Investigating the roles of homeobox containing transcription factors Iroquois 3/5 in mammalian heart development and electrophysiology

by

Jieun Kim

A thesis submitted in conformity with the requirements for the degree of Masters of Science
Department of Molecular Genetics
University of Toronto

© Copyright by Jieun Kim 2010
Investigating the roles of homeobox containing transcription factors Iroquois 3/5 in mammalian heart development and electrophysiology

Jieun Kim
Masters of Science
Department of Molecular Genetics
University of Toronto
2010

Abstract

Iroquois homeobox (Irx) family members, a group of highly conserved homeodomain containing transcription factors, are involved in the patterning and the proper functions of vertebrate organs. They can act as transcriptional activators or repressors in a context-dependent manner. Preliminary data indicated that Irx3 and Irx5 are functionally redundant during cardiac morphogenesis, and they physically interact with other cardiac transcription factors. At E14.5, outflow tract septation failure and ventricular septation failure were observed in Irx3/5DKO mouse hearts. Loss of Irx3/5 in neural crest and endothelial cell lineages led to outflow tract septation failure and ventricular septal defect. In adult mice, Irx3 is expressed in the atrioventricular conduction system, and loss of Irx3 leads to slower ventricular conduction velocity. qRT-PCR analysis and immunofluorescence staining revealed that the expression of gap junction proteins, Cx40 and Cx43, are affected by the loss of Irx3. Over-expression of Irx3 and a dominant repressor form of Irx3, Irx3-EnR, resulted in Cx40 upregulation, indicating that Irx3 acts as an indirect positive regulator of Cx40. Irx3-EnR over-expression in vivo resulted in postnatal onset of atrial enlargement, ventricular hypertrophy, and conduction failure. Taken together, this study demonstrates the significance of Irx3/5 in both cardiovascular development and cardiac electrophysiology.
Acknowledgments

I would like to thank all members of the Hui Lab for their technical supports and providing me a great working environment throughout my study, and Anna Rosen and Han Kim from the Backx Lab for their collaboration on the adult electrophysiology project. I would also like to acknowledge Shoshana Spring for OPT scanning service and Lisa Yu for MicroCT scanning service at TCP. I would like to thank my supervisory committee members, Dr Peter Backx, Dr Andras Nagy, and Dr Norman Rosenblum for their invaluable advices and inputs. Finally, I thank my supervisor, Dr Chi-chung Hui, for his tremendous guidance and support.

This study was funded by Canadian Institutes of Health Research (CIHR) and Hospital for Sick Children Restracomp Graduate Studentship.
# Table of Contents

Abstract ......................................................................................................................... ii
Acknowledgements ......................................................................................................... iii
Table of Contents ........................................................................................................... iv
List of Figures ................................................................................................................ vi
List of Abbreviations ...................................................................................................... viii

Chapter 1: Introduction
1.1 Iroquois in development and physiology .............................................................. 1
1.2 Mammalian cardiovascular development ............................................................. 3
1.3 Mammalian cardiac conduction system ............................................................... 5
1.4 Cardiac transcription factors in cardiac morphogenesis and conduction system ... 7
1.5 Gap junction proteins in cardiac morphogenesis and conduction system .......... 8
1.6 Potential involvement of Irx3 and Irx5 during cardiac morphogenesis ............... 9
1.7 Potential involvement of Irx3 in the cardiac conduction system ......................... 11

Chapter 2: Materials and methods
2.1 Mouse lines ............................................................................................................ 13
2.2 Ink injection ........................................................................................................... 13
2.3 MicroCT scanning ................................................................................................ 14
2.4 H&E staining ........................................................................................................ 14
2.5 X-gal staining ....................................................................................................... 14
2.6 Electrocardiogram measurements and analysis .................................................. 15
2.7 Tamoxifen injection ............................................................................................... 15
2.8 Immunofluorescence staining and imaging .......................................................... 15
2.9 Optical projection tomography ............................................................................. 16
2.10 Western blot analysis .......................................................................................... 16
2.11 Quantitative real-time PCR analysis .................................................................. 17

Chapter 3: Results – Part 1
3.1 Loss of Irx3 and Irx5 leads to various cardiac phenotypes at E14.5 ...................... 18
3.2 Irx3 is expressed in the neural tube during early cardiac morphogenesis and in the outflow tract region as well as ventricles in late stages of development .................. 20
3.3 Conditional knockout of Irx3/5 in the neural crest and endothelial cell lineages ... 21
3.4 Conditional loss of Irx3 and Irx5 in neural crest cell lineage leads to embryonic lethality and cardiac phenotypes at E14.5 ................................................................ 21
3.5 Conditional loss of Irx3 and Irx5 in endothelial cell lineage leads to embryonic lethality and cardiac phenotypes at E14.5 ................................................................ 21
Figures for Chapter 3 .................................................................................................... 23

Chapter 4: Results – Part 2
4.1 Irx3 is highly expressed in the atrioventricular conduction system and the endocardium of both ventricles .................................................................................. 31
4.2 Loss of Irx3 leads to reduced expression of Cx40 in the ventricular conduction system ................................................................................................................. 31
4.3 In vitro and in vivo approaches for studying of the functions of Irx3 activator form and repressor form.................................................................32
4.4 Ubiquitous over-expression of Irx3EnR using inducible Cre shows heart rate variability..................................................................................................................33
4.5 Cardiac-specific over-expression of Irx3EnR using promoter-driven aMHC-Cre results in high mortality, ventricular conduction failure, and atrial enlargement in adult mice..........34
4.6 Cardiac-specific over-expression of Irx3EnR has an early onset phenotype in postnatal hearts but not in neonatal hearts ........................................................................................................................................35
4.7 Western blot analysis shows that cardiac-specific over-expression of Irx3EnR leads to misregulation of Cx40 in postnatal hearts.............................................................................................................36
Figures for Chapter 4................................................................................................................37

Chapter 5: Discussion and future directions
5.1 Irx3 and Irx5 are necessary for outflow tract septation and ventricular septation.........48
5.2 Irx3 and Irx5 activities are required in various lineages and signalling pathways for outflow tract development and ventricular septation process........................................................................49
5.3 Irx3 and Irx5 may function as cardiac transcription factor complex during cardiac morphogenesis................................................................................................................................51
5.4 Irx3 is expressed and essential in the atrioventricular conduction system of adult mouse heart........................................................................................................................................52
5.5 Irx3 mainly acts as a transcriptional repressor for Cx40 regulation and gain of function mutation leads to atrial and ventricular phenotypes..........................................................................................52
5.6 Possible mechanism of Irx3 in the regulation of mammalian cardiac conduction system and morphogenesis........................................................................................................................................54

References........................................................................................................................................57
List of Figures

Chapter 1: Introduction
Figure 1: Iroquois family in *Drosophila* and Mammalian genomes ............................................... 2
Figure 2: Diagram of Cardiovascular system .................................................................................. 3
Figure 3: Major steps in mammalian cardiac morphogenesis ......................................................... 4
Figure 4: *Wnt1-Cre* conditional knockout of Sonic hedgehog signalling component and *Tie2-Cre*
conditional over-activation of ERK1/ERK2 pathway lead to OFT defects ........................................ 5
Figure 5: Mammalian cardiac conduction system and electrocardiogram ....................................... 6
Figure 6: Preliminary data show that *Irx3/5DKO* has double outlet ventricle
syndrome at E14.5 ....................................................................................................................... 10
Figure 7: Loss of Irx3 leads to the slowing of ventricular conduction velocity ......................... 12
Figure 8: qRT-PCR shows that Cx40 mRNA expression level is positively regulated by Irx3 ....... 12

Chapter 3: Results – Part 1
Figure 9: *Irx3/5DKO* mice display various levels of gross morphology defects and cardiac
phenotypes at E14.5 ................................................................................................................... 23
Figure 10: *Irx3/5DKO* mice present abnormal positioning of aorta and pulmonary artery with
respect to ventricles ............................................................................................................... 24
Figure 11: *Irx3/5DKO* mice present blockage between the ventricles and the OFT ................. 25
Figure 12: *Irx3/5DKO* shows the misorientation of pulmonary artery and aorta with respect to
each other ................................................................................................................................... 26
Figure 13: *Irx3/5DKO* presents ventricular septation defect (VSD) and double outlet right
ventricle (DORV) syndrome at E14.5 ...................................................................................... 27
Figure 14: *Irx3LacZ* is expressed in extracardiac and intracardiac regions
during embryogenesis ............................................................................................................. 28
Figure 15: Experimental design for cell lineage analysis of Irx3/5 activity in outflow tract
development and ventricular septation .................................................................................... 28
Figure 16: Loss of Irx3 and Irx5 in neural crest cell lineage leads to cardiac defects as well as
craniofacial defects ............................................................................................................. 29
Figure 17: Loss of Irx3 and Irx5 in endothelial cell lineage leads to cardiac defects as well as
craniofacial defects ............................................................................................................. 30

Chapter 4: Results – Part 2
Figure 18: *Irx3LacZ* is expressed in the atrioventricular conduction system and the endocardium
in adult mice ............................................................................................................................. 37
Figure 19: Immunofluorescence staining of Cx40 and Cx43 in *Irx33/4* and *Irx33/2LacZ/2LacZ* ........ 38
Figure 20: Effect of activator and repressor forms of Irx3 on Cx40 and Cx43 expression in
cultured neonatal cardiomyocytes .......................................................................................... 39
Figure 21: Cre-LoxP systems to induce Irx3EnR expression in vivo ........................................... 40
Figure 22: Tamoxifen-induced, ubiquitous expression of Irx3EnR leads to heart rate
variability ................................................................................................................................... 41
Figure 23: Promoter-driven, cardiac-specific expression of Irx3EnR in vivo leads to early
lethality ...................................................................................................................................... 41
Figure 24: Promoter-driven, cardiac-specific expression of Irx3EnR in vivo leads to abnormal
cardiac electrophysiology ....................................................................................................... 42
Figure 25: ECG analysis of *Rosa26Irx3EnR* and *aMHC-Cre3/2;Rosa26Irx3EnR* adult mice ....... 43
Figure 26: Irx3EnR was expressed in both atria and ventricles of $aMHC-Cre^{+/-}:Rosa26^{Irx3EnR}$ animals. 

Figure 27: Over-expression of Irx3EnR leads to atrial enlargement and ventricular hypertrophy in adult mice.

Figure 28: Over-expression of Irx3EnR leads to atrial enlargement with no abnormal valve morphology at four weeks.

Figure 29: Over-expression of Irx3EnR does not lead to observable phenotypes at neonatal stage.

Figure 30: Cx40 expression level is increased in the ventricles with Irx3EnR over-expression.
List of Abbreviations

\textit{aMHC: alpha-myosin heavy chain} gene

AO: aorta

ASD: atrial septal defect

AV: atrioventricular

Cx40: connexin 40

Cx43: connexin 43

\textit{EnR: Engrailed repressor} domain

\textit{Esr1: estrogen receptor alpha} gene

HOS: Holt-Oram Syndrome

\textit{Irx: Iroquois} gene

IVS: interventricular septum

LA: left atrium

LV: left ventricle

OFT: outflow tract

PA: pulmonary artery

RA: right atrium

RV: right ventricle

Shh: Sonic Hedgehog

VSD: ventricular septal defect
1 Introduction

1.1 Iroquois in development and physiology

Proper organogenesis is crucial for both viability and physiological functions of an organism. Transcription factors precisely control the tissue-specific gene expression for differentiation and specification during development. After morphogenesis is complete, some of these transcription factors maintain their roles in tissue-specific gene activity regulation for the functions of each tissue as well.

Iroquois homeobox (Ir) gene family members encode transcription factors containing a highly conserved homeodomain with the three-amino acid length extension (TALE) superclass at their N-terminus (Burglin 1997). Irx was first identified in Drosophila melanogaster as loss of Iro complex led to misregulation of wing notum specification genes, resulting in patterning defects in lateral bristle formation (Gomez-Skarmeta and Modolell 1996). There are six Irx genes in both mouse and human genomes while three Irx genes are present in the Drosophila genome (Houwelling et al., 2001; Peters et al., 2000). They are found in two clusters: Irx1, 2, and 4 in Cluster A (mouse chromosome 13 and human chromosome 5), Irx3, 5, and 6 in Cluster B (mouse chromosome 8 and human chromosome 16) (Peters et al., 2000; Bosse et al., 2000) (Figure 1).

