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Global profiling of Rbm24 bound RNAs uncovers a multi-tasking RNA binding protein

Yu Lin[a,1], Kar Tong Tan[b,1], Jing Liu[a], Xu Kong[a], Zhengrong Huang[d,**], Xiu Qin Xu[a,c,*]

[a] The Institute of Stem Cell and Regenerative Medicine, Medical College, Xiamen University, 361000, PR China
[b] Cancer Science Institute of Singapore, National University of Singapore, Singapore
[c] ShenZhen Research Institute of Xiamen University, PR China
[d] Department of Cardiology, The First Affiliated Hospital of Xiamen University, Fujian Province, 361000, PR China

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ABSTRACT

RNA binding proteins serve as critical molecular switches in a multitude of post-transcriptional regulatory processes. In the heart and muscles, the tissue specific RNA binding protein, Rbm24, is known to play important developmental roles via driving different post-transcriptional processes. Nonetheless, the currently identified molecular targets and regulatory pathways seem inadequate to completely explain the observed developmental effects upon Rbm24 knockdown/knockout. Here, by performing RNA Immunoprecipitation and coupling it to microarrays (RIP-Chip), we have generated an atlas of the mRNA binding repertoire of Rbm24. Further functional evaluation of its targets led to the elucidation of novel roles for Rbm24 in post-transcriptional processing, besides its already known roles in regulation of mRNA stability and alternative splicing. Interestingly, Rbm24 is found to cause the destabilization of Chrm2 via binding to an element in the coding region. In addition, Rbm24 is also found to have an uncharacterized role in driving the generation of isoforms with alternative transcriptional start sites. We have, for the first time, demonstrated that Rbm24 is a multi-tasking RNA binding protein capable of regulating its bound targets via a range of mechanisms.

1. Introduction

Post-transcriptional processes are critically important in orchestrating the proper spatial and temporal expression of different mRNA transcripts in the developing heart. Following transcription, an mRNA molecule embarks on a remarkably complex voyage towards the hub of translation where it serves as a template of instruction for protein synthesis. Along the way, the RNA molecule is subjected to a whole array of regulatory processes which are critical determinants of whether it is eventually translated into a protein. Indeed, post-transcriptional regulatory processes serve multi-layered and multi-functional roles in regulation of an mRNA transcript. Importantly, it should be noted that post-transcriptional regulatory processes function to a great degree via the action of RNA binding proteins (RBPs) (Gerstberger et al., 2014).

RBPs are proteins that bind directly to single or double stranded RNAs via direct interaction with RNA via their RNA binding domains (RBDs). Examples of RBDs include the RRM, KH domain and Zinc fingers domain which allow for the specific recognition and binding of different RNA sequences and structures. It should be noted that many RBPs are often expressed in a tissue specific manner to drive important tissue developmental processes. The developing heart is a particular case in point where there has been a growing appreciation of the role of cardiac-specific RBPs in heart development and heart diseases (Liao et al., 2016). Cardiac RBPs are probably one of the newest and most exciting classes of factors linked to cardiomyopathy which is an increasingly prevalent and deadly disease. One of the earliest RBPs associated with cardiomyopathy was Rbm20, where mutations within this gene were found to be associated with dilated cardiomyopathy in human patients (Brauch et al., 2009; Li et al., 2010a). A mechanistic understanding was reached when Rbm20 was found to be involved in defective splicing of Titin in a spontaneously occurring rat strain exhibiting symptoms of dilated cardiomyopathy (Guo et al., 2012). At approximately the same time, we also characterized a novel heart specific RBP, Rbm24, and noted the strong resemblance between our *rbm24* knockdown zebra-fish model and dilated cardiomyopathy (Poon

* Corresponding author at: he Institute of Stem Cell and Regenerative Medicine, Medical College, Xiamen University, Chengzhi Building, Xiang'an Campus, Xiamen, Fujian Province, 361100, PR China.
** Corresponding author at: Department of Cardiology, the First Affiliated Hospital of Xiamen University, 361000, P.R.China.
E-mail addresses: huangzhengrong@xmu.edu.cn (Z. Huang), xuxq@xmu.edu.cn (X.Q. Xu).
[1] These authors contributed equally.

