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Post-Transcriptional Modification by Alternative Splicing in Cardiovascular Development and Congenital Heart Defects

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ABSTRACT

Advancements in genomics, bioinformatics and genome editing have uncovered new dimensions in gene regulation. Post-transcriptional modifications by the alternative splicing of mRNA transcripts are critical regulatory mechanisms of mammalian gene expression. In the heart, there is an expanding interest in elucidating the role of alternative splicing in transcriptome regulation. Substantial efforts have been directed towards investigating this process in heart development and failure. However, few studies have shed light on alternative splicing products and their dysregulation in congenital heart defects (CHDs). While elegant reports have shown the crucial roles of RNA binding proteins (RBPs) in orchestrating splicing transitions during heart development and failure, the impact of RBPs dysregulation or genetic variation on CHDs has been fully addressed. Herein, we review the current understanding of alternative splicing and RBPs' roles in heart development and CHDs and discuss the impacts of perinatal splicing transition and its dysregulation in CHDs. We further summarize

discoveries made of causal splicing variants in key transcription factors that have been implicated in CHDs. Improved understanding of the roles of alternative splicing in heart development and CHDs may potentially inform novel preventive and therapeutic advancements for newborn infants with CHDs.

KEYWORDS

Posttranscriptional Modification, Alternative Splicing, Congenital Heart Defects, Transcriptome, Splicing Variants, Genome.

NONSTANDARD ABBREVIATIONS

CHDs (Congenital Heart Defects)

RBPs (RNA Binding Proteins)

E–C (Excitation-Contraction)

TOF (Tetralogy of Fallot)

RVOT (Right Ventricular Outflow Tract)

TAV (Tricuspid Aortic Valve)

BAV (Bicuspid Aortic Valve)

HLHS (Hypoplastic Left Heart Syndrome)

ARVD (Arrhythmogenic Right Ventricular Dysplasia)

LVNC (Left Ventricular Noncompaction)

RBPMS (RNA-Binding Protein with Multiple Splicing)

INTRODUCTION

The genome era has introduced new opportunities to understand novel mechanisms of gene regulation, including post-transcriptional regulation by alternative splicing mechanisms. Orchestrated by the splicing machinery, RNA splicing is a highly regulated post-transcriptional modification process by which introns are removed from nascent pre-mRNAs, leading to the generation of mature mRNAs for translation and protein synthesis [**Figure 1.A**]. In contrast to canonical “constitutive” splicing, alternative splicing exhibits temporal regulation during cellular

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differentiation, orchestrating tissue homeostasis and organ development by fine-tuning their cellular properties, physiological functions, and developmental trajectories. Meanwhile, dysregulated splicing networks may lead to impaired organ formation and function. Diverse physiological conditions and environmental stimuli may alter splicing decisions, leading to the generation of multiple mRNA isoforms from a single gene in tissue-specific and context-dependent manners. This supports the concept that alternative splicing plays crucial roles in proper organ formation and function during critical stages of mammalian development.

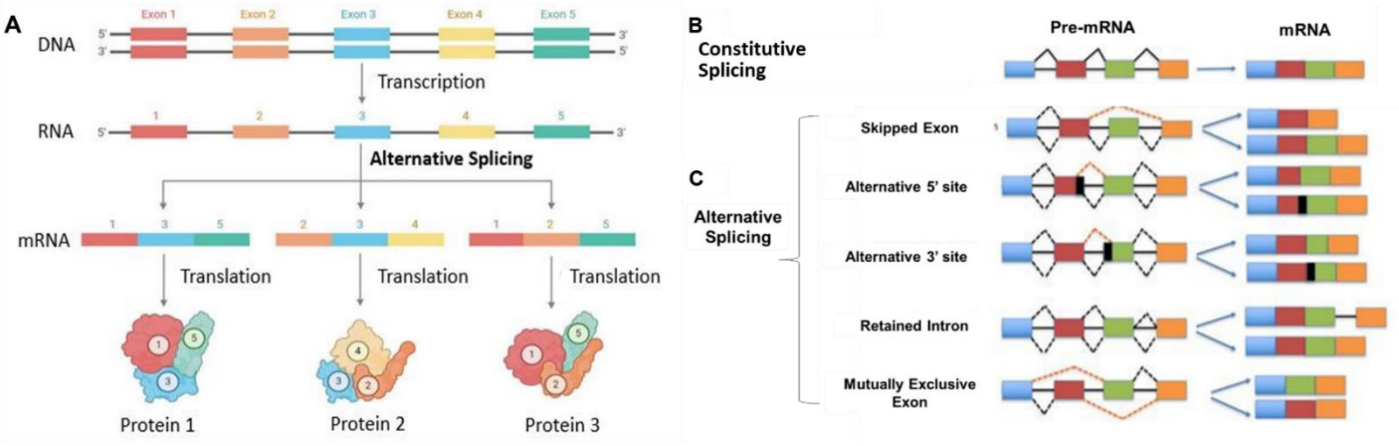

Figure 1. Schematic Representation of Alternative Splicing Process and patterns. A. Alternative splicing leads to functional diversity of transcriptome and proteome by creating a diverse array of protein isoforms from a single gene. **B.** Constitutive splicing. **C.** Alternative splicing modes: Skipped exon, (2) mutually exclusive exon usage, alternative 5' splice site selection, alternative 3' splice site selection, retained intron, and mutually exclusive exon.

