

**The molecular pathogenesis of FUS
mutations in amyotrophic lateral sclerosis
using *in vivo* and *in vitro* models**

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterised by degeneration of both upper and lower motor neurons. Most ALS occurs in a sporadic manner, though 5-10% is familial. Fused in sarcoma (FUS) is an RNA binding protein, and mutations in the *FUS* gene are responsible for approximately 4% of familial and <1% of sporadic ALS cases. This work uses *in vitro* and *in vivo* models to examine the cellular effects of *FUS* mutations and the potential mechanisms of motor neuron toxicity.

The first section describes a family with a severe clinical form of ALS due to a *FUS* truncation mutation p.G504Wfs*12. This mutation demonstrates a severe phenotype *in vitro*, with mislocalisation of mutant FUS from the nucleus to the cytoplasm, and co-localisation with stress granules. Comparing these results with other mutations highlights the correlation between FUS mislocalisation and clinical phenotype, suggesting that the distribution of FUS plays a role in the pathogenesis of ALS.

The next section explores the phenotype of primary motor neurons from a FUS BAC transgenic mouse model expressing low levels of wild-type or P525L human FUS. P525L-FUS shows marked mislocalisation to the cytoplasm and co-localisation with stress granules, with evidence of reduced survival and alterations in stress granule dynamics. This suggests that motor neurons expressing mutant FUS show inherent vulnerability.

The final section explores transcript levels and alternative splicing in both FUS and TDP-43 BAC transgenic mouse models. Key proteins such as TDP-43, FUS and

SMN show alterations in mRNA levels in CNS tissue, and some subtle splicing alterations are described.

This work suggests that mutations in *FUS* are likely to affect motor neurons even when expressed at very low levels. Alterations in FUS distribution, stress granule dynamics, and regulation of transcription may all play a part in conferring a susceptibility to developing ALS.

DECLARATION

The work presented in this thesis was carried out by the author except where acknowledgment is made. The work was performed in the Nuffield Department of Clinical Neurosciences, University of Oxford during 2011-2015. No part of this work has been submitted for any other degree or any other University or Institute of learning.

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PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS WORK

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TABLE OF CONTENTS

TITLE PAGE	i
ABSTRACT	ii
DECLARATION	iv
ACKNOWLEDGEMENTS	v
PUBLICATIONS AND PRESENTATIONS ARISING	vi
CONTENTS	viii
ABBREVIATIONS	xv
LIST OF FIGURES AND TABLES	xxi
CHAPTER 1: INTRODUCTION	1
1.1 Motor Neuron Diseases	
1.1.1 Amyotrophic lateral sclerosis	1
1.1.2 Spinal muscular atrophy.....	3
1.2 ALS Genetics	
1.2.1 Overview of ALS genetics	4
1.2.2 <i>FUS</i> mutations in ALS.....	8
1.2.3 <i>TARDBP</i> mutations in ALS	10
1.2.4 <i>EWS</i> and <i>TAF15</i> mutations in ALS	11
1.3 Function of the key proteins	
1.3.1 <i>FUS</i>	12
1.3.2 <i>TDP-43</i>	17
1.3.3 <i>EWS</i> and <i>TAF15</i>	21
1.3.4 <i>SMN</i>	22

1.4 Stress granules

1.4.1 Introduction to stress granules	23
1.4.2 The stress granule cycle	24
1.4.3 Types of cell stress.....	26
1.4.4 Stress granule components.....	26
1.4.5 Stress granules and neurodegeneration	27

1.5 The pathophysiology of ALS

1.5.1 Mislocalisation of mutant protein and recruitment into stress granules	28
1.5.2 Protein underexpression or overexpression	31
1.5.3 RNA processing	32
1.5.4 Protein metabolism and interaction	34
1.5.5 Review of other hypotheses	35
1.6 Aims of this work	36

CHAPTER 2: MATERIALS AND METHODS

2.1 Genotyping

2.1.1 Patient data	38
2.1.2 PCR and sequencing	38
2.1.3 pGEM-t confirmation	39

2.2 Culture of immortalised cell lines

2.2.1 Cell culture and transfection.....	39
2.2.2 Stress treatment of immortalised cells.....	40

2.3 Construct generation

2.3.1 pcDNA constructs and site-directed mutagenesis	40

2.3.2 BAC constructs.....	41
2.4 Primary neuronal cultures	
2.4.1 Primary motor neuron culture	41
2.4.2 Primary cortical culture	42
2.4.3 Transfection of primary neurons.....	43
2.4.4 Stress treatment of primary neurons	44
2.4.5 Survival assay	44
2.4.6 Genotyping	44
2.5 Immunocytochemistry	45
2.6 Western blotting	
2.6.1 Protein extraction	46
2.6.2 Bicinchonic acid (BCA) Protein assay	47
2.6.3 Western Blot.....	48
2.6.4 Densitometry	48
2.7 Microscopy	
2.7.1 Immunofluorescence microscopy	49
2.7.2 Confocal microscopy	50
2.8 Animal work	
2.8.1 Mouse models.....	52
2.8.2 Tissue for primary cultures	53
2.8.3 Tissue for protein and RNA	54
2.9 qRT-PCR	
2.9.1 RNA extraction and cDNA synthesis	55
2.9.2 Primer design and optimisation	55
2.9.3 qRT-PCR.....	56

2.9.4	Splicing	
2.9.5	PCR	57
2.9.6	Analysis.....	59

CHAPTER 3: IDENTIFICATION AND CHARACTERISATION OF A G504Wfs*12 FUS TRUNCATION MUTATION IN A FAMILY WITH RAPIDLY PROGRESSIVE ALS

3.1 Introduction

3.1.1	Introduction.....	60
3.1.2	Aims of chapter 3.....	60

3.2 Results

3.2.1	Clinical information	61
3.2.2	Confirming the presence of a FUS truncation mutation in this case of fALS.....	65
3.2.3	The creation of pcDNA constructs bearing the G504Wfs*12-FUS truncation mutation	71
3.2.4	G504Wfs*12-FUS is mislocalised to the cytoplasm in HEK293 and NSC-34 cells	77
3.2.5	G504Wfs*12-FUS co-localises with stress granules in HEK293 and NSC-34 cells	81
3.2.6	G504Wfs*12-FUS is mislocalised to the cytoplasm and co-localises with stress granules in primary neurons	89
3.2.7	FUS truncation mutations cause a severe form of ALS	92
3.3	Discussion.....	94

CHAPTER 4: IN VITRO ANALYSIS OF THE EFFECTS OF *FUS* AND *TARDBP* MUTATIONS ON PRIMARY NEURONS

4.1 Introduction and aims	101
4.2 Results in the FUS BAC transgenic model	
4.2.1 FUS expressed using a BAC construct in HEK293 cells	104
4.2.2 The effect of cell stress on wild-type and mutant BAC-derived FUS in HEK293 cells	108
4.2.3 BAC-derived mutant FUS does not co-localise with SMN	108
4.2.4 Primary motor neuron cultures from the FUS BAC transgenic mouse model	112
4.2.5 Motor neurons expressing P525L-FUS have reduced Survival	117
4.2.6 P525L-FUS is mislocalised to the cytoplasm of primary motor neurons	117
4.2.7 The distribution of FET proteins is not significantly altered by the presence of P525L mutant FUS	123
4.2.8 Stress granule dynamics in P525L-FUS motor neurons	128
4.2.9 Mutant FUS is incorporated into stress granules	135
4.2.10 SMN distribution and gem number is not altered by P525L-FUS expression in motor neurons	138
4.3 Results in FUS BAC transgenic cortical neurons	
4.3.1 Primary cortical cultures from the FUS BAC transgenic mouse model	142
4.3.2 P525L FUS is mislocalised to the cytoplasm of FUS BAC transgenic cortical neurons	142

4.3.3 P525L FUS is incorporated into stress granules in cortical neurons.....	143
4.3.4 Stress granule dynamics in P525L FUS cortical neurons	150
4.4 Results in the FUS knockout model	
4.4.1 Primary motor neuron cultures from the FUS knockout mouse model.....	154
4.4.2 The distribution of FET proteins is not altered by FUS Knockout	158
4.4.3 Stress granule dynamics in FUS knockout motor neurons.....	158
4.4.4 SMN is not altered by FUS knockout	163
4.5 Results in the TDP-43 BAC transgenic mouse model	
4.5.1 Primary motor neuron cultures from the TDP-43 BAC transgenic mouse model	163
4.5.2 The distribution of FET proteins is not altered by mutant TDP-43 expression.....	164
4.5.3 SMN is not altered by mutant TDP-43 expression	164
4.6 Discussion	
4.6.1 FUS BAC transgenic motor neurons	173
4.6.2 FUS BAC transgenic cortical neurons.....	180
4.6.3 FUS knockout motor neurons.....	182
4.6.4 TDP-43 BAC transgenic motor neurons	183

CHAPTER 5: THE EFFECTS OF FUS OR TDP-43 MUTATIONS ON KEY mRNA SPECIES AND ALTERNATIVE SPLICING

5.1 Introduction and aims	186

5.2 Expression levels of key proteins in the FUS and TDP-43 BAC transgenic mouse models	
5.2.1 TDP-43 BAC transgenic mouse model	187
5.2.2 FUS BAC transgenic mouse model.....	197
5.3 Protein levels in the TDP-43 BAC transgenic mouse model.....	206
5.4 Alternative splicing in the FUS and TDP-43 BAC transgenic mouse models	
5.4.1 Splicing in the TDP-43 BAC transgenic mouse model	208
5.4.2 Splicing in the FUS BAC transgenic mouse model	228
5.5 Discussion	
5.5.1 Transcript levels in the TDP-43 BAC transgenic mouse model.....	240
5.5.2 Protein levels in the TDP-43 BAC transgenic mouse model	243
5.5.3 Transcript levels in the FUS BAC transgenic mouse model	246
5.5.4 Alternative splicing in the TDP-43 BAC transgenic mouse model...	249
5.5.5 Alternative splicing in the FUS BAC transgenic mouse model	250
 CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS	
6.1 General discussion	253
6.2 Future directions	257
REFERENCES.....	259
APPENDICES	277

ABBREVIATIONS

ALS	Amyotrophic lateral sclerosis
ALSFRS-R	ALS functional rating scale - revised
BAC	Bacterial artificial chromosome
BCA	Bicinchonic acid
BDNF	Brain-derived neurotrophic factor
bp	Base pair
ChAT	Choline acetyltransferase
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CT	Computer tomography
DNA	Deoxyribonucleic acid
eIF2 α	Eukaryotic Initiation Factor 2
EWS	Ewings sarcoma breakpoint region 1
fALS	Familial ALS
FET	FUS, EWS and TAF15 proteins
Fmr1	Fragile X mental retardation 1
FTD	Frontotemporal dementia
HBSS	Hank's balanced salt solution
HDAC6	Histone deacetylase 6
HEK293	Human embryonic kidney 293 (immortalised cell line)
Het	Heterozygous
HIV	Human immunodeficiency virus
hnRNPA1	Heterogeneous nuclear ribonucleoprotein A1
HRI	Heme-regulated eIF2 α kinase
ICC	Immunocytochemistry
KO	Knockout

LMN	Lower motor neuron
Mag	Myelin-associated glycoprotein
Mapt	Microtubule-associated protein tau
miRNA	micro RNA
MN	Motor neurons
MND	Motor neuron disease
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
mTORC1	Mammalian target of rapamycin complex 1
NLS	Nuclear localisation signal
NMDA	N-methyl-D-aspartate (receptor)
n.s.	Not significant (statistically)
NTg	Non-transgenic
PABP	Poly(A)-binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
Ppp3ca	Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform
PRMT1	Protein arginine methyltransferase 1
PSP	Progressive supranuclear palsy
Ptk2b	Protein Tyrosine Kinase 2 Beta
PY-NLS	Proline-tyrosine nuclear localization signal
RNA	Ribonucleic acid
RRM	RNA recognition motif
QGSY rich	Serine, tyrosine, glutamine and glycine rich

qRT-PCR	Quantitative real-time PCR
RGG	Arginine-glycine-glycine rich
sALS	Sporadic ALS
SARFH	Sarcoma-associated RNA-binding fly homologue (drosophila FUS homologue)
SCA	Spinocerebellar ataxia
SEM	Standard error of the mean
SG	Stress granule
SMA	Spinal muscular atrophy
SMN	Survival motor neuron
snRNP	Small nuclear ribonuclear protein
SOD1	Superoxide dismutase 1
TAF-15	TATA-binding protein-associated factor 15
TARDBP	TAR DNA-binding protein (gene)
TDP-43	TAR DNA-binding protein 43 (protein)
TIA1	T-Cell-Restricted Intracellular Antigen-1
TRAF2	TNF receptor-associated factor 2
tRNA	Transfer RNA
Ttc3	Tetratricopeptide repeat domain-containing protein 3
UMN	Upper motor neuron
UPS	Ubiquitin proteasome system
UTR	Untranslated region
UV	Ultraviolet
WB	Western blot
WT	Wild-type
ZBP1	Z-DNA-binding protein 1
*	p<0.05

**

$p < 0.01$

$p < 0.001$

LIST OF FIGURES AND TABLES

Figures

Figure 1.1 FUS and TDP-43 proteins and their functional regions	7
Figure 1.2 Stress granule cycle	25
Figure 3.1 fALS family tree	64
Figure 3.2 Genotyping results from fALS cases	66
Figure 3.3 The creation of pcDNA constructs bearing FUS mutations	72
Figure 3.4 HEK293 cells transfected with FUS pcDNA	79
Figure 3.5 NSC-34 cells transfected with FUS pcDNA.....	83
Figure 3.6 Quantification of cellular phenotype FUS pcDNA-transfected cells.....	85
Figure 3.7 Primary neurons transfected with FUS pcDNA	90
Figure 4.1 Transfection of HEK293 cells with the FUS BAC construct.....	106
Figure 4.2 Confocal images of HEK293 cells transfected with FUS BAC.	109
Figure 4.3 Quantification of cellular phenotype of FUS BAC-transfected cells.....	111
Figure 4.4 SMN in HEK293 cells transfected with the FUS BAC construct.....	113
Figure 4.5 Primary motor neurons from the FUS BAC mouse model.....	114
Figure 4.6 mCherry in FUS BAC primary motor neurons	119
Figure 4.7 FUS distribution in FUS BAC primary motor neurons	124
Figure 4.8 EWS and TAF15 in FUS BAC primary motor neurons	126
Figure 4.9 Stress granules in FUS BAC primary motor neurons	130
Figure 4.10 mCherry and SGs in FUS BAC primary motor neurons	136
Figure 4.11 SMN in FUS BAC primary motor neurons	139

Figure 4.12 Primary cortical neurons from the FUS BAC mouse model ..	144
Figure 4.13 mCherry in FUS BAC primary cortical neurons	145
Figure 4.14 mCherry and SGs in FUS BAC primary cortical neurons	148
Figure 4.15 Stress granules in FUS BAC primary cortical neurons	151
Figure 4.16 Primary motor neurons from the FUS knockout mouse model	155
Figure 4.17 Cellular phenotype in the FUS knockout mouse model.....	159
Figure 4.18 Cellular phenotype in the TDP-43 BAC mouse model	166
Figure 5.1 Optimisation of primers for qRT-PCR.....	189
Figure 5.2 mRNA expression in the TDP-43 BAC mouse model	191
Figure 5.3 mRNA expression in the FUS BAC mouse model.....	200
Figure 5.4 Protein expression in the TDP-43 BAC mouse model.....	207
Figure 5.5 Splicing analysis in the TDP-43 BAC mouse model.....	212
Figure 5.6 Splicing analysis in the TDP-43 BAC mouse model.....	230
Figure 7.1 Protein expression in HEK293 cells transfected with FUS BAC	280
Figure 7.2 Nuclear area of FUS BAC primary motor neurons	283
Figure 7.3 mCherry fluorescence in FUS BAC primary neurons	284
Figure 7.4 PABP and TIA1 fluorescence in FUS BAC primary neurons...	286
Figure 7.5 TIA1 in FUS BAC primary motor neurons	287
Figure 7.6 mCherry and TIA1 in FUS BAC primary motor neurons.....	288
Figure 7.7 PABP and TIA1 fluorescence in FUS knockout motor neurons	290
Figure 7.8 mRNA expression by line in the FUS BAC mouse model	293
Figure 7.9 Protein expression in the TDP-43 BAC mouse model.....	299
Figure 7.10 Splicing analysis by line in the FUS BAC mouse model.....	302
Figure 7.11 Overview of BAC transgenic TDP-43 mouse model.....	310

Tables

Table 1.1 Genes implicated in ALS	6
Table 3.1 Published fALS cases due to FUS truncation mutations	93
Table 5.1 The effects of the M337V TDP-43 mutation on mRNA levels...	190
Table 5.2 The effects of the P525L FUS mutation on mRNA levels	199
Table 5.3 The effects of the M337V TDP-43 mutation on splicing	211
Table 5.4 The effects of the P525L FUS mutation on splicing.....	229
Table 7.1 Primers used in this study	277
Table 7.2 Antibodies used in this study	279
Table 7.3 Mouse lines used in this study.....	282
Table 7.4 FUS BAC transgenic mice used for RNA analysis	292

CHAPTER 1: INTRODUCTION

1.1 Motor Neuron Diseases

1.1.1 Amyotrophic lateral sclerosis

Motor neuron disease (MND) is a neurodegenerative disease of motor neurons. The most common form of MND is amyotrophic lateral sclerosis (ALS) which comprises both upper and lower motor neuron features. Other forms of MND include primary lateral sclerosis and progressive muscular atrophy, which describe upper and lower motor neuron-specific conditions respectively. Progressive bulbar palsy is used to describe a variant affecting only the bulbar region. ALS is the most common, and even when patients have predominantly upper or lower motor neuron features at presentation, both elements often emerge as the disease progresses.

The incidence of ALS is 2-3 cases per 100,000 person years, with a male predominance of 54-61%. The mean age of onset is between 58 and 65 years with a peak at 65-79 years, though it can occur at almost any age (1-4). Juvenile ALS is usually used to describe disease onset under 25 years of age (5). Limb onset is the most common presentation, comprising about 60% of cases, with bulbar onset, involving the muscles of speech and swallowing, in about 30-35% (3, 6). The remaining cases may present with respiratory onset, or a more generalised picture. Diagnosis is made clinically, though nerve conduction studies and EMG can be used to support the diagnosis, and imaging studies may be used to rule out other pathology. The disease is progressive, and multiple regions usually become involved. In addition, about half of patients with ALS develop detectable cognitive deficits, with 15% fulfilling the criteria for frontotemporal dementia (FTD) (7-9). Median survival is 2.5-3 years from onset (10, 11). Death may be caused by a

combination of factors including respiratory insufficiency, general wasting and immobility, and difficulty in swallowing with increased risk of respiratory infection.

Supportive treatment is the mainstay of care for patients with ALS, ideally carried out in a multi-disciplinary setting (12). For example, percutaneous endoscopic feeding can allow those with swallowing difficulties to obtain their nutritional requirements, and non-invasive ventilation can ease symptoms of respiratory involvement. Aids to assist communication and mobility are also important. The only drug shown to improve survival is riluzole, which increases the median tracheostomy-free survival by 2-3 months (13).

Pathologically, ALS is characterised by neuronal loss, particularly of both upper and lower motor neurons (14). Ubiquitinated cytoplasmic inclusions are visible in the motor neurons that remain. In the majority of sALS and fALS, the main constituent protein within these aggregates is TAR DNA-binding protein 43 (TDP-43), which has been found to be ubiquitinated, hyper-phosphorylated, and cleaved to generate C-terminal fragments (15). This pathology has been described in widespread regions of the central nervous system including neurons and glia of the spinal cord, frontal and temporal cortices, hippocampus, amygdala and brainstem (16, 17). TDP-43 pathology is absent in the presence of some gene mutations, which will be discussed in the next section. Clinical and pathological studies have also suggested that the disease starts focally and then spreads (14, 18-20). This has implications for our understanding of the pathophysiology of disease.

There is a pathological overlap with FTD. In ALS, pathology may be found in many areas of the brain including frontal and temporal cortices. Ubiquitinated TDP-43 pathology is found in about 50% of FTD cases and about 5% of cases have FUS pathology (21, 22). Most of the remaining cases have tau pathology (21, 23).

ALS has often been classified into sporadic (sALS) and familial (fALS). It is worth noting that distinguishing between the two does not necessarily reflect the presence or absence of a disease-causing genetic mutation. Patients with apparently sporadic disease may have *de novo* mutations, and issues of non-paternity and variable penetrance can mask a genetic component. However, the study of fALS has led to the discovery of a large number of disease-causing mutations, which will be discussed in the next section. Environmental factors have been proposed as risk factors for disease, including physical fitness, agricultural activity, head injury, pesticide exposure, electrical injury and smoking (24-27). However, a causative link has not been established. It is thought that sporadic disease is likely to be caused by a combination of complex genetic risk factors along with environmental contributors such as ageing.

1.1.2 Spinal muscular atrophy

Spinal muscular atrophy (SMA) is an inherited disease of lower motor neuron degeneration caused by mutations in the survival motor neuron (*SMN*) gene. Humans possess an *SMN1* gene along with an inverted duplication called *SMN2*. *SMN2* differs from *SMN1* by five nucleotides without amino acid changes, and may be present in differing copy numbers. One of these nucleotide changes leads to an increase in exon 7 exclusion which, in the majority of transcripts, leads to a truncated form of the protein which is subsequently degraded. However, low levels of full length protein are produced by the *SMN2* gene. SMA is caused by homozygous mutations in the *SMN1* gene which prevent protein synthesis (28-30). *SMN* protein is therefore only produced by the *SMN2* gene, and disease severity is roughly proportional to *SMN2* copy number and hence the quantity of functional *SMN* (31).

Disease severity varies considerably, with the most severe presenting at birth with weakness, hypotonia, areflexia and respiratory compromise, and the less severe presenting in later life with progressive proximal weakness but a normal life expectancy. Incidence is calculated between 1 in 10,000 and 1 in 25,000, depending on whether predictions are based on clinical presentation or allele frequency (32, 33). Treatment is generally supportive, including respiratory, mobility and nutritional input. Research is focused on treatments such as genetic therapies to increase exon 7 inclusion.

1.2 ALS genetics

1.2.1 Overview of ALS genetics

The majority of ALS is sporadic, but 5-10% of patients have a family history of the condition (1-3, 6). Many genes containing ALS-causing mutations have now been identified, and the main ones are listed in table 1.1 (34-46). Other genes have been reported in rare cases, often causing neurodegenerative syndromes that can resemble ALS (reviewed in (47)). The function of the proteins encoded by these genes have a tendency to fall under one of several categories including RNA metabolism, the ubiquitin-proteasome pathway and proteins involved in vesicular transport or cytoskeletal function. This has led to the speculation that disruption of these processes may play an important role in the initiation of ALS at the cellular level.

The first causative gene to be identified was the gene encoding superoxide dismutase (*SOD1*), which contributes to about 20% of fALS (34, 36). Most *SOD1* mutations are missense, and demonstrate autosomal dominant inheritance (34, 36,

48). Neuropathology of SOD1 ALS demonstrates SOD1 positive inclusions with the notable absence of TDP-43 (17). The discovery of this mutation led to an explosion of research into the pathophysiology of disease, including the development of several mouse models. Many potential mechanisms of cellular dysfunction have been found in these models including excitotoxicity, protein misfolding and mitochondrial dysfunction. However, it is not clear how closely these models resemble human disease, and it has proved difficult to differentiate causative mechanisms from secondary effects of cell toxicity, particularly in overexpression models with a severe clinical phenotype (reviewed in (49)).

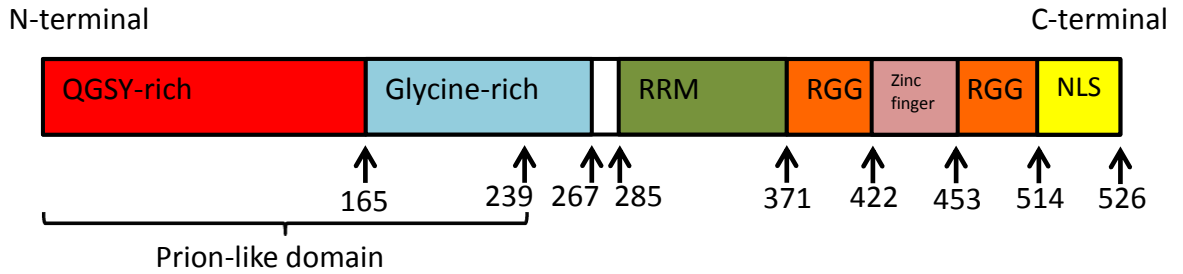
The recently described C9orf72 mutation accounts for the largest known proportion of fALS (60%) and is also present in about 7% of patients with sALS (35). Patients with this dominant mutation have multiple copies of a GGGGCC hexanucleotide repeat located between the first two non-coding exons of the C9orf72 gene (50, 51). Neuropathological studies in these cases demonstrate typical TDP-43 pathology. Inclusions negative for TDP-43 but ubiquitin and p62-positive, are also found particularly in the hippocampus, cerebellum, and frontal and temporal cortices. These contain dipeptides produced by repeat-associated non ATG-initiated (RAN) translation (52, 53). Potential triggers for cell toxicity include C9orf72 haploinsufficiency, nuclear RNA foci, RAN translation products, or a combination of these processes (reviewed in (54)).

This study focuses on RNA binding proteins in ALS, particularly FUS. The fact that several RNA binding proteins, which have structural and functional similarities, have been implicated in ALS, provides an interesting insight into the pathophysiology of disease.

Gene	Protein	Frequency	Function	References
C9orf72	C9orf72	40% fALS, 1% sALS	RNA interaction?	Majounie 2012
SOD1	SOD1	20% fALS, 1% sALS	Superoxide metabolism	Cudkowicz 1997 Chio 2008, 2012
TARDBP	TDP-43	4% fALS, <1% sALS	RNA binding protein	Rutherford 2008 Guerreiro 2008 Chio 2012
FUS	FUS	4% fALS, <1% sALS	RNA binding protein	Kwiatkowski 2009 Chio 2012
SQSTM1	p62	1-2% fALS, 1-4% sALS	Ubiquitin binding	Fecto 2011 Yang 2015
VCP	VCP	1-2% fALS, <1% sALS	Ubiquitin/proteasome	Johnson 2010
PFN1	Profilin 2	1-2% fALS, <1% sALS	Actin binding	Wu 2012 Lattante 2013
OPTN	Optineurin	<1% fALS, <1% sALS	Membrane trafficking	Chio 2012 Maruyama 2010
ANG	Angiogenin	<1% fALS, <1% sALS	Angiogenesis	Chio 2012
UBQLN2	Ubiquilin 2	<1% fALS, <1% sALS	Ubiquitin/proteasome	Daoud 2012

Table 1.1. Table describing the main genes implicated in ALS.

FUS



TDP-43

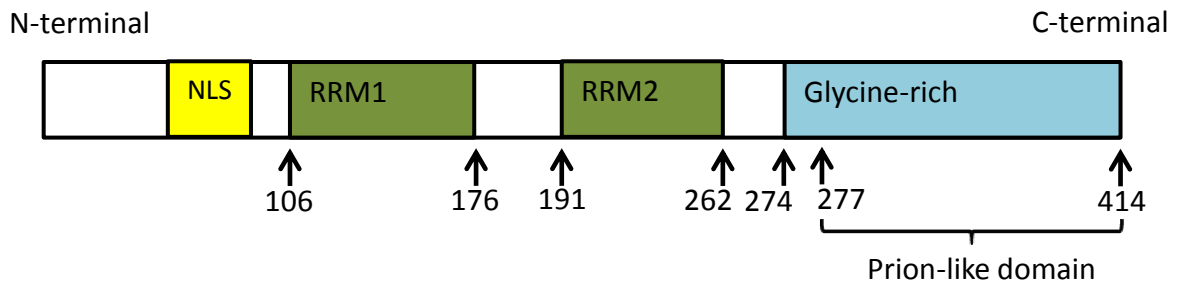


Figure 1.1. A diagram showing FUS and TDP-43 proteins and their functional regions. (QGSY= region enriched in glutamine, glycine, serine and tyrosine residues, RRM=RNA recognition motif, RGG=region rich in arginine/glycine/glycine repeats, NLS=nuclear localisation signal)

1.2.2. *FUS* mutations in ALS

The human *FUS* (fused in sarcoma) gene lies on chromosome 16p11.2 and is also sometimes known as TLS (translocated in liposarcoma). The *FUS* gene is 11.6 kb in length, containing 15 exons which code for a 526 amino acid protein of 75 kDa (figure 1.1). The N-terminal region from amino acids 1-165 is rich in serine, tyrosine, glutamine and glycine residues (QGSY rich), while a glycine-rich region can be found from amino acids 166-267. Most of this region represents a prion domain, which resides between amino acids 1 and 239. An RNA-recognition motif (RRM) is present from amino acids 285 to 371, followed by an arginine and glycine (RGG) rich region to the end of the C-terminal. Within this RGG region is a zinc finger domain between amino acids 422 and 453 (55, 56). A PY-nuclear localisation signal (PY-NLS) is present at the C-terminal (57).

The QGSY-rich region is important in *FUS* aggregation and hence in its function in chromatin binding and activation of transcription in the nucleus (58). Glycine-rich regions often have roles in protein and RNA interaction, for example by forming specific structures upon RNA binding to create target specificity (59). The precise role of this region in *FUS* has not been fully explored, but it has been shown to have a role in recruitment of *FUS* into stress granules (60). The prion-like domain as a whole has a more well defined role in *FUS* aggregation, both as a physiological property, for example around sites of DNA damage to promote repair, and also as a mechanism of toxicity (58, 61, 62).

The RRM has traditionally played a role in RNA binding, and there is some evidence that RNA and DNA binding occurs in this region of *FUS* (63). However, other studies

have found to that this region is not essential for RNA binding (64, 65). This region also appears to play a role in recruitment into stress granules (60).

The RGG domains have been implicated in a range of functions including RNA binding, recruitment to sites of DNA damage and to stress granules, and the modulation of telomere length by binding G-quadruplexes (60, 62, 65-67). In addition, they are required for FUS aggregation and toxicity (61). The zinc finger domain within this region is also involved in RNA binding and recruitment into stress granules, with a role in RNA recognition of specific G-rich sequences (60, 65).

Amino acids 498 to the C-terminal 526 is a predicted PY-NLS, and is required for effective nuclear import of FUS (68-70). Further detail about this aspect of FUS is discussed in the next section.

Mutations in the gene encoding FUS are responsible for around 4% of fALS and <1% sALS. Most mutations are missense. Although most cases of fALS are inherited in an autosomal dominant manner, one family from the Cape Verde Islands demonstrated an autosomal recessive pattern (40). Most *FUS* mutations are clustered in the glycine-rich region and the NLS. *FUS* mutations cause ALS with wide phenotypic variation which is often indistinguishable from sALS. However, *FUS* mutations cause ALS with a lower than average age of onset of 46 years, and account for a relatively high proportion of juvenile and aggressive disease (40, 71-76). These severe forms of disease are often caused by specific mutations such as P525L and truncation mutations. These are less likely to be inherited due to the young age at onset, and are more likely to have occurred *de novo* (77, 78).

Patients with *FUS* mutations display different neuropathological features compared with other forms of the disease. Neuronal cytoplasmic inclusions contain the FUS

protein rather than TDP-43. There is no evidence of post-translational modification of FUS, such as phosphorylation, ubiquitination, or the formation of fragments (79, 80). In addition, some young-onset forms of the disease display FUS positive basophilic inclusions (81). Patients with ALS and FUS pathology almost always have *FUS* mutations, and reported exceptions may be due to a failure of detection using the usual techniques (82). It is worth noting that FUS pathology is found in a subgroup of patients with FTD. However, in the majority of cases of FUS-FTD there is no mutation present (83, 84). Indeed, FTD-FUS may also be distinguished from ALS-FUS in that other FET proteins (EWS and TAF15) are present in the neuronal inclusions in FTD-FUS but not ALS-FUS (85, 86).

1.2.3 *TARDBP* mutations in ALS

The TAR DNA-binding protein (*TARDBP*) gene which encodes TDP-43 is located on chromosome 1p36.22 and contains 6 exons coding for 414 amino acids (figure 1.1). The main transcript is produced from exons 2-6, producing a 43 kDa protein (87). It contains 2 RNA-recognition motif (RRM) regions; the RRM1 region appears to play the largest role in DNA and RNA binding and the functions of TDP-43 associated with this (88, 89). Although the RRM2 does not appear to directly bind nucleic acids, it plays a role in complex formation and alternative splicing (88-90). The C-terminal consists of a glycine-rich region which plays a major role in protein interactions, though also affects cellular localization and splicing regulation and assists in RNA binding (59, 91-93). The C-terminal is highly conserved in different species, suggesting an important role. There is a predicted prion domain between amino acids 277 and 414 at the C-terminal, encompassing most of the glycine rich region (94).

Mutations in *TARDBP* have been found in about 4% of fALS and <1% of sALS (57). They are all inherited in an autosomal dominant manner, and most mutations are missense. They are clustered in the region of the gene encoding the glycine-rich domain at the C-terminal end of the protein (61).

1.2.4 *EWS* and *TAF15* mutations in ALS

FUS is a member of the FET family of proteins, comprising FUS, EWS (Ewing's sarcoma protein) and TAF15 (TATA-associated binding factor 15). These are all RNA-binding proteins and share structural and functional features.

The EWS protein is encoded by the *EWS* gene on chromosome 22. Like FUS, the N-terminal consists of a QGSY domain. There are three RGG recognition motifs, a zinc finger domain and an RRM, regions involved in RNA binding (95). The gene encoding *TAF15* is found on chromosome 17, encoding a protein comprising 16 exons. It has a similar structure to the other FET proteins, with a QGSY domain at the N-terminal, and RNA binding domains consisting of three RGG domains, an RRM and zinc finger domain (96). EWS and TAF15 both contain a prion-like domain at the N-terminal and a PY-motif at the C-terminal which can function as a NLS (97-99).

EWS and *TAF15* variants have been found to be present in ALS patients and absent in healthy controls. These mutant proteins appear to share *in vitro* characteristics with mutant FUS, such as cytoplasmic aggregation (97, 100). It has not been proven that mutations in these genes cause ALS, but they may contribute to the pathophysiology of this disease.

1.3 Functions of the key proteins

1.3.1 FUS

Distribution

FUS is an RNA-binding protein, and it carries out many functions associated with this. It is almost ubiquitously expressed and has a predominantly nuclear localisation, though it can shuttle between the nucleus and cytoplasm (64, 101). Nuclear import occurs via a transportin-mediated mechanism, requiring the C-terminal PY-NLS and this process is modified by the adjacent RGG region (69, 102). The methylation status of FUS, catalysed at least in part by protein arginine methyltransferase 1 (PRMT1), affects the cellular shuttling and distribution of FUS as well as altering its effect on transcription (103-105).

Protein binding

FUS has been found to bind to between 35 and 126 proteins, particularly those involved in RNA processing such as RNA-binding proteins and splicing factors (106-108). Differences in the protein interactome in different studies are likely to reflect differences in the methodologies and cell types used.

Autoregulation

FUS is able to bind to its pre-mRNA in the region of exon 7. This leads to an increase in exon 7 skipping, which causes a premature stop codon in exon 8 and results in nonsense-mediated decay. In this way it is able to autoregulate its own protein levels (109). It is likely that levels of FUS are tightly controlled, and under or over-

expression can have significant effects (110). It has been suggested that autoregulatory mechanism may cross-react between FET proteins as overexpression of the *Drosophila* FUS homologue SARFH (sarcoma-associated RNA-binding fly homologue) appears to reduce FUS and EWS levels, though further clarification is required (111).

Transcription

Regulation of transcription is an increasingly well-characterised role of FUS. Initial studies found that FUS binds to other transcription factors, for example transcription factor IID, PRMT, Spi-1, TDP-43, beta catenin, and nuclear factor kB (NF-kB), modifying expression of their targets (103, 112-116). FUS appears to bind single stranded DNA in the promoter region at transcription start sites, interacting with RNA polymerase II (117, 118). Potential binding motifs include TCCCCGT, AAAGTGTC and AGGTTCTA (118). Chromatin immunoprecipitation detected FUS targets on 48 or 1161 promoter regions, depending on statistical threshold used, with target genes including those involved in the cell cycle, gene expression, and neuronal functions (118).

RNA binding targets

Given that FUS has important roles in RNA processing, defects in the way mutant FUS interacts with particular mRNA species could contribute to the pathophysiology of ALS. It is therefore of interest to look at the specific RNA binding targets of FUS. Several studies have used crosslinkage and immunoprecipitation (CLIP-seq)

techniques in both mouse and human cells to identify mRNAs that interact with FUS and to detect specific binding points.

Around 40-45,000 binding sites of FUS have been identified in mRNA from between 5-7,000 genes (119, 120). These are largely intronic, particularly in long introns, though mutant FUS appears to have fewer intronic targets in HEK293 cells (119-121). Specific sites include highly conserved introns, at the beginning of long introns, and clustered around alternatively spliced introns (119, 121, 122). Experiments involving knockdown of FUS reported alterations in transcript levels and splicing, though this varies with the model used (117, 119, 121). There have not been consistent reports of a target motif, though experiments in both mouse and human brain have proposed that either GGUG, GGU, or GUGGU motifs are enriched in FUS mRNA targets (119, 121, 123, 124). It has also been suggested that FUS binds a specific structure rather than sequence, for example an AU-rich stem loop (120).

Specific targets include mRNAs of other RNA binding proteins, proteins involved in splicing, cell adhesion, and neuronal development and projection (121, 124). It commonly binds to the 3' UTR of proteins involved in neuronal projections and synapses, suggesting a role in transport of the mRNA and perhaps local translation at the synapse itself (119, 125). Some of these targets appear to be involved in neurological disorders or neurodegeneration, for example Mapt, Fmr1, SOD1, UBQLN1/2 and neurofilament proteins (119, 122, 124).

Splicing

FUS is also involved in the regulation of alternative splicing. It is part of the spliceosome machinery and interacts with other splicing factors such as SR proteins

(126-128). Several early studies have shown it to modify alternative splicing of the E1A reporter (113, 115, 123, 128). Larger studies investigating the RNA binding targets of FUS have detected between 116 and over 1000 altered splicing events after FUS knockdown, depending on the model used (119, 121, 124). Targets include those implicated in neuronal processes and neurodegeneration such as Ndrp2, MAPT, Mag and Fmr1 (119, 122, 129). The autoregulation of FUS is also mediated via alternative splicing of its own exon 7 (109).

DNA repair

FUS has an important role in DNA repair. It promotes homologous recombination and the formation of D loops as an essential part of this process, as well as non-homologous end joining (62, 130-132). There is evidence of FUS phosphorylation in response to double stranded DNA breaks, as well as interaction with poly (ADP-ribose) polymerase and histone deacetylase 1, both of which are recruited along with FUS to sites of DNA damage (62, 133-135). FUS knockdown or knockout causes defects in DNA damage repair (62, 133, 136). The FUS C-terminal RGG domain can form a complex with G-quadruplexes at the telomere, interacting with telomeric DNA and RNA and regulate telomere length (67, 137-139).

RNA transport

There is evidence to suggest that FUS is involved in the transport of mRNA and local translation, a process that is particularly important in neurons due to their long processes. It interacts with transport machinery of the cell such as myosin Va, myosin VI and the actin stabilising protein Nd1-L (125, 140-142). It is a constituent of RNA granules, structures that transport mRNA to sites of local translation and which

contain mRNA and associated binding proteins (125, 143-145). Live imaging has shown that FUS can move bidirectionally in dendrites, which requires actin filaments, microtubules and myosin Va, and movement into dendrites is stimulated by glutamate receptor activation (125, 140, 146). This stimulus also increases RNA in the dendrites, supporting the theory that FUS transports mRNA (125). FUS enhances translation, which may be an important step in local protein synthesis in distal neuronal projections (143). Indeed, FUS is often present at the tip of dendritic spines and there is evidence of its involvement in the post-synaptic machinery and NMDA receptor complex (141, 144, 146, 147). FUS knockout neurons have abnormal dendritic spine morphology, implicating FUS in dendritic assembly either directly, via its association with structural components such as actin, or indirectly through transport and translation of other mRNAs important in dendritic organisation (146).

Stress response

FUS has been implicated in cellular stress response. Most studies suggest that wild-type FUS is not found in stress granules after common forms of stress, such as oxidative stress (70, 102, 148). However, it appears that hyperosmolar stress can be an exception and cause redistribution of wild-type FUS from the nucleus to the cytoplasm where it is incorporated into stress granules, improving cell survival compared to FUS knockdown (101, 104). Mutant FUS is much more strongly implicated in stress granule dynamics, which will be discussed in more detail in the next section.

miRNA

There is evidence that FUS is involved in miRNA processing, as it is part of the Drosha complex and can bind to pri-miRNAs (149, 150).

1.3.2 TDP-43

TDP-43, like FUS, is an RNA-binding protein and therefore has many functional similarities to FUS. The 43 kDa TDP-43 is the most common isoform translated from *TARDBP* mRNA, though up to ten further isoforms have been detected in mammalian cells (87, 151). Like FUS, it is found predominantly in the nucleus but is able to shuttle between the nucleus and cytoplasm (90, 93, 152, 153). TDP-43 autoregulation was suggested initially by the fact that heterozygote knockout animal models were reported to have no significant decrease in mRNA nor protein level compared to those with two copies of the *TARDBP* gene (154, 155). *In vitro* experiments have verified the presence of autoregulation. This has shown that TDP-43 binds to the 3'UTR of its own transcript leading to an increased rate of decay, which may involve the exosomal pathway (156, 157). The most likely mechanism appears to be that increased levels of TDP-43 cause splicing of intron 7 in the 3' UTR, creating a transcript with an alternative poly-A site that remains predominantly nuclear and is therefore not translated (158).

DNA and RNA binding

TDP-43 has been shown to bind to DNA and RNA and affect gene expression and alternative splicing. The initial description of TDP-43 was due to its ability to bind the regulatory element TAR DNA of HIV-1, causing HIV-1 transcriptional repression

(159). Other relevant RNA targets include neurofilament, where it binds to the 3'UTR and stabilises the mRNA, and HDAC6 where it maintains levels of this autophagy-associated protein (90, 153).

TDP-43 can regulate splicing by promoting both exon exclusion and inclusion. For example, it can bind to the 3'UTR of the cystic fibrosis transmembrane conductance regulator (CFTR) mRNA, causing exon 9 skipping, and the apolipoprotein A-II mRNA promoting the exclusion of exon 3 (160, 161). It also interacts with SMN transcripts and can promote exon inclusion (89).

Larger cross-linkage, immunoprecipitation and sequencing studies have been carried out in a variety of cell types. In human brain, 3.7 million RNA targets of TDP-43 were identified, with clusters in 7,499 protein-coding genes and the 3'UTR of 1,172 genes (162). In mice, a similar number of genes were targeted, 7,951, and knockdown of TDP-43 caused upregulation of 471 mRNAs and downregulation of 283, demonstrating its role in transcription regulation (119, 157). Interestingly, there appears to be a relatively small overlap with genes targeted by FUS of 2,727, and corresponding up- and down-regulation were seen in only 41 and 45 genes respectively (119). In NSC-34 cells, although mRNA from only 204 genes was found to be bound to TDP-43, functional effects could be seen. For example, the 3'UTR of progranulin mRNA is one such target, and showed increased mRNA degradation and reduced protein levels with increased levels of TDP-43 (163). In HEK cells, expression of 402 genes was altered after TDP-43 knockdown (90). These results demonstrate the ability of TDP-43 to bind to RNA with functional effects, though target detection is variable depending on the cell type or organism studied.

TDP-43 also has widespread effects on splicing. Analysis of SH-SY5Y cells after TDP-43 knockdown revealed 229 altered splicing events, many of which were in

genes involved in neuronal development (162). In mice, TDP-43 knockdown caused 788 splicing changes compared with 374 changes seen after FUS knockdown, with an overlap of only 83 events (119). This again suggests that TDP-43 and FUS tend to have different targets.

GU repeats have been consistently reported to be the favoured mRNA binding targets of TDP-43, with around 80% of 3'UTR targets containing this motif in NSC-43 cells (157, 160-163). Most targets are nuclear, and this is reflected in the fact that most are in introns. Interestingly, binding is often found in introns and the 3'UTR of the same mRNA, suggesting a role in mRNA transport and subsequent local translational processing (162). Targets of TDP-43 include those important in neuronal differentiation and dendrite development (119, 157).

Protein binding

TDP-43 interacts with other RNA binding proteins such as hnRNP A1, which involves the glycine rich region of TDP-43 near the C-terminal (91, 164). More widespread analysis of TDP-43 binding partners in cell lines detected between 52 and 261 proteins that immunoprecipitated with TDP-43, though the larger number may reflect contamination (165, 166). The most common interactors are proteins such as hnRNPs and other RNA binding proteins, reflecting the role of TDP-43 in RNA processing (165, 166). Some studies suggest that TDP-43 and FUS interact, particularly in the nucleus. This suggests that they might share some common pathways, for example they each bind to histone deacetylase 6 mRNA and alter expression levels (108, 114, 166).

mRNA transport

Like FUS, there is evidence that TDP-43 is involved in mRNA transport and local translation. It has been shown to be transported in axons of primary motor neurons, respond to the neurotrophic factor BDNF, and affect axon outgrowth (167). In the dendrites of rat hippocampal neurons, TDP-43 was found in granules displaying features of RNA transport granules, increasing after depolarisation (168). These features, along with the fact that TDP-43 promotes dendritic branching in drosophila, suggest that TDP-43 plays a role in mRNA transport and local translation in neuronal processes (169).

Stress response

There are varying reports about whether TDP-43 can become associated with stress granules, but some experiments suggest that wild-type TDP-43, particularly if overexpressed, can become associated with stress granules (60, 170-172). This requires the RRM1 and a section of the glycine rich region at the C-terminal (60, 172).

Some reports did not find any alterations in stress granule dynamics after TDP-43 knockdown, for example no change was observed in the percentage of cells with stress granules (170). Others have reported that TDP-43 knockdown causes delayed stress granule assembly, a reduction in the percentage of cells with stress granules, and a reduction in stress granule size. During the resolution of stress, reduced numbers and increased size of stress granules was also observed (171, 173). Cell survival has been reported as both normal and reduced in TDP-43 knockdown after

cell stress (171, 172). These differences are likely to reflect different cell types, levels of knockdown and stress granule markers used.

miRNA

TDP-43 has also been implicated in micro RNA (miRNA) synthesis. It interacts with both the Drosha and Dicer complexes involved in miRNA synthesis, and can bind to certain primary miRNAs (174). Depletion of TDP-43 can cause an increase or decrease in the levels of certain miRNAs, and may in part explain the defects seen in neurite outgrowth in TDP-43 knockdown (174, 175).

1.3.3 EWS and TAF15

EWS is a member of the FET family of proteins along with FUS and TAF15. EWS is ubiquitously expressed and nuclear in distribution (101). There is evidence of a role in homologous DNA pairing and DNA damage repair, with knockout mice displaying defects in B lymphocyte development, meiosis, and radiation sensitivity (176, 177). EWS is involved in transcriptional regulation, in part by binding to important parts of the transcription machinery such as transcription factor IID and RNA polymerase II (178). Indeed, knockdown of EWS altered the expression of 407 genes, and has been shown to enhance transcription of hepatocyte nuclear factor, probably acting as a co-activator with its association with CREB-binding protein (179, 180). EWS is able to bind G-quadruplex RNA via the terminal RGG motif, and in HEK293 cells it was found to bind to 19020 RNA clusters (120, 181). The function of EWS in splicing is suggested by its association with the splicing complex (182). The effect of EWS on splicing has been demonstrated by the alteration of cyclin D1 isoforms (183). It is

likely to have a role in microRNA processing as it is part of the DROSHA complex, involved in microRNA synthesis (149). Together these functions of EWS are closely related to those of FUS.

TAF15 is the final member of the FET family. It has a similar cellular distribution to the other FET proteins, being predominantly located in the nucleus (101). It is able to bind both DNA and RNA (184) and it interacts with other proteins, including those involved in transcriptional regulation and miRNA synthesis (116, 149). Like other FET proteins, it has a role in transcriptional regulation. In HEK293 cells, knockdown of TAF15 resulted in the differential expression of 293 genes, but interestingly only 21 of these overlapped with both EWS and FUS (180). Its role in RNA processing has also been demonstrated by its association with cytoplasmic RNA granules (99).

1.3.4 SMN

SMN is a ubiquitous protein found localised in the cytoplasm and in discrete nuclear puncta known as gems (185, 186). The SMN protein is a core component of the SMN complex which consists of SMN bound to the geminin proteins 2-8 (187-193). This complex is able to bind to the small nuclear RNAs and proteins that make up the small nuclear ribonuclear proteins (snRNPS) U1, U2, U5 and U4/6 (187, 194). These subsequently form the spliceosome responsible for splicing of pre-mRNA (195). SMN deficiency therefore has significant effects on both gene expression and splicing patterns, for example in motor neurons of a mouse model of SMA and human iPSC-derived motor neurons (196, 197).

In addition to this core function, SMN appears to play other roles in the cell. It binds to other proteins including RNA binding proteins such as FUS and TDP-43 (198). It also appears to be important in the formation of stress granules, and ability of cells to survive cell stress (199).

Given the fact that SMN deficiency is severely detrimental to motor neurons, causing the disease SMA, studies have looked at specific roles of SMA in motor neurons. It has been suggested that splicing alterations in motor neurons are greater than in other cells in an SMA mouse model (197). SMN is transported along axons and is important in neurite outgrowth, potentially by transporting β -actin mRNA to growth cones (200-202). This implies a function in mRNA transport and local translation, which may be a reason why motor neurons are particularly vulnerable in SMA.

1.4 Stress Granules

1.4.1 Introduction to stress granules

Stress granules are cytoplasmic ribonucleoprotein (RNP) structures that form when a cell is under stress, and their formation is a normal physiological process. A variety of triggers can lead to this response, for example oxidative stress, heat shock and ER stress. Under these conditions, cells focus their resources on synthesising stress response proteins such as heat shock protein which can help the cell to survive the insult (203). The translation of proteins that are not essential for survival is halted and the mRNAs of these proteins are temporarily stored in cytoplasmic structures known as stress granules during the period of cell stress. These granules are active and highly organised to allow sorting of the mRNA to occur, so that when the stress is resolved the cell can rapidly resume normal translation (204).

1.4.2 The stress granule cycle

Stress granule formation is commonly via initiation by a eukaryotic initiation factor kinase (eIF2 α kinase). In the presence of a trigger for cell stress, the eIF2 α kinase causes phosphorylation of eIF2 α at serine residue 51 (205). This reduces the amount of a complex containing eIF2 α and tRNAs that is required for initiation of capped mRNAs, thereby reducing translation of these mRNA species (206). mRNAs with alternative initiation sites are protected from this process and therefore translation can continue. This enables proteins required for protecting the cell during stress, such as heat shock proteins, to be spared from translational repression (203). Stress granules can also form in an eIF2 α -independent manner in the presence of certain triggers such as hippuristanol and pateamine A (207). This mechanism blocks translation by inhibiting eIF4A helicase. The presence of free mRNAs initiates the primary aggregation stage of stress granule formation, also requiring proteins such as G3BP and TIA-1 (204). A secondary aggregation stage involves the recruitment of more proteins such as PABP-1, followed by a tertiary recruitment stage where non-RNA binding proteins are incorporated such as TRAF2 and PMR1 (208, 209). Stress granules then enter an mRNA triage stage involving the selection of mRNAs for storage, export or decay. This is regulated by proteins such as ZBP1 (210). When the insult to the cell is over, stress granules disassemble and normal translation can resume (figure 1.2).

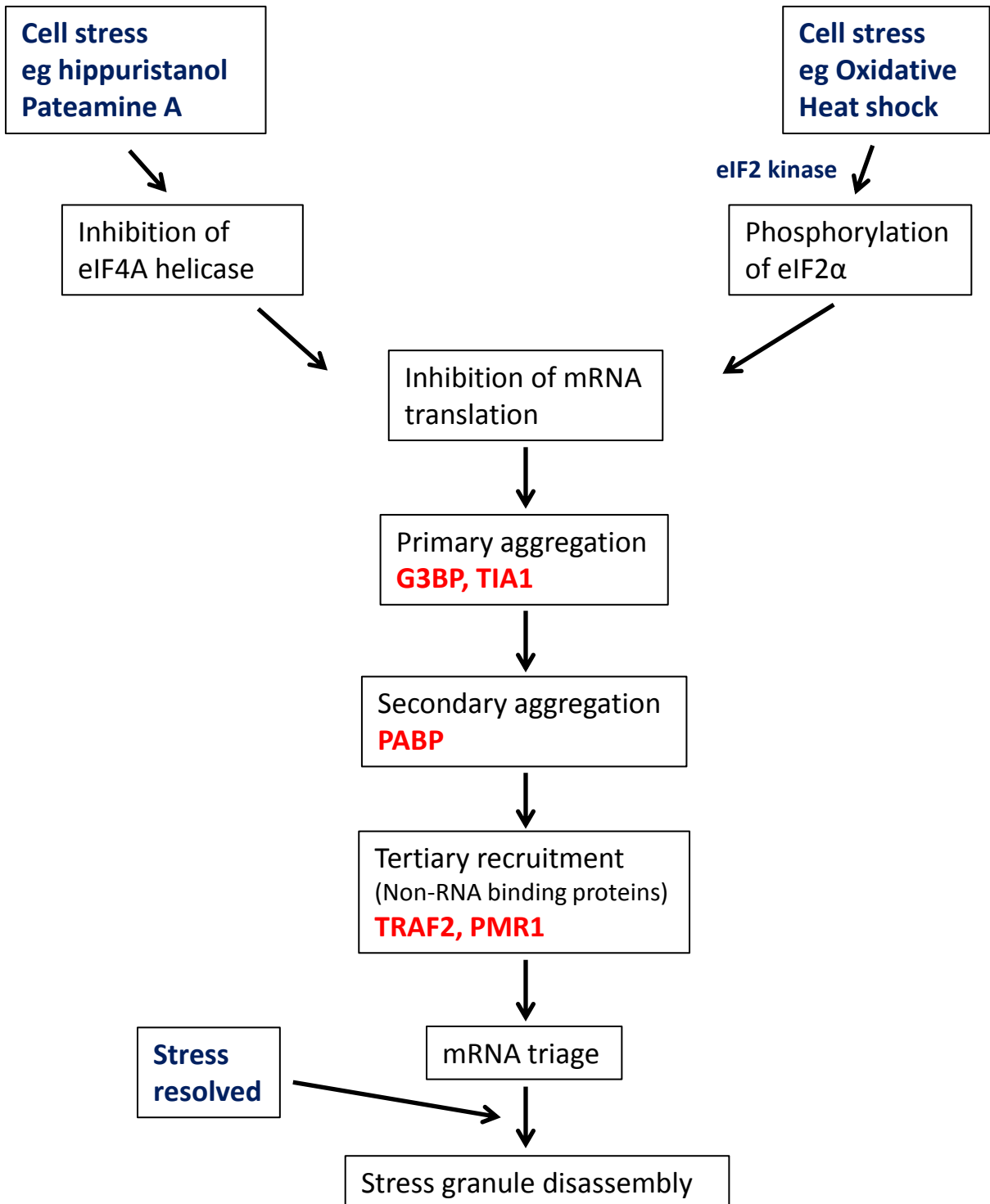


Figure 1.2. A flow chart showing the stress granule cycle from initiation to disassembly. Examples of proteins incorporated at each stage are shown in red (References 204-9).

Several studies have looked at the speed at which cells develop stress granules and subsequently how quickly they disassemble. This may vary with the type of insult and the cell type involved. One study looking at arsenite stress in COS cells demonstrated stress granule formation in about 14 minutes and disassembly in 60-90 minutes (204). Other studies have also suggested that these granules form in 10-15 minutes in both HeLa cells and yeast (211, 212). Studies looking at this *in vitro* may not fully represent what happens *in vivo*. The type of cellular insult is also likely to influence the formation and dissolution of these structures. In addition, the effect on a single cell is likely to depend also on background genetic variations as well as how the cell is associated with neighbouring cells and the surrounding milieu (213).

1.4.3 Types of cell stress

Different types of stress involve different eIF2 α kinase responses. Oxidative stress activates haem-regulated initiation factor 2 α kinase (HRI), which is a method of stressing cells commonly used in this study (214). Heat shock, UV radiation and viral infection trigger protein kinase R (PKR) (215). Endoplasmic reticulum dysfunction activates PRK-like endoplasmic reticulum kinase (PERK) (216). Other eIF2 α kinases include Z-DNA kinase and GCN2 which are triggered by viral infection and amino acid deprivation respectively (217). Each of these triggers lead to eIF2 α phosphorylation and initiation of the stress response.

1.4.4 Stress granule components

Stress granules contain mRNA and other components of polysomes including several eIF kinases and PABP1 (206). Other mRNA binding proteins are also

present, though are not necessarily found in all stress granules. Examples of these proteins include T cell internal antigen-1 (TIA1), TIAR (TIA-1-related) and Ras-GTPase-activating protein SH3-domain-binding protein (G3BP) (204, 218). These proteins are involved in many aspects of RNA processing such as mRNA stability and splicing. There are other proteins found in stress granules that are not linked to RNA processing and are likely to be sequestered into these granules via protein binding, for example PMR1 which binds to TIA1 (209). Interestingly, constituents of stress granules may contribute to cell signalling. The incorporation of pro-apoptotic markers such as TRAF2 into stress granules is likely to have anti-apoptotic effects and the integration of mTORC1 protects the cell from DNA damage (208, 219).

1.4 5 Stress granules and neurodegeneration

The stress response is universal to all cells *in vitro* and *in vivo*, and has been described in prokaryotes as well as eukaryotic cells (220, 221). This process is thought to be protective for cells as it allows the preferential translation of mRNAs that promote survival, and reduces pro-apoptotic factors. However, there are some studies that suggest that this process can be detrimental, especially if it is disrupted. For example, continued activation of eIF2 α caused neurotoxicity in mice (222). Of particular relevance is the link between stress granules and neurodegenerative diseases, where it has been suggested that stress granules play a part in the pathology. Stress granule markers have been found in pathological neuronal inclusions in neurodegenerative diseases such as FTD, Alzheimer's disease, SCA2 and Huntington's disease (60, 223-226). In ALS, TDP-43 and FUS have been shown to co-localise with stress granule markers *in vivo* and *in vitro* (60, 148, 170). This has led to the suggestion that stress granules may be the precursors of these

aggregates, though a direct link has not been proven. It is also possible that proteins aggregate independently of stress granules, but sequester stress granule markers at some point during their formation (223). Either way, stress granules are distinct from pathological aggregates in their dynamic nature and ability to rapidly disperse. The link between ALS-FUS and stress granules will be discussed in further detail later.

1.5 The pathophysiology of ALS

In the last few years, much has been discovered about the genetics of ALS and the normal functions of the proteins involved. However, it is still not entirely clear how mutations in these proteins cause ALS, nor indeed how sporadic disease occurs. This section summarises various aspects of cell biology that are affected by ALS mutations, with a focus on the *FUS* gene. These give an insight into possible mechanisms of pathogenesis of this disease.

1.5.1 Mislocalisation of mutant protein and recruitment into stress granules

As described previously, FUS has a predominantly nuclear distribution and is able to shuttle between the nucleus and cytoplasm. The C-terminal NLS is important in nuclear import. Some forms of mutant FUS, namely those with mutations in the C-terminal, are located to a greater extent in the cytoplasm, with apparent nuclear clearing (40, 71, 148). Interestingly, the degree of mislocalisation appears to correlate with the severity of disease clinically, with particularly marked mislocalisation in the presence of mutations such as P525L-FUS and C-terminal truncations (102). The fact that not all mutations cause mislocalisation suggests that it is not a prerequisite for disease, but the correlation with onset does implicate mislocalisation in the pathophysiology of this condition. For example, a higher

degree of cytoplasmic FUS could increase the likelihood of disease initiation and therefore tend to cause disease at a younger age.

Under conditions of cell stress such as oxidative stress or heat shock, cytoplasmic FUS accumulates in granules in the cytoplasm, which may be mutant FUS, or wild-type FUS with blocked nuclear import (70, 102, 227). It has generally been felt that these are stress granules as they resemble these structures and display the same markers. However, more recent work suggests that there may be some differences between these granules and typical stress granules (143, 228). The RNA binding domain of FUS, the RGG-zinc finger domain, is the most important region for recruitment of FUS into stress granules, though the glycine-rich domain and RRM do contribute (60, 229).

Mislocalisation of FUS and accumulation within stress granules could result in cell toxicity by a loss of function of FUS in the nucleus or gain of toxic function in the cytoplasm, and it may be a combination of both. Evidence to suggest that mutant FUS can sequester wild-type FUS into the cytoplasm and into stress granules, could account for a dominant negative effect (230, 231).

It has been hypothesised that these granules visualised *in vitro* could be the precursors of the large cytoplasmic aggregates seen in patients with ALS. Although there is no direct evidence for this, it is supported by the fact that mutant FUS is found in both structures, and that stress granule markers are present in neuronal aggregates in ALS patients (81, 102, 223). It has been suggested that these FUS-

positive cytoplasmic granules could act as a 'seed' for the development of more robust cytoplasmic aggregates, leading to increasing cellular toxicity.

The presence of mutant FUS appears to alter the dynamics of stress granules themselves, with delayed assembly and larger and more abundant granules (229). There may be a pro-survival role of FUS when present in hyperosmolar-induced granules (104). This adds to the evidence that alterations in stress granules in the presence of mutant FUS may contribute to the pathophysiology of ALS.

The presence of FUS inclusions in ALS-FUS has led to further studies to examine the propensity of FUS to aggregate (58, 61). FUS is aggregation-prone and has the ability to form hydrogels which may have an amyloid-like structure. These could undergo polymeric extension, and even recruit another RNA binding protein (hnRNPA1) (58). The prion-like domain, RRM and first RGG are required for aggregation and toxicity (61). These aggregates are soluble in SDS, making them more reversible than typical prion-like fibres. Despite the fact that *FUS* mutations did not promote aggregation in this study, this characteristic of FUS could be important in the development of cytoplasmic aggregates seen in ALS patients.

It has been suggested that mutant FUS (and indeed other ALS-related proteins) may spread in a prion-like fashion in ALS. Disease initiation would occur in a single cell, which may be at increased risk in the presence of an underlying mutation, but may require several 'hits'. FUS becomes misfolded with a propensity to aggregate. This is supported by the fact that FUS has a prion-like domain and tendency to aggregate. The disease would then spread from the site of onset, which is supported by clinical

and neuropathological studies (18, 19). The mechanism of spread is yet to be elucidated, but there is some evidence of ‘transmissibility’ in the SOD1 mouse model where spinal cord homogenate from paralysed mice induced SOD1 misfolding in susceptible strains (232).

There is evidence that other FET proteins demonstrate similar characteristics to FUS, and could potentially induce disease via similar mechanisms. EWS variants found in ALS patients have been shown to cause mislocalisation of EWS in motor neurons *in vitro* (97). Both EWS and TAF15 have also been shown to accumulate in stress granules, particularly in the presence of mutations in their NLS or with inhibition of transportin-mediated nuclear import (99, 101). In FTD, but not ALS, cytoplasmic aggregates contain these FET proteins (86). In addition to the role of TDP-43 in pathological aggregates in sALS and fALS, there is also evidence that mutations in the *TARDBP* gene cause TDP-43 mislocalisation *in vitro* and accumulation in stress granules (152, 233). This could explain how abnormalities in any of these proteins could lead to motor neuron toxicity via similar pathways.

1.5.2 Protein underexpression or overexpression

Changes in the levels of FUS may contribute to the pathophysiology of ALS, which may also be relevant if changes in FUS distribution cause levels to alter in the nuclear and cytoplasmic compartments. *FUS* mutations in the 3'UTR have been described in ALS patients, which appear to cause FUS overexpression and increased cytoplasmic staining as detected in patient fibroblasts (234). In addition, rodent models of overexpressing wild-type FUS develop neuronal loss, reduced lifespan, and both motor and cognitive phenotypes (110, 235, 236). *In vitro*,

overexpression of FUS alters gene expression, and the fact that mutant FUS has a longer half-life compared to wild-type FUS could lead to increased levels of FUS and changes comparable to overexpression (231, 237).

Reduced levels of FUS can also be detrimental to cells. FUS knockout leads to widespread changes in RNA processing as previously described. FUS knockout in mice results has resulted in defects such as chromosomal instability, and in one model perinatal death (136, 238). The presence of FUS autoregulation supports the fact that levels must be tightly controlled, and this may be disrupted by *FUS* mutations (109).

Autoregulation also occurs with TDP-43, a process that may be disrupted by *TARDBP* mutations (156). TDP-43 knockout is incompatible with life, and knockdown causes widespread effects on RNA processing (157). Overexpression can also cause toxicity (239). Therefore if TDP-43 levels are disturbed in the presence of the mutations, this could contribute to the pathophysiology of ALS.

1.5.3 RNA processing

The effect of altered levels of FUS on a large number of mRNA targets has been described, and may be affected indirectly by *FUS* mutations as a result of altered cellular distribution. It can also be very difficult to distinguish direct effects of the mutation from downstream effects of motor neuron degeneration. This is particularly relevant when looking at patient postmortem samples, when disease is usually advanced. Studies looking at motor neurons from patient post-mortem tissue have reported some changes in transcript and protein levels, for example, one study found

411 aberrantly spliced genes in ALS patients compared to controls, though this could be a downstream effect of cell toxicity and death (119, 240). In a mouse model, RNA-sequencing revealed differential expression of 766 genes and 1814 splicing changes in an R521C mutant compared to wild-type, though at the end stage of disease (231). *In vitro* experiments in HEK293 cells have demonstrated more widespread alterations in gene expression and RNA targets in mutant compared to wild-type FUS, for example wild-type FUS has 78% of its RNA targets in introns, but mutant FUS had 13% intronic and the 61% in 3'UTRs (120, 241). This could reflect the redistribution of mutant FUS to the cytoplasm. It is also worth noting that FUS autoregulation via exon 7 splicing is also disrupted in the presence of *FUS* mutations (109). There is therefore a lot of evidence supporting alterations in RNA expression and splicing in the presence of *FUS* mutations, though it is less clear which of these are direct effects of the mutation and which reflect wider cellular dysfunction.

The role of TDP-43 in RNA processing suggests that mutations in the TARBBP gene could also have a significant effect on transcript levels and splicing. Indeed, when TDP-43 was knocked down in mice, altered levels of 601 mRNAs and 965 altered splicing events were noted (157). Widespread splicing changes were also observed in the cortices of TDP-43 transgenic mice, though there were differences in wild-type and mutant lines compared to non-transgenic (242). RNA targets in the cortices of patients with sporadic FTD with TDP-43 inclusions has also been studied, and demonstrated some alterations in RNA targets of TDP-43, including altered binding to 7 3'UTR and 48 intronic transcripts (162). Again, these results suggest that mutant TDP-43, like FUS, could have an effect on RNA processing. Further studies are

required to determine whether this is an early event in ALS pathogenesis or whether the splicing changes are secondary to more widespread cellular changes.

1.5.4 Protein metabolism and interaction

As well as disruption to RNA processing, there is evidence of alterations in protein metabolism in ALS, particularly in the ubiquitin proteasome system (UPS) and autophagy, processes important in protein degradation. Neuronal inclusions in ALS patients are usually positive for ubiquitin, although there is some variation in ALS with FUS inclusions (40, 71, 243, 244). Mutations in UBQLN2, which encodes ubiquilin2, an ubiquitin-like protein, have been found to cause ALS (245). Mice with motor neuron-specific knockout of proteasomes develop an ALS-like phenotype (246). Although evidence in FUS is lacking, studies looking at TDP-43 suggest the UPS is primarily involved in degrading soluble TDP-43, with autophagy clearing the aggregated form (247). This suggests that clearing of other ALS-related proteins such as FUS may occur via this system. Mutations, for example, causing misfolded FUS or abnormalities in the UPS or autophagy could therefore increase the accumulation of aggregated protein within neurons. Indeed, it may be that a 'double hit' mechanism is required, where defects in both the aggregated protein such as FUS, and in the protein degradation system are required for disease initiation.

Interactions between the proteins of interest might explain how mutations in different genes can cause the same disease. It has been suggested that TDP-43 and FUS share common pathways, for example in the observation that wild-type FUS rescued the phenotype shown in a TDP-43 knockdown drosophila model (248, 249).

Although reports vary, studies have demonstrated an interaction between these

proteins (108). An interaction between FUS and SMN has also been demonstrated (61, 108). This is interesting as mutant FUS enhanced this interaction and appeared to cause SMN dysregulation, manifesting in gem loss and axonal defects (61, 250). This raises the hypothesis that mutant FUS could cause motor neuron toxicity by its interaction with SMN, causing loss of function of SMN and therefore motor neuron loss via a similar mechanism to SMA. Indeed, the interactome of SMN also contains TDP-43 and EWS, potentially linking all forms of ALS in a common mechanism (198). More generalised differences in the protein interactome have been reported in the presence of mutant FUS, which could lead to widespread cellular changes (106).

1.5.5 Review of other hypotheses

There are many other factors that may be involved in ALS pathogenesis, either as an early event or as a result of more widespread cellular dysfunction. There is evidence of altered miRNA levels in ALS patients, as well as *in vitro* evidence of disruption to miRNA synthesis with altered levels of FUS and potentially with *FUS* mutations (150, 251, 252).

Mitochondrial dysfunction has been reported in sALS, fALS and many animal models, particularly mouse SOD1 models, though again this may be a result of cell toxicity rather than a cause (253-255). Excitotoxicity is still a strong candidate for causing or at least modulating neuronal toxicity in ALS. The fact that riluzole, which reduces glutamate levels, is the only drug to improve survival in ALS patients suggest that this mechanism is involved (13, 256). The role of FUS in DNA damage repair suggests that FUS mutations may cause deficits in this pathway and hence toxicity. Defects in this pathway have been demonstrated with *FUS* mutations in cell culture models, mouse models and human patients (132, 133, 231).

Despite the similarities between FUS and TDP-43, there are distinctions between them. Pathologically, TDP-43 inclusions are found in sALS and ALS with *TARDBP* mutation, whereas FUS inclusions are only found in ALS in the presence of *FUS* mutations. In addition, TDP-43 within these inclusions show post-translational modifications to create C-terminal fragments and hyperphosphorylated TDP-43, which are not seen in FUS inclusions (15). Although both are RNA binding proteins, the majority of their targets do not overlap (119). These observations suggest that more generic characteristics of these proteins may be more important than specific functions. For example, the propensity of these proteins to aggregate and their ability to bind proteins and RNA could be the important factor (58, 61). This could lead to neuronal toxicity through mechanisms such as sequestration of cytoplasmic proteins or RNA. They also provide the potential for disease spread via a prion-like mechanism or something similar (232). In order to determine how these mutations cause motor neuron toxicity and death, further work is required to examine early events at disease onset rather than at a late stage of disease. Models that avoid under or over-expression of relevant proteins are more likely to yield results relevant to human disease.

1.6 Aims of this work

The principle aim of this thesis is to examine the role of FUS in ALS, together with other related RNA-binding proteins. Particular emphasis will be placed on determining the effect of low levels of expression of mutant FUS, to distinguish effects of the mutation from those of overexpression. The effects of mutant FUS on protein distribution, transcription and splicing will be examined. The relationship

between FUS and other RNA-binding proteins implicated in ALS will also be examined to look for common mechanisms of neurotoxicity.

The project will be divided into three main sections:

1. The aim of the first section is to confirm the presence of a *FUS* mutation in a family with a severe form of ALS. A construct bearing this mutation will then be generated and used to further investigate the *in vitro* phenotype and study the relationship between *FUS* mutations and the severity of clinical disease.
2. The aim of the next section will be to investigate the effect of low level expression of the P525L *FUS* mutation on primary neurons. Protein distribution and stress granule dynamics will be studied, as well as the relationship between FUS and other RNA-binding proteins implicated in ALS.
3. The final section will focus on the effects of low levels of expression of mutant FUS and TDP-43 on transcription, splicing and protein expression *in vivo* using mouse models. This will examine early changes that occur in the presence of these mutations and whether they act via a common mechanism.

CHAPTER 2: MATERIALS AND METHODS

2.1 Genotyping

2.1.1 Patient data

Clinical information was gathered using the hospital records of the relevant patients. Information was anonymised so that personal identifiable information was removed.

2.1.2 PCR and sequencing

For exon 14 amplification, primers designed by Dr Dirk Bäumer were used (appendix table 7.1). PCR was carried out using Megamix blue (Microzone) in thin-walled PCR tubes (Axygen). PCR reactions were assembled on ice, where 1 µl DNA was added to 17 µl Megamix blue with 1 µl each primer (10 µM). PCR reactions were carried out using a thermal cycler (Veriti 96 well thermal cycler, Applied Biosystems) as follows:

Step	Temperature	Duration
Initialisation	95°C	3 minutes
Denaturation	95°C	30 seconds
Annealing	59°C	1 minute x35
Elongation	72°C	1 minute
Final hold	4°C	

Products were run on a 1.8% agarose TBE gel (Life Sciences) with 500 µg/l ethidium bromide (Invitrogen), against 100bp ladder markers (Bioline) at 120 volts for 2 hours. Products were visualised using UV light (LKB 2011 *Macrovue transilluminator*, Bromma) and the correct 370 base pair product was cut out. DNA was extracted using the QIAquick gel DNA extraction kit (Qiagen). A Sanger DNA

sequencing service using a 48 capillary ABI-3730 DNA analyser was used to sequence the exon 14 PCR products.

2.1.3 pGEM-T confirmation

Exon 14 PCR products were cloned into the TA-cloning vector Promega pGEM-T easy (Promega) to obtain reliable sequencing results. The pGEM-T vector was incubated with the exon 14 PCR product and DNA ligase at room temperature for 1 hour to insert the product into the vector. Highly efficient competent bacteria (JM109 competent cells, Promega) were transformed with 2µl of ligation product using heat shock in a water bath at 42°C for 45 seconds. These were incubated in sterile Luria Broth (LB; 10 g tryptone, 6.4 g yeast extract, 10 g sodium chloride made up to 1 litre distilled water, pH 7.5) for 1.5 hours at room temperature then plated onto LB plates (150 ml LB, 2.25g agar (VWR), 75 µl IPTG (Promega), 150 µl ampicillin (Sigma), 150 µl X-gal (Promega)) and incubated at 37°C overnight. White colonies were selected and further grown in LB. The vector was then purified using Qiaprep miniprep kit (Qiagen). Correct insertion into the vector was verified using restriction digest with the Eco-R1 enzyme (Life Technologies). Exon 14 primers were used to amplify and sequence this region as described above.

2.2 Culture of immortalised cell lines

2.2.1 Cell culture and transfection

HEK293 and NSC-34 cells were used in this study. They were grown in Dulbecco's modified Eagle medium (DMEM, Sigma) with 10% foetal calf serum (FCS, Sigma) and 1% penicillin/streptomycin (10,000 µg/ml Invitrogen). On day 1 of transfection, 5×10^5 HEK293 cells or NSC-34 cells per well were seeded onto a 6 well plate. On

day 2, the DNA (4 µg pcDNA, 3 µg BAC) was incubated in 250 µl OptiMEM (Invitrogen) and 10 µl plus reagent (Invitrogen) per well. After 10 minutes, 250 µl OptiMEM with 10 µl per well lipofectamine (Invitrogen) was added and incubated for 30 minutes at room temperature. Cells were then washed with 2 x 2.5 ml OptiMEM and incubated with 1 ml OptiMEM plus the transfection mix for 3 hours at 37°C. Cells were then washed with 3 x 2.5 ml OptiMEM and left overnight at 37°C in DMEM with 10% FCS per well. Cells were used 24 hours post-transfection for protein extraction or fixed for staining.

2.2.2 Stress treatment of immortalised cells

Oxidative stress: Cells were incubated in 0.5 mM sodium arsenite (Sigma) in normal media for 60 minutes at 37°C. Cells were then fixed immediately in 4% paraformaldehyde in PBS (Sigma).

Heat shock: 20mM HEPES (Sigma) pH 7.4 was added to the media to buffer it.

Plates were then placed in a 42°C water bath for 60 minutes, then immediately fixed.

ER stress: Cells were incubated in normal media containing 1µM thapsigargin (R&D systems) for 60 minutes, then immediately fixed.

Fixation was carried out by incubating cells in 4% paraformaldehyde in PBS for 15 minutes at room temperature, then washing 3 times in PBS. Cells were stored in PBS at 4°C prior to staining.

2.3 Construct generation

2.3.1 pcDNA constructs and site-directed mutagenesis

pcDNA3.1 constructs were kindly donated by Dr Peter Oliver. These contained murine wild-type, R521C and P525L-FUS cDNA. The wild-type *FUS* construct was

used for site-directed mutagenesis. This was carried out using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent) using the primers described in appendix table 7.1 and following the manufacturer's instructions. The new construct was transformed into competent cells, grown in Luria Broth and then extracted using Qiaprep miniprep kit (Qiagen). Products were sequenced as described above to confirm the presence of the mutation. In order to create the human G504Wfs*12-*FUS* mutation using mouse *FUS* cDNA, mutagenesis was carried out in three stages with the product from each stage used for the next.

2.3.2 BAC constructs

The wild-type, R521C and P525L BAC constructs were created by Dr Matthew Thomas and Dr Javier Alegre Abarrategui in Dr Richard Wade-Martins' research lab (figure 4.1a). It is a 59541bp (59.5 kb) construct containing the whole *FUS* gene including introns and exons, along with the promoter region. There is an mCherry tag fused to exon 1.

2.4 Primary neuronal cultures

2.4.1 Primary motor neuron culture

This method was based on a published protocol (257). Briefly, mouse embryos were harvested on embryonic day 13.5. Lumbar cords were dissected in sterile PBS under a microscope and stored in 90 µl Hank's Hank's Balanced Salt Solution (HBSS, Invitrogen) in a sterile 0.5ml microfuge tube on ice. Individual cultures were set up from each embryo to create cultures of each genotype. Remaining tissue was stored at -20°C for genotyping.

When all embryos had been dissected, 10 µl of a 1% trypsin/HBSS solution (Lorne laboratories) was added to each tube and incubated at 37°C for 15 minutes. 10 µl of 1% trypsin inhibitor/HBSS solution (Sigma) was then added, and the cells triturated using a 200 µl tip until dissociated. Cells were transferred to a panning plate coated with an anti-p75 antibody (mouse anti-human p75 monoclonal antibody, Novus Biologicals) containing neurobasal media (Gibco) with 1 x Glutamax I (Gibco). They were incubated for 45 minutes at room temperature on a stable surface.

Panning plates were gently washed 3 times with neurobasal media containing 1 x Glutamax I to remove p75-negative cells and debris. Depolarisation solution (30 mM potassium chloride, 0.8% sodium chloride and 2 mM calcium chloride) was added for 15 seconds to lift the cells, then 2 washes with neurobasal complete medium (neurobasal medium, 10% horse serum (PAA), Glutamax I, 1 x B27 (Invitrogen)) was used to collect the resulting cell suspension. This was centrifuged at 400 g for 10 minutes without acceleration or brake. Supernatant was removed and the cell pellet resuspended in neurobasal complete medium. 100 µl of the solution was plated on each coverslip coated with poly-DL-ornithine hydrobromide (Sigma) and laminin (Invitrogen), and incubated at 37°C. After 1 hour, plates were flooded with neurobasal complete medium with additional 5ng/ml GDNF (Peprotech), BDNF (Peprotech) and CNTF (Peprotech), and 10 nM β-mercaptoethanol (VWRI). Media was changed after 24 to 48 hours, then every 2-3 days. Except where stated, cells were fixed on day 6.

2.4.2 Primary cortical culture

This method was based on a published protocol (258). Mouse pups were sacrificed one day after birth (P1) and individual cultures were set up from each pup. The brain was dissected in sterile PBS to harvest the cortex, removing the hippocampus and

meninges. The cortex was cut into 4 pieces and stored on ice in 1 ml HBSS. A sample of remaining tissue was stored for genotyping. After pooling sufficient cortical tissue, 50 μ l trypsin was added and incubated for 30 minutes at 37°C. 500 μ l of the solution was removed and 50 μ l DNase 1 (Qiagen) added to the remaining solution for 30 seconds. Then 4.5 ml DMEM with 10% FCS was added, mixed and removed twice to wash the tissue. 1ml DMEM+FCS was then added and tissue triturated using a P1000 tip. Cells were counted and a cell suspension was made in DMEM+FCS in order to plate 7×10^4 cells in 100 μ l per coverslip coated with poly-L-lysine hydrobromide (Sigma). These were incubated at 37°C for 1 hour, then the wells flooded with neurobasal medium plus Glutamax I, B27 and penicillin/streptomycin.

Media was changed every 2-3 days, with the addition of 10 μ M Ara-C (Tocris) from day 2 onwards. Cells were fixed on day 7.

2.4.3 Transfection of primary neurons

This procedure was based upon published protocols (200). Neuronal media was replaced 1 hour prior to magnetofection, and the removed conditioned media retained. Plasmid DNA (0.5 μ g) was incubated with 1.75 μ l NeuroMag beads (Oz Biosciences) in 100 μ l Minimum Essential Medium (MEM, Invitrogen) for 15 minutes. Cells were placed on the magnet, and the transfection mix added dropwise. They were incubated at 37°C on the magnet for 15 minutes, and 50% of the media was replaced with the original conditioned media after 1 hour. Cells were fixed 24 hours after transfection.

2.4.4 Stress treatment of primary neurons

On day 6 for motor neurons and day 7 for cortical neurons, cells were stressed where stated with sodium arsenite. Fifty per cent of the media was removed and sodium arsenite, diluted in pre-warmed medium, was then added to 0.5 mM. Cells were incubated for 60 minutes at 37°C. For the 'stressed' condition they were fixed immediately. In the 'post stress' conditions, arsenite was washed off by removing 2 ml media and immediately replacing with 3.5 ml prewarmed normal media. Cells were again incubated for the stated duration at 37°C prior to fixation.

Neurons were fixed by incubating in 4% paraformaldehyde in PBS for 15 minutes at room temperature then washed 3 times in PBS. They were then stored in PBS at 4°C prior to staining.

2.4.5 Survival assay

To assess motor neuron survival, cultures were set up as described in 4.2.1, with care taken to create a consistent cell suspension prior to plating onto 4 separate coverslips. One coverslip was fixed on day 0, at least 2 hours from plating. A further coverslip was fixed on day 2, 4 and 6. Quantification is described below.

2.4.6 Genotyping

Tissue from embryos or pups was used for DNA extraction using genomic DNA extraction kit (Invitrogen). DNA was used to carry out PCR to determine the presence or absence of the BAC construct. 3 primer pairs were used for the BAC construct and one pair for IL-2 as an internal control to detect the presence of DNA (see appendix table 7.1 for primer sequences). PCR was carried out using a HotStarTaq DNA polymerase kit (Qiagen), with 50 ng DNA used per reaction. Non-transgenic genomic DNA was used as a negative control, with the addition of the

BAC construct as a positive control. PCR reactions were carried out in Pqstar 96 Universal Gradient PCR machine (Pqstar) as follows:

Step	Temperature	Duration
Initialisation	95°C	15 minutes
Denaturation	94°C	1 minute
Annealing	57°C/54°C	1 minute x35
Elongation	72°C	1 minute
Final elongation	72°C	10 minutes
Final hold	4°C	Stored

Annealing temperature was 57°C (BAC primers) or 54°C (IL-2 primers). Products were run on a 2% agarose gel with a 100 bp ladder (Bioline).

2.5 Immunocytochemistry

Cells were stored in PBS prior to staining. Cells were blocked using PBS with 10% normal goat serum (NGS, Vector labs) and 0.1% Triton-X100 for 1 hour at room temperature for the majority of experiments. When primary antibodies raised in goat were used, NGS was replaced with donkey serum.

Cells were then incubated with a primary antibody in 2% NGS, 0.02% Triton-X100 in PBS overnight at 4°C. Antibodies and concentrations used are listed in appendix table 7.2. Coverslips were washed 3 times in PBS for 10 minutes, then a secondary antibody conjugated to a 488nm or 564nm fluorophore (Invitrogen) was added at

1:500 concentration in PBS. Cells were incubated for 1 hour at room temperature, protected from light then washed again 3 times in PBS. They were then mounted with a DAPI stain (Vector labs).

Where 2 antibodies were used, staining was carried out sequentially, with the second primary antibody added instead of mounting. In this case, the primary antibody was incubated at room temperature for 2 hours. The washing steps and secondary antibody follow on as described.

2.6 Western blotting

2.6.1 Protein extraction

Mice were culled using schedule 1 methods and relevant tissue was removed. Tissue for protein extraction was placed on dry ice to freeze and stored at -80°C.

To extract the soluble protein fraction, a small piece of tissue was placed in RIPA buffer (Sigma) with protease inhibitor (Roche) and homogenised using a pellet pestle (Sigma). Crude lysates were cleared by centrifugation at 13,000 g at 4°C for 15 minutes. The supernatant was removed and stored at -80°C until required.

To extract the insoluble fraction, the pellet was resuspended in 100 µl urea buffer (8M urea, 2% SDS and 50 mM tris pH 8.5) with protease inhibitors (Sigma). It was incubated on ice for 20 minutes and then homogenised, then centrifuged at 13,000 g for 30 minutes at room temperature. The supernatant was collected and stored at -80°C.

To extract protein from cells *in vitro* (HEK293 or NSC-34 cells), cells were first washed in PBS. Ice cold lysis buffer was then added (RIPA buffer with protease inhibitor) and the solution was incubated on ice for 5 minutes, then centrifuged at 3000 g for 10 minutes at 4°C. Supernatant was collected and stored at -80°C.

To obtain nuclear and cytoplasmic fractions, NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) were used. Cells were washed in PBS then 500 µl trypsin followed by 1 ml PBS were added. Cells were placed in an 1.5 ml microfuge tube and washed twice in PBS by centrifugation (500 g at 4°C for 3-5 minutes) and removal of supernatant. Cells were lysed with the 'CERI' lysis buffer with protease inhibitor, mixed by vortexing and incubated on ice for 10 minutes. 'CER II' solution was added followed by vortexing and incubation on ice for 1 minute. Centrifugation was carried out at 16000 g for 5 minutes at 4°C. Supernatant (soluble fraction) was removed and stored at -80°C. The pellet was resuspended in 'NER' solution followed by incubation on ice for 40 minutes with vortex every 10 minutes. Centrifugation was carried out at 16000 g for 10 minutes at 4°C and supernatant (nuclear fraction) was stored at -80°C.

2.6.2 Bicinchoninic acid (BCA) Protein assay

BCA Protein Assay Kit was used (Life technologies). BCA standards were made up to concentrations of 1000, 800, 600, 400, 200, 100 and 0 µg/ml. Samples were diluted between 1:20 and 1:2 depending on the volume of tissue used for protein extraction. In each well of a 96 well plate, 200 µl working reagent (50:1 reagent A:reagent B) was added to 25 µl standard/sample and incubated at room temperature for 30 minutes. Each test sample was carried out in duplicate. The absorbance was read using the Fluostar optima plate reader (BMG labtech).

Samples were diluted with RIPA buffer used for protein extraction to create samples containing 2 µg/µl total protein for use in Western blots.

2.6.3 Western blot

Samples were made up with LDS loading buffer (Invitrogen) and sample reducing agent (10x, Life Technologies) then heated to 95°C for 10 minutes. Bis-Tris gels (Invitrogen) were loaded with 30 µg protein per well and run at 100 V for 1 hour in SDS running buffer (Invitrogen) at room temperature. Gels were transferred onto a Polyvinylidene difluoride (*PVDF*) membrane (GE Healthcare) in transfer buffer (Invitrogen) running at 25 V for 2 hours at 4°C. The membrane was blocked using 5% skimmed milk powder and 0.1% Tween in PBS for 1 hour at room temperature. It was then incubated with the primary antibody in the blocking solution overnight at 4°C (see appendix table 7.2 for antibody concentrations). The membrane was washed in PBS with 0.1% Tween 3 times for 10 minutes, then incubated with an HRP-conjugated secondary antibody at 1:10,000 in PBS/Tween at room temperature for 30 minutes. The membrane was again washed 3 times, then placed in Saran Wrap with ECL Western blotting detection reagents (VWR) in a developing cassette. Hyperfilm ECL (GE healthcare) was exposed to the membrane in a dark room and then developed using a developing machine (Xograph).

2.6.4 Densitometry

Densitometry was carried out using the Image J program. The relative density of each band compared to actin was initially calculated. The relative density of each sample could then be compared. For appendix figure 7.1, mCherry, endogenous FUS and total FUS were calculated directly from the band density relative to actin. For 7.1e) the mCherry-FUS band was calculated relative to the endogenous FUS

band using the antibody to total FUS. This was then corrected to assume a 100% transfection rate using the results shown in figure 41.b) in order to compare each mutation.

2.7 Microscopy

2.7.1 Immunofluorescence microscopy

Survival analysis was carried out by staining motor neurons with antibodies to detect Tuj1 (Covance) and ChaT (Millipore). Analysis was carried out using an immunofluorescence microscope (DM 2500, Leica) at the 40 x magnification whilst blinded to genotype. Fields were selected blindly and randomly, and 15-20 fields were included per coverslip in order to count at least 100 cells per embryo at day 0. Only neurons with Tuj1 and ChaT staining were included. Coverslips from 0, 2, 4 and 6 days in vitro (DIV) from each embryo were counted. Neurons from 3 embryos per genotype were analysed.

The total number of cells at time points 2, 4 and 6 DIV were calculated as a percentage of cells at day 0 from the same number of fields.

Statistical analysis was carried out using Graphpad Prism. A one way ANOVA test with Tukey post-hoc analysis was used to compare genotypes at each time point. Throughout this work, graphs are plotted to include the standard error of the means (SEM) of the repeated experiments. For the majority this represents the SEM of n=3.

Analysis of the percentage of cells with stress granules and mCherry granules was carried out using immunofluorescence microscopy. Cells stained with the relevant antibody were examined under 100 x magnification. Fields were selected at random

and 100 cells of each condition were counted per genotype. The number of these with mislocalised protein or cytoplasmic granules was recorded and the percentage of cells showing the relevant pathology was calculated. An n of three experiments was carried out, each using a separate embryo.

Analysis of SMN gems was carried out using immunofluorescence microscopy with a 100 x objective. Neurons from 3 embryos per genotype were analysed, both unstressed and stressed. 15 neurons under each condition were counted per embryo. The first 15 neurons visualised were used to count the number of SMN gems per nucleus, with alterations in the focal plane used to scan throughout the nucleus. The average number of gems per cell was calculated. A one way ANOVA test with Tukey post-hoc analysis was used to test for statistical differences between the genotypes.

2.7.2 Confocal microscopy

Confocal microscopy was carried out using a laser-scanning confocal microscope (Zeiss). Images were taken using the 40 x or 60 x objectives, and scale bars are shown for each series of images. Care was taken to check that there was no bleed-through between different channels.

In order to obtain data for further analysis, confocal microscopy was used to obtain images of 15 neurons per condition. Analysis was carried out blinded to genotype. The focus was used to obtain good visualisation of the cytoplasm and nucleus.

These images were used for off-line analysis of the cellular distribution of proteins including mCherry, FUS, TAF15 and EWS using Zen Lite software. The drawing tool was used to select the nuclear and cytoplasmic areas separately. DAPI staining was

used to determine the nuclear area and another stain such as Tuj1 or a stress granule marker was used to visualise the cytoplasmic area. For each, the average fluorescence intensity and the total number of pixels with each fluorescence value was recorded. The background fluorescence recorded from the unstained control was removed. Total fluorescence was then calculated using the average fluorescence multiplied by the number of pixels with fluorescence above background. Total fluorescence of the cell was calculated using the nuclear plus cytoplasmic fluorescence, which was used to determine the percentage of total fluorescence that was nuclear. 15 cells per embryo per condition were analysed, with an n of 3 per genotype.

Confocal images were also used to determine the number of stress granules and mCherry granules per cell. Images were taken as described and analysis carried out blinded to genotype. The counting tool in Image J was used for analysis. Granules were classified as such if they were visibly discrete from the cytoplasmic staining. There was inevitably an element of subjectivity, though each analysis was completed blinded, in a short space of time and by the author to retain consistency. Where possible, 15 neurons per condition per embryo were analysed, with an n of 3 per genotype. The average number of granules per cell was calculated, and one way ANOVA with Tukey post-test used for statistical analysis to compare genotypes.

In order to investigate stress granule dynamics, confocal microscopy was used to compare cytoplasmic fluorescence of stress granule markers between genotypes and conditions. The same settings were used for each image to maintain consistent exposure. For each cell, the average cytoplasmic fluorescence and the number of pixels with each fluorescence value was recorded. The background fluorescence

recorded from the unstained control was removed. Total fluorescence was then calculated using the average fluorescence multiplied by the number of pixels with fluorescence above background. 15 cells per embryo per condition were analysed, with an n of 3 per genotype. Average fluorescence for each condition and genotype was then compared using one way ANOVA and Tukey post-hoc analysis.

Nuclear area was examined using confocal images taken from previous experiments creating an n of 12. Analysis was carried out off-line using Zen Lite software. A tracing tool was used to highlight the boundary of the nucleus and the area was recorded for each neuron. The average area was calculated and expressed relative to non-transgenic. Genotypes were compared using one way ANOVA with Tukey post-hoc analysis.

Co-localisation of 2 proteins of interest was analysed using the Zen co-localisation tool to calculate Pearson's correlation coefficient within the cytoplasm of the neuron. Pearson's correlation coefficient was used as it was important to assess for a linear relationship between the variables. This method provides a rigorous assessment of the overlap between the two patterns of staining.

2.8 Animal work

2.8.1 Mouse models

The *FUS* BAC constructs were created in Dr Richard Wade-Martins' lab. A service provided by Dr Ben Davies' lab was used for pronuclear injections to create founder mice with random integration of the *FUS* BAC construct. The *FUS* BAC mouse model was developed by Dr Thomas Vizard. 5 experimental lines were developed; wild-type *FUS* lines 2 and 3, and P525L lines 1-3. Each line was examined for the

chromosomal location of the construct using FISH, and the number of BAC copies integrated and expression of human FUS using qRT-PCR and Western blotting. High and low expression lines were identified (appendix table 7.3) and cohorts were set up for behavioural, neuropathological and RNA analysis. There is no significant behavioural phenotype at 6 months, and further results are pending. The majority of work was carried out by Dr Thomas Vizard.

A FUS knockout mouse model is commercially available (Velocigene, Regeneron Pharmaceuticals 11866), created using a BAC targeting vector with a LacZ reporter and neomycin cassette inserted into the *FUS* locus. This causes unconditional deletion of 14,268 base pairs, removing the majority of the 15,245 base pair mouse *FUS* gene.

The TDP-43 BAC constructs were created in Dr Richard Wade-Martins' lab. A service provided by Dr Ben Davies' lab was used for pronuclear injections to create founder mice with targeted integration of the TDP-43 BAC construct. The TDP-43 BAC mouse model was developed by Dr David Gordon. 3 lines were developed; non-transgenic, wild-type TDP-43 and M337V mutant TDP-43. Lines could be used to create mice that were hemizygous or homozygous for the TDP-43 BAC construct (appendix table 7.3). Each line was examined for the expression of human TDP-43 using Western blotting. This work was carried out by Dr David Gordon and is therefore not described in detail (see appendix 7.11 for further details).

Mice were housed in a facility at Oxford University in accordance with home office guidelines under the project licence 30/2792 and I worked under the personal licence number 40/10413.

2.8.2 Tissue for primary cultures

In order to create primary motor neuron cultures containing the *FUS* BAC construct, non-transgenic mice were crossed with mice hemizygous for the BAC construct. The genotype of the resulting embryos would therefore be a combination of non-transgenic and those containing the construct.

In order to create motor neuron cultures from the *FUS* knockout lines, mice heterozygous for the null allele were crossed. No neonatal homozygous *FUS* knockout pups were detected by genotyping, suggesting that these mice are not viable consistent with previous reports (238). E13.5 Embryos were a combination of non-transgenic, heterozygous and homozygous knockout.

To develop motor neuron cultures from the TDP-43 BAC transgenic mouse model, mice homozygous for the BAC construct were crossed with non-transgenic mice. Embryos were all hemizygous for the construct, so genotyping was not necessary.

To set up motor neuron cultures, plug dates were recorded and pregnant females were sacrificed at embryonic day 13.5 using schedule 1 methods. Embryos were transferred to the tissue culture lab in Hibernate medium (Invitrogen) to set up motor neuron cultures.

To set up cortical cultures, pups were sacrificed at postnatal day 1 using schedule 1 methods. They were then transferred to the tissue culture lab, as above, to set up cultures.

2.8.3 Tissue for protein and RNA

Mice were sacrificed at the given time points by Dr Thomas Vizard (*FUS* model) and Dr David Gordon (TDP-43 model) using schedule 1 methods. Brain and spinal cord

was removed. Tissue for protein extraction was stored at -80°C. Tissue for RNA extraction was stored at -80°C in RNAlater solution (Invitrogen).

2.9qRT-PCR

2.9.1 RNA extraction and cDNA synthesis

RNA extraction was carried out using miRNeasy Mini Kit (Qiagen) with RNase-Free DNase Set (Qiagen) and stored at -80°C. Spectrophotometry (Nanodrop 1000, Labtech) was used to determine the concentration. cDNA was made using Transcriptor First Strand cDNA Synthesis Kit (Roche). 1 µg of each RNA sample was used in the majority of cases, and as close as possible when concentrations were lower. cDNA was stored at 4°C and used within 48 hours, or at -20°C and used within 2 weeks.

2.9.2 Primer design and optimisation

Primers for qRT-PCR were designed using the Universal ProbeLibrary Assay Design Center website. The mouse or human sequence was inserted into the program, and used to design 3 primer pairs per gene (high purity salt free oligos, Eurofins).

To examine primer efficiency, cDNA from non-transgenic mice was used. cDNA was used both undiluted and diluted in Tris-EDTA buffer at 1/3, 1/9, 1/27 and 1/81.

Reactions were set up using using LightCycler 480 SYBR Green I Master (Roche) as follows:

Master mix	10µl
5 µM Forward and Reverse Primers	2 µl
Nuclease free water	6 µl
cDNA	2 µl

cDNA was used at each concentration and in duplicate. Controls were used with no template and RT-free cDNA samples.

qRT-PCR was carried out using a light cycler (480 II, Roche) with the following cycle:

Step	Temperature	Duration
Pre-incubation	95°C	5 minutes
Denaturation	95°C	10 seconds
Annealing	60°C	10 seconds x45
Elongation	72°C	10 seconds
Melt curve 1	95°C	5 seconds
Melt curve 2	65°C	1 minute
Melt curve 3	Increase to 97°C	Variable
Cooling	40°C	30 seconds

Efficiency of each primer was calculated by the qRT-PCR program and verified using the equation $\text{efficiency} = (10^{(-1/\text{slope})} - 1) \times 100$. The target for efficiency was between 0.8957 and 1.1017, and within 10% of the efficiency of each standard. Amplification and melt curves were assessed for consistency and the presence of primer dimers. Products were run on a 2% agarose gel to confirm the presence of the correct product. Primers were tried in turn until a primer pair for each gene of interest fulfilled the criteria.

2.9.3 qRT-PCR

Each sample was run on a single plate, with separate reactions set up to run it with each primer pair including the standards GAPDH and 18s. The CT values obtained

from each qPCR run were used to determine the relative expression levels of each RNA of interest. Products were again run on a 2% agarose gel to confirm the presence of the correct product.

For the TDP-43 mouse model, an n of 3 was obtained for each genotype at the 3 month and 9 month time point. For the FUS mouse model, the n numbers varied between 2 and 4 for each line depending on tissue availability. When genotypes were pooled, n=2 non-transgenic, n=5 wild-type and n=10 P525L.

The CT values were used for analysis. Delta CT was calculated as the difference between the CT value of the target gene and the average of the GAPDH and 18s standards. This was used to determine relative expression using the formula $2^{-(\Delta \text{CT})}$. Values were then normalised to the average of the non-transgenic values for each tissue and, where stated, time point. Where there was no expression of non-transgenic (ie using human-specific primers), normalisation was carried out relative to wild-type.

Statistical analysis was carried out using one way ANOVA with Tukey post-hoc analysis.

2.10 Splicing

2.10.1 PCR

Primers (Invitrogen) were selected from published work (109, 119). Primers were selected to include splicing changes detected in FUS depletion, TDP-43 depletion, and both. PCR reactions were set up using HotStarTaq (Qiagen) as follows:

10x Buffer	2µl
10 mM dNTPs	0.4 µl
10 µM Forward & Reverse Primers	1 µl
cDNA	2 µl
Taq	0.2 µl
Nuclease free water	14.4 µl

Primers were tested with cDNA from non-transgenic animals initially to confirm annealing temperature and the presence of products of the correct size. The PCR cycle was as follows (for annealing temperatures see appendix table 7.1):

Step	Temperature	Duration
Initialisation	95°C	15 minutes
Denaturation	94°C	1 minute x30
Annealing	Variable	
Elongation	72°C	
Final elongation	72°C	10 minutes
Final hold	4°C	Stored

Products were run on a 3% agarose TBE gel against 100bp ladder markers (Bioline). Good separation was required in order to obtain accurate analysis.

For the TDP-43 mouse model, an n of 3 was obtained for each genotype at the 3 month and 9 month time point. For the FUS mouse model, the n numbers varied between 2 and 4 for each line depending on tissue availability. When genotypes were pooled, n=2 non-transgenic, n=5 wild-type and n=10 P525L.

2.10.2 Analysis

Image J was used for densitometric analysis of the gel images. The relative intensity of each band was calculated for each sample in order to determine the relative abundance of each splice variant in a semi-quantitative manner. The percentage abundance of each variant was calculated. Lines and genotypes were compared for statistical differences using one way ANOVA with Tukey post-hoc analysis.

CHAPTER 3: IDENTIFICATION AND CHARACTERISATION OF A G504Wfs*12 FUS TRUNCATION MUTATION IN A FAMILY WITH RAPIDLY PROGRESSIVE ALS

3.1 Introduction

3.1.1 Introduction

Mutations in *FUS* are responsible for approximately 4% of familial and <1% of sporadic ALS. This subtype of ALS, classified as ALS-FUS, often has an aggressive clinical course, particularly in the presence of certain mutations. These are often point, missense or truncation mutations within the region encoding the NLS (81, 259). Previous *in vitro* studies have shown that mutations that disrupt this region cause mislocalisation of FUS to the cytoplasm from its normal nuclear location, and incorporation into stress granules after cell stress (102, 148). There appears to be a correlation between clinical severity and cellular phenotype, so it is of interest to explore this in more detail to determine potential moderators of toxicity.

3.1.2 Aims of chapter 3

A family with a particularly severe form of fALS were identified in Oxford in the 1990s, and DNA from 2 family members was stored. Recent routine screening at King's College London revealed the presence of a mutation in the *FUS* gene in a DNA sample from an affected family member. The first aim of this chapter was to confirm the presence of the mutation and compare this with previously reported mutations. In addition, DNA from another family member was tested to examine inheritance. Detailed clinical information was gathered to provide a comprehensive

clinical phenotype of all affected family members to examine the clinical syndrome produced by this mutation.

The next aim was to create a construct bearing this mutation for *in vitro* analysis. This construct was used to characterise the cellular phenotype of this mutation in several cell types, and compare it to other disease-causing mutations. The results were used to examine the hypothesis that there is a genotype-phenotype correlation and help to determine the characteristics of mutant FUS that contribute to motor neuron toxicity.

3.2 Results

3.2.1 Clinical information

Case 1, Index case

The index case was a 33 year old right handed housewife who had been previously well. She had no relevant family history, and lived with her husband and 2 children aged 9 and 16. She noted in early March 1991 that her left thumb would 'drop' but did not seek medical attention. In mid-March she had a traumatic minimally displaced fracture of her left radial head and the arm was in a cast for seven days. No neurological or vascular deficit was detected at this stage. However, in June 1991 at follow-up she reported loss of function of her left hand. On examination, there was thenar eminence wasting and weakness of finger flexors and some intrinsic hand muscles.

She was seen by a neurologist in July 1991 and was found to have weakness of both upper limbs, and areflexia throughout. By October, all four limbs displayed a lower motor neuron pattern of weakness and she was unable to walk. There were no

upper motor neuron, bulbar or sensory signs and plantar reflexes were unresponsive.

Blood tests, cerebrospinal fluid and CT myelogram were all normal.

Electrophysiology revealed acute denervation in all four limbs with no conduction block, though central motor conduction time was prolonged at 23.4 msec which indicates upper motor neuron dysfunction. She was diagnosed with a lower motor neuron form of motor neuron disease. She died in January 1992 aged 34, 10 months from disease onset.

Case 2

The daughter of the index case was previously well, but developed symptoms in May 1997 aged 15 consisting of a limp and an ache in her left hip and ankle. She also had difficulty swallowing, weight loss and a change in voice. In July 1997 she developed shortness of breath and was treated for a chest infection. Six days later she had a respiratory arrest and was admitted to intensive care. Examination revealed normal tone with weak upper and lower limbs bilaterally but no wasting or fasciculations. Reflexes were brisk in her right lower limb with upgoing plantar responses, and sensation was normal. Her tongue was wasted with fasciculations. She had a tracheostomy in place for respiratory support.

Blood tests, cerebrospinal fluid, CT and MRI scans of the brain were normal.

Electrophysiological studies revealed evidence of fasciculations with signs of acute and chronic partial denervation. She was diagnosed with fALS and died in February 1998 aged 15, 9 months after onset.

