UNIVERSITY OF CALIFORNIA, SAN DIEGO

Regulation of RNA splicing in the heart by rbFox1l

A thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Michael Scott Dickover

Committee in charge:

Professor Neil Chi, Chair
Professor Deborah Yelon, Co-chair
Professor Yishi Jin

2011

The Thesis of Michael Scott Dickover is approved and it is acceptable in quality and form for publication on microfilm and electronically:

(Co-Chair)

(Chair)

University of California, San Diego

2011

This thesis is dedicated to everyone who has helped me and supported me throughout college, especially my parents.

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ACKNOWLEDGMENTS

I would first like to acknowledge Dr. Neil Chi for giving me the opportunity to work in his lab and for constantly inspiring me and motivating me to do great science.

Next I would like to acknowledge all the members of the Chi lab, past and present, who have taken the time to teach me and help me progress in the lab.

I would also like to acknowledge Mike Lovci and Gene Yeo for inviting us to work on this project with them and for the advice and data they have provided us.

I would like to acknowledge Yi-Fan Lin and Deborah Yelon for the *Tg(cmlc2:α-actinin-GFP)* line. It was extremely useful in our studies.

Finally, I would like to acknowledge Julia Lipianskaya, Neil Tedeschi, and Justin Sun for their help in creating the *rbFox1*, *rbFox1l, and rbFox2* RNA probes.

ABSTRACT OF THE THESIS

Regulation of RNA splicing in the heart by rbFox1l

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Alternative splicing of primary RNA transcripts greatly diversifies the amount of proteins encoded by an animal's genome. Through alternative splicing, a single gene sequence can produce several structurally and functionally different proteins. Because changes in splicing can result in proteins with different functions, improper splicing can have drastic effects on a cell. Therefore, splicing must be closely regulated by both *cis*- and *trans*-acting factors. To

understand how splicing factors may impact cardiac development and function, we used an antisense morpholino oligonucleotide to knock down the expression of the muscle-specific splicing regulator, *rbFox1l*, which is normally expressed in the developing heart and skeletal muscle of zebrafish embryos. *rbFox1l* morphant embryos develop dysmorphic hearts with decreased cardiac function. In this thesis, we begin to elucidate the underlying mechanisms of how rbFox1l impacts alternate splicing in order to regulate cardiac development and function.

I.

Introduction

Alternative Splicing

The health and survival of a cell depends on the synthesis of properly functioning proteins because these molecules are responsible for carrying out the majority of tasks within a cell. Gene sequences in the DNA encode the design for each protein. However, a single gene can produce several different forms of a protein and each form may have a different function (Wahl et al. 2009). This is accomplished through alternative splicing of the primary RNA transcript (pre-mRNA). This transcript includes all non-protein-coding introns and protein-coding exons of the gene. During splicing of the pre-mRNA, the introns are removed and different combinations of exons are reconstituted into the final mRNA transcript. This inclusion or exclusion of specific exons greatly increases the possible protein products encoded by each gene.

The pre-mRNA sequence contains *cis*-acting factors that dictate which regions are excised and which are retained. The 5' and 3' ends of each intron contain consensus sequences that act as the 5' and 3' splice sites. Upstream of the 3' splice site is the branch point sequence. A hydroxyl group from an adenosine residue in this sequence first attacks a phosophodiester bond in the 5' splice site, releasing the 5' end of the transcript, and creating a lariat structure in the intron attached to the 3' end of the transcript. Next the free hydroxyl group at the end of the 5' exon attacks a phosphodiester bond at the 3' splice site, releasing the intron lariat structure, and ligating the 5' exon and the 3' exon together. After each intron is spliced out in this manner, the result is a continuous

strand of coding sequence with an open reading frame that can be directly translated into a protein.

Various *trans*-acting factors mediate the proper handling and splicing of pre-mRNA transcripts. A ribonucleoprotein complex known as the spliceosome catalyzes the splicing reaction (Wahl et al. 2009). There are four small nuclear ribonuclearprotein (snRNP) complexes that comprise the spliceosome: U1, U2, U4/U6, and U5. These components recognize sequences in the pre-mRNA located at the intron-exon junctions and thus mediate the splicing reaction. The U1 snRNP binds to the 5' splice site through base pair recognition between the U1 snRNA and the splice site sequence. After this complex is stabilized through the recruitment of other small proteins, the U2 snRNP binds to the branch point sequence in an ATP-dependent manner. Once again, other small nuclear proteins stabilize this interaction. The U4/U6 and U5 complexes are preassembled in the nucleus and are the last to bind to the transcript and finalize the formation of the spliceosome. U1 and U4 are then released which results in the activation of the spliceosome. The remaining components catalyze the transesterification reactions that lead to the removal of the intron and the joining of the flanking exons. This process requires the cooperation of many different nuclear proteins and is often differentially regulated.

Other *cis*-acting factors in the pre-mRNA transcript can influence the behavior of *trans*-acting factors, which may ultimately affect the efficiency of splicing around an exon. While some exons are constitutively included in the

mRNA transcript, others may be alternatively spliced, meaning they may be included or excluded from the transcript depending on the context. Two important groups of *trans*-acting factors are the SR proteins and the heterogenous nuclear ribonucleoproteins (hnRNPs). The SR proteins have a serine and arginine rich domain and a conserved RNA recognition motif. They are generally thought to bind to exon splicing enhancers at the end of exons and promote the binding of the U1 snRNP to the proximal 5' splice site. The sequence of some 5' splice sites may have a low affinity for U1 binding, but the presence of an SR protein may increase this affinity, enhancing the use of the proximal 5' splice site. The hnRNPs are thought to have a repressive effect on a splice site. They generally bind to exon splicing silencers and decrease the use of any proximal splice sites. The levels of SR proteins and hnRNPs vary between cell types, allowing these *trans*-acting factors to influence how particular transcripts are spliced.

