Developments in RNA Splicing and Disease

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Pre-mRNA processing, including 5'-end capping, splicing, editing, and polyadenylation, consists of a series of orchestrated and primarily cotranscriptional steps that ensure both the high fidelity and extreme diversity characteristic of eukaryotic gene expression. Alternative splicing and editing allow relatively small genomes to encode vast proteomic arrays while alternative 3'-end formation enables variations in mRNA localization, translation, and stability. Of course, this mechanistic complexity comes at a high price. Mutations in the myriad of RNA sequence elements that regulate mRNA biogenesis, as well as the trans-acting factors that act upon these sequences, underlie a number of human diseases. In this review, we focus on one of these key RNA processing steps, splicing, to highlight recent studies that describe both conventional and novel pathogenic mechanisms that underlie muscle and neurological diseases.

Deep sequencing and microarray analyses of the human transcriptome have revealed that >90% of our genes undergo alternative splicing, which permits a limited genome to encode the vast proteomic repertoire required for the human interactome (Blencowe 2006; Wang and Burge 2008; Wang et al. 2008). From a biochemical viewpoint, the RNA splicing reaction is a relatively simple process that consists of two transesterification reactions (Black and Grabowski 2003; Konarska and Query 2005; House and Lynch 2008). However, this apparent simplicity is compounded by the fact that exons are generally buried within much larger introns and both authentic, and many more false, splice sites exist in genes. In addition, multiple alternative splicing modes are utilized in vivo (Fig. 1). Finding the majority

of these sites is the function of the major spliceosome, which is composed of five small nuclear ribonucleoprotein complexes (snRNPs) and ~170 proteins (Deckert et al. 2006; Behzadnia et al. 2007; Bessonov et al. 2008; Wahl et al. 2009). Conserved sequence elements at exon-intron boundaries, the 5' and 3' splice sites of each exon, and the branchpoint facilitate spliceosome recruitment via base-pairing interactions with five spliceosome-associated snRNAs (U1, U2, U4/6, U5) (Fig. 2). A minor spliceosome, which recognizes variant splice sites and contains a unique set of snRNAs (U11, U12, U4atac, U6atac) in addition to U5, also exists in the nucleus (Will and Luhrmann 2005; Steitz et al. 2008). Spliceosomal recognition of these core elements is modulated by a myriad of additional sequence elements in

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both exons and introns that either activate (exonic splicing enhancer, ESE; intronic splicing enhancer, ISE) or repress (exonic splicing silencer, ESS; intronic splicing silencer, ISS) spliceosome recruitment by interacting with a variety of splicing factors, including serine-arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs) (Fig. 1) (Martinez-Contreras et al. 2007; Long and Caceres 2009). The density of these sites is astonishing, and current estimates suggest that splicing regulatory motifs comprise >75% of the nucleotides in an average exon (Chasin 2007).

thus provides a vital link between the actin-myosin contractile apparatus and the extracellular matrix (ECM). A number of single nucleotide mutations result in skeletal muscle degenerative diseases, such as the severe Duchenne (DMD) and the milder Becker (BMD) muscular dystrophies, as well as X-linked dilated cardiomyopathy. For Duchenne, mutations often disrupt the DMD open reading frame, sometimes by altering splicing enhancers and silencers, while the reading frame, and some dystrophin function, is mostly preserved in BMD. Although every tissue requires flexibility in gene expression, the significance of alternative splicing to human function is particularly striking in the central nervous system. For example, *CACNA1H*, which encodes a T-type low-voltage-activated calcium channel and is a candidate gene for idiopathic generalized epilepsies, is extensively alternatively spliced to potentially generate >4,000 mRNA variants

Figure 2. Spliceosome dynamics and retinopathy. The assembly/disassembly cycle of the spliceosome (adapted with permission from Wahl et al. 2009) during splicing with only the pre-mRNA (blue and yellow boxes, exons; thick black line, intron) and snRNP (grey) complexes illustrated to highlight the roles of U4-associated (PRPF3/hPrp3, PRPF31/hPrp31) and U5-associated (PRPF8/hPrp8, SNRNP200/hBrr2) proteins as well as several other steps that require the DExD/H-type RNA-dependent ATPase/helicase. Steps 1 and 2 refer to the first and second catalytic steps of splicing.

