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Tailoring of Membrane Proteins by Alternative Splicing of Pre-mRNA

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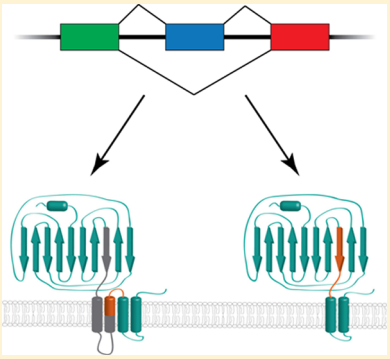
ABSTRACT: Alternative splicing (AS) of RNA is a key mechanism for diversification of the eukaryotic proteome. In this process, different mRNA transcripts can be produced through altered excision and/or inclusion of exons during processing of the pre-mRNA molecule. Since its discovery, AS has been shown to play roles in protein structure, function, and localization. Dysregulation of this process can result in disease phenotypes. Moreover, AS pathways are promising therapeutic targets for a number of diseases. Integral membrane proteins (MPs) represent a class of proteins that may be particularly amenable to regulation by alternative splicing because of the distinctive topological restraints associated with their folding, structure, trafficking, and function. Here, we review the impact of AS on MP form and function and the roles of AS in MP-related disorders such as Alzheimer's disease.



INTRODUCTION TO SPLICING OF PRE-MRNA

In 1941, Beadle and Tatum provided data that led to the "one gene, one protein" paradigm.[1] This paradigm persisted until researchers began to explore the human genome in depth, at which point it was discovered that the number of protein-encoding genes is much lower than originally predicted, leading to the "one gene, many proteins" hypothesis. Seminal work with adenovirus[2,3] first led to the notion that multiple transcripts can arise from the same precursor RNA molecule. In 1978, a new mechanism, now known as alternative splicing (AS), was proposed[4] for the generation of proteomic diversity from a single gene in eukaryotes, and experimental confirmation quickly followed.[5] During the maturation of mRNA in eukaryotes, RNA is spliced to remove regions of pre-mRNA termed introns.[6−11] The remaining segments, termed exons, are linked and can act in both protein-coding and noncoding functions. Differential splicing, in which different exons or regions of exons are included or excluded in construction of the final coding mRNA, provides the basis for allowing a relatively small number of genes to encode a large and diverse number of proteins. In addition to providing diversity to the proteome, AS may also regulate translation through the introduction of premature stop codons (via several possible mechanisms) that promote nonsense-mediated decay (NMD) of the alternatively spliced transcript (see refs 12−16).

While the removal of introns is common to all eukaryotic life forms, the percentage of genes that undergo splicing varies by species. Generally, the abundance of AS events is proportional to organismal complexity, with AS being most prevalent in more complex organisms such as vertebrates.[17] In higher eukaryotes, AS is ubiquitous, with at least 90% of human gene transcripts being subject to this process.[18] Additionally, typical exon length decreases with increasing organism complexity,[19] with 80% of human exons being <200 bp in length,[20] which means that many exons encode only short segments of the protein product. In addition to differences among species, there is also diversity of AS within an organism. The number of potential AS products varies by gene, and the expressed isoform(s) of any given protein can vary by cell type and even state of differentiation.[21]

There are several types of AS that can take place within a given transcript, including exon skipping (ES), alternative donor sites (AD), alternative acceptor sites (AA), intron retention (IR), and the rare complex event known as mutually exclusive (ME) exon splicing. Other rare complex events, such as the skipping of multiple exons in a row, also occur.[22] In a recent analysis of the human AS landscape, it was demonstrated that by far the most common AS events are the four "simple" modes of AS (ES, AD, AA, and IR, with ES accounting for approximately 45% of AS events[23]) (see Figure 1). However, more complex types of AS, such as the skipping of two exons in a row (∼6−12% of total AS events), skipping of three exons in a row (∼2−3% of total AS events), and ME (∼3−5% of total AS events), do account for roughly 30% of total AS outcomes, making complex modes of AS important contributors to genomic diversity. ME exons often encode the same structural domain of the protein with slight variations,[24] giving this mode of AS important roles in tuning protein structure and function.

Splicing occurs by two consecutive transesterification reactions[25] that are catalyzed by the spliceosome, a ∼3 MDa macromolecular machine composed of four snRNAs (small nuclear RNAs) and proteins (see Figure 2). In a popular model based on in vitro studies, this apparatus is postulated to assemble in a stepwise and dynamic fashion.[26,27] In the process of spliceosome assembly, several intermediary complexes form (see Figure 2).[6−11,28] The spliceosome recognizes three

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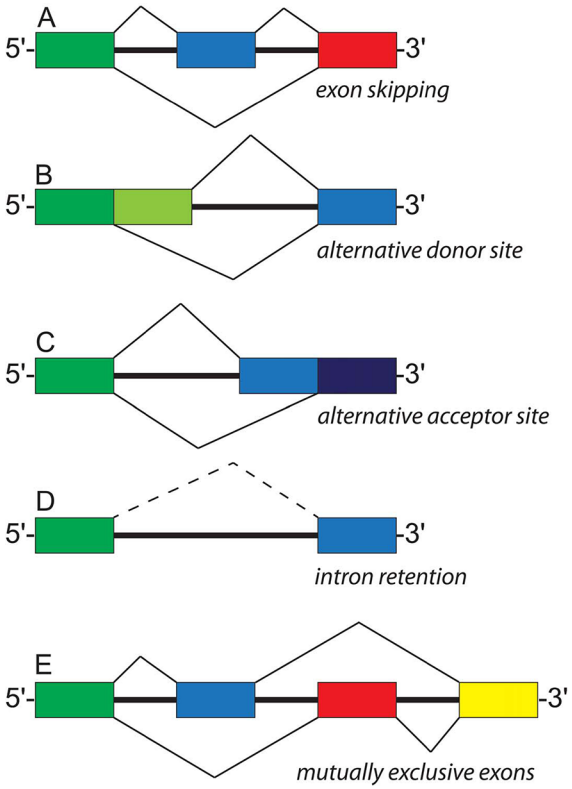

Figure 1. Modes of AS. (A) Exon skipping: (top) canonical splicing and (bottom) removal of the blue exon. (B) Alternative donor sites: (top) canonical splicing and (bottom) use of an alternative 5′ donor site. (C) Alternative acceptor sites: (top) canonical splicing and (bottom) use of an alternative 3′ acceptor site. (D) Intron retention. In cases of intron retention, canonical splicing (−−−) does not occur and the intron is included in the processed mRNA. (E) Mutually exclusive exon splicing: (top) inclusion of blue exon and exclusion of red exon and (bottom) exclusion of blue exon and inclusion of red exon.

primary splicing signals: the 5′ and 3′ splice sites and the branch site. In the first transesterification reaction, the 2′-OH of a conserved adenosine nucleotide within the intervening intron region performs a nucleophilic attack, resulting in the release of the 5′ exon containing a free 3′-OH and the formation of the lariat intermediate structure. In the second step, the 3′-OH of the 5′ exon serves as the nucleophile at the 3′ splice site, producing the ligated exons and releasing the 2′−5′ branched lariat intron. Spliceosome assembly begins with the recognition of the 5′ splice site and branch point sequences of the pre-mRNA by the U1 snRNP and the U2 snRNP, respectively. After the U4/U6·U5 tri-snRNP has bound, the U4/U6 snRNA duplex is replaced by a U2/U6 snRNA duplex. Furthermore, the U1 snRNA base pairing at the 5′ splice site is disrupted and exchanged for base pairing between the 5′ splice site and the U6 snRNA. The subsequent addition of another complex, the NTC (nineteen complex), and the release of the U1 and U4 snRNPs mark the transition from an inactive to an active spliceosome composed of the NTC, and the U5 and U2/U6 snRNPs. Then 5′ splice site cleavage and lariat formation, followed by 3′ splice site cleavage and exon ligation, occur within the activated spliceosome. After completion of the second transesterification step and ligation of the two exons, the postspliceosomal complex is dismantled and the lariat intron is released (see Figure 2). In each step of spliceosome assembly, catalysis, and disassembly, there are a number of RNA helicases and other enzymes that are needed to aid and regulate the many RNA−RNA, RNA−protein, and protein−protein interactions.[9]

Most introns contain conventional consensus sequences that delineate the primary splice sites.[8−10,29] The 5′ splice site typically begins with a conserved GU, which is flanked by three residues on the exon side and seven residues on the intron side that are generally similar among 5′ splice sites. The 3′ splice site usually follows the trinucleotide CAG or UAG (or, more rarely, AAG). The first nucleotide of the adjacent exon is also partially conserved. In addition to these consensus sequences, a tract of pyrimidine-rich bases upstream of the 3′ splice site also contributes to recognition by the spliceosome. The final primary determinant of splice site recognition is the branch site consensus sequence, typically UACUAAC, where the final A serves as the nucleophilic branch site nucleotide and forms the lariat structure depicted in Figure 2. While this sequence is the most preferred by the splicing machinery, variation does occur around the branch site in certain cases. There is also another, less common, class of introns present in higher eukaryotes that utilizes both distinct consensus sequences (|ATATCCTT at the 5′ splice site, CCTTRACCY at the branch site, and YAC| at the 3′ splice site, where the vertical line indicates the actual site of the splicing event) and distinct spliceosomal components (the U11, U12, U4atac, and U6atac snRNPs, which are analogous to the U1, U2, U4, and U6 snRNPs, respectively). This minor spliceosome form proceeds through a stepwise reaction pathway identical to that depicted in Figure 2 and accounts for <1% of introns in human cells.

