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Insulin regulates titin pre-mRNA splicing through the PI3K-Akt-mTOR kinase axis in a RBM20-dependent manner

Chaoqun Zhu[a], Zhiyong Yin[a,c], Bihua Tan[d], Wei Guo[a,b,*]

[a] Animal Science, University of Wyoming, Laramie, WY 82071, USA
[b] Center for Cardiovascular Research and Alternative Medicine, University of Wyoming, Laramie, WY 82071, USA
[c] Department of Cardiology, Xi Jing Hospital, Fourth Military Medical University, Xi'an 710032, China
[d] Department of Pediatrics, Pennsylvania State University College of Medicine, Hershey, PA 17033, USA

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ABSTRACT

Titin, a giant sarcomeric protein, is largely responsible for the diastolic properties of the heart. It has two major isoforms, N2B and N2BA due to pre-mRNA splicing regulated mainly by a splicing factor RNA binding motif 20 (RBM20). Mis-splicing of titin pre-mRNA in response to external stimuli may lead to altered ratio of N2B to N2BA, and thus, impaired cardiac contractile function. However, little is known about titin alternative splicing in response to external stimuli. Here, we reported the detailed mechanisms of titin alternative splicing in response to insulin. Insulin treatment in cultured neonatal rat cardiomyocytes (NRCMs) activated the PI3K-Akt-mTOR kinase axis, leading to increased N2B expression in the presence of RBM20, but not in NRCMs in the absence of RBM20. By inhibiting this kinase axis with inhibitors, decreased N2B isoform was observed in NRCMs and also in diabetic rat model treated with streptozotocin, but not in NRCMs and diabetic rats in the absence of RBM20. In addition to the alteration of titin isoform ratios in response to insulin, we found that RBM20 expression was increased in NRCMs with insulin treatment, suggesting that RBM20 levels were also regulated by insulin-induced kinase axis. Further, knockdown of p70S6K1 with siRNA reduced both RBM20 and N2B levels, while knockdown of 4E-BP1 elevated expression levels of RBM20 and N2B. These findings reveal a major signal transduction pathway for insulin-induced titin alternative splicing, and place RBM20 in a central position in the pathway, which is consistent with the reputed role of RBM20 in titin alternative splicing. Findings from this study shed light on gene therapeutic strategies at the molecular level by correction of pre-mRNA mis-splicing.

1. Introduction

Titin is the largest known myofibril protein that is emerging as a promising target to regulate heart ventricular wall stiffness due to its elastic properties that are responsible for diastolic function during left ventricular filling [1–6]. These functions are partially dictated by the expression pattern of titin. Titin is encoded by a single gene *TTN* with 363 exons and exists as an array of variants resulting from alternative splicing. Two major classes of isoforms from this array of variants are known as N2B and N2BA. The size of these isoforms range from approximately 3.0 to 3.7 MDa resulting from alternatively used exons [7–9]. N2B isoform is produced from a single splicing pathway with a size of approximately 3.0 MDa [8,9]. N2BA isoforms are produced from multiple splicing pathways, and detectable N2BA isoforms are N2BA-A1 (adult form), A2 (adult form), N1 (embryonic and neonatal form) and N2 (embryonic and neonatal form) with sizes of about 3.4, 3.2, 3.7 and 3.6 MDa respectively [8,9]. Based on size differences, elastic properties of these two major classes of titin isoforms are different. The larger N2BA isoform is more compliant and develops lower passive tension, while the smaller N2B isoform is stiffer and develops higher passive tension [7,10]. Therefore, expression ratio of N2B to N2BA in the heart is a crucial determinant of titin-based passive tension. In healthy human left ventricle, expression ratio of N2BA to N2B isoforms is about 30:70 [11]. However, abnormal ratios of titin isoforms resulting from titin mis-splicing have been found in heart failure. An increased ratio of N2BA to N2B has been found in systolic heart failure [11–13] and a decreased ratio in diastolic heart failure [14–17]. Hence, it is critical to decipher the molecular mechanism(s) of titin splicing whereby titin regulates ventricular wall stiffness for treatment of heart failure by manipulation of abnormal titin isoform ratios resulting from titin pre-mRNA mis-splicing.

RNA binding motif 20 (RBM20), a muscle specific splicing factor, has recently been identified as a major regulator of titin splicing in striated muscles (cardiac and skeletal muscle) [18]. RBM20 is a

* Corresponding author at: Animal Science, University of Wyoming, Laramie, WY 82071, USA.
E-mail address: wguo3@uwyo.edu (W. Guo).

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member of SR proteins that assemble in spliceosome and mediate splicing of pre-mRNA. RBM20 knockout mice and rats expresses the only largest titin isoform named as N2BA-G with a size of approximately 3.9 MDa [7–9,25]. Mutations with loss-of-function found in *RBM20* lead to larger N2BA isoform expression [18] and the patients with *RBM20* mutations have been found associated with idiopathic dilated cardiomyopathy [19–22]. In adult cardiac muscle, titin smaller isoform N2B is predominantly expressed when RBM20 is present. However, when RBM20 is absent, only the larger N2BA isoform is expressed [18]. These results suggest that RBM20 is a master regulator of titin slicing in cardiac muscle in a dosage dependent manner. Before RBM20 has been cloned and identified as a splicing factor in titin splicing, studies have shown that titin splicing can be changed in response to external stimuli such as insulin or thyroid hormone [23,24]. However, these studies didn't depict the detailed mechanisms of how external stimuli can regulate titin splicing. This study was designed to examine the role of RBM20 in insulin-induced titin splicing, and how RBM20 connects external stimuli to titin pre-mRNA splicing in the nucleus.

2. Material and methods

2.1. Experimental animals

This study was performed with wild type (*Rbm20*$^{+/+}$, WT), heterozygous (*Rbm20*$^{+/-}$, HT) and homozygous (*Rbm20*$^{-/-}$, HM) rats. The *Rbm20*-deficient (HT and HM) rats were derived from a spontaneous mutant [18,25]. Rats used in the current work were crosses of Sprague-Dawley (SD) × 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.

