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Coordinated Regulation of Alternative Splicing and Alternative Polyadenylation

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ABSTRACT

In higher eukaryotes, alternative splicing (AS) and alternative polyadenylation (APA) have been reported as highly specialized mechanisms to regulate transcriptome and proteome diversity from a limited number of genes to support physiological functions. It was widely believed that AS and APA are independent regulatory mechanisms, however, recent studies provide compelling evidences that AS and APA are often coupled simultaneously, or competitively regulated to promote gene expression. Therefore, fine-tuned coordination and spatiotemporal expression of AS and APA regulatory factors are critical to accomplish transcriptome and proteome diversity that higher eukaryotes have acquired in course of evolution. Here, we discuss recent advances and current understanding in this area, which could be helpful to provide important insights and directions for future research.

Keywords: *Alternative splicing, alternative polyadenylation, RNA-binding protein*

Abbreviations: *AS, alternative splicing; APA, alternative polyadenylation; snRNP, small nuclear ribonucleoprotein; RBP, RNA-binding protein; hnRNP, heterogeneous nuclear ribonucleoprotein; SS, splice site, UTR, untranslated region; CPSF, cleavage and polyadenylation specificity factor; CstF, cleavage stimulation factor*

INTRODUCTION

The intricate mechanism of eukaryotic pre-mRNA processing, achieved by alternative splicing (AS) and alternative polyadenylation (APA), explains the discrepancy between an estimated ~24,000 human genes versus the ~100,000 different proteins that are postulated to be translated[1]. Alternative splicing ensures appropriate removal of introns and inclusion/skipping of exons through differential selection of splice sites in the pre-mRNA transcripts[2]. In contrast, alternative polyadenylation produces distinct ends in pre-mRNA transcripts, through mechanisms including transcription termination, cleavage and polyadenylation[3].According to recent studies, more than ~95% of human transcripts are believed to undergo alternative splicing[4,5,] whereas, ~70% of human genes undergo alternative polyadenylation[6,7]. To support the physiological and cellular demands, AS and APA are evolved as fine-tuned mechanisms that often function in developmental stage-specific or tissue-specific manner.

As most APAs are found at or near the 3' untranslated regions (3' UTRs), it was widely predicted that APA is coupled with AS to influence mostly the last intron removal. Recent studies, however, suggest that upstream APAs are also abundant, which may influence AS choices at upstream exons[8,9,10].

In recent years, it has become increasingly evident that AS and APA are coupled regulatory processes, although the detail mechanisms or the extent of coupling are not extensively dissected. In this review we will provide insights on the coupled, if not competitive, regulation of these two key RNA metabolic processes based on our current understanding.

DISCUSSION

Regulation of Alternative Splicing Choices in a Pre-mRNA Transcript

A dynamic macromolecular complex composed of proteins and RNA, known as'spliceosome', mediate the splicing reaction by removing the intron and joining the ends of two exons. Spliceosome is comprised of five small nuclear ribonucleoproteins (snRNPs) and hundreds of other proteins that cooperate to catalyze splicing reaction[11], and is assembled in a sequential manner. The assembly begins when U1 snRNP recognizes and binds to the 5' SS, SF1 protein binds to the branch point (BP) and U2AF heterodimer (U2AF65 and U2AF35) bind to the polypyrimidine tract (PPT) and 3' AG, respectively. The formation of this initial complex is ATP-independent, and referred to as an E-complex. In the next step, SF1 is replaced by U2 snRNP at the BP in an ATP-dependent manner. At this stage, the complex is called A-complex. Subsequent employment of U4/U6.U5 tri-snRNPs promote formation of a B-complex . At this point, the whole spliceosome undergoes extensive remodeling and conformational changes and releases U1 and U4 snRNPs to form the catalytically active C-complex. Consequently, the intron makes a lariat structure and gets excised, followed by ligation of the two neighboring exons to complete the splicing reaction.

