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Rita Sattler · Christopher J. Donnelly
Editors

RNA Metabolism in Neurodegenerative Diseases

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Rita Sattler • Christopher J. Donnelly
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RNA Metabolism in Neurodegenerative Diseases

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Editors
Rita Sattler
Department of Neurobiology and Neurology
Dignityhealth St. Joseph's Hospital
Barrow Neurological Institute
Phoenix, AZ, USA

Christopher J. Donnelly
Department of Neurobiology
University of Pittsburgh
Pittsburgh, PA, USA

Live Like Lou Center for ALS Research
University of Pittsburgh Brain Institute
Pittsburgh, PA, USA

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Preface

Neurodegenerative diseases are rapidly increasing in prevalence in the growing population of the elderly worldwide. With this comes an increased financial and social burden to care for the affected individuals. There are no effective treatments for these diseases and the mechanistic underpinnings of these progressive and often fatal disorders remain unclear. This emphasizes the urge to better understand the underlying disease mechanisms, which will then allow for the development of new and more effective treatments. RNA metabolism denotes a variety of distinct processes RNA undergoes to promote cellular health and function. It has become apparent in recent years that altered RNA metabolism significantly contributes to disease pathogenesis in numerous neurodegenerative disorders.

Dysfunction in RNA processing is a prevailing theme in neurodegeneration. This was first highlighted as a key pathobiology following the discovery of TDP-43 neuropathology in nearly all amyotrophic lateral sclerosis (ALS) patients and almost half of patients diagnosed with frontotemporal dementia (FTD). In healthy cells this protein is predominantly nuclear; however, in ALS/FTD patients TDP-43 is aggregated in the cytoplasm within the affected neurons. This neuropathology has since been identified in 30% of patients diagnosed with Alzheimer's disease (AD) and nearly 80% of patients diagnosed with chronic traumatic encephalopathy (CTE). Subsequent studies uncovered a number of autosomal dominant mutations in ALS patient populations within the *TARDBP* gene, which encodes the TDP-43 protein. TDP-43 is an RNA-binding protein that functions in a variety of aspects of RNA processing, including splicing, mRNA transport, RNA stability, and micro-RNA processing to name a few. Since this discovery, nearly two dozen mutations in genes that encode RNA-binding proteins have been identified in ALS patients. FUS protein, an RNA-binding protein that exhibits remarkable functional similarity to TDP-43, demonstrates a similar neuropathology in a subset of FTD patients and autosomal dominant mutations in this gene have been identified in ALS patients. In addition to mutations in genes encoding RNA-binding proteins, microsatellite repeat expansions have recently been identified as a causative mutation in subsets of ALS/FTD patients. One mechanism underlying their pathobiology is the generation of non-coding RNA species that are considered "toxic" since they disrupt a variety of RNA/

RBP interactions. This was initially described in patients with myotonic dystrophy but has since been observed in other genetic neurodegenerative disorders including spinocerebellar ataxia type 8 (SCA8) and C9orf72 ALS/FTD. The production of noncoding RNA species is, in part, due to pathogenic epigenetic modifications that allow for the transcription of these RNA species. A-to-I RNA editing defects have been identified in ALS neurons and this can disrupt the function of calcium channels and promote hyperexcitability, a known pathophysiology of ALS motor neurons. Beyond ALS/FTD, RNA mis-splicing and improper ribonucleoprotein (RNP) assembly are common features of spinal muscular atrophy (SMA) due to the genetic mutation responsible for this developmental neurodegenerative disease. Abnormal RNP assembly/disassembly and RNA stability are similarly implicated in a wide range of neurodegenerative disorders, including ALS/FTD, AD, and Huntington's disease (HD) to name a few. Together, these highlight an intrinsic deficit in RNA metabolism across a wide range of genetic and nongenetic neurodegenerative conditions. This common etiology supports the need for a better understanding of these dysfunctions to uncover novel therapeutic targets for drug development for a large patient population with unmet needs.

With this book series on *RNA Metabolism in Neurodegenerative Diseases* we aim to provide review articles addressing the different aspects of RNA pathobiology that contribute to neurodegeneration. These articles summarize the most recent and novel studies highlighting our current understanding of pathologic RNA metabolism. Specifically, we focus on epigenetic changes that alter coding and noncoding RNA expression; abnormal splicing and editing events; impaired RNA transport, RNA stability, and RNP assembly that promotes protein misfolding; the genetic mutations that generate toxic RNA species or dysfunctional RBPs; and deficits in the translation of mRNAs.

The first three chapters of this series provide examples of how direct RNA modifications contribute to disease pathogenesis in varying neurodegenerative diseases. In the first chapter, Veronique Belzil and colleagues review the critical aspects of epigenetic regulations in the ALS and FTD disease spectrum (Chap. 1). Epigenetics generally defines modifications of gene expression without the alteration of the underlying primary nucleotide sequences. Increasing evidence suggests that cell-specific and tightly regulated aberrant RNA processing events of noncoding RNAs are contributing to the epigenetic changes observed during neurodegenerative disease development and progression. This can result in the production of coding and noncoding RNA species that perturb neural function. The following review by Ravindra Singh and Natalia Singh provide a comprehensive overview on spinal muscular atrophy (SMA), the most common genetic cause of infant death. Specifically, they focus on how mutation-induced RNA splicing deficits of the *survival motor neuron 1 (SMN1)* gene lead to disease pathogenesis (Chap. 2). This chapter focuses on the role of cis-elements and transacting factors regulating *SMN* splicing and highlights how these exemplary studies led to the first FDA-approved therapeutic treatment for SMA patients. Notably, this recent therapeutic breakthrough is the only effective treatments to significantly halt disease progression for any neurodegenerative disorder and functions by targeting SMN2 RNA. The next

chapter to address RNA modifications, authored by Rita Sattler and colleagues (Chap. 3), summarizes the role of RNA editing in neurodegenerative diseases including ALS, AD, HD, and others. RNA editing increases the diversity of translated protein variants and can thereby significantly alter the function of target genes, which is critically relevant for many aspects of central nervous system (CNS) function, as discussed in this review.

The next four chapters summarize mechanisms of RNA transport and stability in neurodegenerative diseases. The transport of RNA from the nucleus to the cytoplasm is required for the translation of mRNAs. Recent evidence suggests that nucleocytoplasmic transport is impaired in a variety of genetic and nongenetic neurodegenerative disorders. The chapter by Boehringer and Bowser reviews common cellular mechanism of nucleocytoplasmic RNA transport and further discusses how these pathways are perturbed in neurodegeneration (Chap. 4). RNA stability is of greatest necessity to ensure cellular function and is therefore tightly regulated via numerous complementary mechanisms. Kaitlin Weskamp and Sami Barmada provide a comprehensive overview of these mechanisms and summarize exciting studies presenting evidence to support that aberrant RNA degradation and turnover contribute to neurodegenerative disease pathogenesis (Chap. 5). Ribonucleoprotein complex assembly is a critical step in the formation of mRNP transport particles and RNA containing membraneless organelles, including stress granules. In the next chapter Wilfried Rossoll and colleagues discuss the mechanisms underlying abnormal RNP transport granule assembly in SMA. They also discuss the consequence of dysfunction of this pathway on physiological cellular growth, neuronal maturation, and synaptic plasticity (Chap. 6). In the final chapter of this section, Ross Buchan and colleagues describe the role of ribonucleoprotein granules in ALS (Chap. 7). During cellular stress, stress granules (SGs) form and function to halt translation until the stressor is removed. Many ALS-causing mutations are found in genes whose protein products comprise these RNP granules and, when incorporated, alter SG dynamics. Persistent SG formation is hypothesized to induce protein misfolding and a key event in the seeding of TDP-43 neuropathology.

The final four chapters provide in-depth reviews of disease-causing mutations that disrupt RNA metabolism. Auinash Kalsotra and colleagues first describe how noncoding microsatellite repeat expansions alter RNA processing due to sequestration of RBPs as observed in myotonic dystrophy (Chap. 8). Interestingly, similar intronic repeat expansions were recently identified as the most common genetic cause of ALS/FTD and are thought to act through similar mechanisms. The following chapter by Janice Robertson and colleagues discusses a variety of proposed mechanisms on how the RNA-binding protein TDP-43 might contribute to disease pathogenesis in ALS and FTD (Chap. 9). Mutations in TDP-43 lead to ALS, but the interesting fact is that up to 97% of ALS patients and up to 45% of FTD patients exhibit cytoplasmic aggregation of wild-type TDP-43, independent of disease etiology. This discovery has made TDP-43 one of the most studied RNA-binding proteins in neurodegenerative diseases and different mechanisms leading to these aggregations have been proposed since. This chapter provides a comprehensive overview of the nuclear and cytoplasmic function of TDP-43 and how defects in

these functions might lead to neurodegeneration. Craig Bennett and Albert LaSpada next provide an in-depth discussion on the consequence of mutations in the Senataxin protein on neural health (Chap. 10). Senataxin is a DNA-RNA helicase and mutations in the *SETX* gene are associated with a variety of neurodegenerative diseases, including juvenile-onset ALS and cerebellar ataxia with oculomotor apraxia type 2. This exciting chapter describes the role of Senataxin on RNA processing. We conclude this book with a chapter by Erik Lehmkuhl and Daniela Zarnescu with a focus on the final step in mRNA processing—translation (Chap. 11). Here, the authors provide an in-depth review on the mechanisms of translational inhibition in the context of a variety of neurodegenerative disorders and the implication of dysfunction of this process on cellular proteostasis.

In conclusion, these chapters provide a current view into the rapidly growing areas of investigation of the pathobiology of RNA metabolism and RBP dysfunction in neurodegenerative diseases. This book includes in-depth reviews on a variety of RNA-binding proteins across multiple disorders. We hope the contents of this book stimulate provocative discussions and inspire novel avenues of investigation to further broaden our knowledge of pathologic RNA metabolism with the ultimate goal of translating these discoveries into therapeutic programs for patients.

Phoenix, AZ, USA — Rita Sattler
Pittsburgh, PA, USA — Christopher J. Donnelly

Contents

Contributors

Sami J. Barmada Neuroscience Graduate Program and Department of Neurology, University of Michigan School of Medicine, Ann Arbor, MI, USA

Veronique V. Belzil Department of Neuroscience, Mayo Clinic, Jacksonville, FL, USA

Department of Neurology and Neurosurgery, McGill University, Montreal, QC, Canada

Craig L. Bennett Department of Neurology, Duke University School of Medicine, Durham, NC, USA

Department of Neurobiology, Duke University School of Medicine, Durham, NC, USA

Department of Cell Biology, Duke University School of Medicine, Durham, NC, USA

Ashley Boehringer Department of Neurobiology, Barrow Neurological Institute, Phoenix, AZ, USA

School of Life Sciences, Arizona State University, Phoenix, AZ, USA

Robert Bowser Department of Neurobiology, Barrow Neurological Institute, Phoenix, AZ, USA

J. Ross Buchan Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ, USA

Mark T. W. Ebbert Department of Neuroscience, Mayo Clinic, Jacksonville, FL, USA

Nichole Eshleman Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ, USA

Nikita Fernandes Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ, USA

Auinash Kalsotra Department of Biochemistry, University of Illinois, Urbana-Champaign, IL, USA

Carl R. Woese Institute of Genomic Biology, University of Illinois, Urbana-Champaign, IL, USA

Rebecca J. Lank Department of Neurology, University of Michigan, Ann Arbor, MI, USA

Erik M. Lehmkuhl Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ, USA

Feikai Lin Department of Biochemistry, University of Illinois, Urbana-Champaign, IL, USA

Ileana Lorenzini Department of Neurobiology, Barrow Neurological Institute, Dignity Health, St. Joseph's Hospital and Medical Center, Phoenix, AZ, USA

Chaitali Misra Department of Biochemistry, University of Illinois, Urbana-Champaign, IL, USA

Stephen Moore Department of Neurobiology, Barrow Neurological Institute, Dignity Health, St. Joseph's Hospital and Medical Center, Phoenix, AZ, USA

Interdisciplinary Graduate Program in Neuroscience, Arizona State University, Tempe, AZ, USA

Dmytro Morderer Department of Neuroscience, Mayo Clinic, Jacksonville, FL, USA

Phillip L. Price Department of Neuroscience, Mayo Clinic, Jacksonville, FL, USA

Department of Cell Biology, Emory University, Atlanta, GA, USA

Janice Robertson Tanz Centre for Research in Neurodegenerative Diseases and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada

Wilfried Rossoll Department of Neuroscience, Mayo Clinic, Jacksonville, FL, USA

Rita Sattler Department of Neurobiology and Neurology, Dignityhealth St. Joseph's Hospital, Barrow Neurological Institute, Phoenix, AZ, USA

Marc Shenouda Tanz Centre for Research in Neurodegenerative Diseases and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada

Natalia N. Singh Department of Biomedical Sciences, Iowa State University, Ames, IA, USA

Ravindra N. Singh Department of Biomedical Sciences, Iowa State University, Ames, IA, USA

Albert R. La Spada Department of Neurology, Duke University School of Medicine, Durham, NC, USA

Department of Neurobiology, Duke University School of Medicine, Durham, NC, USA

Department of Cell Biology, Duke University School of Medicine, Durham, NC, USA

Duke Center for Neurodegeneration & Neurotherapeutics, Duke University School of Medicine, Durham, NC, USA

Anna Weichert Tanz Centre for Research in Neurodegenerative Diseases and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada

Kaitlin Weskamp Neuroscience Graduate Program and Department of Neurology, University of Michigan School of Medicine, Ann Arbor, MI, USA

Daniela C. Zarnescu Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ, USA

Department of Neuroscience, University of Arizona, Tucson, AZ, USA

Department of Neurology, University of Arizona, Tucson, AZ, USA

Ashley B. Zhang Tanz Centre for Research in Neurodegenerative Diseases and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada

About the Editors

Rita Sattler is an associate professor of neurobiology at the Barrow Neurological Institute in Phoenix, AZ. She received her master's and doctorate degree in neurophysiology from the University of Toronto in Toronto, Canada, where she studied mechanisms of neurodegeneration in stroke. As a postdoctoral fellow at Johns Hopkins University, Dr. Sattler focused her research on studies of synaptic biology and glutamate receptor function. Her current research combines her expertise in neurodegeneration and synaptic function and is aimed at the elucidation of synaptic dysfunction in neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). The Sattler laboratory primarily uses human patient-derived induced pluripotent stem cells differentiated into neurons and glial cells as a disease model and employs state-of-the-art molecular, biochemical, physiological, and imaging technologies to identify novel disease pathways and therapeutic targets.

Christopher J. Donnelly Christopher Donnelly is an assistant professor of neurobiology and a member of the Live Like Lou Center for ALS Research at the University of Pittsburgh School of Medicine in Pittsburgh, PA. He received his Ph.D. in molecular biology and genetics at the University of Delaware where he studied RNA trafficking and local translation during axon regeneration. As a postdoctoral fellow at Johns Hopkins University School of Medicine, Dr. Donnelly employed patient-derived induced pluripotent stem cell (iPSCs) neurons to study the pathogenic

mechanisms underlying mutations that cause amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). These studies revealed RNA-based mechanisms of neurotoxicity and defects in the nucleocytoplasmic transport pathway as drivers of disease. Dr. Donnelly's lab at the University of Pittsburgh currently focuses on employing human iPSC-derived cultures and Drosophila models to elucidate the pathogenic mechanism that contribute to neurodegeneration. Specifically, his lab studies how genetic mutations alter nucleocytoplasmic transport of RNA and proteins and developed a photokinetic approach to understand the triggers and consequences of intracellular protein aggregation that are pathological hallmarks of neurodegenerative diseases.

Abbreviations

3′UTR	3′ Untranslated region
5′UTR	5′ Untranslated region
5caC	5-Carboxylcytosine
5fC	5-Formylcytosine
5hmC	5-Hydroxymethylcytosine
$5HT_{2c}$	Serotonin receptor subunit 2C
5mC	Methylation of carbon 5 on cytosine
A site	Acceptor site
A/I	Adenosine to inosine
AD	Alzheimer's disease
ADARs	Adenosine deaminase acting on double-stranded RNA
ALS	Amyotrophic lateral sclerosis
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	AMPA receptor, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
ARE	Adenylate-uridylate-rich element
ASD	Autism spectrum disorder
AUF1	ARE RNA-binding protein 1
c9ALS	Amyotrophic lateral sclerosis patients carrying a pathogenic *C9orf72* repeat expansion
c9FTD/ALS	Frontotemporal dementia and amyotrophic lateral sclerosis patients carrying a pathogenic *C9orf72* repeat expansion
C9orf72	Chromosome 9 open reading frame 72
carboxy-DCFDA	5-(and-6)-Carboxy-2′,7′-dichlorofluorescin diacetate
CBC	Cap-binding complex
CDE	Constitutive decay element
CH	Cerebellar hypoplasia
CHMP2B	Chromatin modifying protein 2B
CNS	Central nervous system
CpG	Cytosine followed by guanine in cis
CPSF	Cleavage/polyadenylation specificity factor

DENN	Differentially expressed in normal and neoplastic cells
DM	Myotonic dystrophy
DM1	Myotonic dystrophy type 1
DM2	Myotonic dystrophy type 2
DMPK	Myotonic dystrophy protein kinase
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferases
DPR	Dipeptide repeat
dsRBDs	Double-stranded RNA-binding domains
E Site	Exit site
EEJ	Exon-exon junction
Eif2α	Eukaryotic initiation factor 2 alpha
EJC	Exon junction complex
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
eRNAs	Enhancer RNAs
FMR1	Fragile X mental retardation gene 1
FMRP	Fragile X mental retardation protein
FTD	Frontotemporal dementia
FTO	Fat mass and obesity-associated protein
FUS	Fused in sarcoma
FXTAS	Fragile X-associated tremor ataxia syndrome
G_4C_2	GGGGCC nucleotides
GABA*	Gamma-aminobutyric acid
GBM	Glioblastoma
GFP	Green fluorescent protein
GluA2	Glutamate ionotropic receptor AMPA type subunit 2
GRN	Gene encoding progranulin
H2A	Histone 2A
H2B	Histone 2B
H3	Histone 3
H4	Histone 4
HATs	Histone acetyltransferases
HD	Huntington's disease
HDACs	Histone deacetylases
HERV	Human endogenous retroviruses
HMTs	Histone methyltransferases
HpC	Hippocampus
hPGRN	Human progranulin
HRE	Hexanucleotide repeat expansion
hSOD1	Human superoxide dismutase 1
HTT	Huntingtin gene
IP	Inferior parietal lobe
iPSCs	Induced pluripotent stem cells
IRES	Internal ribosome entry site

Juvenile ALS	ALS4
KDMs	Lysine demethylases
KO	Knockout
$K_v1.1$	Potassium voltage-gated channel subfamily A member 1
lincRNAs	Large intergenic noncoding RNAs
lncRNAs	Long noncoding RNAs
m^5C	RNA methylation at cytosine
m^6A	RNA methylation at adenine
m^7G	RNA methylation at guanine
MAPT	Microtubule-associated protein tau
MBNL	Muscleblind
mCA	Methylation of cytosine followed by adenine
mCG	Methylation of cytosine followed by guanine
miRNA	Micro-RNA
miRNAs	Micro-RNAs
MRE	miRNA recognition element
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
ncRNA	Noncoding RNA
nDNA	Nuclear DNA
NES	Nuclear export sequence
NGD	No-go decay
NLS	Nuclear localization sequence
NMD	Nonsense-mediated decay
NMJ	Neuromuscular junction
NPC	Nuclear pore complex
NSD	Nonstop-mediated decay
NXF1	Nuclear export factor 1
Orf/c9orf72	Open reading frame
P site	Peptidyl site
PAB2	Polyadenylate binding protein 2
PARs	Promoter-associated RNAs
P-bodies	Processing bodies
PCH1	Pontocerebellar hypoplasia type 1
PCR	Polymerase chain reaction
PD	Parkinson's disease
PERK	PRKR-like ER kinase
piRNAs	Piwi-interacting RNAs
poly(A)	Long chain of adenine nucleotides
pre-miRNAS	Precursor miRNAs
pri-miRNAs	Primary miRNAs
PRMTs	Protein arginine methyltransferases
PSP	Progressive supranuclear palsy
PTC	Premature stop codons
RAN	Repeat associated Non-AUG

RAN translation	Repeat-associated non-AUG translation
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNAseq	RNA sequencing
RNP	Ribonucleoprotein particle
ROS	Reactive oxygen species
RRBS	Reduced representation bisulfite sequencing
RRM	RNA recognition motif
rRNA	Ribosomal RNA
RUST	Regulated unproductive splicing and translation
S6K1	S6 kinase 1
sALS	Sporadic amyotrophic lateral sclerosis
SBS	Staufen-binding site
SCA8	Spinocerebellar ataxia type 8
SCI	Spinal cord injury
SG	Stress granule
siRNA	Small interfering RNA
siRNAs	Small interfering RNAs
SKAR	S6 kinase 1 Aly/REF-like target
SLBP	Stem loop-binding protein
SLC1A2	Solute carrier family 1 member 2
SMD	Staufen-mediated decay
SMTG	Superior middle temporal gyri
SOD1	Superoxide dismutase 1
sRNAs	Short RNAs
Stau1	Staufen-1
SUnSET	Surface sensing of translation
SURF	Surveillance complex
TARDBP	TAR DNA-binding protein 43
TDP-43	TAR DNA binding protein—43 kilodaltons
TE	Transposable element
TRAP	Tagged ribosome affinity purification
TREX complex	Translation export complex
tRNA	Transfer RNA
TTP	Tristetraprolin
uORF	Upstream open reading frame
UPR	Unfolded protein response
UTR	Untranslated region
VCP	Valosin-containing protein
VEGF	Vascular endothelial growth factor A

Chapter 1
An Epigenetic Spin to ALS and FTD

Mark T. W. Ebbert, Rebecca J. Lank, and Veronique V. Belzil

Abstract Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are two devastating and lethal neurodegenerative diseases seen comorbidly in up to 15% of patients. Despite several decades of research, no effective treatment or disease-modifying strategies have been developed. We now understand more than before about the genetics and biology behind ALS and FTD, but the genetic etiology for the majority of patients is still unknown and the phenotypic variability observed across patients, even those carrying the same mutation, is enigmatic. Additionally, susceptibility factors leading to neuronal vulnerability in specific central nervous system regions involved in disease are yet to be identified. As the inherited but dynamic epigenome acts as a cell-specific interface between the inherited fixed genome and both cell-intrinsic mechanisms and environmental input, adaptive epigenetic changes might contribute to the ALS/FTD aspects we still struggle to comprehend. This chapter summarizes our current understanding of basic epigenetic mechanisms, how they relate to ALS and FTD, and their potential as therapeutic targets. A clear understanding of the biological mechanisms driving these two currently incurable diseases is urgent—well-needed therapeutic strategies need to be developed soon. Disease-specific epigenetic changes have already been observed in patients and these might be central to this endeavor.

Keywords Amyotrophic lateral sclerosis · Epigenetic modifications · Frontotemporal dementia · Methylation · RNA-mediated regulation

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Mark T. W. Ebbert and Rebecca J. Lank contributed equally to this work.

M. T. W. Ebbert
Department of Neuroscience, Mayo Clinic, Jacksonville, FL, USA

R. J. Lank
Department of Neurology, University of Michigan, Ann Arbor, MI, USA

V. V. Belzil (✉)
Department of Neuroscience, Mayo Clinic, Jacksonville, FL, USA

Department of Neurology and Neurosurgery, McGill University, Montreal, QC, Canada
e-mail: Belzil.Veronique@mayo.edu

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1.1 Introduction

Amyotrophic lateral sclerosis (ALS) is the most prevalent motor neuron disease, causing progressive degeneration of upper and lower motor neurons in 2–3 individuals per 100,000 worldwide [1, 2]. Clinically, ALS is characterized by rapidly progressing muscle weakness and spasticity leading to paralysis and eventually respiratory failure [3]. Patients present with symptoms at a mean age of 55 years and die within 2–4 years [4].

Patients with ALS are also frequently affected by the second most common form of early-onset dementia known as frontotemporal dementia (FTD). While patient symptoms will start with either ALS or FTD, 13–15% of patients will eventually develop symptoms for the other disease [5–9]. This strong link between ALS and FTD was observed before researchers were able to link them genetically. FTD has a prevalence of 15–22 individuals per 100,000 worldwide [10] and is characterized by disturbances in behavior, personality, and language as a result of neurodegeneration in the frontal and temporal lobes. Cognitive impairments are usually detected around age 58, and it is fatal within 6–10 years after symptoms onset [11].

Historically, researchers have extensively searched for the underlying genetic etiologies for these conditions, yet, despite strong efforts to uncover genetic causes, the vast majority of ALS/FTD patients have no known genetic cause and no family history. The discovery of the *C9orf72* DNA GGGGCC (G_4C_2) repeat expansion in ALS and FTD patients brought to light the possibility of RNA-mediated toxicity in disease which could be controlled by epigenetic mechanisms. In this chapter, we will summarize the major epigenetic regulation mechanisms. Then, we will review the current knowledge of epigenetic regulation as it relates to ALS and FTD (Fig. 1.1).

1.2 Epigenetic Mechanisms

The epigenome is generally defined as the collection of heritable but dynamic mechanisms that largely dictate access to the DNA for templated functions such as gene transcription, DNA synthesis, and repair without altering the primary nucleotide sequence; there is, however, a valid argument that chimeric DNA, which does modify the primary nucleotide sequence, is facilitated by the epigenome. These intricacies regarding how far-reaching the epigenome's involvement is in nucleotide sequence will be continually clarified as the field matures. Here, we will focus on general regulation processes.

Post-transcriptional regulation of RNA function and metabolism—by noncoding RNAs (ncRNAs), such as microRNA, or chemical modifications to ribonucleotides—is now also considered part of epigenetic control. Whereas an individual's genome is mostly established at conception, the individual's inherited epigenome continues to change throughout embryonic, fetal, and postnatal life in response to

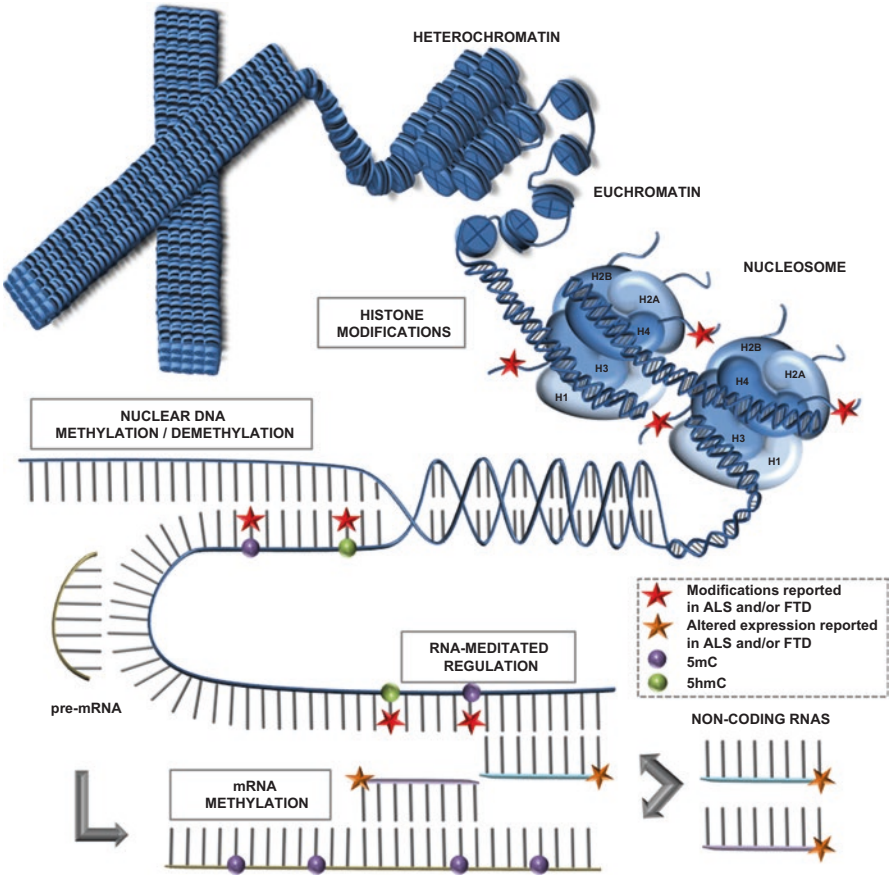

Fig. 1.1 Schematic representation of the major epigenetic regulatory mechanisms and their interactions. The major epigenetic regulatory mechanisms are framed. Red stars mark epigenetic changes known to be involved in ALS/FTD. Orange stars indicate that altered expression of these RNAs has been reported in ALS/FTD. Purple spheres represent 5mC marks while green spheres represent 5hmC marks

cell-intrinsic mechanisms and environmental input. Epigenetic changes are cell-specific and are presumed to be adaptive responses to internal and external stimuli.

As next-generation sequencing is becoming more commonplace, the epigenome is garnering greater attention in many fields, from developmental biology to environmental epidemiology, because it offers a potential mechanistic link between the environment and phenotype. For ALS/FTD researchers, epigenetics is a particularly interesting field that may explain the clinical variability within families sharing ALS/FTD-causing genetic variants, and the cause of disease in patients affected sporadically.

Epigenetic control of genomic functions occurs by regulating nucleosome position and density via histone chaperones and chromatin remodeling complexes [12].

Nucleosomes are the fundamental repeating unit of chromatin, and consist of nucleoprotein complexes containing two copies each of four core histone proteins (H2A, H2B, H3, and H3) and 147 base pairs of DNA wrapped 1.67 turns counterclockwise around this globular structure [13]. Key signals directing nucleosome positioning and chromatin structure include chemical modifications to the DNA and histones, as well as ncRNA species. Epigenetic regulation is also exerted via post-transcriptional regulation of RNA function and metabolism. This section will briefly review our current understanding of these epigenetic mechanisms.

1.2.1 Nuclear DNA Methylation

DNA methylation is the biochemical addition of a methyl group to a nucleotide, often resulting in altered transcription factor binding, chromatin remodeling, and ultimately altered gene expression. Methylation of the carbon 5 on cytosine (5mC) is the most studied and best understood DNA modification.

5mC most frequently occurs at cytosine-guanine nucleotide sequences (mCG), often referred to as CpG sites. Genomic regions enriched in CpG sites, referred to as CpG islands, are often associated with gene promoters. Whether located near transcription initiation sites or distant from annotated promoters, CpG islands play a central role in destabilizing nucleosomes and recruiting proteins that initiate chromatin remodeling [14–16]. Generally, methylation in a CpG island is associated with the reduced expression of a nearby gene.

Recent research has found 5mC marks at cytosine-adenine sequences (mCA). It is believed that mCA may be particularly important in the central nervous system, as the number of mCA and mCG marks detected in the adult brain are approximately equal [17]. Although both mCA and mCG are associated with transcriptional repression, early evidence suggests mCA may be particularly important for more precise transcription regulation—1–2-fold expression changes compared to 100–1000-fold changes observed with mCG [18].

DNA methylation is catalyzed by DNA methyltransferases (DNMTs) 1, 3A, and 3B. While DNMT3A and DNMT3B are associated with de novo methylation, DNMT1 is mainly responsible for maintaining methylation across cell generations by methylating the daughter strand of DNA during cell division, thus preventing hemimethylation of double-stranded DNA [19]. Because of DNMT1's maintenance function, DNA methylation is considered a fairly stable epigenetic mark. Further research into the differentiating roles of DNMT3A and DNMT3B are warranted, as early evidence suggests DNMT3A may be primarily responsible for mCA methylation. Its proper regulation may be especially critical in the brain [20–22].

Bisulfite conversion is currently the preferred method to detect cytosine methylation. Briefly, bisulfite ion deaminates unmethylated cytosines, which result in its chemical conversion to uracil upon alkaline desulfonation [23]. As bisulfite converts 5mCs much more slowly, there is a selective conversion favoring unmethylated cytosines. After DNA amplification, all unconverted cytosines are considered

methylated. Importantly, standard bisulfite conversion does not differentiate methylated cytosines from cytosines that have been demethylated, which is the topic of the next section.

1.2.2 Nuclear DNA Demethylation

Although DNA methylation is considered a stable epigenetic change, it is now well understood that it is closely and dynamically regulated not only by epigenetic "writers," such as DNMTs, but also by "eraser" mechanisms, which keep CpGs unmethylated. Importantly, demethylation is of particular interest in neurodegeneration research because, among all organs, its footprints are most abundant in the brain and further increase with age [24].

DNA demethylation can occur as a passive or active process. Passive demethylation occurs when the process of copying methylation marks onto the daughter strand of DNA is impaired. As a result, future cell generations will lose methylation marks by dilution. Active demethylation is mediated by TET family of dioxygenases, which catalyze the oxidation of 5mC in a stepwise manner, resulting in 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). Oxidation is followed by replication-dependent dilution or thymine DNA glycosylase-mediated base excision and repair, resulting in full demethylation and restoration to unmodified cytosine [25]. 5fC- and 5caC-containing regulatory elements show very limited overlap and are therefore believed to play distinct regulatory roles. 5caC sites are more active than 5fC sites, and both are more active than regions decorated by 5hmC [26].

Our current understanding is that DNA methylation and demethylation processes must be highly orchestrated to appropriately regulate the transcriptome. Further research is necessary to elucidate the exact functions of 5hmC, 5fC and 5caC as it is still difficult to estimate the extent to which these events occur and appreciate their full potential as therapeutic targets.

1.2.3 Mitochondrial DNA Methylation

While nuclear DNA (nDNA) methylation is well recognized, mitochondrial DNA (mtDNA) methylation is still controversial. Using restriction enzyme cleavage, early studies estimated that about 2–5% of mtDNA CCGG sequences are fully methylated whereas the remainder of mtDNA is unmethylated [27]. Mass spectrometry confirmed the presence of methylated bases in human mtDNA [28]. Later, using affinity-based methods, both 5mC and 5hmC modifications were found in the D-loop region of human mtDNA at both CpG and non-CpG dinucleotides [29, 30]. Other studies used bisulfite conversion coupled with sequencing or pyrosequencing and claimed that mtDNA methylation may be as high as 2–18% in the D-loop region

[31, 32]. Recent reports however suggest that mtDNA methylation levels may have been greatly overestimated due to the circular structure of mtDNA, which affects bisulfite conversion efficiency [33–35]. Nonetheless, the reported presence of methyltransferases DNMT1 and DNMT3A inside mitochondria suggests that methylation may take place in mtDNA [30, 36]. Since nDNA and mtDNA interact to modulate the transcriptome [37, 38], future studies need to better characterize mtDNA modifications and their potential role in the regulation of gene transcription.

1.2.4 RNA Methylation

Epigenetic regulation goes beyond transcription. RNA is also under epigenetic regulation and can be methylated or otherwise modified. In fact, methylation occurs in most RNA classes, but the exact function of RNA modifications remains unclear due to limitations in detection and quantification.

Whereas DNA methylation has mainly been observed at cytosines, RNA methylation can occur at cytosine (m^5C), adenine (m^6A), and guanine (m^7G). m^7G (N^7-methylguanine) at the 5′ cap structure is the most widely studied RNA methylation and is necessary for the translation of most messenger RNAs (mRNAs). m^7G cap also mediates nuclear transport of some mRNAs, preserves mRNA stability by protecting it from degradation, and facilitates other processing such as the addition of poly(A) tails to mRNAs [39, 40]. m^7G cap methylation is reversible and can either go through the process of "decapping," where the entire m^7G cap is removed [41], or potentially through m^7G demethylation.

In contrast to the extensively studied 5mC in DNA, the m^5C (N^5-methylcytosine) RNA modification has not been thoroughly explored. While it is still unclear how m^5C is regulated by DNMTs, a study suggested that mouse Dnmt2 RNA methyltransferase may be required for epigenetic heredity [42]. Early studies also demonstrated that m^5C affects interactions of long noncoding RNAs (lncRNAs) with chromatin-associated protein complexes [43].

The most abundant mRNA modification is m^6A (N^6-methyladenine). Dominissini et al. found that silencing m^6A methyltransferase—mostly acting at highly conserved long internal exons, stop codons, and 3′UTRs—alters gene expression and alternative splicing patterns [44]. The same group conducted affinity-based m^6A profiling and identified proteins that bind specifically to m^6A. Their findings suggest that RNA methylation, through the binding of these m^6A-specific binding proteins, may disturb RNA binding proteins' affinity to associate with partner unmethylated RNAs. Importantly, these m^6A-binding proteins recruit additional factors that facilitate functions such as alternative splicing, nuclear export, and mRNA stability [40, 44]. One example is the m^6A-binding protein YTHDC1, which facilitates the export of methylated mRNA from the nucleus to the cytoplasm in vitro. Knockdown of YTHDC1 results in retention of nuclear m^6A-containing mRNA, where transcripts accumulate in the nucleus and are depleted from the cytoplasm [45]. Of interest,

m^6A has been the first reversible modification identified in coding and noncoding RNAs after the discovery that the fat mass and obesity-associated protein (FTO) act as a m(6)A demethylase [46–48], suggesting that, analogous to reversible DNA, reversible RNA methylation may affect gene expression and downstream regulation of RNA-related cellular pathways.

1.2.5 RNA-Mediated Regulation

While 70–90% of the genome is transcribed, only 1–3% of these transcripts encode proteins [49, 50]. As such, the transcriptome is mainly composed of ncRNAs having infrastructural and regulatory functions. These transcripts are gaining increased attention, not only for their recognized roles in transcriptional and post-transcriptional regulation, but also for their targeted effects on gene expression, making them attractive therapeutic targets.

ncRNAs are divided into short (<30 nucleotides) and long ncRNAs (>200 nucleotides), and each subtype of ncRNA fulfills very specific regulatory roles. Specifically, short RNAs (sRNAs) are sub-categorized into micro-RNAs (miRNAs), piwi-interacting RNAs (piRNAs), and small interfering RNAs (siRNAs). lncRNAs are generally sub-classified according to their proximity to protein coding genes—sense, anti-sense, bidirectional, intronic, and intergenic. Apart from their size, sRNAs and lncRNAs have largely different functions in transcription regulation. Two new classes of ncRNAs have also been recently recognized: enhancer RNAs (eRNAs) and promoter-associated RNAs (PARs).

Mature miRNAs are single-stranded 20–24 nucleotide sequences derived from precursor miRNA (pre-miRNAS). These are ~70 nucleotide transcripts with distinct hairpin structures initially derived from nuclear primary miRNA (pri-miRNAs). Mature miRNAs pair with complementary sequences on target mRNA transcripts through the 3′ untranslated region (3′UTR) and repress the targeted mRNA translation after recruiting the RNA-induced silencing complex (RISC) [51, 52]. Mature miRNAs may have several target mRNAs, but their effect on their targets is a modest 1–2-fold change in expression [53]. Although miRNAs seem to initiate modest changes in their targets, changes in expression of miRNAs themselves associate with widespread mRNA expression changes, indicating that miRNAs are central to global expression regulation [53]. In fact, a large screening of the mammalian genome found hundreds of mRNAs with conserved pairing to specific miRNAs, with an enrichment of genes involved in transcriptional regulation [54]. Specifically, several miRNAs target DNMTs that can have far-reaching impact on global methylation levels, which has been implicated in several cancers and autoimmune diseases [55]. The story becomes more complicated when one considers that miRNA expression can in turn be modulated by DNA methylation and histone modifications [55, 56], suggesting that transcriptional regulation needs to be well orchestrated by an epigenetic–ncRNA feedback loop. This highlights the complexity of epigenetics and the interdependence of these mechanisms to regulate gene expression.

piRNAs are 24–31-nucleotide sequences able to form complexes with Piwi proteins of the Argonaute family. They are part of a complex population of small RNAs highly enriched in male gonads [57]. Their main function is to silence transposable elements. These mobile elements are autonomous sequences of DNA that replicate and insert themselves into the genome, potentially introducing detrimental DNA damage. Some results also suggest a role for piRNA in transcriptional regulation and deadenylation-mediated mRNA degradation [58].

Mature siRNAs are 20–24 nucleotide sequences that modulate a given gene's expression after binding to its complementary nucleotide sequence through a process called RNA interference (RNAi). siRNAs mediate post-transcriptional silencing in a way similar to miRNAs, and may also suppress transposon activity in a way similar to piRNAs [59, 60].

lncRNAs are sequences longer than 200 nucleotides characterized by low nuclear expression and low conservation across species. One exception is highly conserved large intergenic noncoding RNAs (lincRNAs), which recruit histone-modifying complexes and transcription factors to transcriptionally modulate targeted chromosomal regions [61, 62]. The majority of lncRNAs are transcribed as complex networks of sense and anti-sense transcripts overlapping protein-coding genes.

eRNAs are sequences of about 800 nucleotides on average that are derived from regions enriched in RNA Polymerase II and transcriptional co-regulators. What makes the genomic sites encoding eRNAs different from those of other lncRNAs is their specific histone methylation signature at histone 3 lysine 4 (H3K4), which is typical of enhancer sites. Similar to lincRNAs, eRNAs are evolutionarily conserved but have a short half-life. eRNA expression levels correlate with the expression of nearby genes, and is thus dynamically regulated upon signaling [63, 64]. eRNAs are believed to act as transcriptional activators.

PARs are short half-life transcripts that can be classified as short or long ncRNAs—their size range from 16 to over 200 nucleotides. PARS are either expressed near transcription start sites or from elements of the promoter [65], from both strands and in divergent orientation with respect to the transcription start site. Most PARS associate with highly expressed genes while being weakly expressed themselves. Increasing evidence demonstrates that PARs may associate with both transcriptional activation and repression [66–69].

1.2.6 Histone Modifications

As discussed above, the density of nucleosomes determines the accessibility of DNA to protein complexes performing templated functions. Heterochromatin contains tightly wound DNA, where gene transcription is repressed. Euchromatin is a looser conformation of the DNA, which is conducive to gene transcription. Chromatin conformation is dynamic and is regulated in part by covalent posttranslational histone modifications, which mainly occur at amino acid residues within unstructured histone "tails." These modifications include acetylation and

methylation of lysines, methylation of arginines, and phosphorylation of serines and threonines, among others. Specific functions have been assigned to several modifications, which led to the formulation of the histone code hypothesis [70]. It is our current understanding of the roles of histone modifications that their combinations and association with transcription factors and other chromatin regulators define epigenetic states, which can be discovered and assigned to genomic regions by machine learning [71].

Enzymes such as histone acetyltransferases (HATs), histone methyltransferases (HMTs), protein arginine methyltransferases (PRMTs), and kinases facilitate the addition of chemicals on amino acid residues (writers). For instance, HATs brings in a negative charge that neutralizes the positive charge on the histones. This chemical change decreases the interaction of the N termini of histones with the negatively charged phosphate groups of DNA, resulting in a more relaxed structure. This structure allows transcription factors to access DNA and initiate gene transcription. Other enzymes such as histone deacetylases (HDACs), lysine demethylases (KDMs), phosphatases, and deubiquitylases catalyze the removal of these epigenetic marks (erasers). As such, the process of histone acetylation is reversed by HDACs. Acetyl group removal leads to a more condensed chromatin state and transcriptional gene silencing. Proteins containing DNA methyl-binding domains, chromodomains, bromodomains, and Tudor domains recognize histone modifications and recruit other chromatin modifiers and remodeling proteins to ultimately regulate DNA-dependent processes (readers).

Functional groups affixed to histones can also initiate chromatin remodeling through cis or trans effects. This way, histone marks can modulate the chromatin by directly affecting histone-histone and histone-DNA interactions, or by recruiting non-histone proteins via specific binding domains that recognize particular modifications [72].

1.3 Epigenetic Changes in Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD)

The 2011 discovery that an expanded hexanucleotide (G_4C_2) repeat within a noncoding region of the *C9orf72* gene causes both ALS and FTD highlighted the genetic link between these two diseases and recognized this mutation as the most common genetic cause of ALS and FTD identified to date [73–75], yet more than 80% of all ALS and FTD cases remain genetically unexplained [76]. While other unknown genetic mutations are certainly at play, more researchers are recognizing that epigenetic changes may contribute to these two diseases based on their potential to (1) explain the phenotypic variability observed across family members carrying the same ALS/FTD-associated mutation, and (2) explain why the majority of patients develop disease without any family history of ALS or FTD. Very few epigenetic studies have been conducted on ALS and FTD before the finding of the

pathogenic *C9orf72* G_4C_2 repeat expansion in 2011. This section summarizes the current knowledge on the epigenetics of ALS and FTD. A full summary can also be found in Table 1.1.

1.3.1 ALS/FTD Epigenetic Studies

1.3.1.1 Nuclear DNA methylation/demethylation

Approximately a decade ago, researchers interested in better understanding the etiology of ALS and FTD started interrogating epigenetic mechanisms using cell models, animal models, and biospecimen obtained from patients.

Early studies on ALS evaluated blood and brain promoter DNA methylation status of a few genes known to be implicated in disease including *SOD1*, *VEGF*, and *SLC1A2* [136, 137], but no changes in methylation were detected.

The methylation status of *SOD1*, *FUS*, *TARDBP*, and *C9orf72* promoters has also been evaluated from the blood of ALS patients carrying not fully penetrant *SOD1* mutations, but again, no methylation variations have been detected at these specific regulatory regions [77]. Of interest, Coppede et al. used an enzyme-linked immunosorbent assay (ELISA) to also evaluate global methylation levels in ALS patients carrying not fully penetrant *SOD1* mutations and observed a significant overall DNA methylation increase [77].

Similarly, others compared brain methylation levels of sporadic ALS (sALS) patients to control cases using Affymetrix GeneChip Human Tiling 2.0R Arrays and identified 38 differentially methylated genomic regions in patients. Further analysis of these 38 regions shed light on specifically altered biological pathways involved in calcium homeostasis, neurotransmission, and oxidative stress [78].

Using ELISA assays, Figueroa-Romero et al. identified global 5mC and 5hmC changes in sALS patients' spinal cords—these changes were not observed in blood [79]. Then, using high-throughput microarrays, the same group conducted genome-wide 5mC and expression profiling and identified loci-specific differentially methylated and expressed genes. The 112 genes identified were highly associated with immune and inflammation responses [79].

In the hope of finding differently methylated regions that might act as modifiers of age of onset in ALS, Tremolizzo et al. evaluated DNA methylation levels in both early onset (<55 years) and late onset (>74 years) ALS patients. They found a global 25–30% increase in DNA methylation levels in whole blood that was independent of age of onset [80]. While no significant difference was found between early onset and late onset ALS, the methylation increase detected in blood is consistent with previous observation in the central nervous system (CNS) of ALS patients [36, 99].

Early epigenetic studies on FTD analyzed the progranulin-encoding gene (*GRN*) known to be mutated in 5–20% of familial FTD and 1–5% of sporadic FTD patients [138–140]. Two groups independently reported that increased *GRN* promoter methylation negatively correlates with *GRN* mRNA levels in FTD subjects [81, 82], a

Table 1.1 Overview of ALS and FTD epigenetic studies

Epigenetic Modification	Overall Findings	Implications for disease	References
Nuclear DNA methylation/ demethylation	Overall DNA methylation is increased in ALS patients carrying not fully penetrant *SOD1* mutations.	Patients with *SOD1* mutations may have unique methylation profiles.	[32]
	Differentially methylated regions are found in sporadic ALS subjects.	Affected genes are associated with calcium homeostasis, neurotransmission and oxidative stress, suggesting a role for these mechanisms in ALS pathogenesis.	[125]
	Global changes in 5mC and 5hmC levels are found in ALS postmortem spinal cords.	Expression changes in 112 genes associate with immune and inflammatory responses.	[51]
	Increased DNA methylation levels are found in ALS blood, independently from age of onset.	While not a modifier of age of onset, DNA methylation levels may act as an ALS marker of epigenetic dysfunction.	[165]
	Increased *GRN* promoter methylation negatively correlates with *GRN* mRNA levels in FTD subjects.	Altered methylation may contribute to *GRN* haploinsufficiency.	[56,11]
	Specific DNA methylation signatures in FTD peripheral blood associate with tauopathy.	Specific DNA methylation signature may be a risk factor for neurodegeneration.	[103]
	DNMT1 and DNMT3A protein levels are increased in motor neurons of ALS patients.	Increased DNMT1 and DNMT3A protein levels may explain the global increase in methylation observed.	[24]
	DNMT3A is present in human cerebral cortex pure mitochondria. Mitochondrial Dnmt3a protein levels are significantly reduced in mice skeletal muscles and spinal cords.	Reduced mitochondria Dnmt3a protein levels may associate with a loss of mtDNA methylation.	[172]
RNA-mediated regulation through miRNAs	Expressing mutant *SOD1* (p.G39A) in mouse muscles alters expression of both miRNAs and genes associated with myelin homeostasis in spinal cords.	Results suggest an epigenetic regulation interplay between muscle cells and neighboring neurons.	[41]
	miRNAs are aberrantly regulated in ALS spinal cords.	Mature miRNAs are globally reduced and miRNA processing is altered.	[52]
	Altered microRNAs have RNA targets part of pathways previously associated with ALS.	Altered miRNA expression may result in altered regulation of key pathological pathways involved in ALS.	[52]
	ALS-associated miRNA expression changes in response to nuclear clearance and cytoplasmic aggregation of TDP-43.	Re-localization of TDP-43 alters RNA-mediated regulation.	[52]
	Altered expression of specific miRNAs in *SOD1* p.G93A mice and patients carrying *TARDBP* p.A90V or p.M337V mutations.	miRNAs and/or their targets may potentially serve as biomarkers or therapeutic targets.	[164,113,183]
	miR-29b regulates human progranulin.	miR-29b may be therapeutically targeted to rescue FTD-associated *GRN*-haploinsufficiency.	[88]
	Expression of FTD-associated *CHMP2B* in mouse brain decreases miR-124 expression.	Altered regulation of miR-124 may lead to altered regulation of AMPAR receptor subunits.	[57]

(continued)

Table 1.1 (continued)

***C9orf72* Epigenetic Studies**	DNA methylation of the *C9orf72* promoter and expanded repeat region in c9FTD/ALS.	Hypermethylation of the *C9orf72 genomic region may contribute* to C9ORF72 loss of function. Evidence for neuroprotective effects and reduced *C9orf72*-associated pathology.	[174,107,12,177,176]
	5mC and 5hmC are both present at the *C9orf72* promoter in *C9orf72*-associated brain tissues.	Estimates of *C9orf72* promoter methylation includes both 5mC and 5hmC modifications.	[49]
	Repressive histone marks at the *C9orf72* locus in c9FTD/ALS.	Reduced expression of *C9orf72*.	[14]
	Differentially methylated regions are abundant in c9ALS and sALS patients and affect many genes and biological pathways involved in ALS.	Altered methylation may result in altered regulation of key pathological pathways involved in ALS.	[45]
	c9ALS and sALS patients have distinct but overlapping brain DNA methylation profiles affecting genes with similar biological functions.	Suggests a conserved pathobiology between c9ALS and sALS.	[45]
Potential drivers of epigenetic changes	Increased incidence of ALS in Chamorro indigenous people of Guam.	Dietary consumption of neurotoxins may induce epigenetic changes through ROS and increase the risk for ALS.	[10]
	Associations between ALS and diet, cycad neurotoxins and ROS.	Neurotoxins may initiate epigenetic changes and increase the risk for ALS.	[16,37,27,134,142]
	Monozygotic twins discordant for ALS.	Supports evidence for environmental contribution to disease onset.	[175,120,180]
	C9orf72 positive identical twins discordant for ALS.		[175,120]
	Stress-induced histone modifications repress transposable elements and other coding and non-coding RNAs.	Acute stress impairs genomic stability and may initiate cognitive impairments.	[82,80,141,48,90,117]
	Exposure to heavy metals such as aluminum sulfate, mercury, lead, and selenium associates with ALS. Heavy metals cause cellular stress and toxicity through ROS.	May drive epigenetic changes, protein denaturation and aggregation and prevent proteasomes from eliminating dysfunctional proteins.	[122,9,30,89,28,50,133]
	Increased risk for ALS in workers exposed to low frequency magnetic fields.	Electromagnetic fields may cause epigenetic changes leading to neurodegeneration.	[70]
	Increased risk for ALS in people exposed to pesticides and herbicides containing organophosphates.	Exposure to organophosphates may initiate epigenetic changes and increase toxicity through ROS.	[115,33,154,124,167,40,89,121,157]
	Increased ALS incidence in athletes and war veterans.	Drug use and ischemia from head injuries may increase ROS and drive ALS.	[25,78,123,137,163]

very interesting finding since *GRN* haploinsufficiency has been recognized as a major cause of FTD [141].

Global DNA methylation levels in blood were also assessed in tau-associated progressive supranuclear palsy (PSP) and FTD patients by Li et al. The major known risk locus for PSP and other neurodegenerative diseases is the H1 haplotype at 17q21.31, a genomic region in linkage disequilibrium with an inverted chromosomal sequence of about 970 kb [142–144]. It was shown that this disequilibrium resulted from an inversion at the H2 haplotype relative to the H1 human reference allele and from a lack of recombination between inverted and non-inverted chromosomes [145]. Li et al. found that the H1/H2 locus may affect the risk for tauopathies through methylation alterations not only at the *MAPT* locus, a region known to be mutated in FTD, but also in at least three neighboring genes. The 17q21.31-associated DNA methylation signature Li et al. identified was unique to tau-associated PSP patients and, to a lesser extent, to tau-associated FTD [83].

As DNA methyltransferases catalyze DNA methylation, researchers have been interested in assessing their expression in disease. Chestnut et al. found that protein levels of DNMT1 and DNMT3A were increased in the motor cortex of ALS patients [36]. Wong et al. went further and confirmed the presence of both Dnmt3a isoform in pure mitochondria of human cerebral cortex and mouse CNS, and 5mcs in mouse mitochondria of neurons and skeletal muscle myofibers, supporting an epigenetic regulation of brain mtDNA [84]. Wong et al. then evaluated mitochondrial Dnmt3a isoform levels in the CNS of different *SOD1* transgenic mouse models including a line hemizygous for a low copy number of hSOD1 -p.G37R mutant allele, a line that expressed high levels of normal wild-type human *SOD1* gene, and a line with skeletal muscle-restricted expression of hSOD1 -p.G37R, -p.G93A, and -wild-type variants. After studying all the lines at presymptomatic or early to middle stages of disease, they found that Dnmt3a protein levels in mouse skeletal muscle and spinal cord mitochondria were significantly reduced early in disease, this even before symptoms' onset. They also found Dnmt1 bound to the outer mitochondrial membrane of the same mice. They observed that 5mC immunoreactivity became aggregated and sequestered into autophagosomes of transgenic mice motor neurons [84].

1.3.1.2 RNA-mediated regulation through miRNAs

It is well established that ncRNAs such as miRNAs are key regulators of RNA functions and metabolism. As such, many have been interested in assessing their potential contribution to ALS/FTD pathogenesis.

Dobrowolny et al. found that selective expression of the human *SOD1* mutation p.G39A after injecting mouse muscles not only leads to hypomyelination in the sciatic nerve, it also alters spinal cord expression of miRNAs and mRNAs known to be involved in myelin homeostasis. This finding suggests that RNA and epigenetic alterations observed in motor neurons may result from changes initiated in neighboring non-neuronal cells [85].

Figueroa-Romero et al. then interrogated spinal cord tissues obtained from post-mortem ALS patients and found that mature miRNA levels are globally reduced. They also identified altered microRNAs having RNA targets part of pathways previously associated with ALS. Knowing that TDP-43 is central to ALS and FTD pathogenesis—TDP-43 pathological signature is observed in about 97% of ALS and 50% of FTD patients [146, 147]—and that TDP-43 plays a central role in miRNA biogenesis [89, 148–152], the same group used transfected cells to determine whether TDP-43 mediates miRNA-induced regulation. They observed ALS-associated miRNA expression changes in response to nuclear clearance and cytoplasmic aggregation of TDP-43, suggesting that TDP-43 pathology may alter the expression or function of endogenous miRNAs and their downstream targets [86].

Several studies have identified alternatively expressed miRNAs in ALS and FTD. Marcuzzo et al. studied the brain of pre-symptomatic and late stage *SOD1* p.G93A transgenic mice and found that expression levels of miR-9, miR-124a, miR-19a, and miR-19b were all altered in late stages of disease. Moreover, the expression analysis they conducted identified miRNA/target gene pairs that were differentially expressed in this mouse model [87]. Toivonen et al. found miR-206 altered in the blood of both *SOD1* p.G93A transgenic mice and ALS patients [88], whereas Zhang et al. found levels of miR-9 decreased in induced pluripotent stem cells (iPSCs)-derived neurons of patients carrying either p.A90V or p.M337V *TARDBP* mutations [89].

Of interest, Jiao et al. found that miR-29b regulates human progranulin (hPGRN, *GRN*) through 3′UTR binding. They demonstrated in vitro that ectopic expression of miR-29b decreased hPGRN expression and knockdown of endogenous miR-29b increased it. Their findings suggest that miR-29b may possibly be therapeutically targeted to rescue the haploinsufficiency observed in FTD patients carrying a *GRN* mutation [90]. Moreover, expression of FTD-associated mutant *CHMP2B* in cerebral cortices of mice has initiated a decrease in miR-124 expression—brain-enriched miR-124 is especially important for the proper regulation of AMPA receptor (AMPAR) subunits. Ectopic expression of miR-124 in the prefrontal cortex of these mice restored AMPAR levels and rescued the behavioral deficits previously observed in the animals [91].

1.3.2 C9orf72 *Epigenetic Studies*

Decreased expression of one or multiple *C9orf72* transcript variants has been observed in various human biospecimen carrying the pathological *C9orf72* G_4C_2 repeat expansion. These biospecimen include frontal cortex, motor cortex, cerebellum, cervical spinal cord, lymphoblastoid cell lines, iPSCs and neurons differentiated from iPSCs, all obtained or derived from ALS and FTD patients [98, 153–159]. In an attempt to better understand the biological mechanism underlying the reduced *C9orf72* expression in *C9orf72*-associated ALS and FTD (c9FTD/ALS) patients,

methylation status of the regions flanking or encompassing the repeat expansion has been evaluated.

Using bisulfite sequencing and restriction enzyme assays, Rogaeva's group found that the CpG island upstream of the G_4C_2 repeat expansion is hypermethylated in the brain and blood of about 36% of ALS and 17% of FTD cases [94, 96]. Lee's group not only confirmed these results, but also uncovered that hypermethylation associates with reduced accumulation of intronic *C9orf72* RNA and reduced burden of *C9orf72*-associated pathological signature (RNA foci and dipeptide repeat accumulation). They also found that demethylation increases cell vulnerability to oxidative and autophagic stress, suggesting that *C9orf72* promoter hypermethylation may mitigate downstream molecular aberrations associated with the pathological G_4C_2 repeat expansion [93]. As the methods initially used to estimate DNA methylation in c9FTD/ALS cases could not differentiate 5mC from 5hmC, Esanov et al. were able to confirm the presence of 5hmC within the *C9orf72* promoter in post-mortem brain tissues of hypermethylated patients [97]. This finding suggests that the previous estimates by Rogaeva's and Lee's groups included both 5mC and 5hmC modifications.

However, considering that all c9FTD/ALS patients show a 50% reduction in total *C9orf72* RNA expression [73] and only approximately one third of patients are found with a hypermethylated CpG island, many details were missing. As such, Rogaeva's group attempted to assess whether it is the repeat expansion that is hypermethylated in patients and consequently drives the reduced expression. For this purpose, they developed a new qualitative assay that was independently validated by a methylation-sensitive restriction enzyme assay, and found that the *C9orf72* repeat expansion was indeed hypermethylated in all ALS and FTD cases carrying more than 50 G_4C_2 copies [95]. This finding was later confirmed by Bauer [92]. In addition, Belzil et al. investigated the brain of c9FTD/ALS patients and found that all patients carried repressive histone marks at the *C9orf72* locus [98]. As such, methylation of the repeat expansion together with repressive histone marks at the *C9orf72* locus likely explains the 50% *C9orf72* reduced expression observed in c9FTD/ALS patients.

A recent multi-omic study aimed to better understand the molecular mechanisms initiating RNA misregulation in *C9orf72*-associated c9ALS and sALS combined RNA and DNA methylation data obtained from brain next-generation RNA sequencing (RNAseq) and reduced representation bisulfite sequencing (RRBS). They found an abundance of differentially methylated cytosines in c9ALS and sALS patients, including changes in many genes and biological pathways known to be involved in ALS. They also observed that c9ALS and sALS patients have generally distinct but overlapping brain DNA methylation profiles that differ from control individuals. Of importance, they found that the c9ALS- and sALS-affected genes and biological pathways have very similar biological functions, suggesting a conserved pathobiology between c9ALS and sALS [99].

Several studies aimed to assess whether DNA methylation is a clinical modifier of ALS and FTD but so far, few correlations have been identified. Among these, hypermethylation of the CpG island upstream of the *C9orf72* repeat expansion has

been found to correlate with shorter disease duration in ALS [96], but was found associated with longer disease duration and later age of death in FTD [160].

1.3.3 Potential Drivers of Epigenetic Changes

The epigenome is a dynamic, complex machinery that plays a critical role in coordinating cellular functions. It is constantly changing to address cellular needs or to react to environmental threats, such as infections. A prime example is heat-shock proteins. These proteins were discovered in the early 1960s by Ferrucio Ritossa when he noticed a "puffing" pattern—now known to be a sudden increase in RNA transcription—in *Drosophila* cells when one of his lab mates increased his incubator's temperature [161]. Many discoveries have resulted from this unintended finding, but one of the most intriguing discoveries was that the "puff" Ritossa described was observable within 2–3 min of heat exposure, demonstrating how agile the epigenomic machinery is. Understanding not only which epigenetic modifications affect disease, but what drives these epigenetic changes is critical to better understanding human health and disease.

As demonstrated by Ritossa's landmark discovery that "heat shock" can induce an immediate response from the epigenetic machinery, environmental influences are a clear driver. "Environment" has broad implications, however, and can include a cell's internal or external influences, such as neighboring cells, as research has shown that epigenetic changes can be transmitted from cell to cell [162]. Many factors affect the dynamic interaction between environmental influences and the epigenome, including exercise, age, diet, and toxic exposures.

Researchers observed a clear example of environmental factors driving ALS in the indigenous Chamorro people of Guam, who experience high ALS incidence because their diet is enriched in cycad neurotoxins. The Chamorro diet includes the flying fox, which has high levels of cycad neurotoxins because it feeds on cycad seeds [100–102]. Additional studies have found associations between ALS and cycad neurotoxins or reactive oxygen species (ROS) [102–104, 163–165]. Exactly how these neurotoxins are driving disease is unknown, but epigenetic modifications are a primary suspect. Importantly, diet has also been shown to induce epigenetic changes across other diseases [105].

The most striking support for epigenetic contribution is perhaps the reports of ALS-discordant monozygotic twins (monozygotic twins where one has disease and the other is unaffected), implicating environmental and epigenetic factors in disease [106–108]. One study identified monozygotic twins that both carry the *C9orf72* repeat expansion, but only one has developed disease. The other study could not find a clear genetic factor that caused disease. A third study by Young et al. identified thousands of large between-twin differences at CpG sites in five monozygotic twin pairs. Young et al. conducted biological pathway analysis, which revealed that impairments in GABA signaling were common to all ALS individuals. Other altered pathways were also identified, including some relevant to ALS such as glutamate

metabolism and the Golgi apparatus [108]. Importantly, Young et al. applied to their 450K data the Horvath algorithm of epigenetic age [166]—an aging clock of chromatin states derived from the characterization of 353 CpG sites—and found that ALS-affected twins were epigenetically older than their unaffected co-twins, confirming previous findings that ALS is characterized by accelerated brain aging [108, 167]. In all cases, the other twin may develop disease in time, but the question would still remain regarding why a significant time gap in onset exists.

Stress is also an environmental variable that has received increased attention in recent years. Both histone methylation and acetylation modifications have been observed in rodents because of stress after social defeat [168]—acute and chronic stress has been shown to activate and repress genes through histone modifications [169]. Interestingly, transposable elements are repressed during acute stress, as are hippocampal coding and noncoding RNA as a result of stress-induced histone modifications. These expression changes have been suggested to impair genomic stability and give rise to cognitive impairments [109–114].

ROS, a species of free radical, can be induced through environmental signals, causing oxidative stress and, ultimately, cause a range of epigenetic modifications altering gene expression [162, 170–172]. Heavy metals are believed to cause cellular stress and toxicity through ROS, driving protein denaturation and aggregation and preventing proteasomes from eliminating dysfunctional proteins [120]. One study used carboxy-DCFDA (5-(and-6)-carboxy-2′,7′-dichlorofluorescin diacetate) to quantify stress-induced ROS production from metal sulfates in human neurons [121] and found aluminum sulfate induced the most ROS.

Repetitive electromagnetic field exposure is also believed to trigger epigenetic changes, including DNA methylation and histone modifications, as was suggested by a study of a large cohort of workers [122]. Resistance welders had a higher incidence of Alzheimer's Disease and ALS, potentially because they are exposed regularly to low frequency magnetic fields [122]. Other studies have suggested that exposure to other heavy metals such as mercury, lead, and selenium, plus pesticides and herbicides containing organophosphate may increase risk for ALS, though no clear association has been found [115–119, 123–130].

As science continues to demonstrate the environmental effects of some high-contact sports on mental health, additional studies to explore the increased ALS incidence in athletes that play American football and soccer [131, 173], and in war veterans [132, 133] are needed. These data further suggest that some ALS cases arise from environmental exposures, potentially from epigenetic consequences of violent jarring in the brain [134]. While various methods, including illicit drug use and ischemia from head injuries, have been proposed to increase ROS production and drive dementia [135], the exact molecular mechanism leading to ALS and FTD needs to be clearly mapped through future rigorous studies.

1.3.4 Therapeutic Potential

The ultimate goal in ALS and FTD research is to develop therapeutic interventions for these diseases, allowing those who are affected to live a long and healthy life. Here, we discuss potential effects of epigenetic therapeutics.

Targeted epigenetic modifiers capable of regulating expression of both the normal and mutant alleles are an exciting possibility. Although significant work must be done in this area, this concept was successfully demonstrated in 1994, suggesting epigenetic therapy is feasible [174]. Since that discovery, other research has been performed in neurodegeneration and cancer in an effort to translate this for clinical use [175, 176]. A recent ALS-specific study utilized bromodomain small molecule inhibitors to increase mRNA and pre-mRNA expression for the normal *C9orf72* allele without destroying the epigenetic markers that repress expression of the expanded allele [177]. Increasing expression of the normal allele without increasing expression for the disease-causing allele is a significant achievement and demonstrates the reality of epigenetic therapy.

DNA methylation effects have been extensively evaluated across many diseases. One study found DNA methylation changes may be good ALS biomarkers for disease and potentially future therapeutic targets [79]. DNMTS have been shown to promote apoptosis and increase 5mC levels in motor neurons, and administering Dnmt inhibitors in a motor neuron-degenerative mouse model mitigated both apoptosis and 5mC levels in the motor neurons [36].

The therapeutic potential of oligonucleotides targeting miRNAs has also been evaluated by researchers using mouse models of ALS. Two groups showed that oligonucleotides able to inhibit either miR-155 or miR-29a extended the lifespan of *SOD1* p.G93A transgenic mice [178, 179]. Similarly, Morel et al. found that injecting oligonucleotides targeting miR-124a in the same transgenic mouse model prevented the pathological loss of EAAT2/GLT1 (encoded by human *SLC1A2*), an astroglial glutamate transporter known to be implicated in ALS [180].

Researchers have shown that histone marks at the *C9orf72* locus are associated with reduced gene expression in ALS and FTD patients carrying a repeat expansion when compared to controls [98]. They were then able to increase *C9orf72* mRNA expression by treating patient-derived fibroblasts with 5-aza-2-deoxycytidine (a demethylating agent). While this study demonstrates a proof of concept, increasing the mutated allele might not be a good therapeutic approach for c9ALS/FTD, as results from others suggest that *C9orf72*-associated hypermethylation may actually be neuroprotective in patients [92, 93]. Nonetheless, similar epigenetic strategies have been developed for cancer therapy, where HDAC inhibitors have reversed the effects of cancer-induced epigenetic changes [181]. These techniques have also been applied in ALS both in vitro and in an animal model, and later proceeded to clinical trials. Specifically, sodium phenylbutyrate (NaPB) prolonged *SOD1* p.G93A mouse survival [182], and was subsequently tested in a phase 2 clinical trial. The participants of this clinical trial tolerated this treatment well, and increased histone

acetylation in participant blood samples [183]. These results present an exciting and realistic opportunity to treat ALS and FTD using targeted epigenetic therapeutics.