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et al., 2012).

Rbm24 was identified in a previous genome-wide expression profiling of cardiomyocytes from a human embryonic stem cells (ESC) cardiac differentiation model we developed, Rbm24 was found to be strongly upregulated during cardiac differentiation and specifically expressed in the mouse heart (Xu et al., 2009). The *rbm24* gene was subsequently functionally interrogated in a zebra-fish *rbm24* model where we found that *rbm24* was essential for sarcomere assembly and cardiac contractility (Poon et al., 2012). Molecularly, Rbm24 was shown to enhance mRNA stability of the p21 transcript by binding to its 3′-untranslated region (UTR). Conversely, Rbm24 was also shown to destabilize the p63 mRNA via binding to its 3′UTR (Xu et al., 2014). Seb4/Rbm24 is a downstream target of MyoD, one of the early skeletal muscle markers that expresses in precursors of muscle mesoderm, and is required for myogenesis during Xenopus early development (Li et al., 2010b). Also, Rbm24 was known to be a myogenesis regulator via regulation of the expression of myogenin (Grifone et al., 2014; Jin et al., 2010). A recent report also highlights the role of Rbm24 as a major regulator of alternative splicing in muscles during vertebrate development in an Rbm24 knockout mouse model (Yang et al., 2014). More recently, we found that Rbm24 functions as a post-transcriptional regulator that governed the cell-type specific splicing during ESC cardiac lineage differentiation (Zhang et al., 2016). However, despite all the mechanisms and functions that has been proposed for Rbm24, there lacks a global study of the RNAs that are directly bound by Rbm24 and the mechanism in which it regulates these mRNA transcripts. To systematically establish the mRNA targets that Rbm24 directly binds to and regulates, we performed RNA immunoprecipitation of Rbm24 and its bound RNAs in a mammalian heart cell line. Interestingly, we found that Rbm24 is capable of regulating its bound targets via a range of mechanisms, reinforcing the notion that Rbm24 is a multi-tasking RBP.

2. Materials and methods

2.1. HL-1 and H9C2 cell culture

HL-1 cells derived from the AT-1 murine cardiomyocytes were a gift from W. Claycomb (Louisiana State University Medical Center, New Orleans, La). HL-1 cells were maintained as described (Claycomb et al., 1998). Briefly, cells were cultured on gelatin (0.02%, w/v)/fibronectin (10 μg/ml)-coated plates. The cells were maintained in Claycomb medium (Sigma Aldrich, Saint Louis, Missouri, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, New York, USA), 2 mM L-glutamine (Gibco, Grand Island, New York, USA), 0.1 mM Norepinephrine (Gibco, Grand Island, New York, USA), 100 U/ml penicillin (Gibco, Grand Island, New York, USA), and 100 μg/ml streptomycin (Gibco, Grand Island, New York, USA). The culture medium was changed with fresh medium every 24 h. The cells were grown at 37 °C in an atmosphere of 5% CO2 in an incubator.

H9C2 cells (ATCC CRL1446, cardiac myoblasts from rat) were grown at a density of about 10^5 cells/cm^2 and cultured as monolayers in DMEM (Gibco, Grand Island, New York, USA) medium supplemented with 10% fetal bovine serum (FBS, Gemini, Foundation Bio-products, Argentina), penicillin (100 U/ml, Gibco, Grand Island, New York, USA), and streptomycin (100 μg/ml, Gibco, Grand Island, New York, USA) under an atmosphere of 5% CO_2 at 37 °C. The medium was replaced by fresh medium every 2 days.

2.2. 293FT cell culture and transfection

293FT cells were propagated in DMEM supplied with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO2. For transfection experiment, 293FT cells were seeded at 10^4 cells per well in a 12-well plate for 24 h, and then 100 ng of plasmid was transfected using PEI. 24 h after transfection, medium was changed and cells were grown for another 24 h before assay.

