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REVIEW

Alternative polyadenylation regulation in cardiac development and cardiovascular disease

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Abstract

Cleavage and polyadenylation of pre-mRNAs is a necessary step for gene expression and function. Majority of human genes exhibit multiple polyadenylation sites, which can be alternatively used to generate different mRNA isoforms from a single gene. Alternative polyadenylation (APA) of pre-mRNAs is important for the proteome and transcriptome landscape. APA is tightly regulated during development and contributes to tissue-specific gene regulation. Mis-regulation of APA is linked to a wide range of pathological conditions. APA-mediated gene regulation in the heart is emerging as a new area of research. Here, we will discuss the impact of APA on gene regulation during heart development and in cardiovascular diseases. First, we will briefly review how APA impacts gene regulation and discuss molecular mechanisms that control APA. Then, we will address APA regulation during heart development and its dysregulation in cardiovascular diseases. Finally, we will discuss pre-mRNA targeting strategies to correct aberrant APA patterns of essential genes for the treatment or prevention of cardiovascular diseases. The RNA field is blooming due to advancements in RNA-based technologies. RNA-based vaccines and therapies are becoming the new line of effective and safe approaches for the treatment and prevention of human diseases. Overall, this review will be influential for understanding gene regulation at the RNA level via APA in the heart and will help design RNA-based tools for the treatment of cardiovascular diseases in the future.

Keywords Alternative polyadenylation • Heart development • Cardiovascular disease • 3′UTR length and gene regulation

1. Introduction

Cleavage and polyadenylation is a process where the 3′ end of eukaryotic pre-mRNAs are cleaved at a specific 'CA' dinucleotide site known as poly(A) site (PAS) followed by the addition of a poly (A) tail of ∼100–200 adenosine nucleotides at the 3′ end of pre-mRNAs.[1] 3′ cleavage and polyadenylation of pre-mRNAs are critical for nuclear export, mRNA stability, localization, and translation.[2–9] Cleavage and polyadenylation are tightly coupled with transcription and splicing.[10,11] Genome-wide studies revealed that 60–70% of human genes have multiple PASs.[12,13]

Alternative polyadenylation (APA) is defined as the differential use of multiple PASs within a given pre-mRNA. Depending on the location of these PAS, APA can directly affect gene expression or generate diverse mRNA isoforms, providing an additional layer of regulation.[14] APA regulates gene expression and function in a cell and tissue-specific manner, and its dysregulation is frequently observed under diseased conditions. Therefore, deciphering APA regulation in health vs. disease will help understand organism development and provide new therapeutic options for diseases in which APA regulation goes awry.

2. Impact of APA on gene regulation

There are different types of APA: tandem-APA, splicing-APA (Coding Region-APA),[15,16] and intronic-APA (IPA).[2,17,18] Tandem-APA is the most common type, in which PASs reside in different locations within the 3′UTR, resulting in transcripts with different lengths of 3′UTR. In this type of APA, the protein-coding region is not affected (*Figure 1*). In splicing-APA, both coding region and 3′UTR length are affected (*Figure 1*). In intronic-APA, the PAS is in the intron, leading to the generation of truncated proteins (*Figure 1*). In this review, we will mostly focus on tandem-APA regulation.

2.1 Tandem-APA in gene expression and protein output

In tandem-APA, proximal PAS (pPAS) or distal PAS (dPAS) is used preferentially to generate transcripts with short or long 3′UTRs, respectively (*Figure 2*). mRNAs with long 3′UTRs often harbour *cis*-acting elements for microRNA (miRNA) binding sites or/and RNA-binding protein (RBP) sites that are missing in transcripts with short 3′UTR isoforms, so they are regulated differently[19] (*Figure 2*). miRNAs bind to 3′UTRs and affect mRNA translation or stability[20,21] (*Figure 2*). RBPs can also bind to the *cis*-acting elements within the 3′UTR to regulate mRNA stability, mRNA translation, or mRNA localization.[22,23] Moreover, RBPs and miRNAs could interact with each other to potentiate or antagonize their functions through binding to 3′UTRs of a given mRNA.[24–26] Tandem-APA regulates mRNA stability[3,27–31] and translation[3,6,30,32–34] by modulating positive or negative *cis/trans*-acting factors that bind 3′UTRs (*Figures 1* and *2*).

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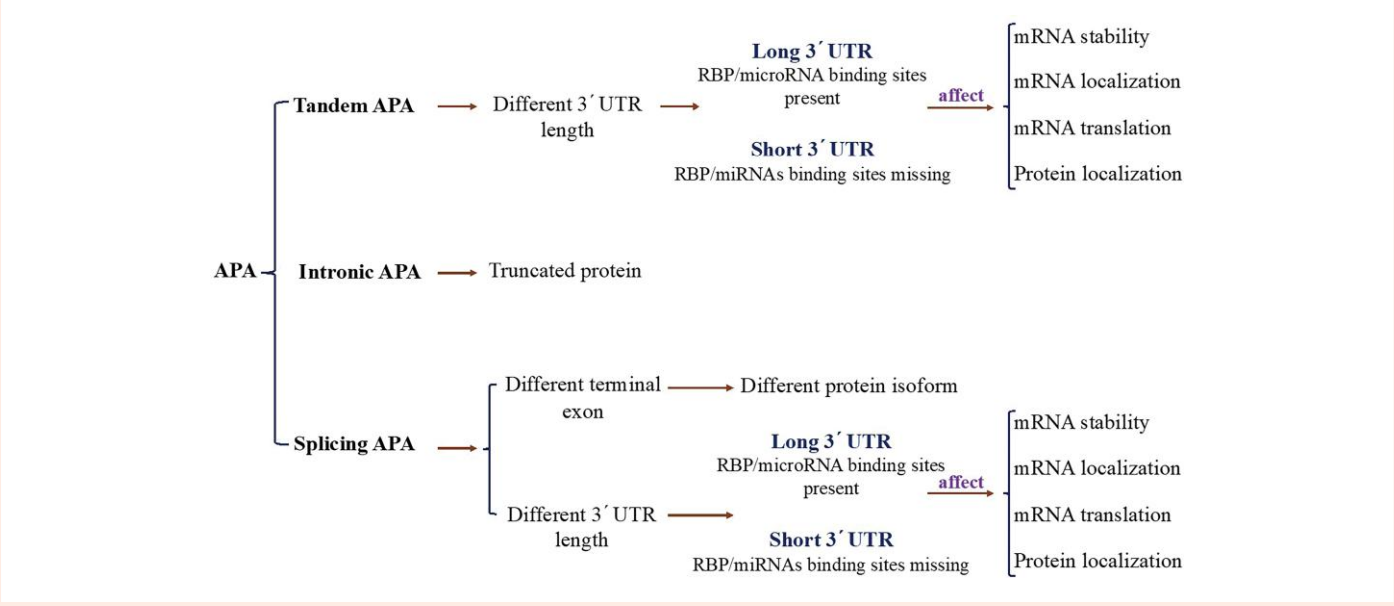

Figure 1 Different types of APA.