Case 3

In 1998, the son of the index case was 23 years old and working as a machine operator. In April of that year he developed progressive difficulty swallowing,

shortness of breath, weak limbs and neck, and speech disturbance. He had fasciculations over the left orbicularis oris and bilateral facial weakness. His tongue was spastic and wasted with fasciculations, and he had a brisk jaw jerk reflex. He displayed paradoxical respiration with a drop in vital capacity on lying flat, indicating weakness of the diaphragm. He had wasting and fasciculations visible in his limb muscles, with weakness of all four limbs. Limb reflexes were brisk with downgoing plantar responses, and sensation was normal.

Blood tests revealed a raised creatine kinase but were otherwise normal.

Electrophysiology showed widespread fasciculations and chronic partial denervation.

His condition gradually deteriorated and he had a gastrostomy and tracheostomy inserted. He died in November 1998 aged 23, 7 months from onset.

Case 4

The mother of the index case developed symptoms in the year 2000 aged 68. She had difficulty using her left arm and had falls, which progressed such that she was unable to walk. On examination in 2010 she had a dystonic facial appearance with reduced upgaze by about 30% but preserved in other directions. She had axial and limb rigidity and some impairment of speech and swallowing. There was no wasting or weakness. She was diagnosed with progressive supranuclear palsy, though there were some atypical features such as relatively well preserved eye movements and slow progression. She died 12 years from onset in 2012 aged 80.

The family tree is shown in figure 3.1.

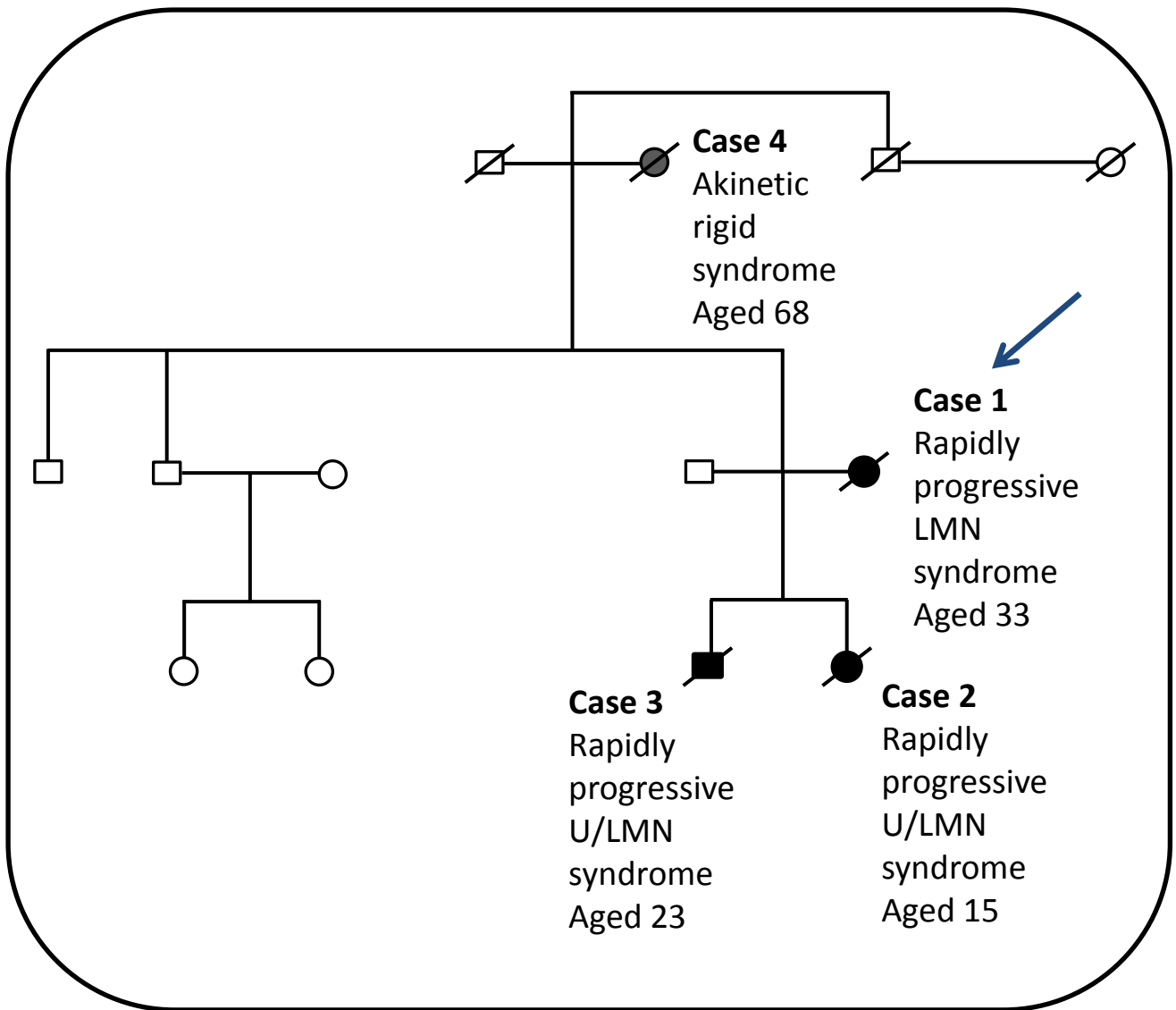


Figure 3.1. Family tree demonstrating the relationship between the described cases and the age of onset. The arrow denotes the index case. (UMN=Upper motor neuron, LMN=lower motor neuron)

3.2.2 Confirming the presence of a *FUS* truncation mutation in this case of fALS

DNA was stored from cases 3 and 4 for use by our research group. DNA from case 3 was sent to Kings College London as part of a separate study, and they sequenced several genes known to contain disease-causing mutations. They found a mutation in exon 14 of *FUS*.

Confirmation of the presence of the mutation was initially carried out. Primers designed by Dr Dirk Bäumer were used to amplify exon 14 of *FUS* from DNA samples from case 3 and a control; a BAC construct containing the wild-type human *FUS* gene (figure 3.2a). The product was sequenced using an in-house sequencing service. This demonstrated the presence of a 2 base pair AG deletion of nucleotides 1509 and 1510 in exon 14 of the *FUS* gene that was not present in the wild-type sequence (figure 3.2b). DNA from case 4 was also available and underwent the same procedure. Sequencing results matched that of wild-type *FUS*, suggesting that the mutation was not present in case 4 (figure 3.2b).

In order to confirm this result and provide more accurate sequencing of each allele, exon 14 products from the wild-type *FUS* BAC construct, case 3 and case 4 DNA were inserted into a pGEM-T vector. This was amplified by bacterial transformation and purification of the vector. *EcoRI* restriction sites were present either side of the insertion site, and *in silico* analysis using Bioedit confirmed the absence of *EcoRI* restriction sites within the exon 14 insertion. Restriction analysis using *EcoRI* was therefore used to determine the size of the insertion, and the products separated on an agarose gel (figure 3.2c). This experiment was repeated three times to obtain an adequate number of products.

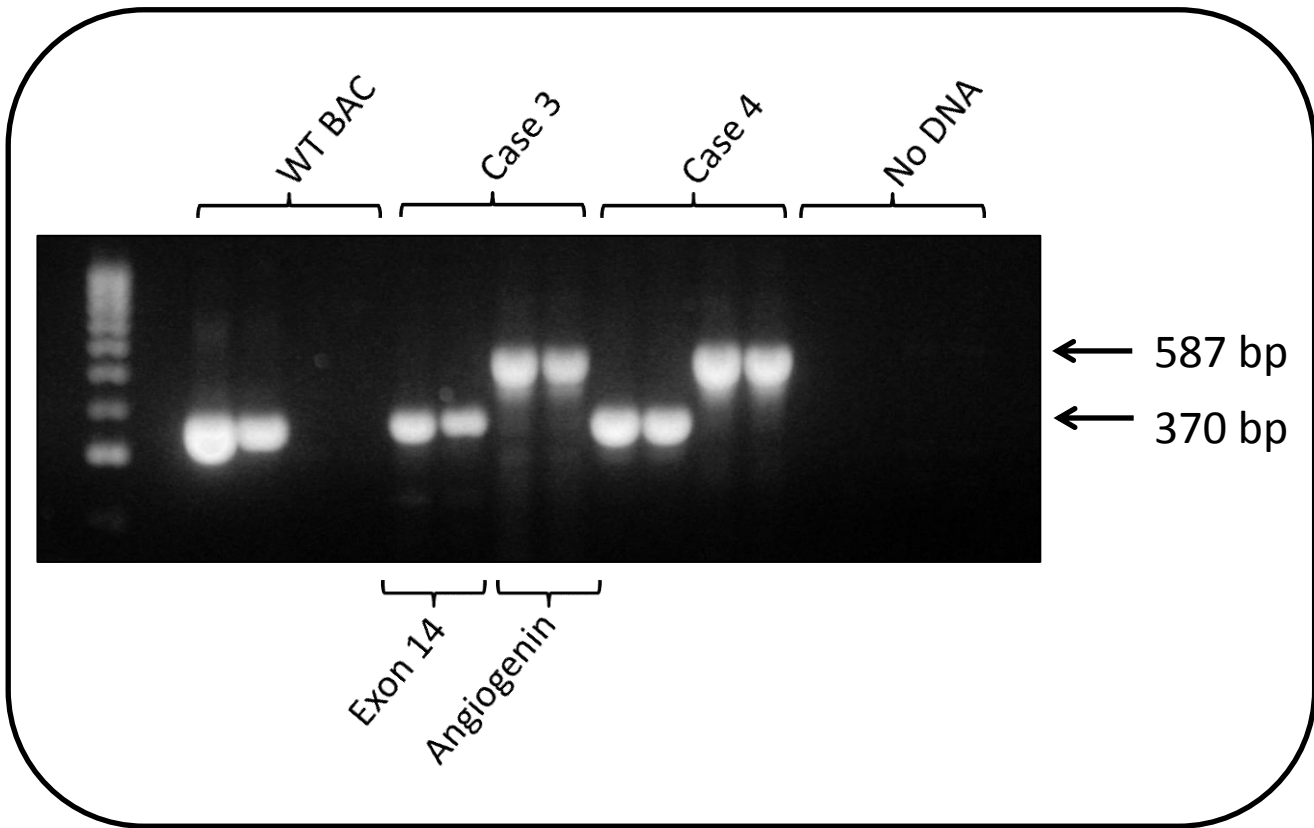
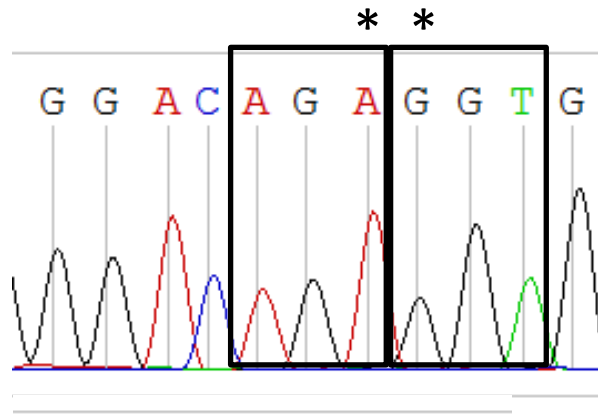
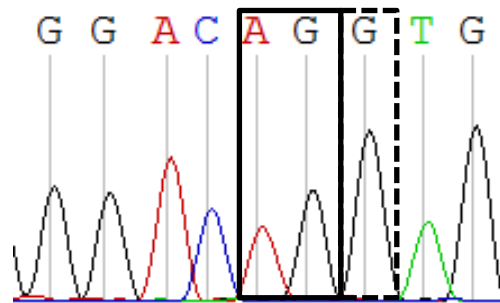


Figure 3.2a) PCR products from the wild-type BAC construct, case 3 and case 4 using primers to amplify exon 14 and control (angiogenin), run on a 1% agarose gel with ethidium bromide. Each reaction is in duplicate. The products were then extracted from the gel and used for sequencing.

Wild-type



Case 3



Case 4

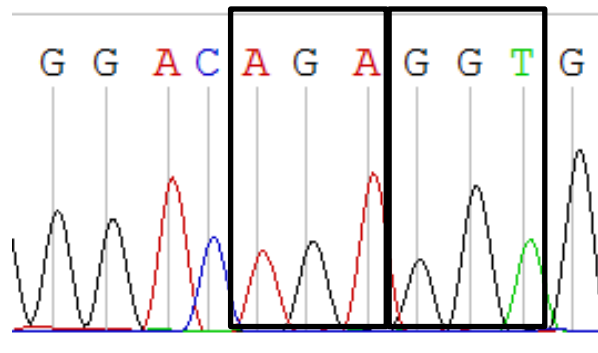


Figure 3.2b) Sequencing results showing the region of interest of exon 14 of FUS. They confirm that case 3, but not case 4, carried the c.1509_1510delAG (p.G504Wfs * 12) mutation. The deleted nucleotides are highlighted by *. The boxes illustrate the affected codons.

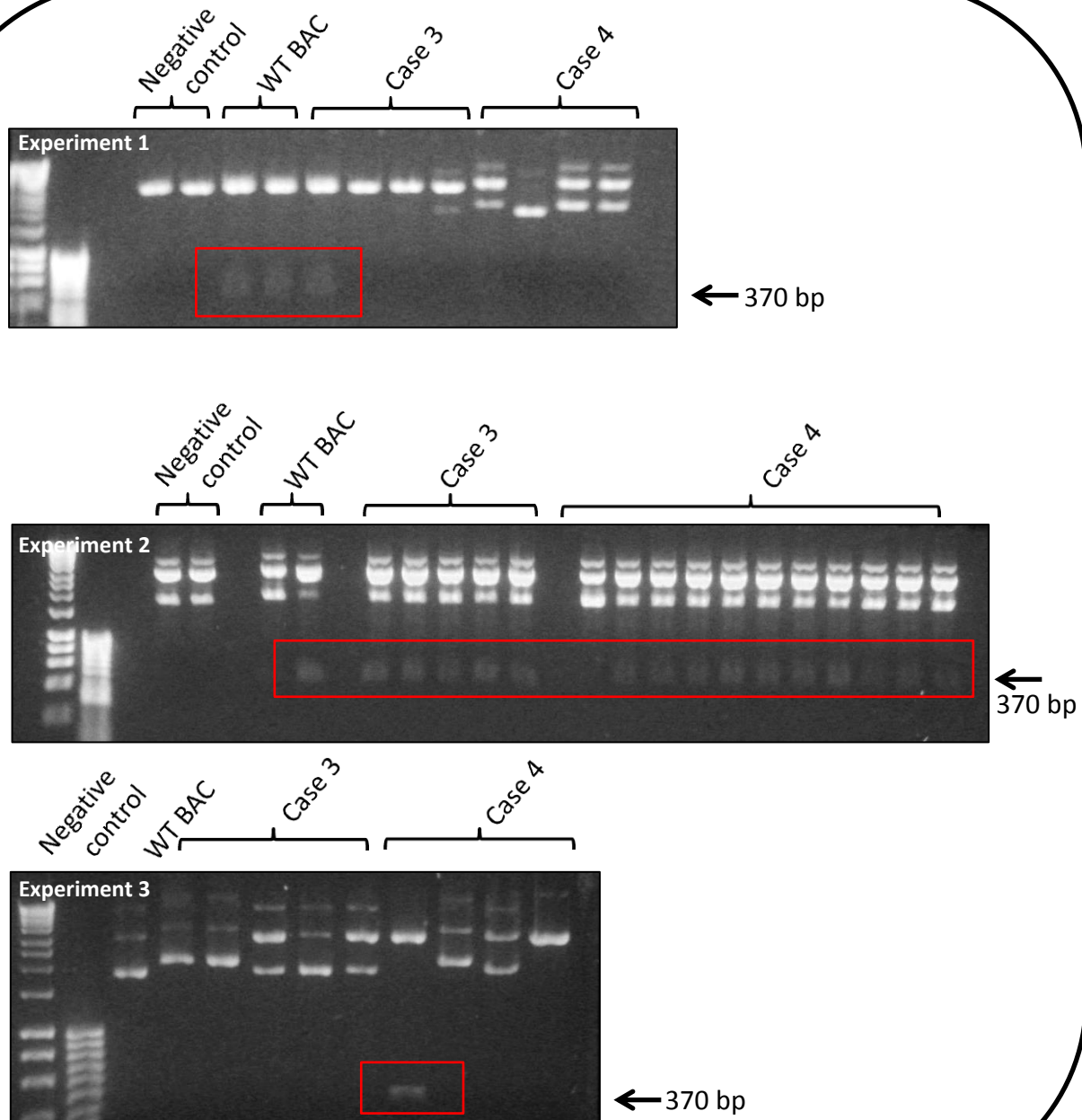


Figure 3.2c) Products of ECO-R1 digest of the pGEMt vector after insertion of exon 14 PCR products, in each of 3 repeated experiments. These are shown on a 1% agarose gel with ethidium bromide. The presence of a 370 bp product suggests exon 14 insertion in the correct site (red boxes). These samples were taken forward for exon 14 sequencing.

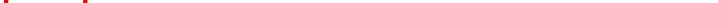
Wild type **A**GGGGG**C**CGG GGTGGTGGGG **A**CAGAGGTGG

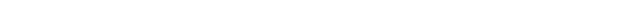
[illegible]

Case 3 2

Case 3

Case 3 4

Case 4 1 

Case 4 2. 

[illegible]

Case 4

Case 4 5

Case 4 6

Figure 3.2d) Results of exon 14 sequencing of the pGEMt constructs with insertion of exon 14 products from wild-type BAC, case 3 and case 4 DNA. It confirms the presence of wild-type and mutant alleles in case 3. The mutation was not detected in case 4 from any of the 6 sequenced samples.

Wild-type FUS

GAC	AGA	GGC	GGT	TTT	GGC	CCT	GGC	AAG	ATG	GAC	TCC	AGG	GGC	GAG	CAC
asp	arg	gly	gly	phe	gly	pro	gly	lys	met	asp	ser	arg	gly	glu	his

AGA	CAG	GAT	CGC	AGG	GAG	AGG	CCA	TAT	TAG
arg	gln	asp	arg	arg	glu	arg	pro	tyr	Stop

G504Wfs*12 FUS

GAC	AGG	TGG	CTT	TGG	CCC	TGG	CAA	GAT	GGA	TTC	CAG	GGG	TGA
asp	arg	trp	leu	trp	pro	trp	gln	asp	gly	phe	gln	gln	Stop

Figure 3.2e) This illustrates the C-terminal nucleotide and amino acid sequences in both wild-type FUS and the G504Wfs*12 mutant. The deleted nucleotides are highlighted in red. The AG deletion leads to a missense amino acid sequence and a premature stop codon 12 amino acids after the mutation. This leads to a truncated form of the protein lacking the nuclear localisation signal.

In total, the correct product length of 370 base pairs was present in two wild-type, six case 3, and eleven case 4 vectors. Exon 14 was sequenced in each of these, and the results shown in figure 3.2d). For case 3, 3 out of 4 products successfully sequenced contained wild-type *FUS* and 1 product contained the g.1509_1510delAG mutation. This result reflects the presence of both alleles in the DNA of the patient. For case 4, 6 products were successfully sequenced and all were wild-type. This confirmed that the mutation was present in case 3 and absent in case 4, suggesting that the mutation occurred *de novo* in the index case and was transmitted to her children.

Further research into the effect of this g.1509_1510delAG mutation revealed that the 2 base pair deletion spans the amino acids arginine and glycine at codons 504-505 of the *FUS* protein. This causes a frameshift, altering the next 12 residues (amino acids 505-516). A premature stop codon is created, leading to the truncated protein p.G504Wfs*12 (figure 3.2e). The majority of the NLS, which is located from amino acids 498 to the C-terminus at 526, is effectively lost in the presence of this mutation.

3.2.3 The creation of pcDNA constructs bearing the G504Wfs*12-*FUS* truncation mutation

In order to study the effects of this mutation in more detail, a pcDNA-based construct was created bearing this mutation. Dr Peter Oliver kindly donated pcDNA constructs encoding mouse *FUS* with an N-terminal c-myc tag. This included wild-type *FUS* and those with R521C and P525L *FUS* mutations. In order to create a construct with the same amino acid sequence as the human G504Wfs*12 mutation, site-directed mutagenesis was carried out using the wild-type pcDNA construct.

Mouse wild-type FUS pcDNA

GAC	AG A	G GC	GGT	T TT	GGC	CCT	GGC	AAG	ATG	GAC C	TCC	AGG	GG C
asp	arg	gly	gly	phe	gly	pro	gly	lys	met	asp	ser	arg	gly

Mouse G504Wfs*12 FUS pcDNA

GAC	AGG	T GG	T TA	TGG	CCC	TGG	CAA	GAT	GGA	T TC	CAG	GGG	T GA
asp	arg	trp	leu	trp	pro	trp	gln	asp	gly	phe	gln	gln	Stop

Human G504Wfs*12 FUS

GAC	AGG	TGG	CTT	TGG	CCC	TGG	CAA	GAT	GGA	TTC	CAG	GGG	TGA
asp	arg	trp	leu	trp	pro	trp	gln	asp	gly	phe	gln	gln	Stop

Figure 3.3a) This illustrates the amino acid sequence in the region of the mutation. The mouse G504Wfs*12 pcDNA sequence shows the nucleotide changes made by mutagenesis in red. It matches the human G504Wfs*12 amino acid sequence.

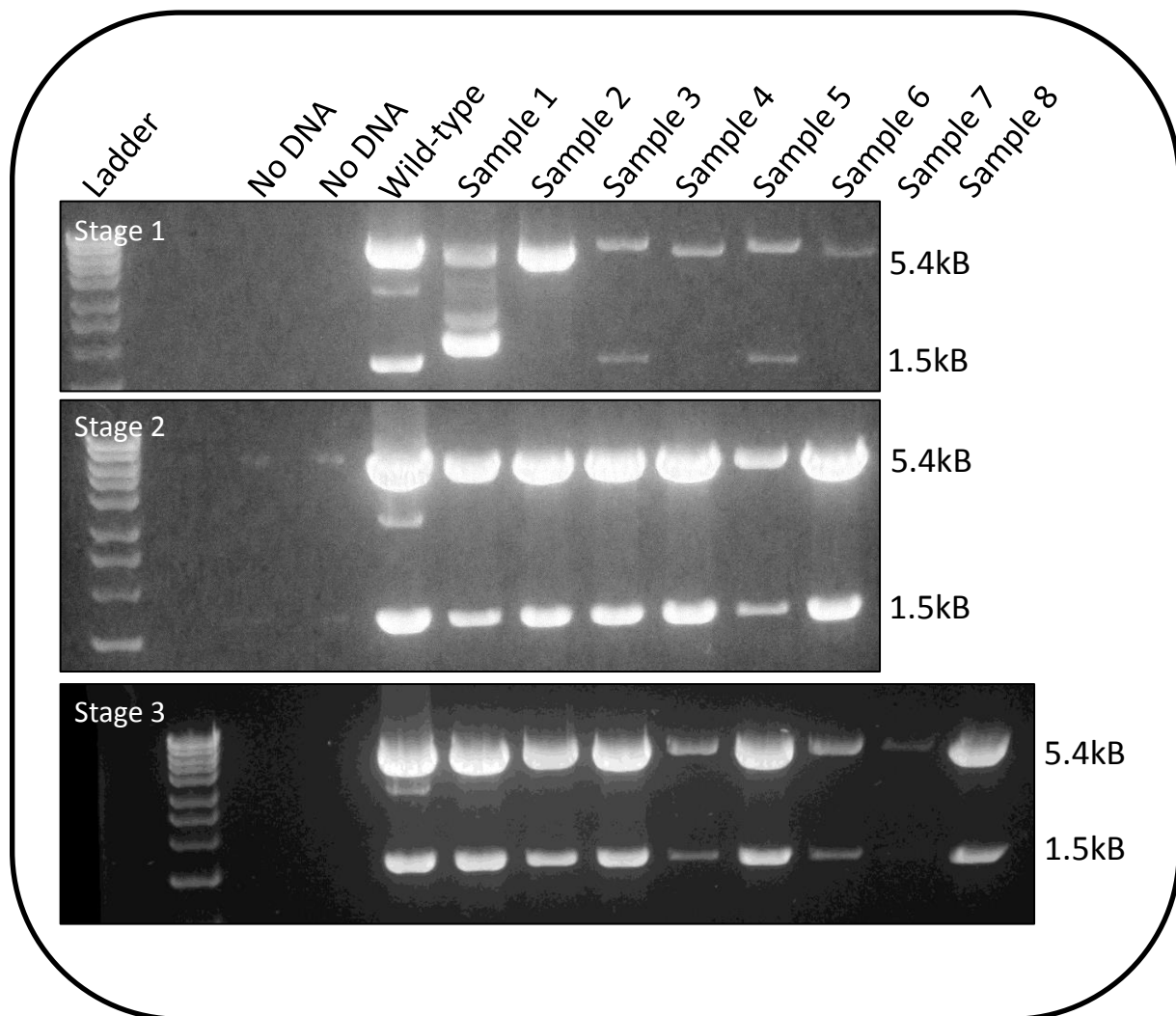
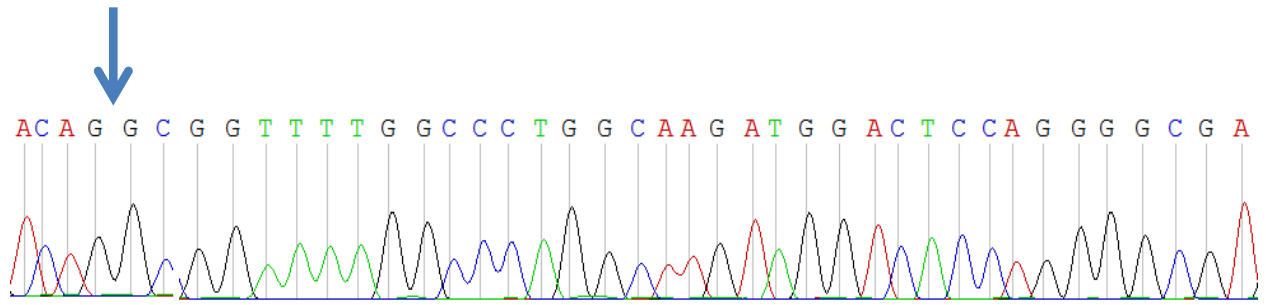
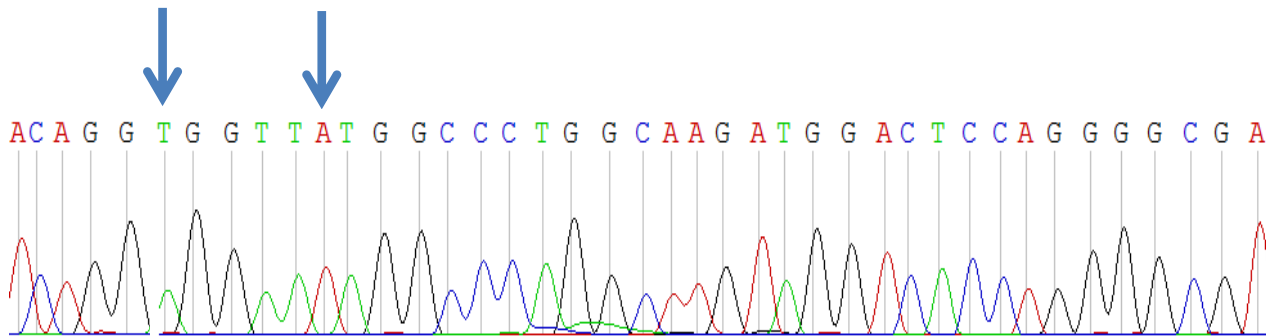


Figure 3.3b) Restriction digest of the pcDNA construct with Kpn1 and Xba1 enzymes after each stage of G504Wfs*12 mutagenesis. Samples with 5.4 kb and 1.5 kb products underwent exon 14 sequencing.

Stage 1



Stage 2



Stage 3

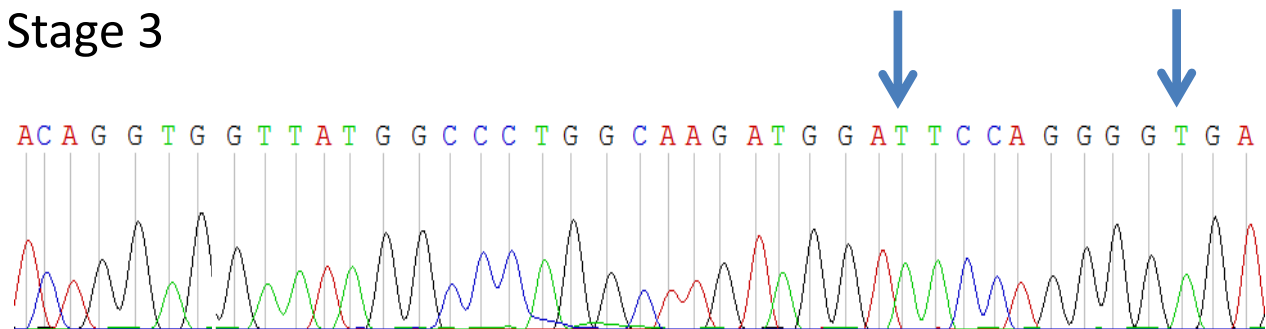


Figure 3.3c) Extracts of exon 14 sequencing results after each stage of G504Wfs*12 mutagenesis. Stage 1: AG deletion (g.1485_1486delAG) at amino acid 495. Stage 2: p.R496W (g.1486C>T) and p.F497L (g.1491T>A) mutations. Stage 3: p.L504F (g.1510C>T) and p.R507* stop mutation (g.1519C>T). The desired mutations are present after each stage.

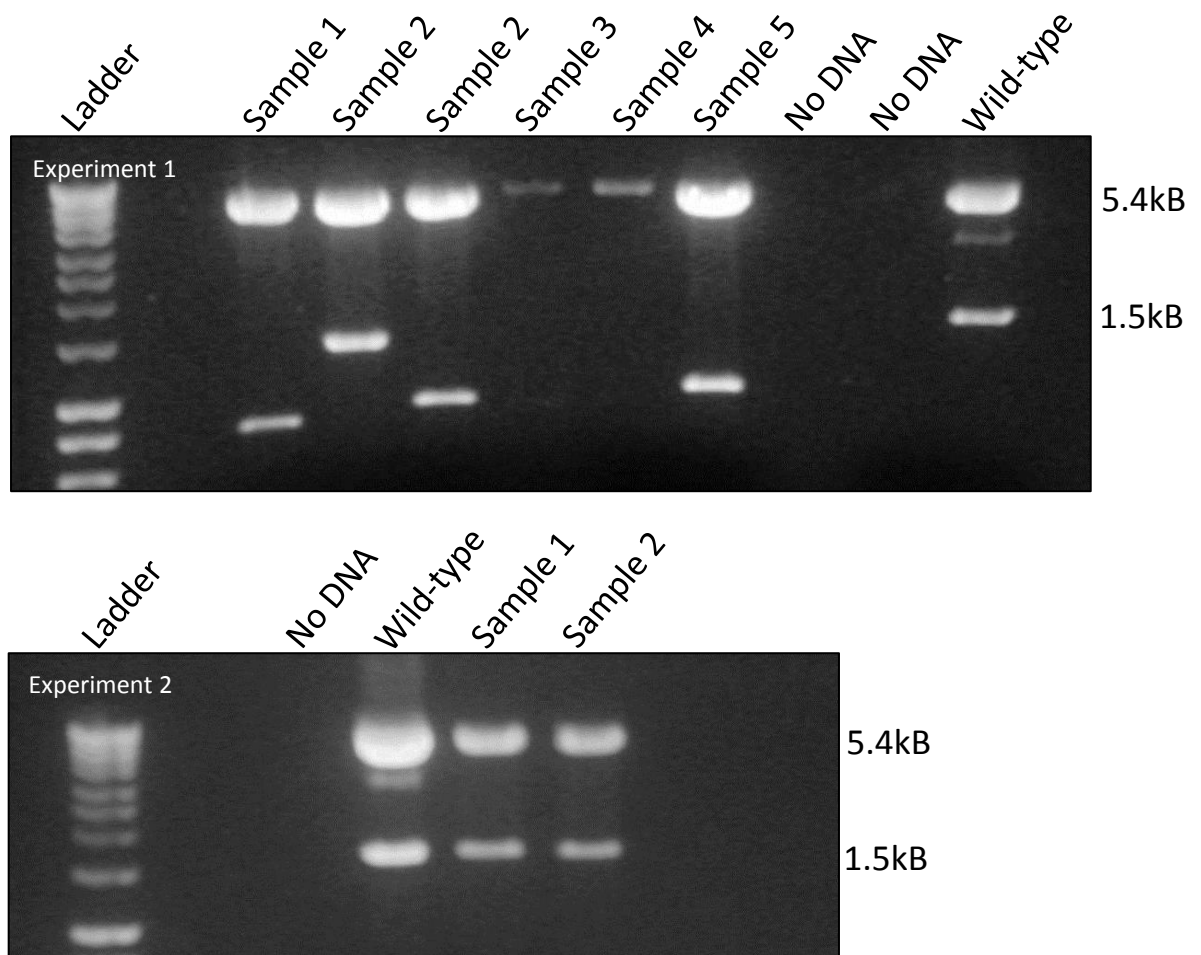
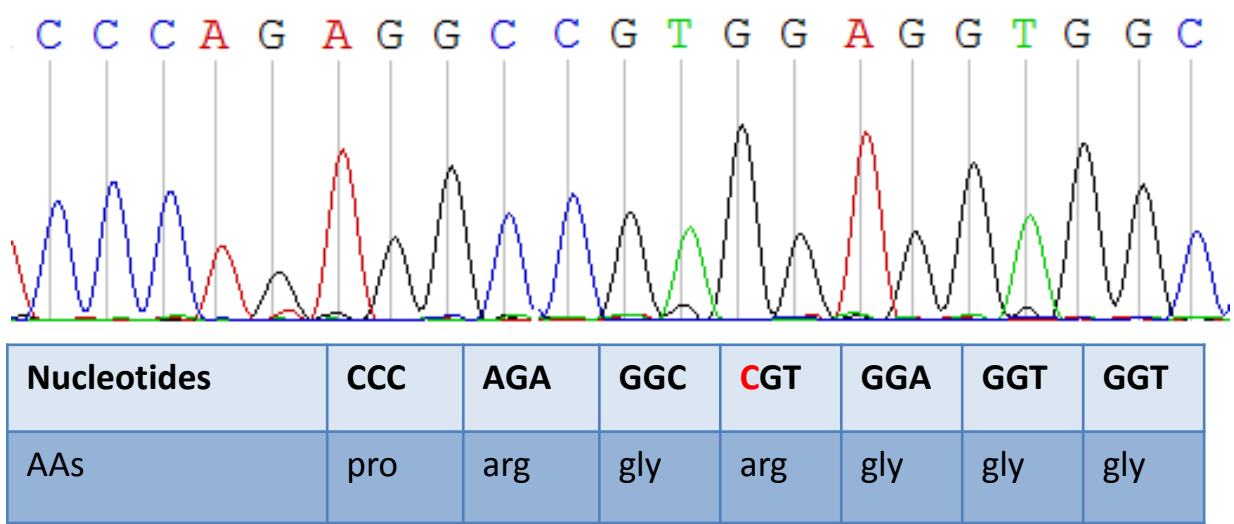


Figure 3.3d) Restriction digest of the pcDNA construct with Kpn1 and Xba1 enzymes after R244C mutagenesis. Samples with 5.4 kb and 1.5 kb products underwent exon 6 sequencing. This experiment was repeated in experiment 2 to obtain the correct product.

Wild-type FUS pcDNA



R244C-FUS pcDNA

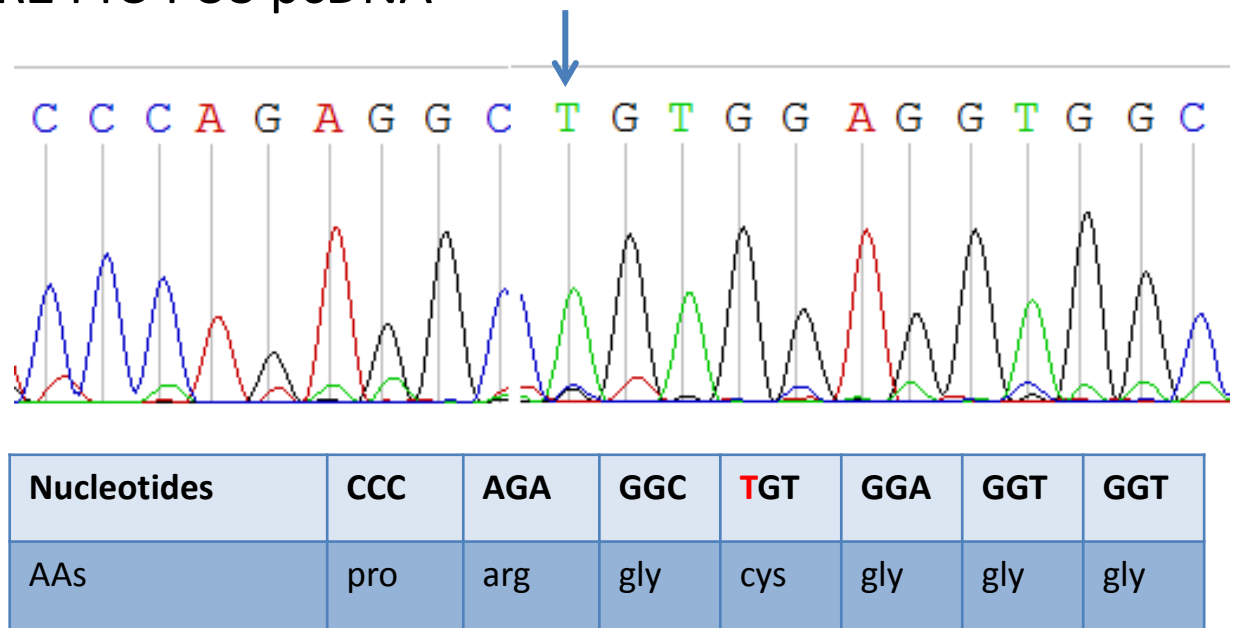


Figure 3.3e) Extracts of exon 6 sequencing of the pcDNA construct showing the successful production of R244C-FUS mutant pcDNA after mutagenesis. The tables below show the nucleotide and amino acid sequences. The nucleotide change made by mutagenesis is shown in red.

Due to considerable sequence differences at the amino acid level, deletion of the nucleotides AG in mouse *FUS* would not recapitulate the same amino acid changes that form the human mutation. Therefore three stages of site-directed mutagenesis were required to alter nucleotides in different locations within the C-terminal in order to create the same amino acid sequence as the human mutation. The first stage created the AG deletion of nucleotides 1485-1486 at amino acid 495 of the mouse gene (g.1485_1486delAG). The second stage created p.R496W (g.1486C>T) and p.F497L (g.1491T>A) mutations, and the third stage created p.L504F (g.1510C>T) and p.R507* (g.1519C>T) mutations, resulting in a stop codon in the appropriate location (figure 3.3a). After each stage, a double restriction digest using *KpnI* and *XbaI* enzymes was used to check that the process had not created any unwanted insertions or deletions (Figure 3.3b). Samples with the correct product sizes of 5.4 kb and 1.5 kb underwent exon 14 sequencing to confirm the presence of the correct mutation at each stage (figure 3.3c).

In addition, further site-directed mutagenesis was carried out using the wild-type *FUS* pcDNA construct to create R244C mutant *FUS* (figures 3.3d&e). This exon 6 mutation is within the glycine-rich region and has been reported to cause a relatively milder clinical phenotype, so is useful for comparison with wild-type *FUS* and *FUS* with C-terminal mutations (40).

3.2.4 G504Wfs*12-FUS is mislocalised to the cytoplasm in HEK293 and NSC-34 cells

In order to investigate the effects of this mutation, the pcDNA constructs were transfected into HEK293 cells using lipofectamine. HEK293 cells were chosen as an initial model as they are resilient, inexpensive and easy to transfect. They could

therefore be used to confirm that these constructs express FUS in transfected cell lines, and to study the effects of the mutation. Wild-type, R521C, P525L and G504Wfs*12-FUS were compared. Cells were fixed 24 hours after transfection, and antibodies to c-myc were used to detect FUS expression driven by the construct. Wild-type FUS was predominantly nuclear, as was the majority of R521C-FUS. However, P525L and G504Wfs*12-FUS showed marked mislocalisation to the cytoplasm leading to diffuse staining throughout the cell (figure 3.4a). Quantification of the percentage of cells with mislocalised c-myc FUS highlights the significant increase in P525L and G504Wfs*12-FUS compared to wild-type (figure 3.6a). There was also a statistically significantly higher percentage of cells with mislocalised R521C-FUS compared to wild-type ($p < 0.01$), though the effect was not as marked as P525L and G504Wfs*12-FUS.

This experiment was repeated in NSC-34 cells, a cell line derived from the fusion of embryonic mouse spinal cord cells with mouse neuroblastoma. This immortalised mouse cell line shares characteristics with motor neurons, and was chosen to assess whether the observations described in HEK293 cells also occur in a more disease-relevant cell type. The R244C-*FUS* pcDNA construct was included to allow comparison between mutations that disrupt the NLS (P525L and G504Wfs*12) and a disease-causing mutation that does not affect the NLS (R244C). Similar results were obtained, with a significantly greater percentage of cells with mislocalised P525L and G504Wfs*12-FUS compared to wild-type (figures 3.4b & 3.6c). The pattern of R244C-FUS localisation, however, is indistinguishable from wild-type FUS.

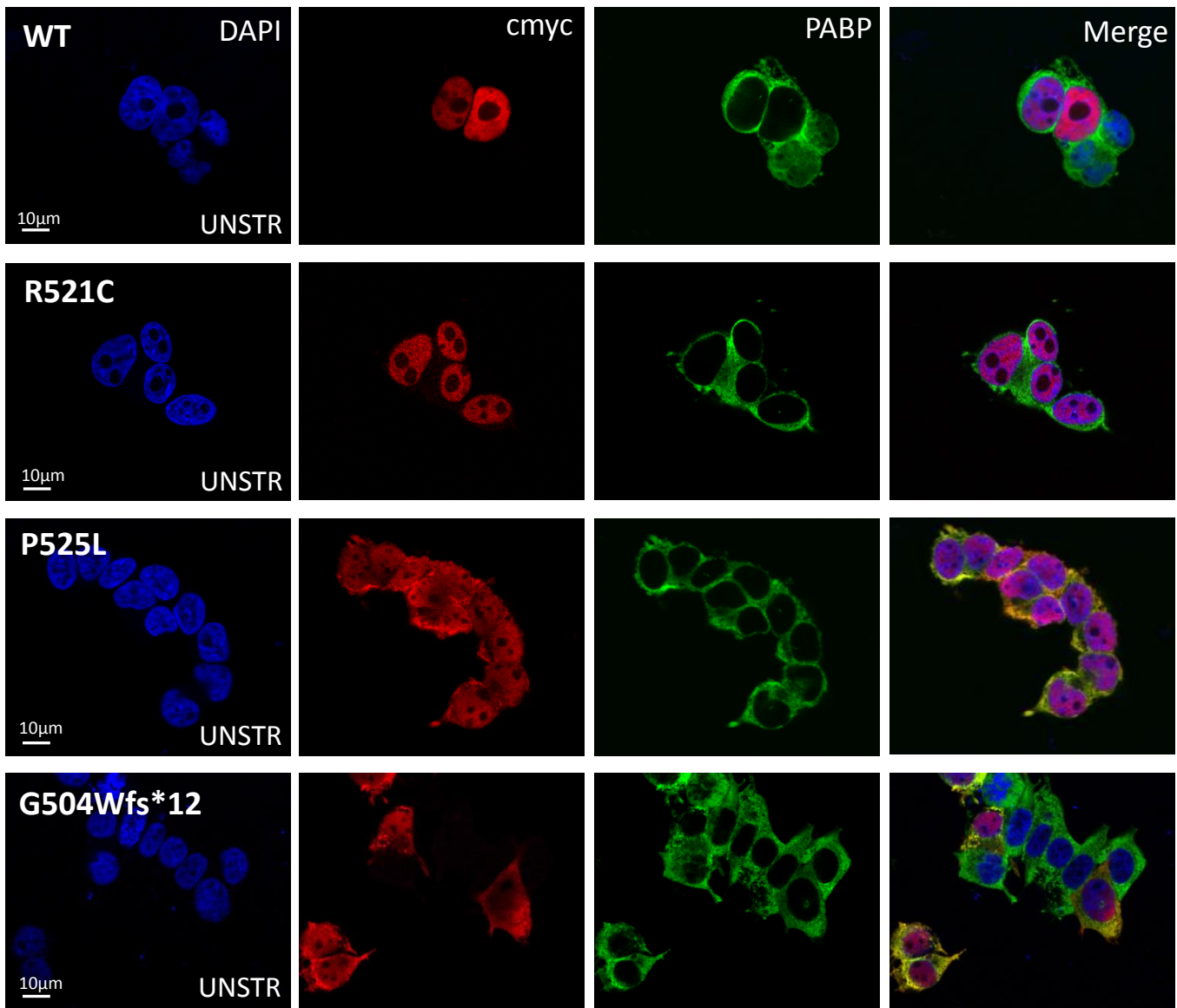


Figure 3.4a) Confocal images of HEK293 cells transfected with the given FUS pcDNA construct showing the cellular distribution of c-myc-tagged FUS. Wild-type and R521C-FUS remain nuclear whilst P525L-FUS and G504Wfs*12-FUS have diffuse nuclear and cytoplasmic expression. ('UNSTR' denotes untreated cells.)

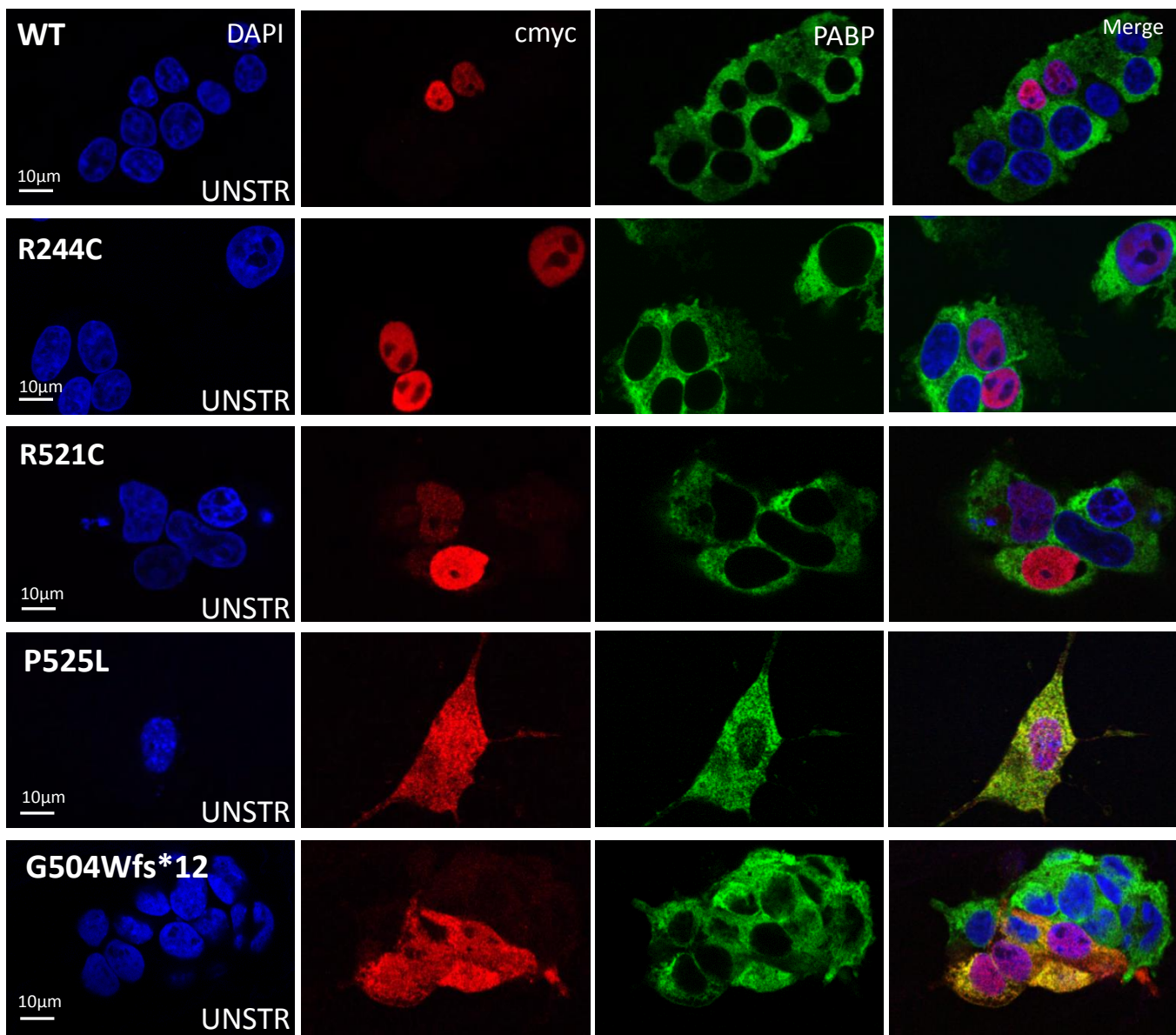


Figure 3.4b) Confocal images of NSC-34 cells transfected with the given FUS pcDNA construct showing the cellular distribution of c-myc-tagged FUS. Wild-type, R244C and R521C-FUS remain nuclear whilst P525L-FUS and G504Wfs*12-FUS have diffuse nuclear and cytoplasmic expression. ('UNSTR' denotes untreated cells.)

R521C-FUS has a trend towards mislocalisation but does not reach statistical significance in NSC-34 cells ($p>0.05$). It is notable that some cells contain cytoplasmic granules that are positive for c-myc and the stress granule marker PABP in the absence of any treatment that may induce cell stress (figure 3.6b&d).

3.2.5 G504Wfs*12-FUS co-localises with stress granules in HEK293 and NSC-34 cells

Previous studies have reported that some forms of mutant FUS are incorporated into stress granules (70, 102, 148). It was therefore of interest to assess whether G504Wfs*12-FUS displayed this characteristic. HEK293 and NSC-34 cells transfected with the *FUS* constructs were stressed with 0.5 mM sodium arsenite for 60 minutes to induce oxidative stress and stress granule formation. The reason this treatment was chosen was because it has been used widely to reliably induce stress granules without causing significant cell death. It has been used in similar experiments looking at the incorporation of FUS and TDP-43 into stress granules, so the results could be compared with published literature (148, 172, 212, 227, 260). It was also practical so could be carried out in a consistent manner between experiments. Different conditions were used at a later stage (figure 3.6). Untreated cells are referred to as 'unstressed'.

Figure 3.5 shows that cells treated with sodium arsenite developed discrete cytoplasmic stress granules positive for the stress granule marker PABP (poly(A) binding protein). Wild-type, R244C and the majority of R521C-FUS remained nuclear and did not co-localise with these granules. However, P525L and G504Wfs*12-FUS formed granules in the cytoplasm after cell stress which co-localised with stress granules (figure 3.5a&b). This was quantified by calculating the percentage of cells

with cytoplasmic granules positive for both c-myc and PABP (figure 3.6b&d). This demonstrated a statistically significant increase in the percentage of cells with this phenotype in P525L-FUS and G504Wfs*12-FUS compared to wild-type ($p<0.01$ and $p<0.001$). There was again a trend towards an increase in cells with R521C-FUS co-localising with stress granules, though this was not statistically significant ($p>0.05$).

The same pattern of results was observed in both HEK293 and NSC-34 cells.

To assess the potential clinical relevance of these results, the average age of onset of ALS with each of these mutations was examined (figure 3.6e) (3, 40, 78). Age of onset can be used as a marker of disease severity, with younger onset denoting a more severe manifestation of disease. Comparing the age of onset with mislocalisation of FUS and co-localisation with stress granules revealed that mutations associated with a lower age of onset displayed a much more marked *in vitro* phenotype.

Stress granules develop as a response to a variety of different forms of cell stress. It was therefore of interest to determine whether certain insults are a greater trigger for the incorporation of mutant FUS into stress granules compared with others. To examine this, NSC-34 cells were stressed for 60 minutes with 0.5 mM sodium arsenite, 1 μ M thapsigargin, or heat shock at 42°C in order to induce oxidative, endoplasmic reticulum or thermal stress, respectively. Cells were again stained for c-myc and PABP, and the percentage of cells with cytoplasmic c-myc was calculated (figure 3.6f). P525L and G504Wfs*12-FUS showed a significantly greater percentage of cells with mislocalised c-myc compared to wild-type FUS. This was true for all conditions examined. In addition, R521C-FUS had a significantly greater percentage of cells with mislocalised mutant FUS compared to wild-type FUS after the heat shock treatment, with a trend towards this in other treatments.

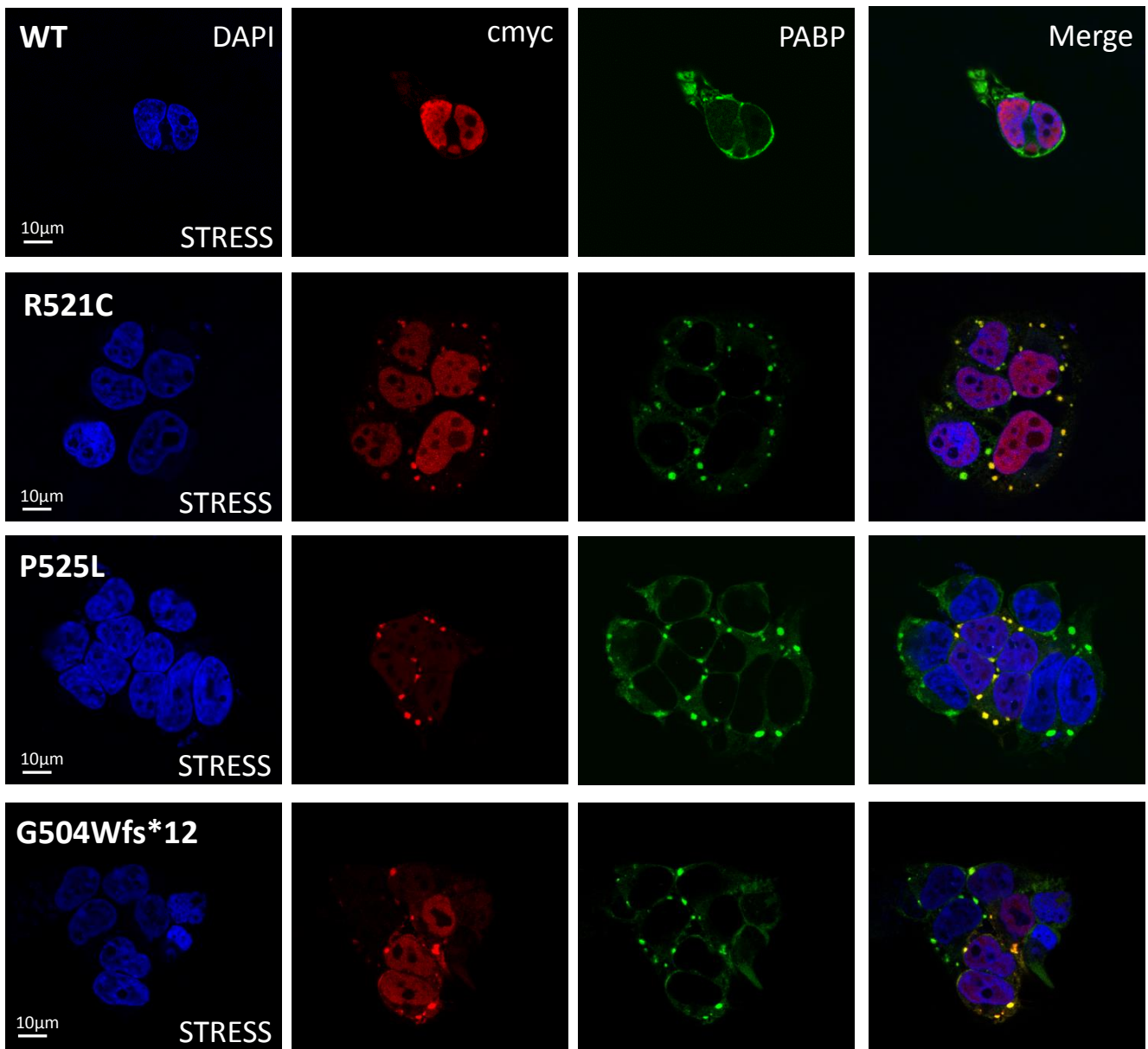


Figure 3.5a) Confocal images demonstrating the cellular distribution of c-myc-tagged FUS in HEK293 cells expressing the given construct after treatment with 0.5 mM sodium arsenite. Stress granules are present in the cytoplasm as demonstrated by PABP staining. Wild-type remains nuclear. R521C-FUS, P525L-FUS and G504Wfs *12-FUS form cytoplasmic granules that co-localise with PABP. ('STRESS' denotes cells treated with sodium arsenite.)

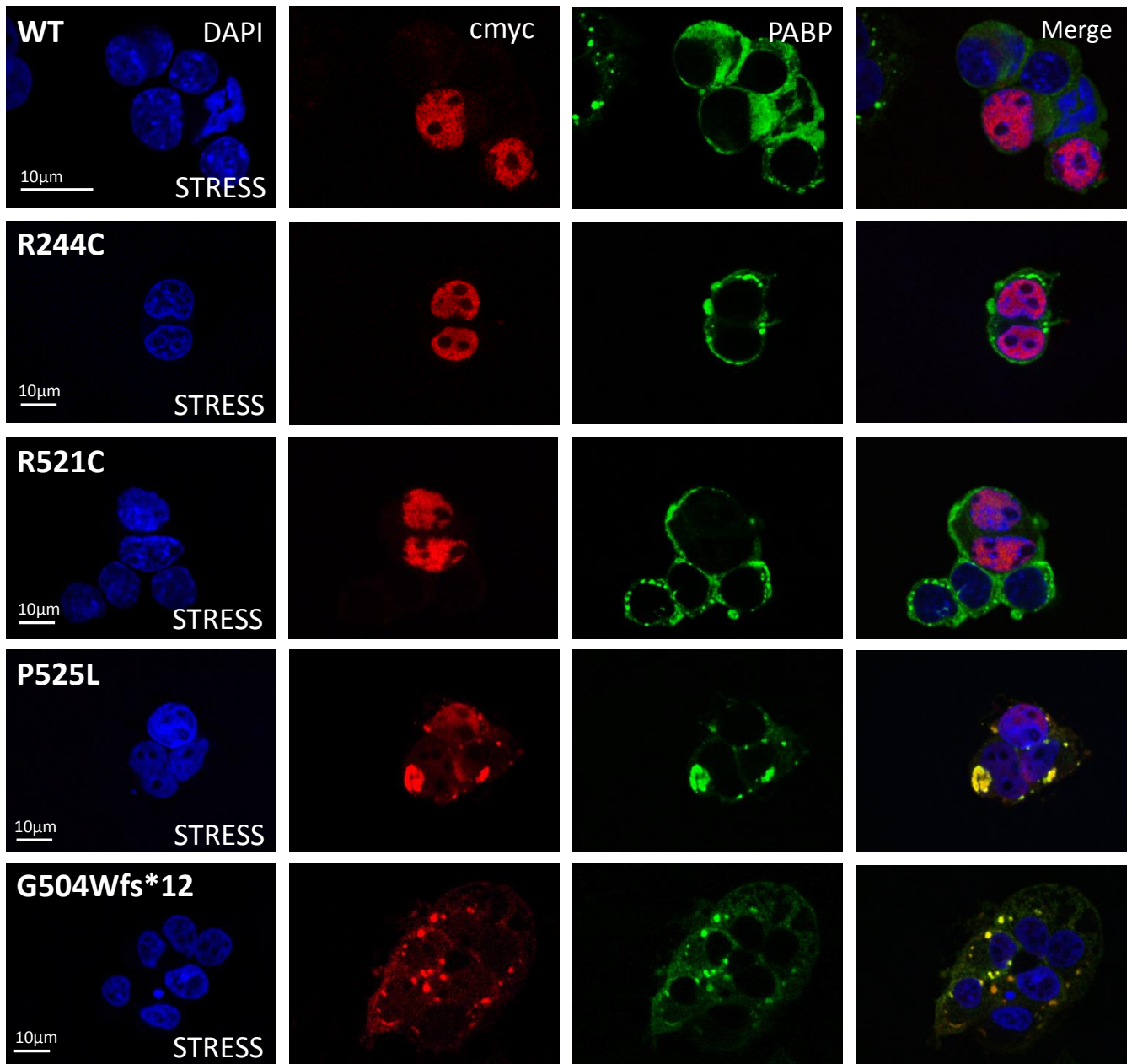


Figure 3.5b) Confocal images demonstrating the cellular distribution of c-myc-tagged FUS in NSC-34 cells expressing the given construct after treatment with 0.5 mM sodium arsenite. Stress granules are present in the cytoplasm as demonstrated by PABP staining. Wild-type, R244C and R521C-FUS remain nuclear. P525L-FUS and G504Wfs *12-FUS form cytoplasmic granules that co-localise with PABP. ('STRESS' denotes cells treated with sodium arsenite.)

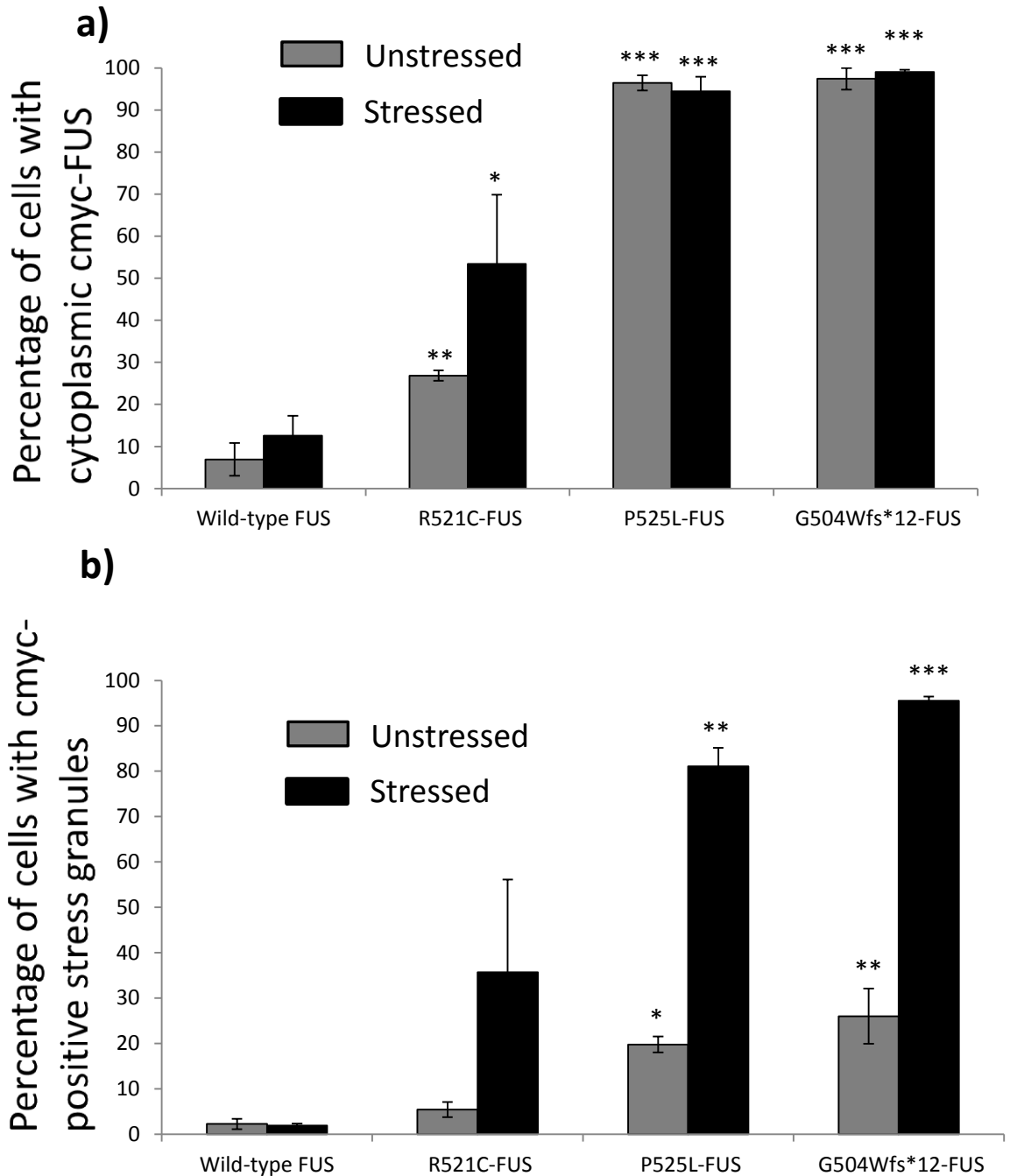


Figure 3.6a) The mean percentage of transfected HEK293 cells that demonstrated cytoplasmic c-myc-FUS, both in the unstressed (grey) and stressed (black) state. **b)** The mean percentage of cells with cytoplasmic granules that stained positive for c-myc and PABP, both in the unstressed (grey) and stressed (black) state. (The error bars represent the standard error of the mean. N=3 experiments, 100 transfected cells per condition each time. One way ANOVA. Tukey post-test. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to wild-type FUS.)

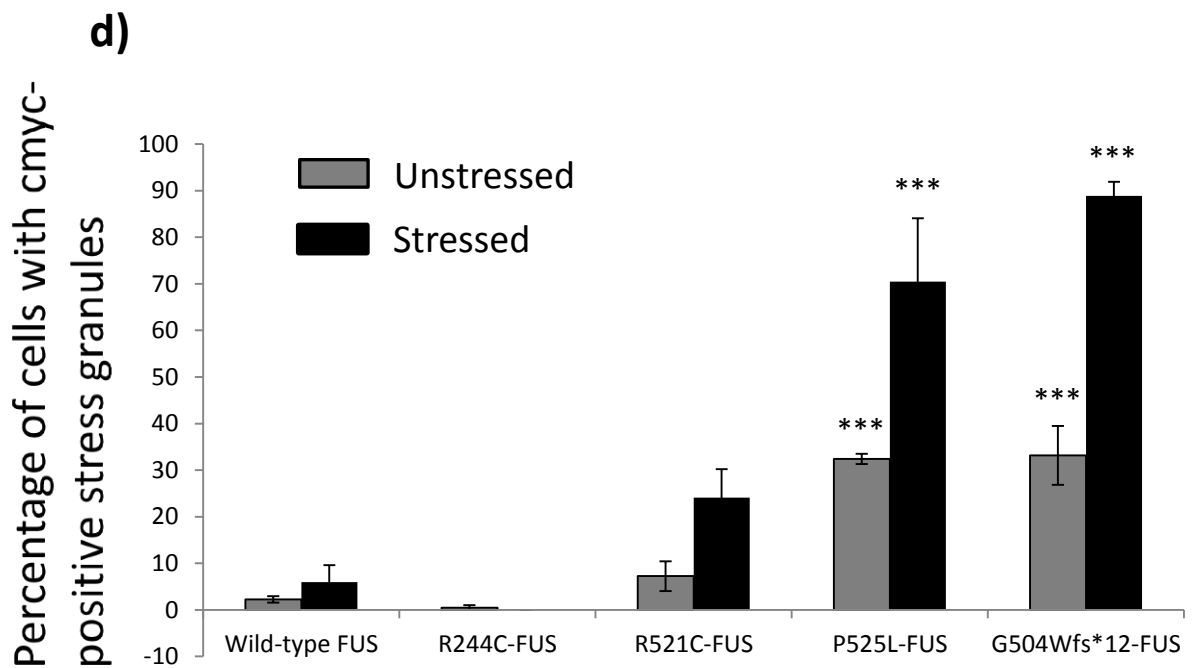
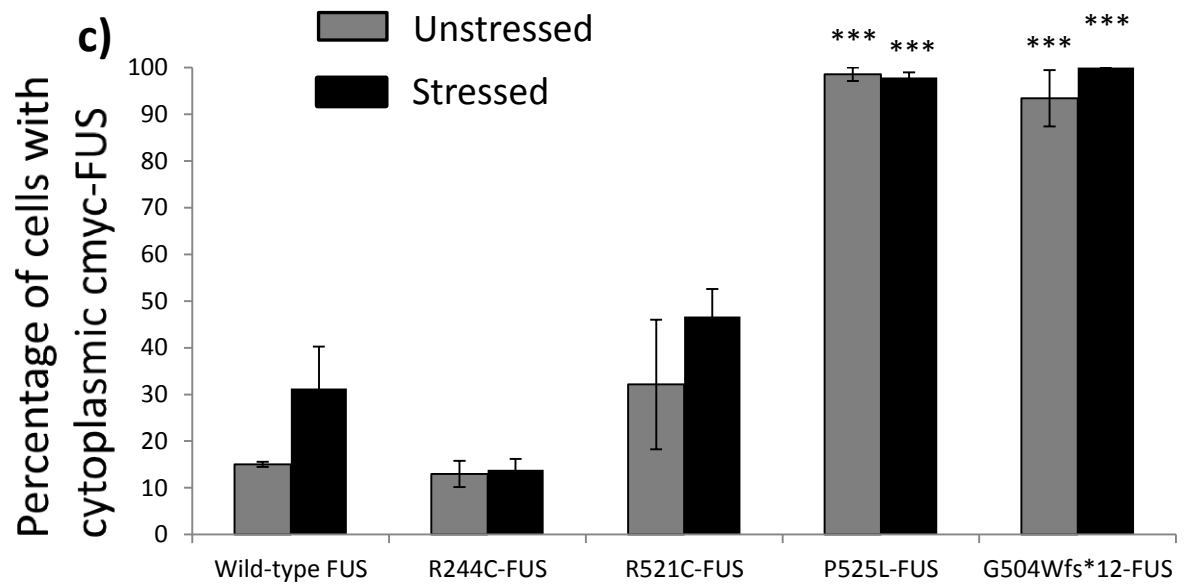


Figure 3.6c) The mean percentage of transfected NSC-34 cells that have cytoplasmic c-myc-FUS, both in the unstressed (grey) and stressed (black) state. **d)** The mean percentage of cells with cytoplasmic granules that stain positive for c-myc and PABP, both in the unstressed (grey) and stressed (black) state. The error bars represent the standard error of the mean. (The error bars represent the standard error of the mean. N=3 experiments, 100 transfected cells per condition each time. One way ANOVA. Tukey post-test.)

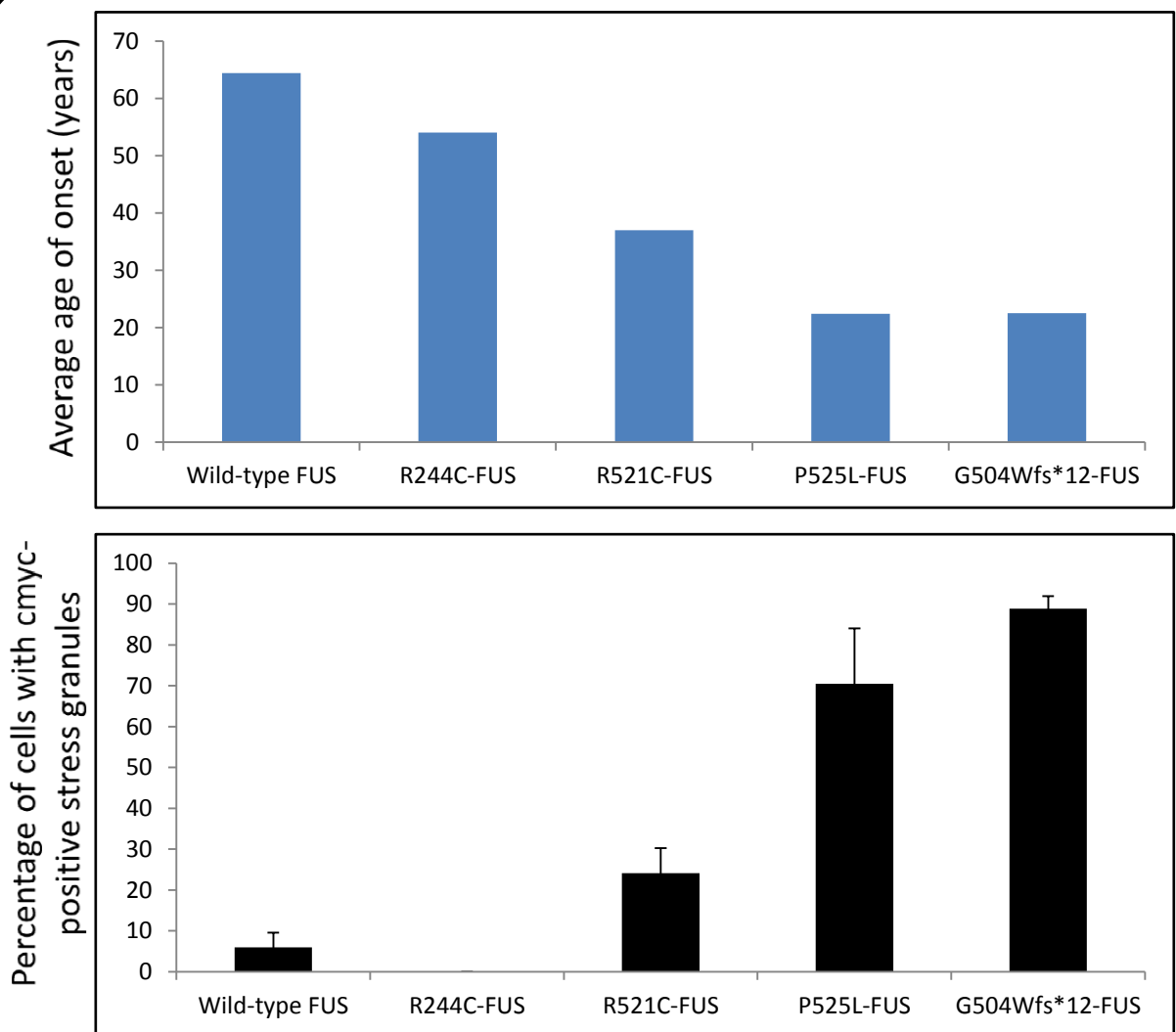
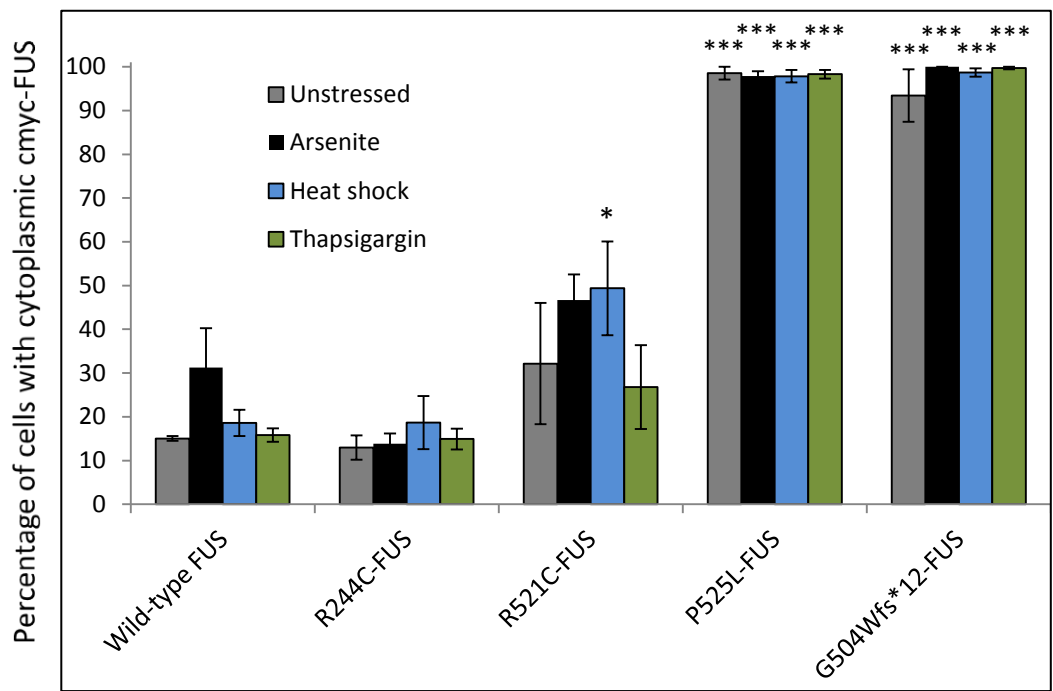


Figure 3.6e) The upper graph shows the average age of onset of ALS with the given mutation (references 5,7,8). For wild-type FUS, the average of onset of sALS has been used. The lower graph is a copy of the graph from figure 3.6b) showing the percentage of NSC-34 cells with c-myc positive stress granules in the presence of each form of FUS. There is an inverse relationship between age of onset and cellular phenotype, suggesting that the extent of cellular phenotype reflects disease severity.

f)



g)

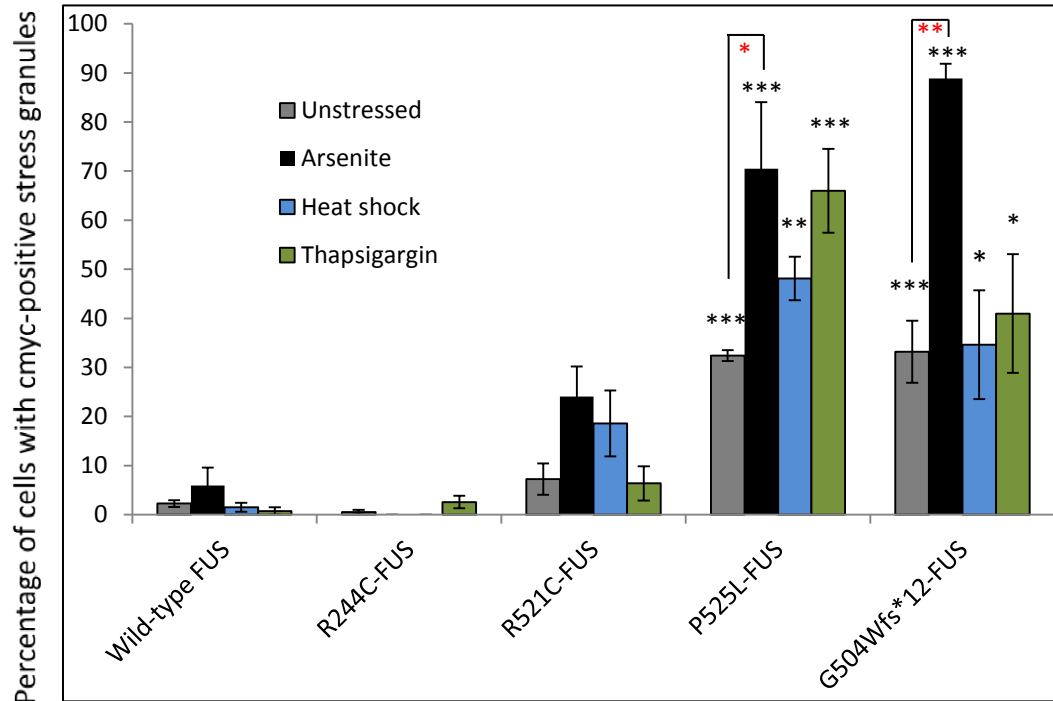


Figure 3.6 Graph showing the effect of different forms of cell stress on NSC-34 cells. (Error bars = SEM. N=3 experiments, 100 transfected cells per condition each time. One way ANOVA. Tukey post-test. Black stars compared to wild-type FUS under the same condition, red stars compared to unstressed within the same mutation.)

f) The percentage of cells with mislocalised FUS under each condition. P525L and G504Wfs*12 FUS have a significantly higher percentage of cells with mislocalised cmyc-FUS compared with wild-type. There is no significant difference between each treatment within each individual mutation. **g)** The percentage of cells with cmyc-positive stress granules. P525L and G504Wfs*12-FUS have a significantly higher percentage of cells with cmyc positive stress granules compared with wild-type under all conditions. Within each mutation, arsenite is the only treatment leading to a significant difference compared to unstressed, and only in the P525L and G504Wfs*12 mutations.

There were no significant differences between each condition in experiments using the same construct, demonstrating that mislocalisation was not significantly altered by any of these forms of cell stress.

The percentage of cells with c-myc positive stress granules was also calculated (figure 3.6g). Again, P525L and G504Wfs*12-FUS had a significantly greater percentage of cells with c-myc positive stress granules compared to wild-type in each condition. However, there was variability between conditions. For the P525L and G504Wfs*12 mutations, there was a significant increase in c-myc granules after arsenite treatment compared to unstressed. However, there was no significant difference between unstressed and either of the other two treatments.

This suggests that arsenite is the most robust trigger for this phenomenon.

3.2.6 G504Wfs*12-FUS is mislocalised to the cytoplasm and co-localises with stress granules in primary neurons

Neurons are the predominant cell type affected in ALS, so it was important to examine whether the G504Wfs*12 mutation displayed a similar phenotype in neuronal cells. Primary cortical neurons were prepared from non-transgenic mouse embryos at embryonic day (E) 16.5. On day 7 in culture they were transfected with *FUS* pcDNA constructs using magnetic beads. After a further 24 hours, cells were stressed with sodium arsenite. Staining with antibodies to c-myc and PABP were again used to allow stress granules and c-myc tagged FUS to be visualised. Again, wild-type FUS was localised predominantly in the nucleus. P525L and G504Wfs*12-FUS appeared to be more cytoplasmic in distribution and co-localised with PABP-positive stress granules (figure 3.7a&b).

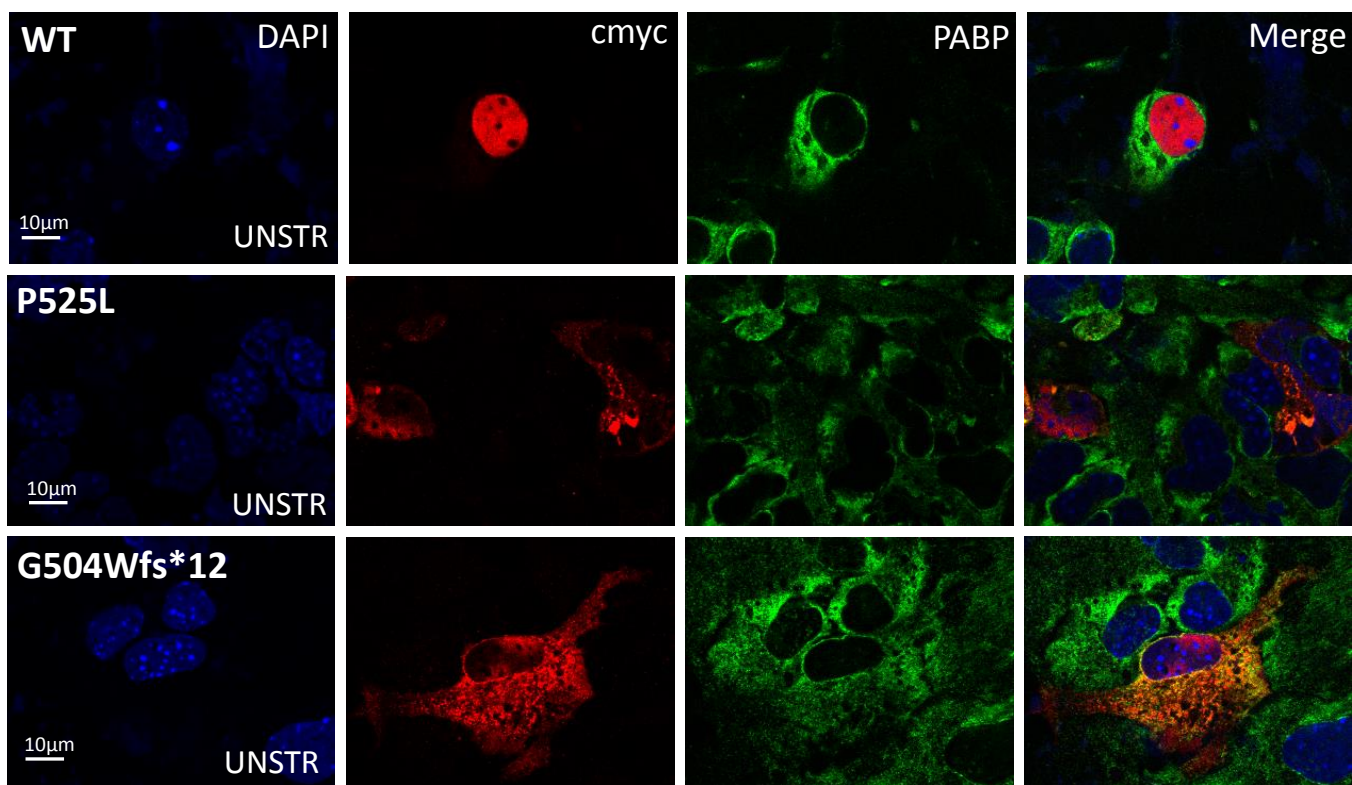


Figure 3.7a) Primary cortical neurons transfected with wild-type, P525L or G504Wfs*12-FUS constructs and stained for c-myc and PABP. Wild-type FUS is nuclear but P525L and G504Wfs*12-FUS have a more diffuse cellular distribution.

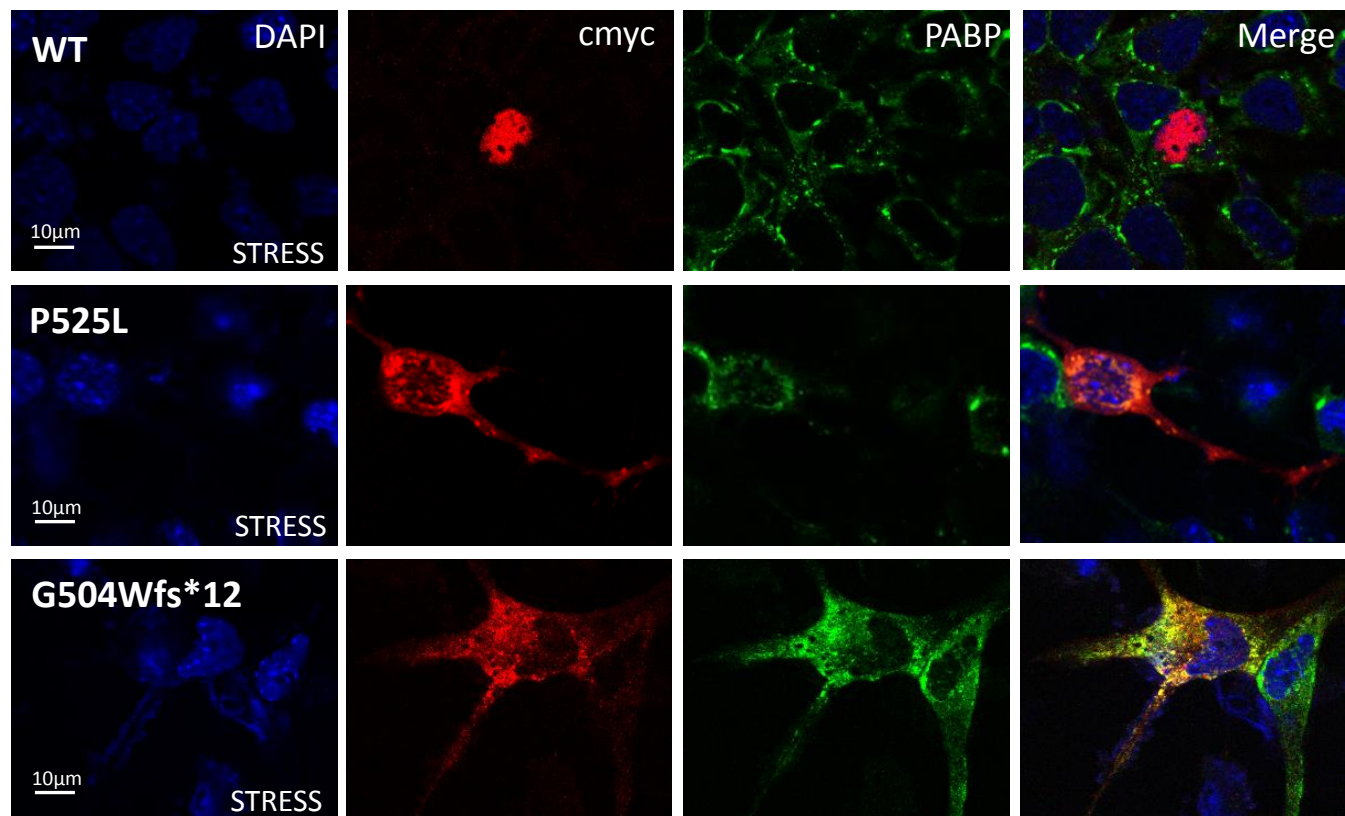


Figure 3.7b) Cortical neurons were stressed with sodium arsenite. PABP staining illustrates the formation of stress granules. Wild-type FUS is nuclear. P525L and G504Wfs*12-FUS have a more cytoplasmic distribution and form granules that co-localise with PABP.

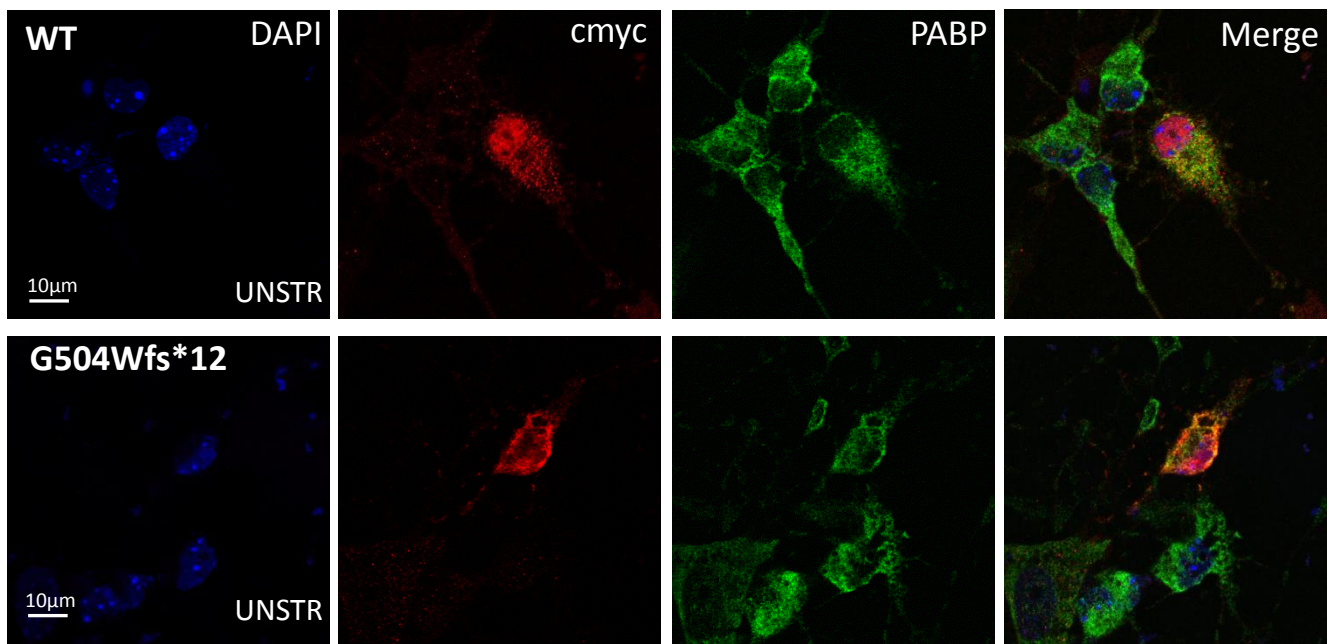


Figure 3.7c) Primary motor neurons transfected with wild-type or G504Wfs*12-FUS constructs and stained for c-myc and PABP. Wild-type FUS is predominantly nuclear but G504Wfs*12-FUS has a more diffuse cellular distribution.

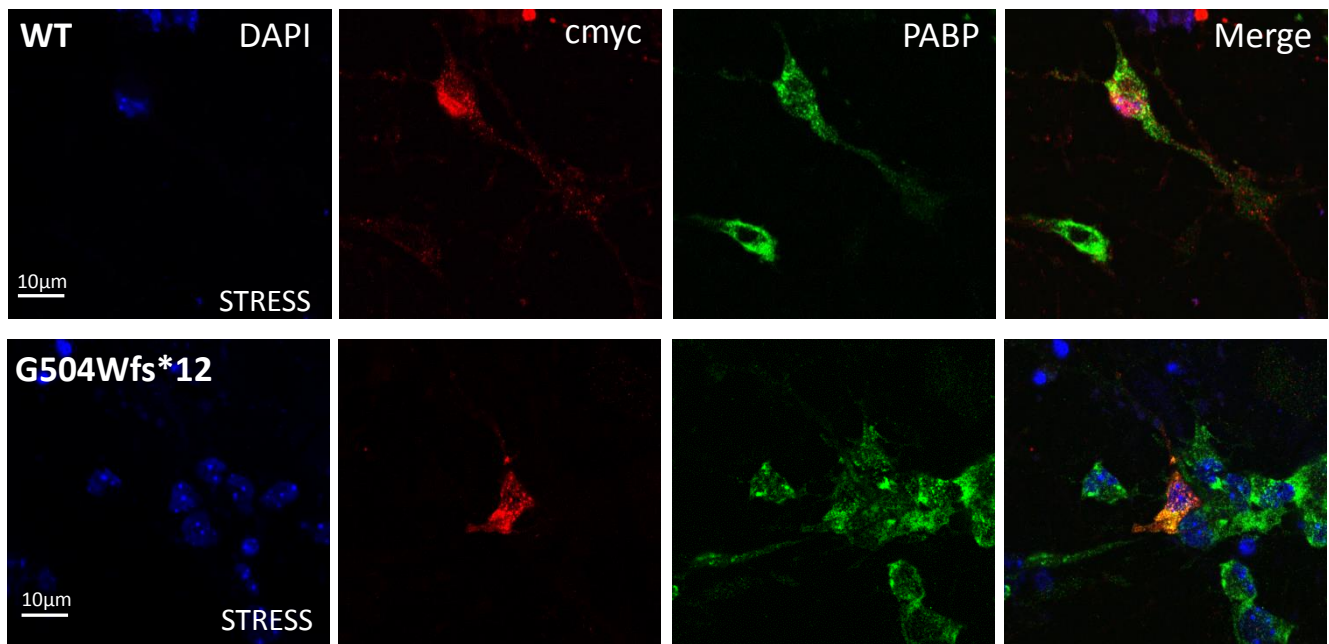


Figure 3.7d) Cells were stressed with sodium arsenite and stained for c-myc and PABP. PABP staining illustrates the formation of stress granules. Wild-type FUS is predominantly nuclear whilst G504Wfs*12-FUS has a more cytoplasmic distribution and co-localises with PABP.

Primary motor neurons were cultured from non-transgenic mouse embryos at E13.5. These were transfected with wild-type and G504Wfs*12-*FUS* pcDNA constructs using magnetic beads. There was again mislocalisation of G504Wfs*12-*FUS* and co-localisation with PABP-positive stress granules in contrast to wild-type *FUS* which appeared mainly nuclear (figure 3.7c&d). Findings in primary neurons therefore reflected observations described in experiments using immortalised cell lines.

3.2.7 *FUS* truncation mutations cause a severe form of ALS

The G504Wfs*12 truncation mutation clearly displayed a severe clinical phenotype in the cases described. It is of interest to determine whether this is a common characteristic of *FUS* truncation mutations in order to further assess how mutations in *FUS* cause motor neuron toxicity. A literature search was carried out to review the characteristics of other reported cases of fALS due to *FUS* truncation mutations. These details are displayed in table 3.1. Clinical phenotype is heterogeneous, even within the same family, with variability in site and age of onset, and disease duration (261, 262). However, the average age of onset is universally low (35.8 years, see table 3.1) and disease duration short compared with sporadic disease (263). Truncation mutations in fALS therefore tend to cause a severe and rapidly progressive form of disease.

Mutation at nucleotide level	Mutation at amino acid level	Familial distribution	Mean age of onset in years [range]	Mean duration in months [range]	Onset [number known]	Reference
c.1432_1478delGGCTATGATCGAGGCGGCTACCGGGGCCGCGGCGGGGACCGTGGAGG	p.G478LfsX23	1 parent, 1 offspring	23.5 [21-26]	23.5 [23-24]	Bulbar [2]	Waibel 2013 (259)
c.1449_1488delCTACCGGGGCCGCGGCGGGGACCGTGGAGGCTCCGAGGG	p.Y485AfsX514	4 cases 1 parent, 3 offspring	-	-	-	Yan 2010 (264)
c.1483delC	p.R495EfsX527	4 cases & 1 carrier 3 generations	47.5 [23-72]	30 [12-48]	Bulbar [1]	Yan 2010 (264)
c.1483C>T	p.R495X	3 cases 3 generations	34.7 [31-37]	14 [12-18]	Bulbar [3]	Waibel 2010 (74)
c.1483C>T	p.R495X	5 cases & 2 carriers 3 generations	29.3 [14-44]	30.6 [9-98]	Bulbar [2] Limb [1]	Yan 2010 (264)
c.1485delA	p.G497AfsX527	3 cases 1 parent, 2 offspring	23.7 [13-29]	14.3 [12-18]	UL [2] LL [1]	Yan 2010 (264)
c.1509_1510delAG	p.G504Wfs*12	3 cases 1 parent, 2 offspring	23.7 [15-33]	8.3 [6-10]	Limb [3]	This report Zou 2013 (76)
c.1527insTGGC	p.K510WfsX517	2 cases 1 parent 1 child	34.5 [23-46]	12.5 [12-13]	Bulbar [2]	Yan 2010 (264)
c.1581delA	p.X527YextX	2 siblings	44 [-]	-	Limb [1]	Kwon 2012 (265)

Table 3.1 illustrates all published fALS cases due to FUS truncation mutations, with clinical details included where available. There is clinical heterogeneity, although the age of onset is consistently low.

3.3 Discussion

This study has identified a *FUS* truncation mutation to be the cause of an aggressive form of autosomal dominant- inherited fALS. A *FUS* construct was generated that recapitulated the human G504Wfs*12 mutation and its cellular phenotype characterised. This mutation has not previously been described in familial disease, but a sporadic case due to a *de novo* mutation has been reported, causing an aggressive form of ALS with onset aged 19 years (78). Truncation mutations have been reported in both sALS and fALS, and they are generally associated with rapidly progressive clinical disease and a young age of onset (table 3.1) (74, 78, 264, 265). It is notable that in many of the familial cases, such as the example described here, the disease only survives one or two generations (figure 3.1). This is not surprising given the young age of onset and therefore decreased likelihood of having had children before the disease develops. However, there is some clinical heterogeneity even within each family.

Cases 2 and 3 would conventionally be described as juvenile ALS, occurring in an individual under 25 years old (5). *FUS* mutations account for a relatively high proportion of young-onset cases, again reflecting the fact that *FUS*-ALS can lead to particularly severe disease (75, 78, 266, 267).

Cases of ALS, particularly familial forms, can be associated with a broad phenotype, which can overlap with other neurodegenerative diseases such as Parkinson's disease (268). This heterogeneity can occur even within the same family (264, 269, 270). Although this is mainly reported with mutations in other genes such as *SOD1*, features such as learning difficulties, Parkinsonism and FTD have been reported in

patients with *FUS* mutations (81, 264, 271). It is therefore possible that this G504Wfs*12 *FUS* mutation could cause the clinical syndrome described in case 4. However, these results show that case 4 does not carry the G504Wfs*12 mutation (figures 3.3 & 3.5). This fits more closely with her clinical syndrome which resembles progressive supranuclear palsy (PSP). This neurodegenerative condition causes rigidity, falls, gaze limitation, Parkinsonism and cognitive impairment. The vast majority of cases are sporadic, as is likely in this case (272). The neuropathology of PSP is most commonly a tauopathy, though case 4 did not consent to an autopsy so the neuropathology of her condition is not known. The patient did display some atypical features such as long duration of illness, but her syndrome most closely resembles PSP clinically. This is a marked contrast to the rapidly progressive motor neuron syndrome occurring in the other cases in her family. In addition, learning difficulties are the only additional feature reported in fALS with *FUS* truncation mutations (74, 78, 259, 264). All reported cases of this particular mutation have caused a young onset, rapidly progressive motor neuron syndrome (78). These facts support the conclusion that case 4 does not carry this mutation but incidentally developed PSP in older age. These results do not rule out other genetic risk factors for neurodegeneration which could have increased the risk or severity of neurological disease in all family members. However, given the toxicity of reported *FUS* truncation mutations then the G504Wfs*12 mutation is likely to be the primary cause of disease in cases 1-3 described here.

It may be inferred that this mutation occurred *de novo* in the index case. Though cases 1 and 2 have not been tested directly, they may be assumed to carry the same mutation given the rarity of disease and similar clinical course. The father of

the index case had no history of neurological disease and died in his 70s from cancer. It is highly unlikely, though not impossible, that he carried this aggressive mutation to late life without developing disease. The mutation occurred *de novo* in the only other reported case with this mutation (78).

G504Wfs*12-FUS overexpression *in vitro* resulted in a severe cellular phenotype with significantly increased FUS mislocalisation and co-localisation with stress granule markers compared to wild-type FUS, in a pattern similar to P525L-FUS. This was true for HEK293, NSC-34, primary cortical and primary motor neurons. Co-localisation determined by confocal microscopy implies that mutant FUS is accumulating within these granules. The presence of FUS in the cytoplasm appears to be a prerequisite for this phenomenon, as mutations that did not cause FUS mislocalisation, such as the R244C mutation, did not develop FUS positive stress granules. Wild-type FUS does not usually accumulate in stress granules, though this phenomenon has been reported when it is localised to the cytoplasm by restricting nuclear entry (102). This suggests that mutations in the NLS do not themselves cause FUS to enter stress granules, but rather they increase cytoplasmic FUS where it is available for incorporation into stress granules after the appropriate stimulus. This is supported by the fact that mislocalisation of FUS is not altered after cell stress (figure 3.6f). This adds to the evidence that suggests that FUS is not drawn from the nucleus into stress granules but must be present in the cytoplasm in order to accumulate in these structures (60, 148).

These results suggest that mutations with a more severe cellular phenotype produce clinical disease with a younger age of onset (figure 3.6e). Age of onset is used here

as a measure of disease severity, with a younger onset indicative of a more severe clinical manifestation. Severity can be subjective and difficult to quantify. However, various measures may be used as markers of severity such as disease duration from onset to death, decline in ALSFRS-R score and decline in respiratory function. Age of onset is the most commonly reported in scientific papers, so is used in this study to illustrate the relationship with cellular phenotype.

Previous studies using cell lines have documented FUS mislocalisation and co-localisation with stress granules in P525L-FUS and various truncation mutations (102, 148). These all have a severe cellular phenotype compared with other mutations studied. Indeed, mutations in the NLS in general appear to cause more mislocalisation. This is intuitive since this is the region involved in the transport of FUS from the cytoplasm to the nucleus (102). If this process is disrupted and FUS is less able to enter the nucleus, cells will display an increase in cytoplasmic FUS as shown in this study.

The development of c-myc positive stress granules in P525L and G504Wfs*12-FUS was observed after a range of different forms of cell stress, but is also observed to some extent in unstressed cells. Arsenite was the only form of stress to cause a significant increase in c-myc positive stress granules compared to unstressed. This may be due to fewer stress granules forming after thapsigargin or heat shock treatment, or a lack of ability to incorporate mutant FUS into stress granules. It may also be that activation of HRI, the eIF2 α kinase response to oxidative stress, is a more sensitive pathway than others in initiating phosphorylation of eIF2 α and hence the formation of stress granules (214). If cell stress is involved in disease

pathogenesis in humans, then these results suggest that oxidative stress via the activation of HRI may be a greater threat to motor neurons than other forms of cellular stress.

The presence of stress granules in some cells without additional cell stress does suggest that the presence of these mutations alone, without additional stress, may be toxic to cells (figure 3.6b&d). However, the cell culture environment, the process of transfection and overexpression of FUS may all cause some degree of stress even in the absence of further treatments. Further work, for example using models that avoid FUS overexpression and studying this mutation *in vivo* can overcome some of these limitations and help to determine whether the mutation alone causes cell toxicity.

These results support the hypothesis that *FUS* mutations with a more severe cellular phenotype and greater FUS mislocalisation may be more toxic to motor neurons, causing more aggressive clinical disease. Mutations in other regions such as the glycine-rich region are still able to cause ALS in some families (40). This implies that the region of FUS involved does not affect the potential for causing ALS, but can modify toxicity in some way. One hypothesis to explain this is that *FUS* mutations affecting the NLS cause mislocalisation of FUS from the nucleus to the cytoplasm even prior to disease onset, as seen these experiments. If disease is initiated after a 'second hit' causing misfolded FUS, then the presence of cytoplasmic FUS could increase the likelihood of this initiation event occurring. This would tend to cause disease at a young age. Once the process has started, the presence of high levels of FUS in the cytoplasm could lead to rapid recruitment of this protein to form

cytoplasmic aggregates. It may also cause disease to spread more rapidly between neurons. Cell death occurs quickly, creating a more rapidly progressive disease. Truncation mutations lack most or all of the NLS, often causing the most aggressive forms of disease (78, 259). In contrast, when mutations are not in the NLS, for example the R244C mutation, FUS is present predominantly in the nucleus. The potential for disease is present, but the chance of disease initiation is less compared to when FUS is in the cytoplasm which would generally lead to disease at an older age. Once disease is initiated, FUS begins to accumulate in the cytoplasm. This process is slower due to the lack of cytoplasmic FUS, and may cause slower spread of disease, leading to slower disease progression. This would explain how the distribution of FUS could modulate toxicity.