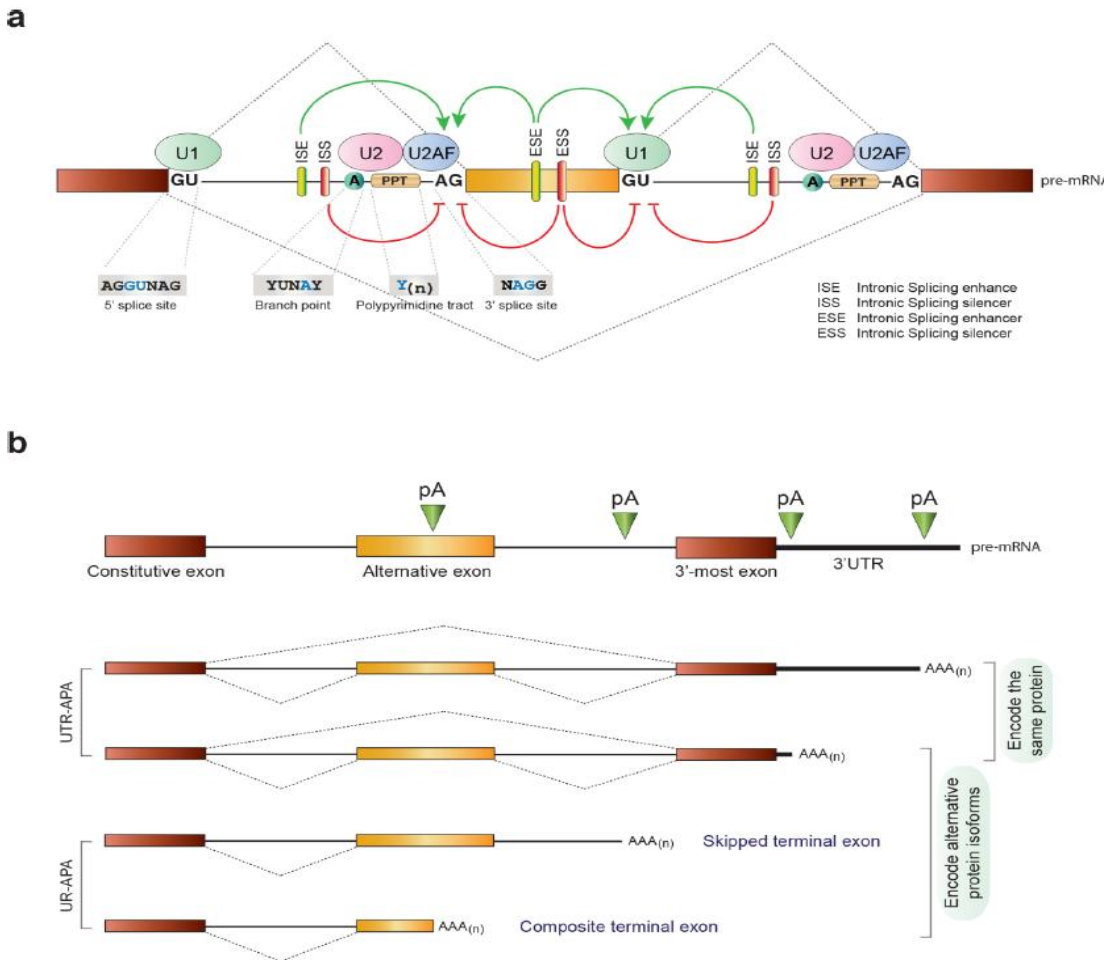

Figure1. *Alternative splicing (AS) and alternative polyadenylation (APA) are closely related posttranscriptional mechanisms to regulate gene expression. (a) Schematic of essential splicing regulatory cis-elements and trans-acting RNA-binding proteins (RBPs) in the pre-mRNA transcript to facilitate or suppress spliceosomal machinery at splice sites. Exons and introns of a hypothetical gene are shown as colored boxes and solid lines, respectively. Invariant GU (5'SS) and AG (3' SS) dinucleotides mark the border of an intron. U1snRNP initially binds to the 5' SS. U2AF proteins (U2AF65 and U2AF35) interact with the polypyrimidine tract (PPT) and the 3' SS, respectively and help to recruit U2 snRNP at the branch point (BP, usually an 'A' nucleotide). The binding of these snRNPs subsequently facilitates further spliceosomal assembly and pairing of the splice sites to ensure efficient intron removal. An alternative splicing event may involve competition between multiple weak splice sites, which are regulated by auxiliary cis-elements and their cognate auxiliary trans-acting factors. These auxiliary cis-elements include intronic and exonic splicing enhancer (ISE, ESE) or silencer (ISS, ESS) elements, which recruit activator or repressive RBPs at these sites, respectively. These RBPs*

