Cellular and Molecular Neurobiology (2020) 40:1–14
https://doi.org/10.1007/s10571-019-00717-0

REVIEW PAPER

Alternative Splicing of ALS Genes: Misregulation and Potential Therapies

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Received: 9 April 2019 / Accepted: 31 July 2019 / Published online: 5 August 2019

Abstract

Neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), Parkinson's, Alzheimer's, and Huntington's disease affect a rapidly increasing population worldwide. Although common pathogenic mechanisms have been identified (e.g., protein aggregation or dysfunction, immune response alteration and axonal degeneration), the molecular events underlying timing, dosage, expression, and location of RNA molecules are still not fully elucidated. In particular, the alternative splicing (AS) mechanism is a crucial player in RNA processing and represents a fundamental determinant for brain development, as well as for the physiological functions of neuronal circuits. Although in recent years our knowledge of AS events has increased substantially, deciphering the molecular interconnections between splicing and ALS remains a complex task and still requires considerable efforts. In the present review, we will summarize the current scientific evidence outlining the involvement of AS in the pathogenic processes of ALS. We will also focus on recent insights concerning the tuning of splicing mechanisms by epigenomic and epi-transcriptomic regulation, providing an overview of the available genomic technologies to investigate AS drivers on a genome-wide scale, even at a single-cell level resolution. In the future, gene therapy strategies and RNA-based technologies may be utilized to intercept or modulate the splicing mechanism and produce beneficial effects against ALS.

Keywords Amyotrophic lateral sclerosis (ALS) · Alternative splicing (AS) · ALS genes · Splicing machinery · Splicing factors · RNA-binding protein (RBP)

Abbreviations

ALS	Amyotrophic lateral sclerosis
AS	Alternative splicing
RBPs	RNA-binding proteins
fALS	Familial amyotrophic lateral sclerosis
sALS	Sporadic amyotrophic lateral sclerosis
FTD	Frontotemporal dementia
ES	Exon skipping
IR	Intron retention
C9	C9ORF72
snRNA	Small nuclear RNA
snRNPs	Small nuclear ribonucleoproteins
PPIases	Peptidyl-propyl *cis*/*trans* isomerases
ESEs	Exonic splicing enhancers
ISEs	Intronic splicing enhancers
ESSs	Exonic splicing silencers
ISSs	Intronic splicing silencers
PrLD	Prion-like domain
hnRNP	Heterogenous nuclear ribonucleoprotein
PTB	Polypyrimidine tract-binding protein
MN	Motoneuron
SMaRT	Transcription of spliceosome-mediated RNA
siRNA	Small interfering RNA

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Background

An increasing number of debilitating human illnesses (cancer, muscular dystrophies, and neurodegenerative disorders) are caused by RNA metabolism defects. One of the pivotal participants in the RNA processing is alternative splicing (AS) whose task is to control and diversify gene expression, monitoring the protein productions of more than 90% of the exon-coding genes (Wang and Cooper 2007). Splicing process abnormalities (i.e., mutations in the intron–exon boundaries or in the exonic/intronic RNA regulatory silencer and enhancer elements) and defects in the spliceosome machinery or in the RNA-binding proteins (RBPs) are known to influence disease pathogenesis, and can represent a direct cause or possible modulators of disease susceptibility and severity (La Cognata et al. 2015; Nissim-Rafinia and Kerem 2005; Vanderweyde et al. 2013; Verma et al. 2018). Despite the considerable role of AS program in all aspects of neuronal development (from neurogenesis to mature synaptic functions) and the remarkable efforts of the scientific community to decipher the complexity of splicing regulation, our knowledge about AS in the ALS context is still not completely elucidated.

Herein, we summarize the AS regulation of ALS-related genes and highlight the contribution of splicing changes in pathology. We also discuss the regulation of splicing mechanisms by epigenomic and epi-transcriptomic events, introducing the new technological advances that enable to investigate AS isoforms at a single-cell level.

RNA-based therapeutic applications are quickly becoming a reality in the treatment of complex diseases (i.e., cancer, ocular and cardiovascular diseases, spinal muscular atrophy, Duchenne's muscular dystrophy, and Alzheimer's disease) (Baralle and Buratti 2017; Jyotsana and Heuser 2018; Sardone et al. 2017; Wasser and Herz 2016). Investigating the splicing mechanisms involved in ALS could pave the way to new interesting perspectives for developing novel therapeutic approaches and raising hope for this devastating and still incurable pathology.

The Alternative Splicing Program: Molecular Mechanism and Regulation

AS is the main mechanism of gene expression regulation that increases transcriptomic and proteomic diversity in eukaryotic cells. It works through five main different events: exon skipping (ES), mutually exclusive exon, alternative 3′ splice site, alternative 5′ splice site, and intron retention (IR) (Tazi et al. 2009).

Splicing process is performed by the spliceosome, a dynamic machine able to identify the exon–intron splice sites and to catalyze the cut-and-paste reactions for the intron removal and the exon junction (Matera and Wang 2014). This machinery is composed of a large number of elements, including small nuclear RNA molecules (snRNAs), some of which play a structural role (i.e., the small nuclear ribonucleoproteins, snRNPs), are regulators of the reaction (i.e., SR proteins, rich in Ser and Arg), or work as ATPasi or RNA-helicases (Matera and Wang 2014; Valadkhan 2010).

The splicing process is regulated by auxiliary *cis*-acting elements, named exonic or intronic splicing enhancers (ESEs or ISEs) and silencers (ESSs or ISSs), which guarantee the correct exon/intron recognition through their binding sites for the SR proteins and operate together with their specific *trans*-acting RBPs (Fig. 1) (Kapeli et al. 2017; Ram and Ast 2007). Other players of splicing regulation are the hnRNP proteins (heterogeneous nuclear ribonucleoproteins) that promote skipping by binding silencers located in the proximity of the exon–intron junctions (Geuens et al. 2016) (Fig. 1). An example of hnRNPs is the PTB (polypyrimidine tract-binding protein), whose spliced PTBP1 isoform (nPTB1) is able to suppress the neural splicing of specific targets, producing the stop of the neuronal differentiation process (Boutz et al. 2007).

