Nanopore sequencing reveals full-length Tropomyosin 1 isoforms and their regulation by RNA binding proteins during rat heart development

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ABSTRACT

Alternative splicing (AS) increases the variety of the proteome by producing multiple isoforms from a single gene. Although short-read RNA sequencing methods have been the gold standard for determining AS patterns of genes, they have a difficulty in defining full length mRNA isoforms assembled using different exon combinations. Tropomyosin 1 (TPM1) is an actin binding protein required for cytoskeletal functions in non-muscle cells and for contraction in muscle cells. *Tpm1* undergoes AS regulation to generate muscle versus non-muscle TPM1 protein isoforms with distinct physiological functions. It is unclear which full length *Tpm1* isoforms are produced via AS and how they are regulated during heart development. To address these, we utilized nanopore long-read cDNA sequencing without gene-specific PCR amplification. In rat hearts, we identified full length *Tpm1* isoforms composed of distinct exons with specific linkages. We showed that *Tpm1* undergoes AS transitions during embryonic heart development such that muscle-specific exons are connected together generating predominantly muscle specific *Tpm1* isoforms in adult hearts. Nanopore sequencing combined with polyA sequencing revealed that the RNA binding protein RBFOX2 controls AS of muscle specific *Tpm1* isoforms and expression of TPM1 protein via terminal exon splicing impacting its polyadenylation. Furthermore, RBFOX2 regulates *Tpm1* AS antagonistically to PTBP1. In sum, we defined full length *Tpm1* isoforms with different exon combinations that are tightly regulated during cardiac development and provided insights into regulation of muscle specific isoforms of *Tpm1* by RNA binding proteins. Our results demonstrate that nanopore sequencing is an excellent tool to determine full-length AS variants of muscle enriched genes.

Introduction

Gene regulation by AS is an important contributor to development and tissue identity (1). AS not only controls gene expression but also increases the proteome output by generating differentially spliced isoforms. Genome-wide analyses indicate that the majority of human genes undergo AS (2). Currently, many computational approaches are available to investigate AS patterns based on short read RNA-sequencing. However, with these techniques it can be difficult to determine the connectivity of multiple exons in a given transcript. This becomes more challenging if a given gene has many transcripts. Recent advances in nanopore sequencing technology allow sequencing of long DNA sequences up to 100,000 bases (3). Oxford MinION sequencer is a portable device that provides real-time, high-throughput and long-read sequencing with <10% error rate (4,5). This technology, therefore, is very attractive to study complex AS patterns in the context of full-length transcripts.

TPM1 is a coiled coil protein that forms polymers along with the actin filament. TPM1 is the predominant tropomyosin gene expressed in cardiac muscle and plays a significant role in muscle contraction (6). TPM1 is required for myofibril organization (7), myocardial contraction (8), and cardiac development (9). Mutations or aberrant expression of *TPM1* are associated with familial hypertrophic cardiomyopathy (10-13), dilated cardiomyopathy (14,15) and heart failure (16). *TPM1* has many gene isoforms generated via AS that are tissue specific and developmentally regulated. These multiple isoforms render distinct functions including cytoskeleton support and muscle contraction in the heart (17-20). However, it is still unclear what mechanisms dictate highly coordinated AS of *Tpm1* that impacts its expression and function in a cell- and development-specific manner.

RBFOX2 is an RNA binding protein, which regulates AS by binding to a highly conserved motif ((U)GCAUG) in pre-mRNAs (21-24). RBFOX2 is important for muscle differentiation (25), maintaining muscle mass (26), and sustaining muscle function (27). We and other groups have shown that RBFOX2 is involved in cardiovascular diseases including hypoplastic left heart syndrome (28-30), heart failure (31), and diabetic cardiomyopathy (32,33). RBFOX2 binding sites are enriched near alternative exons that are

developmentally regulated postnatally in the heart (34), suggesting a role for RBFOX2 in regulation of AS during postnatal heart development.

Using nanopore cDNA sequencing, we identified full length *Tpm1* isoforms with unique exon combinations that are regulated during rat heart development. We found that muscle and non-muscle specific *Tpm1* isoforms was generated via AS of specific exons during rat heart development. Nanopore sequencing combined with polyA sequencing we uncovered that RBFOX2 regulates not only AS of internal exons but also AS of terminal exons of *Tpm1* affecting expression levels of TPM1 protein via modulating its polyadenylation. Furthermore, RBFOX2 and PTBP1 antagonistically control AS of muscle specific *Tpm1* isoforms. Overall, our results reveal that *Tpm1* spliced isoforms are tightly regulated during rat cardiac development and that RNA binding proteins RBFOX2 and PTBP1 have opposing roles in controlling developmentally regulated *Tpm1* AS. Our findings have broad implications in defining complex alternative splicing patterns of abundant cardiac muscle-enriched genes using nanopore cDNA sequencing without gene specific PCR amplification.

Results

Nanopore sequencing identified full length *Tpm1* isoforms that undergo alternative splicing transitions during rat heart development

Tpm1 has 15 exons, several of which undergo AS regulation, generating different *Tpm1* isoforms (35). To determine the exact combination of exons in full length *Tpm1* isoforms in the heart at different developmental stages, we used total RNA from embryonic day 20 (E20, n=3) and 6-month (6M, n=3) rat hearts and generated cDNA for nanopore sequencing with MinION. We picked late embryonic and adult stages because between these stages the heart undergoes structural and functional changes important for cardiac output and contractility relevant to TPM1 function.

We obtained ~310’000-740’000 reads spanning 3000-4300 unique mRNAs, 190-413 of which had a coverage of greater than 100 reads that were mapped to the rn6 genome using Minimap2 (36). Each horizontal line represents a single *Tpm1* transcript with different exon combinations (Figure 1A). Reads

that were mapped to each *Tpm1* exon are in black. In E20 rat hearts, we identified *Tpm1* isoforms generated via AS of exons 1a/1b, 2a/2b, 6a/6b, 9a, and 9b/9d (Figure 1A, top panel), consistent with previous findings that these mutually exclusive exons are alternatively spliced (37,38). Strikingly, at this developmental stage *Tpm1* transcripts displayed two distinct 3' ends (3´UTR and coding region) (Figure 1A, top panel). The differences in the 3` length of *Tpm1* was generated via AS of terminal exons 9b and 9d, which possess different polyadenylation sites within the 3`UTR (Figure 1A). Inclusion of exon 9b resulted in short *Tpm1* isoforms whereas inclusion of exon 9d generated long *Tpm1* isoforms due to the differential usage of annotated polyadenylation sites present within these exons (Figure 1A, top panel). It has been previously shown that *Tpm1* isoforms that contain exon 9b are primarily expressed in striated muscle (muscle-specific isoform), and that contain exon 9d are expressed in smooth muscle and other cell types (non-striated muscle isoform, in short non-muscle) (37,38). Cardiac output increases and contractions become more coordinated at adult stages in comparison to embryonic stages. Consistent with this, muscle specific *Tpm1* isoforms that end with exon 9b were predominantly expressed at adult stages. On the other hand, non-muscle *Tpm1* isoforms that end with exon 9d (Figure 1A, bottom panel) were present in embryonic hearts but were dramatically decreased in adult hearts.

In addition to the differences at the 3´end of *Tpm1* isoforms, we found unique linkages between internal and terminal exons in a given *Tpm1* transcript. For example, exon 9a was present in almost all muscle specific *Tpm1* transcripts that ended with exon 9b in rat hearts (Figure 1A). This is in agreement with predominant expression of *Tpm1* isoforms that contain both exon 9a and 9b at adult stages when the heart contracts more coordinately in comparison to the embryonic heart. Similar to exon9a-9b linkage, we found that exon 2a was almost always included in non-muscle *Tpm1* isoforms that ended with exon 9d (Figure 1A, top panel). Exon 2a-9d containing isoforms were barely detectable in adult rat hearts (Figure 1A, bottom panel). Also, *Tpm1* isoforms that contain exon 6a were present scarcely at E20 rat hearts, but they were diminished in adult rat hearts (Figure 1A).