The presence or absence of a certain exon changes the structure of the resulting protein, and as such, changes in splicing can have a significant effect on a cell. Other splicing factors exist that can more tightly regulate the process, and the expression of some of these factors is restricted spatially and temporally. The neuron specific factor Nova-1, for example, is required for neuronal survival and alternative splicing of specific exons for some inhibitory receptor pre-mRNAs (Jensen et al. 2000). Loss of Nova-1 resulted in motor function failure and death in postnatal mice. Aberrant splicing of exons in the glycine and $GABA_A$ receptor pre-mRNAs was also found. In striated muscle, exon 5 of the cardiac Troponin T

(cTNT) transcript is differentially spliced during development (Cooper and Ordahl 1985). In embryonic striated muscle, this exon is included in the mRNA, but in adult tissue the exon is excluded. The two different protein products have different calcium sensitivity, which affects the contractile properties of the myofibrils. A conserved muscle-specific splicing enhancer site flanking the exon is targeted by the splicing factor ETR-3, a member of the CELF protein family (Ladd et al. 2001). ETR-3 itself changes from a low molecular weight form to a high molecular weight form during heart development through alternative splicing of its pre-mRNA. This switch in isoforms coincides with the switch to exon 5 skipping in the heart. This suggests that ETR-3 developmentally and tissue specifically regulates splicing of cTNT. Alternative splicing appears to be a highly conserved and tightly regulated process in metazoans.

Alternative splicing is a concerted effort between multiple *cis* and *trans*-acting factors and mutations in either can have drastic effects. Some cases of cystic fibrosis are exacerbated by mutations in a splice site in intron 8 of the *CFTR* gene (Buratti et al. 2004). A string of TG repeats in this region sometimes becomes expanded and increases instances of exon 9 skipping. This results in a nonfunctional protein and increases the severity of the disease. In the neurodegenerative disease myotonic dystrophy type 1 (DM1), an expansion of CTG repeats in the *DMPK* gene results in a mutant RNA with long CUG tracts that form hairpin structures (Lee and Cooper 2009). These CUG repeats are bound by a splicing factor called muscleblind-like1 (MBNL1), causing them to

become sequestered by the mutant RNA and unable to regulate alternative splicing of other RNAs in the nucleus. Several targets of MBNL1 are spliced incorrectly in individuals with DM1 suggesting that sequestration of MBNL1 is at least partially responsible for some symptoms of the disease. These examples demonstrate the severity of possible effects that improperly regulated splicing can have on cells.

The fox-1 family of splicing factors

The *fox-1* (*feminizing locus on X*) gene was originally identified as a gene involved in *C. elegans* sex selection, where it regulates splicing of other genes in the pathway (Meyer 2000). High expression of *xol-1* in nematodes causes the animal to develop as a male. This gene has three transcripts, but only one of these produces a functional protein. Fox-1 most likely represses expression of the functional mRNA by influencing splicing of the *xol-1* pre-mRNA. The Fox-1 family of proteins is highly conserved throughout vertebrates (Jin et al. 2003). Mice and humans have three members: Fox-1 (A2BP1), Fox-2 (RBM9), and Fox-3 (HRNBP3, NeuN). Zebrafish have several homologues due to the genome duplication that has occurred in teleosts. Of these, only Fox-1 like (rbFox-1l) has been well described. The proteins all contain a very highly conserved RNA recognition motif (RRM). The N and C termini are less conserved, although the C terminal is required for the regulation of splicing (Fukumura et al. 2007). The Fox

proteins have been shown to be a highly conserved family of tissue-specific splicing regulators.

Fox-1 is expressed in the brain and striated muscle (skeletal and cardiac muscle) of vertebrates (Jin et al. 2003). *In vitro* selection experiments and gel shift analyses revealed that zebrafish rbFox1l binds specifically to the sequence GCAUG in RNA transcripts. This sequence has previously been identified as a necessary component of exon EIIIB inclusion in the fibronectin gene and it was later confirmed that Fox-1 regulates this splicing event by binding to its consensus sequence in the downstream flanking intron (Huh and Hynes 1994, Jin et al. 2003). Fox-1 binding was also found to regulate splicing of several other exons that are dependent on GCAUG elements. Skipping of a non-muscle specific exon in human mitochondrial ATP synthase γ subunit (F1γ) transcript occurs when Fox-1 binds to its consensus sequence in the upstream intron. Fox-1 also allows switching from the non muscle-specific exon to a muscle-specific exon in the rat α-actinin transcript. Thus, Fox-1 is able to promote inclusion, skipping, or switching of exons in pre-mRNA transcripts containing GCAUG sequences.

Nervous system conditional Fox-1 knockout mice (Nestin:Cre x Fox-$1^{loxp/loxp}$) are susceptible to spontaneous seizures, suggesting that Fox-1 regulates a splicing program important for electrical excitability (Gehman et al. 2011). An exon junction microarray was performed in order to determine splicing changes in Fox-1 null brain tissue. Twenty transcripts were found in which splicing was

significantly altered in the absence of Fox-1. Several of these targets were L-type calcium channel subunits, other ion channels, and their binding partners. Taken together with the aberrant neuronal excitability of the Fox-1 knockout mice, these data suggest that Fox-1 may regulate electrical conduction by influencing splicing of select target pre-mRNAs.

Fox-2 also binds GCAUG sequences via its highly similar RRM, regulates alternative splicing, and is also expressed in brain and muscle tissue (Damianov and Black 2010). Cross-linking immunoprecipitation and subsequent high throughput sequencing (CLIP-seq) of Fox-2 bound RNA targets done in human embryonic stem cells (hESCs) revealed that Fox-2 binds to many RNA binding proteins, splicing factors, and serine/threonine kinases (Yeo et al. 2009). These targets included several hnRNPs, FGF receptors, and tissue-specific splicing factors. Fox-2 also binds to transcripts of *Fox-1* and itself, as each is known to have multiple splice forms. Knockdown of Fox-2 with shRNA followed by RT-PCR was used to verify Fox-2's role in splicing of some of these targets. Its general mechanism of splicing regulation appears consistent with that of Fox-1, wherein binding to GCAUG elements upstream of an exon represses expression of that exon while Fox binding to downstream elements promotes inclusion of the alternatively spliced exon. In addition, it was discovered that depletion of Fox-2 in hESCs causes cell death through apoptosis. Depletion of the protein causes cell death in multiple hESC lines but not in other cell types suggesting that Fox-2 may be required for stem cell survival. Nervous system-specific Fox-2 knockout mice

are not susceptible to spontaneous or kianic acid induced seizures in comparison to the Fox-1 knockouts (Gehman et al. 2011). Fox-1 and Fox-2 appear to have different targets and do not play redundant roles in the regulation of alternative splicing despite their similar expression patterns.