Recent discoveries have shown that AS is powerfully conditioned by epigenomic and epi-transcriptomic regulation, based on histone modifications, chromatin structure, and transcription rate changes (Prasad et al. 1999; Zhu et al. 2018). The action of hnRNPs, SR proteins and the activity of kinase/phosphatase enzymes rely on post-translational modifications (phosphorylation and de-phosphorylation reactions) that are essential to promote splicing (Bedford and Richard 2005; Blackwell and Ceman 2012). The advancement in the understanding of splicing regulation by epigenomic/epi-transcriptomic processes will provide new molecular insights into the function of individual RNA modifications.

Alternative Splicing Regulation of ALS Genes

ALS is a neurodegenerative disease characterized by the progressive degeneration of both upper and lower motor neurons (MN) in spinal cord and motor cortex (Brown 1997), arising from the complex interaction of several molecular and cellular phenomena, including oxidative stress, mitochondrial dysfunction, axonal transport alteration, inflammation, excitotoxicity, and protein aggregation (Mendonca et al. 2012; Rothstein 2009).

Several genes are known to play a role in ALS pathogenesis (such as *TBK1*, *SOD1*, *VCP*, *TARDBP*, *FUS*, *GRN*,

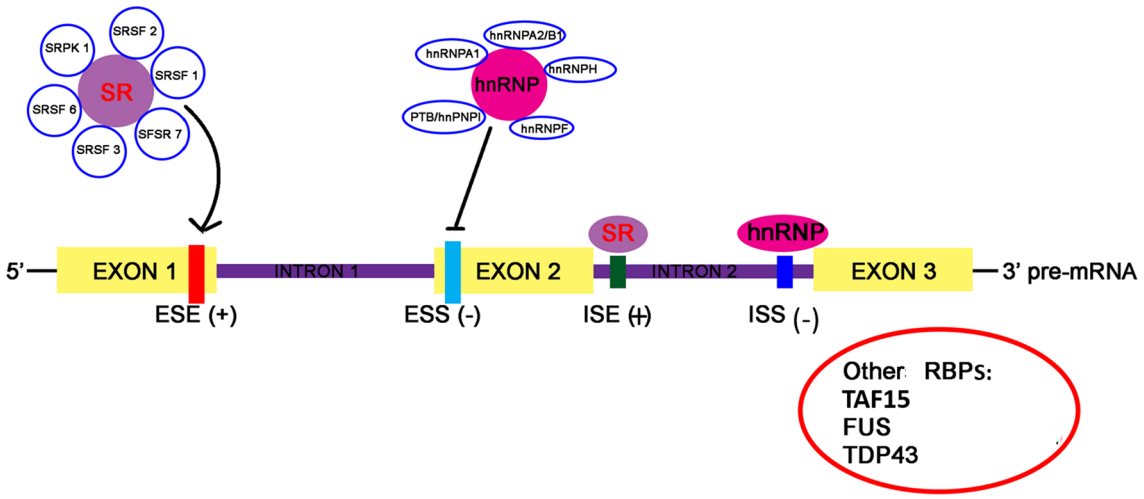

Fig. 1 RNA-binding proteins regulating splicing events. Serine and Arginine-rich proteins (SR proteins) are known to bind exonic or intronic splicing enhancer (ESEs/ISEs), while heterogeneous nuclear ribonucleoproteins (hnRNPs) bind intronic or exonic splicing silencers (ISSs/ESSs). The splicing process is enhanced by SR proteins and repressed by hnRNPs. In the blue circles we report a small list of RS proteins and hnRNP while in the red circle we list RBPs that have been associated with various diseases, including neurological diseases. For further details, please refer to the databases of RNA-binding protein specificities (RBPDB, http://rbpdb.ccbr.utoronto.ca/), which collects RNA-binding proteins associated to neurological diseases

MAPT, *CHCHD10*, and *TUBA4A*) [for an updated review, the reader is referred to (Volk et al. 2018)], and a complete and updated list is accessible in the Online genetic database of amyotrophic lateral sclerosis (ALSoD, http://alsod.iop.kcl.ac.uk/). Among the numerous listed genes, *TARDBP*, *ELP3*, *ANG*, *TAF15*, and *FUS* deserve particular attention because they are directly involved in RNA processing (transport control, stability, and translocation) (Baumer et al. 2010; Greenway et al. 2006; Kabashi et al. 2008; Mackenzie and Neumann 2012; Zhao et al. 2018).

A clear relationship between ALS-related genes and splicing factors is revealed by a bioinformatic analysis performed with STRING, a free web resource collecting known and predicted protein–protein interactions (https://string-db.org/) (Fig. 2). To build the network, we screened genes from both ALSoD database and SpliceAid-F database (http://srv00.recas.ba.infn.it/SpliceAidF/), which collect experimentally validated splicing factors (many ALS genes are splicing factors themselves) and related binding sites. Among RNAs or proteins involved in the splicing machinery or regulation, we included those associated with neurodegenerative, neuromuscular, and neurological diseases (i.e., spinal muscular atrophy, frontotemporal dementia, muscular dystrophy, neurofibromatosis type 1, myotonic dystrophy, fragile X syndrome, congenital myasthenic syndrome, and paraneoplastic encephalomyelitis). Figure 2 shows a number of existing potential connections and protein–protein interactions between the elements, strengthening the hypothesis of the AS contribution in ALS pathogenesis.

In the following sections, ALS genes (listed in Table 1) will be divided in two classes: we will first focus on the splicing regulation of those genes that have their own specific pathology and contribute to a loss-of-function mechanism in ALS (*TARDBP*, *FUS,* and *C9ORF72*), and then we will discuss other susceptibility or ALS-related genes (Conlon and Manley 2017).

