GATA4 Partners in Cardiac Cell Proliferation

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Abstract

Cardiovascular diseases are the leading cause of death in humans throughout the world and "congenital heart defects" (CHDs) are the major cause of infant mortality and morbidity. GATA4 is one of the most critical and intensely studied cardiac transcription factor. It is important for proliferation of cardiomyocytes as well as their survival and adaptive response. The focus of the following thesis was to identify GATA4 mediators and cofactors in cardiac growth. The first part focused on cyclin D2 (CycD2), a growth inducible cell cycle protein. We identified Ccnd2 (gene encoding CycD2) as a direct transcriptional target of GATA4 in postnatal cardiomyocytes and Ccnd2 cardiomyocyte specific overexpression in Gata4 heterozygote mice was able to rescue their heart size and function. We further uncovered a novel regulatory loop between GATA4 and CycD2. CycD2 enhanced GATA4 activation of its target promoters. GATA4 was able to physically interact with CycD2 and its cyclin dependent kinase CDK4 suggesting that GATA4 recruits CycD2/CDK4 to its target promoters. Together, our data uncover a role of CycD2 in the developing and postnatal heart and provide novel insight for the potential of targeting the cell cycle in cardiac therapy. The second part of the project focused on KLF13, a cell specific cofactor of GATA4. KLF13 is a member of the Krüppel-like transcription factors that are important regulators of cell proliferation and differentiation. Klf13 is highly enriched in the developing heart where it is found in both myocardial and endocardial cells. To determine its role in the mammalian heart, we deleted the Klf13 gene in transgenic mice. $Klf13^{-/-}$ mice were born at 50% reduced frequency and presented with variable cardiac phenotypes. Epithelial-mesenchymal transformation (EMT) was affected in these mice and reduced cell

proliferation was evident in the AV cushion. These data uncover a role for a new class of transcription factors in heart formation and point to KLF13 as a regulator of endocardial cell proliferation and a potential CHD causing gene. Future discovery of more cardiac regulators and understanding the molecular basis of CHDs is essential for preventions of these defects and possible development of therapeutic approaches for myocardial repair.

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List of Abbreviations

aa	Amino acid
AHF	Anterior heart field
ANF	Atrial natriuretic factor
ANP	Atrial natriuretic peptide
Ao	Aorta
ASD	Atrial septal defect
ATP	Adenosine triphosphate
AV	Atrioventricular
AVC	Atrioventricular canal/cushion
AVS	Atrioventricular septum
BAF	BRG1-associated factor
BAV	Bicuspid aortic valve
BMP	Bone morphogenic protein
BNP	Brain natriuretic peptide
BRG1	Brahama-like gene 1
Brm	Brahama
BSA	Bovine serum albumin
BW	Body weight
CD, CycD	Cyclin D
CDK	Cyclin dependent kinase
CHD	Congenital heart disease
CO	Cardiac output
CREB	cAmp response element binding protein
CVD	Cardiovascular disease
DCM	Dilated cardiomyopathy
cDNA	Complementary Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic acid
DORV	Double outlet right ventricle
Dox	Doxorubicin
EC	Endocardial cushion
ECG	Electrocardiogram
EF	Ejection fraction
EMT	Epithelial mesenchymal transformation
FGF	Fibroblast growth factor
FOG	Friend of GATA
FS	Fractional shortening
GSK	Glycogen synthase kinase
HA	Haemagglutinin
Has	Hyaluronic acid synthase

