



Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN

Scienze Biomediche e Biotecnologiche

CICLO XXIX

COORDINATORE Prof. Paolo Pinton

**Splicing regulatory role of RNA binding proteins in
suppressing the toxic Ex17b inclusion in human Sort1 mRNA**

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Anni 2013/2016



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DI FERRARA
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29

Titolo della tesi:

Splicing regulatory role of RNA binding proteins in suppressing the toxic Ex17b inclusion in human Sort1 mRNA

Titolo della tesi (traduzione):

Ruolo delle proteine leganti l'RNA nella regolazione dell'inclusione dell'esone 17b nello splicing dell'mRNA umano di Sort1.

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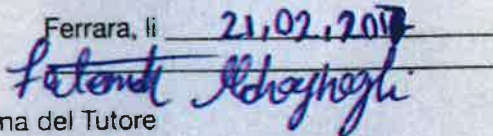
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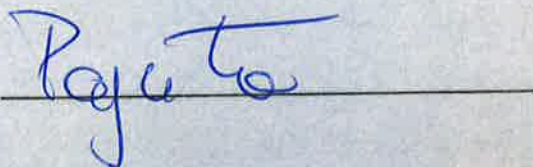
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Acknowledgement

My sincerest thanks and appreciation goes to Prof. Francisco E. Baralle who gave me a great chance to join his team at International Centre for Genetic Engineering and Biotechnology (ICGEB) as a PhD student, and who generously provided me the access to his laboratory and research facilities. Definitely without his precious support it would not be possible for me to carry out this research project.

Besides Prof. Baralle, I would also like to declare my sincere gratitude to my Supervisor Prof. Emanuele Buratti for his unlimited support of my PhD study and related research, for his motivation, patience, and immense knowledge. His guidance helped me in all over my PhD research and in writing of this thesis. I could not have imagined having a better supervisor and educator than him for my PhD study.

I would like to thank my tutor Prof. Franco Pagani and my coordinators Prof. Francesco Bernardi and Prof. Paolo Pinton in University of Ferrara for their encouragement, scientific and moral support all during my PhD study.

Moreover, I would like to thank the thesis reviewers: Prof. Boris Rogelj and Prof. Vincent Anquetil, for their insightful comments and encouragement, and also to the other members of my thesis committee for the questions which made me think to widen my research from different perspectives.

I must also acknowledge all the nice administrative assistants working in the Fellowships Unit of ICGEB specially Ms. Nerina Brandolin, Ms. Tiziana Feriani, and Ms. Barbara Argenti and in the Ufficio Dottorato di Ricerca of Ferrara University specially Ms. Lena Fabbri and also Ms. Claudia La Mela for keeping things running well.

I would like to appreciate all the technical supports from our research technician, Ms. Cristiana Stuani, who enlightened me the first glance of my PhD project.

Acknowledgement

I would like to express my deep thanks to all of my fellow labmates specially Elisa, Kasia, and Flavia for the stimulating discussions, for making friendly scientific environment in the lab, and for all the fun we have had in all over my PhD study. In particular, I am very grateful to Elisa who helped me in accurate translation of my thesis abstract to Italian.

My special thanks goes to my family: my parents and my brothers and sisters for supporting me spiritually throughout working and writing this thesis and my life in general.

Last but not the least, I would love to appreciate all the motivation and encouragement I have been receiving from Prof. Abolhassan Ahmadiani. He is the one professor/teacher who truly made a difference in my life. It was under his tutorship that my interest in research developed and I became highly motivated to continue my academic education in a very high-qualified research center such ICGEB. As a research assistant in Neuroscience Research Center, Shahid Beheshti University of Medical Sciences, he provided me with all the supports possible and became more of a father and friend, than a professor. It was because of his understanding, wisdom and kindness that I was encouraged to apply for a high quality graduate training. I doubt that I will ever be able to convey my appreciation fully, but I owe him my eternal gratitude.

Abstract

Mislocalization and aggregation of RNA-binding proteins, especially TAR DNA-binding protein 43 (TDP-43) and fused in sarcoma (FUS), can impair RNA metabolism and this is a key feature of several neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and Frontotemporal lobar degeneration (FTLD).

In particular it has been shown that TDP-43 could regulate sortilin 1 (Sort1) pre-mRNA splicing. Sort1 encodes a receptor able to regulate the levels of an important neurotrophic growth factor known as progranulin. Indeed, progranulin deficiency is certainly linked to FTLD cases with TDP-43 positive inclusion bodies (FTLD-TDP).

In the past, it has been shown that abnormal deposition of TDP-43 in the cytosolic aggregates leads to inclusion of exon 17b (Ex17b) in Sort1 mRNA. In humans, but not in mouse, this exon contains a premature stop codon and following its inclusion may induce the production of a truncated Sort1 receptor. This short Sort1 receptor can impair progranulin metabolism and help to induce cytosolic TDP-43 aggregate formation, setting up a vicious circle that might eventually cause neuronal death.

In this thesis, I have therefore set out to investigate the splicing elements responsible for differential regulation of Ex17b inclusion in mice as opposed to humans.

By studying mouse and human Sort1 Ex17b and their flanking intronic sequences I have therefore provided a novel insight into the complexity of the RNA-binding protein network that regulates the inclusion of the toxic human exon 17b.

My study has revealed that this differential regulation between human and mouse is achieved in two major ways. First of all, serine/arginine (SR)-rich proteins bound only to mouse and not human Sort1 context and they enhanced Ex17b inclusion. The lack of these splicing enhancers

Abstract

in human sequence is one of the major factors that result in the normal exclusion of toxic Ex17b from human Sort1 mRNA. Secondly, I demonstrated that the inhibition of Ex17b inclusion in Sort1 mRNA was also considerably affected by co-operation of TDP-43 with additional RNA-binding proteins, including hnRNP L, PTB/nPTB, and hnRNP A1/A2 that differentially bound the human exon sequence compare to the mouse exon. Taken together, my results showed that the splicing regulatory elements have been differentially evolved in human vs. mouse Sort1 Ex17b sequences.

Interestingly, mRNA expression of hnRNP A1/A2 and PTB/nPTB was significantly increased in the temporal lobes of FTLD-TDP patients. This observation suggests that in conditions of TDP-43 aggregation the expression of these splicing repressor proteins may increase to help downregulation of the toxic human exon inclusion. Even more in general, my study showed for the first time the importance of other hnRNPs beside TDP-43 in regulation of the disease-related events.

For the future, these data suggest that a comprehensive evaluation of RNA-binding protein network will promote our current knowledge about the role of TDP-43 in the disease pathogenesis and its consequences. This will hopefully open a new window towards obtaining better diagnostic and therapeutic capabilities for FTLD-TDP.

Contents

Acknowledgement.....	1
Abstract.....	3
List of figures.....	10
List of tables.....	12
Abbreviations	13
1 Introduction.....	17
1.1 Frontotemporal lobar degeneration (FTLD).....	17
1.2 FTD/FTLD–disease hallmarks.....	18
1.2.1 Clinical features.....	18
1.2.2 Pathological features.....	19
1.2.2.1 Microtubule-associated protein tau (MAPT).....	19
1.2.2.2 TAR DNA- binding protein 43 (TDP-43)	19
1.2.2.3 Dipeptide-repeat (DPR) species.....	19
1.2.2.4 Fused in sarcoma (FUS)	19
1.2.3 Genetical features	20
1.2.3.1 Microtubule-associated protein tau (MAPT).....	22
1.2.3.2 Progranulin (PGRN)	22
1.2.3.3 C9orf72 repeat expansions.....	23
1.2.3.4 Causative rare genes.....	24
1.3 FTLD Diagnosis	26
1.4 FTLD Treatment.....	27
1.5 Progranulin (PGRN)	27
1.5.1 Physiological roles of PGRN	28
1.5.2 PGRN and nervous system.....	29
1.5.2.1 Neurite outgrowth.....	29
1.5.2.2 PGRN and neuronal ceroid lipofuscinosis (NCL)	29
1.5.3 PGRN and FTLD	30
1.5.3.1. Diagnosis of FTLD-TDP by plasma PGRN levels.....	30
1.6 PGRN receptors	31
1.6.1 TNF receptors.....	31
1.6.1.1 PGRN-TNFR connection and neuronal viability	32
1.6.2 Sort1 Receptor	32
1.7 Sort1 as PGRN receptor in nervous system	34
1.7.1. PGRN regulates neutrite outgrowth independent of Sort1 receptor	34

Contents

1.7.2 Sort1 regulates extracellular PGRN levels.....	34
1.7.3 Sort1 mediates intracellular functions of PGRN.....	35
1.8 Sort1 a therapeutic target for FTLD-TDP	35
1.8.1 Increase of extracellular PGRN levels by manipulation of PGRN-Sort1 axis	35
1.8.2 Does Sort1-PGRN disconnection impair lysosomal function?	36
1.9 RNA metabolism defects and diseases; mis-splicing can lead to neurodegeneration	36
1.10 Misregulation of SORT1 mRNA splicing and FTLD	37
1.11 Pre-mRNA Splicing.....	39
1.12 Alternative splicing.....	41
1.12.1 SR proteins	43
1.12.1.1. SR proteins as splicing enhancers.....	43
1.12.2 hnRNP Proteins.....	43
1.12.2.1 hnRNPs as splicing suppressors	43
1.12.3 Context dependence of alternative splicing regulatory factors	44
1.12.4 Additively and cooperatively function of splicing regulatory factors	44
1.13 TDP-43	45
1.13.1 TDP-43 Pathology.....	46
1.13.2 TDP-43 interacting proteins.....	47
1.13.2.1 Functional consequences of TDP-43 interaction with hnRNPs.....	48
1.14 PGRN deficiency and cytosolic TDP-43 aggregation.....	50
1.15 Nuclear depletion of TDP-43 impairs PGRN metabolism through Sort1 mRNA mis-splicing in human	51
1.16 High Sort1 Ex17b mRNA levels in FTLD-TDP patients	55
1.17 Human and mouse Sort1 Ex17b are differentially evolved regarding to RNA binding proteins	57
1.18 FTLD-TDP mouse model.....	57
1.19 The aim of this study	57
2 Materials and methods	60
2.1 Standard reagents and solutions	60
2.1.1 Chemical sources	60
2.1.2 Solutions	60
2.1.3 Synthetic oligonucleotides.....	61
2.1.4 Enzymes.....	61
2.1.4.1 Restriction enzymes	61
2.1.4.2 DNA modifying enzymes	61
2.1.4.3 Nucleases	61
2.2 Bacteria	61
2.2.1 Bacterial culture media and strain.....	61
2.2.2 Bacterial competent cells.....	62
2.2.3 Bacterial transformation	62
2.3 Plasmid preparation.....	62
2.3.1 Small-scale preparation of plasmid DNA from bacterial cultures (miniprep).....	62
2.3.2 Large-scale preparation of plasmid DNA from bacterial cultures (midiprep).....	63
2.4 Quality and quantity control of the obtained nucleic acids.....	63
2.4.1 Nucleic acid concentration.....	63
2.4.2 UV fluorescence of intercalated ethidium bromide.....	63
2.5 Enzymatic DNA modifications.....	64
2.5.1 Plasmid DNA digestion	64

Contents

2.5.2 Large fragment of E. Coli polymerase I (Klenow), T4 polynucleotide kinase (PNK) and Calf Intestinal Alkaline Phosphatase (CIP).....	64
2.5.3 T4 DNA ligase	65
2.6 Electrophoretic separation of nucleic acids	65
2.6.1 Agarose gel electrophoresis of DNA.....	65
2.6.2 Elution and purification of DNA fragments from the agarose gel	65
2.7 DNA sequencing	65
2.8 Human and mouse SORT1 Ex17b minigene constructs	66
2.9 human-mouse SORT1 Ex17b hybrid minigene constructs	66
2.9.1 Human exon mutations	66
2.9.2 Human upstream intron modification	66
2.10 Cell culture	67
2.10.1 Cell lines used for the experiments.....	67
2.10.2 Maintenance and analysis of cells in culture.....	68
2.11 Transient transfection of the cell lines	68
2.11.1 In Hep-3B, SH-SY5Y, SK-N-BE, and N-muli cells.....	68
2.11.2 In NSC-34 and Neuro-2a cell lines.....	69
2.11.3 Transient co-transfection of Neuro-2a cell lines.....	69
2.12 RNA extraction.....	70
2.13 mRNA analysis by RT-PCR.....	70
2.13.1 Reverse transcription (RT)	70
2.13.2 RT-PCR amplification	71
2.14 Endogenous mouse Sort1	71
2.15 Densitometric analysis of RT-PCR bands	71
2.16 Small interfering RNA (siRNA) transfections.....	72
2.16.1 The siRNA sense sequences used for gene knockdown.....	72
2.16.2 In Hep-3B and N-muli cell lines	72
2.16.3 In NSC-34 cells.....	73
2.16.4 In SH-SY5Y, SK-N-BE, and Neuro-2a cell lines.....	73
2.16.5 siRNA co-transfection of Neuro-2a cell lines.....	74
2.17 Protein extraction	75
2.18 SDS-page gel electrophoresis	75
2.19 Western blot and antibodies.....	76
2.20 pull down analysis.....	77
2.20.1 DNA templates.....	77
2.20.2 In vitro RNA transcription.....	78
2.20.3 Pull down analysis.....	79
2.21 qRT-PCR analysis on human brain samples.....	80
2.22 Statistical analysis.....	81
3 Results	82
3.1 TDP-43 splicing regulation of Sort1 Ex17b in different mouse and human cell contexts .82	82
3.1.1 Human and mouse Sort1 Ex17b minigenes constructed in pTB vector backbone	82
3.1.2 Transfection of human and mouse Sort1 Ex17b minigene constructs in different human and mouse cell lines following TDP-43 depletion.....	84
3.2 Identification of hnRNPs that differentially bind to human and mouse Sort1 Ex17b 88	88
3.2.1 Subcloning of distinct exonic and intronic regions of human and mouse Sort1 Ex17b in pBluescript II KS+ plasmid	88

Contents

3.2.2 Pull down assay to map hnRNPs binding sites in human and mouse Sort1 Ex17b contexts	90
3.2.3 Similar binding patterns of hnRNPs to both human and mouse Sort1 Ex17b contexts	91
3.2.4 Different binding patterns of hnRNPs to human and mouse Sort1 Ex17b contexts	93
3.3 Binding of hnRNP A2 to human Ex17b correlates with splicing repression	94
3.3.1 Substitution of exclusive mouse exonic nucleotides in human, enhanced Ex17b inclusion in the human context	94
3.3.2 Substitution of exclusive mouse exonic nucleotides in human, abolished hnRNP A2 binding to human Ex17b	96
3.4 hnRNP proteins work co-operatively with TDP-43 to repress Ex17b inclusion	98
3.4.1 Single knock down of hnRNPs did not affect Sort1 Ex17b splicing	98
3.4.2 Simultaneous knock down of hnRNPs with TDP-43 affected Sort1 Ex17b splicing regulation	101
3.4.2.1 TDP-43 could repress inclusion of Ex17b more efficiently through co-operation with other hnRNPs	101
3.4.2.2 Co-knock down of other hnRNPs in the presence of TDP-43; TDP-43 the main splicing regulatory factor for Sort1 Ex17b	104
3.4.2.3 Co-operative function of TDP-43 and other hnRNPs to suppress inclusion of endogenous Sort1 Ex17b	106
3.5 Identification of a Sort1 Ex17b splicing enhancer in the mouse upstream intron	109
3.5.1 Binding of splicing enhancers to the upstream intronic region of mouse Sort1 Ex17b ...	109
3.5.2 Splicing enhancing function of SR proteins following binding to the mouse upstream intron	111
3.5.2.1 Generation of human-mouse hybrid minigenes by human upstream intron modification	111
3.5.2.2 Increase of Sort1 Ex17b inclusion in human-mouse hybrid following TDP-43 depletion	114
3.5.2.3 Splicing enhancing function of SRp40/SRSF5 following TDP-43 depletion	116
3.5.2.4 Splicing enhancing function of Tra-2 β following TDP-43 depletion	118
3.6 Altered mRNA levels of hnRNP proteins in the brain of FTLTDP patients	120
3.7 New insights to splicing and neurodegeneration; the other related projects in our laboratory	122
3.7.1 hnRNPs function as general TDP-43 toxicity modifiers	122
3.7.2 Gene variants that are involved in Parkinson's disease can affect pre-mRNA splicing ..	124
4 Discussion	125
4.1 Impairment of splicing regulation and FTLTDP disorder	125
4.2 Strike splicing regulation of Sort1 Ex17b inclusion in human	127
4.3 Mapping the binding sites of expected hnRNPs and SR protein in mouse and human Sort1 Ex17b context	127
4.3.1 hnRNPs bound to human Sort1 Ex17b in a higher extend	127
4.3.2 SR protein binding to mouse upstream intron of Ex17b	128
4.4 Splicing evolution toward repression of Sort1 Ex17b in human; human Ex17b splicing silencers versus mouse Ex17b splicing enhancers	129
4.5 Co-operation of TDP-43 with hnRNPs to repress Sort1 Ex17b inclusion	130
4.5.1 TDP-43, the only repressive hnRNP that can inhibit Sort1 Ex17b inclusion directly and independently	130
4.5.2 Redundant splicing function of hnRNPs with TDP-43 to inhibit Ex17b inclusion	130
4.6 The model presenting binding of splicing regulators to human and mouse Sort1 Ex17b and their functions	132
4.7 Compensatory expression of hnRNPs in the brain of FTLTDP patients	133
4.8 Conclusion	135

Contents

Bibliography	136
Appendix	155
Publications related to this Project.....	155

List of figures

Figure 1.1. Genetic and pathological features of FTL and ALS sometimes overlap.....	21
Figure 1.2. PGRN structurally is made of 7.5 granulin units, including p, G, F, B, A, C, D, and E.	29
Figure 1.3. Three main domains of Sort1 receptor.	33
Figure 1.4. Chemical reactions mediated by spliceosome in order to remove the introns and join vicinal exons;	40
Figure 1.5. Schematic of splicing regulation.	42
Figure 1.6. Schematic of TDP-43 structure with different amino acidic domains.	46
Figure 1.7. The presented Pie chart shows the proportion of different TDP-43 protein interactors. (Freibaum et al. 2010).	48
Figure 1.8. Inclusion of mouse Sort1 Ex17b in the mature mRNA.....	52
Figure 1.9. Human and mouse Sort1 Ex17b nucleotide sequence homology.	53
Figure 1.10 The effect of TDP-43 knockdown on human and mouse Ex17b inclusion in human HeLa cells.....	54
Figure 1.11. Based on the loss of function theory, nuclear depletion of TDP-43 through Sort1 mRNA mis-splicing impairs PGRN metabolism in human.	56
Figure 3.2. Sort1 Ex17b splicing regulatory role of TDP-43 in different cellular contexts.	88
Figure 3.3. Subcloning of human and mouse Sort1 Ex17b in pBluescript II KS+ plasmid.	89
Figure 3.4. Similar binding patterns of hnRNPs to human and mouse Ex17b contexts.....	92
Figure 3.5. Different binding patterns of hnRNPs to human and mouse Sort1 Ex17b contexts.	93
Figure 3.6. Mouse exclusive exonic nucleotides enhance Ex17b splicing in human context. .	95
Figure 3.7. Mouse exclusive exonic nucleotides abolished binding of hnRNP A2 to human Ex17b sequence.	97
Figure 3.8. Single knock down of hnRNPs without affecting the basal human or mouse Sort1 Ex17b inclusion levels.	101
Figure 3.10. Effects on human and mouse Ex17b recognition following knock down of hnRNPs in the presence of TDP-43.	106
Figure 3.11. Effects of knocking down diverse hnRNPs on endogenous mouse Sort1 Ex17b in Neuro-2a cells.	109
Figure 3.12. Binding sites identification of SR proteins in the mouse upstream intron of Sort1 Ex17b sequence.....	110
Figure 3.13. Intronic sequences used to generate human-mouse Sort1 Ex17b hybrids.	112

List of Figures

Figure 3.14. Generation of human-mouse Sort1 Ex17b hybrids.	113
Figure 3.15. Identification of a splicing enhancer of Ex17b in the mouse upstream intron... ..	115
Figure 3.16. Overexpression of SR protein factors and Tra-2 β in Neuro2a cells to determine their effects on endogenous mouse Ex17b expression levels.	117
Figure 3.17. Silencing of Tra-2 β in Neuro-2a cells to determine its effect on endogenous mouse Ex17b inclusion levels.	119
Figure 3.18. The RNA levels of several hnRNP proteins is altered in the brain of FTLD-TDP patients.	121
Figure 3.19. Rescue of TDP-43 controlled pre-mRNA splicing.	124
Figure 4.1. Comparison of the splicing regulatory proteins binding to the human and mouse 17b exons.....	133

List of tables

Table 1.1. Sort1 mRNA isoforms in different placental mammals.	38
Table 2.1. Oligonucleotides used for Ex17b amplification from Sort1 minigenes.	66
Table 2.2. Oligonucleotides used to construct human-mouse Sort1 minigenes	67
Table 2.3. Oligonucleotides for endogenous Sort1 amplification.	71
Table 2.4. Oligonucleotides used for different gene knockdown.	72
Table 2.5. Oligonucleotides used for subcloning of separated Sort1 sequences in pBluescript II KS+.	78
Table 2.6. T7 polymerase sequence included oligonucleotides used to make DNA templates for pull down assay.	78
Table 2.7. Oligonucleotides used to amplify hnRNPs in patients brain by qRT-PCR	81

Abbreviations

All the abbreviations are indicated also in the text when introduced for the first time.

ALS: amyotrophic lateral sclerosis

apoAII: apolipoprotein A-II

APS: ammonium persulfate

BBB: blood–brain barrier

BPS: branch point sequence

bvFTD: behavioral variant of frontotemporal dementia

C9orf72: chromosome 9 open reading frame 72

CBD: corticobasal degeneration

cDNA: Complementary DNA

CHCHD10: coiled-coil-helix-coiled-coil-helix domain containing protein 10

CHMP2B: charged multivesicular body protein 2B

CIP: Calf Intestinal Alkaline Phosphatase

CIP: Intestinal Alkaline Phosphatase

CK-1: Casein kinases 1

CLIP: cross-linking immunoprecipitation

CSF: cerebrospinal fluid

CTFs: C-terminal fragments

DENN: differentially expressed in normal and neoplasia

DMEM: Dulbecco's Modified Eagle Medium

DMSO: dimethyl sulfoxide

DPR: Dipeptide-repeat

Abbreviations

DR3: Death receptor 3
ELISA: enzyme-linked immunosorbent assay
ER: endoplasmic reticulum
ESE: exonic splicing enhancer
ESS: exonic splicing silencer
Ex17b: Exon 17b
FBS: fetal bovine serum
FTD: frontotemporal dementia
FTLD-U: ubiquitin-positive FTLD
FTLD: Frontotemporal lobar degeneration
FUS: Fused in sarcoma
GRD: glycine-rich domain
GRNS: granulins
HEK-293: Human embryonic kidney cells 293
hnRNPs: heterogeneous nuclear ribonucleoprotein
iPSC: induced pluripotent stem cells
ISE: intronic splicing enhancer
ISS: intronic splicing silencer
LRPPRC: leucine-rich pentatricopeptide repeat containing protein
M-MLV: Moloney murine leukemia virus
MAPT: Microtubule-associated protein tau
MND: motor neuron disease
MPEP: 1-[2-(2-tert-butyl-5- methylphenoxy)-ethyl]-3 methylpiperidine
NCL: neuronal ceroid lipofuscinosis
NES: nuclear export signal
nfvPPA: non-fluent variants of primary progressive aphasia
NLS: nuclear localization signal
NMD: nonsense-mediated decay
nPTB: neural Polypyrimidine tract-binding protein
NT3: neurotensin-3

Abbreviations

OD: optical density
OH: hydroxyl group
OPA buffer: one for all buffer
OPTN: optineurin
PD: Parkinson's disease
PGRN: progranulin
PNK: polynucleotide kinase
PPT: polypyrimidine tract
PPT: polypyrimidine tract
Ps.Ex: pseudoexon
PSP : progressive supranuclear palsy
PTB: Polypyrimidine tract-binding protein
qRRM: quasi-RNA recognition motif
RBP: RNA-binding protein
RIP: RNA-immunoprecipitation
ROS: reactive oxygen species
RRM: RNA-recognition motif
RT-PCR: reverse transcription-polymerase chain reaction
RT: Reverse transcription
SAHA: suberoylanilide hydroxamic acid
SDS: sodium dodecyl sulfate
SLPI: secretory leukocyte peptidase inhibitor
SOD1: superoxide dismutase 1
Sort1: sortilin 1
SQSTM1: sequestosome 1
SRP: serine/arginine-rich protein
SRrps: Serine/Arginine-rich related proteins
SRSF: Serine/Arginine-rich Splicing Factor
svPPA: semantic variants of primary progressive aphasia
TBK1: TANK binding protein 1

Abbreviations

TDP-43: Transactive response DNA-binding protein 43 KDa

TEMED: tetramethylethylenediamine

TGN: trans-Golgi apparatus network

TL1A: TNF-like protein 1A

TNFR: tumor necrosis factor receptor

U2AF: U2 axillary factor

UBQLN2: ubiquilin 2

UPS: Ubiquitin-proteasom system

VCP: valosin-containing protein

VSP10p: vacuolar protein sorting 10 protein

WT: wild type

Chapter 1

1 Introduction

1.1 Frontotemporal lobar degeneration (FTLD)

In 1892 for the first time Arnold Pick, a neurologist from Czech Republic, described a rare disorder similar to Alzheimer's disease that was called Pick's disease for a century (Pick 1892).

However, in the early 90's by continuous efforts and experiments of various research groups mostly in Sweden and UK (The Lund and Manchester Groups. 1994), it was discovered that not only this disease is not rare among human population but also is distinguishable from Alzheimer's disease because it happens at the younger ages between 45 to 65 with estimated 6 to 11 survival years after the symptom onset (Hodges et al. 2003) (Onyike & Diehl-Schmid 2013).

Now this disease is known as frontotemporal dementia (FTD) or frontotemporal lobar degeneration (FTLD), because eventually leads to atrophy and degeneration of frontal and/or temporal cerebral lobes (Bang et al. 2015b) (Pottier et al. 2016).

FTLD is a general name for a group of disorders that are pathologically, clinically and genetically heterogeneous but morphologically share brain shrinking (Bang et al. 2015b) (Pottier et al. 2016).

After Alzheimer's disease and dementia with Lewy bodies, FTLD is the third prevalent neurodegenerative dementia that occurs around 1-26 cases per 100,000 persons (Bang et al. 2015a).

FTLD patients are mostly sporadic, without having any other affected person in the family and without clear-known reasons. The rest of the FTLD patients (about 20-40%) are familial cases that show heredity with more than one family member affected. (Snowden et al. 2002) (Chow et al. 1999). Low inherited percentage may refer to autosomal dominant genes involved in this disease. The inherited FTLD mostly is linked with genes on Chromosomes 3, 9, and 17 (Brown et al. 1995) (Foster et al. 1997) (Hosler et al. 2000).

1.2 FTD/FTLD–disease hallmarks

1.2.1 Clinical features

There are three main clinical subtypes of FTLD, including behavioral variants, language variants, and motor variants. According to the affected brain regions from frontal to anterior temporal lobes, different behavioral and language changes would appear.

There are different symptoms in behavioral variant (bvFTD), including ignorance to social conventions, lack of empathy, impulsive or risky behaviors, and personality impairments (Rascovsky et al. 2011) (Rascovsky et al. 2011) (Seelaar et al. 2011) (Sieben et al. 2012).

There are two types of language variants: semantic and non-fluent variants of primary progressive aphasia (svPPA and nvPPA), the former with difficulties in language comprehension and the latter with speech impairment (Neary et al. 1998) (Riedl et al. 2014).

Symptoms in motor variants overlap with corticobasal degeneration (CBD) and progressive supranuclear palsy (PSP) in Parkinson and motor neuron diseases (MND) such as amyotrophic lateral sclerosis (ALS) diseases (Riedl et al. 2014). This shows the complexity of FTLD and reflects the heterogeneity of pathological features of this disease (Seelaar et al. 2011).

Parkinsonian symptoms in PSP includes bradykinesia, postural instability, and axial rigidity and in CBD, akinetic rigidity, language difficulties, and limb apraxia (Bak & Hodges 2008).

In ALS, the most prevalent type of motor neuron disorders, progressive degeneration of upper and lower motor neurons leads to symptoms such as atrophic paralysis, muscle twitch, increased muscular tonus, and difficult breathing caused by weakness of the respiratory muscles. About 15% of FTLD cases develop phenotype of ALS. Instead, 5% of ALS patients show the full clinical symptoms of FTLD (Lomen-Hoerth et al. 2003) (Murphy et al. 2007).

1.2.2 Pathological features

Pathologically, all FTLD variants share degeneration of the frontal and/or temporal lobes. However, in the affected regions at the molecular levels, there are different pathological features with various protein aggregations. In this case, FTLD subtypes are categorized according to these inclusion bodies:

1.2.2.1 Microtubule-associated protein tau (MAPT)

This protein is mainly expressed in neurons and is responsible for stabilizing microtubules and axonal transport and in aggregation forms is not normally functional (van Swieten & Spillantini 2007).

1.2.2.2 TAR DNA- binding protein 43 (TDP-43)

TDP-43 is an RNA binding protein and regulates different aspects of RNA processing from transcription to translation. The cytoplasmic aggregations of this protein lead to loss of its normal functions in the nucleus and instead gain of some toxic functions in the cytoplasm (Arai et al. 2006) (Neumann et al. 2006).

1.2.2.3 Dipeptide-repeat (DPR) species

These are abnormal peptides produced by hexanucleotide repeat expansion located in a non-coding region of chromosome 9 open reading frame 72 (C9orf72). In a non-ATG translation process, using both forward and reverse directions of these pathologic transcripts in all three reading frames, eventually five different peptides are produced that are also known as c9RAN. (Ash et al. 2013), (Mori et al. 2013), (Zu et al. 2013).

1.2.2.4 Fused in sarcoma (FUS)

FUS is an RNA binding protein that in FTLD could aggregate in the neurotoxic inclusion forms (Seelaar et al. 2010) (Sieben et al. 2012) (Urwin et al. 2010).

In conclusion, there is a pathological classification for FTLD according to the neuropathological hallmarks: FTLD-tau (35-45 %), FTLD-TDP together with FTLD-TDP with DPR (45-60 %), FTLD-FUS (5-10 %). In addition there is another subtype with the minimum prevalence, containing ubiquitin-positive but tau- and TDP-43-negative pathology known as FTLD-U (1 %) (Götzl et al. 2016) (Fig. 1.1).

Introduction

There are several reasons for formation of FTLD's inclusion bodies:

1. Proteasomal and/or autophagic and lysosomal impairments recognizable by ubiquitin and p62 positive signs (Arai et al. 2003)(Korolchuk et al. 2009).
2. Any alterations in nuclear import/export signals of some RNA binding proteins such as TDP-43 or FUS that impair their normal shuttling between nucleus and cytoplasm could eventually lead to their cytoplasmic aggregation (Dormann & Haass 2011).
3. Production of prone protein variants due to some gene mutations (Edbauer & Haass 2016).

Controversial evidence about toxicity or protective effects of aggregations is reported. Taking in consideration that probably in the early stages of aggregate formation, toxic species are produced but once the inclusion bodies are formed could protect the cells from the misfolded proteins (Ross & Poirier 2005).

1.2.3 Genetical features

In FTLD, there is a correlation between different gene mutations and protein aggregates, therefore normally any mutation leads to a specific molecular pathology (Sieben et al. 2012) (Pottier et al. 2015).

As shown in Figure 1.1, the main familial FTLD causing reasons are MAPT and progranulin (PGRN) mutations and C9orf72 repeat expansions (Sieben et al. 2012) and rare gene mutations have been reported in valosin-containing protein (VCP)/p97, charged multivesicular body protein 2B (CHMP2B), optineurin (OPTN), TANK binding protein 1 (TBK1), sequestosome 1 (SQSTM1)/p62, and ubiquilin 2 (UBQLN2). Interestingly these rare genes are directly involved in protein degradation so their mutations end up with protein aggregations (Götzl et al. 2016).

Introduction

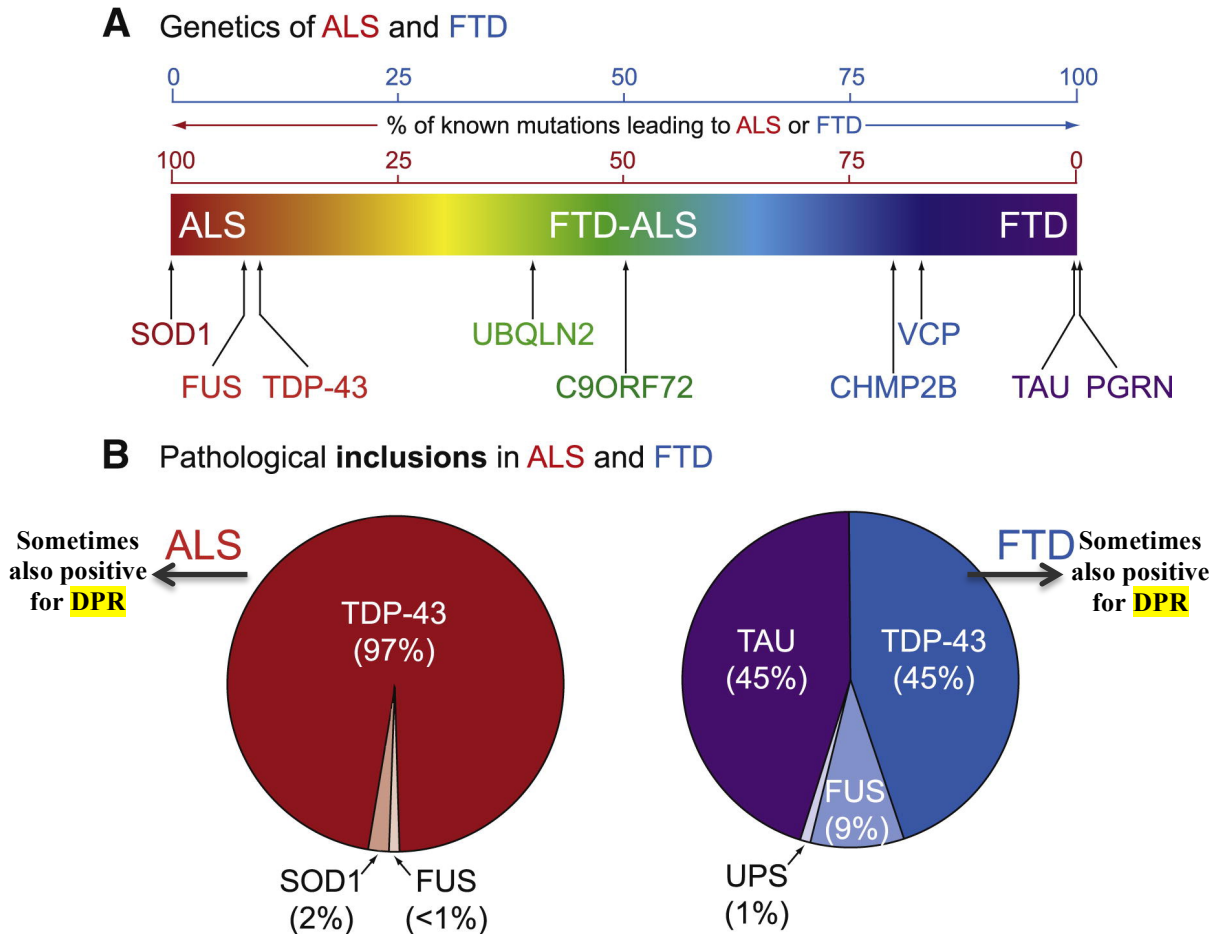


Figure 1.1. Genetic and pathological features of FTLD and ALS sometimes overlap.

A) The schematic spectrum for causative gene mutations involved in familial FTLD and ALS. Main gene mutations leading to FTLD occur in PGRN and TAU genes, while mutations in superoxide dismutase 1 (SOD1), FUS, and TDP-43 genes are mostly involved in ALS. Note that C9orf72 repeat expansions are almost equally contributed for inducing both diseases.

B) A schematic diagram for pathological features of FTLD and ALS. Mostly cytoplasmic inclusions in FTLD are positive for TDP-43 and tau and in ALS are mainly positive for TDP-43. Note that C9orf72 repeat expansions can lead to both TDP-43 and dipeptide-repeat (DPR) cytosolic inclusions. Ubiquitin-proteasom system (UPS) are special ubiquitin positive inclusions negative for TDP-43 or tau. (Ling et al. 2013).

1.2.3.1 Microtubule-associated protein tau (MAPT)

Located on chromosome 17q21, approximately 44 various mutations have been identified in MAPT gene, which are the main cause for 5-20% of familial FTLT and rarely in sporadic cases (Ghetti et al. 2015). MAPT mutants induce heterogeneous clinical appearance with tau-positive inclusion bodies specific for FTLT-tau (Dickson et al. 2011).

MAPT protein is responsible for controlling cytoskeletal dynamics by assembling microtubules (Wang & Mandelkow 2016). There are 6 cerebral isoforms of this protein due to mRNA alternative splicing of exons 2, 3 and 10 and the full mRNA isoform has 15 exons (NCBI Reference Sequence: NM_001123066).

The ability for interaction with microtubules is because of four repeat sequences in the C-terminal of tau protein (Gustke et al. 1994) that are encoded by MAPT exons 9, 10, 11, and 12. Alternative splicing of exon 10 due to mutations can encode more efficient repeats for binding to microtubules (Goedert & Jakes 1990) that contribute in formation of tau aggregations and in nervous system impair axonal transport (Goedert & Jakes 1990) (Riedl et al. 2014).

On the other hand, missense mutations in MAPT coding regions can abolish its binding to microtubules by producing pathological tau filaments (Hong et al. 1998) leading to increased unbound tau available to form inclusions (Goedert et al. 1999).

Cells and animal models (mice, flies, worms, and zebrafish) are available for FTLT-tau showing pathological and behavioral abnormalities similar to patients. (Rockenstein et al. 2015) (Denk & Wade-Martins 2009) (Brandt et al. 2005) Recently a more feasible model using induced pluripotent stem cells (iPSC)-derived neurons (obtained from FTLT-tau patients that carry MAPT mutations) is developing (Lee & Huang 2015).

1.2.3.2 Progranulin (PGRN)

Nearby MAPT locus on chromosome 17, around 70 distinct mutations, including frameshift, nonsense, missense and splice mutations, and rare partial or complete deletion have been discovered in PGRN gene linked to FTLT (Mackenzie et al. 2006) (Gijselinck et al. 2008). These gene mutations lead to tau-negative, ubiquitin-positive inclusions made of TD-P-43 referring to FTLT-TDP patients (Gijselinck et al. 2008).

Mutations on PGRN gene are one of the most important reasons for autosomal dominant FTLD (Baker et al. 2006) with frequency of 5-20% for familial and 1-5% for sporadic cases (Rademakers et al. 2012). Majority of PGRN mutations are loss of function mutations, causing reduced PGRN mRNA and protein levels in the periphery making a good biomarker for FTLD diagnosis that will be explained better later (Finch et al. 2009).

Compare to other mutant carriers, FTLD-TDP patients show the highest variable clinical phenotype (Benussi et al. 2015).

1.2.3.3 C9orf72 repeat expansions

It has been reported that expanded hexanucleotide (GGGGCC) repeat in the non-coding region of chromosome 9 open reading frame 72 gene (C9orf72) is the main genetic known reason for familial FTLD and ALS and sometimes both diseases are referred to c9FTD/ALS (DeJesus-Hernandez et al. 2011) (Renton et al. 2011) (Fig. 1.1). These patients show hundreds to thousands copies of a hexanucleotide repeat (GGGGCC), while healthy and normal gene carriers have just 30 or less copies (DeJesus-Hernandez et al. 2011). This gene provides evidence for existence of overlapping biological pathways between FTLD and ALS (Fig. 1.1). Several mechanisms are identified to explain the toxicities made by this repetition. C9orf72 repeat expansions may result in RNA-mediated toxicity through the formation of RNA foci that probably isolate RNA-binding proteins or start non-ATG translation that cause synthesis of aggregation-prone c9RAN proteins (DeJesus-Hernandez et al. 2011). These repetitions may also result in decrease of C9orf72 mRNA levels in the brain, showing that loss of C9orf72 function can also be involved in disease pathogenesis (DeJesus-Hernandez et al. 2011) (Renton et al. 2011).

Around 25% of familial FTLD and 5% of sporadic FTLD show GGGGCC hexanucleotide expansions. This frequency is even more in ALS cases with familial 33% and sporadic 5%. However, reports from Asia show much less numbers of patients having these toxic repeats (Ishiura & Tsuji 2015).

Like other types of FTLD, the clinical phenotypes of C9orf72 repeat expansion carriers are heterogeneous (Benussi et al. 2015). While the mutation carriers show positive inclusions for TDP-43 (Rademakers et al. 2012) and for this reason this type of FTLD is referred to FTLD-TDP, all of them have other inclusions positive for proteins of the ubiquitin proteasome

system (UPS) such as ubiquitin and p62 with no trace of TDP-43. These aggregates that are mostly found in cerebellum and hippocampus contain some dipeptide repeats (DPRs) that are produced from the GGGGCC expansions by unusual non-ATG translation (Ash et al. 2013) (Mori et al. 2013). There are controversial studies about whether DPRs or TDP-43 inclusions are toxic or not (Gendron et al. 2015).

In general, there are two hypotheses explaining how GGGGCC repeat expansions lead to FTLN. The first theory is the loss of function of C9orf72 gene. This gene contains 12 exons and encodes a not very well characterized protein with homology to differentially expressed in normal and neoplasia (DENN) proteins that controls endosomal trafficking and with interaction to Rab proteins regulates autophagy and endocytic transport (DeJesus-Hernandez et al. 2011). If there are pathological repeats of GGGGCC located in a non-coding part between two exons, C9orf72 transcripts will be reduced and patients will have loss of function of the related protein (DeJesus-Hernandez et al. 2011).

According to the loss of function model, while knockdown of C9orf72 in cells showed endocytosis and autophagy impairment and in zebrafish and worm led to motor phenotypes (Farg et al. 2014) (Ciura et al. 2013) (Therrien et al. 2013), knockout mice did not have any motor or behavioral phenotypes (Koppers et al. 2015).

In this point, second theory is the gain of function where DPRs or TDP-43 inclusions are considered toxic and harmful for cells. Also in the affected brain regions, nuclear RNA foci from the expanded GGGGCC repeats are produced and correlate with patients' clinical phenotypes (Mizielinska et al. 2013). RNA foci are considered toxic for cells because they sequester important RNA-binding proteins such as hnRNP A1, hnRNP H, and SC35 from their RNA targets (Mizielinska & Isaacs 2014).

1.2.3.4 Causative rare genes

CHMP2B

Located on chromosome 3p11.2, mutations in this gene have been seen in a large Danish cohort of FTLN (Holm et al. 2009). There is only one mutation identified for charged multivesicular body protein 2B (CHMP2B) that leads to FTLN-U with p62 positive inclusions but negative for TDP-43 (Rohrer et al. 2009). Being a part of endosome sorting system in the cells, the protein is responsible for vesicle fusion with target membranes (Babst et al. 2002).

VCP

Valosin-containing protein (VCP): located on chromosome 9p13.3, mutations in this gene are shown to be associated with FTLT-DTP and inclusions include both TDP-43 and p62 (Watts et al. 2004).

VCP belongs to AAA-ATPase family and it involves in protein refolding, protein quality control through ubiquitination, protein recycling and degradation via the proteasome, and elimination of aggregate prone proteins through multi-vesicular body formation (Weihl et al. 2006).

Mutations in VCP and CHMP2B together explain about 1% of the familial FTLT (Riedl et al. 2014).

SQSTM1

Around 3% of FTLT cases carry sequestosome 1(SQSTM1) mutations. This gene encodes p62 protein, a receptor involved in autophagy and proteasome degradation and a mediator for protein ubiquitination (van der Zee et al. 2014).

TBK1

Loss of function mutations in TANK binding kinase 1, involved in autophagy and neuroinflammation (Weidberg & Elazar 2011) cause FTLT-ALS (Freischmidt et al. 2015) that sometimes show positive inclusion for TDP-43 (FTLT-TDP) (Pottier et al. 2015) (Gijssels et al. 2015). The mutations are responsible for 2-3% of FTLT-TDP (Pottier et al. 2015) and 2% of clinical FTLT-ALS (Gijssels et al. 2015).

TARDBP and FUS

There is a known clinical and pathologic overlap between ALS, a disorder with motor neuron degeneration, and FTLT (Fig. 1.1). However, while mutations in TARDBP that encode TDP-43 and mutations in FUS are among the main causes of ALS (Ng et al. 2015), they are rarely enrolled in FTLT. In Sardinia there is the highest population of TARDBP mutation carriers with 21.7% of all familial FTLT cases in this region (Quadri et al. 2011). However, by now no FUS mutations have been linked to FTLT-FUS.

CHCHD10

Recently, studies in Asia showed that around 7.7% of FTLT patients carry mutations in coiled-coil-helix-coiled-coil-helix domain containing protein 10 (CHCHD10) gene, while each

of mutations in MAPT and C9orf72 genes cause only 3% of this disease in the same population. This statistics point out CHCHD10 mutations as the main reason of FTLN in Asia (Jiao et al. 2016).

Functional CHCHD10 is needed for proper mitochondrial construction and mutations can cause respiratory chain deficiency and apoptosis inhibition (Genin et al. 2016).

Finally it is considerable that other mutations in several genes can be found in clinical FTLN patients with much lower frequency (Pottier et al. 2016).

1.3 FTLN Diagnosis

Because of the complexity, the latency from the symptom onset and the correct diagnosis of FTLN is almost 3 to 4 years (Diehl-Schmid et al. 2007). Sometimes patients do not complain of any symptoms and their relatives and friends may suspect they have psycho problems rather than a neurodegenerative disease (Pijnenburg et al. 2004).

A cognitive profile and a detailed psychiatric examination of suspicious cases may help to discriminate FTLN from other causes of dementia, particularly Alzheimer's disease and depression.

The next step is laboratory tests, neuroimaging, and cerebrospinal fluid analysis.

While in the early stages of FTLN structural changes are subtle, imaging studies may reveal grey matter indicating atrophy in the frontal and temporal lobes (Schroeter et al. 2007).

So far almost no periphery trustable biomarker exists. High cerebrospinal fluid phospho-tau could be helpful to discriminate FTLN from Alzheimer's disease that instead is positive for α - and β -Amyloid (Bibl et al. 2011).

Laboratory blood tests in Mayo Clinic Jacksonville showed significantly reduced levels of PGRN in FTLN-TDP patients caused by PGRN mutations (Finch et al. 2009).

The consequence of measuring PGRN levels in plasma could be identifying FTLN patients likely to have mutations in PGRN; Therefore after measuring plasma PGRN levels, in suspicious cases PGRN gene could be sequenced to find the exact mutation. Although plasma PGRN levels may not correctly refer cerebral PGRN levels and even if in normal humans these levels are variable, developing simple screening tests would be great advance to help FTLN diagnostics (Bird 2009) (Carrasquillo et al. 2010).

1.4 FTLT Treatment

So far there is no appropriate therapy to cure FTLT patients and available drugs can only modify behavioral symptoms. For example because of the impairment in serotonergic system (decrease in 5-HT_{1A} and 5-HT_{2A} receptors) (Huey et al. 2006) or dopaminergic system (decrease in dopamine metabolites and presynaptic dopamine receptors) (Sjögren et al. 1997) (Rinne et al. 2002), some severe behavioural symptoms like aggressiveness, agitation, and impulsivity can be reduced by selective serotonin reuptake inhibitors or Dopaminergic drugs like dextroamphetamine (Lebert et al. 2004).

The cholinergic system is impaired in Alzheimer's disease but not in FTLT (Procter et al. 1999) (Huey et al. 2006) and it has been shown that cholinesterase inhibitors are worsening the clinical symptoms in FTLT patients (Boxer et al. 2013).

Inhibiting the formation of inclusion bodies is another strategy. For example, it has been shown that methylene blue can inhibit tau aggregation and also prevent TDP-43 toxicity in animal models and now this drug is under clinical trial process (NCT01626378) (Vaccaro et al. 2012).

Recently studies about antisense oligonucleotides against toxic C9orf72 mRNAs (Lagier-Tourenne et al. 2013) (Jiang et al. 2016) and compounds increasing progranulin serum concentrations, such as nimodipine that is under clinical trial (NCT01835665), are developing (Capell et al. 2011).

1.5 Progranulin (PGRN)

Highly conserved among different species, for the first time PGRN was recognized as an autocrine growth factor (Wright et al. 1989) (Zhou et al. 1993). Although primary secreted size of PGRN is around 69 kDa, due to glycosylation this growth factor is detectable around 90 kDa on a sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Liu & Bosch 2012) (Zhou et al. 1993).

Encoded by chromosome 17q21 in humans, this glycoprotein contains 593 amino acid with 7.5 cysteine-rich granulin domains that could be individually released by proteolysis (Wang et al. 2015) (De Muynck et al. 2013).

Introduction

These granulins (GRNs), from the N-terminus to C-terminus of PGRN are named: granulins p, G, F, B, A, C, D, and E, respectively while “p” indicates the half-length motif (Cenik et al. 2012) (Fig. 1.2)

After releasing in a full-length, PRGN can be cleaved in the periphery to the individual granulins. Two essential enzymes regulate this process: elastase and secretory leukocyte peptidase inhibitor (SLPI). Elastase as a protease breaks down PGRN into distinct GRNs and SLPI inhibits the protease activity of elastase (Fig. 1.2).

Both PGRN and GRNs are biologically functional and sometimes show distinct functions either in physiologic or in pathologic conditions (Lewis & Golde 2010) (Bateman & Bennett 2009).

1.5.1 Physiological roles of PGRN

PGRN is mostly expressed in epithelia, immune cells, bone marrow, and nervous system (Lewis & Golde 2010) and its main biological functions can be categorized into growth factor-like activities and regulation of immune responses (Liu & Bosch 2012).

PGRN can accelerate proliferation through triggering ERK, PI3K, and Akt signaling pathways (Feng et al. 2010) (Xu et al. 2011) and its overexpression is reported in many human tumors (Cenik et al. 2012).

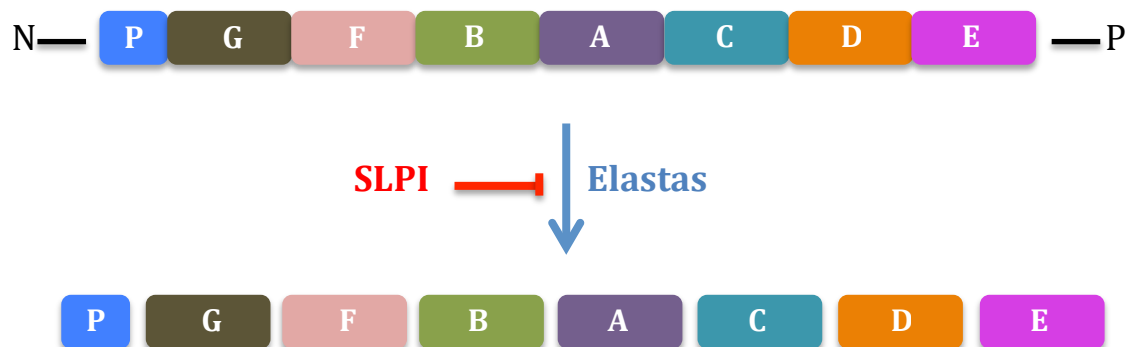


Figure 1.2. PGRN structurally is made of 7.5 granulin units, including p, G, F, B, A, C, D, and E.

There is an equilibrium between PGRN and GRNs levels through two enzymes; Elastase that cleaves PGRN to produce individual GRNs and on the other hand, Secretory leukocyte peptidase inhibitor (SLPI) that inhibits the cleavage activity of elastase.