TARDBP

TAR DNA-binding protein 43 (TDP-43), encoded by *TARDBP* localized on chromosome 1, is one of the components of ubiquitinated protein aggregates found in familial and sporadic ALS patients with different degree of severity (Tsuji et al. 2012). It is an essential splicing factor, since it is a member of the hnRNPs family and thus is involved in the RNA metabolism processes (splicing, transport, and translation) (Buratti and Baralle 2008). TDP-43 is composed of several functional domains: an N-terminal domain (NTD), two-tandem RNA recognition (RRM1-2), and a glycine-rich term prion-like domain located at the C-term (PrLD). This latter is particularly important for AS regulation, since it is involved in mediating the phase transitions underpinning RNP granule assembly (Shorter and Taylor 2013), but on the other hand acts as a pathogenic mutation site rendering TDP-43 prone to misfolding into conformers that accumulate in pathological inclusions (Harrison and Shorter 2017). The absence of PrLD domain prevent aberrant misfolding and toxicity events (Ash et al. 2010; Johnson et al. 2009) and

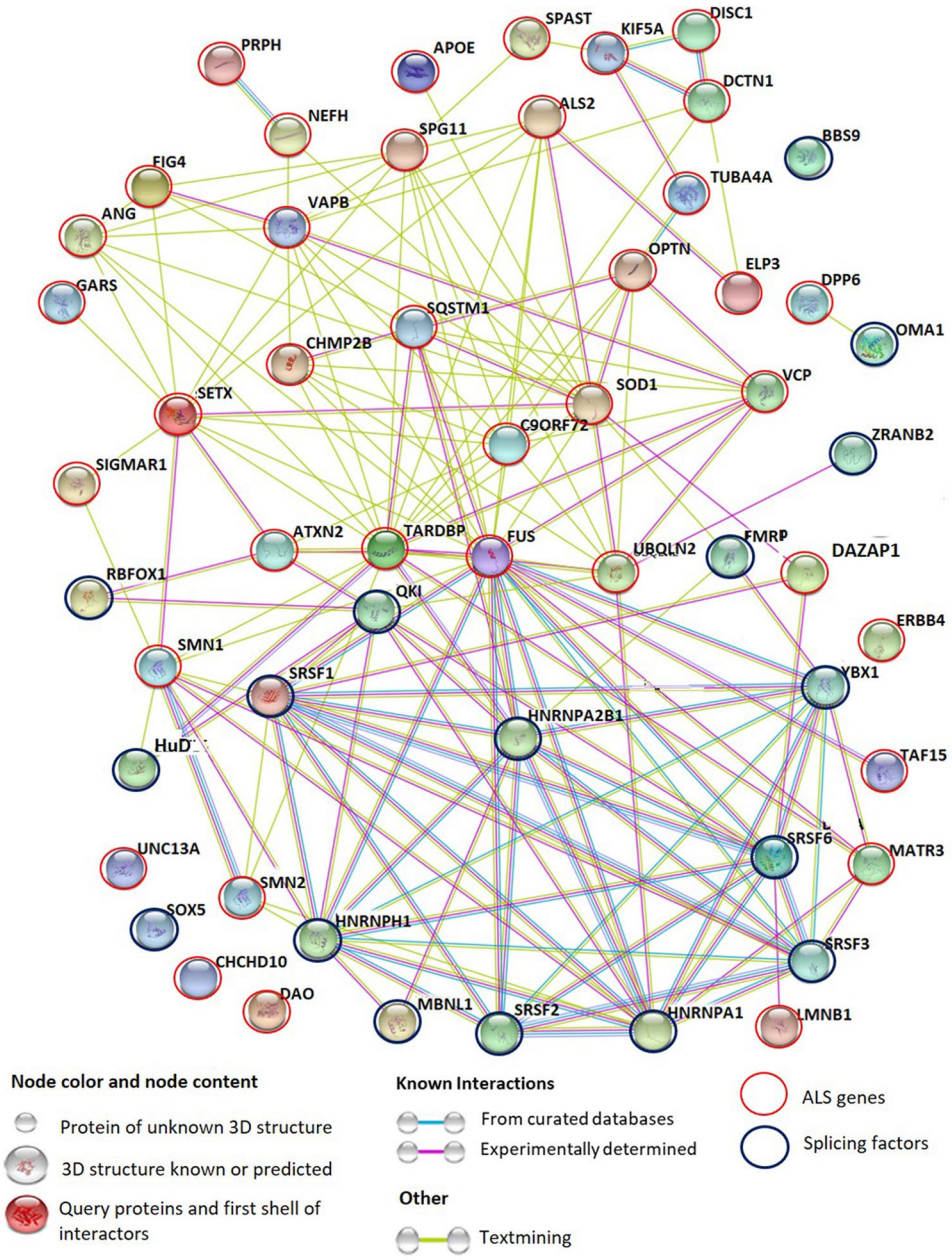

Fig. 2 Representation of known and predicted molecular interactions between ALS genes and splicing regulators. A network created with STRING (https://string-db.org/) shows ALS disease-causing or related genes with known or predicted interactions with splicing factors, as reported in the legend. Red circles represent ALS genes, while blue circles represent splicing factors. For the STRING analysis we used a confidence interaction score of 0.400, focusing on specific and meaningful associations

could be an interesting target for an oligonucleotide therapeutic strategy.