HL1	Immortalized atrial cardiomyocyte cell line
HOS	Holt Oram Syndrome
HR	Heart rate
HW	Heart weight
IAS	Interatrial septum
IVS	Interventricular septum
IVSd	Interventricular septum thickness at diastole
IVSs	Interventricular septum thickness at systole
Kb	Kilo base
KDa	Kilo Daltons
LA	Left atrium
LV	Left ventricle
LVEDD	Left ventricular end-diastolic dimension
LVESD	Left ventricular end-systolic dimension
LVPWd	Left ventricular posterior wall thickness at diastole
LVPWs	Left ventricular posterior wall thickness at systole
MAPK	Mitogen activated protein kinase
MEF	Myocyte enhancer factor
MHC	Myosin heavy chain
Min	Minute
ml	Millilitre
µl	Microlitre
MLC	Myosin light chain
mM	Milimolar
mRNA	Messenger RNA
MV	Mitral valve
NFAT	Nuclear factor of activated T cells
NIH3T3	Mouse embryonic fibroblast cell line
NLS	Nuclear localization signal
OFT	Outflow tract
PA	Pulmonary artery
PCR	Polymerase chain reaction
PDA	Patent ductus arteriosus
PDGF	Platelet derived growth factor
PFO	Patent foramen ovale
PHF	Primary heart field
PPAR	Peroxisome proliferator-activated receptor
PTA	Persistent truncus arteriosus
QPCR	Quantitative PCR
RA	Right atrium
RALDH	Retinaldehyde dehydrogenase
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
RV	Right ventricle
SA	Sinoatrial
SERCA	Sarcoplasmic endoplasmic reticulum Ca^{+2} ATPase
SHF	Secondary heart field

SHH	Sonic Hedgehog
SV	Stroke volume
SWI/SNF	ATP-dependent chromatin-remodeling complex
TAD	Transactivation domain
TGA	Transposition of the great arteries
TOF	Tetralogy of Fallot
TV	Tricuspid valve
VEGF	Vascular endothelial growth factor
VSD	Ventricular septal defect

I. Introduction

A. Embryonic Heart

The heart is the first functional organ in the developing embryo where it has a crucial role in circulation and waste removal. The use of animal models and biochemical approaches in the past decade has shed more light on our understanding of cardiac morphogenesis. Defects occurring before birth and affecting the heart's structure and/or function are referred to as "congenital heart diseases" (CHDs). These account for the loss of around 25% of human embryos and around 1-2% of newborns have some form of cardiac malformations (30, 239). Identifying the different regulators of heart development will help understand the genetic basis of CHDs.

i. Stages of heart formation (figure I.1)

The complex process of heart formation starts prior to the end of gastrulation. Endoderm, mesoderm and ectoderm are the three germ layers that form during gastrulation. The mesodermal layer arises from the primitive streak (which forms the midline of the embryo) through ingression of cells of the epiblast that have undergone epithelial to mesenchymal transformation. Cells from the anterior lateral plate mesoderm become committed to the cardiac fate due to inductive signals from the underlying endoderm. Later, the precardiac mesoderm migrates anterio-laterally and forms two bilateral fields on both sides of the embryonic midline; these cells then extend across the midline and organize themselves into the "cardiac crescent," referred to as the "primary heart field." Precursors

from the primary heart field, located ventrally in the cardiac crescent, then fuse along the ventral midline forming the beating linear heart tube, which represents the first functional organ in the developing embryo (E7.5-8.0 in mice; 3rd week of gestation in humans) (1, 217, 239, 326). The linear heart tube is composed of an outer myocardial layer, an inner endocardial layer and an extracellular matrix separating the two layers. At this stage, the contraction system is not organized with only very few contractile proteins and blood flowing from the venous inflow tract to the arterial outflow tract, located caudally and cranially, respectively (147, 217).

