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**Rbm24-mediated post-transcriptional regulation of skeletal and cardiac muscle development, function and regeneration**

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Running title: Rbm24 function in striated muscles

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## **Abstract**

RNA-binding proteins are critically involved in the post-transcriptional control of gene expression during embryonic development and in adult life, contributing to regulating cell differentiation and maintaining tissue homeostasis. Compared to the relatively well documented functions of transcription factors, the regulatory roles of RNA-binding proteins in muscle development and function remain largely elusive. However, deficiency of many RNA-binding proteins has been associated with muscular defects, neuromuscular disorders and heart diseases, such as myotonic dystrophy, amyotrophic lateral sclerosis, and cardiomyopathy. Rbm24 is highly conserved among vertebrates and is one of the best characterized RNA-binding proteins with crucial implication in the myogenic and cardiomyogenic programs. It presents the distinctive particularity of displaying highly restricted expression in both skeletal and cardiac muscles, with changes in subcellular localization during the process of differentiation. Functional analyses using different vertebrate models have clearly demonstrated its requirement for skeletal muscle differentiation and regeneration as well as for myocardium organization and cardiac function, by regulating the expression of both common and distinct target genes in these tissues. The challenge remains to decipher the dynamic feature of post-transcriptional circuits regulated by Rbm24 during skeletal myogenesis, cardiomyogenesis, and muscle repair. This review discusses current understanding of its function in striated muscles and its possible implication in human disease, with the aim of identifying research gaps for future investigation.

Key words: RNA-binding protein, post-transcriptional regulation; striated muscle; myogenesis, cardiomyocyte; muscle regeneration; cardiac defects

## Introduction

Among the three main types of muscle (skeletal, cardiac and smooth muscles), skeletal muscle is the most abundant tissue of the body and generates forces for the voluntary movements; cardiac muscle or myocardium allows the involuntary contraction of the heart, which is the first functional organ formed during embryogenesis. Skeletal muscle progenitors derive from the paraxial mesoderm, which condenses to form segmented somites (McGrew and Pourquié 1998). Myotomal cells within the somite enter into the myogenic program to differentiate and fuse into multinucleated functional myofibers (Buckingham 2006; Chal and Pourquié 2017). The heart precursors are located within bilateral fields in the anterior lateral plate mesoderm, known as the first and second heart fields (Buckingham et al. 2005). These cells then converge toward the midline to form a beating heart tube, which subsequently undergoes elongation and rightward looping to become an asymmetric organ with four chambers in mammals (Christoffels and Jensen 2020). Smooth muscles originate from a wide variety of embryonic structures; they are present in the walls or in the vicinity of numerous hollow organs, such as circulatory, respiratory, digestive and reproductive systems (Donadon and Santoro 2021).

Both skeletal and cardiac muscles are striated muscles characterized by the formation of highly repetitive structures called sarcomeres, which are the smallest functional units of a myofibril. Each sarcomere is composed of thin actin and thick myosin filaments connected by the giant protein Titin (Rassier 2017). However, there are many differences between these muscles, such as shape, length, and nucleus content (Shadrin et al. 2016), as well as selective gene expression (Lindskog et al. 2015). In addition, skeletal and cardiac muscles differ in their potential and mechanism of regeneration (Shadrin et al. 2016). Adult skeletal muscle tissue has a remarkable ability of regeneration by re-activating the myogenic program in muscle stem cells, commonly known as satellite cells (Motohashi and Asakura 2014; Relaix et al. 2021). The adult mammalian heart, however, shows no or a very limited regenerative capacity, because cardiomyocytes irreversibly exit cell cycle in the first week after birth. Nevertheless, the heart from early postnatal mice and adult lower vertebrates (zebrafish and newt) can fully regenerate

after ventricular resection through proliferation of existing cardiomyocytes, with no or minor contribution of cardiac stem cells (Brezitski et al. 2021; de Wit et al. 2020; Porrello et al. 2011; Zheng et al. 2021). These suggest that skeletal and cardiac muscles employ both common and distinct mechanisms to accomplish structural and functional maturation.

RNA-binding proteins (RBPs) make an important contribution to the proteomic diversity and protein abundance within a cell (Gebauer et al. 2021; Glisovic et al. 2008). They are critically involved in muscle development, regeneration and disease (Hinkle et al. 2019; Shi and Grifone 2021). Nevertheless, compared with transcription factors, their implication in the control of the myogenic and cardiomyogenic programs are largely underestimated (Vicente-García et al. 2022). A better understanding of the intrinsic post-transcriptional circuits involving RBPs in skeletal and cardiac muscle development and function should help to define therapeutic strategy for related human genetic diseases (Cornelius et al. 2022; Kelaini et al. 2021; Shi 2024; Völkens et al. 2024; Weskamp et al. 2021). RNA-binding motif protein 24 (Rbm24) is an evolutionarily conserved and a best-characterized muscle RBP with highly restricted expression in developing and adult striated muscles (Fetka et al. 2000a; Grifone et al. 2014, 2021; Maragh et al. 2011, 2014; Miller et al. 2008; Poon et al. 2012; Saquet et al. 2024). It also displays dynamic subcellular localization during myogenic differentiation, suggesting multifaceted functions (Grifone et al. 2021). At present, although no mutation of the *RBM24* gene has been linked to any human congenital defect, functional analyses in different animal models indicate that the loss of Rbm24 affects proper development of all tissues in which it is expressed, including skeletal muscle, heart, lens, and inner ear hair cells (Grifone et al. 2020; Shi et al. 2022). In addition, *RBM24* expression is reactivated following heart failure (D'Antonio et al. 2022), suggesting an important role in cardiac function and homeostasis.