Possibly through antagonistic effect on TNF- α receptor, PGRN can play as an anti-inflammatory mediator, decrease reactive oxygen species (ROS) production by activated neutrophils, and abolish TNF- α induced IL-8 release (Tang et al. 2011) (Liu & Bosch 2012).

On the other hand pro-inflammatory effects of PGRN, through activation of TNF-R2 could induce protective effects in inflammatory arthritis and joint erosion (Faustman & Davis 2010) (Liu & Bosch 2012). In the early phase of wound healing, together with other pro-inflammatory mediators, PGRN is triggering immune cell defensive reaction (He et al. 2003).

Interestingly there are studies showing that intact PGRN could have anti-inflammatory effects, while individual granulins can induce pro-inflammatory cascade. Therefore, the balance between the precursor protein and granulins is important to control neuroinflammation (Ahmed et al. 2007).

1.5.2 PGRN and nervous system

1.5.2.1 Neurite outgrowth

It has been reported that Neurite outgrowth, projections from the neuronal cell body, are significantly decreased in homozygous GRN-deficient neurons compared to the wild type (WT) neurons and PGRN overexpression could rescue the neurotrophic properties of PGRN (Gass et al. 2012). Such properties are probably related to some specific GRNs, because inhibition of PGRN cleavage, could block its neurotrophic activities (Gass et al. 2012).

1.5.2.2 PGRN and neuronal ceroid lipofuscinosis (NCL)

Several gene mutations mainly encoding lysosomal enzymes are linked to Neuronal ceroid lipofuscinosis (NCL), a disorder with lysosomal accumulated lipofuscin (toxic oxidized cross-linked proteins and lipids) that impairs lysosomal function (Kohlschütter & Schulz 2009).

Interestingly, two cases that carry homozygous PGRN mutations have been reported with no detectable plasma PGRN and symptoms related to NCL including retinal degeneration,

progressive loss of vision, cerebellar ataxia, and seizures (Smith et al. 2012). Therefore, possibly PGRN plays an important role in the lysosomes for lipofuscin-related substrates.

However, in FTLD-TDP patients that are heterozygous for PGRN mutations, no positive inclusion for lipofuscin has been reported by now.

This evidence could show a possible overlap between PGRN related NCL and FTLD disorders that needs to be clarified better (Cotman et al. 2013).

1.5.3 PGRN and FTLD

In 2006, mutations in PGRN were identified as the main cause of familial FTLD-TDP (Baker et al. 2006). After that, up to 70 heterozygous autosomal dominant mutations have been found in 226 families affected by dementia (Carrasquillo et al. 2010). These loss of function mutations decrease PGRN protein levels because they introduce a premature stop codon in the transcript that eventually undergoes nonsense-mediated decay (NMD). Since patients also carry a normal allele of PGRN gene, the normal copy continues production of normal PGRN protein. So there is an expectation of around 50% reduction in total PGRN protein levels (PGRN haplo-insufficiency) (Bird 2009).

Because of the general neurotrophic properties of PGRN protein, its missense mutations could also be responsible for Alzheimer disease (Brouwers et al. 2008), ALS (Slegers et al. 2008), and Parkinson disease (Nuytemans et al. 2008).

1.5.3.1. Diagnosis of FTLD-TDP by plasma PGRN levels

The PGRN levels in plasma, serum, and cerebrospinal fluid (CSF) are detectable using an enzyme-linked immunosorbent assay (ELISA) (Carrasquillo et al. 2010). Human physiological range of PGRN is roughly 100–400 ng/mL and 8–12 ng/mL in the peripheral and central nervous system respectively (Baker et al. 2006). In pathological conditions such as FTLD due to loss of function mutations, plasma PGRN levels are reduced compare to the control individuals (Finch et al. 2009) (Coppola et al. 2008). After determination of reduced or borderline plasma PGRN levels in suspected cases, the next step would be sequencing of the PGRN in order to find the possible mutations.

Developing such a screening test would be a major advance to help FTLD diagnostics (Sassi et al. 2016) (Bird 2009). However, one should note that due to different genetic and

environmental factors, there are wide ranges of plasma PGRN levels even in normal individuals (Carrasquillo et al. 2010).

Also it should be considered that sometimes the plasma levels of PGRN in FTLN cases are not reduced; for example in some FTLN cases the conversion of PGRNs to GRNs is impaired and while PGRN is high in plasma, lack of GRNs induces the disease (J. Wang et al. 2010). Moreover, plasma might not correctly reflect the cerebral PGRN level and care should be taken when interpreting results from these screening analyses (Sassi et al. 2016) (Carrasquillo et al. 2010) (Nicholson et al. 2014).

1.6 PGRN receptors

Two main receptors for PGRN have been identified on the cell surfaces: tumor necrosis factor receptor (TNFR) (Tang et al. 2011) and sortilin 1 (Sort1), (Hu et al. 2010).

1.6.1 TNF receptors

PGRN as a ligand can bind to three TNF receptors including TNFR1, TNFR2, and Death receptor 3 (DR3). It has been discovered that PGRN mainly through its F, A, C granulin regions (Atsttrin is the available engineered molecule from these motifs) binds to these receptors (Tang et al. 2011). Interaction with TNFR2 triggers beneficial pathway that protects joints from inflammatory processes. On the other hand PGRN is also protective by antagonistic effects on TNF- α and TNF-like protein 1A (TL1A) by TNFR1 and DR3 blockage respectively. (Faustman & Davis 2010), (Wang et al. 2015).

From different studies we can conclude that the balance between TNF- α and PGRN pathways is important to regulate inflammatory cascades and any disturbance in their ratio could lead to different diseases.

Therefore, Atsttrin as the main mediator of PGRN is a good candidate to produce new generation of anti-inflammation drug (Wang et al. 2015). Of course from the other view, one should consider that PGRN as an antagonist of TNF- α could also promote tumor formation because TNF- α can destroy tumors by necrosis (Verhoef et al. 2007).

1.6.1.1 PGRN-TNFR connection and neuronal viability

In FTLT-DTP, loss of function mutations and PGRN haplo-insufficiency results in over-activity of TNF- α . Increase in TNF- α activity over-stimulates inflammatory cascades that are lethal for the neurons and lead to cell death. However, it is not clear how increase in TNF- α activity could result in a specific neurodegenerative phenotype like FTLT-DTP (Cenik et al. 2012).

Engineered molecules derived from PGRN are developing as FTLT therapy candidates. They could diminish the TNF α response to the physiological conditions by holding the inflammatory TNFR signaling under the control to avoid reduced outgrowth and eventual neuronal death (Gass et al. 2012).

1.6.2 Sort1 Receptor

Encoded by chromosome 1 (1p13.3), sortilin is a membrane receptor with a crucial role in intracellular trafficking and sorting (Zhong et al. 2016). With 700 amino acids, the extracellular domain of this receptor folds into 10-bladed β -propeller and belongs to vacuolar protein sorting 10 protein (VSP10p) domain family in mammals (Schmidt & Willnow 2016).

Full form of Sort1 receptor in humans contains 831 amino acid with several evolutionary conserved domains among species, including an endoplasmic reticulum (ER) import signal, an extracellular VSP10 domain that binds to ligand, a single pass trans-membrane domain, and a C-terminal cytosolic domain that directs the ligand to the lysosome (Fig. 1.3) (Prudencio et al. 2012) (Mazella et al. 1998).

Introduction

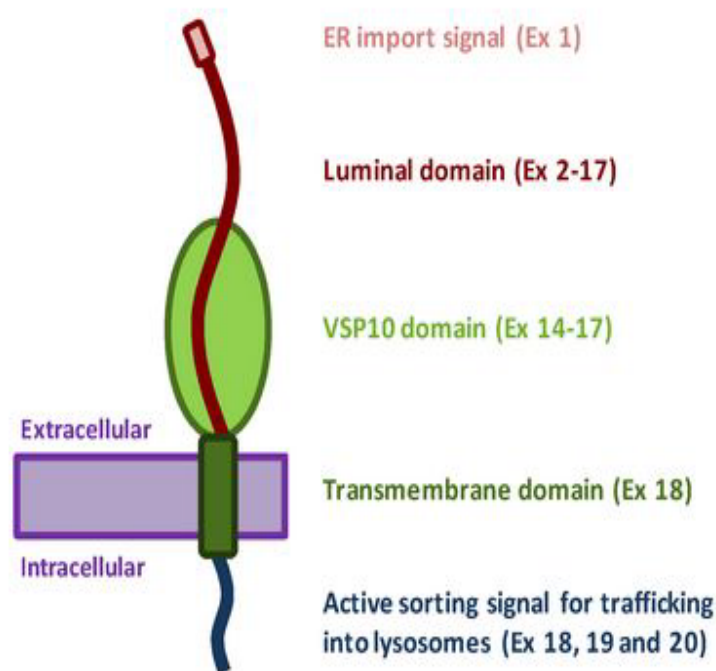


Figure 1.3. Three main domains of Sort1 receptor.

Three main domains of Sort1 receptor, including extracellular, transmembrane, and intracellular domains encoded by different Sort1 transcript exons. After binding to VSP10 domain, PGRN will be directed to the lysosomes through cytosolic domain of Sort1 (Prudencio et al. 2012).

Sort1 receptor is mostly located on the trans-Golgi apparatus network (TGN) to direct newly produced proteins to the early endosomes (Hermey 2009). Around 10% of these receptors are localized on the cell membrane to internalize the ligands such as LDL, and apolipoprotein into the cells (endocytosis) (Hermey 2009) and deliver them to the lysosomes for eventual degradation (Nilsson et al. 2008).

Sort1 that was identified in human brain as an orphan receptor in 1997 (Petersen et al. 1997), was the first cell surface receptor recognized for PGRN ligand (Hu et al. 2010).

Since in nervous system, the most important ligand for Sort1 is neuropeptide neurotensin-3, sometimes this receptor is called NT3 (Zhong et al. 2016). Structurally, NT is very similar to PGRN. In fact they compete for binding to the same amino acid sequence on VSP10 domain (Hu et al. 2010) (Quistgaard et al. 2009). Functionally, after binding to Sort1 receptor, NT could mediate anti-apoptotic effects (Béraud-Dufour et al. 2009).

In nervous system, Sort1 is also a co-receptor for proneurotrophin and unlike NT, is not a competitor for PGRN (Lewis & Golde 2010) and could trigger cell death (Hu et al. 2010).

1.7 Sort1 as PGRN receptor in nervous system

1.7.1. PGRN regulates neurite outgrowth independent of Sort1 receptor

The evidence shows that interaction between Sort1 and PGRN is not needed to induce neuronal survival activities like neuronal outgrowth. For example, in Sort1^{-/-} culture, PGRN can still induce neuronal outgrowth in the absence of Sort1 receptor (Gass et al. 2012). Moreover, pharmacologic interference between GRN E (located on the C-terminal, the main interactor site of PGRN with Sort1) and Sort1 or deletion of GRN E binding site on Sort1 receptor do not inhibit PGRN's neurotrophic activities (De Muynck et al. 2013).

PGRN knocked down in zebrafish embryos defects axonal outgrowth. However, reduction of Sort1 receptor by injection of morpholinos in zebrafish did not change the spinal motor axons' outgrowth, emphasizing that PGRN binding to Sort1 receptor is not required to trigger neuronal outgrowth in vivo (Chitramuthu et al. 2010).

These data propose other responsible receptor(s) for PGRN-induced neuronal outgrowth and TNF receptors are among the candidates (Gass et al. 2012). In this respect, as mentioned before, PGRN is a ligand for TNFR as well and following binding to TNFR antagonizes and inhibits downstream TNF α -mediated inflammatory signaling. Therefore, it is predictable that the loss or reduced levels of PGRN during a long-term will increase cell death by enhancing pro-inflammatory signaling, which is detrimental for neuronal viability (Gass et al. 2012).

1.7.2 Sort1 regulates extracellular PGRN levels

Once Sort1 binds to PGRN, rapidly internalizes and delivers it to the lysosomes. By this, the extra cellular levels of PGRN will be under the control (Lewis & Golde 2010). Indeed, mice that completely lack Sort1 had higher brain and serum PGRN but mice partially lacking Sort1 because of the functional allele, showed normal PGRN levels similar to the wild type ones (Lewis & Golde 2010).

1.7.3 Sort1 mediates intracellular functions of PGRN

After internalizing into the cells through Sort1, PGRN is targeted to the lysosomes and accumulates within the lysosomal compartment of neurons. In fact in both physiological and pathological conditions, while PGRN is mostly expressed in microglial cells, it is also present in neuronal cytoplasm within the lysosomes (Mackenzie et al. 2010).

Perhaps PGRN rather than getting simply internalized into the lysosomes, functions within autophagosomal/lysosomal pathways. Under this hypothesis, PGRN-Sort1 interaction is necessary for clearance of the pathological inclusion bodies such as TDP-43 aggregations in FTLD-TDP through autophagosomal/lysosomal mechanisms (Mackenzie et al. 2010) (X. Wang et al. 2010).

On the other hand, PGRN cleavage into individual GRNs due to lysosomal localization may affect the functional balance between PGRN and GRNs and therefore regulate pathophysiology of FTLD. To confirm this hypothesis, further studies comparing neuronal activities of GRN versus PGRN are needed (Hu et al. 2010).

1.8 Sort1 a therapeutic target for FTLD-TDP

1.8.1 Increase of extracellular PGRN levels by manipulation of PGRN-Sort1 axis

As described before around 50% reduction of PGRN levels can lead to FTLD-TDP. Therefore PGRN-Sort1 pharmacological targeting could be a good therapy candidate for this disease and any interference between ligand and receptor could normalize PGRN levels.

Indeed, GRN+/- mice lacking Sort1 expression have an equal or more PGRN levels compare to the wild type mice. This enrichment is specific for PGRN and other Sort1 ligands are not affected (Hu et al. 2010).

As discussed before, neurotrophic effect of PGRN is not Sort1 dependent (Gass et al. 2012) (De Muynck et al. 2013), so the disconnection between ligand and receptor does not stop neurite outgrowth, assuring PGRN-Sort1 a right target for FTLD-TDP therapy achievement.

It is particularly important to consider that so far no practical way for pharmacological manipulation of PGRN levels in the brain has been proved. Because of the big size, the recombinant PGRN is not able to cross the blood–brain barrier (BBB) for protein replacement. A recent study showed three different ways to interfere PGRN-Sort1 interaction and increase PGRN levels (Lee et al. 2014):

1. A bioactive compound, 1-[2-(2-tert-butyl-5- methylphenoxy)-ethyl]-3 methylpiperidine (MPEP), that decreases Sort1 levels in mammalian cell lines in a dose dependent manner.
2. Other Sort1 ligands including NT as extra cellular PGRN competitors.
3. Suppressing active C-terminal of PGRN that is responsible for Sort1 binding by BVFP. This latest approach specifically stops PGRN and is suitable to avoid unwanted side-effects on other Sort1 ligands. Moreover, this motif is not located on the functional PGRN motifs and does not interfere with the activities of this protein.

1.8.2 Does Sort1-PGRN disconnection impair lysosomal function?

As already discussed, complete depletion of PGRN leads to NCL disease due to lysosomal dysfunction (Smith et al. 2012). By manipulation of PGRN-Sort1 connection extracellular PGRN levels increases to maintain neuronal viability through other receptors. However, inhibition of PGRN endocytosis via SORT1 would decrease PGRN levels in the lysosomes and may triggers lysosomal disorders such as NCL. While more studies needs to be done, it seems that intracellular PGRN levels, produced by biosynthetic pathways, are sufficient to control normal lysosomal activities (Gieselmann 2007).

1.9 RNA metabolism defects and diseases; mis-splicing can lead to neurodegeneration

All the processes related to RNA transcription, splicing, transportation, stabilization, translation, and in general regulation are known as RNA metabolism (Alaqeel et al. 2015).

Normal expression of RNAs, including protein-coding RNAs and non-coding RNAs that regulate RNA processing from transcription to splicing and translation, is very essential for the accurate function of the cells. Functional RNAs in the cells are found in the complexes

together with proteins as ribonucleoprotein (RNPs) made of massive RNA-binding proteins plus one or more RNAs (Dreyfuss et al. 1993) (Glisovic et al. 2008).

The normal activity of RNPs is very vital for the cells as any impairment in its composition or any disturbing mutations either in RNAs or in proteins can lead to a disease (Lukong et al. 2008). Obviously RNA has an extremely important role in the normal function of the neural system as well and non-functional RNAs or RNPs are a source of various neurodegenerative disorders (Alaqueel et al. 2015).

Various mutations in RNA or RNA-binding proteins that control splicing are a key cause of numerous human diseases. Such diseases are the results of defective regulation of pre-mRNA splicing and include general (e.g. cancer, Hutchinson–Gilford progeria syndrome, cystic fibrosis) and neurodegenerative (e.g. Spinal muscular atrophy, FTLN, ALS) diseases (Douglas & Wood 2011) (Chabot & Shkreta 2016).

1.10 Misregulation of SORT1 mRNA splicing and FTLN

As described in Table 1.1 some alternative spliced Sort1 transcripts with different exon numbers are reported in placental mammals (data from Ensembl Genome Browser). In humans the normal-physiological transcript variant contains 20 exons.

However, Interestingly in normal mice there are two alternatively spliced Sort1 transcripts, one with an extra exon between exon 17 and exon 18 (Ex17b) that encodes a region placed between the VSP10 and transmembrane domains. Instead in healthy humans, the chance of having this extra exon is almost zero. Because in humans this exon due to containing a premature stop codon could produce a truncated and non-functional protein (Prudencio et al. 2012). TDP-43, that in FTLN following PGRN mutation aggregates in the cytoplasm, has been discovered as a splicing regulator of Sort1 transcript and it inhibits Ex17b inclusion (Prudencio et al. 2012).

Introduction

Table 1.1. Sort1 mRNA isoforms in different placental mammals.

Data are obtained from the Ensembl Genome Browser: www.ensembl.org (Prudencio et al. 2012). Of our interest, in some species such as mice, in addition to 20 exon transcript that is also normally found in humans, 21 exon transcripts are reported as well. The 21 exon transcript contains an extra exon between exon 17 and 18 and therefore is called Ex17b.

Species	Ensembl Name	Transcript ID	Length (bp)	Exon number	Protein length (aa)	Protein ID
Armadillo	-	ENSDNOT00000012343	2130	18	709	ENSDNOP00000009581
Mouse	Sort1-001	ENTUMUST00000102632	6796	20	825	ENSMUSP00000099692
Mouse	Sort1-003	ENTUMUST00000135636	1629	9	333	ENSMUSP00000123564
Mouse	Sort1-201	ENTUMUST00000090564	2578	21	858	ENSMUSP00000088052
Mouse	Sort1-002	ENTUMUST00000129755	925	2	-	-
Rat	-	ENSRNOT00000049127	6690	20	825	ENSRNOP00000051102
Rat	D4A4E0-RAT	ENSRNOT00000042377	6789	21	858	ENSRNOP00000042760
Guinea Pig	-	ENSCPOT00000003227	2265	19	755	ENSCPOP00000002884
Kangaroo rat	-	ENDSOT00000004036	2496	20	831	ENDSDORP00000003769
Squirrel	-	ENSSTOT00000011212	2487	20	828	ENSSTOP00000010055
Rabbit	-	ENSOCUT00000013810	2196	22	731	ENSOCUP00000011881
Pika	-	ENSOPRT00000008847	2190	19	729	ENSOPRP00000008099
Tree shrew	-	ENSTBET00000002502	2184	25	727	ENSTBEP00000002166
Macaque	-	ENSMMUT00000008147	2566	22	821	ENSMMUP00000007661
Chimpanzee	-	ENSPTRT00000001967	2103	19	700	ENSPTRP00000001807
Gibbon	-	ENSNLET00000003496	6953	21	830	ENSNLEP00000003330
Gorilla	-	ENSGGOT00000006588	6972	21	832	ENSGGOP00000006418
Gorilla	-	ENSGGOT00000025772	7026	20	729	ENSGGOP00000023598
Human	SORT1-001	ENST00000256637	7028	20	831	ENSP00000256637
Human	SORT1-002	ENST00000485149	442	3	-	-
Human	SORT1-003	ENST00000466471	521	3	-	-
Human	SORT1-004	ENST00000483508	759	2	-	-
Human	SORT1-005	ENST0000049577	391	2	-	-
Human	SORT1-006	ENST00000471996	469	3	-	-
Human	SORT1-007	ENST00000482236	615	5	-	-
Human	SORT1-008	ENST00000493736	583	6	-	-
Human	SORT1-201	ENST00000538502	2656	20	694	ENSP00000438597
Cat	-	ENSFCAT00000015266	2190	19	729	ENSFCAP00000014154
Dog	XM_537041.2	ENSCAFT00000031550	2446	19	729	ENSCAFP00000029371
Cow	-	ENSBTAT00000011652	2471	20	832	ENSBTAP00000011652
Pig	-	ENSSSCT00000007482	1952	15	645	ENSSSCP00000007286
Dolphin	-	ENSTTRT00000009560	2496	20	831	ENSTTRP00000009060
Horse	-	ENSECAT00000016625	2167	18	722	ENSECAP00000013435
Elephant	-	ENSLAFT00000017144	2190	19	729	ENSLAFP00000014375

To understand better the connections between Sort1 transcript splicing, PGRN haplo-insufficiency, TDP-43 inclusion bodies, and FTLT-TDP first splicing and splicing regulatory factors should be described.

1.11 Pre-mRNA Splicing

In the nucleus of eukaryotes, the new produced mRNA (pre-mRNA) undergoes several transcript processing including RNA 5' capping, RNA 3' end polyadenylation, and RNA splicing before exporting the mature RNA to the cytoplasm (Zheng 2004).

A pre-mRNA is a combination of two consecutive nucleotide sequences including introns and exons. In splicing process, introns are removed while the remained exons are joined to produce the mature-RNA (Zheng 2004) (Fig. 1.4). Totally there are around 26,564 genes in human with 233,785 exons and 207,344 introns; in the other word we have 8.8 exons and 7.8 introns per gene. About the size, exons are mostly less than 200 bp and introns between 100 to 10,000 bp (Sakharkar et al. 2004).

The responsible machinery that mediates splicing is known as spliceosome, a very developed complex made of around 100 essential proteins and 5 small nuclear RNAs (snRNAs), including U1, U2, U4, U5, and U6 (Zhou et al. 2002)

Intron recognition by spliceosomes depends on some highly conserved sequences in each intron, including 5' splice site (5'ss) that starts with dinucleotide GU, 3' splice site (3'ss) that ends with dinucleotide AG, the branch point sequence (BPS) that is located around 18 to 40 nucleotides upstream from 3'ss, and the polypyrimidine tract (PPT) between BPS and 3'ss (Zheng 2004) (Fig. 1.4) (Fig. 1.5).

To begin splicing, U1 binds to 5'ss, splicing factor 1 (SF1) to BP, and U2 axillary factor 1 (U2AF1) together with 2 (U2AF2) to 3'ss and PPT (Guth & Valcárcel 2000), where they function as a binding platform to direct U2 to the BPS in order to replace SF1. The last step requires ATP hydrolysis (Reed 1996). Two important factors that can increase the efficiency of splicing are the high sequence complementarity of 5'ss to U1 and the long PPT at 3'ss (Hertel 2008). After binding, U1 cleaves 5'ss to trigger a loop formation, known as lariat, through guanine –adenine pairing (Fig. 1.4). This process is mediated by transesterification reaction, in which a hydroxyl (OH) group of the adenine performs a nucleophilic attack on the guanine nucleotide at 5'ss. The vicinity of the BPS and 5'ss is guaranteed by U2 and U4/U6. Next, U5 contributes to bring 3'ss to this region and cleaves it to join two exons by covalently bound. This last step also is mediated by transesterification interaction and an OH group from 3' end of the exon attacks the phosphodiester bond in 3'ss (Konarska et al. 1985).

Introduction

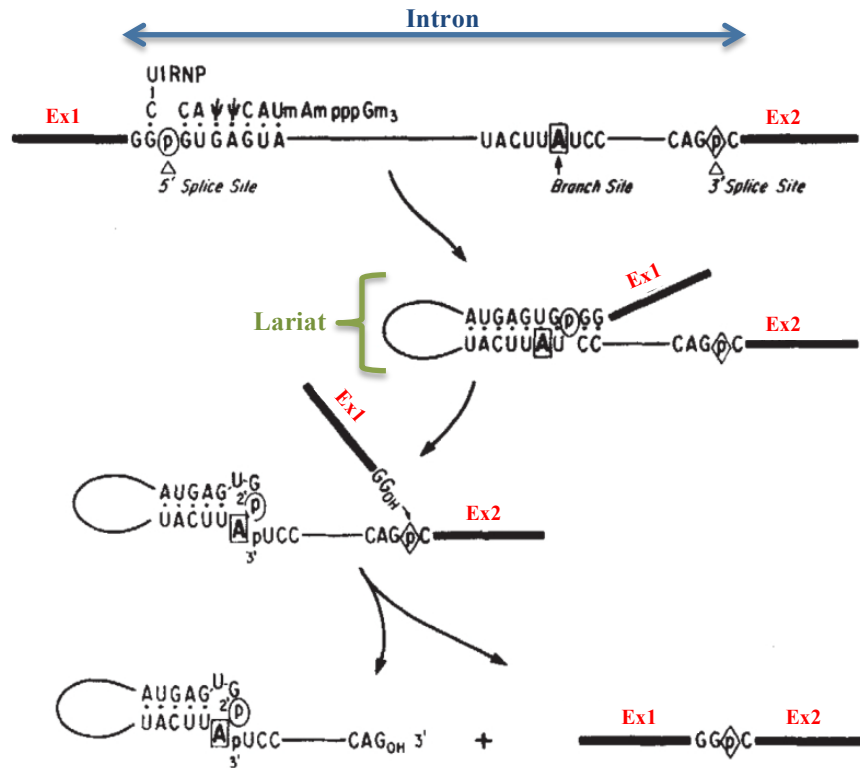


Figure 1.4. Chemical reactions mediated by spliceosome in order to remove the introns and join vicinal exons;
(Ex1 and Ex2) (Konarska et al. 1985).

1.12 Alternative splicing

Alternative splicing is a highly evolved process to increase the coding capacity of eukaryotic genome. This means that a single gene by various combinations of exons could produce different transcript isoforms and eventually different proteins (Johnson et al. 2003) (Fig. 1.5).

The main alternative splicing forms are schematically shown in Figure 1.5B.

Alternative splicing is evolved by special exonic and intronic sequences, known as cis-regulatory elements that can help the spliceosome to recognize the splice sites (Zhang et al. 2003). These sequences are including intronic or exonic splicing silencer (ISS or ESS) and intronic or exonic splicing enhancer (ISE or ESE) elements (Fig. 1.5A). Enhancer elements induce exon inclusion while silencer elements induce exon exclusion (skipping) from the final mature-mRNA (Sharma & Black 2006).

These cis-regulatory elements are recognized by different trans-regulatory elements that are the main mediators of alternative splicing (Zheng 2004).

Two main trans-regulatory factors are serine/arginine (SR)-rich protein family and heterogeneous nuclear ribonucleoprotein (hnRNPs) that usually formers bind to the enhancer elements and latters to the silencer elements (Johnson et al. 2003) (Fig. 1.5A)..

Introduction

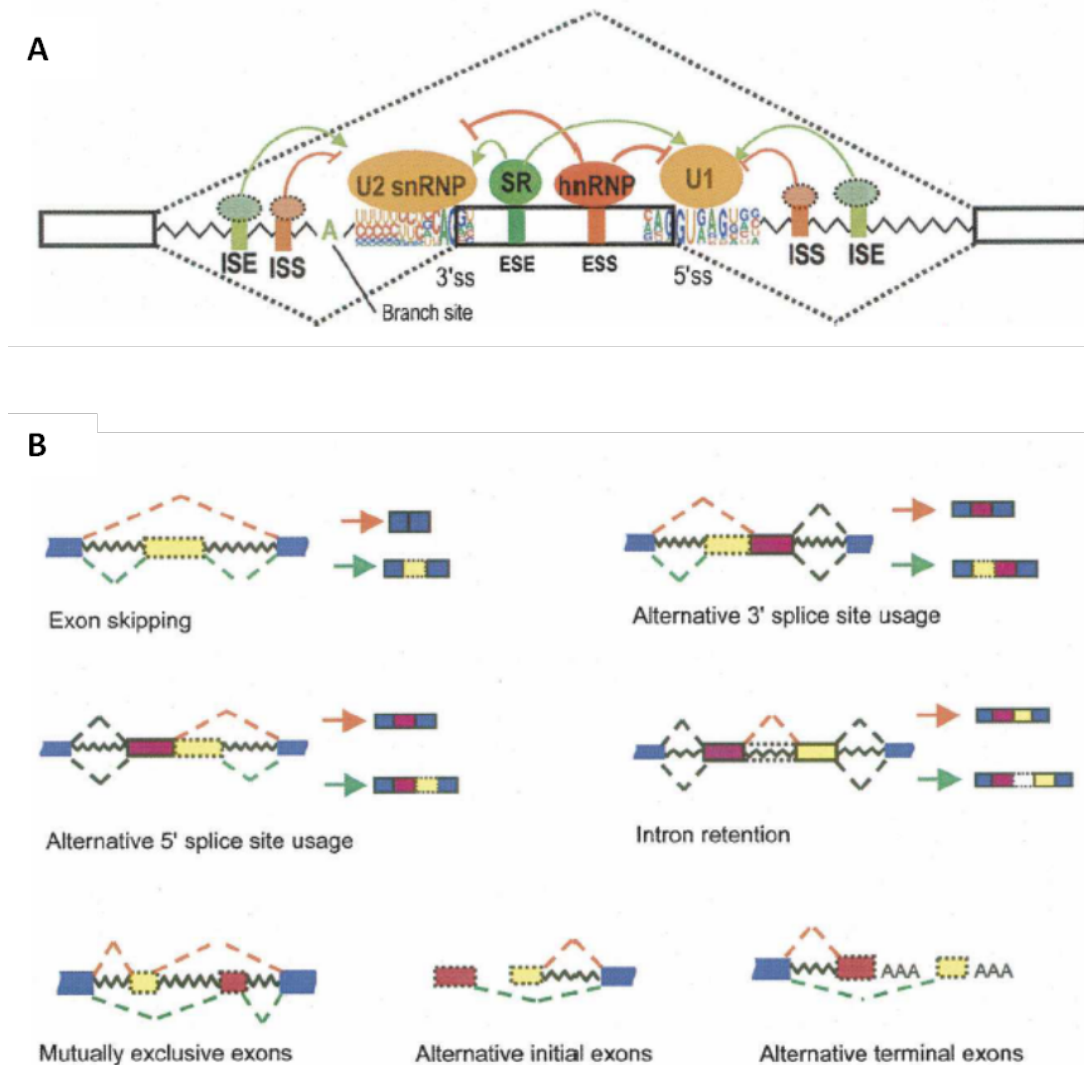


Figure 1.5. Schematic of splicing regulation.

A) Splicing regulation by several factors that bind on different nucleotide sequences; where exons are white boxes, introns are jagged lines.

Through up and down dashed lines, splicing machinery can produce two different alternative spliced transcripts with exon (middle white box) skipping or inclusion respectively. Alternative splicing is controlled by cis-elements (ESE, ESS, ISS, and ISE) that are exonic and intronic sequences and trans-elements (SR proteins, hnRNPs) that are RNA binding proteins.

B) Common sub-types of alternative splicing that can produce different RNA isoforms from a single pre-mRNA (Wang & Burge 2008).

1.12.1 SR proteins

The name of these regulatory factors comes from their structure that contains arginine and serine dipeptides repetitions (arginine/serine (RS) domain) located in their C-terminal region (Busch & Hertel 2012). Functionally these motifs mediate protein–protein interactions essential for recruiting the spliceosomal components (Tacke & Manley 1999).

Moreover, they contain at least one RNA-recognition motif (RRM) in their N-terminal domain to recognize enhancer and silencer sequences (Krainer et al. 1990). So far, in humans around 12 SR proteins are characterized: *Serine/Arginine- rich Splicing Factor, (SRSF) 1–12* (Busch & Hertel 2012). In addition, there is a subclass of RS domain containing factors called SR-related proteins (SRrps) such as Tra and Tra2 with similar functions (Fu 1995).

1.12.1.1. SR proteins as splicing enhancers

RS domain, located in C-terminal of SR proteins, interacts with other splicing factors in order to recruit spliceosomal components to splice sites such as direction of U1 to 5'ss or U2AF to 3'ss. Therefore, by exon definition, SR proteins function as splicing activators (Graveley 2000) (Lam & Hertel 2002) (Fig. 1.5A).

1.12.2 hnRNP Proteins

Consist of RNA and protein components, hnRNPs shuttle between nucleus and cytoplasm with mainly presence in the nucleus (Wang & Burge 2008). At least around 30 hnRNPs has been identified (Krecic & Swanson 1999) involved in a wide range of biological functions, including polyadenylation, translation, mRNA stability and splicing. Like SR proteins, they contain RRM domains usually for binding to splicing silencer sequences on pre-mRNA. They mostly carry RGG boxes (Arg-Gly-Gly tripeptides), and additional glycine-rich, acidic or proline-rich domains (Dreyfuss et al. 1993). Because of modularity, hnRNPs are structurally and functionally diverse and unlike SR proteins are not totally characterized (Busch & Hertel 2012).

1.12.2.1 hnRNPs as splicing suppressors

There are not very well known mechanisms by which hnRNPs inhibit splicing but protein-protein interaction mediated by RGG boxes, glycine-rich, acidic or proline-rich domains is a crucial starting point for splicing regulation.

hnRNPs can block spliceosome formation (Fig. 1.5A), for example PTB/hnRNP I inhibits interaction between U1 and U2 (Izquierdo et al. 2005) or hnRNP A1 can loop out the exon (Martinez-Contreras et al. 2006).

1.12.3 Context dependence of alternative splicing regulatory factors

The location of SR proteins and hnRNPs can affect their ability to activate or suppress the splicing. For example, by increasing the distance between enhancer elements (SR proteins) and the splice sites, the probability of exon inclusion can decrease (Graveley et al. 1998).

On the other hand the location of splicing regulatory sequences (cis-regulatory elements) are important as well. For example, if GGG sequence is located in the introns it will enhance the splicing (McCullough & Berget 1997), but when is located in the exons inhibits splicing (Chen et al. 1999).

Every exon is evolved with a unique group of SR and hnRNP protein binding sites, typically close to the exon/intron junctions, leading to correctly interplay between them to assemble or disassemble the spliceosome (Hertel 2008).

1.12.4 Additively and cooperatively function of splicing regulatory factors

Splicing regulatory factors function additively and more number of them increases their efficiency (McCullough & Berget 1997) (Zhang & Chasin 2004), because more copies elevate the affinity of the associated factors or increases the recruited factors in a synergistic fashion (Dominguez & Allain 2006). Moreover, there is evidence showing that splicing regulatory factors can cooperatively regulate alternative splicing. For example, exon 19 of neuronal NMDA R1 is skipped by cooperative function of regulatory factors binding on exonic UAGG and intronic GGGG motifs at 5'ss region (Han et al. 2005).

The appropriate interaction between splicing regulatory factors can guarantee the physiological conditions for the cells. Recent data have shown that co-overexpression of hnRNP U and hnRNP A1/A2 can inhibit TDP-43-induced neuronal cell death in NSC34 cells (Suzuki et al. 2015). Similarly in *Drosophila*, the ability of TDP-43 to suppress CGG RNA-

Introduction

associated neurotoxicity is dependent on its ability to interact with two fly homologs of hnRNP A2/B1, and overexpression of these factors could attenuate TDP43-induced cell death (He et al. 2014) (Romano et al. 2014).

Finally, due to critical importance of alternative splicing in eukaryotes for production of different protein isoforms, any impairments like mutations of either cis- or trans- regulatory elements can lead to a serious disorder (Cooper et al. 2009).

1.13 TDP-43

Transactive response DNA-binding protein 43 KDa (TDP-43) is one of the most important hnRNPs regarding to FTLN disease, because its cytoplasmic inclusion bodies often are detectable in both sporadic and familial FTLN cases (Neumann et al. 2006). Primary identified as HIV-1 transcription suppressor (Ou et al. 1995), TDP-43 can bind to both DNA and RNA and regulate transcription, pre-mRNA splicing, mRNA stability, microRNA biogenesis, transport and translation (Godena et al. 2011).

Structurally, this 43-kDa protein with 414 amino acids contains an N-terminal nuclear localization signal that retains TDP-43 dominantly inside the nucleus (Budini et al. 2012) (Fig. 1.6). It has two RNA recognition motifs, RRM1 (the main interactor) and RRM2, in order to recognize the binding sites on the RNA (usually UG repeats at 3'ss). The RRM-2 domain carries a nucleus export signal; therefore by this domain and N-terminus nuclear localization signal, TDP-43 continuously shuttles between the nucleus and the cytoplasm in the normal condition (Budini et al. 2012).

TDP-43 has a very important part in C-terminal region that makes up almost half-length of the protein. This domain contains glycine-rich and Glutamine/Asparagine-rich motifs important for protein-protein interactions and possibly responsible to form the inclusion bodies in pathologic conditions (Budini et al. 2012).

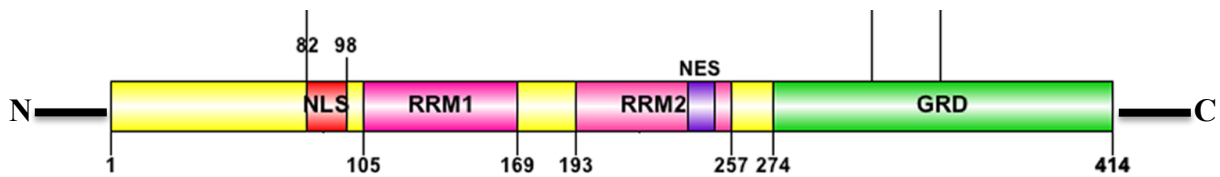


Figure 1.6. Schematic of TDP-43 structure with different amino acidic domains.

The N-terminal contains a nuclear localization signal (NLS) that maintains TDP-43 mainly in the nucleus. By two RNA binding motifs, RRM1 and RRM2, TDP-43 can recognize RNA binding sites. RRM2 domain contains a nuclear export signal (NES) that directs TDP-43 to the cytoplasm. C-terminal domain with glycine-rich domain (GRD) contributes in TDP-43 interaction with other proteins such as hnRNPs (Liu-Yesucevitz et al. 2010).

1.13.1 TDP-43 Pathology

Normally, TDP-43 is mostly localized in the nucleus (Godena et al. 2011) while in the pathological conditions like ALS and FTLT, it is mainly shifted to the insoluble aggregations inside the cytoplasm (Neumann et al. 2006).

Although TDP-43 proteinopathies is a hallmark of ALS and FTLT-TDP diseases, it is also involved in some other neurodegenerative diseases, including Alzheimer's disease and Lewy body disorders (Mackenzie et al. 2010). TDP-43 pathology, as a primary event in ALS and FTLT-TDP and secondary event in other diseases, remains to be determined whether can worsen the extent of neurodegeneration in the patients.

As reviewed by Lee et al. TDP-43 pathology includes abnormal phosphorylation and ubiquitylation, accumulation of insoluble TDP-43 inclusions, the presence of truncated TDP-43 C-terminal fragments (CTFs) mostly in cerebral cortex with molecular weight between 20 to 25 kDa, mislocalization of TDP-43 protein from nucleus into cytoplasmic inclusion bodies,

and nuclear reduction of TDP-43 expression (Lee et al. 2011). This complex changes in TDP-43 physiology makes it complicated to understand which of these events are mainly the causes of neurodegeneration and which are the secondary consequences of the diseases.

All together, there are two theories believing that TDP-43 loss (loss of normal nuclear TDP-43 and therefore loss of its nuclear functions) or gain (toxic TDP-43 aggregates in the cytoplasm) of functions may lead to neurodegeneration (Chen-Plotkin et al. 2010) (Lagier-Tourenne et al. 2010).

1.13.2 TDP-43 interacting proteins

The protein interaction network of TDP-43 has been analyzed in Human embryonic kidney cells 293 (HEK-293) cells by global proteomic approach. After transfection of cells with FLAG-TDP-43, TDP-43 was immunoprecipitated and co-purified proteins were recognized by mass spectrometry. The detected interactor proteins are mostly associated with RNA, including hnRNPs, splicing factors, RNA helicases, proteins responsible for mRNA transport and stability, and translation regulatory factors (Freibaum et al. 2010) (Fig. 1.7). Among TDP-43 interactors, there are also a few number of DNA binding proteins like transcription factors and some DNA repair proteins (Ou et al. 1995).

In general TDP-43 binding proteins can be categorized in two main groups: cytoplasmic proteins that are mostly involved in translation and nuclear proteins such as hnRNPs and SR proteins that mostly regulate RNA splicing (Freibaum et al. 2010). Since TDP-43 is shuttling between nucleus and cytoplasm, this wide range of interactors was predictable (Ayala et al. 2008) reflecting the multiple roles for TDP-43 in RNA metabolism regulation.

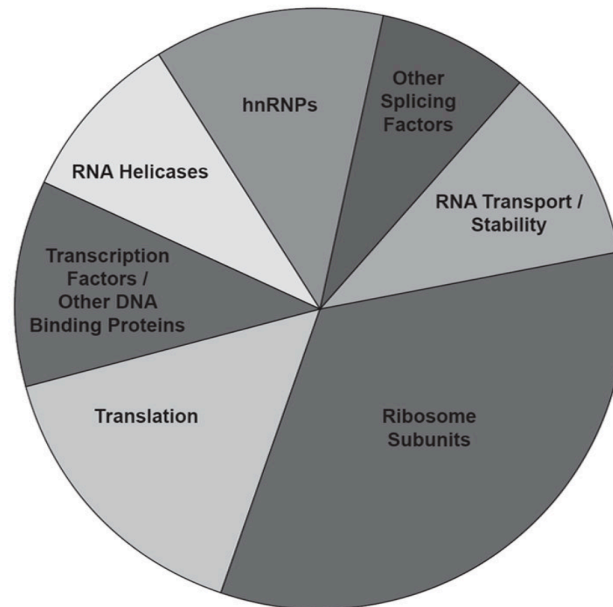


Figure 1.7. The presented Pie chart shows the proportion of different TDP-43 protein interactors. (Freibaum et al. 2010).

1.13.2.1 Functional consequences of TDP-43 interaction with hnRNPs

TDP-43 can interact with other hnRNPs mainly through its C-terminal domain that contains Gln/Asn-rich region between residues 321 to 366 (prion-like domain) (Budini et al. 2012). There are some studies pointing out that these interactions can modulate some functional consequences of TDP-43.

In *Drosophila* TBPH is the homolog of human TDP-43 and these two proteins are functionally replaceable in both fly and human context (Ayala et al. 2005).

It has been shown that interaction with TDP-43 is important for splicing inhibitory effect of Hrp38, hnRNP A1 homolog in *Drosophila*, and TDP-43 knockdown in HEK293 cells can abolish suppression of apolipoprotein A-II (apoAII) exon 3 inclusion by Hrp38 overexpression (Romano et al. 2014).

More interestingly simultaneous knockdown of TBPH (TDP-43 homolog) and Hrp38 (hnRNP A1 homolog) in *Drosophila* worsens the locomotor deficit and reduces life span in higher extends expected for an additive effect of individual knockdowns for each gene (Romano et al. 2014).

Introduction

It looks that cooperation between TDP-43 and other hnRNPs are evolutionary conserved not only in humans but also in *Drosophila* and have fundamental consequences *in vivo* (Romano et al. 2014).

In another work in *Drosophila*, TDP-43 can rescue neurodegeneration induced by CGG repeat expansion in the 5' UTR of the fragile X gene (FMR1) through interaction with hnRNP A2/B1 (fly homologs are Hrb87F and Hrb98DE) (He et al. 2014). In this study, transgene expression of a toxic 90 CGG repeat expansion targeted to the *Drosophila* eye induces eye degeneration. TDP-43 overexpression can abolish this degeneration and ameliorate eye phenotype. Interestingly after deletion of C-terminal residues 321-366 that are crucial for TDP-43 to interact with other hnRNPs specially with hnRNP A2/B1 (D'Ambrogio et al. 2009), TDP-43 loses its neuroprotective function against CGG repeat toxicity (He et al. 2014). On the other hand, knockdown of *Drosophila* hnRNP A2/B1 homologs, Hrb87F and Hrb98DE, also blocks TDP-43 for suppressing CGG repeat toxicity (He et al. 2014).

Finally it looks that CGG repeats can mis-splice hnRNP A2/B1 mRNA targets and this could be compensated by TDP-43 overexpression (He et al. 2014).

There are reports showing that nuclear overexpression of TDP-43 leads to caspase-mediated cell death through several toxic pathways such as JNK/c-Jun signaling, upregulation of CHOP and Bim expression and downregulation of Bcl-xL expression (Suzuki et al. 2011) (Suzuki & Matsuoka 2012) (Suzuki & Matsuoka 2013). Interestingly this toxic effect of overexpressed nuclear TDP-43 can be ameliorated by interaction with hnRNP U (Suzuki et al. 2015).

Immunoprecipitation assays has turned out that hnRNP U is one of the strongest TDP-43 interactors in the nucleus of neuronal cell line, NSC34. Further interaction assessments using wild type and mutant hnRNP U constructs showed that 90 amino acids located in C-terminal of hnRNP U are crucial for TDP-43 interaction (Suzuki et al. 2015).

While knockdown of hnRNP-U additively increases nuclear overexpressed TDP-43-induced neuronal cell death, overexpression of hnRNP U moderates this toxic effect by decreasing caspase-3 cleavage.

Toxicity regulation of hnRNP U is dependent on the presence of its C-terminal with 90 amino acids involved in TDP-43 interaction and mechanistically is through JNK/c-Jun signaling modification. Obviously the protective effect of hnRNP-U overexpression has a limit and

more expression of this binding protein above a protective threshold can be toxic itself (Suzuki et al. 2015).

Either knockdown or overexpression of TDP-43 in the nucleus decreases variant 1 of human POLDIP3 mRNA and increases variant 2 in which exon3 is skipped (Shiga et al. 2012) (Suzuki et al. 2015) It has been shown that while co-overexpression of hnRNP-U and TDP-43 did not have any effect on the reduced levels of variant 1, it could decrease the TDP-43-induced variant 2 elevations (Suzuki et al. 2015).

Finally in the same study was discovered that simultaneous overexpression of hnRNA-A2 with TDP-43 could rescue neuronal cell death as well (Suzuki et al. 2015).

1.14 PGRN deficiency and cytosolic TDP-43 aggregation

In FTLTDP patients following PGRN haplo-insufficiency, because of PGRN mutations and degradation of produced mRNA by NMD, TDP-43 mis-localizes in the cytoplasm in the aggregated forms known as TDP-43 inclusion bodies (Bird 2009) (Götzl et al. 2016).

In this situation, TDP-43 undergoes several post-translational modifications such as proteolytic cleavage ending up with production of two low-molecular-weight ~25 and ~35 kDa TDP isoforms, phosphorylation, and ubiquitination that eventually through oligomerization, fibrilization, and blocking degradation by ubiquitin–proteasome system lead to TDP-43 inclusion formation that according to the gain function theory could be toxic and harmful for the cell health (Zhang et al. 2009) (Zhang et al. 2007).

It has been shown that PGRN haplo-insufficiency through caspase activation, triggers TDP-43 proteolytic cleavage and produces two short isoforms ~25 and ~35 kDa (involved in aggregate formation) and directs the cells to the programmed cell death (Zhang et al. 2007). It is considerable that elevated activate caspase-3 has been detected in both FTLTDP and ALS patients (Su et al. 2000).

The mechanisms by which reduction of PGRN can activate caspase-3 and stimulate TDP-43 cleavage are not well described. So far, it has been demonstrated that SLPI that blocks PGRN proteolysis, can also inhibit caspase-3 and PGRN reduction disturbs caspase-3 regulatory role of SLPI (Zhu et al. 2002).

Here, there are some recent studies focusing on eliminating the accumulated TDP-43 in the cytoplasm caused by PGRN deficiency.

Lack of PGRN in lymphoblasts can increase cell cycle activity through stimulation of the ERK1/2 signaling (Alquézar et al. 2014) and induce cytosolic TDP-43 pathology appearance (Alquezar et al. 2015).

It has been shown that increasing expression of PGRN by suberoylanilide hydroxamic acid (SAHA) (Cenik et al. 2011), or inhibition of ERK1/2 activity by selumetinib can reduce TDP-43 cytosolic aggregates in PGRN deficient cells with no change of TDP-43 levels in the normal conditions. Therefore, these compounds could be appropriate therapy candidates for FTLTDP patients in future (Alquezar et al. 2015).

In the next study they targeted Casein kinases 1 (CK-1) as a TDP-43 phosphorylation mediator that triggers TDP-43 cytosolic inclusions (Alquezar et al. 2016).

They found that CK-1 inhibitors (able to cross blood brain barrier) could normalize mis-localization of TDP-43 in lymphoblasts that carry PGRN mutations.

Moreover, these kinase inhibitors could protect human neuroblastoma cell line from the toxic effects of TDP-43 phosphorylation induced by ethacrynic acid (Alquezar et al. 2016).

1.15 Nuclear depletion of TDP-43 impairs PGRN metabolism through Sort1 mRNA mis-splicing in human

In humans TDP-43 pathology according to the loss of function theory, can mis-regulate mRNA splicing of Sort1 that is the main receptor to control the extracellular PGRN levels and also mediate some internal cellular functions of PGRN as mentioned before (Lewis & Golde 2010) (Mackenzie et al. 2010) (J. Wang et al. 2010).

In mice, TDP-43 regulates Sort1 mRNA splicing and PGRN metabolism (Polymenidou et al. 2011). In fact TDP-43 inhibits inclusion of Exon 17b (located between exon 17 and exon 18) in the mature Sort1 transcript (Prudencio et al. 2012).

Of interest, in the physiological conditions in mice, two isoforms of Sort1 are detectable with or without Ex17b. The transcript isoform included Ex17b in mice encodes a functional

Introduction

receptor having an extra region between the VSP10 and trans-membrane domains able to regulate PGRN endocytosis (Prudencio et al. 2012) (Fig. 1.8).

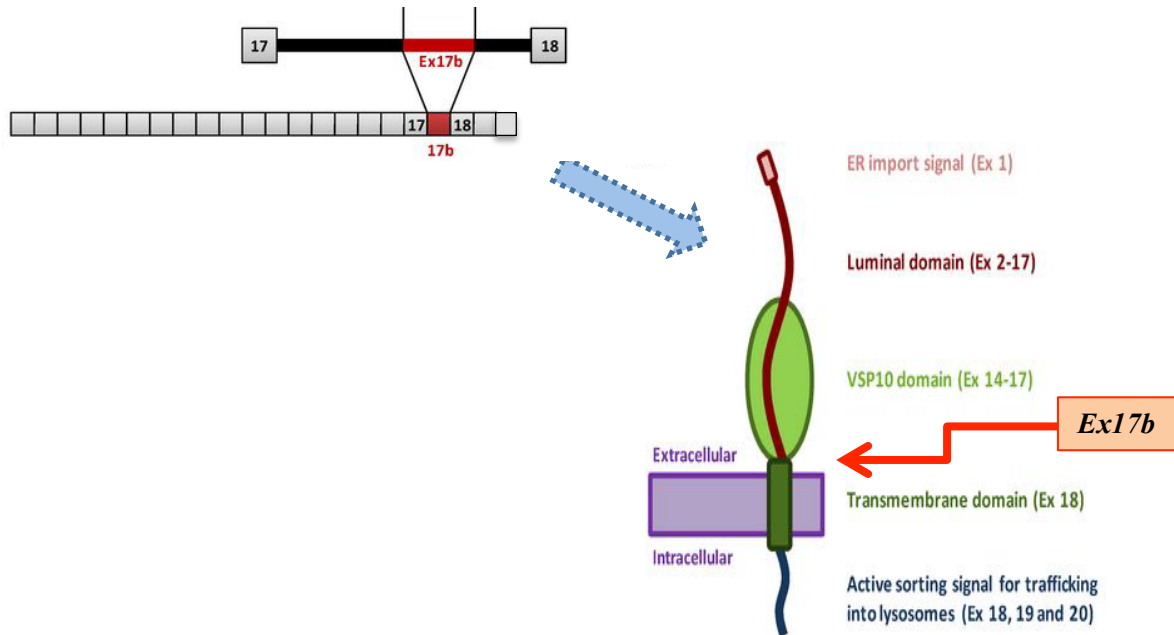


Figure 1.8. Inclusion of mouse Sort1 Ex17b in the mature mRNA.

