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# RNA binding proteins in the regulation of heart development

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## Abstract

*In vivo*, RNA molecules are constantly accompanied by RNA binding proteins (RBPs), which are intimately involved in every step of RNA biology, including transcription, editing, splicing, transport and localization, stability, and translation. RBPs therefore have opportunities to shape gene expression at multiple levels. This capacity is particularly important during development, when dynamic chemical and physical changes give rise to complex organs and tissues. This review discusses RBPs in the context of heart development. Since the targets and functions of most RBPs - in the heart and at large - are not fully understood, this review focuses on the expression and roles of RBPs that have been implicated in specific stages of heart development or developmental pathology. RBPs are involved in nearly every stage of cardiogenesis, including the formation, morphogenesis, and maturation of the heart. A fuller understanding of the roles and substrates of these proteins could ultimately provide attractive targets for the design of therapies for congenital heart defects, cardiovascular disease, or cardiac tissue repair.

### Keywords

RNA binding protein; heart; development; morphogenesis; RNA processing; regulation

## 1. Introduction

Growing interest in the functional repertoire of RNA binding proteins (RBPs) has emerged as their potential to regulate gene expression has become more broadly appreciated. While the old paradigm of gene expression focused on the activation of transcriptional programs by DNA binding proteins, the roles of RBPs in post-transcriptional regulation have recently been given greater scrutiny. Post-transcriptional regulatory mechanisms have been identified at all levels of the life cycle of a transcript: regulation of pre-mRNA alternative splicing (Kelemen et al., 2013), mRNA editing (Chateigner-Boutin and Small, 2011), transcript stability (Schoenberg and Maquat, 2012), transcript localization (transport and sequestration) (Medioni et al., 2012), and regulation of translation (Kong and Lasko, 2012). RBPs are involved in the regulation of each of these processes [for an overview see (Glisovic et al., 2008)]; some specific examples are illustrated in Figure 1. Some of these



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functions are nuclear, while others are cytoplasmic, or take place within other organelles such as mitochondria. There are several different types of RNA binding domains, which divide RBPs into structurally distinct families. While many employ beta sheets as interaction surfaces to interface with client transcripts and utilize aromatic residues and base stacking interactions to achieve recognition of targets, a detailed understanding of the binding properties of many RBPs remain to be elucidated (Lunde et al., 2007). While their RNA binding domains can help catalog these proteins, RBP families are not characterized by unified functions. Individual RBPs may perform several functions within the same cell, and may have different functions in different cell types. The ability of RBPs to circumvent the transcription machinery allows them to quickly and selectively fine-tune expression, and this capacity has been recognized as especially important in developmental and pathological systems (Masuda et al., 2009; Misquitta et al., 2001; Siomi and Dreyfuss, 1997). For example, during early zygotic development, when maternal transcripts are translated but the transcription machinery is silent, RBPs provide robust mechanisms for regulating gene expression to direct processes such as pattern formation and cell-type specification (Lee and Schedl, 2006).

The post-transcriptional regulation of gene expression by RBPs during development also has evolutionary consequences. Because of their generally small size and ability to rely on diffusion for tissue oxygenation, many invertebrate species lack a defined circulatory system. The invertebrate heart is typically a simple, beating tube or sac that moves fluid through the body via peristaltic contractions. Vertebrates, on the other hand, have closed circulatory systems with multi-chambered hearts. It has been proposed that vertebrates exhibit a greater degree of cellular and organismal complexity than invertebrates due in large part to expansion of the transcriptome (without proportional expansion of the genome) via an increase in alternative RNA processing, particularly pre-mRNA alternative splicing (Ast, 2004; Maniatis and Tasic, 2002). Consistent with this, several RBP families involved in alternative splicing regulation are differentially expanded in vertebrates compared to invertebrates, whereas basal splicing machinery proteins are generally invariant among all eukaryotes (Barbosa-Morais et al., 2006; Pascual et al., 2006).

The development of the heart is a complex and finely orchestrated process. RBPs have been shown to be involved in nearly every step of heart development, from the establishment of cardiac lineages to the maturation of the heart after birth (Figure 2). In addition, there are RBPs that are known to be expressed in the heart at some stage, but whose molecular and biological roles in heart development have not yet been determined. This review will focus on the expression and functions of RBPs that have been implicated in the formation, morphogenesis, and maturation of the heart (Figure 3). It should also be noted that a set of RBPs known as Argonaute proteins interact with microRNAs (miRNAs) within the RNA-induced silencing complex (RISC) to destabilize or inhibit the translation of target mRNAs that possess sequence complementarity (Amiel et al., 2012). Although miRNA-mediated gene expression changes are important in the heart during normal development and disease (Chen and Wang, 2012; Ono et al., 2011), we will not discuss Argonaute proteins further in this review, as it is generally the miRNAs rather than their associated RBPs that are developmentally regulated.

## 2. Cardiac cell fate, heart tube formation, and differentiation

The heart is the first functional organ to form in the vertebrate embryo. Induction of cardiogenesis begins in the pregastrula embryo (Ladd et al., 1998; Yatskievych et al., 1997), and precardiac cells become specified within the nascent mesoderm as it arises during gastrulation (Brand, 2003). As presumptive cardiogenic cells migrate out of the primitive streak, the heart-forming regions are found on either side of the midline. Both positive and

negative signals from nearby tissues act to induce cells in the heart-forming regions to migrate rostrally and medially to form the cardiac crescent (i.e., the primary heart field). A second cohort of cells (i.e., the secondary heart field) arising from splanchnic mesoderm at the arterial pole also contributes to the formation of the heart (Brand, 2003; Dunwoodie, 2007). Cell migration, along with flexion and folding of the embryo, bring the heart-forming regions together at the midline where they fuse to form the primitive heart tube: an outer, myocardial layer surrounding an inner, endocardial layer. The linear heart tube is arranged from the sinoatrial segment (inflow tract) at the posterior end to the presumptive left and right ventricles, and finally the conus arteriosus (outflow tract) at the anterior end. Completion of the heart tube coincides with the assembly of nascent myofibrils in the cardiac myocytes, and beating begins. In vertebrates, the primitive heart tube is later transformed into a multi-chambered heart.

The inductive signals that establish cardiac cell fates have been long studied, and include Wnt-βcatenin, fibroblast growth factor (FGF) and Hedgehog proteins, and members of the transforming growth factor β (TGFβ) superfamily such as the bone morphogenetic proteins (BMPs) (Brand, 2003; Dunwoodie, 2007). These growth factors initiate signaling cascades that activate important cardiogenic transcription factors of the NKX, TBX, GATA, and MEF2 families (Brand, 2003; Dunwoodie, 2007). Much less is known about the contributions of post-transcriptional programs to the establishment of cardiogenic cell lineages, but several RBPs have been shown to participate in heart tube formation or cardiac cell differentiation (Figure 2).