To validate the nanopore sequencing data and assess expression of different *Tpm1* isoforms in embryonic versus adult rat hearts, we designed primers to determine the inclusion of exons 6a, 6b, 9b and

9d (Table S1). Consistent with the nanopore sequencing data, the expression of *Tpm1* transcripts that include exons 6b and 9b gradually increased as the heart developed from E13 till adulthood (Figure 1B). Exons 9a and 9b are important determinants for actin binding affinity of TPM1 and interactions with troponin complex in the presence or absence of Ca^{2+} (39,40). The exon 6a is required for actin binding and replacing exon 6b with exon 6a increases TPM1 actin binding affinity (41). The differences in inclusion of internal exon 6a, exon 2a and terminal exons 9a-9b and 9d in *Tpm1* isoforms expressed in rat hearts correlates with the actin binding activity of *Tpm1* during muscle contraction at different developmental stages.

The RNA binding protein RBFOX2 controls AS and mRNA levels of muscle specific *Tpm1* isoforms that are developmentally regulated

RBFOX2 is an AS regulator abundantly expressed in skeletal and heart muscle (32,33,42,43). RBFOX2 binding sites are enriched in introns flanking alternative exons that are postnatally regulated in mouse hearts (44). To determine the role of RBFOX2 in developmentally regulated AS of *Tpm1*, we depleted RBFOX2 in embryonic rat heart derived H9c2 cells and performed nanopore sequencing. Control H9c2 cells displayed 22 different *Tpm1* mRNA isoforms (Table S2) including both muscle and non-muscle *Tpm1* isoforms that end with either exon 9d or exon 9b, similar to that observed in E20 rat hearts (Figure 2, top panel vs Figure 1A, top panel). In RBFOX2 knocked down (KD) cells only 9 different *Tpm1* mRNA isoforms were detected (Table S3), non-muscle *Tpm1* isoforms that end with exon 9d were almost exclusively expressed. Strikingly, muscle specific short *Tpm1* isoforms that end with exon 9b disappeared upon RBFOX2 depletion (Figure 2A, bottom panel). To determine how RBFOX2-mediated AS of terminal exons affects polyadenylation of *Tpm1*, we analyzed our poly(A)-ClickSeq data from RBFOX2 KD H9c2 cells that identified genome wide alternative polyadenylation changes (45,46). We found that polyA (p(A)) usage in exon 9b was abolished in RBFOX2 KD cells compared to control cells (Figure 2B), in agreement with exclusion of this exon in full length *Tpm1* transcripts (Figure 2A, bottom panel), validating our nanopore sequencing data.

We also identified specific exon connections in H9c2 cells. Similar to the linkage of exon 9a to the muscle-specific exon 9b in rat hearts (Figure 1A), exon 9a was predominantly included in muscle specific short *Tpm1* isoforms but only in a few non-muscle *Tpm1* isoforms in H9c2 cells (Figure 2A). Exon 6b and 6a were mutually exclusively spliced such that inclusion of exon 6a was favored in RBFOX2 depleted cells (Figure 2A). We also performed RT-qPCR to validate AS of mutually exclusive exons 9b vs 9d and 6b vs 6a. RBFOX2 depletion led to a decrease in expression of muscle specific *Tpm1* transcripts that end with exon 9b (Figure 2C) while did not affect non-muscle *Tpm1* transcripts that end with exon 9d (Figure 2D). Importantly, the depletion of RBFOX2 resulted in downregulation of TPM1 protein correlating with low levels of muscle specific *Tpm1* isoforms in these cells (Figure 2E). *Tpm1* isoforms including exon 6a was dramatically increased upon RBFOX2 depletion (Figure 2F). These results show that RBFOX2 KD altered AS and protein levels of *Tpm1*.

The RNA binding protein PTBP1 has been shown to regulate *Tpm1* AS (35,47-50). RBFOX2 has been shown to regulate AS and mRNA levels of PTBP family member (51). To rule out the possibility that the effect of RBFOX2 on AS of *Tpm1* was mediated via changes in PTBP1, we examined PTBP1 protein levels in control and RBFOX2 depleted H9c2 cells. RBFOX2 KD did not affect PTBP1 protein levels (Figure 2G). In sum, these results indicate that RBFOX2 is critical for controlling AS of *Tpm1*.

RBFOX2 depletion does not affect developmentally regulated AS patterns of TPM2 and TPM3

There are four family members of TPM in vertebrates namely TPM1, TPM2, TPM3 and TPM4. TPM1 and TPM2 are predominantly expressed in muscle and are involved in contraction (52-55), whereas TPM3 and TPM4 are enriched in non-muscle cells supporting actin cytoskeleton (17,18,52,53,56-61). To determine whether AS of other *TPM* genes were also regulated during rat heart development, we examined our nanopore sequencing data. *Tpm2* and *Tpm3* AS patterns were noticeably different between embryonic and adult stages in rat hearts (Figure 3A and 3B top vs bottom panels). Similar to *Tpm1*, *Tpm2* exons 6a/6b and 9b/9d differentially spliced during rat heart development (Figure 3A vs Figure 1A). On the other hand, AS of *Tpm4* was not regulated during rat heart development (Figure 3C). We investigated if RBFOX2

regulates AS of other *Tpm* gene family members. Nanopore sequencing revealed that AS of *Tpm2*, *Tpm3* and *Tpm4* were not obviously affected by loss of RBFOX2 in H9c2 cells (Figure 4A, 4B, 4C). These results indicate that RBFOX2 mediated AS regulation is specific to *Tpm1*.

Ectopic expression of RBFOX2 partially rescues developmentally regulated AS of *Tpm1* in RBFOX2 depleted cells

We next tested if ectopic expression of RBFOX2 can rescue *Tpm1* AS changes in RBFOX2 depleted cells. We expressed GFP tagged RBFOX2 in RBFOX2 depleted H9c2 cells and found that GFP-RBFOX2 protein was expressed at low levels similar to the endogenous RBFOX2 levels in RBFOX2 KD cells (Figure 5A, lane 1 vs 3). Consistent with our results in Figure 2, depletion of RBFOX2 decreased mRNA levels of muscle specific *Tpm1* isoforms (Figure 5B). Ectopic expression of GFP-RBFOX2 partially rescued expression of muscle specific *Tpm1* isoforms (Figure 5B). There was no significant change in expression levels of non-muscle specific *Tpm1* long transcripts upon RBFOX2 KD. Expression of GFP-RBFOX2 did not affect mRNA levels of non-muscle *Tpm1* transcripts (Figure 5C), restating that RBFOX2 controls only muscle specific *Tpm1* isoforms. We also tested if ectopic expression of GFP-RBFOX2 can rescue AS changes in *Tpm1* exons 6a/6b in RBFOX2 KD cells. Expression of GFP-RBFOX2 also partially rescued AS changes of *Tpm1* exon 6a/6b (Figure 5D). Overall, our findings strongly suggest that RBFOX2 is a regulator of AS of developmentally regulated *Tpm1* alternative exons.

RBFOX2 and PTBP1 antagonistically control developmentally regulated AS of *Tpm1*

We found that AS of *Tpm1* exons 6a/6b and terminal exons are regulated by RBFOX2. The RNA binding protein PTBP1 was identified as a regulator of *Tpm1* AS of mutually exclusive exons 2a/2b (35,47) and exons 6a/6b (48) and terminal exons (49,50). To validate these results in H9c2 cells, we knocked down PTBP1 and PTBP2 to deplete PTBP proteins in H9c2 cells. PTBP depletion increased muscle specific *Tpm1* transcripts (Figure 6A), opposite to that in RBFOX2 depleted cells (Figure 2A and 2C). There was no significant change in the generation of non-muscle *Tpm1* isoforms in PTBP1 KD cells, suggesting that like

RBFOX2 PTBP also regulates muscle specific *Tpm1* isoforms (Figure 6B). Inclusion of *Tpm1* exon 6a was inhibited in PTBP depleted H9c2 cells (Figure 6C), contrary to what was observed in RBFOX2 depleted H9c2 cells (Figure 2F). These findings indicate that RBFOX2 and PTBP1 antagonistically control developmentally regulated AS of *Tpm1* contributing to the generation of muscle specific *Tpm1* isoforms.