Sort1 Ex17b (located between exon 17 and exon 18) in mice encodes a region between VSP10 and transmembrane motifs and produces a functional receptor (Prudencio et al. 2012).

Instead in humans in the normal conditions this alternative isoform of Sort1 transcript including Ex17b is absent because it contains a premature stop codon and is potential to produce a truncated and non-functional Sort1 receptor that binds but does not internalize PGRN (Prudencio et al. 2012) (Fig. 1.9).

This is a critical evolutionary difference in Sort1 splicing between human and mouse, and in addition to other differences it can somehow explain why FTLN mice models do not show the phenotypes of human FTLN (Prudencio et al. 2012).

Introduction

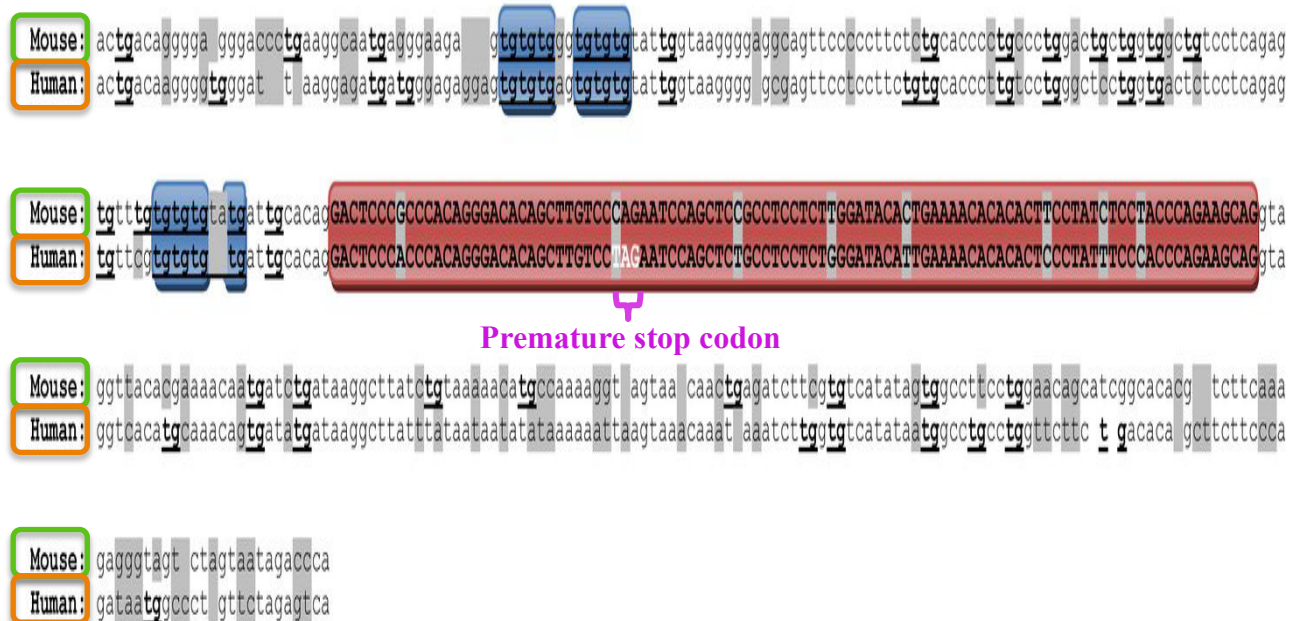


Figure 1.9. Human and mouse Sort1 Ex17b nucleotide sequence homology.

There is a high nucleotide homology between mouse (upper line) and human (lower line) Ex17b (red high-lighted region) and their flanking introns. However, human Ex17b contains a premature stop codon (TAG) that can lead to production of a truncated Sort1 receptor. TDP-43 can inhibit inclusion of Ex17b by binding to intronic splicing silencer rich in “tg” repeats (blue high-lighted regions). (Prudencio et al. 2012).

The mouse Ex17b inclusion is increased following TDP-43 knockdown, the fact that is confirmed in the mouse cell lines, primary cultures and in vivo. This splicing inhibitory regulation is dependent on the RRM, glycine-rich domain, and nuclear localization signal of TDP-43 (Prudencio et al. 2012).

While TDP-43 depletion led to a significant increase of Ex17b inclusion, TDP-43 knockdown in human HeLa cells only caused a mild increase of EX17b inclusion (Prudencio et al. 2012) (Fig. 1.10). The obtained data were surprising since the sequences of Ex17b and its flanking introns in mice and humans are highly homologs (Fig. 1.9).

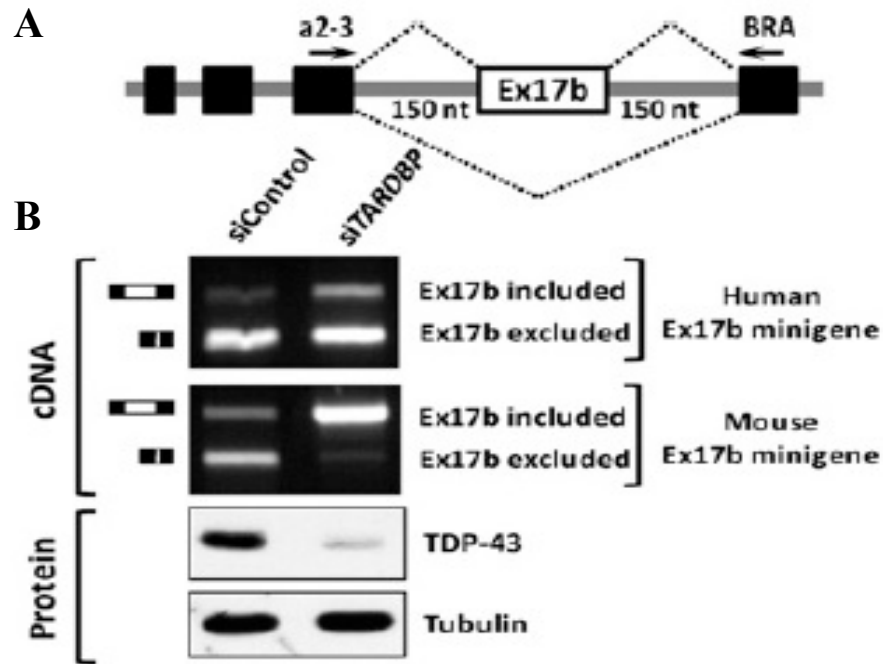


Figure 1.10 The effect of TDP-43 knockdown on human and mouse Ex17b inclusion in human HeLa cells.

A) Schematic presentation of human/mouse Ex17b minigenes transfected in HeLa cells after TDP-43 silencing. **B)** Using BRA and α2-3 primers splicing pattern of Ex17b minigenes are detected on agarose gels. While there is a huge increase in mouse Ex17b inclusion after TDP-43 knockdown, only a mild increase of human Ex17b inclusion is detectable after the same experiments. The lowest panel shows successful TDP-43 depletion in HeLa cells (Prudencio et al. 2012).

These results are very important because they suggest existence of other splicing regulatory factors apart from TDP-43 that tightly control inhibition of Ex17b in humans and probably that is how in physiological conditions Ex17b is highly excluded from human Sort1 transcript. Therefore one could assume that in the pathological conditions, loss of nuclear TDP-43 function following aggregation of this protein in the cytoplasm and possibly additional impairment of other splicing regulatory factors may result in increase of Sort1 Ex17b mRNA levels and final imbalance between extracellular and intracellular PGRN levels.

According to some in vitro experiments, using cell expression of human and mouse Sort1 alternative isoforms and the supplemented media with recombinant exogenous PGRN, while both mouse produced receptors are locational and functionally normal, the human truncated Sort1 receptors are mostly detectable in the extracellular regions and behaving like a decoy receptor meaning that they are potential to compete with the wild type Sort1 receptors and reduce PGRN endocytosis and signaling (Prudencio et al. 2012)(Fig. 1.11).

1.16 High Sort1 Ex17b mRNA levels in FTLD-TDP patients

Brain samples from FTLD-TDP patients show high levels of Sort1 Ex17b mRNA in the temporal cortex but not in the other brain regions such as cerebellum that is not affected by this disorder. In fact the levels of Sort1 Ex17b mRNA in the cerebellum are not detectable. Also considerably, Sort1 Ex17b mRNA is not detectable in the brain of FTLD-tau cases with positive inclusion bodies for tau but not TDP-43 (Prudencio et al. 2012).

All together it looks that mis-regulation of Sort1 mRNA splicing is specific for FTLD-TDP and the affected tissue is cerebral cortex.

Interestingly higher levels of wild type Sort1 transcript are also detectable in FTLD-TDP compare to the control healthy brains. This increase in wild type Sort1 isoform is also found in TDP-43 knocked down human cells and possibly implies the compensatory reaction to the decoy effect of truncated Sort1 Ex17b receptors in order to improve PGRN uptake and signaling. However, on the other hand increase of wild type Sort1 could also worsen the condition by over reduction of extracellular PGRN that has other vital duties through other signaling pathways by binding to TNF receptors (Lee et al. 2014) (Prudencio et al. 2012) (Gass et al. 2012). This shows the crucial importance of PGRN levels regulation in the brain; therefore any disturbance of Sort1 as a PGRN receptor may have detrimental consequences for the brain.

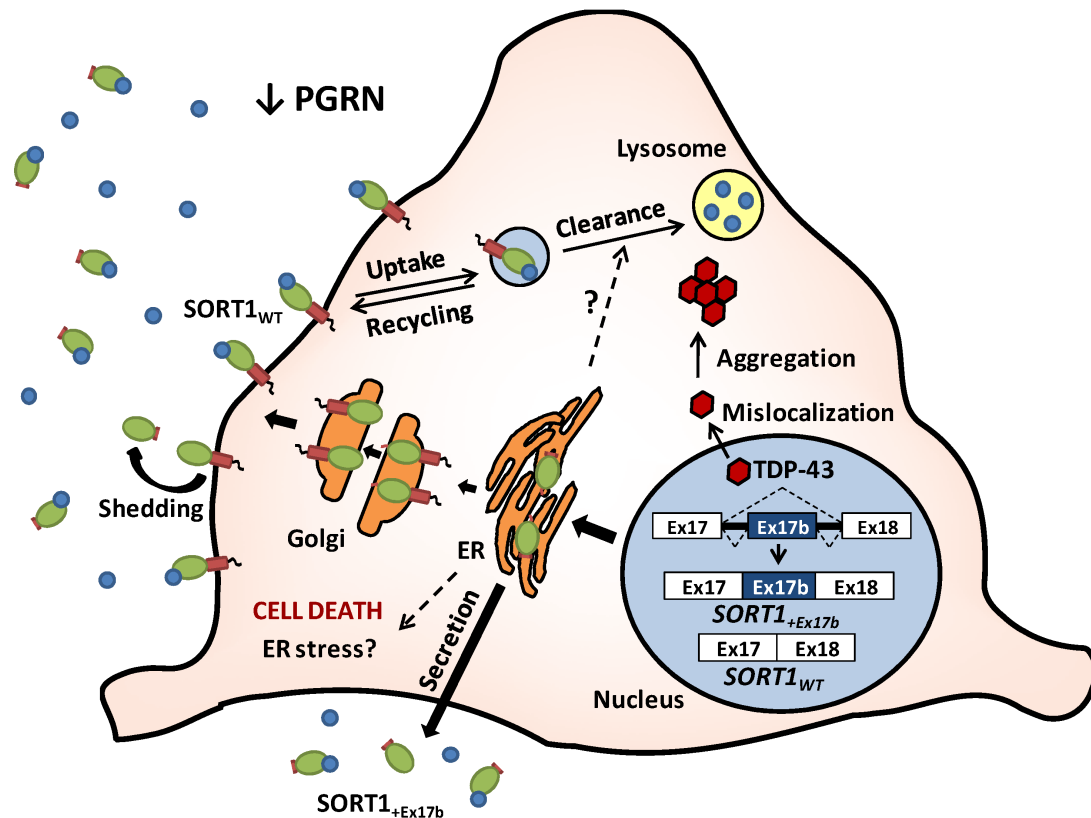


Figure 1.11. Based on the loss of function theory, nuclear depletion of TDP-43 through Sort1 mRNA mis-splicing impairs PGRN metabolism in human.

Due to PGRN haplo-insufficiency (caused by PGRN mutations), TDP-43 is mis-localized in the cytoplasm in the aggregate forms. Nuclear depletion of TDP-43 increases inclusion of Ex17b in Sort1 transcript that is potential to produce truncated Sort1 receptors able to bind to PGRN but unable to internalize it. Therefore the extracellular levels of PGRN will drop more that consequently worsens TDP-43 aggregate formation. On the other hand, by preventing PGRN internalization, truncated Sort1 receptors can also interfere with lysosomal pathways and probably with TDP-43 clearance (Prudencio et al. 2012).

1.17 Human and mouse Sort1 Ex17b are differentially evolved regarding to RNA binding proteins

Bioinformatics analyses have revealed a very high degree of homology between Ex17b and its flanking introns' sequences across the different species, taking in consideration of an in-frame stop codon in human and other primates made by a nucleotide mismatch in Ex17b sequence (Prudencio et al. 2012) (Fig. 1.9).

Since in the physiological conditions, Sort1 Ex17b mRNA is not detectable in species with a premature stop codon in Ex17b sequence, it could be assumed that evolutionary changes have been in favor of Ex17b exclusion in those cases.

Binding of splicing enhancers such as SRp40 and Tra2 to mouse but not human Sort1 sequence somehow reflects the evolutionary hypothesis claiming that evolution has been in a direction to suppress the inclusion of this toxic exon in human (Prudencio et al. 2012).

1.18 FTLT-TDP mouse model

So far there is no developed animal model for FTLT-TDP. While in mice by peripheral axotomy, TDP-43 mislocalizes from nucleus to the cytoplasm in motoneurons and mice show some FTLT/ALS-like pathology (Moisse et al. 2009), none of PGRN or Sortilin knockout mice in any age show complete FTLT-TDP pathological features (Ahmed et al. 2010) (Jansen et al. 2007).

The lack of a proper animal model to study FTLT-TDP leaves understanding the exact relation among PGRN reduction, neurodegeneration, and TDP-43 pathology unclear. Therefore, there is still a strong need to have a valid animal model for FTLT-TDP in order to discover the exact molecular mechanisms involved in the disease and also to look for the right and effective therapies.

1.19 The aim of this study

As previously described, emerging evidence indicates that Sort1 as an important PGRN receptor is a key player in regulation of neuronal viability and its splicing aberration due to

Introduction

impairment of PGRN endocytosis and signaling can probably contribute to induce neurodegeneration (Lewis & Golde 2010) (Mackenzie et al. 2010) (X. Wang et al. 2010).

Tightly regulation of Sort1 mRNA splicing is crucial for humans because inclusion of Ex17b can produce a truncated receptor that through competing with normal receptors can inhibit PGRN internalizing and signaling and therefore is toxic for the cells (Prudencio et al. 2012). For this reason, evolutionally it is important for human Sort1 transcript to have its own special RNA binding protein network dedicated for inhibition of toxic Ex17b inclusion in the mature mRNA.

According to the mice experiments, TDP-43 is one of the main candidates that can suppress Ex17b inclusion in human as well. But surprisingly, unlike mouse Ex17b, cells depleted from this splicing suppressor, show a mild increase in human Ex17b inclusion, pointing the existence of other possible regulatory factors responsible for splicing regulation of this extra exon in human (Prudencio et al. 2012). Thus, a better understanding of all possible splicing regulatory factors for Sort1 Ex17b is of great importance.

The previous study by Prudencio et al. showing the importance of TDP-43 in suppression of Ex17b was only done in Hela cells (Prudencio et al. 2012); Therefore first of all there is a need to extend the splicing regulatory role of TDP-43 in inclusion of human and mouse Ex17b in different cell contexts.

Then the next step is a precise mapping of all the regulatory splicing factors including hnRNPs and SR proteins and their binding sites that may act independently or in a combinatorial manner with TDP-43 to increase or decrease human/mouse Ex17b inclusion. The obtained data may contribute to explain the differential inclusion of Sort1 Ex17b in human vs. mice.

By this project I will provide a better splicing-history of Ex17b in different species and useful data with regards to the adaptation of splicing mechanisms through evolution.

At the end for the clinical point of view by collaboration with Department of Neuroscience, Mayo Clinic Jacksonville in USA in order to determine whether a change in hnRNP protein expression might be associated with FTLD-TDP, their transcript levels are evaluated in the temporal cortex of FTLD-TDP patients, an affected brain region in which a significant increase in Ex17b inclusion has been already observed (Prudencio et al. 2012).

Introduction

All in all, I can extend the results of this project to determine how these regulatory splicing factors/sequences functionally control Ex17b recognition in various human and mouse cell lines/tissues, the degree of conservation of these elements in different species, and especially the potential pathological connection between different RNA binding proteins and Sort1 in disease conditions.

Therefore, the major aim of this thesis will be providing a better understanding of cis- and trans-splicing elements involved in exclusion of toxic Ex17b in human that is potential to induce several pathological implications like FTLT-TDP.

Chapter 2

2 Materials and methods

2.1 Standard reagents and solutions

2.1.1 Chemical sources

Chemical reagents were provided from different companies, including Gibco-BRL, Life Technologies Inc., Sigma-Aldrich, Merck, boehringer mannheim corporation, Fluka, Riedel-Haell, Amersham Biosciences, BioRad, Invitrogen, Qiagen, Roche Diagnostics, and Euroclone.

2.1.2 Solutions

1. PBS: 137 mM NaCl, 2,7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4
2. TBS: 50 mM Tris, 150 mM NaCl, pH 7.6.
2. 10X TBE: 108 g/l TRIS, 55 g/l Boric Acid, 9.5 g/l EDTA
3. 5X DNA sample buffer: 0.5 g/ml Sucrose, 0.24 g /ml Urea, 0.5 ml TBE 10X, 0.01 ml 1% bromophenol blue
4. 5X SDS protein sample buffer: 250 mM Tris/HCl pH 6.8, 10% w/v SDS, 30% v/v Glycerol, 5% v/v β -mercaptoethanol, 0.002% v/v bromophenol blue
5. 10X Transfer buffer: 30.3 g/l TRIS, 144 g/l Glycine
6. 1X SDS-PAGE running buffer: 50mM TRIS, 0.38 Glycine, 0.1% w/v SDS

2.1.3 Synthetic oligonucleotides

All oligonucleotides used in this project were synthesized by Sigma-Aldrich.

2.1.4 Enzymes

2.1.4.1 Restriction enzymes

All the restriction enzymes were obtained either from Promega or New England Biolabs.

2.1.4.2 DNA modifying enzymes

Taq Polymerase and T4 DNA ligase were obtained from Roche Diagnostics, Klenow fragment of *E. coli* DNA polymerase I, T4 Polynucleotide kinase and Calf Intestinal Alkaline Phosphatase (CIP) were obtained from New England Biolabs and M-MLV reverse transcriptase was purchased from Invitrogen.

2.1.4.3 Nucleases

RNase A was obtained from Sigma-Aldrich. The 10 mg/ml stock solution of RNase A was prepared in ddH₂O and boiled for 10-20 min to destroy trace amounts of DNase activity.

DNase I recombinant was purchased from Roche Diagnostics. All enzymes were used according to the manufacturer's instructions.

2.2 Bacteria

2.2.1 Bacterial culture media and strain

The *E. coli* K12 strain DH5 α was always used for transformation by the interested plasmid in order to amplification. For the short term, the agar plates containing appropriate antibiotic (ampicillin) were used to keep the bacterial colonies at +4°C. But for the long term after adding sterile glycerol to a final 30% v/v concentration to the liquid bacterial cultures, they were stored at -80 °C.

When needed, an overnight culture of bacteria was grown in Luria-Bertani medium (LB) made of 10 g/l Difco Bactotryptone, 5 g/l Oxoid yeast extract, 10 g/l NaCl, pH7.5. For proper screening of wanted bacteria, 100 μ g/ml of ampicillin was added to LB medium.

2.2.2 Bacterial competent cells

Competent cell preparation was according to the method described by Chung (Chung et al. 1989). First, *E. coli* strains were grown overnight at 37°C in 5 ml of LB. Then the following day, 100 ml of fresh LB medium was added to this pre-inoculum and again they were grown at 37°C. Every half an hour for 4-5 hours, using a spectrophotometer, the absorbance at the wavelength of 600 nm was under the control until OD₆₀₀ reached to 0.3-0.4.

In this step by putting the LB medium on the ice, bacteria stop growing. They should be centrifuged for 10 minutes at +4°C at 1000 rpm to obtain the pellet that contains only bacteria. The pellet was resuspended in 10 ml of cold TSS solution (10% w/v PEG, 5% v/v DMSO, 35mM MgCl₂, pH=6.5 in LB medium) and rapidly aliquoted and frozen in liquid nitrogen and stored at -80°C.

Bacterial ability for competition was satisfactory if more than 100 colonies were observed after transformation by 0.1 ng of pUC19 plasmid.

2.2.3 Bacterial transformation

For the ligation reactions, half of the reaction volume and for transformation of clones, 20 ng of plasmid DNA was incubated with 60 µl of competent cells for 30 minutes on ice, then immediately a heat shock at 42°C was performed for 2 minutes. After another 5 minutes of incubation on ice, 60 µl of LB were added and the bacteria allowed to recover for 20 minutes at 37°C. Bacteria were then cultured on the agar plates containing the appropriate antibiotic (ampicillin) and the plates were incubated overnight at 37°C.

2.3 Plasmid preparation

2.3.1 Small-scale preparation of plasmid DNA from bacterial cultures (minipreparation)

For fast DNA extraction, an inoculation of a single colony in 3 ml of LB medium supplemented with the appropriate antibiotic (ampicillin) was grown overnight at 37°C. Quick small-scale preparation (miniprep.) was performed using the Wizard plus SV minipreps DNA purification system (Promega) according to the manufacturer's instructions.

2.3.2 Large-scale preparation of plasmid DNA from bacterial cultures (midipreparation)

To obtain a larger amount of DNA extraction, a single colony was inoculated overnight in 50 ml LB medium supplemented with a proper antibiotic (ampicillin) at 37°C. The following day the JetStar purification kit (Genomed) was used for DNA extraction, according to manufacturer's instructions.

2.4 Quality and quantity control of the obtained nucleic acids

2.4.1 Nucleic acid concentration

Using NanoDropTM 1000 spectrophotometer, an optical density (OD) of 1.0 at 260 nm is usually considered equivalent to a concentration of 50 µg/ml for double stranded DNA and 40 µg/ml for single stranded DNA and RNA.

The ratio of values measured at 260 nm and 280 nm indicates the purity of the samples with optimum pureness numbers including 1.8 for DNA and 2 for RNA. Any protein contamination will decrease the optimum pureness numbers (Evans 1990). Therefore by this technic not only the concentration but also the purification of the obtained nucleic acid could be evaluated.

2.4.2 UV fluorescence of intercalated ethidium bromide

DNA could be visualised after staining with ethidium bromide (0.5 g/ml). Ethidium bromide's fluorescence will increase by intercalation between bases in a double stranded nucleic acid. For this reason visually is possible to estimate nucleic acid concentration on a gel by comparing the intensity of UV-induced fluorescence of the sample with a known standard sample.

On a denaturing agarose gel, high-quality RNA should show two main bands, including 18S and 28S ribosomal RNA with a smear from 0.5 to 12kb.

2.5 Enzymatic DNA modifications

2.5.1 Plasmid DNA digestion

For DNA digestion, every type of restriction enzyme was accompanied with the appropriate digestion buffer created by the same company. In general, for an analytical digestion (only for visually control) 100-500 ng of DNA was digested in a final volume of 50 μ l containing 5 units of the restriction enzyme of interest and 2-3 hours incubation was performed at the optimal temperature indicated by the manufacturer. Instead for a preparative digestion (the digested DNA will be used for the following steps) 3-5 μ g of DNA was used in the same mentioned condition, in a final reaction volume of 100 μ l.

2.5.2 Large fragment of E. Coli polymerase I (Klenow), T4 polynucleotide kinase (PNK) and Calf Intestinal Alkaline Phosphatase (CIP)

These enzymes were used to blunt-end ligation during construction of recombinant plasmids. The large fragment of DNA polymerase I (Klenow) is a proteolytic product of E. coli DNA polymerase I. This polymerizing enzyme has 3'→5' but not 5'→3' exonuclease activity; Therefore Klenow is a good candidate for digesting specific residues added by Taq DNA polymerase at the 3' terminal and generating compatible ends for blunt ligations.

To start, 2.5U of Klenow together with the appropriate buffer (at the final concentration 1X) and dNTPs 5mM, were added to 30 μ l of initial product. The mix was incubated 15 minutes at 25°C and then the reaction was blocked by EDTA with final concentration of 10mM. Finally, Klenow was inactivated through heating at 75°C for 15 minutes.

In the next step in order to phosphorylate the 5'-hydroxyl terminal of polynucleotides 10U of T4 polynucleotide kinase (PNK) together with ATP to a final concentration of 1mM and proper quantity of kinase buffer were added. The mix was incubated for 30 minutes at 37°C and then was stored at -20°C for further use.

Calf Intestinal Alkaline Phosphatase (CIP) dephosphorylates 5' and 3' ends of DNA and RNA phosphomonoesters in nonspecifically in order to prevent religation of linearized plasmid DNA. The enzyme acts on 5' protruding, 5' recessed and blunt ends.

For dephosphorylation, 1 μ g of vector (25 μ l) was mixed with 5X OPA (one for all) buffer and

4 µl of a dilution of the CIP 1:100 in final volume of 50 µl. After 60 minutes incubation at 37°C, CIP was deactivated by heating at 80°C for 15 minutes.

2.5.3 T4 DNA ligase

In ligation reaction T4 DNA ligase joins double stranded DNA fragments of compatible sticky or blunt ends. For ligation about 20 ng of digested vector were ligated with a 5-10 fold molar excess of digested insert in a total volume of 30 µl containing 1X ligase buffer and 1U of T4 DNA ligase. The reaction mix was incubated at room temperature for 4-12 hours.

2.6 Electrophoretic separation of nucleic acids

2.6.1 Agarose gel electrophoresis of DNA

DNA samples were size-fractionated by electrophoresis on the agarose gels ranging in concentration from 1% to 2% for large and small fragments respectively in TBE1X buffer. The samples prepared in DNA loading buffer 1X were loaded on the gels containing ethidium bromide (0.5 µg/ml) and these gels were electrophoresed at 80-100mA in 1X TBE running buffer. DNA was finally visualized by UV trans-illumination and the result recorded by digital photography. The DNA bands were sized according to 1 kb DNA ladder (Invitrogen).

2.6.2 Elution and purification of DNA fragments from the agarose gel

After agarose gel electrophoresis, under UV light, the interested DNA bands were cut and DNA extraction was performed using the EuroGold gel extraction kit (Euroclone) according to the manufacturer's instructions. The approximate levels of recovered DNA were calculated visually by UV fluorescence of intercalated ethidium bromide in an agarose gel electrophoresis.

2.7 DNA sequencing

For sequence analysis, 2 µg of each plasmid DNA preparation was sent to MacroGen Inc. Company. If needed, the own primers also were sent at concentration of 5 pmol/µl in deionized water, 2 µl per each run.

2.8 Human and mouse SORT1 Ex17b minigene constructs

pTB plasmid is the backbone of the cell transfected human and mouse Sort1 Ex17b minigene constructs. This plasmid is a modified version of the hybrid construct containing exons from the α -globin and fibronectin, under the control of the α -globin promoter.

The intronic region among the fibronectin exons contains a unique NdeI site where 99 bp of Sort1 Ex17b and 150 bp of its flanking intronic regions were inserted.

Following cell minigene transfection, the splicing pattern of Ex17b was analyzed by RT-PCR amplification using two forward and reverse primers located on pTB vector, including Alfa2-3 and Bra2 respectively (Table 2.1). By these two primers two 246-bp and 345-bp bands were detectable on agarose gel referring to Ex17b exclusion and inclusion respectively.

Table 2.1. Oligonucleotides used for Ex17b amplification from Sort1 minigenes

Name	Sequence from 5' to 3'
Alfa2-3	caacttcaactcctaagccactgc
Bra2	gtcaccaggaagttggttaaata

2.9 human-mouse SORT1 Ex17b hybrid minigene constructs

2.9.1 Human exon mutations

Human to mouse ('h to m': 1 h to m, 2 h to m, 3 h to m, 4 h to m) substitutions in the human Ex17b minigene were provided from the previous study (Prudencio et al. 2012). They made all the hybrid constructs by PCR-directed mutagenesis with specific primers.

2.9.2 Human upstream intron modification

Taking advantage of a unique NdeI site in pTB vector and also NdeI site included designed primers for amplification of upstream intron of EX17b; human-mouse hybrids were subcloned into the pTB backbone in order to generate three hybrid minigene constructs, including H1, H2, and H3.

Using proper primers designed for three distinct parts of mouse upstream intron (Table 2.2) together with human Sort1 Ex17b minigene as a template, three human-mouse Sort1 hybrid

Materials and methods

constructs were amplified by PCR.

After purification from the gel, PCR products and pTB vector were digested by NdeI in order to produce sticky ends for ligation.

Table 2.2. Oligonucleotides used to construct human-mouse Sort1 minigenes

Name	Sequence from 5' to 3'
HF 99	aacatatgagggactgacaaggggtgggattaag
pTB 1950 As	ccgaagaggcttaagttgccac
Part1 S	aattaacatatgacactgacaggggagggaccctgaaggcaatgaggggaagagt
Part2 S	tgtgtgggtgtgtgtattggtaaggggagggcagttcccccttctctgcacccctgccttgactgctg
Part2 As	cagcagtcagggcaggggtgcagagaagggggaactgcctcccccttaccaatacacacaccacaca
Part3 S	tggtggctgtcctcagagtgtttgtgtgtgtatgattgcaca
Part3 As	tgtgcaatcacacacacaaacactctgaggacagccacca

2.10 Cell culture

2.10.1 Cell lines used for the experiments

Different human and mouse cell lines with neuronal or non-neuronal origins were used in this project.

- Hep-3B, human hepatocellular carcinoma-derived cell line
- SH-SY-5Y, human neuroblastome cell line
- SK-N-BE, human neuroblastome cell line
- N-muli, mouse liver derived cell line
- NSC-34, mouse motor neuron-like hybrid cell line
- Neuro-2a, neuroblastome cell line

2.10.2 Maintenance and analysis of cells in culture

All different cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM from Gibco) supplemented with Glutamax I, sodium pyruvate, pyridoxine and 4.5 g/l glucose, together with 10% fetal bovine serum (FBS) (Euroclone) and antibiotic/antimycotic (Sigma) to prevent mycoplasma, bacterial and fungal contamination according to the manufacturer's instructions.

Cells were maintained in an incubator at 37°C and 5.5 % CO₂ in humid atmosphere. Cell cultures were tested for mycoplasma at regular intervals. All the cell lines used were adherent and were kept in 100 mm Petri dishes.

In order to seed the cells, dishes containing a confluent monolayer of cells were washed twice with PBS solution, then treated with 1-2 ml of PBS/EDTA/trypsin solution (PBS containing 0.04% w/v EDTA and 0.1% w/v trypsin) at 37°C for about 30 seconds until the cells were dislodged.

After trypsin inactivation with adding DMEM (in a volume corresponding to 3 times of trypsin volume used), cells were collected by centrifugation for 5 minutes at 1000 rpm at room temperature. Next cells were resuspended in 3 ml of DMEM and 0.5 ml of resuspended cells was seeded in a new dish in total 10 ml volume of medium. Before final incubation at 37°C, a gentle mixture was done.

In order to have stocks from each cell line, cells were resuspended in FBS supplemented with 10 % dimethyl sulfoxide (DMSO), slowly cooled overnight at -20°C, and then for short time kept at -80°C and for long-term storage in liquid nitrogen.

2.11 Transient transfection of the cell lines

2.11.1 In Hep-3B, SH-SY5Y, SK-N-BE, and N-muli cells

Apart from the mouse neuroblastoma cell lines (NSC-34 and Neuro-2a), Liposome-mediated transfections of 2.5×10^5 cells were performed using Effectene reagent. To begin 0.5 µg of minigene construct together with 4 µl of Enhancer (Qiagen) were added to 150 µl of EC buffer (Qiagen) for each transfection and after pipetting the mixture it was incubated at room temperature for 5 minutes to allow the condensation of the DNA.

Then 5 μ l of Effectene (Qiagen) were added to the mixture and another incubation of 10 minutes has been performed again. After adding 500 μ l of complete culture medium (Gibco) the mixture was added to the cells in 1.5 ml of the same medium and incubated at 37°C. 20 hours later the cells were harvested for further evaluation.

2.11.2 In NSC-34 and Neuro-2a cell lines

For mouse neuroblastom cell lines, including NSC-34 and Neuro-2a transfections were done using lipofection with Lipofectamine 2000 reagent (Invitrogen). For transfection DNA:Lipofectamine 2000 ratio (μ g: μ L) was 1:2 for both cell lines. Ratios were established empirically to optimize the efficiency of transfection before the main experiments.

In 6-well plates according to the specific growth rate for each cell, around 5×10^5 cells in 2 mL of DMEM were seeded one day before the transfection. When cells roughly reached to 90 % of confluency they were transfected.

For each well, the required amount of Lipofectamine 2000 was diluted in 250 μ L of Optimem medium (Gibco) and after 5 minutes incubation at RT it was added to the DNA previously mixed with 250 μ L of Optimem. The mix was incubated at room temperature for 20 minutes to allow DNA- liposomes complexes formation. Meanwhile cells were washed twice with PBS and 1 mL of Optimem was added to each well. After 20 minutes of incubation, 1 mL of DNA-liposome complexes was added to the relevant well. 20 hours later the cells were harvested for further evaluation.

2.11.3 Transient co-transfection of Neuro-2a cell lines

In co-transfection experiments, 4 μ g of minigene construct together with 4 μ g of the overexpression plasmids (SRp75/SRSF4, SC-35/SRSF2, Tra-2 β , and SRp40/SRSF5) were simultaneously transfected by Lipofectamine 2000 (Invitrogen) into Neuro-2a cells following the same protocol mentioned above. In this case the required amount of Lipofectamine 2000 was doubled.

2.12 RNA extraction

After washing the cells twice with PBS, cells were collected by Eurogold Trifast (Euroclone) in order to extract RNA. Next chloroform was added to each sample and after mixing well samples were incubated for 5 minutes at room temperature. Then samples were centrifuged for 20 minutes at 13000 rpm at +4°C.

The mix was separated into three phases upon centrifugation. The aqueous phase containing the RNA, the interphase the DNA and the organic phase the protein. The upper phase, containing RNA was gently isolated and transferred in a new tube and RNA was precipitated by adding an equal volume of isopropanol.

After 10 minutes incubation at +4°C, another 20 minutes of centrifugation at 13000 rpm at +4°C was done. The obtained RNA pellet was washed with 70% ethanol and then resuspended in ddH₂O.

Integrity of extracted RNA was verified on 1 % agarose gel and the quantity was measured by NanoDrop™ 1000 spectrophotometer as previously described.

2.13 mRNA analysis by RT-PCR

2.13.1 Reverse transcription (RT)

Complementary DNA (cDNA) was synthesized by reverse transcription of total RNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase 100U (Invitrogen), according to manufacturer's instructions.

1 µg of total extracted RNA from each sample was mixed with random primers (Pharmacia) in a 20 µl final volume for cDNA synthesis. The mix was incubated at 95°C for 5 minutes in order to RNA denaturation. Meanwhile a reaction mix containing 1X first strand buffer (Invitrogen), 10mM DTT, 5mM dNTPs each and M-MLV 100U (Invitrogen) was prepared. The mix was added to each cooled denatured RNA and incubated at 37°C for 1 hour. For each RT-PCR analysis 3 µl of cDNA was used in a total volum of 50 µl.

2.13.2 RT-PCR amplification

For reverse transcription-polymerase chain reaction (RT-PCR) briefly, 47 μ l of a reaction mix consist of 30 picomoles of each primer, 1X of Taq buffer (New England Biolabs), dNTPs mix 5mM each, and Taq DNA polymerase 2.5U (New England Biolabs) was added to 3 μ l of cDNA of each sample.

PCR amplification was carried out for 35 cycles (94°C 2 minutes, 94°C 30 seconds, 56°C 1 minute, 72°C 45 seconds, 72°C 10 minutes).

2.14 Endogenous mouse Sort1

In order to evaluate endogenous Ex17b inclusion, mouse SORT1 in Neuro-2a cell line was amplified using two sense and antisense oligonucleotide primers located on Sort1 exons 17 and 19 respectively (Table 2.3).

Table 2.3. Oligonucleotides for endogenous Sort1 amplification

Name	Sequence from 5' to 3'
Mouse Sort1 Ex17 S	caaatgccaaagtgaggatgaa
Mouse Sort1 Ex 19 As	ttgaatccaaagcctctacgcc

2.15 Densitometric analysis of RT-PCR bands

The scanned RT-PCR bands were quantified using ImageJ software to compare the density of Ex17b included or excluded bands from each experimental sample on an agarose gel. For final statistical comparisons, the average percentage of Ex17b inclusion was calculated for each experimental sample.

2.16 Small interfering RNA (siRNA) transfections

2.16.1 The siRNA sense sequences used for gene knockdown

In Table 2.4 the sense sequences of oligonucleotides used for gene silencing (siRNA) are presented.

Table 2.4. Oligonucleotides used for different gene knockdown

Name	Sequence from 5' to 3'
Human/Mouse Luciferase (control)	uaaggcuaugaagagauac (Sigma)
Human TDP-43	gcaaagccaagaugagccu (Sigma)
Mouse TDP-43	cgaugaacccauugaaaaua (Sigma)
Mouse hnRNP-A1	cgaugaacccauugaaaaua (Sigma)
Mouse hnRNP-A2	ggaacaguuccguaagcuc (Sigma)
Mouse PTB-P1	aacuuccaucauuccagagaa (Sigma)
Mouse PTB-N1	gagaggauccugacgaacua (Sigma)
Mouse hnRNP-L	uauggcuuggaucaaucua (Sigma)
Mouse Tra-2 α	ggaucuucgugaaguauuu (Sigma)
Mouse Tra-2 β	ggaggauacagaucacguu (Sigma)
Mouse Dazap-1	gagacucugcgcagcuacu (Darmacon)
Mouse hnRNP-H	ggaaauagcugaaaagcu (Darmacon)
Mouse hnRNP-F	Sequence not provided (Ambion)

2.16.2 In Hep-3B and N-muli cell lines

In hepatoma cell lines, including Hep-3b and N-muli siRNA transfections were performed by Oligofectamine Reagent (Invitrogen) according to the manufactures instructions.

Briefly in 6 well plates (35 mm), 40-50% confluent plates were prepared. In one tube 2 μ l of 40 μ M siRNA was added to 178 μ l of Opti-MEM medium and in another tube, 2 μ l Oligofectamine was added to 18 μ l of Opti-MEM medium.

Materials and methods

The mixes were incubated for 5 minutes at room temperature. Then the Oligofectamine mix was added to pre-complexed siRNA mix and incubated for more 20 minutes at room temperature. Finally, 200µl mix was dropped on the cells maintained in 800µl of Opti-MEM without antibiotics and FBS.

After 24h, a second siRNA transfection was performed with the same protocol described above and if needed was followed by the transfection with the different minigene constructs.

20 hours after the transfection, cells were harvested for RNA extractions, RT-PCR analyses and Western blots. Western blot was performed in order to check the efficiency of protein silencing.

2.16.3 In NSC-34 cells

For NSC-34 cell line siRNA transfection was done by Lipofectamine 2000 reagent (Invitrogen). For transfection siRNA:Lipofectamine 2000 ratio (µg:µL) was 1:2. Ratios were established empirically to optimize the efficiency of transfection before the main experiments.

In 6-well plates around 5×10^5 cells in 2 mL of DMEM were seeded one day before the transfection. For each well, the required amount of Lipofectamine 2000 was diluted in 250 µL of Optimem medium (Gibco) and after 5 min incubation at RT it was added to the siRNA previously mixed with 250 µL of Optimem. The mix was incubated at room temperature for 20 minutes to allow siRNA- liposomes complexes formation. Meanwhile cells were washed twice with PBS and 1 mL of Optimem was added to each well. After 20 minutes of incubation, 1 mL of siRNA-liposome complexes was added to the relevant well.

After 24 hours, a second siRNA transfection was performed with the same protocol described above and if needed was followed by the transfection with the different minigene constructs.

20 hours after the transfection, cells were harvested for RNA extractions, RT-PCR analyses and Western blots. Western blot was performed in order to check the efficiency of protein silencing.

2.16.4 In SH-SY5Y, SK-N-BE, and Neuro-2a cell lines

In SH-SY5Y, SK-N-BE, and Neuro-2a cell lines, siRNA transfections were performed by HiPerFect Reagent (Qiagen) according to the manufactures instructions.

Materials and methods

In the first day of silencing, 6 μ l of 40 μ M siRNA was added to 200 μ l of Opti-MEM medium. After 5 min incubation at room temperature, 12 μ l of HiPerFect were added directly to the mix and incubated for 10 minutes more at room temperature. Meanwhile about 5×10^5 cells were seeded in 60mm plates with complete DMEM. Then while the cells were in resuspension form in the plate, the prepared mix was gently dropped on them.

After the second day of rest, on the third day the cells were washed twice with PBS solution and then they were incubated with 500 μ l of PBS/EDTA/trypsin solution (PBS containing 0.04% w/v EDTA and 0.1% w/v trypsin) at 37°C until they dislodged. Meanwhile 6 μ l of 40 μ M siRNA was added to 200 μ l of Opti-MEM medium. After 5 min incubation at room temperature, 12 μ l of HiPerFect was added directly to the mix and incubated for 10 minutes more at room temperature.

Dislodged cells were collected and trypsin was inactivated by adding DMEM (with a volume corresponding to 3 times of trypsin volume used). After centrifugation for 5 minutes at 1000 rpm at room temperature cells were collected and then resuspended in 2 ml of DMEM and seeded. Then while the cells were in resuspension form in the plate, the prepared mix was gently dropped on them.

Next, if needed cells were transfected with different minigene constructs. 20 hours after the transfection, cells were harvested for RNA extractions, RT-PCR analyses and Western blots. Western blot was performed in order to check the efficiency of protein silencing.

2.16.5 siRNA co-transfection of Neuro-2a cell lines

Like single transfection, co-transfection of siRNAs against different hnRNPs and TDP-43 was performed by HiPerFect Reagent (Qiagen) according to the manufactures instructions explained before.

To keep the silencing efficiency of siRNAs in simultaneous silencing, the volume of HiPerFect Reagent (Qiagen) was increased to 18 μ l.

2.17 Protein extraction

After resuspension in PBS and 1X protease inhibitor (Roche cocktail), cells were sonicated with Bioruptor® Plus sonication device with high power mode for 10 cycles (sonication cycle: 30 seconds ON, 30 seconds OFF) according to manufacturer's instructions. The obtained total cell proteins were quantified by Bradford test using Biorad reagent (Bio-Rad Laboratories GmbH). Finally for each sample, 20 µg of protein was loaded on an acrylamide gel.

2.18 SDS-page gel electrophoresis

SDS-PAGE was used for protein separation of each sample. Before loading, protein samples were mixed with protein sample buffer 5X at a final concentration of 1X and denatured by heating at 95°C for 10 minutes.

Polyacrylamide gels were made using 40 % (w/v) acrylamide/methylene bis-acrylamide solution (29:1 ratio, GERBU). SDS-PAGE was performed in the vertical gels with the appropriate percentage of polyacrylamide depending on the case.

For 20 mL of 10 % running gel, 5 mL of 40 % acrylamide (GERBU), 5 mL of 1.5 M Tris-HCl pH 8.8, 10 mL of dH₂O, 200 µL of 10 % SDS, 200 µL of 10 % ammonium persulfate (APS) were mixed together and 20 µL of tetramethylethylenediamine (TEMED) was added to initiate the polymerization. Gels were cast in vertical electrophoresis chamber and covered by isopropanol to provide a sharp interface.

After around 10 minutes at room temperature, when the running gel was polymerized isopropanol was flushed with water and 4 % stacking gel was cast. For 5 mL of 4 % stacking gel 0.5 mL of 40 % acrylamide (GERBU), 2.5 mL of 0.5 M Tris-HCl pH 6.8, 2 mL of dH₂O, 50 µL of 10 % SDS, and 50 µL of 10 % APS were mixed and 10 µL of TEMED were added to initiate polymerization.

The cooled denatured proteins were loaded on the gels and run at 25-30 mA in 1X SDS running buffer.

2.19 Western blot and antibodies

After running, SDS-PAGE gels were blotted on standard nitrocellulose membrane (Whatman PROTRAN) in 1X blotting buffer (blotting buffer 10X: 30 g Tris base, 147 g Glycine, water to 1 L) containing 10 % methanol. After blotting, transfer was verified by Ponceau S staining (0.1 g Ponceau S, 5 mL of acetic acid, water to 100 mL, the dye is reused). Then, Ponceau S was washed with water from the membranes and incubated in blocking solution (PBS, 0.1% Tween, 5% milk) for half an hour at room temperature. In case of SR proteins blocking solution was containing 0.5X western blocking reagent (Roche) and TBS.

Then the membranes were incubated overnight at +4°C with different primary antibodies diluted in 2% milk PBS plus 0.1% Tween. In case of SR protein primary antibodies were diluted in 1X western blocking reagent (Roche) and TBS. Tubulin was used as total protein loading control.

The following day, membranes were washed three times in washing solution (PBS and 0.1% Tween) or in case of SR proteins with TBS and 0.1% Tween each time for 10 minutes. Then the membranes were incubated with the appropriate HRP-secondary antibodies (anti-mouse or anti-rabbit, Dako) in 2% milk PBS plus 0.1% Tween, or in case of SR proteins in 1X western blocking reagent (Roche) and TBS for 1 hour at room temperature.

After incubation with secondary antibodies, the membranes were washed three times with PBS and 0.1% Tween or in case of SR proteins with TBS and 0.1% Tween each time for 10 minutes.

For protein detection, the membranes were stained with a chemiluminescence kit (ECL; Pierce Biotechnology). Finally, protein bands were exposed in a darkroom by an autoradiography taken on Kodak Biomax XAR films.

The primary antibodies used in this study are:

- α -TDP-43: available in-house
- α -hnRNP A1: available in-house
- α -hnRNP A2: available in-house
- α -hnRNP H: available in-house
- α -hnRNP F: available in-house

- α - hnRNP C1/C2: available in-house
- α -Dazap1: available in-house
- α -PTB: available in-house
- α - tubulin: available in-house
- α - hnRNP L: Abcam
- α -SRp40/SRSF5: Invitrogen
- α -SRSF2: Invitrogen
- α - Tra2 β : Generous gift from G. Dreyfuss and I.C Eperon

2.20 pull down analysis

2.20.1 DNA templates

Since in vitro RNA transcription needed for pull down assay was performed by T7 RNA Polymerase, DNA templates containing T7 promoters were obtained by two different ways:

1. Human and mouse Sort1 Ex17b and their flanking intronic regions were separately subcloned in a pBluescript II KS+ plasmid (Stratagene) containing a T7 promoter. The oligonucleotides used for these subclonings are present in Table 2.5.

The cloning site was between two SacI and XbaI restriction sites to ensure that the 5' end of the sequence to be transcribed is placed as close as possible to the end of the T7 promoter while the 3' end of the same interested sequence is close to a suitable restriction enzyme (Bam HI) to linearize the plasmid and obtain the minimum possible length of plasmid-related RNA that was eventually transcribed from the sequence of interest.

2. Different human to mouse ('h to m': 1 h to m, 2 h to m, 3 h to m, 4 h to m) hybrid Ex17b sequences were amplified using a forward primer carrying a T7 polymerase target sequence (5'- taatacgaactcactatagg -3') at the 5' followed by 10 complementary nucleotides and a reverse primer carrying 18 nucleotides of the target sequence (Table 2.6).

Materials and methods

Table 2.5. Oligonucleotides used for subcloning of separated Sort1 sequences in pBluescript II KS+

Name	Sequence from 5' to 3'
HF 99 bp	aacatatgagggactgacaaggggtgggattaag
H17 upint As	ctgtgcaatcacacacacga
H17b S	gactcccacccacagggaca
H17b As	ctgcttctgggtgggaaata
H17 doint S	gtaggtcacatgcaaacag
HR 99 bp	aacatatgtgactctagaacagggccattatct
MF 99 bp	aacatatgacactgacaggggagggaccctgaagg
M17 upint As	ctgtgcaatcatacacacacaa
M17b S	gactcccgccac
M17b As	ctgcttctgggtaggagata
M17 doint S	gtaggttacacgaaaacaa
MR 99 bp	aacatatgtgggtctattaactagactaccctct

Table 2.6. T7 polymerase sequence included oligonucleotides used to make DNA templates for pull down assay.

Name	Sequence from 5' to 3'
h-Ex17b-T7 S	taatacgactcactataggcacaggactc
h-Ex17b-T7 As	acctacctgcttctgggt

2.20.2 In vitro RNA transcription

5 µg of DNA template was transcribed using T7 RNA Polymerase (Stratagene). This amount of DNA was added to a mix containing 12 µl of transcription buffer 5X (Stratagene), 6 µl of NTPs 15mM, 6 µl of DTT 100mM, 1.5 µl of T7 Polymerase (Stratagene), and H₂O to reach the final volume of 60 µl.

Materials and methods

After 2 hours incubation at 37 °C, for DNA degradation, samples were treated with 6 µl DNase I (Roche Diagnostics) and incubated for 30 minutes more incubation at 37°C.

The next step was RNA purification. 140 µl of water was added to reach the final volume of the mix to 200 µl. Then 200µl of acid phenol plus chloroform (1:1) were added and the samples were mixed strongly using vortex for 10 sec, followed by a centrifugation at 13000 rpm for 15 minutes at +4°C.

Again 200 µl of chloroform was added to the collected upper phase of the last step and the tubes were centrifuged at 13000 rpm for 5 minuets at +4°C.

The upper phase containing RNA was mixed with 600 µl of Ethanol 100% and 20 µl of NaOAc 3M pH 5.2 for RNA precipitation. The samples were incubated in dry ice for 20 minutes.

After a centrifugation at 13000 rpm for 20 min at +4°C the supernatant was removed and the RNA pellet was washed with Ethanol 70%. The RNA was finally resuspended in 41 µl of sterile water. 1 µl of obtained RNA was loaded on 1 % agarose gel for visualization.

2.20.3 Pull down analysis

A total 15 mg of transcribed RNA was oxidized with sodium m-periodate in a 400 ml reaction mixture (100mM Sodium acetate pH 5.2 and 5mM Sodium m-periodate). This reaction was done in the dark (each test tube wrapped in aluminium foil) for an hour at room temperature in a rotator wheel.

RNA was then ethanol precipitated according to standard protocols, washed once with Ethanol 70% and resuspended in 200 µl of 100mM sodium acetate pH 5.2.

Meanwhile, 100 µl of adipic acid dehydrazide-agarose beads 50% slurry (Sigma) for each RNA sample to be conjugated were washed twice with 10 ml of 0.1 M sodium acetate pH 5.2. After the final wash, the beads pellet was resuspended in 350 µl of 0.1 M sodium acetate pH 5.2 for each RNA sample. After mixing well, the prewashed beads were added to each oxidized RNA volume and incubated overnight at +4°C on a rotator (each test tube wrapped in aluminium foil). This RNA-Bead covalent link formation was also performed in the dark.