### 2.1. Formation of the heart tube

Unlike the multi-chambered vertebrate heart, the heart of the fruit fly *Drosophila melanogaster* remains a simple linear tube. The *Drosophila* heart tube is subdivided into an anterior “aorta” and a larger posterior cavity. Although the fly heart is structurally much simpler, gene expression programs important for cardiogenesis are largely conserved from flies to man. This conservation, along with the ease of performing large-scale genetic screens, has made *Drosophila* a powerful model system for the identification of important regulatory genes. For example, the importance of NK-class homeodomain-containing transcription factors (such as *Nkx-2.5*) in heart formation was first uncovered in studies of the fly paralog, *tinman* (Bodmer, 1993). Similarly, RBPs have been identified in flies that are important in the developing heart.

Held out wings (How) is a heterogeneous nuclear ribonucleoprotein (hnRNP) K-homology (KH)-domain protein (Figure 3) involved in muscle function (Baehrecke, 1997). It is most similar to the mouse Quaking (QK) and *Caenorhabditis elegans* GLD1 proteins, which belong to the signal transduction and activation of RNA (STAR) subfamily of KH-domain proteins. Members of this subfamily have been described to link signal transduction pathways to RNA metabolism in different tissues (Lasko, 2003). In flies, MAPK/ERK-dependent phosphorylation of How promotes its RNA binding activity, and How is phosphorylated *in vivo* in embryonic cardioblasts (Nir et al., 2012). A maternal *how* transcript is found broadly in the *Drosophila* zygote, although no detectable protein product is detected (Zaffran et al., 1997). A zygotic transcript is activated in later stages in the presumptive mesoderm, and then in cells of myogenic lineages and in epidermal muscle attachment cells (Baehrecke, 1997; Zaffran et al., 1997). Later in development, *how* transcripts are restricted to the heart and to muscle attachment sites. This expression pattern is maintained during and following morphogenesis, with the addition of expression in adult muscle precursor cells attached to the wing imaginal discs, resulting in the phenotype for which the gene is named (Baehrecke, 1997; Zaffran et al., 1997). Despite this broad early expression, histological analysis of mutants suggests that perturbation of *how* expression does not result in disruption of striated muscle formation, but rather leads to functional

aberrations including reduced cardiac beat rate and weakened contraction (Zaffran et al., 1997). A recent study has suggested that How regulates the expression of sarcomeric proteins, although it is unclear whether this relationship is direct or indirect (Nir et al., 2012). How has also been shown to be part of a genetic pathway with the extracellular matrix protein Slit, its receptor Robo, and dystroglycan that controls formation of the cardiac lumen during heart tube morphogenesis (Medioni et al., 2008).

The mouse QK has nuclear and cytoplasmic isoforms generated by alternative splicing (Kondo et al., 1999). QK binds to consensus UACU(C/A)A hexanucleotide sequences (Ryder and Williamson, 2004), and regulates the alternative splicing of transcripts encoding proteins involved in myelination in the adult brain (Wu et al., 2002). An allelic series of mice bearing different *Qk* mutations, many of which are embryonic lethal, has revealed roles for QK in formation of the heart and vasculature (Justice and Hirschi, 2010). The specific targets of QK, and the specific functions of different QK isoforms, within the developing heart have yet to be elucidated. Another related STAR protein, Sam68, is also found in mouse heart, among a variety of other tissues, but its contributions to cardiac development or function have not been studied (Richard et al., 2005).

Heart and RRM expressed sequence (hermes), the homolog of the human RNA binding protein with multiple splicing type 1 (RBP-MS type 1), is an RNA recognition motif (RRM)-containing RBP (Figure 3) expressed in a variety of embryonic tissues including heart (Gerber et al., 1999). In *Xenopus laevis*, *hermes* is first detected in the developing heart concomitant with the first cardiac differentiation markers shortly before the coalescence of the linear heart tube, and remains high throughout morphogenesis (Gerber et al., 2002; Gerber et al., 1999). Likewise, in the chicken embryo *HERMES* is first detected in a crescent of Nkx-2.5-positive cardiac precursor cells, before expanding to encompass the entire myocardial layer of the heart tube (Wilmore et al., 2005). At later stages, however, its expression is curtailed in the outflow tract and persists primarily in the ventricles and atria (Wilmore et al., 2005). In the mouse, *Hermes* expression is absent in the early heart tube, but becomes widespread following looping, after which its expression becomes increasingly atrial (Gerber et al., 1999). Hermes likely regulates cardiac cell fate, as over-expression in the *Xenopus* embryo leads to dramatic reductions in the expression of cardiac markers, including cardiac α-actin and Nkx-2.5, and failure of heart tube formation (Gerber et al., 2002). This effect seems to be specific to the role of hermes in heart, since over-expression in somites does not result in aberrant skeletal muscle gene expression.

### 2.2. RBPs in cardiomyocyte differentiation and myofibril development

Recent studies investigating transcriptome-wide changes in alternative splicing during differentiation of pluripotent stem cells have highlighted the importance of post-transcriptional RNA processing in cell fate decisions and differentiation (Brandenberger et al., 2004; Cloonan et al., 2008; Pritsker et al., 2005; Salomonis et al., 2009; Salomonis et al., 2010; Yeo et al., 2007). In a comparison of human embryonic stem cells before and after differentiation into either cardiac or neural progenitors, Salomonis and colleagues identified alternative splicing events common to both differentiation pathways, but also events that were specific for the cardiac lineage (Salomonis et al., 2009). To what extent these alternative splicing events direct cardiomyocyte differentiation, and the RBPs responsible for their regulation, however, remain unknown. Knockdown/knockout experiments in fish, frogs, and rodents have identified several RBPs that are important during development for cardiomyocyte-specific gene expression, myofibril assembly, and myocardial function *in vivo* (Figure 2).

RNA binding motif (RBM) proteins are a subgroup of loosely related RRM-containing proteins (Figure 3) with varying functions, including splice site selection and nonsense-

mediated RNA decay (Sutherland et al., 2005). *RBM24* is up-regulated in both human and mouse embryonic stem cells upon differentiation into cardiomyocytes (Miller et al., 2008; Xu et al., 2009). Orthologs of *RBM24* are expressed first in cardiac precursors, and then in the differentiated myocardium in zebrafish, frog, and mouse embryos (Fetka et al., 2000; Maragh et al., 2011; Miller et al., 2008; Xu et al., 2009). Knockdown of *rbm24a/b* in zebrafish embryos results in reductions in sarcomeric proteins, profound disorganization of the myofibril, looping defects, changes in heart rate, and reduced circulation (Maragh et al., 2011; Poon et al., 2012). A second RBM protein, Rbm20, has been shown to regulate the splicing of transcripts encoding titin, a large sarcomeric protein (Guo et al., 2012; Li et al., 2013). Mutations in *RBM20* have been linked with dilated cardiomyopathy in humans, consistent with an important role in regulating myofibril structure and/or function (Brauch et al., 2009; Li et al., 2010; Refaat et al., 2012). Loss of another RBM protein, Rbm15, in mice leads to a variety of embryonic defects, including cardiac abnormalities and heart failure (Raffel et al., 2009), though its role in heart development remains poorly defined.