2.2. Primary cultures of neonatal rat cardiomyocytes (NRCMs)

Primary cultures of NRCMs were prepared from one-day old neonatal rats from three genotypes: WT, HT and HM, using the neonatal cardiomyocyte isolation system (Worthington) as described previously [18]. Cells were re-suspended in complete medium (M199/DMEM-high glucose (in a ratio of 1:4) medium supplemented with 10% horse serum, 5% foetal bovine serum and 1% penicillin/streptomycin), plated at a density of 1×10^5 cells per cm^2, and maintained in 5% CO_2 incubator at 37 °C. Cells were cultured for 24 h in complete medium, and then switched to serum-starved medium. The serum-starved medium was supplemented with one or two of the following components for three or five days: insulin (175 nmol/L), LY294002 (LY) (10 μmol/L) as described in [23], rapamycin (50 nmol/L and/or 100 nmol/L), and KU0063794 (100 and/or 300 nmol/L).

2.3. Diabetic rat model with streptozotocin (STZ) treatment and sample preparation

WT, HT and HM rats were used for a single injection of STZ at the dose of 50 mg/kg. Each group contained 6 three-month old rats. Glucose tests were carried out 24 h after injections. Blood glucose for normal rats ranges from 60 to 110 mg/dL; if blood glucose is higher than 126 mg/dL, diabetes may be present [26]. The higher than normal blood glucose in STZ injected rats indicates type-1 diabetes was successfully induced by STZ (Fig. 2A and B). One-month after injection, the animals were anesthetized with isoflurane and killed by cervical dislocation. The heart tissues were collected immediately and snap frozen in liquid nitrogen, and then stored at − 80 °C for future analysis.

2.4. Hyperthyroidism and hypothyroidism rat models with thyroid hormone (T3, triiodothyronine) and propythiouracil (PTU) treatment respectively and sample preparation

The detailed procedure was described in our previous study [27].

2.5. Protein preparation and sodium dodecyl sulphate (SDS)-agarose gel electrophoresis

Titin isoforms were resolved using a vertical SDS-1% agarose gel electrophoresis (VAGE) system [28]. Rat heart tissues and NRCM cultures were homogenized in urea-thiourea buffer (8 M urea, 2 M thiourea 75 mM DTT, 3% SDS, 0.05% bromophenol blue, 0.05 M Tris, pH 6.8) as described previously [28]. Protein bands were visualized by silver staining, scanned and analysed densitometrically with NIH ImageJ software. Average titin isoform ratios were calculated from a minimum of three replicates per experimental treatment.

2.6. Western blot analysis

Total protein was separated by SDS-PAGE gel, and transferred onto a PVDF membrane. The membrane was probed with antibodies against RBM20 (home-made) which was validated in our previous studies [9,18,27,43], Akt (Cell Signaling), phospho(p)-Akt(Ser473) (Cell Signaling), p70S6K1(Cell Signaling), phosphor(p)-p70S6K1 (Thr389) (Cell Signaling), and 4E-BP1 (Cell Signaling). Rabbit anti-mouse IgG-conjugated with horseradish peroxidase (Fisher Scientific) was served as the secondary antibody. The blot was developed with ECL western blotting substrate (Bio-Rad) and exposed to CL-Xposure film (Thermo Scientific). Anti-Histone3 (Cell Signaling) and GAPDH (Santa Cruz) were served as the protein loading control.

2.7. p70S6K1 and 4E-BP1 siRNA treatment

siRNAs were purchased from Dharmacon (Accell SMARTpool). Primary cultures of NRCMs from WT, HT and HM were plated in growth medium 24 h prior to transfection. At the density of 65–75% confluency, primary cultures of NRCMs were transfected with siRNA against rat p70S6K1, 4E-BP1, or non-targeting control siRNA pools at a concentration of 1 μM in serum free medium following the manufacturer's instructions. After 72-hour transfection, siRNA medium was replaced with culture medium, cells were incubated for another 24 h, then cells were subjected to protein sample preparation for immunoblot analysis and gel electrophoresis.

2.8. Statistics

GraphPad prism software was used for statistical analysis. Results were expressed as means ± SEM. Statistical significance between two groups was determined using an unpaired Student's *t*-test or Mann-Whitney test. Kruskal-Wallis test was used for comparing means between more than two groups. 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.

3. Results

3.1. Insulin-regulated titin splicing is RBM20-dependent in cultured neonatal rat cardiomyocytes (NRCMs)

Insulin has been reported to regulate titin splicing by which elevated insulin levels promote N2B isoform expression [23]. Since RBM20 has been found to be a master regulator for titin splicing [18], insulin-triggered titin splicing could be linked to RBM20. To test this hypothesis, we isolated and cultured NRCMs from one-day old WT, HT and HM rats in serum-starved medium supplemented with 175 nmol/L

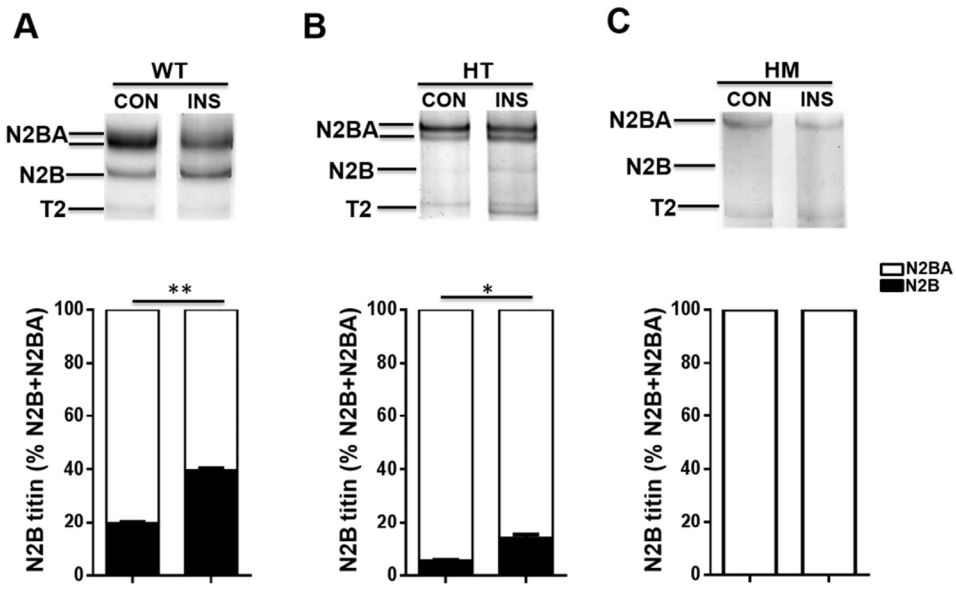

Fig. 1. Effect of insulin on titin splicing in WT, HT and HM NRCMs. (A) and (B) Titin isoform separation and quantitative analysis in WT and HT NRCMs treated with insulin. (C) Titin isoform separation and quantitative analysis in HM NRCMs treated with insulin. CON, control; INS, insulin; T2, degraded titin bands; bars show means ± SEM ($n = 3$); $^{*}P < 0.05$, $^{**}P < 0.01$.

