RBFox1-mediated RNA splicing regulates cardiac hypertrophy and heart failure

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RNA splicing is a major contributor to total transcriptome complexity; however, the functional role and regulation of splicing in heart failure remain poorly understood. Here, we used a total transcriptome profiling and bioinformatic analysis approach and identified a muscle-specific isoform of an RNA splicing regulator, RBFox1 (also known as A2BP1), as a prominent regulator of alternative RNA splicing during heart failure. Evaluation of developing murine and zebrafish hearts revealed that RBFox1 is induced during postnatal cardiac maturation. However, we found that RBFox1 is markedly diminished in failing human and mouse hearts. In a mouse model, RBFox1 deficiency in the heart promoted pressure overload–induced heart failure. We determined that RBFox1 is a potent regulator of RNA splicing and is required for a conserved splicing process of transcription factor MEF2 family members that yields different MEF2 isoforms with differential effects on cardiac hypertrophic gene expression. Finally, induction of RBFox1 expression in murine pressure overload models substantially attenuated cardiac hypertrophy and pathological manifestations. Together, this study identifies regulation of RNA splicing by RBFox1 as an important player in transcriptome reprogramming during heart failure that influence pathogenesis of the disease.

Introduction

RNA splicing is a ubiquitous posttranscriptional process for all multiexon genes in eukaryotes. Alternative mRNA splicing from a single gene yields multiple mature transcripts, which contributes to the total complexity of the human transcriptome and is important for refining cellular identity and function (1, 2). This posttranscriptional regulatory process is essential to cellular specificity, with an estimated approximately 100,000 intermediate to high abundant alternative splice events identified in major human tissues (3). Alternative RNA splicing is regulated by cis-regulatory enhancers and silencers located within pre-mRNAs interacting with trans-acting splicing factors. Misregulated alternative RNA splicing events have a significant role in several human diseases (4–8).

It is well established that alternative mRNA splicing affects a broad spectrum of cardiac genes during normal development as well as pathological manifestation of heart diseases (9–13). For example, the central fructose-metabolizing enzyme in heart is ketohexokinase, which is shown to be regulated during pathological cardiac hypertrophy through the splicing factor SF3B1 (14). Several splicing factors have also been implicated in the regulation of cardiac function. Inactivation of the heterogeneous nuclear ribonucleoprotein U (hnRNP U) in mouse hearts causes lethal dilated cardiomyopathy (15). In addition, mutations of the splicing regulator RBM20 are associated with dilated human cardiomyopathy, and the reduced expression of RBM20 has been suggested to affect several cardiac genes shown to be involved in DCM, including titin (16–19). However, the underlying molecular mechanisms and the specific contribution of RNA splicing to the pathogenesis of stress-induced heart failure (HF) still remain to be fully explored.

In this report, we analyzed the global RNA splicing events in pressure overload–induced failing mouse hearts and identified a muscle-specific isoform of RBFox1 (also known as A2BP1) as a key trans-acting RNA splicing regulator in cardiomyocytes during hypertrophy and HF. RBFox1 has enriched expression in brain and striated muscle cells. RBFox1 expression in brain is associated with autism (20). In addition, RBFox family members, most frequently RBFox1 and RBFox2, appear to be important for heart and skeletal muscle development and function (21, 22). However, the role of RBFox1 in postnatal cardiac function under normal physiological and pathological conditions of induced HF as well as the downstream targets meditated by RBFox1 in the heart remain to be explored. In this study, we found that RBFox1 expression was significantly diminished in both mouse and human failing hearts. RBFox1 expression was essential for normal heart function in the developing zebrafish, and loss of RBFox1 expression significantly aggravated pressure overload–induced cardiac hypertrophy and failure in mice. At a mechanistic level, we found that RBFox1 had a global effect on cardiac mRNA splicing in cardiomyocytes and directly regulated an isoform switch from α1 to α2 in splice variants of the transcription factor family MEF2. RBFox1-mediated Mef2 splicing contributed
to cardiomyocyte hypertrophy and pathological gene induction. Most remarkably, cardiac-specific reexpression of RBFox1 could significantly attenuate pressure overload–induced pathological hypertrophy and HF in mice. Therefore, our study reveals for what we believe to be the first time that RBFox1-dependent RNA splicing, in particular an isoform switch of Mef2 gene splice variants, is a regulatory circuit in cardiac transcriptional reprogramming, with a significant effect on the pathogenesis of HF.