The last member of the Fox family to be identified is the neural nuclei specific marker NeuN (Fox-3, Kim et al. 2009). Although the monoclonal antibody, α-NeuN, has been used to label neuronal nuclei for many years, the identity and function of the protein that it targets has only been recently elucidated. Immunoprecipitation of the protein with α-NeuN was used to isolate it from mouse brain nuclear extracts. Mass spectrometry revealed that the protein is in fact Fox-3. Unlike the other Fox genes, transcripts of Fox-3 were detectable only in brain tissue and not in any muscle tissues. Fox-3 is required for the inclusion of exon N30 in nonmuscle myosin heavy chain (NMHC) II-B. Inclusion of this exon is dependent on an upstream intronic sequence that includes two GCAUG elements (Kawamoto 1996). An interaction between Fox-3 and polypyrimidine tract binding-associated splicing factor (PSF) is necessary for this splicing event to occur (Kim et al. 2011). PSF does not bind to the transcript directly, but is recruited to the site through its interaction with Fox-3. Depletion of PSF significantly decreases the level of N30 inclusion. Again, this mechanism of splicing regulation is consistent with that of Fox-1 and Fox-2 in which binding of the protein to an upstream element promotes inclusion of the alternative exon.

Although regulation of NMHC II-B splicing by Fox-3 has been well described, a more global analysis of its targets and binding partners remains to be realized.

The Fox-1 family of splicing factors seems to play an important role in the regulation of alternative splicing. The RRM, which binds to GCAUG elements on pre-mRNAs, has been highly conserved from *C. elegans* to humans. Fox-1 itself appears to regulate splicing of many genes involved in calcium handling (Gehman et al. 2011). Although its function in the central nervous system has been well described, its role in the heart, which relies heavily on proper calcium handling, has not yet been studied. Calcium is the critical component that links cardiomyocyte excitation to contraction. As a cardiomyocyte experiences an action potential, there is an influx of Ca^{2+} into the cell through voltage dependent L-type calcium channels. This Ca^{2+} influx activates ryanodine receptors on the sarcoplasmic reticulum causing a larger amount of Ca^{2+} to be released into the cytosol. The sarcomeres are the contractile fibers of striated muscle and they require the binding of Ca^{2+} for contraction. During contraction, myosin walks along filamentous actin, but in the resting state, the myosin binding sites on the actin filaments are blocked by the protein tropomyosin. It is held in this position by the troponin complex. During a wave of conduction through the myocyte, Ca^{2+} binds to the troponin complex causing a conformational change and a shift in the position of tropomyosin. The result is the exposure of the myosin binding sites on the actin filaments, which allows for contraction of the sarcomeres. Following this process, Ca^{2+} must then be pumped back out of the cytosol. Much of it is

returned to the sarcoplasmic reticulum for the next cycle. This sequence of events is repeated continuously in the heart as it pumps blood through the body during the entire span of an animal's life. Therefore, accurate handling of ions in cardiomyocytes is critical for proper function of the heart.

It is quite possible that defects affecting splicing of ion channels and interacting partners could lead to cardiac arrhythmias. For example, Timothy syndrome can occur due to changes in the alternative splicing of the L-type calcium channel alpha subunit (CACNA1C, Stump et al. 2011, Tang et al. 2011). This results in cardiac arrhythmias, long QT syndrome, and structural defects in the heart. Although mutations in ion channels and their binding partners have been described that cause heart diseases, it may be of interest to determine the affect of defective splicing of these genes in the heart. To this end, we have begun to study the role of the tissue specific splicing factor Fox-1 in the heart.

Zebrafish animal model system

We have chosen the zebrafish as a vertebrate model system to study *rbFox1l* function. In particular, zebrafish embryos are useful for studying developmental processes and molecular interactions. The embryos are easily accessible and they undergo rapid external development. Therefore, analyses can easily be done in high numbers. In addition, many powerful genetic and molecular tools have been developed for use in zebrafish. Microinjection of nucleic acids into embryos can easily be done for transgenesis, RNA expression,

or RNA knockdown. Fluorescent reporter proteins can be used to mark individual cells or proteins and can be observed under the microscope in live animals. Many new methods have also been developed to spatially and temporally control the expression of such reporter proteins. Each of these techniques can be used to study cardiac development and function in zebrafish (Stainier 2001). The heart of a zebrafish develops very quickly. Two bilateral fields of cardiac precursor cells in the lateral plate mesoderm are specified early on in the developing embryos. By 24 hours post-fertilization (hpf), these cells have merged at the midline, formed a linear heart tube, and have already begun beating in a peristaltic manner. Over the next 24-hour period, the heart tube loops and the cardiac chamber regions balloon out, creating a single atrium and a single ventricle separated by the atrioventricular canal. Thus, by 48 hpf the embryos have developed a fully functioning heart. Although it has fewer chambers than mammalian hearts, many of the processes and molecular pathways driving the formation of the zebrafish heart are highly conserved (Fishman and Stainier 1994, Yelon and Stainier 1999). Therefore, zebrafish have become a strong model for answering questions in vertebrate biology and cardiac development. Using these tools, we have knocked down *rbFox1l* function in developing zebrafish embryos in order to further investigate the role of Fox-dependent alternate splicing in the heart.

II.

Results

Conservation of Fox-1 proteins

Fox-1 has been highly conserved throughout evolution. The RNA recognition motif (RRM) exhibits the highest degree of conservation, followed by the carboxy terminus, with the amino terminus being the least conserved portion (Figure 1A). The RRM of mouse and human Fox-1 are in fact identical and the carboxy termini are 95% similar. Since the carboxy terminus is required for the regulation of splicing, it is expected that this region would be conserved fairly well. Zebrafish rbFox1 contains an RRM that is 99% similar to that of human FOX-1, while the RRM of the zebrafish gene duplication product rbFox1l is only 92% similar. The human FOX-1 RRM is only 78% similar to the original fox-1 protein of *C. elegans*. Despite slight divergence of the other domains in Fox-1, the RRM has maintained a high level of conservation from *C. elegans* through humans.