To better understand how *Tpm1* muscle specific isoforms are regulated by RBFOX2 and PTBP1 during rat heart development, we checked RBFOX2 and PTBP1 protein levels during rat heart development. PTBP1 and RBFOX2 protein levels were abundant at embryonic stages (Figure 6D) when both muscle and non-muscle specific *Tpm1* isoforms were present (Figure 1A, top panel). Since RBFOX2 promotes expression of *Tpm1* muscle specific isoforms, high level of RBFOX2 in embryonic hearts (Figure 6D) is consistent with the abundance of muscle specific *Tpm1* isoforms at embryonic stages (Figure 1A, top panel). Because PTBP1 is a repressor of muscle specific *Tpm1* isoforms, low levels of PTBP1 in adult rat hearts (Figure 6D) correlated well with predominant expression of *Tpm1* muscle specific isoforms in adult hearts (Figure 1A, bottom panel). These results suggest that when both RBFOX2 and PTBP1 are present at embryonic stage, RBFOX2 as a positive regulator is an important determinant of *Tpm1* muscle specific isoform generation at embryonic stages. However, at adult stages downregulation of PTBP1 protein as a repressor has more influence in generation of muscle specific *Tpm1*

Discussion

Tpm1 is an essential gene required for myofibril organization (7), myocardial contraction (8), and heart development (9). *Tpm1* has 15 exons, in which internal exons 1a/1b, 2a/2b and 6a/6b and terminal exons 9a, 9b, 9c and 9d are alternatively spliced (17,47,50,62-65). AS of *Tpm1* isoforms are tissue- and development- specific and exhibit distinct physiological functions (17-20) including cytoskeleton support for almost every eukaryotic cells and muscle contraction for striated muscle cells (52-55). There have been technical challenges to precisely define full length *Tpm1* isoforms that are generated via extensive AS regulation in different cell types and at different developmental stages using short read RNA sequencing methods. It is because short read RNA sequencing is unable to provide direct information about how

different exons are connected and incorporated into full length transcripts to generate different *Tpm1* isoforms. In addition, bias is generated from fragmentation during library preparation. Using nanopore long read sequencing by MinION, we were able to detect full length isoforms of abundant cardiac genes including tropomyosin family members without extensive RNA manipulation and gene specific PCR amplification.

Nanopore sequencing identified full length *Tpm* isoforms with different internal and terminal exon combinations in rat hearts that are regulated during development. We defined striated muscle vs non-striated muscle *Tpm1* isoforms based on the specific exon combinations and internal-terminal exon linkages in embryonic and adult hearts. We also observed that striated muscle specific *Tpm1* isoforms were abundantly expressed at both embryonic and adult stages, but they become the predominant isoform in adult hearts. Loss of non-muscle specific *Tpm1* isoforms in adult rats is in agreement with the increased muscle contraction capability of adult hearts in comparison to embryonic hearts.

We identified RBFOX2 as a critical regulator of developmentally regulated AS of *Tpm1* using nanopore sequencing. This is consistent with a recent study has shown that TPM1 undergoes an AS change in a mouse model where RBFOX2 was conditionally ablated in neural crest cells (66). We showed that RBFOX2 controls AS of muscle specific terminal exons and alters the polyadenylation pattern of *Tpm1*, generating a shorter isoform with a different 3` end. We observed the same change in *Tpm1* polyadenylation pattern in RBFOX2 depleted cells by analyzing our polyA sequencing data (45,46). Our results both sequencing methods combined with RT-PCR validations demonstrate that RBFOX2 is an important determinant of *Tpm1* AS and TPM1 protein levels. Our work provide strong evidence that changes in *Tpm1* AS and proteins levels are one of the likely contributors to heart and muscle defects observed in RBFOX2 loss of function studies (25,26,27, 28-30,31,32,33).

Importantly, RBFOX2 regulates AS of muscle specific *Tpm1* isoforms in opposition to PTBP1. We demonstrated that AS of exons 6a/6b and 9b/9d of *Tpm1* are developmentally regulated in rat hearts and that these exons were controlled antagonistically by RBFOX2 and PTBP. Notably, *Tpm1* last exons 9a and 9d primarily determine the actin binding affinity of tropomyosin and affect interaction of troponin with

tropomyosin in the presence or absence of Ca^{2+} (39,40). The exon 6a/6b is required for cooperative actin binding (41). Therefore, the dynamic and synergic regulation of specific internal exon 6a/6b and last exons 9b/9d via RBFOX2 and PTBP during cardiac development are critical to ensure selective *Tpm1* isoforms expressed in equilibrium to exert specific actin binding activity during different states of muscle contraction.

Our results support the idea that RBFOX2 is a repressor of *Tpm1* exon 6a while is an activator of terminal exon 9b inclusion. Conversely, PTBP1 is an activator of exon 6a but a repressor of terminal exon 9b, consistent with the previous reports (48,49,50). The expression levels of both PTBP1 and RBFOX2 were high at embryonic stages but both were decreased at adult stages in rat hearts. While the predominant expression of muscle specific *Tpm1* isoforms at adult stages was consistent with downregulation of PTBP1, the abundance of muscle specific *Tpm1* isoforms at embryonic stages was in agreement with high levels of RBFOX2. The interplay between these RNA binding proteins in regulating *Tpm1* AS correlates well with their different roles as repressors or activators of exon inclusion.

It is quite common that cardiac structural genes undergo cooperative AS and alternative polyadenylation patterns that determine their specific functions and expression profiles during cardiac development. Mutations in these structural genes including *TPM1* are linked to human heart diseases. Our results using nanopore sequencing provide as an efficient way to reveal full length isoforms of cardiac structural genes and their regulation during heart development. In addition, our work may pave the way for future studies to determine the functional consequences of non-coding mutations on post-transcriptional regulation of cardiac structural genes using nanopore sequencing.

Experimental Procedures

Cell culture

H9c2 cells (ATCC CRL-1446) were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) (ATCC 30-2002), supplemented with 10% fetal bovine serum (FBS, ATCC 30-2020) and 100 units/ml penicillin and streptomycin (Thermofisher Scientific 15140122).

Transfections

For siRNA-KD experiments, H9c2 cells were seeded at 10^6 cells per 100mm dish and transfected with 20nM scrambled siRNA (Invitrogen AM4611), *Rbfox2* siRNA (Invitrogen siRNA ID# s96620) or PTBP siRNAs (Qiagen cat# SI02649206 and SI04255146) using Lipofectamine RNAiMAX (Thermofisher Scientific). Cells were harvested 72 hours post-transfection for RNA or protein extraction. For rescue experiments, $3X10^6$ H9c2 cells were transfected with eGFP (Sigma-Aldrich), human GFP-RBFOX2 (transcript variant 3) (Addgene, plasmid #63086) or empty vector (pcDNA 5) together with scrambled or *Rbfox2* specific siRNAs using Neon Nucleofection System (Thermofisher Scientific) as described previously (67). RNA was harvested 48 hours post-transfection.

Nanopore sequencing with MinION

RNA was extracted from cells using TRIzol (Invitrogen 15596-018) by following the manufacturer's protocol. For nanopore sequencing, three sets of E20 and three sets of 6M rat heart RNA (Zyagen) or RNA from two sets of control and RBFOX2 KD H9c2 cells were used. Total cellular RNA was first poly(A) enriched (New England Biolabs S1550S) and then amplified using oligo-dT primers and template switching oligos using Oxford Nanopore Technologies (ONT) cDNA-PCR sequencing kit (PCS108) as described by the manufacturer. Samples were multiplexed using ONT barcodes. Pooled samples were sequencing on R9.4 flowcells for 36. Reads were demultiplexed and base-called using Albacore and mapped to the rat genome (rn6) using the –splice function of minimap2 as described previously (68). Analysis pipeline is shown in Supplemental Figure 1.