Transcripts from the vast majority of protein-coding genes in mammals are subject to one or more types of alternative splicing [1, 2]. Several alternative splicing types or patterns have been described [Figure 1. B]. Among them five patterns are most commonly encountered: (1) exon skipping (SE), (2) mutually exclusive exon (MEX) usage, (3) alternative 5' splice site selection (5'SS), (4) alternative 3' splice site selection (3'SS), and (5) intron retention (IR) [3]. Remarkably, ES is the most prevalent pattern, in which specific exons, referred to as cassette exons, are either included or skipped from the mature transcript depending on splicing decisions. MEX is less common than ES. In this pattern one cassette exon is included, while the other is skipped in the mature transcript. The usage of alternative splice start sites or end sites affects the 5' or 3'ends respectively, resulting in shorter or longer forms of exons from the same transcripts. Finally, IR occurs when intronic intervals are retained in the mature transcript, which may be translated or processed by a nonsense-mediated decay mechanism. Alternative splicing reactions are catalyzed by the spliceosome [4]. Spliceosome assembly involves the complex interplay

of small nuclear ribonucleoprotein particles (snRNPs, U1, U2, U4/U6, and U5) and other associated proteins. The formation of spliceosomes and their mechanism of action were elegantly investigated and characterized using cryo-electron microscopy studies [5-8].

Alternative splicing is a ubiquitous process throughout organs, tissues, and cell types. In humans, transcripts of more than 95% of the protein-coding genes are estimated to undergo alternative splicing leading to proteome complexity [9]. In contrast to promoter activity regulation by transcription factors that predominantly affect transcript abundance, alternative splicing events affect mRNAs' structure and their translation potentials to functional protein isoforms that can exert diverse tissue specificity, cellular localization, binding ability, or enzymatic activity [10]. Furthermore, by altering the reading frame, alternative splicing may affect mRNA localization or translation leading to protein isoforms of diverse, and sometimes opposing, functions [11]. The prevalence of alternative splicing has raised questions about its biological importance and functional outcomes. Therefore, establishing the partition of this process in human organ development and disease remains challenging. Indeed, not all splicing products lead to functionally intact protein isoforms at the translational level due to several reasons, amongst them: (1) the splicing event may produce a non-coding transcript lacking a functional open reading frame; (2) the splicing event may lead to a functional non-coding transcript that modulates chromatin accessibility or competes with other RNAs; (3) the splicing event may affect transcript stability leading to antisense mediated decay; (4) the splicing event may alter the subcellular localization of the mature mRNA impairing its translation or function; and (5) the splicing events may be overestimated as a result of amplification artifacts.

Alternative splicing events are relatively poorly conserved, however, alternatively spliced exons that exhibit tissue specificity or distinct regulation patterns in response to changes in physiological status or external cues tend to be more conserved at the protein level, suggesting putative functional outcomes [12]. Furthermore, large-scale analyses of AS atlases across seven different mammalian species during development suggest that dynamically regulated alternative splicing transitions during organ development tend to be more evolutionarily conserved than the nondynamic, more frequent, splicing events [13]. Nevertheless, the extent of alternative splicing and

the selection splicing patterns tend to be different across organs, developmental periods, and types of cassette exons [13].

Regulation of Alternative Splicing

Operated by the splicing machinery, or the spliceosome, alternative splicing events are driven by various cis-regulatory elements located at the exon-intron boundaries. These splicing enhancer sequences orchestrate splicing decisions by recruiting RNA-binding proteins (RBPs) and other *trans*-acting factors that bind to the RNA molecule and define the exon-intron junctions [14]. Among the most investigated RBPs that contribute to the exon-intron definition are the serine/arginine (SR)-rich proteins (SF2/ASF, SRp20, SRp40, SRp55, and SRp75), the heterogeneous ribonucleoprotein (hnRNP) family of proteins (PTB, hnRNPA1, hnRNP C, hnRNP D, hnRNP E, hnRNP F/H, hnRNP G, and hnRNPH), and the RBPs containing RNA recognition motif (RRM), K homology domain (KH), and zinc-finger domains [15, 16].