influence splice site recognition, or pairing, within the splicesome. Consensus sequences of the essential cis-elements are shown below (Y = C/U, R = G/A, N = any nucleotide). Activities of splicing enhancer elements are shown as green arrows, and activities of splicing silencer elements are shown as inhibitory red markings. (b) Schematic portraying alternative cleavage and polyadenylation of a hypothetical gene. Alternative poly(A) sites (APAs) may be present at the 3'-most exon within the 3'UTR (UTR-APA), or in upstream introns and exons (UR-APA). Constitutive, alternative and 3'-most exons are depicted as colored boxes. APA sites are indicated as green triangles. Introns and 3' UTR are shown in thin and thick solid lines, respectively. UTR-APAs generate transcripts of different lengths, but would encode the same protein isoform. In contrast, UR-APAs generate truncated transcripts that do not comprise terminal exonic or intronic sequences, consequently influencing splicing choices to produce different transcripts encoding different protein isoforms. Constitutive and alternative splicing choices are shown in dotted selection lines.

The spliceosome recognizes exon/intron boundary and catalyzes a transesterification reaction to excise an intron and join the neighboring exons. Specific *cis*-regulatory elements/motifs, including 5' splice site (5' SS), a branch point (BP), a polypyrimidine tract (PPT) and a 3' splice site (3' SS), bound with their cognate *trans*-acting factors, and guide the recognition of exon/intron boundary (Figure 1a). Since these elements are highly degenerative, proper recognition of exon/intron boundary often rely on multiple auxiliary *cis*-elements, as well as auxiliary *trans*-acting factors that cooperate with the spliceosome for an efficient splicing reaction. Auxiliary *cis*-elements that positively modulate inclusion of an exon are termed as intronic/exonic splicing enhancers (ISEs/ESEs), whereas negatively modulating elements that enhance skipping of an exon are termed as intronic/exonic spicing silencers (ISSs/ESSs). Cognate auxiliary *trans*-acting factors recognize and bind to these elements and modulate the inclusion or skipping of a particular exon, hence are often referred to as 'enhancer'or'silencer' factors, respectively. For example, the majority of the serine/arginine-rich (SR) RNA-binding proteins function as splicing enhancers[12,13,14], whereas, majority of the heterogeneous nuclear ribonucleoproteins (hnRNPs) function as splicing silencers[15]. The complex interplay between these *cis*-elements and trans-acting factors, which varies among different physiological, developmental stage-specific or tissue-specific conditions, leads to altered splice site choices generating alternatively spliced isoforms from the same pre-mRNA transcript.

Regulation of Alternative Polyadenylation at the 3' end of a pre-mRNA Transcript

The 3' end of the majority of eukaryotic pre-mRNAs undergo an endonucleolytic cleavage, followed by addition of a polyadenylated tail. Four multisubunit protein complexes constitute the 3' end processing machinery, including the cleavage and polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), cleavage factor I and II (CFI & CFII)[3,8]. Initially, the canonical poly(A) signal AAUAAA (or other close variants[16]) located approximately 10~30 nucleotides upstream of the cleavage site is recognized and bound by CPSF. Subsequently, CstF64 subunit binds to a U/GU-rich region downstream to the cleavage site. In contrast, CFI binds to a third site (UGUA motif) typically located upstream of the poly(A) signal (PAS). These interactions promote the cleavage reaction. RNA is typically cleaved before transcription termination, as CstF also interacts with RNA polymerase II and slips it off of the RNA transcript[17]. Cleavage also involves the protein CFII, although the mechanism is not well understood[18]. When the RNA is cleaved, a single subunit poly(A) polymerase (PAP) catalyzes the addition of a poly(A) tail of variable lengths.

