RBPMS2 Is a Myocardial-Enriched Splicing Regulator Required for Cardiac Function

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BACKGROUND: RBPs (RNA-binding proteins) perform indispensable functions in the post-transcriptional regulation of gene expression. Numerous RBPs have been implicated in cardiac development or physiology based on gene knockout studies and the identification of pathogenic RBP gene mutations in monogenic heart disorders. The discovery and characterization of additional RBPs performing indispensable functions in the heart will advance basic and translational cardiovascular research.

METHODS: We performed a differential expression screen in zebrafish embryos to identify genes enriched in nkx2.5-positive cardiomyocytes or cardiopharyngeal progenitors compared to nkx2.5-negative cells from the same embryos. We investigated the myocardial-enriched gene RNA-binding protein with multiple splicing (variants) 2 [RBPMS2] by generating and characterizing rbpms2 knockout zebrafish and human cardiomyocytes derived from RBPMS2-deficient induced pluripotent stem cells.

RESULTS: We identified 1848 genes enriched in the nkx2.5-positive population. Among the most highly enriched genes, most with well-established functions in the heart, we discovered the ohnologs rbpms2a and rbpms2b, which encode an evolutionarily conserved RBP. Rbpms2 localizes selectively to cardiomyocytes during zebrafish heart development and strong cardiomyocyte expression persists into adulthood. Rbpms2-deficient embryos suffer from early cardiac dysfunction characterized by reduced ejection fraction. The functional deficit is accompanied by myofibrillar disarray, altered calcium handling, and differential alternative splicing events in mutant cardiomyocytes. These phenotypes are also observed in RBPMS2-deficient human cardiomyocytes, indicative of conserved molecular and cellular function. RNA-sequencing and comparative analysis of genes mis-spliced in RBPMS2-deficient zebrafish and human cardiomyocytes uncovered a conserved network of 29 ortholog pairs that require RBPMS2 for alternative splicing regulation, including RBFOX2, SLC8A1, and MYBPC3.

CONCLUSIONS: Our study identifies RBPMS2 as a conserved regulator of alternative splicing, myofibrillar organization, and calcium handling in zebrafish and human cardiomyocytes.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: alternative splicing • human induced pluripotent stem cells • myocytes, cardiac • RNA-binding proteins • zebrafish
Akerberg et al RBPMS2 Regulates RNA Splicing in Cardiomyocytes

Optimal cardiomyocyte physiology is paramount to vertebrate animal fitness. Alterations in cardiomyocyte function stemming from inherited or de novo mutations,3–5 or environmental exposures to cardiotoxins such as cancer-related treatments and alcohol,6,7 are frequent causes of primary cardiomyopathies in the pediatric and adult populations. Through the identification of cardiomyopathy-causing mutations and fundamental studies involving genetically engineered cells or animal models, much is already known about the gene activities required for establishing, maintaining, and safeguarding cardiomyocyte function. However, the catalog of genes with documented and indispensable functions in human cardiomyocytes remains incomplete. This is evident when considering that a significant percentage of familial cardiomyopathies lack a genetic diagnosis because

the underlying mutations exist in essential but unknown genes or regulatory sequences outside of clinically recognized cardiomyopathy loci.8 Expanding the catalog of known genes with essential functions in human cardiomyocytes will identify novel candidate loci, biomarkers, and treatment opportunities for cardiomyopathies.

RBPs (RNA-binding proteins) facilitate 5′ capping, splicing, polyadenylation, subcellular localization, stability, and translational efficiency of messenger RNAs.9 Disruptions to RBP function can cause broad and profound defects in post-transcriptional RNA processing that compromise cellular health and function. Accordingly, numerous monogenic diseases in the human population, including some heart diseases (see below), are caused by damaging mutations in RBP genes.10

Using RNA interactome capture, 2 laboratories recently purified hundreds of RBPs from cultured or primary rodent cardiomyocytes,11,12 many of which were cardiomyocyte-specific, suggesting that they perform specialized functions in heart muscle. Among those identified were several RBPs with previously documented roles in cardiomyocytes, primarily as mediators of alternative splicing, which dictate the properties of target RNAs and the activities of encoded proteins based on exon composition.13 One such splicing regulator, RBM24 (RNA-binding motif protein 24), is a cardiomyocyte-enriched RBP required for alternative splicing of several sarcomere genes, including TTN (titin) and ACTN2 (actinin alpha 2).14–17 In the absence of RBM24, sarcomeres fail to form or be maintained leading to cardiomyocyte and cardiac dysfunction.

Novelty and Significance

What Is Known?

• Alternative pre-mRNA splicing is a fundamental mechanism of post-transcriptional gene regulation that creates lineage-specific functional diversity in the proteome.
• Although heart-specific alternative splicing events are known to ensure the establishment or maintenance of cardiac function, the essential regulatory factors remain incompletely characterized.
• RBPMS2 (RNA-binding protein with multiple splicing [variants] 2) is an RNA-binding protein expressed selectively in the myocardium, but its function was not previously explored through genetic loss-of-function studies.

What New Information Does This Article Contribute?

• We report that human cardiomyocytes or zebrafish embryos devoid of RBPMS2 suffer from sarcomere organization, calcium handling, and cardiac function defects.
• We also demonstrate that RBPMS2-knockout cells or embryos mis-splice several genes, many of which are known to be involved in sarcomere structure, calcium handling, or cardiac function.
• We discovered that a conserved network of orthologous genes in both species relies on RBPMS2 for alternative splicing regulation in cardiomyocytes.

Our collective understanding of the natural mechanisms that support and safeguard cardiac function remains incomplete. We describe a new and essential genetic regulatory mechanism for ensuring robust ventricular myocardial function. The importance of this regulatory mechanism is evident in the conservation of several RBPMS2 splicing targets, which have persisted in humans and zebrafish despite 450 million years of evolutionary divergence. Further research on RBPMS2 and other cardiac-enriched splicing factors will highlight novel opportunities for diagnosing, preventing, and treating heart disease.

Nonstandard Abbreviations and Acronyms

| ALPM | anterior lateral plate mesoderm |
| ASE | alternative splicing event |
| DKO | double knockout |
| hiPSC-CM | human induced pluripotent stem cell–derived cardiomyocyte |
| RBP | RNA-binding protein |
| rMATS | replicate multivariate analysis of transcript splicing |
| RRM | RNA recognition motif |

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Another RBP, QKI (quaking), performs a similar role in supporting sarcomere function and cardiomyocyte contractility by regulating alternative splicing of sarcomere genes, including ACTN2. The cardiomyocyte-enriched RBM20 (RNA-binding motif 20) protein acts predominantly as a splicing repressor to exclude specific exons of several genes from mature mRNAs, including TTN. Missense mutations in RBM20 cause familial DCM, features of which are recapitulated in genetically engineered pigs and hiPSC-CMs (human induced pluripotent stem cell-derived cardiomyocytes). Of note, in addition to defects in pre-mRNA processing, RBM20 mutations also induce the accumulation of sarcomplasmic ribonucleoprotein granules, which are likely to contribute to disease pathogenesis in a pleiotropic fashion.

Newborns with hypoplastic left heart syndrome (HLHS) were found to carry de novo mutations in the RBP gene RBFOX2 (RNA binding Fox-1 homolog 2). Cardiomyocyte-specific ablation of RBFOX2 in the mouse embryo induced a structural heart defect with some features of HLHS. The authors also documented differential alternative splicing of a gene network, including 2 genes that regulate RhoGTPase cycling. In the postnatal heart, expression of RBFOX2 in cardiomyocytes protects the heart from dilated cardiomyopathy by regulating alternative splicing of numerous genes and transcription of mir-34a. Moreover, misexpression of aberrant RBFOX2 isoforms contributes to heart disease in the context of diabetes and myotonic dystrophy. The related cardiomyocyte-enriched RBP gene RBFOX1 regulates alternative splicing of a network of genes, including MF22 (myocyte enhancer factor 2) family members, and confers protection from pressure overload-induced hypertrophic cardiomyopathy in mice.