Previous studies illustrated that Irx genes are necessary for proper patterning of Drosophila and vertebrate central nervous system and heart (Cavodeassi et al., 2001; Cohen et al., 2000; Matsumoto et al, 2004; Lebel et al., 2003; Bao et al., 1999; Bruneau et al., 2001). Interestingly, Irx genes have also been shown to play important roles in physiological functions of various organs. Loss of Irx5 results in the misregulation of a potassium current gene, Kv4.2, and this leads to higher susceptibility to ventricular tachycardia upon mechanical stress in adult mice (Costantini et al., 2005).
Another interesting feature of *Irx* genes is their dual function as transcription factors. They can act as transcriptional activators or repressors in a context-dependent manner. In particular, *Irx2* can act as a transcriptional activator or repressor in the Fgf signaling pathway for chick hindbrain patterning, depending on its phosphorylation states (Matsumoto et al., 2004). Phosphorylation of *Irx2* at its N-terminus by MAP-Kinase activity allows the activator function of *Irx2* to override while *Irx2* becomes a repressor when the C-terminus is phosphorylated. A dominant repressor form of *Irx2* fused with *Drosophila* Engrailed repressor domain (EnR) in the hindbrain represses the hindbrain-specific gene *Gbx2*, leading to the enlargement of the tectum, which replaces the cerebellum formation.

*Irx4*, on the other hand, regulates the levels of ventricular and atrial genes in a chamber-specific manner as an activator or a repressor, respectively, in chick and mouse cardiovascular system (Bao et al., 1999; Bruneau et al., 2001). Injection of full-length *Irx4* into chick atria led to ectopic expression of *ventricular myosin-heavy chain gene* (*VMHC*) in the atria, while over-expression of *Irx4EnR* led to reduced *VMHC* mRNA expression in the ventricles but increased *atrial myosin-heavy chain* gene (*AMHC*), suggesting that endogenous *Irx4* acts as an activator for *VMHC* expression and a repressor for *AMHC*. Interestingly, *Irx5* has been shown to repress a potassium channel gene, *Kv4.2*, in adult mouse heart, regulating proper transmural gradient across the ventricles (Costantini et al., 2005). How *Irx* transcription factors act as transcriptional activators or repressors in the development and physiology of the mammalian cardiovascular system remains as an interesting question.
1.2 Mammalian cardiovascular development

Mammalian heart development is a complex process involving numerous cell lineages to ensure that all four chambers – left and right atria (LA and RA), and left and right ventricles (LV and RV) – are formed, oriented, and separated properly (Figure 2). The great arteries, aorta (AO) and pulmonary artery (PA) arise from a single tubular structure known as the outflow tract (OFT). The connectivity between AO and LV as well as between PA and RV is crucial for the circulatory function of the heart. Correct orientation of the four chambers, function of the valves between chambers, and connectivity of the great arteries are all crucial requirements of a properly functioning heart.

![Diagram of Cardiovascular System](adopted from NIH website)

The initial structure of the developing heart, the cardiac crescent, is formed at E7.5 in mouse embryo (Figure 3). It contains the first heart field (FHF), which later gives rise to the LV, and the secondary heart field (SHF) contributing to the development of RV, RA, LA, and OFT. The crescent shape of the presumptive heart structure undergoes differentiation and cell migration to become a linear heart tube at E8.0, and the rightward looping of the heart tube occurs at E9.0, allowing formation of a common atrial chamber and a common ventricular chamber. At E10,
chamber formation is more prevalent, and endocardial cushioning between the presumptive LV and RV within the common ventricular chamber occurs. Also, OFT septation is initiated, and further lengthening and differentiation gives rise to AO and PA at later stages. At E14.5, mouse cardiovascular development is complete with RA, LA, RV, and LV from separated from one another, and with AO and PA structures ascending from the LV and RV, respectively.

Figure 3: Major steps in mammalian cardiac morphogenesis (Bruneau 2008)

OFT septation involves contributions from neural crest cell descendants and endothelial cell descendants (Kirby et al., 1983; Jiang et al., 2000; Kisanuki et al., 2001). Endothelial cells are the primary source of mesenchyma that contribute to the initial stage of OFT septation while neural crest descendants are involved in the later stage of OFT septation (Kisanuki et al., 2001; Jiang et al., 2000). Knockout studies in mice have demonstrated that specific gene functions in both lineages are important for OFT development. Experiments using Wnt1-Cre to ablate specific gene activity for Sonic hedgehog signaling pathway, Pax3, Msx2, or p53 in neural crest cell descendants have resulted in OFT septation failure (Goddeeris et al., 2007; Kwang et al., 2002; Morgan et al., 2008) (Figure 4A). Also, the use of Tie2-Cre to disrupt the ERK1/ERK2...
pathway in endothelial cell descendants resulted in a single OFT phenotype (Krenz et al., 2008) (Figure 4B).

![Figure 4: Wnt1-Cre conditional knockout of Sonic hedgehog signaling component (Smo\textsuperscript{smoothened}) and Tie2-Cre conditional over-activation (Shp2-Q79R) of ERK1/ERK2 pathway lead to OFT defects (from Goddeeris et al., 2007 and Krenz et al., 2008)](image)

1.3 Mammalian cardiac conduction system

The establishment of a functioning cardiac conduction system also requires faithful gene regulation throughout the development and function of the cardiovascular system. The electrical signal is initiated from the pacemaker, known as the sinoatrial (SA) node, composed of specialized pacemaker cells located in the right atrium (Figure 5A). The signal then propagates to the atrioventricular (AV) node in the AV junction between the atria and the ventricles. The AV junction consists of the AV bundle, which extends to the bundle of His, followed by Purkinje fibers that extend down the interventricular septum (IVS).

On the right side of IVS, the right bundle branch (RBB) continues to the working myocardium of right ventricle, while the left bundle branch (LBB) continues to the working myocardium of the left ventricle. The working myocardium of the ventricles is composed of three myocardial layers, based on their proximity to the IVS. The endocardium is the innermost layer, the myocardium is in the middle, and the epicardium is the outermost layer. There are electrophysiological heterogeneities within the heart, such as a transmural gradient between the apex and the base, and between the endocardium and the epicardium (reviewed in Boukens et al., 2009).
Figure 5: Mammalian cardiac conduction system and electrocardiogram

The electrical activity of the cardiac conduction system can be recorded by electrocardiogram (ECG). An ECG measures the voltage difference across the membrane of cardiomyocytes. A typical ECG recording of each conduction system cycle consists of a P wave, a QRS complex, and a T wave (Figure 5B). The P wave represents the depolarization of atria as the electric signal is initiated in SA node and propagates to the AV node. QRS complex displays the rapid depolarization of right and left ventricles. The ST wave represents the repolarization of the ventricles as the membrane potential goes back to its resting potential. RR interval is a measurement of the duration between two conduction cycles.

1.4 Cardiac transcription factors in cardiac morphogenesis and conduction system

To understand the molecular mechanisms leading to congenital heart defects and to understand mammalian cardiac morphogenesis, several key transcriptional regulators have been identified (reviewed in Harvey 2002; reviewed in Srivastava 2006; reviewed in Bruneau 2008). They
include Nkx2.5, Tbx5, and Gata4, which regulate specific gene expression throughout heart development. Mutations of these cardiac transcription factors have been linked to human congenital heart defects (reviewed in Bruneau 2008). Interestingly, these cardiac transcription factors are necessary for the maturation of the cardiac conduction system as well as the responsive hypertrophy mechanism, indicative of the developmental basis for proper cardiac conduction system function.

The Nkx2.5 homeobox gene is one of the earliest cardiac markers; it was first identified as tinman for its role in Drosophila melanogaster dorsal mesoderm (Bodmer 1993). Mutations in human NKX2.5 have been correlated with failure in proper separation of the atria (atrial septal defects, ASD) and atrioventricular blockages (AV block) (Schottet et al., 1998). Loss of Nkx2.5 in mice affects the junction between the atrial and ventricular chambers, possibly contributing to the AV conduction system failure (Hatcher and Basson 2009). Postnatal deletion of Nkx2.5 by inducible Cre-mediated recombinase activity causes conduction failure (Briggs et al., 2008), indicating that in addition to cardiac morphogenesis, Nkx2.5 activity is also crucial for proper cardiac electrophysiology.

Tbx5 is a T-box containing transcription factor, which is linked with Holt-Oram syndrome (HOS) in humans (reviewed in Bruneau 2008; Postma et al., 2008). HOS is characterized with cardiac defects and limb deformities, and displays ASD or ventricular septal defects (VSD). Heterozygous Tbx5 mutant mice exhibit various degrees of electrophysiological deficits such as AV blocks and SA node dysfunction (Bruneau et al., 2001). Tbx5 is crucial for the maturation of bundle branches in the postnatal cardiac conduction system; slower PQ interval, which is an indicator for AV conduction failure, was observed in these mice, along with morphologically less distinguished AV node and AV bundle (Moskowitz et al., 2004).

Gata4 is a zinc-finger domain-containing transcription factor that binds to a specific consensus DNA sequence. Mutations in GATA4 have been linked to septation defects in humans (Garg et al., 2003). Interestingly, in hypertrophic condition, Gata4 target gene expression is elevated and the cardiac target genes that are expressed during cardiac morphogenesis are re-activated (Oka et al., 2006).

These cardiac transcription factors physically interact with one another. In particular, Tbx5 and Nkx2.5 interact with each other (Hiroi et al., 2001), and Nkx2.5 and Gata4 also interact with
each other to induce cardiac-specific gene expression in a synergistic manner (Durocher et al., 1997). The physical interactions between them suggest that they form a cardiac-specific transcription complex, and coordinate precise cardiac-specific gene activity in the developing heart. Other co-factors, which may be part of the transcription complex, remain to be investigated.

1.5 Gap junction proteins in cardiac morphogenesis and conduction system

Gap junction proteins control intercellular transport of ions and small molecules. The movement of ions across the membrane and between cells establishes electrical potential, contributing to the proper electrophysiology within the conduction system. There are several gap junction proteins in the mammalian heart. Distinguished by their molecular weights, they are designated as: Connexin40 (Cx40), Connexin43 (Cx43), and Connexin45 (Cx45).

In adult mouse heart, Cx40 and Cx43 are the main gap junction proteins. Cx40 is abundantly expressed in both right and left atria but has low expression in both ventricles, while Cx43 is more globally expressed in four chambers. Cx40 is the only gap junction protein detected in the proximal ventricular conduction system, while it is absent in the AV node (Delorme et al., 1995) as well as in the distal IVS. Cx40 is highly expressed in both RA and LA, while it is not expressed in the ventricular myocardium. Cx43, on the other hand, is the most abundantly expressed gap junction protein in all four chambers. However, previous studies on expression analysis revealed that Cx43 is not present in the His bundle and proximal region of the bundle branches while the distal region of the bundle branches has high Cx43 expression (reviewed in Gros and Jongsma 1996).

While loss of Cx40 in mice results in slower atrial conduction velocity (Hagendorff et al., 1999), it has been found that Cx40 can also contribute to proper atrial or ventricular septation during cardiac morphogenesis (Kirchoff et al., 2000; Gu et al., 2003). In vitro studies demonstrated that Tbx5, Nkx2.5, and Gata4 regulate the transcription of Cx40 by physically binding to Cx40 promoter regions (Linhares et al., 2004). Interestingly, in Tbx5\(^+/\);Nkx2.5\(^+/\) mice, Cx40 expression was missing, and ventricular conduction delays were reported, indicating that Tbx5 and Nkx2.5 act synergistically to regulate Cx40 in vivo (Moskowitz et al., 2007). Polymorphism
in Cx40 has been reported in a human population susceptible to atrial fibrillation as well (Firouzi et al., 2004).

Cx43 also serves important roles in cardiac morphogenesis and the cardiac conduction system. Complete loss of Cx43 in mouse led to cardiac morphogenesis defects, in particular, obstruction of the right ventricular outflow tract, which resulted in death at birth due to failure of pulmonary gas exchange process (Reaume et al., 1995). Further investigation determined that delay in the looping of the ascending part of the heart tube, which later gives rise to RV and OFT, was observed in the absence of Cx43 (Ya et al., 1998). Interestingly, heterozygous Cx43 mutant mice displayed significantly longer QRS duration, indicating slower ventricular conduction velocity due to the haploinsufficiency of Cx43 activity in the conducting system (Guerrero et al., 1997). These findings suggest that the absence of Cx43 can be compensated by other gap junction proteins during embryogenesis to allow the embryo to be viable up to birth; yet, the role of Cx43 in the conduction system is more specialized and cannot be replaced by other gap junction proteins.