In addition to the primary determinants discussed above, there are other sequence signals outside of the conventional splice sites that regulate splicing. For instance, splicing enhancers and suppressors either increase or decrease the incidence of splicing at a particular site.[30,31] The relative strengths of multiple splice sites in the vicinity of the same sequence can also play roles in determining which site the spliceosome recognizes. RNA secondary structure also often appears to play a role in splice site selection.[32−34] In another surprising mechanism, histone modifications present on the nucleosomes binding to exonic regions of DNA have also been directly linked to splicing outcomes.[35] Together, these extra determinants outside the canonical splice site sequences provide additional ways for the spliceosome to distinguish the "correct" splice site from similar, but inappropriate, sites found elsewhere in the gene. Although these extra determinants complicate the "splicing code", making it difficult to untangle which transcripts form from various genes, they serve as an important mechanism for "fine-tuning" splice site selection, providing the flexibility to precisely regulate which sequences are spliced or retained

With the currently available understanding about splice site consensus sequences and other signals that affect splicing, one might expect there to be robust computational approaches that would accurately predict exon−intron boundaries. Indeed, this is the goal of several online splice site locator and gene structure prediction tools;[36−39] however, the additional signals that regulate splicing discussed above often make these predictions complicated and occasionally inaccurate. A recent study[40] has taken further steps to incorporate regulatory information into the prediction algorithms allowing the prediction of tissue-specific alternative splicing. That study utilized information about experimentally determined splice variants from four types of major tissues in mice, as well as information regarding the potential splicing code. This code

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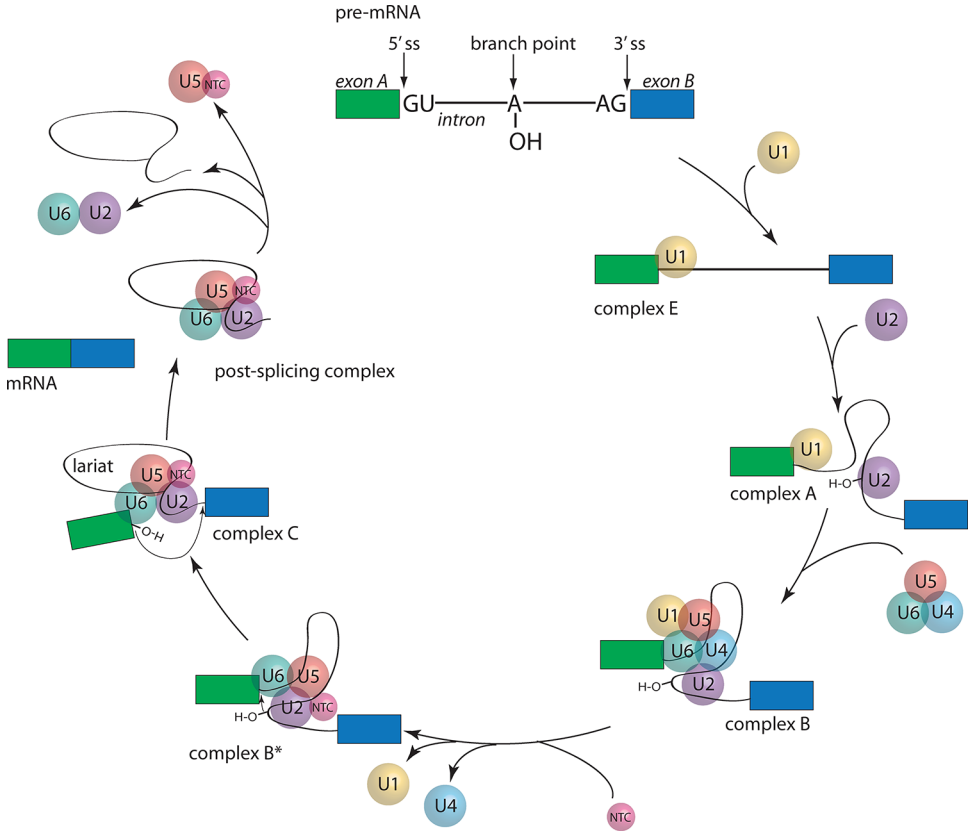

Figure 2. Spliceosome assembly and splicing reaction. In a clockwise manner from the top, U1 snRNP associates with the 5′ splice site to make complex E. U2 snRNP is recruited and brings the branch site near the 5′ splice site, forming complex A. The U4/U6·U5 tri-snRNP is recruited to form precatalytic complex B. The arrival of the nineteen complex (NTC) and the release of U1 and U4 snRNAs mark the conversion to complex B*, an active spliceosome. Complex C forms after the first transesterification reaction and conducts the second transesterification reaction. The mature message is released, and the postsplicing complex is recycled.

includes sequences (1) shown to recruit regulatory proteins, (2) that are enriched around splice sites, (3) containing RNA secondary structure, and (4) that contain spatial relationships among exons, introns, and other sequence motifs.[40,41] As a result of that study, an online tool (Web site for Alternative Splicing Prediction, or WASP) was developed for de novo gene structure prediction as well as de novo prediction of the splice variants present in four major tissues (http://genes.toronto.edu/wasp/). We are hopeful that the continued updates of WASP to reflect our improving and growing knowledge of the splicing code will eventually lead to an accurate tool for predicting gene structure and cell- and tissue-specific profiles of transcript variant expression. Finally, it should be noted that in addition to the computational algorithms for de novo prediction of gene structure and alternative splicing events, there are several databases that compile known splice variants and tissue expression profiles of genes,[36] including a database (TMSPLICE) that specifically documents alternative splicing events in MPs within the mouse genome.[42]

Alternative splicing provides an important mechanism by which higher eukaryotes can both increase genetic diversity and regulate protein activity. Splicing variation affects the structure, function, and expression levels of protein products. Exploring alterations in physiology caused by AS is important for improving our understanding of the ramifications of misregulated AS and, ultimately, the role of AS in disease processes.[43−46] This review will focus on how AS impacts membrane proteins (MPs).

RNA SPLICING AND MEMBRANE PROTEINS (MPS)

MPs play key roles in numerous cellular functions, including cell signaling, transport, and energy transduction, as well as in the organization and stabilization of cellular structure. MPs differ from soluble proteins in terms of native environment, folding topology, and organization of domains and subunits. It is well established that RNA splicing can have a wide range of effects on soluble protein expression, localization, folding, structure, and function.[47] There are also many examples of the role of AS in regulating MPs. However, there is currently no published survey of how AS impacts membrane proteins in ways that reflect the unique properties of this class of proteins. Here, we present a number of examples to illustrate how RNA splicing can alter MP expression, distribution, folding, topology, structure, and function. Care has been taken to highlight examples that represent a cross-function of MP types, including channels, transporters, receptors, structural proteins, and enzymes. The wide range of effects exerted by AS on MPs suggest that AS acts as a little-recognized global regulator of MP structure and function in higher organisms and thus has important implications for human disease.

AS Can Alter MP Topology or Produce Soluble Proteins. The number and arrangement of transmembrane (TM) segments in a MP define the orientation of a protein and are often closely integrated with function and stability. Alternative or aberrant splicing can disrupt TM segments when splice sites are found within a TM-encoding region of the transcript. Likewise, it is possible that removal of an entire TM

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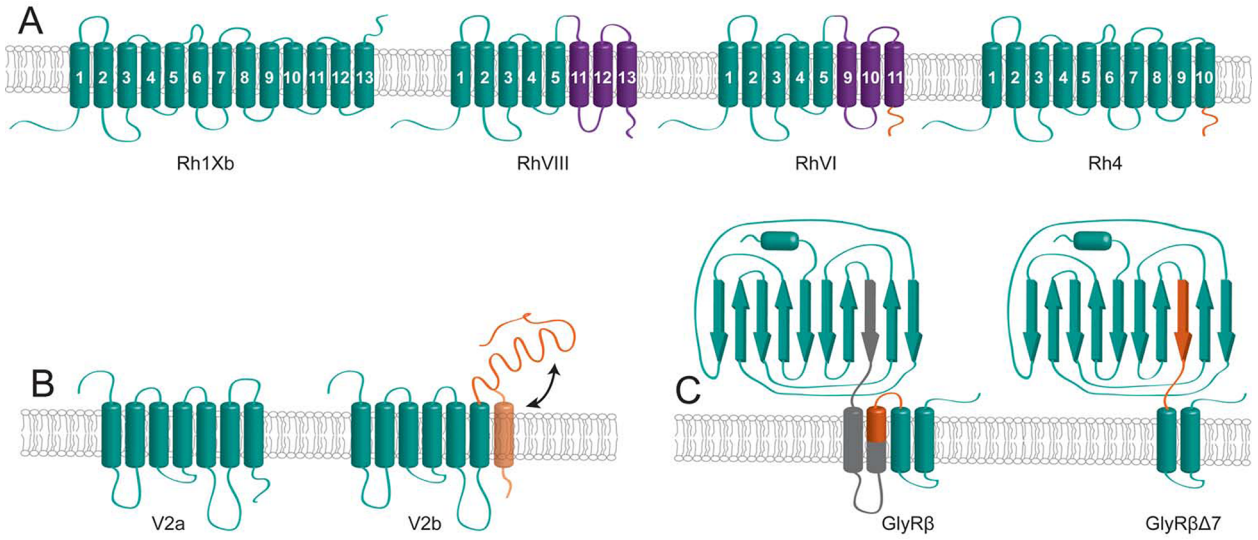

