UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of *T-box 20* in Midgestation Cardiomyocytes

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by

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The Dissertation of Xiaoming Zhu is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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LIST OF ABBREVIATIONS

ASD	atrial septal defect
CHD	congenital heart disease
ChIP-seq	Chromatin Immunoprecipitation-Sequencing
CNV	copy number variant
Cre	Cre recombinase
EndoMT	endothelial-mesenchymal-transition
FHF	first heart field
H3K4me3	trimethylation of lysine 4 of histone H3
OFT	outflow tract
SHF	second heart field
TF	transcription factor
Rosa tdTom	*Rosa26 flox-stop-flox tdTomato*
RNA-seq	RNA sequencing
VSD	ventricular septal defect

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ABSTRACT OF THE DISSERTATION

The Role of *T-box 20* in Midgestaion Cardiomyocytes

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Professor Sylvia Evans, Chair

T-box transcription factor 20 (TBX20) is a transcription factor belonging to the highly conserved T-box family. During cardiogenesis, *Tbx20* is expressed in multiple cell lineages critical for heart formation, including cardiac progenitors, endoderm, cardiomyocytes, endocardial cells and endothelial cells. Mutations in *TBX20* are associated with congenital heart disease (CHD)

including atrial septal defects (ASDs), ventricular septal defects (VSDs) and mitral stenosis (MS). Global or endocardial ablation of *Tbx20* results in cardiac malformations and embryonic lethality. The potential function of *Tbx20* in midgestation cardiomyocytes has yet to be explored. To address this question, cardiomyocyte-specific inducible *Tnnt2-rtTA; TetO-Cre* was used to ablate *Tbx20* by inductions at E8.5, resulting in mutants termed *Tbx20 cKO*. *Tbx20 cKO*s exhibited embryonic lethality at E14.5, with mutant hearts displaying ASDs, VSDs, and hypoplasia of ventricles and left atria. Proliferation was significantly reduced in lineage-traced cardiomyocytes of *Tbx20 cKO*s. An intersection of RNA-seq analyses of purified cardiomyocytes with TBX20-GFP ChIP-seq of embryonic hearts, in conjunction with RNA in situ analyses, revealed that TBX20 in cardiomyocytes directly regulated genes required for myocyte proliferation, and multiple aspects of cardiomyocyte identity and patterning. Of note, TBX20 was discovered to directly regulate *COUP-TFII*, a key regulator of atrial identity. *Tbx20 cKO* atria exhibited decreased expression of *COUP-TFII* and other atrial markers, as well as overexpression of ventricular-specific gene. Our work sheds light on mechanisms by which mutations in TBX20 cause congenital heart disease and highlight a pivotal cell autonomous role for TBX20 in atrial myocyte development.

Chapter 1 Introduction

1.1 Etiology of congenital heart disease

Congenital heart disease (CHD) refers to structural or functional abnormalities of the heart presented at birth that affect blood circulation [1]. CHD is the most common birth defect among all major congenital abnormalities, with 10% occurrence in still births and about 1% in infants. This incidence might be higher due to late manifestation of mild lesions [2]. CHD can result in infantile death, but with advanced prenatal diagnosis and corrective surgery, infantile mortality has substantially declined. However, the population of adults living with CHD is growing at about 5% per year [3, 4]. Although some CHDs can be repaired, they still greatly impact the newborn and its family. Patients may or may not be able to function normally later in life, and are also more susceptible to other cardiac diseases, such as pulmonary hypertension and cardiac arrhythmias [3, 4]. Given the high frequency of CHD and its impact on patients' lives, understanding the etiology of CHD is necessary, as it will help to identify at risk populations, facilitate prevention and better diagnosis during pregnancy, and may lead the way to targeted therapies.

CHD arises from abnormal cardiac development that leads to heart malformations [1]. To identify the origin of the defect, one must first appreciate the normal process of heart development, which comprises a complex set of events [5]. At mouse embryonic day 7.5 (E7.5), cardiac progenitor cells from anterior lateral plate mesoderm form the horseshoe-shaped cardiac crescent,

also known as first heart field (FHF) [6-8]. Cardiac crescent contains both myocardial and endocardial lineages [6, 8, 9] . By E8.5, the two limbs of the cardiac crescent move towards the midline and unite to form a primitive linear heart tube [6]. The heart tube continues to grow with addition of cells from the second heart field (SHF) to the inflow and outflow poles. The SHF is located medial to the cardiac crescent providing rapidly proliferating cells contributing to the heart tube [10-13].

During this time, the linear heart tube also undergoes rightward looping to place its future chambers into their relative spatial positions [14, 15]. Subsequently, at E9.5, endocardial cushions start to form within the outflow tract (OFT) and atrioventricular canal (AVC) regions. These cushions are composed of cardiac jelly between the endocardial lining and myocardial layer, and later populated by mesenchymal cells from endocardium via endothelial-to-mesenchymal transition (EndoMT) [16].

As development continues, endocardial cushions help to septate the heart into four chambers and divide the OFT into aorta and pulmonary trunk. Moreover, AVC endocardial cushions develop into mitral and tricuspid valves, while OFT endocardial cushions develop into aortic and pulmonic valves [16, 17]. Meanwhile, the early conduction system begins to form.

Two sources outside of the cardiac crescent and SHF also contribute to the growing heart: the neural crest gives rise to cells later populating the distal OFT cushion, and the proepicardial organ gives rise to cells that become the epicardial layer of the heart [8, 14, 18]. At later stages, the heart continues to

undergo morphological remodeling and maturation. Eventually, the heart has four mature chambers, properly aligned and divided inflow and outflow, functional valves and conduction system [7].

It is important to note that different lineages contribute to different parts of the heart. Lineage tracing experiments in mice demonstrated that the cardiac crescent lineage contributes to the myocardium of the atria and left ventricle [9, 19]; while the SHF lineage contributes to myocardium of the atria, right ventricle and OFT [12, 13, 20-22].

Complexity of heart development demands that these events are tightly regulated. CHD can arise if regulation of heart development is disturbed. Causes of CHD are generally divided into two categories: non-genetic and genetic. Within the non-genetic category, environmental teratogens (dioxins, polychlorinatedbiphenyls, pesticides) [23], maternal exposures (alcohol, isotretinoin, thalidomide, antiseizure medications) [24], and infectious agents (rubella) [25] are well-recognized factors. Additionally, antiretroviral medications and obesity related to diabetes and hypercholesterolemia are also considered to be risk factors of CHD [26-29]. However, the major cause of CHD is thought to be genetic: mutation in regulators of heart development during embryogenesis [30].

The first recognized genetic cause of CHD was chromosomal aneuploidy [1]. CHD occurs in almost all cases of both trisomy 13 and trisomy 18, indicating that changes in dosage of certain genes can lead to CHD [31]. Advanced technologies have revealed that a frequent etiology of chromosomal

aneuploidy is “copy number variants (CNVs)” that arise from amplification or removal of DNA segments resulting from faulty DNA recombination [1]. Since CNVs change the dosage of neighboring genes, syndromic CHDs are often the result of CNVs [1]. For example, Williams-Beuren syndrome (supravalvar aortic stenosis, developmental delays, gregarious personality, elfin facies, and hypercalcemia) is a result of deletion of 1.5-Mb of chromosome 7q11.23 which alters the dosage of over 25 genes [32]. Although it is uncommon for CNVs to produce nonsyndromic (isolated) CHDs, CNVs have been reported in Tetralogy of Fallot, hypoplastic left heart syndrome, and other sporadic cases of CHD [33-35].

Another cause of genetic CHD is point mutations in important cardiogenesis genes [1]. Point mutations can lead to both syndromic and isolated CHDs. Syndromic CHDs arise from point mutations that change the dosage of genes functioning in developmental pathways that are broadly used in organogenesis [1]. For example, the RAS-MAPK pathway is important in cell proliferation, differentiation and survival. Gain-of-function in any one of 11 genes involved in this pathway: *PTPN11*, *SOS1*, *RAF1*, *KRAS*, *BRAF*, *MEK1*, *MEK2*, *HRAS*, *NRAS*, *SHOC2*, and *CBL* can lead to Noonan syndrome (pulmonary stenosis, ASD, coarctation of the aorta, facial dysmorphism, short stature, pectus deformity, cubitus valgus, neck webbing and developmental delays) [36, 37].

There are four categories of genes in which point mutations can result in isolated CHD, including genes encoding proteins involved in transcriptional

regulation, signal transduction, epigenetics, or genes encoding structural proteins [1].

Transcriptional regulation of cardiac development is governed by genes encoding key transcription factors, such as *Nkx2.5*, N*kx2.6*, *Gata4*, *Gata6* and *Tbx5* etc [1]. Mutations in each of these genes can cause reduced levels or loss-of-function of encoded proteins, or disrupt interactions of encoded transcription factors with their cofactors. Proper cardiac development demands transcription factor action in a highly specified temporal-spatial manner [1]. Loss of important genes encoding transcription factors can ultimately lead to malformation of the heart.

Cardiac development also relies on proper signal transduction. Point mutations within genes encoding critical molecules within developmental signaling pathways can result in CHD. For example, the Notch signaling pathway is key for epithelial-to-mesenchymal transition required during normal valvulogenesis [38], and point mutations in *Notch1* causes malformation of aortic valves [39, 40].

The importance of genes encoding epigenetic modifiers has been highlighted by recent studies using exome sequencing that identified de novo point mutations in several histone-modifying genes. In particular, mutations were identified in five genes involved in writing, erasing, or reading of a particular histone modification, trimethylation of lysine 4 of histone H3 (H3K4me3) [41]. As H3K4me3 is mainly associated with transcriptionally

active genes, its regulation is likely to be crucial for most cellular processes and many aspects of cardiac differentiation.

CHDs arising from point mutations in genes encoding cardiac structural proteins are rare. Mutations in *Myh6* and *Myh7* (α and β cardiac myosin heavy chains, respectively) are causal for atrial septal defects (ASDs), Ebstein anomaly, and other CHDs [42-45].

Researchers have gained a plethora of knowledge of disease genes in CHD through linkage studies in patients, fine-mapping of patient genomes, and by generation and study of animal models. Identification of CHD causative genes improves our current understanding of human cardiac development and CHD molecular pathology. New preventive strategies and new treatments may be developed based on this knowledge. Moreover, this knowledge can be utilized in cardiomyogenic stem cell differentiation, and may eventually help toward development of regenerative therapies for the heart. In inherited cases of CHD, identification of disease genes will also aid the genetic counseling process for CHD-associated families [1].

1.2 Transcription factors and CHD

Cardiac developmental signals rely on transcriptional regulation of gene expression during normal heart development. A group of transcription factors are critical in heart development: the homeodomain protein Nkx2.5, the GATA family of zinc finger proteins including GATA 4, GATA5, and GATA 6, MEF2 factors and SRF (MADS box proteins), T-box factors, including TBX1, TBX2, TBX3, TBX5, TBX18, and TBX20, and the Lim-homeodomain protein ISL1 [46].

These factors control multiple stages of heart development by interacting with each other and with other transcription factors/co-factors [46]. Among these genes, we are particularly interested in the T-box family, because many of its members are core players within the cardiac development circuit.

T-box (*Tbx*) genes are conserved in diverse genomes, with 18 *Tbx* genes identified in mammals [47]. The common feature of *Tbx* genes is that their encoded proteins share a DNA binding domain, the T-box domain, that binds DNA in a sequence-specific manner [48]. T-box transcription factors can function as repressors or activators, and some function as both repressors and activators in different cellular or promoter contexts [49, 50].

During cardiac development, *Tbx* genes mentioned previously are involved in multiple stages of heart development, including specification of cardiac mesoderm, regionalization of the primitive heart tube into chamber and non-chamber myocardium, formation of valves and septa that separate the chambers, recruitment of SHF cells to the OFT, and formation of the cardiac conduction system [47]. *Tbx* genes regulate cardiac gene expression, and can also crosstalk among each other. Deficiency of individual *Tbx* genes in mouse embryos results in cardiac defects [47]. Mutations of *TBX* genes in human are associated with syndromic and isolated CHD: *TBX1* (DiGeorge syndrome) [51], *TBX3* (ulnar mammary syndrome) [52], *TBX5* (Holt-Oram syndrome) [53] and *TBX20* (atrial and ventricular septal defects and other cardiac abnormalities) [54-56].

