent on U2 snRNP include those related to mitochondrial function (Lopez-Gonzalez et al., 2016). This is significant in that energy homeostasis failures have been identified as a key dysfunction in C9-ALS/FTD, and this suggests a link between this and DPR production. Additionally, genes have been identified related to neurite growth, transcription and other splicing factors required for U2 snRNP recruitment to pre-mRNA. Deficits further exacerbate splicing errors in C9-ALS/FTD, showing once again how the interplay between disease mechanisms creates a snowball effect. A recent chemical screen aiming to identify compounds to reduce poly-PR toxicity found that compounds identified had nucleolus-related functions. Specifically, BET Bromodomain inhibitors rescue poly-PR toxic and alleviate nucleolar stress and deficits relating to mRNA splicing (Corman et al., 2019).

1.6.3.4 DPRs induce mitochondrial damage and disrupt energy homeostasis in C9-ALS/FTD

Oxidative phosphorylation deficits are common across ALS, but comparatively C9-ALS/FTD cases only see a relatively small reduction in ATP production (Debska-Vielhaber et al., 2021). Deficits in Ca^{2+} buffering capacity is seen, with C9-ALS/FTD patient-derived motor neurons vulnerable to excitotoxicity due to increased permeability of the Ca^{2+} AMPA receptor. This reduces expression of Ca^{2+} importers MICU1 and MICU2, decreasing the ability of mitochondria to take up Ca^{2+} . Removing the repeat expansion using CRISPR/Cas9 rescues this phenotype indicating Ca^{2+} buffering abnormalities are specific to C9-ALS/FTD (Dafinca et al., 2020). Importantly, failures in Ca^{2+} buffering and corresponding Ca^{2+} influx is linked to triggering of the Integrated Stress Response (ISR) and increased RAN translation (Westergard et al., 2019), thereby producing more DPRs that do further damage to mitochondrial function. This further highlights the crucial role that stress has in ALS onset and progression, and the existing literature indicates that there are multiple contributing factors and points of intersection between stress and ALS pathogenesis (Bozzo et al., 2017).

DPRs provide C9-ALS/FTD specific routes to mitochondrial dysfunction. Poly-GR binds directly to mitochondrial proteins and this is linked to increased oxidative stress (Lopez-Gonzalez et al., 2016). In *Drosophila*, poly-GR causes MICOS dysfunction, causing structural changes and disrupting ion homeostasis and overall mitochondrial activity. MICOS, the mitochondrial contact site and cristae organising system, is a dynamic structure responsible for regulation of proteins associated with energy production, and may represent another route for DPR-specific degeneration in C9-ALS/FTD (Li et al., 2020).

In an inducible mouse model of C9-ALS/FTD, mitochondrial damage is a very early-stage event, with dysfunction observed as early as 3 months. Deficits are observed in

the inner mitochondrial membrane, cristae are lost, and activity of both complex I and V decreases. Poly-GR also interacts with mitochondrial complex V component ATP5A1. Targeting poly-GR for reduction rescues this dysfunctional mitochondrial phenotype even after symptom onset, providing robust evidence for poly-GR induced disruption to energy homeostasis as one of the most important early-stage events in C9-ALS/FTD (Choi et al., 2019).

Astrocyte dysfunction provides another link between C9-ALS/FTD and mitochondrial dysfunction, with C9ORF72 mutant astrocytes showing decreased delivery of anti-oxidants to neurons with corresponding increases in oxidative stress (Birger et al., 2019).

1.6.3.5 DPRs contribute to abnormal phase separations

Arginine-containing DPRs have been implicated in the process of abnormal phase separations that contribute to transitions from soluble to aggregate-prone states. Proteins with arginine-rich motifs can modulate LLPS (Aguzzi & Altmeyer, 2016). Arginine-rich DPRs therefore have a large impact on abnormal phase separations and most research thus far has therefore focused on these over other DPR species. However this does not rule out that all DPRs may contribute to abnormal phase separations and this is worthy of further investigation. Poly-PR and poly-GR accelerate transitions from liquid to solid reminiscent of the process of forming pathological aggregates; this represents a novel pathophysiological mechanism for DPRs in C9-ALS/FTD (Boeynaems et al., 2017; Lee et al., 2016). Arginine-rich DPRs induce abnormal phase separations in proteins linked to RNA metabolism or nucleocytoplasmic transport, and can induce spontaneous assembly of stress granules (Boeynaems et al., 2017).

DPRs interact directly with RBPs, especially with those containing low-complexity domains (Lee et al., 2016), which are responsible for mediation of phase separations and the formation of membrane-less compartments. Many of the proteins that interact with arginine-rich DPRs are components of membrane-less organelles, and their aberrant DPR interactions impair these dynamics and the function of such organelles (Lee et al., 2016). This provides a link between DPR-induced mechanisms, as these compartments such as nuclear speckle domains are the location of splicing events. Therefore, abnormal phase separation could disrupt these thereby influencing splicing errors due to propensity for aggregation of RBPs due to DPR interactions (Yin et al., 2017).

Arginine-rich DPRs have been shown to alter the phase separation of NPM1, a nucleolar protein responsible for maintaining liquid-like constitution of the nucleolus. Poly-PR binds directly with acidic residues in NPM1's intrinsically disordered region to alter phase separation dynamics, thereby disrupting organisation of ribosomal proteins and RNAs within the nucleus. This poly-PR induced disturbance of nucleolar organisation was found to be dependent on DPR repeat length (White et al., 2019). It has been hypothesised that effects are determined by DPR concentration, with moderate DPR concentrations linked to decreased nucleoli fluidity and only higher concentrations linked to sequestration of proteins into phase-separation inhibited complexes. Overall, the effect is to disrupt ribosome biogenesis induced by DPRs through abnormal phase separations (White et al., 2019).

DPR repeat length plays an important role in DPR-induced toxicity through abnormal phase separation. Longer DPRs have a greater ability to influence abnormal phase separations, which is directly linked to previously reported thresholds for the G4C2 repeat expansion length needed to induce toxicity *in vitro* (Jafarinia et al., 2020). This highlights not only the importance of abnormal phase separations as a key pathogenic

mechanism, but of the production of DPRs in particular as driving C9-ALS/FTD compared to other proposed disease mechanisms.

1.6.4 Relative contributions of toxic gain-of-function mechanisms to C9-ALS/FTD

Several studies have helped unpick the relative contributions of gain-of-function mechanisms of RNA toxicity in C9-ALS/FTD. It is likely that both mechanisms, along with the loss-of-function hypothesis, contribute to the disease. The haploinsufficiency hypothesis is insufficient to cause the disease alone. However, it likely contributes to and exacerbates toxic gain-of-function mechanisms. It may be that haploinsufficiency mouse models without motor deficits are due to a lack of gain-of-function elements of the disease, and that it is the exacerbation between them that drives the disease phenotype. This lack of unity in loss and gain of function models may help explain why many gain of function animal models also fail to recreate many key clinical aspects of the disease.

Understanding the driving force behind ALS onset and progression is essential if a viable treatment strategy is to be developed. Levels of DPRs do not correlate well with disease severity in post-mortem tissue (Mackenzie et al., 2015), perhaps suggesting an upstream mechanism such as the formation of RNA foci. Foci formation shows a better correlation with disease severity, however in one study, two transgenic *Drosophila* lines were developed containing the G4C2 repeat expansion and provided strong support for DPRs as the primary source of toxicity in C9-ALS/FTD. One line has the expanded repeat, therefore producing RNA foci and DPRs, and a neurodegenerative phenotype was seen. In the other line, regular stop codons were included so that RNA foci still formed, but DPRs could not be produced. Here, no degeneration was seen indicating it is the production of DPRs and not RNA foci that is the driver of degeneration (Sarah Mizielińska et al., 2014). This was supported by a

further study in *Drosophila*, which had no neurodegenerative phenotype with many RNA foci, and no DPR production. Foci were found at very high levels, but no signs of dysfunction related to RNA biogenesis, the transcriptome or nucleolar dynamics were seen (Tran et al., 2015). This again implicates DPRs, rather than foci, as the main driver of pathogenesis in C9-ALS/FTD. *Drosophila* has been commonly used as a model for ALS and the majority of ALS-related genes have *Drosophila* orthologues and exhibit a disease phenotype including locomotor and morphological deficits. So far, screens using *Drosophila* models have led to the identification of several important disease-related genes and pathways (Yamaguchi et al., 2021). While *Drosophila* studies could be criticised for lacking relevance to the human disease, many of the findings from these studies have been validated in other models. Arguably the strongest approach in C9-ALS/FTD would take a combined approach using both *Drosophila* and patient-derived cells or tissue to help reveal further disease mechanisms (Yuva-Aydemir et al., 2018).

The central role of DPRs in C9-ALS/FTD is undermined by the lack of correlation with clinical severity and pathological burden in post-mortem tissue. Although, it is of course difficult to track progression of DPR pathology using post-mortem studies. Further, mechanisms are not mutually exclusive, with most cells suffering from the effects of both foci formation and DPR production. A summary of the key dysfunctions seen in C9-ALS/FTD associated with foci and DPRs are shown in Figure 1.4. As this chapter has explored, mechanisms do not exist in isolation and instead interact with each other to create disease cascades that continue to exacerbate one another until the point of cell death. However, there is clear and widespread evidence for the toxicity of DPRs and their many deleterious effects specific to C9-ALS/FTD. Therefore, this thesis will focus on the production of DPRs as a key driver of C9-ALS/FTD pathogenesis, although certainly not the only contributing factor, and focus on this process as a target for a potential therapeutic strategy. A strategy focused around DPR production should target mechanisms underlying their synthesis, to reduce their

abundance and associated toxic effects. Therefore, next this thesis will provide an overview of mechanisms underlying both canonical translation and the unconventional translation mechanism associated with production of DPRs.

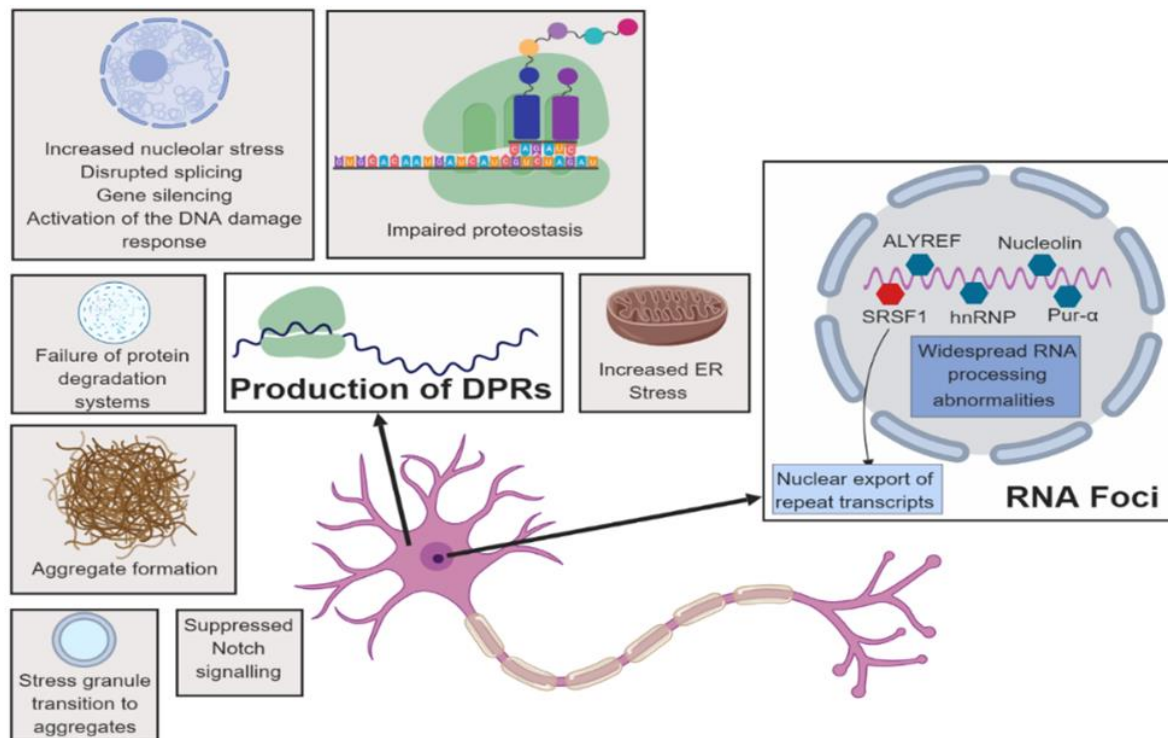


Figure 1.4: Summary of toxic gain-of-function mechanisms associated with RNA foci and DPR production in C9-ALS/FTD. Sequestration of RNA binding proteins by RNA foci causes widespread splicing abnormalities and SRSF1 enables inappropriate nuclear export of repeat-containing mRNA transcripts to the cytoplasm for translation into DPRs. DPRs form insoluble aggregates and are associated with increased ER stress, impaired proteostasis, widespread dysfunction in RNA metabolism, failure of protein degradation systems, suppressed notch signalling and abnormal phase separations that contribute to the further formation of insoluble pathological aggregates. Figure made with Biorender.

1.7 Mechanisms of Canonical translation

1.7.1 Key mechanisms in canonical translation initiation

Three key mechanisms are used to initiate canonical translation in eukaryotes. Typically, AUG-driven cap dependent translation is used for most mRNAs, but

additionally cap-independent translation or canonical translation using near-cognate start codons as alternatives to AUG can be used. The large protein family of eIFs, or eukaryotic initiation factors, is responsible for regulating this process. eIFs have been identified as a rate-limiting step in translation (Hinnebusch, 2017). In canonical AUG-driven translation, the 40S ribosomal subunit is recruited to mRNAs ahead of the translation start site, requiring multiple initiation factors. A closed-loop complex forms whereby eIF4G links poly(A) binding tail protein PABP with cap-binding protein eIF4E. PABP binds to RNA and forms the 48S pre-initiation complex with eIF2, eIF3, eIF5, the 40S ribosome and an attached eIF2 α methionine tRNA. The 48S pre-initiation complex proceeds to scan the mRNA 5'UTR until an AUG start codon is reached. This requires a complex of eIF4A and co-factors eIF4B and eIF4H which are needed to resolve mRNA secondary structures such as G-quadruplexes (Castelli et al., 2021).

G-quadruplexes are dynamic nucleic acid structures comprised of vertically stacked guanine-tetrads, joined by lateral loops and stabilised by hydrogen bonding and monovalent cations (Mendoza et al., 2016; Rhodes & Lipps, 2015). They are essential for fine control of gene expression, with over 700,000 G-quadruplex motifs found throughout the human genome, in particular in promoter regions and UTRs (Bedrat et al., 2016; Chambers et al., 2015; Huppert & Balasubramanian, 2005).

When an AUG is reached, eIF5B recruits the 60S ribosomal subunit, initiating the 80S complex. Initiation factors are then displaced ready for the next mRNA to be translated. Regulation of this processes is therefore highly dependent on the activity of eIFs, and the identification of the AUG start codon, selection of which is dependent on the Kozak consensus element (Castelli et al., 2021).

initiation factors. Overall, use of a near-cognate start codon decreases translation efficiency compared to the standard AUG initiator (Castelli et al., 2021).

1.7.2 eIF4A1 and eIF4A2 are DEAD-box helicases required for translation initiation

While ribosomes have a limited capacity to resolve RNA secondary structures, efficient translation requires DEAD-box helicase eIF4A to resolve 5'UTR secondary structures and allow scanning of translational machinery (Svitkin et al., 2001; Takyar et al., 2005). DEAD-box helicases are proteins which catalyse the unwinding of RNA duplexes and structural rearrangement of RNA/protein complexes in an ATP-dependent manner. RNA helicases are grouped into superfamilies SF1 and SF2, with DEAD box helicases part of SF2. They contain a helicase core which is comprised of two RecA-like domains which are responsible for ATP or RNA binding and unwinding functions (Linder & Jankowsky, 2011). This helicase core can act as a kind of molecular switch, with the affinity for RNA altering in a nucleotide-dependent state (Lorsch & Herschlag, 1998). Structurally, the helicase core is comprised of at least 12 characteristic sequence motifs (Linder & Jankowsky, 2011). Some are conserved across the entire family of DEAD-box helicases while others show unique alterations in individual helicases. Sequence and structural organisation of these motifs are reviewed by Fairman-Williams and colleagues (Fairman-Williams et al., 2010). Structure of eIF4A, showing the topology of the eIF4A complex and spatial arrangement of its RNA-binding sites is shown by Marintchev and colleagues (Marintchev et al., 2009).

RNA helicases are linked with numerous aspects of RNA metabolism including splicing, nuclear export, ribosome biogenesis, translation, and transcript degradation. Many helicases contribute to multiple steps; details of RNA helicases and their role in gene expression is found reviewed in (Castelli et al., 2022)

RNA unwinding by DEAD-box helicases is achieved through a series of conformational changes, transitions between states are important regulatory checkpoints in the catalytic cycle of eIF4A (Andreou & Klostermeier, 2013). In the absence of RNA, the helicase core adopts an open conformation with separate RecA-like domains. RNA or ATP binding alters this towards a rigid, closed conformation (Theissen et al., 2008). A number of methods have been used to assess helicase activity and function, including single-molecule FRET experiments (Theissen et al., 2008), RNA-unwinding assays (Karow et al., 2007), RNAi assays and polysome profiling (Galicía-Vázquez et al., 2012), use of systems such as CRISPR/Cas9 to generate cell lines lacking helicase expression (Galicía-Vázquez et al., 2015), and NMR spectroscopy to determine helicase binding sites (Oberer et al., 2005).

eIF4A comprises a core region of 294-359 amino acids with conserved helicase motifs, and unusually has only a short N-terminal of 50 amino acids, with no C-terminal extension (Rogers et al., 2002). For this reason it is considered a minimal DEAD-box helicase, as most others contain N and C-terminal extensions that flank the helicase core and help mediate a number of functions relating to ATP and RNA interactions (Andreou & Klostermeier, 2013).

The ATP hydrolysis activity of eIF4A and associated conformational changes are the rate limiting step in unwinding activity (Lorsch & Herschlag, 1998). It has been described as a bidirectional helicase in that it can unwind RNA/DNA duplexes of either orientation at the same rate, with unwinding rate dependent on duplex thermodynamic stability rather than length or sequence (Rogers et al., 1999).

Human eIF4A shows strong sequence homology to mouse eIF4A (Schmid & Linder, 1992). Eukaryotes express three isoforms of eIF4A: eIF4A1, eIF4A2 and eIF4A3. While eIF4A1 and eIF4A2 show up to 95% sequence homology with one another, eIF4A3 has only approximately 60% homology (Nielsen & Trachsel, 1988; Owttrim et

al., 1991). eIF4A3 is ten-fold less abundant than eIF4A1 and does not play a role in translation initiation and is instead part of the exon junction complex (Li et al., 1999).

So far, the vast majority of structural-based studies have specifically focused on eIF4A1 (Andreou & Klostermeier, 2013). This was first determined in yeast (Caruthers et al., 2000). The conserved helicase core of eIF4A1 contains motifs I and II which are implicated in ATP binding and hydrolysis, motif III which is associated with coupling ATPases with duplex separation functions, motif IV with RNA binding, and a Q motif linked to ATP binding and specificity (Andreou & Klostermeier, 2013). While crystallography has been used to reveal the quaternary structure of human eIF4A1 (Iwasaki et al., 2019) structural studies thus far have generally either focused only on eIF4A1, or on eIF4A with no distinction made between eIF4A1 and eIF4A2 isoforms. One study of comparative analysis has been carried out, showing crystal structures of domains from eIF4A1, eIF4A2 and nine other DEAD-box helicases. All showed Rec-A domains, with some flexible regions and other regions of high sequence conservation between helicases. eIF4A2 in particular showed some unique confirmations, specifically a longer α -helix 4 structure and a P-loop described as unusually closed, resulting in an ATP binding site not visible on the surface of the protein's structure (Schütz et al., 2010). Some further work investigating differences in domain organisation between eIF4A1, eIF4A2 and eIF4A3 is reviewed by Xue and colleagues (Xue et al., 2021).

eIF4A1 and eIF4A2 are differentially expressed, with eIF4A1 associated with cells undergoing mitosis and eIF4A2 with quiescent cells. This clearly has some relevance in a post-mitotic neuronal cell population. The role of eIF4A1 in post-mitotic neurons is currently poorly characterised and will be explored further in this thesis. eIF4A isoforms have been described as functionally interchangeable (Andreou & Klostermeier, 2013). However, this is a premature judgement: as this thesis will explore, eIF4A1 and eIF4A2 have more divergence than has previously been

assumed in the context of C9-ALS/FTD RAN translation. Depletion of eIF4A1 increased eIF4A2 mRNA and protein levels, while protein synthesis remains suppressed even though eIF4A2 protein levels rose high enough that they should be able to compensate. This suggests a link between eIF4A1 and eIF4A2 expression and indicates that there are functional differences between them (Galicia-Vázquez et al., 2012).

As there has traditionally been an assumption of interchangeability between eIF4A1 and eIF4A2, many studies simply refer to eIF4A and make no distinction between the isoforms. There is very limited research into the differential roles of eIF4A1 and eIF4A2 in relation to promotion or inhibition of translation. eIF4A1 has been shown to be essential for translation initiation, while eIF4A2 is not (Galicia-Vázquez et al., 2015) indicating they do have distinct functions related to translation. Some differential roles have been explored in relation to some other functions. For example, eIF4A2 but not eIF4A1 is essential for miRNA-mediated gene silencing and has been identified as a crucial modulator of miRNA-mediated repression of a number of gene targets. Its depletion leads to alleviation of gene silencing but has no impact on cell viability or global translation. This may be a function unique to eIF4A2 and not found in other members of the eIF4A family (Galicia-Vázquez et al., 2015).

In addition to its role as a helicase, eIF4A has been linked to modulation of RNA condensation and stress granule formation (Tauber et al., 2020). eIF4A limits recruitment of RNAs into stress granules, and that ATP-dependent binding between eIF4A and its RNA targets can prevent stress granule formation and limit RNA condensation. This suggest an important function for eIF4A outside of translation initiation is to limit inappropriate interactions between RNAs (Tauber et al., 2020).

1.7.3 eIF4A1 and eIF4A2 helicase activity is stimulated by eIF4B and eIF4H

eIF4A shows RNA unwinding functions that are regulated by other translation factors through stimulation of ATPase or mediation of interactions with RNA (Parsyan et al., 2011; Rogers et al., 2001). eIF4A forms a complex with other closely related proteins such as eIF4G and eIF4H (Marintchev et al., 2009). Alone, eIF4A1 has only weak RNA helicase activity, but this is stimulated by co-factors eIF4B and eIF4H, which also act to stabilise single stranded regions of the 5'UTR and prevent reannealing, thereby promoting 5' – 3' unwinding activity (Lindqvist et al., 2008; Marintchev et al., 2009). It has also been hypothesised based on the structure of eIF4A1 with the large separation of the core helicase domains, that co-factors may exert their helicase activity through facilitating formation and stabilisation of the closed conformational structure (Caruthers et al., 2000).

eIF4B is poorly conserved compared to other translation initiation factors, with only 25-30% homology between different species (Metz et al., 1999). It comprises an N-terminal region with an RNA recognition motif, although this is not necessary for its interaction with eIF4A1. It has a C-terminal region with another RNA binding domain, and a DRYG domain (rich in aspartic acid, arginine, tyrosine and glycine) to mediate its interactions with eIF3 (Methot et al., 1996; Méthot et al., 1996). The C-terminal domain RNA binding region has been linked to eIF4A1 dependent duplex unwinding in the presence of eIF4G (Özeş et al., 2011).

eIF4H has a core region and RRM in common with eIF4B but lacks the N and C-terminal domains. eIF4B and eIF4H expression levels vary, which has led to a hypothesis that they show transcript-specific or tissue-specific regulatory activity of translation initiation. As eIF4B and eIF4H enhance the function of eIF4A, this has potentially substantial implications for this work and for their role in translation initiation more broadly. There may be alterations in the support they provide which could differ

for eIF4A1 or eIF4A2, or depending on brain region or translational context in terms of initiating RAN or canonical translation. Interaction sites of eIF4B and eIF4H to eIF4A1 overlap and show mutually exclusive binding patterns but no sequence similarity (Richter et al., 1999; Rozovsky et al., 2008). Functionally, the divergence between eIF4B and eIF4H is unclear, although it has been shown that eIF4H is unable to fully substitute for eIF4B to assemble the translation initiation complex in mRNAs with 5'UTR secondary structures (Dmitriev et al., 2003). Both eIF4B and eIF4H increase affinity between eIF4A1 and its RNA targets to enhance unwinding activity and ATP hydrolysis although there is some evidence that the effect of eIF4H is smaller than that of eIF4B (Özeş et al., 2011). The complex between eIF4A1 and its co-factors requires mediation by eIF4G (Nielsen et al., 2011). eIF4G provides a dynamic scaffold for a number of interactions of proteins involved in translation initiation, and has binding sites for PABP, eIF4E, eIF3, as well as eIF4A1 (Prévôt et al., 2003).

1.7.4 DHX36 is an RNA helicase that is a major resolver of G-quadruplexes

DHX36 is a highly conserved member of the DEAH/RHA family of RNA helicases (Chen et al., 2015; Gueddouda et al., 2017) and is a major source of G-quadruplex resolving activity *in vitro* (Vaughn et al., 2005). It has robust DNA and RNA unwinding abilities (Creacy et al., 2008). It specifically unwinds G-quadruplexes with a conventional helicase mechanism with very high levels of efficiency (Yangyuoru et al., 2018). Binding affinity between DHX36 and its DNA and RNA G-quadruplex targets is extremely high compared to other proteins known to target G-quadruplexes (Tseng et al., 2021b).

The gene product of DHX36 is RHAU (RNA helicase associated with AU rich element), it has nuclear and cytoplasmic isoforms, with the nuclear form the more abundant of the two (Iwamoto et al., 2008). It comprises a helicase core, an N and C-terminal domain (Abdelhaleem et al., 2003; Chen & Ferré-D'Amaré, 2017). The core helicase

domain is responsible for ATP binding or hydrolysis, and unwinding activity, the flanking N-terminal domain is necessary for recognition of G-quadruplexes. The N-terminal domain contains an RNA binding domain, providing DHX36's high affinity for RNA over DNA and for parallel over antiparallel G-quadruplexes (Meier et al., 2013; Tippana et al., 2016). In addition, it contains a specific motif that can fold into a DNA-binding induced α -helix. This shows selective binding to G-quadruplexes, resulting in ATP-independent helicase core opening which rotates the C-terminal domain and unwinds G-quadruplexes residue by residue. This is followed by ATP-dependent RNA refolding (Chen et al., 2018; Tippana et al., 2019). Processing of G-quadruplexes relies on both sequence and single stranded DNA accessibility (Yangyuoru et al., 2018). DHX36 also exerts regulatory control through its interactions with long non-coding RNAs which may stabilise G-quadruplexes unwound by DHX36 during remodelling of RNAs (Booy et al., 2016).

1.8 Mechanisms of DPR production by RAN translation

1.8.1 Introduction to RAN translation

RAN translation involves the production of dipeptide repeat proteins (DPRs) from expanded repeat sequences without the need for a canonical AUG start codon. RAN translation has now been identified in several neurodegenerative diseases containing a repeat expansion, including ATXN8 CAG repeat transcripts in SCA8 (Zu et al., 2011), CGG repeats in FXTAS (Todd et al., 2013) and CAG repeats in Huntington's Disease (Bañez-Coronel et al., 2015). The *C9ORF72* G4C2 hexanucleotide repeat undergoes bi-directional RAN translation in all reading frames to produce five neurotoxic DPRs (Castelli et al., 2021).

1.8.2 Near-cognate start codons are used to initiate RAN translation and a frameshift produces DPRs in all three reading frames

In the context of the *C9ORF72* hexanucleotide repeat expansion, secondary structures such as G-quadruplexes influence initiation of RAN translation through altered ribosomal scanning, leading to recruitment of ribosomes to near-cognate start codons. Initiation occurs at a methionine tRNA at a Kozak-embedded CUG codon, 24 nucleotides upstream of the G4C2 repeats (Castelli et al., 2021).

In a *C.elegans* model expressing 75 G4C2 repeats, DPRs are produced with corresponding neurodegeneration, locomotor deficits and early death. A mutation in the near cognate start codon CUG, upstream of the repeat expansion, reduces poly-GA production and rescues the phenotype (Sonobe et al., 2021). Here, CRISPR/Cas9 genome editing was used to generate *C.elegans* with 75 repeats and a nanoluciferase reporter in the poly-GA reading frame, with either a CUG non-canonical translation initiation codon or a mutated AUG version. Similarly plasmids were constructed with or without a replacement of the CUG start codon with UAG to perform *in vitro* work in immortalised cell lines (Sonobe et al., 2021). While these studies may be criticised for lacking relevance to RNA molecules *in vivo*, there is a good level of consistency seen in the literature. C9-RAN DPRs and associated proteins including translation initiation factors have also now been characterised using proteomics approaches and mass spectrometry (F. Liu et al., 2022; May et al., 2014; Moens et al., 2019).

Different DPRs are produced from the CUG start codon by a frameshift mechanism. This is supported by mutations in the CUG or Kozak sequence, which abolishes production of all three sense DPRs (R. Tabet et al., 2018). RAN translation of the G4C2 transcripts are influenced by several factors including length of the repeat expansion, which confers different sensitivity to the three reading frames. It is not yet fully clear if there is a specific repeat length that enhances production of particular

DPRs, or if the abundance of particular DPRs can be predicted from the length of the repeat sequence. A thorough review exploring the current state of knowledge on translation mechanisms for DPR production has recently been published (van't Spijker & Almeida, 2023). At shorter repeat lengths, RAN translation efficiency is reduced in the +1 poly-GA and +3 poly-GR reading frames, but completely abolished for poly-GP. This probably reflects secondary structure formation and the availability of RNA helicases to resolve them (R. Tabet et al., 2018), with longer repeat lengths having greater propensity to form secondary structures and therefore requiring more helicase activity to resolve. Secondary structures such as G-quadruplexes can also induce frameshifting (Yu et al., 2014), (Kapur et al., 2017). Initiation of RAN translation at a non-cognate start codon and the subsequent frameshifting that occurs can also be influenced by factors such as ion concentrations or the interactions between RBPs and secondary structures (R. Tabet et al., 2018). The very large repeat length seen in some C9-ALS/FTD patients makes it likely that additional mutations could also occur. These additional mutations could influence ribosomal frameshifting, or lead to the production of interrupted repeat sequences (Viterbo et al., 2016). DPRs cause further exacerbation of this through DNA damage. Interrupted sequences lead to production of DPRs of different lengths or with unique N or C terminal sequences, chimeric proteins, or DPRs incorporating other amino acids (Gao et al., 2017).

1.8.3 RAN translation depends on the resolution of RNA secondary structures

The mechanisms of RAN translation in C9-ALS/FTD are dependent on the propensity of the repeat sequence to form RNA secondary structures such as G-quadruplexes and R-loops. The formation of secondary structures has also been shown to influence RAN translation in other disorders involving a repeat expansion (Bañez-Coronel et al., 2015). G-quadruplexes are common due to the G-rich nature of the hexanucleotide repeat, and adopt an antiparallel conformation, comprised of four G-quartets with three lateral loops (Brčić & Plavec, 2017). G-quadruplexes play an important role in fine

control of gene expression and their formation impairs polymerase processing, physically blocking translational machinery thereby resulting in premature termination of RNA transcripts (Fratta et al., 2012; Reddy et al., 2013; Šket et al., 2015). R-loops are also found in *C9ORF72* hexanucleotide repeat sense and antisense RNAs. Similarly to G-quadruplexes, they play an important role in regulation of gene expression under normal conditions, but in the context of the *C9ORF72* repeat expansion they accumulate and cause translational arrest (Gan et al., 2011; Nishida et al., 2017; Sanz et al., 2016). In order for RAN translation to proceed and for DPRs to be produced, these secondary structures must be unwound by helicases. Therefore the formation of these secondary structures and the helicases able to unwind them underpin the production of DPRs from the *C9ORF72* hexanucleotide repeat expansion.

1.8.4 The role of eIF4A1 and eIF4A2 in RAN translation initiation

The DEAD-box RNA helicases eIF4A1 and eIF4A2 have been identified as key initiators of canonical and RAN translation through their ability to unwind complex secondary structures in the 5'UTR during ribosomal scanning. Without the helicase activity of eIF4A1 and eIF4A2, translation is inhibited (Bordeleau et al., 2006; Cruz-Migoni et al., 2012; Wolfe et al., 2014). eIF4A has been shown to be necessary for RAN translation of the *C9ORF72* repeat expansion in a cap-dependent manner using CUG as a start codon (Green et al., 2017). Green and colleagues used a series of reporters containing the G4C2 repeat expansion, with a downstream NanoLuciferase reporter and a mutated AUG start codon. eIF4A involvement was assessed through use of the specific eIF4A inhibitor hippuristanol which led to substantial translation reduction of over 900-fold, making a clear link between eIF4A activity and C9-RAN translation (Green et al., 2017).

For sense G4C2 transcripts, a 5'-3' cap-dependent scanning mechanism and CUG start codon upstream of the repeat expansion is used, with methionine as the initiator

amino acid (Ricardos Tabet et al., 2018). There is evidence that RAN translation of C9ORF72 sense repeat expansion mRNA can be dependent or independent of the 5' cap, although DPR production mostly arises from uncapped and spliced intronic RNAs, bypassing the need for an AUG start codon. It also does not require the cap-binding protein eIF4E, similarly to IRES mediated translation. The rate of RAN translation of sense transcripts are approximately 15-35% of a 5' capped RNA of the same length (Cheng et al., 2018).

For C4G2 antisense RAN translation, mechanisms are less clear at present, although investigations into the RNA crystal structure have revealed an α -form like double helix conformation (Dodd et al., 2016). eIF4A has two isoforms, eIF4A1 and eIF4A2, which share over 96% homology and differ by only approximately 10 amino acids (Andreou & Klostermeier, 2013) and the functional variations in which have been largely overlooked by the field of RAN translation thus far.

1.8.5 eIF4A1 and eIF4A2 require co-factors eIF4B and eIF4H for initiation of RAN translation

eIF4A1 and eIF4A2 form a complex with co-factors eIF4B and eIF4H to initiate RAN translation, shown below in Figure 1.6.

C9ORF72 G4C2

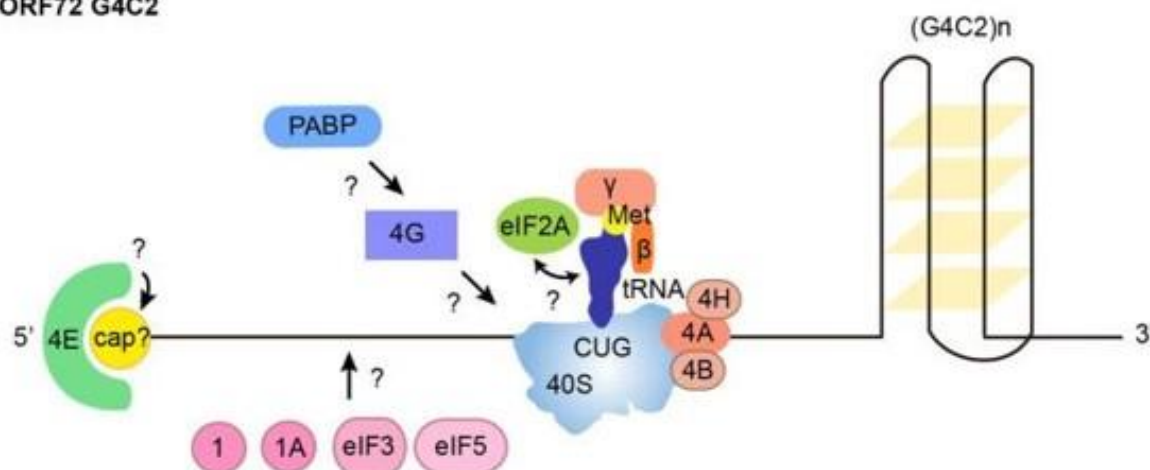


Figure 1.6: Known structures involved in RAN translation initiation of sense G4C2 transcripts.

Adapted from (Castelli et al., 2021). RAN translation of the G4C2 repeat expansion in C9-ALS/FTD require the eIF4A complex, including co-factors eIF4B and eIF4H, to recruit the 40S ribosomal subunit and the met-tRNA to the near cognate CUG start codon. These are RNA helicases which unwind G-quadruplexes to enable RAN translation initiation. The role of PABP, eIF4G, eIF4E, eIF1, eIF1A, eIF3 and eIF5 remain unclear.

Helicase activity of eIF4A1 and eIF4A2 is enhanced by eIF4B and eIF4H (Harms et al., 2014; Sun et al., 2012). In many types of cancer including breast and cervical, high expression of eIF4A1 has been shown to promote cell proliferation and a malignant phenotype while eIF4A1 silencing decreases proliferation and melanoma invasion (Xue et al., 2021). Similarly, eIF4A2 inhibition suppresses invasion and cell migration in colorectal cancer (Xue et al., 2021). eIF4B and eIF4H have also been shown to indirectly promote a cancer malignant phenotype through their ability to enhance translation, with reduction in eIF4B expression linked to decreases in proteins that enhance tumour cell survival in diffuse large B-cell lymphoma (Horvilleur et al., 2014).

eIF4B and eIF4H contain RRM-domains and interact directly with G4C2 RNA transcripts (Cooper-Knock et al., 2014a). eIF4B and eIF4H strengthen the eIF4A mediated unwinding associated with long, complex 5'UTRs (Rozovsky et al., 2008; Sen et al., 2016). It is thought that in the context of G4C2 RAN translation, eIF4B and eIF4H activity mediates scanning of the 5'UTR and frameshifting from the near-

cognate CUG start codon (Green et al., 2016; R. Tabet et al., 2018; Walker et al., 2013).

Both eIF4B and eIF4H have been shown to independently stimulate helicase activity of eIF4A during translation and directly interact with G4C2 RNA transcripts, providing clear evidence for their role in RAN translation (García-García et al., 2015);(Harms et al., 2014; Nielsen et al., 2011; Rogers et al., 2001). There are structural differences between the two, with eIF4B containing a regulatory carboxyl domain with multiple sites for phosphorylation (Raught et al., 2004) while eIF4H is always active and not regulated by phosphorylation state (Goodman et al., 2019).

In *Drosophila* models of C9-ALS/FTD which express the hexanucleotide repeat expansion, eIF4B and eIF4H have been identified as key modulators of RAN translation. Down regulation reduces poly-GR production by RAN translation and rescues the neurodegenerative phenotype. In post-mortem tissue from C9-ALS/FTD patients, eIF4H was downregulated in patients with the *C9ORF72* hexanucleotide repeat expansion (Goodman et al., 2019). This may be as a compensatory mechanism reflecting the cell's attempts to prevent toxic poly-GR production. Reduced eIF4H RNA levels in C9-ALS/FTD may also be a result of RBP protein sequestration by G4C2 RNA foci. Loss of eIF4B and eIF4H do not prevent global translation from occurring in numerous model systems including yeast and *Drosophila* (Coppolecchia et al., 1993; Hernández et al., 2004), although eIF4H knockout mice have growth and behavioural dysfunction (Capossela et al., 2012) while depletion *in vitro* has been linked to stress granule formation (Mokas et al., 2009).

Other closely related proteins have also been shown to regulate DPR production, such as eIF2D in RAN translation of sense transcripts (Sonobe et al., 2021). eIF4E has also shown to be required for RAN translation initiation in some studies (Green et al., 2017), while in others eIF4E was depleted but RAN translation was maintained (Cheng et al.,

2018). This suggests there may be some sequence specific differences that impact regulation of RAN translation.

1.8.6 RAN translation is enhanced by the ISR

Under normal cellular conditions RAN translation is inhibited by an upstream ORF located in intron 1 and flanked by an AUG start codon with two downstream stop codons. However, the activation of the ISR leads to phosphorylation of eIF2 α , which results in down-regulation of canonical translation through poor 60S subunit recruitment to the ribosome and a decrease in ternary complex levels. There is widespread reduction in canonical translation with selective increases in translation of specific RNAs (Holcik & Sonenberg, 2005); this has been reported in a number of neurodegenerative diseases with a repeat expansion mutation, such as Huntington's (Leitman et al., 2014).

Normally, the ISR plays an important role in neuronal plasticity and memory consolidation (Bellato & Hajj, 2016). However, in the context of C9-ALS/FTD, RAN translation is initiated at the downstream CUG (Ricardos Tabet et al., 2018). RAN translation is enhanced by activation of the ISR via eIF2 α phosphorylation, which alters the start codon fidelity. The activation of the ISR has also been shown to maintain translation of poly-GA while also decreasing canonical translation, and that poly-GA production triggers further activation of the ISR (Sonobe et al., 2018). This suggests that the *C9ORF72* repeat expansion increases cellular stress through DPR production which in turn stimulates increased RAN translation of further DPRs (Green et al., 2017). Several studies have been suggestive of a positive feedback loop driving neurodegeneration whereby increased ISR down regulates canonical translation in favour of RAN translation.

This is significant in that RAN translation is generally inefficient, so this mechanism explains how it is able to make a meaningful contribution to neurodegeneration. Cap-independent RAN translation is upregulated by stressful stimuli which triggers phosphorylation of eIF2 α . Importantly pathological TDP-43 has been shown to elevate eIF2 α phosphorylation thereby enhancing RAN translation, and this can be inhibited by blocking eIF2 α phosphorylation (Cheng et al., 2018). It has been hypothesised that eIF2 α phosphorylation enhances RAN translation through a reduction in global translation, some of the rate-limiting components may become more available for initiation of RAN translation (Cheng et al., 2018); this may include members of the eIF4 family. Targeting phosphorylation of eIF2 α reduces DPR production, and excitotoxic stress promotes RAN translation of DPRs using a non-AUG start codon. Many of these stress pathways including oxidative stress or ER stress converge on the ISR, further linking DPR production with eIF2 α phosphorylation. Cell specific tolerances to stress may further influence rates of DPR production (Westergard et al., 2019).

1.8.7 Depletion of helicase DHX36 suppresses RAN translation

Studies of DHX36 binding to RNAs in human cell lines finds a pattern of preferential interactions with over 4500 mRNA G-rich sequences with high propensity for G-quadruplex formation (Sauer et al., 2019). Only a small number of helicases have been identified as resolvers of G-quadruplex activity (Sauer & Paeschke, 2017). Knockout of DHX36 results in increased levels of target mRNAs, while ribosome occupancy and corresponding protein synthesis levels fall, suggesting a translational failure. Translationally incompetent mRNAs containing G-quadruplexes accumulate, and preferentially localise to stress granules, increasing stress granule formation. This links DHX36 activity with cellular stress, as resolving stress induced by G-quadruplexes would normally prevent the accumulation of these translationally incompetent RNAs (Sauer et al., 2019).

Depletion of DHX36 suppresses RAN translation in all three reading frames, dependent on repeat-length while overexpression of wild-type DHX36 enhances RAN translation. A mutant form of DHX36, lacking helicase activity, fails to enhance RAN translation (Tseng et al., 2021a). Furthermore, DHX36 depletion suppresses RAN translation and additionally prevents increased rates of RAN translation that is usually triggered by the ISR (Tseng et al., 2021a). This provides strong evidence that DHX36 regulates RAN translation of the *C9ORF72* hexanucleotide repeat expansion in C9-ALS/FTD, through binding and resolving RNA secondary structures and enhancing translation initiation from a non-AUG site. There were no changes in the levels of C9-repeat RNA in response to either depletion or overexpression indicating the stimulatory effect of DHX36 is post-transcriptional. DHX36 was also found to have lower affinity for C9-repeat DNA than for pure G4 DNA, by up to 100 fold, which may account for the lack of effect on transcript levels (Tseng et al., 2021a). These findings were shown both *in vitro* and in human cells and mouse spinal cord lysates but is limited in that it mostly relies on reporter assays and transfected plasmids. Tseng's work cannot rule out the existence of endogenous RNAs forming additional, dynamic secondary structures such as hairpins which could complicate the role of DHX36 and other helicases in the mechanisms of RAN translation.

Through its role in stress granule dynamics, DHX36 can also influence G4C2 RNA stability, localisation as well as translation (Sauer et al., 2019; Tseng et al., 2021a). Modulation of G-quadruplex structures at the RNA level through helicases targeting G-quadruplexes has an ability to impact RAN translation of the G4C2 repeat expansion which has important implications for a therapeutic strategy focused on reducing DPR production. DHX36 binding to *C9ORF72* G4C2 repeat RNA transcripts is specific to G-quadruplexes (Tseng et al., 2021a).

Knockdown of DHX36 is sufficient to trigger stress granule formation (Sauer et al., 2019) although it is not clear exactly how. DHX36 plays an integral role in the activation of the ISR (Chalupníková et al., 2008). Activation of the ISR lowers initiation codon fidelity (Green et al., 2017), making RAN translation initiation easier to achieve. In addition, DHX36 depletion can trigger the formation of stress granules without requiring any endogenous stressor (Tseng et al., 2021a). DHX36 is thought to specifically influence elongation. Given the role of the ISR in lowering start codon fidelity for RAN translation (Green et al., 2017), DHX36 depletion may slow translation through ribosomal stalling, even though initiation remains enhanced (Tseng et al., 2021a). An alternative hypothesis is that the G4C2 repeat expansion triggers an 'IRES-like' mechanism of RAN translation, enhanced by the ISR, and that DHX36 plays a role in generating the structure allowing for internal ribosome entry (Tseng et al., 2021a).

Further studies have supported DHX36 as a positive regulator of RAN translation of the *C9ORF72* G4C2 repeat expansion. The study is consistent in its findings that DHX36 binds directly to G4C2 repeat RNA G-quadruplex structures with high affinity, and unwinds them with high efficiency, making it therefore essential for RAN translation to proceed. In iPSCs and patient-derived motor neurons, DHX36 depletion reduces DPR production, indicating helicases facilitate DPR production by RAN translation via a mechanism of resolving G-quadruplexes (Liu et al., 2021).

1.9 Treatment strategies for C9-ALS/FTD

There are many therapeutic strategies for ALS/FTD that are currently in development; these are summarised in Figure 1.7.

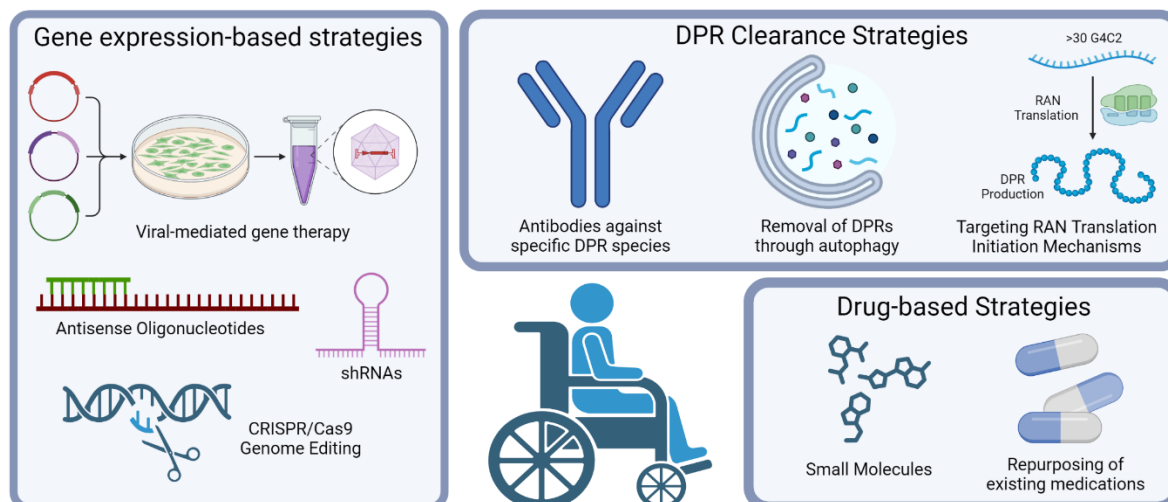


Figure 1.7 Therapeutic Strategies currently in development for ALS/FTD. Some of the many strategies currently being used or tested to treat ALS/FTD are summarised here. These include gene-expression or RNA-interference based strategies including viral mediated gene therapy, use of shRNAs or miRNAs, antisense oligonucleotides or CRISPR/Cas9 genome editing approaches. Other methods include drug-based strategies including development of novel small molecules, or repurposing of existing licenced medications. Finally, newer strategies for C9-ALS/FTD have focused on clearing DPRs directly, through use of antibodies that bind to specific DPR species, more efficient removal of DPRs through protein clearance mechanisms such as autophagy, or targeting proteins or mechanism responsible for initiating RAN translation. Figure made with Biorender.

1.9.1 Viral mediated gene therapy for C9-ALS/FTD

The aim of gene therapy is to silence, restore or otherwise modify genetic material that is causative for a disease. The main approach is the use viral vectors that can cross the blood-brain barrier to deliver the therapy, which could be for example a shRNA or a miRNA. Adenoviruses are typically used as they are capable of delivering small noncoding RNAs across the BBB, thus avoiding the repeated intrathecal delivery that is necessary for other approaches such as use of ASOs (Amado & Davidson, 2021; Corcia et al., 2022). Timing of the intervention and route of administration are some of the many considerations in viral mediated gene therapy, and significant effort is being made to optimise this treatment strategy (Camu et al., 2022).

1.9.1.1 Introduction to adeno-associated virus mediated gene therapy

AAVs are small *Parvoviridae* viruses, comprised of a 4.7kb single stranded genome flanked by two specific palindromic inverted terminal repeats (ITRs). They are not associated with human disease (Colella et al., 2018). They are highly diverse, with 12 serotypes and 108 serovars (Balakrishnan B Fau - Jayandharan & Jayandharan, 2014). AAVs are a suitable choice for gene therapy as they show a strong capability for transduction of neurons and are capable of producing long-lasting transgene expression (Schultz & Chamberlain, 2008; Weinberg et al., 2013). Use of self-complementary AAVs (scAAVs) is associated with increased expression of a transgene without the need for second-strand synthesis, which is normally the rate-limiting step associated with use of AAVs (Colella et al., 2018). Unlike wild type viruses, recombinant AAVs such as the scAAVs used in gene therapy do not integrate into host DNA. Random integration occurs only very rarely, meaning the risk of genotoxicity is low using this strategy (Smith, 2008). However, the minority of integration events that occur do so in sites close to CpG sequences, which is relevant to the CpG rich *C9ORF72* repeat expansion.

Expression patterns of the transgene depend on factors such as viral structure, vector purity, or promoter selection and therefore must be carefully considered (Klein et al., 2008; Weinberg et al., 2013). Gene therapy advances have led to the development of cell specific promoters and novel capsids (Lawlor et al., 2009; Weinmann & Grimm, 2017) while high throughput screening methods have helped identify the most efficient choices for particular cell types (Kotterman & Schaffer, 2014). Use of an integrated AAV and CRISPR/Cas9 system is an innovative method enabling gene regulation with minimal off-target effects (Moreno et al., 2018; Yin et al., 2016). As advancements increase, more efficient methods for transgene delivery and expression will be identified.

1.9.1.2 Limitations associated with use of AAV in viral-mediated gene therapy

AAVs are limited in that maximum cargo capacity of transgenes is 4.7kb in AAVs, and only 2.3kb in scAAVs (Daya & Berns, 2008; McCarty, 2008), although larger genes can be delivered through use of oversized or dual AAV vectors (Dong et al., 2010; Grieger & Samulski, 2005). CNS delivery is a significant challenge due to the blood-brain barrier (BBB), and methods to enable transport across it can be invasive. Peripheral delivery may be more practical as a clinical strategy and intravenous (IV) delivery of scAAV9 can cross the BBB and transduce neurons (Foust et al., 2009; Klein et al., 2008). However, the virus is distributed widely in high doses to multiple peripheral organs. IV delivery can therefore lead to unintended peripheral effects although use of tissue specific microRNA target sites within the expression cassette can help prevent translation in non-target tissue (Xie et al., 2011). Generally, in neurodegenerative disease research, intrathecal injection into the CSF via lumbar puncture is preferred. Additional challenges include managing an immune response, achieving stable long-term transgene expression, and determining an appropriate dose to ensure efficacy with minimal off-target effects. There is a very narrow window in which humans are AAV-antibody naïve, as new-born babies acquire maternal AAV antibodies and wild type exposure typically occurs around the age of 2 years (Calcedo et al., 2011). Immune responses to AAVs vary and it is not yet clear why or when a patient has an immune response (Manno et al., 2006; Mingozzi et al., 2007). This is a key issue in gene therapy as immune responses may lead to loss of transgene expression, and it also must be considered that the transgene itself could provoke an immune response (Martino et al., 2011). Transgene expression can be controlled in various ways for example using a tetracycline-off promoter (Manfredsson et al., 2009). Careful design of the AAV vector and regulatory elements are essential for stable expression of the transgene (Choi et al., 2014; Papadakis et al., 2004), while use of drugs or mutations of the capsid residue can prevent degradation of the vector (Gabriel et al., 2013; Monahan et al., 2010; Zhong et al., 2008). Transgene expression can also

be improved by modifications to the genome, codon optimisation, and elimination of structures such as hairpins, alternative open reading frames, and splice sites (Gao et al., 2006; Presnyak et al., 2015).

1.9.2 Other therapeutic strategies in C9-ALS/FTD include antisense

oligonucleotides, small molecules, re-purposing of existing drugs, genome editing and anti-DPR antibodies among others

Aside from viral mediated gene therapy, other therapeutic strategies currently being tested for C9-ALS/FTD include the use of antisense oligonucleotides (ASOs), small molecules aiming to alleviate the effects of RNA toxic gain-of-function by preventing sequestration of RNA binding proteins or decreasing RAN translation of DPRs, and CRISPR-mediated gene editing. Targeting downstream deficits associated with the C9ORF72 repeat expansion are also possible, for example several strategies have been designed to target NCT dysfunction, or developing neurotrophic factors to provide support and increase motor neuron survival (Hautbergue et al., 2021).

ASOs are small single stranded sequences of <50 bases that target a specific mRNA. They do not cross the blood-brain barrier and therefore must be repeatedly given through intrathecal injection. (Corcia et al., 2022). ASOs have been designed to target *C9ORF72* RNA to reduce foci formation, glutamate excitotoxicity, NCT dysfunction and TDP-43 mislocalisation. ASOs for C9-ALS/FTD are currently in development and are reviewed in (Gagliardi et al., 2020); (Corcia et al., 2022).

Small molecules have been developed that bind to and stabilise secondary structures in *C9ORF72* hexanucleotide repeat expansion RNA. This has shown success at reducing RNA foci and DPR formation in patient-derived neurons and improves survival in *Drosophila* (Simone et al., 2018). Other small molecules selectively bind to

the G4C2 hairpin structure, and this was found to prevent RBP sequestration and to inhibit RAN translation of poly-GP (Wang et al., 2019b) although it is not clear what impact this might have on more toxic, arginine rich DPRs. Finally, high-throughput screening methods have been developed to attempt to find other small molecules that act as inhibitors of RAN translation (Green et al., 2019). Small molecule development is reviewed in (Gagliardi et al., 2020).

In addition to development of new small molecules, some efforts have been made to repurpose existing drugs, which have the advantage of already passing licencing and safety requirements. Metformin is prescribed for diabetes, but in C9-ALS/FTD it inhibits protein kinase R, which reduces DPR production (Hautbergue et al., 2021). Other strategies have centred around reducing DPRs through stimulating their clearance. Heat shock protein family B member 8 (HSPB8) promotes DPR clearance by autophagy in motor neurons (Cristofani et al., 2018) and although this approach is encouraging, it does not tackle the underlying disease mechanisms that cause DPRs to be produced in the first place. Other repurposed drugs being trialled for ALS are reviewed by Corcia and colleagues (Corcia et al., 2021) and include Tauro-ursodeoxycholic acid (TUDCA), Tamoxifen, Levosimendan among others.

Base genome editing to treat ALS is achieved using CRISPR/Cas9. This allows for correction of disease-causing mutations such as the *C9ORF72* repeat expansion, but can be limited by low efficiency or off-target effects (Miccio et al., 2022). Clinical application of CRISPR/Cas9 gene editing is still in its early days, but some promising attempts have been made to target G4C2 repeat DNA and have succeeded in decreasing foci and DPR production *in vitro* (Pinto et al., 2017). Other approaches have used RNA endonuclease fused to Cas9, enabling an RNA-specific CRISPR system to target expanded RNA repeats in C9-ALS/FTD and other microsatellite repeat expansions (Batra et al., 2017). Use of CRISPR/Cas9 systems are reviewed in (Camu et al., 2022; Gagliardi et al., 2020). Other gene editing approaches centre

around nuclease-based approaches using zinc-finger nucleases and transcription activator-like effector nucleases (Miccio et al., 2022).

In C9-ALS/FTD, DPRs have been targeted directly with antibodies (Camu et al., 2022), for example anti poly-GA antibodies have been developed which reduce spinal cord poly-GA inclusion formation, TDP-43 mislocalisation and microglia activation, and rescue mice motor deficits (Zhou et al., 2020). However, there are questions around which DPRs are most appropriate to target, the timing of the intervention, and how to manage any potential off-target effects or autoimmune responses. There is a possibility that depletion of one DPR species may promote increased expression of another and therefore researchers must take a strategic approach to targeting DPR production. Alternatively, a therapeutic approach that targets all DPR species may be too challenging to realistically achieve, or require a higher dose of a newly developed drug which could be associated with more side effects. Therefore careful consideration should be given to which DPRs are targeted, with arginine-rich species a clear initial target given they are most associated with toxic effects (Radwan et al., 2020). Another approach targeting DPRs directly with antibodies used the C9ORF72 BAC transgenic mouse model with 500 G4C2 repeats and found that antibodies delivered peripherally can cross the BBB and reduce DPR production with corresponding decreases neurodegeneration, alleviation of behavioural dysfunction and increased survival time. Interestingly, the anti poly-GA antibody also reduced poly-GP and poly-GR, suggesting a passive immunotherapy strategy could be an effective way to target DPRs, possibly through improved proteasome function (Nguyen, Montrasio, et al., 2020).

Finally, RNA interference strategies have been developed to target RNA gain-of-function toxicity in C9-ALS/FTD. These include siRNAs, shRNAs and miRNAs (Gagliardi et al., 2020). Several strategies have been developed to target RAN translation and DPR production directly (S. Guo et al., 2022). Transcriptional

machinery has been identified as a therapeutic target, with an aim to prevent production of toxic G4C2 repeat expansion RNA and therefore prevent downstream impacts of the repeat expansion (Gagliardi et al., 2020). Some groups are starting to look at targeting RAN-associated RNA helicases, for example in the case of DDX3X, which binds directly to sense but not antisense C9ORF72 repeats. DDX3X reduction is associated with increased DPR production and associated toxicity in *Drosophila*, while elevating DDX3X expression decreased DPR production (Cheng et al., 2019). This thesis will build on this work on RAN-associated RNA helicases as central to a therapeutic strategy to alter RAN translation of DPRs in C9-ALS/FTD.

1.10 Introduction to the PhD project

As detailed above, substantial research efforts have focused on reducing RAN translation in C9-ALS/FTD, reflecting the key role DPR production plays in disease onset and progression. DPR production is unlikely to be the sole mechanism driving C9-ALS/FTD, but the current evidence strongly implicates it as a key contributor, with neurotoxic effects of DPR accumulation associated with almost every major cellular function, notably RNA metabolism, energy homeostasis and mitochondrial function, proteostasis and protein degradation systems. This PhD project has both a discovery and translational focus, and this introduction has set out the case for focusing on DPR production as a potential therapeutic strategy in C9-ALS/FTD.

Emerging advances in our understanding of mechanisms of RAN translation have highlighted RNA helicases as a clear choice for focusing a therapeutic strategy around. The rationale behind this is that RNA helicases are necessary for resolving mRNA secondary structures such as G-quadruplexes, which must be unwound before RAN translation of DPRs can initiate. Therefore, without helicase activity secondary structures will remain unwound and RAN translation of DPRs will not be able to proceed.

This project will focus on members of the eIF4A family that are some of the key helicases involved in RAN translation initiation. Some research in the field of C9-ALS/FTD has already commenced focusing on inhibition of these helicases, with promising results. For eIF4A, the inhibitor hippuristanol reduces RAN translation of G4C2 repeat expansions (Green et al., 2017), and use of FL3, an eIF4A specific inhibitor, reduced DPR production in HEK293T cells without impacting transcript levels (R. Tabet et al., 2018). This provides clear evidence that eIF4A is required for RNA translation initiation and DPR production. However, unlike this project, these studies do not differentiate between eIF4A1 and eIF4A2 and instead treat them as the interchangeable eIF4A, which may overlook important differences in the function of these proteins in the context of RAN translation. Depletion of co-factors eIF4B and eIF4H suppress the neurodegenerative phenotype in *Drosophila* (Goodman & Bonini, 2019). There is also a clear link between DHX36 depletion and reduction of RAN translation of DPRs, as previously discussed.

This project will therefore focus on the key initiator of RAN translation, eIF4A. To build on previous work in this field, we will not assume eIF4A isoforms are interchangeable and will instead investigate eIF4A1 and eIF4A2 separately. This will be the first work considering the activity of these isoforms separately in the context of RAN translation. In addition, we will investigate co-factors eIF4B and eIF4H, as well as DHX36, due to it being the major resolver of RNA secondary structures.

1.10.1 Aims and Hypothesis

The overall hypothesis for this project is that targeting RAN-associated RNA helicases for depletion will reduce RAN translation of neurotoxic DPRs, thereby providing neuroprotection in pre-clinical models of C9-ALS/FTD. C9ORF72 repeat expansions are particularly prone to forming G-quadruplexes due to their G-rich nature, so we

hypothesise that it will be highly reliant on potential RNA helicase activities to resolve these structures and initiate RAN translation.

The first aim for this project is to develop tools to deplete RAN-associated RNA helicase expression and to test their ability to target specific helicases at both protein and mRNA level through western blot and RT-qPCR, respectively. This specificity is crucial as we aim to investigate potential functional differences in RAN translation initiation associated with eIF4A isoforms eIF4A1 and eIF4A2, which differ by only ten amino acids. Tools developed to target RAN-associated RNA helicases will be shRNAs cloned into a variety of vectors, to deplete helicase expression. Secondly, RAN-associated RNA helicase mutants, where protein expression will remain at normal levels, but helicase activity will be non-functional.

Next, we aim to measure the effects of these tools targeting RAN-associated RNA helicases on RAN translation of DPRs using *in vitro* models of C9-ALS/FTD. HEK293T cells and patient-derived motor neurons will be used for transfection with shRNAs or RNA helicase dominant negative mutants. As studies so far have focused almost entirely on sense DPR production, we will also include investigations into antisense DPRs as mechanisms underlying their synthesis are currently poorly understood.

We aim to measure impact on toxicity through *in vitro* cell proliferation assays. We hypothesise that targeting one RAN-associated RNA helicase individually will be non-toxic, while targeting multiple helicases will confer toxicity due to the impact on global translation. Further, we hypothesise that RAN-associated RNA helicase depletion will rescue toxicity associated with DPR production. We aim to reduce DPR production without conferring toxicity resulting from depletion of essential helicases. We hypothesise that RAN translation occurs at a much slower rate than canonical translation, so it would be possible to reduce the former without causing widespread translational arrest. We aim to deplete RAN-associated RNA helicases by

approximately 50% as we hypothesise that this will reduce RAN translation sufficient to alleviate DPR-induced toxicity without causing toxic effects through reductions in canonical translation.

Finally, we aim to investigate RAN-associated RNA helicase localisation, expression, and CNS distribution in post-mortem human tissue. We hypothesise that the key helicases involved in RAN translation that are the focus on this project may show alterations between control and ALS patient groups that inform their suitability as therapeutic targets, or advance our understanding of their role in RAN translation.

Overall, we aim to investigate relative contributions of eIF4A1, eIF4A2, co-factors eIF4B and eIF4H, and DHX36 on RAN translation of DPRs, and to investigate the effects of their depletion on DPR production in pre-clinical *in vitro* models of C9-ALS/FTD. We intend to establish which RAN-associated RNA helicase, or combination of helicases, are most suitable as therapeutic targets in C9-ALS/FTD. This work advances our knowledge of RAN translation initiation by RNA helicases in the context of C9-ALS/FTD. We explore the possible viability of targeting RAN-associated RNA helicases as a therapeutic strategy and show novel post-mortem data revealing insights into the role of helicases in ALS.

2 Materials and Methods

2.1 Materials

2.1.1 Cell Lines

Human embryonic kidney cells (HEK293T) were used for all transfections shown in this report. DH5 α *E.coli* cells were used during transformation of plasmids. Details of patient-derived neuronal cell lines used for immunofluorescence staining are shown in Table 2.1.

Table 2.1: Details of patient-derived neuronal cell lines used for immunofluorescence staining.

Sample	Genetic remarks	Sex	Age at biopsy collection (years)	Source Tissue	Type of reprogrammed cells	Ethnicity	Disease Duration (months)
161	Non-ALS Control	M	31	Fibroblasts	iNPCs	Caucasian	N/A
17	sALS	M	47	Fibroblasts	iNPCs	Caucasian	72
183	C9orf72 ALS	M	50	Fibroblasts	iNPCs, iPSCs	Caucasian	27

2.1.2 Design of oligonucleotides for eIF4A1, eIF4A2, eIF4B and DHX36

Initial input sequences were taken from the NCBI GenBank® genetic sequence database. The best miRNA targets for human eIF4A1, eIF4A2, eIF4B and DHX36 were identified based on the target sequence, see Tables 2.2, 2.4, 2.6 and 2.8. Sequences were selected based on the fewest number of mismatches between the human and mouse gene, as this will allow for future *in vivo* studies without requiring re-design of the shRNAs. Once sequences were identified, oligonucleotides were designed with the following features: a linker, mature miR RNAi sequence, a loop sequence, and the 1-8, 11-21 of 21mer target. See Figure 2.1 below for a schematic diagram of the miR RNAi.

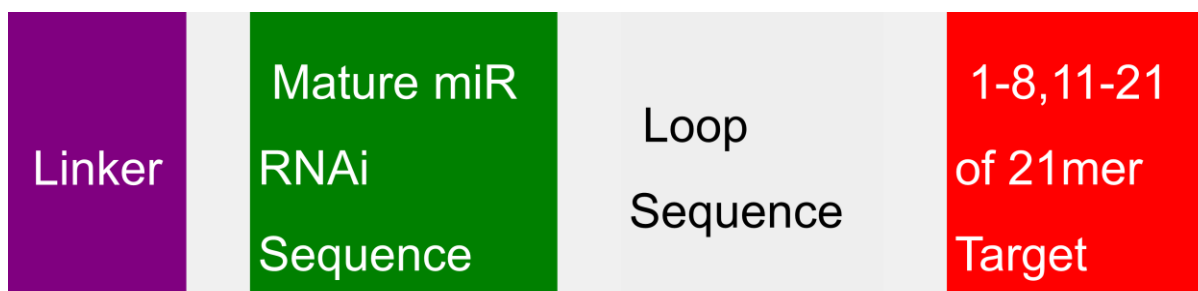


Figure 2.1 schematic diagram of major features in oligonucleotides designed to target RAN-associated RNA helicases

Table 2.2 Best miRNA targets for human eIF4A1. Sequences from ten potential targets are shown and two selected sequences are highlighted

No.	Start	Sequence	GC content %
1	86	CGCTCTATAAGTTGTCGATAG	42.86
2	111	GCACTCCGCCCTAGTTTCTAA	52.39
3	951	TCACCCAGGCAGTCATCTTCA	52.39
4	957	AGGCAGTCATCTTCATCAACA	42.86
5	1001	ACCGAGAAGATGCATGCTCGA	52.39
6	1025	TTCAGTGTATCCGCCATGCAT	47.62
7	1026	TCACTGTATCCGCCATGCATG	52.39
8	1041	TGCATGGAGATATGGACCAAA	42.86
9	1072	CGTGATTATGAGGGAGTTTCG	47.62
10	1375	GCCAAGGGATGGACATCTTGT	52.39

From these targets, two sequences were selected, highlighted in the above Table. Oligonucleotides for eIF4A1 designed based on the miRNA targets can be seen in Table 2.2. In addition to eIF4A1, miR 1 and 2 also target eIF4A2 due to the high degree of similarity between their base sequences.

Table 2.3 Oligonucleotide design to target eIF4A1 and eIF4A1 + 2

Name	No.	Start	Oligonucleotide Type	Oligonucleotide Sequence
miR1	1	957 (CDS)	Top Strand	5'-TGCTGTGTTGATGAAGATGACTGCCTGTTTTGGCCACTGACTGACAGGCAGTCCTTCATCAACA -3'
			Bottom Strand	5'-CCTGTGTTGATGAAGGACTGCCTGTCAGTCAGTGGCCAAAACAGGCAGTCATCTTCATCAACAC -3'
			ds Oligo	5'-TGCTGTGTTGATGAAGATGACTGCCTGTTTTGGCCACTGACTGACAGGCAGTCCTTCATCAACA -3'
				3'-CACAACTACTTCTACTGACGGACAAAACCGGTGACTGACTGTCCGTCAGGAAGTAGTTGTGTCC -5'
miR2	10	1375 (3'UTR)	Top Strand	5'-TGCTGACAAGATGTCCATCCCTTGGCGTTTTGGCCACTGACTGACGCCAAGGGGGACATCTTGT -3'
			Bottom Strand	5'-CCTGACAAGATGTCCCCCTTGGCGTCAGTCAGTGGCCAAAACGCCAAGGGATGGACATCTTGT -3'
			ds Oligo	5'-TGCTGACAAGATGTCCATCCCTTGGCGTTTTGGCCACTGACTGACGCCAAGGGGGACATCTTGT -3'
				3'-CTGTTCTACAGGTAGGGAACCGCAAAACCGGTGACTGACTGCGGTTCCCCCTGTAGAACAGTCC -5'
miR3: designed by Meijer et al.			Top Strand	5'-CACCGTGGAACGAGAGGAGTGGAAGCGAACTCCACTCCTCTCGTTCCAC-3'
			Bottom Strand	5'-AAAAGTGGAACGAGAGGAGTGGAAGTTCGCTTCCACTCCTCTCGTTCCAC-3'
			ds Oligo	5'-CACCGTGGAACGAGAGGAGTGGAAGCGAACTCCACTCCTCTCGTTCCAC-3'
				3'-CACCTTGCTCTCCTCACCTTCGCTTGAAGGTGAGGAGAGCAAGGTGAAAC-5'

Although we were able to design oligonucleotides to target both eIF4A1 and eIF4A2, this project aims to investigate the individual impact of each helicase's activity on RAN translation and the production of DPRs in C9-ALS/FTD, to assess which will be the most effective therapeutic target. Therefore, it was necessary to target each helicase in a highly specific manner. As such a third target was also selected, which is exclusive to eIF4A1. miR 3 was hand designed to target eIF4A1 and has shown to result in efficient silencing in a paper published by Meijer and colleagues (Meijer et al., 2013). Following oligonucleotide design to target eIF4A1 alone, we next looked for sequences that would specifically target eIF4A2.

Table 2.4 Best miRNA targets for human eIF4A2. Sequences from ten potential targets are shown and the selected sequence is highlighted

No.	Start	Sequence	GC content %
1	115	AGCAACTGGAATGAGATTGTT	38.1
2	167	CTCTCCTTCGTGGCATCTATG	52.39
3	221	AGAGAGCTATTATTCCCTGTA	38.1
4	427	GCCACTTGTCATGCCTGCATT	52.39
5	484	CAGGCTGAAGCACCACATATT	47.62
6	519	CGGGAGAGTGTTTGATATGTT	47.86
7	520	GGGAGAGTGTTTGATATGTTA	38.1
8	597	TGAAATGTTGAGCCGTGGTTT	42.86
9	1002	AGGGTCAAGTCGTGTTCTGAT	47.62
10	1055	TGCAACAAGTGTCTTTGGTTA	38.1

From these targets, No. 7 was selected as the best and oligonucleotides designed based on that sequence, seen in Table 2.5 below as miR 1. miR 1 shows significant overlap with an oligonucleotide designed by Meijer and colleagues. This oligonucleotide works effectively and specifically targets eIF4A2 and not eIF4A1. Given the overlap between this and miR 1, we would expect similarly efficiency and specificity. In addition, the new miR 2 has been designed to exclusive target eIF4A2 and not 4A1.

Table 2.5 miR 1 and miR 2 to target eIF4A2

Name	No.	Start	Oligonucleotide type	Oligonucleotide Sequence
miR1	7	520	Top Strand	5'-TGCTGTAACATATCAAACACTCTCCCGTTTGGCCACTGACTGACG GGAGAGTTTGATATGTTA -3'
			Bottom Strand	5'-CCTGTAACATATCAAACACTCTCCCGTCAGTCAGTGGCCAAAACGGGA GAGTGTTTGATATGTTAC -3'
			ds Oligo	5'-TGCTGTAACATATCAAACACTCTCCCGTTTGGCCACTGACTGACG GGAGAGTTTGATATGTTA -3'
				 3'-CATTGTATAGTTTGTGAGAGGGCAAACCGGTGACTGACTGCCCT CTCAAACATACAATGTCC -5'
miR2	New	1796 (3'UT R)	Top Strand	5'-TGCTGATTAAACCCTACCTAGGATTGGTTTTGGCCACTGACTGACC AATCCTATAGGGTTTAAT -3'
			Bottom Strand	5'-CCTGATTAAACCCTATAGGATTGGTCAGTCAGTGGCCAAAACCAAT CCTAGGTAGGGTTTAATC -3'
			ds Oligo	5'-TGCTGATTAAACCCTACCTAGGATTGGTTTTGGCCACTGACTGACC AATCCTATAGGGTTTAAT -3'
				 3'-CTAATTTGGGATGGATCCTAACCAAAACCGGTGACTGACTGGTTAG GATATCCCAAATTAGTCC -5'
miR3	Designed by Meijer et al		Top Strand	5'-CACCGTTTGATATGTTAAACAGAAGACGAATCTTCTGTTTAACATAT CAAA-3'
			Bottom Strand	5'-AAAATTTGATATGTTAAACAGAAGATTCGTCTTCTGTTTAACATATC AAAC-3'
			Ds Oligo	5'-CACCGTTTGATATGTTAAACAGAAGACGAATCTTCTGTTTAACATAT CAAA-3'
				 3'-CAAACATACAATTTGTCTTCTGCTTAGAAGACAAATTGTATAGTTT AAAA-5'

As with eIF4A1, Meijer and colleagues (Meijer et al., 2013) have designed oligonucleotides specifically targeting eIF4A2 (see miR 3 in Table 2.5), which have demonstrated efficient gene knockdown. Next, we moved on to designing oligonucleotides for eIF4B.

Table 2.6 Best miRNA targets for eIF4B Sequences from three potential targets are shown and the selected sequence is highlighted

No.	Start	Sequence	Region	GC Content %
1	1529	AGAAGTAAGTCAGACCAGGAT	ORF	42.86
2	1532	AGTAAGTCAGACCAGGATGCA	ORF	47.62
3	1534	TAAGTCAGACCAGGATGCACG	ORF	52.39

No. 1 was selected, with Table 2.7 showing the miR 1 oligonucleotide that was designed based on this sequence.

Table 2.7 Oligonucleotide to target eIF4B

Name	No.	Start	Oligonucleotide type	Oligonucleotide Sequence
miR 1	1	1529	Top Strand	5'- TGCTGATCCTGGTCTGACTTACTTCTGTTTTGGCCACTGACTGACAG AAGTAACAGACCAGGAT -3'
			Bottom Strand	5'- CCTGATCCTGGTCTGTTACTTCTGTCTAGTCAGTGGCCAAAACAGAAG TAAGTCAGACCAGGATC -3'
			ds Oligo	5'- TGCTGATCCTGGTCTGACTTACTTCTGTTTTGGCCACTGACTGACAG AAGTAACAGACCAGGAT -3'
				3'- CTAGGACCAGACTGAATGAAGACAAAACCGGTGACTGACTGTCTTCA TTGTCTGGTCCTAGTCC -5'
miR 2	Designed by Meijer et al		Top Strand	5'- CACCACGATGACGATGTGTATAGGGCGAACCCCTATACACATCGTCAT CG-3'
			Bottom Strand	5'- AAAACGATGACGATGTGTATAGGGTTCGCCCTATACACATCGTCATC GT-3'
			ds Oligo	5'- CACCACGATGACGATGTGTATAGGGCGAACCCCTATACACATCGTCAT CG-3'
				3'- TGCTACTGCTACACATATCCCGCTTGGGATATGTGTAGCAGTAGCAA AA-5'

As before, Meijer and colleagues (Meijer et al., 2013) have designed an oligonucleotide targeting eIF4B with high efficiency (see miR 2). Finally, we designed oligonucleotides to target DHX36.

Table 2.8 Best miRNA targets for human DHX36 Sequences from ten potential targets are shown and the selected sequences are highlighted

No.	Start	Sequence	GC Content %
1	829	GCTGCAGAAAGGGCAGAATCT	52.39
2	832	GCAGAAAGGGCAGAATCTTGT	47.62
3	954	GCTCCAGTCAGACCCGTATTT	52.39
4	1329	GCCAGATTATGTAAGGGAAC	42.86
5	1505	GCACTTTACATGATCTCTTGA	38.1
6	1996	GCAGTGTTACTCTCCATAAGA	42.86
7	2025	GGAGCTGAACGCTTTGGATAA	47.62
8	2028	GCTGAACGCTTTGGATAACA	42.86
9	2068	GGAGTCCACTTGGCACGATTA	52.39
10	2932	GCAGTACTGTCAGCTATTATA	38.1

Oligonucleotide design based on these sequences is shown in Table 2.9.

Table 2.9 Oligonucleotide design to target DHX36

Name	No.	Start	Oligonucleotide type	Oligonucleotide Sequence
miR 1	2	832	Top Strand	5'- CACCGCAGAAAGGGCAGAATCTTGTCGAAACAAGATTCTGCCCTTTCTGC -3'
			Bottom Strand	5'- AAAAGCAGAAAGGGCAGAATCTTGTTTCGACAAGATTCTGCCCTTTCTGC - 3'
			ds Oligo	5'- CACCGCAGAAAGGGCAGAATCTTGTCGAAACAAGATTCTGCCCTTTCTGC -3'
				3'- CGTCTTTCCCGTCTTAGAACAGCTTTGTTCTAAGACGGGAAAGACGAAAA - 5'
miR 2	4	1329	Top Strand	5'- CACCGCCAGATTATGTAAGGGAACTCGAAAGTTCCTTACATAATCTGGC - 3'
			Bottom Strand	5'- AAAAGCCAGATTATGTAAGGGAACTTTCGAGTTCCTTACATAATCTGGC - 3'
			ds Oligo	5'- CACCGCCAGATTATGTAAGGGAACTCGAAAGTTCCTTACATAATCTGGC - 3'
				3'- CGGTCTAATACATTCCCTTGAGCTTCAAGGGAATGTATTAGACCGAAAA - 5'
miR 3	8	2028	Top Strand	5'- CACCGCTGAACGCTTTGGATAAACACGAATGTTTATCCAAAGCGTTCAGC - 3'
			Bottom Strand	5'- AAAAGCTGAACGCTTTGGATAAACATTCGTGTTTATCCAAAGCGTTCAGC - 3'
			ds Oligo	5'- CACCGCTGAACGCTTTGGATAAACACGAATGTTTATCCAAAGCGTTCAGC - 3'
				3'- CGACTTGCGAAACCTATTTGTGCTTACAAATAGGTTTCGCAAGTCGAAAA - 5'

2.1.3 shRNA design for insertion into pENTR/H1-TO-MCS, scAAV-KAS/H1 and LV-H1-TO vectors

The shRNAs were designed in order to silence endogenous expression of RAN-associated RNA helicases eIF4A1, eIF4A2, eIF4B and DHX36, thereby preventing initiation of RAN translation and the synthesis of toxic DPRs. Following selection of miRNAs, Thermo Fisher Scientific's BLOCK-iT™ RNAi designer was used to establish the shRNA design, using the miRNA to shRNA feature. The design tool can be found at <http://rnaidesigner.thermofisher.com/rnaiexpress/>. Following preliminary testing, we

selected eIF4A1 miR3, eIF4A2 miR1 and miR2, eIF4A1+2 miR1, eIF4B miR1, and DHX36 miR2.

The orientation of the sequence strand was set to sense stem – loop – antisense stem. GC content ranged from 38.1 – 52.39 %. Key shRNA features are shown in Figure 2.2.

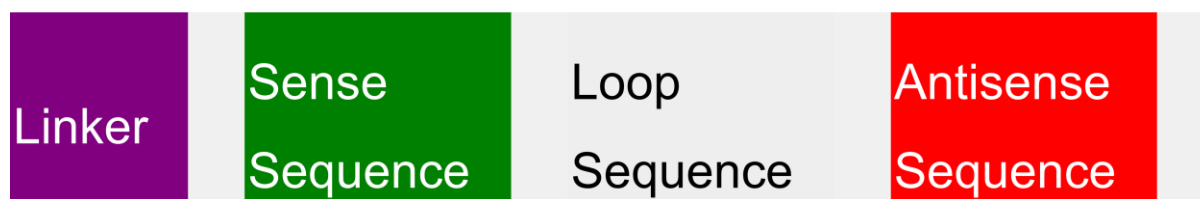


Figure 2.2 schematic diagram of major features in shRNAs designed to target RNA helicases

Oligonucleotides top and bottom strands were annealed to produce a double stranded oligonucleotide with 4-nucleotide overhanging ends. This can be directly cloned into the chosen vectors pENTR/H1-TO-MCS and scAAV-KAS/H1. pENTR clones were subsequently sub-cloned into LV-H1/TO vectors.

Design of shRNA oligonucleotides for eIF4A1 insertion into pENTR/H1-TO-MCS

Table 2.10 pENTR/H1-TO-MCS/ eIF4A1 shRNA3 (design against human - 1 mismatch with mouse)

4A1_29m-shR3_fwd	Restriction Enzyme Site cut XhoI	TCGA
	sense/target shRNA	G CCTTCTGATGTGCTTGAGGTGACCAAGA
	Linker	TTCAAGAGA
	antisense shRNA	TCTGGTCACCTCAAGCACATCAGAAGGC
	RNAPIII term	TTTTTT
4A1_29m-shR3_rev	Restriction Enzyme Site cut HindIII	A
	sense/target shRNA	GCCTTCTGATGTGCTTGAGGTGACCAAGA
	Linker	TCTCTTGAA
	antisense shRNA	TCTGGTCACCTCAAGCACATCAGAAGG
	RNAPIII term	AAAAAA
	Restriction Enzyme Site cut HindIII	AGCTT

Design of shRNA oligonucleotides for eIF4A1 insertion into scAAV-KAS/H1

Table 2.11 scAAV-KAS/ H1-eIF4A1 shRNA_EF-1-eGFP (design against human - 1 mismatch with mouse)

4A1-shR_scAAV_fwd	Restriction Enzyme Site cut BamHI	GATCC
	sense/target shRNA	GCCTTCTGATGTGCTTGAGGTGACCAAGA
	Linker	TTCAAGAGA
	antisense shRNA	TCTTGGTCACCTCAAGCACATCAGAAGGC
	RNAPIII term	TTTTTT
	Restriction Enzyme Site cut HindIII	A
4A1-shR_scAAV_rev	Restriction Enzyme Site cut BamHI	G
	sense/target shRNA	GCCTTCTGATGTGCTTGAGGTGACCAAGA
	Linker	TCTCTTGAA
	antisense shRNA	TCTTGGTCACCTCAAGCACATCAGAAGGC
	RNAPIII term	AAAAAA
	Restriction Enzyme Site cut HindIII	AGCTT

Design of shRNA oligonucleotides for eIF4A2 insertion into pENTR/H1-TO-MCS

Table 2.12 pENTR/H1-TO-MCS/ eIF4A2 shRNA1 (fully conserved between human and mouse)

4A2_29m-shR1_fwd	Restriction Enzyme Site cut XhoI	TCGAG
	sense/target shRNA	CGATGGTGTTCATCGAGAGCAACTGGAATG
	Linker	TTCAAGAGA
	antisense shRNA	CATTCCAGTTGCTCTCGATGACACCATCG
	RNAPIII term	TTTTTT
	Restriction Enzyme Site cut HindIII	A
4A2_29m-shR1_rev	Restriction Enzyme Site cut XhoI	C
	sense/target shRNA	CGATGGTGTTCATCGAGAGCAACTGGAATG
	Linker	TCTCTTGAA
	antisense shRNA	CATTCCAGTTGCTCTCGATGACACCATCG
	RNAPIII term	AAAAAA
	Restriction Enzyme Site cut HindIII	AGCTT

Table 2.13 pENTR/H1-TO-MCS/ eIF4A2 shRNA2 (fully conserved between human and mouse)

4A2_29m-shR2_fwd	Restriction Enzyme Site <i>cut</i> XhoI	TCGAG
	sense/target shRNA	CAGTCAGGTACTGGCAAGACAGCCACATT
	Linker	TTCAAGAGA
	antisense shRNA	AATGTGGCTGTCTTGCCAGTACCTGACTG
	RNAPIII term	TTTTTT
	Restriction Enzyme Site <i>cut</i> HindIII	A
4A2_29m-shR2_rev	Restriction Enzyme Site <i>cut</i> XhoI	C
	sense/target shRNA	CAGTCAGGTACTGGCAAGACAGCCACATT
	Linker	TCTCTTGAA
	antisense shRNA	AATGTGGCTGTCTTGCCAGTACCTGACTG
	RNAPIII term	AAAAAA
	Restriction Enzyme Site <i>cut</i> HindIII	AGCTT

Design of shRNA oligonucleotides for eIF4A2 insertion into scAAV-KAS/H1

Table 2.14 scAAV-KAS/ H1-eIF4A2 shRNA_EF-1-eGFP (29mer-shRNA2, design against human - 1 mismatch with mouse)

4A2-shR_scAAV_fwd	Restriction Enzyme Site <i>cut</i> BamHI	GATCC G
	sense/target shRNA	CAGTCAGGTACTGGCAAGACAGCCACATT
	Linker	TTCAAGAGA
	antisense shRNA	AATGTGGCTGTCTTGCCAGTACCTGACTG
	RNAPIII term	C TTTTTT
	Restriction Enzyme Site <i>cut</i> HindIII	A
4A2-shR_scAAV_rev	Restriction Enzyme Site <i>cut</i> BamHI	C G
	sense/target shRNA	CAGTCAGGTACTGGCAAGACAGCCACATT
	Linker	TCTCTTGAA
	antisense shRNA	AATGTGGCTGTCTTGCCAGTACCTGACTG
	RNAPIII term	AAAAAA G
	Restriction Enzyme Site <i>cut</i> HindIII	AGCTT

Design of shRNA oligonucleotides for eIF4B insertion into pENTR/H1-TO-MCS

Table 2.15 pENTR/H1-TO-MCS/ eIF4AB 29mer-shRNA (fully conserved between human and mouse)

4B-shR_pENTR_fwd	Restriction Enzyme Site cut XhoI	TCGAG
	sense/target shRNA	AATTGACCGTTCCATCCTTCCCACTGCTC
	Linker	TTCAAGAGA
	antisense shRNA	GAGCAGTGGGAAGGATGGAACGGTCAATT
	RNAPIII term	TTTTTT
	Restriction Enzyme Site cut HindIII	A
4B-shR_pENTR_rev	Restriction Enzyme Site cut XhoI	C
	sense/target shRNA	AATTGACCGTTCCATCCTTCCCACTGCTC
	Linker	TCTCTTGAA
	antisense shRNA	GAGCAGTGGGAAGGATGGAACGGTCAATT
	RNAPIII term	AAAAAA
	Restriction Enzyme Site cut HindIII	AGCTT

Design of shRNA oligonucleotides for eIF4B insertion into scAAV-KAS/H1

Table 2.16 scAAV-KAS/ H1-eIF4B 29mer-shRNA_EF-1 α -eGFP

4B-shR_scAAV_fwd	Restriction Enzyme Site cut BamHI	GATCC
	sense/target shRNA	AATTGACCGTTCCATCCTTCCCACTGCTC
	Linker	TTCAAGAGA
	antisense shRNA	GAGCAGTGGGAAGGATGGAACGGTCAATT
	RNAPIII term	TTTTTT
	Restriction Enzyme Site cut HindIII	A
4B-shR_scAAV_rev	Restriction Enzyme Site cut BamHI	G
	sense/target shRNA	AATTGACCGTTCCATCCTTCCCACTGCTC
	Linker	TCTCTTGAA
	antisense shRNA	GAGCAGTGGGAAGGATGGAACGGTCAATT
	RNAPIII term	AAAAAA
	Restriction Enzyme Site cut HindIII	AGCTT

Design of shRNA oligonucleotides for DHX36 insertion into scAAV-KAS/ H1

Table 2.17 scAAV-KAS/ H1-DHX36 shRNA1_EF-1-eGFP (1 mismatch with mouse, 24nt mouse eIF4B shRNA , 25nt human eIF4B shRNA)

DHX36-shR1_scAAV_fwd	Restriction Enzyme Site cut BamHI	GATCC
	sense/target shRNA	GCAGAAAGGGCAGAATCTTGTGGCA
	Linker	TTCAAGAGA
	antisense shRNA	TGCCACAAGATTCTGCCCTTTCTGC
	RNAPIII term	TTTTTT
	Restriction Enzyme Site cut HindIII	A
DHX36-shR1_scAAV_rev	Restriction Enzyme Site cut BamHI	G
	sense/target shRNA	GCAGAAAGGGCAGAATCTTGTGGCA
	Linker	TCTCTGAA
	antisense shRNA	TGCCACAAGATTCTGCCCTTTCTGC
	RNAPIII term	AAAAAA
	Restriction Enzyme Site cut HindIII	AGCTT

2.1.4 Vectors

First, we generated pENTR/H1-TO-MCS plasmid maps for eIF4A1 shRNA3, eIF4A2 shRNA2, eIF4A1+2 shRNA 2, eIF4B 29mer shRNA and DHX36 shRNA 1, including major features and restriction sites. In total, three sets of plasmids were used: pENTR, scAAV and LV, all of which allow for mammalian expression of shRNAs, although only LV vectors would be required for translation in pre-clinical neuronal models.