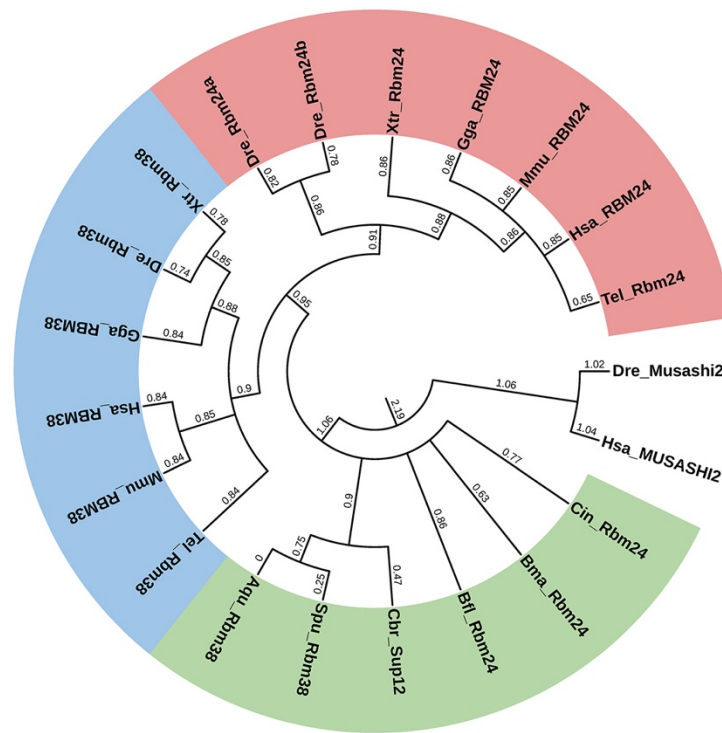
There is accumulating evidence demonstrating that Rbm24 can function in multiple aspects of post-transcriptional gene regulation, ranging from pre-mRNA alternative splicing to mRNA stability, cytoplasmic polyadenylation and translation (Jin et al. 2010; Shao et al. 2020; Yang et al. 2014; Zhang et al. 2018). Thus, it can promote lineage-specific cellular proliferation, differentiation and function through distinct mechanisms, which may be dependent on its

subcellular localization, post-translational modification, and interaction with protein partners or target transcripts. Both past and recent works have provided significant mechanistic insights into the implication of Rbm24 in striated muscle differentiation, regeneration and function. This review discusses its regulatory roles in these processes, and proposes future directions for further understanding Rbm24 functions in skeletal and cardiac muscle biology.

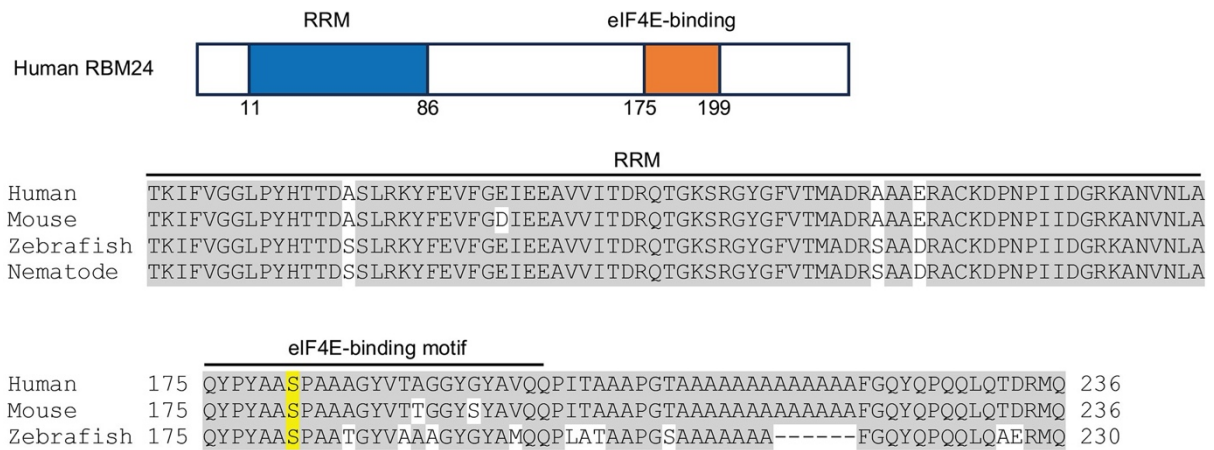
### **Rbm24 protein and its interaction partners**

Vertebrate Rbm24 was originally discovered in *Xenopus* as a false positive in a double-hybrid screening for identifying partners of the forkhead protein XFD-12' and was previously known as Seb4 (Fetka et al. 2000b). The *C. elegans* homolog of vertebrate *Rbm24* gene is *sup-12*, which was cloned by screening mutations that suppress muscle defects resulted from decreased expression of muscle-specific actin depolymerizing factor (ADF)/cofilin (Anyanful et al. 2004). Phylogenetic analysis indicates that Rbm24 is highly conserved from invertebrates to vertebrates. Interestingly, vertebrate Rbm24 and its close paralog, Rbm38, form two distinct clades likely duplicated from an ancestral gene, whereas invertebrates possess only a single protein known as either Rbm24 or Rbm38 (Figure 1). Rbm24 protein harbors a single RNA recognition motif (RRM) at the N-terminal region (Figure 2), which shows a high level of sequence identity from *C. elegans* to mammals (Grifone et al. 2020). The RRM includes two characteristic consensus ribonucleoprotein (RNP) sequences (Afroz et al. 2015) and binds AU/U-rich ligands present in a wide spectrum of transcripts (Ray et al. 2009). Though relatively divergent, the C-terminal half of vertebrate Rbm24 also contains several characteristic motifs. Notably, a highly conserved short stretch of amino acids (residues 175-199 in human RBM24) containing a phosphorylatable serine mediates the interaction of Rbm24 with eukaryotic initiation factor 4E (eIF4E) and prevents the assembly of translation initiation complex (Zhang et al. 2018). However, phosphorylation of the serine residue within this eIF4E-binding motif releases the binding of Rbm24 with eIF4E. Therefore, the functionality of Rbm24 may be regulated through interaction with its partners (Zhang et al. 2018). Rbm38 also contains the eIF4E-binding motif (Lucchesi et al. 2019). It has been shown that glycogen synthase kinase 3 (GSK3) can

function to phosphorylate Rbm38 (Zhang et al. 2013). This raises the possibility that GSK3 may be also involved in regulating Rbm24 post-translational modification and activity.