1.3 *Tbx20* in cardiac development

Tbx20 is an ancient member of the *Tbx* family that is closely related to *Tbx1* based on their DNA binding domain homologies [47]. *Tbx20* was first discovered in Drosophila and zebrafish as *H15* and *hrT* respectively [57-59]. These genes are both expressed in the heart region during development [57-59]. Subsequently, *Tbx20* orthologs with similar cardiac expression patterns were identified in frog, chicken, mice and humans [58, 60-62]. In mouse, *Tbx20* is expressed in pharyngeal endoderm, cardiac progenitors of the second heart field, myocardium and endocardium during heart development [62-66]. In adult, *Tbx20* expression remains in all four chambers of the heart and the interventricular septum [67]

TBX20 can act as an activator or repressor of target genes in a context dependent manner [63, 68, 69]. The T-box DNA-binding domain of TBX20 can also interact with homeodomain factor Nkx2-5 and zinc finger factor Gata4 [63] to synergistically regulate gene expression.

Owing to its expression in heart during embryonic development, *Tbx20* cardiac function has been explored and its importance in heart development has been demonstrated in different organisms by genetic modification of *Tbx20* expression. In zebrafish and *Xenopus*, loss- or gain-of-function of Tbx20 causes dysmorphic hearts [63, 70, 71]. In zebrafish, injection of morpholinos to *Tbx20* results in an unlooped heart tube, malformed chambers, abnormal atrial expression of ventricular-specific myosin heavy chain, and upregulation of *Tbx5* [71]. In *Xenopus*, *Tbx20* morphants have reduced cardiac mass; however, no TBX20 downstream targets were identified, and

Tbx5 expression was normal [70]. Global and cardiac tissue specific deletions of *Tbx20* have also been performed in mouse as discussed in the next section.

1.4 Genetic mouse models with *Tbx20* mutations

To study TBX20 function in mammalian heart development, global and tissue specific ablations of *Tbx20* have been conducted in mice. Global deletion of *Tbx20* (*Tbx20* $^{null/null}$ or *Tbx20* $^{lacZ/lacZ}$) results in early embryonic lethality with severe cardiac defects [64, 66, 72].. Mutant hearts are hypoplastic at E9.5, do not loop, and exhibit an “hourglass” shape. In situ hybridization analyses of a number of marker genes indicated that anterior posterior patterning of the heart tube is established in mutants, but that expression of some genes marking “chamber” versus “non-chamber” (OFT, AV, SV) myocardium is altered [72]. In particular, the atrioventricular canal (AVC)-specific gene *Tbx2* is upregulated throughout the entire heart of mutants.

Cell proliferation is significantly reduced in *Tbx20* null mutants, likely contributing to the smaller heart phenotype, as no increased cell death was observed. Cell proliferation genes *Mycn* and *Ccna*2 are downregulated in mutants, likely providing mechanistic explanation of the phenotype [66]. TBX2 was found to directly bind to *Mycn* to repress its expression and consequently cell proliferation. These early studies suggested that TBX20 regulates cell proliferation in early heart development primarily by restricting *Tbx2* expression to the AVC region, and that in the absence of *Tbx20* ectopic expression of *Tbx2* in future chambers of the heart resulted in decreased

proliferation. However, subsequent studies with simultaneous knockout of both *Tbx20* and *Tbx2* did not rescue decreased proliferation observed in mutant hearts, suggesting that upregulation of Tbx2 alone could not account for reduced proliferation in *Tbx20* null hearts [73]

Overall, results with *Tbx20* null mutant analyses demonstrated a critical role for *Tbx20* in early cardiac development in promoting cardiomyocyte proliferation, and establishing chamber versus non-chamber gene programs [64, 66, 72]. However, these early studies did not fully explore mechanisms of TBX20 action during early heart development, required for full understanding of etiologies of CHD caused by mutations in TBX20. As stated above, TBX20 is expressed in multiple cell types critical for early heart formation, and global knockout of *Tbx20* did not address cell specific requirements for TBX20 in observed phenotypes. Additionally, a more global understanding of direct targets downstream of Tbx20 remains to be explored.

Tbx20 is highly expressed in AVC myocardium that connects the atrial and ventricular chambers. AVC myocardium is less differentiated and has distinct conduction properties from those of atrial or ventricular myocardium [5]. To explore the potential role of TBX20 in AVC myocardium, *Tbx2-Cre* was utilized to ablate *Tbx20* [74, 75]. *Tbx2-Cre* is selectively active in AVC myocardium beginning at E8 [74]. *Tbx2-Cre*; *Tbx20* $^{fl/fl}$ mutants were embryonic lethal by E10.5. Mutant hearts did not form the AVC constriction, and endocardial EMT was also perturbed. Gene expression analyses demonstrated reduced *Bmp2* expression in mutants, and re-expression of

Bmp2 in AVC myocardium partially rescued the EMT phenotype. BMP2 is an early AVC marker [76-78], plays a role in initiation of EMT, and promotes *Tbx2* expression in AVC to repress chamber-specific gene expression during AVC formation [76-78]. Surprisingly, in *Tbx2-Cre*; *Tbx20* $^{fl/fl}$ mutants, *Tbx2* expression was not significantly reduced, despite loss of expression of *Bmp2.* Authors of this study suggested that *Tbx2* expression in AVC is a balancing act between direct repression of *Tbx2* by TBX20 and TBX20 indirectly activating *Tbx2* by activation of *Bmp2.* In this *Tbx2-Cre*; *Tbx20* $^{fl/fl}$ mutants, loss of activation of *Tbx2* by BMP2 was compensated by loss of TBX20 repression, thereby maintaining TBX2 expression in AVC. This study revealed that TBX20 is critical for AVC development by regulation of *Bmp2* [75].

Endocardial cushions are important in septation and valve development during cardiogenesis [5], and patients with mutations in TBX20 exhibit valve defects [54]. siRNA knockdown studies in chick endocardial cushion explants demonstrated a critical role for Tbx20 in mesenchymal cell proliferation [79]. In mouse, endocardial ablation of *Tbx20* was performed utilizing endocardial specific *Nfatc1-Cre* [80, 81]. *Nfatc1-Cre*; *Tbx20* $^{fl/-}$ mutants died from E14.5 to E16.5. Mutant hearts exhibited shorter and blunt valve leaflets as compared to controls, and perturbations in normal valve function. Reduced proliferation of endocardial cells contributed to this phenotype. RNA-seq experiments demonstrated decreased expression of genes important for extracellular matrix (ECM) and Wnt/β-catenin signaling, including the β-catenin co-factor *Lef1.* Wnt/β-catenin signaling is crucial for endocardial EMT and valve

development [82], and TBX20 was shown to bind to enhancer regulatory regions of *Lef1*. In summary, this study demonstrated that endocardial TBX20 promotes valve elongation by regulating ECM gene expression, and endocardial cell proliferation, the latter hypothesized to occur by direct regulation of *Lef1* [81].

In another report, *Tbx20* was ablated in endocardium using *Tie2-Cre. Tie2-Cre; Tbx20* $^{fl/-}$ mutants died at E14.5 [83]. At E13.5, mutant hearts displayed OFT and AV cushion defects and were missing the dorsal mesenchymal protrusion, required for atrial septation. Loss of *Tbx20* in *Tie2-Cre* lineages affected multiple cellular processes crucial for OFT and cushion development. Mutant endocardial lineage traced cells were able to undergo EMT and contributed to OFT and atrioventricular cushions. However, they were abnormally clustered and exhibited migratory defects following EMT. Myocardialization, a process in which OFT cardiomyocytes invade cushion mesenchyme to achieve OFT rotation, was defective in mutants [83]. OFT cushion proliferation was also decreased in mutants. This study demonstrated that endocardial TBX20 cell autonomously regulates endocardial cell proliferation and migratory behavior, and non-cell autonomously affects cardiomyocyte invasion of OFT cushions. In keeping with observed phenotypes, RNA-seq analyses of *Tie2-Cre* lineage traced mutant cells, demonstrated that TBX20 regulates expression of ECM and migration genes. This study also used transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) assays [84] to identify accessible chromatin within

endocardial lineages. To identify direct targets of TBX20 in endocardium, ATAC-seq results were intersected with TBX20-GFP ChIP-seq of whole heart, and chromatin loop maps in the literature. This approach revealed that TBX20 regulates expression of the critical ECM gene, *Vcan,* by binding to a long-range enhancer. Hypomorphic mutants of *Vcan* exhibit phenotypes very similar to those observed in *Tie2-Cre; Tbx20* mutants [85]. This study provided further mechanistic insight into mechanisms by which altering functions of TBX20 in endocardium could result in outflow tract and valve defects observed in patients with *TBX20* mutations [83].

Additionally, a recent study showed that *Tbx20* is also required for maintenance of adult cardiomyocyte phenotype and function [67], which highlights an ongoing role for TBX20, not only during cardiogenesis, but also in adult cardiomyocytes. These results may be of relevance for cardiomyopathies observed in patients with TBX20 mutations [54, 86].

The foregoing studies of global and conditional ablations of *Tbx20* during heart development have helped us to better understand TBX20 regulatory functions in early heart development, and in specific cardiac tissues/lineages, and how mutations in *TBX20* might contribute to CHDs.

Chapter 2 *Tbx20* function in midgestation cardiomyocytes

2.1 Introduction

TBX20 is a transcription factor expressed in pharyngeal endoderm, cardiac progenitors of the second heart field, myocardium and endocardium

during development [63-66]. Global ablation of *Tbx20* in mouse embryos results in early lethality (E10) and heart developmental arrest prior to heart looping [64, 66, 72, 87]. Additionally, *TBX20* mutations are associated with human congenital heart disease (CHD), with patients exhibiting defects in cardiac septation and valvuogenesis [54-56]. These findings confirm that TBX20 plays a major role in mammalian cardiac development. To dissect the function of TBX20 in specific cardiac tissues during development, TBX20 has previously been ablated by endocardial-specific Cres (*Nfatc1-Cre* and *Tie2-Cre*) [81, 83] and atrioventricular canal (AVC) myocardium-specific Cre (*Tbx2-Cre*) [75].These studies demonstrated that endocardial *Tbx20* is required for cushion maturation, cardiac septation, valve elongation and proper outflow tract (OFT) development [81, 83], while AVC myocardial *Tbx20* is required for forming the AV constriction [75]. *Tbx20* is also expressed in adult mouse myocardium, and is required for maintenance of adult cardiomyocyte function [67].

Although *Tbx20* is broadly expressed in all cardiac myocytes during early heart development, no prior studies have examined the cell autonomous function of TBX20 in myocytes throughout the heart in early development. To address this question, we used inducible *Tnn2-rtTA*; *TetO-Cre* to ablate *Tbx20* in midgestation myocardium, generating *Tbx20 cKOs*. TBX20 has dual roles as activator or repressor of downstream target genes, depending on the context [63, 66, 87]. To identify TBX20 downstream targets and examine TBX20 regulation of these targets in cardiomyocytes, we performed RNA-seq

analysis of *Tbx20 cKO* and control cardiomyocytes to identify genes that were differentially expressed. To determine whether the differentially expressed genes were direct targets of TBX20, previously reported TBX20-GFP ChIP-seq data [83] was used to assess TBX20 binding to genes of interest. Results of this study uncover TBX20 function in midgestation cardiomyocytes and reveal mechanisms by which TBX20 is required cell autonomously for multiple aspects of cardiomyocyte development and patterning.

2.2 Material and methods

2.2.1 Mouse strains

Mice were maintained on a BlackSwiss (NIHBL(S); Taconic Biosciences) outbred background. *Tbx20* conditional [66], *Tbx20-GFP* [67] and *Tnnt2-rtTA; TetO-Cre* [88] have been previously described. *Rosa26 flox-stop-flox tdTomato* (*Rosa tdTom*) were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were identified by genotyping on genomic DNA extracted from tail clippings using the following primers: *rtTA*-P1: 5'-TCGACGCCTTAGCCATTGAGAT-3'; *rtTA*-P2: 5'-GGCTGTACGGCGGACCC ACTTTC-3'; *Cre*-P1: 5'-GGCGCGGCAACACCATTTT-3'; *Cre*-P2: 5'-TCCGG GCTGCCACGACCAA. *Tbx20*-P1: 5'- AGTGCTACCCTCTGCGCTGCAAA-3'; *Tbx20*-P2: 5'- AGTAGGAAGGAGCTGGGAAGAGTA-3'; *Tbx20*-P3: 5'-CAGAAAATGACACGCGGATGGTGG -3'.

