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Rbm24 Regulates Alternative Splicing Switch in Embryonic Stem Cell Cardiac Lineage Differentiation

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Key Words. Rbm24 • Alternative splicing • Embryonic stem cell • Cardiac • RNA binding protein

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Received July 20, 2015; accepted for publication February 23, 2016; first published online in STEM CELLS *EXPRESS* March 17, 2016.

1066-5099/2016/$30.00/0

http://dx.doi.org/10.1002/stem.2366

ABSTRACT

The transition of embryonic stem cell (ESC) pluripotency to differentiation is accompanied by an expansion of mRNA and proteomic diversity. Post-transcriptional regulation of ESCs is critically governed by cell type-specific splicing. However, little is known about the splicing factors and the molecular mechanisms directing ESC early lineage differentiation. Our study identifies RNA binding motif protein 24 (Rbm24) as a key splicing regulator that plays an essential role in controlling post-transcriptional networks during ESC transition into cardiac differentiation. Using an inducible mouse ESC line in which gene expression could be temporally regulated, we demonstrated that forced expression of Rbm24 in ESCs dramatically induced a switch to cardiac specification. Genome-wide RNA sequencing analysis identified more than 200 Rbm24-regulated alternative splicing events (AS) which occurred in genes essential for the ESC pluripotency or differentiation. Remarkably, AS genes regulated by Rbm24 composed of transcriptional factors, cytoskeleton proteins, and ATPase gene family members which are critical components required for cardiac development and functionality. Furthermore, we show that Rbm24 regulates ESC differentiation by promoting alternative splicing of pluripotency genes. Among the Rbm24-regulated events, Tpm1, an actin filament family gene, was identified to possess ESC/tissue specific isoforms. We demonstrated that these isoforms were functionally distinct and that their exon AS switch was essential for ESC differentiation. Our results suggest that ESC's switching into the differentiation state can be initiated by a tissue-specific splicing regulator, Rbm24. This finding offers a global view on how an RNA binding protein influences ESC lineage differentiation by a splicing-mediated regulatory mechanism. STEM CELLS *2016; 00:000—000*

SIGNIFICANCE STATEMENT

This study reports a novel function and post-transcriptional regulatory mechnism of an RNA binding protein Rbm24 in embryonic stem cells (ESC) differentiation. Our results suggest that ESC switching into the differentiation state can be initiated by a tissue-specific splicing regulator. To our knowledge, this is the first report describing an RNA binding protein controlling ESC commitment to cardiac lineage specification by engaging a splicing mechanism. Our findings would significantly add to the understanding of the mechanisms in ESC differentiation and heart development, and uncover potential novel pathways operating to direct differentiation of ESCs into cardiomyocytes for envisioned regenerative therapies.

INTRODUCTION

Embryonic stem cells (ESCs), with their ability to differentiate into multicell lineages, hold remarkable potential for cell replacement therapies. The potential of these cells in therapeutic application can be further enhanced through better understanding of the crucial molecular switches that regulate early ESC differentiation. ESC differentiation involves changes in transcription, and transcription factor networks have been shown to play a major role in reorganizing the transcriptome of ESCs in response to differentiation signals [1]. But little is known about the post-transcriptional changes during early lineage differentiation.

Alternative splicing (AS) of RNA, a major form of post-transcriptional gene regulation, is important in mammalian development [2, 3]. AS is a process by which splice sites in primary transcripts are differentially selected to produce structurally and functionally distinct

mRNA and protein isoforms, thus providing an additional mechanism which controls cell fate [2, 4–8]. However, the role of AS in self-renewal, pluripotency, and tissue lineage specification of ESC remains largely unknown.

Recent studies based on genome-wide changes associated with AS during differentiation of ESCs have highlighted the importance of post-transcriptional RNA processing in cell fate decision and differentiation [5, 9, 10]. In a comparison of human ESCs before and after differentiation into cardiac or neural progenitors, Salomonis et al. [5] identified hundreds of AS events between ESCs and differentiated cells, with observed conservation of AS events between mouse and human. Dramatic splicing changes are frequently found during ESC differentiation, and RNA-binding proteins (RBPs) are known to be essential for AS regulation in shaping gene expression during the dynamic process of development [11]. However, it remains unclear to what extent these AS events direct lineage-specific differentiation, and which RBPs are responsible for their regulation.

We have previously reported that RNA binding motif protein 24 (Rbm24) is enriched in human ESCs-derived cardiomyocytes and is highly expressed in human and mouse heart [12, 13]. We further characterized the functional role of Rbm24 in the regulation of cardiac gene expression, sarcomeric assembly, and cardiac contractility in a zebrafish loss-of-function model. Rbm24 deficiency results in diminished heart contractility and the absence of circulation in zebrafish embryos [14]. Grifone et al. [15] also reported the expression of Rbm24 in mouse heart and showed that Rbm24 was required in myogenesis of skeletal muscle in chick. These findings revealed the novel role of the tissue-specific Rbm24 in heart and skeletal development.

Recently, Yang et al. [16] reported that targeted inactivation of Rbm24 disrupted development in mouse. This was consistent with our previous observations in zebrafish [14] where Rbm24 deficiency led to severe sarcomeric disarrangement in striated muscles of the mouse heart [16]. Furthermore, the authors found that Rbm24 null mice died between embryonic day E12.5 and E14.5, showing multiple cardiac malfunctions. RNA-seq analysis of the knockout (KO) hearts identified 68 Rbm24-dependent splicing events, in which several of them were implicated to be responsible for a defective heart phenotype [16]. However, it remains unclear if the observed Rbm24 KO phenotype is the consequence of early cardiogenesis defects which accumulated until late development, leading to embryo lethality.

Our previous study showed that the expression of Rbm24 was upregulated in day 3 of human ESCs differentiation into cardiomyocytes, and the mouse Rbm24 transcript was detected in the cardiac mesoderm at E7.0, the earliest stage analyzed in the study [17]. Notably, in zebrafish, Rbm24 expression was detected as early as 16-cell stage before gastrulation [18]. Based on these observations, we hypothesized that Rbm24 might play a crucial role in the early differentiation of ESCs to cardiomyocytes.

To dissect the cellular and the molecular mechanism by which Rbm24 acts during cardiac specification, an inducible Rbm24 expression system using mouse ESCs was constructed [19]. High throughput RNA-seq analysis was used to analyze the genome-wide transcriptome and the AS events regulated by Rbm24. Our results indicate that Rbm24 is crucial for ESC early differentiation and required for more than 200 splicing events. We further show that overexpression of Rbm24 promotes, whereas knockdown (KD) of Rbm24 inhibits ESC cardiogenesis. Importantly, we demonstrate that Rbm24 directs ESC early differentiation toward the cardiac lineage via activation of specific splicing of cardiac-related RNAs coding for transcription factors, cytoskeletal proteins, and ATPase components. Furthermore, we show that Rbm24 controls proper splicing of ESC/tissue specific isoforms encoding for Tpm1. These isoforms function distinctly in ESC switching from pluripotency to differentiation. KD of ESC-specific the Tpm1 isoform markedly affected the maintenance of ESC pluripotency.

Materials and Methods

Generation of Doxycycline Inducible Rbm24 ESC Line

Human Rbm24 was sequenced and subcloned into p2Lox-Mesp1-Flag-IRES-GFP plasmid [19] in place of Mesp1 to obtain p2Lox-Rbm24-Flag-IRES-GFP plasmid. This construct was electroporated in A2Lox cells as described previously [19, 20]. Two days after electroporation, the recombined cells were selected by using 300 μg/ml of G418 for 1 week.

ESC Culture and Differentiation

A2Lox ESC and R1 ESC lines were routinely maintained on mouse embryonic fibroblasts (MEFs) as described [20, 21]. Medium composition and differentiation protocol are described in Supporting Information Experimental Procedures.

Rbm24 KD ESC Construction

R1 ESCs (ATCC, Manassas, VA, http://www.atcc.org) were infected with lentivirus expressing short hairpin RNA (shRNA) targeting mouse Rbm24 gene (Santa Cruz Biotechnology, Dallas, TX, http://www.scbt.com) and selected with puromycin for 4 days, nontargeting viral particles were used as a control (Ctl). R1 cells were differentiated into embryoid bodies (EBs)s and screened at days 0, 3, 6, 9, and 12, for KD efficiency by both quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot.

RNA-seq

A2Lox ESCs were cultured feeder-free prior to doxycycline (Dox) induction. Total RNAs isolated from biological duplicates of Rbm24 induced (+Dox) for 48 hours and noninduced (−Dox) were used to prepare RNA-seq library according to manufacturer's instruction (Illumina, http://www.illumina.com). The cDNA library was sequenced on the Illumina sequencing platform (Illumina HiSeq 2500, San Francisco) using paired-end technology. Sequencing reads in FASTQ format were mapped to the reference genome, and splice junctions were identified using TopHat [22]. Cufflinks and Cuffdiff were used for transcript reconstruction, quantification, and differential expression analysis [23]. Abundances were reported in FPKM (fragments per kilobase of transcript per million fragments mapped). Further details are documented in Supporting Information Experimental Procedures.