(Zhong et al. 2006). Therefore, it is not surprising that errors in splicing regulation have been linked to a number muscle and neurologic diseases and this number is likely to increase in the future.

Since a number of excellent reviews have been published recently that survey how defects in splicing are associated with a broad range of human diseases (Licatalosi and Darnell 2006; Cooper et al. 2009; Tazi et al. 2009), this review focuses on only a few hereditary diseases in which mutations result in unusual pathogenic mechanisms, which have led to some intriguing insights into splicing regulatory mechanisms. Given the essential nature of the splicing

reaction in all tissues, we begin by examining the paradox of why mutations in select spliceosomal components cause retinal degeneration while loss of a snRNP assembly factor leads to a lower motor neuron disease and proximal muscle atrophy. In addition to the core spliceosome, numerous trans-acting factors recognize pre-mRNA sequence elements to orchestrate spliceosome recruitment and alternative splicing. Thus, we next review the roles of both tandem repeats and dispersed repetitive elements in modulating splicing regulation and the emerging roles of repeat polymorphisms and reiterative element mutations on the pathogenesis of specific diseases.

M.G. Poulos et al.

GHOSTS IN THE MACHINE

The spliceosome is a remarkably dynamic macromolecular machine that depends on the recognition of loose consensus sequence elements at splice sites and the branch point (Fig. 2) (Wahl et al. 2009). This splice site detection process is facilitated by a number of other splicing factors that promote snRNP recruitment (Wang and Burge 2008; Licatalosi and Darnell 2010). For splicing to occur, snRNPs and the pre-mRNA undergo a series of association-dissociation steps and conformational transitions and, although the splicing reaction involves only two catalytic steps, intron removal must be accomplished with great precision in all tissues. While splicing errors underlie a large number of human diseases, it is puzzling that some diseases, which are characterized by the degeneration of only certain cell types, are caused by mutations either in ubiquitously expressed spliceosome components or depletion of factors important for snRNP assembly.

The Spliceosome and Retinopathies

Autosomal dominant retinitis pigmentosa (adRP) is characterized by progressive degeneration of predominantly rod photoreceptor neurons resulting in visual impairment. Some forms of adRP are caused by mutations in four pre-mRNA processing factors (PRPF3, PRPF8, PRPF31, SNRNP200) that are components of the ubiquitously expressed U4/U6.U5 tri-snRNP, which is recruited as a preassembled complex to form the functional spliceosome (Fig. 2) (Mordes et al. 2006; Wahl et al. 2009; Zhao et al. 2009). Another gene, PIM1-associated protein (*PAP1*), which encodes a protein that interacts with PRPF3 to regulate splicing, also causes adRP (Maita et al. 2004; Maita et al. 2005). Why do mutations in these specific genes cause a specific eye disease? One possible explanation relates to the unusual metabolic requirements of photoreceptor cells, including circadian oscillation of transcript levels (von Schantz et al. 1999), and the importance of tri-snRNP dynamics during splicing. Although remodeling of spliceosomal subunits is common

during splicing, the U4/U6.U5 tri-snRNP presents an extreme case since catalytic activation of the spliceosome requires disruption of the extensively base-paired U4/U6 duplex to allow U6 snRNA interactions with U2 and the pre-mRNA (Wahl et al. 2009). Evidence that tri-snRNP remodeling is particularly important for photoreceptors comes from recent reports that SNRNP200 mutations cause adRP (Li et al. 2009; Zhao et al. 2009). SNRNP200, also known as hBrr2, is a DExD/H-box protein that is required for both unwinding of the U4/U6 duplex and spliceosome disassembly. Interestingly, the SNRNP200 mutation associated with more severe adRP causes a larger defect in U4/U6 unwinding activity (Zhao et al. 2009). Of course, it is possible that PRPF3, PRPF8, PRPF31, SNRNP200, and PAP1 have nonsplicing functions that are particularly critical for retinal function, but this possibility seems less likely given their common association with the tri-snRNP.