Figure 3. AS can alter MP topology. (A) The membrane topology of the RhCcEe protein is altered because of AS. RhX1b is the canonical isoform; helices are numbered per this isoform. Exon removal in RhVIII results in the removal of TM segments 6−10. The original TM segments 11−13 (purple) cross the membrane with inverted topology. RhVI, as a result of exon removal, is missing the residues that form TM segments 6−8 and also displays inverted topology in canonical TM segments 9−11. Additionally, RhVI has another deletion due to exon removal. This deletion causes a frameshift and results in a different C-terminal sequence with a premature stop codon. The Rh4 isoform undergoes exon removal that induces a frameshift resulting in a different C-terminal sequence and a premature stop codon at the same location as in RhVI. The new C-terminal sequence generated by the frameshift in RhVI and Rh4 encodes the same 14 amino acids (orange), indicating that the sequence likely is important. (B) G-Protein-coupled receptor V2 vasopressin receptor. V2a is a stably expressed seven-TM segment (7-TM) protein found at the plasma membrane. Conversely, V2b differs in sequence from V2a in the C-terminal region after the sixth TM segment. This alteration in the C-terminus results from the use of an alternate 3′ splice site 76 bp downstream of the canonical 3′ splice site, which causes a frameshift. The V2b protein isoform was experimentally shown to significantly populate two topologies: a 7-TM topology similar to that of canonical GPCRs with the C-terminus located intracellularly and a 6-TM topology with the C-terminus oriented to the extracellular matrix. (C) The glycine receptor β subunit is alternatively spliced to remove exon 7 ($\beta\Delta7$). The long isoform of the β subunit including exon 7 possesses four-TM topology. The $\beta\Delta7$ variant has a predicted two-TM segment topology due to the removal of the tenth β-strand in the extracellular domain, TM segment 1, the first intracellular loop, and a portion of TM segment 2 (gray). The remaining portion of TM segment 2 is predicted to substitute for the tenth β strand in the extracellular domain (orange).

segment via splicing can significantly alter the topology of a MP or even eliminate membrane anchoring altogether. In this section, we review placement of splice junction locations within MP gene structure and examine examples where splicing significantly alters MP topology. We also briefly discuss the impact of these AS events on MP function.

Intron Junctions Tend Not To Be Localized within TM-Encoding Segments. The aberrant introduction of splice site junctions within TM segments would likely lead to serious disruption of the encoded membrane protein structure. Indeed, computational studies assessing the likelihood of an intronic division within a TM region have found that the probability of a TM not being divided by an intron was slightly higher than the expected probability for a random 22-mer amino acid sequence.[42] This difference was most pronounced in single-pass MPs, where TM regions were encoded on single exons 84.6% of the time, in comparison with the random sequence expectation of 58.5%. Thus, there seems to be evolutionary pressure for MPs, especially single-pass MPs, to keep introns out of regions encoding TMs within pre-mRNA.

AS Can Produce MPs with Altered Topology. Rh blood group antigens are important in blood-typing for blood transfusion and organ transplantation. These antigens are typically predicted to have 12 TM segments.[48] The RhCcEe proteins represent an example of MPs whose topologies are altered because of AS.[49] The characterization of Rh cDNA clones led to the conclusion that at least four different transcripts are produced from the RhCcEe gene by AS (Figure 3). The removal of an exon in one of the isoforms (RhVIII) results in the removal of residues 163−313, which form five TM segments. As a result, three of the helices are predicted to cross the membrane in reverse orientation relative to the original full-length transcript. Inversion of TM segment topology also occurs in isoform RhVI, which, as a result of exon removal, is missing residues 163−267, which form three TM segments. The reversal alters the residues that are surface-exposed and may alter antigenicity (see Figure 3A). Additionally, RhVI has another deletion (corresponding to residues 359−384) due to exon removal. This deletion causes a frameshift and results in a different C-terminal sequence with a premature stop codon. The Rh4 isoform is missing amino acid residues 314−358 as a result of exon removal; this exon deletion event induces a frameshift resulting in a different C-terminal sequence and a premature stop codon at the same location as in RhVI. Interestingly, the new C-terminal sequence generated by the frameshift in RhVI and Rh4 encodes the same 14 amino acids, indicating that this sequence is likely important, although its function is not yet known (see Figure 3).

Another example of altered TM topology is found in the V2 vasopressin receptor, a G-protein-coupled receptor (GPCR). Its two isoforms, V2a and V2b, were found to have different topological states and different levels of stability[50] (Figure 3B). V2a is a stably expressed seven-TM segment (7-TM) protein found at the plasma membrane. Conversely, V2b differs in sequence from V2a in the C-terminal region after the sixth TM segment. This altered C-terminal tail results from the use of an

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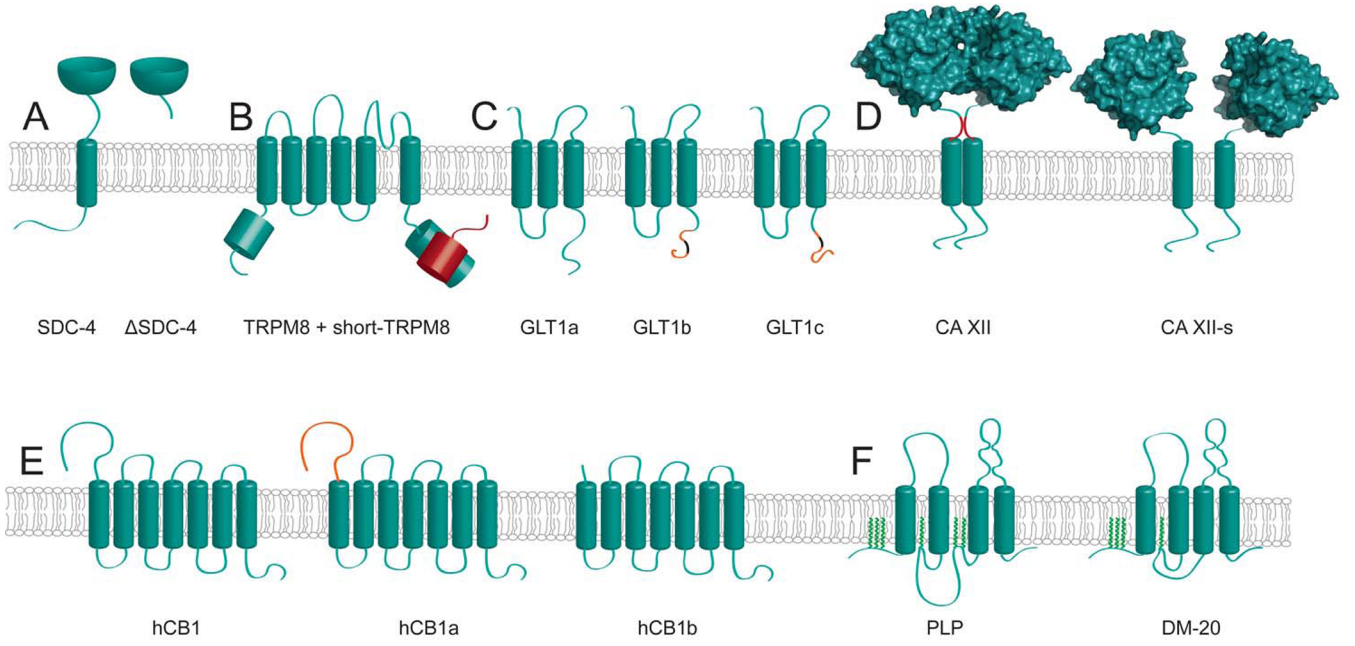

Figure 4. AS can alter MP function. (A) AS of syndecan-4 results in an mRNA that encodes only the ectodomain. This AS event may serve a purpose similar to that of proteolytic shedding of ectodomains. (B) AS of TRPM8 creates a short isoform (red) that regulates TRPM8 through interaction with the C-terminal domain, acting to stabilize the closed form of the channel. (C) AS of GLT1 results in isoforms with different C-terminal tails. GLT1b and GLT1c contain different C-terminal sequences (orange), but both include a PDZ domain binding motif (black) that presumably functions to recruit proteins that contain PDZ domains. GLT1a does not possess this motif. (D) AS of CA XII results in the removal of a GXXXG motif (red) that mediates dimerization. This may prevent homodimerization and influence the activity of the enzyme. (E) AS hCB1 results in different N-termini (orange) and altered receptor responses to canonical ligands, presumably through an allosteric effect. (F) Alternative splicing of PLP results in removal of a portion of a loop that includes sites for post-translational lipid modifications. Removal of these sites may influence the trafficking of protein to lipid raft domains.

alternate 3′ splice site 76 bp downstream of the canonical 3′ splice site, which results in a frameshift. The V2b protein isoform was experimentally shown to significantly populate two different topologies: a 7-TM topology similar to that of canonical GPCRs with the C-terminus located intracellularly and a 6-TM topology with the C-terminus oriented to the extracellular matrix (Figure 3B). The V2b receptor also differs in that it is localized to the ER and Golgi apparatus rather than trafficking to the plasma membrane. Retention of the protein in the ER and Golgi could be due to the splicing-based deletion of C-terminal forward-trafficking motifs or may reflect sequestration of the truncated protein by MP folding quality control.