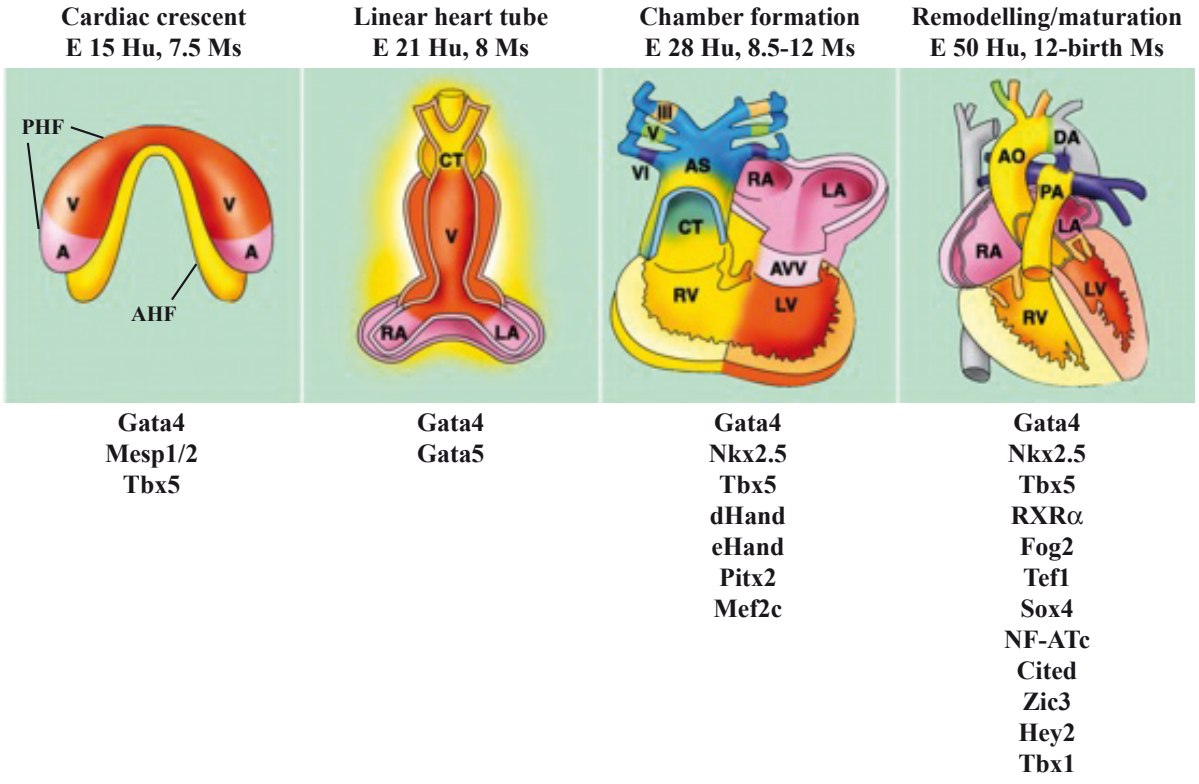

Figure I.1 Schematic diagram of the main stages of mamalian heart devlopment. Cells from the primary heart field (PHF) arrange themselves into the cardiac crescent with the anterior heart field (AHF) cells located medially and anterior to the PHF. The PHF gives rise mainly to the left ventricle (LV) and most of the atria. The SHF is at the origin of the right ventricle (RV), the OFT and parts of the atria. Human (Hu) and corresponding mouse (Ms) embryonic days (E) are indicated. The important transcription factors at each stage are mostly identified using mouse genetics. CT: conotruncus; V: ventricle; AVV: atrioventricular valves; AS: aortic sac; LA: left atrium; RA: right atrium; Ao: aorta; DA: ductus arteriosus; PA: pulmonary artery. Modified from Srivastava, 2006; Nemer, 2008; Rose et al, 2010 (239, 283, 314)**.**

Directly after formation, the heart tube elongates dramatically by two means: division of myocardial cells and addition of cells at the arterial and venous poles. The heart undergoes hence after a series of complex remodeling events to form the four-chambered heart. Researchers have discovered that the progenitor cells at the arterial pole originate from a separate population of cardiac precursors in the pharyngeal mesoderm, termed the "secondary or anterior heart field." These are located directly adjacent to the cardiac crescent and were found to give rise mainly to the outflow tract and right ventricle (33, 141, 348).

The first step of cardiac remodeling is to convert the heart from a linear anterior/posterior orientation to an asymmetrical left-right organization of two atrial and two ventricular chambers, where the heart assumes a spiral shape. This involves the rightward looping of the primitive heart tube upon receiving right-left signals from the developing embryo (E8.0-8.5 in mice; E23 in humans) (134, 142). The inflow region then moves antero-dorsally and positions itself at almost the same rostro-caudal level as the outflow complex; this step is crucial for establishing a correct communication between the future atria and ventricles (64, 217). Realignment of the heart tube is then followed by the formation of inward and outward curvatures due to differential growth along the dorsoventral and anteroposterior axes (46). The chamber or working myocardium forms by ballooning out from the outer curvature of the heart in specified regions; this working myocardium ultimately gives rise to atria and ventricles. Myocytes of the chamber myocardium possess a fast conduction phenotype and a high proliferation rate. Cells from the outer layer of the ventricles migrate into the lumen forming trabeculae; these then become compressed within the ventricle wall forming a thick compacted myocardium able to contract (44, 64, 349). On the other hand, pectinated muscles are formed in the atria. These are present mainly in the right atrium where they line its anterior wall and inner surface as a series of interwoven