insulin. After 5-day treatment, NRCMs were harvested and protein samples were prepared from WT, HT and HM NRCMs. Titin has two major classes of isoforms N2B and N2BA from alternative splicing [29], so two isoforms N2B and N2BA were separated by 1% SDS agarose gel electrophoresis. N2B and N2BA gel band densitometry was quantified using NIH ImageJ. The ratios of N2B to N2BA isoforms were increased significantly by insulin addition in HT and WT NRCMs when compared to control respectively. The N2B-percentage was increased by approximately 18.7% ± 0.91 in WT (Fig. 1A), and 9.7% ± 1.25 in HT (Fig. 1B) respectively. Interestingly, no N2B isoform expression was observed with insulin treatment by comparing to control in HM NRCMs (Fig. 1C). These results suggest that insulin-induced titin splicing requires RBM20 and is RBM20-dependent.

3.2. RBM20 plays an indispensable role in titin splicing in streptozotocin (STZ)-induced diabetic rats

To further confirm this observation, we made an STZ-induced diabetic rat model with HM, HT and WT rats. On the second day after STZ injection, non-fasting glucose test indicated that the glucose level in rats receiving STZ is significantly higher than that in rats only receiving vehicle (Fig. 2A and B), showing impaired glucose uptake and type I diabetes. Heart tissues from STZ-induced diabetic rats were used for titin isoform separation and quantitative analysis with 1% SDS agarose gel electrophoresis and ImageJ. The results revealed that N2B titin isoform percentage was significantly decreased from approximately 92.88% ± 0.84 to 81.9% ± 0.67 in WT and almost undetectable in HT rats (Fig. 2C and D). However, no N2B titin has been detected and no N2BA isoform was increased in STZ treated HM rats when compared to control HM rats (Fig. 2E). These results indicate that titin splicing occurs in diabetic rats with reduced insulin level when RBM20 is present, and further confirm that RBM20 is an essential factor for insulin-induced titin splicing.

3.3. PI3K-Akt-mTOR kinase axis links insulin to RBM20 in titin splicing

Insulin initially stimulates the PI3K/Akt signalling pathway. To examine whether the PI3K signalling connects insulin to RBM20 in the regulation of titin splicing, we treated the HM, HT and WT NRCMs with PI3K inhibitor LY294002. 10 μmol/L of LY294002 was used to treat NRCMs grown in serum-free medium. Cells were harvested after 3-day treatment and utilized to prepare the protein samples for titin isoform and western blot analysis. N2B isoform percentage is significantly reduced approximately 5.9% ± 0.91 in WT (Fig. 3A and B). In HT NRCMs, however, the N2B isoform percentage among control and treatment groups were too low to be detected (Fig. 3B). Increased N2B isoform by elevated insulin level was dampened by LY294002, and was restored to basal level in the control group (Fig. 3A and B). This alteration was not observed in HM NRCMs with/without LY294002 treatment (Fig. 3C). Since PI3K is the major mode of Akt activation [30], we also tested Akt phosphorylation with western blot, and observed increased Akt phosphorylation with insulin treatment and diminished Akt phosphorylation with LY294002 treatment in NRCMs (Fig. 3D, E and F). These results suggest that RBM20 is the downstream substrate of PI3K/Akt signalling pathway activated by insulin.

Furthermore, it is still unknown how the PI3K/Akt signalling triggers RBM20 to regulate titin splicing. It has been well studied that the PI3K/Akt pathway activates the mTOR signalling that promotes gene expression and protein synthesis [31], so we postulate that insulin links RBM20 in titin splicing via the PI3K/Akt/mTOR signalling pathway. To test this possibility, we blocked mTOR activity by adding the pharmacological drug inhibitor, rapamycin and a small molecule inhibitor, KU0063794 to insulin treated or untreated NRCMs. Rapamycin inhibits mTOR complex1 (mTORC1) that is a master growth regulator and promotes protein synthesis [32], while KU0063794 inhibits mTOR complex 2 (mTORC2) that can activate Akt for cellular survival as well as mTORC1 [33]. These two inhibitors will partially or fully reduce mTOR activity. Treatment of both inhibitors for 3 days diminished insulin-induced N2B expression in WT and HT NRCMs (Fig. 4A and B), and significantly reduced N2B basal level in WT and HT NRCMs without insulin treatment (Fig. 4D and E). As was expected, N2B isoform was not changed at all with whichever treatment in HM NRCMs in the absence of RBM20 (Fig. 4C and F). Further, p70S6K1 or S6 kinase 1 is the downstream substrate of mTOR that can promote protein synthesis and cell growth, so we also examined the activity of p70S6K1 through phosphorylation at Thr389 by mTORC1. The phosphorylation level of p70S6K1 at Thr389 was remarkably elevated by insulin in primary cultures of WT, HT and HM NRCMs (Fig. 4G, H and I), while p70S6K1 phosphorylation was diminished in NRCMs with mTOR inhibitors, rapamycin and KU0063794 supplements (Fig. 4G, H, I, J, K and L). From these data, we confirmed that insulin triggers the PI3K-Akt-mTOR kinase axis for titin splicing, and RBM20 is an essential protein in downstream of the mTOR signalling pathway to link insulin to titin splicing.