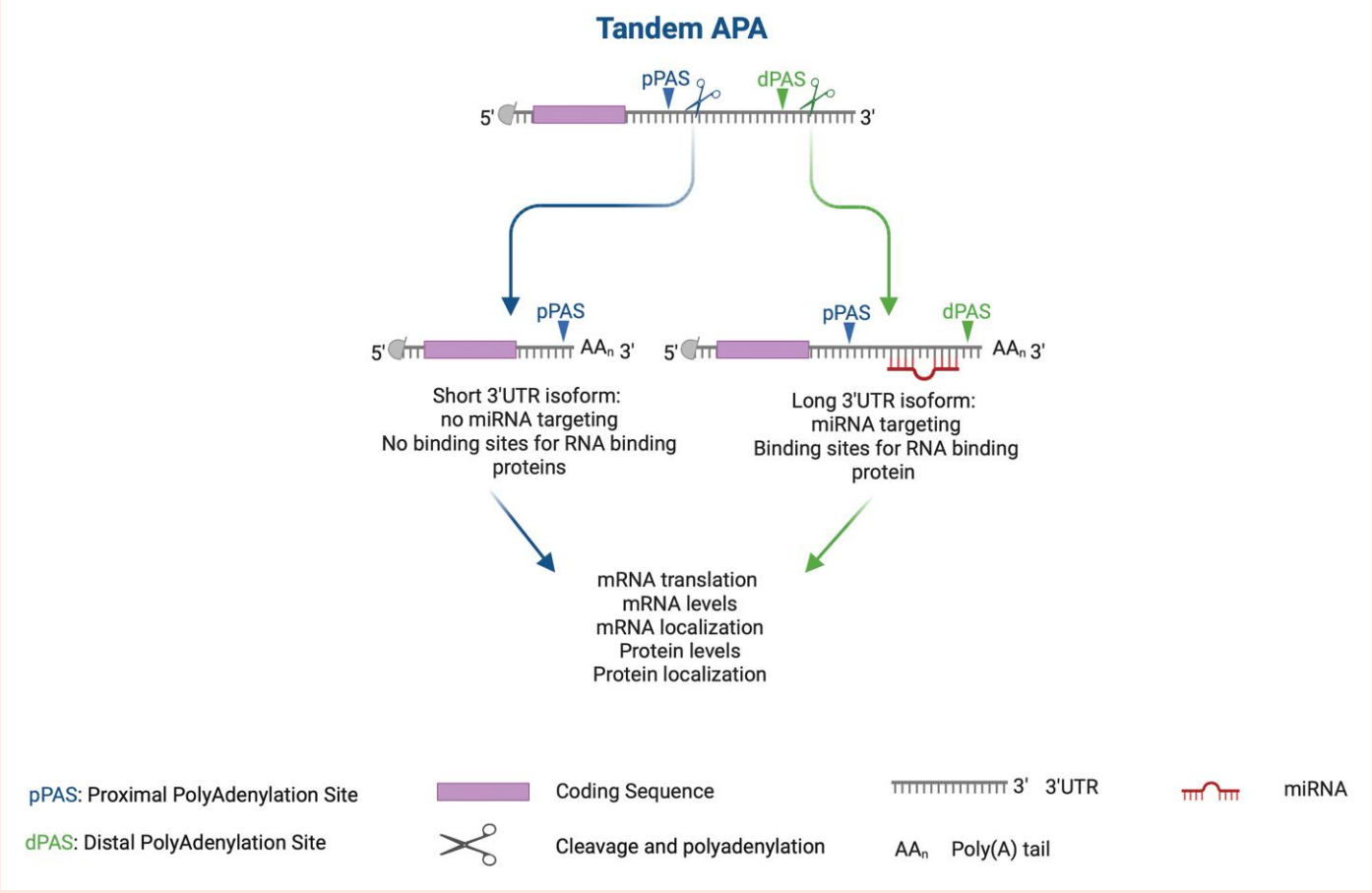

Figure 2 Tandem-APA. A gene that contains >1 PAS in its 3′UTR is susceptible to APA regulation. In this example, this gene has 2 PASs: proximal poly(A) site (pPAS) and distal poly(A) site (dPAS) in the same 3′UTR. The usage of pPAS or dPAS generates short or long 3′UTR isoforms, respectively. Image created using Biorender.

In general, pPAS usage shortens 3′UTR length enabling an escape from miRNAs/RBPs mediated regulation (*Figure 2*).[2,3] A study showed that mRNA transcripts with >1 kb 3′UTR had shorter mRNA half-lives.[27] Other studies also showed a negative correlation between 3′UTR length and mRNA abundance.[3,28–30] However, there is also evidence that longer 3′UTR isoforms are more stable.[6,31] miRNAs[21,35] and some RBPs, such as TIA1,[36] TTP,[26,37] and AUF-1,[38] bind to the 3′UTR and destabilize mRNAs, whereas RBP HuR can stabilize transcripts.[39] The different actions of these RBPs can explain the differences in these studies.

3′UTR also can act as competing endogenous RNAs (ceRNAs) to regulate gene expression.[40–42] When 3′UTRs shorten, they lose miRNA target sites allowing these miRNAs to bind to other genes.[40–42] For instance, *PTEN* and *EPS15* share the same miRNA binding sites and thus are ceRNA partners. Shortening of 3′UTR of *EPS15* frees up miRNAs, making them available to bind to the 3′UTR of *PTEN* mRNA in turn repressing *PTEN* expression.[42]

mRNA isoforms with shorter 3′UTRs are associated with polysomes, leading to increased protein production.[43] All these studies demonstrate that tandem-APA-mediated changes in 3′UTR length can affect both mRNA levels and mRNA translation.