Several lines of evidence suggest the crucial role of TDP-43 in splicing regulation and ALS onset, encompassing both mutations in the genetic sequence of *TARDBP* itself and the AS regulation of specific targets (*SMN2*, *APOAII*, *CFTR HNRNPA1*, *POLDIP3*, *ATG4B*, *STMN*) (Butti and Patten 2018; Deshaies et al. 2018; Torres et al. 2018). About the genetic sequence, two heterozygous missense variants have been described in exon 6 of *TARDBP* that modulate AS and are likely involved in ALS onset or progression (Van Deerlin et al. 2008),. With regard to targets, TDP-43 depletion interfere with the AS of hnRNPA1 pre-mRNA, determining the inclusion of exon7B and culminating in a longer hnRNAP A1B isoform that is prone to aggregation and cytotoxic (Deshaies et al. 2018). Similarly, downregulation of TDP-43 results in an increase of cryptic sites in *ATG4B* (autophagy related 4B cysteine peptidase) causing an impairment of autophagy (Torres et al. 2018). Another identified TPD-43 downstream target is *POLDIP3* (Polymerase delta-interacting protein 3), which is involved in regulating splicing efficiency (Shiga et al. 2012). *POLDIP3* isoform 2 is rarely expressed in healthy tissues and its concentration increases when TDP-43 is depleted, making it a candidate biomarker for TDP-43 dysfunction (Shiga et al. 2012). Furthermore, TDP-43 regulates the splicing of STMN2 (Stathmin-2),

Table 1 Human ALS genes with RefSeq number, gene locus, number of alternative transcript variants, number of exons, and biological processes involved by the encoded protein

Gene name	RefSeq	Chromosomal location	Number of known transcripts	Number of exons	Biological process
TARDBP	NM_007375	1p36.22	32	6	RNA processing: transcriptional repressor; alternative splicing regulator
FUS	NM_001170937 NM_004960 NM_001170634	16p11.1	13	15	RNA processing
C9ORF72	NM_001256054 NM_018325 NM_014005	9p21.2	8	11	Endosomal trafficking
OPTN	NM_001008211 NM_001008213 NM_001008212 NM_021980	10p13	12	16	Cell death; autophagy
NEK1	NM_012224 NM_001199397 NM_001199398 NM_001199400 NM_001199399	4q33	9	34	Cell cycle regulation
SPG11	NM_025137 NM_001160227	9p21.2	25	40	Axon maintenance; proteins trafficking; gene expression regulation
KIF5A	NM_004984	15q21.1	3	29	Intracellular transport
SETX	NM_001351527 NM_015046	12q3.3	5	26	DNA/RNA processing
DCTN1	NM_004082 NM_001190837 NM_001190836 NM_023019 NM_001135040 NM_001135041	2p13.1	27	32	Retrograde transport; microtubule stability
CHCHD10	NM_001301339 NM_213720	22q11.23	5	4	Oxidative phosphorilation
VAPB	NM_0011956771 NM_0047384	20q13.32	6	6	Vescicle trafficking
TAF15	NM_139215 NM_003487	17q12	15	16	RNA processing: RNA polymerase II transcription
ANXA11	NM_145869 NM_001278407 NM_001157 NM_145868 NM_001278409	10q22.3	10	17	Vesicle trafficking, apoptosis, exocytosis, and cytokinesis
EAAT2	NM_004171 NM_001252652 NM_001195728	11p13	50	11	Glutamate reuptake
ADAR2	NM_001112 NM_001346687 NM_015833 NM_001160230 NM_001346688 NM_015834	21q22.3	15	11	A-to-I RNA editing

necessary for normal growth and axonal regeneration. TDP-43 knockdown leads to a decreased STMN2 expression, probably through splicing in a cryptic exon (Klim et al. 2019).

Further evidence shows that TDP-43 dysfunctions alter the splicing efficiency by modifying U snRNP biogenesis and leading to neuronal death (Yahara et al. 2017). In addition, TDP-43 depletion leads to a co-depletion of U12

snRNA, altering snRNPs assembly and providing a potential role for the minor spliceosome machinery in ALS (Ishihara et al. 2013). Taken together, all these data suggest that TDP-43 depletion leads to the inclusion of different cryptic exons, and the maintenance of a homeostatic level of this protein is crucial for AS regulation.

FET-Proteins: FUS, EWSR and TAF15

FUS (*Fused in Sarcoma*), *EWSR* (Ewing sarcoma breakpoint region), and *TAF15* (TATA-Box Binding Protein Associated Factor 15) belong to the family of FET-proteins (Mackenzie and Neumann 2012). These proteins are composed of an N-terminal domain rich in Gln, Gly, Ser, and Tyr, a conserved RNA-binding domain (RBD), the Arginine-Glycine-rich (RGG) domain that may influence RNA-binding, and a Cys_2–Cys_2 zinc finger motif binding nucleic acids (Morohoshi et al. 1998). Similarly to TDP-43, FUS and TAF15 aggregates characterize different neurodegenerative conditions including ALS (Lagier-Tourenne et al. 2010). FET-proteins bind single or double RNA/DNA strands and many experiments suggested their possible implication in transcription, RNA transport and pre-mRNA splicing (Lagier-Tourenne et al. 2010; Wu and Green 1997). Interestingly, FUS is enclosed in spliceosomal complex and interacts with several SR proteins and minor splicing factors, as well as with the U1 snRNP (Butti and Patten 2018; Kapeli et al. 2016; Reber et al. 2016; Shang and Huang 2016). FUS depletion is known to affect the splicing of genes involved in neurogenesis (*PPP2R2C*), dendritic development (*ACTL6B*), and action potential transmission in skeletal muscles (*SCN8A* and *SCN4A*) (Reber et al. 2016). Moreover, FUS is able to interfere with the AS of genes involved in axonal growth and cytoskeletal organization, including *MAPT*, *NTNG1*, *NRCAM*, and *ABLIM1* genes (Butti and Patten 2018).

Genomic *FUS* mutations determine an alternatively spliced exon 7, inducing a frameshift and the consequent splicing variants degradation (Zhou et al. 2013). Moreover, it is involved in some back-splicing events that regulate the formation of circular RNAs (circRNAs) in murine embryonic stem cell-derived motor neurons, causing a reduction in circRNA expression levels (Errichelli et al. 2017).