Experimental Assay of AS

Specific primers flanking predicted sites of AS were used for RT-PCR amplifications. PCR products were separated by

high-resolution agarose gel electrophoresis. All primers used in assay are listed in Supporting Information Experimental Procedures.

RT-PCR, qRT-PCR, Immunostaining, Western-Blot, FACS, and Electrophysiology

Detailed protocols are provided in the Supporting Information Experimental Procedures.

Statistical Analysis

All of the results are presented as mean $\pm$ SD. All data were statistically analyzed by Student's t test, p value $< .05$ was considered to be statistically significant.

Results

Overexpression of Rbm24 Promotes Differentiation of ESCs

We first examined Rbm24 expression during mouse ESC differentiation by performing qRT-PCR after 0, 1, 2, 3, 6, and 12 days of differentiation (Fig. 1A). Pluripotency markers Oct4 and Nanog were rapidly downregulated while the mesoderm marker T-brachyury (T-Bra) and cardiac mesoderm marker Mesp1 were upregulated. The expression of Rbm24 was also upregulated from day 3 when cardiomyocyte differentiation markers start to be detected. Its expression increased by day 6 and continued to elevate along with early cardiomyocyte-specific transcription factor Nkx2.5.

In an attempt to identify Rbm24-regulated molecules that could potentially be involved in early ESC differentiation, we generated a recombinant A2Lox ES cell line in which the expression of the recombinant flag-tagged version of Rbm24 with an IRES-GFP (green fluorescent protein) can be temporally and specifically induced with Dox (Fig. 1B; Supporting Information Fig. S1) [19–21]. In the absence of Dox, no expression of the transgene was detected by fluorescence microscopy (Fig. 1C). Upon Dox induction, GFP expression was clearly detected (Fig. 1C; Supporting Information Fig. S1B). Western blot showed that Rbm24 protein was pronouncedly expressed at 12 hours post-Dox administration in ESCs and increased after 24 hours postinduction (Fig. 1D). Flow cytometry analysis demonstrated that up to 98.23% of ESCs expressed the GFP reporter gene (Fig. 1E).

To determine if Rbm24 directly promotes ESC differentiation, Rbm24 was overexpressed in ESCs cultured in the feeder-free ESC medium without leukemia inhibitory factor (LIF). We then monitored the morphology of ESC clones. Seventy-two hours after LIF withdrawal, ESCs treated with Dox displayed a differentiated morphology, whereas untreated ESCs did not (Fig. 1F). Notably, Rbm24 overexpression resulted in the reduction of alkaline phosphatase (Fig. 1G) and Oct4 staining (Fig. 1H), suggesting Rbm24 overexpression affected ESC pluripotency.

Rbm24 Promotes Cardiac Lineage Specification from ESCs

We used the Rbm24 inducible system to identify the role and mechanism of action of Rbm24 in ESC lineage differentiation. A schematic diagram of the experimental strategy is illustrated in Figure 2A. We first examined whether overexpression of Rbm24 could promote ESC differentiation toward specific lineages when ESCs were cultured under mild differentiated condition in the feeder-free ESC medium without LIF. qRT-PCR was used to analyze the expression of markers specific for ESC lineage differentiation. As shown in Figure 2B, upon Dox induction, mesoderm marker T-Bra, cardiac mesoderm marker Mesp1, and cardiac progenitor markers Nkx2.5 and Mef2c were significantly upregulated. This suggests that Rbm24 promoted ESC differentiation to mesoderm and cardiac lineage. It is noted that expression of pluripotency markers Oct4 and Nanog remained almost unchanged, while Lefty1 was downregulated, suggesting ESCs were undergoing partial differentiation triggered by Rbm24 overexpression. The expression of early endoderm marker Sox17 was moderately upregulated, but late marker AFP was dramatically downregulated, suggesting Rbm24 could contribute to mesoendodermal differentiation of ESCs, but could have a negative impact on the late endoderm lineage. The expression of ectoderm marker Nestin and Sox1 remained unchanged, suggesting Rbm24 did not influence ectoderm differentiation. Together, these results demonstrate that Rbm24 overexpression promotes ESC transition to cardiac differentiation.

We next investigated whether Rbm24 stimulates ESCs to generate cardiac cells more efficiently. We performed cardiac differentiation using the EB method as described [19]. As shown in Figure 2C, the induction of Rbm24 resulted in an accelerated cardiac differentiation, which occurred at day 8 of cardiac differentiation, a day earlier than in untreated cells. Close observation of the EB cultures revealed an increased number of beating zones within EB cultures that have been stimulated with Rbm24 overexpression (Fig. 2C; Supporting Information Videos). Immunofluorescence staining demonstrated the increased expression of cardiac specific markers Nkx2.5, Tpm, Actn2, Tnnt2, Myh6, Mlc2a, and Mlc2v, following Rbm24 induction (Fig. 2D). Confocal microscopy imaging revealed characteristic cross-striation typical of sarcomeric structures in some Rbm24-induced cells, as exemplified in Supporting Information Figure S2A.

To further provide a quantitative assessment of the cardiac differentiation efficiency, we performed fluorescence activated cell sorting (FACS) analysis on the expression of Actn2 after 12 days of differentiation. As shown in Figure 2E, Rbm24-stimulated cells generated about six times more cardiac cells. Time-course qRT-PCR assay further highlighted that Rbm24 induction increased the expression of cardiac specific markers during differentiation (Supporting Information Fig. S2B). These results demonstrated that forced expression of Rbm24 in ESCs enhanced the efficiency of cardiac differentiation.

To characterize the electrophysiological properties of cardiomyocytes generated from ESCs, we performed patch clamp assay on beating cells to assess action potentials (APs) at day 12 and 19 after differentiation. Three major types of APs (nodal-like, atrial-like, and ventricular-like) were detected in both Rbm24 induced beating cells (day 12, $n = 14$; day 19, $n = 16$) and uninduced controls (day 12, $n = 8$, day 19, $n = 9$). The majority of cells under both conditions were atrial-like or ventricular-like. Both groups displayed similar AP pattern for day 12 and 19 beating cells, and proportions of atrial and ventricular-like cells had no statistically significant differences