2.2 Tandem 3′UTR APA in mRNA and protein localization

Tandem-APA can regulate mRNA localization. Unique sequences within the 3′UTRs can function as 'barcodes' for RBP recognition and transport to specific subcellular compartments.[44,45] Genome-wide analysis revealed that mRNAs with shorter 3′UTRs are more enriched in the cytoplasm than the nucleus.[4] There is also evidence that mRNAs with long 3′UTRs are more likely to be localized to the endoplasmic reticulum (ER), whereas shorter 3′UTR isoforms are often localized to the mitochondrial envelope and to ribosomal subunits.[5]

It has been recently discovered that 3′UTR can drive protein localization independent of mRNA localization. *CD47*, which encodes for integrin-associated protein, has two isoforms with short and long 3′UTR. Long 3′ UTR of *CD47* can direct GFP protein localization to the plasma membrane, whereas the short 3′UTR to the ER, independent of their mRNA localization.[9] Subsequent studies identified a meshwork assembled by RBP TIS11B intertwined with ER facilitating 3′UTR's interactions with RBPs HuR and SET, controlling protein localization.[46,47] Altogether, these results indicate that tandem-APA-mediated 3′UTR length changes can affect mRNA localization as well as protein localization independent of mRNA localization within the cell.

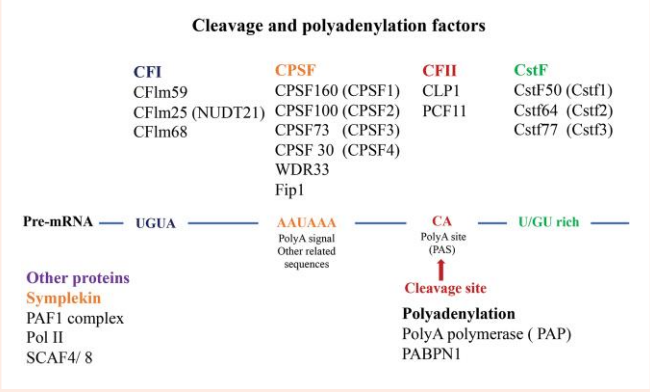

Figure 3 *Cis*-acting RNA sequences and proteins necessary for cleavage and polyadenylation of pre-mRNAs. *Cis*-acting elements include upstream 'UGUA' and downstream 'U/GU-rich' auxiliary elements as well as 'AAUAAA' poly(A) signal and 'CA' cleavage site where polyA is added. Proteins include CFIm, CPSF, CstF, CFII complexes, Symplekin, PAP, and others.

2.3 Splicing-APA in gene regulation

Splicing-APA is mediated by alternative splicing (AS) of exons that contain PASs,[2] which usually involves the last exon definition.[48] PASs can also be present in introns.[49] If PASs are within introns, a truncated protein is generated. In some cases, genes with intronic PASs are expressed at low levels.[50]

Preferential inclusion or exclusion of introns and exons with PASs via AS can modulate protein output as well as mRNA levels. Based on the different locations of PASs in exons/introns, splicing-APA can impact gene expression, mRNA isoform generation, and protein output (*Figure 1*).[14,51]

A well-known example of splicing-APA is the immunoglobulin (Ig) M heavy-chain (μ) gene. IgM has two different isoforms generated via splicing-APA, one is a surface antigen receptor (μ_m) and the other is a secreted isoform (μ_s).[16,52] When B cells become plasma cells, differential IgM splicing leads to predominantly pPAS usage, generating soluble IgM (μ_s)[16,52] In sum, splicing-APA increases protein diversity and influences gene expression.

3. Regulation of APA

3.1 Cleavage and polyadenylation factors

There are several *cis*-acting sequences in the 3′UTR required for cleavage and polyadenylation of pre-mRNAs in mammals. The essential elements include a hexameric poly(A) signal (typically AAUAAA) and a cleavage site which usually is a 'CA' dinucleotide located 10–30 bases downstream of the poly(A) signal (*Figure 3*).[2] In mammals, there is a variable U/G-U-rich region (downstream sequence element) located at 20–40 bases downstream of the PAS (*Figure 3*). A conserved UGUA or UAUA sequence and a U-rich element (upstream sequence element) are usually located upstream of the poly(A) signal[2] (*Figure 3*).

These sequences are recognized by 3′ end processing proteins. The metazoan 3′ end processing machinery includes a large protein complex composed of cleavage and polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), cleavage factor I (CFIm), cleavage factor II (CFIIm), poly(A) polymerase (PAP), poly(A)-binding protein (PABP), and Symplekin[53,54] (*Figure 3*). At the initiation step, components of CPSF and CstF bind to the polyA signal and the U/G-U-rich region, respectively, determining the region for cleavage.

CPSF has six subunits: CPSF-160 (CPSF1), CPSF-100 (CPSF2), CPSF-73 (CPSF3), CPSF-30 (CPSF4), WDR33, and hFIP1.[55] CPSF-30 and WDR33 proteins recognize and bind to the poly(A) signal.[56] CPSF-73 is the endonuclease, cleave the mRNA at the 3′ end, 20–40 nt downstream of the poly(A) signal[57,58] (*Figure 3*). CPSF-73 is associated with CPSF-100 and Symplekin proteins as a stable complex on 3′ end of pre-mRNA to guide polyadenylation.[59]

CstF has three subunits: CstF-50 (CSTF1), CstF-64 (CSTF2), and CstF-77 (CSTF3).[60] CstF-64 binds directly to the GU-rich region, whereas Cstf-50 and Cstf77 associate with both CstF-64 and Symplekin, reinforcing the binding of CPSFs and CstFs to the pre-mRNAs.[2,61,62]

CFIm complex has three proteins CFIm25 (CPSF5/NUDT21), CFIm59 (CPSF7), and CFIm68 (CPSF6), which bind to the UGUA element in the pre-mRNA.[63,64] CFIIm is a heterodimer complex that consists of polyadenylation factor hCLP1 and PCF11.[17,65] They target the 'CA' cleavage site, and weakly or transiently regulate 3′ end processing.[66] The mechanism by which these proteins regulate APA has been extensively reviewed.[16,67,68]

3.2 Co-transcriptional regulation of APA

Cleavage and polyadenylation occur co-transcriptionally. RNA polymerase II (Pol II) is required for efficient 3′ end processing. Factors that influence transcriptional elongation can affect APA. In general, slow transcription elongation is associated with pPAS usage, generating shorter 3′UTR

isoforms.[69,70] Pol II ChIP-seq analysis revealed that Pol II binding was enriched in PASs that are being utilized for 3′ end processing, coincident with where CstF77 is recruited to the PASs.[71] This indicates that Pol II deposition may influence PAS usage and APA.