Both loss-of-function and gain-of-function models have been proposed to explain why mutations in multiple components of the tri-snRNP result in retinal disease (Mordes et al. 2006). For loss-of-function models, tri-snRNP-associated mutations result in haploinsufficiency that only cause overt disease in photoreceptors because these neurons are unusually sensitive to decrements in splicing function due to the high demand for specific proteins, such as rhodopsin (RHO mutations also cause adRP). For example, more than 40 *PRPF31* mutations have been reported and the majority of these create a premature termination codon (PTC), transcript degradation via the nonsense-mediated decay (NMD) pathway, and reduced mutant allele expression (Rio Frio et al. 2008). Additionally, the incomplete penetrance noted for *PRPF31* mutations may reflect variations in expression from the normal allele (Vithana et al. 2003; Rivolta et al. 2006). A potential problem with this haploinsufficiency model is that other neurons (e.g., Purkinje cells) and other tissues (e.g., regenerating muscle) also place considerable demands on transcription and RNA processing. Indeed, a recent study generated mice carrying either a human ADRP

mutation in *Prpf31* (*Prpf31*^{A261P/+} knockin) or *Prpf31*^{+/-} heterozygous knockout mice. Although the mouse retina is enriched in rod photoreceptors compared to humans, these lines fail to model adRP and do not develop retinal degeneration, although the corresponding homozygous knockin and knockout mice are embryonic lethal (Bujakowska et al. 2009). In contrast, heterozygous *Rho*^{+/-} mice, which express lower levels of rhodopsin, develop retinal degeneration (Lem et al. 1999). While this is a single study and may reflect functional differences in the rodent versus human retina, the failure to model adRP in mice will impede further testing of the haploinsufficiency model. A caveat is that *Prpf31*^{A261P/+} knockin mice were generated on a mixed genetic background, so it is possible that mutant *Prpf31* alleles will show a retinal phenotype when placed on a different background (Buchner et al. 2003). Additionally, mouse models should be generated for other adRP mutations, particularly *Snrnp200*. For gain-of-function models, mutant tri-snRNP proteins might alter the spliceosome so that it generates, or fails to efficiently proofread, incorrectly spliced mRNAs (Mordes et al. 2006; Zhao et al. 2009). Another pathogenic mechanism, characteristic of some neurodegenerative diseases, is that U4/U6.U5 tri-snRNP mutations lead to the synthesis of aggregation-prone proteins, which are particularly toxic to photoreceptors.

snRNP Biogenesis and Motor Neuron Disease

The spliceosome requires the functions of multiple snRNPs, which contain a seven-member ring of Sm proteins that assemble onto the Sm site of each snRNA in the cytoplasm with the exception of U6. A critical factor in this assembly process in vivo is the survival of motor neurons gene 1 (*SMN1*), which is ubiquitously expressed in human tissues (Monani 2005; Burghes and Beattie 2009; Chari et al. 2009). In addition to *SMN*, this ATP-dependent *SMN* “assemblyosome” complex consists of several GEMINS (*SIP1/GEMIN2*, *DDX20/GEMIN3*, *GEMINS4-8*) and *STRAP/UNRIP*

(Paushkin et al. 2002; Burghes and Beattie 2009). Loss of *SMN1* expression, due to either mutation or deletion, causes the autosomal recessive disease spinal muscular atrophy (SMA), a leading cause of infantile mortality. SMA is associated with the degeneration of lower motor neurons in the ventral horn of the spinal cord and muscle atrophy, which ranges from severe Type I disease, characterized by an early (<6 months) age of onset and death (<2 years of age), to Type III disease, which manifests later during development (>18 months) and patients may have a normal lifespan (Monani 2005). In a cruel twist of fate, the human genome contains the nearly identical gene *SMN2*, but this copy fails to compensate for *SMN1* loss due to a C → T transition in *SMN2* exon 7. This single nucleotide change blocks inclusion of exon 7 and results in inhibition of *SMN* protein oligomerization, and/or the generation of a C-terminal degradation signal, leading to low levels of functional protein (Monani 2005; Burghes and Beattie 2009; Cho and Dreyfuss 2010).

