RBM20, a gene for hereditary cardiomyopathy, regulates titin splicing

Wei Guo, Sebastian Schafer, Marion L. Greaser, Michael H. Radke, Martin Liss [...], Norbert Hübner, Michael Gotthardt

Supplementary Figures

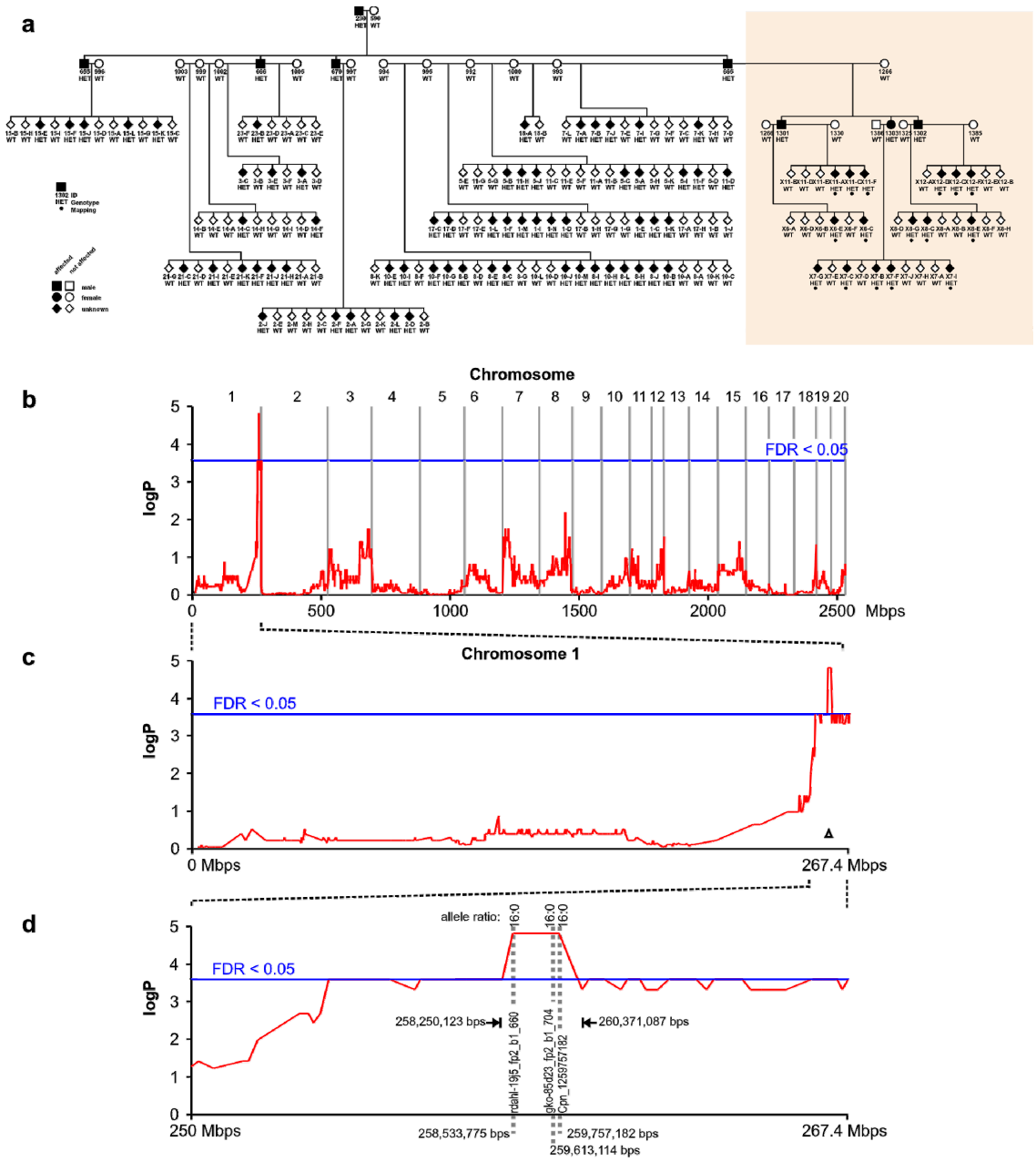


Figure 1 (supplement)

Supplementary Figure 1 Co-segregation of the titin splice defect with the deletion in Rbm20. **(a)** Affected animals on a mixed Sprague Dawley/Fisher F344 background (SD/F) were backcrossed

with Fisher F334 (left) or Brown Norway rats (right). Genotypes of 191 animals are indicated as WT for wildtype and HET for heterozygote. All 86 affected rats expressing the long titin isoform were heterozygous for the deletion. Animals that were backcrossed with Brown Norway rats (BN) and used for genetic mapping are labeled with a black dot. (**b)** The splice defect maps to the long arm of rat chromosome 1. With FDR (False Discovery Rate) set to 0.05, linkage is restricted to a 2.1 Mbps interval on chromosome 1 (**c** – black triangle), that includes 3 SNPs with shared heterozygosity in all 16 animals indicated by the dashed gray lines (**d**). Based on the location of the flanking SNPs the locus containing the candidate gene was assigned to Chr.1: 258,250,123 to 260,371,087 bps.

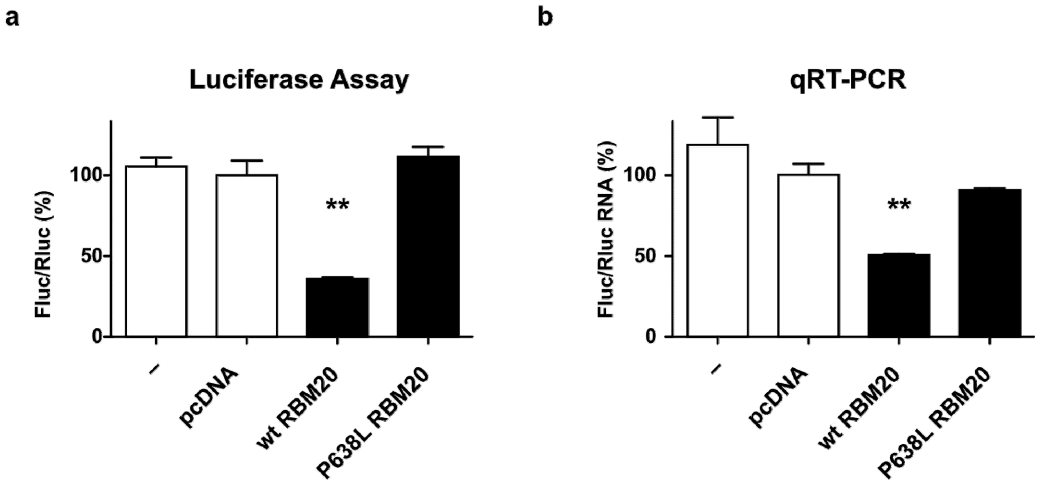


Figure 2 (supplement)

Supplementary Figure 2 Validation of the Luciferase assay. (**a**) 293 cells were transfected with reporter only (-), the empty pcDNA vector (pcDNA) or wildtype and mutant RBM20 (wt RBM20 and P638L RBM20). The wildtype RBM20 leads to reduced inclusion of the firefly luciferase (Fluc/Rluc <50%). (**b**) Analysis of the same groups by qRT-PCR. Firefly and Renilla Luciferase RNA was amplified using SYBR-green chemistry to quantify RNA levels. The calculated fold changes (Fluc/Rluc RNA) values were similar to changes in luciferase ratios (a).

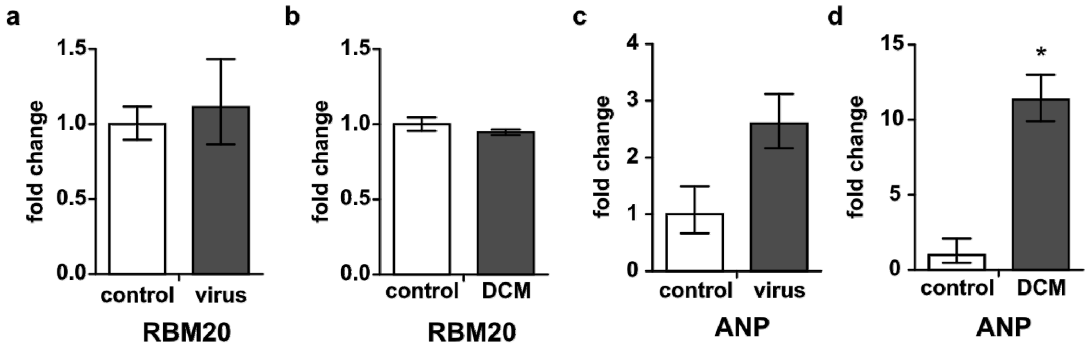


Figure 3 (supplement)

Supplementary Figure 3 Rbm20 expression in murine experimental cardiomyopathy. In both inflammatory cardiomyopathy secondary to the infection with Coxsackievirus B3 (**a**) and DCM that results from a titin-deletion (**b**) cardiac Rbm20 mRNA levels were unchanged. Either form of cardiomyopathy was associated with increased ANP levels (**c, d**). n=6 (virus n=6) (t-test, $p < 0.05$).

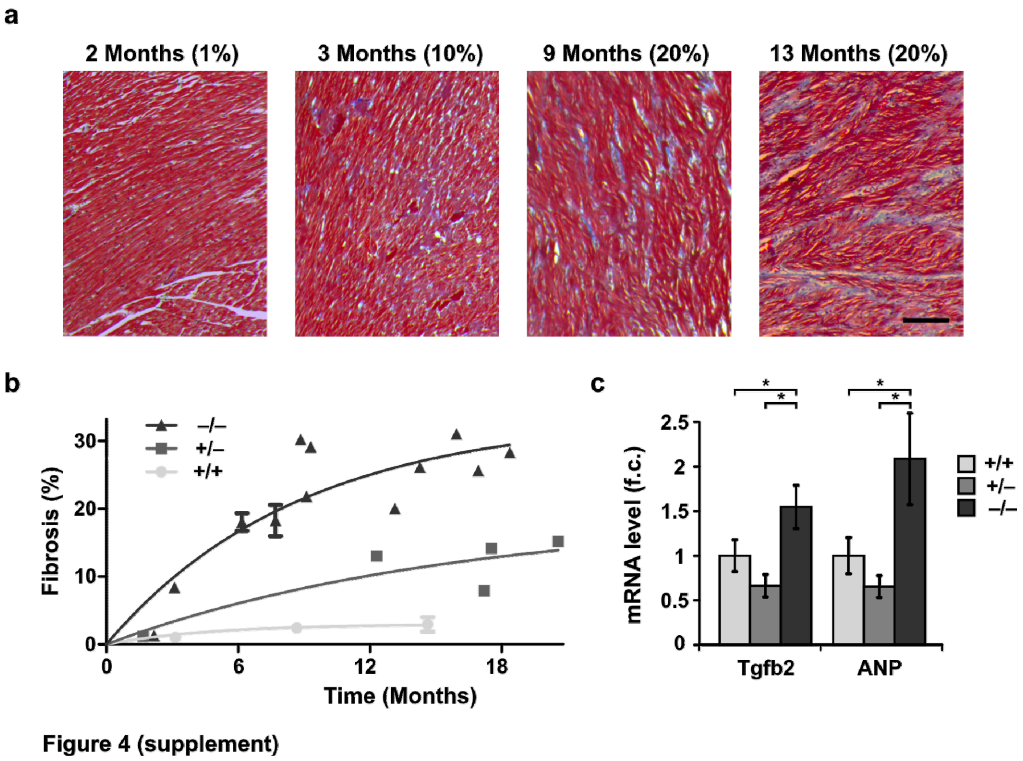


Figure 4 (supplement)

Supplementary Figure 4 Development of myocardial fibrosis in the Rbm20 deficient rat. Electrical conductance in Rbm20 deficient hearts. (**a**) Trichrome staining (blue) of homozygous RBM20 deficient left ventricles at 2, 3, 9, and 13 months of age with increasing fibrosis from 1 to 20%. Size bar = 50 µm. (**b**) Quantification of the amount of fibrosis. The area of fibrosis is intermediate in heterozygotes, which never exceed 15%. This level is reached in homozyogtes by 6 months of age, well before arrhythmia develops in both homozygous and heterozygous mutants at 9 months of age. (**c**) MeSH analysis of differentially expressed mRNAs in RBM20 deficient rat hearts revealed 2 genes related to fibrosis or extracellular matrix. At the early time point before collagen is significantly increased we find TGFbeta2 and ANP upregulated in the homozygous RBM20 deficient rat (-/-). The upregulation of TGFbeta2 would be consistent with an increase in fibrosis.