ridges in a comb-like structure. They provide a voluminous nature to the right atrium and a great contraction strength with minimum muscle mass. In the left atrium, the pectinated muscles are confined to the inner surface of the atrium and are fewer and smaller than the ones of the right atrium (116). The remaining part of the heart tube, consisting of the inner curvature, the inflow tract, the outflow tract and the atrioventricular canal (AVC), retain the primary phenotype of low conduction velocity and contraction (69). This primary myocardium is essential for the formation of some components of the conduction system- the sinoatrial and atrioventricular nodes form from the inflow tract and AVC, respectively- as well as for septation and valve formation (44, 64).

The three principle cardiac septation events that take place during development include: atrial septation, ventricular septation, and primary heart tube septation through cardiac cushions formation. The primary atrial septum (septum primum) forms from the dorsal region of the common atrial wall toward the atrioventricular (AV) cushions by active proliferation of myocardial cells. At this stage, the atria communicate with each other through an opening underneath the primary septum called “ostium primum.” The primary septum then fuses with the AV cushions, thus, closing the ostium primum. In the meantime, the dorsal part of the septum primum disintegrates forming the “ostium secondum,” allowing continuous right to left flow of oxygenated blood. Later on in development, a secondary incomplete atrial septum “septum secondum” forms to the right of the septum primum with an opening called “foramen ovale.” The two septae fuse shortly after birth forming one complete septum (64, 217, 218, 355). The interventricular septum (IVS) on the other hand is a single structure with both muscular and mesenchymal components. The muscular part of the IVS appears as an outgrowth of the ventricles at the outer curvature; the mesenchymal component results from the fusion of the conotruncal and atrioventricular endocardial

cushions. Researchers have discovered that cardiomyocytes from both the left and right ventricles contribute equally to the IVS myocardium in its initial stages of formation (mouse E9.5-E11.5); at E12.5 and onward, cardiomyocytes from the left ventricle are more dominant particularly in the dorsal portion of the IVS (69, 78, 218). The adult IVS myocardium has therefore been suggested to have primarily left ventricular properties (78).

The atrioventricular (AV) and outflow tract (OFT) valves are crucial to ensure a proper flow of blood between the systemic and pulmonary circulatory systems and to prevent mixing of oxygenated and deoxygenated blood. The cardiac valves are completely functional only at late gestation and become fully mature after birth (95). As mentioned earlier, an extracellular matrix or cardiac jelly (composed of elastin, collagen, and glycosaminoglycans) separates the endocardial from the myocardial layer in the primary heart tube. A subpopulation of cells from the endocardial layer in the AV canal (AVC) and OFT, in response to inductive signals from the overlying myocardium, undergoes epithelial-to-mesenchymal transformation (EMT), thus, cellularizing the cardiac jelly and forming the endocardial cushions (EC) (mouse E9.5) (195). The process of cardiac valve formation as described by Armstrong and Bischoff is divided into four overlapping steps: a specification step whereby a subset of the endocardial cells in the AVC and OFT is fated to undertake EMT. These cells then delaminate and start the process of mesenchymal transdifferentiation and migration into the cardiac jelly. The cushions finally undergo extensive remodeling from localized swellings into thin narrow fibrous sheets. Various signaling molecules govern this process of cardiac valve formation including VEGF, Notch, wnt/β-catenin, TGF-β, BMP and hyaluronic acid signaling (figure I.2) (8). Studies have shown that OFT endocardial cushions also contain cells derived from the neural crest and secondary heart field (147). The endocardial cushions in the AVC are essential for the formation of the mitral and tricuspid

