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CELF1, PTBP1, and RBFOX2-mediated alternative splicing regulation in cardiovascular diseases

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CELF1, PTBP1, and RBFOX2-mediated alternative splicing regulation in cardiovascular diseases

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch

May, 2019

Acknowledgements

First, I would like to thank my mentor Dr. Muge Kuyumcu-Martinez for her guidance and support throughout my graduate studies. She has helped me grow as a scientist and discover my passion for writing. Second, I would like to thank my committee members Dr. Andrew Routh, Dr. Eric Wagner, Dr. Shelton Bradrick, and Dr. Joel Neilson. Their valuable feedback helped shape my research and scientific development. Special thanks are due to Dr. James Lee and Dr. Eric Wagner who in their respective roles as Directors of the Biochemistry and Molecular Biology graduate program were influential in my graduate career success. They offered me support and believed in me when I needed it most. I would also like to thank the members of the Kuyumcu-Martinez laboratory, Dr. Sunil Verma and Dr. Jun Cao. I am grateful for the training they provided me and their insightful feedback on my work.

CELF1, PTBP1, and RBFOX2-mediated alternative splicing regulation in cardiovascular diseases

Publication No._____

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The University of Texas Medical Branch, 2019

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Alternative splicing (AS) is dysregulated in Type 1 diabetic (T1D) and hypoplastic left heart syndrome (HLHS) patient hearts but the mechanisms responsible are unclear. Here, we provide evidence that in these patient's hearts that dysregulation of the RNA binding proteins (RBPs) CELF1, PTBP1, and RBFOX2 contribute to AS changes. Utilizing genome-wide approaches, we identified extensive changes in AS patterns in T1D mouse hearts. We discovered that many aberrantly spliced genes in T1D hearts have CELF1 and PTBP1 binding sites. CELF1-regulated AS affects key genes within signaling pathways relevant to diabetes pathogenesis. Disruption of CELF1 binding sites impairs AS regulation by CELF1. We show that a spliced variant of PTBP1 that is highly expressed in normal newborn mouse hearts is aberrantly expressed in adult T1D mouse hearts. We also demonstrated that inducible expression of diabetes-induced PTBP1 spliced variant has less repressive splicing function. Notably, PTBP1 antagonizes RBFOX2-mediated AS of selected mRNA targets in this context. In summary, our results indicate that CELF1 and

PTBP1 target RNAs are aberrantly spliced in the T1D heart, leading to abnormal gene expression.

RBFOX2 is significantly associated with HLHS. In HLHS, three damaging *de novo* RBFOX2 mutations (nonsense, frameshift, and splice site) have been identified. Here we provide evidence that the nonsense and frameshift RBFOX2 mutants are unable to promote proper splicing of their target genes despite normal subcellular localization of these mutants. Further, we show that the nonsense mutant interacts with a subset of proteins that wildtype RBFOX2 does not interact with, indicating that this nonsense mutant may constitute a gain of function. These discoveries pave the way for targeting RBPs and their RNA networks as novel therapies for cardiac complications of diabetes and HLHS.

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A3SS	Alternative 3' splice site
A5SS	Alternative 5' splice site
AFE	Alternative first exon
ALE	Alternative last exon
ANOVA	Analysis of variance
AS	Alternative splicing
BP	Binding protein
CELF	CUG binding protein embryonically lethal abnormal vision-like
cFLIP	caspase inhibitor FLICE-like protein
CHD	Congenital heart defect
CKO	Conditional knockout
CLIP-seq	Cross-linking immunoprecipitation followed by RNA-seq
CTD	C-terminal domain
cTNT	Cardiac troponin T
CX43	Connexin 43
DAB1	Disabled homologue 1
DAS	Differential alternative splicing
DAVID	Database for annotation, visualization, and integrated discovery
DEG	Differentially expressed gene
DES	Differential gene expression
DM1	Myotonic dystrophy type 1

DMEM	Dulbecco's Modified Eagle Medium
DMPK	Dystrophia myotonia protein kinase
DN	Dominant negative
DNA	Deoxyribonucleic acid
E18	Embryonic day 18
EC	Excitation-contraction
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
ES	Cassette exon
ESS	Exonic splicing silencer
FBS	Fetal bovine serum
FDR	False discovery rate
FGF	Fibroblast growth factor
FGFR2c	Fibroblast growth factor receptor 2c
FHL2	Four and a half LIM domain-containing protein 2
FS	Frameshift
FXR1P	Fragile X related 1 protein
GFP	Green fluorescence protein
GO	Gene ontology
GRE	GU-rich element
HDAC	histone deacetylase
HDAC7	Histone deacetylase 7
HLHS	Hypoplastic left heart syndrome

hnRNP	heterogeneous ribonucleoprotein
HOPX	Homeodomain only protein X
IF	Immunofluorescence
IgG	Immunoglobulin G
IP	Immunoprecipitation
IR	Intron retention
IRES	Internal ribosome entry site
ISE	Intronic splicing enhancer
ISS	Intronic splicing silencer
LASR	Large assembly of splicing regulators
LV	Left ventricle
ME	Mutually exclusive
NanoLC-MS/MS	Nanoflow liquid chromatography-tandem mass spectrometry
NB	Newborn
NLS	Nuclear localization signal
NMD	Nonsense mediated decay
NOD	Non-obese diabetic
NOVA2	Neuro-oncological ventral antigen 2
NS	Nonsense
NTD	N-terminal domain
OFT	Outflow tract
PBX	Pre-B cell leukemia transcription homeobox
PKC	Protein kinase C

PKG	Protein kinase G
PRC2	Polycomb complex 2
PSI	Percent spliced in
PTBP1	Polypyrmidine tract binding protein 1
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RBFOX	RNA binding protein fox-1 homolog
RBP	RNA binding protein
RNP	Ribonucleoprotein
RRM	RNA recognition motif
RT	Room temperature
RV	Right ventricle
SF1	Splicing factor 1
SLC2A4	Solute carrier family 2 member 4
snRNPs	Small nuclear ribonucleoprotein particles
SRF	Serum response factor
SS	Splice site
STZ	Streptozotocin
SXL	Sex-lethal
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TAC	Transverse aortic constriction
Tmem184b	Transmembrane protein 184b
TPM1	α-tropomyosin

TPM2	β-tropomyosin
U2AF	U2 auxiliary factor
UTR	Untranslated region
WB	Western blot
WT	Wildtype
ZF	Zinc finger

Chapter 1: Introduction

My dissertation focuses on three RNA binding proteins (RBPs): CELF1, PTBP1, and RBFOX2, all of which are involved in alternative splicing (AS). These AS regulators are important for heart development and contribute to dysregulated cardiac gene expression in a number of cardiovascular diseases including hypoplastic left heart syndrome (HLHS) and diabetic heart disease. Examining the function of CELF1, PTBP1, and RBFOX2 in heart development and disease can provide insights into their emerging roles in HLHS and diabetic heart disease.

RNA splicing and alternative splicing regulation

Pre-mRNA splicing

RNA splicing is a process that removes introns from pre-mRNA and joins together the exons [2]. The process involves a stepwise assembly of small nuclear ribonucleoprotein particles (snRNPs) and accessory splicing factors that make up the spliceosome. The initial step in RNA splicing involves U1 snRNP recognizing the 5' splice site (SS), splicing factor 1 (SF1) binding to the branch point, and U2 auxiliary factor (U2AF) binding to the 3' SS and polypyrimidine tract to form complex E. U2AF recruits the U2 snRNP to form the pre-spliceosome complex A. Subsequently, the U4, U6, and U5 snRNPs are recruited together to form the immature spliceosome complex B [3]. Then the U1 and U4 snRNP are released while the U6 snRNP associates with the 5' SS and U2 snRNP to form the catalytically active spliceosome, complex C, that goes on to perform two transesterification reactions.

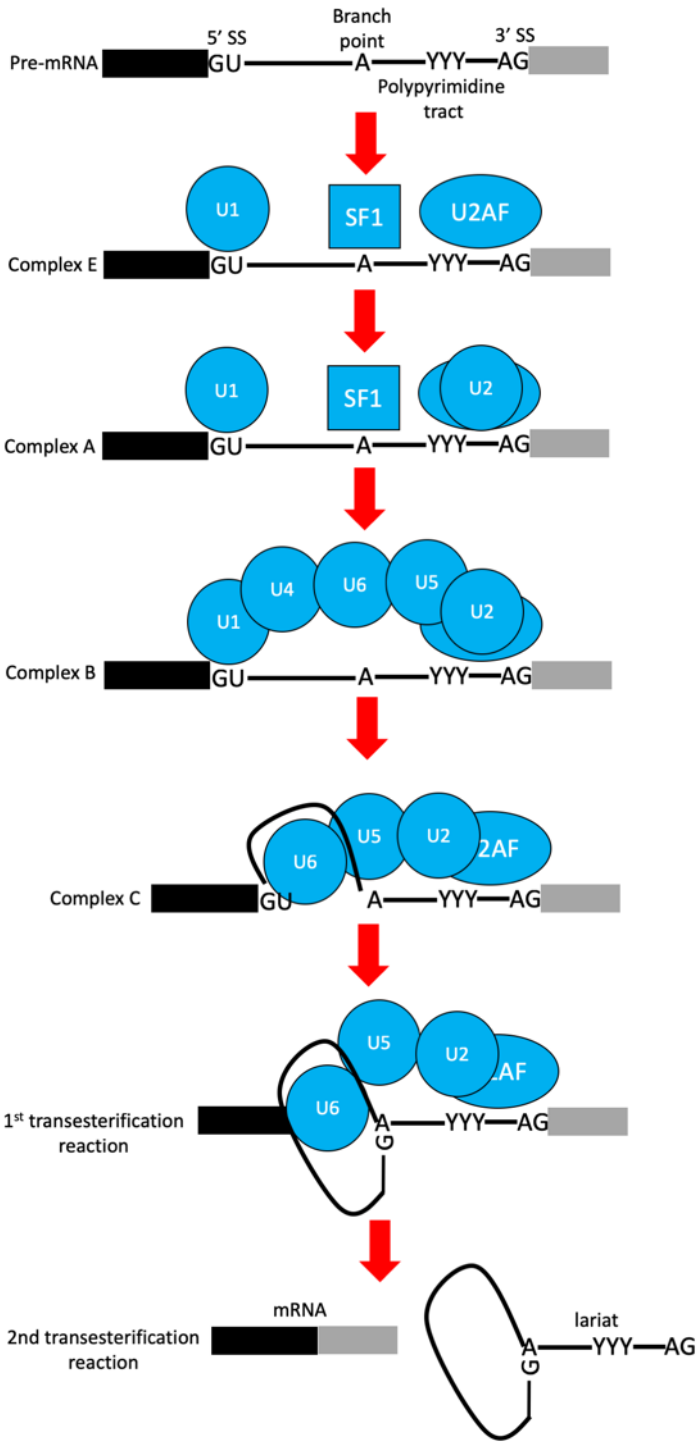

Illustration 1. RNA splicing mechanism. Process of RNA splicing highlighting the important snRNPs involved, the complex rearrangements, and transesterification reactions. Modified from [1].

During the first reaction, nucleophilic attack of the branch site A to the 5' SS G leads to removal of the 5' end of the intron. The second reaction removes the intron releasing it as a lariat and joins together the 5' and 3' ends of the exons to make mRNA that can go on to be translated (Illustration 1) [1, 2, 4].

Alternative splicing

Mammalian splice sites contain conserved splice sites [5] that are marked by a 5' GU and a 3' AG sequence [1]. Exons that are easily recognized by the spliceosome due to the presence of these highly conserved splice sites are constitutively spliced (Illustration 2). On the other hand, alternative exons characterized by the presence of weak splice sites are not readily

recognized by the spliceosome, so they undergo AS. AS allows for the formation of multiple transcripts by selective inclusion or exclusion of alternative exons and introns, thereby increasing proteome diversity [6-9]. Alternative splicing helps explain the discrepancy between the number of human protein coding genes (24,000) and the number of different proteins (100,000) produced [10]. There are seven types of alternative splicing including alternative first exon (AFE), alternative last exon (ALE), cassette exon, mutually exclusive (MXE), alternative 5' splice site (A5SS), alternative 3' splice site (A3SS), and intron retention (Illustration 2) [11, 12]. Cassette

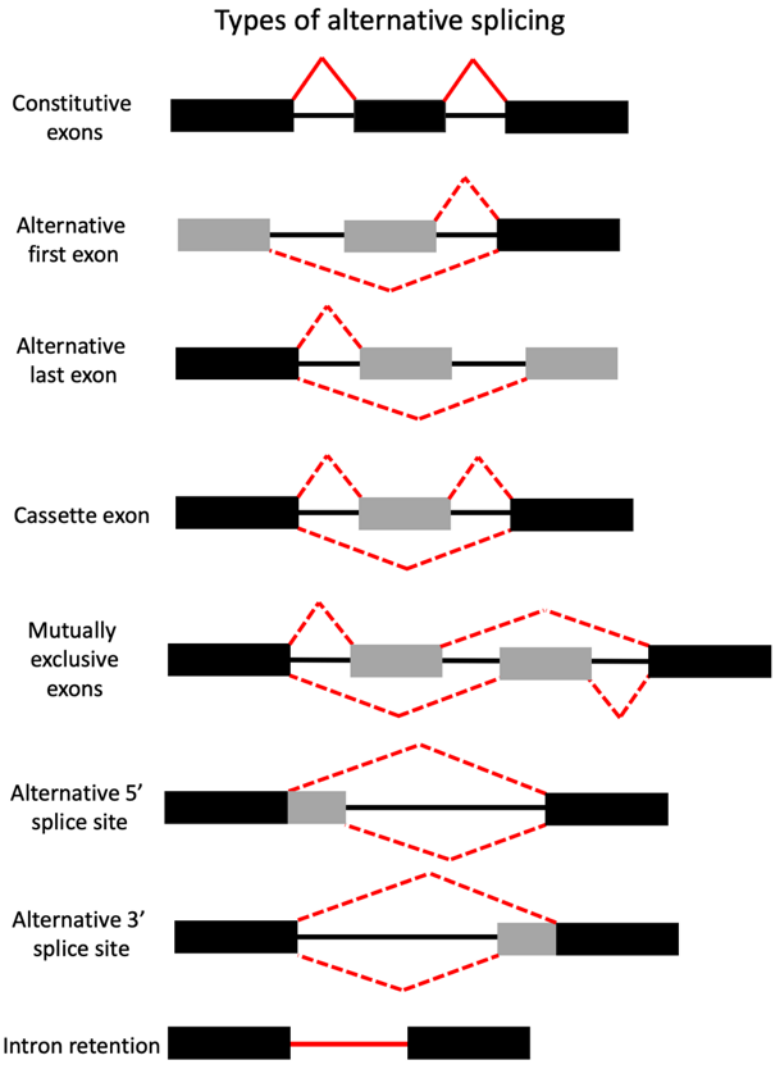

Illustration 2. Seven basic modes of alternative splicing. Black boxes indicate constitutive exons and grey boxes indicate alternative exons. Solid red lines indicate constitutive splicing while dashed red lines indicate alternative splicing. Black lines are spliced intron. Red line represents a retained intron.

exons are the most common type of AS and account for at least one-third of alternative exons [13].

AS has been hypothesized to underlie to the evolution of organism complexity [14]. Surprisingly, there is a lack of correlation between the number of genes in an organism and its complexity. For example, C. *elegans* have more genes than *Drosophila* but the former organism is considered less complex than the latter. AS can help account for this discrepancy [15]. In higher eukaryotes, AS is believed to contribute to cellular complexity [13, 16, 17]. However, incidence of AS throughout the phylogenetic tree is generally correlated with the stage of evolution. Importantly, most higher-level eukaryotes use AS while many lower level eukaryotes do not. Additionally, the number of genes and exons that undergo AS is more abundant in higher-level eukaryotes. The ancient AS mechanism used is termed intron definition. This process involves the splicing machinery being assembled across introns. In higher eukaryotes, the mechanism is known as exon definition and involves the splicing machinery being assembled across exons. Intron definition may have existed to keep introns short while exon definition likely constrains the length of exons. This is reflected in the fact that lower eukaryotes have short introns and long exons while vertebrates have long introns and short exons [18]. Exon length is very important for AS. A short exon is more likely to be included than a large exon [19]. Interestingly, during evolution exon length has decreased [20].

Further, AS is associated with the recent creation or loss of an exon [21, 22]. Therefore, young exons are more often alternatively spliced. There are two ways to create new exons including duplication and exonization of part of the intron [15, 23]. Creating new exons is a major route for increasing functional diversity throughout evolution [15].

The different types of AS also vary across species. For instance, intron retention is common in metazoans, fungi, and protozoa while exon skipping is more common in higher eukaryotes [24]. This indicates that exon skipping may be the type of AS that contributes the most to cellular complexity in higher eukaryotes.

Nearly 95% of all human multi-exon genes are alternatively spliced [25, 26]. Wang et al. analyzed alternative splicing in 15 different human tissues and cell types. They performed deep sequencing of the cDNA to identify the gene and mRNA isoforms. Looking at the reads that mapped to exon-exon junctions, they estimate that 92-94% of human genes are alternatively spliced. They looked at breast cell lines and different human tissues and found that the cell lines clustered separately from the tissue in regard to which mRNAs were alternatively spliced. This indicates that AS is regulated independently between cells and tissues. Further, the pattern of AS was correlated between tissues indicating an overarching coordination in the regulation of these patterns [25].

An additional study by Pan et al. further supports that nearly 95% of all human multi-exon genes are alternatively spliced. They used the Illumina Genome Analyzer system to analyze splicing complexity from mRNA sequencing data and EST-cDNA sequencing data in healthy human tissues including whole brain, cerebral cortex, skeletal muscle, heart, lung, and liver. Using genes with similar sequencing read densities and different number of exons, they determined the median number of AS events per exon in these genes. This study found a linear relationship between the number of exons in a gene and the number of AS events. They estimate that each human multi-exon gene has at least 7 AS events. Further they estimate there is about 100,000 alternative transcripts in human tissues. Finally, they estimate that 92-97% of human multi-exon genes undergo AS [27].

These two studies provide support that nearly 95% or human multi-exons genes undergo AS.

In addition to increasing proteome diversity, AS is important as it regulates development. Splicing of multiples genes at the same time is coordinated during development and changes in alternatively spliced isoforms give rise to different adult tissues and function [28]. For example, the disabled homologue 1 (*DAB1*) gene undergoes AS during brain development. In the embryonic brain DAB1 is present as a short isoform lacking exons 7b and 7c. However, these exons become included in the adult brain. The shorter DAB1 isoform is important for regulation of neuronal migration through an incompletely understood mechanism involving regulation of reelin signaling. When the DAB1 long isoform is expressed it antagonizes the DAB1 short isoform and thus induces neuronal migration defects. Neuronal migration is important for proper brain development into a highly organized laminar structure. Developmental of this structure occurs through coordinated waves of neuronal migration [29]. Importantly, misregulation of neuronal migration can lead to cognitive defects and lissencephaly. DAB1 AS is regulated by the RBP neuro-oncological ventral antigen 2 (NOVA2). When NOVA2 is present it induces the short DAB1 isoform, but when it is absent it induces the long DAB1 isoform [28, 29]. This is just one example of how critical AS is for development and highlights how dysregulated AS can lead to disease.

A classic example of how AS contributes to development is the sex determination in *Drosophila*. The *sex-lethal* (*Sxl*) gene determines sex in *Drosophila*. When *Sxl* is activated it induces female development. When *Sxl* is inactive, male development takes place. *Sxl* encodes an RNA binding protein that controls splicing, translation, and stability

of downstream genes that contribute to female or male development [30]. Further, the doublesex (*DSX*) gene in somatic cells of *Drosophila* is alternatively spliced giving rise to female and male isoforms. The two different isoforms vary at their C-terminal domain (CTD) which ultimately affects their function as a transcription factor. The male DSX isoform represses female differentiation genes, while the female DSX isoform represses some male differentiation genes. These isoforms ultimately contribute to the sexual differences in *Drosophila* including pigmentation patterns, sex specific genitals, and the sex comb [31]. This classic example highlights how important alternative splicing is in control of development.

AS is tissue specific and occurs more frequently in transcripts expressed in complex tissues such as the brain and testis [32, 33]. Further, cells with more complex functions, such as immune cells, also display more AS [34]. Tissue specific AS is partially controlled by the differential expression of splicing regulators, including RBPs, and the downstream regulation of their corresponding transcripts [6, 35]. Wang et al. found a high frequency of tissue specificity for all the major types of AS. 72% of skipped exons were tissue regulated, as were 71% of retained introns, 72% of A5SS, 74% of A3SS, 66% of MXE, 63% of AFE, 52% of ALE, and 80% of tandem 3' UTRs. Importantly, this indicates that almost all AS events displayed tissue specificity. Overall, they found that there were 22,000 tissue-specific alternative transcripts in humans [25]. They also looked at whether these differences were due to tissue-specific or individual-specific variation and found that most of the differences were due to tissue-specific variation. Only 10-30% of alternative transcripts showed individual-specific variation [25]. This is consistent with previous reports that about 21% of alternative transcripts are affected by polymorphisms that

contribute to the relative expression differences among individuals [36]. This work indicates that tissue-specific AS is very common and more prominent than individual-specific variation.

Important for this work, the human heart is composed of several distinct cell types including fibroblasts, cardiomyocytes, endothelial cells, and vascular smooth muscle cells. The two primary cell populations are cardiomyocytes, which account for 30% of the heart, and fibroblasts, which account for 66% of the cells found in the heart [37-40]. Cardiomyocytes and fibroblasts have different functions in the heart. Cardiomyocytes generate contractile force in the heart and control rhythmic beating through regulation of calcium signaling [41]. Fibroblasts contribute to the formation of the extracellular matrix (ECM) and act as a mechanical scaffold necessary for pumping [42, 43]. During heart development, 90% of the AS and gene expression changes in cardiomyocytes are completed by postnatal day 28. In contrast, almost 50% of AS and gene expression changes in fibroblasts occur after postnatal day 28 [40]. These data are consistent with the fact that cardiomyocytes stop proliferating by postnatal day 7 [44, 45]. During development, cardiomyocytes and fibroblasts undergo reciprocal changes in proliferation, cell adhesion, metabolism, transcription, and chemotaxis [40]. For example, solute carrier family 2 member 4 facilitates glucose (*Slc2a4*), a gene involved in mitochondrial metabolism, decreases 2-fold during fibroblast development when the levels at postnatal days 1-3 and at adult maturity were compared. In contrast, Slc2a4 increases 15-fold during cardiomyocyte development. During postnatal development, a greater number of AS events occur in cardiomyocytes (809) than in fibroblasts (326) [40]. Postnatal cardiomyocyte-specific splicing events regulate genes related to membrane organization

and vesicular trafficking, and two RBP families, CELF and MBNL, have been identified as regulators of these changes. More specifically, the family members CELF1 and MBNL1 regulate AS that is important for calcium handling, vesicular trafficking, and transverse tubule organization [40]. However, the direct roles of these two proteins in these processes needs to be further investigated.

Up to this point, I have described how AS is important, including how AS increases proteome diversity by expanding the number of proteins created from a limited number of genes. AS has also contributed to evolutionary complexity throughout the phylogenetic tree. Finally, AS has critical roles in development and tissue specificity. It is clear that AS is an important biological process so it is important to touch upon how this process is regulated.

Alternative splicing regulation

Alternative splicing is regulated by *trans*-acting factors and *cis*-acting sequences that mediate the localized assembly of the splicing machinery [46]. Recognition of the splice sites requires *cis*-acting sequences and the splicing machinery, including general splicing factors, accessory proteins, and regulatory proteins. There are four categories of *cis*-acting sequences including intronic and exonic splicing enhancers and silencers. Trans-acting factors such as RBPs enhance splice site recognition by binding to *cis*-acting sequences and recruiting the necessary spliceosome components including U1 snRNP and U2 snRNP. *Trans*-acting factors can also inhibit splice site recognition by blocking splicing machinery or preventing activators from interacting with enhancer sequences [12, 28, 47].

To regulate AS, RBPs bind to specific RNA sequences through several distinct RNA-binding domains. Among the RNA-binding domains found in nature, the most

common are the RNA recognition motif (RRM), the C3H1 zinc-finger (ZF) domain, and the heterogeneous ribonucleoprotein (hnRNP) K-homology domain [48-50]. RBPs with the same RNA-binding domain do not necessarily bind the same RNA sequence. RBPs have additional auxiliary functional domains that mediate interaction with other proteins and further diversify RBP binding and function. The various binding activities and protein interactions of RBPs give rise to unique ribonucleoprotein (RNP) compositions that differentially modulate downstream RNA metabolism [51].

Much work has been directed towards the elucidation of a "splicing code," or a general set of rules to predict AS based on regulation by *cis*- and *trans*-acting factors. The code is complicated by the fact that AS decisions are tissue- and development-specific and reflect the combinatorial control of the various RBPs found within the cell [52-55]. Further, RBPs can compete for binding to the same sequence motif, thereby contributing another layer of regulation. Therefore, recognition of specific sequence motifs by an RBP can vary by tissue [50, 56].

However, substantial research has begun to decipher the splicing code. RBPs act as trans-acting factors that regulate AS when they bind to *cis*-regulatory elements that are close to splice sites. Importantly, RBPs can both positively and negatively regulate AS, and the outcome is often position specific. The general splicing code suggests a rule where sequence specific RBPs promote inclusion of the alternative exon when they bind to the downstream intron. Conversely, RBPs promote exclusion of the alternative exon when they bind to the upstream intron (Illustration 3) [50]. However, predictions of splicing based on the general splicing code are still of limited practical utility because exons are usually under the control of multiple RBPs at the same time. Therefore, evaluating the binding of an

individual RBP in isolation is an oversimplification of a complex and tightly regulated process. Analysis that includes *cis*-acting factors and RBP concentration as additional regulating components is needed to make accurate splicing predictions [50, 52, 55, 57].

Illustration 3. RBP splicing code.
Grey boxes indicate alternative exons. When RBPs bind downstream of an alternative exon they are predicted to promote inclusion. When they bind upstream of an exon they are predicted to promote exclusion

Alternative splicing regulator CELF1

CELF1 belongs to the evolutionary conserved CUG binding protein (BP) and embryonically lethal abnormal vision-like (CELF) family of RBPs. This family includes six members (CELF1-6). CELF1 and CELF2 are expressed in the heart, skeletal muscle, and brain, while CELF3-6 are specific to the nervous system [58].