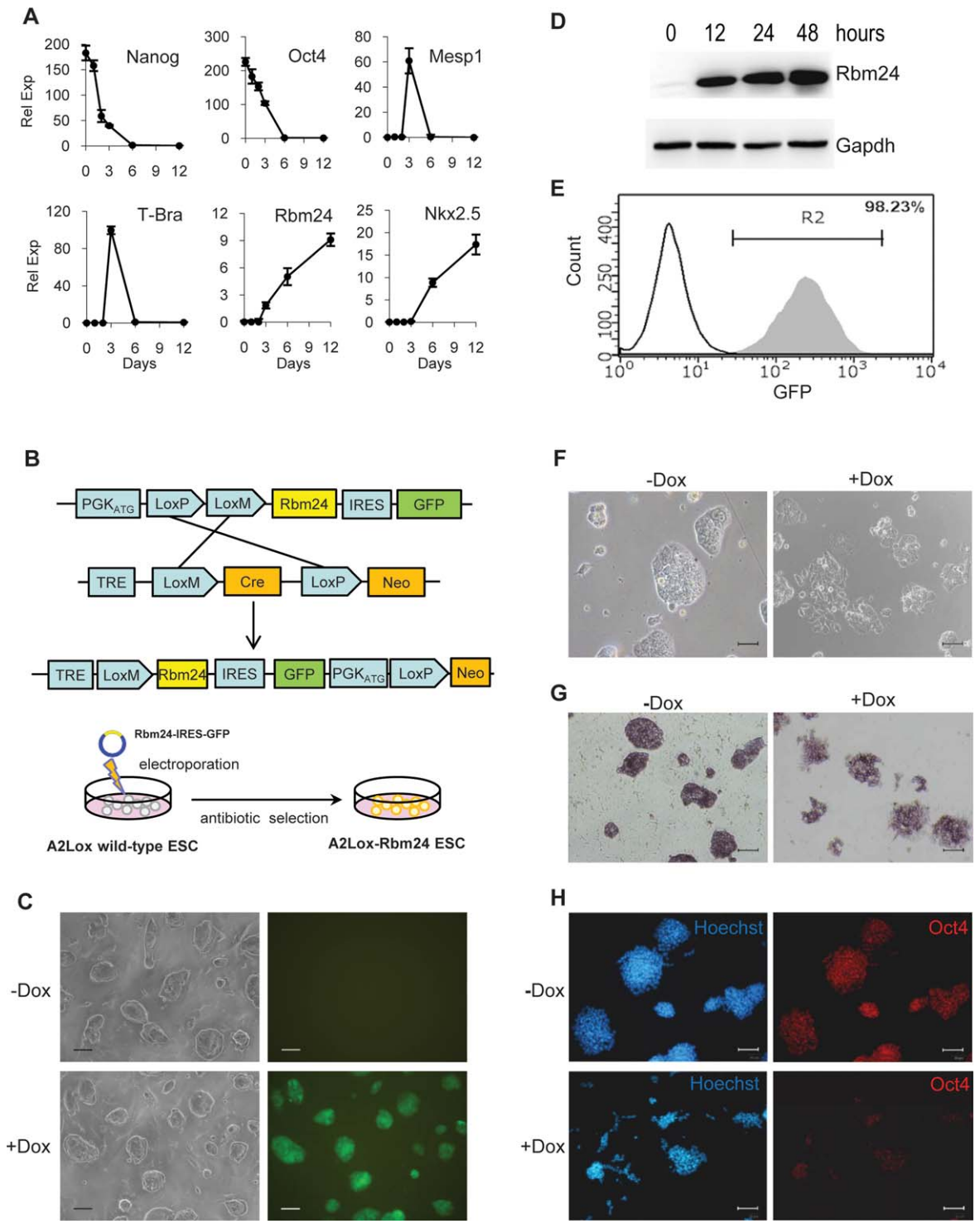

Figure 1. Rbm24 regulates differentiation of ESCs. **(A)**: Expression profiles of pluripotency markers (Oct4 and Nanog), mesodermal markers (Mesp1 and T-Bra), Rbm24, and cardiac marker (Nkx2.5) during normal ESC differentiation by embryoid body (EB) formation as measured by quantitative real-time polymerase chain reaction. Data were normalized to Gapdh. Error bars represent mean ± SD from three biologically independent experiments. **(B)**: Schematic diagram depicting the methodology for generating an ESC line for inducible expression of Rbm24 by DOX. Upper panel: Inducible cassette exchange recombination in A2Lox ESCs (Supporting Information Fig. S1A); lower panel: experimental procedure. **(C)**: Rbm24-IRES-GFP expression following induction of A2lox ESCs with doxycycline (+Dox) for 48 hours. No GFP expression was detected in uninduced cells (−Dox). Scale bar = 200 μm (Supporting Information Fig. S1B for 24-hour Dox induction). **(D)**: Kinetics of Rbm24 expression using Western blot with anti-Rbm24 antibody. Rbm24 was almost undetectable in uninduced condition but was rapidly upregulated following Dox stimulation. **(E)**: GFP expression as measured by flow cytometry after Dox induction for 48 hours in ESCs. **(F)**: Overexpression of Rbm24 for 3 days by Dox induction leads to an altered morphology of ESCs. **(G)**: Alkaline phosphatase staining and **(H)** immunofluorescence staining of Oct4 at day 3 following induction of Rbm24 by Dox in the absence of feeder cells and LIF. Scale bar = 200 μm. Abbreviations: Dox, doxycycline; ESC, embryonic stem cell; GFP, green fluorescent protein; T-Bra, T-brachyury.

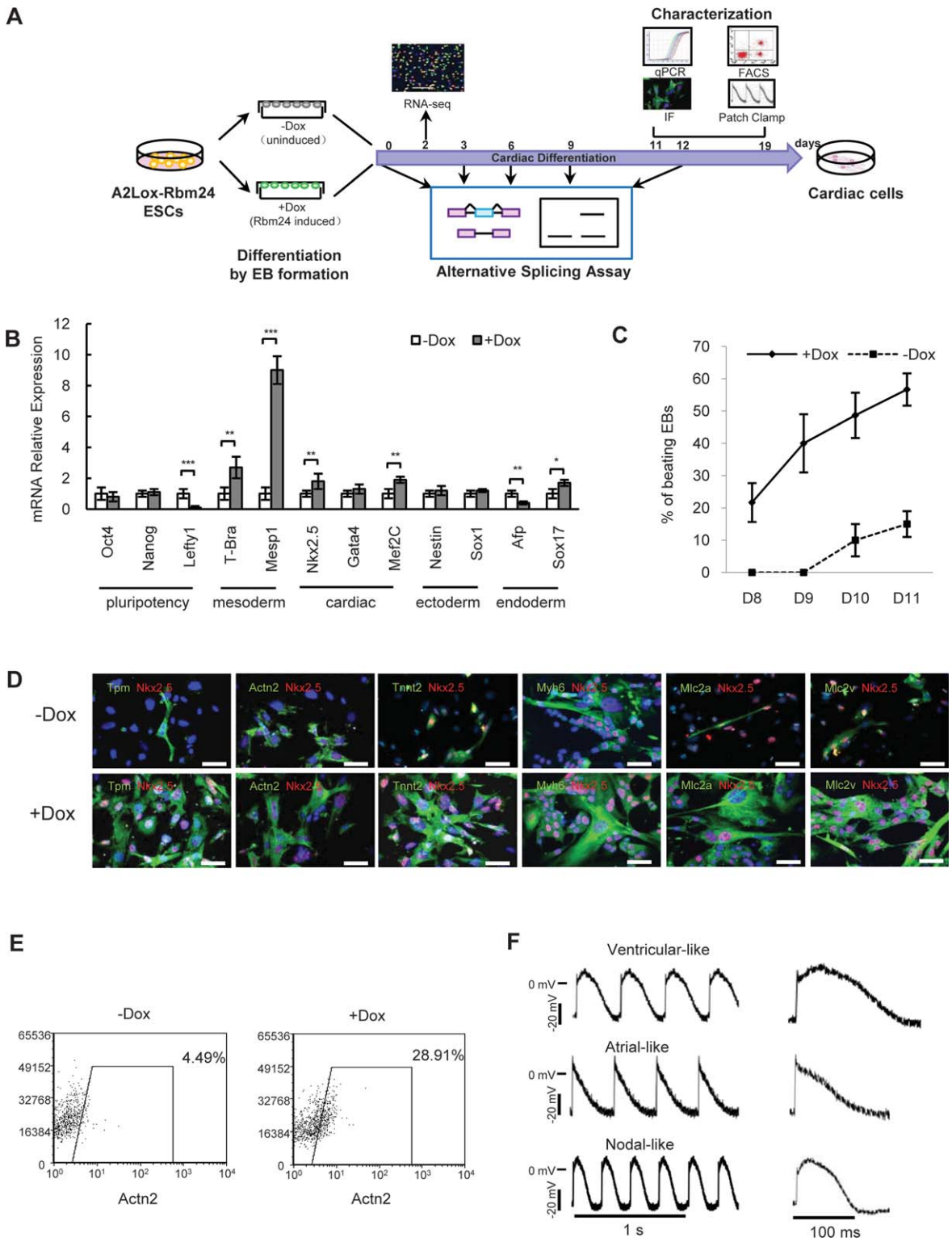

Figure 2. Rbm24 overexpression promotes cardiac differentiation of ESCs. **(A)**: Schematic overview of experimental strategy. Assays were performed at different time points as described in the Results section are as indicated in the diagram. **(B)**: Quantification of pluripotency markers (Oct4, Nanog, and Lefty1), lineage markers (Nestin, Sox1, Sox17, AFP, T-bra, and Mesp1), and cardiac markers (Nkx2.5, Mef2c and Gata4) analyzed by qRT-PCR after 48 hours of Rbm24 induction in ESC medium in the absence of leukemia inhibitory factor. Note that Rbm24 induces the expression of cardiac mesoderm markers Mesp1 and T-bra significantly. Data represent relative expression of transcripts compared to untreated ESCs as mean $\pm$ SD of three biologically independent experiments. (*, $p < .05$; **, $p < .01$; ***, $p < .001$). **(C)**: Rbm24 overexpression promotes cardiac commitment in EB cultures as detected by the precocious appearance and enhancement of beating areas, compared to uninduced cells (Supporting Information video). Error bars represent mean $\pm$ SD from five biologically independent experiments. **(D)**: Immunofluorescence staining of beating cells. Day 11 EBs were dissociated and plated on chamber slides and stained with Nkx2.5, Tpm, Actn2, Tnnt2, Myh6, Mlc2a, and Mlc2v antibodies, the specific markers for cardiomyocyte. Scale bar $= 200\,\mu$m. **(E)**: Quantification of cardiomyocytes by flow cytometry. At day 12 after cardiac differentiation of ESCs, EBs were dissociated and stained for cardiac-specific marker Actn2. **(F)**: Patch-clamp electrophysiology showed representative ventricular-like, atrial-like, and nodal-like action potentials of ESC-derived cardiomyocytes. Beating EBs were enzymatically dissociated to single cells and action potentials (APs) were assessed. Types of APs were recorded in cardiomyocytes isolated from 19 days EBs after differentiation with Rbm24 induction. The same types of APs were recorded in control cells. Abbreviations: Dox, doxycycline; EB, embryoid body; ESCs, embryonic stem cells; FACS, fluorescence activated cell sorting; qPCR, quantitative polymerase chain reaction.