valves; while those in the OFT are at the origin of the pulmonary and aortic valves. The OFT endocardial cushions also septate the OFT into the pulmonary artery and aorta (95, 282). The correct alignment of the pulmonary artery with the right ventricle and that of the aorta with the left ventricle is critical for the proper flow of deoxygenated blood through the pulmonary artery to the lungs for re-oxygenation and oxygenated blood through the aorta to the rest of the body (217). The coronary circulation on the other hand originates mainly from the epicardium, with a slight contribution from the neural crest (64). The epicardium arises from the proepicardium through migration of proepicardial cells from the dorsal body wall. The proepicardium forms from the mesothelial cells near the caudal boundary of the pericardial cavity. Thickening of the compact region of the ventricles causes the diffusion distance of oxygen from the ventricular lumen to increase, thus triggering the need for a coronary circulation within the developing embryo. Cells delaminating from the epicardium undergo epithelial-mesenchymal transformation and differentiate into the coronary endothelial, smooth muscle and fibroblast cells. Endothelial cells form vascular tubes that fuse forming the coronary arteries and veins. The coronary vessels then connect to the ascending aorta and the right atrium. Smooth muscle and fibroblast cells are recruited and the formation of the coronary vasculature is established (249, 339).

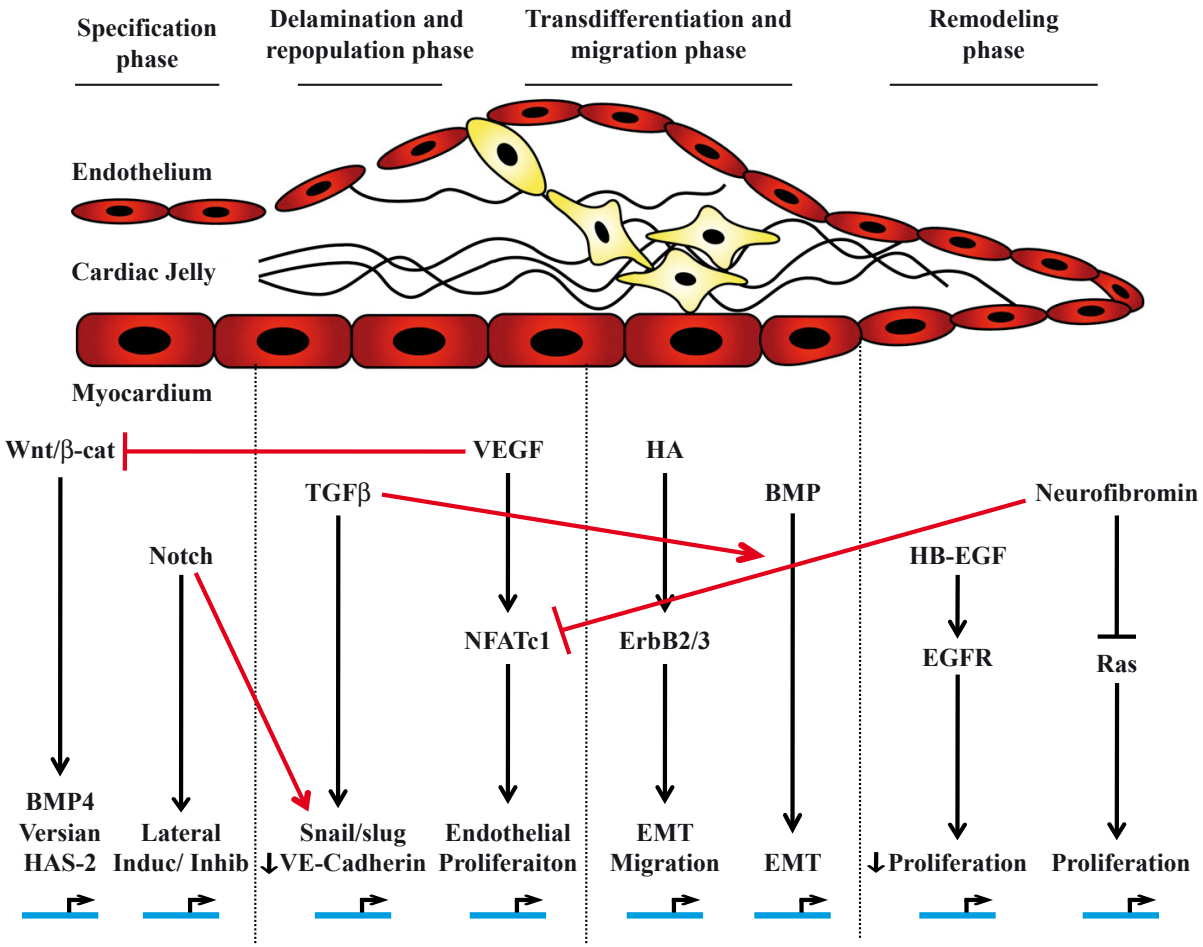

Figure I.2 Overview of cardiac valve formation. A subset of endothelial cells undergoes epithelial mesenchymal transformation (EMT) and populates the cardiac jelly. Localized swellings of cardiac jelly and mesenchymal cells termed cardiac cushions undergo extensive remodeling to form the thin-layered valves. Various signaling molecules and transcription factors are involved at the different stages of valve development. Red and blunted arrows indicate synergistic and inhibitory interactions between pathways respectively. Adapted from Armstrong and Bischoff, 2004 (8).

Components of the cardiac conduction system also form during this period of embryonic development. The sinoatrial (SA) and atrioventricular (AV) nodes form within the slow-conducting, non-chamber myocardium arising from the primary myocardium, where the SA node forms within the inflow tract and the AV node within the AVC. The SA node becomes the pacemaker of the heart. Action potential transmits from the SA node through the atria to the AV node. The AV node delays transmission to the ventricles, thus organizing atrial diastole and ventricular systole. The ventricular conduction system