The CELF proteins control gene expression at the post-transcriptional level by regulating AS and polyadenylation in the nucleus, and mRNA translation and decay in the cytoplasm. To regulate these processes, CELF proteins bind to the consensus motif UGU(G/A) [59]. CELF was originally identified to bind CUG repeats but was later found to preferentially bind to GU-rich elements (GRE) in the RNA [60, 61]. CELF1 regulates AS in a position-dependent manner. When CELF1 binds within 250 nucleotides upstream of an alternative exon, it represses exon inclusion, and when it binds within 250 nucleotides downstream of an alternative exon, it promotes exon inclusion (Illustration 4) [62, 63].

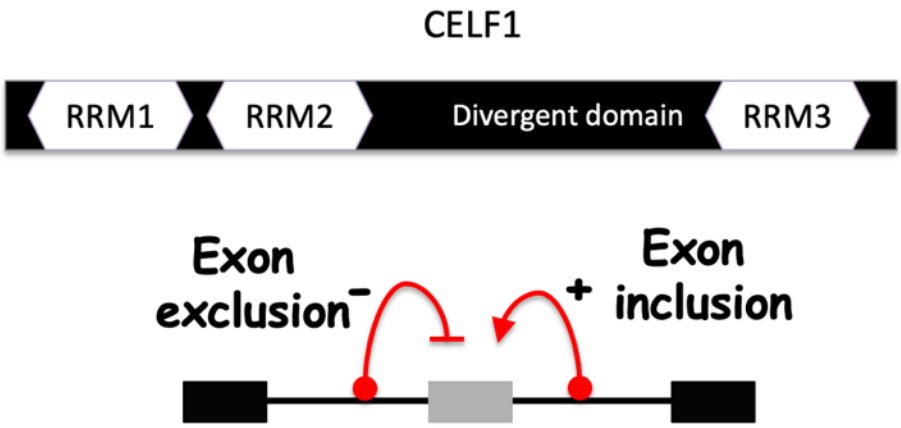

Illustration 4. Schematic representation of CELF1 domain organization and position dependent alternative splicing.
When CELF1 binds upstream of an alternative exon it promotes exclusion and when it binds downstream it promotes inclusion.

CELF1 has a total of three RRMs (Illustration 4). Conserved among all CELF proteins are two N-terminal RRMs and one C-terminal RRM that are separated by a divergent linker region known as the divergent domain [64, 65]. The divergent domain is 160-230 amino acids long and has a role in AS [66, 67]. Recent studies suggest that the divergent domain partially mediates RNA binding. Specifically, in a yeast-three hybrid experiment, deletion of the divergent domain had more of an effect on RNA binding than deletion of the RRMs [61, 65]. Further, deletion of the CELF4 and CELF2 divergent domains leads to a loss of splicing activity [68, 69]. It was found that CELF4 can activate AS of cardiac troponin T (cTNT) when RRM1 and RRM2 or RRM2 alone are paired with the first 66 amino acids from the divergent domain [66]. A minimum of 20 of these amino acids from the divergent domain are needed for CELF4 to promote inclusion of cTNT exon 5 [66]. RRM1 and RRM2 cooperatively bind CUG repeats but are unable to bind independently [65, 70]. RRM3 is also capable of binding RNA when combined with the

divergent domain. However, RRM3 does not bind CUG repeats, but it does bind GU-rich sequences more strongly than RRM1 or RRM2 [58, 65].

The activity of CELF1 is regulated by phosphorylation and microRNAs. CELF1 has multiple predicted phosphorylation sites. Phosphorylation of CELF1 by protein kinase C (PKC α/β) leads to an increase in CELF1 half-life and steady-state levels and thus an increase in CELF1 protein expression [71, 72]. Importantly, work from our lab shows that a nonphosphorylatable CELF1 with 8 predicted PKC phospho-sites mutated is unable to regulate AS of its known targets [71].

Alternative splicing regulator PTBP1

The polypyrimidine tract binding protein 1 (PTBP1) is a well-known repressor of exon inclusion and is part of the hnRNP family of RBPs that bind to exonic and intronic splicing silencers (ISSs and ESSs) to promote skipping of exons [73-78]. There are three tissue-specific isoforms including PTBP1 (hnRNP I), PTBP2 (neuronal PTB), and PTBP3 (ROD1) that bind to polypyrimidine-rich stretches of RNA. PTBP1 is ubiquitously expressed while PTBP2 is predominantly expressed in the brain, muscle, and testis, and PTBP3 is predominantly expressed in hematopoietic cells [79-81].

Although PTBP1 is recognized mainly as a repressor of exon inclusion, it has also been shown to enhance the inclusion of specific exons [82, 83]. One model suggests that in general, PTBP1 binds upstream when it represses exon inclusion and that it binds downstream when it promotes exon inclusion [82, 84]. PTBP1 binds to ISSs, which leads to global repression of weak exons [82, 84]. PTBP1 works in concert with other proteins such as Raver1 and Raver2 to repress stronger exons [85]. Raver1 and Raver2 are two

PTBP1 co-repressors. Three general models of PTBP1 repression have been proposed. In the first model, PTBP1 is hypothesized to compete with U2AF65 for binding at the 3' SS (Illustration 5) [85]. The second model proposes that PTBP1 represses exons by forming a zone of silencing across the alternative exon [81, 86, 87]. This zone of silencing can be formed by a PTBP1 monomer or multimer protein that leads to sequestration of the alternative exon. It may also involve the PTBP1 co-repressor Raver1 [80]. The third model suggests that PTBP1 repression is caused by RNA looping out of the branch point A which is critical for the AS mechanism [80].

PTBP1 has four RRMs, a bi-partite (non-canonical) N-terminal nuclear localization signal (NLS), and an N-terminal nuclear export signal [88]. The function of PTBP1 is dependent on cellular localization. PTBP1 is found in both the nucleus and the cytoplasm. This mechanism of dual-localization allows PTBP1 to regulate AS and polyadenylation in the nucleus and to mediate mRNA stability and translation in the cytoplasm. Generally, PTBP1 is shuttled to the cytoplasm when the cell is under stress, which occurs in the context of viral infections, apoptosis, and toxic exposure [89-91]. RNA binding activity of PTBP1 is not necessary for PTBP1 to shuttle between the nucleus and cytoplasm [92]. However, RRM1, RRM2, and the N-terminal NLS of PTBP1 are essential for shuttling the protein between the nucleus and the cytoplasm, and this shuttling happens in an energy-dependent and transcription-independent manner. However, with just RRM1 and the NLS, PTBP1 is predominantly nuclear. Thus, RRM2 is critical for nuclear export of PTBP1 [80, 81, 92]. RRM2 of PTBP1 is critical for PTBP1 dimerization and interactions with other proteins [93-95]. Interestingly, deleting RRM3 and RRM4 of PTBP1 increases the rate of shuttling between the nucleus and the cytoplasm [80, 81, 92]. RRM1 and RRM4 are

important for PTBP1 binding to RNA [91, 94, 95]. Thus, the shuttling of PTBP1 may be due to protein interactions rather than RNA binding.

PTBP1 is organized into 15 exons which are alternatively spliced to form four different isoforms (Illustration 5) [86, 96-98]. In humans, isoform 1 of PTBP1 (PTB1) has all four RRMs but lacks exon 9. Exon 9, which is located between RRM2 and RRM3, is important because it changes the regulatory capabilities of PTBP1 such that inclusion of this exon increases PTBP1 repression [99]. Exon 9 skipping reduces PTBP1 repression in neurogenesis leading to activation of brain-specific AS patterns. Interestingly, engineered skipping of this exon in chickens resulted in similar AS patterns to the ones in mammals. Thus, AS of PTBP1 exon 9 contributes to evolutionary differences between species in developmentally regulated AS [99].

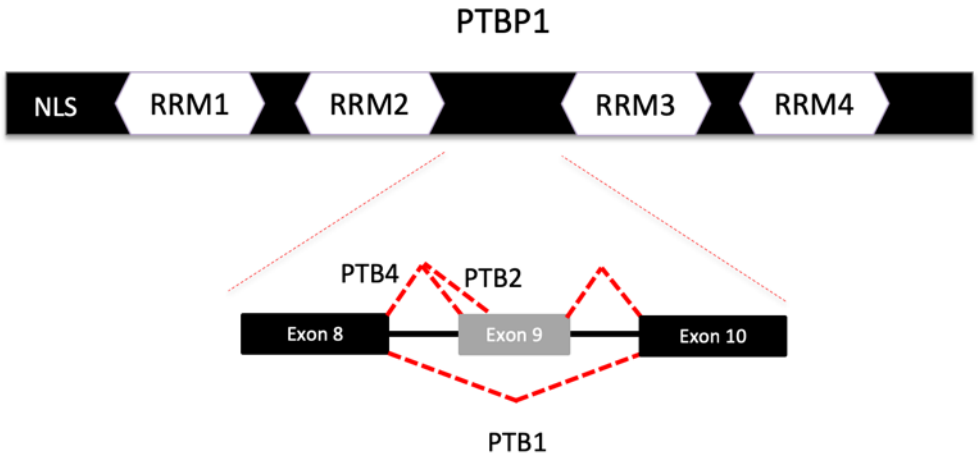

Illustration 5. PTBP1 mechanism of repression.
Proposed mechanisms of PTBP1 repression include 1) competition with U2AF for binding to the polypyrimidine tract 2) oligomerization of PTBP1 across the alternative exon resulting in a zone of silencing and 3) PTBP1 causes looping out of branch point

Isoform PTB2 contains exon 9 and the associated regulatory capabilities. PTB4 is slightly longer than PTB2 as there are two alternative 3' SSs in exon 9 important for its role in AS and internal ribosome entry site (IRES)-dependent translation. IRES-dependent

translation allows for cap-independent initiation of translation [100]. PTBP1 has a role in regulating some IRES mRNAs [89, 91]. Finally, PTB3 lacks RRM1 and RRM2 which negatively affects its role in AS [80, 81]. All four of the RRMs of PTBP1 are involved in RNA binding, but each RRM also has unique functions [101]. RRM1 and RRM2 directly interact with spliceosome U1 snRNA to regulate AS. RRM2 interacts with the PTB co-repressor Raver1 and is involved in PTBP1 dimerization [101]. RRM3 competes with U2AF65 splicing factor for binding to polypyrimidine stretches. In solution, RRM3 and RRM4 interact extensively forming one globular domain important for looping of RNA. Looping of the RNA may be a means by which PTBP1 prevents splicing factors from binding to RNA [77].

Alternative splicing regulator RBFOX2

RBFOX2 belongs to the RNA binding fox (RBFOX) family of RBPs that also includes RBFOX1 (A2PB1) and RBFOX3 (NeuN). RBFOX2 is predominantly expressed in muscle, heart, and neuronal tissues. RBFOX1 is primarily found in skeletal muscle and the heart while RBFOX3 is primarily found in neurons. Collectively, the RBFOX family contributes to neuronal and skeletal muscle development and plays a role in several diseases including autism, schizophrenia, and epilepsy [102]. RBFOX2 contributes to fundamental cellular processes including proliferation, apoptosis, and the epithelial to mesenchymal transition [103-105].

RBFOX2 regulates AS when in the nucleus and mRNA stability when in the cytoplasm [106-108]. Therefore, RBFOX2 can directly affect gene expression and function by generating different protein isoforms [109]. RBFOX2 has an NLS at both the CTD and

N-terminal domain (NTD) that regulate its cellular localization (Illustration 6). RBFOX2 binds to the (U)GCAUG motif in pre-mRNA via its highly conserved RRM to regulate AS in a position-dependent manner [110]. Similar to CELF1, RBFOX2 acts as a silencer when it binds upstream of an alternative exon, and it acts as an activator when it binds downstream of an alternative exon [109-111]. RBFOX2 binds to ISEs to activate exons directly while it represses exons indirectly or as part of a large assembly of splicing regulators (LASR) [112-114]. Splicing activation by RBFOX2 requires self-aggregation mediated by tyrosines in the CTD of RBFOX2. These tyrosines are also needed for RBFOX2s interaction with LASR [115]. Further, although the CTD of RBFOX2 is sufficient to promote exon inclusion, both the CTD and RRM are required for exon repression [109]. The mechanism of RBFOX2s regulation on selected targets has been reported. For example, RBFOX2 acts on protein 4.1R exon 16 by facilitating recruitment of U1 snRNP to the weak 5' SS. Consequently, inclusion of the exon is promoted. When RBFOX2 represses exon inclusion, it blocks recognition of the 3' SS and branch site and inhibits formation of the E complex [109, 110, 116, 117].

RBFOX2 itself is alternatively spliced giving rise to brain and muscle-specific isoforms. The M43 exon of RBFOX2 is exclusively found in heart and skeletal muscle while the B40 exon is primarily found in the brain [118]. M43 lacks residues needed for higher order assembly with the LASR. Specifically, tyrosines in the CTD of RBFOX2 are needed for higher order assembly, and three critical tyrosines are missing in M43 [115]. Multiple promoters drive RBFOX2 expression, and their activation is cellular context-specific [118]. RBFOX2 has two distinct translation start sites that alter the NTD of the protein [119]. The use of these translation start sites is tissue- and development-specific

[118, 120]. The NTD of RBFOX2 that results from the first translation start site has a classical NLS, an inhibitor of the apoptosis protein binding motif, and an MYND domain. However, these features are lost in the RBFOX2 isoform that is translated from the second translation start site (Illustration 6) [119]. Additional differences in abundance, post-translational modifications, and multi-protein complex formation affect the tissue-specific regulation of RBFOX2.

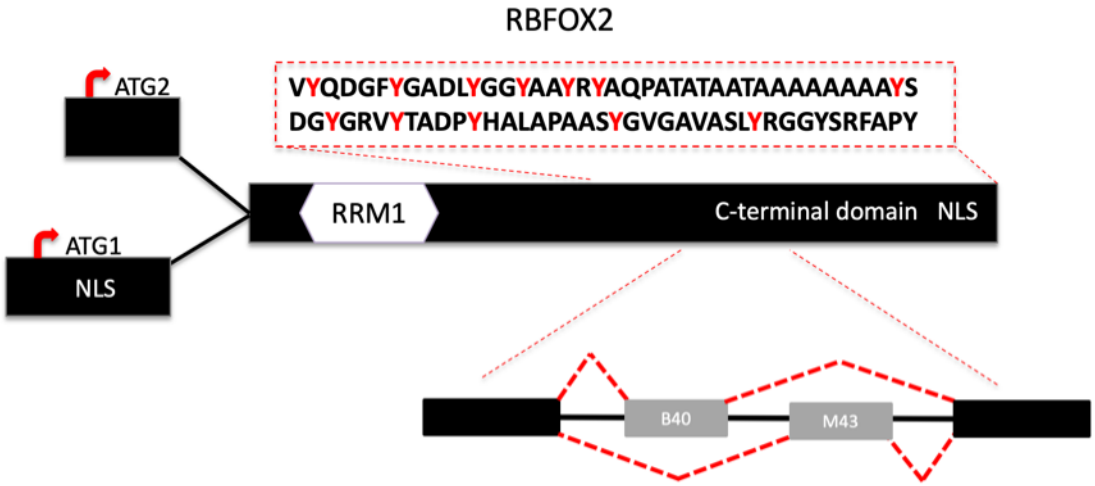

Illustration 6. Schematic representation of RBFOX2 domain organization.
RBFOX2 has one RRM and two alternative promoters. RBFOX2 is alternatively spliced itself to give rise to tissue specific isoforms. The B40 exon is found predominantly in the brain and the M43 exon in heart and skeletal muscle. The CTD of RBFOX2 has tyrosines (shown in red) that are needed for higher order assembly.

Important for this work, RBFOX2 autoregulates its own splicing of exon 6 to form a protein isoform lacking the second half of the RRM. This results in an in-frame mRNA deletion and production of a stable dominant negative (DN) isoform. This DN isoform displays reduced RNA binding capability but can repress wildtype RBFOX2 dependent splicing [118]. Previous work from our lab demonstrates that this DN RBFOX2 isoform is upregulated in the diabetic heart and inhibits AS of RBFOX2 targets [121].

CELF1, PTBP1, and RBFOX2 as regulators of cardiac development and disease

RBPs are important regulators of heart development and have been implicated at various stages including formation, morphogenesis, and maturation of the heart [122]. The heart undergoes extensive remodeling during development, particularly before and after birth, when it must adapt to new stresses and assume adult functionality [123]. The post-birth remodeling process involves transcriptional and post-transcriptional adaptions that primarily occur between postnatal day 1 and 28 [40]. Cardiomyocyte proliferation ends by postnatal day 7 and occurs concurrent to a decrease in the cell cycle and DNA replication within the heart [40, 44, 45]. Later increases in heart size are attributed to cellular hypertrophy [40]. The mature cardiomyocytes also transition from a carbohydrate to fatty acid metabolism that is consistent with the upregulation of genes related to fatty-acid metabolism, the mitochondria, and oxidation-reduction reactions [40, 124, 125]. Interestingly, the majority of genes with altered expression during cardiovascular development are independently regulated by differential AS or gene expression. Differential AS regulates cell structure and motility genes. Differential gene expression regulates signal transduction and oxidative metabolism genes [63]. Binding motifs for known splicing regulators were found flanking developmentally regulated exons and included: CELF, muscleblind-like protein (MBNL), PTB, signal transduction and activation of RNA (STAR), heterogenous nuclear ribonucleoprotein L (hnRNPL), and RBFOX2. Interestingly, the RBFOX and CELF1 motifs adjacent to developmentally regulated exons in the heart are conserved across eight species [63].

There is a dynamic relationship between cardiovascular development and disease. A key component in cardiovascular disease is the reversion from an adult splicing program

to a fetal program [71, 123, 126-128], which underscores the importance of RBPs. Numerous RBPs are altered during cardiovascular disease and induce dysregulated splicing which contributes to changes in cardiac gene expression. Importantly, RBPs regulate cellular phenotype changes that can contribute to cardiovascular disease. For example, the progression from cardiomyocytes to hypertrophic cardiomyocytes in cardiac hypertrophy is regulated by RBM20, CRIP, and SRSF1 [128-131]. In addition to cardiomyocytes, RBPs are key regulators of gene expression in a number of other cells that are integral to cardiovascular development including vascular smooth muscle cells, perivascular stromal cells, endothelial cells, monocytes, and macrophages [128]. Notably, endothelial dysfunction is associated with cardiovascular disease acceleration. Nitric oxide is a regulator of vascular tone, and activated endothelial cells produce less nitric oxide, which causes vasoconstriction [132, 133]. The RBPs QKI and HuR regulate the eNOS enzyme and subsequently nitric oxide production. QKI is also essential for maintaining the barrier function through mRNA transcript stabilization [134, 135]. In endothelial cells, RBPs also regulate growth factors including VEGF, endoglin, and HIF1α [136-138]. Additionally, through macrophage and monocyte regulation, RBPs affect inflammation that promotes cardiovascular disease [139].

RNA splicing efficiency decreases during heart failure and contributes to disease pathogenesis by impairing normal cardiac gene expression [140]. In failing hearts, the transcription factor serum response factor (SRF) is alternatively spliced to form a dominant-negative isoform that represses genes that are important for contractile function [141]. In ischemic cardiomyopathy, dilated cardiomyopathy, and aortic stenosis, important

sarcomeric genes undergo AS including cardiac troponin T and I, titin, filamin C gamma, and myosin heavy chain 7. AS of titin leads to reduced myofibrillar stiffness [140, 142].

Increasing evidence suggests that CELF1-, PTBP1-, and RBFOX2-regulated AS is important in cardiovascular development and disease. Important for my work, binding motifs for CELF1, PTBP1, and RBFOX2 are found flanking developmentally regulated exons in the heart [63]. Additionally, CELF1, PTBP1, and RBFOX2 contribute to gene expression changes that are important for cardiogenesis and heart function [122, 143].

An increased understanding of RBP function during healthy periods of change, such as development, can lead to novel insights about their contribution to disease pathogenesis. In the next sections, the roles of CELF1, PTBP1, and RBFOX2 in cardiovascular development and disease will be discussed (summarized in Table 1).

Table 1. CELF1, PTBP1, and RBFOX2 in cardiovascular diseases

RBP implicated	**Cardiovascular disease**	**References**
CELF1	Diabetic heart disease/cardiomyopathy	[144]
CELF1	Dilated Cardiomyopathy	[145, 146]
CELF1	Neonatal cardiac dysfunction	[147]
CELF1	Heart failure	[145, 148]
CELF1	Cardiac complications of myotonic dystrophy type 1	[149]
PTBP1	Cardiac Hypertrophy	[150]
RBFOX2	Pressure overload-induced heart failure	[151]
RBFOX2	Hypoplastic Left Heart Syndrome	[152] [153]
RBFOX2	Conotruncal Defects	[154]
RBFOX2	Diabetic heart disease	[121]

CELF1 in heart development

CELF1 has important roles in embryogenesis and heart development [155, 156]. Importantly, in the developing myocardium CELF1 is highly expressed, and its expression changes throughout development by more than 10-fold. CELF1 expression is high in the embryonic heart and low in the adult heart [63]. Although decreased CELF1 expression begins 6 days after birth, the mRNA levels do not change, indicating that post-transcriptional modifications cause decreased protein levels [63]. CELF1 is phosphorylated by PKC in embryonic and newborn hearts, which contributes to increased CELF1 protein expression [72]. The postnatal downregulation of CELF1 expression is regulated by reduced PKC phosphorylation and the microRNA miR-23a/b [157].

During development, CELF1 is found in both the nucleus and the cytoplasm of cardiac cells indicating that CELF1 has multiple roles in post-transcriptional gene regulation during heart development [156]. CELF1 activity is critical for cardiac development and function including calcium signaling, vesicular trafficking, and transverse tubule organization [40, 63]. CELF1 regulates at least 45 AS events in the neonatal heart including genes with roles in chromatin organization, cytoskeleton function, and lipid/glucose metabolism [147]. In the postnatal heart, low CELF1 expression induces AS transitions of genes that have roles in vesicular trafficking, endocytosis, and membrane organization [40]. Thus, CELF1 mediated AS impacts cardiac function.

CELF1 binds to 3' UTRs and promotes mRNA decay [67, 158, 159]. In the neonatal heart, CELF1 regulates mRNA stability of genes with important roles in the cell cycle by binding to their 3' UTRs. CELF1 also indirectly regulates genes related to proliferation, ion transport, circadian rhythm, and the immune response [147]. CELF1 downregulation

during development is implicated in mRNA stabilization and activation of multiple pathways. There is a tetracycline-inducible transgenic mouse model (TRECUGBP1/MHC) that exhibits CELF1 overexpression in cardiomyocytes. Analysis of these mice revealed that 41% of the genes that were upregulated during development were downregulated 72 hours after CELF1 induction. This indicates that 41% of the genes that are increased in relative expression during heart development are controlled by CELF1 through mRNA stabilization [40]. The affected genes have diverse roles in ion transport, fatty-acid metabolism, oxidation-reduction, heart contraction, hypertrophy, and mitochondria. Interestingly, 14% of the genes that are decreased during development are also regulated by CELF1 [40, 145]. Collectively, this indicates that CELF1-mediated AS and mRNA decay play important roles throughout cardiac development.

CELF1 in cardiovascular disease

Dysregulation of the developmental expression pattern of CELF1 leads to cardiac defects. Homozygous knockout of *CELF1* in neonates induces cardiac dysfunction characterized by reduced heart weight, abnormal repolarization, reduced ejection fraction (the amount of blood pumped out of the ventricles during each heartbeat), and fractional shortening (a shortening of the left ventricle diameter between diastole and systole) [147]. Interestingly, cardiac dysfunction induced by *CELF1* knockout resolves with age, perhaps indicating some as-yet-undefined redundancy in these regulatory processes [147]. As previously mentioned, CELF1 regulates mRNA stability of genes with important roles in the cell cycle by binding to their 3' UTRs. When *CELF1* is knocked out, these cell cycle genes increase in expression. This indicates that CELF1 contributes to the maintenance of

mRNA levels by destabilizing transcripts during neonatal heart development. It is possible that CELF1 acts in concert with other regulators to control transcript stability that contribute to heart development and that these other regulators become more dominant after birth, accounting for the cardiac resolution [147].

Overexpression of a dominant negative CELF (MHC-CELFΔ) in the postnatal heart muscle causes cardiac hypertrophy, dysfunction, and fibrosis, in addition to dilated cardiomyopathy and premature death. These DN CELF mice display dysregulated AS by postnatal week three, and gene expression profiling revealed that contraction and calcium signaling were most significantly affected [160]. Interestingly, crossing the DN CELF mice with mice overexpressing wild-type CELF1, rescued the dysregulated AS and cardiac defects, indicating that CELF1 regulates AS events that are critical to cardiac function. Specifically, overexpression of wildtype CELF1 partially restored heart weight, the incidence of hypertrophy, and survival in these mice. Additionally, it partially restored splicing of *Mtmr1*. Mtmr1 is a member of a phosphatase family and dysregulation of its splice isoforms may contribute to defects in striated muscle [161]. Therefore, regulation of proper *Mtmr1* splicing is critical for normal muscle function.

Gene expression profiling of MHC-*CELF*Δ hearts revealed that a number of serum response factor network (SRF) members were also significantly altered in the MHC-*CELF*Δ mice. SRF is an important cardiac transcription factor, and many of its downstream targets were upregulated in these transgenic mice. Specifically, two well-known inhibitors of SRF activity were downregulated. These proteins were homeodomain only protein X (HOPX) and four and a half LIM domain-containing protein 2 (FHL2). Interestingly, SRF

expression was unchanged. Conversely, overexpression of CELF1 reduced SRF downstream targets and induced FHL1 expression [160].