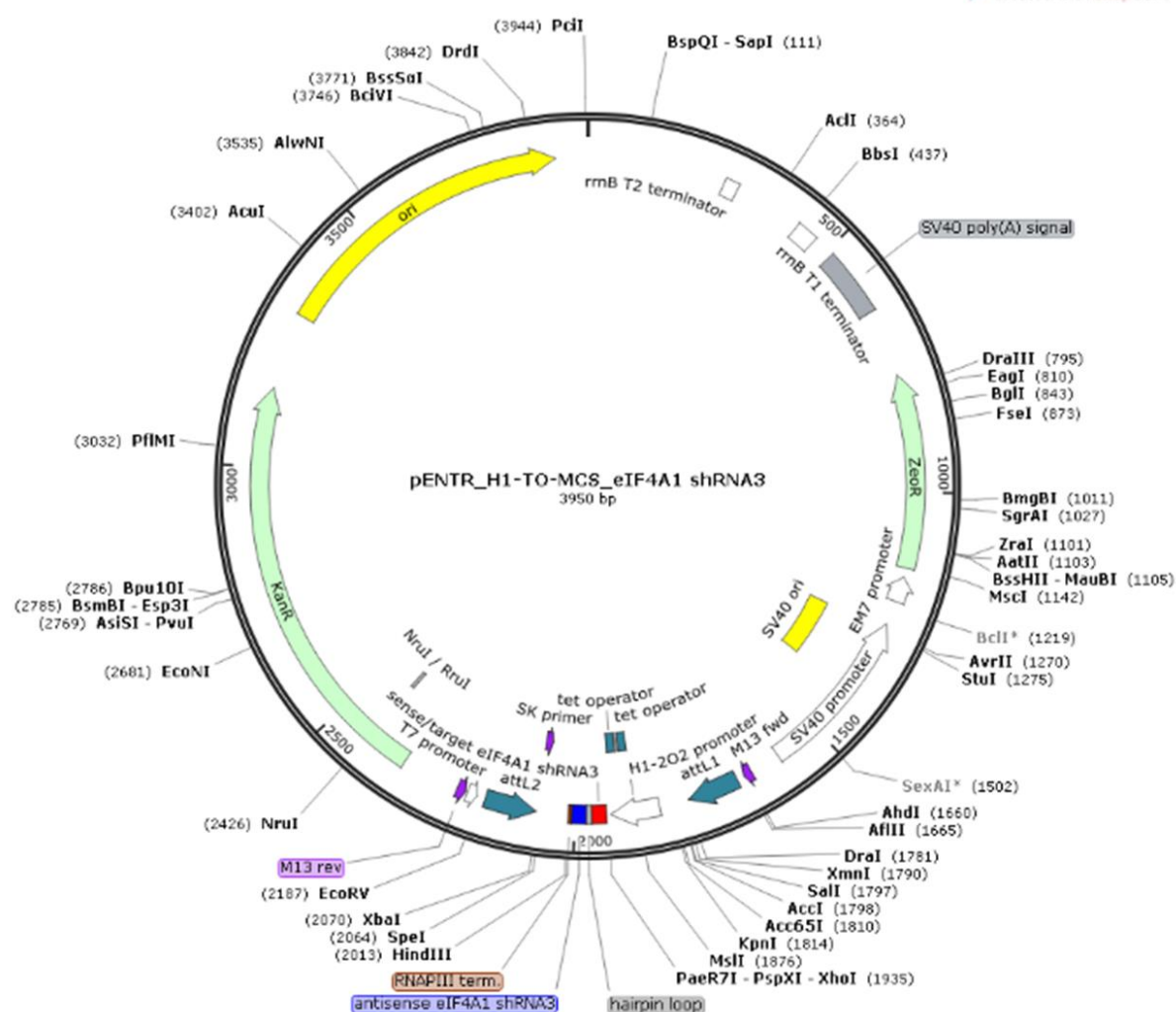


Figure 2.3 Plasmid map for pENTR eIF4A1 shRNA-3

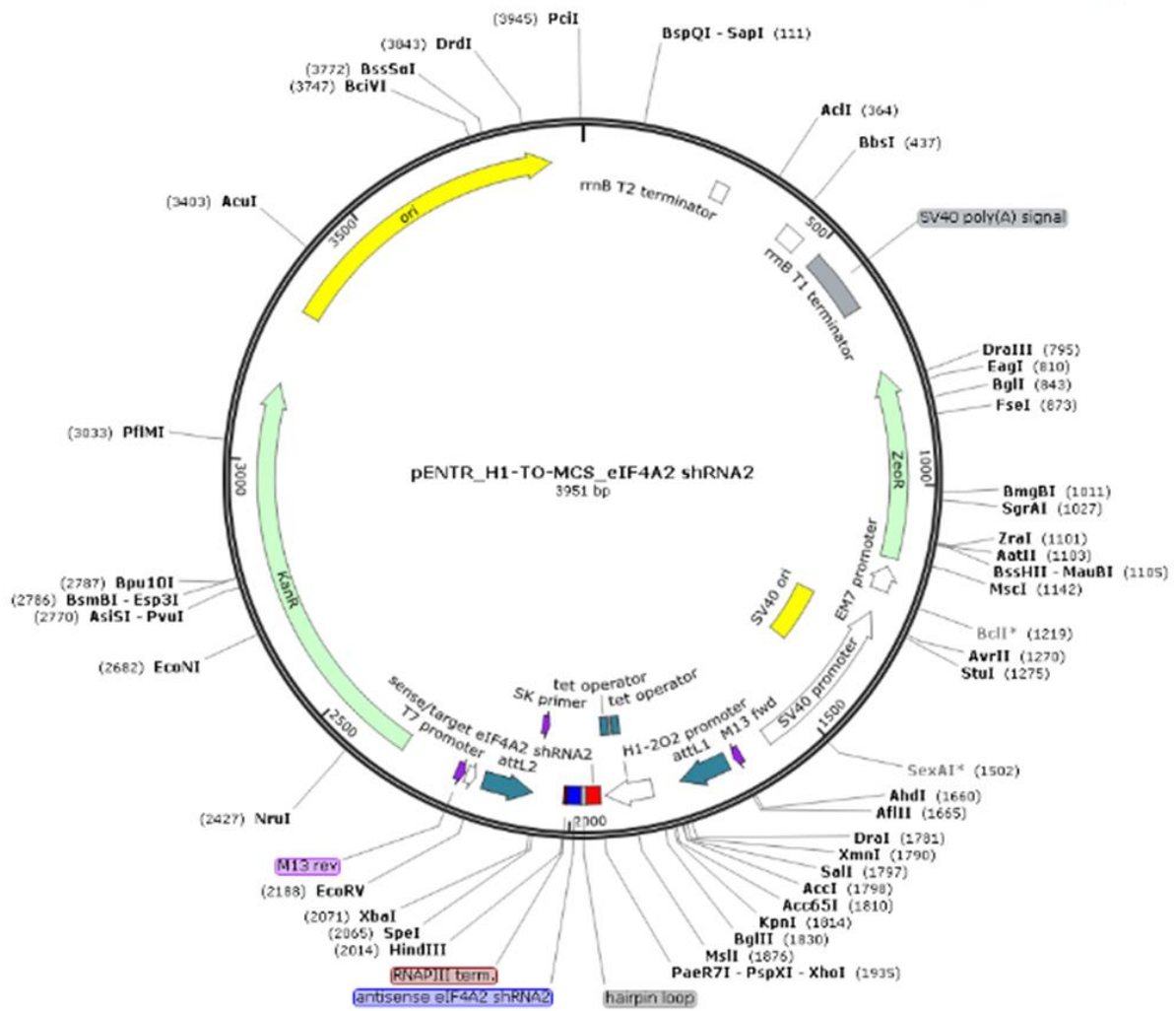


Figure 2.4 Plasmid map for pENTR eIF4A2 shRNA-2

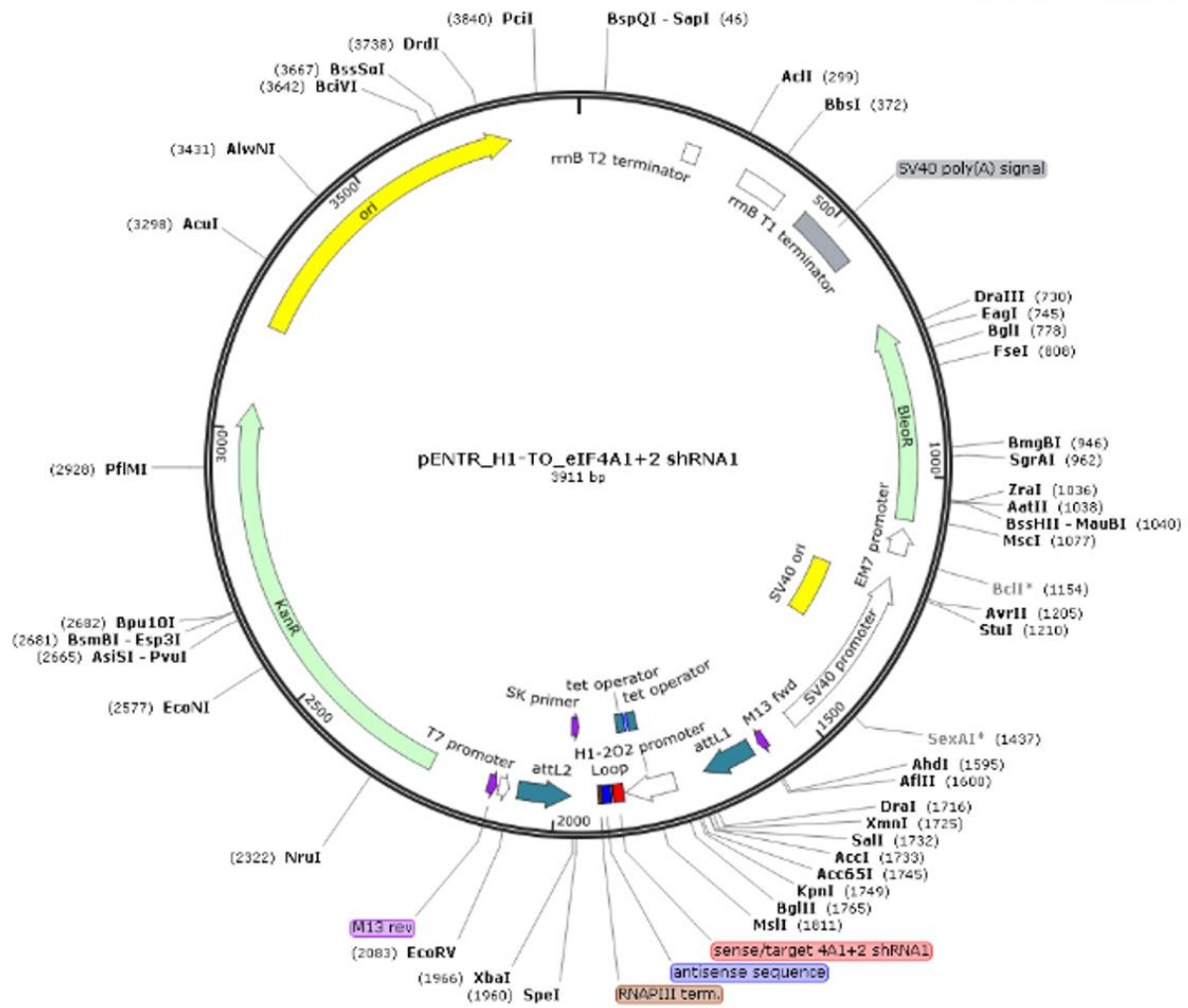


Figure 2.5 plasmid map for pENTR eIF4A1+2 shRNA-1

98 | Page

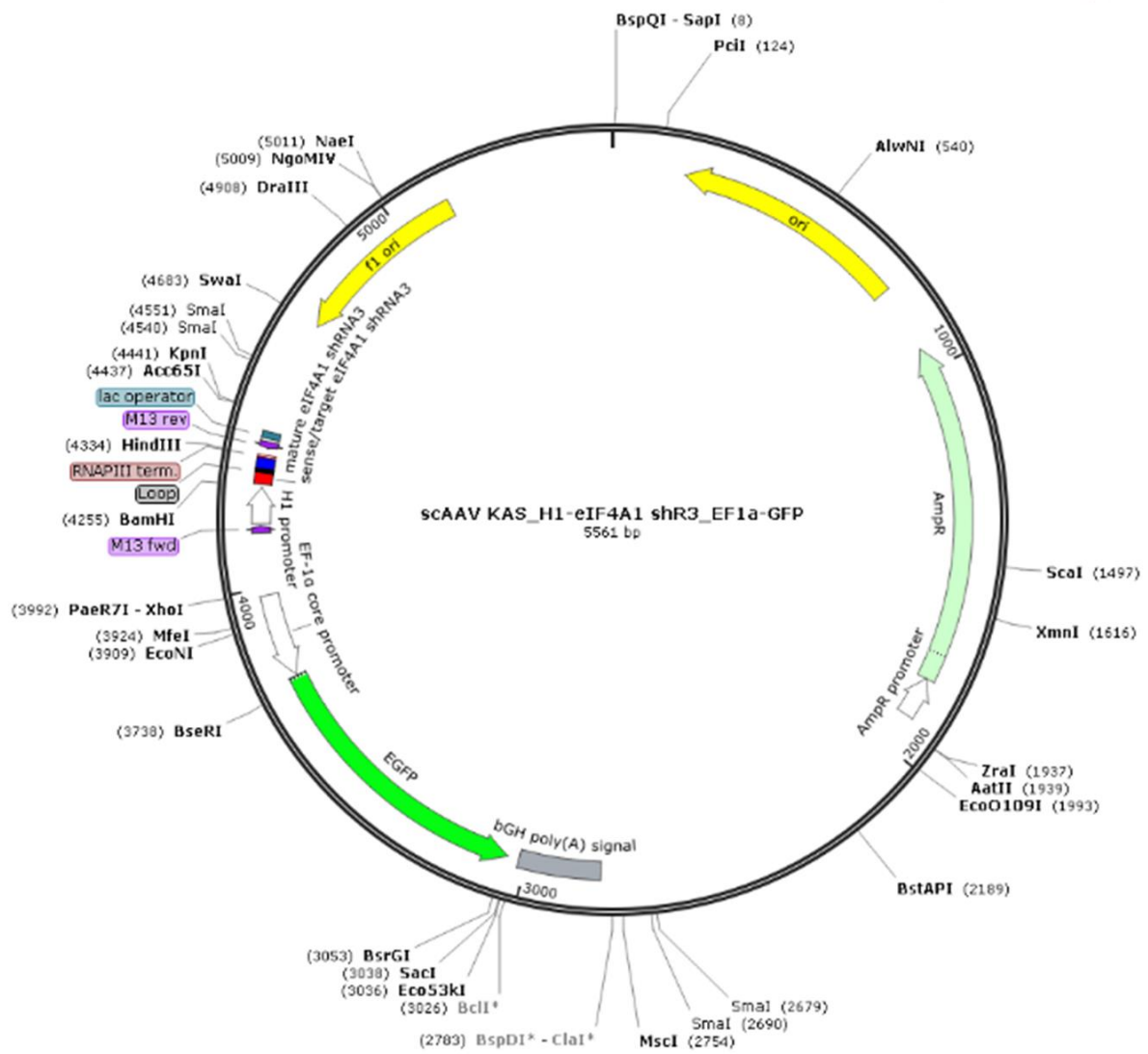


Figure 2.8 Plasmid map for scAAV eIF4A1 shR-3

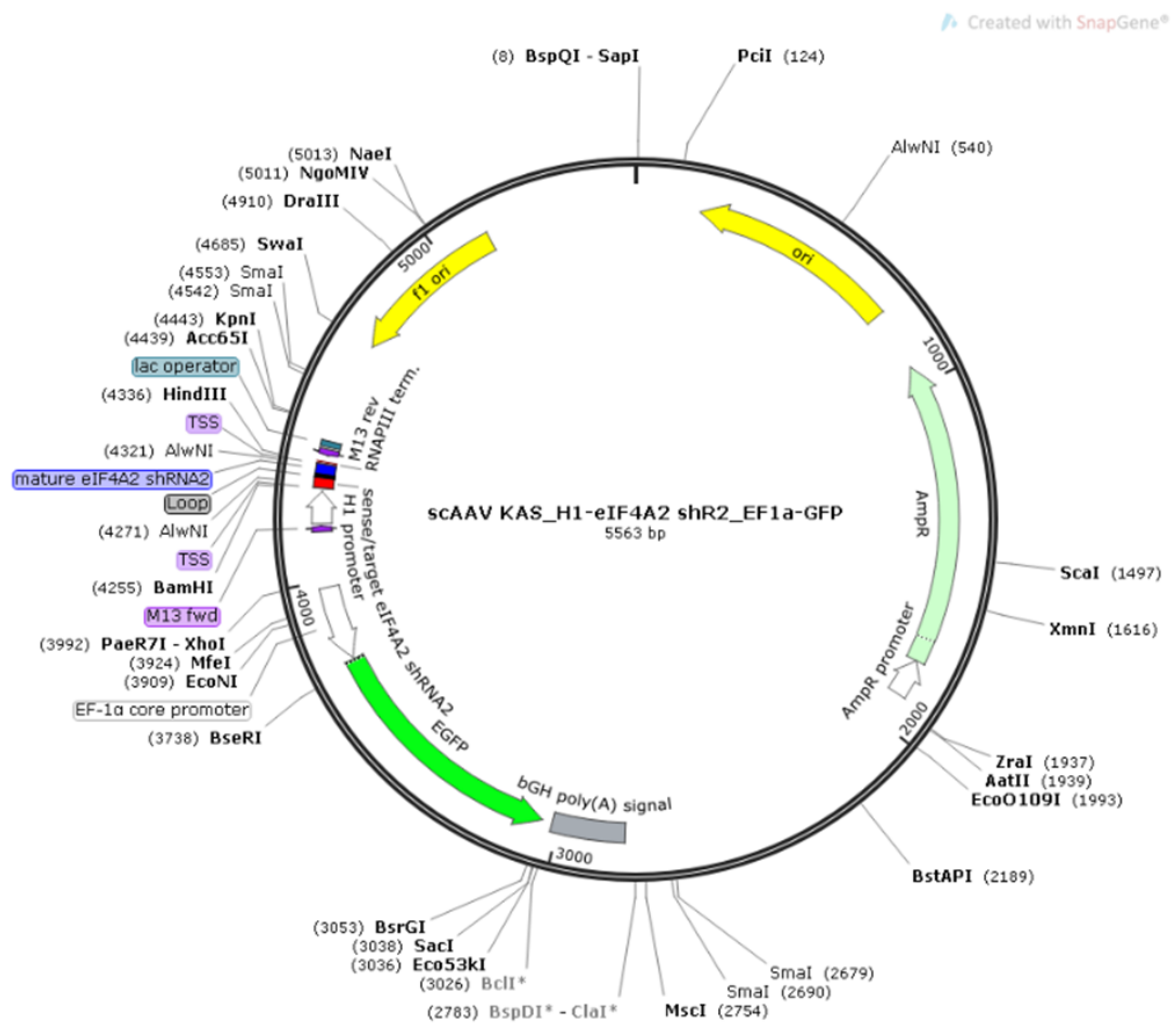


Figure 2.9 Plasmid map for scAAV eIF4A2 shR2

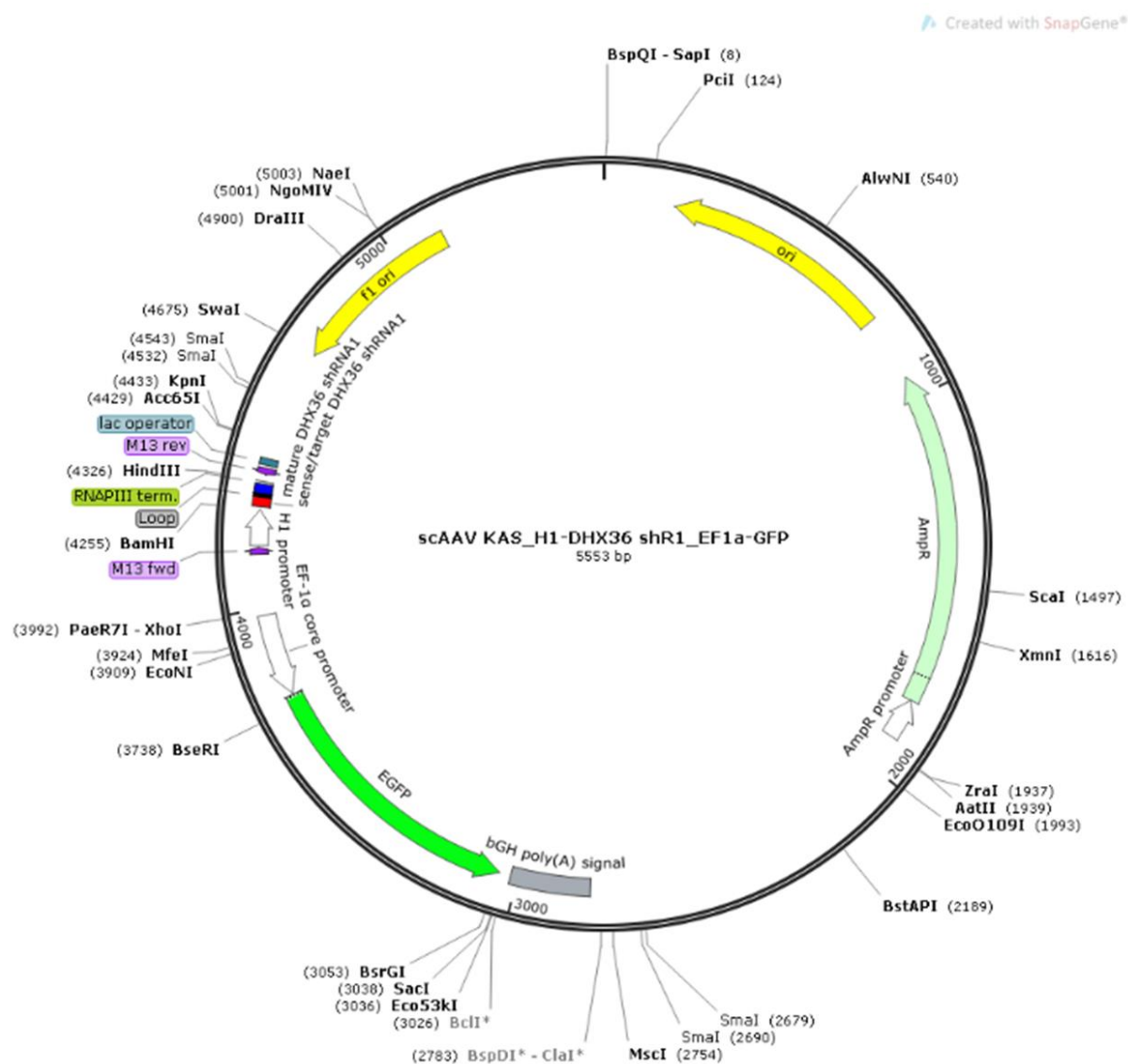


Figure 2.10 Plasmid map for scAAV DHX36 shR1

The final set of plasmid maps include LV-H1-TO plasmid maps for eIF4A1 shR3, eIF4A2 shR 2, eIF4A1 + 2 shR 1 and DHX36 shR 1, including major features and restriction sites.

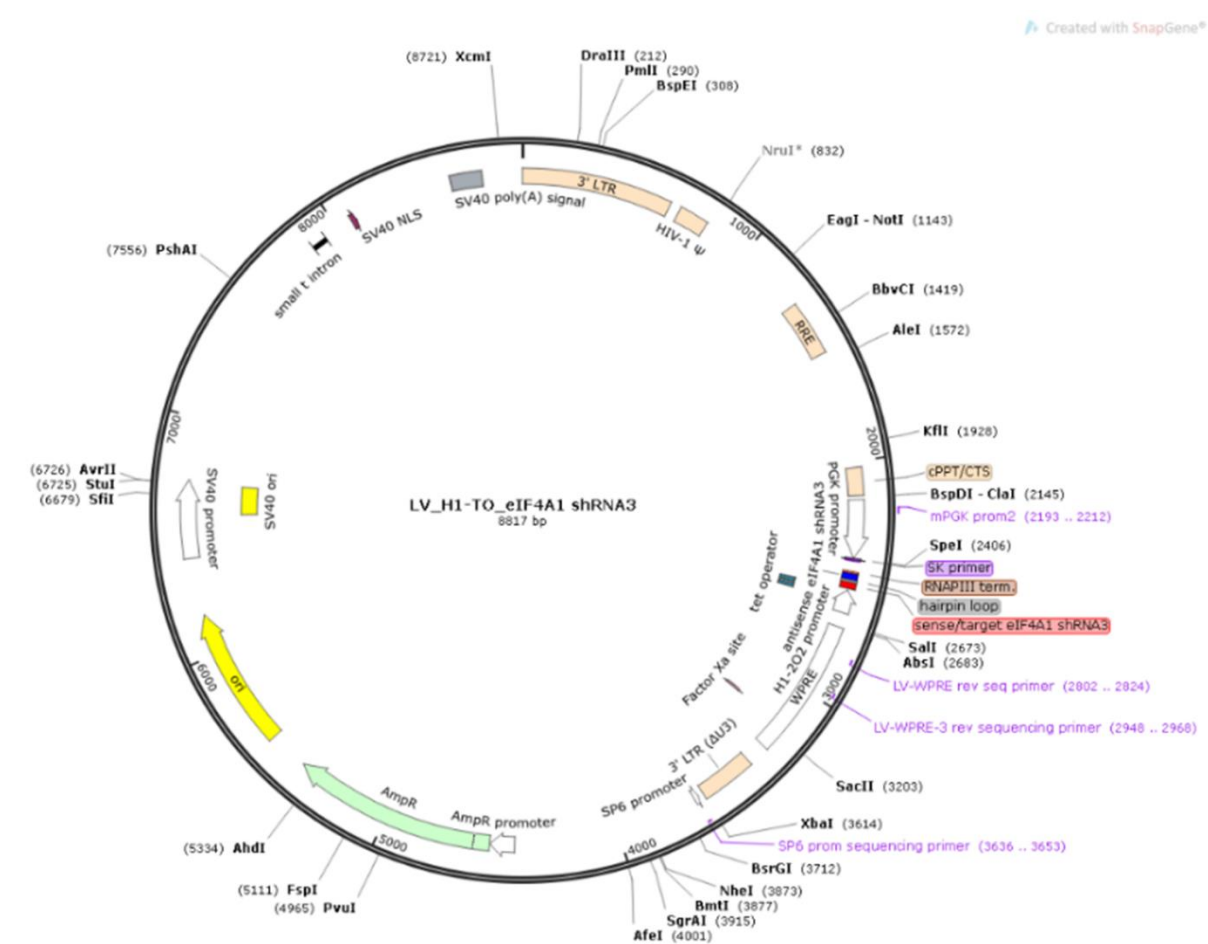


Figure 2.11 Plasmid map for LV-eIF4A1 shRNA3

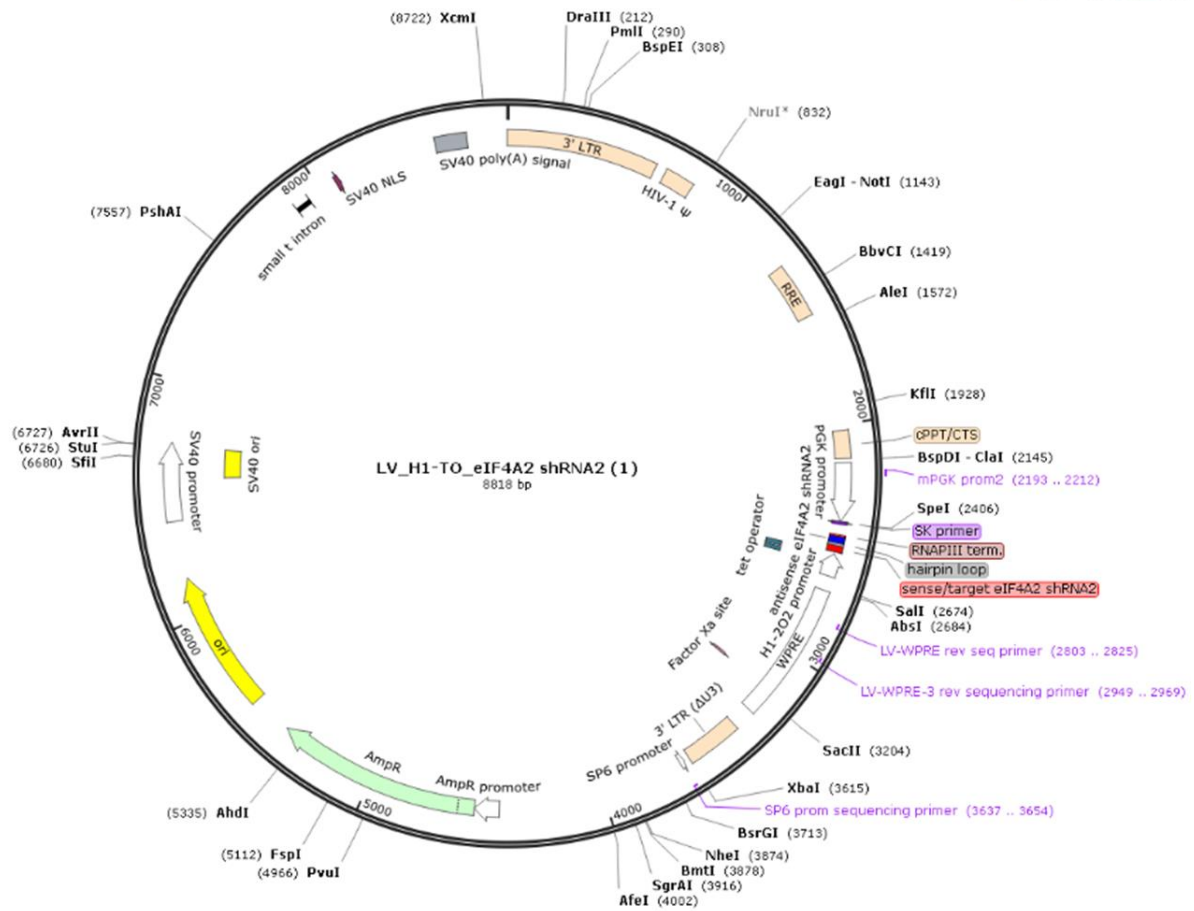


Figure 2.12 Plasmid Map for LV-eIF4A2 shRNA2

105 | Page

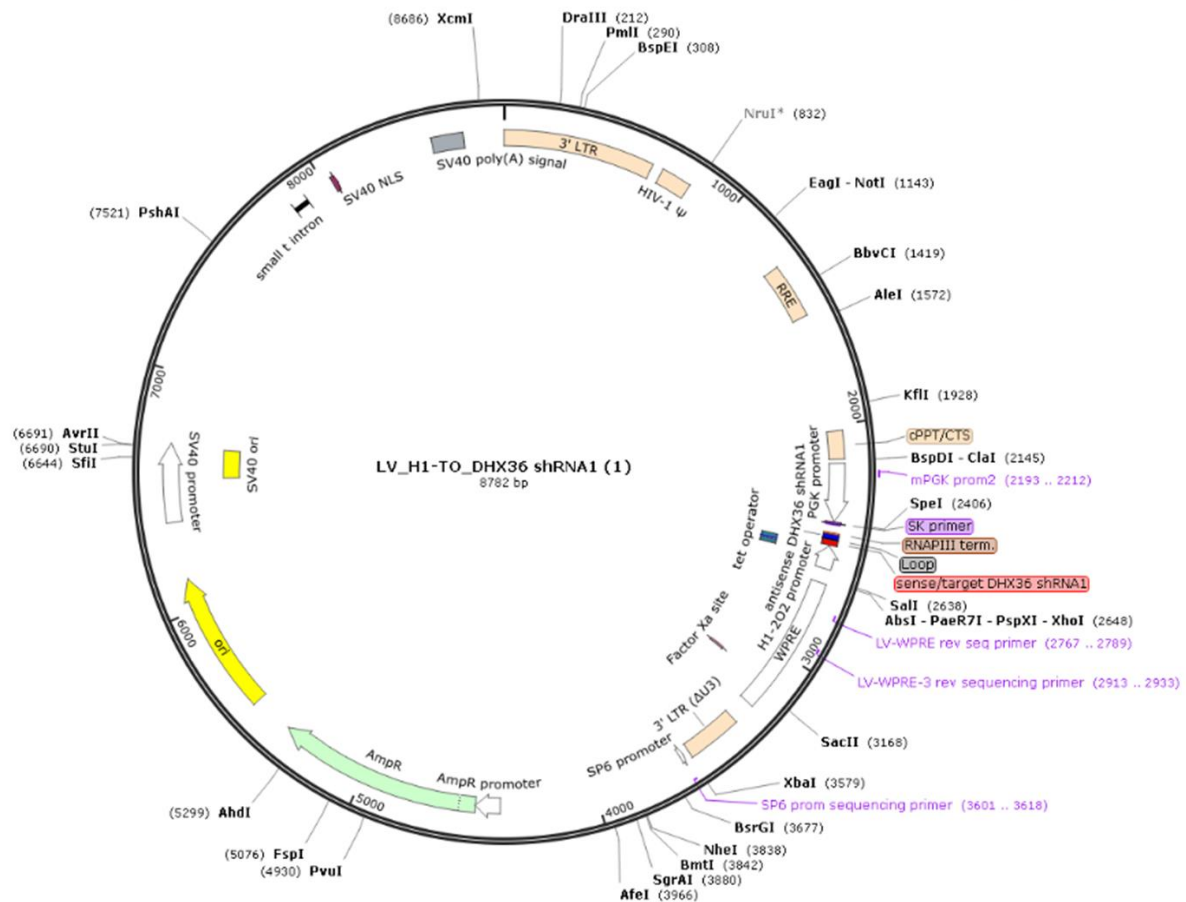


Figure 2.14 Plasmid Map for LV-DHX36 shRNA1

2.1.5 Antibiotics

Table 2.18 Antibiotics use for inoculations

Vector	Antibiotic	Dilution
pENTR/H1-TO-MCS	Kanamycin	1:1000
scAAV-KAS/H1	Ampicillin	1:1000
LV-H1-TO	Ampicillin	1:1000

2.1.6 Buffers and Solutions

Cloning and Restriction Digest

QIAquick Gel Extraction Kit

T4 DNA Ligase buffer: BioLabs 10 X buffer for T4 DNA ligase with 10 mM ATP

#B0202S

Fast Digest Green Buffer: Thermo Fisher Scientific Lot 00638694

T4 Polynucleotides Kinase Reaction Buffer: BioLabs 10 X buffer #B0201S

Mini and Midi Preparations of plasmid DNA

QIAprep Spin Mini Prep Kit

QIAGEN Plasmid Plus Midi Kit

Transfections

Optimem 1 x Reduced Serum Medium + L-Glutamine Gibco Ref 11058-21

Polyethylenimine (PEI) 1mg/ml

Western Blot

Running Buffer 1 x: 30 g Tris, 144 g Glycine, 10 g SDS up to 10 L dH₂O

Transfer Buffer 5 x: 150 g Tris, 720 g Glycine up to 10 L dH₂O

TBS-T: Tris-buffered saline (21.6 g Tris, 79.2 g NaCl up to 10L dH₂O pH 7.6) with 2 ml/litre 0.1 % Tween-20

Lysis buffer: 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% Triton x 100, 50 mM HEPES, 10 % Glycerol, pH 7.5

Stacking gel buffer: 30.3 g Trizma/Tris, 2 g SDS, up to 500 ml dH₂O, pH 6.8

Resolving gel buffer: 90.85 g Trizma/Tris, 2 g SDS, up to 500 ml dH₂O, pH 8.8

Tetramethylethylenediamine (TEMED): N,N,N',N'-tetramethylethane-1,2-diamine, C₆H₁₆N₂

Ammonium Persulfate (APS): (NH₄)₂S₂O₈

Protease Inhibitor Cocktail (PIC) complete Tablets EDTA free, 1 tablet/2ml dH₂O

Phenylmethanesulfonyl fluoride (PMSF) 100 mM

1 x working reporter buffer: diluted from Promega 5 x reported buffer in dH₂O + 20 µl per ml PMSF + PIC

Enhanced Chemiluminescence (ECL): ECL1: 1 ml 1 M Tris-HCl, 100 µl 250 mM luminol in DMSO, 44 µl 90 mM p-coumaric acid in DMSO, up to 10 ml dH₂O

Immunohistochemistry

Hydrogen Peroxide: 3 % H₂O₂ + Methanol; Fisher Chemicals H/1750/15

TBS: Tris-buffered saline (21.6 g Tris, 79.2 g NaCl up to 10 L dH₂O pH 7.6)

Harris Haematoxylin

Scott's Tap Water (8.75 g Sodium Bicarbonate, 50 g Magnesium Sulphate, 2.5 l tap H₂O)

DPX Cell Path mounting medium

VectaMount Aqueous mounting medium

Immunofluorescence

PBS: Phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 2 mM KH₂PO₄)

Triton-X 100

Paraformaldehyde 4%

Hoechst 33342 (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate): Thermo Fisher 62249, 1:5000

Vectorshield mounting medium with DAPI

MTT assays

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MTT buffer: 50 % DMF, 20 % SDS

RT-qPCR

RNAse-free lysis buffer 50 ml: 7.5 ml NaCl, 2.5 ml 10% Triton, 50 µl DTT, 100 µl

EDTA 0.5M, 5 ml HEPES, 5 ml 10% Glycerol

Phenylmethanesulfonyl fluoride (PMSF) 100 mM

PureZol RNA isolation reagent BioRad Cat.7326890

ETOH (RNAse free)

DNAse I recombinant Incubation Buffer: Roche Diagnostics 04716728001

dNTP 10 mM mix: Bioline DM1-717106

dN6 oligo: Sigma HA05153767-010

5 x First Strand buffer: Invitrogen Y02321

DTT 0.1 M: Invitrogen Y00147

2 x brilliant III SYBR Green QPCR master mix: Agilent Technology Cat #600882

2.1.7 Enzymes

T4 DNA ligase: Thermo Scientific #EL0011 Lot 00429450

Alkaline Phosphatase Calf Intestinal (CIP): BioLabs #M0290S

T4 Polynucleotides Kinase: BioLabs #M0201S

DNAse I recombinant enzyme: Roche Diagnostics 04716728001

M-MLV RT enzyme: Invitrogen 28025-021

Table 2.19 Restriction Enzymes

Plasmid	Restriction Enzyme	Company	Product Code	Restriction Site					
ENTR/H1-TO-MCS	<i>HindIII</i>	Thermo Scientific	FD0504	5'	A	A	G	C	T T 3'
				3'	T	T	C	G	A A 5'
	<i>XhoI</i>	Thermo Scientific	FD0694	5'	C	T	C	G	A G 3'
				3'	G	A	G	C	T C 5'
	<i>DraI</i>	Thermo Scientific	FD0224	5'	T	T	T	A	A A 3'
				3'	A	A	A	T	T T 5'
	<i>EcoRV</i>	Thermo Scientific	FD0303	5'	G	A	T	A	T C 3'
				3'	C	T	A	T	A G 5'
	<i>XbaI</i>	Thermo Scientific	FD0684	5'	T	C	T	A	G A 3'
				3'	A	G	A	T	C T 5'
scAAV-KAS/H1	<i>BamHI</i>	Thermo Scientific	FD0054	5'	G	G	A	T	C C 3'
				3'	C	C	T	A	G G 5'
	<i>HindIII</i>	Thermo Scientific	FD0504	5'	A	A	G	C	T T 3'
				3'	T	T	C	G	A A 5'
	<i>KpnI</i>	Thermo Scientific	FD0524	5'	G	G	T	A	C C 3'
				3'	C	C	A	T	G G 5'
LV-H1-TO	<i>BclI</i>	Thermo Scientific	FD1254	5'	A	C	T	A	G T 3'
				3'	T	G	A	T	C A 5'
	<i>SaI</i>	Thermo Scientific	FD0644	5'	G	T	C	G	A C 3'
				3'	C	A	G	C	T G 5'

2.1.8 Antibodies

Table 2.20: Primary Antibodies

Target Protein	Company Name	Product Code	Species	Dilution	Concentration mg/ml
eIF4A1	Cell Signalling	#2490	Rabbit	1:1000	0.088
eIF4A1	Abcam	Ab31217	Rabbit	1:1000	0.9
eIF4A2	AbCam	Ab31218	Rabbit	1:1000	1.0
eIF4B	AbCam	Ab68474	Rabbit	1:2000	0.219
eIF4B	Invitrogen	MA5-17071	Rabbit	1:1000	1
eIF4H	Cell Signalling	#3469	Rabbit	1:1000 western blot, 1:1500 IHC	
DHX36	Santa Cruz	Sc377485	Mouse	1:1000	0.2
DHX36	AbCam	Ab226813	Rabbit	1:1000	0.6
SRSF1	AbCam	Ab38017	Rabbit	1:1000 western blot; 1:2000 IHC	0.9
SRSF1	Cell Signalling	#14902	Rabbit	1:1000	0.114
TDP-43 C Terminal	Proteintech	12892-1-AP	Rabbit	1: 500	0.65
TDP-43 Phospho	Biolegend	SIG-39852	Rat	1: 1000	1
TDP-43 Phospho	Affinity Biosciences	AF365	Rabbit	1:1000	1
p62	BD Biosciences	BD610833	Mouse	1:200	0.25
FLAG	Sigma	F1804	Mouse	1:2000	1
V5	AbCam	Ab27671	Mouse	1:2000	1
α -tubulin	Santa Cruz	Sc32293	Mouse	1:2000	0.2
β -Actin			Mouse	1:1000	
TUJ1 (Anti-beta III Tubulin)	Millipore	Ab9354	Chicken	1:1000	0.3

Table 2.21 Secondary Antibodies

	Company Name	Product Code	Dilution	Concentration mg/ml
Anti-Mouse	Promega	W402B	1:5000	1
Anti-Rabbit	Promega	W401B	1:5000	1
Anti-Rat 488	Alexa Fluor	Ab150157	1:1000	2
Anti-Rabbit 594	Alexa Fluor	Ab150080	1:1000	2
Anti- Chicken 488	Alexa Fluor	Ab150169	1:1000	2

Biotinylated secondary antibodies used for immunohistochemistry were included in IHC kits, detailed in section 2.1.9.

2.1.9 Kits for Immunohistochemistry

DAB staining

Vecastain Elite ABC Kit Rabbit IgG Peroxidase PK-6101

Vecastain Elite ABC Kit Mouse IgG Peroxidase PK-6102

Vecastain Elite ABC Kit Rat IgG Peroxidase PK-4004

DAB Substrate Kit (Peroxidase) SK-4100

Alkaline Phosphatase Staining

Vectastain ABC-AP Rabbit IgG AK-5001

Vector Red Substrate Kit (AP) SK-5100

ImmPact AMEC Red Substrate Kit, Vector Laboratories SK-4285

Avidin/Biotin Blocking Kit, Vector Laboratories SP-2001

2.1.10 Post-Mortem Human Tissue Donor Information

All post-mortem tissue came from Sheffield Brain Tissue Bank (SBTB). Tissue was released under SBTB REC number 19/SS/0029 with ethical approval for the project confirmed either by an external REC or by the SBTB Management Board, in compliance with current Codes of Practice from the Human Tissue Authority.

Table 2.22 Details of Post-Mortem Human Tissue Donors

Blinded Case No.	Case ID	Clinical Diagnosis	Genetics	Age years	Sex	Disease Duration (months)	Post Mortem Delay (hours)
1	LP043/2005	sALS		31	M	66	33
2	LP085/2007	Control		59	F	N/A	5
3	LP098/2007	Control		68	M	N/A	63
4	LP048/2012	sALS		42	M	34	48
5	LP020/2013	sALS		78	M	80	36
6	PA135/2018	Control		85	F	N/A	72
7	LP040/2011	fALS/FTD	C9ORF72 HRE	66	F	56	10
8	LP046/2010	FTLD-MND	C9ORF72 HRE	67	F	43	24
9	LP041/2004	sALS	C9ORF72 HRE	62	M	20	48
10	LP039/2011	fALS/FTD	C9ORF72 HRE	71	M	26	96
11	NA019/1991	Control		54	M	N/A	8
12	LP005/2011	sALS		76	F	52	40
13	NA014/1999	Control		84	F	N/A	52
14	LP005/2010	sALS		40	M	17	48
15	LP066/2008	fALS/FTD		59	F	43	28
16	LP042/2009	fALS		53	F	28	22

In some cases, insufficient tissue was available, so the following substitutes were included:

Table 2.23 Details of additional post-mortem human tissue used when main cohort was unavailable

Case ID	Clinical Diagnosis	Genetics	Age	Sex	Disease Duration/ months	Post Mortem Delay / hours	Notes
PA224/2016	Control		82	F	N/A	Unknown	Case 11 only had spinal cord available; motor cortex from PA224/2016 was used as an alternative
LP005/2007	Control		63	M	N/A	Unknown	Case 12 only had spinal cord available; motor cortex from LP005/2007 was used as an alternative
LP81/2009	FTLD-MND	C9ORF72 HRE	59	M	27	60	Case 16 only had spinal cord available; motor cortex from LP81/2009 was used as an alternative
PA125/1994	Control		75	F	N/A	31	Frozen tissue was not available from other controls used in IHC; PA125/1994 was used as an alternative

2.1.11 Mouse Spinal Cord

Mice used in this study were C9-500 BAC Transgenic mice (N=3) and non-transgenic counterparts (N=3). Details of the mouse model are described by Liu and colleagues (Liu et al., 2016)

2.1.12 Imaging and Software

Immunohistochemistry images were taken on a Nikon Eclipse DS-Ri1 Microscope and/or scanned using the Hamamatsu Nano Zoomer XR. Immunohistochemistry

analysis was performed using Hamamatsu NDP.view2 Image viewing software. Immunofluorescence images were captured using the Olympus B53 microscope with 20x or 40x objectives, edited and merged using Image J.

2.2 Methods

2.2.1 Cloning for pENTR/H1-TO-MCS and scAAV-KAS/H1 plasmids

2.2.1.1 Restriction Digest

In a 1.5 ml conical microcentrifuge tube, 5 µg vector DNA (i.e. scAAV-KAS/H1 or pENTR/H1-TO-MCS), was mixed with 5 µl 10 X Fast Digest Green buffer and 36 µl ultra-pure water. Finally, 2 µl of each restriction enzyme was added last. See Table 2.19 for restriction enzymes used. Samples were mixed thoroughly gentle inversion, and pulsed in the Thermo Micro CL-17 centrifuge to remove any bubbles. Samples were placed in a 37°C Flowgen Bioscience Digital Dry Bath heating block for two hours to ensure the plasmids were fully digested.

2.2.1.2 Vector dephosphorylation

1 µl CIP was added to the vectors to prevent self-ligation. They were returned to the 37°C heating block for a further 15 minutes followed by 2 minutes at 90°C in order to deactivate the CIP.

2.2.1.3 Annealing of Oligonucleotides

First, 38 µl of annealing buffer was added to a 500 µl PCR tube with 6 µl of forward and reverse oligonucleotides. Samples were vortexed to mix thoroughly. To facilitate hybridisation, samples underwent a programme of heating and cooling using a GS1

Thermo Cycler. Samples underwent an initial 4 minute heating step at 94°C, followed by 10 minutes at 70°C and a slow cooling period back to room temperature of 40 minutes.

2.2.1.4 PNK Phosphorylation

Following annealing, 7 µl oligonucleotides were added to a 1.5 ml conical microcentrifuge tube with 1 µl forward PNK buffer, 1 µl 10 mM ice-cold ATP, and 1µl PNK enzyme. Samples were placed in a 37°C heating block for 45 minutes, to enable addition of a phosphate group to the 5' end of oligonucleotides in order to make subsequent ligations more efficient. Samples were moved to a 90°C heating block for 5 minutes and left to cool to room temperature for 15 minutes.

2.2.1.5 Ligation

In order to fuse the phosphorylated oligonucleotide to the digested vector, 1 µl of vector was placed in a 1.5 ml conical microcentrifuge tube with 10 µl oligonucleotide, 6 µl ultra-pure H₂O, 2 µl T4 DNA ligase buffer, and 1µl T4 DNA ligase. Samples were placed in a 37°C heating block for one hour.

2.2.1.6 Transformation and Plating

The ligated reaction samples were transformed into competent DH5-α *E.coli*. Ligated samples were put on ice for 15 minutes and 10 µl was mixed with 90 µl DH5-α cells. Samples were mixed by gentle inversion and put on ice for 10 minutes. Samples were then quickly moved to a 37°C heating block for 5 minutes, to ensure a sudden enough change in temperature to form pores in DH5-α cell walls. Next, 1 ml LB broth was added and samples were returned to the 37°C heating block for one hour. Cells were plated on kanamycin or ampicillin Agar plates. See Table 2.18 for the antibiotic

required for each plasmid. Samples were placed in a Thermo Scientific MicroCL 17 Centrifuge at 6,000 rpm for 5 minutes. Subsequently, 1 ml of supernatant was removed and discarded, and the pellet resuspended in the remaining supernatant. Cells were spread on Agar plates using a sterilised spreader and left at 37°C overnight to allow colonies to grow.

2.2.1.7 Inoculation of Colonies

First, 3 ml of LB broth was added to a 15 ml universal sample tube with the appropriate antibiotic in a 1:1000 dilution. See Table 2.18 for antibiotics used. A single colony was collected from the Agar plates using a pipette tip, and dropped into the universal sample tube containing the LB broth and antibiotic. Colonies were left to grow overnight on a 37°C SciQuip Midi Incu-shaker.

2.2.2 Isolation of Plasmid DNA

2.2.2.1 Rapid Mini Prep

First, 1 ml of the overnight culture was placed in a 1.5 ml conical microcentrifuge tube and placed in the Thermo Scientific MicroCL 17 Centrifuge for 3 minutes at 13,000 rpm. The supernatant was discarded and the pellet resuspended in 200 µl buffer P1 from the QIAprep Spin Mini Prep Kit. The bacterial cells were lysed with 200 µl buffer P2 and gently mixed by inversion. The samples were incubated for 3 minutes, and the lysis was stopped by the addition of 300 µl buffer S3. The samples were gently inverted until a colour change from blue to white was observed, and then centrifuged for 10 minutes at 13,000 rpm. Supernatant was transferred to a fresh conical microcentrifuge tube and the pellet discarded. 700 µl isopropanol was added and samples were incubated for 10 minutes at room temperature. Samples were then centrifuged at

13,000 rpm for a further ten minutes. The supernatant was discarded and the pellet left to air-dry before being re-suspended in 50 µl EB buffer.

2.2.2.2 Restriction Digest and Agarose Gel Electrophoresis

Following rapid mini preps, 5 µl of each sample was added to a conical microcentrifuge tube with 1 µl restriction buffer, 3 µl H₂O, and 0.5 µl each restriction enzyme. See Table 2.19 for details of restriction enzymes used. Samples were placed in a 37°C heating block for one hour. A 3 % agarose gel was used (6 g Agarose + 200 ml 1 x Tris-Acetate EDTA TAE buffer + 4 µl ethidium bromide) with a 30-well comb. Next, 5 µl Gene ruler DNA Ladder Mix was loaded into the first lane. The 10 µl sample following restriction digest was loaded and run using a BioRad PowerPac Basic Power Supply at 80 V for 50 minutes.

2.2.2.3 Inoculation of colonies for Midi Prep

First, 100 ml LB broth was placed in a 500 ml flask with the appropriate antibiotic in a 1:1000 dilution. Next, 200 µl of overnight culture was added to the flask, which was left on a 37°C shaker overnight.

2.2.2.4 Midi Prep

Of the 100 ml overnight culture, half was poured into a 50 ml universal sample tube, and placed in a Sigma 3-16PK Centrifuge for 4,500 rpm for 10 minutes. The supernatant was discarded in disinfectant and the other half of the overnight culture was poured over the pellet, and centrifuged for a further ten minutes at 4,500 rpm. The supernatant was discarded in disinfectant and the DNA extracted from the pellet using the QIAGEN Plasmid Plus Midi Kit. The pellet was re-suspended in 4 ml Buffer P1 and

vortexed to mix. 4 ml of buffer P2 was added and the samples were inverted gently until a colour change to blue was observed. Samples were incubated for 3 minutes and 4 ml buffer S3 was added. Samples were inverted until a colour change to white was observed. Samples were transferred to a QIAGEN filter cartridge and incubated for 10 minutes at room temperature before being passed through the filter. 2 ml of binding buffer was added to each sample, which were placed in the -20°C freezer for 10 minutes. The lysate was transferred 700 µl at a time to a QIAGEN plasmid plus spin column, which was centrifuged for 30 seconds at 10,000 rpm. The flow-through was discarded and a further 700 µl lysate added until the sample was finished. Samples were washed with 700 µl buffer ETR and centrifuged for 1 minute at 10,000 rpm, followed by a further wash with 700 µl buffer PE + ethanol, and a further 1 minute centrifuge at 10,000 rpm. The flow through was discarded and the tube centrifuged for a further 3 minutes at 10,000 rpm to remove excess wash solution. Samples were then placed in a 37°C heating block for 3 minutes. Finally, 200 µl buffer EB was added to the centre of the tube and incubated for 5 minutes. To elute the plasmid, samples were centrifuged for 3 minutes at 10,000 rpm and the flow-through collected.

2.2.2.5 Measurement of nucleic acid concentrations by spectrophotometry

Nucleic acid concentration in each sample was analysed using a ND-1000 Nanodrop Spectrophotometer according to manufacturer's instructions.

2.2.2.6 Sequencing

Plasmids were sent for sequencing at the University of Sheffield Medical School, using an Applied Biosystems' 3730 DNA Analyser. We supplied 10 µl of plasmid DNA in water >100 ng/µl and M13 Fwd/Rev primers.

2.2.3 Sub-Cloning

2.2.3.1 Restriction Digest

As previously described in section 2.2.1.1.

2.2.3.2 Agarose Gel Electrophoresis

A 1 % agarose gel was used for vector LV-H1-TO (2 g agarose + 200 ml TAE buffer + 4 µl ethidium bromide). A 2 % gel was used for RNA helicase inserts eIF4A1, eIF4A2 and DHX36 (4 g Agarose + 200 ml TAE buffer + 4 µl ethidium bromide). A 10 well comb was used and 5 µl Gene ruler DNA Ladder Mix was loaded into the first lane. The 50 µl sample following restriction digest is loaded and run using a BioRad PowerPac Basic Power Supply at 80 V for 50 minutes. The band was removed using a scalpel blade and UV-transilluminator to visualise the band.

2.2.3.3 Gel Extraction

The Qiagen gel extraction kit was used to extract the DNA from the gel bands. First, 600 µl buffer QG was added and each sample was placed in a heating block at 37°C for 10 minutes, or until the gel had melted completely. Next, 200 µl of isopropanol was added and the samples mixed by inversion. A QIAquick column was placed in a collection tube and 700 µl of the sample was added and centrifuged at 4,000 rpm for 1 minute. The flow through was discarded and 700 µl more of the sample was added, until no more sample remained. Next, 500 µl buffer QG was added to wash, and centrifuged at 10,000 rpm for one minute. Flow through was discarded and 750 µl buffer PE with added ethanol was added to the column and centrifuged at 10,000 rpm for 1 minute. Flow through was discarded and the column was centrifuged for an additional minute at 10,000 rpm. The column was placed in a 1.5 ml conical

microcentrifuge tube and 30 µl buffer EB was added to the centre of the column, in order to elute the DNA. The sample was incubated at room temperature for 4 minutes and centrifuged for a final minute at 10,000 rpm.

2.2.3.4 Ligation

In a 500 µl PCR tube: 1 µl LV-H1-TO vector is mixed with 16 µl insert. 2 µl 10 x LB ligation buffer was vortexed and added with 1 µl T4 DNA ligase. The vector and insert were left to ligate overnight and kept at a constant 16°C using GS1 Thermo Cycler.

2.2.3.5 Transformation and Plating

As previously described in section 2.2.1.6.

2.2.4 Western Blots

2.2.4.1 Transfection of shRNAs into HEK293T cells

HEK293T cells were maintained following the manufacturer's recommendations. Cells were plated on 6-well or 24-well plates at a density of 200,000 or 50,000 cells per well, respectively. For 24-well plates, 2 – 3 wells were used per experimental condition. Approximately 24 hours after plating, cells were co-transfected with the shRNA plasmid and either sense or antisense V5-tagged DPRs. For 6-well plates 1 µg of each plasmid was transfected, for 24-well plates 350 ng of each plasmid was transfected. PEI was used as a transfection reagent, at 7 µg/well for 6-well plates and 1.75 µg/well for 24 well plates. Opti-MEM was used at 200 µl per well for 6-well plates and 50 µl per well for 24-well plates. This helped ensure transfection efficiency by maintaining the cells in reduced serum conditions. Plasmids, PEI and Opti-MEM were mixed

thoroughly by pulse vortexing 15 times, and left to incubate for ten minutes at room temperature before adding dropwise to cells. Plates were returned to a 37°C incubator, and after 72 hours, cells were harvested, and protein extracted.

2.2.4.2 Protein Extraction from 6-well plates

Media was removed from each well and discarded in disinfectant. Each well was washed with 1 ml sterile PBS. Cells are lysed with 200 µl lysis buffer, with 20 µl Protease Inhibitor Cocktail and PMSF added per ml Lysis buffer. Cells were dislodged from each well by scraping with a Fisherbrand disposable cell scraper, and the lysates were transferred into a 1.5 ml conical microcentrifuge tube. Samples were incubated for 5 minutes to ensure lysis was complete. Each lysate was passed through a 1 ml syringe with 25G x5/8 needle ten times to ensure cells were fully detached. Samples were placed in a Thermo Scientific MicroCL 17R Centrifuge at 13,000 rpm at 4°C for 5 minutes. The pellet was discarded while the supernatant containing the extracted protein is transferred to a fresh conical microcentrifuge tube. 2 µl of each sample was added to 1 ml of 1 x Bradford's reagent. Absorbance was recorded using the S1200 Diode Array Spectrophotometer and used to calculate total protein concentration for each sample.

2.2.4.3 Protein Extraction from 24-well plates

Media was removed from each well and discarded in disinfectant. Each well was washed with 200 µl sterile PBS. Cells were lysed with 50 µl lysis buffer with 20 µl Protease Inhibitor Cocktail and PMSF added per ml lysis buffer. Cells were dislodged from each well by scraping with a P-200 pipette tip. Lysate from wells of the same experimental condition were mixed in one well, and then transferred into a 1.5 ml conical microcentrifuge tube. Samples were incubated for 5 minutes to ensure lysis

was complete. Each lysate was passed through 1ml syringe with 25G x5/8 needle ten times to ensure cells were fully detached. Samples were placed in a Thermo Scientific MicroCL 17R Centrifuge at 13,000 rpm at 4°C for 5 minutes. The pellet was discarded while the supernatant containing the extracted protein was transferred to a fresh conical microcentrifuge tube. Next, 2 µl of each sample was added to 1 ml of 1 x Bradford's reagent. Absorbance was recorded using the S1200 Diode Array Spectrophotometer and used to calculate total protein concentration for each sample.

2.2.4.4 Protein Extraction from frozen post-mortem tissue

Frozen tissue was placed in a 1.5 ml screw-close tube with approximately 10 homogeniser beads. First, 600 µl working reporter lysis buffer was added to samples and homogenised at 5500 rpm for 20 seconds x2. Samples were left on ice for 10 minutes then homogenised at again at 5500 rpm for 20 seconds x2. Samples were then centrifuged at 4°C for 10 minutes at 17,000 g. The cell lysates were moved to new tubes and stored at -20°C; beads and remaining tissue was stored at -80°C.

2.2.4.5 SDS Page

SDS Page was run using a Bio-Rad Mini-PROTEAN Tetra Cell System. Glass plates were cleaned thoroughly with 70 % IMS and water before use. Plates were clamped in the cassette and gels were prepared. Throughout, 12 % gels were used as standard resolving gels, with 15 % gels used for V5-tagged DPRs. All 12 % gels were prepared as follows: 3.5 ml distilled H₂O, 4 ml of 30 % acrylamide, 2.5 ml resolving lower buffer, 50 µl 10 % APS, 20 µl of TEMED. All 15 % gels were prepared as follows: 2.5 ml distilled H₂O, 5 ml 30 % acrylamide, 2.5 ml resolving lower buffer, 50 µl 10 % APS, 10 µl of TEMED. Approximately 4.5 ml of the gel was added to each cassette, with 100 µl of isopropanol to remove any bubbles, and left for 30 minutes to fully polymerise.

Isopropanol was then removed from the surface of the gel with filter paper. The 5 % stacking gel was prepared with 2.9 ml distilled H₂O, 0.85 ml 30 % acrylamide, 1.25 ml stacking upper buffer, 25 µl 10% APS, 10 µl TEMED. The stacking gel was added above the resolving gel until it reached the top of the glass plates. Before polymerisation was complete a 15-well comb was added to create sample wells. Samples were prepared by adding 5 µl of 4x Laemmli buffer and denatured for 5 minutes at 90°C. Samples were then centrifuged for approximately 10 seconds in the MicroCL 17 Centrifuge. Finally, 20 µl of sample was added to each well with 4 µl of pre-stained PM in lane one. Samples were run at 150 V for approximately 1 hour.

2.2.4.6 Transfer of Western Blots

Proteins were transferred from the gel to a nitrocellulose membrane using a Biometra Fastblot analytic jena transfer machine, according to manufacturer's protocol. Gels were transferred for 1 hour at 0.15 A per gel. Membranes were then blocked for one hour in 5 % dried skimmed milk TBS-T at room temperature on the roller mixer. Membranes were incubated overnight in primary antibody at 4°C on the roller mixer. Membranes were washed three times for 10 minutes in TBS-T. Membranes were incubated in secondary antibody for one hour at room temperature on the roller mixer. Three ten-minute washes were repeated.

2.2.4.7 Imaging of Western Blots

Membranes were exposed to equal quantities of ECL 1 and ECL 2 (enhanced chemiluminescence) for one minute and imaged using a Syngene G-Box with GeneSys G-Box Chemi XT software.

2.2.5 MTT Cell Proliferation Assays

Cells were transfected on a 6-well plate for 72 hours as previously described. For a 24-well plate, 50 µl per well MTT was added to cells, which were then incubated at 37°C for one hour. At room temperature, 550 µl MTT buffer was added to each well and mixed thoroughly with cells until purple formazan speckles were no longer visible. Cells were placed in the dark on a shaker for one hour at room temperature. Samples were then analysed using the PHERAstar microplate reader (programme: MTT toxicity; plate : Greiner 24; wavelength set at 595). All readings were averaged per experimental condition and calculated as a % of the control.

2.2.6 Reverse Transcriptase qPCR

2.2.6.1 RNA Extraction

First, 40 µl DEPC-PIC, 40 µl PMSF and 2 µl RNase inhibitor was added to 2 ml lysis buffer. HEK293T cells were lysed as previously described, using the RNase free lysis buffer. Next, 600 µl trizol was then added and incubated with cells at room temperature for 10 minutes. Following addition of trizol, 160 µl chloroform was added, samples shaken for 20 seconds and incubated at room temperature for a further 5 minutes. Samples were centrifuged at 4°C at 10,000 g for 10 minutes. The upper layer from each sample was removed without disturbing the phenol/chloroform lower layer. An equal volume of isopropanol was added to each sample with 1 µl glycogen, followed by sodium acetate equal to 10 % of the total volume. Samples were inverted to mix and incubated for 30 minutes at room temperature, then centrifuged at 4°C at maximum speed for 30 minutes. The supernatant was removed and discarded and 200 µl 70% RNase free ETOH was added to each sample. Samples were centrifuged for 5 minutes at maximum speed followed by pulses in the centrifuge to totally remove all supernatant. The pellet was left to air-dry at room temperature for 15 minutes, then

22 µl DEPC-treated water was added to each sample to re-suspend the pellets. Next, 2.5 µl 10 x DNase I recombinant incubation buffer and 0.5 µl DNase I recombinant enzyme was added to each sample. Samples were then incubated at 37°C for 30 minutes and 75°C for 10 minutes, then RNA was analysed using a Nanodrop spectrophotometer as previously described in section 2.2.2.5. Samples were stored at -80°C until ready to use.

2.2.6.2 cDNA Synthesis

In a PCR tube 2000 ng RNA, 1 µl dNTP mM mix, 1 µl dN6 40 µM primer was added and samples made up to 13 µl in DEPC-treated water. Samples were incubated in a PCR machine at 70°C for 5 minutes then placed on ice. Samples were made up in duplicate with each having a +RT and a -RT. To +RT samples 4 µl 5 x FS buffer, 2 µl 0.1 M DTT and 1 µl M-MLV RT enzyme was added, the -RT samples received the same apart from RT enzyme was replaced by 1 µl DEPC-treated water. Samples were placed in the PCR machine and the RT programme was run at 25°C for 10 minutes, 42°C for 1 hour and 85°C for 5 minutes, before being held at a constant 10°C. Finally, 40 µl DEPC-treated water was added to each sample.

2.2.6.3 PCR

A master mix was made up comprising 5 µl per well of 2 x brilliant III SYBR Green QPCR master mix, 3 µl per well DEPC-treated water, 1 µl per well of primer mix made up of forward + reverse primers. A primer was used for each RNA of interest with U1 snRNP as a control. Each primer was assigned 2 wells of the 96-well plate per cDNA +RT sample. -RT samples were included as a control row. Each well contained 1 µl cDNA + 9 µl master mix. Each plate was centrifuged for one minute and run in a BioRad CFX96 Thermal Cycler overnight (denaturation: 95°C for 30 seconds, annealing 50°C for 30 seconds, elongation 72°C for 2 minutes, 30 cycles). CQ values were used to

calculate Delta CT, normalised to U1 controls and calculated as a % of the control value.

2.2.7 Immunohistochemistry

2.2.7.1 3,3'-diaminobenzidine (DAB) staining

FFPE tissue was sectioned on a microtome to 5 µm. Sections were dewaxed by immersion in xylene for 2 x 5 minutes, then rehydrated in absolute alcohol then 95 % alcohol for 2 x 5 minutes each. Endogenous peroxidase activity was blocked by immersing sections in 3 % H₂O₂ + methanol for 20 minutes at room temperature. Antigen retrieval was carried out in a pressure cooker in pH 6 citrate buffer at 120°C at 20 psi for 30 minutes. Sections were washed in tap water for one minute followed by TBS for 5 minutes. Sections were then blocked in normal serum, made up according to manufacturer's instructions. Primary antibodies were diluted appropriately according to previous optimisation, shown in Table 2.20, and sections were incubated overnight at 4°C. Sections were washed in TBS for 2 x 5 minutes and incubated for 30 minutes at room temperature in secondary antibody made up according to manufacturer's instructions. At the same time, ABC reagent was made up according to manufacturer's instructions and avidin: biotin complexes were allowed to form during secondary antibody incubation. Sections were washed in TBS 2 x 5 minutes and incubated in ABC for a further 30 minutes at room temperature. Sections were washed in TBS 2 x 5 minutes. The reaction was visualised with diaminobenzidine (DAB) made up according to manufacturer's instructions. Sections were incubated from 6 – 10 minutes, depending on primary antibody used, until a colour change was visible. Sections were rinsed with distilled water and washed in tap water for 5 minutes. Nuclei were counterstained for 30 – 60 seconds in Harris haematoxylin, washed in tap water for 1 minutes, immersed in Scott's tap water for 10 seconds then returned to tap water for a final 1-minute wash. Finally, sections were dehydrated through 70%, 95%

and absolute alcohols for 1 minute each, before being transferred to the fume hood for covering with a coverslip using DPX.

2.2.7.2 Double Staining DAB + Alkaline Phosphatase

DAB staining was performed as previously described in section 2.2.7.1, but final counterstaining and all subsequent steps were not carried out. Instead, sections were incubated in avidin and biotin blocks for 15 minutes each, using an ABC kit detailed in section 2.1.10. Normal serum was made up corresponding to the species of the 2nd primary antibody and sections were blocked in this for 30 minutes at room temperature. Next, sections were incubated in the 2nd primary antibody overnight at 4°C. Final staining steps including secondary antibody incubation and ABC blocking were then carried out as described in section 2.2.7.1, using the alkaline phosphatase kit according to manufacturer's instructions. Sections were then counterstained, dehydrated and covered with coverslips as previously described in section 2.2.7.1.

2.2.7.3 Double Staining DAB + ImmPact AMEC Red

Sections were stained used the alkaline phosphatase kit according to manufacturer's instructions and as previously described in section 2.2.7.1. Sections were counterstained and washed as normal but not immersed in alcohol or xylene and instead covered with a coverslip using aqueous mounting medium. Slides were scanned and then the coverslip was removed by immersion overnight in water at 40°C. Red pigment was dissolved using 100 % ethanol for 10 minutes then rehydrated in 95 % and 70 % alcohol. Sections were washed in TBS for 5 minutes then DAB staining was carried out as described previously in section 2.2.7.1.

2.2.8 Immunofluorescence Staining of patient-derived neurons

Patient-derived neurons were generated from control, sALS and C9-ALS/FTD patient fibroblasts. They were cultured and differentiated over a 2-week period by Dr. Ya-Hui Lin. In a 24-well plate containing coverslips, two wells were used per experimental condition. All steps were carried out at room temperature unless otherwise noted. Cells were washed in 500 μ l PBS and fixed for 20 minutes with 100 μ l 4% paraformaldehyde. Paraformaldehyde was removed and cells were washed in PBS, then permeabilised for 10 minutes using 0.2 % Triton X-100 in PBS. The permeabilisation solution was removed, cells were washed in PBS, and then blocked for 1 hour in 3 % horse serum and 0.05 % Triton X-100 in PBS. Primary antibodies were prepared to the appropriate dilution in blocking solution, and a 50 μ l drop was added to parafilm. Coverslips were removed from the 24-well plate and placed cell-side down onto the primary antibody solution. Cells were incubated in their primary antibody overnight at 4°C. Coverslips were replaced in the 24-well plate, and washed 3x in PBS. Subsequent steps were performed under low light conditions. Coverslips were incubated in secondary antibody for one hour at room temperature, then washed 3x in PBS. Next, 500 μ l Hoechst 33342 Fluorescent Stain was added to coverslips for 10 minutes, followed by 2 x PBS washes. Finally, coverslips were mounted onto slides cell-side down using Vectashield fluorescence mounting medium with DAPI.

2.2.9 Statistical Analysis

Syngene Gene Tools software was used to quantify Western Blot images. GraphPad Prism was used to analyse quantification and create graphs. A one-way or two-way ANOVA was used throughout cell work to analyse statistical significance of normally-distributed data which involved more than two experimental groups and results were plotted on a bar chart (NS = Non significant, $p > 0.05$; *= $p < 0.05$; **= $p < 0.01$; ***= $p < 0.001$; ****= $p < 0.0001$).

3 Identification and manipulation of RAN translation factors in human cell models of C9-ALS/FTD

3.1 Background, aims and rationale

This chapter explores the hypothesis that RAN-associated RNA helicases involved in general translation initiation may also be involved in RAN translation of pathological *C9ORF72* repeat expansions, due to their high level of secondary structure formation. Previous literature had shown that in cancer, eIF4A contributes to unwinding of highly structured mRNAs encoding oncogenes, thus promoting their translation (Wolfe et al., 2014). This role linking eIF4A to unwinding activities and translation led to the hypothesis that targeting eIF4A could reduce RAN translation.

The aim for the first stages of this project was to further investigate the relationship between helicases and RAN translation of DPRs; the hypothesis was that reducing helicase expression or activity will prevent initiation of RAN translation and therefore provide neuroprotection through decreased production of DPRs. To achieve this, a series of tools were developed to test in cell models of C9-ALS/FTD, allowing for investigation of the therapeutic potential of targeting RAN-associated RNA helicases in the context of the *C9ORF72* repeat expansion. These comprise shRNAs cloned into various vectors enabling mammalian and lentiviral expression of shRNAs. This chapter shows work exclusively in a HEK293T cell model, with data from additional cell models shown in chapter 5 of this thesis. Initially work started with pENTR vectors with shRNAs targeting eIF4A1, eIF4A2 and eIF4B, then an eIF4A1+4A2 shRNA, allowing for investigation into the impact on RAN translation of targeting both helicases simultaneously. With scAAV vectors, a shRNA targeting DHX36 was introduced, as DHX36 has been identified as a major resolver of mRNA secondary structures *in vitro*. Finally for LV vectors, the study was expanded to include eIF4H shRNA as along with eIF4B this is another key co-factor of eIF4A1/eIF4A2. The efficiency and specificity of

shRNAs was tested through assessing their ability to knock down their target protein through western blot, and at the mRNA level through RT-qPCR.

Following cloning and testing of shRNA constructs, a second strategy was used to investigate RAN-associated RNA helicase involvement in RAN translation. Two eIF4A1 and eIF4A2 helicase dominant negative mutants were used to test the effect of preventing helicase activity without reducing helicase levels. Overall, in this chapter we show two methods to reduce DPR production, through either an expression or depletion-based strategy targeting eIF4A1, eIF4A2 and their co-factors. Through these experiments, the data in this chapter provides evidence that eIF4A1, eIF4A2, eIF4B and eIF4H are necessary for DPR production, and that disrupting their expression or helicases activity is a viable strategy to reduce RAN translation of DPRs.

3.2 Cloning of shRNAs into mammalian and viral vectors

This project was started with cloning shRNAs targeting RAN-associated RNA helicases into three vectors: pENTR, scAAV and LV. Primers for eIF4A1, eIF4A2, eIF4B, eIF4H and DHX36 have been designed to produce plasmids capable of mediating down-regulation of helicase expression. Additionally, a shRNA targeting eIF4A1+2 was used, so that the option was available to knock down eIF4A1 and eIF4A2 individually or in tandem. Figure 3.1 provides an overview of the cloning methodology and strategy used in this chapter.

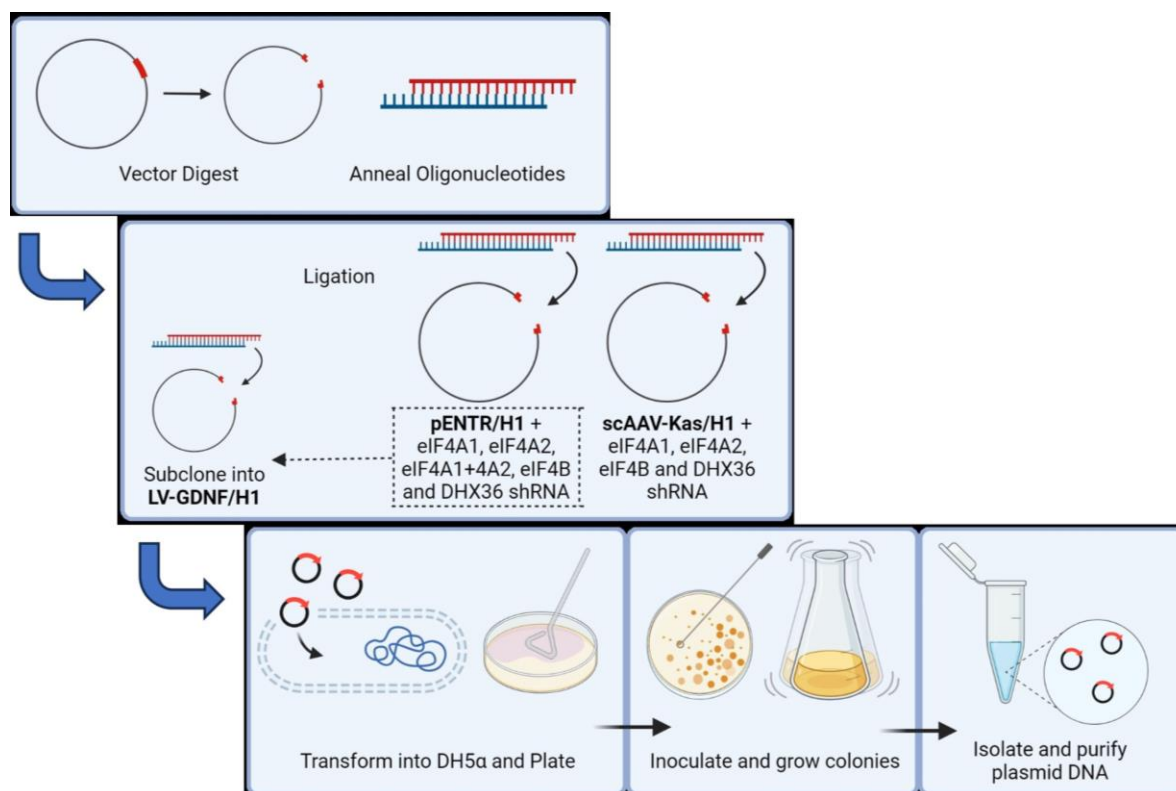


Figure 3.1 An overview of the cloning method and strategy. shRNAs targeting eIF4A1, eIF4A2, eIF4A1+eIF4A2, eIF4B and DHX36 were cloned into pENTR/H1, followed by sub-cloning into LV-GDNF/H1. shRNAs targeting eIF4A1, eIF4A2, eIF4B and DHX36 were cloned into scAAV-Kas/H1. Vectors underwent restriction digest, oligonucleotides were annealed and ligated into each vector, then transformed into competent DH5- α *E.coli*. Cells were plated, colonies inoculated and grown before finally isolation and purification of plasmid DNA is carried out. Figure made with Biorender.

3.2.1 pENRT/H1-TO-MCS-eIF4B shRNA

shRNAs to target eIF4B were cloned into pENRT/H1-TO-MCS between XhoI and Hind III restriction sites. Details of their design are shown in materials section 2.1.3. pENTR shRNAs targeting eIF4A1, eIF4A2 and eIF4A1+2 were established tools being used by the group, and had been cloned prior to this project starting, so only pENTR eIF4B shRNA was cloned for this work. Relevant restrictions sites are highlighted on the plasmid map shown in materials section 2.1.4. This vector allows expression of shRNAs under a H1 promoter using an RNAPIII terminator. H1 is a commonly used promoter for type 3 RNA polymerases, which was crucial when considering these small sequences of nucleotides which will be unlikely to express in a standard RNAPII

system. H1 promoters are frequently used in research for expression of small noncoding RNAs in the context of RNAi. They provide robust levels of transcription in a wide variety of cell types, and have critical elements located upstream of transcriptional initiation sites meaning almost any small RNA sequence can be expressed (Gao et al., 2021).

Plasmids were transformed into competent DH5- α *E.coli* cells, which were grown on agar plates selective against kanamycin. Six colonies were picked and inoculated in medium containing kanamycin. Plasmid DNA was isolated by mini prep, and plasmid DNA was digested using *Dra*I and *Xba*I restriction enzymes. Next, samples were loaded onto an agarose gel to determine if the shRNA had successfully ligated into the pENRT/H1-TO-MCS backbone, with a band expected for the pENTR-shRNA at 406 bp. An empty pENTR vector with only the H1 promoter and no shRNA insert was used as a control and a band for this was expected at 352 bp.

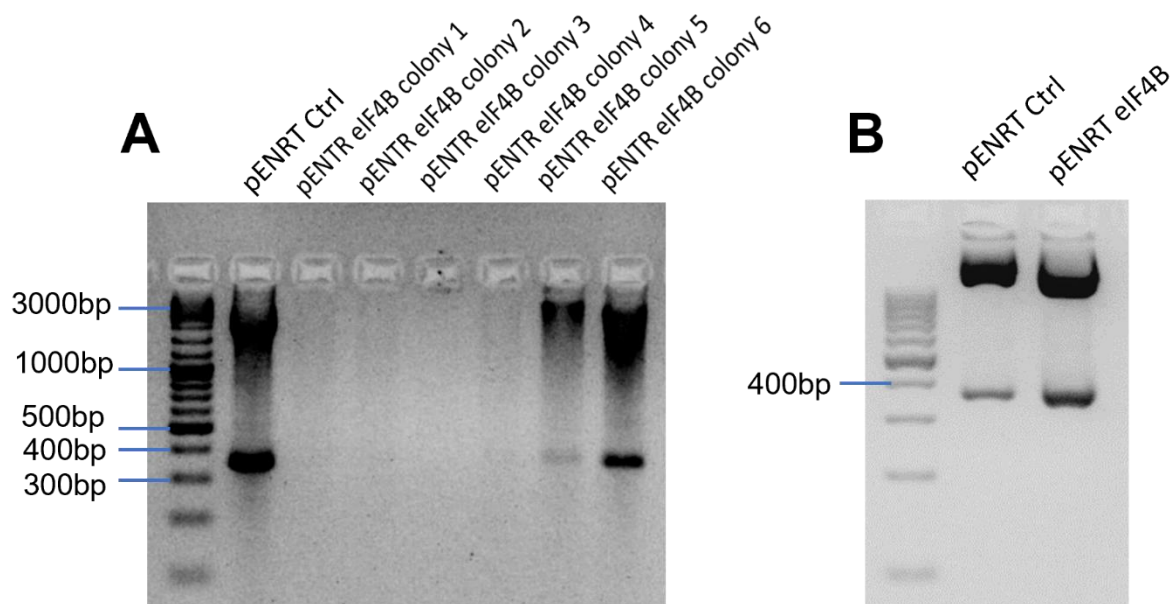


Figure 3.2 Agarose Gel Electrophoresis for cloning of eIF4B shRNA into a pENTR backbone. (A) shows agarose gel following mini prep digest to determine in which of the inoculated colonies the eIF4B shRNA has successfully incorporated into the pENTR backbone. An empty pENTR was loaded as a control followed by 6 colonies loaded sequentially, where 5 and 6 have successfully incorporated the plasmid. A band was expected at 406 bp for pENTR-shRNA and at 352 bp for the pENTR-Control. (B) confirms successful integration of colony 6 eIF4B shRNA into the pENTR backbone. Plasmids were digested with *Dra*I and *Xba*I and run on a 3% agarose gel at 80 V for 50 minutes.

Figure 3.2A shows that of the six colonies, clone 5 and clone 6 were successful in incorporating the eIF4B shRNA into the pENTR backbone. Colony 6 was selected to prepare mini-preps of plasmid DNA due to its more prominent band.

Following mini prep, pENTR eIF4B shRNA was digested once again with restriction enzymes *DraI* and *XbaI* and samples were loaded to a further agarose gel. This confirmed the successful integration of the shRNA into the pENTR/H1-TO-MCS vector and is shown in Figure 3.2B. As expected, a band was observed at approximately 400bp confirming the successful integration of the shRNA into the pENTR/H1-TO-MCS vector; plasmids were later sequenced to confirm successful cloning. Next, the efficiency of these shRNAs in knocking down their target proteins was tested, shown in Figure 3.3

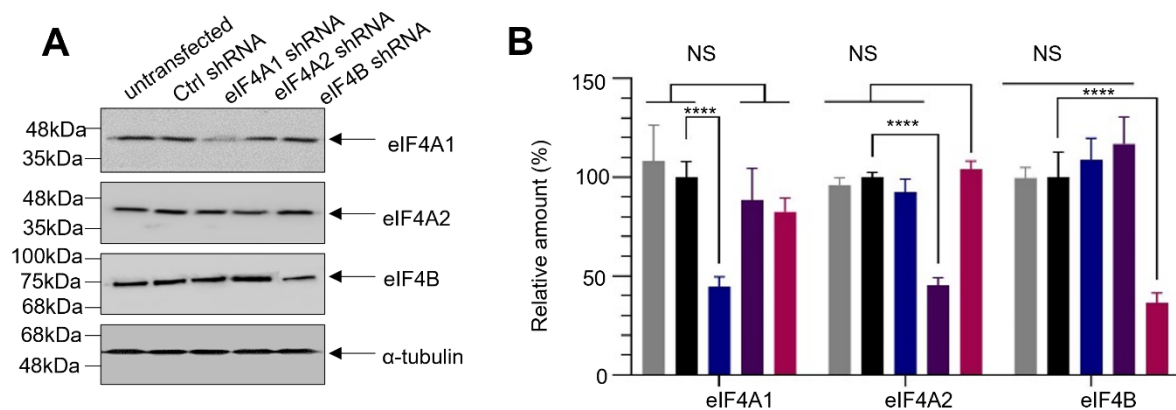


Figure 3.3 Testing pENTR-eIF4A1, eIF4A2 and eIF4B shRNAs. (A) shows a representative western blot from HEK293T cells transfected for 72 hours with pENTR eIF4A1, eIF4A2 or eIF4B shRNA. Blots were probed for eIF4A1, eIF4A2, eIF4B and α -tubulin as a loading control. eIF4A1 and eIF4A2 were predicted to be 46 kDa and eIF4B 80 kDa. (B) shows results quantified in independent biological triplicates, mean + SEM analysed by one-way ANOVA with Tukey's multiple comparisons. eIF4A1 is shown in blue, eIF4A2 in purple and eIF4B in red. Untransfected cells are shown in grey and control shRNA in black. NS: not significant, ****: $p < 0.0001$.

Each shRNA was specific in that only knockdown of the target RAN-associated RNA helicase was detected. pENTR eIF4A1, eIF4A2, and eIF4B shRNA reduced protein levels by 55% for eIF4A1 and eIF4A2, and 65% for eIF4B. In this and all other

experiments in this chapter, chemiluminescence was used over fluorescence due to its greater sensitivity, and to avoid autofluorescence (Alegria-Schaffer et al., 2009).

3.2.2 scAAV-KAS/H1 eIF4A1, eIF4A2, eIF4B and DHX36

Next, shRNAs targeting eIF4A1, eIF4A2, eIF4B and additionally DHX36 were cloned into the scAAV-KAS/H1 vector between *Bam*HI and *Hind*III restriction sites. Plasmid maps with relevant restriction sites highlighted can be seen in materials section 2.1.4. Plasmids were transformed into competent DH5- α *E.coli* cells and grown on agar plates containing ampicillin. For eIF4A1, eight colonies were selected and inoculated against ampicillin, while for eIF4A2 and DHX36 two colonies were picked due to low rates of overnight colony growth. Plasmid DNA was isolated using mini prep. *Bam*HI and *Hind*III restriction enzymes were used to digest plasmid DNA, and samples were run on an agarose gel to determine if the shRNAs had successfully incorporated into the scAAV-KAS/H1 vector. scAAV-KAS/GFP-SRSF1 shRNA was used as a control plasmid. A band at 174bp was expected for all scAAV plasmids including the control.

For eIF4A1, only one colony showed successful integration of the shRNA into the vector, so it was automatically selected. For scAAV-KAS/H1-eIF4A2 and DHX36, multiple colonies showed bands indicating successful integration. In this case the more visible band on the agarose gel indicated the colony selected while colonies with fainter bands were not selected. Following colony selection, an Inverted Terminal Repeat test was carried out. ITRs are sequences of 145 base pairs, forming stable hairpin structures, which are located flanking the insert in each AAV vector. While these are crucial for the shRNA expression, they also are associated with problems in cloning due to their high instability. Bacterial cells do not easily tolerate plasmids with ITRs and they can easily be mutated or deleted (Bao & Cagan, 2006), leading to reduced efficiency of the cloned plasmids. Therefore, it was essential to test for integrity of ITRs during cloning. Next, samples were once again digested with *Bam*HI

and *HindIII* restriction enzymes and run on an agarose gel to confirm successful incorporation of shRNAs into scAAV-KAS/H1 vectors. Figure 3.4 shows a band was observed at 174 bp confirming the successful integration of the shRNA into the scAAV-KAS/H1. Samples were sequenced to confirm cloning was successful.

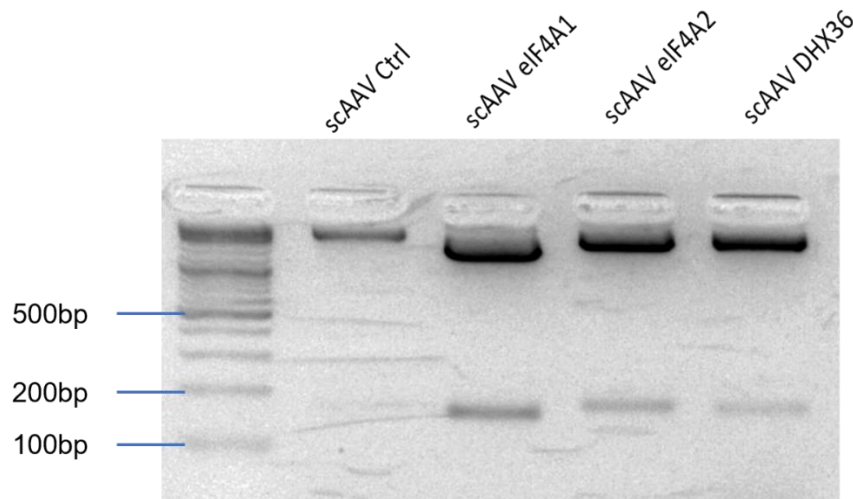


Figure 3.4 Agarose gel electrophoresis to confirm cloning of eIF4A1, eIF4A2 and DHX36 shRNAs into an scAAV vector. Plasmids were digested with *Bam*HI and *Hind*III and run on a 3% agarose gel at 80 V for 50 minutes. A band was expected at 174 bp.

3.2.3 Cloning of shRNAs targeting RAN-associated RNA helicases into a lentiviral plasmid

Following their design, pENTR-H1-eIF4A1, eIF4A2, eIF4A1+2 and DHX36 shRNAs were sub-cloned into the LV-GDNF-H1 vector. Again, a H1 promoter was chosen for expression of short transcripts like shRNAs. The vector and shRNA inserts were digested using *Bcl*I and *Sa*II restriction enzymes. shRNA inserts were run on an agarose gel and LV-GDNF-H1, bands were cut out for DNA extraction and overnight ligation. The vector and inserts are shown prior to gel extraction in Figure 3.5.

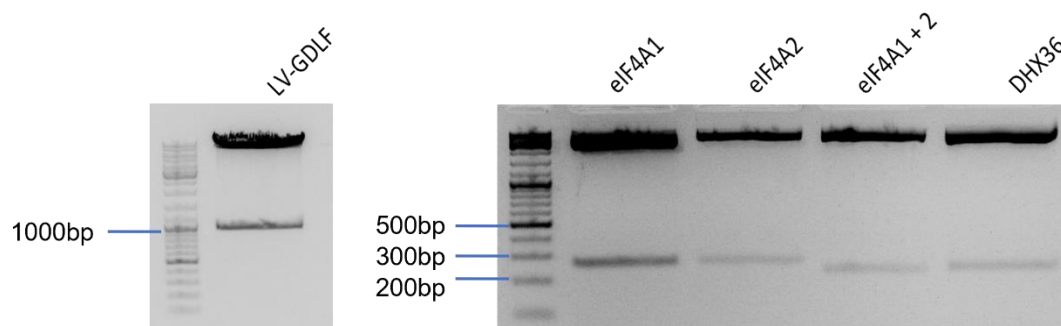


Figure 3.5 Gel extraction of LV-GDNF vector and eIF4A1, eIF4A2, eIF4A1+2 and DHX36 shRNAs. The vector was run on a 1% agarose gel and shRNA inserts on a 2% gel, both for 50 minutes at 80 V. For LV-GDNF the larger, top band was removed and for eIF4A1, eIF4A2, eIF4A1+2 and DHX36 shRNA inserts the smaller, lower band was removed.

Plasmids were transformed into DH5- α competent *E.coli* cells, and grown on agar plates selective against ampicillin. Six colonies were picked and inoculated against ampicillin, and a mini prep was used to isolate plasmid DNA. Plasmid DNA was digested with *BcuI* and *SalI* restriction enzymes, and samples were run on an agarose gel to determine if the shRNAs had successfully integrated into the LV-GDNF backbone. This is shown in Figure 3.6.

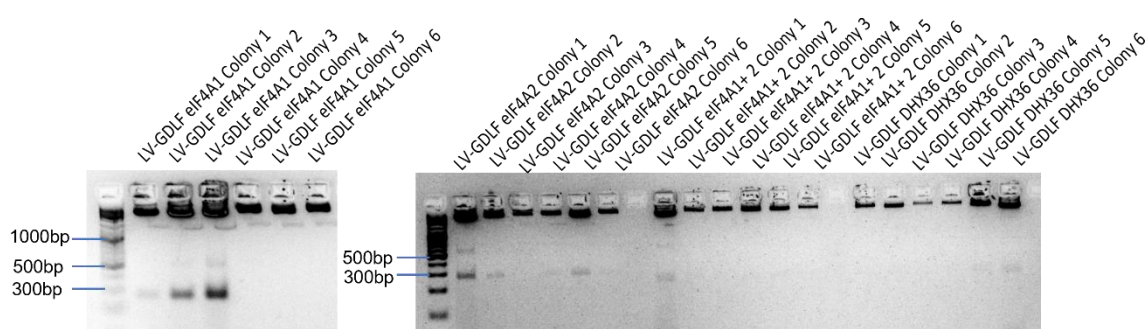


Figure 3.6 Agarose gel electrophoresis for cloning of eIF4A1, eIF4A2, eIF4A1+2 and DHX36 shRNA into an LV-GDNF vector. Plasmids were digested with *BcuI* and *SalI* restriction enzymes and run on a 3% agarose gel at 80 V for 50 minutes to determine which of the inoculated colonies had successfully incorporated the LV backbone. Visible bands indicate colonies with successful integration of the shRNA into the LV vector.

Figure 3.6 shows that for eIF4A1 shRNA, colonies 1-3 have successfully integrated into the LV-GDNF vector. For eIF4A2 shRNA, successful clones are seen in colonies 1, 2 and 5; for eIF4A1+2 shRNA only colony 1 is successful; for DHX36 shRNA colonies 5 and 6 have faint bands indicating successful clones. In cases where multiple colonies were successful, clones were selected with the most prominent band. For eIF4A1 shRNA this was colony 3, for eIF4A2 shRNA colony 1, and for DHX36 shRNA colony 6. For eIF4A1+2 shRNA colony 1 was chosen by default. Mini prep was used to isolate the plasmid DNA for each of these clones. Samples were then digested once again with *BcuI* and *SalI* and run on an agarose gel, to confirm the successful integration; shown in Figure 3.7 with the presence of bands in the expected location. Samples were sent for sequencing to confirm successful cloning.

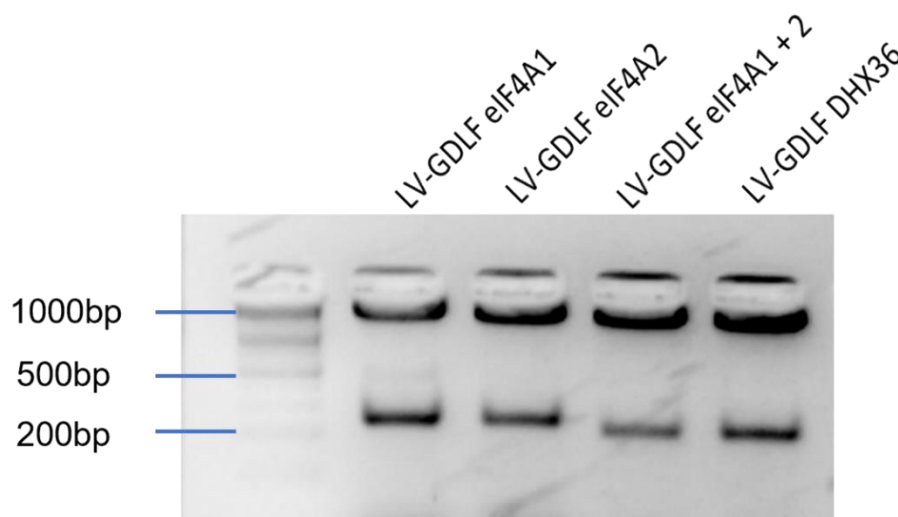


Figure 3.7 Agarose gel electrophoresis to confirm cloning of eIF4A1, eIF4A2, eIF4A1+2 and DHX36 shRNAs into LV-GDNF. Plasmids were digested with *BcuI* and *SalI* and run on a 3% agarose gel at 80 V for 50 minutes.

LV-shRNAs described in this section did not produce a specific and consistent knockdown of target RAN-associated RNA helicases when tested by western blot. The reasons for this are unclear; it is possible that this may be attributed to technical errors as the LV-shRNAs worked effectively in some cases but were hampered by inconsistencies. Overall, they were considered too unreliable for experimental use and therefore LV-shRNAs were purchased from Origene. In addition to increased

efficiency and a guarantee of reliability, these shRNAs also come with a GFP tag allowing for easy checking of transfection efficiency.

3.2.4 Testing Origene eIF4A1, eIF4A2, eIF4B and eIF4H shRNAs

shRNAs targeting eIF4A1, eIF4A2, eIF4B and eIF4H were purchased from Origene. While a shRNA targeting eIF4H had yet to be included for this project, the increased efficiency of using purchased shRNAs led to the decision to broaden the scope of the project to now include eIF4H. The shRNA's ability to successfully and specifically knockdown their target proteins was tested with an initial western blot of HEK293T cells transfected with each shRNA. A representative blot is shown in Figure 3.8 and demonstrates that a substantial shRNA induced knockdown of eIF4A1, eIF4A2, eIF4B and eIF4H successfully occurred.

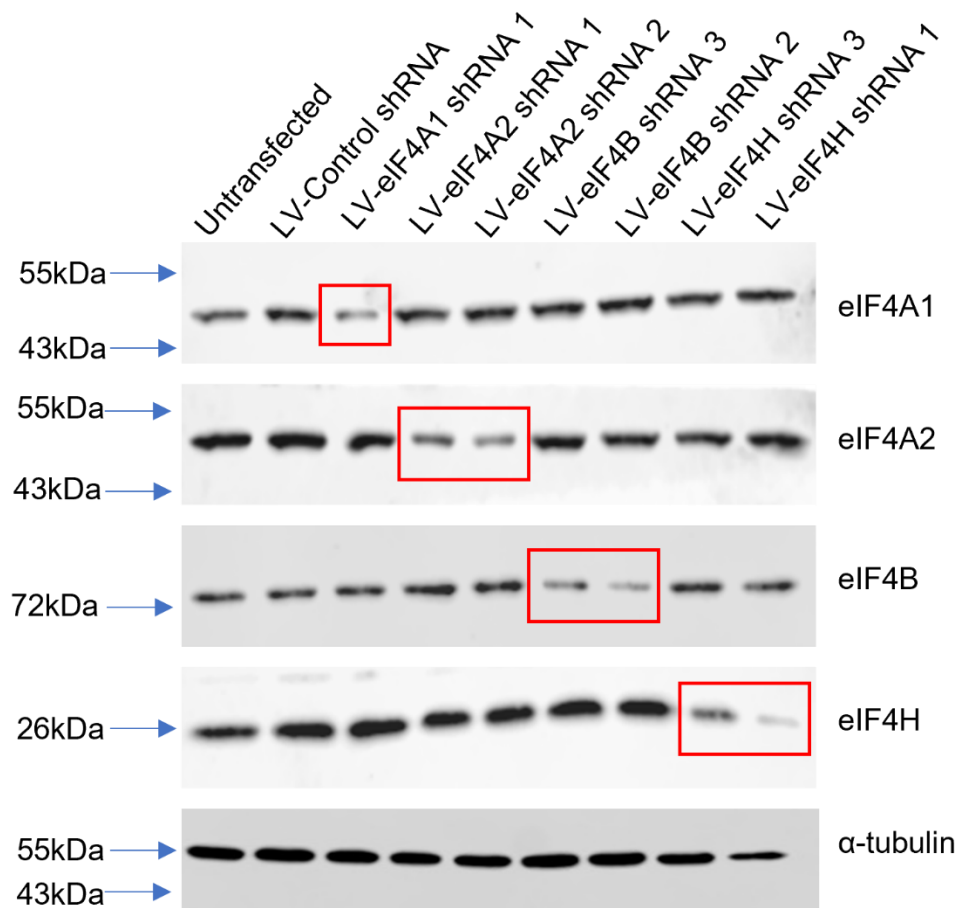


Figure 3.8 Western Blot to show knockdown of eIF4A1, eIF4A2, eIF4B or eIF4H by shRNA. HEK293T cells were transfected with each shRNA and probed for eIF4A1, eIF4A2, eIF4B and eIF4H. α -tubulin was used as a loading control. The bands indicating the knockdown of each protein are highlighted in red. Molecular weight was expected to be 46 kDa for eIF4A1 and eIF4A2, 80 kDa for eIF4B, 27 kDa for eIF4H and 55 kDa for α -tubulin.

Next, the shRNAs were tested through RT-qPCR in HEK293T cells to see if RAN-associated helicase mRNA expression had decreased consistent with the protein expression. Results of this are shown in Figure 3.9. Each shRNA was able to specifically reduce the target helicase mRNA, leading to a statistically significant decrease in expression without impacting any other helicase. Some variability was seen: each shRNA achieved a decrease in the mRNA expression of their target helicase of between 54 and 70%. Each RAN-associated RNA helicase had two shRNA options with the exception of eIF4A1, which had only one option available; the helicase shRNA achieving the most substantial knockdown was selected to take forward to

future experiments. These included investigating the impact on DPR production, shown later in this chapter, and MTT assays investigating the impact of shRNAs on cell viability, shown in chapter 5.

Figure 3.9 A shows results for eIF4A1 shRNA, where eIF4A1 shRNA 1 was selected by default with a mean knockdown of 65%. Figure 3.9 B shows eIF4A2 shRNAs, where shRNA 2 was selected as it achieved a better knockdown of eIF4A2, of 71% compared to 61% from shRNA 1. Figure 3.9 C shows eIF4B shRNAs where shRNA 2 was chosen, resulting in a 69% knockdown compared to 55% from shRNA 3. Figure 3.9 D shows eIF4H shRNA, here shRNA 1 was chosen with a 68% decrease while shRNA 3 resulted in a 61% reduction.