Lastly, the RBP genes CELF1 (CUGBP elav-like family member 1) and MBNL (muscleblind-like splicing regulator) 1) are reciprocally down and upregulated, respectively, in the hearts of neonatal mice to regulate a fetal-to-adult transition in the alternative splicing of numerous genes including many important for vesicular trafficking. Differential alternative splicing between fetal and adult hearts in humans has also been reported. Taken together, these studies underscore the essential and dynamic nature of alternative splicing regulation in cardiomyocytes, which supports cardiomyocyte physiology and protects the heart from dysfunction over the life of a vertebrate animal.

The founding member of the conserved RNA-binding protein with multiple splicing (variants) 2 (RBPM2S2) gene family was discovered in a differential expression screen designed to identify cardiac-enriched transcripts in Xenopus laevis (reviewed in Akerberg et al). The encoded protein contains an RRM and binds directly to RNA. In situ hybridization confirmed strong enrichment of RBPM2S2 expression in cardiomyocytes of amphibian and avian embryos. Enriched expression of RBPM2S2 has since been reported in the zebrafish embryonic heart and in human cardiomyocytes profiled by single-cell RNA sequencing (Single Cell Type Atlas). The consistent enrichment of RBPM2S2 expression in vertebrate hearts is suggestive of a specialized and essential function in cardiomyocytes, but this hypothesis has yet to be explored through gene knockout studies.

Here, we describe the first genetic loss-of-function study designed to explore the role of RBPM2S2 in zebrafish and hiPSC-CMs. Specifically, we show that rbpms2-deficient zebrafish embryos suffer from cardiac dysfunction characterized by reduced ejection fraction. The functional deficit is accompanied by myofibrillar disarray and calcium handling defects in mutant cardiomyocytes. Rbpms2-deficient animals also exhibit differential alternative splicing of 150 genes, including several responsible for sarcomere structure and calcium handling. Lastly, RBPM2S2-deficient human cardiomyocytes display virtually identical cellular phenotypes and share a conserved network of 29 mis-spliced genes with rbpms2-deficient zebrafish. Ultimately, our study identifies a novel and conserved RBP that is indispensable for the health and function of cardiomyocytes.

**METHODS**

**Data Availability**

All data and materials will be made publicly available upon publication. RNA-sequencing datasets were deposited into the NCBI (National Center for Biotechnology Information) gene expression omnibus (GEO) repository under accession numbers GSE167416, GSE167414, and GSE207681. Please see the Major Resources Table in the Supplemental Materials.

**Zebrafish Husbandry and Strains**

Zebrafish were bred and maintained following animal protocols approved by the Institutional Animal Care and Use Committees at Massachusetts General Hospital and Boston Children's Hospital. All protocols and procedures followed the guidelines and recommendations outlined by the Guide for the Care and Use of Laboratory Animals. The following zebrafish strains were used in this study: TgBAC(-36nkv2.5:ZsYellow)67,68 Tg(my7:GFP)69 [formerly Tg(cmlc2:GFP)67,68] Tg(my7:NLSeGFP)69 [formerly Tg(cmlc2:nucGFP)67,68] Tg(my7:actn3b-EGFP)69,70 [formerly Tg(cmlc2:actinin3-EGFP)69,70 Tg(cmlc2:actin3-EGFP)69,70]. rbpms2a and rbpms2b (this study), and rbpms2b (this study).

**Generation and Genotyping of rbpms2a and rbpms2b Mutant Alleles**

Customized TALENs (transcription activator-like effector nucleases) were used to induce double-stranded breaks in regions of the rbpms2a and rbpms2b loci that encode the RMM (RNA-recognition motifs). TALENs were designed to bind the sequences 5'-TGCGTGTGATCAACG-3' (+) and 5'-TGAAGGGTCTGAAACGC-3' (−) in exon 2 of rbpms2a and 5'-TGCCCAACATATCCAC-3' (+) and 5'-TTAAAGTGTCAAAGTGG-3' (−) in exon 2 of rbpms2b. TALEN constructs were constructed as described (Sanjana et al).
et al., 2012). Messenger RNAs encoding each TALEN pair were produced in vitro and co-injected into one-cell stage zebrafish embryos as described.\textsuperscript{46} Germ-line transmission of TALEN-induced mutations were detected using fluorescent polymerase chain reaction (PCR) and DNA-fragment analysis as described.\textsuperscript{49} The nucleotides deleted in the mutant alleles \textit{rbpms2a\textsubscript{chb}} and \textit{rbpms2b\textsubscript{chb}} are shown (Figure S4A and S4B). The primers utilized to distinguish wild-type \textit{rbpms2a} and \textit{rbpms2b} from the mutant alleles \textit{rbpms2a\textsubscript{chb}} and \textit{rbpms2b\textsubscript{chb}} by fluorescent PCR and DNA-fragment analysis are also shown (Figure S8). The wild-type alleles produce amplicons of 97 base pairs (bp) (\textit{rbpms2a\textsubscript{chb}} and \textit{rbpms2b\textsubscript{chb}}).

\textbf{hiPSC Maintenance and Genome Editing}

All hiPSC lines were maintained at 37 °C, 5% CO\textsubscript{2} in Essential 8 medium (catalog no. A1517001, Thermo Fisher Scientific) and passaged every 3 to 4 days using Versene (catalog no. 8 medium (catalog no. A1517001, Thermo Fisher Scientific). We previously described methods\textsuperscript{50} was synthesized using the T7 MegaShortScript transcription kit (Thermo Fisher Scientific). We previously described methods\textsuperscript{50} to generate a doxycycline-inducible Cas9 line in the WTC-11 iPSC background (Coriell Institute). Targeted integration of Cas9 into the AAVS1 (adeno-associated virus integration site 1) locus was performed using Addgene plasmids no. 7350 and no. 48139. This WTC-11 AAVS1 Cas9 hiPSC line was treated with doxycycline (2 μg/mL) for 16 hours before transfection to induce expression of an nuclear localization sequence (NLS)-SpCas9 fusion protein. After induction, \textasciitilde0.5\times10\textsuperscript{5} cells were transfected with 10 μg guide RNA using an Amaxa nucleofector (Lonza Technologies). Cells were seeded at a low density to pick single clones. Positive clones were confirmed by Sanger and amplicon sequencing. Stem cell marker expression was validated in isolated cell lines by flow cytometry using anti-SSEA4 (stage-specific embryonic antigen 4)-FITC (catalog no. 130-098-371, Miltenyi Biotec) and anti-lgG-FITC (catalog no. 130-104-611, Miltenyi Biotec) for negative control. Normal karyotypes were confirmed for both wild-type and \textit{\Delta RBPMS2} hiPSCs by KaryoStat (Thermo Fisher).

\textbf{RESULTS}

\textbf{Enriched Expression of the RBP Genes \textit{rbpms2a} and \textit{rbpms2b} in Zebrafish Cardiomyocytes}

Lineage-enriched genes often perform specialized and essential functions in the development, maintenance, or function of the cell populations they mark. To generate a candidate list of zebrafish genes with essential activities in cardiopharyngeal progenitors or embryonic cardiomyocytes, we sought to identify mRNAs with restricted expression to the subset of anterior lateral plate mesodermal (ALPM) cells marked by the \textit{nkx2.5;ZsYellow} transgene. This population contains cardiopharyngeal progenitors for multiple cell types including second heart field-derived ventricular cardiomyocytes and outflow tract lineages, a subset of pharyngeal muscles, and endothelium of the pharyngeal arch arteries.\textsuperscript{42,51–55} It also contains early-differentiating ventricular cardiomyocytes derived from the first heart field.\textsuperscript{51,53,54}