2.2.2 Induction protocol

Experiment control and mutants were generated by the following setup: Inducible *Tnnt2-rtTA; TetO-Cre*; *Tbx20* $^{+/fl}$ males were crossed with *Tbx20* $^{fl/fl}$;

Rosa tdTom females. Timed matings were performed and the day of a female vaginal plug was considered embryonic day E0.5. Doxycycline (MP Biomedicals, cat# 198955) was dissolved in drinking water at 1mg/mL and fed to pregnant females at E8.5 until the day of embryo collection.

2.2.3 Histology

Embryos were isolated in cold 1xPBS, fixed in 4% paraformaldehyde in 1x PBS at 4°C overnight. Tissue was dehydrated using ethanol gradient (50%, 70%, 80%, 90%, 95%, 3x100%; 5 minutes each), cleared in Histoclear (National Diagnostics) for 15-30 minutes, incubated 3 x 10 minutes in Paraplast X-tra (Leica biosystems) and embedded for sectioning. For cryosections, tissue was dehydrated in a sucrose gradient (5% and 12%, 1 hour each; 20%, 12 hour) embedded in 20% Sucrose: OCT and freeze with dry ice. 10 um sections were used for H&E staining or immunostaining as described previously [67].

2.2.4 Embryonic heart chamber circumference measurement

Image J software [89] was used to trace the perimeter of each chamber of the heart in sections. Multiple representative sections of three controls and three *Tbx20* cKO E11.5 hearts were used for measurement. The sum of perimeter measurements of each chamber was reported as the perimeter of that chamber. T test was performed to examine whether the perimeters of *Tbx20* cKO chambers were significantly different than the respective control chambers.

2.2.5 Cell proliferation quantification experiments

Cell counting experiments were performed using Volocity Software (Perkin Elmer). *In vivo* quantification of cell number and EdU labeling was performed by counting all lineage traced cardiomyocytes in every 4 sections of E9.5, E10.5 and E11.5 hearts.

2.2.6 Immunostaining

Antibodies used for immunostaining were Cleaved caspase-3 (#9664; 1:200; cell signaling) and ACTN2 (A7811; 1:200; Sigma Aldrich). 200ul EdU (3g/L) was injected intraperitoneal in pregnant females 2 hours prior to embryo isolation. EdU staining was performed following instructions (C10337; Molecular probes). DAPI was used to label cell nucleus (1:1000).

2.2.7 Cell cycle Experiment

200ul 5-Ethynyl-2'-deoxyuridine (EdU; 3g/L) was injected intraperitoneal in pregnant females 2 hours prior to embryo isolation. Embryonic hearts were harvested in ice-cold PBS with 0.5Mm EDTA. Cells were dissociated as described previously [83] using enzymatic digestion by collagenase (10mg/ml; Worthington) and dispase (10mg/mL, Invitrogen). Cells were fixed and permeabilized with Cytofix/cytoperm fixation/permeabilization kit (#554714; BD). DAPI (1:1000) were used to label cell nucleus. Flow cytometry was performed with dissociated cardiac cells with standard protocol of stem cell core facility, University of California, San Diego. The results were analyzed with FlowJo software (Tree Star Inc., Ashland, OR)

2.2.8 FACS sorting

Hearts were harvested, dissociated and stained with DAPI to label dead cells. *Tnnt2-rtTA; TetO-Cre* lineage cells were sorted on Influx Cell sorter (BD Biosciences) and collected in TRIZol reagent (Ambion) for RNA extraction.

2.2.9 RNA extraction, RNA-seq and qRT-PCR

RNA was extracted from ~10,000 FACS-sorted cells pooled from E11.5 hearts using TRIZol and phenol-chloroform. TruSeq mRNA stranded kit (Illumina) was used to make libraries from 50ng/RNA from each pool of cells. RNAseq was performed and differential gene expression was determined as previously described [68]. Four *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{+/fl}$ and four *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{fl/fl}$ were used for RNAseq. For quantitative RT-PCR (qRT-PCR), RNA was extracted from FACS sorted cells using TRIZol and cDNA was generated using SuperScript VILO (invitrogen). qRT-PCR was performed using FastStart SYBR Green Master Mix (Roche) on a Bio-Rad CFX96 Real-Time PCR system. *Tbx20* exon 2-P1: 5'-AGATGGCTAAAATCGC CTGC-3'; *Tbx20* exon 2-P2: 5'-AGCGGTATCTCTTGTTGTCC-3'.

2.2.10 RNA in situ

Embryos for RNA in situ hybridization were collected between E9.5 – E11.5, fixed overnight at 4°C in 4% paraformaldehyde in PBS. Tissue was dehydrated using methanol (50%, 70%, 2x100%; 15 minutes each) and store in 100% methanol at -20°C. Whole-mount in situ was performed based to described protocol [90] with probes targeting RNA of the following genes : mouse *tdTomato*, *Isl1*, *Mycn*, *Erbb2*, *Bmp2*, *Bmp10*, *Tbx5*, *Gja5*, *Nkx2.5*, *COUP-TFII*, *Hey1*, *Hey2*, *Irx4*, *MLC2a* and *MLC2v* [75, 81, 91].

2.3 Results

2.3.1 *Tbx20* exon 2 is efficiently ablated by *Tnnt2-rtTA*; *TetO-Cre* in cardiomyocytes during midgestation

TBX20 is an essential regulator of cardiogenesis, and is expressed in cardiomyocytes throughout heart development. To study roles of *Tbx20* in mid gestation cardiomyocytes, *Tnnt2-rtTA; TetO-Cre* was used to specifically ablate *Tbx20* from cardiomyocytes. Using doxycycline, *Cre* expression was induced from E8.5 onwards. Using genetic lineage tracing, specificity and efficiency of the induction was confirmed by in situ hybridization at E11.5 in *Tnnt2-rtTA; TetO-Cre;Rosa*$^{+/tdTom}$ RNA (Figure 2.1A). Additionally, qRT-PCR on FACS sorted cardiomyocytes demonstrated that expression of *Tbx20* exon 2 was diminished in *Tbx20 cKO* cardiomyocytes compared to littermate controls (Figure2.1B). These data demonstrate that induction of *Tnnt2-rtTA; TetO-Cre* at E8.5 resulted in specific and efficient ablation of *Tbx20* in developing cardiomyocytes.

2.3.2 Ongoing requirement for *Tbx20* in midgestation cardiomyocytes

To establish whether *Tbx20* is required in cardiomyocytes during mid gestation, the induction protocol described above was used to ablate *Tbx20* from E8.5 onwards. Mutant embryos were harvested at multiple stages between E9.5 and E14.5. Embryos with heterozygous deletion of *Tbx20* by *Tnnt2-rtTA; TetO-Cre* were recovered at expected Mendelian ratios. However, by E14.5, embryos with homozygous deletion of *Tbx20* (*Tbx20 cKO*) were markedly absent ($P = 0.042$; Table 2.1). Gross morphological analysis of

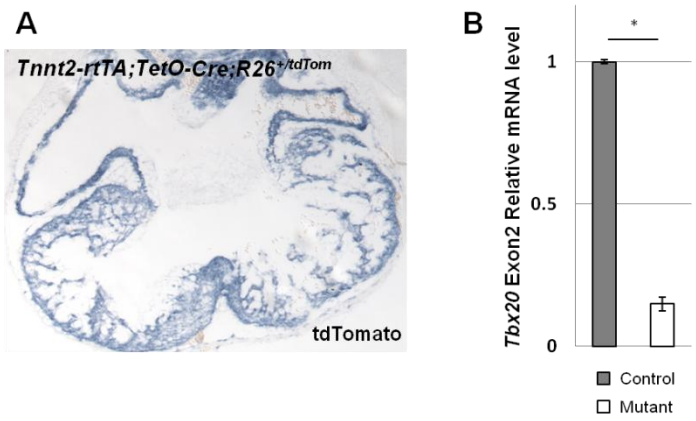


Figure 2.1 *Tnnt2-rtTA*; *TetO-Cre* ablates *Tbx20* efficiently in midgestation cardiomyocytes. (A) RNA in situ for *tdTomato* mRNA on sections of E11.5 *Tnnt2-rtTA; TetO-Cre;R26*$^{+/tdTom}$ hearts. **(B)** Quantitative RT-PCR of the floxed *Tbx20* exon 2 in FACS sorted *Tnnt2-rtTA;TetO-Cre* lineage traced cardiomyocytes from E11.5 hearts in either *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{+/fl}$ (Controls) versus *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{fl/fl}$ (*Tbx20* cKOs) .

Table 2.1 Genotypes of embryos recovered from crossing *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{+/fl}$ males to *Tbx20* $^{fl/fl}$ female mice. Expected numbers in parentheses. Chi-square test was performed and P values were calculated for each embryonic stage. *, P < 0.05 was considered statistically significant.

	Without *Cre*		*Tnnt2-rtTA;TetO-Cre*		
Stage	***Tbx20*** $^{+/fl}$	***Tbx20*** $^{fl/fl}$	***Tbx20*** $^{+/fl}$	***Tbx20*** $^{fl/fl}$	χ^2
E9.5	27 (27.5)	20 (27.5)	32 (27.5)	31 (27.5)	*P* = 0.35
E10.5	27 (33)	33 (33)	42 (33)	30 (33)	*P* = 0.28
E11.5	149 (133.25)	128 (133.25)	124 (133.25)	132 (133.25)	*P* = 0.44
E12.5	7 (6.25)	8 (6.25)	3 (6.25)	7 (6.25)	*P* = 0.50
E13.5	14 (10)	8 (10)	13 (10)	5 (10)	*P* = 0.14
E14.5	9 (4.25)	3 (4.25)	4 (4.25)	1 (4.25)	**P* = 0.042

Tbx20 cKO embryos at E13.5 revealed dysmorphic hearts and pericardial effusion indicating cardiac insufficiency. These data suggested an ongoing requirement for *Tbx20* in mid gestation cardiomyocytes.

Up until E11.5, *Tbx20 cKO* embryos were of similar size as their control somite-matched littermates, and no gross morphological defects were observed (Figure 2.2). Further detailed analysis of mutant hearts revealed that at E9.5, *Tbx20 cKO* hearts were properly looped and visually indistinguishable from control hearts. At both E10.5 and E11.5, mutant hearts were overall smaller than control hearts. Most notably, the size of left atria in *cKO* hearts was severely reduced (Figure 2.2 and Figure 2.3). Circumferential measurements of all four cardiac chambers confirmed that left atrium and both ventricles were smaller; while no consistent change in right atrial size was observed (Figure 2.4). Histological sections of E10.5 and E11.5 also revealed that *Tbx20 cKO* hearts had under-developed atrial and interventricular septa (Figure 2.3). Additionally, venous valves of *Tbx20 cKO* hearts were smaller at E10.5 and thinner at E11.5. Endocardial cushions, in which *Tbx20* is also highly expressed, did not appear to be affected (Figure 2.3).