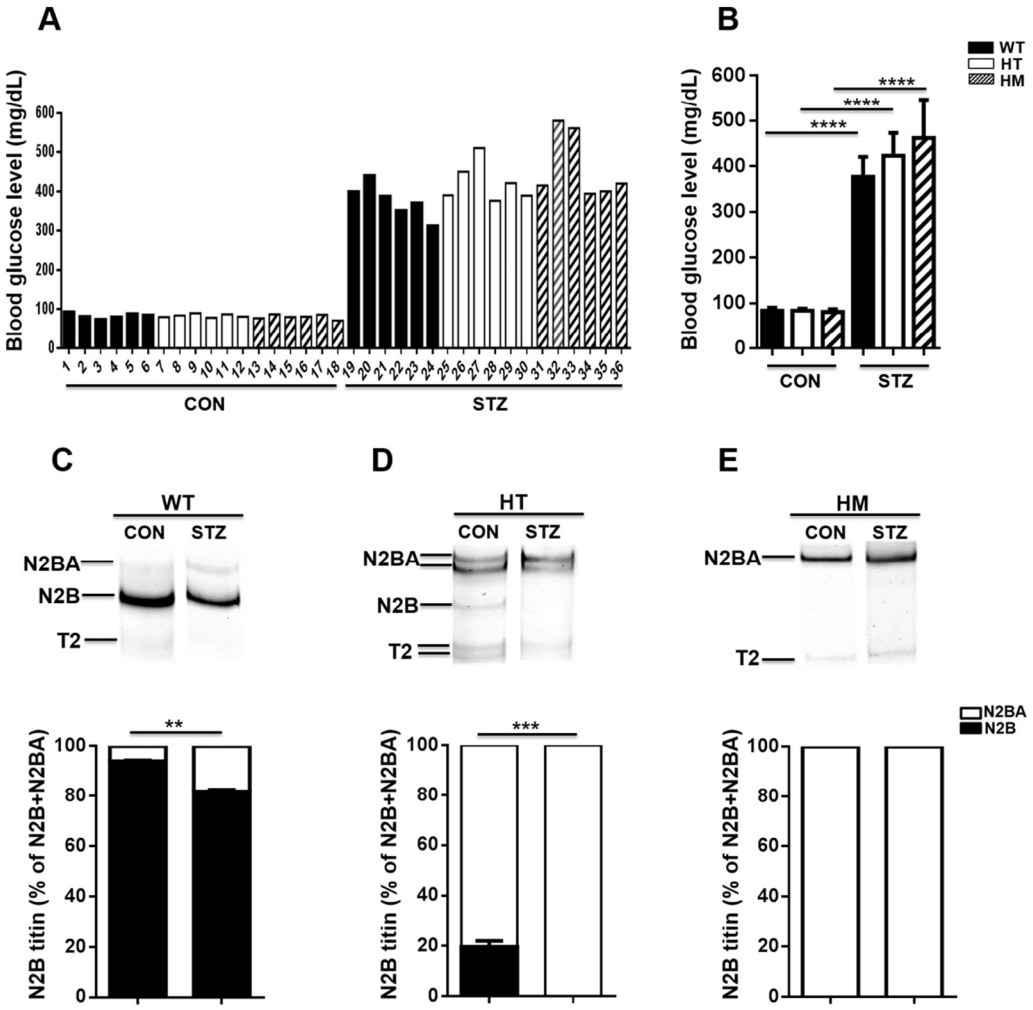

Fig. 2. Role of RBM20 in titin splicing in STZ-induced diabetic rats. (A) Glucose levels of individual rats treated with STZ in three groups of WT, HT and HM. (B) Averaged glucose levels between three groups. (C)–(E) Titin isoform separation and composition analysis in diabetic rat heart tissues; T2, degraded titin bands; STZ, streptozotocin; CON, control; bars show means ± SEM ($n = 6$); $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

3.4. Titin splicing through insulin-induced PI3K-Akt-mTOR kinase axis is due to increased RBM20 expression

Although our results indicate that RBM20 is essential for insulin-induced titin splicing via the PI3K-Akt-mTOR kinase axis, the detailed mechanism of how RBM20 links insulin to titin splicing is still unclear. It is well studied that the PI3K/Akt/mTOR signalling pathway is a master growth regulator that promotes protein synthesis [31], so we presumed that the PI3K/Akt/mTOR pathway governs the expression of RBM20 and thus altered RBM20 expression level regulates titin splicing. We next tested the expression level of RBM20 in WT and HT NRCMs with insulin and inhibitors treatment. Total RBM20 protein levels were significantly elevated with insulin supplementation (Fig. 5A, B, C and D), but dramatically reduced by the PI3K or mTOR inhibitors (LY294002, rapamycin and KU0063794) when compared to control (Fig. 5A, B, C, D, E and F). Decreased RBM20 protein level controlled by the insulin signalling was further confirmed with STZ-induced diabetic rats that have reduced insulin level (Fig. 5G and H). These results demonstrated that insulin-induced titin splicing was mediated by elevated RBM20 level via the PI3K/Akt/mTOR signalling pathway.

Additionally, our previous data have shown that thyroid hormone (T3) can also regulate titin splicing via the PI3K/Akt pathway [27]. Using the rats treated with T3 and propylthiouracil (PTU, a drug reducing thyroid hormone level in vivo), we observed elevated RBM20 level via the activated PI3K/Akt pathway by T3, and reduced RBM20 level through the down-regulated PI3K/Akt pathway by PTU (Fig. 5G and H). Altogether, these data firmly confirm that titin splicing can be regulated by the PI3K-Akt-mTOR kinase axis triggered by either insulin or thyroid hormone, but controlled by RBM20 protein levels through this pathway.

3.5. RBM20 expression is controlled by two downstream antagonistic proteins of mTOR kinase

Two mTOR downstream substrates, p70S6K1 and 4E-BP1 are antagonistic proteins that regulate protein translation and synthesis. Activated mTOR phosphorylates p70S6K1 that promotes gene expression, while activated mTOR inhibits 4E-BP1 through phosphorylation that up-regulates gene expression. To determine whether p70S6K1 and 4E-BP1 govern RBM20 expression, and in turn titin splicing, we knocked down p70S6K1 and 4E-BP1 in WT, HT and HM NRCMs with siRNA transfection. The results demonstrated that these siRNAs efficiently reduced p70S6K1 and 4E-BP1 levels respectively (Fig. 6A, B, and C). RBM20 level was significantly reduced in WT and HT NRCMs with down-regulated p70S6K1 by siRNA (Fig. 6D and E), while RBM20 level was remarkably elevated with down-regulated 4E-BP1 by siRNA (Fig. 6D and E). As expected, titin N2B level was increased with elevated RBM20 expression in 4E-BP1 down-regulated NRCMs, while it was decreased with reduced RBM20 expression in p70S6K1 down-regulated NRCMs (Fig. 6F and G). No changes of titin isoform ratios were observed in both down-regulated p70S6K1 and 4E-BP1 HM NRCMs (Fig. 6H). These data clearly indicate that two antagonistic proteins p70S6K1 and 4E-BP1 govern RBM20 expression through the PI3K-Akt-mTOR kinase axis, and thus, regulate titin splicing.