In yeast, mutated Rpb1(Pol II) slowed transcription and resulted in global mRNA 3′UTR shortening.[72] Moreover, both global and reporter-based nuclear run-on assays showed that highly expressed genes tend to display Pol II pausing at pPASs in cells, and human and mouse tissues.[13]

Transcription factors and Pol II-associated proteins affect APA via interactions with cleavage and polyadenylation factors. Transcription factor TFIID interacts with CPSF complex.[16] CDC73, a component of the Pol II and chromatin-associated human Paf1 complex, interacts with both CPSF and CstF complexes and affects mRNA 3′ end processing.[73] These results, and others not discussed here, demonstrate a strong association between APA regulation and transcription.

3.3 The role of splicing factors and regulators in APA regulation

Spliceosome components U1 snRNP,[74] U1A,[75,76] U2 snRNP, and its auxiliary factor U2AF65[77,78] are involved in the recruitment of 3′ end processing factors and stimulation of mRNA cleavage and polyadenylation. Excess levels of U1A inhibit PAP activity and 3′ end processing.[79] U1 snRNP recruits CPSF160 to the 3′UTR and stabilizes the interaction and enhances cleavage and polyadenylation of pre-mRNAs.[74] U1 snRNP is important in protecting pre-mRNAs from premature cleavage and polyadenylation at cryptic intronic PASs. Depletion of U1 snRNA at 5′ splice site disrupted U1 snRNP function leading to widespread premature 3′ end cleavage and polyadenylation.[80] Consistently, antisense morpholino oligonucleotide (AMO) against U1 snRNA promoted cryptic intronic PAS usage leading to premature cleavage and polyadenylation.[81] Reducing the expression of U1 by AMO without affecting its splicing function led to widespread 3′UTR shortening.[82] These studies reveal that U1A and U1 snRNP play multiple roles in regulating APA.

U2 snRNP directly interacts with CPSF and regulates 3′ processing.[77,78] The subunit of U2 snRNP auxiliary factor U2AF65 binds to CFIm and stimulates 3′ end cleavage and polyadenylation when it is tethered to the 3′ end of adenovirus L3 pre-mRNA.[83] RBPs with roles in pre-mRNA splicing and AS,[84,85] such as SRp20, SRSF7,[55] hnRNPA2/B1,[86] hnRNP1,[87] RBFOX2,[88] CELF2,[89] MBNL,[90] NOVA,[91] bind 3′UTRs and/or affect APA patterns.[18] These results collectively indicate that splicing factors/regulators can regulate APA.

3.4 Epigenetic regulation of APA

Epigenetic regulation can influence APA.[92] Allele-specific DNA methylation affects APA of imprinted gene *H13*.[93] *H13* has five PASs[93] (*Figure 4*). DNA methylation downstream of PASs inhibits specific PAS usage, determining allele-dependent *H13* and MCTS2 expression[93] (*Figure 4*), linking methylation to APA and gene expression.

Genetic ablation of DNA methyltransferases DNMT1 and DNMT3b led to APA changes in 546 genes in HCT116 cells. Majority (75%) of these genes displayed 3′UTR shortening.[94] DNA methylation between pPAS and dPAS within the 3′UTRs interrupted CTCF-mediated chromatin remodelling.[94] CTCF regulates transcription by recruiting cohesion, forming a chromatin loop.[95,96] CTCF-binding sites located between two PASs are often associated with cohesin complex component RAD21 and Pol II binding.[94] Notably, depletion of RAD21 or CTCF-binding site restored dPAS usage. A model was proposed in which DNA methylation between pPAS and dPAS can prevent CTCF binding to that region supporting transcription elongation to reach to the dPAS.[94] These studies support a strong link between epigenetic regulation and APA.

3.5 Role of RNA modifications on APA regulation

Methylation and pseudouridylation of RNAs can influence APA.[97] m^6A modification mediated by VIRMA and METTL3 can affect APA, likely through the association of VIRMA with CFIm25 and CFIm68.[98] Depletion of YTHDC1, m^6A reader protein, resulted in genome-wide APA changes in mouse oocytes,[99] likely through interactions with CFIm68. m^6A methyltransferase subunit WTAP promoted intron retention, polyadenylation, and exon skipping at potential G-quadruplex-forming sequences, which altered splicing-APA.[100]