There are two different MHC-*CELF*Δ lines. The MHC-*CELF*Δ-10 line is the "severe line" and expresses more dominant-negative CELF. The MHC-*CELF*Δ-574 line is the "mild line" and expresses lower amounts of dominant negative CELF. The MHC-*CELF*Δ-574 line has less severe cardiac hypertrophy. These two lines have varying degrees of dysregulation downstream of CELF activity. The "severe line" has greater impairment of cardiac function including myocytolysis, fibrosis, and increased mortality, defects that are not exhibited by the "mild line". Mild expression of MHC-*CELF1*Δ in juvenile mouse hearts caused cardiac problems that resolved with age, even though the dominant-negative CELF expression and AS dysregulation persisted [162]. Interestingly, gene expression changes that were present in the mild MHC-*CELF*Δ line improved as the mouse aged. Specifically, genes related to calcium handling were dysregulated at 3 weeks of age leading to reduced calcium release and poor contractile performance. *Pln* and *Ryr2m* mRNA levels are expressed at reduced levels in both the mild and severe MHC-*CELF*Δ lines. However, this discrepancy is less severe in the mild line by 24 weeks and corresponds with improved calcium release and contractility. Additionally, the SRF transcriptional program that is dysregulated in juvenile mouse hearts partially resolves in older animals. The mechanism of recovery from a genetically induced juvenile cardiomyopathy is unknown [162].

Re-induction of CELF1 in the adult heart causes a reversion to fetal AS and gene expression patterns and is implicated in multiple cardiovascular pathologies including heart failure, dilated cardiomyopathy, and the cardiac complications of myotonic dystrophy [145, 146, 148]. Dilated cardiomyopathy is a myopathy characterized by left ventricle

dilation, impaired systolic function, and reduced ejection fraction. Overexpression of CELF1 in mouse hearts causes dilated cardiomyopathy and heart failure. On the molecular level, CELF1 overexpression results in defective splicing and a reversion to fetal splicing. Specifically, CELF1 developmentally regulates *Fxr1* and *Mtmr1* and causes an AS reversion. In dilated cardiomyopathy, re-induction of CELF1 dysregulates gap junction integrity important for cardiac contractility and conduction [145, 148]. CELF1 binds to the 3' UTR and promotes mRNA degradation of the major gap junction found in the heart, connexin 43 (Cx43) [145, 148]. Cx43 is integral to cardiac contractility and conduction such that downregulation of it correlates with cardiomyopathy, arrhythmia, and heart failure [145, 163, 164]. Therefore, abnormal expression of CELF1 in the adult heart dysregulates cardiac contractility and conduction and is associated with dilated cardiomyopathy and heart failure.

CELF1 upregulation is implicated in the pathogenesis of myotonic dystrophy type 1 (DM1) [146]. DM1 is caused by toxic CUG repeats present in the 3' UTR of the dystrophia myotonia protein kinase (DMPK) gene. Mutant transcripts aggregate in the nucleus and sequester RBPs leading to dysregulated AS [165]. Cardiac complications are the second leading cause of mortality in DM1 patients and affect 80% of patients. These include defects in cardiac conduction, atrioventricular blockage, dilated cardiomyopathy, arrhythmias, and left ventricular systolic dysfunction. Defects in cardiac conduction can lead to fatal arrhythmias, and for this reason, 30% of all DM1 patients die due to cardiac complications [40].

CELF1 protein levels are elevated in DM1 patients, and studies now indicate that CELF1 contributes to the cardiac complications associated with DM1 [166]. Toxic CUG

repeats in DMPK activate PKC, which then phosphorylates CELF1 and induces a 2-4-fold increase in relative CELF1 expression [146]. The exact mechanism of how these toxic CUG repeats in DMPK activate PKC is unknown [167]. Human CELF1 overexpression in adult mouse hearts resembles the functional (echocardiographic and electrocardiographic) and molecular abnormalities seen in DM1 [146]. The conduction abnormalities that are observed include cardiac conduction abnormalities including PR interval elongation and QRS complex widening. Additionally, CELF1 overexpression causes cardiac dysfunction including left ventricular systolic dysfunction and dilation. These abnormalities are indicative of dilated cardiomyopathy and are similar to the features of DM1 observed in patients [146]. In the mice, dilated cardiomyopathy occurred in concert with necrosis and myocardial fiber loss, and the effects were observed eight days after CELF1 induction. Left ventricular ejection fraction and posterior wall thickness were also decreased in mice overexpressing CELF1. The defects caused by CELF1 overexpression were so severe they caused premature lethality within two weeks of induction [146]. Inhibition of PKC reduces CELF1 phosphorylation and protein expression, which correlates with restored cardiac function [166].

A reversion to fetal AS patterns is a hallmark of DM1. Heart or cardiomyocyte-specific overexpression of CELF1 reproduces a reversion to fetal AS patterns similar to that in DM1 patients [63, 146]. Interestingly, these effects were observed in the absence of toxic CUG repeats, indicating that CELF1 independently contributes to DM1 pathogenesis [146]. Collectively, these data indicate a causative role for CELF1 upregulation in the dysregulated splicing that is observed in DM1 [146].

Important for this work, we have previously found that CELF1 is upregulated in diabetic patient hearts. Diabetes is a metabolic disease characterized by hyperglycemia due to either a lack of insulin production (Type 1: T1D) or lack of insulin sensitivity (Type 2: T2D). The major cause of mortality in diabetic patients is due to cardiovascular complications [168, 169]. Ultimately, cardiac complications in diabetes can progress into heart failure [170].

CELF1 is phosphorylated by PKC leading to upregulation of its expression in diabetic hearts [71]. Upregulation of CELF1 occurs concurrently with defects in developmentally regulated AS. We found that overexpression of CELF1 leads to splicing patterns similar to those in neonatal hearts. In both T1D and T2D, splicing patterns revert to a fetal pattern. Importantly, phosphorylation of CELF1 is necessary for this splicing pattern indicating that CELF1 upregulation via PKC phosphorylation contributes to splicing dysregulation in the diabetic heart [71]. However, the global consequences of increased CELF1 expression on gene expression changes in diabetic hearts still remain unknown. My findings on how CELF1 contributes to splicing changes in the diabetic heart will be discussed in Chapter 2. Briefly, this chapter describes 138 CELF1 targets that are aberrantly spliced in T1D, leading to changes in gene expression. Importantly, these CELF1 targets have roles in muscle contraction and protein kinase G (PKG) signaling [144].

PTBP1 in heart development and disease

The regulation of PTBP1 during heart development is controversial. Some studies suggest that protein levels of PTBP1 are decreased in expression during heart

development in the absence of corresponding changes at the level of mRNA expression. Specifically, Zhang et al., found that PTBP1 levels rapidly decrease after birth and reach undetectable levels by postnatal day 90 [171]. Other studies suggest that PTBP1 expression does not change during heart development [63].

PTBP1 expression is regulated through a caspase-dependent cleavage mechanism during heart development [172]. PTB is cleaved via a pathway involving histone deacetylases (HDAC). HDACs are known to have important roles in regulating gene expression in striated muscle. HDAC4 and HDAC5 bind to and inhibit the transcriptional activity of MEF2. MEF2 is a transcription factor involved in skeletal and heart muscle development. Thus, HDAC4 and HDAC5 negatively regulate skeletal and heart muscle development through MEF2. Mice deficient in HDAC5 had decreased levels of PTB in their hearts, which, suggested a causal relationship between HDACs and PTB. However, this effect was specific for HDAC5 and knockout of HDAC1, HDAC2, or HDAC9 did not inhibit PTB expression [172].

During heart development, HDAC activates caspase inhibitor FLICE-like protein, (cFLIP) which inhibits caspase activity. Caspase-dependent signaling occurs in the fetal myocardium, but is downregulated during heart differentiation [171, 173]. When caspases are active, PTB is cleaved, triggering its degradation in the proteasome [172]. Interestingly, this effect is specific to heart cells because cardiomyocytes treated with HDAC inhibitors exhibit cleaved PTB, but this effect is not recapitulated in HEK293 cells. Consistent with these data, in HDAC5-deficient mice, cFLIP and PTB are downregulated. Conversely, cFLIP overexpression prevents cleavage of PTB. Additional evidence supporting caspase-dependent regulation of PTB during heart development arises from caspase-3 and caspase-

7 heart-specific knockout mice. These mice exhibited increased PTB when compared to wild-type controls. In both groups, the PTB transcript was unchanged [172]. Because cFLIP is an inhibitor of caspase activity, it interferes with heart development and thus, is downregulated during cardiomyocyte differentiation.

Reduced PTBP1 expression during heart development results in the inclusion of exons that are normally repressed by PTBP1 [172]. Through this mechanism, PTBP1 affects α-tropomyosin (*TPM1)*, β-tropomyosin (*TPM2*), and the MEF2 family of transcription factors. MEF2 is an essential transcription factor for heart muscle differentiation and cardiac stress adaptation while α-tropomyosin and β-tropomyosin are cardiac structural proteins important for muscle function and general cytoskeleton functions [82, 172, 174, 175]. Mef2 is alternatively spliced in striated muscle and brain tissues such that the short exon beta is more included during differentiation. This beta exon encodes for a glutamic acid rich sequence that promotes stronger transcriptional activity when compared to other MEF family proteins. *In vitro*, PTB regulates skipping of the beta exon in both HeLa cells and C2C12 myoblasts [82, 176]. During heart development, this exon becomes more prevalent, and its increased levels correlate with decreased PTB protein expression [172]. More specifically, overexpression of PTB in neonatal cardiomyocytes induced skipping of the beta exon in Mef2 (*MEF2a* and *Mef2d)*. Further evidence for PTB regulation of beta exon skipping was indicated by studies with HDAC-inhibited cells. The HDAC-inhibited cells, which had attenuated PTB expression, exhibited increased inclusion of the Mef2 beta exon. Despite this correlation, PTB only weakly crosslinks to Mef2 in HeLa cell studies, suggesting that PTB indirectly regulates Mef2

[172]. Nonetheless, these results suggest that PTBP1 contributes to important developmental transitions in the heart through regulation of AS.

α-tropomyosin and β-tropomyosin are cardiac structural proteins that have mutually exclusive exons regulated by PTB knockdown [82, 172, 174, 175]. The *TPM2* mutually exclusive exons are exons 6 and 7. The *TPM1* mutually exclusive exons are exon 8 and 9. Notably, the alternative exons of *TPM1* and *TPM2* were included in neonatal cardiomyocytes that overexpressed PTB. CLIP-sequencing also identified PTB motifs around the TPM alternative exons. During postnatal heart development PTB is downregulated and the PTB-repressed exons of *TPM1* and TPM2 are included at increased frequency. A PTB regulatory mechanism of TPM exon-inclusion was supported by different mouse models. In HDAC5-deficient mice, where PTB expression was low, the TPM alternative exons were included at greater frequencies. Conversely, in caspase 3 and caspase 7 deficient mice, where PTB expression is high, inclusion of the alternative exons was less frequent [172].

PTBP1 also has a role in regulating apoptosis during cardiomyocyte differentiation, possibly through IRES-dependent translation [171, 172]. Apoptosis is an important component of cardiac development, with roles in the formation of the outflow tract, conducting system, cardiac valves, and coronary vasculature. Specifically, apoptosis contributes to vessel regression in the vasculature formation [177]. Apoptotic machinery is expressed in the embryonic myocardium and is silenced after birth coinciding with downregulation of PTBP1 [173, 178]. Importantly, PTBP1 overexpression was found to increase expression of components of the apoptotic machinery [171]. Collectively, these data indicate that PTBP1 promotes apoptosis during heart development.

Although PTBP1 is important in cardiovascular development, less is known about its role in cardiovascular disease. However, PTB deficiency in mice results in embryonic lethality concomitant with proliferation and differentiation defects [179]. Additionally, gene expression analysis in mice indicates that PTBP1 is increased in relative expression during cardiac hypertrophy, but downregulated during development [150]. Thus, there is a need to further explore PTBP1's role in cardiovascular diseases.

My findings on a developmentally regulated spliced variant of PTBP1 present in the diabetic heart and its functional consequences on AS will be detailed in Chapter 3. In summary, this chapter describes that a developmentally regulated PTBP1 isoform with less repressive function is upregulated in the T1D heart. Importantly, I identified a group of PTBP1 targets that are dysregulated in diabetes and demonstrate that the PTBP1 isoform with less repressive function regulates AS of these transcripts [180]. This provides the first evidence that PTBP1 is modulated in the T1D heart and provides a potential role for PTBP1 in gene expression changes in the diabetic heart.

RBFOX2 in heart development

RBFOX2 expression is high in the neonatal heart and only slightly attenuated in the adult heart [63, 181]. RBFOX2 protein expression slightly decreases beginning at postnatal day 6 and is accompanied by a parallel decrease in RBFOX2 mRNA [63]. In whole heart samples, RBFOX2 is predominantly nuclear [63]. However, in cardiomyocytes which make up 30% of the heart [37-40], RBFOX2 is predominantly cytoplasmic, indicating that RBFOX2 has multiple roles in regulation of gene expression

in the heart as a whole. [182]. This is not surprising as RBFOX2 has well-established roles in RNA metabolism in both the nucleus and cytoplasm [106-108].

RBFOX2 contributes to heart function through regulation of AS, microRNAs, and polycomb-mediated transcriptional repression [151, 181-183]. RBFOX2 regulates splicing of genes with important roles in heart function including calcium handling, cytoskeleton reorganization, channel activities, proteasome function, mitochondrial activity, and cellular signaling [151, 181]. Interestingly, RBFOX2 may initiate a cascade of gene regulation as it also regulates genes with roles in splicing (MBNL1) and transcription (MEF2) [151, 181]. Importantly, a subset of these genes is developmentally regulated in the postnatal heart. For example, RBFOX2 mediates repression of *Epb4.1* and *Atp5c1* during postnatal heart remodeling to enhance cardiac function [151]. *Epb4.1* encodes the cytoskeletal protein 4.1R that interacts with spectrin and actin to promote membrane stability and is required for the expression of a number of cell surface transmembrane proteins. *Epb4.1* has been shown to regulate calcium signaling and repolarization in the heart [184]. Previous research demonstrates that RBFOX2 directly regulates *Epb1*.4. Specifically, RBFOX2 activates exon 16 of *Epb4.1*, which is repressed in the absence of RBFOX2. Inclusion of exon 16 increases 4.1R affinity for spectrin and actin [114]. Spectrin and actin are essential for proper targeting of transmembrane ion transporters and contribute to the regulation of intracellular calcium signaling [184, 185].

ATP5c1 encodes for the ATP synthetase γ subunit F1γ. Extensive research has demonstrated that the RBFOX family of RBPs regulate *ATP5c1*. Exon 9 of F1γ is excluded in heart and skeletal muscles [186], and RBFOX1 induces skipping of this exon by

preventing E complex formation [117]. Importantly splicing of ATP synthetase γ subunit F1γ enhances energy production in cardiac muscle mitochondria [186].

While RBFOX2 is a well-known regulator of AS, it also contributes to the cardiac phenotype through regulation of transcription. RBFOX2 acts as a repressor of transcription by interacting with polycomb repressive complex 2 (PRC2) and nascent RNA [182]. Importantly, RBFOX2 has an important role in regulating excitation-contraction coupling in the post-natal heart via regulating t-tubules [182, 183]. Specifically, RBFOX2 induces a set of microRNAs that dysregulate excitation-contraction coupling. One of these microRNAs, miR34a, targets Jph2, which is an excitation-contraction coupler. Jph2 links T-tubules with the sarcoplasmic reticulum, which allows for coordination between electrical signals on the membrane and the contractile apparatus [183]. Important for this work, the RRM domain of RBFOX2 is not required for interaction with PRC2 while the CTD is required [182]. Collectively, these data indicate that RBFOX2-mediated AS and transcription play important roles in cardiac function.

Unpublished results from Dr. Sunil Verma in our lab further demonstrate that RBFOX2 is necessary for heart development. Conditional knockout (CKO) of RBFOX2 in mice results in embryonic lethality by embryonic day 11.5 (E11.5). At E10.5 RBFOX2 CKO embryos display hemorrhaging, edema, and underdeveloped hearts (unpublished results). These embryos have reduced contractile tissue. At E9.5, RBFOX2 CKO embryos do not display overt phenotypic differences. However, these embryos have reduced cardiac tissue and proliferation defects. Cardiac transcriptome analysis at E9.5 identified 1140 AS events changed in the RBFOX2 CKO mice. Gene ontology (GO)analysis of the cassette exons revealed that genes related to cellular adhesion and cytoskeleton were affected.

RBFOX2 depletion adversely affects endothelial cell adhesion while leading to faster migration. These data indicate that RBFOX2 is essential for normal cardiac development and function.

RBFOX2 in cardiovascular disease

Dysregulation of the developmental expression pattern of RBFOX2 leads to cardiac defects. RBFOX2 knockout in embryonic cardiomyocytes does not prevent live birth, but it causes dilated cardiomyopathy that progresses to heart failure mice at postnatal stages [151]. These mice appear normal at week 5, but by week 9 they display enlarged cardiac chambers and thin ventricular walls. Their condition progresses to dilated cardiomyopathy by week 22, and only 5% of these mice live past a year [151]. These data indicate that RBFOX2 is critical for post-natal heart function.

Cardiomyocytes isolated from RBFOX2 knockout hearts exhibit calcium-handling defects that impaired whole-cell twitch. These phenomena were confirmed in cultured RBFOX2 knockout neonatal cardiomyocytes. Reduced transient peaks and increased calcium sparks, in addition to spontaneous transient frequency, collectively indicated defective excitation-contraction coupling in the RBFOX2 knockout heart [151]. RBFOX2 is decreased early in heart failure, and this is correlated with cardiac stress that is characterized by dysregulated excitation-contraction coupling. Excitation-contraction coupling is important for the transmission of electrical signals to the cardiac muscle contraction apparatus, and depletion of RBFOX2 induces a set of microRNAs that dysregulate this coupling [183]. Thus, depletion of RBFOX2 contributes to compromised cardiac function through dysregulation of excitation-contraction coupling.

RBFOX2 knockout in mice causes dysregulated AS of genes that have previously been implicated in heart development and disease. Included in this set of dysregulated genes are two genes that are important for the structural integrity of the cardiac contractile apparatus (*Pdlim5* and *Ldb3*) and four genes that were previously linked to cardiomyopathy (*Abcc9, Sorbs1, Sorbs2,* and *Enah*) [151]. Notably, *Abcc9* is an ATP-sensitive potassium channel and *Enah* (mena) is associated with cytoskeletal organization and remodeling [187, 188]. Additionally, RBFOX2 knockdown caused dysregulated AS of two critical cardiac transcription factors, Mef2a and Mef2d that correlated with a decrease in Mef2a protein expression [151].

The importance of RBFOX2 in heart development has additionally been demonstrated in a zebrafish model. RBFOX2 knockout zebrafish embryos displayed normal phenotypes despite having dysregulated AS. When RBFOX1 and RBFOX2 were knocked-out together using morpholinos, these double morphants exhibited heart defects such as reduced heart rate and pericardial edema. Importantly, re-expression of RBFOX2 could rescue heart function [189]. Further, RBFOX2 expression rescued dysregulated AS caused by the single knockout of RBFOX1. This indicates that RBFOX2 is important for healthy heart development and function and that RBFOX2 may be able to compensate for RBFOX1 activity in heart function [189]. However, it is important to note that morpholinos are known to have many off-target effects and that a CRISPR-based knockout zebrafish model has not been developed. Therefore, there is a great need to develop better models of RBFOX2 knockout to determine its role in heart function.

RBFOX2 is implicated in multiple cardiovascular pathologies including pressure overload-induced heart failure, HLHS, and diabetic heart disease [121, 151, 152].

RBFOX2 is decreased early in heart failure and causes dysregulation of genes with important roles in cardiac function. Decreased RBFOX2 expression in heart failure causes cardiac stress that is characterized by dysregulated excitation-contraction (EC) coupling. Cardiac specific ablation of RBFOX2 led to defective EC coupling [151]. EC coupling is important for the transmission of electrical signals to the cardiac muscle contraction apparatus, and depletion of RBFOX2 induces a set of microRNAs that dysregulate this coupling [183]. Thus, miRNAs induced by RBFOX2 contribute to compromised cardiac function in heart failure.

Rbfox2 in diabetic heart disease: Important for this work, our lab has previously shown that RBFOX2 regulates transcripts that are aberrantly spliced in the diabetic heart [121]. Of patients diagnosed with diabetes, 5-10% have T1D [190] and 90-95% have T2D [191]. The lack of insulin production in T1D is due to auto-immune mediated loss of β-islet cells in the pancreas that are responsible for producing insulin [192]. T2D is mainly caused by environmental factors (obesity and a sedentary lifestyle) and genetic factors. Obesity and a sedentary lifestyle cause insulin resistance in the muscle and liver that leads to decreased uptake of glucose by cells ultimately causing hyperglycemia. Insulin resistance in combination with a genetic predisposition can lead to β-islet cell failure that leads to reduced insulin production [193].

The major cause of mortality in diabetic patients is due to cardiovascular complications [168, 169]. Diabetic patients are at an increased risk of developing cardiovascular disease because they often have risk factors such as obesity, hypertension, and dyslipidemia [194]. These cardiovascular diseases can include atherosclerosis, microangiopathy, cardiovascular autonomic neuropathy, diabetic cardiomyopathy, and

myocardial infarction [170, 194]. Diabetes leads to cardiac muscle cell dysfunction characterized by alterations to cellular architecture, metabolism, and calcium signaling that induces hypertrophy, apoptosis, and fibrosis [170]. Oxidative stress, endothelial dysfunction, and increased coagulability present in diabetic patients also increase their risk of cardiovascular disease [194]. Ultimately, cardiac complications in diabetes can progress into heart failure [170].

In the diabetic heart, RBFOX2 regulates genes that have important roles in heart function including metabolism, apoptosis, protein trafficking, and calcium homeostasis. RBFOX2 becomes dysregulated during the onset of disease pathogenesis. Interestingly, RBFOX2 expression is increased in diabetes, but the functional activity is decreased. A DN form of RBFOX2 is produced in diabetes, which blocks wild-type RBFOX2 mediated splicing. Expression of the DN RBFOX2 impairs intracellular calcium release in cardiomyocytes [121]. Thus, as an AS regulator, RBFOX2 contributes to diabetic transcriptome changes.

Importantly, many transcripts that are aberrantly spliced in diabetes have roles in heart function including *MTMR3*, *FXR1*, *MEF2a*, and *PBX3*. Inclusion of the alternative exon of all four these genes is decreased in Type 2 diabetic patient hearts. CLIP-sequencing revealed that *MTMR3*, *FXR1*, and *MEF2a* display RBFOX2 CLIP clusters in the introns surrounding their alternative exons. These data suggest that RBFOX2 may regulate their splicing [121]. Collectively, this data indicates that RBFOX2 regulates many genes with important roles in the heart in diabetes.

RBFOX2 in congenital heart defects: RBFOX2 has been previously implicated in the congenital heart defect HLHS. HLHS is an often-lethal congenital heart defect in which babies are born with underdeveloped left ventricles and/or aorta resulting in severely compromised systemic circulation (Illustration 7) [195, 196]. HLHS accounts for ~25% of deaths among infants with congenital heart defects [197, 198]. Without surgical interventions HLHS is fatal in the first few days of life [199, 200]. The burden on the right ventricle over time can cause lifelong cardiac complications. Hypoplasia of the left heart is thought to be due to defective cardiomyocyte proliferation [201]. There is limited information regarding the etiology and pathogenesis of HLHS. Thus, there is an urgent need to determine the pathogenic mechanisms of HLHS to develop better therapies [153].

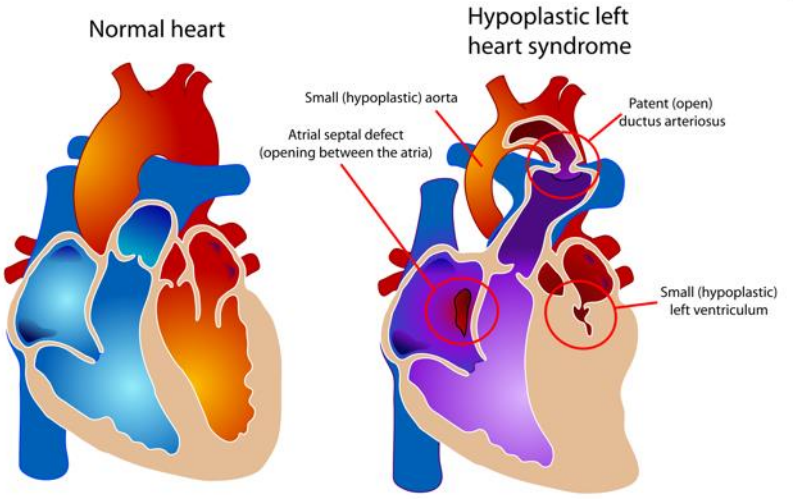

Illustration 7. HLHS specific heart changes.
Image from public domain.

HLHS is multigenic and heterogenous meaning multiple genes and mutations can lead to the same phenotype. This has made it very difficult to determine a genetic etiology of HLHS and create mouse models to study the disease. The first mouse HLHS mouse line (*Ohia)* revealed that HLHS was digenic and identified 8 different mouse lines. Exome sequencing from these mice identified 330 individual mutations confirming that HLHS is

genetically heterogenous. Mutations in *Sap130* and *Pcdha9* led to the HLHS phenotype in these mice. Mutations in Notch-signaling related genes were also identified. This data indicates that HLHS has complex genetics and may arise through multiple mutations in different genes [202].

The AS regulator RBFOX2 is significantly associated with the HLHS phenotype [203]. In one of the largest CHD patient genetic studies (1,213 parent offspring trios), exome sequencing identified multiple damaging *de novo* mutations that accounted for 20% of patients with CHD associated with other coexisting diseases and 2% of patients with isolated CHD. These damaging do novo mutations occurred in 4,420 genes that are highly expressed in the heart. D*e novo* mutations in 392 of these genes are expected to contribute to CHD. Twenty-one of these genes had multiple damaging *de novo* mutations. Important for this work, three *de novo* RBFOX2 mutations (nonsense, frameshift, and splice site) that were identified from congenital heart disease patients are strongly associated with HLHS left ventricular obstruction phenotype [153].