2.3.3 *Tbx20* is required for cardiomyocyte proliferation and cell cycle progression

To understand how *Tbx20* ablation leads to observed decreases in chamber sizes, proliferation of cardiomyocytes was analyzed at E9.5, E10.5 and E11.5. EdU incorporation was quantified in *Tnnt2-rtTA;TetO-Cre;Rosa*$^{+/tdTom}$ lineage traced cardiomyocytes (Figure 2.5). Notably, at all

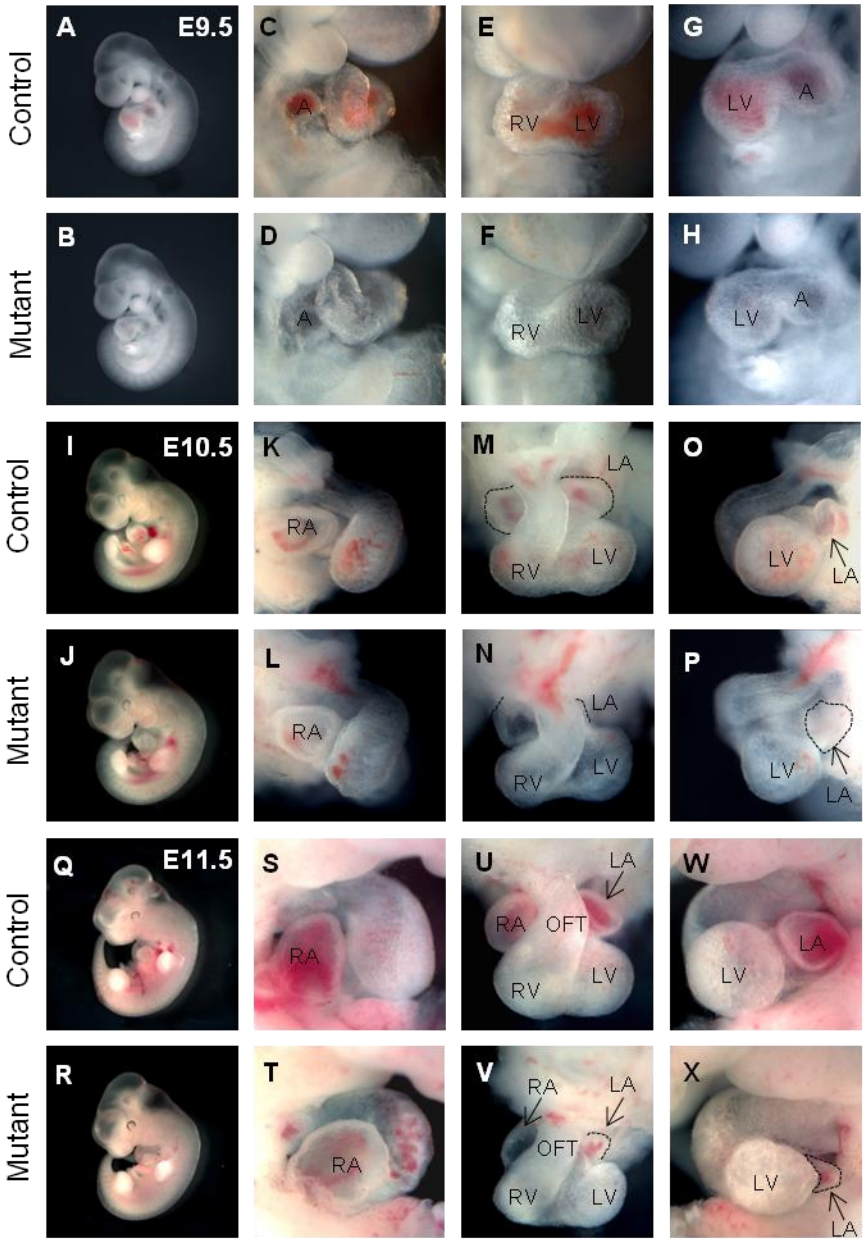


Figure 2.2 Cardiac morphology of *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{+/fl}$ and *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{fl/fl}$ embryos at E9.5, E10.5 and E11.5.(A-H) E9.5; **(I-P)** E10.5; **(Q-X)** E11.5. From left to right in each row: whole embryo, right side view, ventral view and left side view of hearts. Control: *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{+/fl}$; Mutant: *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{fl/fl}$; A: atrium; RV: right ventricle; LV: left ventricle; RA: right atrium; LA: left atrium; Dotted line indicates outline of RA and LA as labeled.

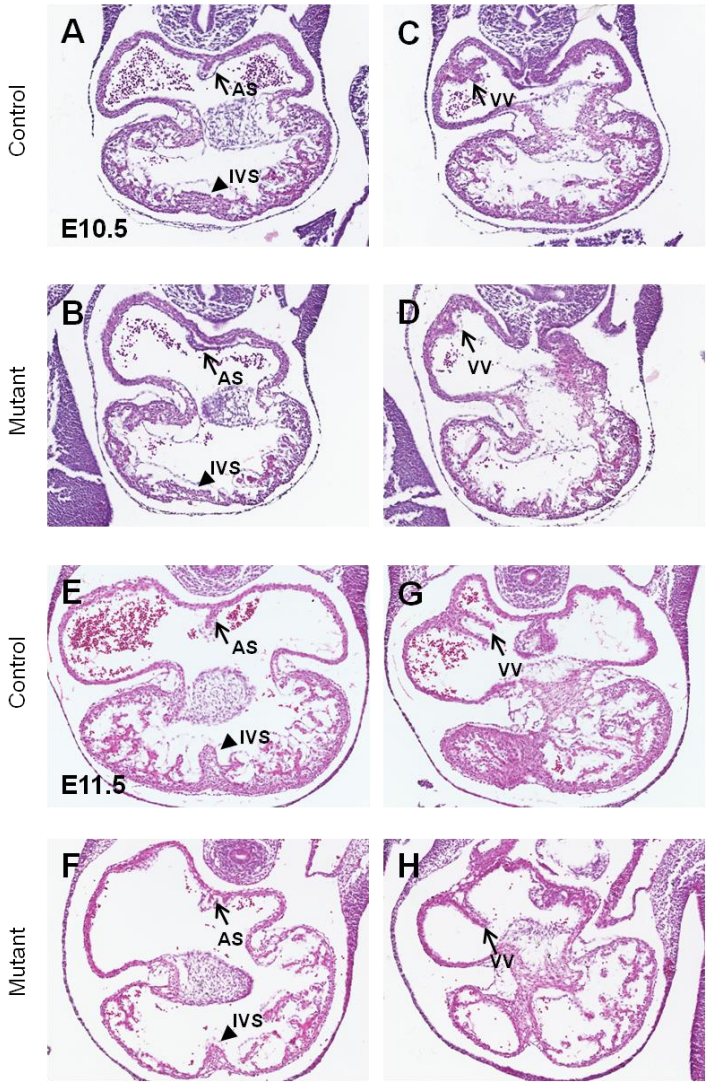


Figure 2.3 Histological analysis of cardiac phenotypes. Hematoxilin and eosin stained sections of *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{+/fl}$; and *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{fl/fl}$ hearts at E10.5 **(A-D)** and E11.5 **(E-H)**. Arrows in A, B, E and F indicate atrial septum (AS). Arrow heads in A, B, E and F indicate interventricular septum (IVS). Arrows in C, D, G and H indicate venous valves (VV) in more posterior sections. Control: *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{+/fl}$; Mutant: *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{fl/fl}$.

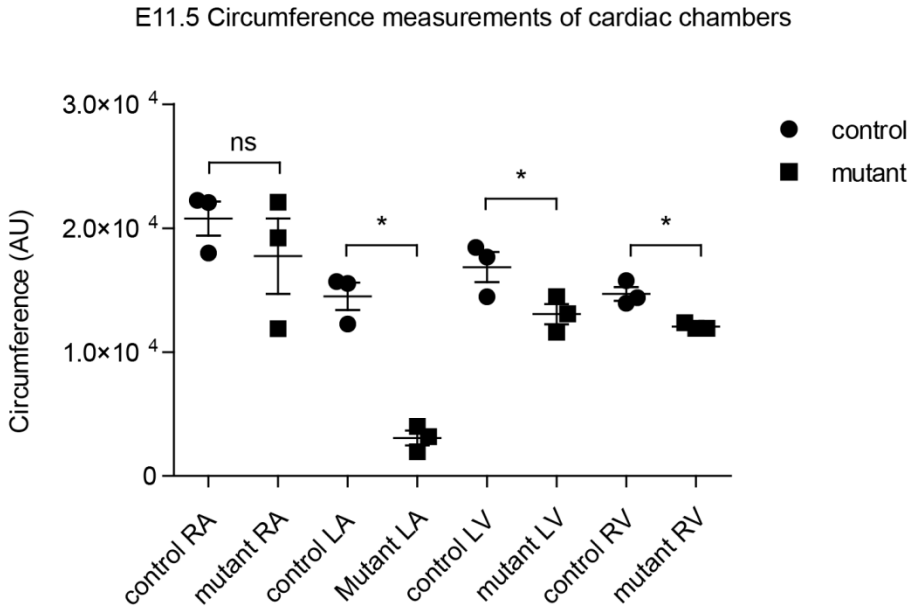


Figure 2.4 Chamber circumference measurements at E11.5. Circumference measurement of right atrium, left atrium, right ventricle and left ventricle in *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{+/fl}$ and *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{fl/fl}$ hearts: *, P<0.05; ns, not significant; *n* = 3. Control: *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{+/fl}$; Mutant: *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{fl/fl}$. RA: right atrium; LA: left atrium; RV: right ventricle; LV: left ventricle.

stages examined, there was a significant reduction in proliferation in *Tbx20 cKO* cardiomyocytes when compared to those of somite-matched control littermates. To understand whether proliferation was affected similarly in all chambers, EdU incorporation rates were quantified per compartment in E10.5 hearts. Interestingly, proliferation of cardiomyocytes was decreased in all four compartments, with left atrial cardiomyocytes displaying the most striking decrease. Proliferation of non-lineage traced cells in endocardial cushions was not affected (Figure 2.5 G & H), suggesting that decreased proliferation of mutant cardiomyocytes was a direct effect of loss of *Tbx20* expression. These results suggested that reduced proliferation in myocardium contributed to cardiac malformations in *Tbx20 cKO* mice.

To gain further insight as to mechanisms by which TBX20 regulates cell cycle progression in mid-gestation cardiomyocytes, flow cytometric analysis of cell cycle progression was performed using quantification of EdU incorporation and DNA content in control and *cKO* cardiomyocytes (Figure 2.6 A). Results demonstrated that a higher percentage of *cKO* cardiomyocytes were in G1 phase, while a lower percentage of them were in S phase relative to cardiomyocytes of controls, suggesting that G1 to S phase progression was affected in the mutant population (Figure 2.6 B). Finally, cleaved Caspase 3 immunostaining was performed to establish whether increased apoptosis might underlie reduced heart size in *Tbx20 cKO* mutants. However, cleaved Caspase 3 staining did not reveal appreciable levels of apoptosis in either control or mutant hearts (data not shown).

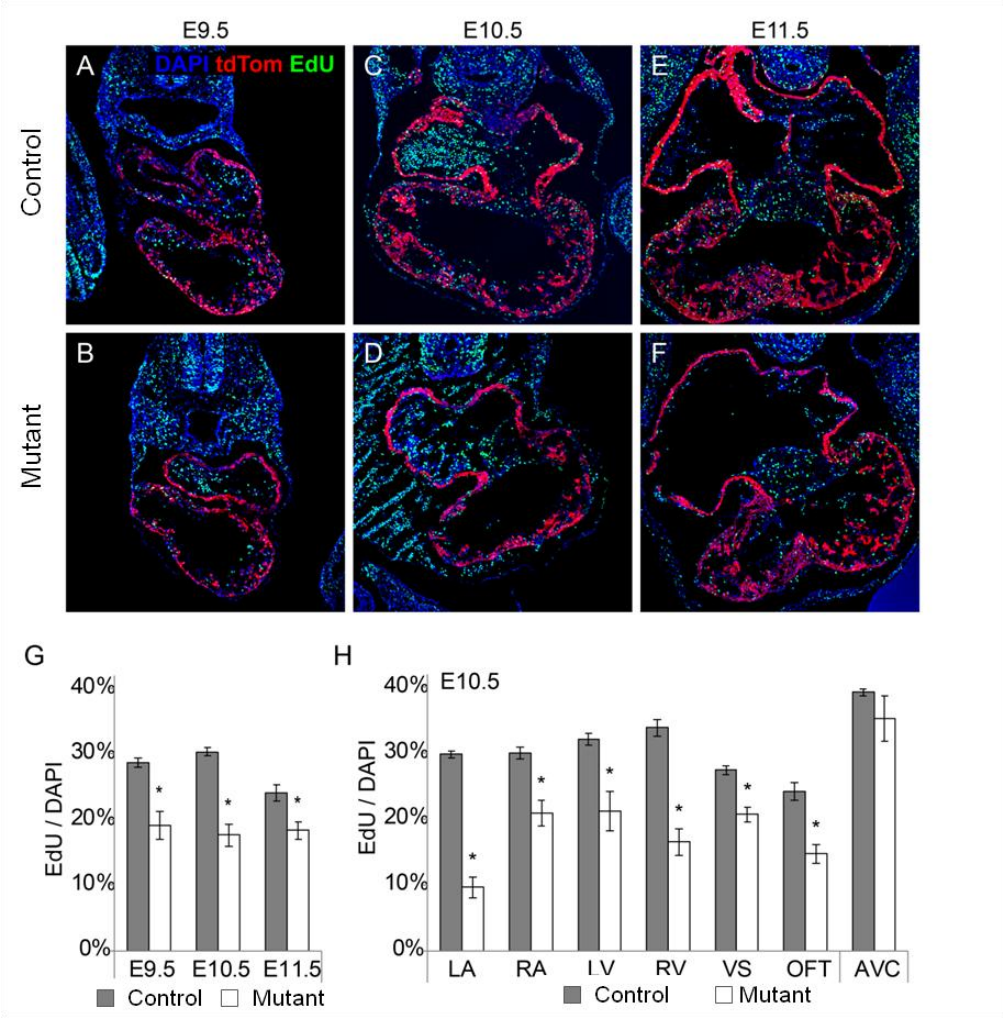


Figure 2.5 EdU labeling of lineage traced cardiomyocytes at E9.5, E10.5 and E11.5. (A-F) Representative sections of *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{+/fl}$ (Control) and *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{fl/fl}$ (Mutant) hearts labeled with EdU. **(G)** Percentages of EdU + lineage traced cardiomyocytes in E9.5, E10.5 and E11.5 control and *Tbx20* mutant hearts. **(H)** Percentages of EdU + lineage traced cardiomyocytes of different compartments of E10.5 controls and *Tbx20* mutants. *, $P < 0.05$; n=3.