Intriguingly, a given gene can transcribe pre-mRNA transcripts with multiple PASs. These alternative PASs (APA) can locate at the 3' untranslated regions (3'UTRs), or even in upstream introns and exons (Figure 1b). A recent study on the mouse transcriptome demonstrates that about 68% of mRNA transcripts have APA in the 3'-most exon, whereas, about 42% have APA in upstream regions[7]. Alternative PASs in the 3'-most exon would generate variable 3' UTR lengths, but will encode the same protein. This subset of APAs is also known as UTR-APAs. In contrast, alternative PASs in upstream introns or exons may potentially result in changes in the coding sequence (CDS). This subset of APAs is also known as upstream-region APAs (UR-APAs).

The 3' End Processing Factors as Potential Alternative Splicing Regulators

The poly(A) factor CstF64 or it's paralog CstF64τ is a subunit of a trimeric CstF complex including CstF77 and CstF50[19,20,21]. As mentioned above, CstF recognizes a specific U/GU-rich downstream element (DSE) in the transcript downstream to the cleavage site to

facilitate polyadenylation. Intriguingly, recent studies provided compelling evidence that apart from its role in polyadenylation, CstF64 also plays a potential regulatory role in alternative splicing (AS) choices. One striking example of CstF-mediated AS regulation is the regulation of immune globin M (IgM) heavy chain gene[22]. During the transition of a resting B cell to an activated state, IgM heavy chain gene undergoes an alternative splicing switch from a membrane bound isoform to a secreted isoform. IgM heavy chain gene has two APA sites. The secreted isoform is produced from the usage of the proximal PAS, while the membrane-bound isoform is produced from the usage of the distal PAS. Over expression of CstF in activated B cells enhances the usage of the proximal PAS, which induce the switch from membrane-bound to secreted form of IgM heavy chain.

In a recent study, it has been reported that knockdown of CstF64 is associated with a large number of AS events[23]. They identified increased CstF64 binding coverage in the upstream intronic region around alternatively spliced exons; however, no measurable differences were identified for the downstream intronic sequences. Interestingly, strong CstF64 binding signals were observed at APA sites in the upstream introns of the splicing regulatory protein hnRNP A2/B1. The 3' UTR of hnRNP A2/B1 harbors three potential APA sites. Selection of the proximal PAS generates hnRNP A2 transcript, which does not differ between wild-type and CstF64 knockdown conditions. In contrast, lack of CstF64 promotes the selection of the most distal PAS, which can potentially generate multiple splicing isoforms, including hnRNP B1 transcript that undergoes nonsense-mediated mRNA decay (NMD)[24], causing a decrease in hnRNP B1 protein levels. Since hnRNP A2/B1 are splicing regulatory proteins, it is likely that the observed AS changes after CstF64 knockdown is due to the altered expression of hnRNP A2/B1 proteins. Genome wide analysis revealed that about one third of AS events upon CstF64 knockdown, are also affected by hnRNP A2/B1 knockdown. This observation suggested that most of the AS changes influenced by CstF64, were likely due to indirect effects. Interestingly, splicing regulatory proteins hnRNP C, hnRNP H3, SRSF5 and SRSF6 were also identified as CstF64 targets in the same study.

Poly(A) factor CFI consists of two CFI25 subunits and two large subunits of either CFI59 or CFI68[25]. CFI25 Nudix domain is involved in specific interaction with UGUA motif in the RNA[26]. In contrast, CFI59 and CFI68 contains an N-terminal RNA recognition motif (RRM) similar to well-known splicing regulatory SR proteins, and are detected in purified spliceosome[27,28]. CFI has also been shown to interact with U2AF65, implicating a correlation between splicing and the 3' end processing[29,30]. Recently, it has been shown that changes in APA is correlated with changes in AS upon knockdown of CFI25[23]. For example, an inhibitor of matrix metalloproteinases, TIMP-2 (encoded by *TIMP2*), has two functional PASs[23]. It was shown that the distal PAS is preferentially used in HeLa cells. However, down regulation of CFI25 favors the selection of the proximal PAS. Interestingly, depletion of CFI25 resulted in a depletion of intron retention event at the terminal exon of *TIMP2* gene. Thus, alternative PAS selection may often correlate with alternative splicing outcomes.

