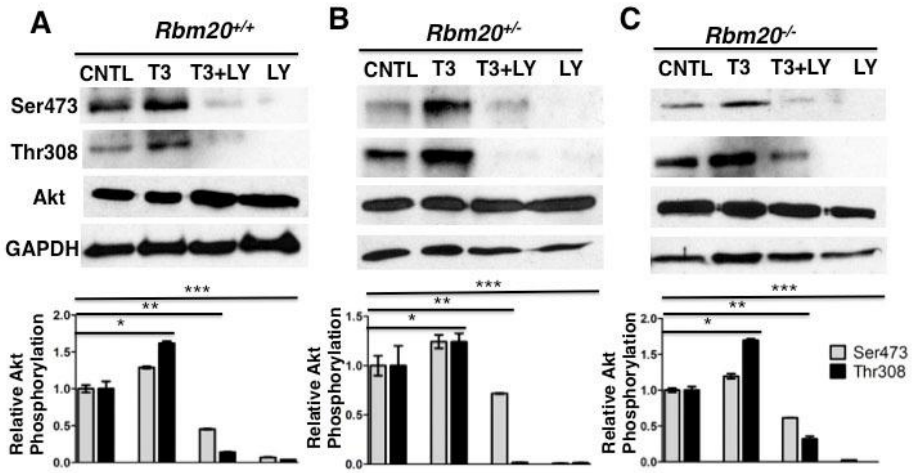
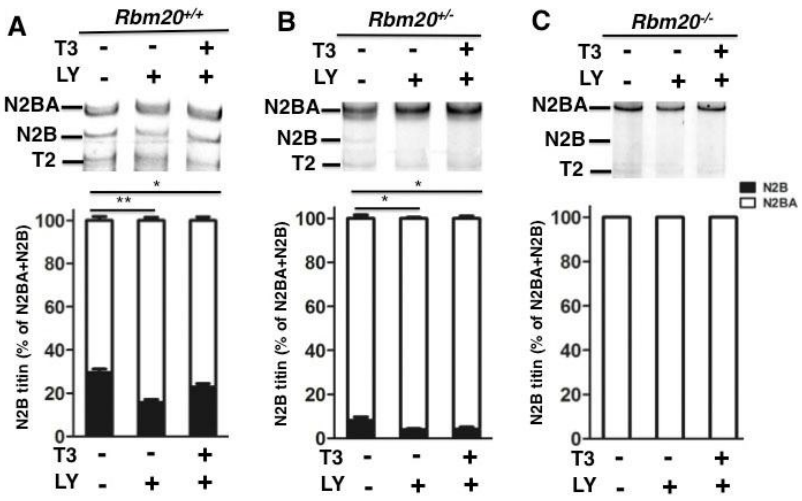
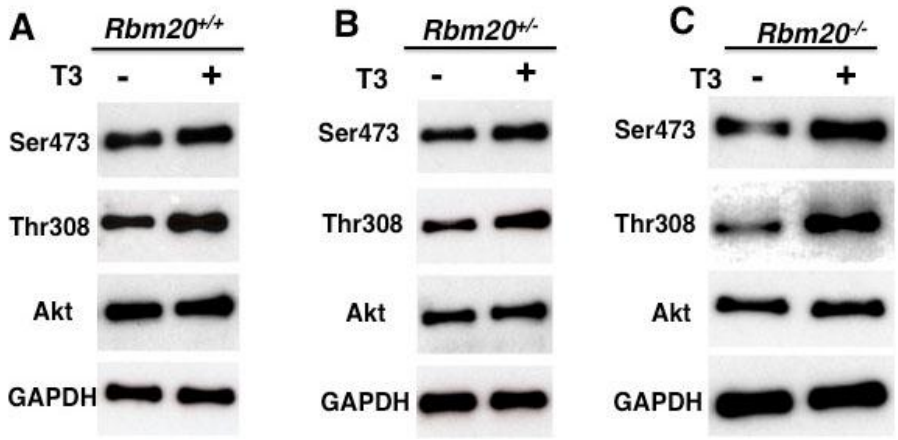
Supplementary Figures