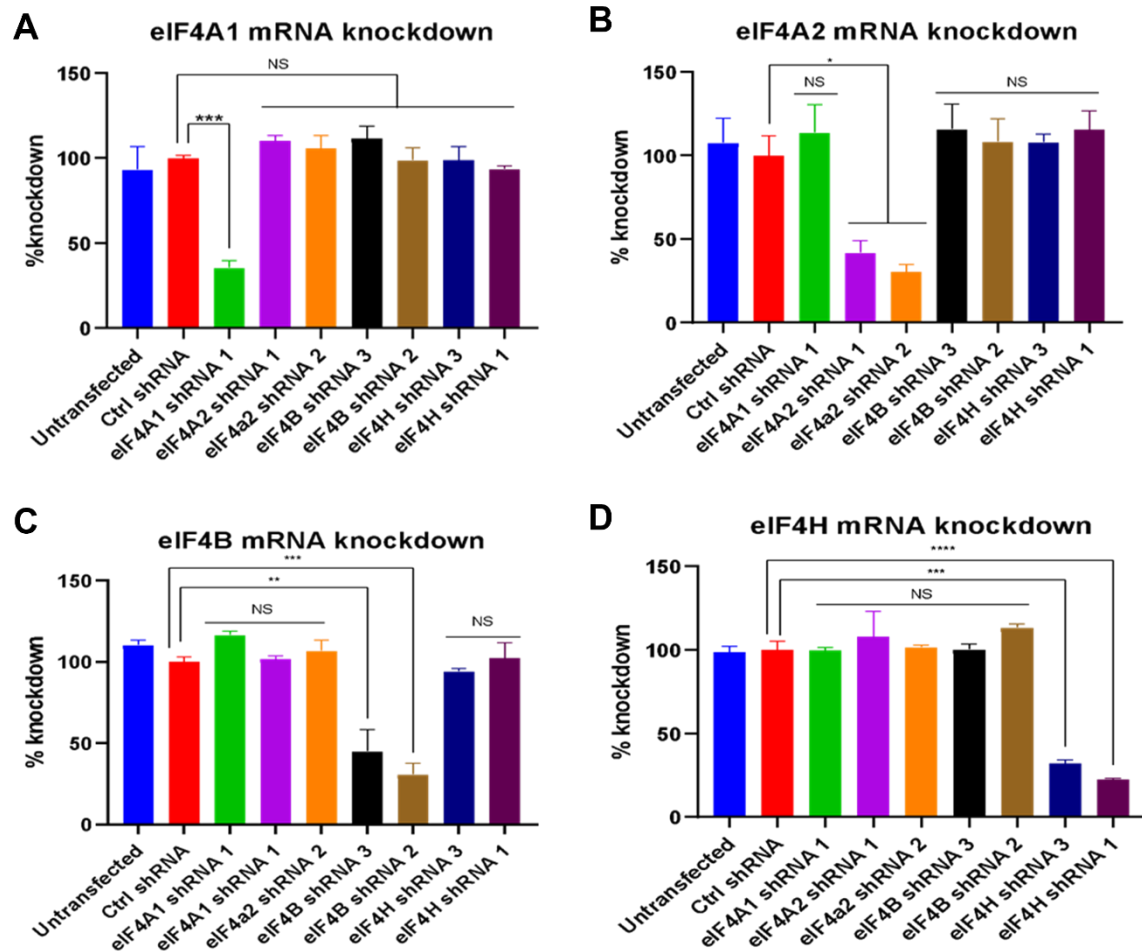


Figure 3.9 RT qPCR data showing the strength and specificity of eIF4A1, eIF4A2, eIF4B and eIF4H shRNA knockdown in HEK293T cells. (A) shows eIF4A1 shRNA; (B) shows eIF4A2 shRNA; (C) shows eIF4B shRNA; (D) shows eIF4H shRNA. N=3, Mean + SEM; analysed by one-way ANOVA with Tukey's correction for multiple comparisons; NS: not significant, *:p<0.05, **:p<0.01, ***:p<0.001, ****p<0.0001.

Following shRNA selection, western blot testing was repeated, shown in Figure 3.10 This confirms the chosen shRNAs were consistently producing a specific decrease in protein expression of their target RAN-associated RNA helicase.

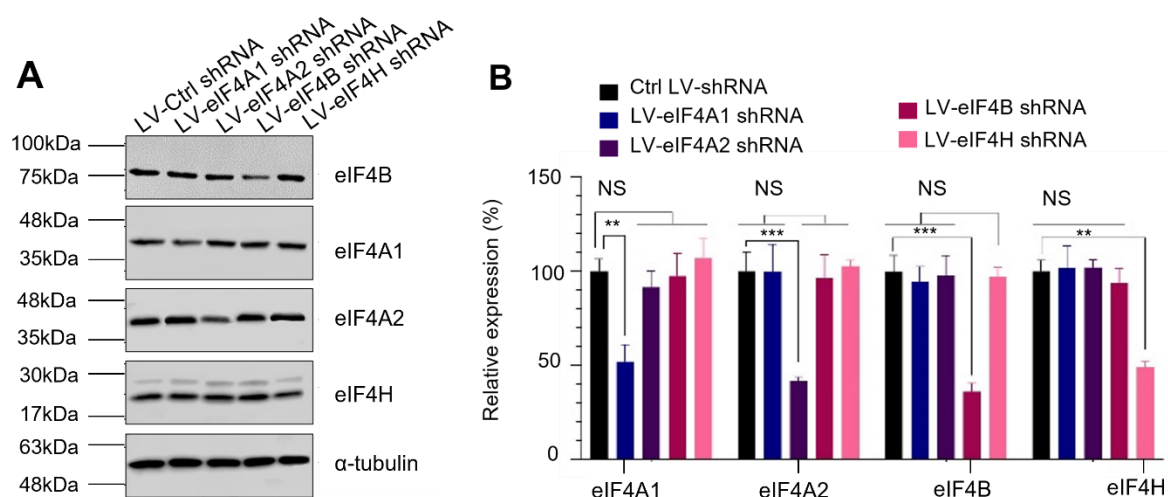


Figure 3.10 Western Blot to confirm consistent RAN-associated helicase knockdown using Origene LV-shRNAs (A) shows a representative western blot from HEK293T cells transfected for 96 hours with either Ctrl shRNA, eIF4A1 shRNA (V3LHS_410768), eIF4A2 shRNA (V3LHS_376084), eIF4B shRNA (V2LHS_227515) or eIF4H shRNA (V3LHS_404933). Blots are probed for eIF4A1, eIF4A2, eIF4B, eIF4H and α-tubulin as a loading control. Molecular weight was expected to be 46 kDa for eIF4A1 and eIF4A2, 80 kDa for eIF4B, 27 kDa for eIF4H and 55 kDa for α-tubulin. (B) shows (A) quantified in independent biological triplicates, Mean ± SEM; one-way ANOVA with Tukey's correction for multiple comparisons; NS: not significant; *: p<0.05; **: p<0.01; ***: p<0.001

3.3 Strategies to target RAN-associated RNA helicases and reduced DPR

reduction in HEK293 cell models of C9-ALS/FTD

After all necessary tools to target RAN-associated RNA helicases had been developed, they were next tested in HEK293T cell models of C9-ALS/FTD. The first strategy comprised using shRNAs to reduce helicase expression and determine if this results in a decrease in DPR production. Secondly, two sets of dominant negative helicase mutants were used. Details of these can be found in section 3.3.2. The principle of this strategy was not to knock-down helicases, but instead to render their helicase activity non-functional and test if this leads to a corresponding decrease in DPR production.

3.3.1 Transfection with RAN-associated RNA helicase shRNAs leads to a decrease in DPR production in HEK293T cell models of C9-ALS/FTD

Initially, pENTR eIF4A1, eIF4A2 and eIF4B shRNAs were tested by western blot to determine if they had an impact on RAN translation of DPRs in HEK293T cells co-transfected with helicase shRNAs and either 45 G4C2 (Sense) or C4G2 (antisense) C9ORF72 repeats tagged with V5. The use of the V5 tag allows for detection of DPRs in all three reading frames. For a control, un-transfected cells with no DPRs and a pENTR plasmid expressing a scrambled shRNA sequence and H1 promoter were used.

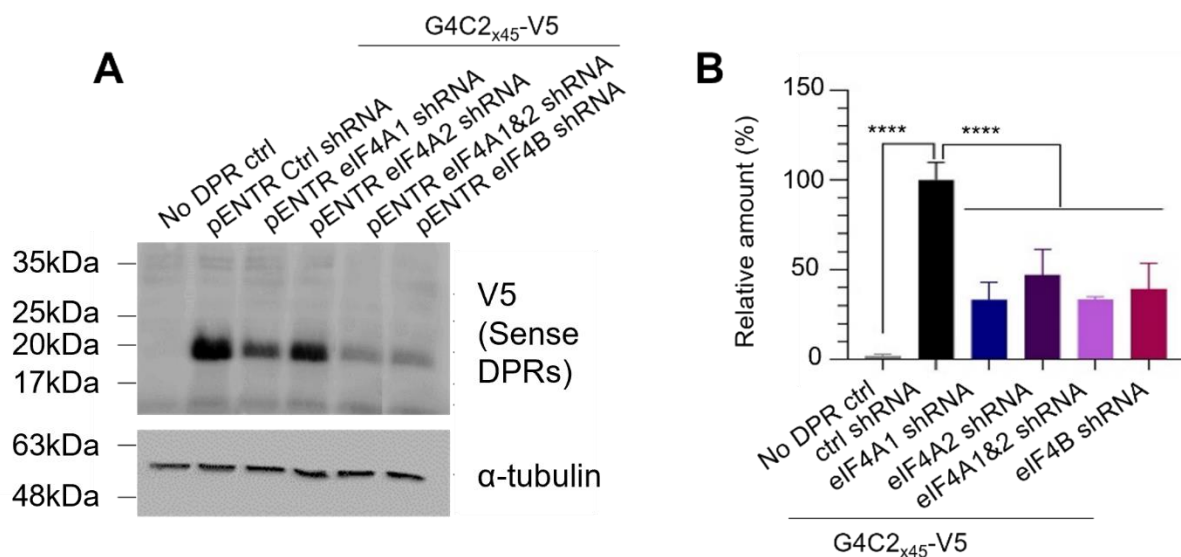


Figure 3.11 Western Blots for HEK293T cells co-transfected with pENTR shRNAs and Sense DPRs. (A) shows a representative western blot of HEK293T cells co-transfected with control, eIF4A1, eIF4A2, eIF4A1+4A2, eIF4B shRNA, and 45 G4C2 V5 tagged repeats. Blots were probed for V5 which detects sense DPRs in all three reading frames and α-tubulin as a loading control. A band was expected at 20 – 25 kDa for V5-tagged DPRs and at 55 kDa for α-tubulin. (B) shows results from (A) quantified in independent biological triplicates, mean ± SEM, one-way ANOVA with Tukey's multiple comparisons, ****.p<0.0001.

Figure 3.11 shows HEK293T cells co-transfected with pENTR shRNAs and sense DPRs, while pENTR shRNAs and antisense DPRs are shown in figure 3.12. For both

sense and antisense DPRs, shRNAs targeting all RAN-associated helicases resulted in a highly significant decrease in DPR production. For both sense and antisense DPRs the trend was broadly similar, with eIF4A2 shRNA producing the smallest decrease in DPR production of 50%. eIF4A1 showed a similar reduction of both sense and antisense DPRs by 70%. eIF4B was slightly more effective at reducing antisense DPRs than sense, with decreases of 75% and 60% respectively. Despite targeting two RAN-associated helicases, eIF4A1+4A2 shRNA did not produce a larger reduction in DPR production than targeting each helicase individually. No other combination of helicases was used for these experiments. For both sense and antisense DPRs, knockdown of eIF4A1+4A2 reduced DPR production by 65%.

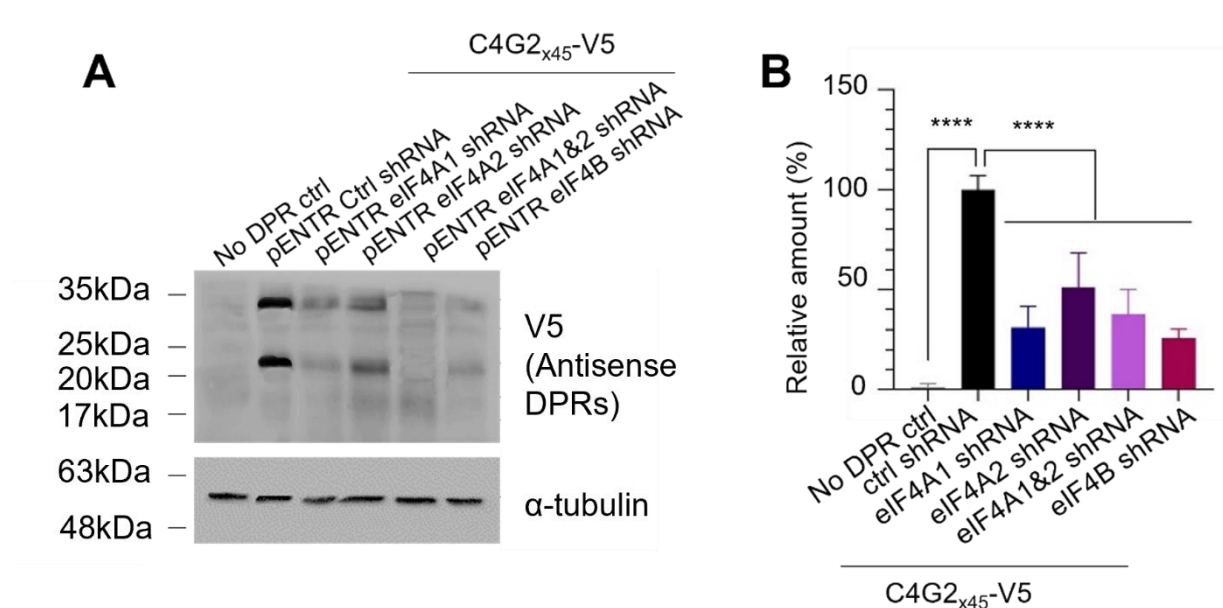


Figure 3.12 Western Blots for HEK293T cells co-transfected with pENTR shRNAs and antisense DPRs. (A) shows a representative western blot of HEK293T cells co-transfected with control, eIF4A1, eIF4A2, eIF4A1+4A2, eIF4B shRNA, and 45 C4G2 V5 tagged repeats. Blots were probed for V5 which detects antisense DPRs in all three reading frames and α -tubulin as a loading control. A band was expected at 20 – 25 kDa for V5-tagged DPRs and at 55 kDa for α -tubulin (B) shows results from (A) quantified in independent biological triplicates, mean $\pm$ SEM, one-way ANOVA with Tukey's multiple comparisons, ****: $p < 0.0001$.

pENTR / scAAV eIF4A1, eIF4A2, and DHX36 shRNAs were co-transfected into HEK293T cells with V5-tagged sense or antisense DPRs. The use of the V5 tag allows

for detection of DPRs in all three reading frames. Representative western blots and quantification of results in triplicate is shown in Figure 3.13 for sense DPRs and Figure 3.14 for antisense. For a control, a pENTR plasmid expressing a scrambled shRNA sequence from an H1 promoter was used. Additionally, scAAV-SOD1 shRNA and scAAV-SRSF1 shRNA were used as negative and positive controls. SOD1 is a gene with a known ALS-causing mutation, but is not associated with C9ORF72, RAN translation or the production of DPRs. Therefore, SOD1 shRNA was not predicted to be associated with any decrease in DPR production. However, in Figure 3.13 quantification of western blots indicates sense DPR reduction is associated with SOD1 knockdown. This is not seen for antisense DPRs in Figure 3.14 which follows the expected pattern of results. The reason for the reduction of sense DPRs associated with SOD1 knockdown is unclear, but it is most likely due to a technical error. Alternatively, SOD1 transfection may have been particularly toxic in this case, undermining crucial cell function and therefore indirectly limiting its ability to carry out RAN translation. In contrast, SRSF1 shRNA should lead to a decrease in DPR production; this was found to be consistently the case with both sense and antisense DPRs. This is because SRSF1 is required for the export of repeat-containing C9ORF72 mRNA transcripts to the cytoplasm for RAN translation into DPRs (Hautbergue et al., 2017).

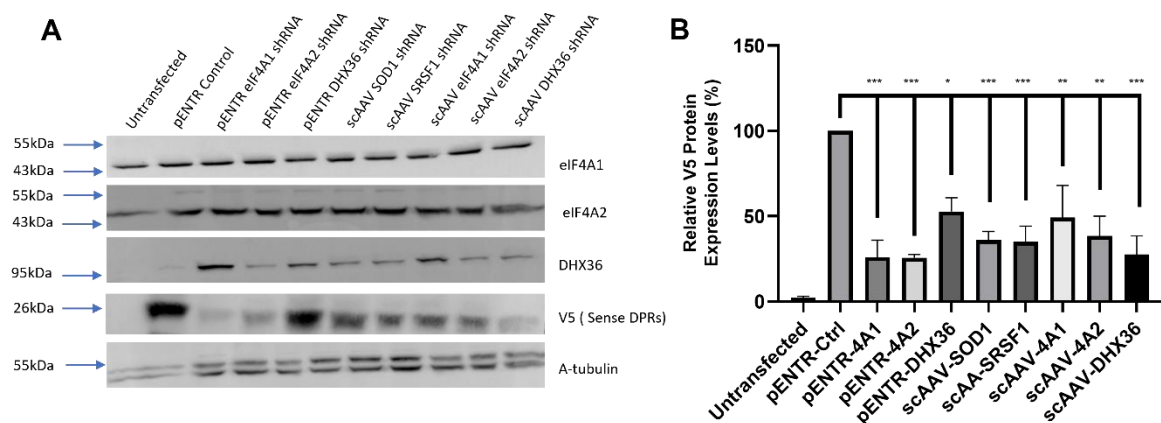


Figure 3.13 Western Blot and quantified results for HEK293T cells co-transfected with RAN-associated RNA helicase shRNAs and Sense DPRs. (A) shows a representative western blot which has been probed for eIF4A1, eIF4A2, DHX36 and V5 which detects sense DPRs in all three reading frames, and α -tubulin as a loading control. A band was expected for eIF4A1 and eIF4A2 at 46 kDa, for DHX36 at 115 kDa, for V5-tagged DPRs at 20 – 25 kDa and for α -tubulin at 55 kDa. (B) shows quantified results, mean + SEM analysed by one-way ANOVA with Dunnett's corrections for multiple comparisons. N=3, *:p<0.05, **:p<0.01, ***:p<0.001.

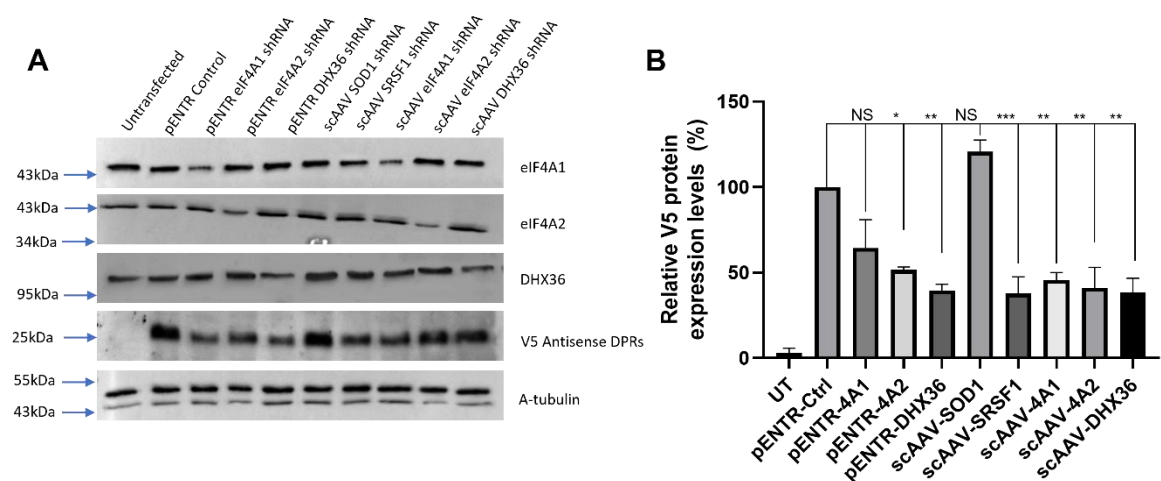


Figure 3.14 Western Blot and quantified results for HEK293T cells co-transfected with RAN-associated RNA helicase shRNAs and Antisense DPRs. (A) shows a representative western blot which has been probed for eIF4A1, eIF4A2, DHX36 and V5 which detects antisense DPRs in all three reading frames, and α -tubulin as a loading control. A band was expected for eIF4A1 and eIF4A2 at 46 kDa, for DHX36 at 115 kDa, for V5-tagged DPRs at 20 – 25 kDa and for α -tubulin at 55 kDa. (B) shows quantified results, mean + SEM analysed by one-way ANOVA with Dunnett's corrections for multiple comparisons. N=3, NS: not significant, *:p<0.05, **:p<0.01, ***:p<0.001.

First, we established that shRNAs were functioning as expected and that both pENTR and scAAV shRNAs led to the knock-down of their target helicase eIF4A1, eIF4A2, or DHX36. Both pENTR and scAAV shRNAs appear more effective at reduction of antisense DPRs compared to sense DRPs, although in all cases V5 levels fell by at least 50%. Next, we examined the impact of RAN-associated RNA helicase knockdown on DPR production. Both pENTR and scAAV shRNAs were successfully able to reduce relative V5 protein expression levels. This was the case for both sense and antisense DPRs.

In antisense DPRs, for scAAV eIF4A1, eIF4A2 and DHX36 shRNA there was a statistically significant fall in DPR production of between 55-60%. Decreases in antisense DPRs were broadly consistent regardless of which RAN-associated helicase was targeted for scAAV plasmids. pENTR vectors performed less well with only pENTR-eIF4A2 and DHX36 shRNA reaching a statistically significant fall in DPRs. Antisense DPRs decreased in expression by between 40% for eIF4A1 shRNA and 60% for DHX36 shRNA. Despite not reaching statistical significance pENTR-eIF4A1 shRNA still shows a clear downward trend in antisense DPR production.

3.3.2 Transfection with RAN-associated RNA helicase mutants leads to a decrease in DPR production in HEK293T cells models of C9-ALS/FTD

In conjunction with our collaborators Professor Martin Bushell and colleagues, two sets of RAN-associated RNA helicase mutants were designed, with their key features shown in a schematic diagram in Figure 3.15. eIF4A1 Q339E and eIF4A2 Q340E have a mutation in their helicase C-terminal domain, rendering their helicase activity non-functional and have been previously described by Cruz and colleagues (Cruz-Migoni et al. 2012). Q339E and Q340E mutants have a substitution of Glutamine 339 or 340 into glutamic acid. The glutamine moiety is part of the catalytic triad of the active site conferring RNA helicase activity (Cruz-Migoni et al., 2012). eIF4A1 and 4A2 PRRVAA

were a kind gift from Professor Bushell, and are enhanced RNA binding mutants. First, eIF4A1 Q339E and eIF4A2 Q340E, and secondly eIF4A1 PRRVAA and eIF4A2 PRRVAA were used to test the impact of these mutants on synthesis of both sense and antisense DPRs. In each experiment, only one set of mutants was used: either Q339E/Q340E or PRRVAA. In no instances were cells transfected with helicases carrying double mutations of both Q339E/Q340E and PRRVAA at the same time.

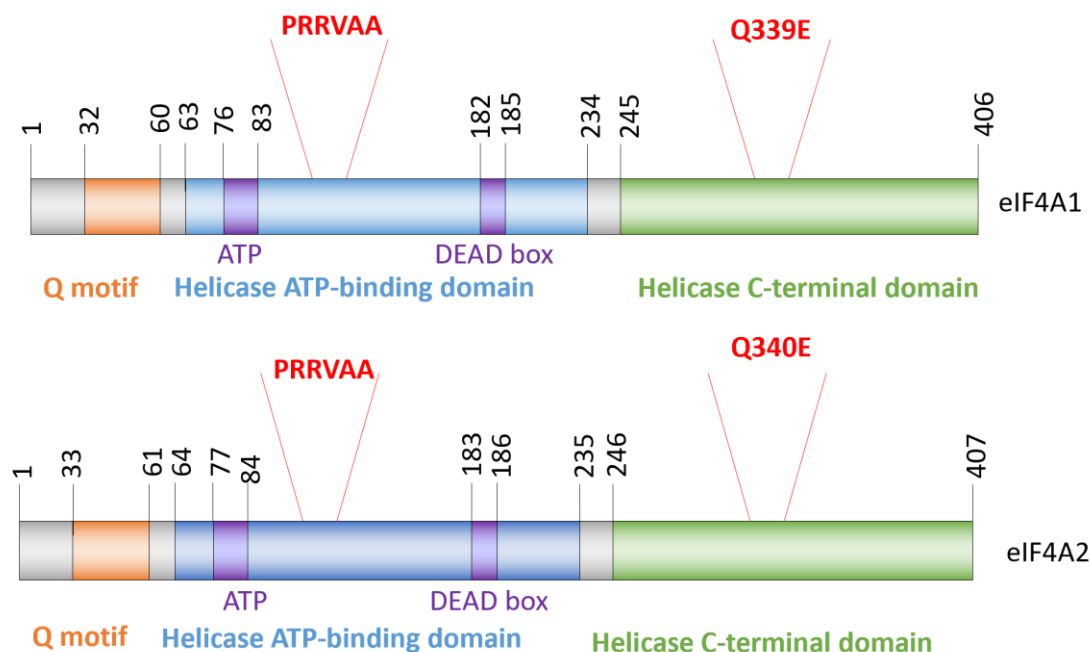


Figure 3.15 Schematic diagram of RAN-associated helicase mutants eIF4A1 Q339E/PRRVAA and eIF4A2 Q340E/PRRVAA indicating the relevant regions with the location of the mutations shown in red

3.3.2.1 RNA Helicase mutants eIF4A1 Q339E and eIF4A2 Q340E are associated with decreased production of sense and antisense DPRs

FLAG eIF4A1 or eIF4A2 and mutant FLAG eIF4A1 Q339E or eIF4A2 Q340E were co-transfected in HEK293T cells with V5-tagged sense or antisense DPRs. V5 tags detect DPRs in all three reading frames. For controls, un-transfected cells and a 3X-FLAG plasmid with no helicase insert were used. Representative western blots with

quantified results showing decreased DPR production is shown in Figure 3.16 for sense DPRs and Figure 3.17 for antisense DPRs.

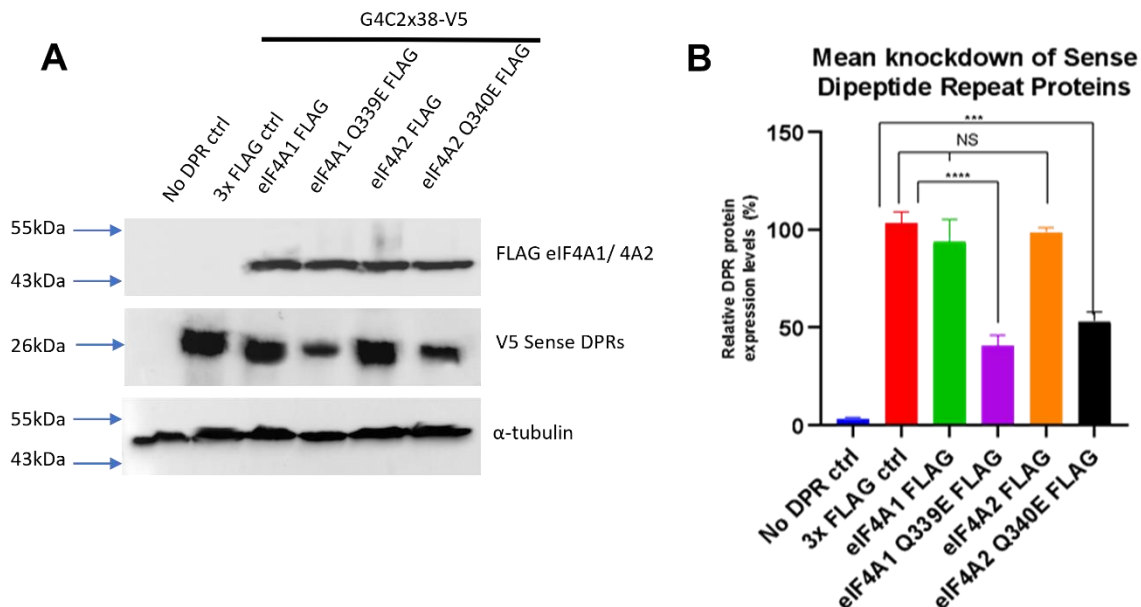


Figure 3.16 Expression of RAN-associated RNA Helicase mutants eIF4A1-Q339E and eIF4A2-Q340E leads to decreased production of sense DPRs in HEK293T cells. (A) shows a representative western blot of HEK293T cells co-transfected with sense DPRs and either normal eIF4A1 and eIF4A2 or helicase mutants eIF4A1 Q339E and eIF4A2 Q340E. Blots were probed for FLAG detecting eIF4A1/eIF4A2 and V5 which detects sense DPRs in all three reading frames. α-tubulin was used as a loading control. A band was expected for eIF4A1 and eIF4A2 at 46 kDa, for V5-tagged DPRs at 25 kDa and for α-tubulin at 55 kDa. (B) shows results of (A) quantified in independent biological triplicates, with mean + SEM analysed by one-way ANOVA with Tukey's corrections for multiple comparisons. NS: not significant, ***: p<0.001, ****: p<0.0001.

HEK293T cells transfected with mutant helicases eIF4A1 Q339E or eIF4A2 Q340E result in a highly significant decrease in production of sense DPRs. DPR production shows a slightly greater fall associated with eIF4A1 Q339E than eIF4A2 Q340E, of 60% compared to 45%. As expected, over expression of wild-type eIF4A1 and eIF4A2 is not associated with any change in sense DPR production. Despite HEK293T transfection with normally functioning helicases acting as an overexpression model, there is no increase in sense DPR production associated with transfection of normal eIF4A1 or eIF4A2.

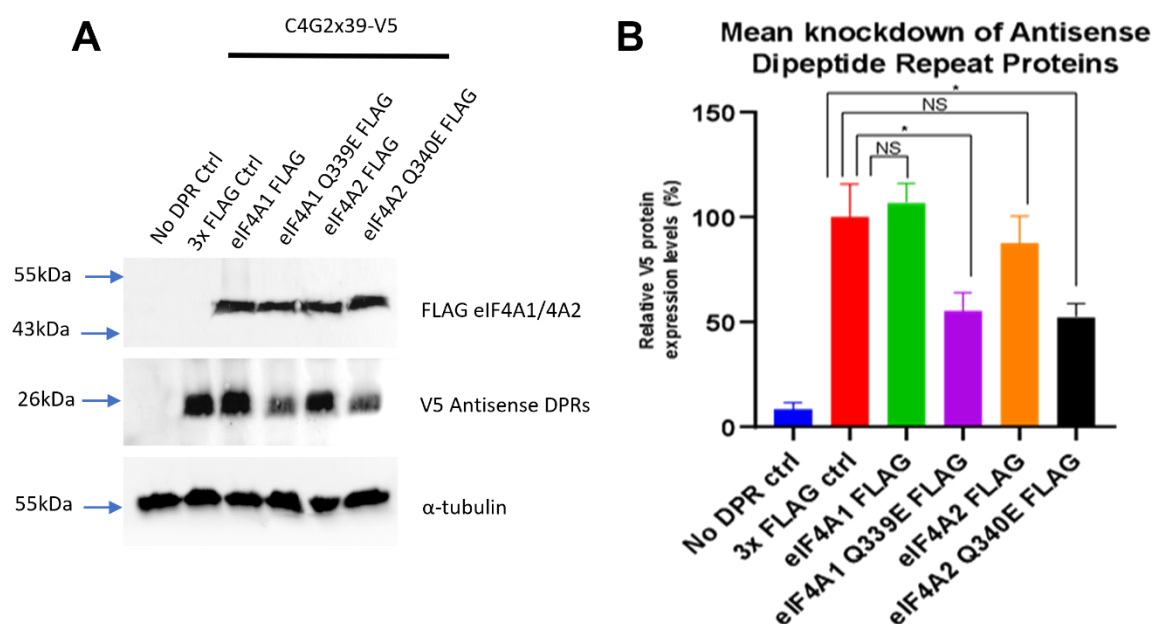


Figure 3.17 RAN-associated RNA Helicase mutants eIF4A1-Q339E and eIF4A2-Q340E result in decreased production of antisense DPRs in HEK293T cells. (A) shows a representative western blot of HEK293T cells co-transfected with antisense DPRs and either normal eIF4A1 and eIF4A2 or helicase mutants eIF4A1 Q339E and eIF4A2 Q340E. Blots were probed for FLAG detecting eIF4A1/eIF4A2 and V5 which detects antisense DPRs in all three reading frames. α -tubulin was used as a loading control. A band was expected for eIF4A1 and eIF4A2 at 46 kDa, for V5-tagged DPRs at 25 kDa and for α -tubulin at 55 kDa. (B) shows results of (A) quantified in independent biological triplicates, with mean + SEM analysed by one-way ANOVA with Tukey's corrections for multiple comparisons. NS: not significant, *:p<0.05.

HEK293T cells transfected with mutant helicases eIF4A1 Q339E or eIF4A2 Q340E result in a significant decrease in production of antisense DPRs. As with sense DPRs, non-mutated, normally functioning eIF4A1 and eIF4A2 are not associated with any decrease or increase in antisense DPR production.

3.3.2.2 RAN-associated RNA Helicase mutants eIF4A1 PRRVAA and eIF4A2

PRRVAA are associated with decreased production of sense and antisense DPRs

Next, the same set of experiments was conducted with RNA binding mutants eIF4A1 PRRVAA and eIF4A2 PRRVAA, which were a kind gift from Professor Martin Bushell, Beatson Institute. FLAG eIF4A1 or eIF4A2 and mutant FLAG eIF4A1 PRRVAA or eIF4A2 PRRVAA were co-transfected in HEK293T cells with V5-tagged sense or antisense DPRs. V5 tags detect DPRs in all three reading frames. As before, for controls un-transfected cells and a 3X-FLAG plasmid with no helicase insert were used. Representative western blots with quantified results showing decreased DPR production is shown in Figure 3.18 for sense DPRs and Figure 3.19 for antisense DPRs.

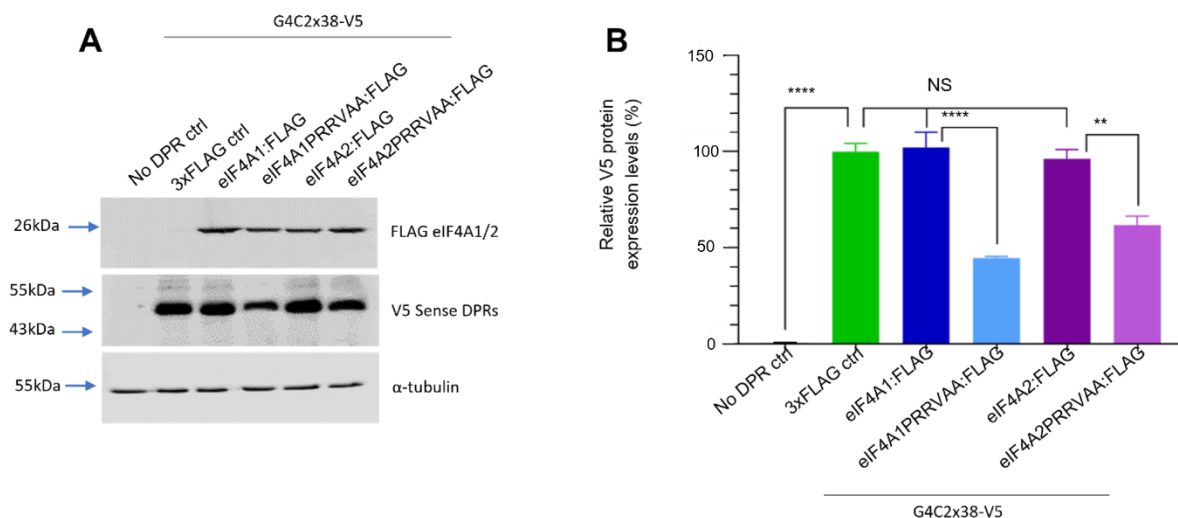


Figure 3.18 RAN-associated RNA Helicase mutants eIF4A1 PRRVAA and eIF4A2 PRRVAA result in decreased production of sense DPRs in HEK293T cells. (A) shows a representative western blot of HEK293T cells co-transfected with sense DPRs and either normal eIF4A1 and eIF4A2 or helicase mutants eIF4A1 PRRVAA and eIF4A2 PRRVAA. Blots were probed for FLAG detecting eIF4A1/eIF4A2 and V5 which detects sense DPRs in all three reading frames. α -tubulin was used as a loading control. A band was expected for eIF4A1 and eIF4A2 at 46 kDa, for V5-tagged DPRs at 25 kDa and for α -tubulin at 55 kDa. (B) shows results of (A) quantified in independent biological triplicates, with mean + SEM analysed by one-way ANOVA with Tukey's corrections for multiple comparisons. NS: not significant, **:p<0.01, ****: p<0.0001.

HEK293T cells transfected with mutant helicases eIF4A1 PRRVAA or eIF4A2 PRRVAA result in a highly significant decrease in production of sense DPRs. This appears to affect the different sense DPRs consistently with no evidence of selective expression of DPRs shown in these experiments. DPR production shows a slightly greater fall associated with eIF4A1 PRRVAA than eIF4A2 PRRVAA, of 55% compared to 40%. As shown above, wild-type eIF4A1 and eIF4A2 is not associated with any decrease in sense DPR production. Over expression of normal eIF4A1 or eIF4A2 is not associated with any increase in DPR production and there is no evidence from these experiments that over expression of helicases leads to selective DPR expression.

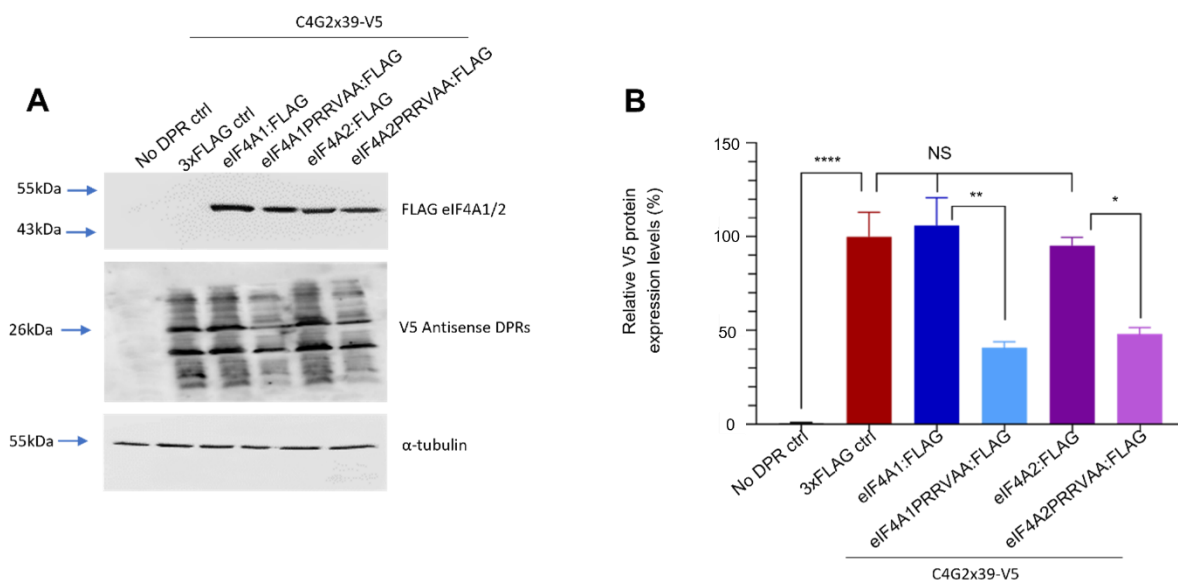


Figure 3.19 RAN-associated RNA Helicase mutants eIF4A1 PRRVAA and eIF4A2 PRRVAA result in decreased production of antisense DPRs in HEK293T cells. (A) shows a representative western blot of HEK293T cells co-transfected with antisense DPRs and either normal eIF4A1 and eIF4A2 or helicase mutants eIF4A1 PRRVAA and eIF4A2 PRRVAA. Blots were probed for FLAG detecting eIF4A1/eIF4A2 and V5 which detects antisense DPRs in all three reading frames. α -tubulin was used as a loading control. A band was expected for eIF4A1 and eIF4A2 at 46 kDa, for V5-tagged DPRs at 25 kDa and for α -tubulin at 55 kDa. (B) shows results of (A) quantified in independent biological triplicates, with mean + SEM analysed by one-way ANOVA with Tukey's corrections for multiple comparisons. NS: not significant, *:p<0.05, **:p<0.01, ****: p<0.0001.

HEK293T cells transfected with mutant helicases eIF4A1 PRRVAA or eIF4A2 PRRVAA result in a significant decrease in production of antisense DPRs. Figure 3.19 shows a number of bands detected by the anti-V5 antibody indicating antisense DPRs, in contrast to sense DPRs shown in Figure 3.18, which produce only one band. It is not clear why multiple bands are seen for antisense DPRs. They may be attributed to non-specific binding indicating insufficient blocking steps during this experimental protocol, or excess loaded protein combined with an over-sensitive detection system. Alternatively, sample degradation may have occurred in this case, or the DPRs may have undergone post-translational modifications or cleavages giving rise to additional unexpected bands. In this case, all bands were quantified. As with sense DPRs, non-mutated, normally functioning eIF4A1 and eIF4A2 are not associated with any decrease or increase in antisense DPR production.

3.4 Chapter Summary and Discussion

The aim of this project was to explore the role of RNA helicases in RAN translation initiation as a potential therapeutic target in C9-ALS/FTD. RAN translation leads to the production of neurotoxic DPRs, through a poorly understood mechanism which urgently requires more research attention. Despite DPR production being identified as a key pathogenic mechanism driving C9-ALS/FTD, currently no therapeutic strategies have been developed that focus on targeting RAN translation to prevent DPR production. In this project, we focus on RNA helicases as important regulators of RAN translation, investigating mechanisms of RAN initiation with a long-term aim of developing a robust treatment strategy centring on these proteins.

3.4.1 Development of tools to target RAN-associated RNA helicases

To start this project, we initially developed tools required to target RAN-associated RNA helicases in cell models of C9-ALS/FTD. We have shown successful cloning of

shRNAs targeting RAN-associated RNA helicases into three different vectors, allowing plasmid-based mammalian but also viral expression for potential downstream therapeutic approaches: pENTR, scAAV and LV. shRNAs were chosen over miRNA as they are generally more specific, and with only 10 amino acid differences between eIF4A1 and eIF4A2 this was an important consideration. This project has been undertaken with a view to future experiments and clinical translation potential. shRNAs are beneficial for future therapies in that they have fewer off-target effects than miRNAs, and generally benefit from a more favourable regulatory environment.

Prior to starting this project, the Hautbergue laboratory had used RNA immunoprecipitation to confirm direct binding of eIF4A1 and eIF4A2 to expanded *C9ORF72* repeats. Then, they established pENTR shRNAs targeting eIF4A2, eIF4A2, eIF4A1+2. At the early stage of the project this was expanded to include pENTR-eIF4B shRNA, as there was established data in the literature reporting that eIF4B was linked to DPR production in *Drosophila*. eIF4B was identified following a genomic screen for modifiers of poly GR-induced toxicity in *Drosophila* expressing the G4C2 repeat expansion mutation. Downregulation of eIF4B significantly reduced levels of poly-GR (Goodman et al., 2019). eIF4H was also identified by Goodman and colleagues as a modifier of poly-GR production and was also included at a later stage of the project. We aimed to see if this result was also consistently found in mammalian cell models of C9-ALS/FTD. scAAV shRNAs were produced as a direct clone from pENTR shRNAs. While scAAV plasmids are generally seen as less efficient at transfection in *in vitro* models, they are good vectors for animal studies. Again, looking to future translation to clinical gene therapy trials, scAAV vectors show good transgene expression, low toxicity and an ability to cross the blood-brain barrier. In the more immediate future, developing scAAV shRNAs plasmids will allow for trials in *in vivo* mouse models of C9-ALS/FTD, without requiring any redesign of shRNAs.

During cloning experiments, it does appear from the agarose gel in Figure 3.2 that the control vector produced a slightly larger band, which was unexpected. The exact location of bands for the control vector and the shRNA-containing vector differ by only approximately 50 base pairs and are therefore hard to differentiate between; use of a different gel concentration may have aided in this. As the plasmids were sequenced, confirming correct incorporation of the shRNA in all cases, it is most likely these band locations reflect technical anomalies in running the agarose gel. This could occur in sample preparation, incomplete digestion by restriction enzymes, contamination of some samples or dye, poor gel or buffer preparation, or faulty equipment with fluctuations in voltage resulting in failure to run an even current across all sample wells of the gel. Alternatively, the bands may have run to the expected location but issues with visualisation may have occurred. For this reason, all samples were sent for sequencing to validate the cloning carried out in this thesis. Sequencing results confirming correct incorporation of shRNAs provide increased confidence in the plasmids produced for future experiments.

At this stage, scAAV-DHX36 shRNA was also introduced to the project. DHX36 has been identified in the literature as the most important resolver of G-quadruplexes *in vitro* (Vaughn et al., 2005). This role makes it highly relevant to this project, as the G-rich nature of the C9ORF72 repeat expansion mutation is particularly prone to formation of G-quadruplexes. We therefore decided to investigate the relationship between DHX36 and DPR production in the context of C9-ALS/FTD. Results were promising, with shRNAs targeting DHX36 associated with a substantial decrease in DPR production. However, we are yet to ascertain if this effect will also be observed in an *in vivo* disease model.

shRNAs were next subcloned into lentiviral vectors, which were eventually purchased from Origene due to a lack of consistency in target helicase knockdowns from previously cloned LV-shRNAs. Both pENTR and scAAV as well as LV vectors

containing shRNAs showed some inconsistency and variation in terms of their ability to knock down their target proteins. However, when RAN-associated helicase knockdown was achieved there was a corresponding decrease in DPR production. At this point, we extended the scope of the project to include eIF4H shRNA. eIF4H is a known co-factor for RAN-associated helicases we were already investigating, eIF4A1, eIF4A2 and eIF4B and has also been linked to DPR production in *Drosophila* (Goodman et al., 2019). We therefore considered it important to include to thoroughly understand the role these helicases play in RAN translation initiation. The extra convenience offered by commercially sourced shRNAs allowed expansion of our proteins of interest to include eIF4H along with eIF4A1, eIF4A2 and eIF4B. For future experiments, knock-down of additional translation factors could be used as a control to determine the extent to which helicases are necessary for DPR production, but the resources required for these experiments were not available for this project. Purchased LV-shRNAs also offered the advantage of a GFP tag, allowing for easy detection of transfection efficiency. While we had previously achieved good results with DHX36 shRNA, leading to efficient reduction of DPRs, it is not part of the eIF4 complex. Although certainly worthy of further investigation, the scope of this project focuses on the role of eIF4A1/4A2 and their co-factors in RAN translation initiation.

3.4.2 Limitations associated with HEK293T cell models of C9-ALS/FTD

Lentiviral vectors were selected for their ability to transduce neurons, allowing for future experiments in patient-derived neuronal cell models. This is a necessary step before moving forward with *in vivo* C9-ALS/FTD models. The work shown in this chapter was carried out exclusively in HEK293T cells. This is an immortalised cell line, relying on transfection of overexpressed proteins, and therefore is associated with some limitations. It was selected for initial experiments as it is robust, easy to maintain and quick to grow, and relatively easy to transfect. However, while it shows proof-of-principle, it lacks disease relevance. It is possible that RAN-associated RNA helicase

activity and expression may differ in non-proliferative motor neurons. Therefore, we next aimed to repeat these experiments in a more sophisticated cell model of patient-derived neurons, which better reflects the true conditions of these helicases in C9-ALS/FTD.

3.4.3 Strategy surrounding partial depletion of RAN-associated RNA helicases to reduce DPR production *in vitro*

After cloning or purchasing shRNAs in the pENTR, scAAV and LV vectors, we first tested their ability to knockdown their target RAN-associated helicase before moving on to investigate the impact on DPR production. It was crucial that we established any drop in DPRs produced was due to the specific helicase targeted. This is particularly important given the high levels of homology between them. For pENTR and scAAV we used western blot on HEK293T cells transfected with shRNAs and quantified falls in the target protein. For LV shRNAs, we investigated both at the protein level and the mRNA level, using RT-qPCR. Our project strategy did not involve a total knockdown of any helicase. Given their crucial roles in canonical as well as RAN translation, this would undoubtedly be highly toxic and not viable as a therapeutic strategy. Instead, we intended to achieve a knockdown that reduced RAN translation of DPRs to a non-toxic level, while preserving essential canonical translation. The exact level at which this can be achieved remains to be fully determined, and crucially next steps of the project will involve investigations into toxicity associated with RAN-associated helicase knockdown. Additional future experiments should also investigate helicase levels and the relationship with DPR production and cell survival over a set time. Cells were transfected with shRNAs targeting helicase levels for 72 hours, but it is not known how helicase levels fell over that time or what impact a longer period would have on cell viability.

Although some variability was seen across biological repeats, shRNAs in all vectors were able to produce a knockdown of their target helicases of between 50-65% at the protein level and 65 – 70% at the mRNA level, which is in line with our intentions for this work. For pENTR and scAAV vectors, eIF4A1 and eIF4A2 protein levels were reduced by 55%, for eIF4B it was 60%. For Origene LV-shRNAs, protein levels decreased by 50% for eIF4A1 and eIF4H, 60% for eIF4A2 and 65% for eIF4B. When decreases in mRNA was investigated, the decrease of transcripts produced was detected at slightly greater than at the protein level, at 65% for eIF4A1, 70% for eIF4A2, 69% for eIF4B and 68% for eIF4H. This is likely reflective of the fact that helicases are highly abundant, stable proteins, and the transfection period was only 72 hours. Therefore, some of the proteins detected by western blot were already produced prior to shRNA transfection and simply hadn't yet degraded, hence why the knockdown at the protein level appears slightly less effective. There is also the possibility that post-translational compensatory mechanisms could contribute.

After establishing the shRNAs were working as intended, we investigated their effects on sense and antisense DPR production. HEK293T cells were co-transfected with the (Emma et al., 2021) shRNAs and V5-tagged G4C2 or C4G2 C9ORF72 repeats. Some variety of repeat lengths were used, ranging from 38 to 45 repeats. These differences are small enough to be unlikely to be biologically relevant, given that in patient populations repeats can vary by hundreds or thousands of repeats, and yet converge on the same set of symptoms. The repeats used in these experiments are crucially all considered a pathogenic repeat length, given that the 'tipping point' for repeat length toxicity is generally accepted in the field to be around 30 repeats (Emma et al., 2021). The use of the V5 tag allows for detection of DPRs in all three reading frames, important given it can be technically challenging to produce specific antibodies for DPRs. This is due to their low complexity, meaning poor specificity and cross-reactivity with other DPR species are common issues to contend with. Additionally, the compact

structure of aggregated DPRs can result in the epitope being obscured (Jambeau et al., 2022).

For pENTR shRNAs, eIF4A1, eIF4A2 and eIF4B were all effective at reducing V5 levels, producing a statistically significant decrease for both sense and antisense DPRs. Comparing each RAN-associated helicase, the trend is generally consistent between sense and antisense DPRs. It was not known whether any differences between sense and antisense DPR production would be likely, as most published research studies so far generally only consider sense DPRs and do not include antisense DPRs in their work. A general trend across vectors and sense and antisense DPRs was that eIF4A1 shRNA produces a larger fall in DPR levels than eIF4A2 shRNA. For sense DPRs, eIF4A1 shRNA achieves a 68% decrease, while it was 70% for antisense DPRs, while for eIF4A2 the decrease is 55% and 50% respectively. eIF4B shRNA resulted in a 60% decrease in sense DPRs, but was the best performer for antisense DPRs, leading to a 75% reduction in V5 levels. That eIF4A1 shRNA produced the more substantial reduction of both sense and antisense DPRs than eIF4A2 suggests it has the more central role in RAN translation initiation. It may not follow that eIF4A1 is the most suitable helicase to target for a therapeutic strategy. There may be too large an impact on canonical translation, and a co-factor may be a more viable target. The data presented here confirms that eIF4B has an essential role in RAN translation initiation. It is unknown to what extent eIF4B can function independently of eIF4A1 or eIF4A2 to initiate RAN translation. eIF4B led to the greatest overall knockdown of antisense DPRs. This may simply be because eIF4B is a less abundant protein to start with, so any decrease has a more severe impact. Alternatively, it has been suggested that eIF4A1 and eIF4A2 activity can compensate for each other, which is a mechanism that may not be available for eIF4B, leaving the cell more vulnerable to the effects of decreasing levels. It may also be possible for cells to take a flexible approach and use one of several helicases as a co-factor to help initiate RAN translation, which would represent a challenge for this therapeutic

strategy. It may also be the case that RAN translation initiation mechanisms differ in proliferative and non-proliferative cell types, hence why this work will be expanded to a more disease relevant neuronal model. Interestingly, the use of eIF4A1+4A2 shRNA did not result in a greater decrease in DPRs than targeting eIF4A1 and eIF4A2 independently. This was the only combination of helicase tested and future experiments should also consider other combinations, such as eIF4A1 + eIF4B shRNAs, which could provide further useful insight into helicase function in the context of DPR production, but were beyond the scope of this thesis. The result from eIF4A1 + eIF4A2 shRNA suggests a high level of flexibility from the cell, with helicases acting as co-factors, possibly eIF4B, able to compensate for the loss of either or both eIF4A1 and eIF4A2. If no compensatory mechanism was in place, we would expect targeting both eIF4A1 and eIF4A2 together to lead to close to double the DPR reduction of targeting them alone. This highlights the importance of understanding the full range of regulators of RAN translation initiation and how they interact when designing an effective therapeutic strategy. For scAAV vectors, the reduction in antisense DPRs was generally more consistent and to a greater extent than shRNAs in pENTR vectors.

3.4.4 Use of RAN-associated RNA helicase dominant negative mutants to reduce DPR production *in vitro*

Our next strategy involved the use of RAN-associated RNA helicase mutants, to test if non-functional helicases would reduce DPR production similarly to knocking down helicases with shRNAs. Two sets of mutants were used, Q339E/ Q340E had mutations in their helicase domain, and PRRVAA in their RNA binding domain. The former have been used in published work, although the impact on global protein translation resulting from these mutants was not investigated (Cruz-Migoni et al., 2012). However, in a study investigating a different set of eIF4A dominant negative mutants in a rabbit reticulocyte translation system, use of the mutants was found to be associated with strong inhibition of translation. This repression could be rescued by

addition of functional eIF4A, although not by its co-factor eIF4B. All mRNAs investigated were repressed and the effect was described as dramatic, with a 30-fold inhibition of translation associated with some eIF4A mutants (Pause et al., 1994). These findings indicate that any repression of activity of the key helicases involved in translation initiation is likely to have widespread effects on global translation. However, this study did not account for the eIF4A1 and eIF4A2 isoforms and it is possible that this dramatic effect only arises from targeting both helicase simultaneously, which is supported by the work presented in this thesis.

The mechanism of the PRRVAA mutation is not fully characterised, but it is thought to involve excessively high affinity between RNA and the RNA-binding domain effectively stalling the helicase's unwinding of secondary structures. This strategy has shown promising results and confirmed the necessity of functional helicase activity in RAN translation. HEK293T cells were co-transfected with normally functioning or mutant helicases, and either 38/39 G4C2 / C4G2 sense or antisense V5-tagged DPRs. Overall cells transfected with mutant helicases showed a consistent and significant decrease in V5 levels, indicating a reduction in RAN translation of DPRs.

For Q339E/ Q340E mutants, sense DPRs showed a greater reduction with eIF4A1 mutants than eIF4A2 mutants, with V5 reductions of 60% and 45% respectively. For antisense DPRs results are more consistent between eIF4A1 and eIF4A2, with a 45% decrease associated with eIF4A1 mutants and 50% for eIF4A2 mutants. Consistent with previous results, this indicates eIF4A1 helicase activity has the more important role in initiating RAN translation. The larger decrease in sense DPR production than antisense suggests differences in underlying RAN translation mechanisms. For example, sense G4C2 transcripts may be more prone to forming secondary structures such as G-quadruplexes than antisense C4G2 transcripts. Therefore, they would be more reliant on helicase activity to resolve these and initiate RAN translation. This would mean cells were more sensitive to loss of helicase activity when it came to

producing DPRs from sense transcripts than antisense, leading to a bigger fall in DPR levels.

For the PRRVAA mutation, again both sense and antisense DPRs showed a statistically significant fall in response to transfection with mutant helicases. For sense DPRs, mutant eIF4A1 led to a 55% reduction, while mutant eIF4A2 led to a 40% reduction. For antisense DPRs, levels fell by 60% for mutant eIF4A1 and 55% for mutant eIF4A2. This contrasts with Q339E/ Q340E mutants, where sense transcripts showed a greater DPR decrease than antisense transcripts. Overall, these experiments have not provided clear insight into whether sense DPR transcripts are easier to reduce than antisense, or if there is any meaningful difference. However, the observed difference in results does suggest some mechanistic differences in production of sense and antisense DPRs, otherwise it would be expected that a more similar decrease would be seen. The result is not consistent with the Q mutants in that eIF4A1 Q339E resulted in a greater decrease in sense DPRs than antisense; the opposite is true for eIF4A1 PRRVAA, where antisense DPRs showed a greater decrease than sense. Interestingly eIF4A2 did show consistency across mutations, with both mutations resulting in a larger decrease in antisense DPRs than sense. It could be that the actions of different RAN-associated RNA helicases impact sense and antisense DPR production differently in addition to there being mechanistic differences in how sense and antisense transcripts are translated.

As well as mutant helicases, cells were also transfected with normally functioning helicases. This therefore represented an over expression model, but no increase in DPR production was seen associated with these helicases. This data shows that although helicases are required for RAN translation, they are not the rate limiting step, as increasing their levels does not produce a corresponding increase in DPRs the way decreasing helicase activity decreases DPR production. This highlights the complex picture of RAN translation initiation, with its multiple key helicases and co-factors,

possible alterations in different cell types, and possible mechanistic alterations when processing sense and antisense repeat mRNA transcripts.

3.4.5 Limitations associated with protein detection by Western Blotting

Throughout this and subsequent chapters of this thesis, Western Blotting has been used to detect alterations in helicase protein levels resulting from shRNA-mediated partial knockdown. This technique has a number of potential technical pitfalls and limitations associated with it, which are outlined in several publications (Alegria-Schaffer et al., 2009) (Mahmood & Yang, 2012) (Ghosh et al., 2014) (Bass et al., 2017). Several of these limitations may be relevant to the work presented in this thesis. In figure 3.3, the knockdown of eIF4A2 appears smaller in magnitude than knockdown of other helicases. The band seen for eIF4A2 on the Western Blot is visibly larger than other helicases, however upon analysis the knockdown appears equivalent. Similarly in figure 3.11, the graph produced from quantifying the bands appears not to represent the original blot itself very well. In other blots, the band is slightly shifted from its expected location, despite a validated antibody showing specific detection of the protein. This raises questions about the method of signal detection and its reliability. A chemiluminescence signal has been used for detection in all blots shown in this thesis. Here, luminol is oxidised in the presence of an HRP enzyme, emitting light as a result (Alegria-Schaffer et al., 2009). Chemiluminescent signals strength and duration depends on various factors such as the ratio between enzyme and substrate, with over-application of the enzyme conjugate linked to signal variability or poor sensitivity. This can be a result of excessive enzyme causing a rapid signal peak and consequently the substrate is used up too quickly. Alternatively, HRP could become inactive due to prolonged exposure to the substrate, as an abundance of free radicals are produced during oxidation. These bind to HRP preventing its interaction with the substrate, also leading to poor signal quality (Alegria-Schaffer et al., 2009). Other researchers have highlighted the challenges and possible limitations of detecting high

and low protein concentrations on the same blot, which is likely to be highly relevant to this work (Pillai-Kastoori et al., 2020). Other issues associated with Western Blotting may include membrane saturation, where laying of abundant proteins causes underestimation of band strength, as the antibody may only be able to access and bind the top layer (Pillai-Kastoori et al., 2020). This may be a particularly relevant to this work as helicases are highly abundant. Chemiluminescent detection systems are also prone to inaccuracies through signal saturation, where band intensity exceeds the upper limits of the detection system. Expansion of this work should also consider alternatives to chemiluminescence, such as fluorescence, which may offer better reliability and overcome several problems associated with quantification of chemiluminescence (Bass et al., 2017; Janes, 2015; Pillai-Kastoori et al., 2020).

Even if the detection of Western Blot bands is optimal, methods to quantify the results may be responsible for the inconsistencies seen in some of the figures in this chapter. Several publications highlight potential problems with quantification (Bass et al., 2017), for example software detecting different amounts of protein producing the same signal intensity measurements (Bell, 2016). Methods suggested to overcome this include automated, systematic quantification systems, more intricate optimisation of other potential sources of variability during the Western Blot protocol, and use of alternative controls to housekeeping proteins, such as total cellular protein stains (Bass et al., 2017; Bell, 2016) (Pillai-Kastoori et al., 2020). Other researchers have suggested the use of dot blots or alternative figures to bar graphs to better present data showing quantification of Western Blots (Kroon et al., 2022). Other issues surrounding quantification of Western Blot data include normalisation of the target protein to loading controls. Here, the loading control may fail to produce signal intensity in proportion to protein abundance, therefore leading to inaccurate assessment of protein levels upon quantification (Pillai-Kastoori et al., 2020). Quantification methods may also fail to properly account for background signal, and newer analysis software and methods are currently in development to improve this (Bass et al., 2017) (Pillai-

Kastoori et al., 2020). It is essential that these strategies should be used for future work carried out beyond this thesis, to resolve the inconsistencies seen between Western Blots and their corresponding analysis. Further validation methods should also be used, such as the qPCR data shown in Figure 3.9, to confirm protein knockdown at the mRNA level and to support Western Blotting data. Alternatives to Western Blots should also be considered for future work on helicase knockdowns, with ELISAs, protein arrays or mass spectrometry suggested as a common method to quantify protein abundance (Bass et al., 2017)

4 Characterising the expression pattern of RAN translation factors at tissue and cellular levels in human post-mortem CNS

4.1 Introduction

4.1.1 Background, aims and rationale

The aim of this chapter was to use immunohistochemistry to characterise the distribution and localisation of RAN translation factors identified in the previous chapter in post-mortem human brain and spinal cord. The focus was on RAN-associated RNA helicases previously discussed: eIF4A1, eIF4A2, eIF4B and eIF4H. Additionally, SRSF1 was included due to its established role in the pathogenesis of the *C9ORF72* repeat expansion mutation. SRSF1 is required for export of repeat-containing mRNA transcripts from the nucleus to the cytoplasm (Hautbergue et al., 2017). Therefore, despite not being a helicase or other initiator of RAN translation, it is essential for RAN translation to occur, justifying its investigation as part of this thesis.

A key aim for this work was to identify any alterations in staining patterns of these proteins between ALS patient and neurologically normal control groups that may provide insight into their role in RAN translation in the context of C9-ALS/FTD, or to the pathogenesis of ALS more broadly. Further, this work aims to identify any alterations that may influence their suitability as therapeutic targets in C9-ALS/FTD. The majority of the existing research on members of the eIF4 family has come from the field of cancer biology, with little attention from neurodegenerative disease researchers. Several groups have published immunohistochemistry patterns of eIF4A1 in various types of cancer including oral squamous cell carcinoma (OSCC) (Zhao et al., 2019), colorectal cancer (Li et al., 2017) and breast cancer (Modelska et al., 2015). Here, eIF4A1 shows strong cytoplasmic staining with expression upregulated in tumours. eIF4A2 immunohistochemistry has been reported in lung

(Shaoyan et al., 2013) and breast (Yan et al., 2011) cancer as also highly expressed in the cytoplasm. In oesophageal cancer, eIF4A2 is also cytoplasmic with expression levels associated with clinical prognosis (Lyu et al., 2020). eIF4B also shows moderate cytoplasmic staining in breast cancer (Modelska et al., 2015). In lung tumours, eIF4H was highly expressed in contrast to low expression in healthy lung tissue, and had a cytoplasmic localisation (Vaysse et al., 2015).

Overall, the cancer literature points to eIF4 helicases as highly expressed in a wide range of tissue types, with a generally strong cytoplasmic staining pattern. SRSF1 immunohistochemistry has also been performed outside the field of neurodegeneration, for example in bronchi in asthma (Y.-I. Guo et al., 2022). Here, SRSF1 was highly expressed and localised predominantly to the nucleus. Although this study was carried out in a mouse model, and given the homology between mouse and human, one would anticipate this localisation pattern to be applicable to other human tissues. It is currently unknown to what extent eIF4A1, eIF4A2, eIF4B and eIF4H helicases are expressed in the brain, and if this is consistent across different brain regions, highlighting the need for further investigations. Importantly for this study, no work has yet been published exploring any alterations in relation to ALS. Several factors should be considered that could rule out targeting a particular RAN-associated RNA helicase as a viable therapeutic approach. For example, poor expression in a relevant brain area for ALS such as the motor cortex, or a mis-localisation associated with disease onset which may impact the function of the helicase. Overall, this work aims to address the lack of characterisation of these helicases in the brains of individuals who have died from ALS/FTD.

4.1.2 Post-mortem human tissue strategy

This work assessed three main features of eIF4A1, eIF4A2, eIF4B, eIF4H and SRSF1 in post-mortem human brain. First, the protein expression levels through western

blotting and immunohistochemistry across different brain regions. Second, immunohistochemistry to show protein distribution comparing ALS patient and control groups. Finally, protein localisation within neurons and glia in these brain regions comparing patient and control groups.

Although this work focused on the *C9ORF72* mutation, tissue from sporadic ALS and neurologically normal controls was also included to establish if any differences were specific to the *C9ORF72* mutation or applicable to ALS more broadly. The total cohort for immunohistochemistry included 16 cases: Five neurologically normal controls, five sporadic ALS and six C9-ALS/FTD. For western blotting, 12 cases were used, with four per diagnostic group. Wherever possible, tissue from the same individuals were used for both immunohistochemistry and western blotting. This cohort size was considered to be the smallest number of cases that were likely to show a true representation of staining patterns in the different diagnostic groups; a larger cohort would have been preferable but was not possible due to the precious nature and limited availability of this post-mortem tissue. For quantitative work, researchers were blinded to case diagnosis until analysis of this work was complete, to avoid introducing bias.

Brain regions were selected as most relevant in ALS/FTD: prefrontal and temporal lobes, hippocampus, and motor cortex in addition to the spinal cord. The cerebellum was also included as although this region has functions relating to fine motor control and motor co-ordination, it has generally been considered to be well preserved with minimal involvement in ALS (Prell & Grosskreutz, 2013). In the context of C9-ALS/FTD it contains DPR inclusions (Al-Sarraj et al., 2011) meaning any alterations to RAN-associated RNA helicase immunohistochemistry associated with this unique pathology can be identified. A summary of the brain regions used for immunohistochemistry is summarised in Figure 4.1.

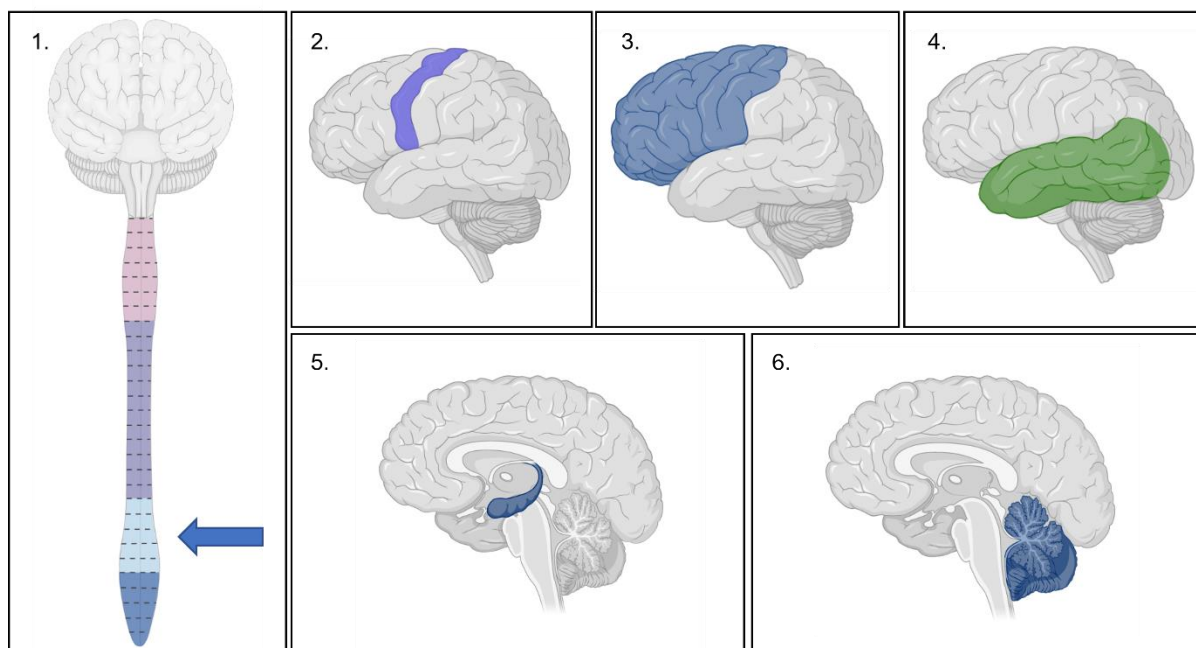


Figure 4.1 Summary of brain regions examined in post-mortem control, sporadic ALS and C9-ALS/FTD patients. Schematic diagrams showing the regions included in immunohistochemical staining for eIF4A1, eIF4A2, eIF4B, eIF4H and SRSF1. Numbers indicate the order in which staining was carried out. First, (1) Lumbar spinal cord; (2) Motor cortex; (3) Frontal Lobe; (4) Temporal Lobe; (5) Hippocampus; (6) Cerebellum.

Not all brain regions were examined in all cases. Spinal cord and motor cortex was examined in all 16 cases. However, the other regions (prefrontal cortex, temporal lobes, hippocampus and cerebellum) were limited to six cases in total. This comprised two controls, one sALS and three C9-ALS/FTD. Throughout immunohistochemistry experiments, a negative control was included where one case underwent the same protocol, but the primary antibody was not added.

4.2 Antibody Optimisation

All antibodies were optimised to find the most suitable conditions for immunohistochemistry. All antibodies were optimised using sporadic ALS parietal lobe, as such tissue is in relatively low demand from the brain bank. Antibody dilutions were initially selected based on manufacturer recommendations or published research.

Antigen retrieval was carried out in a pressure cooker at 120°C at 20 psi for 30 minutes in pH 6 buffer for all antibodies; pH 9 buffer and no antigen retrieval were also tested but deemed less suitable. Samples that did not undergo antigen retrieval had weaker staining patterns with poor contrast between the positive labelling and background than those that had received pressure cooker treatment. Appropriate conditions were selected with the help of a qualified neuropathologist; these are indicated in bold with an asterisk in Figures 4.2 to 4.6. Selection was based on which conditions gave the greatest contrast between the stained protein of interest and the background, where the positive labelling was strong enough to consistently detect without over-saturation that would have prevented identification of differences across cases and diagnostic groups.

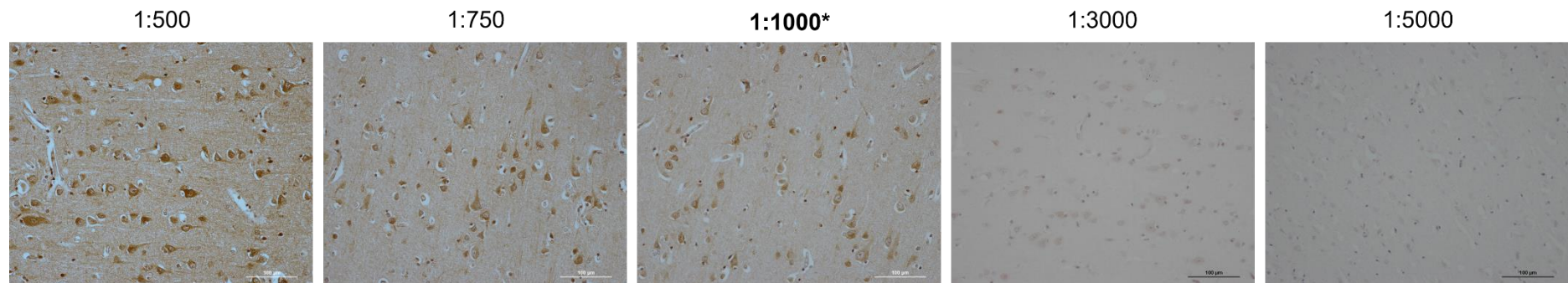


Figure 4.2 eIF4A1 antibody optimisation for IHC. FFPE sALS parietal lobe stained with Abcam eIF4A1 with antigen retrieval at pH 6; dilutions left - right 1:500, 1:750, 1:1000, 1:3000, 1:5000. Final selected dilution 1:1000. Magnification is 20x, scale bar = 100µm.

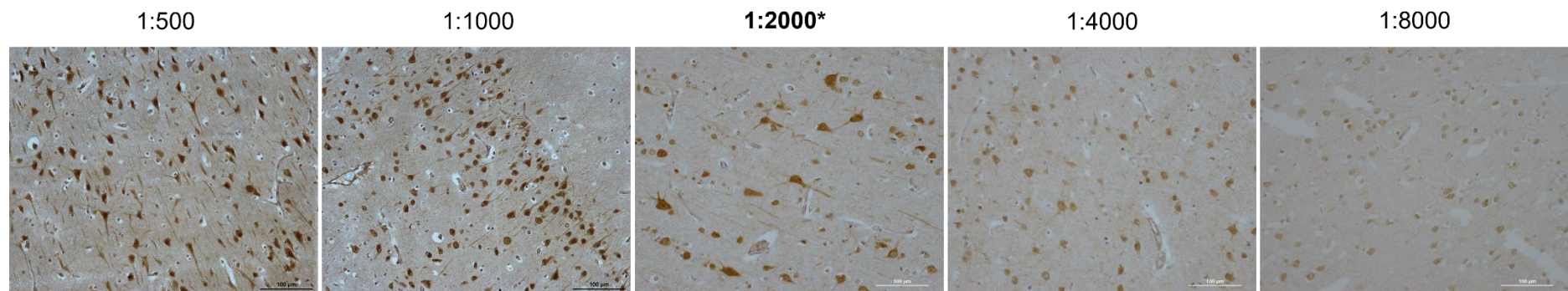


Figure 4.3 eIF4A2 antibody optimisation for IHC. FFPE sALS parietal lobe stained with Abcam eIF4A2 with antigen retrieval at pH 6; dilutions left - right 1:500, 1:1000, 1:2000, 1:4000, 1:8000. Final selected dilution 1:2000. Magnification is 20x, scale bar = 100µm.

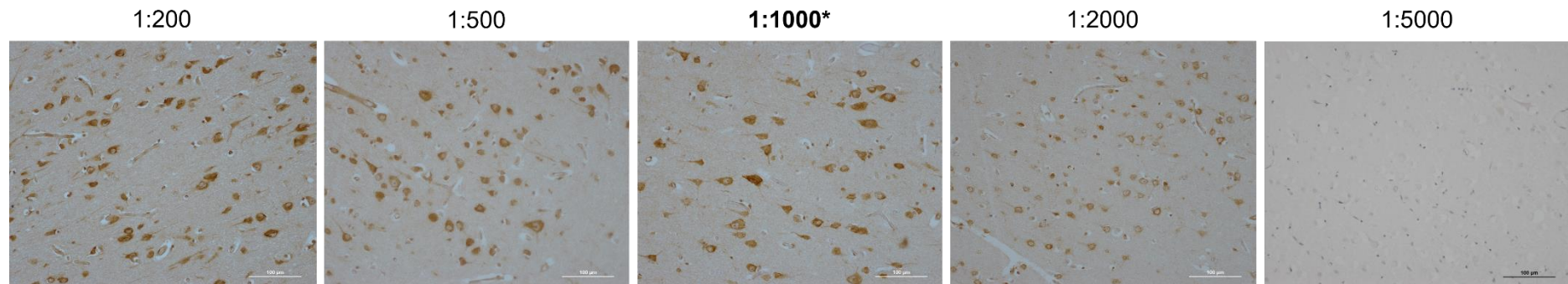


Figure 4.4 eIF4B antibody optimisation for IHC. FFPE sALS parietal lobe stained with Abcam eIF4B with antigen retrieval at pH 6; dilutions left - right 1:200, 1:500, 1:1000, 1:2000, 1:5000. Final selected dilution 1:1000. Magnification is 20x, scale bar = 100µm.

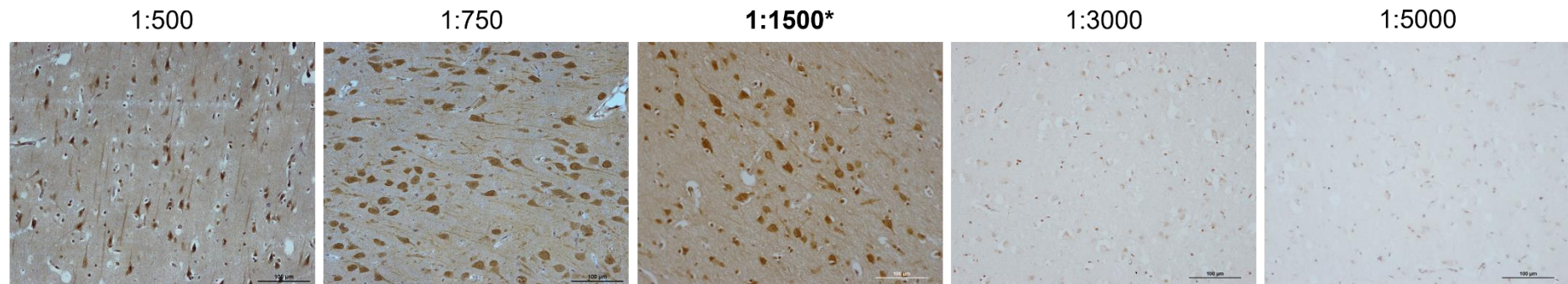


Figure 4.5 eIF4H antibody optimisation for IHC. FFPE sALS parietal lobe stained with Abcam eIF4H with antigen retrieval at pH 6; dilutions left - right 1:500, 1:750, 1:1500, 1:3000, 1:5000. Final selected dilution 1:1500. Magnification is 20x, scale bar = 100µm.

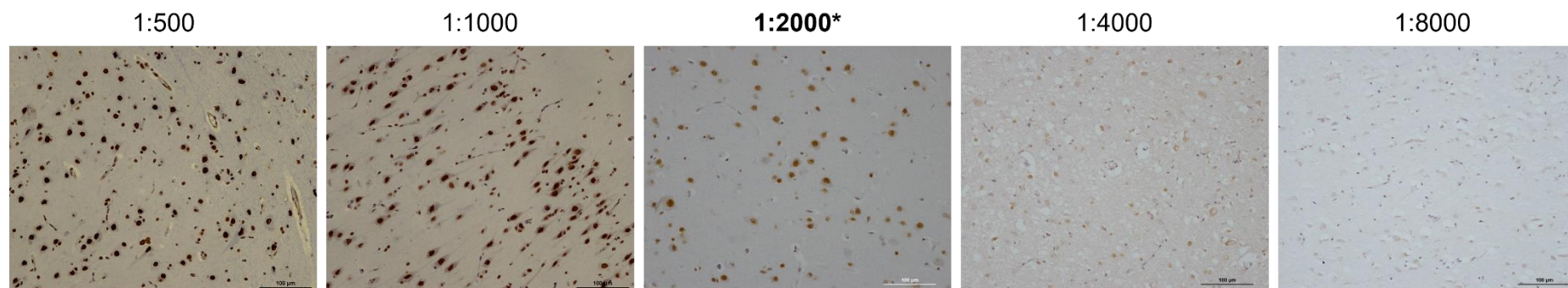


Figure 4.6 SRSF1 antibody optimisation for IHC. FFPE sALS parietal lobe stained with Abcam SRSF1 with antigen retrieval at pH 6; dilutions left - right 1:500, 1:1000, 1:2000, 1:4000, 1:8000. Final selected dilution 1:2000. Magnification is 20x, scale bar = 100µm

4.3 eIF4A1, eIF4A2, eIF4B, eIF4H, DHX36 and SRSF1 protein levels in human post-mortem tissue

Prior to immunohistochemistry, western blotting was used to establish protein expression levels in frozen spinal cord and motor cortex tissue. Blots were probed for the key helicases used in this study, eIF4A1, eIF4A2, eIF4B, eIF4H, DHX36, and splicing factor SRSF1. For eIF4B and SRSF1 two alternatives for primary antibodies were tested. For eIF4A1, eIF4A2, eIF4H and DHX36 only one antibody was available and/or no further testing was required as this antibody was found to work well. Probing for eIF4B and SRSF1 resulted in blank blots with no protein detectable by these antibodies; DHX36 was detectable in the motor cortex but not spinal cord.

Initial optimisation showed that unlike previous work on HEK293T cells, α -tubulin was not a suitable loading control and instead showed substantial variation between cases. Two alternatives were tried: TUJ1, and β -actin; both were deemed more appropriate loading controls for post-mortem tissue (Figure 4.7) as they produced less variation in protein expression between cases.

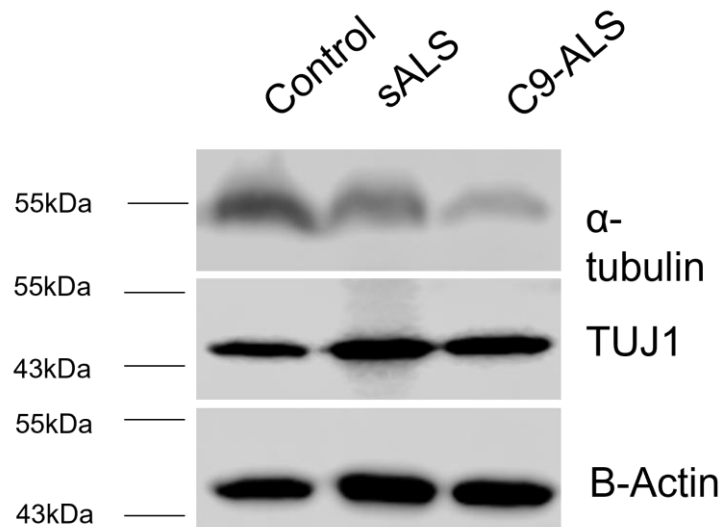


Figure 4.7 Trials of alternative loading controls in frozen post-mortem human spinal cord. 30μg protein loaded; blots were probed for α-tubulin, TUJ1 or β-actin and revealed consistent results with TUJ1 and β-actin but not α-tubulin. The expected molecular weight for α -tubulin and TUJ1 was 50-55 kDa, and for β -actin 42 kDa.

4.3.1 Western blots in frozen human sporadic and C9-ALS/FTD spinal cord

tissue

After establishing appropriate loading controls, western blots were first carried out on spinal cord tissue from control, sALS and C9-ALS/FTD patients. Spinal cord was chosen as it is considered one of the most biologically relevant areas in ALS, with the most pathology. Therefore, any alternations in RAN-associated RNA helicase expression between control and ALS cases was predicted to be most likely observable in this area. First, western blots were probed for eIF4A1 and eIF4A2 (Figure 4.8) with levels normalised to controls.

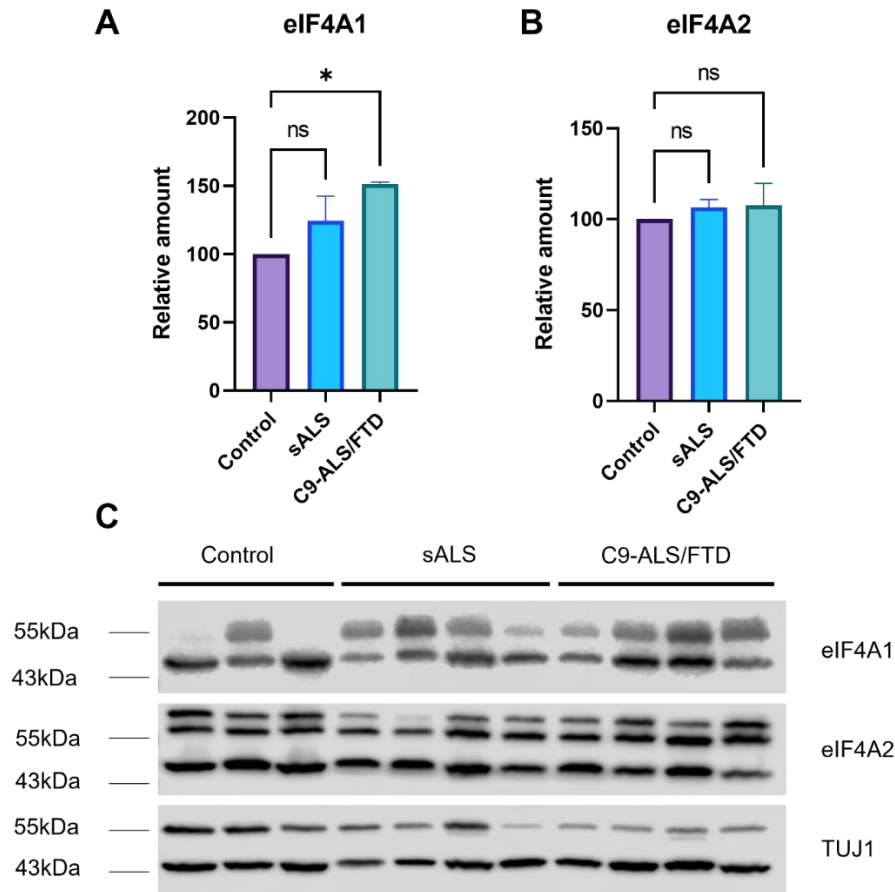


Figure 4.8 Western Blot from frozen post-mortem human spinal cord probed for eIF4A1 and eIF4A2. (A) shows eIF4A1 expression and (B) shows eIF4A2 expression, as a % relative to the control. Blots were quantified in independent biological triplicates, mean $\pm$ SEM, one-way ANOVA with Tukey's correction for multiple comparisons. NS = not significant, * = $p < 0.05$. (C) shows representative western blot, frozen post-mortem spinal cord, blots were probed for eIF4A1 and eIF4A2 with TUJ1 as a loading control. The expected molecular weight is 48 kDa for eIF4A1 and eIF4A2 and 50-55 kDa for TUJ1.

In spinal cord there was no difference in eIF4A2 expression between controls and ALS patient cases. For eIF4A1 greater expression was seen in both ALS groups compared to controls; this was statistically significant for C9-ALS/FTD but not for sALS.

Next, spinal cord western blots were probed for eIF4H (Figure 4.9). The same cases were used as in Figure 4.8, detailed in Table 2.22 and 2.23. Significantly less eIF4H was seen in ALS compared to controls with sALS and C9-ALS/FTD cases showing a 50% and 75% reduction respectively.

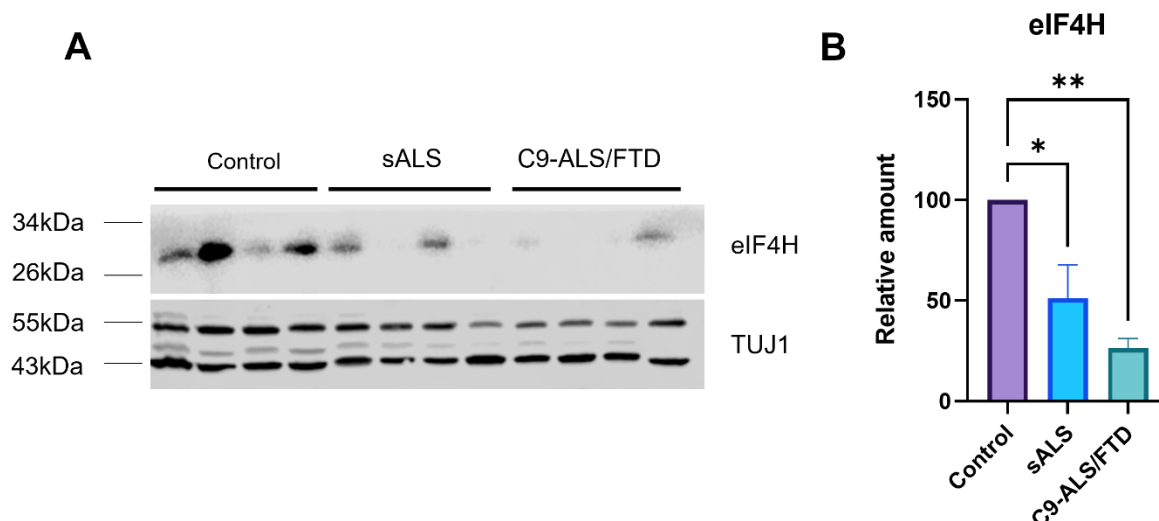


Figure 4.9 Western Blot from frozen post-mortem human spinal cord probed for eIF4H. (A) shows representative western blot from frozen post-mortem spinal cord, blots were probed for eIF4H with TUJ1 as a loading control. The expected molecular weight was 27kDa for eIF4H and 50-55 kDa for TUJ1. (B) shows eIF4H results quantified in triplicate expressed as % relative to control; Mean $\pm$ SEM, one-way ANOVA with Tukey's correction for multiple comparisons, * = $p < 0.05$, ** = $P < 0.01$.

4.3.2 Western Blots of helicases in frozen human sporadic and C9-ALS motor cortex

Detection of some proteins from spinal cord western blots proved challenging: eIF4B, DHX36, and SRSF1 were undetectable in this region with the available antibodies. Spinal cord is also precious and in high demand by ALS researchers. Therefore, motor cortex was next assessed to establish if results were consistent with spinal cord. eIF4B, SRSF1 and DHX36 detection in spinal cord may have failed due to lack of protein expression in this region, or it may be attributed to the antibodies tested which may not be able to detect their target proteins through western blot. A good antibody should recognise the target protein without displaying additional bands as a result of non-specific interactions. Some additional bands may also appear as a result of post-translational modifications so their appearance should not discount the use of an antibody. Many proteins show somewhat unexpected migration patterns on western blots, including in this thesis, so antibodies should also be able to detect these

adequately (Ghosh et al., 2014). A good antibody should also be able to detect low-expression proteins with high sensitivity. In post-mortem human tissue, there is an added complication of epitopes being masked by the structure of the tissue, which can hinder antibody performance, although antigen retrieval methods can help overcome this issue.

Blots were probed for helicases eIF4A1, eIF4A2, eIF4B, eIF4H and DHX36, as well as splicing factor SRSF1. eIF4B and SRSF1 remained undetectable by any of the antibodies available. Initially all blots were loaded with 30µg of total protein. To rule out very low protein expression preventing detection by western blot, a repeat was attempted with 100µg total protein loaded. eIF4B and SRSF1 blots remained blank with no signal detected with any available antibody. Blots were carried out in triplicate for eIF4A1, eIF4A2, eIF4H and DHX36 (Figure 4.10). For each diagnostic group, four cases were included, detailed in Table 2.22 and 2.23. In cases where multiple bands were seen, all were included during quantification of these blots.

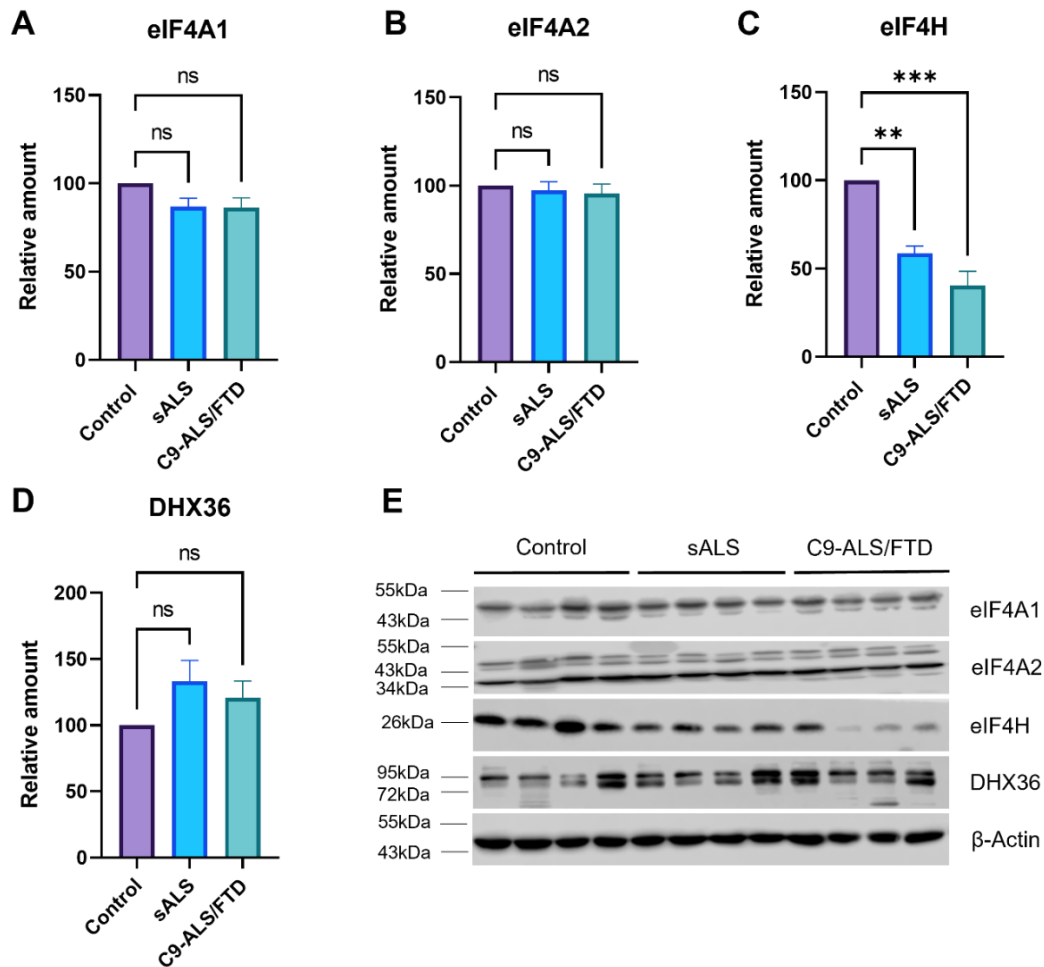


Figure 4.10 Western Blot from frozen post-mortem human motor cortex (A) shows eIF4A1, (B) shows eIF4A2, (C) shows eIF4H, (D) shows DHX36, quantified in triplicate expressed as % relative to control; Mean $\pm$ SEM, one-way ANOVA with Tukey's correction for multiple comparisons. NS = not significant, ** = $p < 0.01$, *** = $p < 0.001$. (E) shows representative western blot, frozen post-mortem motor cortex, blots were probed for eIF4A1, eIF4A2, eIF4H and DHX36 with TUJ1 as a loading control. The expected molecular weight was 48 kDa for eIF4A1 and eIF4A2, 27 kDa for eIF4H, 110kDa for DHX36 and 42kDa for β -actin.

Western blots from the motor cortex showed no statistically significant difference in eIF4A1 or eIF4A2 expression or banding pattern between control and ALS groups. Consistent with spinal cord, eIF4H motor cortex shows a significantly lower expression in both sALS and C9-ALS/FTD, with the latter showing the greatest deficit. For DHX36, while greater mean levels were seen in ALS groups compared to controls, neither was significant.

4.4 eIF4A1 and eIF4A2 immunohistochemistry in human brain and spinal cord

Immunohistochemistry was first performed for eIF4A1 and eIF4A2 as these have previously been identified as the key helicases involved in RAN translation initiation. First, spinal cord was stained before moving onto other brain regions: motor cortex, prefrontal and temporal lobes, hippocampus, and cerebellum. eIF4A1 and eIF4A2 staining was carried out side-by-side to detect any changes specific to either isoform, as they only differ by 10 amino acids.

4.4.1 eIF4A1 but not eIF4A2 forms neuronal cytoplasmic inclusions in spinal cord motor neurons of C9-ALS/FTD and sALS but not in control cases

In controls, eIF4A1 showed strong cytoplasmic staining in spinal cord motor neurons, while the nucleus and nucleolus remained unstained. eIF4A1-positive cytoplasmic inclusions were found in motor neurons throughout the spinal cord in every ALS case of both subgroups (Figure 4.11). Five sALS and six C9-ALS/FTD cases were examined in addition to five controls. No eIF4A1-positive cytoplasmic inclusions or other eIF4A1-positive pathology was detected in any of the control cases. eIF4A1 immunohistochemistry appeared to show a loss of cytoplasmic staining in inclusion-positive cells compared to those that were inclusion-negative, or in control cases. However, this was not quantified so cannot be definitively determined and was not shown in western blots from this region.

Spinal cord eIF4A2 immunohistochemistry showed very strong cytoplasmic motor neuron labelling; nuclei were negative for eIF4A2. Controls and ALS groups showed similar staining. No eIF4A2-positive inclusions were seen in any case (Figure 4.12).