RNA binding protein, fox-1 homolog (RBFOX) proteins are RRM-containing proteins (Figure 3) that specifically bind to (U)GCAUG elements and regulate alternative splicing (Kuroyanagi, 2009). In addition to the RRM domain, RBFOX1 (also known as A2BP1) possesses two Sm domains, also found in small nuclear ribonucleoproteins, suggesting additional roles in RNA processing or transport (Shibata et al., 2000). RBFOX1 and RBFOX2 (also known as RBM9) are highly expressed in the developing heart, skeletal muscle, and brain, and (U)GCAUG motifs have been found to be enriched in transcripts that are alternatively spliced in these tissues (Bland et al., 2010; Brudno et al., 2001; Das et al., 2007; Gallagher et al., 2011; Jin et al., 2003; Sugnet et al., 2006; Zhang et al., 2008). The splicing activity of RBFOX proteins and splicing patterns of RBFOX targets in the heart are highly conserved across species (Gallagher et al., 2011; Jin et al., 2003; Venables et al., 2012). Although knockdown of individual Rbfox proteins in zebrafish does not result in gross defects, *rbfox1/rbfox2* double morphant embryos exhibit severe skeletal and cardiac muscle defects, including myofibrillar disorganization, disrupted swimming, and reduced cardiac function (Gallagher et al., 2011). Expression of *rbfox2* alone is sufficient to rescue wild type myocardial structure and function, suggesting that it can complement the functions of *rbfox1* in cardiac myofibril development (Gallagher et al., 2011).

For proper assembly and maintenance of myofibrils, and for cardiomyocyte function, connections must be formed and maintained between apposing myofibrils, between myofibrils and the plasma membrane, and between neighboring cardiomyocytes. The RBP Fragile X mental retardation, autosomal homolog 1 (FXR1), has recently been shown to be involved in the proper regulation of these connections (Whitman et al., 2011). FXR1, a homolog of FMR1, which is lost in Fragile X Syndrome, contains two KH domains and an RGG box (Figure 3) (Burd and Dreyfuss, 1994; Siomi et al., 1995). A role in transport and translation regulation of mRNA targets has been proposed for fxr1 during somitogenesis in *Xenopus* (Huot et al., 2005). *Fxr1*-null mice have disorganized cardiac and skeletal muscle, and die within a few hours of birth, likely due to cardiac and respiratory insufficiency (Mientjes et al., 2004). Using these mice, Fxr1 was shown to regulate the expression of Talin2, which is found at the costameres (i.e., myofibril-membrane anchors), and Desmoplakin, which is found at the desmosomes (i.e., cell-cell junctions), in cardiac muscle at the level of translational repression (Whitman et al., 2011). Dysregulation of these proteins results in disruption of desmosome, costamere, and sarcomere structures at the microscopic level, and in overt cardiomyopathy and muscular dystrophy at the macroscopic level (Whitman et al., 2011). The expression and function of Fxr1 in the developing heart and skeletal muscle is conserved in zebrafish (Engels et al., 2004), where knockdown of *fxr1* using antisense morpholinos likewise results in abnormal myotome formation and severe embryonic cardiomyopathy (Van't Padje et al., 2009).

## 3. Cardiac morphogenesis

Following formation of the primitive heart tube, the heart undergoes extensive morphogenesis to transform from a simple tube into a multi-chambered pump that is capable of directing pulmonary and somatic circulation. Left-right asymmetry, established in the early embryo by a combination of asymmetrically deposited signaling molecules and the asymmetric activation of transcription programs, culminates in the heart in cardiac looping, in which right-handed bulging and twisting of the heart tube gives rise to a C-shaped tube with the inflow and outflow tracts both oriented rostrally (Brand, 2003). Differential proliferation distinguishes the “outer curvature” from the “inner curvature” of the heart, and contributes to subsequent expansion and definition of the chambers (Wagner and Siddiqui, 2007). RBPs have been implicated in the regulation of morphogenesis of the valves and septa that then divide the chambers, as well as morphogenesis of the developing myocardium (Figure 2).

### 3.1. Endocardial cushion development

The looped heart tube is subdivided into a four-chambered heart through the formation and remodeling of structures called endocardial cushions in the atrioventricular canal (AVC) and outflow tract (OFT) regions (DeLaughter et al., 2011; Person et al., 2005). In the AVC and OFT, the endocardium is pushed away from the myocardium by the localized expansion of extracellular matrix. The cushions are cellularized by the invasion of mesenchymal cells produced from a subpopulation of endocardial cells via epithelial-to-mesenchymal transition (EMT). Subsequent remodeling into the valves and septa involves condensation and differentiation of the cushion mesenchyme at post-EMT stages (Kirby, 2007). The AVC cushions give rise to the mitral and tricuspid valves, as well as to the atrioventricular septum, and contribute to the atrial and ventricular septa (Kirby, 2007). The OFT cushions give rise to the aortic and pulmonary valves, and transiently septate the OFT during early morphogenesis (Qayyum et al., 2001). Dysregulation of EMT in the heart can lead to valve and septal defects (Person et al., 2005). Although the tissue interactions, growth factors, signaling pathways, and transcription factors involved in regulating endocardial cushion EMT have been well studied (DeLaughter et al., 2011; Person et al., 2005), the roles of post-transcriptional RNA processing in regulating EMT and post-EMT remodeling are much less well understood.