The other members of the Fox family can also be found in the zebrafish genome (Figure 1B). As previously mentioned, the *rbFox1* gene has been duplicated in zebrafish, resulting in *rbFox1* and *rbFox1 like* (*rbFox1l*), which encode a 373 and 382 amino acid protein, respectively. *RbFox2* is found on chromosome 6 and encodes a 241 amino acid protein and *rbFox3* is found on chromosome 12 and encodes a 364 amino acid protein. The entire known Fox family is found in zebrafish and it has previously been shown that *rbFox1l* can also regulate alternative splicing (Jin et al. 2003, *rbFox1l* is referred to as *Fox-1* in this reference).

Expression of *rbFox* genes in developing zebrafish

In mammals, the *Fox-1* genes have been shown to be expressed and to regulate splicing in the central nervous system and striated muscle (Damianov and Black 2010, Kim et al. 2009). Duplicated genes in zebrafish are often expressed in the same tissues and perform redundant roles. However, we observed that *rbFox1* is expressed only in the developing brain, while *rbFox1l* is expressed in the developing somites and cardiac tissue (Figure 2A-2F). *RbFox1l* expression can be observed in the bilateral heart fields and somites at the 16-somite stage (Figure 2A). It is expressed throughout the linear heart tube and the somites at 24 hpf (Figure 2B). By 48 hpf, *rbFox1l* expression appears to be more restricted to the ventricular chamber of the heart and somitic expression appears to be decreasing (Figure 2C).

Although *Fox-2* expression has been detected in the heart and brain of mammals, we only detected its expression in the developing brain of zebrafish embryos (Figure 2D-F). The pattern of *rbFox3* expression has not been determined in zebrafish. Fox-1 function in the developing heart has not yet been described, but the specific expression of *rbFox1l* in the heart and skeletal muscle of zebrafish allows us to more closely study its role there.

Knockdown of *rbFox1l*

We used an antisense morpholino oligonucleotide (MO) targeted against the ATG start site of *rbFox1l* in order to block its translation. By 48 hpf, *rbFox1l* morphants exhibited pericardial edema and many had bent tails (Figure 3A-B). By 72 hpf, wild-type hearts form distinct atrial and ventricular chambers separated by the atrioventricular (AV) canal and the chambers have taken on a rounded, kidney-like shape (Figure 3C). However, the cardiac chambers of *rbFox1l* morphants appear dysmorphic at 72 hpf (Figure 3D). The atria appear to be elongated instead of ballooning outward as in the wild-type heart. The ventricles appear to be smaller than those in the wild-type heart. The improper formation of the heart's chambers in *rbFox1l* morphants may be responsible for the observed pericardial edema, which increases between 2-5 days post-fertilization (dpf). *rbFox1l* may play an important role in cardiac chamber formation by regulating alternative splicing of exons in the heart.

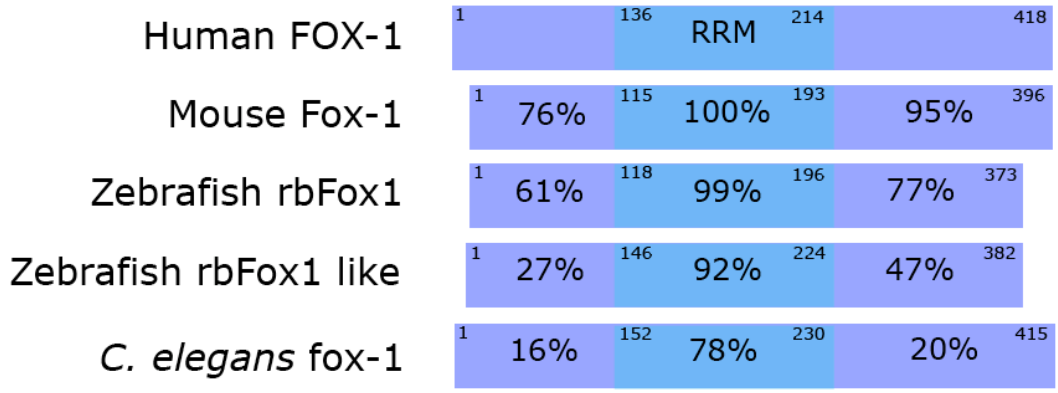
To determine whether rbFox1l depletion may result in mis-specification of the heart, we analyzed the expression patterns of several genes that mark distinct regions in the heart (Figure 4). Whole-mount *in situ* analysis revealed that these markers were similarly expressed in both wild-type and *rbfox1l* morphants. *cmlc2* was expressed throughout the heart (Figure 4A-B). *vmhc* and *amhc* expression remained restricted to the ventricle and atrium, respectively (Figure 4E-F and I-J). *anf* was expressed in the outer curvature of each chamber (Figure 4C-D), whereas *bmp4* and *tbx2b* expression both remained restricted to the AV

canal (Figure 4G-H and K-L). These data suggest that misspecification of cardiomyocyte cell types does not underlie the observed phenotype in the heart. Because aberrant numbers of cardiomyocytes may change the shape of the heart, we will compare the number of cardiomyocytes between rbFox1l and wildtype hearts using the cmlc2:dsRed-nuc line, which labels the nuclei of cardiomyocytes with red fluorescent protein.

Cardiac function in *rbFox1l* morphant embryos

In addition to being dysmorphic, *rbFox1l* morphant embryos also appeared to have decreased cardiac function. By 48 hpf, a slight pericardial edema was observed (Figure 3B), and by 5 dpf, 71.7% (86/120) of embryos injected with *rbFox1l* MO exhibit significant pericardial edema (Figure 5A). In addition to being dysmorphic, the contraction in morphant hearts was significantly decreased compared to the wild-type heart and by 5 dpf, 10.0% (12/120) of *rbFox1l* morphant ventricles stopped beating entirely (Figure 5A). However, atrial heart rates remained similar to wild type between 2-5 dpf (Figure 5B). Ventricular heart rates mostly remained comparable to that in wild type, but since some ventricles stopped beating at 5 dpf, the overall average for that time point is significantly lower for *rbFox1l* morphants (Figure 5C). We plan to measure the fractional shortening of the ventricles in *rbFox1l* morphants and compare it to that of wild type ventricles to gain a better idea of how contractility is affected.

A.



B.