Given the epigenome's dynamic and targetable nature combined with its apparent involvement in disease, it is a primary target for additional therapeutic efforts. As a field, researchers studying epigenetics in neurodegenerative diseases have made significant progress characterizing their involvement, but it is unclear whether the observed epigenetic modifications are driving disease or whether they are just a consequence. For example, researchers recently observed clear transcriptomic and epigenetic differences between c9ALS and sALS brains [99, 184], but it has not been shown whether reversing them would rescue neuronal health. If researchers can establish that the epigenetic dysregulation is driving these changes and that reversing them rescues neuronal behavior, epigenetic therapeutics would revolutionize ALS and FTD treatment.

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Chapter 2
Mechanism of Splicing Regulation of Spinal Muscular Atrophy Genes

Ravindra N. Singh and Natalia N. Singh

Abstract Spinal muscular atrophy (SMA) is one of the major genetic disorders associated with infant mortality. More than 90% cases of SMA result from deletions or mutations of *Survival Motor Neuron 1* (*SMN1*) gene. *SMN2*, a nearly identical copy of *SMN1*, does not compensate for the loss of *SMN1* due to predominant skipping of exon 7. However, correction of *SMN2* exon 7 splicing has proven to confer therapeutic benefits in SMA patients. The only approved drug for SMA is an antisense oligonucleotide (Spinraza™/Nusinersen), which corrects *SMN2* exon 7 splicing by blocking intronic splicing silencer N1 (ISS-N1) located immediately downstream of exon 7. ISS-N1 is a complex regulatory element encompassing overlapping negative motifs and sequestering a cryptic splice site. More than 40 protein factors have been implicated in the regulation of *SMN* exon 7 splicing. There is evidence to support that multiple exons of *SMN* are alternatively spliced during oxidative stress, which is associated with a growing number of pathological conditions. Here, we provide the most up to date account of the mechanism of splicing regulation of the *SMN* genes.

Keywords SMN · SMA · Splicing · ISS-N1 · ISS-N2 · Cryptic splice site · U1 snRNA

2.1 Introduction

Pre-mRNA splicing is an essential process in eukaryotic cells during which noncoding (intronic) sequences are removed and coding (exonic) sequences are joined together to generate mRNA. The complex reaction of splicing is catalyzed by a spliceosome, a macromolecular machinery [1]. The most critical step of a splicing reaction is the accurate determination of the 5′ and 3′ splice sites (5′ss and 3′ss) that

R. N. Singh (✉) · N. N. Singh
Department of Biomedical Sciences, Iowa State University, Ames, IA, USA
e-mail: singhr@iastate.edu

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mark the beginning and end of an intron, respectively [2]. All intron-containing human genes have potential to be alternatively spliced, generating multiple mRNA isoforms from a single gene [3]. Decision to include or exclude an exon during pre-mRNA splicing is dictated by a combinatorial control of *cis*-elements and transacting factors. The same *cis*-element when presented in a different context may have different effects on splicing [4, 5]. Hence, the relative impact of a *cis*-element cannot be accurately predicted, it [the impact] requires experimental validation. Interpreting the consequences of a splicing-associated mutation remains a puzzle, since a single mutation can cause at least one of the following changes: loss of a positive element, gain of a negative element, change of a structural context, and nonsense-mediated decay (NMD) due to creation of an in-frame premature termination codon (PTC) [6, 7]. Rules of splicing are quite flexible and are heavily influenced by the relative abundance of various splicing factors in different tissues [8]. Further, splicing is coupled to other events including transcription, the 5′ capping, and the 3′ polyadenylation [6, 9]. Therefore, deciphering the mechanism by which a given exon is alternatively spliced remains a daunting task. A growing number of disorders are linked to aberrant splicing [10, 11]. Each case of aberrant splicing calls for an in-depth analysis of the context-specific rules so that strategies to manipulate splicing could be devised in a gene-specific manner.

Humans carry two near identical copies of the *Survival Motor Neuron* gene: *SMN1* and *SMN2* [12]. Both *SMN* genes code for SMN, a multifunction protein essential for the survival of all animal cells. The ability of SMN to interact with nucleic acids and proteins allows it to participate in various cellular processes, including but not limited to transcription, splicing, translation, macromolecular trafficking, and signal transduction [13]. The critical difference between *SMN1* and *SMN2* is the splicing of exon 7. Unlike *SMN1* exon 7, *SMN2* exon 7 is predominantly skipped in most tissues, except in testis [14]. The exon 7-skipped transcript generated by *SMN2* codes for SMNΔ7, a partially functional and unstable protein [15–17]. Loss of *SMN1* creates SMN deficit, leading to spinal muscular atrophy (SMA), a major genetic disease of children and infants [18, 19]. Aberrant expression and/or localization of SMN have been associated with several other diseases, including amyotrophic lateral sclerosis (ALS), metabolic disorders, male infertility, and stress-associated disorders [14, 20–22]. Correction of *SMN2* exon 7 splicing has proven to confer therapeutic benefits in mouse models of SMA [23, 24]. The first approved drug for SMA, Nusinersen (Spinraza™), is an antisense oligonucleotide (ASO) that promotes inclusion of *SMN2* exon 7 by sequestering an inhibitory *cis*-element called Intronic Splicing Silencer N1 or ISS-N1 [25, 26]. In this review, we describe studies that culminated in the discovery of ISS-N1 and analyze how the characterization of ISS-N1 paved the way for a better understanding of pre-mRNA splicing in the context of a human disease. We summarize the role of various cis-elements and transacting factors that regulate *SMN* exon 7 splicing. We also discuss how lessons learnt from the *SMN* genes will help find effective therapies for genetic diseases associated with aberrant splicing.

2.2 Organization of Human *SMN* Genes

The presence of two *SMN* genes in humans is attributed to the intrachromosomal duplication of ~500 kb segment at the 5q13.3 locus on chromosome 5 (Fig. 2.1a; [12, 35, 36]). Despite conservation of the coding region of *SMN* between human and rodents, there are substantial differences in the promoter, intronic, and the untranslated regions (UTRs). The abundance of Alu elements in human *SMN* genes suggests a distinct regulation of transcription and splicing of *SMN* in primates. Both *SMN* genes are ~34 kb long including ~6 kb long promoter sequence. Several mutations within the promoter region distinguish *SMN1* from *SMN2*, suggesting that transcription of these genes might be differentially regulated under certain conditions, such as stress (Fig. 2.1b). Each *SMN* gene is comprised of 10 exons, that is, 1, 2A, 2B, 3, 4, 5, 6, 6B, 7 and 8 (Fig. 2.1c). About 2/3rd of exon 1 serves as the 5′UTR, whereas the remaining 1/3rd serves as the coding sequence. Exon 8 is the longest exon that encodes the 3′UTR. *SMN2* intronic sequences flanking exon 7 contain several substitutions and a 5-nt deletion (Fig. 2.1c). A C-to-T substitution at the sixth position (C6U) of exon 7, a G-to-A substitution at the -44th position (G-44A) of intron 6, and an A-to-G substitution at the 100th position (A100G) of intron 7 are associated with skipping of *SMN2* exon 7 [37–40]. Recently discovered exon 6B is generated by exonization of an Alu element within intron 6 [33]. Another alternative transcript is generated by intron 3 retention. It codes for a short protein called axonal SMN or aSMN [34]. Considering intron 3 is conserved between human and mouse, expression of aSMN has been detected in mice as well. SMN contains several functional domains and interacts with various proteins. All isoforms of SMN possess identical N-terminus that is involved in interactions with both proteins and nucleic acids (Fig. 2.1d; [13]).

Recent reports reveal that two antisense transcripts, which function as long noncoding RNAs (lncRNAs), are generated from *SMN* locus. One of these lncRNAs termed *SMN-AS1* is ~1.6 kb long; it starts and finishes within intron 1 (Fig. 2.1a; [27]). Other one termed *SMN-AS1** is ~10 kb long; it starts within intergenic region downstream of exon 8 and extends till intron 5 (Fig. 2.1a; [28]). These lncRNAs are specific to humans and their expressions appear to downregulate SMN levels through transcriptional control. The significance of fine-tuning of SMN levels within cells is underscored by a recent study that showed the pathogenesis of osteoarthritis caused by aberrantly high expression of SMN [41]. Factors that regulate *SMN* transcription and splicing modulate SMN levels in a cell-specific manner. Testis happens to be one of the tissues with a very high SMN demand. This demand is met by an entirely different set of rules that govern transcription and splicing of the *SMN* genes in testis. Here, we describe a critical role of the context-specific *cis*-elements in *SMN* splicing and outline the emerging rules that are likely to be applicable in most cell types.

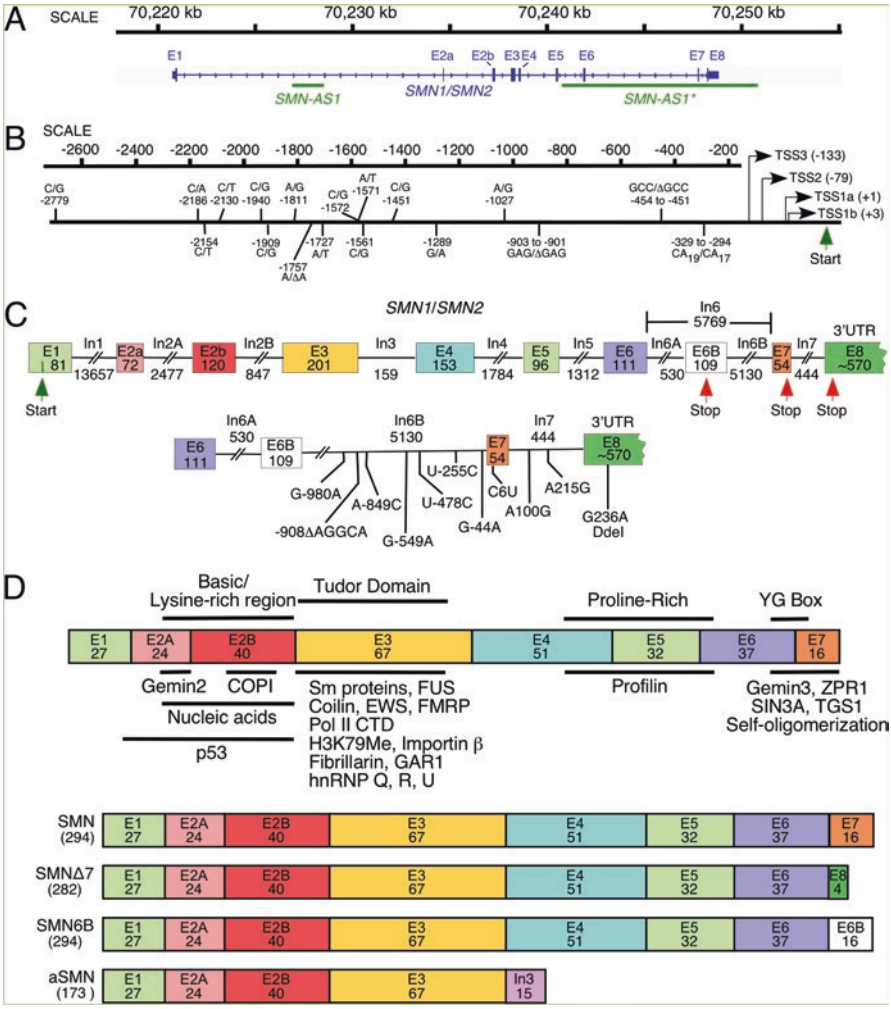

Fig. 2.1 Organization of *SMN* gene. (**a**) A view of human *SMN1*/*SMN2* gene(s) located on chromosome 5. Exons and introns are shown as boxes and lines, respectively. Loci of antisense RNAs, SMN-AS1 [27], and SMN-AS1* [28] are marked with bars. (**b**) Diagrammatic representation of human SMN promoter region. Multiple transcription start sites (TSS) identified so far are indicated using arrows. Numbers in brackets correspond to their position relative to TSS1a (+1). TSS1a and TSS2 were identified in [29] as transcription start sites preferentially used in adult and fetal tissues, respectively. TSS1b was mapped in Echaniz-Laguna et al. [30], and TSS3 was identified in Monani et al., [31]. Nucleotide differences between the *SMN1* and *SMN2* promoters are indicated based on Monani et al., [31]; [29, 32]), where nucleotide positions were calculated from TSS1a. Translation initiation site is marked as Start. (**c**) Diagrammatic representation of the *SMN1*/*SMN2* pre-mRNA. Exons and introns are shown as boxes and lines, respectively. Sizes of exons and introns are indicated in nucleotides (nts). The translation initiation and termination sites are marked as Start and Stop, respectively. Exon 8 is mostly used as the 3′ untranslated region (UTR). The bottom panel indicates nucleotides differences between *SMN1* and *SMN2* in the region located downstream of exon 6B. The last position of intron 6B is designated as −1. For exons 7 and 8, as

2.3 Regulation of *SMN* Exon 7 Splicing

Our understanding of *SMN* exon 7 skipping is continuing to evolve as more and more regulatory elements are being discovered within this relatively short exon and its flanking intronic sequences. Early studies established that the C6U substitution is the primary cause of *SMN2* exon 7 skipping [38, 39]. It was also shown that the 3′ss of *SMN2* exon 7 is weakened by the C6U substitution; but the usage of this 3′ss was enhanced when the downstream 3′ss of exon 8 was blocked [42]. Based on bioinformatics predictions and in vitro studies, it was proposed that C6U abrogates an enhancer associated with SRSF1 (ASF/SF2), a member of the highly conserved family of serine/arginine (SR)-rich proteins (Fig. 2.2a; [46]). However, this simple "SRSF1 abrogation" hypothesis did not hold true in a subsequent cell-based study, where the depletion of SRSF1 did not cause the expected enhancement of *SMN1* exon 7 skipping (Fig. 2.2a; [47]). A more recent study suggests a surprising dual role of *SRSF1* in regulation of *SMN2* exon 7 splicing, as both overexpression and depletion of *SRSF1* caused enhanced skipping of *SMN2* exon 7 [48]. An alternative hypothesis that C6U creates a silencer associated with hnRNP A1/A2 was proposed to explain the skipping of *SMN2* exon 7 [47]. Supporting this hypothesis, depletion of hnRNP A1/A2 promoted *SMN2* exon 7 inclusion [47, 49, 50]. Subsequent studies implicated the role of multiple hnRNP A1/A2 sites in the regulation of *SMN* exon 7 splicing [37, 51–53]. These findings brought additional complexity to the interpretations of the hnRNP A1/A2 depletion experiments, since the observed effect could be attributed to abrogation of hnRNP A1/A2 binding to any/all of these sites within *SMN2* pre-mRNA. Interestingly, *hnRNP A1* knockout mice show muscle-specific developmental defects [54]. Hence, depletion of hnRNP A1 cannot be exploited for a potential therapy of SMA.

The hnRNP A1/A2 model has been subsequently modified to include Sam68 as an additional factor associated with the inhibitory effect of C6U (Fig. 2.2a; [55]). Consistent with the role of hnRNP A1 and Sam68 in *SMN2* exon 7 splicing, low extracellular pH that increased the nuclear concentrations of hnRNP A1 and Sam68 was found to enhance *SMN2* exon 7 skipping [56]. Another mechanism by which C6U might affect *SMN2* exon 7 splicing is through creation of an extended inhibitory context (Exinct) that consists of overlapping negative motifs [57]. Interestingly, C6U also strengthens a predicted terminal stem-loop structure, TSL1 (Fig. 2.2b). Supporting the distinct inhibitory role of TSL1, mutations that disrupted TSL1 without abrogating C6U-associated hnRNP A1/A2 motif promoted *SMN2* exon 7

Fig. 2.1 (coninued) well as intron 7, counting starts with the first position of the respective exon or intron. (**d**). Diagrammatic representation of SMN protein isoforms. Protein regions encoded by each exon are shown as colored boxes with the number of amino acids given. In the top panel, protein domains are indicted above, while SMN interacting partners are shown below the diagrammatic representation of the full-length SMN. For further details see Singh et al. [13]. The bottom panel shows the known SMN isoforms as compared to the full-length SMN protein. These isoforms are generated either due to exon 7 skipping or exonization of a region within intron 6 [33] or intron 3 retention [34]. The size of each isoform (in amino acids) is given in brackets. Abbreviations are given in Table 2.2

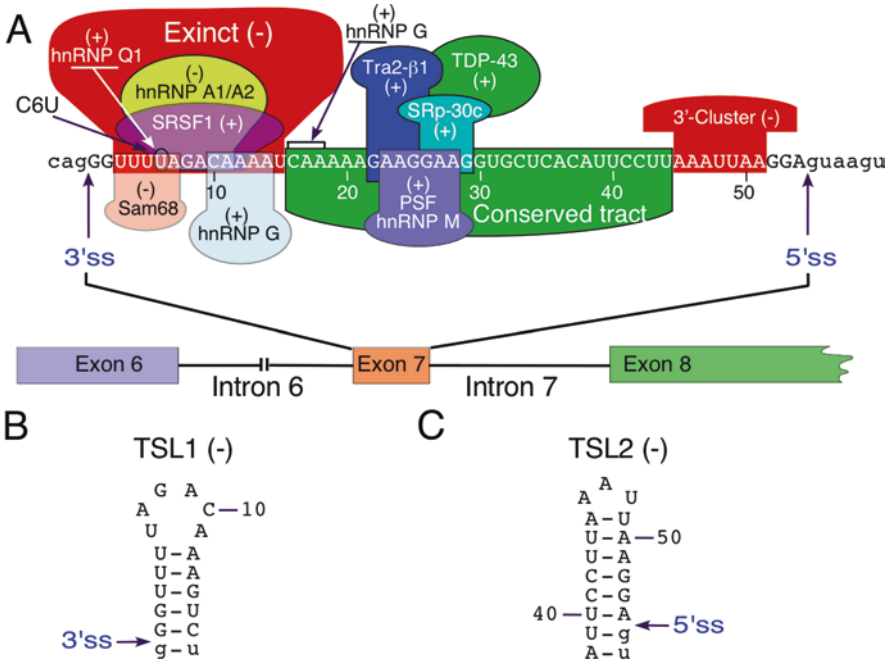

Fig. 2.2 Exon 7 splicing regulation. (**a**) Diagrammatic representation of cis-elements and trans-acting factors that modulate *SMN* exon 7 splicing. Positive and negative elements are indicated by (+) and (−), respectively (For further details see [43]). Numbering of nucleotides starts with the first position of exon 7. Exonic and intronic sequences are shown in upper- and lower-case letters, respectively. The 3′ and 5′ss are indicated by arrows. (**b**) Terminal stem-loop structure, TSL1, formed at the beginning of *SMN2* exon 7 as determined by enzymatic structure probing [44, 45]. Both TSL1 and TSL2 are marked by (−) because they contribute toward exon 7 skipping. Numbering of nucleotides starts with the first position of the exon. Exonic and intronic sequences are shown in upper- and lower-case letters, respectively. The 3′ ss is indicated by an arrow. (**c**) Terminal stem-loop structure, TSL2, formed at the end of *SMN2* exon 7 as determined by enzymatic structure probing [44, 45]. (−) indicates that TSL1 contributes to exon 7 skipping. Numbering of nucleotides starts with the first position of the exon. Exonic and intronic sequences are shown in upper- and lower-case letters, respectively. The 5′ss is indicated by an arrow. Abbreviations are given in Table 2.2

inclusion [57]. It should be noted that the proposed hypotheses associated with the inhibitory effect of C6U are not mutually exclusive. Recent years have witnessed a shift in the debate as critical roles of several other negative elements located away from the C6U site have been discovered.

As per the exon definition model, positive factors bridge cross-exon interactions before splicing takes place [58]. An early study implicated SFRS10 (Tra2-beta1) as one of the factors that interacts directly with a GA-rich sequence located in the middle of exon 7 (Fig. 2.2a; [59]). Several other proteins, including TDP43, SRSF9 (SRp30c), PSF and hnRNP M, were subsequently shown to stimulate exon 7 inclusion through a direct or indirect interaction with exon 7 (Fig. 2.2a; [60–64]). Surprisingly, a follow-up study in a mouse model of SMA established that SFRS10

is dispensable for *SMN* exon 7 splicing [65]. This finding underscored the complexity of splicing regulation when the loss of a positive factor could be tolerated due to the presence of other factors with redundant/overlapping functions. Thus far, studies suggest that skipping of *SMN2* exon 7 is driven largely by the occurrence of negative interactions. The list of factors that regulate *SMN2* exon 7 is large and continues to grow (Table 2.1). However, interaction sites for most of the identified transacting factors remain unknown. There have been very limited attempts to correlate the effect of the naturally occurring mutation within a given factor and splicing of *SMN* exon 7.

Table 2.1 Factors tested for an effect on *SMN2* exon 7 splicing

			Effect of overexpression		Effect of depletion		
Factor (Gene)	Effect on exon 7 splicing	Binding Location	*SMN2*^{m}	*SMN2*^{g}	*SMN2*^{m}	*SMN2*^{g}	References
ASF/SF2 (*SRSF1*)	Positive Neutral	Exon 7	No	Yes	No	Yes No(	[46, 47, 66, 37, 53, 67, 68, 48]
SC35 (*SRSF2*)	Negative Neutral	–	No	Yes	ND	Yes	[64, 47, 48]
SRp20 (*SRSF3*)	Negative Neutral	–	No	Yes	ND	Yes	[64, 48]
SRp75 (*SRSF4*)	Negative	–	ND	No	ND	Yes	[48]
SRp40 (*SRSF5*)	Negative Neutral	–	No	Yes	ND	Yes	[64, 48]
SRp55 (*SRSF6*)	Negative Neutral	–	No	No	ND	Yes	[64, 48]
9G8 (*SRSF7*)	Negative Neutral	–	No	Yes	Yes	Yes	[47, 64, 69, 48]
SRp30c (*SRSF9*)	Positive Neutral	–	Yes	No	ND	No	[64, 67, 48]
SRp38 (*SRSF10*)	Neutral	–	ND	No	ND	No	[48]
SRSF11 (*SRSF11*)	Negative	–	ND	Yes	ND	Yes	[48]
Tra2-β1 (*TRA2B*)	Positive	Exon 7	Yes	Yes	No	ND	[59, 64, 60, 70]
ZIS/ZNF265 (*ZRANB2*)	Negative	–	Yes	ND	ND	ND	[71]
hnRNP A1 (*HNRNPA1*)	Negative	Exon 7, Intron 7, 3′ss	ND	Yes	Yes	Yes	[47, 66, 37, 53, 60, 67, 52, 51, 69, 50]
hnRNPA2/B1 (*HNRNPA2/B1*)	Negative	–	ND	ND	Yes	Yes	[47, 37, 67, 52, 69, 50, 48]
hnRNPC (*HNRNPC*)	Positive Negative Neutral	I6-E7 junction	ND	ND	No; Yes	Yes	[68, 69, 48]

(continued)

Table 2.1 (continued)

			Effect of overexpression		Effect of depletion		
hnRNP D (*HNRNPD*)	Neutral	–	ND	ND	ND	No	[48]
hnRNP F (*HNRNPF*)	Neutral	–	ND	ND	No	No	[69, 48]
hnRNP G (*RBMX*)	Positive	Exon 7	Yes	ND	ND	ND	[63, 72, 70, 60, 73]
hnRNP H (*HNRNPH1*)	Neutral	–	ND	ND	Yes; No	No	[51, 69, 48]
hnRNP K (*HNRNPK*)	Neutral	–	ND	ND	No	ND	[69]
hnRNP L (*HNRNPL*)	Neutral	–	ND	ND	No	ND	[69]
hnRNP M (*HNRNPM*)	Positive	–	Yes	ND	Yes	Yes	[67, 69, 62]
RALY (*RALY*)	Neutral	–	ND	ND	No	ND	[69]
hnRNP Q (*SYNCRIP*)	Positive	Exon 7	Yes	Yes	Yes	ND	[67]
hnRNP U (*HNRNPU*)	Negative	–	ND	Yes	ND	Yes	[69, 48]
CHERP (*CHERP*)	Negative	–	ND	ND	Yes	ND	[69]
HuR (*ELAVL1*)	Negative	3′-UTR	ND	ND	ND	Yes	[48]
PSF (*SFPQ*)	Positive	Exon 7	Yes	Yes	ND	Yes	[67, 62]
PUF60 (*PUF60*)	Negative	3′ss	ND	ND	Yes	Yes	[69, 74]
TDP-43 (*TARDBP*)	Positive	–	Yes	ND	No	ND	[60]
TIA1 (*TIA1*)	Positive	Intron 7	Yes	Yes	Yes	Yes	[75]
RBM10 (*RBM10*)	Negative	–	ND	ND	Yes	Yes	[69, 76]
Sam68 (*KHDRBS1*)	Negative	Exon 7	Yes	ND	Yes	ND	[55]
SF1 (*SF1*)	Negative	Branch Point	ND	ND	Yes	ND	[69]
SmD3 (*SNRPD3*)	Positive	–	ND	ND	ND	Yes	[77]
SON (*SON*)	Negative	–	ND	ND	Yes	ND	[69]
U1-70K (*SNRNP70*)	Positive	–	ND	ND	ND	Yes	[77]
U2AF35 (*U2AF1*)	Negative	–	ND	ND	Yes	Yes	[69]
U2AF65 (*U2AF2*)	Negative	3′ss	ND	ND	Yes	Yes	[67, 69, 74]
U2B″ (*U2B″*)	Positive	–	ND	ND	ND	Yes	[77]

Abbreviations: positive, positive effect on exon 7 splicing; negative, negative effect on exon 7 splicing; neutral, neutral effect on exon 7 splicing; 3′ss, 3′ splice site; 3′-UTR, 3′-untranslated region; ND, not performed or assayed; Yes, observed; No, not observed; *SMN2*^{m}, *SMN2* minigene; *SMN2*^{g}, Endogenous *SMN2* gene

Table 2.2 Abbreviations and terminology used in this study

Abbreviation	Full name	Relevant figures
3′ss	3′ splice site	2
3′-UTR	3′ untranslated region	
5′ss	5′ splice site	2
5′-UTR	5′ untranslated region	
ALS	amyotrophic lateral sclerosis	
ASO	Antisense oligonucleotide	5
bp	Base pair	
C6U	A C-to-U substitution at the sixth position of *SMN2* exon 7	2
Element 1	Negative *cis*-element located within *SMN* intron 6	3
Element 2	Positive *cis*-element located within *SMN* intron 7	3
FTD	Frontotemporal dementia	
eU1	Engineered U1 snRNA	6
hnRNP	Hetero-nuclear ribonucleoprotein	2
ISS-N1	Intronic splicing silencer N1 (located within *SMN* intron 7)	3, 5
ISS-N2	Intronic splicing silencer N2 (located within *SMN* intron 7)	4, 5
ISTL1	Internal stem formed by LDI-1 (located within *SMN* intron 7)	4, 5
ISTL2	Internal stem formed by LDI-2 (located within *SMN* intron 7)	4
ISTL3	Internal stem formed by LDI-3 (located within *SMN* intron 7)	4
ISTL4	Internal stem formed by LDI-4 (located within *SMN* intron 7)	4
nt	Nucleotide	
LDI	Long-distance interaction (located within *SMN* intron 7)	3, 4, 5
lncRNA	Long non-coding RNA	1
Nusinersen	An ASO drug that targets ISS-N1 sequence (synonym of Spinraza™)	5
PMD	Pelizaeus–Merzbacher disease	
PLP1	Proteolipid protein 1	
SMA	Spinal Muscular Atrophy	
SMN (Italics)	Survival motor neuron gene or transcript	
SMN-AS1 (Italics)	Antisense transcript (lncRNA) generated from *SMN* locus	1
$SMN2^{m}$	*SMN2* minigene	
$SMN2^{g}$	Endogenous *SMN2* gene	
*SMN-AS1** (Italics)	Antisense transcript (lncRNA) generated from *SMN* locus	1
SMN	Survival motor neuron protein	
SMN6B	SMN6B protein	7
Spinraza™	An ASO drug that targets ISS-N1 sequence (synonym of Nusinersen)	5
TSL1	Terminal stem-loop 1 located within *SMN* exon 7	2

(continued)

Table 2.2 (continued)

Abbreviation	Full name	Relevant figures
TSL2	Terminal stem-loop 2 located within *SMN* exon 7	2, 4, 5
TSS	Transcription start site	1
U1 or U1 snRNA	U1 small nuclear RNA	5, 6
U1 snRNP	U1 small nuclear ribonucleoprotein	5, 6
URC1	U-rich cluster 1 located within intron 7	3, 5
URC2	U-rich cluster 2 located within intron 7	3, 5
URC3	U-rich cluster 3 located within intron 7	3, 5
UTR	Untranslated region	
WDM	Welander distal myopathy	
wt	Wild-type	

2.3.1 In Vivo Selection of Exon 7

In vivo selection is a powerful method to determine the position-specific role of every exonic residue on splicing of a given exon. The feasibility of in vivo selection for an entire exon was first demonstrated in the context of *SMN1* exon 7 [78]. The method employed a partially randomized exon 7 and repeated rounds of selection for sequences that promoted exon 7 inclusion [78]. The approach was modeled on in vitro selection of a large sequence used for the simultaneous identification of *cis*-elements and structural motifs critical for RNA-protein interaction [45, 79]. The results of in vivo selection confirmed the presence of "Exinct" in the beginning of exon 7 (Fig. 2.2a; [78]). The findings of in vivo selection also uncovered the role of a "conserved tract," a long stretch of nucleotides in the middle of exon 7 that constituted a number of overlapping positive *cis*-elements (Fig. 2.2a; [78]). In addition, the results of in vivo selection revealed the existence of a negative *cis*-element, the "3′-cluster," located toward the end of exon 7 (Fig. 2.2a; [78]). Of note, the "3′-cluster" overlaps with the exonic region that is not conserved between human and rodents, suggesting that human *SMN* exon 7 acquired this negative regulator of splicing after the divergence from the common rodent ancestor ~80 million years ago. Major findings of in vivo selection were independently confirmed by an antisense microwalk as well as by a machine-learning-based simulation study [80, 81].

The most surprising finding of in vivo selection was the overwhelming selection of a non-wild type G residue (A54G) at the last position of exon 7 [78]. Validating experiments confirmed the strong stimulatory effect of A54G substitution on *SMN2* exon 7 splicing. For instance, substitutions abrogating various positive *cis*-elements of exon 7 were fully tolerated in the presence of 54G. Numerous mechanisms by which 54G imparts such a strong stimulatory effect on *SMN2* exon 7 splicing could be envisioned. For example, 54G is predicted to disrupt an inhibitory structure (terminal stem loop 2 or TSL2) that sequesters the 5′ss of exon 7 (Fig. 2.2c). In addition, 54G increases the base pairing between U1 snRNP and the 5′ss of exon 7. Indeed, both of these predictions turned out to be true [82]. Hence, findings of

in vivo selection had a transformative effect on our understanding of *SMN* exon 7 splicing. In particular, they revealed that the 5′ss of exon 7 is weak in both *SMN1* and *SMN2*. Subsequent studies focused on the mechanism that defines the 5′ss of exon 7 [43, 44, 83, 84]. These studies culminated in discoveries that led to the first therapy for SMA.

2.3.2 Effect of Terminal Stem Loop 2

In order to demonstrate the role of an RNA structure in pre-mRNA splicing, one must first perform structure probing to definitively confirm the existence of a specific RNA structure. In addition, using site-specific mutagenesis one must then show a correlation between disruption of the structure and altered splicing. Validating experiments must also demonstrate that the splicing pattern is restored when the structure is reinstated. Thus far only a handful of studies have fulfilled the above-mentioned requirements to conclusively establish the role of an RNA structure in pre-mRNA splicing. Inspired by the results of in vivo selection, we performed a systematic study uncovering the role of the terminal stem-loop 2 (TSL2) predicted to partially sequester the 5′ss of exon 7 in splicing regulation of this exon (Fig. 2.2c). Enzymatic structure probing confirmed the existence of both TSL1 and TSL2 [82]. Supporting the inhibitory role of TSL2, U40G or A54C substitution that disrupted TSL2 was found to promote *SMN2* exon 7 inclusion. As expected, when U40G and A54C substitutions were combined to reinstate the TSL2 structure, a strong inhibitory effect on *SMN2* exon 7 splicing was restored [82]. These results unequivocally confirmed that TSL2 plays the inhibitory role in the regulation of *SMN* exon 7 splicing. One of the mechanisms by which TSL2 prevents *SMN2* exon 7 inclusion is through poor recruitment of U1 snRNP at the 5′ss of exon 7. Consistent with this argument, a mutated U1 snRNA with extended complementarity to the 5′ss of exon 7 was found to restore *SMN2* exon 7 inclusion [82]. Independently validating these findings, an ASO-mediated depletion of endogenous U1 snRNP was found to promote skipping of exon 7 from both *SMN1* and *SMN2* [49]. However, the effect of U1 snRNP depletion was less pronounced in case of *SMN1* exon 7 than *SMN2* exon 7. This could be due to C6U substitution strengthening TSL1 and as a consequence stabilizing TSL2. It is also possible that the stimulatory factor(s) interacting with *SMN1* exon 7 disrupt TSL2.

2.3.3 Effect of Intronic Splicing Silencer N1

In an effort to identify additional *cis*-elements that might suppress the recognition of the 5′ss of *SMN2* exon 7, we analyzed the intronic sequences immediately downstream of the 5′ss of exon 7. Using the *SMN2* minigene we generated a set of mutants with overlapping deletions and tested their splicing pattern. Our results

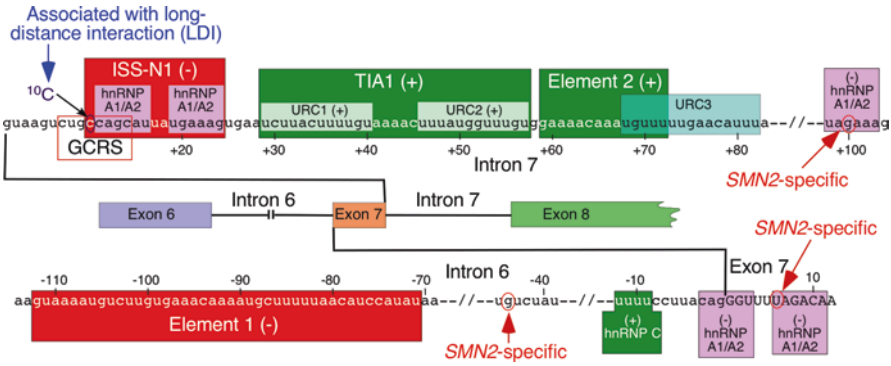

Fig. 2.3 Diagrammatic representation of intronic cis-elements and transacting factors that modulate *SMN* exon 7 splicing. Positive and negative elements are indicated by (+) and (−), respectively. Positive and neutral numbers indicate nucleotide positions within intron 7 and exon 7, respectively, starting with the first intronic/exonic position. Negative numbers indicate nucleotide positions within intron 6, starting with the last intronic position. Exonic and intronic sequences are shown in upper- and lower-case letters, respectively. Exons and introns are also shown as colored boxes and lines. *SMN2*-spesific single nucleotide substitutions are indicated. Intron 7-located ISS-N1, the overlapping GC-rich sequence (GCRS) and ^{10}C contribute to skipping of exon 7 [43]. ISS-N1 harbors two hnRNP A1/A2B1-binding sites that are highlighted in pink. An *SMN2*-specific C6U substitution in exon 7 and A100G substitution in intron 7 create additional binding sites for hnRNP A1 [37, 47]. Another hnRNP A1-binding site is located at the junction of intron 6 and exon 7 [51]. Element 2 and U-rich clusters (URC1 and URC2) are positive *cis*-elements [75, 86]. TIA1 interacts with URC1 and URC2 and promotes exon 7 inclusion [75]. Intron 6-located Element 1 is highlighted in red [87]. It serves as a binding site for PTB and FUSE-BP [88]. A binding site for the stimulatory hnRNP C1/C2 within intron 6 is highlighted in green [68]

revealed that the sequence spanning from the 10th to 24th positions of intron 7 is highly inhibitory for exon 7 inclusion [85]. We termed this sequence as intronic splicing silencer N1 or ISS-N1 (Fig. 2.3; [85]). ISS-N1 deletion obviated the requirement for several positive *cis*-elements responsible for *SMN* exon 7 inclusion. We next employed type 1 SMA patient fibroblasts (GM03813) to validate the inhibitory effect of ISS-N1 in the context of the endogenous *SMN2*. Of note, GM03813 cells carry only *SMN2* and offer an invaluable tool to examine the effect of compounds on spicing of *SMN2* exon 7. As expected, an ASO that blocked ISS-N1 fully restored *SMN2* exon 7 inclusion in GM03813 cells [85]. Importantly, ISS-N1-targeting ASO had a pronounced stimulatory effect on *SMN2* exon 7 splicing even at a low concentration of 5 nM. This could be due to strong inhibitory nature of ISS-N1 combined with its high accessibility for an ASO that targets it.

Among several hundred targets examined thus far, ISS-N1 remains the most effective target for an ASO-mediated stimulation of *SMN2* exon 7 inclusion [89]. Numerous studies employing various mouse models have independently validated the in vivo efficacy of ISS-N1-targeting ASOs [23]. The recently approved ISS-N1-targeting drug for SMA, Nusinersen (synonyms: ISIS-SMNRx, IONIS-SMNRx and Spinraza™), is a modified oligonucleotide that carries phosphorothioate backbone and encompasses methoxyethyl modification at the 2′-hydroxyl position of the

sugar moiety [23]. The above-mentioned modifications are known to enhance the in vivo stability of oligonucleotides. Multiple reports published recently discuss different aspects of the drug development process that led to the FDA approval of Nusinersen [25, 26, 49, 90–93]. More than a dozen independent studies employing ASOs with different chemistries have validated the stimulatory effect of ISS-N1 sequestration on *SMN2* exon 7 splicing [89, 94]. An in-depth analysis of these studies for an improved future ASO-based therapy is beyond the scope of this review.

Several studies have been performed to uncover the mechanism of ISS-N1 function. The inhibitory effect of ISS-N1 was only partially maintained in a heterologous background, suggesting that the context of *SMN2* makes ISS-N1 a strong negative regulator of splicing [85]. An early report implicated two putative-binding sites of hnRNP A/A2 within ISS-N1 as the major cause of the inhibitory effect of this *cis*-element (Fig. 2.3; [52]). This model has been recently revised to suggest that two RNA-recognition motifs (RRMs) of a single hnRNP A1 molecule interact with two putative sites within ISS-N1 [95]. Noticeably, the cytosine residue at the first position (^{10}C) of ISS-N1 does not fall within the putative hnRNP A1/A2-binding site. Yet, sequestration of ^{10}C was found to be absolutely critical for an ASO-mediated splicing correction of *SMN2* exon 7 (Fig. 2.3; [96]). It has been also confirmed that ASO-mediated sequestration of two putative hnRNP A1/A2-binding sites within ISS-N1 is not enough to produce a stimulatory effect on *SMN2* exon 7 splicing [50, 96]. Overall, several studies suggest a more complex mode of ISS-N1 action. Furthermore, motifs upstream and downstream of ISS-N1 appear to be involved in it as well [49, 50, 96, 97].

In search for the shortest ASO that effectively restores *SMN2* exon 7 inclusion, we performed an ultra-refined antisense microwalk within and around ISS-N1 sequence [97]. Of note, ASO sizes and their respective targets in our ultra-refined antisense microwalk differed by single nucleotides. Such approach unequivocally guarantees success for the identification of the shortest therapeutic ASO [98]. Our results showed that sequestration of a GC-rich sequence (GCRS) by an 8-mer ASO fully restored *SMN2* exon 7 inclusion (Fig. 2.3; [97]). Interestingly, GCRS-targeting ASO was found to be more specific than an ISS-N1-targeting ASO, particularly at higher concentrations [97]. This is not entirely surprising, since long ASOs can tolerate mismatched base pairs, whereas as shorter ASOs require total complementarity. Subsequent studies confirmed the therapeutic efficacy of a GCRS-targeting ASO in both mild and severe mouse models of SMA [99]. Although GCRS partially overlaps with ISS-N1, it may represent a distinct negative element. Future studies will determine if a specific factor associates with GCRS.

2.3.4 Effect of U-Rich Clusters Within Intron 7

SMN intron 7 contains multiple U-rich clusters (URCs). URC1 and URC2 are located next to each other immediately downstream of ISS-N1 (Fig. 2.3). Element 2, the very first intronic *cis*-element shown to promote exon 7 inclusion, is located

downstream of URC2 [86]. It partially overlaps with the third U-rich cluster, URC3 (Fig. 2.3). Overlapping deletions in the *SMN2* minigene confirmed the strong stimulatory nature of the above URCs and Element 2. Subsequent experiments linked the stimulatory effect of URC1 and URC2 with TIA1, a glutamine-rich RNA-binding protein [75]. TIA1 and its related protein TIAR generally interact with URCs immediately downstream of a 5′ss and stimulate exon inclusion by promoting recruitment of U1 snRNP to suboptimal 5′ss [100]. However, the context of TIA1/TIAR interactions in *SMN2* intron 7 is somewhat different due to the presence of ISS-N1 between the 5′ss of exon 7 and URC1/URC2 sites to which TIA1 binds. Overexpression of TIA1 fully restored *SMN2* exon 7 inclusion, suggesting that factors that interact with ISS-N1 interfere with recruitment of TIA1 to URC1/URC2 [75]. Supporting the role TIA1 in *SMN* exon 7 splicing in the context of a human disease, Welander distal myopathy (WDM) patients carrying a TIA1 mutation display an elevated level of *SMN* exon 7 skipping [101]. Recently, mutations in TIA1 have been also linked to frontotemporal dementia (FTD) and ALS [102]. However, it is not known if FTD/ALS patients carrying TIA1 mutations display *SMN* exon 7 skipping in any of their tissues. Notably, nervous tissue of *Tia1* knockout mouse shows dysregulated expression of lipid storage and membrane dynamics factors [103]. However, effect of *Tia1* deletion on *SMN2* exon 7 splicing cannot be evaluated because mice lack *SMN2*. To obviate this problem, we generated a *Tia1* knockout mouse in the context of a mild SMA model harboring *SMN2* alleles [104]. Interestingly, loss of *Tia1* in this mouse model did not show changes in *SMN2* exon 7 splicing, although the severity of the SMA disease was affected in a gender-specific manner [104]. Several reasons may account for the discrepancy between the effects of *Tia1* deletion (in mouse) and *TIA1* mutation (in human). For instance, TIA1 is involved in various types of protein-protein and RNA-protein interactions during pre-mRNA splicing, stress granule formation, and mRNA trafficking [105, 106]. It is likely that a mutant TIA1 protein perturbs protein-protein and RNA-protein interactions in the above-mentioned processes. On the other hand, the complete loss of *Tia1* in the mouse model is tolerated due to the presence of its related protein Tiar and/or other glutamine-rich RNA-binding protein.

2.3.5 Effect of Long-Distance Interactions Within Intron 7

Splicing of *SMN* exon 7 is modulated by a unique RNA structure formed by long-distance interactions (LDI) within intron 7 [43, 50, 96]. This structure is termed as "Internal-Stem formed by LDI 1" or ISTL1 (Fig. 2.4; [50]). Chemical structure probing confirmed the formation of ISTL1 along with several other structures within intron 7 (Fig. 2.4). Two strands of ISTL1 are separated from each other by 279-nts, of which 189 residues are located within the independently folded modules. The 5′ strand of ISTL1 overlaps with the 5′ss of exon 7 as well as ^{10}C, which occupies the first position of ISS-N1. It appears that the formation of ISTL1 strengthens TSL2. Consistently, F14, a 14-mer ASO that sequesters the first 14 residues of ISS-N1,

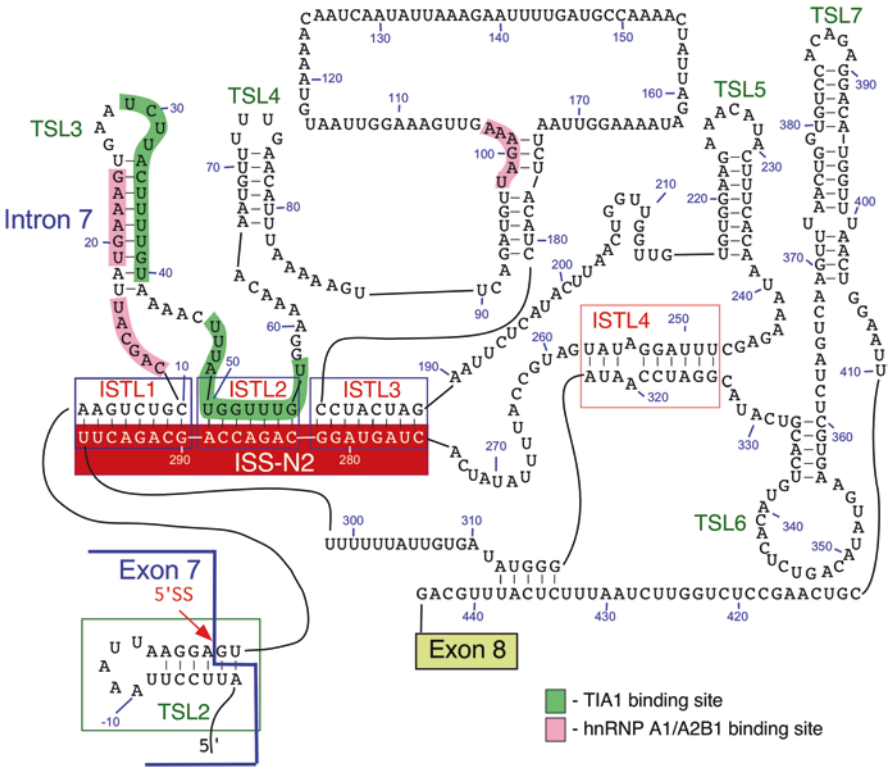

Fig. 2.4 Secondary structure of *SMN2* intron 7 derived from chemical probing. Numbering starts from the first position of intron 7. Negative numbers represent upstream sequences within exon 7. TSLs, ISTLs and binding sites for TIA1 and hnRNP A1/A2B1 are shown and highlighted. ISS-N2 is composed of the 3′ strands of ISTL1, ISTL2 and ISTL3 [43, 50]. The 5′ss of exon 7 is indicated by a red arrow. Abbreviations are given in Table 2.2

including ^{10}C, destabilizes both ISTL1 and TSL2 [50, 96]. On the contrary, L14, a 14-mer ASO that sequesters the last 14 residues of ISS-N1, but not ^{10}C, strengthens both ISTL1 and TSL2. Consequently, F14 and L14 have opposite effects on *SMN2* exon 7 splicing: F14 promotes *SMN2* exon 7 inclusion, while L14 causes skipping of this exon [50, 96]. The opposite effects of F14 or L14 were found to be independent of the oligonucleotide chemistry, suggesting that ASO-induced structural rearrangement at the 5′ss of exon 7 was the driving force behind the splicing outcomes [96]. This is a rare example in which two ASOs of identical size annealing to sequences differing only by a single nucleotide produce opposite effects on pre-mRNA splicing.

The 3′ strand of ISTL1 overlaps with ISS-N2, a negative element located deep within intron 7 (Fig. 2.4; [50]). ISS-N2 also participates in the formation of ISTL2 and ISTL3, other intra-intronic structures formed by LDIs (Fig. 2.4). Formation of ISTL2 sequesters URC2, one of the binding sites of TIA1. Similar to ISS-N1, deletion or an ASO-mediated sequestration of ISS-N2 restores *SMN2* exon 7 inclusion.

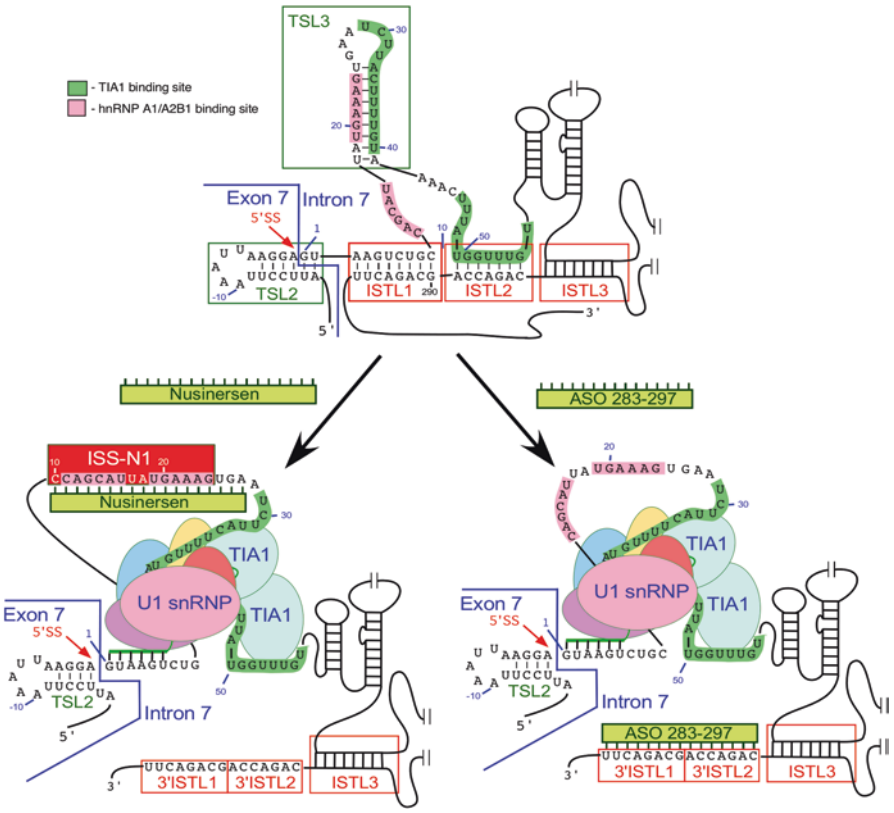

Fig. 2.5 ASO-based mechanism of *SMN2* exon 7 splicing correction. Only the relevant sequences of exon 7/intron 7 are given. Nucleotide numbering starts from the first position of intron 7. ISS-N1 and the binding sites for TIA1 and hnRNP A1/A2B1 are marked by colored boxes. The 5′ ss of exon 7 is indicated by a red arrow. The annealing positions of U1 snRNA to this 5′ ss are shown. TSL2 and 3 are local RNA secondary structures, while ISTL1, 2 and 3 are the structures formed by long-distance interactions. These structures are boxed. Nusinersen and ASO 283–297 are shown as yellow bars [25, 107]. Their annealing positions within intron 7 are indicated. Targeting of the corresponding intronic sequences by Nusinersen and ASO 283–297 causes massive structural rearrangements, including disruption of TSL3 and ISTL1. As the results TIA1-binding sites become accessible, the recruitment of U1 snRNP to the 5′ ss of exon 7 is increased and, in case of Nusinersen, the binding of hnRNP A1/A2 to ISS-N1 is blocked. Abbreviations are given in Table 2.2

Interestingly, ASO-mediated sequestration of ISS-N1 and ISS-N2 brings the similar structural changes at the 5′ss of *SMN2* exon 7, suggesting a common mechanism of action. It appears that both ISS-N1- and ISS-N2-targeting ASOs promote inclusion of *SMN2* exon 7 through abrogation of ISTL1 and an improved recruitment of TIA1 (Fig. 2.5). In vivo study with an ISS-N2 targeting ASO was recently shown to confer gender-specific therapeutic benefits in a mild mouse model of SMA [107].

2.3.6 Extension of Exon 7 by the Activation of a Cryptic 5'ss

Various instances of SMA caused by enhanced exon 7 skipping triggered by mutations at the 3′ or the 5′ss of *SMN1* exon 7 have been reported [12, 108, 109]. Such patients cannot benefit from Nusinersen or any other therapeutic approach requiring the fully functional splice sites of exon 7. However, these patients can take advantage of an engineered U1 snRNA (eU1)-based approach aimed at the activation of a cryptic 5′ss located downstream of the natural 5′ss of exon 7. The proof of principle has recently been established in the context of a pathogenic G-to-C mutation at the first position (G1C) of *SMN1* intron 7 (Fig. 2.6; [49]). As expected, *SMN1* exon 7 carrying G1C substitution undergoes complete skipping of exon 7 with or without an ISS-N1-targeting ASO. However, eU1s targeting ISS-N1 or sequences upstream or downstream of this *cis*-element activate a cryptic 5′ss (Cr1) leading to the inclusion of an "extended" exon 7. Of note, another cryptic 5′ss, Cr2, located within URC2 could also be activated by a different set eU1s, albeit with less efficiency [49]. Cr1 and Cr2 usage increases the length of exon 7 by 23 and 51 nts, respectively (Fig. 2.6). Since the stop codon of SMN is located within exon 7, activation of Cr1 or Cr2 will have no consequences for the protein. Indeed, the activation of Cr1 in *SMN1* construct carrying pathogenic G1C mutation led to the production of SMN, confirming that transcripts generated by Cr1 activation are stable and translation competent (Fig. 2.6; [49]).

The discovery of Cr1 and Cr2 brings new perspective to our understanding of *SMN* exon 7 splicing regulation. Cr1 partially overlaps with ISS-N1, suggesting that the factors interacting with ISS-N1 are likely to suppress the activation of Cr1 as well. Interestingly, Cr1 is efficiently activated even by those eU1s that did not anneal to Cr1 directly [49]. Also, activation of Cr1 does not require assistance of the endogenous U1 snRNP, suggesting that usage of Cr1 can occur in the absence of the typical RNA:RNA duplex formed between the 5′ss and the U1 snRNA. This finding has broad implications as it suggests that the U1 snRNP can affect selection of a 5′ss from distance. It appears that positive *cis*-elements required for inclusion of *SMN* exon 7 are dispensable for Cr1 activation. For instance, point mutations that activated Cr1 in *SMN2* tolerated the loss of the enhancer associated with Tra2-beta1. Further, eU1s targeting Cr1 prevented skipping of exon 7 associated with the pathogenic mutation at the 3′ss of *SMN1* exon 7. Overall, these findings suggest that the activation of Cr1 might employ an entirely different set of rules.

2.3.7 Role of cis-Elements Within Intron 6

Various mutations at the 3′ss of *SMN1* intron 6 have been found to be associated with SMA pathogenesis [12, 110, 111]. However, very limited studies have been done to uncover the role of cis-elements within *SMN* intron 6. Element 1, an extended inhibitory sequence situated immediately upstream of the 3′ss of exon 7, was the first cis-element to be reported within intron 6 (Fig. 2.3; [87]). Deletion or

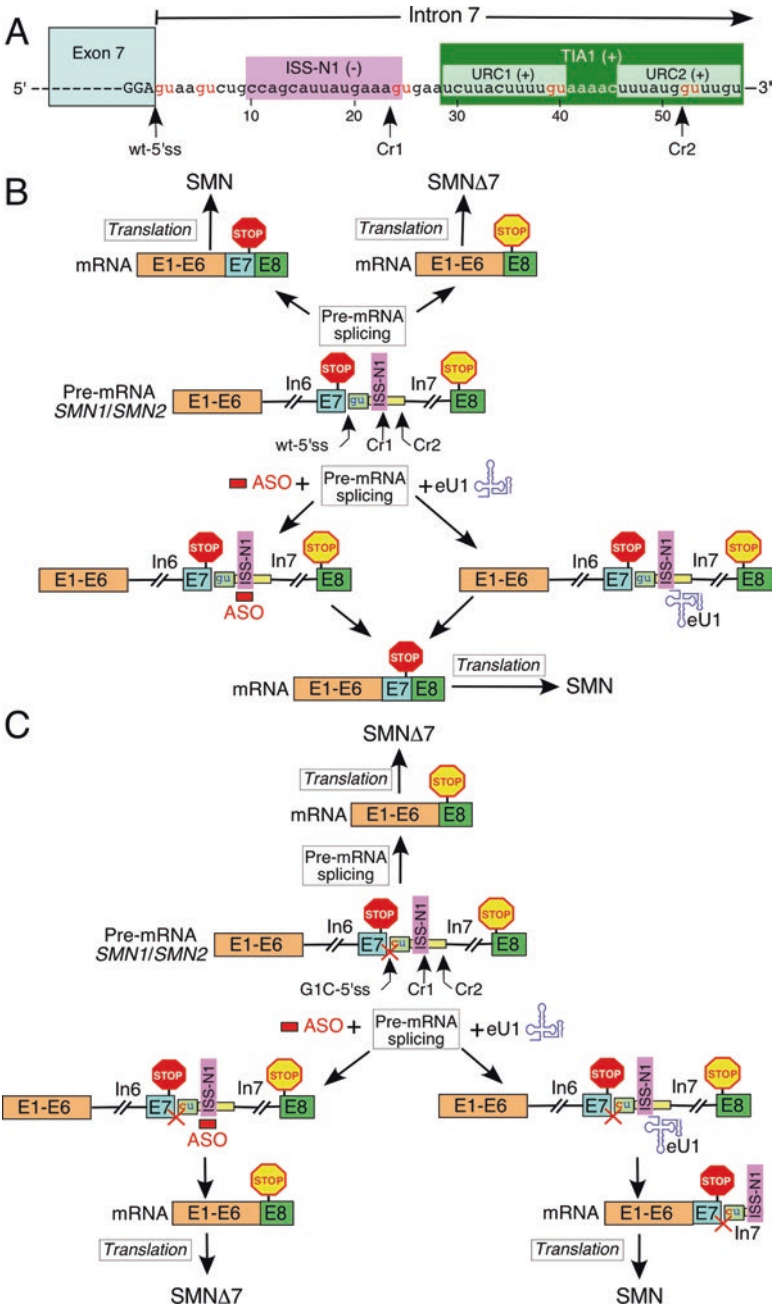

Fig. 2.6 Effect of an ASO and eU1 on splicing of exon 7. (**a**) Diagrammatic representation of exon 7/intron 7 junction. Exonic and intronic sequences are shown in upper- and lower-case letters, respectively. Exon7 is also shown as a blue box. Nucleotide numbering starts from the first position of intron 7. ISS-N1 and URC1 and URC2 are marked by colored boxes. The wild type and the cryptic

an ASO-mediated sequestration of Element 1 promoted *SMN2* exon 7 inclusion [87, 112]. A recent report demonstrated an in vivo efficacy of an Element 1-targeting ASO in a severe mouse model of SMA [112]. Another negative *cis*-element at the junction of intron 6 and exon 7 has been suggested to constitute a binding site for hnRNP A1 (Fig. 2.3; [51]). The location of this site right next to the other hnRNP A1-binding site created by the C6U mutation within exon 7 strikingly resembles the arrangement of two putative hnRNP A1 sites within ISS-N1. As recently proposed, close proximity of the two hnRNP A1 sites is conducive for a tight interaction involving two RRMs of a single hnRNP A1 molecule [95]. The polypyrimidine tract (PPT) at the 3′ss of exon 7 has been suggested to harbor a positive element associated with hnRNP C (Fig. 2.3; [68]). However, the role of hnRNP C in *SMN* exon 7 splicing could not be independently validated by depletion experiments [48, 113]. Interestingly, an A-to-G substitution at the -44th position (A-44G) of intron 6 has been found to promote *SMN2* exon 7 inclusion (Fig. 2.3; [40]). The A-44G substitution is naturally present in human population and SMA patients carrying A-44G substitution show mild phenotype [40].

2.4 Exonization of an Intronic Alu-Element

Alu elements are primate-specific transposable elements encompassing ~300 bp bipartite motifs derived from the 7SL RNA, an essential component of the protein signal recognition complex [114]. Insertion of Alu elements has played a significant role in primate evolution due to their drastic effect on chromatin remodeling, transcription and generation of novel exons [115, 116]. Multi-exon skipping detection assay (MESDA) is a powerful technique that simultaneously detects most *SMN* splice isoforms in a single reaction [117]. Employing MESDA, we have recently reported a novel exon, exon 6B, generated by the exonization of an Alu element located within intron 6 [33]. Expression of exon 6B-containing transcripts has been confirmed in various tissues of a mouse model of SMA as well as in human tissues examined [33]. Both *SMN1* and *SMN2* produce exon 6B-containing transcripts. Generally, the right arm of an antisense sequence of an Alu is used for exonization

Fig. 2.6 (continued) 5′ ss of exon 7 (Cr1 and 2) are indicated by arrows. GU dinucleotides are highlighted in red. (**b**) Model of how in the context of the intact 5′ ss of exon 7 an ASO and eU1 promote production of the full-length SMN protein (Adapted from [49]). The ASO block ISS-N1 and eU1 activates usage of the wild-type 5′ ss of exon 7. Exons and introns are indicated by the colored boxes and lines, respectively. The ASO is shown as a red bar, and eU1 as a blue structure. ISS-N1, stop codons in exon 7 and 8 and the 5′ ss of exon 7, wild type and cryptic, are indicated. (**c**) Model of how in the context of the mutated 5′ ss of exon 7 only eU1 promotes production of the full-length SMN protein (Adapted from [49]). The G to C mutation at the first position of intron 7 is shown in red. The inactivation of the 5′ ss is signified by a red cross. The ASO blocks ISS-N1 and eU1 activate usage of the cryptic 5′ ss of exon 7, Cr1. Exons and introns are indicated by the colored boxes and lines, respectively. The ASO is shown as a red bar, and eU1 as a blue structure. ISS-N1, stop codons in exon 7 and 8 and the 5′ ss of exon 7, wild type and cryptic, are indicated. Abbreviations are given in Table 2.2

[118]. However, the 109-nt long exon 6B originated from the left antisense arm of an Alu element. The low expression of exon 6B-containing transcripts is attributed to various factors, including suppression by hnRNP C and degradation by Nonsense Mediated Decay (NMD). An overwhelming 39% of *SMN* sequence is occupied by >40 Alu elements located within introns. Exon 6B is the first and only known example of *SMN* exon derived from the exonization of an intronic Alu element. Due to its location upstream of exons 7, it is likely that splicing of exon 6B is influenced by exon 7 and vice versa. However, the mechanism of exon 6B splicing regulation remains to be determined.

Amino acids coded by exon 7 define the critical C-terminus of SMN and confer protein stability. The loss of amino acids coded by exon 7 is the primary reasons why SMNΔ7 is less stable than SMN (Fig. 2.7; [16, 119]). Irrespective of exon 7 inclusion or skipping, the exon 6B-containing transcripts code for SMN6B protein in which the last 16 amino acids are coded by exon 6B. The altered C-terminus makes SMN6B less stable than SMN. However, SMN6B was found to be more stable than SMNΔ7, suggesting that the altered C-terminus of SMN6B is not deleterious as observed in case of SMNΔ7 (Fig. 2.7; [33]). As expected, SMN6B retains the ability to interact with Gemin2, a key protein required for most SMN functions. Similar to SMN, SMN6B localizes to both, nuclear and cytosolic compartments. Hence, it is likely that SMN6B will be able to ameliorate SMA pathology if expressed at sufficient levels.

2.5 Alternative Splicing of Other *SMN* Exons

The diversity of *SMN* splice isoforms is best demonstrated by MESDA, which captures susceptibility of various *SMN* exons to skipping under normal and stress-associated conditions [117]. Low levels of exon 3 and exon 5-skipped transcripts are generated under normal conditions in most tissues from both *SMN1* and *SMN2* [117]. *SMN2* exons 5 and 7 become highly susceptible to skipping under the conditions of oxidative stress, although skipping of *SMN1* exon 5 is also enhanced by oxidative stress. A recent study examined the effect of paraquat, an oxidative-stress-causing agent, on splicing of various *SMN2* exons in different tissues of a transgenic mouse model harboring *SMN2* [120]. Findings of this study revealed tissue-specific effect of oxidative stress on splicing of various *SMN2* exons. For instance, skipping of *SMN2* exons 3, 5, and 7 was found to be substantially increased under oxidative stress in lung as compared to brain and spinal cord, which instead showed significant enhancement of *SMN2* exons 5 and 7 skipping. The study also captured individual differences of the effect of oxidative stress on splicing of various *SMN2* exons. For example, one of the four animals examined showed enhanced co-skipping of exons 3, 4, 5, 6 and 7 in liver at 8 h post paraquat treatment. Another animal showed enhanced co-skipping of exons 3, 5, 6 and 7 in liver at 12 h post paraquat treatment. While reasons for these individual differences remain unknown, findings underscore that the rules of stress-associated splicing regulation should be interpreted with caution.

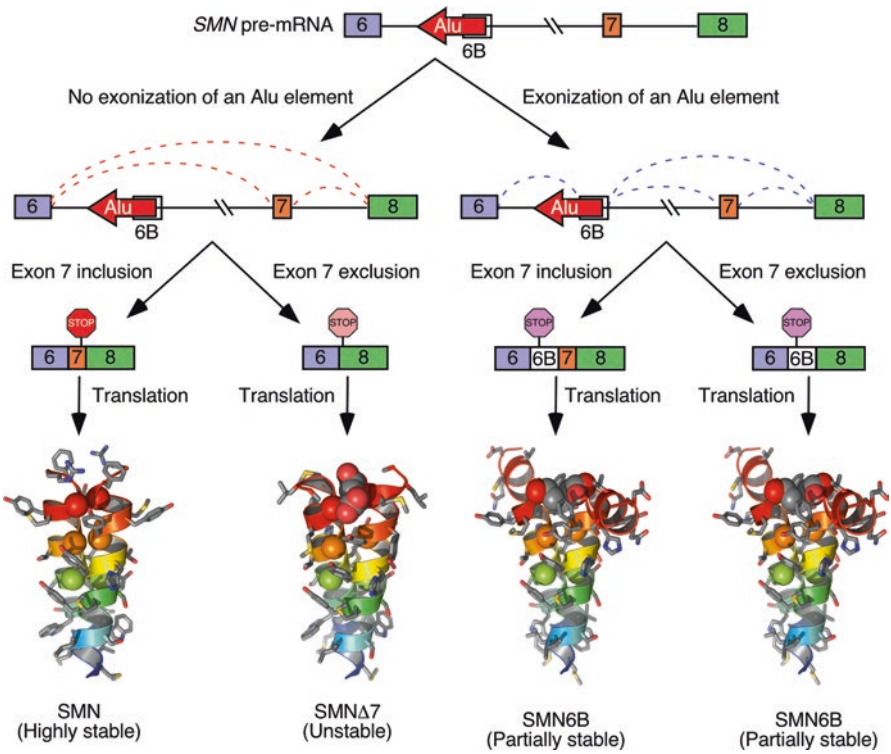

Fig. 2.7 A model showing skipping and inclusion of *SMN* exon 6B. Exon 6B is derived from an Alu element located within *SMN* intron 6 [33]. Transcripts that include exon 7 but exclude exon 6B produce full-length SMN, a highly stable protein. Transcripts that lack both exons 6B and exon 7 produce SMNΔ7, an unstable and partially functional protein. Transcripts that include exon 6B produce SMN6B protein irrespective of inclusion or exclusion of exon 7. SMN6B protein is more stable than SMNΔ7 [33]

Depletion of U1 snRNP creates a stress on the splicing machinery as well as on other co-transcriptional events dependent upon the availability of U1 snRNP [121]. A diverse set of *SMN* transcripts is generated upon depletion of U1 snRNP by an ASO that sequesters the 5′ end of endogenous U1 snRNA [49]. MESDA profile of *SMN* transcripts generated under U1 snRNP depletion condition is distinct from those observed under the conditions of oxidative stress. For example, splicing of all exons was affected under U1 snRNP depletion, whereas splicing of *SMN2* exons 5 and 7 was the most affected under oxidative stress condition [49, 117]. Interestingly, skipping of exon 6 was the least among all other internal exons of *SMN* under both U1 snRNP depletion and oxidative stress conditions [49, 117]. This could be attributed to relatively high accessibility of the 5′ss of exon 6 coupled with a strong duplex between U1 snRNP and the 5′ss of exon 6.