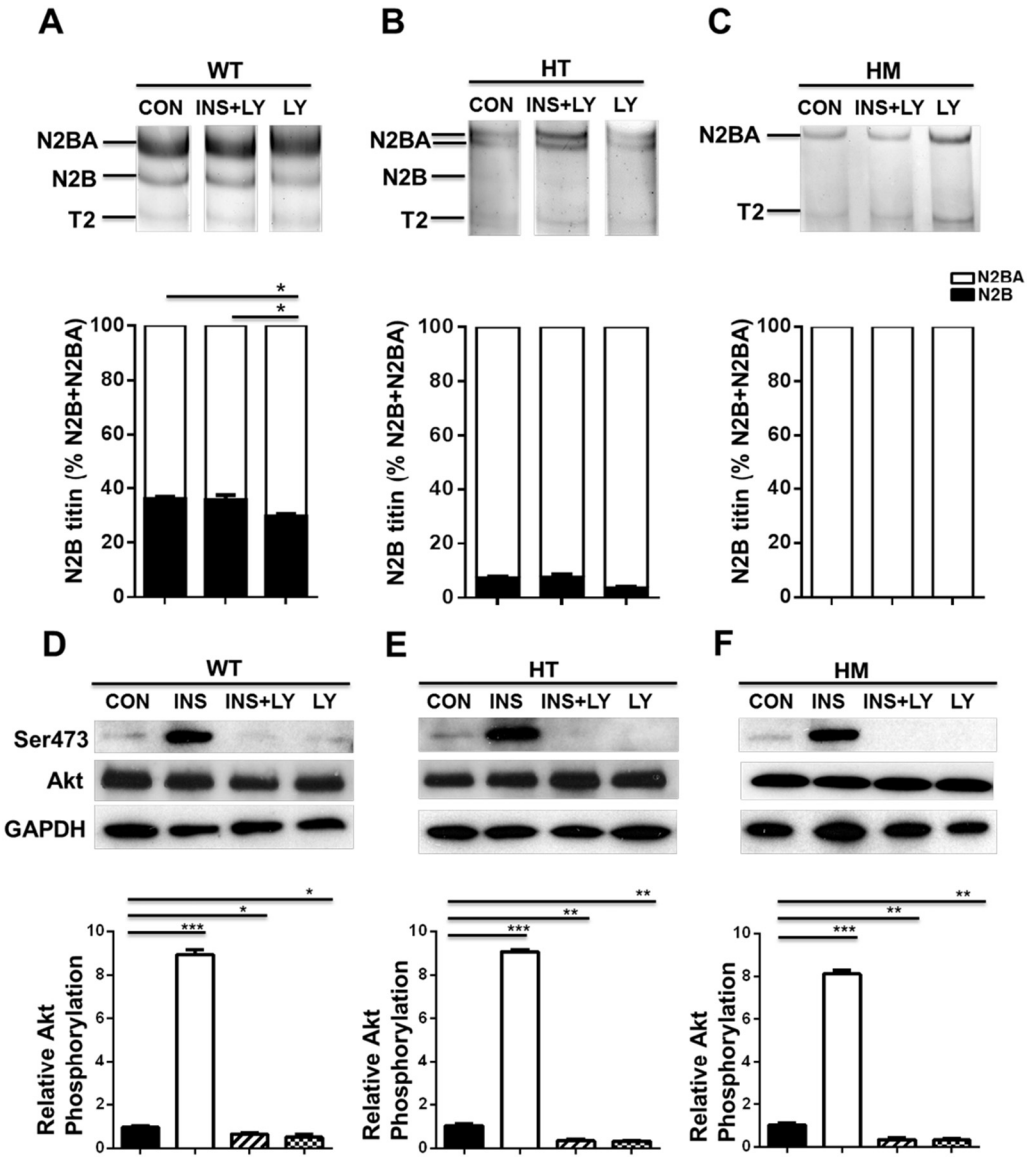

Fig. 3. Titin splicing in WT, HT and HM NRCMs with PI3K inhibitor treatment. (A)–(C) Titin isoform separation and composition analysis for insulin and inhibitor treated NRCMs. (D)–(F) Western blot analysis on Akt phosphorylation with insulin and inhibitor treatment; GAPDH was used as loading control and anti-Akt represents total Akt protein; CON, control; INS, insulin; LY, LY294002; T2, degraded titin bands; bars show means ± SEM ($n = 3$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

4. Discussion

Pre-mRNA splicing is a nearly ubiquitous and prevalent process that allows production of multiple proteins from a single gene in mammalian genome [34,35]. It requires a very large ribonucleoprotein (RNP) complex known as the spliceosome comprised of > 100 associated proteins [36,37]. To unambiguously recognize splice sites, this core splicing complex needs assistance from *cis*-regulatory sequences and *trans*-regulatory factors including Ser/Arg-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs) [38–40]. Mis-regulation of this process may lead to human disease. Organisms regulate alternative splicing in response to an external stimulus commonly by changing the concentration and activity of splicing regulatory proteins which can be achieved by protein synthesis, intracellular localization and phosphorylation. In the last decade, increasing studies have indicated that cell signalling pathways are responsible for such splicing changes, though the studies of the mechanisms by which signalling pathways result in inducible changes in splicing, have lagged far behind [41]. Our salient findings reveal that pre-mRNA splicing of titin gene and the newly identified regulatory splicing protein RBM20 are changed in response to hormone stimuli such as insulin and thyroid hormone in primary cultured cardiomyocytes and heart tissues from animal models, and the splicing factor RBM20 plays an essential role in titin splicing process.