Differently from *FUS*, *TAF15* plays a minimal role in AS altering the splicing of few known genes: *GPCPD1* (glycerophosphocholine phosphodiesterase 1), *KCNMA1* (the alpha-1 gene of the calcium-activated potassium channel subunit), and *GRIN1* (*N*-methyl-D-aspartate receptor subunit NR1) (Kapeli et al. 2016).

Given the essential role that FUS plays in splicing regulation, further studies deserve to be carried out to better understand the consequence of the loss of function of *FUS* (and of other FET-proteins) on RNA splicing and its potential contribution to ALS pathogenesis.

C9ORF72

Hexanucleotide GGGGCC (G_4C_2) repeat expansion in *C9ORF72* (C9) is the main cause of ALS and Frontotemporal Dementia (FTD). This G_4C_2 expansion results in a dipeptide repeat protein (DPR), that accumulates in the cerebellum, cortical region and hippocampus of ALS patients (Gijselinck et al. 2012; Liscic 2015).

The differential use of transcription alternative start and termination sites in C9 is known to produce at least three RNA variants, encoding a long protein isoform (called isoform A) of approximately 54 kDa and a short isoform (named isoform B) of about 24 kDa (Barker et al. 2017). Expansion carriers exhibit a reduction in both *C9ORF72* mRNAs and protein levels, suggesting that toxicity may be mediated by a loss-of-function mechanism (Barker et al. 2017).

Mis-splicing of the expanded C9 transcript may play a role in C9-mediated toxicity; whereas *C9* is able to sequester several members of the hnRNP family (such as hnRNP A1, hnRNP A3, hnRNP H) resulting in altered splicing patterns of their RNA targets (Conlon et al. 2016, 2018; Lee et al. 2013; Mori et al. 2013).

A number of studies described global splicing alterations in C9 expansion carriers. Transcriptome analysis in lymphoblastoid cells and motor neurons of C9-FTD/ALS cases revealed an increased occurrence of splicing errors (most evident among patients with faster disease progression) and an enrichment of upregulated transcripts involved in RNA splicing, thus suggesting that such increased error rate could be a consequence of RBPs sequestration into foci, which in turn would contribute to disease progression and severity (Cooper-Knock et al. 2015). Brain transcriptome profiling analysis showed extensive AS and alternative polyadenylation defects in the cerebellum of C9ALS subjects, involving also ALS-associated genes (e.g., *ATXN2* and *FUS*) (Prudencio et al. 2015). Experiments conducted on lymphoblastoid cell lines revealed an increased number of aberrant splicing events, especially in the ALS patients with a rapid disease progression (Prudencio et al. 2015).

Interestingly, the G-quadruplex structure of C9 is able to sequester the splicing factor *SRSF2* (Serine and arginine SF factor 2) (Conlon et al. 2016; Zhang et al. 2015), which in turn helps the binding of U1 snRNP to the 5′ splice site or other factors to the 3′ splice site of target genes (Mure et al. 2018). Very recently, DPRs were found to block spliceosome assembly associating and interfering with U2 snRNP, causing a global splicing alteration in ALS patients (Yin et al. 2017).

Alternative Splicing Regulation of ALS-Related Genes

A substantial number of other ALS-related genes are susceptible to splicing-based regulation. In the next sections, we will focus on *SETX*, *OPTN*, *NEK1*, *SPG11*, *VAPB*, *DCTN1*, *CHCHD10*, *KIF5A*, *EEAT2,* and *ADAR2*.

SETX

Senataxin (*SETX*) encodes for an RNA-binding protein with a highly conserved helicase domain involved in regulation of RNA transcription (Bennett et al. 2018) and associated with both ALS4 (a form of juvenile ALS) and AOA2 (ataxia with oculomotor apraxia type 2). Sequencing of *SETX* coding regions and genetic engineering experiments revealed mutations correlated to an exonic cryptic donor site activation and an ESSs creation, resulting in MNs degeneration (Bennett et al. 2018; Tripolszki et al. 2017).

OPTN

Optineurin (OPTN) is localized on chromosome 10p13 and encodes for a protein involved in membrane and vesicle trafficking, transcription activation and cellular morphogenesis. This gene is mainly known to be responsible for hereditary primary open-angle glaucoma (POAG) and ALS, but its mutations were recently described to cause mRNA downregulation and degradation (Maruyama et al. 2010; Toth and Atkin 2018). In particular, an intronic mutation is known to activate a cryptic exonic donor site, resulting in ES of exon 6 (Del Bo et al. 2011); a non-sense mutation causes a stop codon and generates a frameshift due to exon 5 deletion (Maruyama et al. 2010); additional OPTN variants were reported to create either an ESEs or an ESSs (Johnson et al. 2012).

NEK Family

NIMA-related kinases *(NEK)* protein family are players in several fundamental biological processes, including cell cycle regulation (Moniz et al. 2011). These proteins share a common kinase domain, a basic domain, one or more coiled-coil motifs (CC), and two nuclear export sequences (NES). Eleven mammalian NIMA-related kinases are currently known, and among these, NEK10 is the only that does not contain the N-terminal catalytic domain, while NEK4, 6, and 7 do not have the coiled–coiled motifs. A growing number of NEKs are implicated in DNA damage response, some of which have tissue-specific functions, such as NEK3 and NEK7 in neurons (He et al. 2016; Shi et al. 2016).

A recent whole exome sequencing study suggested an association between NEK1 mutations and ALS, emphasizing a loss-of-function mechanism in 0.8% of ALS patients and highlighting a link between NEK1 and other known ALS genes (*SOD1*, *TBK1*, *C21orf2*) (Brenner et al. 2016; Cirulli et al. 2015; Kenna et al. 2016). To the best of our knowledge, there are currently no evidence correlating NEK1 splicing alterations to ALS, although it is known that the other NIMA-related proteins undergo splicing regulation. Specifically, *NEK4* isoform is engaged in mRNA processing mediated by the spliceosome and is localized in nuclear speckles and substrates containing snRNPs (Basei et al. 2015). NEK2 phosphorylates SRSF1 and, therefore, modulates SRSF1's target genes controlling important AS events (Naro et al. 2014). Moreover, SF phosphorylation and *NEK2* silencing negatively affect the splicing (Naro et al. 2014). Given the importance of splicing regulation in components of the NEK family, it is possible that splicing in *NEK1* is involved in ALS pathology.