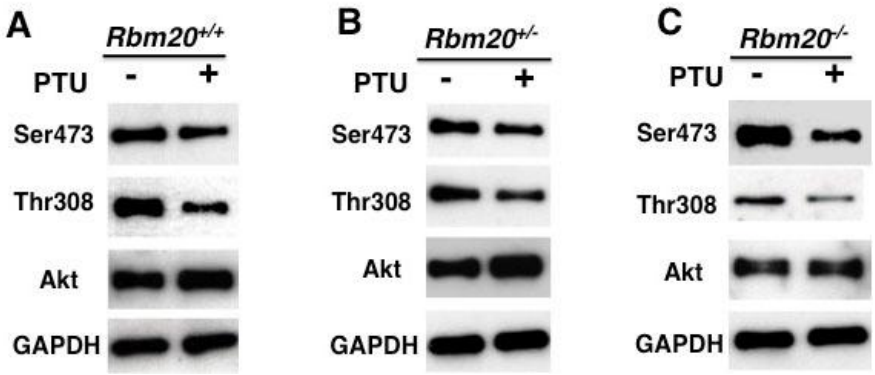
Supplementary Figure S1. Akt phosphorylation in primary cultures of *Rbm20*$^{+/+}$, *Rbm20*$^{+/-}$ and *Rbm20*$^{-/-}$ NVCMs treated with T3, T3+LY and LY respectively. A, B and C, Akt was phosphorylated on both sites of p-Akt Ser473 and p-Akt Thr308 by T3 addition and de-phosphorylated on both sites by LY addition in all three genotypes; the phosphorylation on both sites of p-Akt Ser473 and p-Akt Thr308 increased significantly with T3, but significantly decreased with LY. CNTL, control; Ser473, western blot for one of the Akt phosphorylation sites; Thr308, western blot for another Akt phosphorylation site; Akt, western blot for total Akt expression; GAPDH, protein loading control; Bars show means±SEM (n=3); *P<0.05; **P<0.01; ***P<0.001.



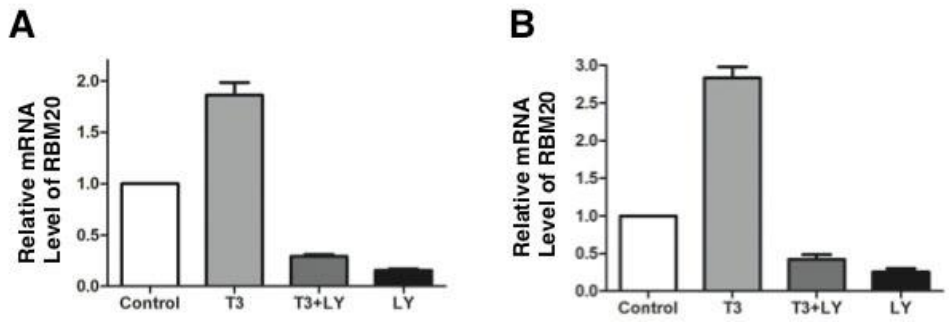
Supplementary Figure S2. Effect of LY on titin isoform transition in primary cultures of *Rbm20*$^{+/+}$, *Rbm20*$^{+/-}$ and *Rbm20*$^{-/-}$ NVCMs. A, N2B-titin isoform decreased with both T3 and LY addition or LY addition only when compared to control without T3 and LY in primary cultures of *Rbm20*$^{+/+}$ NVCMs; B, N2B-titin isoform decreased in both treatments when compared to control without T3 and LY in primary cultures of *Rbm20*$^{+/-}$ NVCMs; C, the ratios of N2B to N2BA titin isoforms were not affected by either LY itself or LY+T3 in *Rbm20*$^{-/-}$ NVCMs, T2, degraded titin bands; Bars show means ±SEM (n=3); *P<0.05, **P<0.01.