There is still some debate about whether the disease 'spreads' and how this might occur. An alternative to the above hypothesis is that there is no 'spread' of disease. Each motor neuron is pre-programmed with a mutation, with additional modifying factors in the genome which could be altered by environmental factors. Mutant FUS could cause some disruption to the cell, but the cell is able to compensate, for example by using autophagy to remove aggregated protein as described for TDP-43 (247). However, a threshold is reached where cells can no longer compensate, resulting in motor neuron dysfunction, and clinical onset of disease. In this model, toxicity lies solely in the mutated protein itself, and does not rely on further 'hits' or spread. In reality, there may be some combination of the two mechanisms involved. Either way, it is clear that some mutations in *FUS* are consistently more toxic than others, and that disruption of the NLS is a good predictor of severe disease.

There are limitations of this study. It uses an overexpression model, and overexpression of wild-type FUS is not physiological and has been shown to cause neurodegeneration in rodent models (110, 235). Further work will aim to assess the effects of mutant FUS expressed at levels similar to that of endogenous FUS, to avoid the pitfalls of overexpression. The treatments used to cause stress are used as a way of examining their responses to extreme conditions rather than as a reflection of what they experience *in vitro*, which are likely to be more subtle. It can be used as a model to examine the differences between mutant and wild-type FUS, but it does not prove that these processes are occurring in humans with *FUS* mutations. Primary neurons reflect the cell type of interest more closely. However, results are limited by the relatively low yield of cells per experiment, particularly with motor neurons. Transfection occurs at low rates in these cells, and creates variable levels of expression within the cell population. Quantification is therefore difficult. Further work using motor neurons from transgenic models expressing mutant FUS provide a more homogenous population of neurons to study.

CHAPTER 4: IN VITRO ANALYSIS OF THE EFFECTS OF *FUS* AND *TARDBP* MUTATIONS ON THE LOCALISATION OF RNA-BINDING PROTEINS IMPLICATED IN ALS, FTD AND SMA.

4.1 Introduction and aims

FUS overexpression is detrimental to neurons and does not provide a realistic model of ALS (110). However, transfection with pcDNA constructs leads to variable and increased expression of exogenous compared to endogenous *FUS*. In order to tackle these limitations, a BAC construct was developed by Richard Wade-Martins' research group in Oxford (figure 4.1a). This large 59.5 kb construct was designed to contain the whole human *FUS* gene and regulatory elements including the promoter region, together with an N-terminal mCherry tag. The aim of this construct was to express *FUS* at levels comparable to endogenous *FUS*, as is likely to occur in human disease. This was used for *in vitro* studies and to create a *FUS* transgenic mouse model expressing either wild-type or P525L mutant *FUS*. This chapter contains 5 aims which will address; 1) initial characterisation of *FUS*-BAC expression in a human cell line, 2) characterisation of primary neurons from the novel *FUS*-BAC mouse, 3) the effect of genetic ablation of *FUS* on motor neurons, 4) interaction of TDP-43 with FET family members, and 5) the consequence of *FUS* mutation on SMN in neurons from the *FUS*-BAC mice.

Aim 1: Characterisation of a *FUS*-BAC cellular phenotype in an immortalised cell line

The first aim of this chapter was to investigate the cellular phenotype of immortalised cell lines transfected with this construct. Although transfection was likely to create variable levels of expression between cells, the presence of regulatory elements

should limit overexpression. These experiments first examined whether these constructs successfully express human FUS. Cellular phenotype was then examined to see whether lower levels of mutant FUS lead to similar changes to those observed with pcDNA constructs, such as mislocalisation of mutant FUS.

Aim 2: Characterisation of primary motor and cortical neurons from a novel FUS-BAC mouse

ALS is predominantly a disease of motor neurons, so these cells are the most relevant model to study the mechanisms of disease. There are limitations in transfecting these neurons as the rates of transfection are low and the transfection process itself may damage these cells. In addition, transfection tends to cause overexpression and variable levels of FUS expression between cells which again reduces its relevance to disease. In order to tackle these limitations, the FUS-BAC mouse model was used to create primary motor neuron cultures to create a population of motor neurons with a more homogenous expression of FUS, avoiding the need for transfection. These neurons expressed low levels of FUS from the construct, avoiding the complications associated with overexpression. The aim of this work was to characterise the phenotype of motor neurons from this mouse model in order to examine potential mechanisms of toxicity associated with the *FUS* P525L mutation. Cortical neurons from this model were also used to examine whether changes are motor neuron specific or more widely observed. Cortical neuronal cultures also allowed a greater number of conditions to be examined as more cells could be produced.

Aim 3: Examination of the effect of FUS-deficiency in primary motor neurons

The mechanism of toxicity of mutant FUS is still not confirmed, though it has been hypothesised that mutations cause loss of function of FUS (107, 230). The potential mechanism of this is the sequestration of FUS into aggregates within the cytoplasm resulting in a lack of availability to carry out its normal cellular functions. In order to examine this further, primary motor neuron cultures from a FUS knockout mouse model were created. The aim of this was to examine the cellular phenotype of these neurons and compare them with neurons expressing mutant FUS. Should similar changes be observed in each model, it would support the hypothesis that there is a loss of function of FUS in the presence of a mutation.

Aim 4: Examination of the interaction of TDP-43 with FET family members

TDP-43 is an RNA binding protein with similar functions to FUS. This has led to the hypothesis that they cause ALS via a similar mechanism or even through the same pathway (166, 273, 274). A BAC transgenic mouse model expressing human wild-type or M337V mutant TDP-43 was used in this study. In this model, mutant TDP-43 has been shown to display increased mislocalisation to the cytoplasm of motor neurons compared to wild-type TDP-43 (Gordon et al, in progress). Motor neurons from this model were used to examine the hypothesis that mutant TDP-43 sequesters FET proteins into the cytoplasm, causing a functional deficiency of FUS or the related family members EWS and TAF-15. This would link TDP-43 and FUS-ALS in a common mechanism.

Aim 5: Addressing the consequence of mutant FUS on SMN

Deficiency of the SMN protein causes degeneration of motor neurons in the condition SMA (29). FUS and SMN are able to interact, and it has been hypothesised that *FUS* mutations sequester SMN in cytoplasmic FUS-positive inclusions, creating functional SMN deficiency (108). A loss of nuclear gems has been reported in SMN deficiency and in the presence of *FUS* mutations. However, studies to examine this hypothesis have mainly been in cell lines overexpressing SMN, FUS or both (107, 108). The aim of this work was to examine the relationship between FUS and SMN in a system without such overt overexpression, using mutant FUS expressed via the BAC construct.

4.2 Results in the FUS-BAC transgenic mouse model

4.2.1 FUS expressed using a BAC construct in HEK293 cells

A diagram of the BAC construct is shown in figure 4.1a). The first stage of this work was to transfect these FUS-BAC constructs into immortalised cell lines to confirm the ability of the BAC construct to express FUS and examine the cellular phenotype.

Transfection using lipofectamine was not successful in NSC-34 cells (data not shown) but was possible using HEK293 cells. Rates of transfection were relatively low at about 4-5%, with no difference between each construct (figure 4.1b).

Expression of mCherry-FUS in transfected lines was confirmed by Western blotting using whole cell lysates prepared from transiently transfected HEK293 cells (figure 4.1c). An antibody to total FUS (endogenous and BAC-derived) revealed the presence of endogenous FUS in each line (75kDa) with an additional 104 kDa band in transfected cells, representing mCherry-FUS. Quantification of mCherry-FUS

expression using an antibody raised to the mCherry tag suggested that mutant FUS was expressed at a higher level than wild-type, despite similar transfection rates (appendix 7.1). There is also a tendency for endogenous FUS to be expressed at lower levels in cells transfected with FUS-expressing BAC constructs, which could be explained by transcriptional levels of endogenous FUS being autoregulated by human FUS expressed via the BAC construct (109). An estimate of mCherry-FUS protein expression relative to endogenous FUS was made using the transfection rates and expression levels (methods 2.6.4 & appendix 7.1d). This suggested that FUS expression from the BAC was between 0.2 and 2.4 times that of endogenous. These are relatively low levels from a construct and, although only an estimate, support the theory that FUS expressed using these BAC constructs avoids the large amount of overexpression induced with pcDNA constructs.

Native fluorescence from the mCherry tag was used to assess the cellular distribution of BAC-derived FUS. Wild-type, R244C and R521C-FUS were nuclear, whilst P525L-FUS showed mislocalisation to the cytoplasm (figure 4.2a).

Quantification of the percentage of cells with mislocalised mCherry-FUS demonstrated this marked phenotype in the P525L-FUS expressing cells (figure 4.3a; $p < 0.001$, P525L FUS versus wild-type FUS), similar to that observed with pcDNA-derived P525L-FUS. Some cells expressing R521C-FUS showed mislocalised FUS, but this was not significantly different from wild-type and not as prominent as that observed with the pcDNA construct.

Some P525L-FUS expressing cells developed mCherry-positive stress granules in the absence of additional treatment to stress the cells, though this does not reach statistical significance compared to wild-type FUS (figure 4.3b).

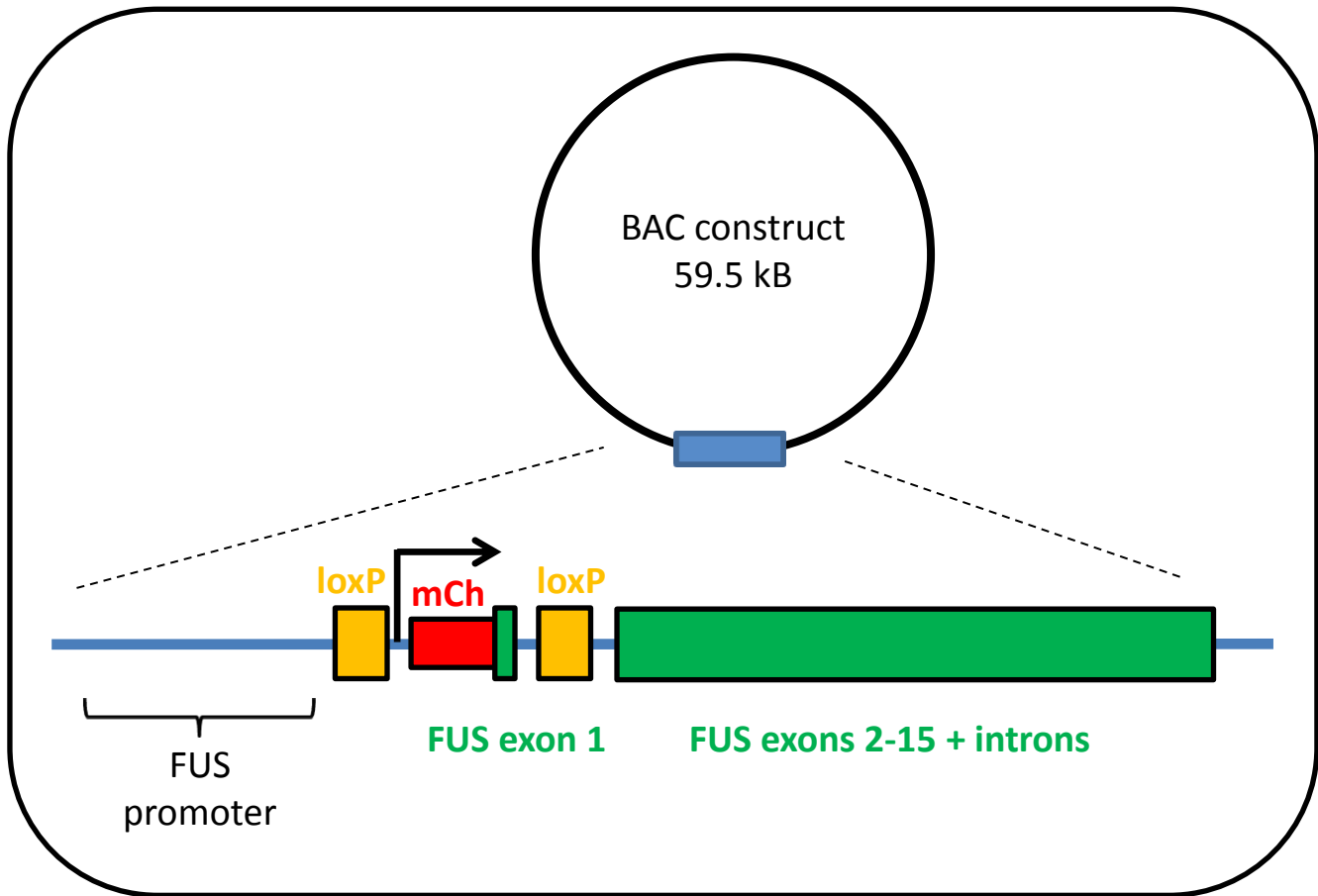


Figure 4.1a) Diagram of the FUS BAC construct. It encodes the whole human FUS gene including regulatory regions in order to express human FUS with an mCherry tag. 4 constructs were created to express wild-type, R244C, R521C and P525L-FUS.

Percentage of cells transfected
with the BAC construct

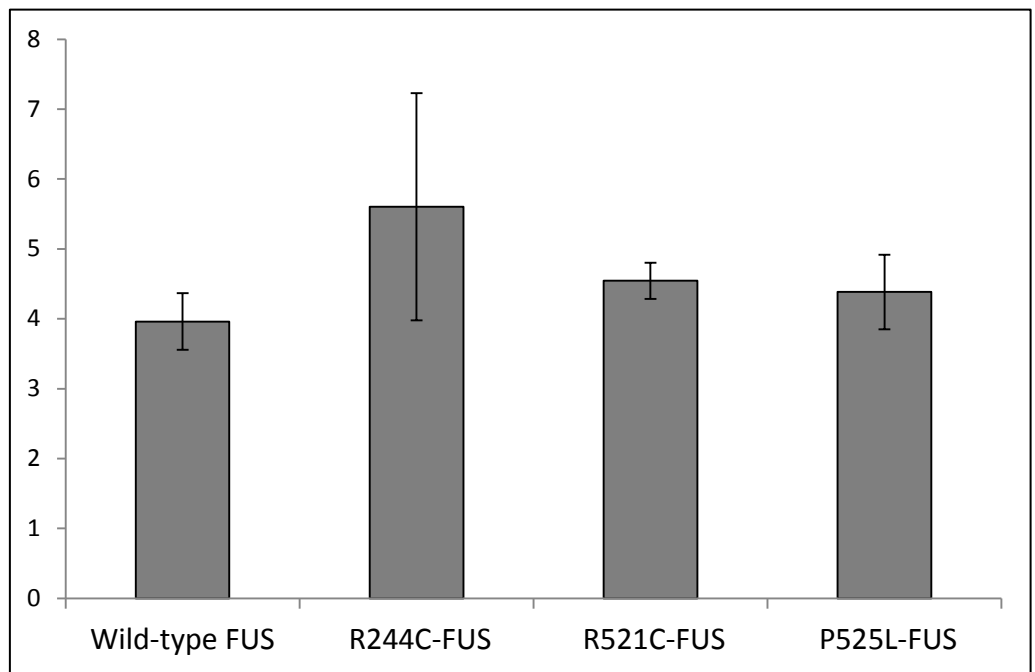


Figure 4.1b) Transfection rates of HEK293 cells transfected with the given FUS BAC construct using lipofectamine. There is no significant difference between the constructs. (N=3 experiments, 100 cells each. Error bars =SEM. One way ANOVA, Tukey post-test.)

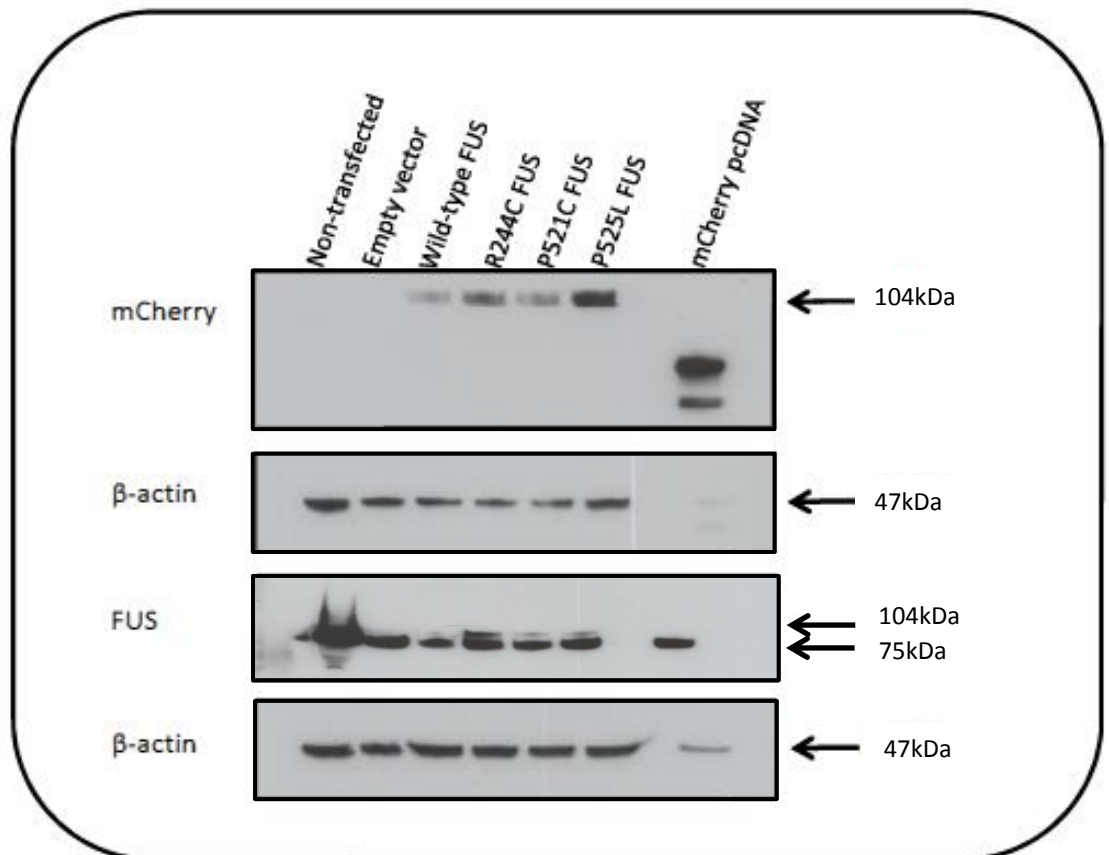


Figure 4.1c) Western blots of cell lysate from HEK293 cells transfected with the given BAC construct. mCherry is expressed in all lines transfected with the construct. mCherry-FUS produces a 104kDa protein detected using an antibody against FUS. 107

4.2.2 The effect of cell stress on wild-type and mutant BAC-derived FUS in HEK293 cells

Cells were stressed with 0.5 mM sodium arsenite for 60 minutes to induce the formation of stress granules as demonstrated by PABP staining. Within the sodium arsenite-treated cells wild-type, R244C and R521C-FUS remained localised almost exclusively within the nucleus of transfected cells. P525L-FUS formed cytoplasmic granules that co-localised with PABP, suggesting incorporation of mutant FUS into stress granules (figure 4.2b). This was quantified by assessing the percentage of cells with mislocalised mCherry-FUS and with mCherry-positive stress granules, in both untreated cells and cells treated with arsenite (figure 4.3). After cell stress, mislocalisation remained at a similar level but the majority of cells expressing P525L-FUS developed mCherry-positive stress granules ($p < 0.001$). These results match the observations made using pcDNA-FUS constructs.

4.2.3 BAC-derived mutant FUS does not co-localise with SMN

To examine the hypothesis that mutant FUS can sequester SMN in cytoplasmic granules, HEK293 cells were transfected with wild-type and P525L-BAC constructs. The P525L mutation was examined as it forms more cytoplasmic granules in response to stress, which are potential sites of FUS-SMN interaction. Confocal microscopy was used to assess the distribution of mCherry and SMN.

SMN was distributed as expected, with staining in the cytoplasm and in nuclear gems (figure 4.4). Nuclear gems were visible when present in the given focal plane. Wild-type FUS remained nuclear so, as expected, it did not co-localise with cytoplasmic SMN. In the unstressed P525L-FUS expressing cells, FUS was more cytoplasmic in distribution.

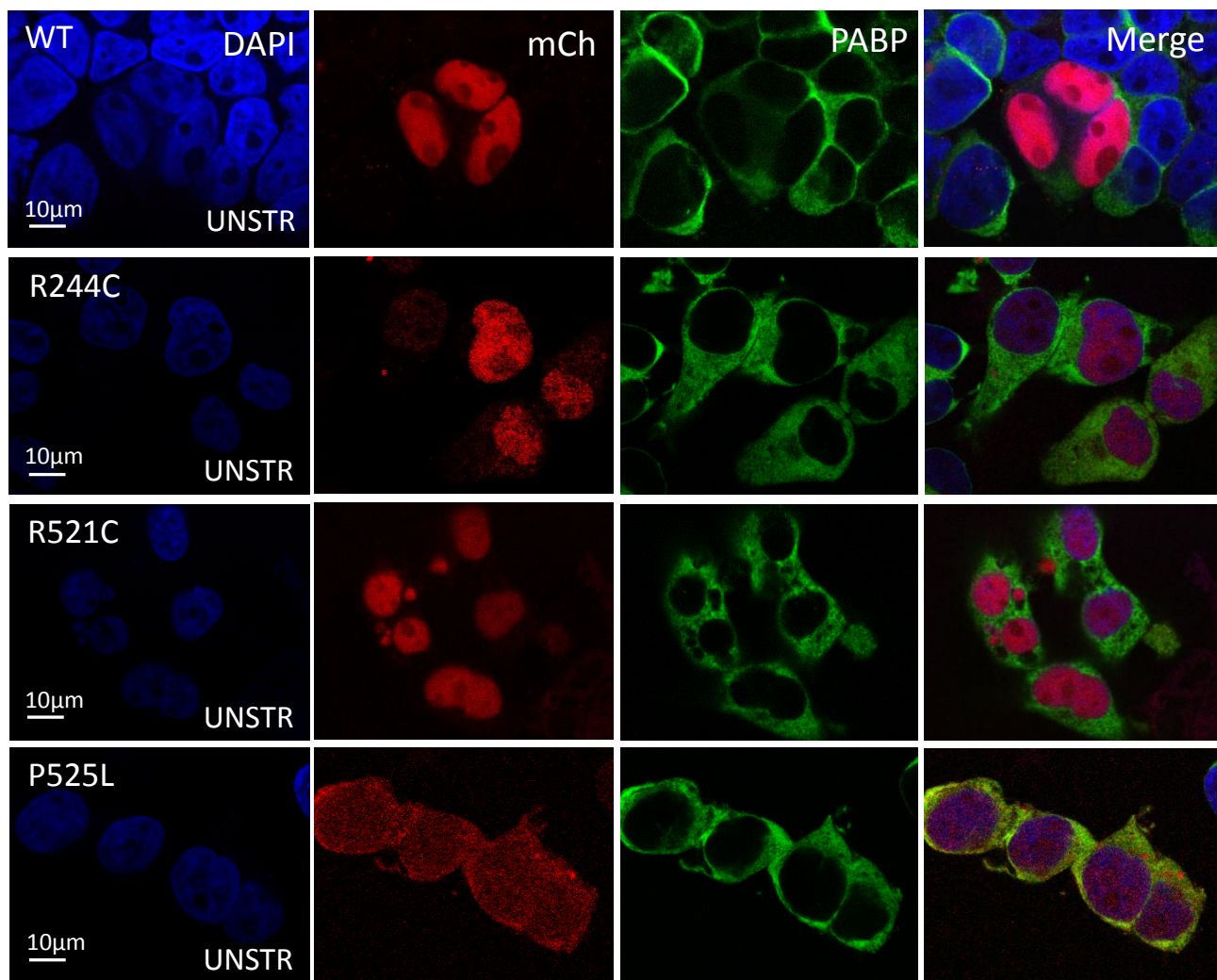


Figure 4.2a) Confocal images of HEK293 cells transfected with the given BAC construct then stained for DAPI, mCherry and PABP. Transfected cells are detected by the presence of mCherry. Wild-type, R422C and R521C-FUS remain nuclear. P525L-FUS is mislocalised to the cytoplasm with evidence of nuclear clearing.

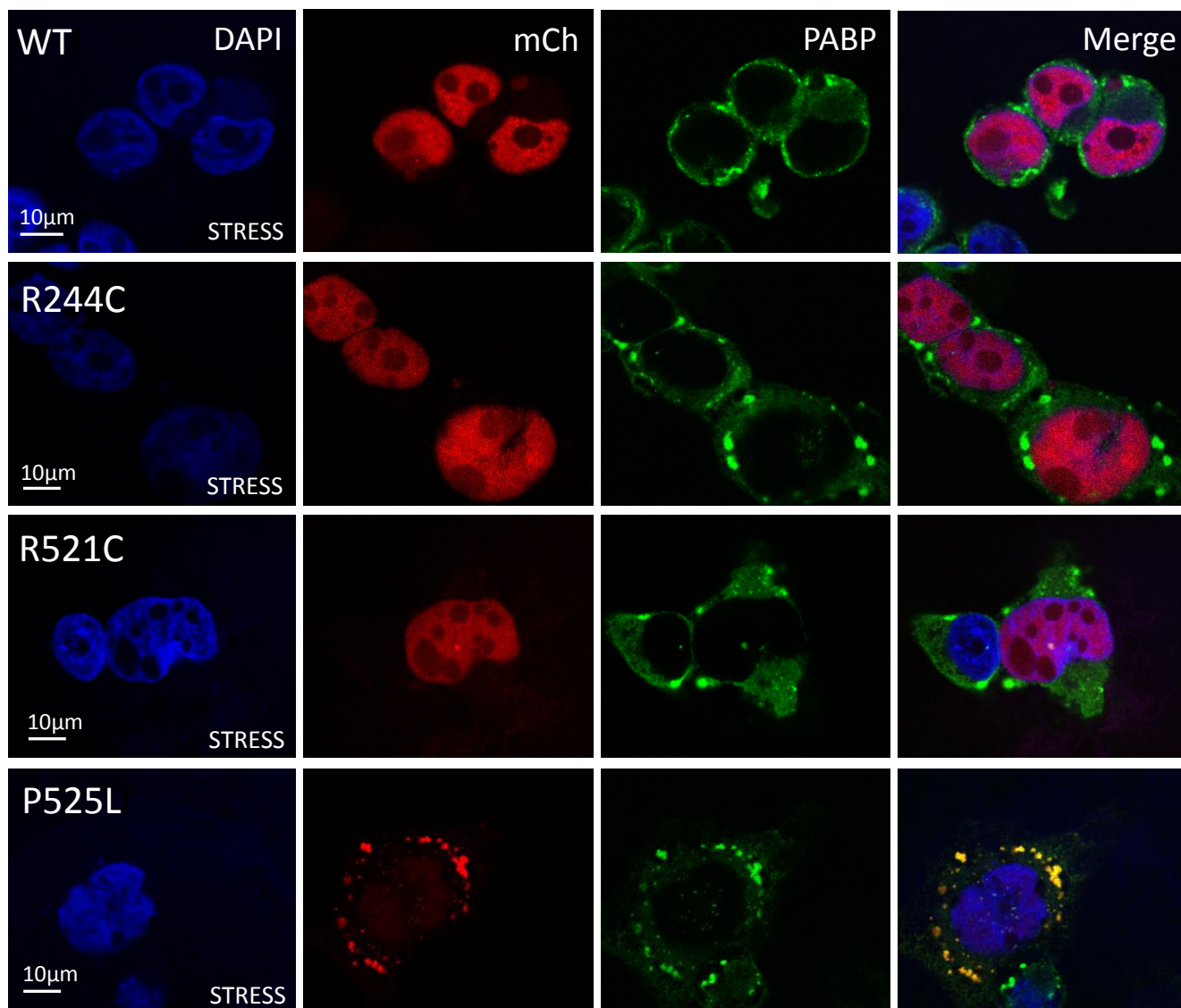


Figure 4.2b) Confocal images of HEK293 cells transfected with the given BAC construct, stressed with 0.5 mM sodium arsenite for 60 minutes, then stained for DAPI, mCherry and PABP. PABP-positive stress granules are visible in the cytoplasm. WT, R244C and R521C-FUS are nuclear in distribution. There is nuclear clearing of P525L-FUS and it is visible in cytoplasmic granules that co-localise with PABP.

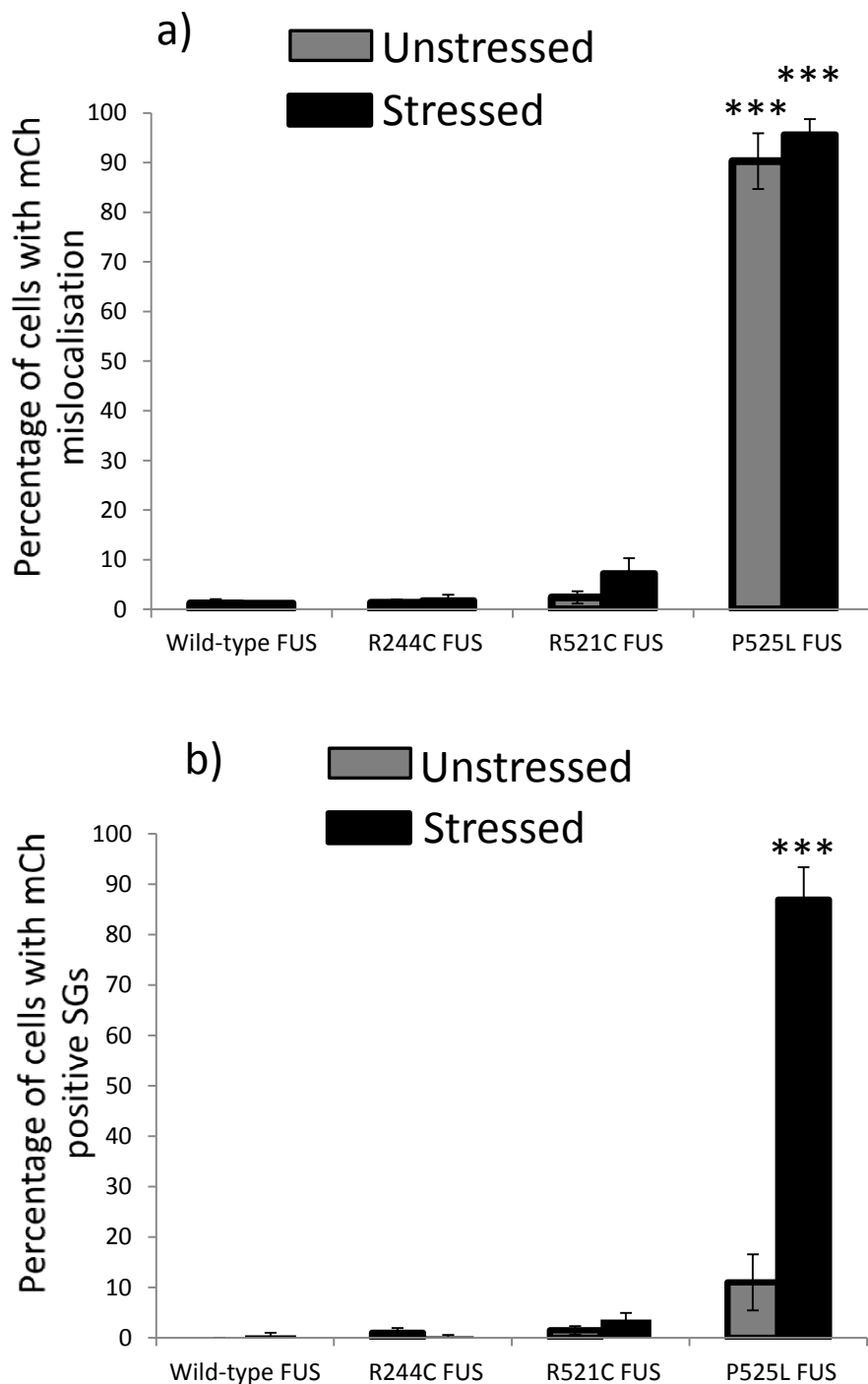


Figure 4.3 Quantification of the phenotype of HEK293 cells transfected with the given BAC construct. **a)** The percentage of cells with mislocalised mCherry-FUS as detected by immunofluorescence microscopy. There is a significant increase in P525L-FUS compared to the other constructs, both in the unstressed state and after sodium arsenite treatment (stressed). **b)** The percentage of cells with mCherry-positive stress granules as detected by immunofluorescence microscopy. After sodium arsenite treatment there is significant increase in P525L-FUS expressing cells compared to the other constructs. (N=3 experiments, 100 transfected cells each. One way ANOVA. Tukey post-test. Compared to wild-type under the same condition.)

In most cells this was diffuse and therefore there was some overlap with SMN. However, there was very little overlap between the SMN and mCherry staining, suggesting that the proteins did not co-localise. In the P525L-FUS stressed cells, despite the visible P525L-FUS and SMN granules, these were distinct and did not co-localise (figure 4.4). The exception to this was in two larger granules in the unstressed P525L condition, where there was co-localisation. This suggests that in larger, more aggregate-like, granules there may be some potential for interaction, though this type of granule was rarely observed.

4.2.4 Primary motor neuron cultures from the FUS-BAC transgenic mouse model

In order to study a more disease-relevant cell type, primary motor neuron cultures were created using the FUS-BAC transgenic mouse model (see methods 2.8.1 and appendix table 7.3). Wild-type line 2 and P525L line 1 low expressing lines were used so observed effects would be due to the mutation itself. Transgenic mice hemizygous for the BAC insertion were crossed with non-transgenic mice to produce both non-transgenic and BAC-positive embryos. Primary motor neuron cultures were set up using the p75^{NTR}-immunopanning method, and embryos were cultured individually due to their unknown genotype. The remaining tissue from the embryos was used for DNA extraction and genotyping (figure 4.5a).

Cultured neurons displayed neuronal morphology and were positive for the neuronal marker Tuj1 and the motor neuron marker ChaT, confirming that these cultures contained a population of motor neurons (figure 4.5b).

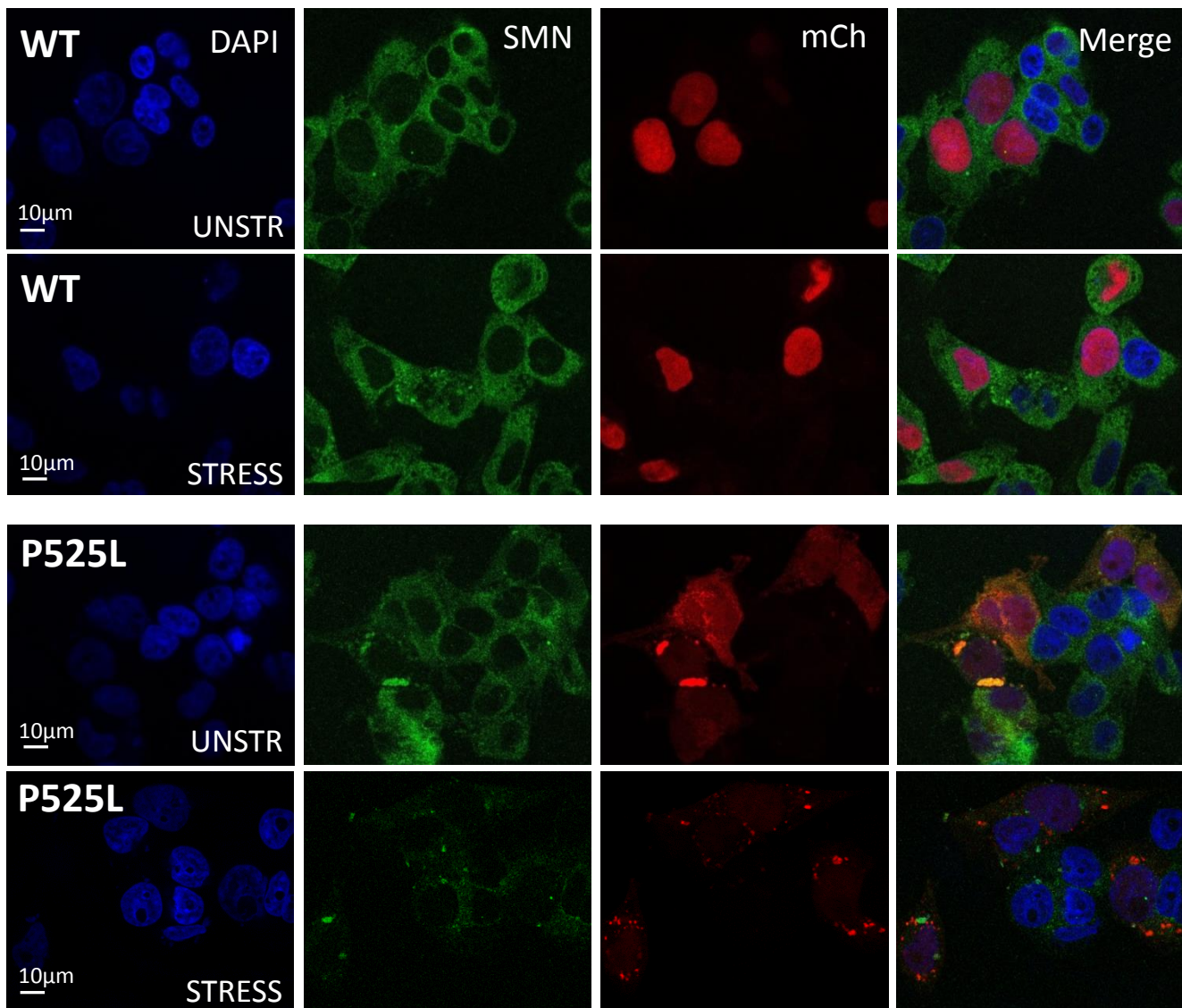


Figure 4.4. HEK293 cells transfected with the given BAC construct and stressed where stated with 0.5 mM sodium arsenite for 60 minutes, then stained for DAPI, SMN and mCherry. SMN has a diffuse cytoplasmic localisation with some visible nuclear gems. After stress, it is occasionally seen in cytoplasmic granules. There is no co-localisation with wild-type FUS which remains nuclear. In the P525L unstressed state, the diffuse cytoplasmic location of each protein leads to some apparent co-localisation. This is particularly apparent in 2 large cytoplasmic granules. However, in the stressed condition the SMN and mCherry-FUS granules do not co-localise.

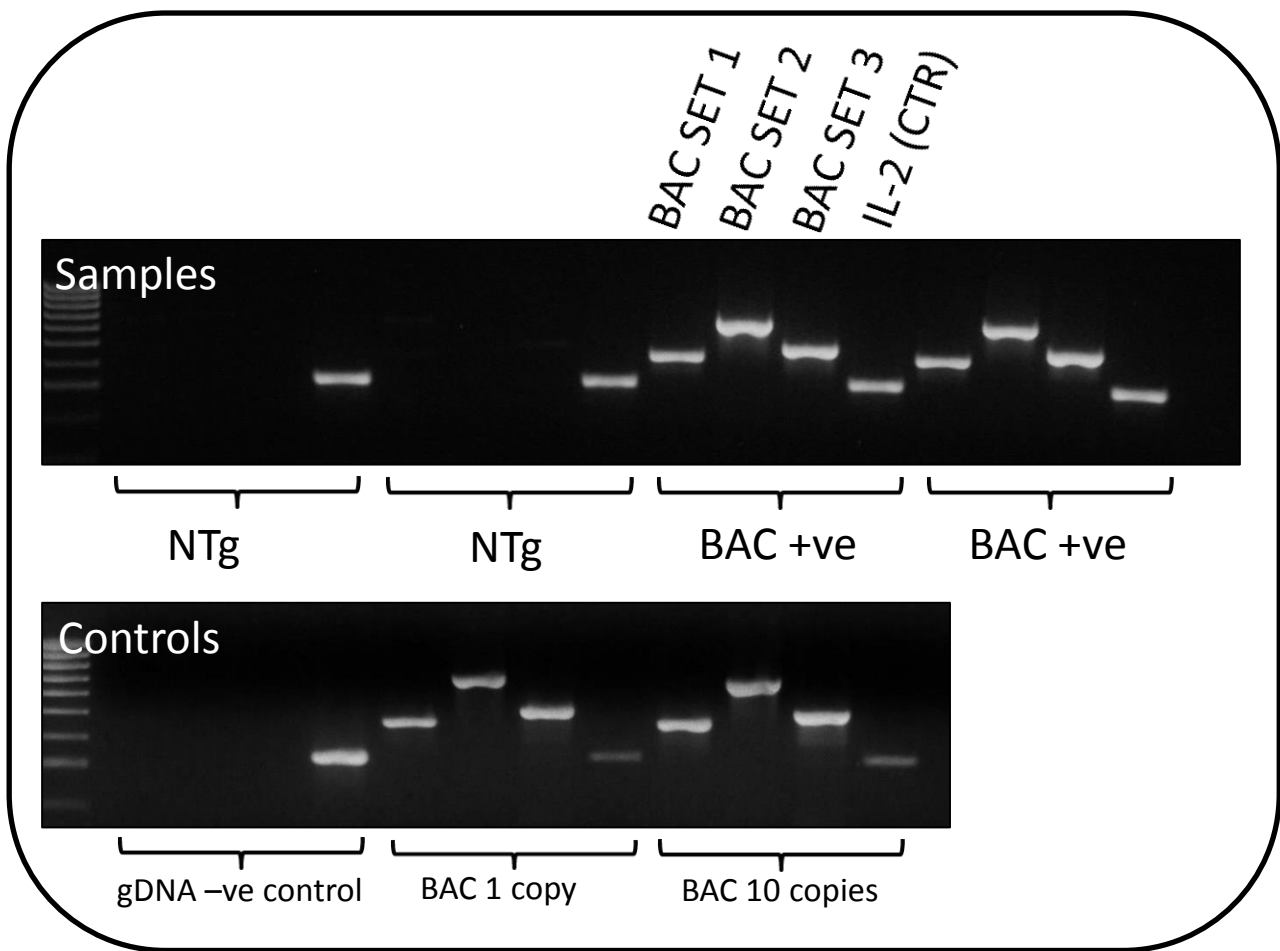


Figure 4.5a) An example of genotyping results from embryos used to set up primary motor neuron cultures from the FUS BAC transgenic mouse model. PCR was used to detect 3 regions of the BAC construct, and IL-2 primers were used as a control to detect the presence of genomic DNA. 2 embryos shown are non-transgenic and 2 carry the BAC construct. Controls were prepared with NTg gDNA and spiked with the BAC construct.

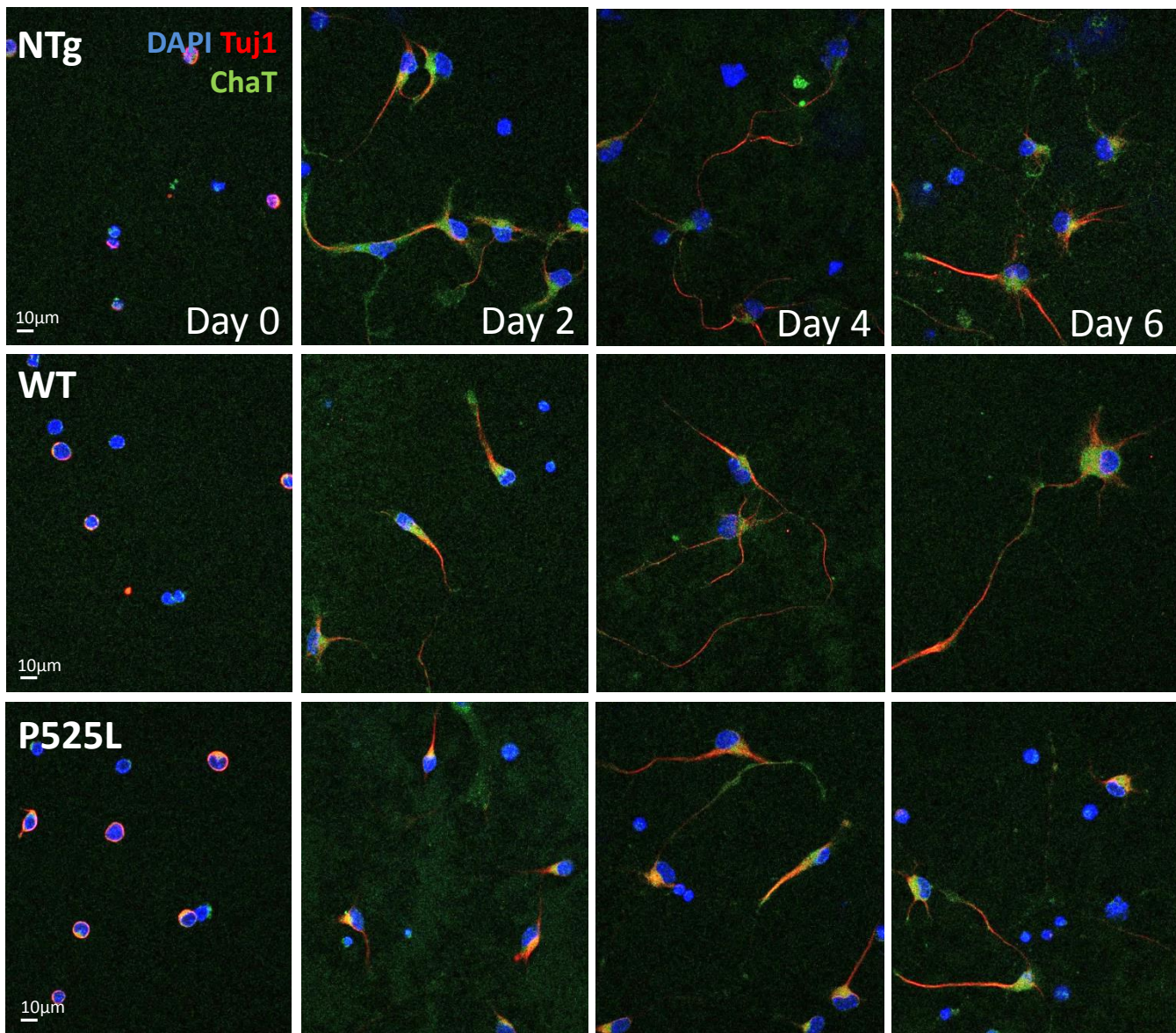


Figure 4.5b) Motor neurons prepared from the FUS BAC transgenic mouse model and fixed at 0, 2, 4 and 6 DIV. Cells develop increasingly long projections and have a neuronal morphology. The presence of motor neurons is verified by positive Tuj1 and ChaT staining. Cell counts were used to determine survival.

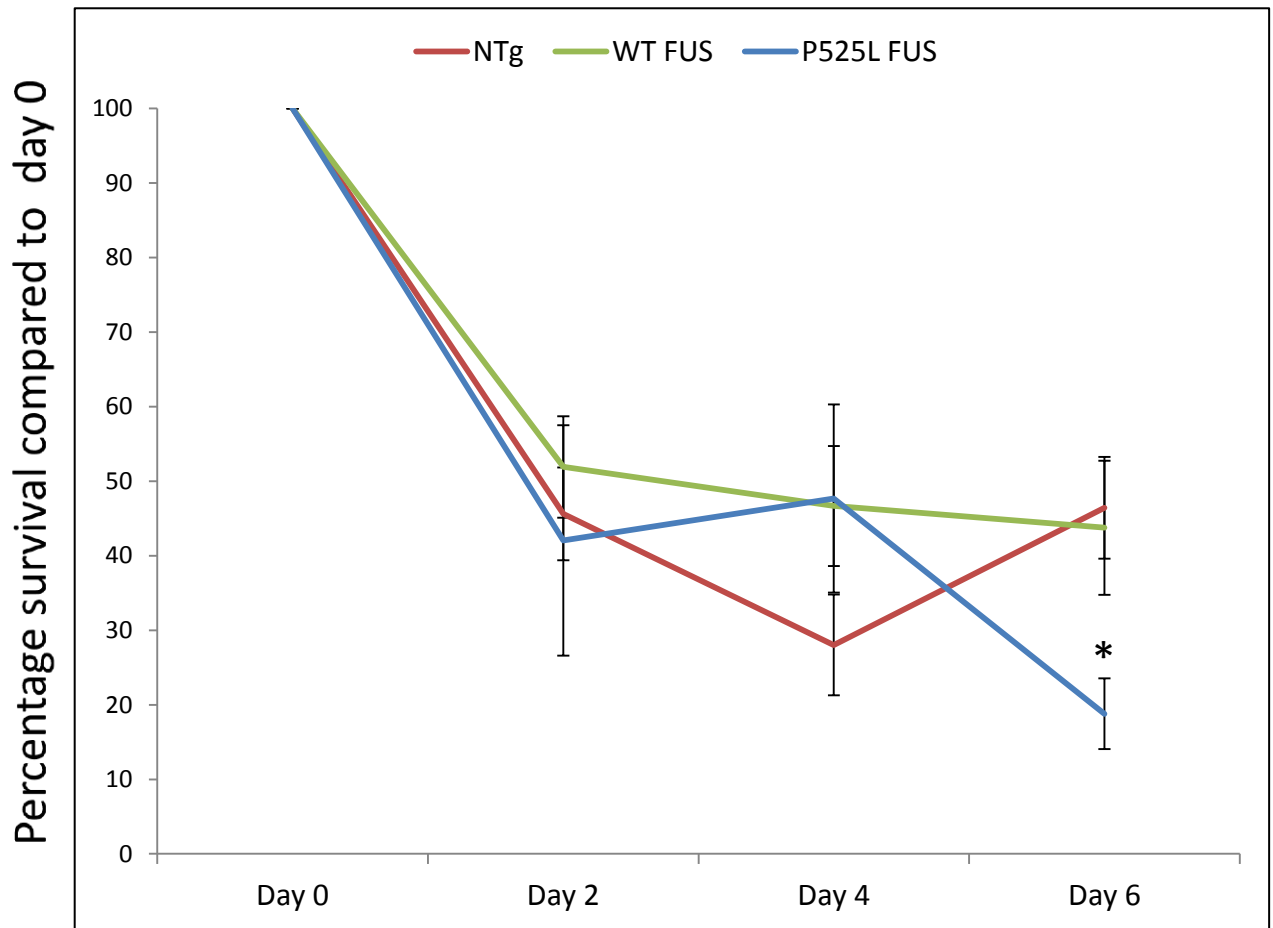


Figure 4.5c) Survival of motor neurons from the FUS BAC transgenic mouse model expressed as a percentage of day 0. It shows a significant reduction in survival of P525L-FUS motor neurons compared to non-transgenic after 6 days in culture. (N=3 experiments, >100 cells per embryo on day 0. Error bars = SEM. One way ANOVA. Tukey post-test. Compared to NTg.)

4.2.5 Motor neurons expressing P525L-FUS have reduced survival

Motor neuron survival was assessed on day 0, 2, 4 and 6 in culture using antibodies against Tuj1 and ChaT. There was a significant reduction in survival of the P525L-FUS transgenic motor neurons compared to non-transgenic on day 6 ($p < 0.05$) (figure 4.5c). Neurons of all genotypes displayed neuronal morphology and neurite projection (figure 4.5b). There was no significant difference in nuclear area between motor neurons of each genotype (appendix 7.2).

4.2.6 P525L-FUS is mislocalised to the cytoplasm of primary motor neurons

Confocal microscopy was carried out to determine whether native mCherry fluorescence was detectable in primary motor neurons post-fixation, which would make additional mCherry immunocytochemistry unnecessary. Transgenic motor neurons did not demonstrate any residual native mCherry fluorescence compared to background signal in non-transgenic neurons (appendix 7.3a). The advantage of this being that a secondary antibody conjugated to a fluorophore with a similar spectral range as mCherry could be used to detect other proteins if required.

Primary motor neurons were therefore stained using an antibody to mCherry. The initial mouse mCherry antibody was unsuccessful and could not distinguish non-transgenic from transgenic lines (appendix 7.3b). A rabbit mCherry antibody, however, showed clear fluorescence in the transgenic lines compared to non-transgenic. This showed that wild-type FUS was nuclear in distribution as expected from previous experiments. P525L-FUS was mislocalised to the cytoplasm (figure 4.6a).

Quantification demonstrated a significant reduction in the proportion of nuclear mCherry-FUS in the P525L-FUS expressing neurons compared to wild-type ($p < 0.001$) (figure 4.6a). When cells were stressed with sodium arsenite, the extent of mislocalisation was unchanged (figure 4.6b).

After treatment with sodium arsenite, cytoplasmic P525L-FUS formed cytoplasmic granules, and the percentage of cells with granules was quantified (figure 6c). As expected, very few wild-type FUS-expressing cells showed this characteristic as mCherry-FUS was predominantly nuclear. A significantly greater percentage of cells expressing P525L-FUS had granules compared to wild-type FUS, even without sodium arsenite treatment ($p < 0.05$). This increased significantly after cells were stressed, so that 66% of cells showed this pattern ($p < 0.01$) (figure 4.6c). This result shows that the majority of P525L neurons demonstrated cytoplasmic mCherry-FUS granules after sodium arsenite treatment.

Characteristically, stress granules tend to resolve when stress is removed (104). It was therefore of interest to determine whether these granules dissipated after arsenite treatment. Cells were incubated for 60 minutes in 0.5 mM sodium arsenite, then for a further 60 minutes in normal media prior to fixation. This is referred to as the 'post-stress' condition. There was no apparent resolution of these granules after the removal of stress, and quantification revealed no significant difference compared to those fixed directly after sodium arsenite treatment (figure 4.6c). This shows that P525L-FUS granules did not resolve within 60 minutes of the removal of stress.

The number of mCherry-FUS granules per cell was assessed using confocal microscopy. As expected, this was very low in wild-type FUS-expressing cells. In P525L neurons, there was an increase after cell stress. Although there was a slight reduction when stress was removed, this was not significant (figure 4.6d).

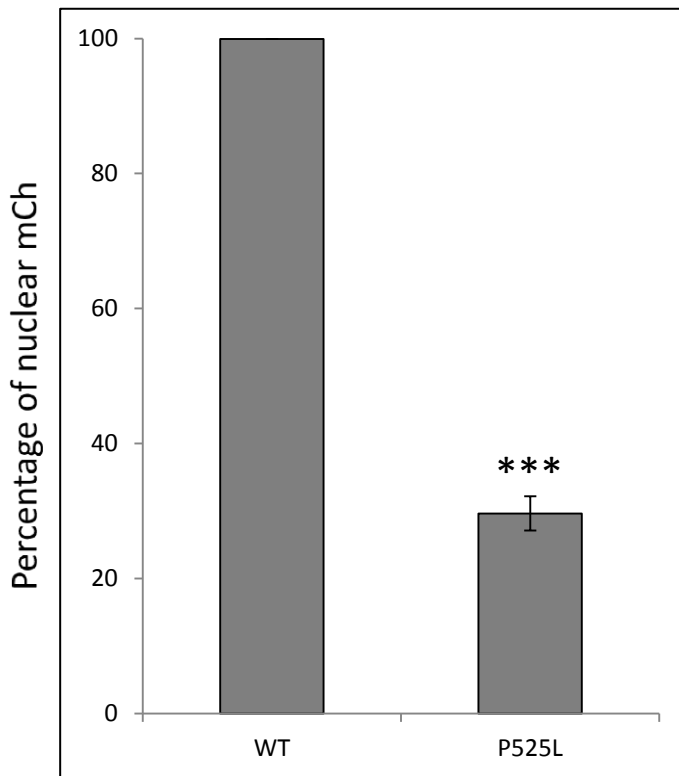
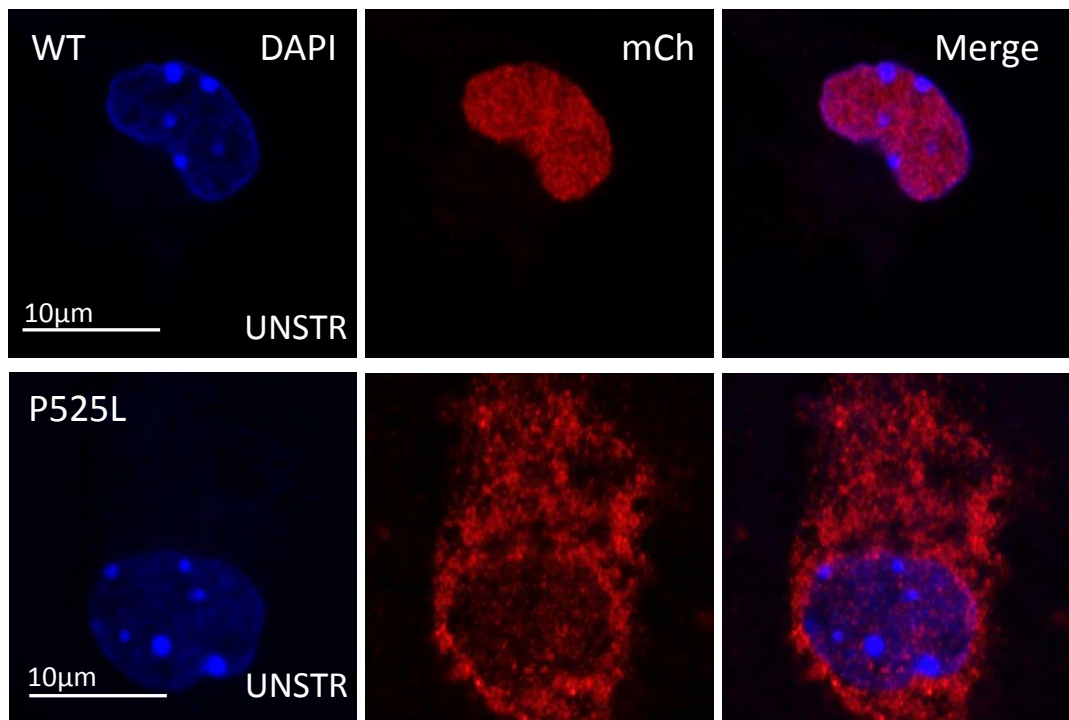


Figure 4.6a) Confocal images of primary motor neurons from the FUS BAC transgenic mouse model show that mCherry-tagged P525L-FUS is mislocalised to the cytoplasm with nuclear clearing. Nuclear and cytosolic localisation was quantified using confocal microscopy. The total fluorescence intensity of the red channel in both the nuclear and cytoplasmic compartments was calculated, and the nuclear signal expressed as a percentage of the total. The proportion of nuclear mCherry-FUS is significantly reduced in the P525L-FUS line compared to wild-type, reflecting mislocalisation to the cytoplasm. (N=3 experiments, 15 cells each. Unpaired t test. Error bars =SEM.)

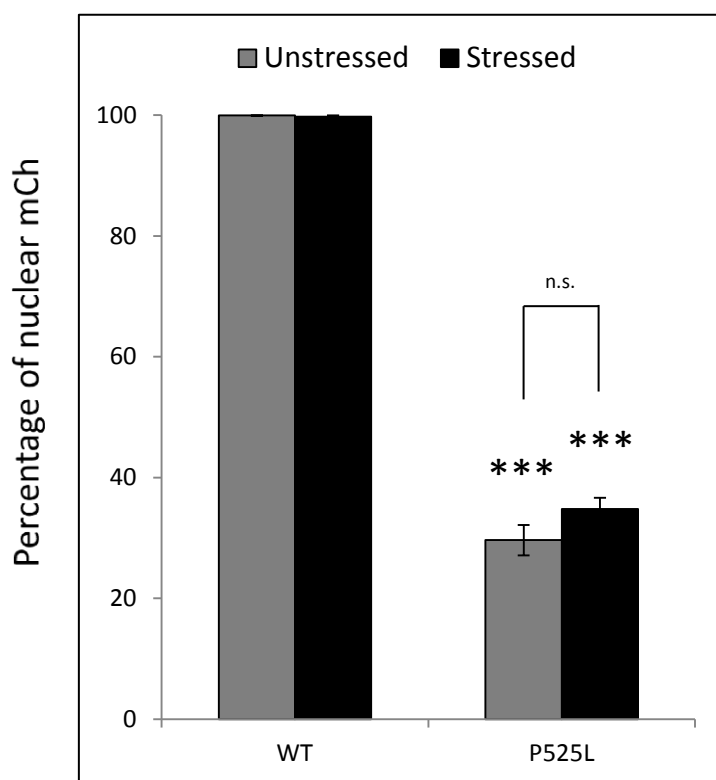
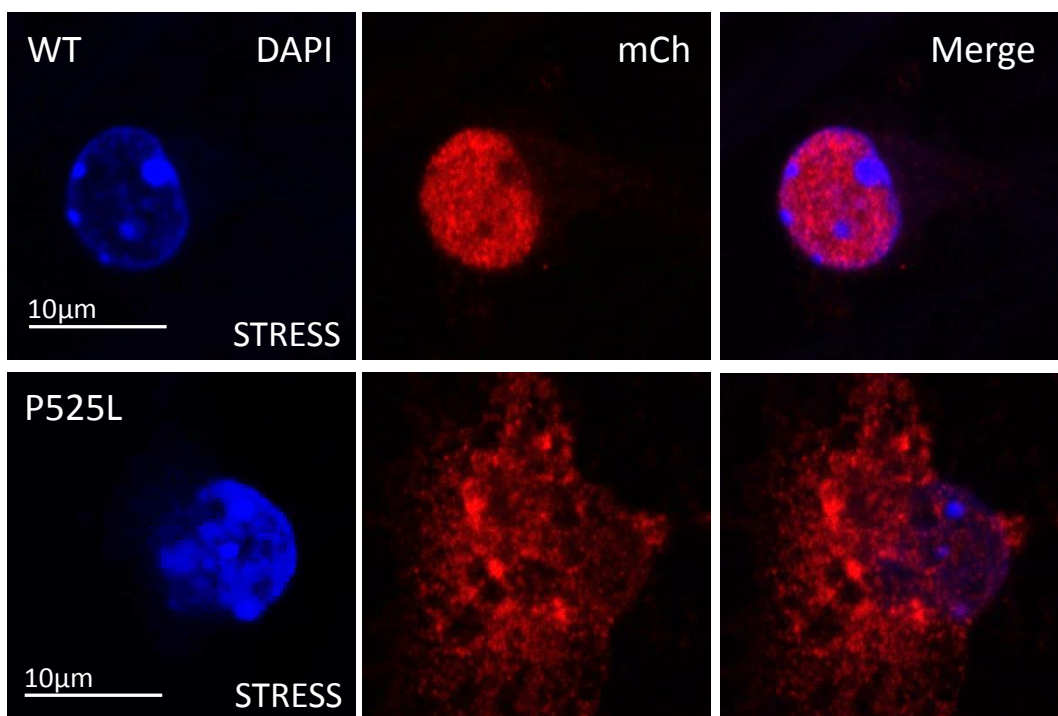


Figure 4.6b) Motor neurons from the FUS BAC transgenic mouse model were stressed with 0.5 mM sodium arsenite for 60 minutes. The distribution of mCherry-FUS between the nuclear and cytoplasmic compartments is unaffected by this treatment. However, P525L-FUS is visible in cytoplasmic granules after cell stress. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test. Stars denote significance compared to wild-type under the same condition. n.s.=not significant)

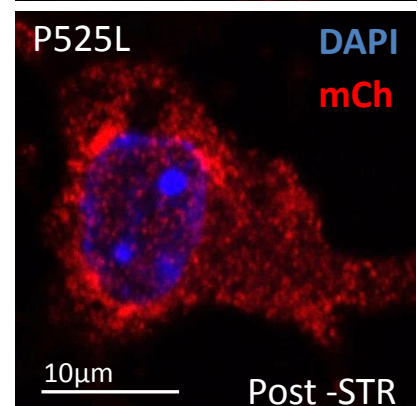
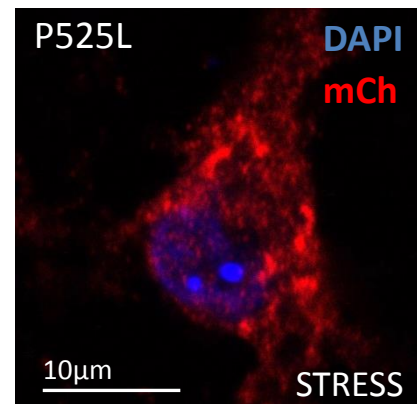
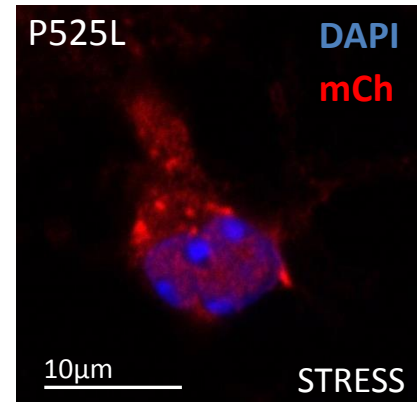
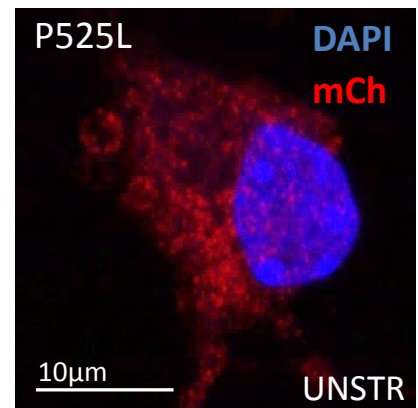
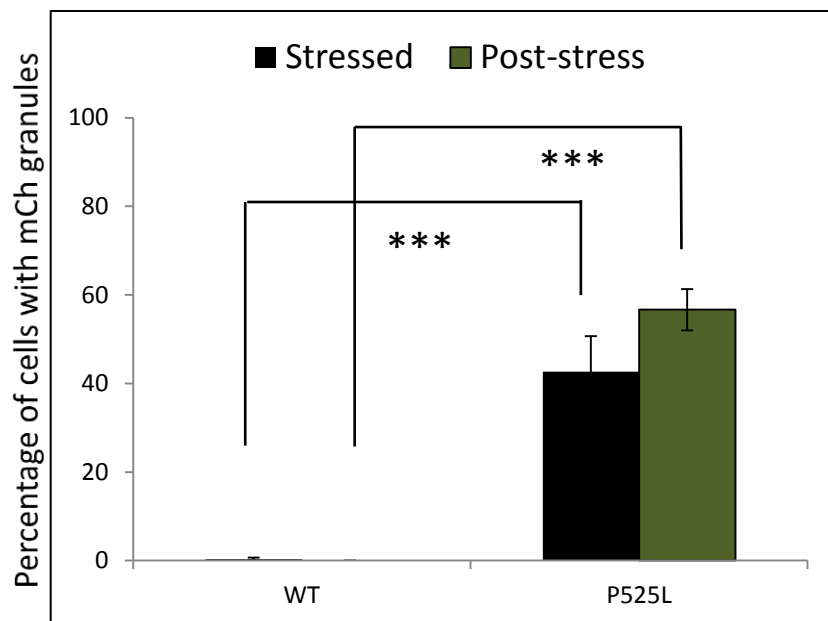
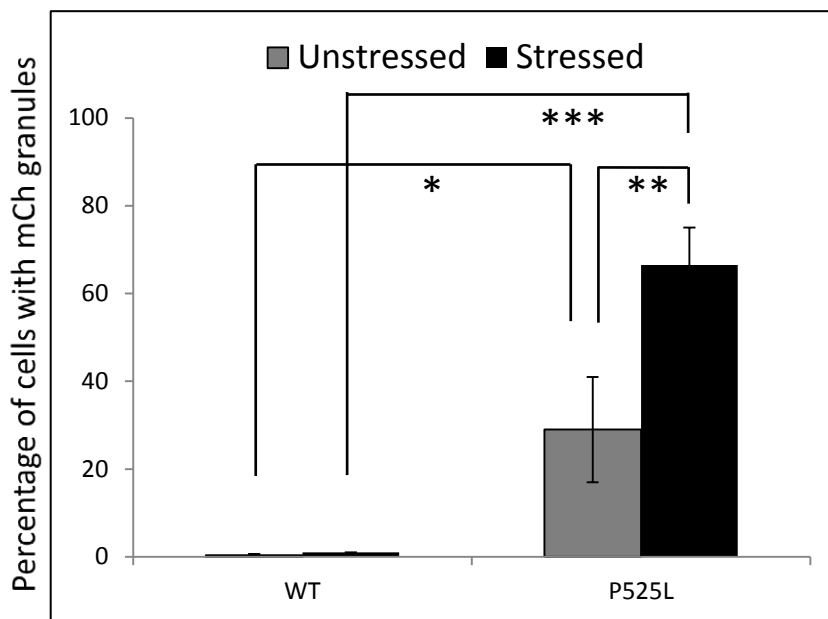


Figure 4.6c) Confocal microscopy showing the distribution of mCherry-FUS in P525L motor neurons in the unstressed condition, after sodium arsenite treatment ('stressed') and after the removal of sodium arsenite for 60 minutes ('post-stress'). mCherry-FUS granules are present after sodium arsenite treatment. The graphs show a significant increase in the percentage of neurons with these granules in P525L motor neurons compared to wild-type under all conditions. There is also an increase in the P525L neurons after sodium arsenite treatment. There is no significant change when stress is removed. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test)

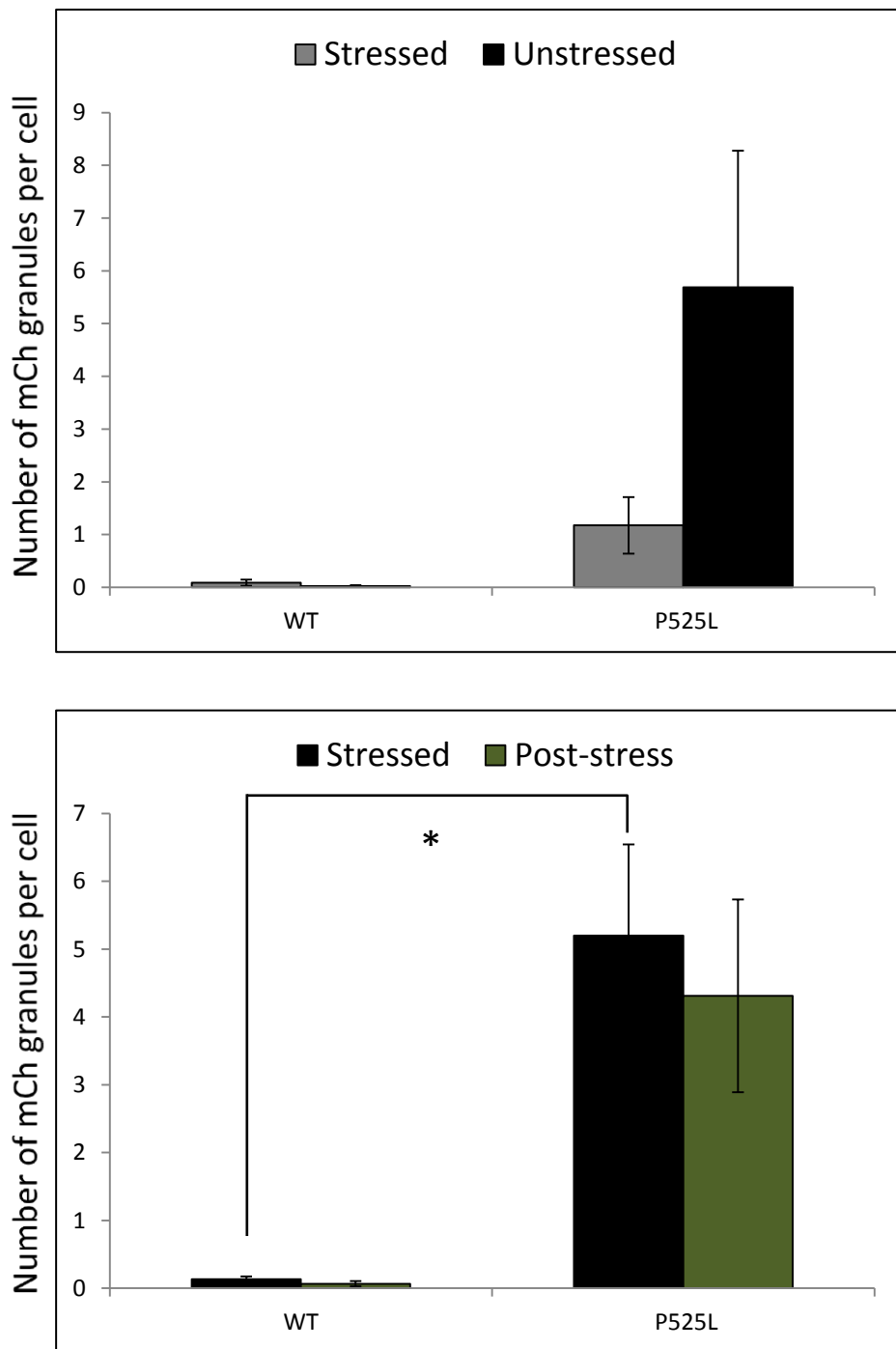


Figure 4.6d) Graphs showing the average number of mCherry-FUS cytoplasmic granules per cell in motor neurons from the FUS BAC transgenic mouse model. Wild-type neurons have very few granules. There are significantly more mCherry-FUS granules in P525L neurons compared to wild-type, which reaches statistical significance in the 'stressed' condition. There is a trend towards a reduction in the P525L neurons when stress is removed, but this is not statistically significant. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test)

This corroborates the results of figure 4.6c) above that suggest that these granules do not dissipate within 60 minutes of the removal of stress.

4.2.7 The distribution of FET proteins is not significantly altered by the presence of P525L mutant FUS

The next stage was to determine the proportion of total FUS that was mislocalised in P525L-FUS expressing neurons, in view of the very low levels of BAC-derived FUS compared to endogenous in these lines (appendix table 7.3). FUS was predominantly nuclear in location in all lines, which was verified by quantification (figure 4.7a). There was a very subtle trend towards a reduction in nuclear total FUS in the P525L-FUS expressing line, but this did not reach statistical significance.

After sodium arsenite treatment FUS remained predominantly nuclear in all lines (figure 4.7b). However, in the stressed cells the trend towards cytoplasmic P525L-FUS was more marked. This suggests that there may be some cytoplasmic mislocalisation detectable in these cells compared to wild-type FUS, though not at a sufficient volume to reach statistical significance. Interestingly, when the exposure was increased such that nuclear FUS was over-saturated, some cells expressing P525L-FUS demonstrated cytoplasmic FUS (figure 4.7c). It is difficult to determine the extent of this in unstressed cells due to the presence of background fluorescence. However, in the stressed neurons this fluorescence appeared in granules in many of the cells in a pattern similar to that of mCherry staining. This suggests that mCherry-FUS was detected by this antibody, but expression levels were too low to reach significant levels when this was quantified.

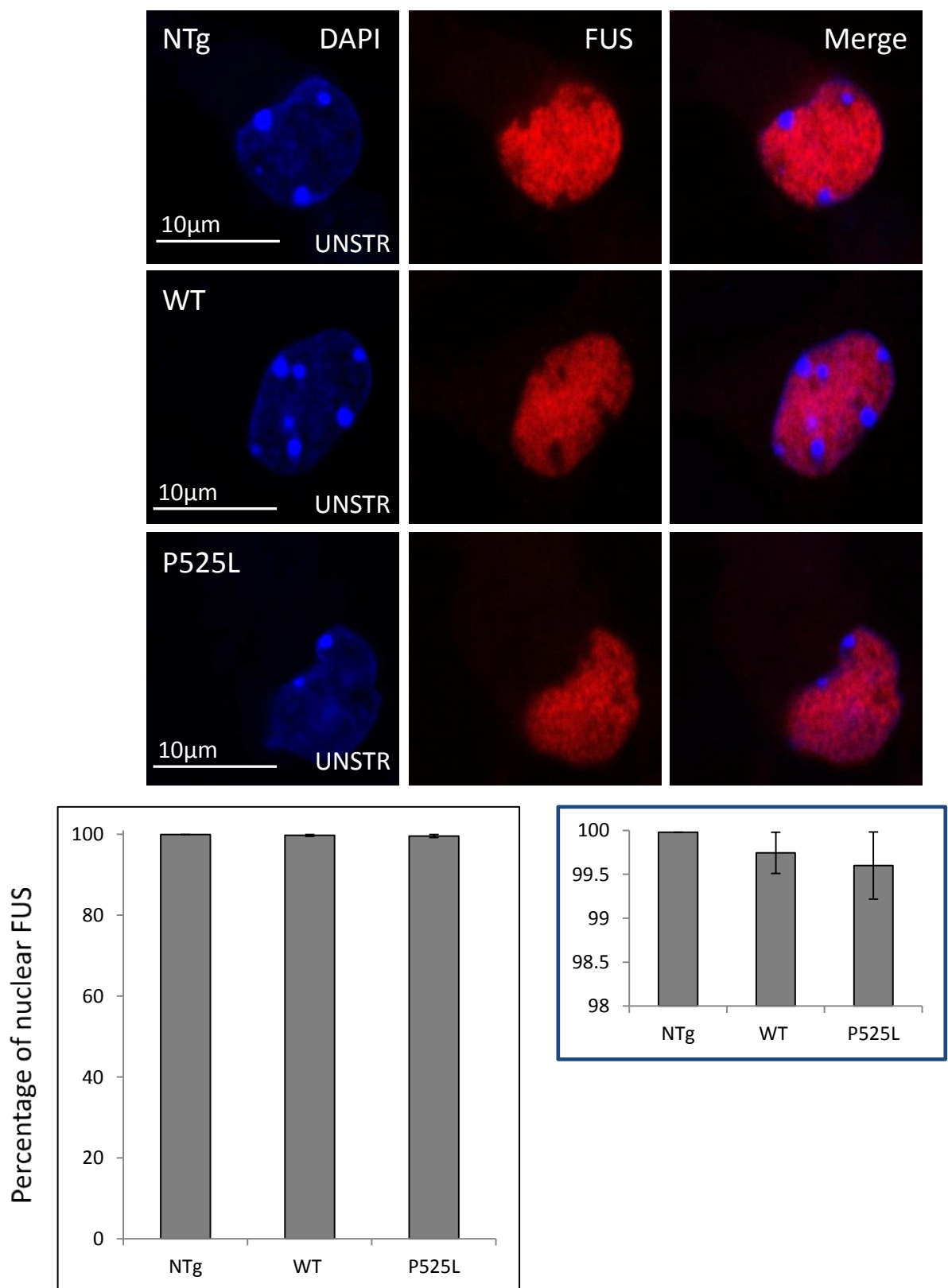
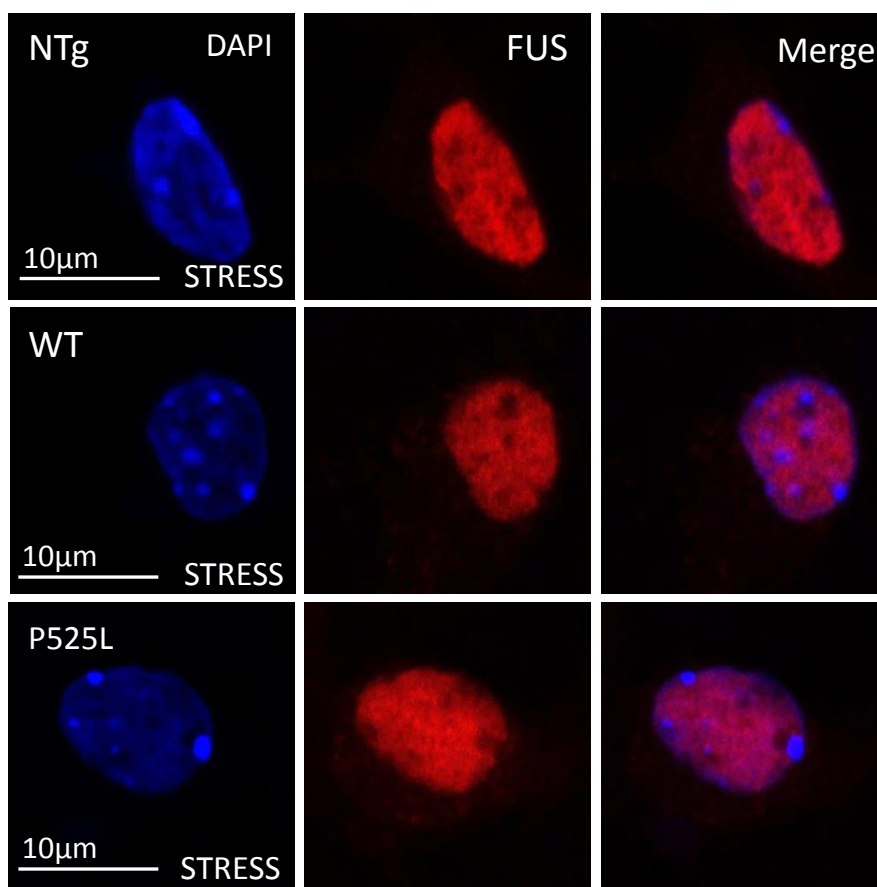
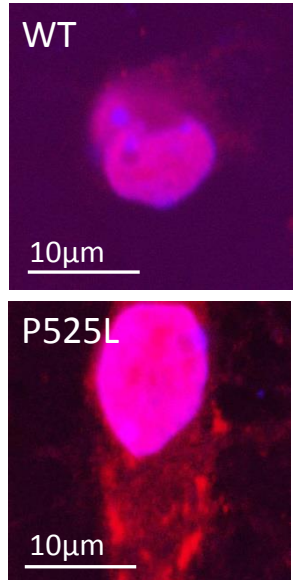


Figure 4.7a) Motor neurons from the FUS BAC transgenic mouse model stained using an antibody to total FUS. Confocal microscopy shows that FUS is predominantly nuclear. Quantification of FUS distribution does not reveal any significant difference between the lines. However, there is a small non-significant reduction in nuclear FUS in the P525L motor neurons. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test). 124

b)



c)



Percentage of nuclear FUS

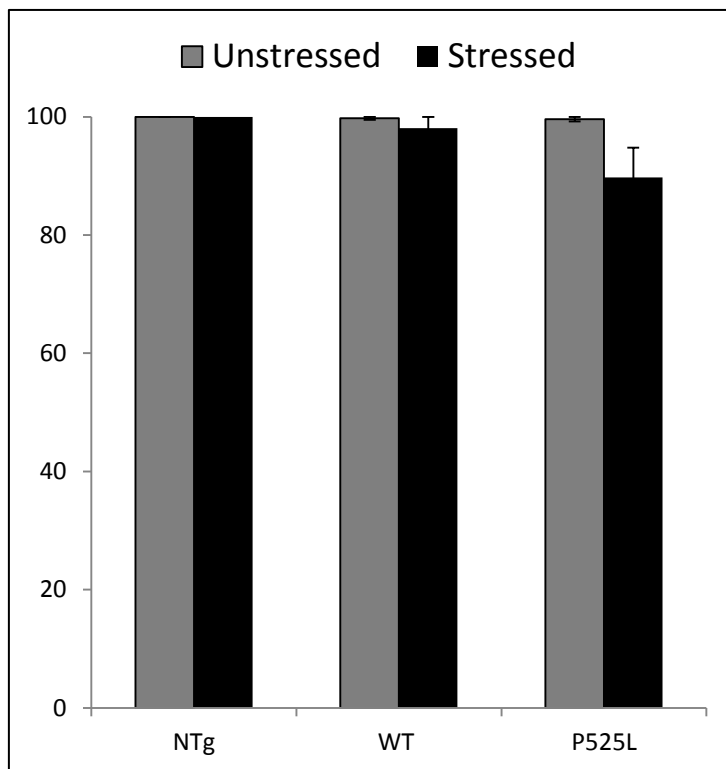


Figure 4.7b) After treatment with sodium arsenite, FUS appears to remain nuclear. Quantification reveals no significant difference between the lines, though there is a trend towards a reduction in nuclear FUS in the P525L motor neurons. **c)** FUS distribution in neurons using confocal microscopy with high exposure. Cytoplasmic granules are visible in the P525L motor neurons that are not visible under normal exposure. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test)

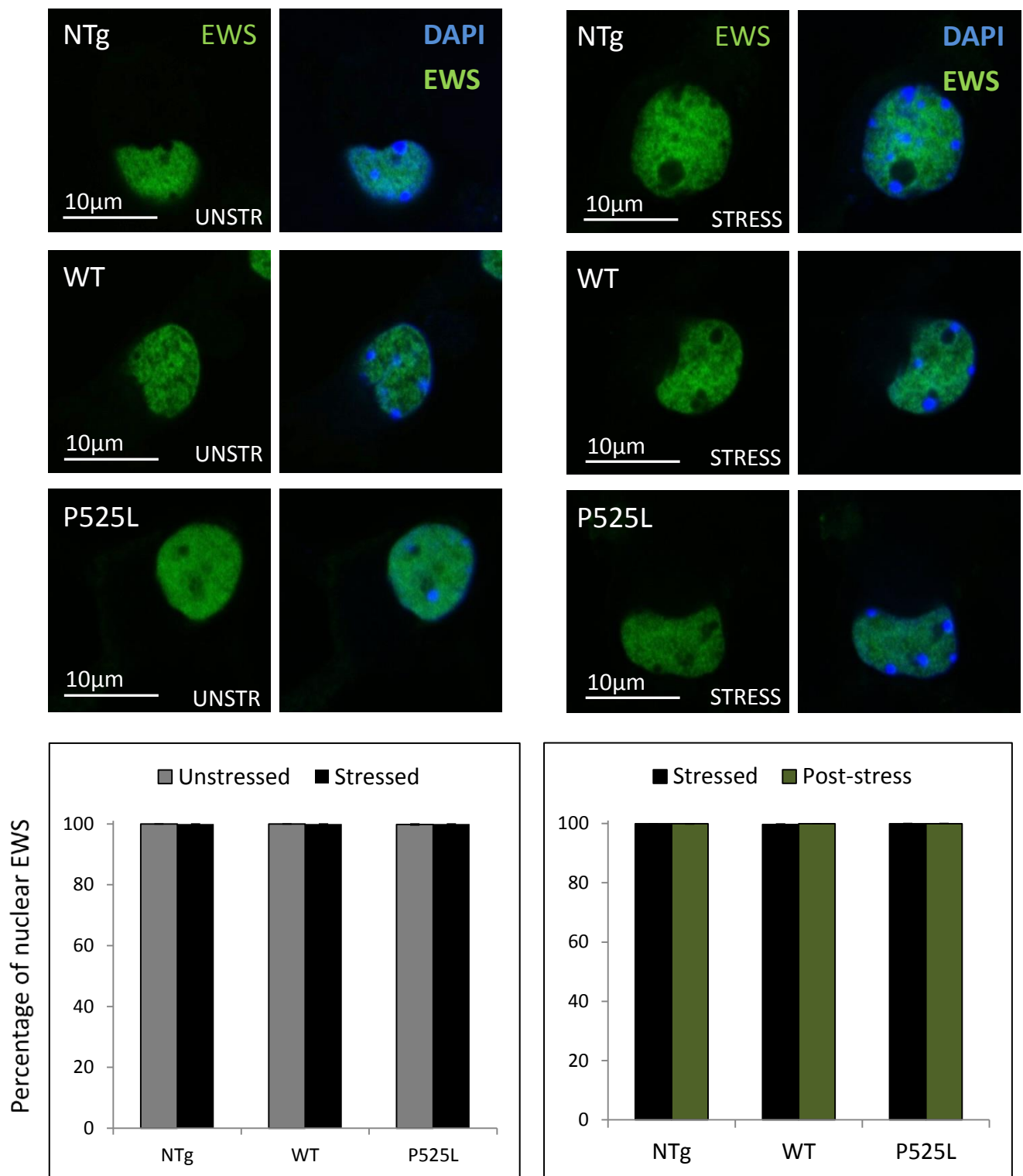


Figure 4.8a) Motor neurons from the FUS BAC transgenic mouse model stained with an antibody to EWS, with quantification of cellular distribution using confocal microscopy. There is no significant difference in EWS distribution between the lines. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test)

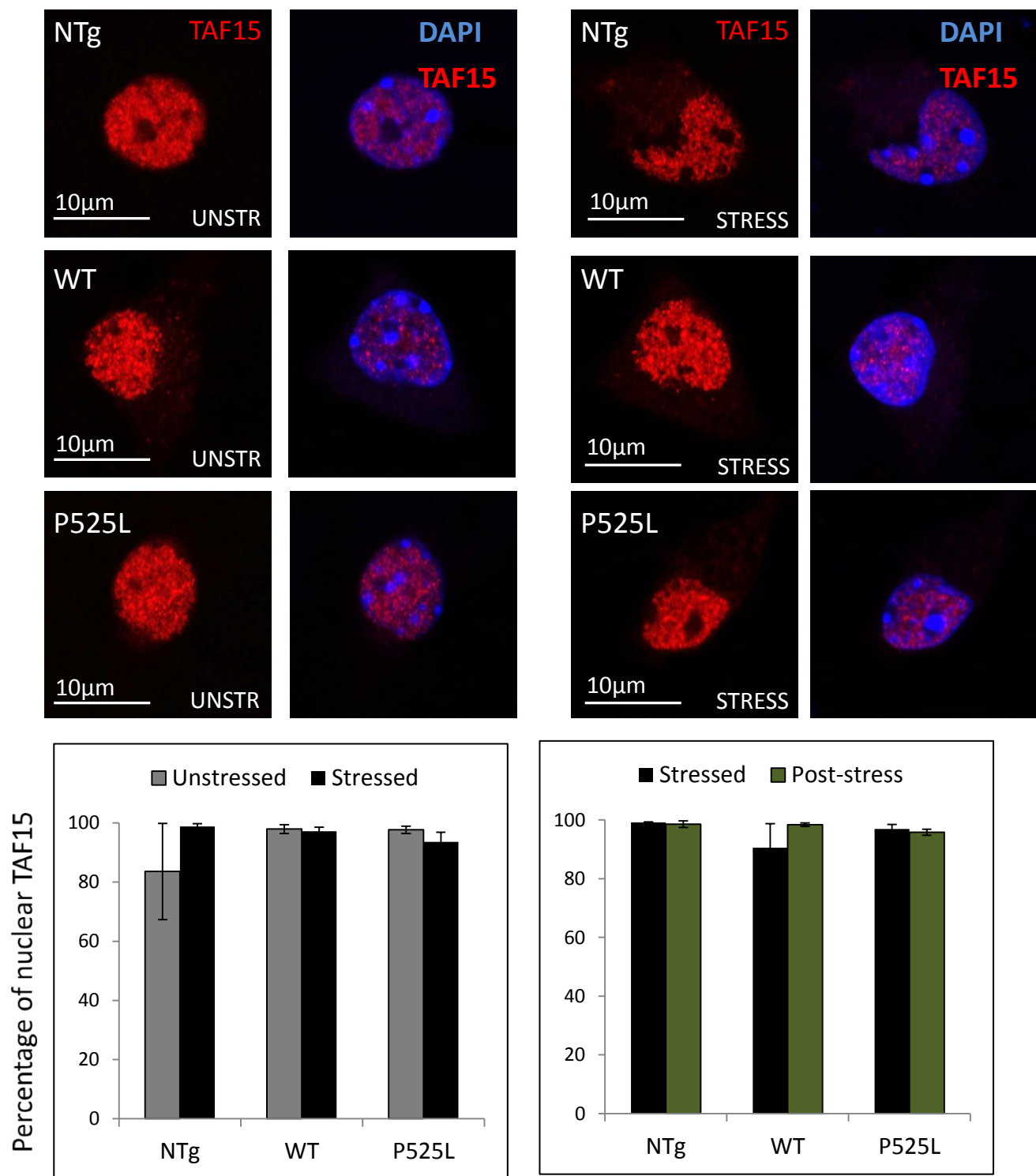


Figure 4.8b) Motor neurons from the FUS BAC transgenic mouse model stained with an antibody to TAF15, with quantification of cellular distribution using confocal microscopy. There is no significant difference in TAF15 distribution between the lines. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test)

An mCherry immunoreactive band corresponding to the size of mCherry-FUS was detected by Western blot using this antibody, which confirms that it detects mCherry-FUS expressed by the construct (Vizard et al, in progress).

In FTD with FUS inclusions (FTD-FUS), FUS positive aggregates contain EWS and TAF15. However, in ALS-FUS, EWS and TAF15 are absent from neuronal inclusions (86). It was therefore important to examine the distribution of the other FET proteins in this model. Nuclear fluorescence as a percentage of total fluorescence was calculated for each of these proteins under each condition. EWS and TAF15 were predominantly nuclear in all lines. There was no significant difference in EWS or TAF15 distribution in P525L motor neurons compared to wild-type FUS in the unstressed, stressed or post-stress conditions (figure 4.8a&b). This suggests that mutant FUS was not able to sequester EWS and TAF15 into the cytoplasm and that these FET family members are not incorporated into normal, or pathological FUS-containing stress granules. However, given that FUS is not detectably mislocalised in these experiments then this does not fully assess the ability of FUS to sequester EWS and TAF15 into the cytoplasm, and cannot rule it out. In addition, it is possible that there is mislocalisation of EWS and TAF15 below levels that can be detected using these techniques.

4.2.8 Stress granule dynamics in P525L-FUS motor neurons

After establishing P525L-FUS mislocalisation and incorporation into stress granules, the effect of P525L-FUS on stress granule dynamics was then addressed. The percentage of cells with stress granules in the unstressed, stressed and post-stress condition was assessed. PABP, G3BP and TIA1 antibodies were all used in order to

detect stress granules at different stages of their cycle, and assess whether results were consistent between antibodies.

Staining with PABP showed no significant differences between the lines under each condition (figure 4.9a). In each line there was a significant increase in cells with stress granules after sodium arsenite treatment compared to unstressed ($p < 0.001$).

In the post-stress condition there was a trend towards a reduction, particularly in non-transgenic and wild-type lines, though this did not reach statistical significance.

Results were very similar using the G3BP antibody (figure 4.9b). The only exception was that in the stressed condition there was a decrease in the percentage of cells with stress granules in the P525L-FUS neurons compared to non-transgenic ($p < 0.05$). Although this is not observed with the PABP antibody, it could suggest subtle deficiencies in stress granule formation in the presence of P525L-FUS.

Results were much more variable using the TIA1 antibody as fluorescence appeared more diffuse and speckled rather than in discrete granules. However, there were again no detectable differences between each line (appendix 7.5).

The number of stress granules per cell was quantified using confocal microscopy.

Staining with the PABP antibody revealed a reduction in the number of stress granules per cell in both wild-type and P525L expressing neurons in the stressed condition compared to non-transgenic ($p < 0.001$). There was a further reduction in the P525L neurons compared to wild-type ($p < 0.001$). In contrast, in the post-stress condition there was an increase in stress granules per cell in P525L neurons compared to non-transgenic and wild type ($P < 0.001$) (figure 4.9c). This suggests that there may be defects in both stress granule formation and resolution at this stage of the stress granule cycle.

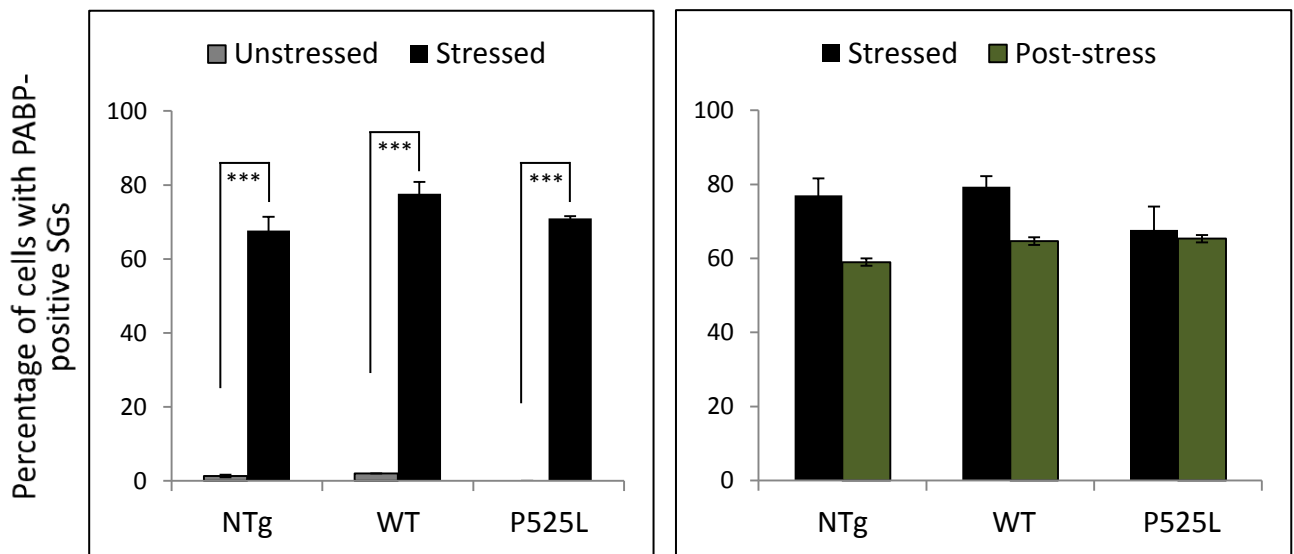
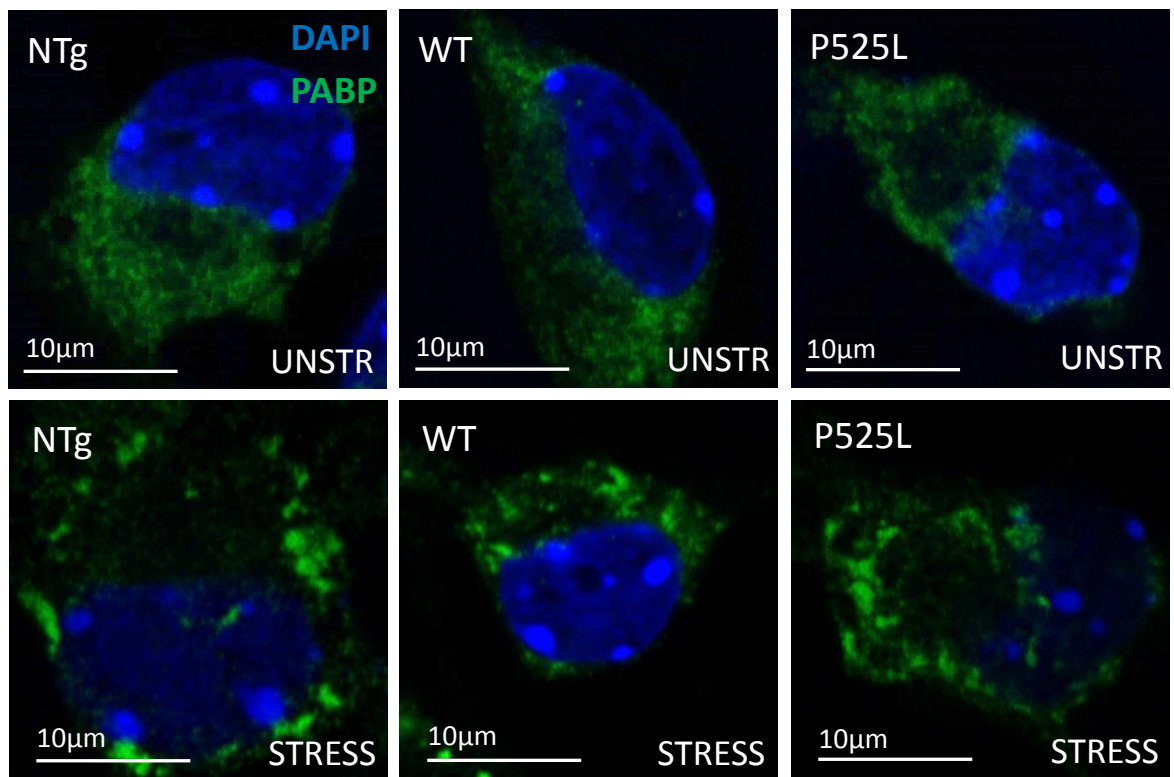


Figure 4.9a) Cells from the FUS BAC transgenic mouse model stained with PABP in the unstressed condition ('unstressed') after sodium arsenite treatment ('stressed') and after the removal of sodium arsenite for 60 minutes ('post-stress'). Confocal microscopy reveals the development of stress granules after sodium arsenite treatment. Quantification of the percentage of cells with stress granules reveals an increase after sodium arsenite treatment, but no significant change when stress is removed. There is no difference between each line. (N=3 experiments, 100 cells each. One way ANOVA. Tukey post-test)

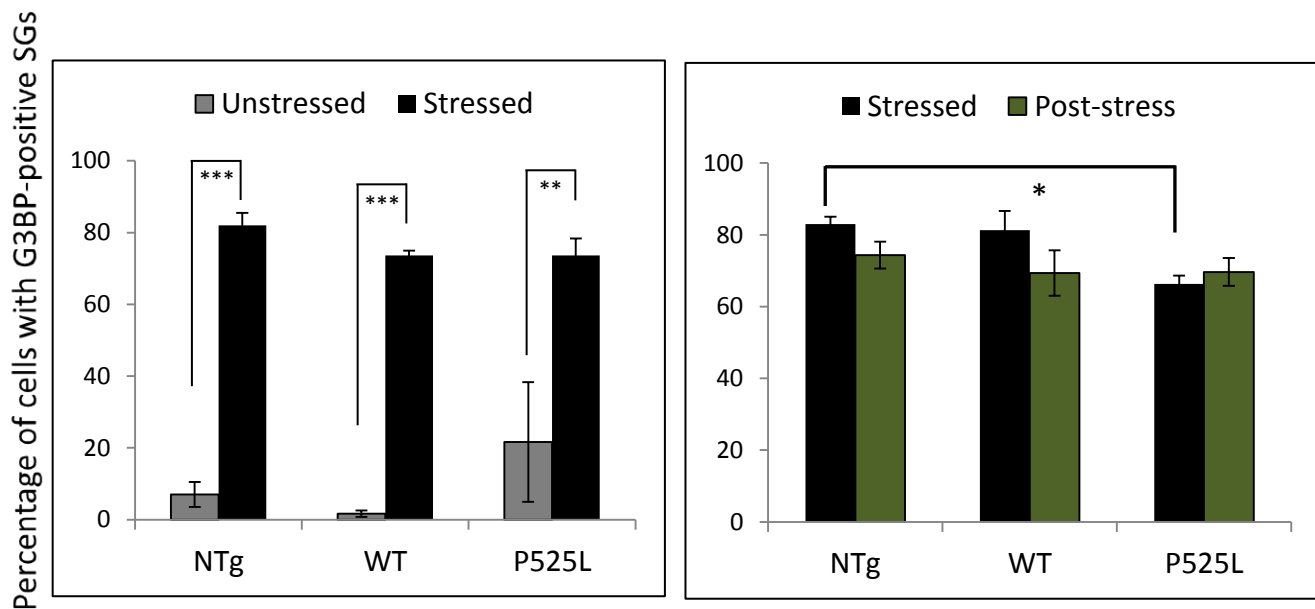
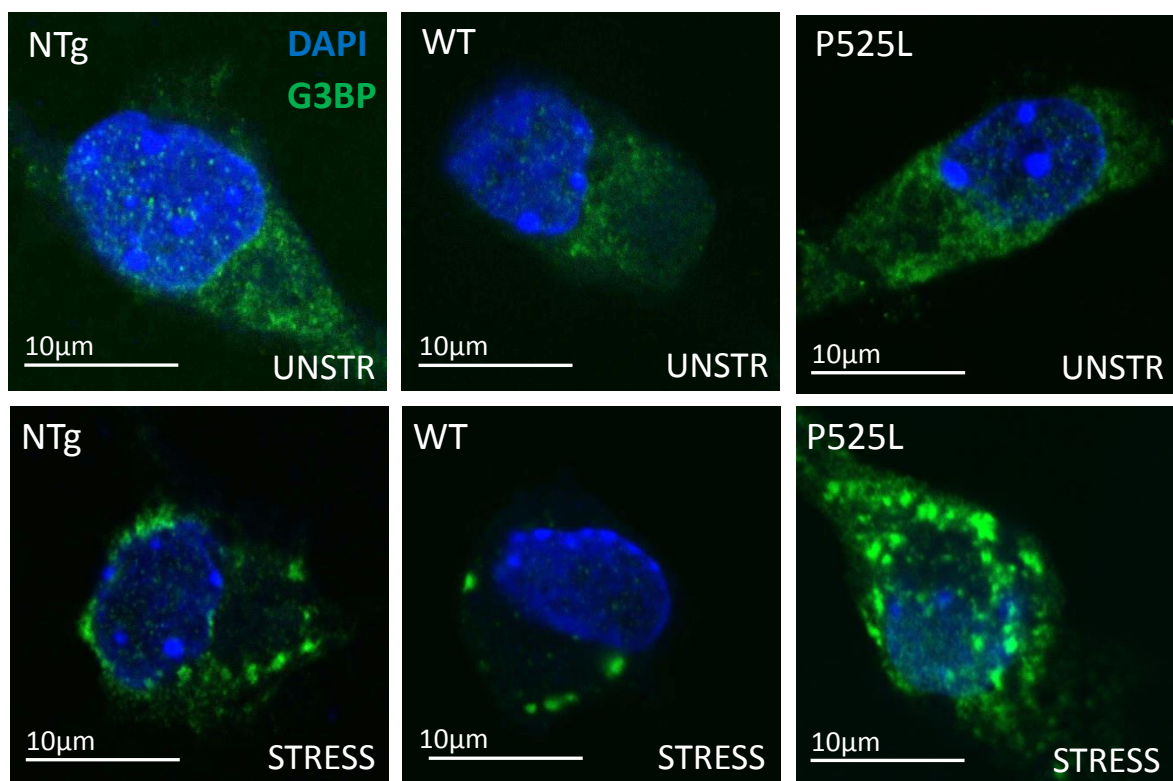


Figure 4.9b) Staining with the G3BP antibody reveals an increase in the percentage of cells with stress granules after stress. There is a reduction in the percentage of P525L neurons with G3BP positive stress granules compared to non-transgenic in the stressed condition of the second experiment. (N=3 experiments, 100 cells each. One way ANOVA. Tukey post-test)

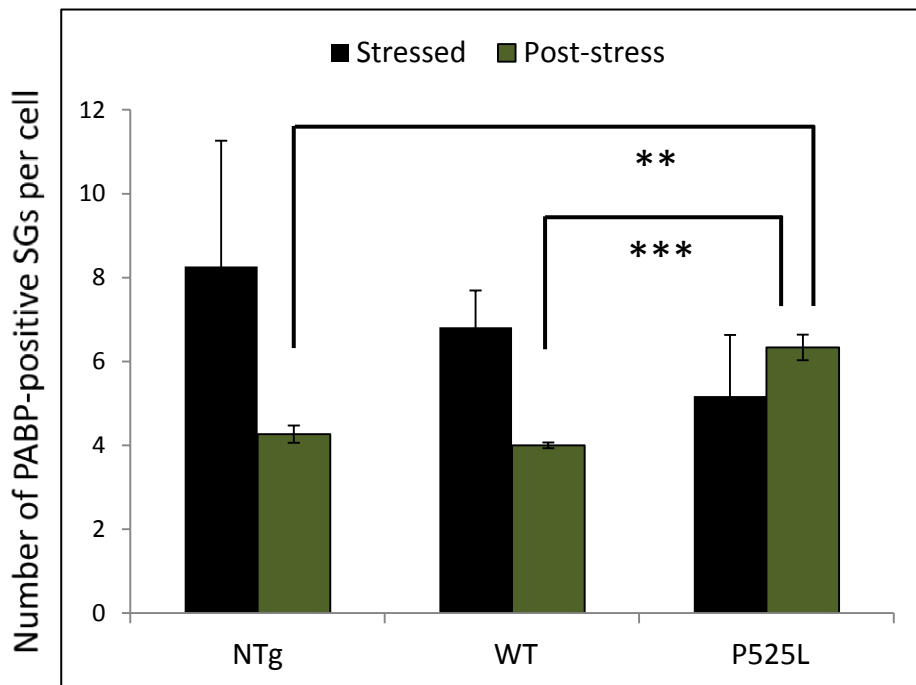
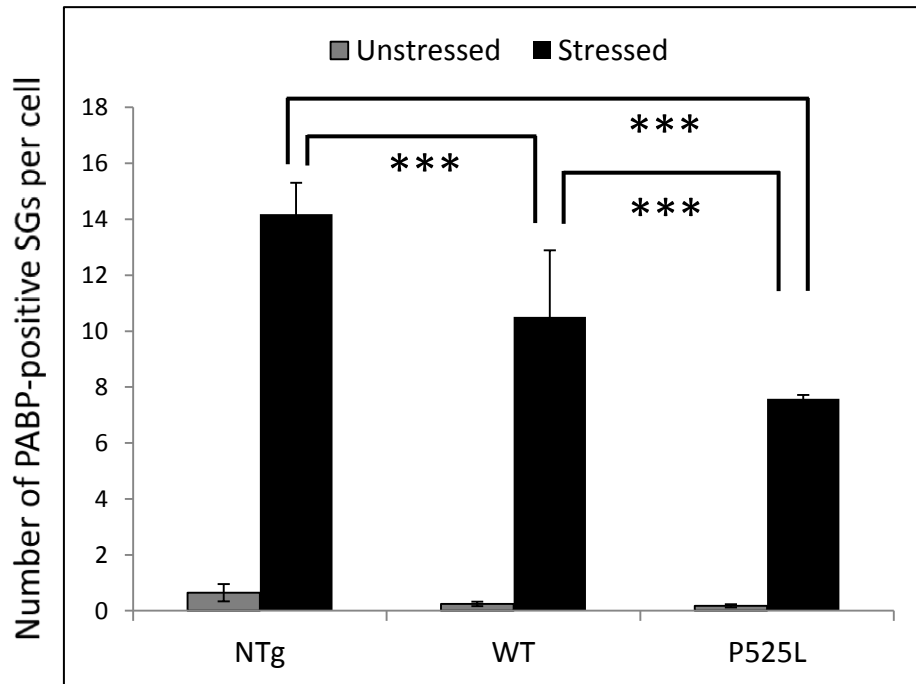


Figure 4.9c) Quantification of the number of PABP-positive stress granules per motor neuron. There are fewer granules in the P525L neurons compared to non-transgenic and wild-type in the stressed condition. Post-stress, there is an increase in PABP-positive stress granules in the P525L neurons. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test).