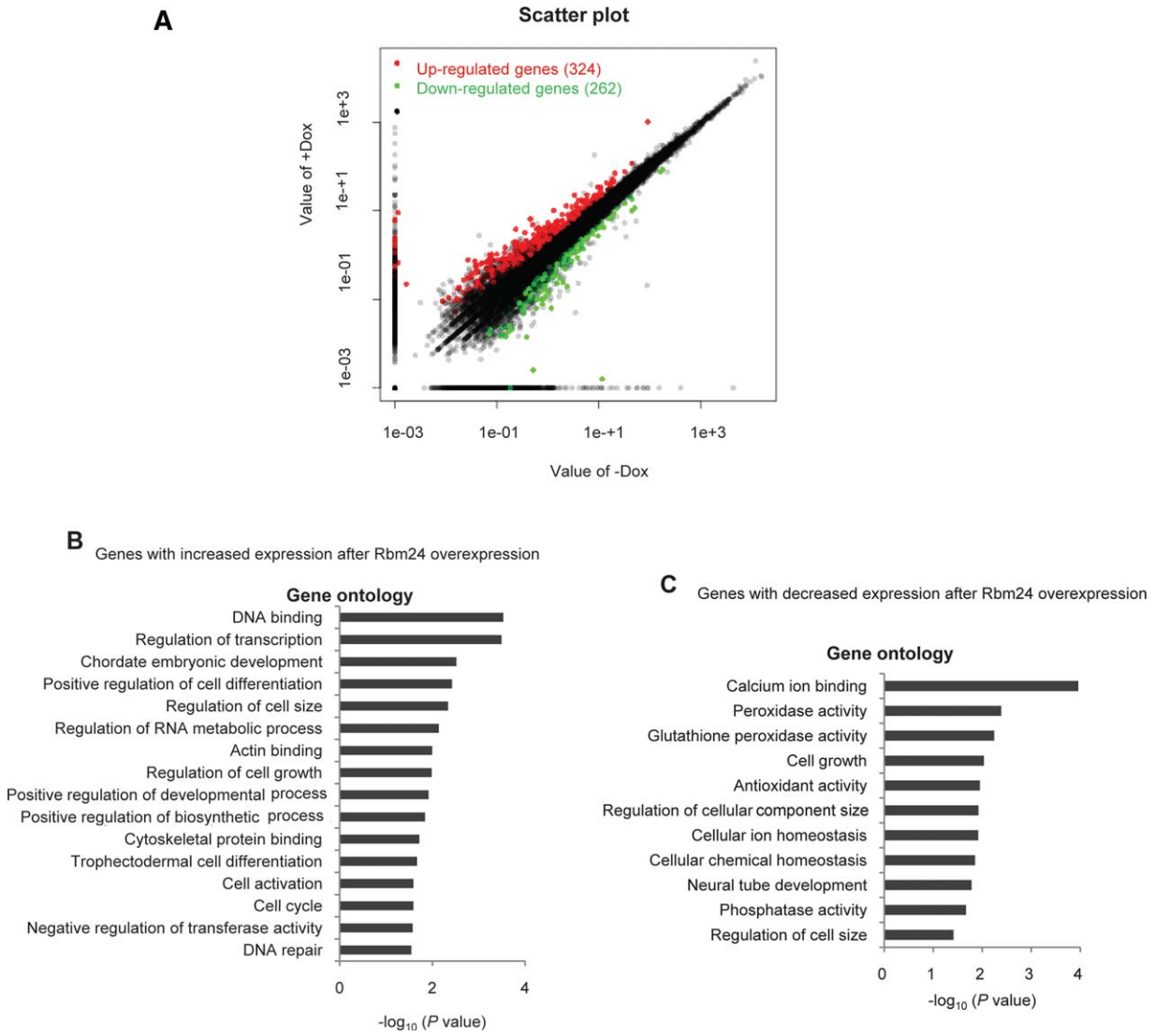

Figure 3. RNA-seq analysis of gene expression regulated by Rbm24. **(A)**: A scatter plot depicting the differentially expressed genes following Dox induction. Red dots represent upregulated genes, and green dots represent downregulated genes. **(B)**: Gene ontology analysis of upregulated genes after 48 hours Rbm24 induction. **(C)**: Gene ontology analysis of downregulated genes after 48 hours Rbm24 induction. Abbreviation: Dox, doxycycline.

between two groups. Representative APs from the Rbm24 induced group on day 19 are shown in Figure 2F.

Genome-Wide Profiling Identifies Rbm24-Regulated Genes

To uncover the molecular mechanisms by which Rbm24 induced ESC differentiation, we performed a genome-wide analysis of Rbm24-regulated genes using high throughput RNA sequencing (RNA-seq) technology. This allows us to determine transcriptomic changes and assess the extent to which ESC-differential AS events are controlled by Rbm24.

Firstly, we examined the differential gene expression to identify genome-wide molecular phenotype alteration in response to Rbm24 overexpression. Approximately 586 genes exhibited a 1.5-fold or greater change in response to Rbm24 overexpression (Fig. 3A; Supporting Information Dataset1). Notably, overexpression of Rbm24 increased mRNA of sarcomeric protein Actn2, which is a cardiac marker, by about eightfold. This result was verified by qRT-PCR (data not shown), suggesting that Rbm24 may directly regulate mRNA level of Actn2.

To obtain a functional overview of the genes responsive to overexpression of Rbm24, we grouped expression data into Gene Ontology (GO) categories using DAVID (http://david.abcc.ncifcrf.gov/). Genes that show increased expression are enriched in development, differentiation, actin-binding, and cytoskeletal protein binding (Fig. 3B), and genes that show decreased expression are enriched in antioxidant activity which is known to inhibit cardiac differentiation (Fig. 3C) [24]. This further highlights that ESCs have transitioned to undergo differentiation to cardiomyocytes upon Rbm24 induction.

Identification of Rbm24-Regulated AS Events and Associated Pathways in ESC Differentiation

To identify the AS events following Rbm24 overexpression, an algorithm based on Cufflinks was used to assign reads to known transcripts and calculate their abundance (Fig. 4A) [23]. We selected the transcripts greater than fourfold change upon Rbm24 induction and FPKM ≥ 4 at least in one sample. Approximately 247 differentially spliced transcripts belong to 230 genes were identified to be AS regulated by overexpression of Rbm24 (Supporting Information Dataset2). To assess the reliability of the data, we checked the isoform-switch of Naca, a previously known target of Rbm24 [16], in our RNA-seq data. As shown in Supporting Information Figure S3, Rbm24 overexpression significantly increased the expression of cardiac-isoform skNac, but not the αNac, in accordance with the early report [16]. We further validated four AS events using RT-PCR. As shown in Figure 4B, Dox induction changed the isoform patterns of each detected gene, giving us high confidence in our RNA-seq analysis.

To further validate our in vitro ESC differentiation observation, we compared 230 genes with Rbm24-regulated AS events which were from the RNA-seq data of the Rbm24 knockout mouse [16]. Approximately 11 genes overlapped with the previously reported Rbm24-dependent 68 AS events in the heart (calculated as 16% overlapping) including Naca, Myo18a, Cad, Add1, Rnps1, Cald1, Actb, Cox7a2l, Atp5c1, Slc25a3, and Tpm1 [16]. This suggests the fidelity of our RNA-seq data and reinforces the splicing role of Rbm24 in regulating heart development. Notably, these 11 overlapping AS genes were detected in two different biological systems, which are Rbm24 knockout mouse heart [16] and Rbm24 overexpressing ESCs (this study) by independent RNA-seq assays, indicating these genes might be the direct splicing targets of Rbm24.

To assess the potential functionality of Rbm24-regulated AS genes, we grouped them into GO categories. The 230 genes were classified according to GO annotations. Figure 4C presents subcategories ($p < .05$) of molecular components, in which "cytoskeleton" and "kinase activity" were reported significantly enriched in terms of AS during ESC differentiation [8]. Other subcategories, such as RNA binding and translation factor activity, are predominantly related to post-transcriptional regulations. As shown in Figure 4D, the analysis also pointed to processes involved in cardiac differentiation of ESCs such as cytoskeleton organization, cell division, cell cycle, and response to stress. Finally, significant ($p < .05$) subcategories of cellular components are shown in Figure 4E. Our analysis pointed to components involved in cardiac cell structure and function such as cytoskeleton and mitochondrion.

We also searched our RNA-seq data against the Kyoto Encyclopedia of Genes and Genomes database (KEGG, http://www.kegg.com [25]). As shown in Supporting Information Table S1, nine pathways were identified when applying software-defined significance criteria ($p < .05$) including spliceosome, regulation of actin cytoskeleton, cardiomyopathies, adherens junction, and mRNA surveillance, indicating an essential role of Rbm24 in RNA regulation and heart function. Taken together, these results demonstrate that Rbm24 is a major splicing factor regulating AS events important in ESC cardiac differentiation.