A member of the muscleblind-like (MBNL) protein family, MBNL1, has been implicated in regulating endocardial cushion EMT (LeMasters et al., 2012; Vajda et al., 2009). MBNL proteins bind to RNA via two conserved pairs of zinc knuckle domains (Figure 3), and regulate pre-mRNA alternative splicing, RNA localization, and mRNA stability (Ho et al., 2004; Masuda et al., 2012; Wang et al., 2012). In the chicken embryo, *MBNL1* expression is not detected in the linear heart tube prior to cardiac looping, but is detected in the looped heart prior to the formation of the endocardial cushions (LeMasters et al., 2012; Vajda et al., 2009). In the AVC and OFT, *MBNL1* is strongly expressed in the endocardium (LeMasters et al., 2012; Vajda et al., 2009). Knockdown of *MBNL1* in chick AVC or OFT explants induces enhanced EMT *ex vivo*, indicating that MBNL1 is a negative regulator of EMT in the endocardial cushions (LeMasters et al., 2012; Vajda et al., 2009). Active, secreted TGFβ3 levels are also elevated following MBNL1 knockdown (LeMasters et al., 2012). TGFβ proteins are important for inducing EMT (Arthur and Bamforth, 2011; Kruithof et al., 2012), suggesting MBNL1 may regulate EMT in part via modulation of EMT-inducing signals. MBNL1 preferentially binds to YGCY motifs *in vitro* and *in vivo* (Goers et al., 2010; Wang et al., 2012), and these motifs are highly enriched in known MBNL1-responsive transcripts (Gates et al., 2011; Ho et al., 2004; Wang et al., 2012). Strikingly, a recent study investigating alternative splicing in breast cancer cells found an enrichment of YGCY motifs near exons that exhibited decreased inclusion following EMT (Shapiro et al.,

2011). This suggests MBNL1 may play a general role in regulating alternative splicing transitions during EMT.

A paralog of MBNL1, MBNL2, is also expressed in the embryonic heart (Fardaei et al., 2002; Fernandes et al., 2007; Kanadia et al., 2003b; Liu et al., 2008), but its temporal and spatial distribution within the developing heart, and whether it plays a role in EMT, have not yet been investigated. Knockdown of *mbnl2* in zebrafish does result in cardiac abnormalities, however, including dilation and disorganization of the myofibril, and mis-regulated alternative splicing of *tnnt2*, a known target of MBNL proteins in the myocardium (Machuca-Tzili et al., 2011).

Additional RBPs have also been implicated in regulating alternative splicing during EMT. Epithelial splicing regulatory proteins (ESRP) 1 and 2 are RRM-containing RNA binding proteins that have been shown to regulate hundreds of alternative splicing events during EMT (Dittmar et al., 2012; Warzecha et al., 2009a; Warzecha et al., 2009b). Knockdown and over-expression studies have suggested that changes in ESRP expression are determinative for EMT and its converse, MET (Reinke et al., 2012; Shapiro et al., 2011; Warzecha et al., 2010), but in a recent study neither *Esrp1* nor *Esrp2* were detected in the embryonic heart (Revil and Jerome-Majewska, 2013). Motif analysis of intronic regions flanking EMT-regulated cassette exons not only suggests common regulatory roles for MBNL and ESRP proteins in EMT-specific alternative splicing, but also members of the polypyrimide tract binding protein (PTB), hnRNP, and RBFOX families (Shapiro et al., 2011). None of these have yet been interrogated for a role in endocardial cushion development.

### 3.2. Myocardial trabeculation and compaction

Myocardial cells are highly dynamic during cardiac morphogenesis, undergoing epithelialization, proliferation, and compaction as trabeculae form and then coalesce in the ventricular wall (Harvey and Rosenthal, 1999). The trabeculae consist of protrusions into the lumen of the heart, and are made up of poorly developed but strongly coupled cells, which contribute to the ventricular conduction system (Moorman and Christoffels, 2003). The trabeculae also increase the surface area of the thickening tissue, which is critical for oxygenation of the heart before the coronary arteries form. Although the anatomical changes that occur during trabeculation and compaction have been well described, the mechanisms that control these processes are not well understood.

Cardiac helicase activated by MEF2 protein (CHAMP) is an RNA helicase expressed specifically in the myocardium during prenatal and postnatal development (Liu et al., 2001). CHAMP is a member of the RNA helicase superfamily I, and shares several conserved motifs with helicases involved in DNA replication, transcription, and RNA processing (Figure 3) (Liu et al., 2001). Embryonic expression of CHAMP begins in the linear heart tube following the initiation of MEF2C expression. During trabeculation, CHAMP is strongly expressed in the non-proliferative trabecular cardiomyocytes, but not in the proliferative compact zone (Liu et al., 2001). This regional localization, as well as spatiotemporal similarities between the expression of CHAMP and both neurotrophin-3 and its receptor, Trk C, which regulate cardiomyocyte proliferation (Lin et al., 2000), led to speculation that CHAMP plays a role in repressing cardiomyocyte proliferation and growth during myocardial morphogenesis (Liu et al., 2001). Although this has not been experimentally confirmed *in vivo*, over-expression of CHAMP in primary neonatal cardiomyocytes inhibits cellular hypertrophy and leads to the up-regulation of the cell cycle inhibitor p21 (Liu and Olson, 2002).

Interestingly, CHAMP is a variant of the testis-specific helicase, MOV10 like-1 (MOV10l1), generated by alternative promoter usage, using a start codon within exon 14 (Liu et al., 2001). A second cardiac-specific helicase, cardiac-specific isoform of Mov10l1 (Csm), is also generated by alternative promoter usage from the MOV10l1 locus; its start codon lies within exon 16 (Ueyama et al., 2003). Despite their similar origins, the two helicases have distinct effects. While Csm potentiates cardiomyocyte hypertrophy induced by phenylephrine treatment (Ueyama et al., 2003), CHAMP over-expression is able to block this hypertrophy (Liu et al., 2001). The expression and role of Csm in embryonic heart development has not been investigated.

## 4. Postnatal maturation of the heart

Although the four-chambered architecture of the heart is established during embryogenesis, the rerouting of circulation to the lungs, switch from hyperplastic to hypertrophic growth, and increase in workload on the heart after birth prompts extensive molecular and cellular remodeling during early postnatal life. This remodeling involves changes in the expression of growth factors, cell cycle regulators, contractile and cytoskeletal proteins, and ion channels (Chen et al., 2004; Harrell et al., 2007; MacLellan and Schneider, 2000; Siedner et al., 2003), and is not limited to changes in transcription, but also includes changes in post-transcriptional RNA processing.