A third protein whose TM topology is affected by alternative splicing is the glycine receptor,[51] which assembles into heteropentameric ($\alpha_2\beta_3$) ion-conducting pores and acts to mediate neurotransmissions in the central nervous system. It was found that the β subunit of this receptor was alternatively spliced to remove exon 7 ($\beta\Delta7$). The long isoform of the β subunit including exon 7 possesses a predicted four-TM topology, while the $\beta\Delta7$ variant possesses a predicted two-TM segment topology because of the removal of TM segment 1, the first intracellular loop, and a portion of TM segment 2 (see Figure 3C). Interestingly, the extracellular domain is kept intact, and the protein is stably expressed in the cerebral cortex and can hetero-oligomerize with the glycine receptor α subunits or with the glycine receptor-anchoring protein gephyrin. Because TM segment 2 (which is shortened in $\beta\Delta7$, where it is instead predicted to form the tenth β strand in the large extracellular domain) is part of the ion conduction pathway, the functional properties of glycine receptors containing $\beta\Delta7$ may be different. Indeed, experiments showed that the $\alpha_1\beta\Delta7$ complexes did not exhibit resistance to the channel-blocking agent picrotoxin while $\alpha_1\beta$ complexes display this property,[51] suggesting that the pore is formed by only the α subunits.

AS Can Alter the Signal Peptide. Signal peptides are important for membrane targeting and determination of protein subcellular localization and are often altered as a result of AS. In a computational analysis of the mouse transcriptome, 40% of transcriptional units (sets of transcripts derived from the same gene) were shown to have signal peptide variation, with the majority of transcript variation being the consequence of AS.[52] These alterations are important for targeting soluble proteins to various subcellular compartments (lumen of the endoplasmic reticulum vs the cytosol, for example). Signal peptides also play an important role in the targeting and integration of proteins into the membrane, where they help determine membrane protein topology. The same study investigated a high-confidence set of 782 transcriptional units, where the presence of alternative transcription initiation start and termination sites in transcripts was independently confirmed, with variations in membrane organization. While this set most likely excludes most examples of AS based on the selection criteria, it is important to note that AS may accomplish a similar task in cells based on alternative initial exons. In the same set of transcripts, there were 41 instances of signal peptide removal that resulted in a switch from type I (C-terminal cytosolic) TM topology to type II (N-terminal) TM topology in single-pass TM proteins. It is likely that the use of alternative initial exons, which make up 58% of the mechanisms of variation among the set of transcriptional units with signal peptide variation investigated in this study, could result in a similar total inversion of topology. It seems feasible that AS

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could also result in complete inversion of topology of multispan membrane proteins. There are MPs of this class that are known to populate opposite topologies,[53] although splicing has not been established as the driving mechanism.

Removal of the signal peptide by AS has been documented in the parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor.[54] The gene for this GPCR can be transcribed from two different transcription start sites. Use of a novel promoter in the kidney results in the generation of three alternatively spliced transcripts (types I–III), of which the signal peptide is replaced with a hydrophilic sequence in the type III splice variant. This splice variant, which was shown to retain reactivity, was expressed only minimally on the cell surface, with the majority being localized to intracellular compartments, likely as a result of replacement of the signal peptide. The type III receptor could be reactive to an intracellular form of PTHrP or may simply represent the outcome of a downregulation mechanism.

AS Can Result in the Production of Soluble Proteins from MP-Encoding Genes. In addition to an altered TM topology, alternative splicing can eliminate TM domains from transcripts that would otherwise encode MPs. This results in soluble protein isoforms that may have vastly different roles in (or out) of the cell. A survey[55] of a portion of the human MP transcriptome revealed that nearly 40% of the 464 surveyed alternatively spliced single-pass MP genes have isoforms that completely lack a TM domain (Figure 4A). For example, the alternatively spliced soluble isoform of the syndecan-4 receptor (SCD-4) is very similar to the ectodomain of the canonical isoform. Syndecan receptors normally function as coreceptors by concentrating and presenting a variety of ligands (e.g., extracellular matrix proteins, growth factors, cytokines, and cell adhesion molecules) to other cell surface receptors, or they act to internalize ligands, sequestering them from other receptors, thereby modulating signaling. The ectodomain of SCD-4 can be proteolytically cleaved, an event that is regulated by multiple signaling pathways, and this solubilized domain can still bind ligand, affecting signaling by competing with the membrane-bound SCD-4 counterpart.[56] It is possible that the AS soluble isoform may play a ligand sequestration role similar to that played by the ectodomain generated by proteolytic cleavage.

Another example of production of a soluble isoform from a MP transcript is cadherin-7, a cell adhesion TM glycoprotein. In developing chickens, it was found that a soluble form of cadherin-7 was expressed[57] and that this isoform can interact with the corresponding domain in the TM variant of this same protein, resulting in inhibition of cell–cell adhesion by interfering with homomeric interactions of the TM form.

AS Can Alter MP Function. *Channels.* Ions cannot cross the hydrophobic lipid bilayer via diffusion. Cells therefore express numerous types of channels, both to regulate cytosolic ion content and, in the case of specialized cells such as neurons and muscle cells, for signaling purposes. Ion channel activity can be regulated by AS. For example, transient receptor potential (TRP) potassium channels are heavily regulated by AS.[58] The functional impact can range from altered conductance to changes in selectivity or activation. More drastic changes include the generation of dominant negative isoforms that prevent native channel function through heteromultimerization with the canonical isoforms. Consider the cold- and menthol-sensitive TRPM8 channel. Bidaux et al.[59] have described the activity of two newly discovered short isoforms of TRPM8 (dubbed short TRPM8α and short TRPM8β). These two short isoforms contain only the cytosolic N-terminal domain and thus are not capable of forming functional channels but bind to and regulate canonical long-form TRPM8 channel tetramers (Figure 4B). Specifically, these variants stabilize the closed form of the channel, reducing channel activity and sensitivity to cold.

The TRPM3 channel is alternatively spliced to produce different sequences within the presumed pore domain, generating different cation selectivities. For instance, TRPM3α1 and TRPM3α2 are both outwardly rectifying cation channel isoforms, meaning they allow higher current flux out of the cell than into the cell, whereas other TRPM3 variants are inwardly rectifying. TRPM3α2 is highly permeable to divalent cations, while TRPM3α1 favors monovalent conductance. Additionally, monovalent cations block currents through TRPM3α2 but not TRPM3α1. These TRPM3 variants clearly demonstrate a role for AS in regulating channel ion permeability, selectivity, and regulation.[60]

Transporters. Transporters facilitate the passage or flip-flop of solutes across the membrane, often in an energy-consuming manner against the TM concentration gradient (active transport). Transporter activity is typically tightly regulated, with AS being one mechanism of regulation.

An important family of regulated transporters consists of the P-type ATPases. This family includes the plasma membrane calcium pump, which is present in all eukaryotic cells and helps to maintain appropriate Ca^{2+} levels within the cytosol by moving calcium ions from the cytosol to the extracellular matrix. The calcium pump is alternatively spliced such that its calmodulin binding regulatory domain is altered to reduce its net positive charge, resulting in a lower calmodulin affinity.[61] The reduced affinity of the ATPase for calmodulin results in a reduced affinity of the transporter for cytosolic Ca^{2+} ions.

Another example is the glutamate transporter family. After release of neurotransmitter into the synaptic cleft, transporters are responsible for transmitter reuptake by the cell and regulation of the volume of these vital molecules at the synapse. Levels of glutamate, the major excitatory neurotransmitter in the brain, are regulated by glutamate transporters, the most prominent of which is GLT1 (EEAT2 in humans). The three alternatively spliced isoforms of this protein (GLT1a, GLT1b, and GLT1c) differ in their C-termini,[62] with both GLT1b and GLT1c containing a PDZ domain recognition motif. GLT1a lacks this sequence (Figure 4C). Inclusion of a PDZ domain binding sequence likely has functional consequences for the different GLT1 isoforms, because they may then recruit PDZ domain-containing proteins that modulate transporter function, trafficking, or associated signaling. GLT1a and GLT1b have similar expression profiles and are primarily expressed in the brain, while GLT1c is expressed in the retina.