All of the identified HLHS-specific RBFOX2 mutations are within the CTD and are predicted to either truncate the protein or target the mRNA for degradation (Illustration 8). The CTD of RBFOX2 is necessary for its nuclear localization and subsequent AS function because it mediates interaction with other RBPs and spliceosome components. In

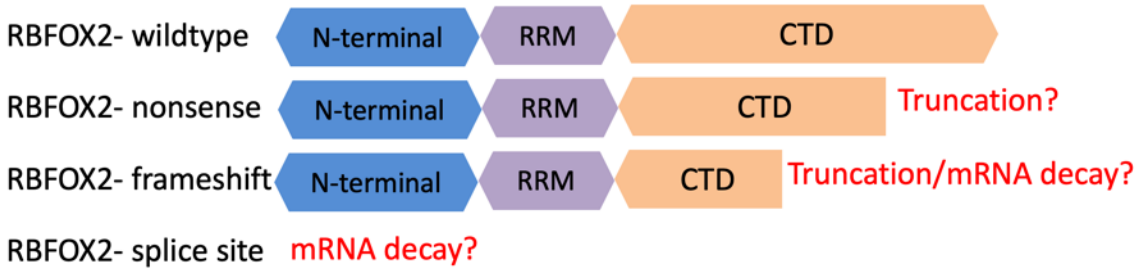

Illustration 8. Predicted effects of HLHS specific RBFOX2 mutations.

HLHS patients, RBFOX2 has a lower molecular weight, which indicates that it is a truncated form or a different isoform [152]. The nonsense RBFOX2 mutant forms cytoplasmic puncta and is unable to regulate AS of its targets [152]. Seventy percent of altered transcripts in HLHS patients have RBFOX2 binding sites, which suggests that RBFOX2 mutations contribute to aberrant gene expression and HLHS development [152]. Relevant to the disease, RBFOX2 mutations contribute to gene expression changes in HLHS to disrupt cell cycle and metabolism contributing to defective cardiomyocyte proliferation that may underlie the hypoplasia of the left heart [152].

Interestingly, RBFOX2 mutations are also associated with another group of congenital heart defects, which are classified as conotruncal defects. Conotruncal defects can include Tetralogy of Fallot, double-outlet right ventricle, aortic arch abnormalities, membranous ventricular septal defects, and truncus arteriosus. These defects are associated with an RBFOX2 missense mutation [154]. Although all the HLHS-specific RBFOX2 mutations are within the CTD, the conotruncal defect-specific mutation is specifically located within the RRM [153, 154].

Further research is needed to identify how mutations in different domains of RBFOX2 contribute to different congenital heart defects. My preliminary findings on the functional consequences of RBFOX2 mutations in HLHS will be explained in Chapter 4. In summary, I found that the HLHS specific RBFOX2 mutants are unable to control splicing of their targets despite not displaying altered cellular localization. Further, these mutants have altered interactions with their binding partners such that the nonsense mutant gains interactions. Thus, HLHS specific mutants may alter RBFOX2 function in RNA metabolism and gene expression.

Co-regulation of splicing between RBFOX2, CELF1, and PTBP1

Cell-specific regulation of splicing is determined by a combination of positive and negative antagonistic effects on splice site use by splicing activators and inhibitors [204]. Each alternative splicing event is under the control of multiple RBPs, and each cell type expresses different levels of RBPs. Among RBPs, the RBFOX2, CELF, and PTB families are implicated in coordinated alternative splicing transitions during heart development [63, 71, 205]. RBFOX2, CELF1, and PTBP1 are expressed at high levels in the embryonic heart. RBFOX2 and CELF1 are reduced in relative expression in the adult heart while PTBP1's expression is controversial. Notably, many of the same alternative splicing events that are co-regulated by these three RPB's during heart development are also present in cardiovascular disease.

Evidence suggest that PTBP1 and CELF1 can regulate AS of some targets in a similar pattern [206] while they antagonistically regulate AS of other targets [207, 208]. PTBP1 and RBFOX2 have been shown to antagonistically control AS [206, 209]. Similarly, CELF1 and RBFOX2 have been shown to antagonistically regulate AS.

Cardiac troponin T (cTNT) is a regulatory protein in the heart that controls calcium-dependent actin and myosin interactions [210]. Importantly, cTNT has a developmentally regulated exon that is controlled by CELF and PTB proteins [204]. Due to the small size of this exon (30nt) and its noncanonical 5' splice site, it is weakly recognized [204]. Its alternative exon, exon 5, is included in the embryonic heart at a greater frequency and is included at lower levels in the adult heart [204, 211, 212]. CELF promotes inclusion of the alternative exon by binding to introns downstream of exon 5, while PTB promotes

exclusion by binding to silencing sites that are located both upstream and downstream of the intron. When the cTNT exon 5 is included, myofibrils are more sensitive to calcium [211]. As CELF1 usually promotes inclusion of this alternative exon it becomes less included in adult hearts when CELF1 is downregulated. Conversely, PTB levels may remain high into adulthood. Thus, enhancers of exon inclusion are downregulated in conjunction with the maintenance of repressors and contribute to the exclusion of cTNT in the adult heart.

Fibroblast growth factor (FGF) signaling has a role in regulating proliferation and differentiation of cardiomyoblast in the myocardium during midgestation heart development. FGF9, FGF16, and FGF20 derived from the endocardium signal to the myocardium through fibroblast growth factor receptor 2c (FGFR2c) and FGFR1c [213]. FGFR2 has two mutually exclusive exons (IIIB and IIIC) in its CTD that affect its ligand binding characteristics [78]. These mutually exclusive exons are alternatively spliced in a tissue-specific pattern. Exon IIIb is predominantly found in epithelial cells, while exon IIIC is predominantly found in mesenchymal cells. AS of these mutually exclusive exons is regulated by RBFOX2 and PTBP1. PTBP1 has been shown to promote exon IIIc inclusion and represses exon IIIb inclusion by binding to upstream and downstream intronic silencing sequences [78]. RBFOX2 promotes exon IIIb inclusion and repression exon IIIc inclusion [103]. Therefore, RBFOX2 and PTBP1 antagonistically regulate FGFR2 important for proliferation, differentiation, and EMT in the developing heart.

CELF1 and RBFOX2 antagonistically regulate AS. Interestingly, CELF1 CLIP peaks were located near cassette exons regulated by RBFOX2 in myotubes. Further, RBFOX2 knockout mice and CELF1 heart-specific overexpression displayed co-

regulation of 22-30% of AS events. The majority of these co-regulated alternative splicing events were similarly more or less included. Compared with other RBPs, the CELF family was found to coregulate the largest number of AS events with RBFOX2. Importantly for this work, RBFOX2 and CELF1 bind together on exons regulated during heart development and disease. Roughly seventeen percent of exons regulated during heart development are cobound by RBFOX2 and CELF1. Interestingly, CELF1 and RBFOX2 coregulated 11.3% of dysregulated alternative splicing in the type 1 diabetic heart [214]. CELF1 and RBFOX2 are phosphorylated in the fetal heart and in differentiated cardiac cells increasing their protein expression [71].

These three RBPs are also regulated similarly to each other. For example, CELF1, PTBP1, and RBFOX2 are all regulated by microRNAs such that knockout of Dicer in adult myocardium results in increased protein expression of these 3 proteins [157].

Summary

CELF1, PTBP1, and RBFOX2 regulate gene expression at the post-transcriptional level through AS. Increasing evidence suggests that CELF1, PTBP1, and RBFOX2 have important roles in heart development and disease. This dissertation will further elucidate how CELF1, PTBP1, and RBFOX2 are dysregulated in cardiovascular diseases, specifically diabetes and HLHS, and how changes in these RBPs contribute to dysregulated AS changes.

Chapter 2: CELF1 contributes to aberrant alternative splicing patterns in the Type 1 Diabetic heart

Modified in part from:

CELF1 contributes to aberrant alternative splicing patterns in the Type 1 diabetic heart

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Published:
Biochemical and Biophysical Research Communications
2018 Sep 18
18;503(4):3205-3211. doi: 10.1016/j.bbrc.2018.08.126
Epub 2018 Aug 27.

Introduction

Type 1 diabetes results from autoimmune-mediated obliteration of pancreatic beta islet cells that produce insulin. Uncontrolled diabetes can lead to complications that impair quality of life and even result in death [215-217]. Cardiovascular complications are the major causes of mortality among diabetic patients [168, 170, 218]. In addition to hypertension and atherosclerosis, diabetic conditions adversely affect the cardiac muscle cells leading to heart failure [219-221] [170, 221]. Diabetic conditions dramatically affect gene expression and AS contributing to disease pathogenesis [222, 223], but there is limited information on AS regulators responsible for such changes [223, 224].

AS allows selective inclusion or exclusion of alternative exons, introns, or parts of exons and introns into the mature mRNA; thereby controlling gene expression and generating different protein isoforms [7-9]. Precise regulation of AS is critical for heart function and development [225, 226]. AS is controlled by RBPs that bind RNA and interact with the splicing machinery. Although RBPs have emerging roles in diabetes, only a few RBPs are associated with cardiac complications of diabetes [71, 121, 224].

We have previously shown that the RBPs RBFOX2 and CELF1 are altered in T1D hearts. We have demonstrated the consequences of RBFOX2-mediated changes in diabetes cardiac pathogenesis [71]. However, the role of CELF1 in T1D-induced AS changes still remains unknown. CELF1 regulates AS, mRNA translation and mRNA decay [64, 227, 228]. Overexpression of CELF1 in mouse hearts leads to dilated cardiomyopathy and heart failure associated with defective AS patterns [146]. Therefore, in this study we comprehensively analyzed the cardiac transcriptome and tested whether CELF1 contributes to AS changes in T1D hearts. Our results indicate that many putative CELF1 targets with important functions in the heart, displayed altered AS patterns in T1D hearts. Importantly we show that CELF1 regulates AS of these transcripts that are differentially spliced in T1D hearts.

RESULTS

Widespread AS and gene expression changes in Type 1 diabetic mouse hearts

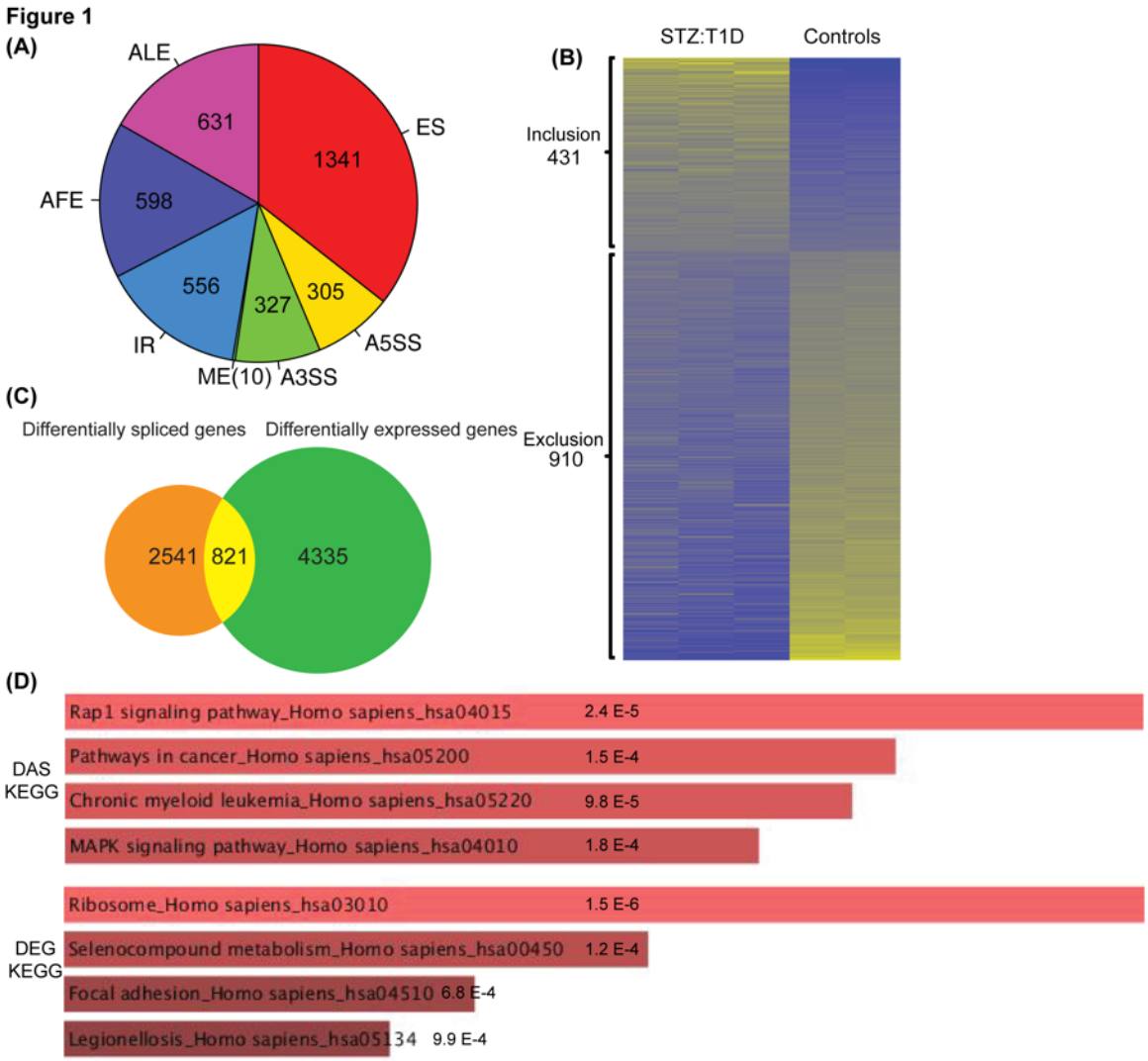

Figure 1. DAS and DEG analysis in T1D mouse hearts.

A) DAS analyses were performed using the RNA-seq data from STZ:T1D and mock treated, non-diabetic (Control) mouse hearts. AS changes in STZ:T1D mice were grouped into seven different categories based on the type of AS event: cassette Exon Splicing (ES), Alternative 5' Splice Site (A5SS), Alternative 3' Splice Site (A3SS), Mutually Exclusive exon (ME), Intron Retention (IR), Alternative First Exon (AFE) and Alternative Last Exon (ALE). The pie chart depicts the distribution of different splicing types identified in T1D hearts based on the criteria that $|\Delta\Psi| > 0.05$ and $q < 0.05$. The total DAS events was 3768. B) Heat map of alternative exons that are either included or excluded in T1D vs control mouse hearts based on percent spliced in (PSI) values. Yellow represents high PSI values and blue indicates low PSI values. C) Venn diagram comparison of DAS and DEG in STZ:T1D and control mouse hearts. 3362 DAS genes (some have multiple AS events) were compared to 5156 genes with a change in mRNA levels (>2-fold increase or decrease, and $q < 0.05$). D) The KEGG categories for DAS or DEG changes in T1D were identified using Enrichr (http://amp.pharm.mssm.edu/Enrichr). Significance is represented on the charts.

We have previously identified cassette exon splicing changes in T1D hearts [223]. In the current study, we extended our analyses and determined differentially expressed genes (DEG) as well as all forms of differential alternative splicing (DAS) in the T1D heart using our RNA-Seq data obtained from STZ:T1D mouse hearts (GSE80664) [121]. We identified seven major types of AS events including cassette exon splicing (ES), alternative 5' splice site (A5SS), alternative 3' splice site (A3SS), alternative first exon (AFE), alternative last exon (ALE), mutually exclusive exons (ME) and intron retention (IR) in diabetic hearts. We determined percent spliced in (PSI, Ψ) and found a total of 3768 statistically significant AS events altered in T1D hearts (Fig. 1A) with the criteria that percent spliced in $|\Delta\Psi| > 0.05$ and $q < 0.05$. We calculated the q-values from the p-values in the likelihood ratio test by the Benjamini-Hochberg procedure. All major types of AS patterns were affected in T1D hearts, with the most common being the cassette exon splicing (1341/3768: 35.6% ES) (Fig. 1A). Of 1341 cassette exon splicing events detected in diabetic hearts, 910 were exon exclusion and 431 were exon inclusion events (Fig. 1B).

We identified 5156 differentially expressed genes in T1D hearts (>2-fold change and $q < 0.05$). Next, we checked whether mis-spliced genes display mRNA levels changes. Only 21.8% of mis-spliced transcripts (821 out of 3768) exhibited changes in mRNA levels (Fig. 1C). The majority of the mis-spliced transcripts that exhibited mRNA levels changes were cassette exon skipping events. KEGG pathway analysis of DAS genes identified Rap1 signaling, pathways in cancer, and MAPK signaling (DAS, Fig. 1D) while analysis of DEG in T1D hearts identified ribosomes, selenocompound metabolism, and focal adhesion processes to be affected in T1 hearts (DEG, Fig. 1D).

Since the majority of cassette exon splicing leads to removal of the protein coding

regions affecting the protein output in T1D hearts, we focused on the top AS changes that favor exon exclusion. We validated three top changing exon exclusion events (*Ablim3* exon 18, *Per1* exon 16, and *Gpr116* exon 12) that were not identified in our original AS analysis and displayed mRNA level changes [121]. As expected, these pre-mRNAs displayed enhanced exon exclusion in independent STZ:T1D mice when compared to controls based on the number of reads mapped to these exons (Fig. 2A) and by qRT-PCR (Fig. 2B). AS of these three genes were also affected in a different T1D mouse model known as NOD mice, favoring alternative exon exclusion (Fig. 2C). In sum, our DAS analysis successfully identified new AS events that were validated using two different T1D mouse models.

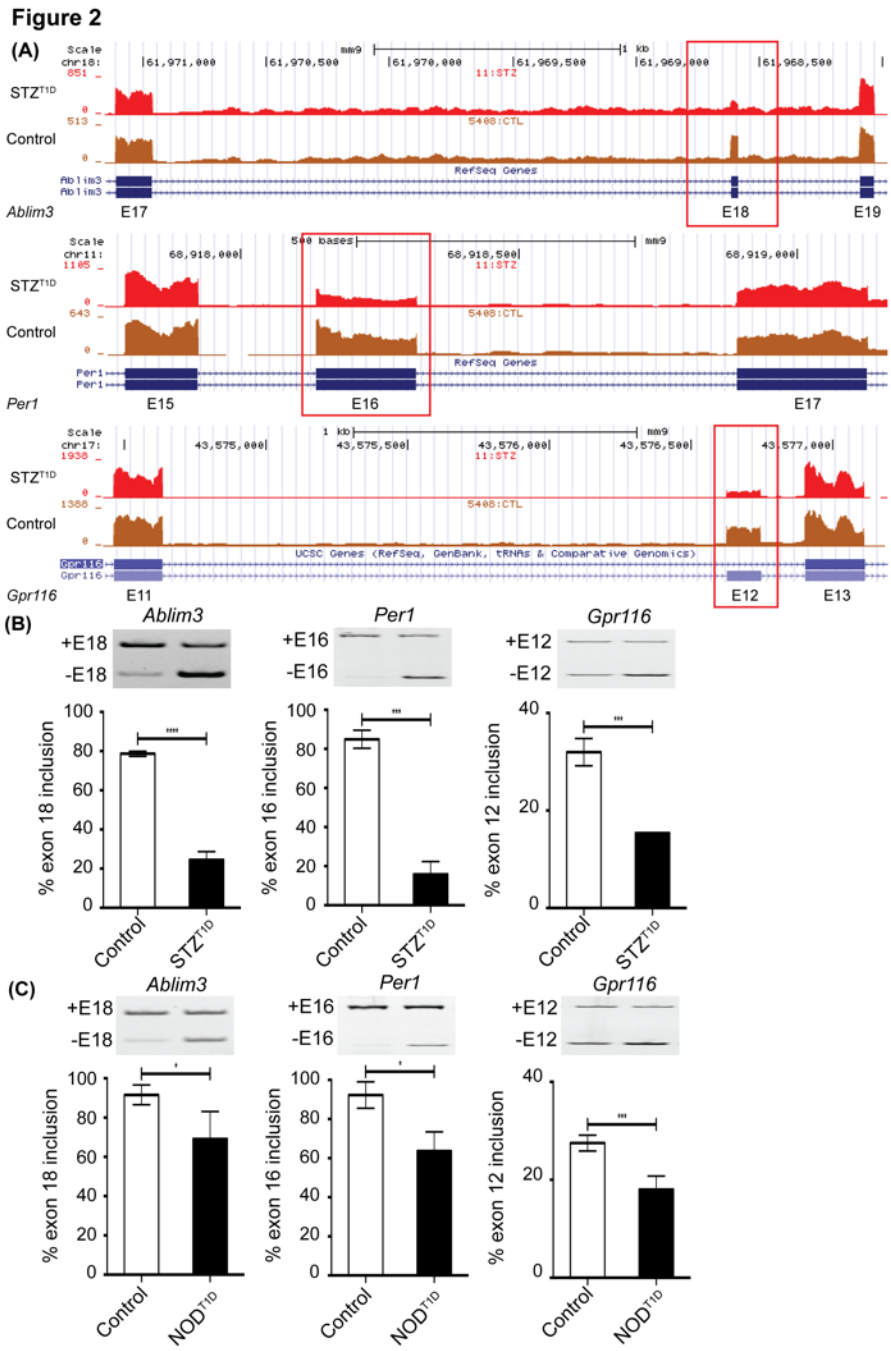

Figure 2. Validation of newly identified DAS events in two different T1D mouse models.

Representative genome browser images of AS of *Ablim3* exon 18, *Per1* exon 16, and *Gpr116* exon 12 in mock treated, non-diabetic (Control) or STZ:T1D mice left ventricles based on RNA-Seq reads. % inclusion of *Ablim3* exon 18, *Per1* exon 16, and *Gpr116* exon 12 was determined by qRT-PCR in B) Control or STZ:T1D (n≥3) and C) in non-diabetic (Control) or NOD:T1D mice left ventricles (n≥3). In AS gel figures +E# = Exon # inclusion and -E# = Exon # exclusion. Data represent means ± SD. P-values are represented as **** < 0.0001, *** < 0.001, * < 0.05.

Transcripts that display CELF1 binding sites undergo AS changes in Type 1 diabetic hearts

Since CELF1 protein is elevated in T1D hearts [71], we tested if CELF1 contributes to AS changes in T1D hearts. *In vivo* targets of many RBPs have been identified by several methods including CLIP-seq [229]. Thus, we used the CELF1 CLIP-seq dataset obtained from mouse hearts [64] to determine CELF1 binding sites within 250nt of the upstream and downstream intronic sequences and 50nt of the 5' and 3' ends of the alternative exons, mis-spliced in T1D hearts. Out of 1318 differential cassette exon (ES) events (with one variable exon/event), 138 transcripts with aberrant AS patterns in T1D hearts displayed CELF1 binding sites (Fig. 3A). Importantly, 72% of CELF1 targets displayed aberrant exon exclusion in T1D hearts (Fig. 3B), impacting the protein coding region of these genes. KEGG pathway analysis of CELF1 targets identified the cGMP-PKG signaling pathway, aldosterone synthesis and vascular smooth muscle contraction as top categories (Fig. 3C). We first validated the AS pattern of putative CELF1 target histone deacetylase 7 (*HDAC7*) identified by our CELF1 CLIP-seq vs RNA-seq comparison. *HDAC7* pre-mRNA has CELF1 binding sites indicated by the mapped CELF1 CLIP-seq reads (Fig. 3D, black rectangle). HDAC7 controls transcription by affecting chromatin remodeling and myocyte enhancer factor proteins [230] and is implicated in vascular integrity [231]. In T1D hearts, *Hdac7* exon 8 was more excluded (Fig. 3E). This AS change is expected to remove an exon within the histone deacetylase domain affecting its function. To validate whether CELF1 regulates *HDAC7* AS, we depleted CELF1 in heart-derived H9c2 cells and examined endogenous *HDAC7* AS. CELF1 knockdown induced almost complete exclusion of *HDAC7* exon 8, suggesting that CELF1 regulates this exon. These results validate our

CLIP-seq and RNA-seq comparison and show that CELF1 target RNAs undergo AS changes in T1D hearts.

Figure 3

(A)

Binding Sites	Transcripts mis-spliced in diabetes
CELF1	138/1318

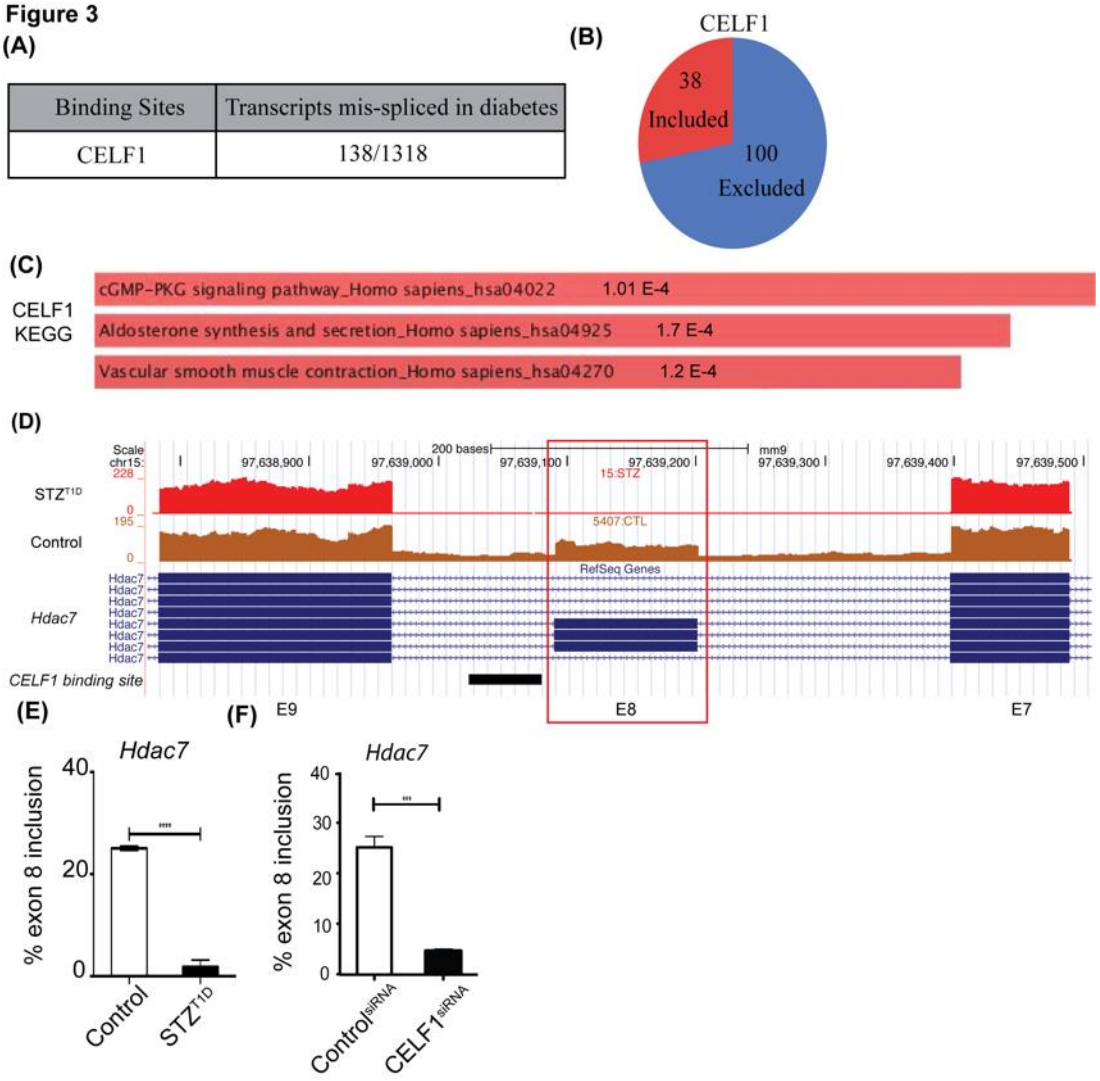

Figure 3. CELF1 binding sites are present within transcripts mis-spliced in T1D hearts.