Furthermore, two essential factors for processing the 3' end of mRNA, cleavage and polyadenylation specificity factor (CPSF) and Symplekin (SYMPK), regulate AS of internal exons[31]. CPSF and SYMPK, as RBFOX2 cofactors regulate binding of the early intron recognition factors, U1 snRNP and U2AF. Genome-wide analysis uncovered that CPSF also mediates AS of many internal exons in the absence of RBFOX2. Intriguingly, CPSF/SYMPK also found to be cofactors of splicing regulatory RNA-binding proteins NOVA2 and hnRNP A1.Thus, recent studies reveal an unanticipated role for the 3' end processing factors in global promotion of AS.

Splicing Regulatory Proteins in APA Regulation

Recent studies have provided unexpected new insights into the role of splicing regulatory factors in APA regulation. In one such study, we have recently reported that splicing regulatory protein hnRNP H/F and the 3' end processing factor CstF64 regulate AS and APA in a competitive regulatory fashion to determine the human *ACHE* isoforms (Figure 2)[32]. HnRNP H/F binds to two specific G-runs in exon 5a of *ACHE* gene and activates the selection of a distal 3' SS to produce the brain and muscle specific $ACHE_T$ transcript. Apart from the canonical PAS located at the 3' UTR of *ACHE* gene, we identified a cryptic/weak PAS with a sequence of UAUAAA[16] inside the alternatively spliced exon 5a. We located the cleavage site immediately upstream to the splicing regulatory G-runs. Knockdown of hnRNP H/F enhanced

the activation of the cryptic PAS, suggesting its role in influencing APA regulation. Interestingly, a GU/U-rich element was identified downstream to the cleavage site, which shared an overlapping sequence with the splicing regulatory G-runs in exon 5a. Further studies confirmed that hnRNP H/F and CstF64 compete for binding to the overlapping sites and determines the fate of the pre-mRNA transcript. When hnRNP H/F is abundant, it inhibits the binding of CstF64 to the cryptic PAS ensuring that the transcript is elongated up to the canonical PAS. Additionally, hnRNP F/H activates the distal 3' SS to generate $AChE_T$ isoform. In contrast, lack of hnRNP H/F enables the binding of CstF64 to the GU/U-rich motif to activate the cryptic PAS, generating a truncated pre-mRNA transcript that can only produce $AChE_H$ and $AChE_R$ isoforms (Figure 2). Intriguingly, global analysis of CLIP-seq and RNA-seq data in hnRNP H downregulated cells[33] revealed that hnRNP H competitively regulates alternative 3' SS selection and alternative PAS regulation in several other genes also. Thus, being initially described as splicing regulator, hnRNPs H and F have additional regulation in APA.