Results

RBFox1 is a key splicing regulator in cardiomyocytes, mediating a large number of splicing events during cardiac stress. In a previous study, we profiled transcriptome-wide alternative RNA splicing events associated with pressure overload–induced HF in mice (23). In an attempt to further analyze the RNA sequencing (RNA-seq) result, a random subset of 32 alternative splicing events (out of 28 genes) identified to be markedly changed in the failing mouse heart was tested by qRT-PCR in neonatal, normal adult, and pressure overload–induced mouse failing hearts (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI84015DS1). All of the splicing events showed reciprocal expression changes during postnatal cardiac maturation versus HF (Supplemental Figure 1). This observation reveals a “fetal-like” alternative RNA splicing pattern in the failing heart parallel to a similar pattern of expression for pathological genes.

To investigate the underlying molecular basis of such coordinated RNA splicing regulation, we performed a de novo motif discovery analysis for all affected exons in the proximal regions (500 bp of upstream and downstream introns and exons) for the known trans-acting RNA splicing regulators (24). A number of significantly enriched binding motifs, including CELF, TIA, ASF, hnRNPL, and RBFox1/2, were identified (Figure 1A and Supplemental Table 2). The mRNA expression levels for Celf, Tia, Asf, and Hrnrpl were not significantly changed in failing mouse hearts compared with those in normal controls (Figure 1B); neither were expression levels for 22 other known RNA splicing regulators, including those previously implicated in cardiac RNA splicing and the pathogenesis of cardiomyopathy, such as RBM20 (18, 19, 25), SC35 (13), and MBNL2 (refs. 26 and Supplemental Figure 2). Consistent with a recent report (27), RBFox2 expression was only modestly reduced at the protein level in mouse hypertrophic hearts but not significantly affected at the mRNA level (Figure 1, B, D, and F). In contrast, the cardiac isoform of RBFox1 expression showed a significant reduction at both mRNA and protein levels (Figure 1, B, C, E, G, and H). Reduced RNA polymerase II occupancy at the Rbfox1 (but not Rbfox2) locus supports a transcriptional mechanism of RBFox1 repression in the diseased heart (ref. 28 and Figure 1I). In contrast to its diminished expression in the diseased heart, RBFox1 was significantly increased during cardiac development in both zebrafish and mice (Supplemental Figure 3). Therefore, RBFox1 is a potential RNA splicing regulator, with a dynamic expression pattern parallel, with “fetal-like” RNA splicing changes in HF.

In order to identify potential downstream RNA splicing events regulated by RBFox1 in the heart, we performed RNA-seq analysis in neonatal rat ventricular myocytes (NRVMs) at basal conditions (with low endogenous RBFox1 expression) and upon adenovirus-mediated RBFox1 expression. A global shift of RNA splicing pro-
direct effect on MeF2 alternative splicing in the intact heart and promotes pressure overload–induced HF in vivo.

**RBFox1 mediates cardiomyocyte hypertrophy response.** Our data indicate that loss of cardiac RBFox1 expression significantly contributes to pathological hypertrophy and HF. To demonstrate the remodeling, and pathologic marker gene expression (Figure 3, B–H; Supplemental Figure 9, B and C; and Supplemental Figure 10). Similar cardiac defects were observed in zebrafish by morpholino inactivation of RBFox1 (Figure 4). These data suggest that loss of RBFox1 expression, as observed in the failing heart, has a
Mef2c genes, which were also significantly blunted by RBFox1 expression (Figure 5E and Supplemental Figure 12).