Receptors. G-Protein-coupled receptors (GPCRs) are an essential subset of MPs, the target of almost 40% of the drugs on the market today,[63] and represent an interesting example in terms of AS. Although many GPCR-encoding genes do not contain introns, some GPCR genes do.[64–66] Besides the V2R and PTHrP receptors discussed above, classic examples include the D_2 dopamine receptor and the rat type I pituitary adenylate cyclase-activating peptide receptor (PAC_1 receptor). The D_2 receptor is present in one of two isoforms in the central nervous system (CNS): short (D_{2S}) and long (D_{2L}). The D_{2S} receptor lacks 29 residues within the third intracellular loop, likely resulting in different signaling properties because of the

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role of the intracellular loops in interaction with its cognate G-protein.[64] The PAC_1 receptor is expressed in five different isoforms, all with variations in the third intracellular loop resulting in different signaling properties. The alternative forms of this loop differentially regulate activation of the downstream effectors adenylate cyclase and phospholipase C,[64] again, most likely by altering interactions and specificity between the receptor and heterotrimeric G-proteins.

In the case of cannabinoid receptor hCB1, two splice variants have been identified (hCB1a and hCB1b) in addition to the canonical receptor. These variants have altered amino termini; hCB1a has an altered N-terminal sequence, and hCB1b is missing the first 33 N-terminal amino acid residues. In both cases, AS occurs through the use of different splice site donor and acceptor sites. hCB1a and hCB1b display altered pharmacological properties in relation to those of the canonical hCB1 receptor (Figure 4E). Their affinity for the native ligand anandamide is dramatically reduced, and their affinity for 2-arachidonoylglycerol is somewhat decreased. Additionally, it was found that instead of acting as an agonist, as it does in the hCB1, 2-arachidonoylglycerol functions as an inverse agonist when interacting with hCB1a and hCB1b.[67] Because the N-termini are not in the ligand binding site, this example points to a function of AS in the allosteric regulation of receptors.

Enzymes. TM enzymes are also affected by AS. Carbonic anhydrase XII (CA XII) is an enzyme whose alternative splicing is associated with cancer.[68] There are two alternative transcripts of CA XII, the longer of which encodes 11 additional amino acid residues immediately preceding the predicted TM domain. Both transcripts are found in healthy tissue, but the longer isoform normally dominates. However, in astrocytomas, the level of expression of the shorter isoform is greatly increased and the longer isoform is rarely detected. Why the shorter isoform is more prevalent in cancer cells is not well understood, although it is likely that the shorter isoform of CA XII may have difficulty in forming its normal quaternary structure. This is because a portion (GXXX) of the GXXXG motif, important for homodimerization of the enzyme, is missing because of alternative splicing (Figure 4D). Thus, quaternary structure disruption likely contributes to catalytic dysfunction. For additional examples of enzymes affected by AS, see Alzheimer's Disease.

Structural Proteins. The proteolipid protein (PLP) is the most abundant protein in CNS myelin and is thought to maintain the structural integrity of myelin and support myelin compaction, although it may have additional roles in signaling.[69] PLP is a tetraspan MP with an intracellular "principal loop" connecting TM helices II and III. DM-20 is a splice variant of PLP and lacks a large portion (34 amino acids) of the principal loop,[70] including two palmitoylation sites[71] (Figure 4F). It is likely that this region allows for differential regulation of the functions of PLP and DM-20, altering their trafficking, structure, and/or signaling functions. It is interesting to note that AS-based elimination of two palmitoylation sites may also reduce the propensity of DM-20 to interact with cholesterol-rich membrane domains often termed "lipid rafts" or other specialized membrane domains in myelin membranes.

Regulation of Protein–Protein Interactions. Differences in alternatively spliced isoforms can impact the ability of the protein product to interact with partners. As discussed above, the GLT1 transporter isoforms differ in their possession of a PDZ domain binding motif, a motif likely used to bind protein partners containing PDZ domain binding domains. Another example is the *N*-methyl-D-aspartate (NMDA) receptor. Yotiao, an A-kinase-anchoring protein, forms a scaffold to the NR1A splice variant of the NR1 subunit of the NMDA receptor but not to the NR1C splice variant.[72] This interaction is modulated by either the presence or the absence of a subdomain encoded by an alternatively spliced C1 exon cassette that is included in NR1A but not the NR1C splice isoform. Additional research suggests that Yotiao may mediate a ternary complex of itself, the NMDA receptor, and cAMP-dependent protein kinase II. This interaction is postulated to provide a mechanism by which cAMP modulates NMDA receptor signaling.[73] Similarly, calmodulin interacts avidly with the receptor through binding of NR1A to the region encoded by the very same C1 exon cassette, thereby inhibiting NMDA receptor function.[74,75]

Tissue-Specific AS. Through tissue-specific isoform expression, AS can alter the function of the protein product of a gene to better suit the requirements of its environment. For example, TMEM16A is thought to function as a calcium-dependent chloride channel (CaCC) with eight proposed TM segments. Recent studies of the patterns of alternative splicing that produce distinct isoforms of this protein show that differential exclusion or inclusion of exons 6b, 13, and 15 in the mRNA transcript occurs in different organs.[76] The liver, placenta, prostate, thyroid, and trachea tend toward inclusion of exon 6b and exclusion of exon 15. However, in the brain, the mRNA is most often missing exon 6b but contains exon 15. Exon 13 skipping was found to occur only in brain and skeletal muscle. These changes in mRNA structure are reflected in the protein structure with functional consequences. For instance, skipping of exon 6b results in a deletion of a 22-amino acid intracellular segment in the N-terminus of TMEM16A, leading to a much higher affinity of TMEM16A for intracellular Ca^{2+} ions. Deletion of exon 13 results in removal of four amino acids in the intracellular loop between TM segments 2 and 3, reducing the voltage dependence of the Cl^- currents.

AS can also involve noncoding regions of the mRNA. For instance, peripheral myelin protein 22 (PMP22), a protein involved in the inherited peripheral neuropathy Charcot-Marie-Tooth disease, is spliced in a tissue-specific manner. The first exon in the 5′ untranslated region (UTR) is alternatively spliced to include exon 1a or exon 1b.[77] Exon 1a dominates in myelinating Schwann cells, while exon 1b is common to non-neural tissues expressing PMP22. Evidence suggests that expression of these two alternative transcripts is actually regulated by two different upstream promoters in the DNA, with the resultant pre-mRNA containing either exon 1a or 1b. The resultant proteins are identical, and it appears that the different promoters and the different 5′ exons act to differentially regulate PMP22 expression in a tissue-specific manner at both the transcriptional and the translational stages.

Impact of AS on Trafficking and Subcellular Distribution. Trafficking sequence motifs play key roles in directing the trafficking and subcellular distribution of proteins within the cell. AS can result in the removal or retention of these signals to directly affect protein trafficking and organelle sublocalization. For instance, there are two sets of *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors (SNAREs). v-SNAREs are single-pass MPs embedded in the membranes of vesicles, while t-SNAREs are embedded in the membranes of the target organelle. Together, these SNAREs work to fuse vesicles to their target destination, releasing the vesicular contents. Importantly, there are two isoforms of the well-characterized v-SNARE vesicle-associated MP-1 (VAMP-1),

VAMP-1A and VAMP-1B, that differ in sequence at their C-termini, presumably through use of an alternative 3′ exon. The VAMP-1B isoform is targeted to the mitochondria via conferral of a positive charge to the C-terminus and shortening of the hydrophobic membrane-embedded portion of the protein by four amino acid residues, while the VAMP-1A isoform is localized to the plasma membrane and the endosomes.[78] Consequently, AS not only affects the trafficking of these important proteins but also likely affects the destination of the vesicular contents of VAMP-directed vesicles (to the mitochondrial matrix for VAMP-1B and to the extracellular matrix for VAMP-1A). In another example, lysosome-associated MP (LAMP) 2, a major component of the lysosomal membrane, has three splice variants (LAMP-2a, -2b, and -2c) that are trafficked either to lysosomal compartments (LAMP2c) or to the cell surface (LAMP-2a and -2b). These differences are dictated by variation of their C-terminal targeting sequence,[79] which is governed by alternative splicing of the last exon in the transcript, presumably through a mutually exclusive splicing event of the 3′ exon.

Impact of AS on Folding. MP folding is still poorly understood in comparison to soluble protein folding, and evidence of the impact of alternative splicing on MP stability, folding pathways, and interactions with chaperones is currently sparse. Some AS events do result in the retention of specific variants (but not others) in the ER, which may indicate that AS sometimes results in changes in protein structure that are perceived by the protein folding quality control as signatures of misfolding. For instance, the V2b variant of the vasopressin receptor[50] is retained in the ER, as are two alterative isoforms of the TM aspartyl protease β-site APP cleavage enzyme 1 (BACE1), BACE1 I-476 and I-457, which are also far less active than canonical BACE1 isoform I-501.[80] It has also been specifically suggested that V2b might represent a destabilized form of the V2 vasopressin repressor,[50] which is not surprising considering that the V2b isoform has an altered topology (see Figure 3). The extent to which these apparent AS-encoded "misfolding" events are important for normal cell biology (and are therefore evolutionarily selected for) remains to be seen. In any case, as the technology for examining MP folding and misfolding becomes more accessible, care should be taken to investigate the possible impact of AS on MP trafficking and folding efficiency.