SPG11

SPG11 (Spastic Paraplegia 11), localized on chromosome 15q13-15, encodes for spatacsin protein, which plays a pivotal role in axonal maintenance, synaptic vesicle transport, and autophagy. Spatacsin is essential for neuronal survival and is ubiquitously expressed in the nervous system, prominently in the cerebellum, cerebral cortex and hippocampus. *SPG11* mutations are considered causative for both hereditary spastic paraplegia (HSP) and the autosomal recessive juvenile ALS (ARJALS) form (Orlacchio et al. 2010). ARJALS is a rare disease that occurs before the age of 25 years with a slowly progressive course. Interestingly, HSP and ARJALS have many similarities in clinical presentation, molecular genetics, and cellular pathology. This overlap suggests that the same genetic variants may contribute to a common pathogenic pathway. Mutations in *SPG11* affect different splice donor region variants (Pippucci et al. 2009; Yu et al. 2016).

VAPB

Vesicle-associated membrane protein (VAMP)—associated protein B (also known as *VAPB*) plays a role in cellular stress response of the endoplasmatic reticulum (ER) and in unfolded protein response (UPR) (Suzuki et al. 2009; Walker and Atkin 2011). VAPB is ubiquitously expressed in eukaryotic cells and is involved in cellular calcium homeostasis regulation, protein transport, phospholipidic metabolism and viral infections (Kanekura et al. 2009). It is composed of three structural domains: a MSP (major sperm proteins)

conserved domain, a central amphipathic helicoidal structure and a C-terminal transmembrane domain. Five *VAPB* splice variants are known, all expressed in the human nervous system, which accumulate after proteasomal inhibition and contribute to ALS onset (Nachreiner et al. 2010). The identified variants lack specific exons of wt-*VAPB*: exon 2 (*VAPB-2* isoform), exons 4 and 5 (*VAPB-4,5* isoform), exon 3 (*VAPB-3* isoform), exons 3 and 4 (*VAPB-3,4* isoform) (Nachreiner et al. 2010).

DCTN1

DCTN1 (Dynactin subunit 1) is located on chromosome 2p13 and encodes the dynactin protein (a component of the ubiquitous dynactin complex) that binds both microtubules and cytoplasmic dynein. The complex is involved in vesicle retrograde transport processes (endosomes and lysosomes), axonogenesis, ER-Golgi transports, and chromosomes shift. The AS of this gene results in a brain-specific and an ubiquitously expressed isoform (Lazarus et al. 2013). AS of *DCTN1* determine both distal hereditary motor neuropathy type VIIB (HMN7B), also known as distal spinal and bulbar muscular atrophy, and neurodegenerative disorders such as ALS (LaMonte et al. 2002). Different studies reported *DCTN1* missense mutations and splicing changes in familial and sporadic ALS patients, mainly in Caucasians (Couthouis et al. 2014; Munch et al. 2004, 2005). Recently, a reduction of DCTN1 mRNA was reported in sALS motor cortex and spinal cord samples (Kuzma-Kozakiewicz et al. 2013).

CHCHD10

CHCHD10 (Coiled-Coil-Helix-Coiled-Coil-Helix Domain Containing 10) encodes a mitochondrial protein, localized in the intermembrane space and involved in mitochondrial organization maintenance. Mutations of this gene cause ALS2 and FTD. A recent study on fALS patients revealed a heterozygous missense variation that actives an acceptor cryptic site (Teyssou et al. 2016). Moreover, two mutations that cause about 50% reduction in CHCHD10 protein levels and contribute to motoneuronal disease were identified (Brockmann et al. 2018).

KIF5A

KIF5A (Kinesin Family Member 5A) encodes a protein of the kinesin family, principally expressed in neurons and involved in axonal and organelle transport. Heterozygous missense mutations in *KIF5A* cause monogenic spastic paraplegia (HSP10) and Charcot-Marie-Tooth type 2 (CMT2), while frameshift mutations are known to cause neurodevelopmental syndromes (Nicolas et al. 2018). Recently, two studies described the association between the increase of splice site mutations in the *KIF5A* C-term region and fALS (Brenner et al. 2018; Nicolas et al. 2018), definitively demonstrating that *KIF5A* mutations interfere with axonal transport and contribute to motor neuron degeneration.

ANXA11

Annexin A11 (ANXA11) is located on chromosome 10q22.3 and encodes for a member of the annexin family, a group of cellular proteins working in calcium-dependent modality (Tawani and Kumar 2015). It is involved in vesicle trafficking, exocytosis, endocytosis, signal transduction, cytokinesis, and apoptosis. *ANXA11* is susceptible to phosphorylation in the amino-terminal region (Furge et al. 1999) and a variety of interactions influence its subcellular localization. *Annexin A11* has been associated with cancer, autoimmune disorders (such as systemic lupus erythematosus), and multisystem autoimmune disease (sarcoidosis) (Hofmann et al. 2008).

ANXA11 has been recently implicated in ALS (Smith et al. 2017). In particular, the p.D40G mutation was associated to annexin A11-positive protein aggregates in spinal cord motor neurons and hippocampal neuronal axons. *ANXA11* is one of the genes most frequently mutated in sALS Chinese patients, where a splice site mutation in exon 6 causes a partial deletions in amino-acidic protein sequence (Zhang et al. 2018).