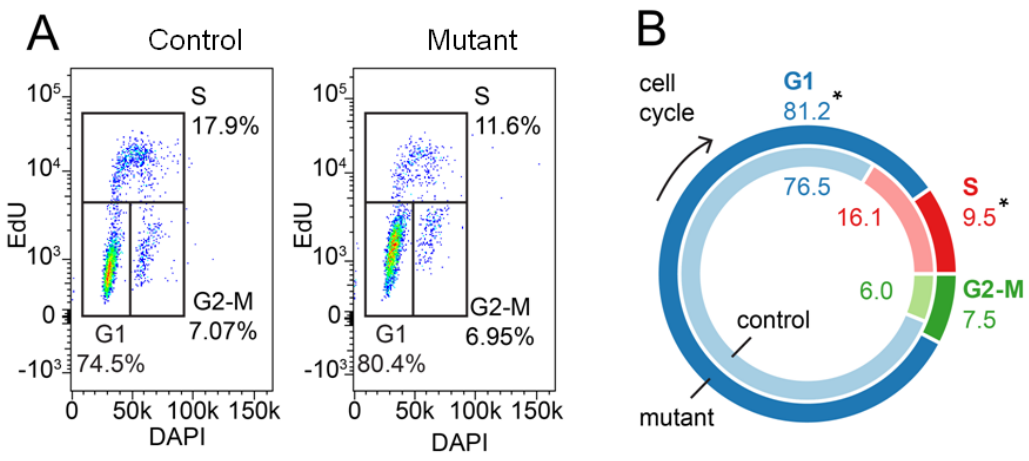


Figure 2.6 Cardiomyocyte cell cycle analysis using flow cytometry. (A) Representative FACS plot showing analysis of cell cycle stage using EdU incorporation and DNA content (DAPI). Percentage of cells at each phase is labeled. **(B)** Relative distribution of cardiomyocytes at each cell cycle stage in *Tnnt2-rtTA;TetO-Cre;Tbx20* $^{fl/fl}$ (mutant) and *Tnnt2-rtTA;TetO-Cre;Tbx20* $^{+/fl}$ (control) littermates (*n=3)*. The inner and outer circle represents controls and mutants respectively. *, $P < 0.05$.

2.3.4 Global transcriptome analysis reveals TBX20 regulates pathways associated with cell cycle and cardiac morphogenesis

To further understand the functional impact of *Tbx20* ablation on midgestation heart, RNA-seq analyses were performed with FACS-purified *Tnnt2-rtTA;TetO-Cre;Rosa*$^{+/tdTom}$ cardiomyocyte lineages from E11.5 *Tbx20 cKO* and somite-matched littermate control hearts. Gene Ontology (GO) analysis of differentially expressed genes revealed that genes related to cell cycles were highly enriched among downregulated genes (Figure 2.7A). Genes within this GO term include *cell division cycle 6* (*Cdc6*), *chromatin licensing and DNA replication factor 1* (*Cdt1*) and *cyclin A2* (*Ccna2*). During cell cycle, *Cdc6* and *Cdt1* are involved in the formation of the pre-replication complex that is necessary for DNA replication [92]. Cdc6 and Ccna2 activate *cyclin dependent kinase 2* (*Cdk2),* which is the G_1 CDK required for the onset of chromosomal DNA replication in mammalian cells [93, 94]. Downregulation of *Cdc6*, *Cdt1* and *Ccna2* in *Tbx20 cKO cardiomyocytes* is in line with our observation that mutant cardiomyocytes display decreased G1 to S transition and provides support for the hypothesis that *Tbx20* regulates cell cycle progression in midgestation cardiomyocytes. Gene ontology categories that were enriched in upregulated genes include “anatomical structure morphogenesis”, “cardiovascular system development” and “regulation of cell communication” (Figure 2.7B). Genes that are critical for cardiac development within this group included *Tbx2*, *Isl1*, *Fgf10*, *Hopx*, *Bmp2* and *Bmp10*. These

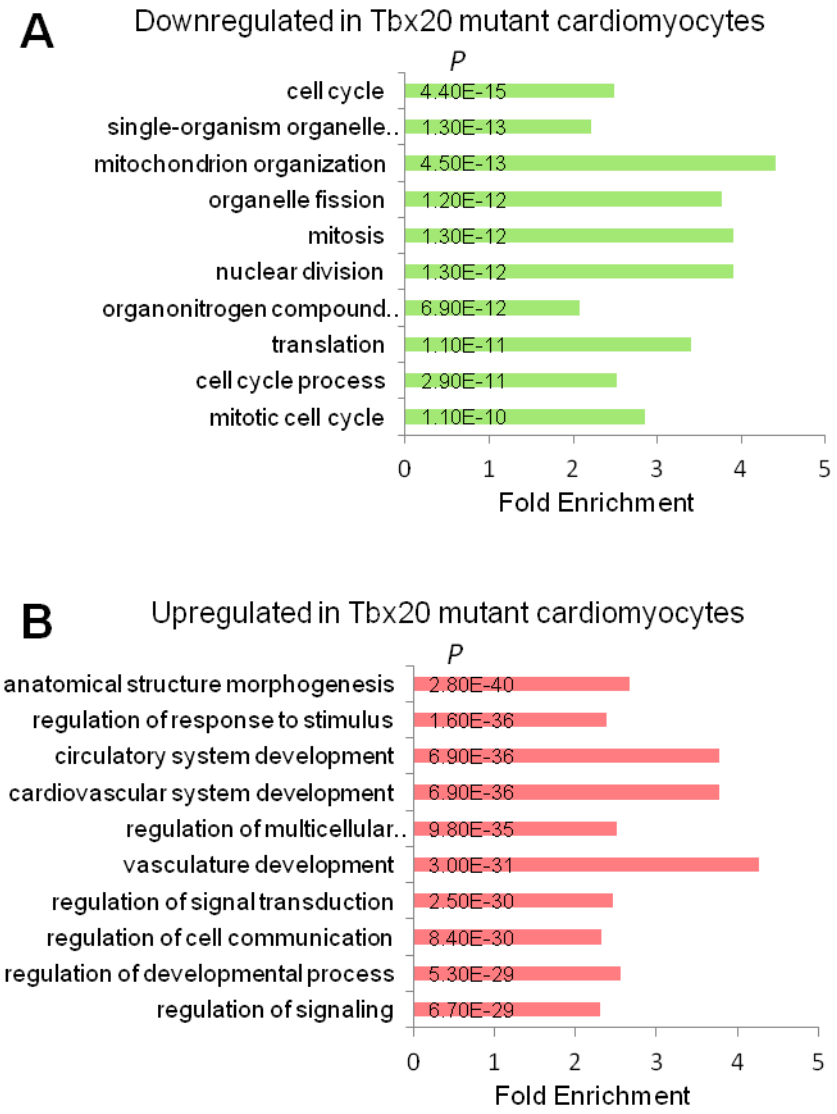


Figure 2.7 Gene ontology analysis of differentially expressed genes in E11.5 *Tnnt2-rtTA;TetO-Cre;Tbx20*$^{fl/fl}$ mutant cardiomyocytes versus *Tnnt2-rtTA;TetO-Cre;Tbx20*$^{+/fl}$ control littermates.(A) Gene ontology terms of downregulated genes in *Tbx20* mutant cardiomyocytes. **(B)** Gene ontology terms of upregulated genes in *Tbx20* mutant cardiomyocytes.

data provided further support for the idea that *Tbx20* is essential for spatiotemporal regulation of cardiomyocyte gene expression.

2.3.5 Intersection of RNA-seq and TBX20 ChIP-seq reveals critical direct targets of TBX20

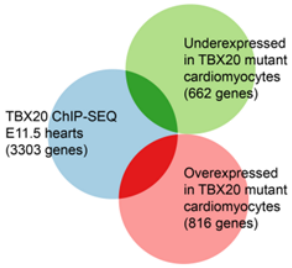
TBX20 is a transcription factor that can bind DNA to activate or repress expression of its target genes [47]. To identify TBX20 binding sites in E11.5 mouse heart, ChIP-sequencing (ChIP-seq) was previously performed using a transgenic mouse line expressing a TBX20-GFP fusion protein under the control of *Tbx20* regulatory sequences [83]. To identify putative direct targets of TBX20 in mid-gestation cardiomyocytes, we selected differentially expressed genes from E11.5 hearts which also had TBX20 binding peaks as identified from ChIP-seq analyses. With this approach we identified 199 downregulated and 349 upregulated direct targets of TBX20. GO term analysis of these genes revealed that TBX20 directly regulates processes involved in embryonic heart function, cardiac muscle contraction, cardiac cell differentiation and heart morphogenesis at midgestation (Figure 2.8).

2.3.6 TBX20 regulates proliferation by direct activation of *Mycn* and *Erbb2*

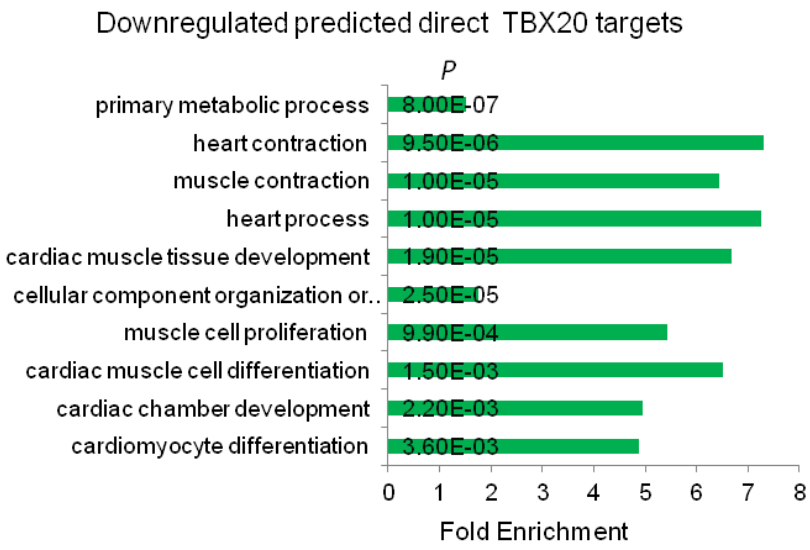
Decreased proliferation and interrupted cell cycle in *Tbx20* cKO mutant hearts suggested downregulation of genes important for cell cycle regulation in cardiomyocytes. *Mycn* and *Erbb2* are both required for myocardial proliferation and are essential for heart development [95, 96]. RNA-seq and RNA in situ showed that *Mycn* and *Erbb2* were down regulated in myocardium of *cKO*

Figure 2.8 Identification of pathways directly regulated by TBX20 in cardiomyocytes. **(A)** Intersection of RNA-seq and ChIP-seq; **(B-C)** Gene ontology analysis of differentially expressed genes in E11.5 *Tnnt-rtTa;TetO-Cre;Tbx20*$^{fl/fl}$ mutant cardiomyocytes versus *Tnnt-rtTa;TetO-Cre;Tbx20*$^{+/fl}$ control littermates that were also predicted direct targets genes based on TBX20-GFP ChIP-Seq in E11.5 mouse hearts. Downregulated direct targets include genes for muscle development and function and cardiac chamber formation.