Rbm24-Regulated AS Contributes to ESC Cardiogenesis

Notably, as demonstrated by GO and KEGG analysis, the global RNA-seq analysis identified classes of genes with cell-type-specific functions, that appear to be constitutively expressed in ESCs. As exemplified in Supporting Information Figure S4, some of them have high mRNA expression levels comparable to pluripotency genes Oct4 and Nanog. These genes are well-documented to play a role in the heart and/or muscle, for example, tropomyosin protein Tpm1, 2, 3 [26], Tnnt1 [27], and Ak1 [28]. By contrast, little data is available regarding the expression and role of these genes in undifferentiated or differentiated states of ESCs.

From our GO analysis, we found that Rbm24 regulated transcripts were enriched in cytoskeleton and mitochondrion function (Figs. 4E, 5A). Heart and skeletal muscle cells have specific cytoskeleton and actin-binding systems to support their contractile function and specific mitochondrion to supply high levels of energy. Given that Rbm24 is specifically expressed in muscle cells, we speculated that our identified Rbm24-associated constitutive genes could be spliced by Rbm24 during differentiation to produce specific isoforms to implement tissue-specific function.

To test our hypothesis, we selected eight genes to investigate whether their splicing patterns are correlated with Rbm24 regulation during ESC differentiation toward cardiomyocytes. Among the eight genes, Naca could be spliced to produce skNac which is a muscle-specific transcription factor [29], while Tpm1, Capzb, and Tpm3 are related to the cytoskeleton. Itga6 is an actin binding protein and modulates the expression of a large number of cell migration-related genes [30]. Atp5c1 (ATP synthase gamma-subunit) has an AS isoform specifically expressed in both heart and skeletal muscle [31]. Enah is localized to the Z-disc and is required for beta-actin assembly at Z-disc of cardiac muscles [32]. Sun1 plays an essential role in proper myonuclear positioning in mouse skeletal muscle [33].

Firstly, we investigated whether these Rbm24-regulated AS events could occur during the onset of ESC differentiation. As shown in Figure 5B, all AS events were detected in ESCs upon Dox induction, indicating that these transcripts are pre-existing and spliced by Rbm24 in the course of ESC transition into differentiation. Subsequently, we investigated whether Rbm24-regulated AS events occur during ESC differentiation into cardiomyocytes. As shown in Figure 5C, all genes displayed an Rbm24-regulated AS pattern at day 6 and increased at day 12, along with upregulation of Rbm24 and early cardiomyocyte-specific transcription factor Nkx2.5 (Fig. 1A). This indicates that Rbm24-regulated AS patterns are related to cardiac differentiation of ESCs. It is interesting to note that the splice isoforms of these genes are also present in normal mouse cardiac/muscle tissues (see Supporting Information Result and Fig. S5). Enah gene displayed similar AS patterns as verified by qRT-PCR (Supporting Information Fig. S6). In all, our results suggest that Rbm24 promotes ESC differentiation toward cardiomyocytes by splicing constitutive genes (such as Capzb, Naca, Sun1, and Atp5c1) or muscle-specific genes (such as Tpm1, Tpm3, and Enah) to produce cardiac isoforms.

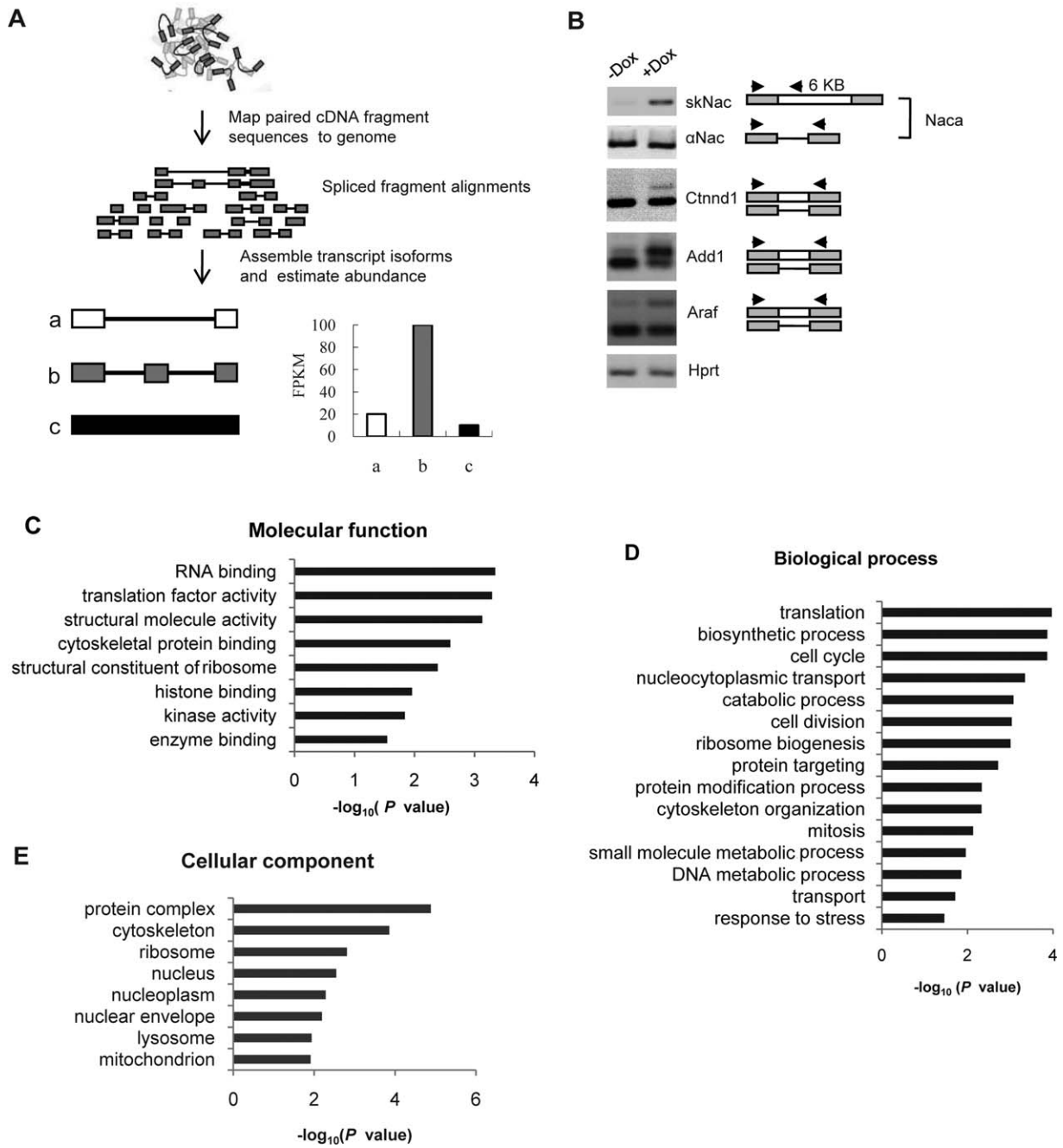

Figure 4. RNA-seq analysis of alternative splicing (AS) events regulated by Rbm24. **(A)**: Overview of algorithm to analyze Rbm24-regulated AS events. Sequences of cDNA fragments generated from high throughput sequencing were aligned to the genome by a spliced aligner (Tophat) [22]. Cufflinks was then used to assemble transcript structures and estimate transcript abundance [23]. **(B)**: Validation of RNA-seq data by real-time polymerase chain reaction (RT-PCR). Naca has two isoforms, skNac and αNac, generated by alternative splicing a 6 kb exon 2. Two primer pairs were used to amplify these two isoforms, respectively. Hprt was used as an internal control. 30 cycles of PCR were performed. **(C)**: Gene ontology analysis of Rbm24-regulated AS events (molecular function). **(D)**: Gene ontology analysis of Rbm24-regulated AS events (biological process). **(E)**: Gene ontology analysis of Rbm24-regulated AS events (cellular component). Abbreviation: Dox, doxycycline; FPKM, fragments per kilobase of transcript per million fragments mapped.