Similar to adRP, both splicing and non-splicing models have been proposed for SMA pathogenesis to explain how loss-of-function mutations in a ubiquitously expressed gene result in selective effects on specific cell populations. Splicing models emphasize the possibility that lower motor neurons may be particularly sensitive to SMA-associated declines in *SMN* activity and relative snRNP abundance, which causes mis-splicing of critical pre-mRNAs. In support of this possibility, there is a strong correlation between phenotypic severity, *SMN* levels, and snRNP assembly activity in mouse models of SMA (Gabanella et al. 2007). Loss of *SMN* in these models also causes cell-type specific changes in snRNA levels, with minor spliceosome snRNAs particularly affected in brain and spinal cord (Gabanella et al. 2007; Zhang et al. 2008). Exon microarrays have been used to detect widespread splicing abnormalities in the *Smn*^{-/-}; *SMN2*; *SMNΔ7* mouse SMA model, with a maximum lifespan of 14 days, at end-stage disease (postnatal day 11, P11), which led to the suggestion that SMA is a pan-splicing disease (Zhang et al. 2008).

M.G. Poulos et al.

This analysis has been challenged since symptomatic mice were analyzed, suggesting that alternative splicing changes may represent secondary effects due to disease progression. Indeed, a comparison of presymptomatic (postnatal day 1, P1), early-symptomatic (P7), and late-symptomatic (P13) mice showed that the majority of splicing changes in spinal cord were only observed in late-symptomatic mice (Baumer et al. 2009). Nevertheless, it is possible that many gene expression and RNA splicing changes have been missed in presymptomatic mice using microarrays so it will be important to re-examine these mice using RNA-Seq technologies.

Efforts to develop effective SMA therapies have employed several elegant splicing-related strategies designed to enhance *SMN2* exon 7 splicing levels (Khoo and Krainer 2009; Sendtner 2010). Prominent among these are approaches based on bifunctional antisense oligonucleotides (ASOs) or U7 snRNAs composed of sequences complementary to *SMN2* exon 7 for targeting coupled to splicing enhancer (ASO-ESE, U7-ESE), or minimal RS domain peptide, motifs to increase exon 7 inclusion. Evidence that this strategy is effective in vivo has been provided recently. SMA model mice (*Smn*^{-/-}; *hSMN2*^{+/+}), which also express a U7-ESE transgene, show a dramatic improvement in survival over *Smn*^{-/-}; *hSMN2*^{+/+} mice alone (Meyer et al. 2009). Importantly, extensive ASO tiling analyses across exon 7 and flanking introns 6 and 7 revealed additional splicing silencers, and ASO-mediated antisense masking of these sequence motifs increases exon 7 inclusion in several tissues of human *SMN2* transgenic mice (Hua et al. 2007; Hua et al. 2008).

Non-splicing functions of SMN have also been reported. Indeed, this protein has been proposed to be a master assembler of RNP complexes (Terns and Terns 2001). For example, the Sm-like (LSm) proteins also form ring structures that function in cytoplasmic RNA turnover and RNA–RNA interactions, but it is not known if SMN activity is essential for this assembly process (Wilusz and Wilusz 2005). Other proposed SMN functions include

transcriptional regulation and axonal mRNP transport and translation (Monani 2005; Burghes and Beattie 2009).

REPETITIVE ELEMENTS AND SPLICING

Studies on trans-acting factor mutations have expanded our understanding of spliceosome assembly and function. However, many other diseases are caused by mutations in pre-mRNA sequence elements, and there is a growing appreciation for the importance of reiterative sequences in splicing regulation. Repetitive sequence elements comprise >50% of the human genome and are broadly divided into two categories, dispersed and tandem repeats (Richard et al. 2008). Dispersed repeats include tRNA genes, gene paralogs, and transposon/transposon-derived elements such as long- and short-interspersed elements (LINEs, SINEs) and LTR retrotransposons. Among these, transposable elements (TEs) may cause disease either by insertional mutagenesis or nonallelic homologous recombination between TEs (Belancio et al. 2009). An intriguing example of how these elements can affect splicing regulation is provided by the short interspersed element (SINE) *Alu*. *Alu* is a small (~300 nt) primate-specific non-LTR retrotransposon that is the most abundant (>10⁶ copies) repetitive element in the human genome (Keren et al. 2010). *Alu* elements are dimeric structures composed of left and right arms joined by an A-rich linker region and are preferentially located in introns in gene-rich regions often in the antisense orientation. During evolution, *Alu* mutations have resulted in the appearance of regulatory sequences that are recognized by the spliceosome so that these elements are sometimes “exonized” or included in the mRNA product as internal alternative exons (Lev-Maor et al. 2003; Keren et al. 2010). Other mutations can affect *Alu*-associated splicing events. For example, a C→T transition in intron 6 of the *CTDP1* gene, which encodes a phosphatase associated with the C-terminal domain in RNA polymerase II, generates a 5′ splice site while also activating an upstream cryptic 3′ splice site in an antisense *Alu* element, leading