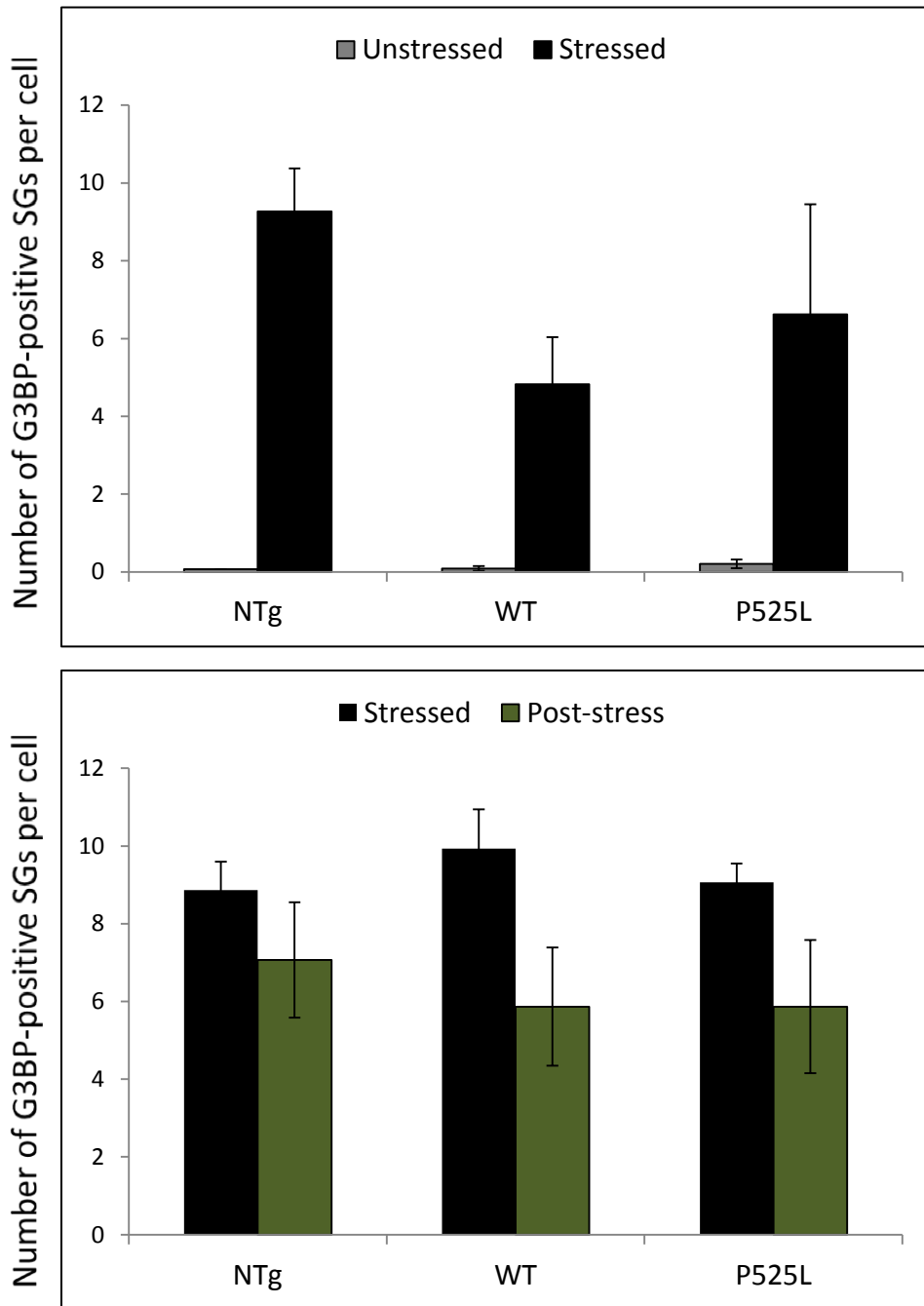


Figure 4.9d) Quantification of the number of G3BP-positive stress granules per motor neuron. There are no significant differences detected between each line. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test).

Staining with the G3BP antibody did not reproduce these results, and there were no significant differences between each line. Interestingly, each line showed a more consistent reduction in the number of stress granules per cell after the removal of stress compared to the results using the PABP antibody (figure 4.9d). The disparity in results may be due to the detection of stress granules at different stages of the cycle, or to differences in the binding and specificity of the antibodies.

The number of TIA1 positive stress granules per cell was not quantified due to the speckled appearance of the staining and difficulty in detecting individual granules, leading to unreliable results.

Fluorescence intensity of stress granule markers in the cytoplasm was used as an alternative method of detecting differences in stress granule formation. The rationale behind this experiment was that stress granule formation would lead to increased fluorescence of stress granule markers.

Using the PABP antibody, there was a trend towards an increase in fluorescence after cell stress in all lines. However, this did not reach statistical significance as there was wide variability in results (appendix 7.4a). Interestingly, there did appear to be a non-significant reduction in fluorescence in the P525L neurons compared to non-transgenic and wild-type FUS. This may reflect the observation that fewer stress granules form per cell in the P525L mutant neurons. Results using the TIA1 antibody were more variable, with no consistent increase in fluorescence after cell stress (appendix 7.4b). These results may reflect natural variations in fluorescence between coverslips, so it was not used to draw any firm conclusions about stress granule dynamics.

4.2.9 Mutant FUS is incorporated into stress granules

The next step was to determine whether mCherry-FUS granules co-localised with stress granules. As expected, wild-type FUS was nuclear and did not co-localise with G3BP. In the unstressed state, G3BP and mislocalised P525L-FUS were both cytoplasmic with evidence of co-localisation detected by yellow fluorescence, despite the absence of stress granules. After cell stress, both proteins formed cytoplasmic granules which co-localised (figure 4.10a). The correlation coefficient of co-localisation was calculated and wild-type FUS showed no evidence of a correlation between mCherry-FUS and G3BP staining. In both the unstressed and stressed conditions there was a correlation between P525L mCherry-FUS and G3BP that was greater than wild-type FUS, reaching statistical significance in the 'stressed' and 'post stress' conditions ($p < 0.001$ and $p < 0.01$) (figure 4.10b). There was a trend towards increased co-localisation in stressed P525L neurons compared to unstressed, and compared to post-stress, though this did not reach statistical significance. This suggests that there is a greater amount of co-localisation within cytoplasmic granules compared to the rest of the cytoplasm. Results are similar for TIA1 and mCherry co-localisation, though the correlation coefficient is lower (appendix 7.6). This may be again due to the lack of such discrete granules detected by the TIA1 antibody compared to G3BP.

These results support the published observation in cell lines that P525L mutant FUS is incorporated into stress granules (102, 148).

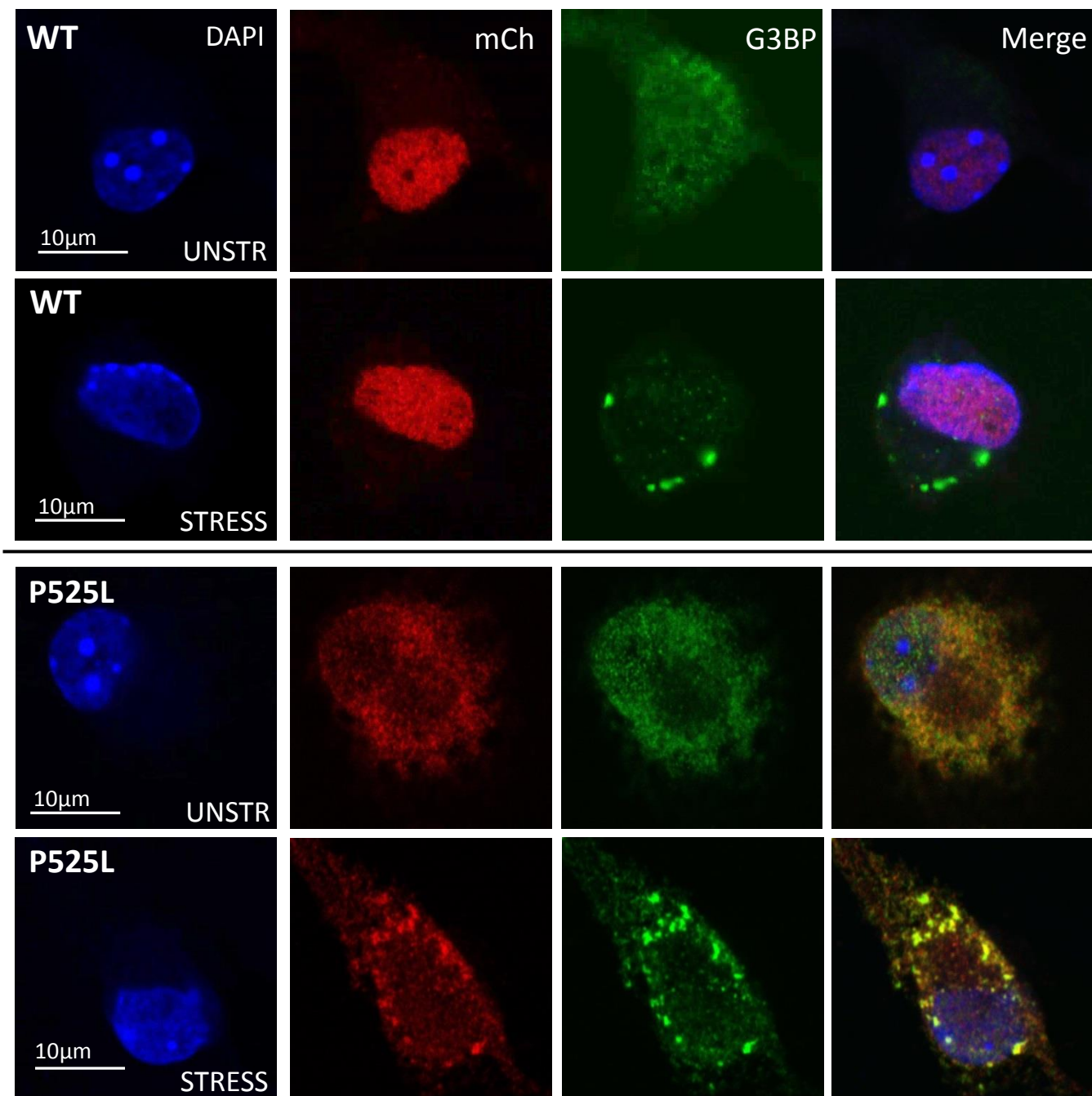


Figure 4.10a) Motor neurons from the FUS BAC transgenic mouse model stained with antibodies to mCherry and G3BP demonstrate co-localisation of P525L but not wild-type mCherry-FUS with G3BP positive stress granules.

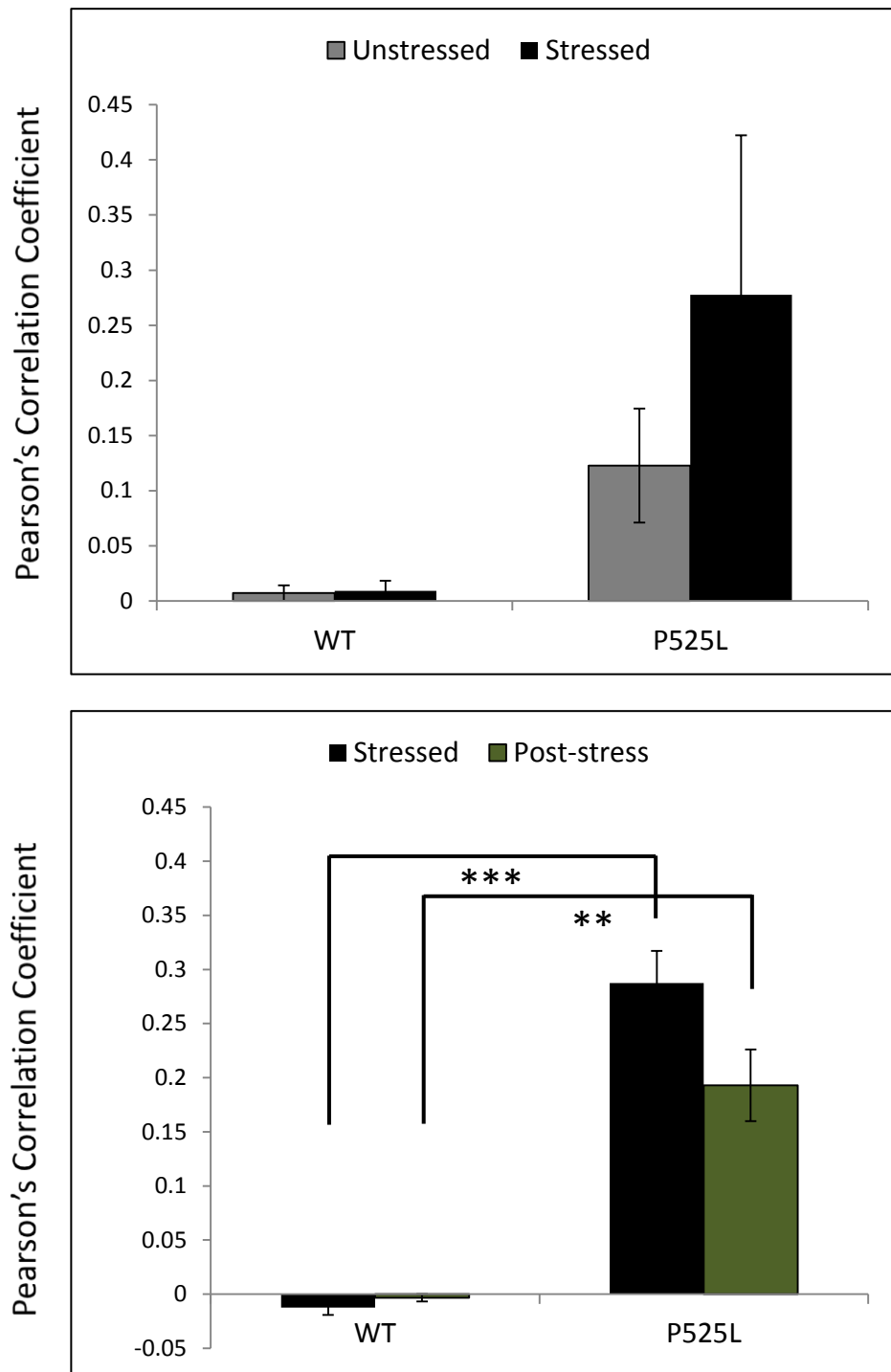


Figure 4.10b) Quantification of G3BP and mCherry-FUS co-localisation in motor neurons from the FUS BAC transgenic mouse model using confocal microscopy to calculate Pearson's correlation coefficient. There is increased co-localisation in P525L neurons compared to wild-type. (N=3 experiments, 15 cells each. One way ANOVA, Tukey post test.)

4.2.10 SMN distribution and gem number is not altered by P525L-FUS

expression in motor neurons

In order to examine whether mutant FUS co-localises with SMN in cytoplasmic granules, unstressed and stressed neurons were stained for SMN and mCherry (figure 4.11a). As expected, wild-type mCherry was nuclear and P525L mCherry mislocalised to the cytoplasm, forming granules after oxidative stress. SMN did not form granules after oxidative stress and remained diffusely distributed in the cytoplasm. There was no visible co-localisation above that expected from two cytoplasmic proteins. Co-localisation analysis verified this, with a low correlation between the localisation of SMN and mCherry staining and no significant difference between wild-type and P525L-FUS lines (figure 4.11b). The lack of SMN granules also shows that SMN is not incorporated into cytoplasmic granules of mutant FUS. SMN is enriched in nuclear foci known as gems. Several studies using cell lines have reported loss of nuclear SMN gems due to an association with mutant FUS (107, 108). To examine whether this occurred in these mouse motor neurons, SMN gem number was assessed in non-transgenic, wild-type and P525L-FUS motor neurons. There was no difference in the number of nuclear gems between each line, both in the unstressed and stressed conditions (figure 4.11b). There was no evidence from these results that P525L-FUS causes mislocalisation or functional deficiency of SMN.

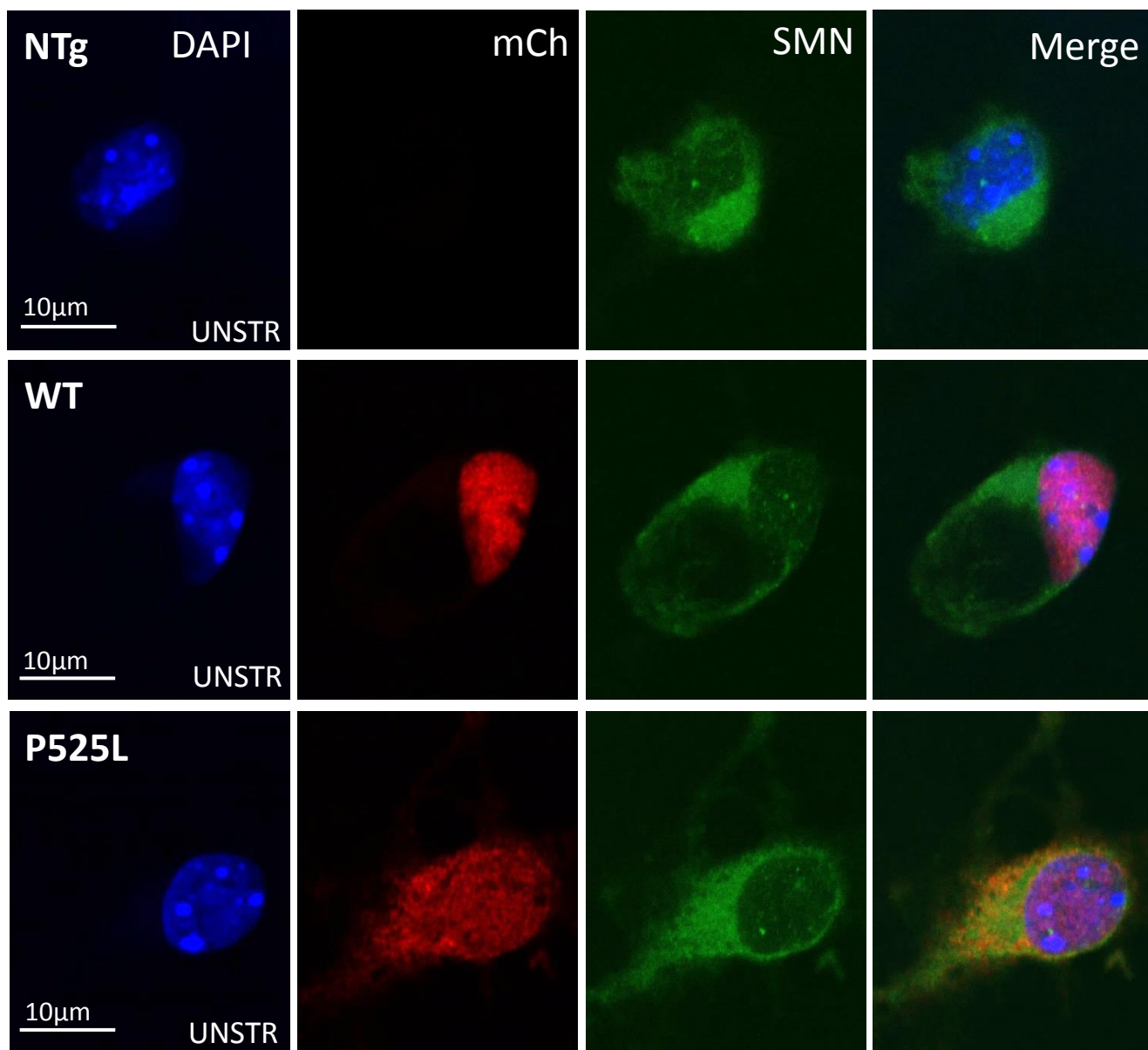


Figure 4.11a) Motor neurons from the FUS BAC transgenic mouse model in the unstressed and stressed conditions stained for mCherry and SMN. There are no cytoplasmic SMN granules after cell stress, and no evidence that SMN is incorporated into mutant FUS cytoplasmic granules (see next page).

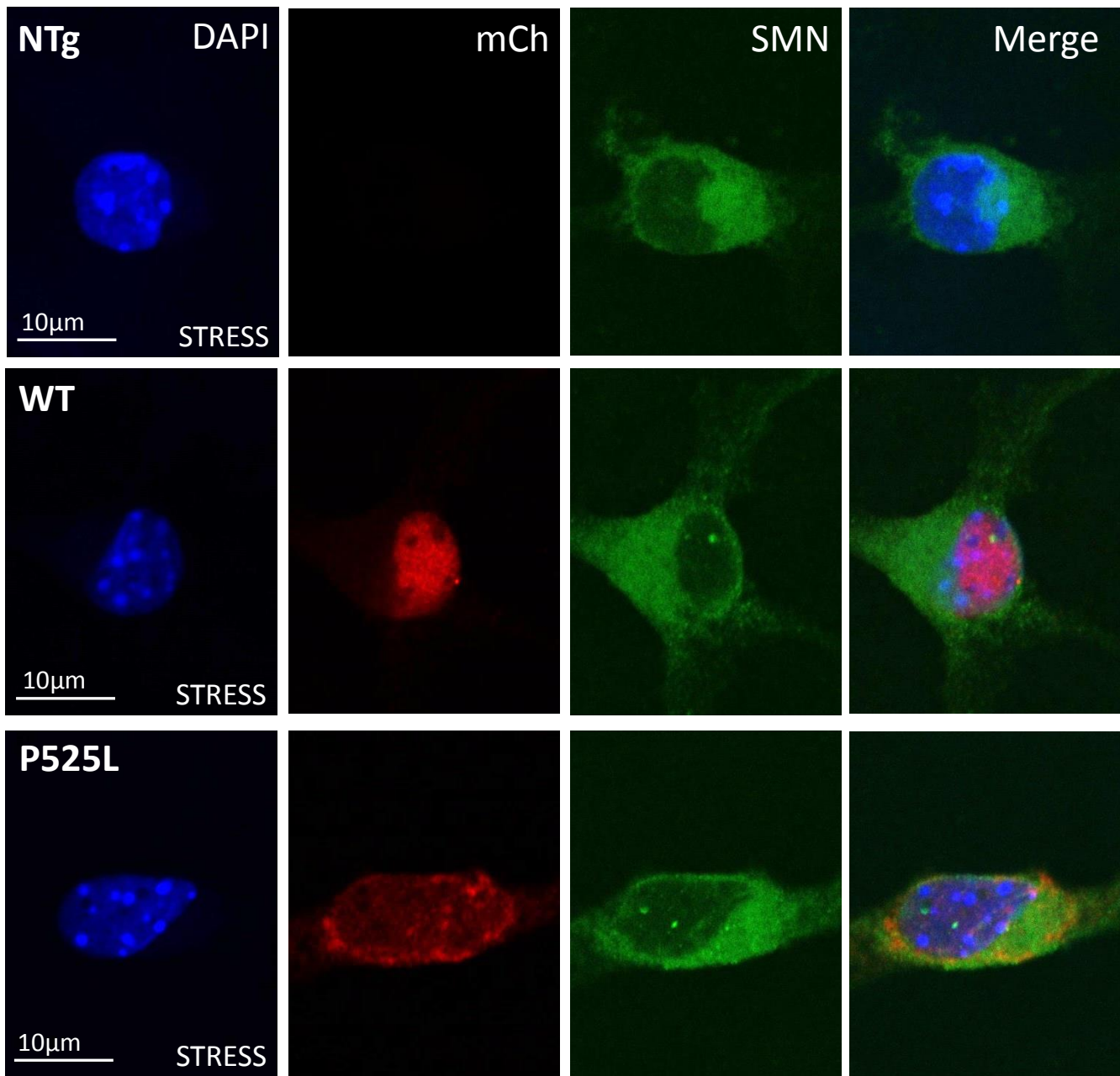


Figure 4.11a) Continued.

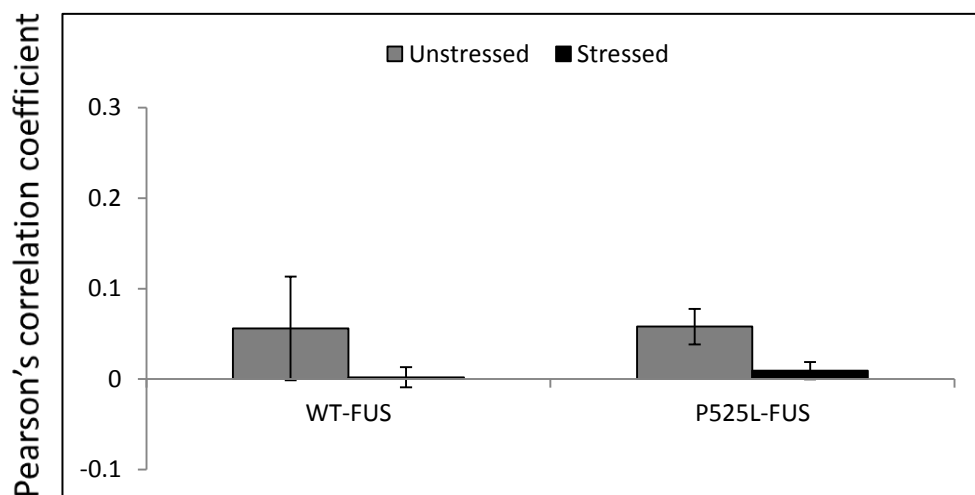


Figure 4.11b) Co-localisation analysis of SMN and mCherry in primary motor neurons from the FUS BAC transgenic mouse model. Pearson's correlation coefficient is low, and there is no significant difference between wild-type and P525L mutant lines. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test)

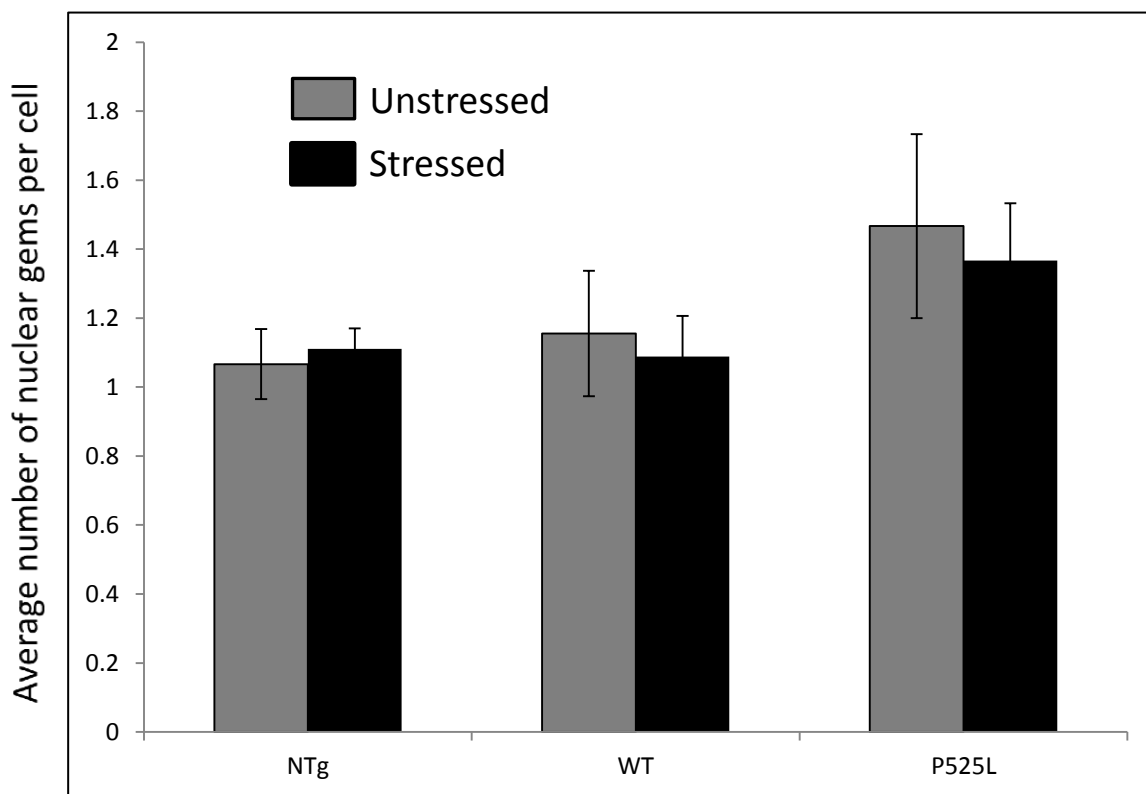


Figure 4.11c) Quantification of the number of SMN gems per motor neuron of the FUS BAC transgenic mouse model. There is no significant difference between each line. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test).

4.3 Results in FUS-BAC transgenic cortical neurons

4.3.1 Primary cortical cultures from the FUS-BAC transgenic mouse model

Cortical cultures were set up using FUS-BAC transgenic wild-type FUS line 2 and P525L-FUS line 1. Cultured cells demonstrated neuronal morphology and were positive for the neuronal marker Tuj1 (figure 4.12).

4.3.2 P525L FUS is mislocalised to the cytoplasm of FUS-BAC transgenic cortical neurons

Cortical neurons stained with an antibody raised against mCherry revealed significant mislocalisation of P525L mCherry-FUS compared to wild-type, with only an average of 33% remaining in the nucleus ($p < 0.001$) (figure 4.13a).

The availability of more cells using cortical compared to motor neuron culture enabled further experimental conditions to be examined. To examine the effect of the *FUS* mutation on the disassembly of stress granules, sodium arsenite was removed and replaced with fresh medium for 1 hour and 3 hours before cells were fixed.

There was a significantly lower proportion of nuclear mCherry-FUS in P525L neurons compared to wild-type FUS under all conditions ($p < 0.001$), but there was no significant change after sodium arsenite treatment (figure 4.13b).

Cytoplasmic granules of P525L-FUS were visualised by immunocytochemistry using the mCherry antibody after cells were stressed. There was a significant increase in the percentage of cells with these granules under all stressed conditions compared to unstressed ($p < 0.001$) (figure 4.13c). There was a non-significant increase in the percentage of cells with mCherry-FUS granules between the stressed condition and 1 hour post-stress, suggesting that granules are still forming at this time point. There

was a significant reduction between the 1 hour and 3 hour post-stress time points suggesting that during this time period these granules were starting to dissolve ($p < 0.01$) (figure 4.13c).

Analysis of the number of mCherry-FUS granules per cell revealed very few or no granules visible in the wild-type FUS neurons (figure 4.13d). There were some granules present even in the unstressed state in the P525L cells. After stress there was a significant increase in the number of granules per cell compared to wild-type and to the unstressed condition ($p < 0.001$ and $p < 0.01$). There was a non-significant reduction in the number of granules per cell when stress was removed, with gradual reduction at 1 hour then 3 hours.

These results together show that P525L-FUS was mislocalised to the cytoplasm of cortical neurons even at these low expression levels, reflecting the observations in motor neurons. When stress was removed, there was a trend towards a reduction in the percentage of cells with stress granules and the number of granules per cells, particularly 3 hours after the removal of stress. This suggests that the cortical neurons are able to dissolve these granules over time when the stress insult is no longer present.

4.3.3 P525L FUS is incorporated into stress granules in cortical neurons

Confocal microscopy was used to assess co-localisation between mCherry-FUS and G3BP within the cytoplasm of cortical neurons cultured from the BAC mice (figure 4.14a&b). In the P525L neurons there was co-localisation, particularly after cell stress, which was significantly greater than wild-type FUS ($p < 0.001$ after stress).

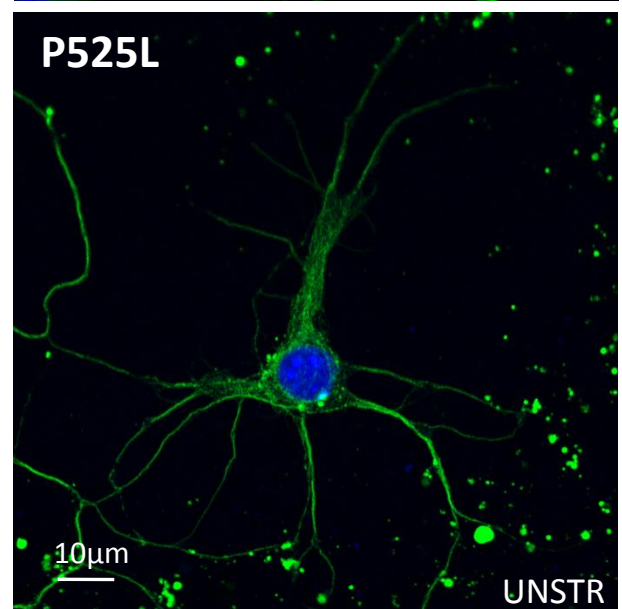
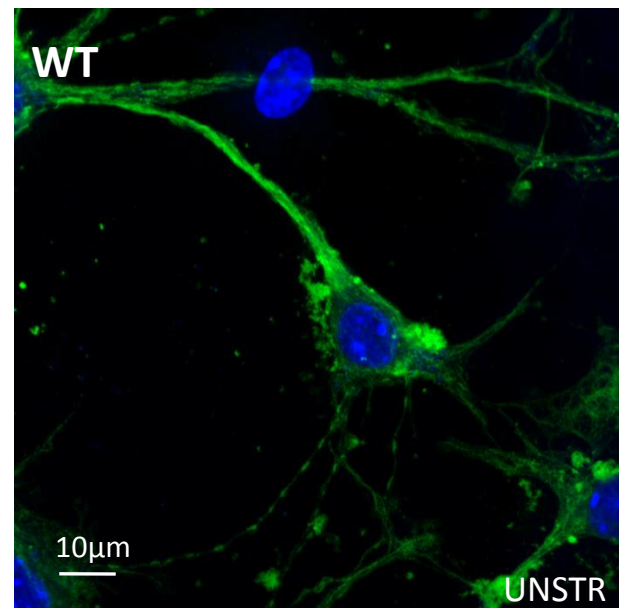
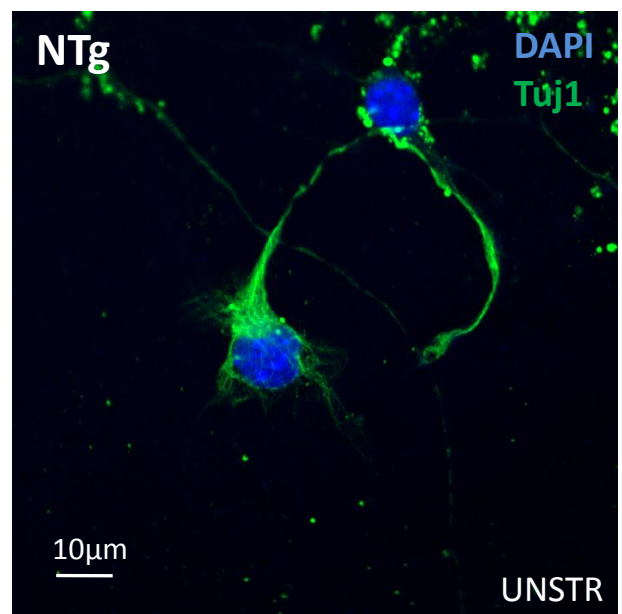


Figure 4.12. Primary cortical neurons from the FUS BAC transgenic mouse model display neuronal morphology and the neuronal marker TuJ1.

Figure 4.13a) Primary cortical neurons from the FUS BAC transgenic mouse model stained for mCherry and DAPI. They demonstrate cytoplasmic mislocalisation of P525L-FUS compared to wild-type. (N=3 experiments, 15 cells each. Unpaired t test.)

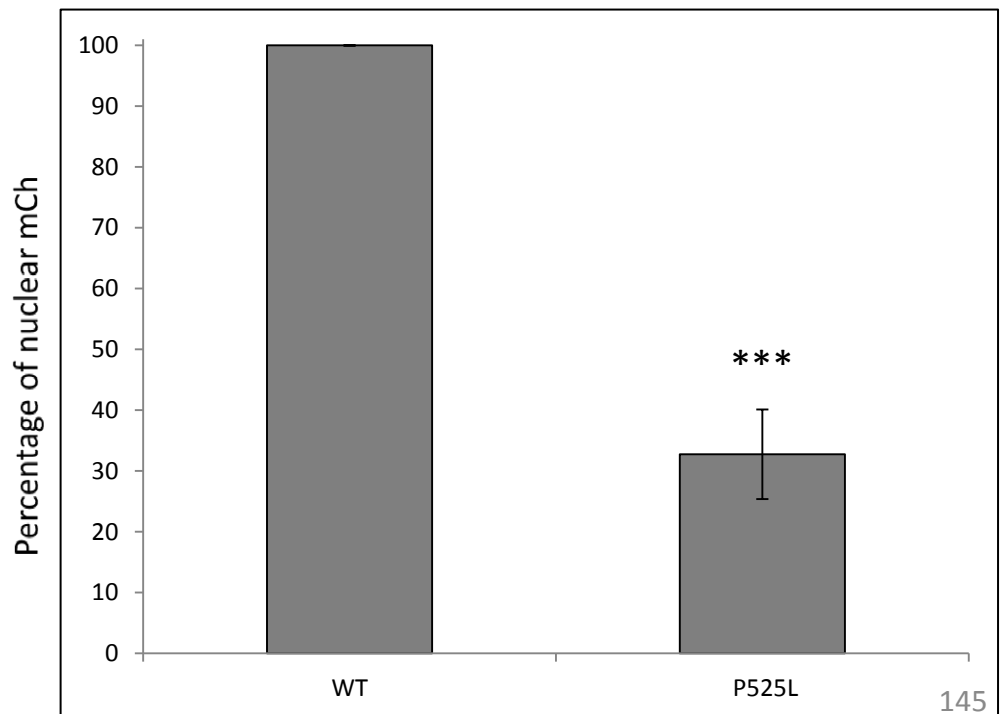
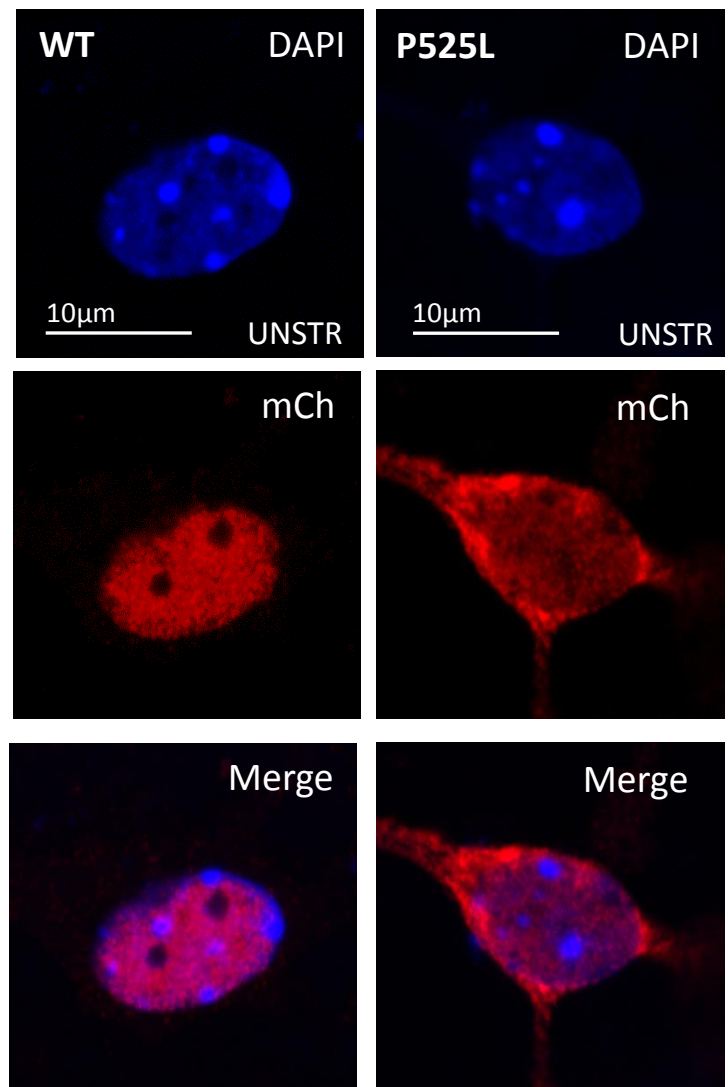
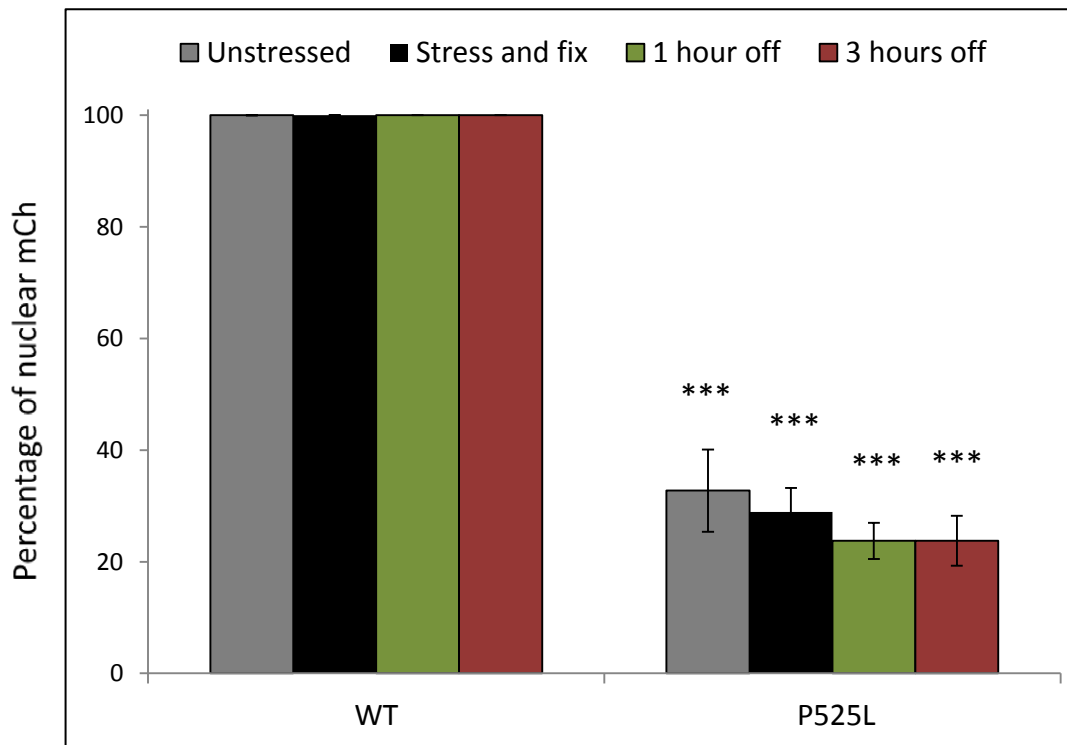
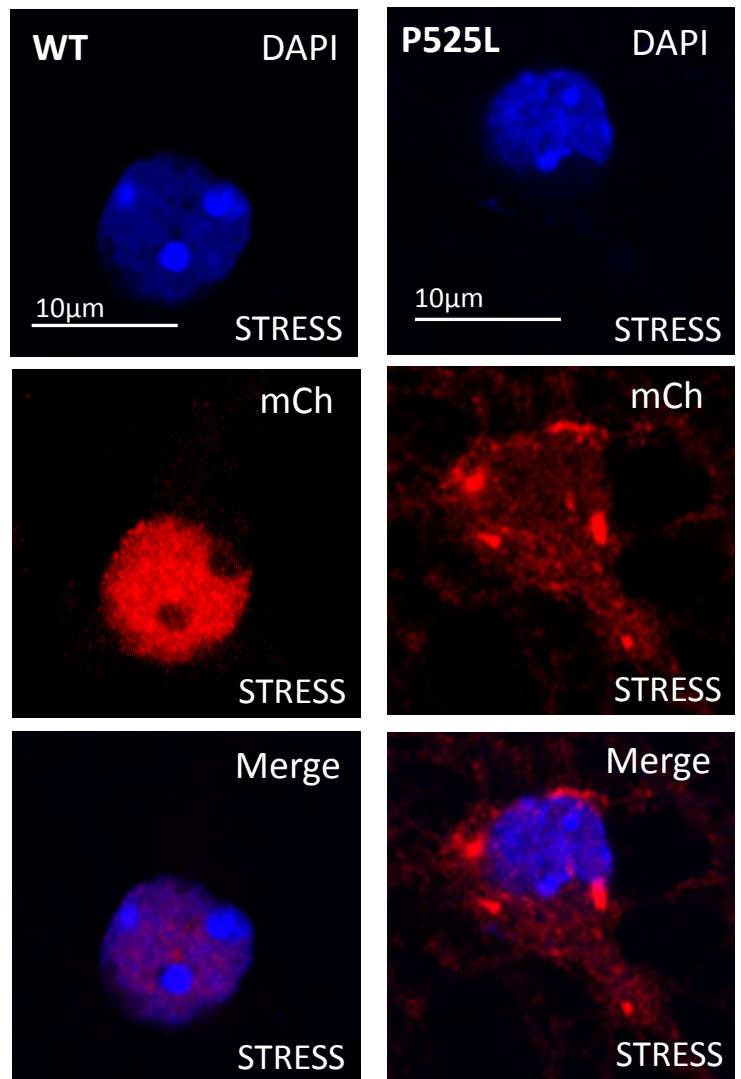


Figure 4.13b) Primary cortical neurons from the BAC transgenic mouse model were untreated ('unstressed'), treated for 60 minutes in 0.5mM sodium arsenite and immediately fixed ('stress and fix'), treated with sodium arsenite then incubated for 1 hour in normal media ('1 hour off'), and treated with sodium arsenite then incubated for 3 hours in normal media ('3 hours off'). Wild-type FUS-mCherry remains nuclear in all conditions. P525L FUS-mCherry is mislocalised to the cytoplasm, with no significant difference between each treatment. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test. Stars denote statistically significant differences compared to wild-type under the same condition).



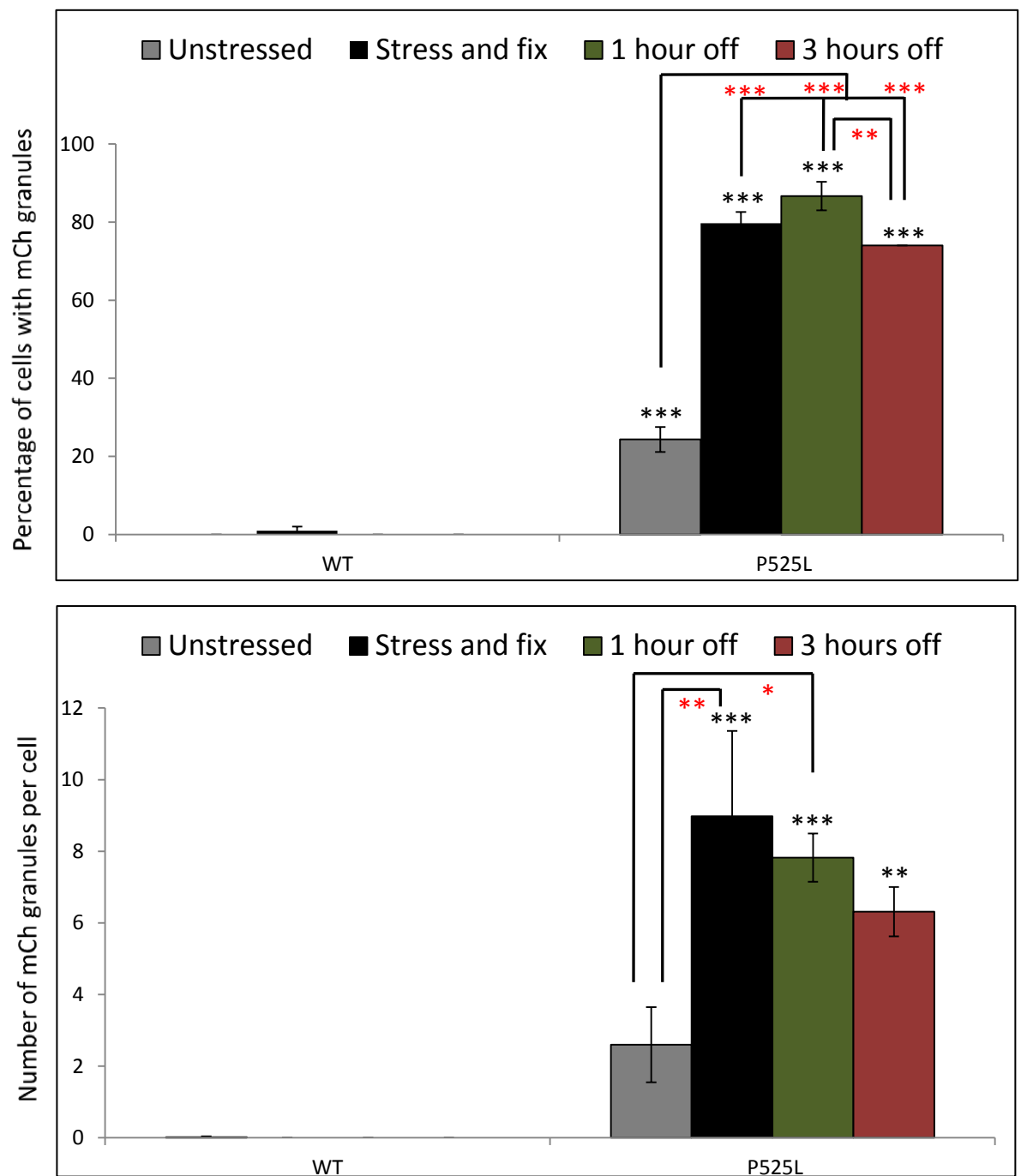


Figure 4.13c) Quantification of the percentage of cells with mCherry-FUS granules in primary cortical neurons from the FUS BAC transgenic mouse model. There are very few wild-type cells with granules. Some P525L neurons display granules without stress, though the percentage significantly increases after stress. There is a small but significant decrease in the percentage of cells with granules between the 'stress and fix' and the '3 hours off' conditions. **d)** Quantification of the number of mCherry-FUS granules per cell in primary cortical neurons from the FUS BAC transgenic mouse model. There are very few granules in wild-type neurons. There is a significantly greater number in P525L neurons after stress, and a trend towards a reduction when stress is removed. (N=3 experiments, 100 cells each. One way ANOVA. Tukey post-test. Black stars denote statistically significant differences compared to wild-type under the same condition).

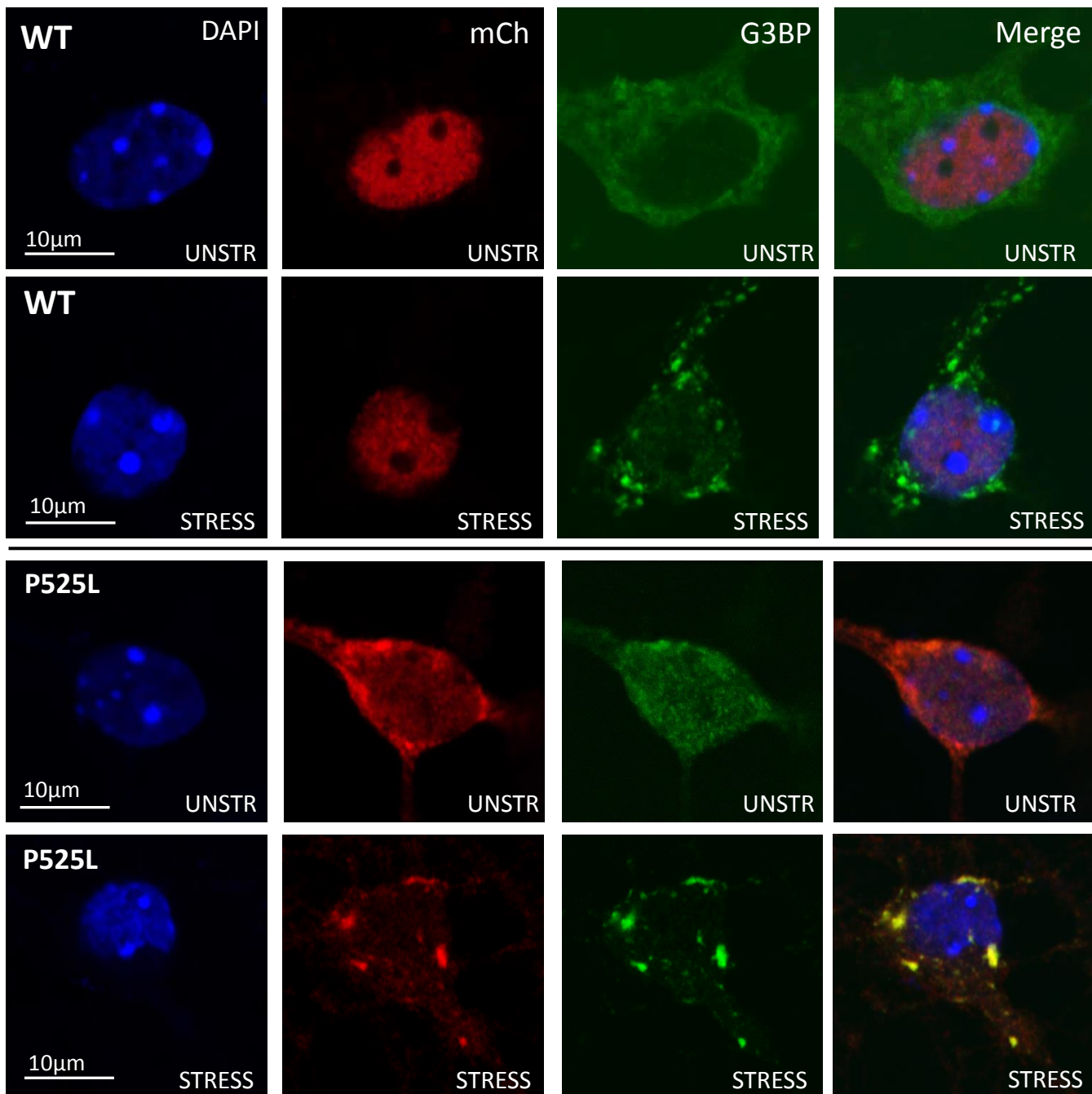


Figure 4.14a) Confocal microscopy of cortical neurons from the FUS BAC transgenic mouse model stained with DAPI, mCherry and G3BP. They demonstrate co-localisation between G3BP and mCherry-FUS in P525L but not wild-type neurons, occurring predominantly in stress granules.

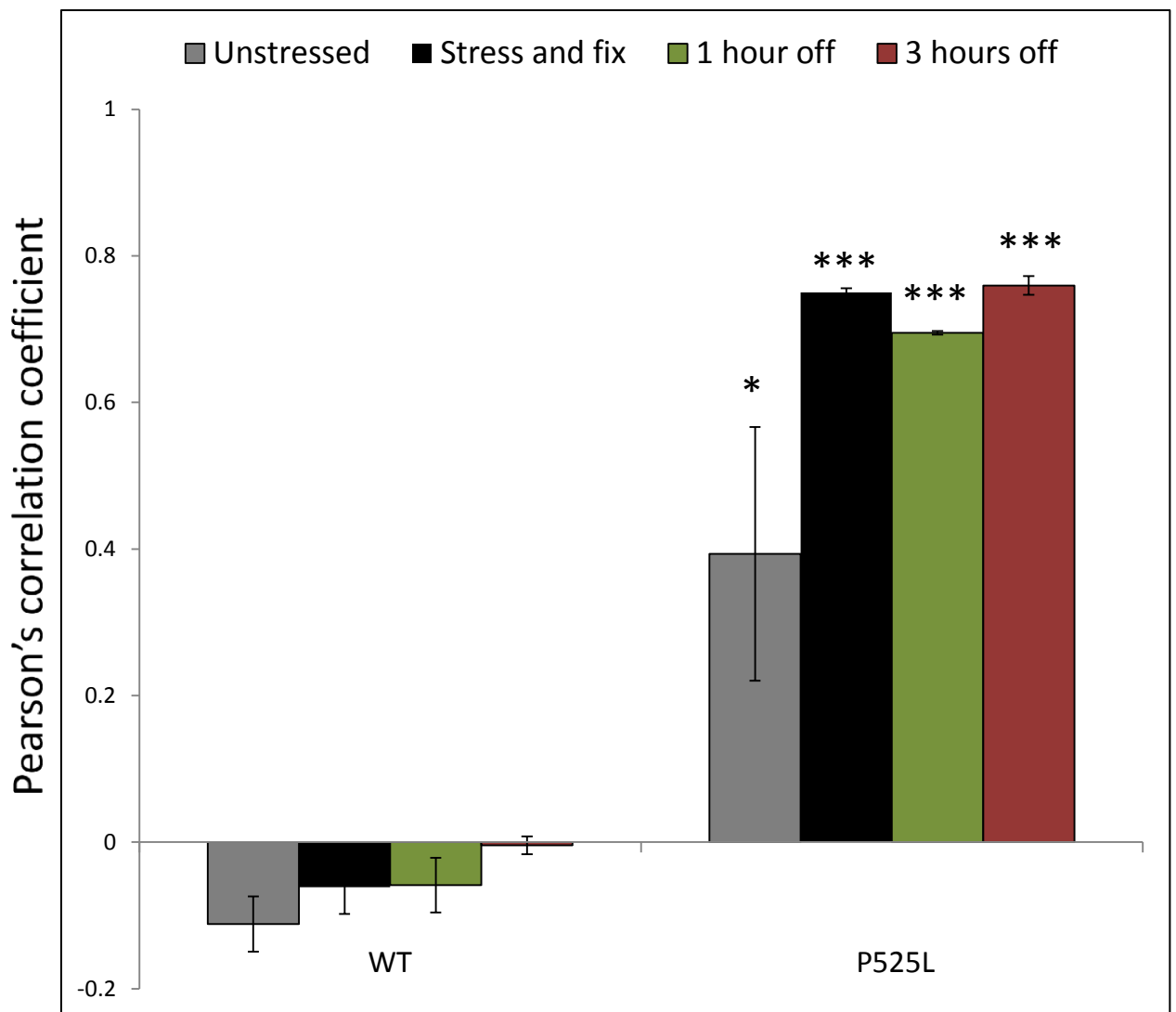


Figure 4.14b) Quantification of mCherry-FUS and G3BP co-localisation in cortical neurons from the FUS BAC transgenic mouse model using confocal microscopy to calculate Pearson's correlation coefficient. Wild-type neurons have no evidence of co-localisation. P525L neurons show significantly increased co-localisation, particularly after stress. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test. Stars denote statistically significant differences compared to wild-type under the same condition).

There was a non-significant increase in the correlation coefficient in the stressed compared to unstressed conditions. This suggests that most co-localisation may occur in the stress granules, as observed in the images themselves.

4.3.4 Stress granule dynamics in P525L FUS cortical neurons

After treatment with sodium arsenite, the percentage of cells with G3BP-positive stress granules increased significantly in each line ($p < 0.001$) (figure 4.15a).

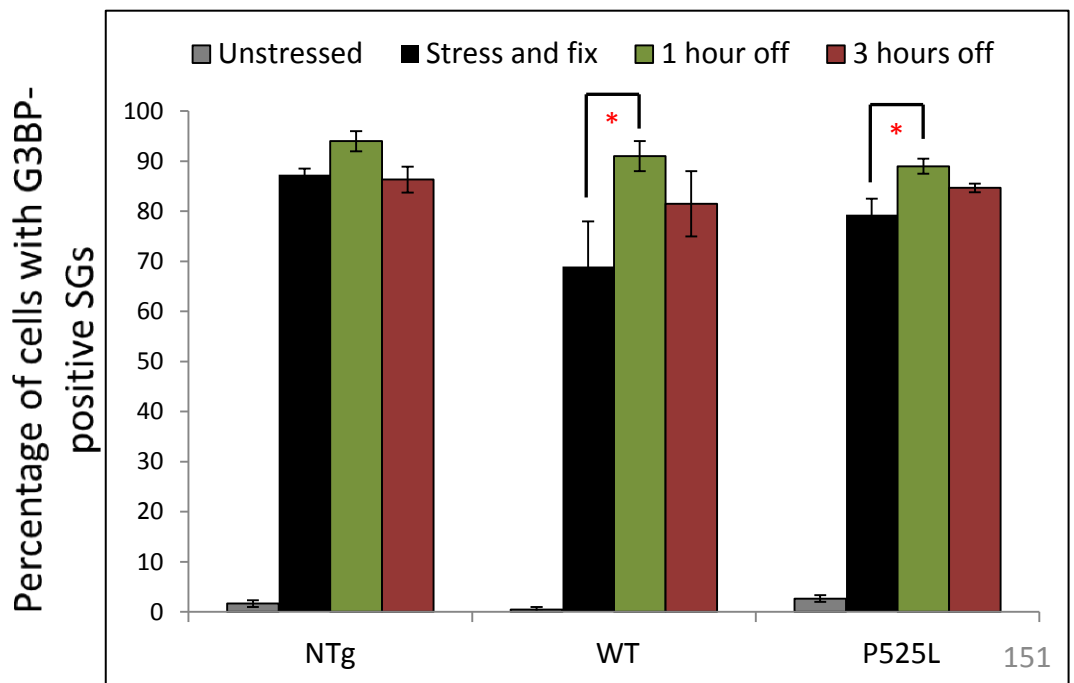
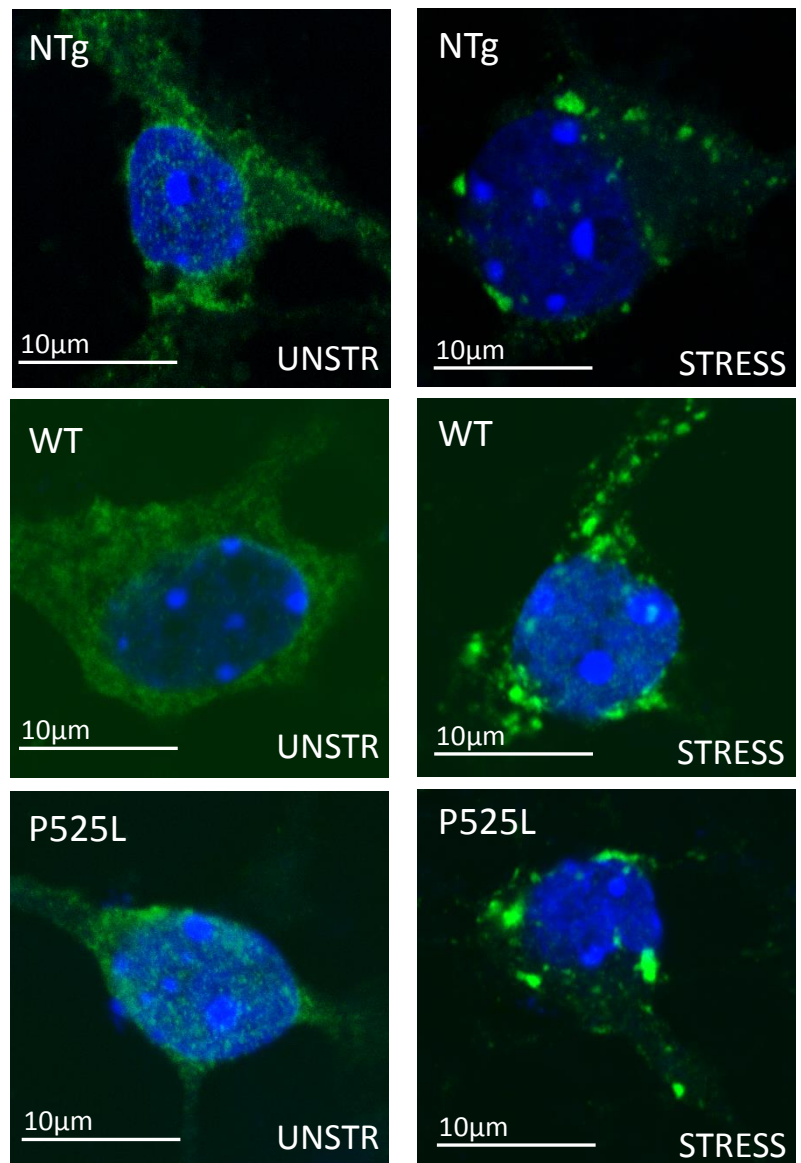
Interestingly, there was a significant increase between the stressed and the 1 hour post-stress conditions in the wild-type and P525L neurons ($p < 0.05$). This suggests that 1 hour after stress, granules were still forming in the transgenic neurons, similar to the observation in mCherry granules (figure 4.13c). 3 hours after stress, a reduction is observed suggesting granules are starting to dissolve. There was no significant difference between each genotype in a given condition using this antibody.

There was a significant increase in the percentage of cells with PABP-positive stress granules after stress compared to unstressed in all genotypes (NTg and P525L $p < 0.001$, WT $p < 0.01$) (figure 4.15b). The trend towards an increase at 1 hour compared to the stressed condition was also observed, though was only significant in the wild-type cells ($p < 0.01$). There was again a reduction at 3 hours compared to 1 hour, though this was only significant in the non-transgenic neurons ($p < 0.05$). Comparing the lines using this antibody revealed some differences. In the unstressed state, the P525L neurons had significantly more cells with stress granules compared to the other 2 genotypes ($p < 0.001$).

Figure 4.15a)

Images of cortical neurons from the FUS BAC transgenic mouse model show the presence of G3BP positive stress granules after cell stress.

Quantification of the percentage of cells with stress granules shows a significant increase after stress (not illustrated) but an increase 1 hour post-stress where shown, and no difference between genotypes. (N=3 experiments, 100 cells each. One way ANOVA. Tukey post-test).



Percentage of cells with PABP-positive SGs

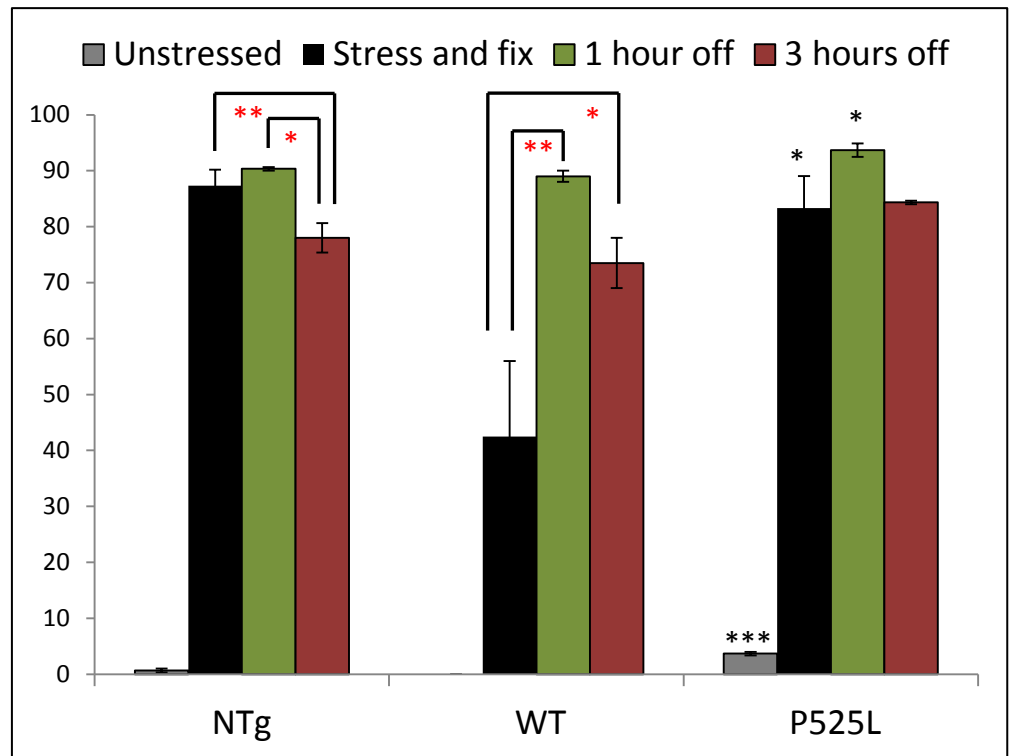


Figure 4.15b) Quantification of the percentage of cortical neurons from the FUS BAC transgenic mouse model with PABP-positive stress granules. There is a significant increase after stress (not illustrated) and a trend towards a reduction 3 hours after treatment compared to 1 hour. There are some small but significant changes in the P525L compared to wild-type neurons. (N=3 experiments, 100 cells each. One way ANOVA. Tukey post-test. Black stars denote statistically significant differences compared to wild-type under the same condition).

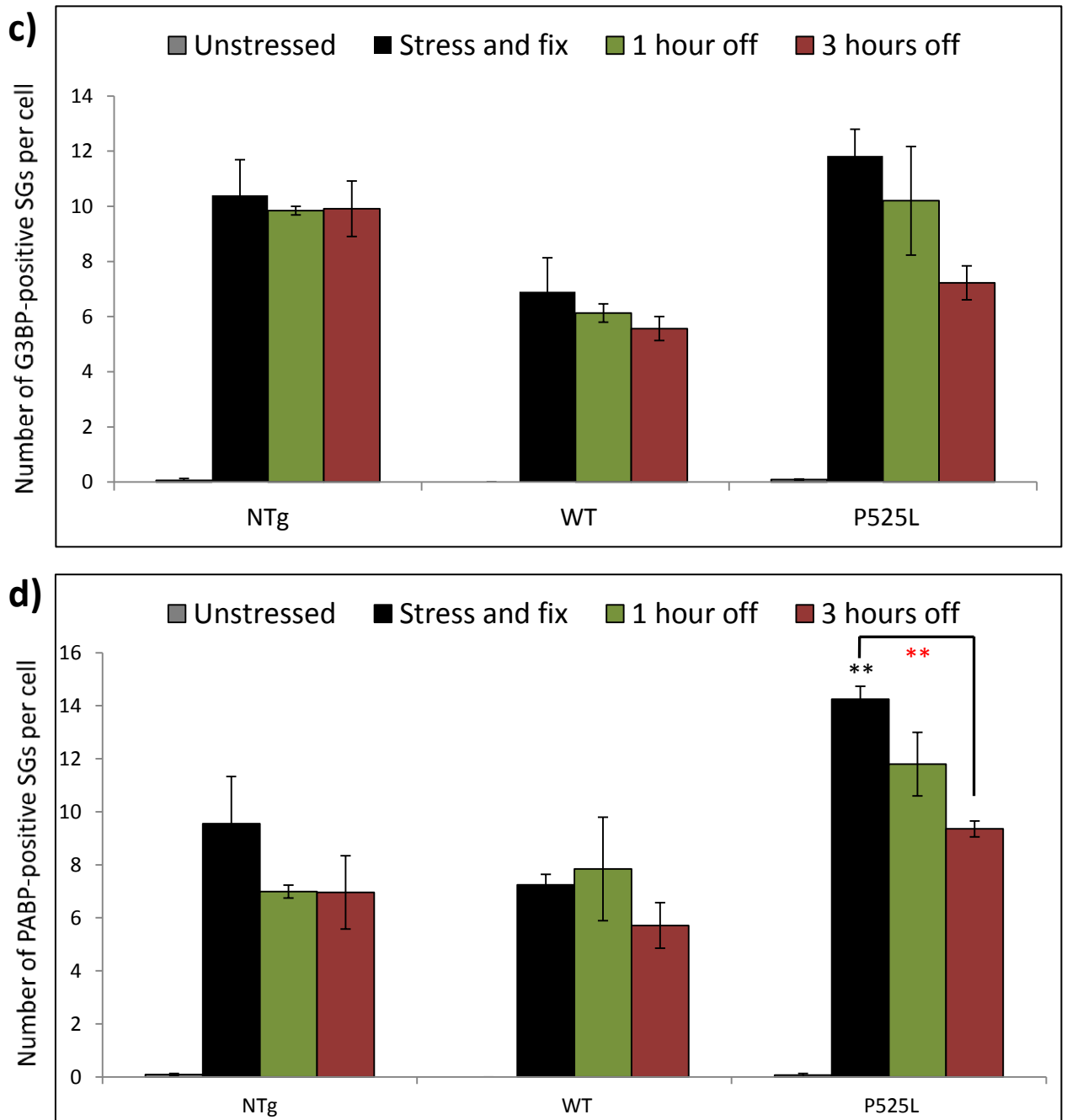


Figure 4.15 Quantification of the number of stress granules per cortical neuron from the FUS BAC transgenic mouse model. **c)** G3BP-positive stress granules. There is a significant increase after stress in each genotype, but no significant difference between the genotypes under each condition. There is a trend towards a reduction when stress is removed, particularly in the P525L neurons. There is also a non-significant increase in granules in the P525L compared to wild-type neurons in each condition. **d)** PABP-positive stress granules. There is a significant increase after stress in each genotype, and in the P525L neurons there is a reduction in the '3 hours off' compared to the 'stressed' condition. There is also an increase in granules in the P525L neurons compared to wild-type in the 'stressed' condition. These could reflect alterations in stress granule dynamics in the presence of the mutation. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test. Black stars denote statistically significant differences compared to wild-type under the same condition).

This suggests that cells may have been under stress due to the presence of the mutation, or that cytoplasmic mCherry induced the formation of stress granules. In the stressed and 1 hour post-stress conditions, P525L neurons again displayed an increased percentage of cells with stress granules compared to wild-type ($p < 0.05$). This may be due to low values for the wild-type FUS in this experiment. However, it does suggest that the mutation may alter stress granule dynamics by increasing stress granule formation.

The average number of stress granules per cell was determined using G3BP and PABP antibodies (figure 4.15c&d). Both antibodies revealed a significant increase in stress granules per cell in stressed compared to unstressed in all lines ($p < 0.001$). There was an increase in stress granules per cell in the P525L compared to wild-type cells, reaching statistical significance with the PABP antibody ($p < 0.01$). P525L neurons did show a clear reduction when stress is removed, but values were still greater than in wild-type FUS neurons. This again suggests that the mutation may be causing changes in neuronal stress response.

4.4 Results in the FUS knockout model

4.4.1 Primary motor neuron cultures from the FUS knockout mouse model

A non-conditional FUS transgenic knockout model was used for these experiments. This is a commercially available model generated by deletion of the entire FUS coding region by insertion of a lacZ-neomycin cassette through homologous recombination (VelociGene). Complete knockout of FUS using this model caused embryonic lethality between embryonic day 14 and 18, so no knockout pups were born.

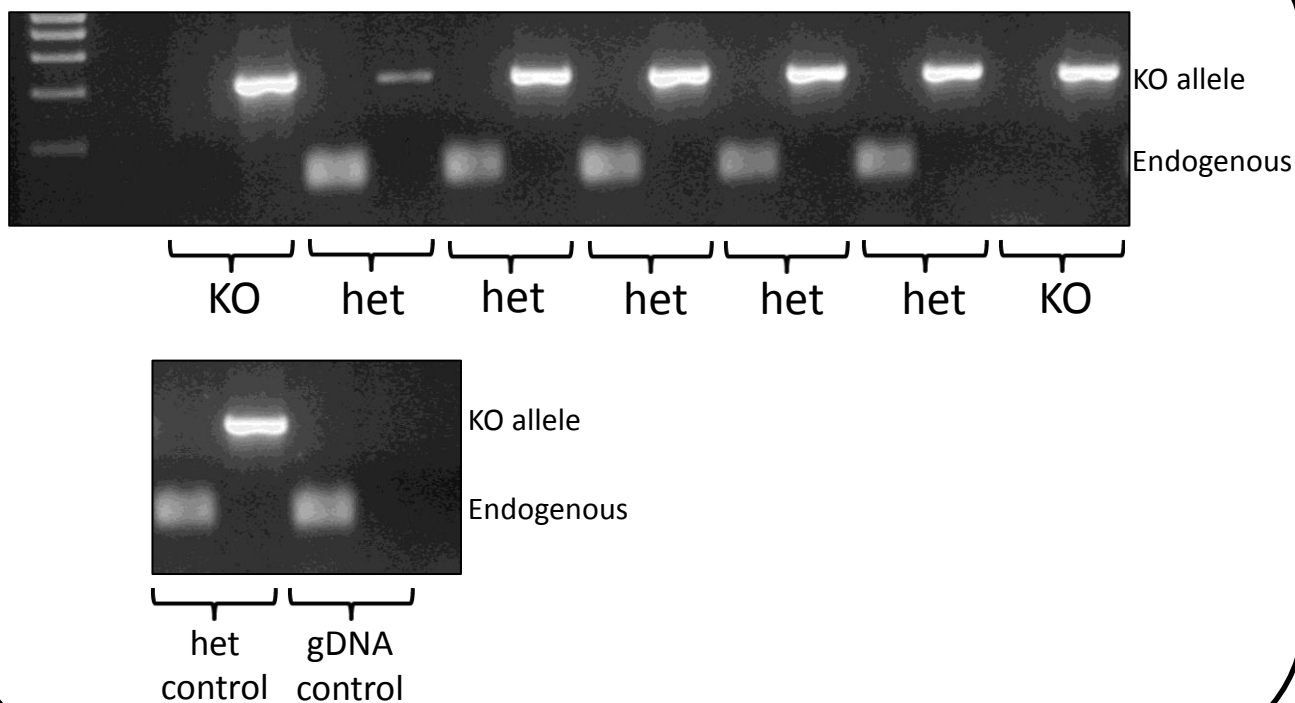


Figure 4.16a) Genotyping results of embryos from the FUS knockout mouse model used for primary motor neuron cultures. PCR was used to detect both the endogenous allele and the insert used to create the knockout. Embryos may be non-transgenic, heterozygous knockout or homozygous knockout.

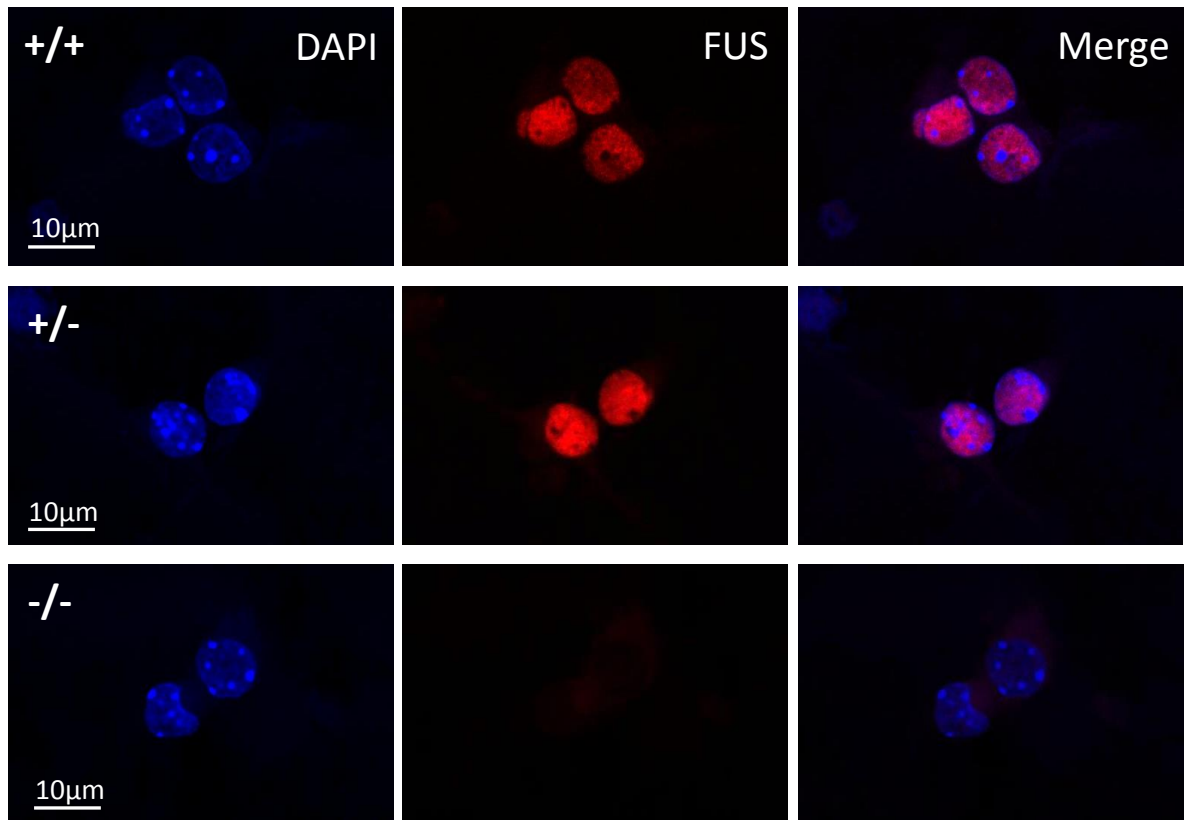


Figure 4.16b) Primary motor neurons from the FUS knockout mouse model were stained for FUS. Confocal images demonstrate that FUS is detectable in non-transgenic and heterozygous but not homozygous knockout motor neurons. (+/+ = non-transgenic, +/- = heterozygous knockout, -/- = homozygous knockout)

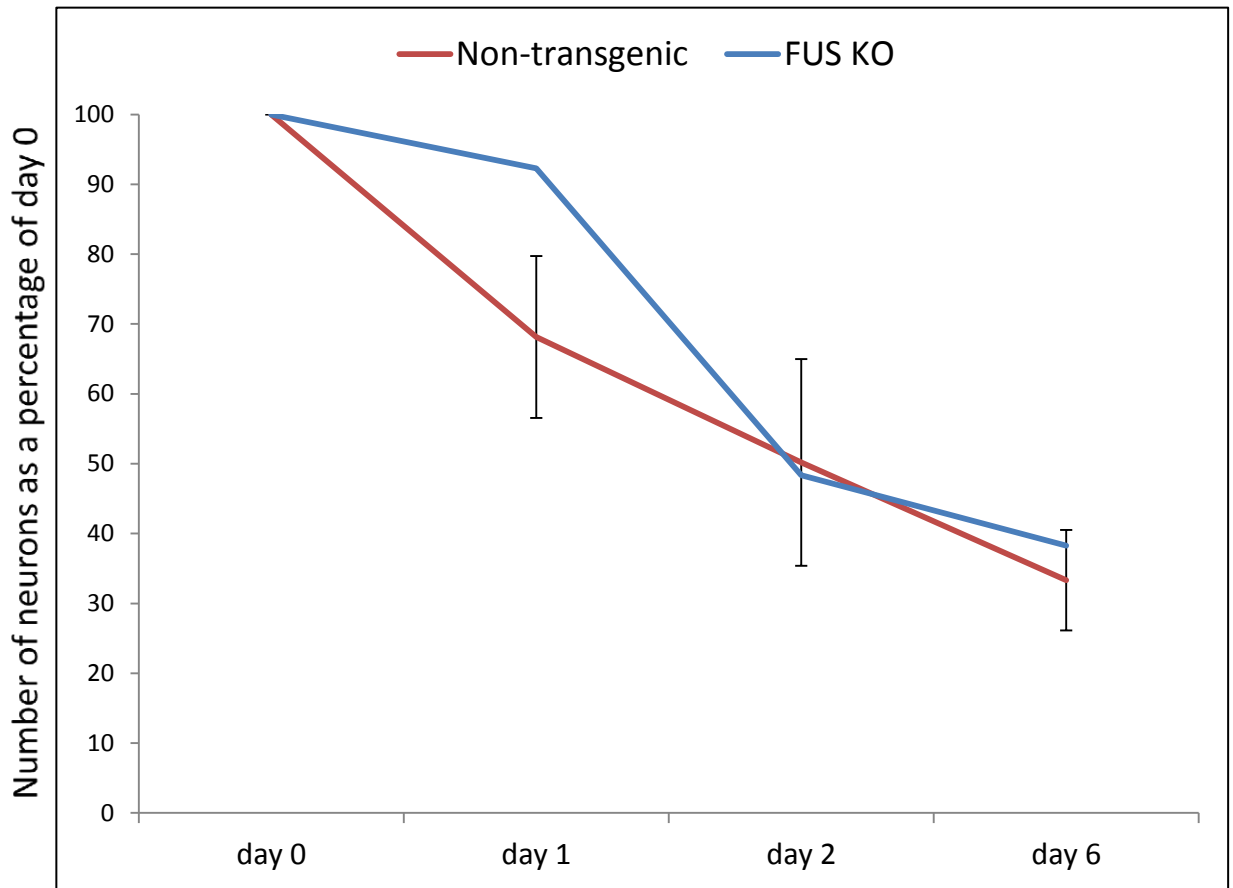


Figure 4.16c) Survival curves comparing non-transgenic with FUS homozygous knockout motor neurons does not detect a difference in survival. (n=3 non-transgenic, n=1 KO, 100 cells per experiment day 0. Unpaired t-test)

Mice heterozygous for the knockout allele were crossed to create embryos which were non-transgenic, heterozygous or homozygous for the null allele. Primary motor neuron cultures were set up using individual embryos and the remaining tissue was then used to determine the genotype by using primers specific for endogenous FUS and the insert (figure 4.16a). Embryos of all genotypes were found, showing that it is possible for knockout embryos to survive to embryonic day 13.5.

Neurons were stained for endogenous FUS, which confirmed that FUS was absent in homozygous knockout neurons (figure 4.16b).

Survival of FUS-knockout neurons was compared with non-transgenic, however no differences were observed, suggesting that FUS knockout does not impair motor neuron survival (figure 4.16c).

4.4.2 The distribution of FET proteins is not altered by FUS knockout

In order to examine whether FUS knockout resulted in mislocalisation of the other FET proteins, neurons were stained with antibodies to EWS and TAF15. This revealed no significant difference between the lines, suggesting that FUS knockout did not affect the distribution of other FET proteins (figure 4.17a).

4.4.3 Stress granule dynamics in FUS knockout motor neurons

PABP was used to detect stress granules in these neurons both in the unstressed and stressed conditions. There was no difference in the percentage of cells with stress granules in homozygous knockout compared to non-transgenic neurons, but there was an increase in the heterozygous lines ($p < 0.01$) (figure 4.17b).

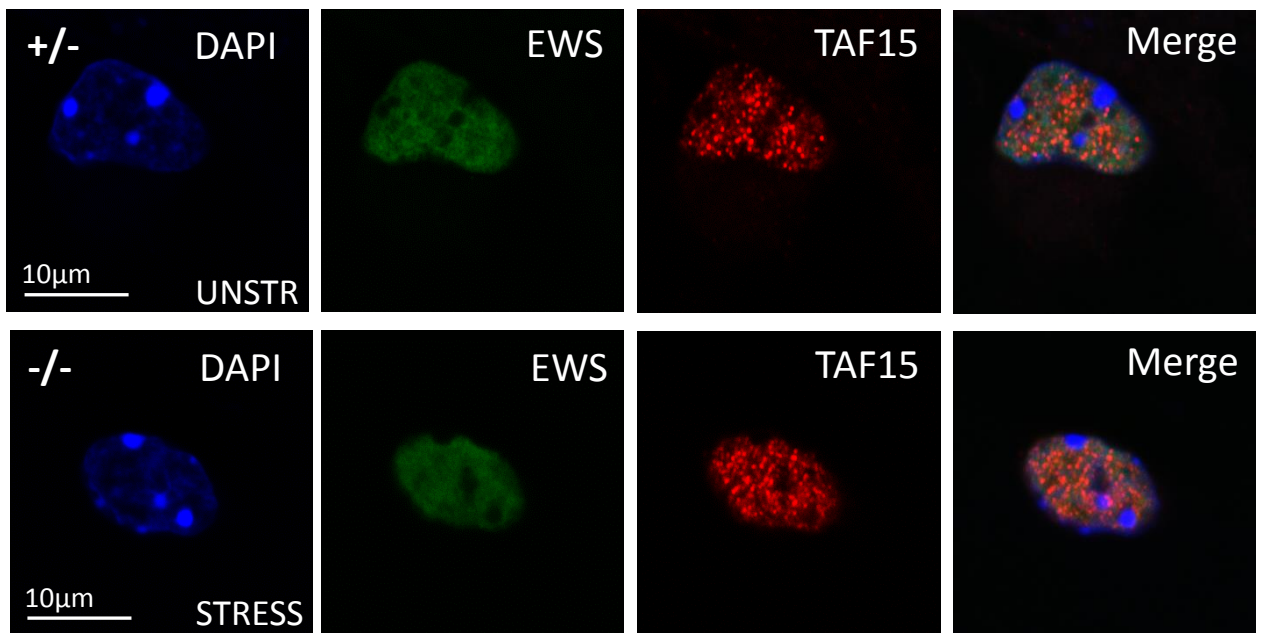


Figure 4.17a)

Analysis of the distribution of EWS and TAF15 in primary motor neurons from the FUS knockout mouse model. Both proteins are predominantly nuclear, with no difference detected between the lines. (n=3 +/+, n=3 +/-, n=2 -/-, 15 cells per experiment. One way ANOVA. Tukey post-test).

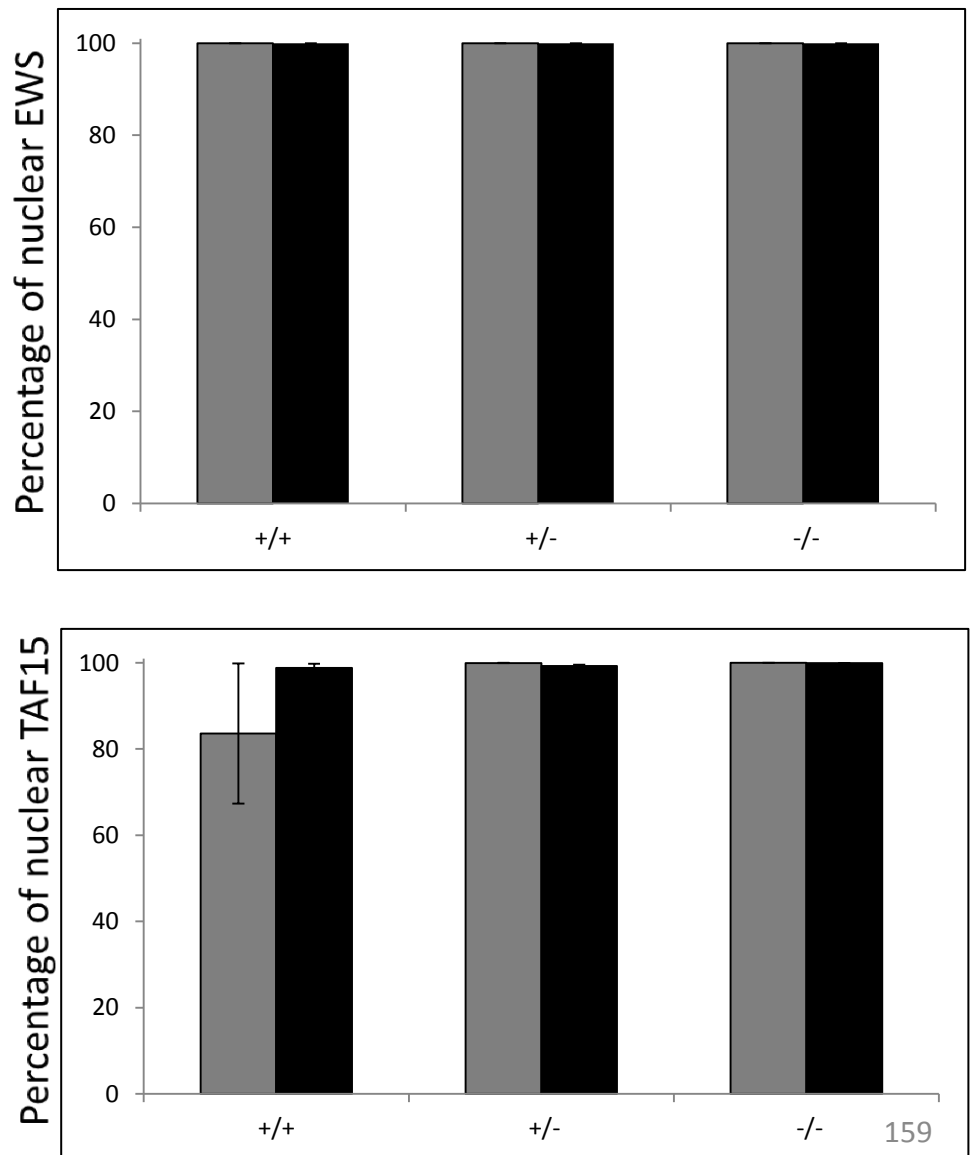
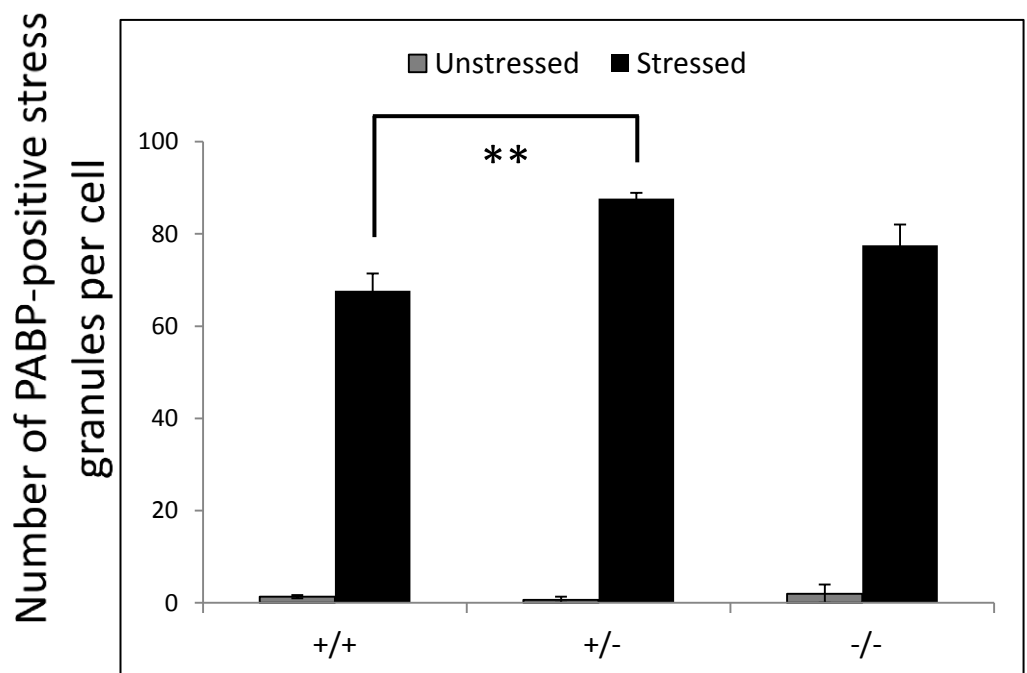
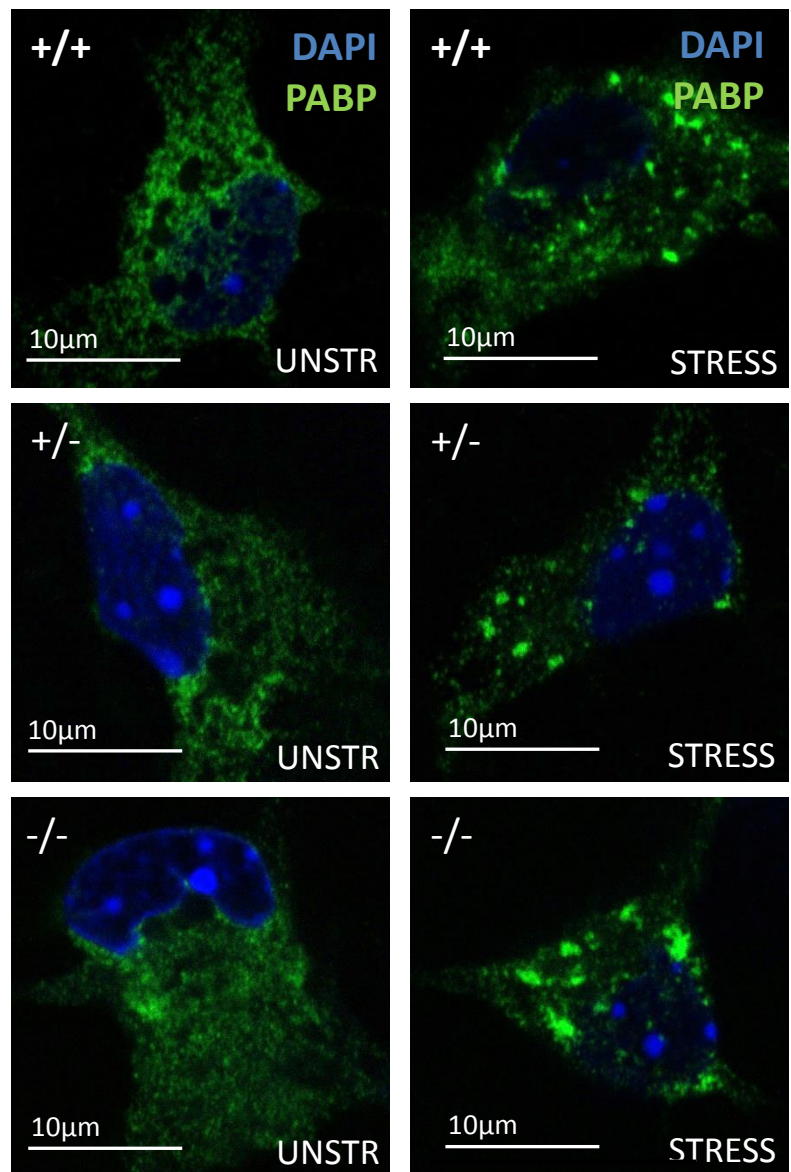


Figure 4.17b) Motor neurons from the FUS knockout mouse model were used to quantify the percentage of cells with PABP-positive stress granules in the unstressed and stressed condition. There is no significant difference observed between FUS knockout and non-transgenic neurons, though there is an increase in the heterozygous knockout neurons compared to non-transgenic. (n=3 +/+, n=3 +/-, n=2 -/-. 100 cells per experiment. One way ANOVA. Tukey post-test).