■ AS OF MPS AND DISEASE

Alternative splicing of soluble proteins has long been documented as a factor contributing to disease.[43−46] Here, we review two disorders in which aberrant RNA splicing has a pathological impact on MPs.

Myotonic Dystrophy Type 1. Myotonic Dystrophy Type 1 (DM1) is an inherited multisystem progressive disorder affecting 1 in 8000 people. DM1 is characterized by myotonia, heart defects, cataracts, gastrointestinal defects, insulin resistance, muscle wasting, and neuropsychiatric disorders.[81,82] DM1 appears to be triggered by expansion of a CUG repeat in the 3′ untranslated region (3′-UTR) of the mytonic dystrophy protein kinase (DMPK) gene transcript.[82] The number of CUG repeats correlates with disease severity.[83] The CUG-expanded RNA accumulates in nuclear foci[84] and likely gains a toxic function mediated by hyperphosphorylation. This leads to stabilization of CUG repeat binding proteins, such as CUG binding protein (CUG-BP),[85,86] a member of the CELF family of splicing regulators, and sequestration of muscleblind (MNBL).[84,87] Sequestration of MNBL and stabilization of CUG-BP aberrantly alter AS of their downstream target transcripts (see Figure 5). As such, DM1 can be classified as

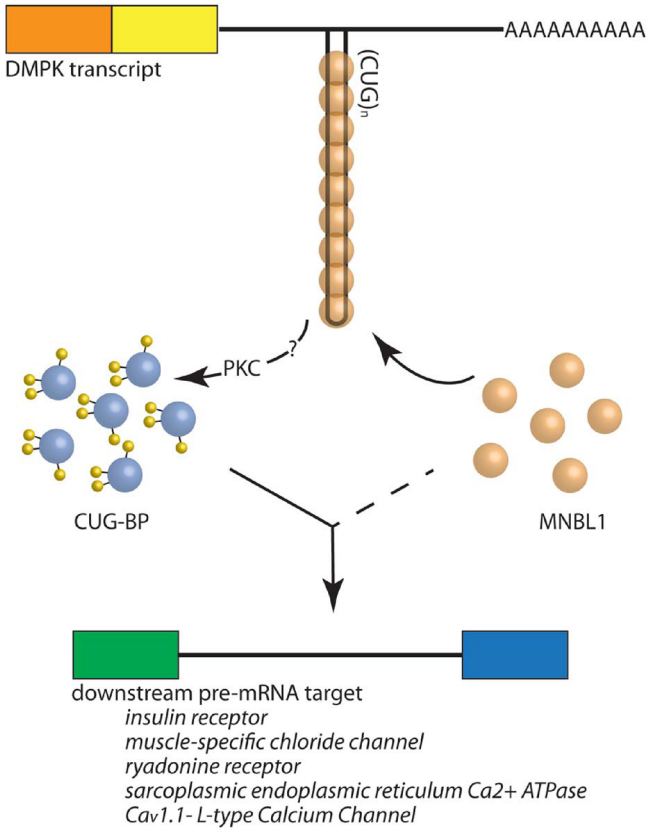

Figure 5. CUG repeats in the DMPK gene cause dysregulation of AS. Mutation of the DMPK gene to include CUG repeats sequesters the MNBL1 protein and stimulates hyperphosphorylation of CUG-BP through a PKC-mediated mechanism. These changes result in dysregulated splicing of downstream targets of MNBL1 and CUG-BP.

an indirect spliceopathy because, while no splice sites are altered, AS of multiple proteins is dysregulated. While both soluble and MPs are adversely affected, below we review the AS of MPs affected in this disease.

Insulin Receptor. Aberrant AS of the insulin receptor (InsR) results in the insulin resistance phenotype observed in DM1 patients. The insulin receptor functions as a heterotetramer with two α and two β subunits. In DM1, there is a switch from the normal production in skeletal muscle of the IR-B splice variant of the α subunit, which includes a 12-residue C-terminus, to the production of the IR-A variant lacking this terminus.[88,89] Omission of the C-terminus results in a weakened responsiveness of the InsR to insulin and reduced kinase activity (and, hence, signaling) for the IR-A isoform that is expressed in DM1.[90,91]

Muscle-Specific Chloride Channel. One of the most common symptoms of DM1 is myotonia, a delay in the relaxation of muscle after contraction. Hyperexcitability is likely caused by loss of function of chloride channels. It is thought that this is due to aberrant alternative splicing of the muscle-specific chloride channel (ClC-1), which results in decreased levels of the channel.[92] Three aberrant splice variants of ClC-1 were found in the skeletal muscle of DM1 patients. All three of these disease-generated isoforms contain premature stop

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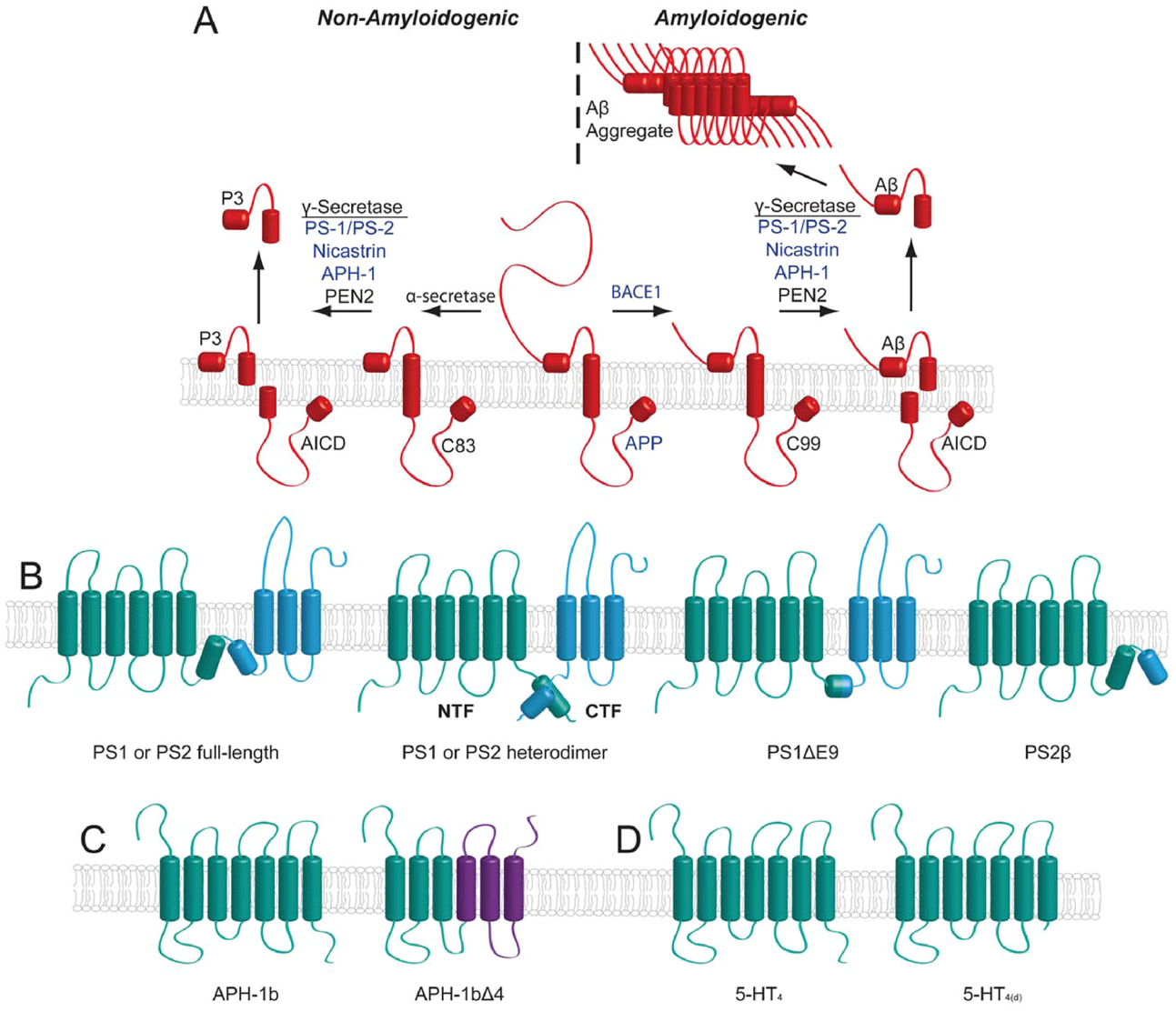