It is likely that the energy (ATP) deficit created by oxidative stress downregulates the biogenesis of snRNPs, particularly U1 snRNP, which is generally maintained at

a higher level than other snRNPs. It has been recently shown that the depletion of DHX9, an RNA helicase that resolves the double-stranded RNA structures, enhances the Alu-induced RNA processing defects, including aberrant pre-mRNA splicing and circRNA production from transcripts harboring Alu repeats [122]. Similar to snRNP biogenesis, RNA helicases require ATP for their function. Therefore, it is likely that large RNA:RNA duplexes formed by Alu elements positioned in opposite orientations in *SMN* pre-mRNA are not appropriately resolved by RNA helicases under the conditions of oxidative stress. Preliminary analysis of the publicly available circRNA database suggests production of circRNAs by *SMN* [123]. However, it is not known what fraction of *SMN* transcripts make circRNAs and which of the circRNAs are predominantly expressed in most cell types. Future studies will determine how Alu elements might impact generation of *SMN* circRNAs under normal and stress-associated conditions in a cell-specific manner.

2.6 Effect of Transcription on Splicing of Various *SMN* Exons

Transcription requires opening of chromatin structure followed by recruitment of transcription initiation factors [9]. Transcription in vivo is coupled to splicing through two likely mechanisms: “recruitment coupling” and “kinetic coupling” [124]. These two mechanisms are not mutually exclusive and it is often difficult to conclusively distinguish one mechanism from the other. In case of recruitment coupling, RNA polymerase II (pol II) recruits splicing factors at the promoter site and then transports it to the splice sites. In case of kinetic coupling, the rate of transcription elongation influences the outcome of splicing. The evidence that transcription affects splicing of *SMN* exon 7 comes from a promoter-swapping experiment performed in minigene systems. In particular, the replacement of the wild-type *SMN* promoter with CMV or TK promoter caused enhanced skipping of exon 7 in both *SMN1* and *SMN2* minigenes [117]. These results suggested that wild-type promoter harbors sequences that are stimulatory for exon 7 splicing.

Additional evidence that transcription affects *SMN* splicing comes from small molecules that affect the activity of histone acetylases (HATs) and histone deacetylases (HDACs). The former and the latter enzymes activate and suppress transcription, respectively. Various HDAC inhibitorsm, including trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), and benzamide M344, have been shown to modulate splicing of *SMN* exon 7 [125]. Another mechanism by which transcription could modulate splicing of *SMN* exons is through the regulation of the formation of loops within pre-mRNA. PTB and hnRNP A1/A2 have been implicated in deciding splicing outcomes through looping out specific sequences [126, 127]. In particular, looping out of an exon promotes its skipping, whereas looping out of an intra-intronic sequence promotes exon inclusion. Furthermore, a slow elongating pol II might delay the formation of a specific loop. Considering that *SMN* pre-mRNA contains binding sites for the loop-forming hnRNP A1/A2 protein, it is highly likely that splicing of various *SMN* exons is regulated by transcription.

2.7 Conclusions

SMA is one of the leading genetic diseases associated with infant mortality. As soon as the association of SMA with *SMN1* deletion/mutations was established in 1995, attempts began to find a potential cure/therapy for this disorder. Since *SMN2* is almost universally present in SMA patients, it offers an obvious therapeutic target for exon 7 splicing correction. The major breakthrough came when the critical role of the context-specific *cis*-elements located away from the pathogenic mutations, such as C6U, was beginning to be established. In particular, the discovery of the intronic *cis*-element, ISS-N1, reported in 2006 produced an effective target, sequestration of which fully corrected *SMN2* exon 7 splicing and restored SMN levels in SMA patient cells. General interest in ISS-N1 combined with subsequent independent validations of its therapeutic potential paved a way to the first FDA-approved drug for SMA. In addition, the detailed characterization of ISS-N1 led to the discovery of a unique RNA structure formed by long-distance intra-intronic interactions that contributes to exon 7 skipping. Interestingly, abrogation of a similar structure within intron 3 of the proteolipid protein 1 (*PLP1*) gene has been recently suggested to cause X-linked Pelizaeus–Merzbacher disease or PMD [128]. Growing evidence suggests that splicing of various exons is differentially regulated under the normal and stress-associated conditions. It is also becoming obvious that the intronic Alu elements are capable of increasing the diversity of *SMN* splice isoforms and may play an important role in the generation of circRNAs [123]. Furthermore, new findings that two antisense transcripts are produced from the *SMN* locus highlight the existence of an addition layer of *SMN* transcription and potentially splicing control. The development of novel tools and reliable assays that accurately capture transcription-coupled splicing events would tremendously advance our understanding of how expression of the *SMN* gene is regulated, including the pre-mRNA splicing step. This advancement would also uncover the likely mechanisms of the tissue-specific modulation of splicing of various *SMN* exons under the normal and stress-associated conditions. A better understanding of *SMN* splicing has implications for several diseases impacted by the low levels of the SMN protein. Lessons learnt from *SMN* would also provide unique insights into our understanding of a growing number of human diseases associated with aberrant splicing.

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Disclosures and Competing Interests

The ISS-N1 target (US Patent# US7838657) was discovered in the Singh laboratory at UMass Medical School (MA, USA). Inventors, including RN Singh, NN Singh and UMASS Medical School, are currently benefiting from licensing of the ISS-N1 target to Ionis Pharmaceuticals and Biogen. Iowa State University holds intellectual property rights on GC-rich and ISS-N2 targets. Therefore, inventors including RN Singh, NN Singh and Iowa State University could potentially benefit from any future commercial exploitation of GC-rich and ISS-N2 targets.

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Chapter 3
RNA Editing Deficiency in Neurodegeneration

Ileana Lorenzini, Stephen Moore, and Rita Sattler

Abstract The molecular process of RNA editing allows changes in RNA transcripts that increase genomic diversity. These highly conserved RNA editing events are catalyzed by a group of enzymes known as adenosine deaminases acting on double-stranded RNA (ADARs). ADARs are necessary for normal development, they bind to over thousands of genes, impact millions of editing sites, and target critical components of the central nervous system (CNS) such as glutamate receptors, serotonin receptors, and potassium channels. Dysfunctional ADARs are known to cause alterations in CNS protein products and therefore play a role in chronic or acute neurodegenerative and psychiatric diseases as well as CNS cancer. Here, we review how RNA editing deficiency impacts CNS function and summarize its role during disease pathogenesis.

Keywords RNA editing · MARCH · AMPA · GluA2 · 5HT receptors · K channels · Excitotoxicity · Neurodegeneration · Psychiatric diseases · Cancer

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Ileana Lorenzini and Stephen Moore contributed equally to this work.

I. Lorenzini
Barrow Neurological Institute, Department of Neurobiology, Dignity Health, St. Joseph's Hospital and Medical Center, Phoenix, AZ, USA

S. Moore
Barrow Neurological Institute, Department of Neurobiology, Dignity Health, St. Joseph's Hospital and Medical Center, Phoenix, AZ, USA

Interdisciplinary Graduate Program in Neuroscience, Arizona State University, Tempe, AZ, USA

R. Sattler (✉)
Department of Neurobiology and Neurology, Dignityhealth St. Joseph's Hospital, Barrow Neurological Institute, Phoenix, AZ, USA
e-mail: rita.sattler@dignityhealth.org

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3.1 Introduction: RNA Editing Overview

RNA editing is a molecular process that allows changes in the sequence of specific RNA transcripts to increase the diversity of different RNAs that can be generated from the genome. This can result in translation of different protein variants, but can also alter alternative splicing events and micro RNA-binding efficiencies [1]. RNA editing occurs during or after transcription through two distinct mechanisms: (1) chemically modifying a nucleotide, and therefore, altering the nucleotide sequence; (2) inserting or deleting nucleotides and changing the length of the mRNA. This chapter will focus on the most common form of RNA editing, the Adenosine to Inosine (A/I) nucleotide modification of RNA catalyzed by a family of enzymes known as adenosine deaminase acting on double-stranded RNA (ADARs). We will summarize how the dysfunction of these RNA editing enzymes and the subsequent substrate alterations contributes to central nervous system (CNS) diseases [2–5].

While other RNA editing events such as C-to-U or G-to-A exist, the catalytic deamination of Adenosine into Inosine is the most prevalent [6, 7]. There are three ADAR gene family members in mammals: ADAR1, ADAR2, and ADAR3 (These enzymes have also been referred to as ADAR, ADARB1, and ADARB2 respectively). All ADAR proteins contain double-stranded RNA-binding domains (dsRBDs), a nuclear localization sequence (NLS), and a C terminal deaminase domain [8] (see Fig. 3.1). There are two ADAR1 isoforms, ADAR1 p150 containing two additional Z DNA-binding domains and an NES and ADAR1 p110 a truncation isoform maintaining one Z DNA-binding domain and no NES [9]. ADAR1 is widely expressed throughout the body and to a lesser extent in the CNS. It has been shown to bind to over 10,000 genes and is necessary for normal development [10, 11].

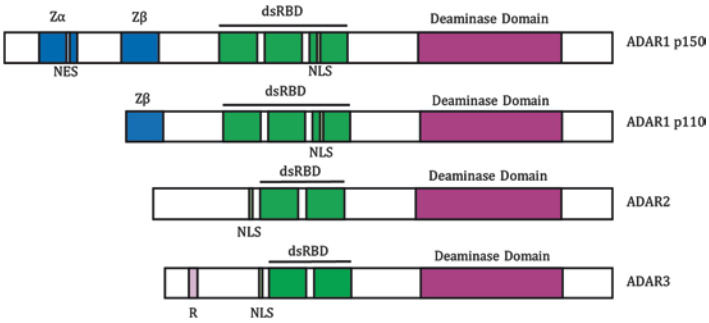

Fig. 3.1 ADAR domain structures. ADAR family members do share certain domain structures, including a C-terminal Deaminase Domain, dsRNA-binding domains (dsRBD), and a nuclear localization domain (NLS). ADAR1 comes in two isoforms, ADAR1 p150 and p110. ADAR1 p150 has two Z-DNA-binding domains, Zα and Zβ, in addition to a nuclear export sequence, which explains why ADAR1 p150 can be found both in the nucleus and in the cytoplasm. ADAR1 p110 only has a Zβ domain, and is only expressed in the nucleus. ADAR3 differs from ADAR1 and ADAR2 by the existence of a N-terminal RG-rich region. ADAR1 and ADAR2 are ubiquitously expressed throughout the body, while ADAR3 is CNS specific. The chromosomal locations of the *ADAR1–3* genes are 1q21.3, 21q22.3, and 10p15.3, respectively

ADAR1$^{-/-}$ mice are embryonic lethal and die around day E11.5 [10]. The mouse embryos undergo widespread apoptosis and show severe liver disintegration due to the loss of ADAR1 [10, 11]. ADAR2 is highly expressed in the CNS, and to a lesser extent in peripheral tissues [12]. ADAR2 has been shown to be responsible for the A/I editing of transcripts that are most actively edited. Knockout mouse models have shown that ADAR2 is required for normal development and ADAR2$^{-/-}$ mice die by P20 and become progressively seizure prone [13]. The third and final member of the family, ADAR3, is thought to have no RNA editing activity [14]. ADAR3 contains an additional arginine-rich domain [14]. Unlike its family members, ADAR3 is expressed exclusively in the brain [15]. Because no ADAR3 editing activity has been reported, the function of the enzyme is still an area of debate. There is a growing amount of evidence to suggest that ADAR3 acts as a negative regulator of overall RNA editing by binding and sequestering editing substrates of ADAR1 and ADAR2 [15, 16].

How does RNA editing work? The hydrolytic deamination of adenosine by the catalytic activity of ADAR1 and 2 disrupts the canonical Watson and crick base pairing of adenosine and as a result the edited inosine will be interpreted by the translational machinery as a guanosine (see Fig. 3.2). Therefore, RNA A/I editing events that fall within protein-coding regions can potentially alter the codon and allow the translational machinery to introduce amino acid changes into the protein that were not encoded by the genome. This can allow for important variation of protein products produced by a single strand of RNA (e.g. serotonin receptor [17]). Editing also occurs in noncoding regions of the transcriptome where the location of the edited nucleotide can regulate splicing, retain edited mRNA in the nucleus, or prevent micro-RNA processing [7, 18–25]. Historically, the estimation of total RNA editing sites was difficult and RNA editing was studied utilizing the serendipitous discovery of A/I sites [26]. With the ever-increasing capabilities of sequencing technologies, it is now possible to analyze RNA editing sites with far greater detail [19, 26, 27]. There are conflicting reports on the total number of RNA editing events in the human genome with reports claiming over one hundred million editing sites spanning the majority of the transcriptome [7, 18, 19, 26, 27]. The majority of these RNA editing events are found within *Alu* repetitive elements. These genomic elements are approximately 300 bp in lengths and are primate-specific transposable elements that comprise approximately 10% of the human genome [28]. These repetitive elements form long dsRNA secondary structures that make them ideal targets for ADARs. ADARs edited sites and levels of RNA editing, as well as ADAR proteins themselves are thought to be evolutionary conserved and play a role in environmental adaptation [29].

A/I RNA editing has been recognized as a significant event during CNS cortical development [30]. An increasing RNA editing pattern is observed during deep cortical layer formation suggesting these events occur at a critical period in neuronal maturation. As previously stated knockout mouse models of both ADAR1 and ADAR2 have shown that mice deficient in these deaminases form severe developmental phenotypes, emphasizing the importance of A/I RNA editing during CNS

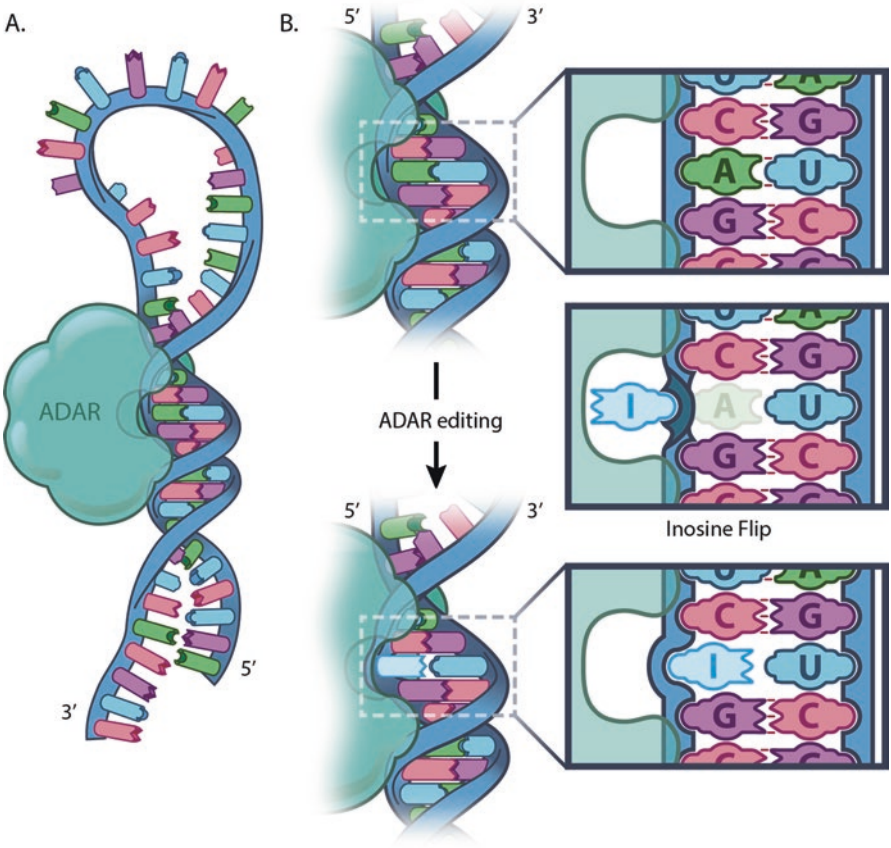

Fig. 3.2 ADAR A/I RNA editing. (**a**) ADAR enzyme (light green structure) acting on double-stranded RNA. (**b**) ADAR dsRNA-binding domains act on dsRNA editing sites and its catalytic domain converts adenine to inosine. Within the catalytic domain an amino group on the adenine base is replaced by an oxygen and converted to inosine

development [13]. At mature states, neurons show higher ADAR expression and editing activity than non-neuronal cells suggesting a limited involvement of other brain cells in RNA editing [4, 30]. Editing events may occur in response to environmental factors or to maintain normal CNS physiology. It can alter the function of target genes such as α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors for fast excitatory neurotransmission, serotonin-$5HT_{2C}$ receptors, or potassium channels $K_v1.1$ for modulation of neuronal excitability [31, 32]. Due to the regulation of these ion channels by ADARs, RNA A/I editing is considered crucial for proper neuronal function.

3.1.1 Major CNS RNA Editing Targets

To illustrate the importance of RNA editing in the CNS, we decided to introduce briefly three major RNA editing targets, which have shown to play a role in disease pathogenesis of several of the CNS disorders discussed below.

3.1.1.1 AMPA Receptors

AMPA receptors are ionotropic glutamate receptors responsible for fast synaptic transmission in the CNS [33]. The functional properties of AMPA receptors are greatly dependent on its subunit composition, GluA1–4, determining its role in synapse formation, stabilization, and synaptic plasticity [34]. The GluA2 subunit has the ability to regulate the calcium (Ca^{2+})-permeability of AMPA receptors [35–37]. Most AMPA receptors become permeable to Ca^{2+} by lacking the GluA2 subunit, and these GluA2-lacking receptors are thought to contribute to normal brain function, especially synaptic plasticity [33, 37–41]. However, there are numerous reports suggesting that GluA2-containing AMPA receptors become Ca^{2+}-permeable due to a lack of editing of the GluA2 Q/R site, although in the brain, almost 100% of GluA2 mRNA is present in its edited form [42–46] (see Fig. 3.3). This unique element of GluA2 is regulated by ADAR2-mediated A/I RNA editing [31]. Mice lacking ADAR2 can be rescued by expression of a forced edited GluA2 subunit [13]. This provides evidence that this single editing event is essential for normal development and survival. It further supports the idea that unedited Ca^{2+}-permeable GluA2-containing AMPA receptors do not have a physiological role similar to GluA2-lacking AMPA receptors. In this chapter, we will discuss the role of AMPA receptor GluA2 Q/R editing in the context of the role of glutamate excitotoxicity in neurodegenerative diseases, especially Amyotrophic lateral sclerosis (ALS; see below).

3.1.1.2 Serotonin Receptors

Serotonin 5-hydroxytryptamine (5-HT) receptors are a family of chemical messengers that produce a wide variety of physiological responses including circadian rhythms, mood, memory, cognition, and possibly peristalsis in the gastrointestinal tract [47–49]. There are 15 unique receptors divided into seven subgroups (5-HT1–7), all subgroups are classified as G-protein coupled receptors with the exception being the 5-HT3 receptors that are ionotropic [50–52]. The 5-HT2C receptor subtype is expressed throughout the CNS [53, 54]. There are five ADAR-meditated RNA editing sites on the 5-HT2C mRNA, designated sites A through E [17]. These five editing sites are located within 13 base pairs and are responsible for three codons allowing for significant variation in the protein isoforms [17, 55]. With only 7% of 5-HT2C mRNA lack editing at any of the five sites, the majority of transcripts are exposed to ADAR-mediated A/I editing, the most prevalent showing editing at

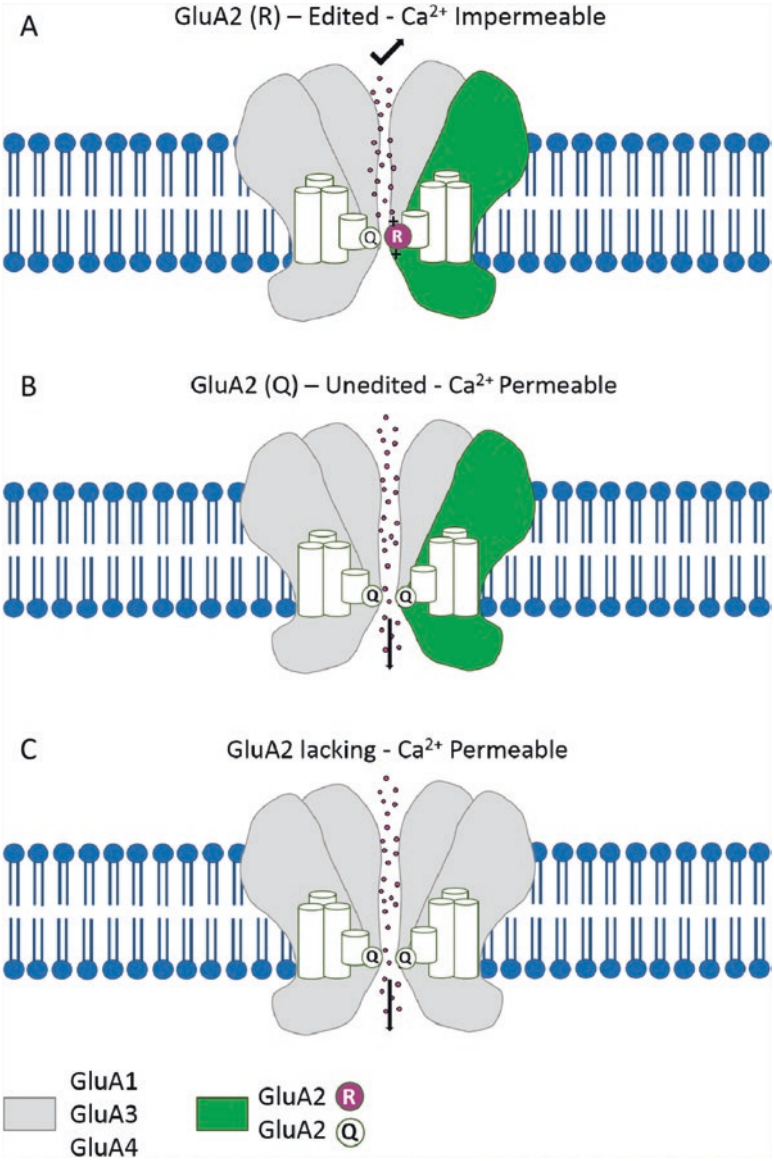

Fig. 3.3 Role of GluA2 in AMPA receiver Ca^{2+} permeability. (**a**) AMPA receptors containing fully edited GluA2 (R) are impermeable to calcium due to the positively charged arginine in the channel pore. (**b**) When GluA2 (Q) is unedited, this positive charge is removed with the presence of the glutamine, and AMPA receptors become permeable to calcium. (**c**) Calcium permeability is also present when AMPA receptors lack GluA2 (Q) altogether and are composed of other AMPA receptor subunits instead

the ABC and D sites [17]. Editing of this receptor alters binding affinity and functional potency of receptor agonists, and thereby affection receptor function during synaptic signaling. The fully edited 5-HT2C receptor isoforms have been shown to have a 40-fold decrease in serotonergic potency, decreasing inositol phosphate accumulation and calcium release [56–58]. The role of serotonin receptor editing is mostly relevant for neuropsychiatric disorders, such as schizophrenia and depression (see below).

3.1.1.3 Voltage Gated Potassium Channels

Voltage gated potassium channels (Kv channels) are the largest subgroup of potassium channels [59, 60]. Comprised of 12 subgroups (Kv1–12) these six transmembrane domain subunits form tetrameric Kv channels containing an inner pore and external voltage sensor domains allowing for the conversion of voltage across the membrane to be transferred into mechanical work [60]. The Kv1 family (Kv 1.1,2, and 4) has been shown to localize to soma, axons, synaptic terminals, and proximal dendrites [59, 61]. The Kv1.1 channel plays an important role in the regulation of neuronal excitability [62]. An ADAR2-mediated A/I editing site lies within the ion pore of the Kv1.1 subunit, mediating an isoleucine to valine substitution [63]. No differences were observed between the voltage-dependent activation of edited and unedited Kv1.1 channels [63]. In contrast A/I editing at this site has been proposed to target the process of fast inactivation [63]. Fast inactivation of Kv1 channels is mediated by the inactivating ball domain on the Kvβ1 subunit [64]. Regulation of this mechanism by RNA editing will have profound effects on regulation of neuronal excitability.

3.2 RNA Editing Deficits in Neurodegeneration

As summarized above, the post-transcriptional modification of RNA transcripts by ADARs through RNA editing generates protein diversity regulating many critical aspects of CNS function. Therefore, if the RNA editing process fails it could lead to CNS diseases, or exacerbate acute injury and chronic disorders. In the following sections, we will discuss RNA editing deficits for chronic and acute neurodegenerative disorders, neuropsychiatric diseases, and brain cancers.

3.2.1 RNA Editing in Chronic Neurodegenerative Diseases

Alzheimer's disease (AD) accounts for 60–80% of dementia cases [65]. It is the most prevalent form of dementia characterized by a progressive loss of memory and cognitive dysfunction. The neuropathological hallmarks comprise of plaques and tangles known to play a critical role in neurodegeneration [65, 66]. Areas of the

hippocampus, pre-frontal, and temporal cortex play a significant role in AD pathophysiology [67]. Studies done by Akbarian et al. associated deficits in RNA editing of the α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluA2 in the pre-frontal cortex of AD patients with changes in intracellular Ca^{2+} which could lead to neuronal dysfunction and neurodegeneration due to excessive Ca^{2+} permeability [68]. The authors showed that the pre-frontal cortex of Alzheimer's patients has approximately 1.0% of all GluA2 RNA molecules unedited. In healthy states the pre-frontal cortex shows less than 0.1% of all GluA2 RNA molecules are unedited and more than 99.9% are edited. Other studies found lower RNA editing levels at the GluA2 Q/R site in the hippocampus and caudate of sporadic AD patients and Apo E4 carriers, independent of clinical diagnosis. Interestingly, ADAR levels were decreased only in the caudate region of the patient's brains [69]. The E4 allele of the apolipoprotein ApoE gene has been recognized as a major genetic risk factor for AD and it has been suggested that ApoE plays a role in hippocampus AMPA receptor dynamics and glutamate regulation [70–72]. Interestingly, studies performed in the triple-transgenic AD mouse model (3×Tg-AD, PS1(M146 V); APP(Swe); tau (P301L)), a widely used transgenic mouse model for AD which exhibits both plaques and tau pathology, showed decreased levels of all AMPA receptor subunits, except for GluA2, while no editing deficiencies were detected [73]. A study aimed at analyzing the hippocampal transcriptome of normal aged mice using RNA sequencing, also examined age-related RNA editing changes as a mechanism to generate alternative transcripts [74]. In 29 months old mice, 41 out of 682 editing sites were significantly changed, which corresponded to 35 genes. One of the genes exhibiting increased editing was the serotonin receptor 2c, which has previously been found showing altered RNA editing in a mouse model of impaired memory function [75]. A comprehensive study on RNA editing in postmortem AD patient tissue revealed significant loss of RNA editing in the hippocampus, and to a lesser extent in the temporal and frontal lobes [76]. Most of the editing changes showed hypo-editing, including the serotonin receptor 2c, which in contrast to what was found in the aging mice discussed above showed less RNA editing in the hippocampus, temporal and frontal lobes. Surprisingly, the authors were unable to find a true correlation between the editing deficits and the expression levels of neither ADAR1 nor ADAR2, suggesting that ADAR dysfunction could be caused by mechanisms other than decreased transcription.

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder where the progressive death of both the upper and lower motor neurons leads to atrophy of skeletal muscles and ultimately death due to respiratory failure [77]. The known genetic contribution to ALS is relatively little, only 10% of the ALS cases are believed to be familial. The remaining 90% of ALS is designated as sporadic ALS in which there is no familial history of the disease [78]. While the etiology remains largely unknown there have been great strides in understanding the pathology of the disease attributed to the advances in genomic sequencing capabilities [79].

Early studies identified the dysregulation of astrocytic glutamate transporters in ALS as the leading cause for increased levels of glutamate at the synapse [80]. Pyramidal tract projection into the spinal cord uses glutamate as the excitatory neu-

rotransmitter and motor neurons expressing abundant glutamate receptors are most vulnerable to exaggerated glutamate stimulation, supporting excitotoxicity as a major mechanism for motor neuron loss in ALS [81]. A likely contributor to the mechanism behind neuronal excitotoxicity is the dysfunction of the AMPA receptor leading to exaggerated calcium influx and slow neuronal death [82, 83]. As mentioned previously, elevated calcium influx through the AMPA receptors can occur through the absence of GluA2 from the receptor complex or through RNA editing of the GluA2 Q/R site. Initial studies addressing the role of AMPA receptors in motor neuron cell death supported both of these mechanisms [84–88]. Over the years, Kwak and colleagues provided accumulating evidence that spinal motor neurons from sporadic ALS patients showed reduced GluA2 Q/R editing efficiencies, leading to increased Ca^{2+} permeability of AMPA receptors and subsequent excitotoxic motor neuron cell death [84, 89, 90]. The group further showed that these editing deficits are accompanied by a downregulation of ADAR2 [91], and transgenic mice with specific motor neuron knockdown of ADAR2 exhibited inefficient GluA2 Q/R editing and decreased motor function, which was rescued when the mice were crossed with transgenic mice overexpressing a fully edited version of GluA2 [92]. Interestingly, the oculomotor neurons, which are generally not affected in ALS patients, of these mice were not degenerated despite a loss of ADAR2 and a decrease in GluA2 Q/R editing. Also, the motor neurons of the ADAR2 conditional knockout mice exhibited classical TDP-43 pathology, and similar co-pathologies were found in sporadic ALS patient spinal cord motor neurons [93]. The authors propose that Ca^{2+} influx via unedited GluA2 containing AMPA receptors leads to activation of calpain, which in turn triggers TDP-43 pathology and nucleocytoplasmic transport deficits, in addition to excitotoxicity [94, 95].

The loss of GluA2 Q/R editing efficiency has not been demonstrated in other subgroups of ALS, while decreased ADAR2 levels were reported in spinal motor neurons of a single patient carrying a FUS mutation [96]. A recent transcriptome study using deep RNA sequencing technology reported that while spinal cord tissue shows decreased GluA2 Q/R editing efficiencies compared to other brain regions, there was no detectable difference of GluA2 Q/R editing deficits between control spinal cord patient tissue and sporadic ALS patient tissue [97]. One explanation for this discrepancy could be the use of spinal cord tissue lysate versus laser-captured motor neuron analysis, or, the use of RNA sequencing versus a restriction digest-based RNA editing technique. Future studies are required to address these conflicting results. Finally, Donnelly et al. described sequestration of ADAR3 to C9orf72 repeat RNAs in postmortem C9orf72 ALS patient tissue and patient-derived human-induced pluripotent stem cells differentiated into motor neurons (hiPSC-MNs) [98]. Additionally, the hiPSC-MNs showed increased susceptibility to glutamate toxicity, which was mimicked by siRNA knockdown of ADAR3. Ongoing studies in our laboratory are aimed at understanding how ADAR3 dysfunction could regulate ADAR2 function and subsequent excitotoxicity in C9orf72 ALS/Frontotemporal Dementia (FTD), and whether ADAR2 function itself is altered in C9orf72 ALS/FTD patients.

Huntington's disease (HD) and Parkinson's disease (PD) have not been investigated much in regards to RNA editing deficits. HD is an autosomal dominant mutation caused by an abnormal trinucleotide CAG repeat expansion in the huntingtin gene (HTT). Carriers of this mutation produce an unusual polyglutamine sequence that causes disease by a toxic gain of function of the protein huntingtin. Even though HD impacts the entire brain, the most affected regions are the basal ganglia and striatum composed of the caudate nucleus and putamen. To a lesser extent areas of the cerebellum, substantia nigra, hippocampus, and layer III, V and IV of the cerebral cortex are affected [99]. Very early research in HD, often referred to as Huntington's chorea, suggested that aberrant glutamate homeostasis might be involved in HD disease pathogenesis [100]. As an example, researchers used intrastriatial injections of glutamate or kainic acid to mimic biochemical changes observed in HD [101, 102]. With the cloning and discovery of glutamate receptors, the role of glutamate and excitotoxicity becomes a major disease mechanism for HD [103] and the first study examining RNA editing of glutamate receptors subunits GluA2, 5 and 6 noted no difference in the RNA editing efficiency between healthy control and HD patient brain tissue samples [104]. A later study provided the first evidence to support little, yet significant changes in GluA2 editing in the striatum on HD patient tissue [68]. Nearly 5% of GluA2 Q/R was unedited, which still leaves a large percentage of edited GluA2, but could nevertheless contribute to increased Ca2+ permeability and neuronal death. Interestingly, a more recent study decreased immunostaining for GluA2 in the striatum of HD patient tissue when compared to control tissue, suggesting that an overall lack of GluA2 might further contribute to glutamate excitotoxicity in HD [105].

PD is the second most common neurodegenerative disorder affecting nearly 1% of the population [106]. Patients with PD exhibit crippling motor deficits or bradykinesia (or slowness of movement), rigidity, resting tremor, and postural instability also known as the four cardinal manifestations of PD [107]. These symptoms arise due to the degeneration of dopaminergic neurons in the substantia nigra [106, 108]. Similar to HD, among the many proposed cellular dysfunctions [108] excitotoxicity has been suggested to play a role in the degeneration of the dopaminergic neurons [109]. However, despite the proposed role of excitotoxicity there has been little evidence that suggests any known RNA editing deficits [110]. With the increase in RNA sequencing capabilities the ability to study RNA editing events by whole transcriptome sequencing is allowing for more complex analysis of A/I editing sites in disease. One whole transcriptome study associated Parkinson's disease with changes in Alu insertions the largest target of the ADAR family of proteins [111]. Due to ADARs RNA editing of micro RNAs and Long noncoding RNA and alterations in these RNAs in PD, RNA editing is hypothesized to play a role in disease pathogenesis [110], but only future studies will prove whether this hypothesis is correct. An intriguing new concept has just been proposed in regards to utilizing endogenous ADAR2 editing activity to repair a PD disease causing mutation in PINK1 [112]. A G-to-A mutation in PINK1 introduces a premature stop codon and shortens the protein's C-terminus including its kinase domain. The authors designed guideRNAs to enable endogenous ADAR2 to edit and recode the user-defined mRNA target.

This was successfully achieved in mammalian cell lines and showed a functional rescue of PINK1/Parkin-mediated mitophagy [112].

3.2.2 RNA Editing in Acute Neurodegeneration

Epilepsy is a neurological disorder characterized by abnormal neuronal hyperexcitability of a subpopulation of cells resulting in unprovoked recurrent seizures [113, 114]. The mechanisms responsible for this neuronal hyperexcitability are multifaceted and include genetic predispositions, acute brain injuries, as well as epigenetic changes alterations. Overstimulated cells have a prolonged increase in intracellular Ca^{2+} concentrations, which has been suggested to contribute to the mechanisms of hyperexcitability seen in epilepsy. AMPA receptors are involved in fast excitatory neurotransmission and are therefore thought to play a key role in the generation of seizures. Various studies present evidence that connects deficits in AMPA receptor editing with seizure vulnerability. Transgenic mice expressing a fully unedited GluA2 Q/R site die around 3 weeks of age and develop severe seizures [115]. Interestingly, GluA2 knockout mice, while similarly showing premature death, do not show signs of seizures, but instead show increased susceptibility to absence seizures [116]. ADAR2 knockout mice behave very similar to the GluA2 Q/R unedited mice and develop seizures before prematurely dying at 21 days of age [13]. These mice are rescued by crossing the ADAR2 KO mice with transgenic mice overexpressing a fully edited GluA2 Q/R site [13]. RNA editing analyses of epileptic brain tissue resulted in contradictory results, with studies showing no altered RNA editing at the GluA2 Q/R site (while there were RNA editing changes in GluA5 and GluA6) [117]. Only one study examined ADAR2 expression from needle biopsy samples obtained from hypothalamic hamartoma tissue and found loss of nuclear immunostaining of ADAR2 concomitant with lower RNA editing efficiency at the GluA2 Q/R site [118]. A recent genome-wide analysis of epileptic and healthy mouse hippocampus revealed a correlation between seizure frequency and differential RNA editing [119]. Functional enrichment analysis revealed that pathways relevant for epilepsy showed the highest degree of differential RNA editing, e.g., neuron projection, synapse, seizures. More work needs to be done to fully understand whether RNA editing plays a significant role in this disorder.

Stroke patients suffer from a spontaneously disrupted blood supply to the brain resulting in a loss of oxygen and nutrients to affected regions. Accounting for 85% of all strokes an ischemic stroke occurs when blood flow to part of the brain is obstructed. After an ischemic attack and loss of blood supply, cells are immediately unable to sustain normal homeostasis leading to massive irreversible cell death [120]. Because of the rapid neuronal loss in stroke victims immediate and effective treatment is crucial to minimize damage [121, 122]. Post-ischemic excitotoxicity results from consumption of ATP, failure of ATP synthesis, and dysregulation of the ionic concentration across the plasma membrane leading to rapid rise in intracellular calcium concentrations and death of the cell [123]. Historically, the increase in

calcium permeability of neurons affected by ischemia was thought to be due the downregulation of GluA2 following ischemia commonly referred to as "The GluA2 hypothesis" [36]. However, in 2006 unedited GluA2 was found in the CA1 pyramidal neurons of rats following ischemia [124]. The calcium permeability of AMPA receptors in the CA1 pyramidal neurons is 18-fold higher following ischemia when compared to control groups [125]. In addition, loss of ADAR2 expression increases neuronal sensitivity to ischemia and can be rescued by expression of a fully edited GluA2(R) [124, 125]. These studies suggest that loss of RNA editing contributes to the disruption in neuronal homeostasis following ischemic stroke and immediate prevention of these deficits may protect against neuronal damage.

Spinal Cord Injury (SCI) is defined as damage to the spinal cord causing reduced or complete loss of motor function [126, 127]. It generally affects glutamatergic tracts descending from varying brain regions and serotonergic tracts descending from the brainstem. Serotonin signaling is critical in the spinal cord by providing neuromodulation to motor neuron and recent studies showed reduced A → I RNA editing of the $5HT_{2c}R$ serotonin receptor after SCI, which was suggested to contribute to loss of motor neuron function [126–129]. These studies demonstrated that RNA editing deficiency for $5HT_{2c}R$ was due to a decrease in the ADAR2 expression suggested to be caused by a continuous inflammatory response during injury. In addition to $5HT_{2c}R$, the authors also found reduced RNA editing of potassium channel Kv1.1, an additional ADAR2 target. Additional studies strongly support the fact that microglial cells and immune infiltrating cells are involved in the dysfunction of A → I RNA editing in SCI [4, 128, 129], suggesting that at least during spinal cord injury, RNA deficits of neuronal targets are triggered by non-cell autonomous mechanisms. Future studies are needed to test the hypothesis that these non-cell autonomous mechanisms also occur in other neurodegenerative diseases characterized by RNA editing deficits.

3.3 A → I RNA Editing Dysfunction in Psychiatric Diseases

Depression and Schizophrenia. Depression is a long term mood disorder that affects a person's thoughts and feelings as well as daily activities such as working, eating and sleeping [130]. This disorder is caused by a combination of genetic, biological and environmental factors. Serotonin or 5-hydroxytryptamine (5HT), a monoamine neurotransmitter has been implicated in this psychiatric disease [131, 132]. Patients suffering from depression have lower levels of serotonin or an increase in the number of serotonin receptors. Selective serotonin reuptake inhibitors are frequently used to treat depression to maintain serotonin for longer periods at the synapse. Schizophrenia is classified as a chronic mental disorder where the patients lose contact with reality and present psychotic behaviors (positive symptoms), disruption of normal behaviors (negative symptoms), poor executive function and poor

working memory (cognitive symptoms) [133]. Similar to depression, schizophrenia is caused by genetic aberrations and environmental factors.

5HT-serotonergic receptors are relevant to mental disorders such as depression, anxiety, and schizophrenia. The $5HT_{2c}R$, a G-protein couple receptor, is known to undergo RNA editing post-transcriptional modification [32, 134–136]. Altered editing of $5HT_{2c}R$ pre-mRNA occurs in the pre-frontal cortex of depressive and schizophrenic patients. A/I RNA editing of the $5HT_{2c}R$ occurs at five sites (A-to-E) causing protein and functional diversity. Previous studies have shown that depressive and schizophrenic patients have reduced expression of ADAR2 with a decrease or increase in RNA editing in some of the five $5HT_{2c}R$ sites [137, 138] making it difficult to elucidate how RNA editing is associated with these psychiatric disorders. These studies suggest that RNA editing is not only disease-specific, but it may also be determined by the severity of the psychiatric diseases.

Cocaine addiction. An estimated 18.3 million people between the ages of 16–64 used cocaine in 2014 making it one of the most common illicit drugs in the world (National Institute on Drug Abuse 2016; [139]). Numerous health risks are associated with cocaine use such as cognitive impairment, respiratory disease, cardiovascular disease, congenital malformations, and premature mortality [140]. Approximately 20% of recreational users will develop a dependence for cocaine within 5 years [141]. Drug-seeking behavior is thought to be influenced by limbic cortical-ventral striatal circuitry which afferents to the basolateral amygdala and nucleus accumbens providing the circuitry for stimulus-reward pathway that reinforces drug seeking behavior [142]. Increased calcium permeable AMPA receptors in the nucleus accumbens have been associated with drug-seeking behavior [143]. These alterations may be due to increased GluA1 in the nucleus accumbens [144]. However, downregulation of ADAR2 and GluA2 Q/R editing deficits have been identified in the nucleus accumbens shell in rats following cocaine self-administration [145]. Both upregulation of GluA1 and misediting of the GluA2 Q/R site could explain alterations in the nucleus accumbens that leads to the reinforcement of drug-seeking behavior.

Considered a multi-factorial disorder *autism spectrum disorder* (ASD) is a range of neurological abnormalities affecting one in 68 children in the United States [146]. Children affected by ASD exhibit reduced eye contact, facial expression, and body gestures [147]. Due to the heterogeneity of the classification of the disease the etiology is still widely unknown. Genetic causes have only been identified in 10–20% of individuals. Deep whole transcriptome sequencing of 30 patients with ASD identified RNA A/I editing alterations in 20 of 25 sites analyzed [148]. In contrast to other neurodegenerative disorders discussed in this chapter, RNA editing levels in ASD were found to be significantly higher than control groups [148]. Interestingly, the editing at the GluA2 Q/R site is not altered in ASD [148, 149]. Alterations in RNA A/I editing in ASD have been explained by alterations in ADAR2 self-regulation and loss of fmr1 [148–150].

3.4 Brain Cancer

Glioblastoma multiforme (GBM) is a tumor generated from astroglial cells generally localized in the cerebral hemispheres, and to a lesser extent in other regions of the brain or spinal cord. A transcriptome study using RNA sequencing for global A-to-I editing events in human revealed that genes with predicted editing events were significantly enriched for cancer-related genes, suggesting that RNA editing plays a significant role in the development of cancer [151]. This was later confirmed by Hwang and colleagues, who showed via gene ontology analyses that there was a selective change in the pattern of RNA editing in gliobastomas [30] (also recently reviewed in [152]). Indeed, early studies found a significant reduction in the GluA2 Q/R and the serotonin receptor 5-HT(2C) editing efficiency in malignant human brain tumors, which correlated with decreased ADAR2 self-editing activity [153]. These studies were confirmed when significantly reduced editing in Alu sequences was found in brain tissues [154]. All three ADAR genes showed lower RNA levels and the reduced ADAR3 levels correlated with the grade of malignancy of glioblastoma multiforme. Along those lines, high grade astrocytomas equally show lack of ADAR2 editing activity when grown in vitro, as well as in vivo via a flank tumor growth model in nude mice [155, 156].

As previously discussed, A-to-I editing also affects miRNAs, ~22 nucleotide long noncoding RNAs known to silence gene expression by binding to the 3′untranslated region (3'UTR) of mRNAs. miRNAs can undergo A-to-I RNA editing at premature states when the miRNA has a double-stranded structure. Analyses of high grade gliomas revealed reduced editing of miRNA-376 [157]. The authors found a strong correlation between the extent of unedited miRNA-376 and tumor spread, which was measured using magnetic resonance imaging of the patient's brains. The authors further confirmed these results in xenograft mouse models, showing that unedited miRNA-376 promoted glioma growth and spread, while edited miRNA-376 was protective. Similar results were recently reported on miRNA-589-3p [158]. A more recent study showed that A-to-I miRNA editing is enhanced at the seed region of the miRNA, an area critical to bind its target mRNA [159]. The authors further confirmed by RNA sequencing of GBM patient tissue that a significant reduction of miRNA editing occurs in GBM tissue and is correlated with the reduction of ADAR2 expression [159].

Interestingly, one study found elevated levels of ADAR3 in GBMs when compared to control brain tissue [16]. The authors suggested ADAR3 as a potential regulator of the Q/R editing site by binding to GluA2 subunit pre-mRNA and thereby inhibiting editing by ADAR2 in GBM. They hypothesized that an elevated expression of ADAR3 and reduced GluA2 editing will induce calcium permeability through the glutamate receptor, which in turn accelerates cell migration and tumor invasion into surrounding peri-tumoral tissue.

3.5 Conclusions

RNA editing, with now an estimate of over a million editing sites in primates and humans, has gained increasing interest as an important mechanism of RNA processing, not only during development, but also in disease. Given its ability to contribute to the molecular complexity in the human body, including the brain, it is of importance that we learn more about the regulation of RNA editing and how it can contribute to disease pathogenesis. It will be important to fully understand temporal and spatial regulation, of specific brain regions and likely also cell types, of the individual ADAR editing enzymes. This knowledge will be especially critical if we consider targeting ADAR enzymes for therapeutic purposes in any of the discussed diseases, as well as any non-CNS disorders.

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Chapter 4
RNA Nucleocytoplasmic Transport Defects in Neurodegenerative Diseases

Ashley Boehringer and Robert Bowser

Abstract In eukaryotic cells, transcription and translation are compartmentalized by the nuclear membrane, requiring an active transport of RNA from the nucleus into the cytoplasm. This is accomplished by a variety of transport complexes that contain either a member of the exportin family of proteins and translocation fueled by GTP hydrolysis or in the case of mRNA by complexes containing the export protein NXF1. Recent evidence indicates that RNA transport is altered in a number of different neurodegenerative diseases including Huntington's disease, Alzheimer's disease, frontotemporal dementia, and amyotrophic lateral sclerosis. Alterations in RNA transport predominately fall into three categories: Alterations in the nuclear membrane and mislocalization and aggregation of the nucleoporins that make up the nuclear pore; alterations in the Ran gradient and the proteins that control it which impacts exportin based nuclear export; and alterations of proteins that are required for the export of mRNA leading nuclear accumulation of mRNA.

Keywords RNA · TREX · Exportin · Nuclear pore complex · Amyotrophic lateral sclerosis · Alzheimer's disease · Huntington's disease · Frontotemporal dementia

4.1 Introduction

In eukaryotic cells, transcription and translation are compartmentalized by the nuclear membrane, or nuclear envelope. The nuclear membrane separates the nucleus, where transcription takes place, from the cytoplasm, where translation

A. Boehringer
Department of Neurobiology, Barrow Neurological Institute, Phoenix, AZ, USA

School of Life Sciences, Arizona State University, Phoenix, AZ, USA

R. Bowser (✉)
Department of Neurobiology, Barrow Neurological Institute, Phoenix, AZ, USA
e-mail: robert.bowser@dignityhealth.org

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occurs. Transport between the two compartments is tightly regulated via trafficking through nuclear pores that are contained within the nuclear membrane. While molecules, including both proteins and nucleic acids, with a molecular mass below 40 kDa may diffuse freely through the pores, most larger molecules are actively transported using numerous carrier proteins [1–3]. RNA transport is predominantly mediated by either NXF1 (Nuclear RNA Export Factor 1), also known as TAP, or members of the exportin family of proteins. The export adaptor used is largely dependent on the type of RNA, with mRNA predominantly relying on NXF1 [4, 5]. All other types of RNA require a member of the exportin family as an adaptor, along with a gradient of the GTPase Ran. rRNA [6–8], snRNA, and some mRNAs utilize CRM1 (exportin-1, XPO1) [9, 10], and tRNA and miRNA require exportin-t and exportin-5 respectively [11–14]. In each case, the transport carrier protein is required to move the RNA, in the form of a ribonucleoprotein particle (RNP), through the nuclear pore and release it on the cytoplasmic side. In this chapter, we will review the canonical pathways for transport of RNA from the nucleus to the cytoplasm under normal conditions, as well as explore the alterations in RNA transport that have been identified in neurodegenerative diseases. These alterations predominantly fall into three categories; alterations in the nuclear envelope as well as mislocalization of the proteins making up the nuclear pore, alterations in the Ran gradient, and deficits in the export of mRNA, identified in both models of neurodegenerative disease and tissue from patients who suffered from these diseases.

4.2 Export of RNA from the Nucleus

4.2.1 Nuclear Pores Regulate Transport Between the Nucleus and the Cytoplasm

Transport between the nucleoplasm and cytoplasm is controlled by a protein structure called the nuclear pore complex (NPC). The NPC is approximately 125 MDa in size in vertebrates and comprised of a group of proteins known as nucleoporins [15]. The geometric structure of the nuclear pore consists of eight spokes connecting radially to form concentric rings and exhibits an eightfold symmetry, formed from over 500 copies of up to 30 different nucleoporins [15, 16]. The NPC can be broken into three regions; the central channel, nuclear basket, and cytoplasmic filaments. The central channel that is embedded within the nuclear envelope allows cargoes to move in and out of the nucleus. The nuclear basket is found on the nuclear side of the pore, and functions to bind transport competent mRNPs (messenger ribonucleoprotein particles) and direct them to the pore. Cytoplasmic filaments guide both proteins into the nuclear pore, and RNA cargoes which are exiting the pore, toward the translational machinery. The pore forms a central channel approximately 50–100 kDa/40 nm in size and is lined with nucleoporins-containing phenylalanine-glycine (FG) repeat domains. These FG repeats both fill the channel

of the pore as well as comprise both the cytoplasmic filaments and nuclear basket. An estimated 6 MDa of FG repeats are found in a single pore and these domains provide both a barrier to diffusion, as well as docking sites for transport factors as they are trafficked through the pore [17].

4.2.2 Exportin-Mediated Export of RNA

Different types of molecules (proteins, mRNA, rRNA, tRNA, miRNA) rely on a host of different transport factors to transverse through the nuclear pore. Some mRNA as well as most other types of RNA, including rRNA, tRNA, and miRNA, require an exportin protein to facilitate their export [18]. Exportins are a family of seven proteins including: CRM1 (XPO1), CSE1L (XPO2), XPOt (XPO3), XPO4, XPO5, XPO6, and XPO7 which function in export from the nucleus (Fig. 4.1). Much like the nuclear import transporters importins, exportins require a small GTPase called RanGTPase (Ran) to function. Export via exportins requires a gradient of Ran to exist in which GTP bound Ran (RanGTP) is concentrated in the nucleus, and both GDP bound Ran (RanGDP) and its GTPase activator RanGAP1 are concentrated in the cytoplasm [19]. Of the seven known exportins, CRM1 is required for the export of some mRNAs as well as rRNA, in addition to being a primary transporter of proteins [18]. CRM1 does not directly bind RNA but instead relies on a series of RNA-binding adaptor proteins which bind RNA and then CRM1 for RNA export (Fig. 4.1) [20–22]. These adaptor proteins require a Nuclear Export Sequence (NES), for CRM1 is HX2–3HX2–3HXH, where H is a hydrophobic

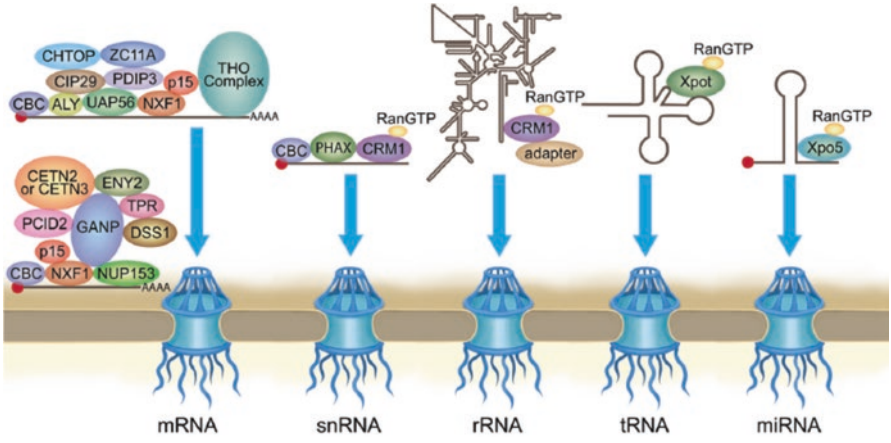

Fig. 4.1 Canonical RNA export pathways. Export of mRNA predominantly requires the TREX and TREX-2 pathways. snRNA and rRNA export requires exportin CRM1 bound to RanGTP along with the adaptor PHAX for snRNA and specific adaptors for different subunits of rRNA. Export of tRNA and miRNA require the RanGTP bound exportins, XPOt and XPO5 respectively

amino acid (i.e., isoleucine, leucine, methionine, phenylalanine, or valine) X is any amino acid [23, 24]. The binding of CRM1 to a NES-containing protein is cooperative with its binding to RanGTP [25]. After transport through the nuclear pore, GTP hydrolysis occurs which helps to dissociate its cargoes. In addition to a small subset of mRNAs, CRM1 is necessary for the export of rRNAs. Both the pre-60S subunit and the pre-40S subunit can be exported via CRM1 and an adaptor (Nmd3 or Lvt1 respectively). The pre-60S can also be exported by exportin-5 while the pre-40S subunit seems to rely solely on CRM1 [6, 7]. Other types of RNAs are also exported in a similar Ran-dependent process using other exportins, with export of tRNA requiring exportin-t (XPOt) and export of miRNA requiring exportin-5 (XPO5) [11–14, 26] (Fig. 4.1). Binding between pre-miRNA and XPO5 is mediated by the pre-miRNA structure rather than sequence with the recognition of a two-nucleotide 3′ end overhang structure and the double-stranded stem found in pre-miRNA [27]. In both cases the RNA is bound by a GTP-bound exportin that allows for its trafficking through the pore.

4.2.3 NXF1 Is the Primary Transporter of mRNA

Nucleocytoplasmic trafficking of mRNA through the nuclear pore mainly occurs via the transport factor NXF1. NXF1 is loaded onto mRNA via a series of handoffs involving the TREX (TRanscription and EXport) complex. Transport of mRNA is intricately linked with transcription and all stages of pre-mRNA processing including splicing. The TREX complex is made up of the THO complex-containing Thoc1 (Hpr1), Thoc2, Thoc3 (hTEX1), Thoc5, Thoc6, and Thoc7 as well as UAP56 (ddx39b), and Aly (AlyRef) [28] (Fig. 4.1). Unlike exportin-mediated export, TREX does not rely on a Ran gradient, but rather ATP hydrolysis.

The specificity of mRNA to TREX is mediated by its link to RNA polymerase II transcription, as well as a length requirement mediated by hnRNPC. hnRNPC interacts with the 5′ end of RNA if it is longer than 300 bp, preventing the recruitment of export factors other than TREX to the mRNP [29]. During transcription, proteins necessary for capping of the 5′ end, splicing, 3′ end cleavage, and polyadenylation bind to the nascent RNA. In metazoans, TREX has been shown to be predominantly coupled to splicing, whereas in yeast it has been shown to be more associated with transcription [30]. In human cells, TREX proteins have been shown to be recruited to the 5′ end of pre-mRNA near the cap-binding complex (CBC) which consists of the proteins CBP80 and CBP20 [31]. Aly binds closest to the CBC followed by UAP56 which binds downstream of Aly but upstream of the exon junction complex (EJC) (Fig. 4.1). This interaction is thought to be mediated by protein-protein interactions between Aly and CBP80 [31]. Interestingly, binding of mRNA to Aly and TREX complex member Thoc2 has been shown to require capped and spliced mRNA, suggesting that the recruitment of Aly to mRNA requires more than just binding to CBP80 [31].

Binding of Aly and RNA to UAP56 has been shown to stimulate the intrinsic ATPase activity of UAP56, which aids in its dissociation from the complex. The dissociation of UAP56 from the mRNP constitutes the handover of the mRNP to Aly. Aly along with a co-activator, Thoc5 or Chtop, are required for the binding of NXF1 to RNA [32]. NXF1 functions as a heterodimer with p15 (NXT1), and has very little RNA-binding activity in its native state. Upon binding with Aly and a co-activator, NXF1 is remodeled to expose its RNA-binding domains [32]. At this stage, the mRNP is turned over to NXF1 for trafficking though the nuclear pore.

Another export complex, TREX-2, also has a role in the export of mRNA via the NXF1 transporter. TREX-2 is built upon a scaffold protein GANP (Germinal-center-associated nuclear protein), which subsequently binds ENY2, PCID2, and DSS1 [33] (Fig. 4.1). The exact role of TREX-2 is unclear, though in yeast it has been shown to be involved in localizing a subset of actively transcribing genes to the pore [34]. In metazoans however, it has been shown to be involved in chaperoning mature mRNPs from processing centers to the pore for export [33]. It is unclear whether TREX and TREX-2 work cooperatively on the same mRNPs or transport different subsets of mRNPs, though some cooperation between the two complexes is thought to occur in mammalian cells [33]. One proposed model suggests that TREX-2 attaches to the mRNP after it is transferred from Aly to NXF1 and mediates its transport to and interaction with the nuclear pore [33].

4.3 Alterations in RNA Export and in Proteins Required for RNA Export Have Been Identified in Neurodegenerative Diseases

Many groups, including ours, have recently emphasized the role that alterations in nucleocytoplasmic trafficking play in a number of neurodegenerative diseases [35–40]. While initial studies focused on defects in protein trafficking, likely due to the common pathology of protein aggregation in the cytoplasm observed in many of these diseases, evidence for defects in RNA trafficking has recently come to light [39, 41]. These RNA trafficking alterations in disease states predominantly fall into three categories of defects; alterations in the localization of nucleoporins and abnormal nuclear envelope architecture, defects in the Ran gradient and alterations in the proteins that are responsible for maintaining it, and alterations in TREX proteins as well as mRNA retention within the nucleus (Fig. 4.2). It is important to note that alterations in protein trafficking are intricately linked to alterations in RNA trafficking due to the use of common regulatory proteins in nuclear export of proteins and RNA. Alterations in nucleoporins and the nuclear envelope as well as loss of the Ran gradient are likely to influence all forms of transport in and out of the nucleus. While export of mRNA via the TREX/NXF1 pathway is Ran independent, it requires members of the export process to be imported back into the nucleus to function, which is a Ran-dependent process.

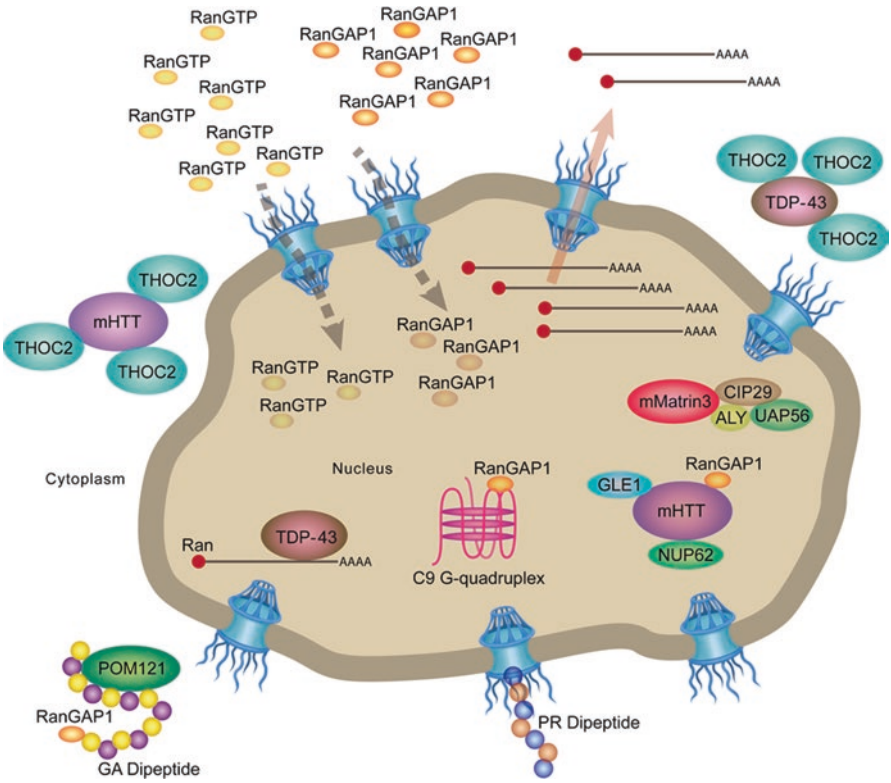

Fig. 4.2 RNA export defects identified in neurodegenerative diseases. Altered nuclear membrane morphology, interactions between DPRs and the nuclear pore, mislocalization and sequestration of export proteins into pathological aggregates, mutant proteins or pathological RNA species, interactions of TDP-43 with Ran mRNA as well as modifications to the Ran and RanGAP1 gradients, and decreased levels of mRNA export are all seen in a host of different neurodegenerative diseases

4.3.1 Nuclear Pore Alterations in Neurodegenerative Diseases

The earliest evidence for RNA transport alterations is the mislocalization of nucleoporins away from the nuclear envelope where they function, as well as abnormal nuclear envelope morphology which is often highlighted by nucleoporin immunostaining. These phenotypes have been identified in both animal models and patient tissue from several different neurodegenerative diseases, including Alzheimer's disease, Huntington's disease, Amyotrophic Lateral Sclerosis (ALS), and Frontotemporal Dementia (FTD) (Fig. 4.2) [35–37, 39, 40].

In Alzheimer's disease tissue, nuclear envelope abnormalities were noted in the hippocampus by immunostaining for Nup62, an FG-containing nucleoporin normally localized to the central channel of the nuclear pore [35]. In control tissue, Nup62 immunoreactivity forms a smooth circle in the nuclear envelope whereas in Alzheimer's patients it forms a tortuous and uneven nuclear envelope. It is important

to note that these alterations in the nuclear envelope were not accompanied by positive staining for caspase-3 or TUNEL suggesting that this is not a consequence of apoptotic cell death [35].

Nuclear envelope defects were detected in two mouse models of Huntington's disease. The first, expressing physiological levels of ~175 CAG trinucleotide repeat expansion within one or both huntingtin (*Htt)* alleles which survive on average approximately 90 weeks and exhibit a tremor by 33 weeks, exhibited an age-dependent increase in the number of cells with abnormal nuclear envelopes, which was more severe in mice expressing two copies of Q175 compared to those expressing a single expanded copy, as observed using staining against Lamin B1 in the cortex and striatum. In this model, 89% of cells in the cortex and 62% in striatum have abnormal nuclear envelopes at 24 months of age [36]. This phenotype was also present in the cortex of mice expressing a 23 kDa human exon 1 fragment of *Htt* with a 120–125 repeat polyglutamine expansion (R6/2 mice) which have an age of onset of 9–11 weeks and a lifespan of approximately 10–13 weeks. In R6/2 mice, 89% of cortical cells had altered nuclear envelopes by 3 months of age [36]. This same mouse model of *Htt* was shown by others to exhibit intranuclear inclusions of Nup62 that colocalized with mHtt aggregates in the striatum and cortex [37]. In the zQ175 mouse model of Huntington's disease which contains the human *Htt* exon 1 sequence with a 193 CAG repeat which replaces the mouse *Htt* exon 1 within the mouse *Htt* gene, the nucleoporin Nup88 was identified in intracellular inclusions that colocalized with mHtt aggregates [37]. Abnormal nuclear envelopes were also seen in iPSC (induced pluripotent stem cell) derived neural progenitors from Huntington's patients, and in the motor cortex of patient tissue [36]. Components of the nuclear pore complex including Dbp5, a protein necessary at the terminal step of mRNA export to remove proteins from mRNAs after they have been transported through the pore, and RanBP3, a Ran-binding protein that acts a cofactor for CRM1-mediated export, were also identified in polyglutamine aggregates isolated from a cell culture model of Huntington's disease [42].

In ALS mutant SOD1 mouse models of ALS, alterations of NPC components include increased immunoreactivity of the nucleoporins GP210 and Nup205 [38]. This staining was reminiscent of staining patterns in sporadic ALS patients which showed increased staining for GP210 in the nuclear envelope and cytoplasm [38]. Others have also identified nuclear envelope irregularities as denoted by Nup62, Nup88, and Nup153 immunoreactivity in SOD1 mice that worsened with age as well as in both sporadic ALS (sALS) and familial ALS (fALS) patient tissue [43].

In a genetic screen performed in a *Drosophila* model of C9orf72, loss of function of Nup50 enhanced the phenotype of the C9 repeat expansion, as did a dominant negative form of Ran, whereas loss of function of Nup107 and Nup160 suppressed the phenotype [39]. When a *Drosophila* model expressing codon optimized PR DPRs was used for a genetic screen, knockdown of fly orthologs of the nucleoporins TPR, SEH1, NUP62, and NUP93 enhanced the phenotype while NUP50, NUP197, and NUP155 suppressed the phenotype [44]. Interestingly, NUP50 appears to modify the phenotype of the repeat expansion differently than the phenotype of the PR DPRs. These results suggest that altered subcellular distribution of nucleoporins

may have a functional role in disease pathogenesis rather than being a consequence of the disease pathology, and that these alterations could have both loss of function and toxic gains of function phenotypes. This phenotype was accompanied by nuclear envelope irregularities as well as puncta of Nup107 in the salivary glands of flies [39]. As Nup107 is both found in aggregates and puncta, and its loss of function suppresses the disease phenotype in flies, it is possible that these aggregates and puncta of NPC can be toxic to cells. The mechanism by which these alterations in the nuclear envelope and mislocalization of NPC proteins induce disease is unknown, but a number of hypotheses have been proposed. PR dipeptides, formed from RAN (repeat-associated non-ATG) translation of the C9orf72 repeat expansion (DPRs), were found to bind to the FG repeat of the central channel of the nuclear pore complex and keep them in a polymerized state, possibly physically blocking movement through the nuclear pore (Fig. 4.2) [45]. Nuclear transport proteins including nuclear pore complex components and transport proteins such as CRM1 were found to interact with the DPRs PR and GR, produced from the C9orf72 repeat expansion, and CRM1 was also found to be an enhancer of a GR viability phenotype in *Drosophila* [46]. Another group suggests that cytoplasmic protein aggregates lead to the mislocalization of NPC proteins [47]. This hypothesis was tested using an artificial, aggregation prone β-sheet protein which led to the accumulation of NPC proteins in the cytoplasm and defects in both protein import and export [47].

4.3.2 Alterations in the RanGTPase Gradient Have Been Identified in Neurodegenerative Diseases

Another theme common among neurodegenerative diseases is alterations in the Ran gradient or its binding partners and regulators. As noted above, a high nuclear to cytoplasmic ratio of RanGTP is required for nuclear export where RanGTP is needed to bind to the exportin family of proteins within the nucleus.

In mice expressing mutant *Htt,* Gle1, part of the terminal step of mRNA export, as well as RanGAP1 are found co-aggregated with *Htt* [36]. RanGAP1 (Ran GTPase Activating Protein) is necessary for activating the GTPase function of Ran leading to its conversion to a GDP bound state Both RanGAP1 and Nup62 were found in inclusions in Htt R6/2 mice and RanGAP1 and Nup88 were found in mHtt inclusions in zQ175 Htt mice [37]. RanGAP1 was also mislocalized and concentrated in perinuclear puncta, and Nup62 was mislocalized in the frontal cortex and striatum of Huntington's patients [37]. Higher levels of RanGTP are required in the nucleus compared to the cytoplasm to fuel active transport via exportins. In iPSC derived neurons from Huntington's patients, the nuclear to cytoplasmic ratio of Ran is decreased [37]. Interestingly, expression of either RanGAP1 or Ran ameliorated cell death in cells expressing mutant Huntingtin, suggesting that at least part of the mechanism of action may be a loss of function of these proteins [37].

In Alzheimer's disease, cytoplasmic aggregates of NTF2, part of the import pathway required for importing Ran into the nucleus, were found in patient tissue [35]. Nuclear levels of Ran were also found to be decreased both in a mouse model of FTD based on knockout of the gene-encoding progranulin, GRN [48]. Mice lacking GRN exhibit increased levels of ubiquitin immunoreactivity in the form of amorphous granular cytoplasmic staining in neurons of the posterior thalamus, CA2-4 regions of the hippocampus, midbrain and brainstem. Mice also exhibited increased lipofusin granules and vacuolation in the habenular nucleus and CA2-3 regions of the hippocampus. Both microgliosis and astrogliosis were found in mice lacking GRN which was most evident in the brainstem and thalamus, and focal neuronal loss was found in the CA2-3 region of the hippocampus at 23 months of age [48]. These defects in the nuclear levels of Ran were also present in FTD patients carrying GRN mutations [49, 50].