Zebrafish Fox-1 family	Chromosome	Protein Length
rbFox1	3	373 amino acids
rbFox1 like	16	382 amino acids
rbFox2	6	241 amino acids
rbFox3	12	364 amino acids

Figure 1. RbFox1 conservation. (**A**) Schematic representation of mouse, zebrafish, and *C. elegans* Fox-1 proteins as compared to human FOX-1 protein. Percentages indicate the similarity of amino acid sequences in the amino-terminus, RNA recognition motif (RRM), and carboxy-terminus of each protein compared to the human FOX-1 protein. Smaller numbers at the top of each diagram denote the positions in the amino acid sequence of each domain. (**B**) *rbFox1* family members found in zebrafish listed with their associated chromosome and protein length in amino acids.

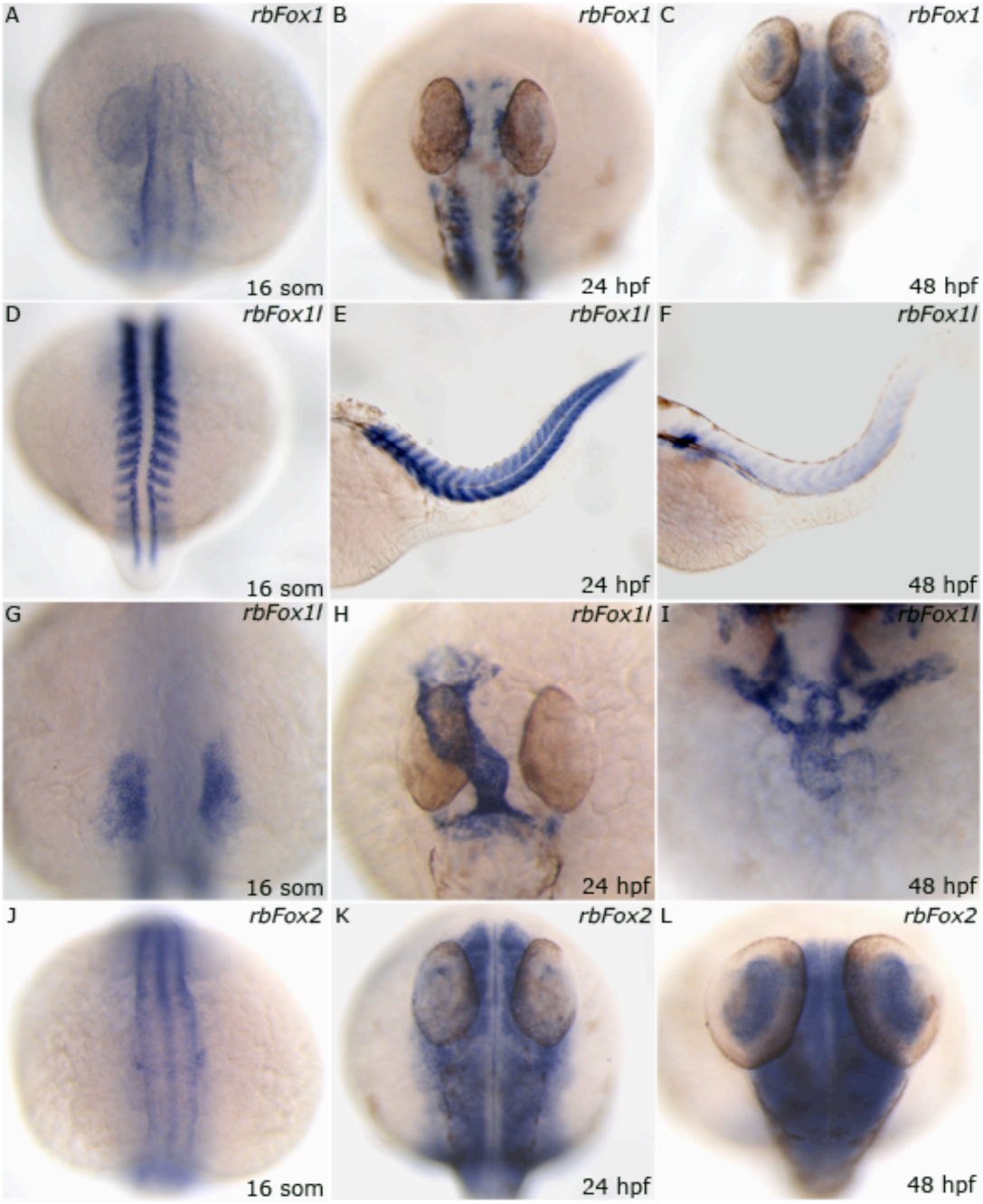


Figure 2. Expression of *rbFox1* family members in zebrafish embryos. Whole-mount *in situ* hybridization in wild-type embryos of (**A-C**) *rbFox1*, (**D-I**) *rbFox1l*, and (**J-L**) *rbFox2* mRNA in embryos at (**A,D,G,J**) 16 somites, (**B,E,H,K**) 24 hpf, (**C,F,I,L**) 48 hpf. *rbFox1* expression is restricted to the developing brain. *rbFox1l* expression is restricted to (**D-F**) somites and (**G-I**) the heart. *rbFox2* expression is also restricted to the developing brain.