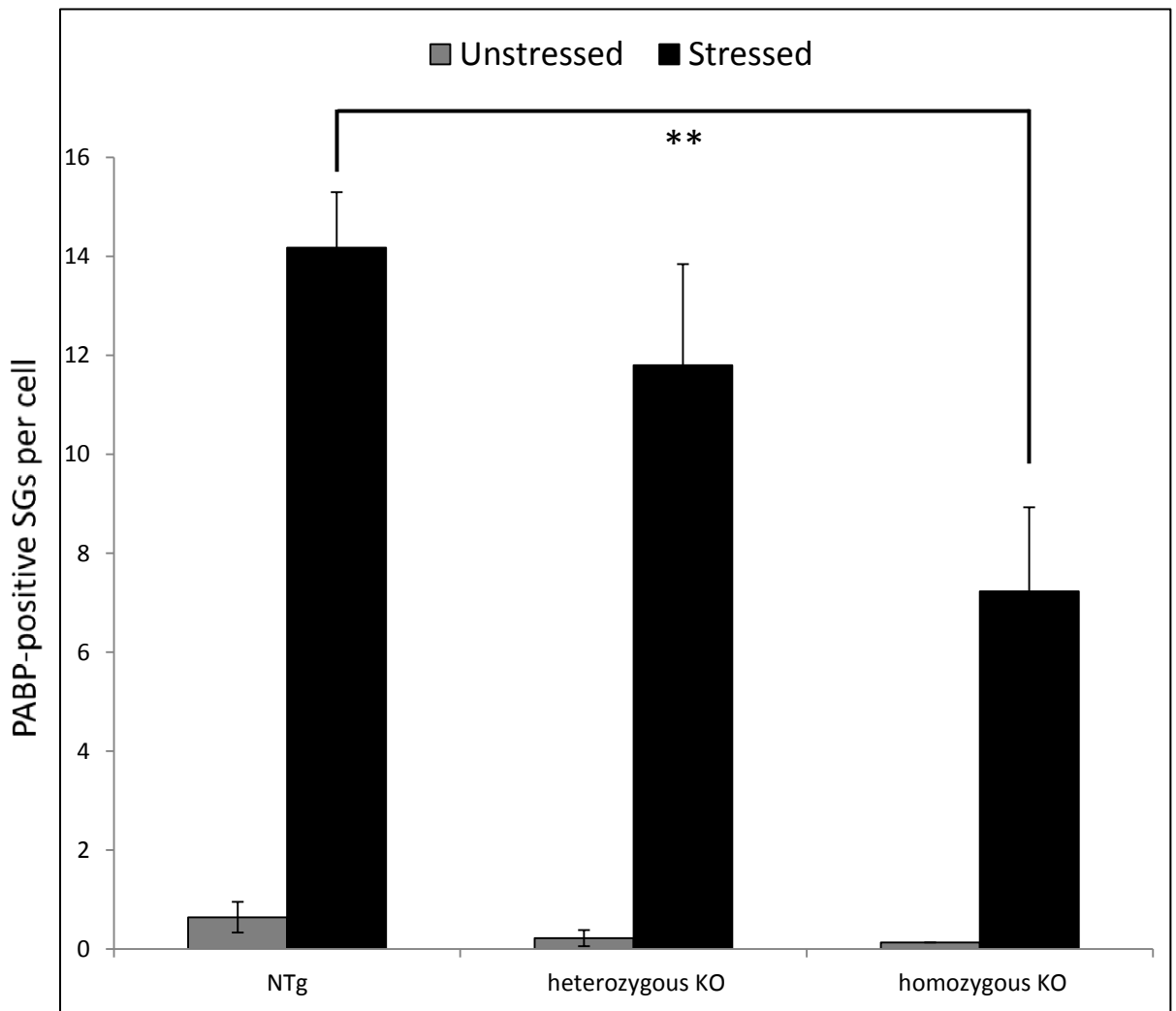
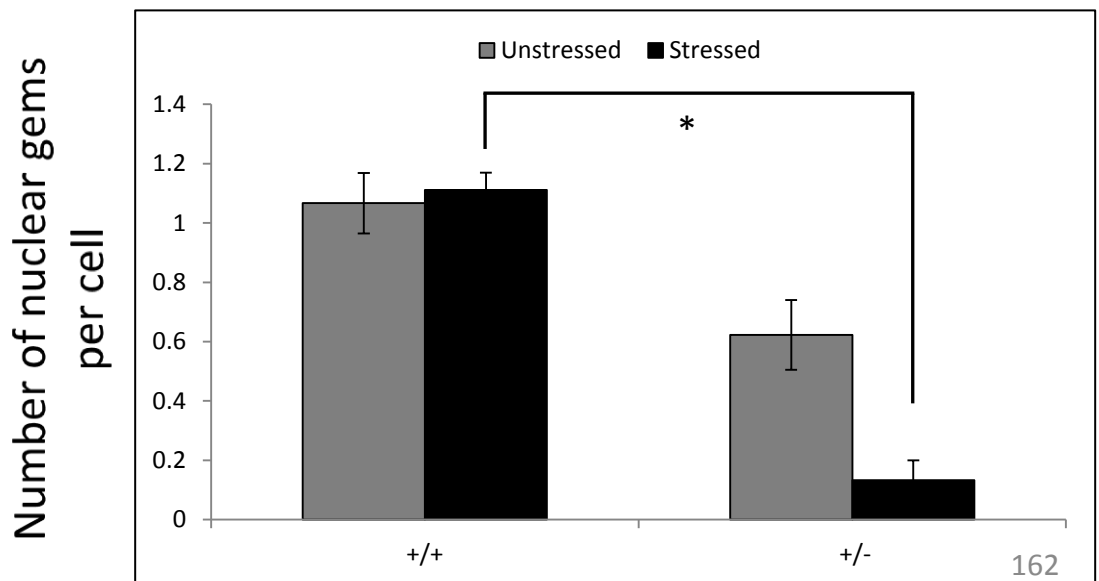
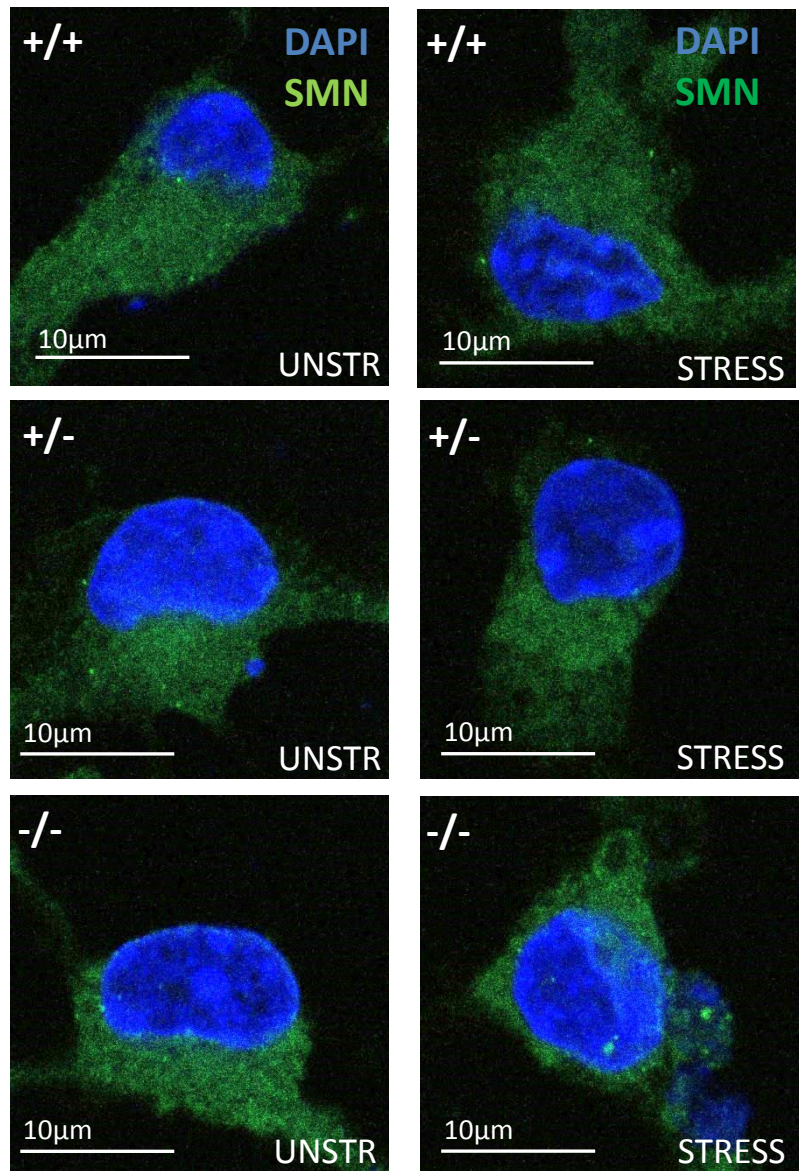


Figure 4.17c) Quantification of the number of PABP-positive stress granules per neuron from the FUS knockout model. There is a significant reduction in the number of stress granules per cell in the homozygous knockout compared to non-transgenic. (n=3 +/+, n=3 +/-, n=2 -/-. 15 cells per experiment. One way ANOVA. Tukey post-test).

Figure 4.17d)

Quantification of the number of SMN gems per neuron from the FUS knockout model. This shows a reduction in gem number in the heterozygous neurons compared to non-transgenic after cell stress. (n=3, 15 cells per condition. One way ANOVA. Tukey post-test). Data for -/- genotype cannot be analysed due to insufficient numbers.



This suggests that there may be alterations in FUS in the heterozygotes that affect stress granule dynamics. Using an antibody to TIA1 did not reveal any difference between the lines (appendix figure 7.7c). Quantification of the number of PABP-positive stress granules per cell revealed a significant reduction in number in FUS KO neurons compared to non-transgenic ($p < 0.01$) (figure 4.17c). Interestingly, this closely matches the data showing a reduction in granules per cell in P525L motor neurons (figure 4.9c).

4.4.4 SMN gems may be altered by FUS knockout

Images of FUS knockout motor neurons stained for SMN did not reveal any alterations in SMN distribution in the absence of FUS (figure 4.17d). Heterozygous knockout neurons had significantly fewer gems than non-transgenic after cell stress ($p < 0.05$). This suggests that alterations in FUS levels may have an effect on SMN, particularly after cell stress. There was no significant difference in the number of gems per cell in the FUS homozygous knockout, though there was a trend towards a decrease compared to non-transgenic (data not shown). However, there were insufficient embryos available to carry out reliable statistical analysis on the homozygous knockout lines.

4.5 Results in the TDP-43 BAC transgenic mouse model

4.5.1 Primary motor neuron cultures from the TDP-43 BAC transgenic mouse model

The TDP-43 BAC transgenic mouse model (see appendix 7.11 for overview) was used to examine the effect of mutant TDP-43 on FUS and the other FET proteins.

Mice homozygous for the BAC construct were crossed with non-transgenic mice, creating hemizygous embryos which were used for cultures. Non-transgenic, wild-type and M337V lines were used, the latter two expressing human TDP-43 with a GFP tag. GFP was not, however, detectable without additional staining with an antibody raised to the GFP tag (Gordon et al, unpublished). These embryos were again cultured individually despite having a known genotype in order to maintain continuity.

4.5.2 The distribution of FET proteins is not altered by mutant TDP-43 expression

Primary motor neurons from the TDP-43 BAC transgenic mouse model demonstrate cytoplasmic mislocalisation of mutant M337V TDP-43 but not wild-type TDP-43 expressed by the construct (Gordon et al, in preparation, see appendix figure 7.11). The distribution of the FET proteins FUS, EWS and TAF15 were examined in TDP-43 transgenic motor neurons to determine whether mutant TDP-43 sequesters these FET proteins into the cytoplasm. Motor neurons were stained for FUS, EWS and TAF15. There was no difference in the distribution of any of these proteins between each line, suggesting that mislocalisation of FET proteins does not occur in this model (figure 4.18 a-c).

4.5.3 SMN is not altered by mutant TDP-43 expression

It has been observed that M337V mutant TDP-43 has increased cytoplasmic mislocalisation compared with wild-type in primary motor neurons (Gordon et al, in progress). It was therefore of interest to examine whether this might affect SMN, for

example via the sequestration of SMN into the cytoplasm of neurons expressing mutant TDP-43. Primary motor neurons from the TDP-43 BAC transgenic lines were stained for SMN and GFP. There were no visible differences between the lines apart from a lack of GFP expression in the non-transgenic lines as expected. There was no visible accumulation of SMN or GFP-tagged TDP-43 in any of the lines. Co-localisation analysis was carried out between SMN and GFP staining. The correlation coefficient between the distribution of SMN and GFP was low, with no significant difference between wild-type and M337V TDP-43 (figure 4.18 d-e). There was therefore no evidence that mutant TDP-43 sequestered SMN in the cytoplasm of these neurons.

In order to examine whether mutant TDP-43 affects gem number, the number of nuclear gems per cell was quantified. This showed no significant difference in the number of gems per cell between each line (figure 4.18f).

Figure 4.18a)
Primary motor neurons from the TDP-43 BAC transgenic mouse model were stained for FUS. FUS remained nuclear in all genotypes in both the unstressed and stressed condition, with no significant mislocalisation. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test)

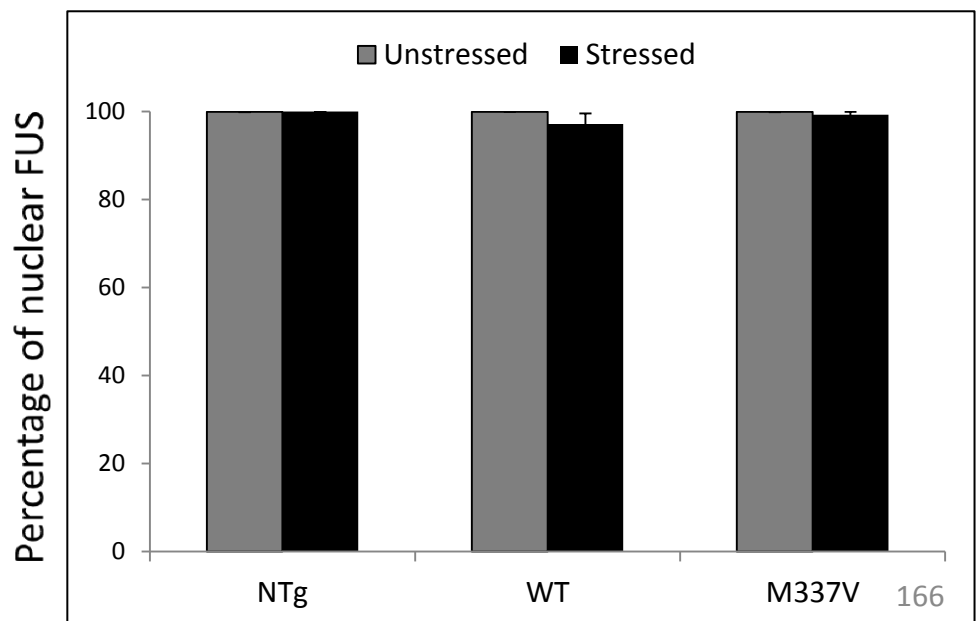
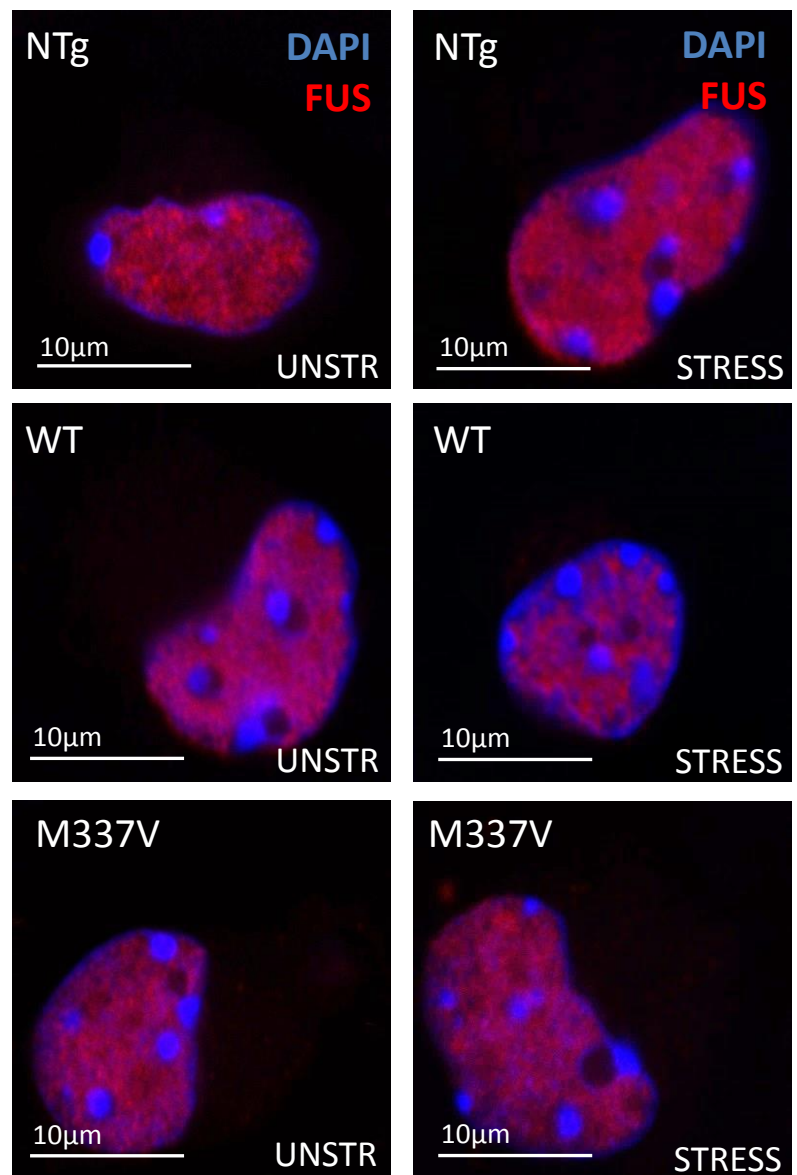


Figure 4.18b)

Primary motor neurons from the TDP-43 BAC transgenic mouse model were stained for EWS. EWS remained nuclear in all genotypes in both the unstressed and stressed condition, with no significant mislocalisation. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test).

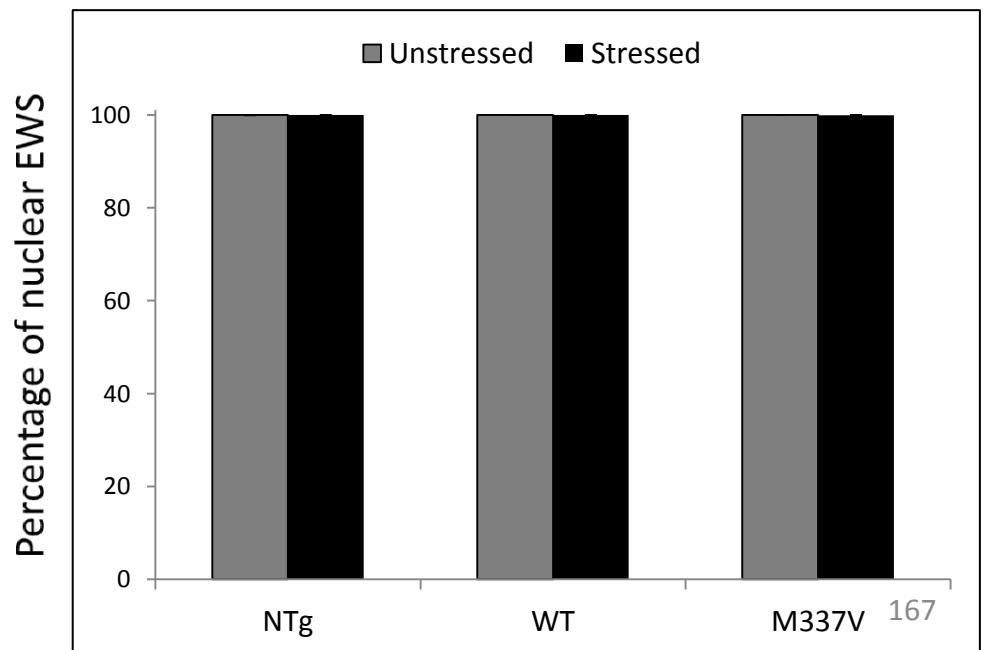
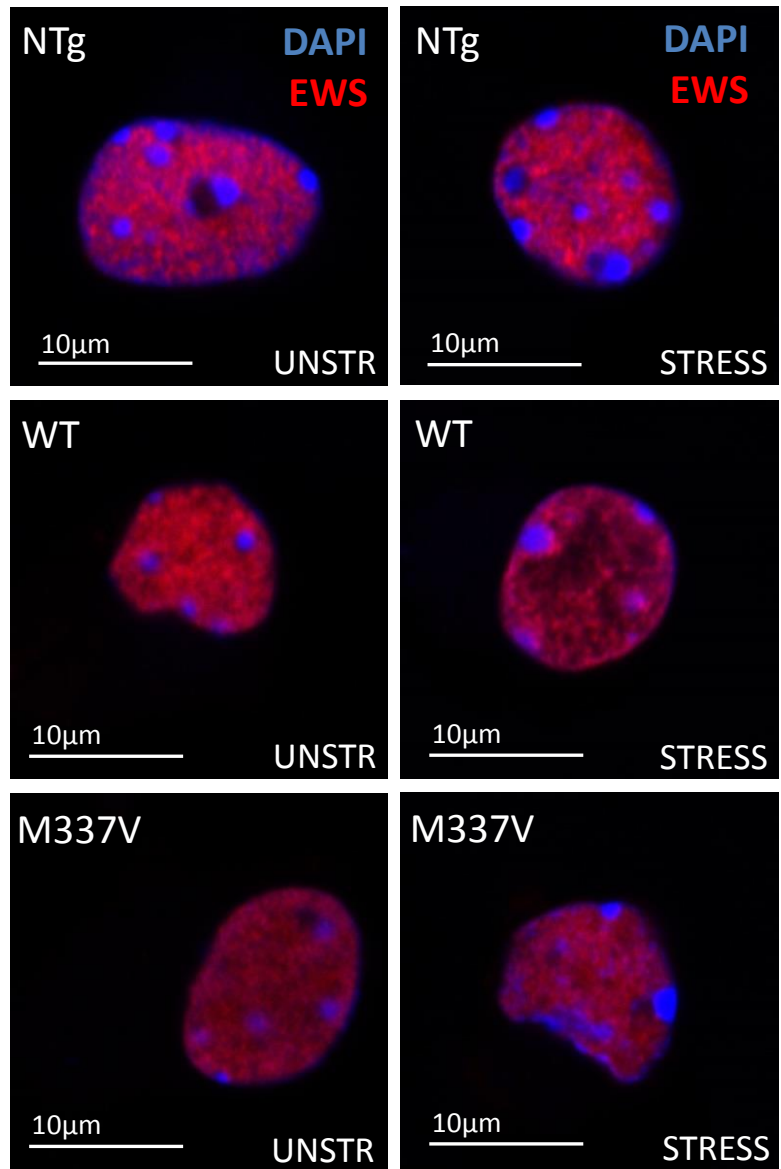
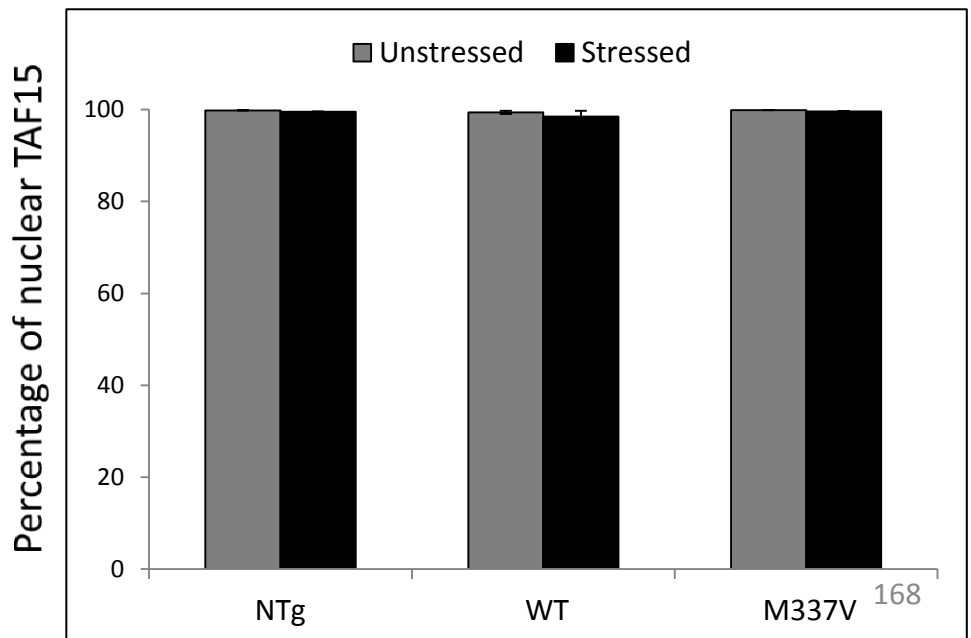
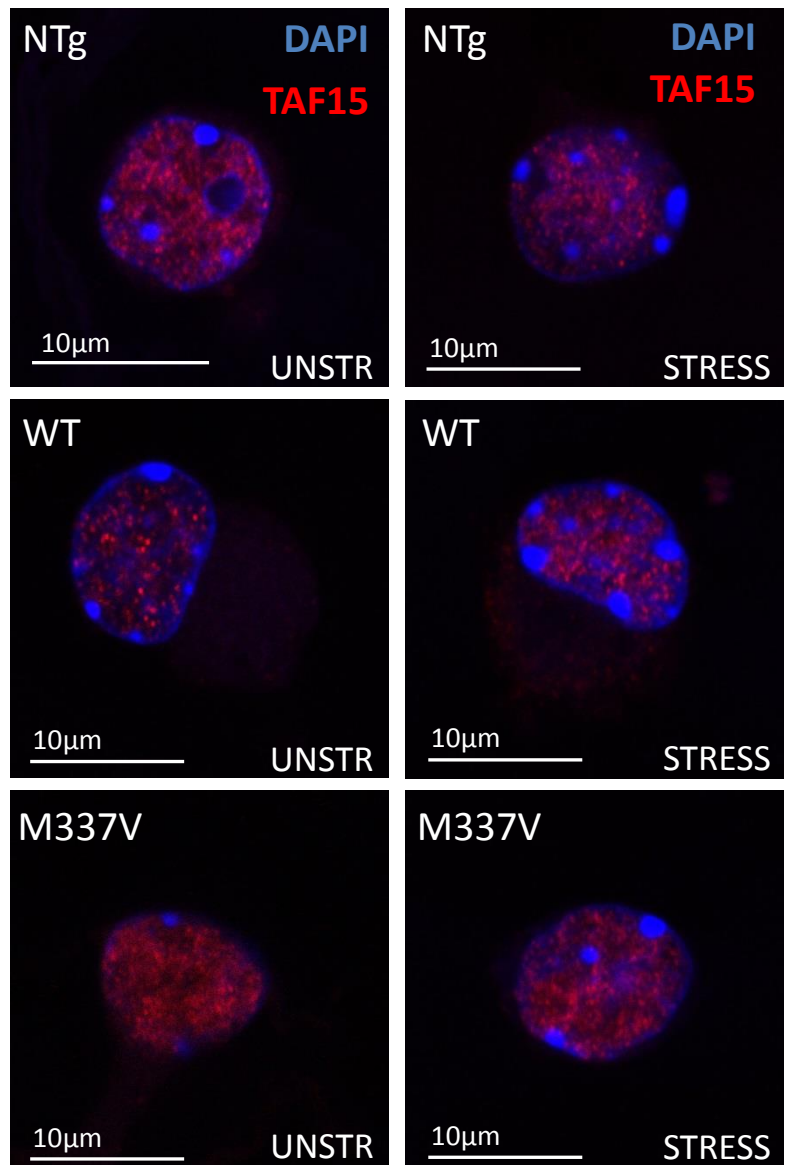


Figure 4.18c)
Neurons were stained for TAF15. TAF15 remained nuclear in all genotypes in both the unstressed and stressed condition, with no significant mislocalisation. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test).



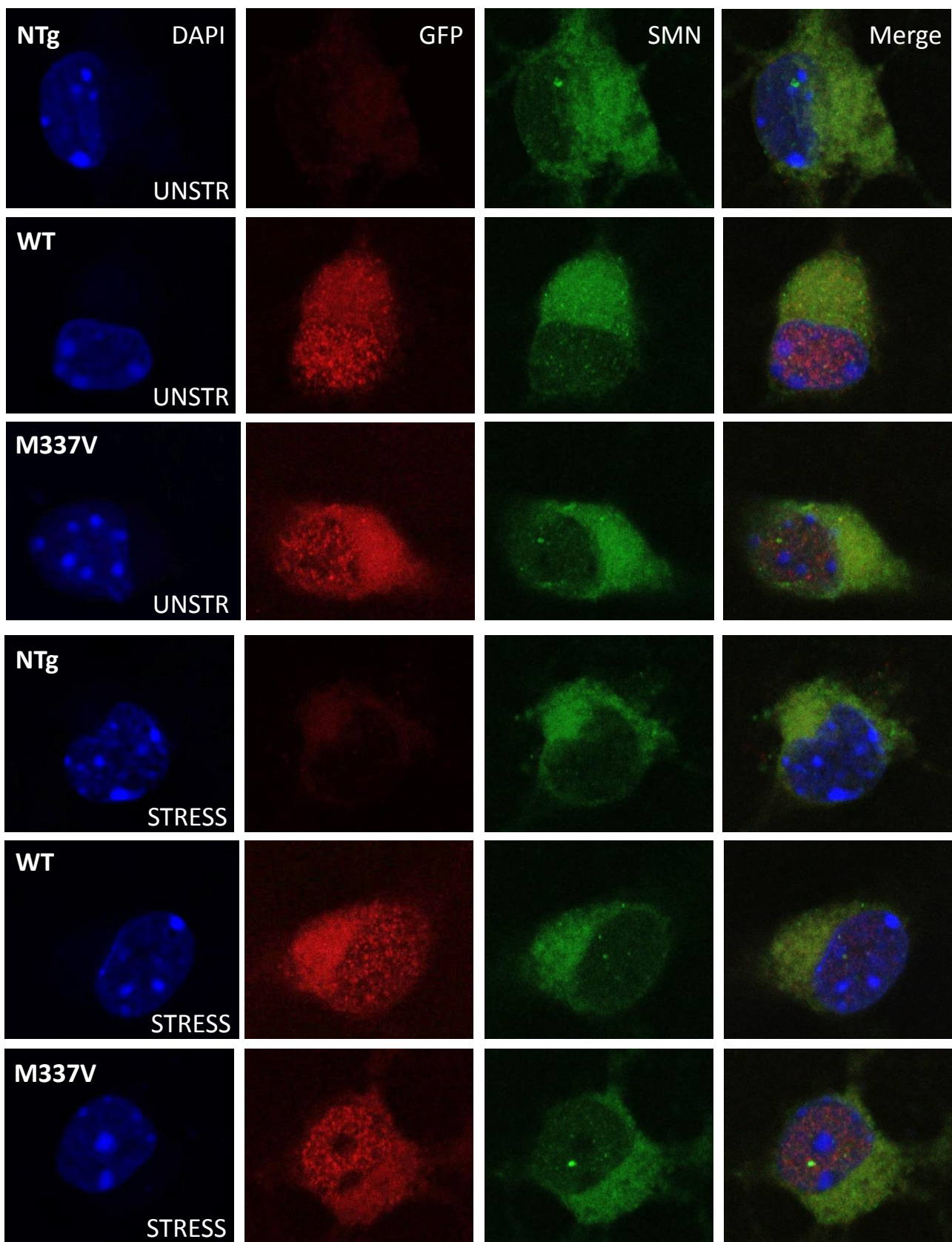


Figure 4.18d) Motor neurons from the TDP-43 BAC transgenic mouse model stained for GFP and SMN. There is no evidence that SMN or GFP are incorporated into cytoplasmic granules.

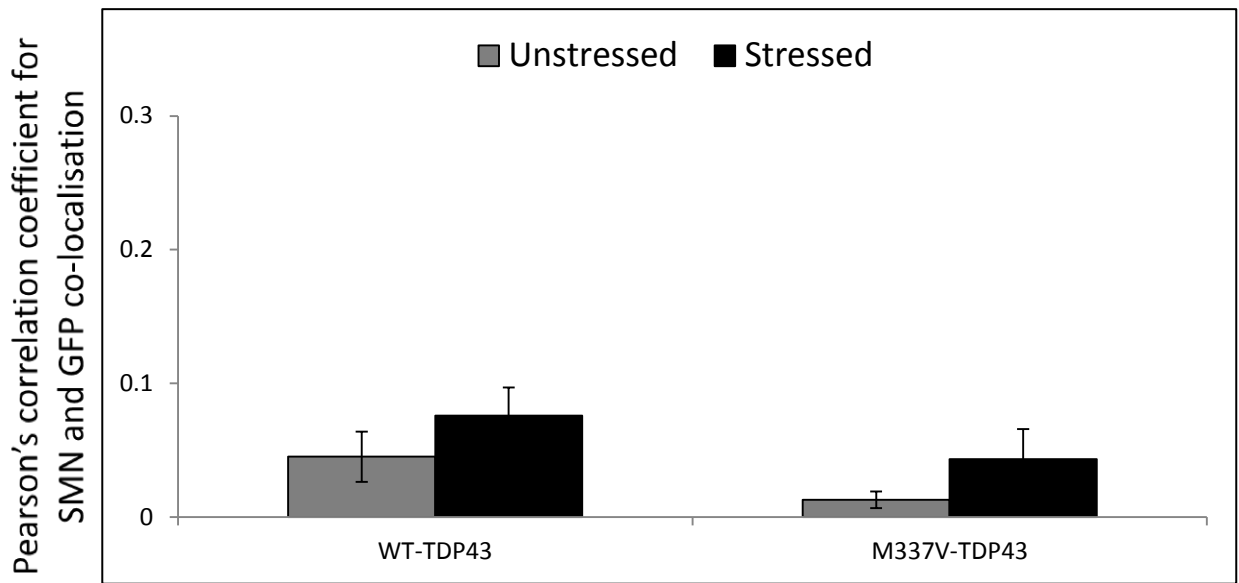


Figure 4.18e) Co-localisation analysis of SMN and GFP in primary motor neurons from the TDP-43 BAC transgenic mouse model. Pearson's correlation coefficient is low, and there is no significant difference between wild-type and M337V mutant lines. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test)

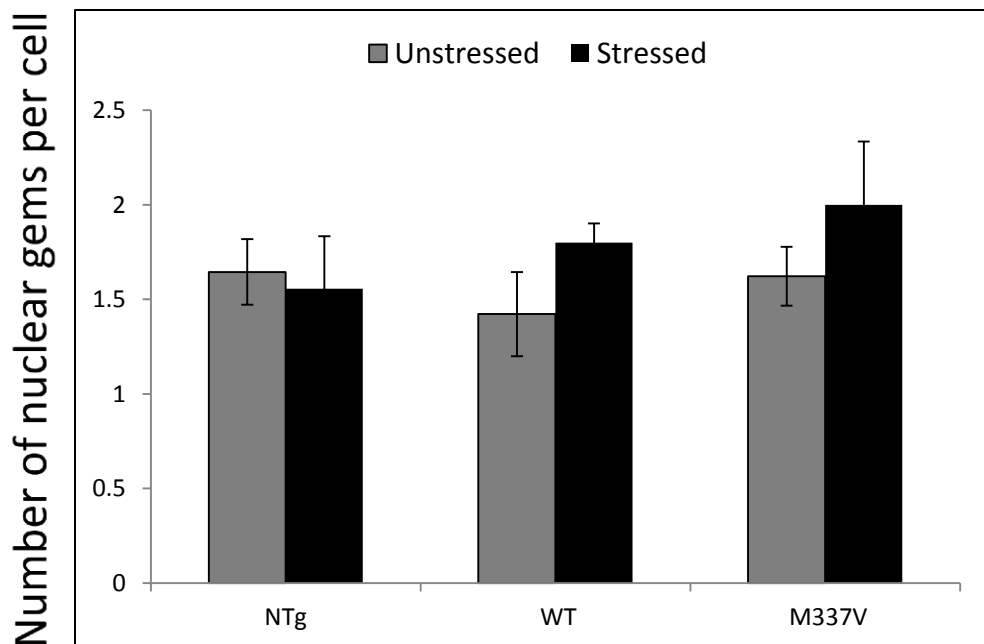


Figure 4.18f) Motor neurons derived from the TDP-43 BAC transgenic mouse model showed no difference in the number of nuclear gems per cell. (N=3 experiments, 15 cells each. One way ANOVA. Tukey post-test)

Summary of findings

1. P525L-FUS expressed in HEK293 cells using the BAC construct demonstrates mislocalisation of mutant FUS and incorporation into stress granules. This reflects the changes observed using the pcDNA construct.

FUS BAC transgenic mouse model

2. Motor neurons expressing P525L-FUS demonstrate reduced survival compared to non-transgenic.
3. Mutant FUS is mislocalised to the cytoplasm and incorporated into stress granules.
4. There is no evidence of mislocalisation of endogenous FET proteins.
5. There is some evidence of altered stress granule dynamics in the presence of P525L FUS, such as a reduced number of stress granules per cell after sodium arsenite treatment.
6. SMN does not co-localise with mutant FUS, nor is there a significant alteration in gem number in the presence of mutant FUS.
7. Results in cortical neurons reflect those in motor neurons.

FUS knockout mouse model

8. FUS is absent in motor neurons derived from the FUS homozygous knockout embryos. There is no detectable alteration in the distribution of EWS or TAF15.
9. There is no significant difference in stress granule dynamics in the homozygous knockout motor neurons, though there were some changes detected in the heterozygous neurons.

TDP-43 BAC transgenic mouse model

10. The distribution of endogenous FET proteins is not altered by the presence of M337V mutant TDP-43.
11. There is no evidence of altered gem numbers nor co-localisation of SMN with mutant TDP-43.

4.6 Discussion

4.6.1 FUS BAC transgenic motor neurons

The FUS-BAC construct can provide a useful tool to model ALS by containing the regulatory elements of the gene. This is more realistic to human disease, and can also help to avoid overexpression of FUS, which itself causes motor neuron toxicity (110). The large size of the 59.5 kb BAC construct is likely to be the cause of the low transfection rates in HEK293 cells, and the inability to transfect NSC-34 cells.

Nevertheless, in cells that did express FUS driven by the construct, P525L-FUS was shown to be mislocalised to the cytoplasm and to co-localise with stress granule markers, which is likely to reflect incorporation of mutant FUS into these structures. This supports other studies investigating this mutation, and also mimics the phenotype observed in pcDNA-transfected cells described in chapter 3 (figure 3.4) (102). This occurs despite the fact that estimates using Western blotting suggest that expression is at most 2.5 times that of endogenous FUS, which avoid the overexpression that occurs with pcDNA constructs (appendix 7.1). This supports the hypothesis that the P525L mutation itself causes mislocalisation by disruption of the C-terminal NLS, and corroborates previous studies that have also endeavoured to examine lower levels of mutant FUS (148).

Interestingly, cellular localisation of R521C-FUS does not differ significantly from wild-type FUS in this model, in contrast to pcDNA transfection which did display a mild phenotype. This is likely to be a reflection of lower levels of mutant FUS expression in the BAC-transfected cells. This suggests that some clinically less severe mutations may require over-expression to develop this phenotype. This would support a 'second hit' hypothesis which may be required in order to cause pathology in the presence of some mutations (230). The more severe mutations, such as

P525L, may cause this phenotype without the need for a 'second hit', leading to early onset disease (78). The observation that the extent of mislocalisation is proportional to clinical severity is supported by previous research demonstrating that mutations in the NLS of FUS alter its binding affinity to transportin (275). The reduction in binding affinity was shown to be proportional to the extent of cytoplasmic mislocalisation and disease severity. The extent to which transportin binding is disrupted may, in part, explain the variation in mislocalisation seen with different mutations in these experiments.

A limitation of this model is that expression levels are likely to vary between cells, which is overcome by the use of primary neurons from BAC transgenic mice.

Motor neurons from BAC transgenic mice displayed motor neuron markers and morphology (figure 4.5b). P525L-FUS expressing motor neurons had a mild but significantly reduced survival compared to non-transgenic neurons *in vitro* (figure 4.5c). This suggests that the presence of the mutation is toxic to motor neurons. In many ways this is not surprising, given the marked mislocalisation of mutant protein and severity of disease in humans with this mutation. However, it is interesting that humans tolerate this mutation for a number of years before clinical onset of disease. This suggests that a compensatory mechanism may be occurring prior to disease onset.

P525L mCherry-FUS displayed marked mislocalisation to the cytoplasm, with only about 30% of total mCherry-FUS present in the nucleus of unstressed cells (figure 4.6a). It reinforces the theory that mutations in the NLS affect Transportin-mediated nuclear import of FUS (102, 275). However, some import must occur in order for the

30% to be present in the nucleus. Cytoplasmic mutant FUS (as demonstrated by mCherry staining) formed granules after cell stress, similar to the phenotype observed in pcDNA-transfected cells (figure 4.6c & 3.5). This is important as it illustrates that this phenomenon takes place even in the presence of very low levels of mutant protein. These granules did not dissipate within an hour of the removal of sodium arsenite. This is within the range suggested for the dissolution of stress granules of 60-90 minutes, though may be longer (204). These granules may be stress granules that have incorporated mutant FUS within them, potentially increasing the time for dissolution. It is important because if they formed in motor neurons *in vivo*, they are likely to have effects on the cell lasting at least 60 minutes, and may have lasting effects on the cell.

P525L but not wild-type mCherry-FUS becomes incorporated into stress granules as demonstrated by co-localisation with stress granules markers, particularly after cell stress (figure 4.10). Although the percentage of cells with stress granules and with mCherry granules does not significantly change when stress is removed (figure 4.6c & 4.9b), there is a trend towards a reduction in the number of stress granules and mCherry-FUS granules per cell (figure 4.6d & 4.9d). If co-localisation is occurring predominantly within stress granules, as it appears from the confocal images, then the reduction in co-localisation when stress is removed fits in with the trend towards a reduction in the number of granules per cell and suggests that some stress granule disassembly is occurring. This corroborates previous studies that suggest both that co-localisation occurs in stress granules, and that they dissolve in 60-90 minutes (60, 204).

Staining motor neurons with an antibody to total FUS only detects cytoplasmic FUS when the confocal gain settings are increased, saturating the nuclear FUS signal. The antibody is able to detect mCherry-FUS, as shown on the Western blots. The fact that mislocalisation is not detected at significant levels using this antibody is likely to be due to the low levels of mCherry-FUS expression compared to endogenous (appendix table 7.3). It also suggests that mutant FUS does not sequester endogenous FUS to the cytoplasm in a significant amount, as some previous studies have suggested (230). It is likely that higher levels of mutant FUS, as seen in overexpression models, is required for this to occur. However, it does not rule out the possibility of mutant FUS sequestering endogenous FUS at low levels that do not reach statistical significance.

The fact that EWS and TAF15 remain nuclear in the presence of mutant FUS reflects the neuropathology of human ALS-FUS in which EWS and TAF15 are not mislocalised (86). This is in contrast to FTD-FUS in which *FUS* mutations have not been detected and neuropathology shows mislocalised FUS, EWS and TAF15 (86). Again, this does not rule out the possibility of FUS sequestering these proteins at undetectable levels in this model.

It has been shown in one study that mutant FUS can alter stress granule dynamics, causing a delay in stress granule assembly and larger, more numerous granules (229). The study used doxycycline-inducible mutant FUS in HEK293 cells, and levels were within 1-2 times that of endogenous FUS, so the pitfalls of overexpression were reduced. However, this cell type may not reflect the physiology of motor neurons. Stress granules are part of the physiological response to stress and are thought to be protective (104). Alterations in stress granule dynamics caused by the

pathological incorporation of mutant FUS within these granules could impair the response of the cell to stress with detrimental effects. A variety of antibodies to detect stress granules were used in this study, targeting proteins incorporated into these structures at different stages of the stress granule cycle. G3BP is involved in stress granule nucleation and primary aggregation, whilst PABP is incorporated during the secondary aggregation phase (276). These were the most robust antibodies, detecting discrete cytoplasmic granules after arsenite treatment. Throughout these experiments, TIA1 staining was more diffuse and speckled in appearance compared to PABP and G3BP. This implies that it is less able to detect discrete stress granules, which may be due to reduced binding specificity. TIA1 is linked to translational silencing and is not a core stress granule marker, so it may be that it is not consistently present in stress granules formed in this study (276). Although the general trends may be taken into account using the TIA1 antibody, it has not been used to draw firm conclusions.

Treatment with sodium arsenite caused stress granule formation in motor neurons. PABP and G3BP antibodies showed almost no cells with stress granules in the unstressed state and approximately 70-80% of cells with stress granules after sodium arsenite treatment (figures 4.9a & b). This demonstrates that these primary motor neurons are capable of forming stress granules, though there were generally no differences in stress granule formation between the lines. The exception to this was a reduction by approximately 17% in the number of cells with stress granules in the P525L neurons compared to non-transgenic in the stressed conditions using the G3BP antibody. It could be argued that there is a slight trend towards this in the other experiments, but it is not a consistent finding. It suggests that there may be a subtle deficiency in stress granule formation in the presence of P525L-FUS.

When stress was removed, there was a trend towards a reduction in the percentage of cells with G3BP and PABP positive stress granules, though this did not reach statistical significance. This is interesting, as previous studies suggest that stress granules are able to dissolve within minutes of the removal of stress (104, 276). The persistence of these granules may reflect the toxic effect of the sodium arsenite which takes longer than 60 minutes to resolve, or incomplete clearing of the arsenite when the media was changed. Motor neurons may require a longer time period for the dissolution of these granules. Interestingly, the trend towards a reduction in the percentage of cells with stress granules was much less in the P525L-FUS motor neurons compared to the other genotypes, though it did not reach statistical significance. This could reflect deficiencies in the ability of these cells to dissolve stress granules in the presence of mutant FUS, which could contribute to toxicity.

Analysis of the number of stress granules per cell yields some interesting results. G3BP and PABP staining tended to show discrete cytoplasmic granules which could be counted individually. There is an element of subjectivity in this process. However, experiments were blinded and analysed in one sitting in order to improve consistency and avoid bias. Results were relatively consistent between experiments as shown by the error bars. The results using the PABP antibody show a reduction in the number of stress granules per cell in the presence of P525L-FUS, suggesting that the formation of stress granules is impaired. This could be due to a reduction in the formation of stress granules, the merging of granules to create a lower total number, or altered expression of stress granule components leading to a reduction in total number per cell. When stress was removed this observation was reversed, suggesting that there may be an impaired ability of the neurons to dissolve stress granules in the presence of mutant FUS (figure 4.9c). This could be due to the

binding of FUS to protein or RNA within these granules, reducing their ability to dissolve (106, 119). This would be consistent with the hypothesis that these granules are the precursors of the FUS-positive aggregates seen in human disease, which may be initiated by stress granules that failed to dissipate (81).

Results using the G3BP antibody failed to detect this change, however, with no significant difference between the lines in any condition. The reason for the contrast with PABP staining may be because PABP is incorporated at a different stage of stress granule formation compared to G3BP (204). Potentially, expression of these stress granules markers may be altered by the presence of mutant FUS.

Alternatively it may reflect a difference in antibody binding and specificity, or chance variation between experiments.

Previous studies have suggested that FUS interacts with the SMN complex, raising the hypothesis that mutant FUS in the cytoplasm sequesters SMN and leads to a functional deficiency (107, 108). There have also been reports of SMN becoming incorporated into stress granules after cell stress, which could potentially be a site of this sequestration (277). There is also evidence that FUS mutations disrupt SMN gem formation, leading to a reduction in nuclear gems (108). The results described here suggest that cytoplasmic SMN granules can occasionally form after cell stress in HEK293 cells, but these are not present in every cell and do not co-localise with P525L-FUS (figure 4.4). This appears to be a cell-type specific response as there was no evidence of the formation of SMN granules after cell stress in motor neurons. There was some overlap between P525L-FUS and SMN as expected, as both proteins are present diffusely in the cytoplasm. However, SMN does not co-localise with wild-type or P525L-FUS nor become incorporated into FUS cytoplasmic granules. In addition there is no evidence of gem loss in the presence of mutant

FUS (figure 4.11). There is therefore no evidence in this study of SMN dysfunction in the presence of mutant FUS.

The differences between these results and those previously reported may be due to the cell type studied. Motor neurons are the cell type predominantly affected in ALS, so results from these cells are likely to be more relevant to human disease. The differences observed could also be explained by the low level of expression of mutant FUS protein in this study. There may be a threshold of mutant protein expression which leads to defects in gems, which is not reached in these motor neurons. Primary neurons may also be less tolerant to SMN depletion compared to cell lines, leading to cell death prior to the end point of the experiment. It would be useful to examine motor neurons with a range of levels of mutant protein expression to examine this further.

4.6.2 FUS BAC transgenic cortical neurons

Results from the cortical neurons reflect those in the motor neurons in several respects. P525L-FUS mislocalisation is observed using the mCherry antibody, with the development of mCherry-FUS cytoplasmic granules (figure 4.13). The addition of the 3 hours post-stress time point in the cortical cultures suggests that these granules are beginning to dissipate by this time point. Again, their co-localisation with stress granules is verified in these neurons, particularly after stress (figure 4.14). Indeed, the correlation coefficient is greater than in the motor neurons. This may reflect more consistent stress granule formation in cortical neurons, which appears to be the main site of co-localisation.

There is again some evidence of subtle disruption of the normal stress response in the presence of the P525L mutation in cortical neurons, with an increase in the percentage of cells with PABP-positive stress granules in P525L cortical neurons compared to wild-type. The 3 hours post-stress time point is also useful as it demonstrates a reduction in the percentage of cells with stress granules and the number of stress granules per cell compared to 1 hour post-stress. This shows that these granules are able to dissolve, even in the presence of the mutation, but it may be that full dissolution occurs beyond the end-point of the experiment (figure 4.15).

An important reason to examine cortical neurons was to detect differences between cortical and motor neurons in their response to mutant FUS, which could help to explain motor neuron susceptibility in ALS. Motor neurons in this study showed a reduction in the number of stress granules per cell in the P525L neurons, particularly using the PABP antibody. In cortical neurons, there was a trend in the opposite direction. This may reflect a phenotype specific to motor neurons reflecting an inadequate response to stress. This could make the neurons more vulnerable, and contribute to their reduced survival. Cortical neurons on the other hand, may be pushed into increasing their stress granules per cell in order to mount an adequate stress response in the presence of the mutation (figures 4.9c & 15b). This is supported by previous work suggesting that FUS deficiency affects cellular stress response and subsequently cell survival (104).

These defects in cortical neurons in the presence of P525L-FUS may also help to explain why some patients with this mutation have learning difficulties, which could result from impaired cortical development (78, 81).

4.6.3 FUS knockout motor neurons

Studies of FUS knockout motor neurons were undertaken in order to compare the cellular phenotype with that of P525L mutant neurons. FUS deficiency did not reduce cell survival in this study (figure 4.16c). However, FUS knockout in the mouse embryo is lethal as no knockout pups were born. It is not clear which role or combination of roles of FUS are essential for cell viability, but these results suggest that embryonic lethality it is not due to death of motor neurons. One role of FUS that might be essential for survival is DNA repair (62). This appears to be responsible for the phenotype of male sterility and radiation sensitivity in another FUS knockout model (136). However, the widespread roles of FUS in RNA binding and regulation may also be an important factor (119).

Interestingly, the percentage of cells with stress granules and gem counts per cell were altered in the FUS heterozygous knockout compared to non-transgenic (figure 4.17b&e). FUS is present in the FUS heterozygotes, as shown by the FUS staining (figure 4.16b). FUS autoregulation means that levels may be unchanged in the heterozygote (109). However, it is not clear whether the loss of one allele can be compensated for by this mechanism. There may be some level of FUS deficiency causing these changes in the heterozygous neurons, which do not occur in the complete absence of FUS. These changes are not observed in P525L mutant FUS motor neurons, suggesting that observations in P525L neurons are not due to FUS loss of function.

FUS knockout motor neurons are comparable to P525L motor neurons in demonstrating a reduction in the number of PABP-positive stress granules per cell after sodium arsenite treatment (figures 4.9c & 4.17c). This on its own may support this observation as a loss of function effect of the *FUS* mutation. However, it does

not rule out a different mechanism in each of these genotypes. For example, FUS knockout may lead to defective production of stress granules and P525L FUS may cause several granules to merge. Each of these scenarios would lead to a reduction in the total number of granules. The combination of results in the FUS knockout model that both match and contrast with those seen in the FUS mutant neurons suggests that there may be some loss of function effects of the *FUS* mutation, but that this is not likely to be the sole mechanism of toxicity.

4.6.4 TDP-43 BAC transgenic motor neurons

Studies have suggested that the pathways of mutant FUS and TDP-43 toxicity may converge to cause ALS, particularly as they are both RNA binding proteins and share similar functions (166, 273, 274). In neurons from the TDP-43 BAC model, M337V mutant TDP-43 does not cause mislocalisation of any of the FET family of proteins. There is no evidence of co-localisation between TDP-43 and SMN, nor gem deficiency in these neurons (figure 4.18). This suggests that mutant TDP-43 in this model does not sequester FET proteins or SMN in the cytoplasm. This reflects what occurs in human disease, where the majority of studies have found that TDP-43 and FUS inclusions do not occur together (71). However, it does not rule out a common pathway in causing toxicity, which will be examined further in the next chapter.

Summary of Findings

Aim 1: Characterisation of a FUS-BAC cellular phenotype in an immortalised cell line revealed that, like pcDNA-expressed FUS, P525L-FUS was mislocalised to

the cytoplasm and was incorporated into stress granules during oxidative stress.

There was no evidence that SMN was also incorporated into these granules.

Aim 2: The characterisation of primary motor and cortical neurons from a novel FUS-BAC mouse showed that the main phenotype observed in BAC transgenic neurons was the marked mislocalisation of mutant FUS compared to wild-type, which is likely to be due to the loss of a functional nuclear localisation signal (102). Mutant FUS was incorporated into stress granules and caused some alterations in stress granule dynamics such as a reduction in the number of granules per cell. There was also evidence of a defect in the resolution of stress granules after cell stress was removed. Motor neuron survival after 6 days in culture was reduced in the presence of the P525L mutation. These defects could all play a part in increasing the risk of motor neuron degeneration in the presence of a *FUS* mutation.

Aim 3: Examination of the effect of FUS deficiency in primary motor neurons showed no survival disadvantage in FUS knockout neurons at 6 days *in vitro*. There were no changes detected in the distribution of the other FET proteins. Some changes were observed in the heterozygous or homozygous knockout neurons in Stress granule dynamics and SMN gem number. This suggests that there may be subtle alterations in cellular processes when FUS levels are altered. Although there were some similarities to the results in P525L-FUS neurons, these were not sufficient to suggest that P525L-FUS has solely a loss of function effect.

Aim 4: Examination of the interaction of TDP-43 with FET family members revealed no alteration in the distribution of the FET family proteins or SMN, and difference in gem number, in the presence of mutant M337V TDP-43. This suggests that TDP-43 does not exert its toxic effect by sequestering these proteins into the cytoplasm.

Aim 5: The consequences for mutant FUS on SMN in neurons from the FUS-BAC transgenic model revealed no visible change in SMN distribution in the presence of P525L-FUS, or significant alterations in gem numbers. This suggests that mutant P525L-FUS is not sequestering SMN into stress granules, nor creating a functional SMN deficiency.

These results may be used to hypothesise how *FUS* mutations cause ALS. Under normal conditions, wild-type FUS is nuclear as supported by this study. Neurons may undergo events leading to an increase in cell stress and the development of stress granules. However, it is likely that these granules do not contain wild-type FUS and dissipate with time when stress is removed (276). In the presence of mutant FUS, there is evidence that there may be some alterations in neurodevelopment, but without overt toxicity or ALS (78, 81). However, these results show that a significant proportion of mutant FUS is likely to be cytoplasmic. If the cell is placed under stress, mutant FUS is incorporated into stress granules and causes alterations in their formation and dissolution. Deficient formation of stress granules may cause toxicity, which may be reflected in the reduced survival of P525L motor neurons in this study (104). In addition, defects in stress granule disassembly along with the continuing presence of mutant FUS may lead more resistant granules. These have been proposed to be the precursors of the FUS-positive aggregates observed in patients with ALS-FUS mutations (223). The results from this study support elements of this hypothesis. Importantly, they also demonstrate that very low levels of mutant FUS are sufficient to observe differences in mutant compared to wild-type FUS.

CHAPTER 5: THE EFFECTS OF *FUS* OR *TDP-43* MUTATIONS ON KEY mRNA SPECIES AND ALTERNATIVE SPLICING

5.1 Introduction and aims

FUS and *TDP-43* both play important roles in RNA processing. Studies looking at RNA targets of these proteins have detected over 5,500 RNA targets at over 40,000 binding sites. Knockdown of these proteins caused altered levels of over 600 mRNA species (119, 157). This demonstrates the widespread effect these RNA binding proteins have on RNA processing. The majority of these targets are distinct to each protein, though there is some overlap. However, this study and others have used knockdown experiments to study the effects of these proteins (119, 124). Given that mutations in these genes cause ALS, it is more relevant to human disease to study the effect of mutations, rather than knockdown, on RNA processing.

Proteins such as *FUS*, *TDP-43*, *SMN* and *FET* have all been implicated in the degeneration of motor neurons. It has been hypothesised that interactions between these proteins may enable them to act via a common mechanism (86, 108, 114). It was therefore of interest to examine whether mutations in *FUS* or *TDP-43* cause altered mRNA transcription of these or other proteins implicated in motor neuron degeneration.

The first aim of this chapter was to examine mRNA levels of *FUS*, *TDP-43*, *SMN*, *TAF15* and *EWS* in CNS tissue from the BAC transgenic mouse models. This was compared with protein expression using Western blots.

In addition to altered transcript levels, over 370 altered splicing events have been reported as a result of *FUS* knockdown, with over 950 after *TDP-43* knockdown (119, 157). Some of these targets are proteins involved in neuronal function and

neurodegeneration, and this was studied in more detail by Lagier-Tourenne et al (119). RNA targets with both FUS and TDP-43 binding sites and roles in neuronal function or neurodegeneration were selected to study the effect of FUS and TDP-43 knockdown on the relative abundance of splice variants. Some of these targets were altered by both FUS and TDP-43 knockdown, whilst others were specific to one or the other. These targets provide a useful representation of the effect of these proteins on splicing. However, it is more relevant to ALS to examine the effect of mutations on these targets. The second aim of this chapter was to examine whether there were alterations in splicing of known targets of FUS and TDP-43 in the presence of mutations, using the BAC transgenic mouse models. This was compared to the effects of knockdown described by Lagier-Tourenne et al to assess whether the mutation has evidence of a loss of function effect (119).

5.2 Expression levels of key proteins in the FUS and TDP-43 BAC transgenic mouse models

5.2.1 TDP-43 BAC transgenic mouse model

qRT-PCR primers were designed to detect all transcripts of the human TDP-43 transgene, and to amplify a region contained within all known transcripts of total TDP-43, FUS, SMN, TAF15 and EWS. These were tested to ensure that they had satisfactory efficiency and specificity (figure 5.1). The relative expression of each mRNA species was examined in cortex and spinal cord collected at 3 and 9 month time points from wild-type and M337V TDP-43 mouse lines. These time points were selected as they reflect pre-symptomatic and early symptomatic time points of the

M337V mutant mouse model. A summary of the observed changes in the M337V mutant mouse tissue compared to wild-type is described in table 5.1.

Human TDP-43 mRNA levels in the homozygous lines was approximately double that detected in the hemizygous lines at both time points in cortex and spine, reaching statistical significance in the M337V lines in the 3 month cortex ($p < 0.01$, figure 5.2a). This suggests that transcription is proportional to the number of inserted copies of human TDP-43 from the BAC construct. When using primers that detect both human and endogenous TDP-43 (total TDP-43) there was a trend towards an increase in total TDP-43 in the mutant lines at 3 months. This trend may be explained by the increased expression of human TDP-43, but it is seen in both mutant lines. Interestingly, the reverse was seen at 9 months with a significant decrease in both mutant lines compared with non-transgenic ($p < 0.05$). This was also demonstrated to a lesser degree in the wild-type TDP-43 (figure 5.2b).

Endogenous FUS expression showed a significant increase in transcript levels at 3 months ($p < 0.01$) and trend towards a decrease at 9 months in the M337V homozygous mutant lines, and to a lesser extent in the hemizygous line compared to non-transgenic (figure 5.2c). This decrease at 9 months was also observed in wild-type TDP-43 cortical tissue, with a significant difference in the wild-type TDP-43 homozygous line compared to non-transgenic ($p < 0.05$, figure 5.2c). SMN expression showed a similar pattern of expression with a more marked increase in SMN transcript level at 3 months and decrease at 9 months in the mutant lines, particularly in the homozygous line (figure 5.2d). This decrease at 9 months was also observed in the wild-type TDP-43 lines, particularly in homozygous TDP-43 cortical tissue which was significantly less than non-transgenic ($p < 0.05$, figure 5.2d).

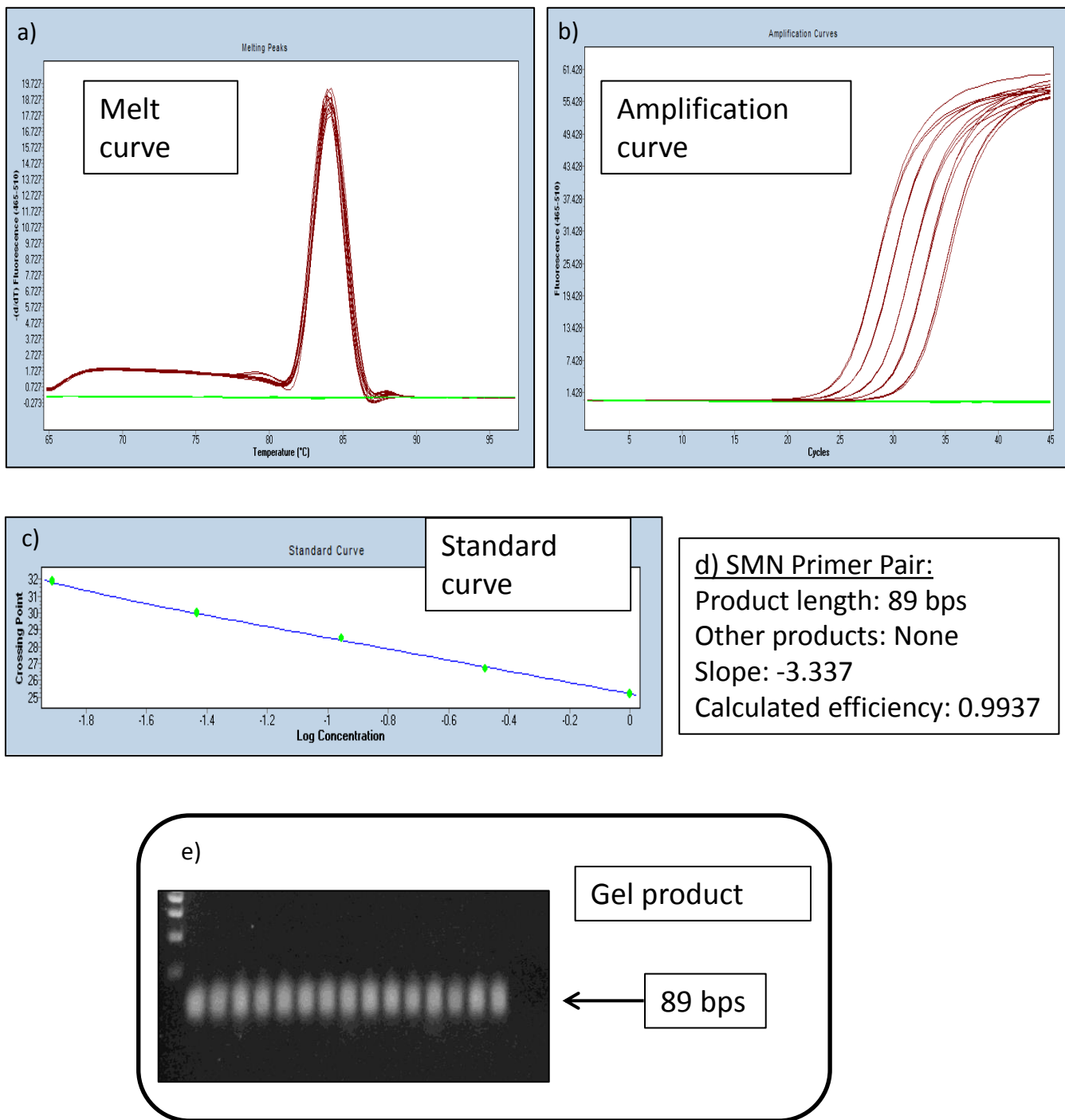


Figure 5.1. Optimisation of primers for qRT-PCR, using the SMN primer pair as an example. **a)** The melt curve demonstrates continuity of melting temperature for all samples, showing that the same product is generated. **b)** The amplification curve shows differences in the amount of product, reflecting the differences in initial cDNA concentrations. **c)** The standard curve shows a good line of best fit, used to calculate the slope and efficiency of the primer pair. **d)** Summary of the data for the SMN primer pair. **e)** The qRT-PCR product was run on an agarose gel and shows the expected product size of 89 base pairs.

	TDP-43 3 months		TDP-43 9 months	
	Cortex	Spine	Cortex	Spine
Human TDP-43	↑ in homozygous	↑ in homozygous	↑ in homozygous	↑ in homozygous
Total TDP-43	↑ in M337V	↑ in M337V	↓ in M337V	↓ in M337V
Endogenous FUS	↑ in M337V	↑ in M337V	↓ in M337V	(↓) in M337V
Endogenous SMN	↑ in M337V	↑ in M337V	↓ in M337V	↓ in M337V
Endogenous TAF15	(↑) in M337V	↑ in M337V	→	→
Endogenous EWS	(↑) in M337V	(↑) in M337V	(↓) in M337V	(↓) in M337V

Table 5.1: Summary of the effects of the M337V mutation on mRNA levels in the TDP-43 BAC transgenic model, compared to wild-type. Arrows in brackets denote small effects.

Human TDP-43

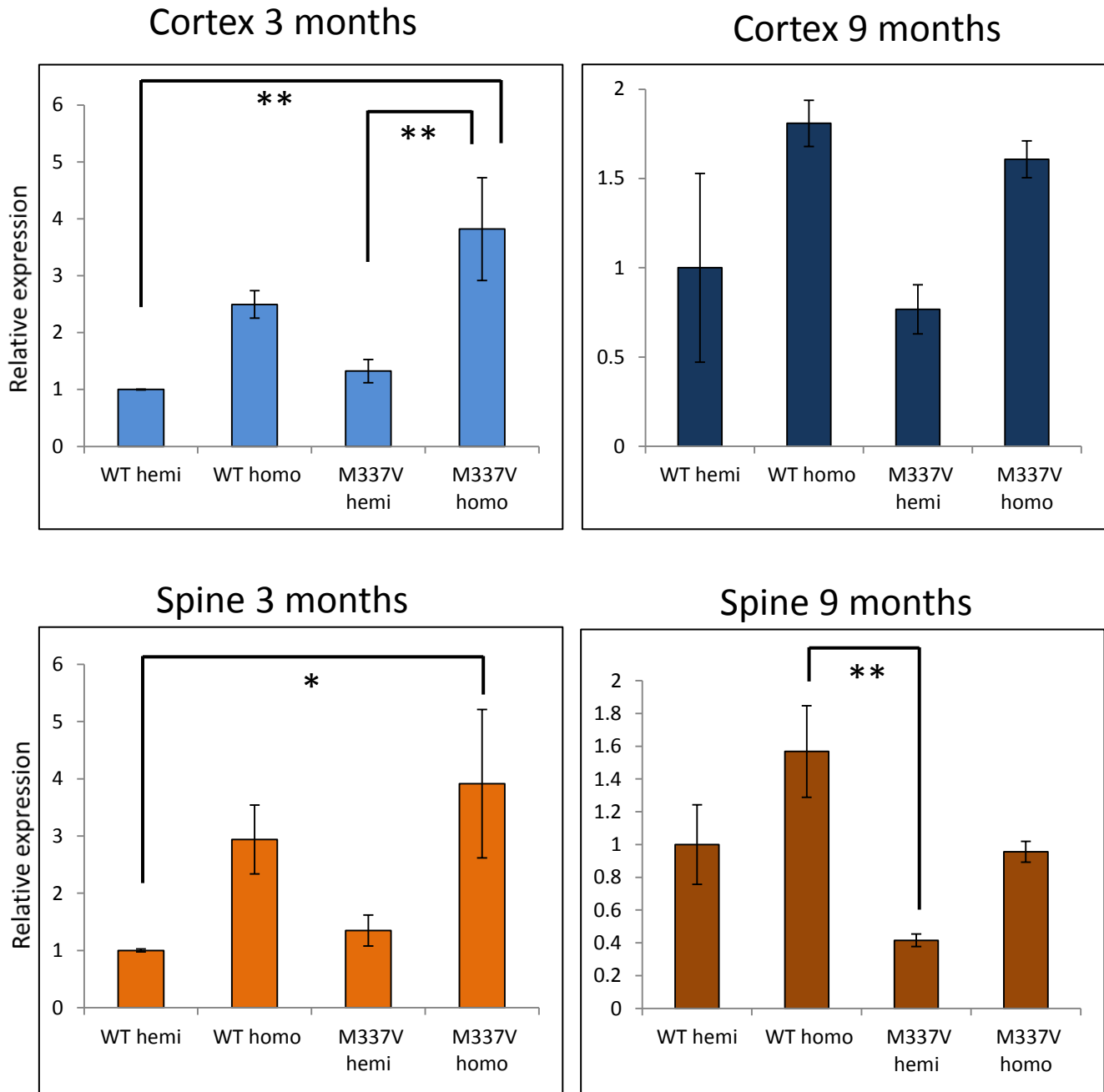


Figure 5.2. mRNA expression in cortex and spine of 3 and 9 month old animals from the TDP-43 BAC transgenic mouse model, detected using qRT-PCR. (N=3 mice per genotype. One-way ANOVA. Tukey post-test. *=p<0.05, **=p<0.01, ***=p<0.001).

a) Expression of human TDP-43 relative to wild-type hemizygous. There is increased human TDP-43 expression in homozygous lines compared to hemizygous, reflecting the number of copies of the human TDP-43 transgene.

Total TDP-43

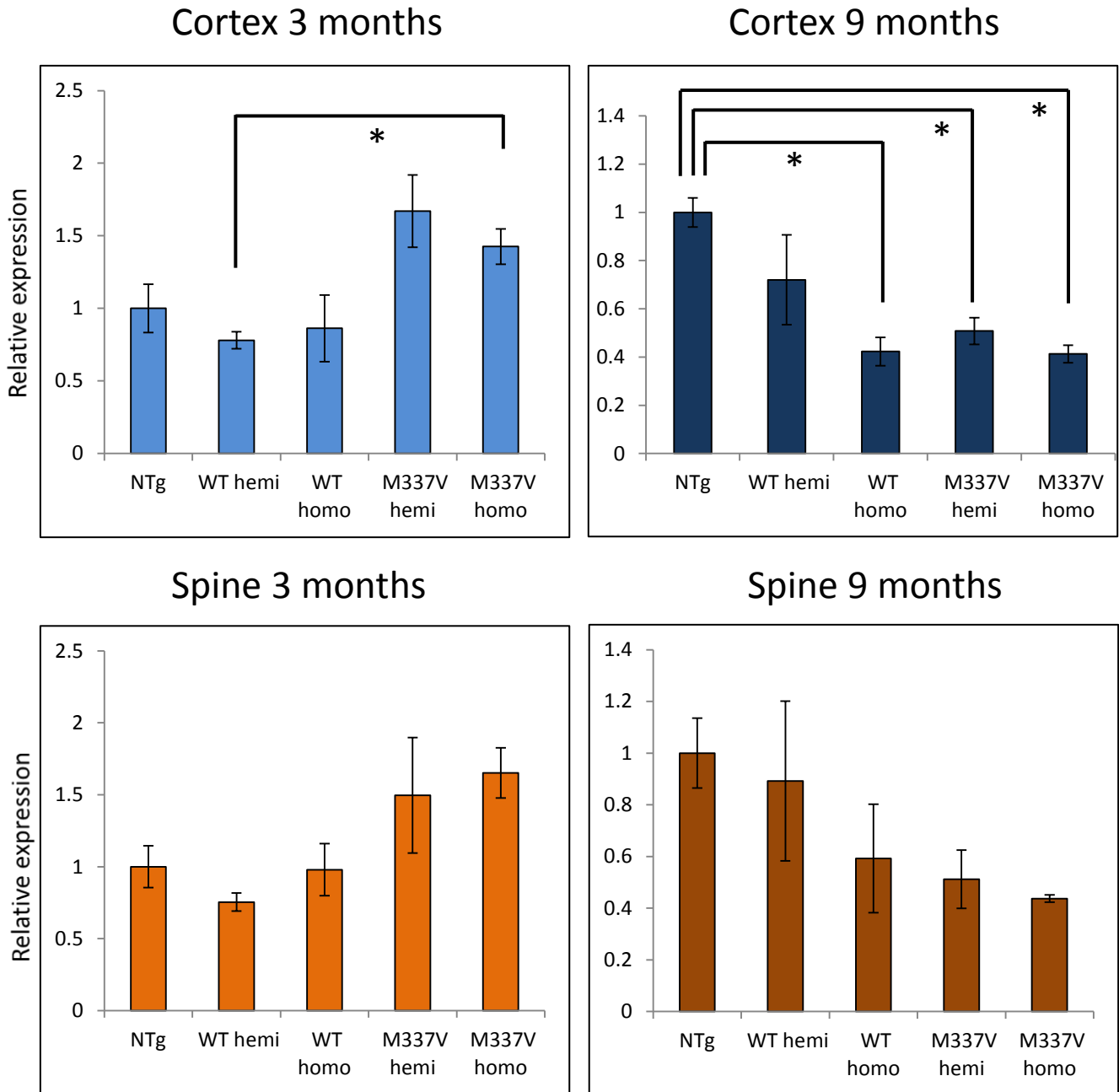
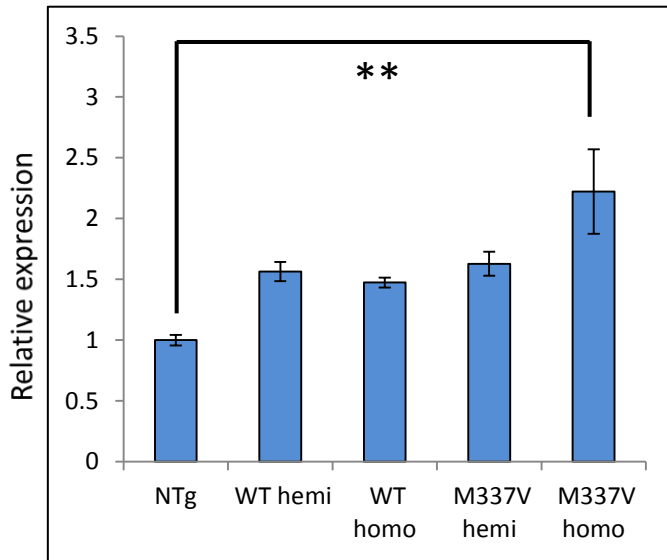


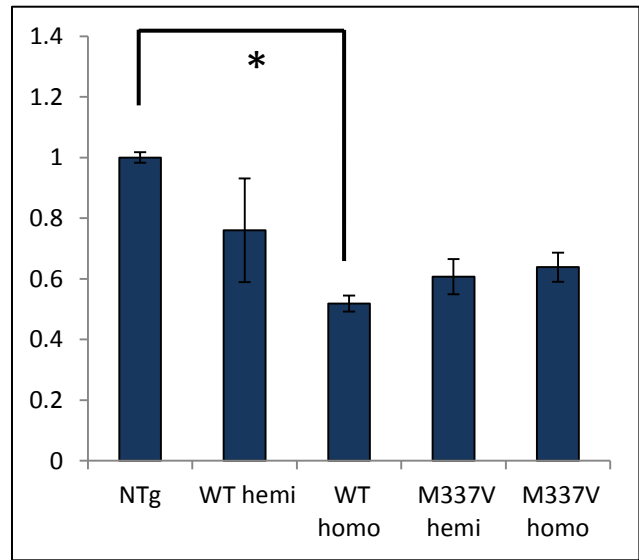
Figure 5.2b) Expression of total TDP-43 relative to non-transgenic in the TDP-43 BAC transgenic mouse model. This reflects expression of endogenous and human TDP-43. There is a trend towards an increase in total TDP-43 in the mutant lines at 3 months and a reduction at 9 months.

Endogenous FUS

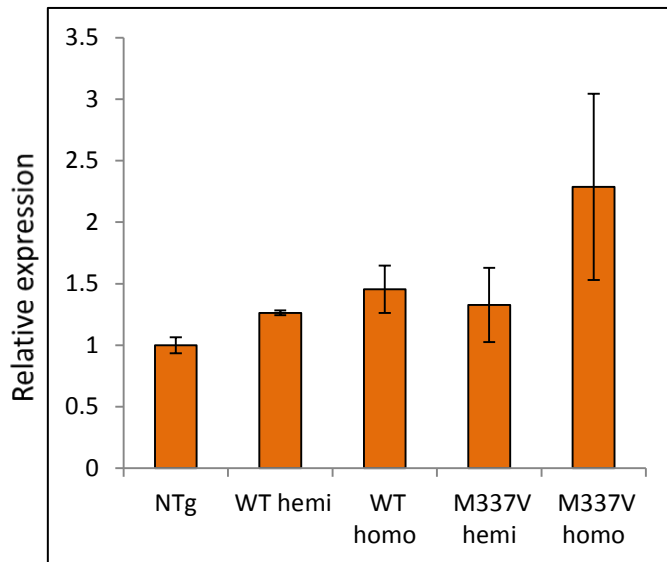
Cortex 3 months



Cortex 9 months



Spine 3 months



Spine 9 months

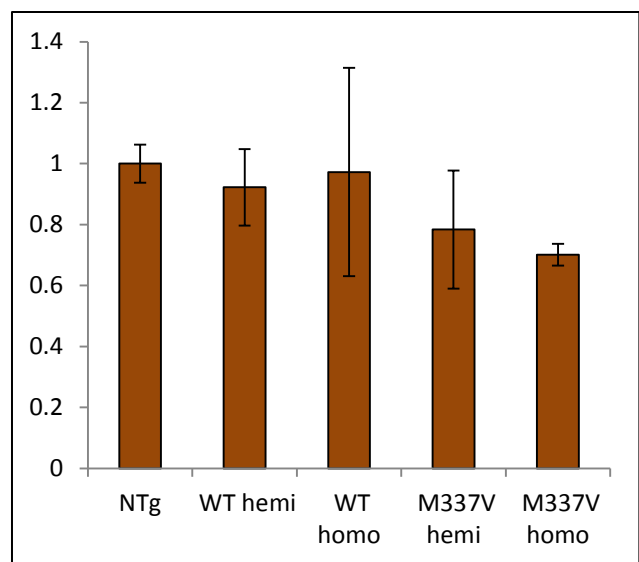


Figure 5.2c) Expression of endogenous FUS relative to non-transgenic in the TDP-43 BAC transgenic mouse model. There is a trend towards an increase in FUS in the mutant lines at 3 months and a reduction at 9 months.

Endogenous SMN

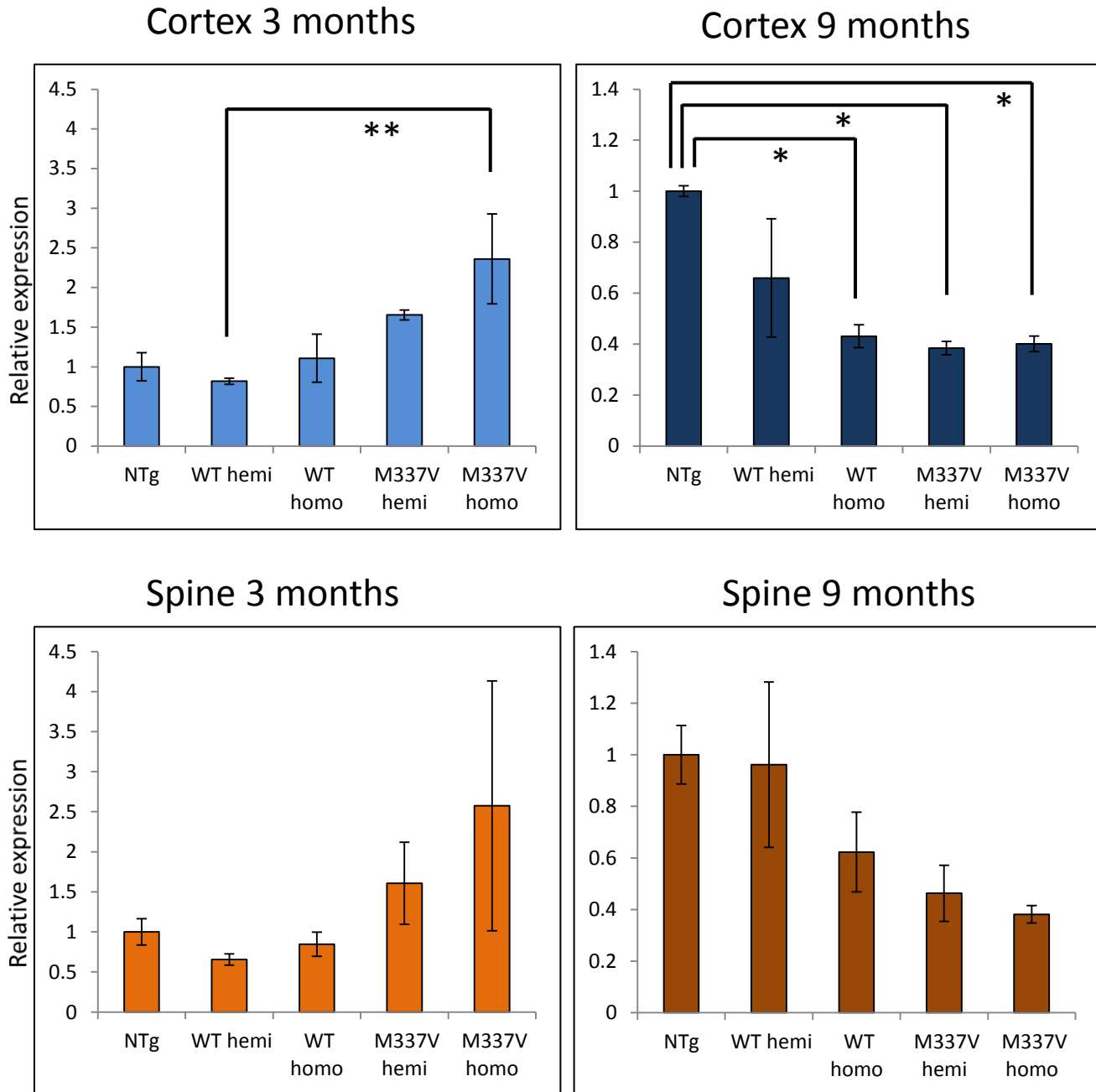
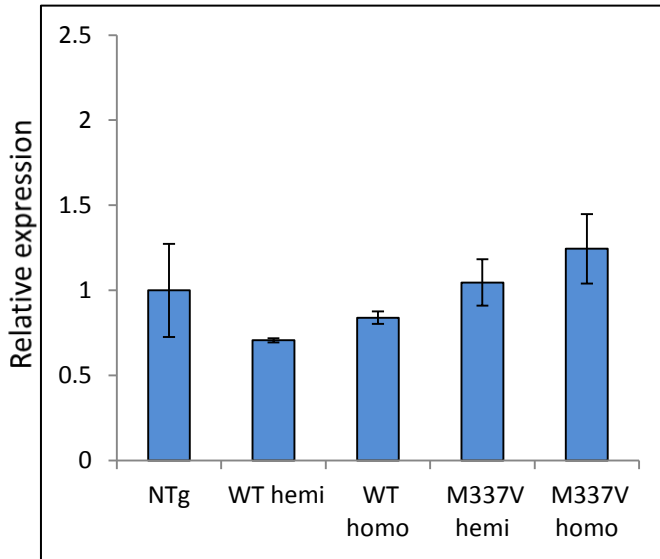


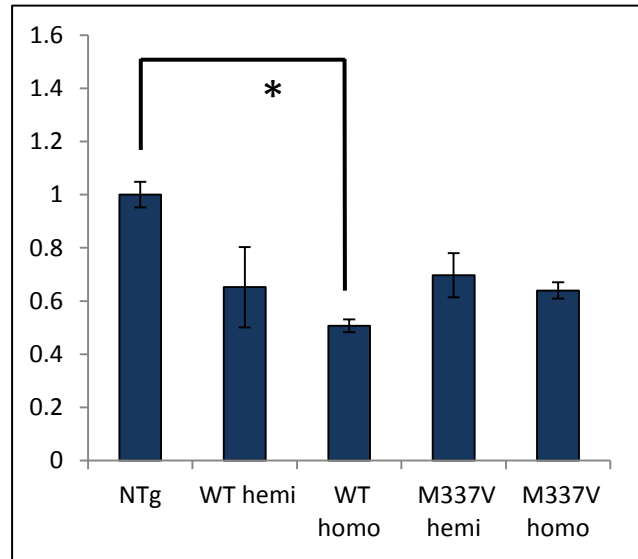
Figure 5.2d) Expression of SMN relative to non-transgenic in the TDP-43 BAC transgenic mouse model. There is a trend towards an increase in SMN in the mutant lines at 3 months and a reduction at 9 months.

Endogenous TAF-15

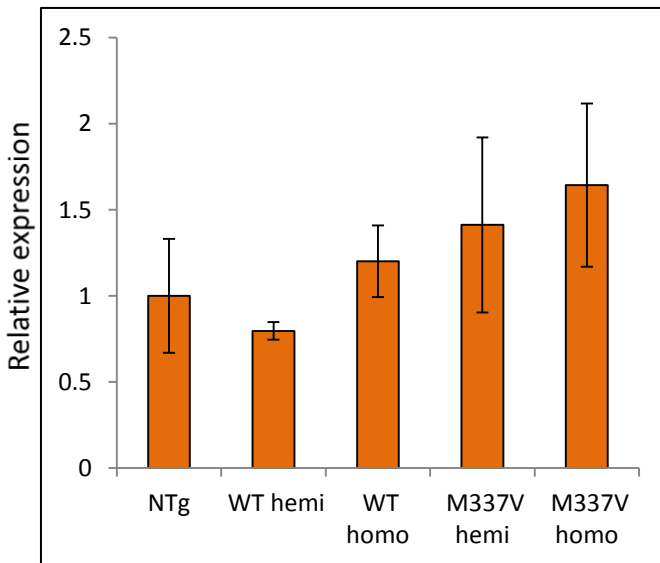
Cortex 3 months



Cortex 9 months



Spine 3 months



Spine 9 months

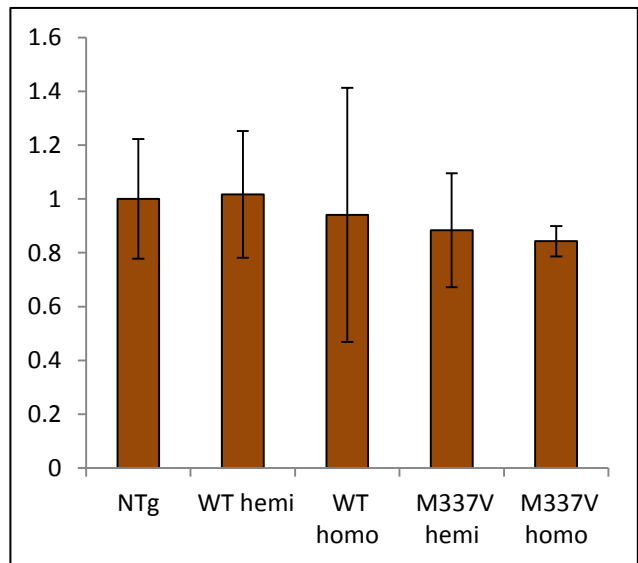


Figure 5.2e) Expression of TAF15 relative to non-transgenic in the TDP-43 BAC transgenic mouse model. Levels are relatively stable with the exception of a reduction in TAF15 expression in the wild-type homozygous line compared to non-transgenic in the cortex at 9 months.

Endogenous EWS

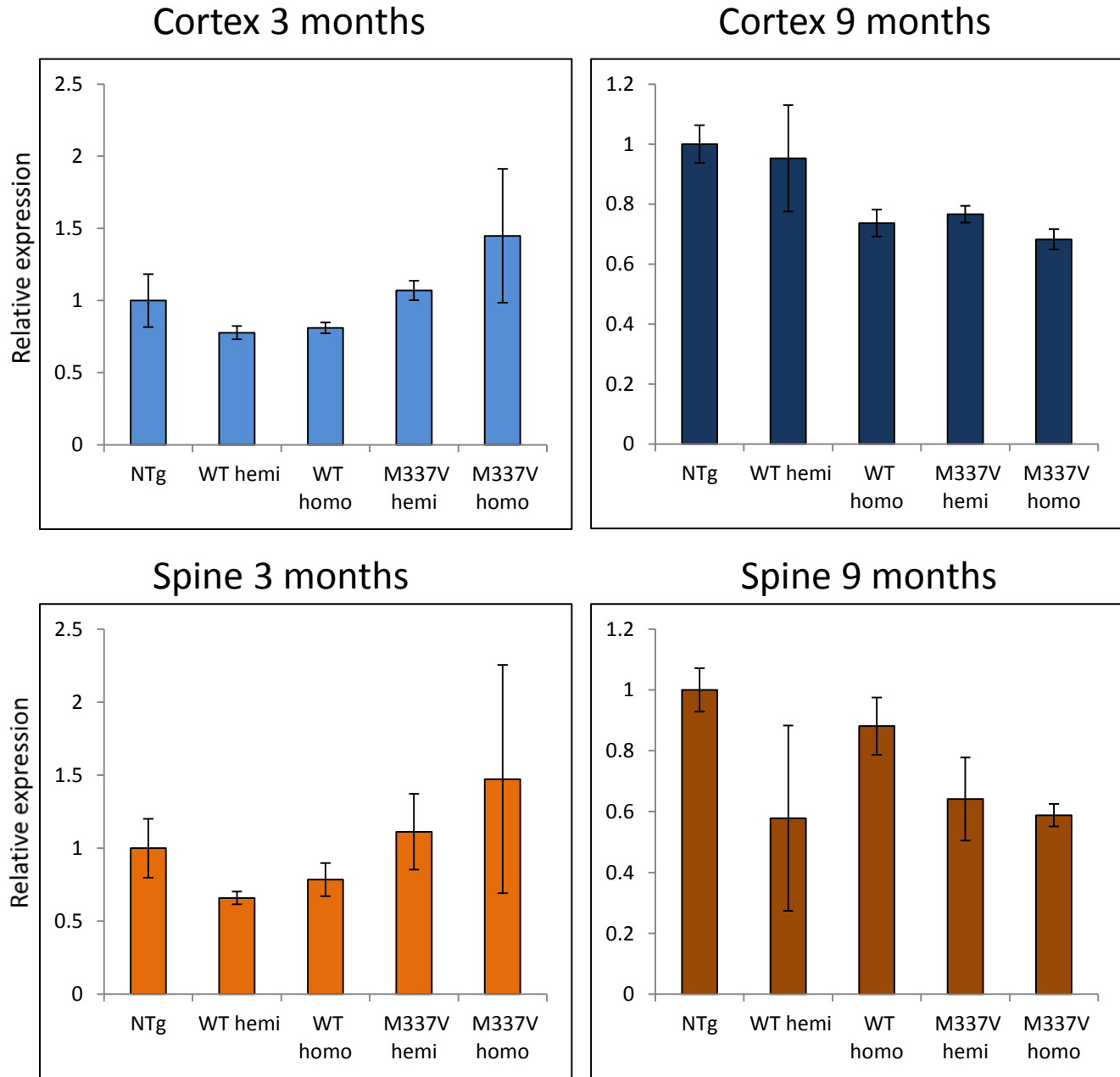


Figure 5.2f) Expression of EWS relative to non-transgenic in the TDP-43 BAC transgenic mouse model. Levels are relatively stable with no significant differences between the lines.

TAF15 and EWS mRNA expression levels were relatively consistent between the lines. Although there was a slight trend towards an increase in mRNA expression at 3 months and decrease at 9 months in the mutant lines, this was subtle and did not reach statistical significance (figures 5.2 e and f).

5.2.2 FUS BAC transgenic mouse model

qRT-PCR primers designed to detect all known transcripts of total FUS, endogenous FUS, TDP-43, SMN, TAF15 and EWS were used to examine the relative expression of these mRNAs within CNS tissue from the FUS BAC transgenic model. The ages of the mice varied due to sample availability, though the majority of non-founder mice used were approximately 10 weeks old (appendix table 7.4). All FUS BAC transgenic lines were used, including those expressing higher and lower levels of human FUS protein. This allowed results to be compared by line (figure 5.3) and also pooled by genotype (appendix 7.8), and expressed relative to non-transgenic. The changes observed in the P525L mouse lines compared to wild-type are summarised in table 5.2.

Total FUS mRNA expression was greater in both transgenic lines compared to non-transgenic, with no significant difference between the transgenic lines (figure 5.3a). When results were separated per line, WT-3 and P525L-3 showed the greatest expression (appendix figure 7.8a). These lines contain the greatest number of FUS-BAC copies and protein levels, suggesting that the greater number of copies of the human *FUS* transgene result in higher levels of transcription and therefore higher levels of protein.

Quantification of endogenous FUS mRNA levels revealed interesting results. Results by genotype showed an increase in endogenous FUS transcript in wild-type FUS

tissue compared to each of the other genotypes (p values range between <0.05 and <0.001 , figure 5.3b). Results by line showed a similar trend, though only displayed a significant increase in the WT-2 line in the cortex compared to non-transgenic ($p<0.05$, appendix figure 7.8b). Although data cannot be directly compared with total FUS transcript levels, the levels of total FUS and endogenous FUS in the non-transgenic tissue should remain constant. Therefore these results suggest that most of the 2-3 fold increase in total FUS is explained by a roughly 2 fold increase in endogenous FUS in the wild-type FUS tissue. Endogenous FUS mRNA in the P525L tissue did not differ significantly from non-transgenic, so it may be inferred that most of the 3 fold increase in FUS is likely to be due to mRNA from the BAC construct. The significant difference between wild-type and P525L lines (p values range from <0.05 to <0.001) suggests that the mutation has an effect on endogenous FUS transcription.

Endogenous TDP-43 and SMN mRNA levels showed a similar pattern of expression, with increased mRNA levels in wild-type FUS compared to non-transgenic and P525L CNS tissue (figures 5.3c&d). The results for TAF15 and EWS showed more consistency between genotypes and lines. Here there was a similar pattern of mRNA expression to SMN and FUS, particularly in TAF15 expression, but with much smaller differences in mRNA levels between genotypes. However, few values were significantly different so there was insufficient evidence to draw firm conclusions. There were also no significant differences between wild-type FUS and P525L tissue, suggesting that the mutation itself did not have a detectable effect on transcription of these mRNA species (figures 5.3e&f).

	Cortex	Cerebellum	Spine
Human TDP-43			
Total TDP-43	↓ in P525L	↓ in P525L	↓ in P525L
Total FUS (hu+endog)	↑ in P525L	→	↓ in P525L
Endogenous FUS	↓ in P525L	↓ in P525L	↓ in P525L
Endogenous SMN	↓ in P525L	↓ in P525L	↓ in P525L
Endogenous TAF15	↓ in P525L	→	↓ in P525L
Endogenous EWS	→	→	↓ in P525L

Table 5.2: Summary of the effects of the P525L mutation on mRNA levels in the FUS BAC transgenic model, compared to wild-type.

Total FUS

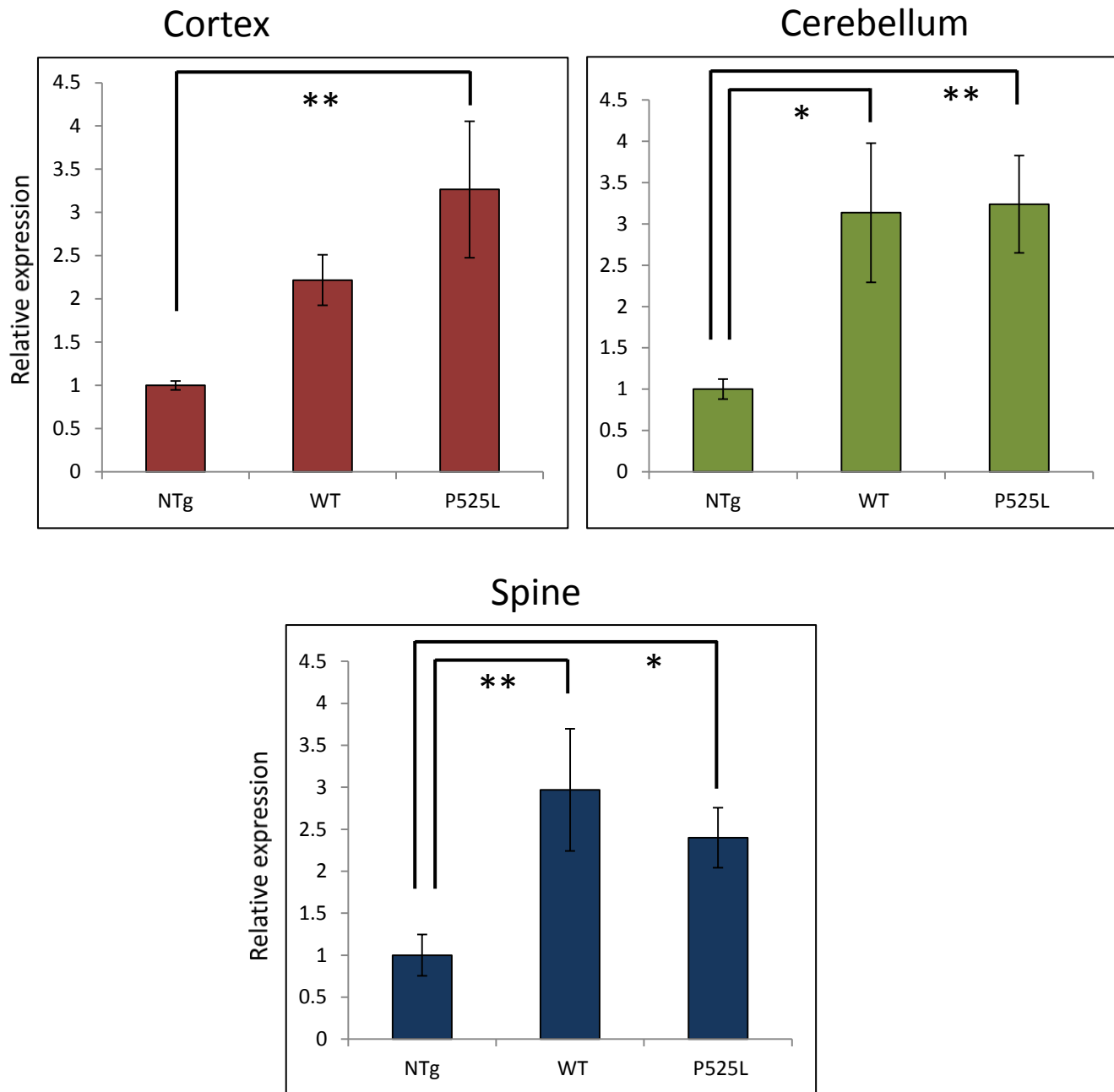


Figure 5.3. mRNA expression in the cortex, cerebellum and spine from the FUS BAC transgenic mouse model detected using qRT-PCR. Results are shown by genotype. (n=2xNTg, 3xWT-2, 2xWT-3, 3xP525L-1, 4xP525L-2, 3xP525L-3. One-way ANOVA with Tukey post-test. *=p<0.05, **=p<0.01, ***=p<0.001).

a) Expression of total FUS relative to non-transgenic. Endogenous and BAC-derived FUS are detected. It shows an increase in total FUS in both transgenic lines.

Endogenous FUS

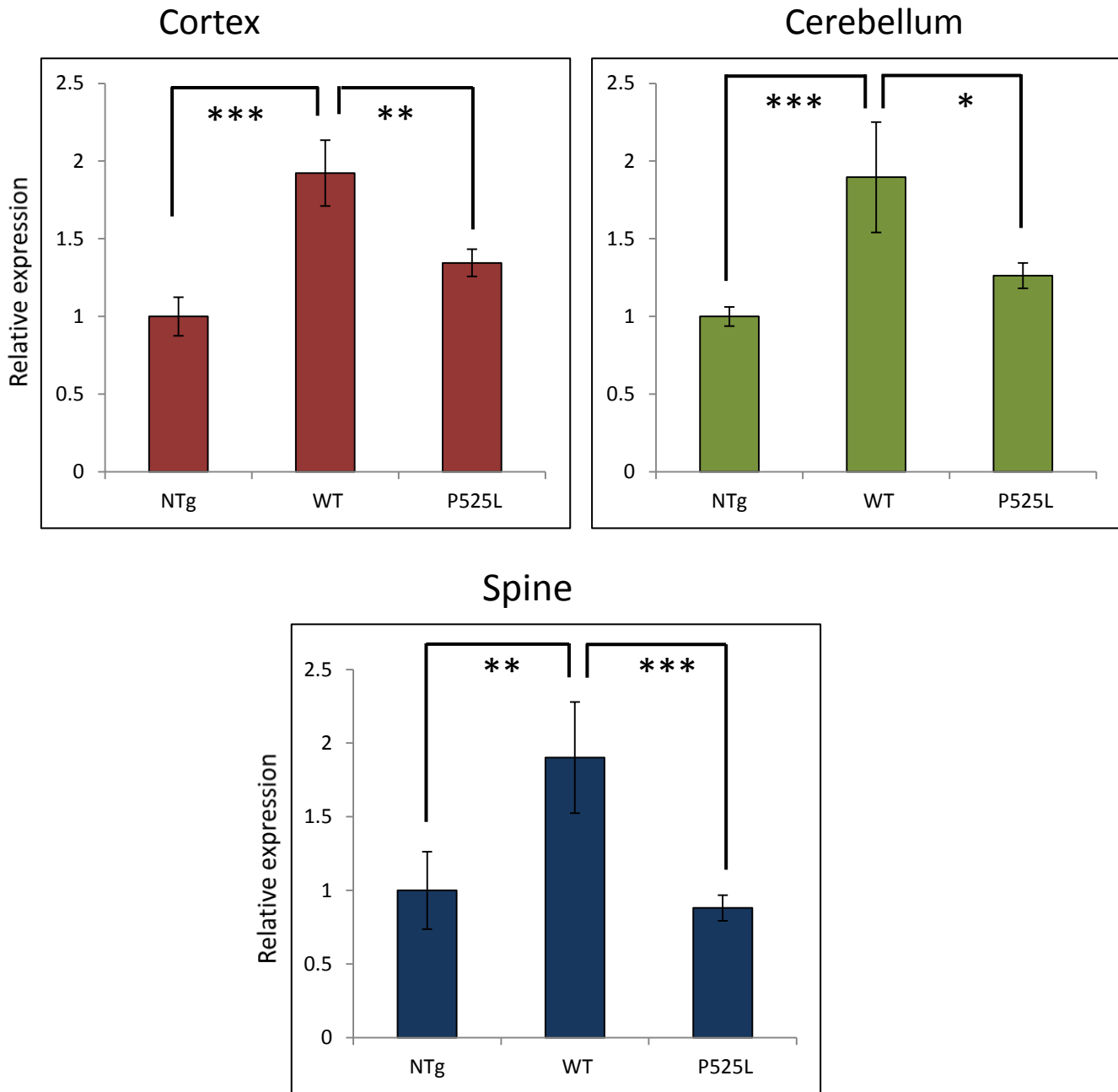


Figure 5.3b) Expression of endogenous FUS relative to non-transgenic in the FUS BAC transgenic mouse model. Wild-type FUS BAC tissue has increased endogenous FUS expression compared to non-transgenic and P525L.

Endogenous TDP-43

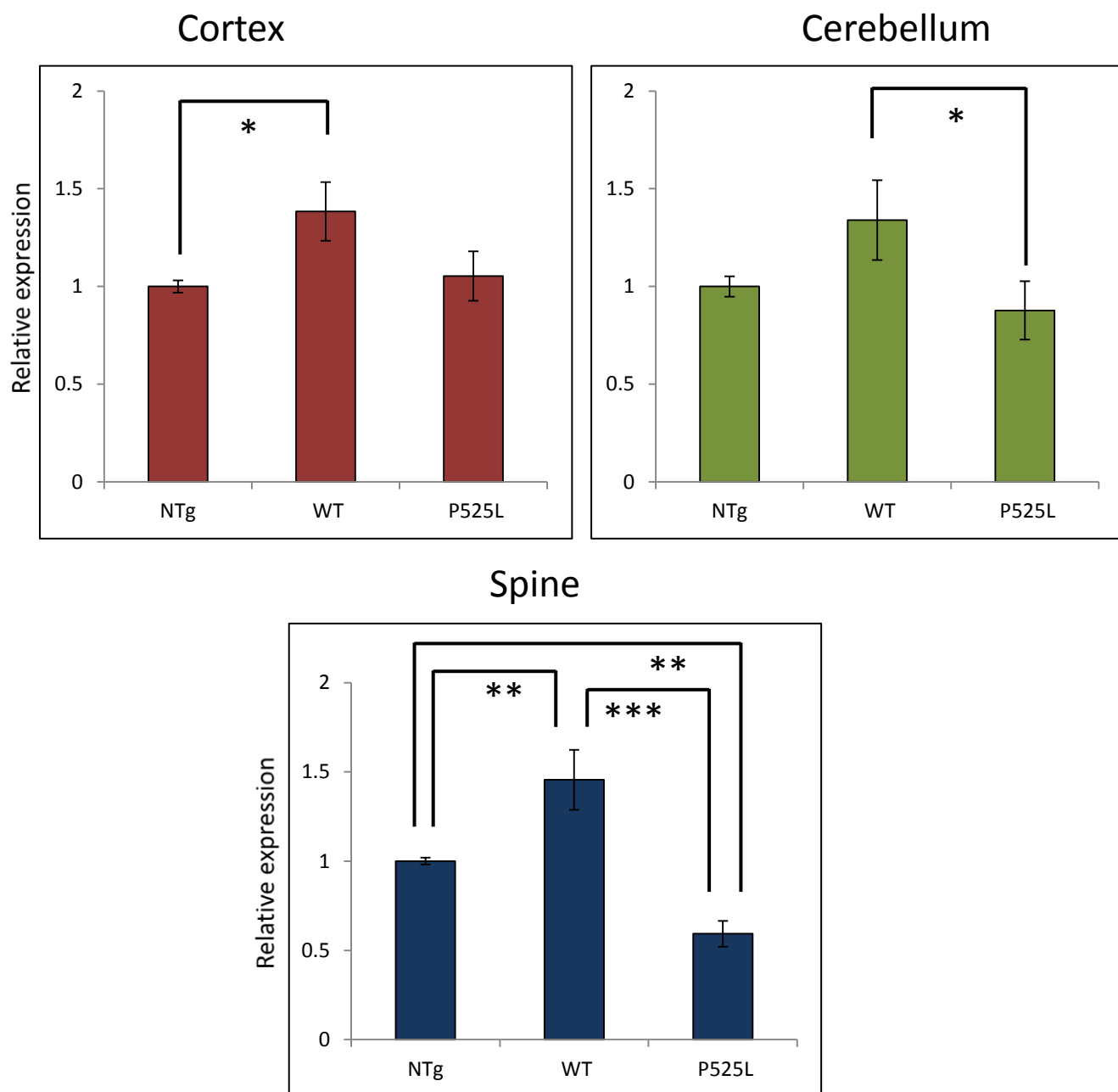


Figure 5.3c) Expression of endogenous TDP-43 relative to non-transgenic in the FUS BAC transgenic mouse model. Wild-type FUS tissue has increased endogenous TDP-43 expression compared to non-transgenic and P525L.