Insulin and thyroid hormone increase synthesis and block the degradation of proteins through activation of the mTOR signalling pathway. mTOR is the major downstream effector of the PI3K/Akt signalling pathway [42]. Whether the regulatory splicing protein RBM20 expression in response to hormone stimuli is through the activation of the PI3K-Akt-mTOR kinase axis remains unknown. The data presented here reveal that both insulin and thyroid treatments activate the PI3K and in turn Akt and mTOR. The activation of mTOR increases RBM20 expression, while inhibition of either PI3K or mTOR kinase significantly reduces the protein level of splicing factor RBM20. These results strongly support that insulin or thyroid hormone stimuli trigger RBM20 expression through activation of PI3K, Akt and thus mTOR. Previous studies have reported that splicing factor RBM20 is a repressor of titin splicing, wherein increased RBM20 promotes exon skipping of titin gene, and reduced RBM20 level suppresses exon skipping [18,43]. Therefore, altered RBM20 level is a key for insulin or thyroid hormone-induced titin splicing. In addition, activation of mTOR results in phosphorylation of p70S6K1 and translational repressor 4E-BP1, wherein phosphorylation of these two proteins promotes protein synthesis [44]. With mTOR inhibition and down-regulation of p70S6K and 4E-BP1, our data further depicted an essential role of RBM20 expression through activation of downstream effectors of mTOR

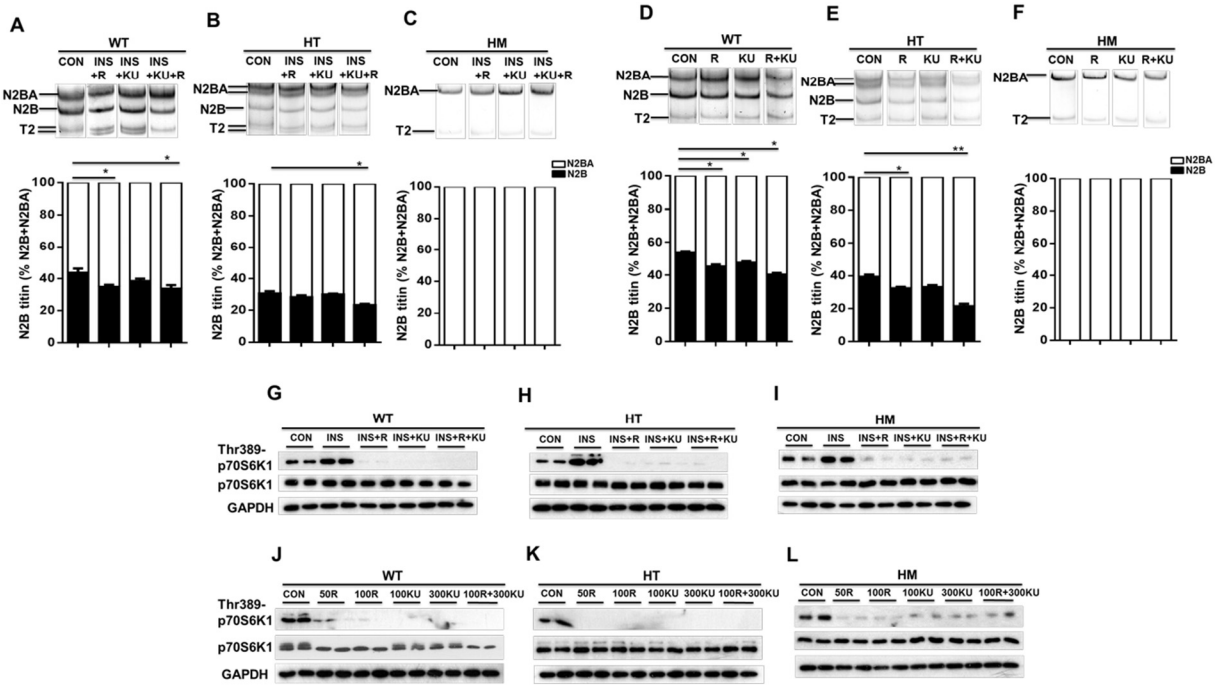

Fig. 4. Titin splicing in WT, HT and HM NRCMs with mTOR inhibitor treatment. (A)–(C) Titin isoform separation and composition analysis with insulin and mTOR inhibitors rapamycin and KU0063794 treatment. (D)–(F) Titin isoform separation and composition analysis with mTOR inhibitor treatment only. (G)–(L) Western blot analysis on p70S6K1 phosphorylation at position Thr389 with insulin and mTOR inhibitor treatment; GAPDH was used as loading control; anti-p70S6K1 represents total protein; CON, control; INS, insulin; R, rapamycin; KU, KU0063794; T2, degraded titin bands; bars show means ± SEM ($n = 3$); $^{*}P < 0.05$, $^{**}P < 0.01$.