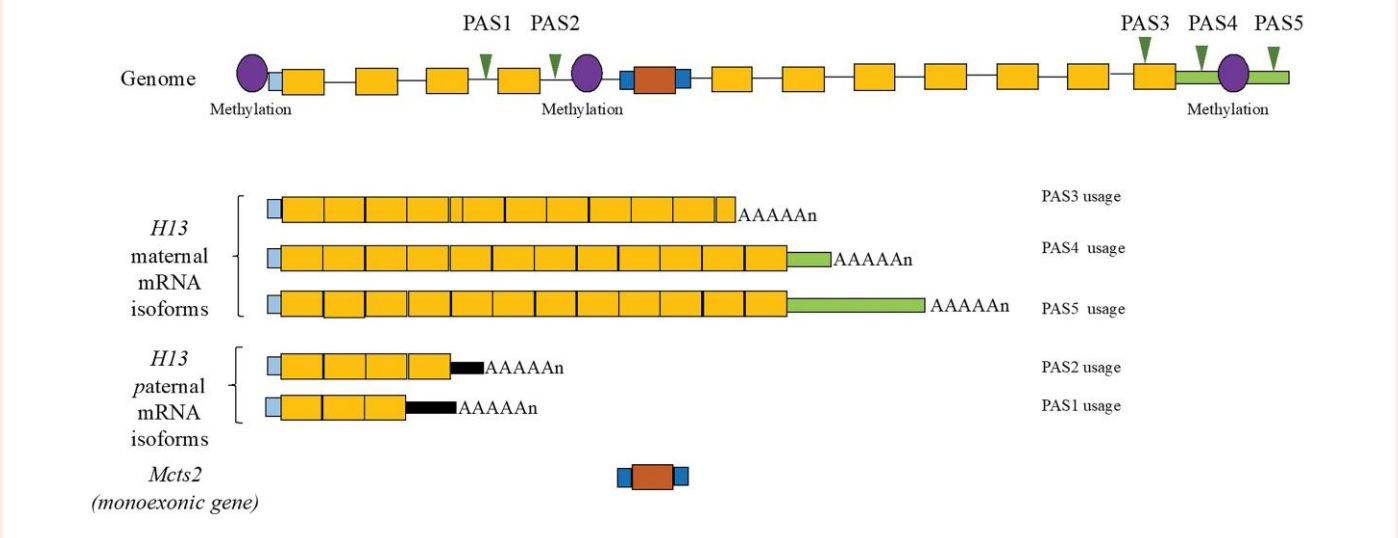

Figure 4 Gene structure and mRNA isoforms generated from *H13/Mcts* locus. Monoexonic gene *Mcts2* resides in the fourth intron of *H13* gene. Two PASs (PAS1 and PAS2) of *H13* gene are located before *Mcts2*, whereas three (PAS3, PAS4, and PAS5) are located on the last exon of *H13*. There are three CpG methylation sites: *H13* promoter, *Mcts2* promoter, and one between PAS4 and PAS5. Among these three CpG islands, *H13* promoter is not methylated in both maternal and paternal alleles in neonatal mouse brain, while *Mcts2* promoter and *H13* 3′ end were methylated in the maternal allele but not in the paternal allele. Correspondingly, PAS3, PAS4, and PAS5 were primarily utilized in maternal, and PAS1 and PAS2 were utilized in paternal alleles.

Thirty per cent 3′UTRs[101,102] in humans are pseudouridylated, suggesting a potential role for pseudouridylation in APA regulation. Depletion of pseudouridine synthases PUS1, PUS7, RPUSD4, and TRUB1, which bind to 3′UTR, led to 3′UTR lengthening via APA in HepG2 cells.[103] These studies suggest that RNA modifications may play an important role in regulating APA through mechanisms that remain to be elucidated.

3.6 Influence of ubiquitination on APA

Ubiquitination of cleavage and polyadenylation factors can impact APA. Depletion of ubiquitin hydrolase USP22 disrupted recruitment of CPSF-73 to the 3′ end of *IRF1* pre-mRNA and resulted in defective cleavage and polyadenylation of *IRF1* pre-mRNA.[104]

Another study unveiled a role for ubiquitin ligase MAGE-A11 in APA regulation.[105] MAGE-A11 is primarily expressed in male germline but is aberrantly activated in cancer cells, promoting tumour progression.[105] Activation of MAGE-A11 led to ubiquitination of PCF11, which disassociated CFIm25 and resulted in 3′UTR shortening of mRNAs.[105] 3′UTR shortening mediated by MAGE-A11 led to elevated oncogene expression.[105] About 50% of 3′UTR shortening events regulated by MAGE-A11 overlapped with CFIm25 depletion, showing that PCF11 ubiquitination but not degradation led to CFIm25 dissociation from 3′ end of pre-mRNAs. These studies indicate that ubiquitination can act as an additional regulatory layer for APA control.

4. APA in heart development and cardiovascular disease

The role of APA in cancer and other human diseases was extensively reviewed.[17,68,106] APA-mediated gene regulation in the heart is being recognized as an important contributor to cardiac gene expression and function. In this section, we summarized APA regulation during heart development and in diseased hearts.

4.1 APA regulation during heart development

APA is highly regulated during cell proliferation[28,32,107] and differentiation,[108,109] processes essential for heart development. APA is regulated in a tissue-specific manner depending on the availability of cleavage and specificity factors as well as APA regulators.[110–113]

A study used mouse embryos to profile global poly(A) usage and found that genes underwent 3′UTR lengthening as the embryos developed.[114] Consistently, single-cell RNA (scRNA) sequencing revealed global 3′UTR lengthening in all cell types between embryonic day 9.5 (E9.5) and E13.5 stages during mouse embryo development.[115] This result is not surprising, as 3′UTR lengthening occurs during cell differentiation.[114,116] However, it is not well understood how (i) APA affects cardiac gene expression during development, (ii) APA is regulated during heart development, and (iii) cleavage and polyadenylation machinery or RBPs involved in APA are regulated during heart development.

Using long-read cDNA sequencing and poly(A)click-seq (PAC-Seq), we examined splicing-APA-mediated regulation of *Tropomyosin* (*Tpm1*) by RBPs during rat heart development.[88] *Tpm1* is an actin-binding protein required for cytoskeletal functions and for contraction in muscle cells. *TPM1* is necessary for heart development and mutations or changes in *TPM1* is linked to human heart diseases. *Tpm1* has four terminal exons which are differentially spliced in a tissue-specific and development-dependent manner.[117–121] Muscle-specific *Tpm1* isoforms generated via splicing-APA were increased during rat heart development,[88] correlating with the high demand for contraction of adult hearts. We showed that developmentally regulated RBPs RBFOX2 and PTBP1 antagonistically regulate muscle-specific *Tpm1* isoforms via splicing-APA in rat myoblasts.[88,122]

Transcription factor NKX2-5 is implicated in APA regulation in the embryonic heart. *Nkx2-5* is necessary for heart development[123] and establishing transcriptional networks for cardiac differentiation.[124] *NKX2-5* is mutated in patients with congenital heart defects.[125–128] A recent study linked *Nkx2-5* to APA regulation.[129] Essential cardiac genes cardiac troponin T (*Tnnt2*) and ATPase sarcoplasmic/endoplasmic reticulum Ca2+ Transporting 2 (*Atp2a2*) mRNAs displayed longer 3′UTRs in *Nkx2-5* mutant hearts and in *Nkx2-5* depleted embryonic cardiomyocytes.[129] Genomic analysis showed *Nkx2-5* occupancy in transcription start sites as well as downstream genomic regions near PAS, suggesting a possible role for *Nkx2-5* in co-transcriptional APA regulation.[129] Supporting this, *Nkx2-5* depletion promoted serine 2 phosphorylation of Pol II and its consequent binding to downstream regions of *Tnnt2* and *Atp2a2*, which facilitated the recruitment of cleavage and polyadenylation machinery to the dPAS, resulting in 3′UTR lengthening.[129] Phosphorylation of Pol II-serine 2 mediates transcription-coupled 3′ end processing through promoting binding of cleavage and polyadenylation factors to PASs.[130]

A recent study successfully identified non-polyadenylated and polyadenylated transcripts in single cells of mouse embryos during heart morphogenesis using VASA-seq, contributing to better understanding APA regulation during heart development.[131] These results suggest that transcription factors and RBPs can influence APA-mediated cardiac gene expression during heart development.