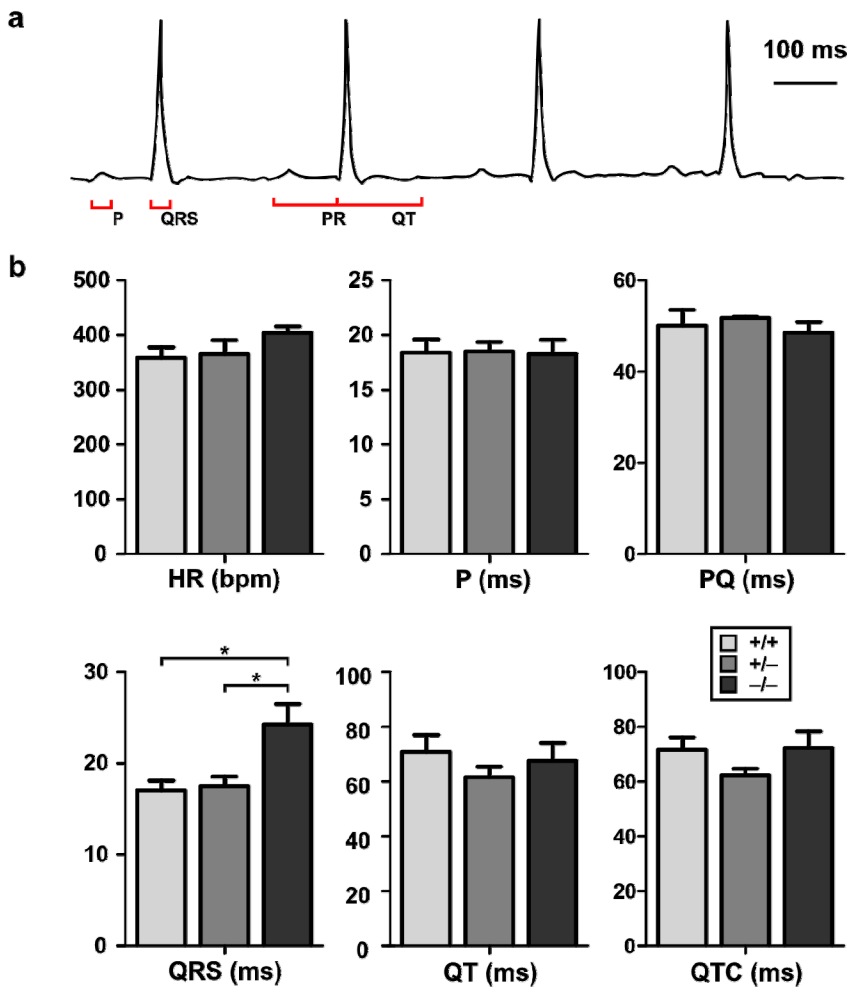


Figure 5 (supplement)

Supplementary Figure 5 Electrical conductance in Rbm20 deficient hearts. (**a**) Trace of the rat surface ECG indicating the P- interval representing atrial depolarization, the QRS complex largely resulting from ventricular depolarization, the PR and QT interval as the time from atrial to ventricular depolarization and ventricular depolarization to the end of repolarization, respectively. (**b**) Depolarization and repolarization as well as conduction times were normal between genotypes except for a widened QRS complex only in the homozygous Rbm20 deficient animals under basal conditions (n = 4 per group).

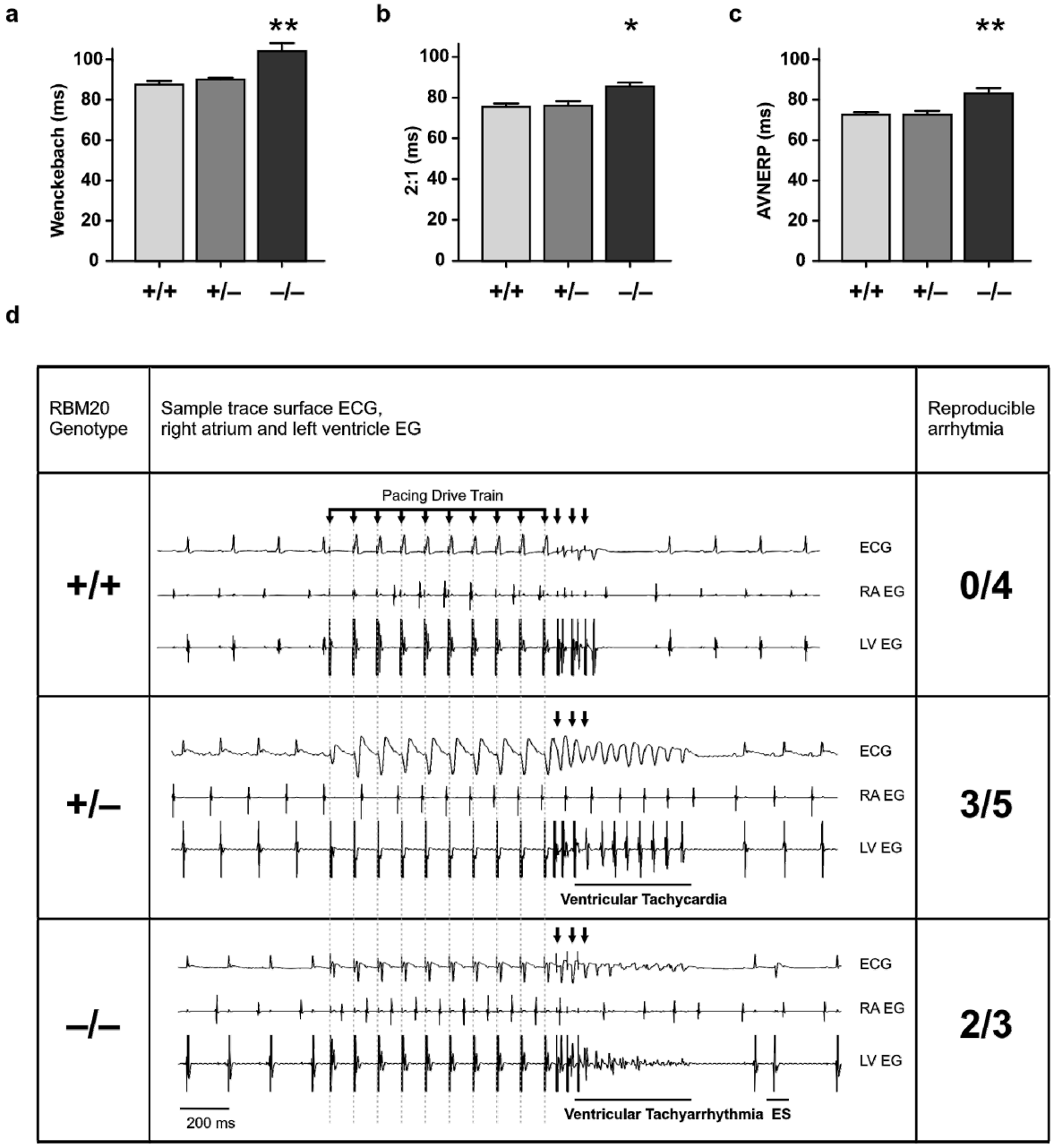


Figure 6 (supplement)

Supplementary Figure 6 Electrophysiological phenotype as determined by EP catheter. Rbm20 mutant animals showed signs of impaired atrioventricular conduction as indicated by the increased Wenckebach and 2:1 AV cycle length (**a, b**) and the increased Atrio-Ventricular Node

Effective Refractory Period (**c**) as compared to wildtype and heterozygote animals. (**d**) Inducibility of ventricular arrhythmias by *in vivo* programmed electrical stimulation. Representative results of programmed electrical stimulation from all genotypes are depicted with one ECG lead as well as the endocardial right-atrial (RA) and left-ventricular (LV) electrograms. Delivered electrical stimuli are indicated as arrows, the pacing drive train as dotted lines. The number of animals with reproducible arrhythmia over the total number of animals tested is indicated on the right. Both heterozygote and homozygote Rbm20 deficient animals show ventricular tachyarrhythmia in response to pacing. ES = extrasystole. (n = 12).

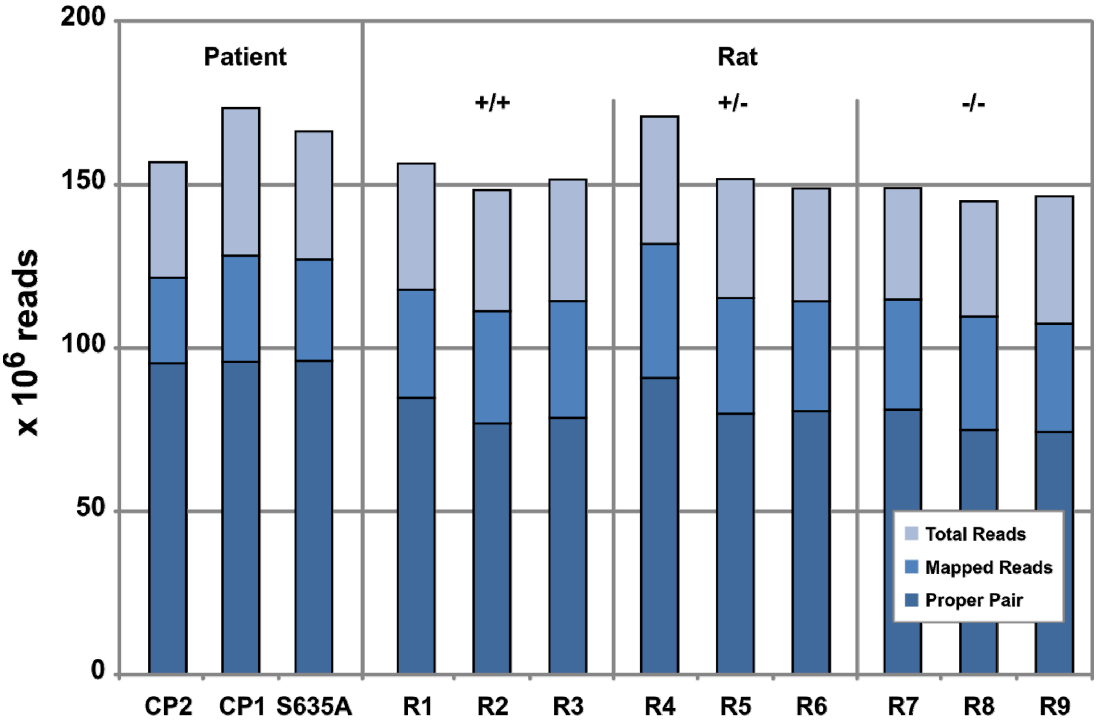


Figure 7 (supplement)

Supplementary Figure 7 Sequencing output of 12 Illumina HiSeq runs. For the three patients (left) and the 3 rats per genotype, we obtained ~120 mio reads each. Of these >100 mio each were mapped and >75 mio were considered proper pairs (reads that map in expected distances on the reference genome). Reads (100 bp each) were paired-end and mapped against the reference genomes (rn4 or hg19) with TopHat.