The following day, the beads with the bound RNA were pelleted by 5 minutes centrifufation at 4000 rpm, washed twice with 1 ml of 2 M NaCl, and spun down at 4000 rpm for 5 minutes.

Materials and methods

Then, the beads were washed three times with 1.0 ml of SolD 1X buffer (20 mM HEPES pH 7.5, 100 mM KCl, 0.2 mM EDTA pH 8, 0.5 mM DTT, 6% Glycerol), spun down at 4000 rpm for 5 minutes and supernatant was discarded each time.

They were then incubated on a rotator with HeLa cell nuclear extract (Cilbiotech) for 30 minutes at room temperature, in 400µl final volume in a 1x binding buffer containing 40 µl SolD 10X (200 mM HEPES pH 7.5, 2mM EDTA, 5mM DTT, 60% Glycerol) plus 40 µl KCl 1M, 100 µl HeLa nuclear extract (approx. 10-15 µg/µl), 218.5 µl H₂O, and 1.5 µl Heparin 200 µg/µl (Heparin final concentration: 0.75 µg/µl).

The beads were then pelleted by centrifugation at 4000 rpm for 5 minutes at room temperature and washed 4 times with 1 ml of SolD 1X washing buffer. After adding washing buffer, they were incubated each time for 10 minutes on a rotating wheel at room temperature then spun down with a centrifugation at 4000 rpm to remove the supernatant.

Finally 50 µl of 5X SDS protein sample buffer was added and after denaturation at 95°C for 10 minutes the samples were loaded on a SDS-PAGE gel.

2.21 qRT-PCR analysis on human brain samples

This part of the project was done by collaboration with Department of Neuroscience, Mayo Clinic Jacksonville in USA. Available RNA collected from temporal cortex of FTLD-TDP patients from the previous study (Prudencio et al. 2012) was used to determine the mRNA levels of hnRNPs by qRT-PCR.

Briefly, cDNA (diluted 1:40) from temporal cortex of 20 FTLD-TDP and 19 age-matched controls (Prudencio et al. 2012) was used for qRT-PCR in an HT7900 analyzer (Applied Biosystems). Each 5 µl reaction contained: 2 µl of cDNA diluted 1:40, 0.5 µl primer mix (1 µM for each primer) and 2.5 µl SYBR green (Invitrogen).

The qRT-PCR program was as follows: 50°C 2 minutes, 95°C 2 minutes, 40 cycles of 95°C 15 s and 60°C 1 minutes. mRNA values were normalized to either GAPDH or RPLP0 values, endogenous transcript controls, resulting in similar values; Therefore finally mRNA levels of the different transcripts were normalized to GAPDH to present on the graphs.

The sequences of the primer pairs used for qRT-PCR are present in Table 2.7.

Materials and methods

Table 2.7. Oligonucleotides used to amplify hnRNPs in patients brain by qRT-PCR

Name	Sequence from 5' to 3'
TARDBP S	atctggtgtatgttgcaactatcc
TARDBP As	gaacttctccaaaggtactaaaatactc
hnRNP A2B1 S	tttggggatggctataatgg
hnRNP A2B1 As	ccataaccggggctacct
hnRNP L S	agcggctcaagactgacaa
hnRNP L As	gtgcgggtcatcgtagttct
PTBP1 S	atgacaagagccgtgactacac
PTBP1 As	gagaggctgagattataccaggtg
GAPDH S	gttcgacagtcagccgcac
GAPDH As	ggaatttgccatgggtgga
RPLP0 S	tctacaaccctgaagtgcttgat
RPLP0 As	caatctgcagacagacactgg

2.22 Statistical analysis

The quantified percentage of Sort1 Ex17b inclusion was determined by densitometric analysis using ImageJ software (n=3). Statistical differences were calculated by one-way ANOVA test (post-test Tukey). $P < 0.05$ was considered significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Statistical differences in transcript levels between FTL-D-TDP cases (n=20) and controls (n=19) were calculated by Student's t-tests. $P < 0.05$ was considered significant (* $P < 0.05$, ** $P < 0.005$).

Chapter 3

3 Results

3.1 TDP-43 splicing regulation of Sort1 Ex17b in different mouse and human cell contexts

3.1.1 Human and mouse Sort1 Ex17b minigenes constructed in pTB vector backbone

To study the alternative splicing regulation of human and mouse Sort1 Ex17b, two minigenes constructed in pTB vector backbone were used. Human or mouse Sort1 Ex17b sequences (99 bp) together with 150 nucleotides of upstream and downstream flanking introns were inserted in NdeI restriction site of pTB vector (Fig. 3.1A).

Ex17b expression was under α -globin promoter control, moreover SV40 enhancer sequence was used to increase expression efficiency (Fig. 3.1A). By two forward (Alfa2-3) and reverse (Bra2) primers, designed on pTB backbone, two 246-bp and 345-bp PCR bands were detectable on the agarose gel referring to Ex17b exclusion and inclusion respectively (Fig. 3.1A and 3.2).

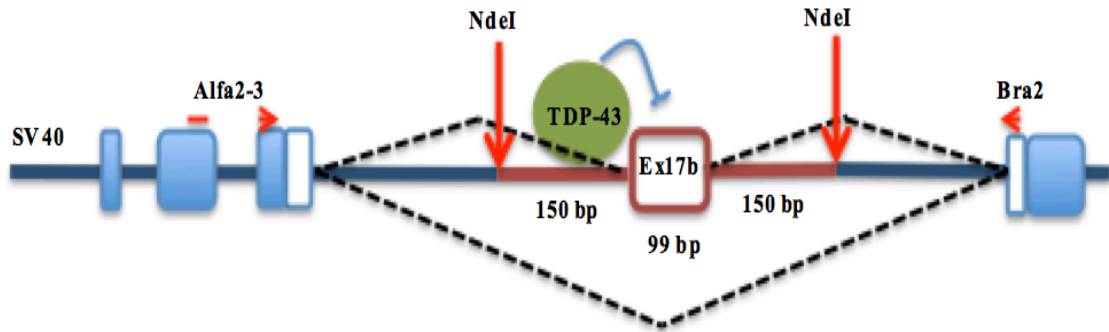
In Figure 3.1B, the sequences of human and mouse SORT1 Ex17b and their upstream and downstream flanking introns are shown. Considering the nucleotide differences between human and mouse Sort1 Ex17b regions that are highlighted in the figure, it is clear that there is a high homology between human and mouse Sort1 Ex17b sequences.

Results

There is a “tg” rich region in the upstream intron of both human and mouse Sort1 EX17b that act as TDP-43 binding site (Lukavsky et al. 2013). Through binding to tg-repeat elements located near the 3’ss of Ex17b, TDP-43 can play a negative splicing regulatory role for the exon (Fig. 3.1).

As shown in Figure 3.1B one of the nucleotide differences between human and mouse sequences is highly important because of producing a premature stop codon “TAG” in human Ex17b (specified in a red bracket) that is absent in mouse sequence. This premature stop codon in human Ex17b is potential to encode a truncated Sort1 receptor that can function as a decoy receptor for the normal Sort1 receptors and therefore induce toxicity for the cells (Prudencio et al. 2012).

A



B

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Human: agggactgacaaaggggggga ttaaggagatgatgggagaggagtgtgtgggtgtgtgtattggaagggg ggcagttcctcctctctgtgcacct
Mouse:  acactgac aggggagggacccctgaaggcaatga gggga agagtggtgtgggtgtgtgtattggaaggggaggcagttcccctctctgtgcacct

Human: tgcctgggctcctgggtgactctcctcagagtgttctgtgtgtg tgattgcacagGACTCCCACCCACAGGGACACAGCTTGTCCAGAAATCCAGCTCT
Mouse:  tgcctgggctcctgggtgactctcctcagagtgttctgtgtgtgtatgattgcacagGACTCCCACCCACAGGGACACAGCTTGTCCAGAAATCCAGCTCC

Human: GCCTCCTCTGGGATACATTGAAAACACACACTCCCTATTTCCCACCCAGAAGCAGgtaggtcacatggcaaacagtgatgataaggcttatgtataata
Mouse:  GCCTCCTCTGGGATACACTGAAAACACACACTTCCTATCTCCTACCCAGAAGCAGgtaggtcacagaaaacaatgatctgataaggcttatgtataata

Human: atataataaaaaattaagtaaaacaaataa atcttggtgtcatataatggcctcctgggtctctt gacacagcttcttccagataatggcct gtt
Mouse:  agatgccaaaaggta gtaa caactgagatcttctgtgtcatatagggcctcctgggaacagcatcggcaca cgtcctcaagagggtagtc tagta

Human: cttaggtca
Mouse:  atagaccga

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Results

Figure 3.1. Human and mouse Sort1 Ex17b minigene constructs.

A) Schematic presentation of the exon minigenes used in the transfection experiments. After SV40 transcription enhancer sequence, the α -globin and fibronectin EDB are shown as blue and white boxes, respectively. Sort1 Ex17b with 99 nucleotides and its flanking intronic regions with 150 nucleotides are indicated between NdeI restriction sites. The binding location of TDP-43 near the 3'ss of both human and mouse exons is also shown. Possible splicing events are indicated by up (exon inclusion) and down (exon skipping) dashed lines. Two forward (Alfa2-3) and reverse (Bra2) primers on pTB vector backbone, were used for PCR amplification.

B) The sequences of human (upper sequence) and mouse (lower sequence) SORT1 Ex17b (bold uppercase letters) and their flanking introns (lowercase letters) are shown. Nucleotide differences between human and mouse are highlighted. The red bracket indicates the premature stop codon (TAG) in the human Ex17b sequence.

3.1.2 Transfection of human and mouse Sort1 Ex17b minigene constructs in different human and mouse cell lines following TDP-43 depletion

Using Sort1 minigene system, it has been revealed that in HeLa cells TDP-43 depletion increases inclusion of Ex17b. However, the increase of the mouse Ex17b inclusion was dramatically significant compare to the human Ex17b inclusion under the same condition Fig. 1.10 (Prudencio et al. 2012).

These data explain that human Ex17b inclusion is tightly under the control compare to the mouse. The major difference between the human and mouse Ex17b sequences is that human carries a translational stop codon (Fig. 3.1B) and human Ex17b inclusion could result in formation of a truncated Sort1 receptor that is not properly functional as a PGRN receptor; Therefore it is predictable that human Ex17b inclusion should be more restricted.

However, data from qRT-PCR studies showed increase in endogenous Ex17b following TDP-43 knockdown in human M17 cell line (Prudencio et al. 2012).

In my thesis I used the previously explained human and mouse minigene constructs (Fig. 3.1), to analyze the effect of TDP-43 knockdown on Sort1 Ex17 splicing in a variety of human and mouse neuronal (SK-N-BE, SH-SY-5Y, NSC-34, and Neuro-2a) and non-neuronal (Hep-3B

Results

and N-muli) cell types. By this approach primarily I wanted to extend our knowledge about TDP-43 splicing regulatory role of human and mouse Ex17b to different cell context.

After TDP-43 knockdown, cells were transfected with human or mouse Sort1 Ex17b minigenes. Around 20 hours after minigene transfection, cells were divided in two parts and the first part was evaluated for protein detection by western blotting in order to prove that TDP-43 was efficiently knocked down in each sample. In Figure 3.2, western blot images are represented in the lower panels of each related RT-PCR image.

The second parts of the collected cells were used to evaluate the splicing pattern of human and mouse Ex17b minigene constructs by RT-PCR amplification. As described before, by specific primers (Alfa2-3) and (Bra2), two 246-bp and 345-bp bands for Ex17b exclusion and inclusion respectively were produced (Fig. 3.2).

As shown in Figure 3.2, under the normal conditions in the presence of TDP-43, low levels of Ex17b were detected in all cell lines tested, whether of human or mouse, neuroblastoma or non-neuroblastoma origin (Fig. 3.2). This finding refers to existence of some splicing regulatory elements for both species.

Interestingly in the normal conditions, minigene evaluations showed that the splicing pattern for mouse Ex17b was more variable than that of the human. For example in human neuronal cell lines (SK-N-BE, SH-SY-5Y), high levels of mouse Ex17b inclusion were seen while in non-neuronal Hep3B cell line very low levels of mouse Ex17b inclusion were detected. Moreover, having a look to any cell line separately, it was obvious that in the normal conditions and in the presence of TDP-43, human Ex17b was less included compare to mouse Ex17b. These results are in consistent with the previous indications where they showed that the inclusion of human Ex17b is more heavily repressed compare to its mouse homologue (Prudencio et al. 2012).

As mentioned before, to further define the regulatory role of TDP-43 in this critical splicing event, all the analyses were carried out also under TDP-43 knock down conditions through siRNA treatment.

Figure 3.2 shows that human Ex17b inclusion was only minimally affected by silencing of TDP-43 in most of the cell lines. However, there was an exception in case of mouse

Results

neuroblastoma Neuro-2a cells where a statistically significant increase in human Ex17b inclusion was observed after TDP-43 depletion (see Figure 3.9A for quantification of this event).

On the other hand, TDP-43 silencing had a very positive effect on the inclusion levels of mouse Ex17b with just one exception, in the human hepatocellular carcinoma-derived Hep-3B cell line (Fig. 3.2).

Taken together, these data demonstrate that inclusion of the human Ex17b is much more heavily repressed than mouse Ex17b, a conclusion that is in agreement with the previous findings (Prudencio et al. 2012). However, two facts that mouse Ex17b inclusion varied considerably depending on the cell line tested (e.g. compare Hep-3B and SH-SY-5Y cell lines, Fig. 3.2) and human Ex17b mostly remained without any changes following TDP-43 depletion, suggest that additional splicing regulatory factors can modulate this splicing event.

Results

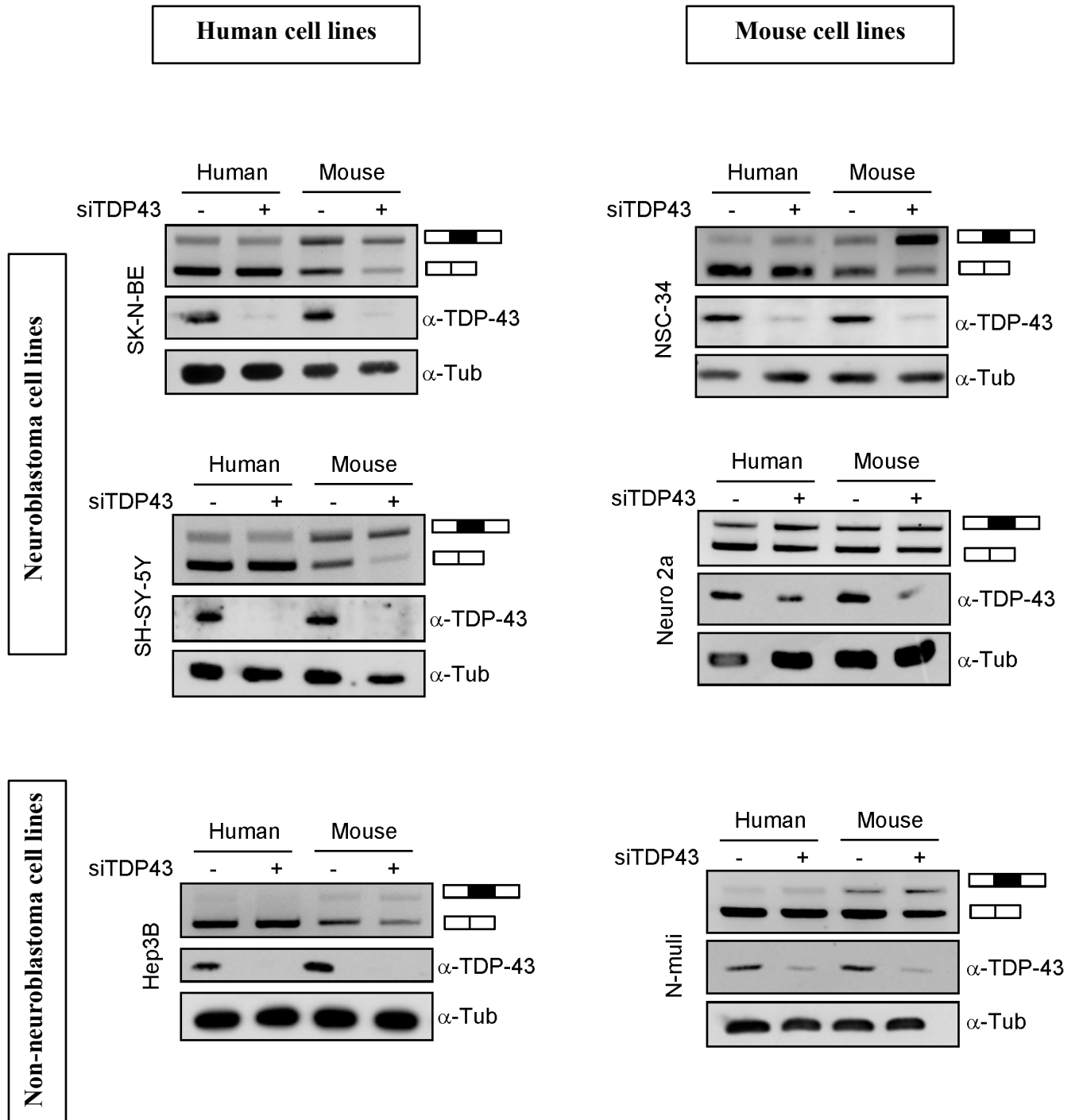


Figure 3.2. Sort1 Ex17b splicing regulatory role of TDP-43 in different cellular contexts.

Primarily, all the cells were transfected either by Luciferase siRNA (as control) or TDP-43 siRNA. Then they were transfected by human or mouse Sort1 Ex17b minigene constructs. 20 hours later, cells were collected and divided into two distinct parts for further protein and RNA evaluation.

Basic splicing pattern and eventual effects of TDP-43 depletion on human or mouse SORT1 Ex17b inclusion are shown in different human neuronal (SK-N-BE, SH-SY-5Y) and non-neuronal (Hep-3B) and also mouse neuronal (NSC-34, and Neuro-2a) and non-neuronal (N-muli) cell lines.

Upper panels show the final images for RT-PCR samples that were stained with ethidium bromide and run on a 1.5% agarose gel. A schematic diagram of the two minigene spliced transcripts, including Ex17b inclusion (containing a black box) versus Ex17b skipping is reported on the right of each agarose gel.

The lower panels are the western blot images that show the efficiency of TDP-43 knock down in each cell and tubulin was used as loading control.

3.2 Identification of hnRNPs that differentially bind to human and mouse Sort1 Ex17b

3.2.1 Subcloning of distinct exonic and intronic regions of human and mouse Sort1 Ex17b in pBluescript II KS+ plasmid

Previously through pull down analyses it was described that both human and mouse Sort1 Ex17b constructs (in a complete form including exons and intronic regions) could bind almost equally to the splicing repressors, including TDP-43, hnRNP A1, hnRNP A2, and hnRNP B1 (Prudencio et al. 2012).

To further map the precise binding site of hnRNPs, in the first steps of this thesis project the human and mouse Sort1 Ex17b regions using appropriate oligonucleotide primers were split into upstream intron, exon, and downstream intron and then they were subcloned in the pBluescript II KS+ plasmid.

As shown in Figure 3.3 each nucleotide sequence of Sort1 Ex17b construct was placed between SacI and XbaI restriction sites in pBluescript II KS+ plasmid backbone. In this place the 5' end of the sequence to be transcribed is placed near the end of the T7 promoter while its 3' end is close to a BamHI restriction enzyme. This was very important because later by BamHI enzyme, plasmid was linearized to be ready for in vitro transcription by T7

Results

polymerase that recognizes T7 promoter. By this I obtained the minimum possible length of plasmid-related RNA, needed to start pull down assessments.

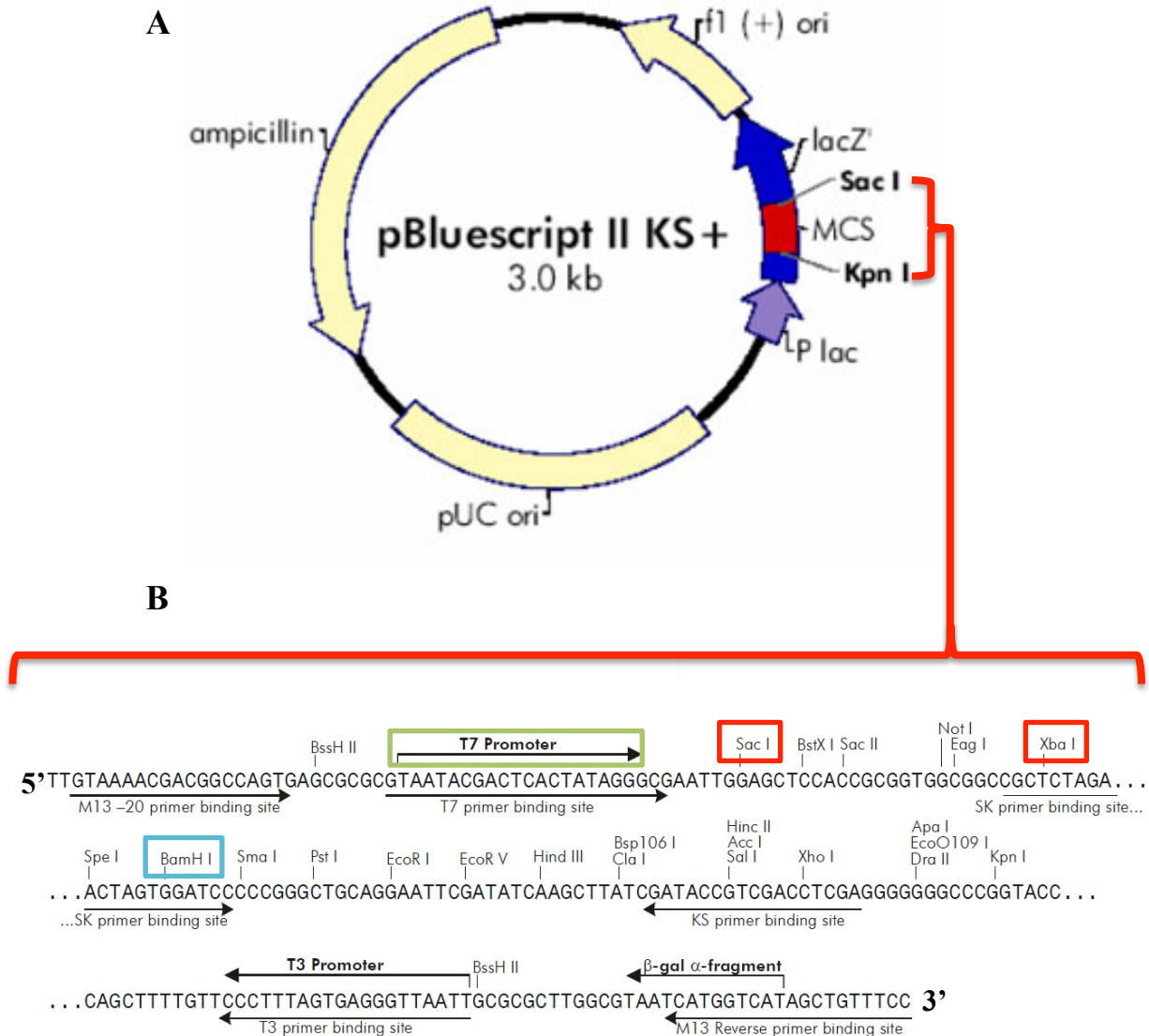


Figure 3.3. Subcloning of human and mouse Sort1 Ex17b in pBluescript II KS+ plasmid.

A) A scheme of the ampicillin resistance pBluescript II KS+ plasmid, a phagemid derived from pUC19. LacZ transcription proceed from kpn I to SacI. **B)** pBluescript II Ks+ multiple cloning site region (sequence shown 598-826). Distinct Sort1 Ex17b regions were cloned between SacI and XhoI restriction sites (red boxed), so that 5' end of the sequence to be transcribed is placed near the end of the T7 promoter (green box) and its 3' end is close to BamHI restriction site (blue box).

3.2.2 Pull down assay to map hnRNPs binding sites in human and mouse Sort1 Ex17b contexts

To identify the splicing factors that contribute to the regulatory action of TDP-43 on Ex17b splicing, I used a previously described affinity pull down system that allows the identification of the binding location of such factors in the Ex17b context.

Primarily, all 6 plasmid constructs containing distinct human and mouse Ex17b and flanking intronic regions were linearized by BamHI restriction enzyme. Then in vitro transcription was performed using T7 RNA Polymerase and the obtained RNA from each distinct intronic and exonic region was phenol and chloroform purified.

Affinity RNA-binding protein (RBP) purification was performed using RNA-coated adipic acid dehydrazide beads and for protein interactions RNA samples were incubated with HeLa cell nuclear extract.

After several washings, the ability of the following hnRNP proteins to bind to these regions was tested by Western blot: TDP-43, hnRNP H, hnRNP F, PTB (hnRNP I), hnRNP A1, hnRNP A2, DAZAP1, hnRNP L, and hnRNP C1/C2.

The specific hnRNPs selection was based on the identified hnRNPs (TDP-43, hnRNP A1, hnRNP A2) as binding factors into the Ex17b context as described previously (Prudencio et al. 2012). In addition, we also included the following hnRNPs because of some reasons:

1. hnRNP H is a group of hnRNPs containing hnRNP H and hnRNP F that are highly homolog in their sequences and mediate mRNA processing. Structurally they have three identical repeats of RNA binding motif known as quasi-RNA recognition motif (qRRM) and two glycine rich domains for protein interactions (Bent et al. 1995) (Matunis et al. 1994). These factors have been followed in my thesis due to the fact that the high number of G-repeats in the upstream introns of mouse and human Sort1 Ex17b represent well known binding sites for these factors (Caputi & Zahler 2001).
2. As described before, intron recognition by spliceosomes depends on some highly conserved sequences such as polypyrimidine tract (PPT) that is located between BPS and 3'ss. This sequence could be recognized by different splicing regulatory factors including Polypyrimidine tract-binding protein PTB (hnRNP I). Following PTB binding to the certain polypyrimidine tracts, adjacent exon will be skipped from the final alternative transcript. I

evaluated PTB binding sites in human and mouse Sort1 constructs because several known binding “cucc” sequences are present in introns and in the 17b exon (Singh et al. 1995).

3. Like other hnRNPs, hnRNP L structurally contains two RNA recognition motifs (RRMs) and glycine- proline rich domain for protein interaction. This RNA binding protein is potential to regulate Sort1 mRNA splicing through recognition of ca-repeats located in the 17b exonic region (Hui et al. 2003).(Hui et al. 2003)

4. Dazap1, There are no well-known binding sequences for Dazap1 but this protein was chosen because it preferentially binds downstream of 5’splice sites and has been described to act in concert with hnRNP A/Bs to regulate alternative splicing (Pastor & Pagani 2011).

5. hnRNP C was also tested because it is a very abundant hnRNP protein that binds u-rich sequences (Görlach et al. 1994).

3.2.3 Similar binding patterns of hnRNPs to both human and mouse Sort1 Ex17b contexts

As shown in Figure 3.4, affinity purification showed that TDP-43 bound to the upstream intronic region of both human and mouse exon 17b sequences. This result is congruent with the presence of a ug-rich region in the intron upstream of Ex17b, as TDP-43 presents high binding affinity to such regions (Lukavsky et al. 2013).

Additionally, other hnRNP proteins displayed similar binding patterns to both human and mouse Ex17b contexts (e.g. hnRNP H, hnRNP F, hnRNP A1, Dazap1, and hnRNP L) or no binding (hnRNP C1/C2).

In particular, TDP-43, hnRNP H, and hnRNP F mostly bound to the upstream intronic region of Ex17b while hnRNP A1 and Dazap1 bound to both the upstream and downstream introns and binding of hnRNP L was detectable in all the exon and intronic parts (Fig. 3.4).

Of interest these hnRNPs mostly bound to the intronic sequences both in mouse and human. However, hnRNP L in addition of binding to the upstream and downstream introns also bound to Ex17b in both human and mouse context. This could refer to the higher importance of hnRNP L in controlling the inclusion of Ex17b in the final transcript. Therefore this hnRNP was more evaluated in this project that will be explained later.

Results

The similar binding patterns between mouse and human Sort1 sequences were predictable based on the high sequence similarity between their regions, especially within the exon (see Fig. 3.1B).

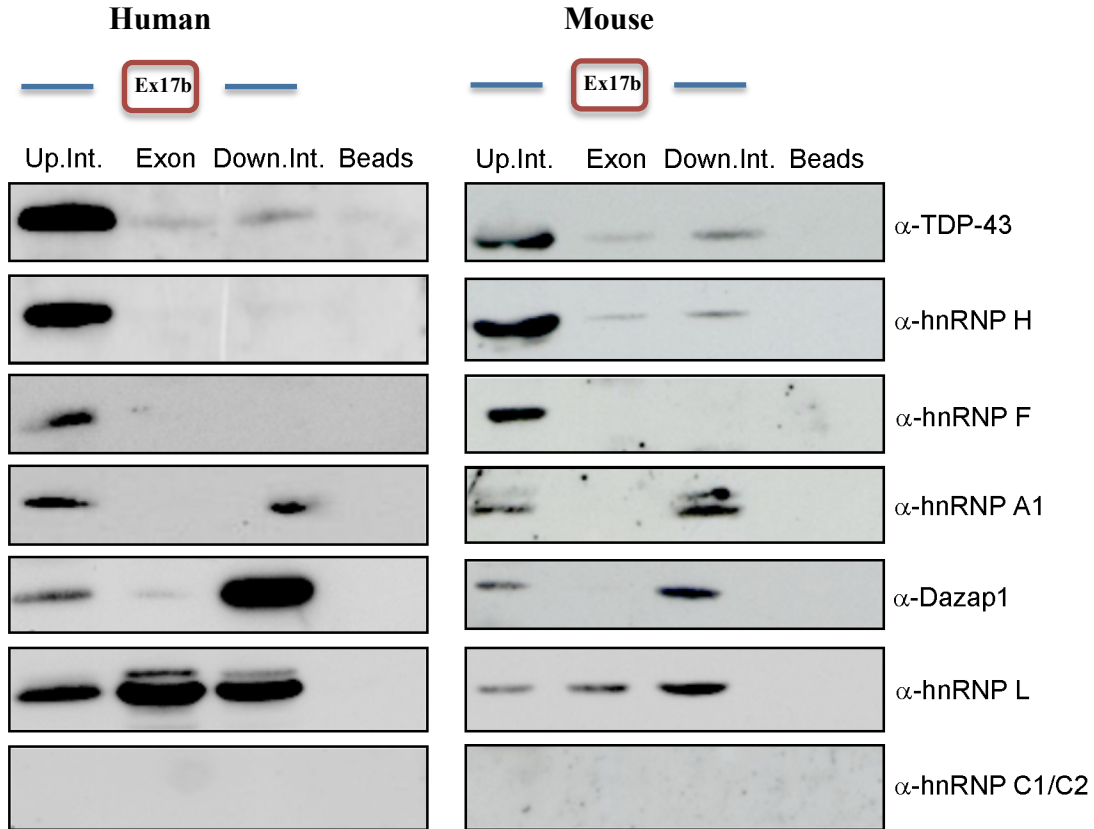


Figure 3.4. Similar binding patterns of hnRNPs to human and mouse Ex17b contexts.

The binding efficiency of several hnRNP proteins, including TDP-43, hnRNP H, hnRNP F, hnRNP A1, Dazap1, hnRNP L, and hnRNP C1/C2 was assessed by western blot after pull down of in vitro transcribed RNAs from human (on the left) and mouse (on the right) Sort1 Ex17b exons (red boxes) and their flanking introns (blue lines).

As expected, according to the high sequence homology between human and mouse Sort1 sequences, there was a similar hnRNPs binding pattern between their exon and intronic regions. Moreover, it is notable that TDP-43 bound to the upstream intronic regions of both human and mouse Ex17b sequences as predictable due to repeats in those regions.

3.2.4 Different binding patterns of hnRNPs to human and mouse Sort1 Ex17b contexts

As shown in Figure 3.5, two RNA binding factors including hnRNP A2 and PTB exhibited striking differences in the binding patterns between human and mouse Sort1 Ex17b contexts.

Although hnRNP A2 bound to both human and mouse upstream and downstream intronic regions, it bound within Ex17b in the human but not in the mouse context. PTB showed binding to human upstream and downstream introns to a greater extent than the corresponding mouse intronic regions (Fig. 3.5).

These differences are highly important because hnRNP A2 and PTB are known as splicing repressors (Clower et al. 2010) and interestingly here they bound strongly to human sequences compare to mouse. Therefore, it seems that in human Ex17b inclusion is more strictly under the control via these two splicing repressors.

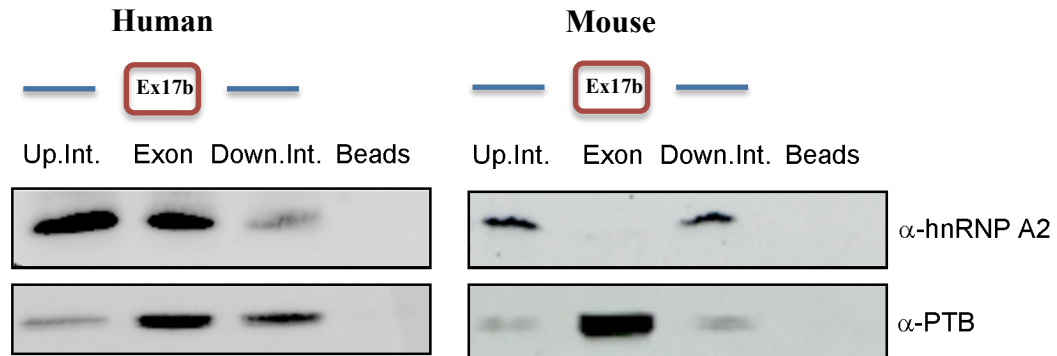


Figure 3.5. Different binding patterns of hnRNPs to human and mouse Sort1 Ex17b contexts.

The binding efficiency of hnRNP A2 and PTB was assessed by western blot after pull down of in vitro transcribed RNAs from human (on the left) and mouse (on the right) Sort1 Ex17b exons (red boxes) and their flanking introns (blue lines).

hnRNP A2 bound to Ex17b in the human but not in the mouse context. PTB bound to human upstream and downstream introns to a greater extent than the corresponding mouse intronic regions.

3.3 Binding of hnRNP A2 to human Ex17b correlates with splicing repression

3.3.1 Substitution of exclusive mouse exonic nucleotides in human, enhanced Ex17b inclusion in the human context

Despite of high homology between human and mouse Sort1 Ex17b sequences shown in Figure 3.1B, pull down analyses showed that two RNA binding proteins, including hnRNP A2 and PTB differentially bound to the human and mouse sequences.

To better understand the differential binding preference of these splicing repressors for the human versus mouse Sort1 Ex17b contexts, I took advantage of a series of human-mouse hybrid minigenes generated in the previous study (Prudencio et al. 2012).

Having a look to the human and mouse Ex17b sequences shown in Figure 3.6A, 8 nucleotide differences existing between these sequences are detectable. Trough substitution of mouse exclusive exonic nucleotides in human sequence, four human-mouse hybrids including 1 h-to-m, 2 h-to-m, 3 h-to-m, and 4 h-to-m were generated as shown in Figure 3.6A.

It was previously reported that following transfection of these mutated constructs in HeLa cells, two mutants including 1 h-to-m and 4 h-to-m include Ex17b more than the wild type human construct (Prudencio et al. 2012).

Therefore, first of all I transfected human and mouse wild types and human-mouse hybrid constructed minigenes in the neuronal Neuro-2a cells to see if the results previously observed in HeLa cells are replicable.

20 hours after transfection of wild type human and mouse Sort Ex17b plus all four human mutant constructs, cells were collected and through RT-PCR alternative spliced transcripts were evaluated on the agarose gel.

The obtained data from Neuro-2a cells showed that the splicing patterns of different mutants are following the patterns already seen in HeLa cells. This means that compare to the wild type human, inclusion of Ex17b was increased in mutants 1 h-to-m and 4 h-to-m (Fig. 3.6B).

Results

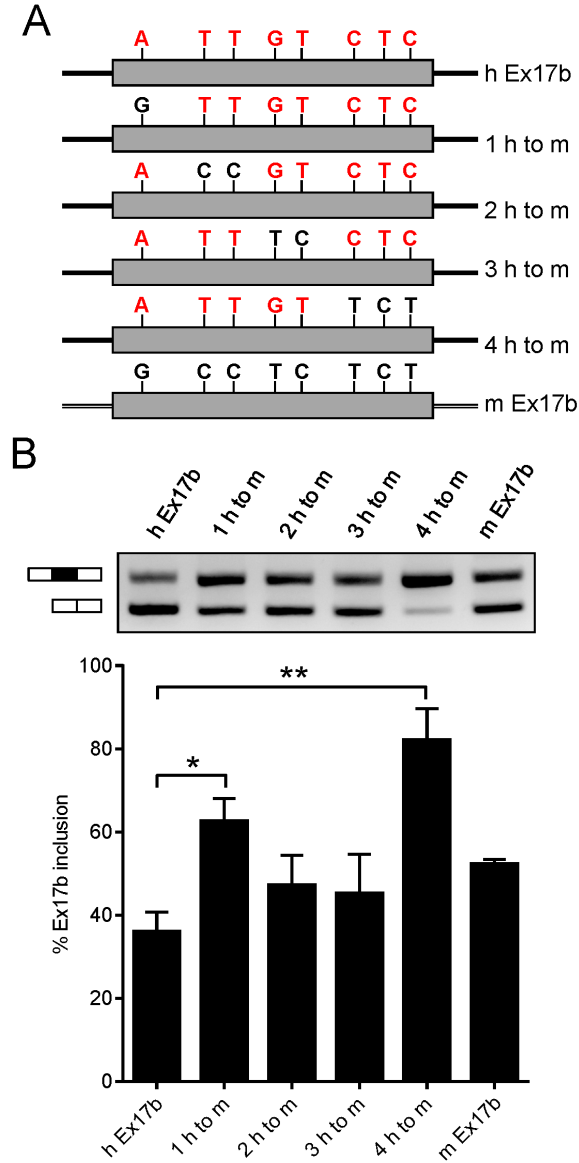


Figure 3.6. Mouse exclusive exonic nucleotides enhance Ex17b splicing in human context.

A) Schematic diagram of the wild type 17b exons and human to mouse (h to m) hybrid constructs based on the human Ex17b sequence. **B)** Transfection of the wild type and various h-to-m constructs in Neuro-2a cells. The inclusion of Ex17b was statistically increased in the case of 1 h-to-m and 4 h-to-m compare to the wild type human construct. The RT-PCR samples are stained with ethidium bromide and run on a 1.5% agarose gel. A schematic diagram of the two minigene spliced transcripts (17b inclusion containing a black box vs. 17b skipping) is reported on the left of the agarose gel. The quantified percentage of Sort1 Ex17b inclusion was determined by densitometric analysis using ImageJ software. Statistical differences were calculated by one-way ANOVA test (post test Tukey). *P < 0.05, **P < 0.01 (n = 3).

3.3.2 Substitution of exclusive mouse exonic nucleotides in human, abolished hnRNP A2 binding to human Ex17b

Regarding the data from figures 3.4 and 3.5 we showed that three hnRNPs including hnRNP A2, hnRNP L, and PTB, bind to human 17b exon sequence. To determine whether the nucleotide substitutions from the mouse exonic sequences to human would affect the occupancy of hnRNP factors, we performed pull down analyses for the wild type and human-mouse hybrid minigenes described in Figure 3.7A and looked for protein occupancy of the hnRNP factors hnRNP A2, PTB, and hnRNP L. DNA templates for pull down assessments were amplified through designing exonic primers containing T7 promoter sequence needed for in vitro transcription by T7 polymerase.

Interestingly, binding of hnRNP A2 was completely abolished in both the 1 h-to-m and 4 h-to-m mutants (Fig. 3.7B) and these two mutants according to the previous minigene transfection data in Figure 3.6B displayed the highest levels of exon inclusion. Conversely, hnRNP A2 was still able to bind the remaining 2 h-to-m and 3 h-to-m mutants (Fig. 3.7B), which again according to the minigene transfection data, showed similar levels of exon inclusion to the wild-type human sequence (Fig. 3.6B).

These findings indicate that binding of hnRNP A2 through a critical Adenin nucleotide and “ctc” sequence in human Sort1 17b exon sequence is very important to repress the inclusion of human Ex17b.

In addition, minor changes were observed in the case of hnRNP L and PTB binding to the mutant compare to the wild type sequences (Fig. 3.7B). This finding was not surprising since according to Figure 3.4A, both hnRNP L and PTB proteins displayed similar binding patterns to both the mouse and human 17b exons.

While changes in PTB-binding do not appear to correlate perfectly with the splicing patterns, it should be noted that in the 1 h-to-m and 4 h-to-m hybrid minigenes transfected into Neuro-2a cells, PTB binding will still occur in the downstream human intron (Fig. 3.5). This may eventually affect the splicing by multimeric assembly of PTB complexes, as previously described by Spellman & Smith (Spellman & Smith 2006), in ways that are not easy to predict.

Results

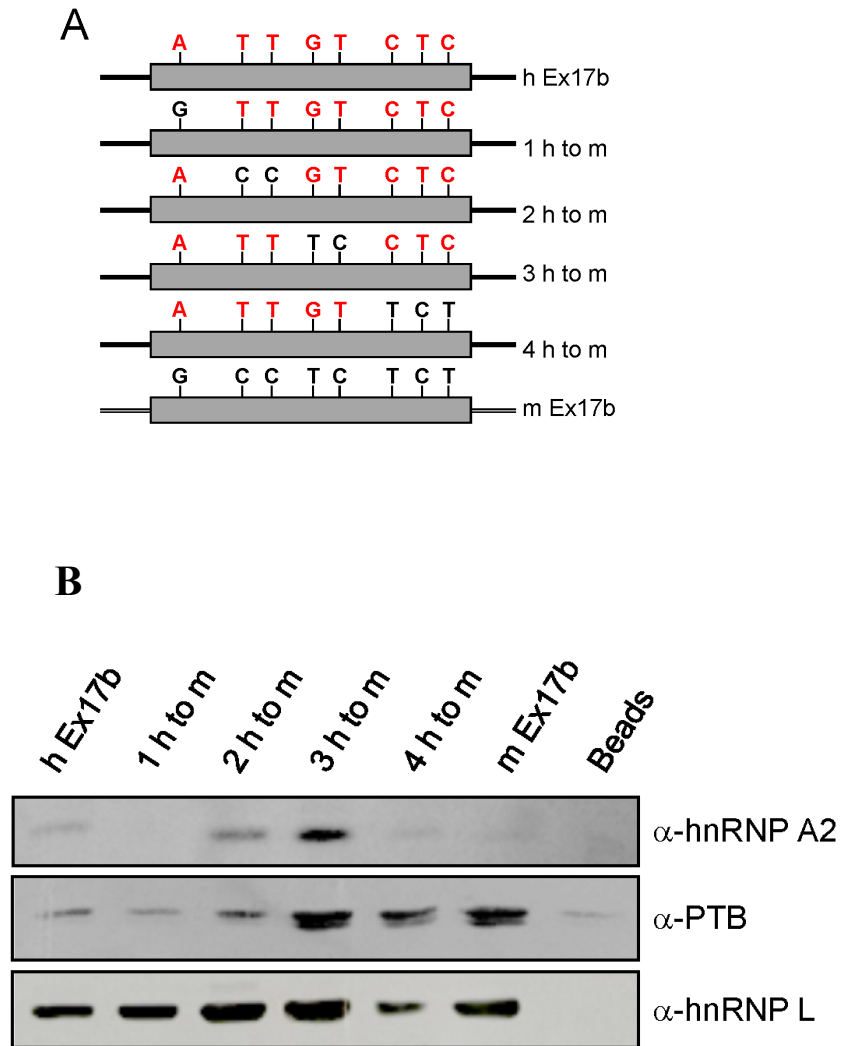


Figure 3.7. Mouse exclusive exonic nucleotides abolished binding of hnRNP A2 to human Ex17b sequence.

A) Schematic diagram of the wild type 17b exons and human to mouse (h to m) hybrid constructs based on the human Ex17b sequence. **B)** In vitro-transcribed RNA from wild type and mutant exonic sequences were incubated with human HeLa nuclear protein extracts to assess their ability to bind the following proteins: hnRNP A2, hnRNP L and PTB. As seen in the western blot images, while binding of hnRNP L and PTB to the hybrid constructs was almost unaffected, binding of hnRNP A2 was abolished in 1 h-to-m and 4 h-to-m mutants.

3.4 hnRNP proteins work co-operatively with TDP-43 to repress Ex17b inclusion

3.4.1 Single knock down of hnRNPs did not affect Sort1 Ex17b splicing

After determining several hnRNPs that are able to bind to human and mouse Sort1 17b exons or flanking intronic regions (see Figure 3.4 and 3.5), next step was to dissect the individual contributions of different hnRNP proteins in the exon 17b splicing event.

To achieve this aim, neuronal Neuro-2a cells were transfected with siRNA against described potential hnRNPs that can bind to Sort1 Ex17b sequences (Figure 3.4 and 3.5). After hnRNPs knockdown, cells were transfected with human or mouse Sort1 Ex17b minigene constructs.

20 hours after minigene transfection, cells were collected and divided in two parts and primarily efficient gene knock down was evaluated by western blot technic.

Once depletion of hnRNPs expression was approved by western blot analysis, alternative spliced Sort1 transcripts were detected on an agarose gel after cDNA amplification through RT-PCR.

Final evaluation of slicing patterns showed that Ex17b inclusion following single knock down of various hnRNP proteins was unaffected. In fact while in Neuro-2a cells TDP-43 knock down alone was capable of increasing Ex17b inclusion in both human and mouse minigenes (Fig. 3.2 and 3.9A), single knock down of other hnRNPs including hnRNP A1/A2, hnRNP L, PTB/nPTB, hnRNP H, hnRNP F, and DAZAP1 (Fig. 3.8) had very minimal effect on the basal levels of either human or mouse Ex17b inclusion.

All together these results confirmed that TDP-43 is the crucial splicing regulatory factor for Sort1 Ex17b and it acts as a splicing repressor for both human and mouse 17b exons.

Of note, from the technical point of view in order to better silence hnRNP A1 expression, I have co-transfected Neuro-2a cells with both siRNA against hnRNP A1 and hnRNP A2. The reason for this co-transfection was that the previous data showed upregulation of hnRNP A2 levels following hnRNP A1 knock down (Bonomi et al. 2013).

In fact hnRNP A1 can regulate the hnRNP A2 levels through binding to its 3' UTR region (an untranslated region located after the translation stop codon with a critical role in translation termination) to mediate degradation of hnRNP A2 pre-mRNA by a pathway known as

Results

nonsense-mediated mRNA decay (NMD) (Bonomi et al. 2013). So one of the consequences of hnRNP A1 knock down could be upregulation of hnRNP A2 levels.

In addition, because of a high homology between hnRNP A1 and A2 they can contribute in the same mRNA target splicing regulation and sometimes they show overlapped and redundant functions (Bonomi et al. 2013) (Biamonti et al. 1994).

Therefor to obtain a better cell context with the lowest possible expression of hnRNP A, I decided to co-knock down both hnRNP A1 and hnRNP A2 before human and mouse Sort1 minigenes transfection.

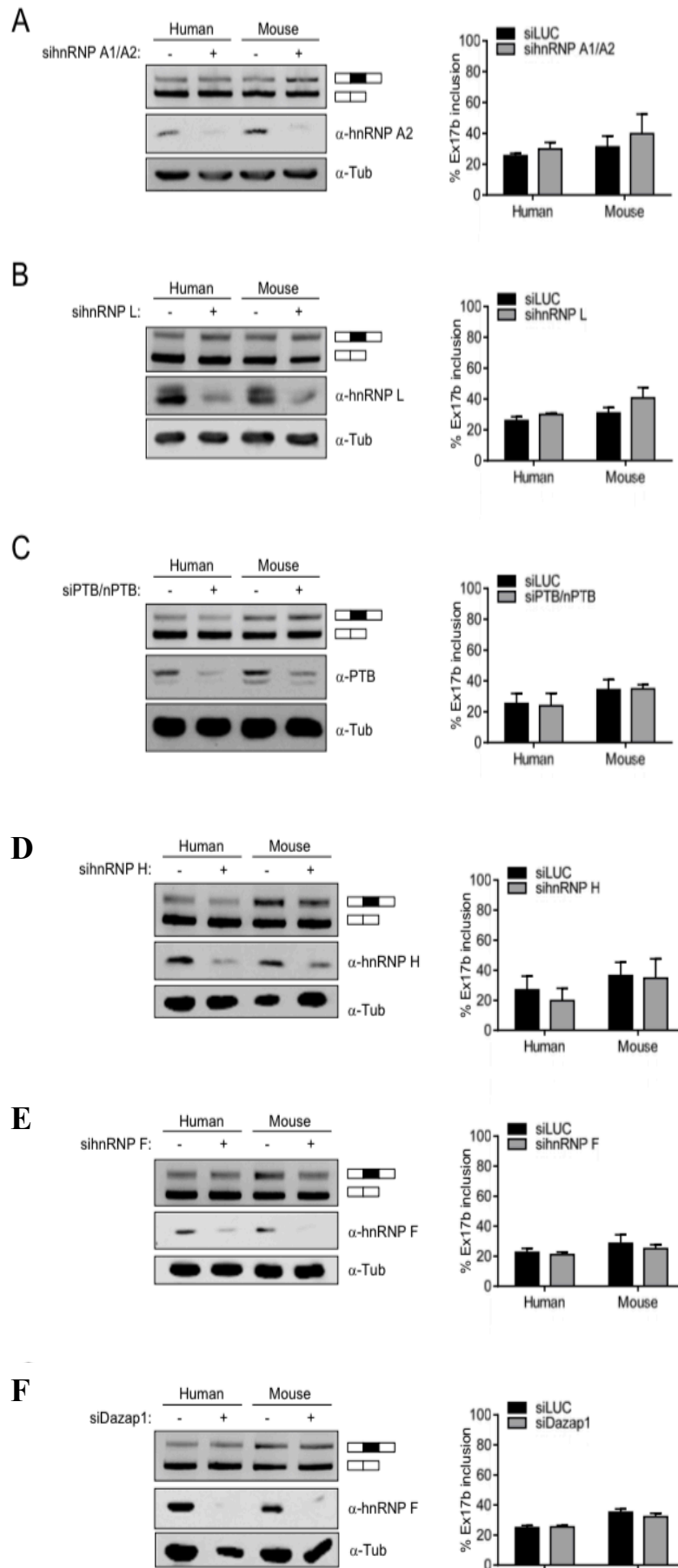
On the other hand, nPTB (neural PTB) is a homolog of PTB (hnRNP I) but despite of PTB that is expressed broadly in human tissues, nPTB is mainly expressed in the brain (Spellman et al. 2007). Both PTB and nPTB can recognize RNA binding sites enriched in “cucc” sequences (Spellman et al. 2007).

It has been shown that PTB knock down consequently resulted in upregulation of nPTB. From the mechanistic point of view, depletion of PTB led to more inclusion of exon 10 in nPTB transcript that avoids its degradation by NMD (Spellman et al. 2007).

Of interest, it has been shown that knockdown of PTB and nPTB together could induce greater changes in regulation of alternative splicing of PTB’s targets compare to knock down of PTB alone (Spellman et al. 2007).

Therefore also in the case of PTB knock down I decided to co-transfect Neuro-2a cells with both PTB and nPTB siRNA to eliminate any upregulation of nPTB that may have overlapped or redundant splicing functional effects.

Results



Results

Figure 3.8. Single knock down of hnRNPs without affecting the basal human or mouse Sort1 Ex17b inclusion levels.

A-F) Knockdown of hnRNP A1/A2 (**A**), hnRNP L (**B**), PTB/nPTB (**C**) hnRNP H (**D**), hnRNP F (**E**) and Dazap 1 (**F**) in the Neuro-2a cell line. None of the single depletions could change splicing pattern of Sort1 Ex17b.