Upon binding the cis elements at intron-exon junctions, RBPs promote or repress splice site interaction. Thus, they regulate splice site selection at an early stage of spliceosome formation [17, 18], although they may contribute to advanced stages of spliceosome assembly as well [4]. Initially, U1 ribonucleoprotein binds to the 5′splice site (5'ss), while U2AF, a conserved heterodimer that plays a vital role in defining functional 3′ splice sites (3'ss) in pre-mRNA splicing, binds to the 3′ss and the poly-pyrimidine tract (YYY-rich). These early interactions lead to the recruitment of U2 snRNP to the pre-mRNA splice site, followed by the addition of tri-snRNP particles composed of U4/U6 and U5 snRNPs [Figure 2]. Subsequently, RNA helicases facilitate rearrangements of snRNP interactions in the assembled spliceosome, which in turn catalyzes the splicing event [4]. Remarkably, mutagenesis of minigene reporters has demonstrated that splicing efficiency requires cooperative interactions of many RBPs that bind with ‘‘multivalent’’ motifs proximal to alternative exons to precisely carry out the splicing reaction [18, 19].

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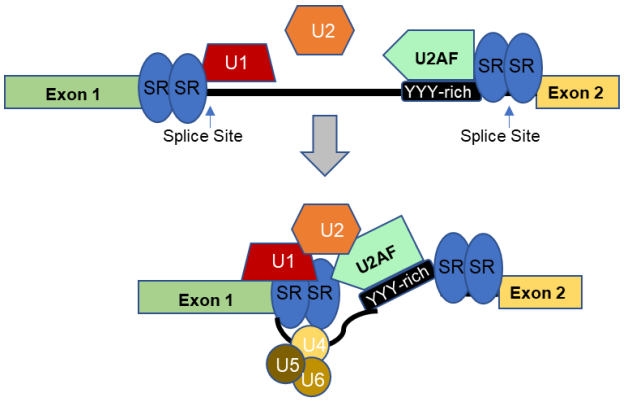

Figure 2. Schematic Illustration of Spliceosome Assembly. Alternative splicing (AS) of a pre-mRNA is carried out by the spliceosome complex. U1 ribonucleoprotein binds to the 5′splice site while U2AF binds to the 3′splice site and the poly-pyrimidine tract (YYY-rich). RNA-binding proteins (RBPs) such as SR proteins bind to the splicing recognition motif. U2 ribonucleoprotein mediates the interaction between U1 ribonucleoprotein and U2AF leading to a conformational change of the RNA promoting binding of the tri-ribonucleoprotein complex (U4–U5–U6).

In summary, alternative splicing regulation is carried out by multiple regulatory factors, acting in *cis* or *trans* to achieve a precise definition of splicing sites. However, to date, the repertoires of proteins that control alternative splicing are not fully characterized. Employing long read sequencing and examining RNP covalent interactions in post-transcriptional gene regulation, alongside the current advances in functional genomics and CRISPR-based approaches for modulating splicing, are expected to unfold the complexity of alternative splicing mediated transcriptome regulation [20- 26]. New sequencing technologies, such as single-cell RNA seq and Nanopore sequencing, have already been implemented in neuroscience and cancer biology [27-29], and more recently in cardiovascular disease [30], revealing cell-type specific alternative splicing events and their functional impacts on cell behavior and fate. Considering that impaired splicing can lead to various human diseases [31-36], efforts tailored to baseline understanding of tissue-specific and cell-specific alternative splicing processes and their physiologic roles are essential to fully reveal their contribution to human disease.

Alternative Splicing Transition During Heart Development

Heart development is a highly dynamic process during which significant transcriptome remodeling occurs in a spatial-temporal regulated manner [37, 38]. These changes are driven mainly by transcriptional and post-transcriptional modification mechanisms, including alternative mRNA splicing.