A


Underexpressed
in TBX20 mutant
cardiomyocytes
(662 genes)
TBX20 ChIP-SEQ
E11.5 hearts
(3303 genes)
Overexpressed
in TBX20 mutant
cardiomyocytes
(816 genes)

B


Downregulated predicted direct TBX20 targets
P
primary metabolic process
heart contraction
muscle contraction
heart process
cardiac muscle tissue development
cellular component organization or..
muscle cell proliferation
cardiac muscle cell differentiation
cardiac chamber development
cardiomyocyte differentiation
8.00E-07
9.50E-06
1.00E-05
1.00E-05
1.90E-05
2.50E-05
9.90E-04
1.50E-03
2.20E-03
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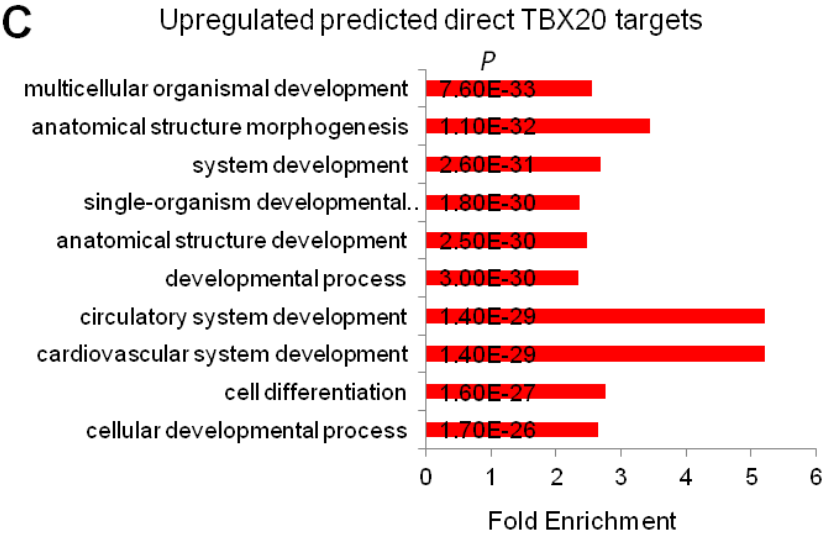
7
8
Fold Enrichment

C


Upregulated predicted direct TBX20 targets
P
multicellular organismal development
anatomical structure morphogenesis
system development
single-organism developmental..
anatomical structure development
developmental process
circulatory system development
cardiovascular system development
cell differentiation
cellular developmental process
7.60E-33
1.10E-32
2.60E-31
1.80E-30
2.50E-30
3.00E-30
1.40E-29
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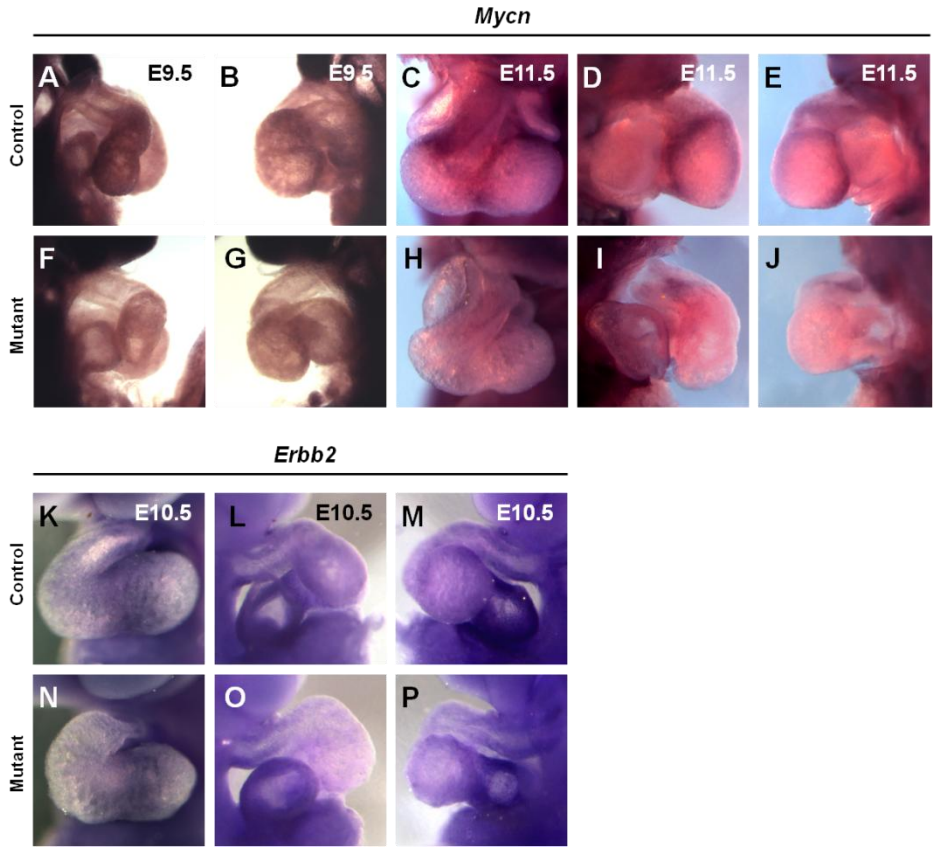


Figure 2.9 TBX20 regulates cardiomyocyte proliferation genes. (A-J) *Mycn* expressio*n* is reduced at ventricles of *Tbx20* mutant at E9.5 and E11.5. **(K-P)** *Erbb2* expression is reduced at ventricles of TBX20 mutant at E10.5. A, D, F, I, L & O: right side view of the heart; B, E, G, J, M & P: left side view of the heart; C, H, K & N: ventral view of the heart.

mutants relative to controls (Figure 2.9). In addition, *Mycn* and *Erbb2* were identified as direct TBX20 targets in our ChIP-seq experiments, indicating that TBX20 directly activates *Mycn* and *Erbb2* to promote cardiomyocyte proliferation.

2.3.7 TBX20 represses a cardiac progenitor gene program in cardiomyocytes

Amongst upregulated direct target genes were an intriguing number of key SHF genes, including transcription factor, LIM/homeodomain (*Isl1*), fibroblast growth factor 10 (*Fgf10*), and HOP homeobox (*Hopx*). *Isl1* regulates proliferation, survival and migration of undifferentiated cardiac progenitors, and is downregulated as progenitors enter the heart and differentiate towards cardiomyocytes [13]. *Fgf10* marks the anterior second heart field and promotes proliferation of cardiac progenitor cells that contribute to the arterial pole of the heart and, as for *Isl1*, is downregulated during differentiation [97]. *Hopx* has recently been identified as a marker of a pool of cardiac progenitor cells that is committed to myocyte cell fate but not yet differentiated [98]. To validate sustained expression of a cardiac progenitor gene program in cardiomyocytes after ablation of *Tbx20*, RNA in situ hybridization was performed for *Isl1* and *Fgf10*. Results with *Fgf10* showed no differences between control and *cKO*s (Supplementary Figure 1). At E11.5, where *Isl1* expression is typically restricted to the distal OFT, in *Tbx20 cKO* mutants, *Isl1* was expanded into the proximal OFT and right ventricle (Figure 2.10).

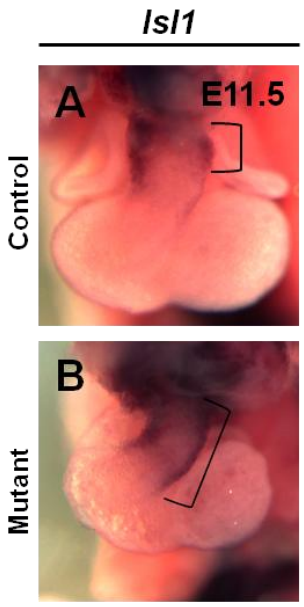


Figure 2.10 TBX20 regulates second heart field marker *Isl1.* (A-B) Expression of *Isl1* is expanded from outflow tract to right ventricle at E11.5 *Tbx20* mutant. A-B: ventral view of the heart. Arrow in A & B indicates *Isl1* expression. Brackets indicate the expansion of *Isl1* in comparable regions.

2.3.8 TBX20 directly alters expression of genes important in cardiac development

RNA-seq and ChIP-seq data suggested that several important cardiac genes were differentially expressed in *Tbx20 cKO* mutants or direct targets of TBX20. Expression of these genes was further examined in *Tbx20 cKO* and controls by whole mount RNA in situ hybridization. *Bmp2* and *Bmp10* were direct targets of TBX20 that were upregulated in mutants. *Bmp2* is an atrio-ventricular canal (AVC) myocardium marker and is critical for early AVC development [76, 77]. BMP2 activates *Tbx2* in AVC myocardium to repress a chamber myocardial phenotype [75]. Normally, *Bmp2* expression is restricted to the AVC region. In *Tbx20 cKO*s, *Bmp2* expression was normal within the AVC but was aberrantly extended into atrial myocardium (Figure 2.11). This finding suggested that TBX20 might directly repress *Bmp2* in atrial tissue. *Bmp10* is a critical gene for trabeculation and growth of the ventricular wall [99]. Expression levels of *Bmp10* were not affected in ventricles of *Tbx20* cKOs. However, aberrant upregulation of *Bmp10* was observed in right atria of mutants. Although previous studies have shown upregulation of Bmp10 in hypertrabeculated hearts from embryos lacking FK506 binding protein 12 (FKBP12) [100], potential effects of *Bmp10* upregulation in right atrium are not known.

Embryos lacking *Tbx5* have abnormal heart tube formation and severely hypoplastic atria, whereas over-expression of *Tbx5* inhibits ventricular maturation [101, 102]. As ChIP-Seq analysis identified *Tbx5* as a

direct target of TBX20 [53], we examined expression of *Tbx5* in *Tbx20 cKO*s. At E9.5, *Tbx5* expression was comparable between controls and mutants, (Supplementary Figure 2). However, at E11.5 *Tbx5* was significantly upregulated in *Tbx20 cKO* atria when compared to controls (Figure 2.11). In chick embryos, *Tbx5* overexpression inhibits myocyte proliferation [103]. The foregoing suggests that overexpression of *Tbx5* in *Tbx20 cKO* atria might contribute to reduced atrial proliferation in *Tbx20 cKO*s.

Upregulation of Tbx5 in both atria did not appear to explain why left atrial proliferation was more severely affected than right atrial proliferation. To investigate potential pathways accounting for increased severity of the left atrial phenotype, we investigated potential alterations in expression of *Pitx2,* a major regulator of left-right asymmetry in the heart [104]. *Pitx2* inhibits proliferation of the left atrium, with mutants showing right atrial isomerism [105, 106]. Moreover, our ChiP-seq data suggested *Pitx2* as a direct target of TBX20 in embryonic heart. To understand whether alterations in *Pitx2* expression might underlie differences in atrial sizes in *Tbx20* cKOs, we examined *Pitx2* expression in E9.5 and E11.5 hearts. Notably, no differences in *Pitx2* expression levels or pattern were observed in E9.5 and E11.5 mutants (Supplementary Figure 3), indicating that left-right differences in *Tbx20 cKO* hearts occur independently of alterations in *Pitx2* mRNA expression.

Expression of other potential direct targets of TBX20 that are important for heart development was also examined, including *Gja5* (Connexin 40;

chamber marker) and *Nkx2*.5 (pan-cardiac marker). *Gja5* and *Nkx2*.5 expression were comparable between controls and mutants (Figure 2.11).