RT-PCR samples are stained with ethidium bromide and run on a 1.5% agarose gel. A schematic diagram of the two minigene spliced transcript (17b inclusion containing a black box vs. 17b skipping) is reported on the right of each agarose gel. Luciferase siRNA treated cells have been used as control and correct depletion of each factor was determined by western blot assay (extracts were normalized using tubulin).

The graph on the right side of each experiment presents the quantified percentage of human and mouse Ex17b inclusion that determined by densitometric analysis using ImageJ software. Statistical differences were calculated by one-way ANOVA test (post test Tukey) (n=3).

3.4.2 Simultaneous knock down of hnRNPs with TDP-43 affected Sort1 Ex17b splicing regulation

3.4.2.1 TDP-43 could repress inclusion of Ex17b more efficiently through co-operation with other hnRNPs

As shown before in Figure 3.2, using minigene systems, depletion of TDP-43 in neuronal Neuro-2a cells increased the inclusion of human and mouse Sort1 Ex17b. Next to evaluate the significance of this enhanced inclusion, I quantified the percentage of Sort1 Ex17b inclusion in three different experimental groups by densitometric analysis using ImageJ software and as presented in Figure 3.9A this increase in inclusion was statistically significant in the case of human Ex17b.

Of our interest no changes in splicing regulation of Sort1 Ex17b was detected following single knockdown of different hnRNPs that were already shown being capable of binding to the related sequences (Fig. 3.8). Therefore the next aim was to understand whether TDP-43 could co-operate with these hnRNPs in order to repress inclusion of the toxic human Sort1 Ex17b in the mature transcript.

Through immunoprecipitation assays, it has been already reported that TDP-43 can interact with other hnRNPs (Freibaum et al. 2010). These interactions eventually could affect TDP-43

Results

splicing regulatory roles as demonstrated in several studies (He et al. 2014) (Suzuki et al. 2015) (Romano et al. 2014).

Therefore in the next step to investigate the potential co-operative repressive role of hnRNP proteins and TDP-43, I performed a series of hnRNP knock down experiments in conjunction with TDP-43 knock down. After co-siRNA transfection, I transected Neuro-2a cells with human and mouse Sort1 Ex17b minigene constructs.

20 hours after minigene transfection, cells were collected and divided in two parts and primarily efficient gene knock down for both TDP-43 and hnRNP was evaluated by western blot technic.

Once the co-depletion of gene expression was approved by western blot analysis, alternative spliced Sort1 transcripts were evaluated by RNA extraction and RT-PCR. The amplified products were run on an agarose gel and after detection, the scanned RT-PCR band images were quantified by densitometric analysis using ImageJ softwar and the percentage of Sort1 Ex17b inclusion in three independent experimental groups was statistically evaluated.

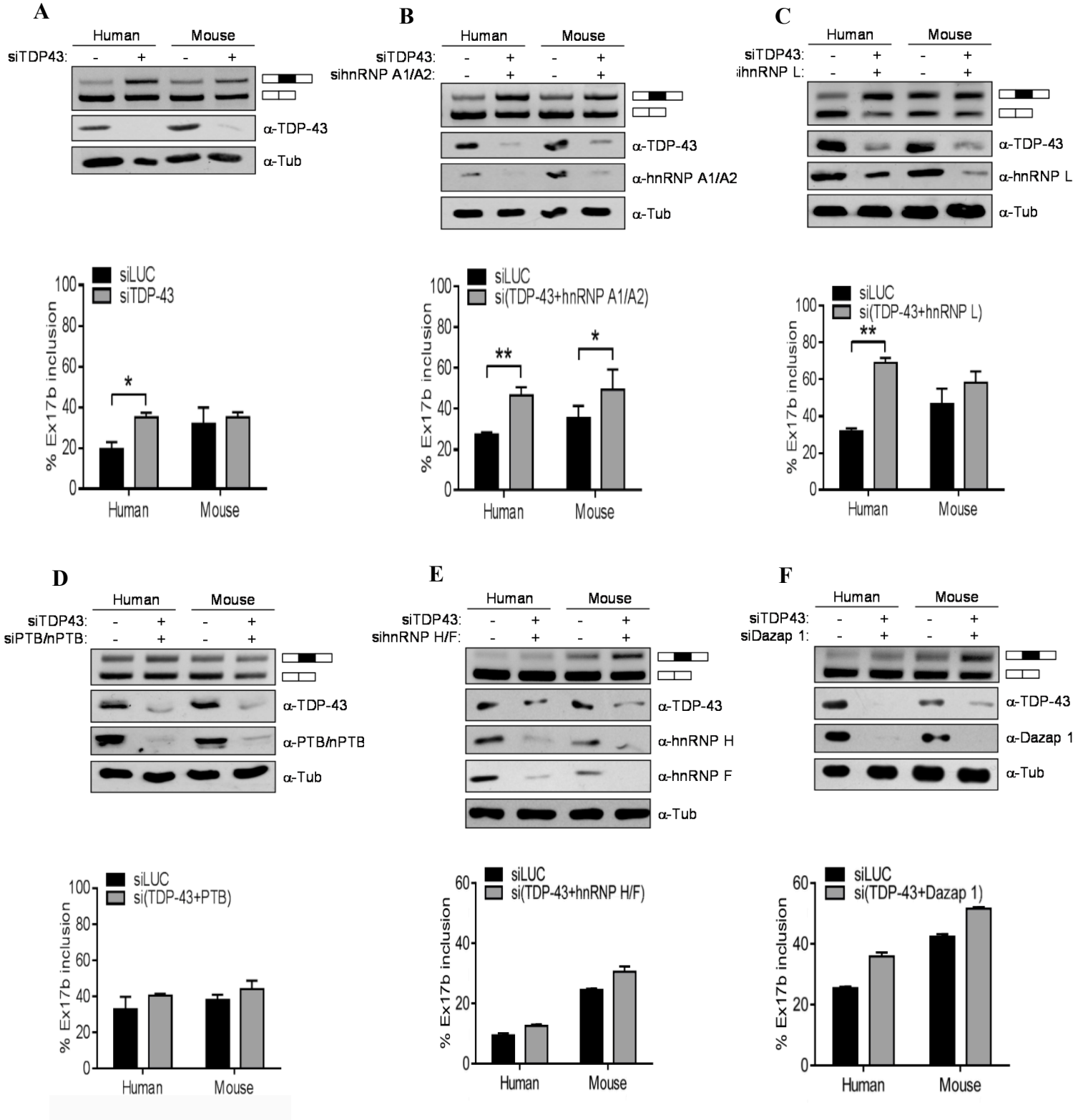
Interestingly, knockdown of both hnRNP A1/A2 and TDP-43 significantly increased the levels of human and mouse Ex17b inclusion (Fig. 3.9B) in a higher levels compare to single TDP-43 knockdown (Fig. 3.9A).

This result is consistent with the data showed before in Figure 3.7 where I proved the presence of a silencer element bound by hnRNP A2 in the human Ex17b that upon mutation abolished the binding of this splicing repressor.

In addition, while no statistically significant increases were observed when TDP-43 was knocked down together with PTB/nPTB (Fig. 3.9D), hnRNP H/F (Fig. 3.9E), or Dazap1 (Fig. 3.9F), I detected a significant increase in Ex17b inclusion when both TDP-43 and hnRNP L were simultaneously knocked down (Fig. 3.9C).

The increased levels of Ex17b inclusion following depletion of both TDP-43 and hnRNP-L (Fig. 3.9C) was much higher than single TDP-43 depletion (Fig. 3.9A) and even higher than co-knock down of TDP-43 together with hnRNP A1/A2 (Fig. 3.9B).

Results



Results

Figure 3.9. Co-operation of TDP-43 and hnRNP L or hnRNP A2 to repress SORT1 Ex17b inclusion in the human and mouse minigene systems.

A) TDP-43 knockdown, alone or simultaneously with hnRNP A1/A2 (**B**), hnRNP L (**C**), PTB/nPTB (**D**), hnRNP H/F (**E**) or DAZAP1 (**F**) in Neuro-2a cell line. Co-knock down of TDP-43 together with hnRNP A1/A2 or with hnRNP L could increase the inclusion of Ex17b in higher levels compare to TDP-43 knock down alone.

RT-PCR samples are stained with ethidium bromide and run on a 1.5% agarose gel. A schematic diagram of the two minigene spliced transcript (17b inclusion containing a black box vs. 17b skipping) is reported on the right of each agarose gel. Luciferase siRNA treated cells have been used as control and depletion efficiency was determined by western blot assay (extracts have been normalized using tubulin).

The graph on the bottom of each experiment presents the quantified percentage of human and mouse Ex17b inclusion that determined by densitometric analysis using ImageJ software. Statistical differences were calculated by one-way ANOVA test (post test Tukey). *P < 0.05, **P < 0.01 (n = 3).

3.4.2.2 Co-knock down of other hnRNPs in the presence of TDP-43; TDP-43 the main splicing regulatory factor for Sort1 Ex17b

To understand the importance of TDP-43 as a crucial regulator for Sort1 Ex17b splicing, I started to co-silence different hnRNPs simultaneously in the presence of TDP-43 to see if any co-knock down of hnRNPs could increase inclusion of Ex17b.

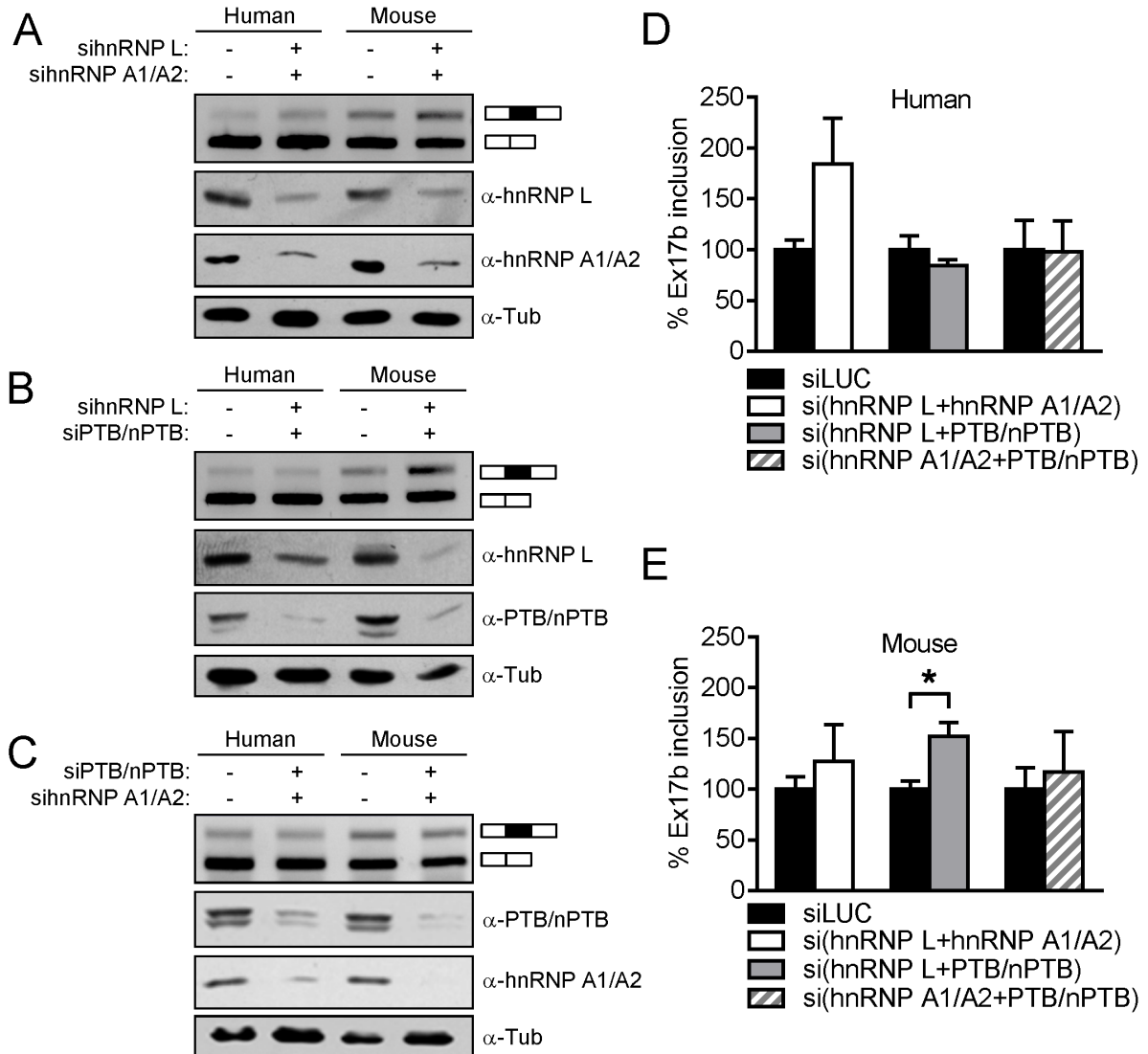
According to Figure 3.9C, first I evaluated if hnRNP L knock down would achieve similar results when a different hnRNP protein was concomitantly knocked down. However, I observed a modest increase of human Ex17b inclusion following double knock down of hnRNP L and hnRNP A1/A2 (Fig. 3.10A and D), but not when hnRNP L was knocked down with hnRNP PTB/nPTB (Fig. 3.10B and D).

Conversely for mouse Ex17b inclusion, a statistically significant increase was found following hnRNP L and hnRNP PTB/nPTB knock down (Fig. 3.10B and E), while no effect was observed when hnRNP L was knocked down with hnRNP A1/A2 (Fig. 3.10A and E).

However for both mouse and human Ex17b inclusion, no effect was observed when hnRNP A1/A2 was knocked down together with hnRNP PTB/nPTB (Fig. 3.10C-E).

Results

In general the splicing changes in hnRNPs co-knock down experiments in the presence of TDP-43 did not show dramatic changes compare to TDP-3 depleted experiments; Therefore TDP-3 remains to be the key splicing regulator of Sort1 Ex17b and as described before and it co-operates with other hnRNPs to repress the inclusion of this toxic exon in human (see Fig. 3.9A-C).



Results

Figure 3.10. Effects on human and mouse Ex17b recognition following knock down of hnRNPs in the presence of TDP-43.

The following knockdowns were performed: hnRNP L together with hnRNP A1/A2 **(A)**, PTB/nPTB **(B)**, or PTB/nPTB with hnRNP A1/A2 **(C)** in Neuro-2a cell line. RT-PCR samples are stained with ethidium bromide and run on a 1.5% agarose gel. A schematic diagram of the two minigene spliced transcript (17b inclusion containing a black box vs. 17b skipping) is reported on the right of each agarose gel. Luciferase siRNA treated cells have been used as control and depletion efficiency was determined by western blot assay (extracts were normalized using tubulin). **(D, E)** The quantified percentage of human **(D)** and mouse **(E)** Ex17b inclusion was determined by densitometric analysis using ImageJ software. Statistical differences were calculated by one-way ANOVA test (post test Tukey). *P < 0.05 (n = 3).

3.4.2.3 Co-operative function of TDP-43 and other hnRNPs to suppress inclusion of endogenous Sort1 Ex17b

Using Sort1 human and mouse Ex17b minigene construct in Neuro-2a cells, I already proved that the only single gene knock down that could affect Sort1 Ex17b inclusion was TDP-43 (Fig. 3.8 and 3.9A). In fact following TDP-43 depletion, Ex17b was more included while individual depletion of other hnRNPs including hnRNP A1/A2, hnRNP L, PTB/nPTB, hnRNP H, hnRNP F, and DAZAP1 did not affect Sort1 Ex17b splicing pattern (Fig. 3.8 and 3.9A).

Moreover, I showed that simultaneously knock down of TDP-43 together with hnRNPs could strengthen the negative splicing regulatory role of TDP-43 (Fig. 3.9) and the maximum inhibition of Sort1 Ex17b was seen when both TDP-43 and hnRNP L were depleted in Neuro-2a cells (Fig. 3.9C).

By this project I also pointed TDP-43 as the key splicing regulator for Sort1 Ex17b, because in the presence of this inhibitory factor co-knock down of any other hnRNP proteins could not dramatically change the splicing pattern of Ex17b (Fig. 3.10).

In order to generalize the effects observed when Sort1 Ex17b minigene systems were used and to understand whether these effects are happening endogenously in the same cells (Neuro-2a cells), I also analyzed the endogenous Sort1 Ex17b inclusion following the same experimental conditions.

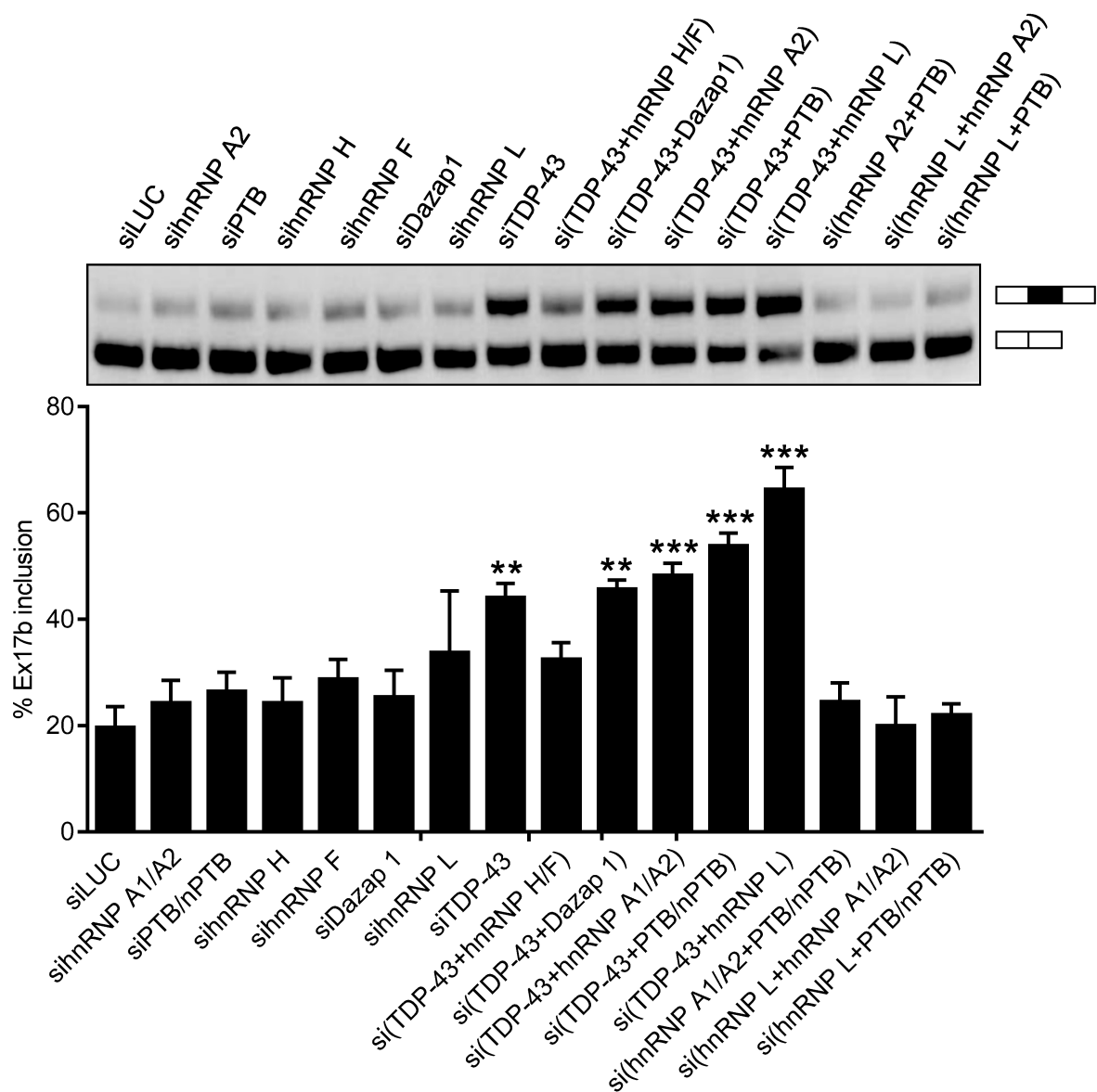
Results

For the endogenous Sort1, TDP-43 and other hnRNPs including hnRNP A1/A2, hnRNP L, PTB/nPTB, hnRNP H, hnRNP F, and DAZAP1 were singularly or concurrently knock down in Neuro-2a cells. After cell collection, alternative spliced Sort1 transcripts were evaluated by RNA extraction and RT-PCR. The amplified products were run on an agarose gel and after detection, the scanned RT-PCR band images were quantified by densitometric analysis using ImageJ software and the percentages of Sort1 Ex17b inclusion in three independent experimental groups was statistically evaluated.

Confirming the results obtained from the minigene system (Fig. 3.8, 3.9, and 3.10), knock down of either TDP-43 alone, or in conjunction with hnRNP L or hnRNP A1/A2, were the most efficient combinations in promoting endogenous Ex17b inclusion (Fig. 3.11). Moreover, co-depletion of TDP-43 and DAZAP1 or PTB/nPTB increased inclusion of endogenous Ex17b significantly while any other co-depletion in the presence of TDP-43 could not change splicing pattern of endogenous Ex17b.

In conclusion, these findings demonstrate that while TDP-43 plays a central role in repressing Ex17b inclusion, additional factors within the hnRNP network function with TDP-43 to regulate this critical splicing event.

Results



Results

Figure 3.11. Effects of knocking down diverse hnRNPs on endogenous mouse Sort1 Ex17b in Neuro-2a cells.

In order to amplify endogenous Sort1 Ex17b, cDNA prepared from the extracted RNA was subjected to RT-PCR using primers in exons 17 and 19 of mouse Sort1. Endogenous experiments confirmed the results obtained by minigene system; the only single gene knock down increasing the endogenous Ex17b inclusion was TDP-43. Co-knock down of TDP-43 with hnRNPs could increase the inclusion of Sort1 Ex17b compare to TDP-43 knock down alone, while co-knock down of hnRNPs in presence of TDP-43 did not change splicing pattern of endogenous Sort1.

RT-PCR samples are stained with ethidium bromide and run on a 1.5% agarose gel. A schematic diagram of the two minigene spliced transcript (17b inclusion vs. 17b skipping) is reported on the right of the agarose gel. The quantified percentage of Sort1 Ex17b inclusion was determined by densitometric analysis using ImageJ software. Statistical differences were calculated by one-way ANOVA test (post test Tukey). **P < 0.01, ***P<0.001 (n = 3).

3.5 Identification of a Sort1 Ex17b splicing enhancer in the mouse upstream intron

3.5.1 Binding of splicing enhancers to the upstream intronic region of mouse Sort1 Ex17b

In the previous study it was shown that two splicing enhancer factors, SRp40/SRSF5 and Tra-2 β , bound to the mouse Sort1 Ex17b minigene sequence much more efficiently than human sequence (Prudencio et al. 2012). Therefore they proposed that the mouse Sort1 Ex17b minigene contains one or more enhancer splicing sequences that are absent in human minigene (Prudencio et al. 2012).

In my thesis project to further investigate these data and better binding site mapping of splicing enhancers, primarily the human and mouse Sort1 Ex17b regions were split into upstream intron, exon, and downstream intron in a pBluescript II KS+ plasmid containing a T7 promoter as described in Figure 3.3.

Next by T7 RNA Polymerase, these sequences were separately in vitro transcribed and pull down of SR proteins was performed using RNA-coated adipic acid dehydrazide beads. Finally

Results

western blot of the pulled down proteins was performed to detect SRp40/SRSF5, Tra-2 β , and SRSF1 by specific anti bodies.

Pull down analysis showed that SRp40/SRSF5 and Tra-2 β bound mainly to the upstream intron of the mouse Sort1 (Fig. 3.12) and there was no binding of SRSF1 (used as negative control) to neither human nor mouse exon 17b contexts.

As shown in Figure 3.12, the obtained results from RNA-protein interactions not only confirmed the already observed finding by Prudencio et al. about higher affinity of SR proteins to bind to the mouse Sort1 context (Prudencio et al. 2012) but also provided more precise binding site of SRp40/SRSF5 and Tra-2 β as splicing enhancers in the upstream intron of mouse Sort1.

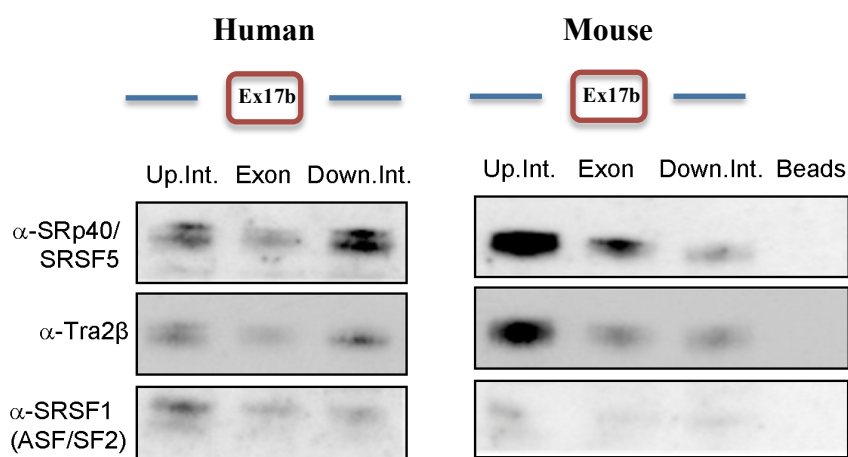


Figure 3.12. Binding sites identification of SR proteins in the mouse upstream intron of Sort1 Ex17b sequence.

The binding efficiency of splicing enhancers, including SRp40/SRSF5, Tra-2 β and SRSF1/(ASF/SF2) was assessed by pull down analysis of in vitro transcribed RNA from human (on the left) and mouse (on the right) SORT1 Ex17b exons (red boxes) and their flanking introns (Up. and Down Int , blue lines). Bound proteins were then tested by western blot using specific antibodies.

SRp40/SRSF5 and Tra-2 β were mainly bound to the upstream intronic region of mouse Sort1 Ex17b context. SRSF1 as a negative control did not bind to any mouse or human sequences.

3.5.2 Splicing enhancing function of SR proteins following binding to the mouse upstream intron

3.5.2.1 Generation of human-mouse hybrid minigenes by human upstream intron modification

In order to further pinpoint the upstream intronic region of mouse Sort1, where SR proteins bind (Fig. 3.12) and to test splicing functionality of this region, I created three human to mouse mutants.

In Figure 3.13A the sequences of human and mouse upstream intronic region of Sort1 Ex17b are presented with nucleotide differences between human and mouse that are highlighted.

In this project I replaced 150 nucleotides of human intron with three selected parts of mouse intronic regions in order to create three different mutants, including mutant H1, mutant H2, and mutant H3.

Human mutant H1 contained the first 43 upstream intronic nucleotides of the homolog mouse region while H2 contained nucleotides from 43 to 111 and H3 contained nucleotides from 110 to 150. (Fig. 3.13B).

As described in Figure 3.14 to start generating the human mutated constructs, I designed different primers according to three distinct parts of mouse upstream intron. I also took advantage of a primer designed on pTB backbone that was available in the lab (pTB 1950 As). A sense primer was also designed according to human upstream intron (HF99).

Using human construct as PCR template, the amplified regions were then purified and digested by NdeI restriction enzyme in order to produce sticky ends as the ones in pTB plasmid backbone in order to ligation.

After overnight incubation of transformed bacteria in the agar plates containing ampicillin, some clones were picked and a small-scale of plasmids was obtained by miniprep and sent for sequencing.

The received sequences reads were evaluated and the right sequences were chosen and the right plasmids as the final human mutants were transfected in Neuro-2a cells.

Results

A

```
Human: agggactgacaaggggtggga ttaaggagatgatgggagaggagtgtgtgggtgtgtgtattggttaagggg gcgagttccctccttctgtgcacct  
Mouse: acactgac aggggagggaccctgaaggcaatga gggga a gagtgtgtgggtgtgtgtattggttaaggggagggcagttccctccttctgtgcacct  
  
Human: tgtcctgggctcctgggtgactctcctcagagtgttctgtgtgtg tgattgcacag  
Mouse: tgcctgggactcctgggtgctgtcctcagagtgttctgtgtgtgtatgattgcacag
```

B

Part 1	Human: agggactgacaaggggtggga ttaaggagatgatgggagaggagt Mouse: acactgac aggggagggaccctgaaggcaatga gggga a gagt
Part 2	Human: gtgtgggtgtgtgtattggttaagggg gcgagttccctccttctgtgcaccttgtcctgggctc Mouse: gtgtgggtgtgtgtattggttaaggggagggcagttccctccttctgtgcaccttgcctgggactg
Part 3	Human: ctgggtgactctcctcagagtgttctgtgtgtg tgattgcacag Mouse: ctgggtggctgtcctcagagtgttctgtgtgtgtatgattgcacag

Figure 3.13. Intronic sequences used to generate human-mouse Sort1 Ex17b hybrids.

A) Human (upper line) and mouse (lower line) upstream intron sequences with high homology. The nucleotide differences between human and mouse Sort1 upstream intronic regions are gray highlighted. **B)** Three distinct parts of mouse upstream intron sequence were selected to replace in human Sort1 Ex17b construct.

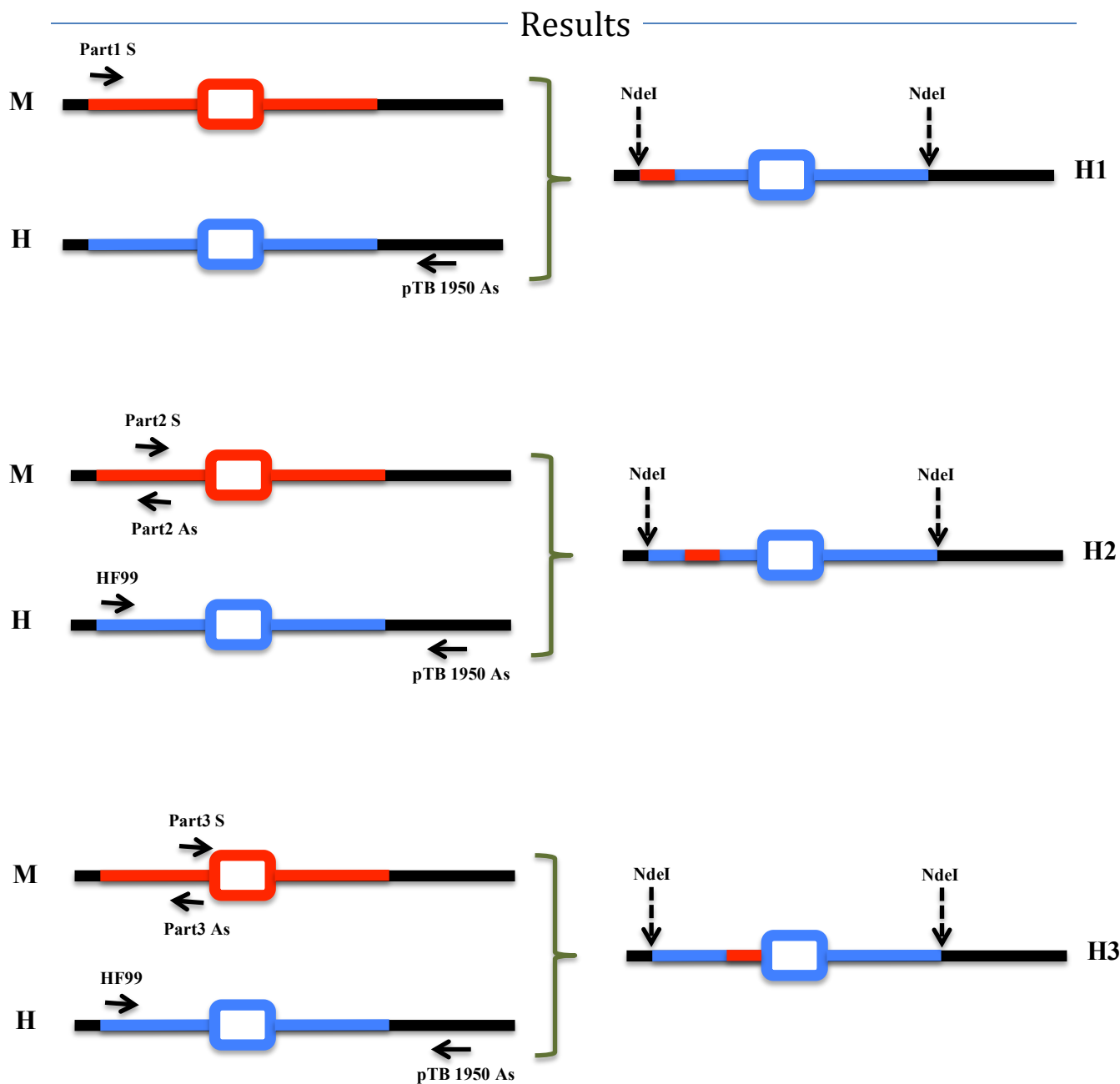


Figure 3.14. Generation of human-mouse Sort1 Ex17b hybrids.

Different primers were designed according to mouse (M, red box and lines) and human (H, blue box and lines) Ex17b constructs' sequences. pTB 1950 As primer is a partial complement of pTB plasmid backbone (black line). Human mutants, including H1, H2, and H3 were generated by PCR amplification of human Ex17b sequence using the specific primers.

PCR-products were purified and like pTB vector were digested by NdeI in order to produce sticky ends for ligation.

3.5.2.2 Increase of Sort1 Ex17b inclusion in human-mouse hybrid following TDP-43 depletion

The obtained human-mouse hybrid constructs are schematically shown in Figure 3.15A. Following TDP-43 knock down I transfected Neuro-2a cells with both human wild type and the generated human mutant constructs. Luciferase siRNA was transfected in the same cells as control.

20 hours after minigene transfection, cells were collected and divided into two parts. After checking the first parts for gene expression depletion through western blot, RNA was extracted from the second parts in order to evaluate alternative spliced Sort1 transcripts.

Following TDP-43 knock down the inclusion of wild type human Sort Ex17b was increased (Fig. 3.15B). This was expected as shown before that in Neuro-2a cells TDP-43 is the main repressor of human Ex17b inclusion (Fig 3.9A)

RT-PCR results also showed that in the absence of TDP-43, mutant H2 (human minigene containing the middle part of the upstream intron from mouse minigene) contained the highest levels of Sort1 Ex17b (Fig 3.15B). The density of the included Ex17b in H2 mutant on the agarose gel was higher than wild type human Sort1 context (Fig 3.15B).

Moreover the quantified percentage of Sort1 Ex17b inclusion through densitometric analysis using ImageJ software and further statistical analysis showed that depletion of TDP-43 resulted in statistically increase of Sort1 Ex17b in H2 mutant compare to wild type human Sort1 context (Fig 3.15C).

This result showed that 43-111 nucleotide replacement had introduced an enhancer element in the human context. This pointed the importance of the middle part of mouse upstream intron as a cis-enhancer element that through binding to SRp40/SRSF5 and Tra-2 β factors enhanced inclusion of Sort1 Ex17b.

Results

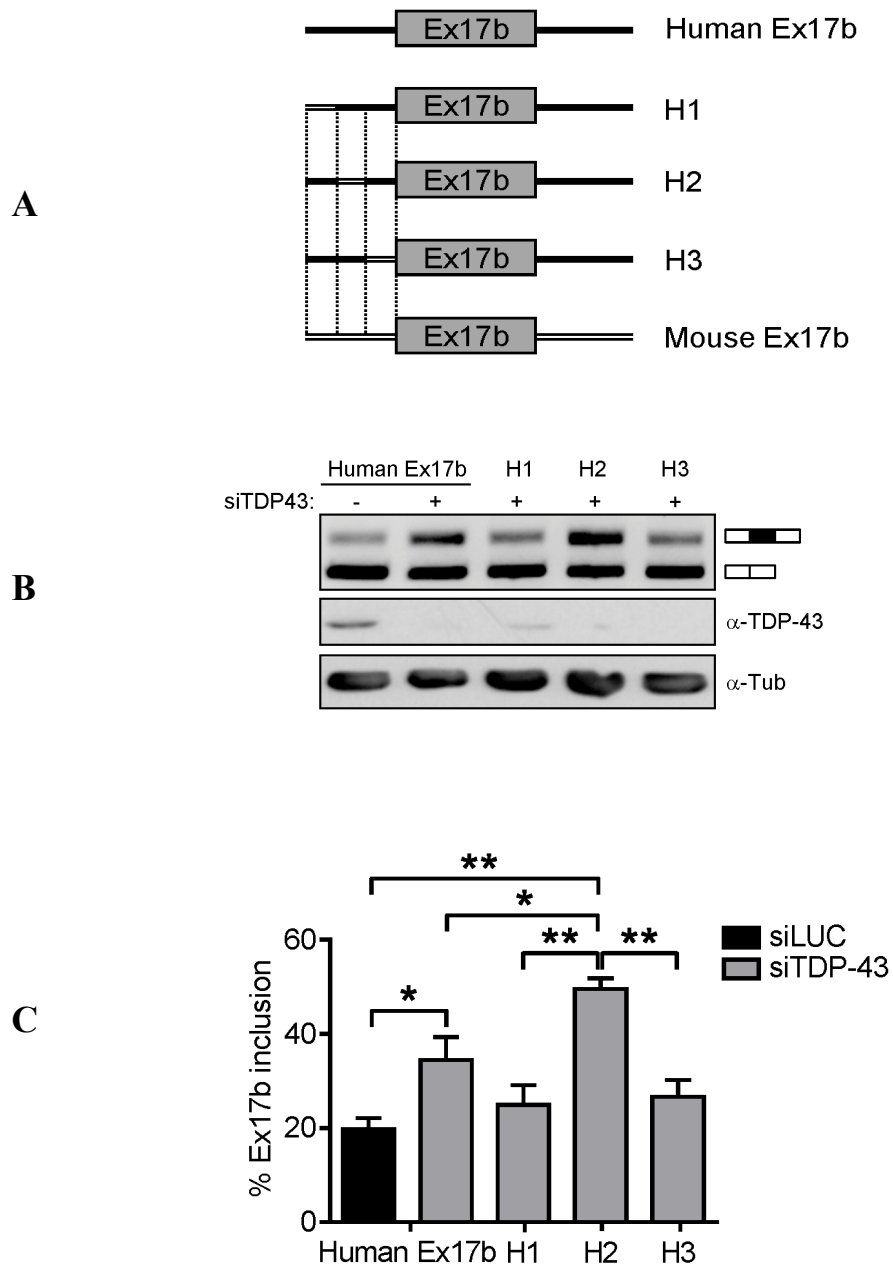


Figure 3.15. Identification of a splicing enhancer of Ex17b in the mouse upstream intron.

A) Schematic representation of hybrid human Ex17b minigenes constructed to assess the importance of mouse upstream intron in inclusion of Ex17b. **B)** Hybrid constructs were expressed in Neuro-2a cells after TDP-43 knockdown. H2 mutant, showed the highest levels of Ex17b. RT-PCR samples are stained with ethidium bromide and run on a 1.5% agarose gel. A schematic diagram of the two minigene spliced transcript (17b inclusion containing a black box vs. 17b skipping) is reported on the right of the agarose gel. Luciferase siRNA treated cells were used as control and correct depletion was determined by western blot assay (extracts were

Results

normalized using tubulin). C) The quantified percentage of SORT1 Ex17b inclusion for each construct was determined by densitometric analysis using ImageJ software. Statistical differences were calculated by one-way ANOVA test (post test Tukey). *P < 0.05, **P < 0.01 (n = 3).

3.5.2.3 Splicing enhancing function of SRp40/SRSF5 following TDP-43 depletion

As described before, in the mouse context I found intronic splicing enhancer for Sort1 Ex17b (Fig. 3.15). Usually SR proteins enhance exon inclusion by binding to the exonic sequences and the presence of SR-binding splicing enhancer elements in intronic sequences is a rather rare but a previously described phenomenon for some genes, such as b-tropomyosin exon 6A (Gallego et al. 1997), CT/CGRP gene exon 4 (Lou et al. 1998), and Tra2 (Hastings et al. 2001).

Moreover, while in these genes the enhancer sequences are present in the downstream intron of the affected exonic region, in Sort1 the enhancer sequence is localized in the upstream intron.

Therefore, in order to better define the properties of this upstream enhancer element in mouse Sort1 context, I overexpressed the SRp40/SRSF5 and Tra-2 β proteins in Neuro-2a cells and determined their effect on the inclusion of the endogenous mouse Ex17b.

This was performed both under normal conditions (Fig. 3.16A) and following TDP-43 knock down by siRNA transfection in Neuro-2a cells (Fig. 3.16B).

After TDP-43 silencing cells were transfected with overexpression plasmids for SRp75/SRSF4, SC-35/SRSF2, Tra-2 β , and SRp40/SRSF5 and the day after cells were collected and RNA was extracted in order to evaluate alternative spliced Sort1 transcripts.

On the agarose gel after RT-PCR, interestingly SRp40/SRSF5 had the expected effect of promoting mouse Ex17b inclusion. However, this occurred only in the absence of TDP-43. In contrast, overexpression of Tra-2 β had no effects on Ex17b inclusion in both conditions.

As showed in the graph in Figure 3.16C, the quantified percentage of Sort1 Ex17b inclusion through densitometric analysis using ImageJ software and further statistical analysis showed that the increased inclusion of Sort1 Ex17b following SRp40/SRSF5 in the absence of TDP-43 was statistically significant.

Results

Moreover, no effect could be observed following overexpression of other SR factors (SRp75/SRSF4 and SC-35/SRSF2), which do not bind within the Ex17b context (Prudencio et al. 2012) and were used as negative controls.

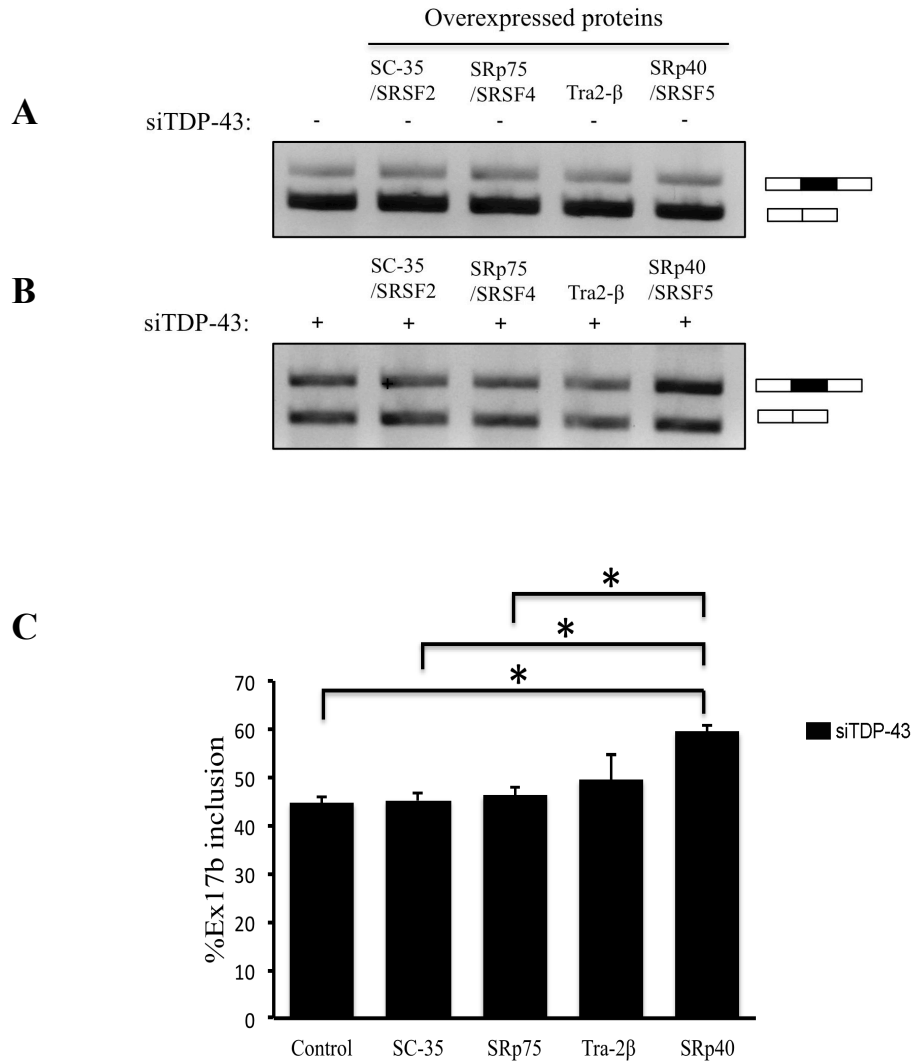


Figure 3.16. Overexpression of SR protein factors and Tra-2 β in Neuro2a cells to determine their effects on endogenous mouse Ex17b expression levels.

Overexpression of SRp75/SRSF4, SC-35/SRSF2, Tra-2 β , and SRp40/SRSF5 in the presence (**A**) or absence (**B**) of endogenous TDP-43. SRp40/SRSF5 overexpression significantly increased Ex17b inclusion in the absence of TDP-43. RT-PCR samples were stained with ethidium bromide and run on a 1.5% agarose gel. A schematic diagram of the two transcripts (17b inclusion containing a black box vs. 17b skipping) is reported on the right.

Results

The graph (C) shows the quantified percentage of endogenous mouse Ex17b inclusion following each overexpression following knock down of TDP-43, as determined by densitometric analysis using ImageJ software. Statistical differences were calculated by one-way ANOVA test (post test Tukey). *P < 0.05.

3.5.2.4 Splicing enhancing function of Tra-2 β following TDP-43 depletion

As described in Figure 3.16, overexpression of Tra-2 β neither in presence nor in the absence of TDP-43 did not significantly affect the splicing pattern of endogenous Sort1 Ex17b in Neuro-2a cells.

However, since I had revealed before that this splicing enhancer could bind to the mouse upstream intron of Sort1 Ex17b (Fig. 3.12), I decided to further investigate the possible splicing enhancing regulatory role of this factor.

To achieve this aim, I decided exploring the splicing of Ex17b in the absence of Tra-2 β . In addition to singular Tra-2 α/β and TDP-43 knock down, I co-transfected Neuro-2a cells with siRNA against Tra-2 α/β and TDP-43 and for control Luciferase siRNA treated cells were prepared as well.

To obtain a better silencing of Tra-2 β , Tra-2 α was silenced as well because Tra-2 β depletion upregulates Tra-2 α and these two factors regulate splicing of some overlapped targets (Best et al. 2014).

After gene silencing following the right protocol, cells were collected and RNA was extracted in order to evaluate endogenous alternative spliced Sort1 transcripts.

On the agarose gel after RT-PCR, as expected TDP-43 depletion caused in increase of Sort1 Ex17b inclusion confirming the previous observation explained in Figure 3.9A.

Interestingly Tra-2 α/β silencing decreased Ex17b inclusion and simultaneous knock down of TDP-43 and Tra-2 α/β could improve the elevated endogenous Ex17b inclusion following TDP-43 depletion. In the other word depletion of Tra-2 β not only alone but also in the absence of TDP-43 could lower endogenous mouse Ex17b inclusion (Fig 3.17A).

While the included Ex17b was still higher than the control group, the quantified percentage of Sort1 Ex17b inclusion through densitometric analysis using ImageJ software and further statistical analysis showed that Tra-2 β depletion could significantly decrease the elevated level of Ex17b inclusion following TDP-43 knock down (Fig. 3.17B).

Results

In conclusion these data demonstrate the splicing regulatory role of Tra-2 β as an enhancer for Sort1 Ex17b inclusion.

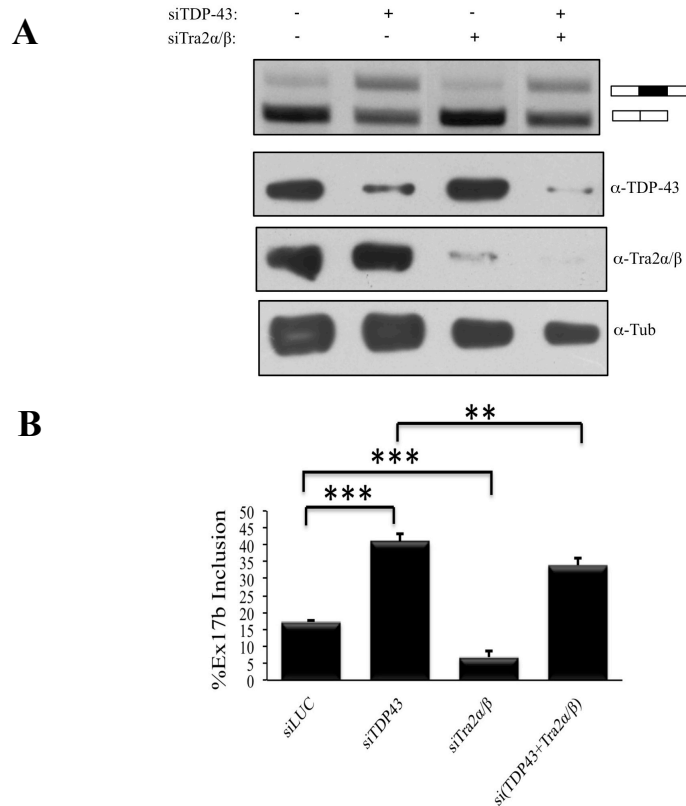


Figure 3.17. Silencing of Tra-2 β in Neuro-2a cells to determine its effect on endogenous mouse Ex17b inclusion levels.

A) Depletion of Tra-2 β decreased Ex17b inclusion and improved the elevated level of Ex17b inclusion following TDP-43 knock down. RT-PCR samples are stained with ethidium bromide and run on a 1.5% agarose gel. A schematic diagram of the two minigene spliced transcript (17b inclusion containing a black box vs. 17b skipping) is reported on the right of each agarose gel. Luciferase siRNA treated cells have been used as control.

B) The graph shows the quantified percentage of endogenous mouse Ex17b inclusion following two independent silencing experiments, as determined by densitometric analysis using ImageJ software. Statistical differences were calculated by one-way ANOVA test (post test Tukey). **P < 0.01, ***P<0.001.

3.6 Altered mRNA levels of hnRNP proteins in the brain of FTLD-TDP patients

Previously cerebral samples from FTLD-TDP patients showed high levels of Sort1 Ex17b mRNA in the temporal cortex (Prudencio et al. 2012). However, Sort1 Ex17b mRNA was not detectable in the temporal cortex of FTLD-tau where there is no TDP-43 pathology (Prudencio et al. 2012).

In FTLD-TDP patients, TDP-43 is accumulated in the cytosolic aggregates and due to loss of function of TDP-43 in the nucleus, could mediate toxicity for the cells (Neumann et al. 2006). One of the crucial nuclear roles of TDP-43 that is affected in FTLD is mRNA splicing regulation (Chen-Plotkin et al. 2010).

In my thesis loss of TDP-43 function was induced by cell transfection of siRNA against TDP-43 that due to increase in inclusion of Sort1Ex17b could be toxic for human. In fact human Sort1 transcript containing Ex17b is potential to generate a truncated receptor that is not functional (Prudencio et al. 2012).

Of interest, I found that other hnRNPs could co-operate with TDP-43 in order to repress inclusion of human Sort1 Ex17b.

In the next step collaborating with Department of Neuroscience, Mayo Clinic Jacksonville in USA we sought to determine whether a change in hnRNP protein expression might be associated with FTLD-TDP.

hnRNPs mRNA levels were evaluated in the temporal cortex, a brain region in which a significant increase in Ex17b inclusion had been seen in FTLD-TDP patients (Prudencio et al. 2012). Using quantitative RT-PCR (qRT-PCR), a significant increase in the splicing repressors *HNRNP A2B1* (hnRNP A1/A2) and *PTBP1* (PTB) in FTLD-TDP patients relative to controls was observed (Fig. 3.18).

In addition, consistent with previous reports (Prudencio et al. 2012), no significant differences were detected in *TARDBP* expression levels between FTLD-TDP cases and controls.