In a model of Parkinson's disease based on administration of the drug 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), mice that lacked one copy of the Ran-binding protein, Ranbp2, had a more severe disease course and slower recovery [51]. Interestingly, in mice lacking any other genetic modifications, knockdown of Ranbp2 in Thy1 positive motor neurons led to motor deficits, respiratory distress, and premature death [52].

Many models of ALS also exhibit similar defects in either the Ran gradient or Ran-binding proteins. TDP-43 is a protein mutated in rare forms of fALS as well as present in pathological aggregates in most ALS, FTD and subsets of patients in a number of other neurodegenerative diseases, and has been shown to bind the 3' UTR of Ran mRNA and regulate its levels (Fig. 4.2) [50]. Loss of nuclear TDP-43 correlated with loss of Ran in the frontal gyrus of patients with FTD caused by mutations in progranulin (GRN) and led to overall decreased levels of Ran in the cortex [49, 50]. In addition, knockdown of TDP-43 in SH-SY5Y cells, which models the loss of nuclear TDP-43 commonly seen in ALS patients, leads to decreased levels of RanBP1 [53], and TDP-43 knockdown in Neuro2a cells led to decreased levels of Ran mRNA and protein [50]. In mice expressing mutant SOD1 an upregulation and nucleoplasmic mislocalization of RanGAP1 were observed [38]. A similar increase in RanGAP1 staining was seen in tissue from sALS patients [38].

The RanGAP1 protein has also been shown to bind to the G-quadruplex structure formed by the RNA of the C9orf72 repeat expansion, and there is a reduced nuclear to cytoplasmic ratio of Ran in iPSC motor neurons derived from C9-ALS patients and immortalized cell lines (S2 cells) expressing the 30 G_4C_2 repeats (Fig. 4.2) [40]. Both iPSC derived motor neurons and motor cortex tissue from ALS patients carrying the C9orf72 expansion exhibited discontinuous nuclear envelope staining for RanGAP1 as well as mislocalization and puncta that occasionally colocalized with Nup107 and Nup205 [40]. In a mouse model of C9orf72 expressing the GA DPR, both RanGAP and Pom121, a transmembrane nucleoporin involved in anchoring the NPC to the membrane, were found in nuclear and cytoplasmic puncta that often colocalized with the poly(GA) aggregates (Fig. 4.2) [54]. Interestingly, in two *Drosophila* models of C9orf72, genetic screens identified RanGAP1 as a modifier of the disease phenotype. Both screens were performed by expressing constructs in

the eye and then co-expressing targets and looking for modification of the eye phenotype. Codon optimized, ATG-mediated expression of the DPR PR was coupled with expression of RNAi lines leading to the discovery that knockdown of RanGAP1 enhanced the toxicity caused by PR [44]. When 30 G_4C_2 repeats were similarly expressed in the *Drosophila* eye, RanGAP1 overexpression suppressed the toxicity accompanied by the repeat, whereas RanGEF enhanced the toxicity [40]. Importantly, in this model system the phenotype of the altered Ran gradient (which likely inhibits the export of both proteins and RNA) could be partially rescued by a variety of treatments. The Ran gradient phenotype was rescued with antisense oligonucleotides against the C9orf72 repeat, by destabilizing the G quadruplex structure the repeat forms, or by inhibiting CRM1, suggesting both that these defects may be induced by the repeat, and that drug strategies currently being employed for the repeat might modulate these defects [40]. Conversely, knockdown of the splicing factor SRSF1, which also has a role in TREX-mediated mRNA export, has been proposed as a possible therapeutic strategy in C9orf72 ALS as cytoplasmic localization of the repeat transcript is necessary for the production of DPRs and both knockdown of SRSF1 or blockage of the interaction between SFSF1 and NXF1 was protective in multiple models of C9orf72 [55].

4.3.3 Nuclear mRNA Retention in Neurodegenerative Diseases

While mislocalization of nucleoporins and defects in the Ran gradient and Ran-binding proteins are likely to cause alterations in the nuclear export of RNA, recent studies have identified deficits in the export of mRNA in models of neurodegenerative disease.

In Huntington R6/2 mice, the TREX complex component Thoc2 is mislocalized and found in inclusions, and mRNA was found to be retained within the nucleus of these cells (Fig. 4.2) [36, 47]. The same phenotypes of Thoc2 aggregation and nuclear mRNA retention were found in cells expressing Htt86Q as well as C-terminal fragments of TDP-43 or even an artificial aggregation prone β-sheet construct [47]. In mice expressing a ~175 CAG trinucleotide repeat of *Htt* (Htt^{Q165}), mRNA accumulated within nuclei by RNA-FISH (fluorescence in situ hybridization) using an oligo dT probe, in a dose-dependent manner [36]. In addition to phenotypes in models of neurodegenerative disease, this phenotype of mRNA nuclear accumulation has been identified in the cortex in tissue from Huntington's patients [36].

Some rare forms of fALS are caused by mutations in Gle1, a protein that is an integral component of the release of mRNA from transport machinery in the cytoplasm. While the mechanism by which these mutations cause disease is not completely understood, it has been suggested that haploinsufficiency of Gle1 is to blame, suggesting a role for mRNA transport defects in this disease [56].

Expression of an ALS causing variant of SOD1 (G93A) in NSC-34 cells causes retention of RNA within the nucleus, as measured by an increased nuclear to cytoplasmic ratio of RNA transcripts identified using RNA-seq [57]. This retention was

not accompanied by an increase in transcripts-containing introns suggesting that the nuclear retention was not linked to defects in splicing, but rather likely due to defects in nuclear trafficking [57].

Recently, multiple groups have shown interactions between the C9orf72 repeat or its products with proteins involved in mRNA nuclear export. Multiple nucleoporins, as well as CRM1 and SRSF7 have been identified as protein interactors of the dipeptide repeats PR and GR [46]. In a genetic screen in *Drosophila,* in which 8, 28, or 58 copies of the G_4C_2 repeat are expressed in the eye using the GMR-GAL4 driver, aimed at discovering modifiers of the C9orf72 phenotype, proteins involved in mRNA export were identified. The strongest suppressor was found to be Aly, with partial loss of function of NXF1, CHTOP, NCBP2, ARS2, Gle1, and CRM1 enhancing the phenotype. Importantly, expression of the repeat in cells led to an accumulation of poly(A) + mRNA within the nucleus, which can be decreased with Aly knockdown [39]. Others have also shown the accumulation of poly(A) + mRNA within the nucleus of cells transfected with the C9orf72 repeat accompanied by the nuclear accumulation of PABPC1 with binds to the C9orf72 RNA. PABPC1 accumulation is a phenomenon reminiscent of viral infection where nuclear PABPC1 nuclear accumulation is sufficient to cause nuclear mRNA retention [58].

Recently, we have shown that Matrin 3, a nuclear matrix protein mutated in rare forms of ALS, binds to many TREX components and proteins involved in nuclear RNA export including, Aly, UAP56 and Sarnp in cell culture as well as nuclear spinal cord lysates [41]. The expression of ALS linked mutations in Matrin 3 in cell lines also causes the accumulation of poly(A) + mRNA within the nucleus. These mutations also caused nuclear accumulation of mRNAs of ALS-relevant proteins TDP-43 and FUS linking mRNA nuclear retention to disease pathology (Fig. 4.2) [41].

4.4 Conclusions

Alterations in nucleocytoplasmic transport have been identified by numerous groups in a wide range of neurodegenerative disorders including Alzheimer's disease, Huntington's disease, FTD, and ALS (Table 4.1). The identification of these alterations in such a wide span of neurodegenerative diseases suggests neuronal survival depends upon proper regulation of trafficking to and from the nucleus. While altered protein nucleocytoplasmic transport has been well documented in many neurodegenerative diseases, the only direct evidence for defective RNA transport has been the accumulation of poly(A) + mRNA within the nucleus in patient-derived tissue and various disease models. However, the alterations in both the localization and levels of nucleoporins and the loss of the Ran gradient and mislocalization of Ran-binding proteins strongly suggests defects occur in the transport of all RNA subtypes. Further studies are necessary to explore how other RNA subtypes are mislocalized in neurodegenerative diseases. While it is unclear why defects in nucleocytoplasmic trafficking preferentially affect neurons, there is evidence to

Table 4.1 Summary of defects identified in RNA export in neurodegenerative diseases

Phenotype	Disease/model supporting data	Citation
Nuclear membrane structural abnormalities	*AD*: Identified by Nup62 immunostaining in patient hippocampal tissue	[35]
	HD: Identified with Lamin B1 immunostaining in cortex and striatum of 175 CAG repeat expansion mice, in cortex of R6/2 mice, in iPSC-derived neuronal progenitors, and in the motor cortex of HD patients	[36]
	ALS: Nuclear envelope abnormalities denoted by Nup62, Nup88, and Nup153 immunoreactivity in spinal cord tissue from mutant SOD1 G93A mice and ALS patients	[43]
	ALS: Identified with Lamin C immunostaining in salivary gland cells of *Drosophila* expressing the C9orf72 repeat expansion	[39]
Puncta and aggregates of nucleoporins and RNA export proteins	*HD*: R6/2 mice exhibited Nup62 positive inclusions that co-localize with mHtt aggregates in striatum and cortex	[37]
	HD: zQ175 mice exhibited Nup88 and RanGAP1 positive intracellular inclusions that co-localized with mHtt aggregates	[37]
	ALS: Intranuclear Nup107 positive puncta identified in fly salivary gland cells expressing the C9orf72 repeat expansion	[39]
	ALS + *HD*: Expression of an aggregation prone C-terminal fragment of TDP-43 or the 96 CAG repeat Huntington results in mislocalization of THOC2 to cytoplasmic puncta	[47]
	HD: Dbp5 and RanBP3 identified in polyglutamine aggregates isolated from cells expressing a 96 CAG repeat expansion	[42]
	HD: Gle1 and RanGAP1 identified in mHtt aggregates in cortex of 175 CAG repeat expansion mice	[36]
	HD: RanGAP1 was mislocalized and concentrated in perinuclear puncta and Nup62 was mislocalized in frontal cortex and striatum of HD patients	[36]
	ALS: Discontinuous nuclear immunostaining as well as mislocalization and puncta that occasionally co-localized with Nup107 and Nup205 in iPSC derived motor neurons and motor cortex tissue from ALS patients carrying the C9orf72 repeat expansion	[40]
	ALS: In mice expressing the GA DPR (C9orf72), RanGAP1 and Pom121 were identified in nuclear and cytoplasmic puncta which often co-localized with GA aggregates	[54]

(continued)

Table 4.1 (continued)

Phenotype	Disease/model supporting data	Citation
Increased NPC protein immunoreactivity	*ALS*: SOD1 G93A mice showed increased immunoreactivity for GP210 and Nup205 in spinal cord tissue and patient tissue showed increased GP210 immunoreactivity in the nuclear envelope and cytoplasm	[38]
NPC and RNA export proteins identified as disease modifiers in *Drosophila* genetic screens	*ALS*: Loss of function of Nup50 and dominant negative Ran enhanced an eye phenotype in flies expressing the C9orf72 repeat expansion, loss of function of Nup107, and Nup160 suppressed phenotype	[39]
	ALS: Knockdown of fly orthologs of TPR, SEH1, NUP62, and NUP93 enhanced an eye phenotype in flies expressing the DPR PR (which is created by the C9orf72 repeat expansion), Nup50, Nup197, and Nup155 suppressed the phenotype	[44]
	ALS: CRM1 knockdown enhanced viability phenotype of flies expressing the DPR GR (from C9orf72 repeat expansion) and Nup205 knockdown suppressed phenotype	[46]
	ALS: RanGAP1 was identified as a modifier of an eye phenotype in two Drosophila models of C9orf72: 1) Flies expressing the PR DPR in which RanGAP1 knockdown enhanced toxicity; 2) Flies expressing 30 G_4C_2 repeats in which RanGAP1 overexpression suppressed toxicity and RanGEF enhanced toxicity	[40, 44]
	ALS: Loss of function of Aly, NXF1, CHTOP, NCBP2, ARS2, Gle1, and CRM1 suppressed the eye phenotype of the C9orf72 repeat	[39]
Polymerization of FG nucleoporins	*ALS*: PR DPR formed from C9orf72 repeat expansion bound to FG repeat of the central channel of the nuclear pore keeping them in a polymerized state	[45]
RNA export proteins interact with disease linked proteins or dipeptide repeats	*ALS*: CRM1 and Aly identified as interactors of the DPRs PR and GR (from C9orf72 repeat expansion) expressed in HEK293 cells	[46]
	ALS: Aly, UAP56, and Sarnp identified as interactors of mutant Matrin 3 in NSC-34 cells and spinal cord extracts	[41]

(continued)

Table 4.1 (continued)

Phenotype	Disease/model supporting data	Citation
Altered levels or localization of Ran and Ran binding proteins	*HD*: iPSC derived neurons from Huntington's patients exhibited decreased nuclear to cytoplasmic Ran ratios	[37]
	FTD: FTD patients with progranulin mutations as well as a mouse model of FTD based on progranulin knockout exhibited decreased nuclear Ran	[49, 50]
	ALS: Knockdown of TDP-43 in SH-SY5Y cells led to decreased levels of Ranbp1 and TDP-43 knockdown in Neuro2a cells lied to decreased levels of Ran mRNA and protein	[50, 53]
	PD: Mice lacking one copy of Ranbp2 had a more severe disease course and slower recovery in MPTP model of Parkinson's	[51]
	ALS: RanGAP1 immunostaining is increased in sALS patient tissue and is upregulated and mislocalized to the nucleoplasm in mice expressing mutant SOD1	[38]
	ALS: Reduced nuclear to cytoplasmic ratio of Ran was identified in iPSC motor neurons derived from ALS patients carrying the C9orf72 repeat expansion as well as immortalized cells expressing 30 G_4C_2 repeats	[40]
Nuclear retention of RNA and mRNA	*HD*: mRNA is retained within the nucleus of cells in both the R6/2 and 175 CAG repeat mouse models of Huntington's disease, as well as in cells expressing 86 polyglutamine repeats and in the cortex of tissue from Huntington's patients	[36, 47]
	ALS: mRNA was retained within the nucleus of cells expressing a C-terminal fragment of TDP-43 or an artificial aggregation prone β-sheet construct	[47]
	ALS: An increased nuclear to cytoplasmic ratio of RNA was identified by RNA-seq when G93A mutant SOD1 was expressed in NSC-34 cells	[57]
	ALS: Increased nuclear retention of RNA was identified in *Drosophila* salivary gland cells from flies expressing the C9orf72 repeat expansion. This phenotype was partially rescued by Aly knockdown	[39]
	ALS: Accumulation of mRNA within the nucleus of cells was identified in cells expressing the C9orf72 repeat expansion	[58]
	ALS: Expression of ALS linked mutations in Matrin 3 leads to increased nuclear to cytoplasmic ratios of both total mRNA and TDP-43 and FUS mRNA	[41]
Mutations of Gle1 in ALS patients	*ALS*: mutations in Gle1 which likely lead to haploinsufficiency are a rare genetic cause of ALS	[56]

AD Alzheimer's disease, *HD* Huntington's disease, *FTD* frontotemporal dementia, *ALS* amyotrophic lateral sclerosis, *PD* Parkinson's disease

suggest that post-mitotic cells including neurons may be more susceptible to age-related defects in nucleocytoplasmic transport. The proteins of the NPC are normally replaced during cell division where they are disassembled and reassembled with newly synthesized proteins during mitosis [59]. In post-mitotic cells such as neurons, the NPC is not completely disassembled and proteins such as Nup107 and Nup160 do not appear to turn over, suggesting that they are some of the longest-lived proteins in the body [60, 61]. The longevity of the NPC makes it vulnerable to the buildup of damage over time and unsurprisingly is subject to age-related dysfunction [60]. The susceptibility of neurons as post-mitotic cells to defects in the NPC, as well as the age-related nature of neurodegenerative diseases, could explain the contribution of nucleocytoplasmic trafficking defects in these diseases. While there is clear evidence that these defects are present in neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, FTD, and ALS, the mechanism by which these defects occur as well as the role that these defects play in disease onset and pathogenesis remains unknown and merits continued study.

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Chapter 5
RNA Degradation in Neurodegenerative Disease

Kaitlin Weskamp and Sami J. Barmada

Abstract Ribonucleic acid (RNA) homeostasis is dynamically modulated in response to changing physiological conditions. Tight regulation of RNA abundance through both transcription and degradation determines the amount, timing, and location of protein translation. This balance is of particular importance in neurons, which are among the most metabolically active and morphologically complex cells in the body. As a result, any disruptions in RNA degradation can have dramatic consequences for neuronal health. In this chapter, we will first discuss mechanisms of RNA stabilization and decay. We will then explore how the disruption of these pathways can lead to neurodegenerative disease.

Keywords RNA · Decay · Alternative splicing · Transport · Stress granule · Exosome · Disease · Neurodegeneration

5.1 Mechanisms to Maintain RNA Stability

Following transcription, the newly formed transcript can be stabilized in several ways (Fig. 5.1). Most RNA that codes for protein, also referred to as coding or messenger RNA (mRNA), undergoes several processing steps that prevent degradation, assist in export from the nucleus, and aid in translation. Additionally, both coding and noncoding RNA (ncRNA) are stabilized by the adoption of unique secondary structures or sequestration in cytoplasmic ribonucleoprotein particles when the cell is under stress.

K. Weskamp · S. J. Barmada (✉)
Neuroscience Graduate Program and Department of Neurology, University of Michigan School of Medicine, Ann Arbor, MI, USA
e-mail: sbarmada@umich.edu

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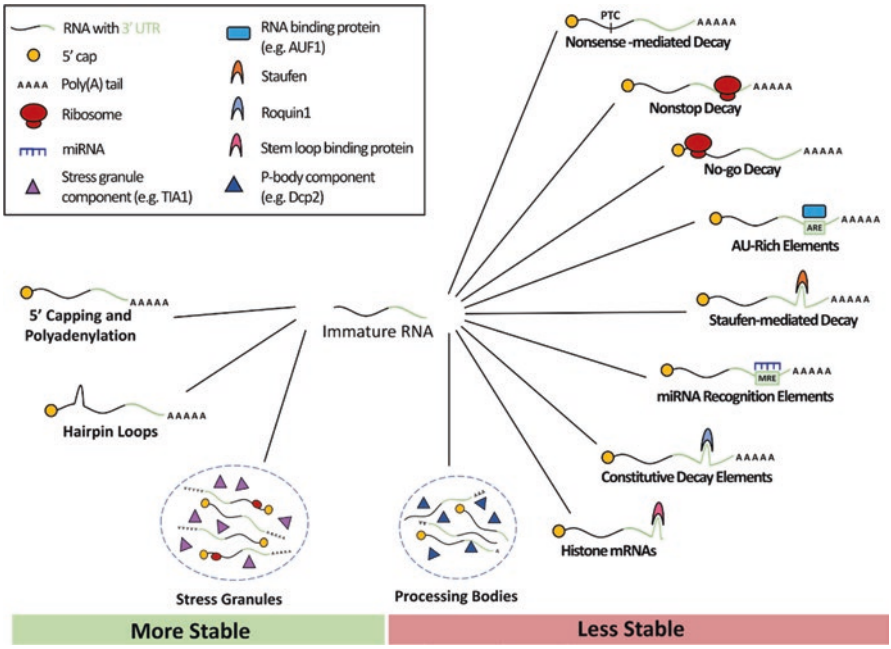

Fig. 5.1 Pathways responsible for RNA homeostasis. RNA stability is promoted by two key mechanisms (left). Following transcription, nascent RNA is stabilized by the addition of a 5′ cap and poly(A) tail, as well as the formation of secondary structures. Transcripts are also sequestered and stabilized in stress granules upon exposure to cellular stress. In contrast, RNA degradation pathways target faulty transcripts for removal (right). Transcripts that contain premature stop codons are targeted by nonsense-mediated decay. When translation fails to stop or start, the associated transcripts are degraded by nonstop decay and no-go decay, respectively. RNA decay mechanisms also regulate transcript abundance through several elements located within the 3′ UTR, including AU-rich elements, Staufen binding sites, miRNA recognition elements, and constitutive decay elements. Lastly, P-bodies sequester and destabilize RNA transcripts

5.1.1 Polyadenylation

Polyadenylation refers to the addition of a series of adenosine monophosphates to the 3′ end of mRNA transcripts [1]. This poly(A) tail protects nascent mRNA from enzymatic degradation [2, 3], facilitates nuclear export [4], and assists in translation [3]. Polyadenylation begins when a complex of several proteins recognizes a binding site on the mRNA transcript. An enzyme in this complex, cleavage/poyadenylation specificity factor (CPSF), cleaves the 3′ end of the transcript, and a second component, polyadenylate polymerase, adds sequential adenosine monophosphate units to create the poly(A) tail [5]. As the poly(A) tail grows longer, polyadenylate-binding protein 2 (PAB2) is recruited, which further increases the affinity of polyadenylate polymerase to the RNA [6]. Additional poly(A)-binding proteins then associate with the tail and facilitate nuclear export, stabilization of the RNA, and translation [7].

Many transcripts harbor more than one polyadenylation site. The site that is ultimately utilized primarily affects the length of the 3′ untranslated region (UTR), with little direct influence on protein translation or function [8]. However, the 3′ UTR may also encode microRNA recognition elements [9], DNA methylation sites [10], or motifs recognized by regulatory RNA-binding proteins [11, 12]. Thus, where a poly(A) tail starts can significantly influence the likelihood of transcript degradation. Moreover, in some cases alternative poly(A)-binding sites occur within the coding region, and their usage results in truncation of the translated protein [13]. Poly(A) tails are gradually eroded over time, and transcripts with shorter tails are both less likely to be transcribed and more likely to be degraded [14]. This process can be accelerated by the binding of microRNA to the 3′ UTR or through the removal or degradation of poly(A)-binding proteins [15].

5.1.2 Methylguanine Cap

The majority of coding RNAs undergo a second processing step that involves the addition of a methylguanine cap to the 5′ end of the transcript. This cap stabilizes the transcript by preventing exonuclease-mediated degradation [16–18], and is also required for the translation of most mRNAs [19, 20]. Additionally, the 5′ cap assists in splicing [21–25], nuclear export [24, 25], and possibly polyadenylation [26].

The capping process is initiated before transcription is complete, and begins when RNA triphosphatase removes one of the 5′ terminal phosphate groups [27]. mRNA guanylyltransferase then catalyzes the addition of guanosine triphosphate to the remaining terminal biphosphate to create an unusual 5′–5′ triphosphate linkage. This guanosine is then methylated by a methyltransferase [27]. The cap-binding complex (CBC) binds to the methylated 5′ cap, which is in turn recognized by the nuclear pore complex and exported into the cytoplasm [28, 29]. Once there, the CBC is replaced by the translation factors eIF4E and eIF4F, which are recognized by other translation initiation machinery components, including the ribosome [30, 31].

Binding of the CBC and translation factors also stabilize transcripts by blocking the binding of decapping enzymes [32–34]. When these decapping enzymes outcompete the translation factors, they hydrolyze the 5′ cap and expose the 5′ monophosphate. The resulting decapped transcripts are subject to rapid degradation by 5′ exonucleases [35].

5.1.3 Secondary Structure

DNA primarily forms double helices, but the single-stranded nature of RNA and its propensity to form hydrogen bonds allows it to form more complex structures that can directly affect transcript stability. The most common RNA secondary structure is the hairpin loop, created when two complementary regions of the same strand

base-pair to form a double helix that ends in an unpaired loop [36]. These loops are found in pre-microRNA, transfer RNA (tRNA), and mRNA, and their stability depends on several factors, including length, degree of complementarity in the stem, and guanine to cytosine base pair content. Hairpin loops stabilize mRNA [37–40] and in many cases increase translation efficiency [39, 40]. This may occur by blocking exonuclease activity, but the precise mechanism remains unclear. Hairpin loops may also act as binding sites for proteins that direct mRNA transport and localization [41–43].

The combination of several hairpin loops forms a multiloop; the most abundant example of this structure is found in the cloverleaf-shaped tRNAs that assist in protein translation. The relative stabilities of multiloops vary based on size, number of loops, and complementarity [44]. Hairpin loops can also form pseudoknots, in which at least two hairpin loops are linked by single stranded loops. Pseudoknots are relatively stable, they form the catalytic core of some ribozymes [45, 46] and telomerases [47], and may also be involved in translation, though little is known about their functional significance [48]. Other structures, such as G-quadruplexes and R-loops, are more often associated with disease and will be discussed below.

5.1.4 Stress Granules

Cells undergo a wide range of molecular changes in response to environmental stressors, including the inhibition of conventional translation [49, 50] and the formation of stress granules (SGs). SGs are cytoplasmic ribonucleoprotein particles rich in mRNA, RNA-binding proteins, and stalled translation initiation complexes [51–53]. SG coalescence effectively sequesters the attached mRNAs and the 40S ribosome subunit [54, 55], preventing further translation and stabilizing the bound mRNAs. Proteins unrelated to the original translation initiation complex are also recruited, and their composition helps determine SG dynamics and longevity [56]. Which proteins participate is often dependent on their posttranslational modifications and the specific stressor involved [57–61], providing a rapid and reversible way for the cell to modulate SG formation and composition. Many RNA-binding proteins found in SGs contain low-complexity domains that are inherently flexible; the ability of these domains to form reversible homo- and heterotypic interactions with one another via their low-complexity domains may be responsible for the dynamics of SG formation and dissociation [62, 63]. Additionally, SGs often contain a number of proteins that promote RNA stability and regulate translation [64]. Moreover, deadenylation is largely inhibited in stress granules [65–67]. When the stressor has passed, several RNA-binding proteins catalyze SG disassembly [68–70], and the transcript is either degraded or released to resume translation. These observations suggest that SGs serve two basic functions: preventing the translation of unnecessary transcripts during stress, and protecting these transcripts from degradation until the stress has subsided.

5.2 Mechanisms of RNA Decay

The typical life of an mRNA transcript includes a complex sequence of events including transcription, capping, adenylation, splicing, and export. When mistakes occur during this process, quality control mechanisms exist to recognize and eliminate defective transcripts that may give rise to dysfunctional or toxic proteins (Fig. 5.1). However, these pathways do more than ensure the fidelity of RNA transcripts. They also serve important regulatory roles, enabling rapid modulation of steady-state RNA levels—and therefore protein production—in response to changes in the intracellular or extracellular environment.

5.2.1 RNA Degradation Machinery

There are three major classes of intracellular RNA-degrading enzymes: endonucleases that cut RNA internally, 5′–3′ exonucleases that degrade RNA from the 5′ end, and 3′–5′ exonucleases that hydrolyze RNA from the 3′ end. These enzymes may work independently or within a complex such as the exosome, a versatile structure for the degradation of immature or abnormal RNA. The core of the eukaryotic exosome complex is formed by nine proteins, six of which are members of the RNase PH-like family [71]. These form a ring that is capped by three additional proteins with RNA-binding domains [72]; this structure bears remarkable similarity to the 26S proteasome [73], which consists of a central proteolytic barrel (the 20S core) capped on either end by 19S regulatory subunits. The exosome is primarily composed of 3′–5′ exoribonucleases, and RNAs are degraded by removing terminal nucleotides from the 3′ end of the transcript. This occurs through the cleavage of phosphodiester bonds, either through RNase PH-like protein-mediated phosphorolytic cleavage or hydrolytic cleavage by proteins associated with the exosome [74]. Several other proteins bind to the exosome to regulate its activity and specificity [75–77]. The exosome also processes small nuclear RNAs, small nucleolar RNAs, and ribosomal RNAs [78], though how these molecules are targeted to and released from the exosome remains unclear.

5.2.2 Nonsense-Mediated Decay

Occasionally, errors introduced during transcription, insertions, deletions, or nonsense mutations uncover premature stop codons (PTCs) within the coding sequence of an mRNA. If translated, PTC-containing transcripts would encode truncated proteins that may have toxic gain-of-function or dominant-negative activities. Nonsense-mediated decay (NMD) is a surveillance mechanism that eliminates transcripts containing PTCs, thereby preventing the synthesis of proteins that could be detrimental to the cell.

mRNA transcripts undergo splicing following transcription, during which introns are removed and exons are spliced together. The resulting exon-exon junctions (EEJs) are occupied by a complex of proteins (the exon junction complex, or EJC) that assist in splicing until they are displaced by the ribosome during the first, or pioneer, round of translation. If the stop codon is downstream or within about 50 nucleotides of the final EJC, the transcript is translated normally. According to the EJC model of NMD, a stop codon that occurs upstream of an EJC is recognized as a PTC, triggering transcript degradation [79, 80]. When the ribosome stalls at a PTC, the protein UPF1, along with the eukaryotic release factors eRF1 and eRF3, forms the surveillance complex (SURF) and binds adjacent to the PTC. SURF then interacts with two components of the nearby EJC, UPF2, and UPF3B [81–83]. This triggers UPF1 phosphorylation, which causes the complex to move along the mRNA, resolving secondary structure and removing adherent proteins that may inhibit degradation [84, 85]. Phosphorylated UPF1 also binds to SMG6, an endonuclease that directly cleaves the mRNA [86, 87], as well as SMG5 and SMG7, which trigger deadenylation [88], decapping, and further degradation [89]. Additionally, UPF1 may be recruited to transcripts independent of a PTC or adjacent EJC, particularly within long 3′ UTRs [90]. A working theory is that UPF1 preferentially binds long 3′ UTRs and is phosphorylated via an unknown mechanism, triggering transcript decay. However, more work is required to identify the pathway resulting in destabilization of transcripts bearing long 3′ UTRs.

5.2.2.1 Alternative Exon Inclusion and Exclusion

Though NMD is an important quality control mechanism, it also helps regulate the expression of functional mRNA [91], predominantly through alternative mRNA splicing. This phenomenon is remarkably widespread: NMD-related regulation of transcript abundance is involved in cell proliferation [92, 93], immunity [94], stress [95], viral response [96], and neuronal activity [97, 98]. The differential inclusion or exclusion of exons (alternative splicing) enables a single gene to encode multiple transcript and protein isoforms, and in many cases alternatively spliced transcripts are subject to NMD. Because changes in the splicing environment determine which isoforms are produced [99, 100], alternative splicing can regulate gene expression by creating transcripts that are more or less stable. An estimated 33% of alternative transcripts contain PTCs [101], and between 12% and 45% of alternatively spliced transcripts are estimated to be NMD targets [101]. Regulated unproductive splicing (RUST) of this type regulates RNA abundance in relation to neuronal activity levels [102], developmental stage, and cell type [103]. Moreover, there is growing evidence that RUST is utilized by several RNA-binding proteins to regulate their own expression (autoregulation), particularly components of the splicing machinery [104–108].

5.2.2.2 Upstream Open Reading Frames

Upstream open reading frames (uORFs) are mRNA elements that include a start codon in the 5′ UTR that is out-of-frame with the main coding sequence. Because ribosomes bind to the 5′ cap of the mRNA and scan for start codons, uORFs can disrupt or interfere with translation of the downstream coding sequence [109, 110]. Moreover, a stop codon at the 3′ uORF end may be viewed as a PTC within the context of the whole transcript. As predicted by the EJC model of NMD, the presence of uORFs correlates with lower expression levels of the downstream ORF [111, 112], and uORF-bearing transcripts are particularly susceptible to degradation by NMD [113–115].

5.2.3 Nonstop Decay

Nonstop decay (NSD) is a surveillance mechanism involved in the detection and degradation of mRNA transcripts that lack stop codons [77, 116] due to premature polyadenylation or point mutations that disrupt existing terminal codons. Without a recognizable stop codon, the ribosome translates into the poly(A) tail and then stalls, unable to release the mRNA transcript [117].

NSD is activated when Ski7, a component of the exosome complex, binds the empty aminoacyl (A) site of the stalled ribosome via its C-terminal domain [76, 77]. This is supported by the fact that C-terminal deletions of Ski7 result in impaired NSD but do not affect general exosome function [116]. Additionally, the Ski7 C-terminal domain strongly resembles other proteins that bind the ribosome during normal translation, elongation, and termination such as EF1a and eRF3 [118]. After binding, Ski7 releases the stalled ribosome and recruits the exosome to rapidly deadenylate the transcript [77, 116, 119, 120].

5.2.4 No-Go Decay

No-go decay (NGD) is a mechanism that recognizes mRNA transcripts stalled during translation [121–123] due to damaged RNA, stress [124], or strong secondary structure that blocks the progress of translation machinery [121]. NGD is the most recently discovered RNA surveillance pathway, and as such little is known about its mechanism. However, evidence suggests that NGD may degrade mRNA in a manner that resembles translation termination. Two proteins that promote NGD, Hbs1 and Dom34, strongly resemble eRF1 and eRF3, two factors that catalyze the end of translation [121, 125].

Analogous to Ski7 in NSD, Hbs1 possesses the same C-terminal domain that allows EF1a, eRF3, and Ski7 to bind the empty A site on the stalled ribosome [126, 127]. Dom34 is homologous to eRF1 and binds directly to Hbs1 [126, 128]. Upon

binding, the Dom34/Hbs1 complex triggers the release of the nascent peptide and the ribosome is released or degraded. Likewise, the mRNA transcript is targeted for endonucleolytic cleavage and the fragments are subsequently degraded via the exosome or exonucleases [121, 125]. It is not currently known how the Dom34/Hbs1 complex releases the mRNA from the ribosome, but the close relation between Hbs1 and Ski7 suggests that ribosome release may occur in the same manner as NSD. Moreover, NGD can occur independently of the Dom34/Hbs1 complex; further work is needed to identify the other factors involved.

Additionally, it remains unclear why some transcripts are targeted by NGD and not others. Pausing during translation is a normal occurrence [129] and may even serve biological functions [130–132], but only a fraction of transcripts are NGD substrates. Potentially important factors include the degree of ribosome stalling and whether or not the A site is empty to allow Dom34/Hbs1 complex binding. Further studies are needed to clarify this mechanism.

5.2.5 Adenylate-Uridylate-Rich Elements

While some mRNA decay pathways target faulty transcripts, others allow the cell to rapidly modulate gene expression in response to intracellular and extracellular stimuli. Several of these pathways regulate transcript levels via binding sites within the 3′ UTR, including adenylate-uridylate-rich elements (AREs), Staufen-mediated decay, microRNAs, and constitutive decay elements.

AREs are 50–150 nucleotide regions with frequent adenine and uridine bases that generally target the mRNA for rapid degradation [133, 134]. The mechanism underlying this pathway is not well understood, but several RNA-binding proteins interact with these sites and modulate transcript stability. For example, overexpression of hnRNP D, also known as ARE RNA-binding protein 1 (AUF1), destabilizes mRNA-containing AREs [135, 136]. Conversely, AUF1 depletion increases both ARE-containing mRNA stability and abundance of the corresponding proteins [137, 138]. Similarly, ablation of tristetraprolin (TTP), an RNA-binding protein that also recognizes AREs, increases mRNA and protein levels in a variety of cell types [139–141] and transcripts [142–147].

Though the exact mechanism is unclear, the association of ARE-binding proteins to AREs is followed by deadenylation [148–151], decapping, and 3′–5′ degradation via the exosome [152]. Certain subunits of the exosome bind to AREs directly, and several ARE-binding proteins including TTP associate with the exosome in vitro [75, 153], ensuring rapid and preferential elimination of ARE-continuing transcripts. Many ARE-binding proteins are also associated with SGs and P-bodies (discussed later in this chapter), suggesting that 5′–3′ exonuclease-mediated degradation may contribute to the turnover of ARE-containing transcripts as well [154, 155]. However, not all ARE-binding proteins trigger mRNA decay. For example, the Hu family of proteins stabilize bound ARE-containing transcripts [156–159], suggesting that the effect of AREs on RNA stability depends on a combination of factors, including the ARE-binding protein, transcript, and environment.

5.2.6 Staufen-Mediated Decay

Staufen-mediated decay (SMD) also regulates transcript levels via the 3′ UTR. SMD is triggered when Staufen-1 (Stau1) recognizes double-stranded RNA structures that form sufficiently downstream of the termination codon [160, 161]. Staufen-binding sites (SBS) are created by intramolecular hairpin loop formation within the 3′ UTR [161], or intermolecular base-pairing of the 3′ UTR with partially complementary long noncoding RNA [162]. Upon binding to the SBS, Stau1 recruits UPF1, which in turn stimulates mRNA decay [160], likely in much the same way as in NMD. Moreover, given that UPF1 is critical for both SMD and NMD, there may be competition between the two pathways based on the availability of UPF1 [163].

5.2.7 microRNAs

microRNAs (miRNAs) are small, noncoding RNAs that base-pair with complementary sequences within RNA transcripts to trigger their decay and/or translational repression. These 20–25 nt RNAs are produced from an RNA precursor (pri-miRNA) that forms a hairpin loop shortly after transcription [164, 165]. This structure is recognized by the nuclear protein DGCR8, which recruits the enzyme Drosha to cleave the hairpin from the rest of the transcript [166, 167]. The resulting molecule (pre-miRNA) is then exported to the cytoplasm [168] where the enzyme DICER cuts away the looped end [169], leaving a duplex of two short, complementary RNA strands behind. Though either strand can function as a mature miRNA, one is usually degraded [170, 171]. The remaining miRNA associates with the RNA-induced silencing complex (RISC), which assists in orienting the miRNA to its mRNA target, repressing translation of the target transcript and triggering its degradation.

The bound miRNA guides RISC to its binding site (miRNA recognition element or MRE) on the target transcript, most often within the 3′ UTR, though binding can occur within coding regions as well [172, 173]. The degree of miRNA-mRNA complementarity is a major predictor of transcript fate [174]. High degrees of sequence complementarity allow the Argonaute family of proteins—components of RISC [175]—to catalyze RNA decay through an unknown mechanism that may involve deadenylation, decapping, or exonucleolytic degradation [176, 177]. In contrast, miRNAs that bind weakly or with less complementarity induce translational repression [174] through a mechanism that remains unclear.

5.2.8 Constitutive Decay Elements

In addition to AREs, SBSs, and MREs, structured RNA degradation motifs also directly lead to transcript turnover. Constitutive decay elements (CDEs) are stem loop structures located within the 3′ UTR that trigger mRNA decay [178, 179]

through recruitment of the RNA-binding protein Roquin1 [179, 180]. Roquin1 binds to the CDE stem loop structure via two binding sites in its ROQ domain [180], triggering degradation by recruiting the Ccr4-Caf1-Not deadenylation complex [179]. A transcriptome-wide search of 3′ UTRs in mice revealed several unique CDEs that are frequent and highly conserved across vertebrate species. Many, but not all, of these CDEs are Roquin1-associated [179], indicative of potential novel and unexplored pathways responsible for RNA decay.

5.2.9 Histone mRNAs

Much like CDE-containing transcripts, histone mRNAs encode highly conserved stem loop structures within their 3′ UTRs. These hairpins are essential for the rapid synthesis and degradation of histone mRNA during the S phase of the cell cycle, during which the cell undergoes DNA replication and chromosome remodeling [181]. At the end of S phase, histone hairpin loops are recognized by stem loop-binding protein (SLBP), which recruits the proteins necessary to add a short, oligonucleotide tail to histone mRNAs [182]. The oligonucleotide tail forms a binding site for LSM1–7, which triggers degradation via the exosome and endonucleases [182]. Interestingly, histone mRNA decay also requires UPF1 and its interaction with SLBP [183], though the exact role of UPF1 in histone mRNA metabolism remains unclear.

5.2.10 Processing Bodies

Processing bodies (P-bodies) are dynamic cytoplasmic foci comprised of mRNA and RNA-binding proteins. While SGs primarily sequester and protect mRNA until it can resume translation, P-bodies target associated transcripts for translational repression, decapping, and decay. Although P-body assembly is not required for RNA decay [184], it may directly compete with translation initiation; only transcripts that are not engaged in translation can be recruited to P-bodies [185–187], and upon translational inhibition P-bodies increase in number [185, 188]. Conversely, a decrease in P-body components leads to an increase in mRNAs associated with actively-translating polysomes [189]. P-bodies lack translation initiation machinery [185, 187], and are instead primarily composed of proteins associated with translational repression and mRNA decay, including decapping enzymes, exonucleases, and NMD components [190]. This suggests that functional transcripts undergo active translation before they are recruited to P-bodies. Once transferred, the mRNA is no longer translated [189, 191] and is instead degraded by decapping enzymes [192, 193] or other nucleases. However, mRNAs may also escape P-bodies and

resume translation [187, 194], and regulated expression of proteins such as NoBody and MLN51 can drive P-body disassembly [195, 196]. Together, these observations indicate that P-bodies are part of a highly dynamic process characterized by constant flux between pools of mRNA transcripts that are being actively translated, those that are stalled or sequestered in SGs, and those that are being degraded within P-bodies.

5.3 RNA Turnover in Neurodegenerative Disease

The regulation of RNA is critical to cell health, and increasing evidence indicates that disruption of RNA stability may underlie neurodegenerative disease. Alterations in RNA turnover have been identified in several pathways, including RNA sequestration in stress granules or foci, RNA transport, the exosome, alternative splicing, and retrotransposons (Fig. 5.2).

5.3.1 RNA Sequestration

During times of stress, the cell diverts its energy and resources toward survival and recovery. A powerful mechanism to conserve resources is the sequestration of mRNAs in SGs to limit the translation of nonessential proteins. Typically, when the stressor passes, SGs dissolve and stalled mRNAs are released for translation. However, during prolonged periods of stress or disease, SGs sometimes fail to disassemble. This extended sequestration of mRNAs could effectively disrupt the delicate balance between SGs, polysomes, and P-bodies, effectively interrupting mRNA homeostasis, interfering with protein synthesis, and potentially contributing to downstream toxicity in neurodegenerative diseases.

5.3.1.1 Disruption of Stress Granule Dynamics

Of the ~125 proteins identified as components of human SGs, 60% are RNA-binding proteins [197]. This group of proteins is also highly enriched for the low complexity domains that facilitate the reversible aggregation of proteins into membraneless organelles such as SGs. The mutation or mislocalization of several RNA-binding proteins stabilizes SGs, sometimes driving them to form irreversible aggregates that sequester mRNA and RNA-binding proteins indefinitely and disrupt SG homeostasis. Conversely, though the machinery that drives SG disassembly remains unclear, any errors within this pathway may likewise lead to RNA dyshomeostasis and subsequent disease.

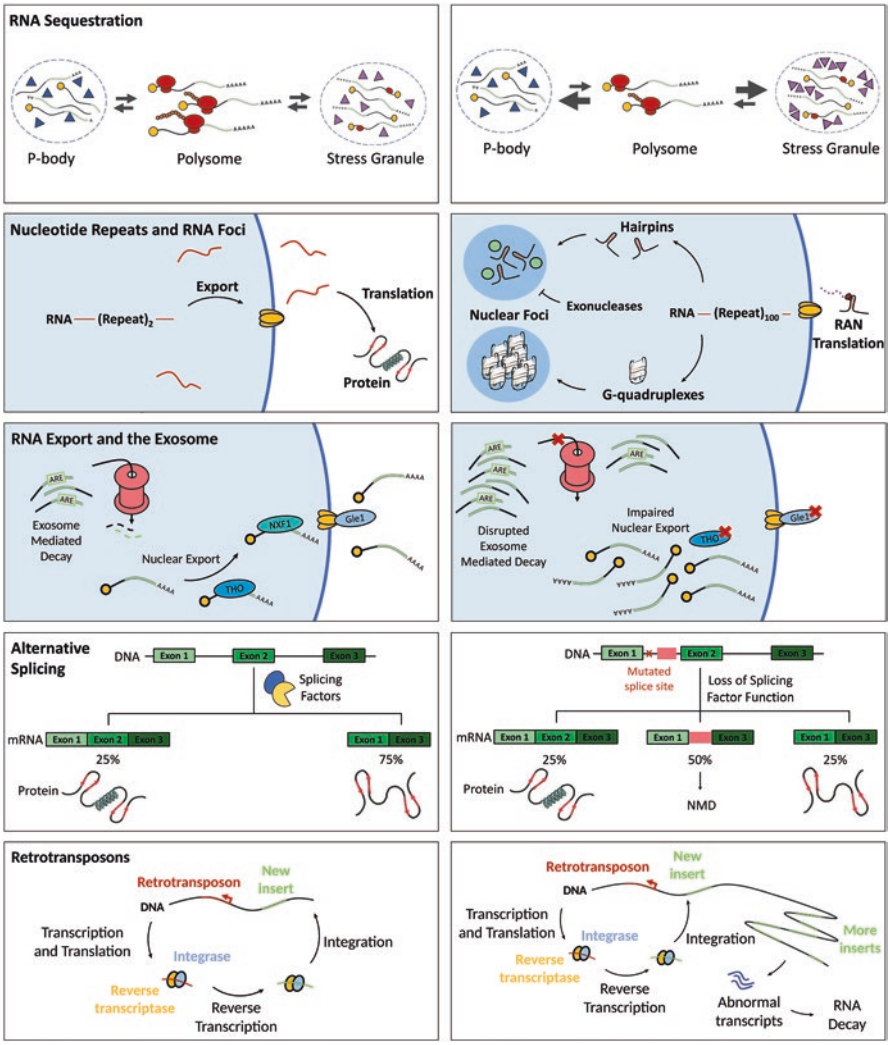

Fig. 5.2 Abnormal RNA stability in neurodegenerative disease. Here, we compare how normal pathways (left column) are disrupted in disease (right column). *RNA Sequestration*: There is constant flux between pools of RNA transcripts that are actively being translated (the polysome), those sequestered in stress granules, and those associated with P-bodies. In disease states, increased stress granule formation or reduced stress granule dissociation disrupts the equilibrium, resulting in fewer transcripts undergoing translation. *Repeat Expansions and RNA Foci:* Transcripts containing repeat expansions form secondary structures such as hairpin loops and G-quadruplexes that are often stabilized in nuclear foci, which also sequester RNA-binding proteins (green circles). These transcripts also generate proteins via RAN translation that can disrupt membraneless organelles involved in RNA splicing and processing. *RNA Transport and the Exosome*: Mutations in *THO*, *Gle1*, and other components of the RNA export pathway result in nuclear RNA retention and degradation via the exosome complex. Mutations in exosome components can inhibit RNA turnover and further disrupt RNA homeostasis. *Alternative Splicing*: Mutations that disrupt splice sites, or splicing regulators such as TDP43, result in the inclusion of unannotated or "cryptic" exons (pink). These transcripts are often targeted for nonsense-mediated decay. *Retrotransposons:* These transposable elements insert themselves into the genome, often disrupting open reading frames or splice sites. The transcripts that are transcribed from these regions are often faulty, and are targeted for RNA decay

RNA-Binding Proteins in Stress Granule Dynamics

TDP43 and FUS are two stress granule components that are integrally involved in neurodegenerative disease, particularly amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Both TDP43 and FUS are primarily nuclear proteins, but their cytoplasmic mislocalization [198–200] and nuclear exclusion [201–203] are characteristic features of ALS and FTD. These proteins are capable of nucleocytoplasmic shuttlingin response to various stressors they associate with cytoplasmic SGs, but when the stress has passed they return to the nucleus [204]. ALS-linked mutations in the genes encoding TDP43 and FUS promote increased association with SGs [202, 205], abnormal SG formation [206], and reduced SG dissociation [207, 208]. TDP43 and FUS play important roles in alternative splicing and the stress response, and their sequestration impacts the processing of several transcripts that are critical for neuronal viability [209, 210]. Likewise, excess cytoplasmic TDP43 and FUS may sequester related RNA-binding proteins within SGs, further disrupting RNA homeostasis [64]. Importantly, TDP43- and FUS-related toxicity relies upon the ability of these proteins to bind RNA. Deletion of the RNA recognition motifs in either protein greatly reduces toxicity without affecting localization [211, 212], suggesting that RNA binding, not localization, imparts toxicity. Furthermore, these observations indicate that the sequestration of mRNAs themselves, not just RNA-binding proteins, is particularly damaging to neurons.

ALS-linked mutations are also found in other RNA-binding proteins such as Matrin3 [213], hnRNPA1, hnRNPA2/B1 [214], and TIA1 [215], all of which associate with SGs. These mutations are often centralized within the proteins' low complexity domains, and evidence indicates that they likewise alter SG dynamics, suggesting a link between SG association/dissociation and pathogenicity.

Stress Granule Disassembly

Though relatively little is known about SG disassembly, evidence suggests that valosin-containing protein (VCP) is crucial for this phenomenon. VCP regulates several cellular processes including autophagy [216], chromatin remodeling [217], and membrane trafficking [216], as well as SG clearance [218]. VCP accumulates in SGs, and its knockdown results in the persistence of SGs even after the stressor has passed [218]. Moreover, mutations in the gene-encoding VCP cause a multisystem proteinopathy that includes ALS and FTD [219], and the overexpression of mutant VCP results in impaired SG disassembly [218]. Thus, pathogenic mutations in the genes encoding VCP, TDP43, and FUS all stabilize SGs, thereby effectively sequestering essential mRNA and RNA-binding proteins within these organelles. As such, altered SG dynamics and abnormal RNA stability may represent a conserved pathway underlying ALS, FTD, and related neurodegenerative diseases.

5.3.2 Nucleotide Repeats and RNA Foci

Microsatellites are repeated tracts of nucleic acids that compose approximately 50% of the human genome [220]. These regions are a source of genomic instability, and expansion mutations that increase the number of repeats above a certain threshold can lead to neurodegenerative diseases such as Huntington's disease (HD), myotonic dystrophy (DM), spinocerebellar ataxias, Freidrich's ataxia, fragile X syndrome, fragile X-associated tremor ataxia syndrome (FXTAS), ALS, and FTD [221, 222]. In most cases, the length of the expanded region is inversely correlated with prognosis—higher repeat number results in earlier onset and more severe symptoms. Repeat expansions have unique pathological implications—they form unique secondary structures that may disrupt translation, sequester RNAs and other proteins into nuclear foci, and serve as a substrate for noncanonical translation.

5.3.2.1 Repeat Expansion Secondary Structure

The majority of expansion mutations associated with disease are trinucleotide CNG repeats, where N is any nucleotide. Due to the high degree of complementarity, CCG, CAG, CUG, and CGG repeats readily form mismatched hairpin loops [223] whose stability increases proportionally with the number of repeats [224]. Tetra-, penta-, and hexa-nucleotide repeats also form hairpins [225], though they appear to be less stable.

Repeat expansions with a high percentage of guanine nucleotides can also form G-quadruplexes. In these structures, four guanine bases associate through Hoogsteen hydrogen bonding to form a square guanine tetrad, and two or more tetrads stack to form a G-quadruplex [226]. Whether or not G-quadruplexes exhibit a physiological function remains unknown, but some evidence indicates that they participate in transcriptional regulation and/or telomere maintenance [227]. They are also observed in association with cancer, copy number variants, and age-related disease, specifically ALS and FTD. The most common mutation responsible for inherited ALS and FTD consists of a GGGGCC (G_4C_2) repeat expansion in the first intron of *C9orf72* [228, 229]. Unaffected individuals have 2–8 (G_4C_2) repeats [230], but tracts of >32 (G_4C_2) repeats lead to ALS, FTD, or both with nearly 100% penetrance by age 80 [231]. These repeats form stable G-quadruplexes [232], which are further stabilized in longer repeat expansions [233].

(G_4C_2) repeat expansions also form structures known as R-loops at the site of transcription, composed of nascently-synthesized RNA hybridized to the complementary DNA strand [234, 235]. The unbound DNA strand may also form hairpins or G-quadruplexes, further stabilizing the loop [236]. In addition to *C9orf72*-related ALS/FTD, R-loops are also observed in fragile X syndrome and Freidrich's ataxia [237] characterized by CGG and GAA trinucleotide repeats, respectively. The abundance of R-loops in these disorders depends on the size of the repeat expansion, with higher repeat number correlating with more frequent R-loops. These structures may contribute to the pathology of expansion diseases in several ways: by blocking

translation [238], disrupting chromatin remodeling [239], or promoting genomic instability at the repeat expansion site [235]. In support of the pathogenic effects of R-loops, mutations in the gene encoding senataxin (*SETX*), a helicase that helps resolve R-loops [240], cause juvenile ALS (ALS4), while SETX overexpression prevents neurodegeneration in ALS models [241].

5.3.2.2 RNA Foci

In addition to their effects on RNA stability and translation, the propensity of repeat expansions to form stable secondary structures contributes to the formation of RNA foci [242, 243]. These nuclear inclusions may drive pathogenesis through the sequestration and nuclear retention of specific RNA-binding proteins. For example, CUG repeat expansions in *DMPK* cause myotonic dystrophy type 1 (DM1), a neuromuscular disease characterized by progressive muscle loss and weakness. This repeat expansion sequesters and disrupts the splicing activity of muscleblind (MBNL) [244, 245], a protein responsible for the processing of several key downstream transcripts [246]. MBNL binds to hairpins that result from repeat expansion mutations in *DMPK* with high affinity [245, 247], and preventing MBNL sequestration via small molecules that recognize CUG hairpin loops restores its splicing activity and helps maintain RNA homeostasis in DM1 models [248]. Additionally, the RNA foci observed in DM1 [249] and myotonic dystrophy type 2 (DM2) [250] sequester several other RNA-binding proteins, suggesting that global disruption of alternative splicing may contribute to DM pathogenesis [251]. RNA foci are also observed in *C9orf72*-linked ALS/FTD [252], where the G_4C_2 repeat transcripts sequester several splicing factors including hnRNPA1, hnRNPH, and SC35, as well as the RNA-binding protein hnRNPA3 and the mRNA export receptor ALYREF [253]. The sequestration of proteins essential to multiple cellular processes by repeat expansion transcripts suggests that these diseases occur, at least in part, through an RNA gain-of-function mechanism.

5.3.2.3 Repeat-Associated Non-AUG (RAN) Translation

Nucleotide repeats can be translated into polypeptides even if they are not located within a traditional open reading frame, via a noncanonical pathway termed repeat-associated non-AUG (RAN) translation. RAN translation may be triggered by hairpin loops formed by repeat-containing stretches of DNA, which effectively stall ribosome scanning and facilitate translational initiation at near-AUG codons [254–256]. This process occurs in multiple reading frames in both the sense and antisense directions, producing several dipeptide repeat-containing proteins (DPRs) [254]. RAN translation products are detected in spinocerebellar ataxia type 8, HD [257], DM1 [254], FXTAS [256], and *C9orf72*-associated ALS/FTD [258], suggesting that RAN translation is a common phenomenon in repeat expansion diseases. In some cases, there appears to be an inverse relationship between RAN translation

and RNA foci formed by repeat expansions. This observation suggests that the repeat-expanded RNA may be sequestered in nuclear foci, precluding nuclear export and subsequent translation [259]. This may serve as a coping response to prevent the translation of DPRs; failure of this coping response over time may result in increased RAN translation and subsequent neurodegeneration [260, 261]. In support of this hypothesis, RNA foci in *C9orf72* mutant mice are abundant yet rarely associated with neurodegeneration [261]. RAN peptides may also affect RNA stability by disrupting membraneless organelles such as the nucleoli [262] and Cajal body [263], which are responsible for ribosomal RNA [264] and spliceosome maturation [265], respectively. Lastly, an increase in SGs and a decrease in P-bodies is observed in neurons expressing RAN peptides [266]; in this case, RAN peptides may act similarly to small proteins such as NoBody [195] that dissolve P-bodies, releasing unstable RNAs to be sequestered by SGs. Additional studies are required to determine the effect of RAN peptides on RNA stability, P-body dynamics, and global RNA homeostasis.

5.3.3 RNA Transport

The diverse functions of RNA are determined, in part, by its subcellular localization. As a result, RNA transport mechanisms are crucial for RNA function, particularly in highly compartmentalized and morphologically complex cells such as neurons. Among the most important of these mechanisms is nucleocytoplasmic transport, in which RNA transcripts are shuttled from the nucleus to the cytoplasm. Several neurodegenerative diseases exhibit deficits in nucleocytoplasmic RNA transport, leading to RNA sequestration in the nucleus and widespread dysregulation of gene expression. Thus, interruption of nuclear export machinery can have severe consequences on neuronal health.

5.3.3.1 Impaired Nuclear Export

Nuclear mRNA export is triggered by deposition of the highly conserved translation export (TREX) complex at the 5′ end of the nascent transcript [267]. The core of this complex, THO, recruits ALYREF and several other nuclear export factors [268–271]. ALYREF then binds to nuclear export factor 1 (NXF1) [272], triggering a shift from a conformation with low RNA-binding affinity to one that readily binds the transcript [273, 274]. NXF1 directs the transcript to the nuclear pore complex (NPC), a large multimeric structure that spans the nuclear envelope and enables the transport of molecules into and out of the nucleus. NXF1 facilitates NPC docking and transcript translocation via interactions with NPC components containing low complexity domains enriched in phenylalanine and glycine residues [275].

Disruption of this pathway leads to nuclear retention of RNA, and which is then rapidly degraded by the nuclear exosome [276, 277]. Interrupting nuclear RNA

export can have severe consequences for neuronal survival, and mutations in nuclear export components are linked to several neurological and neurodevelopmental disorders. Chromosomal translocation and inactivation of THOC2, a subunit of the core TREX complex, leads to cognitive impairment, cerebellar hypoplasia, and congenital ataxia in humans [278]. Additionally, missense mutations in THOC2 have been implicated in fragile X syndrome [279], and mutations in a second THO subunit, THOC6, lead to intellectual disabilities [280]. Moreover, loss-of-function mutations in *Gle1* result in ALS [281] and fetal motor neuron disease [282]. Gle1 is a nuclear export mediator located on the cytoplasmic face of the nuclear pore that facilitates both the release of the transcript from the nuclear pore and its dissociation from export adaptor proteins [283], freeing it to undergo translation. This process may be specific to mRNAs with poly(A) tails, as depletion of Gle1 results in a nuclear accumulation and subsequent degradation of polyadenylated mRNAs [284, 285].

Abnormal nucleocytoplasmic transport is also a characteristic finding in models of ALS [286–288], DM1 [289], and HD [290, 291]. Toxicity in these models can be suppressed by pharmacologic or genetic modulation of nuclear transport components, testifying to the broad significance of this pathway in disease pathogenesis. Moreover, age is a likely contributor to impaired nuclear import, as aged cells display abnormal NPCs and reduced expression of nucleocytoplasmic transport genes [292, 293]; the resulting reduced fidelity in nuclear import/export is consistent with the observed age-dependent risk of nearly every neurodegenerative disease.

5.3.3.2 Disruption of the Nuclear Pore

In addition to disruption of the recruitment of the transcript to the pore, interruption of the pore itself can alter nucleocytoplasmic transport. RAN translation of repeat expansion mutations produces several DPRs. Some of these DPRs, including arginine-rich dipeptides generated from RAN translation of the *C9orf72* G_4C_2 repeat in familial ALS/FTD, clog the nuclear pore, and inhibit the transport of RNA and other macromolecules into and out of the nucleus [294]. Again, this contributes to the nuclear retention of RNAs that are susceptible to exosome-mediated decay [276, 277]. Arginine-containing DPRs are among the most toxic of the dipeptides in ALS/FTD models [262, 295], suggesting that impaired nucleocytoplasmic transport contributes significantly to neurodegeneration in these disorders.

5.3.4 The RNA Exosome Complex

The exosome complex is an RNA degradation mechanism that contributes broadly to RNA turnover, surveillance, and processing. This complex works closely with other pathways to orchestrate the degradation of immature, abnormal, or misplaced RNA.

5.3.4.1 Exosome-Associated Mutations in Neurodegenerative Disease

Due to the importance of the exosome in regulating RNA decay, mutations in this complex can have severe implications. Mutations in *EXOSC3*, the gene encoding the core exosome component RRP40, are linked to autosomal recessive pontocerebellar hypoplasia type 1 (PCH1) [296]. This progressive neurodegenerative disease is characterized by atrophy of the pons and cerebellum and loss of spinal motor neurons, accompanied by developmental delay, muscle atrophy, and difficulty breathing [297]. Thirty-seven percent of PCH1 patients exhibit *EXOSC3* mutations, most of which are heterozygous missense mutations [297]. Disease severity correlates with genotype, as patients with homozygous missense mutation fare better and those with a combined missense and null mutation fare worse [298].

Similarly, mutations in a gene encoding a separate exosome component, *EXOSC8*, result in cerebellar hypoplasia (CH) [299]. This autosomal recessive disorder is also characterized by progressive degeneration of the cerebellum, pons, and spinal motor neurons, as well as abnormal myelination. Though the mechanism is unclear, an increase in exosome substrates, including ARE-containing mRNAs encoding myelin proteins, in CH models suggests that impaired exosome function may contribute to dysmyelination of the involved tracts and subsequent neurodegeneration [299].

5.3.5 Alternative Splicing

Between 92% and 94% of all genes in the human genome are alternatively spliced [300], and the brain expresses more alternatively spliced genes than any other organ [301, 302]. This suggests that alternative splicing is a key regulator of transcript stability and gene expression, and its misregulation can have severe effects on neuronal health [303].

5.3.5.1 Nonsense-Mediated Decay and Unannotated or "Cryptic" Exon Splicing

A primary consequence of alternative splicing is RNA destabilization [101]. As discussed above, in many cases alternative splicing may serve to regulate normal transcript levels. This is supported by the fact that over one third of RNA transcripts are spliced to include PTCs, and these transcripts are likely targeted for degradation via NMD [101]. Mutations that affect splicing and result in either the inclusion of PTC-encoding exons or a shift the reading frame that uncovers "silent" PTCs may destabilize transcripts and lead to disease via gene haploinsufficiency. For example, disease-associated missense *GRN* mutations cause ALS and FTD by altering mRNA splicing, triggering NMD of *GRN* transcripts, and consequent reductions in progranulin protein expression [304–307]. In other cases, mutations that create novel splice sites or the dysregulation of splicing factors leads to the inclusion of unannotated or

"cryptic" exons and the production of faulty transcripts that are eventually targeted for decay. Several regulatory proteins suppress these unannotated exon splicing events, including TDP43. Depletion of TDP43 results in a widespread increase in cryptic exon splicing events, and the inclusion of these exons may lead to NMD [308, 309]. Many of these events are specific to neurons [310], which suggests that the disruption of TDP43-mediated cryptic exon regulation may contribute to ALS and FTD.

NMD can be manipulated through the modulation of specific pathway components: overexpression of UPF1 and UPF3B stimulates NMD, while UPF1 knockdown or the overexpression of UPF3A, an antagonistic paralog of UPF3B that sequesters UPF2, suppresses NMD [311]. Consistent with a potential link between NMD and ALS/FTD pathogenesis, overexpression of UPF1 or UPF2 prevents FUS- and TDP43-mediated neurodegeneration in model systems [312]. One possibility is that UPF1 overexpression in these models prevents cell death by boosting endogenous NMD, thereby enabling the pathway to properly metabolize an overabundance of NMD substrates. However, further investigation is required to confirm and extend these findings.

5.4 Retrotransposons

Transposable elements (TEs) are mobile genetic elements that constitute a large portion of most eukaryotic genomes. Retrotransposons, which encode a reverse transcriptase and an integrase that allow them to "copy and paste" themselves from one region to another, represent approximately 40% of the human genome [313]. Though the vast majority of retrotransposons are inactive [314], some retain the ability to mobilize. Retrotransposition occurs approximately once every 10–100 births [315], and the insertion of these elements near or within active genes is a significant source of genomic instability and cellular toxicity [316, 317]. Though transcription of these regions is downregulated [318, 319], the transcripts that are transcribed are degraded via NMD [320] and other noncanonical pathways [321]. Several mechanisms have also evolved to suppress retrotransposon expression and prevent the resultant large-scale deletions and genomic rearrangements [322], though the efficiency of these mechanisms declines with age [316, 323, 324]. Moreover, the elevated expression of retrotransposons correlates with several neurodegenerative disorders [325–327], suggesting that a reduction in retrotransposon repression may contribute to disease pathogenesis.

5.4.1 Retrotransposons in ALS

As previously discussed, TDP43 aggregation and mislocalization play a fundamental role in ALS and FTD, and TDP43 serves as a key regulator of alternative splicing for hundreds of transcripts. TDP43 also recognizes several TE-derived RNA transcripts [328], and this binding is reduced in FTD patients coincident with elevated TE expression. This suggests that TDP43 normally regulates TE expression, and the

loss-of-functional TDP43 in FTD results in TE overexpression [328]. This is further supported by the finding that TEs are derepressed in ALS/FTD models involving TDP43 overexpression or knockdown [328, 329], suggesting that TE dysregulation may contribute to neurodegeneration in ALS and FTD. This may occur through activation of DNA damage-mediated programmed cell death due to the large-scale deletions and genomic rearrangements that result from de-repressed TEs [329], and there is some evidence to suggest that TDP43 pathology impairs siRNA-mediated gene silencing, an essential system that normally protects the genome from retrotransposons [329].

Human endogenous retroviruses (HERVs) represent a subclass of retrotransposons originating from ancient viral infections that resulted in the integration of viral DNA into the host genome. The most recent of the retroviruses to integrate into the human genome is HERV-K [330]. The HERV-K envelope protein is expressed in both cortical and spinal neurons of ALS patients, suggesting activation of the retrovirus in disease. Furthermore, ectopic expression of the HERV-K envelope protein triggers neurodegeneration and motor dysfunction in mice [331]. Like other retrotransposons, HERV-K is regulated by TDP43, suggesting that HERV-K derepression in TDP43-deficient cells might contribute to neurodegeneration in ALS [331].

5.4.2 Retrotransposons in Aging

Age is a major risk factor for most neurodegenerative diseases, likely due to a reduced ability to regulate protein degradation [332], oxidative stress [333], and DNA damage [334]. While retrotransposons are a significant source of genomic instability, additional evidence suggests that they are more destructive in aging brains. The expression and mobility of several TEs increase with advanced age [316, 324]; these changes, in turn, are linked to progressive, age-dependent memory impairment and shortened lifespan [324]. Thus, the derepression of retrotransposons during normal aging could contribute to the age-related increase in risk for neurodegenerative diseases.

5.5 Conclusions and Future Directions

Neurodegenerative diseases vary widely in clinical presentation, neuropathology, and genetic background. However, it is becoming increasingly clear that alterations in RNA turnover are a key contributor to disease pathogenesis. The magnitude and extent of RNA dyshomeostasis observed in neurodegenerative disease models strongly suggests a fundamental disruption of one or more of the many mechanisms that tightly regulate RNA stability. While compensatory pathways may allow cells to cope with subtle changes in SG dynamics, alternative RNA splicing, or RNA degradation, over time such pathways become less efficient and the ability of the

cell to maintain RNA homeostasis slowly erodes. Mitotic cells evade toxicity by dilution and division, but for long-lived cells such as neurons, the resulting abnormalities eventually lead to cell death. Because altered RNA stability results from the disruption of several related but distinct pathways, it is unlikely that focusing on single transcripts will result in a cure. Instead, a more complete understanding of RNA degradation in both healthy and diseased conditions may highlight common mechanisms and key upstream elements that could be rationally targeted for therapeutic development.

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Chapter 6
RNP Assembly Defects in Spinal Muscular Atrophy

Phillip L. Price, Dmytro Morderer, and Wilfried Rossoll

Abstract Spinal muscular atrophy (SMA) is a motor neuron disease caused by mutations/deletions within the survival of motor neuron 1 (*SMN1*) gene that lead to a pathological reduction of SMN protein levels. SMN is part of a multiprotein complex, functioning as a molecular chaperone that facilitates the assembly of spliceosomal small nuclear ribonucleoproteins (snRNP). In addition to its role in spliceosome formation, SMN has also been found to interact with mRNA-binding proteins (mRBPs), and facilitate their assembly into mRNP transport granules. The association of protein and RNA in RNP complexes plays an important role in an extensive and diverse set of cellular processes that regulate neuronal growth, differentiation, and the maturation and plasticity of synapses. This review discusses the role of SMN in RNP assembly and localization, focusing on molecular defects that affect mRNA processing and may contribute to SMA pathology.