**Figure 1.** Phylogenetic tree of Rbm24 and Rbm38. Multiple alignments of the two proteins were performed using the Clustal W algorithm, and the phylogenetic tree was constructed using the Bayesian method (Zhang et al. 2020a). Invertebrate Rbm24 or Rbm38 are represented in green; vertebrate Rbm24 and Rbm38 are shown in red and blue, respectively. The tree is rooted using human and zebrafish Musashi protein as an outgroup. Numbers at each node represent Bayesian posterior probability values based on three million runs. Hsa, *Homo sapiens*; Mmu, *Mus musculus*; Tel, *Thamnophis elegans*; Gga, *Gallus gallus*; Xtr, *Xenopus tropicalis*; Dre, *Danio rerio*; Cin, *Ciona intestinalis*; Cbr, *Caenorhabditis briggsae*; Spu, *Strongylocentrotus purpuratus*; Aqu, *Amphimedon queenslandica*; Bma, *Bombyx mandarina*; Bfl, *Branchiostoma floridae*.



**Figure 2.** Schematic representation of human RBM24 protein and alignments of the RRM and the C-terminal region including the eIF4E-binding motif in Rbm24 proteins between different species (human, NP\_001137414.1; mouse, NP\_001074894.1; zebrafish, NP\_998030.1; nematode, NP\_508674.1). The phosphorylatable serine residue in the eIF4E-binding motif, which is conserved among vertebrate Rbm24, is highlighted in yellow.

Rbm24 has been also shown to physically interact with other RBPs, such as Rbm20, in regulating the expression of cardiac-specific protein isoforms through alternative splicing (Ito et al. 2016). During lens differentiation in zebrafish, Rbm24 promotes the translational efficiency of *crystallin* mRNAs by controlling their poly(A) tail lengths, likely through interaction with cytoplasmic polyadenylation element-binding protein 1 (Cpeb1) and cytoplasmic poly(A)-binding protein 1 like (Pabpc1l), two components of the cytoplasmic polyadenylation complex (Shao et al. 2020). Therefore, Rbm24 may represent a novel regulator of mRNA cytoplasmic polyadenylation, and it can coordinate tissue-specific gene expression through multiple post-transcriptional mechanisms, by interacting with distinct protein partners.

### Conserved and restricted expression pattern of Rbm24 in striated muscles

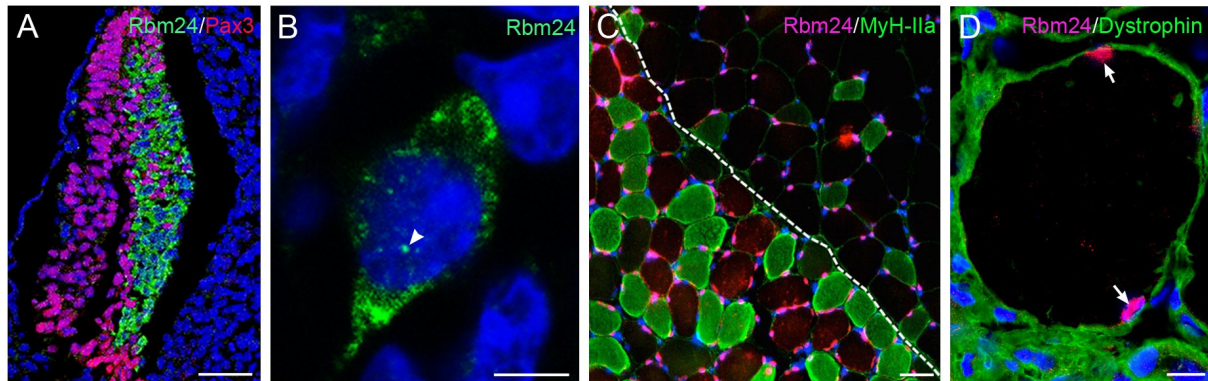
It seems that except zebrafish, all other vertebrates have only a single *Rbm24* gene. Zebrafish has *rbm24a* and *rbm24b* paralogs likely produced by genome duplication. While *rbm24a* exhibits a higher degree of overall identity and shows identical expression pattern with respect to *Rbm24* in other vertebrates, *rbm24b* displays more restricted expression in the somites, with no or very weak expression in the heart primordium (Grifone et al. 2020; Maragh



et al. 2014; Poon et al. 2012; Saquet et al. 2024). Zebrafish *rbm24a* and *Xenopus Rbm24* transcripts are first detected in the paraxial mesoderm at the late gastrula or early neurula stage, in a very similar pattern as *MyoD*. As development proceeds, they are expressed in both segmented somites and the presomitic mesoderm, becoming gradually restricted only to the presomitic mesoderm (Fetka et al. 2010a; Saquet et al. 2024). During organogenesis, the expression of zebrafish *rbm24a* and *Xenopus Rbm24* also appears in the heart primordium, well before the formation of the heart tube (Fetka et al. 2000a; Poon et al. 2012; Saquet et al. 2024). Additionally, at least in *Xenopus*, *Rbm24* transcripts are detected in branchiomic cranial muscles, which also express *MyoD*, but they are not present in *Myf5*-positive/*MyoD*-negative extraocular muscle (Grifone et al. 2014). In the mouse embryo, strong *Rbm24* expression is first evident at E7.5 in the cardiac crescent derived from the first heart field, whereas an obvious expression in segmented somites can be observed around E9.5 (Miller et al. 2008; Xu et al. 2009). From E10.5 to E12.5, *Rbm24* is progressively expressed in head and limb muscles (Grifone et al. 2014). In addition, *Rbm24* protein can be detected in differentiating lens fibers, olfactory receptor neurons and inner ear hair cells (Grifone et al. 2018).