1.6 Potential involvement of Irx3 and Irx5 during cardiac morphogenesis

Previous studies show that Irx genes are expressed in the developing heart as well as in adult hearts (Christoffels et al., 2000; Lebel et al., 2003; Costantini et al., 2005). Irx5 represses the potassium channel gene, Kv4.2, expression in adult mouse heart, setting up a gradient of Kv4.2 with the highest expression in the epicardium and lowest in the endocardium (Costantini et al., 2005). Loss of Irx5 also leads to higher susceptibility to ventricular tachycardia upon mechanical stress induction, indicating its role in regulating the ventricular conduction system.

Irx5 and Irx3 are co-expressed throughout development (Dana Cohen, unpublished data), and Irx5KO mice and Irx3KO mice are viable and fertile with no cardiac morphology defects. This suggests that they are functionally redundant during embryogenesis. At E14.5, Irx3 and Irx5 are co-expressed in the ventricular conduction system, alluding to their possible redundant and non-redundant roles in the conduction system development as well (preliminary data, Bruneau Lab).
Figure 6: Preliminary data show that *Irx3/5DKO* has double outlet right ventricle syndrome at E14.5 (Courtesy of Rong Mo, unpublished data) (A) A transverse section of wild type heart at E14.5, depicting the connection between the right ventricle and the pulmonary artery (B) In the transverse section of *Irx3/5DKO* heart at E14.5, the right ventricle and the pulmonary artery are connected (C) In wild type heart, the aorta is connected to the left ventricle (D) In *Irx3/5DKO* heart at E14.5, the aorta is connected to the right ventricle, resembling a congenital heart defect known as Double Outlet Right Ventricle syndrome (DORV) pa: pulmonary artery, ao: aorta, rv: right ventricle, lv: left ventricle.

However, loss of both Irx3 and Irx5 leads to embryonic lethality at E14.5 with gross phenotypes including exencephaly and hindlimb bone structure deformity (unpublished data, Hui Lab). Interestingly, *Irx3/5DKO* embryos present cardiac defects as well. Preliminary data showed that both aorta and pulmonary artery were connected to the right ventricle in *Irx3/5DKO* heart at E14.5, displaying a similar feature of a human congenital heart defect known as double outlet right ventricle syndrome (DORV) (Figures 6B and D). Further characterization of *Irx3/5DKO* cardiac phenotypes and investigation of whether Irx3/5 activity in the neural crest cell lineage or
endothelial cell lineage is necessary for proper OFT development will be done as a first and foremost step to understand the role of Irx3 and Irx5 in mammalian cardiovascular development.

1.7 Potential involvement of Irx3 in the cardiac conduction system

Interestingly, ventricular conduction system failure was observed in Irx3KO. Heart electrophysiology was analyzed on eight week old Irx3\(3^{tauLacZ/+}\) and Irx3\(3^{tauLacZ/tauLacZ}\) mice. ECG measurements show that QRS elongation was prominent in the absence of Irx3 (Figures 7B and C, Anna Rosen, unpublished data). This was mainly due to the slower conduction velocity from the bundle of His to the Purkinje system within the AV conduction system (Figure 7E). The slowing of conduction velocity can be due to AV block or bundle branch block, which can be caused by physical blockage of the conduction system due to hypoplastic conducting fibers or by changes in electrical properties due to misexpression of channel genes (Morita et al., 2008; Nass et al., 2007). In a fast-paced setting such as cardiac conduction system, a small delay of signal propagation can be detrimental.

Quantitative real-time PCR performed on Irx3\(3^{tauLacZ/+}\) and Irx3\(3^{tauLacZ/tauLacZ}\) neonatal cardiomyocyte culture showed that the expression level of Cx40 was greatly reduced in the absence of Irx3 (Figure 8). Meanwhile, the mRNA expression level of Cx43 did not have significant change in Irx3\(3^{tauLacZ/tauLacZ}\) cardiomyocyte culture. It is hypothesized that Irx3 regulates the expression of Cx40 in cardiac conduction system, thereby coordinating precise signal propagation within the AV conduction system. The spatial distribution of Cx40 and Cx43 will be studied with respect to Irx3 activity in order to understand how the major gap junction proteins of mammalian conduction system are regulated by Irx3.

Since Irx family members can serve as transcription activators or repressors in a context-dependent manner, understanding whether Irx3 functions as an activator or a repressor for downstream target genes in the conduction system is crucial. The affects of Irx3 activator form and Irx3 repressor form will be tested in vitro and in vivo to investigate the nature of Irx3 transcription factor in the mammalian cardiac conduction system.
Figure 7: Loss of Irx3 leads to the slowing of ventricular conduction velocity (Courtesy of Anna Rosen, unpublished data) (A) Normal ECG in 8 week-old Irx3\(^{+/+}\) mice (B) QRS elongation and R notch (R') are observed in 8 week-old Irx3\(^{-/-}\) mice (C) ECG analysis for PR, QRS, and QT interval duration in 8 week-old Irx3\(^{+/+}\), Irx3\(^{+/}\), and Irx3\(^{-/-}\) mice (D) Schematic diagram of different regions within atrioventricular conduction system, AH: atria to His bundle, AV: atria to ventricle, HV: His bundle to the distal branches in the ventricles

<table>
<thead>
<tr>
<th>Interval (msec)</th>
<th>Irx3(^{+/+}) (n=7)</th>
<th>Irx3(^{+/}) (n=11)</th>
<th>Irx3(^{-/-}) (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR</td>
<td>30.2 ± 0.6</td>
<td>35.1 ± 0.8</td>
<td>36.7 ± 5.5</td>
</tr>
<tr>
<td>QRS</td>
<td>9.4 ± 0.3</td>
<td>10.1 ± 0.3</td>
<td>11.6 ± 0.6*</td>
</tr>
<tr>
<td>QT</td>
<td>48.5 ± 1.5</td>
<td>44.1 ± 1.6</td>
<td>43.0 ± 1.0</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>466 ± 19</td>
<td>468 ± 8</td>
<td>450 ± 24</td>
</tr>
</tbody>
</table>

Figure 8: qRT-PCR shows that Cx40 mRNA expression level is positively regulated by Irx3 (Courtesy of Han Kim, unpublished data) Cx40 mRNA and Cx43 mRNA levels were analyzed in neonatal cardiomyocyte culture from \(Irx3^{tauLacZ/tauLacZ}\) (Irx3KO) and neonatal cardiomyocyte culture from wild type transfected with Irx3 construct containing adenovirus (AdIrx3). Cx40 mRNA level was significantly increased in AdIrx3 while significantly decreased in Irx3KO. No statistical significance was observed for Cx43 mRNA levels between AdIrx3 and Irx3KO.
Chapter 2
Materials and methods

2 Materials and methods

2.1 Mouse lines

In accordance with the Toronto Centre for Phenogenomics Animal Care Committee, all mice used in this study were housed in standard vented cages. The following mouse lines were used: Irx3\textsubscript{tauLacZ/+}, Esr1-Cre\textsuperscript{+/}, αMHC-Cre\textsuperscript{+/}, Wnt1-Cre\textsuperscript{+/}, Tie2-Cre\textsuperscript{+/}, Rosa26\textsuperscript{Irx3EnR}, Irx3\textsuperscript{KO/+}Irx5\textsuperscript{EGFP/+}, and Irx3\textsuperscript{floxflo}Irx5\textsuperscript{EGFP/EGFP}. Various crosses were set up to obtain genotypes of interest, and for embryonic phenotype analyses, timed pregnancy method was used. Assuming the mating occurred at midnight, the morning after the mating was denoted as 0.5 day post coitum (0.5dpc or embryonic day 0.5, E0.5). Embryos were dissected at E8.25, E9.0, E10.5, or E14.5 based on the experimental designs. For embryos, yolk sac was removed and digested in 300µL of 50mM NaOH at 95°C for 10 minutes. Addition of 100µL of 0.5M Tris-pH8.0 neutralized the solution. 2µL of DNA solution was used for PCR genotyping. For postnatal animals, ear clips were used for genotyping. All embryo images and whole heart images were acquired using Leica Fluorescence Stereomicroscope with 5.0 mega-pixel colour CCD camera powered by Apple iMac.

2.2 Ink injection

After dissection, embryos were placed into a petri dish with warm 1xPBS. Each embryo was transferred to the gel stand where its limbs were fixated with needles. The chest cavity was exposed after opening the rib cage. The tip of a finely drawn Pasteur pipette with India ink was inserted into the left ventricle of the embryo. The ink was expelled into the chamber by slowly blowing air into the other end of the Pasteur pipette.
2.3 MicroCT scanning

The embryos were fixed in 4% PFA at 4°C overnight after dissection. The embryos were washed in 1xPBS at 4°C for two days, then 1mL of 1% Iodine solution was used to stain the embryos for five hours prior to the scanning. The specimens were scanned at 14µm resolution for 2 hours using Micro-CT scanner (GE eXplore Locus Sp, GE Healthcare, London, ON, Canada). 720 views were acquired through 360° rotation with the x-ray source at 80 kVp and 80µA. After the scanning, the images were viewed and analyzed using GE Health Care MicroView version 2.1.2 for Windows Vista OS.

2.4 H&E staining

Embryos and organs were fixed in 4% PFA in PBS overnight at 4°C. Serial dehydration with different concentration of ethanol and xylene wash was performed. Embryos and organs embedded in paraffin were sectioned using microtome at 7µm and placed on glass slides. The slides were de-waxed and rehydrated, then were stained with hematoxylin for 10 minutes. Followed by rinsing with dH2O, the slides were subjected to serial incubations with 0.5%HCl, 70%EtOH, dH2O and 1% lithium carbonate. Samples were partially dehydrated, stained with 0.5% eosin and dehydrated again by EtOH and xylene. After mounting the slides with permount and xylene, each sample image was captured using Leica DM 2000 microscope with Nikon ACT-1 Version 2.70 on Windows Pentium IV.

2.5 X-gal staining

Embryos and hearts were dissected in cold 1xPBS, and fixed in 2.7% formaldehyde, 0.02% NP-40 in 1xPBS. After fixation, they were washed in 2mM MgCl2, 0.02% NP-40 in 1xPBS at 4°C for four times for 15 minutes each. For whole mount X-gal staining, the embryos were incubated in the X-gal solution containing 1mg/ml X-gal, 2mM MgCl2, 0.02% NP-40, 5mM K4Fe(CN)6-3H2O, 5mM K3Fe(CN)6 in 1xPBS for 1 hours for E8.25, 2 hours for E9.0, and overnight for E10.5 and E14.5 at 37°C. Whole mount X-gal staining of adult hearts was done by incubation in the X-gal solution overnight at 37°C. After X-gal solution incubation, they were dehydrated in methanol prior to imaging. For section X-gal staining, the samples were immersed to 30% sucrose at 4°C overnight after fixation and wash. They were embedded in OCT solution and
frozen with dry ice. 10µm sections were cut using cryostat machine and X-gal solution was applied after the sections were dry then rehydrated. Counter-staining with eosin for 10 minutes was performed afterward, followed by rehydration with EtOH and xylene, and mounting with permount and xylene.

2.6 Electrocardiogram measurements and analysis

Wire leads with Lead III configuration were implanted subcutaneously to adult mice under anesthesia. Body temperature of the animals was monitored to ensure the acquiring of representative electrophysiology measurements. ECG was taken for 10 minutes each time every other day for the period of one to three weeks. The most representative and consecutive 10 second strip from each set was used for statistical analysis.

2.7 Tamoxifen injection

To induce Cre-mediated recombinase activity in Esr1-Cre mice, tamoxifen injection was performed. The concentration of tamoxifen in sesame oil was determined based on the correlation between efficiency and concentration described in the literature (Lavine et al., 2008; Li et al., 2007). 5µL/body weight in grams was administered to 8-week old Esr1-Cre+/--;Rosa26Irx3EnR and wild type littermates for the five consecutive days by inserting the needle through the animal’s esophagus. In order to confirm the efficiency of tamoxifen injection, immunofluorescence staining with anti-GFP antibody was used to stain for GFP reporter protein fused to Irx3EnR.