4.2 Dysregulation of APA in cardiac hypertrophy

Cardiac hypertrophy is the enlargement of the heart in response to physiological stimuli or pathological stress. Pathological cardiac hypertrophy is an early adaptive process to increase heart function, but eventually can lead to heart failure.

Genome-wide APA changes were identified in mouse and rat hypertrophic hearts[132–134] and in human failing hearts;[135] 315 tandem-APA events were identified in transverse aortic constriction (TAC)-induced hypertrophic hearts, and 60% of which resulted in 3′UTR shortening.[132] The hallmark of cardiac hypertrophy is the reactivation of foetal gene expression.[136,137] Importantly, hypertrophic mouse hearts displayed embryonic-like APA patterns.[132]

Another study also showed global 3′UTR shortening in TAC-induced hypertrophic mouse hearts,[134] which mimicked embryonic heart APA. Moreover, 3′UTR length changes resulted in differential association of these mRNAs with translating polysomes.[134] These results suggest that during cardiac hypertrophy APA changes can alter both mRNA levels and translation, affecting protein output.

Calcium homeostasis and cell cycle are important for cardiac development and hypertrophy.[138,139] Genes involved in calcium homeostasis and cell cycle, i.e. *Asph*, *Egfr*, *Pard3*, and *Arpp21*, used different terminal exons at embryonic vs. adult stages.[134,140–142] Thus, APA may contribute to foetal gene reactivation during hypertrophy.

APA regulators are altered during cardiac hypertrophy. Star-PAP is a nuclear polyA polymerase, which regulates 3′ end processing of selective mRNAs under cellular stress.[143] Star-PAP depletion resulted in the loss of the longest 3′UTR isoform of *NQO1*,[133] which is a critical gene for heart function. Both Star-PAP and NQO1 proteins were reduced in hypertrophic rat hearts. Ectopic expression of Star-PAP and longest 3′UTR isoform of *NQO1* reversed hypertrophic gene response in cardiomyocytes.[133] These suggested that APA regulation of *NQO1* via Star-PAP is a contributor to cardiac hypertrophy. Another study also linked Star-PAP to 3′ end processing in hypertrophic hearts. Star-PAP interacted with AS regulator RBM10 and regulated APA of anti-hypertrophic genes including *HAND2, TGFBR3, ERBB3, RHEBL1, COL5A1, CACNA1G*, and *HO-1*, affecting their expression[144] (*Table 1*). Mechanisms by which RBM10 and Star-PAP coordinately mediate APA need further investigation.

Recent advancements in sequencing technology and computational algorithms allow precise determination of APA patterns[151] (*Table 2*). scAPAatlas of human and mouse tissues is a good source to assess APA at the single-cell level.[151] According to the scAPAatlas, *CLOCK*, a transcription factor that regulates circadian rhythm, has four different mRNA transcripts with varying 3′UTR lengths generated via APA[151] (*Table 1*). In atrial cardiomyocytes, predominantly long 3′UTR-containing transcripts of

Table 1 Genes regulated via APA and APA regulators relevant to heart development and cardiovascular diseases

Heart disease	APA types	Genes regulated via APA	APA factors/ regulators or RBPs	References
Cardiac hypertrophy	Tandem - APA; Splicing - APA	*Arfgef2, Lrrc58, Ppm1k, Klf4, NQ0, CLOCK, Gsk3b, Camkk2, Ccnd2, Faf2, Mtus1, Etf1, Utp6, Pdzrn3, Ctc1, Asph, Egfr, Pard3, Arpp2, CACNA1G, Rheb11, HAND2, TGFBR3, ERBB3, RHEBL1, COL5A1, and HO-1*	Star-PAP, RBM10	132–134,143,144
Heart failure	Tandem - APA	*CDC42EP3, PIGK, RCAN1, WEE1, FBRSL1,SERF, COL1A, FN1, and TGFβR1*	PABPN1, CPSF30, PCF11, CSTF64	135,145
Cardiac arrythmias	Intronic -APA	*KCNH2*	HuR, PABPN1	69
Atherosclerosis	Tandem - APA; Splicing - APA; Intronic -APA	*Irf2bp2, Ddx5, Timp2, Ub32n and Flt1*	CPSF6, CFIm68	146,147
Hypertension	Tandem - APA	*ATP1B1* and *SLC7A1*	Undefined	148–150

CLOCK were identified; whereas in ventricular cardiomyocytes, predominantly short 3′UTR isoforms were detected.[151] Single nucleotide polymorphisms in *CLOCK* are associated with obesity[161] and ischaemia/reperfusion.[162] Cardiomyocyte-specific expression of CLOCK mutant under chronic desynchronic light cycle increased expression of cardiac hypertrophy markers,[163] linking APA regulation of *CLOCK* to cardiac hypertrophy.

4.3 APA regulation and APA regulators in heart failure

In heart disease, APA is aberrantly regulated. In a study, genome-wide APA changes were identified in human failing hearts.[135] About half of these APA changes led to 3′UTR shortening.[135] *CDC42EP3*, *PIGK*, *RCAN1*, and *Wee1* displayed significant 3′UTR shortening, whereas *FBRSL1* and *SERF2* showed 3′UTR lengthening in human failing hearts[135] (*Table 1*). Importantly, changes in their 3′UTR lengths correlated with changes in their protein levels, suggesting that 3′UTR length changes are reflected in protein levels. Consistently, dysregulation of 3′ end processing proteins PABPN1, CPSF30, and PCF11 were identified in human failing hearts[135] (*Table 1*). Mechanisms responsible for altered expression of PABPN1, CPSF30, and PCF11 in failing hearts and the consequences of these changes on APA need to be further investigated.

CSTF64 expression was increased in left ventricles and cardiac fibroblasts of human failing hearts.[145] Profibrotic genes including *Col1A*, *Fn1*, and *TGFβR1* exhibited a shift to a pPAS usage rather than dPAS, resulting in increased protein levels of fibrotic genes, contributing to the fibrosis.[145] Loss of CSTF64 in cardiac fibroblasts isolated from heart failure patients led to increased dPAS usage of profibrotic genes reducing their expression[145] (*Table 1*).