Endogenous SMN

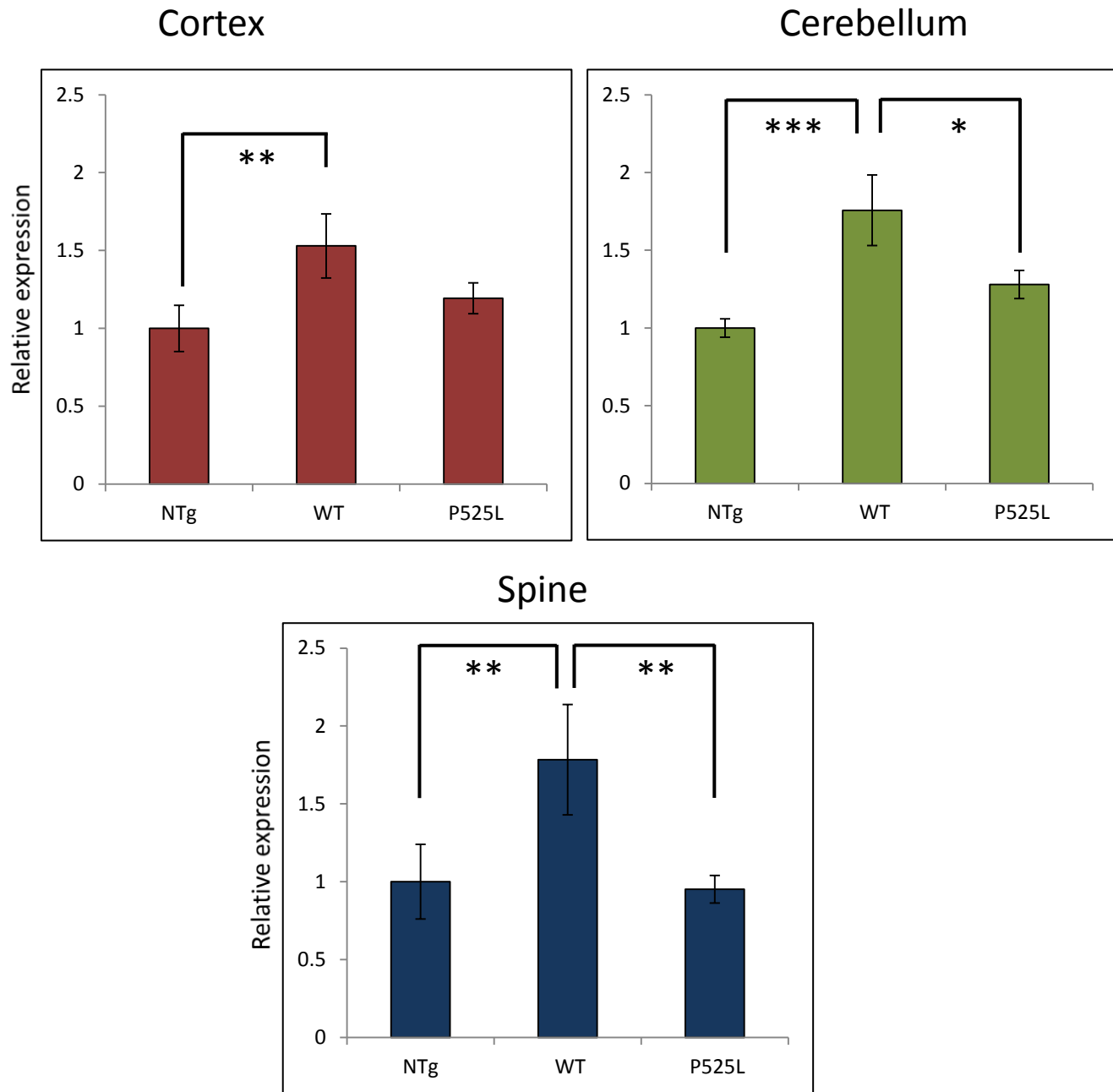


Figure 5.3d) Expression of SMN relative to non-transgenic in the FUS BAC transgenic mouse model. Wild-type FUS BAC tissue has increased SMN expression compared to non-transgenic and P525L.

Endogenous TAF15

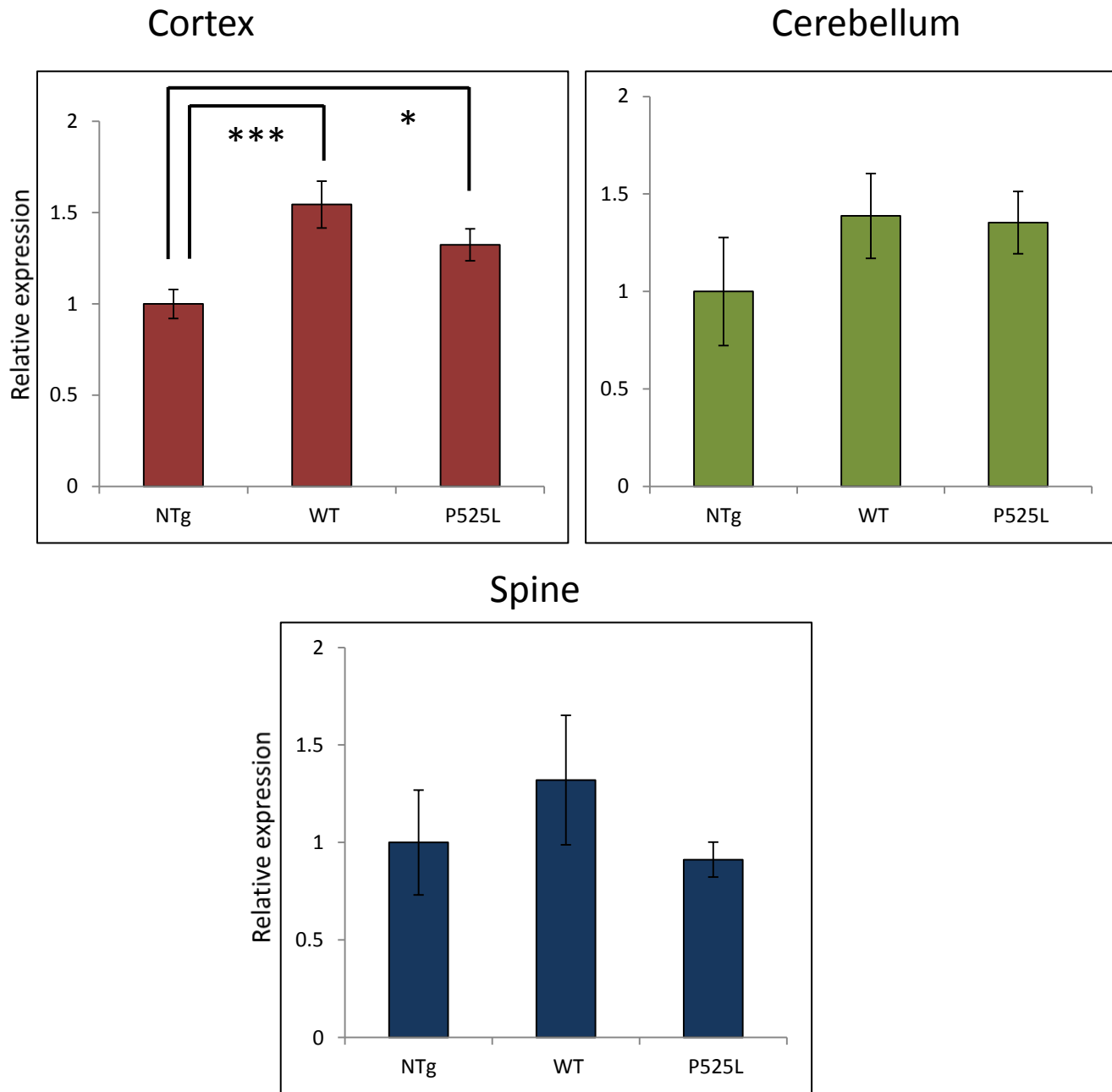


Figure 5.3e) Expression of TAF15 relative to non-transgenic in the FUS BAC transgenic mouse model. In the cortex there is increased TAF15 expression in both transgenic lines compared to non-transgenic, but no difference between wild-type and P525L.

Endogenous EWS

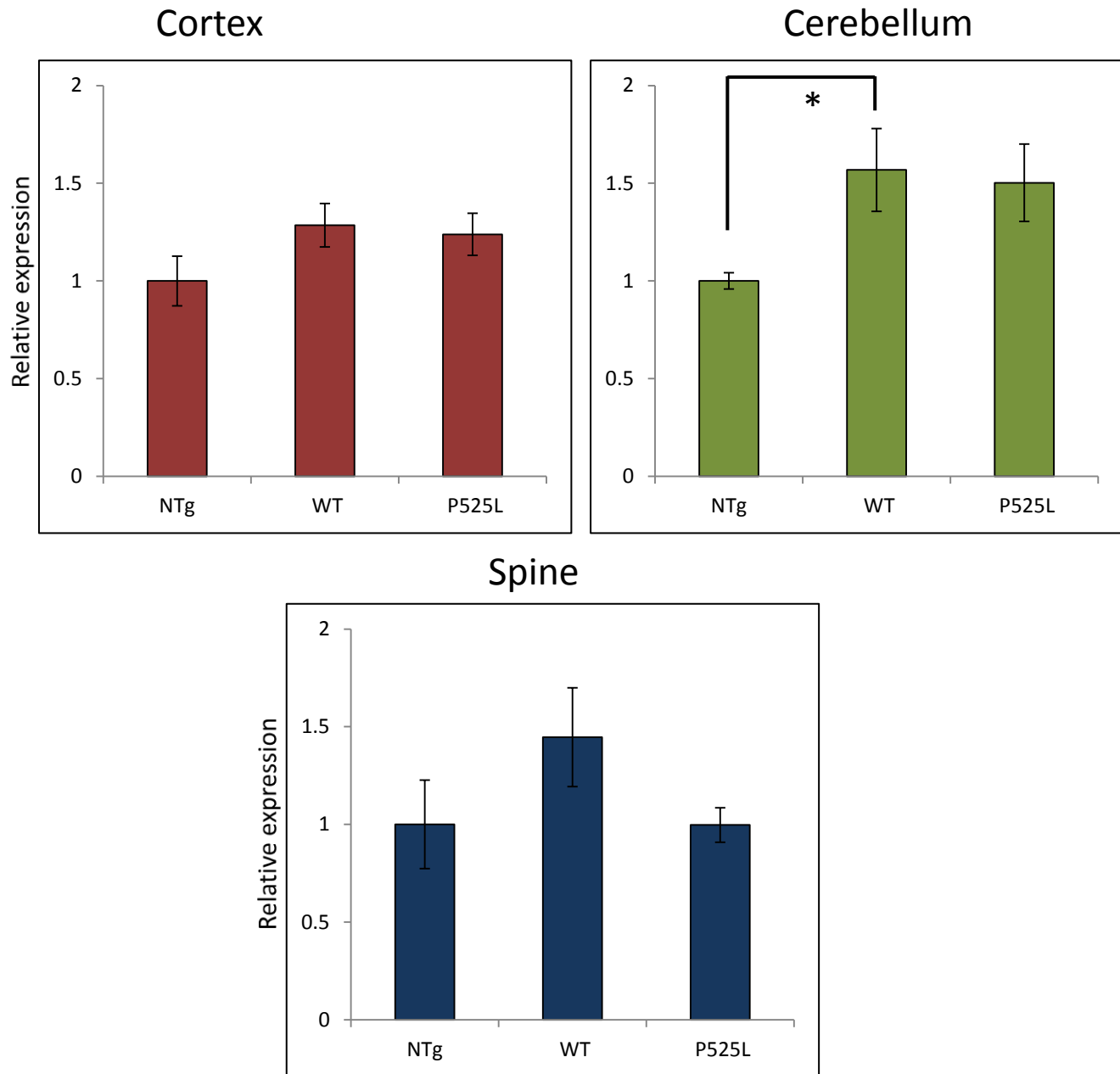
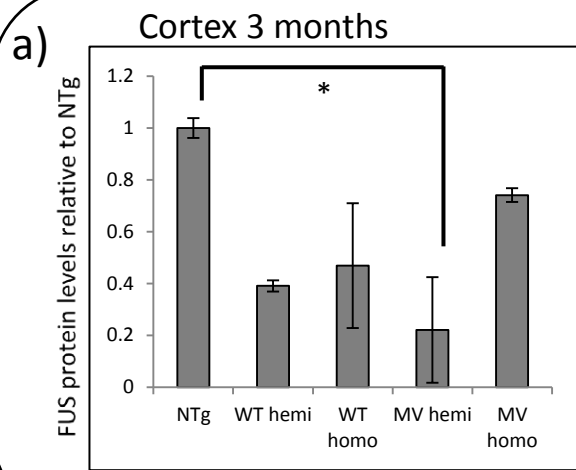


Figure 5.3f) Expression of EWS relative to non-transgenic in the FUS BAC transgenic mouse model. In the cerebellum there is increased EWS expression in wild-type lines compared to non-transgenic, but no difference between wild-type and P525L.

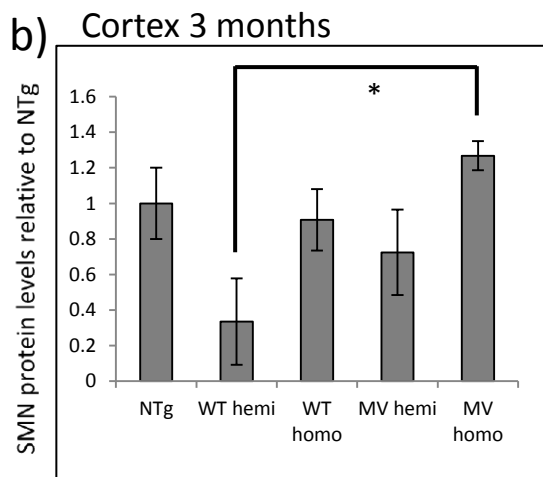
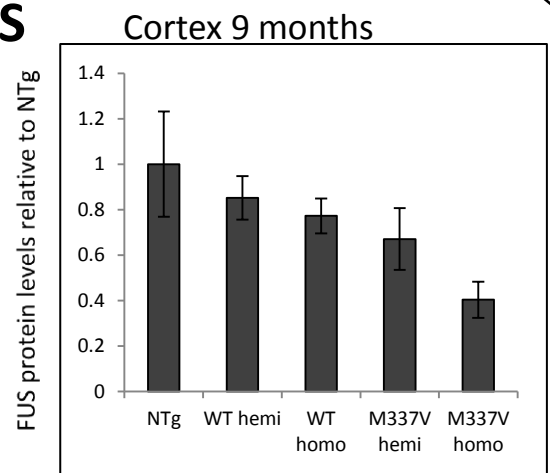
5.3 Protein levels in the TDP-43 BAC transgenic mouse model

FUS expression in each line of the TDP-43 BAC transgenic mouse model was examined using Western blot analysis of whole cell lysates prepared from cortex or spinal cord. In 3 month cortex samples there were no clear differences in FUS protein expression between TDP-43 genotypes, though FUS levels in the transgenic mice were generally lower than non-transgenic, which reached statistical significance in the M337V hemizygous line ($p < 0.05$, figure 5.4a). At 9 months, although there were no significant differences, there were modest decreases in FUS protein expression seen in wild-type hemizygous, wild-type homozygous and M337V hemizygous cortex compared to non-transgenic, and lowest levels seen in M337V homozygous cortex. This reflected the changes observed in FUS mRNA levels at this time point (figure 5.2c). There was insufficient tissue to examine FUS levels in the spine at these time points.

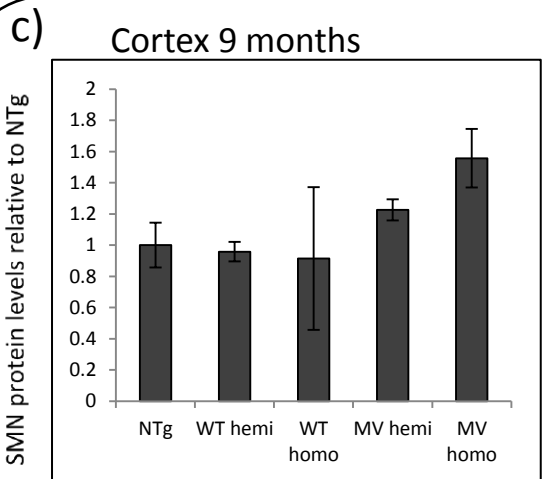
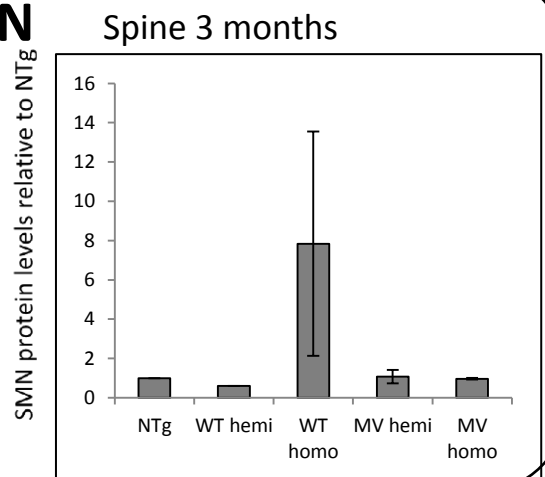
SMN protein levels in the cortex at 3 months showed a significant increase in SMN protein level between the wild-type hemizygous line and the M337V homozygous line (figure 5.4b). Interestingly, there was also an increase in SMN mRNA transcript levels in the M337V homozygous line at this time point (figure 5.2d). In the spine, the wild-type TDP-43 homozygous line had a non-significantly increased level of SMN, though variability was high between samples. At 9 months in the cortex, there was a trend towards an increase in SMN in the M337V lines (figure 5.4c), which is the opposite of the observed increase in SMN mRNA levels at this time point (figure 5.2d). In the spine at 9 months, although the wild-type hemizygous line appeared to have increased SMN levels, this was not statistically significant.



FUS



SMN



SMN

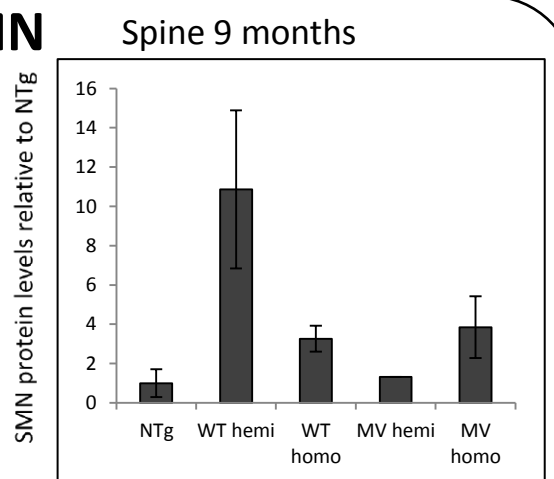


Figure 5.4 Quantification of Western blots using tissue from the TDP-43 BAC transgenic mouse model demonstrating **a)** the expression of endogenous FUS protein relative to actin. There is a trend towards lower levels of FUS in transgenic lines in the 3 month cortex, and a decrease in FUS levels in the M337V mutant lines at 9 months. **b)** The expression of SMN protein relative to actin at 3 months **c)** The expression of SMN protein relative to actin at 9 months (FUS=72 kDa, actin=42 kDa. SMN=40 kDa n=2-3. One-way ANOVA with Tukey post-test.)

Interestingly, there were additional larger bands at 65 kDa and 75 kDa visible in the SMN Western blots, particularly in the M337V mutant tissue (blots from all lines were carried out simultaneously and exposed for the same time period) (appendix 7.9b&c). This suggests that SMN may form complexes with other proteins in the presence of mutant TDP-43.

There was unfortunately insufficient tissue to carry out protein analysis on the FUS transgenic mouse model. However, as the mouse matures then tissue will be collected from each time point to allow analysis to take place.

5.4 Alternative splicing in the FUS and TDP-43 BAC transgenic mouse models

5.4.1 Splicing in the TDP-43 BAC transgenic mouse model

RNA from CNS tissue from the TDP-43 BAC transgenic model at 3 and 9 months was used to examine altered splicing. Primers for the relevant target were selected for their ability to detect each splice variant (119). The relative abundance of each variant was analysed in a semi-quantitative manner by PCR and analysis of the relative intensity of each band present on the gel. A summary of the effects of the M337V mutation on the relative abundance of the full length variant of each target is summarised in table 5.3.

Ndr2 is expressed in glial cells and astrocytes, and upregulation has been associated with Alzheimer's Disease (278, 279). TDP-43 and FUS knockdown have been associated with a reduction in full length transcript and relative increase in the exon 3 skipped variant (119). These results show a small but significant reduction in full length transcript in the M337V homozygous line compared with one of the wild-type TDP-43 lines at both 3 and 9 months (figure 5.5b), consistent with reported changes observed in TDP-43 knockout tissue (119).

TIA1 is an RNA binding protein involved in multiple steps of RNA processing, and is a component of stress granules (204). TDP-43 and FUS knockdown have been associated with an increase in full length TIA1 compared to the exon 5 skipped variant (119). Though there was a small decrease in full length TIA1 in the homozygous lines in the spine at 3 months, there were no significant differences observed between the lines of the TDP-43 BAC transgenic mouse at either time point (figure 5.5c and d).

Mag (myelin associated glycoprotein) is a constituent of CNS myelin and inhibits axonal growth and regeneration (280, 281). Interestingly, few splicing targets studied by Lagier-Tourenne et al had differential effects with TDP-43 and FUS knockdown (119). However, Mag displayed an increase in full length RNA with FUS knockdown and a decrease with TDP-43 knockdown, compared to the exon 8 skipped variant (119). There were no differences in exon 8 splicing observed in the TDP-43 BAC transgenic model.

Ppp3ca encodes a subunit of calmodulin-dependent calcineurin and is therefore involved in many cellular processes mediated by calcium, such as neurotransmitter release (282). It has also been implicated in SOD1 ALS, and a reduction in full length ppp3ca compared to the exon 13 skipped variant has been reported in TDP-43 knockdown, but no effect seen with FUS knockdown (119, 283). The gels depicted in figures 5.5g and h show a distinct difference in the relative abundance of each ppp3ca variant between cortex and spine in the TDP-43 BAC transgenic model.

There were no differences between the lines observed in the spinal cord. However, in the brain there was a significant reduction in the full length variant at 3 months and increase at 9 months in the M337V hemizygous line compared to either wild-type or non-transgenic. It is difficult to draw firm conclusions from this, but it may represent

altered splicing which could change between the pre-symptomatic and symptomatic stages of disease in this model.

Dnajc5 is expressed in neuronal tissue and synaptic vesicles. Mutations in this gene have been implicated the neurodegenerative condition inherited neuronal ceroid lipofuscinosis (284). TDP-43 knockdown has been associated with an increase in the full length splice variant compared to the exon 9 skipped variant (119). Interestingly, an additional 310 base pair product was observed from tissue in this study compared to those previously reported. However, no significant differences between the lines were observed.

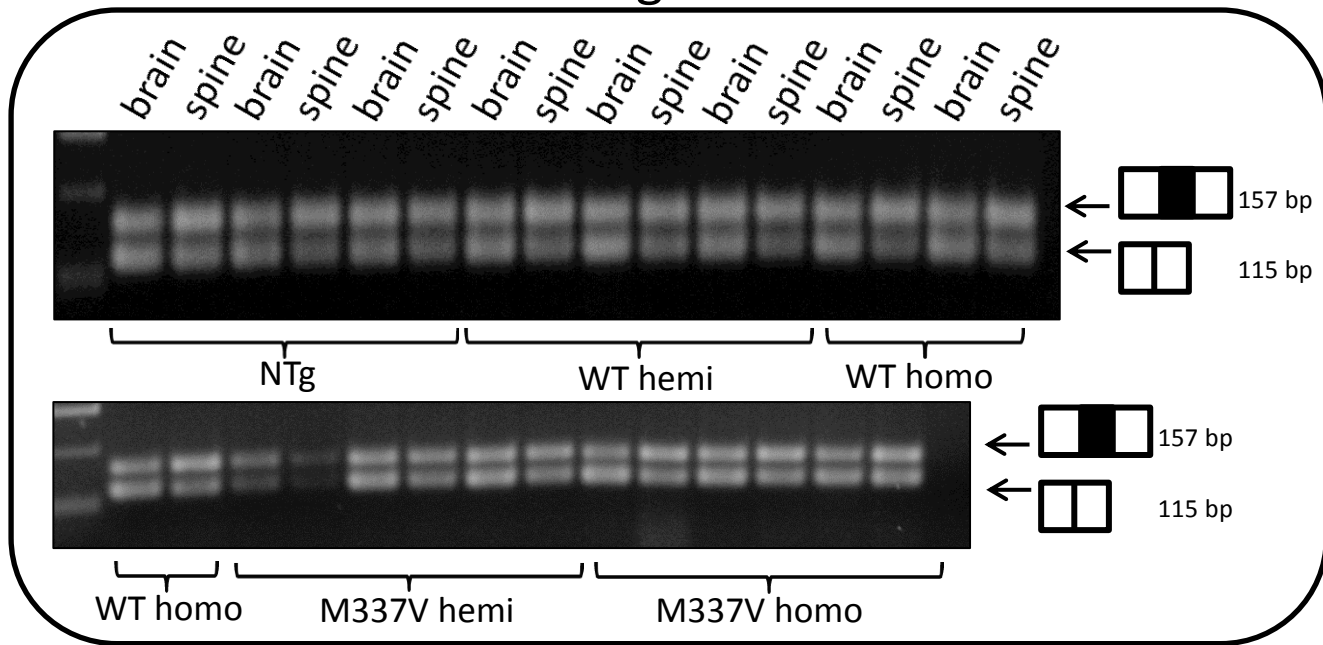
Ptk2b encodes a tyrosine kinase with many functions within the cell including regulation of neuronal activity. It has also been implicated in the pathogenesis of Alzheimer's disease (285). A reduction in full length compared to exon 24 skipped variant has been observed after FUS knockdown, with no effect after reduction of TDP-43 (119). However, no differences were observed in the TDP-43 BAC transgenic model (figure 5.5k and l).

Ttc3 encodes an ubiquitin-protein ligase, which mediates ubiquitination of the survival protein Akt (286). A reduction in full length compared to exon 3 skipped variant has been described after FUS knockdown, with no effect of reduced TDP-43 (119). Interestingly, an increase in full length product was observed in the M337V hemizygous tissue compared to non-transgenic at 3 months, with a similar trend seen in the M337V homozygous tissue (figure 5.5m). However, this was not observed at 9 months (figure 5.5n). Again, it may suggest some early splicing changes in the presence of the TDP-43 mutation.

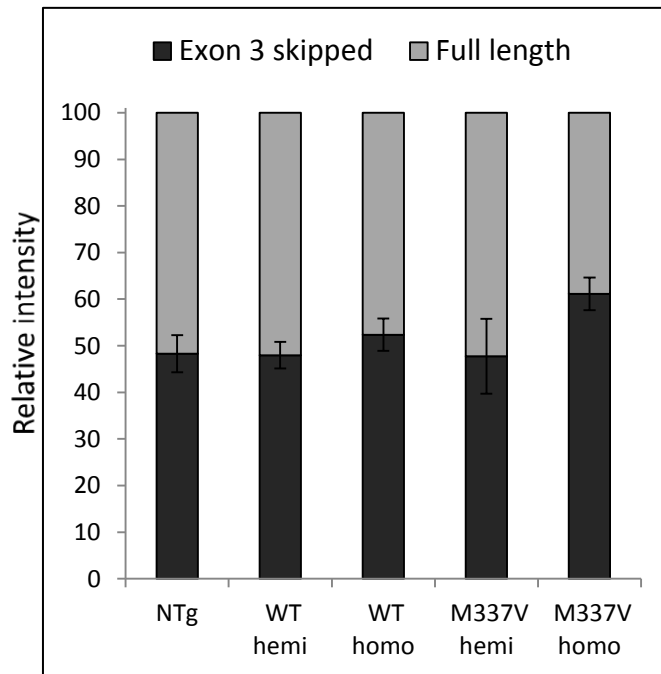
	TDP-43 Knockdown	TDP-43 BAC 3 months		TDP-43 BAC 9 months	
	Brain	Cortex	Spine	Cortex	Spine
Ndrp2	↓ full in KD	→	(↓) full in M337V	(↓) full in M337V	(↓) full in M337V
TIA1	↑ full in KD	→	→	→	→
Mag	↓ full in KD	→	→	→	→
Ppp3ca	↓ full in KD	(↓) full in M337V	→	(↑) full in M337V	→
Dnajc5	↑ full in KD	→	→	→	→
Ptk2b	→	→	→	→	→
Ttc3	→	→	(↑) full in M337V	→	→
FUS		→	(↓) full in M337V	→	→

Table 5.3: Summary of the effects of the M337V mutation on the relative abundance of full length splice variants in the TDP-43 BAC transgenic model, compared to wild-type. The effects in the 'knockdown' column are taken from Lagier-Tourenne et al, 2012. Arrows in brackets denote small effects.

Ndrdg2



Brain



Spine

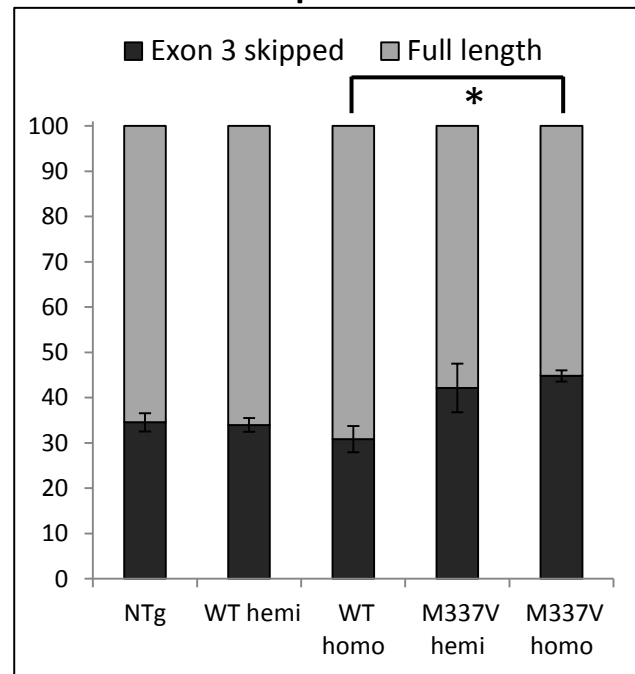
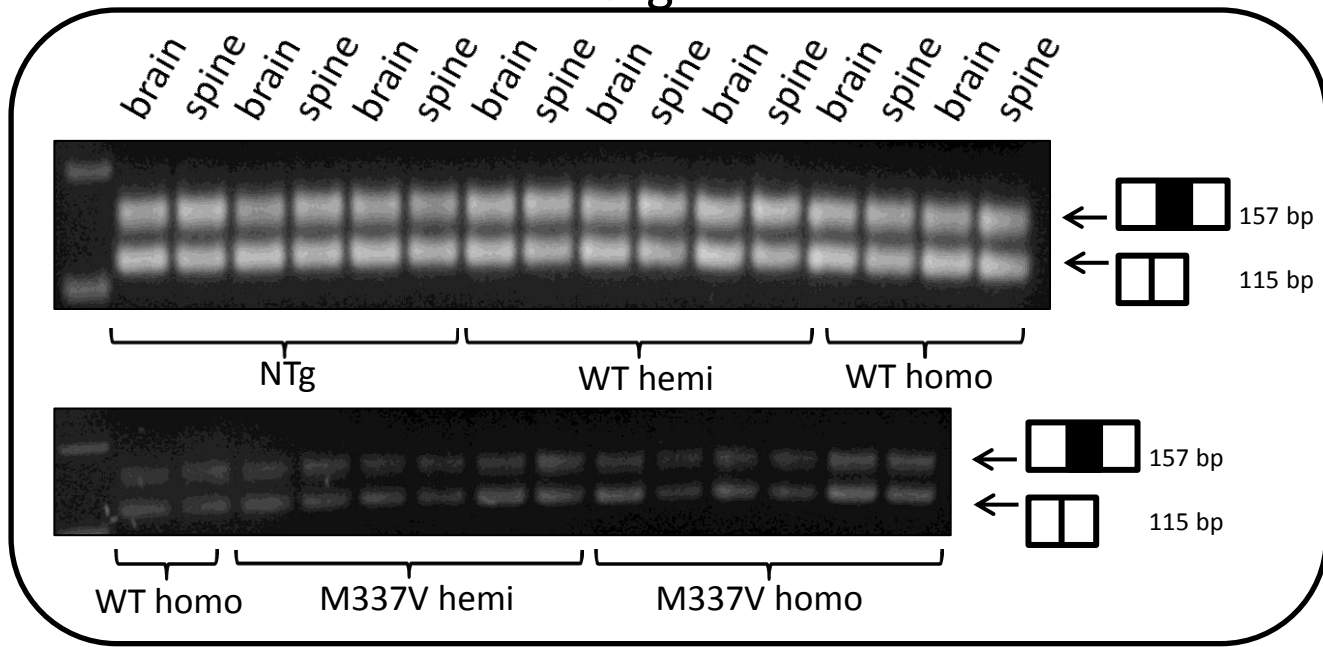


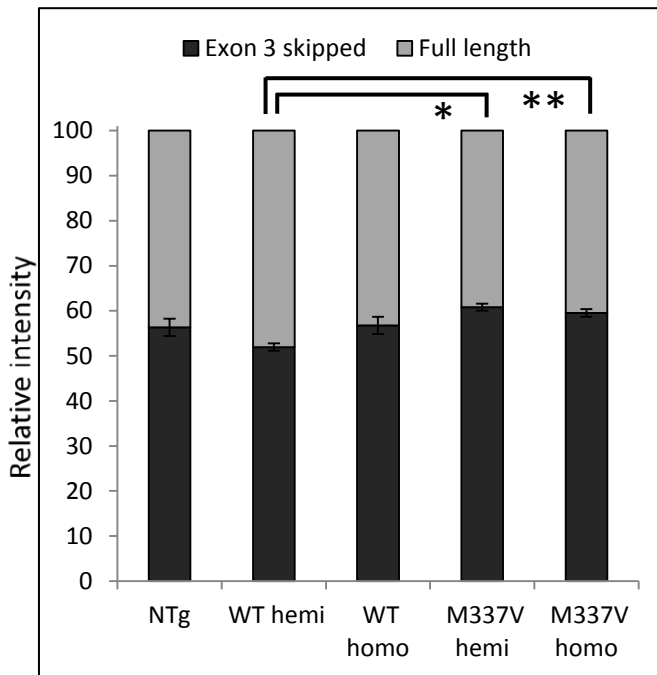
Figure 5.5 Analysis of alternative splicing in the TDP-43 BAC transgenic mouse model using semi-quantitative PCR. An example of the gel is shown above, with quantification below. (NTg = non-transgenic, WT = wild-type, hemi = hemizygous, homo = homozygous. N=3 mice per genotype. One-way ANOVA with Tukey post-test. *=p<0.05, **=p<0.01.)

a) Analysis of alternative splicing of *ndrg2* in the TDP-43 BAC transgenic mouse model at 3 months, using primers to detect exon 3 inclusion. There is a significantly lower proportion of full length *ndrg2* in the M337V homozygous spine compared to wild-type homozygous spine.

Ndrg2



Brain



Spine

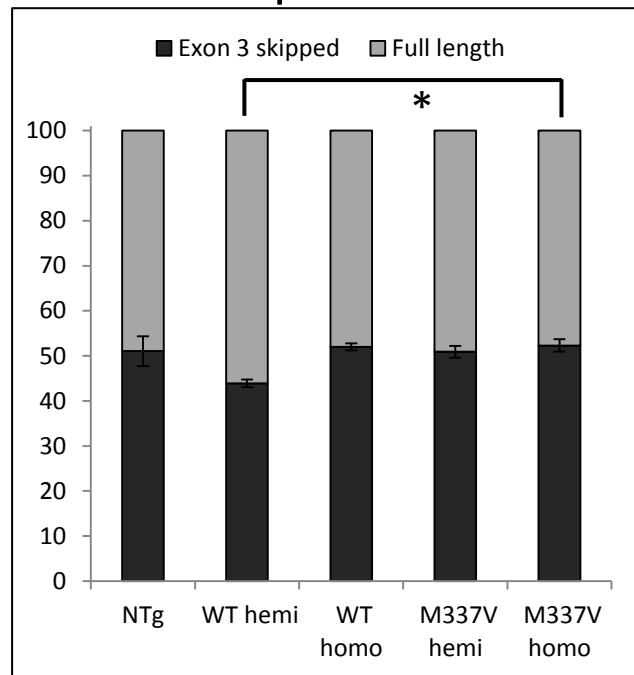
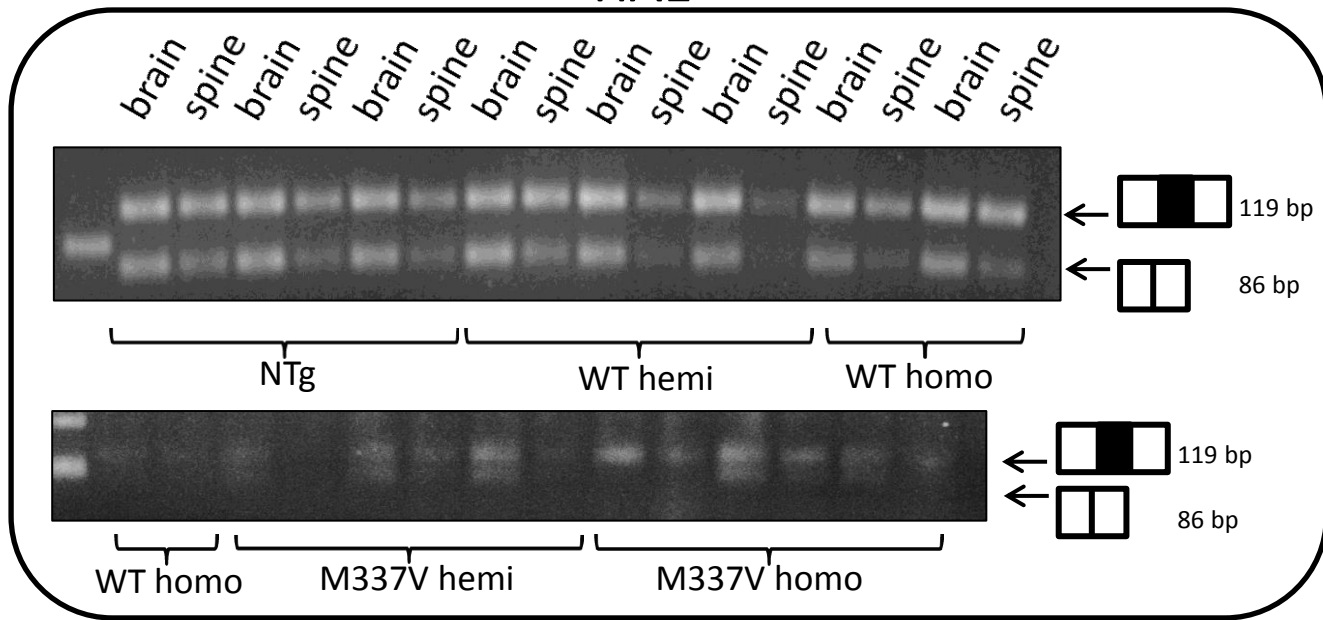
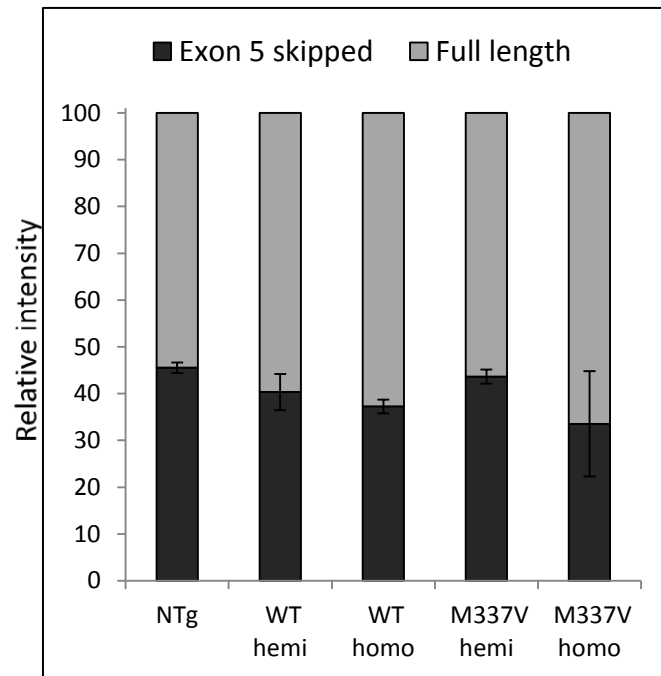


Figure 5.5b) Analysis of alternative splicing of *ndrg2* in the TDP-43 BAC transgenic mice at 9 months, using primers to detect exon 3 inclusion. There is a significantly lower proportion of full length *ndrg2* in the M337V lines compared to wild-type where illustrated.

TIA1



Brain



Spine

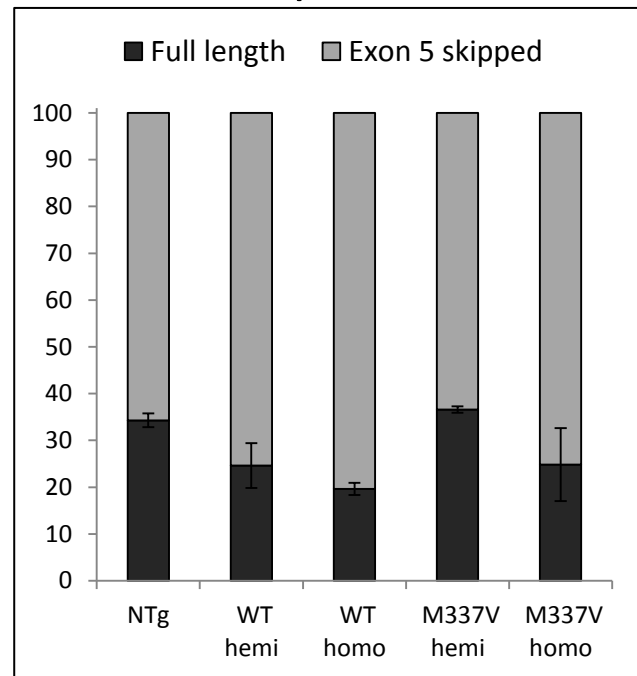
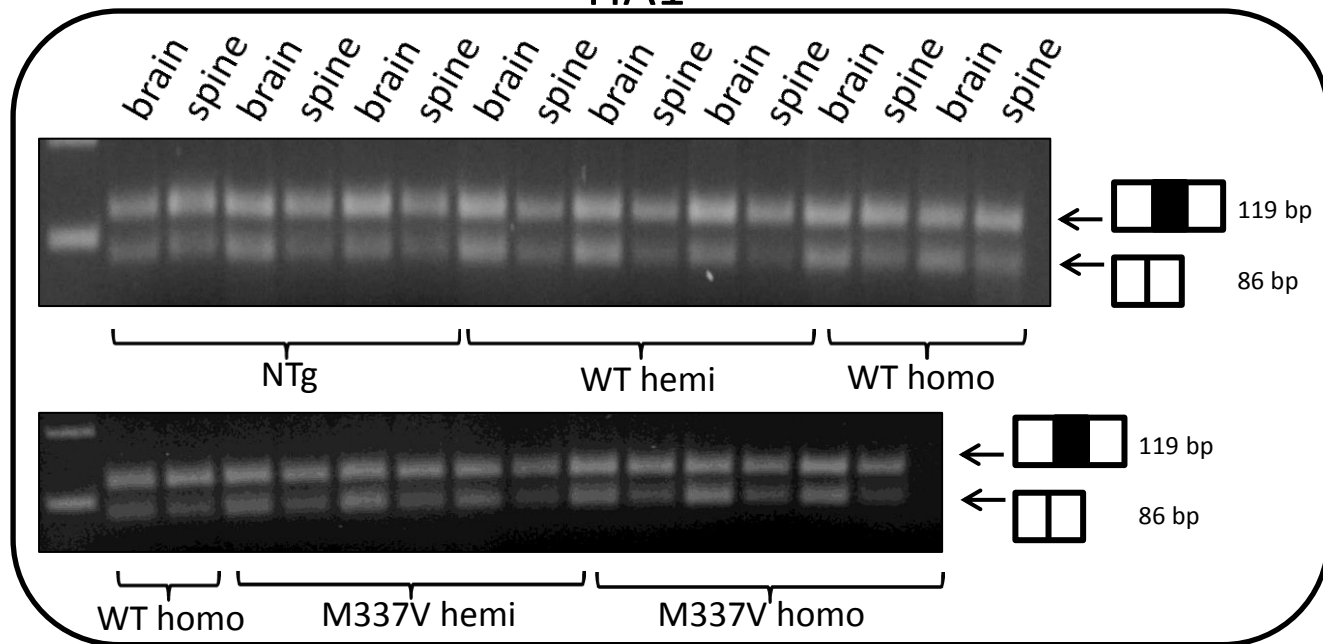
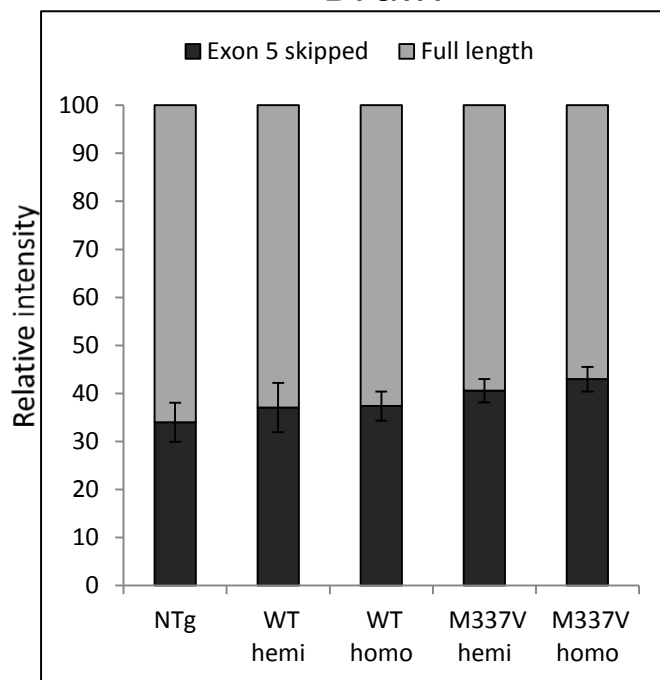


Figure 5.5c) Analysis of alternative splicing of TIA1 in the TDP-43 BAC transgenic mice at 3 months, using primers to detect exon 5 inclusion. There is no significant difference between the lines.

TIA1



Brain



Spine

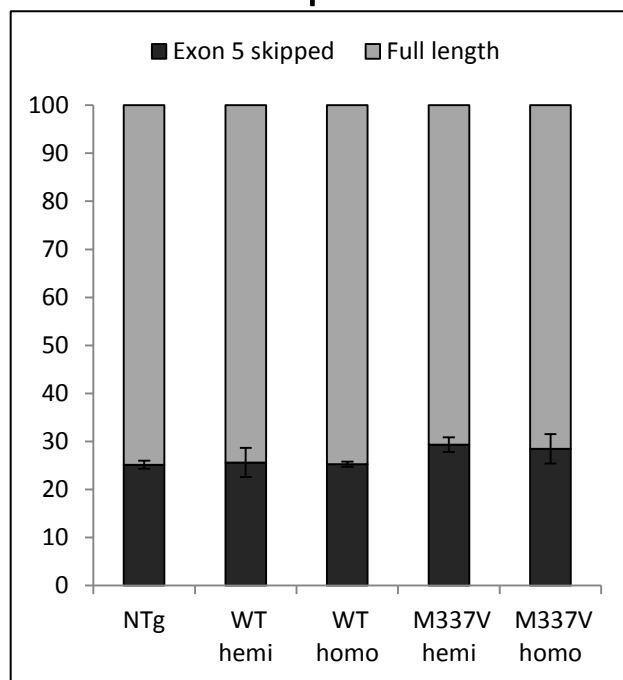


Figure 5.5d) Analysis of alternative splicing of TIA1 in the TDP-43 BAC transgenic mice at 9 months, using primers to detect exon 5 inclusion. There is no significant difference between the lines.

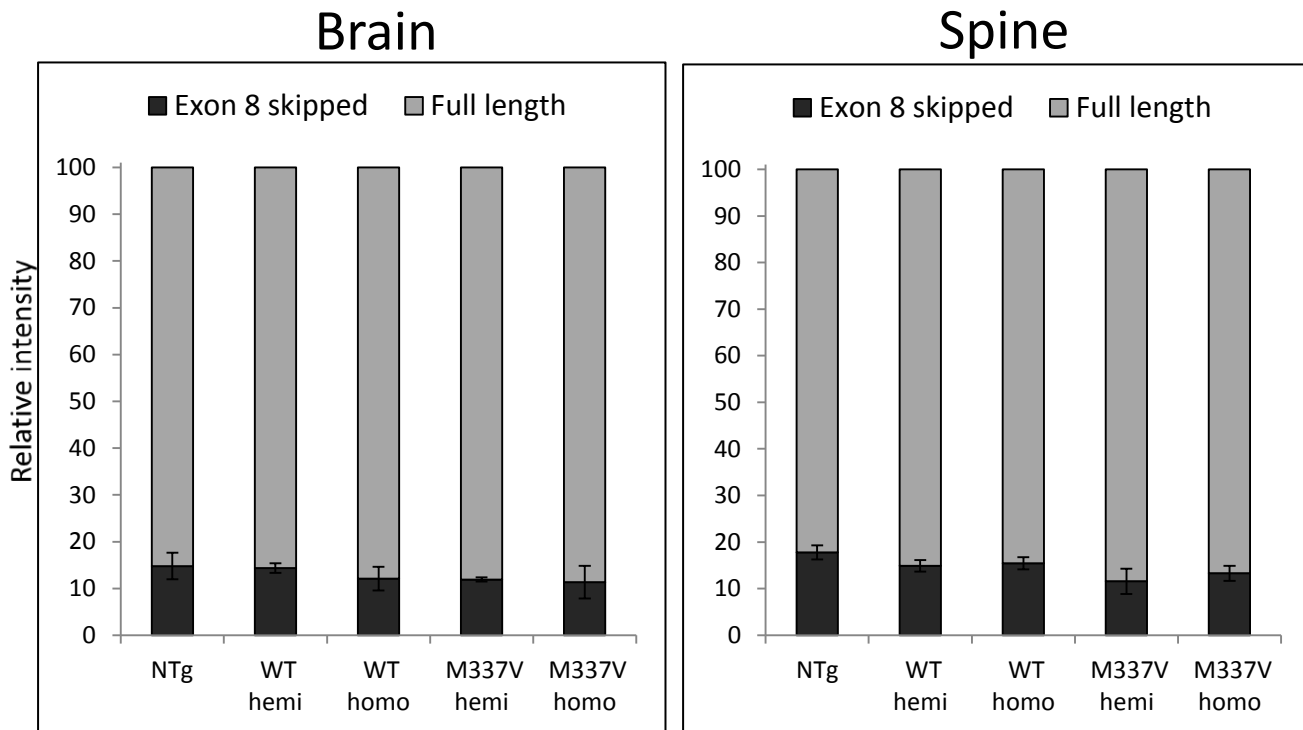
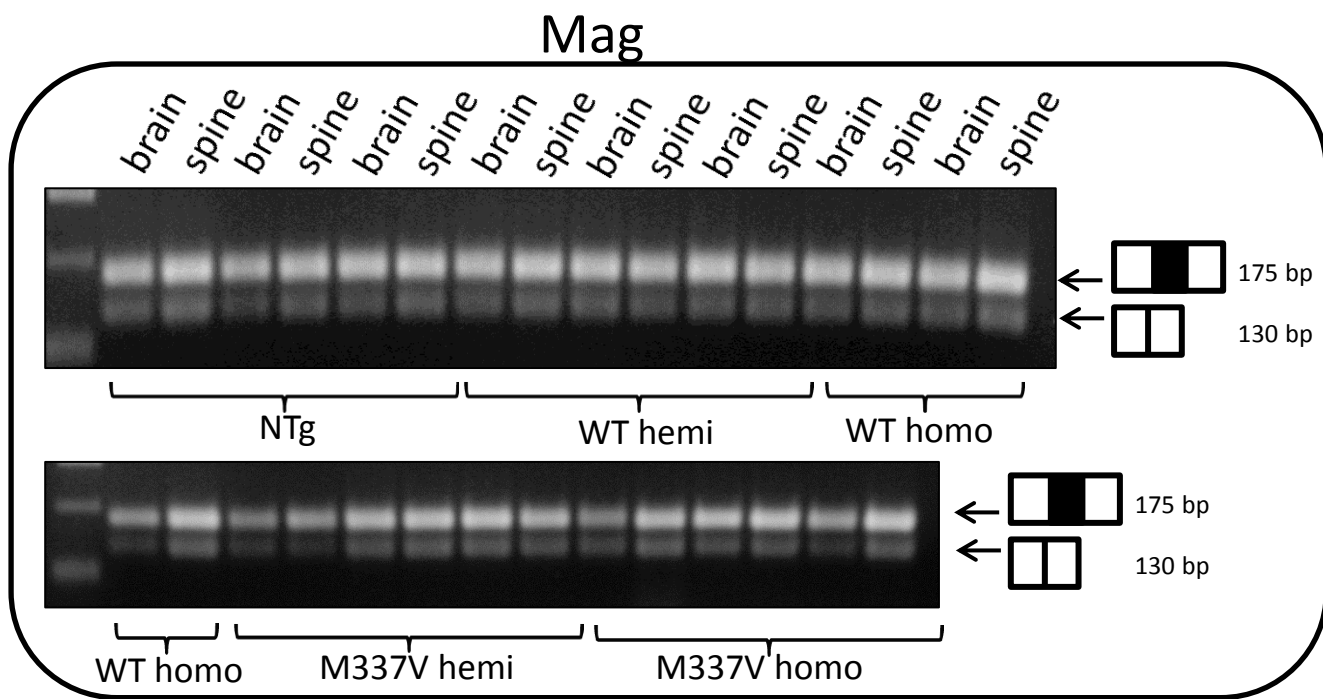
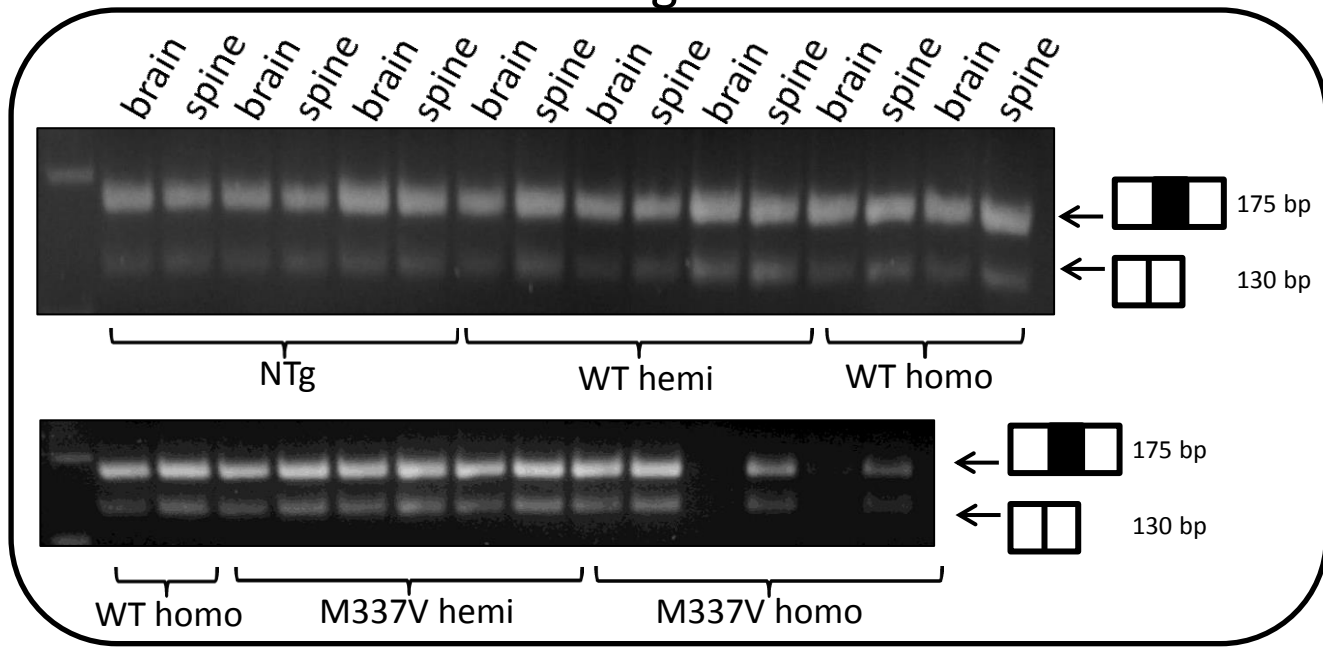
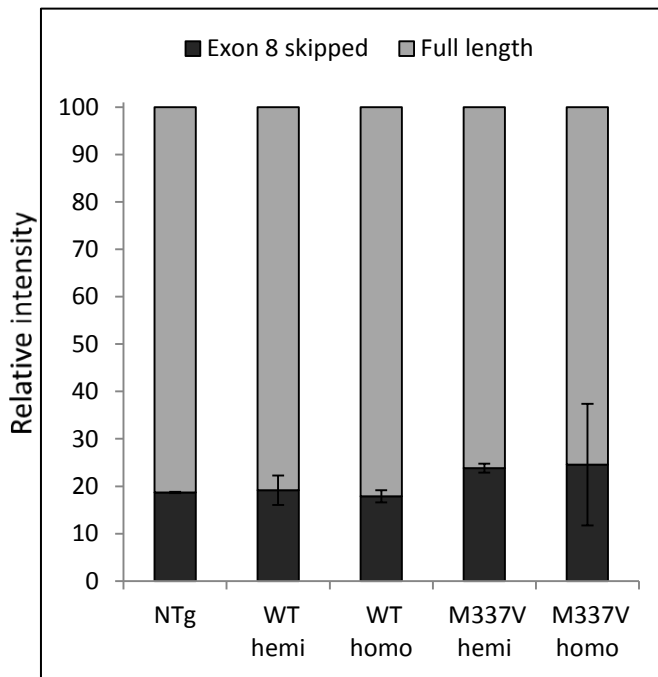


Figure 5.5e) Analysis of alternative splicing of Mag in the TDP-43 BAC transgenic mice at 3 months, using primers to detect exon 8 inclusion. There is no significant difference between the lines.

Mag



Brain



Spine

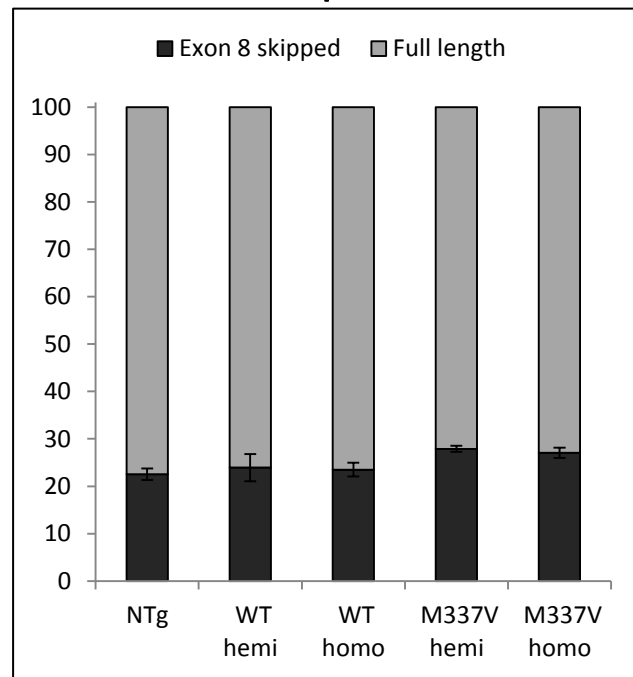
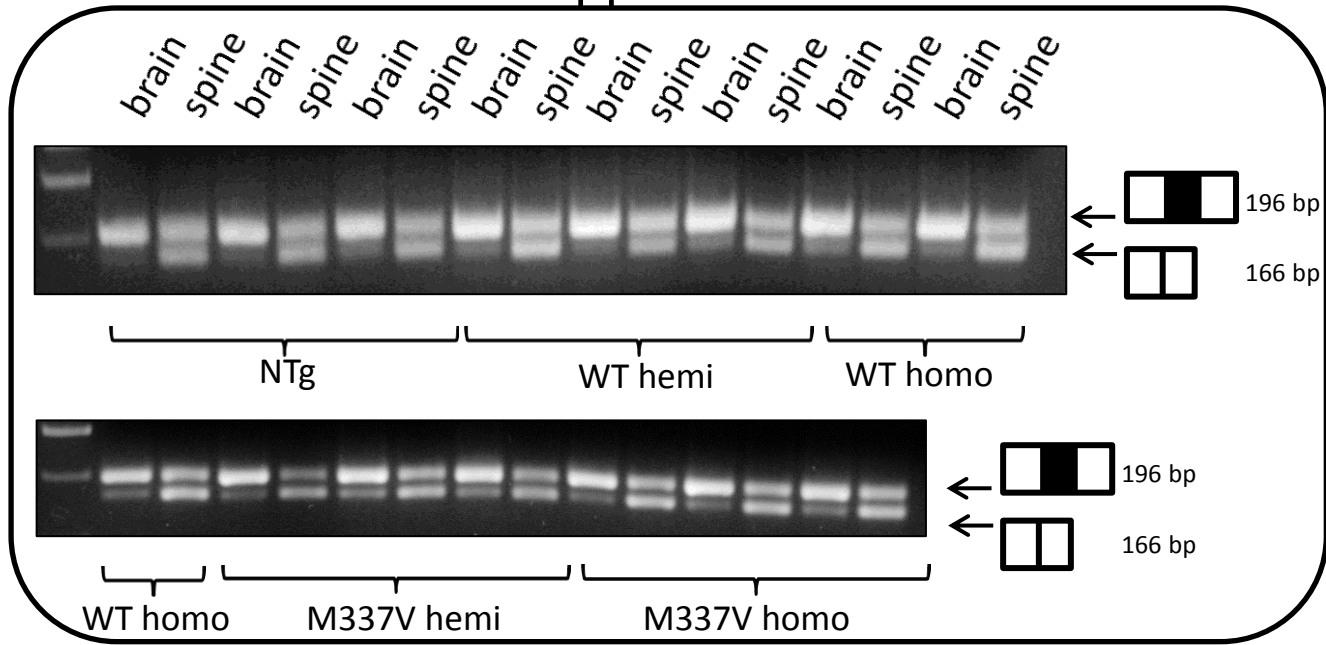
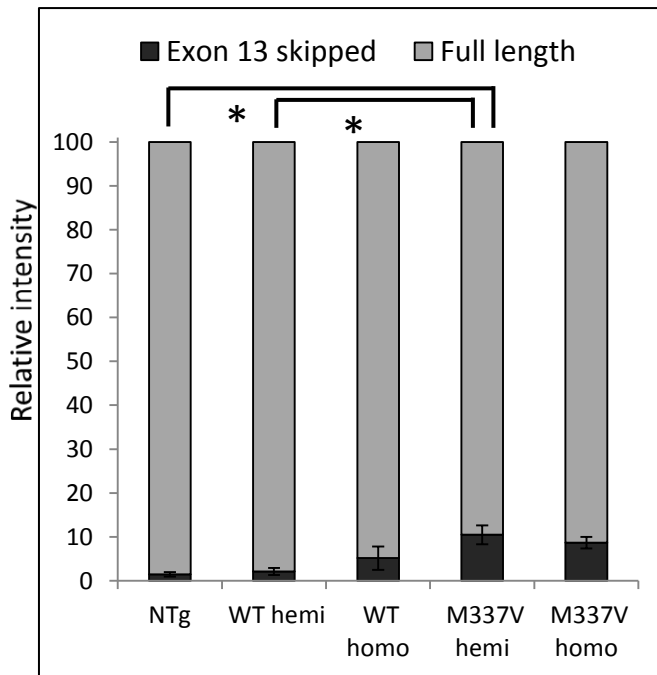


Figure 5.5f) Analysis of alternative splicing of Mag in the TDP-43 BAC transgenic mice at 9 months, using primers to detect exon 8 inclusion. There is no significant difference between the lines.

Ppp3ca



Brain



Spine

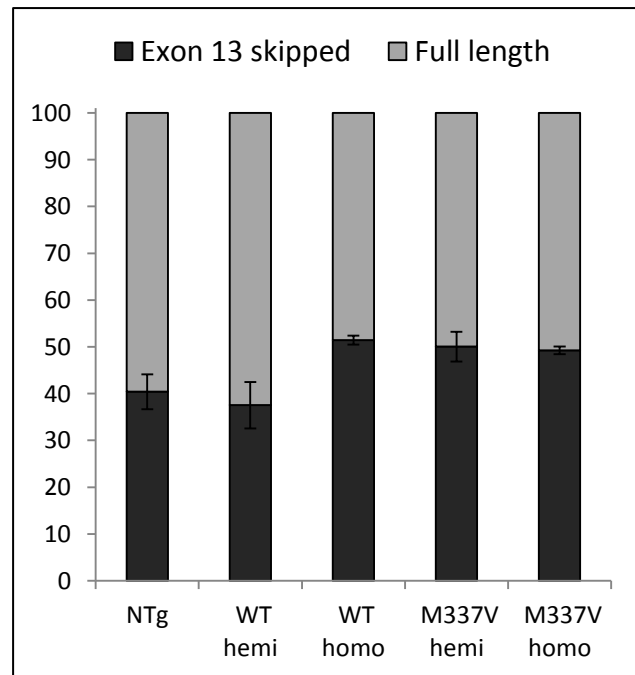
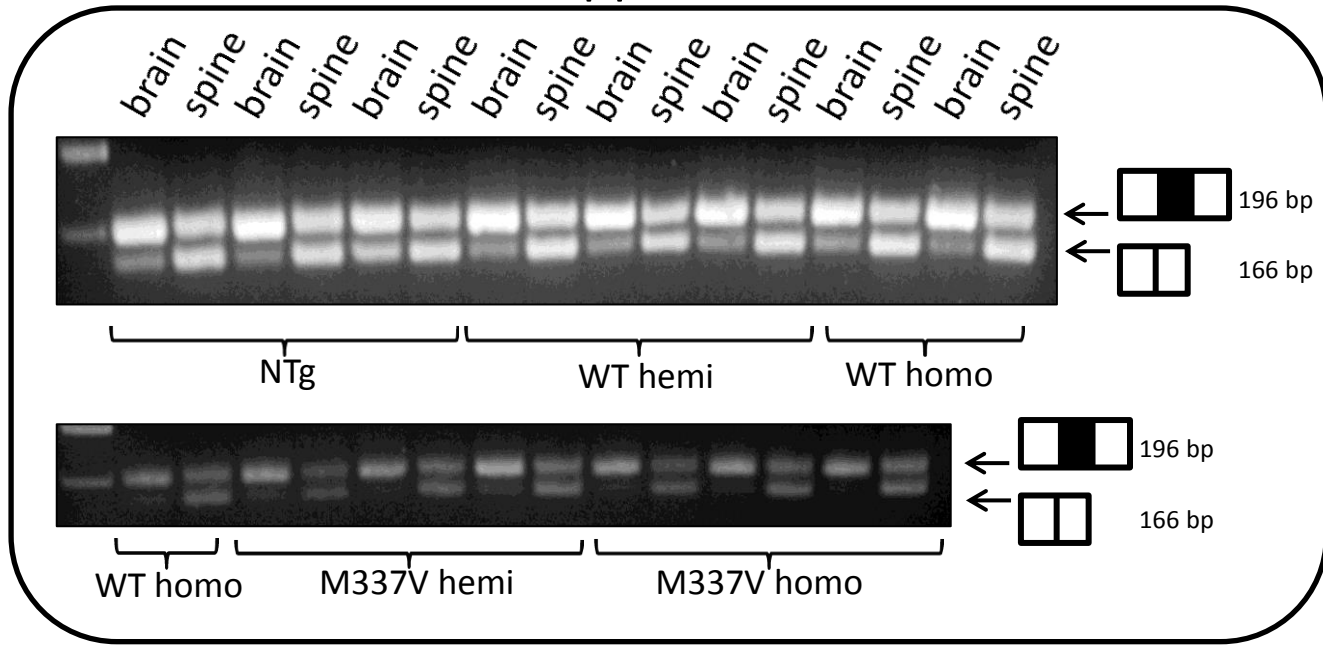
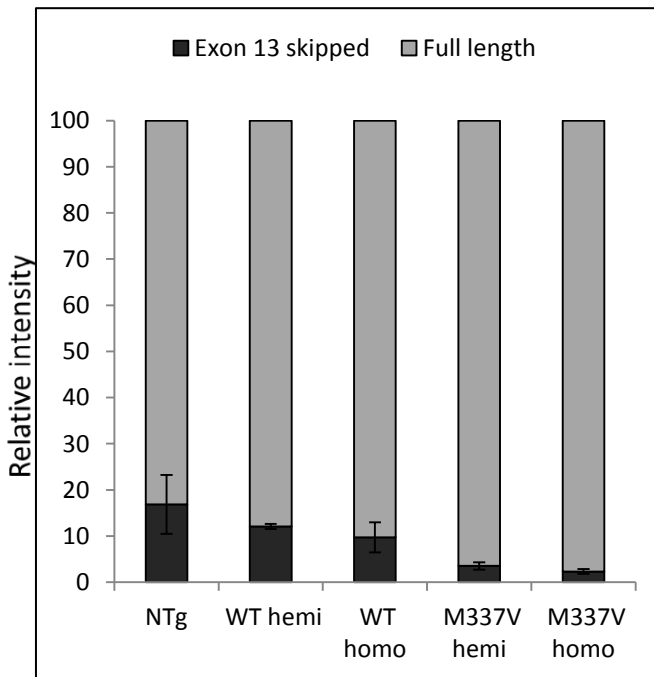


Figure 5.5g) Analysis of alternative splicing of Ppp3ca in the TDP-43 BAC transgenic mice at 3 months, using primers to detect exon 13 inclusion. There is a significant reduction in full length transcript in the M337V hemizygous line compared to non-transgenic and wild-type hemizygous in the brain.

Ppp3ca



Brain



Spine

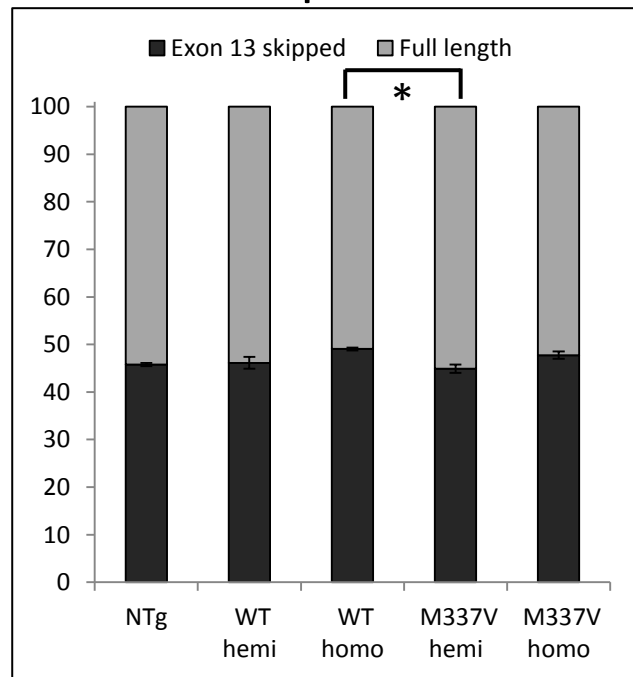
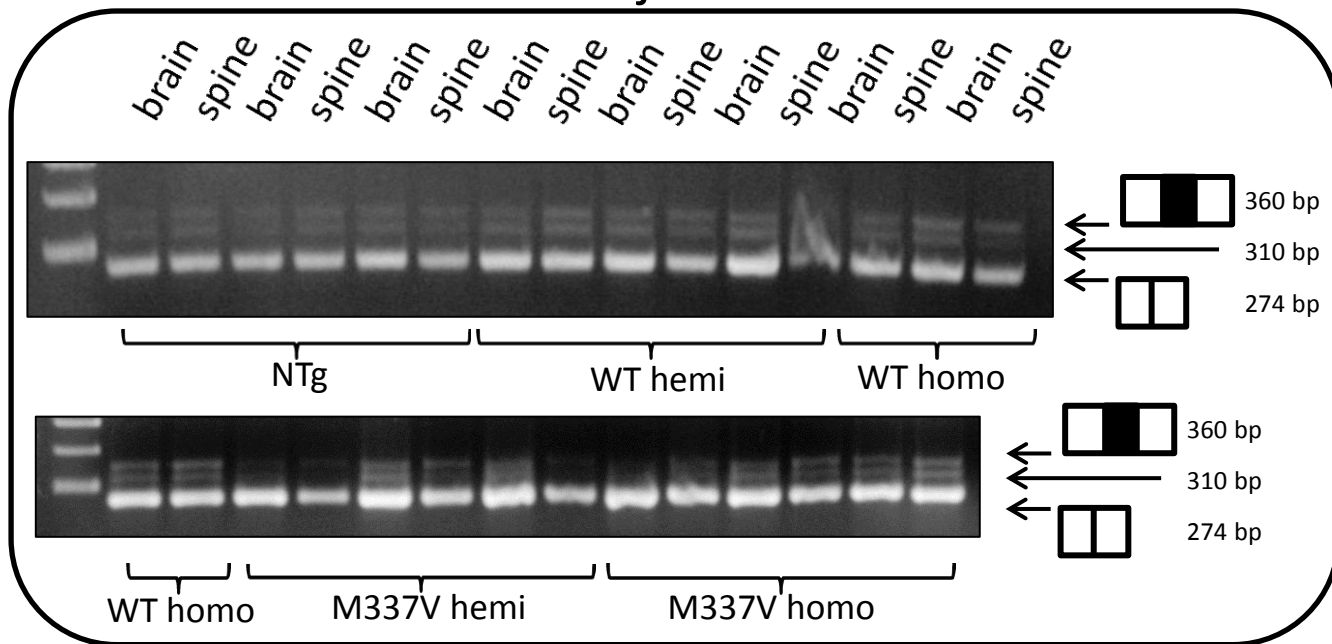
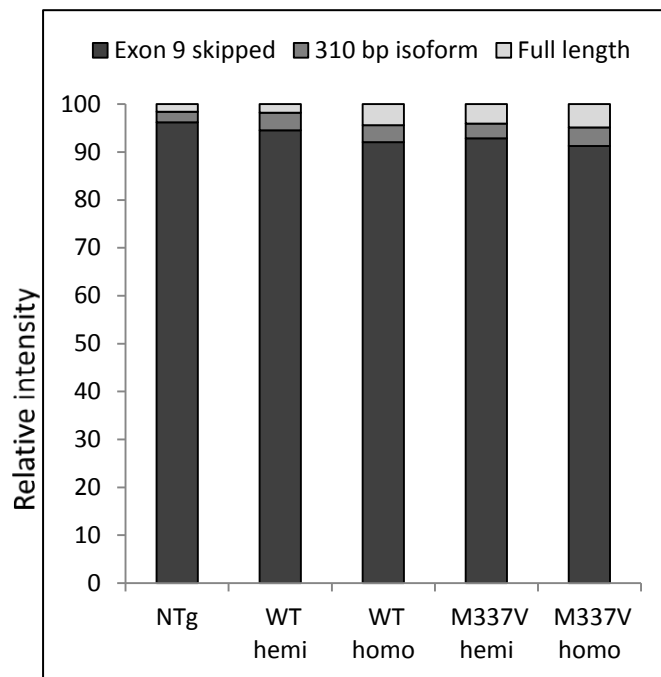


Figure 5.5h) Analysis of alternative splicing of Ppp3ca in the TDP-43 BAC transgenic mice at 9 months, using primers to detect exon 13 inclusion. There is a trend towards an increase in the proportion of full length transcript in the mutant lines in the brain, and a significant increase in the proportion of full length transcript in the M337V hemizygous line compared to wild-type homozygous in the spine.

Dnajc5



Brain



Spine

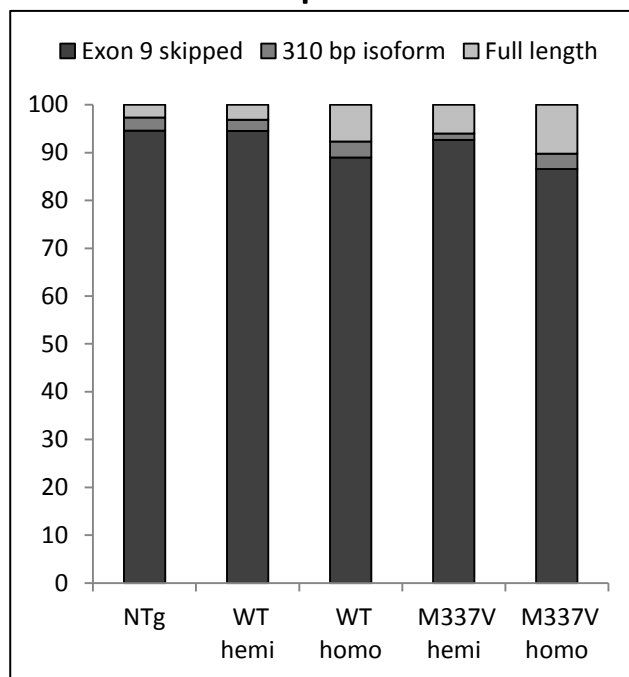
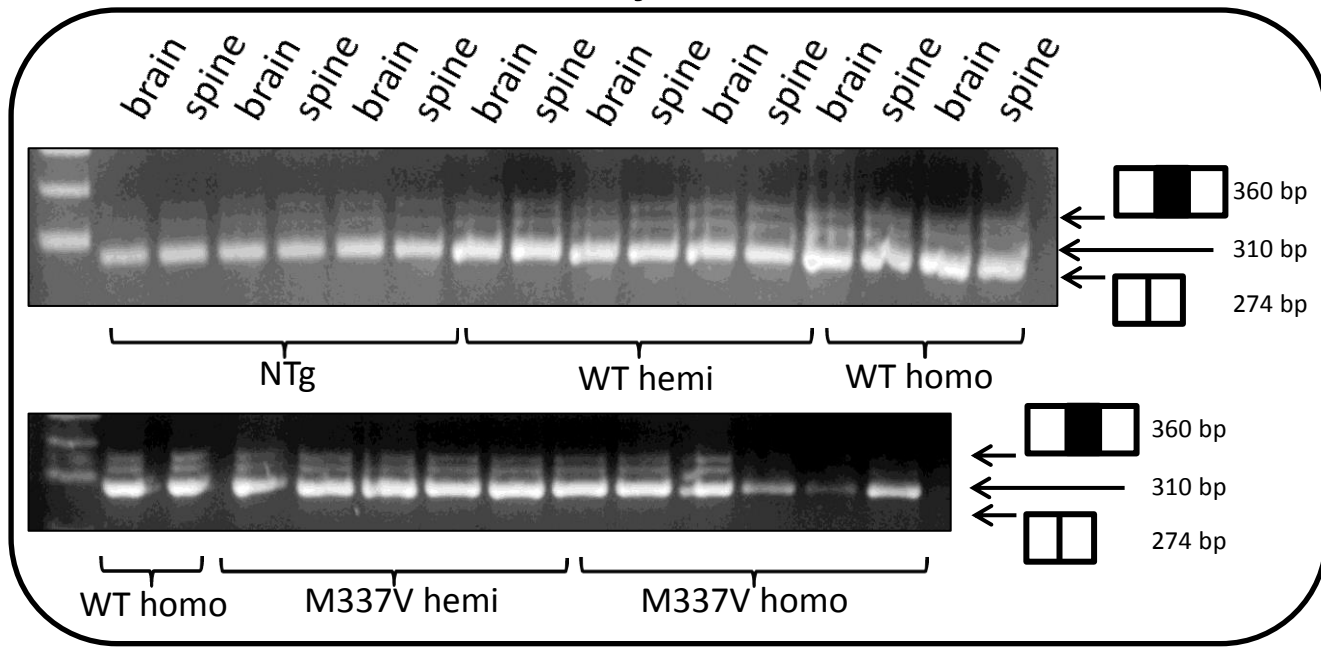
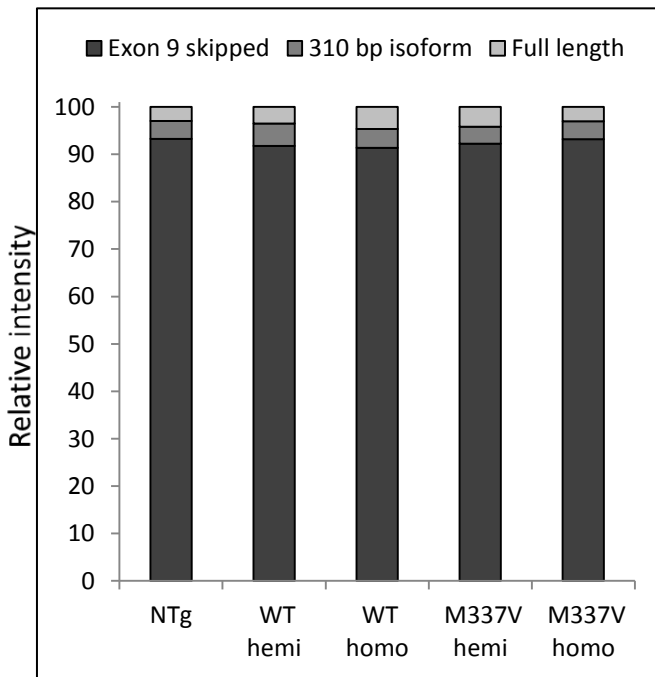


Figure 5.5i) Analysis of alternative splicing of Dnajc5 in the TDP-43 BAC transgenic mice at 3 months, using primers to detect exon 9 inclusion. Three products were formed, with no significant differences between the lines.

Dnajc5



Brain



Spine

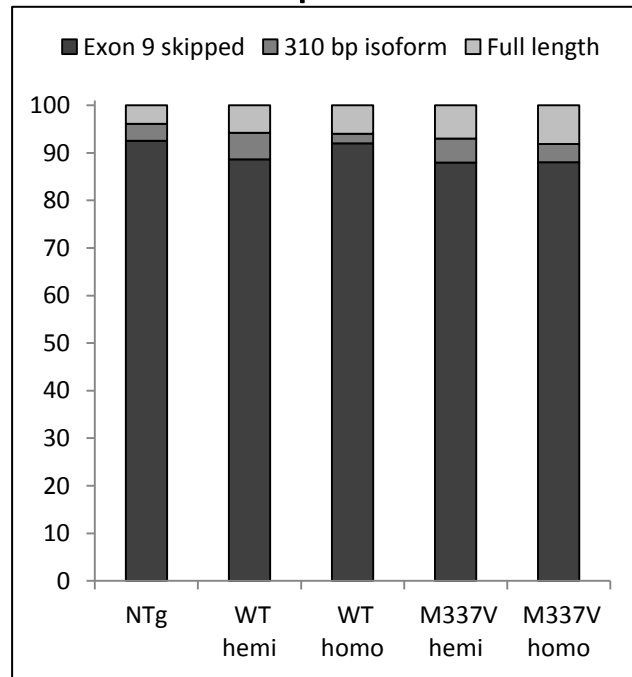
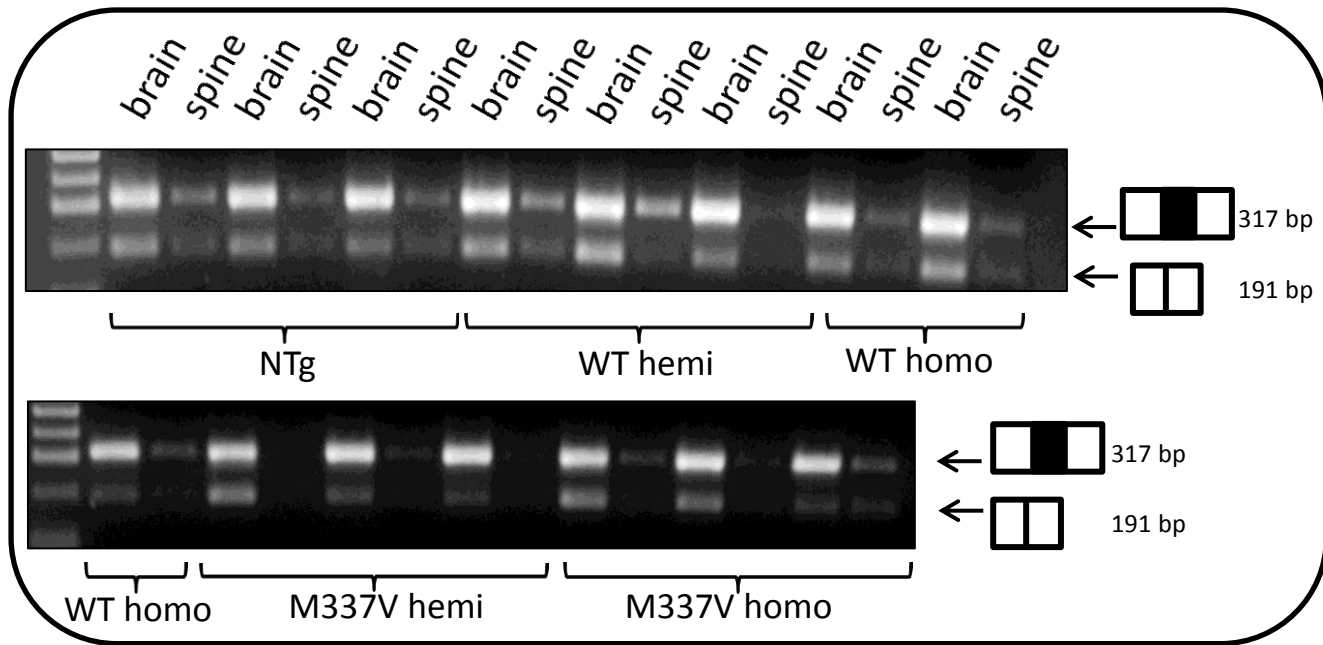
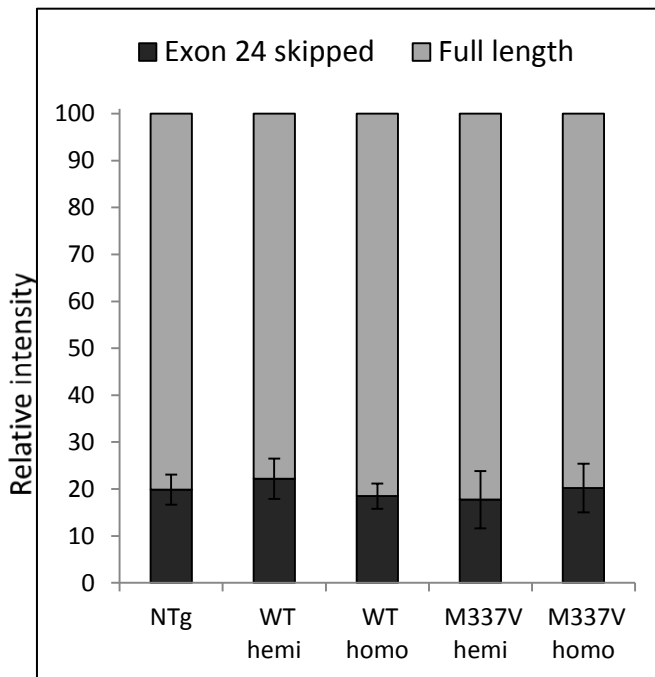


Figure 5.5j) Analysis of alternative splicing of Dnajc5 in the TDP-43 BAC transgenic mice at 9 months, using primers to detect exon 9 inclusion. Three products were formed, with no significant differences between the lines.

Ptk2b



Brain



Spine

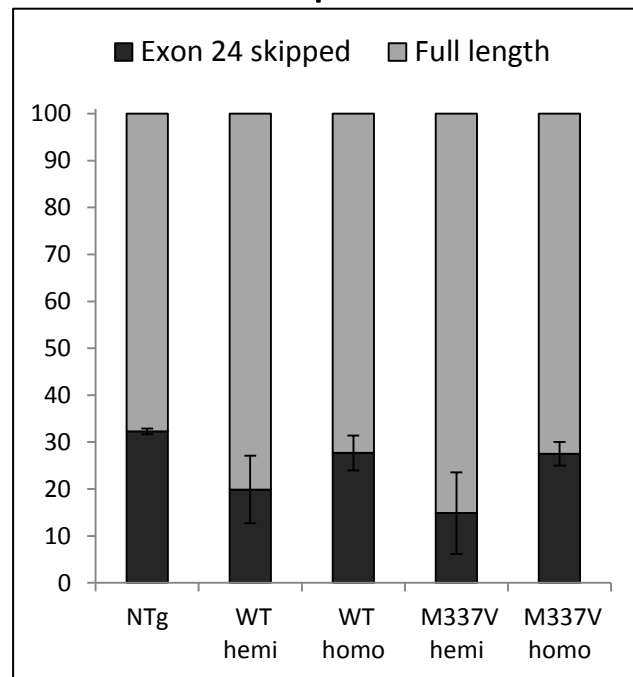
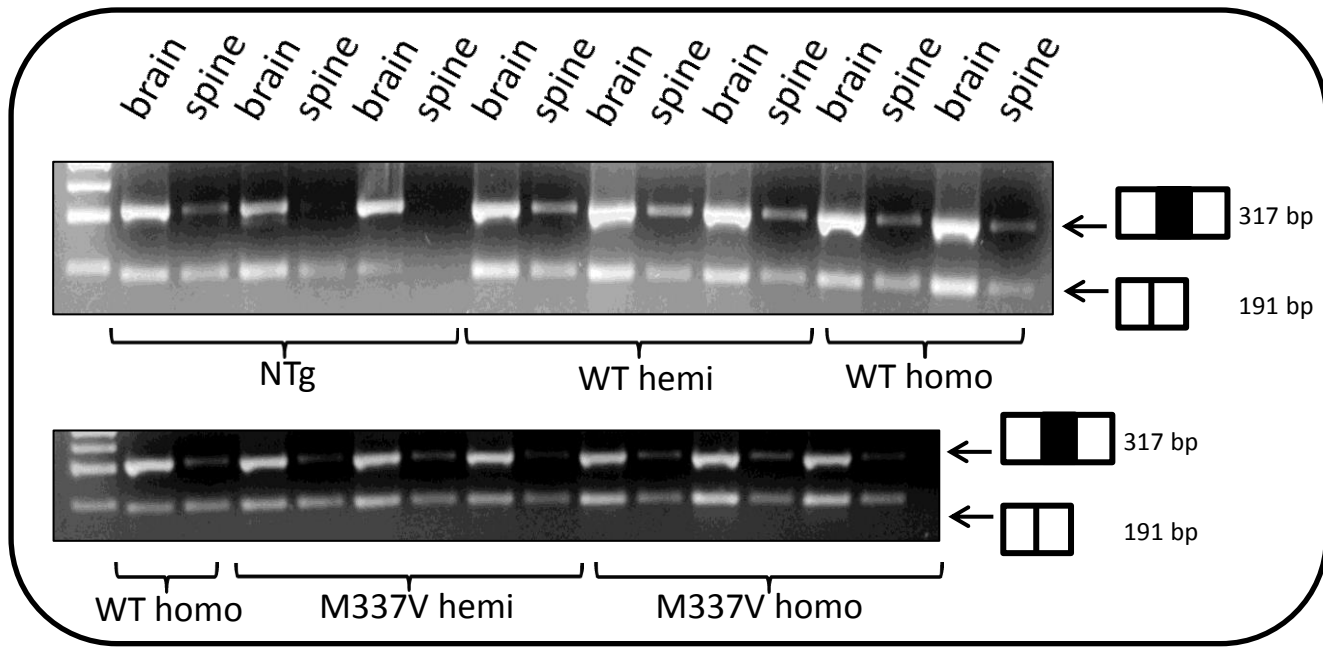
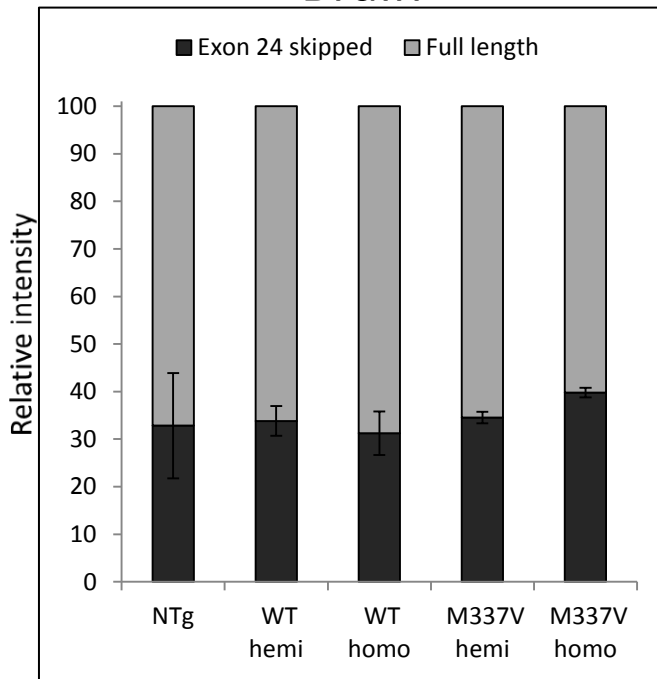


Figure 5.5k) Analysis of alternative splicing of Ptk2b in the TDP-43 BAC transgenic mice at 3 months, using primers to detect exon 24 inclusion. There are no significant differences between the lines.

Ptk2b



Brain



Spine

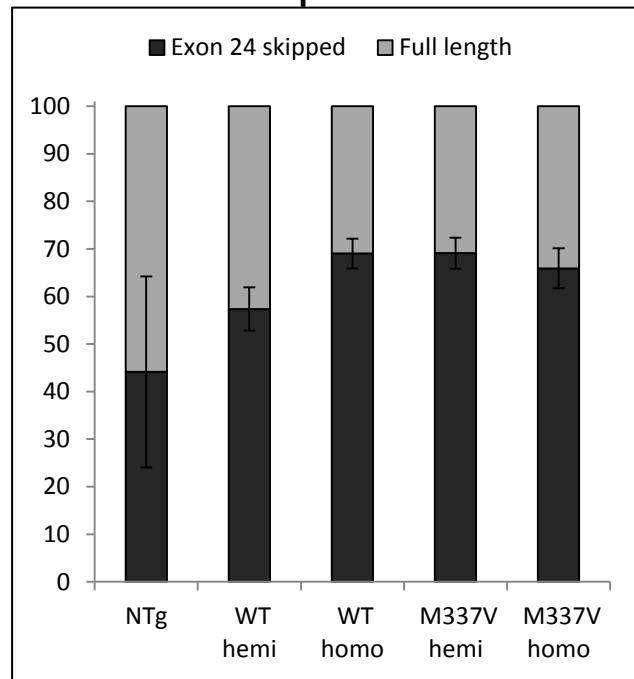
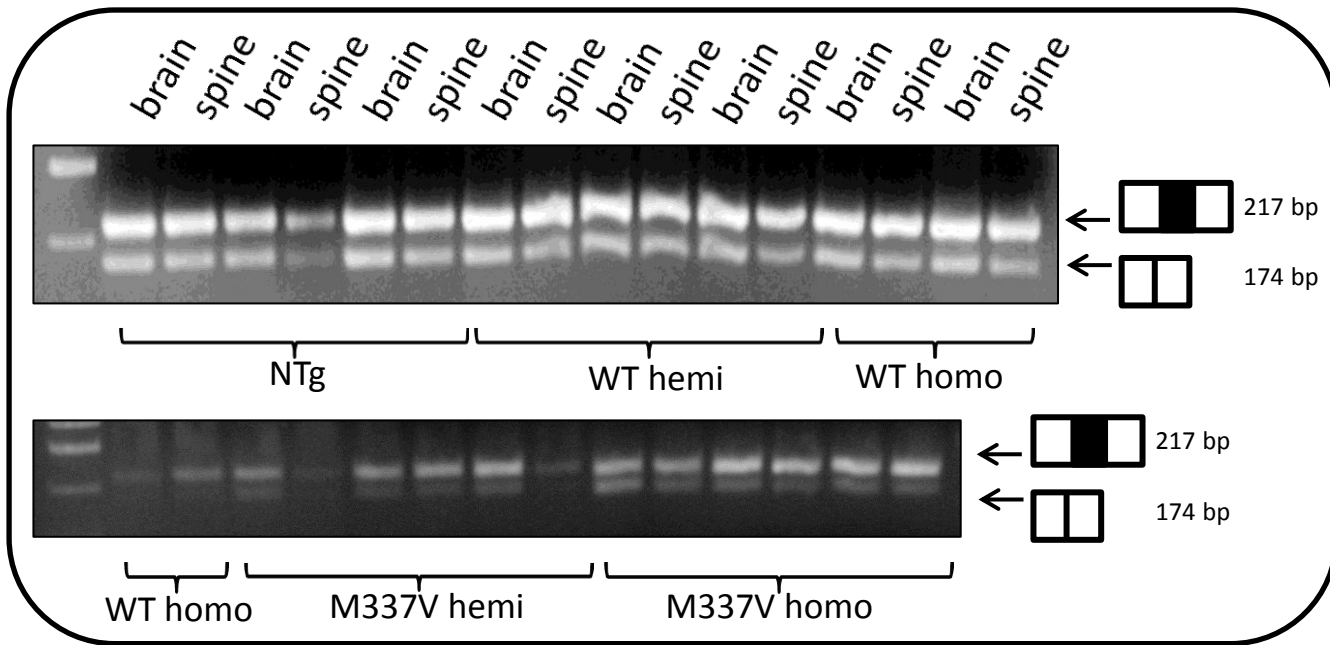
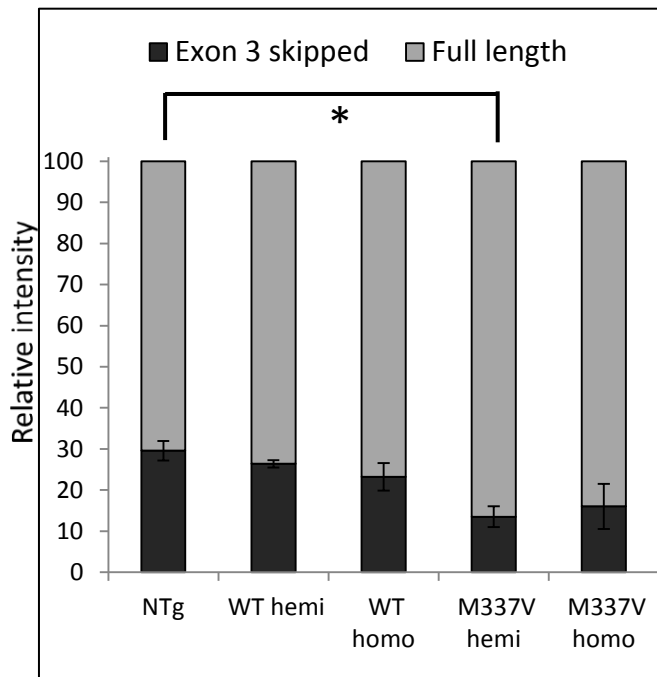


Figure 5.5I) Analysis of alternative splicing of Ptk2b in the TDP-43 BAC transgenic mice at 9 months, using primers to detect exon 24 inclusion. There are no significant differences between the lines.

Ttc3



Brain



Spine

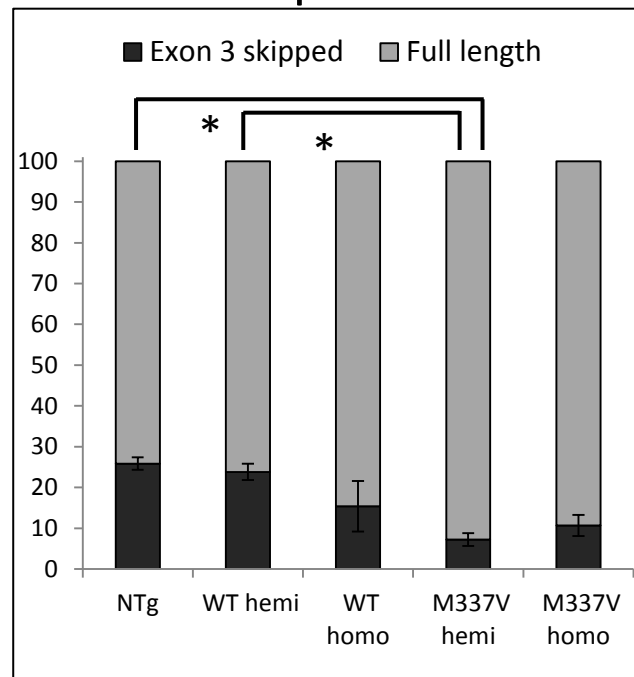
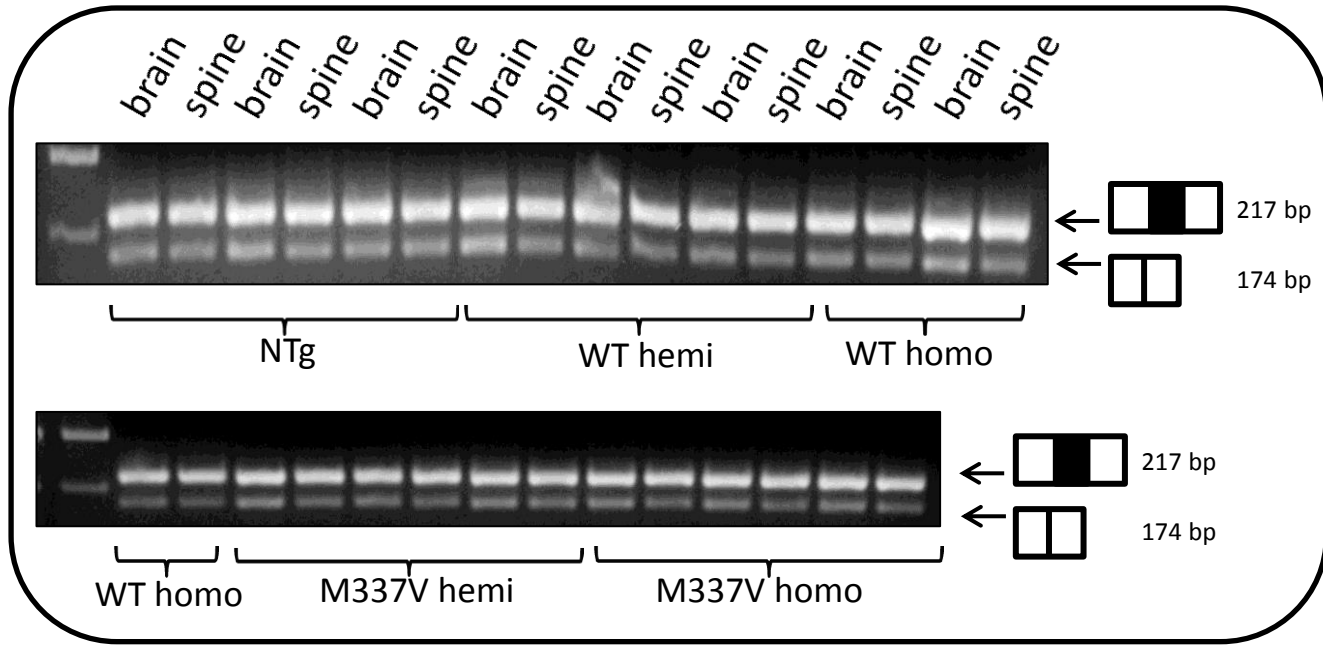
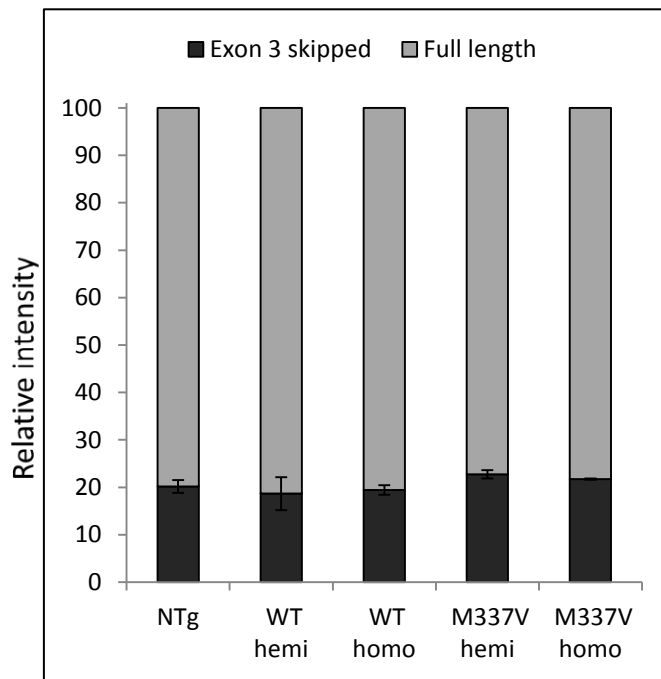


Figure 5.5m) Analysis of alternative splicing of *Ttc3* in the TDP-43 BAC transgenic mice at 3 months, using primers to detect exon 3 inclusion. There is a trend towards an increase the proportion of full length transcript in the mutant lines, reaching statistical significance in the M337V hemizygous lines where indicated.