to its inclusion and the autosomal recessive disorder congenital cataracts facial dysmorphism neuropathy (CCFDN) (Varon et al. 2003).

Tandem repeats include gene tandems, rDNA arrays, and satellite DNAs. The latter group, specifically polymorphic microsatellites, plays a prominent role in hereditary neurological disease and splicing dysregulation. Aside from short repeats, the contraction of a larger repeat, the D4Z4 3.3 kb macrosatellite on chromosome 4q, also appears to underlie at least one muscular dystrophy, and recent studies suggest that contraction-induced alterations in chromatin structure and RNA processing may also be pathogenic.

Microsatellite-induced Disruption of Alternative Splicing

The human genome contains ~253,000 di-/tri-/tetra-nucleotide microsatellite repeats (Richard et al. 2008). These simple sequence repeats may contract or expand due to errors in DNA replication, recombination, and repair. When the latter occurs, transcription of some expanded repeats results in the synthesis of RNA hairpin structures, which are toxic because they affect the activities of several splicing factors. For the neuromuscular disease myotonic dystrophy (DM), the *DMPK* and *CNBP/ZNF9* genes contain CTG and CCTG expansions, respectively. DM type 1 (DM1) is associated with *DMPK* (CTG)_{37–>3,500}, while type 2 disease (DM2) is caused by *CNBP* (CCTG)_{75–~11,000} expansions. DM is an autosomal dominant disease that affects multiple systems including skeletal (myotonia, weakness/wasting) and heart (arrhythmias) muscles, the eye (subcapsular cataracts), and the brain (mental retardation, cerebral atrophy). Moreover, DM is both a congenital (CDM) and adult-onset degenerative disease. Curiously, CDM is only associated with CTG^{exp} mutations in the *DMPK* gene. While the molecular basis for this unique association has not been delineated, one possibility that has not been examined is the potential for problematic transmission of very large *CNBP* CCTG^{exp} (e.g., >1,000) alleles through the

germline, so disease-associated *CNBP* mutant alleles might only be generated postnatally.

Upon transcription of the mutant *DMPK* and *CNBP/ZNF9* genes, the corresponding CUG and CCUG expansion (CUG^{exp}, CCUG^{exp}) RNAs accumulate in nuclear, or ribonuclear, foci and inhibit the muscleblind-like (MBNL) proteins while also increasing CUGBP1/ETR-3-like factor (CELF) levels (Fig. 3). Coordinate MBNL loss and CELF1 (formerly CUGBP1) up-regulation occurs because (C)CUG^{exp} RNAs form RNA hairpins that sequester MBNL proteins while also activating protein kinase C (PKC), which results in elevated CELF1 steady state levels due to hyperphosphorylation. The MBNL and CELF proteins act antagonistically to control alternative splicing during development. MBNL1 promotes adult, while CELF1 increases fetal, splicing patterns for specific exons during the fetal to adult transition, so fetal isoform expression persists in adult DM tissues and causes characteristic disease manifestations. The timing of disease onset in adults could reflect the continued somatic expansion of these repeats, particularly in tissues with postmitotic cell populations (e.g., muscle, brain), so that a pathogenic threshold is crossed. Genome-wide expression and splice junction microarray analyses, as well as a crystal structure of two MBNL1 zinc finger-like domains (Zn3/4) bound to r(CGCUGU), indicate that MBNL1 preferentially recognizes a core YGCY motif consistent with its high affinity binding to CUG^{exp} and CCUG^{exp} RNAs (Castle et al. 2008; Teplova and Patel 2008; Du et al. 2010; Goers et al. 2010). In agreement with studies on the brain-specific Nova family of splicing regulators and the importance of binding site position relative to the regulated exon (Ule et al. 2006; Ule and Darnell 2007), binding of MBNL1 upstream of the 3' splice site promotes skipping of that alternative exon while binding downstream of the 5' splice site favors inclusion (Du et al. 2010). A similar scenario has been described for CELF proteins that recognize a UGUGU motif (Faustino and Cooper 2005; Castle et al. 2008; Daughters et al. 2009). CELF proteins contain three RNA recognition