2.8 Immunofluorescence staining and imaging

Frozen sections were prepared for the immunofluorescence staining. After fixing the perfused samples in 4% PFA upon dissection at 4°C overnight, followed by 30% sucrose incubation at 4°C, the samples were submerged into OCT and frozen in dry ice. Cryostat machine was used to section the frozen OCT blocks containing the samples at 15µm. The sections were then mounted on glass slides, and were dried overnight, then stored at -20°C for the future. Upon defrosting, the samples were rehydrated with 1xPBS wash. After membrane permeabilization with 0.3%H2O2-MeOH, incubation with blocking solution for 30 minutes was performed at room
temperature. The slides were incubated with primary antibodies in blocking solution (1:100 for anti-GFP antibody (invitrogen), 1:100 for anti-Cx43 antibody (sigma-aldrich, and invitrogen), and 1:100 for anti-Cx40 antibody (invitrogen)) at 4°C overnight. After washing with 1xPBS, the slides were incubated with secondary antibodies in blocking solution (1:200) for an hour. The slides were then washed with 1xPBS and mounted with DAPI and vector shield before imaging. Zeiss Axiovert 200 equipped with a Hamamastu Orca AG CCD, spinning disc confocal scan head, and a Hamamatsu C9100-13 EM0CCD 4 separate diode-pumped solid state laser lines (Spectral Applied Research: 405nm, 491nm, 561nm, and 638nm) was used. The equipment was driven by Volocity acquisition software and powered by a Pentium IV processor.

2.9 Optical projection tomography

The Optical projection tomography (OPT) scanning was performed as previously described (Sharpe et al., 2002). The heart samples were fixed at 4%PFA at 4°C overnight after clearing out the blood. 1xPBS wash was performed twice for 30 minutes 4°C then the samples were stored in 70% EtOH at 4°C. The samples were rehydrated in 1xPBS before being embedding into the 1% agarose with 24-28°C melting points. After cooling the agarose gel to 32°C, the samples were suspended in the middle to the individual gel blocks. Each block was placed on top of the mount and attached with glue prior to the scanning. The scanning was performed as previously described (Sharpe et al., 2002). Visualization and analysis of the images were performed with Ontario Consortium for Cardiac Imaging (OCCI) viewer version 1.0.99 for Windows Vista OS.

2.10 Western blot analysis

Atria and ventricles were separated in cold 1xPBS and flash frozen. Total protein lysates were prepared in lysis buffer containing 50mM Tris (pH7.4), 150mM NaCl, 5mM EDTA, 1mM EGTA, 0.1% SDS, 0.5% Doc, 1% NP-40, 25mM sodium pyrophosphate, 1mM sodium orthovanadate, 10mM NaF, 1mM β-glycerophosphate, and EDTA-free complete protease inhibitor cocktail (Roche) followed by centrifugation at 4°C. Proteins were separated by 8% SDS-PAGE and transferred to nitrocellulose for immunoblotting overnight at 4C with Irx3 (generated from Hui Lab), Cx40 (Santa Cruz), Cx43 (Sigma-Aldrich) antibodies.
2.11 Quantitative real-time PCR analysis

Gene expression assay was conducted on 10 ng of template cDNA by Quantitative PCR (qPCR) using Taqman and SYBR Green PCR methods equipped with ABI 7900HT (Applied Biosystems). Primers were designed using the Primer Express® software and the Primer-BLAST in the National Center for Biotechnology Information (NCBI) website, followed by confirmation with nucleotide BLAST. Other Primer sequences were obtained from previously published articles. Primers for SYBR green based PCR, optimized with different concentration and dissociation curve, and validated by the PCR efficiencies near 100% were used for experiments. PCR results described as threshold cycle value ($C_T$) were compared using relative quantitation of gene expression with Comparative $C_T$ Method ($\Delta\Delta C_T$ Method). The amount of target, normalized to an endogenous reference (GAPDH) and relative to a control group, is given by: $2^{-\Delta\Delta C_T}$. 
Chapter 3
Results – Part 1

3 Results – Part 1

3.1 Loss of Irx3 and Irx5 leads to various types of cardiac defects at E14.5

Previously, *Irx3/5DKO* mice were reported to be embryonic lethal at E14.5. To further understand the causes of embryonic lethality and the gross morphological defects, 27 *Irx3/5DKO* embryos were examined at E14.5. Loss of Irx3 and Irx5 resulted in a spectrum of phenotypes (Figure 9). The embryos were categorized into three groups, based on the severity of their gross morphology defects. Group 1, which presents the least severe phenotypes, displayed smaller craniofacial structure and smaller overall body size (Figure 9B, 7/27 (25.9%)). Gross hindlimb structure was comparable to the control littermates. The embryos in Group 2 also displayed reduction in overall craniofacial structure and body sizes. However, in contrast to the first group, their hindlimbs and tails were not curled up, an indication that they may have earlier lethality due to more severe cardiac functions (Figure 9C, 10/27 (37.0%)). In Group 3, the embryos displayed exencephaly in addition to craniofacial defects (Figure 9D, 5/27 (18.5%)). In the most severe cases, *Irx3/5DKO* embryos were already being resorbed at E14.5 with developmental arrest, indicating that these embryos had lethality at an earlier time point (data not shown, 5/27 (18.5%)).

Preliminary data showed that *Irx3/5DKO* displayed double outlet ventricle syndrome. To gain three-dimensional information on the cardiac morphology of *Irx3/5DKO* at E14.5, India ink was injected to visualize the orientation of great arteries with respect to ventricles. There were variable levels of morphology defects in these mutants. In wild type hearts, the ascending aorta rising from the left ventricle is located posterior to the pulmonary artery rising from the right ventricle (Figure 9A’ and A’’). In the least severe case, the relative orientation of ascending aorta and pulmonary artery was switched (Figure 9B’). In human populations, a similar case is known as Transposition of Great Arteries (TGA). Approximately 60% of TGA patients display anterior
ascending aorta on the right side of pulmonary artery. In other Irx3/5DKO hearts, it was more difficult to distinguish the origin of pulmonary artery and ascending aorta (Figures 9C’ and 9D’). The aortic arch, which loops leftward from the ascending aorta to the descending aorta connected to superior vena cava, is not properly formed in these embryos, indicating more severely hindered circulatory failure as a potential cause of embryonic lethality. Narrower ventricular chamber morphology was observed as well (Figure 9D’).

Although India ink injection provided some insights for great artery orientations in Irx3/5DKO, it was difficult to analyze intracardiac phenotypes as well as the inner connectivity between great vessels and ventricles. Therefore, one Irx3/5DKO embryo from Group 1 and Group 2 and one wild type littermate at E14.5 were subjected to microCT scanning.

At the four chamber level, the angle between IVS and the left to right axis in the centre of the body was smaller in these embryos (Figures 10B and 10C). This indicates that the lengthening or rotation of the great arteries may have been disrupted due to the loss of Irx3 and Irx5 activity during cardiac morphogenesis.

A sagittal view of the embryos showed outflow tract obstruction in both of them (Figures 11B’ and 11C’, red arrows). As the obstruction between the ventricles and the outflow tract can hinder the proper circulatory function, this morphological defect may be a contributing factor for the embryonic lethality in Irx3/5DKO. In one embryo, hemorrhaging was also observed in various body parts (Figures 11C and C’).

A coronal view of the embryos was studied to further investigate the orientation of the great arteries with respect to each other. In wild type, the pulmonary artery was located more caudally and on the left side of the aorta (Figure 12A’). However, in Irx3/5DKO, this relative positioning was lost. In one embryo, the pulmonary artery was found on top of the aorta (Figure 12B’), while the great arteries were located on the same level of Z axis in the other embryo (Figure 12C’).

Out of 27 Irx3/5DKO embryos collected, eight representative embryos were subjected to paraffin sectioning and H&E staining to further characterize the cardiac phenotypes. All eight embryos presented various cardiac phenotypes. 87.5% of them (7 out of 8) displayed ventricular septal defects, where there is an opening between the right and left ventricles due to incomplete chamber septation (Figure 13B and 13C). 50% of these embryos also presented double outlet
ventricle syndrome (4 out of 8, Figure 13C). More severe defects, which include indistinguishable ventricular chambers and narrower structure, were observed in 25% of the embryos (2 out of 8, data not shown). MicroCT scanning also revealed such intracardiac phenotypes in one of the Irx3/5DKO mutants analyzed.

3.2 Irx3 is expressed in the neural tube during early cardiac morphogenesis and in the outflow tract region as well as ventricles in late stages of development

Loss of Irx3 and Irx5 led to outflow tract development failure as well as endocardial tissue cushioning defects. To understand how Irx3 and Irx5 may contribute to the development of the outflow tract and endocardial tissue cushioning, the expression pattern of Irx3 was analyzed by performing beta-Gal staining on Irx3\textsuperscript{tauLacZ} whole embryos at various stages. The dynamic expression pattern of Irx3 could be observed. Previously in the lab, a transgenic Irx3\textsuperscript{tauLacZ} mouse line was generated by replacing exon 1 of Irx3 with tauLacZ cassette followed by a stop signal. The expression of tauLacZ is driven by the Irx3 promoter to recapitulate the Irx3RNA expression pattern.

At E8.25, Irx3LacZ was not detected within the developing heart undergoing looping and ballooning processes (Figure 14A). However, high Irx3LacZ expression was found in the neural tube and the pharyngeal arches posterior to the heart (Figure 14A, white arrowhead and yellow arrowhead). At E9.0, Irx3 expression is observed in the developing outflow tract (Figure 14B, white arrowhead). There was a faint expression in the intracardiac region, particularly, the endocardial tissue within the common ventricular chamber (Figure 14B). At E10.5, where the outflow tract development and the endocardial tissue cushioning continue, Irx3 expression was maintained in both outflow tract and within the common ventricular chamber (Figure 14D).

At E14.5, Irx3LacZ expression was found in the septated outflow tract structures, which are pulmonary artery and aorta (Figure 14E). While there was no Irx3LacZ expression in both RA and LA, the AV junction area showed Irx3LacZ expression (Figure 14E), suggesting a possible role for Irx3 in the developing conduction system. Both RV and LV also displayed high level of Irx3LacZ (Figure 14E).
3.3 Conditional knockout of Irx3/5 in the neural crest and endothelial cell lineages

Cardiac neural crest cell descendants and endothelial cell descendants are both necessary for proper outflow tract development and septation. In addition, endothelial cell descendants are also important for endocardial tissue cushioning. Since *Irx3/5DKO* hearts displayed various types of outflow tract defects and ventricular septation defects, it is important to determine which cell lineage is required for Irx3/5-depndant cardiac morphogenesis. To do so, *Wnt1-Cre* and *Tie2-Cre* mouse lines were used to generate conditional knockout of *Irx3/5* in the cardiac neural crest cell descendants and the endothelial cell descendants, respectively, using the *Irx3*\(^{\text{flox/flox}}\) *Irx5*\(^{\text{EGFP/EGFP}}\) mice (Figure 15).

3.4 Conditional loss of Irx3 and Irx5 in neural crest cell lineage leads to embryonic lethality and cardiac phenotypes at E14.5

Loss of Irx3 and Irx5 in the neural crest cell lineage led to early embryonic lethality. At E14.5, *Wnt1-Cre\(^{+/-}\);Irx3\(^{\text{flox/flox}}\)Irx5\(^{\text{EGFP/EGFP}}\) embryos (n=2) exhibited craniofacial defects, similar to *Irx3/5DKO* (Figure 16). These embryos were subjected to H&E staining after coronal sectioning in order to see the orientation of great arteries in these embryos. Interestingly, at the valve level (Figures 16C, D, and E), both conditional mutants showed hyperplasic aortic and pulmonary valve structures (Figures 16D and E). Furthermore, both valves were closed in one conditional mutant heart, suggesting possible valve function defects (Figure 16E). These observations suggest that Irx3 and Irx5 activity in neural crest cell descendants are important for outflow tract development.