Genome-wide sequencing and functional genomics tools have revealed significant splicing transitions during the differentiation of human embryonic stem cells into cardiac precursors [39, 40], and uncovered significant differences in alternative splicing patterns between fetal and adult hearts [41, 42]. Compared to the adult heart, RI events were found to be more predominant in the fetal heart. Furthermore, cellular proliferation processes were enriched in the fetal-specific alternative splicing events. In contrast, adult-specific events were enriched in energy-related categories [3]. Calcium channel beta2 (CACNB2), tropomyosin 1(TPM1), disabled-1 (Dab1) [43, 44] as well as important cell cycle regulators including, pumilio RNA-binding family member 1 (PUM1), calcium/calmodulin-dependent protein kinase 2D (CAMK2D), and anaphase-promoting complex subunit 11 (ANAPC11) exhibit significant splicing differences between fetal and adult hearts [3]. Likewise, sarcomere-related proteins are developmentally regulated via alternative splicing including cardiac troponin T (cTnT). Exon 5 of cTnT is predominantly expressed in the embryonic heart, encoding a protein domain that increases embryonic cTnT-containing myofilament sensitivity to calcium, as compared to the less sensitive adult cTnT myofilaments, thereby modulating the contractile properties of embryonic myocardium [45, 46]. More recently, single-cell RNA sequencing analysis of 996 samples representing the cellular composition of fetal-like (hiPSC-derived cardiac progenitors), healthy adult hearts, and diseased failing hearts further addressed the cellular heterogeneity of fetal and adult hearts [30]. The study also revealed significant reversion of fetal-specific RBPs in the diseased failing hearts that were associated with the re-induction of approximately 1,500 fetal-specific isoforms, compared to healthy adult hearts [30]. Remarkably, the reactivated fetal-specific isoforms tend to harbor RBP binding sites, have canonical splice site sequences, and contain known upstream polypyrimidine tracts.

Like in prenatal development, alternative splicing transition continues to play an important role as a regulatory component of transcriptomes in the early postnatal development of the murine heart. During this period, dramatic hemodynamic changes occur, driving significant alterations in cellular respiration, metabolism, proliferation, and functional properties. These changes are associated with a highly coordinated alternative splicing program that produces substantial protein isoform transitions that are critical for postnatal heart growth and maturation [47]. Using bulk RNA-sequencing, transcriptome dynamics in mouse heart cells, cardiomyocytes and cardiac

fibroblasts, at different prenatal and postnatal stages were recently revealed [48]. While significant splicing changes in cardiomyocytes occur within the first month after birth indicating an important role of alternative splicing in cardiomyocyte maturation, splicing transitions in cardiac fibroblasts continue beyond the first month. Finally, it is worthy to note that alternative splicing products during postnatal heart development are more likely to exert functional consequences when splicing transition occurs simultaneously in more than one organ, such as splicing events in the heart and brain during development [49-53].

Alternative splicing transitions during heart development are regulated by multiple of RBPs that exhibit significant temporal changes in their expression levels, exerting their functions in cooperative or antagonistic manners [54-56]. Out of approximately 1500 RBPs expressed in the heart, 390 cardiac-specific RBPs have been identified [54]. Examples of cardiac RBPs that have been principally investigated in heart development include CELF1 (CUGBP Elav-like family member-1), MBNL1 (muscleblind-like protein-1), RBFOX1, RBFOX2, RBM20 and RBM24, among several others [54-59]. Studies have shown that the MBNL and CELF families play leading roles in splicing transition during pre-and postnatal heart development [47, 49, 60]. Similar to their expression regulation during development, they often lead to reciprocal changes in their shared splicing targets, suggesting antagonistic regulation [49]. While MBNL1 was induced, CELF proteins were suppressed in the postnatal heart. Importantly, both MBNL1 and CELF are regulated by RBM20-mediated alternative splicing during heart development. Correspondingly, RBM20 loss of function in the adult heart reverts their levels to the embryonic splicing pattern. Rbfox1 was also identified as a vital regulator for the conserved splicing process of transcription factor Mef2 family members and was found to be a major player in the reversion of global fetal gene programming in pressure overload heart failure [58].

The identification of the downstream targets of several cardiac RBPs and splicing regulators [Table 1] has provided important insights into how these factors can affect heart developmental decisions, physiology, and function [54-62]. During mammalian heart development, RBPs orchestrate alternative splicing of sarcomere genes that determine the structure and mechanical properties of heart muscle, best exemplified by splicing events in Titin, which contains the largest number of exons that may be alternatively utilized via splicing

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regulation, thereby modulating the titin-based passive tension which determines diastolic ventricular filling. For example, exons 50–219 are shown to be developmentally regulated, with the longer protein isoform (N2BA) predominantly expressed in neonatal hearts, while the shorter protein isoform (N2B) is predominantly expressed in adult hearts [63]. Importantly, this shift toward higher N2B titin isoform levels increases sarcomere-passive tension and myocardial stiffness. Hence, the relative abundance of N2BA isoform compared to N2B isoform determines myocardium elasticity that controls ventricular filling during diastole [63]. Remarkably, in response to pressure overload, the left ventricles of patients with aortic stenosis exhibit a shift in titin isoform expression towards the shorter N2B isoform. Consequently, the higher passive tension upon stretch may lead to a decline in cardiac performance [64]. Importantly, the Titin splicing at the PVEK region, which forms the I band with N2BA, is regulated by RBM20 [63]. Hence, loss-of-function mutations in human RBM20 have previously been shown to cause hereditary cardiomyopathy due to impaired Titin isoform transition and excessive production of N2BA isoform in the RBM20-deficient hearts leading to flaccid titin filaments and replacement fibrosis [63].