2.3. Generation of Rbm24 overexpression HL-1 and H9C2 cell line and Rbm24 knockdown HL-1 cell line

Given that Rbm24 is an RNA binding protein, the accessibility of the epitope to which the immunoprecipitation antibody is detected needs to be considered. Previous studies reported that the epitope-tagged RBPs were more efficient than the endogenous proteins for RBPs isolation, possibly due to the obstruction caused by binding RNAs and other components of RNPs (Keenes et al., 2006; Cassar et al., 2015; Zhou et al., 2015). Therefore, we generated exogenously expressing Rbm24 infused with affinity purified tag (Flag) transgenic lines to conduct RIP experiment as described below.

Human Rbm24 gene was cloned by PCR from plasmid containing full length Rbm24 cDNA (Cat.No SC325643, Origene, Rockville, Maryland, USA). To generate the Rbm24 overexpression HL-1 and H9C2 cell lines, the pCMV-Rbm24-Flag was transfected into HL-1 and H9C2 cells by Lipofectamine 2000 (ThermoFisher, Waltham, Massachusetts, USA) as per the manufacturer's instruction. Briefly, 5×10^5 cells were seeded on gelatin (0.02%, w/v) and fibronectin (10 μg/ml) coated 6-well plates. On the following day, 2 μg pCMV-Rbm24-Flag plasmids were mixed with 4 μl Lipofectamine 2000 in 100 μl Opti-MEM (ThermoFisher, Waltham, Massachusetts, USA) media. The mixture was then added to the cells. 3 days later, the transfected cells were selected under 300 μg/ml G418 for 10 days.

To generate the Rbm24 knockdown HL-1 (sh-Rbm24) cell line, a pool of 3 target-specific Rbm24 shRNA lentiviral vector plasmids was utilized (Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA, Cat.No sc-152735-SH). Each of these 3 different types of plasmids encoded shRNAs was designed to knock down Rbm24 gene expression. In parallel, a non-targeting shRNA plasmid was used to generate a control HL-1 cell line. Lentiviral vectors were produced as previously described (Ling and Arlinghaus, 2005). Briefly, 293FT cells were transiently transfected by a PEI method with a four-plasmid vector system consisting of VSVG, MDL, Rev, and Rbm24 shRNA. The supernatant was collected 2 and 3 days after transfection and ultracentrifuged at 140,000*g* for 1.5 h. The pellet was resuspended in DMEM/10% FBS and frozen at −80 °C. HL-1 cells were then infected with lentivirus expressing shRNA as follows: HL-1 cells were seeded at a density of 3×10^5 cells/well into a 6-well plate. On the following day, infections were performed in the presence of 8 μg/ml of polybrene. Two days later, the infected HL-1 cells were selected with puromycin (3 μg/ml) for 4 days.

2.4. RNA immunoprecipitation followed by microarray (RIP-Chip)

RNA-IP of Rbm24 was performed based on the protocol by Keenes et al. (Keenes et al., 2006). Briefly, HL-1Rbm24-Flag cells were homogenized using polysome lysis buffer supplemented with RNase inhibitors and protease inhibitors, 5% of the cell lysate were used directly for total RNA isolation. In order to reduce the background, the remaining cell lysate was incubated overnight with protein G agarose beads (Millipore, Darmstadt, Germany) at 4 °C for pre-incubation. The supernatant was immunoprecipitated by anti-Flag mouse monoclonal antibody beads (M2; Sigma Aldrich, Saint Louis, Missouri, USA) on the following day. Four hours after the incubation with M2 beads, the RNAs bound by Rbm24 were released by adding 400 μl Trizol reagents (Ambion, Life technologies, Carlsbad, California, USA) directly. RNAs were extracted by standard Trizol protocol with RNase-free DNase I (Thermo fishier scientific, Waltham, Massachusetts, USA).

For RIP-chip experiments, the pulldown RNAs were then converted to biotinylated cRNA using the Illumina RNA Amplification Kit (Ambion, Austin, TX). Samples were purified using the RNeasy kit (Qiagen, Valencia, CA). Samples were then hybridized onto the Illumina MouseRef-8 Expression Beads Chip. Washing and scanning