M.G. Poulos et al.

Figure 3. RNA-mediated disease mechanism in myotonic dystrophy. Errors in DNA replication, recombination, and repair may lead to an increase in a normal length (CTG)₅ (small red box) repeat located in DMPK exon 15 to (CTG)_{>50} expansions (large red box). Following transcription, these mutant mRNAs with CUG^{exp} RNA hairpins sequester the MBNL proteins (red ovals) while also triggering activation of protein kinase C (PKC), which results in hyperphosphorylation (white P in black circle) of CELF1 (green oval). MBNL proteins are required during postnatal development to activate adult splicing patterns (red arrows) while CELF1 promotes fetal exon splicing (green arrows). Only specific exons are regulated by these two antagonistic splicing factors. For example, MBNL induces adult-specific skipping of CLCN1 exon 7a in muscle and GRIN1/NMDAR1 exon 5 in brain, while CELF1 promotes inclusion of these fetal exons. Open red and green arrowheads indicate that the precise roles of MBNL and CELF1 in GRIN1 pre-mRNA splicing have not been determined.

motifs (RRM1-3) and the C-terminal RRM of CELF1 forms specific contacts with the UGU trinucleotide (Tsuda et al. 2009). Both *Mbnl1* knockout and CELF1 transgenic mouse models have been generated, which recapitulate some features of DM disease (Kanadia et al. 2003; Timchenko et al. 2004; Ho et al. 2005). Nevertheless, the relative contributions of MBNL1 and CELF1 misregulation to pathogenesis are

unclear, and the molecular etiology of congenital DM remains a mystery.

The aberrant sequestration of splicing factors by another CG-rich microsatellite has been reported for the late-onset neurological disease fragile X-associated tremor ataxia syndrome (FXTAS). The primary features of FXTAS are action tremor and gait ataxia but cognitive decline, executive function deficits,

and mild parkinsonism have also been noted (Hagerman et al. 2001; Grigsby et al. 2007). This neurodegenerative disorder, which was originally identified in older adult carriers of premutation alleles of the fragile X syndrome (FRAXA) mental retardation gene (FMR1), is caused by (CGG)_{55–200} expansions in the FMR1 5' UTR that are shorter than those observed for FRAXA-associated (CGG)_{>230} repeats (Hagerman and Hagerman 2007). In neurons and astrocytes, transcripts from the mutant *FMR1* gene accumulate in large ubiquitin-positive intranuclear inclusions, together with a number of other proteins. Interestingly, these toxic CGG^{exp} RNAs are expressed in other cell types, including fibroblasts, and during embryonic development, which might explain the neurodevelopmental abnormalities observed in some premutation children (Garcia-Arocena et al. 2010). Several RNA-binding proteins, including CELF1 and hnRNP A2/B1, have been implicated in FXTAS pathogenesis, but a recent study indicates that the splicing factor Sam68, encoded by the *KHDRBS1* gene, is particularly prone to sequestration by CGG^{exp} RNAs (Sellier et al. 2010). Although this is a surprising finding because previous studies have indicated that Sam68 preferentially recognizes A- and U-rich sequences, the Sam68 CGG^{exp} interaction region does not include the central KH RNA-binding domain. Instead, Sam68 interacts with CGG^{exp} RNA via an N-terminal region important for protein–protein interactions, so recruitment to nuclear rCGG^{exp} inclusions is probably mediated by another uncharacterized factor. In support of the hypothesis that Sam68 plays an important role in FXTAS, expression of this protein is required for neuronal differentiation of P19 cells and *Khdrbs1*^{−/−} null mice develop motor coordination deficiencies, as indicated by rotarod and hindlimb beam walking tests, although cerebellar degeneration has not been reported (Lukong and Richard 2008; Chawla et al. 2009). Significantly, Sam68 depletion, either by sequestration in FXTAS brains or by shRNA-mediated knockdowns, results in the alternative splicing of some Sam68 target exons. Unlike DM, where specific mis-splicing events