EAAT2

Oxidative stress is one of the most important mechanisms responsible for motor neuron degeneration (Rothstein 2009). *EAAT2* (Excitatory aminoacid transporter 2) is involved in 90% of glutamate reuptake and undergoes RNA defects: exon 9 skipping and intron 7 retention. Splicing defects observed in *EAAT2* mRNA in motor cortex and spinal cords of sALS patients were associated to alterations in spliceosomal components (Grabowski 1998; Lin et al. 1998). More recently, reduced EEAT2 activity was linked to aberrant EAAT2 mRNA transcripts as a consequence of abnormal RNA splicing (Bristol and Rothstein 1996).

ADAR2

The possibility that RNA processing defects play a role in neurodegenerative diseases is also confirmed by the regulation of *ADAR* (adenosine deaminase acting on RNA), an RNA-regulating protein responsible for binding to double-stranded RNA (dsRNA) and converting (editing) adenosine (A) to inosine (I) (Licht et al. 2016).

Defective *ADARs* were associated to different human pathologies: cancers, metabolic and neurological diseases (Slotkin and Nishikura 2013). Both ADAR enzymes and

splicing elements act on the same substrates, dsRNA, determining the interaction of these two processes. Specifically, editing affects post-transcriptional regulation and introduces or removes splice sites. Several studies have shown that editing machinery affects splicing directly by altering the secondary RNA structures or *cis*-regulatory sequences, or indirectly by binding competitive regions of the dsRNA and, therefore, preventing the access to the splice machinery (Hsiao et al. 2018; Licht et al. 2016). ADAR isoform 2, in particular, catalyzes the adenosine-inosine conversion at the Q/R site of GluA2 pre-mRNA, the most common RNA editing in higher eukaryotes (Hideyama et al. 2012). In ALS patients, downregulation of *ADAR2* and reduction in RNA editing were correlated to FUS-positive cytoplasmic inclusions (Aizawa et al. 2016), suggesting that *ADAR2* downregulation could affect motor neuron physiology in ALS.

Genome-Wide Assessment of AS Events in ALS: New Technologies and Future Perspectives

Genomic technologies (splice-sensitive microarray or RNA sequencing) have revolutionized the way transcriptome research is conducted, enabling analysis of the entire span of transcripts in a biological sample (Colombrita et al. 2015; Hu et al. 2013; Ishigaki et al. 2012; La Cognata et al. 2016; Morello et al. 2017; Shiga et al. 2012).

Two main RNAseq applications are currently raising particular interest for dissecting the complexity of splicing regulation: (i) the study of AS events on a large scale, spanning from the classic evaluation of differential expression between samples until the characterization of gene expression dynamics, gene boundaries, translation efficiency or RNA–protein interaction, and (ii) the single-cell-level isoform studies (Arzalluz-Luque and Conesa 2018; Zucca et al. 2019).

Bulk RNAseq studies are the most widespread and have been applied to investigate the dynamic variations of transcriptomes in in vitro differentiated motor neurons obtained from human control and patient-specific VCP mutant-derived iPSCs (Luisier et al. 2018). Surprisingly, these time-resolved RNAseq experiments revealed increased IR events as a dominant feature during the early differentiation phases, and identified *SFPQ* factor (splicing factor proline and glutamine rich) as the major intron-retaining transcript across diverse ALS-causing mutations (*VCP*, *SOD1* and *FUS*), proposing *SFPQ* IR events as a hallmark biomarker of familial and sporadic ALS (Luisier et al. 2018).

Despite genome-wide RNAseq studies are nowadays feasible, they rely on the experimental average gene expression across populations of cells, excluding the possibility to capture cell-to-cell variability and thus motivating the development of single-cell strategy (Arzalluz-Luque and Conesa 2018). Indeed, single-cell level insights are required to fully understand the biology of AS and represent the new challenge in RNAseq applications (Hwang et al. 2018). Each splice isoforms can be differently expressed depending on the particular cell type, showing a dominant (i.e., very highly expressed) isoform and several others with significantly lower expression values. This raise the question about whether the diverse and complex isoform expression landscape constitutes an additional layer of gene expression regulation contributing to ALS etiology, or if it is solely a result of the stochastic functioning of the AS machinery (Hwang et al. 2018).

Splicing Modulation Therapy

Deregulated AS is emerging as an important area for therapeutic intervention. Gene therapy, in particular, represents a promising pharmacological option for patients with diseases of genetic origins and is mainly based on antisense oligonucleotides (ASOs), spliceosome-mediated RNA trans-splicing (SMaRT) or small interfering RNAs (siRNAs) approaches (Arechavala-Gomeza et al. 2014).

Antisense oligonucleotides (ASOs), which are synthetic single-stranded nucleic acids, are able to bind the pre-mRNA intron/exon junctions and modulate splicing acting on enhancers or repressor sequences, determining exon skipping or including alternatively spliced exons (Havens and Hastings 2016; McClorey and Wood 2015). Multiple exon skipping strategies with ASOs have been already used in a variety of neurodegenerative and neuromuscular disorders, including Duchenne muscular dystrophy (DMD), spinal muscular atrophy (SMA), and ALS (Aartsma-Rus et al. 2017; Benoit-Pilven et al. 2018; McCampbell et al. 2018; Niks and Aartsma-Rus 2017; Ren et al. 2017; Sardone et al. 2017; Tosolini and Sleigh 2017). ASOs can be chemically modified, generating bifunctional ASOs with added RNA or peptide motifs, and specifically target mutant RNAs or AS protein recruitment to the transcript (Singh et al. 2018).

ASO's therapy has already entered into the medical field of neurological disorder. Eteplirsen, a third-generation ASO that hybridizes to exon 51 of DMD pre-mRNA, represents the first FDA-approved ASO for the treatment of Duchenne Muscolar Distrophy. Nusinersen is another FDA-approved ASO-based drug (marketed as Spinraza) used for SMA therapy, and works by promoting the exon skipping of exon 7 in *SMN2* gene by blocking intronic splicing silencer N1 (ISS-N1) located immediately downstream of exon 7 (Verma 2018).