### 4.1. CELF- and MBNL-mediated alternative splicing programs

Many cardiac transcripts undergo fetal-to-adult changes in alternative splicing (Kalsotra et al., 2008; Park et al., 2011). Several families of RBPs have been implicated in fetal-to-adult reprogramming of alternative splicing in the heart (Figure 2). A study using splicing-sensitive microarrays identified fetal-to-adult splicing transitions in the developing mouse heart, many of which were conserved between mammals and birds (Kalsotra et al., 2008). Computational analyses identified several motifs that were highly enriched near the developmentally-regulated exons, including putative binding sites for hnRNP, PTB, STAR, RBFOX, MBNL, and CUGBP, Elav-like family (CELF) proteins (Kalsotra et al., 2008). Consistent with this, some of these RBPs were shown to be developmentally regulated. RBFOX1 transcript and protein levels are robustly up-regulated shortly after birth, whereas those of RBFOX2 decline slightly in the adult heart (Kalsotra et al., 2008). MBNL1 is up-regulated over the course of embryonic and postnatal heart development (Kalsotra et al., 2008; Terenzi and Ladd, 2010). CELF1 and CELF2 exhibit higher protein levels in embryonic than adult heart, and are down-regulated during early postnatal life (Kalsotra et al., 2008; Ladd et al., 2005a). Interestingly, the down-regulation of CELF proteins during heart development occurs without a change in *CELF* transcript levels, indicating that these RBPs are themselves post-transcriptionally regulated. Mechanisms include phosphorylation-driven changes in CELF1 protein stability and miRNA-mediated repression of *CELF1* and *CELF2* translation (Kalsotra et al., 2010; Kuyumcu-Martinez et al., 2007).

Strikingly, over half of the fetal-to-adult splicing transitions identified by Kalsotra and colleagues respond to over-expression of CELF1 or loss of MBNL1 in the hearts of genetically modified mice, suggesting these proteins are determinative for driving these developmental transitions (Kalsotra et al., 2008). A subset of these splicing events is regulated by both CELF1 and MBNL1 in an antagonistic manner. Although a cardiac phenotype has not yet been described for *Mbnl1*-null mice (Kanadia et al., 2003a), over-expression or repression of CELF proteins in the early postnatal myocardium leads to dysregulation of CELF-mediated alternative splicing and rapid onset of cardiac dysfunction in transgenic mice (Koshelev et al., 2010; Ladd et al., 2005b; Terenzi et al., 2009). The juvenile onset of cardiomyopathy, as well as the spontaneous recovery of cardiac function following maturity in a line of mice with mild repression of CELF activity (Terenzi et al.,

2009), further supports a role for CELF-mediated alternative splicing programs specifically in postnatal remodeling.

CELF and MBNL proteins are found in the cytoplasm as well as the nucleus in the developing heart (Blech-Hermoni et al., 2013; Kalsotra et al., 2008; Ladd et al., 2005a), suggesting that the effects of these RBPs in postnatal maturation are likely not restricted to pre-mRNA alternative splicing. Although their cytoplasmic roles have not been investigated in the heart, in developing skeletal muscle CELF1 regulates the translation of *Mef2A* and *p21*, key regulators of muscle-specific gene expression and growth arrest (Timchenko et al., 2004), and both CELF1 and MBNL1 have been shown to regulate the stability of a large number of muscle transcripts (Masuda et al., 2012). Interestingly, CELF1 promotes decay of *Mbnl1* transcripts and MBNL1 promotes decay of *Celf1* transcripts, suggesting that these factors mutually contribute to their reciprocal patterns of expression during myogenesis (Masuda et al., 2012).

### 4.2. Roles of the SR protein family in the maturing heart

Members of the serine/arginine-rich (SR) protein family have also been implicated in fetal-to-adult cardiac reprogramming. There are twelve human SR proteins, SRSF1–12, each characterized by the presence of one or two RRMs and a carboxy-terminal RS domain enriched with arginine/serine dipeptides that functions as a protein:protein interaction domain (Figure 3) (Twyffels et al., 2011). SR proteins regulate splicing of both constitutive and alternative exons, mRNA export, stability, and translation (Shepard and Hertel, 2009; Twyffels et al., 2011). SR proteins also promote the processing of some miRNAs by facilitating cleavage by Drosha (Wu et al., 2010). The subcellular localization and activities of SR proteins are regulated in part through phosphorylation by SR protein-specific kinases (Zhou and Fu, 2013). Depletion of SRSF1 (formerly known as ASF/SF2) induces apoptosis in the DT40 chicken B-cell line (Li et al., 2005; Wang et al., 1996), and germline deletions of *Srsf1*, *Srsf2* (formerly known as *SC35*), or *Srsf3* (formerly known as *SRp20*), result in early embryonic lethality (Jumaa et al., 1999; Wang et al., 2001; Xu et al., 2005). Together, these studies have suggested essential, non-redundant roles for multiple SR proteins in cell viability. Cardiac-specific knockouts of two of these essential SR proteins, however, indicate that they have additional roles in regulating contractile function in developing heart muscle.

Cardiac-specific ablation of *Srsf1* or *Srsf2* was accomplished by crossing mice with floxed alleles with the MLC-2v-Cre transgenic line (Ding et al., 2004; Xu et al., 2005). Both cardiac-specific *Srsf1*- and *Srsf2*-null mice are healthy at birth, but develop early onset cardiomyopathy within the first four weeks of life (Ding et al., 2004; Xu et al., 2005), corresponding to the period of postnatal remodeling. Cardiomyocyte apoptosis was not observed in either model, but both displayed defects in excitation-contraction coupling (Ding et al., 2004; Xu et al., 2005). In cardiac-specific *Srsf1*-null mice this has been attributed at least in part to altered splicing of $Ca^{2+}$/calmodulin-dependent kinase IIδ (CaMKIIδ), and transgenic over-expression of the inappropriate CaMKIIδ splice form was sufficient to phenocopy the defects in calcium handling in these mice (Xu et al., 2005).

SRSF10 (formerly known as SRp38) has also been implicated in regulating calcium handling in the developing heart. Unlike SRSF1, SRSF10 is not essential for viability in DT40 cells, although its loss does impair recovery from stress (Shin et al., 2004). Germline ablation of *Srsf10* does not result in the early embryonic lethality seen in other SR protein gene knockouts, but nonetheless few homozygous *Srsf10*-null fetuses reach full term (Feng et al., 2009). Most *Srsf10*-null embryos die by embryonic day E15.5 and exhibit multiple cardiac abnormalities, including septal defects, thinning of the myocardium, and altered intracellular calcium handling in embryonic cardiomyocytes (Feng et al., 2009). It was

suggested that the calcium handling defects may be due to changes in the level and alternative splicing of *triadin* (Feng et al., 2009), though this is unlikely to contribute to the other developmental defects in *Srsf10*-null mice as *triadin*-null mice are viable with no obvious cardiac malformations (Shen et al., 2007). The earlier onset of cardiac dysfunction and presence of additional defects in *Srsf10*-null versus cardiac-specific *Srsf1*- and *Srsf2*-null mice indicates that although multiple SR proteins are expressed in the heart, they play distinct roles in cardiac development.

## 5. Developmental dysregulation and disease

Dysregulation of developmental programs is often seen in disease states. Disruption of RBP function during embryogenesis can disrupt proper formation of the heart, leading to congenital heart disease. Dysregulation of developmental RBP pathways in the adult heart, however, can also perturb cardiac function and contribute to acquired heart disease.