A) Number of transcripts differentially spliced in T1D mouse hearts that display CELF1 binding sites. CELF1 binding sites were identified by extracting data from CELF1 CLIP-Seq and overlapping these binding sites within transcripts (1318) mis-spliced in T1D hearts. To evaluate the significance of events that are positively or negatively regulated in both datasets, Fisher's exact test was used to examine the enrichment. The Pearson correlation coefficient for regulated AS changes was calculated as 0.74, higher than the correlation coefficient of non-regulated events. **B)** Distribution of cassette exon inclusion versus exclusion in CELF1 target transcripts differentially spliced in diabetes. **C)** The KEGG pathway categories for CELF1-regulated AS events in T1D hearts using *Enrichr*. **D)** Representative genome browser images of putative CELF1 target *Hdac7* exon 8 in Control vs STZ:T1D mice left ventricles. CELF1 binding sites derived from CLIP-Seq data were represented below the genome browser image as a black rectangle. **E)** AS of *Hdac7* exon 8 in Control (n=3) vs STZ:T1D mice left ventricles (n=2). **F)** AS analysis of endogenous *Hdac7* exon 8 in H9c2 cell transfected with scrambled (Control)

CELF1 binding is important for regulation of AS targets mis-spliced in T1D hearts

Since putative CELF1 targets affected in T1D mouse hearts are within the cGMP signaling pathway (Fig. 3C) relevant to cardiac complications of diabetes, we investigated AS of transmembrane protein 184b (*Tmem184b*). The family member of TMEM184B, TMEM184A, was identified as a regulator of cGMP-PKG and MAPK signaling pathways [232] (Fig. 1D). Deletion of TMEM184B in mice leads to cardiovascular defects that include abnormal heart morphology and enlarged hearts (http://www.mousephenotype.org/data/genes/MGI:2445179). Notably, *Tmem184b* pre-mRNA has CELF1 binding sites and has altered AS pattern in T1D hearts according to our DAS analysis. In two different T1D mouse models, *Tmem184b* exon 9 was more skipped (Fig. 4A, right and left panels). To determine the regulation by CELF1, we depleted or ectopically expressed CELF1 in H9c2 cells. CELF1 depletion but not overexpression of CELF1 resulted in increased inclusion of *Tmem184b* exon 9, suggesting that CELF1 is a repressor of this exon (Fig. 4B-C). This may indicate that CELF1 levels are already high in these cells resulting in a maximum level of inclusion that can't be altered by additional CELF1 expression. Proper nuclear localization of GFP-CELF1 was confirmed by GFP-fluorescence (Supp. Fig. 1).

To further investigate CELF-mediated regulation, we generated a splicing minigene reporter by inserting intron9-exon9-intron10 of *Tmem184b* in between the open reading frame of GFP [233]. We also constructed a mutant *Tmem184b* splicing minigene that removed one of the CELF1 CLIP-seq peaks that represents a CELF1 binding cluster. The removal of only one CELF1 binding peak led to more inclusion of exon 9 in

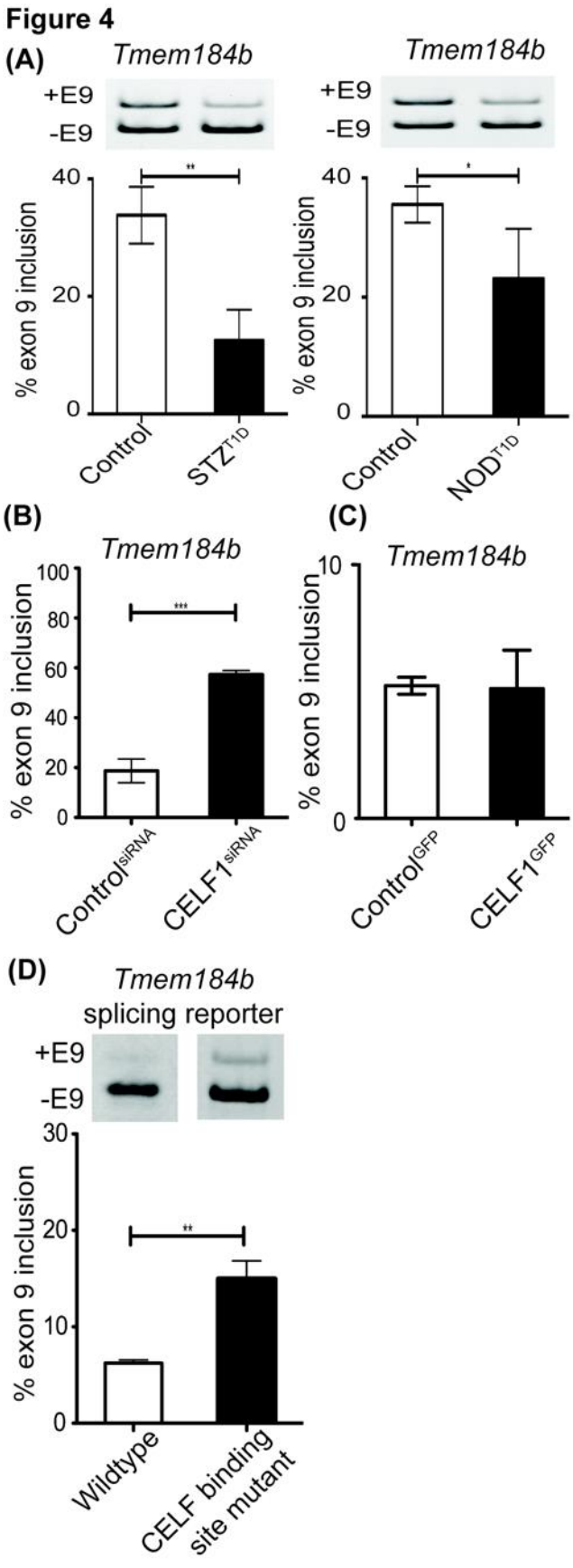

Figure 4: CELF1 contributes to diabetes-induced AS changes.

AS analysis of *Tmem184b* exon 9 **A)** in Control vs STZ:T1D (left graph) or Control vs NOD:T1D mice left ventricles (right graph) (n≥3), **B)** in H9c2 cell transfected with scrambled (Control) or CELF-specific siRNA pools (n=3), **C)** in H9c2 cells transfected with GFP (Control) or $CELF1^{GFP}$ (n=3), and **D)** in H9c2 cells transfected with wild-type or CELF1 binding site mutant *Tmem184b* splicing minigene reporter (n=4, two independent experiments). Data represent means ± SD. P-values are represented as *** < 0.001, ** < 0.01, * < 0.05.

comparison to WT *Tmem184b* (Fig. 4D). Knockdown of CELF1 (Fig. 4B) or removal of CELF1 binding site within *Tmem184b* pre-mRNA (Fig. 4D) led to increased exon inclusion indicating that CELF1 is a repressor of this exon. These results show that CELF1 binding site is important for repression of *Tmem184b* exon 9 inclusion and that CELF1 contributes to the aberrant AS of *Tmem184b* in T1D hearts.

Conclusion

In this study, we comprehensively analyzed transcriptome changes in STZ:T1D mouse hearts and found extensive DAS and DEG changes. We identified different types of AS events that are altered in diabetic hearts: 36% cassette exon splicing, ~15% intron retention, ~16% first exon splicing, and ~17% alternative last exon splicing (Fig. 1A). Although the majority of AS changes in diabetic hearts were independent of changes in mRNA levels (Fig. 1C), 821 transcripts exhibited changes in both AS and mRNA levels (Fig. 1). Of the 1341 cassette exon changes, exon exclusion events (910) that remove protein-coding regions are favored in T1D hearts. Importantly, we validated these AS changes in two different T1D mouse models.

Previously, we have shown that RBFOX2 is a major contributor to AS dysregulation and cardiomyocyte dysfunction in the heart [121]. In this study, our goal was to determine whether other RBPs play a role in AS changes in T1D hearts. Our previous data indicate that CELF1 protein is increased in diabetic hearts [71]. In this study, we identified 138 CELF1 targets mis-spliced in T1D hearts and showed that CELF1 regulates AS of targets with binding sites (Figs. 3 and 4). Importantly, the majority of CELF1 target genes with roles in muscle contraction and PKG signaling display exon exclusion, which likely impacts protein function (Fig. 3B). Importantly, transgenic mice conditionally

overexpressing CELF1 in cardiomyocytes develop dilated cardiomyopathy and heart failure associated with aberrant AS patterns. Therefore, we hypothesize that upregulation of CELF1 in the diabetic hearts could contribute to cardiomyopathy by affecting AS of critical genes.

Although CELF1 is upregulated in T1D hearts, our AS analysis of the CELF1 target *Hdac7* indicate that diabetes-induced AS changes are consistent with CELF1 depletion or low CELF1 splicing activity rather than CELF1 overexpression. RBFOX2 splicing activity is reduced in diabetic hearts despite an increase in its protein levels [71, 121]. It is possible that an inactive isoform of CELF1 is also increased in T1D hearts or CELF1-regulated alternative exons are also controlled by a complex network of RBPs affected in T1D hearts. CELF1 and RBFOX2 oppositely regulate AS [64, 206, 234] and CELF1 and RBFOX2 have overlapping targets mis-spliced in diabetes [234]. Thus, we propose a model that changes in RBFOX2 and CELF1 jointly contribute to genome wide AS changes in the T1D hearts.

Chapter 3: A developmentally regulated spliced variant of PTBP1 is upregulated in Type 1 diabetic hearts

Modified in part from:

A developmentally regulated spliced variant of PTBP1 is upregulated in Type 1 diabetic hearts

KarryAnne Belanger, Curtis A. Nutter, Jin Li, Peng Yu, Muge N. Kuyumcu-Martinez

Published:
Biochemical and Biophysical Research Communications

Introduction

T1D is an autoimmune disease characterized by hyperglycemia due to very little or no insulin production by the pancreas [192]. T1D patients can develop cardiovascular complications that can lead to heart failure [235-237]. Aberrant AS regulation has been implicated in cardiovascular complications of diabetes, however the molecular mechanisms responsible for AS changes in T1D hearts are not well defined. RBPs regulate AS via binding to specific motifs in the intronic and exonic regions of pre-mRNAs and interacting with the splicing machinery. Recent studies indicate that RBPs have emerging roles in systemic complications of diabetes [224].

PTBP1 belongs to a family of RBPs including PTBP1, PTBP2 (also known as neuronal PTB) and PTBP3 (ROD1) that bind to polypyrimidine tract sequences in the RNA. They have well-established roles as repressors of exon inclusion [74-78, 99, 238, 239], though there is evidence that PTBP can activate exon inclusion of certain pre-mRNAs

[83, 240]. PTBP1 is regulated during heart development and implicated in coordinated AS transitions during heart development [63, 171, 172]. It is widely unknown if it is modulated in T1D hearts.

In this study, we found that PTBP1 is differentially spliced in adult T1D hearts increasing the expression of a PTBP1 isoform predominantly expressed in newborn mouse hearts. We identified a group of PTBP1 targets aberrantly spliced in T1D mouse hearts. We found that inducible expression of diabetes-induced PTBP1 spliced variant with less repressive function changes AS patterns similar to that in diabetic hearts. PTBP1 and RBFOX2 antagonistically regulate AS of some of their targets.

Results

PTBP1 is differentially spliced in Type 1 diabetic mouse hearts

We have previously identified genome wide AS changes in STZ induced T1D (STZ^{T1D}) mouse hearts [71, 121]. To determine the mechanism responsible for T1D induced AS dysregulation, we examined T1D heart AS data and identified a splicing change in PTBP1 where exon 8 (exon 9 in human) is more skipped in T1D mouse hearts indicated by lower number of reads mapped to this exon (Fig. 5A). This PTBP1 isoform lacking exon 8 is reported to have low splicing repressor function [99, 241, 242]. qRT-PCR analysis showed increased exon 8 skipping of PTBP1 in two different T1D mouse models: STZ and non-obese diabetic (NOD) when compared to non-diabetic controls (Fig. 5B and 5C).

Figure 5

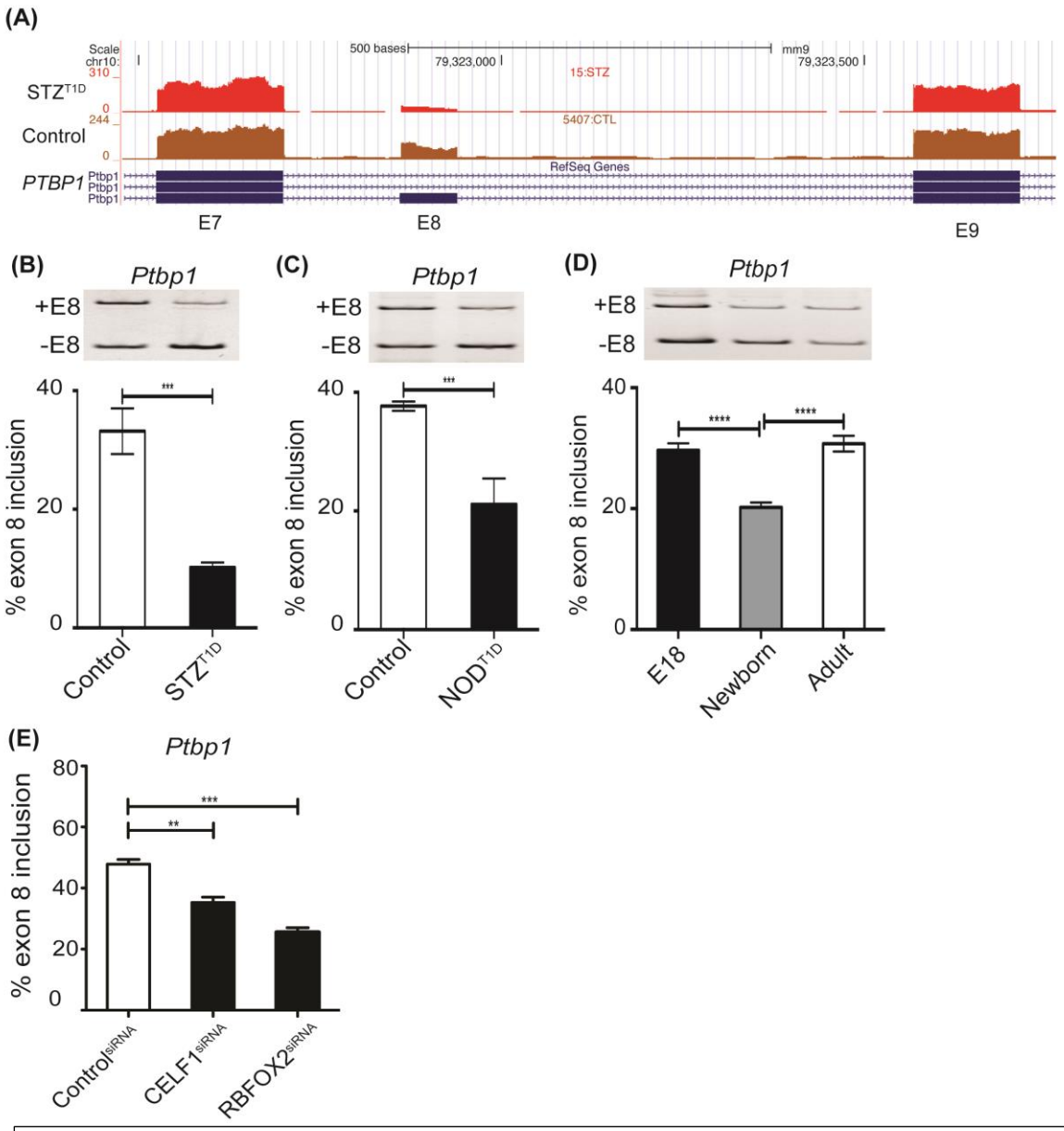

Figure 5. Regulation of PTBP1 splice variant expression in the heart under diabetic conditions and during development.

A) Representative genome browser images of *PTBP1* exon 8 exclusion in diabetic mouse hearts in comparison to the control mouse hearts. qRT-PCR analysis of *PTBP1* exon 8 inclusion in **B)** control or streptozotocin induced diabetic (STZ:T1D), **C)** in control or non-obese diabetic (NOD:T1D) mouse hearts. qRT-PCR analysis of *PTBP1* exon 8 inclusion **D)** during mouse heart development at different stages: embryonic day 18 (E18), newborn (NB), or adult (n≥3) and **E)** in H9c2 cells depleted of RBFOX2 or CELF1. (n≥3). Data represent means ± SD. Statistical significance was calculated using Student's t-test for two groups and one-way ANOVA to compare three different groups. P-values are represented as **** < 0.0001, *** < 0.001 and ** < 0.01.

Regulation of diabetes-induced and developmentally regulated PTBP1 spliced variant in the heart

The expression of this PTBP1 spliced variant has been shown to regulate AS of brain specific genes during neuronal development [99]. For that reason, we tested whether PTBP1 exon 8 splicing is regulated during mouse heart development. We found that PTBP1 exon 8 is more skipped in WT newborn hearts in comparison to WT adult and embryonic hearts (Fig. 5D). To determine how PTBP1 splicing is modulated in T1D mouse hearts and during mouse heart development, we tested whether CELF1 and RBFOX2 are involved. We have previously shown that both CELF1 and RBFOX2 protein levels are modulated in the diabetic heart [71, 121, 144]. These proteins are involved in developmental regulation of AS in the heart [63, 157, 243]. Therefore, we reasoned that changes in PTBP1 AS might be due to alterations in CELF1 and RBFOX2. We depleted CELF1 or RBFOX2 then tested PTBP1 exon 8 AS. We found that depletion of these proteins leads to more exon 8 skipping (Fig. 5E) similar to the AS pattern in diabetic mouse hearts (Fig. 5B and 5C). We expect that a double knockout of CELF1 and RBFOX2 would lead to an additive effect on exon 8 skipping. This result is consistent with low CELF1 and RBFOX2 AS function in the diabetic heart [71, 121].

PTBP1 targets undergo AS changes in diabetic hearts

Since PTBP1 is modulated in diabetic hearts, we wanted to determine the contribution of PTBP1 to genome wide AS changes in the diabetic heart. For that reason, we overlaid our Type 1 diabetic heart RNA-Seq data [71, 144] with the available PTBP1 CLIP-Seq datasets obtained from mouse embryonic stem cells [244]. We examined PTBP1 binding sites within 250nt of the upstream and downstream intronic sequences and 50nt of

the 5' and 3' ends of the alternative exons, which are typically the regions for AS regulation. Out of 1318 differential cassette exon events [144], 58 exhibited PTBP1 binding sites (Fig. 6A). Our result is consistent with results from Blencowe lab, which identified 58 cassette exons regulated by this PTBP1 spliced variant in chicken DT40 B stable cells [99]. When we examined transcripts with PTBP1 binding sites, PTBP1 regulated alternative exons were equally excluded and included in diabetic hearts (Fig. 6B) whereas the 1318 differential cassette exons were more excluded.

Figure 6

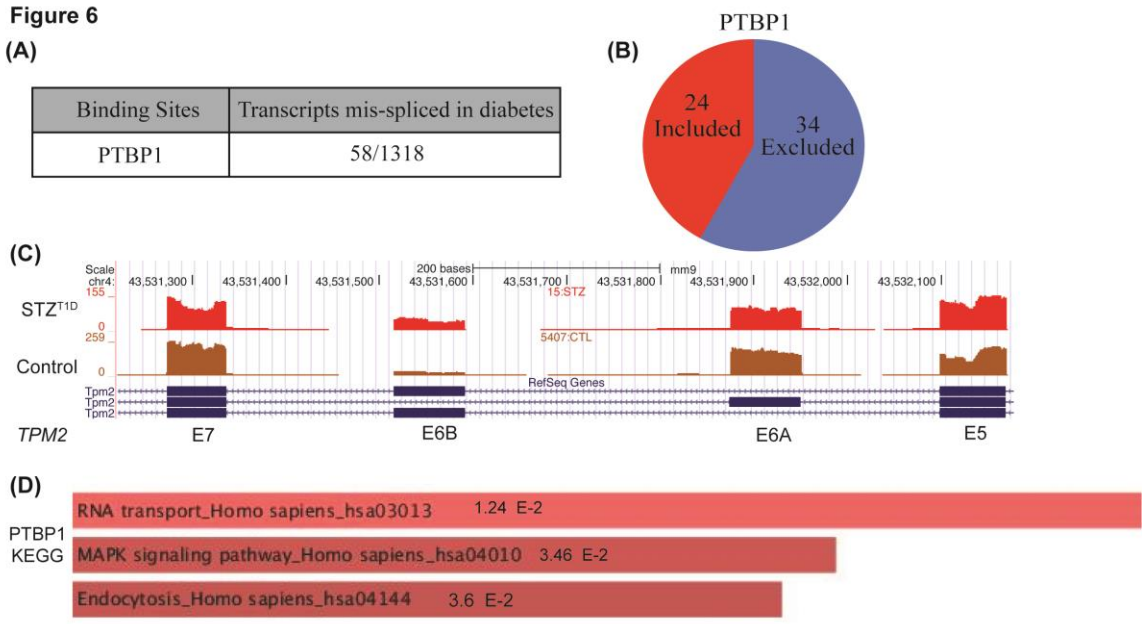

Figure 6. PTBP1 binding sites are present within transcripts that are mis-spliced in Type 1 diabetic mouse hearts.

A) Number of transcripts differentially spliced in Type 1 diabetic mouse hearts that display PTBP1 binding sites. PTB binding sites were identified by extracting data from previously published CLIP-Seq data and overlapping these binding sites within transcripts (1318) mis-spliced in diabetic hearts. 58 of these transcripts exhibit PTBP1 binding sites. **B)** Distribution of cassette exon inclusion or exclusion of PTBP1 targets in diabetic hearts. **C)** Representative genome browser images of a known PTBP1 target *Tpm2* exon 6b (E6B) in control vs STZ:T1D mice hearts. **D)** The KEGG pathway categories for PTBP1-regulated AS events in diabetic hearts using *Enrichr*.

To better comprehend how this spliced variant of PTBP1 impacts AS outcomes, we examined AS of the well-known and extensively studied target of PTBP1 (*Tpm2* exon 6b) in diabetic hearts. PTBP1 is a repressor of *Tpm2* exon 6b inclusion [245], but we find that this exon was more included in diabetic hearts based on RNA-seq reads (Fig. 6C). This is consistent with expression of a PTBP1 spliced variant with low repressor activity. To determine the consequences of PTBP1-mediated AS changes in diabetic mouse hearts, we performed the KEGG pathway analysis of PTBP1 targets using Enrichr (http://amp.pharm.mssm.edu/Enrichr). Top categories for PTBP1-regulated genes (total: 58) are RNA transport, MAPK signaling and endocytosis (Fig. 6D).

Diabetes-induced PTBP1 spliced variant regulates AS of transcripts differentially spliced in diabetic hearts

We identified *CLIP1* gene as a target of PTBP1 differentially spliced in the diabetic heart. The genome browser image shows that *CLIP1* exon 9 is more included in STZ:T1D mouse hearts (Fig. 7A). To determine how diabetes-induced PTBP1 spliced variant regulates AS of *CLIP1*, we generated inducible human 293 cells stably expressing WT PTBP1 that includes exon 9 (exon 8 in mouse): PTBP1ex9 and that excludes exon 9: PTBP1Δex9. We treated these cells with PTBP1/2 siRNA to remove the endogenous PTBP activity, and then induced expression of empty vector or PTBP1 (+ex9, Δex9) using doxycycline. We examined AS patterns of *CLIP1* and *Med23* (a known *PTBP1* target used as a positive control). Depletion of PTBP1/2 led to increased inclusion of alternative exons of *CLIP1* and *MED23*, indicating that PTBP1 is a repressor of these exons (Fig. 7B). Cells stably expressing WT PTBP1+ex9 displayed lower inclusion of *MED23* and *CLIP1* alternative exons consistent with its role as a repressor (Fig. 7B, dark gray bars), whereas expression

of PTBP1Δex9 spliced variant caused more inclusion and thus could not rescue the effect of PTBP1 knockdown (Fig. 7B, black bars vs dark gray bars). We validated the inclusion of PTBP1 exon 9 (Fig. 7C) and the efficiency of knockdown of endogenous PTBP1 by WB (Fig. 7D). These results suggest that a spliced variant of PTBP1 with less repressive function is generated in diabetic hearts and contributes to AS of its targets.