Immunofluorescence staining indicates that *Rbm24* protein undergoes changes in subcellular localization during myogenic differentiation (Figure 3). In the myotome of mouse embryos at E11.5, *Rbm24* protein is accumulated in the cytoplasm of *MyoD*-positive myoblasts entering into the differentiation program, but it is not present in *Pax3*-positive muscle progenitor cells in the dermomyotome (Grifone et al. 2014). In adult mouse skeletal muscles, *Rbm24* protein is restricted to myofiber nuclei, but it is absent in satellite cells that localize between the sarcolemma and the basal lamina (Grifone et al. 2021). Consistent with this cytoplasm to nucleus translocation during skeletal muscle development, *Rbm24*-GFP fusion protein first accumulates in the cytoplasm of mononucleated myoblasts of C2C12 cells and shows nuclear localization in multinucleated myotubes (Grifone et al. 2021). Moreover, *Rbm24* expression in the myonuclei of adult muscles is enriched in slow-twitch myofibers stained by MyHC-I and MyHC-IIa antibodies (Figure 3); it also exhibits linear correlation with the expression of Myogenin in different types of skeletal muscles (Grifone et al. 2021). Since Myogenin is

predominantly expressed in adult slow-twitch muscle fibers (Hughes et al. 1993), this observation raises a possibility that Rbm24 may be differentially involved in regulating the function of adult muscle fiber types.



**Figure 3.** Expression and localization of mouse Rbm24 protein in skeletal myoblasts and myofibers. **(A)** Rbm24 expression in differentiating myoblasts within the myotome but not in Pax3-positive muscle progenitor cells located at the dermomyotome of E11.5 mouse embryo (Grifone et al. 2014). Scale bar: 50  $\mu$ m. **(B)** Rbm24 accumulation in the cytoplasm of fate-committed myoblasts. The signals are also present as nuclear foci (arrowhead). Scale bar: 10  $\mu$ m. **(C)** Rbm24 is present predominantly in type IIa myofibers of the slow-twitch muscle in adult mice (Grifone et al. 2021). Scale bar: 50  $\mu$ m. **(D)** Localization of Rbm24 protein (arrows) in the myonuclei of adult myofibers inside the dystrophin-associated muscle cell membrane. Scale bar: 10  $\mu$ m.

In the developing mouse heart, cytoplasmic localization of Rbm24 protein can be also detected in cardiomyocytes at E11.5 (Grifone et al. 2014). The expression and subcellular distribution of Rbm24 at more late stages of heart development and in the adult heart remain unclear. Thus, it will be of interest to examine the spatiotemporal changes of Rbm24 protein expression during differentiation and maturation of cardiomyocytes. Besides striated muscles, it is worth mentioning that Rbm24 protein also shows cytoplasmic accumulation in differentiating cells within head sensory organs including lens fibers, olfactory receptor neurons and inner ear hair cells, which is clearly correlated with its function in regulating mRNA stability or poly(A) tail

length associated with mRNA translation efficiency (Cheng et al. 2020; Grifone et al. 2018; Shao et al. 2020).

### **Rbm24 is required for myogenic differentiation**

The specific accumulation of Rbm24 protein in fate-committed myoblasts is consistent with its function in myogenic differentiation. At least in *Xenopus* and mouse, *Rbm24* gene harbors multiple E-box (CANNTG) consensus, which are bound and regulated by MyoD. This suggests that *Rbm24* may be a direct transcriptional target of MyoD in embryonic myogenesis (Grifone et al. 2014; Li et al. 2010; Maguire et al. 2012). However, in *MyoD* homozygous null mutant mice, *Rbm24* expression is only completely lost in head muscles and partially reduced in limb muscles, but remains relatively unaffected in the myotomes, raising the possibility that other regulators may contribute to activating *Rbm24* transcription in differentiating trunk muscles (Grifone et al. 2014). Indeed, early B cell factor family members of transcription factors (Ebf2 and Ebf3) have been shown to regulate *Rbm24* expression during myogenesis in the *Xenopus* embryo (Green and Vetter 2011). It is possible that other myogenic factors, such as Myf5, may also function as transcriptional regulators of the *Rbm24* gene (Li et al. 2010).

Functional analyses clearly show that Rbm24 is required for myogenic differentiation. In *Xenopus* and chick embryos, inhibition of Rbm24 function in myotomal cells using antisense morpholino oligonucleotides prevents the formation of muscle cells, as revealed by the decreased expression of muscle-specific myosin (Grifone et al. 2014; Li et al. 2010). In zebrafish, morpholino-mediated knockdown of either *rbm24a* or *rbm24b* affects somitogenesis, resulting in reduced somite numbers in the early embryos (Maragh et al. 2014). Simultaneous knockout of *rbm24a* and *rbm24b* leads to disorganization of myofibers and destabilization of sarcomeric structure in differentiating skeletal muscles (Saquet et al. 2024). Constitutive knockout of *Rbm24* in mice also disrupts skeletal muscle integrity, leading to loss of M-bands in the sarcomere (Yang et al. 2014). Conversely, overexpression of Rbm24 has been also shown to promote differentiation of C2C12 cells (Jin et al. 2010; Miyamoto et al. 2009). These observations suggest that Rbm24 is an important regulator of skeletal myogenesis.