RT-qPCR

Total RNA from cells and rat hearts (purchased from Zyagen) at E13 (pooled), E16 (pooled), E18 (pooled), E20 (pooled), post-natal day1 (D1) (pooled), and 6M (pooled) stages were extracted using Trizol. 2µg of total RNA was used for cDNA synthesis using AMV reverse transcriptase (15 units/µg, Life Biosciences). For RT-qPCR, master mix was set up by mixing 5 µl of cDNA, 3 µl of H_2O, 2 µl of PCR gene specific primer (10X conc) (Table S1) and 10 µl of master mix (Roche 04707516001) in 20 µl reaction. The RT-qPCR was conducted using LightCycler 480 Instrument (Roche) using the following conditions: 95 °C 10 s; 62 °C 15 s; 72 °C 10s for 40 cycles. Melting curve was obtained to ensure single product. ΔCt method was adopted for quantification. Semi-quantitative RT-PCR instead of RT-qPCR was used for determining *Tpm1* short and *Tpm1* long transcript levels due to the alternative splicing of exon 9a that generates two different sized DNA bands after amplification. 2µg of total RNA was used for cDNA synthesis using AMV reverse transcriptase (15 units/µg, Life Biosciences). PCR was performed using 5µl of cDNA, 25µM dNTPs, 100ng of each gene specific forward and reverse primer and 0.2µl of Biolase Taq polymerase (Bioline) in a 20µL reaction. The amplified products were analyzed on 5% acrylamide gel. *Hprt* was used as an internal control for RT-PCR quantification.

Western Blot

The membrane was blocked with 5% dry fat-free milk solution in PBS containing 0.1% Tween (PBST) at RT for 1 hour and then incubated with indicated primary antibodies overnight at 4°C. The membrane was washed with PBST for 15 minutes three times and incubated with HRP-conjugated secondary antibody for 1 hour at RT followed by three washes using PBST. Immobilon Western chemiluminescent (Millipore WBKLS0500) kit was used to detect HRP activity of the secondary antibody. The membrane was then imaged using ChemiDoc Touch imaging system (Bio-rad). Image J software was used for band intensity quantification. Primary antibodies used for this study are as follows: TPM1 (1:1000, Cell Signaling, D12H4), RBFOX2 (1:1000, Abcam, ab57154), PTBP1 (1:5000, a gift from Dr. Mariano Garcia-Blanco) and α-tubulin (1:20000, Sigma-Aldrich, T6074).

Data availability

Nanopore sequencing data was deposited to NCBI SRA database with project number PRJNA517125.

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Conflict of interest

Authors declare no conflict of interest.

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Abbreviations:

AS: alternative splicing

E20: embryonic day 20

6mo: 6-months old

KD: knock down

p(A): poly(A)

TPM: tropomyosin

Figures with figure legends

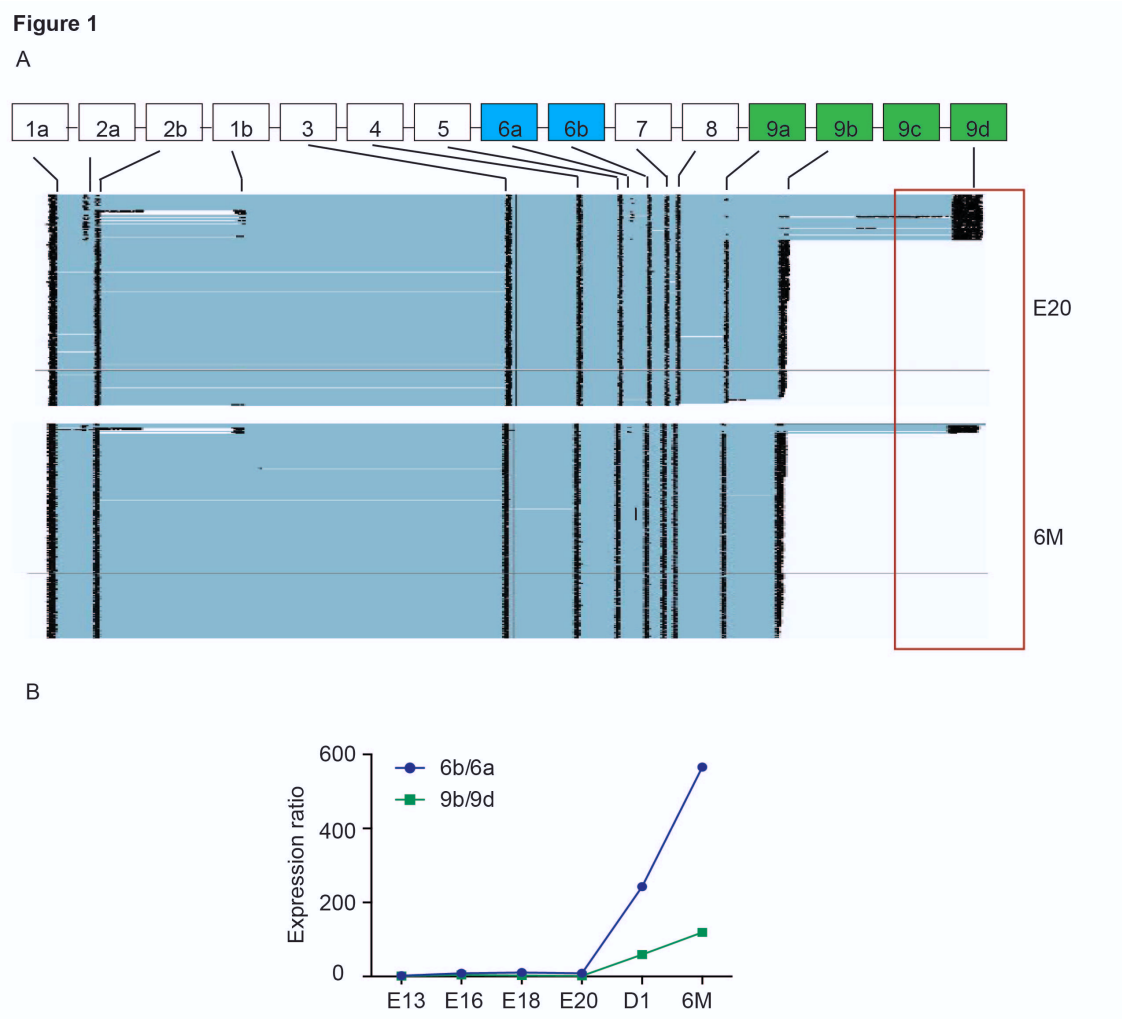

Figure 1. Identification of full length *Tpm1* isoforms generated via alternative splicing during rat heart development using nanopore sequencing. (**A**) Representative images of nanopore sequencing reads mapped to *Tpm1* gene in rat hearts at different developmental stages: embryonic day 20 (E20) and 6-months (6M) old (n=3). (**B**) Relative ratio of *Tpm1* isoforms that include exon 6b vs 6a or exon 9b vs 9d in rat hearts at E13, E16, E18, E20, D1 (1-day old) and 6M.