2.3.9 TBX20 regulates atrial and ventricular identity by direct regulation of *COUP-TFII*, *Hey1*, *Hey2* and *Irx4*

"Cardiac chamber development" was one of the top GO terms of downregulated TBX20 direct targets. Within this group of genes, *COUP-TFII*, *Hey1*, *Hey2* and *Irx4* play critical roles in establishing cardiac chamber identity [91, 107, 108]. *COUP-TFII* is specifically expressed in atria within the heart and is a major determinant of atrial identity [91]. Myocardial knockout of *COUP-TFII* with *Myh6-Cre* results in progressive loss of atrial identity evident from E14.5 to E17.5 [91]. COUP-TFII promotes atrial identity by activating atrial markers such as *MLC2a*, *Sln*, *Kcnj3*, *Tbx5*, *Gja5* and *Hey1*, and by repressing ventricular markers including *MLC2v*, *MLC1v*, *Pln*, *Hey2*, *Irx4* and *Lbh* [91]. In *Tbx20 cKO* mutants, *COUP-TFII* expression in atria was similar to controls at E9.5. However, at E10.5 and E11.5, *COUP-TFII expression* was greatly reduced in *cKO*s relative to controls (Figure 2.12 A-J). The atrial specific gene *Hey1* was absent or reduced in atria of *cKO*s at both E9.5 and E10.5. Expression of the atrial gene *MLC2a* did not appear to be affected in *cKO*s relative to controls (Figure 2.12. K-R). Reduced *COUP-TFII* and *Hey1* expression suggested perturbation of atrial identity in *Tbx20 cKO*s. In myocardial knockouts of *COUP-TFII* ventricular genes *Hey2*, *Irx4, and MLC2v* are upregulated in mutant atria at E14.5 [91]. We examined ventricular identity genes *Hey2*, *Irx4* and *MLC2v* in *Tbx20 cKOs*. *MLC2v* was ectopically

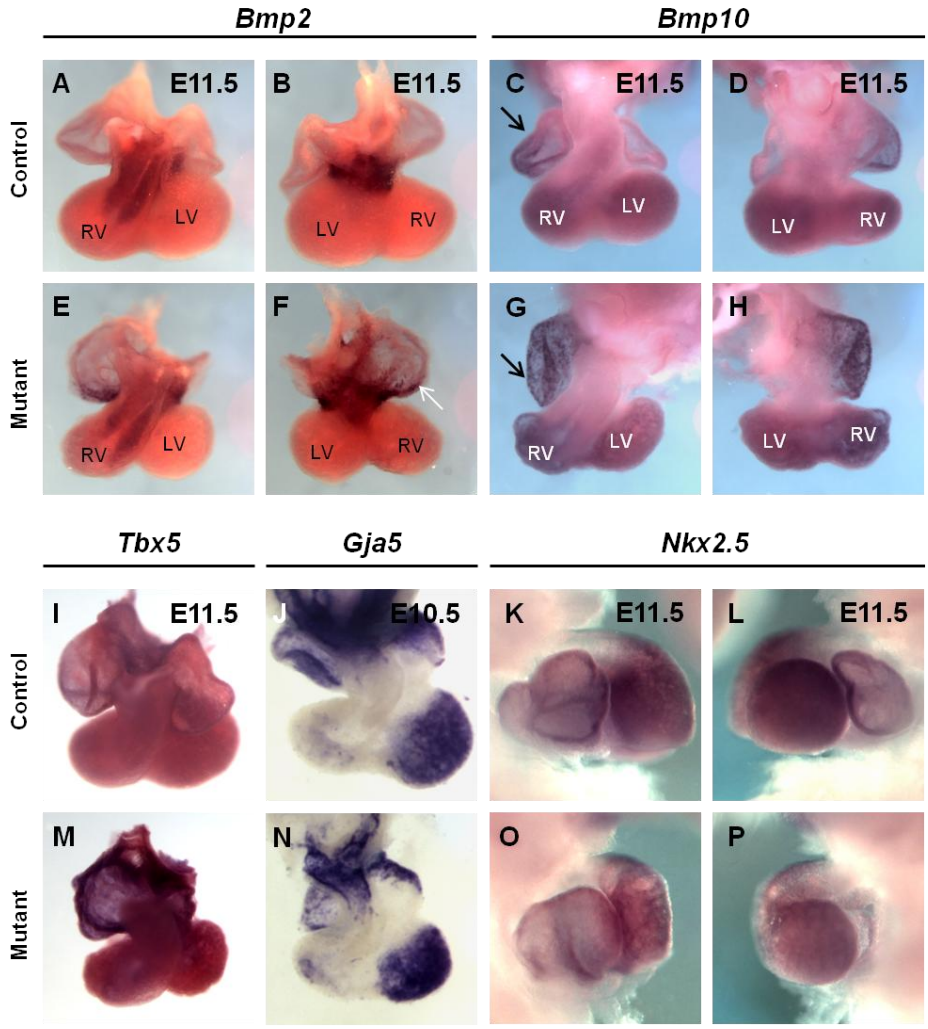
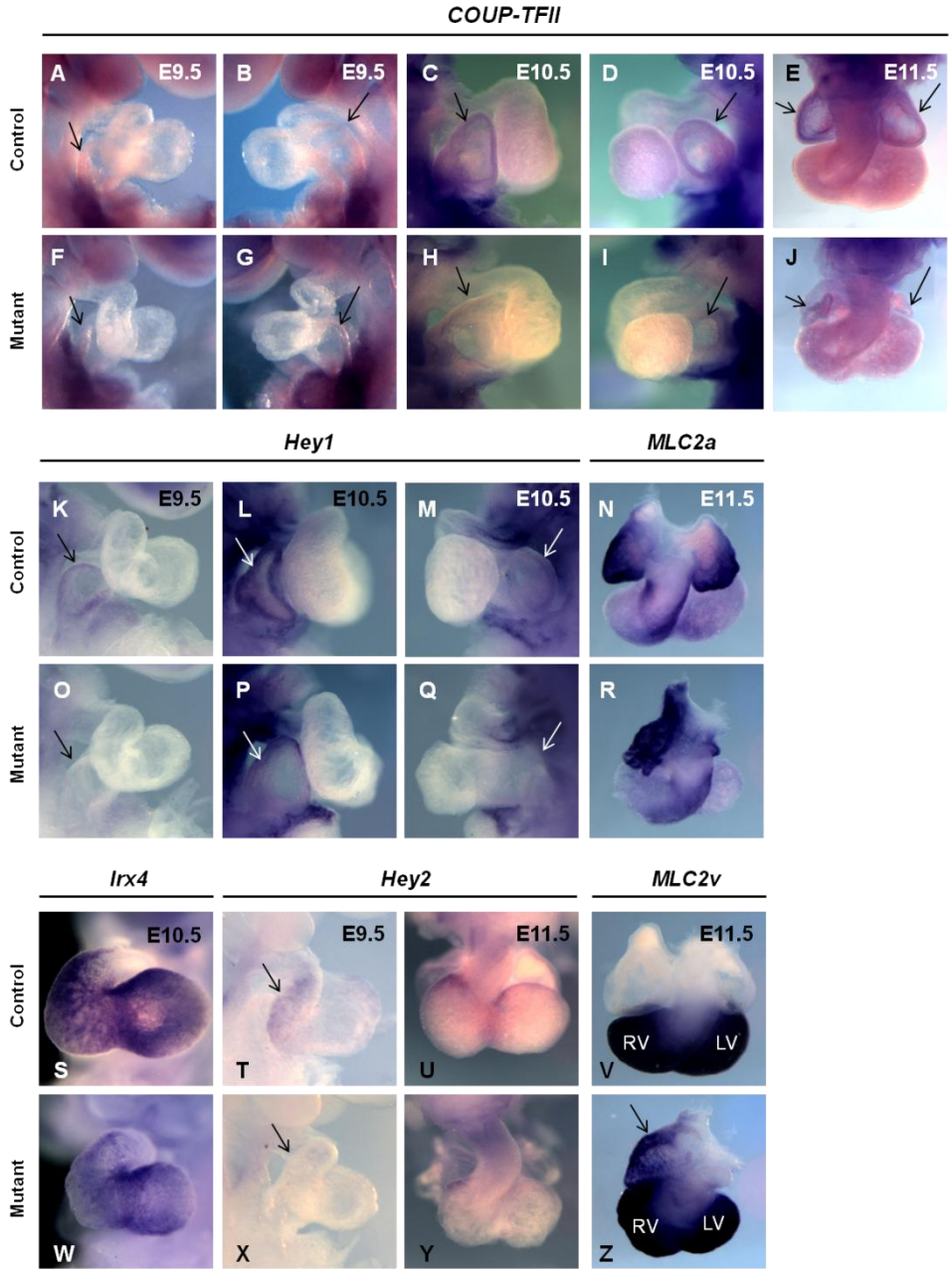


Figure 2.11 TBX20 regulates important cardiac development genes.(A, B, E & F) *Bmp2* expression is expanded to atria in *Tbx20* mutant. White arrow in F indicates *Bmp2* overexpression in the atria. **(C, D, G & H)** *Bmp10* is overexpressed in right atrium of *Tbx20* mutant. Black arrow in C & G indicates *BMP10* expression in the atria. **(I & M)** *Tbx5* is overexpressed in atria of *Tbx20* mutant. **(J & N)** *Gja5* expression is unchanged in *Tbx20* mutant. **(K, L, O & P)** *Nkx2.5* expression is unchanged in *Tbx20* mutant. A, C, E, G, I-J & M-N: ventral view of the heart; B, D, F & N: dorsal view of the heart; K & O: right side view of the heart; L & P: left side view of the heart. RV: right ventricle; LV: left ventricle.

Figure 2.12 TBX20 regulates chamber identity genes. (A-J) *COUP-TFII* expression is downregulated in atria of *Tbx20 cKO* mutant. **(K-M & O-Q)** *Hey1* is missing in atria of *Tbx20 cKO* mutant at E9.5 & E11.5. (**N & R)** *MLC2a* expression is unchanged in *Tbx20 cKO* mutant**. (S & W)** *Irx4* is downregulated in ventricles of *Tbx20 cKO* mutant. **(T-U & X-Y)** *Hey2* is downregulated in ventricles of *Tbx20 cKO* mutant. **(V & Z)** *MLC2v* is overexpressed in right atrium of *Tbx20* mutant. RV: right ventricle; LV: left ventricle. A, C, F, H, K, L, O, P, T, X: right side view of the heart; B, D, G, I, M, Q: left side view of the heart; E, J, N, R, S, U, V, W, Y, Z: ventral view of the heart. Arrows indicate RNA expression in the atria.


COUP-TFII
Control
Mutant
A E9.5
B E9.5
C E10.5
D E10.5
E E11.5
F
G
H
I
J
Hey1
MLC2a
K E9.5
L E10.5
M E10.5
N E11.5
O
P
Q
R
Irx4
Hey2
MLC2v
S E10.5
T E9.5
U E11.5
V E11.5
RV
LV
W
X
Y
Z

expressed in right atrium. At E11.5, although *Hey2* and *Irx4* were not upregulated in *Tbx20 cKO* atria, *Hey2* and *Irx4* were reduced in *Tbx20 cKO* ventricles. *Hey2* and Irx4 are important for regulation of a ventricular specific program [109, 110]. In summary, *Tbx20* plays a critical role in establishing atrial and ventricular identity by direct regulation of genes required to execute atrial and ventricular gene programs.

2.4 Discussion

In this study, a cardiomyocyte-specific inducible *Cre, Tnnt2-rtTA; TetO-Cre*, was used to ablate *Tbx20* in midgestation cardiomyocytes (*Tbx20 cKO*s). This provided an opportunity to study requirements for *Tbx20* throughout cardiomyocytes during early heart development, which has not previously been addressed. Additionally, no global transcriptome analyses or ChIP-Seq analyses have been performed to gain mechanistic understanding of the role of TBX20 in cardiomyocytes during early heart development. Altogether, our analyses revealed previously unrecognized critical gene targets and cell autonomous functions of TBX20 in midgestation cardiomyocytes, illuminating a major role for TBX20 in atrial development and identity.

Cell proliferation on the outer curvature of the heart between E9.5 to E12.5 makes major contributions to the growth of chamber myocardium [111]. *Tbx20* global mutants' evidence decreased cardiomyocyte proliferation and, arrest development at E9.5, with severely hypoplastic, unlooped hearts [64, 66, 72]. However, as *Tbx20* is expressed in multiple cell populations that contribute to or signal to the heart and might affect cardiomyocyte proliferation

non-cell autonomously, including pharyngeal endoderm, cardiac progenitors, endothelium, and endocardium, the cell autonomous requirement of *Tbx20* in cardiomyocyte proliferation remains to be addressed. Ablation of *Tbx20* in AVC myocardium with *Tbx2-Cre* does not affect cardiomyocyte proliferation [75]. However, in contrast to cardiomyocytes of developing chambers, AVC cardiomyocytes display low proliferative activity [111]. To assess the potential role of *Tbx20* in cell autonomous regulation of cardiomyocyte proliferation, we ablated *Tbx20* utilizing a cardiomyocyte-specific inducible *Cre*. *Tbx20* mutant hearts displayed less expanded chambers and septal defects with reduced proliferation in *Tbx20 cKO* cardiomyocytes. These results demonstrated for the first time a cell autonomous requirement for TBX20 in cardiomyocyte proliferation.