3.5 Conditional loss of Irx3 and Irx5 in endothelial cell lineage leads to embryonic lethality and cardiac phenotypes at E14.5

Loss of Irx3 and Irx5 in endothelial cell descendants resulted in embryonic lethality. At E14.5, the conditional mutants displayed craniofacial defects comparable to *Irx3/5DKO* (Figure 17B, n=4). These embryos were subjected to transverse sectioning and H&E staining to examine whether loss of Irx3 and Irx5 in endothelial cell descendants results in endocardial tissue
cushioning defects. At the AO and PA levels (Figures 17C, D, and E), circulatory failure was observed in one mutant (Figure 17E, n=1). As expected, ventricular septal defect was observed at the subpulmonary artery level (Figure 17D, n=1). All four conditional mutants presented hyperplastic mitral and tricuspid valve structures (Figures 17D and E), alluding to possible functional failure as well as pressure-overload in the cardiovascular system. Furthermore, hyperplastic ventricles were observed, indicating the significance of Irx3 and Irx5 in the endothelial cell lineage for the cushioning process (Figure 17E).
Figure 9: *Irx3/5DKO* mice display various levels of gross morphology defects and cardiac phenotypes at E14.5.

27 Irx3/5DKO mutants at E14.5 were categorized into three groups based on the severity of their morphological defects (B, C, and D). They also presented varying degrees of cardiac phenotype. The transposition of great arteries (TGA) was observed in B’ and BB’’ where the aorta was anterior to the pulmonary artery (white arrowhead: AO, aorta, white arrow: PA, pulmonary artery). In embryos with more severe gross morphology defects, the proper connectivity between PA and RV, and AO and LV was not discernible (C’ and D’). Also, unitubular chamber structure was observed as well (D’). A’’, B’’, C’’, and D’’ are the schematic representation of A’, B’, C’, and D’, respectively.
Figure 10: *Irx3/5DKO* mice present abnormal positioning of aorta and pulmonary artery with respect to ventricles.

(A) A transverse view of wild type heart at E14.5 (white dotted line: the central body axis, red arrowhead: left ventricle, yellow arrowhead: right ventricle, green arrow: interventricular septum) (B and C) *Irx3/5DKO* hearts were more shifted towards left side of the embryos away from the central body axes. This indicates that the rotation of OFT structure might have been disrupted due to the absence of Irx3 and Irx5 activity. (C) Clear distinction between left ventricle (red arrowhead) and right ventricle (yellow arrowhead) is not observed, and the chambers are narrower.
Figure 11: *Irx3/5DKO* mice present blockage between the ventricles and the OFT.

(A and A’) A sagittal view of wild type heart at E14.5 from microCT scanning (A’’) A series of the sagittal sections from the right side of the embryo to the left side of the embryo show the proper connection between the pulmonary artery and the right ventricle, and the aorta and the left ventricle (red arrow: pulmonary artery, yellow arrow: aorta) (B, B’ and B’’) A sagittal view of the embryos revealed that the connection between ventricles and the OFT was disrupted (red arrow). (C and C’) Thickening of OFT was observed and blood clots are observed in one Irx3/5DKO (red arrow: outflow tract, green arrow: blood clot). (C’’) A series of sagittal sections show obstruction of aorta (yellow arrow) and multiple blood clots (green arrow).
Figure 12: *Irx3/5DKO* shows the misorientation of pulmonary artery and aorta with respect to each other. (A and A’) In wild type heart at E14.5, the pulmonary artery is located more cranially in comparison with the aorta (yellow arrowhead: pulmonary artery, green arrowhead: aorta). (B and B’) In *Irx3/5DKO*, the pulmonary artery (yellow arrowhead) is completely on top of the aorta (blue arrowhead) (C’ and C’) In another *Irx3/5DKO* embryo, stenosis is observed in both pulmonary artery and aorta while the pulmonary artery is not located more upward compared to the aorta (A’’-C’’). Sets of serial images from microCT scanning (yellow arrowhead: pulmonary artery, green arrowhead: aorta).
Figure 13: *Irx3/5DKO* presents ventricular septation defect (VSD) and double outlet right ventricle (DORV) syndrome at E14.5.

(A) A transverse section of wild type heart at E14.5 (B and C) The interventricular septum has an opening in *Irx3/5DKO* embryos (black arrows). (C) The connection between the aorta and the left ventricle is missing (C). Ao: aorta, PA: pulmonary artery, RV: right ventricle, LV: left ventricle. Scale: 400µm.
*Figure 14*: *Irx3LacZ* is expressed in extracardiac and intracardiac regions during embryogenesis. *Irx3LacZ* expression was observed in the neural tube but not in the heart undergoing looping process at E8.25 (A, white arrowhead: neural tube, yellow arrowhead: pharyngeal arch, red arrowhead: heart). At E9.0, *Irx3LacZ* was expressed in the outflow tract (B, white arrowhead: outflow tract). Faint expression was detected within the common ventricular chamber (B, red arrowhead). At E10.5, *Irx3LacZ* expression was prominent in both outflow tract region and the ventricular septum region (C and D, white arrowhead: outflow tract, red arrowhead: the ventricular septum region, white arrow: pulmonary endoderm). At E14.5, *Irx3LacZ* expression was seen in the pulmonary artery and aorta, the atroventricular junction, and both right and left ventricles (E, white arrow: pulmonary artery and aorta, yellow arrow: atroventricular junction, green arrowhead: right ventricle, red arrowhead: left ventricle).

*Figure 15*: Experimental design for cell lineage analysis of Irx3/5 activity in outflow tract development and ventricular septation.
Figure 16: Loss of Irx3 and Irx5 in neural crest cell lineage leads to cardiac defects as well as craniofacial defects.

Wnt1-Cre\textsuperscript{++}::Ir\x3\textsuperscript{flox/flox}\textsuperscript{Ir\x5\textsuperscript{EGFP/EGFP}} embryos at E14.5 presented smaller and more condensed craniofacial structure (B, white arrow). Frontal sectioning of the embryos revealed that these embryos also had cardiac defects. In comparison with the wild type littermate, the mutant embryos presented hyperplastic valve structures (D and E, black arrow: pulmonary valve, black arrowhead: aortic valve). The orientation of the pulmonary artery and aorta was incorrect as well (D' and E', black arrow: pulmonary artery, black arrowhead: aorta). Scale: 100\textmu m.
Figure 17: Loss of Irx3 and Irx5 in endothelial cell lineage leads to cardiac defects as well as craniofacial defects.

*Tie2-Cre^{+/+};Irx3^{flx/flx}Irx5^{EGFP/EGFP}* embryos at E14.5 presented smaller and more condensed craniofacial structure (B, white arrow). Transverse sectioning of the embryos revealed that they presented ventricular septal defect (D, black arrow) and hyperplasic valve structures (E and F, green arrowhead: tricuspid valve, blue arrowhead: mitral valve). Hyperplasic trabeculation and unitubular structure was observed as well (F). Scale: 400µm.
Chapter 4
Results – Part 2

4 Results – Part 2

4.1 Irx3 is highly expressed in the atrioventricular conduction system and the endocardium of both ventricles

To determine where *Irx3* is expressed in the adult mouse heart at the time of ECG measurements, X-gal staining was performed on eight-week old *Irx3tauLacZ/+* hearts. Long-axis staining of *Irx3tauLacZ/+* heart showed a distinct pattern of *Irx3LacZ* expression in the area of the atrioventricular junction (Figure 18A). *Irx3LacZ* expression was localized to the edge of the common bundle as well as the area near the atrioventricular node. The expression continued along the bundle branches on both right and left sides of the interventricular septum (Figure 18B). Heart electrophysiology data and the expression pattern of Irx3 from both short-axis and long-axis staining results indicate that Irx3 is an important component of the atrioventricular conduction system as well as the ventricular conduction system. Short-axis staining showed high level of *Irx3LacZ* expression in the endocardium of both right and left ventricles while the expression was absent in the epicardium of both ventricles (Figure 18C). This expression pattern is similar to that of Irx5 in adult mouse heart (Constantini et al., 2005). Open-chamber staining approach also displayed *Irx3LacZ* expression in the Purkinje fibers (Figure 18D).

4.2 Loss of Irx3 leads to reduced expression of Cx40 in the ventricular conduction system

The mRNA expression levels of Cx40 in the Purkinje system, endocardium, and epicardium were examined in *Irx3tauLacZ/tauLacZ* hearts to determine whether the regulation of Cx40 had any correlation with *Irx3LacZ* expression pattern. The expression level of Cx40 was significantly reduced in the Purkinje system of *Irx3tauLacZ/tauLacZ* (unpublished data, Anna Rosen). The expression level of Cx40 in endocardium and epicardium of *Irx3tauLacZ/tauLacZ* did not show any significant difference from that of *Irx3tauLacZ/+*. Another gap junction protein, Cx43, which is
more highly and globally expressed in adult mouse heart, did not show any significantly different expression levels in the absence of Irx3.

In conjunction with the qRT-PCR data, immunofluorescence staining experiments were performed to see whether loss of Irx3 leads to misexpression of Cx40 and Cx43 in the atrioventricular system. Immunofluorescence staining with anti-Cx40 antibody and anti-Cx43 antibody was performed on frozen sections of eight-week old Irx3+/+ and Irx3<sup>tauLacZ</sup>/+ hearts.

In wild type hearts, Cx40 expression was observed in the AV bundle of the atrioventricular conduction system (n=2, Figure 19A). However, Cx40 expression was not observed in this region in the absence of Irx3 (n=2, Figure 19B). Cx43, on the other hand, is not expressed in the proximal interventricular septum region. In the distal region, Cx43 expression is localized to the intercalated discs between cardiomyocytes. Immunofluorescence staining with anti-Cx43 antibody revealed that Cx43 is ectopically expressed in the absence of Irx3. In particular, expression was observed in the proximal region of the interventricular septum (Figure 19D), which was absent in the Irx3+/+ hearts (Figure 19C). Cx43 expression was only seen in the distal regions of the Irx3<sup>+/+</sup> hearts (Figure 19C). Taken together, the expression pattern of Cx40 and Cx43 was disrupted in the absence of Irx3, where ectopic expression of Cx43 was observed in the region where Cx40 expression was missing, in comparison with the wild type hearts (Figures 19E and F).

4.3 In vitro and in vivo approaches for studying the functions of Irx3 activator form and repressor form

Irx transcription factors can act as activators or repressors in a context-dependent manner. In collaboration with the Backx Lab, neonatal cardiomyocytes were transfected with adenovirus constructs containing Irx3<sup>Vp16</sup> (an activator form) or Irx3<sup>EnR</sup> (a repressor form) to determine whether Irx3 functions as a transcriptional activator or repressor of Cx40 in the mouse conduction system (Figure 20A). qRT-PCR showed that the over-expression of Irx3<sup>EnR</sup> in cardiomyocyte culture led to the high mRNA expression of Cx40 (Figure 20B). These effects were also observed with the over-expression of untagged and FLAG-tagged Irx3 constructs, suggesting that Irx3 mainly acts as a repressor to regulate Cx40. Interestingly, the over-expression of Irx3<sup>Vp16</sup> led to a reduction of Cx40 mRNA expression (Figure 20B). Western blot
analysis was performed as well to confirm that Cx40 level was increased by over-expression of Irx3 while it was decreased by over-expression of Irx3Vp16 (Figure 20C). These observations suggest that Irx3 upregulates Cx40 expression indirectly by repression of the expression of a transcriptional repressor of Cx40 (Figure 20D).

To test the effect of Irx3EnR in vivo, a conditional transgenic mouse line, Rosa26\textsuperscript{Irx3EnR}, was used. In this transgenic mouse line, Irx3EnR was targeted into the Rosa26 locus and Irx3EnR expression can be activated by Cre-mediated deletion of a LoxP flanked STOP cassette upstream of the Irx3EnR transgene. A GFP ORF fused at the end of Irx3EnR construct encodes a reporter protein to see whether the expression of Irx3EnR is induced. To test the effect of repression of Irx3EnR, two different Cre-LoxP systems were used: inducible Esr1-Cre and promoter-driven αMHC-Cre. Esr1-Cre allows the Cre-mediated recombination upon tamoxifen administration, thereby resulting in the ubiquitous expression of Irx3EnR in Esr1-Cre\textsuperscript{+/−};Rosa26\textsuperscript{Irx3EnR} mice (Figure 21A). In Esr1-Cre\textsuperscript{+/−};Rosa26\textsuperscript{Irx3EnR} mouse, cytoplasmic Cre-recombinase fused with mutated estrogen receptor ligand binding site is expressed. This Cre-recombinase can only enter the nucleus upon the binding of tamoxifen. Upon tamoxifen administration, Cre-recombinase can enter the nucleus and mediate homologous recombination to remove the PGK-neo TpA stop cassette, allowing the expression of Irx3EnR transgene. On the other hand, αMHC expression begins as early as E7.5 in the myocardial layer of developing mouse heart, and its expression continues in postnatal heart. This approach allows Cre-mediated recombination only in cardiomyocytes, thereby expressing Irx3EnR only in cardiomyocytes without tamoxifen administration (Figure 21B).