Figure 2

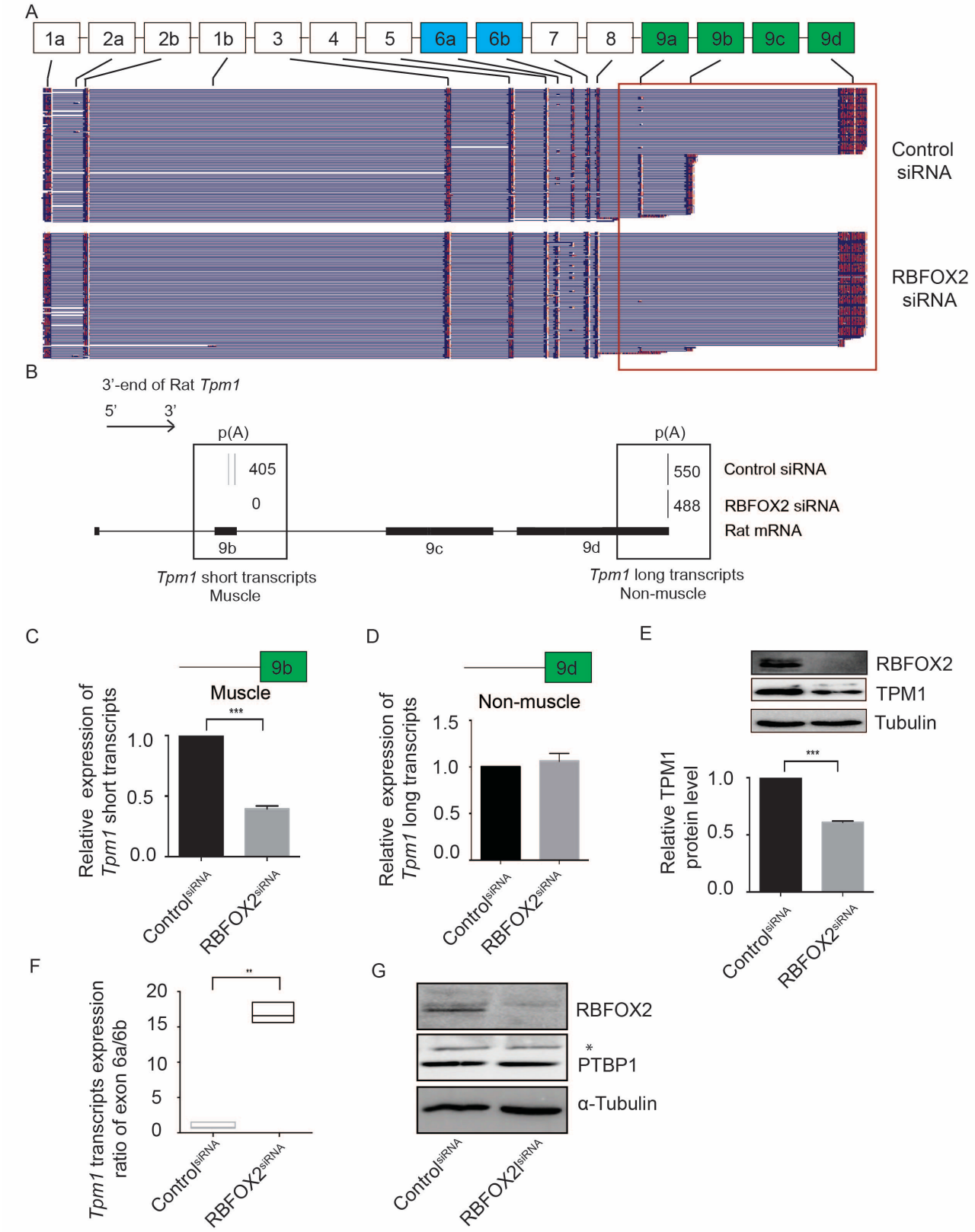
A
1a
2a
2b
1b
3
4
5
6a
6b
7
8
9a
9b
9c
9d
Control siRNA
RBFOX2 siRNA
B
3'-end of Rat Tpm1
5'
3'
p(A)
405
0
9b
9c
9d
p(A)
550
488
Control siRNA
RBFOX2 siRNA
Rat mRNA
Tpm1 short transcripts
Muscle
Tpm1 long transcripts
Non-muscle
C
9b
Muscle
***
Relative expression of Tpm1 short transcripts
1.0
0.5
0.0
Control siRNA
RBFOX2 siRNA
D
9d
Non-muscle
Relative expression of Tpm1 long transcripts
1.5
1.0
0.5
0.0
Control siRNA
RBFOX2 siRNA
E
RBFOX2
TPM1
Tubulin
***
Relative TPM1 protein level
1.0
0.5
0.0
Control siRNA
RBFOX2 siRNA
F
**
Tpm1 transcripts expression ratio of exon 6a/6b
20
15
10
5
0
Control siRNA
RBFOX2 siRNA
G
RBFOX2
*
PTBP1
α-Tubulin
Control siRNA
RBFOX2 siRNA

Figure 2. RBFOX2 regulates AS and expression levels of muscle specific isoforms of tropomyosin 1 (*Tpm1*). (**A**) Full-length *Tpm1* isoforms identified by nanopore sequencing in control vs RBFOX2 depleted H9c2 cells (n=3). (**B**) Polyadenylation pattern of *Tpm1* transcripts in control and RBFOX2 depleted H9c2 cells determined by poly(A)-ClickSeq. (n=3) (**C**) Relative mRNA levels of *Tpm1* muscle-specific (short) transcripts that end with exon 9b in control and RBFOX2 depleted H9c2 cells determined by RT-qPCR. mRNA levels in control cells were normalized to 1. Data represent means ± SD. Statistical significance was calculated using t-test to compare two different groups in three independent experiments (n=3). ***p= 0.0079. (**D**) Relative mRNA levels of *Tpm1* non-muscle transcripts (long) in control and RBFOX2 depleted H9c2 cells. mRNA levels in control cells were normalized to 1. Data represent means ± SD. Statistical significance was calculated using t-test to compare two different groups in three independent experiments (n=3). (**E**) Relative protein levels of TPM1 in control and RBFOX2 depleted H9c2 cells determined by Western blot. α-Tubulin was used as a loading control. Protein levels in control cells were normalized to 1. Data represent means ± SD. Statistical significance was calculated using t-test to compare two different groups in three independent experiments (n=3), ***p=0.0001. (**F**) Ratio of expression levels of *Tpm1* exons 6a to 6b in control and RBFOX2 depleted H9c2 cells determined by RT-qPCR. Expression levels of *Tpm1* exon 6a to 6b in control cells were normalized to 1. Data represent means ± SD. Statistical significance was calculated using t-test to compare two different groups in three independent experiments (n=3). ** p=0.013. (**G**) Representative Western blot images of RBFOX2 and PTBP1 in control and RBFOX2 depleted H9c2 cells. α-Tubulin was used as a loading control.