To isolate ALPM cells labeled with the transgene, \textit{Tg(nkx2.5;ZsYellow)} embryos were dissociated into single cells at a developmental stage when ZsYellow fluorescence is restricted to the ALPM (14–16 somites stage; Figure 1A). Dissociated cells were separated by fluorescence-activated cell sorting into ZsYellow-positive and ZsYellow-negative subpopulations before being subjected to RNA-sequencing and differential expression analysis (Figure 1A; Figure S1A through S1C). From this, we identified 1848 protein-coding RNAs enriched in the ZsYellow-positive population (Figure 1A; Table S1) after applying the inclusion criteria for Gene Ontology (GO) term enrichment analysis (fold change [FC] >1.5; adjusted \textit{P} value \textit{[P adj]<0.05).} Low replicate numbers precluded us from making claims of statistical significance based on the reported \textit{P} values (Table S1). As a result, this gene set is ranked based on log2FC (Figure S1D, Table S1). Nonetheless, among the enriched genes were several previously characterized lateral plate mesodermal markers that encode transcription factors essential for cardiopharyngeal development including \textit{nkk2.5}, \textit{hand2}, \textit{gata4/5/6}, \textit{mef2c/a/b}, \textit{slc8a1a}, \textit{mef2c/a/b}, \textit{actc1a}, \textit{acta2b}, \textit{tnn1b}, \textit{tnnt2a}, \textit{tnnc1a}, \textit{tnp4a}, \textit{tnh2}), calcium handling proteins (\textit{slc8a1a}, \textit{tn2r}, \textit{atp2a2a} [\textit{seca2}], and other factors expressed preferentially in cardiomyocytes (\textit{cx36.7, nppa/al/b}; Figure S1D; Table S1). Due to the presence of early-differentiating cardiomyocytes in the ZsYellow-positive population, we also identified numerous genes encoding sarcomere components (\textit{cmic1}, \textit{myl7} [\textit{cmlc2}], \textit{myh7} [\textit{vmhc}], \textit{myh6} [\textit{amhc}], \textit{acta1b}, \textit{acta1c}, \textit{acta2b}, \textit{tnn1b}, \textit{tnnt2a}, \textit{tnnc1a}, \textit{tnp4a}, \textit{tnh2}), calcium handling proteins (\textit{slc8a1a}, \textit{tn2r}, \textit{atp2a2a} [\textit{seca2}], and other factors expressed preferentially in cardiomyocytes (\textit{cx36.7, nppa/al/b}; Figure S1D; Table S1). As expected, GO term enrichment analysis uncovered overrepresentation of biological process terms “heart development,” “angiogenesis,” “heart contraction,” “actin cytoskeletal organization,” and “sarcomere organization” in the ALPM-enriched gene set (Figure S1E, see Table S1 for complete GO term analysis). The successful identification of ALPM markers with well-documented and indispensable roles in cardiopharyngeal progenitors or cardiomyocytes suggests that novel molecular activities of equal importance are likely to be among the uncharacterized ALPM-restricted genes.

The RBP genes \textit{rbpms2a} and \textit{rbpms2b} stood out among the most highly enriched as having never been implicated in heart development or function (Figure S1D; Table S1). Furthermore, of the 19 ALPM-enriched genes that were annotated with GO terms “RNA processing” and “RNA binding,” \textit{rbpms2a} was the most highly enriched (\textasciitilde14-fold enrichment), and \textit{rbpms2b}
Figure 1. Restricted expression of RNA-binding proteins Rbpms2a (RNA-binding protein with multiple splicing [variants] 2a) and Rbpms2b to the zebrafish myocardium.

A, Workflow used to identify 1848 protein-coding RNAs enriched in ZsYellow-positive (ZsY+) cardiopharyngeal progenitors or cardiomyocytes of the anterior lateral plate mesoderm in comparison to ZsYellow-negative (ZsY−) cells from the same 14–16 somites stage (ss) Tg(nkx2.5:ZsYellow) embryos. B–G, Representative brightfield images of 16 ss (B and E), 28 hours postfertilization (hpf; C and F), and 48 hpf (D,G) embryos processed for in situ hybridization with riboprobes that detect rbpms2a (B–D) or rbpms2b (E–G). n=10/group. H–K, Representative confocal projections of hearts in 24 hpf (H and H'), 33 hpf (I and I'), 48 hpf (J and J'), and 72 hpf (K and K') zebrafish embryos (Continued)
(±5-fold enrichment) ranked sixth based on log2FC (Figure S1F; Table S1). Classified as ohnologs, rbpms2a and rbpms2b encode proteins that are 92% identical (96% in the RRM; Figure S2A and S2B) and perform redundant functions in oocyte differentiation.\(^{30}\) Full-length Rbpm2a and Rbpm2b share >81% identity with human RBPM2S (Figure S2A and S2B), and their RRM domains are over 92% identical (Figure S2A and S2B). Such a high degree of sequence conservation suggests that the molecular function of RBPM2S proteins and the properties of their binding motifs are also likely to be conserved across evolution.

To validate the predicted ALPM localization of rbpms2a and rbpms2b, we performed in situ hybridization for both genes at the same developmental stage (16 somites stage) used for profiling nkx2.5:ZsYellow-positive ALPM cells (Figure 1A). As anticipated, both transcripts localized bilaterally to the ALPM (Figure 1B and 1E), in a pattern mirroring early-differentiating cardiomyocytes at this stage.\(^{56}\) Later-stage analysis localized rbpms2a and rbpms2b to the linear heart tube at 24 to 28 hours post-fertilization (hpf; Figure 1C and 1F; Figure S3A and S3C) and to both the atrium and ventricle at 48 hpf (Figure 1D and 1G; Figure S3B and S3D). Consistent with previous reports,\(^{40,57}\) extra-cardiac expression was observed in the pronephric duct and retina (Figure S3A through S3D).

To investigate the lineage specificity and subcellular localization of Rbpm2a and Rbpm2b in the zebrafish heart, we immunostained wild-type embryos with a monoclonal antibody raised against human RBPM2S, which cross-reacts with zebrafish Rbpm2s (see below). Consistent with the distribution of rbpms2a/b transcripts, Rbpm2s protein was detected in the heart tube (Figure 1H and 1I; Figure S3E and S3F), 2-chambered heart (Figure 1J and 1K; Figure S3G), pronephric duct (Figure S3E through S3G), and retina (Figure S3F and S3G). Double-immunostaining for Rbpm2s and the myocardial marker cardiac troponin T (CT3 antibody) revealed that Rbpm2s localizes to the myocardium at all stages analyzed (Figure 1H', I', J', and K'). Examination of single optical sections through the ventricular wall showed nuclear Rbpm2s signals surrounded by troponin T-positive cytoplasm, confirming myocardial localization (Figure 1L through 1L'''). Adjacent endocardial cells were devoid of Rbpm2s signals, demonstrating that expression in the ventricular chamber is restricted to the myocardial layer (Figure 1L through 1L''''). Weak expression of Rbpm2s also became evident in the outflow tract by 72 hpf (Figure 1K and 1K'). The subcellular distribution of Rbpm2s appeared variable, ranging from a nucleocyttoplasmic distribution to exclusively nuclear (Figure 1H, 1I, 1J, and 1K), but the significance of this observation is unknown. Lastly, we documented Rbpm2s expression, both nuclear and cytoplasmic, in cardiomyocytes of adult zebrafish (Figure 1M and M'), suggesting that Rbpm2s functions in the myocardium continuously throughout the lifespan of zebrafish rather than transiently during embryogenesis.