Ttc3



Brain



Spine

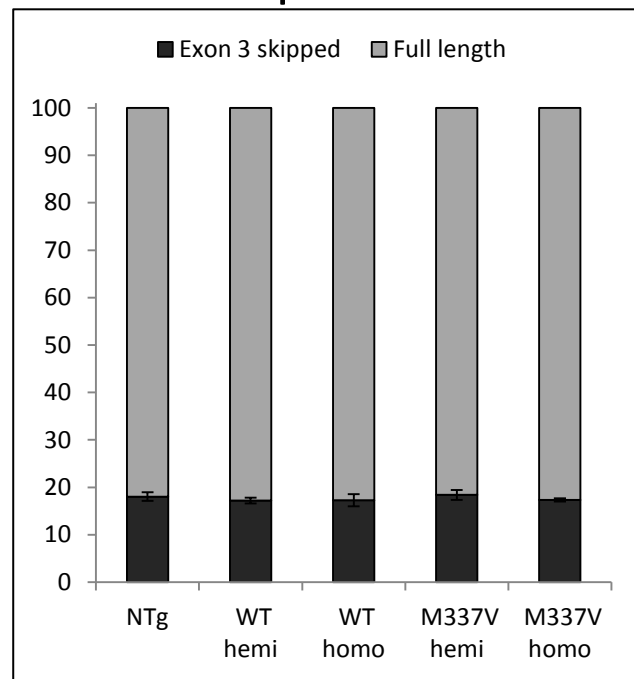
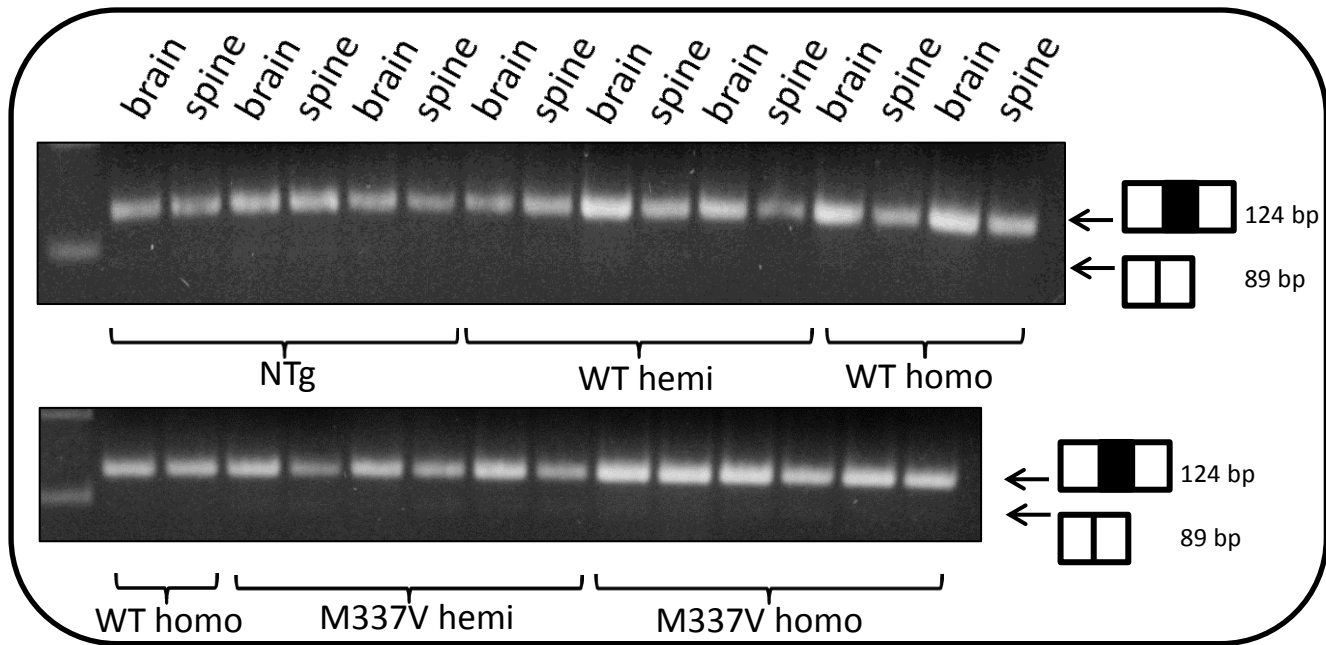
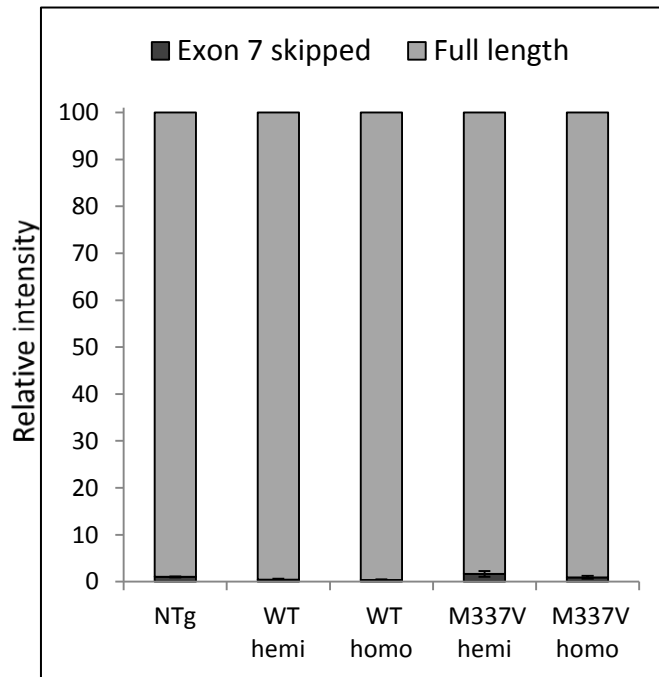


Figure 5.5n) Analysis of alternative splicing of Ttc3 in the TDP-43 BAC transgenic mice at 9 months, using primers to detect exon 3 inclusion. There are no significant differences between the lines.

FUS



Brain



Spine

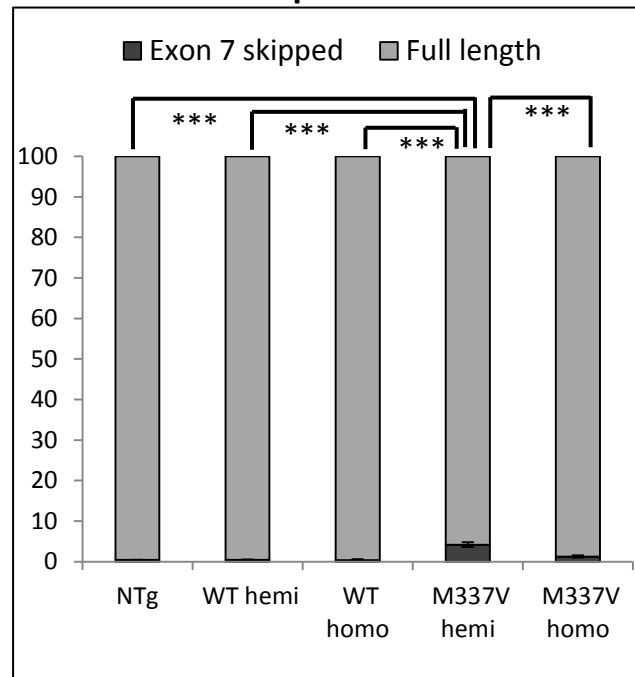
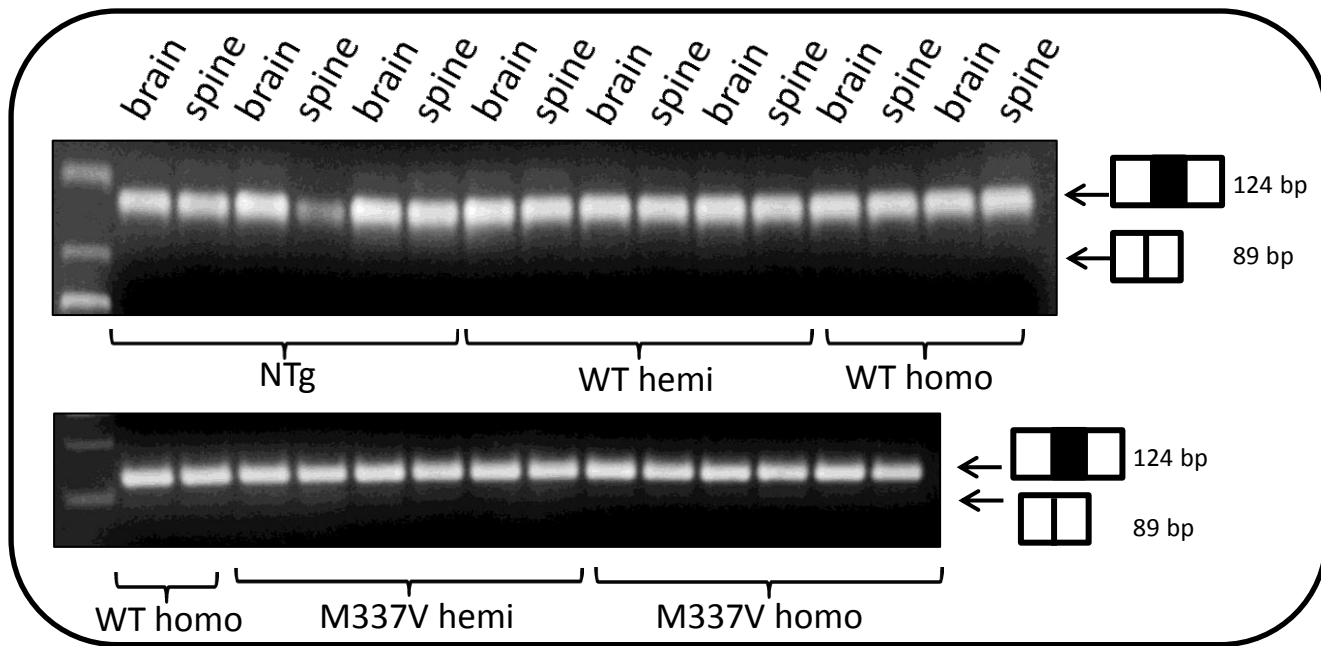
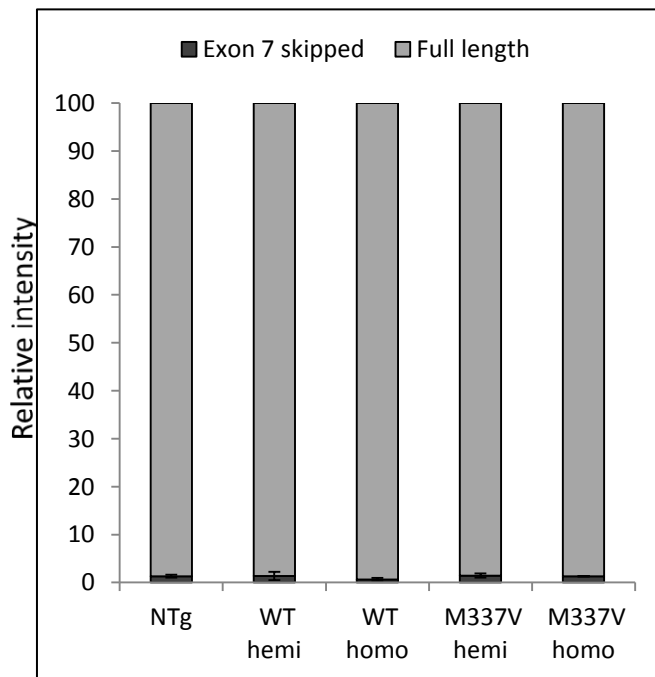


Figure 5.5o) Analysis of alternative splicing of FUS in the TDP-43 BAC transgenic mice at 3 months, using primers to detect exon 7 inclusion. There is a significant increase in the proportion of exon 7 skipped transcript in the M337V hemizygous line compared all other lines in the spine, but no significant differences in the brain.

FUS



Brain



Spine

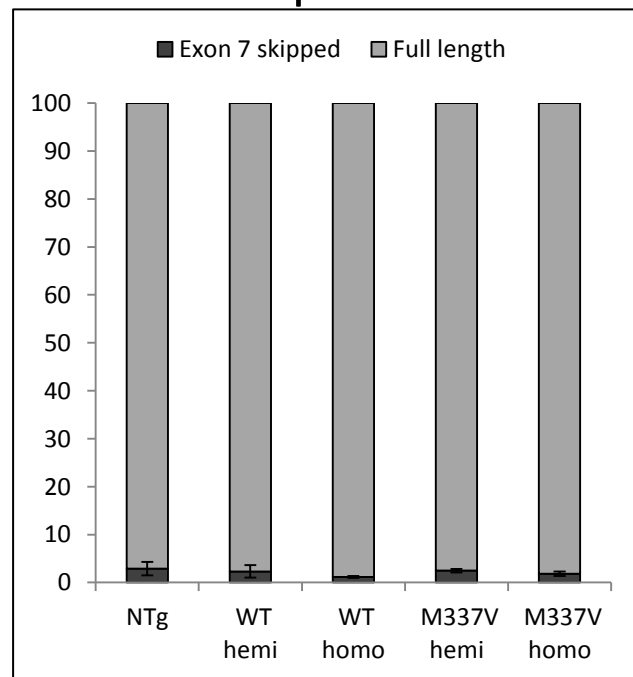


Figure 5.5p) Analysis of alternative splicing of FUS in the TDP-43 BAC transgenic mice at 9 months, using primers to detect exon 7 inclusion. There are no significant differences between the lines.

Primers were also used to detect the relative abundance of full length and exon 7 skipped FUS mRNA (109). An increase in exon 7 skipped variant was observed in the spine of the M337V hemizygous tissue (figure 5.5o and 5.5p). However, the amount of this product was very small, potentially due to its rapid degradation, so it is difficult to draw firm conclusions from this result.

5.4.2 Splicing in the FUS BAC transgenic mouse model

CNS tissue from the FUS BAC transgenic mouse model was used to examine alterations in splicing in the same targets as examined in the TDP-43 BAC transgenic model. These were pooled by genotype (figure 5.6) and also by individual line (appendix 7.10). *Ndr2*, *mag*, and *ppp3ca* showed no difference when the splicing of each was compared between the genotypes (figures 5.6a, c and d, respectively). *Dnajc5* showed a reduction in the exon 9 skipped variant in the presence of the mutation (figure 5.6e). However, the larger products were present in very small amounts, so it is difficult to make firm conclusions from this. *TIA1* showed an increase in the full length product in the P525L compared to non-transgenic tissue (figure 5.6b), which reflects the published observation in FUS knockdown tissue (119). *Ptk2b* and *ttc3* have each been reported to show a reduction in full length with FUS knockdown. Interestingly, this pattern was also observed in the FUS transgenic lines, particularly in *ttc3* (figure 5.6g). These differences are small but significant, and again may suggest some subtle splicing changes in the FUS transgenic mouse model.

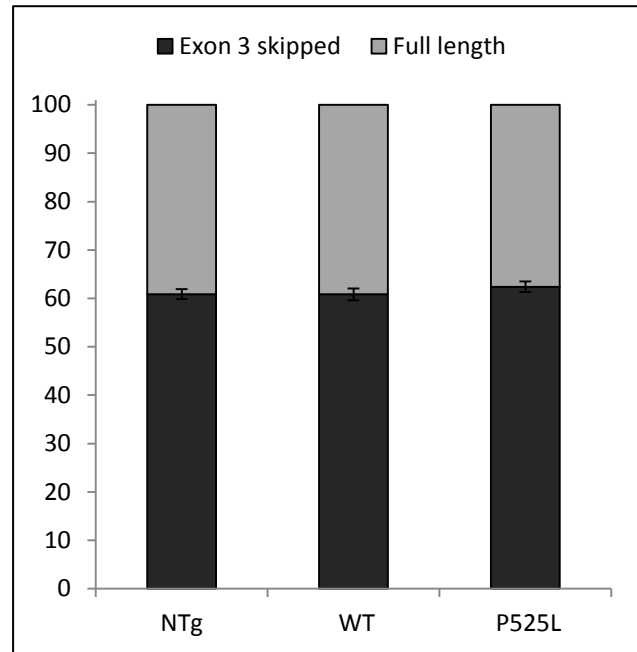
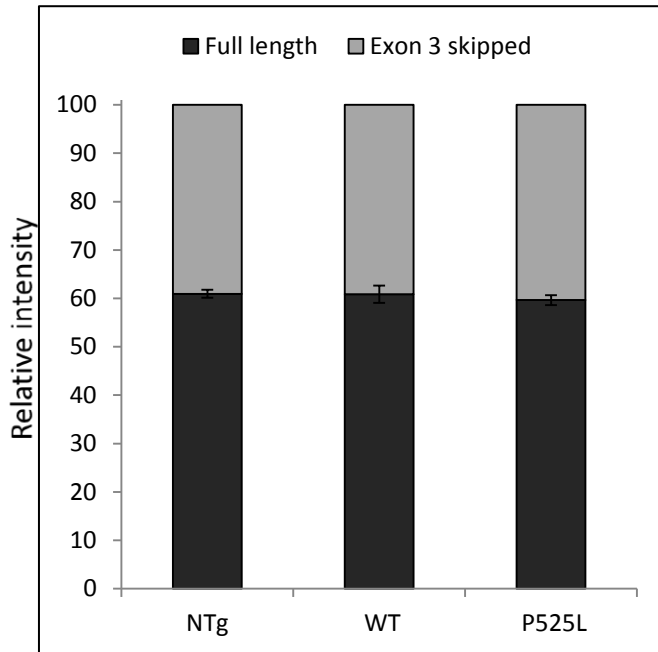
	FUS Knockdown	FUS BAC		
	Brain	Cortex	Cerebellum	Spine
Ndr2	↓ full in KD	→	→	→
TIA1	↑ full in KD	→	→	→
Mag	↑ full in KD	→	→	→
Ppp3ca	→	→	→	→
Dnajc5	→	→	→	↑ full in P525L
Ptk2b	↓ full in KD	→	→	→
Ttc3	↓ full in KD	(↑) full in P525L	→	→
FUS		→	→	(↓) full in P525L

Table 5.4: Summary of the effects of the P525L mutation on the relative abundance of the full length splice variants in the FUS BAC transgenic model, compared to wild-type. The effects in the ‘knockdown’ column are taken from Lagier-Tourenne et al, 2012.

Cortex

Ndr2

Cerebellum



Spine

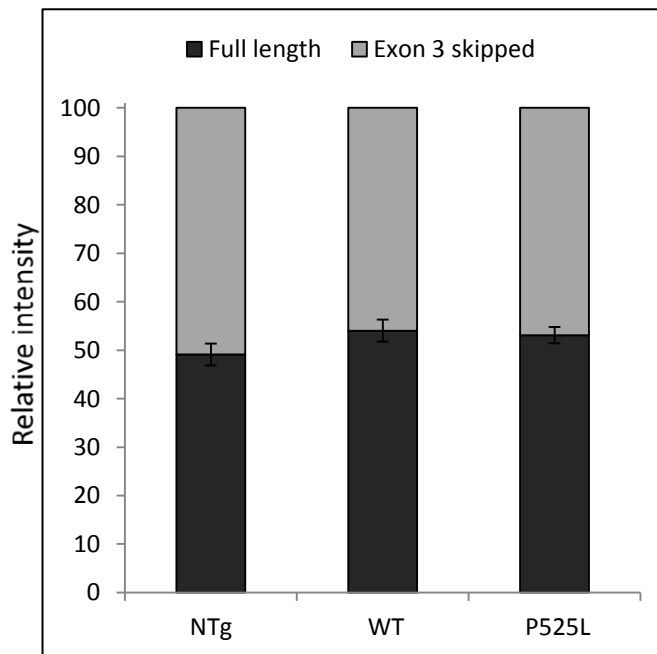
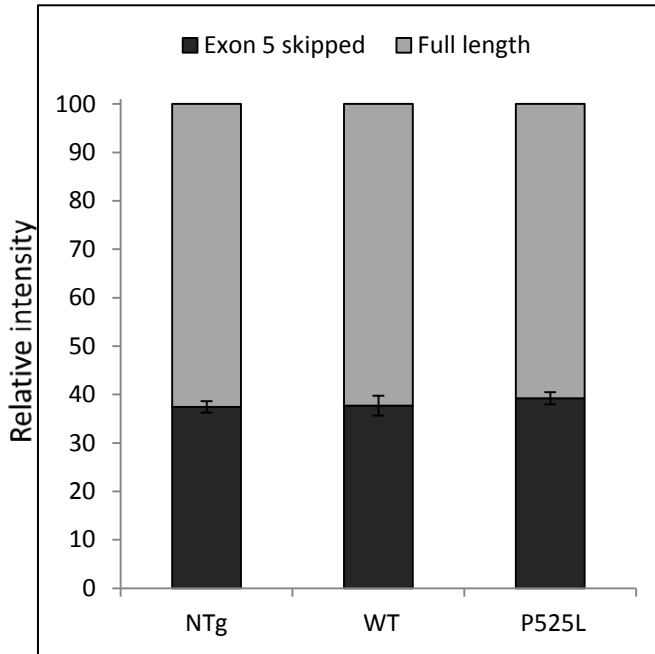


Figure 5.6. Analysis of alternative splicing in the FUS BAC transgenic mouse model using semi-quantitative PCR. Results are expressed by genotype. (NTg = non-transgenic, WT = wild-type. n=2xNTg, 5xWT, 10xP525L. One-way ANOVA with Tukey post-test. *=p<0.05, **=p<0.01, ***=p<0.001)

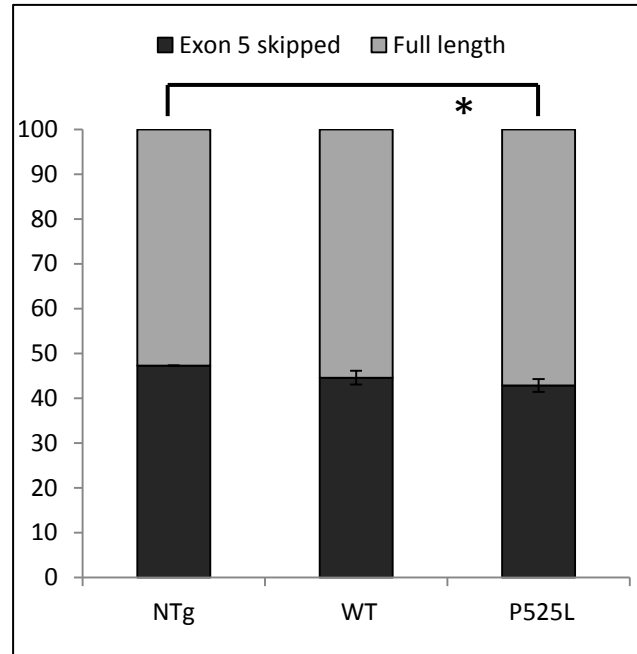
a) Analysis of alternative splicing of Ndr2 from FUS BAC transgenic mice, using primers to detect exon 3 inclusion. There are no significant differences between the lines.

TIA1

Cortex



Cerebellum



Spine

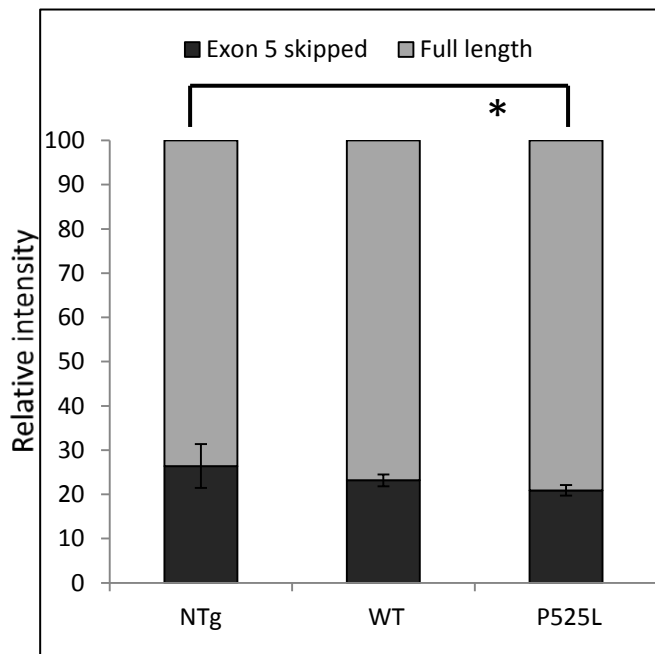
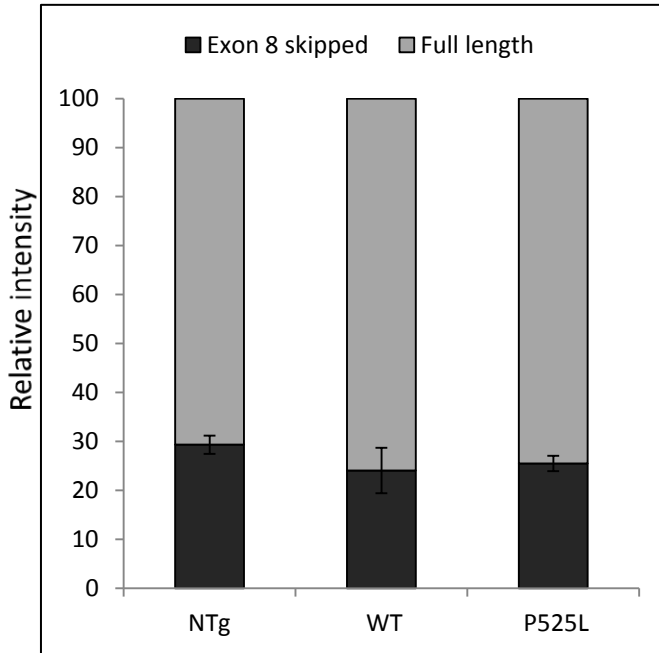


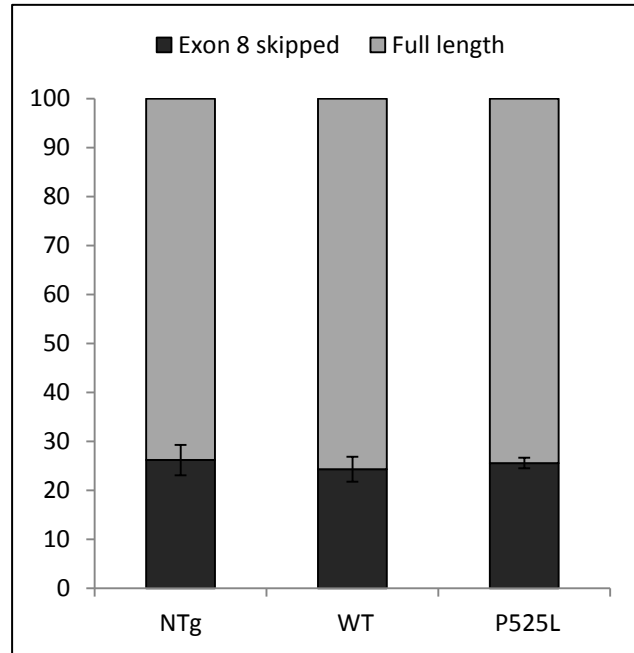
Figure 5.6b) Analysis of alternative splicing of TIA1 in the FUS BAC transgenic mice, using primers to detect exon 5 inclusion. There is a significant increase in full length product in the mutant lines compared to non-transgenic in the cerebellum and spine.

Mag

Cortex



Cerebellum



Spine

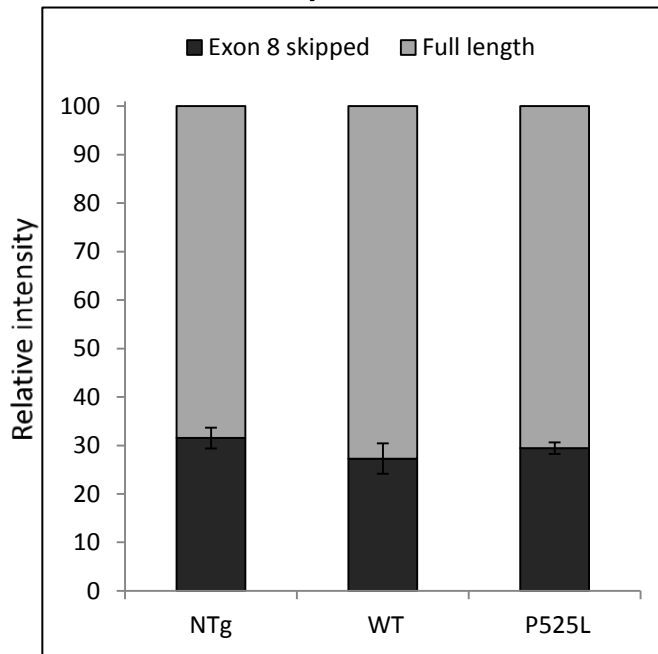
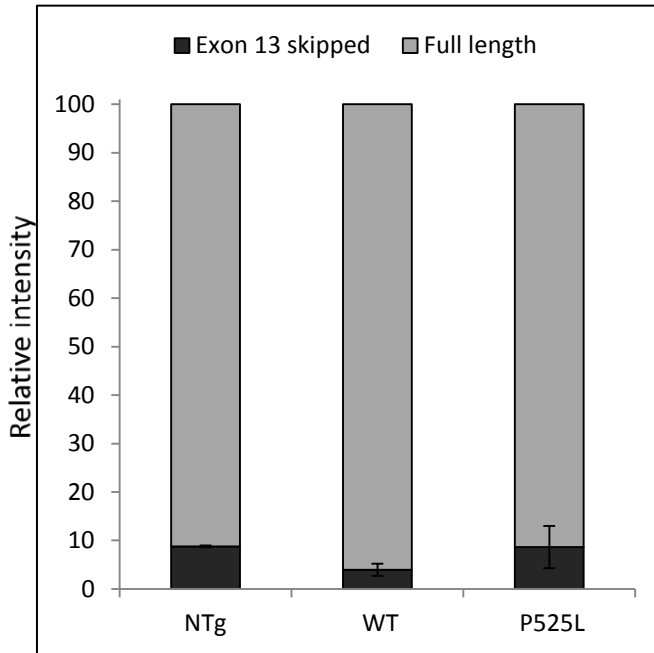


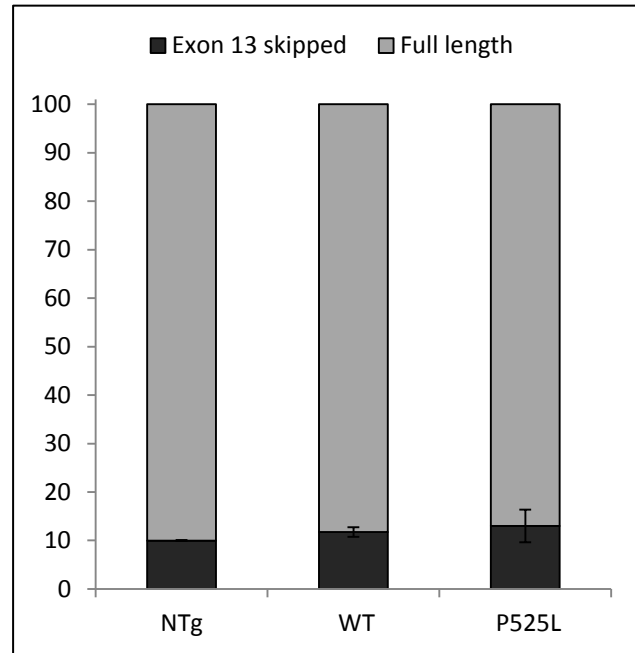
Figure 5.6c) Analysis of alternative splicing of Mag in the FUS BAC transgenic mice, using primers to detect exon 8 inclusion. There are no significant differences between the lines.

Ppp3ca

Cortex



Cerebellum



Spine

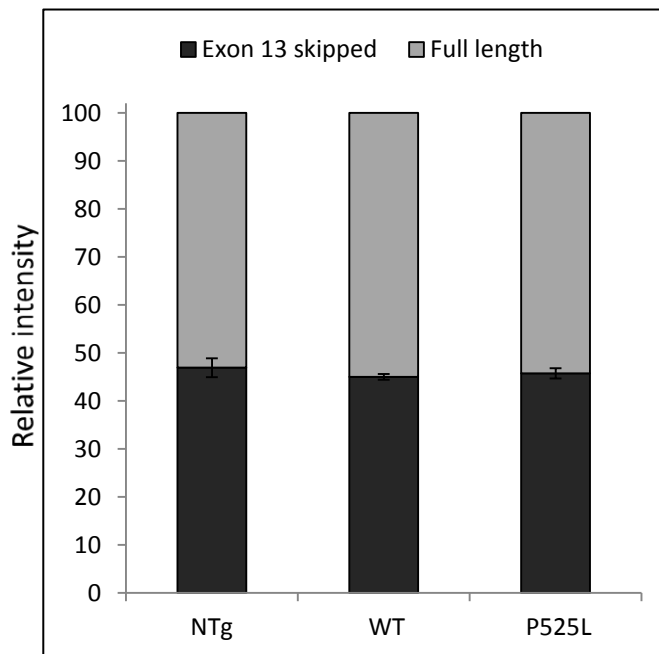
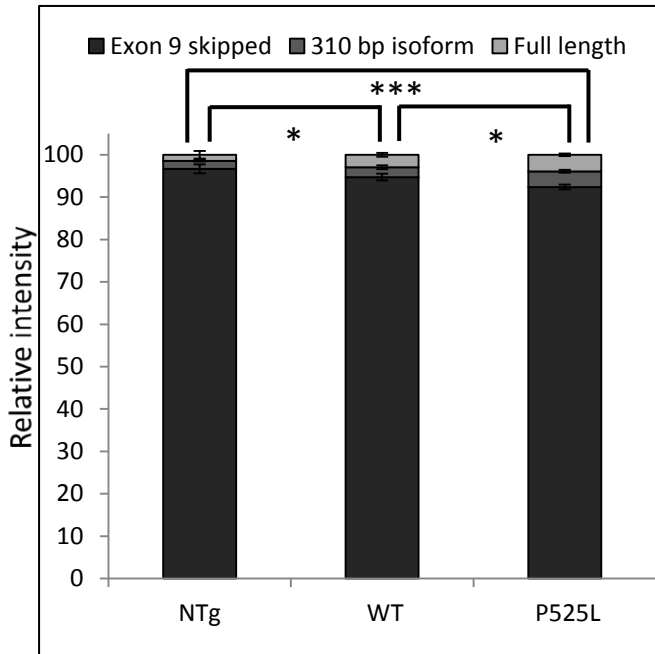


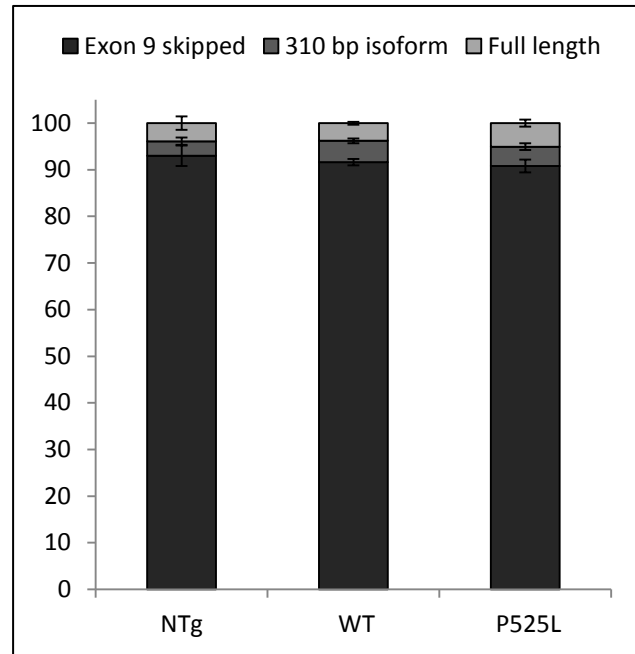
Figure 5.6d) Analysis of alternative splicing of Ppp3ca in the FUS BAC transgenic mice, using primers to detect exon 13 inclusion. There are no significant differences between the lines.

Dnajc5

Cortex



Cerebellum



Spine

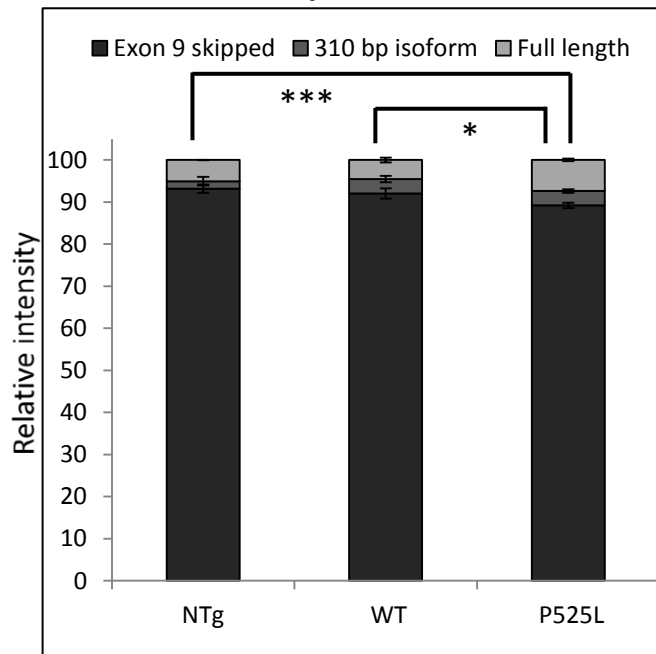
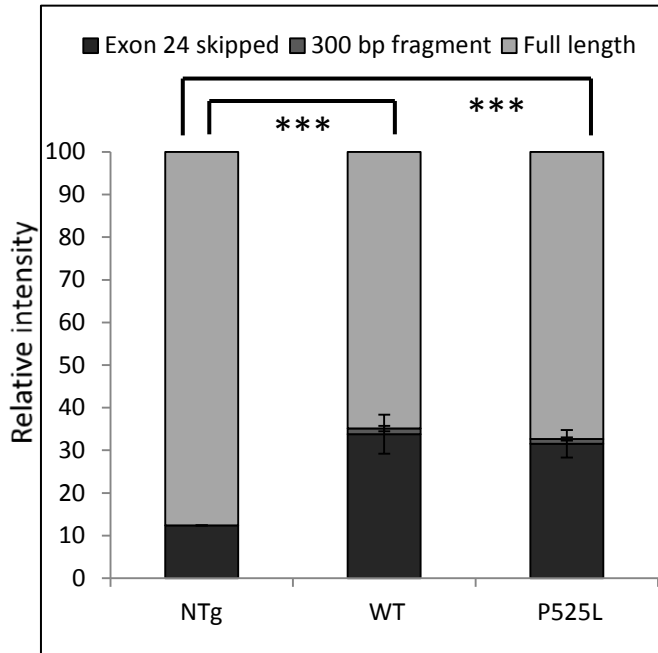


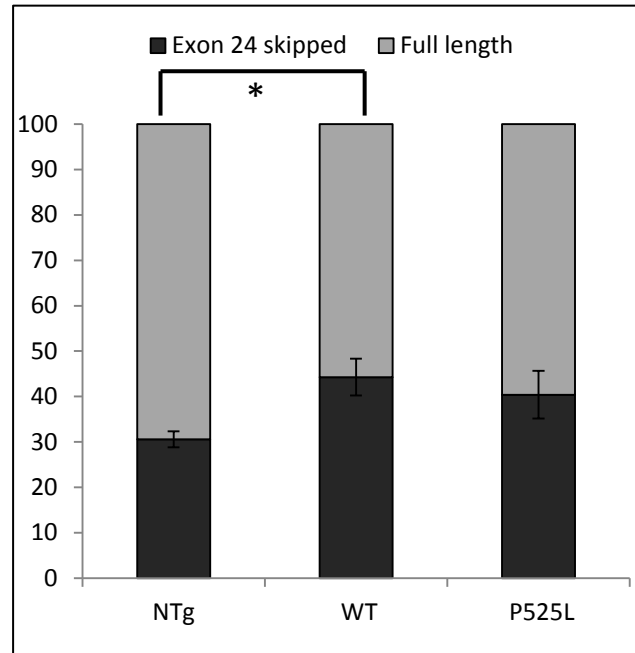
Figure 5.6e) Analysis of alternative splicing of Dnajc5 in the FUS BAC transgenic mice, using primers to detect exon 9 inclusion. There are three products detected. Statistically significant differences in the exon 9 skipped variant are illustrated. (Other statistics: Cerebellum: Nil significant. Cortex: Full length NTg vs WT $p < 0.01$, NTg vs P525L $p < 0.001$, WT vs P525L $p > 0.05$. 310 bp isoform: NTg vs WT $p > 0.05$, NTg vs P525L $p < 0.001$, WT vs P525L $p < 0.01$. Spine: Full length NTg vs WT $p > 0.05$, NTg vs P525L $p < 0.001$, WT vs P525L $p < 0.001$. 310 bp isoform NTg vs WT $p < 0.05$, NTg vs P525L $p < 0.05$, WT vs P525L $p > 0.05$.)

Ptk2b

Cortex



Cerebellum



Spine

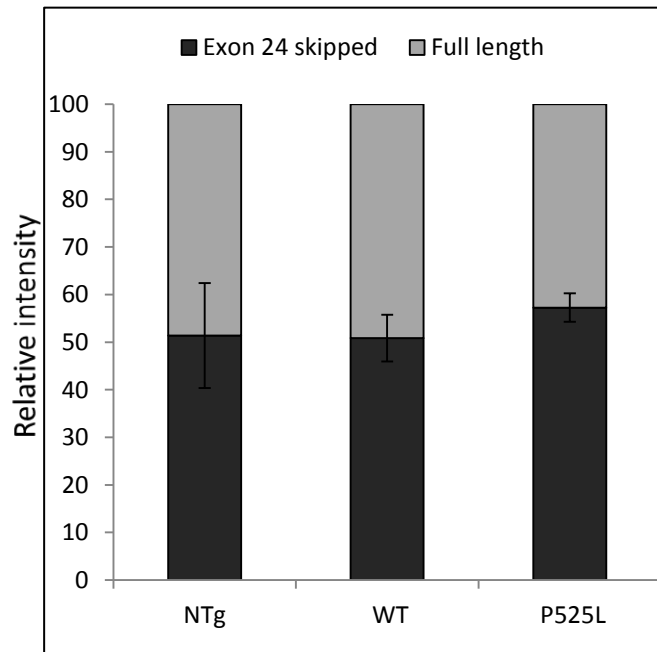
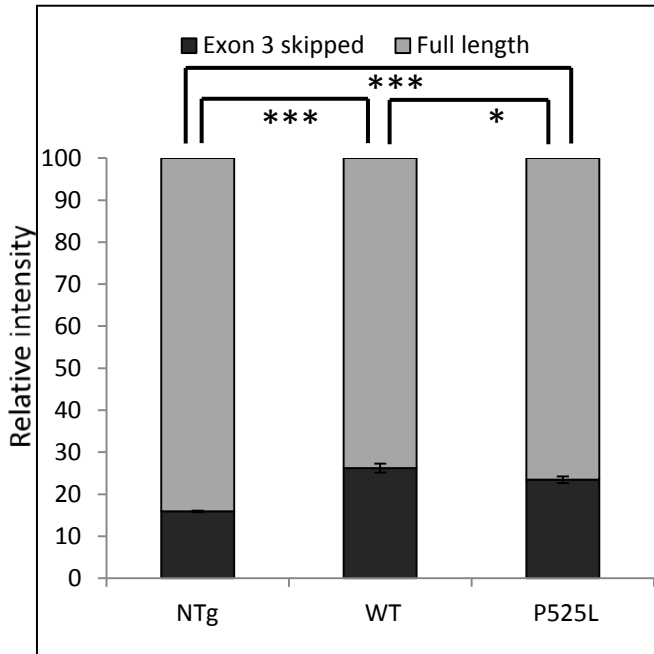


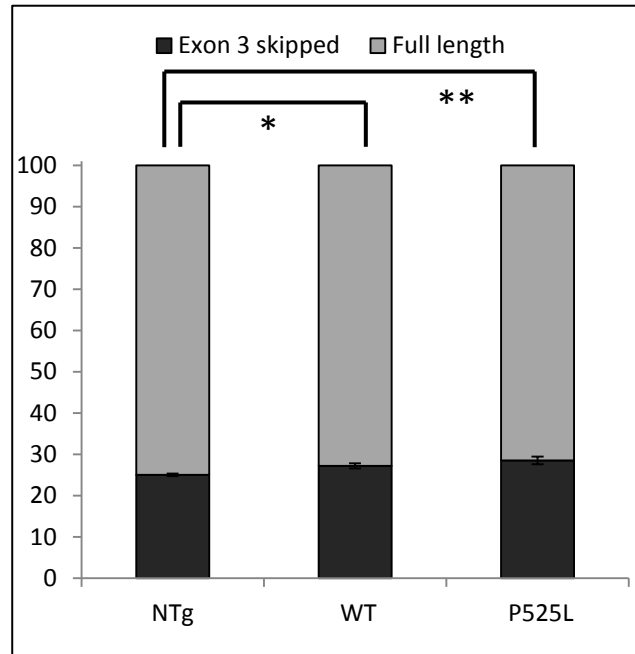
Figure 5.6f) Analysis of alternative splicing of Ptk2b in the FUS BAC transgenic mice, using primers to detect exon 24 inclusion. Three products were detected in the cortex. There is a significant reduction in full length product in the transgenic lines as illustrated.

Ttc3

Cortex



Cerebellum



Spine

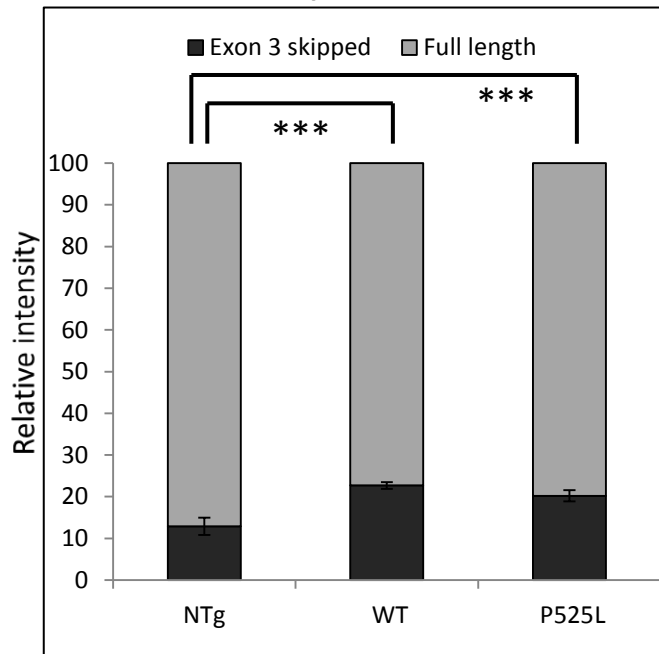
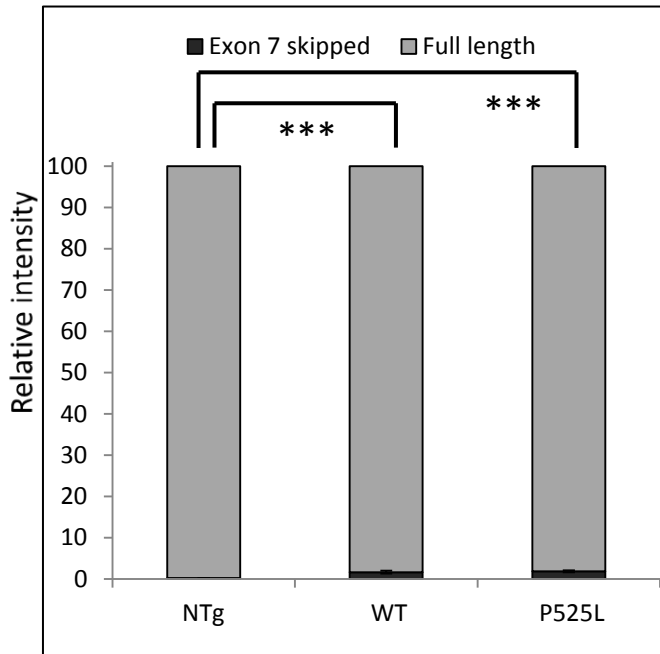


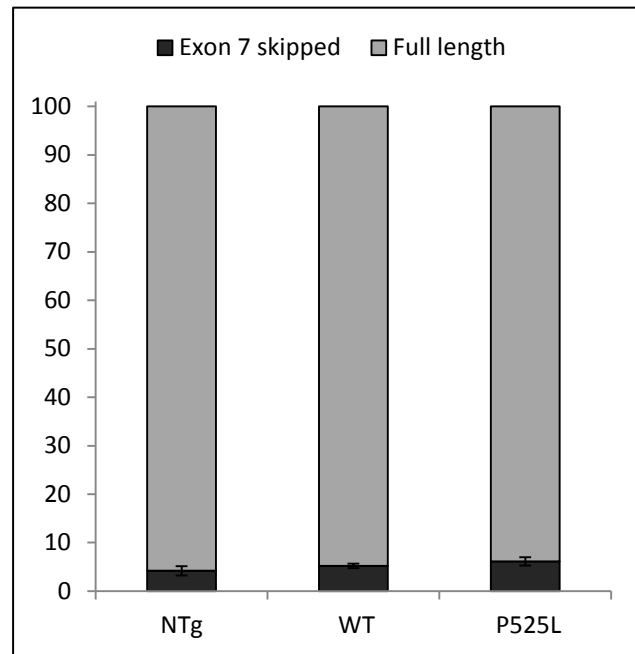
Figure 5.6g) Analysis of alternative splicing of Ttc3 in the FUS BAC transgenic mice, using primers to detect exon 3 inclusion. There is a significant reduction in full length product in the transgenic lines compared to non-transgenic in all tissue.

FUS

Cortex



Cerebellum



Spine

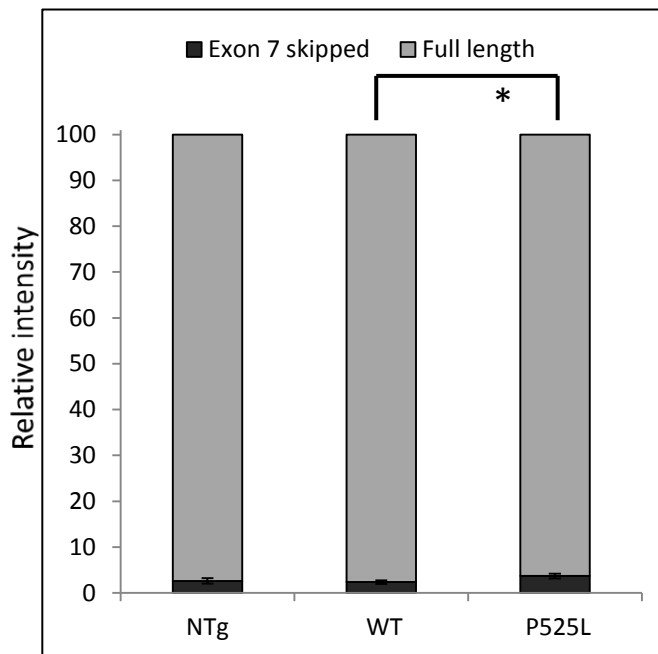


Figure 5.6h) Analysis of alternative splicing of FUS in the FUS BAC transgenic mice. There was a small amount of exon 7-skipped product detected, with statistically significant differences between the lines illustrated.

Summary of findings

TDP-43 BAC transgenic mouse model

- 1.The expression of human TDP-43 is proportional to the number of BAC copies present.
- 2.There is a reduction in total TDP-43 at 3 months and an increase in 9 months in the M337V mutant lines. This suggests that the mutation affects TDP-43 autoregulation.
- 3.FUS and SMN also show changes in mRNA levels in the presence of the M337V mutation, suggesting that this mutation has more widespread effects on RNA processing.
- 4.There is a difference in mRNA levels between the 3 month pre-symptomatic time point and the 9 month time point where the mouse develops detectable abnormalities in motor function.
- 5.There are some subtle splicing defects, for example in Ndr2. However, these are not widespread in the model at the 3 and 9 month time points, suggesting that they may not be an early event in motor neuron dysfunction.

FUS BAC transgenic mouse model

- 6.The levels of total FUS mRNA reflect the number of copies of the BAC construct present in each transgenic line.
- 7.Endogenous FUS levels differ between both non-transgenic and wild-type lines and wild-type compared to P525L-FUS lines. This suggests that the transgene itself alters FUS autoregulation, but that the presence of the mutation has a further effect.

8. TDP-43 and SMN mRNA levels are also significantly different in the wild-type compared to P525L-FUS lines, suggesting that the mutation has more widespread effects on the transcription of other important mRNA species.
9. Some subtle splicing changes are observed. However, these are not widespread and suggest that altered splicing may not be significantly affected by the P525L-mutation at this stage.

5.5 Discussion

5.5.1 Transcript levels in the TDP-43 BAC transgenic mouse model

TDP-43 has been shown to undergo autoregulation at the transcriptional level, with a reduction in TDP-43 mRNA when expression of TDP-43 protein is elevated over a certain threshold. Several studies have investigated the mechanism for this, and the most likely appears to be via binding of TDP-43 to the 3'UTR of its own transcript, creating an alternative poly(A) tail that prevents nuclear export (156-158). These results show human TDP-43 expression occurs relative to the number of BAC copies present, as demonstrated by the increase in homozygous lines compared to hemizygous (figure 5.2a). This suggests that transcription occurs in proportion to the number of copies of the gene. This does not in itself rule out an autoregulatory process, and is compatible with the hypothesis that autoregulation occurs at the transcriptional level.

This becomes more interesting when examining the expression of total TDP-43, consisting of both endogenous mouse and human mRNA. At the 3 month time point, levels of TDP-43 in non-transgenic and wild-type BAC lines are stable. This is consistent with an autoregulatory process to retain stable transcript levels despite the presence of additional TDP-43 genes. However, the mutant lines appear to show increased transcript levels. This reaches statistical significance in the M337V homozygous line compared with wild-type hemizygous ($p < 0.05$). This suggests that the autoregulatory mechanism may be affected by the presence of the M337V mutation. This 3 month time point is pre-symptomatic, so this could be a very early change.

At 9 months, there was a reduction in TDP-43 transcript levels in all transgenic lines, reaching significance in the cortex in wild-type homozygous and both M337V mutant lines compared to non-transgenic ($p < 0.05$). This displays the reverse trend to that at 3 months, and is interesting as total transcript levels are lower despite the presence of human TDP-43 mRNA as well as endogenous. One hypothesis for the mechanism behind this observation is that it is due to the accumulation of mutant protein, suppressing transcript levels by autoregulation. However, the autoregulatory process is likely to occur predominantly in the nucleus, so the mislocalisation of mutant TDP-43 to the cytoplasm would go against this theory (15, 158). Defects in autoregulation itself may explain the reduced transcript levels, for example by altered binding of M337V TDP-43 protein to its mRNA 3'UTR, increasing transcript suppression. Amino acids 321-366 of the C-terminal region appear to be important in autoregulation, which is the region containing the M337V mutation (156). Altered RNA binding has also been demonstrated in the cortices of patients with TDP-43 FTD (162). However, other experiments have failed to show defective RNA binding in the presence of TDP-43 mutations (156). Further studies are therefore required to further study the effects of TDP-43 mutations on autoregulation.

The pattern of FUS and SMN mRNA expression are similar in the TDP-43 BAC transgenic mouse, and both demonstrate a trend towards an increase at the 3 month time point and decrease at 9 months in the mutant lines compared to non-transgenic. There is also a decrease in the wild-type lines at 9 months (figures 5.2c&d). These are general trends with some values reaching significance, and conclusions must therefore be drawn with caution. However, there is evidence that TDP-43 is able to bind to various mRNA species and alter transcript levels, for example neurofilament and CFTR (153, 160). Indeed, there is evidence that TDP-43 can bind to SMN pre-

mRNA and increase full length SMN transcript (89). It has also been shown that TDP-43 can bind to FUS mRNA at the 3'UTR and introns 6 and 7, with TDP-43 knockdown reducing levels of FUS mRNA and protein (157). Given that these reports suggest that increased levels of TDP-43 are associated with increased FUS and SMN proteins, it is reasonable to suggest that alterations in FUS and SMN mRNA levels are as a result of the altered levels of total TDP-43 transcript described in figure 5.2b. However, there may also be other regulatory factors that occur as a result of the mutation.

EWS and TAF15 mRNA levels are relatively stable between each line. TDP-43 has binding sites on each of these mRNAs, and there is evidence that TDP-43 knockdown may cause downregulation of TAF15 (157). This could explain the subtle changes seen in these two mRNAs, though there is not sufficient evidence from these results to suggest that EWS and TAF15 mRNA levels are significantly altered in these transgenic mice.

It is interesting to note the difference in the pattern of mRNA expression between 3 and 9 months. This may reflect the fact that the 3 month time point is pre-symptomatic in the mouse model (Gordon et al, in preparation). Subtle changes at this point, such as increases in total TDP-43, FUS and SMN, may reflect early cellular dysfunction. The changes observed are also more subtle and only present in the mutant model, which could reflect specific effects of the mutation. The 9 month time point reflects an early symptomatic stage. At this point there may be more widespread cellular dysfunction, secondary effects of neuronal degeneration and changes in non-neuronal cells. Effects at this point are also seen with the wild-type model so are not specific to the mutation.

The fact that there are some changes in the wild-type TDP-43 model is likely to reflect the presence of human TDP-43. Interestingly, human TDP-43 in this model is unable to compensate for the loss of endogenous TDP-43 in a knockout model, resulting in the death of endogenous knockout embryos even in the presence of the human TDP-43 transgene (Gordon et al, in preparation). This suggests that human TDP-43 in this model is not expressed in the same way as endogenous, or differs physiologically such that it is unable to rescue the knockout model. In addition, the behavioural phenotype of wild-type TDP-43 lines also show a mild version of the phenotype described in the mutant lines. Indeed, there is frequently an increase in the extent of the observed behavioural phenotype going in turn from wild-type hemizygous to wild-type homozygous, M337V hemizygous, and M337V homozygous (Gordon et al, in preparation). A reflection of this pattern in the results here corroborates the fact that there may be increasing dysfunction with each of these lines.

Findings in the spinal cord and brain are closely matched, which strengthens the results. However, there is more variability in the spinal cord samples so fewer values reach statistical significance. This is likely to reflect the greater variability in tissue in the spinal cord samples, which are much smaller and therefore may become contaminated with other spinal cord regions or small amounts of surrounding tissue.

5.5.2 Protein levels in the TDP-43 BAC transgenic mouse model

Western blot analysis reveals that levels of FUS protein at 3 months show a trend towards a reduction in all transgenic lines, particularly the M337V hemizygous line ($p < 0.05$, figure 5.4a). The reduction in FUS protein in the M337V hemizygous line

shows the opposite trend to the transcript levels in the mutant lines at this time point, which may be an indirect manifestation of an autoregulatory process. However, the results are not consistent enough to be completely convincing. At 9 months there is a stronger trend towards a reduction in FUS protein levels from non-transgenic to the mutant lines, though it does not reach statistical significance. This is the same pattern observed in transcript levels at this time point, and implies that reduction in FUS levels may be due to a reduction in transcript levels. It has been proposed that FUS regulates its own mRNA levels via binding to exon 7 within its own transcript (109). A reduction in protein and transcript levels in this case implies that this mechanism is not functioning effectively in the presence of the mutation at this time point. This may be due to altered transcriptional regulation in the presence of mutant TDP-43. TDP-43 has been shown to regulate transcription of several other proteins, and although direct effects on FUS transcription have not been shown, it can bind to FUS mRNA so it is possible that it regulates FUS mRNA levels (157, 162). It may also be that a greater amount of FUS is in an insoluble form as a result of binding to mutant M337V in the cytoplasm. Insoluble cytoplasmic TDP-43 is described in the majority of ALS cases in humans and also in this mouse model (Gordon et al, in preparation, (15)). This may create lower amount of soluble protein in the mutant lines even with a functioning autoregulatory system.

SMN levels in the cortex at 3 months show a trend towards increased levels in the M337V lines, particularly the homozygous line compared to wild-type hemizygous (figure 5.4b). This shows the same trend to the transcript levels and so suggests that increased levels may be due to an increase in transcription. At 9 months, that trend remains, despite the fact that transcript levels decline at 9 months. This could imply that SMN transcript levels are reduced as part of an autoregulatory response to

excess SMN at 9 months. However, the mechanism proposed for SMN autoregulation at present does not involve alteration of transcript levels. Instead, it is suggested that Geminin5 activates SMN transcription, which increases with a reduction in SMN binding to Geminin5 (287).

The mechanism of increased SMN protein levels in the 9 month cortex is unclear, but mutant TDP-43 could potentially reduce SMN breakdown. TDP-43 has been shown to bind particularly to proteins involved in RNA processing (165). One study showed that TDP-43 was present in the interactome of SMN, though it was also present in one control experiment (198). It is feasible that mutant TDP-43 affects complexes with which SMN interacts, reducing SMN degradation. A more general dysfunction in protein degradation has been reported in ALS, so if this is the case in this mouse model then it could also account for excess SMN in the presence of the TDP-43 mutation (245-247). However, these results only show trends and more detailed studies should be carried out to investigate this further.

The additional bands visible in the SMN blots at 65 kDa and 75 kDa are particularly prominent in the M337V mutant lines. SMN may form complexes with other proteins or even potentially TDP-43 itself forming the band at about 75 kDa, which could be enhanced in the presence of M337V TDP-43 (198). In this way, SMN may be sequestered into the cytoplasm by mutant TDP-43 resulting in functional SMN deficiency. This has been suggested as a potential mechanism of toxicity in the presence *FUS* mutations, so it is possible that TDP-43 has a similar effect (107, 108, 250). This may also fit in with the trend towards the increase in SMN levels in the mutant lines at 9 months, as SMN bound to other cytoplasmic proteins may be more resistant to degradation (247).

It is important to note that the observations using Western blots are mainly trends, and further work is required to determine whether they are robust findings. Samples from the spine were very small, and there was insufficient protein to determine FUS levels. In addition, not all lines had n=3 due to availability of tissue. Further studies will be undertaken to further examine the effect of mutant TDP-43 on SMN and FUS.

5.5.3 Transcript levels in the FUS BAC transgenic mouse model

Total FUS mRNA levels show that a greater number of copies of the *FUS* gene results in higher levels of transcription and therefore higher levels of protein. This suggests that transcription is occurring from the transgene. However, this effect is not directly proportional to the estimated number of copies of the transgene. For example, the P525L-3 line contains 93 copies of the construct but levels of total mRNA are 3-6 fold higher than non-transgenic and FUS from the BAC makes up 10-15 % of total FUS protein. Firstly, this suggests an element of autoregulation, which has already been described for the FUS protein (109). According to this mechanism, FUS protein binds to exon 7 of its own mRNA causing exon 7 splicing, then increased nonsense-mediated decay of the transcript lacking exon 7 reduced protein synthesis. The observation that total levels of FUS protein are not increased in the mouse model suggests that this mechanism is effective (Vizard et al, unpublished). This is contrary to previous models that have tended to introduce a FUS cDNA transgene without any self-regulatory regions, causing overexpression of FUS. These results combined with analysis of protein expression in this model suggest that autoregulation is occurring, which is a significant advantage of this model. However, the fact that FUS from the BAC is reduced to such a small proportion of

total FUS does suggest that there may be some selective downregulation of human FUS compared to endogenous.

Despite apparently effective autoregulation in this model, total FUS transcript levels are still higher in the transgenic lines compared to non-transgenic. This suggests that there may be other mechanisms involved, for example rates of transcription may exceed the rate at which the autoregulatory mechanism can control. In addition, the levels of protein derived from the *FUS* transgene are very low compared to that of endogenous (appendix table 7.3). This suggests that human FUS mRNA from the transgene may be selectively degraded in preference to endogenous FUS mRNA.

This picture becomes more complex when levels of endogenous transcript are taken into account. The fact that wild-type FUS lines show an increase in endogenous FUS is surprising, as it would be expected that endogenous levels of FUS would decrease in the presence of BAC-derived FUS if autoregulation was occurring effectively and without discrimination between human and endogenous FUS mRNA. Due to the lack of human transcript in the non-transgenic line, total FUS represents only endogenous FUS so the levels should be the same for each, though results cannot be directly compared. In the wild-type tissue total FUS transcript levels are 2-3 times greater than non-transgenic, and endogenous FUS transcript levels are just under 2 times greater. It may be inferred from this that much of the increase in total FUS transcript levels comes from the increase in endogenous levels. It may be hypothesised that wild-type human FUS affects the autoregulatory mechanism. It is also possible that transcription from the human transgene does not occur effectively. Further work to assess the impact of the human transgene on FUS autoregulation would help to determine the mechanism behind these results. Interestingly, endogenous FUS in the P525L tissue is not significantly different from non-

transgenic, but is significantly lower than wild-type ($p < 0.01$ in the cortex). This implies that a similar mechanism to wild-type may be in play, as a reduction in endogenous FUS compared to non-transgenic would be expected if autoregulation was occurring effectively. The difference between P525L and wild-type also suggests that the mutation itself has an effect on FUS transcript levels. This is supported by the published observation that *FUS* mutations disrupt the autoregulatory mechanism, particularly those associated with greater mislocalisation of FUS (109).

The increase in TDP-43 and SMN mRNA levels in wild-type FUS lines compared to non-transgenic and P525L is interesting. FUS has been shown to interact with both SMN and TDP-43 proteins (108, 114). In addition, FUS binds to many mRNA species and can alter transcript levels (119). However, although there is some evidence that FUS can bind to TDP-43 mRNA, reports vary, and there is no definitive evidence that FUS binds SMN mRNA (119-122, 124). It is possible that transgenic human FUS alters binding to TDP-43 and SMN mRNA directly. However, it may represent more widespread alterations in transcription regulation, or a secondary effect rather than as a direct binding target. The consistent increase in endogenous FUS, TDP-43 and SMN mRNA in the presence of human wild-type FUS does suggest that the human FUS gene may have widespread effects on transcription rather than effects specific to certain targets. The difference between wild-type and P525L tissue implies that the mutation also has an effect on transcription regulation. This is consistent with previous reports that *FUS* mutations demonstrate altered patterns of RNA binding (120).

TAF15 and EWS show more consistent levels of mRNA between each genotype, though there are small variations. FUS has been shown to bind to these mRNAs,

suggesting it may have a role in transcription regulation of these proteins (119).

There is no evidence from these results that EWS and TAF15 transcription is significantly altered in the presence of human wild-type or mutant FUS.

5.5.4 Alternative splicing in the TDP-43 BAC transgenic mouse model

Analysis of splicing in the TDP-43 BAC transgenic mouse model reveals several targets that are not significantly altered in the presence of transgenic TDP-43; TIA1, mag, dnajc5, and ptk2b. There were some subtle but significant differences in the mutant lines in *ndrg2* which reflected that observed in TDP-43 knockdown. However, results from *ppp3ca* were variable, with differing results in the mutant lines at 3 and 9 months. In addition, there were changes observed in *ttc3* splicing at 3 months, a target that was unaffected by TDP-43 knockdown (119).

Splicing defects have been reported in ALS and in models of the disease (119, 240). However, it is not clear whether these occur as an early phenomenon, playing a primary role in neuronal dysfunction, or whether they occur secondarily, reflecting more widespread neuronal dysfunction and death. In order to examine further, it is useful to examine early pre-symptomatic time points, before there is significant neuronal damage. This may be a period during ALS pathogenesis when the neuron is able to compensate for the presence of the mutation, so direct effects of the mutation may be studied. In the case of the TDP-43 BAC transgenic mouse model, there are no behavioural changes observed at the 3 month time point. By 9 months, however, a behavioural phenotype is starting to emerge (Gordon et al, in preparation). These time points are therefore ideal to compare pre-symptomatic with early symptomatic alterations in cell biology.

Results described here using the TDP-43 BAC mouse model show only small or no differences in the splicing of selected targets, even at the 9 month time point. This suggests that excessive and widespread splicing dysfunction is not present at these time points. Assessing the results as a whole, there is not enough evidence to confirm the hypothesis that widespread splicing changes occur early in disease pathogenesis in this model. The mixed picture when comparing results to that of TDP-43 knockdown does not support a loss of function hypothesis, at least at this stage. However, further work using RNA sequencing will give a much broader picture of splicing patterns at these time points.

5.5.5 Alternative splicing in the FUS BAC transgenic mouse model

Splicing analysis in the FUS transgenic model was carried out at mixed time points due to the availability of tissue, so it is difficult to relate these changes to symptomatic time points. However, no obvious phenotype was observed in any of these animals. These results are therefore most likely to represent either a pre-symptomatic time point or, in the event that no phenotype is observed, it is simply a reflection of the presence of the transgene. This can still yield important information about the pathophysiology of ALS. People with this condition live apparently healthy lives prior to disease onset, even in the presence of mutations. It is therefore of interest to determine whether mutations have an effect on neurons during this time, and if so, how they are able to compensate without any apparent neuronal deficit. Interestingly, 3 of the targets examined showed alterations in the mutant lines that reflected those observed in FUS knockout mice (TIA1, ptk2b and ttc3) (119). They showed differences in the mutant compared to non-transgenic lines and not compared to wild-type, and were relatively small. However, they could reflect some

loss of function effect of the mutation. The loss of function hypothesis explains motor neuron toxicity as a result of functional deficiency of FUS. The redistribution of FUS from the nucleus to the cytoplasm has been shown *in vitro*, and in patients with ALS-FUS there is accumulation in cytoplasmic aggregates (40, 71, 102). This could potentially lead to a loss of the normal nuclear function of FUS. Indeed, the aggregation of FUS in the cytoplasm could also affect its cytoplasmic functions as well, for example in mRNA transport (125). A reduction in FUS is toxic to cells, as demonstrated by FUS knockout models (136, 238). It has proved difficult to determine whether the toxicity of *FUS* mutations is as a result of loss of function of FUS, and one way to examine this is by comparing the phenotype of *FUS* mutations with that of knockdown. The results from this study show some similarities between the splicing targets, so this would support the hypothesis that loss of function of FUS plays a part in toxicity. The fact that the observed changes in splicing are subtle is likely to reflect the fact that this model has low levels of mutant protein expression. Further work to examine the changes in splicing over time would help to determine whether this is an early phenomenon in this model.

Determination of the relative abundance of each splice variant was carried out in a semi-quantitative manner, which limits the conclusions that can be drawn from the results. In order to examine this in more detail, further work using RNA sequencing would be useful. Study of the FUS mouse model is also limited by the availability of tissue at specific time points. This will be tackled by the development of cohorts of mice to examine tissue at 3 monthly intervals. This will also enable behavioural phenotype to be compared with alterations in RNA, protein and neuropathological studies at each time point. Further work will also include more detailed assessment

of autoregulation of FUS and TDP-43 in these models. Detection of the FUS exon 7 skipped variant is limited by its early decay, which is part of the autoregulatory process. Further work using a minigene reporter and methods to reduce nonsense-mediated decay would help to assess this in more detail.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

6.1 General discussion

Overall conclusions

1. A *FUS* truncation mutation has been confirmed in a family with a severe form of ALS. This has been characterised *in vitro*, showing mislocalisation and incorporation into stress granules. This work demonstrates the correlation between the clinical severity of a mutation and the observed *in vitro* phenotype.
2. Motor neurons from the BAC transgenic mouse model express low levels of mutant *FUS*. However, there is still significant mislocalisation of mutant *FUS* and incorporation into stress granules as well as a reduction in survival. There is evidence of altered stress granule dynamics. However, there is no evidence of altered *SMN* or endogenous *FET* proteins. This shows that even with low levels of mutant *FUS*, this phenotype is observed.
3. There is no evidence of altered distribution of *FET* proteins or gem number in motor neurons from the *FUS* homozygous knockout or in the *TDP-43* BAC transgenic mouse model. This provides evidence against mislocalised mutant *FUS* or *TDP-43* sequestering other RNA binding proteins into the cytoplasm.
4. Alterations in mRNA levels of other important species are observed in the *FUS* and *TDP-43* BAC transgenic mouse models. This suggests that the mutations are affecting mRNA processing.
5. There are some subtle alterations in splicing observed in the *FUS* and *TDP-43* BAC transgenic mouse models. However, they do not convincingly mimic those of *FUS* or *TDP-43* knockdown. This suggests that these *FUS* and *TDP-43* mutations do not cause widespread splicing defects at an early stage.

ALS is a neurodegenerative disease primarily of motor neurons. In recent years, much has been learned about the genetics of this disorder. The presence of the C9orf72 repeat expansion in a relatively large number of patients with the disease (7% of sALS and 39% fALS) suggests that the genetic basis of this disease may be significant (35). It is likely that genes are involved in susceptibility to this disease in more than a monogenic manner (288). In the coming years it may become clear how the pattern of our genes, along with epigenetics factors, may play a part in conferring risk of ALS (289). This may be more relevant to patients without a clear family history, which make up the majority of cases of ALS.

The discovery that mutations in genes such as *SOD1*, *TARDBP* and *FUS* can cause ALS has provided much insight and speculation about the pathophysiology of this disease (40, 290, 291). Models, particularly mouse models, with these mutations have reported many potential mechanisms of toxicity such as oxidative stress, mitochondrial dysfunction, inflammation and protein aggregation (292). However, in models with widespread neuronal death, it can be difficult to distinguish between mechanisms that initiate disease and those that are involved early from secondary effects of neuronal dysfunction and death. In addition, it is not clear how closely these models represent human disease. Many drug treatments that have been successful in these models have failed to be of benefit to humans with ALS (293). In addition, the mechanisms of disease are still unclear, particularly in terms of factors that may initiate and perpetuate the disease process, which is vital in order to design appropriate treatments.

For this reason, research should focus on trying to create models that represent ALS as closely as possible, and validate the results in humans whenever it is feasible to do so. In this way, a picture of ALS can be built up that consists of a combination of

background genetic risk and potential mechanisms at play to initiate and propagate disease.

The first section of this work highlights the relationship between the clinical severity of *FUS* mutations and phenotype *in vitro*. This is an important observation. The fact that individuals can live for many years before developing the relatively rapidly progressive motor syndrome suggests that either the mutation confers a background risk that requires another event to initiate disease, or that a tipping point is reached after a certain point and the clinical syndrome develops. The fact that mislocalisation of *FUS* reflects disease severity points towards this feature as an important part of creating this disease risk (102). Mislocalisation of *FUS* could contribute to disease in several ways, for example by increasing the propensity of proteins to aggregate in the cytoplasm or altering RNA processing pathways (119, 294). Greater mislocalisation would therefore increase the likelihood of clinical disease developing, and the age of onset is subsequently lower.

The second section used primary motor neurons from a *FUS* BAC transgenic mouse model to examine the effect of low levels of mutant *FUS* expression on the cell type of most relevance in ALS. Even at low levels of expression, P525L mutant *FUS* was mislocalised from the nucleus to the cytoplasm. Survival is reduced to some degree, though many cells are still viable and the morphology is not visibly altered. There are some defects in stress granule formation and dissolution, but these are not replicated with all markers so it may not be a robust phenomenon. Additionally, there is no significant sequestration of endogenous FET proteins by cytoplasmic P525L-*FUS*. Interestingly, the phenotype of mice expressing human P525L *FUS* is not significantly different from non-transgenic or wild-type at the 6 month time point (Vizard et al, unpublished). Together this points to a cellular phenotype that appear

to be compensated for to a relatively good degree at this stage in this particular model.

The third section of work shows some alterations in transcript levels in both the FUS and TDP-43 BAC transgenic mouse models. However, protein levels are generally not significantly altered. The splicing changes observed are again very subtle. This suggests that there may be some compensation for the alterations observed at the transcriptional level. This may be in part occur via autoregulatory mechanisms of SMN and FUS, or by alterations in other proteins in the cells (109, 287). In addition, if levels of FUS or SMN were significantly increased or decreased it would be expected to cause an RNA processing phenotype with significant alterations in splicing (29, 110, 119, 197, 295). This study has not found evidence of widespread early splicing alterations. This has also been verified using RNA sequencing, which has again showed little evidence of splicing dysfunction presymptomatically (Gordon et al, in preparation). The presence of the remaining two endogenous alleles in both models may also play a part in compensating for the addition of the human mutant gene.

These results as whole suggest a model of ALS consisting of early changes in motor neurons in the presence of mutations, for example mutant FUS or TDP-43 mislocalisation. However, these changes appear to be compensated for early on, at least in part. This could reflect the normal motor function of humans with *FUS* mutations prior to disease onset. However, at some point the balance in humans must be tipped, either by the gradual accumulation of cellular dysfunction or by a further initiating event, leading to clinical disease onset.

It remains to be discovered whether disease onset is triggered by a lack of compensation after years of subclinical neuronal dysfunction, or whether there is an

initiation event that triggers disease (296). There is some evidence of early cellular dysfunction, for example the fact that some individuals with FUS and C9orf72 mutations have been reported to have learning difficulties prior to the development of neurodegeneration later in life (78, 297). However, the fact that the vast majority of individuals with ALS have an apparently normal motor system until onset, followed by a relatively rapidly progressive disease, does point to a distinct disease onset. It has been proposed that trauma could initiate ALS in a certain region, though this is unproven (25, 298). In addition, there is a suggestion that a more active motor system may be linked with disease onset, for example an increased likelihood of disease onset in a dominant hand, and an apparent increase in athletes (299, 300). These features could all potentially put the motor system under more 'stress' in a particular area, making the onset of disease more likely. In recent months, a cancer-like hypothesis has suggested that further 'hits' to the genome may be required to initiate disease onset (301). A motor system functioning at a higher metabolic rate may be more susceptible to such events, which would also explain why a region that suffered trauma, a dominant hand, or an athlete, may be more likely to suffer such events that increase the likelihood of disease onset.

6.2 Future directions

The FUS BAC transgenic mouse model is in its early stages. As it reaches further longitudinal behavioural testing and tissue sampling time points, it will be important to assess clinical phenotype as well as neuropathological features. As more tissue becomes available, further investigation of RNA and protein levels will be carried out at specific time points. The mislocalisation of mutant mCherry is a marked phenotype. In time, this may be used to help to determine whether cytoplasmic

mCherry granules may be the precursors of aggregates observed in disease. In addition, this mouse may provide a good model to test therapies to reduce FUS mislocalisation or aggregation.

Alterations in transcript levels and splicing may be assessed in more details using RNA sequencing to compare profiles of the mice expressing wild-type human FUS with those expressing P525L FUS. In this way, further evidence may be gained about the early RNA processing changes in this model of ALS.

Should the mouse fail to develop a phenotype, additional methods to investigate the initiation of disease may be useful. The FUS BAC transgenic model is currently being crossed onto the FUS knockout background to examine whether the human gene can compensate for the embryonic lethality caused by FUS knockout. This will also help to examine whether a motor phenotype may be unmasked in the absence of any compensatory effect of endogenous FUS. It could also be crossed with the TDP-43 BAC transgenic mouse to produce mice with 2 mutations, a phenomenon that has been observed in humans (302). Alternatively, a mechanism to 'stress' a particular area of the motor system may be used, or a pathological 'seed' introduced to investigate propagation of disease in wild-type and P525L-FUS expressing mice (20). A greater understanding of the processes leading to susceptibility and initiation of disease will allow further progress to be made in understanding the pathophysiology of ALS.

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Description	Target	Primer name	Sequence	Product length (bp)	Annealing T (°C)	Efficiency	Designed by
ANG control	Exon 1	Ang_1 F	AGCAGCAGCGAATAAGTACG	587	59		D. Bäumer
		Ang_1 R	AGCAGGAGTGTGAACCTACC				
FUS exon 14	Exon 14	FUS_14 F	ATGGGTAAGAAAGGCAGACC	370	59		D. Bäumer
		FUS_14 R	CCTAGCCCTCAAAATGAAACC				
SDM truncation 1	AG deletion	del1485-1486	GTGGTGGGGACAGGCGGTTTTGGCCC	n/a			T. Vizard/L. Kent
		del1485-1486-antisense	GGGCCAAAACCGCCTGTCCCCACCAAC				
SDM truncation 2	R496W/F497L	R496W_F497L	GGTGGGGACAGGTGGTTATGGCCCTGGCAA	n/a			T. Vizard/L. Kent
		R496W_F497L_antisense	TTGCCAGGGCCATAACCCACTGTCCCACC				
SDM truncation 3	L504F/R507*	L504F_R507*	CTGGCAAGATGGATTCCAGGGGTGAGCACAGACAGG	n/a			T. Vizard/L. Kent
		L504F_R507*_antisense	CCTGTCTGTGCTCACCCCTGGAATCCATCTTGCCAG				
SDM R244C	R244C	Mut_R244C	GAACCCAGAGGCTGTGGAGGTGGCC	n/a			L. Kent
		AntiMut_R244C	GGCCACCTCCACAGCCTCTGGGTTCC				
BAC set 1	BAC 1	BAC1_F	TTTGTGTTTCTTAGTAGGGGCGGG	450	57		B. Davies
		BAC1_R	AGAGCCCTTGACCCCAAATGAC				
BAC set 2	BAC 2	BAC2_F	AGCGTTGGAAGCTTCATGTCCT	688	57		B. Davies
		BAC2_R	CCATGAGCCACACATGCTTCCAATCT				
BAC set 3	BAC 3	BAC3_F	GAACAGAGGCCATAGGATAACAGG	505	57		B. Davies
		BAC3_R	GGGACATCGATCTTCCAGGAAAGT				
IL-2 control	IL-2	IL2_F	CTAGGCCACAGAATTGAAAGATCT	324	54		B. Davies
	IL-2	IL2_R	GTAGGTGGAAATTCTAGCATCATCTC				
FUS qPCR	FUS cDNA	FUS qPCR A f	GGACAACAGAACCAGTACAACAGT	94		0.83415145	L. Kent
		FUS qPCR A rev	CGCCACCACTCATAGAGGAC				
FUS qPCR	FUS cDNA	FUS qPCR B f	TTATGGACAGACCCAAAACACA	111		0.84573699	L. Kent
		FUS qPCR B rev	TGCTGCCCATAGAAGATTGT				
FUS qPCR	FUS cDNA	FUS qPCR C f	TGGTGGCTATGAACCCAGA	96		0.88278517	L. Kent
		FUS qPCR C rev	GGACCACCAAATTTATTGAAGC				
FUS qPCR	FUS cDNA	FUS qPCR D f	GGCTACTCCCAACAGAGCAG	124		0.88640074	L. Kent
		FUS qPCR D rev	TGTTTTGGGTCTGTCCAT				
FUS qPCR	FUS cDNA	FUS qPCR E f	AATCGGGGTGGTGGAAT	116		0.54832241	L. Kent
		FUS qPCR E rev	CCTCCACTGGGGAATCCT				
TDP-43 qPCR	TDP-43 cDNA	TDP qPCR A f	AATTCTGCATGCCCAAGA	93		n/a	L. Kent
		TDP qPCR A rev	GCATCTGTCTCATCCATTTTCC				
TDP-43 qPCR	TDP-43 cDNA	TDP qPCR B f	GGTCTCCCTGGAAAAACAA	113		0.91887007	L. Kent
		TDP qPCR B rev	AACCTTTTCGAGTGACCAGTT				
SMN qPCR	SMN cDNA	SMN qPCR A f	GCTCCGTGGACCTCATTTT	89		0.99374844	L. Kent
		SMN qPCR A rev	GGGCCGTGAATTTTAGACC				

Table 7.1. The primers used in this study (continued on next page).

Description	Target	Primer name	Sequence	Product length (bp)	Annealing T (°C)	Efficiency	Designed by
EWS qPCR	EWS cDNA	EWS qPCR A f	GGCGTCCACGGATTACAGT	124		0.86261998	L. Kent
		EWS qPCR A rev	TCCATAGCTTTGTTGCCATA				
EWS qPCR	EWS cDNA	EWS qPCR B f	TGGAGAACAGAATGCAACCA	94		0.74142913	L. Kent
		EWS qPCR B rev	ACCTCGTCCACGATCACC				
EWS qPCR	EWS cDNA	EWS qPCR C f	GACCACTGCCACCTACGG	81		1.25414943	L. Kent
		EWS qPCR C rev	GGGGCAGTTGGAGTGCTAT				
EWS qPCR	EWS cDNA	EWS qPCR D f	GCTTCAATAAGCCTGGTGGA	67		0.72659965	L. Kent
			GGATCTATAGGAAGGCCTAGA				
		EWS qPCR D rev	TCA				
EWS qPCR	EWS cDNA	EWS qPCR E f	CCTGCCTACCCACCTATG	79		0.89405467	L. Kent
		EWS qPCR E rev	TCTCAGCAGGCTTGTTACCAT				
			AGGCCTGAATTTATGAGAGGT				
TAF15 qPCR	TAF15 cDNA	TAF15 qPCR A f	G	70		0.96607250	L. Kent
		TAF15 qPCR A rev	CACGGCCTCTATATCCTCCA				
TAF15 qPCR	TAF15 cDNA	TAF15 qPCR B f	TGATGACCTCCATCAGCTA	86		0.91499234	L. Kent
			TGGCAAATGACACTTTTATGAT				
		TAF15 qPCR B rev	G				
GAPDH qPCR	GAPDH cDNA	GAPDH qPCR A f	AGCTTGTCAACGGGAAG	62		0.95986775	L. Kent
		GAPDH qPCR A rev	TTTGATGTTAGTGGGGTCTCG				
GAPDH qPCR	GAPDH cDNA	GAPDH qPCR B f	GCCAAAAGGGTCATCATCTC	60		0.95564677	L. Kent
		GAPDH qPCR B rev	CACACCCATCACAACATGG				
GAPDH qPCR	GAPDH cDNA	GAPDH qPCR C f	TGTCGCTCGTGGAATCTGAC	75		0.97751969	L. Kent
		GAPDH qPCR C rev	CCTGCTTCAACACCTTCTTG				
HPRT1 qPCR	HPRT1 cDNA	HPRT1 qPCR A	CAGGACTGAAAGACTTGCTCG	106		0.96141166	L. Kent
			AGAT				
		HPRT1 qPCR	CAGCAGGTCAGCAAAGAACTT				
			ATAGC				
18s qPCR	18s cDNA	18sJD f	GGACCAGAGCGAAAGCATTTG	113		0.98152838	L. Kent
		18sJD rev	GCCAGTCGGCATCGTTTATG				
Ndr2 splice variants	Exon 3 cDNA	Ndr2 forward	AGGACAAACACCCGAGACTG	115, 157	52		Lagier-Tourenne
		Ndr2 reverse	ATGGTAGGTGAATATCGCCG				
TIA1 splice variants	Exon 5 cDNA	Tia1 forward	TGAAAGTGAATTGGGCAACA	86, 119	51		Lagier-Tourenne
		Tia1 reverse	TGGACTGAGGTCACCAACAA				
Mag splice variants	Exon 8 cDNA	Mag forward	AATTCAGAAATCTCTGGGGCA	130, 175	52		Lagier-Tourenne
		Mag reverse	CCCAGGTCTGAGTGGGAATA				
Ppp3ca splice variants	Exon 13 cDNA	Ppp3ca forward	CTGACACTGAAGGGCCTGAC	166, 196	55		Lagier-Tourenne
		Ppp3ca reverse	GAGGTGGCATCCTCTCGTTA				
Dnajc5 splice variants	Exon 9 cDNA	Dnajc5 forward	CTCTATGTGGCGGAGCAGTT	274, 310, 360	54		Lagier-Tourenne
	(from 7-10)	Dnajc5 reverse	GCTGTATGACGATCGGTGTG				
Ptk2b splice variants	Exon 24 cDNA	Ptk2b forward	AGACCTCCTCCACAGACCAA	317, 191	53		Lagier-Tourenne
		Ptk2b reverse	CATCTTTCCTCTCGCTCAG				
Ttc3 splice variants	Exon 3 cDNA	Ttc3 forward	TGCTGAGGGAGGTCTCAGTT	217, 174.	55		Lagier-Tourenne
		Ttc3 reverse	GGAGAGTGGCTTACTGCACC				
FUS autoregulation	Exon 7 cDNA	FUSex7 forward	AGTGGTGGCTATGAACCCAGA	89, 124	59		Zhou
			GGT				
		FUSex7 reverse	AGTCATGACGTGATCCTTGGTC				
			CC				

Table 7.1 Continued

Antibody	Raised in	Poly/monoclonal	Company	Conc (ICC)	Conc (WB)	Labels
FUS	Rabbit	Polyclonal	Cambridge bioscience	1:500	1:5000	FUS
EWS	Mouse	Monoclonal	Santa Cruz	1:150	n/a	EWS
TAF15	Rabbit	Monoclonal	Abcam	1:250	n/a	TAF15
TDP-43	Rabbit	Polyclonal	Proteintech	1:500	1:2000	TDP-43
SMN	Mouse	Monoclonal	BD biosciences	1:4000	1:2500	SMN
c-myc	Mouse	Monoclonal	Sigma	1:5000	1:5000	Tag (FUS pcDNA)
GFP (& variants)	Rabbit	Polyclonal	Abcam	1:2000	1:1000-5000	Tag (TDP-43 BAC)
mCherry	Mouse	Monoclonal	Abcam	1:500	1:2000	Tag (FUS BAC)
mCherry	Rabbit	Polyclonal	Abcam	1:500	n/a	Tag (FUS BAC)
TIA-1	Goat	Polyclonal	Santa Cruz	1:500	n/a	Stress granules
TIA-1	Mouse	Monoclonal	Santa Cruz	1:1000	n/a	Stress granules
PABP-1	Rabbit	Polyclonal	Abcam	1:500	n/a	Stress granules
G3BP	Mouse	Monoclonal	Abcam	1:500	n/a	Stress granules
Tuj1	Rabbit	Polyclonal	Covance	1:2000	n/a	Neuronal marker
ChAT	Goat	Polyclonal	Millipore	1:100	1:500	Motor neuron marker
β -actin	Mouse	Monoclonal	Sigma	n/a	1:2500	Western blot control

Table 7.2. Primary antibodies used in this study.

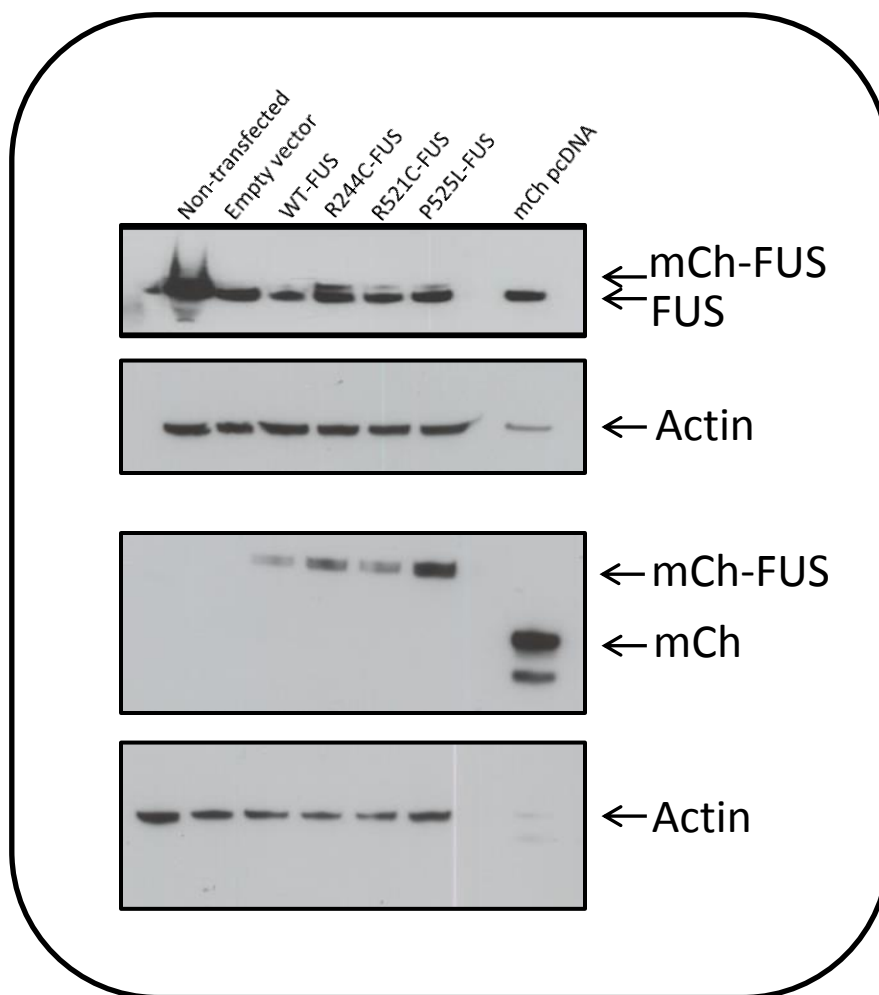


Figure 7.1a). Western blots of cell lysate from HEK293 cells transfected with the given BAC construct.

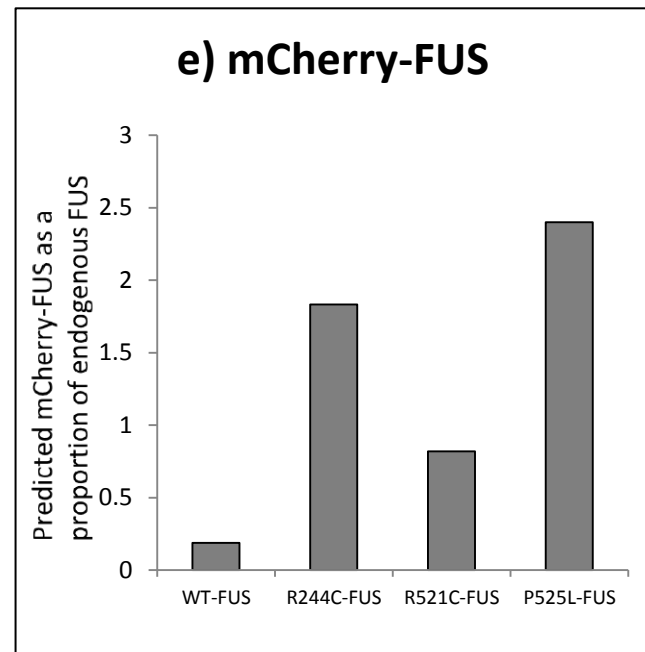
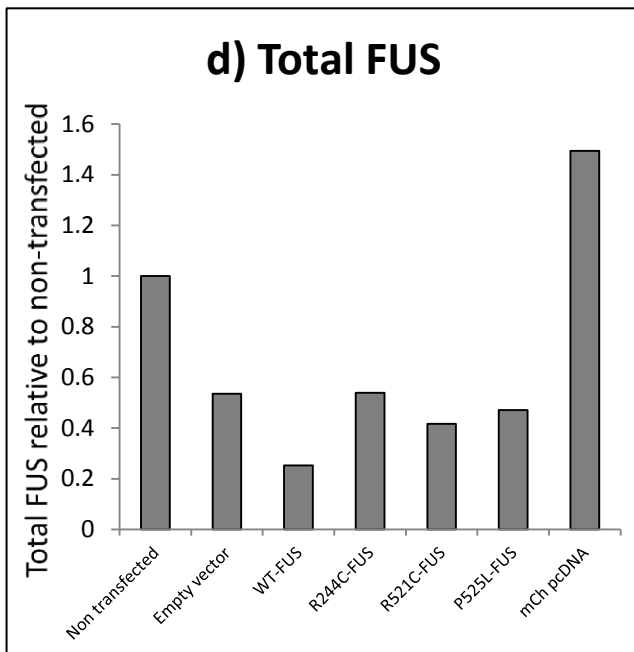
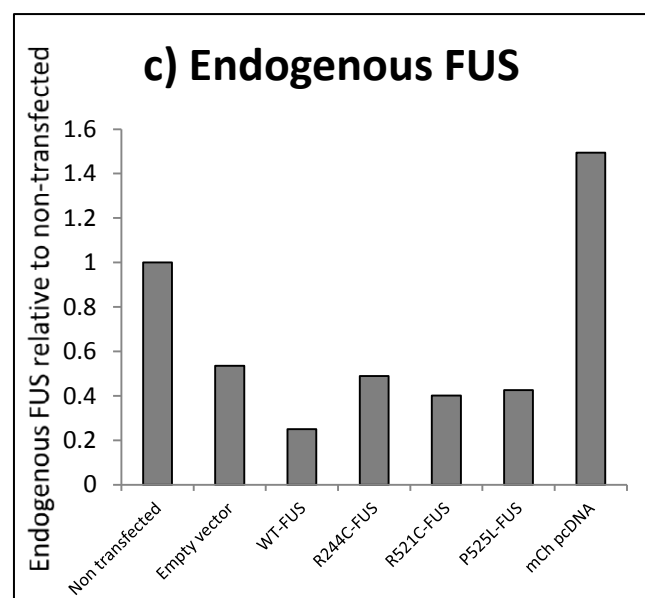
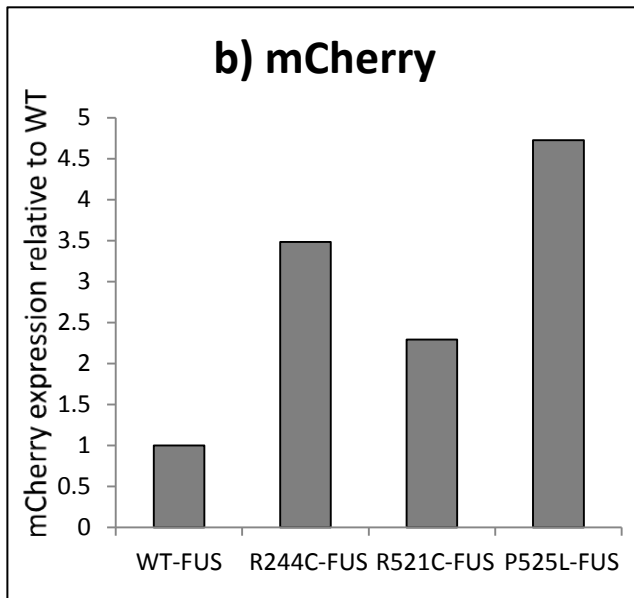


Figure 7.1. Quantification of Western blots **b)** mCherry expression relative to wild-type FUS **c)** Endogenous FUS relative to non-transfected cells, showing that endogenous FUS appears to be decreased in cells transfected with the FUS BAC construct **d)** Total FUS relative to non-transfected cells, which suggests that FUS may be decreased in cells transfected with the FUS BAC construct **e)** Predicted expression of mCherry-FUS relative to endogenous FUS, normalised to a 100% transfection rate. mCherry-FUS and endogenous FUS were detected using an antibody to total FUS. This suggests that there is less than 1-2 fold expression of FUS from the BAC construct compared to endogenous FUS, which avoids marked overexpression. All Western blots were controlled for protein levels by normalising to actin.