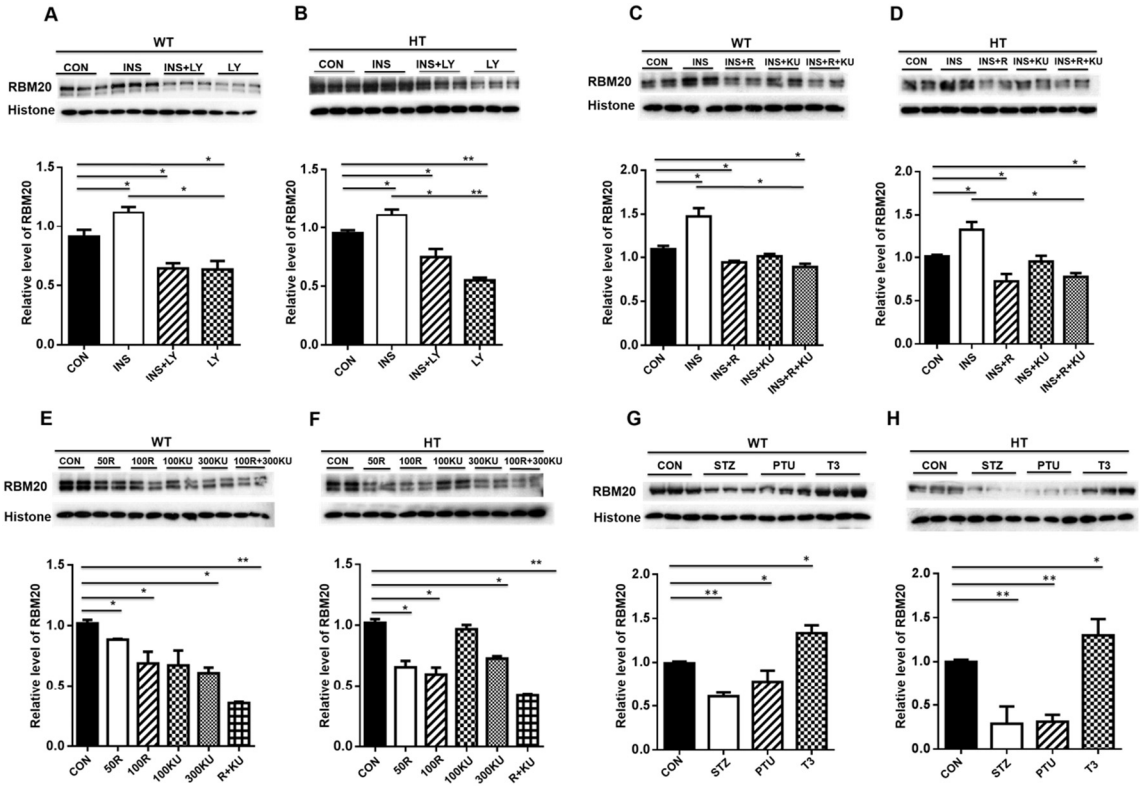

Fig. 5. RBM20 expression levels in WT and HT NRCMs. (A)–(F) Relative RBM20 expression levels by western blot in WT and HT NRCMs treated with insulin, PI3K- and mTOR-inhibitors. (G) and (H) Relative RBM20 expression levels in the heart of WT and HT rats treated with STZ, thyroid hormone (T3) and PTU. Protein load amount was normalized to nuclear protein histone. CON, control; INS, insulin; R, rapamycin; KU, KU0063794; STZ, streptozocin; PTU, propylthiouracil; T3, triiodothyronine; bars show means ± SEM ($n = 3$); $^{*}P < 0.05$, $^{**}P < 0.01$.

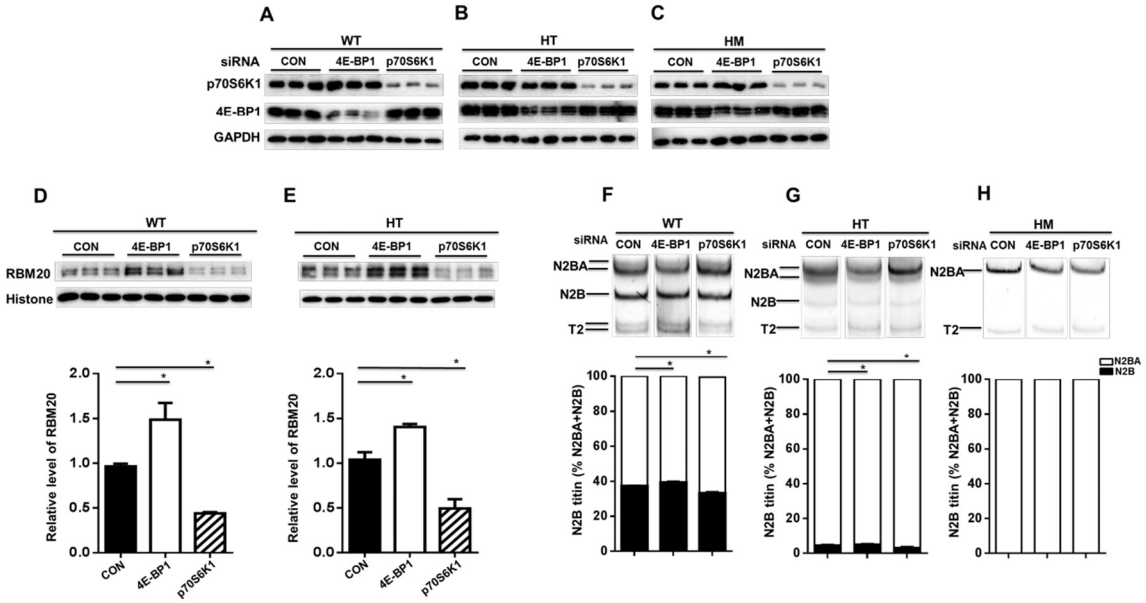

Fig. 6. Effect of mTOR downstream target p70S6K1 and 4E-BP1 on RBM20 expression and titin splicing. (A)–(C) 4E-BP1 and p70S6K1 expression with siRNA treatment in WT, HT and HM NRCMs; protein load amount was normalized to GAPDH. (D) and (E) Relative RBM20 expression analysis in WT and HT NRCMs with siRNA transfection; protein load amount was normalized to nuclear protein histone. (F)–(H) Titin isoform separation and quantitative analysis with protein samples from siRNA treated WT, HT and HM NRCMs; CON, control with non-targeting siRNA; T2, degraded titin band; bars show means ± SEM ($n = 3$); $^{*}P < 0.05$, $^{**}P < 0.01$.