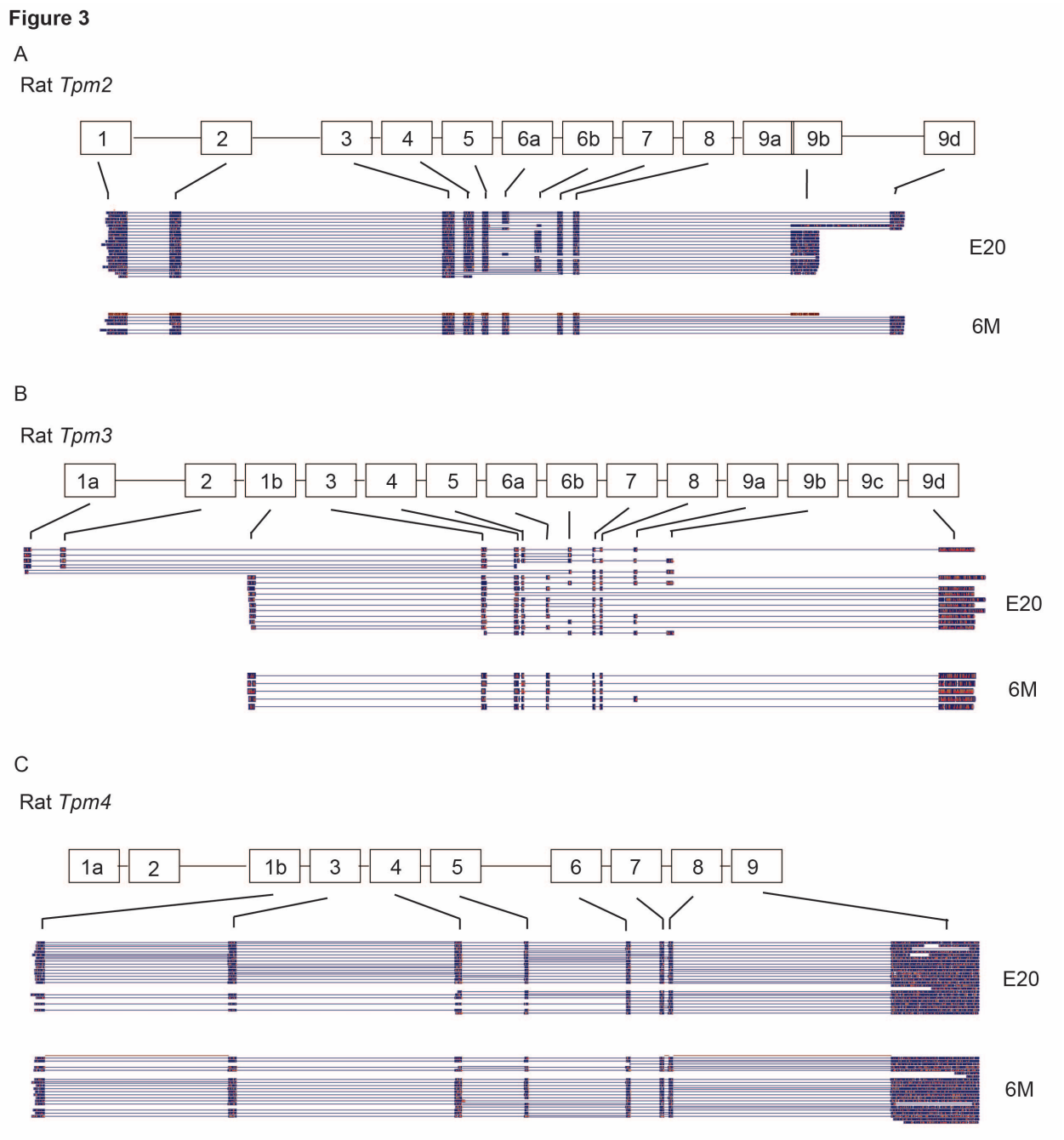

Figure 3. Full-length isoforms of *Tpm2* **(A)**, *Tpm3* **(B)** and *Tpm4* **(C)** identified by nanopore sequencing in rat hearts at E20 and 6M developmental stages.

Figure 4

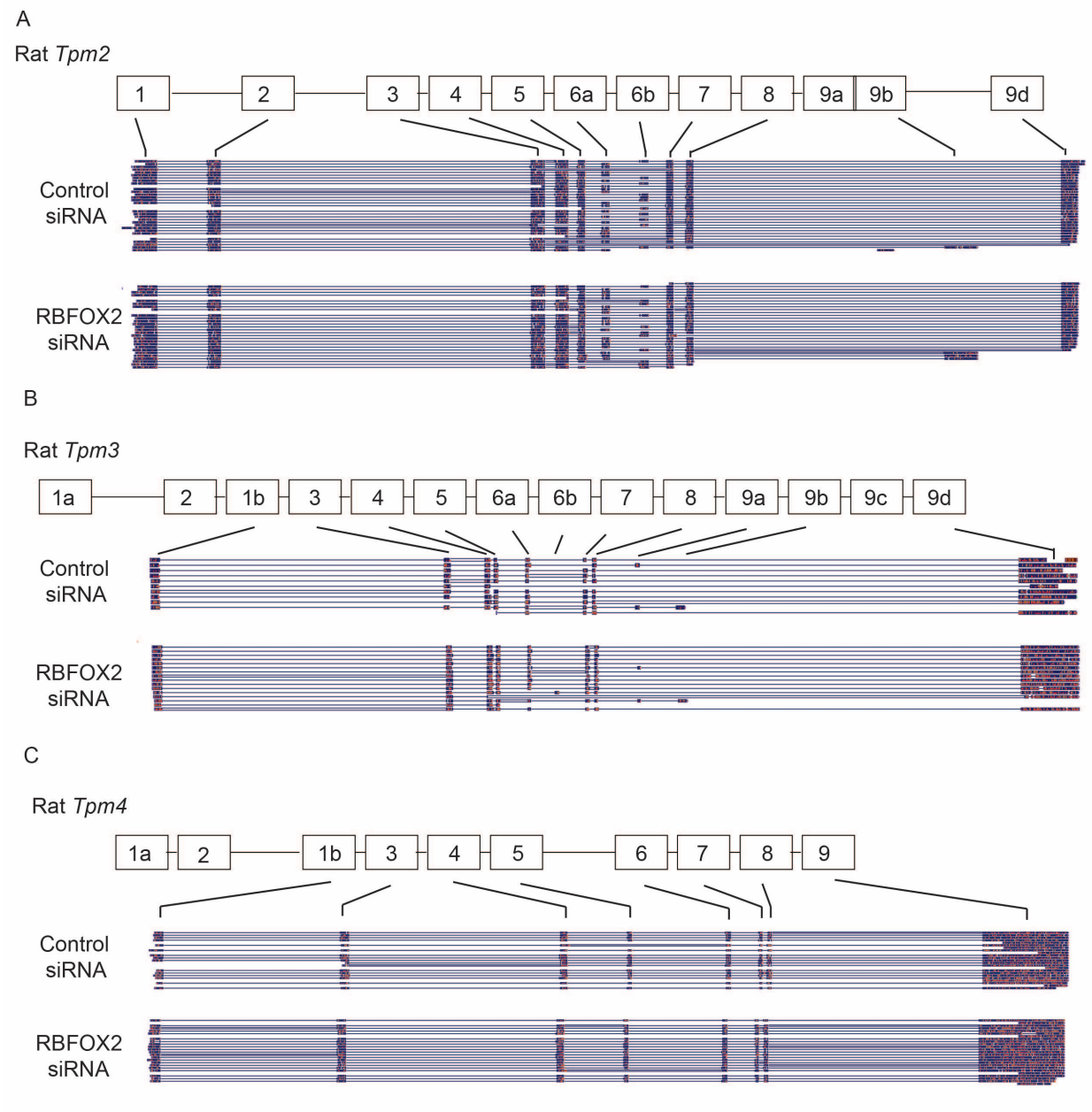

Figure 4. Full-length isoforms of *Tpm2* (**A**), *Tpm3* **(B)**, *Tpm4* **(C)** in control and RBFOX2 depleted H9c2 cells determined by nanopore sequencing.

Figure 5

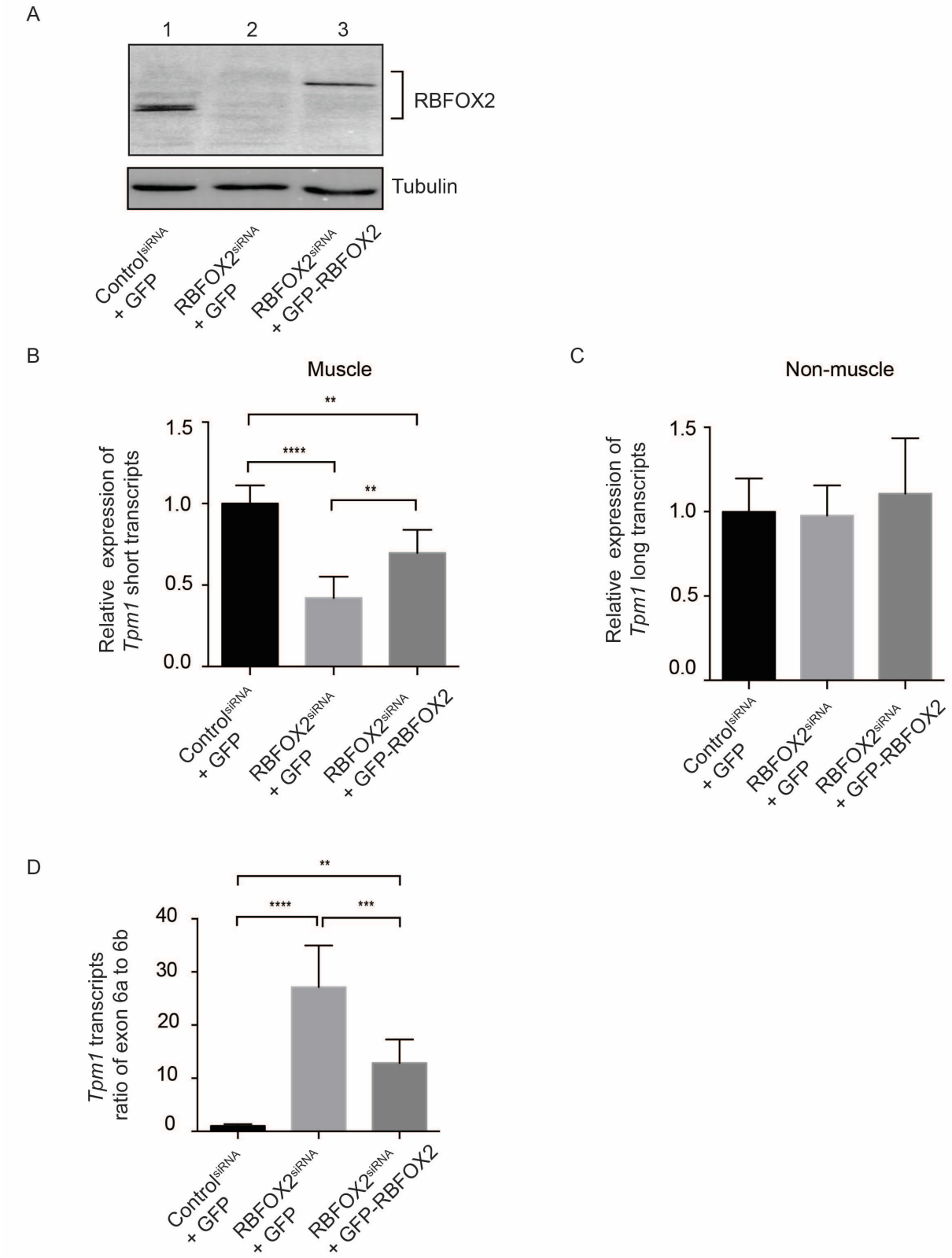
A
1
2
3
RBFOX2
Tubulin
Control siRNA + GFP
RBFOX2 siRNA + GFP
RBFOX2 siRNA + GFP-RBFOX2
B
Muscle
Relative expression of Tpm1 short transcripts
1.5
1.0
0.5
0.0
**
****
**
C
Non-muscle
Relative expression of Tpm1 long transcripts
1.5
1.0
0.5
0.0
D
Tpm1 transcripts ratio of exon 6a to 6b
40
30
20
10
0
**
****
***