In order to establish the role of the α1 to α2 switch of Mef2 in RBFox1-mediated regulation of cardiac hypertrophy, we first tested the contribution of Mef2 splicing variants in cardiomyocyte hypertrophy in vitro. By using a direct impact of RBFox1 expression in cardiomyocytes, we ectopically expressed RBFox1 in NRVMs and observed attenuated cardiomyocyte hypertrophy in response to phenylephrine (PE) treatment (Figure 5, A–C) and hypertrophic gene expression (Figure 5D) in an RNA splicing–dependent manner (Supplemental Figure 11). Consistently, PE induced α2 to α1 switches for the Mef2d and Mef2c genes, which were also significantly blunted by RBFox1 expression (Figure 5E and Supplemental Figure 12).
MEF2D a2–specific siRNA (Supplemental Figure 13), we found that MEF2D a2 variant expression was necessary for RBFox1-mediated attenuation of hypertrophic gene expression in the NRVMs under basal conditions (Figure 6A) as well as PE-treated conditions (Figure 6B). A similar effect was observed for the MEF2C a2 variant (Supplemental Figure 14). Therefore, RBFox1-mediated MEF2C and MEF2D a1 to a2 isoform switch is important for its antihypertrophic effect. In good agreement with an early study in
zebrafish (36), inactivation of RBFox1 led to a change of the \( \text{mef2a} \) and \( \text{mef2d} \) α1/α2 ratio and HF (Figure 6C). Remarkably, the HF phenotype caused by RBFox1 knockdown was markedly rescued by simultaneous knockdown of the \( \text{mef2a} \) α1 variant but not the \( \text{mef2a} \) α2 variant (Figure 6D and Supplemental Figure 15). Conversely, overexpression of zebrafish (\( \text{Mef2a} \)) or mouse (MEF2a) \( \text{Mef2a} \) α1 but not the α2 splicing variant was sufficient to cause a similar HF phenotype in the developing zebrafish (Figure 6E and Supplemental Figure 16). Finally, RNA-seq analysis from zebrafish expressing α1 or α2 Mef2a variants revealed global differences in gene expression (Figure 6F). All these results suggest that RBFox1 is a potent regulator of cardiac hypertrophy and its downstream splicing variants of the \( \text{Mef2} \) genes lead to different effects on the cardiac transcriptome and cardiac pathology.

Restoring RBFox1 expression prevented pathological hypertrophy. In vitro study suggests that RBFox1 expression can protect cardiomyocytes against pathological hypertrophy. To validate this observation in vivo, we generated a transgenic mouse line with cardiac-specific and inducible expression of RBFox1 (ref. 37, Figure 7A, and Supplemental Figures 17 and 18). Consistent with our earlier in vitro observations, doxycycline-induced RBFox1 expression in the heart also resulted in a substantial and specific enhancement of the α2 exon inclusion for all the \( \text{Mef2} \) genes in heart, without affecting the relative inclusion of the β exon (Figure 7B and Supplemental Figure 8). Remarkably, RBFox1 expression significantly attenuated pressure overload–induced cardiac hypertrophy (Figure 7, C and D), contractile dysfunction (Figure 7E), pathological gene expression (Figure 7F), and fibrotic remodeling based on histology and molecular markers (Figure 7G and Supplemental Figure 19). These data support the notion that restoring RBFox1 expression can ameliorate broad features of pathological hypertrophy and HF in the intact hearts.

**RBFox1 contributed to global RNA splicing reprogramming during HF.** Finally, in order to determine the specific contribution of RBFox1 to global RNA splicing reprogramming during HF, we performed a genome-wide RNA splicing profiling study by RNA annealing, selection, and ligation sequencing (RASL-seq) analysis (38) in cardiac tissues from control, RBFox1-CKO, and RBFox1 transgenic (RBFox1-TG) mice following sham surgery or pressure overload (Supplemental Figure 20). We detected a total of 1,140 significantly changed exon splicing events in the TAC-induced failing mouse hearts compared with that in the controls. Remarkably, 505 of them were also affected by cardiac-specific \( \text{Rbfox1} \) knockout (Figure 8, A and C), suggesting that RBFox1 inactivation may contribute up to nearly 44.3% of the exon splicing changes induced by TAC (\( P < 4E^{-64} \)). Similarly, 575 of these exons were also affected in RBFox1-TG hearts, again suggesting that RBFox1 reexpression affects 50.4% of TAC-induced exon splicing events (\( P < 3E^{-50} \)). Most importantly, clustering analysis from significantly changed splicing events shared among all 3 genotypes showed that RNA splicing profiles from RBFox1-CKO and RBFox1-TG hearts were diagonally located at the opposite direction from those of wild-type hearts following TAC (Figure 8B). While tissues from RBFox1-CKO animals expanded and augmented the RNA splicing changes in
wild-type failing hearts, RBFox1-TG tissue showed a reduced or reversed effect on the majority of them. All of these data support the notion that loss of RBFox1-mediated RNA splicing has a major contribution to the global RNA splicing defects observed in the pathologically stressed hearts. Restoring RBFox1 activity can ameliorate a significant degree of global RNA splicing reprogramming, reinforcing its protective effect against hypertrophy and dysfunction.