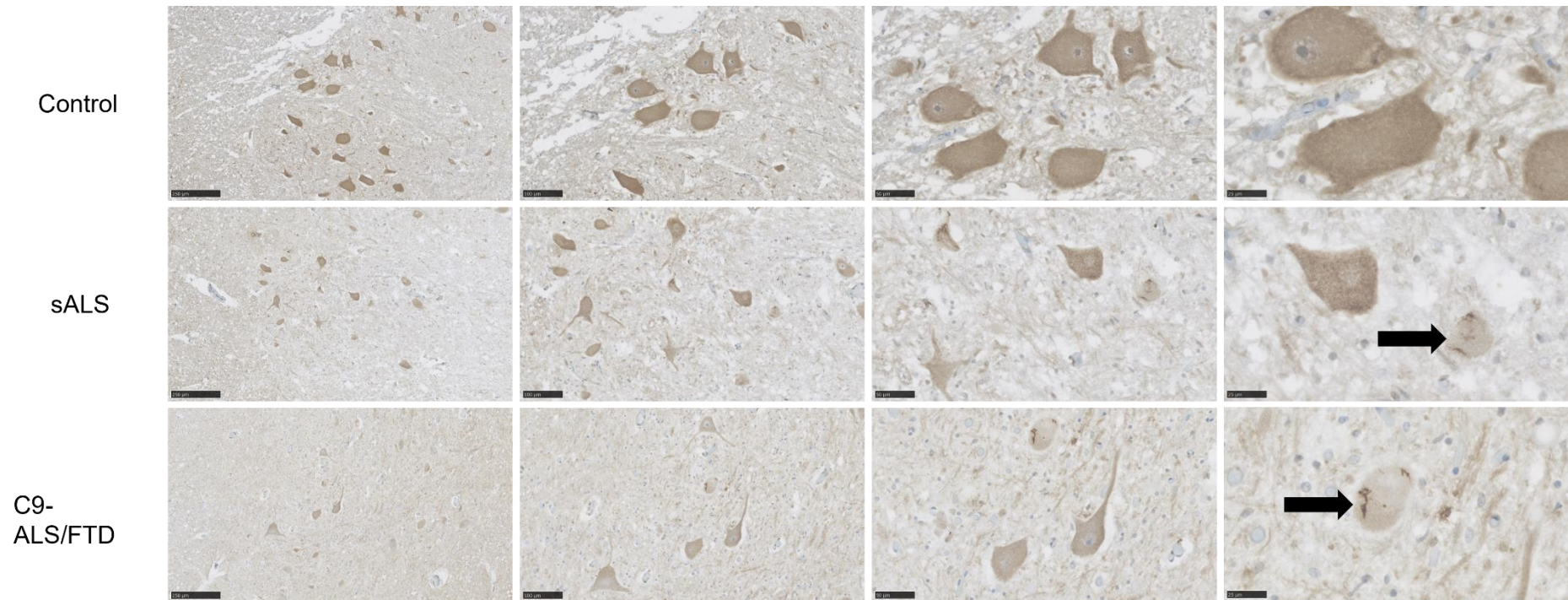


Figure 4.11 **eIF4A1 immunohistochemistry in control and ALS Spinal Cord.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4A1 showed positive cytoplasmic staining of varying intensity, and negative nuclear staining in spinal cord motor neurons. sALS and C9-ALS/FTD cases showed eIF4A1-positive neuronal cytoplasmic inclusions (black arrows) that were absent from controls. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

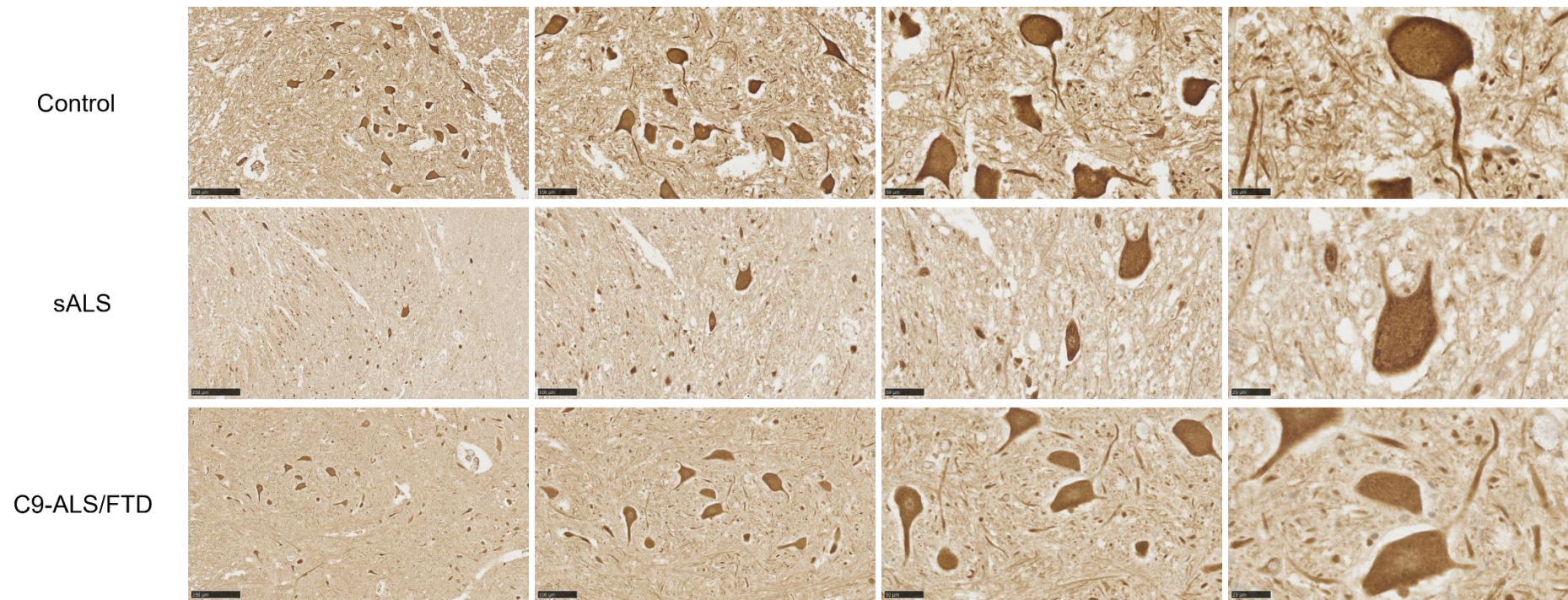


Figure 4.12 eIF4A2 immunohistochemistry in control and ALS Spinal Cord. Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4A2 showed strong staining of the cytoplasm and neuronal processes and negative nuclear staining in spinal cord motor neurons. No eIF4A2-positive inclusions were seen in any case. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

Inclusions that were positive for eIF4A1 were generally observed to be neuronal cytoplasmic inclusions with no evidence of nuclear or glial pathology observed in the cases included in this cohort. Inclusions consistently displayed a filamentous morphology, ranging from small, thin thread-like structures to very large, thick inclusions engulfing almost the entire stained cytoplasm of the neuron. While showing substantial variation in size was seen both within and between cases, there was no clear difference between sALS and C9-ALS/FTD groups overall in terms of inclusion burden, abundance, or morphology. Examples of sALS eIF4A1 positive inclusions are shown in Figure 4.13.

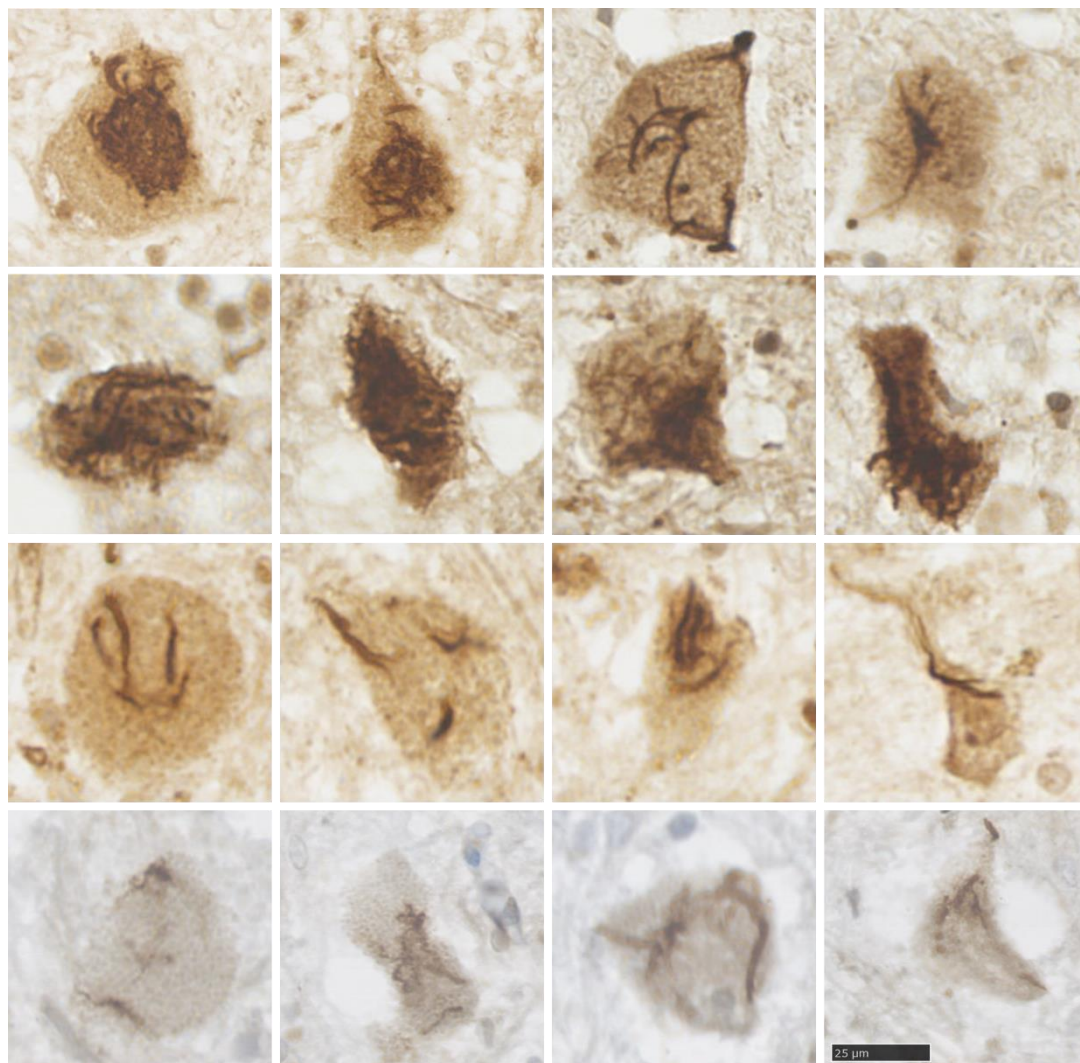


Figure 4.13 Immunohistochemistry showing eIF4A1-positive inclusions in sALS Spinal Cord. Images taken from each of the five sporadic ALS cases included in this study showing different eIF4A1-positive pathology. eIF4A1-positive inclusions in sALS are cytoplasmic, skein-like structures which vary from relatively small, thin threads to large aggregates filling the cytoplasm. Scale bar = 25μm.

Consistent with the variation seen in inclusion size, eIF4A1-inclusion positive neurons also varied in abundance between individuals. In the C9-ALS/FTD subgroup, one case in particular was noted to have very frequent eIF4A1 positive inclusions compared to others (LP041/2004). All eIF4A1-positive inclusions across the cohort were photographed and counted, and this case was an outlier in terms of raw numbers of inclusions identified. Figure 4.16 shows that an average of 7.81% of spinal cord motor neurons were inclusion-positive in C9-ALS/FTD cases, but in case LP041/2004 this was 17.58%. Interestingly, this case was observed to have very strong SRSF1-positive glial staining in the motor cortex compared to other cases, and one of the shortest disease durations of all cases in this study, at only 20 months. Examples of C9-ALS/FTD eIF4A1 positive inclusions are shown in Figure 4.14.

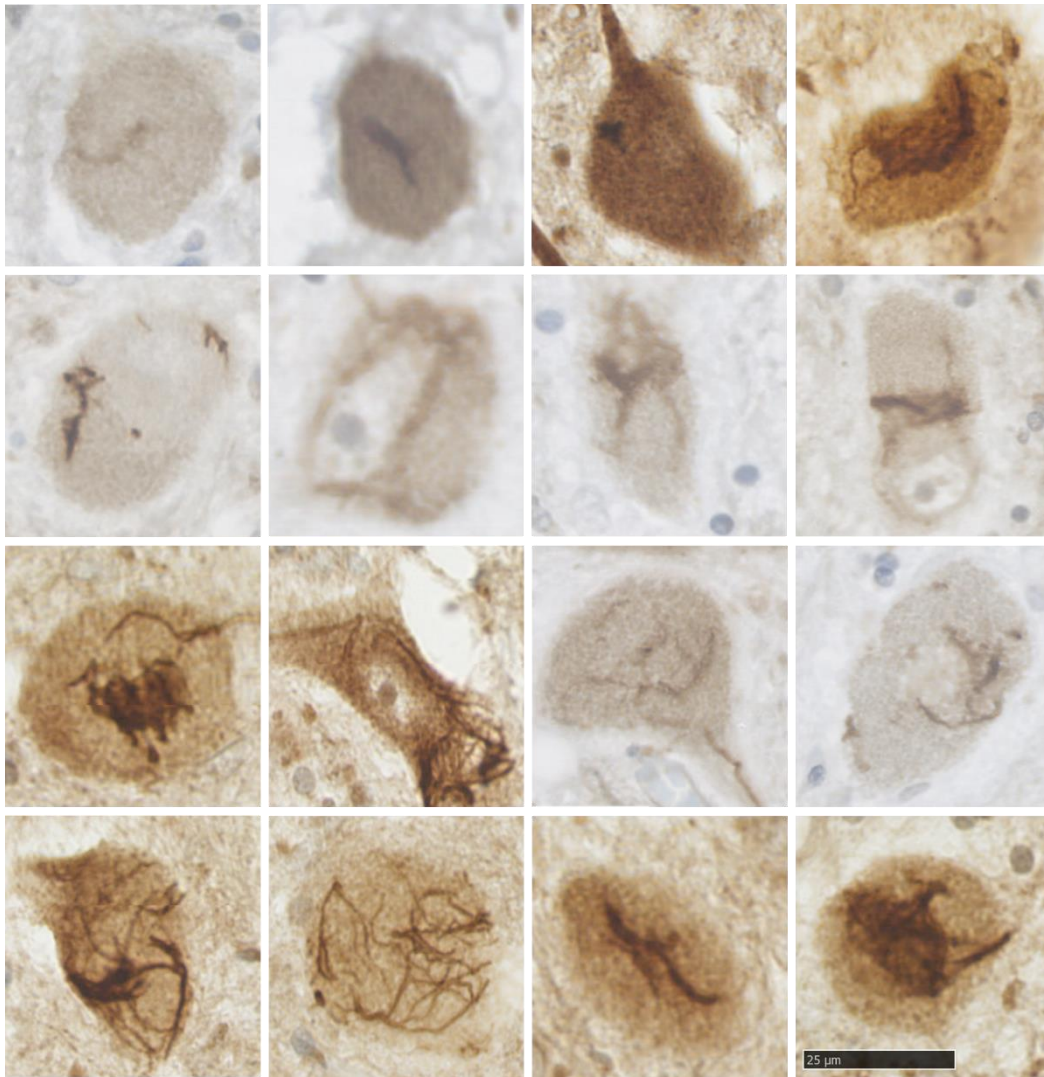


Figure 4.14 Immunohistochemistry showing eIF4A1-positive inclusions in C9-ALS/FTD Spinal Cord. Images taken from each of the six C9-ALS/FTD cases included in this study showing different eIF4A1-positive pathology. eIF4A1-positive inclusions in C9-ALS/FTD are cytoplasmic, skein-like structures which vary from relatively small, thin threads to large aggregates filling the cytoplasm. Scale bar = 25μm.

Next, the eIF4A1 pathology burden in C9-ALS/FTD and sporadic ALS spinal cord was assessed by counting the number of eIF4A1 inclusion-positive motor neurons and calculating this as a percentage of total motor neurons. Following eIF4A1 DAB immunostaining, each motor neuron was photographed and assessed as inclusion positive, negative, or ambiguous. Examples of ambiguous cells are shown in Figure 4.15.

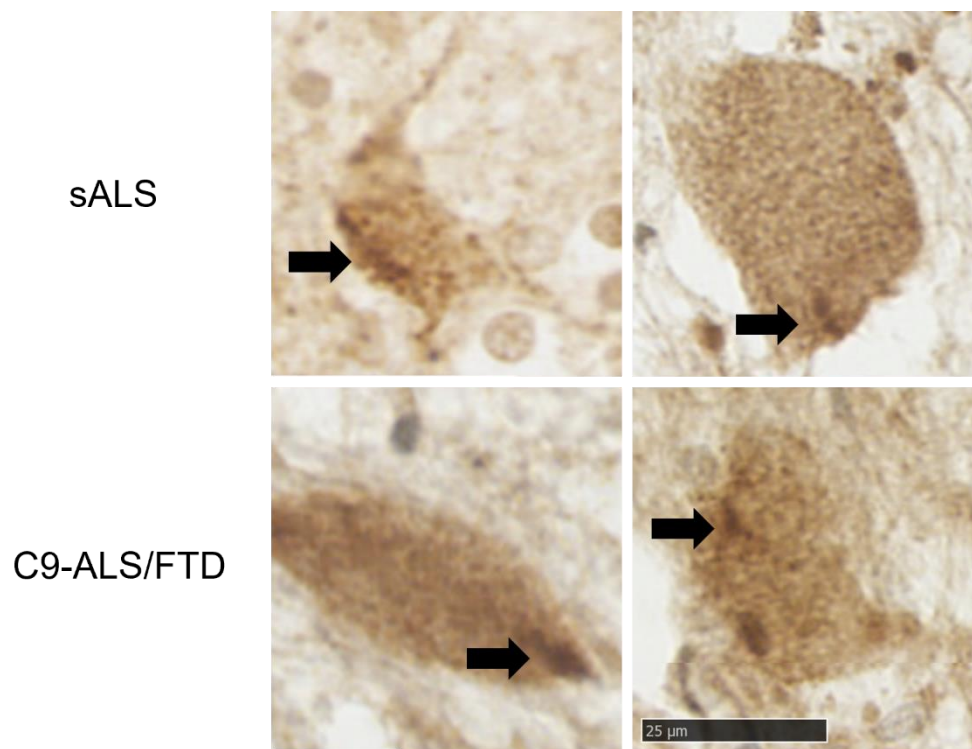


Figure 4.15 Immunohistochemistry in ALS Spinal Cord showing eIF4A1-positive ambiguous inclusions. Top row: sporadic ALS; bottom row: C9-ALS/FTD. Images from four cases showing examples of a subset of eIF4A1-positive inclusions that were identified as ambiguous in that they appeared as possibly pathological, but their morphology was not sufficient to class them as a full inclusion. Scale bar = 25μm.

Inclusions were counted by four independent, blinded operators. Motor neurons were identified with the help of a qualified neuropathologist and only included if they were longer than 25μm and/or had a visible nucleus. Inclusions were categorized as positive or ambiguous depending on the discretion of the operator, with operators agreeing prior to classification that inclusions would be classed as ambiguous if they were judged to be substantially smaller and lacked the defined structure of ‘certain’ inclusions. While this introduced a level of subjectivity into ratings, the use of four rather than only two operators and the level of consistency in ratings between them increased confidence in the scoring system. All blinded operators were in agreement that no eIF4A1-positive pathology was seen in any control case.

The total inclusion burden was broadly similar in both sALS and C9-ALS/FTD groups, with eIF4A1-positive pathology seen in an average of 8.04% of sporadic ALS, and 7.81% in C9-ALS/FTD spinal cord motor neurons. For sporadic ALS cases, this was subdivided into 5.15% for ‘certain’ and 2.89% for ‘ambiguous’ inclusions; for C9-ALS/FTD cases this was 5.65% and 2.16% respectively. There was a large range in inclusion burden within diagnostic groups for both sporadic ALS and C9-ALS/FTD cases, as shown in Figure 4.16.

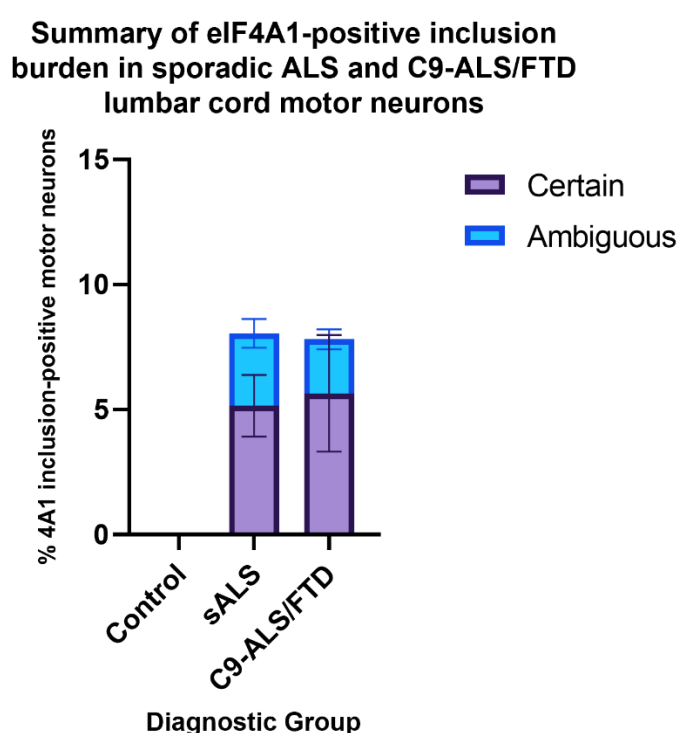


Figure 4.16 Summary of eIF4A1-positive inclusion burden in spinal cord motor neurons. Showing average inclusion burden for five sporadic ALS and six C9-ALS/FTD cases. Inclusion burden expressed as % of total spinal cord motor neurons with eIF4A1-positive pathology; inclusions scored ‘certain’ shown in purple, ‘ambiguous’ in blue.

4.4.2 Relationship between eIF4A1, phosphorylated TDP-43 and p62 pathology

It was observed that eIF4A1-positive inclusions resembled the hallmark pathology in ALS, that of phosphorylated TDP-43. It has been widely reported in the literature that TDP-43 inclusions are able to sequester other proteins. The next aim was to determine

if this was the case for eIF4A1 inclusions, or if they were formed independently of TDP-43 pathology. An initial way to provide evidence to answer this question is to establish the extent of co-localisation between the two pathologies.

First, two TDP-43 antibodies were tested. Appropriate dilutions and antigen retrieval conditions had previously been optimised by other laboratory members (Figure 4.17). Both had manufacturer's guidelines stating they detect phosphorylated TDP-43 as opposed to C-terminal or pan-TDP-43. The first was a rabbit polyclonal IgG antibody from Affinity Biosciences, which binds to phosphorylated serine residue 409. The second was a rat monoclonal IgG2a antibody, from Biolegend, which also binds to a region including phosphorylated serine residues 409 and 410. It was determined that the rat antibody, shown in the bottom panel of Figure 4.17, was more suitable: Staining with this antibody was more specific while the rabbit antibody, shown in the top panel, more closely resembled a pan-TDP-43 antibody with higher background staining rather than detecting only the phosphorylated form of TDP-43.

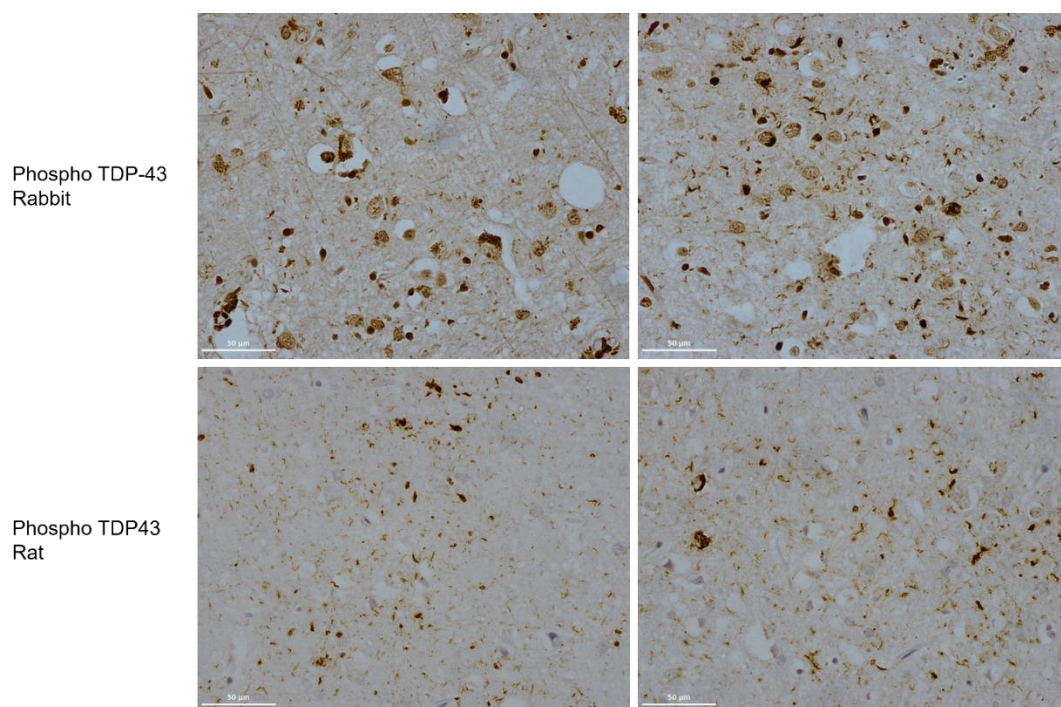


Figure 4.17 Immunohistochemistry for alternative phosphorylated TDP-43 antibodies: Spinal cord tissue confirmed positive for TDP-43 pathology was incubated with rabbit (top panels) or rat (bottom panels) phosphorylated TDP-43 primary antibodies. Scale bar = 50µm.

An additional aim was to determine if the pathological eIF4A1 inclusions were positive for p62. Therefore, a p62 antibody was also tested that had previously been optimised by other laboratory members. Both dilution and specificity of the p62 antibody was suitable for double staining with eIF4A1 (Figure 4.18). P62 immunohistochemistry have previously been performed in the ALS spinal cord, and was found to form a number of inclusion types in motor neurons including skein, basophilic and Lewy body-like inclusions (Mizuno et al., 2006).

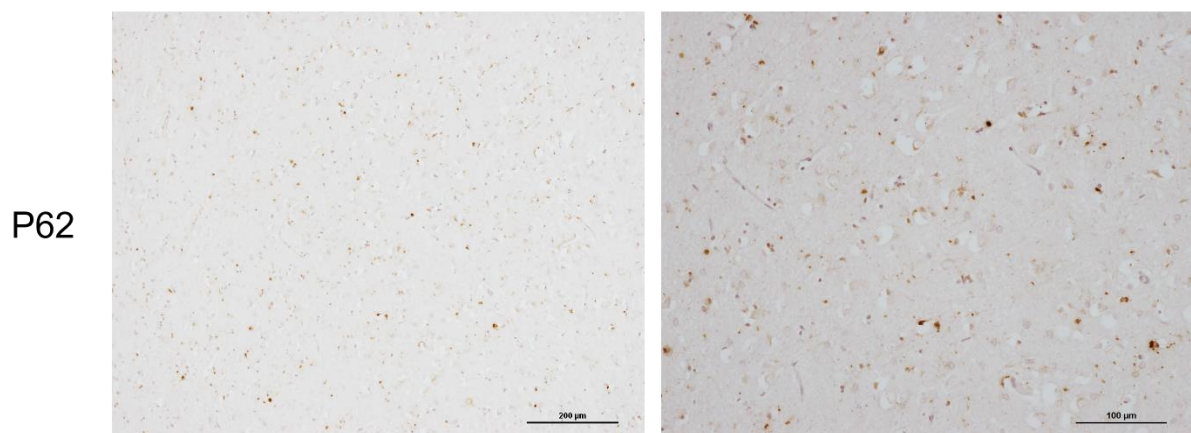


Figure 4.18 Immunohistochemistry to test p62 antibody specificity and dilution staining was carried out on spinal cord tissue confirmed positive for ALS pathology. Scale bar = 200µm (left) and 100µm (right).

Double staining of eIF4A1 and phosphorylated TDP-43 was carried out first using DAB (brown) and the enzyme alkaline phosphatase (pink) respectively on sporadic and C9-ALS/FTD cases (Figure 4.19).

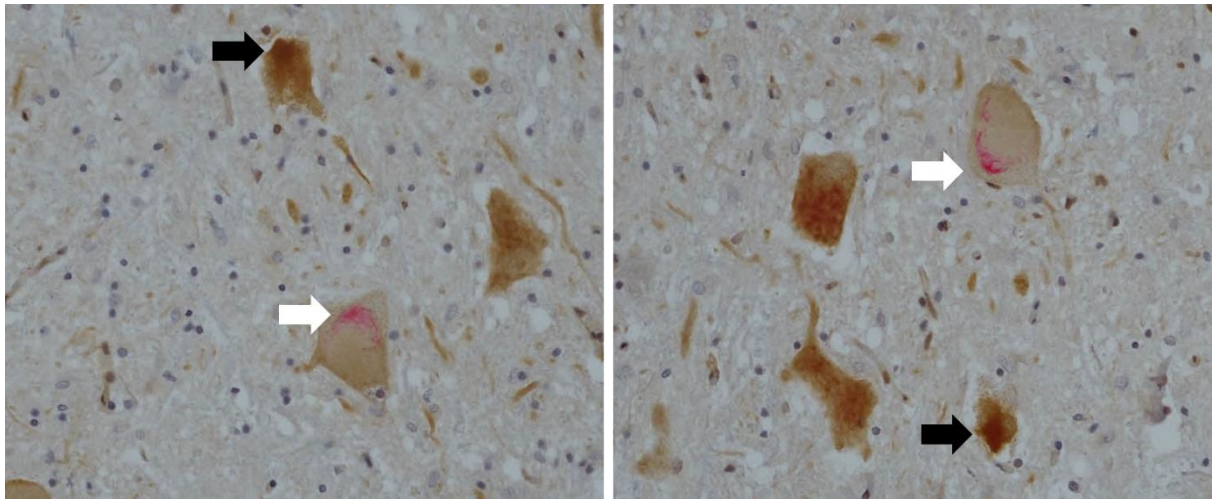


Figure 4.19 Double Immunohistochemistry of eIF4A1 and phosphorylated TDP-43. Extent of co-localisation of eIF4A1 (DAB, brown) and phosphorylated TDP-43 (alkaline phosphatase, pink) pathology is shown here in C9-ALS/FTD spinal cord. Black arrows show eIF4A1-positive pathology which could not be confirmed as TDP-43 positive or negative; white arrows show TDP-43-positive eIF4A1-negative pathology. Magnification = 40x.

Initial double staining revealed neurons with TDP-43-positive and eIF4A1-negative inclusion pathology. With this combination of antibody and chromogen, it could not be determined if eIF4A1-positive inclusions are also TDP-43 positive, indicating co-localisation between the pathologies, or negative. Therefore, the next strategy chosen was to switch the HRP and alkaline phosphatase staining, to see if pathology could be detected that was eIF4A1-positive and TDP-43 negative (Figure 4.20).

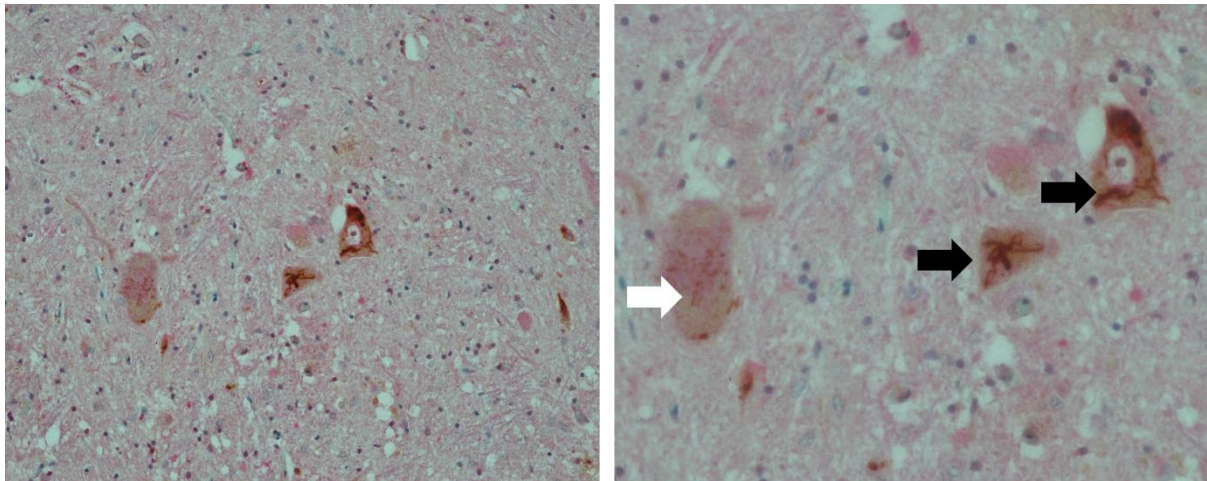


Figure 4.20 Double Immunohistochemistry of eIF4A1 and phosphorylated TDP-43. Extent of co-localisation of eIF4A1 (alkaline phosphatase, pink) and phosphorylated TDP-43 (DAB, brown) pathology is shown here in C9-ALS/FTD spinal cord. This revealed eIF4A1-positive, TDP-43 negative pathology (white arrows) and phosphorylated TDP-43 inclusions (black arrows) which could not be determined as eIF4A1-positive or negative. Magnification = 20x (left) 40x (right).

Figure 4.20 revealed inclusions that were positive for eIF4A1 (alkaline phosphatase) but negative for TDP-43 (DAB). TDP-43 inclusion-positive cells cannot be determined as eIF4A1-positive or negative with this combination of chromogens and antibodies. Inclusions can be seen with pink alkaline phosphatase staining positive for TDP-43 or eIF4A1, as shown in Figure 4.19 and 4.20. These results indicate that eIF4A1 pathology may be able to form independently of TDP-43. However, because brown DAB staining is always stronger and obscures the pink, co-existing pathology can't be determined using this combination of TDP-43/eIF4A1 and DAB/alkaline phosphatase. In order to further understand the relationship between eIF4A1 and TDP-43 inclusions, both pathologies were next assessed in the same cell.

Spinal cord tissue was stained with ImmPact for the first primary antibody, resulting in a red stain, and then slides were scanned using the Hamamatsu Slide Scanner. The coverslip was then removed, the stain dissolved, and DAB staining was used for the second primary antibody, resulting in a brown stain. The slides were then scanned for a second time. Scanned images were examined side-by-side, to show the presence or absence of both pathologies in an individual cell.

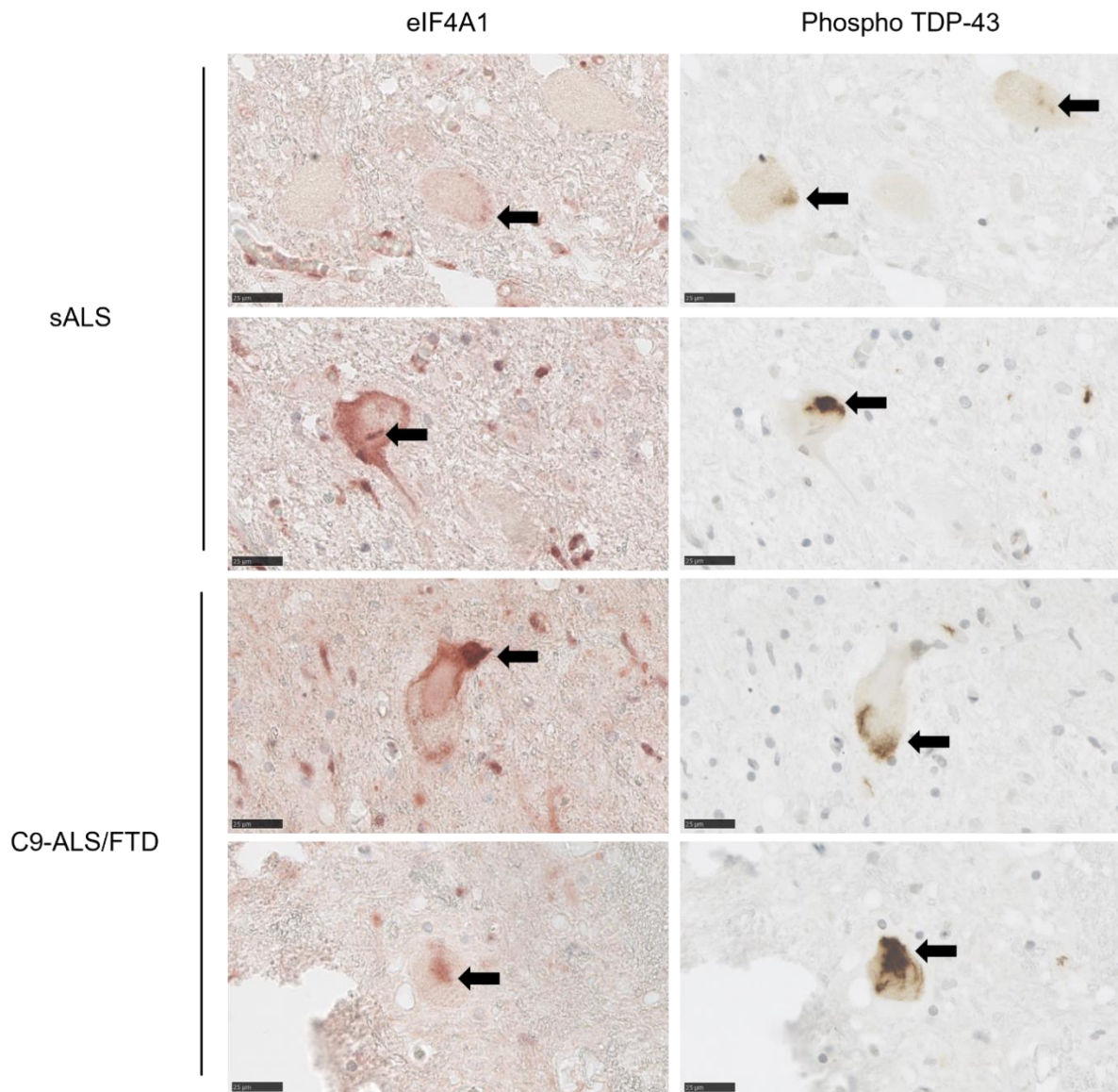


Figure 4.21 Double immunohistochemistry of eIF4A1 and phosphorylated TDP-43 positive pathologies in the same cell. Top panels: ALS spinal cord, bottom panels: C9-ALS/FTD spinal cord. eIF4A1 (ImmPact, red) and phosphorylated TDP-43 (DAB, brown) show instances of separate neurons with eIF4A1-positive, TDP-43 negative pathology or vice versa (black arrows, top panel). Some neurons show both pathologies in the same cell with no apparent co-localisation (black arrows, panels 2 and 3). In other examples co-localisation occurs between eIF4A1 and TDP-43 positive pathology (black arrows, bottom panel). Scale bar = 25µm.

ImmPact staining of eIF4A1 produced a red cytoplasmic stain of varying intensity equivalent to what was previously seen with DAB immunostaining for this helicase. DAB staining of phosphorylated TDP-43 produced weak diffuse cytoplasmic staining with clear pathological inclusions. However, it was observed that eIF4A1 pathology

was not detected to the same extent with ImmPact as with DAB staining; while present, it was generally weaker than shown initially with DAB staining.

While some instances of co-localisation between eIF4A1 and TDP-43 positive inclusions was seen (Figure 4.21, bottom panel), many neurons were positive for TDP-43 pathology with no evidence of eIF4A1 pathology, or vice versa i.e. eIF4A1-positive, TDP-43 negative pathology. Often, inclusions appeared to form in separate cells (Figure 4.21, top panel), although it can't be ruled out that they may be out of the plane of this section and therefore undetectable. In some neurons, both inclusion types occurred in the same cell, but there was no apparent co-localisation. The inclusions were in different areas of the cytoplasm (Figure 4.21, middle panels). There were no differences in patterns of eIF4A1 and TDP-43 co-localisation in sALS and C9-ALS/FTD groups. Overall, this demonstrated variation in co-localisation patterns between eIF4A1 and TDP-43 pathologies, but importantly it showed the eIF4A1 inclusions were able to form independently of TDP-43 inclusions.

Following eIF4A1 and phosphorylated TDP-43 double staining, eIF4A1 and p62 double staining were performed. For this, ImmPact / DAB were used as opposed to alkaline phosphatase / DAB so pathology could be assessed in the same cell. As eIF4A1 pathology was much more easily detectable using DAB, all spinal cord sections were stained first using ImmPact to detect p62 then used DAB to detect eIF4A1 (Figure 4.22).

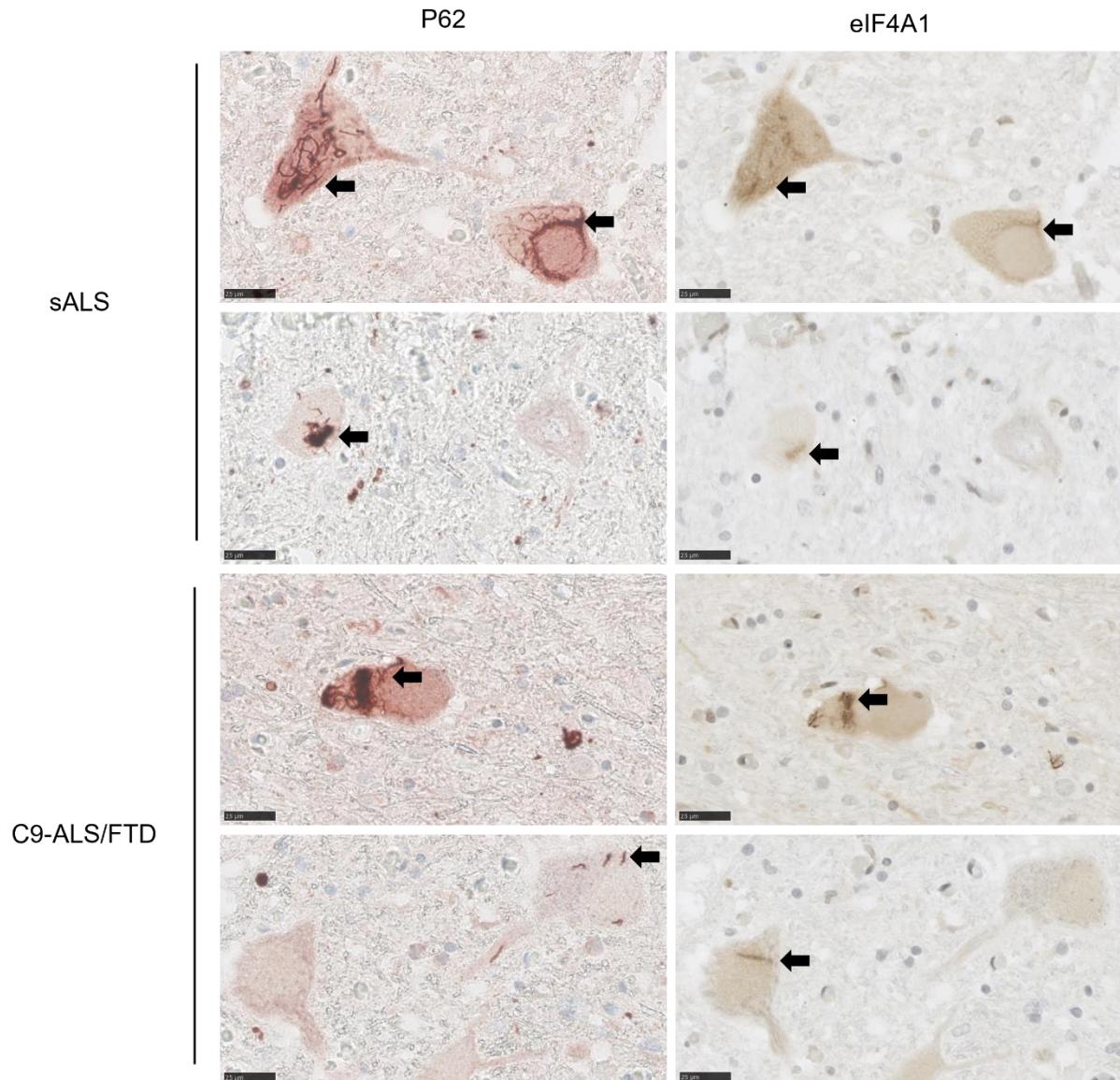


Figure 4.22 Double immunohistochemistry of eIF4A1 and p62-positive pathologies in the same cell. Top panels: ALS spinal cord, bottom panels: C9-ALS/FTD spinal cord. eIF4A1 (DAB, brown) and p62 (ImmPact) show generally good co-localisation between pathology types (black arrows, panels 1-3). However some rare examples were seen of non-overlapping pathology formed in different cells with no evidence of co-localisation (black arrows, bottom panel). Scale bar = 25µm.

In sALS and C9-ALS/FTD, there is strong co-localisation between p62 and eIF4A1 inclusions. Although the majority of eIF4A1 inclusions in sALS and C9-ALS/FTD spinal cord were found to be p62-positive, some irregular examples were also seen in particular in C9-ALS/FTD cases. These included neurons that had eIF4A1-positive, p62-negative inclusions and vice versa (Figure 4.22, bottom panel).

4.5 Helicase immunohistochemistry in control and ALS brains

Following investigations into eIF4A1 in spinal cord, immunohistochemistry was performed in other brain regions: the motor cortex, prefrontal and temporal lobes, hippocampus, and cerebellum. Helicases included in these studies were eIF4A1, eIF4A2, eIF4B, and eIF4H. For eIF4A1 and eIF4A2 immunohistochemistry, the spinal cord and motor cortex, five controls, five sALS and six C9-ALS/FTD cases were examined; for all other regions two controls, one sALS and three C9-ALS/FTD cases were examined. For eIF4B and eIF4H immunohistochemistry, two controls, one sALS and three C9-ALS/FTD cases were examined for all regions.

In brief, eIF4A1 positive inclusions were not observed in any other brain regions than the spinal cord in any ALS or control case examined. No inclusions positive for eIF4A2, eIF4B or eIF4H were found in any region. Helicase staining patterns vary in intensity, but generally were found to show positive labelling in the cytoplasm with variable nuclear labelling. Overall, few differences were observed between control and ALS groups, those that were are noted in Table 4.1, which summarises immunohistochemistry results for each helicase across the six regions. Immunohistochemistry images for eIF4A1, eIF4A2, eIF4B and eIF4H can be found in the appendix section A in Figures 8.1 – 8.26.

Table 4.1 Summary of helicase immunohistochemistry across the spinal cord and CNS regions

	Spinal Cord	Motor Cortex	Frontal Lobe	Temporal Lobe	Hippocampus	Cerebellum
eIF4A1	Cytoplasmic and nuclear labelling of neurons, with eIF4A1 positive cytoplasmic inclusions in sALS and C9-ALS/FTD but not controls	Cytoplasmic and variable nuclear neuronal staining with some extension into processes; variable glial labelling	Weak cytoplasmic labelling with negative nuclear labelling in all cortical layers	Weak cytoplasmic labelling with negative nuclear labelling in all cortical layers	Positive labelling throughout CA1 – CA3 and the dentate gyrus. Clear cytoplasmic and negative nuclear labelling of CA4 hippocampal neurons	Weak cytoplasmic staining in Purkinje cells with some extension into processes and generally negative nuclei
eIF4A2	Strong labelling of neuronal cytoplasm and processes	Cytoplasmic and axonal neuronal labelling throughout all cortical layers; positive glial nuclei labelling	Cytoplasmic labelling of neurons in all cortical layers. Processes and blood vessel endothelial cells also show positive staining.	Cytoplasmic labelling of neurons in all cortical layers. Processes and blood vessel endothelial cells also show positive staining.	strong eIF4A2 staining was seen in CA1, CA2, CA3 pyramidal neurons and the dentate gyrus. CA4 hippocampal neurons showed strong cytoplasmic staining and negative nuclear staining with some labelling of processes. Strong labelling was also seen throughout the granule cell layer of the hippocampus	cytoplasmic eIF4A2 labelling was seen in Purkinje neuron cell bodies and processes
eIF4B	Very weak staining in the neuronal cytoplasm with generally no labelling of the nucleus	Weak staining of neuronal cytoplasm in all cortical layers. Negative neuronal nuclear labelling, with weak axonal staining and some positive glial cell nuclei	Neurons in all cortical layers showed weak cytoplasmic labelling and variable nuclear staining. Observation of slightly stronger neuronal labelling in sALS and C9-ALS/FTD cases compared to controls, where nuclear labelling was generally absent	Positive cytoplasmic labelling, which appeared to be stronger in intensity than staining in the prefrontal lobe, and variable nuclear labelling	Staining was minimal in CA1, CA3 and CA4 regions; staining intensity was slightly greater in the CA2 region. CA4 hippocampal neurons showed a weak cytoplasmic staining pattern, with no labelling of the nucleus, neuronal processes, or glia. There was very weak labelling of cells of the dentate gyrus granule cell layer.	Positive labelling was seen to a moderate strength in the Purkinje cell cytoplasm with limited extension into processes; staining was negative in the nucleus and weak in the granular layer.
eIF4H	Neurons of the dorsal and ventral horns were positive, with variable labelling of the nucleus. Positive labelling of neuronal processes, axons and glial cell nuclei were also seen.	Cytoplasmic labelling and variable nuclear labelling was seen in neurons throughout all cortical layers, with variable glial cell labelling	strong cytoplasmic and weak or absent nuclear labelling is seen in neurons in all cortical layers	strong cytoplasmic and weak or absent nuclear labelling is seen in neurons in all cortical layers	Positive labelling of cells throughout Ammon's horn and the dentate fascia. CA4 hippocampal neurons showed strong cytoplasmic and nuclear labelling	Weak-moderate cytoplasmic and nuclear staining was seen in Purkinje cells, with variable staining of the granular layer

4.6 SRSF1 immunohistochemistry in control, sporadic ALS and C9-ALS/FTD

post-mortem brain and spinal cord regions

Next, brain and spinal cord regions were stained for SRSF1. In the spinal cord and motor cortex, five controls, five sALS and six C9-ALS/FTD cases were examined; for all other regions two controls, one sALS and three C9-ALS/FTD cases were examined. SRSF1 generally showed strong neuronal nuclear labelling with negative cytoplasmic labelling. Figures summarising SRSF1 immunohistochemistry in the motor cortex, prefrontal cortex, temporal lobe, hippocampus and cerebellum are shown in the appendix section A in Figure 8.27 – 8.31.

In sALS and C9-ALS/FTD groups, SRSF1-positive glial labelling was seen in grey and white matter regions of the spinal cord, indicated by blue arrows in Figure 4.23. This labelling was seen in three of five sALS cases and four of six C9-ALS/FTD cases examined, but in none of the five control cases. The glial cell morphology suggests staining was seen in astrocytes and possibly microglia. Extensive staining of the neuropil was also seen, while controls showed no neuropil or glial labelling.

This pattern of SRSF1 glial labelling continued in other brain regions examined. In C9-ALS/FTD cases, strong SRSF1-positive cytoplasmic glial labelling was seen in large patches in all layers of the motor cortex, with a morphology suggestive of astrocytes (Figure 4.24). In total, motor cortex from five out of six C9-ALS/FTD cases showed evidence of SRSF1 glial cell labelling. Motor cortex from five controls and five sALS cases was also examined and no evidence of glial cell staining by SRSF1 was seen in any of them.

In the white matter underlying the motor cortex, some similar glial cell labelling was seen, this time in both sALS and C9-ALS/FTD cases (Figure 4.25). No equivalent

staining was seen in any of the five controls. The glial cell morphology suggests SRSF1-positive staining was again seen in astrocytes. While there was some glial staining in sALS white matter (in contrast to the motor cortex, where there was none) the glial expression was greater in extent in C9-ALS/FTD than in sALS. For sALS white matter, two out of five cases showed strong evidence of SRSF1-positive glial staining, two had limited weak labelling, and one had none. In C9-ALS/FTD, six cases were examined and five showed SRSF1-positive glial cell labelling.

Similar to the motor cortex, underlying white matter regions of the prefrontal cortex (Figure 4.26) showed SRSF1-positive glial cell staining in sALS and C9-ALS/FTD cases, albeit more subtle than in the motor cortex. The glial cell morphology again suggests the staining was seen in astrocytes. In controls, one case of the two examined showed similar glial staining in white matter, but the intensity was weaker and more restricted in the area affected. In C9-ALS/FTD, prefrontal lobe SRSF1 white matter staining was greater than sALS cases.

In white matter underlying the temporal lobe (Figure 4.27) both sALS and C9-ALS/FTD showed SRSF1-positive glial cell labelling that was absent in controls. The morphology suggested SRSF1 labels astrocytes. Only two controls, one sALS and three C9-ALS/FTD cases were examined in these regions, limiting any conclusions that can be drawn. The sALS case showed consistent SRSF1-positive glial cell staining in underlying white matter of the prefrontal cortex and temporal lobes. In the three C9-ALS/FTD cases, two cases showed similar glial labelling across both areas, while one case showed no evidence of positive SRSF1 glial cell labelling in any area.

In the CA4 subfield, hippocampal neurons showed a strong nuclear and negative cytoplasmic SRSF1 staining pattern. SRSF1 also shows very subtle labelling of glial cells in this region in controls and ALS cases (Figure 4.28). The glial cell morphology again suggested astrocyte labelling. In the cerebellum, control and ALS cases of both

groups showed strong nuclear labelling of SRSF1 in Purkinje cells and some granule cells while the cytoplasm and cell processes were negative.

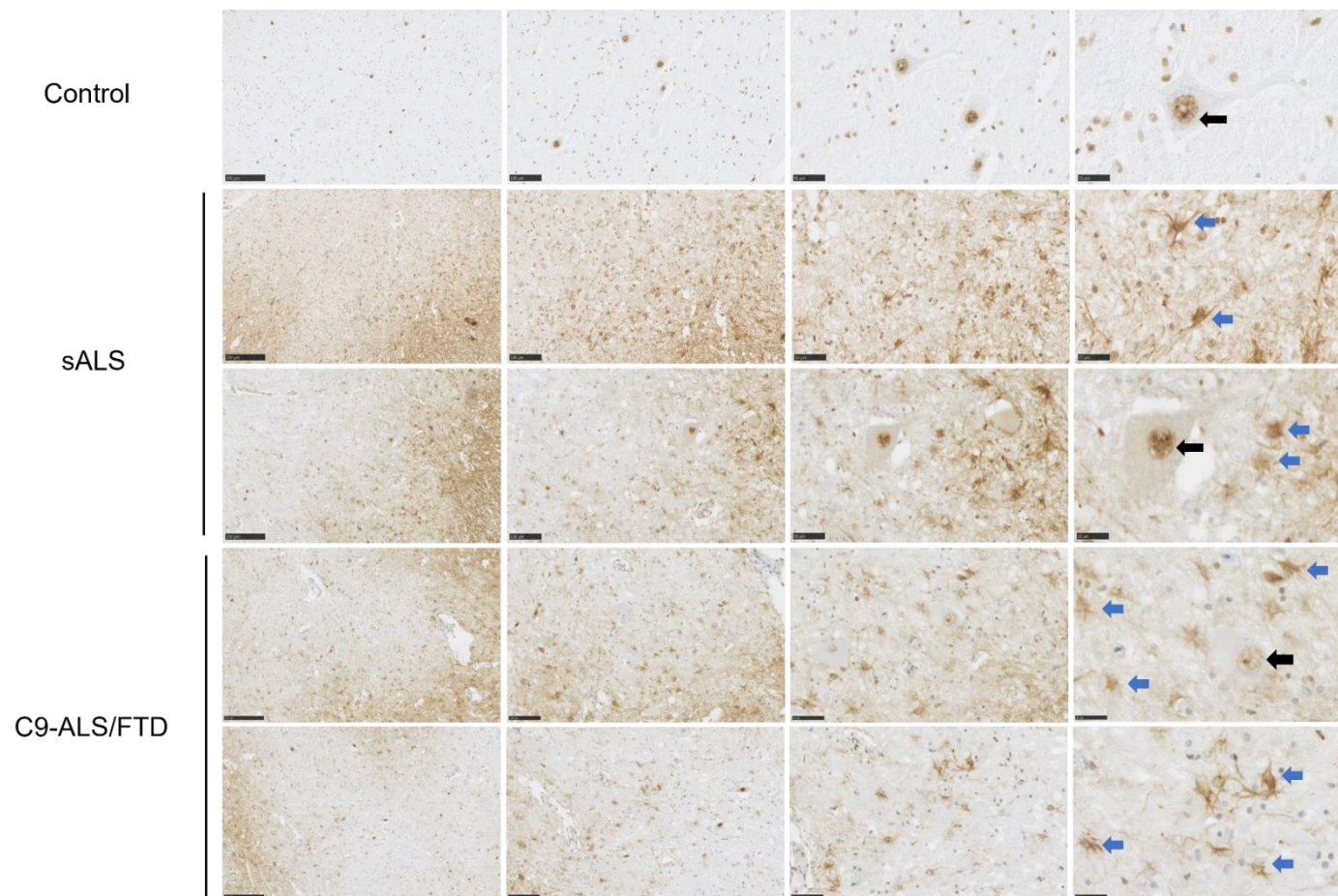


Figure 4.23 **SRSF1 immunohistochemistry in control and ALS spinal cord.** Top row: control; middle two rows: sporadic ALS; bottom two rows: C9-ALS/FTD. SRSF1 showed negative labelling of the neuronal cytoplasm and positive labelling of neuronal nuclei (black arrows) and glia (blue arrows) which have a morphology suggestive of astrocytes and possibly microglia. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

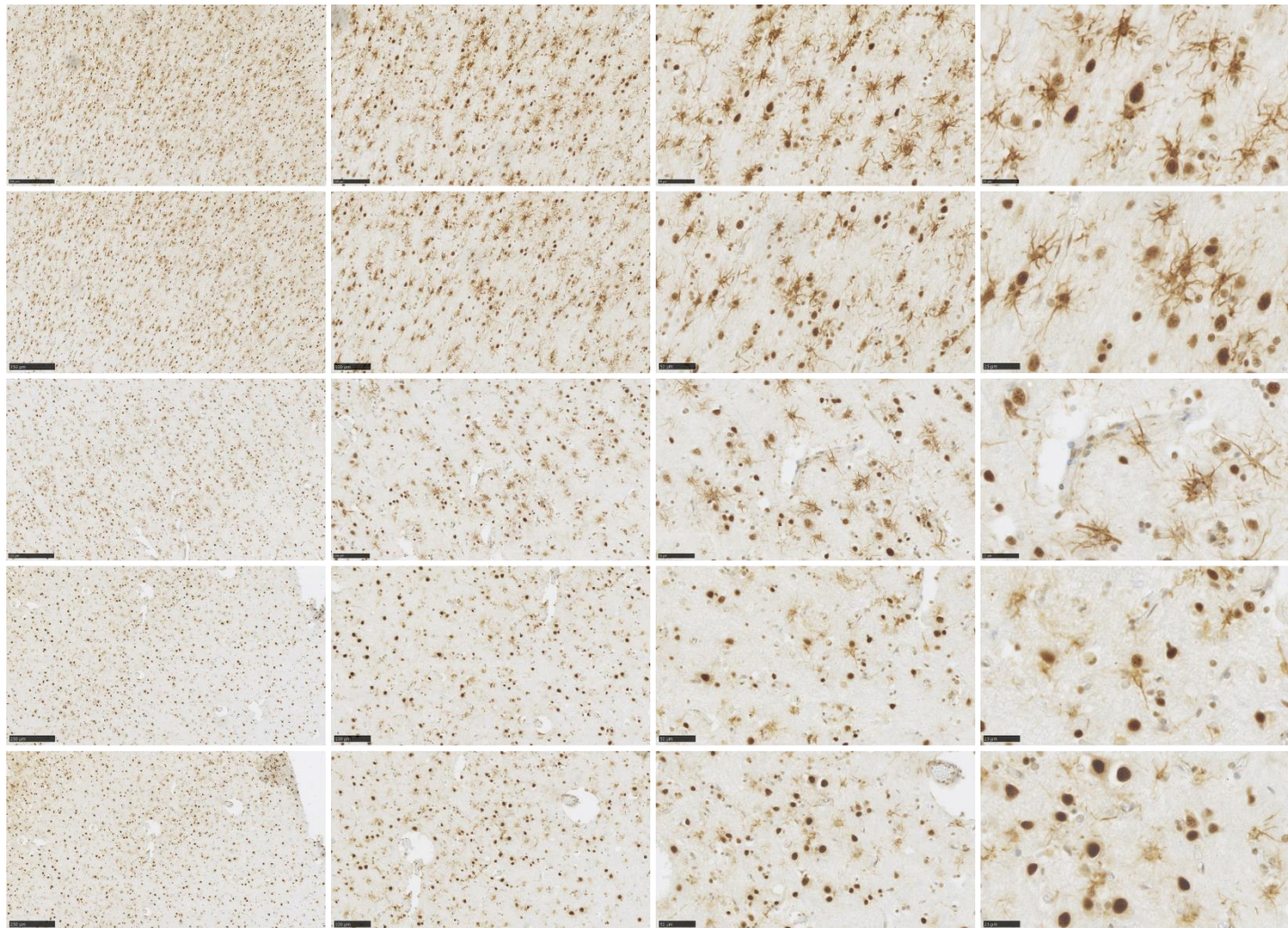


Figure 4.24 **SRSF1-positive glial cell labelling in C9-ALS/FTD Motor Cortex.** Images from two individuals with the *C9ORF72* repeat expansion. C9-ALS/FTD cases showed strong glial labelling throughout all layers in large patches of the motor cortex; positive labelling of neuronal nuclei is also seen. Glial cell morphology appeared astrocytic. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

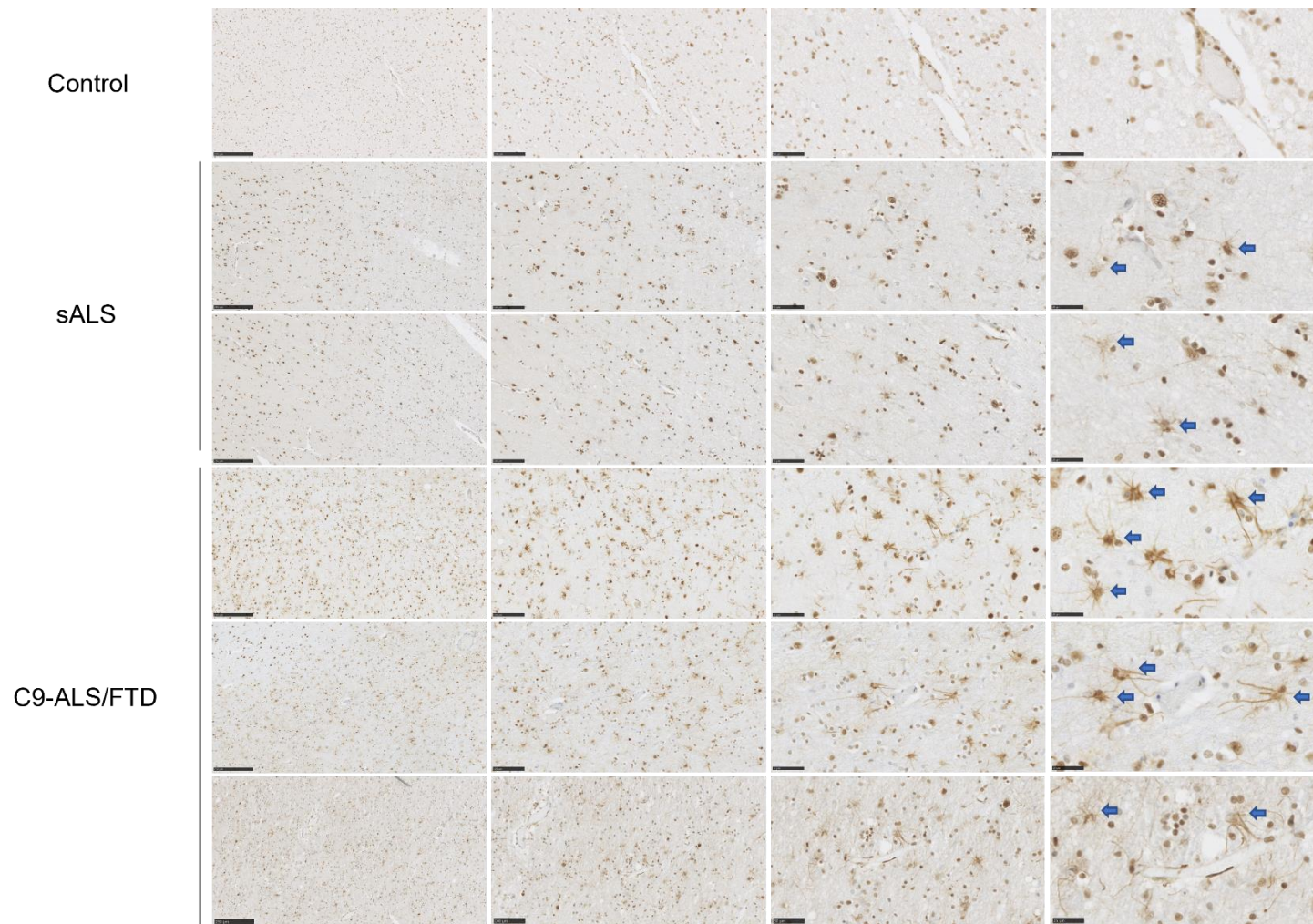


Figure 4.25 **SRSF1-positive glial cell labelling in motor cortex underlying white matter** Top row: control; middle rows: sporadic ALS; bottom rows: C9-ALS/FTD. In sALS and C9-ALS/FTD cases, SRSF1 showed positive glial cell labelling (blue arrows) with a morphology suggestive of astrocytes. No similar glial cell SRSF1 staining was seen in controls. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

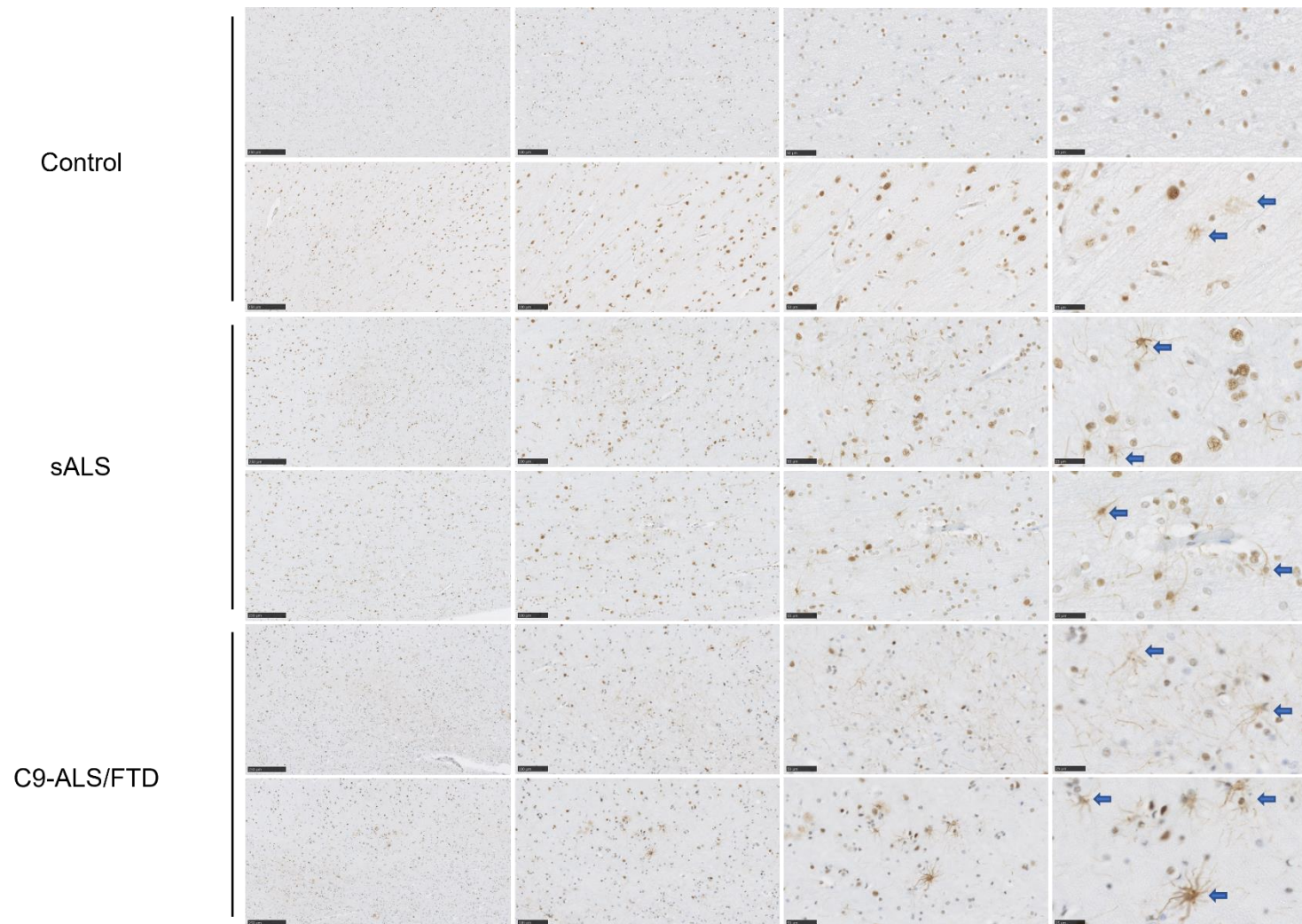


Figure 4.26 **SRSF1-positive glial labelling in Prefrontal Cortex underlying white matter** Top rows: control; middle rows: sporadic ALS; bottom rows: C9-ALS/FTD. In sALS and C9-ALS/FTD cases, SRSF1 showed positive glial cell labelling (blue arrows) with a morphology suggestive of astrocytes. One control case showed similar glial cell SRSF1 staining. Scale bars left-right are 250µm, 100µm, 50µm, 25µm

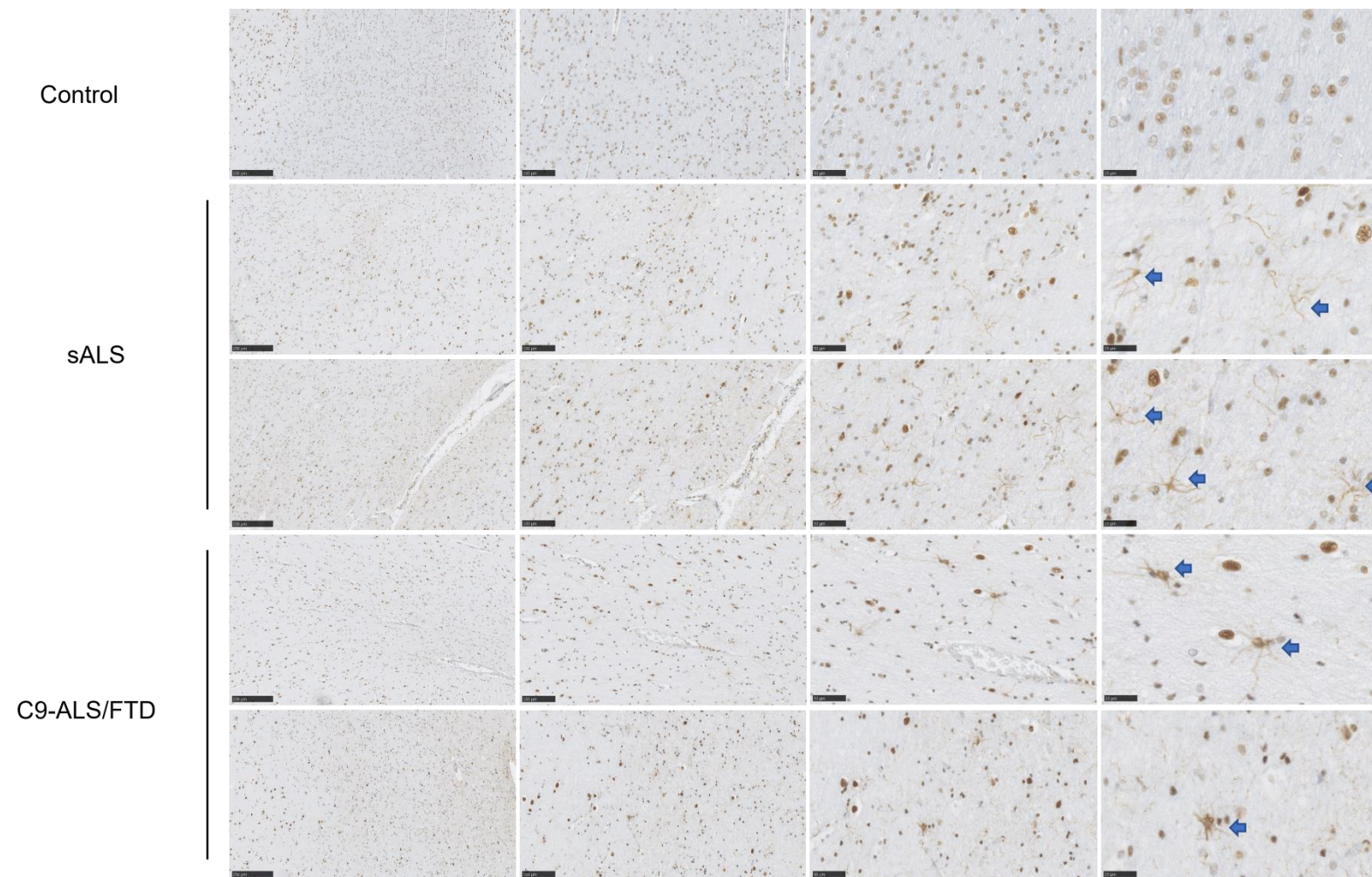


Figure 4.27 **SRSF1-positive glial labelling in Temporal Lobe underlying white matter** Top row: control; middle rows: sporadic ALS; bottom rows: C9-ALS/FTD. In sALS and C9-ALS/FTD cases, SRSF1 showed positive glial cell labelling (blue arrows) with a morphology suggestive of astrocytes and possibly microglia. No similar glial cell SRSF1 staining was seen in controls. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

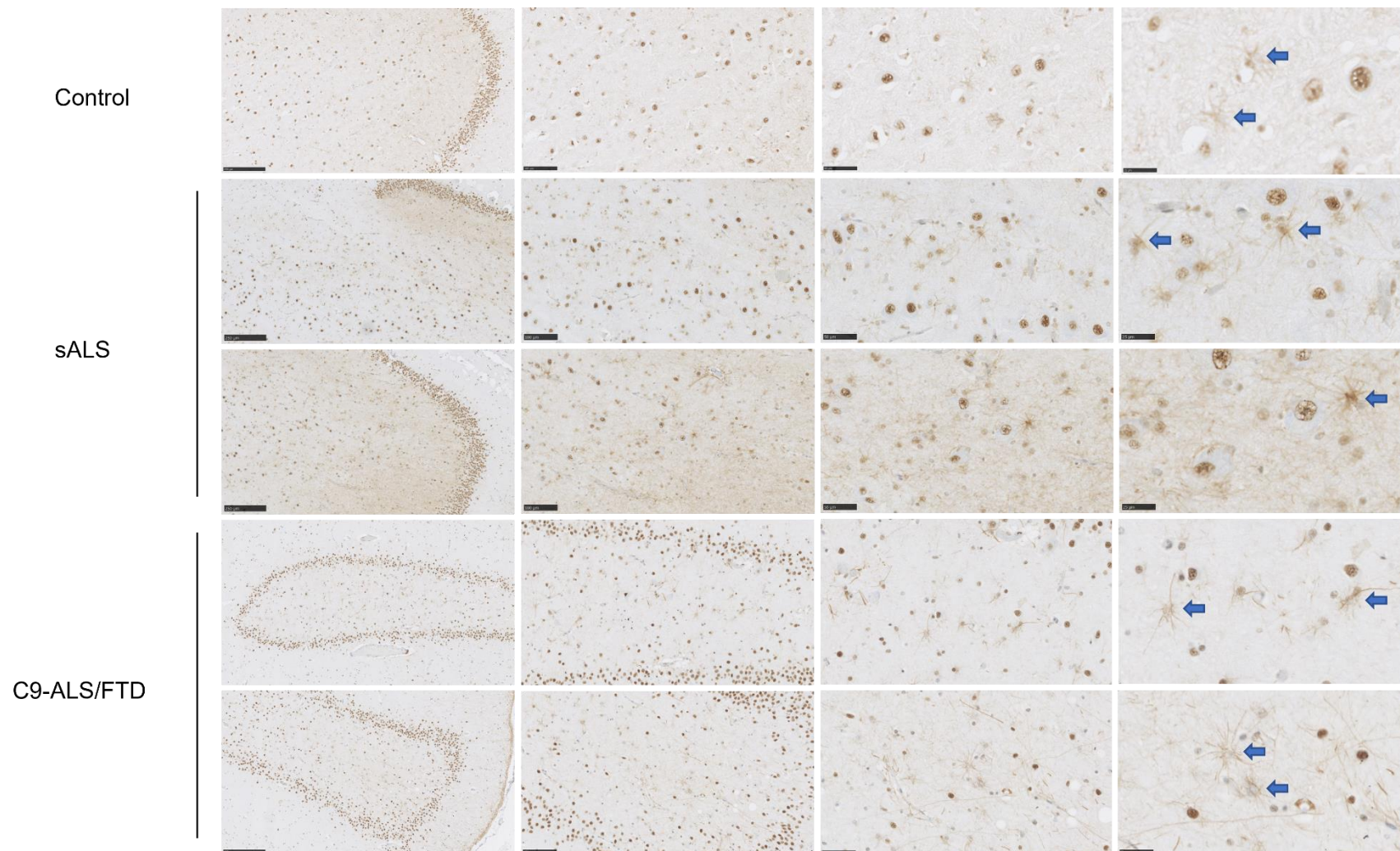


Figure 4.28 **SRSF1 immunohistochemistry in control and ALS Hippocampal neurons.** Top row: control; middle rows: sporadic ALS; bottom rows: C9-ALS/FTD. SRSF1 showed positive CA4 hippocampal neuron nuclear labelling with negative cytoplasmic staining in all cases. Some very subtle glial cell SRSF1 staining was also seen (blue arrows) with a morphology suggesting astrocytes. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

4.7 eIF4A1 and eIF4A2 immunohistochemistry in C9-transgenic mouse spinal cord

Following immunohistochemistry revealing the presence of eIF4A1-positive inclusions in human ALS spinal cord, the next aim was to investigate eIF4A1-positive pathological inclusions in FFPE spinal cord of transgenic *C9ORF72* mutant mice. The aim of this work was to determine if the inclusions seen in the human brain were also present in a mouse model of the disease. Non-transgenic mice without the *C9ORF72* repeat expansion mutation were used as a control. eIF4A2 immunohistochemistry was also performed to establish the specificity of any eIF4A1-positive inclusions detected, and to be consistent with the work in human tissue. The same antibodies were used as previously shown for the staining in human tissue. However, immunostaining required re-optimisation as it was observed that staining intensity was dramatically higher in mouse than in human tissue under the same conditions.

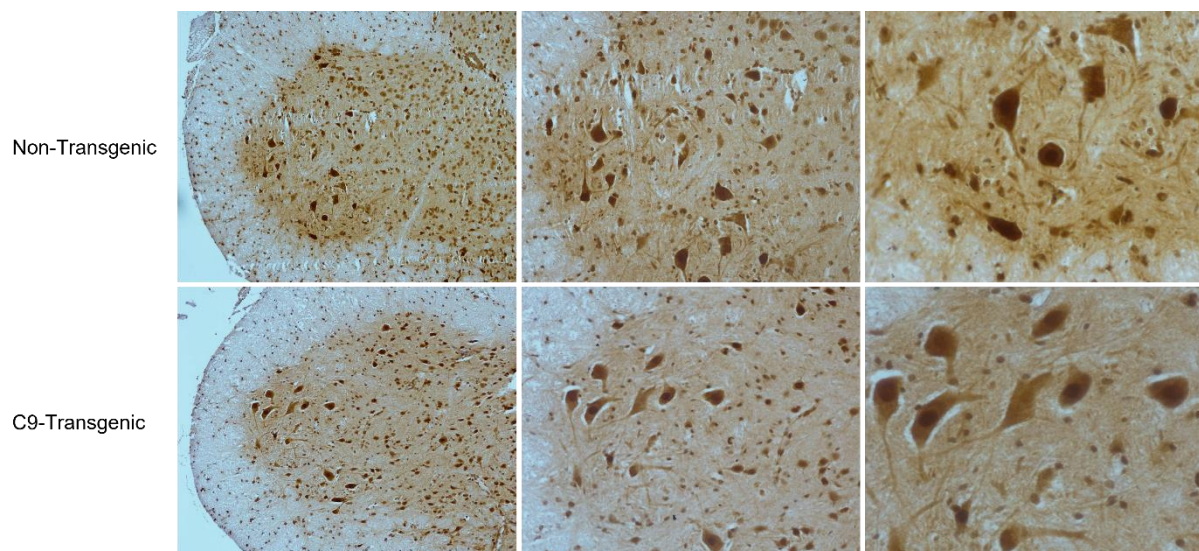


Figure 4.29 eIF4A1 immunohistochemistry in mouse spinal cord. Top row: non-transgenic; bottom row: C9-transgenic mouse; N=3 per group. eIF4A1 showed strong cytoplasmic and nuclear labelling of mouse motor neurons, with positive axonal and glial cell nuclei labelling also seen throughout the spinal cord. No evidence of eIF4A1-positive pathology was seen in any case. Magnification left-right: 10x, 20x, 40x.

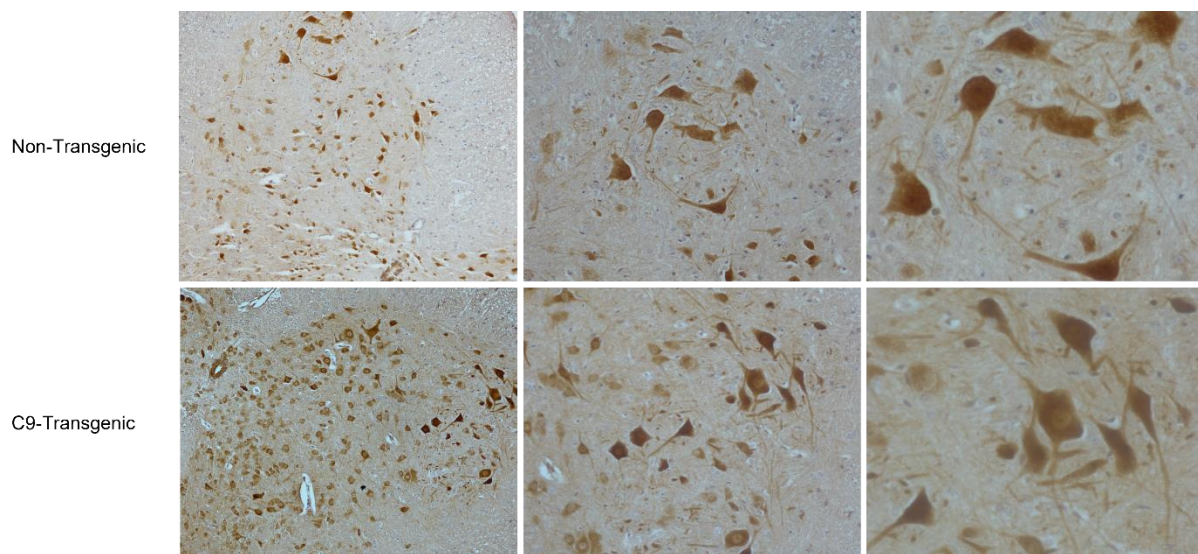


Figure 4.30 eIF4A2 immunohistochemistry in mouse spinal cord. Top row: non-transgenic; bottom row: C9-transgenic mouse; N=3 per group. eIF4A2 showed strong cytoplasmic and nuclear labelling of mouse motor neurons throughout the spinal cord. Neuronal processes and axons were also positive for eIF4A2. Variable labelling of glial cell nuclei was seen. There was no evidence of eIF4A2-positive pathology in any case. Magnification left-right: 10x, 20x, 40x.

eIF4A1 and eIF4A2 immunohistochemistry showed strong cytoplasmic and nuclear staining throughout the spinal cord dorsal and ventral horn neurons. Labelling of neuronal processes, axons and glia was seen for both eIF4A1 and eIF4A2. No evidence of eIF4A1-positive neuronal cytoplasmic inclusions, or any other eIF4A1 or eIF4A2-positive pathology was seen, in any of the six mice used, including three C9-transgenic and three non-transgenic mice.

4.8 Chapter Summary and Discussion

The purpose of this chapter was to characterise RAN-associated RNA helicases in post-mortem human tissue from ALS patients. Although immunohistochemistry had previously been reported in the literature in a number of tissue types across the field of cancer biology, investigations involving neurodegenerative disease, or the CNS were notably absent. The aim of this work was to establish the staining pattern,

distribution, and localisation of RAN-associated RNA helicases across the most disease-relevant areas in ALS and FTD. These comprise the spinal cord, motor cortex, prefrontal and temporal lobes, hippocampus, and cerebellum. ALS and ALS/FTD cases were compared to neurologically normal controls. Both C9-ALS/FTD and sALS cases were included to determine if any variations in staining pattern seen were specific to the *C9ORF72* repeat expansion or to ALS more broadly. Although this thesis is focused on the *C9ORF72* repeat expansion, establishing if any observed pathology is specific to the mutation or more generally applicable can provide useful insight into mechanisms underlying ALS.

This chapter showed western blotting from frozen tissue and immunohistochemistry from FFPE tissue. The helicases examined were eIF4A1, eIF4A2, and co-factors eIF4B and eIF4H. DHX36 was included for western blotting only. SRSF1 was also included as it is of particular interest for our research group due to it being identified as necessary for repeat-containing *C9ORF72* mRNA transcripts to exit the nucleus for RAN translation (Hautbergue et al., 2017) Therefore despite not being a helicase itself, SRSF1 is required for RAN translation to be able to take place.

4.8.1 Investigations into novel eIF4A1-positive pathology in the ALS spinal cord

The main finding was the existence of eIF4A1-positive inclusions in the cytoplasm of spinal cord motor neurons. These were found in every ALS case examined, but no controls, and no other regions apart from the spinal cord. Inclusions were not specific to the *C9ORF72* repeat expansion and were also found in sALS cases. This has important implications for our understanding of the mechanisms underlying RAN translation, and the role of helicases in ALS generally. Not only is the first report of eIF4A1-positive pathology in ALS, but no other helicase has also been found to form pathological inclusions in any subtype of ALS or any other neurodegenerative condition.

This project is novel in its approach to eIF4A1 and eIF4A2: the literature thus far refers to eIF4A, treating the two isoforms interchangeably (Andreou & Klostermeier, 2013). The data in this chapter provide evidence for their divergence in that no eIF4A2-positive inclusions were found in the spinal cord or any other brain regions, despite only differing from eIF4A1 by approximately 10 amino acids. This implies a level of functional difference between eIF4A1 and eIF4A2, which could be cell or tissue-specific, or related to another factor entirely. Co-factors eIF4B and eIF4H also did not show any evidence of inclusion formation in any region.

eIF4A1-positive inclusions were detected by an Abcam anti-eIF4A1 antibody which has been shown in the previous chapter to be highly reliable and specific at detecting eIF4A1. When eIF4A1 was depleted with shRNAs a specific partial knockdown of eIF4A1 only was shown at the protein level through western blotting. A less intense band of the expected size was seen showing a specific depletion for eIF4A1 only with no evidence of non-specific binding with eIF4A2. If eIF4A1 pathology was simply a non-specific interaction it might be expected that eIF4A2, being so structurally similar, would show similar pathology. The lack of eIF4A2 or control pathology provides confidence to support our identification of eIF4A1 as forming novel pathology in ALS spinal cord.

Both sALS and C9-ALS/FTD groups were found to have eIF4A1-positive pathology in approximately 8% of lower motor neurons. No differences were found between diagnostic groups, suggesting eIF4A1-positive pathology may be applicable to ALS generally rather than specific to a subtype of the disease. An interesting further study would be to see if eIF4A1-positive inclusions could be detected in patients with other key ALS mutations such as in *SOD1*, *FUS* or *TARDBP*. There was no evidence of eIF4A1-positive staining in the DPR pathology of the hippocampus and cerebellum

that forms the unique hallmark of C9-ALS/FTD (Freibaum & Taylor, 2017), as no eIF4A1-positive inclusions were observed in these areas.

4.8.2 eIF4A1 double staining with phosphorylated TDP-43 and p62 in the ALS spinal cord

All eIF4A1-positive inclusions showed cytoplasmic localisation with a consistent morphology of thick thread or skein-like inclusions that resembled some ALS TDP-43 inclusions. Compact inclusions, such as those that may be seen with TDP-43 immunohistochemistry, were absent. Therefore, double staining was carried out to detect the extent of co-localisation between phosphorylated TDP-43 and eIF4A1-positive pathology. Consistent co-localisation would suggest TDP-43 inclusions are sequestering cytoplasmic eIF4A1, rather than eIF4A1 forming an independent proteinopathy.

Double staining results revealed that eIF4A1 pathology can form independently of TDP-43 pathology. This was evidenced by the existence of neurons showing either eIF4A1-positive, TDP-43-negative pathology and vice versa. In some cases, neurons were identified that contained both inclusion types that were not co-localised and instead were at opposite ends of the cytoplasm. In other cases, co-localisation was seen between eIF4A1 and TDP-43 pathology.

Further double staining studies were performed between eIF4A1 and p62. Here, co-localisation was observed in the clear majority of cases, but rare examples could be found where no co-localisation was seen. While eIF4A1-positive inclusions were generally p62-positive, eIF4A1 pathology was also able to form independently of p62.

This work has focused primarily on the identification of eIF4A1-positive inclusions, first establishing that they are indeed specific to ALS and then attempting to determine if

they form independently or other key pathologies related to ALS, specifically TDP-43 and P62. These key questions have been successfully answered by this work, with evidence that eIF4A1-positive inclusions are indeed able to form independently of TDP-43 and p62. However, it is not clear from this data how frequently this co-localisation happens and to what extent eIF4A1-positive, TDP-43 or p62-negative inclusions are a rare occurrence. An important future step for this work will be to quantify frequency of co-localisation. Here, cases should be double stained for eIF4A1 and TDP-43 or p62. Inclusions positive for eIF4A1, TDP-43 and p62 should be identified and counted individually. Next, numbers of eIF4A1 inclusions also positive for TDP-43 or p62 should be counted and calculated as a percentage of total eIF4A1-positive inclusions. At least two blinded observers should perform this work with an inter-rater reliability calculation used to increase confidence in the data. However, due to the substantial variation within diagnostic groups observed thus far, it is essential to perform this analysis in a larger cohort that was available. As the aim of this double staining was to establish proof-of-principle that eIF4A1-positive, TDP-43 and p62-negative inclusions were able to form, only two cases were used that had previously been identified as having a large burden of eIF4A1-positive inclusions. Therefore quantification of these results is beyond the scope of this thesis.

4.8.3 eIF4A1 and eIF4A2 immunohistochemistry in C9-transgenic mouse spinal cord

Following identification of eIF4A1-positive pathology in human post-mortem tissue, immunohistochemistry was carried out on the spinal cord of three C9-transgenic and three control mice. Mice used in this study were C9-500 BAC Transgenic mice as described by Liu and colleagues (Liu et al., 2016). The aim was to attempt to identify eIF4A1-positive pathology in this model, but none was seen in any of the six mice included in this study. The lack of identification raises several issues regarding this novel pathology. First, it suggests that development of eIF4A1-positive inclusions

could be a late-stage event in ALS. This is consistent with current literature on TDP-43, where mis-localisation is thought to be a driver of early pathology (Suk & Rousseaux, 2020). In mouse model of ALS, *TARDBP* mutant mice show early motor neuron pathology, indicating TDP-43 dose-dependent neurotoxicity (Ebstein et al., 2019). This suggests that TDP-43 may precede other types of pathology including these novel eIF4A1-inclusions. The mice in this study were sacrificed at only 7 months, which is considered the very earliest point at which the mice could be considered 'aged'. The lack of mouse pathology may also reflect broader problems associated with this model or interspecies differences relating to eIF4A1, which are yet to be fully clarified. Mouse models are frequently used in ALS, but have been criticised for their lack of success in translation to the human disease and the development of effective therapies, which is discussed in depth by Morrice and colleagues (Morrice et al., 2018). In this study, antibodies that had been optimised in human tissue showed hugely over-saturated staining in mouse tissue under the same conditions, indicating higher relative expression of the protein possibly indicating functional differences between the species. Alternatively, there may have been differences in tissue processing that could account for the differences in staining intensity. This could include better preservation of the antigen in mouse tissue, due to the lack of post-mortem delay, better fixation techniques and more efficient perfusion of tissue with less proteolysis (Ramos-Vara & Miller, 2014). It is likely that these sources of variation contributed to the differences seen in mouse and human staining with the same antibody (Fritschy, 2008; Goodman, 2018; Ivell et al., 2014).

4.8.4 SRSF1 and RAN-associated RNA helicase immunohistochemistry in ALS spinal cord and CNS

Aside from the spinal cord and motor cortex, immunohistochemistry was performed for eIF4A1, eIF4A2, eIF4B, eIF4H and SRSF1 on the prefrontal and temporal lobes, hippocampus and cerebellum. This has provided new insight into the previously

uncharacterised patterns of localisation and distribution across the CNS. RAN-associated RNA helicases show a generally cytoplasmic localisation with variable nuclear staining which is consistent with their role as RAN translation initiation factors. Minimal differences in helicase staining patterns were observed between control and ALS groups in the regions examined. The current literature predominantly focused on helicase function rather than a comparison of expression of different helicases, so it is unclear if differential expression occurs in different organs or tissues. This would be an informative study to better understand different helicase staining patterns in the context of different tissues.

Some additional further investigations could be made, for example looking at greater nuclear localisation of eIF4B in the ALS prefrontal cortex compared to controls. However, this would require blinded analysis, with a large case cohort than was available for this study to draw any conclusions.

SRSF1, as a splicing factor, has a nuclear localisation with negative cytoplasmic staining pattern seen in neurons. In all regions examined this staining pattern is highly consistent. SRSF1 immunohistochemistry has also revealed particularly interesting patterns of glial staining, with extensive positive labelling seen in large patches of the motor cortex which is specific to C9-ALS/FTD. Although, similar glial labelling is seen in underlying white matter in both sALS and C9-ALS/FTD cases but not controls. There appeared to be more SRSF1 glial staining in C9-ALS/FTD than in controls; the next step for this study would be to perform blinded quantification of these regions and to use a glial marker to confirm the identity in a large number of cases. Further glial labelling, although much more subtle, was also seen in prefrontal cortex and temporal lobes in sALS and C9-ALS/FTD cases but not controls. Very weak glial SRSF1 labelling was also seen in the hippocampus although there was also some evidence of positive staining in one of two control cases examined. No glial marker was used to confirm glial identity in any region, but staining patterns indicate an astrocytic

morphology. This staining was only seen in a very small number of cases, limiting the reliability of these results, so future studies should focus initially on expanding this cohort.

SRSF1 labelling was expected to be restricted to the nucleus based on previous studies using SRSF1 immunohistochemistry in the field of cancer biology, where generally strong nuclear expression of SRSF1 was seen (G. Broggi et al., 2021; G. A.-O. Broggi et al., 2021). In addition SRSF1 was found to mediate the assembly of nuclear speckles and other transcription-related activities in the nucleus (Tripathi et al., 2012). Therefore, this pattern of glial cytoplasmic staining was unexpected. The antibody used for immunohistochemistry has previously been used for western blotting of cultured cells, providing confidence on the quality and specificity of the antibody (Kurien & Scofield, 2017). It is possible that the labelling is not specific and instead the antibody is detecting another protein upregulated by reactive astrocytes that is more abundant in ALS cases than controls. However, the most prominent areas of SRSF1-positive glial labelling were in large patches of the motor cortex in C9-ALS/FTD cases only. This exclusivity provides additional confidence in the results, as if the labelling was not specific it would be expected to occur in a range of cases across diagnostic groups. An additional western blot on cultured astrocytes would be an important next step to help validate the results of these studies. It is possible that SRSF1 is upregulated in ALS cases compared to controls. If this is the case it has important mechanistic implications for ALS, which are further explored in chapter six of this thesis.

4.8.5 Western blots of eIF4A1, eIF4A2 and eIF4H in frozen ALS spinal cord and motor cortex

Western blotting was carried out on frozen spinal cord and motor cortex tissue to detect any changes in protein expression between control and ALS groups.

Investigations were carried out into use of α -tubulin as a loading control, which was found to show too much variability between individuals to be reliably used Figure 4.7. Instead, TUJ1 or β -actin were found to be more consistent. In the literature, standard loading controls such as α -tubulin have been highlighted as having several serious drawbacks, and that in future total protein should be used as an alternative control in these studies (Moritz, 2017).

In frozen spinal cord, blots probed for eIF4A1 showed significantly greater levels in C9-ALS/FTD groups compared to controls. sALS cases had intermediate levels between C9-ALS/FTD and controls but did not differ from them significantly. In the motor cortex, no difference in eIF4A1 or eIF4A2 expression in controls and ALS groups was seen.