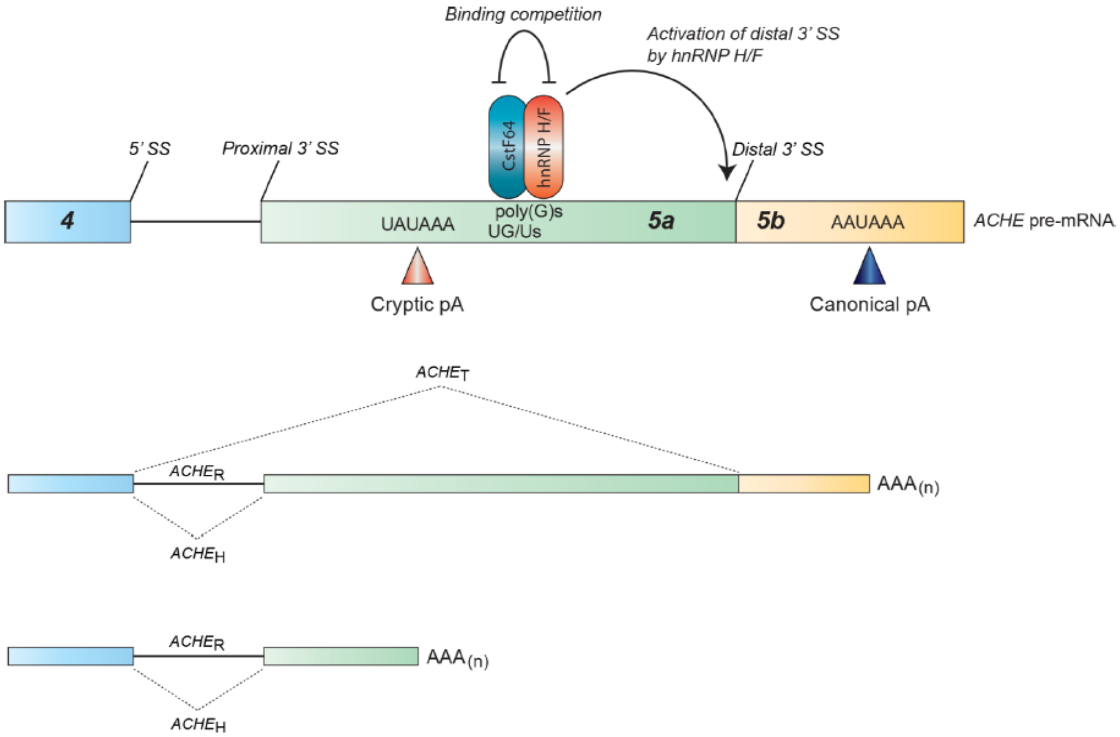

Figure2. *Schematic portraying competitive regulation of AS and APA in human ACHE gene. Exons are shown as colored boxes, and an intron is depicted as solid line. The 3' end of ACHE pre-mRNA has a single 5' donor site (5' SS) at the end of exon 4, but two alternative 3' acceptor sites (3' SSs). Apart from the canonical PAS located in exon 5b (AAUAAA, blue triangle), a cryptic/weak PAS is also located in exon 5a (UAUAAA, red triangle). HnRNP H/F binds to specific G-runs in exon 5a, and activates the distal 3' SS. Polyadenylation factor CstF interacts with specific UG/U-rich motifs in exon 5a, which are overlapping with the splicing regulatory G-runs. HnRNP H/F and CstF64 thus engage in a binding competition for their overlapping binding motifs. When abundant, hnRNP H/F binds to exon 5a to activate the distal 3' SS, additionally ensuring that CstF64 cannot bind to the UG/U-rich motif, to generate the $ACHE_T$ isoform. Lack of hnRNP H/F lets CstF64 to win the binding competition and activates the cryptic PAS to generate a truncated pre-mRNA transcript, which can produce $ACHE_H$ (spliced from the proximal 3' SS) and $ACHE_R$ (read-through) isoforms.*

Many spliceosomal proteins have recently been identified as regulators of APA. Most notably, U1 snRNP was identified as a suppressor of polyadenylation at downstream PASs through inhibition of PAP activity[34,35]. Recently, genome wide analysis disclosed that U1 snRNP protects pre-mRNA from premature cleavage and polyadenylation at cryptic PASs near transcription start site[36]. U1 snRNP levels has also been proposed to control mRNA lengths and expression of alternative isoform by modulating PAS[37]. Consistently, large introns with weak 5' SSs undergo APA at their internal PASs in a greater extent, possibly due to compromised binding of U1 snRNP at those weak 5' SSs[38].In contrast, U2 snRNP has been

implicated to promote the usage of downstream PASs through multiple protein-protein interactions[39,30].