Keywords Spinal muscular atrophy (SMA) · Survival of motor neuron (SMN) · RNA-binding protein (RBP) · Ribonucleoprotein (RNP) · Molecular chaperone · RNA processing · RNA localization

6.1 SMA Clinical Background

Spinal muscular atrophy (SMA) is an autosomal recessive disorder characterized by the early-onset of skeletal muscle atrophy and a progressive degeneration of motor neurons in the anterior horn of the spinal cord [1]. Impairments in synaptic

P. L. Price
Department of Neuroscience, Mayo Clinic, Jacksonville, FL, USA

Department of Cell Biology, Emory University, Atlanta, GA, USA
e-mail: Price.Phillip@mayo.edu

D. Morderer · W. Rossoll (✉)
Department of Neuroscience, Mayo Clinic, Jacksonville, FL, USA
e-mail: Morderer.Dmytro@mayo.edu; Rossoll.Wilfried@mayo.edu

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maturation, sensory-motor circuitry, and synaptic transmission at the neuromuscular junction (NMJ), followed by a dying-back axonopathy, precede muscle denervation and loss of α-motor neurons in the spinal cord. In classical SMA, proximal muscles are more severely affected than distal muscles.

SMA is the leading genetic cause of death in infancy [2]. Across ethnicities, an incidence of 1 in 8,000–20,000 has been estimated [3]. Disease classification is based on the age of onset and clinical severity, the most common classification scheme distinguishes between Types I–IV [4]. The most common form of SMA (Type I; SMA1) typically leads to muscle weakness within the first 6 months and death due to respiratory failure by the age of 2. Type II SMA patients present with signs of muscle weakness during the first 7–18 months. Affected children may crawl and sit unassisted, but often require support for standing and mobility. These patients typically have a life expectancy into early adulthood. Type III and IV SMA are milder forms of the disease, with patients having a normal life expectancy and displaying muscle weakness presenting in adolescence and adulthood. Type III SMA patients are able to stand unsupported and walk with moderate difficulty. Type IV (adult-onset) SMA patients are usually not diagnosed until early adulthood. These patients experience slowly progressing muscle weakness, primarily affecting the legs, hips, shoulders, arms.

6.2 SMA Is Caused by Reduced SMN Protein Levels

In >95% of cases, SMA is caused by homozygous deletions or compound heterozygous mutations in the survival of motor neuron 1 (*SMN1*) gene encoding the SMN protein [5]. Humans possess a nearly identical copy of this gene (*SMN2*), which carries a splice site mutation in exon 7. This C>T transition in *SMN2* promotes the exclusion of exon 7 from the full-length protein, leading to the expression of only 10–20% of full-length SMN protein, and 80–90% of a truncated SMN protein isoform (SMNΔ7) that is rapidly degraded [6–8]. Since SMA patients lack functional *SMN1* genes, SMN protein is expressed only from the *SMN2* gene, leading to reduced levels of full-length SMN protein. Thus, SMA is directly caused by a pathological reduction of functional SMN protein levels below a critical threshold, and disease severity is correlated with *SMN2* copy number [6, 7]. Therapeutic approaches have mainly focused on raising SMN protein levels via gene therapy [8], or via increasing the splicing efficiency of *SMN2* exon 7. Small molecules [9] as well as antisense oligonucleotides (ASOs) have been developed as splicing modifiers of *SMN2* [10], leading to the introduction of the ASO Nusinersen as the first drug approved by the US Food and Drug Administration for the treatment of SMA [11–13]. The high cost for the treatment and the need for administering ASOs via lumbar puncture have created practical challenges that raise important ethical questions [14]. A continued effort will be required to provide more effective, affordable, and accessible treatment options.

6.3 SMN Protein Deficiency Primarily Affects Synapses in the Motor Circuitry

During early development, the assembly and stabilization of highly organized synaptic structures is essential for the maturation and function of the central nervous system. In neurodegenerative diseases, structural and functional abnormalities in synaptic connections often precede neuronal loss and cell death, and are thought to account for early clinical deficits [15–17]. The underlying cause for the vulnerability of motor neurons to reduced SMN protein levels remains unclear. Aside from *Drosophila*, *C. elegans*, and zebrafish SMA animal models, several mouse models with different severity have been engineered to closely recapitulate pathological hallmarks observed in human patients [18].

Unlike humans, mice only possess one gene encoding SMN, and a complete knockout results in early embryonic lethality [19]. To recapitulate the disease phenotype of human SMA, the introduction of the human *SMN2* transgene into the background of a homozygous deletion of murine *Smn1* has allowed for the creation of severe mouse models of SMA [20, 21]. Further mouse models have been generated to represent less severe forms of SMA as important preclinical models for therapy development [22–24].

Although born with a normal number of motor neurons, severe SMA mice experience a 35–40% loss of spinal cord and lower-brainstem motor neurons by day 5 [20]. As summarized in Fig. 6.1, pre-synaptic deficits include the aggregation of neurofilaments in the presynaptic terminal, poor terminal arborization, irregular distribution and positioning of synaptic vesicles, and reduced neurotransmission [25]. In addition, multiple studies in SMA mouse models have observed significant impairments in mitochondrial function and axonal transport, including increased oxidative stress levels and organelle fragmentation [26, 27].

Deleterious effects on the maturation and maintenance of NMJs support the characterization of SMA as an NMJ synaptopathy [28]. As the most commonly studied mouse model of SMA, SMNΔ7 mice carry a homozygous deletion of the murine *Smn1* gene and contain two transgenic constructs, one containing a single copy of the human *SMN2* gene locus, and a second encoding the human *SMN2* promoter driving expression of human *SMN2* cDNA lacking exon 7 (*SMN*Δ7) [22]. These mice have an average life span of 17.7 days, and display several similar phenotypes observed in SMA patients. Although most NMJs remain innervated until late in the disease time course of SMNΔ7 mice, thorough explorations of the synapse electrophysiology and ultra-structure revealed a significant decrease in synaptic vesicle density and release probability [29]. These deficits were found to be associated with a delayed maturation of NMJ terminals and myofibers, and together indicate that NMJ synaptic dysfunction precedes degeneration of the motor axon and finally the loss of motor neurons in severe SMA mouse models [29]. Reduced subsynaptic clefts and lack of synaptic vesicles at the NMJ and abnormal preterminal accumulation of vesicles have also been observed in SMA1 patients [30, 31].

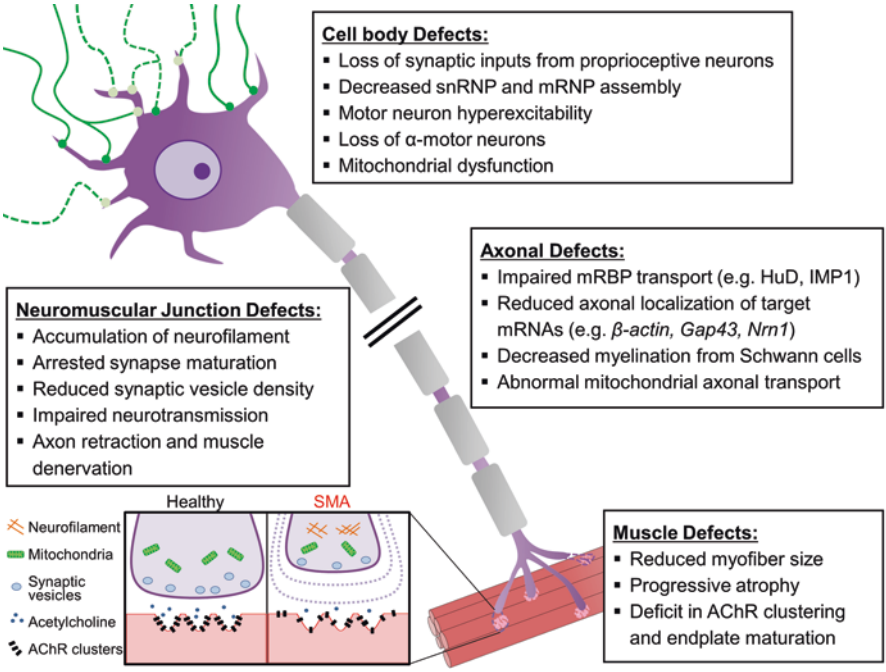

Fig. 6.1 Cellular Defects in SMA motor neurons. Schematic of a spinal motor neuron, highlighting morphological and molecular defects within different cellular compartments

Related to NMJ function, myotubes in SMA1 fetuses can display a significant retardation in growth and maturation [32]. One of the principal prenatal defects observed in mouse models and human SMA1 patients was an arrest in acetylcholine receptor clustering into 'pretzel'-shaped structures during postsynaptic endplate maturation, compromising the structural and functional integrity of the NMJ [30]. In SMA mice, abnormal molecular composition, disruptions in normal satellite cell differentiation, and reductions in myofiber size, have been described in skeletal muscle [22, 33, 34]. Agrin, a protein best known for its role in organizing acetylcholine receptors at the NMJ, is misspliced and reduced in motor neurons of SMA mice [35]. Rescue of the Z+ Agrin isoform prevented the development of several pathological phenotypes, and improved mean survival by 40% [35]. Taken together, this research points to abnormal function and maturation of the NMJ as key contributors to SMA pathogenesis, and as a potential target for therapy [36].

However, notable defects are also present within other cell-types that relay information, support motor neuron function and viability, and contribute to motor circuitry. In SMNΔ7 mice, loss of proprioceptive sensory synaptic input onto spinal motor neurons has been observed in embryonic mice, suggesting that the disruption of the spinal motor circuitry at multiple levels is an early phenotype contributing to motor dysfunction (Fig. 6.1) [37, 38]. Defects in astrocyte activity and myelination may also influence the severity of SMA [39–42]. SMA astrocytes display significant

deficits in stimulating neurite outgrowth and differentiation of motor neurons, but may also display potential toxic gain-of-function properties [43, 44].

The generation of conditional SMA mouse models with promoter-driven depletion or rescue of SMN expression has made it possible to study the pathological effects of selectively reduced SMN levels in specific cell-types and tissues. Using this approach has demonstrated that depletion or restoration of SMN in motor neurons (*Hb9-Cre*; *ChAT-Cre*) significantly alters the functional synaptic output and excitability of the motor unit and retention of sensory-motor synapses [45, 46]. Nevertheless, restoration of SMN solely in motor neurons provides little to no improvement in life span, likely due to abnormal cardiac innervation by the autonomic nervous system in this severe SMA mouse model [45]. Multiple studies using a muscle-specific Cre driver (*Myf5-Cre*; *HSA-Cre*) to restore normal SMN in muscle of SMA mice showed that both replacement or depletion of SMN in muscle had little to no phenotypic effect on the mice [47–49]. The greatest improvement in survival and function is the result of SMN restoration throughout the entire nervous system (*Nestin-Cre* and *ChAT-Cre*; *PrP-Cre*), affecting neurons and glia alike and largely rescuing SMA phenotypes and life span [45, 47]. Although lower motor neurons and their circuitry are the primary targets of SMA pathology, mounting evidence suggests that SMN deficiency may contribute to defects in multiple tissues and across additional peripheral organs [49, 50].

In summary, these studies highlight the necessity of SMN protein during development, and demonstrate the physiological consequences of insufficient levels of SMN on function and survival of various cell-types. While motor neuron degeneration and loss of central synapses and NMJs in the motor circuitry are the primary targets of SMA pathology, restoration of SMN in multiple cell-types may be necessary for a complete rescue of the SMA phenotype. The advent of effective therapies targeting the CNS may lead to the development of multi-organ impairment in surviving SMA patients, requiring systemic delivery of therapies [51].

6.4 SMA Is Caused by Reduced RNP Assembly

SMN is an evolutionarily conserved and ubiquitously expressed protein with an essential role in RNA processing. Complete loss of SMN is lethal in all organisms and depends on maternal contribution across different species, highlighting its importance to cell development and survival [52]. The temporal expression of SMN protein levels is developmentally regulated, with the highest expression levels during the embryonic period and a gradual decrease into the early postnatal period in mice and humans [53]. SMN granules are also present in the axons and growth cones of developing and regenerating motor neurons, and at the postsynaptic endplate of the neuromuscular junctions [54–56]. Active bi-directional fast axonal transport of SMN has been demonstrated in primary forebrain and motor neurons [57, 58].

As its best characterized molecular function, SMN facilitates the assembly of small nuclear ribonuclear proteins (snRNPs), bringing together specific sets of protein and RNA molecules that form the building blocks for spliceosome formation and pre-messenger RNA splicing [59]. More recent studies from multiple laboratories have demonstrated that SMN plays a broader role in the assembly of various RNP complexes with divergent roles in RNA processing, including mRNA splicing, turnover, and trafficking [60]. In contrast to late onset neurodegenerative diseases that are often characterized by the accumulation of RNA-binding proteins into pathological aggregates [61], SMA is set apart by an SMN-dependent deficiency in the formation of RNPs, and is therefore best described as an RNP hypo-assembly disease [62, 63].

SMN associates with eight proteins (Gemins 2–8 and Unrip) to form a complex that is present in the cytoplasm and in discrete nuclear bodies called "gems," for Gemini of Cajal bodies (or coiled bodies) [64–66]. The self-oligomerization of SMN and subsequent formation of the macromolecular SMN complex requires the evolutionarily conserved YG-box. Located at the carboxy-terminus, the YG-box provides a structural basis for the SMN complex to form higher-order complexes ranging from 20S to 80S [69, 70]. A subunit of the SMN complex that includes SMN and Gemin2 recognizes Sm proteins, and assists in the ATP-dependent assembly of the heptameric Sm core complex [67–69]. Spliceosomal Sm proteins belong to a large family of Sm and Sm-like (LSm) proteins that share a conserved Sm motif necessary for protein-protein interaction, and are essential for snRNP biogenesis. Symmetrical dimethylation of a subset of Sm proteins by the protein arginine *N*-methyltransferase 5 (PRMT5) complex enhances their affinity for the conserved Tudor domain within the SMN protein [70, 71]. Gemin3 is a DEAD-box RNA-dependent RNA helicase and ATPase [72]. Gemin5 recognizes and interacts with large, ~50–60 nucleotide sequences or, snRNP codes, on specific spliceosomal U snRNAs [73, 74]. Gemin6 and Gemin7 are thought to possess an Sm protein-like structure, facilitating the recruitment of Sm proteins into snRNPs [75].

6.5 The SMN Complex Is an Assembly Machine for Spliceosomal snRNPs

Although SMN and the associated Gemin 2–8 proteins increase the efficiency and specificity of snRNP complex assembly, they do not become part of the final structure, thus acting as a molecular chaperone [60]. Sm proteins have an intrinsic ability to associate with snRNAs in vitro, forming snRNP complexes with little regard to RNA specificity. The presence of the SMN complex restricts illicit associations of Sm proteins with erroneous RNAs, and promotes the recognition of snRNAs. The assembly of Sm proteins and binding of specific RNA requires a coordinated interaction between the SMN complex and the PRMT5 complex [76]. The PRMT5 complex consists of PRMT5, pICln, and WD45 (Mep50), and pre-assembles specific

sets of Sm proteins via the pICln subunit [68, 77]. pICln is displaced from these recruited Sm proteins by the SMN complex, which promotes the transfer of Sm proteins from an intermediate RNP complex onto snRNA to form U snRNPs [59, 78, 79]. Therefore, the SMN complex functions as an assemblysome that regulates snRNP biogenesis, structure, and function [80]. Recognition and binding of splice sites require the association of several small nuclear RNAs (snRNAs) and Sm proteins. Typically, uridine-rich snRNAs (U1, U2, U4, U5 U6, U11, U12, U4atac, and U6atac) are assembled with a set of seven Sm proteins (Sm B/B′, D1, D2, D3, E, F and G) into different classes of heptameric snRNP core complexes that are essential to the catalytic activity of the spliceosome [69, 81]. The U2-dependent major spliceosome comprised of U1, U2, U4/6, and U5, is the predominant machinery responsible for the accurate removal of canonical "GT-AG" introns from most eukaryotic transcripts, whereas the U12-dependent minor spliceosomal complex comprised of U11, U12, U4atac/U6atac, and U5, removes rare "AT-AC" introns. Despite U12 introns representing only <1% of all human introns, the U12-dependent spliceosome is essential for the viability and development of many multicellular organisms, including humans [82]. U12-type introns have been identified mainly in genes with a role in DNA replication and repair, transcription, RNA processing, and translation, but can also be found in genes related to vesicular transport, cytoskeletal organization and assembly, and voltage-gated ion channel activity [83]. As discussed below, alterations to U12-dependent spliceosomal activity may have particularly deleterious effects on the morphology and physiology of neurons. Of note, a mutation in the gene encoding U12 snRNA has been identified as the potential cause of early onset cerebellar ataxia in one pedigree [84], whereas mutations of core spliceosomal factors are typically associated with severe developmental disorders [85, 86].

Aside from its role in spliceosomal snRNP assembly, SMN has also been shown to be involved in the assembly of related RNP complexes with diverse roles in RNA metabolism [60]. Unlike the U2 and U12 complexes, U7 snRNPs function not in splicing, but in the unique 3′-end processing of replication-dependent histone mRNAs that comprise the most abundant class of intronless and non-polyadenylated transcripts in metazoans [87]. Facilitated by the SMN complex, U7 snRNA associates with Sm-like (LSm) proteins LSm10 and LSm11 instead of SmD1 and SmD2, to form the heptameric Sm core characteristic of snRNPs complexes [88]. Interactions between U7 snRNA and the stem-loop-binding protein (SLBP) mediate the recruitment and positioning of the trans-acting factors that cleave histone pre-mRNA [87]. It remains to be seen whether SMN also plays a role in the assembly of the structurally related but functionally distinct LSm2–8 and LSm1–7 complexes, which play a role in pre-mRNA processing and mRNA decay [89].

While it is well established that the SMN complex promotes snRNP assembly, and ultimately spliceosome formation, several questions regarding the arrangement and association of SMN with Sm proteins remain. Further examination into the structural arrangement and functions of these complexes in vivo and their regulation by cellular signaling pathways are necessary to fully understand the physiological relevance of these complexes in development and disease.

6.6 SMA Deficiency Causes Widespread Splicing Defects

The extensively examined role of SMN in snRNP biogenesis and pre-mRNA splicing led researchers to hypothesize that SMA phenotypes are the result of SMN-dependent alterations in snRNP biogenesis and splicing, and that SMA can be described as a general splicing disease [90]. Evidence supporting this hypothesis is substantial, yet incomplete. A potential direct link between defective snRNP assembly activity and SMA phenotypes was provided by a study showing that injection of purified U snRNPs could rescue embryonic arrest and SMA-like axon degeneration caused by a reduction of SMN or Gemin2 in zebrafish embryos [91], although later studies in zebrafish have not found low Gemin2 levels to cause specific motor axon defects, arguing for a separate role for SMN in the SMA disease process that is snRNP independent [92]. Moreover, studies have shown a reduction in SMN-dependent snRNP activity in SMA patient tissue and animal models, demonstrating a correlation between snRNP activity and disease severity, but no selectivity for vulnerable cell types or tissues was found [81, 90, 93].

As previously described, the SMN complex facilitates snRNP assembly of the major (U2-dependent) and minor (U12-dependent) spliceosomes, as well as the U7 histone processing complex. Accordingly, several studies exploring how SMN deficiency influences the assembly and activity of each pathway have provided insight into the relationship between SMN-dependent snRNP activity and SMA phenotypes. Interestingly, changes in snRNP assembly in mouse models of SMA primarily affect the (U12 dependent) minor spliceosome pathway [93]. Caused by a deficiency in SMN, an inability of U11 snRNP to accumulate and form the U12 spliceosome machinery results in increased U12 intron retention, exon skipping, and aberrant splicing events. In a *Drosophila* model of SMA, mis-splicing of the U12 intron-containing gene *Stasimon* correlated with motor neuron pathology [94]. While overexpression of Stasimon in a *Drosophila* model of SMA rescued axonal pathfinding and outgrowth defects in motor neurons, it failed to restore normal viability and locomotion [94]. Table 6.1 provides a list of selected mRNAs, which are affected by SMN-dependent splicing alterations and have also been suggested to contribute to SMA pathology. SMN depletion may also affect U7 histone mRNA processing. Due to an accumulation of U7 pre-snRNA, U7 snRNP steady-state levels are significantly reduced in SMN-deficient cell lines and SMA mouse tissue, decreasing the post-transcriptional regulation of histone mRNA and resulting in the accumulation of uncleaved, 3′-end-extended histones [88].

Although these experiments emphasize the physiological relevance of snRNP core assembly, it remains unclear whether SMN-dependent alterations to snRNP biogenesis can account for the full spectrum of pathology observed in SMA patients and disease models. Studies in *Drosophila Smn* null mutant larvae showed no appreciable defects in the splicing of mRNAs containing minor-class introns, despite significant reductions in minor-class spliceosomal snRNAs [95]. These findings suggest that SMN's role in snRNP biogenesis can be uncoupled from its effect on viability and locomotion. A comparison of snRNP-dependent and SMN-specific

Table 6.1 Mis-spliced transcripts in models of SMA

Gene	Description	Species	Experimental condition	Cell line/ tissue	Reference	Relevance to SMA pathology
SMN2	Splicing-deficient gene copy encoding SMN	Human	SMA patient	iPS cells	[175]	SMN-deficiency affects *SMN2* splicing in a feedback loop that further reduces SMN protein levels[175]
			SMN1 knockdown	HEK293		
TMEM41b/ Stasimon	Transmembrane protein	*Drosophila*	*smn* loss-of-function	Larvae	[94]	*Stasimon* knockdown in cholinergic neurons increases EPSP amplitude in NMJ, restored by *Stasimon* expression. *Stasimon* expression rescues motor neuron defects in Zebrafish SMA model [94]
		Mouse	*SMN1* knockdown	NIH3T3		
			Moderate SMA model ($SMN2^{+/+};SMN\Delta7^{+/+};Smn^{-/-}$) [22]	Spinal cord, L1 DRG		
Nrxn2a	Pre-synaptic membrane protein	Zebrafish	*SMN* knockdown	Embryo	[176]	Knock-down of *Nrxn2a* results in motor axon defects [176]
		Mouse	Severe SMA model ($Smn^{-/-}/SMN2$) [20]	Spinal cord		
Rit1	G protein	Mouse	*SMN1* knockdown	NSC-34	[177]	Alternatively spliced *Rit1* isoform reduces neuritic length in NSC-34 cells [177]
			Severe SMA model ($Smn^{-/-}/SMN2$) [21]	Lumbar spinal cord		
Agrn	Heparin sulfate proteoglycan; organizer of the NMJ	Mouse	Moderate SMA model ($SMN2^{+/+};SMN\Delta7^{+/+};Smn^{-/-}$) [22]	Motor neurons	[178]	SMN depletion leads to exclusion of Z exons from *Agrn* mRNA [178]. It was previously established that *Agrn* Z^+ isoforms are important for maturation of postsynaptic termini in NMJ [179]. Expression of *Agrn* Z^+ in motor neurons of SMA mice mitigates NMJ defects [35]
			Moderate SMA model ($SMN2^{+/+};SMN\Delta7^{+/+};Smn^{-/-}$) [22]	Motor neurons	[35]	

(continued)

Table 6.1 (continued)

Gene	Description	Species	Experimental condition	Cell line/ tissue	Reference	Relevance to SMA pathology
Uba1	Ubiquitin-like modifier activating enzyme 1	Mouse	Severe SMA model (*Smn*$^{-/-}$/*SMN2*) [20, 21]	Spinal cord	[180]	*Uba1* knockdown disrupts axonal growth and branching, and leads to increase in β-catenin level in Zebrafish. β-catenin levels are also increased in SMA mouse models, and inhibition of β-catenin signaling ameliorates neuromuscular pathology in SMA mice [180]
Anxa2	Ca^{2+}-binding actin regulating protein	Mouse	Moderate SMA model (*SMN2*$^{+/+}$;*SMNΔ7*$^{+/+}$;*Smn*$^{-/-}$) [22]	Spinal cord	[90]	In addition to reported alterations in splicing, *Anxa2* was also shown to be overexpressed in SMA in several proteomic studies [181–183], and *Anxa2* mRNA is mislocalized from neurites in SMN-deficient NSC-34 cells [128]
Cacna1a *Cacna1b* *Cacna1c* *Cacna1e* *Cacna1h*	Voltage-gated Ca^{2+} channel subunits	Mouse	Severe SMA model (*Smn*$^{-/-}$/*SMN2*) [21]	Spinal cord	[184]	In addition to reported alterations in splicing, reduction of Cacna1b in axonal growth cones of motor neurons from another severe SMA model mice [20], accompanied with alterations in their excitability, has been shown [138]

RNA changes in SMA models suggests that defects in snRNP supply are unlikely to be the primary drivers of SMA pathophysiology, at least in *Drosophila* [96]. Despite impairment of snRNP synthesis, endogenous snRNP and snRNA levels were found to be unaltered in SMA1 patient-derived fibroblasts, a chicken cell line, and a severe *Drosophila* mutant, all of which had severely reduced SMN levels [81, 93, 97]. Moreover, despite a significant difference in lifespan between the severe and SMNΔ7 models (~9 days), there was no difference in snRNP assembly activity, suggesting that the difference in disease severity is caused by differential effects on an additional function of the SMN protein [93]. It should be noted that snRNAs that are not associated with Sm cores are unstable, so snRNA levels are similar to snRNP levels [98].

Taken together, this research strongly suggests that while the direct effects of SMN deficiency on altered snRNP assembly and splicing are likely to contribute to SMA phenotypes, it fails to fully explain motor neuron susceptibility and the full spectrum of phenotypes observed in SMA pathology.

6.7 SMN Acts as a Molecular Chaperone for mRNP Assembly

Aside from its role in the assembly of noncoding RNAs into snRNP complexes, SMN has also been implicated in transport and local translation of mRNAs to the distal end of neurites [54, 55, 99–106]. It is based on observations from several labs that SMN associates with multiple mRNA-binding proteins (mRBPs) and regulates the localization of specific transcripts and RNA-binding proteins into axons [103, 104, 106–109]. Localized mRNA create micro-environments of newly synthesized proteins in specific subcellular compartments, promoting autonomous control of local proteomes and stimulus driven adaptive responses [110–112]. Both developing and adult axons contain complex transcriptomes that support the formation and maintenance of neural circuits in vivo [113, 114]. mRNA localization is most commonly achieved through the association of its 3′ untranslated region (3′UTR) with mRBPs, to form messenger ribonucleoprotein (mRNP) transport granules [115]. This assembly into mRNPs serves as a major regulator of multiple steps of mRNA processing, including nuclear export, intracellular trafficking, turnover, and translation. Regulatory sequences within the 3′UTR serve as platforms for the assembly of mRNPs [116] and act as cis-acting localization sequences or “zipcodes” that govern the precise spatiotemporal expression of the transcripts [117, 118]. Directed by these zipcode sequences, mRNPs associate with molecular adaptors and motor proteins to form transport granules, which translocate along microtubules and actin filaments to specific microdomains within the cell. In developing and regenerating axons, RNPs containing growth-promoting mRNA transcripts localize to the growth-cone and allow the cell to navigate and respond to environmental factors [110–112].

Indications of possible splicing-unrelated functions of SMN first came out from studies of its localization in neuronal cell cultures. Besides its principal localization in the cytoplasm and within nuclear gems [119], SMN was also found in axons and dendrites of motor neurons from rat spinal cord sections in association with cytoskeletal components, suggesting potential motor-driven transport [120]. It was also shown that SMN is only partially colocalized with Gemin2 in cytoplasm of mouse cultured embryonic motor neurons, indicating that functions of some SMN subpopulations are not associated with snRNP biogenesis [121]. Cytoplasmic localization of SMN was shown to be exon 7-dependent, which points to possible role of SMN mislocalization in SMA pathology [57]. In addition, SMN was shown to be a part of granules that are actively transported along cytoskeletal structures in neurites of chicken forebrain neurons and in axons of motor neurons [57, 58]. Furthermore, it has been found that overexpression of wild-type SMN was able to promote neurite growth in differentiated PC12 cells [104], and downregulation of SMN leads to axon growth defects in mouse cultured motor neurons [104], zebrafish motor neurons [122], *Xenopus* motor neurons [123], as well as to reduced neurite outgrowth in PC12 cells [124]. Although SMN partially colocalizes with Gemins in cytoplasmic granules in axons and dendrites, the significance of this association is unclear [103, 125, 126].

To date, the most studied non-splicing function of SMN is its role in mRNA localization and transport in neurons. The first evidence for a role in mRNA trafficking was the observed reduction of *β-actin* mRNA in axonal growth cones of cultured motor neurons isolated from a severe SMA mouse model [104]. Similar effects were also observed for the localization of the neurite-outgrowth promoting *neuritin 1* or *candidate plasticity-related gene 15* (*Nrn1/Cpg15*) transcript in cortical neuron neurites upon SMN knockdown [109] and *growth-associated protein 43* (*Gap43*) mRNA in SMN-deficient motor neurons [55]. In addition, a general reduction of poly-A mRNA abundance in axons of anti-*Smn1* shRNA treated mouse motor neurons and a reduction in axonal protein synthesis in *Smn1* shRNA treated cortical neurons have been reported [55, 103]. A transcriptomic microarray analysis using microfluidic chambers to divide axonal and somatodendritic compartments of cultured mouse motor neurons revealed 1189 downregulated probe sets in axonal compartment upon *Smn1* knockdown [127]. Interestingly, while *Smn1* knockdown led primarily to the upregulation of transcripts in the somatodendritic compartment, the overwhelming majority of significantly altered axonal RNAs were downregulated, indicating that these changes resulted from a deficiency in axonal targeting rather than from changes in gene expression rate. The characterization of SMN-associated mRNAs in NSC-34 cells found that transcripts encoding annexin A2 (*Anxa2*) and Cytochrome c oxidase subunit 4 isoform (*Cox4i2*) colocalized with SMN in neurites of differentiated NSC-34 cells and were depleted in neurites upon *Smn1* knockdown [128]. A list of mRNAs that are known to be axonally transported or de-stabilized in an SMN-dependent manner is shown in Table 6.2.

Since SMN does not contain a canonical RNA-binding domain, and unlike Gemin5 and unrip has not been identified as an mRNA-binding protein in large-scale UV-crosslinking experiments [129], it is currently not clear whether its role in

Table 6.2 mRNA components of SMN-dependent mRNPs

Transcript	Species	Experimental condition	Cell line/ tissue	Reference	Type of evidence
β-actin	Mouse	Severe SMA mouse model [20]	Motor neurons	[104]	SMN deficiency reduces *β-actin* mRNA localization in distal axons
		Severe SMA mouse model [22]	DRG sensory neurons	[185]	*β-actin* mRNA is reduced in growth cones of cultured sensory neurons from Smn-deficient embryos
		Smn knockdown	MN-1	[132]	SMN is required for *β-actin* mRNA targeting to RNA granules
	Rat	Transfection with hnRNP R expression constructs	PC12	[104]	Interaction between SMN and hnRNP R modulate *β-actin* mRNA localization in neuritic growth cones
		–	Cortical neurons	[109]	Co-precipitates with SMN
Gap43	Mouse	Severe SMA mouse model [22]	Motor neurons	[55]	*Gap43* mRNA is reduced in axons and growth cones
		Smn knockdown	Motor neurons	[55]	*Gap43* mRNA is reduced in axons and growth cones
		Smn knockdown	MN-1	[132]	SMN is required for recruitment of *Gap43* mRNA to RNA granules
	Zebrafish	*SMN* and *HuD* mutants	Motor neurons	[136]	*Gap43* mRNA levels are decreased in motor neurons in HuD-dependent manner
	Rat	–	Cortical neurons	[109]	Co-precipitates with SMN
Nrn1/Cpg15	Rat	*Smn* knockdown	Cortical neurons	[109]	Co-precipitates with SMN. SMN knockdown affects *Nrn1* mRNA levels in both soma and neurites
Anxa2	Mouse	*Smn* knockdown	NSC-34	[128]	Associates with SMN complex and is reduced in axons upon SMN knockdown
Cox4i2	Mouse	*Smn* knockdown	NSC-34	[128]	Associates with SMN complex and is reduced in axons upon SMN knockdown
Tau	Mouse	*Smn* knockdown	MN-1	[132]	SMN is required for recruitment of *Tau* mRNA to RNA granules
p21	Mouse	Mild SMA mouse model [186]	Spinal cord	[108]	SMN depletion increases *p21* transcript stability

Table 6.3 mRBP components of SMN-dependent mRNPs

Name	Species	Tissue/cell line	Reference	Other supporting evidence
hnRNP R	Human	HEK293	[107]	Interaction with SMN is required for association between *β-actin* mRNA and hnRNP R [104]
	Mouse	Motor neurons, spinal cord extracts	[54]	
KSRP/ FBP2/ MARTA1	Mouse	N2a, spinal cord	[108]	p21 mRNA that is targeted for degradation by KSRP is upregulated in SMA tissues [108]
HuD/ ELAVL4	Rat	Cortical neurons	[109]	SMN is required for HuD targeting into RNA granules [132]. The HuD target mRNA *Gap43* is decreased in motor neurons from *Smn* mutant zebrafish [136]
	Mouse	Spinal cord		
		Motor neurons	[103]	
		MN-1	[132]	
	Zebrafish	Motor neurons	[136]	
IMP1/ZBP1	Rat	Brain	[106]	SMN facilitates association of IMP1 with *β-actin* mRNA [63]
	Mouse	Motor neurons		
SBP2	Human	HEK293	[187]	Levels of several SBP2-dependent selenoprotein mRNAs are reduced in spinal cords from SMA mice [135]

mRNA localization involves its direct interaction with mRNA or is mediated via associated mRBPs. SMN was shown to associate with a large number of mRBPs, including hnRNP R and Q [107, 130], FMRP [131], HuD/ELAVL4 [103, 109, 132], IMP1/ZBP1 [106], KSRP/FBP2/MARTA1 [108], TDP-43 [133], and FUS/TLS [134]. For several of these mRBPs, their association with mRNAs has been shown to be SMN-dependent (Table 6.3). In most cases these interactions are mediated by the Tudor domain of SMN [103, 106, 108, 132]. Some of these mRBPs ensure neuritic mRNA localization, acting in concert with SMN. hnRNP R was shown to modulate *β-actin* mRNA localization in differentiated PC12 neurites, and its SMN-interacting domain was required for this activity [104]. Moreover, hnRNP R directly interacts with *β-actin* mRNA, and SMN facilitates this interaction [104]. Along with their target mRNA, axonal localization of corresponding mRBPs, such as HuD and IMP1, is also affected by SMN deficiency [103, 106]. While the molecular function of SMN in mRNA localization is not as well defined as its role in the snRNP assembly, these results indicated a potentially related role of SMN as an organizer of protein-mRNA complexes (mRNPs) that are then transported within neurites. In support of this hypothesis, SMN was shown to mediate recruitment of HuD and its target mRNAs *Gap43* and *Tau* to RNA granules in differentiated MN-1 cells [132]. Finally, the binding of IMP1 to the *β-actin* 3′UTR was shown to be impaired in motor neurons from an SMA mouse model, and IMP1-containing

mRNP granules were largely reduced in size in SMA1 patient fibroblasts [63]. These data indicate that the function of SMN as a molecular chaperone for RNP assembly [60] is not limited to snRNPs, but also includes other RNP types, including the assembly of mRNP transport granules [63].

More detailed mechanistic aspects of SMN activity in mRNP assembly have yet to be revealed. In particular, it would be interesting to determine if the same components of SMN complex that promote snRNP assembly also act in mRNP formation. The observation that selenocysteine insertion sequence (SECIS)-binding protein 2 (SBP2), which is an mRBP for selenoprotein mRNA, directly interacts with the SMN-complex proteins Gemins 3, 4, 7 and 8, suggests their involvement in the assembly of selenoprotein mRNPs [135]. However, their role in the formation of transport mRNP granules in motor neurons still has to be determined.

6.8 mRNP Assembly Defects Can Contribute to SMA Pathology

It is presently not known whether the defects of mRNP assembly contribute to neurodegeneration in SMA patients, but there are several lines of evidence supporting this hypothesis. As stated above, mRNP granules in fibroblasts from SMA patients are reduced in size, indicating that mRNP assembly is indeed impaired in SMA [63]. Furthermore, these granules show decreased association with the cytoskeleton, indicating that mRBP transport defects may occur in SMA [63]. In addition, downregulation of SMN-dependent mRBPs resemble the effects of SMN downregulation. HuD knockout in Zebrafish results in decreased branching of motor axons, and HuD expression in motor neurons from SMN-mutant Zebrafish rescues its defects [136]. Knockdown of hnRNP R also leads to defects in axonal growth in Zebrafish and mouse motor neurons, resulting in defective clustering of voltage-gated Ca^{2+} channels in axonal growth cones, similar to defects described in motor neurons from SMA mouse models [137, 138]. On the other hand, increased expression of both IMP1 and HuD, which are mislocalized in cellular SMA models, restore *Gap43* mRNA and protein levels in growth cones and rescues axon outgrowth defects in SMN-deficient motor neurons [55]. Taken together, these data indicate a potential role for impaired mRNP formation in SMA pathology.

mRNA localization in neurites has been identified as a key process that determines the enrichment of neuritic compartments for specific proteins [139]. Therefore, disruption of mRNA transport by defective mRNP formation has the potential to dramatically alter the neuritic proteome. It is well established that local translation of *β-actin* mRNA regulates directed growth of axonal growth cones in response to guidance cues [112, 140], and interaction between IMP1 and *β-actin* mRNA is required for these responses [140]. Since association between IMP1 and *β-actin* mRNA is decreased in SMA [63], deficiency in IMP1 mRNP formation may contribute to axonal defects observed in SMA models. Similarly, *Gap43* mRNA

that is targeted to axons by association with mRBPs IMP1 and HuD [141] is reduced in axons and growth cones of SMN-deficient motor neurons [55]. Although local translation of *Gap43* is required for axon elongation, *β-actin* translation is more important for axonal branching [142]. Interestingly, defects in both elongation and branching of axons upon SMN depletion were reported in zebrafish in vivo [122]. Another example for an SMN-dependently localized mRNA is *Nrn1*, which can rescue axonal defects in a zebrafish SMA model [109]. Since neuritin is known to regulate synapse stability [143], lack of this protein due to insufficient mRNA transport could affect NMJs, where the earliest SMA-associated structural changes occur [28]. Of note, limited amount of IMP1 in DRG neurons restricts axonal localization of *Gap43* and *β-actin* mRNA, while limited availability of HuD induces competition between *Gap43* and *Nrn1* mRNA for HuD binding and axonal localization [144, 145]. It is possible that this phenomenon provides the basis for the regulation of specific mRNA in axons depending on neuronal developmental stage or specific conditions, such as response to neuronal injury. Therefore, it would be of interest to establish how SMN depletion affects relative abundances of specific mRNAs and mRBPs in axons and growth cones. Another question is how SMN regulates association of mRNA with distinct mRBPs. While it is well known that binding of HuD provides stabilization of its mRNA targets, such as *Gap43* [146], association with the SMN-interacting mRBP KSRP leads to increased *Gap43* mRNA decay [147].

mRNPs have the ability to form higher-order cytoplasmic mRNP granules in response to certain environmental conditions. Cellular stress can result in the assembly of mRNPs and stalled translation pre-initiation complexes into cytoplasmic stress granules [148]. The sequestration of translationally stalled housekeeping mRNAs and enhanced expression of stress-response factors, such as molecular chaperones, is believed to be protective for cell survival under stress conditions. SMN has been shown to facilitate stress granule formation [149], whereas its downregulation inhibits cellular stress response [150]. Thus, a reduced capacity for stress granule formation could make cells more vulnerable to environmental conditions. One of the described functions of stress granules is the prevention of apoptosis by sequestration of certain molecules, such as MTK1 and mTORC1 kinases, and inhibition of apoptotic signaling [151, 152]. If SMA is associated with a deficiency in stress granule formation, this could contribute to apoptotic mechanisms in neurodegeneration and cell death [153].

6.9 Other mRNA-Processing Functions of SMN

Aside from its role in snRNP and mRNP assembly, there is evidence for the involvement of SMN at other stages of the mRNA life cycle, including transcription and translation (Fig. 6.2). The first evidence for the involvement of SMN in transcription came from the finding that it interacts with bovine papillomavirus transcriptional activator E2 and stimulates E2-dependent transcription [154]. Subsequent studies identified additional SMN interactors involved in transcription, including the tumor

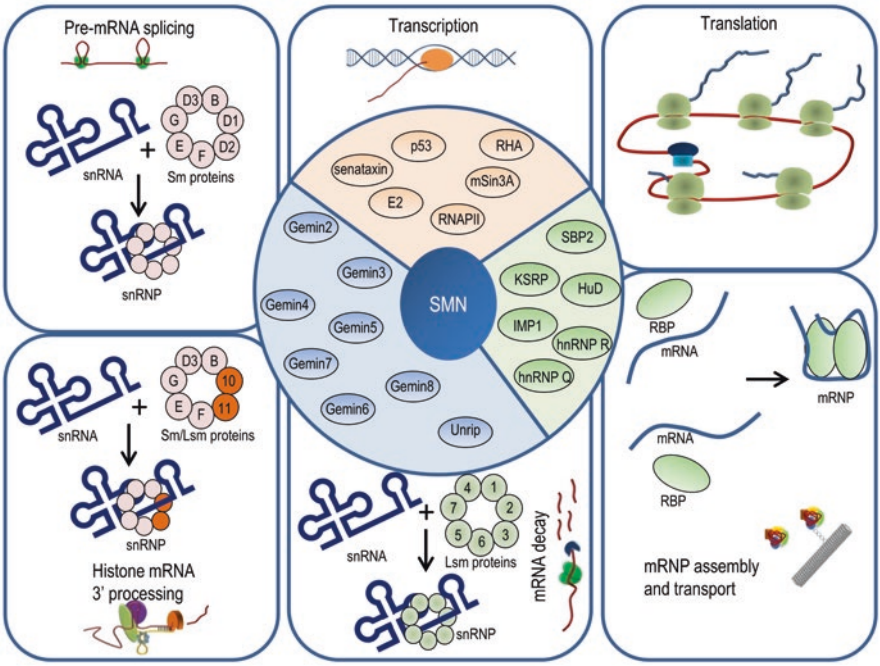

Fig. 6.2 Molecular Functions of SMN in mRNA processing. SMN can associate with a large selection of proteins to regulate snRNP assembly (splicing, histone mRNA processing, mRNA decay), transcription, translation, and mRNP assembly (mRNA transport and local translation)

suppressor and transcriptional activator p53 [155] and the transcription corepressor mSin3A [156]. Moreover, artificial recruitment of SMN to promotor regions resulted in repression of transcription [156]. SMN was found to associate with key components of transcription machinery, such as RNA helicase A and RNA polymerase II. Overexpression of truncated SMNΔN27 results in transcription inhibition and accumulation of these components in gems and coiled bodies [157]. SMN was also shown to interact with the DNA/RNA helicase senataxin [158] and facilitate the association of senataxin and the C-terminal domain (CTD) of RNA polymerase II in a manner that was dependent on CTD symmetric dimethylation [159]. Formation of this complex is required for resolving DNA-RNA loops (R-loops) and proper transcription termination [159]. It has been shown that SMN knockdown in SH-SY5Y cells leads to increased R-loop formation and DNA damage [160]. Of note, senataxin mutations are a rare cause of proximal spinal muscular atrophy [161], juvenile amyotrophic lateral sclerosis [162], and ataxia-ocular apraxia 2 [163], indicating a possible link between the regulation of transcription termination via R-loops and neurodegeneration across different neurodegenerative diseases (for reviews, see [164, 165]).

The role of SMN in mRNP assembly and transport implies that *SMN* mutations can cause defects in local translation due to inefficient localization of mRNA to

their destination sites. Indeed, defects in axonal translation in motor neurons from a mouse model of severe SMA (*Smn*$^{-/-}$;*SMN2*) and in cortical neurons upon SMN knockdown were reported [55, 166]. In addition, there is accumulating evidence that SMN can directly regulate translation. SMN can associate with the translation machinery, and it has been found in polyribosome fractions purified by ultracentrifugation from MN-1 cells [167]. Moreover, SMN can anchor ribosomes to the plasma membrane, since the ribosomal content in plasma membrane fractions was depleted in SMA patient-derived fibroblasts or normal fibroblasts upon SMN knockdown [168]. SMN deficiency in MN-1 cells does not affect overall translation rates but leads to increased translation of CARM1 arginine methyltransferase mRNA, and possibly other specific mRNAs, via currently unknown mechanism [167]. Another study demonstrated that low amounts of SMN in cortical neurons reduce protein synthesis by upregulation of miR-183 microRNA and downregulation of mTOR pathway [105]. In contrast, there were no significant differences in mTOR activation status and protein synthesis rate upon SMN knockdown in human fibroblasts under steady-state conditions. However, when membrane protrusion formation was stimulated in these cells, a decrease in translation rate was observed in *SMN* knockdown fibroblasts, and this difference was mTOR-dependent [168].

An SMN-dependent defect in translation has also been suggested by polysome profiling experiments, which indicated a reduction in the polysome peak of profiles from late-symptomatic SMA mouse tissue [169]. RNA-seq data analysis identified genes associated with translation-related processes as significantly dysregulated in SMA motor neurons, providing evidence for a role of SMN in the regulation of ribosome biogenesis and translational activity [169].

SMN-mediated regulation of translation is an emerging field that needs further studies to elucidate its molecular mechanism and potential role in SMA pathology, but it may very well be related to a function for SMN in assembling mRNA and associated proteins, similar to its role in snRNP and mRNP assembly.

6.10 Open Questions and Future Perspectives

While diverse functions of SMN in regulating different aspects of mRNA processing are well established, the relative contributions of these SMN-dependent pathways and molecular mechanisms that result in disease pathology remain unclear. Studies examining the biological function of SMN are beginning to reveal the dynamic roles that SMN plays as a chaperone in snRNP assembly and splicing, mRNP assembly and mRNA trafficking, and as a regulator of post-transcriptional gene expression. Additional studies to discern which SMN function is critically affected in SMA will be crucial to our understanding of SMN biology and SMA pathology.

Although there is substantial evidence suggesting that low levels of SMN result in motor neuron dysfunction and loss, little is known about the pathological consequences of SMN depletion in other cell-types. It now appears likely that other

neurons in the spinal cord motor circuitry, glia, myofibers, and other tissues outside the CNS may contribute to the pathophysiology of SMA [45–50]. Importantly, the recent introduction of Spinraza™ and the anticipated addition of gene therapy (AVXS-101) as a treatment for SMA is expected to attenuate aggressive aspects of disease pathology in the CNS of SMA I patients, potentially leading to a more chronic disease, and necessitating the characterization of otherwise masked disease phenotypes in peripheral organs and tissues.

As a regulator of snRNP complex assembly, SMN deficiency is known to cause widespread changes in splicing and gene expression in various cellular and animal models of SMA. However, the question if and how a defect in the canonical housekeeping function of SMN in snRNP assembly directly causes the neurodevelopmental and neurodegenerative processes that lead to SMA pathogenesis remains. While the discovery of *stasimon* as a potential regulator of motor neuron circuitry provided the first link between SMN-dependent splicing variations and the development of SMA pathology [94], future studies will be needed to examine and characterize the role of stasimon in human patients. As listed in Table 6.1, research into identified SMN-dependent splicing isoforms will be required to substantiate a direct link between splicing defects and SMA phenotypes. A thorough characterization of aberrant pre-mRNA processing in motor neurons of SMA mouse models and patients will continue to expand our understanding of the down-stream consequences of SMN deficiency that may explain motor neuron susceptibility and disease pathology.

Research from several groups has begun to elucidate the noncanonical functions of SMN in regulating mRNP assembly and trafficking, as well as local and general translation [101, 170]. Understanding SMN-dependent interactions, assembly, and localization of mRNP complexes could elucidate how extensive cell polarity and trafficking demands characteristic of motor neurons contribute to disease vulnerability. Axonal localization defects are prevalent in multiple neurological disorders including amyotrophic lateral sclerosis (ALS), Alzheimer disease (AD), Huntington disease (HD), and Fragile X Syndrome (FXS), with recent studies emphasizing the role of local protein synthesis in regulating synaptic transmission and axon maintenance, and its relevance for human disease [113, 171–173].

While the precise molecular mechanism of SMN-mediated snRNP assembly is well characterized (reviewed in [60]), much less is known about the exact molecular processes that govern mRNP assembly. SMN was shown to interact with several mRBPs using its Tudor domain, and similar to Sm and Lsm proteins, these interactions are triggered by arginine methylation of mRBPs [108, 132]. Indeed, work from several labs has shown that SMN associates with mRBPs known to regulate the axonal localization and synthesis of growth-promoting mRNAs. With a growing body of research linking SMN deficiency to reduced RNP assembly and transport, several questions are beginning to arise regarding the molecular mechanisms and the nature of the SMN interactome. Questions such as: How extensive is SMN's involvement in mRNP assembly and trafficking? By what molecular mechanisms does SMN mediate the association of mRBPs with target mRNAs? While current models for the formation of mRNPs suggest that the interaction of mRNAs bearing

distinct localization elements with mRBPs is sufficient to initiate mRNP assembly [174], a recent study has identified SMN as a chaperone for the assembly of mRNP granules, at least for those containing the IMP1 protein [63]. Do other core components of the SMN complex (Gemins and unrip) contribute to noncanonical functions of SMN? These questions are currently unanswered, and should be addressed in future studies. Another important task is to identify a potential link between hypoassembly of mRNPs and the neurodegeneration of motor neurons in SMA. Comparing the molecular composition of mRNPs in normal cells and in SMA models may offer clues into the components that are necessary to achieve normal biological function. A recent transcriptomic study identified a large number of mRNAs that are mislocalized in axons upon SMN knockdown in cultured mouse motor neurons in vitro [127], and it remains to be seen if similar changes can be identified in vivo.

Selective disruption and rescue of diverse SMN-dependent RNA processing functions, as summarized in Fig. 6.2, should allow us to assess their contribution to SMA pathology. The identification of pivotal pathways and molecules in RNP assembly and transport will expand our understanding of the underlying biology that contributes to organismal development and regeneration, and potentially offer novel strategies to treat and rescue degenerative phenotypes in a wide variety of neurological disorders and disease. While current efforts for the treatment of SMA are mainly focused on raising SMN protein levels, a thorough understanding of SMN's role in the SMA disease process may lead to the identification of additional targets for therapy.

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Chapter 7
Stress Granules and ALS: A Case of Causation or Correlation?

Nikita Fernandes, Nichole Eshleman, and J. Ross Buchan

Abstract Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease characterized by cytoplasmic protein aggregates within motor neurons. These aggregates are linked to ALS pathogenesis. Recent evidence has suggested that stress granules may aid the formation of ALS protein aggregates. Here, we summarize current understanding of stress granules, focusing on assembly and clearance. We also assess the evidence linking alterations in stress granule formation and dynamics to ALS protein aggregates and disease pathology.

Keywords Stress granules · ALS · TDP-43 · FUS · SOD1 · mRNA · Autophagy · C9ORF72 · Chaperones · Cytoskeleton

7.1 Introduction to ALS

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by premature degeneration of upper and lower motor neurons, typically in mid-adult life. Death usually results within 2–5 years due to paralysis and respiratory failure. Approximately 90% of ALS cases are sporadic, with 10% familial [1].

Mutations in >30 genes are linked to ALS onset (Table 7.1), several of which are linked to another neurodegenerative disease, Frontotemporal dementia (FTD). FTD results from neuronal atrophy within frontal and temporal cortices that causes cognitive, behavioral, and language defects [66]. Many patients diagnosed with ALS or FTD exhibit symptoms of the other disease. Given this, and commonalities at the genetic and cellular levels, ALS and FTD are often considered different facets of a neurodegenerative disease continuum [1]. Here, we focus on ALS, though much discussed is also relevant to FTD.

ALS is characterized by cytoplasmic aggregates within affected neurons, often termed "inclusion bodies." In 97% of ALS cases (including all sporadic cases),

N. Fernandes · N. Eshleman · J. R. Buchan (✉)
Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ, USA
e-mail: rbuchan@email.arizona.edu

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Table 7.1 Evidence for ALS-linked mutations affecting SG dynamics

Gene	Location of mutation(s)	Function	In SGs?	Evidence ALS mutations affect SGs		References
				Mutations that increase SG persistence or formation	Mutations that decrease SG persistence or formation	
RNA metabolism						
TDP-43	C-term IDR [2]	Transcription/splicing/ translation regulation/ transport	Y	Mutant ↑ number [3] and size of SG+ inclusions [4]	Mutant (endogenous) in IDR ↓ SG assembly; mutant outside IDR no effect [5]	Mackenzie [2] Yesucevitz [3] Dewey [4] McDonald [5]
FUS	Dispersed [2]	Transcription/splicing/ translation regulation/ transport/DNA damage Repair	Y	Mutant FUS delays SG assembly, once formed ↑ number and size of SG+ inclusions [6, 7]; another mutant ↓ release of FUS from SGs [6]	Mutant FUS ↑ SG disassembly [7]	Mackenzie [2] Baron [7] Ryu [6]
TIA1	IDR [8]	Major SG component/ splicing/translation regulation	Y	Mutant delays SG disassembly [8]	Unknown	Mackenzie [8]
hnRNP A2B1/ hnRNPA1	IDR [9]	Splicing/translation regulation/stability/ transport	Y	Mutant ↑ recruitment of mutant protein into SGs [10]	Unknown	Kapeli [9] Kim [10]
ATXN2	PolyQ expansion [11]	Translation/endocytosis	Y	Involved in SG assembly and recruitment of TDP-43 to SGs [12]	Unknown	Elden [11] Becker [12]
ANG	Dispersed [13]	Angiogenesis	Y	Angiogenin-generated tiRNAs ↑ SG assembly [14]	Mutant ↓ SG assembly [15]	Padhi [13] Lyons [14] Thiyagarajan [15]
ELP3	KAT domain [16]	Translation elongation, histone acetylation	N	Unknown	Unknown	Glatt [16]

Gene	Location of mutation(s)	Function	In SGs?	Evidence ALS mutations affect SGs		References
				Mutations that increase SG persistence or formation	Mutations that decrease SG persistence or formation	
SETX	N-term protein interaction domain, Helicase domain [17]	Predicted helicase	N	Unknown	Unknown	Bennett [17]
MATR3	Dispersed [18]	Nuclear matrix protein	N	Mutant nuclear. Few cells MATR3+ SGs [19]	Unknown	Boehringer [18] Gallego-Iradi [19]
SMN1	Abnormal copy numbers [20, 21]	Biogenesis of snRNPs	Y	WT involved in SG assembly [22]	Unknown	Blauw [20] Corcia [21] Hua [22]
TAF15	C-term Zn finger and RGG domains [9]	Transcription regulation	Y	Unknown	Unknown	Kapeli [9]
EWSR1	Around Zn finger motif, N-term QGSY domain [9]	RNA-binding protein	Y	Unknown	Unknown	Kapeli [9]
SS18L1	Dispersed, 1 CBP binding motif deletion [23]	Transcription regulation, subunit of chromatin remodeling complex	Y	Unknown	Unknown	Kukharsky [23]
GLE1	Dispersed, 1 mutant has C-term extension [24]	RNA export	Y	Regulates SG assembly [25]. Mutants localize to SGs and no effect on SG dynamics [26]	Unknown	Kaneb [24] Aditi [25] Aditi [26]

(continued)

Table 7.1 (continued)

Gene	Location of mutation(s)	Function	In SGs?	Evidence ALS mutations affect SGs		References
				Mutations that increase SG persistence or formation	Mutations that decrease SG persistence or formation	
Protein turnover/vesicular trafficking						
C9ORF72	Intronic hexanucleotide repeat expansion [27]	DENN protein with Rab GEF activity, endocytosis, autophagy	Y	DPRs ↑ stress, ↓ G3BP1 dynamics in SGs, change composition of SGs [28–30]	Unknown	DeJesus-Hernandez [27] Maharjan [28] Boeynaems [30] Lee [29]
VCP	N-term [31]	Proteasomal degradation, autophagy	Y	Disease mutants induce constitutive SGs [32]	Unknown	Ayaki [31] Buchan [32]
UBQLN2	Proline repeat motif (Pxx) in central domain [33]	Proteasomal degradation, autophagy, endosomal trafficking	N	Unknown	Unknown	Deng [33]
p62	Dispersed [34]	Ubiquitination, selective autophagy adaptor	Y	p62 localizes to SGs, KD ↓ SG disassembly [35],OE of p62 reduces TDP-43 [36]	Unknown	Fecto [34] Guo [35] Brady [36]
OPTN	Coiled coil and ubiquitin binding domains, deletion of some exons [37]	Selective autophagy adaptor	N	Unknown	Unknown	Swarup [37]
ALS2	Truncations [11, 38]	Vesicle trafficking (Rab GEF)	N	Unknown	Unknown	Yang [38] Hadano [11]
CHMP2B	I29V, T104N or Q206H [39]	Vesicle trafficking (endocytosis and autophagy)	N		Unknown	Cox [39]
FIG4	Dispersed [40]	Vesicle trafficking	N	Unknown	Unknown	Chow [40]

Gene	Location of mutation(s)	Function	In SGs?	Evidence ALS mutations affect SGs		References
				Mutations that increase SG persistence or formation	Mutations that decrease SG persistence or formation	
VAPB	Major sperm protein domain [41]	Vesicle trafficking, facilitates UPR	N	Unknown	Unknown	Nishimura [41]
TBK1	Dispersed [42]	Regulator of selective autophagy	N		Unknown	Freischmidt [42]
CCNF	Dispersed [43]	Ubiquitination	N		Unknown	Williams [43]
Chaperones						
SIGR1	Ligand binding motif [44]	ER chaperone	N	Mutant induce constitutive SGs [45]	Unknown	Watanabe [44] Dreser [45]
Cytoskeleton						
PFN1	N-term [46]	Actin cytoskeletal dynamics	Y	Mutants induce constitutive SGs, ↓ SG disassembly and ↓ recruitment of Pfn1 to SGs [47]	Unknown	Wu [46] Figley [47]
DCTN1	Dispersed [48, 49]	Interacts with dynein facilitating transport of membrane organelles/ vesicles	Y	Unknown	Unknown	Münch [48] Vilariño-Güell [49]
NEFH	C-term [50, 51]	Structural neurofilament, axonal transport	N	Unknown	Unknown	Al-Chalabi [50] Skvortsova [51]
SPG11	N-term, C-term [52]	DNA damage repair, cytoskeleton stability, synaptic vesicle transport	N	Unknown	Unknown	Couthouis [52]

(continued)

Table 7.1 (continued)

Gene	Location of mutation(s)	Function	In SGs?	Evidence ALS mutations affect SGs		References
				Mutations that increase SG persistence or formation	Mutations that decrease SG persistence or formation	
MAPT	Dinucleotide polymorphism [53]	Microtubule assembly	Y	Unknown	Unknown	Münch [53]
TUBA4A	Dispersed, many C-term [54, 55]	Alpha tubulin	Y	Unknown	Unknown	Smith [54] Perrone [55]
PRPH	Rod domain [56, 57]	Intermediate filament	N	Unknown	Unknown	Corrado [56] Gros-Louis [57]
TRPM7	C-term [58]	Ion channel, Ser/Thr protein kinase	N	Unknown	Unknown	Hermosura [58]
DNA damage repair						
NEK1	Kinase domain, basic domain and coiled coil domain [59]	DNA damage repair	N	Unknown	Unknown	Brenner [59]
C21ORF2	Unclear	DNA damage repair, celia formation, mitochondrial function	N		Unknown	
Other						
SOD1	Dispersed [60]	Superoxide metabolism	N	Mutants ↓ dynamics of SOD1 + SGs [61]	Mutants delay SG assembly [62]	Cleveland [60] Mateju [61] Gal [62]
ERBB4	C-term [63]	Receptor Tyr Protein Kinase	N	Unknown	Unknown	Takahashi [63]
CHCHD10	Dispersed [64]	Associated with MICOS	N	Mutants induce constitutive SGs, colocalization of TDP-43 with SGs [65]	Unknown	Cozzolino [64] Woo [65]

Acronyms: C-term = C-terminal, N-term = N-terminal, IDR = Intrinsically disordered region, SGs = stress granules, DENN = differentially expressed in neoplastic vs. normal cells, MICOS = mitochondrial contact site and cristae organizing system

these aggregates are enriched for 43 kDa TAR DNA-binding protein (TDP-43) [1, 67, 68]. TDP-43 is typically hyper-phosphorylated, ubiquitinated, and N-terminally truncated within these aggregates; phosphorylation may be mediated by Casein Kinase δ1 [69], whereas cleavage is due to Caspase-3 activity [1, 67, 68, 70]. Under normal conditions, TDP-43 is a nuclear RNA-binding protein that regulates many steps of mRNA metabolism including transcription, splicing, export, translation, and mRNA stability [71]. In familial ALS cases lacking TDP-43 pathology, aggregates of either Fused in Sarcoma (*FUS*) [71, 72], another normally nuclear RNA-binding protein whose functions overlap with TDP-43, or Superoxide dismutase (*SOD1*), an antioxidant enzyme, are observed. Mutations in *FUS* and *SOD1* typically drive formation of these aggregates [73–75].

TDP-43, FUS, and SOD1 aggregates may lead to both a toxic gain of function and a loss of function. Many excellent reviews have addressed this [1, 76–79]. However, perturbations in mRNA metabolism caused by these aggregates are often suggested as an underlying cause of ALS pathology [1, 78, 80–84]. This reflects the localization of other RNA-binding proteins (see below) and mRNA [85, 86] within these aggregates, and the fact that TDP-43 and FUS toxicity require RNA-binding activity in numerous model systems [87–90]. Preventing formation or facilitating removal of these aggregates is considered a promising therapeutic approach, hence considerable research is now focused in these areas.

Much interest has focused on whether ALS cytoplasmic aggregates are linked to perturbations of endogenous mRNA-protein (mRNP) granules, particularly stress granules (SGs). It has been hypothesized that SGs may facilitate TDP-43/FUS aggregation, and/or that perturbations of SG dynamics may contribute to the ALS disease mechanism [78, 81–83]. Here, we introduce the reader to SGs, before assessing evidence that SGs may be a component of ALS pathogenesis.

7.2 Introduction to Stress Granules

Various mRNP granules exist in eukaryotic biology, including SGs, P-bodies, and neuronal transport granules (NTGs) [91]. While distinguished by composition, morphology, and cellular context, they are all dynamic, self-assembling structures that lack a limiting membrane. All mRNP granules harbor non-translating mRNPs, including proteins that regulate mRNA translation, localization, and stability.

SGs are cytoplasmic mRNP granules that are conserved throughout eukaryotes. They usually form only during cellular stress, when translation of most mRNAs is repressed. SGs contain polyadenylated mRNA, translation initiation factors, small ribosomal subunits, various RNA-binding proteins (RBPs), and several cell signaling proteins [82, 92–95]. SGs are typically dynamic, with most components examined via kinetic microscopy methods exhibiting residency times of <30s. However, dynamics of SG components can change over time or under different growth conditions, and immobile populations of mRNAs and proteins are also commonly observed, suggestive of a storage role for SGs [94].

SGs may function as "triage" sites for mRNAs, in which some mRNAs may be stored, while others are returned to translation or targeted for decay [93]. This may occur in P-bodies, with which SGs can physically dock and may exchange mRNP components [96, 97]. mRNP localization within SGs is selective, as stress-responsive mRNAs are often excluded from SGs during stress responses [98]. Finally, SGs can regulate signaling pathways by virtue of sequestration of kinases from specific substrate proteins [94], and also have roles in viral defense [99].

7.3 SG Assembly and Disassembly

SG assembly can occur rapidly following cellular stress (<10 min in yeast; <15 min in human cells—[97, 100, 101], and often involves proteins whose importance in assembly varies in a stress-specific manner [95, 102]. However, key principles have emerged.

7.3.1 Non-translating mRNA Is Required for SG Assembly

SG assembly requires non-translating mRNAs. SGs normally exhibit an inverse relationship with translation, requiring translation repression in order to induce SG assembly [92, 103, 104]. Drugs that arrest ribosomes on mRNAs (e.g., cycloheximide) prevent SG assembly if administered before or coincident with a SG-inducing stress, and cause disassembly of already-formed SGs [103, 105]. This suggests that mRNAs undergo regular exchange between SGs and polysomes, and once mRNAs are trapped in polysomes, they cannot nucleate SG assembly. Conversely, SG assembly is enhanced by drugs that dissociate ribosomes from mRNAs (e.g., puromycin), thus increasing the non-translating mRNA pool [103, 106]. Finally, the absence of 60S ribosomal subunits from SGs, and assays to identify sites of active protein synthesis, indicate that SGs are translationally silent [104, 107, 108].

In principle, noncoding RNA molecules (ncRNAs) could help nucleate SG assembly. Many ncRNAs localize in SGs including small ribosomal subunit RNA [108], tRNA fragments ("tiRNAs") [109], miRNAs [110], and long ncRNAs [111]. Interestingly, ncRNAs also co-purify with P-bodies, albeit mRNAs are preferentially enriched [112]. However, no current evidence suggests a direct scaffolding role for ncRNAs in SG assembly, through a precedent exists for paraspeckles [113].

7.3.2 Protein-Protein and Protein-RNA Interactions Drive SG Assembly

Proteins that aid SG assembly often self-interact and form oligomeric complexes. Such interactions may occur via defined structural domains, as with G3BP1 [114], or via intrinsically disordered regions (IDRs; also termed "low-complexity" or "prion-like"

depending on composition), as with TIA1 [115]. Both proteins are considered key to SG assembly. IDRs often aid protein localization in SGs [116–118] and/or facilitate SG assembly through formation of amyloid-like structures [95, 115, 118, 119].

Protein-RNA interactions also drive SG assembly. Approximately half of SG-localizing proteins bind RNA [120], some via IDRs [118, 119], and others via classically recognized RNA-binding domains. RNA binding is key for the ability of several proteins to facilitate SG assembly and/or localize in SGs [114–116, 121].

7.3.3 *Liquid-Liquid Phase Separation May Facilitate SG Assembly*

SG assembly likely occurs in part via a liquid-liquid phase separation (LLPS) process. The phase separation model posits that SGs, and other RNP granules, are driven by both homo and heterotypic protein-protein and protein-RNA interactions once critical local concentrations of interactors are attained [96, 118, 122–126]. Molecules with high valency and binding affinity likely play the most prominent roles in such phase separation. In principle, RNA-RNA interactions may also facilitate SG assembly via an LLPS process. Indeed, RNAs containing G_4C_2 repeat expansions observed in ALS-mutant alleles of *C9ORF72* (Table 7.1) can undergo LLPS [127]. The role of phase separation in SG assembly has been expertly discussed elsewhere [95, 122], thus only key findings are now summarized.

Several full-length SG proteins can phase separate into homotypic liquid-like droplets in vitro, including FUS [124] and hnRNPA1 [118, 119]. Furthermore, IDR domains from various SG proteins, including TDP-43 and TIA1, can also phase separate in vitro into liquid-like droplets [119, 128]. Such phase separation is promoted by low salt, low temperature, crowding agents, and particularly the addition of RNA [118, 119, 129]. Interestingly, these liquid-like droplets can mature over time into less dynamic bodies, sometimes termed hydrogels, or form fibrillar aggregates [117–119, 124]. Rates of phase separation, hydrogel formation, and aggregation can be accelerated if the proteins in question harbor ALS-associated mutations (e.g., FUS, hnRNPA1 [118, 124, 130]).

7.3.4 *SG Assembly: More Than Just a Passing Phase?*

Although many SG proteins can phase separate, several observations suggest that other processes affect in vivo SG assembly. First, super-resolution microscopy indicates that human cell SGs possess substructure, consisting of SG "cores" that are protein dense, and a liquid-like protein shell that surrounds the cores [120]. SG cores can be detected and purified as soon as SGs become microscopically visible, both in yeast and human cells [101, 120]. Second, SG cores are relatively stable, and do not dissolve in dilute lysate as would be predicted for LLPS droplets. Third, SG assembly in human cells is inhibited at low temperature, contrary to enhanced LLPS

and/or hydrogel formation in vitro [117, 118, 126]. Finally, ATP depletion strongly inhibits SG assembly, and reduces the mobility and internal dynamics of already-formed SGs [120]. This suggests the involvement of energy-driven processes in regulating SG formation.