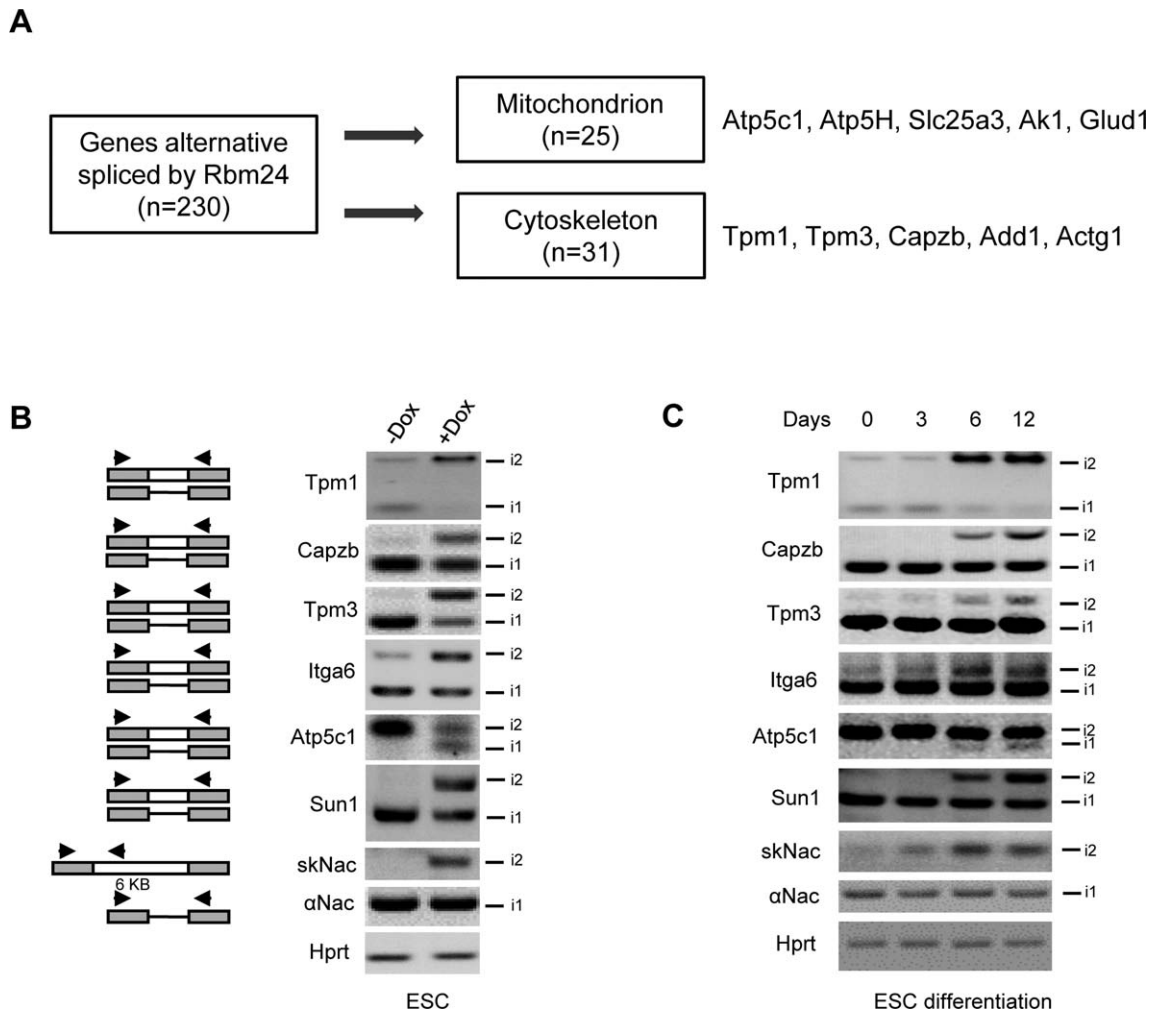

Figure 5. Rbm24-regulated alternative splicing (AS) events are related to cardiac differentiation. **(A)**: Rbm24-regulated AS events are enriched in the mitochondrion and cytoskeleton. **(B)**: Real-time polymerase chain reaction (RT-PCR) analysis of Rbm24-regulated AS events in ESCs with or without Rbm24 induction. RNA was isolated 48 hours after Dox induction. Hprt was used as an internal control. Schematics of all splicing events are shown. i1-exclusion isoform (exon excluded); i2-inclusion isoform (exon included). Upregulation of i2 isoform of Tpm1, Capzb, Tpm3, Itga6, Sun1, and skNac was detected upon Dox treatment. Atp5c1 displayed exon exclusion AS pattern, i1 was upregulated by Rbm24. **(C)**: Time-course analysis of AS events by RT-PCR during A2Lox ESC differentiation toward cardiomyocytes. Differential expression of AS events (observed in Fig. 5B) was detected in EBs 6 and 12 days after cardiac differentiation. i1/i2 are annotated as per Figure 5B. Hprt was used as an internal control. Abbreviations: DOX, doxycycline; ESC, embryonic stem cell.

KD of Rbm24 Inhibits Cardiac Differentiation of ESCs

Next, we investigated whether Rbm24 is required for ESC cardiac differentiation, and whether the observed cardiac-specific AS events are Rbm24 dependant. We generated an Rbm24 KD stable cell line using Rbm24 shRNA-expressing lentivirus in R1 ESCs. As shown in Figure 6A, 6B, shRNA efficiently depleted Rbm24 expression at the mRNA and protein levels during cardiac differentiation. Notably, cardiogenesis in Rbm24-KD ESCs were significantly inhibited, no beating area was detected until day 16, and only a small portion of beating area was observed when differentiation was prolonged to day 17 (Fig. 6C). We further analyzed the expression of markers specific for cardiac differentiation by qRT-PCR. As shown in Figure 6D, expression of cardiac markers Nkx2.5, Gata4, and Myh6 was significantly reduced in Rbm24 KD cells. KD of Rbm24 also decreased the expression of early mesoderm marker Mesp1 and endoderm marker Sox17 but increased the expression of ectoderm markers Nestin and Sox1. This suggests that Rbm24 deficiency impaired endoderm and mesoderm differentiation, inversely correlating with our observations in the Rbm24 overexpression experiments (Fig. 2B; Supporting Information Fig. S2B).

As we have shown the above Rbm24-regulated AS patterns occur during ESC differentiation into cardiomyocytes (Fig. 5C), we then investigated whether Rbm24-regulated AS events were perturbed in Rbm24 KD ESC differentiation. As shown in Figure 6E, Rbm24-regulated AS patterns are dramatically alerted at day 6 and day 12 in the Rbm24 KD cells, suggesting that the expression of alternative isoforms of these genes are Rbm24 dependent and correlated with ESC cardiac differentiation. We further confirmed this observation for four additional genes by qRT-PCR (Supporting Information Fig. S7). Additional AS assays were also performed using alternative cell line of mouse cardiac HL-1 to substantiate this finding (Supporting Information Results; Fig. S8). Together, these results demonstrate that Rbm24 is required for ESC cardiac differentiation by regulating cardiac specific AS events.