In the past, RNA-binding proteins that were known to regulate alternative splicing, were very often recognized as regulators of APA. One of the very first examples was a splicing regulatory protein in brain, NOVA2[40], which was found to repress PAS usage when bound to a nearby region, whereas, binding to more distant site promoted PAS usage. Another widely studied RBP, embryonic-lethal abnormal visual (ELAV) was shown to suppress the usage of proximal PASs to lengthen the 3' UTR in Drosophila[41,42]. Mammalian ELAV homologues, the Hu proteins (HuR, HuB, HuC and HuD), are another good example of RBPs that also regulate APA, as they inhibit the usage of PASs with U-rich elements[43]. Two SR proteins, SRSF3 and SRSF7 have been found to inversely regulate APA. In mouse P19 cells, SRSF3 lengthens 3' UTRs, whereas, SRSF7 shortens those 3' UTRs[44]. A major hnRNP protein, polypyrimidine tract binding protein 1 (PTBP1), was shown to directly compete with CstF for recognition of the PAS, by binding to a pyrimidine-rich downstream sequence element[45]. Nuclear Poly(A)-Binding Protein 1 (PABPN1) was suggested to compete with CPSF for binding to many proximal PASs[46]. Interestingly, RNA-binding proteins hnRNPs H and F, have been shown to regulate APA by a context-dependent manner. Binding of hnRNP H to G-rich elements downstream to a PAS, enhances the recruitment of CstF64 in pre-mRNAs of viral genes including AAV, SVL40, IVA2, and AdL3, as well as, mammalian immunoglobulin M (IgM) and p53 genes[47,48,49]. On the other hand, binding of hnRNP H to either an upstream or a downstream element to a PAS, promotes the recruitment of poly(A) polymerase (PAP) in β-globin pre-mRNA[50]. In contrast, binding of hnRNP F, but not hnRNP H, to G-rich elements downstream to a PAS of the IgM pre-mRNA in B cells, and SVL40 pre-mRNA, suppresses binding of CstF64[51,52]. Thus, many splicing regulatory RBPs also regulate APA in different circumstances.

AS and APA Factors in the Context of Human Genetic Diseases

Fused in sarcoma (FUS) and TAR DNA-binding protein 43 (TDP43) proteins, which are linked to the fatal neurodegenerative disease amyotrophic lateral sclerosis (ALS), interact with CPSF and CstF complexes. Down regulation of FUS leads to activation of promoter-proximal PASs[53]. On the other hand, TDP-43 auto regulates its own expression by directly competing with CstF64 for binding to DSE elements, and in turn suppressing PAS usage[54]. An expansion of the hexanucleotide (GGGGCC) in *C9ORF72* gene has been implicated as a leading known cause of ALS and frontotemporal dementia (FTD). Notably, ALS patients having the expansion had a global shortening of 3' UTRs observed in the cerebellum[55]. One suggested mechanism of how these expansions can lead to ALS and FTD demonstrates the sequestration of hnRNP H into the nuclear foci, but additional studies are required to dissect the role of sequestered RBPs in the shortening of the 3' UTRs[56,57]. Likewise, muscleblind-like (MBNL) RNA-binding proteins are known to be sequestered in expanded CUG repeats in myotonic dystrophy (MD). Intriguingly, MBNL proteins tend to repress PAS recognition when they bind between the cleavage sites and DSEs of core PASs[58]. In contrast, when bound upstream of the PASs, they tend to enhance PAS usage. These examples highlight the significance of understanding the role of RBP-mediated APA regulation in the context of human genetic diseases.

CONCLUSION

Given the significance of AS and APA to promote gene expression and to enable transcriptome and proteome diversity, it is of considerable interest to determine the functional link between these two key regulatory processes. Fine-tuned coordination between AS and APA is critical to support gene expression to maintain physiological integrity, as well as, to support developmental stage-specific or tissue-specific regulation. It is now well established that splicing defects play critical roles in human diseases, including numerous types of neurological disorders[59,60] and cancer[61,62,63]. Likewise, APA also has been demonstrated to have regulatory roles in many human diseases, most notably in cancer[64,65,66,10]. Therefore, coordinated APA and upstream AS could potentially generate alternatively spliced mRNA isoforms that could differ in their cellular functions, and may be related to the underlying pathophysiology in different disease conditions. Therefore, scrutinizing more mechanistic investigations related to AS and APA, and their coupled regulation in disease models, will broaden our understanding about the disease mechanisms by uncovering critical regulatory

players, such as RNAs, RNPs, and auxiliary associated factors. These critical mechanistic insights could enable us to develop rational therapeutic strategies by modulating regulatory targets to reverse the aberrant AS and APA to a physiological state. Whether such approaches could potentially be served in clinical settings in near future still remains to be explored.

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