Figure 5. Ectopic expression of RBFOX2 partially rescued AS changes in *Tpm1* caused by RBFOX2 depletion. (**A**) RBFOX2 protein expression levels in (1) scrambled siRNA treated, (2) RBFOX2 siRNA treated, (3) RBFOX2 siRNA treated H9c2 cells ectopically expressing of GFP or GFP-RBFOX2. (**B**) Relative expression levels of muscle specific *Tpm1* (short) transcripts in scrambled siRNA treated, RBFOX2 siRNA treated, or RBFOX2 siRNA treated H9c2 cells expressing GFP or GFP-RBFOX2. mRNA levels in control cells (1) were normalized to 1. Data represent means ± SD. Statistical significance was calculated using one-way ANOVA to compare three different groups in three independent experiments (n=3). P-value for Control^{siRNA}+GFP vs. RBFOX2^{siRNA}+GFP is $p<0.0001$; for RBFOX2^{siRNA}+ GFP vs. RBFOX2^{siRNA}+ GFP-RBFOX2 is $p= 0.0056$; for Control^{siRNA}+GFP vs. RBFOX2^{siRNA}+ GFP-RBFOX2 is $p=0.0027$. (**C**) Relative expression levels of non-muscle *Tpm1* (long) transcripts in control, RBFOX2 depleted, or RBFOX2 depleted GFP or GFP-RBFOX2 expressing cells. mRNA levels in (1) cells were normalized to 1. Data represent means ± SD. Statistical significance was calculated using one-way ANOVA to compare three different groups in three independent experiments (n=3). (**D**) Ratio of expression levels of *Tpm1* transcripts containing exons 6a vs 6b in control, RBFOX2 depleted, or RBFOX2 depleted GFP or GFP-RBFOX2 expressing cells. Expression ratios in control (1) cells were normalized to 1. Data represent means ± SD. Statistical significance was calculated using one-way ANOVA to compare three different groups in three independent experiments (n=3). P-value for Control^{siRNA}+GFP vs. RBFOX2^{siRNA}+GFP is $p<0.000001$; for RBFOX2^{siRNA}+ GFP vs. RBFOX2^{siRNA}+ GFP-RBFOX2 is $p=0.000683$; for Control^{siRNA}+GFP vs. RBFOX2^{siRNA}+ GFP-RBFOX2 is $p=0.003409$.

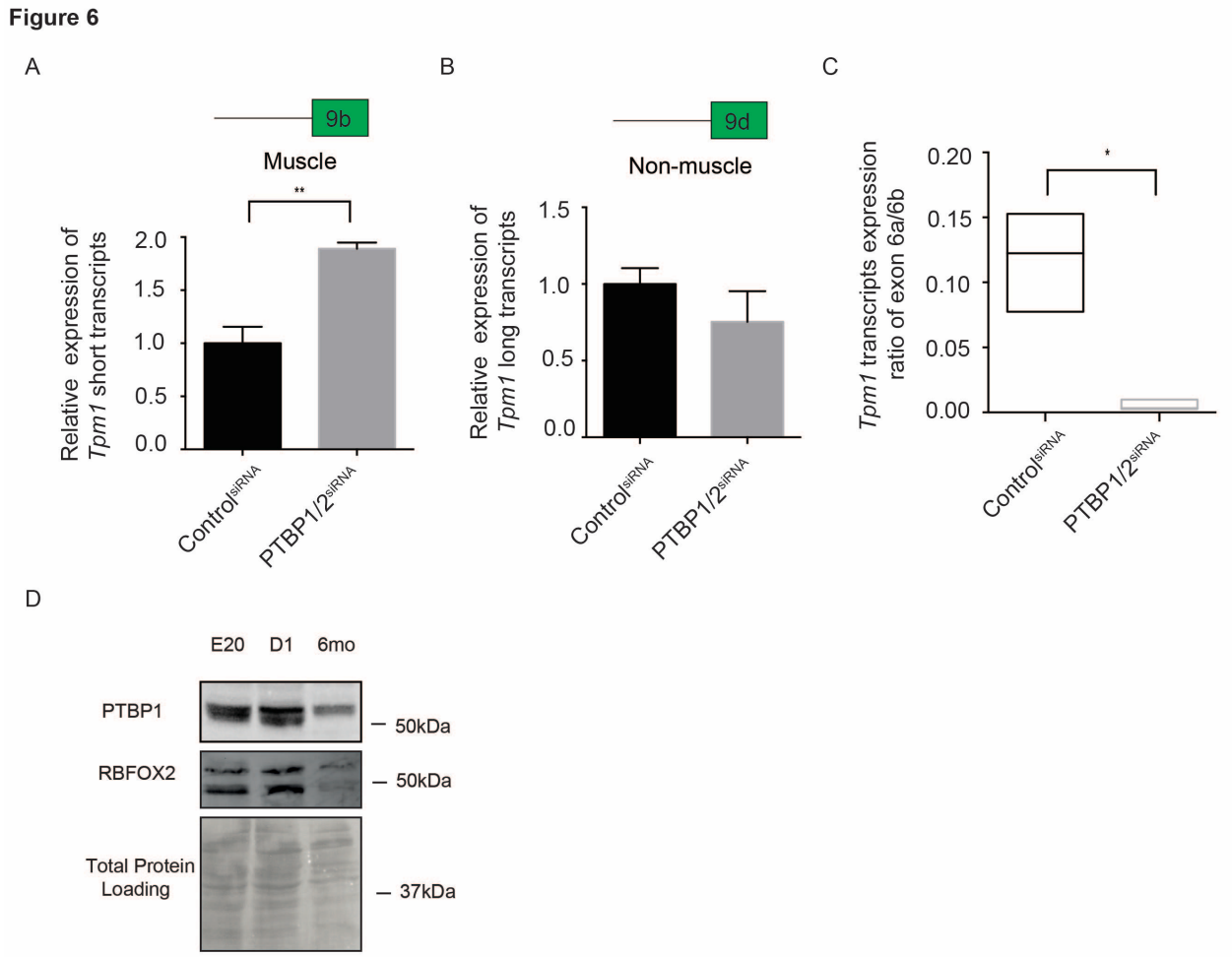

Figure 6. PTBP controls developmentally regulated AS of *Tpm1* antagonistically to RBFOX2. (**A**) Expression of muscle specific *Tpm1* isoforms in H9c2 cells treated with control or PTBP1/2 siRNA. mRNA levels in control cells were normalized to 1. (**B**) Non-muscle *Tpm1* transcript levels in H9c2 cells treated with control or PTBP1/2 siRNAs. mRNA levels in control cells were normalized to 1. (**C**) Expression ratio of *Tpm1* exon 6a vs 6b in H9c2 cells treated with control or PTBP1/2 siRNA. Data represent means ± SD. Expression ratio of *Tpm1* exon 6a to 6b in control cells were normalized to 1. Statistical significance was calculated using t-test to compare two different groups in three independent experiments (n=3). P-values are represented as ** p= 0.0050, * p= 0.0347. (**D**) Western blot image of PTBP1 and RBFOX2 in rat hearts

at different embryonic and postnatal stages. Even protein loading was monitored by Ponceau S stain of the membrane.