Antibodies against eIF4B, DHX36 and SRSF1 did not detect any western blot signal in spinal cord. It is possible that other commercially available antibodies may have been successful in detecting these proteins, but no exhaustive list was screened due to limited resources of this project in terms of costs and time available. Therefore, only one or two antibodies previously used by the Hautbergue lab in molecular biology applications were tested. The anti-DHX36 antibody was viable for use in motor cortex but eIF4B and SRSF1 western blotting was not possible in either region. These antibodies were used successfully to detect these proteins for immunohistochemistry from FFPE tissue, albeit with a very weak staining intensity for eIF4B. The failure to detect SRSF1 or eIF4B using these antibodies in western blots from frozen human post-mortem tissue may reflect the conditions in which these frozen samples have been collected and stored. Samples from FFPE tissue may have been better preserved whereas degradation may have occurred in frozen tissue samples. Antigens for immunohistochemistry are more likely to remain in their tertiary 3-dimensional structure than in western blots, which may explain why the antibody is effective for one application and not the other (Fritschy, 2008; Goodman, 2018; Ivell et al., 2014; Kurien

& Scofield, 2017). Antibodies had also been previously successfully used for western blots from HEK293 cells. Cultured cells benefit from a high level of consistency in terms of preparation and storage compared to frozen human tissue samples. Cultured cell samples are unlikely to be stored long-term and in different circumstances, therefore benefiting from consistency and less likelihood of degradation, unlike post-mortem samples for immunohistochemistry, where substantial variability can arise from tissue processing methods (Ramos-Vara & Miller, 2014).

Alternative antibodies were tested, but no working antibody was found. It was considered that these proteins may be expressed at such a low level as to be undetectable when 30µg protein was loaded, but even when 100µg was loaded no signal was detected through western blotting. As these proteins were successfully detected with immunohistochemistry, it is likely that the lack of signal with western blotting was due to the antibodies available rather than very low expression of the target protein. In the motor cortex, DHX36 expression shows no significant difference between controls and ALS groups.

eIF4H was successfully detected on western blots from both spinal cord and motor cortex. eIF4H showed significantly lower levels in both regions in both ALS groups compared to controls (45 to 50 % in sALS and 60 to 75% in C9-ALS/FTD). This was not reflected in eIF4H immunohistochemistry, which showed strong staining throughout many regions. It is unclear why this discrepancy has arisen and may reflect technical errors during immunohistochemistry such as over-saturation of the eIF4H primary antibody that may have required further optimisation. The eIF4H antibody had previously been validated for molecular biology techniques shown earlier in this thesis and had shown no evidence of non-specific binding. eIF4H staining was not quantified and although the staining intensity did not appear to differ from control cases, analysis of DAB staining intensity as described in several publications may reveal more subtle differences that are hard to distinguish by eye (Crowe & Yue, 2019; Rizzardi et al.,

2012). Although the same cases were used during western blotting and immunohistochemistry, the former used frozen tissue while the latter used FFPE tissue, which may also contribute to observed differences. In a recent paper, eIF4H was also found to be down-regulated in both post-mortem human tissue and patient-derived cell lines in patients with the G4C2 repeat expansion mutation compared to healthy controls (Goodman et al., 2019), adding confidence to the western blot results shown in this thesis. Immunohistochemistry was not used in these investigations. Instead, western blotting was used in patient-derived cell lines and showed a 47.5% reduction in eIF4H levels in four independent cell lines from C9-ALS/FTD patients compared to controls. This was confirmed by qPCR in cerebellar tissue from 66 C9-ALS/FTD patients and 22 controls, which also showed a drop in eIF4H levels in C9-ALS/FTD patients. Next, semi-quantitative analysis of eIF4H immunohistochemistry from these regions should be performed, ideally on a larger cohort, to determine if staining results and western blotting results are consistent in showing lower levels of eIF4H associated with ALS, and if this also applies to other brain regions.

In both spinal cord and motor cortex, substantial differences were seen in different cases, both within and between diagnostic groups. There are several factors which could contribute to this, for example the post-mortem delay which varies from 5 to 96 hours, full details of which can be found in Table 2.22. Other factors that can influence staining results include the fixation and storage of tissue, and variability in preservation of the antigen and proteolysis (Boenisch, 2004; Ramos-Vara & Miller, 2014; Werner et al., 2000).

4.8.6 Limitations associated with the use of post-mortem tissue

Like all research methods, the use of post-mortem human tissue has several limitations. Although it provides the unique opportunity to examine the brains of individuals who have died of ALS or ALS/FTD, it is, by definition, the end-stage of the

disease. Therefore, it does not provide the chance to track development of pathologies such as the eIF4A1-positive inclusions detected here, or the increased SRSF1 glial expression. It also has a large survivorship bias, as any neurons remaining at the point of the patient's death will be those with the lowest pathological burden. Neurons with the most toxic components or pathologies will likely die early in the course of the disease and this could lead to a misinterpretation of the actual pathologies driving disease when researchers examine post-mortem tissue. It also highlights the debate that has continued for many years in the field of neurodegenerative disease research, regarding the nature of protein aggregates and if they are inherently toxic or reflect an attempt at a protective mechanism by the cell (Baloh, 2011). Therefore, the assessment of eIF4A1 pathology burden may not accurately reflect the true progression of the disease. The small numbers of cases used in this study present problems with meaningful quantitative analysis, especially given the high heterogeneity between individuals both within and between diagnostic groups. Large-scale population level cohorts should be established to enable collections of post-mortem tissue on a greater scale, for statistical analysis to establish differences between RAN-associated RNA helicase immunohistochemistry.

5 Manipulating RAN translation factors as potential gene therapeutic targets in C9-ALS/FTD

5.1 Background, aims and rationale

In chapter 3, tools were developed to target key helicases involved in the initiation of RAN translation: eIF4A1, eIF4A2, eIF4B and eIF4H. This has enabled specific reduction of their expression or helicase activity, and this resulted in a corresponding decrease in DPR production by RAN translation. This chapter will explore the therapeutic potential of targeting RNA helicases known to be involved in the initiation of general translation, in the context of the C9ORF72 repeat expansion in ALS/FTD. The aim was to test the toxicity of helicase partial knock down in a HEK293T cell model: if helicases are viable as a therapeutic target, it is essential to rule out any off-target effects or any potential harm associated with targeting them in this way. Toxicity was tested associated with mammalian and lentiviral expression of shRNAs targeting these helicases as shown in the first results chapter. We hypothesised that knocking down individual helicases is non-toxic in HEK293T cell models, while targeting multiple helicases simultaneously is likely to be toxic due to the impact on canonical translation. We hypothesised that because RAN translation happens at a lower rate than canonical translation, the level at which our shRNAs reduce helicase expression means RAN translation of DPRs can be effectively reduced, thus alleviating DPR induced toxicity while preserving essential canonical translation.

Next, further experiments were performed with shRNAs and eIF4A1/eIF4A2 helicase mutants described in chapter 3. We have previously shown that both the use of shRNAs and helicase mutants is effective at reducing DPR production, next we aimed to determine if this is associated with rescuing a cytotoxic phenotype caused by DPRs in HEK293T cell models of C9-ALS/FTD. We hypothesised that both shRNAs and helicase mutants could alleviate toxicity induced by the C9ORF72 repeat expansion,

through preventing RAN translation of DPRs. The next aim was to replicate these results in a more disease-relevant models. First, patient derived astrocytes in co-culture with healthy mouse motor neurons; this model is well-established to show a neurodegenerative phenotype associated with mixing healthy motor neurons with diseased astrocytes. This model system is outlined in full by Varcianna and colleagues (Varcianna et al., 2019). Second, data is shown from an *in vivo Drosophila* model of C9-ALS/FTD. We hypothesised that knock down of helicases in both these models would reduce RAN translation of DPRs thereby rescuing the neurotoxic phenotype.

Chapter 4 showed characterisation of RNA helicases in post-mortem human brain and spinal cord tissue from control, sALS and C9-ALS/FTD patients. This chapter showed for the first time the existence of a novel eIF4A1 positive proteinopathy in the spinal cords of ALS patients, but not controls. Double staining of post-mortem spinal cord tissue provided evidence that showed that eIF4A1 pathology can be TDP-43 positive. However, some exceptions exist of eIF4A1-positive, TDP-43-negative inclusions, suggesting TDP-43 pathology is not necessary for the formation of eIF4A1-positive inclusions. Here, the relationship between eIF4A1 and TDP-43 pathology was explored further in a patient-derived neuronal model. Through immunofluorescence staining of control, sALS and C9-ALS/FTD cell lines, we investigated cellular localisation of eIF4A1 and TDP-43 pathology.

5.2 Exploring cytotoxicity associated with shRNA helicase depletion in HEK293T cell models of C9-ALS/FTD

5.2.1 shRNAs specifically targeting eIF4A1, eIF4A2, eIF4B and eIF4H are not associated with cytotoxicity in HEK293T cells

First, we explored the impact of the LV-shRNAs targeting helicases eIF4A1, eIF4A2, eIF4B and eIF4H on HEK293T cytotoxicity. LV-shRNAs were purchased from Origene

as described in chapter 3. Cytotoxicity was measured using MTT assays, which use cell proliferation and viability as an indirect measure of toxicity. MTT assays have been used in research for many years and are based on the reduction of the MTT reagent, a tetrazolium salt, by the metabolic activity of proliferating cells. This leads to the production of the dark blue formazan which is then measured by spectrophotometry (Mosmann, 1983) (Berridge & Tan, 1993). MTT reduction is carried out in the mitochondria and therefore this assay provides a measure of cell viability through mitochondrial function (Lobner, 2000). Cell proliferation was normalised to that of a control shRNA with a scrambled sequence. Additionally, we used non-transfected cells as an additional control. Use of this control allowed us to establish to what extent the process of transfection caused a fall in cellular proliferation, compared to what could be attributed to the impact of shRNAs themselves.

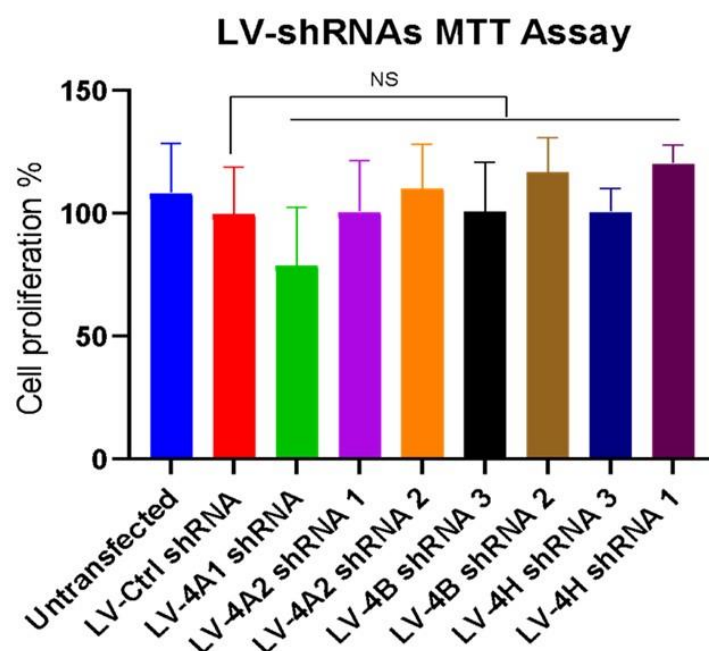


Figure 5.1 MTT assay for LV-shRNAs targeting eIF4A1, eIF4A2, eIF4B or eIF4H. Cell proliferation assay of HEK293T cells transfected for 72 hours with control or helicase shRNAs. Performed with three biological replicates; Mean $\pm$ SEM; one-way ANOVA with Tukey's corrections for multiple comparisons; NS: not significant.

Figure 5.1 shows that in HEK293T cells transfected with Origene LV-shRNAs, compared to control shRNA there was no significant toxicity as measured by decreases in cell proliferation. Each helicase has two shRNA options which were tested, with the exception of eIF4A1 shRNA, where only one option was available. No significant change in cell proliferation was associated with either option. For eIF4A1, a downwards trend in cell proliferation was observed which was distinct from other helicases, indicating a fall of 25%; however, this was not a statistically significant fall.

5.2.2 pENTR-shRNAs are associated with cytotoxicity in HEK293T cells only when multiple helicases are targeted simultaneously.

After establishing that there is no cytotoxicity associated with LV-shRNAs, which target eIF4A1, eIF4A2, eIF4B and eIF4H, we next tested pENTR-shRNAs for eIF4A1, eIF4A2 and eIF4B. Additionally pENTR-eIF4A1 + 4A2 shRNA, which enabled testing the impact of knocking down multiple helicases simultaneously. MTT assays were carried out, using cell proliferation decreases as a measure of cytotoxicity. As previously described, controls were untransfected cells and a scrambled shRNA without a protein target.

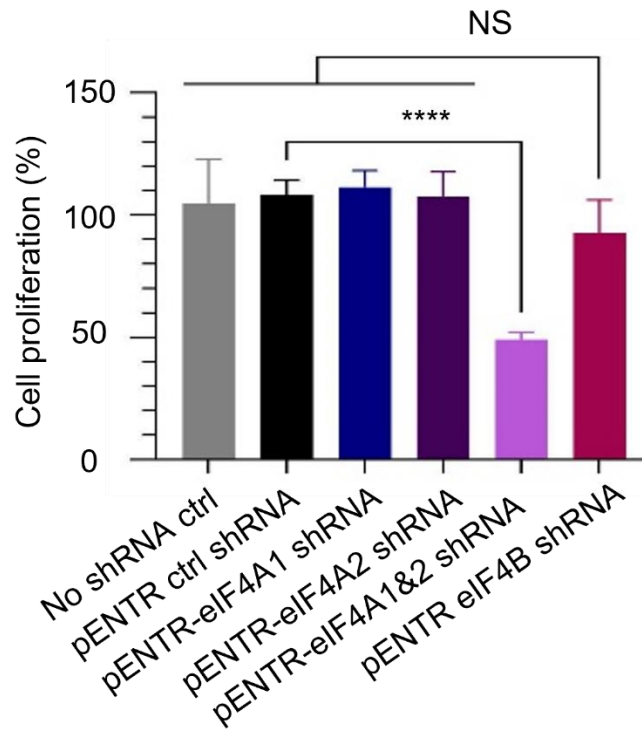


Figure 5.2 MTT assay for pENTR-shRNAs targeting eIF4A1, eIF4A2, eIF4A1+4A2 and eIF4B. Cell proliferation assay of HEK293T cells transfected with control or helicase shRNAs. Performed with three biological replicates. Mean $\pm$ SEM; one-way ANOVA with Tukey's correction for multiple comparisons; NS: not significant; ****: $p < 0.0001$.

Figure 5.2 shows that in HEK293T cells, pENTR-shRNAs knocking down eIF4A1, eIF4A2, or eIF4B were non-toxic compared to control shRNA, as measured by cell proliferation. However, when a shRNA was used targeting both eIF4A1 and eIF4A2 simultaneously, there was a highly significant reduction in cell proliferation. Knockdown of both helicases resulted in a fall in cell proliferation of 50% compared to control shRNA.

5.2.3 Partial depletion of helicases is associated with mild cellular stress

Following MTT assays we used examination of stress granules as an additional measure of cellular stress. Stress granules are a type of transient ribonucleoprotein body that assembly through LLPS in response to cellular stress. Under chronic stress they can become persistent and are thought to act as a site of aggregation for various

ALS-related proteins (Dudman & Qi, 2020). HEK293T cells were treated with toxins sodium arsenate, or BLF1. BLF1 is a bacterial toxin which functions to inactivate eIF4A via deamidation of glutamine 339, thereby leading to widespread cellular translational arrest (Rust et al., 2018). Cruz and colleagues describe the discovery and role of BLF1, called Bpsl1549 (Cruz-Migoni et al., 2012). This was therefore used as an accompaniment to our strategy, as an alternative method to investigate the effects of helicase depletion. For this study, siRNAs were used as opposed to shRNAs. Cells were treated with siRNAs targeting eIF4A1, eIF4A2, and eIF4B individually, and then simultaneously as eIF4A1 + eIF4A2, eIF4A1 + eIF4B, or eIF4A2 + eIF4B siRNAs; or they were treated with toxins sodium arsenate or BLF1. Stress granules were measured and calculated as a percentage of total cells, with cells classed as having 1-2 stress granules, or more than 2. Two controls were used, first a control siRNA with a scrambled, non-targeting sequence. Secondly untransfected cells were used as a control to establish the baseline stress level associated with transfection or treatment of any kind. Immunofluorescence was also carried out to visualise stress granule formation in cells treated with the toxins or siRNAs targeting eIF4A1 and eIF4A1 + eIF4A2. TIAR1 was used as a stress granule marker. This experiment enabled exploration of the link between stress granule formation and translational stalling.

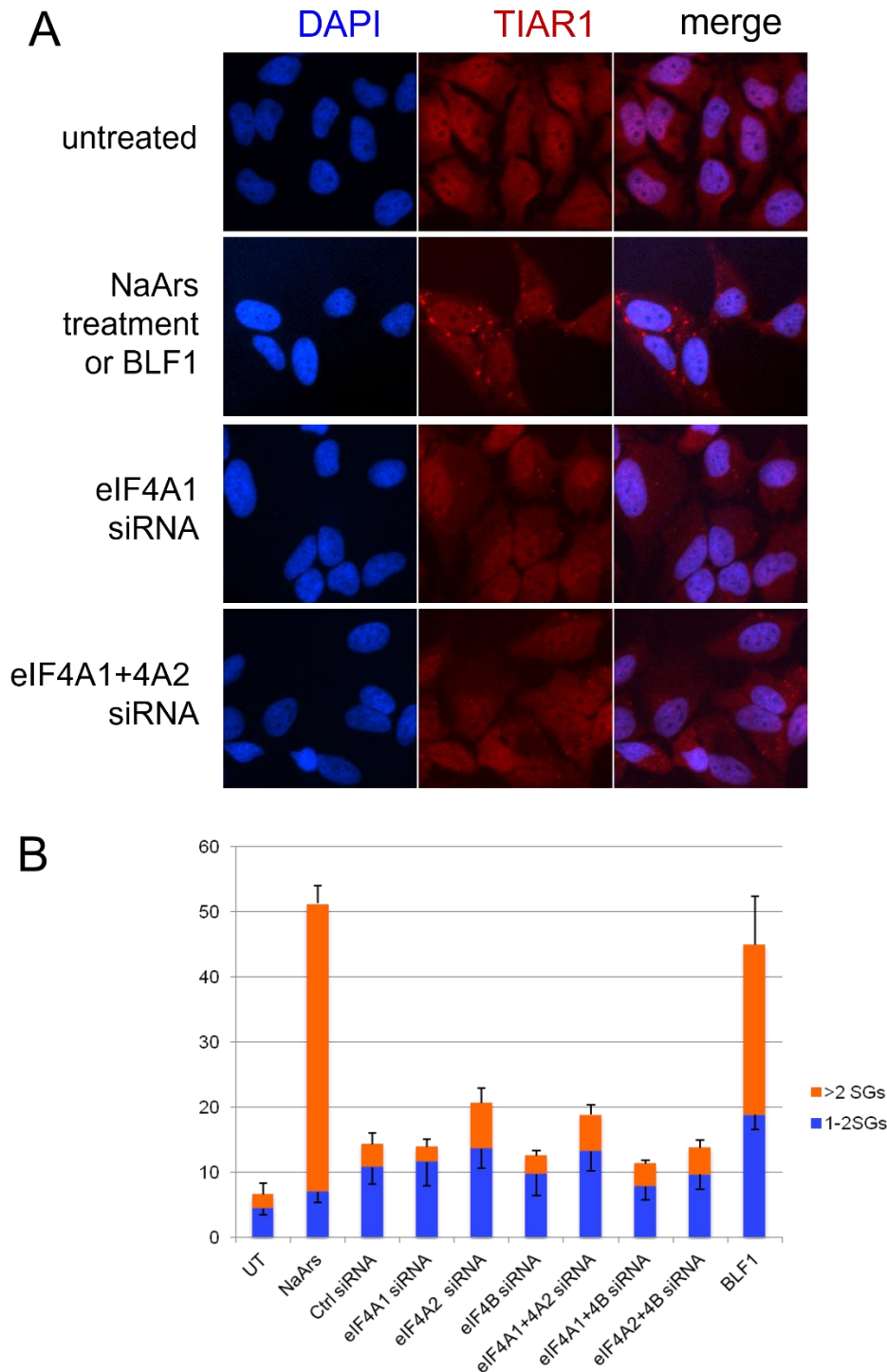


Figure 5.3 Partial depletion of eIF4A1, eIF4A2 and eIF4B causes only mild stress granule formation. (A) shows immunofluorescence of HEK293T cells either untreated or transfected with toxins sodium arsenate or BLF1, eIF4A1 siRNA, or eIF4A1 + eIF4A2 siRNA. The left column shows the nucleus stained by DAPI, the middle column shows stress granule marker TIAR1, the right column shows the merged image. (B) shows breakdown of stress granules as a % of total in cells either untreated, or treated with sodium arsenate, BLF1, or helicase siRNAs. Blue represents % of cells with 1-2 stress granules, orange represents % of cells with greater than two stress granules. This experiment was carried out by Dr. Lydia Castelli.

Figure 5.3 B shows that as expected, cells treated with sodium arsenate or BLF1 showed a large increase in the percentage of cells with more than two stress granules. This can also be seen using immunofluorescence staining for TIAR1 in Figure 5.3 A. For sodium arsenate, 50% of total cells were stress granule positive, while for BLF1 it was 45%. Furthermore, these cells showed an increase in proportion of number of stress granules, with over 80% of positive cells treated with sodium arsenate showing more than 2 stress granules. For BLF1 treated cells, over 50% of the stress granule positive cells were found to have more than 2 stress granules. In contrast in untreated cells, fewer than 10% of cells contained any stress granules, and of those that did more than half contained 1-2 stress granules only. A modest increase in stress granule formation was seen associated with all siRNA transfections, including control siRNA which did not deplete helicases or any other target protein. Control and helicase siRNAs all led to an increase in total stress granule positive cells to be between 10 and 20%. The large majority of these cells were classed as only having one stress granule, with a much smaller proportion classed as having more than 2 stress granules, indicating mild stress only. This was the case regardless of whether helicases were targeted individually or simultaneously. eIF4A2 siRNA was associated with the largest formation of stress granules, with 20% of total cells stress granule positive. Of this, approximately 60% were classed as having 1-2 stress granules.

5.3 Investigating the effects of partial depletion of helicases on alleviation of DPR-induced toxicity

5.3.1 Partial depletion of helicases with shRNAs rescues cytotoxic effects of sense and antisense *C9ORF72* repeat expansion

Following investigations into toxicity associated with knockdown of helicases themselves, we moved on to investigate the ability of helicase depletion to rescue C9ORF72 induced toxicity in HEK293T cell models of C9-ALS/FTD. Cells were co-

transfected with sense or antisense G4C2/C4G2 repeats and pENTR-shRNAs targeting eIF4A1, eIF4A2, eIF4B or eIF4A1 + eIF4A2. A scrambled sequence shRNA was used as a control. Cells not treated with DPRs were used as an additional control, to ensure any observed toxicity was due to the repeat expansion itself and subsequent DPR production. Toxicity was measured using an MTT assay of cell proliferation.

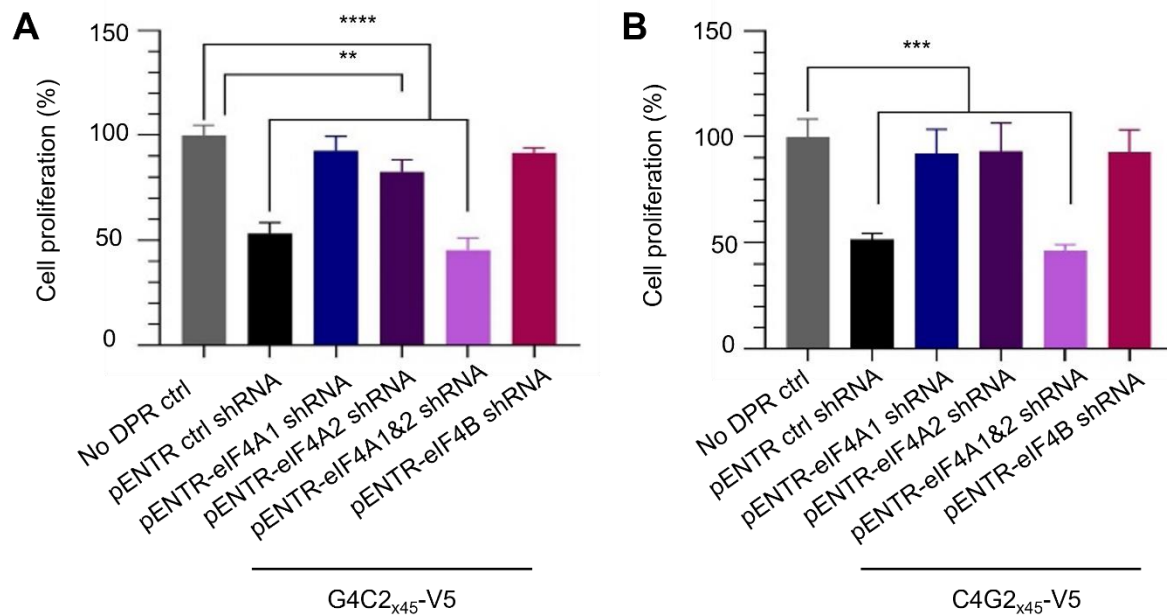


Figure 5.4 MTT assays of HEK293T cells co-transfected with helicase shRNAs and sense or antisense DPRs. HEK293T cells were transfected for 72 hours with V5-tagged sense G4C2x45 (A) or antisense C4G2x45 (B) repeat transcripts and either control-shRNA or shRNAs targeting eIF4A1, eIF4A2, eIF4A1 + eIF4A2, or eIF4B. Performed with three biological replicates; results were quantified in triplicate; mean $\pm$ SEM; one-way ANOVA with Tukey's correction for multiple comparisons. **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

Figure 5.4 shows that in HEK293T cells co-transfected with control shRNA and either sense or antisense DPRs, there is a significant fall in cell proliferation of 50% compared to cells without DPRs. In cells where helicases have been partially depleted by shRNAs, this toxicity is rescued, with cell proliferation returned back to the level of the no DPR control. This is seen only when targeting individual helicases, i.e. eIF4A1, eIF4A2 or eIF4B shRNAs. When a shRNA is used targeting eIF4A1 and eIF4A2 simultaneously, the alleviation of toxicity as measured by cell proliferation is not seen.

Instead, cell proliferation remains decreased by 50%, equivalent to the control shRNA. Results are highly consistent between cells transfected with sense and antisense DPRs.

5.3.2 Non-functional helicase mutants rescue cytotoxic effects of sense and antisense C9ORF72 repeat expansion

5.3.2.1 Q340E / Q339E Mutants

Next, helicase mutants eIF4A1 Q339E and eIF4A2 Q340E were used, to test if these were also associated with rescue of the cytotoxic phenotype associated with sense and antisense DPRs. As described in chapter 3, these have a mutation in their helicase domain rendering their RNA helicase activity non-functional. HEK293T cells were co-transfected with V5-tagged sense or antisense DPR repeats, and either normal or mutant eIF4A1 or eIF4A2. Cell proliferation was used as a measure of toxicity, and results were normalised to those of a control which was untreated with DPRs. Additionally, a FLAG plasmid control was used to determine baseline toxicity associated with co-transfection with DPRs without any alterations to helicases.

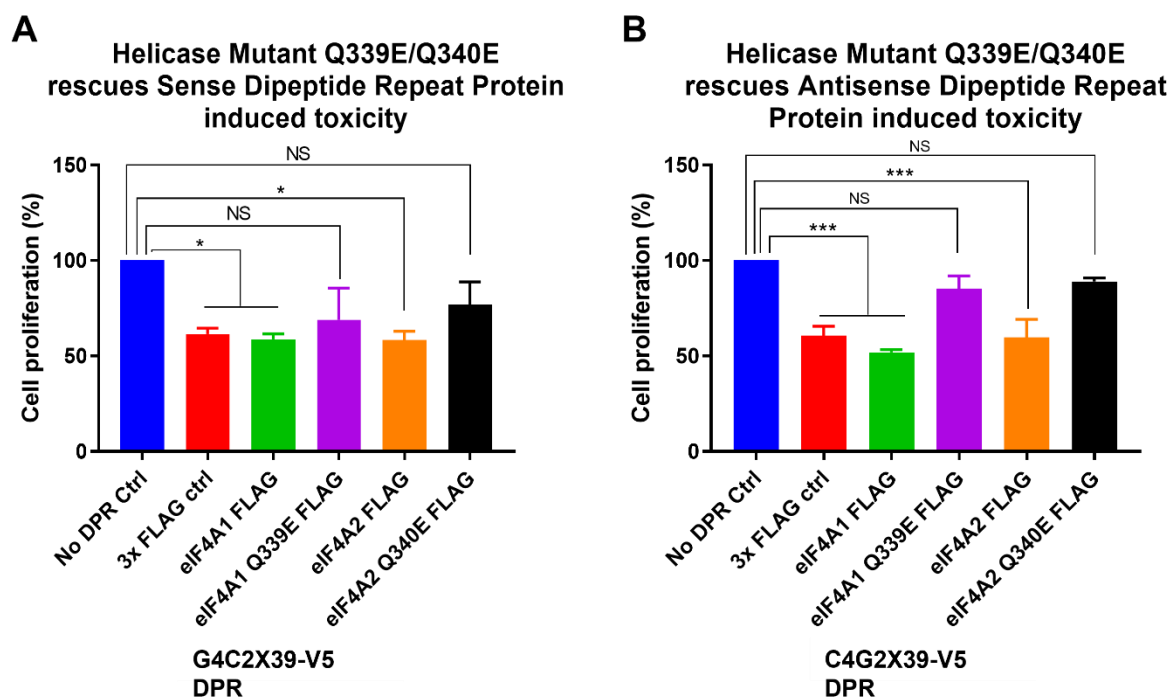


Figure 5.5 MTT Assays of HEK293T cells co-transfected with helicase mutants and sense or antisense DPRs. HEK293T cells were transfected for 72 hours with V5-tagged sense G4C2x39 (A) or antisense C4G2x39 (B) repeat transcripts and either a FLAG control, normally functioning eIF4A1 or eIF4A2, or mutant eIF4A1 Q339E or eIF4A2 Q340E. Performed with three biological replicates. Results were quantified in triplicate; mean $\pm$ SEM; one-way ANOVA with Tukey's correction for multiple comparisons. NS: not significant, *:p<0.05,***:p<0.001

Figure 5.5 shows results from HEK293T cells co-transfected with sense or antisense DPRs and functional or mutant eIF4A1 and eIF4A2. Cells co-transfected with a FLAG control plasmid and sense or antisense DPRs showed a large decrease in cell proliferation, of approximately 40%. In cells co-transfected with DPRs and normally functioning helicases, this fall in cell proliferation is also seen, of between 40-50%. Results are largely consistent between sense and antisense DPRs, although the cell proliferation decrease for antisense DPRs reaches a higher level of statistical significance. For cells co-transfected with sense or antisense DPRs and eIF4A1 or eIF4A2 mutants, toxicity is rescued with cell proliferation increasing to the point where there is no statistically significant difference between these cells and that of the control which lacks DPRs. There is a clear trend towards a greater alleviation of toxicity

associated with antisense DPRs than sense, for both eIF4A1 Q339E and eIF4A2 Q340E.

5.3.2.2 PRRVAA mutants

Next, similar experiments were conducted using PRRVAA mutants, which have a mutation in their ATP-binding domain. As before, HEK293T cells were co-transfected with V5-tagged sense or antisense DPR repeats, and either normal or mutant eIF4A1 or eIF4A2. Cell proliferation was used as a measure of toxicity, and results were normalised to those of a control which was untreated with DPRs. Again, a FLAG plasmid control was used to determine baseline toxicity associated with co-transfection with DPRs without any alterations to helicases.

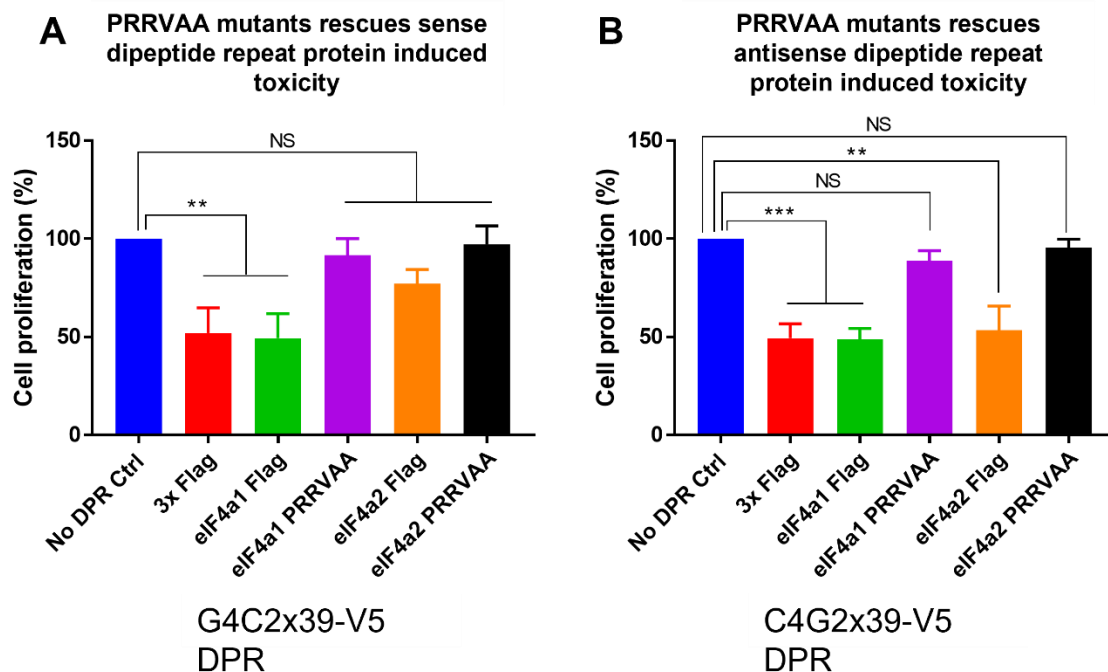


Figure 5.6 MTT Assays of HEK293T cells co-transfected with RNA binding mutants and sense or antisense DPRs. HEK293T cells were transfected for 72 hours with V5-tagged sense G4C2x39 (A) or antisense C4G2x39 (B) repeat transcripts and either a FLAG control, normally functioning eIF4A1 or eIF4A2, or mutant eIF4A1 PRRVAA or eIF4A2 PRRVAA. Performed with three biological replicates. Results were quantified in triplicate; mean $\pm$ SEM; one-way ANOVA with Tukey's correction for multiple comparisons. NS: not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

Figure 5.6 shows results from HEK293T cells co-transfected with sense or antisense DPRs and functional or mutant eIF4A1 and eIF4A2. Cells co-transfected with a FLAG control plasmid and sense or antisense DPRs showed a large decrease in cell proliferation, of approximately 50%. In cells co-transfected with DPRs and normally functioning helicases, this fall in cell proliferation is also seen of approximately 50%. The exception to this was associated with normally functioning eIF4A2 and sense DPRs, where only a small fall in cell proliferation was seen which was not statistically significant. For cells co-transfected with sense or antisense DPRs and eIF4A1 or eIF4A2 PRRVAA mutants, toxicity is rescued with cell proliferation increasing to the point where there is no statistically significant difference between these cells and that of the control which lacks DPRs. There is no difference in alleviation of toxicity associated with antisense DPRs than sense, for both eIF4A1 and eIF4A2 PRRVAA mutants.

5.3.3 Effects of partial depletion of helicases in mouse motor neurons and patient-derived astrocyte co-cultures

Following investigations in HEK293T cell models of C9-ALS/FTD, healthy mouse motor neurons in co-culture with patient-derived astrocytes were used. This model allowed for investigations of motor neuron survival and ALS astrocyte-mediated neurotoxicity (Meyer et al., 2014); (Hautbergue et al., 2017) (Varcianna et al., 2019). Cells were transfected with shRNAs targeting eIF4A1, eIF4A2, eIF4B and eIF4H for 72 hours. Next, iAstrocytes were added to the motor neuron cultures and cells were imaged and motor neuron count assessed. After 48 hours of growth, cells were re-imaged. The use of this live-cell imaging allowed for differences in motor neuron counts to be analyzed, to determine the effects of adding astrocytes on motor neuron survival.

In addition to transfection with helicase shRNAs, SRSF1 miRNA was used as SRSF1 has been of particular interest in this study, and results presented in the previous chapter indicate it may have an upregulation or activation in astrocytes that is relevant in ALS. As a control, a miRNA that did not target any helicases for depletion was used. Motor neuron survival for cells with partial helicase depletion was normalized to that of the control. Initially, only one control patient and one C9-ALS patient line was used, with preliminary results shown in Figure 5.7, below.

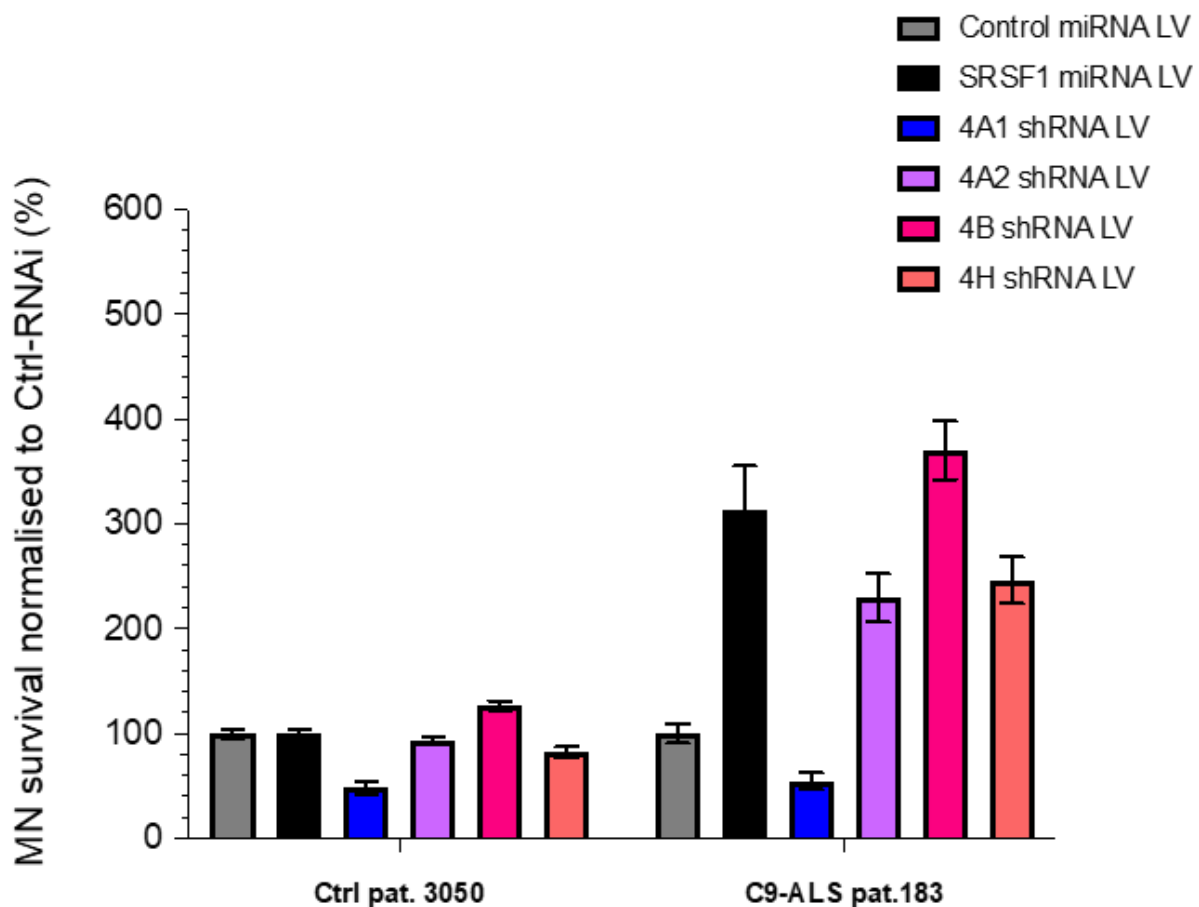


Figure 5.7 Partial depletion of eIF4A1, eIF4A2, eIF4B and eIF4H on healthy mouse motor neurons and patient-derived astrocyte co-cultures. Preliminary data showing one control patient line and one C9-ALS patient line. Cells were transfected with helicase shRNAs and motor neuron survival normalised to that of a control miRNA. This experiment was performed by Dr. Ya-Hui Lin and additional replicates are currently being undertaken.

In these initial experiments shown in Figure 5.7, results are generally consistent with previous HEK293T cell work, with one notable exception of eIF4A1. In the control patient line, SRSF1 miRNA and eIF4A2, eIF4B, and eIF4H shRNA have a very modest

impact on motor neuron survival, or no effect at all compared to control miRNA. Whereas eIF4A1 shRNA leads to a 50% decrease in motor neuron survival. In the C9-ALS patient line, SRSF1 miRNA and eIF4A2, eIF4B and eIF4H shRNAs are associated with a substantial increase in motor neuron survival compared to the control miRNA. eIF4B shRNA is associated with the largest increase, of nearly 300%. This indicates that following addition of iAstrocytes, motor neuron cultures were able to continue growing to a great extent and when treated with eIF4B shRNA in particular their survival was enhanced. This was determined by analysis of motor neuron counts immediately upon addition of iAstrocytes and comparing this to motor neuron counts following 48 hours of growth. In contrast eIF4A1 shRNA leads to a 50% fall in motor neuron survival compared to control miRNA.

5.3.4 Effects of partial depletion of eIF4A1 and eIF4A2 in a *Drosophila* model of C9-ALS/FTD

The final investigations around the effects of partial helicase depletion were carried out in a *Drosophila* model of C9-ALS/FTD. Here, eIF4A was targeted for partial depletion with no differentiation made between eIF4A1 and eIF4A2.

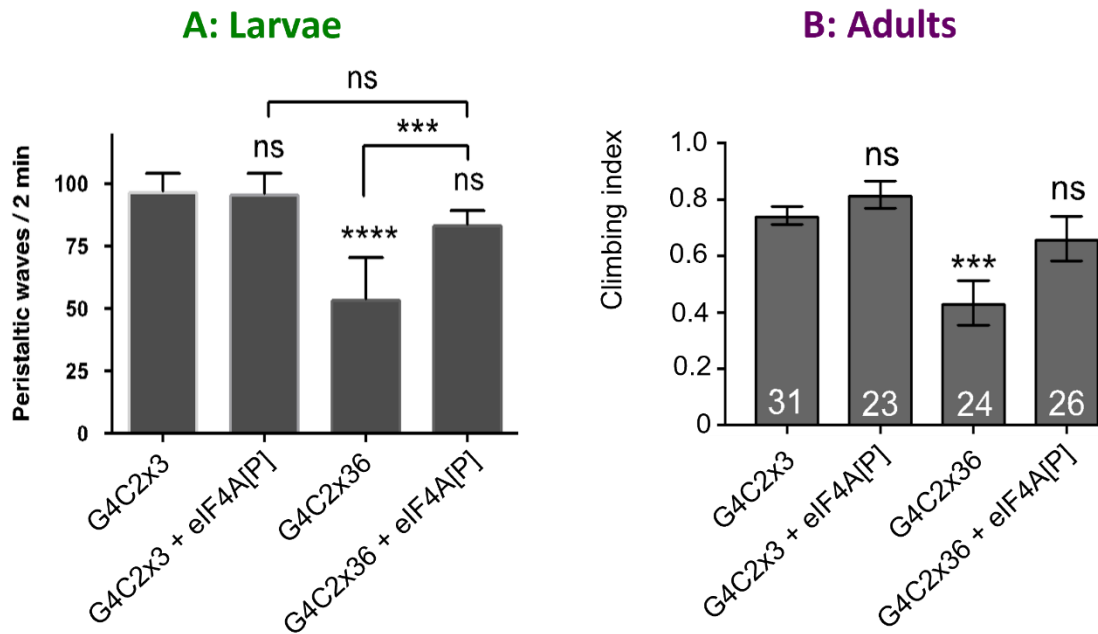


Figure 5.8 Effects of partial depletion of eIF4A in Drosophila. (A) shows larvae where peristaltic waves/ 2 minutes are used as a measure of a neurodegenerative phenotype; (B) shows adult flies where climbing index is used as a measure of a neurodegenerative phenotype. NS: not significant, ***: $p < 0.001$, ****: $p < 0.0001$. This experiment was performed by Dr Alvaro Sanchez-Martinez in Dr Alexander Whitworth's lab (University of Cambridge).

Figure 5.8 results from a Drosophila model with a C9ORF72 G4C2 repeat expansion of 36 repeats, resulting in locomotor deficits in both larvae and adults, indicating a neurodegenerative phenotype. In larvae this is measured through peristaltic waves / 2 minutes, in adults it is measured by climbing index. Control Drosophila were used with only 3 G4C2 repeats, and these did not show the neurodegenerative phenotype associated with the repeat expansion. Depletion of eIF4A by 50% rescued these motor deficits in both larvae and adults indicating that in ALS *in vivo* and *in vitro* models, helicase depletion alleviates C9ORF72 linked neurodegeneration.

5.4 Detection of eIF4A1 positive pathology in patient-derived neuronal lines

For the final set of experiments, three patient-derived neuronal lines were used: one control, one sALS and one C9-ALS/FTD. Details of cell lines used can be found in materials section 2.1.1. We used immunofluorescence staining first to detect TDP-43.

Our aim was to ensure the antibodies we intended to use were specific to C-terminus TDP-43 or the phosphorylated TDP-43 associated with ALS pathology. TUJ1 was used as a neuronal marker. For all cases, results from only one biological replicate were given, as the intention was primarily to demonstrate proof-of-principle regarding cellular localization and presence of pathology for eIF4A1.

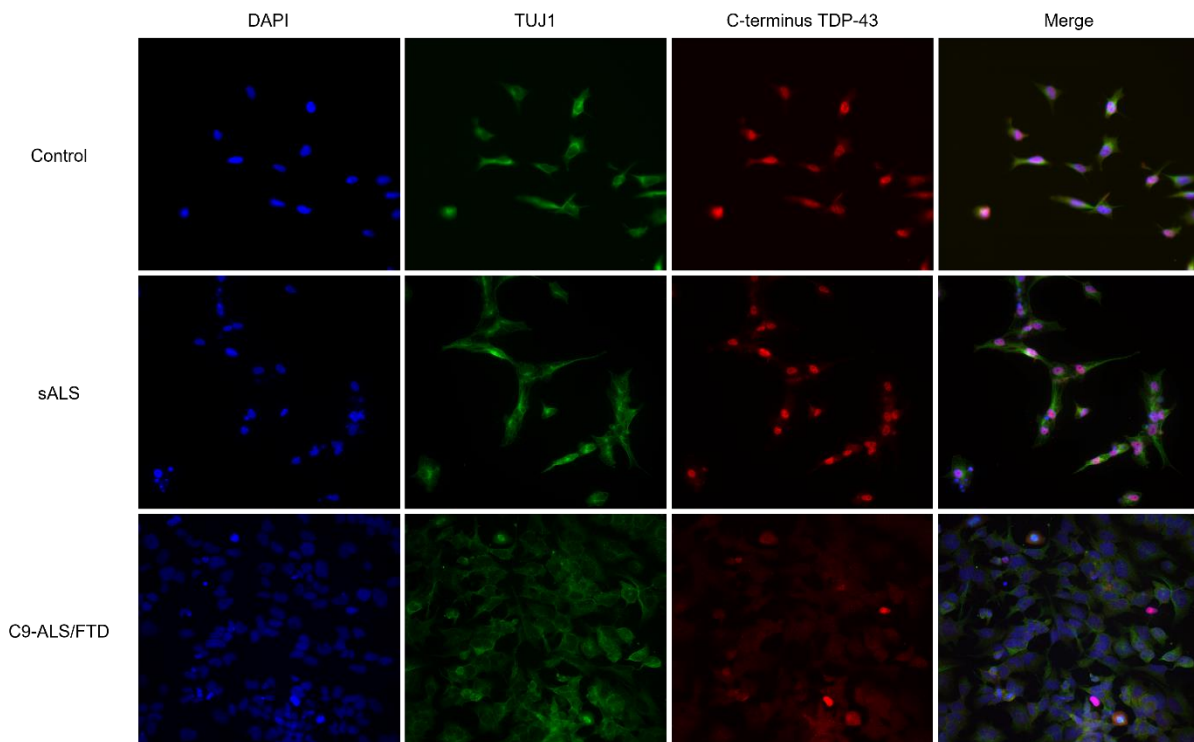
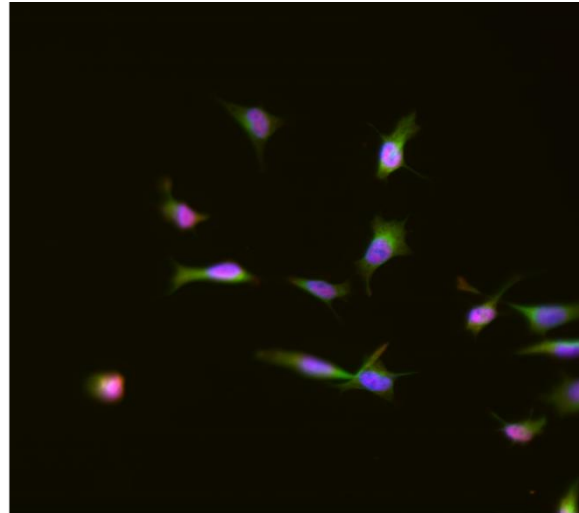
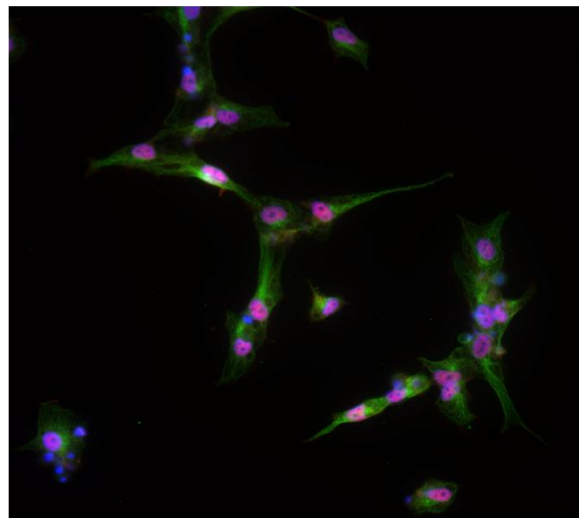


Figure 5.9 Cellular distribution of TDP-43 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons. Representative immunofluorescence images are shown for each neuronal line using antibodies against an epitope in the C-terminal domain of TDP-43 (red), or the pan-neuronal marker TUJ1 (green). DAPI stained the nucleus in blue. Cells were grown by Dr. Ya-Hui Lin and all immunofluorescence staining by Bridget Benson.

Control



sALS



C9-ALS/FTD

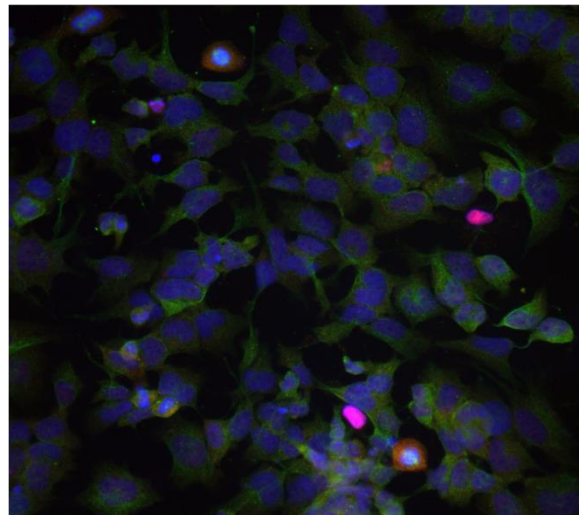


Figure 5.10 Enlarged merged images of cellular distribution of C-terminal TDP-43 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons. Close-up of Figure 5.9 merged immunofluorescence images, shown for controls (top panel) sALS (middle) and C9-ALS/FTD (bottom) neuronal lines using antibodies against C-terminal TDP-43 (red) and pan neuronal marker TUJ1 (green). DAPI stained the nucleus in blue.

As expected, Figure 5.9 shows C-terminus TDP-43 with a nuclear localisation; this can be seen in the merge image co-localised with DAPI. In control neurons, we would anticipate the majority of TDP-43 to be detected by this C-terminus antibody rather than the pathologically, phosphorylated form. This is reflected in Figure 5.9 which shows substantial staining in control neurons of C-terminus TDP-43. This is retained in sALS, but predominantly lost in C9-ALS/FTD neurons, seen in the bottom panel of Figure 5.9. Here, we see minimal C-terminus TDP-43 signal and only a few cells showing co-localisation with DAPI staining indicating the presence of nuclear C-terminal TDP-43.

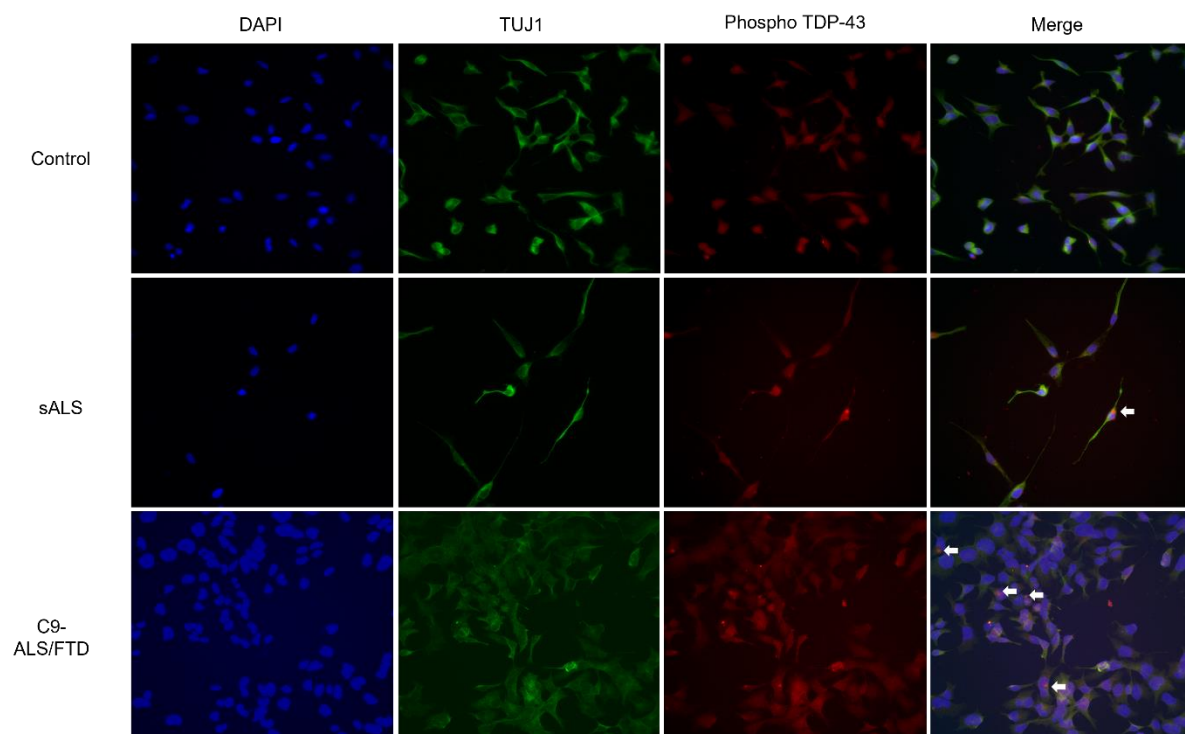


Figure 5.11 Cellular distribution of phosphorylated TDP-43 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons. Representative immunofluorescence images are shown for each neuronal line using antibodies against phosphorylated TDP-43 (red), or the pan-neuronal marker TUJ1 (green). DAPI stained the nucleus in blue. White arrows indicated phosphorylated TDP-43 inclusions. Cells were grown by Dr. Ya-Hui Lin and all immunofluorescence staining by Bridget Benson.

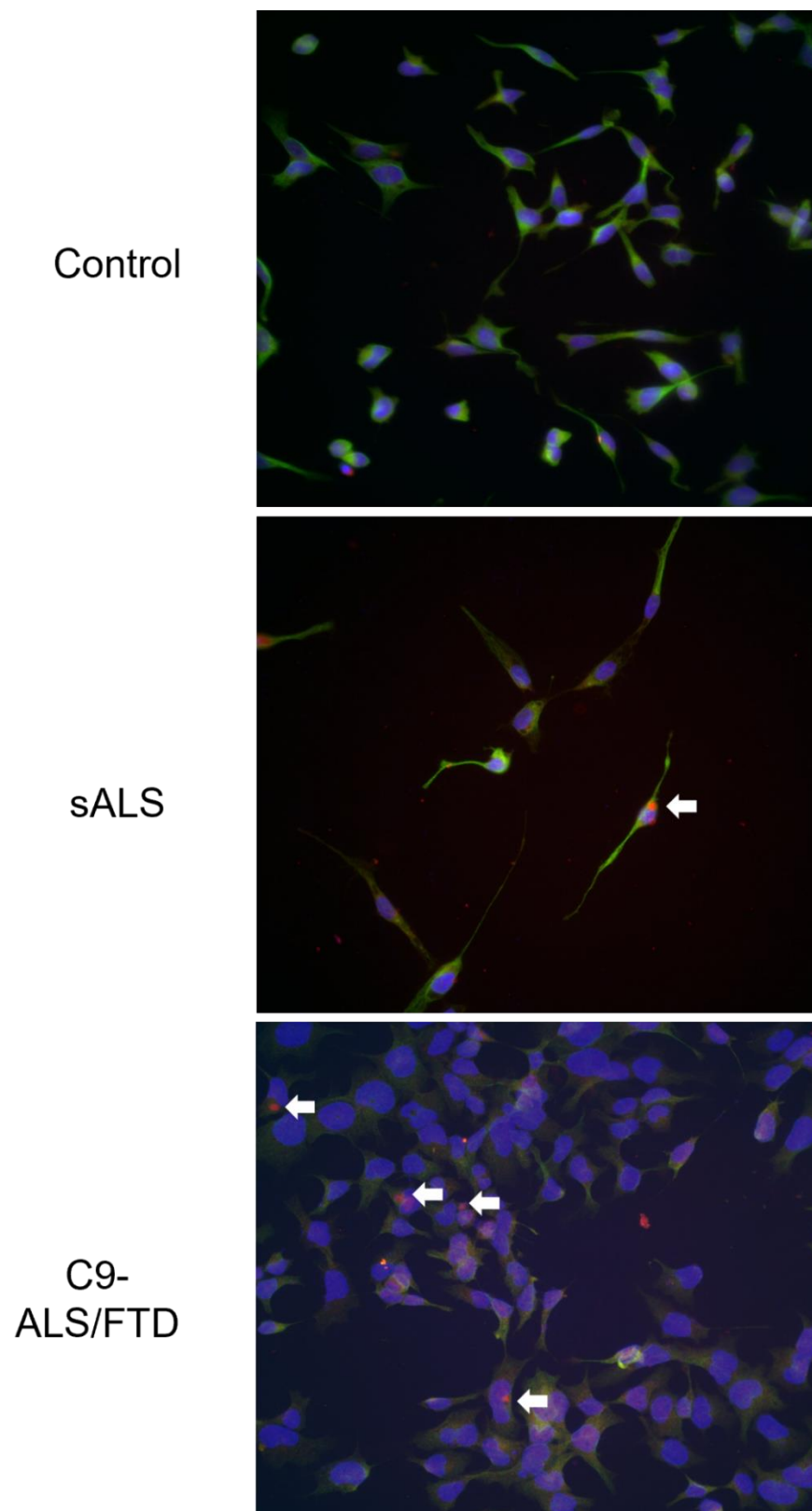


Figure 5.12 Enlarged merged images of cellular distribution of phosphorylated TDP-43 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons. Close-up of Figure 5.11 merged immunofluorescence images, shown for controls (top panel) sALS (middle) and C9-ALS/FTD (bottom) neuronal lines using antibodies against phosphorylated TDP-43 (red) and pan neuronal marker TUJ1 (green). DAPI stained the nucleus in blue. White arrows indicate TDP-43 pathology.

Figure 5.11 shows immunofluorescence staining of pathological, phosphorylated TDP-43. Together with Figure 5.9 we show our antibodies are detecting distinct forms of TDP-43, as here we see the cytoplasmic localization of phosphorylated TDP-43, in contrast to the nuclear localization of C-terminus TDP-43. In sALS and C9-ALS/FTD neurons, but not controls, phosphorylated TDP-43 forms cytoplasmic inclusions which appear as small spherical structures. These are indicated by white arrows in Figure 5.11. Next, we used immunofluorescence to determine if eIF4A1-positive pathology can be detected in these neuronal lines, consistent with results from post-mortem human tissue. TUJ1 was again used as a neuronal marker. We also stained for eIF4A2, expecting to see no inclusions consistent with post-mortem immunostaining.

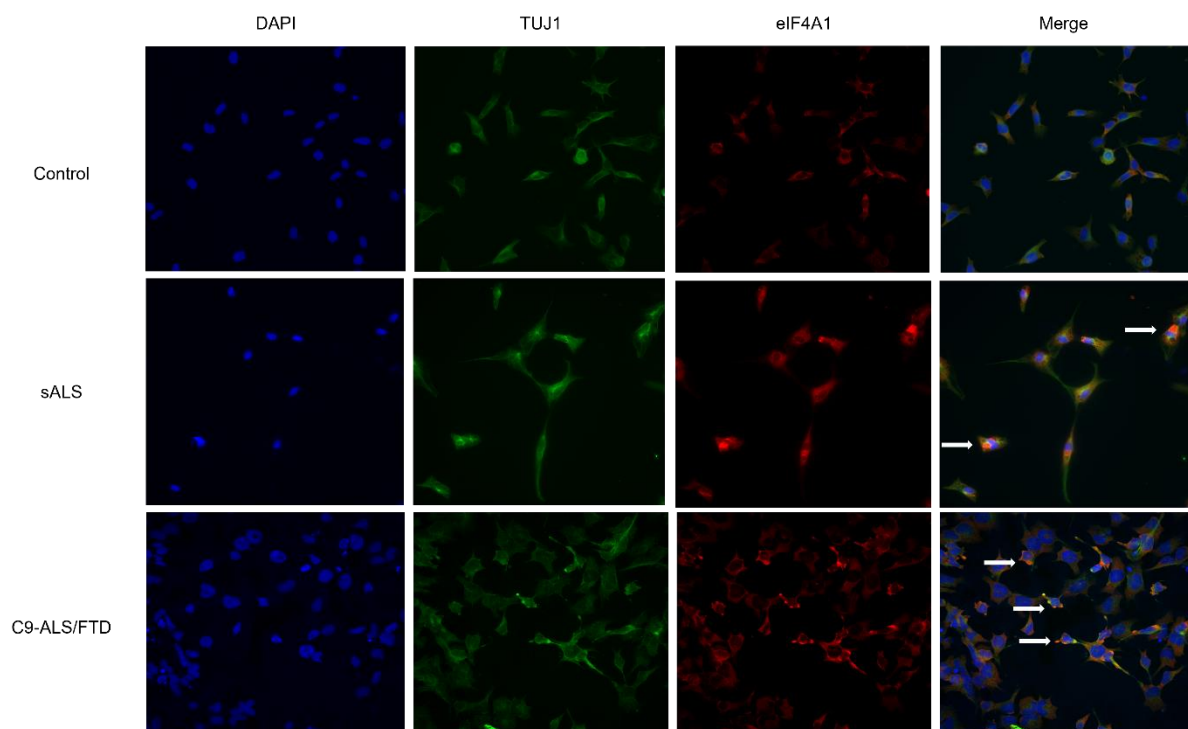


Figure 5.13 Cellular distribution of eIF4A1 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons. Representative immunofluorescence images are shown for each neuronal line using antibodies against eIF4A1 (red), or the pan-neuronal marker TUJ1 (green). DAPI stained the nucleus in blue. White arrows indicate eIF4A1 inclusions. Cells were grown by Dr. Ya-Hui Lin and all immunofluorescence staining by Bridget Benson.

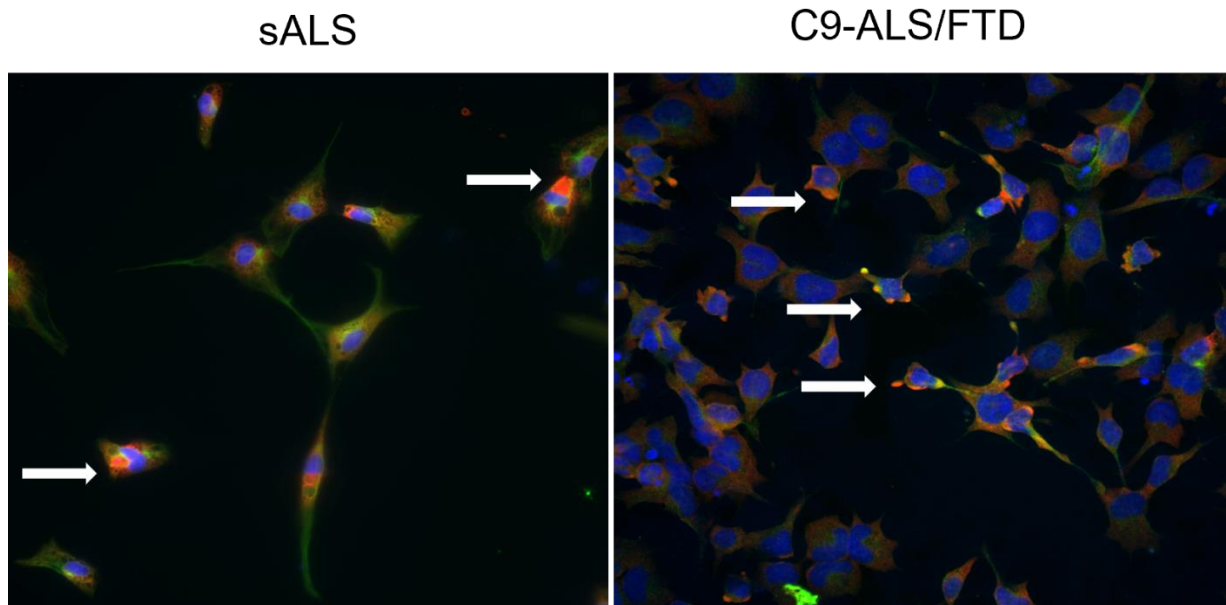


Figure 5.14 Inclusion formation eIF4A1 in sALS and C9-ALS/FTD patient-derived neurons. Close-up of Figure 5.13 merged immunofluorescence images, shown for sALS (left) and C9-ALS/FTD (right) neuronal lines using antibodies against eIF4A1 (red) and pan neuronal marker TUJ1 (green). DAPI stained the nucleus in blue. White arrows indicate eIF4A1-positive pathology. Cells were grown by Dr. Ya-Hui Lin and all immunofluorescence staining by Bridget Benson.

Figure 5.13 confirms that eIF4A1 positive inclusions are detectable in patient-derived sALS and C9-ALS/FTD neurons, but not controls. A larger version of eIF4A1-positive inclusions from Figure 5.13 is shown in Figure 5.14. Similarly to TDP-43 pathology, eIF4A1 inclusions are cytoplasmic and appear as brightly stained spherical structures. White arrows in Figure 5.13 and 5.14 show the location of eIF4A1 positive pathology. It was observed they tended to be larger in the sALS line, but more numerous in the C9-ALS/FTD line.

Figure 5.15 shows that eIF4A2 immunofluorescence staining revealed a cytoplasmic localisation, with no pathological inclusions observed in the control, sALS or C9-ALS/FTD case.

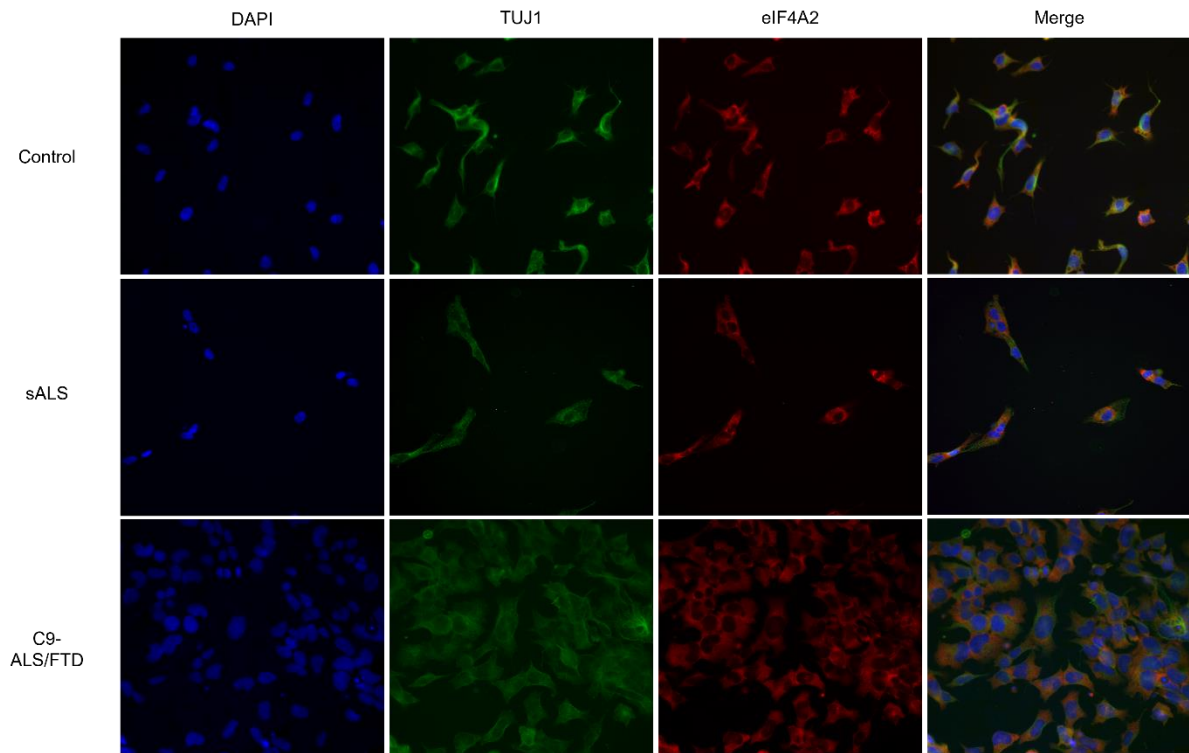


Figure 5.15 Cellular distribution of eIF4A2 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons. Representative immunofluorescence images are shown for each neuronal line using antibodies against eIF4A2 (red), or the pan-neuronal marker TUJ1 (green). DAPI stained the nucleus in blue. Cells were grown by Dr. Ya-Hui Lin and all immunofluorescence staining by Bridget Benson.

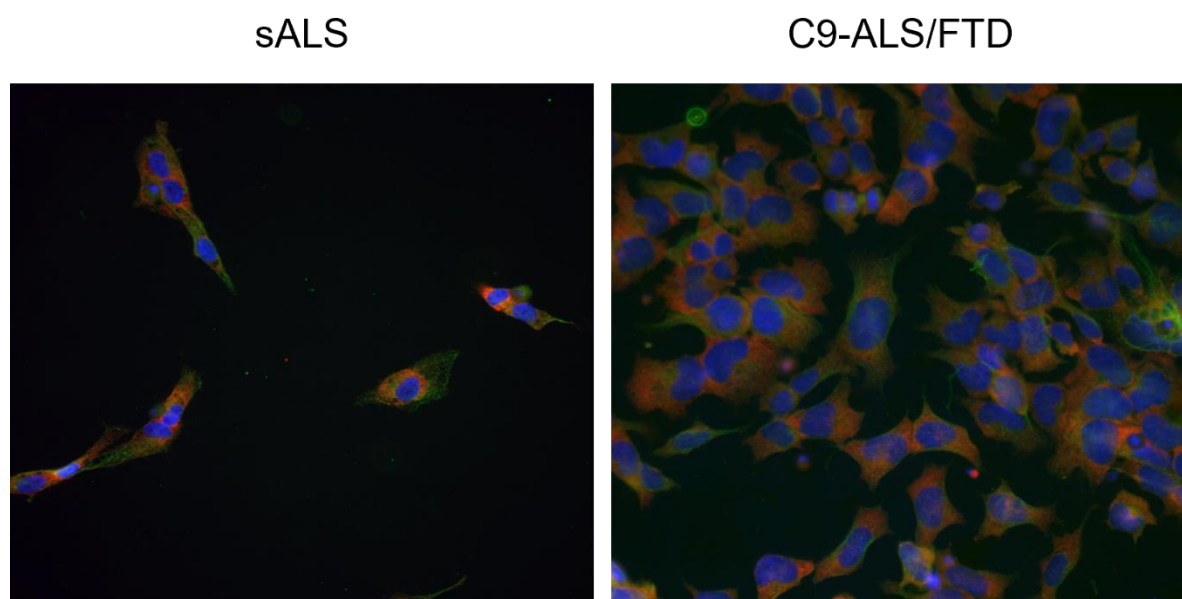


Figure 5.16 Enlarged Merged images showing cellular distribution of eIF4A2 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons. Close-up of Figure 5.15 merged immunofluorescence images, shown for sALS (left) and C9-ALS/FTD (right) neuronal lines using antibodies against eIF4A2 (red) and pan neuronal marker TUJ1 (green). DAPI stained the nucleus in blue.

Following single immunofluorescence staining, which established the specificity of our TDP-43 antibodies and the expected staining pattern of helicases eIF4A1 and eIF4A2, we carried out double staining. This was between phosphorylated TDP-43 and eIF4A1, to determine if pathological inclusions co-localize in these neuronal lines. Double staining between phosphorylated TDP-43 and eIF4A2 was carried out as a control to confirm novel pathology is specific to eIF4A1 in these cell lines.

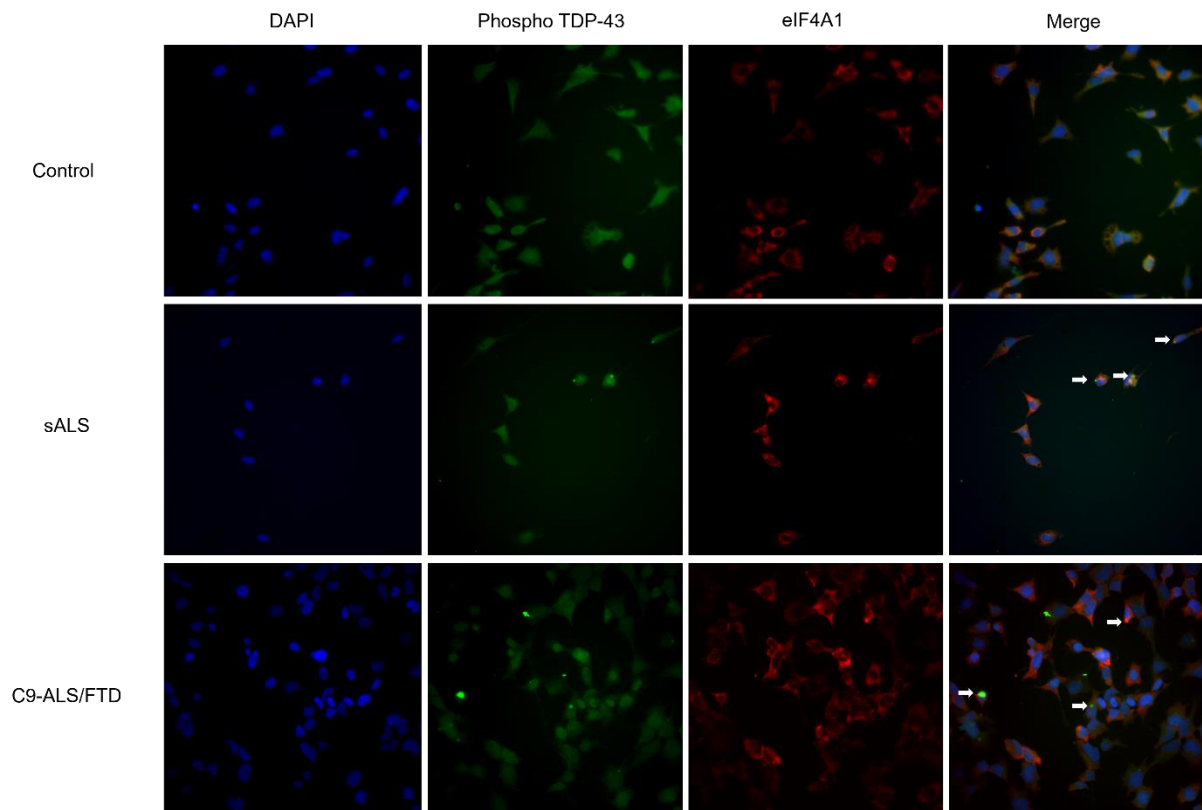


Figure 5.17 Cellular distribution of phosphorylated TDP-43 and eIF4A1 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons. Representative immunofluorescence images are shown for each neuronal line using antibodies against eIF4A1 (red) and phosphorylated TDP-43 (green). DAPI stained the nucleus in blue. White arrows indicate inclusions. Cells were grown by Dr. Ya-Hui Lin and all immunofluorescence staining by Bridget Benson.

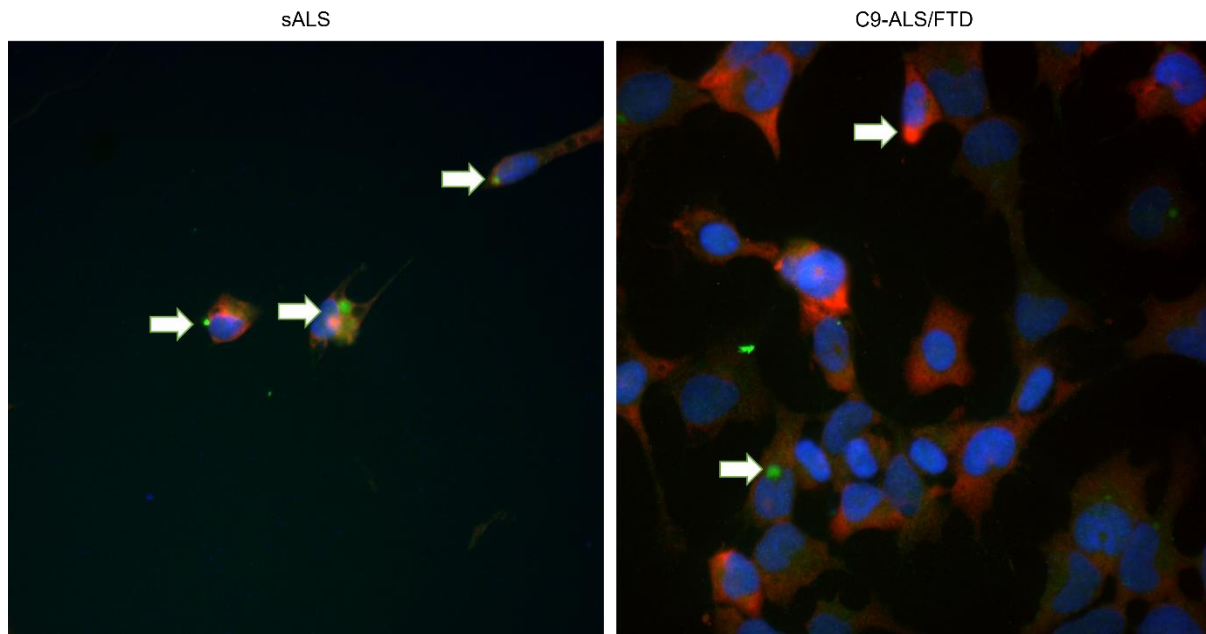


Figure 5.18 Inclusion formation eIF4A1 in sALS and C9-ALS/FTD patient-derived neurons. Close-up of Figure 5.17 merged immunofluorescence images, shown for sALS (left) and C9-ALS/FTD (right) neuronal lines using antibodies against eIF4A1 (red) and phosphorylated TDP-43 (green). DAPI stained the nucleus in blue. White arrows indicate TDP-43 or eIF4A1-positive pathology. Cells were grown by Dr. Ya-Hui Lin and all immunofluorescence staining by Bridget Benson.

Figure 5.17 shows the extent of co-localisation between phosphorylated TDP-43 and eIF4A1. Both TDP-43 and eIF4A1 pathology is visible in sALS and C9-ALS/FTD neurons but not controls, with a close-up image shown in Figure 5.18. Generally, pathology positive for TDP-43 or eIF4A1 is not present in the same neuron. However, on rare occasions both inclusion types are found in the same neuron, we see evidence that the pathology is not co-localized. Inclusions are visualized as small, bright spherical cytoplasmic structures, indicated by the white arrows in Figure 5.17 and 5.18. In sALS neurons, shown on the left panel of Figure 5.18, there is a rare example of a neuron with both inclusion types. Here, the center neuron has two distinct inclusions within the cytoplasm positive for phosphorylated TDP-43 and eIF4A1. These are clearly distinct and non-overlapping. TUJ1, a commonly used neuronal marker, also appears to show inclusions. As a key component on the microtubule network (Mariani et al., 2019) it is likely that these are staining artifacts arising from pathological

disruption to the complex cytoskeleton network. However, further investigation is required to rule out any additional implications.

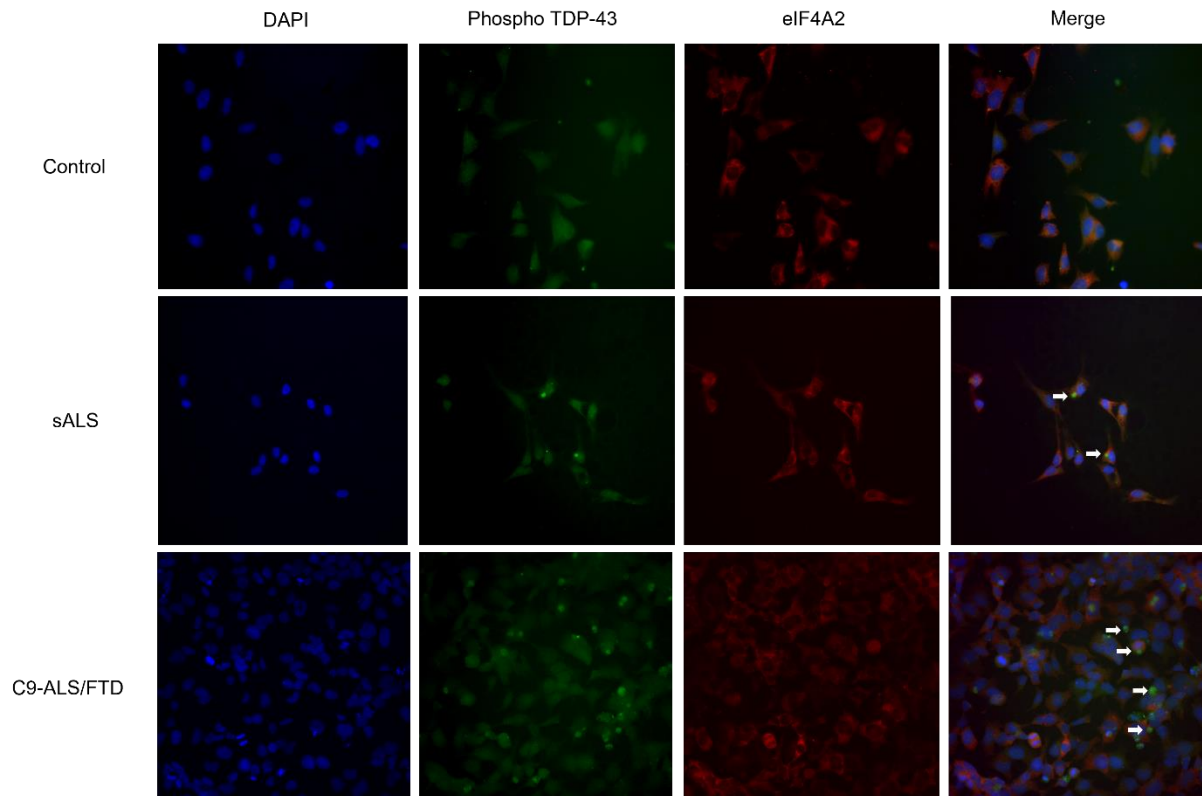


Figure 5.19 Cellular distribution of phosphorylated TDP-43 and eIF4A2 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons. Representative immunofluorescence images are shown for each neuronal line using antibodies against eIF4A2 (red) and phosphorylated TDP-43 (green). DAPI stained the nucleus in blue. White arrows indicate TDP-43 inclusions. Cells were grown by Dr. Ya-Hui Lin and all immunofluorescence staining by Bridget Benson.

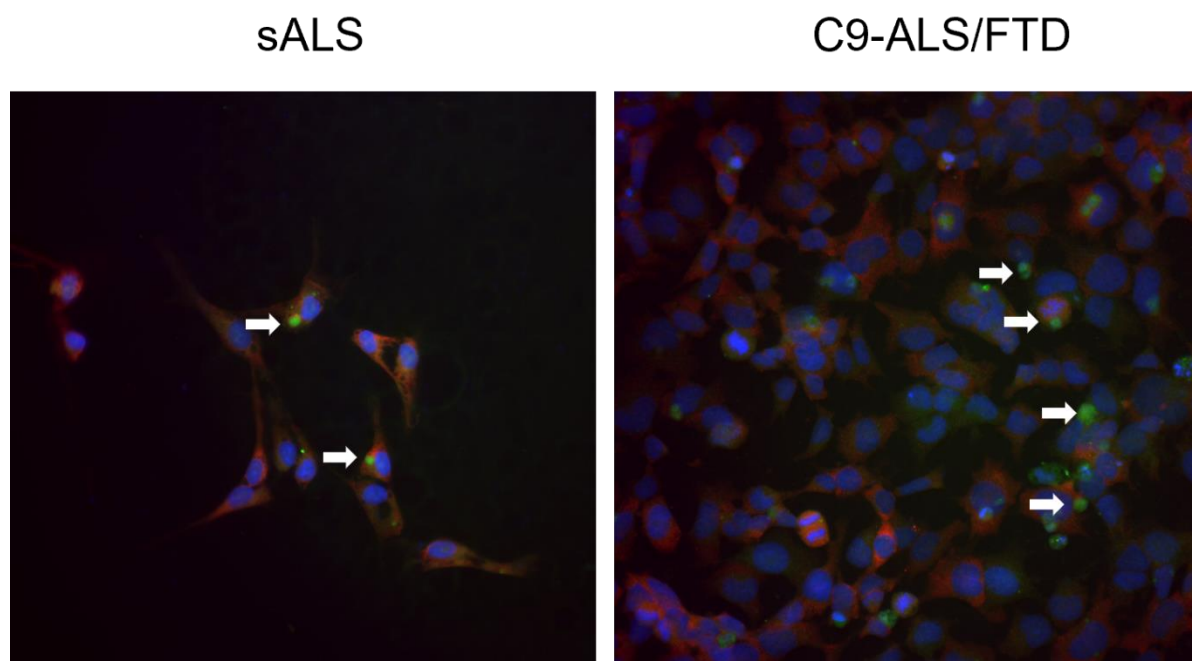


Figure 5.20 Cellular distribution of phosphorylated TDP-43 and eIF4A2 in healthy controls, sALS and C9-ALS/FTD patient-derived neurons. Close-up of Figure 5.19 merged immunofluorescence images, shown for sALS (left) and C9-ALS/FTD (right) neuronal lines using antibodies against eIF4A2 (red) and phosphorylated TDP-43 (green). DAPI stained the nucleus in blue. White arrows indicate TDP-43-positive pathology.

Finally, Figure 5.19 shows results of co-staining between phosphorylated TDP-43 and eIF4A2. Consistent with results seen in post-mortem human tissue, eIF4A2 does not form any cytoplasmic inclusions in any patient neuronal line. TDP-43 pathology is indicated with white arrows.

5.5 Chapter Summary and Discussion

5.5.1 Investigating cytotoxic effects linked to partial depletion of RAN-associated RNA helicases

In this final results chapter, we completed our experiments on RNA helicases through exploration of their associated toxicity and ability to alleviate a neurodegenerative phenotype in *in vitro* and *in vivo* models of C9-ALS/FTD. This work is with a view towards use of these helicases as therapeutic targets. First, we used MTT assays,

which use cell proliferation as a measure of cytotoxicity. We showed that shRNA designed to partially deplete helicases are non-toxic in that they do not significantly decrease cell proliferation compared to control shRNA in a transfected HEK293T cell model of C9-ALS/FTD. As well as initiation of RAN translation, eIF4A1, eIF4A2, eIF4B and eIF4H are responsible for initiation of canonical translation. A potential pitfall in targeting helicases with shRNAs was that if the depletion was too large, the cell's ability to carry out canonical translation of essential proteins would be blocked which would certainly result in cell death. Therefore, as an initial step in designing a therapeutic strategy, it was crucial to rule out the inherent toxicity associated with any depletion of helicases. First, we showed that targeting individual helicases through either LV- or pENTR- shRNAs did not induced cytotoxicity as measured via a cell proliferation assay. As shown in Figure 5.1 LV- eIF4A1 shRNA results in a fall in cell proliferation of approximately 25% compared to control shRNA but this was not statistically significant. This may reflect the central role of eIF4A1 in initiation of both canonical and RAN translation. If eIF4A1 is the key helicase required to initiate translation, the cell is likely to be particularly sensitive to its depletion. This is consistent with work investigating the effects of BLF1, which abolishes the helicase activity of eIF4A and was found to be cytotoxic in cell culture and lethal when given to mice (Cruz-Migoni et al., 2012). However, this paper does not differentiate between eIF4A isoforms which may account for the toxicity seen. There are fewer studies looking at knockdown of eIF4A1 but not eIF4A2, although some from the field of cancer biology reveal that eIF4A1 knockdown is associated with decreased cell proliferation, cell cycle arrest and apoptosis (Liang et al., 2017; Zhang et al., 2022). Further studies are needed to establish the effect of eIF4A1 knockdown in the context of neurodegeneration, but these findings are consistent with eIF4A1's status as a key initiator where depletion will have profound effects on the cell's translational abilities. It remains to be seen if this is a more profound effect than knockdown of eIF4A2 and not eIF4A1.

Alternatively, there may be increased off-target effects and subsequent toxicity associated with the LV-eIF4A1 shRNA in particular, as the pENTR-eIF4A1 shRNA does not show the same trend towards toxicity, as shown in Figure 5.2.

5.5.1.1 Cytotoxicity associated with partial depletion of multiple RAN-associated RNA helicases

Figure 5.2 also reveals that if two helicases, eIF4A1 and eIF4A2, are targeted simultaneously for partial depletion, a significant level of toxicity as measured by cell proliferation is seen. Again, this is likely to be reflective of the importance of these helicases in initiating RAN translation, with depletion of two helicases representing too large of a deficit for the cell to overcome to maintain canonical translation, thereby leading to cell death. This is an interesting and potentially challenging aspect of this therapeutic strategy, in that a balance must be found between depletion of helicases to a great enough extent to halt RAN translation, while maintain canonical translation to a sufficient level for the cell to function properly with no associated cytotoxicity. A better understanding of the exact roles of each helicase in RAN and canonical translation initiation is required for this to be possible, as is a greater understanding of the cells ability to take a flexible approach to translation and compensate mechanistically for the loss of any one helicase. It may be the case that eIF4A1-shRNA has the greatest associated toxicity simply because the cell is less able to compensate for the loss of eIF4A1 function than it is for any other helicase. For this set of experiments, we used shRNA targeting eIF4A1 and eIF4A2 together. Further experiments that could be carried out could target more combinations of helicase. For example, to see the relative effects on cell proliferation of eIF4A1 + eIF4A2 shRNA compared to eIF4A1 + eIF4B shRNA. This would provide evidence about the relative importance of each helicase in translation initiation and suggest any possible compensatory mechanisms that may exist.

5.5.1.2 Stress granule formation as a measure of toxicity associated with partial depletion of RAN-associated RNA helicases

Throughout these studies, MTT cell proliferation assays were used as a measure of toxicity. Future studies could include a translome analysis that would provide a clearer picture of the impact on canonical translation in cells with depleted helicases. Some examples of methods for translome analysis and other related techniques are reviewed in full (King & Gerber, 2016; Stark et al., 2019). We have added a further investigation into cellular stress through investigating TIAR1 stress granule formation associated with helicase depletion. As shown in Figure 5.3, cells treated with sodium arsenate show frequent stress granules indicative of severe cellular stress. This is also the case for cells treated with eIF4A inhibitor BLF1, showing total arrest of the eIF4A initiation complex results in severely stressed cells. This is in contrast to partial depletion of helicases, which results in only small increases in stress granule formation indicating mild stress associated with this strategy. Interestingly, unlike MTT assay results, targeting multiple helicases simultaneously did not result in an increase in stress granule formation compared to targeting individual helicases for depletion. siRNAs targeting multiple combinations of helicases were used, including eIF4A1 + eIF4A2, eIF4A1 + eIF4B, and eIF4A2 + eIF4B. No substantial differences in stress granule formation were associated with any combination.

The depletion most associated with stress granule formation was eIF4A2 siRNA. While this has not been reported before in the literature, another paper has made a link between eIF4A2 and stress granule formation through sumoylation (Jongjitwimol et al., 2016). Here, sumoylation of eIF4A2 occurs on a single residue and increased recruitment to stress granules occurs in response to arsenite treatment, but decreases in response to hippuristanol, an inhibitor of eIF4A1 and eIF4A2. Interestingly, the opposite effect was found for eIF4A1 highlighting the functional divergence between these two proteins in the context of stress granule dynamics. Blocking sumoylation of

eIF4A2 impairs stress granule formation indicating this process plays a key role in the stress response.

5.5.1.3 Partial depletion of RAN-associated RNA helicases rescues DPR-induced deficits *in vitro* and *in vivo*

The final set of MTT assays were used to determine the ability of partial helicase depletion to rescue a neurodegenerative phenotype associated with the C9ORF72 repeat expansion. Two strategies were used as described in chapter 3: pENTR-eIF4A1, eIF4A2, or eIF4A1 + 4A2 shRNAs, and eIF4A1 Q339E / eIF4A2 Q340E mutants. HEK293T cells were co-transfected with shRNAs and either sense or antisense DPRs. A control shRNA was used to establish the level of cell proliferation deficits associated with the DPR production; this was approximately 50%. A further control was included with no transfected DPRs. When helicases were depleted individually with shRNAs, these cell proliferation deficits rose to the equivalent level of the control cells which were not transfected with DPRs. The exception to this was eIF4A1 + eIF4A2 shRNA, where no rescue of cell proliferation deficits was seen. For helicase mutants, similar results were observed. In cells co-transfected with mutant helicases and DPRs, cell proliferation deficits were alleviated to the level equivalent to control. Over-expressed normally functioning helicases were not associated with any increases in cell proliferation. Overall, these results provide strong evidence to support helicases as a necessary component of DPR production by RAN translation. When helicase levels are reduced, either through shRNA depletion, or because a mutation has rendered them non-functional, cells have reduced ability to initiate RAN translation and produce cytotoxic DPRs. Therefore, with reduced levels of helicases the degenerative phenotype associated with the C9ORF72 repeat expansion is alleviated. When using shRNAs, results are highly consistent between cells transfected with sense and antisense DPRs. This consistency was not seen with the helicases mutants. Instead, cells co-transfected with helicase mutants and sense DPRs showed a modest

rescue of toxicity compared to those transfected with antisense DPRs. This may reflect the specific mechanism of the mutation, or it may suggest there could be some alterations in RAN translation associated with sense and antisense transcripts that is worthy of further investigation. If antisense transcripts are more prone to forming secondary structures, they may be more reliant on helicases for RAN translation initiation, and therefore lack of functional helicases will cause a greater alleviation of toxicity. This may not have implications for development of a treatment strategy in that several groups have identified arginine rich DPRs as the most toxic species in C9-ALS/FTD (Radwan et al., 2020). Arginine rich DPRs are produced from both sense and antisense transcripts, so any viable treatment strategy would likely require targeting of both sense and antisense DPR production.

In this chapter, we wanted to introduce a more disease-relevant disease model in addition to previous work carried out in HEK293T cells. Therefore, we next carried out *in vitro* studies of patient-derived astrocytes in co-culture with healthy mouse motor neurons. It is well established that mixing these cell types induces neurodegeneration in the originally healthy motor neurons. We carried out a preliminary motor neuron survival assay on one control and one C9-ALS/FTD patient cell line, investigating the impact of helicase depletion in these cells. While this work requires further experimental evidence, initial results indicate that helicase depletion with shRNAs is neuroprotective in this model, in particular eIF4B depletion. However, only one control and patient line were used so definitive conclusions cannot yet be drawn from this work. Interestingly, the exception to this is eIF4A1 depletion, which showed no associated neuroprotection. This is inconsistent with earlier data provided from HEK293T cell models which showed that partial depletion of eIF4A1 was able to rescue the toxic phenotype associated with the C9ORF72 repeat expansion. The lack of neuroprotection associated with eIF4A1 depletion in this model suggests it is unlikely to be a suitable target for a therapeutic strategy in C9-ALS/FTD. The reason for this is not clear yet, as the roles for eIF4A1 and eIF4A2 remains largely unexplored

in neurons. However, it is likely that it affects the neuron's ability to undertake canonical translation, possibly indicating a lesser role for eIF4A2 or eIF4B compared to eIF4A1. As previously discussed, whole-translatome studies are required to establish the exact nature of the impact on translation ability (King & Gerber, 2016; Stark et al., 2019).