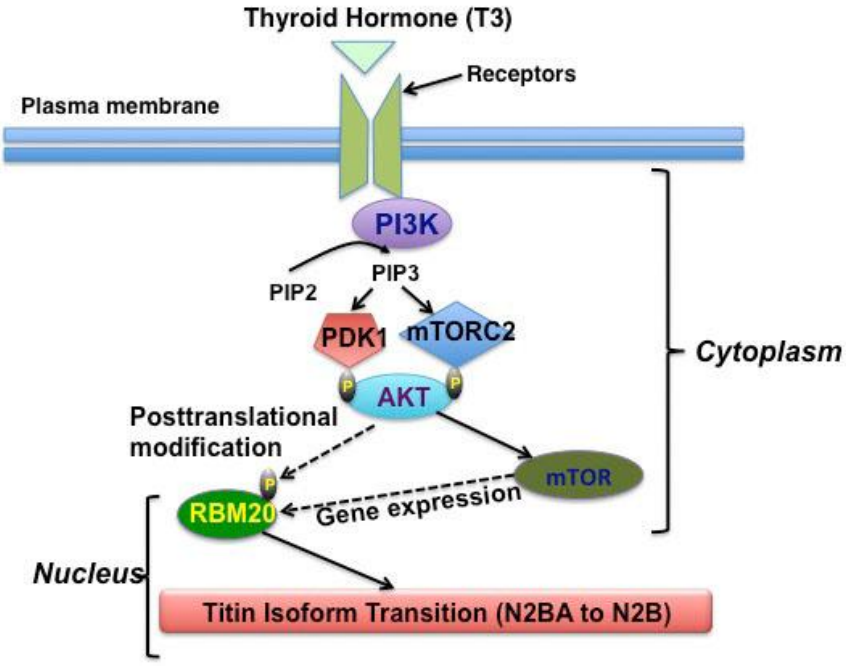
Supplementary Figure S3. Effect of T3 on Akt activity. A, B and C, the phosphorylation of Akt increased with T3 treatment in all three groups with and without RBM20 when compared to control groups without T3 treatment. Ser473, western blot for one of the Akt phosphorylation sites; Thr308, western blot for another Akt phosphorylation site; Akt, western blot for total Akt expression; GAPDH, protein loading control.



Supplementary Figure S4. Effect of PTU on Akt activity. A, B and C, the phosphorylation of Akt was decreased by PTU in all three groups with and without RBM20 when compared to control groups without PTU treatment. Ser473, western blot for one of the Akt phosphorylation sites; Thr308, western blot for another Akt phosphorylation site; Akt, western blot for total Akt expression; GAPDH, protein loading control.



Supplementary Figure S5. Relative expression of RBM20 with real-time PCR. A and B, Real-time RT PCR indicated that the relative mRNA levels of RBM20 increased with T3 supplementation and decreased with LY supplementation when compared to control in *Rbm20*$^{+/+}$ and *Rbm20*$^{+/-}$ NVCMs. Bars show means ±SEM (n=3).



Supplementary Figure S6. Putative regulatory mechanisms of titin isoform transition. The PI3K/Akt signaling pathway was activated by thyroid hormone-T3; the activated Akt could either phosphorylate RBM20 (dotted line in black) and/or alter gene expression of RBM20 (dotted line in black) and, thus, regulate titin isoform transition, and alter the ratios of the two major classes of titin isoforms: N2BA to N2B.

Supplementary Materials and methods

Experimental animals

The study was performed with wild type (*Rbm20*$^{+/+}$, WT), heterozygous (*Rbm20*$^{+/-}$, HT) and homozygous (*Rbm20*$^{-/-}$, HM) rats. The Rbm20-deficient (HT and HM) rats derive from a spontaneous mutant identified in our previous work (Guo et al., 2012). Rats used in the current work were crosses of Sprague-Dawley (SD) X Fisher 344 X Brown Norway (BN) (All strains were originally obtained from Harlan Sprague Dawley, Indianapolis, IN). Animals were maintained on standard rodent chow. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The procedure was approved by the Institutional Animal Use and Care Committee of the University of Wyoming.

Primary cultures of neonatal rat ventricular myocytes (NRVMs)

Primary cultures of NRVMs were prepared from one-day old rats from three genotypes: *Rbm20*$^{+/+}$, *Rbm20*$^{+/-}$ and *Rbm20*$^{-/-}$, using the neonatal cardiomyocyte isolation system (Worthington) as described previously (Guo et al., 2012). The cells were re-suspended in complete medium (M199/DMEM media and 20% fetal calf serum (FCS) supplemented with 1% penicillin/streptomycin), plated at a density of $1x10^6$ cells per cm^2, and maintained in 5% CO_2 at 37°C. Cells were cultured for 2 days in complete media, and then switched to serum-starved (FCS-starved) medium. The serum-starved medium was supplemented with T3 (150nmol/L), LY294002 (LY) (50μmol/L) and T3+LY (150nmol/L+50 μmol/L) as described (Kruger et al., 2008).