### 5.1. RNA binding proteins and congenital heart defects

Several RBPs have been linked with syndromes characterized by congenital heart defects. Although a causal link has not been made in either case, *CELF2* has been proposed as a candidate gene for congenital heart defects associated with two genetic disorders, partial monosomy 10p (Lichtner et al., 2002) and familial arrhythmogenic right ventricular dysplasia (Li et al., 2001). A single case report has linked a partial deletion of the *RBFOX1* gene with complex heart defects (Lale et al., 2011), but further genetic evidence of *RBFOX1* mutations causing developmental defects in humans is lacking.

Holt-Oram syndrome (HOS) is an autosomal dominant disorder characterized by upper limb abnormalities and a spectrum of cardiac birth defects, most typically septal and conduction defects (Mori and Bruneau, 2004). HOS is caused by a variety of mutations in the gene encoding the transcription factor TBX5, but the underlying mechanism of pathogenesis may not be limited to dysregulated transcription. TBX5 has been shown to form an RNA-dependent complex with the SR protein SRSF2, and affect constitutive and alternative splicing of reporter minigenes in cells (Fan et al., 2009). Strikingly, a severe mutation in *TBX5* (G80R) associated with complete penetrance of cardiac defects strongly affects this splicing activity, whereas a less severe mutation (R237Q) associated with incomplete cardiac penetrance does not (Fan et al., 2009).

DiGeorge Syndrome (DGS) is caused in most cases by a deletion at the genomic locus 22q11.2, and is characterized by a constellation of birth defects attributable to disruption of neural crest cell development, including craniofacial and cardiac defects (Keyte and Hutson, 2012). The transcription factor gene *TBX1* is a leading candidate for DGS pathogenesis, but *Tbx1* is not expressed in cardiac neural crest cells in mice (Garg et al., 2001; Vitelli et al., 2002). The *DiGeorge critical region 8* (*DGCR8*) gene also lies within the 22q11.2 region, and encodes a double-stranded RBP essential for miRNA biogenesis (Seitz and Zamore, 2006). Genetic inactivation of *Dgcr8* specifically in neural crest cells in mice leads to cardiac malformations typical of DGS, including persistent truncus arteriosis, aortic arch malformations, and septal defects (Chapnik et al., 2012), supporting the idea that loss of this RBP contributes to the cardiac defects in DGS patients.

Mutations in *RBM10* have been implicated in talipes equinovirus, atrial septal defect, Robin sequence (micrognathia, glossoptosis, and cleft palate), and persistence of the left superior vena cava (TARP), a rare X-linked disorder with severe congenital defects affecting multiple organs including the heart (Gripp et al., 2011; Johnston et al., 2010). Although the molecular functions of RBM10 are not well characterized, it was identified in a proteomic

analysis of the human spliceosome (Rappsilber et al., 2002), and has been shown to regulate the stability of at least one mRNA (Mueller et al., 2009).

### 5.2. The recapitulation of fetal programs during adult heart disease

During heart disease, there is a partial reactivation of both transcriptional and post-transcriptional fetal gene expression programs. Many alternative splicing and alternative polyadenylation site choices found in the hypertrophic heart are more similar to those found at fetal stages than in healthy adults, indicating that patterns of fetal RNA processing are reestablished in response to pressure overload (Ames et al., 2013; Park et al., 2011). These differences are likely mediated by RBPs that become dysregulated during disease. For example, RBFOX1 is down-regulated while PTB is up-regulated during cardiac hypertrophy (Park et al., 2011). Alterations in alternative splicing are associated with heart disease in both mice and humans (Ames et al., 2013; Kong et al., 2010; Park et al., 2011; Song et al., 2012). Polymorphisms that affect alternative splicing of cardiac transcripts have also been linked with susceptibility to myocardial infarction and cardiac hypertrophy (Komamura et al., 2004; Mango et al., 2005).

Dysregulation of mRNA decay is another important driver of gene expression changes during cardiovascular disease. The levels of AUF1 (also known as hnRNP D), an RBP of the hnRNP family (Figure 3) that destabilizes transcripts via binding in the 3′ untranslated region (3′ UTR) (Misquitta et al., 2001), are elevated in cardiomyocytes in response to stress and in the hearts of human patients with heart failure (Glaser et al., 2006; Pende et al., 1996). The increase in AUF1 is strongly associated with a reduction in β-adrenergic receptor mRNA levels and impaired calcium handling in heart failure (Misquitta et al., 2006). AUF1 is expressed in the embryonic mouse heart (Gouble and Morello, 2000), but is normally undetectable in the adult heart (Lu and Schneider, 2004), suggesting that this may also represent the reactivation of a fetal decay program in adult heart disease.

Although the reemergence of fetal gene expression in the heart is generally thought to be compensatory, the reiteration of fetal programs in adult tissues can itself be pathogenic. In myotonic dystrophy (*dystrophia myotonica*, DM), the expression of mutant RNAs containing expanded CUG or CCUG repeats leads to a panoply of symptoms including electrocardiographic and echocardiographic anomalies, skeletal muscle myotonia, muscle wasting and weakness, neurological abnormalities, and endocrine dysfunction (Schoser and Timchenko, 2010). The expression of expanded repeat-containing RNAs disrupts a number of RBPs in DM cells, but pathogenesis is thought to be largely attributable to a gain of CELF1 function and loss of MBNL1 function that mirror embryonic expression patterns of these factors (Schoser and Timchenko, 2010). The reiteration of fetal alternative splicing patterns in adult DM tissues has been linked directly to patient symptoms (Charlet-B. et al., 2002; Savkur et al., 2001). Over-expression of CELF1 or deletion of MBNL1 in mice is sufficient to reestablish fetal CELF/MBNL-mediated alternative splicing patterns and mimic DM phenotypes (Ho et al., 2005; Kanadia et al., 2003a; Koshelev et al., 2010; Timchenko et al., 2004; Ward et al., 2010). Conversely, restoration of MBNL1 levels or repression of CELF activity rescues normal adult alternative splicing patterns and reduces pathogenesis in a DM mouse model (Berger and Ladd, 2012; Kanadia et al., 2006; Warf et al., 2009; Wheeler et al., 2009).