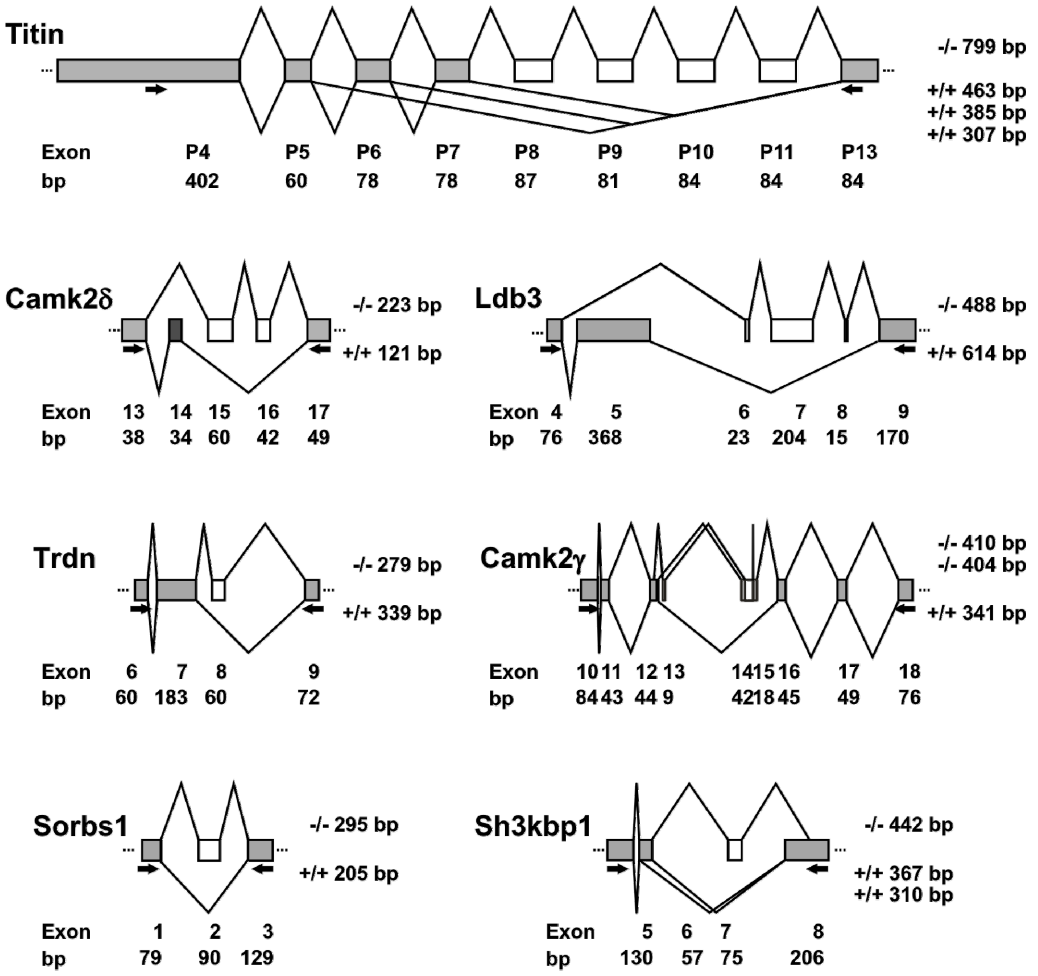


Figure 8 (supplement)

Supplementary Figure 8 Exon structure of alternatively spliced regions in titin, CaMKIIδ, LDB3,Trdn, Camk2γ, and Sorbs1. Primer positions used for PCR-based splice analysis are indicated as black arrows. Exons expressed primarily in Rbm20 deficient animals are indicated in white, regions primarily in wildtype animals are depicted in dark grey. Split reads indicate alternative inclusion of titin PEVK exons P5 and P6 and alternate exon usage in CaMKIIδ. Exon sizes are indicated below the corresponding exon numbers - length of PCR fragments produced in +/+ vs. -/- on the right.

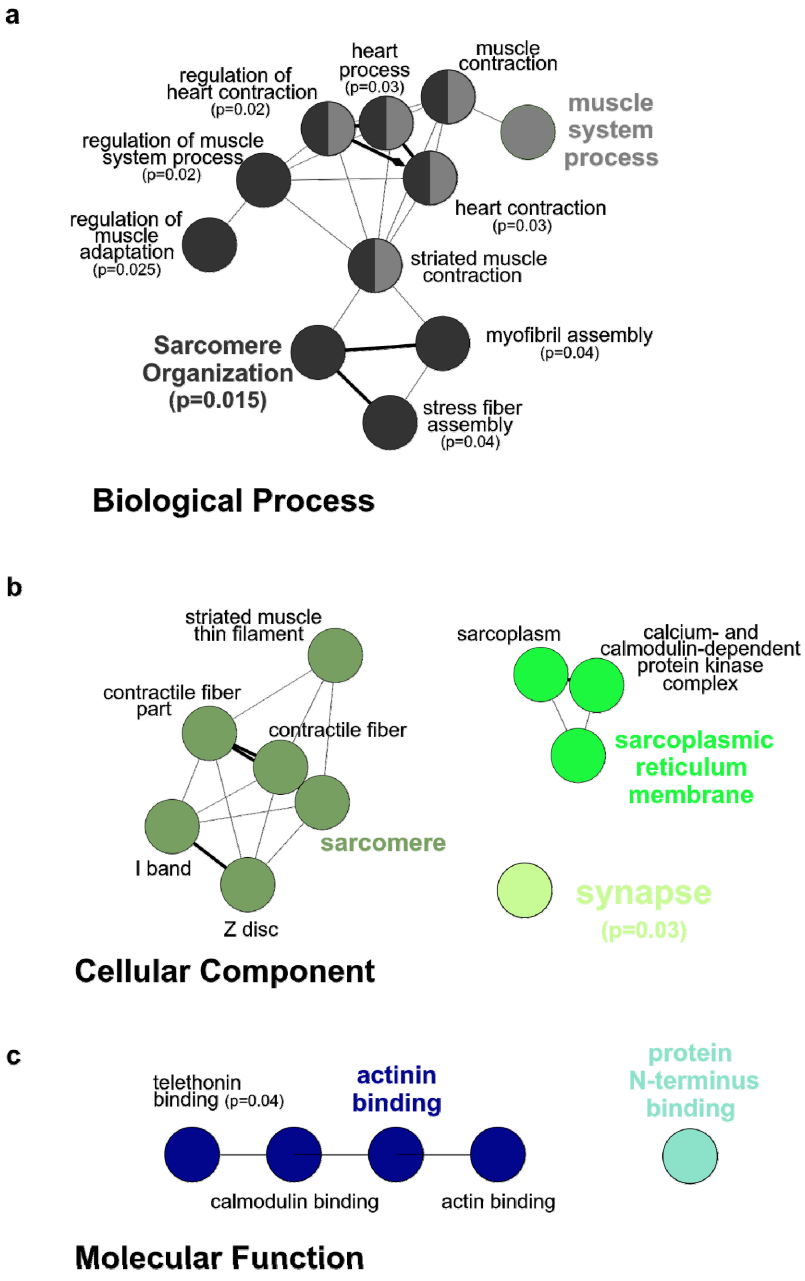


Figure 9 (supplement)

Supplementary Figure 9 Function and cellular location of RBM20 substrates as determined by gene ontology (GO) analysis. For each GO-category the functionally grouped network is

represented with the terms displayed as nodes and linked based on their kappa score level (≥0.3). Only significant enrichment terms were considered (P<0.05; P<0.01 not indicated – compare table S3-5). Labels of the most significant terms for each network are shown in the color of the nodes. Degree of connectivity between terms is calculated using kappa statistics and reflected in the line thickness. (**a**) RBM20 substrates are involved in sarcomere organization, structure, contraction, and adaptation of striated muscle, as well as the regulation of muscle function. (**b**) Enriched genes localize primarily to all major components of the sarcomere (left, dark green) and the sarcoplasmic reticulum (right, intermediate green). The synapse is the only component represented at P>0.01. (**c**) RBM20 substrates can function as adaptor proteins and bind sarcomeric proteins such as actin, α-actinin, and T-cap. Colors correspond to the GO-terms used in Figure 5.

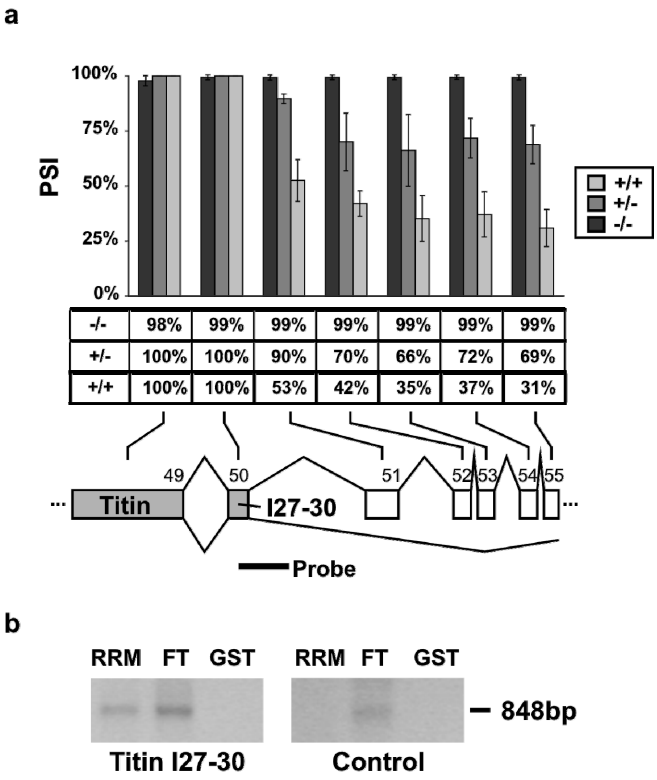
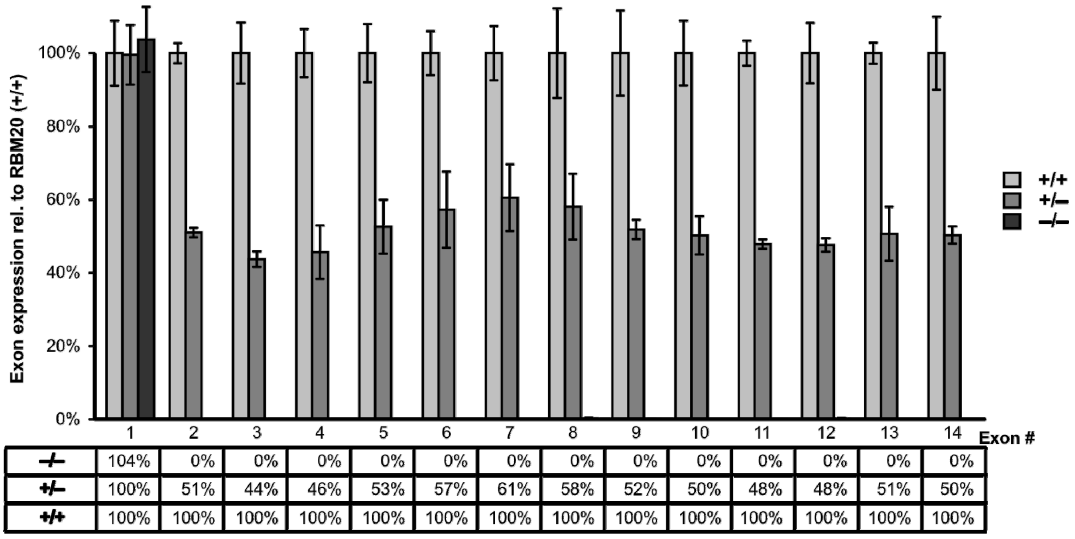