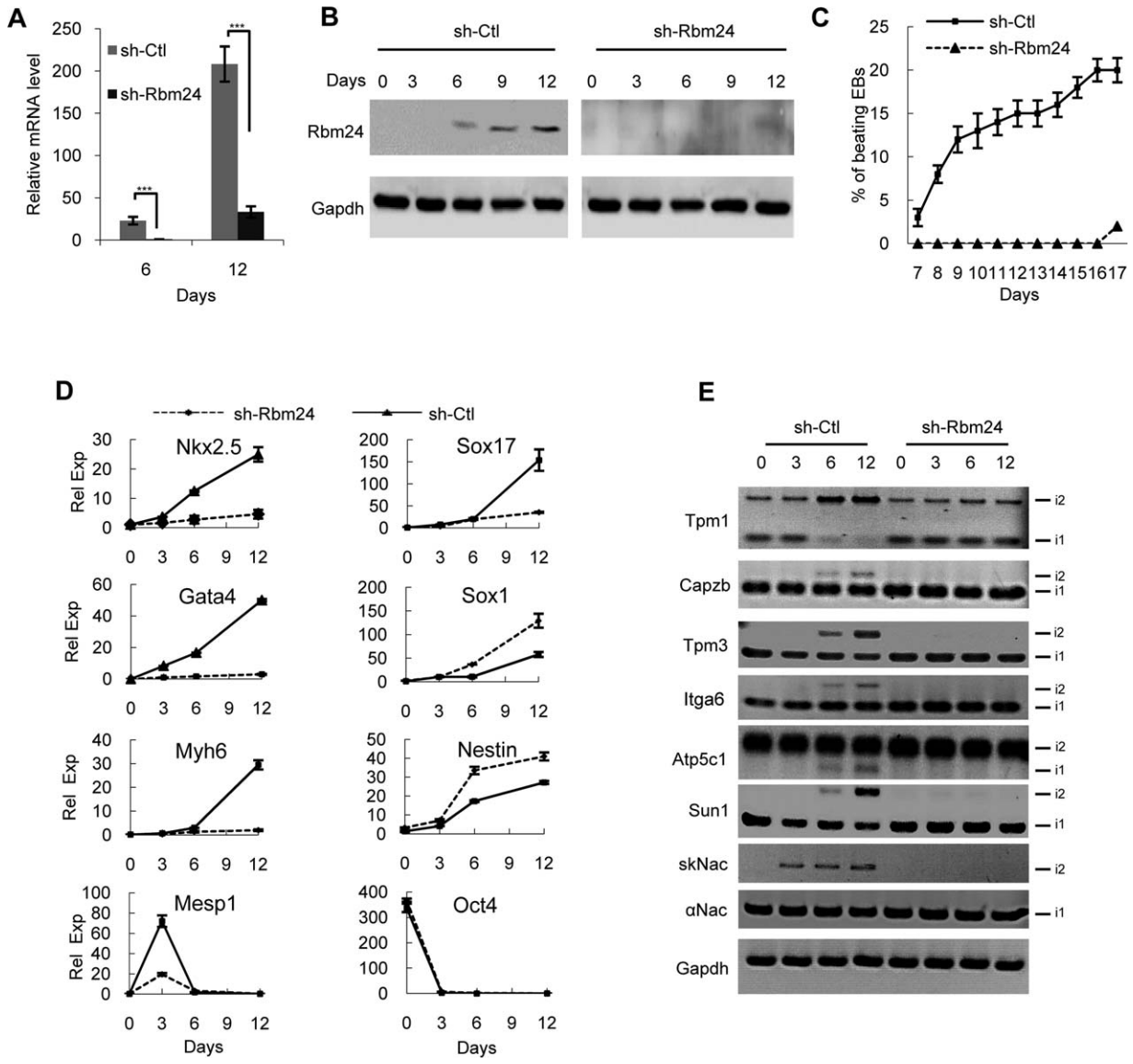

Figure 6. Rbm24 is required for embryonic stem cell (ESC) cardiogenesis. **(A)**: Quantitative real-time polymerase chain reaction (qRT-PCR) of day 6 and 12 EBs after ESC (R1 line) cardiac differentiation showed efficiency of Rbm24 shRNA-lentivirus knockdown (KD) at transcript level. Data were normalized to Gapdh. Error bars represent mean ± SD from three biologically independent experiments (***, $p < .001$). **(B)**: Western blot of day 0, 3, 6, 9, and 12 EBs after cardiac differentiation showed depletion of Rbm24 protein in shRNA KD cells. Gapdh was used as an internal control. **(C)**: Rbm24 KD inhibited ESC cardiac differentiation as demonstrated by barely detectable beating EBs in shRNA KD cells compared to control. Error bars represent mean ± SD from three biologically independent experiments. **(D)**: Temporal analysis of marker expression in ESC differentiating over 12 days in Rbm24 KD versus control cells by qRT-PCR. Data were normalized to Gapdh. Error bars represent mean ± SD from three biologically independent experiments. **(E)**: AS patterns of day 0, 3, 6, and 12 EBs after cardiac differentiation were compared between Rbm24 KD cells and control cells by RT-PCR. i1/i2 are annotated as per Figure 5B. Gapdh was used as an internal control. Abbreviation: EB, embryoid body.

Rbm24 Regulates Differentiation by Promoting AS of Pluripotency Genes

As noted above, overexpression of Rbm24 decreased the pluripotency of ESCs (Fig. 1F–1H), we next examined whether splicing of pluripotency-associated genes would possibly be regulated by Rbm24. We compared Rbm24-regulated AS transcripts with genes essential for ESC maintenance as previously identified by large-scale RNAi screening in human ESCs [34]. Thirteen genes were found to overlap in this comparison (Fig. 7A). We therefore asked whether Rbm24 is potentially involved in the regulation of these splicing events.

Tpm1 was reported to have an ESC-specific isoform (Fig. 7B) [8], so it was selected from the list of 13 pluripotency genes to investigate whether Rbm24 decreased the proportion of ESC isoform. We quantified the ratio of the expression level between isoforms with exon5 (ESC- isoform, Tpm1-E5) and exon6 (tissue-isoform, Tpm1-E6) in ESC cardiac differentiation by qRT-PCR. As shown in Figure 7C, Tpm1-E5 is about twice the expression level in ESCs compared to Tpm1-E6. The ratio of mRNA with Tpm1-E5/Tpm1-E6 progressively decreased during cardiac differentiation, indicating dynamic alternation of expression levels between the two isoforms during ESC transition from pluripotency to a differentiated state. We then checked if the AS regulation of Tpm1-E5

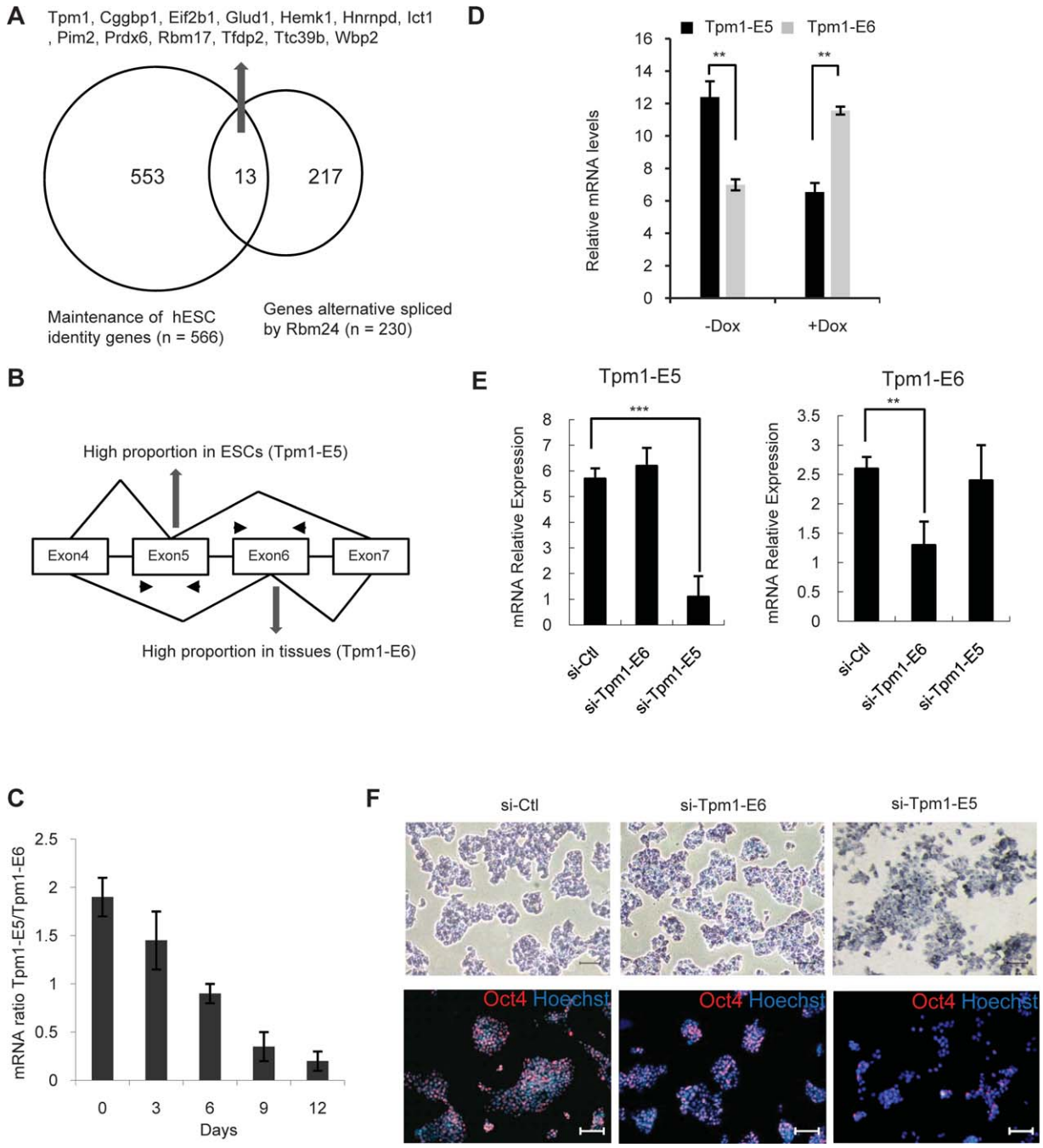

Figure 7. Rbm24 regulates alternative splicing of pluripotency genes. **(A)**: Venn diagram showing the overlap of hESC identity maintenance genes with genes splicing regulated by Rbm24. Genes essential for hESC maintenance were extracted from Supporting Information Table S1 of genome-wide RNAi screen in hESCs [34]. **(B)**: Diagram of Tpm1 splicing patterns in ESCs versus tissues, primers designed for qRT-PCR detection are indicated by black arrows. **(C)**: Quantitative real-time polymerase chain reaction (qRT-PCR) analysis indicates a Tpm1 splicing isoform switch during cardiac differentiation of ESCs. Data were normalized to Gapdh. Note the alternation of expression levels between two isoforms during ESC differentiation as indicated by the decreased ratio of mRNA with exon5 versus exon6. **(D)**: Rbm24 decreased the expression of ESC-specific exon 5 and increased the expression of tissue-specific exon 6. qRT-PCR was used to measure the mRNA level. Data were normalized to Gapdh ($n = 3$; **, $p < .01$). **(E)**: qRT-PCR was used to determine efficiency and specificity of isoform-specific knockdown in ESCs. Data were normalized to Gapdh. The expression levels of Tpm1-E5 or Tpm1-E6 were significantly decreased by E5 or E6 siRNA, respectively ($n = 3$, **, $p < .01$; ***, $p < .001$). **(F)**: The effect of Tpm1 isoform- specific knockdown in ESCs as determined by alkaline phosphatase staining (up panel) and immunofluorescence staining of Oct4 (lower panel), observed at day 3 after electroporation of isoform-specific siRNA. Nuclei were stained with Hoechst (blue). Scale bar = 200 μm. Abbreviations: Dox, doxycycline; hESC, human embryonic stem cell.

and Tpm1-E6 was controlled by Rbm24. As shown in Figure 7D, Rbm24 overexpression decreased the expression of ESC-specific Tpm1-E5 and increased the expression of tissue-specific Tpm1-E6.