## 6. Concluding remarks

RNA binding proteins provide a robust and versatile mechanism for regulating gene expression. In eukaryotes, the regulation of alternative splicing of pre-mRNAs by RBPs underlies a great expansion in the proteome (Nilsen and Graveley, 2010), allowing for the production of multiple gene products from the majority of gene loci (Pan et al., 2008; Wang

et al., 2008). RNA editing by RBPs can not only expand the proteome (Maas, 2010; Rosenthal and Seeburg, 2012), but is also important in the modification of non-coding RNAs (such as snoRNAs and miRNAs) and in viral attenuation (Mallela and Nishikura, 2012). Transport, localization, and regulation of RNA stability by RBPs allow for direct control of the spatial and temporal profiles of gene products (Pratt and Mowry, 2013; Weis et al., 2013), such as in the establishment of zygote polarity. Finally, regulation of translation by RBPs allows for the selective fine-tuning of protein production, and is particularly powerful in early zygotic events, during which maternal transcripts must be translated in the absence of a transcriptional apparatus (Lee and Schedl, 2006). All these mechanisms regulate gene expression without the need to transcribe new RNA, and thus make it possible to respond to external stimuli or developmental cues with remarkable speed and specificity.

This review describes the diverse RBP toolkit employed in the developing heart, which is involved in differentiation, morphogenesis, structure, and function. In contrast to other tissues, such as the nervous system (Boutz et al., 2007; Gao and Taylor, 2012; McKee et al., 2005; Okano and Darnell, 1997; Perrone-Bizzozero and Bolognani, 2002; Yano et al., 2010), the identities and functions of RBPs in the developing heart have been pursued with significantly less vigor. While the importance of the heart cannot be disputed, its early appearance and small size in the embryo make the ability to investigate this developing organ technically difficult. Fortunately, the ability to investigate RNA processing (such as RNA editing, alternative splicing, or transcript occupancy by RBPs) using the small amounts of tissue available from often microscopic embryos has recently become feasible thanks to the introduction of advanced methods such as laser-capture microdissection, high-throughput sequencing, and large-scale bioinformatic and computational analyses (Kishore et al., 2010; Wang et al., 2009).

A key challenge facing investigators as they adapt and apply these new tools is the identification of endogenous targets of the RBPs under study. For instance, while the splicing activity of regulators of alternative splicing has been investigated using artificial minigenes designed to mimic splicing substrates (Cooper, 2005), few *bona fide* targets have been identified for many of these regulators *in vivo*. At the same time, transcriptome analyses have indicated that the vast majority of genes (> 90%) are alternatively spliced (Pan et al., 2008; Wang et al., 2008), but have not linked these events to specific RBPs. Unlike DNA binding proteins, RBPs often do not have specific binding motifs. Instead, they bind with variable affinities to a range of sequences or secondary structures, with some preference for nucleotide content or morphology. This makes computational approaches to finding RBP binding sites challenging. Direct biochemical investigations of RBP binding have focused on identifying the highest affinity interactions, yet it is believed that most RBP:RNA interactions are not strong or are transient, and it is the relationships between RBPs and auxiliary factors that increase binding specificity (Burd and Dreyfuss, 1994; Lunde et al., 2007). The role of weak or suboptimal binding in RNA processing remains to be elucidated (Pickrell et al., 2010).

With a large number of potential targets for each RBP, another important question to address is how these specific targets contribute to developmental processes. In addition to directly affecting the expression of specific protein isoforms involved in cell fate or morphogenesis, RBPs can exert control over key regulatory proteins. For example, the activities of both transcription factors (e.g., Belaguli et al., 1999) and splicing factors (e.g., Terenzi and Ladd, 2010) have been shown to be regulated in the heart by alternative splicing. Thus the identification and elucidation of RBP programs at key developmental stages may shed light on how signaling molecules, their receptors, and their downstream mediators are controlled at multiple levels. The burgeoning appreciation for the importance of RBPs during normal

development and in disease states, along with the rise of the technology necessary to properly interrogate their regulatory programs, will doubtless contribute important new insights into the formation and function of the developing heart. In time, RBPs and the transcripts they regulate during heart development may provide attractive targets for the design of treatments for congenital heart defects, cardiovascular disease, or cardiac tissue repair.

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## Abbreviations

| | |
|---|---|
| **3′ UTR** | 3′ untranslated region |
| **AVC** | atrioventricular canal |
| **CELF** | CUG-BP, Elav-like family |
| **CHAMP** | cardiac helicase activated by MEF2 protein |
| **Csm** | cardiac-specific isoform of Mov10l1 |
| **DGCR8** | DiGeorge Syndrome critical region gene 8 |
| **DM** | dystrophia myotonica (myotonic dystrophy) |
| **DGS** | DiGeorge Syndrome |
| **EMT** | epithelial-to-mesenchymal transition |
| **ESRP** | epithelial splicing regulatory protein |
| **FXR1** | Fragile X mental retardation autosomal homolog 1 |
| **HERMES** | heart and RRM expressed sequence |
| **hnRNP** | heterogeneous nuclear ribonucleoprotein |
| **how** | held out wings |
| **KH domain** | hnRNP K homology domain |
| **MBNL** | muscleblind-like |
| **MET** | mesenchymal-to-epithelial transition |
| **miRNA** | microRNA |
| **OFT** | outflow tract |
| **PTB** | polypyrimidine tract binding protein |
| **RBFOX** | RNA binding Fox-1 homolog |
| **RBM** | RNA binding motif |
| **RBP** | RNA binding protein |
| **RISC** | RNA-induced silencing complex |
| **RRM** | RNA recognition motif |
| **RS domain** | arginine/serine-rich domain |
| **SRSF** | serine/arginine-rich splicing factor |
| **STAR** | signal transduction and activation of RNA |

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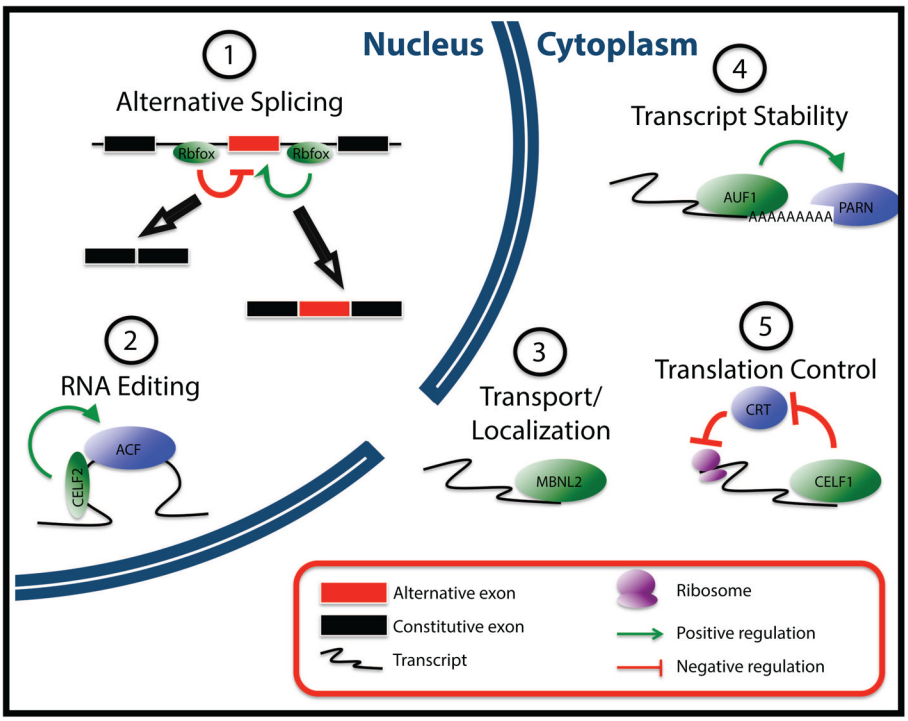