Figure 7

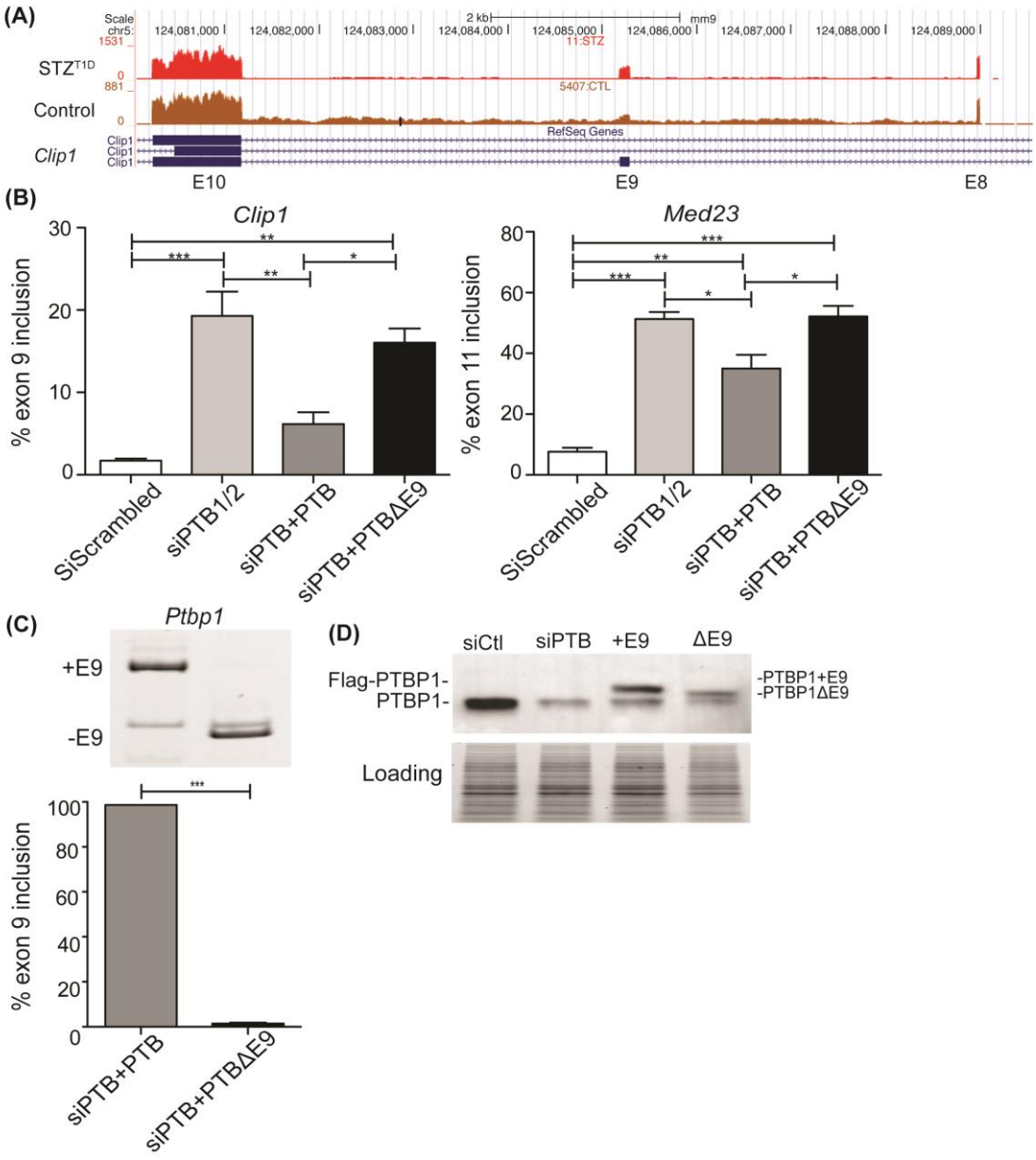

Figure 7. PTBP1 splice variant regulates AS of transcripts differentially spliced in diabetic hearts.

A) Representative genome browser images of PTBP1 target *Clip1* exon 9 in control vs STZ:T1D mice hearts. AS analysis of **B)** *Clip1*, *Med23* and **C)** *PTBP1* in 293 cells depleted of PTBP1/2 and stably expressing inducible WT (PTBP1+ex9) and diabetes-induced spliced variant (PTBP1Δex9) using qRT-PCR. **D)** Validation of endogenous PTBP1 depletion and ectopic expression of PTBP1+ex9 and PTBP1Δex9 by Western blot (WB) using an antibody against PTBP1. Total protein staining was used to confirm even protein loading. Data represent means ± SD. Statistical significance was calculated using one-way ANOVA to compare four different groups. P-values are represented as *** < 0.001, ** < 0.01, * < 0.05.

PTBP1 regulates AS in diabetic hearts antagonistically to RBFOX2

So far, our published results and results shown here indicate that 3 RBPs CELF1 [71] RBFOX2, and PTBP1 are altered in the diabetic heart [121] (Fig. 5). Thus, to determine how changes in these 3 RBPs affect AS decisions in the diabetic heart, we focused on AS of *Tmem184b* gene as *Tmem184b* pre-mRNA exhibits binding sites for all three RNA binding proteins: PTBP1, CELF1 and RBFOX2 ([121] and Fig. 8A). We have shown that CELF1 regulates AS of this exon, which is excluded in T1D hearts [144]. Thus, here we tested the roles of PTBP1 and RBFOX2 in AS of *Tmem184b*. Knockdowns of PTBP1/2 resulted in inclusion of *Tmem184b* exon 9 suggesting that PTBP is a repressor of this alternative exon (Fig. 8B). On the other hand, RBFOX2 overexpression increased exon inclusion, but its knockdown led to exon exclusion, suggesting that RBFOX2 is an activator of this alternative exon (Fig. 8B). In sum, PTBP1 and RBFOX2 antagonistically regulate AS of *Tmem184b*.

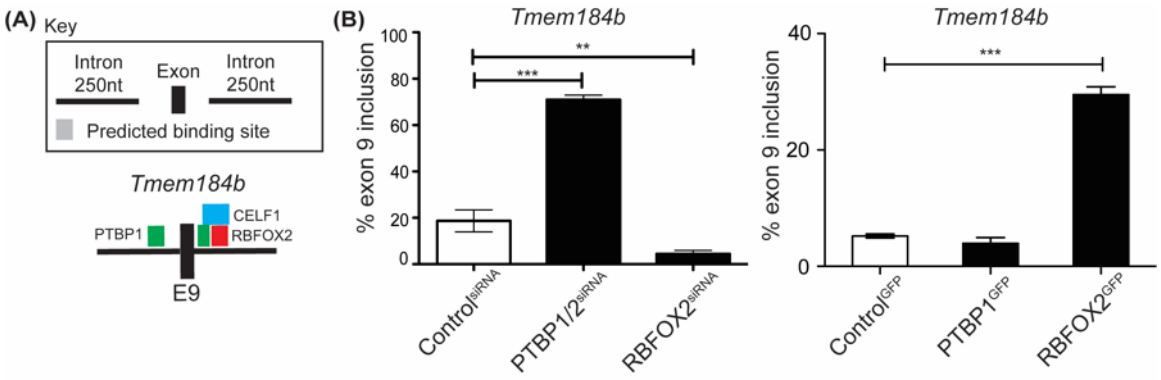

Figure 8. PTBP1 regulates alternative splicing of *Tmem184b* antagonistically to RBFOX2 in the diabetic heart.

A) Diagram of PTBP1 (green boxes), CELF1 (blue box), and RBFOX2 binding sites (red box) within the *Tmem184b* pre-mRNA derived from CLIP-seq data and PTBP1 motif analysis. **B)** AS analysis of endogenous *Tmem184b* exon 9 in H9c2 cells transfected with scrambled (Control) or RBP specific siRNA pools (PTBP1/PTBP2^{siPool} or RBFOX2^{siPool}) (n=3) and transfected with GFP (Control), PTBP1^{GFP} or RBFOX2^{GFP} (n=3). Data represent means ± SD. Statistical significance was calculated using one-way ANOVA to compare three different groups. P-values are represented as *** < 0.001, ** < 0.01

Conclusion

We have previously shown that RBFOX2 and CELF1 are contributors to AS dysregulation in the diabetic heart [121]. In this study, our goal was to determine whether other RBPs play a role in AS changes in the diabetic heart. Indeed, both computational and molecular analyses identified a splicing change in PTBP1 in diabetic hearts. PTBP proteins are splicing repressors with well-defined roles in cancer, neuronal and heart development, and pluripotency of embryonic stem cells [211, 244, 246-249]. Developmentally regulated AS events in the heart are enriched for adjacent PTBP1 binding sites [226]. However, PTBP1's role in AS regulation in diseased hearts is not clear. Here, we provide evidence that PTBP1 contributes to AS changes in the diabetic heart (Fig. 6). Here, we show for the first time that a group of PTBP1 targets are affected in diabetic hearts.

Importantly, we found that PTBP1 undergoes a splicing change in diabetic hearts that generates a spliced variant with less repressive splicing function. Consistent with this finding, a well-known *Tpm2* alternative exon repressed by PTBP1 was more included in diabetic hearts (Fig. 6). Diabetes-induced PTBP1 spliced isoform lacking exon 8 in mouse (exon 9 in human) has been shown to display less repressive splicing function [99, 241, 242] and activation of a group of brain-specific AS networks during neurogenesis [99]. We showed that diabetes-induced spliced variant of PTBP1 displays less repressive function (Fig. 7). In addition, this PTBP1 spliced variant was more favored in the newborn mouse heart (Fig. 5D). Newborn heart undergoes hypertrophic growth during post-natal heart development that increases as an adaptation to stress in adult hearts, but overtime hypertrophy can lead to heart dysfunction [250]. Cardiac hypertrophy is associated with adverse outcomes in diabetic patients [251]. Since we found the PTBP1 spliced variant is

aberrantly expressed in adult diabetic hearts, it is possible that expression of this PTBP1 variant might contribute to cardiac hypertrophy by activating newborn-like AS patterns. In support of this, PTBP1 targets that are mis-spliced in diabetic hearts are within the MAPK signaling pathway, which has a major role in cardiac hypertrophy.

Our results indicate that PTBP1, CELF1, and RBFOX2 are all altered in diabetic hearts [71, 121]. PTBP1 is a well-known repressor of exon inclusion [74-78, 99, 238, 239], but it has been shown to also activate exon inclusion [83, 240]. PTBP1 has been shown to repress differentiated muscle-specific alternative exons [73, 76, 204, 206, 207, 211, 252]. PTBP1 and CELF1 similarly regulate AS of some targets [206], but they also antagonistically regulate AS [207, 208]. PTBP1 and RBFOX2 antagonistically control AS [206, 209]. CELF1 and RBFOX2 oppositely regulate AS [64, 206, 234]. Using the *Tmem184b* exon 9 AS as a model, we found that both CELF1 [144] and PTBP1 are repressors and RBFOX2 is an activator of *Tmem184b* exon 9 inclusion. Thus, we propose the model that modulation of RBFOX2, CELF1, and PTBP1 jointly contribute to genome wide AS changes in the diabetic heart.

Chapter 4: RBFOX2 mutations contribute to dysregulated alternative splicing in hypoplastic left heart syndrome

Introduction

HLHS is a congenital heart defect (CHD) that encompasses a range of malformations due to underdevelopment of the left side of the heart and aorta [195]. It is often a fatal defect that affects 2.6 out of 10,000 births in the USA and accounts for ~25% of deaths among infants with CHDs [197, 198]. In HLHS patients, the left ventricle, aortic and mitral valves, and the ascending aorta are not fully formed, resulting in severely compromised systemic circulation [195]. Without surgical intervention, HLHS is exceedingly fatal in the first few days of life. The two widely accepted hypotheses for HLHS etiology are 1) dysregulation of signaling pathways and gene regulatory networks important for normal heart development [253-255] and 2) aberrant changes in blood flow during cardiogenesis [256-259]. Currently, the only treatments available are either cardiac transplantation or staged functional single ventricular palliation where the right ventricle (RV) pumps blood to the rest of the body and lungs [260, 261]. In HLHS patients who undergo staged reconstruction, the burden on the RV overtime can cause lifelong cardiac complications including arrhythmias and pathological hypertrophy leading to heart failure [260, 262, 263]. The mechanisms underlying left heart hypoplasia during embryogenesis and RV complications in the postnatal period are unknown. Thus, there is an urgent need to elucidate these mechanisms.

Dysregulation of AS and mRNA levels has been identified in HLHS patient hearts [264], but the molecular mechanisms responsible for these changes are unknown. AS regulator RBFOX2 is significantly associated with the HLHS phenotype [203]. In one of

the largest CHD patient genetic studies (1,213 parent offspring trios), exome sequencing identified multiple damaging *de novo* mutations that accounted for 20% of patients with CHD associated with other coexisting diseases and 2% of patients with isolated CHD. These damaging do novo mutations occurred in 4,420 genes that are highly expressed in the heart. D*e novo* mutations in 392 of these genes are expected to contribute to CHD. Twenty-one of these genes had multiple damaging *de novo* mutations. Among these, three damaging *de novo* RBFOX2 mutations (nonsense, frameshift, and splice site) were identified in HLHS patients among other CHDs (Illustration 8) [153]. Additionally, another study identified a CHD proband with a RBFOX2 *de* novo copy number loss also associated with HLHS [265]. It is unknown how these individual mutations impair RBFOX2 function in RNA metabolism and lead to HLHS. Based on the type and location of the mutation, it is predicted that the nonsense and frameshift mutation lead to truncation of the protein.

Previous work from our lab has determined that RBFOX2 is a major contributor to transcriptome changes identified in HLHS patients [152]. Sixty-nine percent of 1348 differentially expressed transcripts in HLHS patient RVs are *in vivo* targets of RBFOX2 identified by crosslinking-immunoprecipitation followed by high throughput RNA sequencing (CLIP-seq) [152]. Compared to this, only about 7% of genes in human embryonic stem cells are *in vivo* targets of RBFOX2 [110]. Notably, gene ontology (GO) analysis revealed that several of the RBFOX2 targets are structural/cytoskeletal genes, cardiac transcription factors, cell cycle regulators, and RBPs that are important for cardiogenesis [152].

Of 1348 differentially expressed transcripts in HLHS, 934 exhibited RBFOX2-CLIP clusters in intronic and exonic sequences consistent with its role as an AS regulator.

We have previously shown that the RBFOX2 nonsense mutant forms cytoplasmic puncta and is unable to regulate AS of one of its targets consistent with aberrant AS patterns identified in HLHS patients. Additionally, we found that RBFOX2 displayed a lower molecular weight in HLHS patient right ventricles indicating that it may in fact be truncated in patients consistent with the frameshift and nonsense mutant predictions [152]. However, further analysis is needed to elucidate the role of the HLHS specific RBFOX2 mutants in dysregulated AS.

In this study, I generated stable cells expressing HLHS specific RBFOX2 mutants by introducing cDNA encoding these mutants. I found that the HLHS-specific RBFOX2 mutants are expressed similarly to each other in both the nucleus and cytoplasm. I demonstrate that the nonsense and frameshift RBFOX2 mutants are unable to control splicing of their target genes. Further, my preliminary data suggest that the nonsense mutant might interact with a subset of proteins that wildtype RBFOX2 does not interact with.

Results

Successful generation of inducible stable cells expressing HLHS specific RBFOX2 mutants

To characterize the functional effects of the HLHS specific RBFOX2 mutants, we generated tetracycline (doxycycline) inducible human 293 stable cells expressing flag-tagged wildtype, nonsense, and frameshift RBFOX2 along with an additional RBFOX2 RRM mutant. The RBFOX2 RRM mutant is unable to bind RNA and was used as a negative control, while WT RBFOX2 was used as a positive control. I also created an empty vector stable cell line to use as an additional negative control. RBFOX2 was cloned into the pcDNA5/FRT/TO plasmid and co-transfected with pOG44 recombinase into the

Flp-in T-REx 293 host cell line. Cells stably expressing the constructs were selected in 100μg/mL hygromycin as previously described [99]. The Flp-in T-Rex 293 cell line system allows for integration of the constructs into the same locus in every cell. This allows us to not have to select individual clones and eliminates the need to use more than one clonal cell line. After 24-hours of doxycycline (1μg/mL) induction, western blot analysis with anti-Flag and anti-RBFOX2 antibodies revealed successful induction of RBFOX2 WT and mutants. Importantly, the nonsense and frameshift RBFOX2 mutants were truncated and displayed lower molecular weight as expected (Figure 9). We noticed that the nonsense mutant was expressed at lower levels in these stable lines. However, the nonsense mutant expression level is still well above endogenous RBFOX2 levels, which are barley detectable by western blot in our model system.

We next verified that the expression of RBFOX2 in these stable cells was tightly regulated by tetracycline. The expression of RBFOX2 was only present when induced by tetracycline (1μg/mL) or doxycycline (1μg/mL). Wildtype, RRM, nonsense, and frameshift RBFOX2 were not detectable in un-induced cells indicating that expression of RBFOX2 was not leaky (Figure 10).

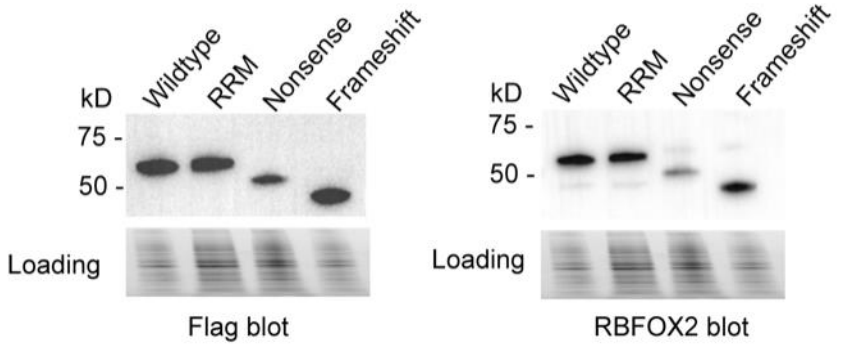

Figure 9. Molecular weight of RBFOX2 mutants is altered.

Validation of RBFOX2 molecular weight by Western blot using an antibody against Flag and RBFOX2. Total protein staining was used to confirm even protein loading.

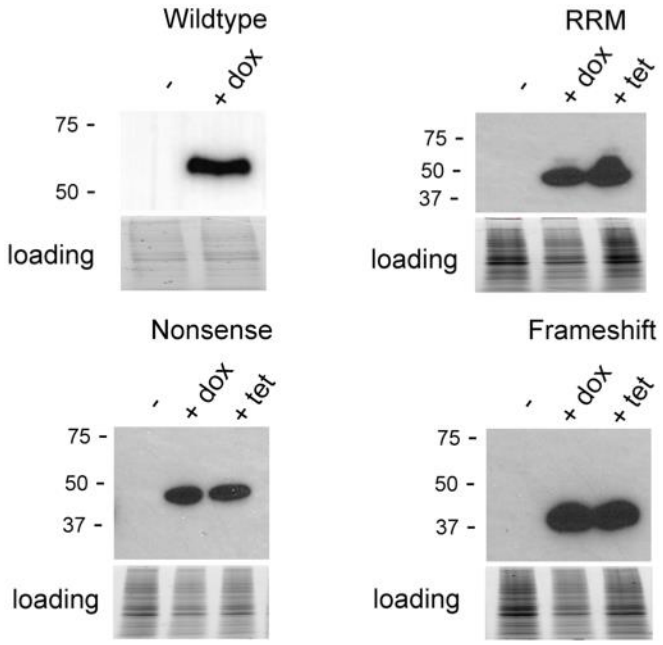

Figure 10. Validation of inducible flag-RBFOX2 expression.

Validation of inducible RBFOX2 expression by Western blot using an antibody against Flag. Total protein staining was used to confirm even protein loading.

RBFOX2 mutants are found in both the nucleus and cytoplasm

Previously published data from our lab suggest that the subcellular distribution of RBFOX2 is altered in HLHS patients [152]. Additionally, ectopic expression of a GFP-tagged RBFOX2 nonsense mutant revealed aberrant localization to punctate bodies in the cytoplasm (in contrast, wildtype RBFOX2 is mostly nuclear). Thus, we performed a nuclear-cytoplasmic fractionation to look at the localization of RBFOX2 in the 293 stable cells. Western blot of protein lysates from cytoplasmic and nuclear fractions using antibodies against alpha-tubulin as a cytoplasmic marker and Histone H3 as a nuclear marker revealed no cross-contamination in the nuclear and cytoplasmic extracts. Western blot analysis of the RBFOX2 mutants with anti-flag antibody revealed that wildtype, nonsense, frameshift, and RRM RBFOX2 are expressed in both the nucleus and cytoplasm. It appears that RBFOX2 frameshift mutant is localized slightly more to the nuclear fraction and for unknown reasons manifests as a doublet on the immunoblot (Figure 11). However, these data are derived only from one replicate and will need further replicates to confirm this finding.

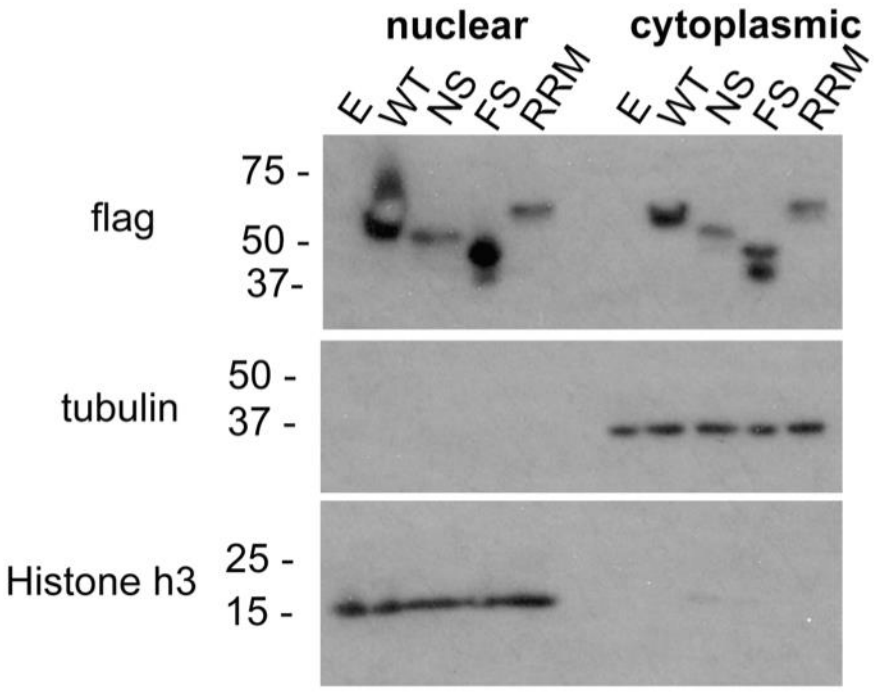

Figure 11. RBFOX2 mutants are found in both the cytoplasm and nucleus.

Western blot analysis of nuclear-cytoplasmic fractionation of RBFOX2 mutant stable cells using an antibody against flag. E= empty vector, WT= wildtype, NS= nonsense, FS= frameshift, RRM- RNA recognition motif mutant.

RBFOX2 mutants are unable to control splicing of target genes

Previous work from our lab has identified that a large group of transcripts altered in HLHS patient hearts have RBFOX2 binding sites. Unpublished work from our lab has identified new targets of RBFOX2 that undergo significant splicing changes in RBFOX2 knockout embryos. The CTD of RBFOX2 has binding sites for splicing regulators and is important for splicing. Importantly, the HLHS specific nonsense and frameshift mutants are predicted to truncate the protein at the CTD. Therefore, we investigated how the HLHS specific RBFOX2 mutants affected AS of RBFOX2 targets. Similar to the RRM mutant RBFOX2, we found that the HLHS specific RBFOX2 mutants are unable to control splicing of five target genes tested (Ank2, Fat1, Fam126, ECT2, and Abi1). Wildtype RBFOX2 did not regulate AS of ARNT exon 5. Therefore, the RBFOX2 mutants did not show a lack of splicing control for this event (Figure 12).

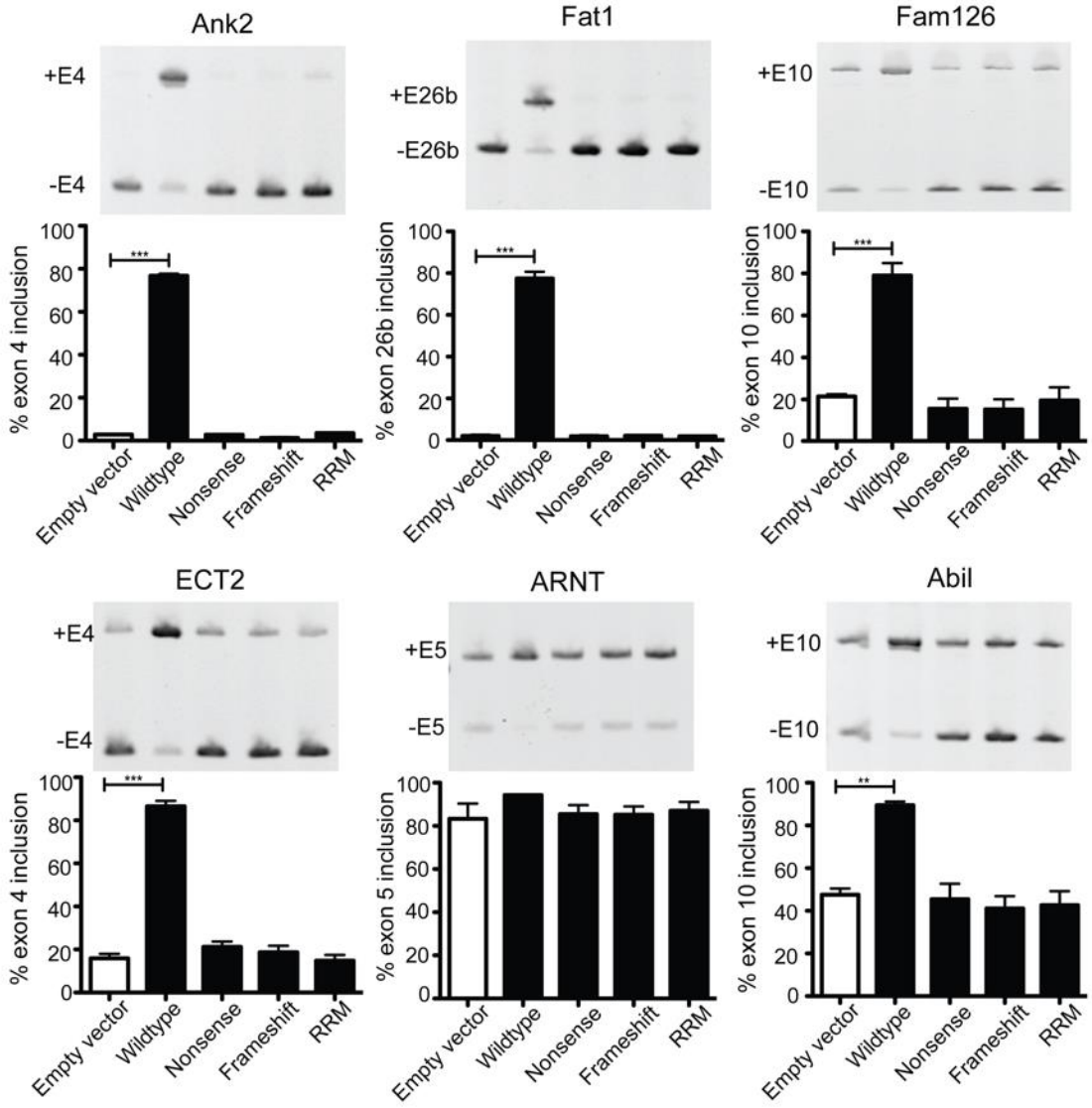

Figure 12. RBFOX2 mutants are unable to control splicing of target genes.