4.4 Ubiquitous over-expression of Irx3EnR using inducible Esr1-Cre results in heart rate variability

\textit{Esr1-Cre}\textsuperscript{+/−};Rosa26\textsuperscript{Irx3EnR} mice were generated by crossing the \textit{Esr1-Cre} line with the \textit{Rosa26\textsuperscript{Irx3EnR}} line. To induce the expression of Irx3EnR, 0.05mg/gram body weight of tamoxifen was administered via the esophagus to eight-week old animals for five consecutive days. At day 14 post tamoxifen injection, in vivo ECG measurements of \textit{Esr1-Cre}\textsuperscript{+/−};Rosa26\textsuperscript{Irx3EnR} showed heart rate variability, abnormal P wave pattern, and extra heart beat (Figures 22B and C). Immunofluorescence staining with anti-GFP antibody was performed to see the expression of the
reporter protein GFP in the hearts from Esr1-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup> mice. Immunofluorescence signal from GFP expression in all four chambers of the heart confirmed that the over-expression of Irx3EnR was induced upon successive injection of tamoxifen to the animals (Figures 22D to G). To prevent the physiological stress brought by anesthesia, a telemetry device was surgically inserted into the animals, and their cardiac conduction activity was further monitored. *Esr1-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup> mice constantly presented with heart rate variability (data not shown).

However, there were two caveats to this approach. First, the expression was not limited to the cardiovascular system. In fact, Irx3 is highly and globally expressed in lungs and also in brain. The over-expression of Irx3EnR in the lungs of *Esr1-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup> might have contributed to the abnormal ECG phenotype, in particular, the heart rate variability phenotype. It can also be speculated that aberrant Irx3 activity within the brain might have contributed to the cardiac conduction failure since the cardiac conduction system is also regulated by the brain stem. Second, tamoxifen injection may have non-specific effects on heart physiology. Tamoxifen injection into *Esr1-Cre<sup>+/−</sup> mice was sometimes lethal, probably due to the physiological stress from the drug toxicity. Out of six wild type control mice, four animals died during the drug administration period due to unknown health problems.

4.5 Cardiac-specific over-expression of Irx3EnR using promoter-driven αMHC-Cre results in high mortality, ventricular conduction failure, and atrial enlargement in adult mice

To induce cardiac-specific over-expression of Irx3EnR, a promoter-driven αMHC-Cre line was crossed with the Rosa26<sup>Irx3EnR</sup> line. αMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup> mice had a very high mortality rate. Six out of seven αMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup> died between the age of seven weeks to 13 weeks (Figure 23). To determine their cardiac conduction system function, ECG was performed on αMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup> and Rosa26<sup>Irx3EnR</sup> mice starting from the fifth week after birth. These mice presented conduction system failure with various levels of severity. The QRS elongation was prominent in these mice (n=7/11, Figures 24B and C), and for the more severe cases, double P wave was observed (n=1/11, Figure 24C). QRS duration was higher in αMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup> than Rosa26<sup>Irx3EnR</sup>, whereas, QT length was lower in αMHC-Cre<sup>+/−</sup>.
Measurements of RR interval and heart rate did not show a significant difference between αMHC-Cre$^{+/+}$;Rosa26$^{Irx3EnR}$ and Rosa26$^{Irx3EnR}$ mice (Figure 25).

Immunofluorescence staining with anti-GFP antibody confirmed that Irx3EnR was expressed in αMHC-Cre$^{+/+}$;Rosa26$^{Irx3EnR}$ mice (Figure 26). All αMHC-Cre$^{+/+}$;Rosa26$^{Irx3EnR}$ mice displayed atrial enlargement and thrombosis (Figures 27A and B). The heart weight to body weight ratio was more than 1.5 fold higher than the control mice (Figure 27C), mainly due to the atrial enlargement (Figures 27D and E). These mice exhibit ventricular hypertrophy (Figures 27F and G), which might have contributed to the higher heart weight to body weight ratio.

4.6 Cardiac-specific over-expression of Irx3EnR has an early onset phenotype in postnatal hearts but not in neonatal hearts

To determine the onset of atrial enlargement phenotype, hearts from the four-week old αMHC-Cre$^{+/+}$;Rosa26$^{Irx3EnR}$ and Rosa26$^{Irx3EnR}$ mice were harvested. Four out of six mice exhibited the atrial enlargement phenotype with various extents (Figures 28A and B), possibly due to mosaicism of the Cre-LoxP system. These animals were smaller than Rosa26$^{Irx3EnR}$ littermates, and had higher heart weight to body weight ratio (Figure 28C).

To understand the ventricular hypertrophy phenotype that was observed in older mice, I hypothesized that the over-expression of Irx3EnR throughout cardiac morphogenesis would lead to structural defects of the aortic and pulmonary valves, given the endogenous expression of Irx3 in the outflow tract region, and cause pressure-overload to the cardiovascular system. To test this hypothesis, these hearts were embedded in paraffin and transversely sectioned, followed by H&E staining to observe the valve structures. The valve structures of αMHC-Cre$^{+/+}$;Rosa26$^{Irx3EnR}$ were comparable to that of Rosa26$^{Irx3EnR}$ (Figures 28 D to G), ruling out the possibility of valve morphology defect due to the over-expression of Irx3EnR.

Neonatal hearts were harvested from αMHC-Cre$^{+/+}$;Rosa26$^{Irx3EnR}$ and Rosa26$^{Irx3EnR}$ mice. At P$_0$, the atrial enlargement phenotype was not observed (Figures 29A and B). The hearts were subjected to optical projection tomography (OPT) to obtain three-dimensional information of the internal structure. OPT analysis revealed that αMHC-Cre$^{+/+}$;Rosa26$^{Irx3EnR}$ heart structure was
comparable to \( \text{Rosa26}^{\text{Irx3EnR}} \), yet mild ventricular hypertrophy was observed (Figures 29C, D, and E). This indicates that \( \alpha\text{MHC-Cre}^{+/-};\text{Rosa26}^{\text{Irx3EnR}} \) atrial phenotype was not due to cardiac morphogenesis defects while ventricular hypertrophy may have been due to functional defects.

4.7 Western blot analysis shows that cardiac-specific over-expression of \( \text{Irx3EnR} \) leads to misregulation of \( \text{Cx40} \) in postnatal hearts

Western blot analysis on the heart lysates from \( \alpha\text{MHC-Cre}^{+/-};\text{Rosa26}^{\text{Irx3EnR}} \) and \( \text{Rosa26}^{\text{Irx3EnR}} \) mice was performed to see whether there is a change of \( \text{Cx40} \) expression similar to the neonatal cardiomyocyte culture data. Since \( \text{Irx3} \) is not expressed in the atria, atria and ventricles were dissected separately and the effects of \( \text{Irx3EnR} \) over-expression in these structures were examined by western blot analysis of \( \text{Cx40} \) expression. In atria, \( \text{Cx40} \) expression was comparable between \( \text{Rosa26}^{\text{Irx3EnR}} \) and \( \alpha\text{MHC-Cre}^{+/-};\text{Rosa26}^{\text{Irx3EnR}} \) (Figure 30A). In ventricles, over-expression of \( \text{Irx3EnR} \) resulted in the increase of \( \text{Cx40} \) expression (Figure 30B), consistent with the neonatal cardiomyocyte culture data. These observations suggest that \( \text{Irx3EnR} \) over-expression leads to atrial-specific, and ventricular-specific regulation of downstream target genes, which will further be discussed in the next chapter.
Figure 18: *Irx3LacZ* is expressed in the atrioventricular conduction system and the endocardium in adult mice. (A) 8 week-old *Irx3LacZ* hearts with beta-gal staining showed *Irx3LacZ* expression in the atrioventricular conduction system. (B) Magnified view of (A) shows specific *Irx3LacZ* expression in the AV junction area as well as the proximal bundle branches. (C) Short-axis staining showed *Irx3LacZ* expression in the endocardium but absent in the epicardium. (D) Purkinje fibers also expressed *Irx3LacZ* in both right and left ventricles (left ventricle shown here). Scales: (A) 2000μm, (B) 500μm, (C) 2000μm.
Figure 19: Immunofluorescence staining of Cx40 and Cx43 in Irx3^{+/+} and Irx3^{tauLacZ/tauLacZ}
(A) Cx40 is expressed in the AV bundle of Irx3^{+/+} adult heart (green, Cx40, area indicated by white box, n=2)
(B) Cx40 is not expressed in the AV bundle of Irx3^{tauLacZ/tauLacZ} (white box, n=2)
(C) Immunofluorescence staining of Irx3^{+/+} does not detect Cx43 expression in the AV bundle (white box).
(D) Cx43 expression was detected in the AV bundle of Irx3^{tauLacZ/tauLacZ} (green, Cx43, area indicated by white box, n=3).
(E) Schematic of Cx40 and Cx43 expression pattern in wild type adult heart
(F) Schematic of Cx40 and Cx43 expression pattern in the absence of Irx3 in the atrioventricular region.
AV bundle: atrioventricular bundle, IVS: interventricular septum.
Scale: 48μm.
Figure 20: Effect of activator and repressor forms of Irx3 on Cx40 and Cx43 expression in cultured neonatal cardiomyocytes
(Courtesy of Han Kim and Vijitha Puvvindran) (A) Schematic of adenovirus constructs for cell transfection assay. (B) qRT-PCR showed Cx40 mRNA expression was increased when untagged Irx3 and Irx3EnR were over-expressed. On the other hand, reduction of Cx40 mRNA expression was observed when Irx3Vp16 was over-expressed. (C) Western blot showed that Irx3EnR and Irx3Vp16 were expressed in the cardiomyocyte culture (Irx3EnR = 150kDa, Irx3Vp16 = 95kDa, indicated by black arrows). Cx40 level was increased in both Irx3 and Irx3EnR transfected cardiomyocyte cultures but decreased in Irx3Vp16 transfected cardiomyocyte culture (red arrow). Cx43 level was reduced in Irx3EnR transfected cardiomyocyte culture but increased in Irx3Vp16 transfected cardiomyocyte culture (blue arrow). (D) Schematic of proposed mechanism on how Irx3 regulates Cx40 and Cx43.
Figure 21: Cre-LoxP systems to induce Irx3EnR expression in vivo. (A) *Esr1-Cre* contains a transgenic Cre-recombinase fused with a binding domain for mutated form of estrogen receptor (orange). Upon binding to tamoxifen, the fusion Cre-recombinase can enter the nucleus and removes the STOP cassette, activating the ubiquitous expression of Irx3EnR. (B) *αMHC-Cre* contains Cre-recombinase transgene. Cre-recombinase expression is driven by αMHC promoter, thereby, removing the STOP cassette, activating the cardiac-specific expression of Irx3EnR.
Figure 22: Tamoxifen-induced, ubiquitous expression of Irx3EnR leads to heart rate variability

(A) Wild type ECG taken 14 days post tamoxifen administration. RR interval duration was consistent and no abnormality was observed. (B) Esr1-Cre+/--;Rosa26Irx3EnR ECG taken 14 day post tamoxifen administration. RR interval duration was inconsistent (each black arrow indicating each RR interval) (n=4). (C) In some Esr1-Cre+/--;Rosa26Irx3EnR animals, an extra heart beat was observed (red arrow). (D) Immunofluorescence staining with anti-GFP antibody (red) showed that Irx3EnR was expressed in Esr1-Cre+/--;Rosa26Irx3EnR hearts (n=3). (D) left atrium. (E) endocardium of left ventricle. (F) interventricular septum. (G) epicardium of left ventricle. Scale: 12.5μm. DAPI: blue, anti-GFP antibody: red.

Figure 23: Promoter-driven, cardiac-specific expression of Irx3EnR in vivo leads to early lethality

αMHC-Cre+/--;Rosa26Irx3EnR mice had much shorter lifespan compared to their wild type littermates (n=4 for Rosa26Irx3EnR, n=7 for αMHC-Cre+/--;Rosa26Irx3EnR).