Taken together, these findings indicate that increased expression of the splicing repressors hnRNP A1/A2 and PTB may represent a compensatory response to the loss of nuclear TDP-43 function as a result of aggregation in FTLD.

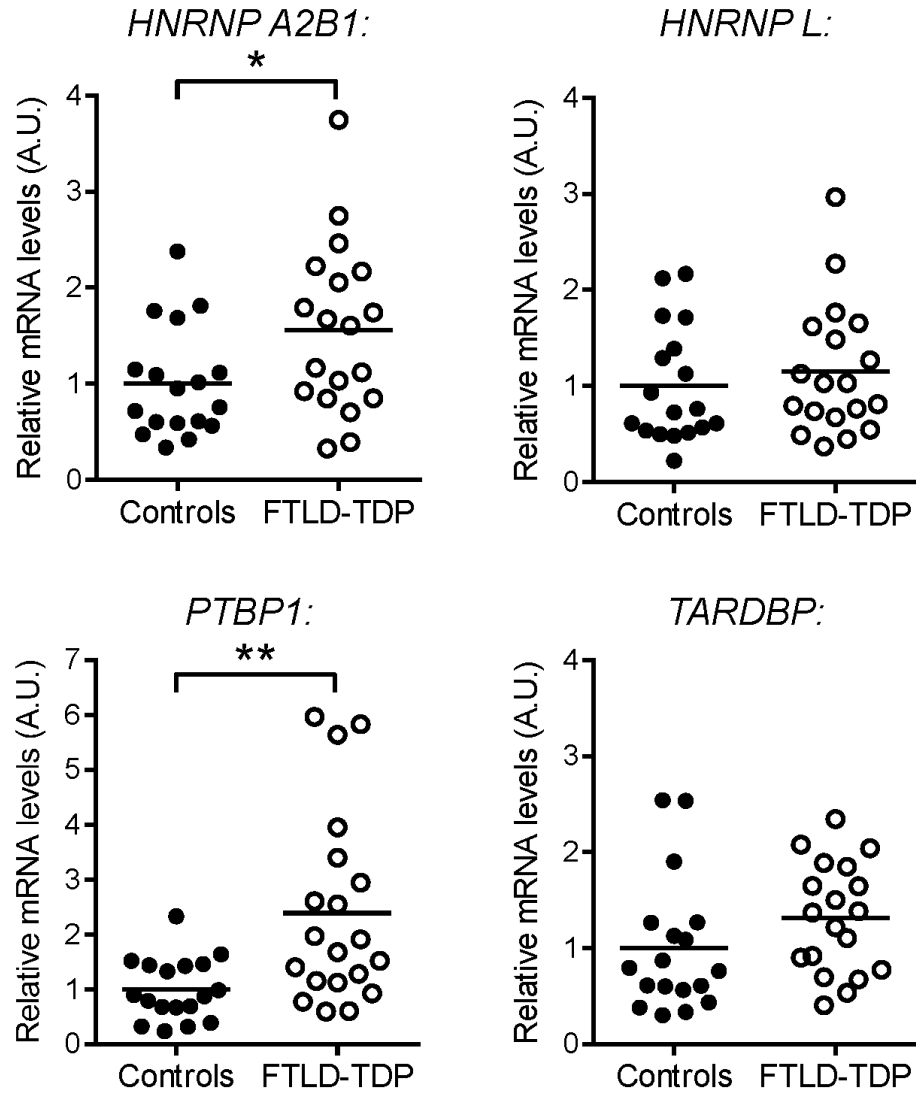


Figure 3.18. The RNA levels of several hnRNP proteins is altered in the brain of FTLD-TDP patients.

Transcript levels of several hnRNPs measured by qRT-PCR in human FTLD-TDP cases (n=20) and age-matched controls (n=19) and normalized to *GAPDH* RNA levels. Statistical differences were assessed by Student *t*-test (* $P < 0.05$, ** $P < 0.005$).

3.7 New insights to splicing and neurodegeneration; the other related projects in our laboratory

3.7.1 hnRNPs function as general TDP-43 toxicity modifiers

By my PhD project I have provided a novel insight into the complexity of the TDP-43 RNA-binding protein network suggesting that a comprehensive assessment of this network will dramatically advance our current understanding about the role of TDP-43 in disease pathogenesis, diagnostic, and therapeutic capabilities. Therefore we may not focus only on TDP-43 as the main protein involved in aggregate formation but also on the other members of the hnRNPs family that could indirectly act to improve/worsen the disease outcome.

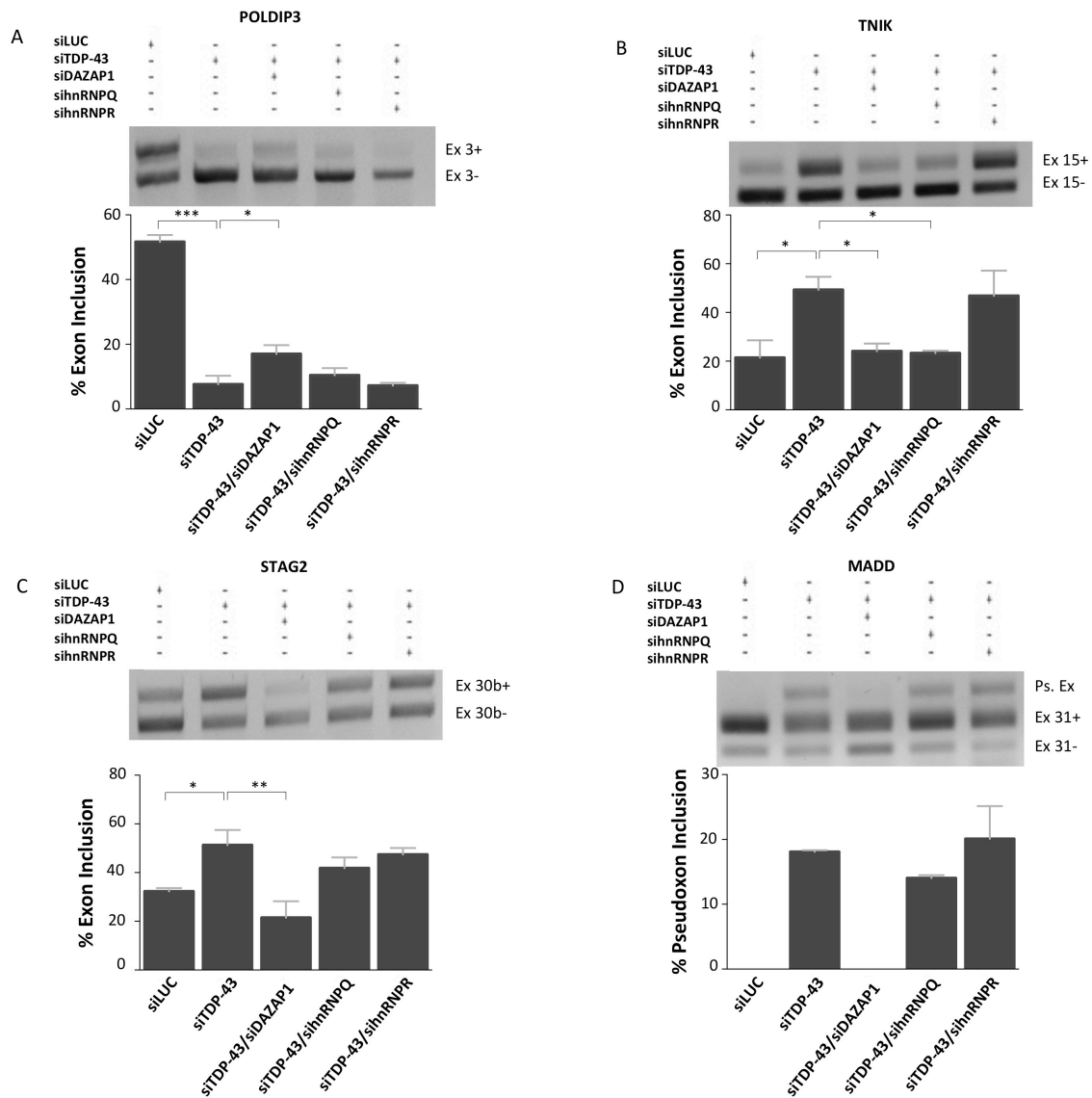
Indeed my PhD project has sparked an idea for a wider project in our laboratory to explore the relationship between TDP-43 and hnRNP proteins in general. In this project using *Drosophila* as a model system we have now performed a functional screening aiming at establishing the effects of known hnRNP proteins on TDP-43 overexpression/depletion phenotypes. As expected, lowering major hnRNP expression together with TDP-43 has generally a harmful effect on flies' locomotor abilities. However, our study has also identified a distinct set of hnRNPs that is capable of rescuing TDP-43 gain of function toxicity in the fly eye. Most importantly, I have shown that removing the human orthologs of these hnRNPs (DAZAP1, hnRNP Q and hnRNP R) in human neuronal SH-SY-5Y cell line can correct pre-mRNA splicing altered by the absence of TDP-43 (Fig. 3.19).

Briefly, as shown in Figure 3.19 DAZAP1 removal significantly rescued the inclusion of POLDIP3 exon 3, the skipping of TNIK exon 15, and the skipping of STAG2 exon 30b. With regards to the MADD gene, although removal of DAZAP1 did not significantly rescue exon 31 inclusion, it did prevent pseudoexon inclusion with a very high efficiency. With regards to hnRNP Q, its removal was effective to rescue exon skipping of TNIK exon 15 but had no effect in all other cases. Finally, removal of hnRNP R had no effect to modify the changes induced by TDP-43 knock down in any of these four genes.

The results of these rescue experiments are summarized in Figure 3.19 and from this figure it is clear that, out of all these three hnRNPs tested, DAZAP1 is the most consistent modifier of the tested TDP-43 targets.

Results

Taken together, the results from this project suggest that modulation of specific sets of hnRNP protein expression can successfully rescue pathological events connected with TDP-43 overexpression or aggregation. The manuscript is currently under consideration in Nucleic Acids Research Journal.



Results

Figure 3.19. Rescue of TDP-43 controlled pre-mRNA splicing.

RT-PCR of SH-SY-5Y cell lines were used to validate the potential effects of hnRNP depletion on the splicing profile of various TDP-43 controlled genes: POLDIP3 exon 3, TNIK exon 15, STAG2 exon 30b, and MADD exon 31. The agarose gel was loaded with the following samples: control siRNA Luciferase transfected cells (lane 1, siLUC), depleted of TDP-43 (lane 2, siTDP-43), depleted of TDP-43 and DAZAP1 (lane 3, siTDP-43/siDAZAP1), depleted of TDP-43 and hnRNP Q (lane 4, siTDP-43/sihhnRNPQ), and depleted of TDP-43 and hnRNP R (lane 5, siTDP-43 /sihnRNPR). The identity of the various transcripts is reported on the right. For the MADD gene, the appearance of a pseudoexon is also reported (Ps.Ex.). *P < 0.05, **P < 0.01, ***P<0.001 (n = 3), calculated by one-way ANOVA.

3.7.2 Gene variants that are involved in Parkinson's disease can affect pre-mRNA splicing

In this work, we have addressed the need to understand the contribution of splicing mutations to Parkinson's disease (PD) pathogenesis. Briefly our minigene assays showed that four LRPPRC (leucine-rich pentatricopeptide repeat containing protein) variants found in PD patients, namely, IVS32-3C>T, IVS35+14C>T, IVS35+15C>T, and IVS9+30A>G, can influence pre-mRNA splicing by modulating the inclusion/exclusion ratio of the respective exons. In addition, an inhibitor of mitochondrial complex I, MPP+, was proven to induce splicing changes of endogenous LRPPRC mRNA that reproduce the effect of the LRPPRC IVS35+14C>T mutation. Finally, we demonstrate that adapted U1 snRNAs could rescue aberrant splicing caused by LRPPRC intronic mutations, thus providing a potentially useful therapeutic strategy. Our results indicate for the first time that several LRPPRC intronic variants can affect normal splicing of this gene and their presence may therefore influence disease risk of PD. The analysis performed in this study has been published in *Neurobiology of Aging* journal (Gaweda-Walerych et al. 2016).

Chapter 4

4 Discussion

4.1 Impairment of splicing regulation and FTLT-TDP disorder

Hundreds of RNA binding proteins (RBPs) are encoded by eukaryotic genome that jointly harmonize and regulate all aspects of RNA life cycle generated within the cells. In fact RNA fate from transcription to translation is under RBPs control, including capping, pre-mRNA splicing, editing, polyadenylation, transport, and RNA stability (Änkö & Neugebauer 2012).

Among all the regulatory steps for RNA metabolism, pre-mRNA splicing often needs a complex network of RBPs that coordinately recognize and bind to specific sequences near or within the exon to be spliced (Wang et al. 2013). By binding to the splicing elements located in the intronic or exonic regions, RBPs can enhance or suppress inclusion of the exons. As a result in eukaryotes, according to the involved RBPs, different alternative spliced transcripts can be generated from a single gene (Sharma & Black 2006) (Zheng 2004).

A correct concentration of RBPs in different cells is a critical factor that will determine whether a particular nucleotide sequence is eventually included in the mature mRNA or not. Because of a high importance of RBP network in regulation of overall RNA processing and metabolism, it is predictable that any changes in RBPs expression levels or mutations could play an active role as causative factors of Mendelian disease in humans (Castello et al. 2013).

Regarding neurodegenerative diseases it has been revealed that changes in the genes involved in RNA/DNA metabolism are associated with FTLT and ALS and this discovery shed light on the RBPs roles in determining disease onset and progression (Ling et al. 2013).

Discussion

Any misregulation in RBPs expression due to RNA metabolism alteration is potential to be very damaging for the neuronal function as described in some reviews (Renoux & Todd 2012) (Ling et al. 2013) (Suzuki et al. 2015).

Therefore more investigation of RBPs regulatory roles in RNA metabolism is expected in order to advance the field of neurodegenerative diseases in the near future (Nussbacher et al. 2015) and an important issue needed to be addressed is how various RBPs including hnRNPs and SR proteins as RNA alternative-splicing regulators may differentially contribute to neurodegeneration.

In my PhD project, I focused on the alternative spliced transcripts of Sort1 gene in human and mouse contexts. The reason was that Sort1 gene by encoding neuronal Sort1 receptor regulates PGRN hemostasis that is an essential neuronal growth factor (Lewis & Golde 2010). It has been shown that in FTLT-TDP low levels of PGRN due to gene mutation causes TDP-43 mislocalization in cytosolic inclusions and therefore loss of TDP-43 nuclear functions as an RBP that eventually impairs splicing regulation of TDP-43 targets (Baker et al. 2006) (Bird 2009) (Chen-Plotkin et al. 2010) (Lagier-Tourenne et al. 2010).

One of the TDP-43 targets is Sort1 and in the temporal lobes of FTLT-TDP cases high levels of an alternative spliced Sort1 mRNA has been detected (Prudencio et al. 2012). Lack of TDP-43 leads to formation of this alternative isoform by including an exon located between exons 17 and 18 known as Ex17b. This extra exon because of containing a premature stop codon is potential to encode a truncated Sort1 receptor that is not functional and due to binding and not internalizing PGRN could induce toxicity for the cells (Prudencio et al. 2012). Therefore Sort1 Ex17b could impair PGRN functions including TDP-43 clearance by autophagosomal/lysosomal pathways (Mackenzie et al. 2010) (X. Wang et al. 2010) and eventually through a vicious circle could worsen the toxicity effects of cytosolic TDP-43 inclusion bodies in FTLT-TDP.

Of interest, mouse Sort1 transcript including Ex17b encodes a functional Sort1 receptor and despite of human, in the physiological conditions mouse contains both transcript isoforms of Sort1 (Prudencio et al. 2012). Therefore this is of high interest to show how in humans splicing regulation has been evolved in favor of Ex17b exclusion.

Based on human and mouse Sort1 transcript comparison, the main aim of my thesis was to better understand the differential splicing regulatory elements that inhibit inclusion of toxic Ex17b in human Sort1 transcript.

4.2 Strike splicing regulation of Sort1 Ex17b inclusion in human

According to the previous study where TDP-43 was introduced as an important splicing regulatory factor to inhibit Sort1 Ex17b inclusion in HeLa and M17 cells (Prudencio et al. 2012), primarily I extended this finding by TDP-43 depletion in different neuronal and non-neuronal human and mouse cells lines.

Minigene system studies in my thesis showed that generally in the presence of TDP-43, low levels of Ex17b were included in both human and mouse Sort1 transcripts in all the tested cell lines. In fact this finding pointed to existence of splicing repressors for both human and mouse Ex17b and as expected in all the tested cell lines the repression force was higher in the case of human Sort1.

Instead after TDP-43 depletion mouse Ex17b was highly included in most of the cell lines while the levels of human Ex17b did not change with an only exception that increased in mouse neuronal Neuro-2a cells.

Confirming the highly restricted inclusion of the human Ex17b compare to mouse and based on variable mouse Ex17b inclusion depending on the cell line tested I sought to explore additional splicing regulatory factors that can regulate Sort1 Ex17b inclusion.

4.3 Mapping the binding sites of expected hnRNPs and SR protein in mouse and human Sort1 Ex17b context

4.3.1 hnRNPs bound to human Sort1 Ex17b in a higher extend

In order to screen the hnRNPs that could regulate Ex17b splicing by binding to the distinct sequences, pull down analyses for Ex17b and its flanking introns of mouse and human were performed separately. Selection of hnRNP for eventual investigation was based on the recent data and the possible binding sites available on Sort1 Ex17b context. For example “ug” and “g” repeats located in upstream intron as TDP-43 and hnRNP H/F binding sites respectively

Discussion

(Lukavsky et al. 2013) (Caputi & Zahler 2001), “cucc” sequences that are present in introns and in the Ex17b as PTB binding sites (Singh et al. 1995), and ca-repeats located in the 17b exonic regions as hnRNP L binding site (Hui et al. 2003). Although no well-known binding sequences for Dazap1 is known, this protein was also investigated because it preferentially binds downstream of 5’ splice sites (Pastor & Pagani 2011). Finally hnRNP C was explored as well because it is a very abundant hnRNP protein that binds u-rich sequences (Görlach et al. 1994).

Eventual obtained pull down analyses showed a similar binding pattern for several hnRNPs including TDP-43, hnRNP H, hnRNP F, hnRNP A1, DAZAP1, hnRNP L, and no binding for hnRNP C1/C2. All of these hnRNPs bound to both human and mouse intronic region except hnRNP L that bound to all exonic and intronic parts pointing important splicing regulatory role of this factor for Ex17b.

However, interestingly two hnRNPs including hnRNP A2 and PTB that are known as splicing repressors (Clower et al. 2010) bound to human Sort1 context more than mouse. In fact in addition to intronic parts hnRNP A2 bound to Ex17b in the human but not in the mouse context. Moreover, PTB showed binding to human upstream and downstream introns to a greater extent than the corresponding mouse intronic regions.

Therefore not only it became clear that in addition to TDP-43 other hnRNPs are involved in splicing regulation of Sort1 Ex17b both in human and mouse contexts but also more strength binding of hnRNP A2 and PTB as splicing repressors reflected higher restricted Ex17b inclusion in humans.

4.3.2 SR protein binding to mouse upstream intron of Ex17b

Since in mice in the physiological condition both isoforms of Sort1 transcript included and excluded Ex17b are detectable we sought to understand the possible binding of splicing enhancers to the distinct intronic and exonic regions of this transcript.

Pull down analyses were also performed looking for splicing enhancers and the results showed that SRp40/SRSF5 and Tra-2 β bound mainly to the upstream intron of the mouse Sort1 and no binding was seen in human Sort1 context. Exclusive binding of SRp40/SRSF5 and Tra-2 β as splicing enhancers (Prudencio et al. 2012) (Graveley 2000) (Lam & Hertel 2002) to the

mouse Sort1 context explains the higher amount of mouse Ex17b inclusion in all the cell lines tested in the first part of my project. Moreover the absence of such enhancers in the human Sort1 contexts reflects the low levels of Ex17b inclusion detected in the same experiments in different cell contexts.

4.4 Splicing evolution toward repression of Sort1 Ex17b in human; human Ex17b splicing silencers versus mouse Ex17b splicing enhancers

Substitution of exclusive mouse exonic nucleotides in human led to increase of Ex17b inclusion because these specific mouse nucleotides abolished hnRNP A2 binding to human Ex17b. In fact in human Sort1 Ex17b, Adenin and “ctc” nucleotides played as exonic cis-silencer elements where hnRNP A2 could bind and inhibit inclusion of this toxic exon in human. Instead replacing these critical human silencers with Guanin and “tct” mouse nucleotides increased Ex17b inclusion by eliminating hnRNP A2 binding sites.

It has been revealed that SR proteins mediate their splicing enhancing effects through binding to the exonic regions. However, in this work I found that SR proteins bound mainly to the intronic region of mouse Sort1. But this was not surprising for us since previously intronic splicing enhancing roles of SR proteins were described for some genes, such as b-tropomyosin exon 6A (Gallego et al. 1997), CT/CGRP gene exon 4 (Lou et al. 1998), and Tra2 (Hastings et al. 2001).

The only difference was that in such experiments the described enhancer sequences were located in the downstream intron of the affected exonic region while in case of Sort1 the enhancer sequence was localized in the upstream intron.

Therefore to prove the functionality of the upstream intronic enhancers by creating human-mouse hybrids I showed that substitution of a partial mouse upstream intron in human Sort1 context could increase inclusion of Ex17b in depletion of TDP-43 pointing the mouse intronic region as splicing enhancer.

Further functional confirmations obtained by overexpression of SR proteins after TDP-43 knockdown. In this case SRp40/SRSF5 could induce inclusion of Ex17b in Neuro-2a cells

while no changes were seen when Tra-2 β was overexpressed. However, knock down of Tra-2 β alone led to significant decrease of Ex17b inclusion and also could rescue the elevated Ex17b inclusion caused by TDP-43 depletion. By these data I could functionally prove that SRp40/SRSF5 and Tra-2 β bind to mouse enhancer sequence (that is absent in human) and increase inclusion of Ex17b in the final Sort1 transcript.

All together despite of high homology between human and mouse Sort1 sequences, it looks that evolution has forced exclusion of toxic human Ex17b by developing splicing silencers in human Sort1 sequence and eliminating the splicing enhancer elements that still exist in mouse sequence.

4.5 Co-operation of TDP-43 with hnRNPs to repress Sort1 Ex17b inclusion

4.5.1 TDP-43, the only repressive hnRNP that can inhibit Sort1 Ex17b inclusion directly and independently

After mapping the binding sites on human and mouse Sort1 Ex17b contexts, all the hnRNPs able to bind to any intronic or exonic regions were silenced in Neuro-2a cells. Although there was a significant increase in inclusion of Ex17b after TDP-43 depletion, none of the other knocked down genes including hnRNP A1/A2, hnRNP L, PTB/nPTB, hnRNP H, hnRNP F, and DAZAP1 could affect Ex17b splicing pattern neither in human nor in mouse. This finding showed that TDP-43 enrolls as the main splicing regulator of Sort1 Ex17b inclusion.

4.5.2 Redundant splicing function of hnRNPs with TDP-43 to inhibit Ex17b inclusion

Immunoprecipitation studies revealed a huge interaction network between TDP-43 and other proteins involved in RNA processing including hnRNPs (Freibaum et al. 2010). After this discovery it became of interest how the association network with hnRNPs could affect splicing functionality of TDP-43. It is predictable that TDP-43 by association and recruiting other

Discussion

hnRNP co-regulates alternative splicing events in order to increase splicing efficiency (McCullough & Berget 1997) (Zhang & Chasin 2004) (Dominguez & Allain 2006).

In fact by now there are a few number studies pointing to co-operative functionality of TDP-43 with other hnRNPs. For example in *Drosophila* TDP-43 interacts with hnRNP A1 to repress inclusion of exon 3 in apolipoprotein A-II (apoAII) transcript (Romano et al. 2014). Also it has been shown that this interaction is important for *Drosophila*'s survival (Romano et al. 2014).

Interaction of TDP-43 and hnRNP A2 is crucial for neuroprotective effects against toxic CGG repeat expansion in the 5' UTR of the fragile X gene (FMR1) (He et al. 2014). Lack of TDP-43 C-terminal region essential for its interaction with hnRNP A2 or knock down of hnRNP A2 abolished the neuroprotective effect of TDP-43 that was further pointing the importance of TDP-43 interaction networks (He et al. 2014).

Finally co-overexpression of TDP-43 with hnRNP U or hnRNP A2 could rescue neuronal cell death induced by high levels of nuclear TDP-43 that activates caspase cascade (Suzuki et al. 2015).

Based on this knowledge and to more explore co-functionality of TDP-43 with hnRNPs, I started to knock down TDP-43 simultaneously with other hnRNPs that I already showed are able to bind Sort1 Ex17b intronic or exonic sequences.

Minigene system in Neuro-2a cells showed efficient co-operation between TDP-43 and hnRNP A1/A2 in order to repress Ex17b. In fact co-depletion of TDP-43 and hnRNP A1/A2 led to higher Ex17b inclusion compare to single TDP-43 knock down. The co-operation between TDP-43 and hnRNP L was even much stronger so that depletion of these two RNA binding proteins together increased the inclusion of human Ex17b to almost 70% compare to the control that was around 30%.

Although using minigene system the increase in Ex17b inclusion was not statistically significant in the case of simultaneous TDP-43 knock down neither with PTB/nPTB nor with DAZAP1, endogenous Ex17b inclusion was dramatically and significantly increased in Neuro-2a cells after the same conditions.

To clear the importance of TDP-43 in inhibiting Sort1 Ex17b in such combinations, I followed co-knock down experiment this time in the presence of TDP-43 to know if any co-depletion of

Discussion

hnRNPs could affect Ex17b splicing. Since the highest inclusion of Ex17b was obtained when TDP-43 was silenced together with hnRNP L both in minigen and endogenous contexts, I sought to know if there is any functional interaction between hnRNP L and hnRNP A1/A2 or PTB/nPTB in repression of Ex17b. Only co-depletion of hnRNP L and PTB/nPTB in the minigene system, could increase mouse (and not human) Ex17b inclusion and no changes were seen in endogenous Sort1 Ex17b inclusion. Moreover, I co-silenced hnRNP A1/A2 together with PTB/nPTB and in this case no increase in Ex17b inclusion was detected neither in minigene nor in endogenous contexts.

Therefore I proved the key splicing role of TDP-43 in repression of Sort1 Ex17b not only independently but also through interaction with other hnRNPs.

4.6 The model presenting binding of splicing regulators to human and mouse Sort1 Ex17b and their functions

All the information obtained by my thesis project is summarized in the schematic diagram shown in Figure 4.1. In this model the recognition site of every detected hnRNP and SR protein is specified. hnRNPs indicated in red including TDP-43, hnRNP A1, hnRNP A2, and hnRNP L negatively control Ex17b splicing both in minigene and endogenous contexts while the ones in black had negative effect only on endogenous Ex17b (DAZAP1 and PTB) or no effect (hnRNP H and hnRNP F).

SRp40/SRSF5 and Tra-2 β that bind only to the mouse upstream intronic region of Ex17b are depicted in green because in my thesis they were recognized as splicing enhancers.

In overall from our proposed model it is clear that the arrangement of RBPs along the evolution has been toward repression of human Ex17b.

Discussion

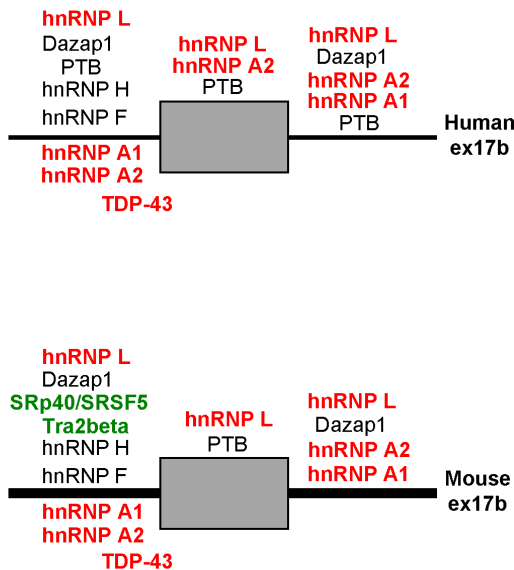


Figure 4.1. Comparison of the splicing regulatory proteins binding to the human and mouse 17b exons.

Schematic models of the human and mouse sortilin Ex17b and the splicing regulatory proteins (hnRNPs and SR proteins) binding to the exons and their flanking introns, as determined by RNA- protein pulldown assays and functional analysis. Splicing negative factors are marked in red font while positive factors are also marked in green font.

4.7 Compensatory expression of hnRNPs in the brain of FTLN patients

The data from my PhD project revealed splicing dysregulation of human Sort1 Ex17b following TDP-43 knock down as well as a number of other hnRNP proteins such as hnRNP A2, hnRNP L, and PTB). Collaborating with Department of Neuroscience, Mayo Clinic Jacksonville in USA, the obtained data from the affected temporal lobes of FTLN-TDP patients were investigated and the findings showed that the expression levels of these RBPs could vary considerably within FTLN-TDP and control individuals.

In addition to prove my obtained data, these findings from the human brains proved that in order to produce the toxic Ex17b included isoform of Sort1 in humans, not only the lack of functional nuclear TDP-43 through aggregation, but also the relative concentration levels of other RBPs as repressive factors are highly important.

Discussion

Of note, the RNA levels of both hnRNP A2 and PTB were significantly upregulated in FTLTDP patients in comparison to control individuals. This fact could reflect activation of some in vivo compensatory mechanisms leading to increase in the expression levels of these splicing repressors trying to counteract the loss of TDP-43 function induced by aggregate formation.

This condition is an approve for the recent study where it has been shown that in NSC-34 cells overexpression of hnRNP U and hnRNP A1/A2 could rescue TDP-43-induced neuronal cell death (Suzuki et al. 2015).

Therefore our findings from the patient brain samples support this general compensatory theory and provide clinical example in which overexpression of hnRNPs following the loss of nuclear TDP-43 function through aggregation may represent a general defense mechanism.

However, the mechanisms through which hnRNP A2 and PTB expression were increased in the temporal lobes of FTLTDP needs to be defined in the future. So far by cross-linking immunoprecipitation (CLIP) assessments, hnRNP A2 and PTB have not been identified as mRNA targets of TDP-43 (Polymenidou et al. 2011), (Tollervey et al. 2011). Using a sort of RNA-immunoprecipitation (RIP) analyses and RNA sequencing it was revealed that possibly TDP-43 could not regulate hnRNP A/B transcripts directly (Sephton et al. 2011). But such data did not ignore the possibility of indirect regulation of TDP-43 through modification of other factors that may affect the transcription or stability of hnRNP A/B and PTB mRNAs.

As reviewed by Volonte et al. (Volonte et al. 2015) it should be also considered that TDP-43 is recognized as a part of Drosha complex and by negative regulation of different miRNA could suppress the expression of other RBPs including hnRNP A2 and PTB.

Thus lack of nuclear TDP-43 due to cytosolic aggregates is not the only reason of pathologic conditions in FTLTDP cases but it is also a result of all the misregulated events controlled by this protein. The results obtained during my PhD project pointed to this complexity and could somehow explain the heterogeneous onset and progression of FTLTDP among different cases. In fact regarding to my data from Ex17b in Sort1 transcript, the extent to which these events will be misregulated also is dependent on the changes in the relative expression levels of other hnRNPs.

4.8 Conclusion

In general data from this project revealed that cis and trans-splicing elements differentially evolved between human and mouse Sort1 Ex17b in a way leading to suppression of this toxic exon in human that is potential to encode a truncated receptor. Though TDP-43 is the main Ex17b splicing repressor, it also co-operates with other hnRNPs in order to more efficient splicing inhibition of Ex17b in Sort1 transcript.

In FTLD-TDP, depending on the misregulated events controlled by TDP-43 including hnRNPs expression levels, the onset and progression of the disease could vary among the patients. Thus a better knowledge of all these eventual connections is expected to have important implications in our understanding of TDP-43 proteinopathies, and represents a very promising area of research for future studies given the therapeutic implications.

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Appendix

Publications related to this Project

Mohagheghi F, Prudencio M, Stuani C, Cook C, Jansen-West K, Dickson DW, Petrucelli L, Buratti E.

TDP-43 functions within a network of hnRNP proteins to inhibit the production of a truncated human SORT1 receptor.

human molecular genetics. 2016, 25(3):534-45.

Gaweda-Walerych K, **Mohagheghi F**, Zekanowski C, Buratti E.

Parkinson's disease- related gene variants influence pre-mRNA splicing processes.

Neurobiology of Aging. 2016, 47:127- 138.

Appocher C*, **Mohagheghi F***, Cappelli S, Stuani C, Romano M, Feiguin F, Buratti E.

Major hnRNP proteins act as general TDP-43 toxicity modifiers both in Drosophila and human neuronal cells.

Nucleic Acids Research, Under Revision.

* Joint First Authors

ORIGINAL ARTICLE

TDP-43 functions within a network of hnRNP proteins to inhibit the production of a truncated human SORT1 receptor

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Abstract

The aggregation and mislocalization of RNA-binding proteins leads to the aberrant regulation of RNA metabolism and is a key feature of many neurodegenerative diseases, including amyotrophic lateral sclerosis and frontotemporal dementia. However, the pathological consequences of abnormal deposition of TDP-43 and other RNA-binding proteins remain unclear, as the specific molecular events that drive neurodegeneration have been difficult to identify and continue to be elusive. Here, we provide novel insight into the complexity of the RNA-binding protein network by demonstrating that the inclusion of exon 17b in the SORT1 mRNA, a pathologically relevant splicing event known to be regulated by TDP-43, is also considerably affected by additional RNA-binding proteins, such as hnRNP L, PTB/nPTB and hnRNP A1/A2. Most importantly, the expression of hnRNP A1/A2 and PTB/nPTB is significantly altered in patients with frontotemporal dementia with TDP-43-positive inclusions (FTLD-TDP), indicating that perturbations in RNA metabolism and processing in FTLD-TDP are not exclusively driven by a loss of TDP-43 function. These results also suggest that a comprehensive assessment of the RNA-binding protein network will dramatically advance our current understanding of the role of TDP-43 in disease pathogenesis, as well as enhance both diagnostic and therapeutic capabilities.

Introduction

Following the discovery of TDP-43 within protein inclusions in patients with frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS), the identification of pathogenic mechanisms triggered by TDP-43 aggregation has been a major research effort hindered by the massive number of RNA processing events regulated by TDP-43 (1,2). Similar to other RNA-binding proteins (RBPs) within the heterogeneous ribonuclear protein (hnRNP) family (3), most of the functional properties displayed by TDP-43 are mediated by binding to specific RNA target sequences (4–7). In addition, given the extensive TDP-43

interactome as indicated by several recent proteomic studies (8,9), including members of the hnRNP protein family, TDP-43 is able to regulate all steps of RNA maturation (transcription, processing, transport, stability and translation) (10). Therefore, the relative expression status of proteins within the TDP-43 interactome could significantly impact TDP-43 function, either through a direct interaction or by acting independently on the same cellular targets. This high degree of complexity may also explain the difficulty associated with pinpointing the specific misregulated events that can be directly linked to disease onset and progression in TDP-43 proteinopathies (11).

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Received: September 28, 2015. Revised and Accepted: November 24, 2015

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Recently, a specific mis-splicing event regulated by TDP-43 that involves mRNA processing of the neurotrophic receptor sortilin 1 (SORT1) within the brain of FTLD-TDP patients was described by us and others (4,12). SORT1 is primarily expressed in neurons and has been shown to regulate the intracellular trafficking of the neurotrophic factor progranulin (PGRN) (13,14). Given that mutations in the PGRN gene (GRN) lead to haploinsufficiency and are a genetic cause of FTLD-TDP, loss of PGRN is associated with disease (15–18), making the regulation of SORT1 expression essential to maintaining PGRN homeostasis. Under normal conditions, TDP-43 heavily represses the inclusion of a specific human SORT1 sequence, exon 17b, that carries a translational stop codon. Loss of TDP-43 promotes inclusion of this exon, leading to the production of a truncated and toxic SORT1 isoform that can bind but not internalize PGRN. Most importantly, the inclusion of this toxic isoform is elevated in FTLD-TDP patients, suggesting a possible link with disease (12). However, the fact that inclusion of exon 17b is not as heavily repressed in species that do not carry a translational stop codon, such as mouse or other rodents (12), indicates the evolution of additional mechanisms to prevent the production of this toxic SORT1 fragment in humans. As TDP-43 binds equally well to a conserved UG-rich region near the 3' splice site (ss) of both human and mouse exon 17b sequences, the existence of other splicing regulatory elements must be present in the human exon 17b to ensure exclusion. In these studies, we identify additional hnRNP splicing factors that work in conjunction with TDP-43 to efficiently repress human exon 17b inclusion and demonstrate that expression of some of these factors is significantly altered in FTLD-TDP patients relative to controls. Overall, these findings indicate that expression of other hnRNP proteins strongly impacts the molecular consequences of decreased nuclear TDP-43 as a result of aggregation in disease.

Results

The splicing of exon 17b upon TDP-43 depletion is determined by the cell context

To study the alternative splicing regulation of human and mouse exon 17b sequences, we used two minigenes that contain either the human or mouse 17b exons flanked by 150 nucleotides of upstream and downstream introns (Fig. 1A). As previously described in human M17 and HeLa cells, TDP-43 binds to UG-repeat elements located near the 3'ss of exon 17b and represses inclusion (12). Here, we extended these analyses to evaluate exon 17b inclusion in a variety of human and mouse neuronal (SK-N-BE, SH-SY-5Y, NSC-34 and Neuro 2a) and non-neuronal cell types (Hep3B and N-mul). Following minigene transfection, the splicing pattern of exon 17b was analyzed by RT-PCR amplification, with specific primers generating either 246-bp and 345-bp bands for exon 17b exclusion and inclusion products, respectively (Fig. 1B and C). As shown in Figure 1, under normal conditions, low levels of exon 17b were detected in all cell lines tested, whether of human (Fig. 1B) or mouse origin (Fig. 1C). Of interest, the splicing pattern for mouse exon 17b was more variable than that of the human, with high levels of mouse exon 17b inclusion in the human neuronal cell lines (SK-N-BE, SH-SY-5Y), and very little inclusion in the human non-neuronal Hep3B cell line. These results are consistent with previous indications that inclusion of human exon 17b is more heavily repressed in neuronal cell lines than its mouse homolog.

To further define the role of TDP-43 in this critical splicing event (12), these analyses were carried out under TDP-43 knock-down conditions following siRNA treatment. Figure 1 shows that human exon 17b inclusion was only minimally affected by

silencing of TDP-43 in most cell lines. The only exception was in Neuro2a cells (see Fig. 4 for quantification of this event), where a statistically significant increase in human exon 17b inclusion was observed. Conversely, TDP-43 silencing had a very positive effect on the inclusion levels of mouse exon 17b with just one exception, in the hepatocellular carcinoma-derived Hep3B cell line (Fig. 1B). Taken together, these data demonstrate that inclusion of the human exon 17b is much more heavily repressed than mouse exon 17b, a conclusion that is in agreement with our previous findings (12). However, the fact that mouse exon 17b inclusion varied considerably depending on the cell line tested (e.g. compare Hep3B and SH-SY-5Y cell lines, Fig. 1B) suggests that additional splicing regulatory factors can modulate this splicing event.

Identification of additional factors that differentially bind human and mouse exon 17b

In order to identify splicing factors that contribute to the regulatory action of TDP-43 on exon 17b splicing, we used a previously described affinity pulldown system that allows the identification of the binding location of such factors in the exon 17b context (12,19–21). The human and mouse exon 17b regions were first split into upstream intron, exon and downstream intron, and these sequences were transcribed separately (Fig. 2). Affinity RBP purification was performed using RNA-coated adipic acid dehydrazide beads, and the ability of the following hnRNP proteins to bind to these regions was tested by western blot: TDP-43, hnRNP H, hnRNP F, PTB (hnRNP I), hnRNP A1, hnRNP A2, DAZAP1, hnRNP L and hnRNP C1/C2. Many of these hnRNP proteins were previously identified as binding factors into the exon 17b context (TDP-43, hnRNP A1, hnRNP A2), as described in our previous publication (12). In addition, we also included the following hnRNPs: (1) hnRNP H/F, due to the fact that the high number of G-repeats in the upstream introns of mouse and human exon 17b represent well-known binding sites for this factor (22), (2) PTB (hnRNP I) since several known binding 'cucc' sequences are present in introns and in the 17b exon (23) and (3) hnRNP L, which may bind to ca-repeats located in the 17b exonic region (24). Although there are no well-known binding sequences for Dazap1, this protein was chosen because it preferentially binds downstream of 5' splice sites and has been described to act in concert with hnRNP A/Bs to regulate alternative splicing (25). Finally, hnRNP C was also tested because it is a very abundant hnRNP protein that binds u-rich sequences (26).

As shown in Figure 2, affinity purification showed that TDP-43 binds to the upstream intronic region of both human and mouse exon 17b sequences. This result is congruent with the presence of a UG-rich region in the intron upstream of exon 17b, as TDP-43 presents high binding affinity to such regions (7,27). Additionally, other hnRNP proteins displayed similar binding patterns to both human and mouse exon 17b contexts (hnRNP H, hnRNP F, hnRNP A1 and hnRNP L) or no binding (hnRNP C1/C2). This was expected based on the high sequence similarity between these human and mouse regions, especially within the exon (see Fig. 1A). However, hnRNP A2 and PTB/nPTB exhibited striking differences in the binding patterns between human and mouse exon 17b contexts. For example, hnRNP A2 showed binding within exon 17b in the human but not in the mouse context, while PTB showed binding to human upstream and downstream introns to a greater extent than the corresponding mouse intronic regions (Fig. 2).

Binding of hnRNP A2 to human 17b exon correlates with splicing repression

To understand the differential binding preference of splicing repressors for the human versus mouse contexts, we took

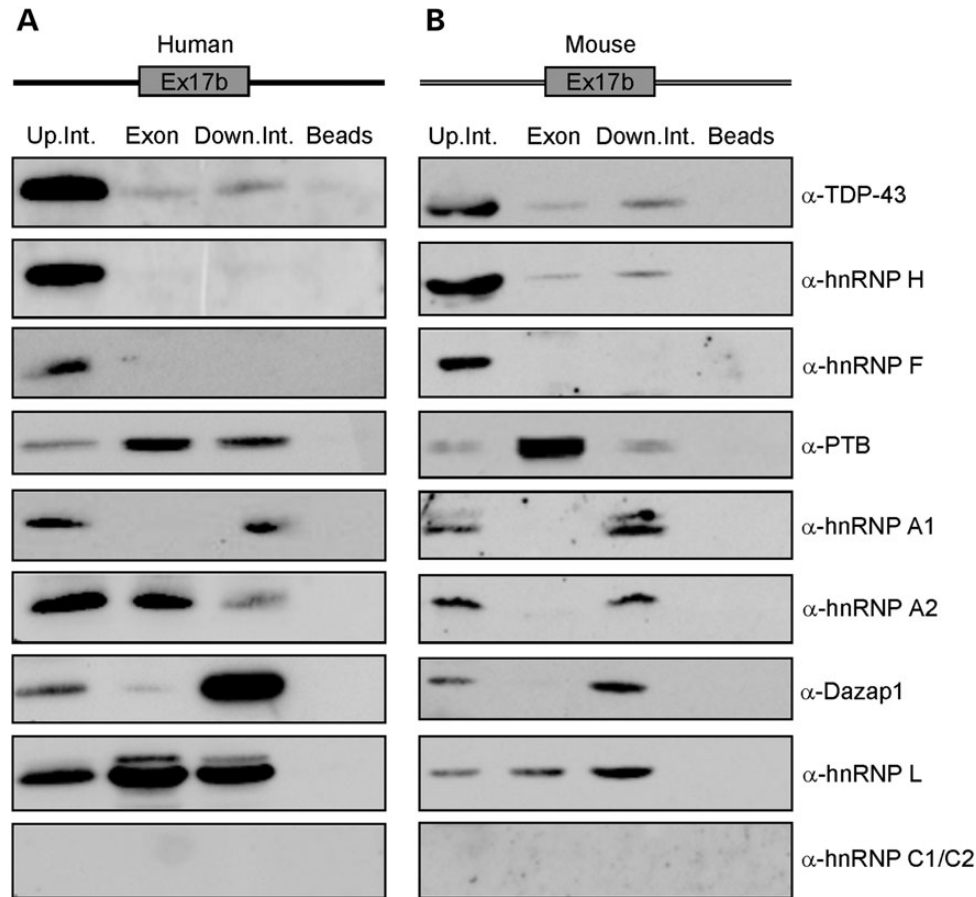


Figure 2. Evaluation of binding efficiency of diverse hnRNPs to human and mouse exon 17b. (A and B) The binding efficiency of several hnRNP proteins was assessed by western blot after pulldown of *in vitro*-transcribed RNAs from human (A) and mouse (B) SORT1 Ex17b exons and their flanking introns.

evaluated levels of exon 17b inclusion following single or double knock down of various hnRNP proteins. While TDP-43 knock down alone was capable of increasing exon 17 inclusion in both human and mouse minigenes (Fig. 4A), single knock down of hnRNP A1/A2, hnRNP L, PTB/nPTB, hnRNP H, hnRNP F and DAZAP1 (Supplementary Material, Fig. S1) had very minimal effect on basal levels of either human or mouse exon 17b inclusion. These results confirm that TDP-43 is indeed the principal repressor of both human and mouse 17b exons.

Next, to investigate the potential co-operative repressive role of hnRNP proteins and TDP-43, we performed a series of hnRNP knock down experiments in conjunction with TDP-43 knock down. Interestingly, knockdown of both hnRNP A1/A2 and TDP-43 significantly increased the level of human and mouse exon 17b inclusion (Fig. 4B). This result is consistent with the presence of a silencer element bound by hnRNP A2 in the human exon 17b that upon mutation abolishes the binding of this protein (Fig. 3). In addition, while no statistically significant increases were observed when TDP-43 was knocked down together with PTB/nPTB (Fig. 4D), hnRNP H/F (Fig. 4E) or Dazap1 (Fig. 4F), we detected a significant increase in exon 17b inclusion when both TDP-43 and hnRNP L were knocked down (Fig. 4C). Based on this finding, we then evaluated whether hnRNP L knock down would achieve similar results when a different hnRNP protein was concomitantly knocked down. We observed a modest increase of human exon 17b inclusion following double knock down of hnRNP L and hnRNP A1/A2 (Fig. 5A and D),

but not when hnRNP L was knocked down with hnRNP PTB/nPTB (Fig. 5B and D). Conversely for mouse exon 17b inclusion, a statistically significant increase was found following hnRNP L and hnRNP PTB/nPTB knock down (Fig. 5B and E), while no effect was observed when hnRNP L was knocked down with hnRNP A1/A2 (Fig. 5A and E). However, for both mouse and human exon 17b inclusion, no effect was observed when hnRNP A1/A2 was knocked down together with hnRNP PTB/nPTB (Fig. 5C–E).

In order to verify that the effects observed when utilizing the minigene system resemble those happening endogenously, we also analyzed exon 17b inclusion of endogenous mouse SORT1 (Fig. 6). Similarly to the results obtained with the minigene system (Fig. 4), knock down of either TDP-43 alone, or in conjunction with hnRNP L or hnRNP A1/A2, was the most efficient combinations in promoting exon 17b inclusion. In conclusion, these findings demonstrate that while TDP-43 plays a central role in repressing human exon 17b inclusion, additional factors within the hnRNP network function with TDP-43 to regulate this critical splicing event.

Identification of a splicing enhancer of exon 17b in the mouse upstream intron

We previously identified two splicing enhancer factors, SRp40/SRSF5 and Tra2 β , which selectively bound to the mouse, but not human, exon 17b context (12). To determine the exact binding

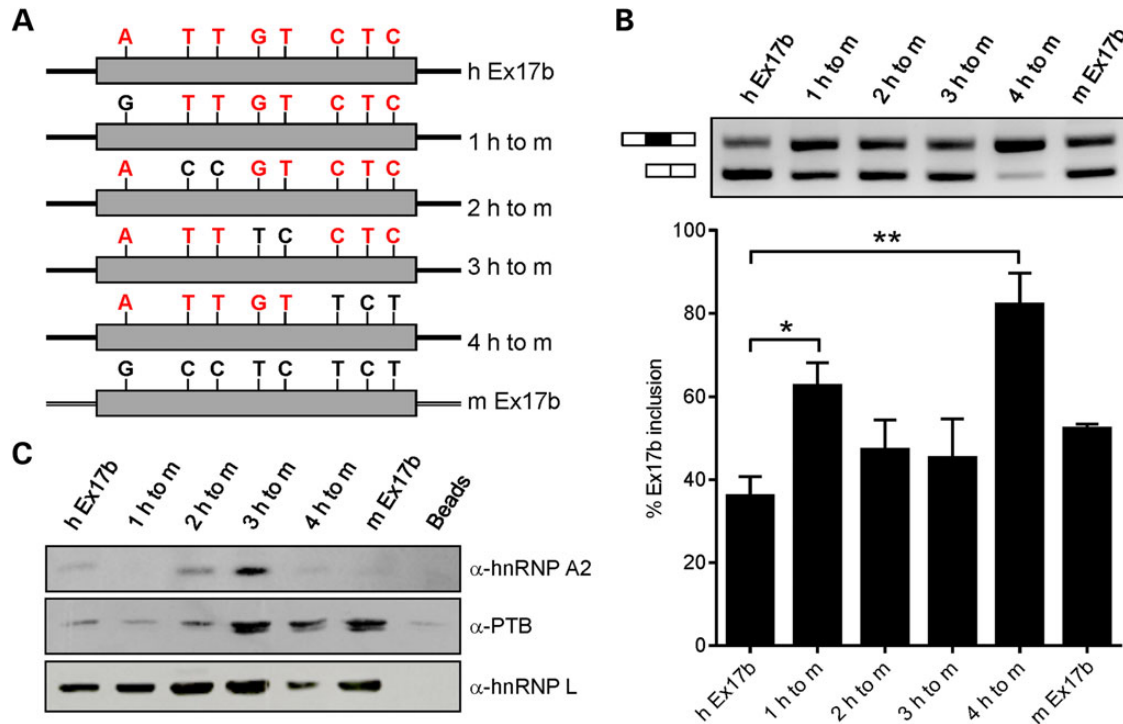


Figure 3. hnRNP A2 binding to the human 17b exon correlates with splicing repression. (A) Schematic diagram of the human to mouse (h to m) hybrid constructs based on the human Ex17b sequence (B) Transfection of the various h-to-m constructs in Neuro2a cells. The RT-PCR samples are stained with ethidium bromide and run on a 1.5% agarose gel. A schematic diagram of the two minigene spliced transcripts (17b inclusion versus 17b skipping) is reported on the left of the agarose gel. The quantified percentage of SORT1 Ex17b inclusion was determined by densitometric analysis using ImageJ software. Statistical differences were calculated by one-way ANOVA test (post-test Tukey). * $P < 0.05$, ** $P < 0.01$ ($n = 3$). (C) *In vitro*-transcribed RNA from wild-type and mutant exonic sequences were incubated with human HeLa nuclear protein extracts to assess their ability to bind the following proteins: hnRNP A2, hnRNP L and PTB.

location for both splicing factors, we performed the same pull-down analysis described in Figure 2. Western blot analyses using antibodies against SRp40/SRSF5 and Tra2 β proteins demonstrate that both factors bind the intron upstream of mouse exon 17b with high efficiency (Fig. 7A). Note, no binding to either human or mouse exon 17b context was found for SRSF1, a negative control. To further pinpoint the region within the upstream intron of mouse exon 17b where these splicing enhancers bind, we created three human to mouse mutants in which we replaced the 150-bp human intron with selected mouse intronic regions: mutant H1 (1–43), mutant H2 (43–111) and mutant H3 (110–150) (Fig. 7B). Mutants were then transfected in Neuro 2a cells and TDP-43 was knocked down by siRNA treatment. As shown in Figure 7C, depletion of TDP-43 resulted in significantly increased exon 17b inclusion in the H2 mutant, suggesting that the 43–111 nucleotide replacement had introduced an enhancer element in the human context.