have been linked to distinct pathological consequences (e.g., myotonia due to aberrant inclusion of *CLCN1* exon 7a), it is not clear if FXTAS is a developmental splicing disease. To address this concern, splicing-sensitive microarray and RNA-Seq analyses should be performed to determine if a FXTAS-specific splicing signature emerges.

Microsatellite expansions and the disruption of developmentally regulated splicing transitions have been implicated in other neurological diseases. The autosomal dominant spinocerebellar ataxias (SCAs) are a group of late-onset and progressive neurodegenerative diseases that are characterized by cerebellar atrophy resulting in ataxia (abnormal limb movement/coordination), tremor, and premature death (Carlson et al. 2009). SCA type 8 (SCA8) is particularly interesting since it is transmitted with reduced penetrance and caused by CTG•CAG repeat expansions in both coding (*ATXN8* gene) and non-coding (*ATXN8OS*) regions since the locus is bidirectionally transcribed (Moseley et al. 2006). For *ATXN8OS*, CUG^{exp} RNAs accumulate in ribonuclear foci that are detectable in the human SCA8 cerebellum, particularly in molecular layer interneurons and Bergmann glia. Ribonuclear foci are also detectable in a BAC transgenic mouse model for SCA8. These mice express a human (CTG•CAG)₁₁₆ expansion and develop characteristic motor deficits possibly due to loss of cerebellar GABAergic inhibition. A recent study confirmed that expression of *ATXN8OS* CUG^{exp} RNAs affects cerebellar inhibitory pathways by altering the activities of developmentally regulated splicing events controlled by CELF1 and MBNL1. Using the cross-linking/immunoprecipitation protocol (CLIP), a number of brain RNA targets for mouse Cugbp1 were revealed including the GABA-A transporter 4, *GAT4/GABT4A*. Expression of CUG^{exp} RNA, which was shown to be coordinately regulated by MBNL1 and CUGBP1, leads to persistence of the fetal *GAT4/GABT4* splicing pattern, chronically elevated *GAT4/GABT4* levels, and loss of GABAergic inhibition in the granular layer of the cerebellum (Daughters et al. 2009).

M.G. Poulos et al.

SCA type 31 (SCA31) has also been reported to be associated with a TGGAA^{exp} insertion in newly identified introns of the *TK2* and *BEAN* genes, which partially overlap and are transcribed in opposite directions on chromosome 16q22.1 (Sato et al. 2009). Similar to DM, mutant SCA31 RNAs accumulate in ribonuclear foci, so this SCA may also be an RNA-mediated disease, although these foci are detectable in Purkinje cells in SCA31. However, the splicing factors involved may be different since UGGAA repeats are not predicted to form stem-loop structures. A clue to the identity of these factors has emerged from studies on repetitive satellite III (SatIII) DNA repeats, which are composed of (GGAAT)_n CAAC(C/A)CGAGT repeats (Valgardsdottir et al. 2008). Thermal and chemical stresses trigger SatIII transcription and these RNAs concentrate in nuclear stress bodies (nSBs), which also contain splicing factors including SFRS1 (ASF/SF2). Interestingly, SFRS1 and SFRS9 (SRp30c) bind to (UGGAA)₈ RNAs in vitro, so it is possible that sequestration of these splicing factors causes misregulation of alternative splicing in Purkinje cells and degeneration (Sato et al. 2009).