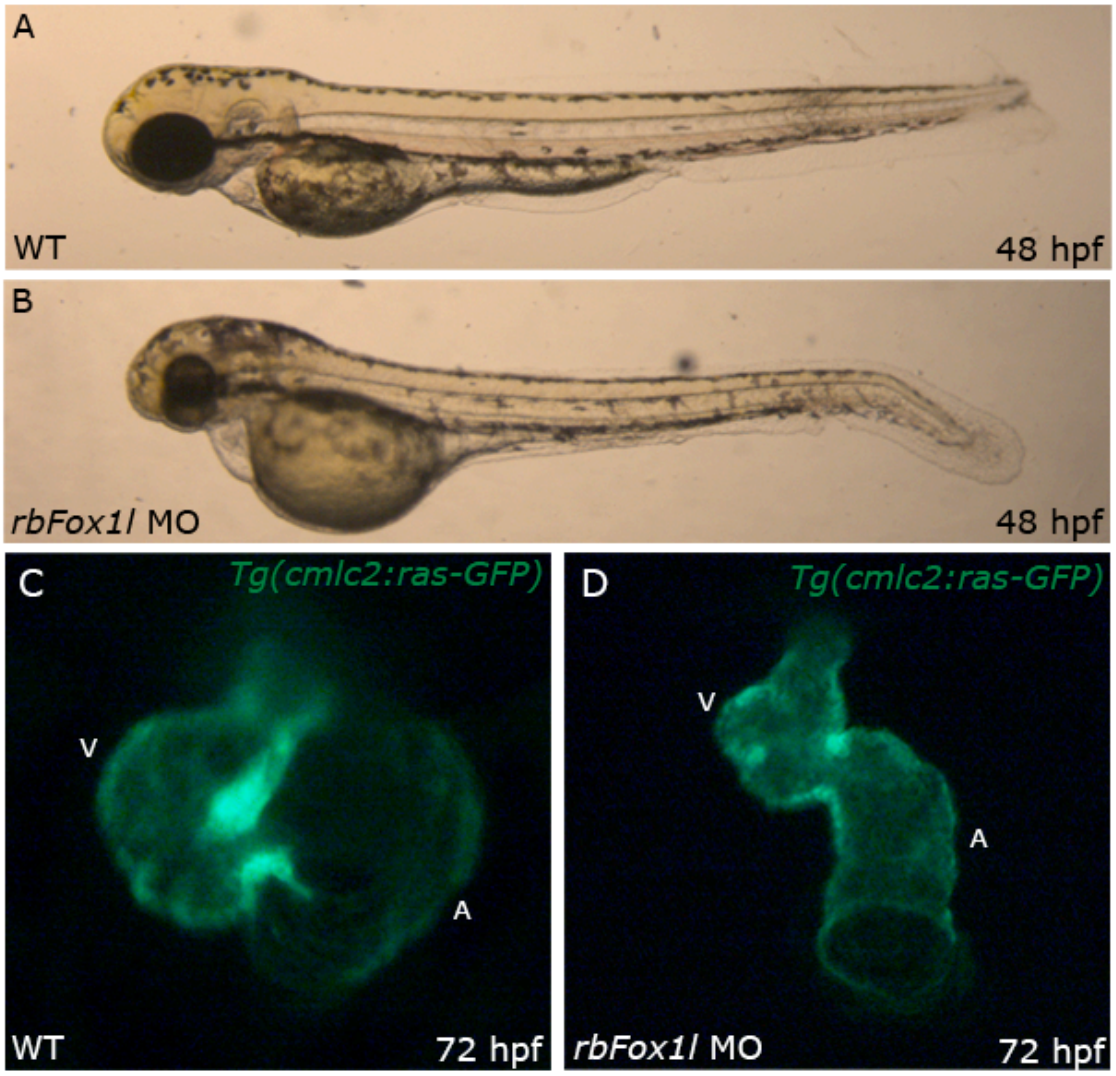


Figure 3. Knock-down of *rbFox1l* expression with an antisense morpholino oligonucleotide (MO). (**A and B**) Brightfield micrographs of (**A**) WT and (**B**) *rbFox1l* morphant embryos at 48 hpf. Morphant embryos begin to exhibit pericardial edema by 48 hpf and many have crooked tails. (**C and D**) Fluorescent micrographs of hearts expressing *Tg(cmlc2:ras-GFP)* in (**C**) WT and (**D**) *rbFox1l* morphant embryos at 72 hpf (A, atrium; V, ventricle). *rbFox1l* morphant hearts appear dysmorphic with long atriums and smaller ventricles.

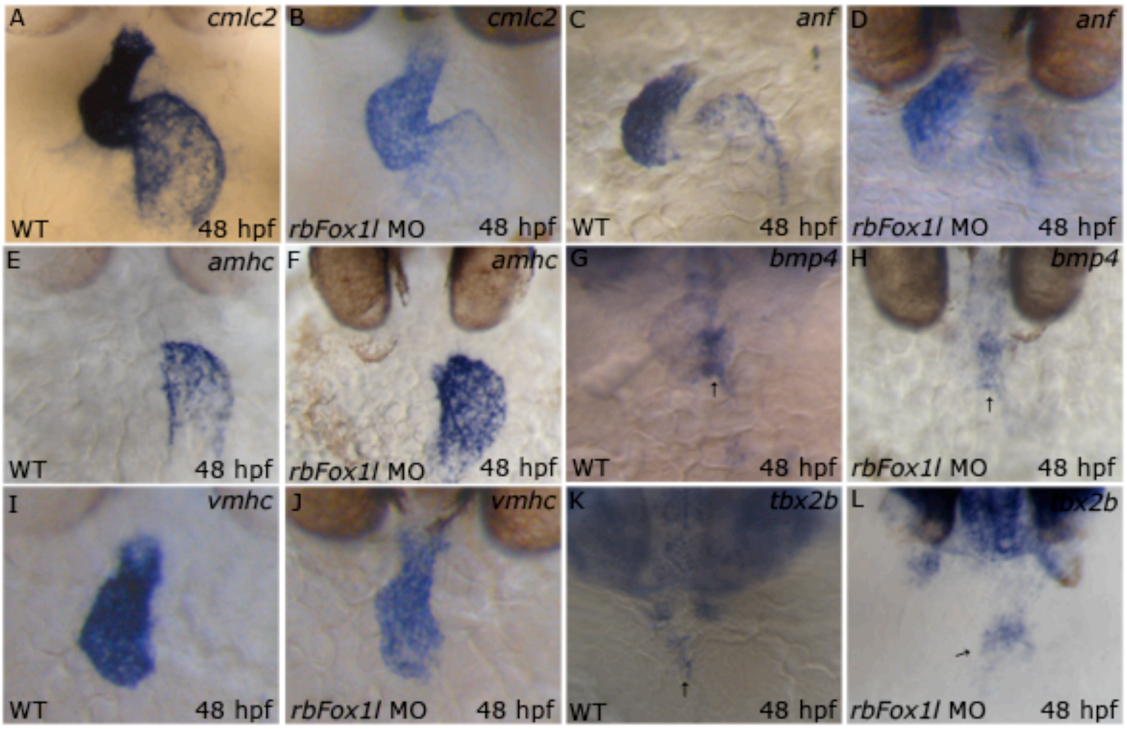


Figure 4. Specification of cardiac cell types in *rbFox1l* morphants. Whole-mount *in situ* hybridization of (**A-B**) *cmlc2*, (**C-D**) *anf*, (**E-F**) *amhc*, (**G-H**) *bmp4*, (**I-J**) *vmhc*, and (**K-L**) *tbx2b* mRNA in (**A,C,E,G,I,K**) WT and (**B,D,F,H,J,L**) *rbFox1l* morphants at 48 hpf. *Amhc* and *vmhc* are expressed in the atrium and ventricle, respectively. *Anf* is expressed in the outer curvature of the cardiac chambers. *Bmp4* and *tbx2b* are restricted to the AV canal (arrows indicate the AV canal region). There appears to be no significant difference in expression of cardiac specification markers between WT and *rbFox1l* morphant hearts.