By fourteen weeks post birth, six out of seven animals died.
Figure 24: Promoter-driven, cardiac-specific expression of Irx3EnR in vivo leads to abnormal cardiac electrophysiology

(A) ECG recording of wild type littermate, Rosa26Irx3EnR showed normal QRS complex pattern (red arrowhead) and P wave pattern (blue arrow). (B and C) ECG recording of αMHC-Cre\textsuperscript{+/−};Rosa26\textsuperscript{Irx3EnR} displayed abnormal QRS complex pattern (red arrowhead, 63.6%, n=11), which suggests QRS elongation. (C) In some ECG recording of other αMHC-Cre\textsuperscript{+/−};Rosa26\textsuperscript{Irx3EnR} animals, double P wave was observed (blue arrow) (9.1%, n=11).
Figure 25: ECG analysis of Rosa26<sup>Irx3EnR</sup> and αMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup> adult mice

ECG analysis revealed that αMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup> animals did not have significantly different RR interval (p=0.33), heart rate (p=0.30), P wave width (p=0.60), or PR interval (p=0.22). On the other hand, they had longer QRS duration (p=0.072) and shorter QT interval (*p=0.054). (Rosa26<sup>Irx3EnR</sup>, n= 4, αMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup>, n=3). RR: RR interval, HR: heart rate, P width: P wave width, PR: PR interval, QRS: QRS complex duration, QT: QT interval.

Figure 26: Irx3EnR is expressed in both atria and ventricles of αMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup> animals

(A) Immunofluorescence staining with anti-GFP antibody showed no Irx3EnR expression in Rosa26<sup>Irx3EnR</sup> atria (left atrium shown). (B) Irx3EnR expression (red) was detected in αMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup> atria (left atrium shown). (C) No Irx3EnR expression was detected in Rosa26<sup>Irx3EnR</sup> ventricles (left ventricle shown). (D) Irx3EnR expression (red) was detected in αMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup> ventricles (left ventricle shown). DAPI: blue, anti-GFP antibody: red.
Figure 27: Over-expression of Irx3EnR leads to atrial enlargement and ventricular hypertrophy in adult mice

(A) Adult Rosa26<sup>Irx3EnR</sup> hearts did not display atrial enlargement (n=7) (B) 8-13 week old aMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup> hearts displayed atrial enlargement for both right atrium (arrowhead) and left atrium (arrow) (n=9) (C) aMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup> animals had significantly smaller body weight but larger heart weight, with almost 2 fold increase in heart weight to body weight ratio (Rosa26<sup>Irx3EnR</sup>, n= 7, aMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup>, n= 9, *p<0.05). (D) H&E staining of Rosa26<sup>Irx3EnR</sup> animals did not show atrial enlargement (E) H&E staining of aMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup> animals showed atrial enlargement (LA shown here) (F) H&E staining of Rosa26<sup>Irx3EnR</sup> ventricles did not show any hypertrophy (indicated by black arrow) (G) H&E staining of aMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup> showed ventricular hypertrophy (indicated by black arrow) Scale: 400μm. RA: right atrium, LA: left atrium, RV: right ventricle, LV: left ventricle, IVS: interventricular septum.
Figure 28: Over-expression of Irx3EnR leads to atrial enlargement with no abnormal valve morphology at four weeks
(A) Four-week old Rosa26\(^{Irx3EnR}\) heart (B) Four-week old \(aMHC-Cre^{+/+};\)Rosa26\(^{Irx3EnR}\) hearts displayed varying degrees of atrial enlargement for both right atrium (black arrowhead) and left atrium (black arrow) (C) Four-week old \(aMHC-Cre^{+/+};\)Rosa26\(^{Irx3EnR}\) animals had smaller body weight, and larger heart weight to body weight ratio. However, no statistically significant difference was observed (p values all greater than 0.05). (D and E) H&E staining of transversely sectioned four-week old Rosa26\(^{Irx3EnR}\) hearts with aortic valve (black arrowhead) and pulmonary valve (black arrow) (leaflets of aortic valve: green arrowheads, leaflets of pulmonary valve: navy arrowheads) (F and G) H&E staining of transversely sectioned four-week old \(aMHC-Cre^{+/+};\)Rosa26\(^{Irx3EnR}\) hearts did not display aortic valve (black arrowhead) and pulmonary valve (black arrow) defects such as valve size, thickness, or leaflet morphology defects (leaflets of aortic valve: green arrowheads, leaflets of pulmonary valve: navy arrowheads). RA: right atrium, LA: left atrium, RV: right ventricle, LV: left ventricle, AoV: aortic valve, PV: pulmonary valve.
Figure 29: Over-expression of Ixr3EnR leads to no atrial phenotype but mild ventricular hypertrophy at birth

(A) Dorsal view of wild type neonatal heart OPT scanning (B) Dorsal view of αMHC-Cre+/−;Rosa26Ixr3EnR neonatal heart showed no obvious atrial enlargement or other structural defects (n=3) (C) Transverse view of wild type neonatal heart (D) Transverse view of αMHC-Cre+/−;Rosa26Ixr3EnR showed mild ventricular hypertrophy (indicated by white arrows) (E) αMHC-Cre+/−;Rosa26Ixr3EnR animals had higher heart weight (n=7 for Rosa26Ixr3EnR, n=3 for αMHC-Cre+/−;Rosa26Ixr3EnR, p=0.0004), comparable body weight (p=0.12), and higher heart weight to body weight ratio (p=0.018). RA: right atrium, LA: left atrium, RV: right ventricle, LV: left ventricle.
Figure 30: Cx40 expression level is increased in the ventricles with Irx3EnR overexpression
(A) Irx3EnR protein expression and endogenous Irx3 protein expression were observed in the atria of αMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup> (n=2). Cx40 protein expression levels in the atria were comparable between Rosa26<sup>Irx3EnR</sup> and αMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup>. (B) Irx3EnR expression was observed in the ventricles of αMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup> (n=2). Endogenous Irx3 expression was increased in αMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup>. Cx40 protein expression was increased in the ventricles of αMHC-Cre<sup>+/−</sup>;Rosa26<sup>Irx3EnR</sup>. 
5 Discussion and Future Directions

5.1 Irx3 and Irx5 are necessary for outflow tract septation and ventricular septation

Irx3/5DKO mutant analysis in this study showed that Irx3 and Irx5 are necessary for proper cardiac morphogenesis. At E14.5, two most prevalent phenotypes were observed in Irx3/5DKO. First, OFT septation failure was observed, where the proper connection between the ventricular chambers and the great arteries was missing. Second, ventricular septal defects were observed, which display an opening between right ventricle and left ventricle, suggesting disrupted endocardial tissue cushioning process during the ventricular septation stage.

Using Irx3tauLacZ/ transgenic mice, the dynamic expression pattern of Irx3LacZ was studied starting from an early time point to further investigate the involvement of Irx3 and Irx5 during earlier cardiac morphogenesis stages. High Irx3LacZ expression was observed in the neural tube at E8.25 while there was no cardiac-specific Irx3LacZ expression. At E9.0, expression was observed in the OFT region as well as the presumptive interventricular septum region. At later stages, these expression patterns are consistent. At the completion of cardiac morphogenesis at E14.5, Irx3LacZ expression is observed in both pulmonary artery and aorta, both right and left ventricles, and AV junction.

Outflow tract septation and ventricular septation require proper contributions from neural crest cell and endothelial cell lineages. Conditional mutants were generated by using promoter-driven Wnt1-Cre and Tie2-Cre lines to ablate the activity of Irx3 and Irx5 in the neural crest cell lineage and the endothelial cell lineage, respectively. The cardiac morphology of these embryos was examined at E14.5. In Wnt1-Cre+/-;Irx3\(^{\text{floox/floox}}\)Irx5\(^{\text{EGFP/EGFP}}\) embryos, hyperplasic aortic valve and pulmonary valve were observed, and some level of obstruction was seen as well. In Tie2-Cre+/-;Irx3\(^{\text{floox/floox}}\)Irx5\(^{\text{EGFP/EGFP}}\) embryos, ventricular septal defect and hyperplasic atrio-ventricular valves were observed. While further experiments are needed to determine how exactly Irx3 and
Irx5 regulate the OFT development and septation, and ventricular septation, these findings suggest that Irx3 and Irx5 are involved in both neural crest and endothelial cell lineages for proper cardiac morphogenesis.

5.2 Irx3 and Irx5 activities are required in various lineages and signaling pathways for outflow tract development and ventricular septation process

The outflow tract defects and ventricular septation defects were the most prevalent in Irx3/5DKO embryos at E14.5. For the outflow tract septation process, the contribution of neural crest cell lineage is crucial (Kirby et al., 1983; Goddeeris et al., 2007; Kwang et al., 2002; Morgan et al., 2008). High Irx3LacZ expression in the neural tube at the early embryonic stage and in the outflow tract in the later stages suggests that extracardiac contribution of Irx3 and Irx5 is necessary for the proper OFT development and septation. The absence of Irx3 expression within the developing heart at early stages is another clear piece of evidence for the importance of extracardiac contribution of Irx3 and Irx5 to OFT. Conditional loss of Irx3 and Irx5 in neural crest cell descendants led to outflow tract defects. This suggests that the OFT phenotypes observed in Irx3/5DKO was partially due to the absence of Irx3 and Irx5 in neural crest cell descendants.

Cardiac morphogenesis requires coordinated activities of various signaling pathways. For instance, proper regulation of Sonic hedgehog (Shh) signaling pathway is necessary for outflow tract septation. The Shh pathway is activated by Shh molecules binding to transmembrane receptors Patched1 and Patched2. There are multiple steps involved in the regulation of Shh pathway activity (Jiang and Hui 2008). Mutations in the components of the Shh signaling pathway display OFT defects (Washington Smoak et al., 2005), and more interestingly, disruption of the signaling pathway within the neural crest cell lineage also leads to OFT defects (Goddeeris et al., 2007). Interestingly, genetic interaction between Irx3 and Shh signaling pathway in developing neural tube is alluded to in the previous studies (Briscoe et al., 2000). In the neural tube, the gradient of Irx3 expression is negatively regulated by Shh.

While much remains to be elucidated as to how exactly Irx3 and Irx5 activity within the neural crest cell lineage contributes to OFT development, it is interesting to ask whether there is an
interaction between Irx3/5 and Shh pathway. To address this question, compound mutants were
generated. Surprisingly, compound mutants with loss of Irx3/5 and reduction of Shh were viable
past E14.5 with properly septated OFT, indicating that there is a genetic interaction between
Irx3/5 and Sonic hedgehog pathway to regulate the proper OFT septation (Jieun Kim,
preliminary data). However, more experiments should be conducted to understand how Irx3/5
activities are coordinated with Shh pathway during heart development.

On the other hand, other populations of cells such as pharyngeal endoderm and cells of the
anterior heart field also contribute to OFT development (Waldo et al., 2005, Mjaatvedt et al.,
2001). Thus, lineage-analysis of these populations with respect to Irx3/5 activity will be
informative to understand the roles of these transcription factors during OFT development.

Previous studies have shown that rotation of the OFT is a crucial factor for proper aortic-
pulmonary septation and positioning as the failure in correct OFT rotation initiation might lead to
misorientation of the great arteries (Bajolle et al., 2006). While Pax3 mutation associated with
DORV might be due to the neural crest cell failure, Pitx2c mutants also display DORV
phenotype despite the presence of neural crest cells, indicating that rotation defect can be a factor
leading to OFT septation failure (Bajolle et al., 2006). There is a possibility that Irx3/5 activity is
involved in the rotation of OFT during early OFT development stage, as some of the embryos
displayed left-shifted heart phenotype. Characterizing of Irx3/5DKO heart at earlier time points
will help determine whether Irx3/5DKO phenotypes observed at E14.5 were also due to OFT
rotation defects. Cardiac neural crest cells migrate from the neural tube into the OFT region and
can contribute to the differentiation of developing OFT myocardium (van den Hoff and
Moorman 2000; Epstein et al., 2000; Le Douarin et al., 2004). Therefore, it is worth investigating
whether the loss of Irx3 and Irx5 leads to defects on neural crest cell migration, and how their
activity within the neural crest cell descendants is necessary for the differentiation and
specification of the OFT myocardium.