These findings suggest that APA factors are susceptible to dysregulation in diseased hearts. The contribution of APA factors/regulators to heart diseases is largely unknown. It would be important to determine whether changes in APA and/or APA regulators are secondary to failing hearts or primary drivers of heart failure.

4.4 APA regulation of ion channels involved in cardiac arrhythmias

Mutations in genes encoding ion channels can lead to cardiac arrhythmias.[164] The Kv11.1 voltage-gated potassium channel *(KCNH2 gene)* mediates the rapid activation of delayed rectifier current (I_{Kr}) and is responsible for action potential repolarization in the heart.[165] Changes in Kv11.1 levels or *KCNH2* mutations are associated with cardiac arrhythmia.[166] *KCNH2* isoforms with different N- and C-terminal termini are identified.[69] Among these isoforms, PASs in intron 9 lead to the generation of a C-terminal domain (CTD) truncated isoform, which is non-functional.[69] Consistently, mutations in *KCNH2* gene that affects the CTD were identified in long QT syndrome (LQTS) patients.[167,168] RBPs HuR[69] and PABPN1[169] were identified as inhibitors of PAS usage in intron 9 and promoters of full-length *KCNH2* expression and proper I_{Kr}. Further studies are needed to determine whether modulating *KCNH2* APA in LQTS could prevent arrhythmias (*Table 1*).

4.5 APA in atherosclerosis

Atherosclerosis is characterized by vascular plaque formation due to inflammation-mediated accumulation of fats, cholesterol, and other substances in the arterial walls. Plaques narrow and clog arteries, limit blood flow, and can potentially burst.[170] mTORC1 pathway is activated during atherosclerosis and has been identified as a critical contributor to atherosclerosis progression. Activation of mTORC-1 resulted in dramatic shortening of 3′UTRs of 846 genes, which in turn increased their polysome association and translation.[146] Of these APA-regulated genes, *Irf2bp2*,[171] *Ddx5*,[172] *Timp2*,[173] and *Ub32n*[174] important for the development of atherosclerosis were up-regulated. mTOR activation regulates phosphorylation and

Table 2 Methods to identify APA patterns

Method	Fragmentation	Analysis	Ref.	Limitations	Advantage
Microarray	Restriction endonucleases	Affymetrix Power Tools, etc.	32,114,152–154	Low resolution and sensitivity	Simple
3′-Seq	Heat shearing	Map reads to ref. seq. by Bowtie and uses (CS_J) $=\sum j=1\pi wj*j$ to determine differential poly(A) usage	107	May cause bias during fragmentation and ligation	Simple, better sensitivity
3P-Seq	Restriction endonucleases	Reads mapping, tailing, etc.	155	May cause bias during fragmentation and ligation, complex experimental process	Simple, better sensitivity
PAS-Seq	RNA Fragmentation Buffer	Reads mapping, poly(A) site clustering, 3′UTR database establishing, etc.	109	May cause bias during fragmentation and ligation	Simple, better sensitivity
3′READS	RNA Fragmentation Buffer	Reads mapping, poly(A) site clustering, 3′UTR database establishing, etc.	116	May cause bias during fragmentation and ligation	Simple, better sensitivity
PolyA-seq	No fragmentation	Reads mapping and tailing, determine poly(A) site with poly(A) score $=\log_{10}\left(\frac{\prod_{i=1}^{10} pA(x_i)}{\prod_{i=1}^{10} l(x_i)}\right)$	12	May cause bias during ligation and size selection	Simple, no fragmentation, better sensitivity
2P-seq	Partial digestion with RNase T1	Reads mapping, poly(A) site clustering, etc.	6	May cause bias during fragmentation, and ligation; complex procedure	Better sensitivity
MAPS	No fragmentation	Reads mapping, etc.	156	May cause bias during capturing and ligation	No fragmentation, better sensitivity
PAT-Seq	Partial digestion with RNase T1	Reads trimming, tail length and poly(A) site determination, etc.	157	May cause bias during fragmentation, size selection, and ligation; complex	Better sensitivity
SAPAS	Heat shearing	Reads mapping, poly(A) site clustering, etc.	158	May cause bias during fragmentation	Better sensitivity
IVT-SAPAS	Heat shearing	Reads mapping, poly(A) site clustering, etc.	159	May cause bias during fragmentation, and ligation; complex	Better sensitivity
PAC-Seq	No fragmentation	Reads mapping, poly(A) site clustering, etc.	160	May cause bias during click it process	Simple, no fragmentation, better sensitivity
Long-read sequencing	No fragmentation	Reads mapping, etc.	88	Coverage issues	Simple, no fragmentation

subcellular localization of CPSF6[18] (*Table 1*), likely contributing to mTORC-mediated APA changes.

Another example of atherosclerosis-associated gene regulated by APA is *Flt1* (*Table 1*). *Flt1* encodes for the vascular endothelial growth factor receptor 1 (VEGFR1).[175] Inhibition of VEGFR1 reduces angiogenesis and atherosclerosis.[176] *Flt1* has a PAS located in intron 13, which generates the soluble form of FLT1.[147] Soluble FLT1 functions as a decoy sequestering VEGF and inhibiting angiogenesis and atherosclerosis.[175,177]

4.6 APA in hypertension

Hypertension or high blood pressure is a major risk factor for heart failure, atrial fibrillation, heart valve disease, atherosclerosis, aortic syndromes, and ischaemia.[178,179] Several genes related to hypertension harbour polymorphisms in their 3′UTRs that affect their APA regulation.[148–150,180] *ATP1B1* encodes for ATPase Na^+/K^+ transporting subunit beta 1, which is important for maintaining Na^+/K^+ ions across the cell membrane. Increased *ATP1B1* expression is linked to hypertension in rats.[181] A polymorphic T-rich sequence is found downstream of the pPAS of *ATP1B1*.[148] This polymorphic T-rich sequence promotes pPAS usage, generating an *ATP1B1* short 3′UTR isoform that increased ATP1B1 protein levels, promoting hypertension[148] (*Table 1*).