### **Rbm24-mediated post-transcriptional regulatory mechanisms in skeletal muscle**

The post-transcriptional mechanisms by which Rbm24 functions in skeletal myogenesis remain elusive. Several lines of evidence suggest that it can regulate mRNA stability and pre-mRNA splicing during muscle development (Table 1). In C2C12 cells, overexpression of Rbm24 has been shown to promote myogenic differentiation by increasing Myogenin expression through binding to the 3'-untranslated region (3'-UTR) and stabilization of its mRNA (Jin et al. 2010). At present, there is still no direct in vivo evidence demonstrating the function of Rbm24 in stabilizing muscle-specific mRNAs. However, RNA-seq analysis indicates that it acts essentially as a major activator of muscle-specific alternative splicing. Indeed, *Rbm24* homozygous mutant mice show exclusion or reduced inclusion of muscle-specific exons (Yang et al. 2014). Several of these Rbm24-regulated genes, such as *Coro6*, *Naca*, *Slc25a3*, *Fxr1*, *Usp25* and *Usp28*, play important roles in skeletal muscle growth, organization, regeneration and function. A majority of splicing alterations observed in *Rbm24*-knockout mice have been also detected in zebrafish *rbm24a* mutants (Shao et al. 2020), suggesting conserved Rbm24 function in regulating muscle-specific alternative splicing events. Consistent with its in vivo function, knockdown of *Rbm24* in primary mouse satellite cells prevents the differentiation of myoblasts by inhibiting the expression of muscle-specific isoforms of *Coro6*, *Fxr1* and *Naca* (Cardinali et al. 2016). These observations suggest that Rbm24 may represent an important regulator of muscle-specific alternative splicing events. It is of note that the nematode SUP-12 has been shown to cooperate with other factors in the control of muscle-specific pre-mRNA splicing (Kuroyanagi et al. 2007; Ohno et al. 2012). Nevertheless, given the cytoplasm to nucleus translocation of Rbm24 during muscle development (Grifone et al. 2021), it may regulate the stability and/or translation of muscle-specific mRNAs to initiate myogenic differentiation but activate muscle-specific alternative splicing to promote myofibrillar organization and muscle function. Further studies will be necessary to examine the temporal functions and regulatory mechanisms of Rbm24 in skeletal myogenesis.

### **Rbm24 is implicated in skeletal muscle regeneration**

The powerful regenerative capacity of skeletal muscle cells mostly relies on the presence of a pool of Pax7-positive quiescent satellite cells. Stimulated by damage-activated

local signals, these cells re-enter the cell cycle to proliferate and then undergo differentiation by fusing with each other or with existing myofibers, thereby repairing the damaged muscle tissues (Michele 2022; Montarras et al. 2013). Rbm24 is required for injury-induced muscle regeneration. Knockout of *Rbm24* in mouse skeletal muscle or satellite cells disrupts myoblast fusion and delays muscle regeneration by inhibiting alternative splicing events of several muscle-specific genes essential for myogenic differentiation and muscle function, including *Mef2d*, *Naca*, *Rock2* and *Lrrfip1* (Zhang et al. 2020b). Transplantation of *Rbm24*-knockdown satellite cells into cardiotoxin-injured tibialis anterior muscles reveals that Rbm24 function is also required for Myogenin expression at early stages of muscle injury, likely by regulating the stability of its mRNA (Grifone et al. 2021). Therefore, Rbm24 may exert multiple post-transcriptional regulatory roles during the muscle repair process. These observations suggest that Rbm24 can maintain the homeostasis of adult muscles and that its dysfunction may cause muscle degenerative disease. Although Rbm24 protein is not present in satellite cells under physiological conditions, it is rapidly accumulated in neoforming myofibers that rebuild the injured muscles (Grifone et al. 2021), raising the possibility that its expression should be reactivated in response to injury-induced signals. Thus, the underlying mechanism merits future investigation.

At present, human *RBM24* has not been identified as a disease-associated gene in skeletal muscle. However, recurrent mutations of MYOD1, a potential upstream transcriptional regulator of *RBM24*, are present in cell/sclerosing rhabdomyosarcoma with aggressive clinical outcomes (Agaram et al. 2019; Rekhi et al. 2016). Thus, it will be of interest to examine whether and how *RBM24* expression and associated post-transcriptional networks are affected in rhabdomyosarcoma. In this regard, it is of note that *RBM24* displays either ant-tumor or pro-tumor activity, likely depending on cellular context and its post-translational modification (Shi 2022).

### **Rbm24 is required for heart development**

The most notable and severe phenotype caused by the loss of Rbm24 function in vertebrate embryos is cardiomyogenic differentiation defects, which are better characterized in

zebrafish and mice. Although zebrafish has a two-chambered heart, the molecular and cellular mechanisms central to cardiac development are largely conserved in this species (Bakkers 2011; Kemmler et al. 2021; Staudt and Stainier 2012). In addition, the external development and transparency of zebrafish early embryos allow direct visualization of defective cardiac development by live imaging. Morpholino-mediated knockdown of *rbm24a* or *rbm24b* has been shown to affect myocardium development, leading to impaired blood circulation and cardiac contractility (Maragh et al. 2011; Poon et al. 2012). In *rbm24a* mutants generated by the CRISPR/Cas9 approach, cardiac edema is visible around 1.5 dpf (days post-fertilization) and becomes progressively severe as development proceeds. Homozygous mutant larvae cannot survive beyond 7 dpf due to the absence of blood circulation, likely accompanied with other abnormalities in head sensory organs such as blindness and balancing deficits that impair their movements and predatory action (Cheng et al. 2020; Shao et al. 2020; Saquet et al. 2024). The mutant heart shows dilated atrium and shriveled ventricle, which is associated with severe disorganization of sarcomeres in cardiomyocytes. Similar cardiac defects are also present in *Rbm24* homozygous mutant mice. From E10.5 onward, defective ventricular septum, reduced myocardial compaction, dilated atria, thinned atrioventricular endocardial cushions, and reduced wall thickness in the atria and ventricles are apparent. These lead to growth retardation and embryonic lethality around E13.5 (Yang et al. 2014; Zhang et al. 2018).