Figure 10 (supplement)

Supplementary Figure 10 Titin pre-RNA binding as determined by RNA retention analysis. (**a**) The I-band coding exon I27-30 is represented in titin isoforms independently of RBM20. A string of exons immediately 3' is skipped in wildtype rats as indicated by the reduced PSI values (heterozygotes with an intermediate effect). PSI values are provided in the table below. The exon-intron junction I27-30 (probe) and a downstream control region were amplified and used for in vitro transcription and RBM20 binding studies. (**b**) Using the bacterially expressed RNA recognition motive of RBM20 (GST-RRM) and the negative control GST alone as compared to the flow through (FT), we were able to retain the *in vitro* transcribed titin mRNA fragment on glutathione beads with RRM as opposed to beads with GST alone. Binding specificity was validated using a control probe downstream.



–/–	104%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
+/–	100%	51%	44%	46%	53%	57%	61%	58%	52%	50%	48%	48%	51%	50%
+/+	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

Figure 11 (supplement)

Supplementary Figure 11 Quantification of RBM20 exon expression *in vivo* by RNA seq. Exons 1-14 of RBM20 are expressed in wildtype animals (+/+, normalized expression of 100%). Heterozygous and homozygous deficient animals express normal amounts of RBM20 exon 1. The deletion of Exon 2-14 leads to expression levels of 50% in the heterozygote and a loss of expression in the homozygote RBM20 deficient rat.

Supplemental Table

Table S1: Summary of clinical and rodent phenotypes

	Dilation	Hypertrophy	Arrhythmia	Sudden Death	Interstitial Fibrosis	Subendocardial Fibroelastosis
Rat +/+	-	-	-	-	-	-
Rat +/-	+	-/+ (n.s.)[1]	+	+	+	+
Rat -/-	+	-/+ (n.s.)[1]	+	+	++	++
DCM-CP1	+	+	+	-	+	n.d.
DCM-CP2	+	+	+	-	+	n.d.
DCM-S635A	+	-	+	-	n.d.	n.d.
DCM-50	+	+	+	+	+	+

- = not present; -/+ = not significant; + = present; ++ increased vs. wildtype and heterozygotes;

n.d.= not determined

Table S2: Conserved RBM20 genotype dependent splicing in human and rat

HumanID	RatID	Human Name	Rat Name
ENSG00000155657	ENSRNOG00000022637	TTN	P97851_RAT
ENSG00000145349	ENSRNOG00000011589	CAMK2D	KCC2D_RAT
ENSG00000122367	ENSRNOG00000011343	LDB3	Ldb3
ENSG00000186439	ENSRNOG00000012609	TRDN	Trdn
ENSG00000137872	ENSRNOG00000004812	SEMA6D	Sema6d
ENSG00000162599	ENSRNOG00000006966	NFIA	Nfia
ENSG00000065526	ENSRNOG00000033556	SPEN	LOC680138
ENSG00000148660	ENSRNOG00000009783	CAMK2G	Camk2g
ENSG00000134775	ENSRNOG00000027230	FHOD3	Q5D003_RAT
ENSG00000095637	ENSRNOG00000015658	SORBS1	Sorbs1
ENSG00000112200	ENSRNOG00000012718	ZNF451	Zfp451
ENSG00000147010	ENSRNOG00000004322	SH3KBP1	Sh3kbp1
ENSG00000151067	ENSRNOG00000007090	CACNA1C	Cacna1c
ENSG00000160145	ENSRNOG00000001706	KALRN	Kalrn
ENSG00000163110	ENSRNOG00000016419	PDLIM5	Pdlim5
ENSG00000134769	ENSRNOG00000016671	DTNA	Dtna
ENSG00000052126	ENSRNOG00000008747	PLEKHA5	Plekha5
ENSG00000173406	ENSRNOG00000042489	DAB1	ENSRNOG42489
ENSG00000187239	ENSRNOG00000008258	FNBP1	Fnbp1
ENSG00000157637	ENSRNOG00000004604	SLC38A10	Q4G035_RAT
ENSG00000103148	ENSRNOG00000020541	NPRL3	Mare
ENSG00000140538	ENSRNOG00000018674	NTRK3	Ntrk3
ENSG00000197959	ENSRNOG00000026490	DNM3	Dnm3
ENSG00000139436	ENSRNOG00000001190	GIT2	Git2
ENSG00000169057	ENSRNOG00000037262	MECP2	Mecp2
ENSG00000137074	ENSRNOG00000006582	APTX	Aptx
ENSG00000120049	ENSRNOG00000018018	KCNIP2	Kcnip2_v2
ENSG00000136828	ENSRNOG00000016910	RALGPS1	Ralgps1
ENSG00000063601	ENSRNOG00000002697	MTMR1	Mtmr1
ENSG00000140416	ENSRNOG00000018184	TPM1	Tpm1_v9
ENSG00000184182	ENSRNOG00000019953	UBE2F	Ube2f

Table S3: Gene Ontology analysis – Biological Process

GOID	GO Term	GO Levels	GO Groups	Nr. Genes	Term P-Value (adj.)*	Group P-Value (adj.)*	Associated Genes
GO 0003012	muscle system process	3	Group 0	8	5.76E-7	Group 0 1.47E-7	CAMK2D, CAMK2G, DTNA, KCNIP2, SORBS1, TPM1, TRDN, TTN
GO 0006936	muscle contraction	4	Group 0 Group 1	7	6.05E-6	Group 0 1.47E-7 Group 1 5.23E-5	CAMK2D, DTNA, KCNIP2, SORBS1, TPM1, TRDN, TTN
GO 0006941	striated muscle contraction	5	Group 0, Group 1	4	2.40E-4	Group 0 1.47E-7 Group 1 5.23E-5	CAMK2D, DTNA, TPM1, TTN
GO 0045214	sarcomere organization	5, 6, 7, 8, 9	Group 1	2	0.015	Group 1 5.23E-5	TPM1, TTN
GO 0008016	regulation of heart contraction	4, 5, 6	Group 0, Group 1	3	0.019	Group 0 1.47E-7 Group 1 5.23E-5	CAMK2D, KCNIP2, TPM1
GO 0090257	regulation of muscle system process	4, 5	Group 1	3	0.023	Group 1 5.23E-5	CAMK2D, CAMK2G, TPM1
GO 0043502	regulation of muscle adaptation	5, 6	Group 1	2	0.024	Group 1 5.23E-5	CAMK2D, CAMK2G
GO 0003015	heart process	4	Group 0, Group 1	3	0.033	Group 0 1.47E-7 Group 1 5.23E-5	CAMK2D, KCNIP2, TPM1
GO 0060047	heart contraction	5	Group 0, Group 1	3	0.033	Group 0 1.47E-7 Group 1 5.23E-5	CAMK2D, KCNIP2, TPM1
GO 0030239	myofibril assembly	4, 5, 6, 7, 8	Group 1	2	0.044	Group 1 5.23E-5	TPM1, TTN
GO 0043149	stress fiber assembly	6, 9	Group 1	2	0.046	Group 1 5.23E-5	SORBS1, TPM1

*Adjusted p-values shown were corrected for multiple testing (Bonferroni). The dotted line separates adjusted P-values <0.01. For graphical display see figure 5a and supplementary figure 9.

Table S4: Gene Ontology analysis – Cellular Component

GOID	GO Term	GO Levels	GO Groups	Nr. Genes	Term P-Value (adj.)*	Group P-Value (adj.)*	Associated Genes
GO 0033017	sarcoplasmic reticulum membrane	4, 5, 6, 7, 8, 9, 10	Group 1	3	3.26E-4	Group 1 0.019	CAMK2D, CAMK2G, TRDN
GO 0005954	calcium- and calmodulin-dependent protein kinase complex	3, 4, 5	Group 1	2	6.23E-4	Group 1 0.019	CAMK2D, CAMK2G
GO 0030017	sarcomere	3, 4, 5, 6, 7, 8, 9	Group 0	4	0.001	Group 0 2.43E-4	LDB3, PDLIM5, TPM1, TTN
GO 0044449	contractile fiber part	2, 3, 4, 5, 6, 7, 8	Group 0	4	0.002	Group 0 2.43E-4	LDB3, PDLIM5, TPM1, TTN
GO 0016528	sarcoplasm	4, 5, 6	Group 1	3	0.003	Group 1 0.019	CAMK2D, CAMK2G, TRDN
GO 0043292	contractile fiber	4, 5, 6, 7	Group 0	4	0.003	Group 0 2.43E-4	LDB3, PDLIM5, TPM1, TTN
GO 0030018	Z disc	3, 4, 5, 6, 7, 8, 9, 10, 11	Group 0	3	0.004	Group 0 2.43E-4	LDB3, PDLIM5, TTN
GO 0031674	I band	3, 4, 5, 6, 7, 8, 9, 10	Group 0	3	0.007	Group 0 2.43E-4	LDB3, PDLIM5, TTN
GO 0005865	striated muscle thin filament	3, 4, 5, 6, 7, 8, 9, 10	Group 0	2	0.009	Group 0 2.43E-4	TPM1, TTN
GO 0045202	synapse	1	Group2	5	0.033	Group 2 =0.003	CAMK2D, DNM3, DTNA, PDLIM5, SH3KBP1

*Adjusted p-values shown were corrected for multiple testing (Bonferroni). The dotted line separates adjusted P-values <0.01. For graphical display see figure 5a and supplementary figure 9.

Table S5: Gene Ontology analysis – Molecular Function

GOID	GO Term	GO Levels	GO Groups	Nr. Genes	Term P-Value (adj.)*	Group P-Value (adj.)*	Associated Genes
GO 0047485	protein N-terminus binding	3	Group2	4	3.23E-4	Group 2 0.012	APTX, KCNIP2, MECP2, PDLIM5
GO 0042805	actinin binding	4	Group3	2	0.008	Group 3 1.84E-4	PDLIM5, TTN
GO 0003779	actin binding	4	Group3	5	0.008	Group 3 1.84E-4	FHOD3, PDLIM5, SORBS1, TPM1, TTN
GO 0031433	telethonin binding	4	Group3	1	0.041	Group 3 1.84E-4	TTN

*Adjusted p-values shown were corrected for multiple testing (Bonferroni). The dotted line separates adjusted P-values <0.01. For graphical display see figure 5a and supplementary figure 9.