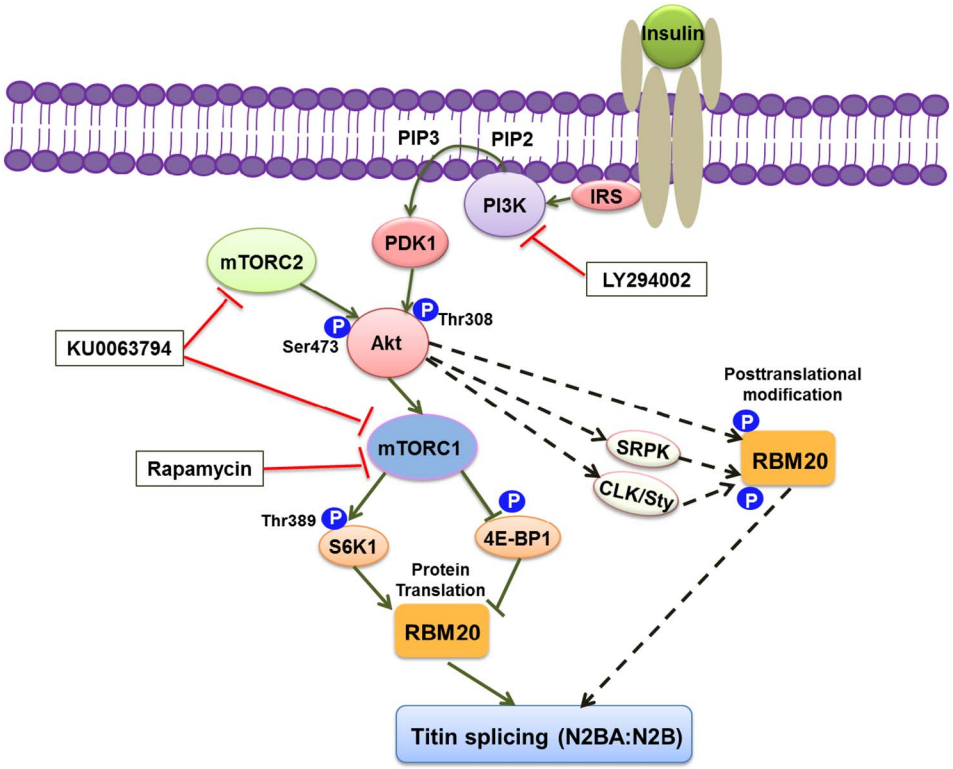

Fig. 7. Schematic diagram depicting the signalling pathways linking insulin-RBM20-titin splicing. Insulin activates the PI3K, and recruits Akt to membrane for phosphorylation and activation by PDK1 and mTORC2; activated Akt then activates mTORC1, and in turn phosphorylates S6K1 and 4E-BP1 that promote RBM20 expression and thus enhance titin exon skipping; PI3K inhibitor LY294002 and mTOR inhibitors rapamycin and KU0063794 reduce RBM20 expression by inhibiting the PI3K-Akt-mTOR kinase axis, and thus represses titin exon skipping; in addition, Akt can directly act on SR proteins or indirectly act on SR proteins via kinase SRPK or CLK/Sty. Since RBM20 is an SR protein, we assume that posttranslational modifications of RBM20 could be another pathway to regulate titin splicing via Akt kinase or the Akt-SRPK/CLK/Sty-SR axis. The dotted lines represent a potential mechanism for titin splicing via RBM20, but need be explored in future studies.

signalling in the regulation of titin splicing (depicted in Fig. 7).

On the other hand, signalling molecules can directly interact with and influence many other components in the splicing machinery. The regulation of splicing is often achieved by the action of SR and hnRNP proteins. Posttranslational modifications of these splicing factors are the major mechanism for altering splicing pathways [45]. The activity of SR proteins is strongly influenced by the phosphorylation state of these proteins [46–49]. Presently, at least four different kinase families have been found to phosphorylate SR proteins. Akt is one of these kinases and modulates the function of the SR proteins that act on exonic splicing *cis*-regulatory elements [50–52]. Activated Akt has been indicated to directly act on SR proteins [52,53] or indirectly relay its signal to the nucleus through SR protein-specific kinases such as SRPK2 [54] or CLK/Sty [53]. Previous work has also placed SR proteins in the growth factors or hormone-induced splicing pathway [53]. Splicing factor RBM20 is an SR protein containing Ser/Arg rich domain. Therefore, in addition to changes of RBM20 expression level through the PI3K-Akt-mTOR kinase axis, whether Akt can directly act on SR protein RBM20 and phosphorylate RBM20 and thus regulate titin splicing remains unclear (Fig. 7). Currently, the phosphorylation state

of RBM20 is completely unknown. Nevertheless, the western blot against anti-RBM20 antibody always shows two bands (Fig. 5). Whether the upper band is due to phosphorylation of RBM20 needs to be further studied. If phosphorylation is the case, future study will be warranted to validate whether the phosphorylation of RBM20 plays a critical role in titin splicing.

Additionally, using the STZ rat model of Type 1 diabetes mellitus, we tested the effects of altered insulin level on titin isoform transition during development of diabetic cardiomyopathy. Studies have found that both Type 1 (STZ) and Type 2 rat models revealed systolic dysfunction [55]. Systolic dysfunction is a later manifestation, usually happening after diastolic dysfunction develops [56]. The systolic dysfunction is characterized with increased myocardial compliance. In our STZ Type 1 diabetic rat model, the expression of N2B titin isoform was remarkably decreased, which could be one of the mechanisms that lead to myocardial wall compliance in systolic dysfunction. Increased left ventricle mass and wall thickness usually occurs in Type 2 diabetes, and may contribute to reduced myocardial compliance and therefore diastolic dysfunction [57]. One of the molecular mechanisms of the diabetic cardiomyopathy has been related to the abnormal insulin signalling in Type 2 diabetes. An increase in IRS1-associated PI3K activity was reported in cardiac biopsies obtained from Type 2 diabetic patients [58]. In our present study, we found that insulin triggered PI3K-AKT-mTOR-RBM20 was a direct signalling pathway that increases titin N2B isoform expression, which may contribute to left ventricular stiffness in Type 2 diabetic cardiomyopathy. Overall, in diabetic patients, the ratio of titin isoform (N2B:N2BA) would be altered under both insulin stimulation in Type 2 diabetes and insulin depletion in Type 1 diabetes, and be a non-negligible factor that contributes to diabetic cardiomyopathy.

In summary, we report for the first time that insulin-induced titin splicing requires splicing factor RBM20 and is RBM20-dependent. Our findings also reveal that insulin activates the PI3K-Akt-mTOR kinase axis. Elevated RBM20 protein level promotes exon skipping of titin gene that generates a smaller titin protein isoform N2B. Our data clearly depict the mechanism by which RBM20 is a key regulator linking insulin to titin splicing via the PI3K-Akt-mTOR kinase axis (Fig. 7). Titin is an important elastic protein in cardiac muscle that contributes to ventricular wall stiffness. Its mis-splicing has been found associated with heart failure. Therefore, our data provide a potential novel therapeutic target contributing to the development of therapies against heart disease caused by mis-splicing.

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Conflict of interests

None.

Transparency Document

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