Alternatively, differences in neuroprotection conferred by eIF4A1 depletion may simply reflect alterations in its role in HEK293T cells compared to cells of the CNS. These data could suggest that there are alterations in helicase function associated with different cell types, and that this should be considered when choosing appropriate disease models for these studies. Better understanding of these mechanistic differences in RAN translation initiation in different cell types is crucial to establish for future work in this area. It is not yet clear if all types of RAN translation rely on the same helicases, or if different helicases are required to initiate RAN translation in different contexts. This may be due to differences in mitotic capability in each cell population: post-mitotic neurons may show some alterations in the helicases that initiate RAN translation compared to HEK293T cells.

The final set of experiments centering around the ability of helicase depletion to rescue a neurodegenerative phenotype came from *in vivo* studies using a C9-ALS/FTD *Drosophila* model. Here, neurodegeneration in larvae was measured through peristaltic waves and adults through climbing ability. In both cases, depletion was of eIF4A1 and eIF4A2. No toxicity was observed associated with only 3 G4C2 repeats, but a degenerative phenotype was associated with 36 G4C2 repeats. This was alleviated through partial depletion of eIF4A in both larvae and adults. This is just an initial set of experiments that does not differentiate between eIF4A1 and eIF4A2, so it is limited in what it can suggest about any functional divergence between these helicases, or which one may be a more viable treatment strategy. However, in

combination with *in vitro* data it provides clear evidence that partial depletion of some helicases rescues neurodegeneration consistently, in multiple models of C9-ALS/FTD.

5.5.2 Characterization of eIF4A1-positive inclusions in control and ALS patient-derived neuronal lines

Finally, this chapter shows immunofluorescence staining of control, sALS and C9-ALS/FTD patient derived neuronal lines. The purpose of these studies was to provide additional evidence to support post-mortem data, following the discovery of novel eIF4A1 pathology in ALS spinal cord. With immunohistochemistry, we showed that eIF4A1 positive inclusions frequently co-localised with TDP-43 pathology, but that formation of eIF4A1 pathology was not dependent on TDP-43, evidenced by examples where pathology did not co-localise. These examples were infrequent, and immunofluorescence staining was used to provide additional evidence exploring the co-localization of eIF4A1 and TDP-43 inclusions. First, we performed single immunofluorescence staining for both C-terminal and phosphorylated TDP-43. C-terminal TDP-43 was found to a large extent in controls as expected. It appeared that more C-terminal TDP-43 was found in sALS cases compared to C9-ALS/FTD, where very little C-terminal TDP-43 staining was seen in the nucleus. It was expected that both sALS and C9-ALS/FTD cases would have little C-terminal TDP-43 staining, and that most TDP-43 would be in its phosphorylated mislocalised form as this is considered the classic pathological hallmark of ALS (Neumann et al., 2006). It is unclear if this variation in cell distribution between sALS and C9-ALS/FTD is a meaningful result or simply a reflection of technical variation in how the cells were cultured and stained. Further biological replicates would be required to establish this as these experiments included only one patient cell line per diagnostic group.

Next, we were able to establish that eIF4A1 inclusions are detectable in both sALS and C9-ALS/FTD cell lines. This is promising for future experiments into this novel pathology in *in vitro* models. We observed that in the majority of cases, TDP-43 and eIF4A1 pathology did not appear within the same cell. In the rare occasions that they did appear together, some examples could be found indicative of co-localization between the different inclusions. However, co-localization is not always seen, as in Figure 5.18, where an example is shown of a single cell with distinct, non-overlapping inclusions positive for TDP-43 and eIF4A1. This is consistent with post-mortem data and provides strong evidence that although inclusions can co-localize TDP-43 is not required for formation of eIF4A1 pathology. As this immunofluorescence work was carried out with the intention of demonstrating proof-of-principle that eIF4A1 pathology could be observed *in vitro*, and independently of TDP-43 pathology, only one biological replicate was used. As such, no quantification has been carried out regarding the percentage of eIF4A1-positive inclusions that form independently of TDP-43. An important next step for this project will be to introduce quantitative analysis. With a minimum of three biological replicates, numbers of TDP-43 and eIF4A1-positive inclusions should be counted in these cell lines, and the percentage of co-localisation calculated between the two inclusion types.

5.5.3 Conclusions

Overall, in this chapter we have shown data providing some insight into mechanisms of RAN translation initiation by helicases in multiple models of C9-ALS/FTD. We have shown evidence to support use of helicases as a viable therapeutic strategy, including that depletion of individual helicases is non-toxic and only induces mild cellular stress. We show that depleting helicases or use of non-functional helicase mutants is able to rescue the toxic phenotype associated with C9-ALS/FTD both *in vitro* and *in vivo*. This supports our initial hypothesis that helicases are necessary for initiation of RAN translation and that targeting them is a viable way to reduce DPR production and their

associated toxicity. In the final chapter of this thesis, we will review all evidence presented thus far and explore in more depth its implications for a therapeutic strategy surrounding targeting of helicases.

6 Discussion and Conclusions

6.1 Overview of project aims and hypothesis

The main hypothesis for this project was that targeting helicases involved in translation initiation would confer potential novel therapeutic strategies of neuroprotection in C9-ALS/FTD through preventing RAN translation of neurotoxic DPRs. This final discussion and conclusions chapter will review the main findings of this project, discuss to what extent the work presented supports the original hypothesis adding key findings to the scientific literature, and explore next steps for this research. The helicases included in this project were eIF4A1, eIF4A2, their co-factors eIF4B and eIF4H, and DHX36 although the latter was restricted to initial cell work only. Additionally, the section of the project characterising RAN translation factors in post-mortem human brain and spinal cord was expanded to include splicing factor SRSF1 due to its crucial role in the production of DPRs.

This work contributes new knowledge to the field in a number of ways: although other groups have successfully targeted proteins involved in translation initiation as a way to prevent RAN translation of DPRs in C9-ALS/FTD (Cheng et al., 2019; Goodman & Bonini, 2019), this is the first time such an approach has been explored for the selective targeting of eIF4A1 and eIF4A2 isoforms as well as their co-factors. The project takes a novel approach through including both sense and antisense DPRs, as the majority of work into RAN translation in C9-ALS/FTD has focused on DPRs derived from sense transcripts only. Additionally, eIF4A1 and eIF4A2 were investigated as separate isoforms, in contrast to other groups working on these helicases. This approach came to fruition especially during the post-mortem tissue section of the work where eIF4A1, but not eIF4A2-positive inclusions were found in ALS spinal cord motor neurons. This is the first time this novel pathology has been reported, and the first time any member of the eIF protein family of eukaryotic translation initiation factors has been reported as able to aggregate in any neurodegenerative disease.

This work was carried out with clinical translation in mind. The project hypothesis was formulated with a view to making targeting helicases the core of a future therapeutic strategy in C9-ALS/FTD, and this translational approach has informed the direction of the work. Overall, the goals for this project have been achieved in that this work has demonstrated that targeting these helicases can successfully reduce DPR production without conferring toxicity. This work could form the basis for future studies continuing to explore the viability of this approach for treating C9-ALS/FTD.

6.2 Targeting RNA helicases reduces DPR production in cell models of C9-ALS/FTD

The initial aim for the project to develop tools to target helicases. A series of shRNAs were cloned into various vectors for testing on pre-clinical cell models of C9-ALS/FTD. First a simple HEK293T cell model was used followed by a more disease-relevant model of healthy mouse motor neurons in co-culture with patient-derived astrocytes. shRNAs were tested for their ability to deplete their target protein at both protein and mRNA level through western blotting and RT-qPCR. In addition, helicase dead mutants for eIF4A1 and eIF4A2 were used as a way to test the impact of non-functional helicase activity without reducing overall helicase expression. Targeting helicases through shRNAs or use of RNA helicase dominant negative mutants was associated with reductions in both sense and antisense DPRs.

For helicase mutants and shRNAs, there was no substantial difference in the relative decrease in levels of sense vs antisense DPRs. This may indicate shared mechanisms in their production, at least relating to helicase activity. Loss of RNA helicase activities of eIF4A1 and eIF4A2 mutants is associated with loss of DPR production, indicating that unwinding the secondary structures of *C9ORF72*-repeat transcripts is critical to RAN translation as postulated in the hypothesis.

Use of shRNAs has also provided clear evidence for eIF4B's role in RAN translation, as depleting this helicase consistently led to falls in DPR production. In initial experiments using mammalian expression of shRNAs designed in the lab, DPR levels decreased by approximately 50% - 70%. There was no single helicase that stood out as leading to a greater fall in DPR production when depleted with shRNAs, instead they all resulted in similar levels of DPR production decreases. Unexpectedly, eIF4A1+eIF4A2 shRNA did not cause a greater decrease in DPR production than eIF4A1 or eIF4A2 shRNA alone. Unfortunately, there is a lack of published data looking at other combinations of double helicase knock-downs, although this would be a very informative future study to carry out. This intriguing result suggests a high level of flexibility associated with RAN translation initiation, functional redundancy or some tissue or cell-specific differences between eIF4A1 and eIF4A2 helicase activity. Alternatively, poor efficacy of shRNAs or poor transfection efficiency. This informed the purchases of commercial LV plasmids, as they had a guaranteed efficacy.

Over expression of wild-type RNA helicases showed no increase in RAN translation of DPRs, demonstrating that although they are necessary for RAN translation initiation, they are not the rate-limiting step. Work on other helicases shows contrasting results, for example that increasing expression of helicase DDX3X reduces DPR production (Cheng et al., 2019). Cells were treated with the small molecular RK-33 which inhibits DDX3X's helicase activity and RAN translation products showed a dose-dependent increase despite no change in overall DDX3X levels. Similarly to the eIF4A1 and eIF4A2 helicase mutant work shown in this thesis, this provides strong support to the hypothesis that it is the helicase activity specifically that modulates RAN translation. Therefore, it is this helicase activity in particular that should be targeted for any future therapeutic approach, rather than some other activity or feature of this class of proteins. That helicase activity is necessary also informs a therapeutic approach as it shows that RNA secondary structures are an essential factor influencing RAN translation

initiation. Cheng and colleagues performed genome-wide CRISPR/Cas9 screens in human cells to identify DDX3X as a modifier of RAN translation without effecting global protein synthesis. This has important implications for this project as it demonstrates a proof-of-principle that interventions targeting helicases can impact RAN translation while preserving canonical translation. The contrary action to eIF4A and its co-factors also highlights the crucial need to better understand the action of helicases and their multiple roles and modes of action in the context of RAN translation as a crucial step in designing an effective therapeutic strategy.

Unlike the data presented in this thesis, whereby depletion of eIF4A, eIF4B, eIF4H and DHX36 activity reduced RAN translation of DPRs, in the case of DDX3X its activity is required for repression of RAN translation. Instead of reducing DPR production, reducing DDX3X increases DPR production and associated toxicity. Elevating DDX3X levels reduces DPRs, alleviates associated toxic effects and improves survival in C9-ALS/FTD patient cells (Cheng et al., 2019). Interestingly, changes to DDX3X levels did not lead to changes in cell proliferation, apoptosis, increased stress granule formation or elevated phospho-eIF2 α in a reporter HeLa cell model. This suggests DDX3X's effects on RAN translation are not linked to the ISR (Cheng et al., 2018). Cheng and colleagues have suggested that DDX3X alters repeat RNA structures to prevent RAN translation occurring (Cheng et al., 2019). This is contrary to a previously proposed mechanism that DDX3X destabilises secondary structures to promote translation (Soto-Rifo et al., 2012). Interestingly, this was found to not be mediated through G-quadruplexes and may instead be through other structures such as hairpins. A recent study reported that hairpin structures are involved in RAN translation of C9-G4C2 repeat RNA (Wang et al., 2019a). This is unlike other closely related helicases such as DHX36. DHX36 was found to act as a strong positive regulator of RAN translation and shows preferential binding to G4C2 repeat RNA G-quadruplexes. DHX36 promotes RAN translation through resolving these G-quadruplexes (Liu et al., 2021) which is consistent with the findings in this thesis that show DHX36 depletion

reduces DPR production. Liu and colleagues also found DHX36 was elevated in patient tissues. Western blots from frozen human motor cortex in this thesis showed a slight increase in DHX36 levels in ALS compared to control samples but cannot be said to support Liu's findings as this did not reach statistical significance. This may be attributable to significant variation in samples arising from lack of consistency in tissue collection, processing and storage, and should be repeated with a larger cohort to provide a more definitive result.

DDX3X was found to bind directly to G4C2 sense repeats, but not C4G2 antisense repeats (Cheng et al., 2019). This is of particular relevance to this work, as most of the published literature only considers sense DPRs and neglects antisense. Cheng's work demonstrates that production of sense and antisense DPRs do have different underlying mechanisms. Additionally, another study looking at DHX36 found that C4G2 antisense RNA does not form G-quadruplexes and does not interact with DHX36 (Liu et al., 2021). While the sense and antisense results were more consistent in this thesis, some subtle differences were seen that should be investigated further in light of these findings. Liu's work is in contrast to some of the results relating to DHX36 in chapter 3, for example shRNA-induced depletion of DHX36 led to statistically significant decreases in both sense and antisense DPR production. If Liu's findings are correct DHX36 must have an additional mechanism through which it reduced antisense DPRs, for example through resolution of another secondary structure other than G-quadruplexes. It may be the case that the helicases involved in this project, or other RAN-associated helicases, have different modes of action on sense and antisense transcripts. This adds an extra layer of complexity in understanding RAN translation as helicases may show differential activity either promoting or inhibiting RAN translation as well as differences associated with sense or antisense transcripts. It may also be the case that eIF helicases, DDX3X, and others may modulate DPR levels in a manner that varies depending on region or cell type. This may have important implications for use of targeting helicases in a therapeutic strategy. This is

especially relevant if only certain DPR species, for example arginine-rich DPRs, are targeted. Overall, this work on eIF4A1, eIF4A2 and their co-factors helps begin to advance our understanding of the complex interaction of multiple RNA helicases in initiating RAN translation.

6.3 Selective depletion of RNA helicase isoforms is non-toxic and rescues DPR-induced defects in cell proliferation and motor neuron survival

The next aim of the project was to establish if targeting helicases was associated with cytotoxicity. This was crucial to establish as the project takes a translational approach with a view to developing the basis for a therapeutic strategy for C9-ALS/FTD. The concern was that any helicases initiating RAN translation were also responsible for initiating canonical translation and impacting the cell's ability to synthesis the proteins it needs for regular function would certainly be highly toxic. Therefore, the strategy was to deplete helicases by approximately 50% rather than a full knock-down, as well as depleting only one isoform (eIF4A1 or eIF4A2). The hypothesis was that this strategy would be adequate to reduce RAN translation of DPRs while preserving canonical translation and would therefore alleviate DPR-induced toxicity without being toxic itself. The impact on canonical translation was not directly investigated in this work and instead measurements of cytotoxicity were used as an indirect measurement, as if canonical translation was blocked this would undoubtedly cause arrest of cell proliferation. The measure of cytotoxicity used in these studies was MTT assays of cell proliferation. The data presented here supports these hypotheses. Targeting individual helicases both reduced DPR production and was non-toxic suggesting that it is possible to find the balance between reducing RAN translation while leaving canonical translation uninhibited. This is a promising result for use of this strategy as a therapy for C9-ALS/FTD. It also provides additional insights into underlying mechanisms as this work demonstrates helicases are not the rate-limiting factor in RAN translation, given that over-expression of helicase did not increase DPR

production. However, it is possible different helicases show differential activity on different mRNA transcripts. They may alter translation in specific ways driven by specific RNA interactions, or dependent on sense or antisense transcripts of various lengths or concentrations. Further work is needed to establish RNA-specific differences in helicase action to better inform the most effective therapeutic approach.

When helicases are targeted simultaneously, a significant reduction in cell proliferation is seen. Only eIF4A1+eIF4A2 shRNA was tested, so the impact of depleting other combinations of helicases is unknown. Additional investigations were carried out into stress granule formation, and here targeting individual or multiple helicases is not associated with any significant increase in stress granule formation. MTT assays could be argued to lack some relevance to a non-proliferative cell population such as neurons, so this is an important result providing further support for helicase depletion as a non-toxic method to reduce DPR production. MTT assays measure mitochondrial activity, so as an alternative the activity of individual mitochondrial respiratory complexes could be assayed to use alterations in metabolic activity as a measure of toxicity (Li et al., 2020). Neuronal survival assays could also be used, which simply calculate the number of viable motor neurons three days post-treatment as a percentage of the pre-treatment viable cells (Varcianna et al., 2019). Longitudinal live-cell imaging using GFP-tagged constructs is an effective and commonly used technique to evaluate *in vitro* toxicity (Wen et al., 2014). Other measures of *in vitro* and *in vivo* toxicity commonly used in C9ORF72 research are reviewed in depth by St. Martin and colleagues (St. Martin et al., 2020).

Data from patient-derived astrocytes in co-culture with healthy mouse motor neurons is not fully consistent with these HEK293T cell results. In co-culture, eIF4A1 depletion reduced motor neuron survival whereas depletion of eIF4A2, eIF4B, eIF4H or SRSF1 was neuroprotective. This indicates there are cell-specific differences in eIF4A1 activity and/or requirement, and that in motor neurons the role of eIF4A1 appears too

crucial for the cell to tolerate any depletion. It also supports previous work in the scientific literature indicating that there are functionally divergent roles between eIF4A1 and eIF4A2. eIF4A1 promotes proliferative and oncogenic functions while eIF4A2 is involved in miRNA-dependent repression of translation and control of pluripotency (Li et al., 2022; Lu et al., 2014; Meijer et al., 2013; Meijer et al., 2019; Modelska et al., 2015). Henceforth researchers should build this into their study design when conducting work in this area. It has been suggested that in addition to the role in translation initiation, eIF4A1 has an RNA chaperone function relating to limiting inappropriate RNA interactions, modulating RNA condensation and limiting recruitment of RNAs to stress granules, thereby preventing stress granule formation (Tauber et al., 2020). It is possible that it is this role that underpins the toxicity associated with eIF4A1 depletion in preliminary co-culture experiments. Although, these co-culture experiments require further biological repeats and validation to confirm that this toxicity associated with eIF4A1 depletion is a true and consistent result.

Further evidence to support use of helicases in treating C9-ALS/FTD is through experiments showing depleting helicases rescues DPR-induced toxicity, measured through the MTT cell proliferation assay. Antisense DPRs show a larger, more significant rescue than is associated with sense DPRs, indicating some differences in RAN translation of sense and antisense transcripts. Importantly, this work was supported in the third results chapter with *in vivo* work using a *Drosophila* model of C9-ALS/FTD. Consistent with results from cell models, depleting helicases in both larvae and adult flies rescued a neurodegenerative phenotype associated with DPR production. Despite lacking a *C9ORF72* homolog, *Drosophila* are considered a good model for C9-ALS/FTD due to their low cost, short lifespan, a genome that is easy to manipulate and track, and the ability to detect and measure a range of complex behaviours (Sharpe et al., 2021). In terms of helicases, eIF4A has a *Drosophila* orthologue (Li & Li, 2006) as do eIF4B and eIF4H (Goodman et al., 2019). Arguably,

Drosophila are as good or better an animal model for studying helicases in C9-ALS/FTD as any other. The *Drosophila* genome has been extensively sequenced and approximately 75% of human genes have an orthologue. Combined with the well-established protocols to perform genetic manipulation or large-scale genetic screens (Sharpe et al., 2021), it is likely that in the future this model could be used to identify more modifiers of RAN translation.

6.4 eIF4A1 forms cytoplasmic inclusions in sALS and C9-ALS/FTD spinal cord motor neurons

This thesis has two main elements: *in vitro* cell models of C9-ALS/FTD exploring helicases in RAN translation, and characterisation of these helicases through immunohistochemistry in post-mortem human brain and spinal cord. This led to the interesting and previously unreported discovery of eIF4A1-positive inclusions in the cytoplasm of sporadic and C9-ALS/FTD spinal cord motor neurons. This was an unexpected finding, as immunohistochemistry studies were initially commenced with the aim of characterising the distribution and localisation of key helicases involved in RAN translation initiation across spinal cord and CNS areas most relevant to ALS and FTD.

No evidence of eIF4A1-positive pathology was found in any other brain region, in any control case, and no other helicase showed positive inclusion staining. It is not clear at this stage why eIF4A2, which shares 96% homology with eIF4A1 and mainly differs by approximately 10 amino acids in the N-terminal region, does not form pathological inclusions in the ALS spinal cord. The answer to this is unclear from this work, but at the core is a fundamental question of why some proteins aggregate and others do not. The factors that influence protein aggregation are being explored by multiple groups. These may be related to protein structure and how this confers properties that make proteins more prone to aggregation, contributions such as chaperones, post-

translation modifications, and external factors such as stress that can influence protein misfolding and aggregation dynamics, and the ability of aggregates to accumulate and avoid degradation pathways. It may also depend on eIF4A1 interactions with different protein factors or RNAs with diverse biological functions (Bäuerlein et al., 2020; Chandel et al., 2018; De & Kleenerman, 2019; Koopman et al., 2022). Recent studies have investigated crystal structure of eIF4A1: RNA complexes (Naineni et al., 2021), or used structure-seq2 and polysome profiling to better understand eIF4A translational mechanisms (Waldron et al., 2019). This has provided important insights into eIF4A activity, for example revealing that eIF4A remodels local 5'UTR structures, and these act as the major limiting factor in translation. eIF4A1 activity is not equal across all mRNAs, and increased propensity for secondary structure formation drives the dependence of eIF4A1 activity for unwinding (Waldron et al., 2019). While these studies are mechanistically informative, they have not yet provided any insight into why this helicase in particular forms aggregates while other similar helicases do not. Further structural based studies should be carried out to consider the features of the eIF4A1 protein that promote increased propensity for aggregation, and other factors that lower the threshold for aggregation to occur. It is crucial to provide an answer explaining the unique mechanisms, structure, and regulators that causes eIF4A1 to aggregate but not eIF4A2 or other helicases.

The data in this thesis provides strong evidence that eIF4A1 pathology is specific to ALS spinal cord, but also raises questions as to why this pathology is not seen elsewhere. This is in contrast to TDP-43 and DPR pathology, which is found across many cortical regions in addition to the spinal cord (Freibaum & Taylor, 2017). The other hallmark ALS pathology other than TDP-43 is the presence of Bunina Bodies (Okamoto et al., 1993), these have a cytoplasmic localisation in motor neurons of the spinal cord but were not investigated for co-localisation with eIF4A1-positive inclusions in this study. In C9-ALS/FTD specifically, the presence of DPR-containing inclusions are seen in the hippocampus, cerebellum and neocortex (Mackenzie et al., 2015). No

evidence of co-localisation with eIF4A1-positive inclusions was observed, nor did they appear to even form in the same brain region. This is expected as the eIF4A1-positive inclusions were not specific to the *C9ORF72* repeat expansion and also appeared in sporadic cases. There is some inconsistency in views regarding which area is most important in ALS and which brain or spinal cord region is the origin of motor neuron degeneration (Ragagnin et al., 2019). Traditionally the spinal cord was seen as the most biologically relevant area, which would be consistent with this as the only site of eIF4A1 pathology, while others in the field consider the motor cortex the disease point of origin. eIF4A1 pathology formation could be linked to other ALS-related dysfunctions known to occur predominately in the spinal cord. For example, neuroinflammation has been linked to various brain regions in ALS (Masrori et al., 2022) but region-specific differences have been found in the spinal cord (Clarke et al., 2019). It could be the case that formation of eIF4A1-positive pathology in the ALS spinal cord was a downstream effect resulting from other dysfunctions that are more responsible for driving disease progression. This would be consistent with the mouse work shown in this thesis, which showed eIF4A1-positive pathology was not present at the age of 7 months, therefore indicating formation of these inclusions is a late-stage event in ALS. Alternative suggestions for the lack of eIF4A1 pathology in the mouse spinal cords is that there are interspecies differences in eIF4A1 between mice and humans. Alternatively, that there are fundamental problems with the phenotype of this mouse model, which has already been reported and responded to (Mordes et al., 2020), (Nguyen, Laboissonniere, et al., 2020).

eIF4A1-positive inclusions were found in approximately 8% of spinal cord motor neurons, with no difference between sALS and C9-ALS/FTD. The identification of eIF4A1 pathology raises the question of functional consequences given that any eIF4A1 bound up in insoluble aggregates will be unable to fulfil its function as a helicase in initiating either RAN or canonical translation. RAN translation is clearly still able to occur, given that DPRs are still produced in C9-ALS/FTD brains. However,

DPRs are rarely formed in the spinal cord (Balendra & Isaacs, 2018), the only location in which eIF4A1-positive pathology is seen. Therefore, it is not obvious that eIF4A1 inclusion formation has no impact on the normal function of eIF4A1. Although eIF4A1 is a highly abundant protein, inclusion formation may leave the neuron vulnerable, with minimal ability to cope with any further loss without impacting translational efficiency. Western blots from ALS frozen spinal cord but not motor cortex showed higher eIF4A1 levels compared to controls, although only results from C9-ALS/FTD spinal cord reached statistical significance. This increase could reflect a compensatory effort by the cell to respond to loss of eIF4A1 bound up in insoluble aggregates, to maintain translation efficiency. The lack of consistency seen within diagnostic groups also raises questions about the reliability of western blot results. In particular, Figure 4.8 and 4.9 show that controls show substantial variability, with a band almost or entirely absent in two of the cases. This may in fact reflect differences in post-mortem sample collection, post-mortem delay, fixation and storage which vary extensively as Table 2.22 shows. It is therefore possible that the variation seen is a result of these technical variabilities rather than a genuine biological phenomenon. A larger cohort of post-mortem cases that were collected and processed under the same conditions would help establish if this is the case. This is unfortunately difficult to achieve with brain banking of human tissue, but more feasible when considering mouse or other animal models. Overall, the links between eIF4A1-positive inclusions and the impact on canonical or RAN translation needs further clarification.

Co-staining was performed to establish the extent of co-localisation between eIF4A1 and TDP-43 pathology, and between eIF4A1 pathology and p62. It was hypothesised that due to the morphology of eIF4A1-positive inclusions, they were likely sequestered by TDP-43 and would therefore be TDP-43 positive. Results of co-staining showed a complex picture in which eIF4A1-positive inclusions are sometimes TDP-43 positive. Interestingly, there were also instances where TDP-43 pathology did not co-localise with eIF4A1 pathology, and instead separate non-overlapping inclusions were seen at

different sides of the cytoplasm. Data from post-mortem tissue was supported by consistent results from immunofluorescence staining of patient-derived neurons showing non-overlapping TDP-43 and eIF4A1-positive inclusions. This indicates eIF4A1 is a novel form of proteinopathy in ALS and is not simply sequestered by existing pathological inclusions.

Although rare exceptions were seen, eIF4A1-positive pathology was almost always also positive for p62. p62 may provide the link between TDP-43 and eIF4A1-positive inclusions and has been suggested to be the point of convergence between molecular mechanisms in C9-ALS/FTD (Davidson et al., 2022). It has been suggested that reduced C9ORF72 results in deficits in autophagy, and therefore increased p62 levels (Sellier et al., 2016). The p62-positive nature of eIF4A1 pathology indicates these inclusions are a toxic species that the cell is trying and failing to remove, as degradation systems become increasingly overwhelmed. As previously discussed, increased cellular stress and the activation of the ISR leads to increased DPR production in C9-ALS/FTD (Westergard et al., 2019). p62 promotes autophagy induction, and furthermore eIF2 α regulates levels of p62 transcripts (Bhardwaj et al., 2020). Production of DPRs has been linked to impairment of protein clearance mechanisms and increases stress (S. Guo et al., 2022). p62 has been described as a 'hub' that modulates many factors involved in aging including resistance to oxidative stress, regulation of protein clearance systems including autophagy and the UPS, apoptosis and cell survival (Fan et al., 2022). When substrates of autophagy or the UPS are not cleared, they accumulate which adds to stress in the cell and can develop into severe disruption of protein clearance systems, leading to a vicious cycle creating additional stress and negatively impacting cell survival (Davidson et al., 2022). p62 homeostasis is essential for neuronal health, with both over expression and depletion of p62 linked with impairment of protein clearance mechanisms (Davidson et al., 2022). Overexpression of p62 enhances TDP-43 aggregation formation in ALS, as well as inducing TDP-43 mis-localisation, altering RNA splicing dynamics, and increasing cell

death (Foster et al., 2021). p62 has been found to frequently co-localise with protein inclusions in ALS/FTD (Foster & Rea, 2020), and in C9-ALS/FTD a distinct set of p62-positive, TDP-43 negative inclusions have been identified in several brain regions including the cortex and hippocampus (Al-Sarraj et al., 2011). Inclusions positive for DPRs have also been found to contain p62 as well as co-localising with an excision repair protein (Riemsлагh et al., 2019) which repairs DNA damage, further highlighting the link between p62 and various measures of cellular stress and dysfunction. The discovery of eIF4A1 and p62 positive inclusions in the ALS spinal cord reveals another pathway through which p62 activity becomes deficient, as p62 incorporated into inclusions will be unable to function adequately, and therefore highlights a novel pathway for p62 contributions to ALS.

It has been suggested that p62 may differentially affect disease progression depending on the stage of the disease. While in early stages of ALS p62 may be effectively used to rapidly form autophagosomes as a kind of emergency response, in later stages p62 is lost to aggregates, exacerbating neuronal dysfunction, protein accumulation and therefore leading to increased stress (Davidson et al., 2022). This work has identified a novel type of p62 positive aggregate. Furthermore, stress granules that form under chronic oxidative stress also are p62 aggregate positive in ALS patient fibroblasts (Ratti et al., 2020). It has been hypothesised that TDP-43 aggregates occur as a result of failures in stress granule dynamics linked directly to autophagy deficits (Monahan et al., 2016). This places p62, along with C9ORF72, as of central importance to several processes that are essential for resolving cellular stress, including eliminating stress granules and maintaining proteostasis, which have been shown to become defective in ALS/FTD. From this work, eIF4A1 also appears to play a role in these dysfunctions. C9ORF72 forms a complex with p62 to clear stress granules for degradation (Chitiprolu et al., 2018). However under chronic stress p62 levels increase, suggesting an impairment in autophagy and stress granule dynamics (Ratti et al., 2020). Aggregates cannot be properly cleared as p62 itself forms

inclusions. Interestingly p62 is also shown to be elevated following traumatic brain injury in a *Drosophila* model of C9-ALS/FTD (Anderson et al., 2018) further emphasising the link between p62 and the ability to respond to stress, which is likely undermined by its incorporation into eIF4A1-positive inclusions.

6.5 Immunohistochemistry of eIF4A1, eIF4A2, eIF4B and eIF4H shows predominantly cytoplasmic localisation

Immunohistochemistry in post-mortem control and ALS tissue has revealed staining patterns for eIF4A1, eIF4A2, eIF4B, eIF4H in spinal cord, motor cortex, prefrontal and temporal lobes, the hippocampus and cerebellum. In the motor cortex, eIF4A1, eIF4A2 and eIF4H showed moderate-strong cytoplasmic staining in neurons throughout all cortical layers, with variable labelling of the nucleus, while eIF4B showed weak cytoplasmic staining and negative nuclear staining. Spinal cord eIF4B was also very weak in staining intensity, localised to the cytoplasm of motor neurons with negative nuclear staining. eIF4H in the spinal cord showed positive cytoplasmic labelling with variable nuclear labelling. In the frontal lobe and temporal lobes eIF4A1 showed a cytoplasm localisation with negative nuclear labelling and was noticeably weaker in intensity here than some other regions, although no quantitative analysis of staining intensity was performed to confirm this observation. eIF4A2, eIF4B and eIF4H showed positive cytoplasmic labelling with variable nuclear labelling throughout all cortical layers. In the hippocampus eIF4A1, eIF4A2 and eIF4H showed positive labelling through Ammon's Horn and the dentate gyrus, while eIF4B labelling in the hippocampal region was minimal. In CA4 hippocampal neurons eIF4A1 and eIF4A2 showed positive labelling of the cytoplasm and variable nuclear labelling. eIF4B showed weak cytoplasmic staining and absent nuclear staining, while eIF4H showed strong labelling of both the cytoplasmic nucleus. In the cerebellum eIF4A1, eIF4A2 and eIF4B and eIF4H labelling is seen in Purkinje cell cytoplasm and processes with generally negative nuclear labelling. No substantial differences in helicase staining

patterns were observed between control and ALS groups in these regions. Overall, the conclusions from this immunohistochemistry data are that there are clear and consistent patterns of generally strong staining of eIF4A1, eIF4A2, eIF4B and eIF4H across all brain and spinal cord regions, with some notable examples such as eIF4B showing weak staining intensity in most regions. Importantly, there appear to be minimal differences in staining patterns between control and ALS patient groups and no features observed that would rule out these helicases as a potential therapeutic target. The notable exception to this is of course the presence of eIF4A1-positive inclusions in ALS spinal cord motor neurons.

6.6 SRSF1 immunohistochemistry reveal positive glial cell staining in the C9-ALS/FTD motor cortex

The link between SRSF1 and astrocytes is intriguing. Although not a helicase, SRSF1 was considered crucial to include in this study as it is necessary for export of repeat-containing transcripts from the nucleus (Hautbergue et al., 2017) and is therefore indirectly required for RAN translation to occur, and has also been identified as a potential therapeutic target in C9-ALS/FTD which complements the overall aims of this project. Immunohistochemistry showed a nuclear localisation for SRSF1 in neurons in all spinal cord and brain regions, which is consistent with its expected location as a splicing factor. No difference in neuronal nuclear expression is seen between control, sALS and C9-ALS/FTD groups. However, these experiments also revealed strong SRSF1-positive cytoplasmic staining resembling immune activated glial cells of the motor cortex of five out of six C9-ALS/FTD cases examined. Although no glial cell marker was used to confirm, the morphology suggested reactive astrocyte labelling.

Astrocytes play a key role in maintaining neuronal function. Their roles include but are not limited to synapse maintenance, regulation of neurotransmitter and ion concentrations, pH and metabolic homeostasis, supply or removal of glucose and

ROS, regulation of neuronal excitability, oxidative stress and excitotoxicity (Halpern et al., 2019). In ALS, astrocytes lose their protective role and shift to a neurotoxic-inducing state (Jiwaji & Hardingham, 2022). This cytoplasmic glial staining was an unexpected result for SRSF1, and no similar results have been reported showing SRSF1 immunohistochemistry in post-mortem ALS/FTD patient brain. There is little in the current literature around SRSF1 in the context of glial expression in ALS/FTD, although one study identified SRSF1 as able to suppress glial TDP-43 toxicity (Azpurua et al., 2021). In a very recent preprint, SRSF1 levels were found to be decreased in perineural glia and astrocytes in a *Drosophila* model of ALS (Krupp S Fau - Tam et al., 2023). This is in contrast to the staining results shown in this thesis, which appear to show an increase in SRSF1 glial expression, or at least a re-localisation from the nucleus to the glial cytoplasm and processes. Further investigation is required to establish the extent of SRSF1 expression in human astrocytes. Krupp and colleagues found that further SRSF1 depletion in astrocytes extends their survival and decreases deficits associated with TDP-43 pathology (Krupp S Fau - Tam et al., 2023). The results shown in this thesis suggest SRSF1 has increased expression in C9-ALS/FTD compared to control astrocytes in regions central to the disease such as the motor cortex. Further studies are needed to confirm, but it is possible that increased SRSF1 in astrocytes contributes to the neurotoxicity in ALS/FTD, which complements Krupp's findings that depletion has protective effects.

The antibody used for this immunohistochemistry has been previously validated through western blots, shown both in this thesis in and in published work (Hautbergue et al., 2017). The demonstration of specific SRSF1 detection in these applications adds confidence to these immunohistochemistry results. In future, western blots from resting and activated astrocyte cultures would be informative.

SRSF1 staining in glial cells was also seen, albeit to a lesser extent, in white matter underlying the motor cortex in both sALS and C9-ALS/FTD cases. Again, one case

each out of the five sALS and six C9-ALS/FTD cases included did not show SRSF1-positive glial cell staining. Subtle staining of SRSF1 in glial cells was also seen in ALS prefrontal and temporal lobes although the staining was relatively weak and only a very small number of cases were examined. With such a small number of cases this limits conclusions that can be drawn regarding SRSF1-positive glial labelling; the cohort would have to be expanded by a large extent with blinded quantification of staining patterns between control and ALS groups to establish if this is a meaningful result. In the motor cortex, the stark difference in staining patterns between C9-ALS/FTD cases and controls is a more robust result indicating a previously unexplored role for SRSF1 in C9-ALS/FTD.

SRSF1 has various roles of fundamental importance to healthy cell function, including relating to transcription activation, alternative splicing, immune regulation, maintenance of mRNA transcript stability, mRNA export from the nucleus, translational control, and miRNA processing (Paz et al., 2021). Splicing of pre-mRNAs by SRSF1 is a crucial element of gene expression control, which also associates with non-coding RNAs to regulate gene expression and maintain RNA stability (Änkö, 2014). SRSF1 also contributes to assembly and activation of the spliceosome (Gordon et al., 2021); (Gehring & Roignant, 2021). Alternative splicing by SRSF1 is linked with regulation of subcellular RNA localisation, (Titus et al., 2021) which have in turn been linked to altered stress granule dynamics and inclusion formation. SRSF1 relies on negative feedback loops to autoregulate its own splicing dynamics which has been identified as critical for maintaining gene regulation. Negative autoregulation should create upper limits for SRSF1 protein concentration, reducing heterogeneity in SRSF1 levels between cells to buffer resulting variations in splicing activity (Ding et al., 2022). The staining results seen in the C9-ALS/FTD motor cortex shows a substantial increase in SRSF1 staining in glial cells that may reflect a failure of this fundamental mechanism. Increased astrogliosis and enhanced astrocyte reactivity has been reported in the brain and spinal cord of ALS patients, as well as in animal and *in vitro* disease models

(Philips & Rothstein, 2014). It is possible that the SRSF1 antibody could be cross-reacting with a protein expressed by activated glia, although as previously shown the specificity of the antibody is well established. Overall, this staining may indicate increased expression that may in turn lead to abnormal activity of SRSF1 in C9-ALS/FTD motor cortex glia. This is likely to have many far-reaching consequences for these cells and the neurons they support. SRSF1 interacts with a vast array of mRNA transcripts (Sanford et al., 2009) so any impact is likely to be substantial.

Several studies have looked at SRSF1 depletion and linked it to increased R-loop formation and genomic instability (Zhong et al., 2009) and RNA binding alterations. The consequences of increased SRSF1 expression is linked to altered exon inclusions (Pandit et al., 2013) and to oncogenesis (Anczuków et al., 2015; Das et al., 2012). In cancer, high levels of splicing factors were linked to energy homeostasis and DNA damage alterations, and importantly distinct patterns of splicing activity (Koedoot et al., 2019). Increased SRSF1 expression has been linked to formation of various isoforms and other abnormal splicing events (Ding et al., 2022). Alterations to splicing patterns have been identified as having adverse effects on cell function and suggested as underlying reduction cellular plasticity during aging (Holly et al., 2013). It is possibly that increased expression of SRSF1 in C9-ALS/FTD motor cortex glial cells could induce aberrant splicing events in these cells that contributed to neuronal dysfunction. Increased SRSF1 could lead to aberrant splicing events in C9-ALS/FTD motor cortex glia such as abnormal alternative splicing, intron retention or exon skipping.

Splicing dysfunction has been linked to several neurodegenerative diseases including ALS (Highley et al., 2014; Nik & Bowman, 2019; Perrone et al., 2020; Titus et al., 2021), and C9-ALS/FTD specifically, where splicing errors correlate with disease severity (Cooper-Knock, Bury, et al., 2015; Prudencio et al., 2015). If the SRSF1 positive staining identified in the C9-ALS/FTD is indeed staining astrocytes, this is an important finding as astrocytes have been linked to ALS/FTD neurodegeneration.

Astrocytes carrying the *C9ORF72* repeat expansion are toxic to motor neurons in culture. Media conditioned by C9-astrocytes increases oxidative stress in wild-type motor neurons without the *C9ORF72* repeat expansion (Birger et al., 2019). It has been hypothesised that astrocyte toxicity to motor neurons could be attributed to lack of support function, non-cell autonomous mechanisms and secretion of neurotoxic factors, or defective glutamate uptake leading to excitotoxicity (Rostalski et al., 2019), (Van Harten et al., 2021), (Murray et al., 2022). SRSF1 may play a contributing role in astrocyte induced toxicity to motor neurons, through increased levels leading to aberrant splicing events which undermine normal astrocyte function. Consistent with the SRSF1-positive glia staining limited mainly to the motor cortex, ALS astrocytes have region specific diversity in miRNA expression, inflammatory profile, and reactive markers (Gomes et al., 2020). It is possible that alterations in SRSF1 expression and activity could contribute to this.

In neurons, SRSF1 enables pathological *C9ORF72* repeat-expanded pre-mRNA transcripts to exit the nucleus for cytoplasmic RAN translation into DPRs (Hautbergue et al., 2017). The increased SRSF1 positive staining in the C9-ALS/FTD motor cortex raises questions regarding the role of SRSF1 on RAN translation in C9 glia. There is some inconsistency in the literature, with some studies reporting no DPRs are found in microglia or astrocytes in C9-ALS/FTD post-mortem brains (Ash et al., 2013; Gendron et al., 2013; Mackenzie et al., 2013), (Saber et al., 2018), while in others rare examples of DPRs in glia have been shown (Rostalski et al., 2019). These studies do not include all DPR species, and it is possible that some DPRs may be present but at lower levels. Alternatively in glia RAN translation may occur at a much lower efficiency, or DPR degradation may be more effective, preventing DPR accumulation. The link between SRSF1 and DPR production in glia and neurons is complex, with recent studies demonstrating the possibility of astrocyte-to-neuron DPR propagation and arguing astrocytes act as a hub for spreading DPRs (Marchi et al., 2022).

Overall, SRSF1-positive staining in glia in the C9-ALS/FTD motor cortex is an interesting finding that may be a contributing factor to astrocyte-induced neurodegeneration. It is intriguing to explore ways in which this finding might complement the existing body of research in this area. However, the findings shown here are preliminary and several factors must be established in the immediate future. Namely, use of glial cell markers in immunohistochemistry to confirm glial cell identity. Secondly, to determine exactly what the SRSF1 antibody is binding to in glia. Finally, to expand the cohort and include more cases with semi-quantitative analysis of glial staining to define the extent of differences between C9-ALS/FTD and controls, which will help provide more confidence in these results to form the basis of further investigations into the link between SRSF1 and glial in C9-ALS/FTD.

6.7 Overall assessment of RAN-associated RNA helicases the most suitable to form a therapeutic strategy for C9-ALS/FTD

One of the broad aims for this work was to identify helicases suitable for taking forward to further studies investigating their potential as therapeutic targets. Results obtained through cell models show that shRNAs targeting eIF4A1 and eIF4A2 together do not lead to a greater reduction in DPR levels but are associated with significant toxicity. This provides evidence that any therapeutic strategy should focus on only one helicase.

A new paper investigating eIF4A1 multimerisation has revealed new insights into the activity of eIF4A1 and its co-factors, with important implications for the work presented in this thesis (Schmidt et al., 2023). It appears that the RNA eIF4A1 interacts with has a strong influence on the function of eIF4A1. Co-factors form a complex with eIF4A1 to modulate its conformational cycle and thereby stimulate its unwinding activity. It is hypothesised that each co-factor operates at a different stage of each catalytic cycle, which has implications for the most effective helicase to target and crucially, at what point. It is also currently unknown how different RNA sequences influence eIF4A1

activity and that of its co-factors, and what consequences this has on mechanisms underlying translation initiation. Understanding these subtleties in the context of G4C2 repeat RNA and RAN translation is of great importance to this area of research. It may be the case that different co-factors have different effects on the unwinding activity of different RNAs by eIF4A1, and that this is likely to influence DPR production. eIF4H in particular shows RNA-sequence specific stimulation of multimeric eIF4A1 complexes thereby facilitating distinct modes of activity.

mRNAs dependent on eIF4A1 unwinding for translation have been found to contain AG-rich motifs in the 5'UTR which specifically activate eIF4A1 helicase functions. This triggers the unwinding of the local RNAs to enable translation to occur and renders these specific mRNAs particularly sensitive to inhibition of eIF4A1 (Schmidt et al., 2023). It is unknown to what extent this mechanism is used in RAN translation or if G4C2/ C4G2 transcripts also show increased sensitivity to eIF4A1-inhibition. This occurs through a process of eIF4A1 multimerisation, with distinct enzymatic activities attributed to different eIF4A1 subunits. Binding of specific RNAs to multimeric eIF4A1 leads to conformational changes in RNA that triggers multimeric complexes to move into an optimal position to achieve efficient ribosomal scanning and unwinding (Schmidt et al., 2023). The length of an mRNA transcript effects eIF4A1 activity which may reveal an important new mechanism to explain why DPRs are produced in such large amounts despite RAN translation occurring less efficiently than canonical translation.

Specific RNA sequence motifs have been identified that are able to trigger eIF4A1 multimerisation, activate eIF4A1-dependent strand separation of RNA structures thereby enabling translation to occur. This is ATP-dependent, and it has been hypothesised that ATPase activity of eIF4A1-complexes controls kinetic stability and thereby unwinding activities rather than strand separation itself. Importantly, eIF4A1-dependent mRNAs are able to use localised RNA structures to overcome translational

repression (Schmidt et al., 2023). This may influence preferential translation of G4C2 repeat containing transcripts in favour of other mRNA transcripts. eIF4A1 appears to operate differently on RNAs depending on their concentration and sequence composition and is preferentially resolves those where multimerisation is able to occur. It is currently unknown to what extent this applies to G4C2 repeat transcripts. This indicates a number of factors influence helicase function including co-factor activity, RNA sequence, length and concentration, and recruitment and assemble of distinct multimeric eIF4A1 complexes, which may also show tissue or cell-type dependent variation (Schmidt et al., 2023). Overall, complex helicase and mRNA-specific networks form to regulate both canonical and RAN translation, and the exact details of these must be determined in order to design an effective therapeutic strategy. Targeting regulation of eIF4A1 multimeric complexes is likely to have a substantial impact on translation, including of repeat-containing transcripts. It may be the case that RNA-specific activation is also seen associated with other helicases such as eIF4A2 or DHX36 although this remains to be elucidated.

eIF4A1, eIF4A2, eIF4B and eIF4H have supporting evidence from shRNA-based depletion showing they are able to disrupt the RAN translation of DPRs *in vitro*. eIF4A1 and eIF4A2 also have results from helicase mutants providing more robust evidence to show the necessity of their helicase activity in DPR production. Depletion of eIF4A2, eIF4B and eIF4H confers neuroprotection in co-culture experiments, however depletion of eIF4A1 was associated with reduced motor neuron survival, indicating it may be unlikely to be suitable as a therapeutic target. While these are preliminary results that need further confirmation, the use of eIF4A1 as a therapeutic target was complicated by the existence of eIF4A1-positive pathology in the ALS spinal cord. This suggests eIF4A1 may have a greater role in ALS progression beyond its role in RAN translation. Before eIF4A1 can be considered as a therapeutic target, it must be clarified what impact eIF4A1 pathology has on protein expression levels, RAN translation efficiency, and normal eIF4A1 function.

eIF4B immunohistochemistry showed weak expression throughout the brain and spinal cord in both ALS and control cases. Confirmation of poor tissue-specific expression is necessary as eIF4B was not detectable through western blot. It is unclear what the implications are for its viability as a therapeutic target. Initial HEK293T and co-culture experiments have suggested eIF4B depletion confers neuroprotective and is sufficient to reduce DPR production. However, potentially poor expression raises questions as to whether this is the primary driver of RAN translation in the human brain. Further, it is possible that depleting a protein already low in abundance may be more likely to have some associated toxicity, as cells may have a lower tolerance for loss compared to more abundant proteins. eIF4B should not be excluded while these factors have yet to be confirmed, but it may not be the best choice for a therapeutic strategy.

eIF4H was able to be successfully detected through western blot in frozen human brain tissue, although with high levels of variation seen both within and between diagnostic groups. This may reflect variability in sample collection, processing and storage as previously discussed. Western blots revealed that in both the spinal cord and motor cortex there was a significant fall in eIF4H levels associated with both sALS and C9-ALS/FTD. The levels were lowest in C9-ALS/FTD, with a 75% reduction compared to controls seen in the spinal cord. However, RAN translation is still able to occur because DPRs have been identified in both regions, albeit rarely in the case of the spinal cord (Freibaum & Taylor, 2017). This suggests that eIF4H has a less important role in DPR production here, although it is not clear if there are region-specific differences in brain helicase activity. eIF4H depletion was neuroprotective in preliminary co-culture experiments, but the western blot results undermine its suitability as a therapeutic target. However, western blot results are not consistent with immunohistochemistry results, where strong staining for eIF4H was seen throughout the brain. Further work is needed to establish eIF4H expression levels in both post-

mortem human brain tissue and cultured cells. That DPRs are produced when eIF4H levels have fallen by such a large extent is good evidence that other helicases are more responsible for driving RAN translation and therefore it would be inadvisable to form a therapeutic strategy around eIF4H, although its role in C9-ALS/FTD should continue to be investigated.

Through a process of elimination, these studies indicate that eIF4A2 may be the best option for future testing as a therapeutic strategy to reduce RAN translation. eIF4A2 has good evidence to support it in this thesis. Depletion of eIF4A2 in HEK293T cells reduces sense and antisense DPR production and rescues DPR induced decreases in cell proliferation. eIF4A2 mutants are also able to rescue DPR-induced toxicity *in vitro*, while *in vivo* eIF4A depletion rescues a neurodegenerative phenotype in *Drosophila*. Co-culture data supports eIF4A2 as neuroprotective in motor neurons. Immunohistochemistry studies show that unlike eIF4A1, eIF4A2 does not form any pathology in ALS brains. Unlike eIF4B, it showed strong staining indicating it is well expressed in the human brain, and unlike eIF4H there is no loss of eIF4A2 associated with ALS in either the spinal cord or motor cortex. Together, these studies highlight eIF4A2 as a suitable candidate for testing in C9-transgenic mouse models with a future hypothesis that depletion will alleviate a neurodegenerative phenotype through reduced RAN translation and DPR production.

6.8 Future studies investigating SRSF1 and RAN-associated RNA helicases in C9-ALS/FTD

The work in this thesis has opened several potential paths for future research studies. First, to include a more in-depth analysis of the impact of helicase depletion on canonical translation, as this is likely to be the source of many of the potential toxicities associated with this therapeutic strategy. Several methods exist for transcriptome-wide profiling which would enable analysis of all ribosome-associated mRNAs in a cell (King

& Gerber, 2016). Further information about the relative contribution of each helicase could be provided by investigating the effects of depletion of different combinations, rather than just eIF4A1 with eIF4A2. Investigations into the neuroprotective potential of helicase depletion should be continued in co-culture experiments. Further, the ability of helicase depletion to rescue a neurodegenerative phenotype should be tested *in vivo* in C9-transgenic mice. As previously outlined, eIF4A2 has been identified as the most promising helicase for initial testing.

Finally, the pathology section of this research has many interesting aspects for further exploration. A quantitative approach to immunohistochemistry should be applied to investigate observed differences between control and ALS cases. The obvious areas of focus are eIF4A1-positive inclusions in the spinal cord and SRSF1-positive glial staining in the motor cortex, the first step of which would be to greatly expand the current case cohort. A variety of methods for quantitative and semi-quantitative immunohistochemical analysis exist, including the development of new methods for digital analysis (Bencze et al., 2021; Jensen et al., 2017; Muraleedharan et al., 2021; Nolan et al., 2020; Rizzardi et al., 2012).

Further investigation into eIF4A1 pathology is one of the most important future studies to conduct. Further investigations could be carried out using C9-transgenic mice but allowing them to age further to determine at what point in the disease process, if any, eIF4A1-positive inclusions appear. This would help establish if eIF4A1 inclusion formation is an earlier or late-stage event, suggesting the degree to which it may be driving ALS progression. eIF4A1 pathology could be studied in cell models of ALS, to see if aggregates could be induced in controls under various cellular conditions such as stress. As eIF4A1 inclusions have been demonstrated as TDP-43 negative, the constituents of the inclusion are important to identify. Mass spectrometry can be used to identify composition of protein aggregates (Adiutori et al., 2021). Mass spectrometry has also been used in the context of Alzheimer's Disease to investigate conformational

changes in protein complex and the process of self-aggregation, and to identify small molecule inhibitors (Young et al., 2016). A similar approach could be used to characterise eIF4A1-positive pathology. *In vitro* characterisation of the eIF4A1 inclusions will also be investigated in reporter cell models and patient-derived neurons using mass spectrometry approaches.

6.9 Summary and Conclusions

Overall, this project has provided support for the hypothesis that depletion of eIF4A1, eIF4A2, eIF4B and eIF4H can confer neuroprotection in C9-ALS/FTD by preventing RAN translation of sense and antisense neurotoxic DPRs. This thesis has demonstrated the development of shRNAs to successfully deplete helicase protein levels by approximately 50%, in order to disrupt RAN translation while preserving canonical translation. shRNA-induced depletion reduces translation of sense and antisense DPRs, which is non-toxic when individual helicases are targeted but toxic when multiple helicases are depleted simultaneously. eIF4A1 and eIF4A2 dead mutants with non-functional helicase activity also decrease DPR production *in vitro*. In HEK293T cell models of C9-ALS/FTD, helicase depletion with shRNAs and helicase mutants is able to rescue DPR-induced toxicity through preventing RAN translation initiation.

Characterisation of helicases in post-mortem human brain and spinal cord revealed a cytoplasmic localisation with variable nuclear labelling. SRSF1 was found to show positive glial labelling with a morphology suggestive of astrocytes in the C9-ALS/FTD motor cortex. eIF4A1 formed cytoplasmic inclusions in approximately 8% of spinal cord motor neurons in sALS and C9-ALS/FTD but not controls. Double staining revealed that eIF4A1-positive pathology could form independent of the hallmark pathology in ALS, that of phosphorylated TDP-43. eIF4A1 inclusions were generally found to be p62 positive.

Together, this study has identified a novel eIF4A1-positive pathology in sALS and C9-ALS/FTD, provided insight into the role of eIF4 helicases in RAN translation initiation, and provided data indicating that helicase depletion has potential as a therapeutic strategy in C9-ALS/FTD.

7 References

- Abdelhaleem, M., Maltais, L., & Wain, H. (2003). The human DDX and DHX gene families of putative RNA helicases. *Genomics*, 81(6), 618-622. [https://doi.org/https://doi.org/10.1016/S0888-7543\(03\)00049-1](https://doi.org/https://doi.org/10.1016/S0888-7543(03)00049-1)
- Abo-Rady, M., Kalmbach, N., Pal, A., Schludi, C., Janosch, A., Richter, T., . . . Sternecker, J. L. (2020). Knocking out C9ORF72 Exacerbates Axonal Trafficking Defects Associated with Hexanucleotide Repeat Expansion and Reduces Levels of Heat Shock Proteins. *Stem Cell Reports*, 14(3), 390-405. <https://doi.org/https://doi.org/10.1016/j.stemcr.2020.01.010>
- Adiutori, R., Puentes, F., Bremang, M., Lombardi, V., Zubiri, I., Leoni, E., . . . Malaspina, A. (2021). Analysis of circulating protein aggregates as a route of investigation into neurodegenerative disorders. *Brain Communications*, 3(3), fcab148. <https://doi.org/10.1093/braincomms/fcab148>
- Aguzzi, A., & Altmeyer, M. (2016). Phase Separation: Linking Cellular Compartmentalization to Disease. *Trends in Cell Biology*, 26(7), 547-558. <https://doi.org/https://doi.org/10.1016/j.tcb.2016.03.004>
- Al-Sarraj, S., King A Fau - Troakes, C., Troakes C Fau - Smith, B., Smith B Fau - Maekawa, S., Maekawa S Fau - Bodi, I., Bodi I Fau - Rogelj, B., . . . Shaw, C. E. (2011). p62 positive, TDP-43 negative, neuronal cytoplasmic and intranuclear inclusions in the cerebellum and hippocampus define the pathology of C9orf72-linked FTL and MND/ALS. (1432-0533 (Electronic)).
- Alberti, S., & Hyman, A. A. (2016). Are aberrant phase transitions a driver of cellular aging? *BioEssays : news and reviews in molecular, cellular and developmental biology*, 38(10), 959-968. <https://doi.org/10.1002/bies.201600042>
- Alegria-Schaffer, A., Lodge, A., & Vattem, K. (2009). Chapter 33 Performing and Optimizing Western Blots with an Emphasis on Chemiluminescent Detection. In R. R. Burgess & M. P. Deutscher (Eds.), *Methods in Enzymology* (Vol. 463, pp. 573-599). Academic Press. [https://doi.org/https://doi.org/10.1016/S0076-6879\(09\)63033-0](https://doi.org/https://doi.org/10.1016/S0076-6879(09)63033-0)
- Almeida, S., Gascon, E., Tran, H., Chou, H. J., Gendron, T. F., Degroot, S., . . . Gao, F.-B. (2013). Modeling key pathological features of frontotemporal dementia with C9ORF72 repeat expansion in iPSC-derived human neurons. *Acta neuropathologica*, 126(3), 385-399. <https://doi.org/10.1007/s00401-013-1149-y>
- Amado, D. A., & Davidson, B. L. (2021). Gene therapy for ALS: A review. *Molecular Therapy*. <https://doi.org/https://doi.org/10.1016/j.ymthe.2021.04.008>
- Anczuków, O., Akerman, M., Cléry, A., Wu, J., Shen, C., Shirole, N. H., . . . Krainer, A. R. (2015). SRSF1-Regulated Alternative Splicing in Breast Cancer. (1097-4164 (Electronic)).
- Anderson, E. N., Gochenaur, L., Singh, A., Grant, R., Patel, K., Watkins, S., . . . Pandey, U. B. (2018). Traumatic injury induces stress granule formation and enhances motor dysfunctions in ALS/FTD models. *Human Molecular Genetics*, 27(8), 1366-1381. <https://doi.org/10.1093/hmg/ddy047>
- Andreou, A. Z., & Klostermeier, D. (2013). The DEAD-box helicase eIF4A: paradigm or the odd one out? (1555-8584 (Electronic)).
- Aoki, Y., Manzano, R., Lee, Y., Dafinca, R., Aoki, M., Douglas, A. G. L., . . . Wood, M. J. A. (2017). C9orf72 and RAB7L1 regulate vesicle trafficking in amyotrophic lateral sclerosis and frontotemporal dementia. *Brain*, 140(4), 887-897. <https://doi.org/10.1093/brain/awx024>
- Arai, T., Hasegawa, M., Akiyama, H., Ikeda, K., Nonaka, T., Mori, H., . . . Oda, T. (2006). TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochemical and Biophysical Research Communications*, 351(3), 602-611. <https://doi.org/https://doi.org/10.1016/j.bbrc.2006.10.093>

- Ash, P. E. A., Bieniek, K. F., Gendron, T. F., Caulfield, T., Lin, W.-L., DeJesus-Hernandez, M., . . . Petrucelli, L. (2013). Unconventional translation of C9ORF72 GGGGCC expansion generates insoluble polypeptides specific to c9FTD/ALS. *Neuron*, 77(4), 639-646. <https://doi.org/10.1016/j.neuron.2013.02.004>
- Atanasio, A., Decman, V., White, D., Ramos, M., Ikiz, B., Lee, H.-C., . . . Lai, K.-M. V. (2016). C9orf72 ablation causes immune dysregulation characterized by leukocyte expansion, autoantibody production, and glomerulonephropathy in mice. *Scientific reports*, 6, 23204-23204. <https://doi.org/10.1038/srep23204>
- Azpuruá, J., El-Karim, E. G., Tranquille, M. A.-O., & Dubnau, J. A.-O. (2021). A behavioral screen for mediators of age-dependent TDP-43 neurodegeneration identifies SF2/SRSF1 among a group of potent suppressors in both neurons and glia. (1553-7404 (Electronic)).
- Bajc Česnik, A., Darovic, S., Prpar Mihevc, S., Štalekar, M., Malnar, M., Motaln, H., . . . Rogelj, B. (2019). Nuclear RNA foci from C9ORF72 expansion mutation form paraspeckle-like bodies. *Journal of Cell Science*, 132(5), jcs224303. <https://doi.org/10.1242/jcs.224303>
- Balakrishnan B Fau - Jayandharan, G. R., & Jayandharan, G. R. (2014). Basic biology of adeno-associated virus (AAV) vectors used in gene therapy. (1875-5631 (Electronic)).
- Balendra, R., & Isaacs, A. M. (2018). C9orf72-mediated ALS and FTD: multiple pathways to disease. (1759-4766 (Electronic)).
- Baloh, R. H. (2011). TDP-43: the relationship between protein aggregation and neurodegeneration in amyotrophic lateral sclerosis and frontotemporal lobar degeneration [<https://doi.org/10.1111/j.1742-4658.2011.08256.x>]. *The FEBS Journal*, 278(19), 3539-3549. <https://doi.org/https://doi.org/10.1111/j.1742-4658.2011.08256.x>
- Bao, S., & Cagan, R. (2006). Fast cloning inverted repeats for RNA interference. (1355-8382 (Print)).
- Baradaran-Heravi, Y., Van Broeckhoven, C., & van der Zee, J. (2020). Stress granule mediated protein aggregation and underlying gene defects in the FTD-ALS spectrum. *Neurobiology of Disease*, 134, 104639. <https://doi.org/https://doi.org/10.1016/j.nbd.2019.104639>
- Bass, J. J., Wilkinson, D. J., Rankin, D., Phillips, B. E., Szewczyk, N. J., Smith, K., & Atherton, P. J. (2017). An overview of technical considerations for Western blotting applications to physiological research [<https://doi.org/10.1111/sms.12702>]. *Scandinavian Journal of Medicine & Science in Sports*, 27(1), 4-25. <https://doi.org/https://doi.org/10.1111/sms.12702>
- Batra, R., Nelles, D. A., Pirie, E., Blue, S. M., Marina, R. J., Wang, H., . . . Yeo, G. W. (2017). Elimination of Toxic Microsatellite Repeat Expansion RNA by RNA-Targeting Cas9. *Cell*, 170(5), 899-912.e810. <https://doi.org/10.1016/j.cell.2017.07.010>
- Bañez-Coronel, M., Ayhan, F., Tarabochia, A. D., Zu, T., Perez, B. A., Tusi, S. K., . . . Ranum, L. P. (2015). RAN Translation in Huntington Disease. (1097-4199 (Electronic)).
- Bedrat, A., Lacroix, L., & Mergny, J. L. (2016). Re-evaluation of G-quadruplex propensity with G4Hunter. (1362-4962 (Electronic)).
- Behrends, C., Sowa, M. E., Gygi, S. P., & Harper, J. W. (2010). Network organization of the human autophagy system. *Nature*, 466(7302), 68-76. <https://doi.org/10.1038/nature09204>
- Bell, G. (2016). Quantifying western blots: none more black. *BMC Biology*, 14(1), 116. <https://doi.org/10.1186/s12915-016-0339-1>
- Bellato, H. M., & Hajj, G. N. M. (2016). Translational control by eIF2 α in neurons: Beyond the stress response [<https://doi.org/10.1002/cm.21294>]. *Cytoskeleton*, 73(10), 551-565. <https://doi.org/https://doi.org/10.1002/cm.21294>
- Belzil, V. V., Bauer, P. O., Prudencio, M., Gendron, T. F., Stetler, C. T., Yan, I. K., . . . Petrucelli, L. (2013). Reduced C9orf72 gene expression in c9FTD/ALS is caused by histone trimethylation, an epigenetic

- event detectable in blood. *Acta neuropathologica*, 126(6), 895-905. <https://doi.org/10.1007/s00401-013-1199-1>
- Bencze, J. A.-O., Szarka, M. A.-O., Kóti, B., Seo, W., Hortobágyi, T. G., Bencs, V., . . . Hortobágyi, T. A.-O. (2021). Comparison of Semi-Quantitative Scoring and Artificial Intelligence Aided Digital Image Analysis of Chromogenic Immunohistochemistry. LID - 10.3390/biom12010019 [doi] LID - 19. (2218-273X (Electronic)).
- Berridge, M. V., & Tan, A. S. (1993). Characterization of the Cellular Reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): Subcellular Localization, Substrate Dependence, and Involvement of Mitochondrial Electron Transport in MTT Reduction. *Archives of Biochemistry and Biophysics*, 303(2), 474-482. <https://doi.org/https://doi.org/10.1006/abbi.1993.1311>
- Bhardwaj, M., Leli, N. M., Koumenis, C., & Amaravadi, R. K. (2020). Regulation of autophagy by canonical and non-canonical ER stress responses. *Seminars in Cancer Biology*, 66, 116-128. <https://doi.org/https://doi.org/10.1016/j.semcancer.2019.11.007>
- Bigio, E. H., Weintraub S Fau - Rademakers, R., Rademakers R Fau - Baker, M., Baker M Fau - Ahmadian, S. S., Ahmadian Ss Fau - Rademaker, A., Rademaker A Fau - Weitner, B. B., . . . Mesulam, M. M. (2013). Frontotemporal lobar degeneration with TDP-43 proteinopathy and chromosome 9p repeat expansion in C9ORF72: clinicopathologic correlation. (1440-1789 (Electronic)).
- Birger, A., Ben-Dor, I., Ottolenghi, M., Turetsky, T., Gil, Y., Sweetat, S., . . . Reubinoff, B. (2019). Human iPSC-derived astrocytes from ALS patients with mutated C9ORF72 show increased oxidative stress and neurotoxicity. *EBioMedicine*, 50, 274-289. <https://doi.org/https://doi.org/10.1016/j.ebiom.2019.11.026>
- Boenisch, T. (2004). Can a More Selective Application of Antigen Retrieval Facilitate Standardization in Immunohistochemistry? *Applied Immunohistochemistry & Molecular Morphology*, 12(2).
- Boeynaems, S., Bogaert, E., Kovacs, D., Konijnenberg, A., Timmerman, E., Volkov, A., . . . Van Den Bosch, L. (2017). Phase Separation of C9orf72 Dipeptide Repeats Perturbs Stress Granule Dynamics. *Molecular cell*, 65(6), 1044-1055.e1045. <https://doi.org/10.1016/j.molcel.2017.02.013>
- Boeynaems, S., Bogaert, E., Michiels, E., Gijssels, I., Sieben, A., Jovičić, A., . . . Van Den Bosch, L. (2016). Drosophila screen connects nuclear transport genes to DPR pathology in c9ALS/FTD. *Scientific Reports*, 6(1), 20877. <https://doi.org/10.1038/srep20877>
- Booy, E. P., McRae, E. K. S., Howard, R., Deo, S. R., Ariyo, E. O., Dzananovic, E., . . . McKenna, S. A. (2016). RNA Helicase Associated with AU-rich Element (RHAU/DHX36) Interacts with the 3'-Tail of the Long Non-coding RNA BC200 (BCYRN1). *The Journal of biological chemistry*, 291(10), 5355-5372. <https://doi.org/10.1074/jbc.M115.711499>
- Bordeleau, M. E., Cencic R Fau - Lindqvist, L., Lindqvist L Fau - Oberer, M., Oberer M Fau - Northcote, P., Northcote P Fau - Wagner, G., Wagner G Fau - Pelletier, J., & Pelletier, J. (2006). RNA-mediated sequestration of the RNA helicase eIF4A by Pateamine A inhibits translation initiation. (1074-5521 (Print)).
- Bozzo, F., Mirra, A., & Carrì, M. T. (2017). Oxidative stress and mitochondrial damage in the pathogenesis of ALS: New perspectives. *Neuroscience Letters*, 636, 3-8. <https://doi.org/https://doi.org/10.1016/j.neulet.2016.04.065>
- Brandmeir, N. J., Geser, F., Kwong, L. K., Zimmerman, E., Qian, J., Lee, V. M. Y., & Trojanowski, J. Q. (2008). Severe subcortical TDP-43 pathology in sporadic frontotemporal lobar degeneration with motor neuron disease. *Acta Neuropathologica*, 115(1), 123-131. <https://doi.org/10.1007/s00401-007-0315-5>

- Breiner, A., Zinman, L., & Bourque, P. R. (2020). Edaravone for amyotrophic lateral sclerosis: barriers to access and lifeboat ethics. (1488-2329 (Electronic)).
- Brettschneider, J., Del Tredici, K., Toledo, J. B., Robinson, J. L., Irwin, D. J., Grossman, M., . . . Trojanowski, J. Q. (2013). Stages of pTDP-43 pathology in amyotrophic lateral sclerosis. *Annals of neurology*, 74(1), 20-38. <https://doi.org/10.1002/ana.23937>
- Brettschneider, J., Van Deerlin Vm Fau - Robinson, J. L., Robinson JI Fau - Kwong, L., Kwong L Fau - Lee, E. B., Lee Eb Fau - Ali, Y. O., Ali Yo Fau - Safren, N., . . . Trojanowski, J. Q. (2012). Pattern of ubiquilin pathology in ALS and FTLD indicates presence of C9ORF72 hexanucleotide expansion. (1432-0533 (Electronic)).
- Broggi, G., Salvatorelli, L., Barbagallo, D., Certo, F., Altieri, R., Tirrò, E., . . . Caltabiano, R. (2021). Diagnostic Utility of the Immunohistochemical Expression of Serine and Arginine Rich Splicing Factor 1 (SRSF1) in the Differential Diagnosis of Adult Gliomas. *Cancers*, 13(9).
- Broggi, G. A.-O., Angelico, G. A.-O., Filetti, V. A.-O., Ledda, C. A.-O., Lombardo, C., Vitale, E. A.-O., . . . Caltabiano, R. A.-O. (2021). Immunohistochemical Expression of Serine and Arginine-Rich Splicing Factor 1 (SRSF1) in Fluoro-Edenite-Induced Malignant Mesothelioma: A Preliminary Study. LID - 10.3390/ijerph18126249 [doi] LID - 6249. (1660-4601 (Electronic)).
- Brčić, J., & Plavec, J. (2017). ALS and FTD linked GGGGCC-repeat containing DNA oligonucleotide folds into two distinct G-quadruplexes. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1861(5, Part B), 1237-1245. <https://doi.org/https://doi.org/10.1016/j.bbagen.2016.11.018>
- Buratti, E., & Baralle, F. E. (2010). The multiple roles of TDP-43 in pre-mRNA processing and gene expression regulation. *RNA Biol*, 7(4), 420-429.
- Bäuerlein, F. J. B., Fernández-Busnadiego, R., & Baumeister, W. (2020). Investigating the Structure of Neurotoxic Protein Aggregates Inside Cells. *Trends in Cell Biology*, 30(12), 951-966. <https://doi.org/https://doi.org/10.1016/j.tcb.2020.08.007>
- Calcedo, R., Morizono, H., Wang, L., McCarter, R., He, J., Jones, D., . . . Wilson, J. M. (2011). Adeno-associated virus antibody profiles in newborns, children, and adolescents. *Clinical and vaccine immunology : CVI*, 18(9), 1586-1588. <https://doi.org/10.1128/CVI.05107-11>
- Camu, W., De La Cruz, E., & Esselin, F. (2022). Therapeutic tools for familial ALS. *Revue Neurologique*. <https://doi.org/https://doi.org/10.1016/j.neurol.2022.10.001>
- Capossela, S., Muzio, L., Bertolo, A., Bianchi, V., Dati, G., Chaabane, L., . . . Pannese, M. (2012). Growth Defects and Impaired Cognitive–Behavioral Abilities in Mice with Knockout for *Eif4h*, a Gene Located in the Mouse Homolog of the Williams-Beuren Syndrome Critical Region. *The American Journal of Pathology*, 180(3), 1121-1135. <https://doi.org/10.1016/j.ajpath.2011.12.008>
- Caruthers, J. M., Johnson Er Fau - McKay, D. B., & McKay, D. B. (2000). Crystal structure of yeast initiation factor 4A, a DEAD-box RNA helicase. (0027-8424 (Print)).
- Castelli, L. M., Benson, B. C., Huang, W. P., Lin, Y. H., & Hautbergue, G. M. (2022). RNA Helicases in Microsatellite Repeat Expansion Disorders and Neurodegeneration. (1664-8021 (Print)).
- Castelli, L. M., Huang, W.-P., Lin, Y.-H., Chang, K.-Y., & Hautbergue, G. M. (2021). Mechanisms of repeat-associated non-AUG translation in neurological microsatellite expansion disorders. *Biochemical Society transactions*, 49(2), 775-792. <https://doi.org/10.1042/BST20200690>
- Chalupníková, K., Lattmann S Fau - Selak, N., Selak N Fau - Iwamoto, F., Iwamoto F Fau - Fujiki, Y., Fujiki Y Fau - Nagamine, Y., & Nagamine, Y. (2008). Recruitment of the RNA helicase RHAU to stress granules via a unique RNA-binding domain. (0021-9258 (Print)).

- Chambers, V. S., Marsico, G., Boutell, J. M., Di Antonio, M., Smith, G. P., & Balasubramanian, S. (2015). High-throughput sequencing of DNA G-quadruplex structures in the human genome. *Nature Biotechnology*, 33(8), 877-881. <https://doi.org/10.1038/nbt.3295>
- Chandel, T. I., Zaman, M., Khan, M. V., Ali, M., Rabbani, G., Ishtikhar, M., & Khan, R. H. (2018). A mechanistic insight into protein-ligand interaction, folding, misfolding, aggregation and inhibition of protein aggregates: An overview. *International Journal of Biological Macromolecules*, 106, 1115-1129. <https://doi.org/https://doi.org/10.1016/j.ijbiomac.2017.07.185>
- Che, M.-X., Jiang, Y.-J., Xie, Y.-Y., Jiang, L.-L., & Hu, H.-Y. (2011). Aggregation of the 35-kDa fragment of TDP-43 causes formation of cytoplasmic inclusions and alteration of RNA processing [\[https://doi.org/10.1096/fj.10-174482\]](https://doi.org/10.1096/fj.10-174482). *The FASEB Journal*, 25(7), 2344-2353. <https://doi.org/https://doi.org/10.1096/fj.10-174482>
- Chen, M. C., & Ferré-D'Amaré, A. R. (2017). Structural Basis of DEAH/RHA Helicase Activity. *Crystals*, 7(8). <https://doi.org/10.3390/cryst7080253>
- Chen, M. C., Murat, P., Abecassis, K., Ferré-D'Amaré, A. R., & Balasubramanian, S. (2015). Insights into the mechanism of a G-quadruplex-unwinding DEAH-box helicase. *Nucleic acids research*, 43(4), 2223-2231. <https://doi.org/10.1093/nar/gkv051>
- Chen, M. C., Tippiana, R., Demeshkina, N. A., Murat, P., Balasubramanian, S., Myong, S., & Ferré-D'Amaré, A. R. (2018). Structural basis of G-quadruplex unfolding by the DEAH/RHA helicase DHX36. *Nature*, 558(7710), 465-469. <https://doi.org/10.1038/s41586-018-0209-9>
- Cheng, W., Wang, S., Mestre, A. A., Fu, C., Makarem, A., Xian, F., . . . Sun, S. (2018). C9ORF72 GGGGCC repeat-associated non-AUG translation is upregulated by stress through eIF2 α phosphorylation. *Nature communications*, 9(1), 51-51. <https://doi.org/10.1038/s41467-017-02495-z>
- Cheng, W., Wang, S., Zhang, Z., Morgens, D. W., Hayes, L. R., Lee, S., . . . Sun, S. (2019). CRISPR-Cas9 Screens Identify the RNA Helicase DDX3X as a Repressor of C9ORF72 (GGGGCC) n Repeat-Associated Non-AUG Translation. *Neuron*, 104(5), 885-898.e888. <https://doi.org/https://doi.org/10.1016/j.neuron.2019.09.003>
- Chia, R., Chiò, A., & Traynor, B. J. (2018). Novel genes associated with amyotrophic lateral sclerosis: diagnostic and clinical implications. *The Lancet Neurology*, 17(1), 94-102. [https://doi.org/https://doi.org/10.1016/S1474-4422\(17\)30401-5](https://doi.org/https://doi.org/10.1016/S1474-4422(17)30401-5)
- Chitiprolu, M., Jagow, C., Tremblay, V., Bondy-Chorney, E., Paris, G., Savard, A., . . . Gibbins, D. (2018). A complex of C9ORF72 and p62 uses arginine methylation to eliminate stress granules by autophagy. *Nature Communications*, 9(1), 2794. <https://doi.org/10.1038/s41467-018-05273-7>
- Choi, J.-H., Yu, N.-K., Baek, G.-C., Bakes, J., Seo, D., Nam, H. J., . . . Kaang, B.-K. (2014). Optimization of AAV expression cassettes to improve packaging capacity and transgene expression in neurons. *Molecular Brain*, 7(1), 17. <https://doi.org/10.1186/1756-6606-7-17>
- Choi, S. Y., Lopez-Gonzalez, R., Krishnan, G., Phillips, H. L., Li, A. A.-O., Seeley, W. W., . . . Gao, F. A.-O. (2019). C9ORF72-ALS/FTD-associated poly(GR) binds Atp5a1 and compromises mitochondrial function in vivo. (1546-1726 (Electronic)).
- Ciura, S., Lattante, S., Le Ber, I., Latouche, M., Tostivint, H., Brice, A., & Kabashi, E. (2013). Loss of function of C9orf72 causes motor deficits in a zebrafish model of amyotrophic lateral sclerosis. *Annals of Neurology*, 74(2), 180-187. <https://doi.org/10.1002/ana.23946>
- Claessen, J. H. L., Kundrat, L., & Ploegh, H. L. (2012). Protein quality control in the ER: balancing the ubiquitin checkbook. *Trends in cell biology*, 22(1), 22-32. <https://doi.org/10.1016/j.tcb.2011.09.010>

- Clarke, B. E., Gil, R. S., Yip, J., Kalmar, B. A.-O., & Greensmith, L. (2019). Regional differences in the inflammatory and heat shock response in glia: implications for ALS. (1466-1268 (Electronic)).
- Colella, P., Ronzitti, G., & Mingozzi, F. (2018). Emerging Issues in AAV-Mediated In Vivo Gene Therapy. *Molecular Therapy - Methods & Clinical Development*, 8, 87-104. <https://doi.org/https://doi.org/10.1016/j.omtm.2017.11.007>
- Conlon, E. G., Lu, L., Sharma, A., Yamazaki, T., Tang, T., Shneider, N. A., & Manley, J. L. (2016). The C9ORF72 GGGGCC expansion forms RNA G-quadruplex inclusions and sequesters hnRNP H to disrupt splicing in ALS brains. *eLife*, 5, e17820. <https://doi.org/10.7554/eLife.17820>
- Conlon, E. G., & Manley, J. L. (2017). RNA-binding proteins in neurodegeneration: mechanisms in aggregate. *Genes & development*, 31(15), 1509-1528. <https://doi.org/10.1101/gad.304055.117>
- Cooper-Knock, J., Bury, J. J., Heath, P. R., Wyles, M., Higginbottom, A., Gelsthorpe, C., . . . Shaw, P. J. (2015). C9ORF72 GGGGCC Expanded Repeats Produce Splicing Dysregulation which Correlates with Disease Severity in Amyotrophic Lateral Sclerosis. *PLoS one*, 10(5), e0127376-e0127376. <https://doi.org/10.1371/journal.pone.0127376>
- Cooper-Knock, J., Higginbottom, A., Stopford, M. J., Highley, J. R., Ince, P. G., Wharton, S. B., . . . Shaw, P. J. (2015). Antisense RNA foci in the motor neurons of C9ORF72-ALS patients are associated with TDP-43 proteinopathy. *Acta neuropathologica*, 130(1), 63-75. <https://doi.org/10.1007/s00401-015-1429-9>
- Cooper-Knock, J., Walsh, M. J., Higginbottom, A., Robin Highley, J., Dickman, M. J., Edbauer, D., . . . Shaw, P. J. (2014a). Sequestration of multiple RNA recognition motif-containing proteins by C9orf72 repeat expansions. *Brain*, 137(7), 2040-2051. <https://doi.org/10.1093/brain/awu120>
- Cooper-Knock, J., Walsh, M. J., Higginbottom, A., Robin Highley, J., Dickman, M. J., Edbauer, D., . . . Shaw, P. J. (2014b). Sequestration of multiple RNA recognition motif-containing proteins by C9orf72 repeat expansions. *Brain : a journal of neurology*, 137(Pt 7), 2040-2051. <https://doi.org/10.1093/brain/awu120>
- Coppolecchia, R., Buser, P., Stotz, A., & Linder, P. (1993). A new yeast translation initiation factor suppresses a mutation in the eIF-4A RNA helicase [<https://doi.org/10.1002/j.1460-2075.1993.tb06078.x>]. *The EMBO Journal*, 12(10), 4005-4011. <https://doi.org/https://doi.org/10.1002/j.1460-2075.1993.tb06078.x>
- Corcia, P., Beltran, S., Bakkouche, S. E., & Couratier, P. (2021). Therapeutic news in ALS. *Revue Neurologique*, 177(5), 544-549. <https://doi.org/https://doi.org/10.1016/j.neurol.2020.12.003>
- Corcia, P., Blasco, H., Beltran, S., Piegay, A. S., & Vourc'h, P. (2022). Treatment of hereditary amyotrophic lateral sclerosis. *Revue Neurologique*. <https://doi.org/https://doi.org/10.1016/j.neurol.2022.09.001>
- Corman, A., Jung, B., Häggblad, M., Bräutigam, L., Lafarga, V., Lidemalm, L., . . . Fernandez-Capetillo, O. (2019). A Chemical Screen Identifies Compounds Limiting the Toxicity of C9ORF72 Dipeptide Repeats. *Cell Chemical Biology*, 26(2), 235-243.e235. <https://doi.org/https://doi.org/10.1016/j.chembiol.2018.10.020>
- Couratier, P., Corcia, P., Lautrette, G., Nicol, M., & Marin, B. (2017). ALS and frontotemporal dementia belong to a common disease spectrum. *Revue Neurologique*, 173(5), 273-279. <https://doi.org/https://doi.org/10.1016/j.neurol.2017.04.001>
- Creacy, S. D., Routh, E. D., Iwamoto, F., Nagamine, Y., Akman, S. A., & Vaughn, J. P. (2008). G4 Resolvase 1 Binds Both DNA and RNA Tetramolecular Quadruplex with High Affinity and Is the Major Source of Tetramolecular Quadruplex G4-DNA and G4-RNA Resolving Activity in HeLa Cell Lysates *. *Journal of Biological Chemistry*, 283(50), 34626-34634. <https://doi.org/10.1074/jbc.M806277200>

- Cristofani, R., Crippa, V., Vezzoli, G., Rusmini, P., Galbiati, M., Cicardi, M. E., . . . Poletti, A. (2018). The small heat shock protein B8 (HSPB8) efficiently removes aggregating species of dipeptides produced in C9ORF72-related neurodegenerative diseases. *Cell Stress and Chaperones*, 23(1), 1-12.
<https://doi.org/10.1007/s12192-017-0806-9>
- Crowe, A. R., & Yue, W. (2019). Semi-quantitative Determination of Protein Expression using Immunohistochemistry Staining and Analysis: An Integrated Protocol. LID - e3465 [pii] LID - 10.21769/BioProtoc.3465 [doi]. (2331-8325 (Print)).
- Cruts, M., Gijselinck, I., Van Langenhove, T., van der Zee, J., & Van Broeckhoven, C. (2013). Current insights into the C9orf72 repeat expansion diseases of the FTLD/ALS spectrum. *Trends in Neurosciences*, 36(8), 450-459. <https://doi.org/https://doi.org/10.1016/j.tins.2013.04.010>
- Cruz-Migoni, A., Hautbergue Gm Fau - Artymiuk, P. J., Artymiuk Pj Fau - Baker, P. J., Baker Pj Fau - Bokori-Brown, M., Bokori-Brown M Fau - Chang, C.-T., Chang Ct Fau - Dickman, M. J., . . . Rice, D. W. (2012). A Burkholderia pseudomallei toxin inhibits helicase activity of translation factor eIF4A. (1095-9203 (Electronic)). <https://doi.org/D> - NLM: UKMS48011 EDAT- 2011/11/15 06:00 MHDA- 2011/12/13 00:00 CRDT- 2011/11/15 06:00 PHST- 2011/11/15 06:00 [entrez] PHST- 2011/11/15 06:00 [pubmed] PHST- 2011/12/13 00:00 [medline] AID - 334/6057/821 [pii] AID - 10.1126/science.1211915 [doi] PST - ppublish
- Dafinca, R., Barbagallo, P., Farrimond, L., Candalija, A., Scaber, J., Ababneh, N. a. A., . . . Talbot, K. (2020). Impairment of Mitochondrial Calcium Buffering Links Mutations in C9ORF72 and TARDBP in iPS-Derived Motor Neurons from Patients with ALS/FTD. *Stem Cell Reports*, 14(5), 892-908.
<https://doi.org/https://doi.org/10.1016/j.stemcr.2020.03.023>
- Das, S., Anczuków O Fau - Akerman, M., Akerman M Fau - Krainer, A. R., & Krainer, A. R. (2012). Oncogenic splicing factor SRSF1 is a critical transcriptional target of MYC. (2211-1247 (Electronic)).
- Davidson, J. M., Chung, R. S., & Lee, A. (2022). The converging roles of sequestosome-1/p62 in the molecular pathways of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). *Neurobiology of Disease*, 166, 105653. <https://doi.org/https://doi.org/10.1016/j.nbd.2022.105653>
- Davidson, Y. S., Flood, L., Robinson, A. C., Nihei, Y., Mori, K., Rollinson, S., . . . Mann, D. M. A. (2017). Heterogeneous ribonuclear protein A3 (hnRNP A3) is present in dipeptide repeat protein containing inclusions in Frontotemporal Lobar Degeneration and Motor Neurone disease associated with expansions in C9orf72 gene. *Acta neuropathologica communications*, 5(1), 31-31.
<https://doi.org/10.1186/s40478-017-0437-5>
- Daya, S., & Berns, K. I. (2008). Gene therapy using adeno-associated virus vectors. (1098-6618 (Electronic)).
- De, S., & Klenerman, D. (2019). Imaging individual protein aggregates to follow aggregation and determine the role of aggregates in neurodegenerative disease. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, 1867(10), 870-878. <https://doi.org/https://doi.org/10.1016/j.bbapap.2018.12.010>
- Debska-Vielhaber, G., Miller, I., Peeva, V., Zusratter, W., Walczak, J., Schreiber, S., . . . Kunz, W. S. (2021). Impairment of mitochondrial oxidative phosphorylation in skin fibroblasts of SALS and FALS patients is rescued by in vitro treatment with ROS scavengers. *Experimental Neurology*, 339, 113620.
<https://doi.org/https://doi.org/10.1016/j.expneurol.2021.113620>
- DeJesus-Hernandez, M., Mackenzie, I. R., Boeve, B. F., Boxer, A. L., Baker, M., Rutherford, N. J., . . . Rademakers, R. (2011). Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron*, 72(2), 245-256.
<https://doi.org/10.1016/j.neuron.2011.09.011>

- Deshaies, J.-E., Shkreta, L., Moszczynski, A. J., Sidibé, H., Semmler, S., Fouillen, A., . . . Vande Velde, C. (2018). TDP-43 regulates the alternative splicing of hnRNP A1 to yield an aggregation-prone variant in amyotrophic lateral sclerosis. *Brain : a journal of neurology*, 141(5), 1320-1333.
<https://doi.org/10.1093/brain/awy062>
- Ding, F., Su, C. J., Edmonds, K. K., Liang, G., & Elowitz, M. B. (2022). Dynamics and functional roles of splicing factor autoregulation. *Cell Reports*, 39(12), 110985.
<https://doi.org/https://doi.org/10.1016/j.celrep.2022.110985>
- Dmitriev, S. E., Terenin Im Fau - Dunaevsky, Y. E., Dunaevsky Ye Fau - Merrick, W. C., Merrick Wc Fau - Shatsky, I. N., & Shatsky, I. N. (2003). Assembly of 48S translation initiation complexes from purified components with mRNAs that have some base pairing within their 5' untranslated regions. (0270-7306 (Print)).
- Dodd, D. W., Tomchick, D. R., Corey, D. R., & Gagnon, K. T. (2016). Pathogenic C9ORF72 Antisense Repeat RNA Forms a Double Helix with Tandem C:C Mismatches. (1520-4995 (Electronic)).
- Dong, B., Nakai, H., & Xiao, W. (2010). Characterization of genome integrity for oversized recombinant AAV vector. *Mol Ther J Am Soc Gene Ther*, 18. <https://doi.org/10.1038/mt.2009.258>
- Douglas, A. G. L. (2018a). Non-coding RNA in C9orf72-related amyotrophic lateral sclerosis and frontotemporal dementia: A perfect storm of dysfunction. *Non-coding RNA Research*, 3(4), 178-187.
<https://doi.org/https://doi.org/10.1016/j.ncrna.2018.09.001>
- Douglas, A. G. L. (2018b). Non-coding RNA in C9orf72-related amyotrophic lateral sclerosis and frontotemporal dementia: A perfect storm of dysfunction. *Non-coding RNA Research*.
<https://doi.org/https://doi.org/10.1016/j.ncrna.2018.09.001>
- Dudman, J., & Qi, X. (2020). Stress Granule Dysregulation in Amyotrophic Lateral Sclerosis [Mini Review]. *Frontiers in Cellular Neuroscience*, 14.
- Ebstein, S. Y., Yagudayeva, I., & Shneider, N. A. (2019). Mutant TDP-43 Causes Early-Stage Dose-Dependent Motor Neuron Degeneration in a TARDBP Knockin Mouse Model of ALS. (2211-1247 (Electronic)).
- Emma, L. v. d. E., Jazmyne, L. J., Adrianna, W., Harro, S., Marka van, B., & John, C. V. S. (2021). Unravelling the clinical spectrum and the role of repeat length in C9ORF72 repeat expansions. *Journal of Neurology, Neurosurgery & Psychiatry*, 92(5), 502. <https://doi.org/10.1136/jnnp-2020-325377>
- Fairman-Williams, M. E., Guenther Up Fau - Jankowsky, E., & Jankowsky, E. (2010). SF1 and SF2 helicases: family matters. (1879-033X (Electronic)).
- Fan, X., Huang, T., Tong, Y., Fan, Z., Yang, Z., Yang, D., . . . Yang, M. (2022). p62 works as a hub modulation in the ageing process. *Ageing Research Reviews*, 73, 101538.
<https://doi.org/https://doi.org/10.1016/j.arr.2021.101538>
- Farg, M. A., Sundaramoorthy, V., Sultana, J. M., Yang, S., Atkinson, R. A. K., Levina, V., . . . Atkin, J. D. (2014). C9ORF72, implicated in amyotrophic lateral sclerosis and frontotemporal dementia, regulates endosomal trafficking. *Human molecular genetics*, 23(13), 3579-3595.
<https://doi.org/10.1093/hmg/ddu068>
- Farg, M. A., Sundaramoorthy, V., Sultana, J. M., Yang, S., Atkinson, R. A. K., Levina, V., . . . Atkin, J. D. (2017). C9ORF72, implicated in amyotrophic lateral sclerosis and frontotemporal dementia, regulates endosomal trafficking. *Human Molecular Genetics*, 26(20), 4093-4094.
<https://doi.org/10.1093/hmg/ddx309>
- Fay, M. M., Anderson, P. J., & Ivanov, P. (2017). ALS/FTD-Associated C9ORF72 Repeat RNA Promotes Phase Transitions In Vitro and in Cells. *Cell Reports*, 21(12), 3573-3584.
<https://doi.org/https://doi.org/10.1016/j.celrep.2017.11.093>

- Feneberg, E., Charles, P. D., Finelli, M. J., Scott, C., Kessler, B. M., Fischer, R., . . . Turner, M. R. (2021). Detection and quantification of novel C-terminal TDP-43 fragments in ALS-TDP
[<https://doi.org/10.1111/bpa.12923>]. *Brain Pathology*, 31(4), e12923.
<https://doi.org/https://doi.org/10.1111/bpa.12923>
- Foster, A. D., Flynn, L. L., Cluning, C., Cheng, F., Davidson, J. M., Lee, A., . . . Rea, S. L. (2021). p62 overexpression induces TDP-43 cytoplasmic mislocalisation, aggregation and cleavage and neuronal death. (2045-2322 (Electronic)).
- Foster, A. D., & Rea, S. L. (2020). The role of sequestosome 1/p62 protein in amyotrophic lateral sclerosis and frontotemporal dementia pathogenesis. (1673-5374 (Print)).
- Fournier, C., Barbier, M., Camuzat, A., Anquetil, V., Lattante, S., Clot, F., . . . Leguern, E. (2019). Relations between C9orf72 expansion size in blood, age at onset, age at collection and transmission across generations in patients and presymptomatic carriers. *Neurobiology of Aging*, 74, 234.e231-234.e238.
<https://doi.org/https://doi.org/10.1016/j.neurobiolaging.2018.09.010>
- Foust, K. D., Nurre, E., Montgomery, C. L., Hernandez, A., Chan, C. M., & Kaspar, B. K. (2009). Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nature biotechnology*, 27(1), 59-65. <https://doi.org/10.1038/nbt.1515>
- Fratta, P., Mizielska, S., Nicoll, A. J., Zloh, M., Fisher, E. M. C., Parkinson, G., & Isaacs, A. M. (2012). C9orf72 hexanucleotide repeat associated with amyotrophic lateral sclerosis and frontotemporal dementia forms RNA G-quadruplexes. *Scientific reports*, 2, 1016-1016. <https://doi.org/10.1038/srep01016>
- Fratta, P., Poulter, M., Lashley, T., Rohrer, J. D., Polke, J. M., Beck, J., . . . Mead, S. (2013). Homozygosity for the C9orf72 GGGGCC repeat expansion in frontotemporal dementia. *Acta neuropathologica*, 126(3), 401-409. <https://doi.org/10.1007/s00401-013-1147-0>
- Freibaum, B. D., Lu, Y., Lopez-Gonzalez, R., Kim, N. C., Almeida, S., Lee, K.-H., . . . Taylor, J. P. (2015). GGGGCC repeat expansion in C9orf72 compromises nucleocytoplasmic transport. *Nature*, 525(7567), 129-133. <https://doi.org/10.1038/nature14974>
- Freibaum, B. D., & Taylor, J. P. (2017). The Role of Dipeptide Repeats in C9ORF72-Related ALS-FTD. *Frontiers in molecular neuroscience*, 10, 35-35. <https://doi.org/10.3389/fnmol.2017.00035>
- Frick, P., Sellier, C., Mackenzie, I. R. A., Cheng, C. Y., Tahraoui-Bories, J., Martinat, C., . . . Neumann, M. A.-O. (2018). Novel antibodies reveal presynaptic localization of C9orf72 protein and reduced protein levels in C9orf72 mutation carriers. (2051-5960 (Electronic)).
- Fritschy, J. M. (2008). Is my antibody-staining specific? How to deal with pitfalls of immunohistochemistry. (1460-9568 (Electronic)).
- Gabriel, N., Hareendran, S., Sen, D., Gadkari, R. A., Sudha, G., Selot, R., . . . Jayandharan, G. R. (2013). Bioengineering of AAV2 capsid at specific serine, threonine, or lysine residues improves its transduction efficiency in vitro and in vivo. *Human gene therapy methods*, 24(2), 80-93. <https://doi.org/10.1089/hgtb.2012.194>
- Gagliardi, D., Costamagna, G., Taiana, M., Andreoli, L., Biella, F., Bersani, M., . . . Corti, S. (2020). Insights into disease mechanisms and potential therapeutics for C9orf72-related amyotrophic lateral sclerosis/frontotemporal dementia. *Ageing Research Reviews*, 64, 101172. <https://doi.org/https://doi.org/10.1016/j.arr.2020.101172>
- Galicia-Vázquez, G., Cencic R Fau - Robert, F., Robert F Fau - Agenor, A. Q., Agenor Aq Fau - Pelletier, J., & Pelletier, J. (2012). A cellular response linking eIF4AI activity to eIF4AII transcription. (1469-9001 (Electronic)).