Table S6: MESH Analysis – Disease Relevance

MESH Term	# of associated Genes	P-Value	P-Value (adj.)*
Cytoskeleton	14	1.21E-8	2.08E-4
Heart Ventricles	8	2.15E-8	3.72E-4
Synapses	9	8.89E-8	1.54E-3
src Homology Domains	11	1.48E-7	2.56E-3
Myocytes, Cardiac	9	2.88E-7	4.98E-3
Heart Failure	**7**	**9.62E7**	**0.016**
Cardiomyopathy, Dilated	**6**	**1.75E-6**	**0.032**
Heart	8	2.24E-6	0.038

*Adjusted p-values shown were corrected for multiple testing (Bonferroni).

Disease related MESH – Terms are highlighted. For graphical display see figure 5a.

Supplementary Methods

Annotation of RBM20. Full length rat Rbm20 (6682 bp) was cloned, sequenced, and annotated (primer information is available upon request). It contains 102 bps of 5' UTR, coding sequence for 1207 amino acids, and 2956 bp of 3' UTR. Rbm20 contains a single RNA-recognition motif (RRM) spanning amino acid positions 522-592, an RS domain (632-666 human vs. 636-658 rat), and two zinc finger domains (412-436; 1161-1192 vs. rat 417-439; 1141-1172). The rat sequence data has been deposited in the NCBI database (Accession number EU562301) and is the source for the provisional reference sequence (NM_001107611). The corresponding human original source and reference sequences are EU822950 and NM_001134363 respectively. The rat and human amino acid sequences are 76% identical with greatest similarities in the RRM and zinc-finger regions.

Genotyping by PCR and Southern blot. For genotyping, template DNA was prepared from rat liver as published previously [2]. For Southern blot analysis, genomic DNA was digested with Hind III overnight and probed with a PCR product generated with primers RBM20 Exon 2 forward (5'-CCAGCTCACCCTCCATCG-3') and RBM20 Exon 2 reverse (5'- GCCATAGTCATAGAACCCTG -3') following standard procedures[2]. Equal loading was validated by ethidium bromide staining of the agarose gel before and after transfer to the Hybond membrane. The deletion was confirmed by PCR (primers P1, 5'-GTCTTCATGATCCTGGAGTG-3; and P2, 5'-GGTGTGGGGTTATGGAGTC-3') (compare Fig 1a). Genotyping of pedigree animals was conducted by PCR multiplexing with two pairs of primers [WT forward (5'-GAAGTCCAATGAGCCGATC -3') and WT reverse (5'- CTCCTTCTTGGTCTCTGTC -3') for wild type, product size 432 bp / M forward (5'- GTCTTCATGATCCTGGAGTG -3') and M reverse (5'-GGTGTGGGGTTATGGAGTC -3') for homozygous mutant, product size 868 bp].

Plasmid Construction. Total RNA was extracted from rat left ventricle (LV) by Trizol regent (Invitrogen). Reverse transcription was done by ImProm-II Reverse Transcription System (Promega) on total RNA with oligo (dT)20 as anchor primer. For antibody production, full length RBM20 was amplified using the forward primer (5'-gcg ccc atg gga atg gtg ctg gct gca gcc at-3') and reverse primer (5'-aag gaa aaa agc ggc cgc tca tag ctt ctt cct ttc caa gtg-3') with left ventricular cDNA as template. RBM20 was cloned in frame with GST and His tag into plasmid pET-41(a) (Novagen) using the NcoI and NotI unique restriction sites. The C-terminus of RBM20 was amplified with forward primer (5'-cat cat cca tgg aca ccg aag cgg agc tga aag-3') and reverse primer (5'-cat cat cat gcg gcc gct agc ttc ttc ctt tcc aag tg-3'). After digestion with the restriction enzymes NcoI and NotI, the C-terminus of RBM20 (RBM20C) was cloned in frame with His tag into plasmid pET-28a(+) (Novagen). For mammalian expression rat RBM20 was amplified with the forward primer (5'-gag cgg ccg cga atg gtg ctg gct gca gc-3') and reverse primer (5'-t cgc ggc cgc gca tag ctt ctt cct ttc caa-3') and cloned in frame into pcDNA3.1(-)/myc-His A (Invitrogen) using the flanking NotI restriction site. Mutations were introduced by site-directed mutagenesis in a two-step cycle PCR approach using the primer pairs listed in the table "RBM20 mutations Cloning Primers" below. The splice reporter construct is based on pcDNA3.1 (Invitrogen) and contains the RBM20 dependent rat titin PEVK exons 4 through 13 (PEVK 12 is not present in rat). Firefly and renilla luciferase coding regions were PCR amplified from pGL3 and pGL4.7 (Promega) and inserted in frame into exon PEVK 8 and 13, respectively. To increase RNA turnover and facilitate readout, we used a RNA destabilization sequence consisting of five tandem AUUUA repeats [3]. All constructs were sequence verified before use.

Tissue culture procedures. Human embryonic kidney cells (HEK 293) were grown in Dulbecco's Modified Eagle's Medium (Lonza) supplemented with 10 % FCS Lot#41Q1274K (Invitrogen). HEK 293 cells with tetracycline-inducible expression of FLAG/HA-tagged RBM20 were established by cotransfecting Flp-In T-REx HEK293 cells with the pFRT/TO/FLAG/HA-DEST vector containing the human RBM20 coding sequence and the Flp recombinase expression plasmid pOG44 using

Lipofectamine 2000 (Invitrogen). Stable transfectants were selected using 100 µg/ml hygromycin B and 15 µg/ml blasticidin.

HL-1 cells[4] were grown in 24 well plates (40,000 cells per well) on Claycomb media (JRH Biosciences) supplemented with 1% penicillin/streptomycin, 1% 10 mM norepinephrine, 1% 200 mM L-glutamine, and 10% fetal calf serum (Sigma-AIdrich). The cells were allowed to proliferate and reach full confluence and subsequently passaged on to a fresh fibronectin coated culture dish every three days at a dilution 1:2. For immunohistochemistry, cells were seeded on fibronectin coated coverslips for two days and fixed with ice cold methanol-acetone and stained with appropriate antibody as described below (see immunofluorescence).

Murine myoblast C2C12 cells were cultured in growth medium, DMEM supplemented with 10% fetal bovine serum. Cells were passaged every two to three days at a dilution of 1:20 or 1:30, respectively. Proliferating C2C12 cells were differentiated into myotubes by switching to a differentiation medium containing 2% horse serum at 80% cell confluence [5]. For immunostaining, C2C12 cells were differentiated on collagen coated (BD Biosciences) coverslips for six days, subsequently fixed by methanol acetone and stained with the appropriate antibody as described below. Cells were harvested at 48 h after transfection for analysis of protein changes.

The primary cultures of Neonatal Rat Ventricular Myocytes (NRVM) were prepared from 1 to 3-day-old rats from Sprague Dawley X Fisher 344 X Brown Norway rats using the Neonatal Cardiomyocyte Isolation System (Worthington) as described in the manufacturer's manual. Briefly, the hearts from neonatal rats are rapidly excised and washed in calcium- and magnesium-free Hank's Balanced Salt Solution (HBSS) to remove blood and debris. Then the ventricles are carefully minced and placed in 10ml of 100ug/ml trypsin in HBSS for overnight at 4°C. On the second day, the tissue was transferred into a 50ml conical tube, and 1ml of 2mg/ml trypsin inhibitor in HBSS was added. After incubating 5min at 37°C, 5ml of 300unit/ml collagenase was added and the tube slowly rotated for 30 to 45 minutes. The tissue was

dissociated into single cells and filtered through a Falcon cell strainer. The cells were allowed to sit in the hood for 1-2 hours and then centrifuged for 5min at 500rpm. The cells were re-suspended in 10ml of M199/DMEM media. Finally the cells are counted and seeded on collagen coated cell Petri plates with around 1 x 10^5 cells per cm^2.

All cultures were supplemented with 1% penicillin/streptomycin and maintained in 5% CO_2 at 37°C.

Generation of recombinant adenovirus (pAd-RBM20) and rescue of the splice phenotype in RBM20 deficient cardiomyocytes. The full length cDNA of Rbm20 was amplified using the forward primer: 5'-cga gcg tcg acc cca cca tgg tgc tgg ctg cag c-3' and the reverse primer: 5'-gcg ata tct tac tat cat agc ttc ttc ctt tcc -3', ligated into the pGEM-T easy vector (Promega), and verified by sequencing. Subsequently, the Rbm20 cDNA was inserted into the pShuttle vector (Stratagene) using SalI and EcoRV. After homologous recombination into the adenovirus vector pADEasy-1, which contains a CMV promoter, the recombinant adenoviral plasmids were amplified and transfected into AD293 cells using FuGENE HD (Roche) according to the manufacturer's instructions. The recombinant adenoviruses were then amplified and purified by ultracentrifugation in a cesium chloride gradient. The concentrations of the viral stocks were determined by plaque assays on Ad293 cells. A second round of amplification by infection of Ad293 cells with the original lysates was used to generate high titer viral stocks. An adenoviral vector carrying the green fluorescent protein (GFP) gene driven by the cytomegalovirus (CMV) promoter was used as a control (generous gift from Dr. G Zhang).

To rescue the phenotype of Rbm20 deficient neonatal rat ventricular myocytes, the cells were plated for 24 hours, treated with recombinant Rbm20 adenovirus (pAd-RBM20) and control pAd-GFP at a multiplicity of infection (MOI) of 50-100 particles per cell. Cells were incubated for 24, 48 and 72 hrs after infection followed by harvesting with 8M urea- SDS buffer. The adenovirus treatment leads to high expression levels and therefore an all- or none effect with cells infected expressing high amounts of RBM20 and cells that are not infected or do not

survive the virus load expressing normal levels of RBM20. The analysis of titin isoform expression by vertical 1% SDS-agarose gel electrophoresis (VAGE) is described below.

Antibodies. Antibodies against Rbm20 (α-Rbm20) were generated in chicken and rabbit with full length Rbm20 as antigen or in rabbit with the C-terminal peptide. N- and C-terminus of RBM20 were expressed by respectively transferring pET41a-Rbm20 and pET28a-Rbm20C into E.coli BL21 (Novagen) and inducing the bacteria with 1mM IPTG at 37 °C for 4 hours. Expressed protein was purified with HisTrap FF (GE Healthcare) based on the manufacture's protocol. Both chicken and rabbit α-Rbm20 were affinity purified by HiTrap affinity column (GE Healthcare) coupled with full length Rbm20. Rabbit anti-Rbm20 was further purified with a HiTrap affinity column coupled with C-terminus of Rbm20. The anti-peptide antibody was raised in rabbit using the synthetic peptide PERSGIGPHLERKKL (rat, amino acids 1193-1207, accession number NP_001101081) as antigen. The antibody was generated and affinity purified by Eurogentec (Liege, Belgium). Specificity of the antibodies was examined by western blotting using wildtype and RBM20 deficient rat heart. Both antibodies produced compatible results by Western blotting indicating specificity. Here we show Western blots and Immunofluorescence staining derived with the anti-peptide antibody.