Discussion
A posttranscriptional gene regulatory circuit we believe is novel in HF. Although RNA splicing is a well-recognized contributor to transcriptome complexity and alternative RNA splicing variants are commonly observed among many cardiac genes during development and diseases, our knowledge regarding their underlying regulatory mechanisms and the functional impact is still very limited. Alternative RNA splicing is regulated by cis-regulatory enhancers and silencers located within pre-mRNAs interacting with trans-acting splicing factors (1, 2, 39–41). Mutations in several splicing factors, including SC35 and RBM20, have been implicated in dilated cardiomyopathy in mice, rats, and humans (18, 42, 43). Other splicing factors are also shown to play important roles in cardiac development and function, including CUGBP1, ETR-like factors,
and HF. While genetic inactivation of Rhfox1 promotes pathological remodeling in stressed mouse hearts and HF in developing zebrafish embryos, RBFox1 expression exerts a marked protective effect on a broad spectrum of cardiac pathological remodeling in pressure-overloaded hearts, including cardiomyocyte hypertrophy and collagen gene expression. In summary, our study uncovered a previously uncharacterized RBFox1/MEF2 regulatory circuit in HF.

**Figure 6.** RBFox1/MEF2 regulatory circuit in cardiomyocyte hypertrophy regulation. (A) Anf and Bnp expression in NRVMs following GFP or RBFox1 expression, with or without coexpressing a scrambled siRNA (ncsiRNA) or an siRNA targeting Mef2d α2 isoform (si-Mef2d-α2) as indicated (n = 3 each sample). (B) Anf and Bnp expression in NRVMs with or without PE treatment or RBFox1 expression or coexpressing a scrambled siRNA or an siRNA targeted to Mef2d α2 isoform (n = 3 each sample). (C) mef2a and mef2d α1/α2 ratio in RBFox1 morpholino–injected hearts compared with control zebrafish embryo hearts (n = 3 each sample). (D) Zebrafish phenotype upon RBFox1 and Mef2a inactivation. Zebrafish embryos were injected with morpholino targeting RBFox1 alone or in combination with Mef2a α1 or α2 isoform–specific morpholino. Embryo phenotype was analyzed at 48 hours after fertilization. Original magnification, ×1 (first column); ×11 (second column). (E) Zebrafish phenotype upon expression of zebrafish Mef2a α1 or zebrafish Mef2a α2, mouse MEF2A α1, and mouse MEF2A α2 at indicated dose imaged at 48 hours after fertilization. Original magnification, ×1 (first column); ×11 (second column). (F) Gene expression profile in the zebrafish embryos 24 hours following expression of individual mef2a α1 or α2 isoforms analyzed by RNA-seq. The heatmap was generated using significantly upregulated (red) and downregulated (green) genes. *P < 0.05, Student’s t test (A–C).
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...different transcriptional activity due to differential phosphorylation by PKA and binding partners acting as corepressors or coactivators (47). Our data from cardiomyocytes (Figure 6) argue for qualitatively different functions for the MEF2 splice variants containing the \( \alpha_1 \) exon or the \( \alpha_2 \) exon. Although MEF2 has a well-established role as a transcriptional activator for cardiogenic and hypertrophic splicing of all the Mef2 genes at their \( \alpha \) exon affects a region adjacent in the 3′ direction to the MEF2- and DNA-binding MADS box domains. A recent study reported that the \( \alpha_1 \) isoform of MEF2D is ubiquitously expressed, whereas the \( \alpha_2 \) isoform is muscle specific. Furthermore, this study found that the two isoforms have similar DNA-binding profiles across the genome in skeletal muscle but different transcriptional activity due to differential phosphorylation by PKA and binding partners acting as corepressors or coactivators (47). Our data from cardiomyocytes (Figure 6) argue for qualitatively different functions for the MEF2 splice variants containing the \( \alpha_1 \) exon or the \( \alpha_2 \) exon. Although MEF2 has a well-established role as a transcriptional activator for cardiogenic and hypertrophic