In contrast to DM and SCA31, Friedreich ataxia (FRDA) is an autosomal recessive disease characterized by atrophy of primary sensory neurons in the dorsal root ganglia as well as hypertrophic cardiomyopathy (Pandolfo 2008). FRDA is caused by noncoding GAA expansions in the first intron of the *FXN* gene, which encodes the frataxin protein that is associated with the mitochondrial matrix and involved in iron homeostasis. Normal *FXN* alleles have up to ~38 GAA repeats that expand to ~70 to >1,000 repeats in FRDA. These GAA expansions are currently believed to be pathogenic because they inhibit transcription by altering DNA structure, by forming non-B DNA triplexes and “sticky” DNA, and by promoting chromatin condensation (Sakamoto et al. 1999; Saveliev et al. 2003). Recently, GAA expansions have also been shown to influence alternative splicing regulation. Insertion of a (GAA)₁₀₀ expansion downstream of an alternative exon in a hybrid minigene splicing reporter resulted in position-dependent effects on exon

skipping, flanking intron retention, and splicing of a cryptic exon containing the (GAA)₁₀₀ repeat (Baralle et al. 2008). More significantly, insertion of (GAA)₁₀₀, but not a complementary (TTC)₁₀₀ repeat, into a frataxin minigene reporter did not alter nascent transcript levels but did inhibit splicing of *FXN* intron 1. Thus, mis-splicing of *FXN* pre-mRNA may be a primary event that leads to decreased *FXN* mRNA levels.

Satellite Contractions: Potential Effects on RNA Processing

While loss-of-function mutations alter cis-acting splicing regulatory sequences in the *DMD* gene to cause exon skipping and frameshifting in DMD/BMD, toxic C(C)UG^{exp} RNAs expressed in DM alter the activities of trans-acting splicing factors that control the expression of developmentally regulated isoforms. A third mechanism that affects splicing regulation may be important in the molecular etiology of another muscular dystrophy, facio-scapulohumeral muscular dystrophy (FSHD). FSHD is caused by the contraction of a D4Z4 macrosatellite repeat array in the subtelomeric region of chromosome 4qter (van der Maarel et al. 2007; Tawil 2008). This 3.3 kb repeat is normally 11–100 D4Z4 units in length but contracts to 1–10 repeats in affected individuals and is preferentially associated with the 4qA161 haplotype. Current position-effect models propose that repeat contraction leads to a decrease in D4Z4 epigenetic modifications resulting in increased expression of genes that are either centromeric, including the spliceosome-associated protein FRG1, or within the repeat, such as the toxic double homeobox protein DUX4. In support of the former proposal, transgenic mice overexpressing FRG1 10- to 50-fold develop several FSHD-associated muscle features including kyphosis and RNA splicing changes (Gabellini et al. 2006). However, microarray expression studies have challenged the possibility that FRG1 is overexpressed in FSHD muscle (Osborne et al. 2007; Klooster et al. 2009). Alternatively, contraction-related changes in chromatin structure, or hypomethylation in the absence of contractions

also known as phenotypic FSHD, may lead to changes in transcript abundance and/or processing from specific D4Z4 repeats that are toxic to muscle cells. Recent studies have focused on the most telomeric D4Z4 repeat, which together with allele-specific flanking sequences, produces transcripts that are processed into si/miRNA-sized fragments as well as alternatively processed DUX4 RNAs that potentially antagonize myogenesis (Dixit et al. 2007; Bosnakovski et al. 2008; Bosnakovski et al. 2009; Snider et al. 2009). Future studies should determine if D4Z4, and perhaps other repetitive element, contractions activate transcription of locus-specific repeat arrays while coordinately altering transcript processing to generate pathogenic RNAs and/or proteins.

Concluding Remarks

Studies designed to clarify the molecular basis of some puzzling hereditary neurological disorders have provided both surprising results and fertile territory for further exploration. The key question of why mutations in genes encoding broadly expressed factors target specific cell types, such as tri-snRNP-associated proteins in adRP and SMN1 in SMA, remains unresolved, but the link to splicing misregulation has been reinforced by recent reports. Dynamic mutations associated with several muscular dystrophies and ataxias have also highlighted a novel disease mechanism, RNA-mediated pathogenesis, and the critical roles of novel alternative splicing factors during development. Future developments should yield more surprises and strengthen the connection between aberrant RNA processing and disease.

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