Global loss of *Tbx20* causes ectopic expression of proliferation repressor *Tbx2* throughout mutant hearts [64, 66, 72]. However, combined loss of *Tbx20* and *Tbx2* does not rescue the hypoplastic heart phenotype, indicating that additional pathways regulated by TBX20, independent of *Tbx2* overexpression, control cardiomyocyte proliferation [73]. In our study, RNA-seq analyses of purified cardiomyocytes demonstrated upregulation of *Tbx2* in *Tbx20 cKO* cardiomyocytes, demonstrating a cardiomyocyte autonomous role of TBX20 in repression of *Tbx2*. Intersection of our RNA-seq data and ChIP-seq data gave new insights into additional mechanisms by which TBX20 cell autonomously regulates myocyte proliferation. As previously found, TBX20 directly suppresses *Tbx2*. Additionally, TBX20 directly activated a number of

genes required to effect cardiomyocyte proliferation, including *Mycn*, *Erbb2,* and genes that regulate G1-S cell cycle progression, including *Cdc6*, *Cdt1* and *Ccna2*. Thus, our data shed new light on pathways by which TBX20 directly and cell autonomously regulates cardiomyocyte proliferation.

Cardiac chamber formation is marked by activation of a cardiomyocyte differentiation gene program in chamber myocardium (atria and ventricles) versus non-chamber myocardium (AVC, OFT and IFT) which retains more primitive characteristics [5]. Multiple *T-box* genes play important roles in different aspects of this process. In chamber myocardium, TBX20 and TBX5 work synergistically with NKX2.5 and GATA4 to promotes chamber differentiation by activating chamber specific genes like *Nppa* and *Gja5* [63, 87, 101, 112], while TBX20 also represses non-chamber specific genes *Tbx2* [64, 66, 72]. In AVC (non-chamber), TBX2 represses chamber specific gene expression to maintain the less differentiated, non-chamber myocardium fate. BMP2 also promotes AVC development by activating *Tbx2*. Studies showed that loss of *Bmp2* in AVC reduces *Tbx2* expression and ectopic expression of *Bmp2* induces *Tbx2* [76-78]. In our study, *Bmp2* was identified as a putative TBX20 direct target that is repressed by TBX20 in atria. Reduced expression of TBX20 direct targets *Hey1* and *Hey2* is also likely to contribute to ectopic BMP2 expression in *Tbx20 cKO* atria, as Hey1 and Hey2 restrict expression of *Bmp2* and *Tbx2* to the AVC [113]. Altogether, our studies demonstrate that TBX20 cell autonomously promotes chamber myocardial fate by suppression of an AVC gene program in chamber myocardium.

RNA-Seq of *Tbx20 cKO* cardiomyocytes revealed increased expression of several cardiac progenitor markers including *Isl1*, *Fgf10* (second heart field markers) and *Hopx* (cardiomyocyte progenitor marker) compared to littermate controls [12, 13, 98]. Notably, these genes were also predicted direct targets of TBX20 in embryonic hearts. *Isl1* is necessary for a subset of undifferentiated cardiac progenitors of the second heart field (SHF) to proliferate, survive and migrate [13]. *Isl1* is downregulated in OFT when cardiac progenitors enter the heart and differentiate. TBX20 has been shown to directly repress *Isl1* in E8.5 myocardium [66]. Our studies demonstrated an ongoing requirement for TBX20 to repress *Isl1* in E11.5 cardiomyocytes. *Fgf10* overexpression in E11.5 TBX20 *cKO* cardiomyocytes as measured by RNA-Seq could not be confirmed by whole mount RNA in situ studies, perhaps owing to the lower sensitivity of the RNA in situ assay. *Fgf10* and *Hopx* expression in *Tbx20* mutant cardiomyocytes will be further assayed by qRT-PCR. Validation of the upregulation of these progenitor genes in *Tbx20 cKO* hearts would provide further support to the hypothesis that TBX20 represses a SHF gene program in developing cardiomyocytes.

Although proliferation of both atria was significantly reduced in *Tbx20 cKO*s, proliferation of left atria was more drastically affected than proliferation of right atria. We examined *Pitx2* expression but found no difference in expression that could explain this phenotype. Left atrial hypoplasia along with other cardiac defects was found in a stillborn baby with a large deletion covering chromosome 15q26.2 that includes *COUP-TFII [114].* Other human

patients with similar 15q26.2 deletion but intact *COUP-TFII* do not show cardiac defects. In mouse, a *COUP-TFII* hypomorphic mutant exhibits left atrial hypoplasia [115], and our *Tbx20 cKO* mutants evidence significant reductions in *COUP-TFII* expression. Given these findings, COUP-TFII insufficiency may underlie the left atrial hypoplasia in *Tbx20 cKO*s.

Atrial and ventricular chambers have unique roles in circulating blood, which requires different contractile and cytoskeletal proteins, ion-channels and conduction system [116-118]. Intersection of *Tbx20 cKO* RNA-seq and TBX20-GFP ChIP-seq data illuminated important cell autonomous roles for TBX20 in setting up both atrial and ventricular identity, revealing mechanistic underpinnings by which it does so. Notably, we discovered that *COUP-TFII* was a direct target of TBX20. COUP-TFII is an orphan nuclear receptor that belongs to the steroid receptor super family [119]. Previous studies demonstrated a pivotal role for COUP-TFII in maintaining atrial chamber identity [91]. During heart development, *COUP-TFII* is expressed in atrial myocardium, but not in ventricular myocardium [115]. Loss of *COUP-TFII* in cardiomyocytes leads to reduced atrial gene expression and ventricularization of atria [91]. In keeping with this, in *Tbx20* cKOs, reduced expression of *COUP-TFII* in both atria was accompanied by reduced expression of the atrial gene *Hey1*, and ectopic atrial expression of the ventricular marker MLC2v. Only a few factors regulating *COUP-TFII* expression have been described from *in vitro* studies [120-123]. Our work has identified TBX20 as a direct regulator of *COUP-TFII in vivo*.

In addition to its critical role in atrial development and identity, our studies revealed a key cell autonomous role for TBX20 in establishing ventricular identity by direct regulation of *Hey2* and *Irx4* in developing ventricular myocytes. Results demonstrated significant reductions in expression of these markers in *Tbx20 cKO*s. Global loss of function of either *Hey2* or *Irx4* results in ectopic expression of atrial markers within embryonic ventricles [109, 110].

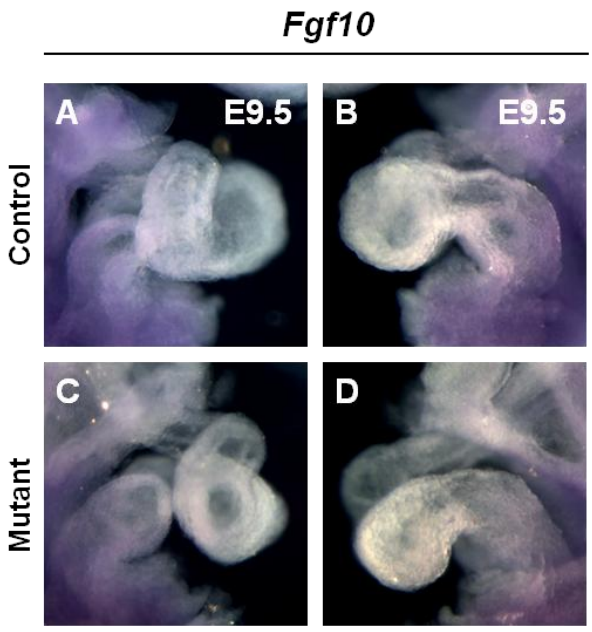
In summary, our study has comprehensively identified multiple pathways directly regulated by TBX20 in a cell autonomous fashion during cardiomyocyte development and patterning. TBX20 cell autonomously and directly regulates genes critical for cardiomyocyte proliferation, the establishment of chamber versus non-chamber myocardium, and atrial and ventricular identity. In particular, our study highlights the previously unsuspected importance of TBX20 in governing atrial development and identity by direct regulation of the transcriptional regulator *COUP-TFII*.

In previous studies, we have identified critical direct targets of TBX20 in adult cardiomyocytes [68] . Future comparison of TBX20 RNA-seq and ChIP-seq data in adult and embryonic cardiomyocytes will enhance our understanding of mechanisms by which TBX20 differentially regulates genes in these two contexts.

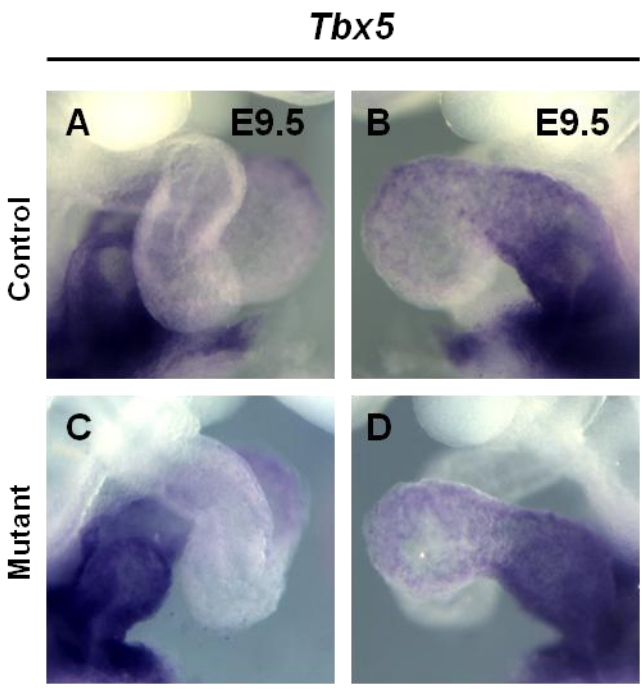
2.5 Acknowledgement

Chapter 2, in part is currently being prepared for submission for the

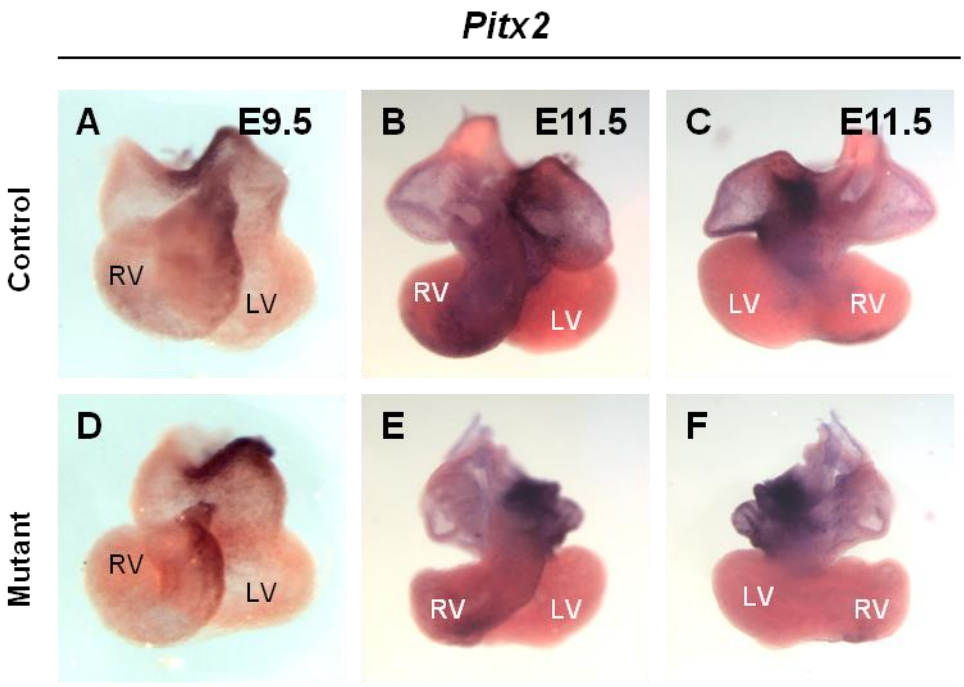
publication of material. Boogerd, Cornelis J.; Sakabe, Noboru; Aneas, Ivy; Nobrega, Marcelo A; Evans, Sylvia M. The dissertation//thesis author was the primary investigator and author of this material.



Supplementary Figure 2.1. *Fgf10* expression in control and *Tbx20 cKO* mutant hearts at E9.5 (A-D) *Fgf10* expression at E9.5 heart remained unchanged in mutants. A & C: right side view of the heart. B & D: left side view of the heart



Supplementary Figure 2.2. *Tbx5* expression in control and *Tbx20 cKO* mutant hearts at E9.5 (A-D) *Tbx5* expression at E9.5 heart remained unchanged in mutants.



Supplementary Figure 2.3. *Pitx2* expression in *Tbx20 cKO* mutant hearts at E9.5 and E11.5 (A-F) *Pitx2* expression pattern at E9.5 and E11.5 hearts remained unchanged in mutants.A, B, D,E: ventral view of the heart; C & F: dorsal view of the heart. RV: right ventricle; LV: left ventricle.

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