- Galicía-Vázquez, G., Chu, J., & Pelletier, J. (2015). eIF4AII is dispensable for miRNA-mediated gene silencing. (1469-9001 (Electronic)).
- Gan, W., Guan, Z., Liu, J., Gui, T., Shen, K., Manley, J. L., & Li, X. (2011). R-loop-mediated genomic instability is caused by impairment of replication fork progression. *Genes & development*, 25(19), 2041-2056. <https://doi.org/10.1101/gad.17010011>
- Gao, F.-B., Richter, J. D., & Cleveland, D. W. (2017). Rethinking Unconventional Translation in Neurodegeneration. *Cell*, 171(5), 994-1000. <https://doi.org/https://doi.org/10.1016/j.cell.2017.10.042>
- Gao, G.-P., Lu, Y., Sun, X., Johnston, J., Calcedo, R., Grant, R., & Wilson, J. M. (2006). High-level transgene expression in nonhuman primate liver with novel adeno-associated virus serotypes containing self-complementary genomes. *Journal of virology*, 80(12), 6192-6194. <https://doi.org/10.1128/JVI.00526-06>
- Gao, Z., van der Velden, Y. U., Fan, M., van der Linden, C. A., Vink, M., Herrera-Carrillo, E., & Berkhout, B. (2021). Engineered miniature H1 promoters with dedicated RNA polymerase II or III activity. (1083-351X (Electronic)).
- García-García, C., Frieda, K. L., Feoktistova, K., Fraser, C. S., & Block, S. M. (2015). Factor-dependent processivity in human eIF4A DEAD-box helicase. *Science*, 348(6242), 1486-1488. <https://doi.org/10.1126/science.aaa5089>
- Gehring, N. H., & Roignant, J.-Y. (2021). Anything but Ordinary – Emerging Splicing Mechanisms in Eukaryotic Gene Regulation. *Trends in Genetics*, 37(4), 355-372. <https://doi.org/https://doi.org/10.1016/j.tig.2020.10.008>
- Gendron, T. F., Bieniek, K. F., Zhang, Y.-J., Jansen-West, K., Ash, P. E. A., Caulfield, T., . . . Petrucelli, L. (2013). Antisense transcripts of the expanded C9ORF72 hexanucleotide repeat form nuclear RNA foci and undergo repeat-associated non-ATG translation in c9FTD/ALS. *Acta neuropathologica*, 126(6), 829-844. <https://doi.org/10.1007/s00401-013-1192-8>
- Gendron, T. F., Chew, J., Stankowski, J. N., Hayes, L. R., Zhang, Y.-J., Prudencio, M., . . . Petrucelli, L. (2017). Poly(GP) proteins are a useful pharmacodynamic marker for C9ORF72-associated amyotrophic lateral sclerosis. *Science Translational Medicine*, 9(383), eaai7866. <https://doi.org/10.1126/scitranslmed.aai7866>
- Gendron, T. F., van Blitterswijk, M., Bieniek, K. F., Daugherty, L. M., Jiang, J., Rush, B. K., . . . Boylan, K. B. (2015). Cerebellar c9RAN proteins associate with clinical and neuropathological characteristics of C9ORF72 repeat expansion carriers. (1432-0533 (Electronic)).
- Ghosh, R., Gilda Je Fau - Gomes, A. V., & Gomes, A. V. (2014). The necessity of and strategies for improving confidence in the accuracy of western blots. (1744-8387 (Electronic)).
- Gomes, C., Sequeira, C., Barbosa, M., Cunha, C., Vaz, A. R., & Brites, D. (2020). Astrocyte regional diversity in ALS includes distinct aberrant phenotypes with common and causal pathological processes. *Experimental Cell Research*, 395(2), 112209. <https://doi.org/https://doi.org/10.1016/j.yexcr.2020.112209>
- Goodman, L. D., & Bonini, N. M. (2019). Repeat-associated non-AUG (RAN) translation mechanisms are running into focus for GGGGCC-repeat associated ALS/FTD. *Progress in Neurobiology*, 183, 101697. <https://doi.org/https://doi.org/10.1016/j.pneurobio.2019.101697>
- Goodman, L. D., Prudencio, M., Srinivasan, A. R., Rifai, O. M., Lee, V. M. Y., Petrucelli, L., & Bonini, N. M. (2019). eIF4B and eIF4H mediate GR production from expanded G4C2 in a Drosophila model for C9orf72-

- associated ALS. *Acta Neuropathologica Communications*, 7(1), 62. <https://doi.org/10.1186/s40478-019-0711-9>
- Goodman, S. L. (2018). The antibody horror show: an introductory guide for the perplexed. *New Biotechnology*, 45, 9-13. <https://doi.org/https://doi.org/10.1016/j.nbt.2018.01.006>
- Gordon, J. M., Phizicky, D. V., & Neugebauer, K. M. (2021). Nuclear mechanisms of gene expression control: pre-mRNA splicing as a life or death decision. *Current Opinion in Genetics & Development*, 67, 67-76. <https://doi.org/https://doi.org/10.1016/j.gde.2020.11.002>
- Green, K. M., Glineburg, M. R., Kearse, M. G., Flores, B. N., Linsalata, A. E., Fedak, S. J., . . . Todd, P. K. (2017). RAN translation at C9orf72-associated repeat expansions is selectively enhanced by the integrated stress response. *Nature communications*, 8(1), 2005-2005. <https://doi.org/10.1038/s41467-017-02200-0>
- Green, K. M., Linsalata, A. E., & Todd, P. K. (2016). RAN translation—What makes it run? *Brain Research*, 1647, 30-42. <https://doi.org/https://doi.org/10.1016/j.brainres.2016.04.003>
- Green, K. M., Sheth, U. J., Flores, B. N., Wright, S. E., Sutter, A. B., Kearse, M. G., . . . Todd, P. K. (2019). High-throughput screening yields several small-molecule inhibitors of repeat-associated non-AUG translation. *The Journal of biological chemistry*, 294(49), 18624-18638. <https://doi.org/10.1074/jbc.RA119.009951>
- Grieger, J. C., & Samulski, R. J. (2005). Packaging capacity of adeno-associated virus serotypes: impact of larger genomes on infectivity and postentry steps. *Journal of virology*, 79(15), 9933-9944. <https://doi.org/10.1128/JVI.79.15.9933-9944.2005>
- Gueddouda, N. M., Mendoza, O., Gomez, D., Bourdoncle, A., & Mergny, J.-L. (2017). G-quadruplexes unfolding by RHAU helicase. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1861(5, Part B), 1382-1388. <https://doi.org/https://doi.org/10.1016/j.bbagen.2017.01.006>
- Guo, S., Nguyen, L., & Ranum, L. P. W. (2022). RAN proteins in neurodegenerative disease: Repeating themes and unifying therapeutic strategies. *Current Opinion in Neurobiology*, 72, 160-170. <https://doi.org/https://doi.org/10.1016/j.conb.2021.11.001>
- Guo, W., Chen, Y., Zhou, X., Kar, A., Ray, P., Chen, X., . . . Wu, J. Y. (2011). An ALS-associated mutation affecting TDP-43 enhances protein aggregation, fibril formation and neurotoxicity. *Nature structural & molecular biology*, 18(7), 822-830. <https://doi.org/10.1038/nsmb.2053>
- Guo, Y.-l., Chen, Z.-c., Li, N., Tian, C.-j., Cheng, D.-j., Tang, X.-y., . . . Zhang, X.-y. (2022). SRSF1 promotes ASMC proliferation in asthma by competitively binding CCND2 with miRNA-135a. *Pulmonary Pharmacology & Therapeutics*, 102173. <https://doi.org/https://doi.org/10.1016/j.pupt.2022.102173>
- Haeusler, A. R., Donnelly, C. J., Periz, G., Simko, E. A. J., Shaw, P. G., Kim, M.-S., . . . Wang, J. (2014). C9orf72 nucleotide repeat structures initiate molecular cascades of disease. *Nature*, 507(7491), 195-200. <https://doi.org/10.1038/nature13124>
- Halpern, M., Brennand, K. J., & Gregory, J. (2019). Examining the relationship between astrocyte dysfunction and neurodegeneration in ALS using hiPSCs. *Neurobiology of Disease*, 132, 104562. <https://doi.org/https://doi.org/10.1016/j.nbd.2019.104562>
- Hardiman, O., & van den Berg, L. H. (2017). Edaravone: a new treatment for ALS on the horizon? *The Lancet Neurology*, 16(7), 490-491. [https://doi.org/https://doi.org/10.1016/S1474-4422\(17\)30163-1](https://doi.org/https://doi.org/10.1016/S1474-4422(17)30163-1)
- Hardiman, O., van den Berg, L. H., & Kiernan, M. C. (2011). Clinical diagnosis and management of amyotrophic lateral sclerosis. *Nat Rev Neurol*, 7(11), 639-649. <https://doi.org/10.1038/nrneurol.2011.153>
- Harms, M. B., Cady, J., Zaidman, C., Cooper, P., Bali, T., Allred, P., . . . Baloh, R. H. (2013). Lack of C9ORF72 coding mutations supports a gain of function for repeat expansions in amyotrophic lateral sclerosis.

- Neurobiology of aging*, 34(9), 2234.e2213-2234.e2232.2234E2219.
<https://doi.org/10.1016/j.neurobiolaging.2013.03.006>
- Harms, U., Andreou, A. Z., Gubaev, A., & Klostermeier, D. (2014). eIF4B, eIF4G and RNA regulate eIF4A activity in translation initiation by modulating the eIF4A conformational cycle. (1362-4962 (Electronic)).
- Harrison, A. F., & Shorter, J. (2017). RNA-binding proteins with prion-like domains in health and disease. *The Biochemical journal*, 474(8), 1417-1438. <https://doi.org/10.1042/BCJ20160499>
- Hashimoto, K., Jahan, N., Miller, Z. A., & Huang, E. J. (2022). Neuroimmune dysfunction in frontotemporal dementia: Insights from progranulin and C9orf72 deficiency. *Current Opinion in Neurobiology*, 76, 102599. <https://doi.org/https://doi.org/10.1016/j.conb.2022.102599>
- Hautbergue, G. M., Castelli, L. M., Ferraiuolo, L., Sanchez-Martinez, A., Cooper-Knock, J., Higginbottom, A., . . . Shaw, P. J. (2017). SRSF1-dependent nuclear export inhibition of C9ORF72 repeat transcripts prevents neurodegeneration and associated motor deficits. *Nature communications*, 8, 16063-16063. <https://doi.org/10.1038/ncomms16063>
- Hautbergue, G. M., Cleary, J. D., Guo, S., & Ranum, L. P. W. (2021). Therapeutic strategies for C9orf72 amyotrophic lateral sclerosis and frontotemporal dementia. *Current Opinion in Neurology*, 34(5).
- He, Y., & Smith, R. (2009). Nuclear functions of heterogeneous nuclear ribonucleoproteins A/B. *Cellular and Molecular Life Sciences*, 66(7), 1239-1256. <https://doi.org/10.1007/s00018-008-8532-1>
- Hernández, G., Vázquez-Pianzola, P., Zurbriggen, A., Altmann, M., Sierra, J. M., & Rivera-Pomar, R. (2004). Two functionally redundant isoforms of *Drosophila melanogaster* eukaryotic initiation factor 4B are involved in cap-dependent translation, cell survival, and proliferation. *European Journal of Biochemistry*, 271(14), 2923-2936. <https://doi.org/10.1111/j.1432-1033.2004.04217.x>
- Hetz, C. (2012). The unfolded protein response: controlling cell fate decisions under ER stress and beyond [Review Article]. *Nature Reviews Molecular Cell Biology*, 13, 89. <https://doi.org/10.1038/nrm3270>
- Highley, J. R., Kirby, J., Jansweijer, J. A., Webb, P. S., Hewamadduma, C. A., Heath, P. R., . . . Ince, P. G. (2014). Loss of nuclear TDP-43 in amyotrophic lateral sclerosis (ALS) causes altered expression of splicing machinery and widespread dysregulation of RNA splicing in motor neurones [\[https://doi.org/10.1111/nan.12148\]](https://doi.org/10.1111/nan.12148). *Neuropathology and Applied Neurobiology*, 40(6), 670-685. <https://doi.org/https://doi.org/10.1111/nan.12148>
- Hinnebusch, A. G. (2017). Structural Insights into the Mechanism of Scanning and Start Codon Recognition in Eukaryotic Translation Initiation. *Trends in Biochemical Sciences*, 42(8), 589-611. <https://doi.org/https://doi.org/10.1016/j.tibs.2017.03.004>
- Hobson, E. V., Harwood, C. A., McDermott, C. J., & Shaw, P. J. (2016). Clinical aspects of motor neurone disease. *Medicine*, 44(9), 552-556. <https://doi.org/https://doi.org/10.1016/j.mpmed.2016.06.004>
- Holcik, M., & Sonenberg, N. (2005). Translational control in stress and apoptosis. (1471-0072 (Print)).
- Holly, A. C., Melzer D Fau - Pilling, L. C., Pilling Lc Fau - Fellows, A. C., Fellows Ac Fau - Tanaka, T., Tanaka T Fau - Ferrucci, L., Ferrucci L Fau - Harries, L. W., & Harries, L. W. (2013). Changes in splicing factor expression are associated with advancing age in man. (1872-6216 (Electronic)).
- Horvilleur, E., Sbarrato, T., Hill, K., Spriggs, R. V., Screen, M., Goodrem, P. J., . . . Willis, A. E. (2014). A role for eukaryotic initiation factor 4B overexpression in the pathogenesis of diffuse large B-cell lymphoma. (1476-5551 (Electronic)).
- Hu, L., Mao, S., Lin, L., Bai, G., Liu, B., & Mao, J. (2022). Stress granules in the spinal muscular atrophy and amyotrophic lateral sclerosis: The correlation and promising therapy. *Neurobiology of Disease*, 170, 105749. <https://doi.org/https://doi.org/10.1016/j.nbd.2022.105749>

- Huppert, J. L., & Balasubramanian, S. (2005). Prevalence of quadruplexes in the human genome. (1362-4962 (Electronic)).
- Hyman, A. A., Weber Ca Fau - Jülicher, F., & Jülicher, F. (2014). Liquid-liquid phase separation in biology. (1530-8995 (Electronic)).
- Ivell, R., Teerds K Fau - Hoffman, G. E., & Hoffman, G. E. (2014). Proper application of antibodies for immunohistochemical detection: antibody crimes and how to prevent them. (1945-7170 (Electronic)).
- Iwamoto, F., Stadler, M., Chalupníková, K., Oakeley, E., & Nagamine, Y. (2008). Transcription-dependent nucleolar cap localization and possible nuclear function of DExH RNA helicase RHAU. *Experimental cell research*, 314(6), 1378-1391. <https://doi.org/10.1016/j.yexcr.2008.01.006>
- Iwasaki, S., Iwasaki, W., Takahashi, M., Sakamoto, A., Watanabe, C., Shichino, Y., . . . Ingolia, N. T. (2019). The Translation Inhibitor Rocaglamide Targets a Bimolecular Cavity between eIF4A and Polypurine RNA. *Molecular Cell*, 73(4), 738-748.e739. <https://doi.org/https://doi.org/10.1016/j.molcel.2018.11.026>
- Jafarinia, H., van der Giessen, E., & Onck, P. R. (2020). Phase Separation of Toxic Dipeptide Repeat Proteins Related to C9orf72 ALS/FTD. *Biophysical Journal*, 119(4), 843-851. <https://doi.org/https://doi.org/10.1016/j.bpj.2020.07.005>
- Jain, A., & Vale, R. D. (2017). RNA phase transitions in repeat expansion disorders. *Nature*, 546(7657), 243-247. <https://doi.org/10.1038/nature22386>
- Jambeau, M., Meyer, K. D., Hruska-Plochan, M., Tabet, R., Lee, C.-Z., Ray-Soni, A., . . . Lagier-Tourenne, C. (2022). Comprehensive evaluation of human-derived anti-poly-GA antibodies in cellular and animal models of C9orf72 disease. *Proceedings of the National Academy of Sciences*, 119(49), e2123487119. <https://doi.org/10.1073/pnas.2123487119>
- Janes, K. A. (2015). An analysis of critical factors for quantitative immunoblotting. *Science Signaling*, 8(371), rs2-rs2. <https://doi.org/10.1126/scisignal.2005966>
- Jensen, K., Krusenstjerna-Hafstrøm, R., Lohse, J., Petersen, K. H., & Derand, H. (2017). A novel quantitative immunohistochemistry method for precise protein measurements directly in formalin-fixed, paraffin-embedded specimens: analytical performance measuring HER2. *Modern Pathology*, 30(2), 180-193. <https://doi.org/10.1038/modpathol.2016.176>
- Jiwaji, Z., & Hardingham, G. E. (2022). Good, bad, and neglectful: Astrocyte changes in neurodegenerative disease. *Free Radical Biology and Medicine*, 182, 93-99. <https://doi.org/https://doi.org/10.1016/j.freeradbiomed.2022.02.020>
- Jongjitwimol, J., Baldock, R. A., Morley, S. J., & Watts, F. A.-O. (2016). Sumoylation of eIF4A2 affects stress granule formation. (1477-9137 (Electronic)).
- Jovičić, A., Mertens, J., Boeynaems, S., Bogaert, E., Chai, N., Yamada, S. B., . . . Gitler, A. D. (2015). Modifiers of C9orf72 dipeptide repeat toxicity connect nucleocytoplasmic transport defects to FTD/ALS. *Nature neuroscience*, 18(9), 1226-1229. <https://doi.org/10.1038/nn.4085>
- Kanekura, K., Yagi, T., Cammack, A. J., Mahadevan, J., Kuroda, M., Harms, M. B., . . . Urano, F. (2016). Poly-dipeptides encoded by the C9ORF72 repeats block global protein translation. *Human molecular genetics*, 25(9), 1803-1813. <https://doi.org/10.1093/hmg/ddw052>
- Kapur, M., Monaghan, C. E., & Ackerman, S. L. (2017). Regulation of mRNA Translation in Neurons—A Matter of Life and Death. *Neuron*, 96(3), 616-637. <https://doi.org/https://doi.org/10.1016/j.neuron.2017.09.057>
- Karow, A. R., Theissen, B., & Klostermeier, D. (2007). Authentic interdomain communication in an RNA helicase reconstituted by expressed protein ligation of two helicase domains [<https://doi.org/10.1111/j.1742->

- [4658.2006.05593.x](#)]. *The FEBS Journal*, 274(2), 463-473.
<https://doi.org/https://doi.org/10.1111/j.1742-4658.2006.05593.x>
- Khosravi, B., Hartmann, H., May, S., Möhl, C., Ederle, H., Michaelson, M., . . . Edbauer, D. (2017). Cytoplasmic poly-GA aggregates impair nuclear import of TDP-43 in C9orf72 ALS/FTLD. *Human molecular genetics*, 26(4), 790-800. <https://doi.org/10.1093/hmg/ddw432>
- King, H. A., & Gerber, A. P. (2016). Translatome profiling: methods for genome-scale analysis of mRNA translation. *Briefings in Functional Genomics*, 15(1), 22-31. <https://doi.org/10.1093/bfpg/elu045>
- Kitamura, A., Nakayama, Y., Shibasaki, A., Taki, A., Yuno, S., Takeda, K., . . . Kinjo, M. (2016). Interaction of RNA with a C-terminal fragment of the amyotrophic lateral sclerosis-associated TDP43 reduces cytotoxicity. (2045-2322 (Electronic)).
- Klein, R. L., Dayton, R. D., Tatom, J. B., Henderson, K. M., & Henning, P. P. (2008). AAV8, 9, Rh10, Rh43 vector gene transfer in the rat brain: effects of serotype, promoter and purification method. *Molecular therapy : the journal of the American Society of Gene Therapy*, 16(1), 89-96.
<https://doi.org/10.1038/sj.mt.6300331>
- Koedoot, E., Smid, M., Foekens, J. A., Martens, J. W. M., Le Dévédec, S. E., & van de Water, B. (2019). Co-regulated gene expression of splicing factors as drivers of cancer progression. *Scientific Reports*, 9(1), 5484. <https://doi.org/10.1038/s41598-019-40759-4>
- Komar, A. A., & Hatzoglou, M. (2011). Cellular IRES-mediated translation: the war of ITAFs in pathophysiological states. *Cell cycle (Georgetown, Tex.)*, 10(2), 229-240.
<https://doi.org/10.4161/cc.10.2.14472>
- Koopman, M. B., Ferrari, L., & Rüdiger, S. G. D. (2022). How do protein aggregates escape quality control in neurodegeneration? *Trends in Neurosciences*, 45(4), 257-271.
<https://doi.org/https://doi.org/10.1016/j.tins.2022.01.006>
- Koppers, M., Blokhuis, A. M., Westeneng, H.-J., Terpstra, M. L., Zundel, C. A. C., Vieira de Sá, R., . . . Pasterkamp, R. J. (2015). C9orf72 ablation in mice does not cause motor neuron degeneration or motor deficits. *Annals of neurology*, 78(3), 426-438. <https://doi.org/10.1002/ana.24453>
- Kotterman, M. A., & Schaffer, D. V. (2014). Engineering adeno-associated viruses for clinical gene therapy. *Nature reviews. Genetics*, 15(7), 445-451. <https://doi.org/10.1038/nrg3742>
- Kriachkov, V., Ormsby, A. R., Kusnadi, E. P., McWilliam, H. E. G., Mintern, J. D., Amarasinghe, S. L., . . . Hatters, D. M. (2022). Arginine-rich C9ORF72 ALS proteins stall ribosomes in a manner distinct from a canonical ribosome-associated quality control substrate. *Journal of Biological Chemistry*, 102774.
<https://doi.org/https://doi.org/10.1016/j.jbc.2022.102774>
- Kroon, C., Breuer, L., Jones, L., An, J., Akan, A., Mohamed Ali, E. A., . . . Weissgerber, T. L. (2022). Blind spots on western blots: Assessment of common problems in western blot figures and methods reporting with recommendations to improve them. *PLOS Biology*, 20(9), e3001783.
<https://doi.org/10.1371/journal.pbio.3001783>
- Krupp S Fau - Tam, O., Tam O Fau - Gale Hammell, M., Gale Hammell M Fau - Dubnau, J., & Dubnau, J. A.-O. (2023). TDP-43 pathology in Drosophila induces glial-cell type specific toxicity that can be ameliorated by knock-down of SF2/SRSF1. LID - 2023.05.04.539439 [pii] LID - 10.1101/2023.05.04.539439 [doi].
- Kurien, B. T., & Scofield, R. H. (2017). Validating Antibody Specificities for Immunohistochemistry by Protein Blotting Methods. In A. E. Kalyuzhny (Ed.), *Signal Transduction Immunohistochemistry: Methods and Protocols* (pp. 61-73). Springer New York. https://doi.org/10.1007/978-1-4939-6759-9_3

- Kwon, I., Xiang, S., Kato, M., Wu, L., Theodoropoulos, P., Wang, T., . . . McKnight, S. L. (2014). Poly-dipeptides encoded by the C9orf72 repeats bind nucleoli, impede RNA biogenesis, and kill cells. *Science (New York, N.Y.)*, 345(6201), 1139-1145. <https://doi.org/10.1126/science.1254917>
- Lai, J. D., & Ichida, J. K. (2019). C9ORF72 protein function and immune dysregulation in amyotrophic lateral sclerosis. *Neuroscience Letters*, 713, 134523. <https://doi.org/https://doi.org/10.1016/j.neulet.2019.134523>
- Lall, D., Lorenzini, I., Mota, T. A., Bell, S., Mahan, T. E., Ulrich, J. D., . . . Baloh, R. H. (2021). C9orf72 deficiency promotes microglial-mediated synaptic loss in aging and amyloid accumulation. *Neuron*, 109(14), 2275-2291.e2278. <https://doi.org/https://doi.org/10.1016/j.neuron.2021.05.020>
- Lashley, T., Rohrer, J. D., Mead, S., & Revesz, T. (2015). Review: An update on clinical, genetic and pathological aspects of frontotemporal lobar degenerations. *Neuropathology and Applied Neurobiology*, 41(7), 858-881. <https://doi.org/10.1111/nan.12250>
- Lawlor, P. A., Bland, R. J., Mouravlev, A., Young, D., & During, M. J. (2009). Efficient gene delivery and selective transduction of glial cells in the mammalian brain by AAV serotypes isolated from nonhuman primates. *Molecular therapy : the journal of the American Society of Gene Therapy*, 17(10), 1692-1702. <https://doi.org/10.1038/mt.2009.170>
- Lee, K.-H., Zhang, P., Kim, H. J., Mitrea, D. M., Sarkar, M., Freibaum, B. D., . . . Taylor, J. P. (2016). C9orf72 Dipeptide Repeats Impair the Assembly, Dynamics, and Function of Membrane-Less Organelles. *Cell*, 167(3), 774-788.e717. <https://doi.org/10.1016/j.cell.2016.10.002>
- Lee, Y.-B., Chen, H.-J., Peres, João N., Gomez-Deza, J., Attig, J., Štalekar, M., . . . Shaw, Christopher E. (2013). Hexanucleotide Repeats in ALS/FTD Form Length-Dependent RNA Foci, Sequester RNA Binding Proteins, and Are Neurotoxic. *Cell Reports*, 5(5), 1178-1186. <https://doi.org/https://doi.org/10.1016/j.celrep.2013.10.049>
- Leitman, J., Barak, B., Benyair, R., Shenkman, M., Ashery, U., Hartl, F. U., & Lederkremer, G. Z. (2014). ER stress-induced eIF2-alpha phosphorylation underlies sensitivity of striatal neurons to pathogenic huntingtin. (1932-6203 (Electronic)).
- Levine, T. P., Daniels, R. D., Gatta, A. T., Wong, L. H., & Hayes, M. J. (2013). The product of C9orf72, a gene strongly implicated in neurodegeneration, is structurally related to DENN Rab-GEFs. *Bioinformatics (Oxford, England)*, 29(4), 499-503. <https://doi.org/10.1093/bioinformatics/bts725>
- Li, D. A.-O., Yang, J., Huang, X., Zhou, H., & Wang, J. A.-O. (2022). eIF4A2 targets developmental potency and histone H3.3 transcripts for translational control of stem cell pluripotency. (2375-2548 (Electronic)). <https://doi.org/2022>
- Li, J., & Li, W. X. (2006). A novel function of Drosophila eIF4A as a negative regulator of Dpp/BMP signalling that mediates SMAD degradation. (1465-7392 (Print)).
- Li, Q., Imataka H Fau - Morino, S., Morino S Fau - Rogers, G. W., Jr., Rogers Gw Jr Fau - Richter-Cook, N. J., Richter-Cook Nj Fau - Merrick, W. C., Merrick Wc Fau - Sonenberg, N., & Sonenberg, N. (1999). Eukaryotic translation initiation factor 4AIII (eIF4AIII) is functionally distinct from eIF4AI and eIF4AII. (0270-7306 (Print)).
- Li, S., Wu, Z., Li, Y., Tantray, I., De Stefani, D., Mattarei, A., . . . Lu, B. (2020). Altered MICOS Morphology and Mitochondrial Ion Homeostasis Contribute to Poly(GR) Toxicity Associated with C9-ALS/FTD. *Cell Reports*, 32(5), 107989. <https://doi.org/https://doi.org/10.1016/j.celrep.2020.107989>
- Li, W., Chen, A., Xiong, L., Chen, T., Tao, F., Lu, Y., . . . Xu, Y. (2017). miR-133a acts as a tumor suppressor in colorectal cancer by targeting eIF4A1. (1423-0380 (Electronic)).

- Li, X., & Manley, J. L. (2005). Inactivation of the SR Protein Splicing Factor ASF/SF2 Results in Genomic Instability. *Cell*, 122(3), 365-378. <https://doi.org/10.1016/j.cell.2005.06.008>
- Liang, S., Ju, X., Zhou, Y., Chen, Y., Ke, G., Wen, H., & Wu, X. (2017). Downregulation of eukaryotic initiation factor 4A1 improves radiosensitivity by delaying DNA double strand break repair in cervical cancer. (1792-1074 (Print)).
- Linder, P., & Jankowsky, E. (2011). From unwinding to clamping — the DEAD box RNA helicase family. *Nature Reviews Molecular Cell Biology*, 12(8), 505-516. <https://doi.org/10.1038/nrm3154>
- Lindqvist, L., Imataka H Fau - Pelletier, J., & Pelletier, J. (2008). Cap-dependent eukaryotic initiation factor-mRNA interactions probed by cross-linking. (1469-9001 (Electronic)).
- Liu, F., Morderer, D., Wren, M. C., Vettleson-Trutza, S. A., Wang, Y., Rabichow, B. E., . . . Rossoll, W. (2022). Proximity proteomics of C9orf72 dipeptide repeat proteins identifies molecular chaperones as modifiers of poly-GA aggregation. *Acta Neuropathologica Communications*, 10(1), 22. <https://doi.org/10.1186/s40478-022-01322-x>
- Liu, H., Lu, Y.-N., Paul, T., Periz, G., Banco, M. T., Ferré-D'Amaré, A. R., . . . Wang, J. (2021). A Helicase Unwinds Hexanucleotide Repeat RNA G-Quadruplexes and Facilitates Repeat-Associated Non-AUG Translation. *Journal of the American Chemical Society*, 143(19), 7368-7379. <https://doi.org/10.1021/jacs.1c00131>
- Liu, J., Hu, J., Ludlow, A. T., Pham, J. T., Shay, J. W., Rothstein, J. D., & Corey, D. R. (2017). c9orf72 Disease-Related Foci Are Each Composed of One Mutant Expanded Repeat RNA. *Cell chemical biology*, 24(2), 141-148. <https://doi.org/10.1016/j.chembiol.2016.12.018>
- Liu, Y., Pattamatta, A., Zu, T., Reid, T., Bardhi, O., Borchelt, David R., . . . Ranum, Laura P. W. (2016). C9orf72 BAC Mouse Model with Motor Deficits and Neurodegenerative Features of ALS/FTD. *Neuron*, 90(3), 521-534. <https://doi.org/10.1016/j.neuron.2016.04.005>
- Liu, Y., Xing, H., Ernst, A. F., Liu, C., Maugee, C., Yokoi, F., . . . Li, Y. (2022). Hyperactivity of Purkinje cell and motor deficits in C9orf72 knockout mice. *Molecular and Cellular Neuroscience*, 121, 103756. <https://doi.org/10.1016/j.mcn.2022.103756>
- Lobner, D. (2000). Comparison of the LDH and MTT assays for quantifying cell death: validity for neuronal apoptosis? *Journal of Neuroscience Methods*, 96(2), 147-152. [https://doi.org/10.1016/S0165-0270\(99\)00193-4](https://doi.org/10.1016/S0165-0270(99)00193-4)
- Longinetti, E., & Fang, F. (2019). Epidemiology of amyotrophic lateral sclerosis: an update of recent literature. *Current opinion in neurology*, 32(5), 771-776. <https://doi.org/10.1097/WCO.0000000000000730>
- Lopez-Gonzalez, R., Lu, Y., Gendron, T. F., Karydas, A., Tran, H., Yang, D., . . . Gao, F.-B. (2016). Poly(GR) in C9ORF72-Related ALS/FTD Compromises Mitochondrial Function and Increases Oxidative Stress and DNA Damage in iPSC-Derived Motor Neurons. *Neuron*, 92(2), 383-391. <https://doi.org/10.1016/j.neuron.2016.09.015>
- Lorsch, J. R., & Herschlag, D. (1998). The DEAD box protein eIF4A. 1. A minimal kinetic and thermodynamic framework reveals coupled binding of RNA and nucleotide. (0006-2960 (Print)).
- Loveland, A. B., Svidritskiy, E., Susorov, D., Lee, S., Park, A., Zvornicanin, S., . . . Korostelev, A. A. (2022). Ribosome inhibition by C9ORF72-ALS/FTD-associated poly-PR and poly-GR proteins revealed by cryo-EM. *Nature Communications*, 13(1), 2776. <https://doi.org/10.1038/s41467-022-30418-0>
- Lu, W. T., Wilczynska, A., Smith, E., & Bushell, M. (2014). The diverse roles of the eIF4A family: you are the company you keep. (1470-8752 (Electronic)).
- Luisier, R., Tyzack, G. E., Hall, C. E., Mitchell, J. S., Devine, H., Taha, D. M., . . . Patani, R. (2018). Intron retention and nuclear loss of SFPQ are molecular hallmarks of ALS. *Nature Communications*, 9(1), 2010. <https://doi.org/10.1038/s41467-018-04373-8>

- Lyu, S., Lu, J., Chen, W., Huang, W., Huang, H., Xi, S., & Yan, S. (2020). High expression of eIF4A2 is associated with a poor prognosis in esophageal squamous cell carcinoma. (1792-1074 (Print)).
- Mackenzie, I. R., Arzberger, T., Kremmer, E., Troost, D., Lorenzl, S., Mori, K., . . . Neumann, M. (2013). Dipeptide repeat protein pathology in C9ORF72 mutation cases: clinico-pathological correlations. *Acta Neuropathologica*, 126(6), 859-879. <https://doi.org/10.1007/s00401-013-1181-y>
- Mackenzie, I. R., Frick, P., Grasser, F. A., Gendron, T. F., Petrucelli, L., Cashman, N. R., . . . Neumann, M. (2015). Quantitative analysis and clinico-pathological correlations of different dipeptide repeat protein pathologies in C9ORF72 mutation carriers. *Acta Neuropathol*, 130(6), 845-861. <https://doi.org/10.1007/s00401-015-1476-2>
- Maharana, S. A.-O., Wang, J. A.-O., Papadopoulos, D. K., Richter, D., Pozniakovsky, A., Poser, I. A.-O., . . . Alberti, S. A.-O. (2018). RNA buffers the phase separation behavior of prion-like RNA binding proteins. (1095-9203 (Electronic)).
- Mahmood, T., & Yang, P. C. (2012). Western blot: technique, theory, and trouble shooting. (1947-2714 (Electronic)).
- Mahoney, C. J., Beck, J., Rohrer, J. D., Lashley, T., Mok, K., Shakespeare, T., . . . Warren, J. D. (2012). Frontotemporal dementia with the C9ORF72 hexanucleotide repeat expansion: clinical, neuroanatomical and neuropathological features. *Brain*, 135(3), 736-750. <https://doi.org/10.1093/brain/awr361>
- Manfredsson, F. P., Burger, C., Rising, A. C., Zuobi-Hasona, K., Sullivan, L. F., Lewin, A. S., . . . Mandel, R. J. (2009). Tight Long-term Dynamic Doxycycline Responsive Nigrostriatal GDNF Using a Single rAAV Vector. *Molecular Therapy*, 17(11), 1857-1867. <https://doi.org/https://doi.org/10.1038/mt.2009.196>
- Mann, J. R., Gleixner, A. M., Mauna, J. C., Gomes, E., DeChellis-Marks, M. R., Needham, P. G., . . . Donnelly, C. J. (2019). RNA Binding Antagonizes Neurotoxic Phase Transitions of TDP-43. *Neuron*, 102(2), 321-338.e328. <https://doi.org/https://doi.org/10.1016/j.neuron.2019.01.048>
- Manno, C. S., Pierce, G. F., Arruda, V. R., Glader, B., Ragni, M., Rasko, J. J., . . . Kay, M. A. (2006). Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med*, 12(3), 342-347. <https://doi.org/10.1038/nm1358>
- Marchi, P. A.-O., Marrone, L. A.-O., Brasseur, L., Coens, A., Webster, C. A.-O., Bousset, L., . . . Azzouz, M. A.-O. (2022). C9ORF72-derived poly-GA DPRs undergo endocytic uptake in iAstrocytes and spread to motor neurons. LID - 10.26508/lsa.202101276 [doi] LID - e202101276. (2575-1077 (Electronic)).
- Mariani, M., Karki, R., Spennato, M., Pandya, D., He, S., Andreoli, M., . . . Ferlini, C. (2019). Class III β -tubulin in normal and cancer tissues. (1879-0038 (Electronic)).
- Marin, B., Boumediene, F., Logroscino, G., Couratier, P., Babron, M. C., Leutenegger, A. L., . . . Beghi, E. (2017). Variation in worldwide incidence of amyotrophic lateral sclerosis: a meta-analysis. *Int J Epidemiol*, 46(1), 57-74. <https://doi.org/10.1093/ije/dyw061>
- Marintchev, A., Edmonds Ka Fau - Marintcheva, B., Marintcheva B Fau - Hendrickson, E., Hendrickson E Fau - Oberer, M., Oberer M Fau - Suzuki, C., Suzuki C Fau - Herdy, B., . . . Wagner, G. (2009). Topology and regulation of the human eIF4A/4G/4H helicase complex in translation initiation. (1097-4172 (Electronic)).
- Martino, A. T., Suzuki, M., Markusic, D. M., Zolotukhin, I., Ryals, R. C., Moghimi, B., . . . Herzog, R. W. (2011). The genome of self-complementary adeno-associated viral vectors increases Toll-like receptor 9-dependent innate immune responses in the liver. *Blood*, 117(24), 6459-6468. <https://doi.org/10.1182/blood-2010-10-314518>

- Masrori, P., Beckers, J., Gossye, H., & Van Damme, P. (2022). The role of inflammation in neurodegeneration: novel insights into the role of the immune system in C9orf72 HRE-mediated ALS/FTD. *Molecular Neurodegeneration*, 17(1), 22. <https://doi.org/10.1186/s13024-022-00525-z>
- May, S., Hornburg, D., Schludi, M. H., Arzberger, T., Rentzsch, K., Schwenk, B. M., . . . Edbauer, D. (2014). C9orf72 FTL/ALS-associated Gly-Ala dipeptide repeat proteins cause neuronal toxicity and Unc119 sequestration. *Acta Neuropathol*, 128(4), 485-503. <https://doi.org/10.1007/s00401-014-1329-4>
- McCarty, D. M. (2008). Self-complementary AAV vectors; advances and applications. *Mol Ther*, 16(10), 1648-1656. <https://doi.org/10.1038/mt.2008.171>
- McEachin, Z. T., Parameswaran, J., Raj, N., Bassell, G. J., & Jiang, J. (2020). RNA-mediated toxicity in C9orf72 ALS and FTD. *Neurobiology of Disease*, 145, 105055. <https://doi.org/https://doi.org/10.1016/j.nbd.2020.105055>
- McGurk, L., Gomes, E., Guo, L., Mojsilovic-Petrovic, J., Tran, V., Kalb, R. G., . . . Bonini, N. M. (2018). Poly(ADP-Ribose) Prevents Pathological Phase Separation of TDP-43 by Promoting Liquid Demixing and Stress Granule Localization. *Molecular Cell*, 71(5), 703-717.e709. <https://doi.org/https://doi.org/10.1016/j.molcel.2018.07.002>
- Meier, M., Patel, T. R., Booy, E. P., Marushchak, O., Okun, N., Deo, S., . . . McKenna, S. A. (2013). Binding of G-quadruplexes to the N-terminal recognition domain of the RNA helicase associated with AU-rich element (RHAU). *J Biol Chem*, 288(49), 35014-35027. <https://doi.org/10.1074/jbc.M113.512970>
- Meijer, H. A., Kong, Y. W., Lu, W. T., Wilczynska, A., Spriggs, R. V., Robinson, S. W., . . . Bushell, M. (2013). Translational repression and eIF4A2 activity are critical for microRNA-mediated gene regulation. *Science*, 340(6128), 82-85. <https://doi.org/10.1126/science.1231197>
- Meijer, H. A., Schmidt, T., Gillen, S. L., Langlais, C., Jukes-Jones, R., de Moor, C. H., . . . Bushell, M. (2019). DEAD-box helicase eIF4A2 inhibits CNOT7 deadenylation activity. (1362-4962 (Electronic)).
- Mendoza, O., Bourdoncle, A., Boule, J. B., Brosh, R. M., Jr., & Mergny, J. L. (2016). G-quadruplexes and helicases. *Nucleic Acids Res*, 44(5), 1989-2006. <https://doi.org/10.1093/nar/gkw079>
- Methot, N., Pickett G Fau - Keene, J. D., Keene Jd Fau - Sonenberg, N., & Sonenberg, N. (1996). In vitro RNA selection identifies RNA ligands that specifically bind to eukaryotic translation initiation factor 4B: the role of the RNA motif. (1355-8382 (Print)).
- Metz, A. M., Wong Kc Fau - Malmström, S. A., Malmström Sa Fau - Browning, K. S., & Browning, K. S. (1999). Eukaryotic initiation factor 4B from wheat and Arabidopsis thaliana is a member of a multigene family. (0006-291X (Print)).
- Meyer, K., Ferraiuolo, L., Miranda, C. J., Likhite, S., McElroy, S., Renusch, S., . . . Kaspar, B. K. (2014). Direct conversion of patient fibroblasts demonstrates non-cell autonomous toxicity of astrocytes to motor neurons in familial and sporadic ALS. *Proceedings of the National Academy of Sciences*, 111(2), 829-832. <https://doi.org/10.1073/pnas.1314085111>
- Miccio, A., Antoniou, P., Ciura, S., & Kabashi, E. (2022). Novel genome-editing-based approaches to treat motor neuron diseases: Promises and challenges. *Molecular Therapy*, 30(1), 47-53. <https://doi.org/https://doi.org/10.1016/j.ymthe.2021.04.003>
- Miller, R. G., Mitchell, J. D., Lyon, M., & Moore, D. H. (2002). Riluzole for amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND). *Cochrane Database Syst Rev*(2), Cd001447. <https://doi.org/10.1002/14651858.cd001447>
- Miller, T., Cudkowicz, M., Shaw, P. J., Andersen, P. M., Atassi, N., Bucelli, R. C., . . . Ferguson, T. A. (2020). Phase 1–2 Trial of Antisense Oligonucleotide Tofersen for SOD1 ALS. *New England Journal of Medicine*, 383(2), 109-119. <https://doi.org/10.1056/NEJMoa2003715>

- Mingozzi, F., Maus, M. V., Hui, D. J., Sabatino, D. E., Murphy, S. L., Rasko, J. E., . . . High, K. A. (2007). CD8(+) T-cell responses to adeno-associated virus capsid in humans. *Nat Med*, 13(4), 419-422. <https://doi.org/10.1038/nm1549>
- Mizielinska, S., Gronke, S., Niccoli, T., Ridler, C. E., Clayton, E. L., Devoy, A., . . . Isaacs, A. M. (2014). C9orf72 repeat expansions cause neurodegeneration in Drosophila through arginine-rich proteins. *Science*, 345(6201), 1192-1194. <https://doi.org/10.1126/science.1256800>
- Mizielinska, S., Grönke, S., Niccoli, T., Ridler, C. E., Clayton, E. L., Devoy, A., . . . Isaacs, A. M. (2014). C9orf72 repeat expansions cause neurodegeneration in Drosophila through arginine-rich proteins. *Science*, 345(6201), 1192-1194. <https://doi.org/10.1126/science.1256800>
- Mizuno, Y., Amari, M., Takatama, M., Aizawa, H., Mihara, B., & Okamoto, K. (2006). Immunoreactivities of p62, an ubiquitin-binding protein, in the spinal anterior horn cells of patients with amyotrophic lateral sclerosis. *Journal of the Neurological Sciences*, 249(1), 13-18. <https://doi.org/https://doi.org/10.1016/j.jns.2006.05.060>
- Mizuno-Yamasaki, E., Rivera-Molina, F., & Novick, P. (2012). GTPase networks in membrane traffic. *Annu Rev Biochem*, 81, 637-659. <https://doi.org/10.1146/annurev-biochem-052810-093700>
- Modelska, A., Turro, E., Russell, R., Beaton, J., Sbarrato, T., Spriggs, K., . . . Le Quesne, J. (2015). The malignant phenotype in breast cancer is driven by eIF4A1-mediated changes in the translational landscape. (2041-4889 (Electronic)).
- Moens, T. G., Niccoli, T., Wilson, K. M., Atilano, M. L., Birsá, N., Gittings, L. M., . . . Isaacs, A. M. (2019). C9orf72 arginine-rich dipeptide proteins interact with ribosomal proteins in vivo to induce a toxic translational arrest that is rescued by eIF1A. *Acta neuropathologica*, 137(3), 487-500. <https://doi.org/10.1007/s00401-018-1946-4>
- Mohagheghi, F., Prudencio, M., Stuaní, C., Cook, C., Jansen-West, K., Dickson, D. W., . . . Buratti, E. (2016). TDP-43 functions within a network of hnRNP proteins to inhibit the production of a truncated human SORT1 receptor. *Human molecular genetics*, 25(3), 534-545. <https://doi.org/10.1093/hmg/ddv491>
- Mokas, S., Mills, J. R., Garreau, C., Fournier, M.-J., Robert, F., Arya, P., . . . Mazroui, R. (2009). Uncoupling Stress Granule Assembly and Translation Initiation Inhibition. *Molecular Biology of the Cell*, 20(11), 2673-2683. <https://doi.org/10.1091/mbc.e08-10-1061>
- Monahan, P. E., Lothrop, C. D., Sun, J., Hirsch, M. L., Kafri, T., Kantor, B., . . . Samulski, R. J. (2010). Proteasome inhibitors enhance gene delivery by AAV virus vectors expressing large genomes in hemophilia mouse and dog models: a strategy for broad clinical application. *Mol Ther*, 18(11), 1907-1916. <https://doi.org/10.1038/mt.2010.170>
- Monahan, Z., Shewmaker, F., & Pandey, U. B. (2016). Stress granules at the intersection of autophagy and ALS. *Brain Research*, 1649, 189-200. <https://doi.org/https://doi.org/10.1016/j.brainres.2016.05.022>
- Mordes, D. A., Morrison, B. M., Ament, X. H., Cantrell, C., Mok, J., Eggan, P., . . . Rothstein, J. D. (2020). Absence of Survival and Motor Deficits in 500 Repeat C9ORF72 BAC Mice. *Neuron*, 108(4), 775-783.e774. <https://doi.org/10.1016/j.neuron.2020.08.009>
- Moreno, A. M., Fu, X., Zhu, J., Katrekar, D., Shih, Y.-R. V., Marlett, J., . . . Mali, P. (2018). In Situ Gene Therapy via AAV-CRISPR-Cas9-Mediated Targeted Gene Regulation. *Molecular Therapy*, 26(7), 1818-1827. <https://doi.org/https://doi.org/10.1016/j.ymthe.2018.04.017>
- Mori, K., Arzberger, T., Grasser, F. A., Gijssels, I., May, S., Rentzsch, K., . . . Edbauer, D. (2013). Bidirectional transcripts of the expanded C9orf72 hexanucleotide repeat are translated into aggregating dipeptide repeat proteins. *Acta Neuropathol*, 126(6), 881-893. <https://doi.org/10.1007/s00401-013-1189-3>

- Moritz, C. P. (2017). Tubulin or Not Tubulin: Heading Toward Total Protein Staining as Loading Control in Western Blots [<https://doi.org/10.1002/pmic.201600189>]. *PROTEOMICS*, 17(20), 1600189. <https://doi.org/https://doi.org/10.1002/pmic.201600189>
- Morrice, J. R., Gregory-Evans, C. Y., & Shaw, C. A. (2018). Animal models of amyotrophic lateral sclerosis: A comparison of model validity. (1673-5374 (Print)).
- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65(1), 55-63. [https://doi.org/https://doi.org/10.1016/0022-1759\(83\)90303-4](https://doi.org/https://doi.org/10.1016/0022-1759(83)90303-4)
- Muraleedharan, R., Nardini, D., Waclaw, R. R., & Dasgupta, B. (2021). Analysis of reactive astrogliosis in mouse brain using in situ hybridization combined with immunohistochemistry. *STAR Protocols*, 2(1), 100375. <https://doi.org/https://doi.org/10.1016/j.xpro.2021.100375>
- Murray, T. E., Richards, C. M., Robert-Gostlin, V. N., Bernath, A. K., Lindhout, I. A., & Klegeris, A. (2022). Potential neurotoxic activity of diverse molecules released by astrocytes. *Brain Research Bulletin*, 189, 80-101. <https://doi.org/https://doi.org/10.1016/j.brainresbull.2022.08.015>
- Méthot, N., Song Ms Fau - Sonenberg, N., & Sonenberg, N. (1996). A region rich in aspartic acid, arginine, tyrosine, and glycine (DRYG) mediates eukaryotic initiation factor 4B (eIF4B) self-association and interaction with eIF3. (0270-7306 (Print)).
- Naineni, S. K., Liang, J., Hull, K., Cencic, R., Zhu, M., Northcote, P., . . . Pelletier, J. (2021). Functional mimicry revealed by the crystal structure of an eIF4A:RNA complex bound to the interfacial inhibitor, desmethyl pateamine A. (2451-9448 (Electronic)).
- Neumann, M., Kwong, L. K., Truax, A. C., Vanmassenhove, B., Kretschmar, H. A., Van Deerlin, V. M., . . . Lee, V. M. (2007). TDP-43-positive white matter pathology in frontotemporal lobar degeneration with ubiquitin-positive inclusions. *J Neuropathol Exp Neurol*, 66(3), 177-183. <https://doi.org/10.1097/01.jnen.0000248554.45456.58>
- Neumann, M., Sampathu, D. M., Kwong, L. K., Truax, A. C., Micsenyi, M. C., Chou, T. T., . . . Lee, V. M. (2006). Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science*, 314(5796), 130-133. <https://doi.org/10.1126/science.1134108>
- Nguyen, L., Laboissonniere, L. A., Guo, S., Pilotto, F., Scheidegger, O., Oestmann, A., . . . Ranum, L. P. W. (2020). Survival and Motor Phenotypes in FVB C9-500 ALS/FTD BAC Transgenic Mice Reproduced by Multiple Labs. *Neuron*, 108(4), 784-796.e783. <https://doi.org/10.1016/j.neuron.2020.09.009>
- Nguyen, L., Montrasio, F., Pattamatta, A., Tusi, S. K., Bardhi, O., Meyer, K. D., . . . Ranum, L. P. W. (2020). Antibody Therapy Targeting RAN Proteins Rescues C9 ALS/FTD Phenotypes in C9orf72 Mouse Model. *Neuron*, 105(4), 645-662.e611. <https://doi.org/https://doi.org/10.1016/j.neuron.2019.11.007>
- Nielsen, K. H., Behrens, M. A., He, Y., Oliveira, C. L. P., Sottrup Jensen, L., Hoffmann, S. V., . . . Andersen, G. R. (2011). Synergistic activation of eIF4A by eIF4B and eIF4G. *Nucleic Acids Research*, 39(7), 2678-2689. <https://doi.org/10.1093/nar/gkq1206>
- Nielsen, P. J., & Trachsel, H. (1988). The mouse protein synthesis initiation factor 4A gene family includes two related functional genes which are differentially expressed. (0261-4189 (Print)).
- Nik, S., & Bowman, T. V. (2019). Splicing and neurodegeneration: Insights and mechanisms [<https://doi.org/10.1002/wrna.1532>]. *WIREs RNA*, 10(4), e1532. <https://doi.org/https://doi.org/10.1002/wrna.1532>
- Nishida, K., Kuwano, Y., Nishikawa, T., Masuda, K., & Rokutan, K. (2017). RNA Binding Proteins and Genome Integrity. *Int J Mol Sci*, 18(7). <https://doi.org/10.3390/ijms18071341>

- Nolan, M., Scott, C., Gamarallage, M. P., Lunn, D., Carpenter, K., McDonough, E., . . . Ansorge, O. (2020). Quantitative patterns of motor cortex proteinopathy across ALS genotypes. *Acta Neuropathologica Communications*, 8(1), 98. <https://doi.org/10.1186/s40478-020-00961-2>
- Oberer, M., Marintchev A Fau - Wagner, G., & Wagner, G. (2005). Structural basis for the enhancement of eIF4A helicase activity by eIF4G. (0890-9369 (Print)).
- Okamoto, K., Hirai, S., Amari, M., Watanabe, M., & Sakurai, A. (1993). Bunina bodies in amyotrophic lateral sclerosis immunostained with rabbit anti-cystatin C serum. *Neuroscience Letters*, 162(1), 125-128. [https://doi.org/https://doi.org/10.1016/0304-3940\(93\)90576-7](https://doi.org/https://doi.org/10.1016/0304-3940(93)90576-7)
- Onyike, C. U., & Diehl-Schmid, J. (2013). The epidemiology of frontotemporal dementia. *Int Rev Psychiatry*, 25(2), 130-137. <https://doi.org/10.3109/09540261.2013.776523>
- Orrell, R. W., & Guiloff, R. J. (2020). Clinical aspects of motor neurone disease. *Medicine*, 48(9), 607-611. <https://doi.org/https://doi.org/10.1016/j.mpmed.2020.06.006>
- Owttrim, G. W., Hofmann S Fau - Kuhlemeier, C., & Kuhlemeier, C. (1991). Divergent genes for translation initiation factor eIF-4A are coordinately expressed in tobacco. (0305-1048 (Print)).
- Pandit, S., Zhou, Y., Shiue, L., Coutinho-Mansfield, G., Li, H., Qiu, J., . . . Fu, X.-D. (2013). Genome-wide Analysis Reveals SR Protein Cooperation and Competition in Regulated Splicing. *Molecular Cell*, 50(2), 223-235. <https://doi.org/https://doi.org/10.1016/j.molcel.2013.03.001>
- Papadakis, E. D., Nicklin, S. A., Baker, A. H., & White, S. J. (2004). Promoters and control elements: designing expression cassettes for gene therapy. *Curr Gene Ther*, 4(1), 89-113.
- Parsyan, A., Svitkin, Y., Shahbazian, D., Gkogkas, C., Lasko, P., Merrick, W. C., & Sonenberg, N. (2011). mRNA helicases: the tacticians of translational control. *Nat Rev Mol Cell Biol*, 12(4), 235-245. <https://doi.org/10.1038/nrm3083>
- Pause, A., Méthot N Fau - Svitkin, Y., Svitkin Y Fau - Merrick, W. C., Merrick Wc Fau - Sonenberg, N., & Sonenberg, N. (1994). Dominant negative mutants of mammalian translation initiation factor eIF-4A define a critical role for eIF-4F in cap-dependent and cap-independent initiation of translation. (0261-4189 (Print)).
- Paz, S., Ritchie, A., Mauer, C., & Caputi, M. (2021). The RNA binding protein SRSF1 is a master switch of gene expression and regulation in the immune system. *Cytokine & Growth Factor Reviews*, 57, 19-26. <https://doi.org/https://doi.org/10.1016/j.cytogfr.2020.10.008>
- Perrone, B., La Cognata, V., Sprovieri, T., Ungaro, C., Conforti, F. L., Andò, S., & Cavallaro, S. (2020). Alternative Splicing of ALS Genes: Misregulation and Potential Therapies. *Cellular and Molecular Neurobiology*, 40(1), 1-14. <https://doi.org/10.1007/s10571-019-00717-0>
- Philips, T., & Rothstein, J. D. (2014). Glial cells in amyotrophic lateral sclerosis. (1090-2430 (Electronic)).
- Pillai-Kastoori, L., Schutz-Geschwender, A. R., & Harford, J. A. (2020). A systematic approach to quantitative Western blot analysis. *Analytical Biochemistry*, 593, 113608. <https://doi.org/https://doi.org/10.1016/j.ab.2020.113608>
- Pinto, B. S., Saxena, T., Oliveira, R., Méndez-Gómez, H. R., Cleary, J. D., Denes, L. T., . . . Wang, E. T. (2017). Impeding Transcription of Expanded Microsatellite Repeats by Deactivated Cas9. *Molecular Cell*, 68(3), 479-490.e475. <https://doi.org/10.1016/j.molcel.2017.09.033>
- Portz, B., Lee, B. L., & Shorter, J. (2021). FUS and TDP-43 Phases in Health and Disease. *Trends in Biochemical Sciences*, 46(7), 550-563. <https://doi.org/https://doi.org/10.1016/j.tibs.2020.12.005>
- Prell, T., & Grosskreutz, J. (2013). The involvement of the cerebellum in amyotrophic lateral sclerosis. *Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration*, 14(7-8), 507-515. <https://doi.org/10.3109/21678421.2013.812661>

- Presnyak, V., Alhusaini, N., Chen, Y. H., Martin, S., Morris, N., Kline, N., . . . Collier, J. (2015). Codon optimality is a major determinant of mRNA stability. *Cell*, 160(6), 1111-1124.
<https://doi.org/10.1016/j.cell.2015.02.029>
- Prpar Mihevc, S., Darovic, S., Kovanda, A., Bajc Cesnik, A., Zupunski, V., & Rogelj, B. (2017). Nuclear trafficking in amyotrophic lateral sclerosis and frontotemporal lobar degeneration. *Brain*, 140(1), 13-26.
<https://doi.org/10.1093/brain/aww197>
- Prudencio, M., Belzil, V. V., Batra, R., Ross, C. A., Gendron, T. F., Pregent, L. J., . . . Petrucelli, L. (2015). Distinct brain transcriptome profiles in C9orf72-associated and sporadic ALS. *Nat Neurosci*, 18(8), 1175-1182.
<https://doi.org/10.1038/nn.4065>
- Prévôt, D., Darlix JI Fau - Ohlmann, T., & Ohlmann, T. (2003). Conducting the initiation of protein synthesis: the role of eIF4G. (0248-4900 (Print)).
- Radwan, M., Ang, C.-S., Ormsby, A. R., Cox, D., Daly, J. C., Reid, G. E., & Hatters, D. M. (2020). Arginine in C9ORF72 Dipolypeptides Mediates Promiscuous Proteome Binding and Multiple Modes of Toxicity*. *Molecular & Cellular Proteomics*, 19(4), 640-654.
<https://doi.org/https://doi.org/10.1074/mcp.RA119.001888>
- Ragagnin, A. M. G., Shadfar, S., Vidal, M., Jamali, M. S., & Atkin, J. D. (2019). Motor Neuron Susceptibility in ALS/FTD. (1662-4548 (Print)).
- Ramos-Vara, J. A., & Miller, M. A. (2014). When tissue antigens and antibodies get along: revisiting the technical aspects of immunohistochemistry--the red, brown, and blue technique. (1544-2217 (Electronic)).
- Ratti, A., Gumina, V., Lenzi, P., Bossolasco, P., Fulceri, F., Volpe, C., . . . Colombrita, C. (2020). Chronic stress induces formation of stress granules and pathological TDP-43 aggregates in human ALS fibroblasts and iPSC-motoneurons. *Neurobiology of Disease*, 145, 105051.
<https://doi.org/https://doi.org/10.1016/j.nbd.2020.105051>
- Raught, B., Peiretti, F., Gingras, A.-C., Livingstone, M., Shahbazian, D., Mayeur, G. L., . . . Hershey, J. W. B. (2004). Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases [<https://doi.org/10.1038/sj.emboj.7600193>]. *The EMBO Journal*, 23(8), 1761-1769.
<https://doi.org/https://doi.org/10.1038/sj.emboj.7600193>
- Reddy, K., Zamiri, B., Stanley, S. Y., Macgregor, R. B., Jr., & Pearson, C. E. (2013). The disease-associated r(GGGGCC)n repeat from the C9orf72 gene forms tract length-dependent uni- and multimolecular RNA G-quadruplex structures. *J Biol Chem*, 288(14), 9860-9866.
<https://doi.org/10.1074/jbc.C113.452532>
- Renton, A. E., Majounie, E., Waite, A., Simon-Sanchez, J., Rollinson, S., Gibbs, J. R., . . . Traynor, B. J. (2011). A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. *Neuron*, 72(2), 257-268. <https://doi.org/10.1016/j.neuron.2011.09.010>
- Rhodes, D., & Lipps, H. J. (2015). G-quadruplexes and their regulatory roles in biology. *Nucleic Acids Research*, 43(18), 8627-8637. <https://doi.org/10.1093/nar/gkv862>
- Richter, N. J., Rogers Gw Jr Fau - Hensold, J. O., Hensold Jo Fau - Merrick, W. C., & Merrick, W. C. (1999). Further biochemical and kinetic characterization of human eukaryotic initiation factor 4H. (0021-9258 (Print)).
- Riemslogh, F. W., Lans, H., Seelaar, H., Severijnen, L.-A. W. F. M., Melhem, S., Vermeulen, W., . . . Willemsen, R. (2019). HR23B pathology preferentially co-localizes with p62, pTDP-43 and poly-GA in C9ORF72-linked frontotemporal dementia and amyotrophic lateral sclerosis. *Acta Neuropathologica Communications*, 7(1), 39. <https://doi.org/10.1186/s40478-019-0694-6>

- Rino, J., & Carmo-Fonseca, M. (2009). The spliceosome: a self-organized macromolecular machine in the nucleus? *Trends in Cell Biology*, 19(8), 375-384.
<https://doi.org/https://doi.org/10.1016/j.tcb.2009.05.004>
- Rizzardi, A. E., Johnson, A. T., Vogel, R. I., Pambuccian, S. E., Henriksen, J., Skubitz, A. P. N., . . . Schmechel, S. C. (2012). Quantitative comparison of immunohistochemical staining measured by digital image analysis versus pathologist visual scoring. *Diagnostic Pathology*, 7(1), 42. <https://doi.org/10.1186/1746-1596-7-42>
- Rogers, G. W., Jr., Komar, A. A., & Merrick, W. C. (2002). eIF4A: the godfather of the DEAD box helicases. *Prog Nucleic Acid Res Mol Biol*, 72, 307-331.
- Rogers, G. W., Jr., Richter Nj Fau - Merrick, W. C., & Merrick, W. C. (1999). Biochemical and kinetic characterization of the RNA helicase activity of eukaryotic initiation factor 4A. (0021-9258 (Print)).
- Rogers, G. W., Jr., Richter, N. J., Lima, W. F., & Merrick, W. C. (2001). Modulation of the Helicase Activity of eIF4A by eIF4B, eIF4H, and eIF4F *. *Journal of Biological Chemistry*, 276(33), 30914-30922.
<https://doi.org/10.1074/jbc.M100157200>
- Rohrer, J. D., Guerreiro, R., Vandrovcova, J., Uphill, J., Reiman, D., Beck, J., . . . Rossor, M. N. (2009). The heritability and genetics of frontotemporal lobar degeneration. *Neurology*, 73(18), 1451-1456.
<https://doi.org/10.1212/WNL.0b013e3181bf997a>
- Rosso, S. M., Donker Kaat, L., Baks, T., Joosse, M., de Koning, I., Pijnenburg, Y., . . . van Swieten, J. C. (2003). Frontotemporal dementia in The Netherlands: patient characteristics and prevalence estimates from a population-based study. *Brain*, 126(Pt 9), 2016-2022. <https://doi.org/10.1093/brain/awg204>
- Rostalski, H., Leskelä, S., Huber, N., Katisko, K., Cajanus, A., Solje, E., . . . Haapasalo, A. (2019). Astrocytes and Microglia as Potential Contributors to the Pathogenesis of C9orf72 Repeat Expansion-Associated FTLD and ALS [Mini Review]. *Frontiers in Neuroscience*, 13.
- Rozovsky, N., Butterworth Ac Fau - Moore, M. J., & Moore, M. J. (2008). Interactions between eIF4A and its accessory factors eIF4B and eIF4H. (1469-9001 (Electronic)).
- Rust, A., Shah, S., Hautbergue, G. A.-O. X., & Davletov, B. (2018). Burkholderia Lethal Factor 1, a Novel Anti-Cancer Toxin, Demonstrates Selective Cytotoxicity in MYCN-Amplified Neuroblastoma Cells. LID - 10.3390/toxins10070261 [doi] LID - 261. (2072-6651 (Electronic)).
- Saberi, S., Stauffer, J. E., Jiang, J., Garcia, S. D., Taylor, A. E., Schulte, D., . . . Ravits, J. (2018). Sense-encoded poly-GR dipeptide repeat proteins correlate to neurodegeneration and uniquely co-localize with TDP-43 in dendrites of repeat-expanded C9orf72 amyotrophic lateral sclerosis. *Acta Neuropathologica*, 135(3), 459-474. <https://doi.org/10.1007/s00401-017-1793-8>
- Sanford, J. R., Wang X Fau - Mort, M., Mort M Fau - Vanduynd, N., Vanduynd N Fau - Cooper, D. N., Cooper Dn Fau - Mooney, S. D., Mooney Sd Fau - Edenberg, H. J., . . . Liu, Y. (2009). Splicing factor SFRS1 recognizes a functionally diverse landscape of RNA transcripts. (1088-9051 (Print)).
- Sanz, L. A., Hartono, S. R., Lim, Y. W., Steyaert, S., Rajpurkar, A., Ginno, P. A., . . . Chedin, F. (2016). Prevalent, Dynamic, and Conserved R-Loop Structures Associate with Specific Epigenomic Signatures in Mammals. *Mol Cell*, 63(1), 167-178. <https://doi.org/10.1016/j.molcel.2016.05.032>
- Sauer, M., Juranek, S. A., Marks, J., De Magis, A., Kazemier, H. G., Hilbig, D., . . . Paeschke, K. (2019). DHX36 prevents the accumulation of translationally inactive mRNAs with G4-structures in untranslated regions. *Nature Communications*, 10(1), 2421. <https://doi.org/10.1038/s41467-019-10432-5>
- Sauer, M., & Paeschke, K. (2017). G-quadruplex unwinding helicases and their function in vivo. (1470-8752 (Electronic)).
- Schmid, S. R., & Linder, P. (1992). D-E-A-D protein family of putative RNA helicases. (0950-382X (Print)).

- Schmidt, T. A.-O., Dabrowska, A. A.-O., Waldron, J. A., Hodge, K., Koulouras, G., Gabrielsen, M., . . . Bushell, M. (2023). eIF4A1-dependent mRNAs employ purine-rich 5'UTR sequences to activate localised eIF4A1-unwinding through eIF4A1-multimerisation to facilitate translation. (1362-4962 (Electronic)).
- Schultz, B. R., & Chamberlain, J. S. (2008). Recombinant adeno-associated virus transduction and integration. *Mol Ther*, 16(7), 1189-1199. <https://doi.org/10.1038/mt.2008.103>
- Schütz, P., Karlberg T Fau - van den Berg, S., van den Berg S Fau - Collins, R., Collins R Fau - Lehtiö, L., Lehtiö L Fau - Högbom, M., Högbom M Fau - Holmberg-Schiavone, L., . . . Schüler, H. (2010). Comparative structural analysis of human DEAD-box RNA helicases. LID - e12791 [pii] LID - 10.1371/journal.pone.0012791 [doi]. (1932-6203 (Electronic)).
- Sellier, C., Campanari, M. L., Julie Corbier, C., Gaucherot, A., Kolb-Cheynel, I., Oulad-Abdelghani, M., . . . Charlet-Berguerand, N. (2016). Loss of C9ORF72 impairs autophagy and synergizes with polyQ Ataxin-2 to induce motor neuron dysfunction and cell death. *Embo j*, 35(12), 1276-1297. <https://doi.org/10.15252/embj.201593350>
- Sen, N. D., Zhou, F., Harris, M. S., Ingolia, N. T., & Hinnebusch, A. G. (2016). eIF4B stimulates translation of long mRNAs with structured 5' UTRs and low closed-loop potential but weak dependence on eIF4G. *Proc Natl Acad Sci U S A*, 113(38), 10464-10472. <https://doi.org/10.1073/pnas.1612398113>
- Shao, Q., Liang, C., Chang, Q., Zhang, W., Yang, M., & Chen, J. A.-O. (2019). C9orf72 deficiency promotes motor deficits of a C9ALS/FTD mouse model in a dose-dependent manner. (2051-5960 (Electronic)).
- Shaoyan, X., Juanjuan, Y., Yalan, T., Ping, H., Jianzhong, L., & Qinian, W. (2013). Downregulation of EIF4A2 in Non-Small-Cell Lung Cancer Associates with Poor Prognosis. *Clinical Lung Cancer*, 14(6), 658-665. <https://doi.org/https://doi.org/10.1016/j.clcc.2013.04.011>
- Sharpe, J. L., Harper, N. S., Garner, D. R., & West, R. J. H. (2021). Modeling C9orf72-Related Frontotemporal Dementia and Amyotrophic Lateral Sclerosis in Drosophila [Review]. *Frontiers in Cellular Neuroscience*, 15.
- Shatunov, A., & Al-Chalabi, A. (2021). The genetic architecture of ALS. *Neurobiology of Disease*, 147, 105156. <https://doi.org/https://doi.org/10.1016/j.nbd.2020.105156>
- Shi, Y., Lin, S., Staats, K. A., Li, Y., Chang, W.-H., Hung, S.-T., . . . Ichida, J. K. (2018). Haploinsufficiency leads to neurodegeneration in C9ORF72 ALS/FTD human induced motor neurons. *Nature Medicine*, 24(3), 313-325. <https://doi.org/10.1038/nm.4490>
- Simone, R., Balendra, R., Moens, T. G., Preza, E., Wilson, K. M., Heslegrave, A., . . . Isaacs, A. M. (2018). G-quadruplex-binding small molecules ameliorate C9orf72 FTD/ALS pathology in vitro and in vivo [https://doi.org/10.15252/emmm.201707850]. *EMBO Molecular Medicine*, 10(1), 22-31. <https://doi.org/https://doi.org/10.15252/emmm.201707850>
- Smith, R. H. (2008). Adeno-associated virus integration: virus versus vector. *Gene Ther*, 15(11), 817-822. <https://doi.org/10.1038/gt.2008.55>
- Sonenberg, N., & Hinnebusch, A. G. (2009). Regulation of translation initiation in eukaryotes: mechanisms and biological targets. (1097-4172 (Electronic)).
- Sonobe, Y., Aburas, J., Krishnan, G., Fleming, A. C., Ghadge, G., Islam, P., . . . Kratsios, P. (2021). A C. elegans model of C9orf72-associated ALS/FTD uncovers a conserved role for eIF2D in RAN translation. *Nature communications*, 12(1), 6025-6025. <https://doi.org/10.1038/s41467-021-26303-x>
- Sonobe, Y., Ghadge, G., Masaki, K., Sandoel, A., Fuchs, E., & Roos, R. P. (2018). Translation of dipeptide repeat proteins from the C9ORF72 expanded repeat is associated with cellular stress. *Neurobiol Dis*, 116, 155-165. <https://doi.org/10.1016/j.nbd.2018.05.009>

- Soto-Rifo, R., Rubilar Ps Fau - Limousin, T., Limousin T Fau - de Breyne, S., de Breyne S Fau - Décimo, D., Décimo D Fau - Ohlmann, T., & Ohlmann, T. (2012). DEAD-box protein DDX3 associates with eIF4F to promote translation of selected mRNAs. (1460-2075 (Electronic)).
- Spiteri, A. G., Wishart, C. L., Pamphlett, R., Locatelli, G., & King, N. A.-O. (2022). Microglia and monocytes in inflammatory CNS disease: integrating phenotype and function. (1432-0533 (Electronic)).
- Sreedharan, J., Blair Ip Fau - Tripathi, V. B., Tripathi Vb Fau - Hu, X., Hu X Fau - Vance, C., Vance C Fau - Rogelj, B., Rogelj B Fau - Ackerley, S., . . . Shaw, C. E. (2008). TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. (1095-9203 (Electronic)).
- St. Martin, J. L., Wang, L., & Kaprielian, Z. (2020). Toxicity in ALS: TDP-43 modifiers and C9orf72. *Neuroscience Letters*, 716, 134621. <https://doi.org/https://doi.org/10.1016/j.neulet.2019.134621>
- Starck, S. R., Jiang, V., Pavon-Eternod, M., Prasad, S., McCarthy, B., Pan, T., & Shastri, N. (2012). Leucine-tRNA Initiates at CUG Start Codons for Protein Synthesis and Presentation by MHC Class I. *Science*, 336(6089), 1719-1723. <https://doi.org/10.1126/science.1220270>
- Stark, R., Grzelak, M., & Hadfield, J. (2019). RNA sequencing: the teenage years. *Nature Reviews Genetics*, 20(11), 631-656. <https://doi.org/10.1038/s41576-019-0150-2>
- Suk, T. R., & Rousseaux, M. W. C. (2020). The role of TDP-43 mislocalization in amyotrophic lateral sclerosis. *Molecular Neurodegeneration*, 15(1), 45. <https://doi.org/10.1186/s13024-020-00397-1>
- Sun, Y., Atas E Fau - Lindqvist, L., Lindqvist L Fau - Sonenberg, N., Sonenberg N Fau - Pelletier, J., Pelletier J Fau - Meller, A., & Meller, A. (2012). The eukaryotic initiation factor eIF4H facilitates loop-binding, repetitive RNA unwinding by the eIF4A DEAD-box helicase. (1362-4962 (Electronic)).
- Svitkin, Y. V., Pause A Fau - Haghighat, A., Haghighat A Fau - Pyronnet, S., Pyronnet S Fau - Witherell, G., Witherell G Fau - Belsham, G. J., Belsham Gj Fau - Sonenberg, N., & Sonenberg, N. (2001). The requirement for eukaryotic initiation factor 4A (eIF4A) in translation is in direct proportion to the degree of mRNA 5' secondary structure. (1355-8382 (Print)).
- Swinnen, B., & Robberecht, W. (2014). The phenotypic variability of amyotrophic lateral sclerosis. *Nat Rev Neurol*, 10(11), 661-670. <https://doi.org/10.1038/nrneurol.2014.184>
- Tabet, R., Schaeffer, L., Freyermuth, F., Jambeau, M., Workman, M., Lee, C.-Z., . . . Lagier-Tourenne, C. (2018). CUG initiation and frameshifting enable production of dipeptide repeat proteins from ALS/FTD C9ORF72 transcripts. *Nature communications*, 9(1), 152-152. <https://doi.org/10.1038/s41467-017-02643-5>
- Tabet, R., Schaeffer, L., Freyermuth, F., Jambeau, M., Workman, M., Lee, C. Z., . . . Lagier-Tourenne, C. (2018). CUG initiation and frameshifting enable production of dipeptide repeat proteins from ALS/FTD C9ORF72 transcripts. *Nat Commun*, 9(1), 152. <https://doi.org/10.1038/s41467-017-02643-5>
- Takyar, S., Hickerson Rp Fau - Noller, H. F., & Noller, H. F. (2005). mRNA helicase activity of the ribosome. (0092-8674 (Print)).
- Talbott, E. O., Malek, A. M., & Lacomis, D. (2016). The epidemiology of amyotrophic lateral sclerosis. *Handb Clin Neurol*, 138, 225-238. <https://doi.org/10.1016/b978-0-12-802973-2.00013-6>
- Tang, D., Sheng, J., Xu, L., Yan, C., & Qi, S. (2020). The C9orf72-SMCR8-WDR41 complex is a GAP for small GTPases. *Autophagy*, 16(8), 1542-1543. <https://doi.org/10.1080/15548627.2020.1779473>
- Tauber, D., Tauber, G., Khong, A., Van Treeck, B., Pelletier, J., & Parker, R. (2020). Modulation of RNA Condensation by the DEAD-Box Protein eIF4A. *Cell*. <https://doi.org/https://doi.org/10.1016/j.cell.2019.12.031>

- Theissen, B., Karow Ar Fau - Köhler, J., Köhler J Fau - Gubaev, A., Gubaev A Fau - Klostermeier, D., & Klostermeier, D. (2008). Cooperative binding of ATP and RNA induces a closed conformation in a DEAD box RNA helicase. (1091-6490 (Electronic)).
- Therrien, M., Rouleau, G. A., Dion, P. A., & Parker, J. A. (2013). Deletion of C9ORF72 results in motor neuron degeneration and stress sensitivity in *C. elegans*. *PLoS One*, 8(12), e83450. <https://doi.org/10.1371/journal.pone.0083450>
- Tippana, R., Chen, M. C., Demeshkina, N. A., Ferré-D'Amaré, A. R., & Myong, S. (2019). RNA G-quadruplex is resolved by repetitive and ATP-dependent mechanism of DHX36. *Nature communications*, 10(1), 1855-1855. <https://doi.org/10.1038/s41467-019-09802-w>
- Tippana, R., Hwang, H., Opresko, P. L., Bohr, V. A., & Myong, S. (2016). Single-molecule imaging reveals a common mechanism shared by G-quadruplex-resolving helicases. *Proceedings of the National Academy of Sciences of the United States of America*, 113(30), 8448-8453. <https://doi.org/10.1073/pnas.1603724113>
- Titus, M. B., Chang, A. W., & Olesnick, E. C. (2021). Exploring the Diverse Functional and Regulatory Consequences of Alternative Splicing in Development and Disease [Review]. *Frontiers in Genetics*, 12.
- Todd, Peter K., Oh, Seok Y., Krans, A., He, F., Sellier, C., Frazer, M., . . . Paulson, Henry L. (2013). CGG Repeat-Associated Translation Mediates Neurodegeneration in Fragile X Tremor Ataxia Syndrome. *Neuron*, 78(3), 440-455. <https://doi.org/https://doi.org/10.1016/j.neuron.2013.03.026>
- Tran, H., Almeida, S., Moore, J., Gendron, T. F., Chalasani, U., Lu, Y., . . . Gao, F. B. (2015). Differential Toxicity of Nuclear RNA Foci versus Dipeptide Repeat Proteins in a *Drosophila* Model of C9ORF72 FTD/ALS. *Neuron*, 87(6), 1207-1214. <https://doi.org/10.1016/j.neuron.2015.09.015>
- Tripathi, V., Song Dy Fau - Zong, X., Zong X Fau - Shevtsov, S. P., Shevtsov Sp Fau - Hearn, S., Hearn S Fau - Fu, X.-D., Fu Xd Fau - Dundr, M., . . . Prasanth, K. V. (2012). SRSF1 regulates the assembly of pre-mRNA processing factors in nuclear speckles. (1939-4586 (Electronic)).
- Tseng, Y.-J., Sandwith, S. N., Green, K. M., Chambers, A. E., Krans, A., Raimor, H. M., . . . Smaldino, P. J. (2021a). The RNA helicase DHX36-G4R1 modulates C9orf72 GGGGCC hexanucleotide repeat-associated translation. *The Journal of biological chemistry*, 297(2), 100914-100914. <https://doi.org/10.1016/j.jbc.2021.100914>
- Tseng, Y.-J., Sandwith, S. N., Green, K. M., Chambers, A. E., Krans, A., Raimor, H. M., . . . Smaldino, P. J. (2021b). The RNA helicase DHX36-G4R1 modulates C9orf72 GGGGCC hexanucleotide repeat-associated translation. *Journal of Biological Chemistry*, 297(2), 100914. <https://doi.org/https://doi.org/10.1016/j.jbc.2021.100914>
- Van Harten, A. C. M., Phatnani, H., & Przedborski, S. (2021). Non-cell-autonomous pathogenic mechanisms in amyotrophic lateral sclerosis. *Trends in Neurosciences*, 44(8), 658-668. <https://doi.org/https://doi.org/10.1016/j.tins.2021.04.008>
- van't Spijker, H. M., & Almeida, S. (2023). How villains are made: The translation of dipeptide repeat proteins in C9ORF72-ALS/FTD. *Gene*, 858, 147167. <https://doi.org/https://doi.org/10.1016/j.gene.2023.147167>
- Varcianna, A., Myszczyńska, M. A., Castelli, L. M., O'Neill, B., Kim, Y., Talbot, J., . . . Ferraiuolo, L. (2019). Micro-RNAs secreted through astrocyte-derived extracellular vesicles cause neuronal network degeneration in C9orf72 ALS. *EBioMedicine*, 40, 626-635. <https://doi.org/https://doi.org/10.1016/j.ebiom.2018.11.067>
- Vaughn, J. P., Creacy, S. D., Routh, E. D., Joyner-Butt, C., Jenkins, G. S., Pauli, S., . . . Akman, S. A. (2005). The DEXH protein product of the DHX36 gene is the major source of tetramolecular quadruplex G4-DNA

- resolving activity in HeLa cell lysates. *J Biol Chem*, 280(46), 38117-38120.
<https://doi.org/10.1074/jbc.C500348200>
- Vaysse, C., Philippe, C., Martineau, Y., Quelen, C., Hieblot, C., Renaud, C., . . . Touriol, C. (2015). Key contribution of eIF4H-mediated translational control in tumor promotion. (1949-2553 (Electronic)).
- Viterbo, D., Michoud, G., Mosbach, V., Dujon, B., & Richard, G. F. (2016). Replication stalling and heteroduplex formation within CAG/CTG trinucleotide repeats by mismatch repair. *DNA Repair (Amst)*, 42, 94-106.
<https://doi.org/10.1016/j.dnarep.2016.03.002>
- Waite, A. J., Baumer, D., East, S., Neal, J., Morris, H. R., Ansorge, O., & Blake, D. J. (2014). Reduced C9orf72 protein levels in frontal cortex of amyotrophic lateral sclerosis and frontotemporal degeneration brain with the C9ORF72 hexanucleotide repeat expansion. *Neurobiol Aging*, 35(7), 1779.e1775-1779.e1713.
<https://doi.org/10.1016/j.neurobiolaging.2014.01.016>
- Waldron, J. A., Tack, D. C., Ritchey, L. E., Gillen, S. L., Wilczynska, A., Turro, E., . . . Le Quesne, J. (2019). mRNA structural elements immediately upstream of the start codon dictate dependence upon eIF4A helicase activity. *Genome Biology*, 20(1), 300. <https://doi.org/10.1186/s13059-019-1901-2>
- Walker, S. E., Zhou F Fau - Mitchell, S. F., Mitchell Sf Fau - Larson, V. S., Larson Vs Fau - Valasek, L., Valasek L Fau - Hinnebusch, A. G., Hinnebusch Ag Fau - Lorsch, J. R., & Lorsch, J. R. (2013). Yeast eIF4B binds to the head of the 40S ribosomal subunit and promotes mRNA recruitment through its N-terminal and internal repeat domains. (1469-9001 (Electronic)).
- Wang, T., Liu, H., Itoh, K., Oh, S., Zhao, L., Murata, D., . . . Wang, J. (2021). C9orf72 regulates energy homeostasis by stabilizing mitochondrial complex I assembly. *Cell Metabolism*, 33(3), 531-546.e539.
<https://doi.org/https://doi.org/10.1016/j.cmet.2021.01.005>
- Wang, Z.-F., Ursu, A., Childs-Disney, J. L., Guertler, R., Yang, W.-Y., Bernat, V., . . . Disney, M. D. (2019a). The Hairpin Form of r(G4C2)exp in c9ALS/FTD Is Repeat-Associated Non-ATG Translated and a Target for Bioactive Small Molecules. *Cell Chemical Biology*, 26(2), 179-190.e112.
<https://doi.org/https://doi.org/10.1016/j.chembiol.2018.10.018>
- Wang, Z.-F., Ursu, A., Childs-Disney, J. L., Guertler, R., Yang, W.-Y., Bernat, V., . . . Disney, M. D. (2019b). The Hairpin Form of r(G(4)C(2))(exp) in c9ALS/FTD Is Repeat-Associated Non-ATG Translated and a Target for Bioactive Small Molecules. *Cell chemical biology*, 26(2), 179-190.e112.
<https://doi.org/10.1016/j.chembiol.2018.10.018>
- Webster, C. P., Smith, E. F., Bauer, C. S., Moller, A., Hautbergue, G. M., Ferraiuolo, L., . . . De Vos, K. J. (2016). The C9orf72 protein interacts with Rab1a and the ULK1 complex to regulate initiation of autophagy. *The EMBO journal*, 35(15), 1656-1676. <https://doi.org/10.15252/emboj.201694401>
- Weinberg, M. S., Samulski, R. J., & McCown, T. J. (2013). Adeno-associated virus (AAV) gene therapy for neurological disease. *Neuropharmacology*, 69. <https://doi.org/10.1016/j.neuropharm.2012.03.004>
- Weinmann, J., & Grimm, D. (2017). Next-generation AAV vectors for clinical use: an ever-accelerating race. *Virus Genes*, 53(5), 707-713. <https://doi.org/10.1007/s11262-017-1502-7>
- Wen, X., Tan, W., Westergard, T., Krishnamurthy, K., Markandaiah, S. S., Shi, Y., . . . Trotti, D. (2014). Antisense proline-arginine RAN dipeptides linked to C9ORF72-ALS/FTD form toxic nuclear aggregates that initiate in vitro and in vivo neuronal death. *Neuron*, 84(6), 1213-1225.
<https://doi.org/10.1016/j.neuron.2014.12.010>
- Werner, M., Chott, A., Fabiano, A., & Battifora, H. (2000). Effect of Formalin Tissue Fixation and Processing on Immunohistochemistry. *The American Journal of Surgical Pathology*, 24(7).

- Westergard, T., McAvoy, K., Russell, K., Wen, X., Pang, Y., Morris, B., . . . Haeusler, A. A.-O. (2019). Repeat-associated non-AUG translation in C9orf72-ALS/FTD is driven by neuronal excitation and stress. *LID - 10.15252/emmm.201809423* [doi] LID - e9423. (1757-4684 (Electronic)).
- White, M. R., Mitrea, D. M., Zhang, P., Stanley, C. B., Cassidy, D. E., Nourse, A., . . . Kriwacki, R. W. (2019). C9orf72 Poly(PR) Dipeptide Repeats Disturb Biomolecular Phase Separation and Disrupt Nucleolar Function. *Molecular Cell*, 74(4), 713-728.e716. <https://doi.org/https://doi.org/10.1016/j.molcel.2019.03.019>
- Witzel, S., Maier, A., Steinbach, R., Grosskreutz, J., Koch, J. C., Sarikidi, A., . . . Ludolph, A. C. (2022). Safety and Effectiveness of Long-term Intravenous Administration of Edaravone for Treatment of Patients With Amyotrophic Lateral Sclerosis. (2168-6157 (Electronic)).
- Wolfe, A. L., Singh, K., Zhong, Y., Drewe, P., Rajasekhar, V. K., Sanghvi, V. R., . . . Wendel, H. G. (2014). RNA G-quadruplexes cause eIF4A-dependent oncogene translation in cancer. (1476-4687 (Electronic)).
- Xiao, S., MacNair, L., McGoldrick, P., McKeever, P. M., McLean, J. R., Zhang, M., . . . Robertson, J. (2015). Isoform-specific antibodies reveal distinct subcellular localizations of C9orf72 in amyotrophic lateral sclerosis. *Ann Neurol*, 78(4), 568-583. <https://doi.org/10.1002/ana.24469>
- Xiao, S., MacNair, L., McLean, J., McGoldrick, P., McKeever, P., Soleimani, S., . . . Robertson, J. (2016). C9orf72 isoforms in Amyotrophic Lateral Sclerosis and Frontotemporal Lobar Degeneration. *Brain Research*, 1647, 43-49. <https://doi.org/https://doi.org/10.1016/j.brainres.2016.04.062>
- Xiao, S., MacNair, L., McLean, J., McGoldrick, P., McKeever, P., Soleimani, S., . . . Robertson, J. (2016). C9orf72 isoforms in Amyotrophic Lateral Sclerosis and Frontotemporal Lobar Degeneration. *Brain Res*, 1647, 43-49. <https://doi.org/10.1016/j.brainres.2016.04.062>
- Xie, J., Xie, Q., Zhang, H., Ameres, S. L., Hung, J. H., Su, Q., . . . Gao, G. (2011). MicroRNA-regulated, systemically delivered rAAV9: a step closer to CNS-restricted transgene expression. *Mol Ther*, 19(3), 526-535. <https://doi.org/10.1038/mt.2010.279>
- Xu, Z., Poidevin, M., Li, X., Li, Y., Shu, L., Nelson, D. L., . . . Jin, P. (2013). Expanded GGGGCC repeat RNA associated with amyotrophic lateral sclerosis and frontotemporal dementia causes neurodegeneration. *Proceedings of the National Academy of Sciences*, 110(19), 7778. <https://doi.org/10.1073/pnas.1219643110>
- Xue, B., Li, R., Ma, H., Rahaman, A., & Kumar, V. (2022). Comprehensive mapping of mutations in the C9ORF72 that affect folding and binding to SMCR8 protein. *Process Biochemistry*, 121, 312-321. <https://doi.org/https://doi.org/10.1016/j.procbio.2022.07.013>
- Xue, C., Gu, X., Li, G., Bao, Z., & Li, L. (2021). Expression and Functional Roles of Eukaryotic Initiation Factor 4A Family Proteins in Human Cancers [Review]. *Frontiers in Cell and Developmental Biology*, 9.
- Yamaguchi, M., Lee, I.-s., Jantrapirom, S., Suda, K., & Yoshida, H. (2021). Drosophila models to study causative genes for human rare intractable neurological diseases. *Experimental Cell Research*, 403(1), 112584. <https://doi.org/https://doi.org/10.1016/j.yexcr.2021.112584>
- Yan, L. X., Wu, Q. N., Zhang, Y., Li, Y. Y., Liao, D. Z., Hou, J. H., . . . Shao, J. Y. (2011). Knockdown of miR-21 in human breast cancer cell lines inhibits proliferation, in vitro migration and in vivo tumor growth. *Breast Cancer Research*, 13(1), R2. <https://doi.org/10.1186/bcr2803>
- Yang, D., Abdallah, A., Li, Z., Lu, Y., Almeida, S., & Gao, F. B. (2015). FTD/ALS-associated poly(GR) protein impairs the Notch pathway and is recruited by poly(GA) into cytoplasmic inclusions. *Acta Neuropathol*, 130(4), 525-535. <https://doi.org/10.1007/s00401-015-1448-6>

- Yangyuoru, P. M., Bradburn, D. A., Liu, Z., Xiao, T. S., & Russell, R. (2018). The G-quadruplex (G4) resolvase DHX36 efficiently and specifically disrupts DNA G4s via a translocation-based helicase mechanism. *J Biol Chem*, 293(6), 1924-1932. <https://doi.org/10.1074/jbc.M117.815076>
- Yeo, G., Holste D Fau - Kreiman, G., Kreiman G Fau - Burge, C. B., & Burge, C. B. (2004). Variation in alternative splicing across human tissues. (1474-760X (Electronic)).
- Yin, H., Song, C. Q., Dorkin, J. R., Zhu, L. J., Li, Y., Wu, Q., . . . Anderson, D. G. (2016). Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. *Nat Biotechnol*, 34(3), 328-333. <https://doi.org/10.1038/nbt.3471>
- Yin, S., Lopez-Gonzalez, R., Kunz, R. C., Gangopadhyay, J., Borufka, C., Gygi, S. P., . . . Reed, R. (2017). Evidence that C9ORF72 Dipeptide Repeat Proteins Associate with U2 snRNP to Cause Mis-splicing in ALS/FTD Patients. *Cell Rep*, 19(11), 2244-2256. <https://doi.org/10.1016/j.celrep.2017.05.056>
- Young, L. M., Saunders, J. C., Mahood, R. A., Revill, C. H., Foster, R. J., Ashcroft, A. E., & Radford, S. E. (2016). ESI-IMS-MS: A method for rapid analysis of protein aggregation and its inhibition by small molecules. *Methods*, 95, 62-69. <https://doi.org/https://doi.org/10.1016/j.ymeth.2015.05.017>
- Yu, C.-H., Teulade-Fichou, M.-P., & Olsthoorn, R. C. L. (2014). Stimulation of ribosomal frameshifting by RNA G-quadruplex structures. *Nucleic Acids Research*, 42(3), 1887-1892. <https://doi.org/10.1093/nar/gkt1022>
- Yuva-Aydemir, Y., Almeida, S., & Gao, F.-B. (2018). Insights into C9ORF72-Related ALS/FTD from Drosophila and iPSC Models. *Trends in Neurosciences*, 41(7), 457-469. <https://doi.org/https://doi.org/10.1016/j.tins.2018.04.002>
- Zhang, D., Iyer, L. M., He, F., & Aravind, L. (2012). Discovery of Novel DENN Proteins: Implications for the Evolution of Eukaryotic Intracellular Membrane Structures and Human Disease. *Front Genet*, 3, 283. <https://doi.org/10.3389/fgene.2012.00283>
- Zhang, L.-L., Chang, W., He, S.-B., Zhang, B., Ma, G., Shang, P.-F., & Yue, Z.-J. (2022). High expression of eIF4A1 predicts unfavorable prognosis in clear cell renal cell carcinoma. *Molecular and Cellular Probes*, 65, 101845. <https://doi.org/https://doi.org/10.1016/j.mcp.2022.101845>
- Zhang, Y. J., Gendron, T. F., Grima, J. C., Sasaguri, H., Jansen-West, K., Xu, Y. F., . . . Petrucelli, L. (2016). C9ORF72 poly(GA) aggregates sequester and impair HR23 and nucleocytoplasmic transport proteins. *Nat Neurosci*, 19(5), 668-677. <https://doi.org/10.1038/nn.4272>
- Zhang, Y. J., Jansen-West, K., Xu, Y. F., Gendron, T. F., Bieniek, K. F., Lin, W. L., . . . Petrucelli, L. (2014). Aggregation-prone c9FTD/ALS poly(GA) RAN-translated proteins cause neurotoxicity by inducing ER stress. *Acta Neuropathol*, 128(4), 505-524. <https://doi.org/10.1007/s00401-014-1336-5>
- Zhao, M., Ding, L. A.-O., Yang, Y., Chen, S., Zhu, N., Fu, Y., . . . Wang, Z. (2019). Aberrant Expression Of PDCD4/eIF4A1 Signal Predicts Postoperative Recurrence For Early-Stage Oral Squamous Cell Carcinoma. (1179-1322 (Print)).
- Zhong, L., Li, B., Mah, C. S., Govindasamy, L., Agbandje-McKenna, M., Cooper, M., . . . Srivastava, A. (2008). Next generation of adeno-associated virus 2 vectors: point mutations in tyrosines lead to high-efficiency transduction at lower doses. *Proc Natl Acad Sci U S A*, 105(22), 7827-7832. <https://doi.org/10.1073/pnas.0802866105>
- Zhong, X.-Y., Wang, P., Han, J., Rosenfeld, M. G., & Fu, X.-D. (2009). SR Proteins in Vertical Integration of Gene Expression from Transcription to RNA Processing to Translation. *Molecular Cell*, 35(1), 1-10. <https://doi.org/https://doi.org/10.1016/j.molcel.2009.06.016>
- Zhou, Q., Mareljic, N., Michaelsen, M., Parhizkar, S., Heindl, S., Nuscher, B., . . . Edbauer, D. (2020). Active poly-GA vaccination prevents microglia activation and motor deficits in a C9orf72 mouse model

- [<https://doi.org/10.15252/emmm.201910919>]. *EMBO Molecular Medicine*, 12(2), e10919.
<https://doi.org/https://doi.org/10.15252/emmm.201910919>
- Zhu, Q., Jiang, J., Gendron, T. F., McAlonis-Downes, M., Jiang, L., Taylor, A., . . . Cleveland, D. W. (2020). Reduced C9ORF72 function exacerbates gain of toxicity from ALS/FTD-causing repeat expansion in C9orf72. *Nature Neuroscience*, 23(5), 615-624. <https://doi.org/10.1038/s41593-020-0619-5>
- Zou, Z. A.-O., Zhou, Z. R., Che, C. H., Liu, C. Y., He, R. L., & Huang, H. P. (2017). Genetic epidemiology of amyotrophic lateral sclerosis: a systematic review and meta-analysis. (1468-330X (Electronic)).
- Zu, T., Gibbens, B., Doty, N. S., Gomes-Pereira, M., Huguet, A., Stone, M. D., . . . Ranum, L. P. (2011). Non-ATG-initiated translation directed by microsatellite expansions. *Proc Natl Acad Sci U S A*, 108(1), 260-265. <https://doi.org/10.1073/pnas.1013343108>
- Zufiría, M., Gil-Bea, F. J., Fernández-Torrón, R., Poza, J. J., Muñoz-Blanco, J. L., Rojas-García, R., . . . López de Munain, A. (2016). ALS: A bucket of genes, environment, metabolism and unknown ingredients. *Progress in Neurobiology*, 142, 104-129. <https://doi.org/https://doi.org/10.1016/j.pneurobio.2016.05.004>
- Änkö, M.-L. (2014). Regulation of gene expression programmes by serine–arginine rich splicing factors. *Seminars in Cell & Developmental Biology*, 32, 11-21. <https://doi.org/https://doi.org/10.1016/j.semcdb.2014.03.011>
- Özeş, A. R., Feoktistova, K., Avanzino, B. C., & Fraser, C. S. (2011). Duplex Unwinding and ATPase Activities of the DEAD-Box Helicase eIF4A Are Coupled by eIF4G and eIF4B. *Journal of Molecular Biology*, 412(4), 674-687. <https://doi.org/https://doi.org/10.1016/j.jmb.2011.08.004>
- Šket, P., Pohleven, J., Kovanda, A., Štalekar, M., Župunski, V., Zalar, M., . . . Rogelj, B. (2015). Characterization of DNA G-quadruplex species forming from C9ORF72 G4C2-expanded repeats associated with amyotrophic lateral sclerosis and frontotemporal lobar degeneration. *Neurobiology of aging*, 36(2), 1091-1096. <https://doi.org/10.1016/j.neurobiolaging.2014.09.012>

8 Appendix

Appendix A: Immunohistochemistry Supplementary Images

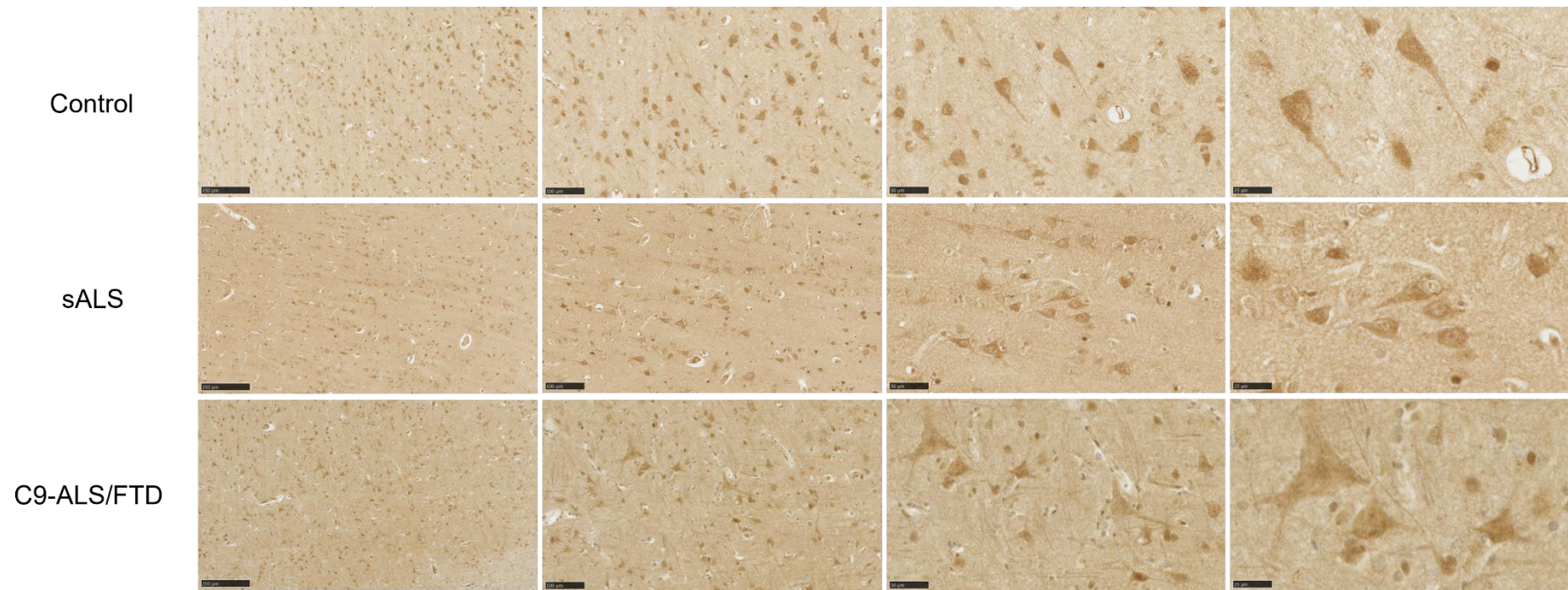


Figure 8.1 **eIF4A1 immunohistochemistry in control and ALS Motor Cortex.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4A1 showed positive cytoplasmic and variable nuclear labelling throughout all layers of the Motor Cortex. Labelling of neuronal processes and glial cell nuclei was also seen. Scale bars left-right are 250µm, 100µm, 50µm, 25µm

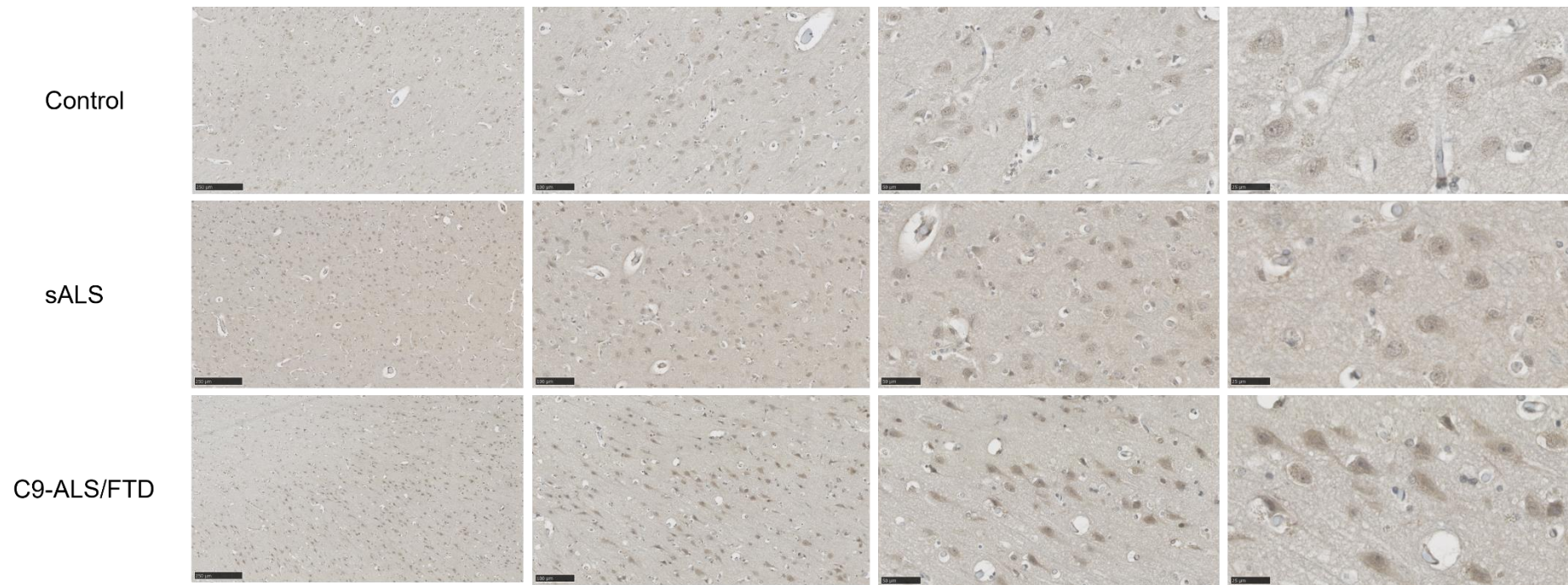


Figure 8.2 **eIF4A1 immunohistochemistry in control and ALS Prefrontal Cortex.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4A1 showed weak cytoplasmic staining of neurons in all layers of the Prefrontal Cortex, with variable labelling of neuronal and glial cell nuclei. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

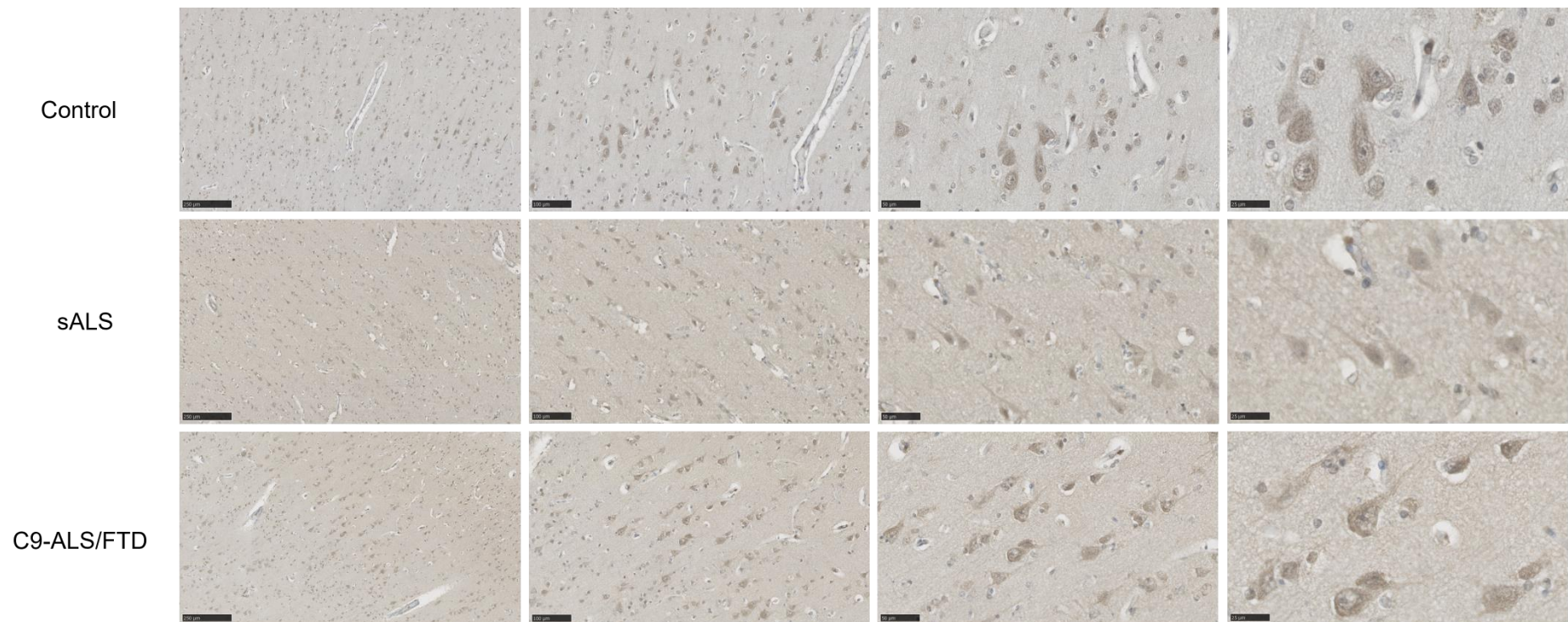


Figure 8.3 eIF4A1 immunohistochemistry in control and ALS Temporal Lobe. Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4A1 showed negative nuclear staining and weak cytoplasmic staining of neurons in all layers of the temporal lobe, with some limited extension into neuronal processes and variable labelling of glial cell nuclei. Scale bars left-right are 250µm, 100µm, 50µm, 25µm

Control



sALS



C9-
ALS/FTD



Figure 8.4 **eIF4A1 immunohistochemistry in control and ALS Hippocampus.** Top row: control; middle rows: sporadic ALS; bottom rows: Positive eIF4A1 staining is seen throughout the hippocampal region, including Ammon's horn and the dentate gyrus. Scale bars left-right: 2.5mm, 1mm, 500μm.