**Figure 1. Mechanisms of RBP-mediated post-transcriptional regulation**

Schematic representations of mechanisms by which a number of proteins described in this review have been shown to regulate gene expression. Note that these are provided as examples; an exhaustive survey of RBP-mediated regulatory mechanisms is beyond the scope of this review. *(1)* RBFOX proteins regulate a variety of alternative splicing events by binding within introns flanking alternative exons. Binding upstream of an exon leads to skipping of that exon, while binding downstream of an exon leads to its inclusion (De Craene and Berx, 2013). *(2)* CELF2 directs the editing of a cytidine in the *Apob* transcript by binding to an AU-rich sequence element upstream of the editing site and recruiting ACF, a component of the editing machinery (Anant et al., 2001). *(3)* MBNL2 regulates the transport and localization of the *Itga2* transcript to the plasma membrane by binding to a zipcode sequence in the 3′ UTR of the transcript (Adereth et al., 2005). *(4)* Multiple mechanisms have been proposed for how AUF1 regulates the stability of target transcripts, including the recruitment of the PARN deadenylase, leading to loss of the poly-A tail and rapid degradation of the RNA (White et al., 2013). *(5)* CELF1 enhances translation of the *p21* transcript by antagonizing a regulatory protein, CRT, which normally blocks ribosome loading (Iakova et al., 2004).

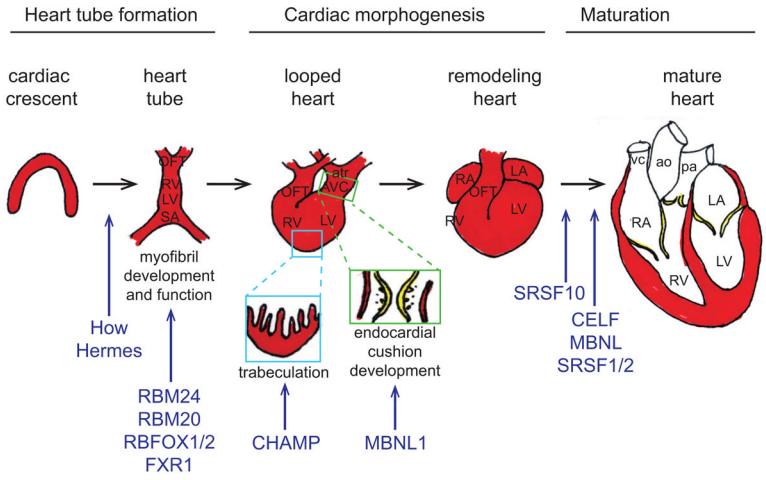


**Figure 2. RNA binding proteins have been implicated in the formation, morphogenesis, and maturation of the heart**

The primitive heart tube forms from precardiac mesoderm within the cardiac crescent. The heart tube undergoes extensive morphogenesis, including cardiac looping, endocardial cushion formation and remodeling, and myocardial trabeculation and compaction. Although the architecture of the heart is established during embryogenesis, maturation of the heart continues through postnatal life. RNA binding proteins that have been implicated in specific steps of heart development are indicated in blue. Abbreviations: OFT, outflow tract; RV, right ventricle; LV, left ventricle; SA, sinoatrial segment; atr, common atrium; AVC, atrioventricular canal; LA, left atrium; RA, right atrium; vc, vena cava; ao, aorta; pa, pulmonary artery.

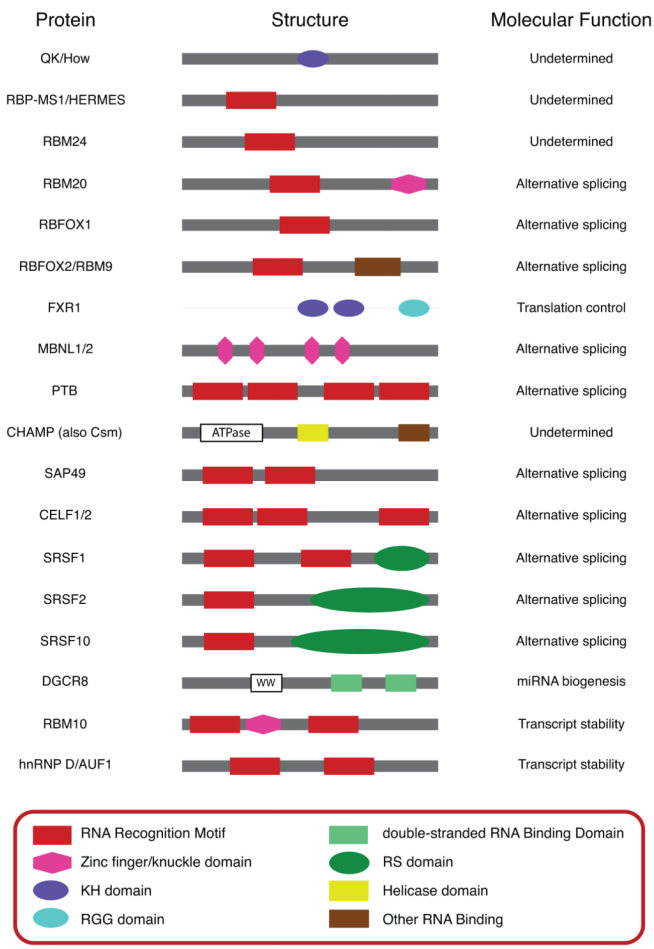


**Figure 3. Domain structure of RNA binding proteins implicated in regulation of heart development**

Schematic representations of the type and position of important domains within the RNA binding proteins described in this review are shown. RNA binding domains and other domains characteristic of these RNA binding protein families are shown in color; other conserved domains are shown in black and white. Proteins and domains are not drawn to scale. Molecular functions of these RNA binding proteins within the heart are indicated, if known; additional functions of these proteins that have been demonstrated in other tissue types are not shown. The domain structures of these proteins are highly conserved across vertebrate species, and the depicted structures represent all homologs described in the text. Note that the structure of QK/How is conserved from human to fly, whereas to date CHAMP/Csm has only been described in mammals.