7.3.5 *ATP-Driven Machines Affect SG Assembly and Disassembly*

Sensitivity of SGs to ATP depletion is likely due to inhibition of numerous ATP-driven chaperones and helicases, which localize within SGs [32, 115, 131, 132], and are purified in SG cores [120]. Genetic analysis of these ATPases indicates contrasting effects on SG assembly and disassembly. For example, inhibition of Hsp70 chaperones and associated cofactors causes SG persistence following alleviation of various stresses [131, 133–136], indicating that Hsp70 aids SG disassembly. In contrast, inhibition of the yeast mini chromosome maintenance (MCM) and RuvB-like helicase complexes causes faster SG disassembly, indicating a role in SG persistence [120]. Inhibition of the Chaperonin-containing T-complex (CCT complex) accelerates SG assembly and increases SG numbers following stress [120], indicating that CCT inhibits SG assembly. An ATPase-deficient allele of yeast Ded1, an RNA helicase, causes strong accumulation of constitutive SGs, within which mRNPs are stalled in translation re-entry [132]. Finally, under certain stresses, the AAA-ATPase Vasolin-containing protein (VCP; Cdc48 in yeast), a "ubiquitin segregase" implicated in proteasomal and autophagic turnover [137], also facilitates SG clearance at least partly via an autophagic mechanism (discussed later [32]). Thus, SGs dynamics likely depend on constant activity from ATP-dependent machines, which may facilitate assembly, disassembly and prevent conversion of SGs proteins into non-dynamic aggregates.

7.3.6 *Posttranslational Modifications Affect SG Dynamics*

Modification of SG proteins often affects their ability to help assemble or disassemble SGs, as well as their localization within SGs [138]. For example, G3BP1 oligomerization, which facilitates SG assembly, is inhibited by a Ras-dependent phosphorylation event [114]. This specifically favors binding to G3BP1 by USP10 at the expense of Caprin1, which limits G3BP1-driven SG assembly [139]. Additionally, SG disassembly is aided by phosphorylation of Grb7 by focal adhesion kinase. Grb7 phosphorylation weakens interactions between SG proteins, such as HuR and TIA1, and their binding to specific mRNAs [140]. Finally, localization of cold-inducible RNA-binding protein and calreticulin to SGs requires methylation and arginylation, respectively [141, 142]. Other modifications implicated in SG dynamics include O-glcnacylation [143], acetylation, ubiquitination [144], neddylation [145], and poly-(ADP) ribosylation [146].

Modifications also affect LLPS processes. In vitro, phosphorylation of the IDR of FUS limits its retention in pre-formed hydrogels of non-phosphorylated FUS [147]. In addition, in vitro droplets of the RNA helicase DDX4 are destabilized by methylation [126].

7.3.7 Role of the Cytoskeleton in SG Dynamics

SG formation is strongly linked to microtubules. Microtubule depolymerizing drugs or knockdown of Dynein microtubule motor proteins limit SG assembly, often resulting in the formation of miniature SGs [144, 148–151]. Localization of specific mRNAs to SGs also requires microtubules [152]. Microtubule disruption or knockdown of Kinesin microtubule motor proteins also impairs SG disassembly [149, 153]. SGs move along microtubules and undergo fusion and fission events, and SG mobility is strongly decreased when microtubules are depolymerized. However, once formed, SGs do not require microtubules to persist [150, 153].

In contrast, a role for actin in SG dynamics is unclear. While one study of actin disruption using cytochalasin B resulted in smaller SGs [149], another using latrunculin B leads to a slight increase in SG size [148], while other latrunculin B studies have reported no effect [144, 153]. Disruption of actin also has no effect on SG disassembly, nor do SGs show localization with actin filaments [153].

7.3.8 Additional Mechanisms of SG Disassembly

SG disassembly can be enacted either by disrupting interactions between proteins and mRNAs that sustain SG formation, or by degrading SG components. The simplest means of SG disassembly is the return of repressed mRNAs within SGs to translation. Indeed, bulk translation levels recover as SGs disassemble following stress alleviation [103, 131, 133], a process often aided by chaperones [133, 134]. However, translational recovery without complete SG disassembly is observed following microtubule disruption [149].

SG components can also be cleared by an autophagic mechanism termed “Granulophagy” [32]. This occurs in yeast and human cell lines in response to certain stresses, or following perturbation (in yeast) of cytoplasmic mRNA decay [32]. Granulophagy involves a diverse set of effectors. In yeast, the Hsp40 Sis1, together with Hsp70, facilitates autophagic targeting of SG components. In contrast, another Hsp40, Ydj1, facilitates SG disassembly with Hsp70 by promoting return of mRNAs to translation [134]. In human cells, the selective autophagy protein p62/SQSTM1 localizes within SGs, and p62 knockdown slows SG clearance [35]. Additionally, Syk kinase facilitates autophagic clearance of SGs in a manner dependent on its catalytic activity and its ability to localize within SGs [154]. Inhibiting autophagy genetically in both yeast, human cells [32], and neurons [6] leads to constitutive SG accumulation in the absence of stress, suggesting a basal level of autophagy helps prevent aberrant SG persistence.

In principle, SGs could also be cleared by the action of the proteasome and mRNA decay enzymes. Although SGs are induced by proteasomal inhibition [133], and G3BP1 itself harbors an endonuclease domain [114], no evidence for a role of either process in SG disassembly is known.

7.4 Hypothesized Role for SGs in Driving TDP-43/FUS/SOD1 Aggregation

A popular model is that SGs may facilitate formation of cytoplasmic aggregates in ALS. Specifically, mutations or cellular conditions that increase SG persistence, either due to excessive SG assembly or impaired SG clearance, increase the chance of SG-localized TDP-43, FUS, or SOD1 stochastically undergoing conversion to a toxic aggregate [78, 81–84, 155]. Following this, other SG components could remain associated with these aggregates, or SGs may dissolve and thus serve as transient nucleators of TDP-43/FUS/SOD1 aggregation. A third possibility is that TDP-43/FUS/SOD1 aggregation occurs independently of SGs entirely (Fig. 7.1). We examine these possibilities below.

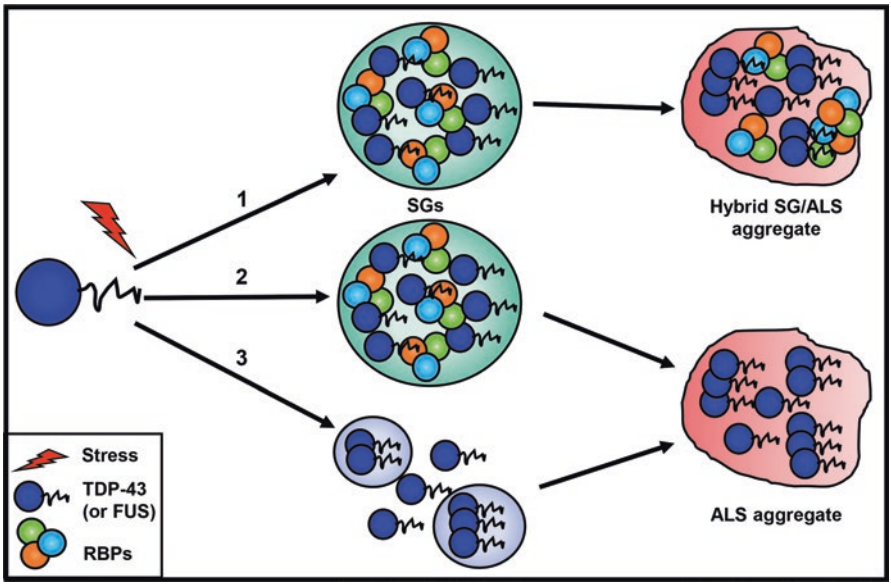

Fig. 7.1 Models for aggregate formation in ALS. (1) After stress induction, SGs form, and TDP 43 (or FUS/SOD1) is recruited. Over time, protein aggregation reduces SG dynamics, leading to formation of persistent SG/ALS aggregate hybrid state, which may sequester numerous mRNP components. (2) As in model 1, except that with time, SG components disassemble, leaving ALS aggregates behind. Thus, SGs nucleate aggregation, but are dispensable for aggregate persistence. (3) After stress induction, ALS protein aggregates begin to form, completely independent of SGs. Over time, ALS aggregates increase and persist

7.5 Localization of TDP-43, FUS, and SOD1 in SGs

If SGs facilitate TDP-43/FUS/SOD1 aggregation, these proteins should localize to SGs, at least transiently, if not throughout the disease. Indeed TDP-43, FUS, and SOD1 can all colocalize with SGs to varying degrees.

7.5.1 TDP-43 Localization to SGs

TDP-43 localizes to SGs in many cellular contexts, and under numerous stress conditions. These include oxidative (arsenite), osmotic (sorbitol), ER (thapsigargin), heat, serum deprivation, proteasome inhibition (MG132), and mitochondrial stress (paraquat) [3–5, 156–158]. Some caveats of these studies, common to many works in the field, include the unclear physiological relevance of these stresses in ALS-afflicted neurons, and that some (not all—[4, 156, 158]) of these studies utilize over-expression of TDP-43 and/or SG proteins, which may drive artifactual SG assembly or TDP-43 aggregation.

However, TDP-43 colocalization in SGs is not always observed. In other studies, arsenite-stressed HEK293 cells [4] and neuroblastoma cells subject to ER (Thapsigargin) or oxidative (SIN-1, Arginine) stress show no TDP-43 localization despite SG induction [159, 160]. SGs lacking TDP-43 are also observed following oxidative (Hydrogen peroxide) and proteasome inhibition (Epoxomicin) stress in multiple cell lines [159].

In several studies, TDP-43 aggregates in ALS and FTD patient spinal cord and brain tissue colocalize with SG markers including TIA1, eIF3, and PABP1 [3, 82, 116, 161]. In other similar studies, such colocalization is not seen [3]. The reason for this discrepancy is unclear, but could reflect differences in the cause or progression of ALS between different patient samples.

7.5.2 FUS and SOD1 Localization to SGs

Endogenous FUS localization to SGs has been reported in HeLa and HEK293 cells following arsenite or sorbitol stress [162, 163]. In contrast, most studies using various cell lines, including rat primary neurons and spinal cord neural cells, show no re-localization of FUS from the nucleus under numerous stresses [116, 163–165]. However, ALS-mutant alleles of FUS often localize to SGs under many stress conditions [116, 162]. Furthermore, spinal cord and hippocampal tissue from familial FUS-mutant ALS and FTD patients with WT FUS exhibits colocalization with the SG marker PABP-1 [166].

SOD1 localization in SGs is poorly studied. However, ALS-mutant SOD1 colocalizes with G3BP1-positive SGs following heat shock in HeLa cells while WT SOD1 does not [61]. Additionally, ALS-mutant SOD1 aggregates in patient-derived

fibroblasts and in motor neurons of *SOD1* transgenic mice colocalized with G3BP1-positive SGs, whereas WT SOD1 did not [62].

In conclusion, there are many instances of colocalization of TDP-43 in cell line and ALS patient tissue, though exceptions are seen. Localization of FUS, and particularly SOD1 to SGs, is less studied, but can occur, particularly with ALS-mutant alleles.

7.6 Is SG Assembly Required for TDP-43, FUS, or SOD1 Aggregation?

The simplest test of whether SG assembly is required for TDP-43, FUS, or SOD1 aggregation would be to impair SG assembly, and then examine if aggregation still occurs under a condition of interest. Surprisingly, this question remains poorly addressed, perhaps because completely blocking SG assembly can be challenging due to redundant assembly mechanisms. However, in arsenite-stressed U2OS cells, Ataxin-2 knockdown, which slows SG assembly rate, is accompanied by fewer cells exhibiting SG colocalizing TDP-43 aggregates over the same time period, compared to WT cells [12]. This suggests that SG assembly may affect TDP-43 aggregation rates.

TDP-43 aggregates can sometimes persist following dissolution of SGs. Specifically, treatment of HeLa cells with Paraquat for 24 h leads to formation of SGs. However, 6 h after stress removal, SGs are mostly gone, whereas endogenous TDP-43 aggregates remain relatively unchanged [158]. Similarly, disassembly of paraquat-induced SGs by a 6 h cycloheximide treatment leaves a significant fraction of endogenous TDP-43 aggregates unaffected [158]. This partially contradicts another study in which TDP-43-GFP aggregates that form due to over-expression in neuroblastoma cells are fully cleared by 1 h of cycloheximide treatment. However expression of a TDP-43-GFP 25 kDa C-terminal fragment, which mimics TDP-43 fragments in ALS aggregates, also generates cycloheximide resistant TDP-43 aggregates [3]. Similarly, we have observed that TDP-43-GFP aggregates in yeast, while initially mostly SG localized, are cycloheximide resistant, unlike yeast SGs [167].

In summary, due to a lack of data concerning the effects of blocking SG assembly on TDP-43, FUS, and SOD1 aggregation, we cannot decisively say whether SG assembly is required for TDP-43/FUS/SOD1 aggregation. The failure to detect colocalization of SG markers with TDP-43 inclusions in some patient tissues could indicate SG-independent assembly, but does not rule out SGs as transient nucleation sites for TDP-43 aggregation.

7.7 Do ALS Mutations Always Perturb SG Formation and Dynamics?

Many ALS-linked mutations occur in SG localizing proteins and/or proteins that affect SG assembly, disassembly, or internal dynamics (i.e., rate at which SG components enter and exit SGs). For example, a commonly held view is that ALS-linked

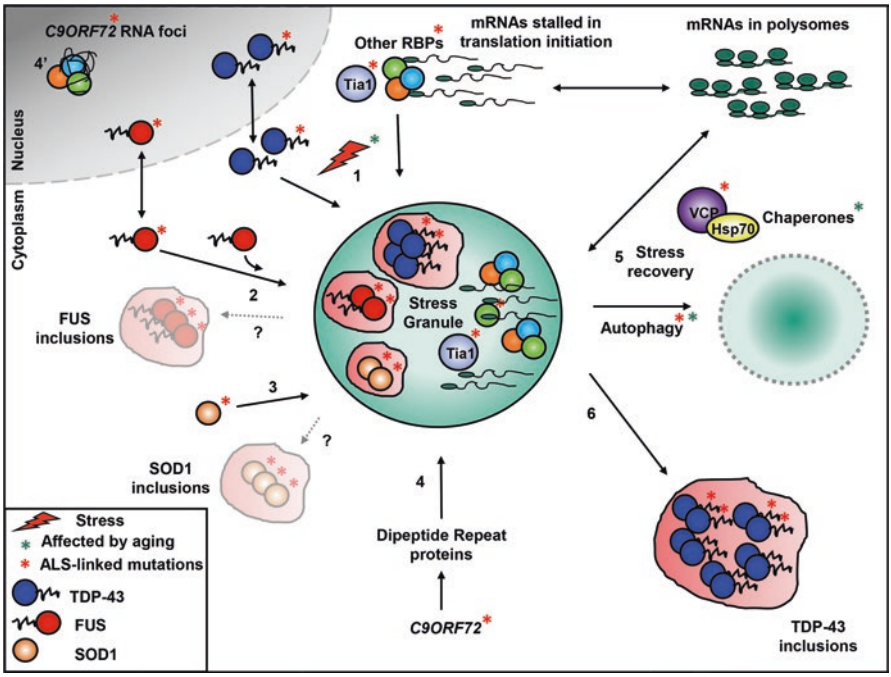

Fig. 7.2 Potential effects of ALS-linked mutations on SG-mediated aggregate formation. (1) Stress induces formation of SGs composed of mRNAs stalled in translation initiation and RBPs, many of which are mutated in ALS. Stress also leads to cytoplasmic localization of WT and/or aggregate-prone TDP-43 mutants and their recruitment to SGs. (2) FUS mutations cause cytoplasmic mis-localization of FUS which, upon stress, is also recruited to SGs. The fate of FUS aggregates, whether they remain within or become independent of SGs, is unclear. (3) SOD1 mutants are recruited to SGs, the fate of which is yet unknown. (4) *C9ORF72* repeat expansions generate dipeptide repeat proteins that induce SG formation and favor their transition to a less dynamic state. (4′) *C9ORF72* repeat expansions also lead to nuclear RNA foci capable of sequestering SG RBPs. (5) Stress recovery leads to SG disassembly via return of mRNAs into translation, or SG clearance via autophagy. (6) TDP-43 aggregates may persist following SG clearance. Alternatively, failure to clear SGs might lead to persistent SG positive TDP-43 inclusions. Aging leads to an increase in oxidative stress and decrease in protein quality control. Additional genetic or stress insults may ultimately drive pathological SG persistence and onset of ALS

mutant RBPs are more aggregation prone than their WT counterparts, and that this facilitates either faster SG assembly, slower SG disassembly, and/or reduced SG internal dynamics [78, 84]. For other ALS-associated genes, connections to SGs are less apparent. The effect of multiple ALS-associated mutations upon SGs is discussed below (also see Fig. 7.2), with select mutant-specific summaries presented in Table 7.2.

Table 7.2 Details of ALS mutants for which effects on SGs have been analyzed

Gene	Type of expression	Allele	Effect on SG localization/ dynamics	References
TDP-43			WT and mutants localize to SGs	
	WT		Facilitates SG assembly	MacDonald [5] Aulas [168]
	OE	G294A (IDR)	Mutation does not increase aggregation potential [169], ↑ percent cells with SG+ TDP-43 inclusions [3]	Johnson [169] Yesucevitz [3]
	OE	A315T (IDR)	↑ percent cells with SG+ TDP-43 inclusions	Yesucevitz [3]
	OE	Q331K (IDR)	Mutation increases aggregation potential [169], ↑ percent cells with SG+ TDP-43 inclusions [3]	Johnson [169] Yesucevitz [3]
	OE	Q343R (IDR)	↑ percent cells with SG+ TDP-43 inclusions	Yesucevitz [3]
	OE	G294A (IDR)	↑ TDP-43 (SG?) granule size	Dewey [4]
	OE	A315T (IDR)	↑ TDP-43 (SG?) granule size	Dewey [4]
	OE	G348C (IDR)	↑ TDP-43 (SG?) granule size and faster assembly	Dewey [4]
	OE	N390S (IDR)	↑ TDP-43 (SG?) granule size	Dewey [4]
	Endo	R361S (IDR)	↓ percent cells with SGs	McDonald [5]
	Endo	D169G (outside IDR)	No effect	McDonald [5]
FUS			Mutants localize to SGs better	
	WT		No effect on SG assembly	Aulas [168]
	OE	R495X (truncation of NLS)	Delays SG assembly, once formed ↑ number and size, ↑ SG disassembly	Baron [7]
	OE	H517Q (mild mis-localization)	No effect	Baron [7]
	OE	R521C (in NLS)	↑ percent cells with FUS+ SGs, ↓ release of FUS from SGs on recovery	Ryu [6]
TIA1			WT and mutants localize to SGs	
	WT		Key SG assembly protein	Gilks [115]
	OE	P362L (IDR)	↓ SG disassembly, TDP-43 recruited to mutant TIA1 SGs	Mackenzie [8]

(continued)

Table 7.2 (continued)

Gene	Type of expression	Allele	Effect on SG localization/ dynamics	References
	OE	A381T (IDR)	↓ SG disassembly, TDP-43 recruited to mutant TIA1 SGs	Mackenzie [8]
	OE	E384K (IDR)	↓ SG disassembly, TDP-43 recruited to mutant TIA1 SGs	Mackenzie [8]
hnRNPA2			WT and mutant localize to SGs	
	WT		Unknown effect on SG formation	
	OE	D290V (IDR)	↑ recruitment of mutant protein to SGs	Kim [10]
ANG			WT or mutant not known to localize to SGs	
	WT		Facilitates SG assembly	Emara [109]
	OE	K40I	Slight ↓ in SG formation	Thiyagarajan [15]
	OE	C39W	No effect	Thiyagarajan [15]
MATR3			WT does not localize to SGs, mutants weakly localize to SGs	
	WT		Unknown effect on SG assembly	
	OE	F115C	Few cells had MATR3 localizing to SGs	Gallego-Iradi [19]
SS18L1			WT and mutants localize to SGs	
	WT		Unknown effect on SG assembly	
	OE	Q388X	No effect on SG localization	Kukharsky [23]
	OE	I123M	No effect on SG localization	Kukharsky [23]
	OE	△222–224	No effect on SG localization	Kukharsky [23]
	OE	A264T	No effect on SG localization	Kukharsky [23]
GLE1			WT and mutants localize to SGs	
	WT		Facilitates SG assembly	Aditi [25]

(continued)

Table 7.2 (continued)

Gene	Type of expression	Allele	Effect on SG localization/ dynamics	References
	Rescue	Mutation causes novel C-term protein—hGle1-IVS14-2A > C	No effect on SG assembly	Aditi [26]
	OE	Mutation causes novel C-term protein—hGle1-IVS14-2A > C	↑ SG size like OE of WT, also formed some independent Gle1 aggregates	Aditi [26]
C9ORF72			WT and mutants localize to SGs	
	WT		Facilitates SG assembly	Maharjan [28]
	OE	GGGGCCx30 added at 5′ end of C9ORF72	Induces constitutive SGs	Maharjan [28]
	OE	GGGGCCx60 added 5′ end of C9ORF72	Induces constitutive SGs	Maharjan [28]
	OE	PR100	DPR localized to SGs, induces constitutive SGs, ↓ SG internal dynamics	Boeynaems [30]
	OE	PA100	No effect	Boeynaems [30]
	OE	GR50	DPR localized to SGs, induces constitutive SGs, ↓ SG internal dynamics	Lee [29]
	OE	PR50	DPR did not localize to SGs, induces constitutive SGs, ↓ SG internal dynamics	Lee [29]
VCP			WT and mutants localize to SGs	
	WT		Facilitates SG clearance	Buchan [32]
	OE	A232E	Induces constitutive TDP-43+ SGs	Buchan [32]
	OE	R155H	Induces constitutive TDP-43+ SGs	Buchan [32]
PFN1			WT and mutants localize to SGs to varying degrees	
	WT		Not essential to SG assembly/disassembly but OE induces constitutive SGs and ↓ SG disassembly	Figley [47]
	OE	C71G	Induces constitutive SGs, also forms separate Pfn1 aggregates; On stress, ↓ Pfn1 recruitment to SGs	Figley [47]

(continued)

Table 7.2 (continued)

Gene	Type of expression	Allele	Effect on SG localization/ dynamics	References
	OE	M114T	Induces constitutive SGs, also forms separate Pfn1 aggregates; On stress, ↓ Pfn1 recruitment to SGs	Figley [47]
	OE	T109M	Induces constitutive SGs	Figley [47]
	OE	G118V	Various stresses, ↓ in Pfn1 recruitment to SGs	Figley [47]
	OE	E117G	↓ SG disassembly	Figley [47]
SOD1			Mutants localize to SGs, WT does not	
	WT		Unknown effect on SG assembly	
	OE	A4V	Delay in SG assembly	Gal [62]
	OE	A4V	Recruitment of mutant SOD1 ↓ SG internal dynamics (FRAP FUS and G3BP1)	Mateju [61]
CHCHD10			WT and mutants not localize to SGs	
	WT		Unknown effect on SG assembly	
	OE	R15L	Induces constitutive SGs, colocalization of TDP-43 with SGs	Woo [65]
	OE	S59L	Induces constitutive SGs, colocalization of TDP-43 with SGs	Woo [65]

Acronyms: SGs = stress granules, OE = overexpression, Endo = endogenous expression, IDR = Intrinsically disordered domain

7.7.1 ALS Mutations in RNA-Binding Proteins

TDP-43: Several ALS-associated TDP-43 mutations, most of which map to the C-terminal IDR, affect SG formation. For example, over-expression of mutant TDP-43 in neuroblastoma cells drives more numerous SG-localizing TDP-43 aggregates than in cells expressing WT TDP-43 [3]. In HEK293 cells, over-expression of mutant TDP-43 induces significantly larger SGs following sorbitol stress than cells expressing WT TDP-43; mutant TDP-43 also enters SGs faster than WT TDP-43 [4]. A common finding in these studies therefore is that TDP-43 ALS mutant alleles facilitate SG assembly better than WT TDP-43. This correlates with an increased aggregation propensity for many TDP-43 mutants in vitro [3, 116, 169, 170].

TDP-43 mutant alleles do not always facilitate SG assembly. In a study of endogenous ALS-linked TDP-43 mutations in ALS patient-derived lymphoblasts [5], arsenite-induced SG assembly was disrupted by a TDP-43 IDR mutant, but not by a

TDP-43 RNA-recognition motif mutant. SG assembly defects with the IDR mutant, or following TDP-43 knockdown, were attributed to a role in maintaining high G3BP1 mRNA levels [168]; similar results have been seen with another TDP-43 mutant [171].

FUS: ALS-associated mutations are dispersed throughout the FUS protein, though most studies focus on mutants that inactivate/truncate a C-terminal nuclear localization sequence (NLS). This causes strong cytoplasmic re-localization of FUS. In one study, a FUS NLS mutant delayed SG assembly in HEK293 cells, but once formed, SGs were larger and more numerous than in WT FUS-expressing cells [7]. Another study of mutant FUS in rodent cortical neurons did not observe this, but did observe stronger localization of mutant FUS in SGs versus WT FUS [6]. Mutant FUS localization in SGs correlates with cytoplasmic mis-localization [164, 165]. FUS mutants that exhibit only mild cytoplasmic mis-localization do not localize to or alter SGs [7]. Additionally, knockdown of endogenous WT FUS does not impair SG assembly [168].

Opposing findings regarding the effect of mutant FUS on SG disassembly and clearance have been observed. Expression of a truncated NLS FUS mutant in HEK293 cells caused increased SG dynamics (TIA1 and G3BP1 mobility increased), and more rapid SGs disassembly after arsenite stress compared to WT FUS-expressing cells [7]. In another study, SGs harboring NLS mutant FUS were impaired in clearance following arsenite stress, and were preferentially targeted by autophagy [6].

TIA1: An ALS-associated mutation was recently identified in the IDR domain of TIA1. This mutation drives TIA1 phase separation in vitro, slows SG disassembly following heat shock in HeLa cells, and decreases TDP-43 mobility in SGs [8]. TDP-43 insoluble aggregates also accumulated in the TIA1 mutant context, consistent with the idea that SG disassembly defects facilitate accumulation of TDP-43 aggregates [8].

hnRNPA2B1 and hnRNPA1: hnRNPA2B1 (A2B1) and hnRNPA1 (A1) localize in SGs [120, 172], and are mutated in a subset of ALS cases. Like TDP-43, these mutations occur in their IDR domains, which drives greater and faster assembly of A2B1 and A1 into fibrils in vitro compared to WT alleles [10]. Notably, mutant alleles can also "seed" fibrilization of their WT counterparts [10]. IDR mutations also induce A2-positive SGs in unstressed conditions and accelerate A2 incorporation into SGs. TDP-43 inclusions in Multi-System Proteinopathy, another degenerative disease, also colocalize with A2B1 and A1 [10]. The effect of WT or mutant A2B1 and A1 on SG disassembly remains unclear.

Ataxin-2: Polyglutamine expansions in Ataxin-2 increase ALS risk [87]. Consequences of polyglutamine-expanded Ataxin-2 include increased TDP-43 binding in an RNA-dependent manner, increased Ataxin-2 stability, and greater cytoplasmic mislocalization of TDP-43 in ALS patient-derived lymphoblasts following heat stress [87]. Additionally, knockdown experiments indicated that WT Ataxin-2 stimulates normal rates of arsenite-induced SG assembly and promotes recruitment of TDP-43 into SGs [12, 173]. Ataxin-2 knockdown also extends lifes-

pan and reduces pathology in a TDP-43 transgenic mouse model [12]. Collectively, these data hint that mutant Ataxin-2 may promote TDP-43 aggregation, possibly within an SG context via altered SG dynamics.

In summary, analysis of the above-mentioned proteins generally supports the hypothesis that ALS-associated mutations (with some exceptions) increase SG persistence, either due to accelerated SG assembly or impaired disassembly. However, the effect on SGs of many RBPs mutated in ALS, and of most specific RBP ALS mutants, remain unknown (Tables 7.1 and 7.2). Analyses of such mutants at endogenous expression levels would help address if existing models are accurate.

7.7.2 ALS Mutations in Protein Quality Control Factors

Several genes implicated in protein quality control, particularly autophagy, are mutated in ALS (Table 7.1). SG clearance can occur via autophagy [6, 32, 35, 154], thus SG accumulation due to autophagic defects may facilitate aggregation of SG-localizing proteins such as TDP-43 [81]. However, only two ALS-associated genes (VCP, p62) implicated in autophagy have been studied for effects on SGs. It remains possible that protein quality control defects could affect TDP-43 turnover independently of SGs. Specific protein quality control genes and their known/possible effects on SGs are now discussed.

VCP: VCP localizes in SGs under various stresses, facilitates efficient SG clearance following heat-shock, participates in Granulophagy, and over-expression of ALS-associated VCP mutants induces constitutive SGs that harbor TDP-43 [32]. Interestingly, VCP is also implicated in SG assembly, with depletion leading to formation of SGs harboring misfolded proteins and 60S ribosomal subunits [174]. ALS-associated VCP mutants may be defective in SG clearance due to a failure to undergo efficient N-terminal SUMOylation [175]. This facilitates SG localization, as assessed by biochemical fractionation, and is required for formation of functional VCP hexamers [175].

Autophagy Factors: Several autophagy "receptors" that can selectively bind substrates and target them for autophagic turnover have been identified as ALS-linked genes [176]. These include p62, Optineurin and Ubiquilin-2, which also facilitates proteasomal turnover. To our knowledge, p62 is the only autophagy receptor that localizes in SGs and facilitates SG clearance [35]. Several other genes that promote autophagic functions, including *TBK1*, *CHMP2B*, *VAPB*, and *FIG4*, are mutated in ALS (Table 7.1), but effects on SG clearance are unknown.

Chaperones and Proteasomal turnover: No ALS-associated mutations in chaperones that localize within or directly act upon SGs have been identified. However, an E102Q mutation in the ER chaperone SigR1, which occurs in juvenile ALS [177], leads to impairment of autophagy, endocytosis and formation of SGs, within which SigR1 localizes [45]. Mechanistic understanding of these effects remains unclear. In addition, SOD1 aggregates can sequester Hsc70 chaperones (constitutive Hsp70s) [178], which impairs endocytosis, but could in principle affect SG disassembly.

Proteasomal inhibition induces SG assembly [133], and has been implicated in TDP-43 turnover [179, 180]. However, besides Ubiquilin-2, we are unaware of any ALS-associated mutants that perturb proteasomal activity and that affect SGs.

In summary, the evidence that defects in protein quality control lead to ALS protein aggregates due to accumulation of SGs remains relatively scant, and warrants further investigation.

7.7.3 ALS Mutations in Cytoskeletal Associated Proteins

Several cytoskeletal-related proteins are mutated in ALS. However, only Profilin, an actin-binding protein that regulates actin filament dynamics, has known effects on SGs. Specifically, Profilin ALS mutants [46] show poor recruitment to SGs, and lead to slower SG disassembly relative to WT Profilin-expressing cells [47]. As SG formation is generally independent of the actin cytoskeleton, mutant Profilin may affect other biological processes. Indeed Profilin genetically interacts with Dynein, and physically interacts with tubulin [47, 181], thus Profilin's mutant effects could be microtubule mediated. Alternatively, mutant Profilin protein also forms SG-distinct aggregates that persist through stress treatment and recovery [47] which may seed formation of TDP-43 aggregates [182].

Mutations in *TUBA4A* (alpha-tubulin subunit) are found in a rare fraction of ALS patients, which destabilizes microtubules [54]. In principle, this could affect SG assembly or disassembly. Other cytoskeletal ALS-linked genes include *DCTN1* (Dynactin subunit 1) [48], *NEFH* [50], and *SPG11* [183] (Table 7.1), though only dynactin subunit 1 has been detected in SGs [120].

In summary, despite a clear role for microtubules in SG assembly and disassembly, the effects of most cytoskeletal-associated ALS-mutant genes on SGs remain poorly characterized.

7.7.4 ALS-Linked C9ORF72 Repeat Expansions

C9ORF72 is the most commonly mutated gene in ALS (Table 7.1). An intronic hexanucleotide repeat expansion of G_4C_2 repeats occurs between exons 1a and 1b (WT range 2–23 repeats), which reduces transcription, protein levels and may cause loss of function [184–186]. Several WT functions and mechanisms of ALS-mutant associated toxicity have been proposed [1, 187–191], some of which affect SGs.

Two *C9ORF72* isoforms are expressed in human cells. The longer possesses a differentially expressed in normal and neoplastic cells (DENN) domain, a hallmark of guanine nucleotide exchange factors for Rab GTPases [192, 193]. Indeed C9ORF72 physically and functionally interacts with several Rab proteins, leading to proposed roles in endocytosis and autophagy [189, 191, 194–196]. Impairment of autophagy, or endocytosis [167] could contribute to impaired TDP-43 turnover and/

or delayed SG clearance. C9ORF72 also localizes in SGs and P-bodies, and stimulates G3BP1 and TIA1 protein levels based on knockdown data [28]. Over-expression of repeat-expanded *C9ORF72* alleles, unlike WT, also induces SG assembly in neuroblastoma and cortical neurons [28]. This may reflect gain-of-function mechanisms discussed below.

G_4C_2 repeats in *C9ORF72* are subject to noncanonical translation, termed repeat-associated non-ATG (RAN) translation. This generates dipeptide repeats (DPRs) from all reading frames of both sense and antisense transcripts [197, 198]. Arginine-containing DPR species are particularly toxic [199, 200], and interact with several SG-localizing proteins containing IDRs [29]. Over-expression of GFP-tagged DPRs, particularly Glycine-Arginine (GR) and Proline-Arginine (PR) dipeptides, causes translation repression and induces spontaneous SGs dependent on eIF2a phosphorylation and G3BP1 [29, 30]. Expression of GR (detectable in SGs) and PR dipeptides also reduces SG internal dynamics (G3BP1 mobility). GR and PR DPRs also enhance hnRNPA1 and TIA1 phase separation in vitro, and reduce the internal dynamics of such phase-separated bodies [29].

Finally, nuclear *C9ORF72* RNA foci accumulate in the brain and spinal cord of *C9ORF72* ALS patients [27, 198, 201]. Paralleling other "toxic-RNA" diseases such as Myotonic Dystrophy, repeat-expanded *C9ORF72* RNA interacts with and may sequester several RNA-binding proteins that could affect SGs. Partially overlapping C9ORF72 interactomes have been described, with other proposed consequences of protein sequestration including defects in nuclear RNA processing, splicing, and nucleocytoplasmic transport (summarized in [202]). Transfection of G_4C_2 RNA into HeLa cells also induces SG assembly and translational repression [203]; whether such effects are direct or stem from RAN translation is unclear.

In summary, *C9ORF72* G_4C_2-derived DPRs may directly modulate SG assembly and dynamics. Roles for *C9ORF72* in autophagy, sequestration, or translation regulation of SG assembly proteins may also be important.

7.7.5 ALS-Linked SOD1 Mutants

SOD1 ALS mutations likely cause a toxic gain of function, as many SOD1 mutations have little or no effect on SOD1 antioxidant activity [204, 205]. In *SOD1* mutant transgenic mice, SOD1 aggregates in spinal cord motor neurons colocalize with G3BP1, as does mutant SOD1 in ALS patient-derived fibroblasts [62]. Additionally, mutant SOD1, but not WT, interacts with G3BP1 in an RNA-independent manner and delays SG assembly following hyperosomotic and arsenite stress when over-expressed in neuroblastoma cells [62]. Additionally, ALS-mutant SOD1 protein is preferentially recruited to SGs over WT SOD1 following heat-stress. This reduces SG internal dynamics (G3BP1 mobility) [61]. Thus, mutant forms of SOD1 can localize in SGs, and modulate SG dynamics, particularly via interactions with G3BP1.

The evidence for ALS mutations in RBPs and C9ORF72 in driving excessive SG assembly, and facilitating SG conversion to a less dynamic state, is reasonably extensive and compelling, although studies are not in uniform agreement (Tables 7.1 and 7.2). Evidence for how ALS mutations in cytoskeletal and protein quality control proteins affect SGs is at a more nascent stage, and the issue of how/if ALS-associated mutations affect SG disassembly is currently understudied.

7.8 Effect of Aging on ALS and SGs

About 90% of sporadic ALS cases have no clear genetic etiology [206]. Additionally, ALS onset typically occurs later in life, between 40 and 70 years of age (average age of onset 55). Interestingly, TDP-43 aggregation within certain brain regions increases during normal aging, albeit the severity and tissue distribution of such aggregates is less widespread than in ALS patients [207–209]. Thus, outstanding questions in the field include whether unidentified mutations, or combinations of mutations are driving disease, and to what extent environmental or age-associated factors affect disease onset [79]. Regarding the later, age-associated defects in protein clearance and accumulation of cellular stress are processes which could increase SG persistence.

7.8.1 Proteostasis in Neurons

Autophagy plays a key role in neuronal cell homeostasis. Supporting this, CNS-specific knockout of ATG5 and ATG7 in mice causes early-onset neurodegeneration, characterized by accumulation of ubiquitinated protein aggregates throughout the CNS, behavioral and motor defects, and premature death [210, 211]. Autophagy activity decreases with age in numerous tissues [212], including the brain as suggested by reduced mRNA and protein expression of core autophagy genes such as BECLIN-1, ATG5, and ATG7 [213–215]. Additionally, inducing autophagy facilitates aggregate clearance and improves cell/organism survival in many models of various neurodegenerative diseases, including ALS [216–218] (and see the therapeutics section 7.10.2).

Autophagic clearance of SGs can occur in neurons, with SGs harboring ALS-mutant FUS being preferentially associated with autophagosomes, versus SGs containing WT FUS [6]. Furthermore, SGs harboring ALS-mutant FUS particularly accumulated in ATG5 knockout mouse embryonic fibroblasts, and ATG7-knockdown neurons [6]. This suggests that SGs harboring ALS-mutant proteins may be particularly dependent on autophagic clearance.

Chaperone function is also thought to decline with age. Supporting this, the ability to increase chaperone levels in response to stress, particularly Hsp70 chaperones, decreases with age in multiple tissues and model systems [219]. Additionally,

multiple chaperone proteins, particularly Hsp40s and ATP-dependent chaperones, decrease in aging human brains, and/or in brains from various neurodegenerative diseases [220]. Interestingly, motor neurons show a paucity of chaperone upregulation in response to stress, or following accumulation of ALS disease aggregates. Specifically, cultured rodent motor neurons show no induction of Hsp70 following heatshock stress, unlike glial cells, and also no upregulation of Hsp70 following expression of ALS-mutant SOD1 protein [221, 222]. Hsp70 and Hsp27 levels are also not elevated in the spinal cord tissue of familial or sporadic ALS patients versus healthy controls [222].

Taken together, a lack of a robust proteostatic mechanism in aged motor-neurons may increase the likelihood of SG persistence and TDP-43/FUS/SOD1 protein aggregation.

7.8.2 Oxidative Stress

The brain exhibits extremely high energy demands compared to other tissues, and consumes 20% of total oxygen within the human body despite accounting for only 2% of body weight [223]. This, coupled with a near complete reliance on mitochondrial-driven oxidative metabolism for energy, means that neurons, relative to other cells, are prone to accumulate reactive oxygen species (ROS) and thus oxidative stress. ROS accumulation generally increases in aging neural tissue, exacerbated by age-associated declines in mitochondrial function [224]. Oxidative stress in sporadic and familial ALS spinal cord and motor cortex tissue is typically even further elevated relative to aged healthy tissue controls [225–228]. Though currently unaddressed, this could induce SG assembly. Numerous other stresses that induce SGs are likely encountered by motor-neurons [229], though whether the levels of such stress would abnormally induce SGs is unclear.

Surprisingly, the effects of aging on SG formation are poorly studied. However, in *C. elegans*, accumulation of insoluble aggregates of SG proteins TIA1 and PABP in non-dynamic SG-like foci occurs in aged worms [230]. This did not occur in identically aged Insulin-like growth factor-1 mutant worms (*daf-2*), which exhibit a two- to threefold increase in lifespan, in part due to reduced protein metabolism and upregulated stress responsive genes [231, 232]. Additional study of SG formation in other aging models therefore seems highly warranted.

7.9 Why Does ALS Affect Motor-Neurons?

Why motor neurons are selectively subject to degeneration in ALS remains contentious, though several ideas based on motor neuron biology have been suggested [81]. First, as long lived post-mitotic cells, motor-neurons may acquire sub-lethal damage (e.g., misfolded protein aggregates, oxidized biomolecules) to a critical

level over time. Cell types in other tissues may avoid this by undergoing cell division and/or being turned over and more efficiently replaced by stem cell populations. Second, motor-neurons are highly enriched in mRNP granules. Besides possessing SGs and P-bodies, the polarized morphology of motor neurons makes them extremely dependent on localized transport and storage of mRNAs in NTGs, which can harbor TDP-43 and FUS including under non-stress conditions [233, 234]. Thus, like SGs, NTGs could theoretically concentrate and increase the likelihood of TDP-43 and FUS forming aggregates. This may be particularly true of NTGs in proximal axonal regions near the cell soma, as super-resolution microscopy techniques indicate a higher concentration of TDP-43 in a more static state compared to TDP-43 found in distal axon NTGs [235]. Notably, ALS-mutant FUS and TDP-43 proteins also inhibit axonal translation and transport of their bound mRNAs in various model systems, which may underlie defects in axon outgrowth [130, 234–236] and neuron degeneration. Finally, the circuit-like nature of nerve tissue, and the ability of ALS-associated protein aggregates to spread between cells either via secretion or following cell lysis [237–239], may lead to more rapid dysfunction than in other tissues [81].

7.10 Are SGs a Promising Therapeutic Target for ALS?

Existing FDA-approved treatments for ALS currently have a limited or an unknown benefit in mitigating ALS symptoms and extending lifespan, and do not offer a cure [240]. While strategies involving antisense oligonucleotides (ASOs) that reduce SOD1 and C9ORF72 expression [241–243], and stem-cell based therapies show promise, we refer readers to other reviews on those topics [243, 244]. Below, we examine whether preventing SG assembly, or facilitating SG clearance, offers a viable therapeutic strategy in ALS.

7.10.1 Limiting SG Assembly

Under physiological conditions, SG assembly typically relies on phosphorylation of the eukaryotic initiation factor 2 (eIF2) α subunit. This results in general translation repression and increased availability of non-translating mRNA to nucleate SG assembly [104]. eIF2α phosphorylation inhibits the ability of eIF2B to promote exchange of GDP for GTP on eIF2, which is necessary for eIF2 to deliver initiator tRNA to the small ribosomal subunit during translation initiation [245, 246]. Four kinases phosphorylate eIF2α in human cells, responding to various stresses including ER protein folding stress (PERK), nutrient stress (GCN2), viral infection (PKR), and heme deprivation (HRI).

In a TDP-43 fly model, TDP-43 expression correlates with increased eIF2α phosphorylation, suggesting that global translation repression is accompanied by

SG formation [247]. Given this, genetic and pharmacological inhibition (GSK2606414; [248]) of PERK was assessed in the TDP-43 fly model. This resulted in reduced eIF2α phosphorylation, presumably limiting translation repression and SG assembly, and led to significant improvements in motor-neuron function in flies. Pharmacological inhibition of PERK also reduced TDP-43 toxicity in primary rat cortical neurons [248]. Unfortunately, GSK2606414 is toxic to pancreatic tissue in mice models. However, ISRIB, which also inhibits eIF2α phosphorylation-dependent SG assembly by rendering eIF2B largely insensitive to eIF2α phosphorylation [249, 250], lacks this problem, and is neuroprotective in mouse prion models [251]. ISRIB may therefore hold promise as an anti-SG assembly, ALS therapeutic agent.

A caveat of the above is that perturbing a global translation repression mechanism may have unexpected off-target effects, and/or limit the ability of cells to effectively adjust their transcriptomes during stress. Targeting of proteins or mechanisms that physically drive SG assembly could be an alternative approach. As previously described, slowing SG assembly by Ataxin-2 knockdown limits TDP-43 aggregation, extended lifespan and improved motor performance in TDP-43 transgenic mice [12]. Targeting other genes that affect SG assembly may be of future interest.

7.10.2 Enhancing SG Clearance

Determining mechanisms by which autophagy and chaperones regulate SG clearance and disassembly could identify new ways to selectively clear SGs that would be of therapeutic benefit. Selectivity may be important, as inducing autophagy non-selectively has produced mixed results in ALS models, sometimes suppressing ALS phenotypes [252, 253], and at other times exacerbating them [254, 255]. The effects on SGs in these studies were not examined. However, one study in rodent cortical neurons has examined how non-selective autophagy induction affects SG clearance rates. Specifically, clearance of SGs harboring ALS-mutant FUS was enhanced by autophagy induction with rapamycin. Clearance of these SGs also coincided with reduced neurite fragmentation and neuronal cell death attributed to ALS-mutant FUS expression [6]. Though correlative, this suggests that accelerating clearance of SGs may offer a viable therapeutic strategy, and is consistent with the notion of SGs as contributors to ALS pathology.

7.11 Summary and Future Directions

In our view, the data summarized above suggests that SGs are probably involved in formation of ALS protein aggregates (especially TDP-43). However, certain views in the field have little supporting data, and key experiments remain to be addressed.

Evidence suggesting that SGs facilitate formation of ALS protein aggregates include: (1) To varying degrees, WT and ALS-mutant TDP-43, FUS and SOD1 localize in SGs under stress, and sometimes colocalize with SG components in patient tissue. (2) Many proteins mutated in ALS also localize in SGs (Table 7.1). (3) By various mechanisms, some ALS-mutants alter SG assembly, disassembly or internal dynamics such that SGs/SG proteins become more persistent and static in nature (Table 7.2). (4) Manipulations that prevent SG assembly (impairing eIF2α-based translation repression; Ataxin-2 knockdown) or facilitate SG clearance (Autophagy upregulation) correlate with improvements in ALS models. However, these manipulations likely affect other cellular processes besides SG formation.

Useful future directions include determining if ALS protein aggregate formation is affected when SG assembly is inhibited or enhanced using robust, targeted means. Also, numerous ALS-associated mutants remain poorly studied regarding their effects on SGs (Tables 7.1 and 7.2). The extent to which SG disassembly and clearance are perturbed in ALS, particularly in cases involving protein quality control mutants, requires further study. Such work would better inform the potential of targeting SG formation for therapeutic purposes.

How ALS aggregates affect disease progression remains unclear. Efforts to purify ALS aggregates to unbiasedly identify what proteins and mRNAs are present within would shed light on the validity of the "perturbed mRNA metabolism" model, and perhaps suggest novel disease mechanisms. In addition, whether SGs affect truncation and modification of TDP-43, which may affect TDP-43 aggregation and toxicity, remains unclear. Finally, greater study of how aging impacts SG formation, particularly in a motor neuron context, may lead to a better general understanding of SG biology, and provide clues as to the age and tissue-specific patterns of pathology seen in ALS.

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Chapter 8
Deregulation of RNA Metabolism in Microsatellite Expansion Diseases

Chaitali Misra, Feikai Lin, and Auinash Kalsotra

Abstract RNA metabolism impacts different steps of mRNA life cycle including splicing, polyadenylation, nucleo-cytoplasmic export, translation, and decay. Growing evidence indicates that defects in any of these steps lead to devastating diseases in humans. This chapter reviews the various RNA metabolic mechanisms that are disrupted in Myotonic Dystrophy—a trinucleotide repeat expansion disease—due to dysregulation of RNA-Binding Proteins. We also compare Myotonic Dystrophy to other microsatellite expansion disorders and describe how some of these mechanisms commonly exert direct versus indirect effects toward disease pathologies.

Keywords Microsatellite repeat expansions · Post-transcriptional gene regulation · RNA toxicity · Alternative splicing and polyadenylation · RNA-binding proteins

8.1 Introduction

Gene expression is a highly coordinated multistep process, which allows organisms to integrate intrinsic and environmental information to exert appropriate cellular functions. The expression of most genes can be regulated at distinct stages of RNA metabolism including synthesis or transcription, post-transcriptional processing or maturation, nucleo-cytoplasmic export, translation, as well as degradation at a rate that is often dictated by transcript- and cell-type-specific cues. Although transcription is a general point of control, many co- and post-transcriptional pre-mRNA processing events add substantial capacity to tune overall gene expression [1]. The

C. Misra · F. Lin
Department of Biochemistry, University of Illinois, Urbana-Champaign, IL, USA

A. Kalsotra (✉)
Department of Biochemistry, University of Illinois, Urbana-Champaign, IL, USA

Carl R. Woese Institute of Genomic Biology, University of Illinois, Urbana-Champaign, IL, USA
e-mail: kalsotra@illinois.edu

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typical pre-mRNA processing events comprise 5′ capping, splicing, and 3′ polyadenylation, which are directly linked to the nucleo-cytoplasmic export and eventual fate of mRNAs. RNA-Binding Proteins (RBPs) are essential in carrying out these processing events in both the nucleus and cytoplasm by interacting with RNA sequence or structural elements and forming distinct mRNA-protein (mRNP) complexes [2]. Disruption of RBP function(s), therefore, frequently results in deleterious RNA metabolism defects that in some cases become pathogenic [3, 4].

Neurodegenerative diseases are a heterogeneous group of neurological disorders characterized by progressive degeneration of structure and function of the central or peripheral nervous systems. Aberrant RNA metabolism is increasingly implicated in neurodegenerative diseases, a subset of which are caused by the expansion of short repetitive elements (microsatellites) within particular genes [5]. The causative repeat expansion mutation for this group of disorders is unstable because the repeat size changes through generations and even within an individual, as different tissues have cell populations with variable repeat length and in some cases the repeat length varies within the same tissue [6]. The severity of a repeat expansion disease is dependent on numerous variables, including the length of the repeat, its sequence context, and the native function of the protein-coding gene with which the repeat is associated. A typical pathogenic feature of these diseases is the accumulation of repeat-containing transcripts into aberrant RNA foci, which can sequester RBPs and prevent them from performing their normal functions [7–9]. Interestingly, once the repeat length cross a critical number, the repeat-containing RNAs can undergo phase separation—partitioning into granules due to multivalent base-pairing between repeat RNAs—or spontaneous gelation to form RNA foci, explaining why disease symptoms appear to be triggered after the expansions have reached a particular threshold number [10].

8.2 Toxicity of Coding and Noncoding Microsatellite Repeat Expansions

Over 25 human genes with tandem repeat expansions have been identified to date, and these disease-causing repeats can occur in the coding or noncoding regions [6] (Fig. 8.1 and Table 8.1). Majority of the microsatellites arise due to the expansion of trinucleotide repeats. However, expanded tetranucleotide, pentanucleotide, and hexanucleotide repeats are also detected. In the early 1990s, two microsatellites were discovered providing the first evidence that simple repeat expansions are linked to human disease. Fragile X Syndrome (FXS)—an X-linked disorder caused by CGG repeat expansions in the 5′ untranslated region (UTR) of the *FMR1* gene—is the most prevalent form of inherited cognitive impairment and mental retardation [11–16]. The repeat expansion in FXS causes loss of *FMR1* gene product FMRP, a polyribosome-associated RBP that binds ~4% of brain mRNAs and regulates their expression—either enhancing or suppressing translation through unknown mechanisms [17–20].

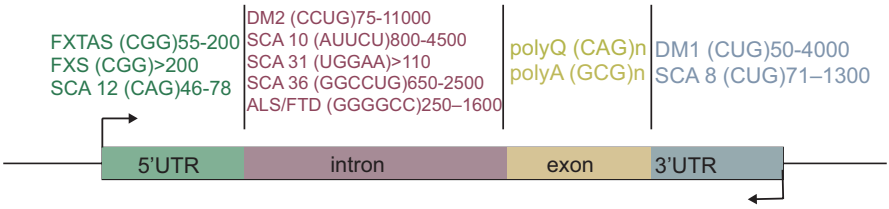

Fig. 8.1 Origin and expansion of microsatellite repeats in human disease. Schematic of the gene location for various disease-associated repeat expansions. Types of repeat expansions are indicated within the parentheses along with the range of expanded repeat numbers (UTR: untranslated region)

Table 8.1 Summary of the tissue-specific symptoms of the repeat expansion diseases with the disease-associated gene

Defected mRNA region	Disease	Defected gene	Tissue-specific clinical symptoms	
			Neuronal tissues	Other tissues
5′UTR	FXTAS	*FMR1* [158]	Ataxia [159], brain atrophy, white matter lesions [160, 161], cognitive decline, parkinsonism [160], peripheral neuropathy, autonomic dysfunction and short-term memory loss [162]	Premature ovarian failure, hypothyroidism in female [159], limb proximal muscle weakness [160]
	FXS	*FMR1* [14]	Autism [163], mental retardation, developmental delay and increased susceptibility to seizures [15]	Macroorchidism [15], cardiac murmur [164], hyperflexible joints, hernias, flat feet [165]
	SCA12	*PPP2R2B* [166]	Ataxia, cerebral and/or cerebellar atrophy [167], seizures [22]	Dysarthria, action tremors in upper limbs [167]
Intron	DM2	*ZNF9* [168]	Cognitive impairment [169], intellectual disability, sleepiness and fatigue [170], brain atrophy, white and grey matter abnormalities [171, 172]	Myotonia, muscle dysfunction, cardiac arrhythmia [40, 173], hypertrophy calf muscles [174]
	ALS	*C9ORF72* [28, 29]	Motor neuron degeneration, frontotemporal lobar dysfunction, dementia and cognitive impairment [175]	Progressive spasticity, muscle wasting, weakness and muscle atrophy [28]
	FTD	*C9ORF72* [28, 29]	Frontotemporal lobar dysfunction, motor neuron dysfunction [176], changes in personality, behavior, and language ability, dementia [175]	Fasciculation, muscle atrophy, weakness [177].

(continued)

Table 8.1 (continued)

Defected mRNA region	Disease	Defected gene	Tissue-specific clinical symptoms	
			Neuronal tissues	Other tissues
Coding region	Polyglutamine (PolyQ) diseases			
	SBMA	*AR* [21]	Lower motor neuron degeneration [178], androgen insensitivity [22]	Muscle weakness, gynecomastia and reduced fertility [22, 178]
	HD	*HTT* [179]	Cognitive decline and dementia [22], dystonia [180]	Chorea [22], movement disorder [181]
	DRPLA	*ATN1* [182–184]	White matter lesion, neural loss, ataxia, seizures, choreoathetosis, dementia [22, 185], myoclonus, epilepsy [184]	Chorea, incoordination [185]
	SCA 1, 2, 3, 6, 7, 17	*ATXN1* [186, 187], *ATXN2* [188–190], *ATXN3* [191], *CACNA1A* [192], *ATXN7* [193], *TBP* [194]	Ataxia, tremor, and dysarthria, parkinsonism (SCA3), retinal dystrophy (SCA7), seizures (SCA17) [22].	Slurred speech (SCA1); hyporeflexia (SCA2); cardiac dysfunction (SCA7) [22]
	Poly Alanine (Poly A) diseases			
	OPMD (OPMD)	*PABPN1* [195]	– (no data)	Eyelid ptosis and dysphagia [195], involuntary muscle weakness [196].
	XLMR	*ARX* [197]	Cognitive impairment [198], mental retardation [199], dysarthria [200]	Involuntary hand movements (MRXS), growth abnormality [200]
3′UTR	DM1	*DMPK* [37]	Neuropsychiatric disturbances, cognitive defeats, sleepiness and fatigue; brain atrophy [169], white and grey matter abnormalities [201], mood disorder, emotion problem and memory problem	Myotonia, muscle wasting, cardiac arrhythmias, insulin resistance, gastrointestinal dysfunctions, posterior iridescent cataracts [54]
	SCA8	*ATXN8* [202]	Cerebellar atrophy [203], progressive ataxia [204]	Limb ataxia, dysarthria, nystagmus, spasticity [22]

Abbreviations: *FXTAS* fragile X-associated tremor/ataxia syndrome, *FXS* fragile X Syndrome, *SCA12* spinocerebellar ataxia type 12, *DM2* myotonic dystrophy type 2, *ALS* amyotrophic lateral sclerosis, *FTD* frontotemporal degeneration, *SBMA* spinal and bulbar muscular atrophy, *HD* Huntington disease, *DRPLA* dentatorubral pallidoluysian atrophy, *SCA* spinocerebellar ataxias, *PolyA* polyalanine diseases, *OPMD* oculopharyngeal muscular dystrophy, *XLMR* syndromic and non-syndromic X-linked mental retardation, *DM1* myotonic dystrophy type 1

Spinal and bulbar muscular atrophy (SBMA)—the other microsatellite disease discovered along with FXS—arises due to a CAG repeat expansion in the coding region of the X chromosome-linked androgen receptor (*AR*) gene [21]. The discovery of SBMA was soon followed by the elucidation of a similar mutation as the basis for a group of disorders now known as the polyglutamine (polyQ) neurodegenerative diseases (Table 8.1). Along with SBMA, the polyQ diseases include Huntington disease (HD), dentatorubral-pallidoluysian atrophy, and six spinocerebellar ataxias (SCA) 1, 2, 3, 6, 7, and 17 [22]. As a group, these nine diseases are among the more common forms of inherited neurodegeneration. The translation of exons containing CAG repeats gives rise to elongated stretches of polyQs in mutant proteins, which aggregate into nuclear or cytoplasmic inclusions in the diseased brain [23–25]. Several observations indicate that the CAG repeat-containing RNAs, in the absence of coding for a protein, may also be a source of toxicity in polyQ diseases [26, 27]. GGGGCC hexanucleotide repeat expansion in the *C9ORF72* gene has gained much attention in the past few years and is now considered the most frequent inherited cause of Amyotrophic lateral sclerosis (ALS) and Frontotemporal dementia (FTD) [28, 29]. Pathology occurs due to the toxicity of expanded repeats, which are transcribed in both the sense and antisense directions and give rise to distinct sets of intracellular RNA and protein aggregates [30–33].

Myotonic Dystrophy (DM) is part of a group of diseases characterized by repeat expansions in noncoding regions of genes. DM is defined in two clinical and molecular forms: myotonic dystrophy type 1 (DM1), and type 2 (DM2), both of which are inherited in an autosomal dominant fashion. The combined worldwide incidence of DM is approximately 1 in 8000 [34, 35]. DM1 is the most prevalent form of adult onset muscular dystrophy [36] and is caused by a CTG repeat expansion in the 3′ UTR of *Dystrophia Myotonica Protein Kinase* (*DMPK*) gene [37, 38]. DM2, on the other hand, is caused by a CCTG repeat expansion in an intron of *Zinc Finger Protein 9* (*ZNF9*) gene [39]. While 5–37 repeats are considered normal, DM1 patients can have up to several thousand CTG repeats, which can reduce expression of DMPK [40] (Fig. 8.2a). *DMPK* is expressed in multiple tissues, and the major symptoms of the disease include muscle hyperexcitability (myotonia), progressive muscle wasting, cardiac defects, insulin resistance, and neuropsychiatric disturbances [41–44]. Table 8.1 provides further description of tissue-specific symptoms observed in DM and other microsatellite expansion disorders.

8.3 RNA Metabolism Defects in Myotonic Dystrophy

Closely after the discovery of repeats, the *DMPK* haploinsufficiency model was put forward to explain the DM1 pathology. However, the removal of *DMPK* gene in mice failed to recapitulate the major neuromuscular symptoms of DM1 [45, 46]. A separate hypothesis proposed that expanded CTG repeats might affect the expression of nearby genes. Although the adjacent gene, *SIX5*, exhibits reduced expression in DM1 patients [47], *Six5* knockout mice also do not reproduce DM1 muscle

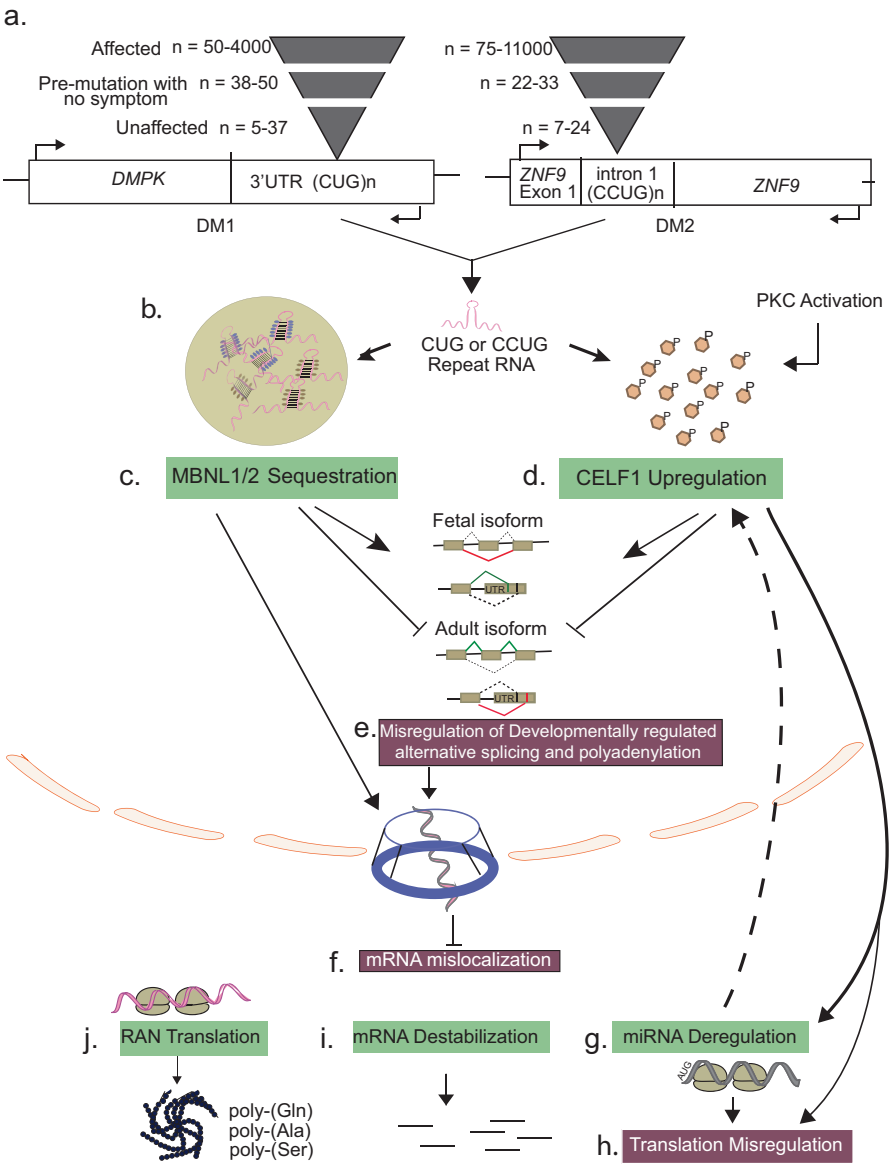

Fig. 8.2 Schematic showing different pathological mechanisms for Myotonic Dystrophy type 1 (DM1) and 2 (DM2). (a) Causative mutation for DM1 is CUG repeat expansion in 3′UTR of *DMPK* gene and for DM2 is CCUG repeat expansion in intron 1 of *ZNF9* gene. The severity of the disease is dependent on the number of repeats. Although these mutations are in two different genes, the disease mechanisms for both diseases are surprisingly similar. Most of the pathology is consistent with the toxic RNA gain-of-function mechanism and affects general RNA metabolism in both the nucleus and cytoplasm. (b) After transcription, the repeat-containing transcripts form stable hairpin loop comprising secondary structures (pink), which aggregate to form ribonuclear foci. (c) Members of the Mbnl family of RNA-binding proteins (RBPs) MBNL1/2 (purple) bind the CUG

pathology [48]. Instead, the CTG repeats alone, regardless of the gene context, are sufficient to induce pathogenic features of DM1 [49, 50]. The predominant pathology of DM1 actually stems from the toxic effects of expanded CUG RNA, which disrupts the normal activity of certain RBPs. Further support for the RNA toxicity model comes from the finding that although the repeat expansion in DM2 is on an entirely different gene, both diseases exhibit similar symptoms.

In both DM1 and DM2, the RNAs with expanded repeats (CUG in DM1; and CCUG in DM2) fold into stable hairpin loops that accumulate as ribonuclear foci in the nuclei of affected tissues [9] (Fig. 8.2b). These expanded RNA transcripts directly trap RBPs such as muscleblind-like proteins (MBNLs) and cause upregulation of CUG-binding protein 1 (CELF1) family of alternative splicing factors [51–54], which results in aberrant splicing of many transcripts and a broad, multi-systemic phenotype (Fig. 8.2c, d). Alternative pre-mRNA splicing generates much of the transcriptome diversity in higher eukaryotes as it enables the production of multiple transcripts with potentially different functions from each individual gene [55]. Alternative splicing decisions are generally influenced by *cis*-acting regulatory elements within pre-mRNAs that promote or inhibit exon recognition, as well as expression/activity of *trans*-acting factors (e.g., MBNL and CELF proteins) that bind to these *cis* elements and regulate the accessibility of the spliceosome to splice sites [56]. The misregulated splicing events in DM are usually developmentally regulated and exhibit an adult-to-embryonic switch in splicing patterns (Fig. 8.2e). Some of these embryonic isoforms fail to meet the adult tissue requirements and thus directly contribute to the overall disease pathology [54].

8.3.1 Misregulation of mRNA Processing

MBNL loss-of-function in DM1 and DM2 is a prominent example of RBP sequestration by disease-associated microsatellite expansion RNAs. The MBNL proteins were initially identified in *Drosophila melanogaster* for their requirement in muscle development and eye differentiation [57], and they were later shown as direct regulators of alternative splicing [58]. There are three MBNL paralogues in mammals, named MBNL1–3. MBNL1 and MBNL2 are widely expressed across many tissues,

Fig. 8.2 (continued) or CCUG repeats and are sequestered in the ribonuclear foci. (d) Hyperphosphorylation by PKC stabilizes another RBP, CELF1, resulting in its gain-of-function. (e) Both MBNL and CELF proteins regulate various aspects of RNA metabolism during normal development. Alterations in their functional levels due to toxic repeat RNA cause adult-to-fetal reversion of splicing and polyadenylation for many pre-mRNAs in the nucleus. (f) MBNL depletion also leads to cellular mislocalization of many mRNAs. CELF1 gain-of-function further affects (g) miRNA metabolism and (h) mRNA translation. (i) Dysregulation of MBNL and CELF activity in the cytoplasm also affects mRNA stability through various mechanisms. (j) Both sense and antisense CUG/CCUG-containing transcripts are subject to RAN translation in all three frames giving rise to homopolymeric polypeptides that accumulate in the cytoplasm and form pathological intracellular aggregates

including brain, heart, muscle, and liver, whereas MBNL3 expression is restricted to the placenta [59]. In a majority of tissues, *MBNL1* and *MBNL2* mRNA levels rise during differentiation [60, 61]. Besides their roles in pre-mRNA processing, MBNLs also influence gene expression by regulating cellular mRNA transport, stability as well as microRNA biogenesis [62–67]. The high expression of MBNL1 in the heart and skeletal muscle is consistent with the most severe DM phenotypes in these tissues. For instance, independent of the repeat expansion, *Mbnl1* deletion in mice reproduces many of the cardinal symptoms of DM1 such as myotonia, myopathy, cataracts, and misregulation of developmentally regulated RNA processing [63, 68].

The expanded repeat-containing RNAs in DM sequester MBNL1, 2, and 3 in nuclear RNA foci [69–71], and this protein redistribution explains the inhibition of their normal functions predominantly in alternative splicing and polyadenylation, microRNA processing, and mRNA localization [58, 62, 67, 72–75]. The MBNL loss-of-function hypothesis is further supported by studies on *Mbnl* single- and compound-knockout mice, which recapitulate many of the DM phenotypes [68, 76–78]. The extent of symptoms, however, varies depending on the tissue context, relative concentrations of MBNL paralogues, and the degree to which they are sequestered [78]. For instance, compared to skeletal muscle, only few splicing defects are observed in the brains of *Mbnl1* knockout mice [63, 79]. Alternatively, *Mbnl2* knockout mice exhibit a number of DM-related central nervous system abnormalities including irregular REM sleep propensity and deficits in spatial memory [76], which is consistent with the observation that MBNL2 expression in the brain is higher than MBNL1 [59]. MBNL2 is directly sequestered by repeat expansions in the brain tissue of human DM patients resulting in misregulation of alternative splicing and polyadenylation of its normal RNA targets [80]. One of the most misspliced mRNA due to loss of MBNL2 is human microtubule-associated protein tau (MAPT) in the DM1 frontal cortex [80]. RNA toxicity mediated through MBNL2 sequestration leads to abnormal expression of tau isoforms and the progressive appearance of neurofibrillary tangles composed of intraneuronal aggregates of hyper-phosphorylated tau protein [81].