Thyroid hormone treatment and sample preparation

Three groups of rats (*Rbm20*$^{+/+}$, *Rbm20*$^{+/-}$ and *Rbm20*$^{-/-}$) were used for triiodothyronine (T3) treatment. Each group contained 8 to 10 three-month old rats. T3 was administered by implanting subcutaneously a T3 pellet (7.5mg, 90 days sustained release; Innovative Research of American, Sarasota, FL). For control groups, placebo pellets were implanted using the same procedure as used when implanting the T3

pellets. Briefly, the rats were anesthetized with 1.6% isoflurane. Hair at the surgical site (the dorsal subcutis slightly caudal to the shoulders) was removed using an animal clipper with a surgical (#40) blade to obtain a close shave without nicks in the skin. An area 3-4 times the size of the T3 or placebo pellet was clipped. All surgical instruments were autoclaved. With a sterile scalpel blade, an incision was made at the site where the pellet was to be implanted. Incision length of approximately 1.5 times the diameter of the pellet was made perpendicular to the long axis of the implant. A closed hemostat was inserted into the incision and the jaws opened to make a suitable implant pocket. The pellets were placed into the subcutaneous pocket. The incision was closed with stables. Animals were placed in a clean, dry cage in a warm place until the animals were recovered. After a three-month treatment, the rats were sacrificed with carbon dioxide. The heart tissues were collected immediately and snap frozen in liquid nitrogen, and then stored at -80°C for further analysis.

Propylthiouracil (PTU) treatment and sample preparation

Equal number of rats with similar age used for the T3 treatment described above was used for the PTU treatment. Rats were on feed with 0.15% PTU (Harlan Tekland Co., Madison, WI) and drinking water with 0.05% PTU for 3 months as described previously (Wu et al., 2007). After a three-month treatment period, the rats were sacrificed with carbon dioxide. The heart tissues were collected immediately and snap frozen in liquid nitrogen, and then stored at -80℃ for future analysis.

Sodium Dodecyl Sulfate-Agarose gel electrophoresis

Titin isoforms were resolved using a vertical sodium dodecyl sulfate (SDS)-1% agarose gel electrophoresis (VAGE) system (Warren et al., 2003b). Protein samples were prepared as described (Guo et al., 2013). Rat heart tissues and NRVM cultures were homogenized in urea-thiourea buffer. Protein bands were visualized by silver staining, scanned and analyzed densitometrically with NIH ImageJ software. Average titin isoform ratios were calculated from a minimum of three replicates per experimental treatment.

Western blot and Akt phosphorylation assay

Western blot analysis was conducted following previous procedures (Guo et al., 2013). Total protein was separated by 10% SDS-PAGE gel, and transferred onto a PVDF membrane. The membrane was probed with antibodies against RBM20, Akt, phospho(p)-Akt(Ser473) and p-Akt(Thr308) (Cell Signaling). Goat anti-rabbit IgG-conjugated with horseradish peroxidase (Fisher Scientific) served as the secondary antibody. Anti-Histone3 (Cell Signaling) and GAPDH (Santa Cruz) served as the protein loading control.

Real-time RT-PCR

Total RNA was extracted using TRIzol reagent (Life Technologies) following the manufacturer's instructions. RNA was reverse-transcribed with SuperScript II reverse transcriptase (Life Technologies). The cDNA was used as a template for SYBR Green quantitative real-time PCR (Bio-Rad) using primers for RBM20 (Forward primer: 5'-CTCAGCTCACCCTCCACC-3' and Reverse primer: 5'-GTTGAAGAGAGGCTGGGAC-3'). The procedure was described in detail previously (Guo et al., 2012; Guo et al., 2013). Three biological replicates for each treatment were analyzed in quadruplicate, with a minimum of two independent experiments. The relative amount of RBM20 mRNA normalized to GAPDH (Forward primer: 5'-AACATCATCCCTGCATCCAC-3' and reverse primer: 5'-CATACTTGGCAGGTTTCTCC-3') was calculated (Pfaffl, 2001).

Statistics

GraphPad prism software was used for statistical analysis. Results were expressed as means ± SEM or SD. Statistical significance between groups was determined using ANOVA and an unpaired Student's *t* test. Significance was considered as probability values of $P<0.05$ indicated by one asterisk, $P<0.01$ indicated by two asterisks, and $P<0.001$ indicated by three asterisks.

Supplementary References:

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