Table 1. RNA Binding Proteins and Their Main Targets In Heart Development.

RNA Binding Protein	Main Target (s)	CHD/Condition	Reference
RBM24	LDB4, CaMKIIδ, TPM, MyoM	Sarcomerogenesis	51; 54-56
RBM20	TTN	Ventricular Elasticity	52;53
CELF1	Fetal-like program	Heart Development/Myofibrinogenesis	57;64;66
MBNL1	Fetal-like Program	Heart Development	57;64;66
SRp38	Triadin	Excitation-Contraction Coupling	62
ASF/SF2	CaMKIIdelta	Excitation-Contraction Coupling	63
TBX5/SC35	RNF	Holt –Oram syndrome	82
RBpms	Pdlim5	Left Ventricle Noncompaction	74
RBFOX2	Rho GTPases	Hypoplastic Left Ventricle	80
RBFOX1	MEF2	Heart Failure/ Fetal-Like Program	44

RBM 24 is another RBP involved in cardiomyogenesis, and together with RBM20, regulates alternative splicing events of various sarcomere genes, including those encoding myomesin, tropomyosins, LDB3, and calcium/ calmodulin-regulated kinase (CaMK) IIδ [62, 65-67]. Changes in isoform expression of these genes have been implicated in dilated cardiomyopathy and impaired calcium handling in cardiomyocytes. Furthermore, the cholinergic receptor muscarinic 2 (CHRM2) has been identified as a target for RBM24, revealing a new

mechanism by which RBM24 variants may modulate cardiac conduction and contractility [66, 67]. SRp38 was also found to regulate cardiomyocyte contractility. Loss of SRp38 in mice disturbed splicing of triadin, a protein that controls calcium release from the sarcoplasmic reticulum during excitation–contraction (E–C) coupling [68], and cardiomyocyte-specific ablation of SF2/ASF resulted in impaired postnatal splicing switch of the Ca(2+)/calmodulin-dependent kinase IIdelta (CaMKIIdelta) leading defect in E-C coupling, dilated cardiomyopathy and heart failure [69]. More recently, Quaking (QKI), an hnRNP protein [35], was identified as a critical alternative splicing regulator in cardiomyocyte differentiation and maturation that is required for heart development and function.

Dysregulated Alternative Splicing in Congenital Heart Defects (CHDs)

I. Splicing Transition in CHDs

Only recently has the role of alternative splicing in CHDs gained further attention. Global transcriptome profiling studies helped examine alternative splicing partitions in different CHD phenotypes. In the following, we highlight some representative studies and reference other equally important literature [Figure 3].

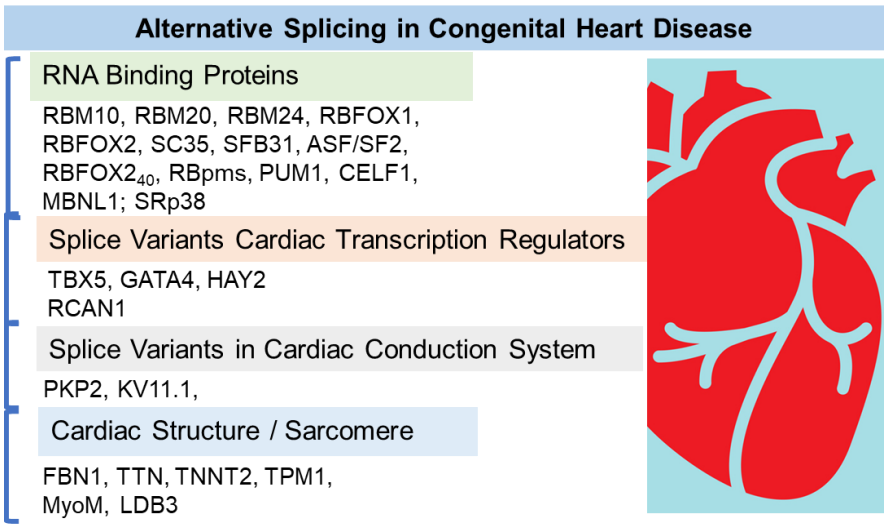

Figure 3. Graphical Summary of Topics Highlighted in the Review. TOP cardiac enriched genes that are members of RNA binding proteins, and other cardiac genes that are affected by alternative splicing or splicing variants are listed based on their functional categories.