More recently, MBNL proteins were found to serve essential roles in poly(A) site selection for many transcripts (Fig. 8.2e). By integrating HITS-CLIP and RNA-seq from MBNL knockout cells and transgenic DM1 mouse model, along with minigene reporter studies, Swanson and colleagues demonstrated that MBNL proteins directly suppress or activate polyadenylation for thousands of pre-mRNAs [75, 80]. Thus, MBNL proteins coordinate multiple pre-mRNA processing steps and their sequestration in DM depletes them from their normal RNA targets.

Besides MBNL loss-of-function, there is accumulation and aberrant sub-cellular distribution of another splicing factor CELF1 in DM. CELF proteins are normally downregulated during postnatal striated muscle development, which facilitates fetal-to-adult splicing transitions in hundreds of muscle transcripts [61, 82]. CELF1 actually does not colocalize with RNA foci [83], and its upregulation in DM1 occurs through two separate mechanisms. First, CELF1 protein is stabilized through its hyper-phosphorylation [84]; and second, reduced levels of microRNAs in DM1 derepress CELF1 protein translation [85, 86] (Fig. 8.2d, g, h). The situation is less

clear in DM2, with conflicting reports of normal [87, 88] and increasing CELF1 protein levels [89] in patient tissues and cells. It is interesting to note that for many pre-mRNAs whose splicing is disrupted in DM1, CELF1 and MBNL1 regulate them in an antagonistic manner [58, 61, 90–92]. The antagonism, however, is not due to direct competition for the binding site as both CELF1 and MBNL1 bind and regulate splicing independently via distinct *cis*-acting RNA motifs.

In addition to MBNL and CELF proteins, other RNA splicing factors are implicated in DM. For instance, hnRNP H binds to *DMPK*-derived CUG-expanded RNAs in vitro and increased hnRNP H levels may also contribute toward DM pathogenesis [93]. hnRNP H forms a repressor complex with MBNL1 and nine other proteins (hnRNP H2, H3, F, A2/B1, K, L, DDX5, DDX17, and DHX9) in normal myoblast extracts but elevated hnRNP H levels in DM1 disrupt the stoichiometry of these complexes which affects splicing of specific pre-mRNAs [94, 95]. Since expanded CUG repeat RNAs fold into hairpin structures [96], the partial recruitment and colocalization of the RNA helicase p68/DDX5 with RNA foci may also have a contributing role in splicing dysregulation. Moreover, p68/DDX5 can modulate MBNL1-binding activity, and its colocalization with nuclear RNA foci can further stimulate MBNL1 binding to repeat RNAs [97].

8.3.2 Misregulation of mRNA Localization and Stability

Following transcription, newly synthesized and fully processed mRNAs are bound by specific RBPs to form export-competent mRNPs, which help their transport through the nuclear pore complex (NPC). Some pre-mRNAs are processed at the speckle periphery before being exported and repeat-containing nuclear foci can colocalize at the periphery of nuclear speckles, a non-membrane bound nuclear assembly of macromolecules including splicing factors. The presence of expanded CUG repeats may, therefore, prevent entry of other RNAs into the nuclear speckle [98, 99]. However, in DM2, the mutant *ZNF9* mRNA is exported normally as the expanded CCUG repeats are removed during splicing. The nuclear foci formed by DM2 intronic repeats are widely dispersed in the nucleoplasm and not associated with nuclear speckles. Also, it is not yet clear whether the DM1 and/or DM2 nuclear foci contain partially degraded fragments of CUG or CCUG repeats or larger intact RNAs respectively.

As discussed above, CELF1 upregulation and MBNL sequestration by the CUG repeats in DM1 cause misprocessing of hundreds of transcripts. Aberrant processing results in nucleocytoplasmic export defects for many of these transcripts. Furthermore, MBNL proteins are localized both in the nucleus and cytoplasm and several studies have demonstrated their direct roles in mRNA localization [62, 100] (Fig. 8.2f). For instance, by interacting with the 3′-UTR of Integrin α3, MBNL2 moves it to the plasma membrane for its local translation [64]. Similarly, MBNL1 also plays major roles in mRNA localization and membrane-associated translation. Transcriptome-wide analyses of subcellular compartments from mouse myoblasts

showed widespread defects in mRNA localization upon combined depletion of MBNL1 and MBNL2 [62]. Many of the mislocalized mRNAs encode for secreted proteins, extracellular matrix components, and proteins involved in cell–cell communication. MBNL depletion in DM can thus have a significant impact on mRNA localization potentially affecting proper neuromuscular junction formation.

In the cytoplasm, MBNLs also regulate mRNA stability [101] (Fig. 8.2i). MBNL1 specifically recognizes YGCY-containing motifs within the 3′-UTR regions and destabilizes the target mRNAs through unknown mechanisms [65, 92]. CELF1, on the other hand, induces mRNA decay of short-lived transcripts through interactions with GU-rich elements (GREs) in their 3′-UTR and possibly recruitment of poly(A)-specific ribonuclease, which promotes deadenylation of target transcripts [102–104]. Many of the CELF mRNA targets with GREs encode proteins essential for muscle cell development and function [105–108]. Interestingly, CELF1 binds to the mRNAs coding for SRP protein subunits and promotes their decay [109]. Signal recognition particle (SRP) is a cytoplasmic ribonucleoprotein complex, which regulates the translation of secreted and membrane-associated proteins. It is likely that the CELF1 overexpression contributes to the faster turnover of SRP mRNAs and the reduced SRP levels thereby attenuate the protein secretory pathway in DM1 [109].

8.3.3 Misregulation of mRNA Translation

CELF1 is additionally involved in the regulation of mRNA translation [106, 110–112] (Fig. 8.2h). The affinity of CELF1 toward its mRNA targets can be modulated through phosphorylation [113]. For instance, phosphorylated CELF1 interacts with a subunit of initiation factor eIF2, leading to the recruitment of translational machinery to target mRNAs [106]. In myoblasts, AKT phosphorylates CELF1 and increases its affinity for *CCND1* mRNA. During myoblast-to-myotube differentiation, cyclinD3-cdk4/6 phosphorylates CELF1, which increases CELF1 interaction with 5′-UTR of *p21* mRNA (a cell cycle inhibitor) and enhances its translation. Myoblasts from DM1 patients show an increased interaction between CELF1 and AKT and have reduced cyclinD3-CDK4/6 levels during differentiation [105]. Moreover, DM1 myoblasts during differentiation show a reduced ability to withdraw from cell cycle, which may be due to the altered translation of P21 or myogenic transcription factor MEF2A by CELF1 [111, 112].

mRNA translation in DM1 is also affected due to microRNA deregulation (Fig. 8.2g). A subset of developmentally regulated microRNAs associated with cardiac arrhythmias is downregulated in the hearts of DM1 patients and mice [67, 86]. Downregulation of these microRNAs recapitulates particular gene expression deficits seen in DM1 hearts including enhanced protein levels of miR-1 targets CX43 and Cav1.2 as well as miR-23a/b target CELF1 [67, 86]. In DM1 and DM2 skeletal muscle biopsies, both the levels and cellular distribution of several evolutionarily

conserved microRNAs are altered affecting their downstream targets [114–117]. Furthermore, specific microRNAs are differentially detected in peripheral blood plasma of DM1 patients, which inversely correlate with skeletal muscle strength and may serve as noninvasive biomarkers [118]. More recently, reduced expression of miR-200c/141 tumor suppressor family was shown to correlate with increased oncologic risk in women with DM1 especially for gynecologic, brain, and thyroid cancer [119].

Besides altering cellular translation through misregulation of RBPs and microRNAs, the microsatellite expansions also promote unconventional translation of repeats in multiple reading frames producing homopolymeric peptides that aggregate in both the nucleus and the cytoplasm [120] (Fig. 8.2j). Designated as Repeat Associated Non-AUG Translation (RAN translation), it was first described for the expanded CAG and CTG repeats that cause spinocerebellar ataxia 8 (SCA8) and DM1, respectively [120]. Interestingly, the efficiency of RAN translation increases with the size of repeats and when RNA forms hairpin-like structures [121]. Additionally, the cells making the toxic RAN protein products are prone to apoptosis as detected in tissues of affected patients, indicating a potential contribution of RAN to pathogenesis. In addition to DM1, Zu et al. recently demonstrated that in DM2 the tetranucleotide expansion repeats are bidirectionally transcribed, and the resulting transcripts are RAN translated, producing tetrapeptide expansion proteins with Leu-Pro-Ala-Cys (LPAC) from the sense strand or Gln-Ala-Gly-Arg (QAGR) repeats from the antisense strand [122]. These RAN proteins were readily detected in the DM2 patient brains; however, the specific roles of these RAN proteins regarding toxicity, mechanism of action, and their regulation are yet to be determined.

Since their original discovery, RAN translation has now been observed in many other repeat-expansion diseases, including ALS/FTD, FXTAS, and Huntington's disease [52, 123]. However, the exact mechanisms initiating translation from these repeats likely differ across diverse sequence contexts [124]. For instance, in case of *FMR1*, expanded CGG repeats in the 5′-UTR initiate CAP-dependent RAN translation upstream of the canonical AUG start codon, producing FMRpolyGlycine and FMRpolyAlanine in FXTAS [123, 125]. In contrast to FXTAS, the expanded repeats in DM1 exist within the 3′ UTR of *DMPK* mRNA, which is not in the normal path of ribosome scanning; thus, unconventional ribosome interactions must contribute in their translation. For *HTT* in Huntington's disease, the CAG repeats are in the ORF, and canonical translation starts at the native AUG codon upstream of the repeats. But in some instances, HTTpolySerine and HTTpolyAlanine proteins are also produced due to RAN-translation and frame shifting from the normal HTTpolyGlutamine frame of the repeats [126]. Finally, in case of ALS/FTD, the GGGGCC repeats are within *C9ORF72* intron, and the RAN-translation generates polyGlycine-Alanine, polyGlycine-Arginine, and polyGlycine-Proline dipeptide products [31, 127]. The RAN translation in this case, however, may occur from the intron retained transcript, spliced lariat, or a 3′ truncated RNA generated due to stalled transcription [124, 128].

8.4 Disrupted Function of RBPs in Other Microsatellite Expansion Disorders

Recent paradigm-shifting advances have established that defective RNA processing through disrupted function of RBPs is central to many other repeat expansion diseases (Table 8.2). For instance, RBP defects occur in both familial and sporadic cases of ALS/FTD [129, 130]. Mutations in *TARDBP* and *FUS* genes respectively encoding TDP-43 and FUS/TLS proteins result in abnormal aggregation of these proteins in neurons and are considered pathogenic for ALS/FTD. TDP-43 and FUS/TLS are RNA/DNA-binding proteins, with noticeable structural and functional similarities.

TDP-43 functions in multiple RNA processing steps including pre-mRNA splicing [131–134], RNA stability [135–137], and transport [138]. Similar to TDP-43, FUS interacts with serine-arginine (SR) proteins that serve diverse roles in splicing [139] and regulates transcription by recruiting other RBPs through noncoding RNAs [140]. Hence, the association of TDP43 and FUS/TLS with ALS and FTD is

Table 8.2 Common postulated pathological mechanisms and associated RNA-Binding Proteins (RBPs) for disease-associated microsatellite repeat expansions

	Diseases name	RBPs	Pathological mechanism
(a)	FXTAS, FXS	FMRP [11–14] Pur α and hnRNP A2/B1 [205, 206], CELF1 [207], Sam68 [208]	mRNP transport and translation [209–213] Nuclear Foci and RBP Sequestration leads to changes in expression and cellular distribution of several proteins [214, 215], RNA Splicing [208, 216].
(b)	DM1/2	MBNL1/2/3 [49, 68, 72–74]; and CELF [39, 83, 84, 90, 91, 111, 217], HnRNP H [93], p68/DDX5 [97]	Nuclear Foci and RBP Sequestration, RNA splicing [58, 61, 62, 82, 218, 219] and polyadenylation misregulation [75, 80], miRNA biogenesis [67, 86, 115], Translation and cellular localization disruption [62, 99], Intracellular aggregation by non-canonical RAN translation [122]
(c)	ALS/ FTD	TDP-43 [220, 221] FUS [222, 223], TAF15 [141, 142], EWSR1 [143, 144] hnRNPA1 and hnRNPA2B1 [146], Ataxin 2 [145], TIA1 [224]	Nuclear foci [225], Splicing misregulation [132, 134, 226], translation, and RNA transport [227], impaired cytoplasmic localization [154, 228, 229], mutated LCD domain mediated cytoplasmic inclusions [146, 230–233]
(d)	SCA 8	MBNL/CELF [234], Staufen [235]	RNA Splicing [234], RAN Translation [120]

Abbreviations: *FXTAS* fragile X-associated tremor/ataxia syndrome, *FXS* fragile X syndrome, *DM1/2* myotonic dystrophy type 1/2, *ALS* amyotrophic lateral sclerosis, *FTD* frontotemporal degeneration, *SCA* spinocerebellar ataxias, *FMRP* fragile X mental retardation protein, *CELF* CUGBP Elav-like family member, *mbnl* Muscleblind like splicing regulator, *hnRNPs* heterogeneous nuclear ribonucleoprotein, *TAF15* TATA box-binding protein-associated factor 15, *EWSR1* Ewing sarcoma breakpoint region 1, *TIA1* T cell intracytoplasmic antigen

redirecting research efforts toward identifying additional RBPs that are mutated in neurological diseases, defining their normal RNA substrates and determining the misprocessed RNAs that underlie particular disease symptoms. In fact, mutations in several other RBPs that are functionally and structurally similar to FUS/TLS such as TAF15 [141, 142] and EWSR1 [143, 144], as well as the less closely related RBPs—Ataxin 2 [145], hnRNPA2B1 [146], hnRNPA1 [146], and Matrin3 [147] were recently identified. Among these RBPs, TDP-43, FUS, and hnRNPA1 harbor low complexity domains (LCDs), which can polymerize and drive phase separation to form dynamic membrane-less organelles or liquid droplets. For instance, a 57-residue segment within the FUS-LCD was recently shown to assemble into a fibril core that promotes phase-separation and hydrogel formation. Interestingly, phosphorylation of the core-forming residues by DNA-dependent protein kinase dissolves the FUS-LCD liquid droplets providing a molecular basis for the dynamics of LCD polymerization and phase separation [148].

Disease-associated mutations within LCDs of RBPs also enhance prion-like properties and accelerate the shift from liquid to solid phase disturbing proper ribonucleoprotein (RNP) formation [127, 149, 150]. These mutations likely trigger protein aggregation due to aberrant self-assembly of LCDs. The cytoplasmic aggregation of RBPs not only affects their typical functions in RNA metabolism but also diminishes general nucleocytoplasmic trafficking, a common consequence of ALS-initiating mutations [151–153]. While the exact reasons impeding nuclear/cytoplasmic transport in ALS are not yet fully established, multiple independent mechanisms have been proposed. For example, nucleocytoplasmic trafficking defects can arise due to proteotoxicity caused by cytoplasmic β-sheet containing protein aggregations [154], direct interactions between repeat RNAs and nuclear import factors [153], or inhibition by RAN translation-products of repeat RNAs [151]. Interestingly, arginine-containing dipeptide repeats produced from RAN translation of hexanucleotide GGGGCC expansions in ALS interact with LCDs of RBPs, which disrupts the dynamics and functions of membrane-less organelle formation by LCDs [155, 156]. Furthermore, subsets of these arginine-containing dipeptides frequently bind to the LCDs encoded by the nuclear pore proteins blocking the transport of macromolecules into and out of the nucleus [157]. Thus, interaction of RAN translation products with LCDs is a yet another pathogenic mechanism that interferes with the normal function of RBPs in microsatellite expansion disorders.

8.5 Conclusions

The past decade has seen remarkable progress in our understanding of the molecular pathogenesis of microsatellite repeat expansion disorders. Although the repeats may vary in terms of their length and location within a gene or the multiple ways through which they cause disease, one commonality of microsatellite expansions is the production of toxic RNA species containing repeats. Mechanistically, the

pathology arises either due to loss-of-function of the affected gene, or gain-of-function of the repeat-containing RNAs. Regarding loss-of-function, the repeats can induce transcriptional silencing of the affected gene through epigenetic modifications or produce a non-functional protein that contains a long stretch of homopolymeric amino acids. In case of gain-of-function, the RNAs with expanded repeats often sequester RBPs and thus disrupt their normal activities. Alternatively, the translated protein with a repetitive stretch of homopolymeric peptide sequence can misfold, aggregate, and trap critical cellular proteins causing nucleo-cytoplasmic export defects and further proteotoxicity. For a number of repeat expansion disorders, there is an intricate overlap of such loss- and gain-of-function mechanisms resulting in complex molecular pathologies. We envision that for many repeat expansions, the future investigations will be geared toward determining the unique versus overlapping disease mechanisms, dissecting direct versus indirect RNA metabolism defects, and finally, understanding whether alterations in RNA metabolism occur early or during late stages of the disease.

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Chapter 9
Mechanisms Associated with TDP-43 Neurotoxicity in ALS/FTLD

Marc Shenouda, Ashley B. Zhang, Anna Weichert, and Janice Robertson

Abstract The discovery of TDP-43 as a major disease protein in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) was first made in 2006. Prior to 2006 there were only 11 publications related to TDP-43, now there are over 2000, indicating the importance of TDP-43 to unraveling the complex molecular mechanisms that underpin the pathogenesis of ALS/FTLD. Subsequent to this discovery, TDP-43 pathology was also found in other neurodegenerative diseases, including Alzheimer's disease, the significance of which is still in the early stages of exploration. TDP-43 is a predominantly nuclear DNA/RNA-binding protein, one of a number of RNA-binding proteins that are now known to be linked with ALS/FTLD, including Fused in Sarcoma (FUS), heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), and heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1). However, what sets TDP-43 apart is the vast number of cases in which TDP-43 pathology is present, providing a point of convergence, the understanding of which could lead to broadly applicable therapeutics. Here we will focus on TDP-43 in ALS/FTLD, its nuclear and cytoplasmic functions, and consequences should these functions go awry.

Keywords TDP-43 · ALS · FTLD · RNA · Granules

9.1 Discovery of TDP-43 as a Disease Relevant Protein in Amyotrophic Lateral Sclerosis and Frontotemporal Lobar Degeneration

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is an adult-onset neurodegenerative disease characterized by loss of motor neurons from the motor cortex, brain stem, and spinal cord, causing progressive paralysis and

M. Shenouda · A. B. Zhang · A. Weichert · J. Robertson (✉)
Tanz Centre for Research in Neurodegenerative Diseases and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON M5T 2S8, Canada
e-mail: jan.robertson@utoronto.ca

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death due to respiratory failure 2–5 years from diagnosis [1]. In populations of European origin, the median incidence of ALS is 2.08 per 100,000 with a median prevalence of 5.4 per 100,000 [2]. Approximately 5–10% of ALS cases exhibit Mendelian inheritance, primarily autosomal dominant, known as familial ALS (fALS), with the remaining 90–95% of sporadic ALS (sALS) cases showing no apparent family history of disease. To date, the genes associated with about 70% of fALS cases have been identified, and a genetic component has also been found in approximately 15% of sALS cases [3]. The major genes accounting for the greatest number of ALS cases are *superoxide dismutase* 1 (*SOD1*; 15–20% of fALS, 1% sALS), *TAR DNA-Binding Protein-43* (*TARDBP*; 4% of fALS, <1% sALS), *Fused in Sarcoma* (*FUS*; 4% of fALS, <1% sALS), and *C9orf72* (30–40% of fALS, 7% sALS) [3]. Clinically, ALS requires involvement of both upper and lower motor neurons with three sites of focal onset, limb (70%), bulbar (25%), and diaphragm (5%), spreading to other regions as the disease progresses [1]. Although manifesting as a motor neuron disease, neuropsychological, neuroimaging, and histological studies have indicated extramotor involvement, and as such ALS has been proposed as a multisystems disorder, affecting other neuronal subtypes [4]. ALS has clear clinical, neuropathological and genetic overlap with frontotemporal dementia (FTD) [5], an umbrella term for a group of disorders affecting the frontal and temporal lobes causing impairments in behavior, language, or executive function [6]. FTD is the second most common form of dementia below the age of 65. There are three major clinical syndromes of FTD: (1) behavioral variant FTD (bvFTD), characterized by changes in personality and social behaviors; and two forms of primary progressive aphasia, (2) semantic dementia (SD) where there is impaired word comprehension and decline in semantic memory; and (3) progressive non-fluent aphasia (PNFA), characterized by impaired speech production [7, 8]. Up to 50% of patients with ALS exhibit clinical signs of frontotemporal dysfunction with 10–15% fulfilling the diagnostic criteria of FTD, usually bvFTD [9]. This is evident at autopsy with neuropathological markers showing degeneration of the frontal and temporal lobes, known as frontotemporal lobar degeneration (FTLD), the term that will be used herein.

In 2006, the clinical overlap between ALS and FTLD was substantiated when two groups independently identified the TAR DNA-Binding Protein-43 (TDP-43) encoded by *TARDBP*, as a core component of the ubiquitinated inclusions pathognomonic of disease [10, 11]. Soon thereafter, primarily autosomal dominant mutations in *TARDBP* were found associated with ~4% of fALS and <1% of sALS cases, indicating a direct role for TDP-43 in disease causation [3, 12, 13]. TDP-43 is a predominantly nuclear DNA/RNA-binding protein that has numerous functions related to RNA metabolism [14, 15]. In ALS, TDP-43 is mislocalized from the nucleus to the cytoplasm of neurons and glia of the primary motor cortex, brainstem motor nuclei, and spinal cord. In the neuronal cytoplasm, pathological TDP-43 appears as non-ubiquitinated diffuse/granules, termed pre-inclusions [16], or as ubiquitinated skein-like inclusions, or compact Lewy body-like inclusions [10, 11, 17]. These structures appear to be a continuum of the same pathology [18]. TDP-43 in pathological inclusions (but not normal nuclear TDP-43) is phosphorylated on

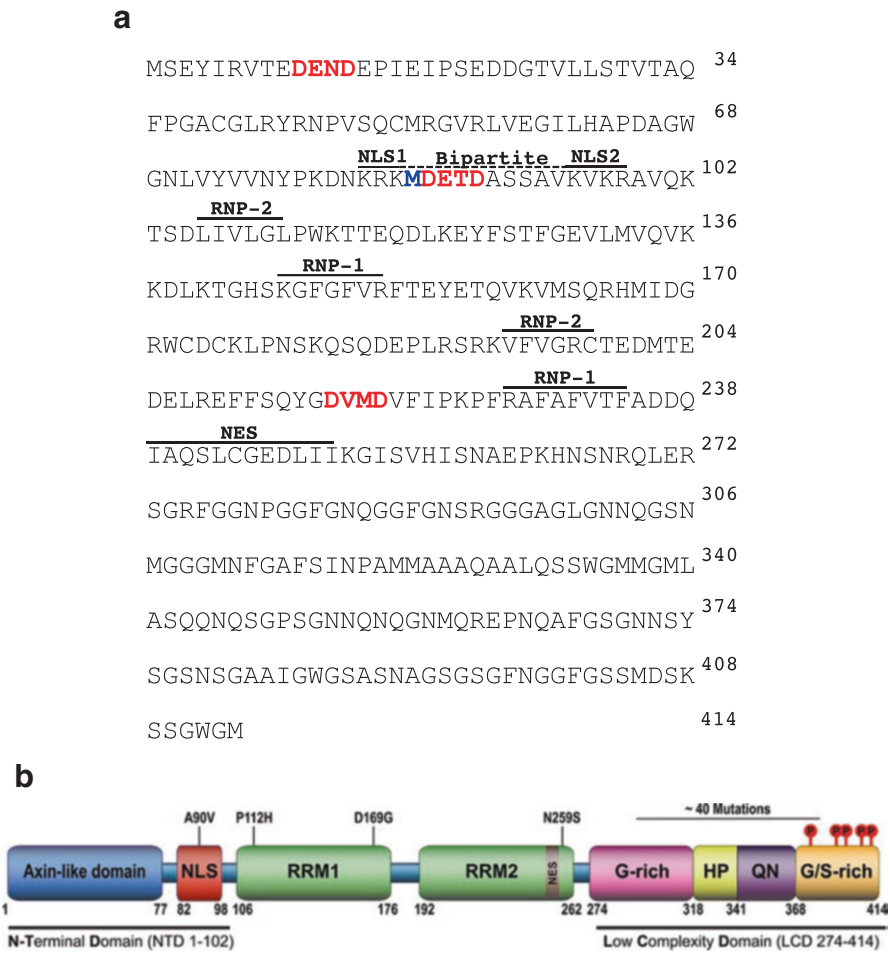

Fig. 9.1 Sequence and domain structure of TDP-43. (**a**) Amino acid sequence of TDP-43 showing location of the NLS (residues 82–98), the NES (residues 239–250), RNP1 and RNP2 motifs, the caspase 3 cleavage sites (DEND, DETD, DVMD in red), alternative translation start site Met 85 (blue). (**b**) Domain structure of TDP-43 with location of pathological phosphorylation sites (serines 379, 403, 404, 409, and 410) shown in red

at least five sites, Ser 379, Ser 403/404, Ser 409/410, all present within the Glycine-Serine-rich domain, and can be detected using specific antibodies (Fig. 9.1) [19]. Evidence suggests that these sites are phosphorylated by casein kinase 1 [19]. TDP-43 pathology is present in over 95% of ALS cases, irrespective of the causal genetic mutation [20], with the key exception of cases caused by mutations in FUS [20, 21] and SOD1, where only rare TDP-43 inclusions are observed [22–24]. Interestingly, a recent study described nuclear clearing of TDP-43 in pyramidal Betz cells in the absence of cytoplasmic inclusions [25]. These cells occurred alongside Betz cells with traces of granular or skein-like TDP-43 pathology as well as Betz cells with

robust pathology [25, 26]. TDP-43 pathology is present in about 50% of FTLD cases (FTLD-TDP), with the other 45% of cases exhibiting tau pathology (FTLD-tau), and the remaining 5% characterized by inclusion bodies comprised of FUS (FTLD-FUS) or other FET (*F*US-*E*wings Sarcoma Protein-*T*AF15) proteins (FTLD-FET) [26].

In FTLD-TDP, there are four main subtypes of TDP-43 pathology that are based on anatomical distribution and morphology, and exhibit clinical and genetic correlations [26–28]. Type A is characterized by compact neuronal cytoplasmic inclusions (NCI), short and thick dystrophic neurites (DN), and occasional lentiform neuronal intranuclear inclusions (NII) primarily in layer II of the neocortex; and is associated with bvFTD or PNFA, and cases caused by mutations in progranulin. Type B exhibits diffuse, granular, and compact NCI as well as abundant "wispy dot-like profiles" throughout all cortical layers, with few DN and NII, and is associated with bvFTD and FTLD-ALS. Type C is characterized by long DN in upper cortical layers with few NCI, and this pathology is associated with SD. Type D is a rare pathology characterized by lentiform NII, short DN, and rare NCI throughout all cortical layers, and is specifically associated with inclusion body myopathy with Paget's disease of bone and frontotemporal dementia caused by mutations in vasolin-containing protein [26, 29]. These distinctive patterns of TDP-43 neuropathology may be reflective of differing disease mechanisms, and this is supported by the association of each subtype with different clinical presentations [27, 28]. An exception are cases caused by G_4C_2 hexanucleotide repeat expansions in C9orf72, where features of Type A and Type B TDP-43 pathology have been observed [29, 30]. The reason for this heterogeneity is uncertain. It is worth noting here that dipeptide repeat (DPR) protein pathology generated through repeat-associated non-ATG (RAN) translation of the G_4C_2 repeats is present in ALS-FTLD cases caused by C9orf72 mutations [31–35]. The anatomical distribution of DPR (poly-GA, poly-GR, poly-PR, poly-PA, poly-GP) pathology differs from TDP-43, with TDP-43 reported as being the better correlate of areas of neurodegeneration [35, 36]. However, poly-GR was recently shown to colocalize with phosphorylated TDP-43 in dendrites of the motor cortex in C9orf72 ALS cases [37].

TDP-43 pathology has also been associated with a number of other neurodegenerative diseases, including Alzheimer's disease and hippocampal sclerosis [39–41], Huntingtons's disease [42], Lewy body-related diseases (Parkinson's Disease, Parkinson-Dementia, Dementia with Lewy bodies) [39, 43–45], argyrophilic grain disease [46], and late stage chronic traumatic encephalopathy [47]. These differing TDP-43 pathologies across numerous diseases are subsumed by the collective term TDP-43 proteinopathy.

9.2 TDP-43 Structure and Function

Ou et al. [48] first identified a protein binding to the pyrimidine-rich motif within the LTR region of HIV-1 by screening a HeLa cell library using a TAR DNA probe. Subsequent analysis using Northern and Western blot identified the protein as TAR

DNA-binding protein of 43 kDa transcribed from a 2.8 kb transcript. Thus, the protein was named TAR-DNA-binding protein of 43 kDa (TDP-43) [48]. Later, TDP-43 was found to function as a splicing regulator by binding to the (TG)m polymorphic repeat region near the 3′-splice site of exon 9 in the cystic fibrosis transmembrane conductance regulator (*CFTR*) pre-mRNA causing exon skipping [49, 50]. Since that time, TDP-43 has been associated with numerous aspects of RNA processing, including transcriptional regulation, pre-mRNA splicing, miRNA biogenesis, lncRNA/ncRNA expression/regulation, as well as mRNA stability, transport and translation (reviewed in [15].

The human TAR DNA-binding protein gene (*TARDBP*) is located on chromosome 1p36, and has six exons with exons 2–6 encoding the 414-aa TDP-43 protein. TDP-43 is evolutionarily conserved in mouse, *Drosophila melanogaster*, and *C. elegans* [51]. TDP-43 is ubiquitously expressed and is essential for embryological development [52–54]. Structurally, TDP-43 contains all the elements of a heterogenous ribonuclear protein (hnRNP) [48–50], comprising of two RNA Recognition Motifs, RRM1 (residues 106–176) and RRM2 (residues 191–262), an N-terminal domain (residues 1–102), and an intrinsically disordered C-terminal domain (residues 274–414) [55] (Fig. 9.1). The RRM domains each contain conserved octamer and hexamer consensus sequences known as Ribonucleoprotein 1 (RNP1) and Ribonucleoprotein 2 (RNP2) [59]. The N-terminal domain (specifically residues 1–77) adopts a Ubiquitin-like [56] or DIshevelled and aXin (DIX)-domain-like fold [57] in solution. The C-terminal domain shares homology with prion-like domains and mediates interactions with other proteins such as hnRNP A/B and hnRNP A2, necessary for the splicing functions of TDP-43 [58–61]. The C-terminal domain is subdivided into four regions: a glycine-rich motif (267–317); a hydrophobic segment (318–340), which adopts a marginally stable α-helical conformation in aqueous solution [62]; an aggregation prone glutamine/asparagine (Q/N)-rich region (341–367), which binds hnRNP A2 [63–66]; and a glycine-serine-rich region (368–414), where the majority of pathological phosphorylation sites are located [19] (Fig. 9.1). The majority of TDP-43 mutations associated with ALS/FTLD are located in the C-terminal domain [20]. TDP-43 is a predominantly nuclear protein, with low levels present in the cytoplasm [67]. The nucleocytoplasmic shuttling of TDP-43 is regulated by a classical bipartite nuclear localization sequence (NLS) at the N-terminus composed of K82RK84 (NLS1) and K95VKR98 (NLS2), and a nuclear export sequence (NES) in RRM2, I239AQSLCGEDLII250 [68, 69].

TDP-43 regulates the expression and splicing of multiple RNA targets, including processing/expression of its own transcript [49, 70–72]. TDP-43 binds with high affinity to UG repeats in target RNAs through the RRM1 and RRM2 domains, with RRM1 indispensable for RNA binding [49, 72]. Native TDP-43 forms functional dimers and oligomers under physiological conditions, interacting through the N-terminal domains, spatially separating the aggregation prone C-terminal domains [73–77]. This dimerization/oligomerization is required for the nuclear splicing activity of TDP-43 [73, 74]. Oligomeric TDP-43 is predicted to provide higher binding affinities for longer contiguous UG-repeats [73]. Furthermore, oligomeric TDP-43 could also bring distal sites into close proximity either within the same

RNA or multiple RNAs, creating loops that potentiate splicing [73, 78]. Three disease mutations have so far been identified in the RNA-binding domain of TDP-43: P112H [79], D169G [12], and N259S [80]. While the D169G mutation does not affect RNA-binding affinity [81], it was found to increase the thermal stability of TDP-43 promoting cleavage by caspase-3 both in vitro and in culture. This produced increased levels of a 35 kDa C-terminal fragment, which enhanced cellular toxicity [82, 83].

RNA targets of TDP-43 have been identified using RNA precipitation techniques in cell culture, mouse brain, and human tissue [71, 84–86]. Using conventional cloning, over 100 TDP-43 RNA targets were identified in SHSY5Y cells, binding predominantly to UG-rich motifs [86]. Binding was mainly to intronic regions (82%), but also to 3′-UTRs and noncoding RNA. Pertinent TDP-43-binding targets included the transmembrane synaptic protein neurexin-1 (*NXRN1*) and the RNA editing enzyme *ADARB2* [86]. In rat primary cortical neurons, 4352 TDP-43 RNA targets were identified, 1971 mapping to introns, 910 to exons, and 1471 to both introns and exons [84]. Gene ontology (GO) analysis of the intronic reads showed enrichment for synaptic formation and function, and regulation of neurotransmitter processes, with *Nrxn1–3* and *Nlgn1–3* (neuroligin*)* identified as top hits. GO terms for exonic targets were related to splicing, RNA processing, and maturation. Notable targets included *Tardbp*, *Fus*, *Grn*, *Mapt*, *Atxn1 and 2*, and *Adarb1* [84]. In mouse brain, over 6000 TDP-43 RNA targets were identified, with TDP-43 binding preferentially to pre-mRNAs with long introns [71]. Depletion of TDP-43 using antisense oligonucleotides led to expression changes in 601 mRNAs and 965 splicing changes. Most of the target genes were downregulated, and GO analysis revealed an enrichment for synaptic activity and function, several genes of which were validated by independent qRT-PCR, including *Nrxn 1 and 3*, and *Nlgn*. Interestingly, pertinent to cholinergic neurons, there was a significant reduction in *Chat* levels [71]. Of the splicing changes, *sortilin 1*, which encodes SORT1 a neuronal progranulin receptor, had the highest splicing score [71]. Subsequent studies have shown that abnormal splicing of human *sortilin 1* caused by TDP-43 depletion generates a non-functional progranulin receptor that acts as a decoy to antagonize progranulin uptake [87]. This is an important link since mutations in progranulin cause haploinsufficiency in FTLD-TDP, and the splice isoform of SORT1 is elevated in FTLD-TDP tissues [87, 88]. Studies in healthy human and FTLD-TDP cortical brain tissue revealed binding of TDP-43 to UG-rich regions in noncoding RNAs and 3′UTRs of mRNAs again with greatest binding to intronic sequences [85]. GO terms of exons regulated by TDP-43 were diverse and included organ morphogenesis, neural tube closure, mitotic cycle, and cell surface receptor linked signaling pathway. Splicing transcripts included those involved in neuronal survival or development, and seven were relevant to neurodegenerative diseases, including *CNTFR* and *KIF1B* [85]. The most significant changes in FTLD-TDP versus healthy controls were increased TDP-43 binding to nuclear paraspeckle assembly transcript 1 (*NEAT1*) and *NEAT2* (*MALAT1*) lncRNAs, and correspondingly these transcripts were increased in FTLD-TDP tissue [85]. The genes with decreased TDP-43 binding were *neurexin 3*

(*NRXN3*) and glial excitatory amino acid transporter-2 (*EAAT2*). EAAT2 levels are decreased in ALS which would cause reduced glutamate clearance at the synaptic cleft and contribute to glutamate excitotoxicty [89, 90]. It is interesting that *NRXN* and/or *NLGN* were consistently identified as TDP-43-binding targets in all four studies, and more generally, genes associated with synaptic function were highly represented [71, 84–86]. Similarly, studies in NSC-34 cells identified TDP-43 mRNA targets enriched for GO terms related to neuron differentiation and dendrite development [91], and *syntaxin 1A* is upregulated in TDP-43 silenced primary neurons [92]. These findings implicate a role for TDP-43 in regulating synaptic integrity/function. TDP-43 has been identified as a neuronal activity responsive factor colocalizing with FMRP and staufen-1 in neuronal transport RNA granules [93]. The number of TDP-43 granules in the somatodendritic compartment of rat primary hippocampal neurons or axons of mouse primary motor neurons increased with neuronal stimulation caused by depolarization or in response to BDNF, respectively [93, 94]. TDP-43 co-associates with RNA and other RBPs in transport granules to deliver translationally dormant mRNAs to synaptic sites, where synaptic activity releases the mRNAs and promotes their localized translation [95–97]. Transport of TDP-43 RNA granules is microtubule-dependent and bidirectional, and involves several motor proteins [98]. Disease-associated mutations in TDP-43 impair this transport of TDP-43 granules and this could lead to synaptic deficits [98]. Indeed, loss or gain of the TDP-43 homolog in Drosophila caused synaptic dysfunction [99–101], and early synaptic loss is a feature of various lines of TDP-43 transgenic mice [102–105]. Collectively, these findings demonstrate the importance of TDP-43 in maintaining synaptic integrity.

Recently, it was demonstrated that TDP-43 represses the splicing of non-conserved cryptic exons which, if expressed, introduce frameshifts and/or premature stop codons causing nonsense-mediated decay [106]. A series of cryptic exons, found to be expressed after knockdown of TDP-43 in HeLa cells, were also found expressed in ALS-FTLD brain tissue [106]. This indicates that aberrant proteins could be expressed or lost as a consequence of TDP-43 depletion, and this could contribute to the disease mechanism. Moreover, the cryptic exons repressed by TDP-43 were highly variable between cell types, and this may give clues to selective vulnerability in disease [107]. To uncover the motor neuron-specific changes caused by abnormal TDP-43, a recent study using translational affinity purification, which allows for the isolation of polysomes from specific cell types, was used to identify the transcripts being actively translated in spinal motor neurons of an A315T mutant TDP-43 transgenic mouse model [108, 109]. Methenyltetrahydrofolate synthetase domain containing (*Mthfsd*) and DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 (*Ddx58*, also known as RIG-1) were identified and found to have altered expression at the protein level in spinal motor neurons of both the A315T TDP-43 mutant mouse and ALS cases [108]. *Mthfsd* is a novel stress granule protein, and was recently ranked in the top ten by IBM® Watson as an ALS-relevant RNA-binding protein [108, 110].

9.3 TDP-43 and Mitochondria

A recent study has shown that TDP-43 is localized to mitochondria, repressing expression of mitochondrial RNAs, and mutations in TDP-43 enhance this localization [111]. Structural damage and fragmentation of mitochondria is thought to be an early event in disease, representing a potential upstream source of motor neuron degeneration [112–114]. The expression of wild type and ALS mutant TDP-43 (M337V, Q331K and A315T) results in vacuolated, fragmented, and aggregated mitochondria [112, 114, 115]. Similarly, the expression of TDP-43 A315T in ALS mouse models and ALS patient fibroblasts has been associated with the loss of mitochondrial cristae [116, 117]. Over-expression of wild-type TDP-43, and in some cases ALS-mutant TDP-43 (Q331K and M337V), alters mitochondrial network dynamics by reducing mitochondrial length in primary motor neurons [114]. In transgenic mice and patient fibroblasts, expression of wild-type or ALS-mutant (M337V and A382T) TDP-43 exhibited altered expression levels of fusion and fission-associated genes, which correlated with anomalous mitochondrial morphology and aggregation [116, 118, 119].

The accumulation of wild-type and ALS-mutant (G298S, A315T and A382T) TDP-43 in mitochondria is mediated by internal mitochondrial targeting sequences in TDP-43 [121]. Wild-type TDP-43 and ALS-mutant TDP-43 (G298S, A315T and A382T) preferentially bind the mRNAs of mtDNA-encoded complex I subunits (ND3 and ND6), and cause the disassembly of complex I by impairing their transcription [121]. Cells expressing wild-type and ALS-mutant (Q331K and M337V) TDP-43 and primary motor neurons expressing TDP-43 M337V were shown to have a reduced mitochondrial membrane potential [124, 125, 120, 121]. TDP-43-associated mitochondrial depolarization is accompanied by a decrease in complex I activity [121]. While studies on patient fibroblasts agree that the expression of TDP-43 G298S and A382T decreases the mitochondrial membrane potential, there is contradictory evidence on whether there is also a decrease in complex I activity, oxygen consumption, and ATP levels [111, 116]. Overall, it is plausible that the gradual depletion of ATP from neurons, which are high energy demanding cells, leads to neuronal degeneration [122].

9.4 TDP-43 Low Complexity Domain and RNP Granules

The C-terminal domain of TDP-43 is a low complexity domain (LCD) sharing homology with prion-like domains, enriched with polar amino acids (asparagine, glutamine, tyrosine, and glycine) characteristic of yeast prions [60]. All but four (A90V, P112H, D169G, N259S) of the over 40 mutations in TDP-43 are clustered in the C-terminal domain (http://alsod.iop.kcl.ac.uk/) (Fig. 9.1b), indicating the importance of this domain in the pathogenesis of ALS/FTLD. A description of these

mutations and their potential functional consequences from animal models and cell culture has been reviewed recently [123, 124]. The C-terminal domain is highly aggregation prone both in vitro and in cell culture, with many disease-associated mutations in this domain enhancing its aggregation propensity [125, 126]. LCDs are common to a number of RBPs, several of which are associated with ALS/FTLD, either through mutation and/or presence in disease pathology, including FUS, hnRNPA1, hnRNPA2, and TIA-1 [21, 127, 128]. RBPs associate with RNAs to form various types of RNP granules, membraneless compartments that have diverse roles in RNA processing, transport, storage, and degradation [129–131]. RNP granules include P-bodies, which store and degrade RNA [132]; stress granules, which triage stalled mRNA translation complexes under various stress conditions, promote translation of proteins necessary for cell survival [133, 134]; and neuronal transport granules, which deliver translationally silenced mRNAs to synapses [131, 135]. RNP granules exhibit properties of liquid droplets, being spherical in shape and undergoing fusion and dissolution, rapidly assembling and disassembling in response to environmental cues [136, 137]. The highly dynamic properties of these structures allow for free diffusion within the granules facilitating rapid exchange with the environment [134]. RBPs appear to form RNP granules by a process liquid-liquid phase separation, a condensed phase distinct from the aqueous phase, mediated by homo- or hetero-oligomerization of their LCDs ([129]. In vitro, the liquid droplets, or hydrogels, formed from the LCDs can undergo a process of molecular aging, or maturation, that appears to be concentration-dependent, transitioning from dynamic structures to amyloid-like fibers [138–143]. This process negatively affects RNA granule function [162]. In TDP-43, the underpinning of this change is linked to an amyloidogenic core region between residues 318–367, which undergoes a structural transformation from α-helix to β-sheet during aggregation [62, 66, 138]. This change in conformation may provide the nidus for pathological TDP-43 aggregation [62].

9.5 TDP-43 and Stress Granules

SGs are formed in response to a variety of stressors such as oxidative stress, osmotic stress, or mitochondrial stress [144–146]. Several hundred proteins are associated with SGs, compositions varying in a cell- and stress-type-specific manner [147, 148]. SGs are considered biphasic, comprising a condensed stable core formed by nucleation of non-translating mRNPs, and surrounded by a less concentrated and highly dynamic shell formed through liquid-liquid phase separation of the composite RNPs [130, 149, 150].TDP-43 is recruited to SGs under a range of environmental stressors in cell culture, including arsenite, paraquat, and sorbitol, and these SGs disassemble when the stressor is removed [151–155]. TDP-43 appears to influence SG assembly/disassembly by regulating expression of G3BP [156]. Moreover, TDP-43 mutations alter the frequency and size of SGs [152, 155, 157]. Interestingly,

a study has shown that paraquat treatment induces TDP-43 SGs in HeLa cells [154]. After removal of paraquat the majority of SGs dissemble, however small amounts of TDP-43 persist, forming ubiquitinated aggregates [154]. This is reminiscent of molecular aging as seen from the in vitro studies of LCDs described above. It has been proposed that defects of SG dynamics are the precursor of TDP-43 pathology in ALS/FTLD. This is based on the observation that TDP-43 pathology in ALS spinal cord is co-labeled with SG markers, TIA-1 and/or eIF3 [158, 159]. However, other studies have failed to show colocalization of SG markers with TDP-43 inclusions [128, 151]. This issue remains unresolved. Stress granules are cleared by autophagy [160]; and it is interesting that a number of genes causing ALS/FTLD affect the autophagic machinery, including VCP, TBK1, SQSTM1, OPTN, and UBQLN2 [161]. This suggests that persistent SG pathology could result as a consequence of impaired autophagy.

9.6 Ataxin 2 Association with TDP-43 and ALS

Ataxin 2 is mainly a cytoplasmic protein with a diverse set of functions ranging from regulating RNA stability and translation to repressing fat and glycogen storage through mTORC1 signaling [162–164]. Polyglutamine tract expansions within the first exon of ataxin 2 cause spinocerebellar ataxia type 2 (SCA2) [165–169]. Normal expansion length is 22–23 glutamines (Q), with >34 Qs causing SCA2. Recently, intermediate length expansions of 27–33 Qs were identified as a risk factor for ALS [170, 171]. Ataxin 2 is a regulator of SG dynamics, with higher levels of ataxin 2 inducing SGs and lower levels reducing SGs [163, 164, 172], including TDP-43 SGs [172]. Downregulation or upregulation of ataxin 2 suppresses or enhances toxicity associated with TDP-43 expression in Drosophila and yeast [170]. Similarly, knockout or knockdown of ataxin 2 extended the life span and reduced pathology of a wild-type TDP-43 transgenic mouse model [172]. The normal life expectancy of the TDP-43 transgenic mouse model used in the study was P24 days with walking deficits apparent at P21 days. Complete knockout of ataxin 2 gave an 80% increase in survival with several animals surviving beyond a remarkable 300 days [172]. One hypothesis for the therapeutic effects of lowering levels of ataxin 2 is that it reduces the number of TDP-43 SGs, the proposed seeds of pathology. Indeed, loss of ataxin 2 reduced the number of TDP-43 aggregates (not defined as SGs) in the brain and spinal cord of the TDP-43 transgenic mouse model used for the study [172]. This supports the idea that abnormal SG dynamics is a key driver of TDP-43 pathology and associated neurodegenerative phenotypes, and that reducing ataxin 2 levels could have therapeutic utility. However, it is possible that ataxin 2 may also be acting through its role as a starvation response factor [162, 173].

9.7 TDP-43 C-Terminal Fragments

In the original studies identifying TDP-43 as a component of pathological inclusion bodies in ALS, a biochemical signature was also described in urea soluble fractions from FTLD-TDP tissues. This comprised the full-length protein at 43 kDa (TDP-43), a species of ~45 kDa corresponding to phosphorylated TDP-43 (P-TDP-43), a higher molecular weight smear corresponding to ubiquitinated TDP-43, and two lower molecular weight species of 24 and 26 kDa corresponding to the C-terminal domain of TDP-43, which separated into at least four species upon dephosphorylation [10, 11]. These lower molecular weight species have been collectively termed TDP-25. Subsequent studies have also identified an N-terminally truncated species of ~35 kDa in ALS or FTLD tissues [83, 174], called TDP-35. Studies have shown that species of 25 and 35 kDa can be generated by caspase 3 cleavage of TDP-43, both in vitro and in cell culture [83, 175]. There are three caspase-3 cleavage sites in TDP-43: DEND (residues 9–12), DETD (residues 86–89), and DVMD (residues 216–219) [83] (Fig. 9.1a). Cleavage at DEND generates a species of ~35 kDa, and cleavage at DETD generates a species of ~25 kDa [83]. Based on these findings, it has been hypothesized that TDP-25 and TDP-35 in ALS/FTLD are generated by caspase-3 cleavage, which has been activated during the neurodegenerative process. Although caspase-3 activation has not unequivocally been shown in disease tissues, this mechanism for the generation of low molecular weight species in ALS/FTLD has become dogma in the field. Using mass spectrometry analysis of disease tissues, additional proteolytic cleavage sites have also been identified, including R208 [176], N291 and N306 [177], and a series of peptides with differing lengths, corresponding to C-terminal domain regions [178, 179]. It is notable that the observed lower molecular weight species from ALS/FTLD tissue extracts correspond to C-terminal regions of TDP-43, and that N-terminal fragments are absent. It is possible that the N-terminal fragments are degraded and the C-terminal spared, perhaps because the C-terminal is pathologically phosphorylated, or that it contains the prion-like LCD, which may be aggregated/misfolded. Indeed, prior studies of human disease tissue have shown that residues 203–209 [180] and residues 341–346 or 341–360 of the Q/N-rich segment are protease resistant [178, 181]. TDP-43 species of 25 and 35 kDa are observed in caspase-3 knockout murine embryonic fibroblasts, indicating that species of these molecular weights can be generated by means other than proteolytic cleavage [182]. This led to the discovery of an alternative translation start site at Met 85, expression from which generates a protein product of 35 kDa [182]. Of note, Met 85 immediately precedes the DETD (residues 86–89) caspase 3 cleavage site (Fig. 9.1a) and as such there are two potential forms of TDP-35, caspase-3 cleaved TDP-35 (C3-TDP-35) and TDP-35 generated by expression from Met 85 (Met-TDP-35) [174]. TDP-35 (either caspase-3 cleaved or Met 85) has a disrupted NLS and mainly localizes to the cytoplasm in transfected cells [182, 183]. Interestingly, TDP-35 spontaneously forms cytoplasmic SGs that recruit nuclear TDP-43 [182]. The TDP-35 SGs are labeled with stress granule markers TIA-1 (Fig. 9.2a–c), G3BP, PABP, and HuR, but not with a P-body marker

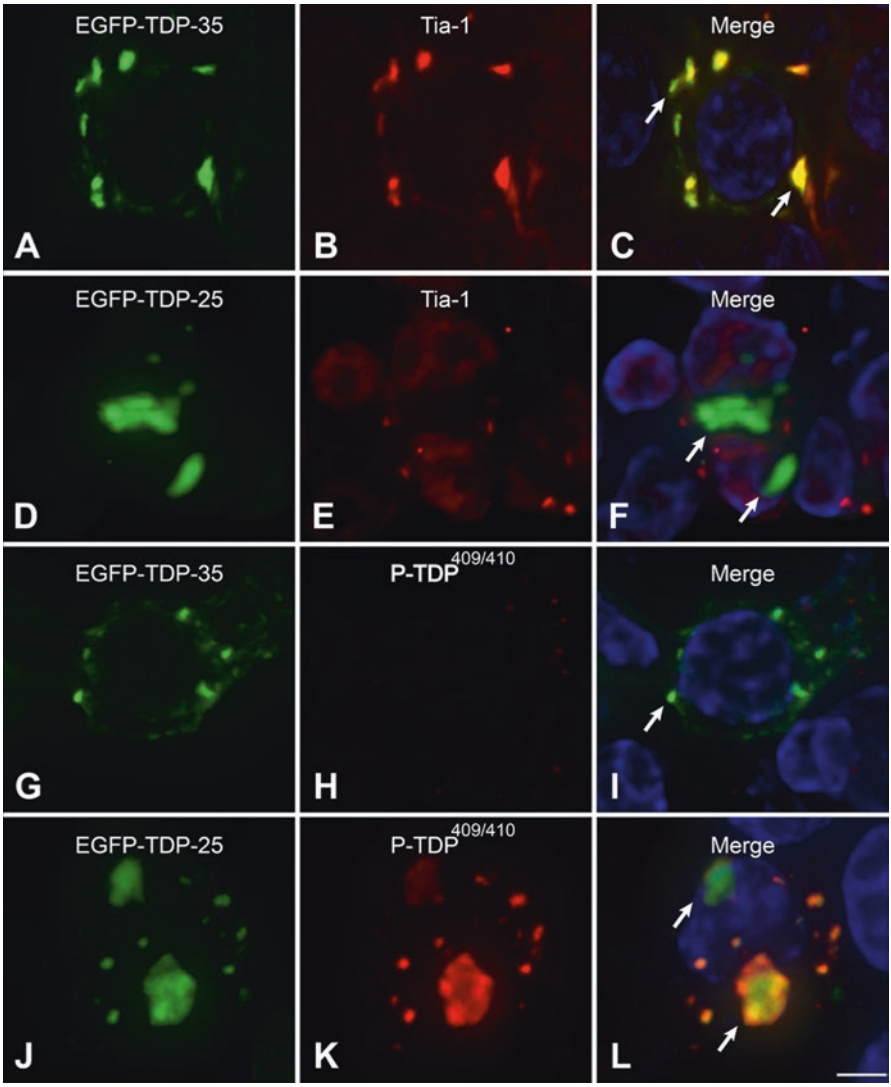

Fig. 9.2 TDP-35 forms stress granules and TDP-25 forms phosphorylated aggregates in transfected HEK 293 cells. HEK 293 cells expressing EGFP-TDP-35 (residues 85–414) (**a**, **g**) or EGFP-TDP-25 (residues 220–414) (**d** and **j**); double-labeling with antibody to TIA-1 (**b**–**e**), or P-TDP-43-409/410 antibody (**h** and **k**) (red); merge with DAPI stain (**c**–**l**). Note cytoplasmic EGFP-TDP-35 (**a**–**c**) but not EGFP-TDP-25 (**d**, **e**) colocalizes with Tia-1 (arrows in **c** and **f**), and EGFP-TDP-25 (**j**–**l**) but not EGFP-TDP-35 (**g**–**i**) colocalizes with P-TDP-43 (arrows in **i** and **l**). Scale bar = 5 μm

DCP1a. The TDP-35 SGs were not labeled with antibody to phosphorylated TDP-43 (phospho 409/410) (Fig. 9.2g–i), but interestingly TDP-35 SGs transformed to a phosphorylated and ubiquitinated aggregated state after treatment with the protease inhibitor MG132 [182]. This finding indicates that TDP-35 SG formation together with deficits in protein quality control can lead to cytoplasmic aggregates with features modeling TDP-43 inclusions in disease. This alludes to a maturation/molecular aging mechanism as described for LCDs above.

TDP-43 autoregulates its own expression and splicing, and a number of TDP-43 splice variants have been reported [70, 71, 184, 185]. A splice variant lacking 91 bp in exon was found upregulated in ALS spinal cord [174]. Expression of this splice variant in cell culture led to the use of the alternate translation start site at Met 85, generating cytoplasmic aggregates of Met 85-TDP-35 that were toxic in primary motor neurons [174]. To verify the genesis of TDP-35 in ALS, neoepitope antibodies were generated that could differentiate between C3-TDP-35 and Met 85-TDP-35 [174]. TDP-43 pathology in ALS spinal cord was labeled with the Met 85-TDP-35 antibody, but not by the caspase 3-TDP-35 antibody, supporting that TDP-35 in ALS is generated through the use of an alternate translation start site (Met 85) and not by caspase 3 cleavage at DETD. Since cytoplasmic TDP-35 can recruit full-length TDP-43, this suggests that TDP-35 could act as a seed for pathological TDP-43 aggregation [68, 174, 182, 186]. It is possible that abnormal splicing of TDP-43 at exon 2 (through means unknown) generates TDP-35 through use of Met 85, which over time forms SGs in a concentration-dependent manner, recruiting full-length TDP-43. As mentioned, TDP-43 functions in the nucleus through oligomerization of its N-terminal domains, thus preventing interaction between the C-terminal domains [73]. Loss of the N-terminal domain, as in TDP-35, would promote interaction between the C-terminal domains, causing aggregation and toxicity [73, 74, 76, 187, 188].

Various other regions and domains of TDP-43 have also been expressed in cell culture [62–64, 67, 69, 74, 75, 114, 151, 152, 189–200]. Expression of C-terminal domains representative of TDP-25 encompassing residues 220–414 [83] or 177–414, 187–414, 197–414 or 208–414 [176], generate cytoplasmic aggregates that are toxic. Unlike TDP-35, these aggregates do not appear to be SGs as they have irregular contours and only very minimally colocalize with SG markers [158, 201] (Fig. 9.2d–f). Instead, the TDP-25 aggregates are ubiquitinated and phosphorylated at 409/410 [83, 176] (Fig. 9.2j–l). Phosphorylation is not necessary for the aggregation of TDP-25 [83]. Instead, evidence suggests that phosphorylation is a defense mechanism against TDP-43 aggregation [202].

9.8 Concluding Remarks

Here, we have given an overview of some of the nuclear and cytoplasmic functions of TDP-43, and the potential consequences if either is perturbed. A major question is what causes TDP-43 to mislocalize from the nucleus to the cytoplasm? Is it a

response to stress, causing nuclear clearing and stress granule formation, with some conformational change in TDP-43 causing aggregation?

Or is it cytoplasmic seeding by disease-associated variants of TDP-43, such as TDP-35? A current view is that TDP-43 pathology could be caused by defects in nucleocytoplasmic transport [203–206]. However, recent studies show that cytoplasmic aggregates of TDP-43 can initiate nucleocytoplasmic transport deficits [207, 208]. These issues remain to be resolved. Finally, there is great interest in prion-like propagation of misfolded/aggregated proteins as a means of spreading disease between cells and between different regions of the brain, as is the case for the microtubule associated protein tau [209, 210] and alpha synuclein [211, 212]. There is evidence that different misfolded conformers, or strains, of these proteins encode strain-specific information generating morphologically distinct types of pathologies [213, 214]. Recent studies suggest that TDP-43 may also spread in a similar fashion [215, 216]. It is tempting to speculate that the types A, B, C, and D TDP-43 neuropathologies observed in ALS/FTLD may be a consequence of different types of TDP-43 strains [215–218].

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Chapter 10
Senataxin, A Novel Helicase at the Interface of RNA Transcriptome Regulation and Neurobiology: From Normal Function to Pathological Roles in Motor Neuron Disease and Cerebellar Degeneration

Craig L. Bennett and Albert R. La Spada

Abstract Senataxin (SETX) is a DNA-RNA helicase whose C-terminal region shows homology to the helicase domain of the yeast protein Sen1p. Genetic discoveries have established the importance of SETX for neural function, as recessive mutations in the *SETX* gene cause Ataxia with Oculomotor Apraxia type 2 (AOA2) (OMIM: 606002), which is the third most common form of recessive ataxia, after Friedreich's ataxia and Ataxia-Telangiectasia. In addition, rare, dominant *SETX* mutations cause a juvenile-onset form of Amyotrophic Lateral Sclerosis (ALS), known as ALS4. SETX performs a number of RNA regulatory functions, including maintaining RNA transcriptome homeostasis. Over the last decade, altered RNA regulation and aberrant RNA-binding protein function have emerged as a central theme in motor neuron disease pathogenesis, with evidence suggesting that sporadic ALS disease pathology may overlap with the molecular pathology uncovered in familial ALS. Like other RNA processing proteins linked to ALS, the basis for SETX gain-of-function motor neuron toxicity remains ill-defined. Studies of yeast

C. L. Bennett
Department of Neurology, Duke University School of Medicine, Durham, NC, USA

Department of Neurobiology, Duke University School of Medicine, Durham, NC, USA

Department of Cell Biology, Duke University School of Medicine, Durham, NC, USA

A. R. La Spada (✉)
Duke Center for Neurodegeneration & Neurotherapeutics, Duke University School of Medicine, Durham, NC, USA

Department of Neurology, Duke University School of Medicine, Durham, NC, USA

Department of Neurobiology, Duke University School of Medicine, Durham, NC, USA

Department of Cell Biology, Duke University School of Medicine, Durham, NC, USA
e-mail: al.laspada@duke.edu

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Sen1p and mammalian SETX protein have revealed a range of important RNA regulatory functions, including resolution of R-loops to permit transcription termination, and RNA splicing. Growing evidence suggests that *SETX* may represent an important genetic modifier locus for sporadic ALS. In cycling cells, SETX is found at nuclear foci during the S/G_2 cell-cycle transition phase, and may function at sites of collision between components of the replisome and transcription machinery. While we do not yet know which SETX activities are most critical to neurodegeneration, our evolving understanding of SETX function will undoubtedly be crucial for not only understanding the role of SETX in ALS and ataxia disease pathogenesis, but also for delineating the mechanistic biology of fundamentally important molecular processes in the cell.

Keywords Senataxin · Helicase · R-Loops · Nuclear exosome · RENT1 · IGHMBP2 · Sen1p · Exosc9 · Sumo · Nucleolus · tRNA

Senataxin (SETX) is now recognized as an important protein in the fields of molecular genetics and neurodegeneration. *SETX* gene mutations lead to two distinct neurological disorders, Ataxia with Oculomotor Apraxia type 2 (AOA2) and Amyotrophic Lateral Sclerosis type 4 (ALS4). AOA2 has uniformly early onset and leads to very severe disability, requiring life-long care [1]. ALS4 is moderate to severe with varying age of onset, but with its average onset at 17-years, ALS4 is considered a juvenile-onset form of familial ALS (FALS) [2]. The *SETX* gene has also attracted recent attention as a potential genetic modifier of sporadic ALS (SALS) [3].

The effort to define key functions of SETX continues as its roles in RNA processing and maintenance of genomic stability are now well established by the molecular genetics community. In early studies, the *SETX* gene was found to be ubiquitously expressed [4, 5], and many functional processes eventually attributed to SETX were originally described for its yeast orthologue, Sen1p. These SETX functions include: (1) RNA polymerase II (RNAP II) transcription termination; (2) the resolution of RNA/DNA hybrids, or R-Loops; (3) processing of noncoding RNAs and mRNA; (4) interaction with the nuclear exosome; and (5) the formation of replication stress-related foci during the S/G_2 transition phase. This last function suggests that SETX may be essential for cell cycling when long genes are being transcribed and RNAP II collides with the replisome. Other important roles for SETX that deserve attention include the regulation of the circadian rhythm genes, *Period* (PER) and *Cryptochrome* (CRY) [6]. Whether these functions are critical to neuron survival is unclear, but highlight the fact that the full spectrum of cellular processes for this helicase is extremely wide-ranging.

In this review, we attempt to clarify the many processes attributed to SETX, and evaluate if SETX gain-of-function toxicity impacts these functions and how this might contribute to motor neuron disease. Detailed proteomics studies have shown that human SETX, like Sen1p, has retained regulatory functions during gene transcription. Interactome analysis of purified TAP-tagged Sen1p identified the RNAP

II subunit Rpo21, along with subunits Rpb2 and Rpb4, as key interactors [7], confirming earlier yeast two-hybrid studies [8]. Furthermore, a number of RNAP I and RNAP III core subunits, elongation factors, and other key components were identified as protein interactors of Sen1p [7]. In contrast, when similar analyses were performed with SETX, no direct interaction with RNAP II was observed, but rather an enrichment within the chromatin fraction, and interaction with RNAP II-related core factors [7]. Importantly, these studies were undertaken with Flag/GFP-tagged SETX, used with HeLa cell stable integration, and at near endogenous levels of protein expression [7]. Hence, human SETX likely retains transcription-related functions, but the regulatory relationships may be quite different in comparison with budding yeast.

In regards to both SETX and Sen1p function, it is crucial to emphasize that these proteins are present at very low abundance; hence, overexpression can lead to aberrant cellular events. Gene duplication can uncover such sensitivity to protein levels as dosage-sensitivity [9, 10]. For example, increased levels of ataxin-2 can distort the cytoplasmic-to-nuclear ratio of TDP-43 and FUS proteins [11], both proteins that are critical in ALS disease. Sen1p is known to be maintained at very low levels in the cell, typically as low as 125 molecules per cell [12], which is much less than its known transcription termination partners, Nrd1p and Nab3p, present at ~19,000 and ~5800 molecules per cell respectively [13]. RNAP II is itself present in yeast at ~14,000 molecules/cell [14, 15]. In a detailed proteomics study using the human U2OS cell line, ~10,000 different proteins were quantified and found to span a concentration range of seven orders of magnitude up to 20,000,000 copies per cell [16]. From this study SETX was found to be in the lowest category of very low-abundant proteins at <500 molecules/cell [16]. Many SETX functional studies have been undertaken with tagged-constructs which will almost assuredly lead to cellular levels of recombinant SETX that are grossly elevated, which means that results of such studies must be interpreted with caution.

10.1 SETX Mutations Cause Both Ataxia with Oculomotor Apraxia and Motor Neuron Disease

Causal links between SETX protein defects and neurological disease were first reported in 2004. We discovered *SETX* gene mutations as the cause of ALS4, a rare, dominantly inherited, juvenile onset form of ALS (OMIM: 602433). Importantly, all 49 affected members of an extended American pedigree were found to carry a L389S mutation [4]. Other sizable European pedigrees were found to segregate R2136H and T3I *SETX* mutations. The L389S mutation was subsequently found in Italian and Dutch pedigrees, confirming the pathogenicity of this mutation in ALS4 [17, 18]. The phenotype of ALS4 is unique compared with classical ALS due to a number of factors, including normal patient life expectancy due to the sparing of the respiratory musculature; absence of bulbar involvement; and the presentation of symmetrical atrophy and weakness [2].

Recessive *SETX* mutations were also reported in 2004 as the cause of a severe Ataxia with Oculomotor Apraxia—type 2 (AOA2; OMIM: 606002) [5]. While dominant mutations are rare, *SETX* recessive mutations are not nearly so rare. AOA2 is considered the third most common autosomal recessive cerebellar ataxia [1, 5], with the most common being Friedreich's ataxia, closely followed by Ataxia-Telangiectasia (A-T). One unique feature shared between AOA2 and A-T is elevated serum levels of alpha-feto-protein (AFP), which are ~9-fold higher in AOA2 patients than normals [19]. A-T is associated with unique DNA repair defects and extra-neurologic features, including a greatly increased cancer risk and immune defects (OMIM: 208900). With AOA2 patients, some groups have reported sensitivity to DNA-damaging agents in patient cells [20], yet others report normal sensitivity [21, 22]. Nonetheless, AOA2 patients do not display an increased cancer risk nor immunological abnormalities, and thus the above features form a differential diagnosis for A-T versus AOA2. AOA2 patients show non-cancer-related extra-neurologic features, such as ovarian failure [23], suggesting tissues other than just the central nervous system (CNS) are susceptible to SETX loss. At the molecular level, Moreira et al. initially reported 15 different *SETX* mutations, ten of which predict premature protein termination. Thus, parental carriers of AOA2 null mutations were carefully examined and found to harbor no neurological phenotypes [1, 5]. Importantly, this suggests that ALS4 dominant mutations possess toxic gain-of-function properties. Now that greater than 150 different *SETX* mutations have been identified to date [24], it is known that missense mutations cluster within either the helicase domain or the amino-terminal domain, confirming the critical nature of these two protein regions [25].

It is of note that within the human genome, only two human proteins exist with significant homology within the helicase domain to SETX: RENT1 (46% similarity) and IGHMBP2 (45% similarity) (Fig. 10.1). RENT1 (the yeast Upf1 orthologue) is an essential component of the nonsense-mediated RNA decay (NMD) pathway for degrading incorrectly spliced or stop codon-containing mRNAs [26], and *Rent1* null mice show embryonic lethality [27], attesting to the importance of this pathway for transcriptome stability. Over-expression of RENT1 (hUpf1) can significantly rescue toxicity in ALS/FTD cell models (TDP-43/FUS) that are likely induced by uncharacterized RNA dysfunction [28]. Recessive mutations of the *IGHMBP2* gene cause a severe spinal muscle atrophy with respiratory distress (SMARD) [29]. This disease has overlap with AOA2 and ALS4, which will be discussed below. Thus, these three human helicase homologs cause either embryonic lethality (Rent1)—due to failure of NMD surveillance of mRNA splicing errors, or specific neuronal vulnerability and neurodegeneration as loss-of-function mutations (SETX and IGHMBP2) or gain-of-function mutations (SETX). Given the known low cellular levels of SETX protein, one could speculate that trace levels of SETX, produced by alternate splicing, might prevent lethality in the human, akin to the minimal levels of normal survival motor neuron protein produced by the SMN2 gene in spinal muscular atrophy [30]. In regards to IGHMBP2, human mutations are known to be homozygous hypomorphic loss-of-function alleles [29], in agreement with the naturally occurring mouse model of SMARD, where ~20% of correctly spliced *Ighmbp2* mRNA is produced [31].