The presence of SR-binding splicing enhancer elements in intronic sequences is a rather rare but a previously described phenomenon for some genes, such as β -tropomyosin exon 6A (29), CT/CGRP gene exon 4 (30) and Tra2 α (31). While in these genes the enhancer sequences are present in the downstream intron of the affected exonic region, in Sort1 the enhancer sequence is localized in the upstream intron. Therefore, in order to better define the properties of this upstream enhancer element, we overexpressed the SRp40/SRSF5 and Tra2 β proteins in Neuro2a cells and determined their effect on the inclusion of the endogenous mouse exon 17b. This was performed both under normal conditions (Supplementary Material, Fig. S2A) and following TDP-43 siRNA knockout (Supplementary Material,

Fig. S2B). Interestingly, SRp40/SRSF5 had the expected effect of promoting mouse exon 17b inclusion. However, this occurred only in the absence of TDP-43. In contrast, overexpression of Tra2 β had no effects on inclusion in both conditions. Moreover, no effect could be observed following overexpression of other SR factors (SRp75/SRSF4 and SC-35/SRSF2), which do not bind within the exon 17b context (12) and were used as negative controls. Finally, to further clarify whether Tra2 β can play a role in exon 17b splicing, we silenced this factor in Neuro2a cells. As shown, in Supplementary Material, Figure S3, silencing of Tra2 α/β could successfully lower endogenous mouse exon 17b inclusion in the absence of TDP-43.

All these findings are summarized in the schematic diagram shown in Figure 8, in which the binding location and effect on exon 17b inclusion is depicted by font colour for the various hnRNP factors (no effect illustrated by black font, while positive or negative influence is depicted by either green or red font, respectively).

Altered expression of hnRNP proteins in the brain of FTLT-TDP patients

In order to determine whether a change in hnRNP protein expression might be associated with FTLT-TDP, we evaluated expression levels in the temporal cortex, a brain region in which we previously observed a significant increase in exon 17b inclusion in FTLT-TDP patients (12). Using quantitative RT-PCR (qRT-PCR), we detected a significant increase in the splicing repressors HNRNP A2B1 (hnRNP A1/A2) and PTBP1 (PTB) in FTLT-TDP patients relative to controls (Fig. 9). In addition, consistent with

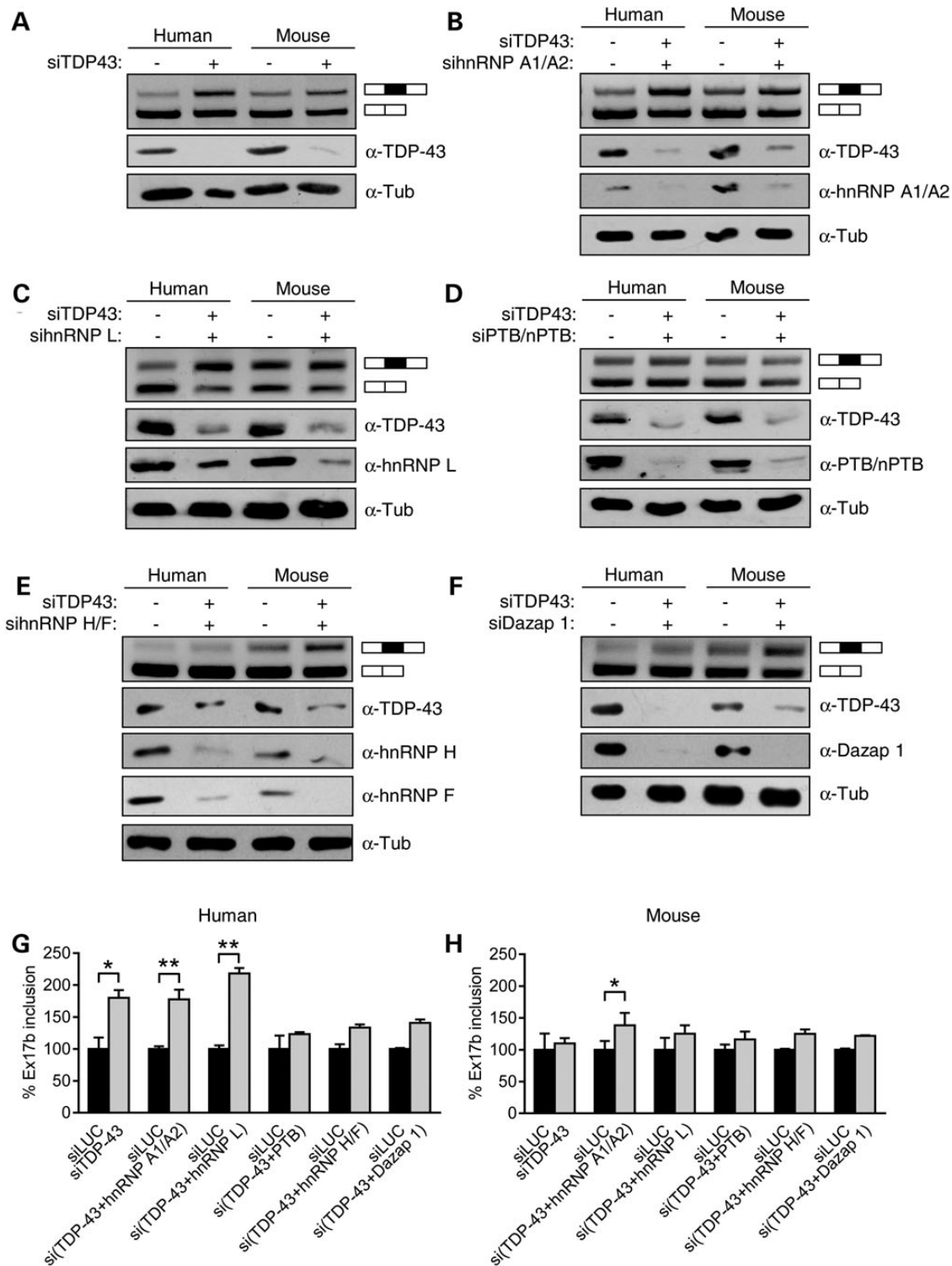


Figure 4. Cooperation of TDP-43 and hnRNP L or hnRNP A2 to repress SORT1 Ex17b inclusion in the human and mouse minigene systems. (A) TDP-43 knockdown, alone or simultaneously with hnRNP A1/A2 (B), hnRNP L (C), PTB/nPTB (D), hnRNP H/F (E) or DAZAP1 (F) in Neuro 2a cell line. RT-PCR samples are stained with ethidium bromide and run on a 1.5% agarose gel. A schematic diagram of the two minigene spliced transcript (17b inclusion versus 17b skipping) is reported on the right of each agarose gel. Luciferase siRNA-treated cells have been used as a control and depletion efficiency was determined by western blot assay (extracts have been normalized using tubulin) (G and H). The quantified percentages of human (G) and mouse (H) Ex17b inclusion were determined by densitometric analysis using ImageJ software. Statistical differences were calculated by one-way ANOVA test (post-test Tukey) * $P < 0.05$, ** $P < 0.01$ ($n = 3$).

previous reports, no significant differences were detected in TARDBP expression levels between FTLD-TDP cases and controls. Taken together, these findings indicate that increased expression

of the splicing repressors hnRNP A1/A2 and PTB may represent a compensatory response to the loss of nuclear TDP-43 function as a result of aggregation in disease.

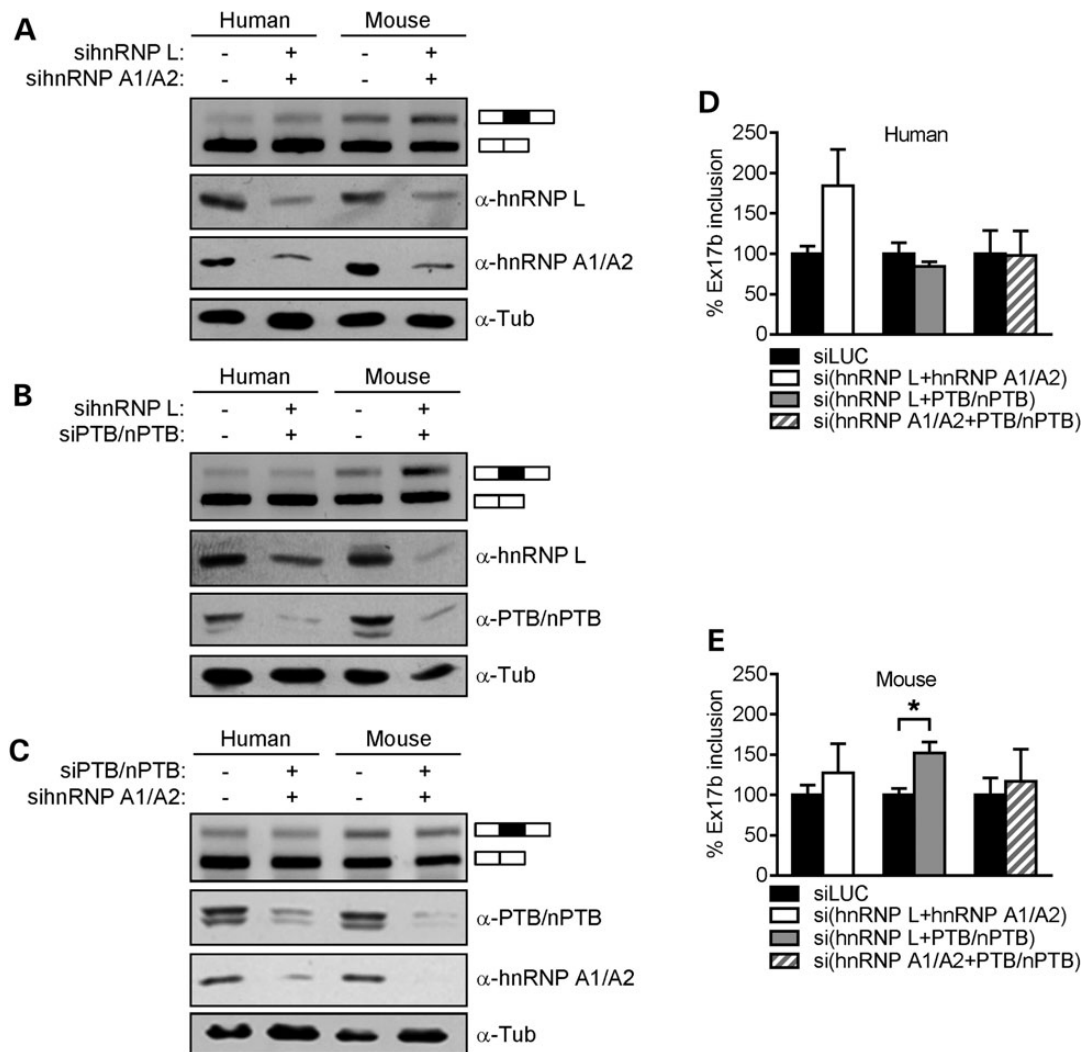


Figure 5. Effects on human and mouse exon 17b recognition following knock down of hnRNPs in the presence of TDP-43. The following knockdowns were performed: hnRNP L together with hnRNP A1/A2 (A), PTB/nPTB (B) or PTB/nPTB with hnRNP A1/A2 in Neuro 2a cell line. RT-PCR samples are stained with ethidium bromide and run on a 1.5% agarose gel. A schematic diagram of the two minigene spliced transcript (17b inclusion versus 17b skipping) is reported on the right of each agarose gel. Luciferase siRNA-treated cells have been used as a control, and depletion efficiency was determined by western blot assay (extracts were normalized using tubulin). (D and E) The quantified percentage of human (D) and mouse (E). Ex17b inclusion was determined by densitometric analysis using ImageJ software. Statistical differences were calculated by one-way ANOVA test (post-test Tukey) * $P < 0.05$ ($n = 3$).

Discussion

The eukaryotic genome encodes hundreds of RBPs that jointly co-ordinate and regulate all aspects of the life cycle of every RNA produced within cells, including transcription, capping, pre-mRNA splicing, editing, polyadenylation, transport, stability and translation (32). Among all these different events, the regulation of pre-mRNA splicing often involves an intricate network of RBPs that bind to particular sequences near or within the exon to be regulated (33,34). The concentration of these factors within different cells is one of the key elements that will determine whether a particular sequence is eventually included in the mature mRNA. Thus, given the importance of the RBP network in maintaining overall RNA processing and metabolism, it is intriguing that mutations or altered expression of RBPs are increasingly being shown to play an active role as causative agents of Mendelian disease in humans (35). In neurodegenerative diseases, insight into the role(s) played by RBPs in determining disease onset and progression has been provided by the discovery that alterations in genes involved in RNA/DNA metabolism are

associated with FTD and ALS (36). In addition, alterations in RNA metabolism caused by misregulation of RBP expression are considered to be very damaging to neuronal function as described in recent reviews (37–41) and observational studies (42–45) on this subject. Therefore, the combined study of RBPs and RNA metabolism is expected to advance the field of neurodegenerative diseases in the near future (46).

Regarding this topic, one specific issue that needs to be addressed is how various hnRNP proteins may differentially contribute to neurodegeneration. In this work, we show that aberrant splicing of human exon 17b in the SORT1 gene is dependent on both TDP-43 as well as a number of other hnRNP proteins (hnRNP A2, hnRNP L, PTB), whose expression levels can vary considerably within FTLD-TDP and control individuals. These findings indicate that in order to produce this toxic exon 17b-containing isoform, not only TDP-43 needs to be removed from the nuclear environment through aggregation but also the relative concentration levels of these other repressive factors need to be decreased. Interestingly, the levels of both hnRNP A2 and PTB/nPTB are significantly

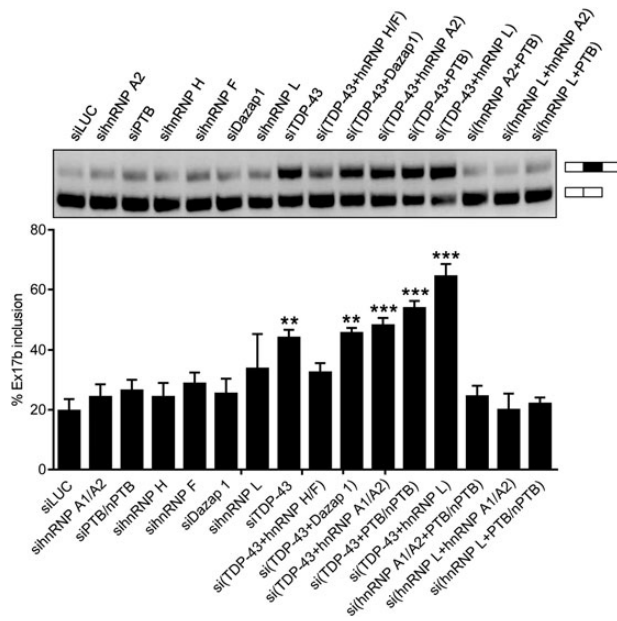


Figure 6. Effects of knocking down diverse hnRNPs on endogenous mouse Sort1 Ex17b in Neuro 2a cells. cDNA prepared from the extracted RNA was subjected to PCR using primers in exons 17 and 19 of mouse Sort1. RT-PCR samples are stained with ethidium bromide and run on a 1.5% agarose gel. A schematic diagram of the two minigene spliced transcript (17b inclusion versus 17b skipping) is reported on the right of the agarose gel. The quantified percentage of Sort1 Ex17b inclusion was determined by densitometric analysis using ImageJ software. Statistical differences were calculated by one-way ANOVA test (post-test Tukey). ** $P < 0.01$, *** $P < 0.001$ ($n = 3$).

upregulated in FTLD-TDP patients with respect to controls, suggesting the activation of *in vivo* compensatory mechanisms which lead to an increase in the expression of these splicing repressors to try and counteract the loss of TDP-43 function. This situation is in keeping with recent data demonstrating that overexpression of hnRNP U and hnRNP A1/A2 can inhibit TDP-43-induced neuronal cell death in NSC34 cells (41). Similarly in *Drosophila*, the ability of TDP-43 to suppress CGG RNA-associated phenotypes is dependent on its ability to interact with two fly homologs of hnRNP A2/B1, and overexpression of these factors could attenuate TDP43-induced cell death (41,47,48). Thus, our findings support this general view and provide a specific example through which overexpression of hnRNP proteins may represent a general defense mechanism following the loss through aggregation of RBPs, such as TDP-43. A key question that will need to be determined in future studies is the mechanism through which this increase in hnRNP A/B and PTB expression occurs. The fact that CLIP analyses have not identified these proteins as mRNA targets of TDP-43 (4,6), with the only exception possibly represented by the hnRNP A/B transcript according to RIP-seq (49), suggests that TDP-43 may not be able to directly repress the transcription of these factors. However, these results do not exclude the possibility that loss of TDP-43 may still lead to alterations in other factors that influence the transcription or stability of hnRNP A/B and PTB mRNAs. Another possibility stems from the fact that TDP-43 has been described to be part of the Drosha complex and negatively alter the expression of several miRNAs, recently reviewed by Volonte *et al.* (50), which might repress the expression of these other hnRNP factors.

The results presented in the current study could also explain differences in disease onset and progression among patients. In fact, it is quite conceivable that the presence of TDP-43 aggregation in FTLD-TDP patients will lead to misregulation of many events

controlled by this protein. However, as shown by our exon 17b results, the extent to which these events will be misregulated also depends on changes in the relative expression levels of other hnRNPs. In this respect, it is also interesting to note that ALS patients have been described to present loss of hnRNP A1 expression in motor neurons that is concomitant with TDP-43 cytoplasmic inclusions, which may represent an additional disturbance in the control of RNA metabolism within these cells (51). As a result, different hnRNP expression levels, which may depend on individual or age-related differences, on top of similar TDP-43 pathologies could potentially account for a different disease course, with regards both to onset and progression. Therefore, a better knowledge of all these eventual connections is expected to have important implications in our understanding of TDP-43 proteinopathies and represents a very promising area of research for future studies given the therapeutic implications.

Materials and Methods

Hybrid minigene constructs

Human and mouse SORT1 exon 17b minigene constructs (hEx17b, mEx17b, 1 h to m, 2 h to m, 3 h to m, 4 h to m) have been previously described (12). Further modifications of human SORT1 Ex17b upstream intron (H1, H2, H3) were generated by PCR-directed mutagenesis with specific primers using human Ex17b plasmids as templates. The products were subcloned inside the NdeI restriction site of the pTB plasmid. Primer sequences for the making of each mutant can be provided upon request.

Cell culture; silencing and transfection

All human (SK-N-BE and SH-SY-5Y, and Hep3B) and mouse (NSC-34, Neuro 2a and N-mu1) cell lines were cultured in DMEM–Gluta-max-1 (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) and Antibiotic-Antimycotic-stabilized suspension (Sigma) in standard conditions. The siRNA sense sequences from Sigma used for silencing the different target proteins were the following: human TDP-43: gcaagccaagaagagccu, mouse TDP-43: cgaugaacc-auugaaaua, hnRNP-A1: cagcugaggaagcucuua, hnRNP-A2: ggaa-caguuccgaaagcuc, PTB-P1: aacuuccaucauuccagagaa, PTB-N1: gagaggauccugacgaacua, hnRNP L: uauggcuuggaucaauca, Tra2 α : ggaucucugagaagauuu, Tra2 β : ggaggauacagacacguu, Luciferase (control): uaaggcuagaagagauac, from Dharmacon: Dazap1: gaga-cucugcgagcuacu and hnRNP H: ggaaauagcugaaagcu, and from Ambion: hnRNP F (the sequence is not provided). Each siRNA was transfected according to manufacturer's instructions. One day after silencing, cells were transfected by the minigenes mainly using Effectene (Qiagen) or by Lipofectamine 2000 (Invitrogen) in the case of mouse neuroblastoma cell lines (Neuro 2a and NSC-34) following the manufacturer's instructions. For overexpression experiments, the relevant plasmids [(SRp75/SRSF4, SC-35/SRSF2), Tra2 β and SRp40/SRSF5] were transfected in Neuro 2a cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. After 20 h post-transfection, cells were collected and divided into two parts: from the first part total-protein extracts were obtained by cell sonication in lysis buffer (1xPBS and 1x Protease inhibitor cocktail, Roche) in order to confirm the gene knock-down efficacy. Tubulin was used as total protein loading control. The other half part of the cells was used for RT-PCR analysis.

RNA extraction and RT-PCR analysis

RNA was obtained using Eurogold Trifast (Euroclone), as per the manufacturer's instructions. One microgram of total RNA was

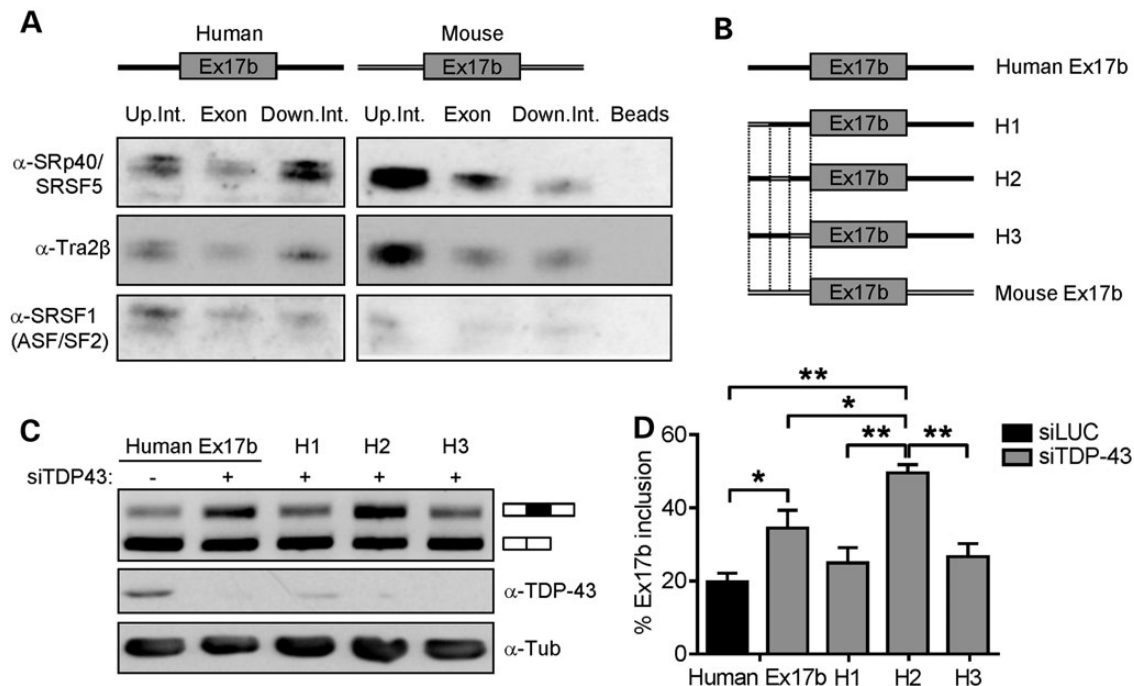


Figure 7. Identification of a splicing enhancer of Ex17b in the mouse upstream intron. (A) Pull-down analysis performed for human and mouse Ex17b exons and their flanking introns (Up. and Down Int.). Bound proteins were then tested for binding of the following factors: SRp40/SRSF5, Tra2 β and SRSF1(ASF/SF2). (B) Schematic representation of hybrid human Ex17b minigenes constructed to assess the importance of mouse upstream intron in inclusion of Ex17b. (C) Hybrid constructs were expressed in Neuro 2a cells after TDP-43 knockdown. RT-PCR samples are stained with ethidium bromide and run on a 1.5% agarose gel. A schematic diagram of the two minigene spliced transcript (17b inclusion versus 17b skipping) is reported on the right of the agarose gel. Luciferase siRNA treated cells were used as a control and correct depletion was determined by western blot assay (extracts were normalized using tubulin). (D) The quantified percentage of SORT1 Ex17b inclusion for each construct was determined by densitometric analysis using ImageJ software. Statistical differences were calculated by one-way ANOVA test (post-test Tukey). * $P < 0.05$, ** $P < 0.01$ ($n = 3$).

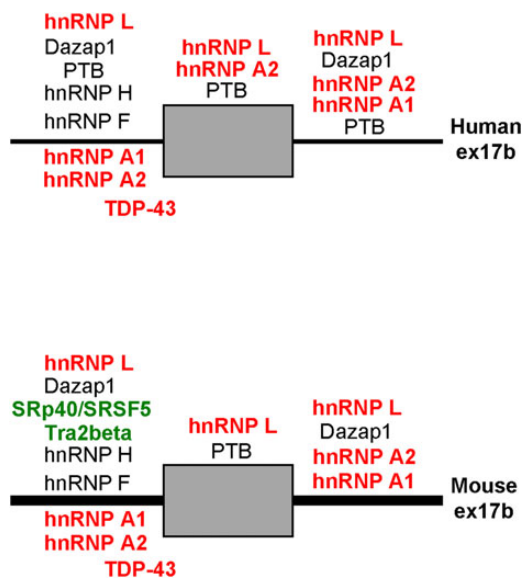


Figure 8. Comparison of the splicing regulatory proteins binding to the human and mouse Ex17b exons. Schematic models of the human and mouse sortilin Ex17b exons and the splicing regulatory proteins (hnRNPs and SR proteins) binding to Ex17b exons and their flanking introns, as determined by our RNA-protein pull-down assays and functional analysis. Negative factors are marked in red font while positive factors are also marked in green font.

used in the retrotranscription reaction with random primers and Moloney murine leukemia virus (M-MLV) Reverse Transcriptase (Invitrogen). Spliced products from the transfected

minigene were obtained using primers Bra2 5' taggatccgggtcac-caggaagtgtggttaaatca 3' and α 2-3 5' caacttcaagctcctaagccactgc 3'. PCR conditions were the following: 94°C for 2 min; 94°C for 30 s, 56°C for 1 min and 72°C for 45 s for 35 cycles and 72°C for 10 min for the final extension. PCRs were optimized to be in the exponential phase of amplification, and products were routinely fractionated in 1.5% (wt/vol) agarose gels. Moreover, the PCR cycling conditions described above and used to detect spliced products from the transfected minigene have been also used to detect endogenous mouse SORT1 product in Neuro 2a cell line using two other primers including: 5' caaatgccaaggtgggat-gaa 3' and 5' ttgaatccaaagcctctacgcc 3' on exons 17 and 19, respectively.

In vitro transcription

RNA templates have been obtained in two different ways. For the experiments reported in Figures 2 and 6, the sequence of interest was cloned in pBluescript KS+ plasmid (Stratagene) that contains a T7 promoter. In this case, the 5' end of the sequence to be transcribed is placed as close as possible to the end of the T7 promoter, and a restriction enzyme site for BamH1 is present at its 3' end to linearize the plasmid. This is to minimize the length of plasmid-related RNA that will eventually be transcribed together with the sequence of interest. On the other hand, for Figure 3, the h-to-m mutants RNA was transcribed from PCR templates amplified from the respective plasmids. A T7 promoter sequence was added towards the 5' end of the template using primers that carried a T7 sequence. For transcription, 5 μ g of DNA template was used in a 60 μ l T7 polymerase (Stratagene) transcription reaction for each sample. The synthesized RNA

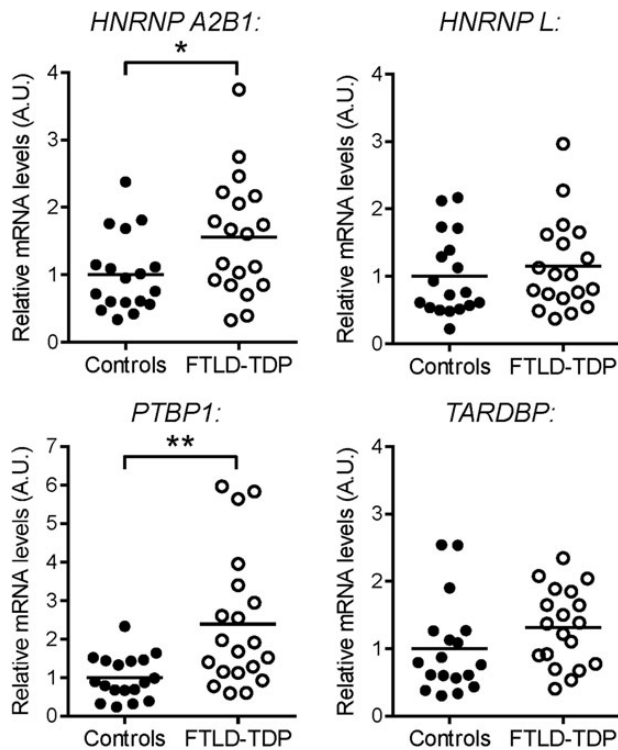


Figure 9. The RNA levels of several hnRNP proteins are altered in the brain of FTLD-TDP patients. Transcript levels of several hnRNPs measured by qRT-PCR in human FTLD-TDP cases ($n=20$) and age-matched controls ($n=19$) and normalized to GAPDH RNA levels. Statistical differences were assessed by Student's *t*-test (* $P < 0.05$, ** $P < 0.005$).

was then purified using standard Acid-Phenol purification method followed by Ethanol precipitation.

Pulldown experiments and western blot analysis

A total of 15 μ g of transcribed RNA was oxidized in the dark for an hour with sodium *m*-periodate in a 400 μ l reaction mixture (100 mM sodium acetate pH 5.2 and 5 mM sodium *m*-periodate). RNA was then ethanol precipitated and resuspended in 200 μ l of 100 mM sodium acetate. Approximately 350 μ l of prewashed equilibrated adipic acid dehydrazide-agarose beads (50% slurry; Sigma) were added to each oxidized RNA volume and placed in the rotor at 4°C for overnight incubation. This RNA-Bead covalent link formation was also performed in the dark.

The beads with the bound RNA were then pelleted 5' 4000 rpm, washed two times with 1 ml of 2 M NaCl and equilibrated in washing buffer (20 mM HEPES pH=7.5, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 6% glycerol). They were then incubated on a rotator with ~1 mg of HeLa cell nuclear extract (Cilbiotech) for 30 min at room temperature, in 400 μ l final volume in a 1 $\times$ binding buffer (200 mM HEPES pH=7.5, 2 mM EDTA, 5 mM DTT, 60% glycerol, this is 10 $\times$ binding buffer), plus 100 mM KCl, heparin was added to a final concentration of 5 mg/ml. The beads were then pelleted by centrifugation at 4000 rpm for 5 min at room temperature and washed four times with 1.5 ml of washing buffer, before addition of SDS sample buffer and loading on a 10% SDS-PAGE gel.

The gel was then electroblotted onto a Nitrocellulose blotting membrane according to standard protocols (Amersham Biosciences) and blocked with 5% skimmed milk (non-fat dry milk in 1 $\times$ PBS and 0.1% Tween-20). Membranes targeted for SR protein recognition were blocked using western blocking reagent (Roche).

Proteins were probed with different antibodies and detected with a chemiluminescence kit (ECL; Pierce Biotechnology).

Primary antibodies against TDP-43, hnRNP H, hnRNP F, hnRNP A1, hnRNP A2, hnRNP C1/C2, Dazap1, PTB and tubulin were already available in-house. The hnRNP L antibody was purchased from Abcam, SRp40/SRSF5 and SRSF2 antibodies were obtained from Invitrogen, and anti-Tra2 β was a generous gift from G. Dreyfuss and I.C Eperon. Rabbit and mouse secondary antibodies were purchased from Dako.

Human sample collection and qRT-PCR analysis

RNA was available from our previous study (12) and was used to determine the mRNA levels of indicated RBPs by qRT-PCR, following previously described procedures (12). Briefly, cDNA (diluted 1:40) from temporal cortex of 20 FTLD-TDP and 19 age-matched controls (refer to (12) for detailed list of cases) was used for qRT-PCR in an HT7900 analyzer (Applied Biosystems). Each 5 μ l reaction contained: 2 μ l of cDNA diluted 1:40, 0.5 μ l primer mix (1 μ M for each primer) and 2.5 μ l SYBR green (Invitrogen). The qRT-PCR program was as follows: 50°C 2 min, 95°C 2 min, 40 cycles of 95°C 15 s and 60°C 1 min. mRNA values were normalized to either GAPDH or RPLP0 values, endogenous transcript controls, resulting in similar values. Note mRNA levels of the different transcripts presented in Figure 9 were normalized to GAPDH. The sequences of the primer pairs used for qRT-PCR are as follows: hnRNP A2B1 5' ttgtgggagtgctataatgg 3' and 5' ccataaccggggctactc 3'; DAZAP1 5' ccagactccctatgtgtca 3' and 5' gcctgaccataagactgtaaacaa 3'; hnRNP L 5' agcggctcaagactgacaa 3' and 5' gtgcgggtcatcgtagtct 3'; PTBP1 5' atgacaagagccgtgactacac 3' and 5' gagaggctgagattaccaggtg 3'; GAPDH 5' gttcgacagtcagccgcac 3' and 5' ggaatttgccatgggtgga 3'; RPLP0 5' tctacaaccctgaagtgtctgat 3' and 5' caatctgcagacagacactgg 3'.

Statistical analysis

The quantified percentage of Sort1 Ex17b inclusion was determined by densitometric analysis using ImageJ software. Statistical differences were calculated by one-way ANOVA test (post-test Tukey). $P < 0.05$ was considered significant ($n = 3$). Statistical differences in transcript levels between FTLD-TDP cases ($n = 20$) and controls ($n = 19$) were calculated by Student's *t*-tests. $P < 0.05$ was considered significant (* $P < 0.05$, ** $P < 0.005$).

Supplementary Material

Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

Funding

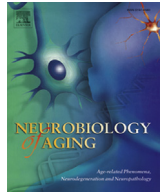
This work was supported by Thierry Latran Fondation (RE-HNPALS) and the EU Joint Programme-Neurodegenerative Diseases JPNB (RiMod-FTD, Italy, Ministero della Sanita') to E.B., National Institutes of Health/National Institute of Neurological Disorders and Stroke (R01 NS077402, P01 NS084974) and National Institute of Environmental Health Services (R01 ES020395) to L.P. and The ALS Association (771DOO) to M.P.

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Parkinson's disease–related gene variants influence pre-mRNA splicing processes

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ARTICLE INFO

Article history:

Received 8 May 2016

Received in revised form 13 July 2016

Accepted 20 July 2016

Available online 28 July 2016

Keywords:

LRPPRC variants
Parkinson's disease
Splicing
Minigene assay
MPP+

ABSTRACT

We have analyzed the impact of Parkinson's disease (PD)–related genetic variants on splicing using dedicated minigene assays. Out of 14 putative splicing variants in 5 genes (*PINK1*, [PTEN induced kinase 1]; *LRPPRC*, [leucine-rich pentatricopeptide repeat containing protein]; *TFAM*, [mitochondrial transcription factor A]; *PARK2*, [parkin RBR E3 ubiquitin protein ligase]; and *HSPA9*, [heat shock protein family A (Hsp70) member 9]), 4 *LRPPRC* variants, (IVS32–3C>T, IVS35+14C>T, IVS35+15C>T, and IVS9+30A>G) influenced, pre-messenger RNA splicing by modulating the inclusion of the respective exons. In addition, 1-Methyl-4-phenylpyridinium ion–induced splicing changes of endogenous *LRPPRC* messenger RNA, reproduced the effect of the *LRPPRC* IVS35+14C>T mutation. Using silencing and overexpression methods, we show that *LRPPRC* exon 33 splicing is negatively regulated by heterogeneous nuclear ribonucleoprotein A1 both in a minigene and endogenous context. Furthermore, exon 33 exclusion due to PD-associated mutation IVS32–3C>T or heterogeneous nuclear ribonucleoprotein A1 overexpression and exon 35 exclusion due to IVS35+14C>T can be rescued by co-expression of modified U1 small nuclear RNAs, providing a potentially useful therapeutic strategy. Our results indicate for the first time that *LRPPRC* intronic variants can affect normal splicing of this gene and may influence disease risk in PD and related disorders.

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1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, defined by cardinal motor features: tremor, rigidity, bradykinesia, and postural instability (Samii et al., 2004). Both genetic and environmental factors are thought to contribute to its pathogenesis (Polito et al., 2016). To date approximately 10% of PD cases arise from mutations in known genes, whereas the remaining cases are idiopathic. Although many variants within splicing regions have been identified in PD patients, very rarely their function has been addressed (Cruts et al., 2012; Samaranch et al., 2010). In this article we analyze functionally selected intronic or synonymous exonic variants identified in PD patients in genes coding for PTEN induced kinase 1 (*PINK1*), heat shock protein family A (Hsp70) member 9 (*HSPA9*), leucine-rich pentatricopeptide repeat containing protein (*LRPPRC*), mitochondrial transcription factor A (*TFAM*), and parkin RBR E3 ubiquitin protein ligase (*PARK2*) (Biswas et al., 2010; De Mena et al., 2009; Gaweda-Walerych et al., 2010; Rakovic et al., 2011; Samaranch et al., 2010; Valente et al., 2004; Zou et al., 2004). All the analyzed variants

encode proteins related to mitochondrial maintenance. Mitochondrial dysfunction has been implicated in both sporadic and genetically caused PD, as reviewed by (Gaweda-Walerych and Zekanowski, 2013b). More specifically, *PINK1* along with parkin are crucial for mitochondrial biogenesis, transport, and dynamics, whereas *TFAM* is responsible for mitochondrial DNA (mtDNA) replication, transcription, and packaging, as reviewed by (Gaweda-Walerych and Zekanowski, 2013a). *HSPA9*-encoded mortalin aids in (re)folding of mitochondrial polypeptides (Deocaris et al., 2006). Its levels are downregulated in PD brains, animal and cellular PD models (Chiasserini et al., 2011; Jin et al., 2006; Shi et al., 2008) and correlate with PD progression (Shi et al., 2008).

The *LRPPRC* localizes predominantly to mitochondria where it stabilizes mitochondrial messenger RNAs (mRNAs), promotes their polyadenylation, and coordinates mitochondrial translation (Chujo et al., 2012; Mili and Pinol-Roma, 2003; Ruzzenente et al., 2012). Mutations in *LRPPRC* cause the French-Canadian type of Leigh syndrome (LSFC), an autosomal, recessive, neurodegenerative disease with the onset in infancy (Mootha et al., 2003) and presence of parkinsonian features (Cooper et al., 2006). At the molecular level, PD and LSFC share deficiency of mitochondrial complex I and IV (Mootha et al., 2003; Mourier et al., 2014; Sasarman et al., 2015; Xu et al., 2004). For this reason, Rakovic et al. sequenced all the 38 exons

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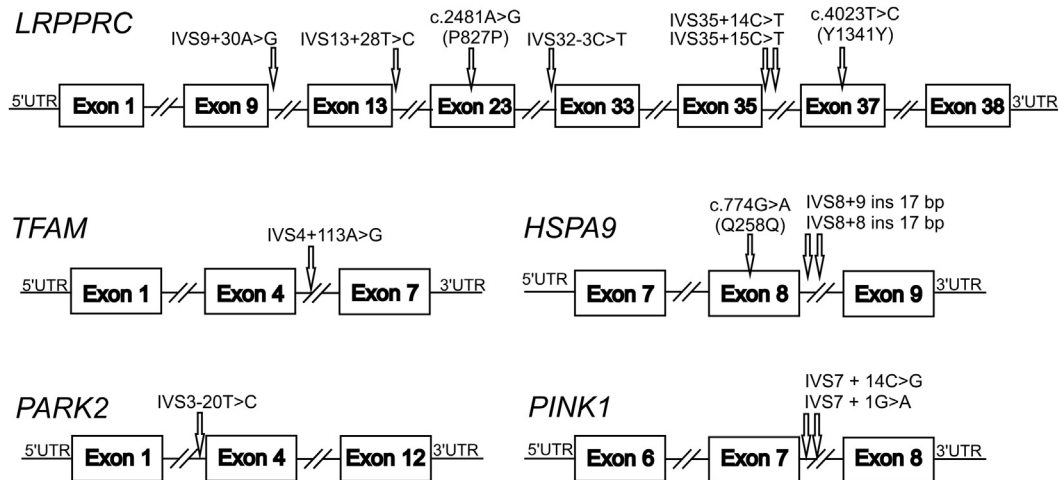


Fig. 1. Schematic representation of the analyzed variants reported in PD patients in the 5 PD-related genes, *LRPPRC*, *TFAM*, *HSPA9*, *PARK2*, and *PINK1*. Exonic sequences are boxed, introns are lines, whereas the arrows indicate the position of genomic variants relative to the splice sites. Abbreviations: *HSPA9*, heat shock protein family A (Hsp70) member 9; *LRPPRC*, leucine-rich pentatricopeptide repeat containing; *PARK2*, parkin RBR E3 ubiquitin protein ligase; *PINK1*, PTEN induced kinase 1; *TFAM*, mitochondrial transcription factor A.

with flanking intronic regions of *LRPPRC* gene in 46 PD patients, revealing over 20 variants (Rakovic et al., 2011) from which we have chosen 7 for further analysis in minigene assay (Fig. 1).

Proteomic analyses showed that mortalin, *LRPPRC*, and *TFAM* interact with some of the well-known PD-related proteins such as *PINK1*, parkin, and DJ-1, indicating that they operate within the same molecular pathway, as reviewed by (Gaweda-Walerych and Zekanowski, 2013b). Thus, it is possible that variants influencing splicing in the previously mentioned genes could ultimately lead to deregulation of various mitochondrial parameters and contribute in this way to PD pathogenesis.

All the variants analyzed in this article can potentially change splicing regulatory sequences such as 5' and 3' splice site (5'ss, 3'ss, respectively) or exonic splicing enhancers and silencers leading to aberrant splicing pattern. Our goal was to determine, using minigene assays, whether these mutations influence pre-mRNA processing, for example, through increased exclusion of the analyzed exon or creation of aberrant mRNA isoforms (Baralle and Baralle, 2005; Baralle et al., 2009). Such events could lead to changed mRNA or protein properties or even result in protein depletion.

Our second aim was to pinpoint major regulatory-splicing factors regulating splicing of the exons influenced by the analyzed mutations, through silencing and overexpression assays.

Finally, we also outline possible new therapeutic venues for rescuing mutation-related aberrant splicing through the expression of adapted U1 small nuclear RNAs (U1 snRNAs) (Fernandez Alanis et al., 2012). The U1 snRNAs along with specific U1 proteins form the U1 small nuclear ribonucleoprotein complex crucial for recognizing the 5' splice site during the first step of the splicing reaction. It is the 5'tail of U1 snRNA that interacts with the 5'ss through its 9-bp tail and recruits the spliceosome machinery on the exon (Aebi et al., 1986). Recently, a novel strategy to correct exon skipping has been developed based on adapted U1 snRNAs with a 5' tail sequence modified so that it is 100% complementary to exon-intron boundary or intronic sequences downstream of canonical donor splice site (Fernandez Alanis et al., 2012). Their overexpression in vitro can successfully correct not only 5'ss mutations where wt U1snRNAs naturally bind but also 3' acceptor splice site mutations (Fernandez Alanis et al., 2012).

2. Materials and methods

2.1. Selection of the variants for the analysis

The variants have been selected based on their association with PD risk (association studies, mutational screening), the lack of detailed functional analysis of their potential influence on splicing (except for *PINK1* IVS7+1G>A for which we try rescue approach), and they have usually higher frequency than in control groups or database (Biswas et al., 2010; Cruts et al., 2012; De Mena et al., 2009; Gaweda-Walerych et al., 2010; Rakovic et al., 2011; Valente et al., 2004). For bioinformatic analysis of splicing impact, we used Human Splicing Finder (Desmet et al., 2009) and Clone Suite Manager v. 9.0.

2.2. Hybrid minigene and U1 snRNA expression constructs

In the case of each wild-type (WT) minigene construct, the appropriate exon with circa 200 bp of flanking intronic sequences was amplified from human DNA using primers with NdeI restriction sites (Table S1). Polymerase chain reaction (PCR) products were passed through Sephacryl S-400 HR gel filtration media (GE Healthcare Life Sciences), cloned blunt end into pUC19 plasmid and subsequently excised and subcloned into pTB plasmid via NdeI restriction site (Baralle and Baralle, 2005). Respective mutations were introduced into WT pTB plasmids by site-directed mutagenesis using specific primers (Table S2). Exon-specific U1 snRNAs were created by site-directed mutagenesis reaction (primers are enlisted in Table S3) on a vector that includes the sequence coding for human snRNA U1 (*RNU1-1*, NCBI ref seq NC_000001.11), kindly provided by Dr F. Pagani (Fernandez Alanis et al., 2012; Pagani et al., 2002). The expected sequence changes for all constructs were confirmed by sequencing.

2.3. Cell culture, transient transfection, silencing, RNA extraction, reverse transcription, and PCR

Hela, HEK293T, and SH-SY5Y cells were grown in standard conditions described previously (Mohagheghi, 2016). Hela and SH-SY5Y cells were treated with 2.5 mM 1-Methyl-4-phenylpyridinium iodide (MPP+) (Sigma), dissolved in standard medium, for 24 hours. For minigene transfection, 0.5–2 µg of pTB constructs (WT or

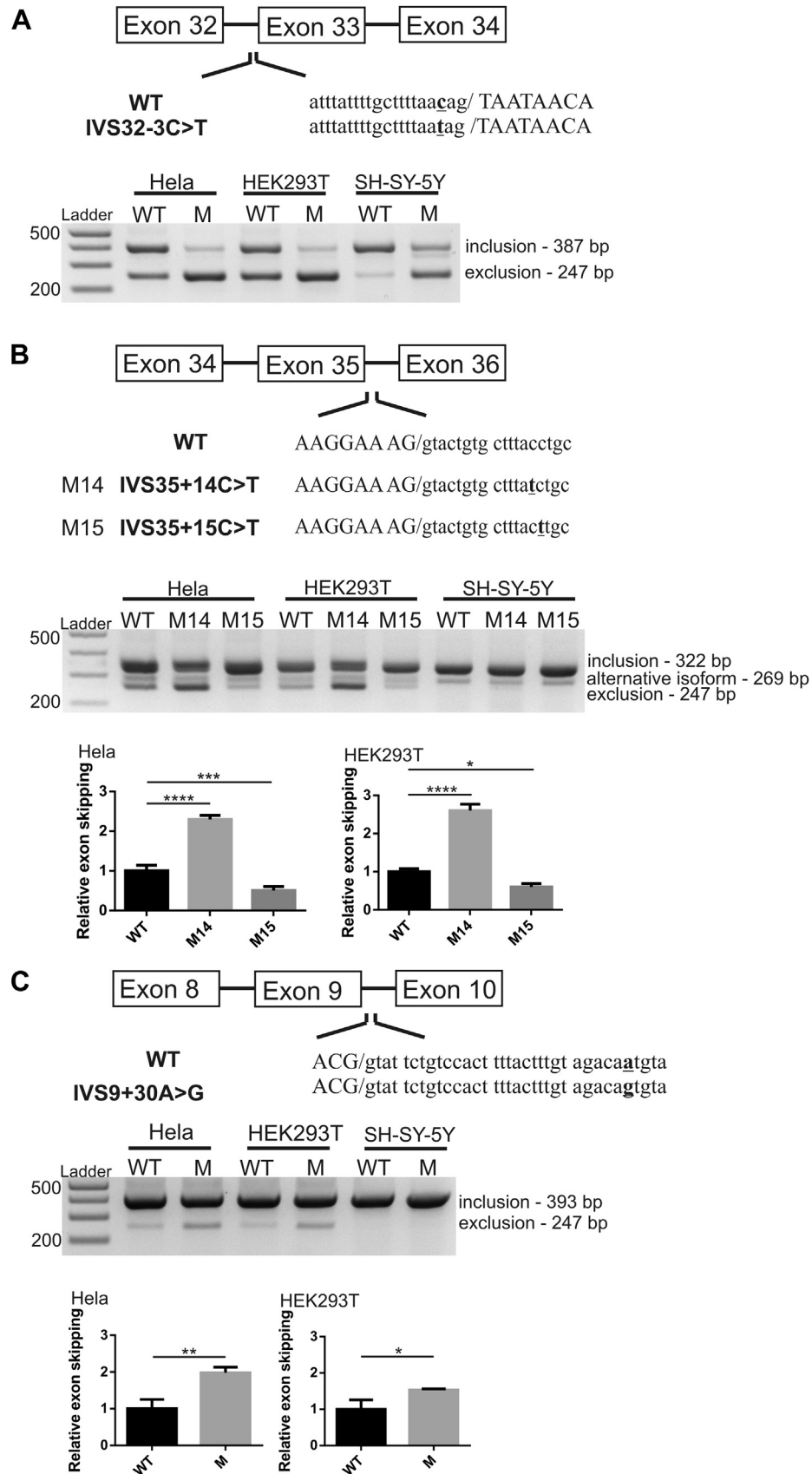


Fig. 2. *LRPPRC* intronic variants (IVS32–3C>T, IVS35+14C>T, IVS35+15C>T, and IVS9+30A>G) influence splicing in minigene system. In upper panels (A), (B), and (C), there is a schematic representation of *LRPPRC* regions affected by splicing mutations. Exonic and intronic sequences are in upper and lower case, respectively. Lower (A), middle (B), and middle (C) panels represent RT-PCR analysis of *LRPPRC* mRNA splicing pattern 24 hours after transfection of WT and MUT minigenes (IVS32–3C>T, IVS35+14C>T, IVS35+15C>T, and

mutated [MUT]) were transfected with Effectene (QIAGEN) or Lipofectamine 2000 (Invitrogen). For co-expression experiments, 0.25–2 µg of each plasmid were co-transfected (Dardis et al., 2014). When required, the amount of DNA was adjusted with the respective empty vectors or pEGFP-C1 vector. Each transfection experiment was performed at least 3 times, and a representative gel is shown. For silencing, HeLa or SH-SY5Y cells were transfected with appropriate siRNA for 2 consecutive days. Then, 6 hours after the second silencing, WT or mutated (MUT) minigenes were transfected. RNA and proteins were collected 24 hours after minigene transfection. Reverse transcription PCR (RT-PCR) analysis of minigene-derived mRNA was done with primers alpha23 and Bra2 (Table S4). RNA extraction (Eurogold Trifast, Euroclone), reverse transcription, PCR with pTB-specific primers (Table S4) or primers for endogenous *LRPPRC* mRNA (Table S5), were performed by standard protocols (Mohagheghi et al., 2016). siRNA sense sequences used for silencing the expression of luciferase (control), human heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), human hnRNP A2 (Darmacon); human SRSF1 (Thermo Scientific) are enlisted in Table S6.

2.4. Inhibition of nonsense-mediated decay

Nonsense-mediated decay (NMD) inhibition was incurred through administration of 50 µg/ml of cycloheximide (CHX) for 3 hours before the collection of RNA after 24 hours and verified by RT-PCR of Down syndrome critical region 1 (*DSCR1*) mRNA. GAPDH expression was used as a reference (Table S7).

2.5. Western blot

Western blot was performed as previously described (Mohagheghi et al., 2016). Proteins were probed with primary antibodies against: hnRNP A1, hnRNP A2 (already available in-house), ASF/SF2 (SRSF1) (anti-mouse, Invitrogen) *LRPPRC* (anti-rabbit, GeneTex Inc), and housekeeping p84 (anti-mouse, Abcam) or tubulin (anti-mouse, Sigma).

2.6. Real-time PCR

For minigene assays, exon exclusion was measured by quantitative real-time PCR using SYBR Green I Master (Roche) and primers complementary exclusively to vector-derived hybrid complementary DNA without the insert (Table S8), using a Biorad Real-Time PCR System, as per manufacturer's instructions. Changes in gene expression were determined with the ΔC_t method using alpha-hemoglobin for normalization (Table S8).