Bicuspid aortic valve (BAV) is a common CHD affecting 0.5–2.0% of the general population and associated with risks for aortic dilatation and dissection. Using Affymetrix exon arrays, Fibronectin (FN) splicing isoforms were

analyzed in dilated and nondilated ascending aorta from human BAV samples (n-69) and normal tricuspid aortic valve (TAV) samples (n=40). An alternatively spliced extra domain A of FN (EDA-FN), which is essential for tissue repair, was found to be correlated with the maximum diameter of TAV, but did not increase in dilated aorta tissues from BAV [70]. Remarkably, transforming growth factor-β (TGFβ) treatment increased EDA-FN isoform expression in cultured cells derived from TAV patients but not in cells derived from BAV patients. Together, this indicates that differences in the TGFβ signaling pathway may explain the impaired inclusion of EDA-FN in BAV patients.

Hypoplastic left heart syndrome (HLHS) is a serious and complex form of CHD, characterized by left ventricle hypoplasia and single ventricle physiology, with compensatory hypertrophy and hemodynamic overload of the right ventricle (RV). Using genome-wide Affymetrix exon array, an extensive transcriptome analysis, including characterizing alternative splicing profiles, of the RV of six HLHS patients and the RV and left ventricle (LV) from control subjects, revealed distinct differential gene expression and alternative splicing events in the RV myocardium of HLHS patients compared to RV and LV from the control heart, representing a unique molecular signature of HLHS, involving 180 differentially expressed genes and 1800 differentially spliced transcripts that were enriched in cell metabolism, cytoskeleton, and cell adhesion. Furthermore, some dysregulated genes can serve as molecular biomarkers for prognostication classification. Such examples include those involved in calcium transporters (SLC8A1 and CACNB2) and energy production (COX4l1and ATP4A1) as well as secreted factors (IFI44 and VEGFA) that can be quantified in plasma samples [71].

Tetralogy of Fallot (TOF) is the most common cyanotic CHD phenotype that is typically associated with significant remodeling of the RV outflow tract (RVOT) due to pulmonary stenosis. By characterizing alternative splicing in RVOT specimens obtained from TOF patients, the small cajal body-specific RNA 1 (scaRNA1) was found to be downregulated in TOF-RVOTs. Mechanistically, scaRNA1 loss in primary cultured cells derived from RV from TOF patients dysregulated splicing important regulators of heart development, including GATA4, NOTCH2, DICER1, MBNL 1, and MBNL2. Mis-splicing of the cardiac development genes was also observed in Zebrafish in response to morpholino-mediated silencing of scaRNA1 and was associated with decreased pseudouridylation

in Spliceosomal RNA U2, potentially leading to reduced communication between the first and second heart fields and conotruncal misalignment, the hallmark in TOF [72].

Ventricular noncompaction is characterized by abnormal ventricular trabeculation and progressive cardiac dysfunction. The genetic cause of this disorder remains elusive. RNA-binding protein with multiple splicing (Rbpms) is highly expressed in the heart and contains a conserved RNA recognition motif (RRM) [73]. A recent report [74] demonstrated that genetic deletion of a novel Rbp with multiple slicing (Rbpms) leads to early lethality in neonatal mice caused by CHDs. Mechanistically, the Rbpms depleted cardiomyocytes undergo early exit from the cell cycle demonstrated by premature binucleation. This cytokinesis defect was also observed in human iPSCs-derived cardiomyocytes that carry RBPMS gene deletion, and found to be associated with dysregulated RNA splicing of genes enriched in cytoskeletal signaling pathways, including the cardiac enriched LIM domain protein 5 (Pdlim5). Specifically, the Rbpms loss resulted in a substantial increase in Pdlim5-short isoforms that impaired cardiomyocyte division and premature binucleation leading to ventricular noncompaction.

DiGeorge syndrome results from a micro-deletion located on the short arm of chromosome 22, which includes the HIRA gene and is commonly associated with TOF and other CHDs. It was shown that an intronic sequence (22k48) transcribed by HIRA opposite strand, but not translated, undergoes alternative splicing [75]. Importantly, haploinsufficiency of this intronic sequence may lead to the stigmata of DiGeorge syndrome, including CHDs indicating that the noncoding intron retention mechanism of splicing can potentially exert pathogenic impacts in CHDs.

II. Role of Pathogenic Variants in RBPs in CHDs

Evidence from human genetics and mouse models has implicated pathogenic variants in RBPs in CHDs by impairing the splicing of their target genes [Table 2]. For example, pathogenic variants in RBM10, a ubiquity expressed RBP, are known causal of TARP (**T**alipes equinovarus, **A**trial septal defect, **R**obin sequence, and **P**ersistent left superior vena cava) syndrome, an X-linked disorder that affects males [76-78]. Pathogenic variants in RBFOX2 have been implicated in HLHS [79]. Furthermore, RBFOX2 may contribute to transcriptome