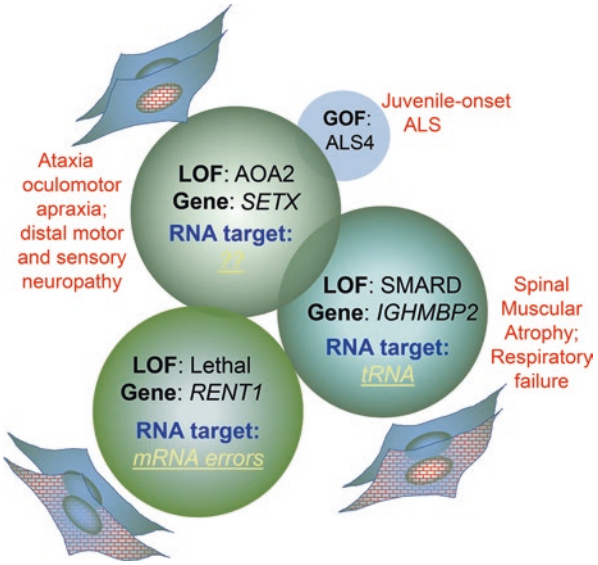

Fig. 10.1 SETX, RENT1, and IGHMBP2 form a helicase subfamily. While there are nearly 100 helicase proteins in the human genome, only two show significant homology to SETX. These three related homologues each perform critical roles in RNA processing, and are essential for survival or for normal central nervous system function. RENT1 is unique in that no human disease results from recessive mutation, likely due to embryonic lethality, and displays a cytosolic localization. The specific RNA targets linked to dysfunction or lethality are known for IGHMBP2 and RENT1, and are listed. In contrast, the specific RNA targets for SETX are unknown, and how altered RNA regulation results in AOA2 or ALS4 remains an open question. SETX resides in the nucleus, while IGHMBP2 localizes to both the nucleus and the cytosol, as shown

10.2 SETX Function: Similarities and Differences with Yeast Sen1p

Soon after *SETX* mutations were discovered, it was noted that SETX and Sen1p shared a highly conserved C-terminal helicase domain. Researchers hypothesized that key functions attributed to Sen1p would likely be retained by human SETX. The *Sen1* gene was named for its suspected function as a splicing endonuclease, but was thereafter found to function primarily in processing a diverse class of noncoding RNAs (ncRNAs) [32, 33]. In further studies, Sen1p was shown to process intron-containing tRNA precursors [34], rRNA precursors [33], 3′-extended forms of some small nucleolar RNAs (snoRNAs), and to a lesser extent, small nuclear RNAs (snRNAs) [33, 35, 36]. Sen1p directly interacts with the RNA-binding proteins Nrd1 and Nab3 [36, 37], and was confirmed as a functioning component of the NRD complex in transcription termination of RNAP II ncRNA transcripts [38, 39]. Taken together, years of dedicated yeast research portray a complex picture of RNA processing, which is essential to yeast survival and directed primarily to the regulation of ncRNAs, as well as a central role in RNAP II transcription termination that may be unique to yeast.

Early SETX work yielded divergent and contradictory views as to what might be its key disease-linked protein function [40]. Readers new to the field could easily be left confused by perusing summaries of the very broad array of proposed SETX functions. An example of one early discrepancy was whether cells lacking SETX were sensitive to DNA-damaging agents. A second function attributed to SETX is a generalized regulation of RNAP II transcription termination. While some reports strongly favor a role for SETX in mediating Xrn2-dependent transcription termination via the formation of R-loops (which are DNA-RNA hybrids formed during the process of transcription) [41], in much the same way as Sen1p, others have concluded that decreased Xrn2 or SETX levels yield only marginal effects on the regulation of transcription termination [42]. However, it is clear that for certain genes and biological processes, SETX is central to transcription termination regulation, as SETX has been shown to control the cyclic expression of the circadian rhythm genes PER and CRY [6]. This work has yielded a model in which recruitment of PER complexes to the elongating polymerase at *Per* and *Cry* termination sites inhibits SETX action, impeding RNAP II release and thereby repressing transcription re-initiation. A third SETX function, reported to be critical for neuron survival, is the resolution of RNAP II-mediated R-loops more generally (independent of termination), which will be discussed later. Fourth, a role for SETX in directing incomplete RNA transcripts to the nuclear exosome has been found in cycling cells when the DNA polymerase machinery collides with active RNAP II transcription.

10.3 R-Loop Resolution Is Not Defective in Setx Null Mice

Another avenue to address the question of which SETX RNA processing functions are most crucial to neuron survival is to create animal models. *Setx* knock-out mice, generated by gene targeted removal of exon four, resulted in near complete loss of SETX protein [43]. However, for a range of reasons possibly including differences in neuroanatomy and lifespan between mice and humans, *Setx* null mice show neither ataxia nor cerebellar degeneration, preventing the possibility to characterize mechanisms of neuron cell death. Failure to recapitulate human recessive ataxias in mice is not without precedent [44], yet a range of critical in vivo studies have been examined in these mice nonetheless. In post-mitotic neurons, R-loops were resolved normally in the cerebellum or brain of mice lacking SETX (similar to wild-type mice), as R-loops could not be detected in wild-type controls or SETX knock-outs. Furthermore, there was no evidence of cells undergoing apoptosis in these tissues in SETX null mice [45]. The authors postulated that the "*major clinical neurodegenerative phenotype seen in AOA2 patients is more likely to be due to a more general defect in RNA processing …rather than a failure to resolve R-loops*". Recent studies from the Libri lab indicate that Sen1p has relatively low processivity on RNA [46]. This is relevant to R-loop removal, R-loops that form in mammals are believed to be very long (>1 kb in humans) [47]. Thus SETX, with its likely low processivity (based upon the Sen1p findings), would not be able to unwind such long structures [46].

10.4 SETX Is SUMO-Modified and Regulates RNAP II Transcription

The multiple RNA processing functions identified for Sen1p undoubtedly require the coordinated efforts of both the amino-terminal protein interaction domain for trafficking, as well as the carboxy-terminal helicase domain for RNA/DNA interaction and processing. In yeast, truncation of the amino-terminal region of Sen1p prevented its proper localization to the nucleolus, though only the helicase domain is required for survival [32]. To better define critical Sen1p protein-binding partners, a yeast two-hybrid (Y2H) screen was employed with follow-up co-immunoprecipitation validation. Only the first 565 residues of Sen1p were required for the identified interactions, again supporting the hypothesis of a crucial function for the amino-terminal domain. Specifically, Sen1p was shown to bind with Rpo21p, the large subunit of RNAP II; Rad2p, a deoxyribonuclease required in DNA repair; and Rnt1p, an endoribonuclease required for RNA maturation [8].

Interestingly, we noted that the Sen1p and SETX protein interaction domains are not conserved at the primary amino acid level [25]. The SETX orthologue in marine vertebrates, such as zebrafish, shows conservation within this domain, but not with fly [25]. However, based upon a hypothesis of functional conservation, we reasoned that a human-specific Y2H screen with the first 650 residues of SETX may identify overlap with Sen1p interactors. The results did bear out some overlap, but to a lesser degree than was expected [48], and was confirmed by a second independent SETX Y2H screen [49]. Our screen also included ALS4 mutants to potentially identify gain-of-function interactors [48], and revealed several important interactor groups that were confirmed with alternate techniques and in other independent proteomics screens [7, 50]. The key interactor groups were: (1) SETX self-interaction or dimerization; (2) critical Sumo/Ubiquitin posttranslational modification; and (3) DNA/RNA-binding proteins, including the exosome component 9 protein (Exosc9).

SETX amino-terminal domain self-interaction and dimerization were validated by purification, size exclusion chromatography, protein cross-linking, and Western blot analysis [48] (Fig. 10.2). Importantly, as ALS is one of a number of conditions in which protein aggregation may drive disease pathogenesis [51], we examined this further. Using techniques including targeted mammalian two-hybrid (M2H) analysis, we found that the SETX mutants L389S (ALS4) and W305C (AOA2) can still engage in self-interaction, and do not lead to excessive aggregation. SETX's ability to dimerize may thus set it apart from Sen1p. Additionally, we found five SETX interactors representing proteins in either the SUMO protein trafficking cascade or the ubiquitin protein degradation pathway. The key interaction with Exosc9 was shown to require SETX SUMOylation [49]. Dramatically, when the exosome components Exosc9 or Exosc10 were depleted by targeted siRNA, this yielded significant co-depletion of SETX [49].

As for the overlap of SETX and Sen1p interactors, several interesting distinctions were noted. The three major Sen1p interactors, Rpo21p, Rad2p, and Rnt1p, were not detected by our Y2H screen. Similarly, but this time in mammalian cells using targeted M2H analysis, we did not detect a direct interaction between SETX and the human

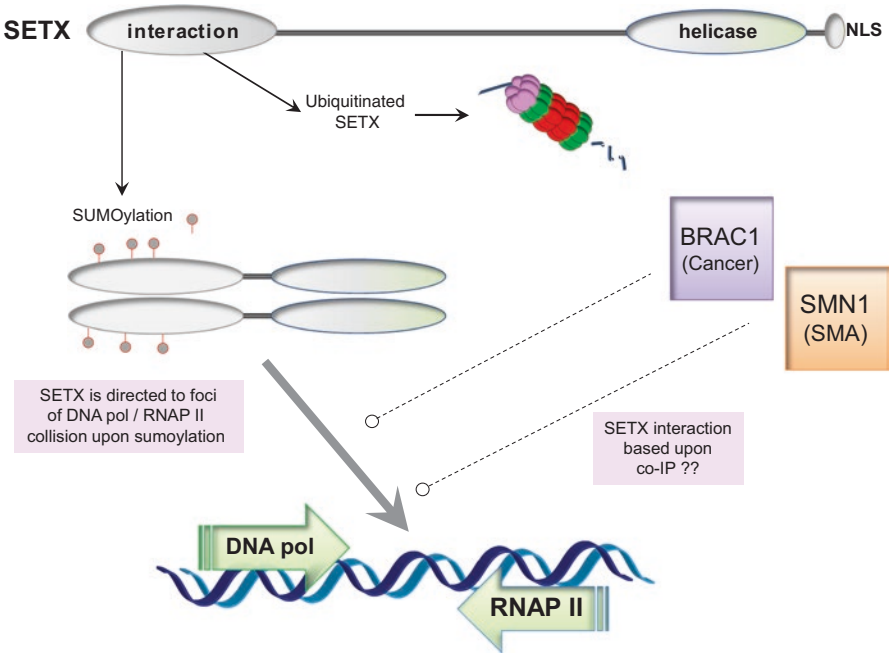

Fig. 10.2 SETX protein domain organization, proposed functions, and protein interactions. The SETX protein is ~303 kDa in molecular mass, and possesses just three known domains: the amino-terminal protein interaction domain, the carboxy-terminal helicase domain, and a nuclear localization signal (NLS) domain. SETX contains amino-terminal sequences that are targets of ubiquitination utilized to degrade SETX protein via the proteasome. The SETX amino-terminal domain possesses regions required for dimerization and for SUMOylation (gray ball and red stem). It is thought that SETX needs to be SUMOylated to direct it to sites of collision between the DNA polymerase-containing replisome and the RNA polymerase-containing transcription machinery. Other key proteins of interest that have been linked with SETX include BRCA1 and SMN1

orthologues to these three proteins (unpublished data). In another proteomics study of Sen1p, tandem affinity purification/mass spectrometry analysis defined this protein as a general transcription factor based upon interactions with RNAP I and RNAP III subunits, as well as with the classic mRNA polymerase complex, RNAP II [7]. This result is consistent with the types of ncRNAs that Sen1p has been previously shown to regulate. Alternatively, proteomics analysis of full-length, GFP/Flag-tagged SETX indicated a general association with the RNAP II complex. A direct interaction was not shown with the core RNAP II subunits (RPB1, RPB2, and RPB3). This suggests that while Sen1p may interact directly with RNAP I, II and III subunits, SETX likely interacts with RNAP II subunits via intermediary associations as periodically directed.

One can conclude that both SETX and Sen1p contain amino acid sequences within the relatively large 500–600 amino-terminal region that are targets for Sumo and Ubiquitin-mediated regulation (Fig. 10.2). For Sen1p, it has been clearly demonstrated that this region is required for signaling its degradation via the ubiquitin proteasome system to maintain low cellular protein levels [52]. Our Y2H screen identified

ubiquitin pathway proteins, Ubc9 and UBC with the amino-terminal region as bait, suggesting similar regulation.

10.5 SETX and Sen1p: A Convincing Role in Connecting RNAP II and the Exosome

Characterizing a specific role for SETX in RNAP II termination has not been convincing, despite some initial reports [50]. A role for Sen1p in this process has been well characterized for ncRNAs, which employ a distinctive mechanism specific for these transcripts in yeast, and not likely used in higher eukaryotes (as noted above). For example, termination of the elongated snoRNA precursors relies upon different machinery than the cleavage and polyadenylation mechanism used for mRNA termination. Rather, ncRNA-specific processing relies on the NRD complex containing the Nrd1 and Nab3 RNA-binding proteins in association with Sen1p [36]. In this case, termination occurs downstream of tetranucleotide motifs, which form binding sites for Nrd1 and Nab3 on the nascent RNA [53]. The Nrd1–Nab3–Sen1p complex, which directly interacts with the RNAP II Carboxy-Terminal Domain (CTD), also directly interacts with the nuclear exosome [54]. Thus, Sen1p as part of the NRD complex forms a bridge between the RNAP II and the exosome to aid in the termination of ncRNA transcripts, such as snoRNAs. In these cases, transcription termination is coupled to 3′–5′ exonuclease trimming by the TRAMP–exosome complex [54]. Extrapolation of the Sen1p termination process to mammals is not readily possible, as the RNA-binding protein Nab3, a critical protein bridging the interaction of Nrd1 and Sen1, has no human homologue [55], and Nab3 has no known role in poly-A-dependent termination [36]. In higher eukaryotes, RNAP II utilizes alternate means of transcription termination [56], and previous studies confirm that SETX is not required for snRNA termination [50].

With regard to transcription, despite likely divergence between the Sen1p and SETX regulation, there is significant evidence to suggest SETX has retained a role for linking RNAP II to the nuclear exosome. As noted above, some degree of SETX co-depletion occurs when major components of the exosome, Exosc9 (Rrp6) and Exosc10 (Rrp45), are depleted. Thus, a model can be proposed that SETX needs to dimerize, and then be SUMOylated as a requirement for its interaction with Exosc9 (and the nuclear exosome) [49], and that transcription-related DNA damage directs the lowly abundant SETX to the exosome in response to such transcription pausing (Fig. 10.3).

10.6 SETX Localization and Function in Cycling Cells

SETX is a large 303 kDa protein that localizes to the nucleus in unsynchronized cell lines. But with different antibodies, several investigators have observed SETX clearly in the nucleolus. Initially, this was not unexpected, as yeast Sen1p was

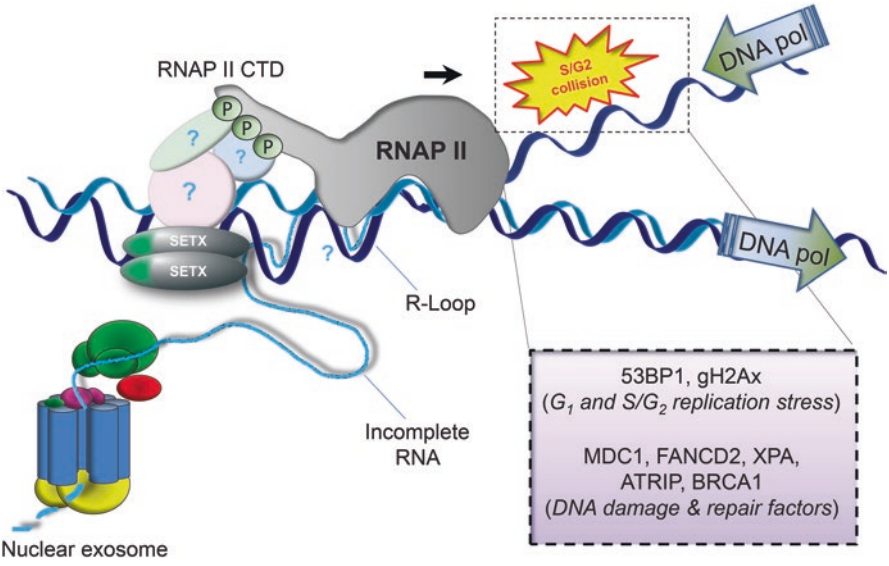

Fig. 10.3 SETX mediates RNA processing and degradation under specific circumstances. The SETX protein interacts with RNAP II via unknown intermediates, but only under certain circumstances, as SETX protein levels are exceedingly low, and SETX may require SUMOylation to direct it to specific foci. SETX foci form at times of replication stress and colocalize with markers such as 53BP1 and γH2Ax. Typical markers of DNA damage and repair factors are found in SETX foci at sites of DNA polymerase and RNAP II collision. According to this model, one likely SETX function is RNA processing via directing incompletely transcribed RNAs to the nuclear exosome for degradation

found to play a major role in the nucleolus, processing rRNA precursors and snoRNAs [33]. Sen1p was also required to maintain the normal crescent shape of the yeast nucleolus, and the temperature sensitive mutant, *Sen1-1*, caused mislocalization of nucleolar proteins, fibrillarin, and Ssb1 [32]. Our initial studies with an affinity-purified SETX antibody revealed strong colocalization with fibrillarin in the nucleolus [57], but further studies are ongoing. A recent publication looking at the possibility of SETX mislocalization found near complete localization to the nucleolus in both control ($SETX^{+/+}$) and patient ($SETX^{R332W/fs}$) fibroblasts [58]. Despite these findings, immunocytochemistry analysis of tagged or endogenous SETX mostly shows a general nuclear localization.

A more detailed analysis of nuclear foci during the cell cycle was recently undertaken with GFP-tagged SETX. These investigators used double thymidine block to synchronize cells and automated wide-field microscopy to visualize SETX dynamic localization. They found that SETX foci were indeed present in the nucleolus at S-phase periodically, but as cells progressed into G_2-phase, SETX became distributed throughout the nucleoplasm [7]. In new studies, evidence was found to link the functions of SETX in potentially directing RNA to the exosome. How does this occur? It should be noted that transcription of large genes can take longer than the

replication phase of the cell-cycle, such that the transcription and replication machinery may collide [59, 60], and SETX has been placed at the sites of these collisions (Fig. 10.3). Upon this backdrop, a range of studies were undertaken to define SETX nuclear foci, in response to phase transitions and drug treatments. In the nucleus, SETX distinct foci were found to be strongest during DNA replication or the S/G_2 phase. When cells were treated with aphidicolin to retard the replication fork, which is a form of replication stress, a two-fold increase in the number of SETX foci resulted [7]. These foci were perfectly colocalized with 53BP1 and γH2AX, markers of spontaneous DNA lesions and transcriptionally active nuclear bodies that form at fragile sites during replication [61] (Fig. 10.3). Then, after treatment of cycling cells with α-amanitin to inhibit RNAP II-mediated transcription, a significant reduction in SETX foci occurred, supporting the idea that coalescence of these distinct foci is dependent on RNAP II transcription. Such SETX-Exosc9 targeted interactions may represent one of the most pivotal roles of SETX, namely to bring functioning exosomes to sites of transcription—replication fork collisions, an interaction that is suggested to depend upon SUMO-2 and SUMO-3 SETX modification [49].

10.7 Lessons from the SETX Homologue IGHMBP2: Role of tRNA Regulation

What is missing from the study of SETX in neurological disease is a smoking gun pointing the way to the RNA pathways that are most affected. Interestingly, the study of SMARD was in a similar quandary, lacking knowledge of the affected RNA pathways; however, recent work has yielded a mechanistic understanding. In 2001, the *IGHMBP*2 gene was identified as the molecular basis of SMARD, when key mutations in six families were reported [29]. SMARD is clinically distinct from SMA, but the IGHMBP2 protein, like SMN1, colocalizes with the RNA-processing machinery in both the cytosol and the nucleus [29]. The mouse model for SMARD is a spontaneous mutant discovered at The Jackson Laboratory known as *nmd* (for neuromuscular degeneration). An important clue to disease mechanism was provided with the discovery that the *nmd* phenotype is suppressed in a semi-dominant fashion by the presence of a modifier region on mouse Chromosome 13 from strain CAST/EiJ [31]. The critical region for rescue is limited to just 166 kb, defined by a BAC clone which contains several tRNA genes, including five $tRNA^{Tyr}$ genes, one $tRNA^{Ala}$ gene, and activator of basal transcription 1 (Abt1) [62]. The *nmd* mice are characterized by motor neuron degeneration with axonal loss leading to neurogenic muscle atrophy and death at 8–12 weeks of age. The phenotypic rescue of *nmd* mice by the Chromosome 13 modifier is dramatic with ventral nerve roots showing completely normal axonal morphology and density at 6–7 weeks of age [63]. Finally, IGHMBP2 has been shown to physically associate with tRNAs, and in particular with $tRNA^{Tyr}$ and the tRNA transcription factor TFIIIC220 [62].

In 2013, further investigations revealed that aberrant tRNA processing can lead to neurodegeneration. The first mammalian RNA kinase to be identified was CLP1, and kinase-dead mice for this protein (*Clp1*$^{K/K}$) were generated [64]. On several genetic backgrounds, *Clp1*$^{K/K}$ homozygous pups were nonviable, but on the CBA/J background, mice survived to ~23 weeks of age. *Clp1*$^{K/K}$ mice display loss of spinal motor neurons associated with axonal degeneration in the peripheral nerves and denervation of neuromuscular junctions and respiratory failure [64]. Transgenic studies demonstrated that CLP1 functions in motor neurons, and that reduced CLP1 activity results in the accumulation of a novel set of small RNA fragments, derived from aberrant processing of pre-tRNA^{Tyr}. In 2014, a CLP1 R140H homozygous missense mutation was reported in five unrelated human families [65]. These patients suffered severe motor-sensory defects, cortical dysgenesis, and microcephaly. Biochemically, these presumed hypomorphic mutations lead to a loss of CLP1 interaction with the tRNA splicing endonuclease complex, greatly reduced pre-tRNA cleavage activity, and accumulation of linear tRNA introns [65].

Many other examples of tRNA biogenesis dysfunction leading to neurodegeneration exist. For brevity, we name just two: (1) an editing-defective tRNA synthetase causes protein misfolding and neurodegeneration in the *sticky* mouse [66]; and (2) a mutation of a CNS-specific tRNA causes neurodegeneration induced by ribosome stalling [67]. These examples serve to support the story of tRNA processing dysfunction in neurological disease and lend further credence to the mechanistic understanding of SMARD caused by IGHMBP2 recessive loss-of-function mutations. Similarly, methods and approaches that will reveal key insights into the most critical SETX RNA processing pathways for neuron health and survival are needed. While the lack of neurological phenotypes in *Setx* knock-out mice prevents the identification of similar modifier effects, other methodologies are likely to emerge to provide similar insight into RNA processing maintenance in neurodegeneration phenotypes caused by loss of SETX function.

10.8 SETX Gain-of-Function Motor Neuron Toxicity in ALS4 and Its Possible Role in Sporadic ALS

Here, we have considered two unique neurodegenerative disorders, AOA2 and ALS4, which represent the genotype/phenotype spectrum resulting from *SETX* mutation, and sought to underscore which RNA processing functions are most relevant to neurodegeneration. We began by recognizing that many functions attributed to SETX were extrapolated from its yeast orthologue Sen1p, which had been thoroughly studied long before *SETX* mutations were first discovered. Notably, Sen1p homology to SETX is restricted to an ~500 amino acid carboxy-terminal helicase domain, with no other regions of the large 303 kDa SETX protein conserved. The SETX amino-terminal protein interaction domain is divergent at the sequence level, but appears functionally conserved (with new protein interactions). Upon detailed examination, not all functions of yeast Sen1p were retained by

mammalian SETX, which functions in a multicellular organism where cell cycling regulation has become much more elaborate. The well-characterized function of *general* transcription termination in Sen1p is not likely conserved in SETX; instead, SETX regulation of transcription termination is restricted to specific genes and cellular pathways, including interestingly circadian rhythm control. Whether disruption of this pathway is relevant to motor neuron health and ALS neurodegeneration remains to be studied. Insofar as future research is concerned, it is important to recognize that Sen1p and SETX proteins are present at exceedingly low levels in the cell. Thus, studies with Sen1p or SETX which utilize massive transient over-expression will likely generate results that are not physiologically relevant.

One key SETX function, conserved from Sen1p, is direct engagement with the nuclear exosome. Two studies demonstrate that SETX interacts with Exosc9 [48, 49], and is regulated by the SUMO and ubiquitin cascade pathways. One group has demonstrated that it is this SUMO-2/3 modification at the amino-terminus that is specifically required for interaction with the exosome and that co-depletion of SETX occurs with either Exosc9 or Exosc10 knock-down [49]. SETX was shown to be present in specific nuclear foci during S/G2-phase human synchronized cells coincident with collision of the DNA replication machinery and the RNA transcriptome [7]. These SETX foci were described as representing replication stress, and at these foci, the SETX interaction with the nuclear exosome was specifically present and enriched [49]. SETX thus appears to play a key role in directing incomplete RNA transcripts to the exosome for degradation (Fig. 10.3). The connection between SETX and exosome regulation deserves further consideration as a possible explanation for how SETX gain-of-function toxicity results in motor neuron disease. Interestingly, recessive loss-of-function mutations in Exosc3 yield infantile-onset motor neuron disease in human pontocerebellar hypoplasia with spinal muscular atrophy type 1B (PCH1B; OMIM 614678) [68], and Exosc3 interacts with matrin-3 [69], a known ALS gene. Furthermore, recessive loss-of-function mutations in Exosc8 yield infantile-onset motor neuron disease in human pontocerebellar hypoplasia with spinal muscular atrophy type 1C (PCH1C; OMIM 616081). These inherited motor neuron degeneration phenotypes highlight that alterations of exosome function are particularly poorly tolerated in cerebellar and motor neurons, two CNS regions where altered SETX function results in neuronal demise. However, another point to consider is that neurons are not cycling cells; hence, the role of SETX in resolving collisions between the replication machinery and the RNAP II transcription complex could actually play out in non-neuronal cells. As glia comprise the bulk of CNS cells, it seems reasonable to propose that neuron demise in ALS4 and AOA2 could be the result of a non-cell-autonomous process occurring in astrocytes or another non-neural CNS cell type.

A final important point to consider when seeking an explanation for SETX neurotoxicity is that SETX belongs to a group of just three homologous proteins, the other two being IGHMBP2 and RENT1. Of this trio, RENT1 is specifically implicated in NMD [70], and its role in NMD appears critical, as loss of function of RENT1 leads to embryonic lethality in mice, with no known human disease correlate. The importance of this helicase protein for RNA toxicity in neurons is suggested by its ability to rescue TDP-43 and FUS ALS-linked cellular pathology [28].

The other member of the trio is IGHMBP2, recessive mutations of which cause SMARD. In this disorder, key processing events for tRNA appear to be the responsible RNA pathway affected. As SMARD is related to autosomal spinal muscular atrophy (SMA), the theme of altered RNA function is reinforced, as SMN protein is essential to spliceosomal snRNP biogenesis and thus the integrity of RNA splicing, and therefore has become a model for understanding RNA dysfunction in neurodegeneration [71]. RNA-binding proteins, such as TDP-43, have also been centrally implicated in ALS, but understanding the role of TDP-43 in motor neuron neurodegeneration is proving to be challenging. There may be multiple dominant, recessive, and toxic mechanisms at play throughout the disease process. However, an intriguing theory based upon loss of function, which necessarily occurs with nuclear clearance of TDP-43, is that of impaired repression of non-conserved cryptic exons [72]. SETX gain-of-function mutations cause ALS4, which is a rare disease, and while several dominant mutations have been linked to ALS4, by far the most penetrant mutation to study is the L389S substitution. Clues to disease mechanism based upon SETX L389S toxic gain-of-function await the description of new mouse models that have been produced and are being characterized. Furthermore, based upon independent SALS exome sequencing reports [3, 73–75], SETX is emerging as a common target for mutation, especially in SALS patients who carry mutations in established pathogenic genes, including C9orf72 repeat expansion carriers [75]. These observations support the hypothesis that these recently discovered disease-linked polymorphisms in SETX could be modifiers of SALS. Hence, future research into SETX normal function and altered action upon gain-of-function mutation holds great potential for advancing our understanding of not just ALS4 motor neuron disease but also for much more common sporadic ALS as well.

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Chapter 11
Lost in Translation: Evidence for Protein Synthesis Deficits in ALS/FTD and Related Neurodegenerative Diseases

Erik M. Lehmkuhl and Daniela C. Zarnescu

Abstract Cells utilize a complex network of proteins to regulate translation, involving post-transcriptional processing of RNA and assembly of the ribosomal unit. Although the complexity provides robust regulation of proteostasis, it also offers several opportunities for translational dysregulation, as has been observed in many neurodegenerative disorders. Defective mRNA localization, mRNA sequatration, inhibited ribogenesis, mutant tRNA synthetases, and translation of hexanucleotide expansions have all been associated with neurodegenerative disease. Here, we review dysregulation of translation in the context of age-related neurodegeneration and discuss novel methods to interrogate translation. This review primarily focuses on amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), a spectrum disorder heavily associated with RNA metabolism, while also analyzing translational inhibition in the context of related neurodegenerative disorders such as Alzheimer's disease and Huntington's disease and the translation-related pathomechanisms common in neurodegenerative disease.

Keywords mRNA · Translation · Ribosome · ALS · FTD · TDP-43 · c9orf72 · RNA-binding proteins

E. M. Lehmkuhl
Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ, USA
e-mail: eriklehmkuhl@email.arizona.edu

D. C. Zarnescu (✉)
Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ, USA

Department of Neuroscience, University of Arizona, Tucson, AZ, USA

Department of Neurology, University of Arizona, Tucson, AZ, USA
e-mail: zarnescu@email.arizona.edu

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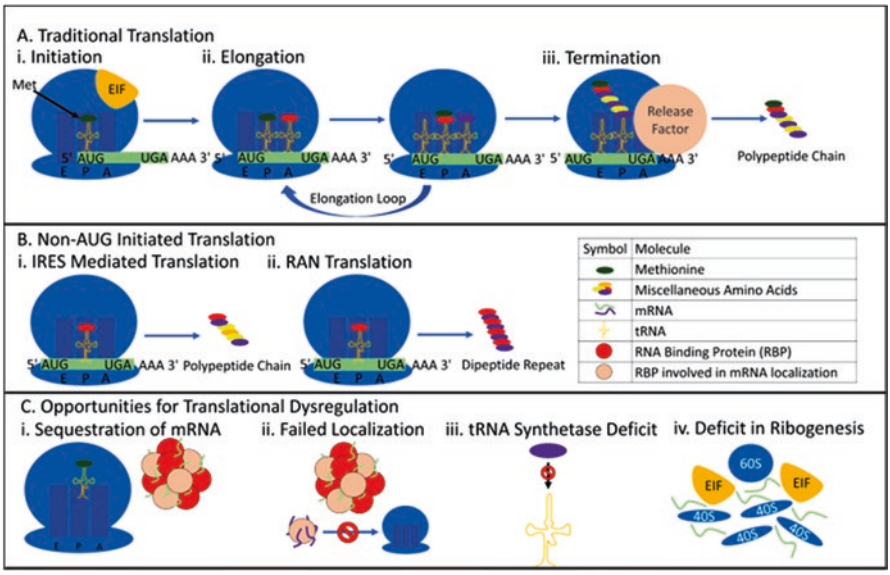

Fig. 11.1 Translation in normal conditions and disease. (**a**) (i) The process of polypeptide synthesis begins with the attachment of the AUG start codon within the mRNA template to the small ribosomal subunit. A transfer RNA (tRNA) specific to the AUG codon positions a methionine (Met) residue on the P (Peptidyl) site of the small ribosomal subunit, forming a translation initiation complex with the aid of translation initiation factors. (ii) Elongation of the peptide chain occurs through the A (Acceptor) site where a tRNA specific to the next codon within the mRNA template recruits the proper amino acid, which is bound to the initial Met at the P site with a peptide bond. (iii) Using this stepwise strategy, the peptide growth continues in an elongation loop until a stop codon (UAA, UAG, or UGA) is reached, causing specialized proteins called release factors to free the mRNA template as well as the newly formed polypeptide. (**b**) Non-AUG Initiated Translation. (i) Internal Ribosome Entry Site (IRES) translation generates normal polypeptide chains. (ii) Repeat Associated Non-AUG (RAN) translation forms toxic dipeptides. (**c**) Errors in translation. (i) Sequestration of mRNA in protein-mRNA granules prevents integration into a ribosome complex and therefore translation. (ii) Trafficking proteins are necessary for proper mRNA trafficking; their absence or dysfunction leads to a lack of mRNA in specific cellular locations. (iii) Deficient synthesis of tRNA prevents polypeptide addition, even with proper translation initiation. (iv) Deficits in ribogenesis reduce the number of actively translating ribosomal complexes

11.1 Introduction

In eukaryotes, normal translation relies on the assembly of a small (40S) and a large (60S) ribosomal subunit into fully assembled (80S) ribosomes. Each subunit comprises several ribosomal proteins and RNAs (rRNAs) that work together to catalyze protein synthesis using messenger RNA (mRNA) as a template (Fig. 11.1a). Translation is a major contributor to protein homeostasis (proteostasis) and its dysfunction has the potential to affect all cellular functions [1].

In addition to canonical AUG-dependent translation, a non-AUG version, known as Internal Ribosome Entry Site (IRES)-mediated translation, can take place via

ribosomal attachment and elongation independent of a start codon (Fig. 11.1bi). Although no consensus sequence is known, the majority of IRESs are located near the 5′ end of the mRNA [2]. Interestingly, tau, one of the major proteins implicated in Alzheimer's disease, has been shown to undergo IRES translation, although the contribution, if any, of this mechanism to pathogenesis remains unknown [3]. A version of IRES translation is Repeat Associated Non-AUG (RAN) translation (Fig. 11.1bii), which has been associated with mutant microsatellite expansions in traditionally noncoding portions of the genome such as untranslated regions (UTRs) or introns, and more recently with coding regions (recently reviewed in [4, 5]). In the absence of an AUG codon, translation is possible because the hairpin structure formed by microsatellite mRNA can mimic the methionine tRNA that initiates translation [6]. Although dipeptide repeats generated via RAN translation and their distinct contribution to disease will be briefly discussed in this chapter, a more in-depth review has recently been published [7].

While the majority of translation occurs within the cytosol or ER bound ribosomes, 13 vital components of the oxidative phosphorylation complex are translated within the mitochondria, which host their own translational machinery including different ribosomal subunits, initiation factors, and tRNAs [8].

Regardless of its type, the complexity of translation as a highly regulated stepwise process provides numerous opportunities for errors caused by inhibition or deficits at any of these stages (see Fig. 11.1). Translation dysregulation has been implicated in several hereditary neurological disorders (reviewed in [9]). For example, loss-of-function mutations in one of five genes encoding subunits for the eukaryotic translation initiation factor eIF2B result in childhood ataxia, characterized by infant encephalopathy and later onset cognitive and motor impairment [10]. In Charcot-Marie-Tooth disease, an inherited neurodegenerative disorder, mutations in glycyl-tRNA synthetase (*GARS*), one of five tRNA synthetases linked to disease, disrupts translation [11]. FMRP, a protein implicated in Fragile X syndrome, regulates translation, in part by associating with ribosomes via direct binding to L5 protein [12]. Furthermore, mutations in *RPS19*, a small ribosomal subunit, have been associated with Blackfan Diamond Anemia, a deficiency in red blood cells that also leads to cognitive dysfunction [13]. Deficient mitochondrial translation has also been implicated in several neurological disorders; non-functional mitochondrial aspartyl or glutamyl-tRNA synthetases lead to leukoencephalopathy associated with ataxia, spasticity, and cognitive decline [9, 14].

Additionally, inhibition of translation can contribute to age-related neurodegenerative disorders. For example, in amyotrophic lateral sclerosis (ALS), TDP-43 sequesters mRNA away from translating ribosomes [15]. In Alzheimer's disease, microRNA-29, which regulates the expression of memory associated mRNAs (e.g., *BACE1*, a secretase implicated in the formation of pathogenic amyloid plaques) is downregulated causing uptranslation of its targets [16]. Translation is also expected to be affected by nucleolar stress and reduced rRNA biogenesis that have recently been associated with hexanucleotide repeat expansion (G4C2 HRE) within the first intron of *c9orf72*, the most common cause of ALS/FTD [17]. The role of translation in progressive neurodegeneration disorders is an exciting, developing field that is

poised to uncover new therapeutic strategies. This chapter will focus on the mechanisms by which errors in translation contribute to age-related neurodegenerative disorders with references to modern methodologies for probing translation deficits in vivo.

11.2 Translational Alterations in Different Types of ALS and ALS/FTD

ALS is a progressive neurodegenerative disorder causing death of motor neurons [18]. Although 90% of ALS cases are sporadic, several genetic loci have been linked to both familial and sporadic cases and have been shown to be involved in a plethora of biological process ranging from ribostasis (e.g., *TARDP*, *FUS*, *senataxin*, *Gle1*) to proteostasis (e.g., *ubiquilin*, *SOD1*, *VCP*) [19]. Although these different gene products contribute directly to various specific aspects of cellular function, translation has been found to be directly or indirectly altered in the context of disease causing mutations, or in the context of wild-type TDP-43 pathology, which represents 97% of ALS and 45% of FTD cases. Recently, several genes linked to ALS were also shown to cause FTD leading to reframing of ALS and FTD as a spectrum disorder [20]. Here, we summarize the current state of the field in regards to translation dysregulation in different types of ALS/FTD.

SOD1—*SOD1* (Superoxide Dismutase) was the first gene associated with ALS in 1993 and remains solely associated with motor neuron disease across the ALS/FTD continuum. In a hallmark paper, Bruijn et al. demonstrated that a gain of toxic function rather than loss of enzymatic activity is responsible for neurodegeneration [21]. Although to date, the pathogenic mechanism of SOD1 has remained elusive, a great deal of evidence provides support for oxidative stress caused by mitochondrial dysfunction. Several hypotheses exist to explain the mechanism by which mutant SOD1 affects mitochondrion dysfunction (reviewed in [22]). Among these, Tan et al. showed that in $SOD1^{G93A}$ ALS mice, mutant SOD1 induces a conformational change in Bcl-2, which leads to reduced permeability of the mitochondrial membrane through altered interactions with Voltage Dependent Anion Channel 1 [23]. The loss of mitochondrial membrane polarity and subsequent oxidative stress lead to an increase in protein misfolding, causing a cascade of secondary effects including an unfolded protein response, which in turn inhibits global translation (reviewed in [24]).

Recently, Gal et al. discovered a novel role for mutant SOD1 pathogenesis [25]. In both $SOD1^{G93A}$ mice and patient-derived fibroblasts, mutated SOD1 was identified in inclusions containing TIA1 and G3BP1, two core components of stress granules [26]. Additional disease-associated variants $SOD1^{A4V}$ and $SOD1^{G85R}$ co-precipitated with G3BP1, indicating that stress granule interaction affects the pathogenesis of multiple SOD1 mutants. Co-precipitation occurs even following RNAse treatment suggesting that the G3BP1-mutant SOD1 interaction is not RNA dependent. Indeed, co-precipitation of truncated G3BP1 mutants and $SOD1^{A4V}$ indi-

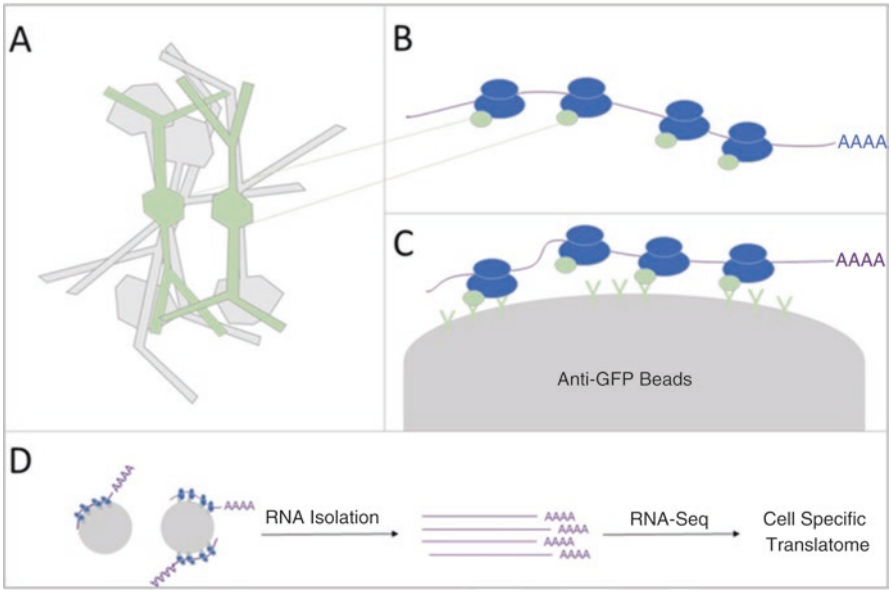

Fig. 11.2 Tagged ribosome affinity purification. (**a**, **b**) Model system-specific expression systems allow expression of tagged ribosomal subunit (RPL10-GFP) to be specifically expressed in cell types of interest (motor neurons or glia, green). (**c**) Using anti-GFP antibodies, the tagged ribosomal subunits are immunoprecipitated out of the whole body lysate. (**d**) Immunoprecipitated mRNA is isolated and subjected to RNA sequencing and bioinformatics to identify cell-specific translatomes normalized to input mRNA levels

cated that the RNA Recognition Motif (RRM) of G3BP1 is necessary and sufficient for the G3BP1-*SOD1*^{A4V} binding. Most importantly, the presence of *SOD*^{A4V} negatively correlated with stress granule formation, indicating a causal relationship between mutant SOD1-G3BP1 binding and stress granule dynamics [25]. Although the mechanism remains unclear, this work establishes a novel relationship between SOD1 mutants and stress granule dynamics, and suggests alterations in translation.

This possibility was addressed in a 2015 study, which examined translational changes that occur in mouse motor neurons, astrocytes, and oligodendrocytes during SOD1 driven ALS pathogenesis [27]. Previous cell-type-specific studies have relied on physical separation of the cell type of interest; this has numerous limitations including potential contamination and exclusion of axons and dendrites. This innovative study employed tagged ribosome affinity purification (TRAP), which allows the identification of cell-specific translatomes from intact, whole organisms (Fig. 11.2).

To define cell-type-specific translatomes, Sun et al. expressed GFP-RPL10 in motor neurons, astrocytes, and oligodendrocytes of *SOD1*^{G37R} mice using cell-specific promoters *Chat*, *Aldh111*, and *Cnp1*, respectively [27]. Importantly, the *SOD1*^{G37R} mutant line recapitulated the expression levels of endogenous SOD1, which is expressed in astrocytes and oligodendrocytes at 30% and 40% of motor

neuron levels, respectively. The spinal cords of the mice were isolated at 8 months of age, corresponding to disease onset when muscle denervation has begun but phenotypes are not overtly present. Immunoprecipitation of GFP-RPL10 and subsequent RNA-seq followed by bioinformatics defined the translatome of each cell type.

At 8 months, Sun et al. observed that motor neurons exhibit upregulated translation of the components of the PERK (PRKR-like ER kinase)-mediated unfolded protein response (UPR) [27]. Protein misfolding induces PERK to phosphorylate eukaryotic initiation factor 2 alpha (eIF2α), which in turn upregulates translation of ATF4, a transcription factor that enhances the UPR response [28]. Translation of both ATF4 and its target transcripts (e.g., heat shock proteins *HSF1* and *HSF2*) was increased. Interestingly, the other components of UPR, namely ATF6 and IRE1, were not induced in disease onset motor neurons.

Laser microdissection was used to isolate motor neurons at the early symptomatic age of 10.5 months. Quantiative PCR experiments showed elevated ATF4 expression indicating that the UPR response continues through disease progression and is not limited to onset. A parallel experiment using $SOD1^{G85R}$ mice concluded that PERK-mediated UPR was also upregulated in these mutants, consistent with the idea that ER stress is common to SOD1 mediated pathogenesis.

Using the TRAP approach, 8 months old mouse astrocytes revealed an upregulation of inflammation related proteins (e.g., transcription factors Cedpb and Cedpd) while mRNAs encoding transcriptional co-activators for metabolic genes (e.g., PRRX1 and SERTAD2) experienced decreased translation. Upregulation of inflammation is characteristic of an astrocyte response to neuron damage. However, the increased translation of transcription factor PGC1α, related to metabolism and nuclear receptors, was also observed. Since upregulation of PGC1α is not characteristic of astrogliosis, it suggests that at least in part, SOD^{G37R} pathogenesis in astrocytes is independent of neuronal damage and may reflect a cell autonomous response to mutations in *SOD1* by astrocytes.

In contrast, oligodendrocytes exhibited minimal translational changes at 8 months, when mice were presymptomatic. However, profiling their translation again at the early symptomatic age of 10.5 months revealed that oligodendrocytes exhibit increased translation of transcripts involved in phagocytosis (e.g., *Rac2* and *Phosophoinositide Phosopholipase C*) accompanied by a predicted decrease in proteins involved in myelination (e.g., CAMK2β).

From their findings, Sun et al. propose a model of SOD1-mediated pathogenesis where mutant SOD1 first induces motor neuron damage through the induction of ER stress [27]. Motor neurons may be selectively vulnerable because of high SOD1 expression and low ER chaperone presence. The effects of motor neuron damage are then amplified by subsequent damage to astrocytes and oligodendrocytes. The translational profiling conducted by Sun et al. provides elegant insights into translatome alterations in vivo, in a cell type and temporal-specific fashion, that highlight the central role of motor neurons in disease.

TDP-43—Encoded by the TAR DNA-Binding (*TARDP*) gene, TDP-43 is an RNA-binding protein comprising two RRM domains [29]. Remarkably, >97% of

patients, regardless of etiology (with a couple of exceptions, including *SOD1* and *FUS* mutations) exhibit proteinaceous aggregates containing the RNA-binding protein TDP-43 [30]. TDP-43 has been implicated in several aspects of RNA processing including mRNA transport and localization to the distal ends of neurites including synapses [31–33]. While TDP-43 is normally required for RNA processing (e.g., splicing) [29], RNA binding is also required for toxicity [34], highlighting the involvement of RNA-based mechanisms in TDP-43 pathogenesis.

Recent studies have shed light into the mechanism by which TDP-43 contributes to ALS pathogenesis. In 2014, Coyne et al. [32] used a previously described *Drosophila* model of ALS [35, 36] based on TDP-43 overexpression to identify *futsch* as physiologically relevant target of TDP-43 regulation. *Futsch* mRNA was shown to be increased in motor neuron cell bodies, but decreased at neuromuscular synapses, consistent with failed mRNA localization. Polysome fractionations of ALS larvae indicated a shift of *futsch* mRNA from actively translating ribosomes to untranslated ribonucleoprotein particle (RNP) fractions consistent with translation inhibition [32]. This combination of defects in RNA localization and translation leads to increased levels of Futsch protein in motor neuron cell bodies, which was confirmed to also occur for its mammalian homolog, MAP1B, in spinal cords from ALS patients. This pathological alteration in Futsch/MAP1B, a microtubule stabilizing protein is consistent with neuromuscular junction (NMJ) instability, which was observed in the fly model. Notably, restoration of *futsch* levels by genetic overexpression mitigates ALS phenotypes including locomotor defects, TDP-43 aggregation, and reduced lifespan suggesting that *futsch* is an important mediator of TDP-43 toxicity in vivo.

TDP-43 knock-down studies in mouse hippocampal neurons have indicated that *Rac1* levels increase at the translational level and this affects spine morphogenesis in dendrites [37]. Together with observations that AMPAR clustering is increased following synaptic stimulation, these findings support a role for TDP-43 in plasticity. The human disease relevance of these observations remains to be established in future studies.

Recently, an interesting mechanistic connection has been identified between ribostasis and proteostasis [15]. Using the same *Drosophila* model of ALS [35, 36] the authors identified *hsc70-4* mRNA as a candidate target of mutant but not wild-type TDP-43 [15]. Hsc70-4 is a conserved member of the Hsc70 family of constitutive chaperones with several roles in protein folding, degradation and various cellular processes including stress response, and chaperone-mediated autophagy [38]. Specifically, Hsc70-4 regulates synaptic vesicle cycling, and just like its cognate mRNA was found to associate preferentially with mutant TDP-43. The consequence of this preferential association with mutant TDP-43 is the sequestration of *hsc70-4* mRNA accompanied by translation inhibition, which in turn leads to defects in the synaptic vesicle endocytosis. A similar post-transcriptional reduction was observed in C9 ALS fly and patient-derived motor neurons, although it remains to be determined whether this is caused by translation inhibition as was the case with TDP-43 models. Notably, restoration of Hsc70-4 through genetic overexpression mitigated ALS phenotypes in a variant-dependent manner suggesting that

although both wild-type and mutant TDP-43 contribute to ALS pathogenesis, they do so through distinct mechanisms [15].

Additional links between TDP-43 and protein synthesis have been uncovered by biochemical studies showing its association with several RNA-binding proteins involved in translation including eukaryotic initiation factors and Fragile X Mental Retardation Protein (FMRP) [39–43]. FMRP overexpression was found to attenuate locomotor dysfunction and increase lifespan in a fly model of ALS based on TDP-43 [42]. Genetic interactions and fractionation experiments collectively led to a model whereby FMRP remodels TDP-43/RNA complexes and releases sequestered mRNA, which can subsequently be translated and mitigate TDP-43 toxicity. Interestingly, FMRP and TDP-43 appear to share translation targets including *Rac1* and *futsch* mRNAs, highlighting previously unknown common mechanisms between neurodevelopmental conditions such as Fragile X syndrome and neurodegenerative diseases like ALS/FTD.

TDP-43 has also been found to regulate translation globally [44]. Using an Affymetrix exon array, Fiesel et al. [44] evaluated splicing variants in HEK293E human embryonic kidney cells following knockdown of TDP-43 with small interfering RNA (siRNA). This study showed that loss of TDP-43 induced alternative splicing of *S6 kinase 1 Aly/REF-like target* (*SKAR*). In addition to being previously associated with spliced mRNA, SKAR also recruits S6 Kinase 1 to protein-mRNA granules to promote translation downstream of mTOR signaling [45]. Knock-down of TDP-43 causes exon 3 exclusion and generation of SKARβ, which results in increased phosphorylation of S6 K1 and its targets, leading to increased global translation [44]. It remains to be determined whether global translation is also altered in patients. The most compelling evidence so far of global translation dysregulation comes from findings that genetic and pharmacological inhibition of eIF2α phosphorylation mitigates ALS phenotypes in fly and cultured cells models [46]. However, given the intimate connections between UPR and global translation that eIF2α mediates, more studies are needed to determine the extent of translation dysregulation in disease pathogenesis.

Given its known interactions with protein partners, TDP-43 appears to be involved in the regulation of translation at multiple steps. Studies have identified both a normal role for TDP-43 in the regulation of global translation in cutured cells, and specific mRNAs targets, including mutant-specific targets in motor neurons, in the context of disease. A distinction needs to be made between TDP-43's normal role in various cell types and how that role changes in disease and more studies are needed to address this important question. The variety of ways in which TDP-43 interacts with the translational machinery leads to additional questions regarding the role of TDP-43 and RNA in ALS pathogenesis.

FUS—Mutations in *Fused in Sarcoma* (FUS), which encodes a nuclear RNA-binding protein, have also been associated with 4% of familial ALS with autopsy showing cytoplasmic inclusions of FUS [47]. Although no specific alterations have been identified in translation in the context of FUS ALS, given the aggregation of mutant FUS^{P525L} in complexes containing nuclear-cytoplasmic shuttling proteins (e.g., hnRNP A1 and A2), spliceosome assembling proteins (e.g., SMN1), and

mRNA [48], it is reasonable to predict indirect changes in the translatome caused by aberrant protein and protein-RNA interactions.

c9orf72—Hexanucleotide repeat expansions (HRE) in *c9orf72* have been recently identified as the most common cause of familial ALS [49, 50]. These G4C2 repeats lie within the first intron of *c9orf72* and range from 2 to 10 in the normal population, and 90 to several hundreds in disease. Repeat expansions as low as 20 have been identified in ALS cases, but a causal relationship has not been established between the size of expansions and disease, and evidence for multiple gene mutation contributions has been found in carrier families [51]. Much research has focused on discerning the normal function of c9orf72, a putative DENN protein [52], and the contribution of hexanucleotide repeat expansions to disease. Although evidence exists to support several disease mechanisms including haploinsufficiency, RNA foci, and dipeptide repeat (DPR)-mediated toxicity, the specific pathomechanism of *c9orf72* remains unclear and subject to controversy [53]. A most remarkable discovery made in regards to *c9orf72* pathomechanism is the finding of nuclear pore alterations and defects in nucleo-cytoplasmic shuttling [17, 54, 55]. Accompanying these phenotypes are defects in RNA SG assembly, translation, and ribosome biogenesis, discussed later.

RNA foci and toxicity—Overexpression of G4C2 HREs of various lengths led to reduced transcription of stress granule proteins TIA-1 and HuR indicating a role for RNA foci in stress granule assembly [56]. However, because these HREs also generated DPRs, the contribution of the latter cannot be excluded. Using elegant live imaging approaches, Schweizer Burguete et al. observed that *c9orf72* HREs foci colocalize with FMRP and translocate bi-directionally within neurites. Interestingly, the presence of HRE increased protein levels for both FMRP and PSD-95, a protein whose translation is facilitated by FMRP, indicating that the *c9orf72* HRE may alter local protein translation [57]. These studies propose that the HREs induce neurodegenerative phenotypes by altering mRNA localization to synapses, a phenotype previously associated with other types of ALS [15, 32, 33].

RAN translation—As mentioned above, RAN (Repeat Associated Non-AUG translation) is a version of IRES-based translation mechanism that causes the repeat expansions to be translated into dipeptide repeats (DPRs). In *c9orf72*-mediated ALS, RAN translation of the G4C2 repeat and its anti-sense mRNA result in poly(GA), poly(GP), poly(GR), poly(PA), and poly(PR) dipeptides, all of which have been detected in patient tissues. One study implicated poly(GA) dipeptides as the primary aggregate inducing DPR [58], however another study concluded that expression of poly(GR) and poly(PR) induced neurodegenerative phenotypes while the three dipeptide products lacking arginine did not [59]. Although which DPRs are toxic remains an actively investigated question in the field, their effect on translation is undisputed.

In the context of ALS, DPRs have been associated with the disruption of translation and nuclear-cytoplasmic transport. Using Surface Sensing of Translation (SUnSET) [60] (Fig. 11.3), Kanekura et al. observed that poly(PR) and poly(GR) DPRs inhibit global translation in NSC34 motor neuron like cells [61]. The arginine-containing DPRs were found to form aggregates containing RNA-binding proteins

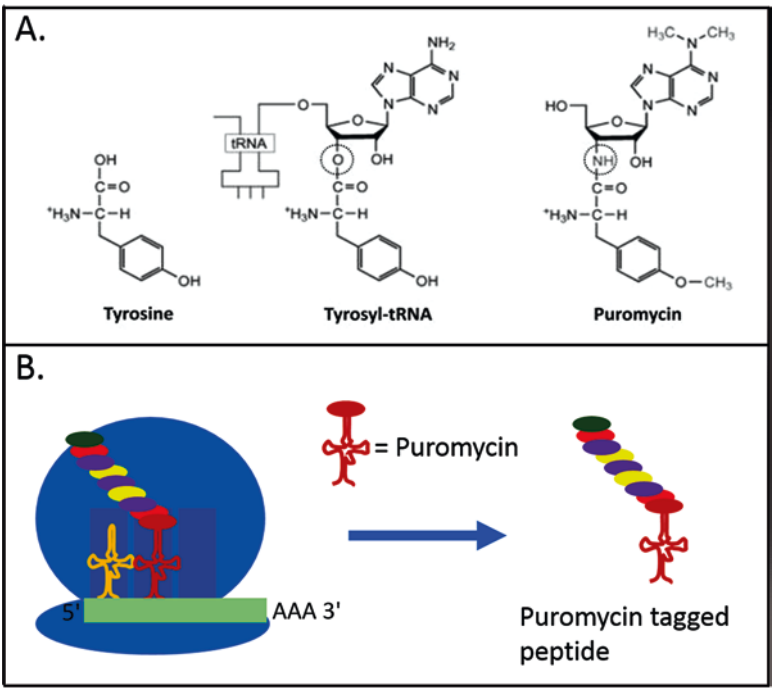

Fig. 11.3 Surface Sensing of Translation (SUnSET). (**a**) Molecular structure of tyrosine, tyrosyl-tRNA, and puromycin. (**b**) Puromycin is a structural analog of tyrosyl-tRNA from the bacterium *Streptomyces alboniger* and can be incorporated into elongating peptide chains. Puromycin attachment releases the peptide chain from the ribosome due to puromycin's non-hydrolyzable amid bond, yielding a puromycin tagged peptide chain. Fluorescent puromycin antibodies can then be used to track translation rates in real time. Traditional sulfur isotope assays were used to verify that puromycin expression does not significantly alter translation rates [60]

(e.g., FUS and TDP-43) along with RNA. The model emerging from this study is that the hydrophobic DPR aggregates block translation by preventing initiation factors from interacting with mRNA.

Poly(PR) and poly(GR) DPRs have also been implicated in post-transcriptional processing, nuclear cytoplasmic transport, and rRNA biogenesis, which can ultimately affect translation. Jovicic et al. also identified several genes related to rRNA biogenesis (e.g., *efg1* and *nsr1*) as significant modifiers of poly(PR) toxicity [17]. Additionally, poly(PR) and poly(GR) peptides colocalize with nucleoli, the site of rRNA synthesis [62]. Although the dysregulation of ribogenesis has been associated with *c9orf72* HRE in multiple studies, the mechanism remains poorly understood. Overall, although the role of *c9orf72* in both healthy and neurodegenerative individuals has been heavily studied in recent years, significant work remains to be done regarding the molecular mechanism and to precisely determine the contributions of G4C2 expanded RNA or the translated dipeptide products to disease. The mixed spectrum of results to date may reflect heterogenous responses to HREs and DPRs among different cell types in the nervous system (Fig. 11.4).

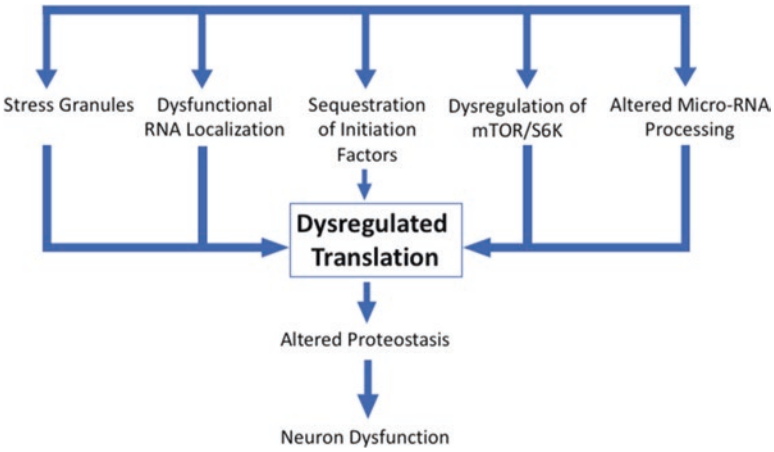

Fig. 11.4 Causes of Translational Inhibition. Cells regulate translation through a robust, complex integration of multiple pathways. The dysregulation of such pathways can alter proteostasis within a cell and lead to neuronal dysfunction

11.3 RAN Translation Beyond ALS/FTD

The first reports of RAN translation were made in association with CAG expanded transcripts in *SCA8* and Muscular Dystrophy type I (DM1) [6]. DM1 is caused by CTG repeat expansions within the myotonic dystrophy protein kinase (*DMPK*). Once transcribed, the expanded CUG mRNA forms a double-stranded structure that sequesters muscleblind (MBNL1), an RNA-binding protein involved in splicing [63]. As a result, CUGBP1 is upregulated, which together with MBNL1 sequestration leads to defects in the fetal to adult splicing transition and disease pathogenesis. Recent studies, however, have proposed an additional pathomechanism whereby the repeat expansions undergo RAN translation [6], albeit the mechanism by which RAN products contribute to disease is unknown. A recent review on this topic provides an excellent overview of the increasingly complex mechanisms behind myotonic dystrophy [64].

Since the initial discovery of RAN translation, additional microsatellite expansion disorders including Fragile X Tremors Ataxia Syndrome (FXTAS) and Huntingtin's disease have been added to the list of conditions in which bidirectional expanded transcripts produce RAN proteins [4, 5]. These novel and unexpected peptides contribute to toxicity challenging existing paradigms about disease mechanisms wherever they are found.

Huntington's disease (HD)—HD is an autosomal dominant neurodegenerative disorder [65]. The expansion of a CAG repeat region within the coding region of the huntingtin gene (*HTT*) leads to disease onset between the ages of 30 and 50 and causes progressive loss of neuron function [65]. The protein product of *HTT*, Huntingtin, is associated with microtubule-based trafficking of vesicles and mRNAs

within neurons [66]. Initial suggestions that protein synthesis may be altered in HD came from fibroblasts showing that in cells cultured from Huntington's patients, RNA accumulates in the nucleus and is not properly translated [67]. A more recent report shows that *HTT* repeat expansions also undergo RAN translation that can drive neurodegeneration through the dysregulation of nuclear-cytoplasmic transport [68]. Similar phenotypes including nuclear envelope morphology, pore architecture, and RNA export defects were found in a parallel study, although no RAN translation products were reported [69]. The discovery of RAN translation by Grima et al. [68] led to proposing a mechanism whereby its products cause these newly discovered phenotypes by specifically altering nuclear pores and inhibiting RANGAP1, a GTPase-activating protein necessary for nuclear cytoplasmic shuttling. Further substantiating this model is the fact that RANGAP1 overexpression or pharmacological restoration of nuclear transport rescued HD phenotypes across multiple model system [68].

Fragile X tremor ataxia syndrome (FXTAS)—FXTAS is caused by the expansion of CGG repeats in the 5′ UTR of the Fragile X Mental Retardation gene 1 (FMR1) [70–72]. The length of the CGG expansions determines the phenotypic outcome, with repeats >200 causing complete loss of transcription and absence of FMRP, while intermediate length repeats (50–200) lead to increased transcript but reduced protein production [73]. The intermediate expansion was associated with intention tremors, ataxia, dementia, and parkinsonism occurring in aging individuals [74]. Mechanistically, CGG repeats were shown to sequester RNA-binding proteins thereby dysregulating their activity within cells [75, 76]. An example relevant to translation regulation is DGCR8, a miRNA processing factor, which binds to expanded *FMR1* mRNA; this leads to decreased levels of mature microRNAs, which in turn can impact the translatome by imparing the translation of their mRNA targets [77] . Several recent studies identify RAN translation products, specifically polyG peptides produced from CGG expanded repeats using an ACG codon as start [78–80]. Elegant experiments in mouse models and patient-derived cells demonstrate that RAN translation-derived polyG peptides but not CGG RNA alone are responsible for FXTAS phenotypes [80].

11.4 Translation Dysregulation in Alzheimer's Disease

Recent studies have associated dysregulation of protein-mRNA complexes with Alzheimer's disease pathology [81]. Tau is a microtubule-associated protein whose aggregation and hyper-phosphorylation is a hallmark of Alzheimer's with pathology predicted to be driven in part by failed axonal transport [82]. Recent studies identified pathological tau in complex with TIA1 [83], a core component of stress granules. Interestingly, tau-TIA1 binding was found to have a positive correlation with stress granule formation suggesting possible consequences on translation that will have to be elucidated in future studies. Additionally, TIA1 mediates translational

inhibition of many stress response genes including P53, a major regulator of DNA damage repair [84]. It remains to be determined if susceptibility to, or DNA damage itself, may be involved in AD pathogenesis. Additionally, a dichotomous relationship was recently observed between TDP-43 and tau levels during Alzheimer's pathogenesis, with TDP-43 being shown to regulate tau protein expression by destabilizing its cognate mRNA [85].

Translation efficiency was also shown to be deficient in Alzheimer's brains and was noted to be an early event in disease. Ding et al. showed that in patient brain extracts, although the same quantity of polyribosome material was produced in control and disease cases, the translational efficiency of the polyribosomes was reduced by >60% in the inferior parietal lobe (IP) and superior middle temporal gyri (SMTG), albeit no significant reduction was observed in the cerebellum [86]. Regarding the mechanism of translation deficiency, $tRNA^{Asn}$ and 5S rRNA were significantly reduced in the IP of Alzheimer's patients together with increased oxidation of 28S rRNA. In contrast, the cerebellum of Alzheimer's patients exhibited increased phosphorylation of eIF2α and p70S6 [86]. While the former is associated with increased unfolded protein response and stress granule formation, the latter is associated with activation of the mTOR pathway and increased translation. Collectively, these findings provide intriguing links between Alzheimer's disease pathogenesis and translation through stress granules, initiation factors, and rRNA; however, the precise involvement of translation in Alzheimer's remains unknown.

11.5 Micro RNAs (miRNAs) and Translation Regulation

miRNAs are noncoding RNAs that can control gene expression by inhibiting mRNA translation or by selective degradation of transcripts (reviewed in [87]). It has been shown that TDP-43 aggregates sequester Dicer and DROSHA, two key RNA-binding proteins required for generating functional miRNA, thus implicating miRNA maturation in ALS pathogenesis [88]. DROSHA was also shown to form aggregates with RAN translation derived DPRs in patient tissues [89]. Additionally, XP05, which is required for precursor miRNA export from the nucleus, was identified as modifier of *c9orf72* HRE and TDP-43-based pathogenesis among other nuclear-cytoplasmic transport proteins [17, 54, 90]. Consequent reduced Dicer and DROSHA activity, and inhibited nuclear cytoplasmic transport potentially explain decreased global miRNA levels observed in multiple forms of ALS (recently reviewed in [91]). Several miRNAs required for synaptic development and maintenance including *miR-9* and *miR-124* were found to be altered in ALS/FTD patient derived cells and tissues suggesting the possibility that specific miRNAs may mediate aspects of toxicity in disease [92, 93]. Although the precise role of miRNAs in disease remains unclear, existing evidence supports the possibility of both a global and target-specific inhibition of miRNA synthesis as a contributor to ALS/FTD pathogenesis (recently reviewed in [91, 94]).

11.6 Concluding Remarks

Recent studies have provided compelling evidence that ALS/FTD and even Alzheimer's disease exhibit defects in multiple steps of RNA processing including protein synthesis. Several neurodegeneration-associated proteins are involved in mRNA export, trafficking, localization, and translation. These processes offer a plausible explanation for the unique pathogeneses observed in neurons, which have dendritic and axonal extremities requiring local translation and mRNA transport across distances vastly larger than the cell body (recently reviewed in [95–97]). The recent discovery that certain ribosomal subunits preferentially translate subsets of mRNA [98] provides an additional layer to the complex mosaic that is translation within neurons.

Evidence exists to support both global and target-specific dysregulation of translation. Globally, activation of the PERK pathway including phosphorylation of eIF2α inhibits global translation at the initiation step by inhibiting the incorporation of eIF2α into ribosomal complexes [99]. The inhibition of PERK has provided encouraging results in attenuating neurodegenerative phenotypes as it mitigated TDP-43-dependent phenotypes in flies and cultured mouse cells [46], and it restored synaptic protein levels and motor function in models of prion-mediated neurodegeneration [100]. Given findings that PERK is also upregulated in SOD1 mice [27], this approach could be extended to additional ALS types. However, pharmacological inhibition of translation did not rescue memory defects in mouse models of Alzheimer's disease [101, 102].

The evidence for specific mRNA targets further substantiates existing hypotheses that axonal transport, neuronal cytoskeleton, and synaptic vesicle cycling are underlying the synaptopathy associated with neurodegeneration. While these provide more specificity to any future interventions, it remains difficult to prioritize which mRNA target carries more physiological significance, and targeting multiple targets simultaneously poses significant challenges. Clearly, Aristotle's famous quote "The more you know, the more you know you don't know" remains relevant today; a lot is yet to be learned about the intricacies of translation dysregulation in neurodegeneration, specific RNA targets and processes, from disease onset to motor neuron failure and death.

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Index

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