Another hypertension-related gene regulated via APA is *SLC7A1* (*Table 1*). *SLC7A1* encodes for high affinity cationic amino acid transporter 1.[182] Decreased SLC7A1 protein is associated with the onset of hypertension. There is a polymorphic SNP ss52051869 upstream of the human *SLC7A1* pPAS.[149,150] Minor *T* allele of the SNP promotes the generation of the *SLC7A1* long 3′UTR isoform, whereas the *C allele* often generates the short 3′UTR isoform.[149,150] The *SLC7A1* long 3′UTR isoform results in decreased SLC7A1 protein, highly associated with the onset of hypertension.[149,150]

5. Identification of global APA patterns

APA was originally detected by microarray, which had limited resolution.[32,152,153] Currently, RNA-sequencing methods are used to determine PAS usage and APA.[6,12,107,109,116,155–160] These methods may include fragmentation and ligation processes, which could generate bias. Correspondingly, several bioinformatic tools were designed to extract

PAS/clusters from RNA-sequencing reads.[183] *Table 2* summarizes the sequencing methods and analyses used for APA detection.

6. APA in cardiovascular disease prevention and therapy

6.1 APA as a biomarker for predicting disease severity

One of the difficulties in treating cardiovascular diseases is that the disease progresses without obvious phenotypes and can irreversibly damage the heart. For example, adults can start accumulating lipids in vessels without symptoms, which can quietly result in atherosclerosis or even thrombosis. These conditions are not easy to treat and might be fatal.[184] Heart failure is typically preceded by cardiac hypertrophy, which initially is an adaptive response to increased cardiac function and shows no significant cardiac dysfunction. However, the development of heart failure leads to high rates of morbidity even with the current treatments.[185]

Since APA patterns are different in normal vs. diseased hearts (*Table 1*), APA profiles in blood cells might be used as biomarkers. APA dysregulation correlates with different grades of tumour and a variety of tumour types, making it a good biomarker for cancer types.[186] Remarkably, three histologically indistinguishable mouse lymphoma subtypes were distinguished with >74% accuracy based on APA signatures.[187] Limited work has been done to profile APA patterns in blood from patients with cardiovascular diseases. APA profiling of blood cells and heart tissues might help better understand molecular mechanisms and gene signatures of cardiovascular diseases. Advancements in scRNA-sequencing provide gene regulatory profiles in a single cell.[188] Future studies are needed to develop scAPAatlas of human heart diseases. A new study generated tools to determine the potential consequences of pathogenic variants of APA in a wide range of pathological conditions.[189] APA profiles and predictions on gene expression in blood might help with the prediction and prevention of cardiovascular diseases at early stages.

6.2 Modulating APA for treatment and prevention of cardiovascular diseases

Correcting APA patterns of disease-causing genes could help restore essential gene expression and function in diseased hearts. For instance, CD47 is increased in human atherosclerotic arteries, and antibody against CD47 ameliorates atherosclerosis.[190] *CD47* has short and long 3′UTR isoforms. The long 3′UTR isoform of *CD47* is the cell surface ligand responsible for immune checkpoint function while the short 3′UTR isoform is not. Preventing the generation of *CD47* long 3′UTR isoform without affecting the short isoform could eliminate pro-atherosclerotic lesions. Thus, APA-targeted gene therapy may provide a specific way to regulate different gene isoforms with different functions.

APA patterns can be modulated using genome editing (CRISPR or Talon), RNA editing, antisense oligonucleotides (ASOs), or small molecules. The polyA signal, PAS or PASs, or the length of 3′UTR can be edited to modulate APA.

CRISPR-mediated genome editing was used to modulate PAS usage of *CCND1* and in turn its expression.[191] The whole region between short and long mRNA 3′UTR was removed to maintain only the short *Calm1* isoform. Removal of *Calm1* long isoform disorganized dorsal root ganglion and hippocampal neuron activation in mice.[192]

Facioscapulohumeral muscular dystrophy (FSHD) patients have increased DUX4 expression in skeletal muscle. Using CRISPR-genome editing *DUX4* APA was modulated to reduce DUX4 expression in FSHD patient-derived myoblasts.[193] Genome editing is an efficient method of manipulating APA. However, potential off-target effects should be considered during design.[194]

ASO-based therapy is another way to alter APA. Modified ASOs that do not cause mRNA degradation is successfully used in clinics to modulate AS of *SMA* gene to treat children with spinal muscular dystrophy.[195,196] ASOs were also used to block the PAS of *DUX4*, which successfully reduced *DUX4* expression in the FSHD patient xenografts model without inducing cell toxicity.[197] ASOs blocking intronic PAS in *Kv11.1* mRNA successfully repressed generation of truncated Kv11.1 isoform and up-regulated Kv11.1 full-length isoform.[198] One of the limitations of ASOs is tissue-specific targeting and delivery.[199] ASOs also might have off-target effects and special consideration need to be taken while performing ASO experiments and two or more ASOs should be used for these studies.[200]

Small molecules can also be used to modulate APA. They can bind specific RNA structures and alter RNA splicing, cleavage of RNA, pre-RNA translation, and deactivate non-coding RNAs.[201] Small molecule derived from *N,N*′-bis(2-quinolinyl)pyridine-2,6-dicarboxamide was able to preferentially stabilize and isolate G-quadruplex DNA and RNA motifs.[202] Small molecules T4 and T5 were identified to promote pPAS usage in many transcripts with longer introns.[7]

7. Concluding remarks

APA has been recognized as an important RNA processing step that can directly affect gene expression and function. It is implicated in a variety of biological processes. It is tightly regulated, but its regulation goes awry in diseased conditions. A large protein complex controls mRNA cleavage and polyadenylation efficiency. Transcription regulation, epigenetic regulation, splicing factors/regulators, RNA modifications, and ubiquitination also closely intertwine with APA.

We touched upon the importance of APA-mediated gene regulation during cardiac development and in heart diseases. There are many unanswered questions that need deeper investigation. Are APA patterns altered similarly in different cardiovascular diseases? Which APA regulators or RBPs are involved in APA dysregulation in cardiovascular diseases of different aetiologies? How can we effectively and specifically target APA regulation of genes in the heart for therapy? How APA-mediated gene regulation contributes to heart development? Thus, understanding the molecular mechanisms responsible for APA dysregulation in cardiovascular diseases will provide new RNA-based therapies with nucleotide-level precision. The translational research using advanced molecular tools to manipulate APA in disease models as well as investigations on APA regulators and APA-related biological pathways in cardiovascular diseases require more attention.

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