Mouse line	Copy no	Expression brain (%)	Expression spine (%)
FUS NTg	0		
FUS WT line 2	8.6	0.07	0.07
FUS WT line 3	23	10.19	9.55
FUS P525L line 1	19	0.35	0.73
FUS P525L line 2			
FUS P525L line 3	93	14.34	10.59
FUS KO NTg			
FUS KO heterozygous			
FUS KO homozygous			
TDP-43 NTg	0	0	0
TDP-43 WT hemizygous	1	17	5
TDP-43 WT homozygous	2	26	9
TDP-43 M337V hemizygous	1	29	9
TDP-M337V homozygous	2	31	20

Table 7.3. Mouse lines used in this study. (Expression of FUS/TDP-43 from the transgene as a percentage of total protein)

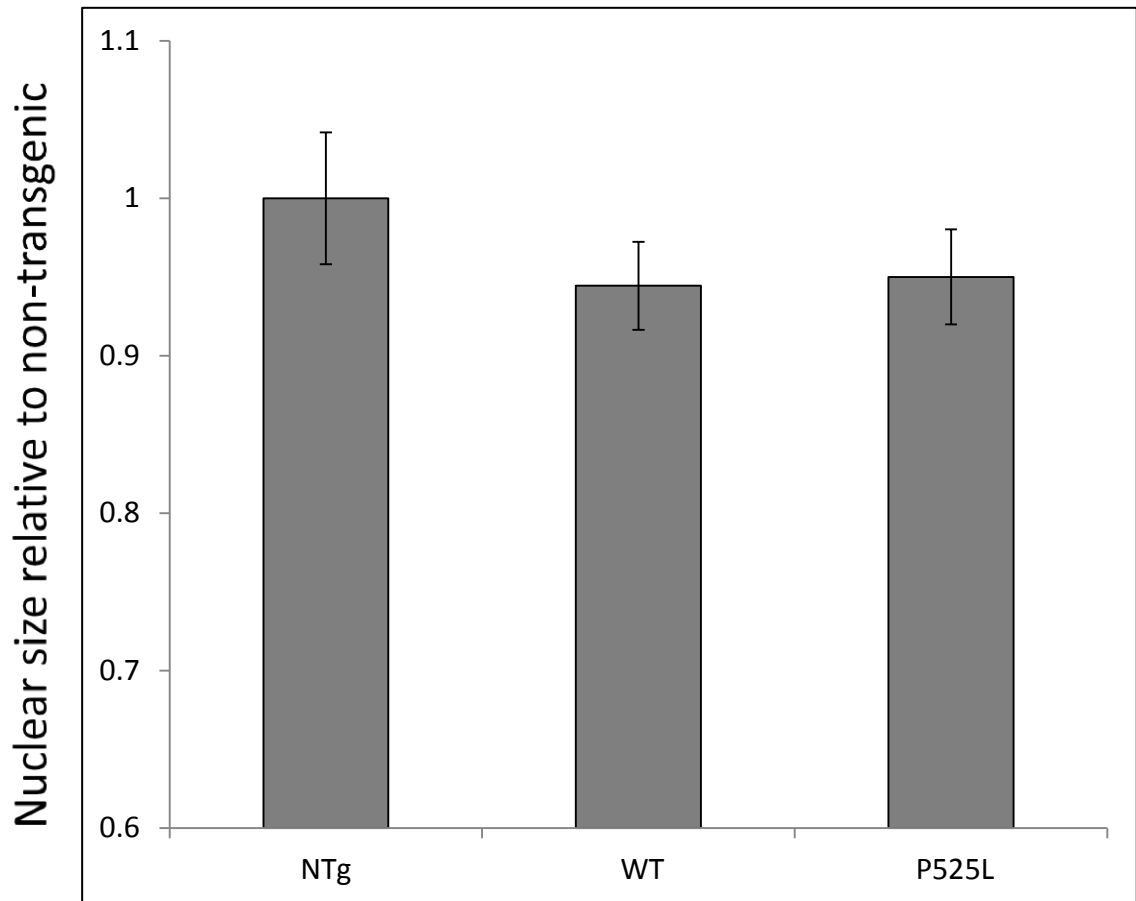


Figure 7.2. Nuclear area in primary motor neurons from the FUS BAC transgenic mouse model as determined by confocal microscopy, relative to non-transgenic. There is no significant difference in nuclear size between each genotype. (N=12. One way ANOVA. Tukey post-test)

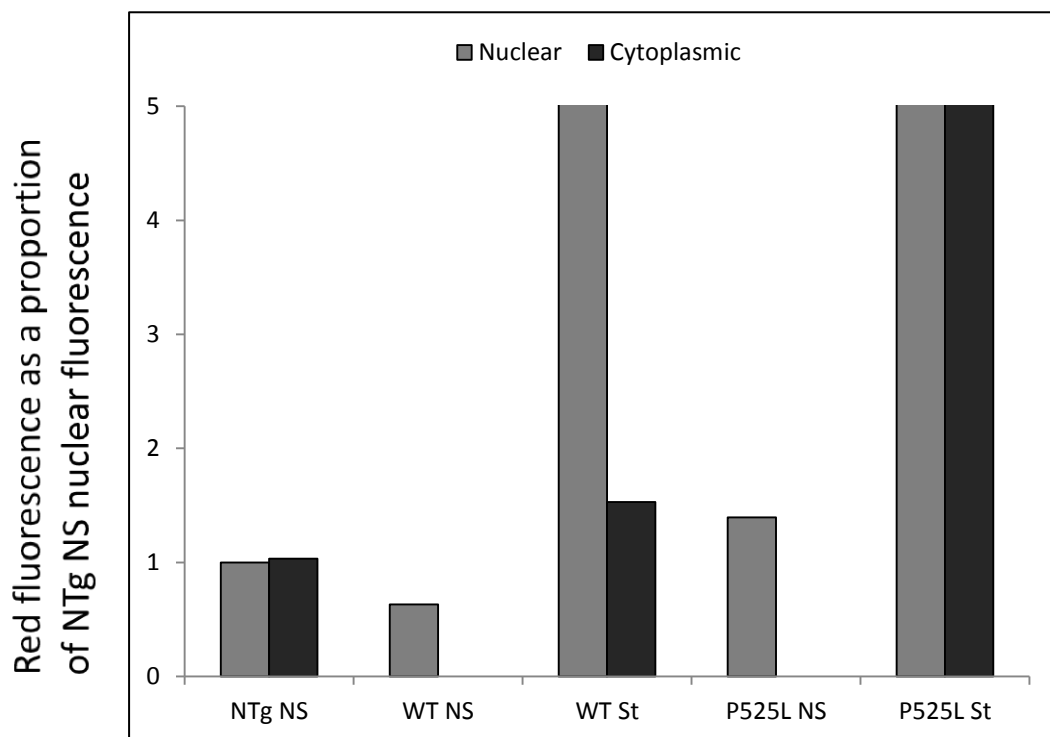
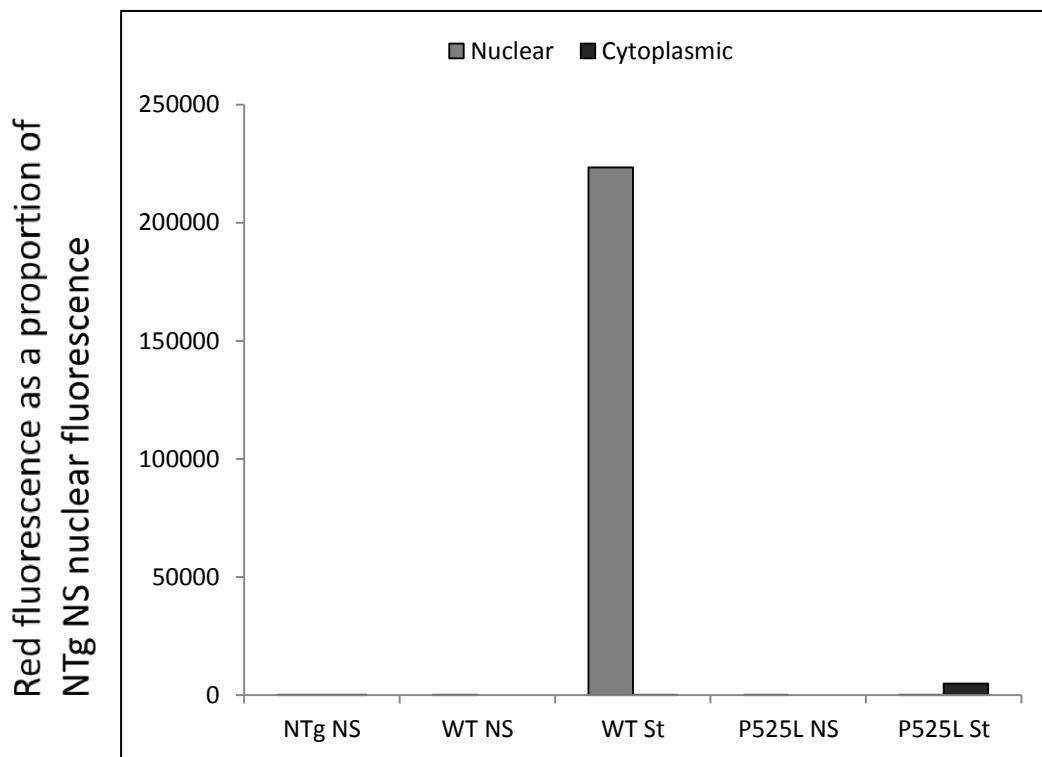


Figure 7.3a) Average red fluorescence in motor neurons from the FUS BAC transgenic mouse model, assessed by confocal microscopy and expressed as a proportion of non-transgenic not stained. Fluorescence is not increased in the transgenic neurons without staining with the mCherry antibody. (NTg = non-transgenic. WT = wild-type. NS = not stained. St = stained with mCherry antibody.) 284

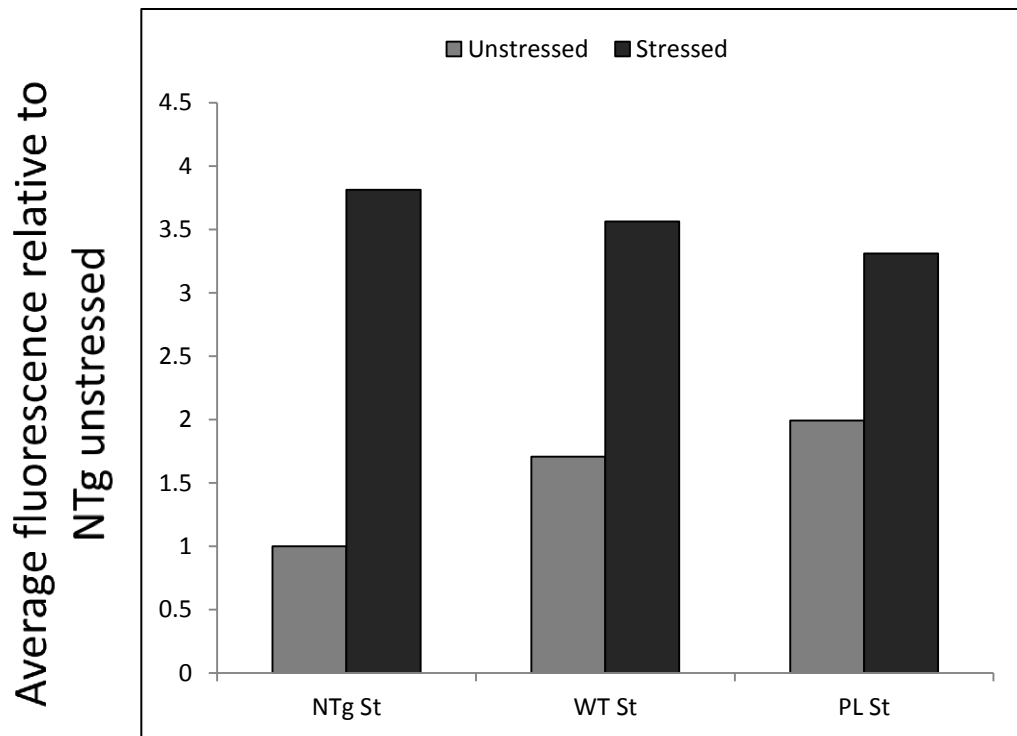


Figure 7.3b) Average fluorescence of primary motor neurons from the FUS BAC transgenic mouse model stained with the mouse mCherry antibody. Fluorescence intensity does not distinguish non-transgenic from transgenic motor neurons. (St = stained. N=1)

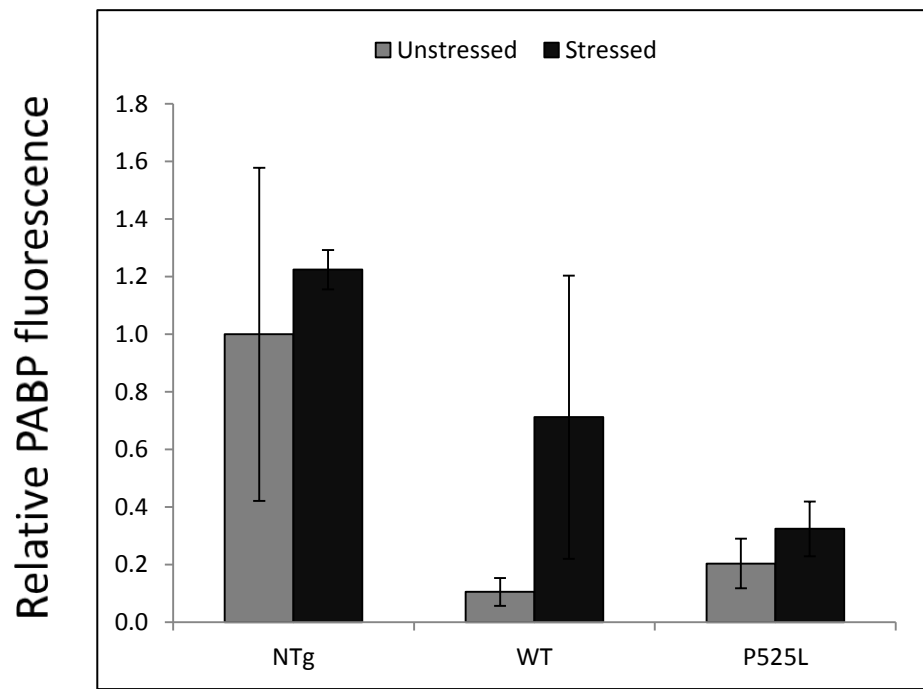


Figure 7.4a). Quantification of PABP fluorescence relative to non-transgenic unstressed in primary motor neurons from the FUS BAC transgenic mouse model. There is a trend towards a reduction in fluorescence in the P525L neurons but no significant difference between the lines. (N=3. One way ANOVA. Tukey post-test)

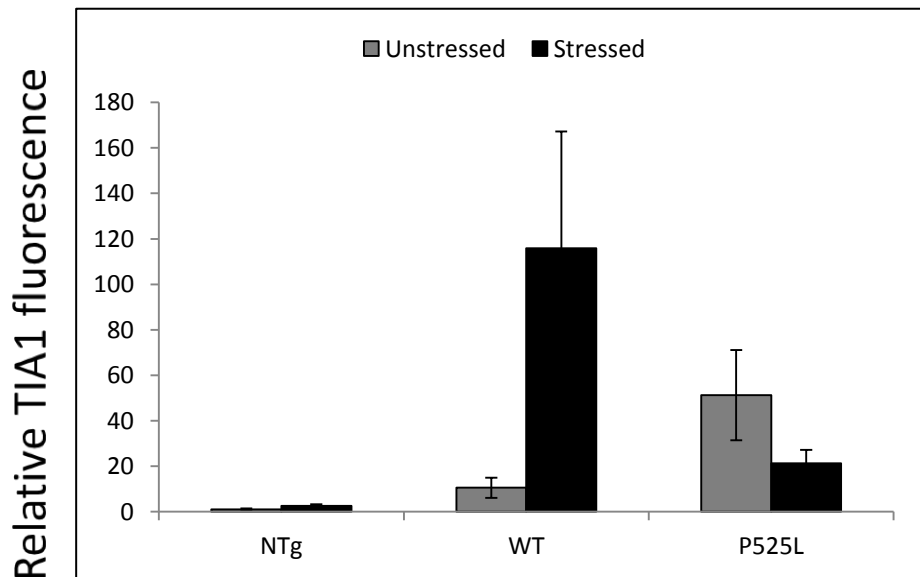


Figure 7.4b) Quantification of TIA1 fluorescence relative to non-transgenic unstressed in primary motor neurons from the FUS BAC transgenic mouse model. There is a lot of variation but no significant difference between the lines.

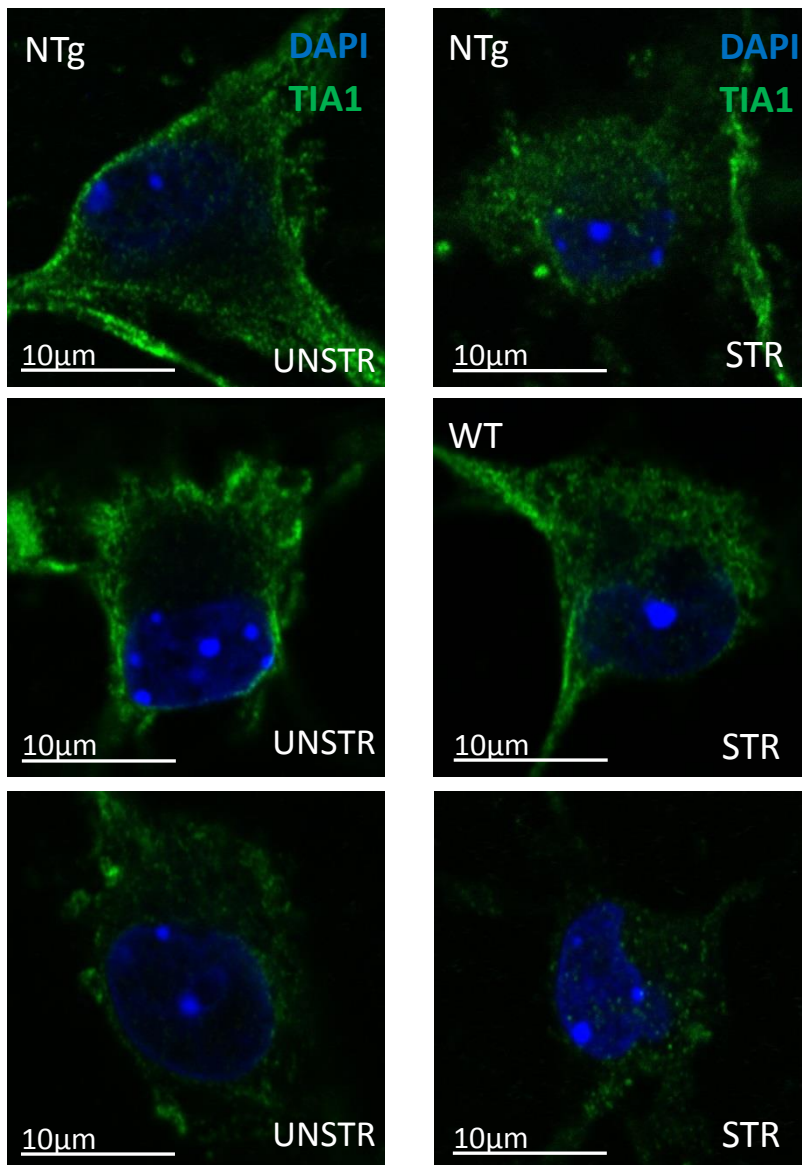
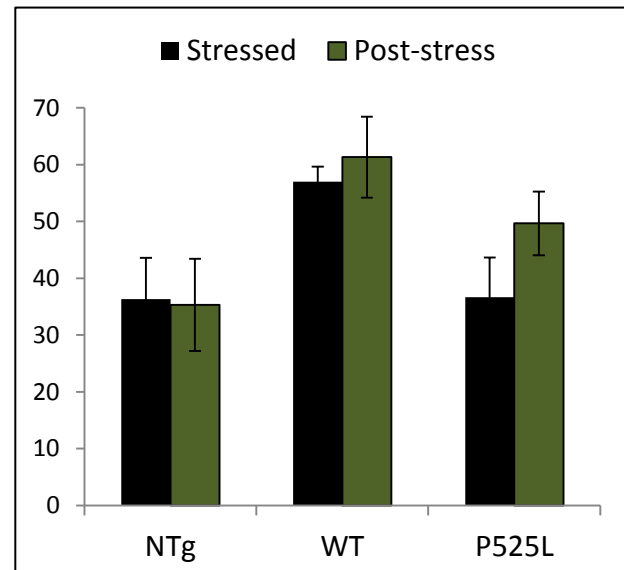
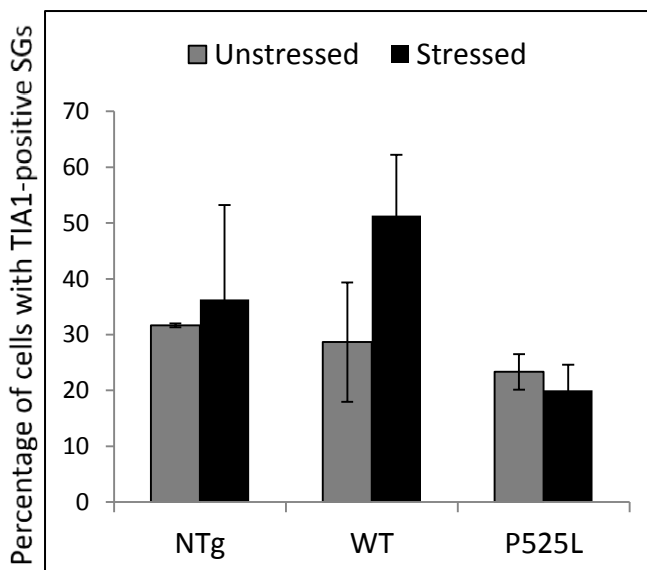


Figure 7.5 Primary motor neurons from the FUS BAC transgenic mouse model were stained with an antibody to detect TIA1. It was more difficult to detect individual stress granules using this antibody and results were more variable. There were no differences between each line. (N=3. One way ANOVA. Tukey post – test)



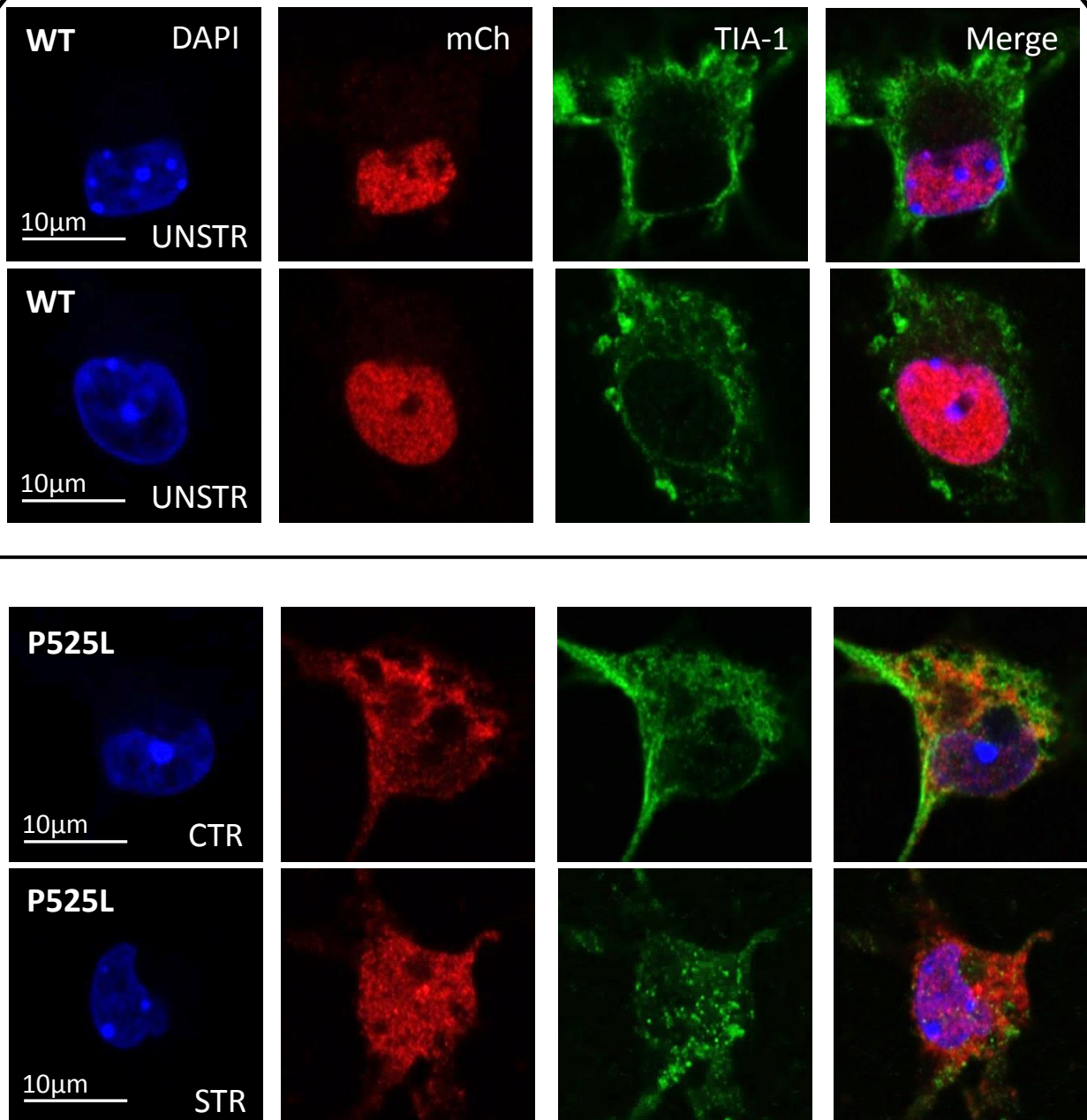


Figure 7.6a) Primary motor neurons from the FUS BAC transgenic mouse model were stained with antibodies to mCherry and TIA1. There is more overlap of P525L-FUS mCherry than wild-type FUS mCherry with TIA1.

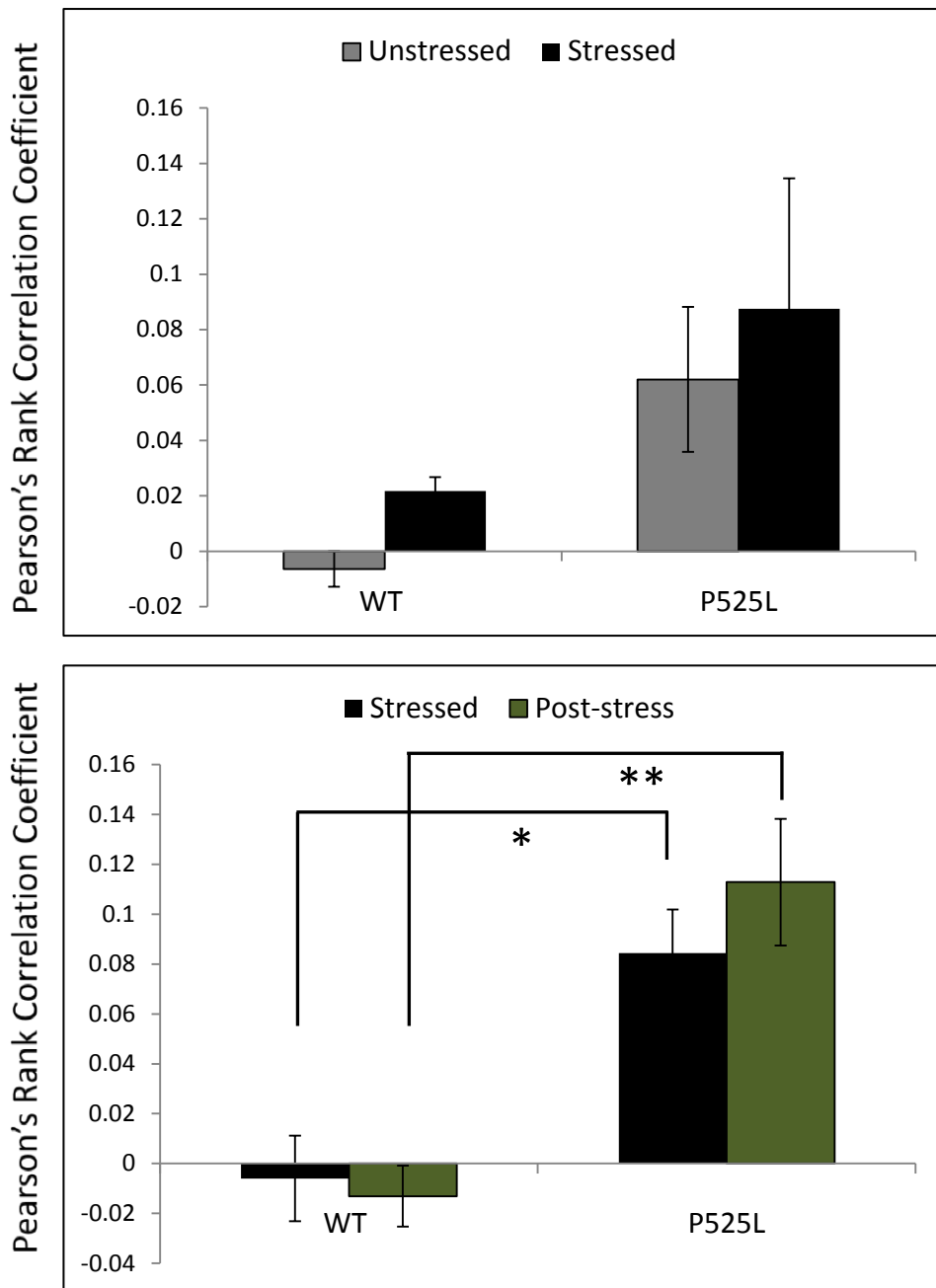


Figure 7.6b) Co-localisation between mCherry and TIA1 in primary motor neurons from the FUS BAC transgenic model, quantified by calculating Pearson's correlation coefficient using confocal microscopy. There is increased co-localisation in P525L neurones compared to wild-type. (N=3. One way ANOVA. Tukey post-test)

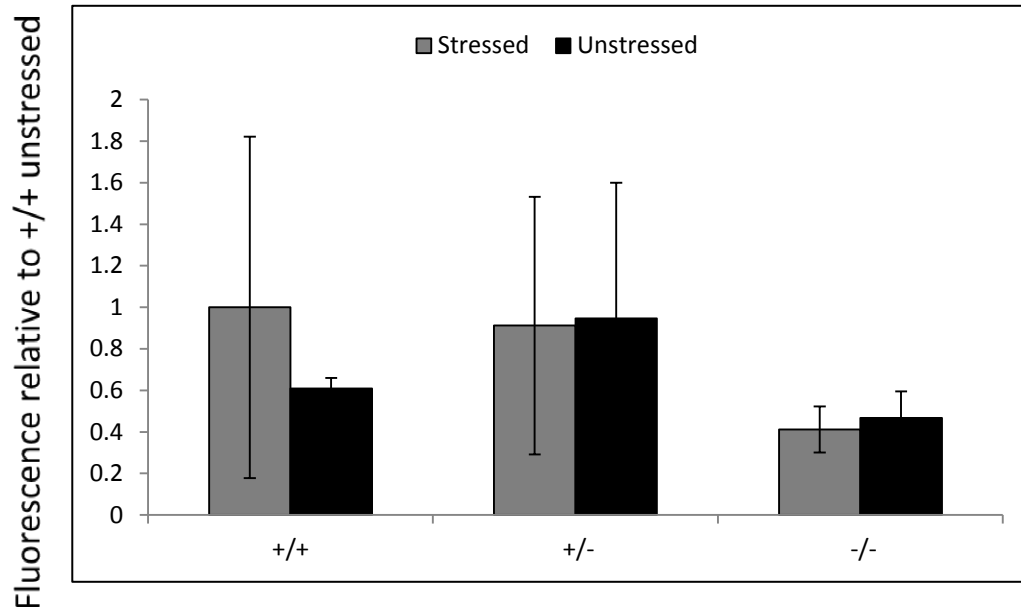


Figure 7.7a) Fluorescence intensity of PABP staining in FUS knockout motor neurons expressed relative to non-transgenic unstressed. There is no significant difference between the lines. (+/+ = homozygous FUS (non-transgenic) $n=3$, +/- = heterozygous knockout $n=3$, -/- = homozygous knockout $n=2$. One way ANOVA. Tukey post-test)

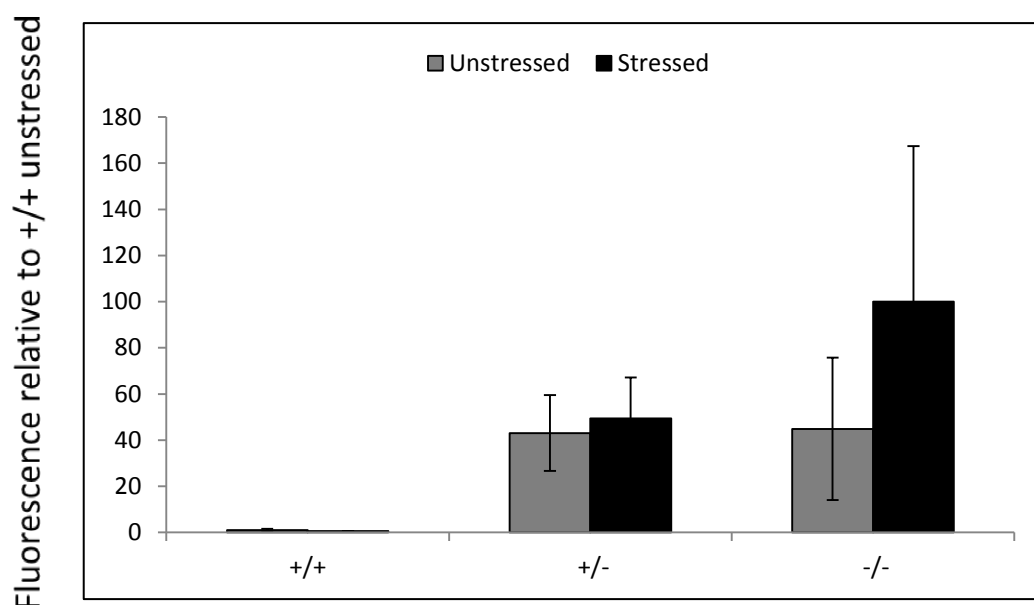


Figure 7.7b) Fluorescence intensity of TIA-1 staining in FUS knockout motor neurons expressed relative to non-transgenic unstressed. There is a lot of variability in fluorescence but no significant difference between the lines.

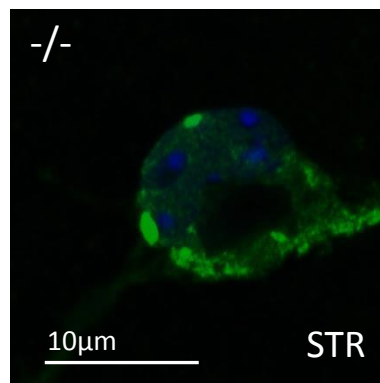
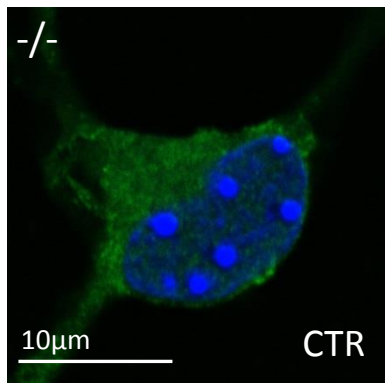
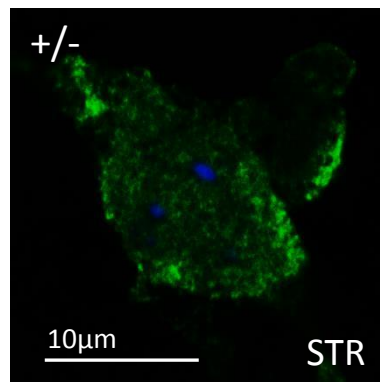
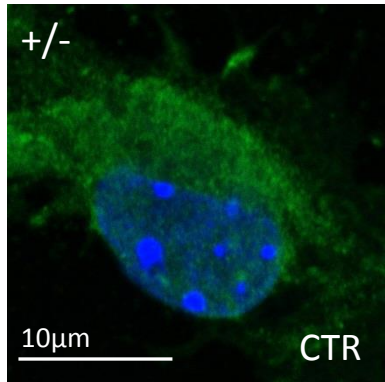
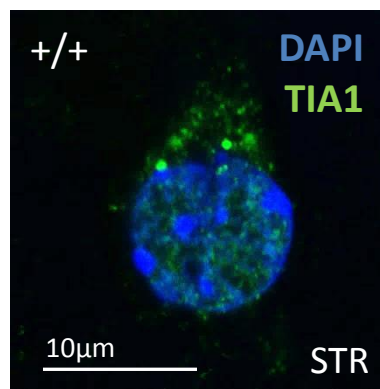
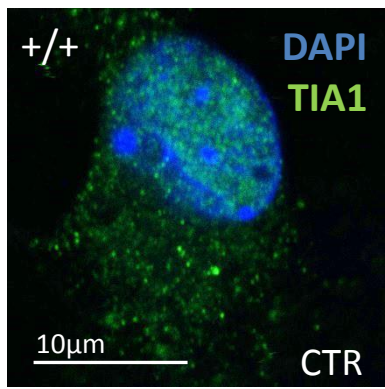
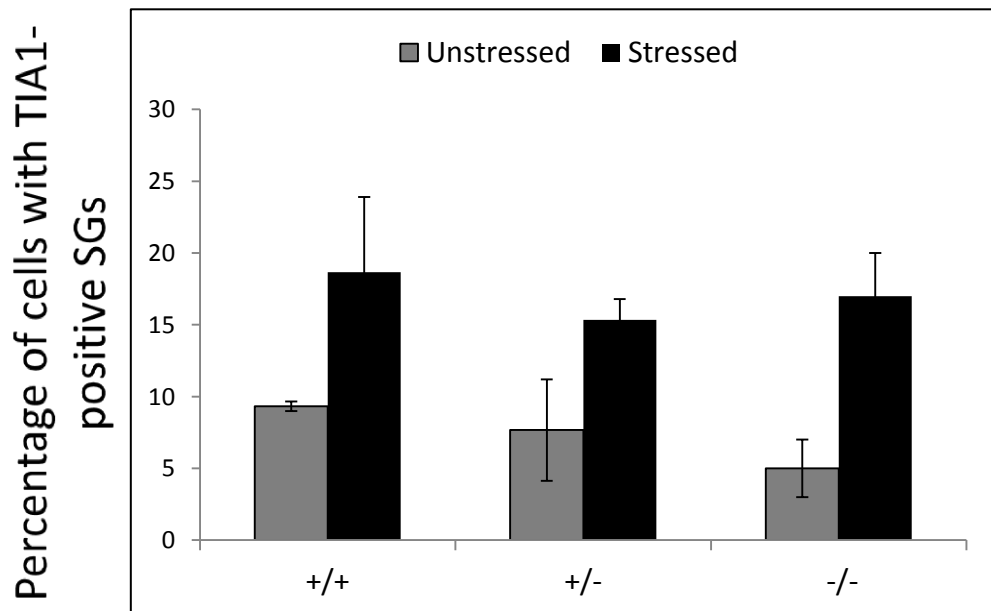


Figure 7.7c)

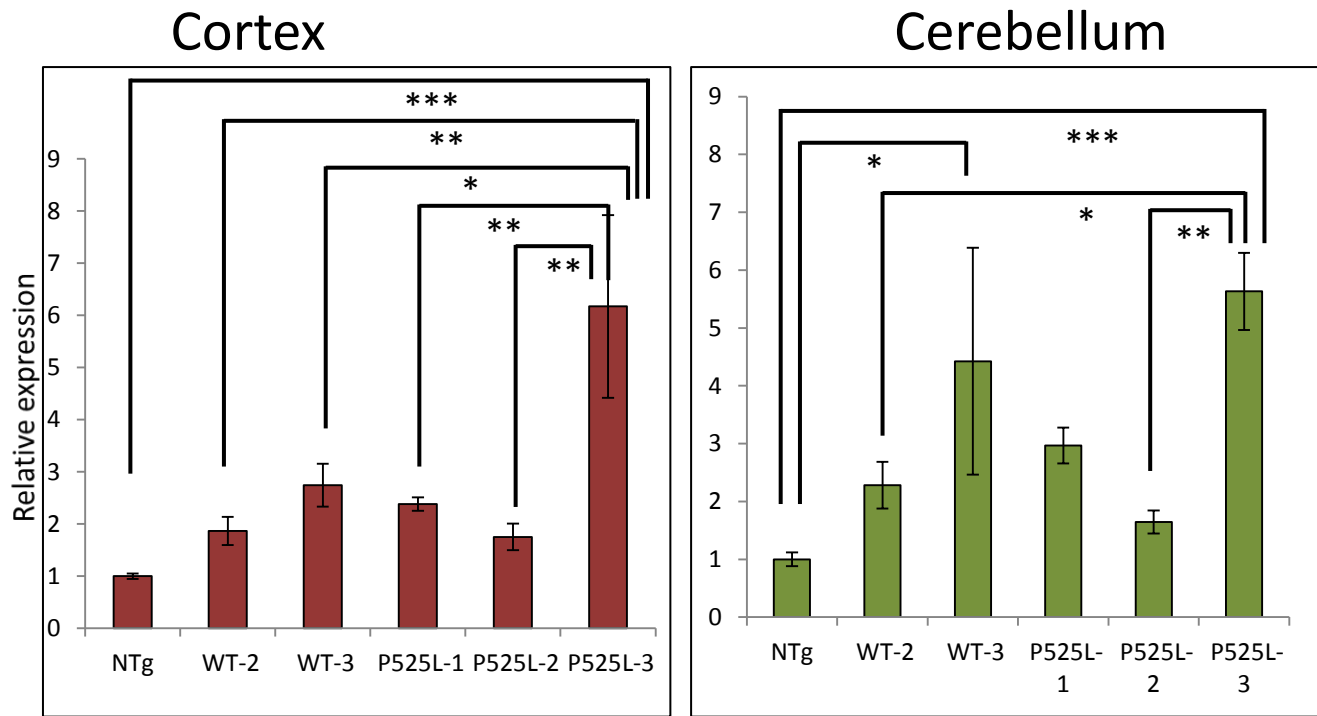
Quantification of the number of TIA1-positive stress granules per motor neuron derived from the FUS knockout model. There is no significant difference between the lines using this antibody.



Line	Mouse ID	Age (days)	Age (full weeks)
NTg	BL6	Unknown	Unknown
	11.1 d	75	10
WT-2	Founder	140	20
	TWFB 2.1g	113	16
	TWFB 4.1F	80	11
WT-3	Founder	140	20
	TWFC 5.1 d	52	7
P525L-1	Founder	492	70
	#55 (TPFA3.1F)	65	9
	TPFA 5.1F	74	10
P525L-2	Founder	421	60
	1.4c	103	14
	5.1e	72	10
	11.1 e	75	10
P525L-3	Founder	421	60
	3.1h	70	10
	5.2d	75	10

Table 7.4. FUS BAC transgenic mice used for RNA extraction for qRT-PCR and splicing analysis.

Total FUS



Spine

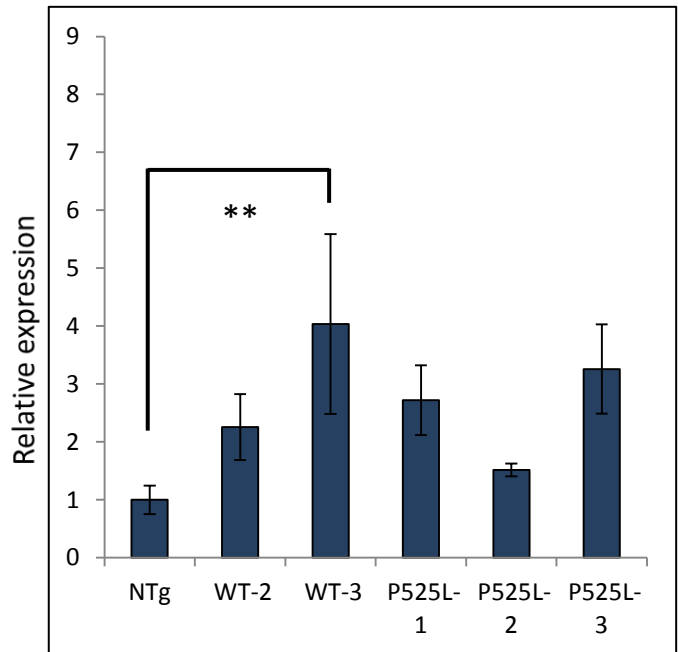
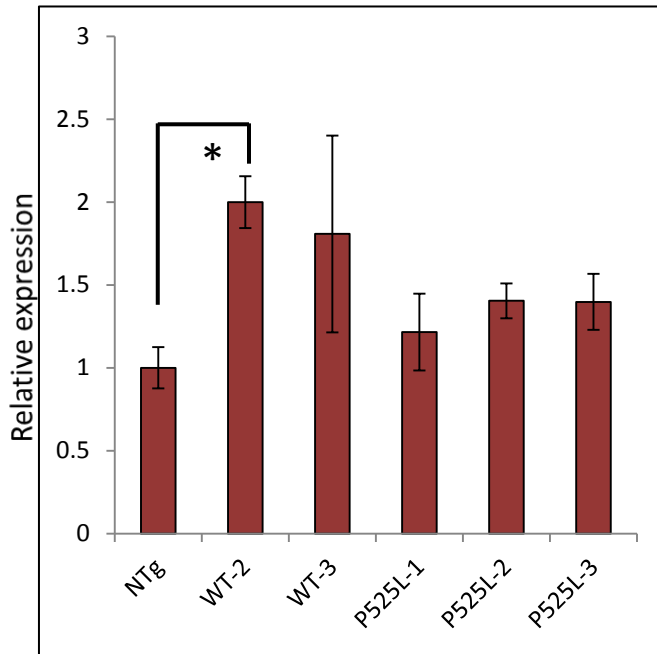


Figure 7.8 mRNA expression in the cortex, cerebellum and spine of the FUS BAC transgenic mouse model as detected by qRT-PCR, showing results by line.

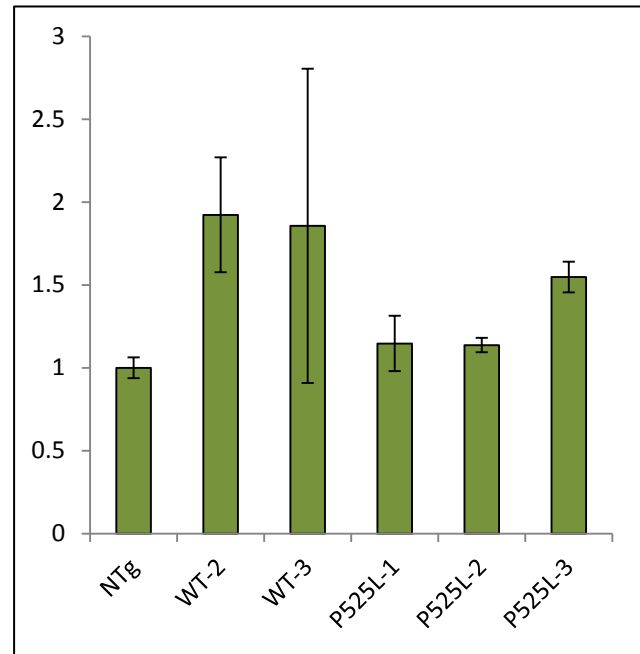
a) Expression of total FUS relative to non-transgenic. There is an increase in total FUS in all transgenic lines, particularly WT-3 and P525L-3 which have the highest copy number of the BAC construct and highest protein level. (N=2-4 per line. One way ANOVA. Tukey post-test)

Endogenous FUS

Cortex



Cerebellum



Spine

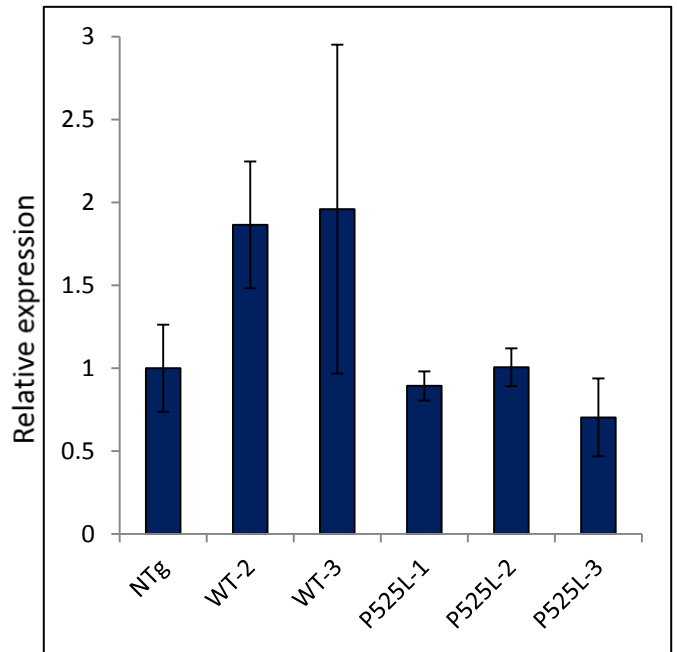
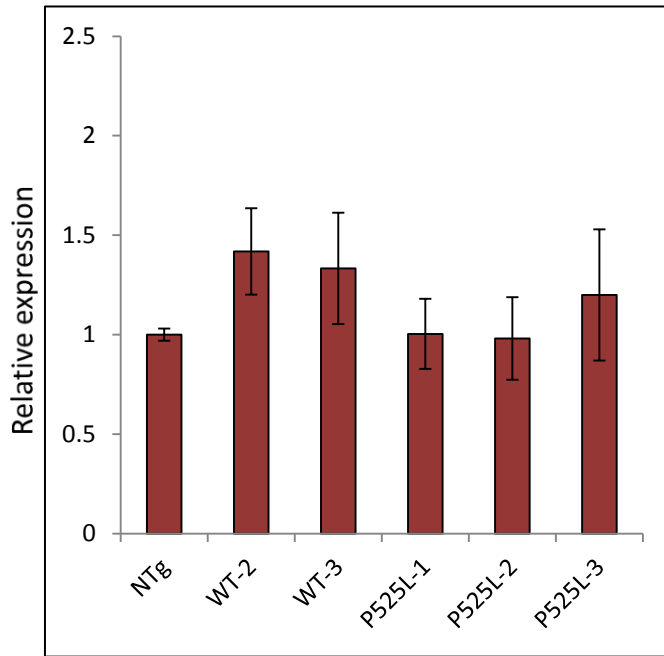


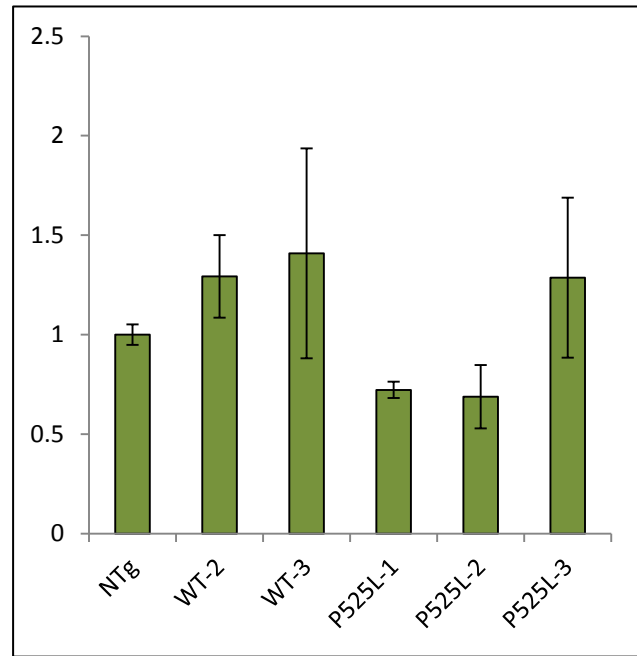
Figure 7.8b) Expression of endogenous FUS relative to non-transgenic in the FUS BAC transgenic mouse model. There is a trend towards an increase in the WT lines compared to non-transgenic and P525L, with a significant increase in endogenous FUS in the WT-2 line compared to non-transgenic.

Endogenous TDP-43

Cortex

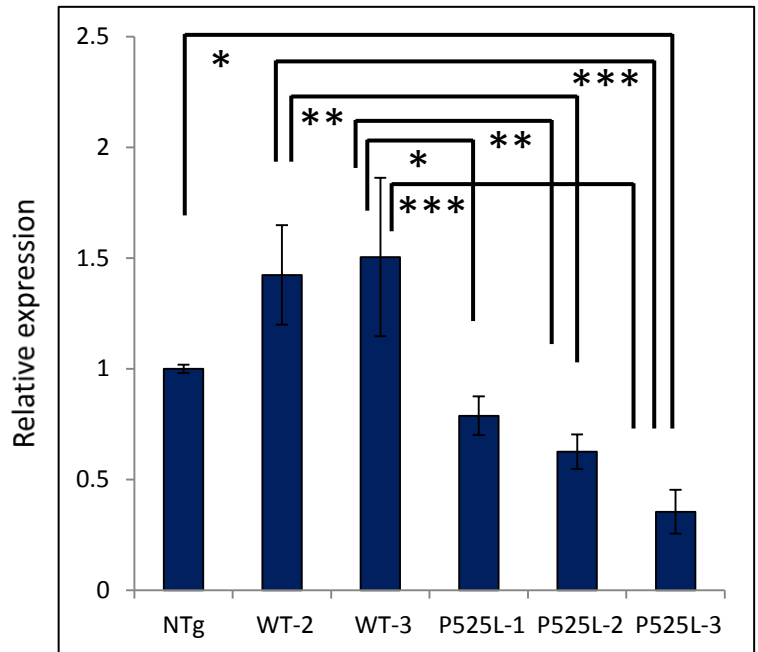


Cerebellum



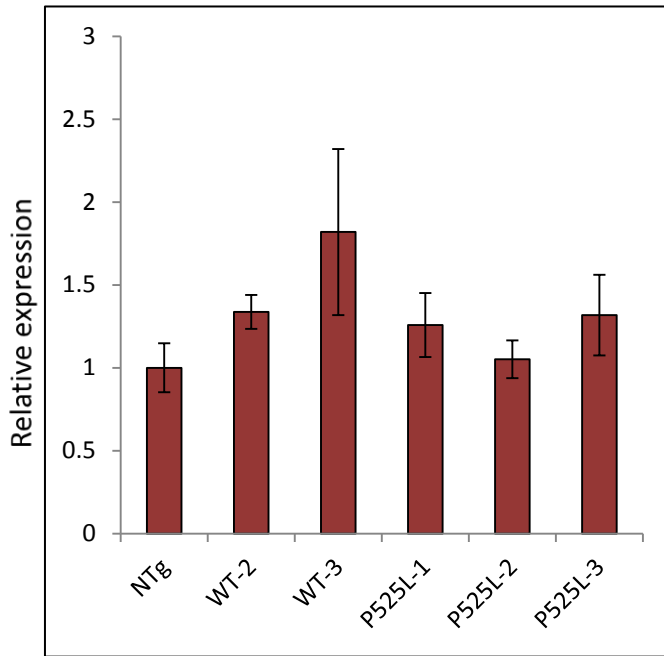
Spine

Figure 7.8c) Expression of endogenous TDP-43 relative to non-transgenic in the FUS BAC transgenic mouse model. There is a trend towards an increase in the WT lines, with significant differences shown in the spine.

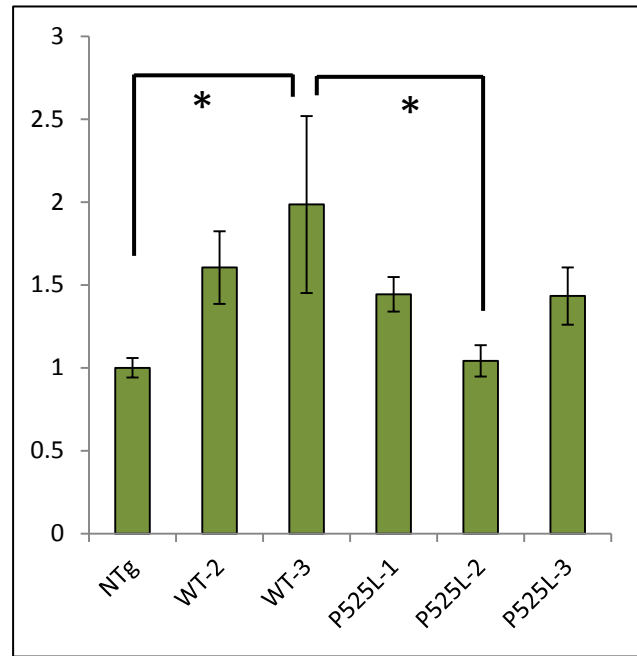


Endogenous SMN

Cortex



Cerebellum



Spine

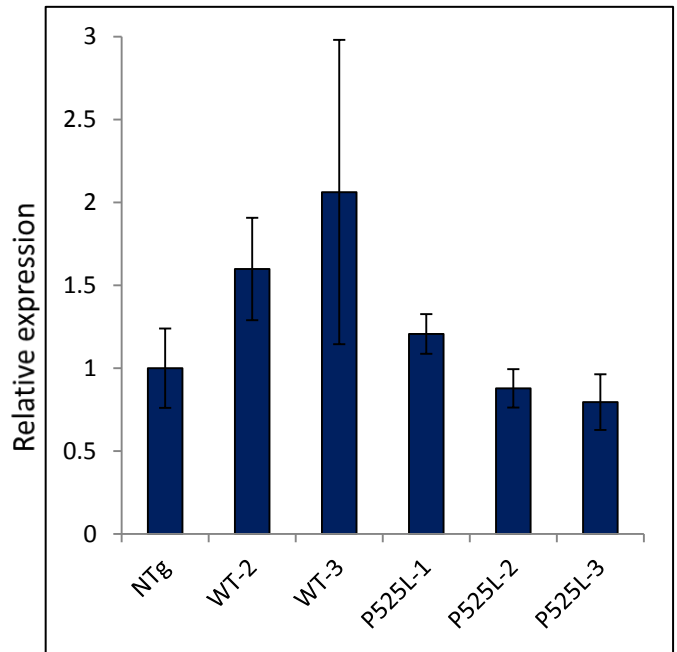
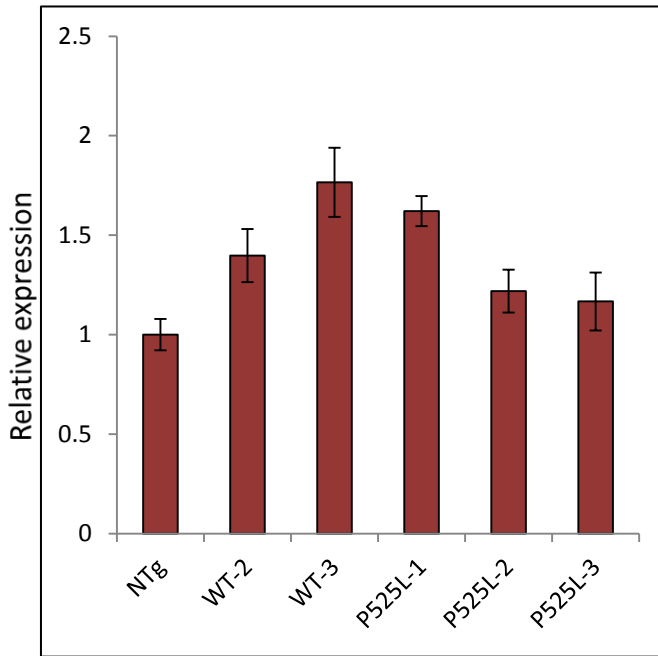


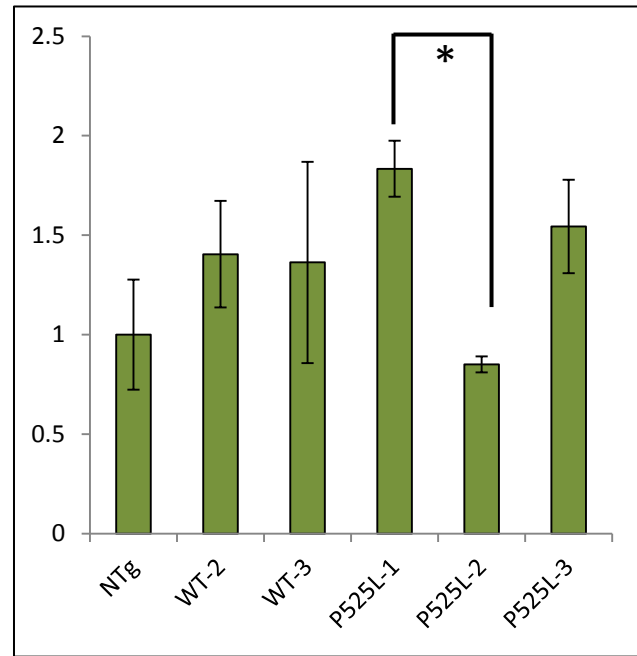
Figure 7.8d) Expression of SMN relative to non-transgenic in the FUS BAC transgenic mouse model. There is a trend towards an increase in wild-type lines compares to non-transgenic and P525L, with a significant increase in the WT-3 line in the cerebellum.

Endogenous TAF15

Cortex



Cerebellum



Spine

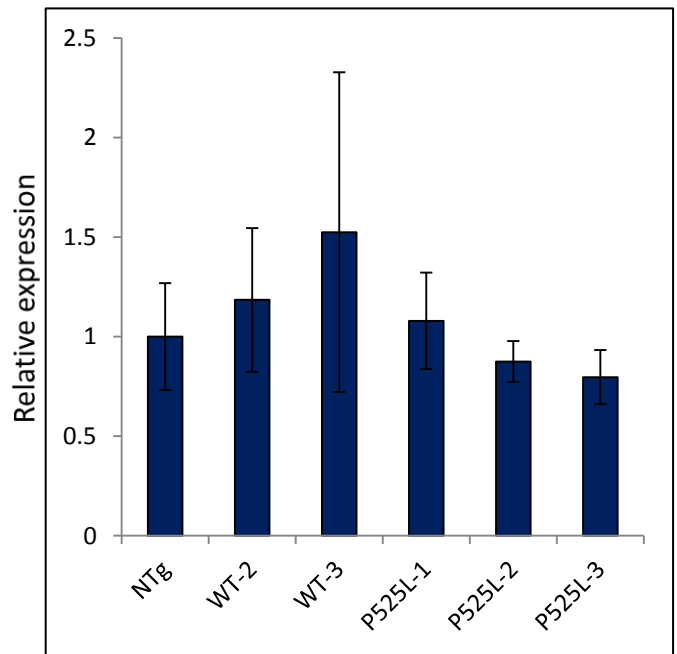
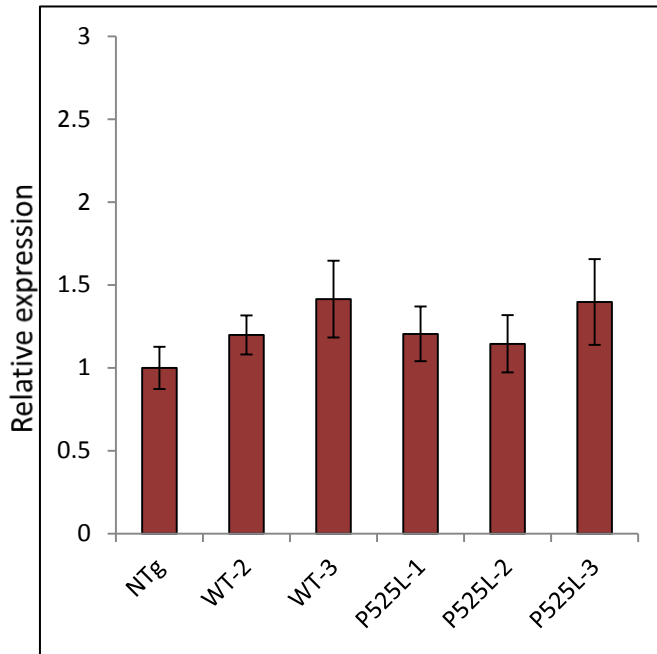


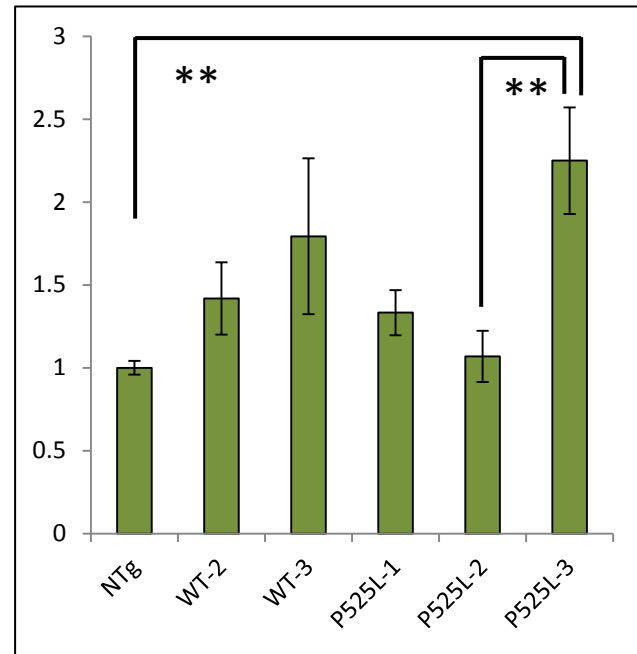
Figure 7.8e) Expression of TAF15 relative to non-transgenic in the FUS BAC transgenic mouse model. The only significant difference is between P525L lines 1 and 2.

Endogenous EWS

Cortex



Cerebellum



Spine

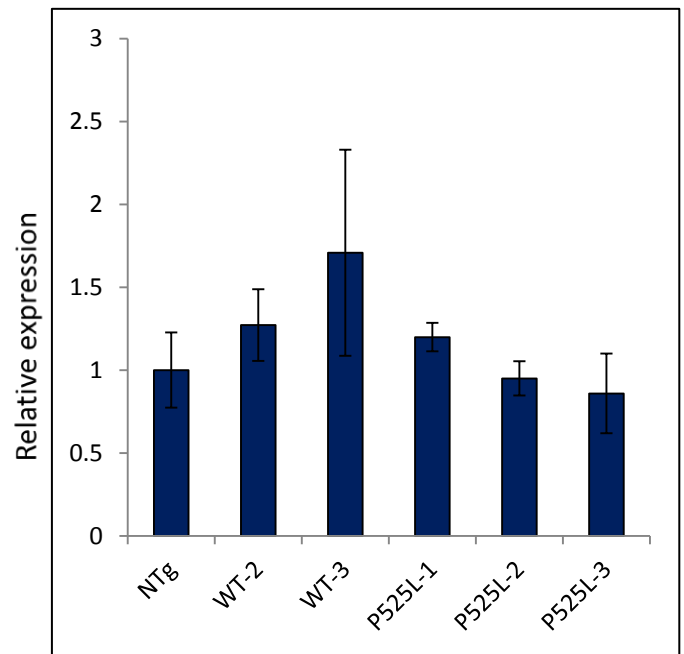
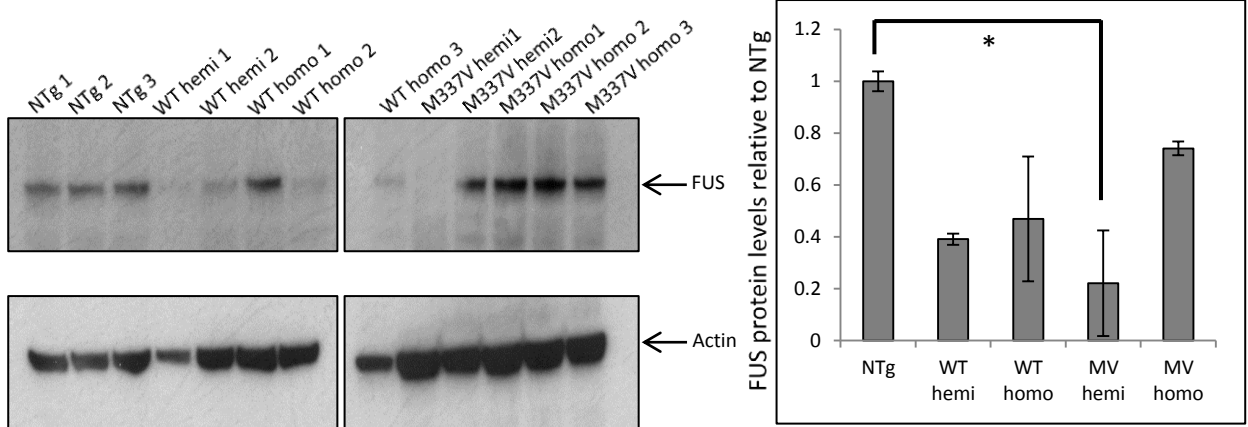


Figure 7.8f) Expression of EWS relative to non-transgenic in the FUS BAC transgenic mouse model. The only significant differences are seen with the P525L-3 line.

FUS

Cortex 3 months



Cortex 9 months

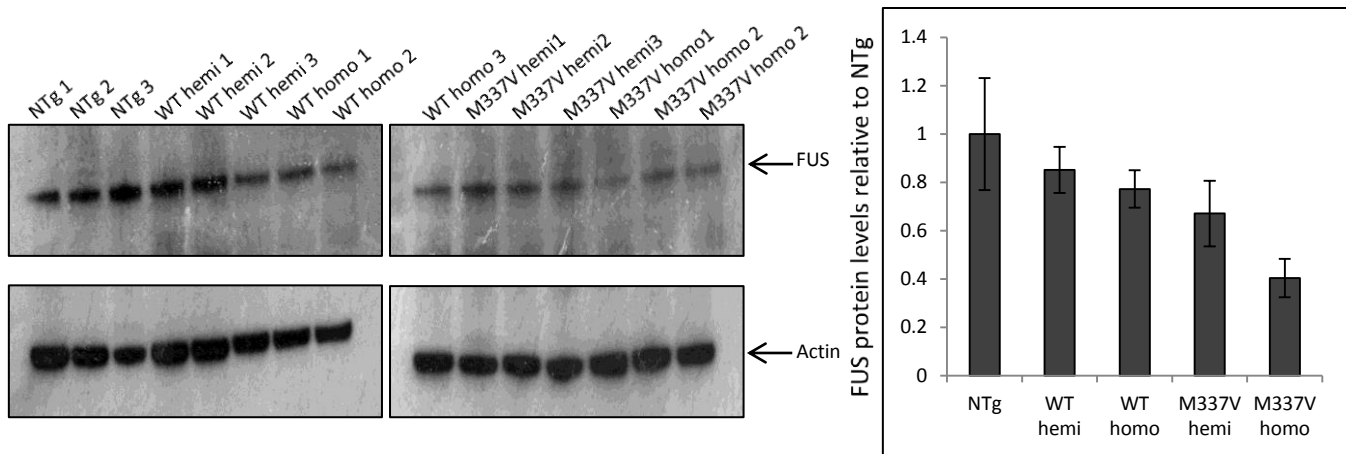
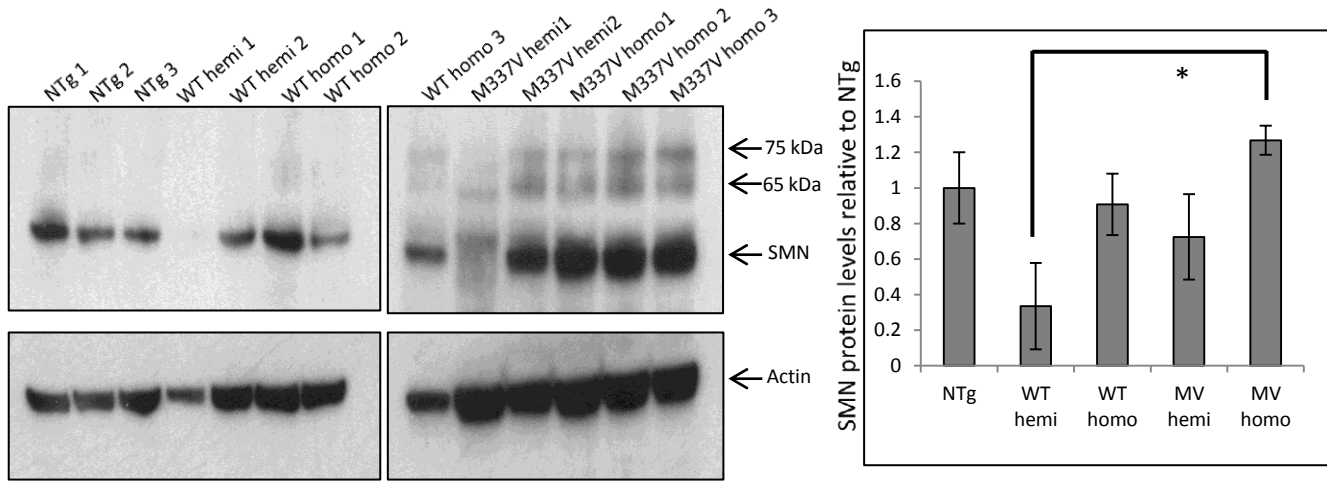


Figure 7.9a) Western blots demonstrating the expression of endogenous FUS relative to actin in the TDP-43 BAC transgenic mouse model. There is a trend towards lower levels of FUS in transgenic lines in the 3 month cortex, and a decrease in FUS levels in the M337V mutant lines at 9 months. (FUS=72 kDa, actin=42 kDa. n=2-3. One-way ANOVA with Tukey post-test. *= $p < 0.05$.)

SMN

Cortex 3 months



Spine 3 months

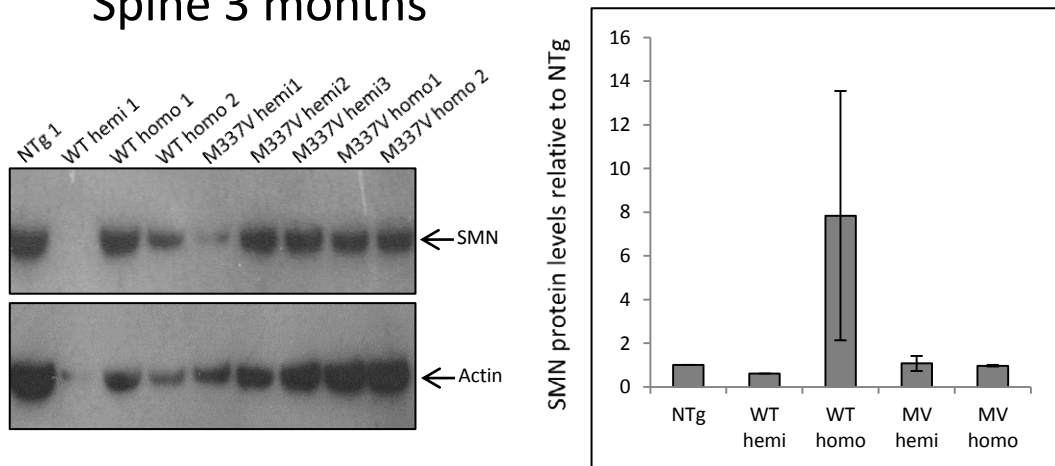
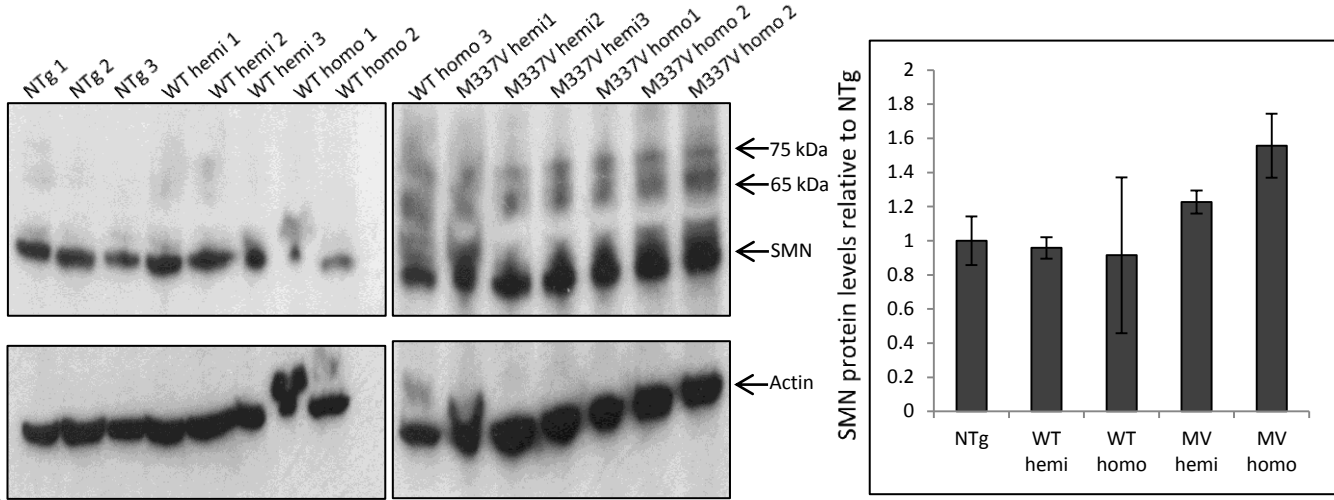


Figure 7.9b) Western blots demonstrating the expression of SMN relative to actin in the TDP-43 BAC transgenic mouse model at 3 months. (SMN=40 kDa)

SMN

Cortex 9 months



Spine 9 months

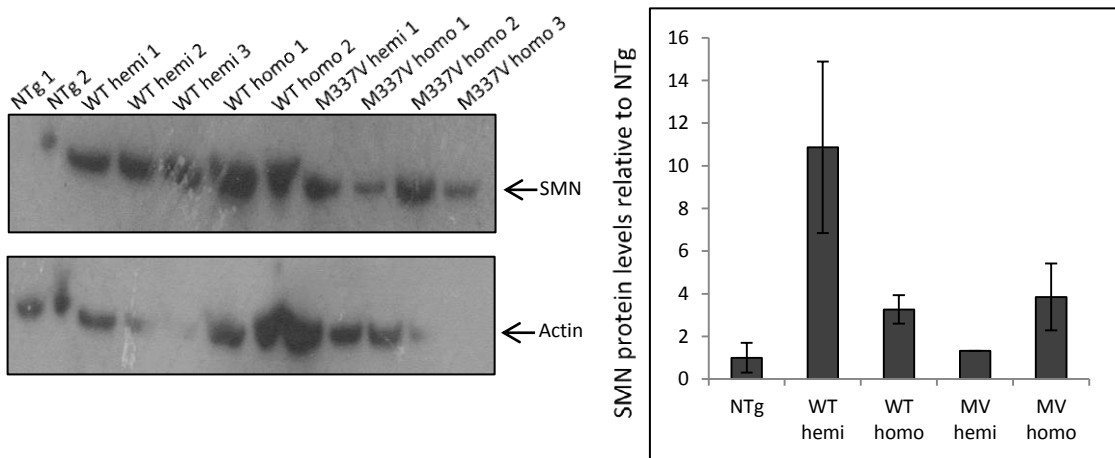
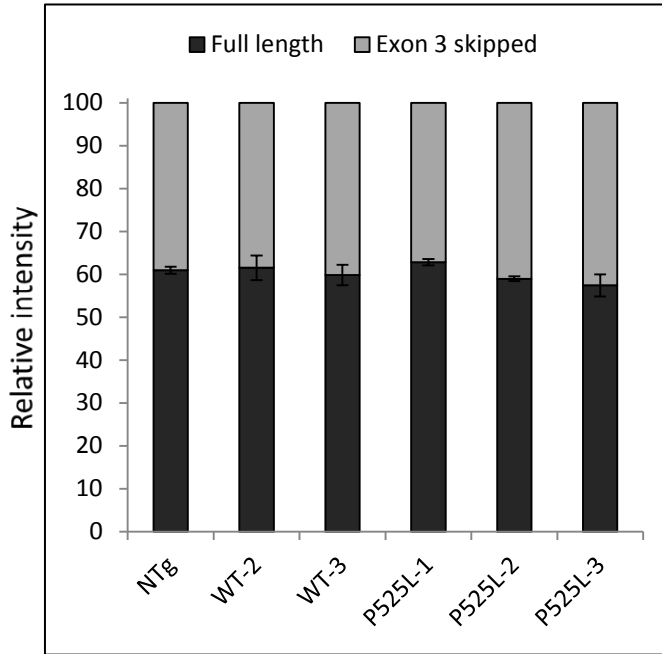


Figure 7.9c) Western blots demonstrating the expression of SMN relative to actin in the TDP-43 BAC transgenic mouse model at 9 months.

Ndrp2

Cortex



Cerebellum

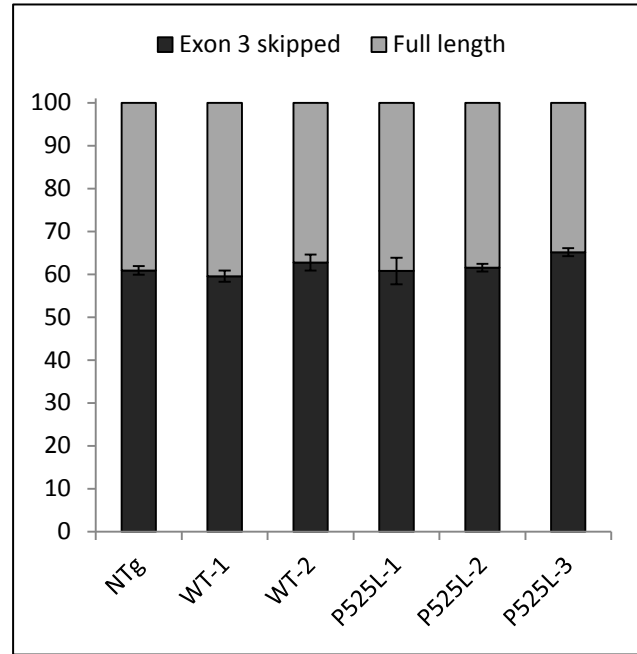
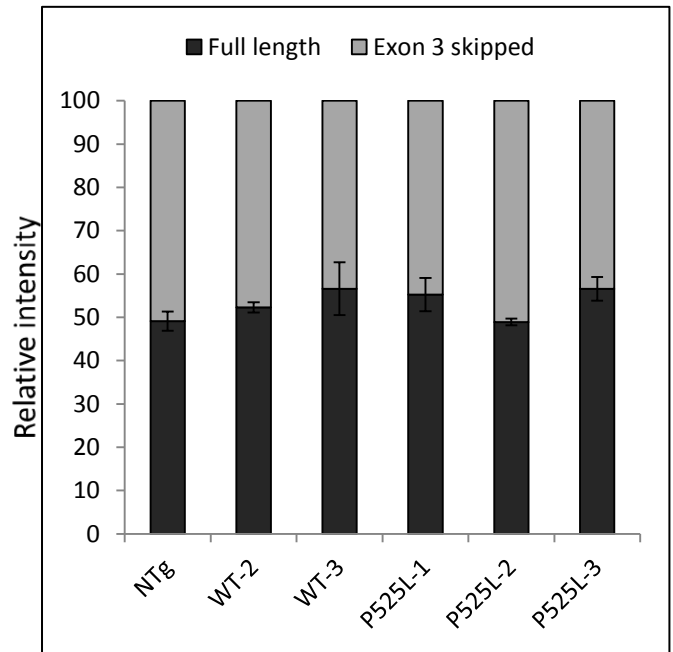


Figure 7.10a) Analysis of alternative splicing in the FUS BAC transgenic mouse model using semi-quantitative PCR. Results are expressed by line. (n=2xNTg, 3xWT-2, 2xWT-3, 3xP525L-1, 4xP525L-2, 3xP525L-3. One-way ANOVA with Tukey post-test. *=p<0.05, **=p<0.01, ***=p<0.001)

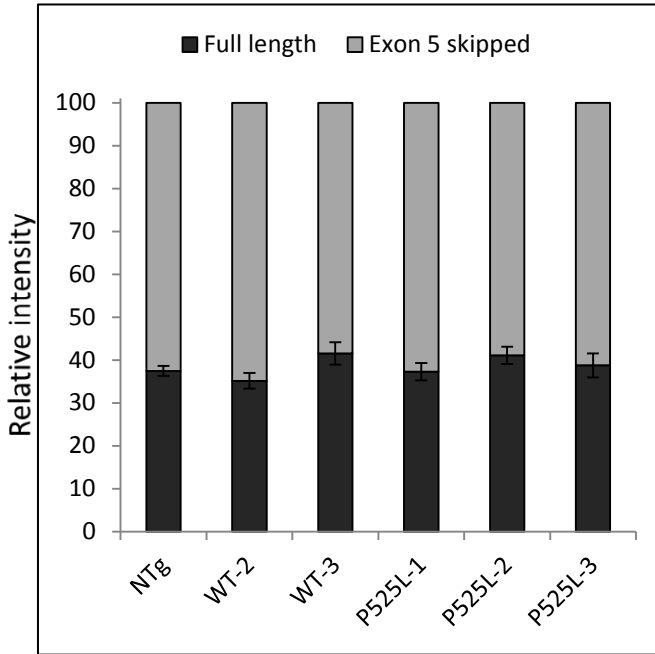
a) Analysis of alternative splicing of Ndrp2 in the FUS BAC transgenic mouse model using primers to detect exon 3 inclusion. There are no significant differences between the lines.

Spine

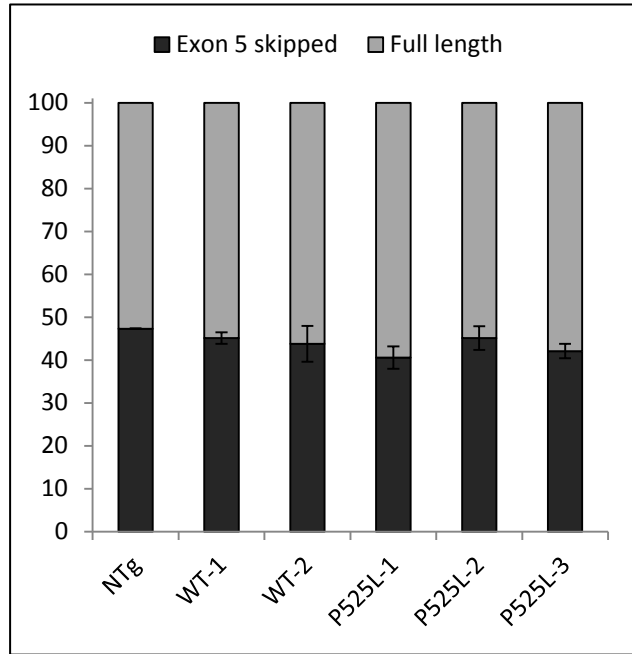


TIA1

Cortex



Cerebellum



Spine

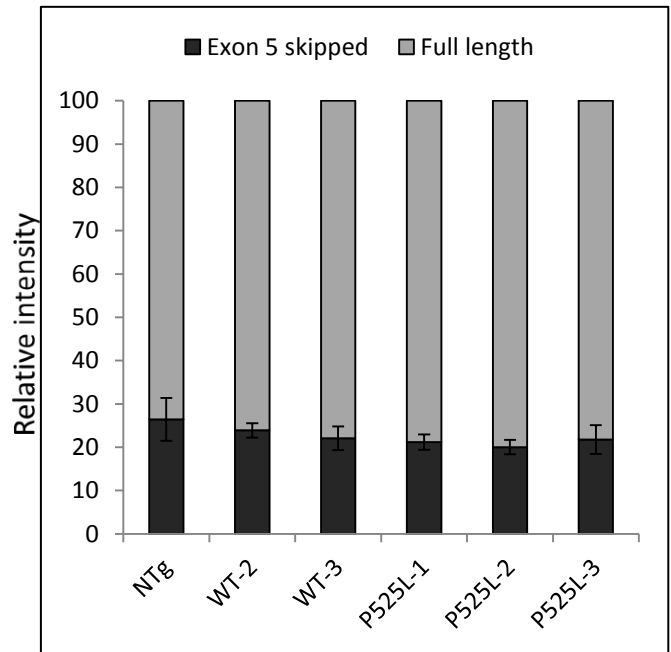
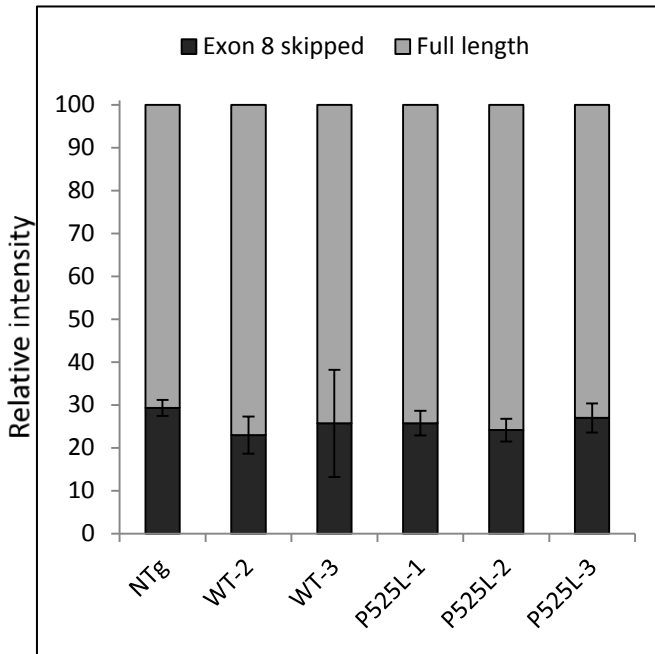


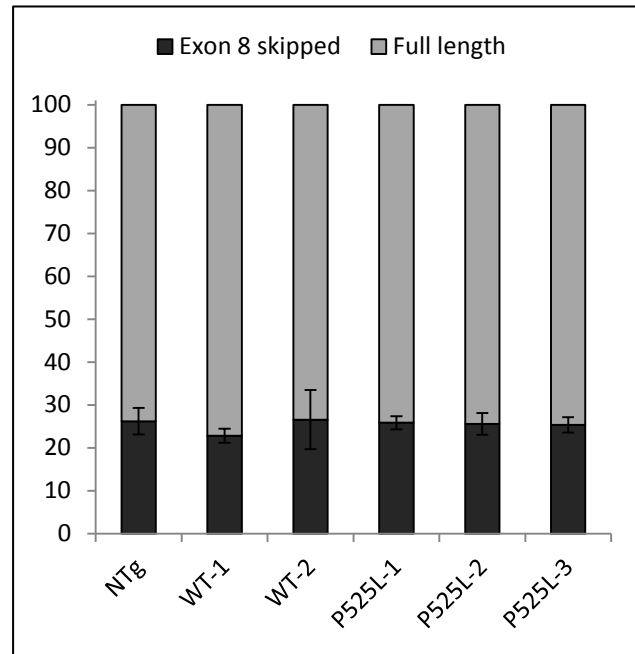
Figure 7.10b) Analysis of alternative splicing of TIA1 in the FUS BAC transgenic mouse model using primers to detect exon 5 inclusion. There are no significant differences between the lines.

Mag

Cortex



Cerebellum



Spine

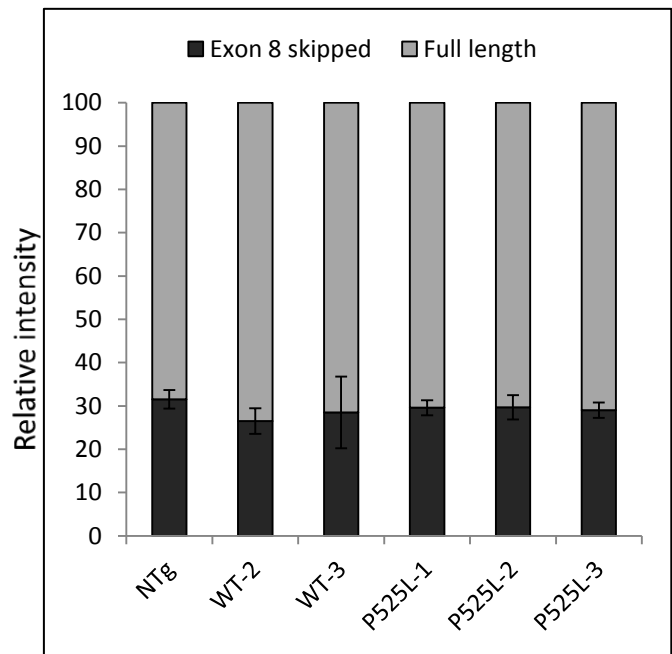
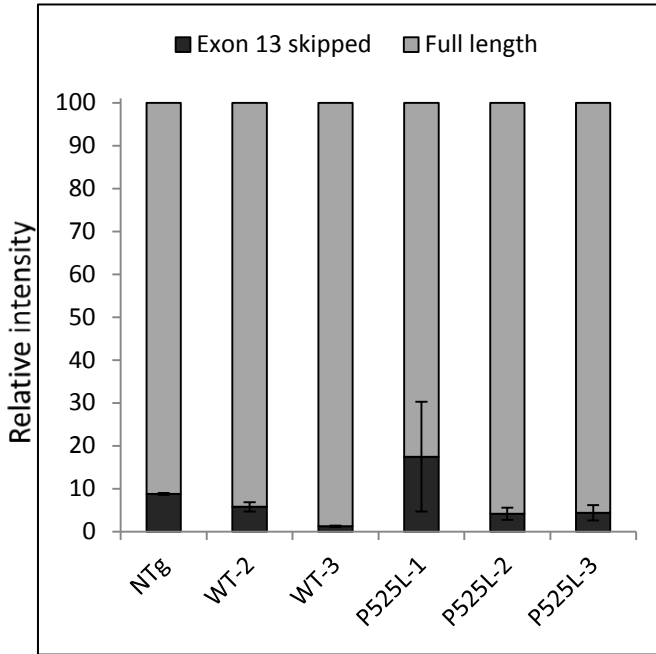


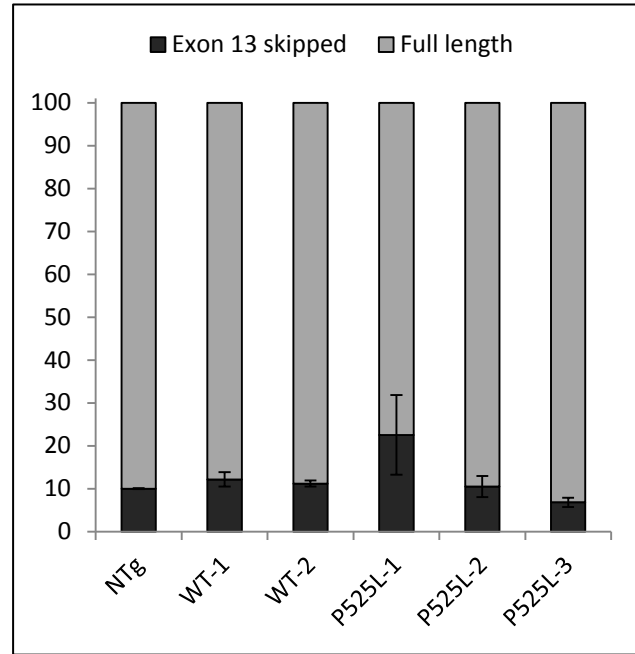
Figure 7.10c) Analysis of alternative splicing of Mag in the FUS BAC transgenic mouse model using primers to detect exon 8 inclusion. There are no significant differences between the lines.

Ppp3ca

Cortex

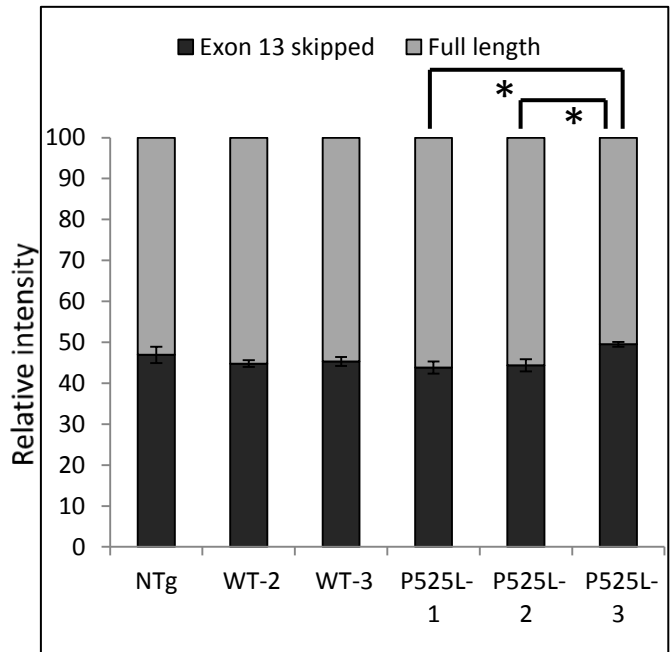


Cerebellum



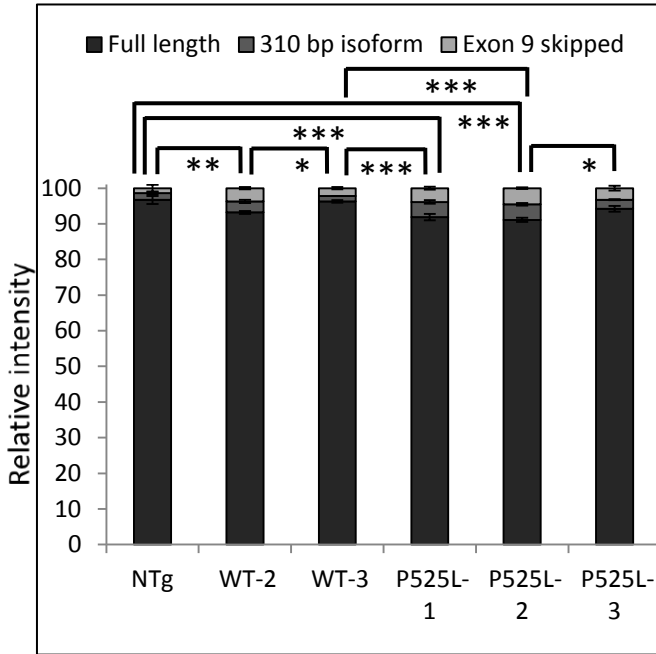
Spine

Figure 7.10d) Analysis of alternative splicing of Ppp3ca in the FUS BAC transgenic mouse model using primers to detect exon 13 inclusion. There is a significant relative decrease in full length product in the P525L-3 line compared to the P525L-1 and P525L-2 lines in the spine.



Dnajc5

Cortex



Cerebellum

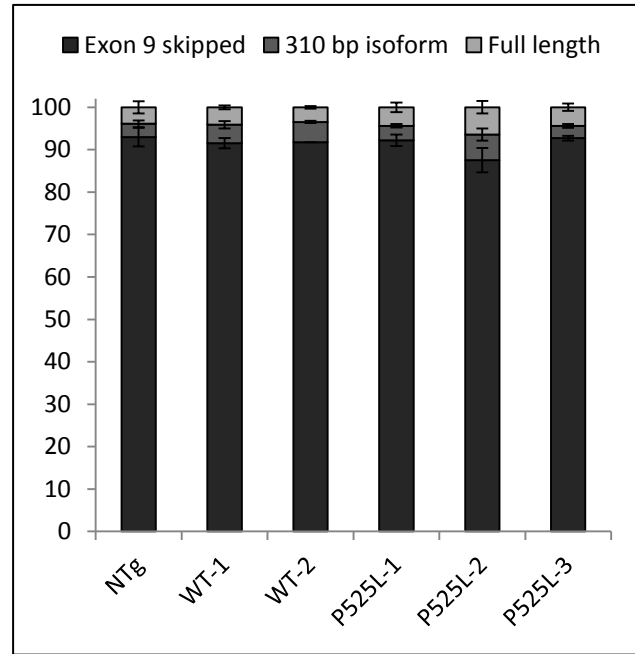
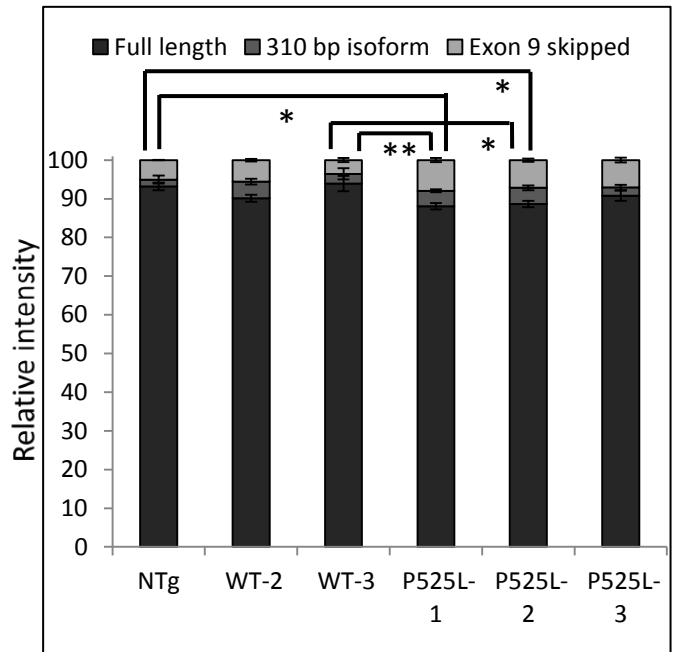
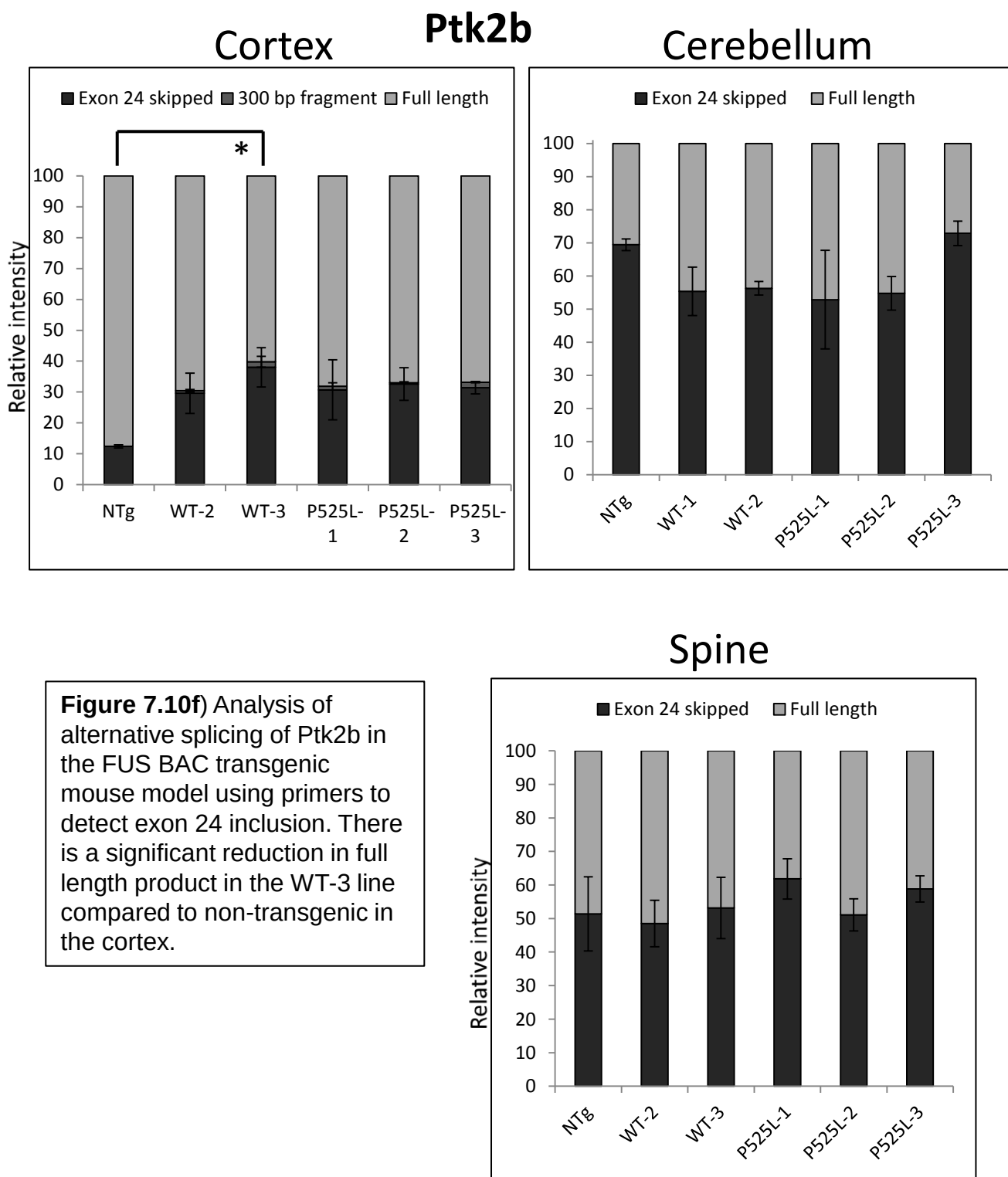


Figure 7.10e) Analysis of alternative splicing of Dnajc5 in the FUS BAC transgenic mouse model using primers to detect exon 9 inclusion. The statistically significant differences between the proportion of exon 9 skipped product are shown.

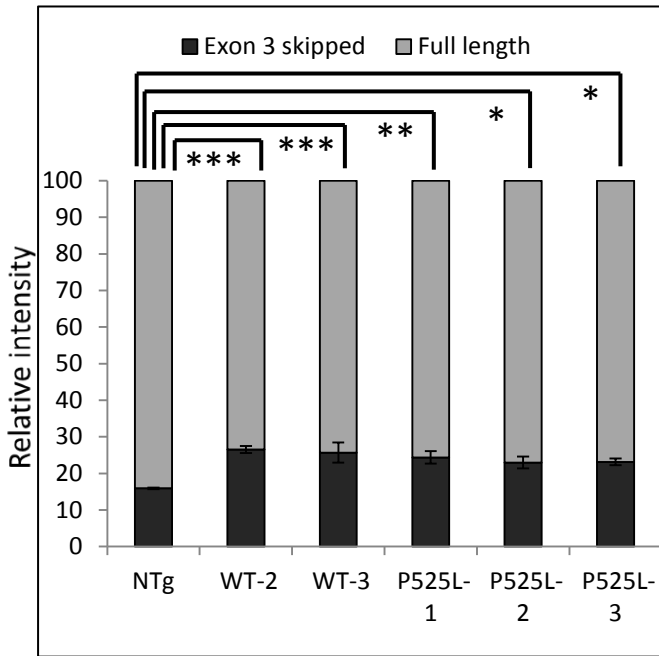
(Other significant results: Cortex: Full length NTg vs WT-2 $P < 0.05$, NTg vs P525L-1 $P < 0.01$, NTg vs P525L-2 $P < 0.001$, WT-3 vs P525L-2 $P < 0.01$. 310 bp product NTg vs P525L-1 $P < 0.001$, NTg vs P525L-2 $P < 0.001$, WT-2 vs WT-3 $P < 0.05$, WT-3 vs P525L-1 $P < 0.001$, WT-3 vs P525L-2 $P < 0.001$, P525L-1 vs P525L-3 $P < 0.01$, P525L-2 vs P525L-3 $P < 0.01$. Spine: Full length NTg vs P525L-1 $P < 0.001$, NTg vs P525L-2 $P < 0.05$, NTg vs P525L-3 $P < 0.05$, WT-2 vs WT-3 $P < 0.05$, WT-2 vs P525L-1 $P < 0.01$, WT-3 vs P525L-1 $P < 0.001$, WT-3 vs P525L-2 $P < 0.001$, WT-3 vs P525L-3 $P < 0.001$. 310 bp product nil significant. Cortex: Nil significant.)

Spine

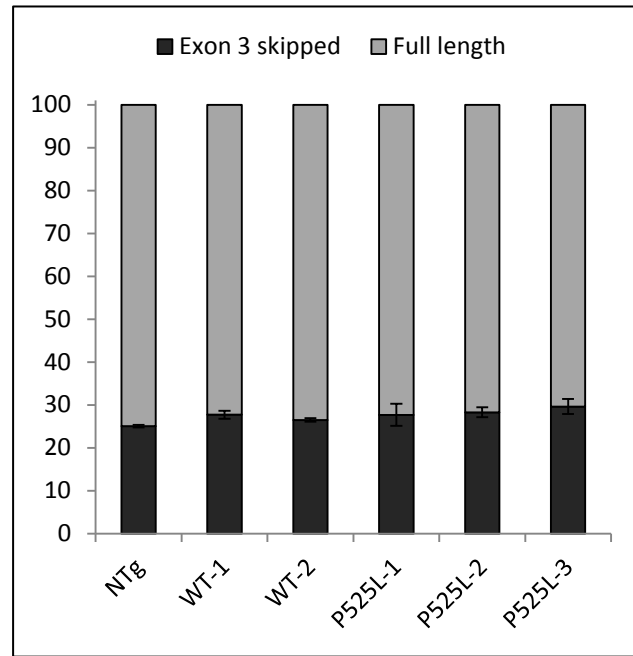




Cortex Ttc3



Cerebellum



Spine

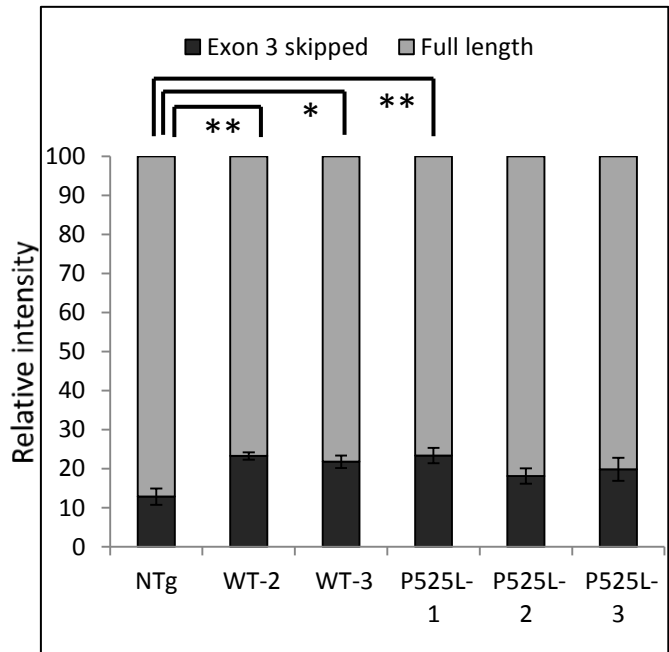
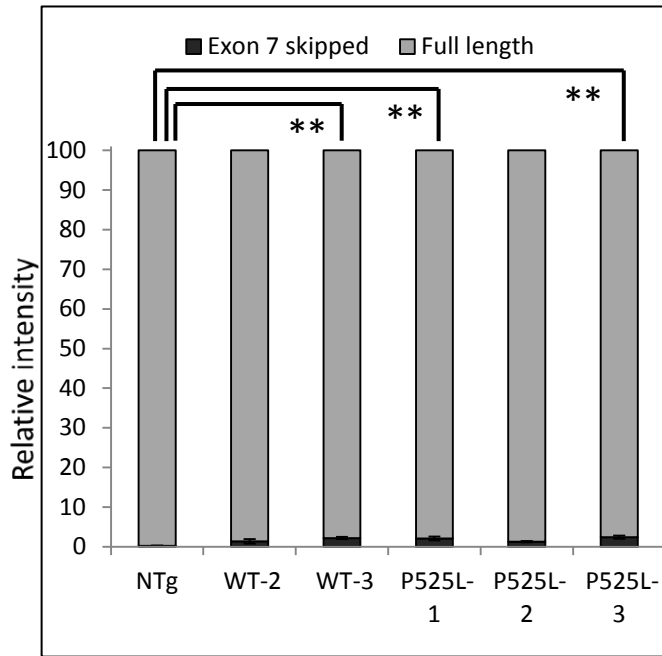


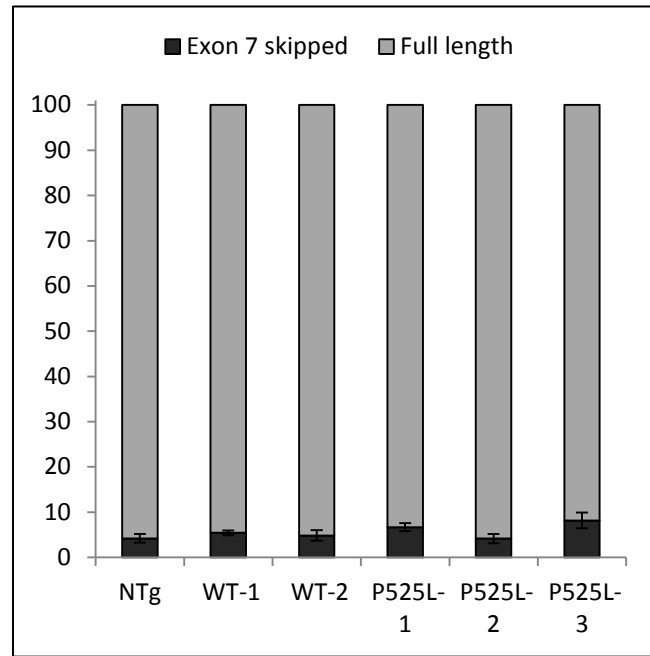
Figure 7.10g) Analysis of alternative splicing of Ttc3 in the FUS BAC transgenic mouse model using primers to detect exon 3 inclusion. There is a significant reduction in full length product in the transgenic lines shown compared to non-transgenic.

FUS

Cortex



Cerebellum



Spine

Figure 7.10h) Analysis of alternative splicing of FUS in the FUS BAC transgenic mouse model. The statistically significant differences between the lines are shown.