Postnatal inactivation of *Rbm24* also causes heart defects with dilated left ventricle and reduced wall thickness, reminiscent of dilated cardiomyopathy (Liu et al. 2019). These observations suggest that *Rbm24* displays strongly conserved function in heart development and that it is required for proper organization and function of cardiac muscle. Nevertheless, how *Rbm24* functions in early cardiac morphogenesis remains unclear. In all vertebrate embryos examined, *Rbm24* is expressed in the heart fields or cardiac crescent before the formation of heart tube (Fetka et al. 2000a; Miller et al. 2008; Saquet et al. 2024; Xu et al., 2009). It is thus important to determine the origin of cardiac phenotypes caused by the loss of its function, which may stem from defects in the differentiation of progenitor cells, looping of the heart tube,

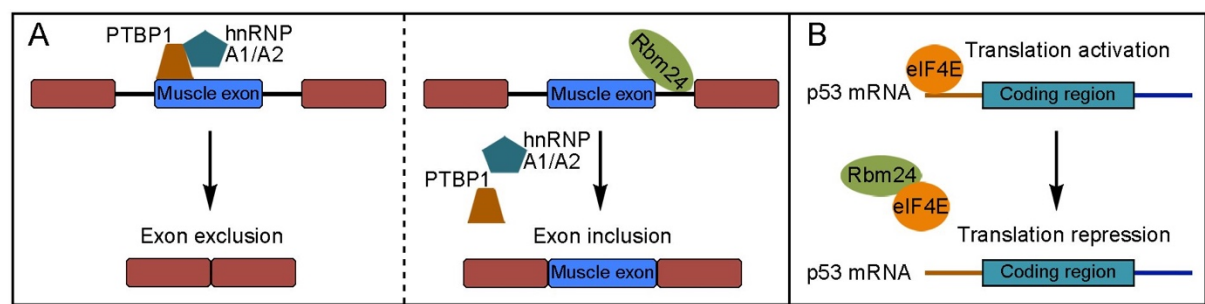
morphogenesis of cardiac chambers or the atrioventricular canal, or establishment of proper cardiac contractile function.

**Table 1.** Rbm24-regulated genes in striated muscles.

Tissues or cells	Target genes	Mechanisms	Functions	References
C2C12	<i>Myogenin</i>	mRNA stability	Promote myoblast differentiation	Jin et al. 2010
Mouse skeletal muscle and satellite cell	<i>Coro6, Naca, Slc25a3, Fxr1, Usp25, Usp28</i>	Alternative splicing	Required for myoblast differentiation and M-band integrity	Cardinali et al. 2016; Yang et al. 2014
Injured mouse skeletal muscle	<i>Coro6, Mef2d, Naca, Rock2</i> and <i>Lrrfip1</i>	Alternative splicing	Required for muscle regeneration	Grifone et al. 2021; Zhang et al. 2020b
Injured mouse skeletal muscle	<i>Myogenin</i>	mRNA stability?	Required for muscle regeneration	Grifone et al. 2021
Mouse heart (E11.5)	<i>Coro6, Naca, Fxr1, Abcc9, Slc25a3, Usp25, Usp28</i>	Alternative splicing	Required for cardiac development and sarcomerogenesis	Yang et al. 2014
Mouse heart (E11.5)	<i>p53</i>	mRNA translation	Inhibit apoptosis and required for heart development	Zhang et al. 2018
Postnatal mouse heart	<i>Titin</i>	Alternative splicing	Required for sarcomerogenesis	Liu et al. 2019
Postnatal mouse heart and human cardiomyocyte	<i>CaMKII<math>\delta</math></i>	Alternative splicing	Regulate QT interval and cardiac rhythm	Liu et al. 2022
Human cardiomyocyte	<i>TITIN, <math>\alpha</math>-ACTININ 2, MYH10, MYBPC3</i>	Alternative splicing	Promote stage-specific myofibrillogenesis	Lu et al. 2022
Rat cardiomyocyte	<i>Enh (Pdlim5)</i>	Alternative splicing	Repress hypertrophic growth	Ito et al. 2016

**Rbm24 promotes sarcomerogenesis and cardiac function by functioning as a splicing regulator**

Transcriptomic analyses of Rbm24-regulated events have provided significant insights into its post-transcriptional regulatory function in heart development (Table 1). In the embryonic heart (E11.5) of *Rbm24* mutant mice, 68 genes show complete exclusion or minor inclusion of muscle-specific exons (Yang et al. 2014). Several of these genes, including *Coro6*, *Naca*, *Fxr1*, *Abcc9*, *Slc25a3*, *Usp25* and *Usp28*, whose defective splicing in *Rbm24* mutant hearts has been experimentally validated, are related to cardiomyogenesis and sarcomere organization or involved in the pathogenesis of dilated cardiomyopathy. Except *Abcc9*, all other genes also show defective alternative splicing in the skeletal muscle of *Rbm24* mutants (Yang et al. 2014), suggesting that they are common targets of Rbm24 in striated muscles. Mechanistically, Rbm24 binds to an intronic splicing enhancer and releases the repression of exon inclusion caused by binding of PTBP1 (polypyrimidine tract binding protein 1) and hnRNP A1/A2 (heterogeneous nuclear ribonucleoprotein A1/A2) to an exonic splicing silencer (Yang et al. 2014). Thus, it acts as an activator of muscle-specific exon inclusion during cardiomyogenesis (Figure 4A). Consistent with this functionality, overexpression of Rbm24 in mouse embryonic stem cells has been shown to induce their differentiation toward cardiac lineage by switching alternative splicing events essential for cardiac muscle organization and contractility (Zhang et al. 2016). It is therefore relatively well documented that Rbm24 regulates the expression of cardiomyocyte-specific structural and functional genes to promote cardiomyogenesis.