With regard to ALS, one of the first ASO-based clinical trials was designed to silence SOD1. Intrathecal administration of the ASO IONIS-SOD1Rx resulted both practical

and safe in SOD1 ALS patients during phase I testing (NCT01041222) (Miller et al. 2013). A phase Ib/IIa trial (NCT02623699) is currently in process to further evaluate safety, tolerability, and pharmacokinetics of IONIS-SOD1Rx (McCampbell et al. 2018).

Among the ALS-related genes that have been discussed in the present review, *C9ORF72* may represent the best candidate for ASOs therapy. As previously described, C9 accumulates in nuclear foci, conferring toxicity associated with the repeat expansion and, therefore, compromising the pre-mRNA processing (Wojciechowska and Krzyzosiak 2011). Given the encouraging results obtained in splicing modulation therapy in other polyglutamic diseases (CAG repeats), such as spinocerebellar ataxia (SCA 2) and Huntington Disease (HD) (Rindt et al. 2017; Tawani and Kumar 2015), it is not surprising to imagine the use of ASOs and/or other exon skipping approaches to restore the reading code or remove exons containing mutations in ALS. Early testing of ASO-based therapeutics for C9ALS was performed on iPSC-derived neurons and fibroblasts (Butti and Patten 2018; Donnelly et al. 2013; Lagier-Tourenne et al. 2013). ASOs were designed to target the repeat expansion or within surrounding N-terminal regions of the C9 mRNA transcript to degrade the transcript or to block the interaction between the repeat expansion and RNA-binding proteins, resulting in a reduction of RNA foci, dipeptide proteins and restored normal gene expression markers (Butti and Patten 2018; Donnelly et al. 2013; Lagier-Tourenne et al. 2013). Recently, ASOs have been also tested in mouse models expressing the expanded C9 (Butti and Patten 2018). Last year, a randomized, double-blind, placebo controlled phase I clinical trial with an antisense oligonucleotide (BIIB078) targeting C9 has been started. The study will involve 80 patients carrying the pathological expansion (ClinicalTrials.gov Identifier: NCT03626012).

Other studies have analyzed the effects of ASO modifications within the oligonucleotide backbone, sugar and heterocycles in order to improve delivery, potency, and stability to target *FUS*, demonstrating that affinities of various nucleic acid binding domains depend on chemical modifications and that ASO–protein interactions influence the localization of ASOs themselves (Bailey et al. 2017). These preliminary data suggest that ASO-based therapy can be a powerful way for treating ALS-relate genes although it is clear that therapeutic outcomes will depend on the stage of disease progression and on the time of intervention.

The SMaRT method has been used for cystic fibrosis and HD (Rindt et al. 2017). SMaRT is based on the correction of alterations at post-transcriptional level through the introduction of an exogenous RNA into targeted cells to induce a trans-splicing event between exogenous RNA and target endogenous pre-mRNA (Berger et al. 2016). There are currently no applications of this approach for ALS.

Finally, SiRNAs, 21–25 nucleotide double-stranded RNA molecules, are becoming an important therapeutic tool for different diseases including tumors or metabolic/genetic disorders due to genetic malfunction and deregulated expression. The siRNA selectively targets and silences the gene by inhibiting the expression of the protein (Borna et al. 2015; Ozcan et al. 2015). Several siRNAs were designed in silico to target the glycine-rich region of *TARDBP* via molecular dynamics and thermo-physical analyses (Bhandare and Ramaswamy 2016). Since siRNAs strategy induces a catalytic process resulting in complete gene knockdown, likely detrimental for the cell, the exon skipping approach may be preferred since it corrects the gene-reading frame.

Conclusion

Many proteins implicated in motor neuron and neurological disorders have a physiological function in different RNA processes (mRNA stability, transcription regulation, transport of RNA granules, splicing, miRNA biogenesis, and RNA editing). A large number of diseases related to splicing were documented, but this is probably under-estimated because the splicing mutation effects are often not considered as a primary cause of the diseases. An increasing understanding of splicing regulation will create new treatment strategies for modulating this process in disease contexts, leading to personalized medicine. The overlap between clinical data, etio-pathogenetic mechanisms, and gene therapy strategies may offer novel solutions by creating rigorous guidelines in clinical trials (Ludolph et al. 2010). This kind of holistic approach seems to be now the most promising to advance the therapy of complex and multifactorial diseases.

Acknowledgements The authors gratefully acknowledge the projects "Un approccio diagnostico multidisciplinare per le malattie neurodegenerative dell'invecchiamento" (DSB.AD009.001) and "Life Analytics, human centric platform per la salute ed il benessere dell'uomo" (DSB.AD008.456), and Angelo Bagalà, Ariangela Belvedere, Benedetto Bruno, Walter Carpino, Tiziana Martire, and Patrizia Rizzuto for their administrative and technical support.

Author Contributions BP and VLC reviewed the literature and wrote the manuscript. FLC participated in revising the manuscripts. TS, CU, SA provided critical inputs to the manuscript. SC conceived, directed, and supervised the project. All authors have read and approved the final version of this manuscript, and agreed to be accountable for all aspects of the work and consent for publication.

Funding This work has been supported by Grants: (i) "Un approccio diagnostico multidisciplinare per le malattie neurodegenerative dell'invecchiamento", DSB.AD009.001; (ii) "Sviluppo ed applicazione di tecnologie biosensoristiche in Genomica" CIP 2014.IT.05. SFOP.014/3/10.4/9.2.10/0008, CUP G67B17000170009, n. 11/2017 "Rafforzare l'Occupabilità nel sistema R&S e la nascita di spin off di ricerca in Sicilia—PROGRAMMA OPERATIVO DEL FONDO SOCIALE EUROPEO REGIONE SICILIA 2014–2020", and (iii)

"Life Analytics, human centric platform per la salute ed il benessere dell'uomo", DSB.AD008.456.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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