Generation of rbpms2-Null Zebrafish

To determine if rbpms2a and rbpms2b are required for myocardial development or cardiac function, we created mutant alleles of both genes using TALENs.\(^{47}\) TALEN pairs were designed to target DNA sequences encoding N-terminal regions of the RRM domains (Figure S4A and S4B). We isolated the mutant alleles rbpms2a\(^{chb}\) and rbpms2b\(^{chb}\), which carry frame-shifting deletions and premature stop codons that truncate the proteins by >80% (Figure 2A and 2B; Figure S4A and S4B). The truncations also remove 82% of the RRM, suggesting that the RNA-binding activities of the mutant proteins are abolished or significantly compromised. As a result, both alleles are likely to be null. Animals homozygous for either rbpms2a\(^{chb}\) or rbpms2b\(^{chb}\) are grossly indistinguishable from siblings during all life stages, which is consistent with a previous study that independently generated mutant alleles of rbpms2a and rbpms2b.\(^{40}\)

To determine if rbpms2a and rbpms2b perform redundant functions in the heart, we generated and examined double homozygous embryos (ie, rbpms2a\(^{chb}\)rbpms2b\(^{chb}\)) animals for cardiac defects. Double knockout (DKO) animals are grossly indistinguishable from control siblings for the first ≈30 hours of life, including normal nkx2.5 expression in the ALPM (Figure S4C through S4E). By ≈33 hpf, they develop a characteristic indentation of the yolk (Figure S4F and S4G), which is indicative of mild pericardial edema. At this stage, double mutants are delayed in initiating blood flow although peristalsis of the heart tube is readily evident. By 72 hpf, pericardial edema is markedly more severe (Figure 2C and 2D), suggesting that rbpms2-null animals suffer from a cardiac malformation or ongoing functional deficit. This phenotype is observed specifically in double-mutant animals, demonstrating that...
rbpms2a and rbpms2b perform redundant functions in the heart, as they do in oocytes. 40

Western blotting and whole-mount immunostaining revealed that the zebrafish epitopes recognized by the anti-human RBPMS2 antibody are completely absent in double mutants (Figure 2E through 2G), demonstrating that the antibody cross-reacts with zebrafish Rbpms2. It also suggests that the mutant mRNAs or proteins are unstable, further suggesting that rbpms2a<sup>chb3</sup> and rbpms2b<sup>chb4</sup> are bona fide null alleles.

To assess double-mutant viability, we monitored the survival of control-sibling and DKO animals from embryonic day 48 (Figure 2H). 41 Representative confocal projections of hearts in 48 hpf CTRL (I and K) or rbpms2-null (J and L) embryos carrying the myl7:GFP (J, n=10/group) or myl7:nucGFP (K and L) transgenes immunostained with an anti-GFP (green fluorescent protein) antibody. M, Dot plots showing ventricular cardiomyocyte (CM) numbers in CTRL (n=6) and rbpms2-null (n=6) hearts at 48 hpf. Error bars show one standard deviation. Statistical significance was determined by an unpaired, 2-tailed Student's t test assuming equal variances. N-Q, Representative single-plane light sheet fluorescence microscopy images of hearts in 48 hpf CTRL (N and O) and rbpms2-null (P and Q) embryos carrying the myl7:GFP transgene. Images of hearts during ventricular diastole (N and P) or systole (O and Q) are shown with Cardiac Functional Imaging Network (CFIN) overlays of the atrial (blue) and ventricular (cyan) lumens.
stages to adulthood. Over 50% of rbpms2-null animals died by 2 weeks of age (Figure 2H). Furthermore, 85% of animals were dead by 60 days postfertilization, and the remaining small percentage of animals did not survive beyond 6 months postfertilization. Independent of age, death was generally preceded by progressive pericardial edema, suggesting that cardiac dysfunction is the underlying cause of premature mortality in rbpms2-null animals.

**Rbpm2-Deficient Animals Exhibit Cardiac Dysfunction Characterized by Reduced Ejection Fraction**

To evaluate DKO embryos for structural heart disease, we examined hearts of 48 hpf control-sibling and rbpms2-null animals carrying the myl7:GFP transgene, which labels the entire myocardium with GFP (green fluorescent protein). Although the 2-chambered heart in double mutants displayed normal left-right patterning, the ventricle was displaced anteriorly relative to the atrium (Figure 2I and 2J), likely reflecting stretching at the cardiac poles caused by pericardial edema. Atrial size was not overtly different in rbpms2-null animals, but their ventricles were smaller by comparison to those in control siblings (Figure 2I and 2J). To determine if this reflected a diminished ventricular cell number, we quantified cardiomyocytes in control-sibling and rbpms2-null animals carrying the myl7:nucGFP transgene, which labels cardiomyocyte nuclei with GFP. Both ventricular and atrial cardiomyocyte numbers were unaltered in double mutants (Figure 2K through 2M; Figure S5A), demonstrating that any deviations in chamber size are not attributable to altered myocardial cell content. It also suggests that rbpms2a and rbpms2b are dispensable for cardiomyocyte differentiation from the first and second heart fields, which is the principal method by which cardiomyocytes are produced before 48 hpf in zebrafish.**43**-**46** Ultimately, these data demonstrate that early cardiac morphogenesis is largely unperturbed in double-mutant animals, with the only apparent anatomic abnormality being a reduction in ventricular size. Because mutant ventricles have normal numbers of cardiomyocytes, we can deduce that the ventricular cardiomyocytes themselves are smaller, which is apparent based on the increased density of cardiomyocyte nuclei in mutant ventricles (Figure 2K and 2L).

To evaluate rbpms2-null hearts for functional deficits, we measured ejection fraction and cardiac output in 48 hpf animals using our previously described Cardiac Functional Imaging Network.**61** Briefly, we used light sheet fluorescence microscopy to capture dynamic Z-stack images of GFP+ hearts in control-sibling and DKO myl7:GFP transgenic embryos over several cardiac cycles. From these data, the Cardiac Functional Imaging Network extracted dynamic chamber volumes over time, including end-diastolic volume (EDV) and end-systolic volume (ESV) as described.**61** Whereas ventricular ESV was unchanged in DKO animals, EDV was significantly reduced (Figure 2N through 2R). As a result, 3 indices of ventricular function, all of which are calculated from EDV and ESV, were also significantly reduced. These include stroke volume SV=EDV-ESV ejection fraction EF=SV/EDV and cardiac output (stroke volume×heart rate; Figure 2R). Heart rate was unchanged in the mutant (Figure 2R). The reduction in EDV is consistent with the diminutive size of the DKO ventricle (Figure 2I through 2L), and the reduced ejection fraction is suggestive of systolic heart failure stemming from compromised cardiomyocyte contraction. Cardiac Functional Imaging Network analysis also revealed an increase in atrial volume (Figure S5B), which was not grossly obvious after fixation (Figure 2I through 2L). This was accompanied by a slight decrease in atrial ejection fraction (Figure S5C). Atrial enlargement is a common secondary consequence of impaired ventricular function.**62** Taken together, these data demonstrate that rbpms2a and rbpms2b are required for robust ventricular function in the zebrafish embryo. This requirement likely extends beyond embryogenesis, because the longest-living DKO animals exhibit signs of cardiac dysfunction such as stunted growth and pericardial edema (Figure S6A). Examination of cardiac sections from these animals revealed hearts with diminutive and fibrotic ventricles as well as massively enlarged atria (Figure S6B through S6E), suggestive of ongoing ventricular dysfunction and reactive atrial dilation.

**Identification of Differential Alternative Splicing Events in rbpms2-Null Zebrafish**

To identify gene expression changes in rbpms2-null embryos, we performed bulk RNA-sequencing and differential expression analysis on control-sibling and rbpms2-null animals at 33 hpf, the earliest developmental stage when DKO animals are distinguishable by morphology (Figure S4C and S4D). We reasoned that early-stage analysis would maximize the chances of highlighting primary molecular defects without interference by secondary responses to cardiac dysfunction. Importantly, we confirmed that the primitive ventricles in 33 hpf rbpms2-null animals exhibit a functional deficit by documenting reduced myocardial wall movement at the arterial pole of the heart tube (Figure S7A). We also confirmed that total cardiomyocyte numbers are unaltered at 33 hpf in double mutants (Figure S7B), reducing the chances that any changes in gene expression would be attributable to altered cellular composition. Lastly, we profiled whole embryos, instead of purified cardiomyocytes, because of the difficulty in recovering sufficient numbers of healthy, pure cardiomyocytes at this stage by embryo dissociation and fluorescence-activated cell sorting.
Although RNA-sequencing and gene-level differential expression analysis did not identify any dysregulated transcripts in rbpms2-null animals (IFC>1.5; P adj<0.05; Figure S8A; Table S2), isoform-level differential expression analysis yielded 105 downregulated and 97 upregulated transcripts (IFC>1.5; P adj<0.05; Figure S8B; Table S3). GO term analysis did not identify overrepresentation of any terms in either gene set (false discovery rate <0.05). However, among the downregulated transcripts, we found three well-characterized myocardial genes including phospholamban (pln/plna), atrial myosin heavy chain 6 (myh6), and natriuretic peptide precursor a ((nppa); Figure S8B; Table S3). We documented trending downregulation of nppa by reverse-transcription quantitative PCR (RT-qPCR) at 33 hpf (Figure S7C). However, this was short-lived because 2 subsequent developmental stages showed statistically significant (48 hpf) or trending (72 hpf) increases in nppa expression (Figure S7C), which is characteristic behavior for nppa in response to cardiac stress (Becker et al66). RT-qPCR analysis of myh6 revealed a downward trend but statistically insignificant change in double mutants (Figure S7D). Ultimately, downregulation of these genes is unlikely to explain the ventricular phenotype in rbpms2-null mutants because nppa-null zebrafish do not exhibit any gross cardiac defects67, and the primary defect in myh6-deficient animals is a silent atrium.68