A.

	Uninjected embryos	rbFox1l MO injected embryos (6ng)
Pericardial edema	0/120 (0%)	86/120 (71.7%)
Ventricular failure (by 5 dpf)	0/120 (0%)	12/120 (10.0%)

B.

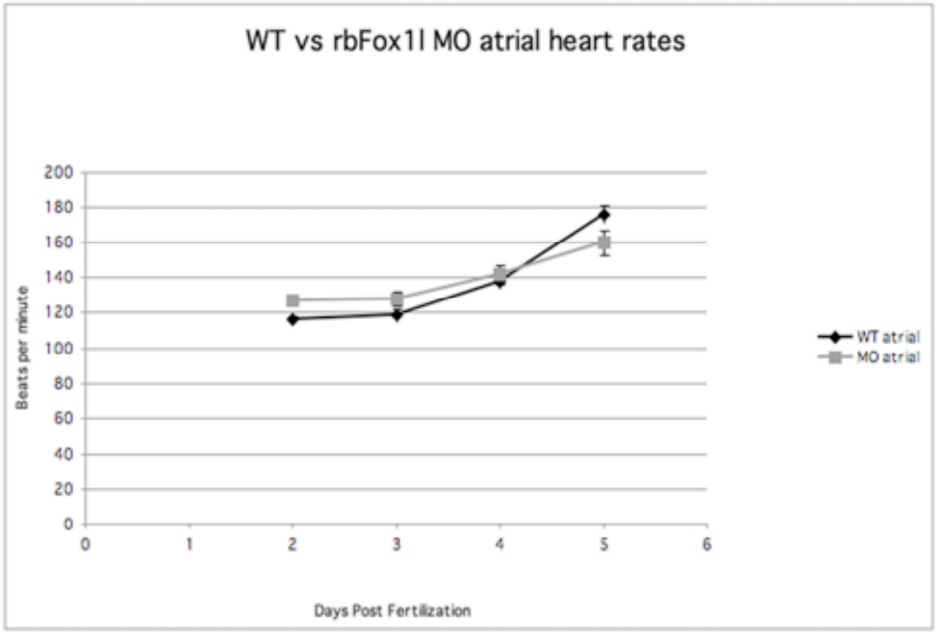


Figure 5. Decrease in cardiac function in *rbFox1l* morphants. (**A**) Pericardial edema was observed in 71.7% of *rbFox1l* MO injected embryos and 0% of uninjected embryos. By 5 dpf, 10% of the morphant hearts exhibited non-contracting ventricles. (**B**) No significant difference in atrial heart rate was observed between WT and *rbFox1l* morphant atrial embryos between 2-5 dpf. (**C**) Ventricular heart rates were similar between 2-4 dpf. Since 10% of *rbFox1l* morphant ventricles stop beating by 5 dpf, there is a significant difference between the average ventricular heart rates of WT and morphant hearts at this timepoint. Error bars represent 95% confidence intervals.

C.

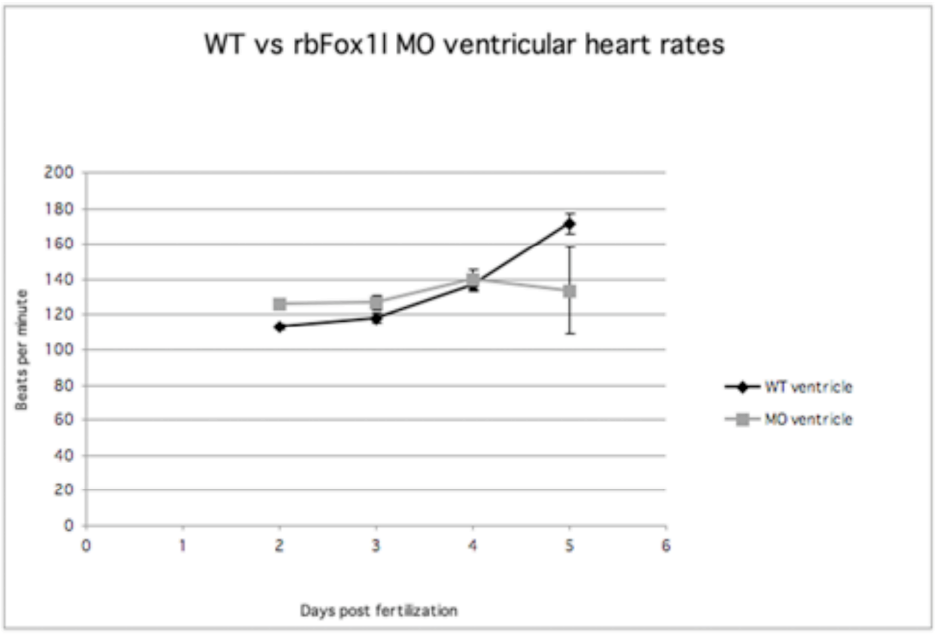


Figure 5, continued.

III.

Discussion

In this study, we have knocked down *rbFox1l* expression in zebrafish embryos to study its impact on cardiac development and function. When *rbFox1l* was knocked down, the cardiac chambers appeared dysmorphic at 72 hpf. Instead of round, kidney-like chambers, the morphant hearts maintained more linear chambers, although cardiac looping appeared to occur normally. By 5 dpf, the morphant hearts exhibited a decrease in cardiac function, with some ventricles failing to contract. It is still unclear whether defects in the conduction system or contractile machinery underlie the decrease in heart function. Preliminary data we have obtained shows that sarcomeres are intact in the morphant hearts. However, this does not ensure that the sarcomeres are properly functioning. We also plan to image calcium transients in the *rbFox1l* morphant hearts to help us determine the state of the conduction system. If conduction is not occurring properly in the heart, it may explain the loss of contraction.