Western Blotting. Western blots were performed with rabbit α-Rbm20 and anti-Actin (A2066, Sigma). Protein samples of left ventricle were prepared with urea-thiourea buffer (8M urea, 2M thiourea, 75mM DTT, 3% SDS, 0.05% bromophenol blue, 0.05M Tris, pH=6.8) from wild type, heterogeneous and homogeneously mutant rats, 3 adults in each genotype. Total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membrane. Membrane was first blocked in phosphate-buffered saline-0.05% Tween 20 (PBST) with 5% nonfat dry milk overnight and then incubated with α-Rbm20 peptide antibody diluted in blocking buffer for 3 hours at room

temperature. After 3 washes with PBST, the membrane was incubated with horseradish peroxidase conjugated secondary antibody in PBST with 5% nonfat dry milk for 2 hours. After another 3 washes, the blot was developed with ECL western blotting substrate (Pierce) and exposed to CL-Xposure film (Thermo Scientific). The same membrane was probed with anti-GAPDH (FL-335, Santa Cruz) or anti-Actin (A2066, Sigma), which serve as a control to show that the total protein loading on different lanes are similar.

Titin isoforms. Protein samples from left ventricles or cultured cells were homogenized in sample buffer (8 M urea/2 M thiurea/0.05 M Tris pH 6.8/ 75 mM DTT/, 3% SDS/ 0.05% bromophenol blue) and titin isoforms were separated using an SDS/agarose gel electrophoresis system [6]. Gels were Coomassie-stained to visualize the titin isoforms N2BA (several sizes, including both the N2A and N2B regions), N2B, and the proteolytic fragment T2. The largest N2BA titin isoform found in the homozygous mutant is 3.83 MDa. We have sequenced cDNAs from all the middle Ig and PEVK regions (exons 49 through 225), and the mutant sequences contain as many or more of these exons as human soleus titin (3.7 MDa). Adding the nearly 100 KDa for the N2B unique region found only in cardiac titin, puts the calculated size close to the estimate derived from the agarose electrophoesis.

Immunofluorescence. HL-1/ C2C12 cells cultured on coverslips were fixed using the cold methanol-acetone (1:1 V/V) fixation procedure at -20°C for 5-10 minutes and air dried. After blocking with normal donkey serum, specific antibodies mouse anti- α-actinin (1:300) (Sigma-Aldrich), mouse anti- Coilin (1:50) (Abcam), mouse anti- CUGBP1 (1:50) (Santa Cruz), mouse anti- SC-35 (1:50) (Abcam), and mouse anti- U2AF65 (1:100) (Sigma-Aldrich), were diluted in PBST (PBS-with 0.5% Triton-X100) and incubated for 2 hours at room temperature or overnight at 4°C. Subsequently, primary antibodies were removed by washing three times in PBST and the coverslips were incubated with secondary conjugates for one hour: goat anti- mouse-Cy3 (1:1000) dilution (Invitrogen) goat anti- rabbit Alexa488 (1:1000) (Invitrogen). Coverslips were

washed, stained with DAPI (Roth) at 1:1000 dilution and mounted on glass slides. Confocal images were acquired using Leica SPE confocal system driven with Leica LAS AF interface software.

Cardiovascular phenotyping (rat). Transthoracic echocardiography was performed using a Sonos 5500 ultrasonograph with a 15-MHz transducer (Philips, Andover MA). For acquisition of two-dimensional guided M-mode images at the tips of papillary muscles and Doppler studies, rats were sedated by IP administration of 25-50 mg/kg ketamine and maintained on a heated platform in a left lateral decubitus or supine position. The chest was shaved and pre-warmed coupling gel applied. Mitral and aortic flows were measured using Doppler pulse wave imaging. End diastolic and systolic left ventricular (LV) diameters as well as anterior and posterior wall (AW and PW respectively) thicknesses were measured on line from M-mode images using the leading edge-to-leading edge convention. All parameters were measured over at least three consecutive cardiac cycles and averaged. Left ventricular fractional shortening was calculated as [(LV diastolic diameter – LV systolic diameter)/LV diastolic diameter] x 100 and LV mass was calculated by using the formula [1.05 x ((diastolic posterior wall + diastolic anterior wall+ LV diastolic diameter)3 – (LV diastolic diameter)3)]. Relative wall thickness was calculated as 2*diastolic posterior wall/LV diastolic diameter.

Surface ECG readings were obtained using light anesthesia with isoflurane (1.6 vol% isoflurane/air) and ECG standard intervals were measured in six-limb leads as described previously [7] (*n* = 12).

In-vivo electrophysiological studies (EPS) with programmed electrical stimulations (PES) were performed as described previously [8]. Briefly, one octapolar 2-French electrode catheter (CIBer mouse cath, NuMed Inc.) was placed via the right jugular vein into the right atrium, a second catheter was placed via the right carotid artery into the left ventricle under electrogram guidance. Electrical stimuli were delivered at twice diastolic threshold with pulse duration of 1.0

ms in right atrium and left ventricle. PES used up to three extra stimuli (S2-S4) following 10 basal stimuli (S1, cycle length 100/120/150 ms). Additionally short episodes of burst pacing (10 sec) with cycle lengths down to 90 ms (ventricle) and 40 ms (atrium) were applied. Only reproducible arrhythmias up from 5 beats were considered. Stimulation protocols were repeated after β-adrenergic stimulation with isoproterenol (10 mg/kg body weight i.p.). All electrograms were digitally stored for off-line analysis, performed by two experienced, independent observers blinded to the animal's genotype.

Histopathology. Hearts were removed from 2 to 20 months old rats and fixed in 4% formaldehyde buffered with PBS. Following dehydration and paraffin embedding, cross sections midway between the base and the apex were cut, de-paraffinized, and stained with Massons trichrome. Images were collected using a Zeiss Axiovert 200 microscope with an AxioCAM HR digital camera and 5X or 20X objectives. Relative area fractions were estimated using the segmentation tool of IPLabs 3.6 (Signal Analytics). With the trichrome stain, the percent areas of blue-green (connective tissue), red (cardiomyocytes), and white (tissue holes) were determined independently. A data set was included in the analysis if the sum of these three measures was in the range from 90-110%. The percent fibrosis was estimated by comparing the blue-green area to the sum of the blue-green and red areas. Four to eight separate images from the interstitial regions were processed for each slide. Sirius red staining was used to confirm the contribution of collagen to the pathology.

Human RBM20 mutation. All studies were approved by the responsible institutional review boards of the Charité-Universitätsmedizin Berlin. All patients gave informed consent. In the patients where RBM20 mutations were detected, myocardial biopsies were obtained from the anterior left ventricular free wall and from the posterobasal wall after performing coronary angiography. Biopsies were immediately frozen in liquid nitrogen.

Clinical data: The female patient [DCM-S635A] suffered from a severe DCM with arrhythmias with an early onset of disease at the age of 23 years. Because of cardiac syncopes and arrhythmias an automated cardioverter/defibrillator (AICD) was implanted. At the age of 29 years, she showed a severe symptomatic disease (NYHA III). Left ventricular dilatation (LVEDD=76 mm, LVESD=60 mm) and cardiac dysfunction (EF=29%) were determined by echocardiography. This presentation is in good concordance with the clinical data published recently. For example, a high rate of ventricular tachycardia and AICD implantation was reported in patients carrying RBM20 mutations.

The female control patient [DCM-CP1 (1785)] was 65 years when the sample was obtained in 2003. In 1992, a dilated cardiomyopathy with severe impairment of left ventricular function was diagnosed. She was treated medically with ACE inhibitors, diuretics, digoxin and beta blockers. In 2000 she was referred for cardiac transplantation to German Heart Institute (DHZB). She presented with NYHA III and atrial fibrillation and ventricular arrhythmia Lown IV b. She had pulmonary artery hypertension (59/ 29, mean 40 mm Hg), elevated pulmonary vascular resistance (PVR752 Dyn*sec*cm-5), cardiac output 1.6 l/min, left ventricular end diastolic pressure 24 mm Hg. Echocardiography revealed left-ventricular dilatation (EDD 69 mm, ESD 60 mm, LVEF 15%), RVEF 20%. VO_2 max was 5.1 ml/min/kg. The patient was first treated conservatively and was transplanted in 2003. At this occasion, LV samples were obtained and immediately frozen in liquid nitrogen.

The male patient [DCM-CP2 (1909) was diagnosed in 2005, at the age of 40, with a dilated cardiomyopathy, left-bundle branch block, ventricular arrhythmia Lown IV b, and heart failure NYHA III. An ICD was implanted. There were multiple incidents of cardiac decompensation. In 2007, pulmonary artery pressure was 50/22 mmHg, mean 31, pulmonary capillary pressure 30 mm Hg, cardiac index 1.9 l/min/kg. The patient was referred for cardiac transplantation to DHZB. At this time, echocardiography revealed LVEF 12-15%, LVEDD 88 mm, RVEF 30%, RVEDD 41 mm, LA 47/50 mm, RA 55 mm, aortic regurgitation I, mitral regurgitation II, tricuspid regurgitation I-II, cardiac output 4 l/min. VO_2max 11.5 ml/min/kg (31%). The patient

was treated with Amiodarone, Candescartan, Digitoxin, beta blockers, Magnesium, Spironolactone, and Torem. He was rated “high urgency” (HU) in the heart transplantation listing. During the transplantation procedure, LV samples were obtained and immediately frozen in liquid nitrogen.

Global Splicing Analysis. We collected left ventricular tissue from 3 rats with Rbm20 deficient homozygous alleles[1], 3 heterozygous and 3 unaffected animals as well as from one human DCM patient with a mutation in the RBM20 gene and two control DCM patients with normal RBM20 status. Tissue was frozen in liquid nitrogen and stored at -80 °C. Total RNA was extracted using the Trizol reagent for nucleic acid purification, RNA quality was estimated (2100 Bioanalyzer, Agilent) and RNA-seq libraries were prepared. Fragmented, rRNA-depleted poly(A)+ mRNA was used to create random-primed double-stranded cDNA molecules. Size selection and adapter ligation was performed using the SPRIworks system (Beckman Coulter) and the final library was amplified with 15 PCR cycles. We then sequenced each sample on one lane on a HiSeq 2000 instrument from Illumina using 2 x 100 bp PE chemistry.

Reads were mapped to the BN reference genome RGSC 3.4 for rat RNA-seq libraries and against GRCh37 for human RNA-seq lbraries using TopHat v 1.3.1[9]. This approach allows alignment of reads across known and predicted splice junctions. We supplied splice junctions annotated in the Ensembl reference database to the pipeline and also enabled de novo splice junction detection. The differential expression of RBM20 exons in wildtype, heterozygotes, and homozygote rats based on the genomic deletion of RBM20 was used as an internal control of the genotype (supplementary figure 11).

In order to detect differential isoform processing in our datasets, we used Cuffdiff, part of the Cufflinks 1.03[10] package, and tested for all genes as annotated by Ensembl. The annotation was supplied by http://cufflinks.cbcb.umd.edu/igenomes.html (the iGenomes project) to allow direct testing of splicing with the cufflinks package without the need to predict isoforms de novo. We then tested for differential splicing in pairwise comparisons for all three groups of animals (RBM20 -/-, -/+ and +/+) as well as for the RBM20 affected patient against two non-affected DCM patients each. Genes which were significantly differentially spliced in all comparisons were considered potential splicing targets of RBM20. Genes are considered orthologs in the human and the rat as defined in the Ensembl database.