Figure 6. AS affects multiple proteins in the amyloidogenic pathway of AlzD. (A) APP processing pathways. On the left, nonamyloidogenic processing is initiated by α-secretase to produce C83, which is further processed by γ-secretase to produce the amyloid precursor protein intracellular domain (AICD) and the p3 peptide. On the right, amyloidogenic processing is initiated by β-secretase to produce C99, which is further processed by γ-secretase to produce the AICD and the Aβ peptide. The Aβ peptide can form toxic oligomers and aggregates. Proteins affected by AS are highlighted in blue. (B) Alternative splicing of presenilin impacts γ-secretase activity. Removal of exon 9 from PS1 results in a constitutively active form of the protein by considerably shortening the autoinhibitory loop and removing the endoproteolytic cleavage site. Exon 5 skipping in PS2 results in protein truncation after the autoinhibitory loop, creating a variant that is missing the CTF that is required for catalytic activity. (C) Removal of exon 4 from APH-1b results in removal of the entire fourth TM segment, causing the inverted topology of the last three TM segments (purple). This also results in the omission of the GXXXG motif that is required for γ-secretase assembly. (D) AS of the 5-HT$_4$ receptor results in a much shorter C-terminal tail, which presumably alters the signaling properties of this GPCR.

codons introduced by the splicing changes, and the transcripts are likely degraded by nonsense-mediated decay. This results in the loss of functional translated ClC-1 in skeletal muscle and thus a decrease in the conductance of chloride across the membrane, causing myotonia.[93]

Ryanodine Receptor and Sarcoplasmic Reticulum Ca^{2+}-ATPase. Perhaps the most debilitating symptom of DM1 is muscle wasting and weakness. It has been shown in other disorders that this symptom can result from changes to calcium homeostasis in muscle cells that increase intracellular Ca^{2+} levels.[94] Two proteins essential to maintaining calcium homeostasis are the ryanodine receptor (RyR1) and the sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase (SERCA). RyR1 is responsible for the release of Ca^{2+} in storage in the sarcoplasmic reticulum during muscle contraction, and SERCA transports Ca^{2+} back across the ER membrane for storage. Research has shown that the transcripts for both of these proteins undergo AS in DM1 patients.[95]

RyR1 has two variants seen in DM1. The neonatal form of RyR1 lacks exon 70 (residues 3481−3485), which corresponds to five residues found in the receptor modulatory region,[96] and the adult form of RyR1 includes exon 70. Adult DM1 patients have been found to have elevated levels of the neonatal RyR1 isoform in their skeletal muscle. This form of RyR1 has lower channel activity than the adult isoform, which would result in an increased rate of depolarization-dependent Ca^{2+} release.[95]

Two SERCA genes, SERCA1 and SERCA2, are also implicated in DM1. SERCA1 has two splice variants. SERCA1a

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is the adult version of the protein and contains exon 22, which encodes seven amino acids at the C-terminus. The neonatal form, SERCA1b, lacks exon 22, and its C-terminus has an eight-residue, highly charged C-terminal tail. In DM1, a switch occurs; the major isoform produced is the neonatal form (SERCA1b) as opposed to the adult form. It was also found that SERCA2 has a novel variant (SERCA2d) with a lower level of expression in DM1. SERCA2d contains intron 19, which results in the addition of 27 amino acids, a frameshift, and a premature stop codon in exon 20. The aberrantly expressed SERCA isoforms both have an altered C-terminus, but how this impacts overall pump function is not known.[95]

$Ca_v1.1$ L-Type Calcium Channel. The voltage-dependent $Ca_v1.1$ calcium channel plays a central and important role in excitation−contraction coupling in skeletal muscle.[97] This channel also undergoes aberrant splicing that is associated with DM1. AS of $Ca_v1.1$ in DM patients results in omission of exon 29 (ΔE29), removing a portion of the extracellular loop near the voltage sensor. As a consequence, the ΔE29 isoform exhibits altered calcium channel gating, which increases Ca^{2+} influx. These defects result in Ca^{2+} overload, likely contributing to muscle weakness and wasting.

Alzheimer's Disease. Alzheimer's Disease (AlzD) is a progressive neurodegenerative disorder affecting >5 million people in the United States alone.[98,99] AlzD can arise sporadically or, in rare cases, can be directly inherited (familial AlzD). While the etiology of the disease remains the subject of intense scrutiny, the amyloidogenic pathway appears to play a central role.[100] Amyloid plaques containing the amyloid-β (Aβ) peptide build up in the brains of AlzD patients and are the histological hallmark of the disease. It is thought, however, that it is soluble Aβ oligomers or aggregates that confer neurotoxicity and are at least partially responsible for AlzD progression.[101] Aβ production occurs through the successive release of the ectodomain of the amyloid precursor protein (APP) by β-secretase followed by γ-secretase cleavage of the remaining 99-residue TM C-terminus to release the Aβ peptides. In a competing (and benign) pathway of APP processing initiated by α-secretase, a nonamyloidogenic peptide known as p3 is generated.[102] The β- and γ-secretase enzymes are TM proteins, with the β-secretase being aspartyl protease BACE1.[103−106] The γ-secretase complex is composed of four TM subunits: presenilin, nicastrin, APH-1, and presenilin enhancer 2 (PEN2).[107] As discussed below, the β-secretase and components of the γ-secretase complex are subject to AS, and this AS may regulate their activities (see Figure 6). Additionally, there is evidence of the involvement of aberrant AS in the progression of AlzD. Finally, APP is itself affected by AS. Here we review instances of AS of MPs in AlzD and discuss the potential impact on disease progression and therapeutics.

Amyloid Precursor Protein. Three major splice isoforms of APP have been documented: APP695 (lacking exons 7 and 8, the predominant neuronal isoform), APP751 (lacking exon 8 and expressed abundantly in non-neuronal CNS tissue and peripheral tissue), and APP770 (containing both exons 7 and 8 and expressed predominantly in peripheral tissues and at only low levels in the CNS).[108,109] Exon 7 encodes a Kunitz protease inhibitor (KPI) domain in the ectodomain of APP that is possibly involved in blocking the α-secretase-initiated nonamyloidogenic APP processing pathway, because inclusion of exon 7 promotes the production of Aβ and inhibits the production of the p3 peptide.[110] However, it should be noted that this domain is a serine-protease inhibitor domain and would be unlikely to directly inhibit the metalloproteinase α-secretase. Exon 8 encodes a region of the protein between the KPI domain and a heparin binding domain. All three APP variants are subject to proteolytic processing along the amyloidogenic and nonamyloidogenic pathways described above. There is much inconsistency in the literature regarding differential expression of these isoforms in non-AlzD versus AlzD brains. Because it is difficult to compare relative values obtained from relative quantification polymerase chain reaction among different studies, definitive in vivo data showing that differential AS of APP predisposes a patient to AlzD are still lacking.

BACE1. The beta-secretase BACE1 is a single-span MP with its catalytic subunit located in the ectoplasm. BACE1 is a prominent target in the as-of-yet unsuccessful search for a drug to lower the rate of amyloid-β production. A recent series of publications[111−114] has described the various isoforms of BACE1 produced through AS. The longest isoform, I-501 (named for the number of amino acids), is both the most active and most commonly produced. However, AS events can also generate a series of shorter isoforms, including I-476, I-455, I-432, and I-127. The I-127 mRNA transcript contains a premature stop codon and is degraded by NMD; in addition, the small amount of translated I-127 protein isoform is subject to degradation by the proteasome.[113] BACE1 I-476, I-457, I-455, and I-432 all exhibit dramatically reduced activity relative to that of I-501, such that promotion of AS to generate the shorter isoforms results in a decreased level of secretion of Aβ.[114] Accordingly, promotion of AS of the BACE1 pre-mRNA may be a viable therapeutic strategy for reducing Aβ production. Recent experiments in the laboratory of M. Wolfe have shown that I-501 production is activated through the binding of heterogeneous nuclear ribonucleoprotein H (hnRNP H) to a G-rich element in the third exon of BACE1 pre-mRNA.[115] Because the 5′ splice site responsible for I-501 production is thought to be weaker than the upstream 5′ splice site,[115] leading to the I-457 and I-453 isoforms, targeting the activation of I-501 production by hnRNP H is a reasonable therapeutic strategy in the search for a way to reduce BACE1 activity, in this case by reducing production of the most active isoform of the enzyme.

Presenilin (PS). PS is the aspartyl protease in the heterotetrameric γ-secretase complex responsible for intramembrane cleavage of a large number of different single-span MP substrates,[116−118] including the 99-residue TM C-terminus (C99) of the APP that is released by BACE1 cleavage. Cleavage of C99 by γ-secretase releases the Aβ peptides (see Figure 6A). After assembly into the γ-secretase, cleavage of autoinhibited presenilin within the intracellular loop located between TM segments 6 and 7 generates active γ-secretase. Prior to its cleavage, the loop that is clipped to generate active presenilin is thought to extend into the active site to block catalysis. The now active presenilin is comprised of a heterodimer of its N-terminal fragment (NTF) and a C-terminal fragment (CTF). The NTF and CTF each contribute an aspartic acid residue necessary for catalysis.[102,119−121]

Inherited mutations of presenilin-1 (PS1) or presenilin-2 (PS2) are one of the known causes of familial (early onset) AlzD. Some of these mutations appear to impact pre-mRNA splicing. Additionally, aberrant splicing of the presenilin transcript has been shown to occur in the more common sporadic AlzD.

Presenilin-1. It has been shown that PS1 is affected by multiple splicing events and that some heritable PS1 mutations modulate splicing. Here, we will discuss three instances in which splicing may play a role in disease progression.