Six target genes were tested, including Ank2, Fat1, Fam126, ECT2, ARNT, and Abi1. Statistical significance was calculated using one-way ANOVA to compare four different groups. P-values are represented as *** < 0.001, ** < 0.01, * < 0.05.

Interactions of HLHS specific RBFOX2 mutants with other proteins

HLHS-specific RBFOX2 nonsense and frameshift mutants are predicted to truncate the CTD domain of this protein, which is important for interaction with splicing factors.

Therefore, it is likely that these mutants lose interactions with proteins important for native RBFOX2 function. We performed a comparative global proteomics analysis to identify the proteins wildtype, frameshift, and nonsense RBFOX2 might interact with. Using the 293 stable cells, we performed flag immunoprecipitation (IP) followed by Western blot to validate the pulldown. A FLAG immunoprecipitation kit (Sigma FLAGIPT1-1KT) was used to pull down FLAG-tagged WT, nonsense, frameshift or RRM mutant RBFOX2 expressed in Flp-in T-REx 293 cells per the manufacturer's protocol. DNase was added to the lysates to ensure the DNA was broken down. Lysates were pre-cleared with mouse IgG agarose beads for 2 hours prior to flag immunoprecipitation. Similar levels of wildtype, RRM, and frameshift RBFOX2 were pulled down. However, the nonsense mutant was expressed at a lower level, so the pull-down of this mutant was proportionally lower after the IP (Figure S2).

We next performed proteomics to determine if there is differential binding of RBFOX2 to its binding partners. Proteomics was performed at the University of Texas Medical Branch Mass Spectrometry Facility. Peptide mixtures were analyzed by nanoflow liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) using a nano-LC chromatography system (UltiMate 3000 RSLCnano, Dionex), coupled on-line to a Thermo Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA) through a nanospray ion source (Thermo Scientific). For the proteomics, we used a very stringent cutoff with a minimum of 5 peptides and a 0.3% false discovery rate (FDR) due to several experimental limitations including high background and low total spectra counts. We ran the empty vector stable cells as a negative control and identified a high background in these cells despite preclearing the lysate with mouse IgG agarose beads. Therefore, we

eliminated all proteins that were present in the negative control empty vector cells as this was considered background. After this, we identified 117 proteins in total. Wildtype RBFOX2 interacted with 32 of these proteins. Nonsense RBFOX2 interacted with 23 proteins in common with wildtype while frameshift only interacted with 6 common proteins. As expected the frameshift RBFOX2 mutant interacted with fewer proteins than wildtype RBFOX2. Interestingly, the nonsense RBFOX2 mutant interacted with more proteins than wildtype RBFOX2 and had 83 novel interactions (Figure 13). A heat map of these interactions is shown in Figure 14. There was a similar depth of coverage between the nonsense, frameshift, and wildtype RBFOX2 samples. However, it is important to note that the normalized total spectra count for the proteins identified in these samples was very low with the highest ones only reaching 15. This may indicate that the overall protein abundance was very low in these samples and is a limitation of this study and that the binding partners identified might not be relevant. Studies needed to be repeated with positive controls (known RBFOX2 targets) for IP.

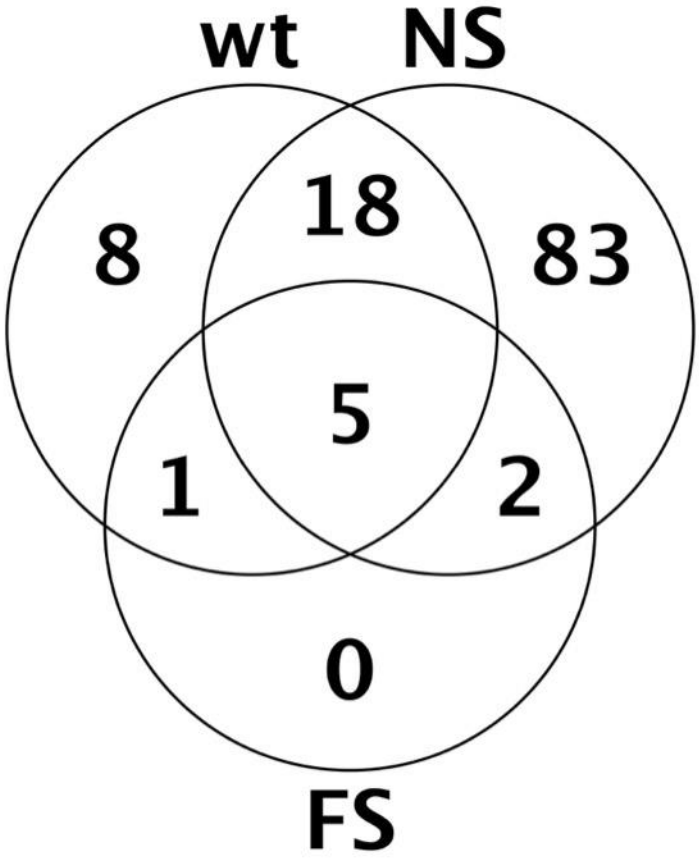

Figure 13. Comparative analysis of RBFOX2 protein interactions.

Proteins were identified at a minimum of 5 peptides and a 0.3% FDR.

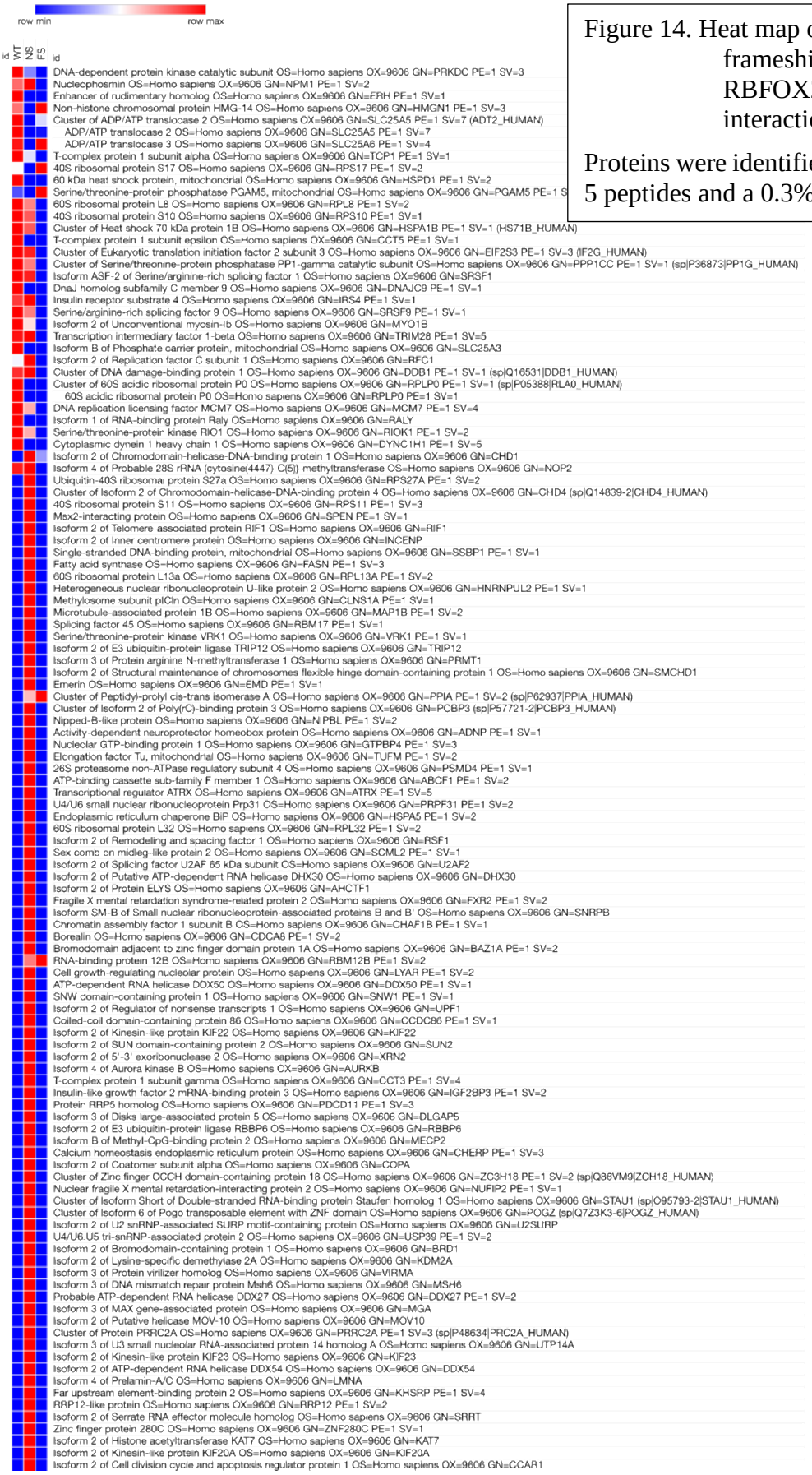

Figure 14. Heat map of wildtype, frameshift, and nonsense RBFOX2 protein interactions.

Proteins were identified at a minimum of 5 peptides and a 0.3% FDR.

With these limitations in mind, we performed KEGG pathway analysis of the 83 novel RBFOX2 nonsense mutant interactions using the Database for Annotation, Visualization and Integrated Discovery (DAVID) to identify what they may be involved in. Top categories for RBFOX2 nonsense mutant novel interactions (total: 88) are spliceosome, ribosome, and mismatch repair (Table 2).

Table 2. KEGG pathway categories for RBFOX2 nonsense mutant novel interactions.

Category	Term	RT	Genes	Count	%	P-Value	Benjamini
KEGG_PATHWAY	Spliceosome	RT		11	9.6	2.8E-8	2.1E-6
KEGG_PATHWAY	Ribosome	RT		8	7.0	5.0E-5	1.9E-3
KEGG_PATHWAY	Mismatch repair	RT		3	2.6	1.2E-2	2.6E-1
KEGG_PATHWAY	Ribosome biogenesis in eukaryotes	RT		4	3.5	2.5E-2	3.8E-1
KEGG_PATHWAY	DNA replication	RT		3	2.6	2.8E-2	3.5E-1

Conclusion

In this study, we analyzed the functional consequences of HLHS specific RBFOX2 mutants in regard to their subcellular localization, role in AS, and interactions with other proteins. Previously we have shown that the RBFOX2 nonsense mutant forms cytoplasmic puncta and is unable to regulate AS of its targets. In this study, we also found that the HLHS specific nonsense and frameshift RBFOX2 mutants were unable to regulate splicing of their targets. However, we did not find that the mutants displayed altered cellular localization, suggesting that factors other than nuclear localization impact RBFOX2's function in AS. Further, the HLHS-specific RBFOX2 mutants were unable to regulate AS similar to the RRM mutant. This indicates that RBFOX2 needs more than just RNA binding to regulate AS.

We found that wildtype, nonsense, and frameshift RBFOX2 were significantly overexpressed in these stable cells compared to endogenous levels. This is important because it allowed us to perform these experiments without knocking down endogenous RBFOX2 as its predicted effect was expected to be negligible. However, it is important to keep in mind that these constructs are exceedingly overexpressed and this may be a

limitation of the study. Future work should include a dose response curve to identify a concentration and timing of doxycycline that induces more physiologically meaninful RBFOX2 levels.

It is known that the RBFOX2 CTD is important for interaction with other splicing factors and that RBFOX2 regulates splicing as part of the large assembly of splicing regulators (LASR) [109, 113]. In this study, we found that the nonsense and frameshift RBFOX2 mutants displayed lower molecular weight likely due to the predicted truncation at the CTD. Therefore, we propose a model where CTD truncation of RBFOX2 prevents its association with splicing factors and ability to regulate AS.

To further analyze this model, we investigated which proteins each RBFOX2 mutant interacts with. As expected, the frameshift mutant displayed fewer interactions than wildtype RBFOX2. However, unexpectedly, the nonsense mutant displayed more interactions than wildtype RBFOX2. To further elucidate the role of the nonsense mutant in HLHS, we performed a KEGG analysis of its novel interactions. The top category was spliceosome followed by ribosome and mismatch repair. This suggests that the RBFOX2 nonsense mutant interacts with more spliceosome components than the wildtype RBFOX2. This is interesting because RBFOX2 is a well-known AS regulator and we recapitulated its ability to regulate splicing of its targets in this study. However, the nonsense RBFOX2 mutant was unable to regulate splicing of its targets even though it appears to be interacting with more spliceosome components. This may indicate that the nonsense RBFOX2 mutant binds and inhibits these spliceosome factors, ultimately leading to dysregulated AS. However, this will need to be further investigated. It is important to note the limitations of this proteomics study. The first limitation of the proteomics was the high background present in the empty vector sample. The second limitation was the low normalized spectra counts for all of the identified proteins indicating generally low abundance. These limitations make it hard to conclusively determine that the nonsense RBFOX2 mutant is

interacting with more proteins than the wildtype, and this issue must be addressed in future investigations.

Chapter 5 Summary of Dissertation

Overview

Aberrant gene expression is a hallmark of cardiovascular diseases. Gene expression can be regulated transcriptionally and post-transcriptionally. RBPs influence post-transcriptional gene expression through controlling AS, polyadenylation, 5' capping, stability, localization, and translation of mRNA [51]. However, the molecular mechanisms that control post-transcriptional gene expression in cardiovascular diseases are not as well understood.

A key component in cardiovascular disease is the reversion from an adult splicing program to a fetal program [71, 123, 126, 127], which underscores the importance of RBPs that control developmentally regulated AS transitions in the heart. Thus, we hypothesized that RBPs contribute to aberrant gene expression in cardiovascular diseases through regulation of AS. My objective was: A) to identify which RBPs regulate aberrant gene expression in cardiovascular diseases specifically in diabetic heart disease and HLHS, and B) to examine how changes in RBP expression and function contribute to these changes. First, I focused on identifying the AS regulators responsible for AS dysregulation in the diabetic heart. Second, I focused on how known mutations in an AS regulator contribute to gene expression changes in HLHS. The work presented here supports the hypothesis that RBPs contribute to aberrant gene expression in cardiovascular diseases through regulation of AS. Our results show that changes in RBP expression and function can contribute to gene expression changes through dysregulated splicing of thousands of targets. Therefore, this work will significantly contribute to our understanding of the molecular mechanisms driving gene expression changes in cardiovascular diseases. Importantly, this research

contributes to a foundation in the search for new therapeutic targets for treatment of these diseases in the future by identifying RBPs that contribute to gene expression changes in cardiovascular diseases. These RBPs may be targets to restore splicing of their many downstream targets.

We have found extensive AS dysregulation in the diabetic heart. In the first section, we sought to determine which RBPs contributed to this AS dysregulation and found that RBFOX2, PTBP1, and CELF1 contribute to dysregulated AS in the diabetic heart. Our previous data indicated that CELF1 was upregulated in diabetic hearts, but the functional consequences on gene expression changes in the diabetic heart were unknown [71]. Therefore, we systematically analyzed which aberrantly spliced genes in the diabetic heart are regulated by CELF1. We found that 138 CELF1 targets were aberrantly spliced in the T1D heart. Importantly, these CELF1 targets had roles in muscle contraction and PKG signaling. We also found that disrupting the CELF1 binding site impaired AS regulation by CELF1 [144]. Our results indicate that CELF1 contributes to dysregulated AS and gene expression in the diabetic heart.

PTBP1 is expressed in the heart, and its binding sites are found in genes that undergo coordinated AS changes during heart development [63, 171, 172]. It was unknown if PTBP1 was modulated in T1D hearts. Here, we found that a developmentally regulated spliced variant of PTBP1 that is normally expressed in newborn hearts is aberrantly expressed in diabetic hearts. This PTBP1 isoform lacking exon 8 (exon 9 in humans) has been reported to have a reduced repressive function [99, 241, 242]. We identified a group of 58 PTBP1 targets that are dysregulated in diabetes and demonstrated that the PTBP1 isoform with reduced repressive function regulates AS of these transcripts [180].

Importantly, these PTBP1 targets have roles in RNA transport, MAPK signaling, and endocytosis [180]. Our results indicate that PTBP1 contributes to dysregulated AS and gene expression in the diabetic heart.

As RBFOX2 and CELF1 are both modulated in the T1D heart and contribute to dysregulated AS we hypothesized that changes in their expression could impact PTBP1 AS [71, 121, 144]. We found that depletion of CELF1 and RBFOX2 led to exclusion of exon 8 in PTBP1 similar to the pattern observed in the T1D mouse heart. Importantly, this result is consistent with the decreased expression of CELF1 and upregulation of a dominant negative RBFOX2 in the diabetic heart [71, 121]. This suggests that RBFOX2, CELF1, and PTBP1 may cross-regulate each other's expression in the diabetic heart. Importantly, many AS events in the T1D heart were co-regulated by RBFOX2, CELF1, and PTBP1. Thus, we propose a model that low RBFOX2 and PTBP1 splicing activities and increased CELF1 protein levels jointly affect AS on a genome-wide scale and increase the complexity of transcriptome regulation in the heart.

In the second section, I described the role of RBFOX2 in HLHS. RBFOX2 was previously found to be significantly associated with HLHS phenotype. Three damaging *de novo* RBFOX2 mutations (nonsense, frameshift, and splice site) were identified in HLHS patients. It is largely unknown how these individual mutations impair RBFOX2 function and contribute to HLHS pathogenesis. Previous work from our lab has determined that RBFOX2 is a major contributor to transcriptome changes identified in HLHS patients [152]. We have also shown that the RBFOX2 nonsense mutant forms cytoplasmic puncta and is unable to regulate AS of one of its targets [152]. However, further analysis was needed to elucidate the role of the HLHS-specific RBFOX2 mutants in dysregulated AS.

My preliminary findings on the functional consequences of RBFOX2 mutations in HLHS indicate that the RBFOX2 mutants are unable to control splicing of their targets despite generally exhibiting similar cellular localization to the wild-type RBFOX2 protein. Further, mutants lacking the CTD of the protein displayed altered interactions with their binding partners. Indeed, the nonsense RBFOX2 mutant appeared to interact with more proteins than wildtype RBFOX2. Thus, it is clear that the HLHS specific mutants alter RBFOX2 function in RNA metabolism and contribute to gene expression changes.

Significance

This dissertation focused on two cardiovascular diseases that without therapeutic intervention can lead to death. There is a pressing need for novel therapies to prevent diabetic heart disease and improve the long-term survival of infants with HLHS. Genome-wide expression changes and aberrant AS regulation have been identified in both diseases. The importance of post-transcriptional gene regulation and its significance in heart development and cardiac complications have been long realized, but the mechanistic details are largely not well understood.

Findings from this dissertation revealed post-transcriptional networks that are responsible for aberrant gene expression in diabetic heart disease and HLHS. Importantly, we identified RBFOX2, CELF1, and PTBP1 as regulators of these changes. RBFOX2 forms a dominant negative isoform before diabetic cardiomyopathy is seen, suggesting it is causal for the disease. Our findings provide novel targets for therapeutic intervention in diabetic heart disease and HLHS. There is potential to use oligonucleotide-based therapies to restore splicing of RBFOX2 and PTBP1 such that a dominant negative RBFOX2 and a less repressive PTBP1 are not formed. Targeting just one of these RBPs has the ability to restore hundreds of targets that are dysregulated in these diseases. We also know that CELF1 expression is increased in the diabetic heart via phosphorylation and that this

phosphorylation is required for its regulation of splicing events altered in diabetes. Additionally, we know that CELF1 regulates 138 transcripts mis-spliced in diabetes. Thus, therapies targeting CELF1's phosphorylation could also rescue hundreds of targets dysregulated in diabetic heart disease. Phosphatase therapies may be able to correct CELF1 expression and downstream alternative splicing. Thus, targeting these 3 RBPs may help ameliorate the genome-wide expression changes and cardiac dysfunction in diabetic heart disease and HLHS.

The three damaging *de novo* RBFOX2 mutations (nonsense, frameshift, and splice site) identified in HLHS patients were originally believed to be loss-of-function [153]. However, in this study, I found that the nonsense mutant, unexpectedly, appeared to be characterized by more interactions than wildtype RBFOX2. In contrast and as expected, the frameshift mutant was characterized by fewer interactions. Although these results must be interpreted with extreme caution, they may suggest that the RBFOX2 nonsense mutant may represent a gain-of-function or even neomorphic allele. This would be a paradigm shift in the understanding of RBFOX2 in HLHS and may help us better understand how the mutation leads to HLHS phenotype. Consistent with this notion, unpublished results from Dr. Sunil Verma in our lab demonstrate that RBFOX2 knockout does not fully recapitulate the HLHS phenotype. Thus, exploring the potential gain of function for the RBFOX2 nonsense mutation may help us better understand the molecular mechanism leading to HLHS.

Finally, we found that a developmentally regulated spliced variant of PTBP1 was increased in expression in the diabetic heart. Specifically, exon 9 of PTBP1 was less included. When exon 9 is skipped, PTBP1 is less repressive. We found that this exon was also developmentally regulated in the heart, such that it was more included in the embryonic and adult heart. Importantly, exon 9 skipping reduces PTBP1 repression in neurogenesis, leading to activation of brain-specific AS patterns. This exon was also found to contribute to evolutionary difference between species in developmentally regulated AS

[99]. This is the first evidence that exon 9 skipping is important for heart development and diabetic heart disease. This suggests that this event may have evolved to tightly regulate transitions in both brain and heart development and that dysregulation of it can contribute to brain and heart disease.

Future Directions

Role of CELF1 and PTBP1 in diabetic heart disease

While it is clear that CELF1 and PTBP1 contribute to AS dysregulation in diabetes, further research is required to fully characterize their contribution to diabetic heart disease. First, the role of CELF1 and PTBP1 in the cardiac pathogenesis of diabetes needs to be determined. We found that a developmentally regulated spliced variant of PTBP1 with less repressive function is elevated in the T1D heart. The engineering and characterization of animal models that recapitulate the upregulation of CELF1 and the developmentally spliced variant of PTBP1 will be critical for this analysis. These models may help determine if the AS dysregulated by these RBPs directly contributes to cardiac pathogenesis. Further, global analysis of the effect of this PTBP1 isoform on its targets is needed to confirm that it the isoform is universally less repressive. Rescue experiments where wildtype PTBP1 is re-expressed will be needed to see if it rescues diabetes-induced AS changes and improves cardiac complications. This will help us better understand the role this PTBP1 isoform plays a role in cardiac pathogenesis.

Work from this dissertation shows that RBFOX2, CELF1, and PTBP1 co-regulate alternative splicing of some targets [144, 180]. Future work will need to determine how these RBPs co-regulate AS of these targets. Analysis of the *Tmem184b* gene demonstrated that all three RBPs influenced AS but not necessarily as one would predict from the general splicing code. To further complicate this matter, we also found that the three RBPs regulate the AS of one another. This highlights the complexity involved in determining how they

co-regulate AS events important for diabetic heart disease, although this remains an important endeavor.

Role of RBFOX2 in hypoplastic left heart syndrome

In this work, we found that nonsense and frameshift RBFOX2 mutants were unable to regulate splicing of five established RBFOX2 targets. Future work is needed to determine how HLHS patient-specific RBFOX2 mutations globally affect splicing. This will require conducting a global analysis with high-depth RNA sequencing to fully characterize AS variation in cells expressing the two mutants. Additionally, CLIP-sequencing approaches should be initiated to determine if the RBFOX2 mutants bind to different RNAs.

Finally, while our results must be interpreted with caution, our proteomics analysis is consistent with the conclusion that the nonsense RBFOX2 mutant interacts with more proteins than wildtype RBFOX2. Since it was has been consistently assumed that all of the HLHS-specific RBFOX2 mutants result in a loss-of-function, a potential for neomorphic function will need to be explored for the nonsense mutant. If this novel function can be robustly established, analysis of the structural differences between nonsense and frameshift RBFOX2 mutants will be needed to determine what underlies this change in function. On the other hand, it must be noted that all three RBFOX2 mutants underlie similar HLHS phenotypes. Thus, further research into how each of these RBFOX2 mutations, perhaps via different mechanisms, lead to the same phenotype is crucial.