**Figure 4.** Mechanisms of Rbm24-mediated post-transcriptional regulation of gene expression in striated muscles. **(A)** Rbm24 promotes the inclusion of muscle-specific exons by preventing the binding of PTBP1 and hnRNP A1/A2 to an exonic splicing silencer (Yang et al. 2014). **(B)** Rbm24 also functions as a repressor of *p53* mRNA translation by interacting with eIF4E and preventing its binding to the 5'-cap (Zhang et al. 2018).



Conditional knockout of *Rbm24* in the postnatal mouse heart mostly affects the developmental isoform transition of sarcomeric proteins, such as Titin, and leads to progressive dilated cardiomyopathy (Liu et al. 2019). Titin is a giant sarcomeric protein that has important structural role and signaling function in cardiac physiology and disease (Eldemire et al. 2021). The human *TITIN* gene includes 363 coding exons and generates variable protein sizes with different functions by alternative splicing (Guo et al. 2010). The loss of *Rbm24* in mice prevents the inclusion of exons coding for the N-terminal Z-repeat domain that binds to  $\alpha$ -ACTININ 2 (Liu et al. 2019); the deletion of *RBM24* in human embryonic stem cells leads to splicing defects of similar exons (Lu et al. 2022). In addition, at early stages of myofibrillogenesis in cardiomyocytes derived from human embryonic stem cells, *RBM24* promotes the inclusion of exon 6 in the  $\alpha$ -ACTININ 2 transcript. In the absence of *RBM24*, the exclusion of exon 6 results in a truncated and unstable  $\alpha$ -ACTININ 2 protein. This impairs the anchoring of its interacting proteins, such as TITIN, to Z-lines and the replacement of non-muscle MYH10 by muscle-specific MYH6 (Lu et al. 2022).

*RBM20* functions as a major splicing regulator for the *TITIN* gene, and its mutations cause dilated cardiomyopathy by disrupting TITIN isoform switch during heart development (Brauch et al. 2009; Guo et al. 2012; Shi 2024). There are two main TITIN isoforms in the cardiac muscle: the stiffer N2B isoform and the more compliant N2BA isoform with a longer extensible I-band region, which serve as short and long molecular springs, respectively (Tharp et al. 2019). Depending on the animals, N2B and N2BA isoforms are coexpressed at variable levels in fetal and adult hearts to confer the passive stiffness of cardiac muscle (Guo et al. 2010; Neagoe et al. 2003). Changes in their ratio can alter cardiac function and cause dilated cardiomyopathy (Loescher et al. 2022). *RBM24* and *RBM20* are differentially implicated in *TITIN* alternative splicing. While *RBM24* promotes inclusion of exons 11 and 12 coding for the N-terminal Z-repeat domain that binds to  $\alpha$ -ACTININ 2 (Lu et al. 2022), *RBM20* represses exons 51 to 218 encoding I-band area by inhibiting intron excision in this region and, therefore, regulates N2B and N2BA isoform switch (Li et al. 2013). Thus, *RBM24* controls TITIN-mediated

sarcomerogenesis, while RBM20 modulates TITIN-based myocardial passive stiffness. Nevertheless, RBM24 and RBM20 can also cooperate to regulate alternative splicing of the same exons in other cardiac genes, such as *Pdlim5* (Ito et al., 2010), suggesting that they function both separately and jointly in heart development.

Rbm24 deficiency in mice and human embryonic stem cell-derived cardiomyocytes also affects calcium handling by disrupting alternative splicing of CaMKII $\delta$  (calcium/calmodulin-dependent kinase II delta). This causes an aberrant expression of the CaMKII $\delta$ -C isoform, resulting in prolonged ventricular cardiac action potential and QT interval (Liu et al. 2022). Thus, Rbm24 may play a role in regulating cardiac rhythm.

### **Rbm24 regulates mRNA translation during heart development**

There is also evidence showing that Rbm24 can be involved in other post-transcriptional processes. As aforementioned, Rbm24 represents a transcriptional target of p53 and in turn plays a role in *p53* mRNA translation. Aberrant activation of p53-dependent apoptosis has been observed in heart tissues of homozygous *Rbm24* mutant mice, which at least in part contributes to endocardial cushion defect, growth retardation and embryonic lethality (Zhang et al. 2018). In vitro studies suggest that Rbm24 interacts with eIF4E and inhibits *p53* mRNA translation by preventing eIF4E from binding to the 5'-cap and the assembly of translation initiation complex (Figure 4B). Thus, Rbm24 may promote cardiac differentiation by inhibiting p53-induced apoptosis of cardiomyocytes in a manner that is dependent on the phosphorylation of its eIF4E-binding motif. It will be of interest to examine whether Rbm24 also regulates other cardiac genes through the same mechanism.

### **Possible role of Rbm24 in cardiac repair and hypertrophic growth**

The implication of Rbm24 in cardiac repair and regeneration has not been investigated using appropriate cardiac injury animal models. However, several lines of evidence suggest that it may be involved in cardiac pathological processes. Overexpression of Rbm24 in the healthy heart of adult mice leads to extensive cardiac fibrosis (van den Hoogenhof et al. 2018), while reduced Rbm24 activity in heterozygous mutant mice can decrease myofilament passive

stiffness (de Groot et al., 2020). Rbm24 has been also shown to repress hypertrophic growth of rat cardiomyocytes by regulating alternative splicing of Enigma homolog (Enh), also known as Pdlim5, through cooperation with Rbm20 (Ito et al. 2016). Enh isoforms are differentially implicated in cardiac hypertrophy. The long Enh1 isoform containing three LIM domains promotes hypertrophy, whereas the short Enh3 and Enh4 isoforms lacking LIM domains function to repress hypertrophy (Yamazaki et al. 2010). Rbm24 and Rbm20 show biochemical interaction and promote the expression of Enh3 and Enh4 (Ito et al. 2016). These observations suggest that Rbm24 may exert a cardioprotective role by repressing pathological hypertrophy. Given the emerging roles of RBPs in heart regeneration (Shi 2023), the contribution of Rbm24 to cardiac regeneration and maintenance of heart homeostasis merits further *in vivo* investigation using appropriate animal models.