Phospholamban is a reversible micropeptide inhibitor of the SERCA2a (Sarco/Endoplasmic Reticulum Calcium ATPase 2a) pump, which removes calcium from the sarcoplasm to facilitate cardiomyocyte relaxation.69 Mutations in PLN cause dilated cardiomyopathy in the human population.68 Publicly available cDNA sequences and annotations of the pln locus in the zebrafish genome (Genome Reference Consortium Zebrafish Build 11 [GRCz11]; Ensembl annotation release 10669) suggest that pln is alternatively spliced. Interestingly, one of the alternatively spliced isoforms of pln (ENSART00000141159; pln-203) was found to be downregulated in rbpms2-null animals (FC=-43.00; P adj=8.24×10^-10; Figure S8B; Table S3). However, 3 additional alternatively spliced pln transcripts (ENSART00000086648, ENSDART00000132012, ENSDART00000133911) were unchanged (Table S3), highlighting the possibility that Rbpm2 functions to promote inclusion of these specific exons into mature transcripts in wild-type embryos.

Despite supporting evidence from differential isoform expression analysis (Figure S8B; Table S3), rMATS did not identify alternative splicing of pln, likely because it involves a terminal exon (Figure S9), which rMATS is not designed to evaluate (Figure 3A). Therefore, we employed the Mixture-of-Isoforms algorithm79 to evaluate alternative splicing of pln in rbpms2-null animals and found strong statistical evidence that the exon 3-containing pln-203 isoform (ENSART00000141159) is uniquely reduced in mutant animals. Specifically, the Bayes factor was largely above the accepted cutoff of 5 for differences in ENSDART00000141159 versus ENSDART00000133911 usage in wild-type and double-mutant animals across all 5 replicate pairs (Table S5), which is strongly suggestive of differential alternative splicing. This conclusion was also supported by genotype-specific differences in read densities across the locus (Figure S9). Collectively, these data demonstrate that rbpms2-null embryos display abnormal splicing of sarcomere and calcium handling genes.

Validation of Differential ASEs in rbpms2-Null Zebrafish

Next, we used splicing-sensitive RT-qPCR to validate differential ASEs that were detected by RNA-sequencing.
Figure 3. Identification of differential alternative splicing events (ASEs) in rbpms2-null embryos.

A. Schematic diagrams of the categories of ASEs investigated by the RNA-sequencing Multivariate Analysis of Transcript Splicing algorithm including skipped exon (SE), alternative 3′ splice site (A3SS), alternative 5′ splice site (A5SS), mutually exclusive exon (MXE), and retained intron (RI).

B. Volcano plot showing the inclusion (Inc) level differences (InclevelCTRL – Inclevelrbpms2-null) and false discovery rates (FDRs) for differential ASEs between 33 hours postfertilization (hpf) control-sibling (CTRL; n=4, 10 pooled embryos per replicate) and rbpms2-null animals (n=5) filtered by 0 uncalled replicates, FDR<0.1, and |Incleveldifference|>0.1. The ASEs shown in bold were validated with splicing-sensitive quantitative PCR (qPCR) or in situ hybridization.

C. Pie chart showing the proportions of differential ASEs in each category in rbpms2-null animals. (Continued)
To that end, we designed 2 primer pairs each for several alternatively spliced transcripts. One pair was designed to measure relative total transcript levels between control-sibling and rbpms2-null animals, independent of the alternatively spliced exon (Figure S10A). The other was designed to quantify relative isoform-specific levels by having a primer-binding site contained within the exon showing reduced inclusion in mutants (Figure S10A). Consistent with the RNA-sequencing, for each of the four genes tested (mybpc3, myom2a, slc8a1, and pln), total transcript levels were not significantly altered in rbpms2-null animals (Figure 3D). However, the specific exon-containing isoforms were reduced by at least 50% in all cases, and by greater than 80% in three cases, demonstrating significant exon exclusion in DKO animals at 33 hpf (Figure 3E). These events were also documented in 48 hpf embryos, ruling out a transient disruption in splicing (Figure S10B and S10C). Exon exclusion was also confirmed independently using semi-quantitative reverse-transcription PCR followed by calculations of percent spliced in (Figure 3F; Figure S11). Taken together, these data validate the findings from rMATs and the Mixture-of-Isoforms algorithm and demonstrate that Rbpms2 functions to promote the inclusion of these specific exons into mature transcripts.

To confirm that the DKO splicing abnormalities localize to the myocardium, we performed splicing-sensitive in situ hybridization analysis for 2 genes with rbpms2-regulated exons large enough to generate effective exon-specific antisense riboprobes (ie, exon 2 of mybpc3 and exon 3 of pln). In a strategy analogous to the splicing-sensitive RT-qPCR we designed a pair of riboprobes for each gene, one probe to detect total transcript pools of mybpc3 or pln, independent of the alternatively spliced exons (Figure S10A), and the other to detect the isoform-specific cassette exon sequences exclusively (Figure S10A). In control-sibling animals at 33 hpf, total mybpc3 transcripts were localized to both cardiac and skeletal muscle (Figure 3G and 3G'), and total pln transcripts were detected exclusively in the heart (Figure 3K and 3K'). As predicted by the validated RNA-sequencing data (Figure 3D), these expression patterns were unaffected by the loss of rbpms2 (Figure 3H, 3H', 3L, and 3L'). By contrast, isoform-specific in situ hybridization localized the rbpms2-regulated mybpc3 and pln exons to the hearts of control-sibling animals (Figure 3I, 3I', 3M, and 3M'), but these exon sequences were undetectable in double-mutant hearts (Figure 3J, 3J', 3N, and 3N'), confirming that these mis-splicing events are cardiac specific. Taken together, these data implicate rbpms2 in the positive regulation of exon inclusion for several transcripts in the myocardium of embryonic zebrafish.