Supporting Information (Figures and Tables)

Supplemental Figure 1.

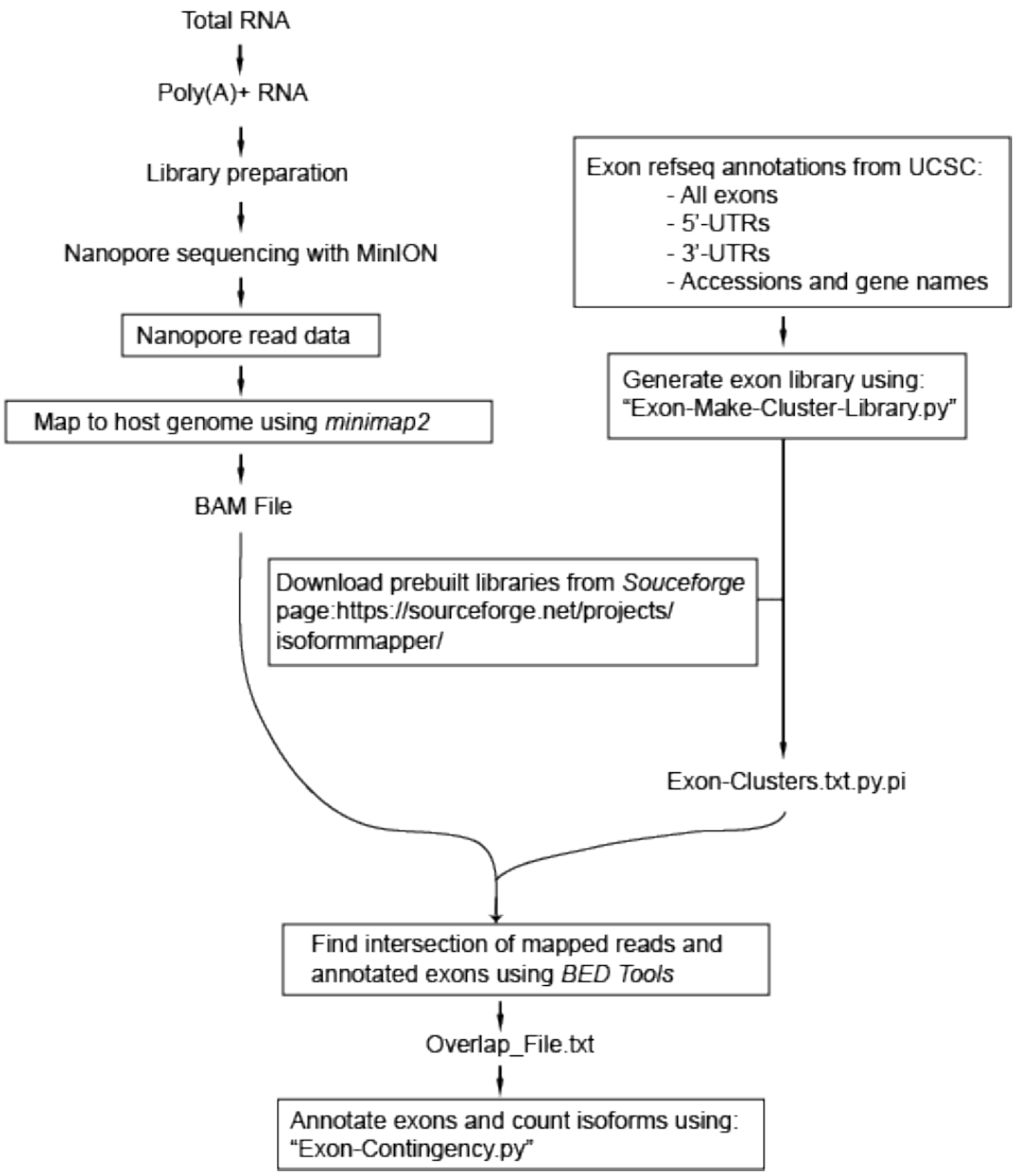

Figure S1. Workflow for nanopore cDNA sequencing.

Supplemental Table 1

Gene name	Species	Gene ID	Analysis target	Forward primer (5' to 3')	Reverse primer (3' to 5')
Tpm1	Rat	ENSRNOG00000018184	Short-*Tpm1* (Exon7-exon9b)	GAAGACAAATATGAAGAGGAGATCAAG	AATAGCAAACAGGAATAGCGTACA
Tpm1	Rat	ENSRNOG00000018184	Long-*Tpm1* (Exon7-exon9d)	GAAGACAAATATGAAGAGGAGATCAAG	CTGGTGCATACTAAGGTTTTCTTCTTT
Tpm1	Rat	ENSRNOG00000018184	Exon4-exon6a	AAGATGCTGACCGAAAGTATGAAG	GCTTTCAAGGTCTGATCCATTATTCT
Tpm1	Rat	ENSRNOG00000018184	Exon4-exon6b	AAGATGCTGACCGAAAGTATGAAG	TGAGCCTCCAGTGACTTCAA
Hprt	Rat	ENSRNOT00000045153.3	Exon2-exon3	CTCATGGACTGATTATGGACAGGAC	GCAGGTCAGCAAAGAACTTATAGCC

Table S1. Primer list for PCR reactions.

Supplemental Table 2

Table S2

Tpm1 transcripts in control H9c2 cells

1	1a	2b	3	4	5	6b	7	8	9d	
2	1a	2b	3	4	5	6b	7	8	9a	
3	1a	2b	3	4	5	6b	7	8		
4	1a	2a	3	4	5	6b	7	8	9d	
5	1a	2b	3	4	5	6b	7	8	9a	9b
6		2b	3	4	5	6b	7	8	9d	
7	1a	2b	3	4	5	6b	7	8	9a	9d
8	1a	2b	3	9d						
9	1a	2b	3	4	5	6a	7	8	9a	
10	1a	2b	3	4	5	7	8	9d		
11	1a	2b	3	4	5	6b	7	9a		
12	1a	2b	3	4	7	8	9a			
13	1a	2a	3	4	5	6a	7	8	9d	
14	1a	2b	3	4	5	6b	7			
15	1a	2b	3	4	5	6b	7	9a	9b	
16	1a	2b	3	4	6b	7	8	9d		
17		2b	3	4	6b	7	8	9d		
18		1b	3	4	5	6a	7	8	9a	
19		2b	3	4	5	6b	7	8	9a	
20	1a	2b	3	4	7	8	9d			
21	1a	2b	3	4	9d					
22	1a	2b	3	4	5	6a	7	8		

Table S2. 22 different *Tpm1* (muscle and non-muscle) isoforms identified in control H9c2 cells using nanopore sequencing.

Supplemental Table 3

Table S3

Tpm1 transcripts in RBFOX2 KD H9c2 cells

1′	1a	2b	3	4	5	6a	7	8	9d
2′	1a	2b	3	4	5	6b	7	8	9d
3′	1a	2b	3	4	5	6a	7	8	
4′	1a	2b	3	4	5	6a	7	9d	
5′	1a	2b	3	4	5	6b	7	8	
6′	1a	2b	3	4	5	6b	7	8	9a
7′	1a	2b	3	9d					
8′	1a	2b	3	4	6a	7	8	9d	
9′		2b	3	4	5	6a	7	8	9d

Table S3. 9 different *Tpm1* isoforms (predominantly non-muscle specific) identified in RBFOX2 depleted H9c2 cells using nanopore sequencing.