Appendix A: Supplementary Figures

Figure S1

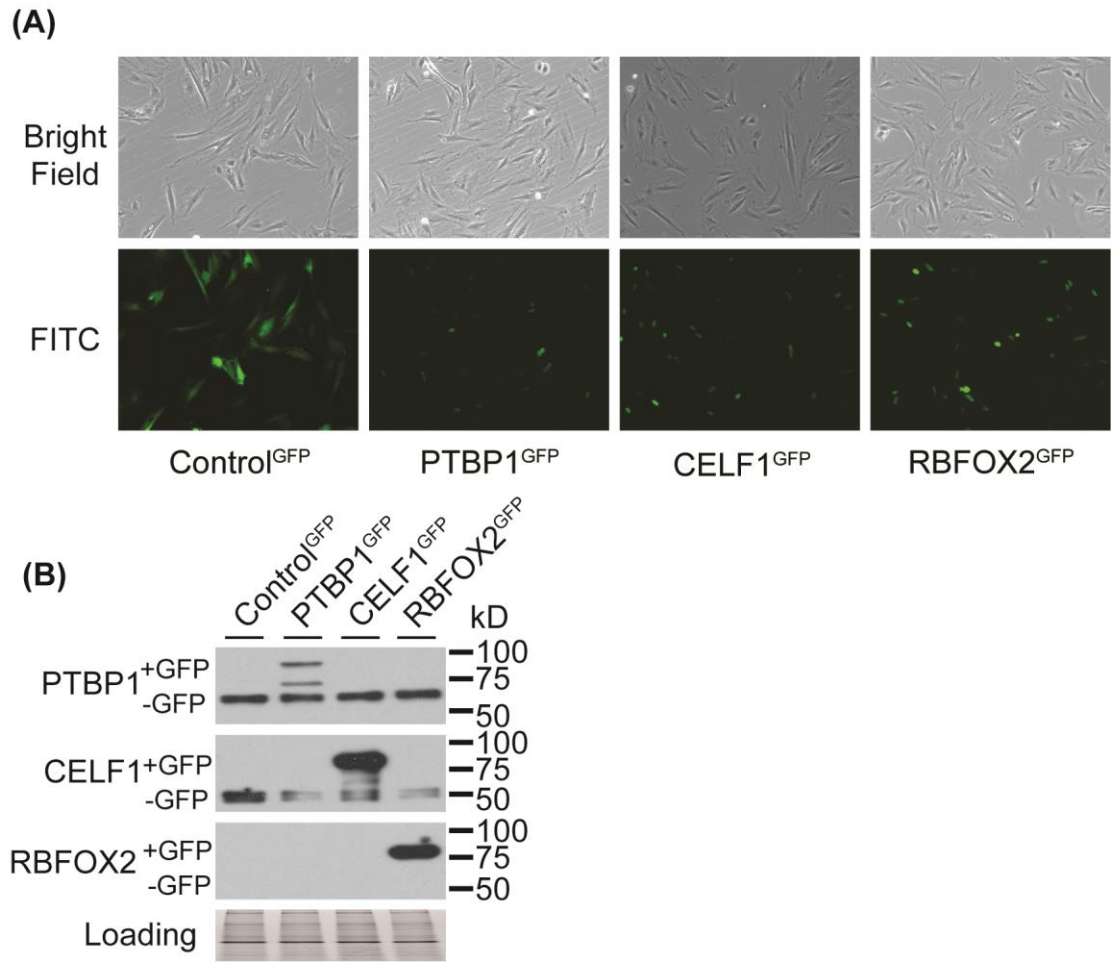

Figure S1. Validation of PTBP1, CELF1, and RBFOX2 overexpression.

A) Representative FITC and bright field images of GFP (Control), $PTBP1^{GFP}$, $CELF1^{GFP}$, and $RBFOX2^{GFP}$ in transfected H9c2 cells (n=3) indicating successful transfection. B) Validation of PTBP1, CELF1, and RBFOX2 GFP overexpression using antibodies (Abs) against PTBP1, CELF1, and RBFOX2. Total protein staining was used to confirm even protein loading

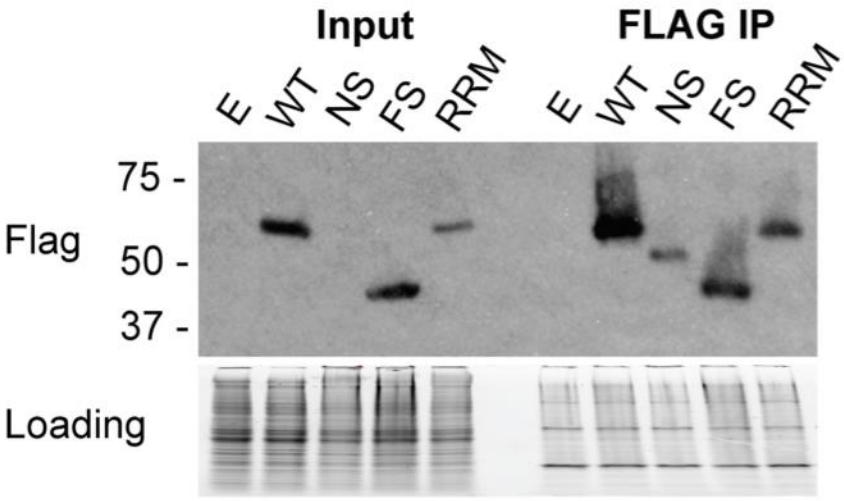

Figure S2. Validation of RBFOX2 pulldown.

Validation of RBFOX2 WT, nonsense, frameshift, and RRM pull down using an antibody against flag. Total protein staining was used to confirm even protein loading

Appendix B: Materials and Methods

ANIMAL EXPERIMENTS

The animal models used for this study are streptozotocin (STZ)-induced T1D and non-obese diabetic (NOD). All animal experiments were conducted in accordance with the NIH Guidelines and approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee (Protocol # 1101001). T1D mouse models were described in [223, 266]. C57BL6J male mice (Jackson Laboratories) were injected with citrate buffer (vehicle control) or 60 mg/kg of STZ daily for five consecutive days. Female NOD (NOD/ShiLtJ) and ICR control mice were obtained from Jackson Laboratories [266]. Mice with hyperglycemia greater than 400mg/dL for at least 3 weeks were sacrificed, and left

ventricles were isolated for RNA extraction. For mouse heart developmental, hearts were isolated from adult (6 months old) mice, embryonic day 18 embryos, and newborn pups.

DIFFERENTIAL ALTERNATIVE SPLICING ANALYSIS

An Illumina HiSeq 1000 system was used for paired-end (2 x 15 cycles) RNA sequencing at the UTMB Next-Generation Sequencing Core facility and yielded ~200 million reads per sample [71, 121]. To identify AS changes, we performed DAS analysis as previously described in [267]. Briefly, raw RNA-Seq reads were first aligned to the mouse (mm9) genome using STAR (version 2.5.1b) [268] with default settings and only uniquely mapped reads were retained for further analysis. The number of reads for each exon and each exon-exon junction in each RNA-Seq file was computed by using the Python package HTSeq [209] with the UCSC KnownGene (mm9) annotation [269]. Dirichlet-multinomial was used to model the counts of the reads aligned to each isoform of each event [270], and the likelihood ratio test was used to test the significance of splicing changes between STZ and control mice. We calculated the q-values from the p-values in the likelihood ratio test by the Benjamini-Hochberg procedure. PSI (Percent Spliced In; Ψ) was used to evaluate the percentage of the inclusion of variable exon relative to the total mature mRNA in the splicing events [271]. The DAS events were identified under $|\Delta\Psi| > 0.05$ and $q < 0.05$.

For DEG analysis, uniquely aligned reads were retained to calculate the read counts for each gene against the UCSC KnownGene annotation (mm9), and a count table was constructed by counting the number of the reads that were aligned uniquely to each of the genes for each sample. Normalization and DEG analysis were performed using DESeq [272]. FDR adjusted q-values were then calculated using the Benjamini-Hochberg

procedure. The log2-fold changes in each comparison were also calculated for each gene. The differentially expressed genes were identified under two-fold changes and $q < 0.05$. Differential cassette exon (ES) events were overlapped with CELF1 binding sites that were extracted from the CELF1 cross-linking immunoprecipitation followed by RNA-seq (CELF1 CLIP-seq) datasets. CELF1 binding sites were examined within the 250bp intronic regions flanking the alternative exon and 50bp downstream of 5' end of the alternative exons. To determine PTBP1-binding sites on transcripts mis-spliced in diabetic hearts, we overlapped the differential cassette exon events identified in our diabetic heart RNA-seq data with the binding sites of PTBP1 that were defined by merging two sets of PTBP1 CLIP-Seq peaks from GSM2259090 and GSM2259091 [244]. PTBP1 binding sites were examined within the 250bp intronic regions flanking the alternative exon and 50bp of the 5' and 3' ends of the alternative exons.

PLASMIDS AND CLONING:

The *Tmem184b* splicing reporter was constructed as follows: pcDNA5-GFP-IL7R splicing reporter plasmid (from Mariano Garcia-Blanco) was mutated from TCTAGA to GATAGA to remove one of the two Xba1 sites. Mutated vector was then digested with Xba1 and Xho1 (Roche) and purified with the QIAquick PCR purification kit. *Tmem184b* genomic sequence that covers intron 9 (500nt)-exon9-intron 10 (316nt) or a mutant version of *Tmem184b* genomic sequence that lacks one of the CELF1 CLIP-seq peaks (62nt) in intron 10 (G-blocks from IDT) were ligated into the vector using T4 DNA ligase (Roche). All constructs were confirmed by Sanger DNA sequencing.

GENERATION OF STABLE CELL LINES

Triple flag-tagged human PTBP1 including exon 9 (PTBP1+ex9) and excluding exon 9 (PTBP1Δex9) in pcDNA5/FRT/TO expression vectors. Flp-in T-REx 293 cells plated on 6-well plates were co-transfected with 4μg pOG44 recombinase and 0.4μg PTBP1+ex9, PTBP1Δex9, or empty vector using Lipofectamine 2000 (Invitrogen). Cells stably expressing the constructs were selected in 100μg/mL hygromycin as described in [99].

Human flag-tagged RBFOX2 nonsense mutant cDNA clone and RBFOX2 mutant lacking binding activity were generated as described previously [152]. RBFOX2 frameshift mutant was generated by replacing C to CT using Quick-change site-directed mutagenesis (Agilent Technologies). Human flag-tagged RBFOX2 nonsense, frameshift, and RRM mutants were cloned into pcDNA5/FRT/TO expression vectors. Flp-in T-REx 293 cells plated on 6-well plates were co-transfected with 4μg pOG44 recombinase and 0.4μg RBFOX2 nonsense, frameshift, RRM, or empty vector using Lipofectamine 2000 (Invitrogen). Cells stably expressing the constructs were selected in 100μg/mL hygromycin as described in [99].

CELL CULTURE AND TRANSFECTIONS

Flp-in T-REx 293 cells stably expressing empty vector, Flag-tagged PTBP1+ex9, or Flag-tagged PTBP1Δex9 were transfected with a pool of 10μM PTBP1/PTBP2 targeting siRNAs (Qiagen siRNA Catalog# SI02649206 and SI04255146) or scrambled siRNA (Invitrogen AM4611) using Lipofectamine RNAiMAX (Invitrogen). 24 hours post-transfection, media was replaced, and expression of PTBP1 was induced with 1μg/mL doxycycline. Cells were harvested 48 hours after induction.

H9C2 cells were cultured and transfected as previously described [71, 121]. H9c2 cells were transfected with a pool of 10μM CELF1 specific (Invitrogen siRNA ID# s168616 and s168617), 10μM PTBP1/PTBP2 targeting siRNAs, RBFOX2 specific siRNA (Invitrogen siRNA ID# s96620), or scrambled siRNAs (Invitrogen AM4611) using Lipofectamine RNAiMAX (Invitrogen) and harvested 72hrs later. H9c2 cells plated on 150mm dish were transfected with 10μg GFP-tagged PTBP1, RBFOX2, CELF1, or 5μg GFP control plasmid using Neon nucleofection system (Invitrogen) and harvested 48 hours post-transfection. H9c2 cells were transfected with 200ng WT, or CELF1 binding site mutant *Tmem184b* splicing reporter and harvested after 72hrs.

QUANTITATIVE RT-PCR

qRT-PCR was performed as described previously [71, 121]. TRIzol (Invitrogen 15596-018) was used to extract RNA from cells and mouse hearts according to the manufacturer's protocol with the modification that RNA was precipitated overnight at -80^{0}C. RNA concentration was determined using the EPOCH Microplate Spectrophotometer (BioTek). Primer information used for qRT-PCR is provided in Supplemental Table 1.

Table S1: Primer sequences used for RT-PCR

Gene Name	Species	Gene ID	Analysis Target	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
Ablim3	Mouse	ENSMUSG00000032735	Exon 18	AAGCTCCTATGCGGATCCTT	GCAGCCCATTTCTCCTGTAG
Per1	Mouse	ENSMUSG00000020893	Exon 16	GCCTCTGATGATGACAAGCA	GTTGGGTCAGGGGCTACTGT
Gpr116	Mouse	ENSMUSG00000056492	Exon 12	GTGTCCCAGTGGGTCTTCTG	GGTCCCGGGTTATTGTTAGG

Tmem184b	Mouse	ENSMUSG00000009035	Exon 9	GGCATGCCTTCACCTACAAG	GTGGACTGCTGCGTGTACTG
Tmem184b	Rat	ENSRNOG00000022802	Exon 9	GGCATGCCTTCACCTACAAG	GTGGACTGCTGCGTGTACTG
GFP				CCACAAGTTCAGCGTGTCCG	CGTCCTTGAAGAAGATGGTG
Hdac7	Mouse	ENSMUSG00000022475	Exon 8	TTCCTCCCCCAGTAGTAGCA	CGAGGGCCTAAAGTTGAATG
Hdac7	Rat		Exon 8	TCCTCCCCCAGTAGTAGCAG	CCAAAGTTGAATGGGTCCTG
PTBP1	Mouse	ENSMUSG00000006498	Exon 8	GCATCGACTTCTCCAAGCTCACC	GGACATTAGGGACAGAGAGGC
PTBP1	Rat	ENSRNOT00000044865.7	Exon 8	GCATCGACTTCTCCAAGCTCACC	GGACATTAGGGACAGAGAGGC
PTBP1	Human	ENST00000035694810	Exon 9	CTCGCTGGACCAGACCAT	TCTGGGTTGAGGTTGCTGAC
Clip1	Human	ENST00000062078 6.4	Exon 9	CCGGGTTGAAGAAGAATCAA	CGGAGCTCAGCTACTTCCTG
Med23	Human	ENST00000354577.8	Exon 11	CCAGGGATATGGTCTGCAAT	CCCATCGTCAAACTTCTCCT

NUCLEAR-CYTOPLASMIC FRACTIONATION

The nuclear-cytoplasmic fractionation was adapted from a previously described protocol [211]. Cells grown on a 150mm plate were rinsed with PBS twice and then scraped into 500µl of lysis buffer (150mM NaCl, 50mM Tris HCl, 1mM EDTA, 1% Triton X-100, protease inhibitors, and PhosSTOP [Sigma]). Samples were kept on ice for the remainder of the procedure. Lysates were passed through a 25G needle three times. Then samples were spun at 600x g at 4° C for 15 minutes to pellet the nuclei. The supernatant was removed and kept as the cytoplasmic fraction. Pellet was then rinsed with lysis buffer and

spun at 600x g at 4° C for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 200µl lysis buffer with 1-2% SDS. Samples then underwent a heat-cool cycle at 90° C for 5 minutes. This was the nuclear fraction.

WESTERN BLOT ANALYSIS

Western blot analyses were performed as described previously [71, 121]. 30-50µg of protein per sample was separated on 10% SDS-PAGE gels and transferred to a PVDF membrane (Immobilon-P, Millipore IPVH00010). Membranes were blocked with 5% dry fat-free milk solution in PBST for 30 minutes and then incubated with primary Ab at 4°C overnight. The primary antibodies used for this study are as follows: anti-RBFOX2 (Abcam ab57154), anti-PTBP1 (From Dr. Mariano Garcia-Blanco), anti-CELF1 (Abcam ab9549), anti-Histone H3 (Abcam ab10799), anti-FLAG (Sigma F7425), and anti-α-TUBULIN (Sigma T6074). Following this, membranes were washed with PBST four times for 15-minute intervals. Finally, the membranes were incubated with HRP-conjugated secondary Abs for 2 hours. Immobilon Western chemiluminescent (Millipore WBKLS0500) or SuperSignal West Femto Chemiluminescent (Pierce 34096) HRP substrate was used to detect HRP signal by exposure to X-ray film.

FLAG IMMUNOPRECIPITATION

A FLAG immunoprecipitation kit (Sigma FLAGIPT1-1KT) was used to pull down FLAG-tagged WT, nonsense, frameshift or RRM mutant RBFOX2 expressed in Flp-in T-REx 293 cells per the manufacturer's protocol. Flp-in T-REx 293 cells were lysed in lysis buffer (150mM NaCl, 50mM Tris HCl, 1mM EDTA, 1% Triton X-100, protease inhibitors, and PhosSTOP [Sigma]). Lysates were sonicated on ice 3 times for 30-second pulses to shear DNA. DNAase was added to the lysates to ensure the DNA was broken down.

Lysates were pre-cleared with mouse IgG agarose beads for 2 hours prior to flag immunoprecipitation.

PROTEOMICS

Sample digestion

The agarose bead-bound purified Flag-tagged proteins were washed several times with 50mM TEAB pH 7.1, before being solubilized with 40uL of 5% SDS, 50mM TEAB, pH 7.55 and incubated at room temperature (RT) for 30 minutes. The supernatant containing proteins was then transferred to a new tube and reduced by making the solution 10mM TCEP (Thermo, #77720) and incubated at 65°C for 10min. The sample is then cooled to RT and 3.75 uL of 1M iodoacetamide acid added and allowed to react for 20 minutes in the dark after which 0.5uL of 2M DTT is added to quench the reaction. 5ul of 12% phosphoric acid is added to the 50uL protein solution. 350uL of binding buffer (90% Methanol, 100mM TEAB final; pH 7.1) is then added to the solution. The resulting solution is added to S-Trap spin column (protifi.com) and passed through the column using a bench top centrifuge (30s spin at 4,000g). The spin column is washed with 400uL of binding buffer and centrifuged. This is repeated three times. Trypsin is added to the protein mixture in a ratio of 1:25 in 50mM TEAB, pH=8, and incubated at 37°C for 4 hours. Peptides were eluted with 80uL of 50mM TEAB, followed by 80uL of 0.2% formic acid, and finally 80 uL of 50% acetonitrile, 0.2% formic acid. The combined peptide solution is then dried in a speed vacuum and resuspended in 2% acetonitrile, 0.1% formic acid, 97.9% water and placed in an autosampler vial.

NanoLC MS/MS Analysis

Peptide mixtures were analyzed by nanoflow liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) using a nano-LC chromatography system (UltiMate 3000 RSLCnano, Dionex), coupled on-line to a Thermo Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA) through a nanospray ion source (Thermo Scientific). A trap-and-elute method was used. The trap column is a C18 PepMap100 (300um X 5mm, 5um particle size) from ThermoScientific. The analytical column is an Acclaim PepMap 100 (75um X 25 cm) (Thermo Scientific). After equilibrating the column in 98% solvent A (0.1% formic acid in water) and 2% solvent B (0.1% formic acid in acetonitrile), the samples (2 µL in solvent A) were injected into the trap column and subsequently eluted (400 nL/min) by gradient elution onto the C18 column as follows: isocratic at 2% B, 0-5 min; 2% to 32% B, 5-39 min; 32% to 70% B, 39-49 min; 70% to 90% B, 49-50 min; isocratic at 90% B, 50-54 min; 90% to 2%, 54-55 min; and isocratic at 2% B, till 65 min.

All LC-MS/MS data were acquired using XCalibur, version 2.1.0 (Thermo Fisher Scientific) in positive ion mode using a top speed data-dependent acquisition method with a 3 second cycle time. The survey scans (*m/z* 350-1500) were acquired in the Orbitrap at 120,000 resolution (at *m/z* = 400) in profile mode, with a maximum injection time of 100 msec and an AGC target of 400,000 ions. The S-lens RF level is set to 60. Isolation is performed in the quadrupole with a 1.6 Da isolation window, and CID MS/MS acquisition is performed in profile mode using rapid scan rate with detection in the ion-trap, with the following settings: parent threshold = 5,000; collision energy = 32%; maximum injection time 56 msec; AGC target 500,000 ions. Monoisotopic precursor selection (MIPS) and charge state filtering were on, with charge states 2-6 included. Dynamic exclusion is used

to remove selected precursor ions, with a +/- 10 ppm mass tolerance, for 15 sec after acquisition of one MS/MS spectrum.

Database Searching

Tandem mass spectra were extracted and charge state deconvoluted by Proteome Discoverer (Thermo Fisher, version 2.2.0388). All MS/MS spectra were searched against a UniProt Human database (version 06-27-2018) using Sequest. Searches were performed with a parent ion tolerance of 5 ppm and a fragment ion tolerance of 0.60 Da. Trypsin is specified as the enzyme, allowing for two missed cleavages. Fixed modification of carbamidomethyl (C) and variable modifications of oxidation (M) and glycosylation were specified in Sequest.

Criteria for Protein Identification

Scaffold (version Scaffold_4.8.7, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 5 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [273]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

STATISTICAL ANALYSIS

Student t-test was used to determine statistical significance between two groups and one-way ANOVA for comparisons of more than two groups with Bonferroni correction using the Prism software.

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5/2017- present	Graduate Assistant Laboratory of Muge N. Kuyumcu-Martinez, Ph.D. University of Texas Medical Branch Galveston, TX
8/2016 – 8/2018	Tutor-Sealy Center for Environmental Health & Medicine University of Texas Medical Branch

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05/2018 – 08/2018	Freelance Writer
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12/2015 – 05/2017	Graduate Assistant
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6/2016 – 8/2016	Course Co-director
	Introduction to the Study of Biological Systems
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6/2014 – 8/2014	Course Instructor
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	Exploration Summer Programs
	Norton, MA
1/2011 – 6/2014	Associate Manager
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6/2013 – 8/2013	Summer Undergraduate Research Program Intern
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RESEARCH ACTIVITIES:

A. Areas of Interest:

1. RNA metabolism in cardiovascular development and disease.
2. Regulation of alternative splicing by RNA binding proteins.

B. Grant Support

1. American Heart Association pre-doctoral fellowship
2. NIEHS Predoctoral Fellow: NIEHS Training Grant (T32-ES07254), 2015-2017
3. Graduate Assistantship: Graduate School of Biomedical Sciences; University of Texas Medical Branch, 2014-2015

COMMITTEE RESPONSIBILITIES:

Wheaton College	Alternative Winter Break Club 2010-2014
	Organization Chair
Wheaton College	Best Buds Club 2010-2012
	Treasurer
UTMB	Global Brigades 2015-2017
	Fundraising Chair
UTMB	Biological Chemistry Student Organization 2016-2017
	Secretary
UTMB	BMB Curriculum Committee

Member

TEACHING RESPONSIBILITIES AT UTMB

A. Teaching: Graduate School (GSBS)

6/2016 – 8/2016	Course Co-Director
	Introduction to the Study of Biological Systems
9/2018 – present	Small group facilitator
	Biochemistry

B. Students/Mentees/Advisees/Trainees

9/2016 – 6/2017	Yunhao Yang	High School Student
9/2017 – 10/2017	John Miller	PhD Rotation Student
9/2017 - 5/2018	Peiru Liu	High School Student
5/2018 - 7/2018	Bobby Varghese	Medical Summer Student
5/2018 - 7/2018	Micah Kerney	Medical Summer Student
3/2018 - 5/2018	Amy Cooper	PhD Rotation Student
5/2018 - 1/2019	Xianxiu Wan	Post-doc
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MEMBERSHIP IN SCIENTIFIC SOCIETIES/PROFESSIONAL ORGANIZATIONS:

2013-2014	Beta Beta Beta National Biological Honor Society
2014-2017	American Academy of Allergy Asthma and Immunology
2016-2017	The Society of Toxicology
2013-2018	Sigma Xi
2016-present	The National Society of Leadership and Success

2017-present American Heart Association

2018-present American Medical Writers Association

HONORS:

2012 Institute for Human Infection and Immunity Award for Research Excellence

2016 Elias Hochman Research Award

2016 Dr. Mary Faggard Kanz Travel Award for Environmental Toxicology

2016 UTMB Legator Endowment Travel Award

2017 Achieving Data Quality and Integrity in Maximum Containment Laboratories Scholarship

2017 UTMB Center for Global Health Education Field Epidemiology Course Scholarship

2017 Jane Welsh Award for Excellence in Cardiovascular Research

2017 Elferink Scholarship for Academic Excellence

2018 The National Society of Leadership and Success dream support grant

2018 Jane Welsh Award for Excellence in Cardiovascular Research

2018 Betty J. William Scholarship

2018 Barbara Bowman Graduate Student Award

PUBLICATIONS:

<u>Papers</u>

1. **KarryAnne K. Belanger**, Bill T. Ameredes, Istvan Boldogh, and Leopoldo Aguilera-Aguirre, “The Potential Role of 8-Oxoguanine DNA

Glycosylase-Driven DNA Base Excision Repair in Exercise-Induced Asthma," *Mediators of Inflammation*, vol. 2016, Article ID 3762561, 15 pages, 2016. doi:10.1155/2016/3762561

2. Bing Tian, Igor Patrikeev, Lorenzo Ochoa, Gracie Vargas, **KarryAnne K. Belanger**, Julia Litvinov, Istvan Boldogh, Bill T Ameredes, Massoud Motamedi, and Allan R Brasier. "NFκB Mediates Mesenchymal Transition, Remodeling and Pulmonary Fibrosis in Response to Chronic Inflammation by Viral RNA Patterns". American Journal of Respiratory Cell and Molecular Biology, 56, pp. 506-520, 2017. doi: 10.1165/rcmb.2016-0259OC. Pubmed: 27911568.
3. **KarryAnne Belanger,** Curtis Nutter, Jin Li, Sadia Tasnim, Peiru Lu, Peng Yu, Muge N. Kuyumcu-Martinez. "Celf1 contributes to aberrant alternative splicing patterns in the type 1 diabetic heart". Biochemical and Biophysical Research Communication, Volume 503, Issue 4, 2018, Pages 3205-3211, 2018. Doi: 10.1016/j.bbrc.2018.08.126.
4. **KarryAnne Belanger**, Curtis A. Nutter, Jin Li, Peng Yu, Muge N. Kuyumcu-Martinez. "A developmentally regulated spliced variant of PTBP1 is upregulated in type 1 diabetic hearts". Biochemical and Biophysical Research Communications, Volume 509, Issue 2, 2019, Pages 384-389. Doi: 10.1016/j.bbrc.2018.12.150.

Abstracts

1. **Belanger K**, Jazmin R, and Ameredes BT. 2015. Decreases in GM-CSF Release and Intracellular Levels of GM-CSF by Human Airway Smooth

Muscle Cells in Response to Carbon Monoxide and Anoxia. J. Allergy Clin. Immunol.

2. **Belanger KK**, Spear WC, Rolls BA, Miller SD, Tian B, Vargas G, Motamedi M, Boldogh I, Brasier AR, Ameredes BT. 2016. Modulation of IL-6 Release Subsequent to Airway Poly I:C Administration. J. Allergy Clin. Immunol.
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