**Figure 7.** Restoring RBFox1 prevented pathological hypertrophy in pressure-overloaded mouse hearts. (A) Western blot analysis of RBFox1 protein in wild-type or single-transgenic hearts (Non-TG) compared with double-transgenic hearts (RBFox1-TG) 6 weeks following sham or TAC operation. (B) Quantification of Mef2 \( \alpha_1/\alpha_2 \) transcript ratio in non-TG and RBFox1-TG hearts after 2 weeks of doxycycline induction (\( n = 3 \) each sample). *\( P < 0.05 \). (C) Heart weight (HW) and body weight ratios in sham-operated mice and non-TG (NTG) and RBFox1-TG mice 6 weeks after TAC (Sham-NTG, \( n = 9 \); Sham-RBFox1-TG, \( n = 9 \); TAC-NTG, \( n = 15 \); TAC-RBFox1-TG, \( n = 16 \)). **\( P < 0.01 \). (D) Cross-sectional area of cardiomyocytes in sham-operated mice and non-TG or RBFox1-TG mice after TAC. Average values were derived from 100 myocytes of from each group. **\( P < 0.01 \). (E) Ejection fraction of sham- and TAC-operated non-TG and RBFox1-TG mice measured by echocardiography up to 5 weeks after TAC (Sham-NTG, \( n = 9 \); Sham-RBFox1-TG, \( n = 9 \); TAC-NTG, \( n = 15 \); TAC-RBFox1-TG, \( n = 16 \)). **\( P < 0.05 \), TAC-NTG vs. TAC-RBFox1-TG. #\( P < 0.05 \), Sham-NTG vs. TAC-NTG. (F) Anf and Bnp mRNA expression levels in non-TG and RBFox1-TG mice following sham surgery or 5 weeks after TAC (\( n = 3 \) each group). *\( P < 0.05 \). **\( P < 0.05 \). (G) Representative images of Masson trichrome–stained ventricular sections from the sham- and TAC-operated non-TG and RBFox1-TG mice as indicated. Original magnification, \( \times 20 \). Data are representative of at least 3 independent experiments. Significant differences between groups were determined by Student’s t test (B) or multiway ANOVA (C–F).
that RBFox2 expression is higher in the neonatal heart but markedly reduced in the mature adult mouse heart, in contrast with that after RBFox1 induction in the postnatal hearts. Therefore, RBFox1 and RBFox2 may have both overlapping and unique functions during cardiac maturation and pathogenesis, with RBFox1 being a predominant player in the adult heart.

The RBFox1/MEF2 circuit in HF regulation and therapy. Our study shows that a single RNA splicing regulator can exert a profound effect on a broad spectrum of pathological features in the diseased heart. Based on both total transcriptome profiling using RNA-seq and RNA splicing profiling by RASL-seq, RBFox1-mediated RNA splicing has a major contribution to the global RNA splicing changes in the diseased heart (Figure 7). This remarkable contribution underscores the significant impact of RBFox1 inactivation.
or reexpression on the pathogenesis of HF. Rbfox1 deletion is associated with a complex form of congenital cardiomyopathy. Most relevantly, the correlated loss of Rbfox1 expression and the splicing changes of the MEF2α exon are observed in the human DCM hearts (Figures 1 and 2). Therefore, Rbfox1-mediated RNA splicing can be a potential new target of intervention against pathological remodeling in the heart and restoring Rbfox1 abundance and function can be explored as a novel therapeutic approach for HF.

Methods

Further details are described in the Supplemental Methods.