First, in human tissues, two isoforms of PS1 differing only in the inclusion or exclusion of four amino acids, VRSQ, in the N-terminal domain are expressed.[122] The VRSQ motif, which is conserved among humans, rats, and mice, provides a putative phosphorylation consensus site for protein kinase C (PKC) at a downstream threonine.[122] This phosphorylation event may play a role in regulating the trafficking of PS1. There is a relative decrease in the level of the longer isoform in some cases of sporadic[123] and familial AlzD,[124] suggesting a link between altered trafficking of PS1 and AlzD etiology.

Aberrant splicing of PS1 can also be induced by mutation. For instance, a mutation within intron 4 was identified in several patients with early onset AlzD,[125,126] which results in production of three aberrant species of PS1. Two of these contain full or partial deletions of exon 4 and result in truncation of the protein product; the third encodes insertion of a single threonine residue between canonical residues 113 and 114.[126] Only the threonine-insertion species was detected in brain homogenates, suggesting the AlzD phenotype most likely results from the insertion species. This is supported by the observation that transfection of the Thr-insertion form of the enzyme into HEK-293 cells resulted in an increased level of $A\beta_{42}$ secretion.[126]

Another interesting aberrant splicing event occurs when a mutation destroys a splice acceptor site at exon 9.[127] This causes the deletion of exon 9, which encodes part of the autoinhibitory loop that includes the site of activating cleavage of presenilin.[128] Because disruption of this loop is required to activate presenilin activity[128,129] and the elimination of the autocleavage site results in constitutively active presenilin,[116] it was originally thought that its elimination via AS is solely responsible for the associated AlzD phenotype. However, it was later demonstrated that the single-point mutation (S290C) that also results from this splicing event may contribute to AlzD pathogenesis by also promoting increased $A\beta_{42}$ production (Figure 6B).[130]

Presenilin-2. For PS2, an aberrant splicing event in sporadic AlzD, resulting in exon 5 skipping (PS2V) and protein truncation.[131−135] The removal of exon 5 causes a frameshift in exon 6 and introduces a premature stop codon. The resulting PS2V mRNA encodes only the amino terminus containing one predicted TM segment and five additional C-terminal residues.[131,132] The PS2V protein is found in the hippocampus and cerebral cortex of patients with sporadic AlzD, accumulates in inclusion bodies that impair the unfolded protein response, and results in increased $A\beta$ production through an undefined mechanism.[132] It is possible that it may exert its effect through associations with other γ-secretase proteins or through disruption of the unfolded protein response.

Recently, a novel splice variant of PS2, termed PS2β, was described to be a γ-secretase inhibitor.[136] Its expression is therefore predicted to be beneficial in terms of reducing the risk of AlzD. This splice variant encodes the entire NTF and the autoinhibitory hydrophilic loop domain that typically resides between TM segments 6 and 7. However, the exons encoding the entire CTF are not included in this isoform, such that this splice variant is catalytically inactive. PS2β was found to weaken the interaction between APH-1 and nicastrin and to inhibit $A\beta$ secretion.[136] It seems then that PS2β may inhibit γ-secretase activity by preventing assembly of the holoenzyme. Perhaps this isoform may represent an endogenous means of repressing γ-secretase activity (Figure 6B).

Anterior Pharynx Defective-1. Another γ-secretase subunit, anterior pharynx defective-1 (APH-1), is a seven-TM-spanning protein encoded by two homologous human genes, APH-1a and APH-1b.[137] APH-1 is known to be responsible for assembly of an initial subcomplex with nicastrin, and together, these two proteins recruit the presenilin and PEN2.[138−142] The APH-1a transcript can be alternatively spliced to produce one of two isoforms, APH-1aS and APH-1aL, which differ only at their C-termini.[137] These splice variants have been shown to reside in distinct, active γ-secretase complexes.[143] Additionally, a novel (third) splice variant, APH-1bΔ4, has been identified that lacks the entire fourth exon, meaning it also lacks the entire fourth TM segment,[144] resulting in predicted inversion of topology for the three C-terminal TM segments. This omitted TM segment also contains a GXXXG motif that is thought to be critical for assembly of the γ-secretase multisubunit complex[145] (Figure 6C). The endogenous level of expression of this variant form of the protein is very low, and the protein is destabilized.[144] Whether alternative or aberrant splicing of APH-1 plays any role in promoting or preventing AlzD has yet to be determined.

Nicastrin. Nicastrin is a highly glycosylated type I MP that participates, along with APH-1, in the assembly of the γ-secretase complex. Two independent genome surveys[146,147] have found evidence of association of sporadic AlzD with the region of chromosome 1 on which the nicastrin gene resides.

In 2005, a novel skipped splice variant of nicastrin was reported to be expressed in rat tissues and a human neuroblastoma cell line.[148] This variant, which encodes a truncated 62-residue protein due to exon 3 removal resulting in a premature stop codon, was shown to be preferentially made in the central nervous system and is subject to degradation by NMD. It is possible that NMD plays a specific role in the healthy CNS to downregulate expression of active γ-secretase, thereby reducing $A\beta$ production.

A second novel nicastrin splice variant was identified, this time lacking the entirety of exon 16.[149] As a result, this splice variant is missing 71 residues preceding the N-terminal region of the TM domain, which was shown to be required for interaction with the γ-secretase complex.[150]

5-HT$_4$ Receptor. Serotonin (5-hydroxytryptamine) receptor 5-HT$_4$, a GPCR, has been implicated in the regulation of APP processing, and receptor agonists have been shown to increase the production of the soluble APP ectodomain released by α-secretase cleavage of full-length APP to initiate the non-amyloidogenic pathway.[151−153] Alternatively spliced isoforms of 5-HT$_4$ have intracellular C-terminal domains of different lengths: a short isoform, 5-HT$_{4(d)}$, that contains only two amino acids after the C-terminal splice site, located after the region encoding TM segment 7 and preceding the intracellular C-terminal tail, and a larger isoform that contains 20 amino acids after the C-terminal splice site, 5-HT$_4$. These isoforms were compared with respect to the promotion of non-amyloidogenic APP processing.[154] It was found that activation of the short 5-HT$_{4(d)}$ isoform by an agonist promotes generation of nonamyloidogenic soluble APPα (sAPPα, the ectodomain released upon α-secretase cleavage) and, unlike that of the longer isoform, stimulation of the short isoform reduces $A\beta$ production. It is possible then that 5-HT$_4$ receptor isoforms may differentially regulate APP processing. Presum-

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ably, this may come from signal transduction differences related to the differing C-terminal tail lengths, possibly involving signaling through arrestins, which become activated when they bind to the multiply phosphorylated C-termini of activated GPCRs. Such arrestin-based signaling would be eliminated in splice variants that removed all or key parts of the C-terminal domain of the 5-HT_4 receptor. Further insight into how 5-HT_4 receptors influence APP processing and their relationship with the secretases is needed to shed light on these data.

CONCLUDING REMARKS

Alternative splicing of pre-mRNA can impact membrane protein trafficking, structure, and function and ultimately contribute to disease. The examples highlighted in this review represent only a sample from what is already a very large pool of literature on this subject. Given that studies of RNA splicing remain in a rapid state of development, these examples represent only modest beginnings. There are undoubtedly new discoveries waiting to be made. It is already clear that splicing can alter almost every aspect of a MP's structure, topology, function, trafficking, and interactions. When MPs from eukaryotes are being studied, the possibility of different isoforms with varying properties should not be overlooked.

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ABBREVIATIONS

AA, alternative acceptor sites; Aβ, amyloid-β; AD, alternative donor sites; AICD, amyloid precursor protein intracellular domain; AlzD, Alzheimer's disease; APH-1, anterior phalanx defective-1; APP, amyloid precursor protein; AS, alternative splicing; BACE1, β site APP cleavage enzyme 1; CA, carbonic anhydrase; ClC1, muscle-specific chloride channel; CNS, central nervous system; CTF, presenilin C-terminal fragment; D_2, dopamine receptor 2; DM1, myotonic dystrophy type 1; DMPK, myotonic dystrophy protein kinase; ER, endoplasmic reticulum; ES, exon skipping; GLT1, glutamate transporter 1; GlyR, glycine receptor; GPCR, G-protein-coupled receptor; 5-HT_4, serotonin (5-hydroxytryptamine) receptor; IR, intron retention; InsR, insulin receptor; KPI, Kunitz protease inhibitor domain; ME, mutually exclusive exon splicing; MP, integral membrane protein; mRNA, messenger ribonucleic acid; MNBL, muscleblind protein; NMD, nonsense-mediated decay; NMDA, *N*-methyl-D-aspartate; NTC, nineteen complex; NTF, presenilin N-terminal fragment; PEN2, presenilin ehancer 2 protein; PKC, protein kinase C; PLP, proteolipid protein; PMP22, peripheral myelin protein 22; PS, presenilin; Rh, Rh blood group antigen protein; RyR1, ryanodine receptor; SDC-4, syndecan receptor 4; SERCA, sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase; snRNA, small nuclear ribonucleic acid; TM, transmembrane; TRP, transient receptor potential; V2R, vasopressin V2 receptor.

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