dysregulation in RVs from HLHS patients [80]. Indeed, it was demonstrated that the majority of the transcripts that are differentially regulated in HLHS compared to control are targets for RBFOX2 with 3'UTR binding sites contributing to mis-splicing [80]. Moreover, conditional deletion of Rbfox2 in mouse embryos led to perturbation of Yolk sac angiogenesis and complex heart defects recapitulating several features of HLHS. Remarkably, Rbfox2 mutant heart-derived transcriptomes analysis identified dysregulated alternative splicing affecting extracellular matrix (ECM) and cellular adhesion networks. This was found to be mediated by Rho GTPases, two of which were identified as targets for Rbfox2 [59]. By affecting the splicing of their downstream sarcomere gene products, such as Titin, pathogenic RBM20 variants can cause human arrhythmogenic dilated cardiomyopathies (DCMs) and cardiac sudden death by disrupting Ca^{2+} handling [63, 81]. However, no disease-associated RBM24 variants have been described to date.

Table 2: Genes Affected by Splicing Variant and Their Contribution to CHDs

Author. Date	Journal	Gene	Variant	Clinical Phenotype
Wang Y et al. 2013	EMBO Mol Med	RBM10	Del (ChrX: 46929367–46930658 bp)	TARP Syndrome/CHD
Tessier SJ et al. 2015	BMC Res Notes	RBM10	tandem donor splice site (GTGGTG) in RBM10 exon 10	TARP Syndrome/CHD
Fan C et al. 2009	J Biol Chem	TBX5/SC35	G80R	Holt -Oram syndrome/CHD
Fusco C et al. 2017	Genes	FBN1	c.6872-24T>A; c.7571-12T>A	Mafan's Syndrome/CHD
Awad MM et al. 2006	Hum Mutat	PKP2	c.2484C>T+ c.2484C>T	ARVD*
Gong Q et al. 2014	Circ Cardiovasc Genet	KV11.1	IVS9-2delA	Long QT Syndrome
Fardoun M et al. 2019	Pediatr Cardiol	HAY2	p.161_190 del	Nonsyndromic VSD
Li X et al. 2018	Pediatr Cardiol	RCAN1	g.482G>T	Nonsyndromic CHD
Bose D et al. 2017	Mutat Res	GATA4	g.83271C>A/M; g.86268A>R	Nonsyndromic CHD
Guo et al . 2012	Nat Med	Rbm20	S635A	Dilated Cardiomyopathy
Refaat et al. 2011	Heart Rhythm	Rbm20	rs942077 and rs35141404	Dilated Cardiomyopathy
Beqqali A et al. 2016	Cardiovascular Res	Rbm20	RBM20 $^{E913K/+}$	Dilated Cardiomyopathy

Among the key core transcription factors in heart development is TBX5, a T-box transcription factor that is required for heart and limb morphogenesis. Pathogenic variants in *TBX5* are a known cause of Holt-Oram syndrome featured by CHDs and forelimb maldevelopment. Other than the known role of TBX5 in transcription regulation, it was also demonstrated that TBX5 plays a role in pre-mRNA splicing, via the forming a complex with the splicing factor SC35, an SR protein that has been shown to be an essential splicing factor involved in spliceosome assembly and as a regulator of alternative splicing [82]. Specifically, TBX5 acted as an RBP with

high specificity to the 5'ss of the ANF minigene but not to the 3'ss. Intriguingly, TBX5 overexpression improved splice site definition and enhanced splicing efficiency of ANF mediated by SC35. Moreover, the pathogenic variant (G80R) that affects TBX5 splicing performance is directly linked to the pathogenesis of Holt-Oram syndrome and featured with complete penetrance of CHDs due to significant mis-splicing of mRNA. In contrast, other variants that do not affect TBX5 splicing performance tend to have incomplete penetrance in CHDs [82].

III. Role of Splicing Site Variants in CHDs

Advances in genomics platforms have improved the detection of rare pathogenic splicing genomic variants that alter canonical splice sites, thus resulting in splicing and functional defects of their genes or impacting their promoter activity and regulatory elements. Nonetheless, establishing the causal roles of these variants and identifying their downstream targets in CHDs remain challenging. Few reports have described pathogenic splicing variants in key cardiac transcription regulators or structural proteins leading to developmental perturbation and CHDs [Table 2]. GATA4, which encodes GATA Binding Protein 4, is a key transcription factor that plays an important role in cardiac development and has been implicated in CHDs. By performing variant analysis of the GATA4 gene in Indian patients with CHDs, two intronic splice site variants ([g.83271C>A/M] and [g.86268A>R]) were predicted to affect intronic splice sites at enhancer and silencer motifs based on *in silico* prediction analysis, indicating that noncoding pathogenic splicing variant can introduce splicing defects leading to CHDs [83].