**Rbfox1-TG and cardiac-specific knockout mice.** Animals in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals (8th ed. The National Academies Press. 2011). The detailed description of the generation of Rbfox1-TG and cardiac-specific knockout mice is provided in the Supplemental Methods.

**Tissue from human nonfailing and failing hearts.** The failing heart samples (n = 4) were obtained from the left ventricular (LV) anterior wall during heart transplantation or implantation of an LV assist device (49). The nonfailing heart samples (n = 4) were obtained from the LV free wall and procured from the National Disease Research Interchange and the University of Pennsylvania. Nonfailing heart donors had no history of macroscopic or laboratory signs of cardiac disease.

**Motif analysis.** Motif analysis was carried out for the exonic regions and 4 intronic regions flanking differentially spliced exons obtained in our previous study (50). The entire exonic region was included. For intronic regions, up to 250 bases from the corresponding exon–intron boundary were included. For introns shorter than 500 bases, only half of the intronic regions were used. Previously described methods (51) were used for motif analysis and are briefly described below.

**Motif conservation.** We analyzed sequence conservation of pentamers in the mouse intronic regions to identify potential splicing regulatory elements. The mouse introns were aligned to 7 other mammalian genomes that have at least 5 sequence coverage in the UCSC 28-way multigenome alignment (51). For each pentamer in each region, a conservation rate (CR) was calculated as the fraction of aligned and conserved occurrences among total occurrences. The significance of the CR of each pentamer was evaluated by comparing it with that of 10 other pentamers with similar expected CRs, calculated using the first-order Markov model. This procedure essentially controls for possible sequence bias in the data set. A P value was calculated by using the binomial distribution.

**Motif enrichment.** In this analysis, to account for background sequence biases, the introns corresponding to each region were binned according to their GC frequency into 10 groups. Expected pentamer frequency was calculated for each pentamer by using the first-order Markov model in introns of each GC group. Pentamer enrichment was then evaluated by comparing the occurrence frequency of each pentamer to the overall expected frequency, calculated by summing up the expected counts of all GC groups. A P value was calculated by using the binomial distribution.

**Pressure overload model of HF in mice.** LV tissues were collected from male C57BL/6 mice 8 weeks after the TAC procedure (which induced HF) and 1 day after birth (neonatal), respectively, and their corresponding sham controls as described previously (23). Doppler velocity measurements of right and left carotid arteries were obtained from TAC-treated mice to confirm the consistency of the surgery procedure. The HF status of the TAC-treated animals was established based on a significant increase in heart weight and a significant reduction in ejection fractions measured by echocardiography. All experimental procedure and echocardiogram analyses were performed blinded to the mouse genotype.

**RNA polymerase II occupancy analysis.** RNA polymerase II occupancy data (28) was visualized at the Rbfox1 locus for sham and TAC conditions using the Integrated Genome Browser (52).

**Gene expression analysis.** Gene expression analysis via RNA-seq, RASL-seq, qRT-PCR, in-situ hybridization, and luciferase reporter gene assays is described in detail in the Supplemental Methods. RNA-seq and RASL-seq data are available in the NCBI’s BioProject database under accession numbers PRJNA295071 and PRJNA294802, respectively.

**Statistics.** Data are expressed as mean ± SD. For comparison between two groups, differences were analyzed by 2-tailed Student’s t test. For comparison of multiple groups, differences were analyzed by 1-way ANOVA. P values ≤ 0.05 were considered as significant.

**Study approval.** Human cardiac tissue collection was approved by the UCLA Institutional Review Board (11-001053 and 12-000207). Written consent from participants or their guardians was obtained.

**Author contributions**

CG conceived the project and designed and performed most of the molecular and genetic studies, data analysis, and interpretation. YW managed funding and participated in experimental design, data analysis, and manuscript preparation. SR performed TAC surgeries and cardiac function studies. JHL, CDR, DJC, MA, TMV, and XX supported bioinformatics analysis and manuscript preparations. JQ, YZ, and XDF contributed RASL-seq analysis. JNC provided support and models for zebrafish analysis. AN provided Nkx2.5-Cre mice as well as support for the phenotypic analysis.

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