For those ASEs validated by RT-qPCR or in situ hybridization (mybpc3, myom2a, pln, and slc8a1a), exon exclusion in DKO hearts preserves their open reading frames, leading to the replacement of full-length proteins with those lacking amino acids encoded by the excluded exons (Figure S10A), which are likely to have altered function. In the case of mybpc3 exon 2, it encodes a domain termed C0, which binds to F-actin and the myosin regulatory light chain.69 C0 is contained exclusively within the cardiac isoform of myosin binding protein C in higher vertebrates (MYBPC3) but is absent from the skeletal muscle isoforms (MYBPC1 and MYBPC2). Similarly, mybpc3 exon 10 encodes cardiac-specific amino acids that become phosphorylated by PKA (protein kinase A) in response to β-adrenergic signaling.69,70 For Myom2a, the short stretch of amino acids absent in DKO animals is of unknown function. For pln, excluding exon 3 shortens the protein by 5 amino acids on the C-terminus, which has been shown to decrease SERCA inhibitory activity in vitro.61 Lastly, the slc8a1a exon with reduced inclusion in DKO animals is one (ie, exon E) of 6 conserved exons that are alternatively spliced in higher vertebrates to control the calcium sensitivity of the transporter.62,63

Zebrafish rbpms2-Null Ventricular Cardiomyocytes Exhibit Myofibrillar Disarray and Calcium Handling Defects

Given the nature of the differential ASEs in rbpms2-null animals, we evaluated 48 hpf control-sibling and double-mutant hearts for myofibrillar defects after immunostaining with antibodies that recognize either thin filaments, thick filaments, or Z-disks. For all epitopes, prominent

Figure 3 Continued. Also shown are the percentages of exons in the skipped exon category that are differentially included or excluded in rbpms2 mutants. D, Bar graph showing the relative total transcript levels in 33 hpf CTRL and rbpms2-null animals that are independent of the alternatively spliced exons. E, Bar graph showing the relative levels of isoform-specific transcripts that contain the indicated Rbpms2-regulated exons in 33 hpf CTRL and rbpms2-null animals. In D and E, single biological replicates (n=6) of 10 pooled embryos, each with 3 technical replicates are shown. Error bars show 1 SD. F, Representative gel images of semiquantitative reverse-transcription PCR (RT-qPCR) amplicons and the average (AVG) percent spliced in (PSI) values for the indicated alternative splicing events in 33 hpf CTRL and rbpms2-null (double knock out [DKO]) animals. Amplicon sizes (in base pairs [bp]; left) and identities (right) are shown. n=6 biological replicates (see Figure S11), 10 pooled embryos/replicate. For D-F, statistical significance was determined by an unpaired, 2-tailed Student’s t test assuming equal variances with Bonferroni-Dunn correction for multiple comparisons (D and E). Significant P values are shown. G–N, Representative brightfield images of 33 hpf CTRL (G, G', I, K, M, and M') and rbpms2-null (H, H', J, J', L, L', N, and N') embryos processed for in situ hybridization (ISH) with riboprobes that detect total pools (G–H, K–L') or specific exon-containing mRNA isoforms (I–J, M–N') of mybpc3 (G–J') or pln (K–N'). The cardiac regions in (G, H, I, J, K, L, and M) and (N) are shown at higher zoom in (G, H, I, J, K, L, M', and N). Red arrowheads in (G and H) highlight expression of mybpc3 in skeletal muscle, which is shown from a dorsal view in the insets. n=10/group. Black arrowheads highlight the linear heart tube. Scale bars=100 μm. ns indicates not significant.
striations were apparent in control-sibling ventricles (Figure 4A, 4A’, 4C, 4C’, 4E), reflecting tandem arrays of sarcomeres within myofibrils, which themselves are aligned transversely and bundled near the cell cortices. By contrast, despite the detection of the epitopes in DKO ventricles, striations were significantly reduced or absent (Figure 4B, 4B’, 4D, 4D’, 4F), indicative of myofibril disarray. We confirmed that myofibril disorganization was also present at an earlier developmental stage of 33 hpf (Figure S7G through S7H’). This defect appears to be specific to ventricular cardiomyocytes because myofibril organization in atrial cardiomyocytes was grossly unperturbed in DKO embryos (Figure S7I through S7J’). Taken together, these data demonstrate that Rbpms2 activity is required for establishing or maintaining the three-dimensional architecture of myofibrils in ventricular cardiomyocytes of the zebrafish embryo.

We also evaluated rbpms2-null hearts for defects in calcium handling by comparing the amplitude and duration of their calcium transients to those in control-sibling hearts. Briefly, control-sibling and mutant hearts were explanted from 48 hpf animals and loaded with the ratiometric calcium-sensitive dye Fura-2. Dual wavelength excitation and high-speed imaging were used to capture the fluctuations in cytosolic calcium occurring throughout the myocardium in control-sibling and mutant hearts over several cardiac cycles. From these images, we measured the amplitude and duration of calcium transients within the atrium and ventricle of each heart. Whereas the atrial values were comparable between control and DKO hearts, (Figure 4G, 4H, 4J through 4L, 4N), the ventricular values were both reduced in double mutant hearts (Figure 4G through 4L; 4K through 4M), demonstrating that Rbpms2 regulates calcium handling in ventricular cardiomyocytes of zebrafish embryos.

Human RBPMS2-Null Cardiomyocytes Exhibit Myofibrillar Disarray and Calcium Handling Defects

To determine if the cellular function of RBPMS2 is conserved in human cardiomyocytes, we used CRISPR/Cas9-mediated genome editing to target the RBPMS2 locus in hiPSCs, which allowed us to generate RBPMS2-deficient hiPSC-CMs through directed differentiation. We targeted exon 3, which encodes amino acids within the RRM (Figure S12A), and isolated a clone (ΔRBPMS2) carrying a biallelic 2 base pair deletion (Figure S12A). The deletion truncates both the full-length protein and its RRM (Figure S2), the resulting aberrant protein is likely to be unstable and lacking in any biological activity.

To investigate whether RBPMS2-null human cardiomyocytes exhibit cellular phenotypes similar to those observed in ventricular cardiomyocytes from rbpms2-null zebrafish (Figure 4A through 4F), we analyzed myofibril structure in wild-type and ΔRBPMS2 hiPSC-CMs by double-immunostaining with antibodies that detect thin filaments or Z-disks. The hallmark striations created by thin filaments, which were readily apparent in control cells (Figure 5D, 5E, and 5G), were almost completely absent in ΔRBPMS2 hiPSC-CMs (Figure 5H, 5I, and 5K). Moreover, whereas the Z-disks in control cardiomyocytes were prominent, well-defined, and arrayed in tandem (Figure 5D, 5F, and 5G), those in ΔRBPMS2 hiPSC-CMs were smaller, punctate, and disorganized (Figure 5H, 5J, and 5K). Taken together, these data uncover a conserved role for RBPMS2 in establishing or maintaining myofibril organization in human cardiomyocytes.

Next, we evaluated wild-type and ΔRBPMS2 hiPSC-CMs for defects in calcium handling. hiPSC-CMs cells were loaded with the calcium-sensitive dye Fluo-4, paced at 1 Hz, and imaged by line scanning confocal microscopy to capture intracellular calcium dynamics (Figure 5L and 5M). Similar to ventricular cardiomyocytes from rbpms2-null zebrafish, ΔRBPMS2 hiPSC-CMs exhibited reductions in calcium transient amplitude (Figure 5N) and duration (Figure 5O and 5P). These phenotypes were accompanied by decreases in time-to-peak (Figure 5Q) and maximum upstroke velocity (Figure 5R). Lastly, without pacing, ΔRBPMS2 hiPSC-CMs contracted at a lower spontaneous beat frequency (Figure 5S). These data demonstrate that human cardiomyocytes rely on RBPMS2 function for optimal calcium handling. Taken together, the overlap in sarcomere and calcium handling phenotypes observed between rbpms2-null zebrafish and ΔRBPMS2 hiPSC-CMs suggest that the cellular function of RBPMS2 is conserved across vertebrate evolution.

Conservation of an RBPMS2-Regulated Alternative Splicing Network Between Zebrafish and Human Cardiomyocytes

To determine if the molecular function of RBPMS2 as a regulator of alternative splicing is conserved in humans, we performed RNA-sequencing on control and ΔRBPMS2 hiPSC-CMs followed by rMATS analysis. We identified 2679 differential ASEs in RBPMS2-deficient hiPSC-CMs when compared to control cells (0 uncalled
Figure 4. Myofibrillar disarray and calcium handling defects in rbpms2-null ventricular cardiomyocytes.

A and B: Representative confocal projections of ventricles in 48 hours postfertilization (hpf) control-sibling (CTRL) or rbpms2-null embryos immunostained with an anti-TroponinT antibody (CT3 [cardiac troponin T]) to visualize thin filaments. Regions demarcated in (A) and (B) are enlarged in (A') and (B'). C and D', Confocal projections of ventricles in 48 hpf CTRL and rbpms2-null Tg(α-actn3b-EGFP) animals immunostained with antibodies that detect tropomyosin (CH1; green) to visualize thin filaments or GFP (magenta) to visualize Z-disks.