To further investigate whether Tpm1 isoforms are functional in ESC pluripotency, we designed isoform-specific siRNAs to KD Tpm1-E5 or E6, respectively in ESCs (Fig. 7E). As

shown in Figure 7F, KD of Tpm1-E5 but not E6 resulted in ESC morphological change, reduction of alkaline phosphatase and Oct4 staining, indicating that ESC specific Tpm1-E5 was functional in ESC and might be involved in the maintenance of pluripotency. These results suggest that Rbm24 reduced ESC pluripotency by switching splicing of the pluripotency transcripts toward tissue-specific isoforms. Together, these data indicate that Rbm24 links splicing with the regulatory components that maintain pluripotency by regulating splicing of transcripts encoding pluripotency related factors.

Discussion

Cellular differentiation requires global changes in regulatory networks at all levels of gene expression. Post-transcriptional gene regulation offers a mechanism for rapid changes in the cellular proteome. In particular, AS represents a wide range acting mode of gene regulation, yet its role in regulating ESC pluripotency and differentiation remains poorly understood. The transition of ESC pluripotency to differentiation is accompanied by an expansion of mRNA diversity and an increase in cellular complexity. Cell type-specific splicing which can generate different transcript variants is a key process underlying post-transcriptional regulation and contributes to proteomic diversity.

A large number of AS events, where primary transcripts are differentially processed to structurally and functionally distinct mRNA isoforms, were detected between ESC/iPSC (induced pluripotent stem cells) and non-ESC/tissues, and were also found to play a role in the transition of ESC fate [5, 6, 9, 10]. These findings highlight the critical role of AS in the specification of ESCs during differentiation. However, only a few tissue-specific splicing factors have been found to play a major role in cell fate determination in ESC [2]. An ESC-specific splicing switch in Foxp1 transcripts has been reported to play a role in the maintenance of pluripotency [6]. More recently, Mbnl proteins were found to inhibit ESC-specific AS [8]. iPSC factor Lin28 plays important roles in pluripotency and development as a repressor of microRNA processing and as a post-transcriptional regulatory factor for a subset of key mRNAs [35].

In this study, we report that RBP Rbm24, a splicing regulator, plays an essential role in modulating post-transcriptional networks between ESC maintenance and cell-type specification. By using an inducible ESC line overexpressing Rbm24 in combination with RNA-seq, we identified more than 200 AS events that were regulated by Rbm24. Our results expand on recent reports establishing the critical roles of Rbm24 in heart development [14, 16]. Rbm24 has been reported as a major regulator of AS in striated muscle [16]. Here, we find that Rbm24 regulates ESC commitment into cardiac lineage differentiation through direct regulation of gene splicing in broad categories. The functional classes of genes that are regulated by Rbm24 splicing comprise both, which are those with widespread homeostatic activities and those with cell-type-specific functions, including transcription factors, sarcomeric proteins for cytoskeleton assembly, and ATPase for fast energy supply. This finding offers a global view about how a RBP such as Rbm24 influences cell fate by AS regulation.

Interestingly, our analysis of overlap between the transcriptome of differentiated cardiomyocytes and undifferentiated ESC reveals a subset of common genes that are highly expressed in both distinct cell types, indicating great diversity in isoforms expression during ESC transition into the differentiated state. This is in line with earlier observation of a genome-wide comparison of AS in human ESCs with neural progenitor cells [9]. We observed that Tpm1, a muscle specific gene encoding a protein in the thin filaments of sarcomere, possessed ESC-specific isoform that was required for maintenance of ESCs. KD of ESC-specific isoform markedly affected the maintenance of ESC pluripotency (Fig. 7). Tpm1 transcript was alternatively spliced into a cardiac-specific isoform by Rbm24 during ESC differentiation into cardiomyocytes (Fig. 7B, 7C). The isoform switch of Tpm1 may contribute to the differentiation phenotype induced by Rbm24. Similar observations were reported for Foxp1 whose DNA-binding preference is changed by AS regulation of RBP Mbnl [8]. The ESC-specific isoform of FOXP1 stimulates the expression of transcription factor genes required for pluripotency, including Oct4, Nanog, Nr5a2, and Gdf3, while concomitantly repressing expression of genes required for ESC differentiation [6].

Given that hundreds of ESC-specific AS events are detected, and many RBPs are differentially expressed between ESCs and differentiated tissues, it is plausible that tissue-specific RBPs could function by AS regulation to produce alternate mRNA isoforms with tissue specific functions, as exemplified by Rbm24 gene here. This mutually exclusive expression of two or more alternative gene products from a single transcript modulated by RBPs could allow for ESC efficient and rapid transition from an undifferentiated to a differentiated state, and probably represents a broad regulatory mechanism in stem cell differentiation and developmental biology.

Interestingly, a growing number of studies revealed that cell adhesion molecules and extracellular matrices could mediate cohesive interaction among stem cells and contribute significantly to their self-renewal and differentiation via cytoskeletal reorganization-signaling [36–39]. We observed that Rbm24 regulated a cluster of AS events associated with the cytoskeleton during cardiac differentiation of ESCs, including Tpm1, Tpm3, and Capzb. We speculate that Rbm24 could trigger the reorganization of the cytoskeleton, and the resultant mechanical force could further shape the stem cells and subsequently alter the extracellular signaling, leading to differentiation of ESC.

Thus far, the roles of RBPs in heart development have been largely constricted to studies during postnatal stage [40], focusing on AS changes that drive fetal-to-adult transitions [2]. Celf1 and Mbnl1 proteins are reported with determinative roles that drive these developmental transitions [41]. Unfortunately, the early molecular mechanism that is responsible for the specification of cardiovascular cell fate from undifferentiated ESCs remains largely unknown [42, 43]. In this study, we show that the process of ESC differentiation allows for the dissection of the cellular and molecular mechanisms underlying cell fate decision. Using an inducible mouse ESC differentiation system, we specifically investigated the earliest molecular events orchestrating the transition of ESCs to a differentiated state. We have found that Rbm24 is required in early ESC cardiogenesis and can direct cardiovascular progenitor specification through AS regulation. To our

knowledge, our study provides the first comprehensive insight into how a tissue-specific RBP directs ESC commitment to cardiac lineage specification by regulating AS.

One potential confounding factor in our approach is that Dox-treatment process itself might somehow alter the gene expression or splicing in cells. The A2Lox inducible ESC line is a well-established system and has been widely used to study the function of targeted genes by Dox induction [19, 44–46], so far none of these studies suggested that Dox had an effect on gene expression. To examine the potential possibility of Dox effect in our study, we analyzed the AS events of Rbm24 target genes by RT-PCR in wild type A2Lox cells, no difference was observed for AS pattern in Dox-treated versus untreated cells (data not shown). Furthermore, the Rbm24-regulated AS events were validated in Rbm24 KD cells using R1 ESCs where no Dox treatment was involved (Fig. 6E). These results combined with the ESC cardiac differentiation functional data indicate that AS events found in our study are result from Rbm24 regulation, not Dox addition itself.

Conclusion

In conclusion, our results identify the regulation of a post-transcriptional switch by Rbm24 as a novel mechanism which alters a constitutively present pool of transcripts to express different isoforms. This leads to the functionalization of cells in tissues during ESC lineage differentiation, which in our case is the differentiation of ESCs into cardiomyocytes. These findings would significantly add to the understanding of the mechanisms in ESC differentiation and heart development, and uncover potential novel pathways operating to direct differentiation of ESCs.

Acknowledgments

We thank Dr. Michael Kyba and Dr. Ceddric Blanpain for their kind donation of the A2Lox ESCs and the p2Lox-Mesp1-tag plasmid, respectively; Dr. Leah Vardy and Yew Mun Lee for critical reading of manuscript. This work was supported by the National Natural Science Foundation of China (NSFC) (Grant no. 81270199), the Major State Basic Research Development Program of China (Grant no. 2014CB965101), Xiamen Joint Research Fund for Major Diseases (3502Z20159014), Research Fund of Xiamen Oceanic Administration (14PYY051SF04), the Natural Science Foundation of Guangdong Province (2015A030310008), the Key Science Foundation of Fujian Province (2013D020), and the Natural Science Foundation of Fujian Province (2012D038).

Author Contributions

T.Z.: conception and design, data analysis and interpretation, and manuscript writing; Y.L.: collection and/or assembly of data and manuscript writing; J.L., Z.R.H., Q.X., and W.H.L.: data analysis and interpretation; W.F., L.Y.G., M.K.Z., Z.G.Z., and L.P.: collection and/or assembly of data; Y.H.L.: administrative support; X.Q.X.: conception and design, financial support, administrative support, final approval of manuscript, and manuscript writing. T.Z. and Y.L. contributed equally to this work.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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