## **Discussion**

The identification of muscle-specific RBPome is rapidly evolving. Therefore, post-transcriptional control of muscle development becomes increasingly important and gains growing interest. Functional studies of key players involved in this regulatory paradigm, including but not limited to RBPs, are essential for better understanding the molecular mechanisms underlying muscle development and disease. Due to their critical roles in skeletal and cardiac muscle development and disease (Blech-Hermoni et al. 2013; de Bruin et al. 2017), RBPs represent a promising therapeutic strategy in the treatment of neuromuscular disorders and cardiovascular diseases (Cornelius et al. 2022; Kelaini et al. 2021; Völkers et al. 2024; Weskamp et al. 2021).

Rbm24 displays highly specific expression in striated muscles and emerges as a multifaceted regulator in muscle differentiation, function and regeneration. Clearly, it coordinates the developmental switch of protein isoforms essential for skeletal and cardiac muscle differentiation, and is required for sarcomere assembly and integrity by regulating alternative splicing of sarcomeric genes. Nevertheless, the post-transcriptional mechanisms by which Rbm24 contributes to establishing the gene expression networks for early steps of myogenesis and cardiomyogenesis remain largely elusive. In the myotome, it is still unclear how cytoplasmic

Rbm24 functions to promote the differentiation of fate-committed myoblasts, although it can regulate myogenic gene expression and cell cycle arrest in C2C12 cells (Jin et al. 2010; Miyamoto et al. 2009). In adult skeletal muscle, it will be intriguing to understand how Rbm24 regulates the differentiation of muscle fiber types and how it is activated following injury. RBPs-mediated muscle type-specific alternative splicing plays an important role in generating muscle diversity (Nikonova et al. 2020). It is of note that *Titin* is differentially spliced in myofiber types, with slow muscle preferentially expressing the long N2A isoform that confers low stiffness (Prado et al. 2005). Interestingly, Rbm24 is enriched in adult slow-twitch muscles (Grifone et al. 2021), thus raising the question as to how it functions in this fiber type. Rbm24 shows early expression in the heart primordium and is reactivated following heart failure (D'Antonio et al. 2022; Miller et al. 2008; Saquet et al. 2024; Xu et al., 2009). Analysis of its subcellular localization in cardiomyocytes with respect to its post-transcriptional activity represents a subject of interest. It is also important to determine the implication of Rbm24 in cardiomyocyte differentiation, heart looping and morphogenesis of cardiac chambers. In the adult heart, the potential contribution of Rbm24 to the maintenance of tissue homeostasis merits future investigation.

Rbm24 likely regulates target gene expression through distinct post-transcriptional mechanisms in a context-dependent manner. It will be of interest to understand how Rbm24 acts in concert with other partners to perform developmental stage-specific functions and maintain homeostasis in adult muscle. In this regard, it is worth mentioning that Rbm24 interacts with key components of the cytoplasmic polyadenylation complex, including *Cpeb1* and *Pabpc1l*, which play an important role in mRNA stabilization and translation activation (Charlesworth et al. 2013). This raises the possibility that Rbm24 may be a component of the cytoplasmic polyadenylation complex and that it may function to regulate mRNA translation through modifications of poly(A) tail lengths during muscle development. Interestingly, members of the *Cpeb* and *Pabpc* families are co-expressed with Rbm24 in muscle tissues and play an important role in controlled changes of mRNA translation during muscle growth and differentiation. In skeletal muscles, *Cpeb1*-mediated reprogramming of the translational

landscape contributes to activating stem cells (satellite cells) during quiescence exit and promoting MyoD protein synthesis (Zeng et al. 2022). The fact that Rbm24 is required for skeletal muscle regeneration opens up an avenue for further exploring the post-transcriptional regulation of this process. In adult cardiomyocytes, the translation of *Pabpc1* mRNA is itself controlled by its poly(A) tail length changes in response to developmental and hypertrophic cues, while Pabpc1 protein in turn functions as a regulator of cardiac physiologic growth by modulating global protein synthesis (Chorghade et al. 2017). Cpeb4 also emerges as a critical RBP involved in cardiomyocyte function by differentially regulating the translation of target mRNAs during pathological remodeling (Riechert et al. 2021). These observations strongly suggest that cytoplasmic polyadenylation plays an important role in the control of muscle growth and regeneration.

Although no mutation of the *RBM24* gene has been associated with any congenital disease in humans, likely due to cardiac defects during early embryonic development, there may be specific cases of pathogenic *RBM24* mutations. Given the strong cardiac phenotype in *Rbm24*-knockout animal models, human *RBM24* may be considered as a potential candidate gene for genetic screenings in cardiovascular diseases (Gaertner et al. 2019). It is also relevant to identify potential *cis*- and *trans*-regulatory elements that control the expression of *RBM24*, which may be responsible for the dysregulation of RBM24 activity in hereditary cardiovascular/skeletal muscle conditions. A better understanding of the mechanism by which RBM24 regulates striated muscle development and function should help to develop therapeutic approaches for the treatment of myopathies and cardiomyopathies.

In conclusion, evidence is accumulating that Rbm24 functions as a critical regulator of skeletal and cardiac muscle development and homeostasis. However, its implication in several important processes of muscle biology, such as myogenic differentiation, muscle stem cell activation, and heart morphogenesis, remains elusive. The post-transcriptional mechanisms by which Rbm24 orchestrates the expression of genes essential for muscle structure and function have been only partially elucidated. Further investigation will be necessary to decipher its dynamic regulatory activity under homeostatic and pathologic conditions.

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## **Contributions**

DSL conceptualized and wrote the work. DSL, RG, XZ and HL contributed to literature search, figure preparation, and editing of the manuscript.

## **Conflicts of Interest**

The authors declare no conflict of interest.

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