E and F, Confocal projections of ventricles in 48 hpf CTRL and rbpms2-null embryos immunostained with an antibody that detects myosin heavy chain (MF20) to visualize thick filaments. For A–F, n=10 animals/group. Scale bars=10 μm. G–N, Color maps (G, H, K, and L) and dot plots (I, J, M, and N) of calcium transient duration (G–J) and calcium release amplitude (K–N) in the ventricle (G–I; K–M) and atrium (G, H, J, K, L, and N) of 48 hours postfertilization control-sibling (CTRL; G, I, J, K, M, and N) and rbpms2-null (H–J; L–N) hearts. Color code depicts localized calcium transient duration in milliseconds (G and H) or the ratiometric fluorescence indicator of calcium release amplitude (K and L). Each dot represents center-chamber measurements from one heart. n=10 hearts/group. Error bars show one SD. Statistical significance was determined by an unpaired, 2-tailed Student’s t test assuming equal variances. Significant P values are shown. A indicates atrium; ns, not significant; and V, ventricle.
replicates; false discovery rate <0.05; |Incleveldifference|>0.1; Figure 6A; Table S6). A majority (52%) of ASEs were skipped exons, followed by mutually exclusive exons (34%), alternative 3′ splice site (6%), retained intron (4%), and alternative 5′ splice site (4%; Figure 6B; Table S6). Within the skipped exon category, exon exclusion was more frequent than exon inclusion in RBPMS2-deficient hiPSC-CMs (Figure 6B; Table S6). GO term enrichment analysis identified overrepresentation of several broad GO terms as well as myofibril, sarcomere, I band, and Z-disc, consistent with the myofibrillar disorganization observed in mutant iPSC-CMs (Table S6).

Lastly, we utilized ExOrthist software to investigate whether a conserved network of genes relies on RBPMS2-mediated alternative splicing in both zebrafish and human cardiomyocytes. First, genes harboring Rbpms2-regulated splicing events in zebrafish were compared to those in hiPSC-CMs (Figure 6C). Of the 150 mis-spliced genes in rbpms2-null zebrafish, 132 have an ortholog in the human genome, and 29 orthologs were found to harbor RBPMS2-regulated exons in hiPSC-CMs (Figure 6C; Table S7). Next, exon-level analysis identified orthologous exons between the zebrafish and human genomes and determined that of the 166 rbpms2-regulated exons...
in zebrafish, 142 reside in genes with high-confidence human gene orthologs (Figure 6C). Seventy-eight have an orthologous exon in the corresponding human gene, and three orthologous human exons are regulated by RBPM2 in hiPSC-CMs (Figure 6C; Table S7). They include exons in slc8a1a (see above), cacnb2a (calcium voltage-gated channel auxiliary subunit beta 2b), and ccser2a (coiled-coil serine-rich protein 2a; Table S7). Cacnb2a is an auxiliary subunit of an L-type voltage-gated calcium channel, andCcser2a is of unknown function. All in all, despite 450 million years of evolutionary divergence between zebrafish and humans, 31 zebrafish exons are contained within a conserved network of 29 ortholog pairs that rely on RBPM2 for splicing in hiPSC-CMs.
DISCUSSION

We report that the RBP RBPMS2 functions as a conserved regulator of alternative splicing, myofibrillar organization, and calcium handling in both zebrafish and human cardiomyocytes. We identified the paralogs rbpms2a and rbpms2b in a differential expression screen designed to uncover mRNAs enriched in a mixed population of cardiopharyngeal progenitors and cardiomyocytes in the zebrafish ALPM. During zebrafish heart development, Rbpms2 localizes selectively to cardiomyocytes, and cardiomyocyte expression continues into adulthood, which is suggestive of a life-long function in the heart. Cardiomyocyte-enriched expression of RBPMS2 was previously documented in amphibians, avians, and humans, which is suggestive of functional conservation throughout evolution. Despite these observations and the prior probability that RBPMS2 is performing a conserved and essential function in cardiomyocytes, this hypothesis had yet to be explored through gene knockout studies.

In a previous study, investigators used morpholinos to knock down expression of the Rbpm2 homolog (ie, hermes) in Xenopus laevis embryos. Although morphant embryos suffered from cardiac defects, the control morpholino induced a similar phenotype, preventing any conclusions to be drawn. In a reciprocal approach, the authors overexpressed rbpms2 and documented a reduction in early myocardial gene expression and failure of heart tube formation, suggesting that rbpms2 serves as a negative regulator of cardiomyocyte differentiation. This function would be consistent with the demonstrated role of RBPMS2 as a negative regulator of smooth muscle cell differentiation when overexpressed in cells of the avian gastrointestinal tract. If rbpms2 were acting as a negative regulator of cardiomyocyte differentiation, then rbpms2-deficient zebrafish would be predicted to exhibit increased cardiomyocyte numbers, which was not observed. This observation reduces the likelihood that RBPMS2 serves as a negative regulator of cardiomyocyte differentiation, at least when expressed at physiologic levels.

Instead, our data demonstrate that RBPMS2 functions on a cellular level to establish or maintain myofibrillar structure in cardiomyocytes. Although RBPMS2-deficient zebrafish and human cardiomyocytes express sarcomere proteins, they lack the characteristic striations observed in control cells, which are created by highly ordered, tandem arrays of sarcomeres within myofibrils. The disordered nature of the contractile apparatus in rbpms2-deficient zebrafish cardiomyocytes almost certainly explains, at least in part, why DKO hearts show a reduction in ejection fraction.

RBPMS2 also functions in cardiomyocytes to optimize calcium handling as evidenced by reductions in the amplitude and duration of calcium transients in RBPMS2-deficient zebrafish and hiPSC-CMs. The dynamics of calcium flux in cardiomyocytes are determined by several independent regulatory mechanisms including the activities of calcium channels and transporters, the calcium-binding capacities of intracellular proteins, sarcoplasmic reticulum stores, and baseline cytosolic calcium. As such, pinpointing the primary molecular cause of altered calcium handling in mutant cardiomyocytes will require further investigation. Nonetheless, given the importance of calcium flux in cardiomyocyte physiology, we presume that the documented abnormalities contribute, at least in part, to the functional deficits since reduced calcium transient amplitude and duration are consistent with systolic dysfunction and reduced ejection fraction. Lastly, because mutations in sarcomere components can alter calcium handling and calcium handling defects can disrupt myofibrils, these phenotypes might be interrelated. Ultimately, the similarities in cellular phenotypes between RBPMS2-deficient cardiomyocytes in two species that diverged ≈450 million years ago suggests that the cellular function of RBPMS2 has been conserved. Interestingly, despite rbpms2 expression in both chambers of the zebrafish heart, the functional, myofibrillar, and calcium handling defects in rbpms2-null embryos mutants are largely confined to the ventricle, for unknown reasons.

Our data also demonstrate that RBPMS2 regulates alternative splicing because numerous differential ASEs were uncovered in rbpms2-null zebrafish and ΔRBPMS2 hiPSC-CMs. In noncardiomyocytes, RBPMS2 regulates the stability, subcellular localization, and translation efficiency of target RNAs (reviewed in Akerberg et al). Despite the observation that amphibian Rbpms2 interacts directly with 2 splicing factors, RBPMS2 itself was never implicated in splicing regulation through genetic loss-of-function studies. Notably, the highly related RBPS2 protein is a master regulator of alternative splicing in smooth muscle cells, which increased the likelihood that RBPMS2 also regulates alternative splicing in some contexts. Consistent with this possibility, RBPMS2 was capable of substituting for RBPS2 in regulating alternative splicing of minigenes containing the RBPS2 RNA recognition element, which is also bound by RBPMS2.