2.7. Statistical analysis

For quantitative densitometric analysis of splicing pattern on agarose gels we used ImageJ software (Schneider et al., 2012). For each band in a gel line, ImageJ assigns intensity value. These values obtained from different biological repeats of the same experiment ($n \geq 3$) were used to calculate the mean percentage of inclusion or exclusion of the analyzed exons. The percentage values obtained from different biological replicates were used to calculate standard

deviations and statistical significance (t -test and 1-way ANOVA test—posttest Tukey, GraphPad Prism 6.0). $p < 0.05$ was considered significant. For densitometric analysis of WB results, intensity value of each *LRPPRC* band was normalized to respective p84 value, then mean, standard deviation, and statistical significance values were calculated based on the individual ones ($n \geq 3$).

3. Results

A schematic diagram of all the variants analyzed in this study is shown in Fig. 1. Each variant was analyzed by preparing a suitable minigene as described in Section 2. Minigenes were then transfected in human cervical cancer-derived HeLa, embryonic kidney HEK293T, and neuroblastoma SH-SY5Y cells to highlight any tissue-specific differences. In all cases, spliced products were detected using minigene-specific primers and RT-PCR. Spliced products were sequenced to confirm their identity.

3.1. *LRPPRC* variants

In the *LRPPRC* gene, (Fig. 1) 4 variants were found to alter the RNA splicing pattern in the minigene assay: IVS32–3C>T, IVS35+14C>T, IVS35+15C>T, and IVS9+30A>G (Fig. 2).

Intron 32 variant (IVS32–3C>T, c.3492–3C>T, rs35113761) has been found in 3 out of 46 patients with PD, a frequency almost 4 times higher than the one reported in 1000G (1000 Genomes project) CEU (Utah residents of northern and western European ancestry) database (0.065 vs. 0.017) (Lill et al., 2012; Rakovic et al., 2011). This substitution, localized within the 3'ss, results in almost complete skipping of exon 33 in all 3 tested cell lines (Fig. 2A). Bioinformatic analysis (reference mRNA NM_133259.3) indicated that skipping of exon 33 would result in frameshift of the *LRPPRC* ORF with the introduction of a premature stop codon likely leading either to NMD or the expression of a truncated protein (1204 aa) (Fig. S1C).

The 2 adjacent variants in intron 35 IVS35+14C>T (c.3900+14C>T, rs3795859), and IVS35+15C>T (c.3900+15C>T, rs76850904) were found in PD patients/1000G CEU database with frequency of 0.15/0.075 and 0.086/0.092, respectively (Rakovic et al., 2011) (Lill et al., 2012). IVS35+14C>T variant led to increased exclusion of exon 35, whereas IVS35+15C>T, on the contrary, caused decreased exclusion compared with WT minigene (Fig. 2B). Real-time PCR with primers designed to measure exclusion in minigene plasmid showed that IVS35+14C>T resulted in 2.3 and 2.6 higher exclusion of exon 35 compared to WT minigene, in HeLa and HEK293T cells, respectively, (Fig. 2B, lower panel). On the contrary, IVS35+15C>T led to lower relative exclusion levels, 0.5 and 0.6 in HeLa and HEK293T cells, respectively, (Fig. 2B, lower panel). Interestingly, in SH-SY5Y cells in minigene system, we observed a predominant band corresponding to exon inclusion (322 bp) but also a lower band (269 bp) corresponding to alternative isoform lacking the first 53 bp of exon 35, confirmed by sequencing (Fig. S1E). Bioinformatic analysis showed that lack of the first 53 bp of exon 35 results in a frameshift and introduction of a premature stop codon (TAG at 3890–92 bp) possibly resulting in NMD or truncated protein (1278 aa) (Fig. S1E). On the other hand, it is worth noting that the entire exon 35 deletion would lead to shorter protein lacking 25 aa (1369 aa) without any frame change

IVS9+30A>G, respectively) in HeLa, HEK293T or SH-SY5Y cells. Lower (B) and (C) panels represent real-time PCR analysis with primers specifically measuring the levels of exclusion in minigene system. IVS35+14C>T (M14) causes increase in exon exclusion (2.3 and 2.6 times), whereas IVS35+15C>T (M15) lowers exclusion levels by about half in comparison to IVS35WT, in HeLa and HEK293T cells, respectively. IVS9+30A>G (M) presence led to 1.98 and 1.53 times higher skipping of exon 9 than WT minigene (WT), in HeLa and HEK293T cells, respectively (C, lower panel). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Abbreviations: *LRPPRC*, leucine-rich pentatricopeptide repeat containing; mRNA, messenger RNA; MUT, mutated; RT-PCR, reverse transcription polymerase chain reaction; WT, wild type.

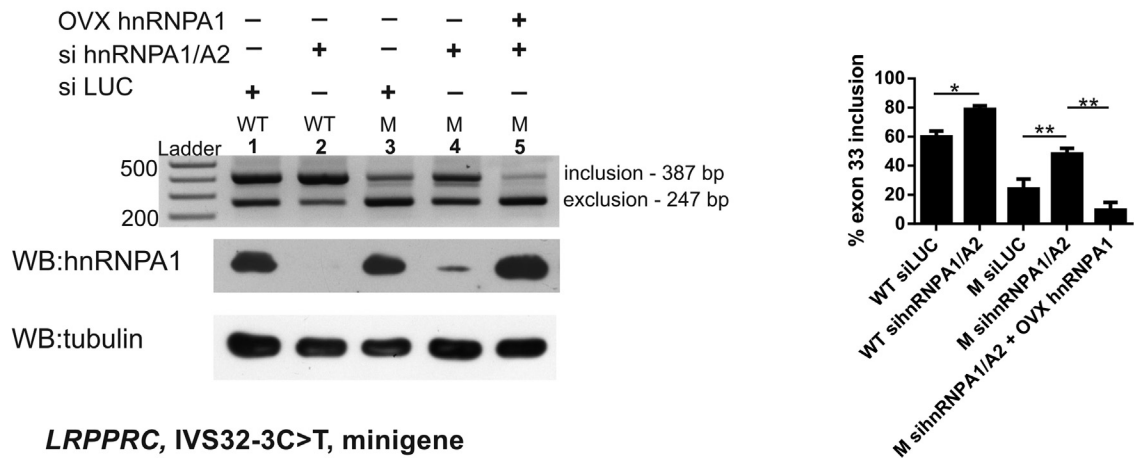
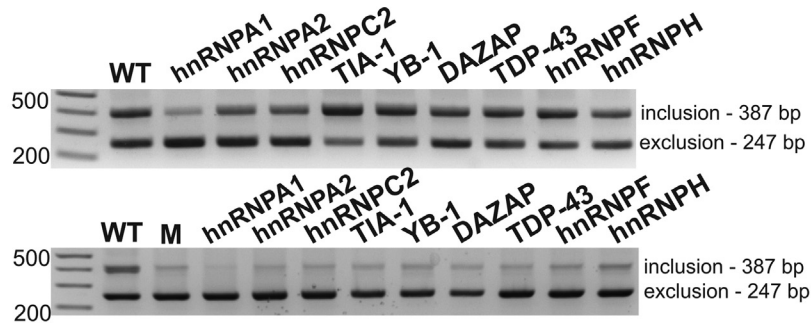
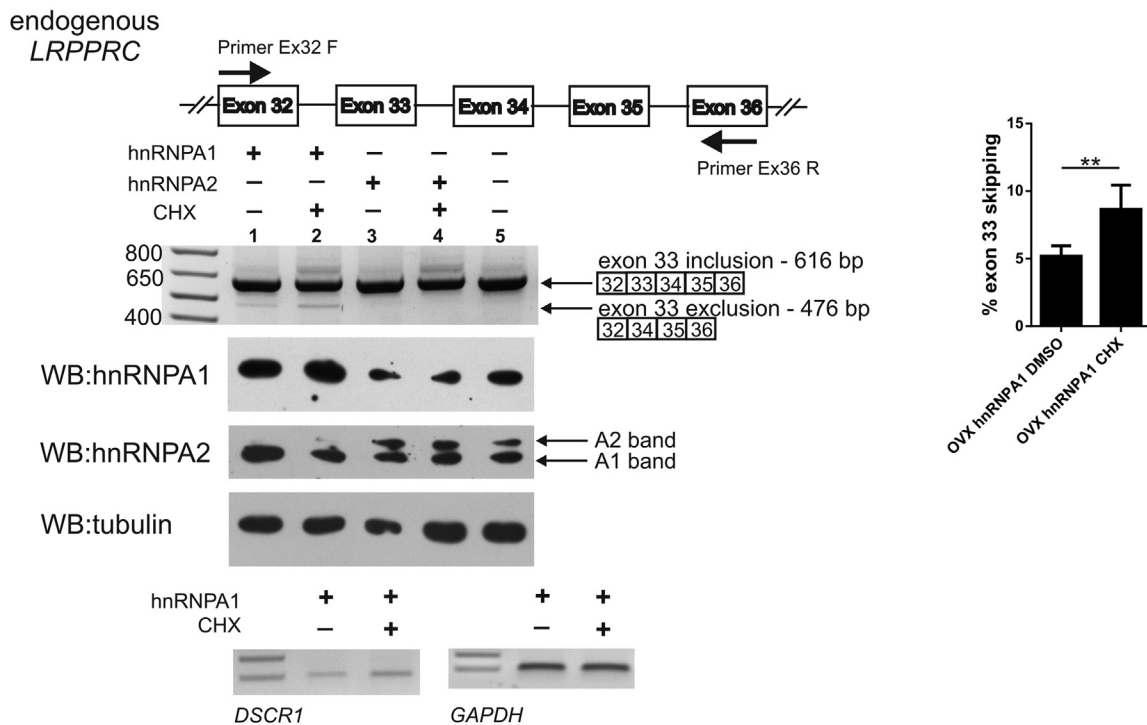
A *LRPPRC*, IVS32-3C>T, minigene**B** *LRPPRC*, IVS32-3C>T, minigene**C** *LRPPRC*, endogenous mRNA

Fig. 3. Negative regulation of *LRPPRC* exon 33 by hnRNP A1 in minigene and endogenous context. (A) Silencing of endogenous hnRNP A1/A2 rescues exon 33 skipping in MUT minigene carrying IVS32–3C>T. WT minigenes (lane 1, 2) and MUT minigenes (lanes 3, 4, 5) were transfected in HeLa cells in the presence of siLUC (lanes 1, 3) or si hnRNP A1/A2 (lanes 2, 4, 5). In addition, overexpression of hnRNP A1 was performed in lane 5. Right panel shows the percentage of exon 33 inclusion determined densitometrically. * $p < 0.05$, ** $p < 0.01$. Left middle and lower panels represent Western blot for hnRNP A1 protein and tubulin, respectively. (B) Overexpression of hnRNP A1 increases exclusion of exon 33 in WT and

(Fig. S1D). Interestingly, NCBI database annotates *LRPPRC* transcript variant X1 (NCBI: XM_011532473.1) with exon 35 skipped, which suggests this exon is alternatively spliced; however, the existence of the truncated protein has been predicted merely bioinformatically (NCBI: XP_011530775.1).

Finally IVS9+30A>G (c.1077+30A>G, rs7593842) in *LRPPRC* resulted in exon 9 skipping (Fig. 2C), 1.98 and 1.53 times higher than in WT minigene, in HeLa and HEK293T cells, respectively, as demonstrated with real-time PCR (Fig. 2C, lower panel). According to bioinformatic analysis, skipping of exon 9 would result in a frameshift change and the introduction of a premature stop codon (TAA, at nucleotide residue 1094–96) likely leading to NMD-mediated degradation of the mRNA or the expression of a truncated protein (346 aa) (Fig. S1B).

The other *LRPPRC* variants analyzed: IVS13+28T>C (c.1582+28T>C, rs62135104); exon 23 c.2481A>G, (P827P, rs115993634); exon 37 c.4023T>C, (Y1341Y) did not have any effect on splicing in our minigene system and were not further investigated (Fig. S2).

3.2. Functional investigation and rescue of the *LRPPRC* IVS32–3C>T variant

Despite of the strong effect of acceptor IVS32–3C>T mutation (almost complete skipping of exon 33 in the minigene system) (Fig. 2A), splicing prediction programs failed to detect a highly significant change in the strength of acceptor splice site following this substitution (HSF score: WT = 84.77, MUT = 77.09; MaxEnt score: WT = 6.92, MUT = 5.26). Thus, we reasoned that in addition to acting on 3' splice strength, the –3C>T substitution may have created or affected an existing splicing regulatory element. One possibility according to HSF prediction is that IVS32–3C>T may have created a new hnRNP1 site. It can be envisaged that hnRNP1 binding to this position could compete with U2AF35 binding to the acceptor site. To test this hypothesis, we silenced hnRNP1 and hnRNP2 (hnRNP1/A2). Although we recovered exon 33 recognition to WT level (Fig. 3A, lane 4), we also observed improved exon recognition in the WT minigene (Fig. 3A, lane 2). In case of the mutated minigene, the increased inclusion was reversed on simultaneous coexpression of si-resistant hnRNP1 after hnRNP1/A2 silencing (Fig. 3A, lane 5). Silencing results (Fig. 3A) are in agreement with the results of overexpression of different hnRNPs where we observed that only hnRNP1 decreased exon 33 inclusion in both WT and MUT minigene (Fig. 3B). Finally, a very similar rescue effect on WT and MUT minigene on silencing of hnRNP1/A2 could also be observed in the SH-SY5Y cell line, indicating that the negative regulation by hnRNP1 is common to different tissues (data not shown).

These results suggest that in addition to the putative motif created by IVS32–3C>T there are other hnRNP1-binding sites already present either within exonic or flanking intronic sequences contributing to the control of exon 33 splicing. Because of the negative regulation of exon 33 inclusion by hnRNP1 in minigene

context (Fig. 3A and B), we further tested if hnRNP1 could also influence endogenous *LRPPRC* mRNA splicing. Only on overexpression of hnRNP1, but not other hnRNPs or SRs, for example, hnRNP2 (or TDP-43, TIA-1, SRSF1, not shown), an additional band appeared corresponding to an isoform with exon 33 skipped, confirmed by sequencing of PCR product (Fig. 3C, lane 1, 2). The isoform underwent degradation by NMD as its expression levels increased following treatment with CHX (Fig. 3C, lane 1, 2). As a positive control for NMD, we used the *DSCR1* gene, a known NMD target (Mendell et al., 2004; Wittmann et al., 2006) and the *GAPDH* gene as a control for equal complementary DNA input (Fig. 3C, lower panel).

Based on the involvement of hnRNP1 in exon 33 splicing regulation, we decided to evaluate different therapeutic strategies to correct the effects of this mutation (Buratti et al., 2006). One of them is overexpression of SR proteins that usually promote exon inclusion and act as general antagonists of hnRNPs repressor functions. To investigate this possibility, we overexpressed several of the major SR protein family members (SRSF1 to SRSF6, and SRSF9) in the presence of the minigene carrying the IVS32–3C>T substitution. However, none of the tested SR proteins was able to increase exon 33 inclusion in MUT minigene (Fig. S3).

An alternative therapeutic strategy would be the coexpression of adapted recombinant U1 snRNA that could improve the recognition of the donor site of exon 33 in the presence of the IVS32–3C>T substitution. To achieve this, we designed 3 modified U1 snRNAs either fully complementary to 5' splice donor site (U1LRP33wt) or to downstream intronic positions +8 (U1LRP33+8) and +12 (U1LRP33+12) in intron 33 as indicated in Fig. 4A. HeLa cells were then cotransfected with WT or MUT minigene and the various U1 constructs (Fig. 4B). As expected, the overexpression of WT U1 snRNA did not have any effect on WT and MUT minigene (Fig. 4B, line 2, 7). Overexpression of (1) U1LRP33wt; and (2) U1LRP33+12 successfully rescued defective splicing in the mutated minigene to WT levels (Fig. 4B, lines 8, 10) whereas U1LRP33+8 gave only partial rescue (Fig. 4B, line 9). Likewise, all the adapted U1 snRNAs improved inclusion of exon 33 in WT minigene, with similar strength as observed for MUT minigene (Fig. 4B, lines 3, 4, 5). In addition, co-expression of U1LRP33+12 was able to reverse the effects of hnRNP1 overexpression in minigene system (Fig. 4C). Most importantly, overexpressing of hnRNP1 in HeLa cells along with U1LRP33+12 in the endogenous context caused the disappearance of the lower band corresponding to the *LRPPRC*-splicing isoform lacking exon 33 (476 bp) (Fig. 4D, lane 3, 4). This suggests that the U1 snRNA constructs can overcome the effects of eventual hnRNP A1 overexpression as well as the presence of the acceptor site mutation, IVS32–3C>T.

3.3. Functional investigation and rescue of the *LRPPRC* IVS35+14C>T mutation

In parallel with the *LRPPRC* IVS32–3C>T rescue experiments, we also attempted to correct the effects of the *LRPPRC* IVS35+14C>T mutation.

MUT minigenes. Panels represent RT-PCR analysis of minigene-derived mRNA. HeLa cells were cotransfected with vectors expressing different hnRNPs and either WT (upper panel) or MUT minigene constructs (lower panel). (C) Overexpression of hnRNP1 causes skipping of exon 33 in endogenous *LRPPRC* mRNA. Vectors expressing hnRNP1 (lane 1, 2) or hnRNP2 (lane 3, 4) or control vector were transfected in HeLa cells. Cells were treated with dimethyl sulfoxide (DMSO) (line 1, 3, 5) or cycloheximide (CHX) (line 2, 4). Upper left panel represents fragment of *LRPPRC* pre-mRNA (exonic sequences are boxed, intronic sequences represented by lines) amplified with primers Ex32F and Ex36R (indicated by the arrows), specific to endogenous mRNA. Middle left panel shows RT-PCR analysis of endogenous *LRPPRC* mRNA. Skipping of exon 33 produces a band shorter by 140 bp, indicated by the arrow. Right panel shows percentage of exon 33 exclusion determined densitometrically; **p* < 0.01. hnRNP1 and hnRNP2 protein levels were monitored by Western blot, whereas tubulin was used as a protein level control. Membrane was first blotted with antibody against hnRNP1 (upper WB panel). Because membrane was not stripped before blotting with antibody against hnRNP2, the middle WB panel shows both the expression of hnRNP2 (upper band) and hnRNP1 (lower band). hnRNP2 is overexpressed in the lane 3, compared to the normal levels in the lane 5—taking into consideration lower tubulin signals in lane 3 versus lane 5. As a positive control for NMD, the Down syndrome critical region 1 (*DSCR1*) gene was used, as a control for even complementary DNA input in RT-PCR *GAPDH* gene was used (lower left and right panel, respectively). Abbreviations: hnRNP1, heterogeneous nuclear ribonucleoprotein A1; *LRPPRC*, leucine-rich pentatricopeptide repeat containing; mRNA, messenger RNA; MUT, mutated; NMD, nonsense-mediated decay; RT-PCR, reverse transcription polymerase chain reaction; WT, wild type.

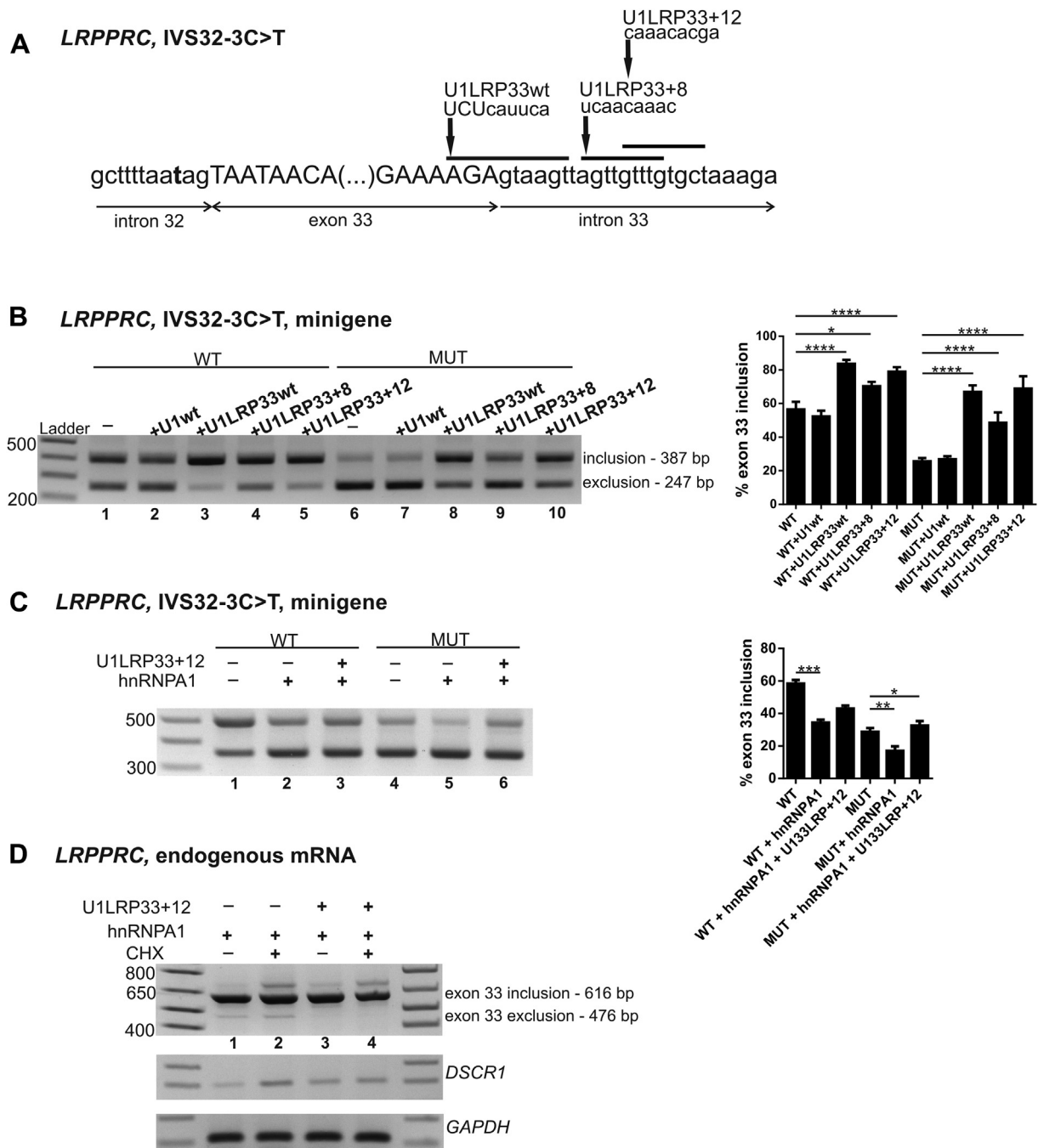


Fig. 4. Overexpression of adapted snRNAU1 complementary to the 5'ss of *LRPPRC* exon 33 can rescue both the minigene and endogenous defective splicing. (A) Schematic representation of the *LRPPRC* pre-mRNA fragment comprising exon 33 (upper case) with flanking intronic sequence (lower case). Arrows indicate the positions to which complementary snRNAU1 has been designed; U1LRP33wt, U1LRP33+8, U1LRP33+12. IVS32–3C>T mutation is in bold; (B) RT-PCR analysis of splicing pattern in HeLa cells cotransfected with WT or MUT minigene vector (carrying IVS32–3C>T mutation) and different snRNAU1 constructs (complementary to the positions shown in A). Right panel shows percentage of exon 33 inclusion determined densitometrically. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. (C) RT-PCR analysis of splicing pattern in HeLa cells cotransfected with WT (line 1,2,3) or MUT minigene vector (carrying IVS32–3C>T mutation, line 4,5,6) and vector overexpressing hnRNPA1 alone or together with snRNAU1-carrying plasmid (U1LRP33+12). Right panel shows percentage of exon 33 inclusion determined densitometrically. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (D) U1LRP33+12 rescued skipping of exon 33 in endogenous context. RT-PCR analysis of the endogenous *LRPPRC* mRNA splicing pattern in HeLa cells on overexpression of hnRNPA1 and cotransfection with snRNAU1-carrying vector (designated as U1LRP33+12) complementary to intronic position IVS33+12. Cells were treated with DMSO (line 1, 3) or cycloheximide (CHX) (line 2, 4) for 3 hours before collecting RNA (after 24 hours). Abbreviations: hnRNPA1, heterogeneous nuclear ribonucleoprotein A1; *LRPPRC*, leucine-rich pentatricopeptide repeat containing; mRNA, messenger RNA; MUT, mutated; RT-PCR, reverse transcription polymerase chain reaction; WT, wild type.

In search for possible auxiliary splicing factors that could regulate exon 35 inclusion, we overexpressed selected hnRNP and SR proteins (Fig. 5A and B). Among hnRNP proteins, only TIA-1 overexpression resulted in increased inclusion of exon 35 in the context of the MUT minigene (IVS35+14C>T), (Fig. 5A, lower panel). Interestingly, TIA-1

overexpression in HeLa cells also increased the level of a truncated alternative isoform lacking first 53 bp of exon 35 as confirmed by sequencing (Fig. 5A, 269 bp band, indicated with an arrow; Fig. S1E).

Among the serine/arginine-rich (SR) proteins tested, overexpression of SRSF1 (ASF/SF2) and SRSF9 (SRp30c) could rescue

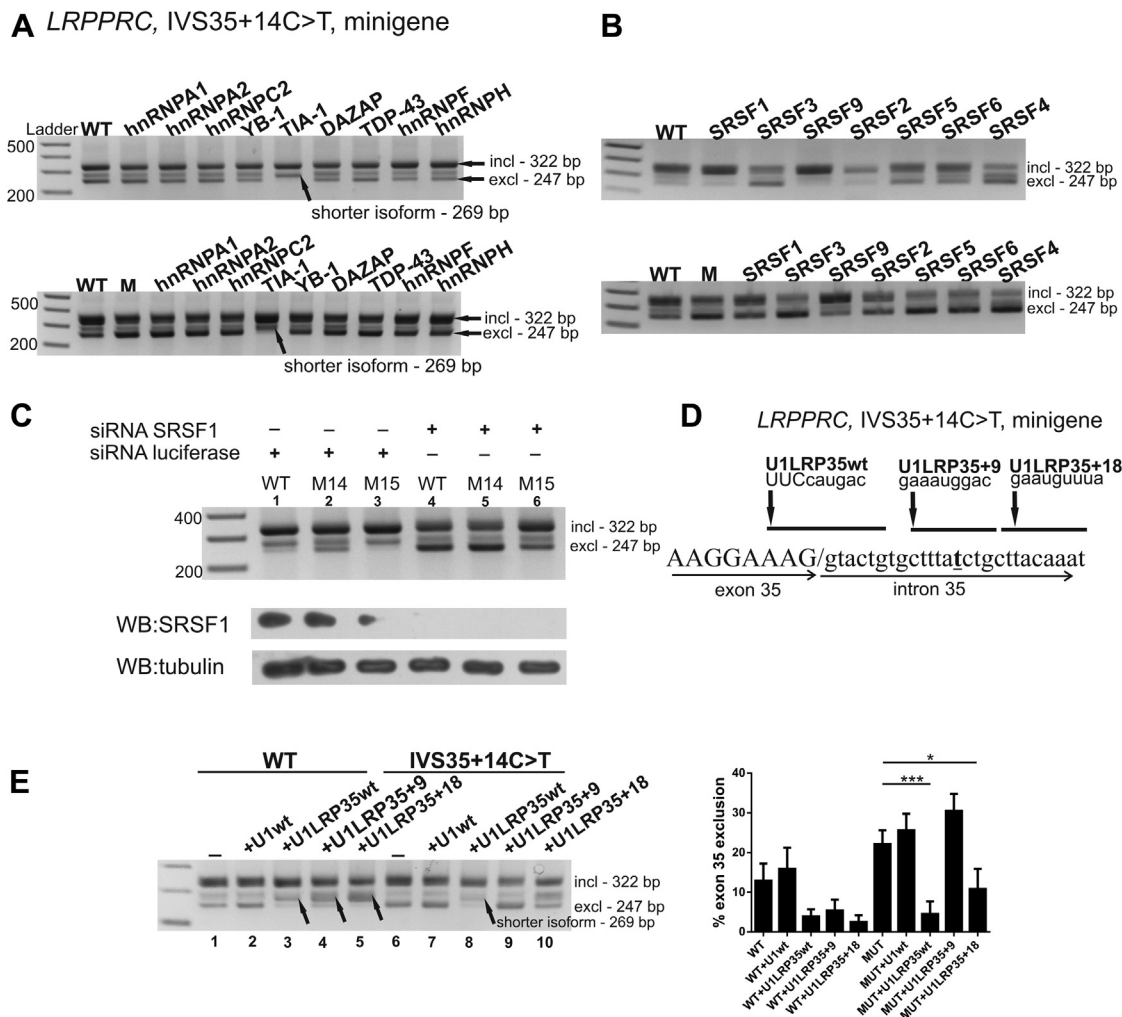


Fig. 5. Overexpression of TIA-1, SRSF1, and SRSF9 decreases exon 35 exclusion. HeLa cells were cotransfected with vectors expressing different hnRNPs (A) or SR proteins (B) and either WT (upper panels) or MUT minigene constructs (lower panels). (A) Apart from increasing exon 35 inclusion, TIA-1 overexpression results in production of alternative isoform lacking first 53 bp of exon 35 (indicated by an arrow) both in the context of WT and MUT minigene; (B) overexpression of SRSF1 and SRSF9 increases exon 35 inclusion without producing the short alternative isoform; (C) Upper panel shows RT-PCR analysis of minigene-derived mRNA WT (lane 1, 4) and MUT (lane 2, 3, 5, 6) in HeLa cells with silenced expression of *SRSF1* gene; *SRSF1* protein level was monitored by Western blot (middle panel), whereas tubulin was used as a protein level control (lower panel); (D) Schematic representation of the *LRPPRC* pre-mRNA fragment comprising exon 35 (upper case) with flanking intronic sequence (lower case). Arrows indicate the positions to which adapted snRNAU1 has been designed, U1LRP35wt, U1LRP35+9, U1LRP35+18; (E) RT-PCR analysis of splicing pattern in HeLa cells cotransfected with WT or MUT minigene vector (carrying IVS35+14C>T mutation) and different snRNAU1 constructs complementary to the positions shown in (D). Right panel shows percentage of exon 35 exclusion determined densitometrically. * $p < 0.05$, *** $p < 0.001$. Abbreviations: hnRNP A1, heterogeneous nuclear ribonucleoprotein A1; *LRPPRC*, leucine-rich pentatricopeptide repeat containing; mRNA, messenger RNA; MUT, mutated; RT-PCR, reverse transcription polymerase chain reaction; WT, wild type.

exon 35 splicing of the MUT minigene, also enhancing inclusion of WT minigene (Fig. 5B). To confirm the overexpression results, we silenced *SRSF1* in HeLa cells and, as expected, we noted increased exclusion for the WT, IVS35+14C>T and IVS35+15C>T minigenes compared to luciferase-silenced controls (Fig. 5C).

In addition, we observed rescue of MUT minigene IVS35+14C>T after coexpression of U1 snRNA fully complementary to 5' splice donor site (U1LRP35wt) but not to downstream intronic positions +9 and +18 (U1LRP35+9, U1LRP35+18), as indicated in Fig. 5D and E. Similarly to the case of TIA-1 overexpression, the overexpression of adapted U1 snRNAs produced also the alternative isoform containing only 22 last bp of exon 35 in addition to rescuing exon 35 splicing, (Fig. 5E, 269 bp band, indicated by arrows). In case of U1LRP35+18 overexpression, although the exclusion band diminished, the alternative band (269 bp) increased instead of WT inclusion band (322 bp) (Fig. 5E).

Taken together, these results suggest that both *LRPPRC* mutations, IVS32–3C>T and IVS35+14C>T, may be rescued using

genetic approaches that are currently being used in preclinical trials to correct splicing mutations (Rogalska et al., 2016).

3.4. MPP+ induces exon 35 skipping accompanied by decrease in *LRPPRC* mRNA and protein levels

Because administration of complex I inhibitor MPP+ is a well-documented model of PD pathogenesis, we wanted to assess its influence on *LRPPRC* splicing. 2.5-mM MPP+ was previously reported to cause cell death in less than 20% of SH-SY5Y cells after 24 hours (Wang et al., 2011). Indeed, both in HeLa and SH-SY5Y cells, we could not see visible signs of cell death under phase-contrast microscope after MPP+ treatment (data not shown). We observed skipping of exon 35 (Fig. 6A, lines 3, 4, 7, 8) accompanied by decrease of wt isoform (Fig. 6A, compare lines 1, 5 vs. 3, 7) in HeLa and SH-SY5Y cells. In HeLa cells, 20% of mRNA displayed skipped exon 35. After CHX treatment, 26% of produced mRNA was WT, 16% corresponded to the isoform with exon 35 skipped, whereas 60% to

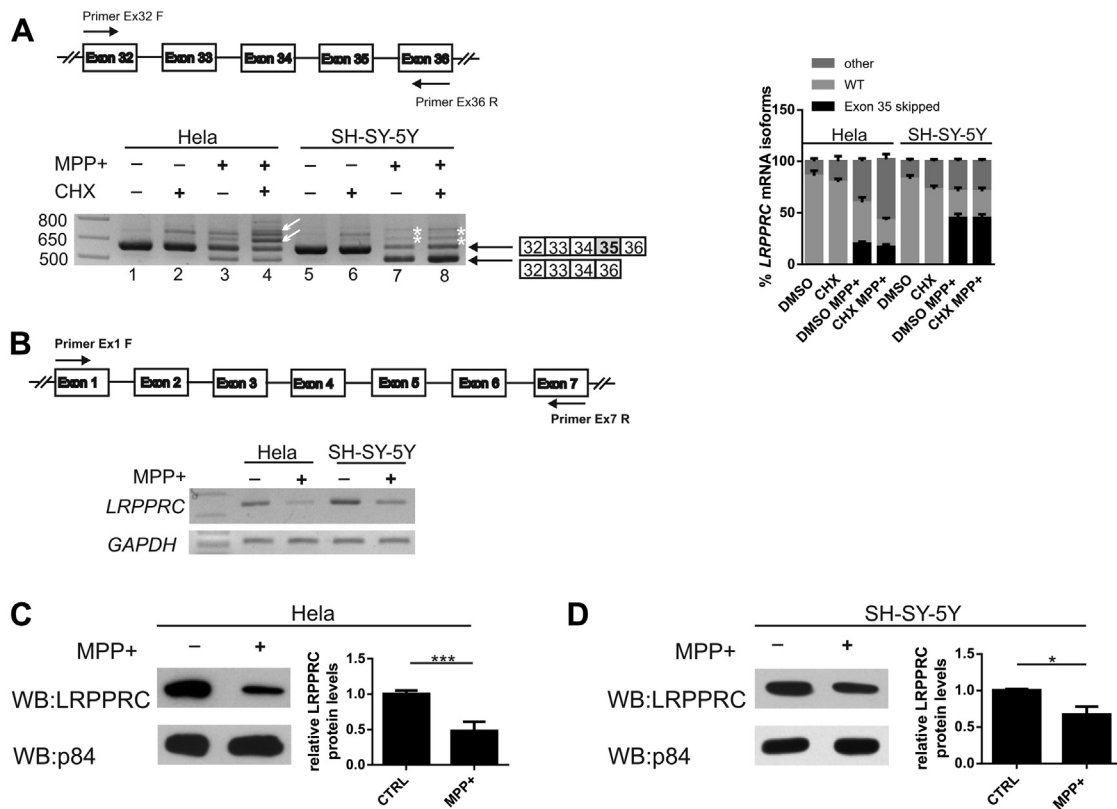


Fig. 6. MPP+ treatment causes skipping of exon 35 in endogenous LRPPRC mRNA and decrease in protein levels. (A) HeLa and SH-SY5Y cells were treated with MPP+ (2.5 mM) for 24 hours and with DMSO (line 1, 3, 5, 7) or cycloheximide (CHX) (line 2, 4, 6, 8) for 3 hours before collecting RNA. Upper panel represents fragment of LRPPRC pre-mRNA amplified with primers specific to endogenous mRNA (indicated by the arrows). Lower panel shows RT-PCR analysis of endogenous LRPPRC mRNA. Skipping of exon 35 produces a band shorter by 75 bp, indicated by the arrow; right panel shows proportions of LRPPRC mRNA isoforms generated after MPP+ treatment, with inhibition of NMD by CHX, determined densitometrically; (B) Upper panel represents fragment of LRPPRC pre-mRNA (exonic sequences boxed, intronic—lines) amplified with primers Ex1F and Ex7R (indicated by the arrows), specific to endogenous mRNA. Lower left panel shows RT-PCR analysis of endogenous LRPPRC mRNA, GAPDH was used as even complementary DNA input control; (C) and (D) LRPPRC protein levels were monitored by Western blot, in HeLa and SH-SY5Y cells, respectively, whereas p-84 was used as a protein level control. Right (C) and (D) panels show densitometric measurement of LRPPRC protein levels obtained from 3 experiments (n = 3). *p < 0.05, ***p < 0.001. Abbreviations: hnRNPA1, heterogeneous nuclear ribonucleoprotein A1; LRPPRC, leucine-rich pentatricopeptide repeat containing protein; MPP+, 1-Methyl-4-phenylpyridinium ion; mRNA, messenger RNA; MUT, mutated; RT-PCR, reverse transcription polymerase chain reaction; WT, wild type.

other alternative isoforms (Fig. 6A, line 4, white arrows) that undergo NMD (compare Fig. 6B). In SH-SY5Y cells after MPP+ treatment, 26% of WT mRNA was produced, 45% corresponded to exon 35-skipped mRNA, and only 29% to other isoforms (Fig. 6A, line 7 and 8, white asterisks). None of these isoforms underwent NMD, as shown by the same proportions without and with CHX treatment. Total mRNA level of LRPPRC decreased after MPP+ treatment as shown by RT-PCR of its 5' region (exons 1–7) (Fig. 6B). For this reason, we expected decreased LRPPRC protein levels, after treatment with MPP+, more pronounced in HeLa than those in SH-SY5Y cells (also due to degradation by NMD), which, indeed, was confirmed by Western blot (Fig. 6C and D).

However, on Western blots, we could not detect 2 bands, corresponding to wt and exon 35-skipped LRPPRC (predicted difference of 2.7 kDa corresponding to 25 aa), even if it is not supposed to undergo NMD and the polyclonal antibody, theoretically, should have recognized both isoforms (the immunogen is within the N-terminal and central part of the protein) (Fig. 6B).

These results suggest that the decrease of LRPPRC protein is due to reduced levels of its mRNA associated with exon 35 skipping.

Furthermore, we checked whether MPP+-induced exon 35 skipping or decrease in LRPPRC protein could be rescued by overexpression of SRSF1, SRSF9, or TIA-1, the splicing factors that we observed to improve exon 35 inclusion in minigene system (Fig. 5A and B). None of these factors could decrease substantially (more

than 10%) exon 35 mRNA skipping or increase LRPPRC protein levels (Fig. S7A and B). However, it must be also taken into account that MPP+ exerts its effect on all the cells in culture, whereas protein overexpression will have lower efficiency. Furthermore, MPP+ influences a plethora of cellular-signaling pathways in parallel: oxidative stress, DNA and protein damage, cell-cycling arrest, apoptosis, autophagy, to name a few (Dagda et al., 2013; Krug et al., 2014; Xu et al., 2005). That is why targeting only one of them might not be sufficient to obtain rescue effect.

3.5. PINK1 variants

PINK1 intronic mutation IVS7+1G>A (g.16378G>A; c.1488 + 1G>A) segregating with PD was detected in a large family pedigree (Samaranch et al., 2010) (Fig. 1). IVS7+1G>A was present either in a compound heterozygous (together with exon 7 deletion, c.1252_1488del) or in a homozygous state. Our minigene vectors transfected in cultured cells reproduced closely the abnormal splicing pattern observed in leukocytes of PD patients by Samaranch et al.: exon 7 skipping and truncated splicing isoform lacking 33 bp due to the use of a cryptic exonic 5'ss (Fig. S4A and B) (Samaranch et al., 2010).

Notwithstanding the G nucleotide in position +1 within 5'ss is highly conserved in humans and aberrant splicing due to its mutation is very difficult to rescue through adapted U1 snRNAs

overexpression in minigene system (Fernandez Alanis et al., 2012; Hartmann et al., 2010; Matos et al., 2014; Schmid et al., 2013), we decided to analyze if this was possible to explore potential therapeutic options. Adapted U1 snRNAs were designed to be either fully complementary to the mutated donor site or to downstream intronic positions as indicated in Fig. S4C. In keeping with previous reports (Fernandez Alanis et al., 2012; Hartmann et al., 2010; Matos et al., 2014; Schmid et al., 2013), we observed that upon co-expression of U1PINK1Ex7WT, and to lower extent U1PINK1Ex7+16, exclusion of exon 7 decreased with simultaneous increase of the alternative inclusion band corresponding to an isoform lacking 33 bp, originating from the use of a cryptic 5'ss with almost as high HSF score (90.18) as WT donor site (95.95). This is in accordance with previous observations suggesting that the ability to rescue 5'ss mutations in position +1 could depend on the strength of adjacent alternative donor sites (Matos et al., 2014). These authors noticed about 50% rescue for *HGSNAT* c.234 +1G>A mutation with traces of alternative isoform being produced, only when the alternative 5'ss had null HSF score compared with the WT one (Matos et al., 2014). So far, U1 snRNA-mediated rescue of mutations in position +1 was successful only in patient's fibroblasts, whereas in minigene system, it led exclusively to undesired alternative splicing pattern, possibly due to the lack of endogenous regulatory context (Hartmann et al., 2010; Matos et al., 2014). Thus, our adapted U1 snRNAs should be tested in fibroblasts from PD patients to find out whether they represent a viable option to rescue this mutation or alternative strategies should be considered. It also remains to be established whether PINK1 protein lacking 11 aa might be harmful for a cell due to, for example, dominant negative effect or it still preserves some residual function. In the latter case, rescue with snRNAU1 might be the way to help patients with compound heterozygous or homozygous IVS7+1G>A mutation. In the analyzed family among 2 carriers of homozygous IVS7+1G>A mutation, one was asymptomatic, whereas the other one had PD (Samaranch et al., 2010). To clear this issue, functional analysis of truncated PINK1 protein is necessary.

Finally, with regards to *PINK1*, we also analyzed variant IVS7+14C>G (c.1488 + 14C>G) detected in 1 early-onset PD patient and absent among 200 control individuals (Valente et al., 2004). However, this mutation did not have any effect on splicing in our minigene system (Fig. S4A and B) and the sequenced band corresponded to correctly spliced exon 7 flanked by minigene sequences.

3.6. TFAM and PARK2 variants

The *TFAM* variant IVS4+113 A>G (c.441+113A>G, rs2306604) that we found previously to be a risk factor for PD (Gaweda-Walerych et al., 2010) has been postulated to create a putative new splice donor and a cryptic splice acceptor in in silico analysis, possibly resulting in the production of a truncated TFAM protein of 102 amino acids (Gunther et al., 2004). A minigene analysis, however, failed to demonstrate any aberrant splicing (Fig. S5A).

The *PARK2* variant IVS3–20T>C (c.413–20T>C, rs4709583) has been detected in a homozygous state in 1 Polish PD patient (personal communication, prof. J. Limon) and was found more frequent in early-onset Parkinson's disease patients than that in control subjects in Chinese population (Zou et al., 2004). Minigene analysis showed mRNA carrying this variant to be processed in an analogous way as to the WT minigene (Fig. S5B). For this reason, none of above changes was further investigated.

3.7. HSPA9 variants

Three variants for *HSPA9* were analyzed, a potentially pathogenic intronic insertion of 17 bp, (IVS8+8; c.879+8 ins or del of

AGAGAGAGGTGAGTTAC, rs41295719), detected in 1 PD patient (age at onset 51 years, without family history) out of 330 PD subjects and 250 controls (De Mena et al., 2009); a synonymous variant c.774G>A in exon 8, (Q258Q, rs41295717) found in 1 PD patient (De Mena et al., 2009) and c.879+9 ins17 bp annotated in NCBI. None of these variants resulted in alteration of RNA processing compared to the WT sequences using the minigene splicing assay (Fig. S6).

4. Discussion

In this work, we have analyzed the impact on splicing of 14 variants detected in PD patients (Fig. 1). Four intronic substitutions in the *LRPPRC* influenced the splicing process of this gene as evidenced by minigene assays (Fig. 2). Exon 33 splicing pattern and the effect of IVS32–3C>T mutation is very similar in all 3 tested cell lines (Fig. 2A). However, the splicing pattern for exon 9 and 35 and the effects of corresponding mutations (IVS9+30A>G, IVS35+14C>T, and IVS35+15C>T) in minigene system depend on the cell line (compare HeLa and HEK293T with SH-SY5Y cells, Fig. 2B and C). This could result from tissue-specific availability of some splicing factors. In addition, it suggests that also in PD patients a given mutation can have different effects depending on the tissue and it underlines the importance of analyzing splicing pattern in various disease-related cell groups.

More specifically, IVS9+30A>G and IVS32–3C>T, resulted in skipping of *LRPPRC* exon 9 and exon 33, respectively. In both cases, this would introduce a premature stop codon in the mature mRNA that could result in its degradation by NMD pathway, and as a consequence, in depletion of *LRPPRC* protein. Such a depletion may be linked to the origin and progression of PD and related diseases (Alzheimer's disease, French Canadian Leigh syndrome, LSFC) by mitochondrial dysfunction. Indeed, consequences of *LRPPRC* depletion are well documented by the analysis of tissues from LSFC patients and various knock-down or silencing approaches in cell lines. They comprise decrease in all mtDNA-encoded mRNAs and mitochondrial respiratory complexes (IV, I, and V) as low as 20%–30% of control levels in fibroblast, liver, and muscle cells from LSFC patients with the most common A354V mutation (Mourier et al., 2014; Olahova et al., 2015; Sasarman et al., 2015; Xu et al., 2004). Pathology in LSFC patients with *LRPPRC* mutations is observed mainly in organs with steady-state low level of *LRPPRC*, such as brain and liver (Xu et al., 2004), and mRNA levels of *LRPPRC* are known to be tissue specific (skeletal muscles>heart>placenta>kidney>liver>lung=brain) (Mili and Pinol-Roma, 2003; Xu et al., 2004). Also, compensatory responses to *LRPPRC* deficiency are tissue specific (Mourier et al., 2014; Olahova et al., 2015; Sasarman et al., 2015). To date, it is not known to what extent *LRPPRC* is involved in post-transcriptional regulation of mitochondrial mRNAs in brain. *LRPPRC* has been linked recently to another late-onset neurodegenerative disease—AD, when Swedish mutant K670N/M671L of amyloid beta precursor protein (APPsw) was shown to downregulate both protein and mRNA levels of *LRPPRC* and its downstream target SLIRP (SRA Stem-Loop Interacting RNA Binding Protein) (Mourier et al., 2014).

Clearly, the clinical impact of *LRPPRC* variants will depend on whether the mutation occurs in a homo- or heterozygous state. It is also worth noting that mutational screening of *LRPPRC* gene in PD patients detected only intronic or exonic synonymous mutations (Rakovic et al., 2011) and none nonsynonymous mutation, the latter ones found, to date, in LSFC patients and other severe early-onset mitochondrial conditions (Mootha et al., 2003; Olahova et al., 2015). Thus, it can be envisioned that even a modest decrease of *LRPPRC* levels or change of its function conferred by splicing mutations (such as IVS32–3C>T, IVS9+30A>G, or IVS35+14C>T) could be further aggravated by aging-related factors (e.g., decrease

in efficiency of some molecular pathways) in the course of PD pathogenesis.

Although LRPPRC does not contain any canonical RNA-binding motifs, its ability to bind RNA has been reported. Mili and Pinol-Roma (2003) have found that the 2 last C-terminal pentatricopeptide (PPR) motifs correspond to minimal RNA-binding domain and are crucial for RNA homopolymers binding (polyU and polyC). It is worth noting that both exons 33 and 35 localize to the linker region between the 2 last PPR motifs. For this reason, lack of the C-terminal domain (due to IVS32–3C>T) or increased exclusion or inclusion of exon 35 (due to IVS35+14C>T and IVS35+15C>T, or MPP+ treatment) could interfere with the RNA-binding properties of LRPPRC. This is a possibility that should be addressed in future work on the functional properties of this protein.

The opposite effect on splicing exerted by the adjacent variants, IVS35+14C>T and IVS35+15C>T (Fig. 2B) is in accordance with higher frequency of the first one in PD patients, as mentioned in Section 3.1. Clearly, this needs to be confirmed by association studies using higher number of affected subjects and proper control groups. At the functional level, increased or decreased inclusion of the same exon has been linked to different neurodegenerative diseases (progressive supranuclear palsy, corticobasal degeneration, argyrophilic grain disease, or Pick's disease, respectively) as evidenced by *MAPT* mRNA isoforms containing or lacking exon 10 (Buee and Delacourte, 1999; Qian and Liu, 2014).

Importantly, we have demonstrated that mitochondrial complex I inhibitor, MPP+, causes skipping of alternatively spliced exon 35 in endogenous mRNA both in SH-SY5Y and Hela cells (Fig. 6A), which mimicks the effect exerted by IVS35+14C>T mutation in minigene system (Fig. 2B). This observation in addition supports the possible involvement of LRPPRC IVS35+14C>T variant in PD pathogenesis, given that administration of complex I inhibitors has become a standard approach in establishing animal and cellular PD models (Bove et al., 2005). MPP+ and annonacin were previously shown to act on splicing, increasing the levels of *MAPT* 4R isoform in human neurons, thought to contribute significantly to progressive supranuclear palsy pathogenesis (Bruch et al., 2014). Our results, therefore, highlight another possible consequence of MPP+ administration that may be related to PD pathogenesis. It remains to be established if LRPPRC mRNA isoform lacking exon 35 is upregulated in PD brains and whether it is accompanied by decrease in LRPPRC protein.

In addition, our study provides also some insight into the splicing regulation of exon 33 and 35. We show that hnRNPA1 is a negative regulator of exon 33 splicing both in minigene and endogenous context in Hela and SH-SY5Y cells, whereas TIA-1 exerts strong effect on exon 35. This could have important implications for neurodegenerative diseases as recent findings indicate that, for example, hnRNPA2B1 levels are upregulated in brains of patients with frontotemporal dementia with TDP-43-positive inclusions (Mohagheghi et al., 2016).

Finally, in this work, we have demonstrated that it is possible to rescue aberrant splicing caused by IVS32–3C>T and IVS35+14C>T by co-expression of adapted U1 snRNAs in a minigene system. The ability to obtain the rescue effect through engineered U1snRNAs targeting non-conserved intronic regions (in our case U1LRP33+12 recovers IVS32–3C>T minigene and more importantly corrects exon 33 skipping in the endogenous mRNA) has the advantage over mutation-adapted or exon-intron boundary complement U1s of reducing undesired off-target events (Dal Mas et al., 2015; Fernandez Alanis et al., 2012).

Although for the remaining minigene constructs we have not observed any differences between WT and MUT version in 3 different cell lines, it should be taken into account that in other tissues or in specific disease-related conditions, the splicing

environment may be substantially altered and lead to different outcomes than the ones we observed.

5. Conclusions

Our results represent the first analysis of the potential functional impact of LRPPRC intronic variants on splicing and strongly suggest that some of these variants could be causative (susceptibility) variants leading to complete (partial) protein depletion or to its structural or functional changes. This could influence PD risk and related disorders (e.g., AD, LSFC), where mitochondrial dysfunction (deficiency of mitochondrial complex I and IV) is claimed to be the one of the main mechanisms of pathogenesis. Our results open a new avenue for future studies on the functional significance of splicing alterations for the properties of the LRPPRC protein and its role in the pathogenesis of PD and related disorders.

Disclosure statement

The authors declare there are no actual or potential conflicts of interest relating to this work.

Acknowledgements

This work was supported by Thierry Latran Fondation (FTLAAP2013/Buratti/REHNPAALS) and the EU Joint Programme-Neurodegenerative Diseases JPND (project RiMod-FTD, MIUR decree n. 1381, dated 30/06/2015, Italy, Ministero della Sanita'). The authors also thank Dr Marco Baralle for critical reading of the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.neurobiolaging.2016.07.014>.

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