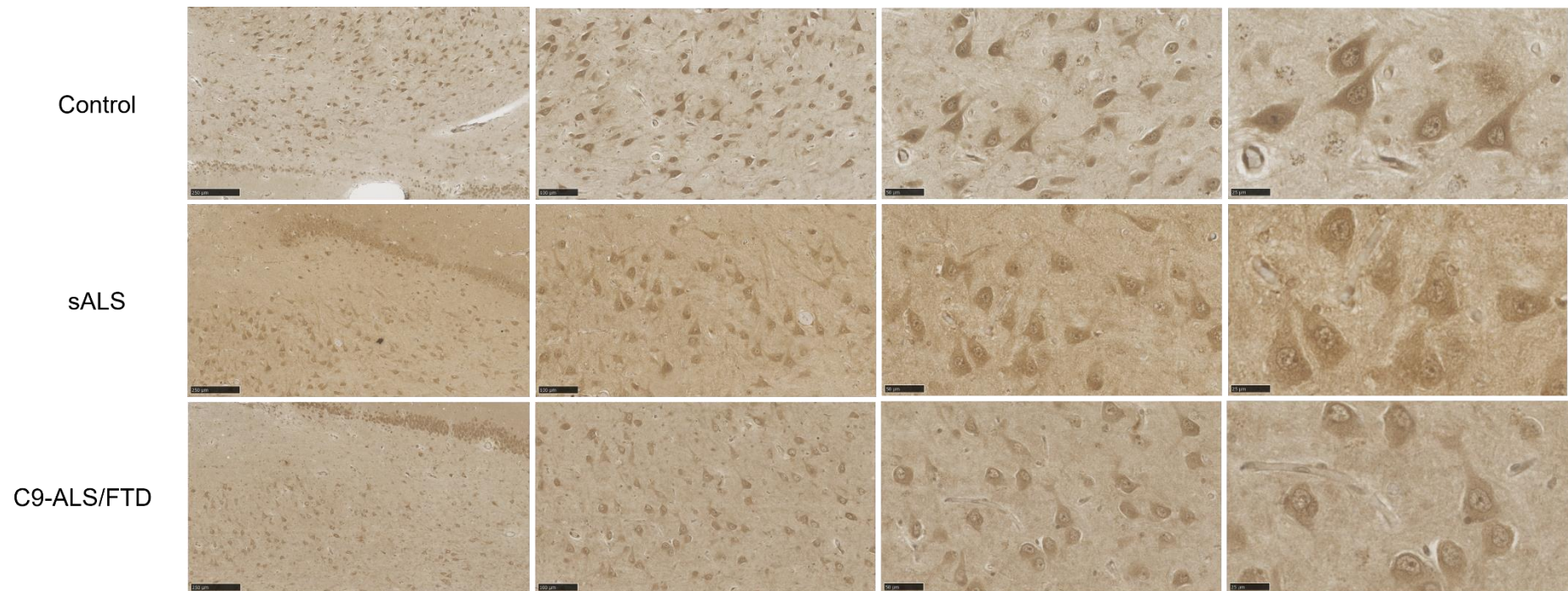


Figure 8.5 **eIF4A1 immunohistochemistry in control and ALS Hippocampal neurons.** Top row: control; middle rows: sporadic ALS; bottom rows: C9-ALS/FTD. eIF4A1 showed positive CA4 hippocampal cytoplasmic staining with some extension into neuronal processes. eIF4A1 staining was negative in the nucleus. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

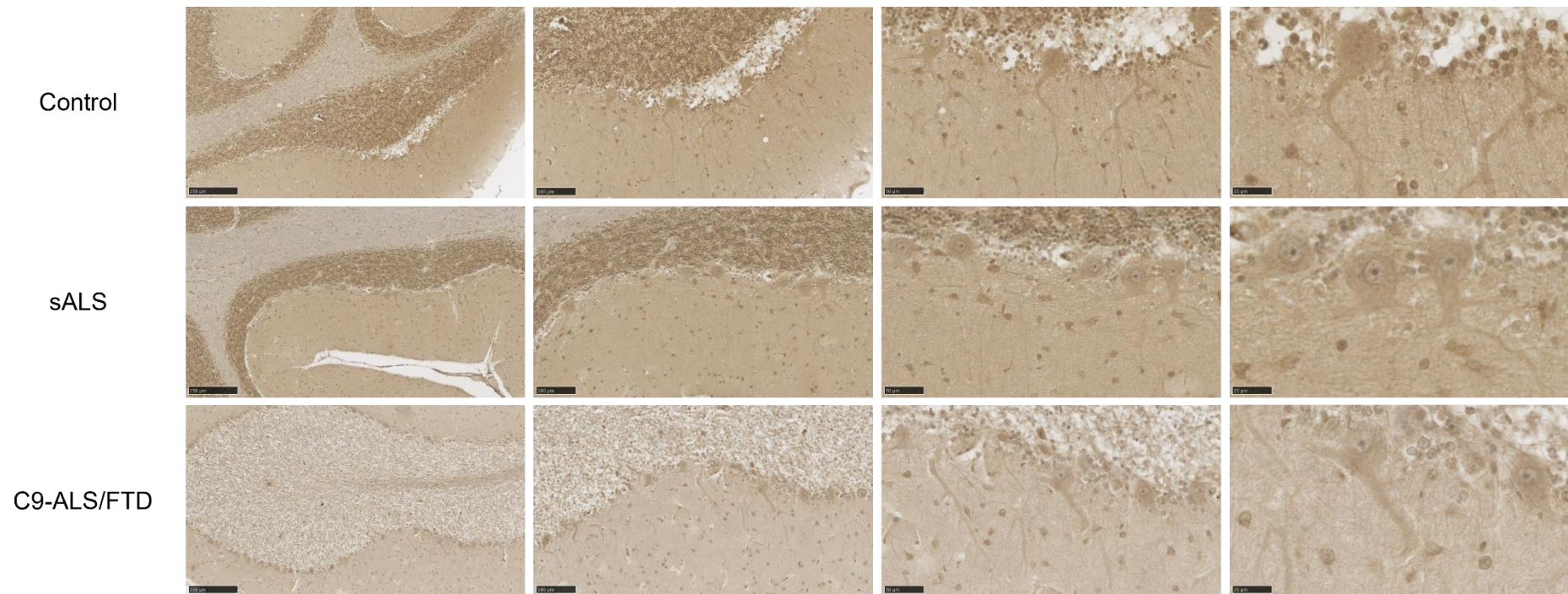


Figure 8.6 **eIF4A1 immunohistochemistry in control and ALS Cerebellum.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4A1 showed weak staining of Purkinje cell cytoplasm and processes, with negative staining of the nucleus. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

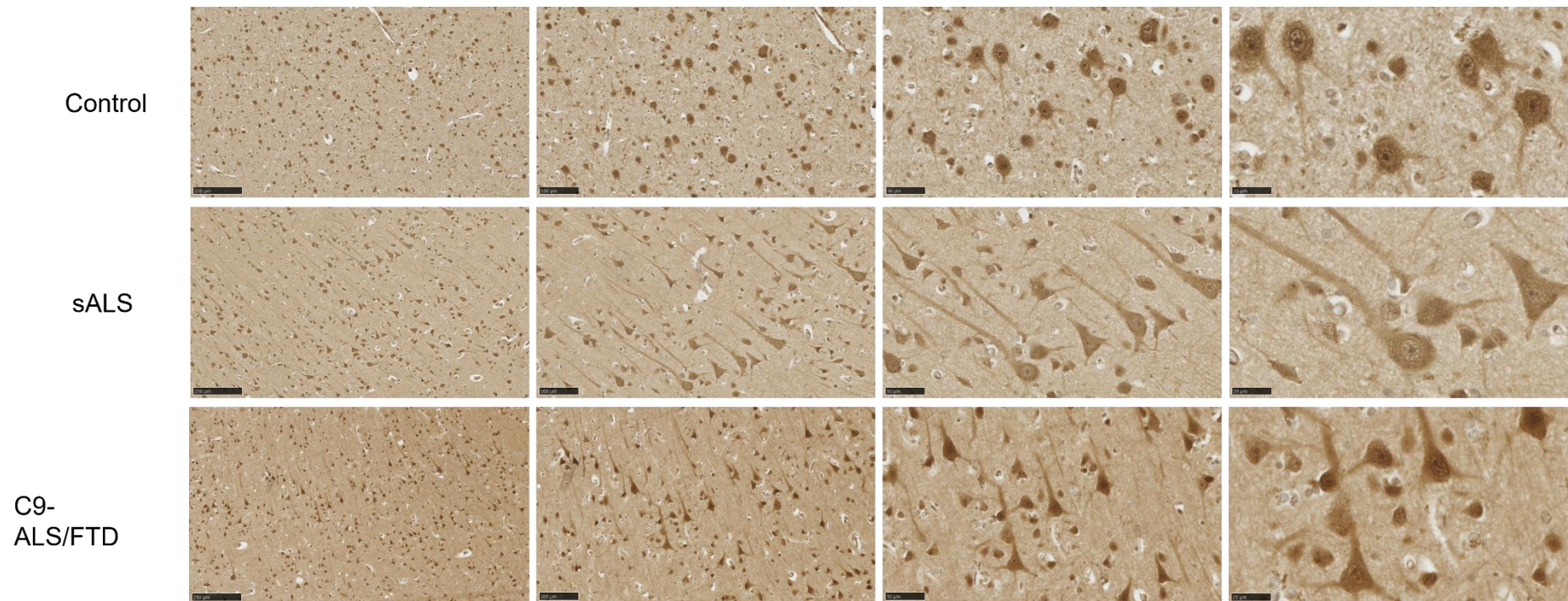


Figure 8.7 eIF4A2 immunohistochemistry in control and ALS Motor Cortex. Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4A2 stained glial cell nuclei and the neuronal cytoplasm and processes throughout all layers of the Motor Cortex. eIF4A2 labelling was generally weaker or absent in the nucleus of motor cortex neurons. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

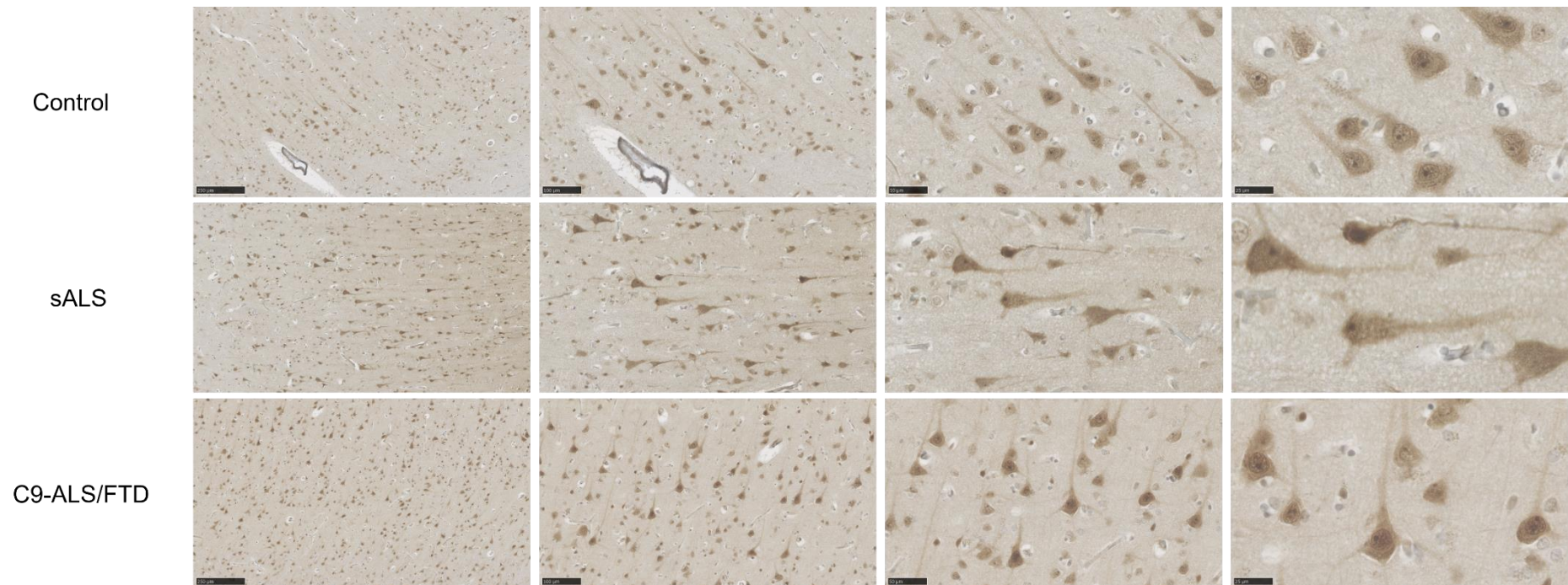


Figure 8.8 **eIF4A2 immunohistochemistry in control and ALS Prefrontal Cortex.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4A2 showed strong staining of neuronal cytoplasm and processes with variable nuclear labelling throughout all cortical layers. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

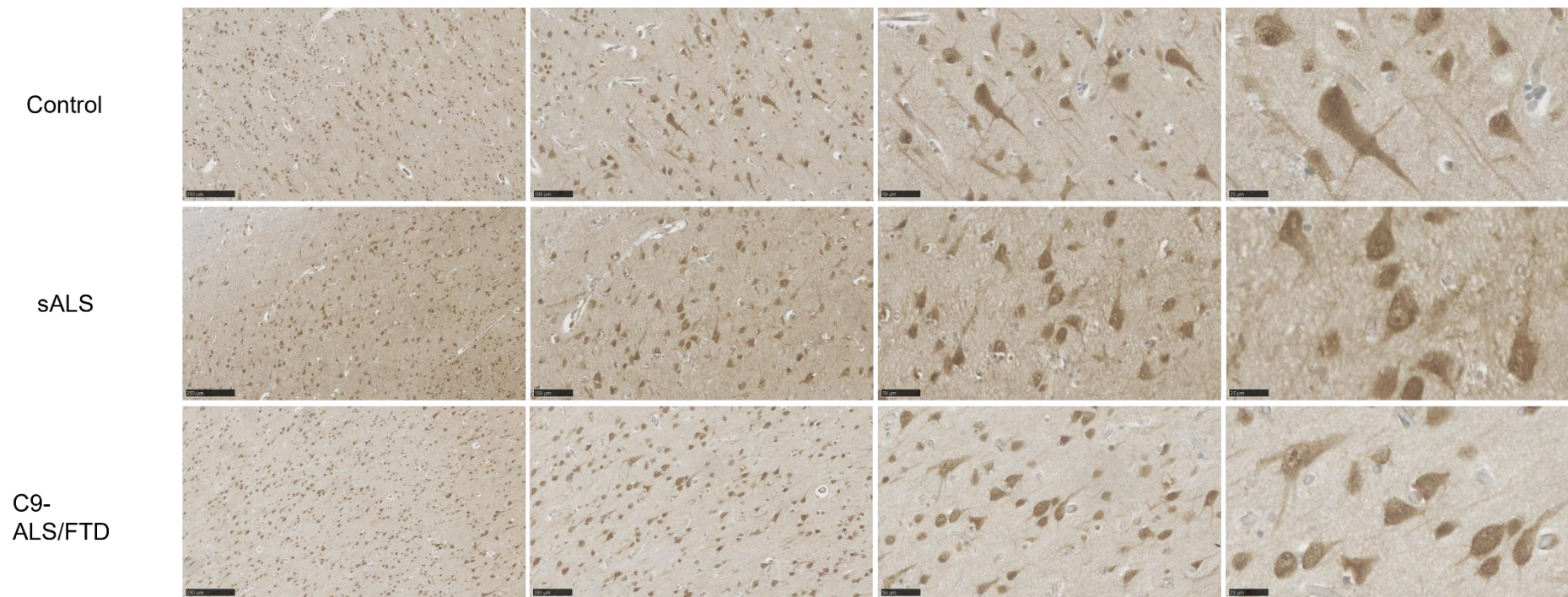


Figure 8.9 **eIF4A2 immunohistochemistry in control and ALS Temporal Lobe.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4A2 showed strong staining of the neuronal cytoplasm and processes with variable nuclear labelling throughout all cortical layers. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

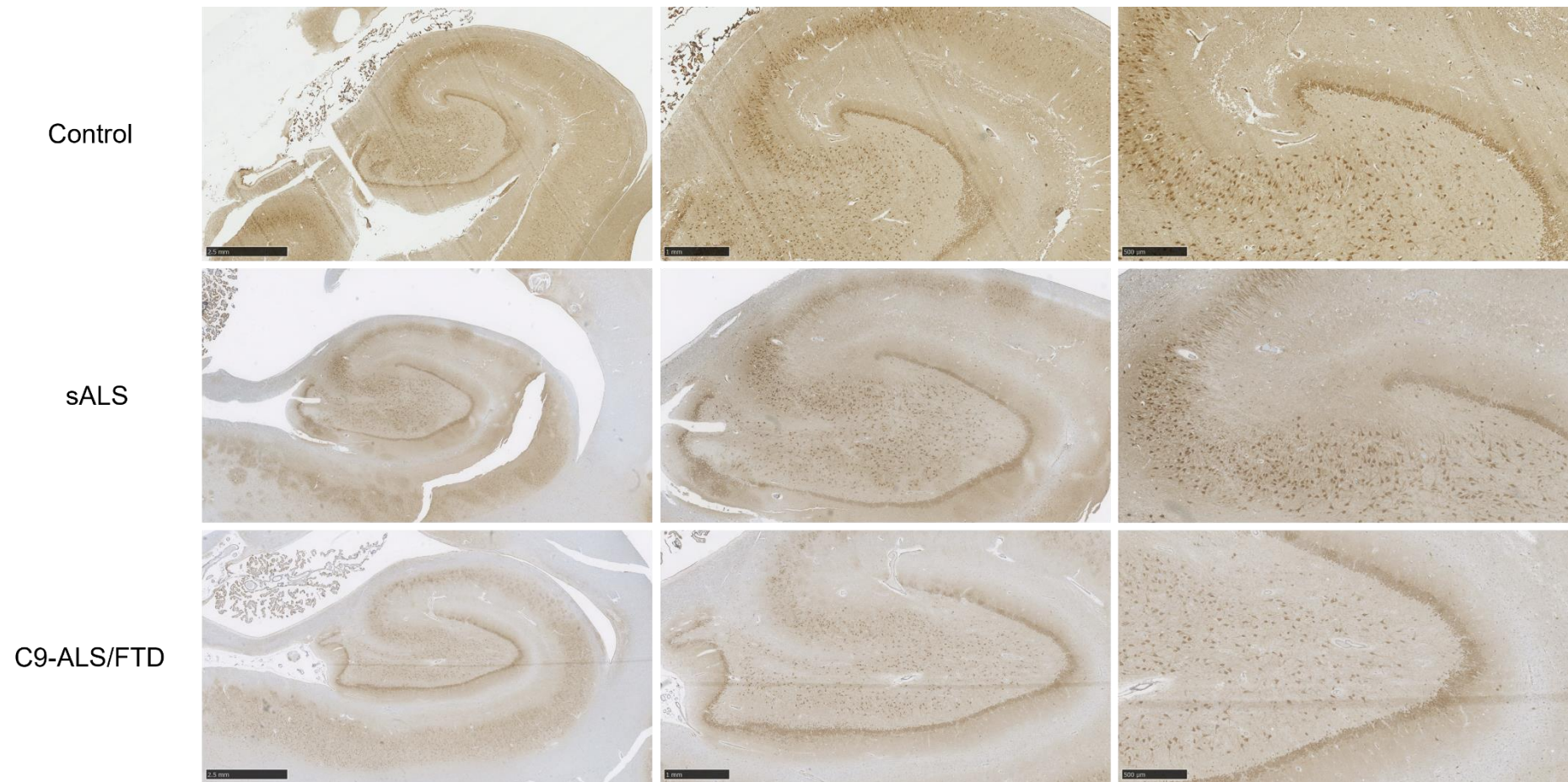


Figure 8.10 **eIF4A2 immunohistochemistry in control and ALS Hippocampus.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. Positive eIF4A2 staining is seen throughout the hippocampal region, including Ammon's horn and the dentate gyrus. Scale bars left-right: 2.5mm, 1mm, 500μm.

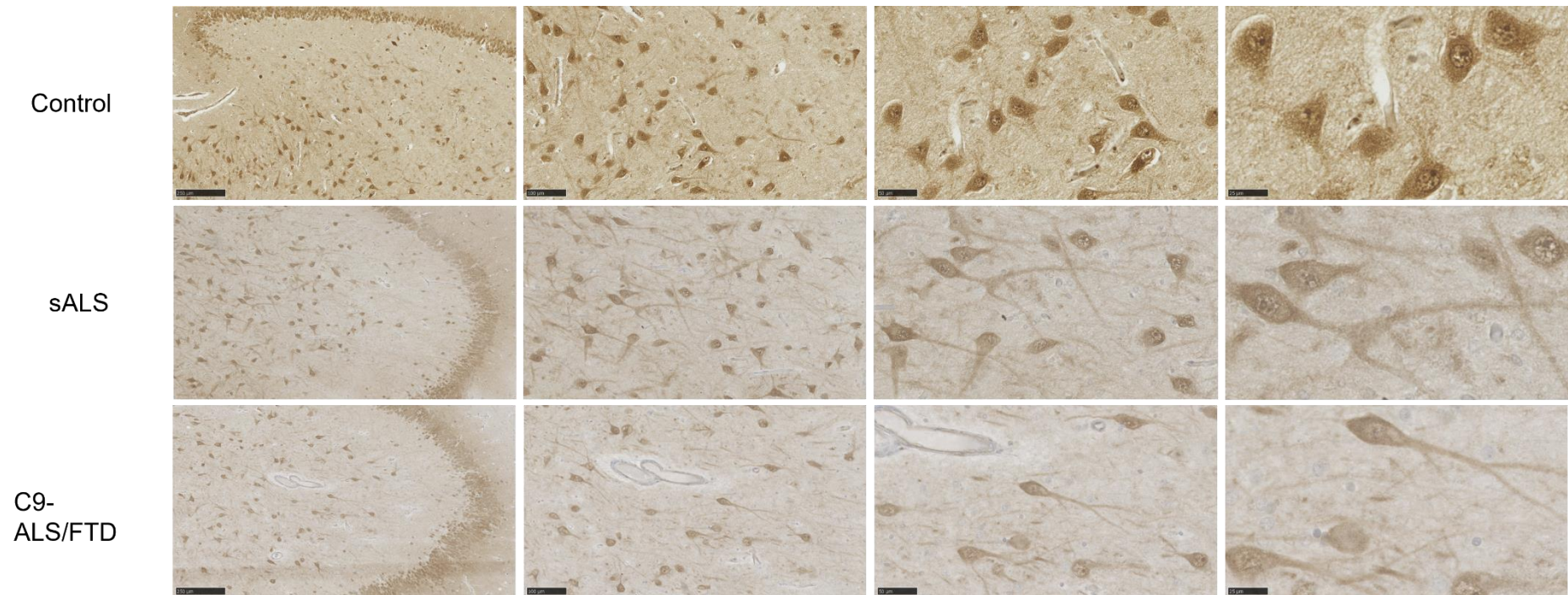


Figure 8.11 **eIF4A2 immunohistochemistry in control and ALS Hippocampal neurons.** Top row: control; middle rows: sporadic ALS; bottom rows: C9-ALS/FTD. eIF4A2 showed positive CA4 hippocampal cytoplasmic staining extending into neuronal processes. eIF4A2 staining was negative in the nucleus. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

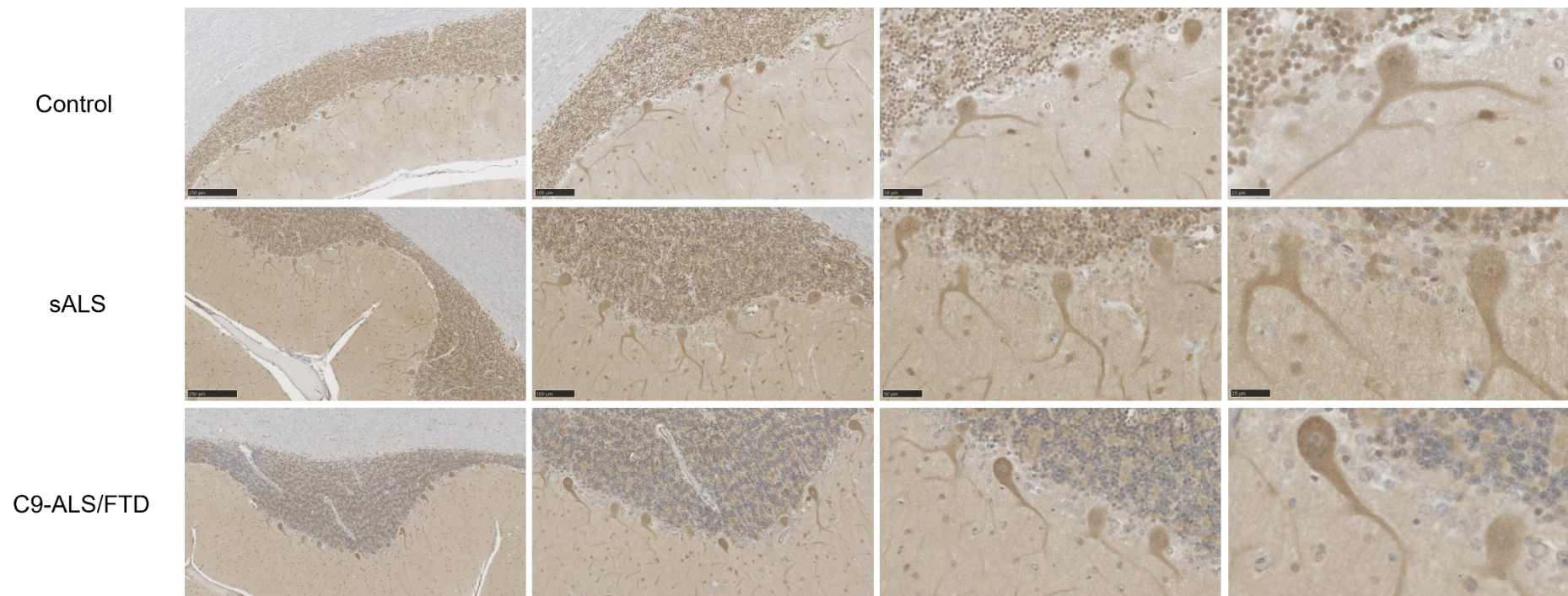


Figure 8.12 **eIF4A2 immunohistochemistry in control and ALS Cerebellum.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4A2 showed strong staining of Purkinje cells cytoplasm and processes, with negative staining of the nucleus. Variable eIF4A2 labelling was seen in the granular layer. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

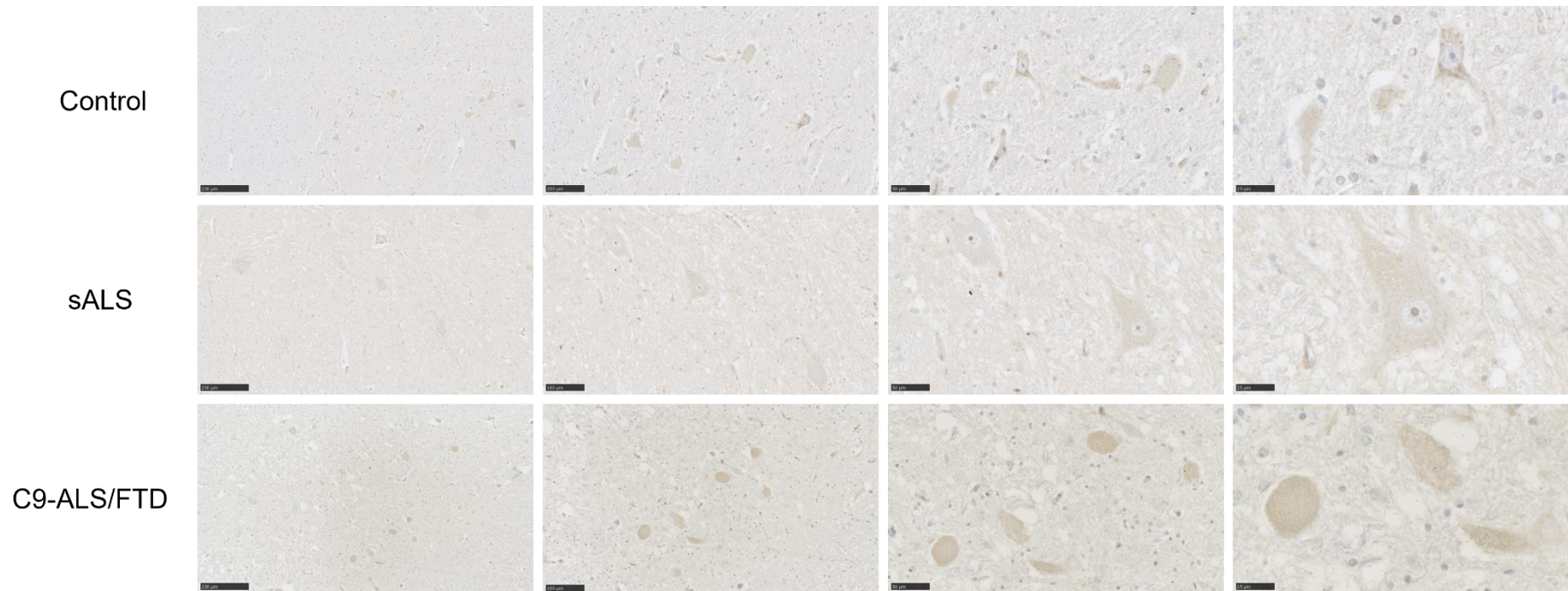


Figure 8.13 **eIF4B immunohistochemistry in control and ALS Spinal Cord.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4B shows very weak cytoplasmic staining with negative labelling of the nucleus in spinal cord motor neurons. Axons and glial cell nuclei were generally negative for eIF4B staining. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

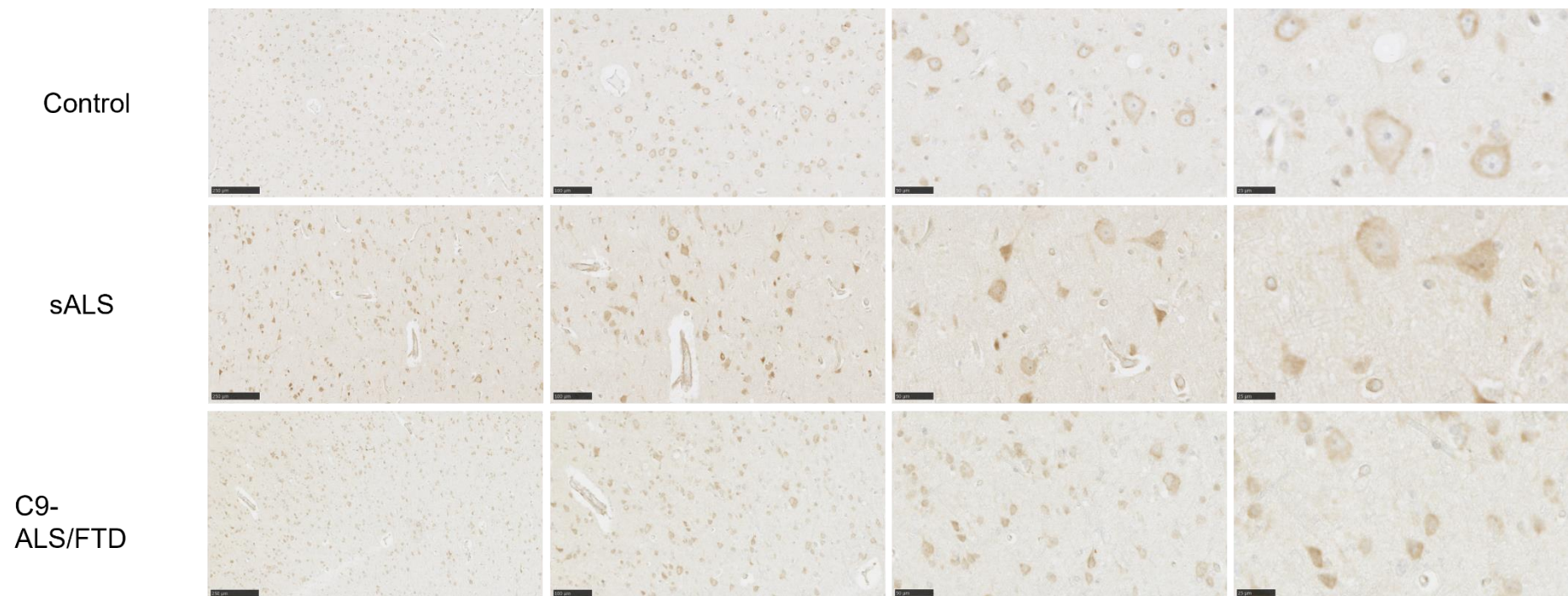


Figure 8.14 **eIF4B immunohistochemistry in control and ALS Motor Cortex.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4B shows weak neuronal cytoplasmic staining with negative labelling of the nucleus in all layers of the motor cortex. Some weak eIF4B labelling of axons and glia cell nuclei are seen. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

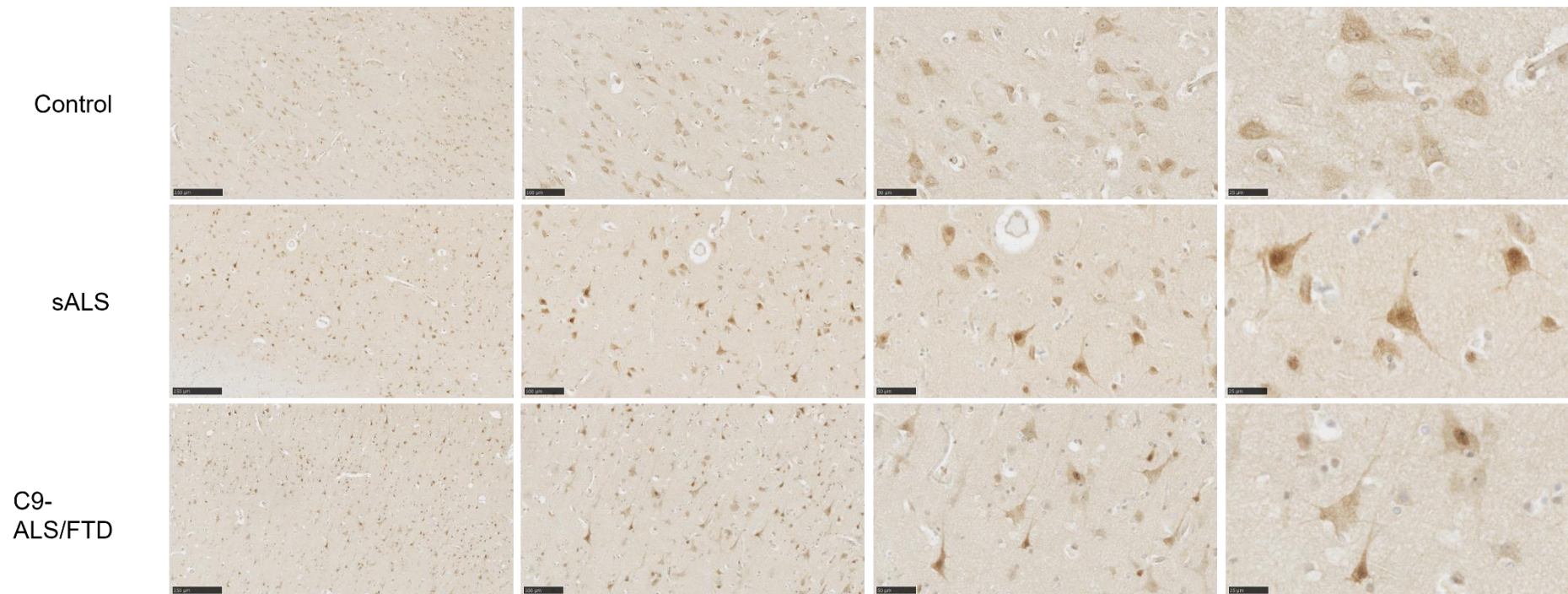


Figure 8.15 **eIF4B immunohistochemistry in control and ALS Prefrontal Cortex.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4B showed weak staining of the neuronal cytoplasm and processes throughout all cortical layers. It appears that in controls, eIF4B labelling of the nucleus was generally negative or only weakly labelled while some stronger positive labelling was seen in sALS and C9-ALS/FTD cases. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

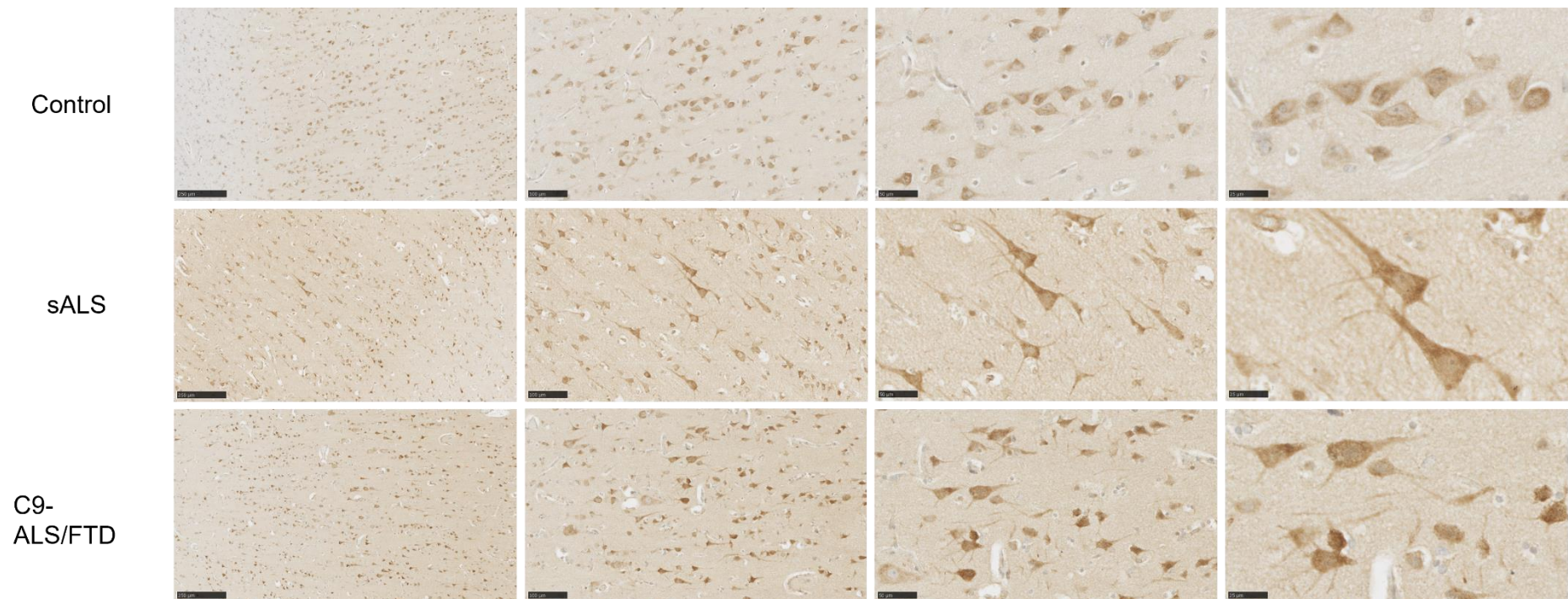


Figure 8.16 **eIF4B immunohistochemistry in control and ALS Temporal Lobe.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4B shows positive neuronal cytoplasmic labelling with variable nuclear labelling in all cortical layers. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

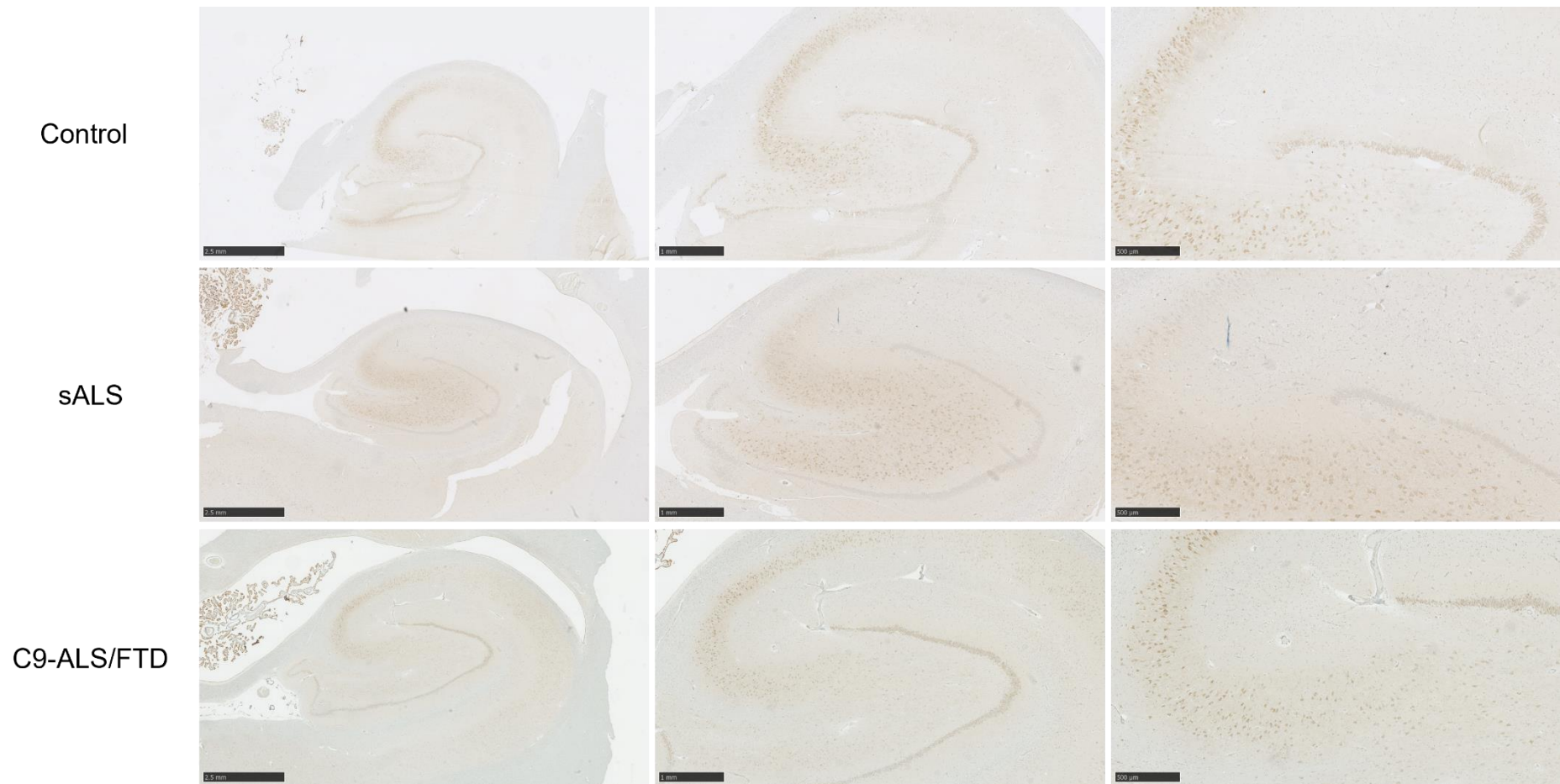


Figure 8.17 **eIF4B immunohistochemistry in control and ALS Hippocampus.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. Minimal eIF4B labelling is seen throughout much of the hippocampal region including CA1, CA3, CA4 and the dentate gyrus. CA2 shows slightly more eIF4B-positive staining. Scale bars left-right: 2.5mm, 1mm, 500μm.

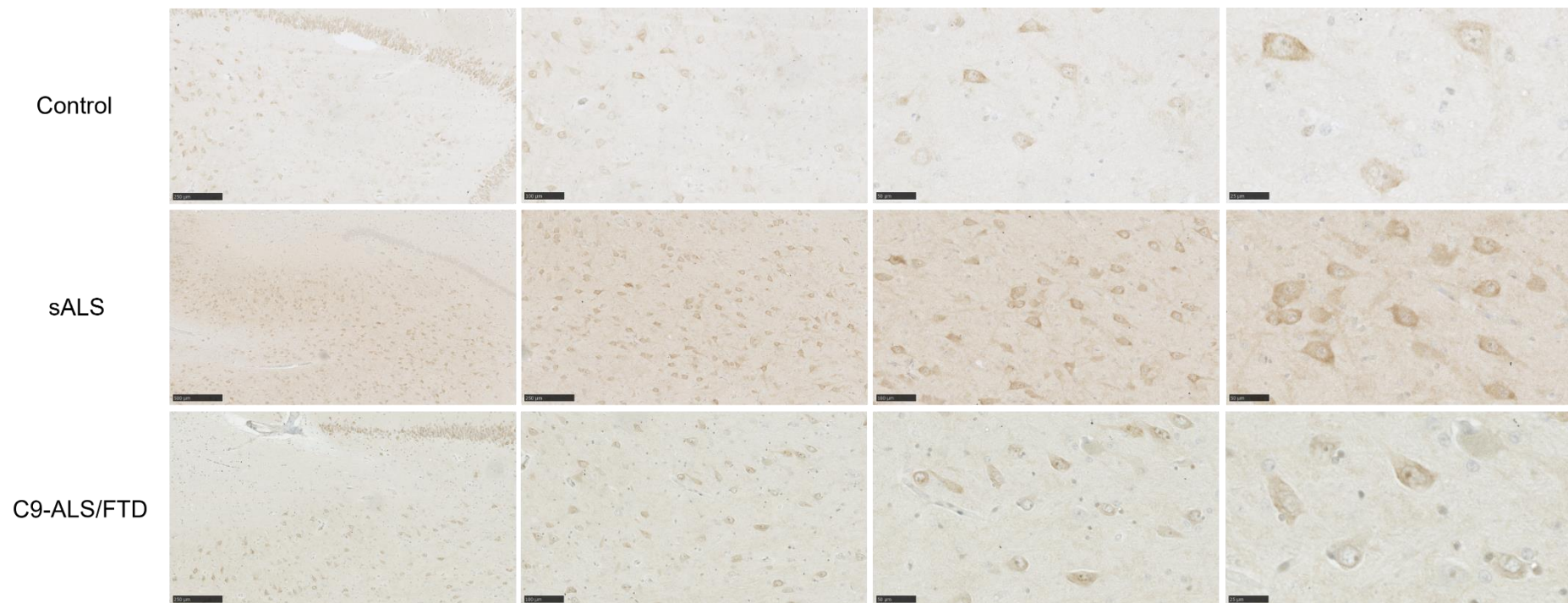


Figure 8.18 **eIF4B immunohistochemistry in control and ALS Hippocampal neurons.** Top row: control; middle rows: sporadic ALS; bottom rows: C9-ALS/FTD. eIF4B showed weak staining of the cytoplasm in CA4 hippocampal neurons; the nucleus, processes and glial cell nuclei were all negative for eIF4B. The granule cell layer showed very weak positive labelling for eIF4B. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

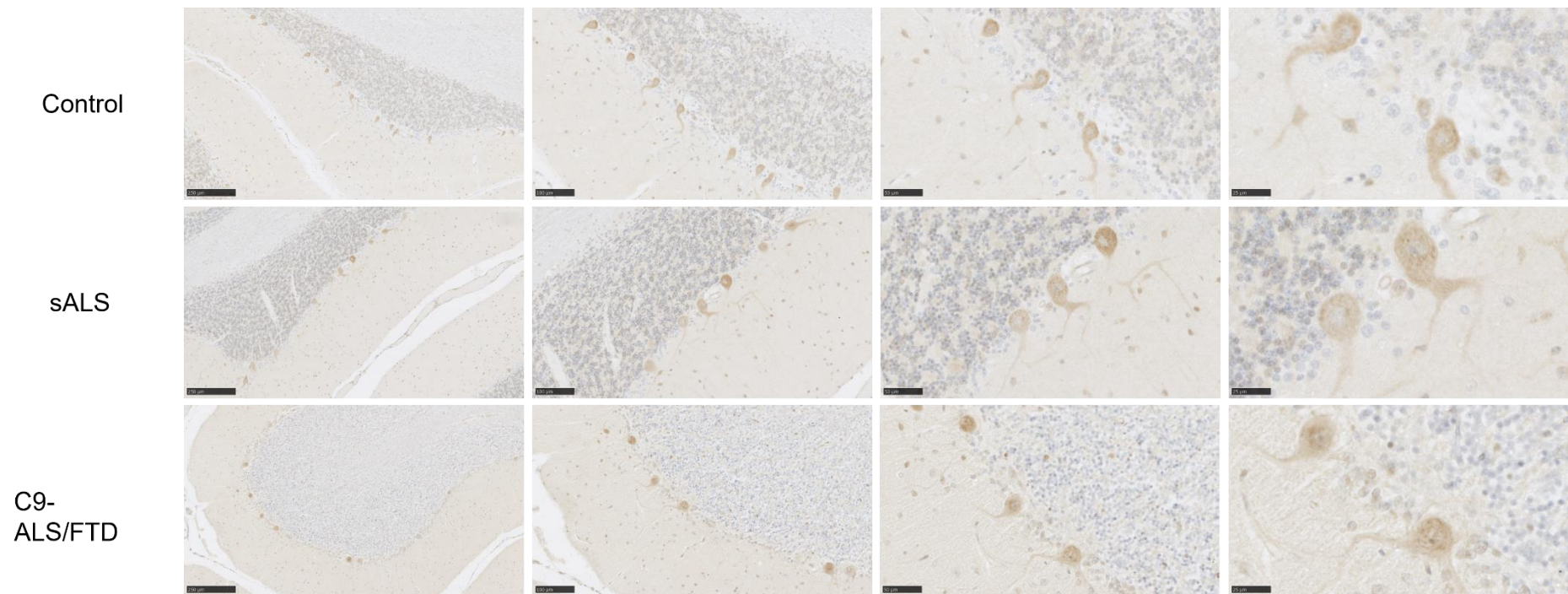


Figure 8.19 **eIF4B immunohistochemistry in control and ALS Cerebellum.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4B showed positive labelling of Purkinje cell cytoplasm and some limited staining of processes, with negative staining of the nucleus. Some weak eIF4B staining was seen in the granular layer. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

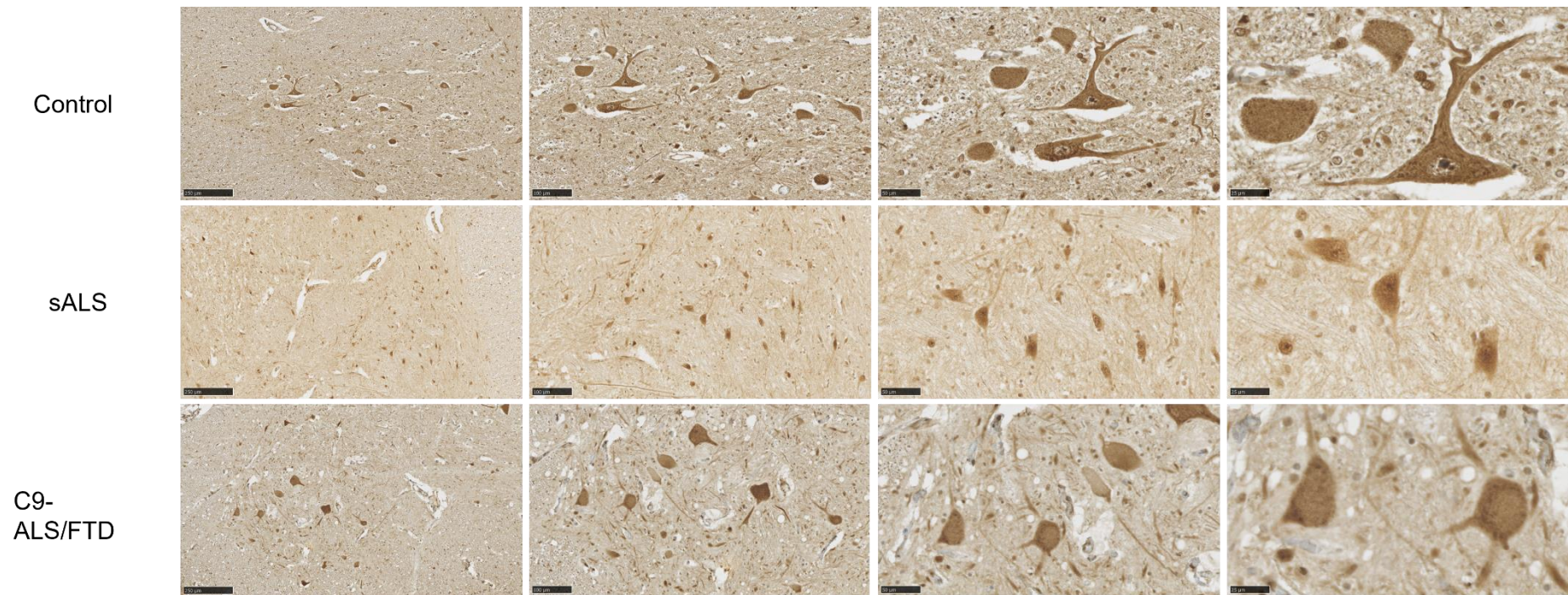


Figure 8.20 **eIF4H immunohistochemistry in control and ALS Spinal Cord**. Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4H showed strong cytoplasmic staining with variable labelling of the nucleus in spinal cord motor neurons. Positive staining was seen in axons and glial cell nuclei. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

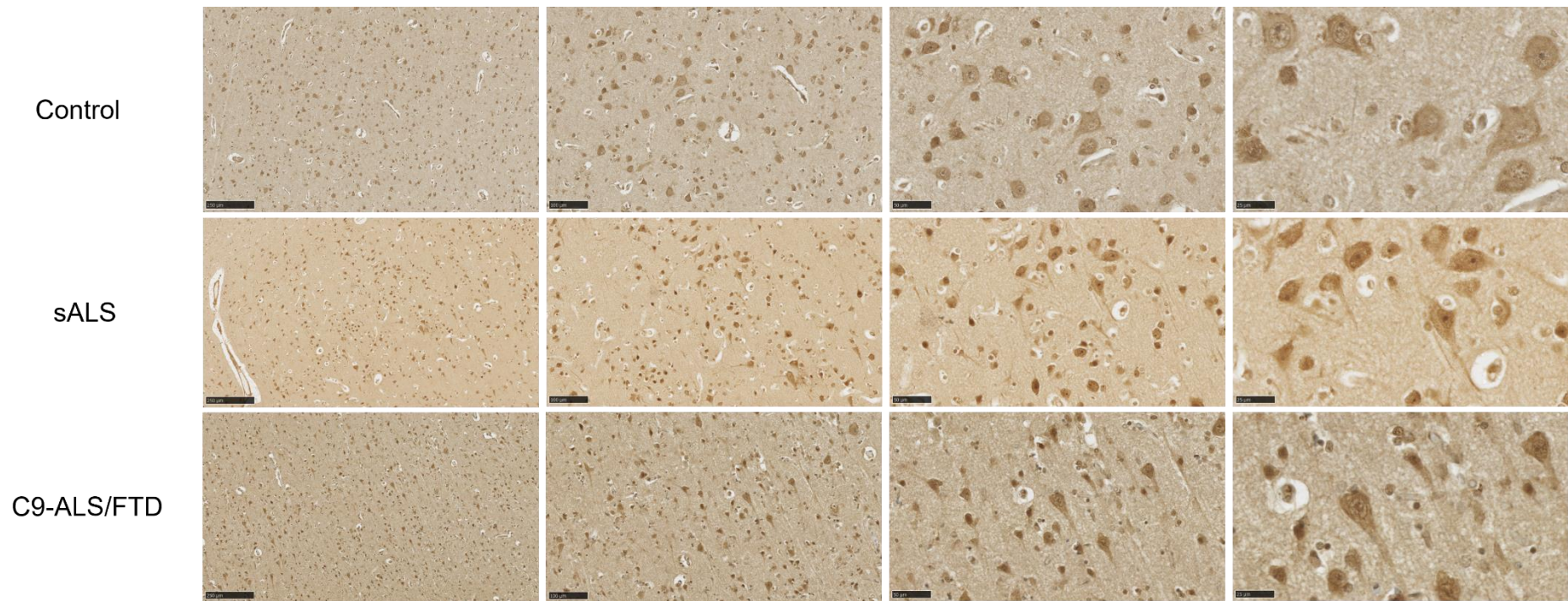


Figure 8.21 **eIF4H immunohistochemistry in control and ALS Motor Cortex.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4H showed positive labelling of neuronal cytoplasmic and processes with variable labelling of the nucleus in all layers of the motor cortex. Some eIF4H-positive labelling of axons and glia cell nuclei were also seen. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

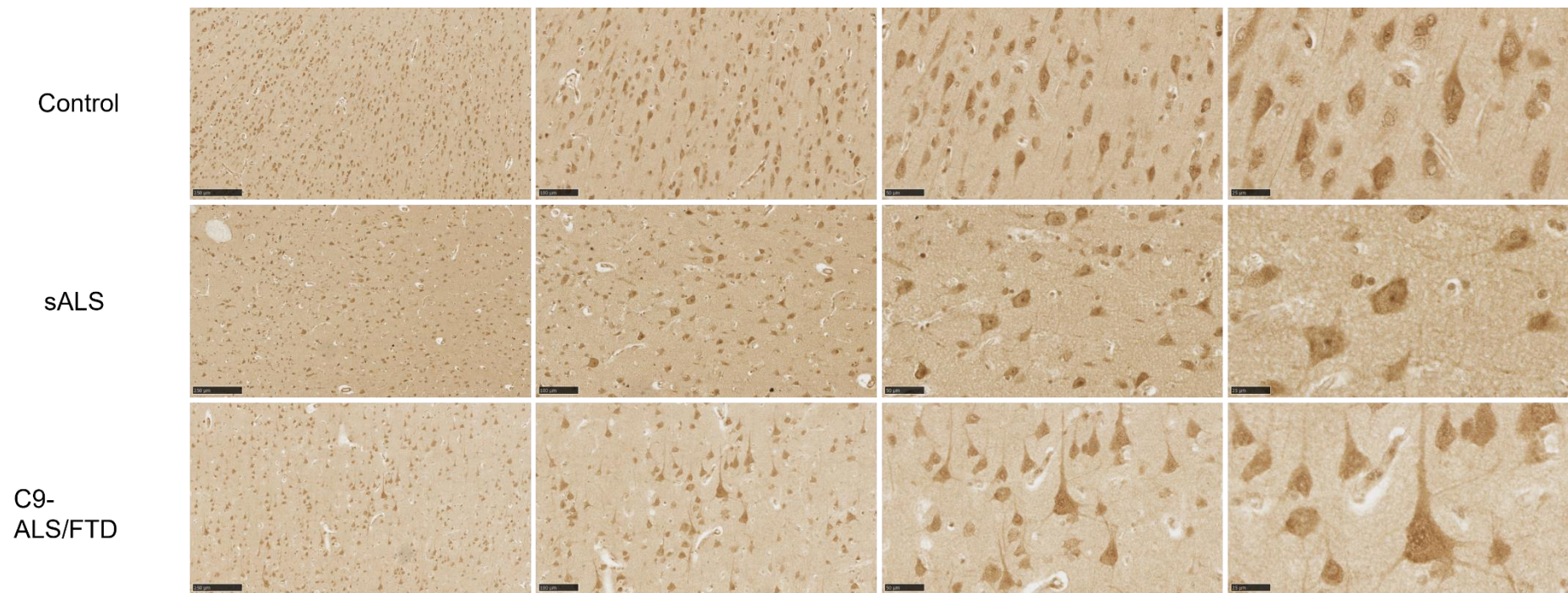


Figure 8.22 **eIF4H immunohistochemistry in control and ALS Prefrontal Cortex.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4H showed strong staining of the neuronal cytoplasm and processes throughout all cortical layers. eIF4H-positive labelling of the nucleus was weak or absent. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

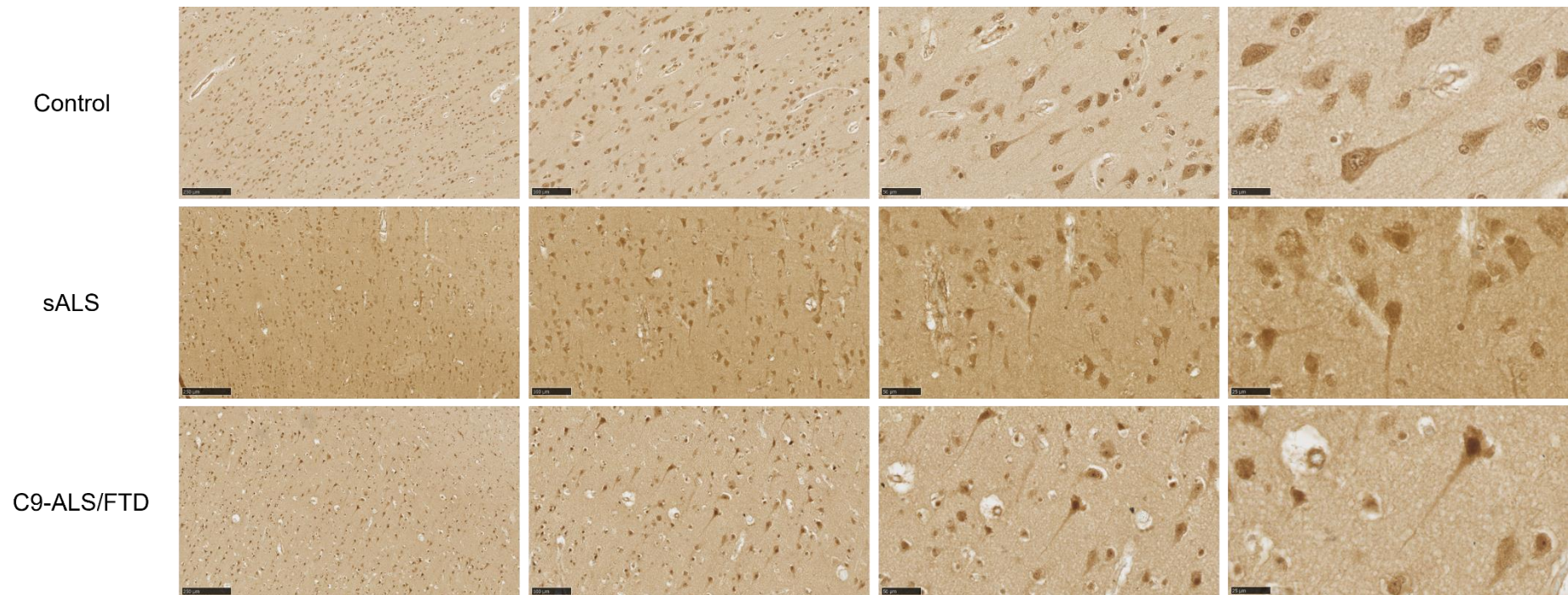


Figure 8.23 **eIF4H immunohistochemistry in control and ALS Temporal Lobe.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4H showed strong staining of the neuronal cytoplasm and processes with variable nuclear labelling, throughout all cortical layers. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

Control



sALS



C9-
ALS/FTD



Figure 8.24 **eIF4H immunohistochemistry in control and ALS Hippocampus.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4H-positive labelling was seen through Ammon's horn and the dentate fascia. Scale bars left-right: 2.5mm, 1mm, 500μm.

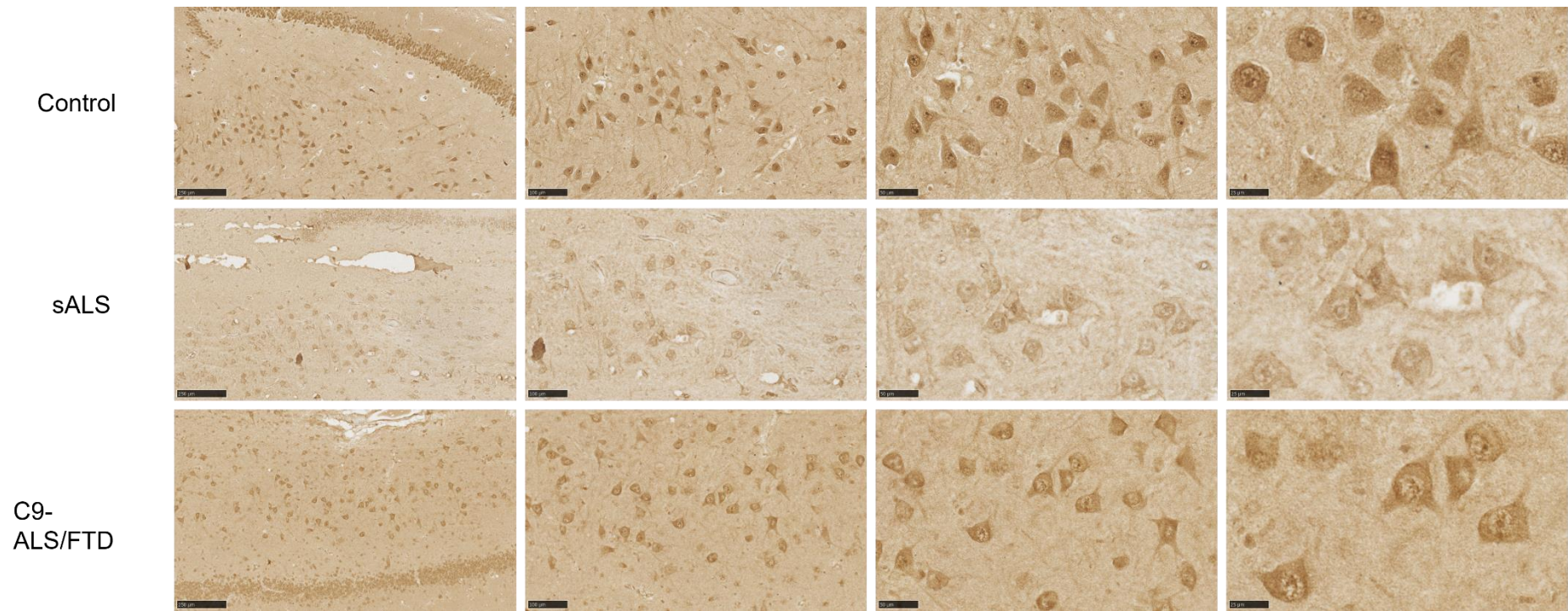


Figure 8.25 **eIF4H immunohistochemistry in control and ALS Hippocampal neurons.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4H showed positive CA4 hippocampal cytoplasm staining, with negative labelling in the nucleus. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

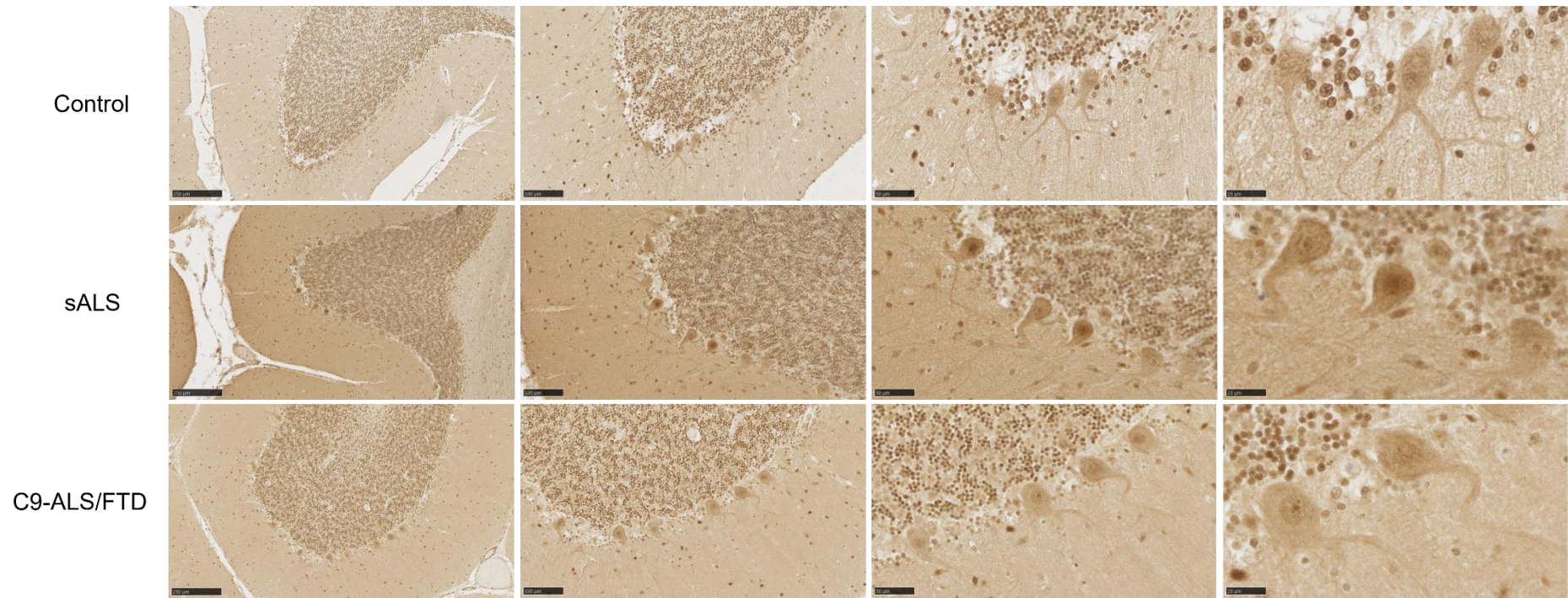


Figure 8.26 **eIF4H immunohistochemistry in control and ALS Cerebellum.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. eIF4H showed positive labelling of Purkinje cell nucleus, cytoplasm, and some limited staining of processes. eIF4H-positive staining was also seen in the granular layer. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

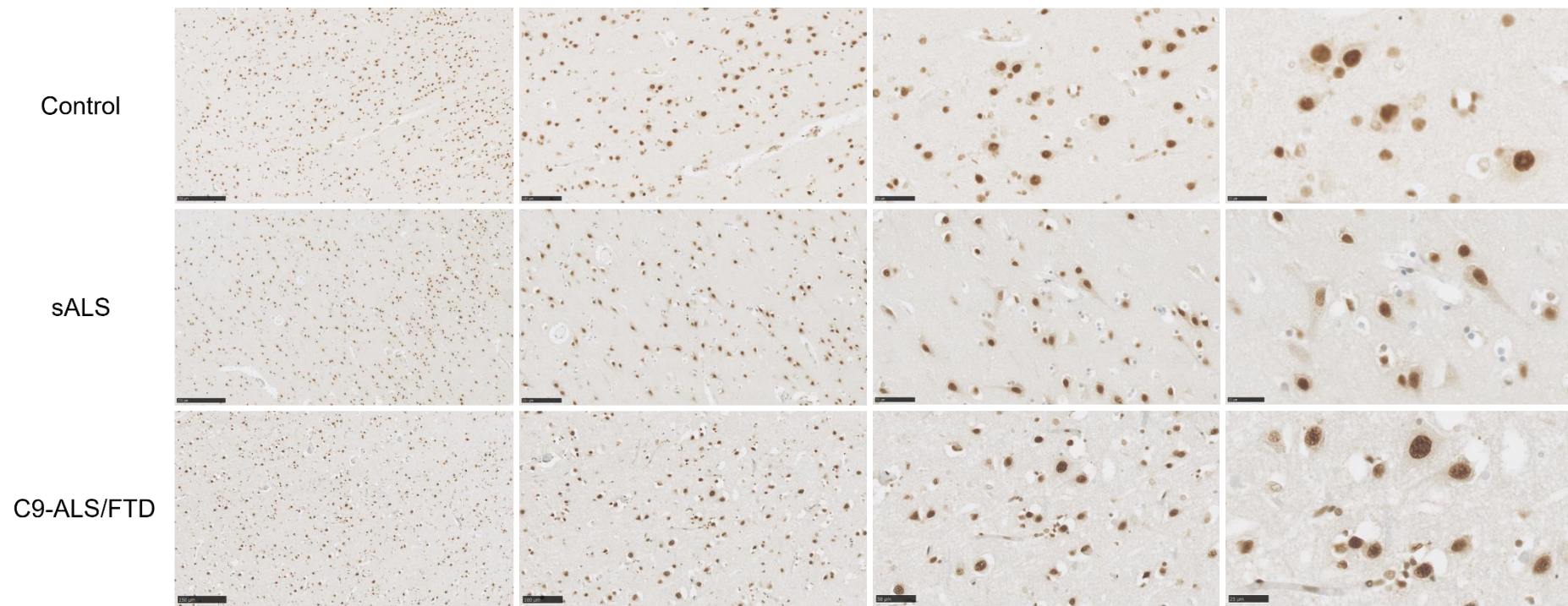


Figure 8.27 **SRSF1 immunohistochemistry in control and ALS Motor Cortex.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. SRSF1 showed negative labelling of the neuronal cytoplasm and strong staining in the nucleus throughout all layers of the motor cortex, consistent between control and ALS cases. SRSF1-positive labelling of glial cell nuclei is also seen. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

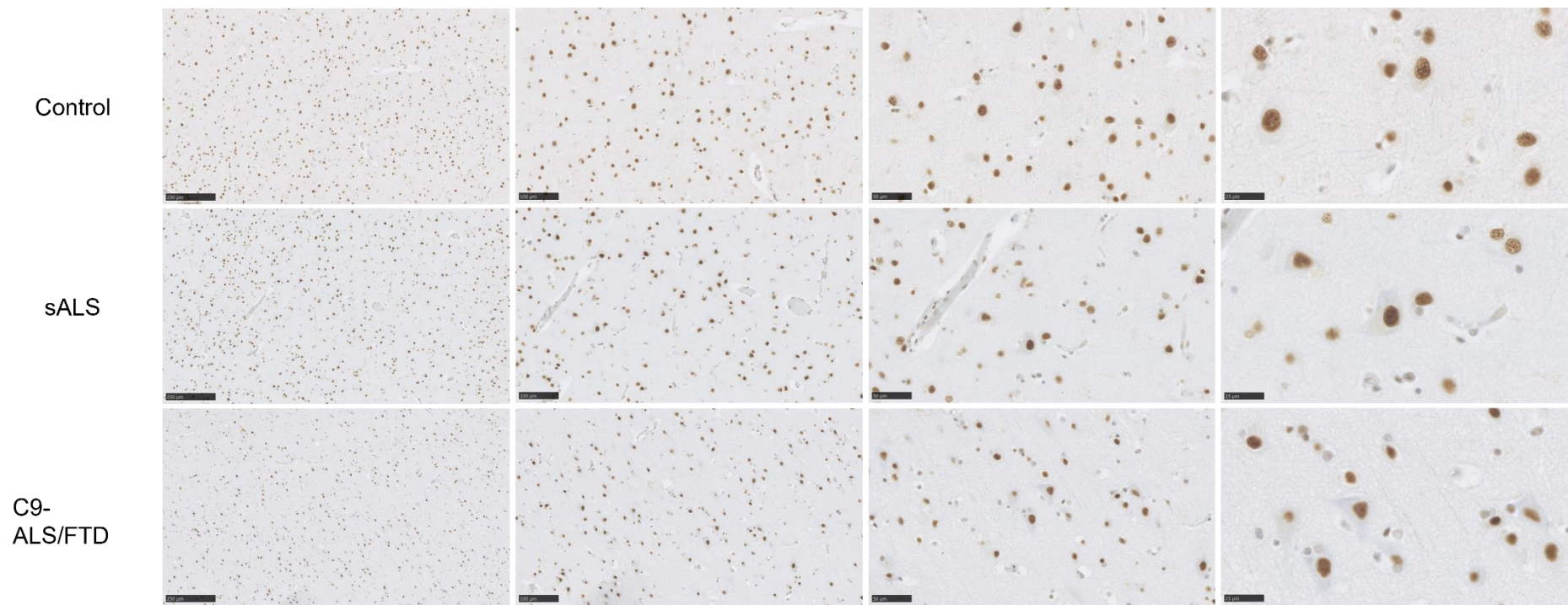


Figure 8.28 **SRSF1 immunohistochemistry in control and ALS Prefrontal Cortex.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. SRSF1 showed negative labelling of the neuronal cytoplasm and strong staining in the nucleus throughout all cortical layers. Scale bars left-right are 250µm, 100µm, 50µm, 25µm

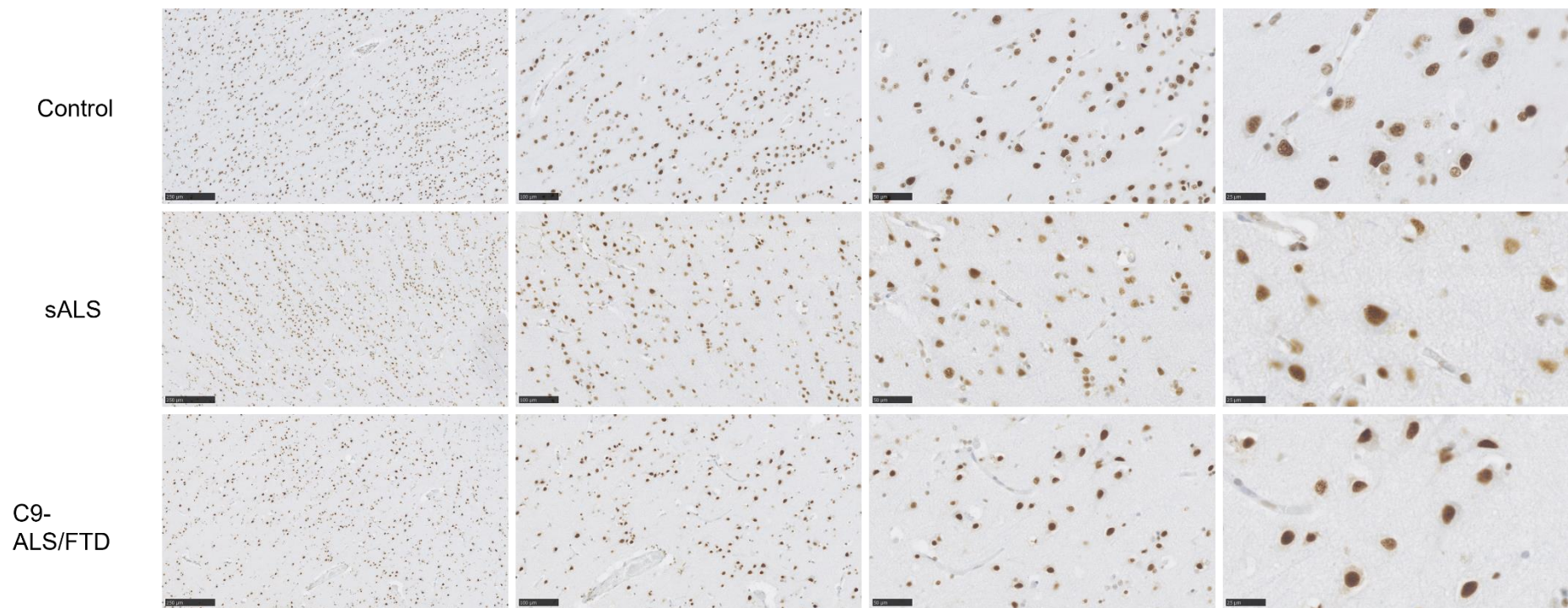


Figure 8.29 **SRSF1 immunohistochemistry in control and ALS Temporal Lobe.** Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. SRSF1 showed negative labelling of the neuronal cytoplasm with strong staining in the nucleus and glial cell nuclei throughout all cortical layers. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

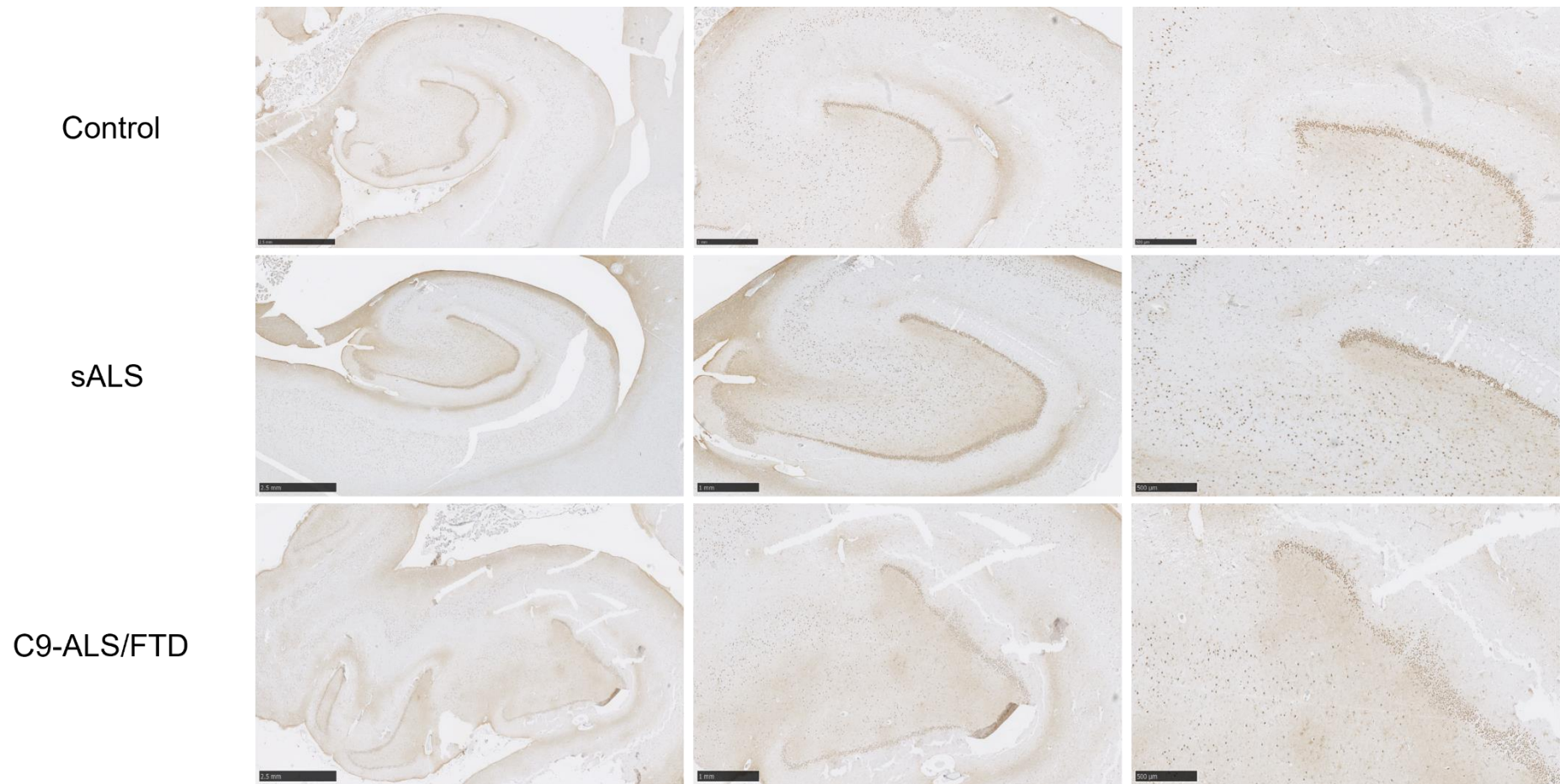


Figure 8.30 **SRSF1 immunohistochemistry in control and ALS Hippocampus.** Top row: control; middle rows: sporadic ALS; bottom rows: Positive SRSF1 staining is seen throughout the hippocampal region, including Ammon's horn and the dentate gyrus. Scale bars left-right: 2.5mm, 1mm, 500µm.

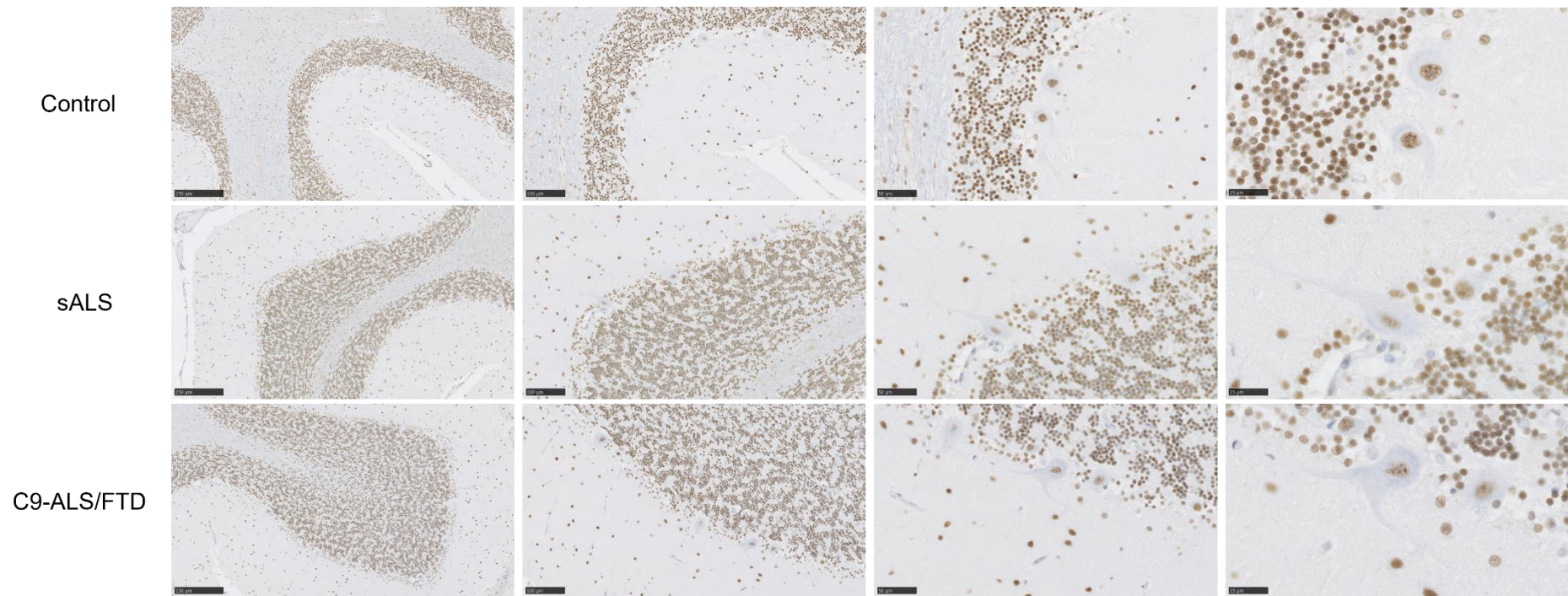


Figure 8.31 **SRSF1 immunohistochemistry in control and ALS Cerebellum**. Top row: control; middle row: sporadic ALS; bottom row: C9-ALS/FTD. SRSF1 showed positive staining in the granular layer, strong nuclear labelling of Purkinje cells with negative cytoplasmic labelling. Scale bars left-right are 250µm, 100µm, 50µm, 25µm.

Appendix B: Copyright Statement

Castelli LM, Benson BC, Huang WP, Lin YH, Hautbergue GM. RNA Helicases in Microsatellite Repeat Expansion Disorders and Neurodegeneration. *Front Genet.* 2022 May 12;13:886563. doi: 10.3389/fgene.2022.886563. PMID: 35646086; PMCID: PMC9133428.

[Copyright](#) © 2022 Castelli, Benson, Huang, Lin and Hautbergue.

This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