Consistent with the observed cellular phenotypes, we documented mis-splicing of genes in rbpms2-null zebrafish that encode sarcomere components (MybpC3 and Myom2a) or proteins that regulate calcium flux in cardiomyocytes (Pln and Slc8a1a). Although we are tempted to draw strong conclusions about how mis-splicing of ≥1 transcripts leads to specific cellular defect, several considerations complicate this task. Because the open reading frames of these four mis-spliced genes remain
intact in DKO zebrafish, the resulting proteins continue to be expressed at normal levels, but without the amino acids encoded by the excluded exons. Therefore, protein activities are likely to be altered, but it is difficult to predict a priori how the missing amino acids will affect protein function. Although it is possible that the mutant proteins are completely devoid of activity, in which case the null phenotypes in zebrafish or other model organisms might be informative, it is equally likely that the protein activities are partially reduced, hyperactivated, or altogether new. In an effort to establish causality of the mis-splicing events, one could recapitulate the mis-splicing events by knocking out the excluded exons in wild-type animals or attempt to rescue the mutant phenotype by adding back properly spliced transcripts. Although these approaches might be feasible for 1 or 2 genes at a time, they would be complicated by the likelihood that the cellular phenotypes arise from the combinatorial effects of multiple mis-splicing events, including those we validated (5) and many that we did not (161). For example, although we validated 5 differential ASEs in four myocardial genes, rbpms2-null zebrafish embryos also differentially splice me2 (malic enzyme), asb5b (ankyrin repeat and SOCS box containing 5b), rbfox2, plekha7a/hadp1 (pleckstrin homology domain containing family member 7a; Table S4), all of which are also expressed in the zebrafish embryonic myocardium.97–99 Importantly, our analysis identified a conserved network of 29 ortholog pairs that relies on RBPMS2 for alternative splicing regulation in zebrafish and human cardiomyocytes. This list includes several genes that are integral to heart development or function including RBFOX2, SLC8A1, and MYBPC3.24,76,78 It remains possible that a greater number of ortholog pairs exist but some evaded detection. This could be due to experimental constraints such as having to perform RNA-sequencing on whole embryos rather than isolated hearts.

The goal of ascribing causality of differential ASEs to cellular phenotypes would be aided by the identification of transcriptome-wide Rbpms2 binding sites in wild-type cardiomyocytes using enhanced cross-linking and immunoprecipitation methodology.100 Unfortunately, this is not feasible in zebrafish embryos because of the difficulty in recovering the large number of purified cardiomyocytes required for analysis (<20 million per replicate). A priority for future studies will be to identify direct targets of RBPMS2 using either transgenic approaches101 or enhanced cross-linking and immunoprecipitation analysis of hiPSC-CMs or cardiomyocytes from adult zebrafish.

Although elevated RBPMS2 expression levels have been detected in gastric cancers102 and chronic intestinal pseudo-obstruction,90 mutations in RBPMS2 have not been implicated in the pathogenesis of human disease to our knowledge. The severity of the myofibrillar defects observed ΔRBPMs2 hiPSC-CMs suggests that human embryos devoid of RBPMS2 would suffer from early cardiac dysfunction and potentially embryonic lethality. Accordingly, based on human sequencing data in the Genome Aggregation Database,103 RBPMS2 appears to be intolerant to loss-of-function mutations because the observed (o) number of predicted loss-of-function single nucleotide variants is significantly lower than expected (e; gnomAD v2.1.1). Whereas 11.2 predicted loss-of-function single nucleotide variants are predicted, only one has been observed in a heterozygous individual of unknown health. This o/e ratio (0.09) and reported CI (0.03–0.42) suggest that RBPMS2 is intolerant to mutations in the human population and may be haploinsufficient in most cases.103 Future studies on hiPSC-CMs heterozygous for RBPMS2 null alleles will provide important insight regarding the potential haploinsufficiency of this gene.

While this manuscript was in revision, Gan et al104 reported that the highly related Rbpms gene is required for maintaining cardiomyocyte diploidy and enabling cardiomyocyte proliferation during mouse heart development, which protects the heart from noncompaction cardiomyopathy. Over 150 differential ASEs were detected in Rbpms-null ventricles, and the cardiac phenotypes were attributed, at least in part, to mis-splicing of the Pdlim5 gene. Together, the study by Gan et al104 and the current study highlight species-specific differences between the expression patterns and functions of Rbpms and RBpms2 in the vertebrate heart. Our study demonstrates that in zebrafish, rbpms2a and rbpms2b are significantly enriched in cardiomyocytes, and knocking out both genes disrupts embryonic heart function. By contrast, rbpms is not enriched in the zebrafish ALPM (Table S1) and does not appear to be expressed in the embryonic heart,105 which suggests that rbpms knockout zebrafish would not exhibit cardiac defects. In the mouse embryo, Rbpms expression is significantly enriched in cardiomyocytes,36,37,104 and knocking it out disrupts heart development.104 To our knowledge, the developmental expression pattern of Rbpms2 has not been published,97 but Rbpms2 knockout mice did not show cardiac defects at 12 weeks of age.105 Taken together, these data suggest that rbpms2 serves an indispensable function in the zebrafish heart, whereas Rbpms performs a similarly important function in the mouse heart. It remains possible that Rbpms2 and Rbpms perform semiredundant functions in the mouse heart, which would be revealed if the cardiac phenotype of Rbpms2, Rbpms2 double mutant mice were more severe than Rbpms-null animals.

It is difficult to compare and contrast the phenotypes of rbpms2-deficient zebrafish and Rbpms2-deficient mice because of species-specific differences in the heart and because the focus of the Gan et al104 study differed from ours. Whereas Gan et al104 evaluated Rbpms-deficient mouse hearts for premature cardiomyocyte polyploidization and noncompaction, we evaluated rbpms2-deficient zebrafish for abnormalities in sarcomere structure and calcium handling. There is little rationale for evaluating rbpms2-deficient zebrafish for premature polyploidization...
and noncompaction because, unlike mouse cardiomyocytes, zebrafish cardiomyocytes remain diploid throughout life and trabecular compaction does not occur in zebrafish. In both species, null animals exhibited defects in alternative splicing. It will be informative in future studies to compare the differentially spliced genes in rbpms2-deficient zebrafish and Rbpms-null mouse hearts to determine how much overlap might exist on a molecular level. Important differences are likely to exist because we did not detect mis-splicing of the Pdlim5 homologs pdlim5a or pdlim5b in rbpms2-null zebrafish (Table S4).

Knocking out either RBPMS2 (this study) or RBPMS2 in hiPSC-CMs elicited phenotypes, suggesting that both genes perform important activities in human cardiomyocytes. The degree of phenotypic overlap between RBPMS2- and RBPMS-null hiPSC-CMs remains unclear because they were examined for different cellular phenotypes in both studies. In future studies, it would be informative to perform a side-by-side comparison of Rbpms2-null and Rbpms-null hiPSC-CMs, together with double mutant hiPSC-CMs, using the same cellular and molecular assays to determine redundant and non-redundant requirements for these genes in human cardiomyocytes.

We propose a model whereby RBPMS2 regulates alternative splicing of its target genes, many of which are likely to be expressed in multiple tissue lineages, to optimize the activities of the encoded proteins specifically for cardiomyocytes. In the absence of RBPMS2, the resulting differential ASEs either disrupt protein production by shifting the reading frame or lead to the production of proteins with suboptimal activities within cardiomyocytes. These molecular abnormalities are likely to interact in a combinatorial fashion to induce the cardiac phenotype. It remains possible that RBPMS2 also performs splicing-independent functions in regulating the stability, subcellular localization, or translational efficiency of target genes in cardiomyocytes, which would also complicate efforts to ascribe the cardiac phenotype to mis-splicing of individual mRNAs. Future attempts to identify transcriptome-wide binding sites for RBPM2S2, coupled with an analysis of how the direct targets behave in mutant animals or cells, will evaluate the possibility of splicing-independent functions for RBPMS2. Ultimately, our study and the recent study by Gan et al begin to highlight the important roles played by a novel family of RBPs in the regulation of vertebrate cardiomyocyte physiology.

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Disclosures
None.

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