Transcript analysis. RNA was prepared from snap frozen tissue using the RNeasy Mini Kit (Qiagen) followed by cDNA synthesis using Thermoscript First-Strand Synthesis System (Invitrogen). Quantitative real-time RT-PCR (qRT-PCR) was performed in triplicates on an ABI 7900 Real Time PCR Instrument (Applied Biosystems) using the SYBR GREEN PCR Master Mix (Applied Biosystems). Data were analyzed with SDS 2.0 software (Applied Biosystems) and Microsoft Excel, using the $2^{-\Delta\Delta Ct}$ method and the expression level of 18S as an internal reference [11]. To confirm alternative splicing by qRT-PCR we normalized to representative exons within the same gene that were determined as not differentially spliced by exon profiling. Primer sequences are indicated below and additional information is available upon request.

***In vivo* cross-linking with immunoprecipitation (CLIP)** was performed as described previously [12]. In brief, HEK 293 cells grown overnight in medium supplemented with 100 µM 4-thiouridine (4SU) were irradiated with 365 nm UV light followed by harvesting and lysis in NP40 lysis buffer. Cell lysates were cleared by centrifugation and RNase T1 treated. FLAG/HA-tagged proteins were immunoprecipitated using the FLAG-specific monoclonal antibody M2 (Sigma) covalently coupled to Protein G Dynabeads (Invitrogen). Immunoprecipitated RNA was radiolabeled by dephosphorylation of RNA with calf intestinal alkaline phosphatase and incubation with T4 polynucleotide kinase and radioactive ATP. The protein-RNA complexes were separated by SDS-PAGE.

RNA retention assay. A genomic fragment at the exon intron junction of the exon encoding I27-30 preceding an alternatively spliced region of titin was amplified using primers ttn I27-30 Af and ttn I27-30 Ar and cloned into pGEM-Teasy. The control was amplified with ttn ctrl Cf and ttn ctrl Cr. The MAXIscript® SP6 or MAXIscript® T7 Kit (Ambion) was used to in-vitro transcribe RNA.

GST-RBM20_aa508-658 contains the RRM domain and was expressed in E.coli Rosetta2 cells. After lysis in 1x REMSA buffer (10 mM HEPES pH 7.4, 20 mM KCl, 1 mM MgCl2, 1 mM DTT) it was captured using glutathione agarose beads (Sigma). 1 ug Sense or antisense RNA was applied per 20 uL settled bead resin and incubated at least 4 h at 4 °C in an end-over-end shaker. Reactions were thoroughly washed in 1x REMSA buffer and incubated with an excess of proteinase K for 30 min at 55°C followed by Phenol/Chloroform extraction. Retained RNA was reverse transcribed using RevertAid™ Premium First Strand cDNA Synthesis Kit (Fermentas). PCR was carried out to detect the retained RNA.

Splice reporter assay. We established a dual luciferase splice reporter assay based on the RBM20 dependent titin PEVK exons 4 through 13 (see *Plasmid Construction*). C2C12 and HEK 293 cells (20000 cells/well on 96 well plates) were transfected 24h after seeding with reporter-plasmid and CMV driven RBM20 or control plasmids (pcDNA3.1, pcDNA3ptbpI CLI, pcDNA3HuDmyc-L - generous gift from Robert Darnell, The Rockefeller University) at the indicated ratios (1:1 or 5- and 25-fold excess of the RBM20 expression construct) using TurboFect (Fermentas) following the manufacturer's instructions. After 48h cells were lysed and assayed using the Dual-Luciferase® Reporter Assay System (Promega) with 8 wells per condition following the manufacturer's instructions and measured with an infinite M200 plate reader (TECAN). Ratios of firefly to renilla luciferase activity were normalized to controls transfected with the reporter construct only. Results were confirmed in three independent experiments and the assay was validated by comparison of light units to Firefly- and Renilla-Luciferase RNA levels (supplementary figure 2). On the RNA level, the alternative reporter isoform expression was determined by sequencing of the RT-PCR products derived from the transfected dual reporter. The isoforms identified indicate that the luciferase containing exon is skipped upon expression of RBM20. The sequences derived document the combination of the following Exons: PEVK4-5-

6-7-13; PEVK4-5-6-13, and PEVK 4-5-13 as indicated in figure 2c. Our RNA seq analysis of the titin PEVK region in the rat heart is consistent with expression of these isoforms and suggests that the inclusion of the luciferase does not alter alternative splicing of titin's PEVK exon 8.

Gene ontology and MESH analysis. Functional enrichment analysis of the RBM20 splice substrates were generated using the Cytoscape 2.8.0 plugin ClueGO v1.4 [13,14]. For the analysis of cellular components and molecular functions, network specificity was set to global, minimum number of identifiers and minimum percentage of mapped identifiers to 1. For the analysis of biological processes, network specificity was set to medium, minimum number of identifiers and minimum percentage of mapped identifiers at 2. Cohen's kappa statistics were used to determine functionally enriched gene-sets. Grouping of terms was assigned by kappa score, with the initial group size of 3 and the percentage of overlapping terms/group for group merge of 50%. The degree of connectivity between terms (edges) is calculated using kappa statistics, which is also used to define functional groups. Enrichment of associated MESH terms to the gene set was calculated using EGAN [15]. The hypergeometric test was used to evaluate significant enrichments for associated terms and we adjust for multiple testing using the Bonferroni method.

General Statistics. GraphPad prism software was used for statistical analysis. Results are expressed as means±SEM. Statistical significance between groups was determined using one-Way ANOVA with a Tukey post test, or a 2-tailed *t*-test for comparison of two groups. The significance level was $P<0.05$=*; $P<0.01$=**; $P<0.001$=***.

Table - PCR primers full length Rbm20

Name	Exon ID/ name	Sequence (5' to 3')	PCR product
P1	5' UTR Exon 2	F: AAGGGGACTGGGTACAGG R: CGAAGCTGGTTGAAGAGAG	515
P2	Exon 2 Exon 2	F: CTCTCTTCAACCAGCTTCG R: TCTCCTTTCATGCTCCCAG	532
P3	Exon 2 Exon 5	F: TGGGAGCATGAAAGGAGAC R: AGAGGAAGCCGAAGGAAAC	619
P4	Exon 5 Exon 9	F: TGTTTCCTTCGGCTTCCTC R: TCTTGTCTTCCCCGTTCTC	555
P5	Exon 9 Exon 11	F: AGAACGGGGAAGACAAGAG R: GTCTGGCTCCATGATGAAG	692
P6	Exon 11 Exon 12	F: CTTCATCATGGAGCCAGAC R: CCCAGGAAGGCAAAGAAAG	638
P7	Exon 12 3' UTR	F: CCTTTCTTTGCCTTCCTGG R: CCTGAGAAGTTGGGTTTTATG	588
P8	3' UTR	F: CTCCTGATTCCTTTAGTCTG	771
	3' UTR	R: ACAGGGTTCTTCTTCACTCC	
P9	3' UTR	F: AGTCATTCATCCTCAGCTAC	704
	3' UTR	R: GAAACTCACACAAGCAAACC	
P10	3' UTR	F: GAAGGCATGAGTACCACTAG	739
	3' UTR	R: TCAATGTATAGCCAAGGGAG	
P11	3' UTR	F: CAGAGGCTGGACATGATTTC	693
	3' UTR	R: GCTAATGGCTGCTGCTCAC	
P12	3' UTR	F: TCTCTCCCCAAGGTTGCTC	438
	3' UTR	R: CTCACAAGCTTTCAAATTCTC	

Table - Sequencing primers Rbm20

Name	Exon ID/ name	Sequence (5' to 3')	Position (bp)
M13	T-easy	F: GTTTTCCCAGTCACGAC R: CAGGAAACAGCTATGAC	2959 176
P2	Exon 2	R: TCTCCTTTCATGCTCCCAG	926
P4	Exon 9	R: TCTTGTCTTCCCCGTTCTC	2062
P5	Exon 11	R: GTCTGGCTCCATGATGAAG	2736
P6	Exon 12	R: CCCAGGAAGGCAAAGAAAG	3355
P8	3' UTR	R: ACAGGGTTCTTCTTCACTC	4601
P10	3' UTR	R: TCAATGTATAGCCAAGGGAG	5766

Table - Primers for Chromosome Walking

Name	Exon ID/ name	Sequence (5' to 3')	PCR Product (bp)
5UTR1		F: GGAAGGGTTAGGAGGTGTG R: TCAACGGCATCAGTCATC	640
5UTR2		F: GTGTCATGTTCTGGGATCTC R: GAGATGAGGAAGCAGAGTG	564
5UTR3		F: CACAGACCTTCAGCCTTTG R: CTTGGTACTGGCTATGGAG	660
5UTR4		F: CACCTTCTTACTCTCATTCC R: GTCCATTGTGAAGTACCAG	751
5UTR5		F: CAATTCCCAGCAACCACATG R: ACACTTCAGAGACATTAGG	798
5UTR6		F: GACAGTACAGCAGCAGGTTC R: TCAGTCTACGTTCATGCCTC	797
5UTR7		F: GTAGTGTCTGTCTTTGTCTC R: AGGAAATCCAGGACTCAATG	790
5UTR8		F: CAGGACAGAGGGACAGATG R: CAGGTCGAGAGTCTACTTC	803
5UTR9		F: CGTTCTTGGCATCACAGTAG R: CTCATTCTTCTTGGGTTGTG	787
5UTR6		F: GACAGTACAGCAGCAGGTTC R: TCAGTCTACGTTCATGCCTC	797
5UTR7		F: GTAGTGTCTGTCTTTGTCTC R: AGGAAATCCAGGACTCAATG	790
5UTR8		F: CAGGACAGAGGGACAGATG R: CAGGTCGAGAGTCTACTTC	803
5UTR9		F: CGTTCTTGGCATCACAGTAG R: CTCATTCTTCTTGGGTTGTG	787
5UTR10		F: CTCCCATCTGCTTTTGCTTG R: CCAGTCAGCTACCACATATC	858
5UTR11		F: CTTCCTGCTTCTGTTCCCTG R: GCTCCCTTGTCTGTTTTCTC	693
5UTR12		F: CGTGTGCTGAAGATTAGAGG R: CCAAGGTCCTGCTGTTCAAG	1100
5UTR13		F: CTTGAACAGCAGGACCTTGG R: CACTCCAGGATCATGAAGAC	739
5UTR14		F: GTCTTCATGATCCTGGAGTG R: GAACCTGCTGCTGTACTGTC	815
3UTR1		F: GAGAAACAGTGTCAAGAAGG R: GGTCTATACATGTCGCTCAC	803
3UTR2		F: GTGTAGGTGTAGGCATAAGG R: TGTGTGGAAAGCAAATCTGG	646
3UTR3		F: TGACTCCATAACCCCACACC R: CTGCTGTTGTCCTCTTTGTG	650
3UTR4		F: CTAGGTCCAGCCCATGTATG R: GTCCCCGTGTCCTCAATTTC	807
3UTR5		F: TCGTATGACTGTCCCTGTTG R: CTCCATAGACCCAAAGAAGC	1005
3UTR6		F: GACTGTTTCCTGTGACTTGG R: TGGACTGGGCACATATCAAG	656
3UTR7		F: GCTGCTTGTGCTTTGAGAG R: CAGGTTCAGTGAGAGGATG	785
3UTR8		F: GCTACTGCTGGGTCAAAAG R: CCAAGCCATCACCAAATAAC	808
3UTR9		F: TCCGGCAGCCAAAGTCATG R: GCTTTCTTCTTTGCCCTCTC	902
3UTR10		F: CAAAAGACACCACCCTGCTC R: GGTGTGGGGTTATGGAGTC	636