The rbFox1 proteins have been shown to regulate alternative splicing of RNA transcripts by binding to GCAUG elements (Jin et al. 2003, Fukumura et al. 2007, Tang et al. 2009). When *RbFox1* was knocked out in the mouse nervous system, the mice were susceptible to spontaneous seizures (Gehman et al. 2011). It was found that the stimulus intensity required to induce excitatory postsynaptic potentials was lower in *RbFox1* knockouts. In addition, aberrant splicing was found in many transcripts involved in electrical conduction, whether they were ion channels themselves or proteins that interact with ion channels. Of

the 20 transcripts of which splicing was most effected in the knockout mice, three of them were subunits of the voltage dependent L-type calcium channel and others included a potassium ion channel, a sodium ion channel, components of SNARE complexes, which interact with several ion channels, and calmodulin binding proteins (Condliffe et al. 2010, Chao et al. 2011). Many of these proteins are also expressed in the heart and are critical for excitation and contraction of cardiomyocytes (Rottbauer et al. 2001, Arnaout et al. 2007, Chao et al 2010, Chopra et al. 2010). RbFox1 has also been shown to regulate splicing of *α-actinin* and tropomyosin, both of which are components of the sarcomeres in cardiomyocytes (Jin et al. 2003, Gehman et al. 2011). Therefore, it is possible that aberrant splicing of RNA transcripts required for conduction, contraction or both may contribute to the decrease in cardiac function.

In order to identify what is responsible for the cardiac phenotype in *rbFox1l* morphants, we first plan to more thoroughly characterize the defects in their hearts. Because the morphology of these hearts appears abnormal, we plan to count the number of cardiomyocytes and determine if it differs significantly from the number in wildtype hearts. We will also analyze the morphology of individual cardiomyocytes to see if there are changes that may cause the heart to be misshapen. If either of these factors is abnormal, we would need to determine which developmental pathways are effected and if any significant components of the pathway have undergone incorrect splicing due to the loss of *rbFox1l*. We also plan to study the conduction in *rbFox1l* morphant hearts using the

Tg(cmlc2:gCamp)^{s883} line (Chi et al. 2008). These fish express a Ca^{2+}-sensitive GFP protein in their cardiomyocytes, which allows us to visualize the frequency and amplitude of Ca^{2+} transients as they move through the heart. If cardiac conduction is abnormal in *rbFox1l* morphants, it would possibly explain the decrease in the heart's function that occurs by 5 dpf. This would lead us to examine the splicing of various ion channels and their binding partners, especially those that were affected in *rbFox1* null mouse brains (Gehman et al. 2011). The work done in the mouse nervous system suggests that *rbFox1* may be an important regulator of genes that influence the conduction properties of a cell. However, the nervous system does not require or express many of the contractile proteins to the same degree as cardiomyocytes. Therefore, *rbFox1* may also be an important regulator of splicing for these genes even though it was not apparent from analyses of *rbFox1* in the nervous system. These studies may help us narrow down which characteristics are most defective and responsible for the phenotype in *rbFox1l* morphant hearts.

Once we have thoroughly analyzed the *rbFox1l* morphant hearts, we next hope to study changes in splicing of various transcripts using a candidate gene approach. Crosslinking and immunoprecipitation of RbFox1 followed by high throughput sequencing of any bound transcripts (CLIP-seq) was performed using mouse cardiomyocytes (Mike Lovci and Gene Yeo, unpublished data). Using data obtained from this experiment, we can find transcripts that are bound strongly by RbFox1. Then, using cDNA synthesized from the RNA of isolated

wildtype and *rbFox1l* morphant hearts in zebrafish, we will perform RT-PCR to determine any changes in splicing of candidate gene transcripts. When we identify transcripts affected by the loss of *rbFox1l*, we can also attempt rescue experiments by injecting the wildtype splicing isoforms of specific genes into *rbFox1l* morphants and observing the phenotype. In this way, we hope to learn which splicing events are regulated by *rbFox1l* in the heart and how they contribute to the phenotype we observe.

Lastly, we would like to determine the expression levels of RBFOX1 in diseased and normal human hearts. It is known that the splicing of many genes is affected in diseased hearts (Kong et al. 2010). If RBFOX1 is responsible for any changes in splicing during heart disease, it may provide a therapeutic target for reversing the effects of certain heart diseases. The phenotype of *rbFox1l* morphant hearts demonstrates the importance of proper splicing regulation in cardiomyocytes. The work described here provides a starting point from which the role of the tissue specific splicing regulator, *rbFox1l*, can be studied in depth, leading to a better understanding of how splicing of critical transcripts is controlled in the heart.

IV.

Materials and Methods

Zebrafish strains

Embryonic and adult zebrafish were raised and maintained under standard laboratory conditions at 28.5°C. Transgenic lines used were *Tg(cmlc2:ras-GFP)*^{s883} (Chi et al. 2008) and *Tg(cmlc2:α-actinin-GFP)*. *Tg(cmlc2:α-actinin-GFP)* was given to us by Yi-Fan Lin and Deborah Yelon (work currently in submission).

***In situ* hybridization**

Whole-mount *in situ* hybridization analyses were performed as previously described (Thisse et al. 2007). *RbFox1* and *rbFoxl* RNA probes were created by Julia Lipianskaya. Other probes used were *rbFox2*, *cmlc2*, *amhc*, *vmhc*, *anf*, *bmp4*, and *tbx2b*. Embryos were mounted in 2:1 Benzyl benzoate:Benzyl alcohol and imaged with a Leica M205FA stereo microscope.

Morpholino mediated knockdown

To knock down translation of *rbFox1l* mRNA, we used an antisense morpholino oligonucleotide (MO) targeted against the ATG start site *rbFox1l*: 5'-GGATCACAGTAGGAGAAGACAACAT -3' (Open Biosystems). Embryos were injected at the one-cell stage with 6 ng of *rbFox1l* MO. Injected embryos were analyzed between 2-5 dpf and compared with uninjected embryos. Injection of up to 10 ng of *rbFox1* MO (5'- CTTGCTCCCTTTTTCCTCCATAAAC -3') caused no visible phenotype, suggesting that defects seen upon injection of *rbFox1l* MO were specific.

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