Splicing variants of key regulatory genes may contribute to CHDs. One example is the Regulator of Calcineurin 1 (RCAN1), which has been linked to CHDs associated with Down syndrome. In order to examine if RCAN1 contributes to non-syndromic cases of CHDs, *RCAN1* exons and flanking regions from 128 patients with nonsyndromic CHD and 150 normal controls were sequenced leading to the detection of six novel heterozygous variants in RCAN1 gene in CHD cases, but were absent in control cases. In particular, the g.482G>T variant was found to enhance RCAN1 promoter activity leading to overexpression of RCAN1.4 isoform, potentially causing CHDs in the absence of Down syndrome [84].

Pathogenic splicing variants in genes encoding structural proteins have also been implicated in CHDs. Encoded by *FBN1*, Fibrillin 1 is a key constituent of ECM. Pathogenic *FBN1* variants are linked to Marfan's syndrome (MFS) and mitral valve-aorta-skeleton-skin (MASS) syndrome. However, *FBN1* variants are mostly enriched in intronic sequences, making the prediction of their pathogenicity and establishing the genotype-phenotype correlations very challenging. A recent study [**85**] reported two *FBN1* deep intronic variants ([c.6872-24T>A] and [c.7571-12T>A]) in two unrelated patients who were affected with MFS/aortic disease and MASS syndrome respectively. Remarkably, both variants led to the retention of intronic regions, resulting in changes in the reading frame and the introduction of premature stop codons. Pathogenic variants of RBPs and splice site variants in CHDs are summarized in [**Table 2**].

III. Splicing Variants Leading to Congenital Conduction Defects (Arrhythmias)

Pathogenic splicing variants may affect ion channel assembly and function, leading to developmental defects in conduction. It has been shown that potassium channel (Kv11.1) isoform switch represents a novel mechanism of congenital long-QT syndrome. A novel splice site variant in KCNH2, which encodes Kv11.1 was detected in an expanded family, affecting the relative abundance of full-length Kv11.1a isoform and the truncated Kv11.1a-USO isoform. This in turn was dictated by the competition between KCNH2 alternative splicing and alternative polyadenylation mechanisms [**86**]. Splicing defects may also impair voltage-gated sodium channels. A recent report demonstrated that a non-muscle isoform of RBFOX2 [$RBFOX2_{40}$] is upregulated in heart tissue from myotonic dystrophy 1 (DM1) patients leading to elevated CELF1 and miRNA suppression [**23**]. By modeling in mice, $Rbfox2_{40}$ isoform overexpression caused mis-splicing of voltage-gated sodium channel transcripts creating a pro-arrhythmic status that altered channel electrical properties leading to conduction defects.

Arrhythmogenic right ventricle dysplasia (ARVD) is a rare inherited disorder that involves the replacement of RV cardiomyocytes with fibro adipose tissues that consequently lead to ventricular arrhythmias. ARVD cases with dominant inheritance and incomplete penetrance are caused by heterozygous PKP2 mutations. Interestingly, the first reported ARVD case with recessive inheritance was caused by a homozygous cryptic splice variant in PKP2 (c.2484C>T), which was initially annotated as a synonymous variant [**87**]. However, further analysis of the

proband's mRNA uncovered the disruption of the PKP2 reading frame and alteration of PKP2 splicing outcomes caused by this cryptic splice site variant leading.

CONCLUSIONS

Alternative splicing is a ubiquitous process that plays important roles in transcriptome regulation and proteome diversity. Current literature evidence supports the important regulatory roles of alternative splicing in cardiovascular development and CHDs. Splicing transition is controlled by a complex and intricate network of RBPs, which orchestrate splicing transition of their targets during heart development and can be dysregulated in CHDs. Pathogenic variants of RBPs may alter splicing decisions of their targets and account for substantial developmental perturbation leading to CHDs. Pathogenic splicing variants of key core cardiac transcription factors and structural genes can be causal of CHDs. Research efforts tailored to examining alternative splicing regulation during development and RBPs dysregulation in CHDs may uncover novel mechanisms of CHDs. In particular, elucidating the pathological impacts of splicing variants may pave the way to novel diagnostic and targeted approaches for CHDs.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable

AVAILABILITY OF DATA AND MATERIALS

Not applicable

COMPETING INTERESTS

No Competing Interests. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

FUNDING

This work was supported by the 2021 UCLA Broad Stem Cell Research Center (BSCRC) T32 Training grant (ZM), and grants from NIH/NHLBI 1R01 HL153853-01 (MT), the Department of Defense-Congressionally Directed Medical Research Programs W81XWH-18-1-0164 (MT), and the 2022 UCLA Academic Senate Research Recovery Fund (MT).

AUTHOR CONTRIBUTIONS

Authors' Contribution: ZM collected references, generated figures and participated in manuscript writing and editing. MT conceived and designed the manuscript, participated in manuscript writing and editing.

ACKNOWLEDGMENTS

Not applicable

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