Table - Splice PCR Primer

PCR name	Exon ID/ name	Sequence (5' to 3')	PCR Product (bp)	
			WT	KO
Titin 98-07	1475398 1475407	F: CAGGAGCAGGTTTCTTTGGA R: GAGCCGTATGAGGAACCGTA	475 391 307	799
CaMKIIδ 37-42	1373037 1373042	F: AAGGGTGCCATCTTGACAAC R: TCGAAGTCCCCATTGTTGAT	242	302
Ldb3	Ldb3 F e4 Ldb3 R e9	F: TCCAAGCGTCCTATCCCCATC R: TGTATTCTGTCCCGGTCATCTG	614 488	614 488
Trdn	Trdn F e6 Trdn R e9	F: CAGAGACAAAGATGGCAGCAA R: CTTCCAGTGATGGTGTTGACA	279 339	279 339
CamKIIg	CamkIIg F e10 CamkIIG R e18	F: CAACGGTCAACAGTGGCATCC R: GTGTAGGCCTCAAAGTCCCCA	341 404/410	341 404/410
Sorbs1	Sorbs1 F e1 Sorbs1 R e3	F: CTTTCAGAATGTGATGTTGGAAG R: CTGACTTGTTTTCATGCTTCG	205 295	205 295
Sh3kbp1	Sh3kbp1 FW e5 Sh3kbp1 RV e8	F: GTGGAGGAAGGATGGTGGGAA R: CCACTTCAATCGACCTTGGTC	310 367	310 367 442
RBM20 7-8	RBM20 F e7 RBM20 R e8	F: GAAGTTGCATGCCCAGAAAT R: CAGCGTCCCTTCTCCTGTAG	82	-
Rbm20 1	RBM20 F e1 RBM20 R e1	F: AGTGAGCGCCTGTGTTCC R: AGGCTGGATTGAGGCTGAG	242	242
PEVK Reporter	PEVK4Bf PEVKEx13rev	F: GACTATGAGGAGATCAAGGTGGAAGC R:CCCTCGAGTCTTCTTTGCCACAGGAACG	300 to 3000	-

Table - Sybr green PCR Primer

PCR name	Exon ID/ name	Sequence (5' to 3')	PCR Product (bp)
ttn 01-98	1475398 1475401	F: CAGGAGCAGGTTTCTTTGGA R: AAGTGGTCCCTGTTCCCATT	348
Ttn 14-15	1475514 1475515	F: AGCTGCGCTCTTGTCTGTCT R: GAAGGGAGATACGGTGTCCA	147
CaMKIIδ 37-39	1373037 1373039	F: AAGGGTGCCATCTTGACAAC R: CTGGTTACCACGTTGGCTTT	113
CaMKIIδ 29-31	1373029 1373031	F: AATCTGCCGTCTCTTGAAGC R: CTGGCATCAGCCTCACTGTA	153
hPTBFluc_Af Fluc_PEVKEx8_r	Firefly	F: GAGTCCTTCGATAGGGACAAGACAATTGC R: AGAGTGCTTTTGGCGAAGAAGG	349
Rluc_PEVKEx13_f hPTBRluc _Ar	Renilla	F: TGCTGGACTCCTTCATCAACTACTATG R: CTCTTCGCTCTTGATCAGGGCGATATCC	424
18S	18Srat F 18Srat R	F: GTCCCCCAACTTCTTAGAG R: CACCTACGGAAACCTTGTTAC	419
Rbm20 mouse	Rbm20-Ex2 F Rbm20-Ex3 R	F: CCTGCCTTTGGGTCTCGGCTTAAC R: CCCTTTCACATGTAGCTCCCAGTC	192
ANP mouse	Mouse ANP F Mouse ANP R	F: CATCACCCTGGGCTTCTTCCT R: TGGGCTCCAATCCTGTCAATC	405

Table - Splice Reporter Cloning Primer

Name	Sequence (5' to 3') *	PCR Product (bp)
luc for luc rew	ccctgcagGAAGACGCCAAAAACATAAAGAA ccgcggccgcCACGGCGATCTTTCCG	1647
Rluc for Rluc rew	ccctcgaGCTTCCAAGGTGTACGACC ccgggcccttaCTGCTCGTTCTTCAGCAC	933
PEVK Ex 4 for PEVK Ex 8 rew	ccggatccaccatgGAGGAGATCAAGGTGGAAGC ccctgcagccCGGTGTGAGAGGCAAAGAAC	1960
PEVK Ex 8 for PEVK Ex 10 rew	ccgcggccgcACCAGCTGTGCACACAAAGA ccctcgagGGGGCTCCACCTTCTTAGG	1590
PEVK Ex 8 for PEVK Ex 13 rew	ccgcggccgcACCAGCTGTGCACACAAAGA ccctcgagTCTTCTTTGCCACAGGAACG	3620
hRLuc_shortl_f hRLuc_shortl_r	ccctcgagCTTCCAAGGTGTACGACCCCG cccgggccctaaattaaattaaattaaattaaatttaCTGC TCGTTCTTCAGCACGCG	973
PEVKint 4-5 fEx11 PEVKint 4-5 rEx13	CTGCCCCTCCGAAAGGTATGCATCCACTGCGTGG GGAGCCTCTGGGACTTTAATAAAAAAAAAAAAAAACA ACACAAG	454
PEVK Ex 8 for PEVK11 r XhoI	ccgcggccgcACCAGCTGTGCACACAAAGA ccctcgagCTTTCGGAGGGGCAGCT	2079
PEVK11 f PEVK Ex 13 rew	TTCCCGAGGCTCCCAAGAAA ccctcgagTCTTCTTTGCCACAGGAACG	138
PEVK Ex 8 for PEVK11 r	ccgcggccgcACCAGCTGTGCACACAAAGA CTTTCGGAGGGGCAGCT	2071
PEVK Ex 8 for PEVK int 10-11 rew	ccgcggccgcACCAGCTGTGCACACAAAGA AGGTGTTCTGGATACCTTTAAAGATAACATTGTCGAA ACAGT	1987
PEVK Int11-13 for PEVK Ex 13 rew	ATGTTATCTTTAAAGGTATCCAGAACACCTGGTGGAG ccctcgagTCTTCTTTGCCACAGGAACG	1561

* restriction sites are indicated in red, start and stop codons introduced in blue, and RNA degradation sequence in green. Grey indicates overlapping sequence for PCR based gene assembly. Regions that are not homologous to the original sequence are provided in small caps.

Table – Cloning Primers in vitro transcription

Name	Sequence (5' to 3')	Position (bp)
ttn I27-30 Af	CCAGCCTGGAGATCCTGAG	Chr3: 59597915-59597934
ttn I27-30 Ar	TCATTTGTTGGGGATTAACAC	Chr3: 59597087-59597107
ttn ctrl Cr	CCTCTGCCCCCTCCTAGATA	Chr3: 59596746-59596765
ttn ctrl Cr	CCCTGACACTGTGCAGAGAA	Chr3: 59595767-59595786

Table – RBM20 mutations Cloning Primers

Name	Sequence (5' to 3')	Position (bp)
rRBM20_V535If	GCACGGAGAATGACaTCATCAACCTGGGGC	1598
rRBM20_V535Ir	GCCCCAGGTTGATGAtGTCATTCTCCGTGC	1627
rRBM20_R634Qf	GGTCCAGAGCGACCACaaTCTCGAAGTCCAATGAGC	1894
rRBM20_R634Qr	GCTCATTGGACTTCGAGAttGTGGTCGCTCTGGACC	1929
rRBM20_R635Af	CCAGAGCGACCACGTgCTCGAAGTCCAATGAGC	1912
rRBM20_R635Ar	GCTCATTGGACTTCGAGcACGTGGTCGCTCTGG	1929
rRBM20_S636Sf	GCGACCACGTTCTtcAAGTCCAATGAGCCG	1903
rRBM20_S636Sr	CGGCTCATTGGACTTgaAGAACGTGGTCGC	1935
rRBM20_S637Gf	CGACCACGTTCTCGAgGTCCAATGAGCCGATCG	1903
rRBM20_S637Gr	CGATCGGCTCATTGGACcTCGAGAACGTGGTCG	1935
rRBM20_P638Lf	GACCACGTTCTCGAAGTCtAATGAGCCGATCGCTCTC	1904
rRBM20_P638Lr	GAGAGCGATCGGCTCATTaGACTTCGAGAACGTGGTC	1940
rRBM20_R716Qf	GGAAACACTATCCCcaGCAGCTGGACAAAGC	2093
rRBM20_R716Qr	GCTTTGTCCAGCTGcTGGGGATAGTGTTTCC	2123

* Regions that are not homologous to the original sequence are provided in small caps.

Supplemental References

1. Greaser, M.L. *et al.* Mutation that dramatically alters rat titin isoform expression and cardiomyocyte passive tension. *J Mol Cell Cardiol* **44**, 983-991 (2008).
2. Gotthardt, M. *et al.* Conditional expression of mutant M-line titins results in cardiomyopathy with altered sarcomere structure. *J Biol Chem* **278**, 6059-6065 (2003).
3. Younis, I. *et al.* Rapid-response splicing reporter screens identify differential regulators of constitutive and alternative splicing. *Mol. Cell. Biol* **30**, 1718-1728 (2010).
4. Claycomb, W.C. *et al.* HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci U S A* **95**, 2979-2984 (1998).
5. Munz, B., Hildt, E., Springer, M.L. & Blau, H.M. RIP2, a checkpoint in myogenic differentiation. *Mol. Cell. Biol* **22**, 5879-5886 (2002).
6. Warren, C.M., Krzesinski, P.R. & Greaser, M.L. Vertical agarose gel electrophoresis and electroblotting of high-molecular-weight proteins. *Electrophoresis* **24**, 1695-1702 (2003).
7. Royer, A. *et al.* Mouse model of SCN5A-linked hereditary Lenegre's disease: age-related conduction slowing and myocardial fibrosis. *Circulation* **111**, 1738-1746 (2005).
8. Fischer, R. *et al.* Angiotensin II-induced sudden arrhythmic death and electrical remodeling. *Am. J. Physiol. Heart Circ. Physiol* **293**, H1242-1253 (2007).
9. Trapnell, C., Pachter, L. & Salzberg, S.L. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* **25**, 1105-1111 (2009).
10. Trapnell, C. *et al.* Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol* (2010).doi:10.1038/nbt.1621
11. Weinert, S., Bergmann, N., Luo, X., Erdmann, B. & Gotthardt, M. M line-deficient titin causes cardiac lethality through impaired maturation of the sarcomere. *J Cell Biol* **173**, 559-570 (2006).
12. Landthaler, M. *et al.* Molecular characterization of human Argonaute-containing ribonucleoprotein complexes and their bound target mRNAs. *RNA* **14**, 2580-2596 (2008).
13. Smoot, M.E., Ono, K., Ruscheinski, J., Wang, P.-L. & Ideker, T. Cytoscape 2.8: new features for data integration and network visualization. *Bioinformatics* **27**, 431-432 (2011).
14. Bindea, G. *et al.* ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* **25**, 1091-1093 (2009).
15. Paquette, J. & Tokuyasu, T. EGAN: exploratory gene association networks. *Bioinformatics* **26**, 285-286 (2010).