Another interesting finding from this study was the significance of Irx3 and Irx5 activity in the
endothelial cell lineage. In particular, the observation of ventricular septal defects and
hyperplasic valve formation in mice lacking Irx3/5 in endothelial cells indicate that Irx3 and Irx5
are necessary for the proper endocardial tissue cushioning process at the AV junction as well as
IVS. In order to explain the similar phenotypes shared by conditional loss of Irx3 and Irx5 in the
endothelial cell lineage and neural crest cell lineage, we can speculate that the requirement of Irx3 and Irx5 activity in these two cell lineages may be different in a spatiotemporal manner. In accordance with the previous findings from the literature, the significance of Irx3 and Irx5 may be greater within endothelial cell lineage at an early stage of cardiac morphogenesis while more significant in neural crest cell lineage at a later stage. It is hypothesized that the loss of Irx3 and Irx5 in the endothelial cell lineage at an early stage could attribute to the phenotypes similar to Irx3/5DKO at E14.5 as the requirements of Irx3 and Irx5 activity at an earlier time point was not met. On the other hand, the loss of Irx3 and Irx5 in the neural crest cell lineage at a later stage could disrupt the fine-tuning of development, leading to the phenotypes observed in Irx3/5DKO at E14.5.

5.3 Irx3 and Irx5 may function as part of cardiac transcription factor complex during cardiac morphogenesis

Congenital heart diseases in human populations with mutations in any of the cardiac transcription factors, TBX5, GATA4, or NKX2.5, often consist of multiple defects in various regions or different types and severity of defects from one patient to another (reviewed in Bruneau 2008). These cardiac transcription factors form a transcription complex and regulate cardiac-specific genes during morphogenesis, and the mutation in any of these factors could cascade into varying degrees of phenotype as the final outcome of cardiac morphogenesis since the combination between cardiac transcription factor activity and downstream target genes could vary in a spatiotemporal manner.

There was a noticeably different level of gross morphology severity among 27 Irx3/5DKO embryos at E14.5, indicating a variable correlation between the genotype and the expected phenotypes. This suggests that Irx3 and Irx5 have complex interactions with other factors and regulations of downstream target genes throughout embryogenesis. The trend of various penetrance levels was also observed in the cardiac morphology of eight representative embryos.

Co-IP data suggest that Irx3 and Irx5 physically bind with other cardiac transcription factors in both Cos1 cell culture and cardiomyocyte culture (unpublished data, Hui Lab). Therefore, further studies are needed to understand the potential involvement of Irx3 and Irx5 in the cardiac transcription factor complex machinery during cardiac morphogenesis.
5.4 Irx3 is expressed and necessary in the atrioventricular conduction system of adult mouse heart

QRS elongation and slower ventricular conduction velocity were the prominent features of adult Irx3tauLacZ\textsuperscript{+/+} mice. In conjunction with these findings, the expression pattern of Irx3 was studied by using Irx3\textsuperscript{tauLacZ/tauLacZ} heart sections. High expression of Irx3LacZ was observed in the endocardium as well as the AV junction and bundle branches of the conduction system. qRT-PCR experiments revealed that Cx40, in particular, is positively regulated by Irx3 within the Purkinje system. Immunofluorescence staining revealed that Cx43 is ectopically expressed in the proximal region of atrioventricular conduction system in the absence of Irx3. On the other hand, the expression level of Cx40 was missing in the AV bundle of atrioventricular conduction system.

5.5 Irx3 is an indirect positive regulator of Cx40 and dominant negative mutation of Irx3 leads to atrial and ventricular phenotypes

In vitro assays were performed with cardiomyocytes transfected with adenovirus containing Irx3, FLAG-Irx3, Irx3Vp16 (activator form), Irx3EnR (repressor form). qRT-PCR and western blot experiments demonstrated that both the endogenous and repressor forms of Irx3 led to upregulation of Cx40, suggesting that Irx3 indirectly regulates Cx40, potentially repressing a repressor upstream of Cx40. To further investigate the role of Irx3 repressor form, gain of function analysis was performed by over-expressing Irx3EnR in vivo. An inducible, ubiquitous Esr1-Cre line and a promoter-driven, cardiac-specific αMHC-Cre line were used to allow the temporal or spatial control of Irx3EnR expression in vivo, respectively.

To induce the ubiquitous over-expression of Irx3EnR, tamoxifen was administered to animals carrying Esr1-Cre and Rosa26\textsuperscript{Irx3EnR}. Esr1-Cre\textsuperscript{+/+};Rosa26\textsuperscript{Irx3EnR} mice displayed heart rate variability upon the drug administration. However, there were concerns with this experimental approach for the two following reasons. First, tamoxifen administration was lethal to some of Rosa26\textsuperscript{Irx3EnR} littermate control mice. Therefore, it was difficult to conclude that the heart rate variability phenotype was entirely due to the over-expression of Irx3EnR. It is possible that the
drug toxicity may have contributed to the phenotype. Second, high expression of Irx3LacZ in lungs suggests that the ubiquitous over-expression of Irx3EnR may also have effects on the thoracic system. Potential misregulation of lung-specific genes may have been a factor for the heart rate variability phenotype.

The use of αMHC-Cre line, on the other hand, showed that the over-expression of Irx3EnR leads to aberrant atrial and ventricular conduction system activities. ECG analyses demonstrated that adult αMHC-Cre<sup>+/−;Rosa26<sup>Irx3EnR</sup> mice had QRS elongation and may also have ST wave elevation, which are the indicators of ventricular conduction system failure. In some case, double P wave was observed as a sign of atrial conduction system failure. These animals also had significantly shorter life span. In all animals, atrial enlargement and ventricular hypertrophy were observed, suggesting that the over-expression of Irx3EnR leads to electrical and structural remodeling of the conduction system and other functional failure.

To determine the onset of the phenotypes, four week old, three week old, and neonatal mice were examined. The atrial enlargement phenotype was observed in some of the four week and three week old animals, while neonatal atria were comparable to the littermate control animals’ atria. However, mild ventricular hypertrophy was observed in the neonatal animals. In order to address the ventricular hypertrophy phenotype, valve stenosis was hypothesized to be a potential cause. However, within the four week old mutant mice and neonatal mutants examined, no abnormal valve structure was observed. Taken together, these suggest that the over-expression of Irx3EnR leads to physiological defects such as atrial enlargement after birth as well as congenital defect such as ventricular hypertrophy upon birth.

In conjunction with the in vitro data, Cx40 was upregulated in the ventricles of adult αMHC-Cre<sup>+/−;Rosa26<sup>Irx3EnR</sup> while the Cx40 level within the atria was comparable to that of the wild type littermate. The mutant animals presented ventricular conduction failure, such as QRS elongation, during ECG measurements prior to being subjected to the western blot analysis, suggesting that aberrant Cx40 expression level may have contributed to the abnormal electrophysiology.

However, many questions remain to be answered. As αMHC-Cre<sup>+/−;Rosa26<sup>Irx3EnR</sup> animals have postnatal phenotypes, determining the onset of the aberrant Cx40 expression level would be informative to link the Cx40 misregulation and ventricular conduction system phenotype. Another interesting aspect is the atrial phenotype. While most of αMHC-Cre<sup>+/−;Rosa26<sup>Irx3EnR</sup>...
displayed the postnatal atrial enlargement phenotype, the expression level of Cx40 within atria was comparable to the wild type. While Irx3LacZ expression was not observed in Irx3\(^{tau\:LacZ/+}\) atria, Cx40 is known to be highly expressed in the atria. Therefore, it can be speculated that the over-expression of Irx3EnR within the atria leads to misregulation of other atrial-specific genes, leading to the enlargement phenotype. Further studies are required to verify other downstream target genes in the atria.

5.6 Possible mechanism of Irx3 in the regulation of mammalian cardiac conduction system and morphogenesis

The specific expression pattern of Irx3LacZ in the AV junction and bundle branches as well as in the Purkinje fibers suggests that Irx3 regulates downstream target genes within these regions to propagate the electrical signal properly down the IVS. Along with qRT-PCR data on the regulation of Cx40 and Cx43 by Irx3, immunofluorescence staining result pinpoints the regions of Cx40 and Cx43 regulation by Irx3. Cx40 and Cx43 possess different electrical properties (Valiunas et al., 2002). The findings from this study, where the ectopic expression of Cx43 is seen in the original expression area of Cx40 in the absence of Irx3 as a potential underlying mechanism for ventricular conduction failure, provide good evidence that Cx40 and Cx43 present different electrical properties for ventricular conduction system. As Cx40 expression is reduced in the AV bundle region and ectopic Cx43 expression is observed in the same region instead in Irx3\(^{KO}\) heart, this suggests that the property of the conduction system circuitry may have changed due to loss of Irx3.

Another interesting finding was the indirect positive regulation of Cx40 by Irx3. Over-expression of Irx3EnR resulted in the increase of Cx40 expression in both neonatal cardiomyocyte culture and \(\alpha MHC-Cre^{+/};Rosa26^{Irx3EnR}\) ventricles while resulting in atrial enlargement phenotype. This suggests that Irx3 may be a novel regulator of atrial and ventricular-specific genes, similar to Irx4, which acts as a transcriptional activator for ventricular genes and a repressor for atrial genes in chick heart (Bao et al., 1999). While Cx40 expression was increased in the ventricles of \(\alpha MHC-Cre^{+/};Rosa26^{Irx3EnR}\), over-expression of Irx3EnR did not affect Cx40 expression level in the atria. While endogenous Irx3 was not expressed within atria from embryonic stages to the adult stage, the expression of Irx3EnR using cardiac-specific \(\alpha MHC-Cre\) led to the expression of
endogenous Irx3 in the atria. The aberrant atrial expression of Irx3 and Irx3EnR may be the cause of atrial enlargement as well as atrial fibrillation observed in some of the animals. Therefore, $aMHC-Cre^{+/−};Rosa26^{Irx3EnR}$ could be a good model to study regulation of atrial-specific genes by providing an environment where the atrial-specific gene activity is disrupted.

The indirect positive regulation of Cx40 by Irx3 provides another perspective to understand the role of Irx3 in cardiac conduction system. Other cardiac transcription factors, Nkx2.5 and Tbx5, positively regulate Cx40. Tbx5 regulates Tbx3, which represses Cx40 expression (Hoogaars et al., 2004). Tbx3 is a candidate repressor downstream of Irx3 and upstream of Cx40. In preliminary RT-PCR experiment, $Tbx3$ mRNA expression was increased in $Irx3KO$ neonatal cardiomyocyte culture (Han Kim, unpublished data). While many experiments need to be performed, this preliminary data suggest that Irx3 may represses Tbx3, to positively regulate Cx40 expression. Also, there is a possibility that in the absence of Irx3, Tbx3 level is increased, hence Cx40 level is decreased.

However, while Tbx3 could be a potential target of Irx3 to regulate Cx40, it is possible that there are unknown factors downstream of Irx3. Therefore, a high-throughput approach such as microarray analysis should be performed to score multiple candidate genes. More informative would be the microarray data on $aMHC-Cre^{+/−};Rosa26^{Irx3EnR}$ at various time points. In particular, using an inducible $aMHC-Cre$ line to temporally express Irx3EnR at the time points of endogenous Irx3 expression would eliminate the potential artifacts from ectopic expression. This will not only allow us to understand the mechanism of Irx3 for Cx40 regulation but also to determine what other factors are affected by Irx3 repressor to result in the observed phenotypes such as ventricular hypertrophy, which has shown to be regulated by Gata4 (Oka et al., 2006). Also, the atrial fibrillation susceptibility is higher when Tbx3 is ectopically expressed (Hoogaars et al., 2007), suggesting that the atrial phenotype of $aMHC-Cre^{+/−};Rosa26^{Irx3EnR}$ could somehow be linked with the Tbx3 upregulation due to Irx3EnR over-expression.

As previously mentioned, Irx3 may be a co-factor for the cardiac transcription complex. The spatiotemporal requirements of such transcription complex may vary at different developmental and physiological stages. Tbx3 mutants present defects in heart development (Mesbah et al., 2008). Also, Cx40 and Cx43 are involved in the cardiac morphogenesis. The mechanism of Irx3 on regulation of these gene activities may be conserved during cardiac morphogenesis.
Therefore, both embryonic study on Irx3/5 and physiology study on Irx3 described here suggest that these transcription factors may play a conserved role in coordinating the activities of the transcription complex at various time points.
References


Reaume AG, de Sousa PA, Kulkarni S, Langille BL, Zhu D, Davies TC, Juneja SC, Kidder GM,