originates from a particular ring of myocardium surrounding the interventricular foramen; the “bundle of His” forms from the myocardium at the dorsal part of the ring whereas the bundle branches derive from the cells at the tip of the IVS. The “his bundle” and its branches drive the action potential away from the AV node and connect to the Purkinje fibers that are formed from the trabecular myocardium of the ventricles. Purkinje fibers are connected to cardiomyocytes through gap junctions. The autonomic nervous system eventually innervates the SA and AV nodes (10, 64, 217, 218).

ii. Cardiogenesis and Morphogenesis

An evolutionary conserved network of signaling molecules and transcription factors act in combination to control induction of cardiogenesis and formation of the vertebrate heart. The spatio-temporal regulation of the expression of various genes is essential for proper heart development and chamber specification.

The induction for cardiogenesis starts from the anterior visceral endoderm and neighboring tissues via positive and negative signaling molecules that act on mesodermal cells rendering only a specific population of them cardiogenic (64, 348). Among the positive signals are fibroblast growth factor 8 (FGF8), bone morphogenetic protein 2 (BMP2), Crescent, and Wnt11; the inhibitory or negative signals include anti-BMPs as *Noggin,* and the Wnt ligands Wnts 3a and 8 (348). Additionally, BMP2 and FGF8 expressed in the outflow tract and caudal pharynx as well as Sonic Hedgehog (SHH) and Wnt-βcatenin (which requires inhibition) signaling pathways control induction of the secondary heart field. Where as BMPs are required for the early induction of mesodermal cells into cardiogenic precursors, FGFs are essential for their proliferation and differentiation into cardiomyocytes (348, 376). These signals induce the expression of various transcription factors that act in

combination to direct mesodermal cells to the cardiogenic lineage. Such cardiac transcription factors include GATA4, TBX 2,3 and 5, NKX2-5, MEF2 and others (239, 348).

Differential expression of various genes along the anteroposterior, dorsoventral and left-right axes is essential for cardiac cells to acquire positional information corresponding to their future location and for proper chamber formation. Cardiac pattering along the anteroposterior (AP) axis occurs very early on in development, even prior to the primary heart tube formation and is controlled by retinoic acid signaling. Excess retinoic acid results in an expansion of the atrial (posterior) compartment; where as deficiency leads to expansion of the anterior or ventricular region (359, 377). The expression levels of several transcription factors are affected by retinoic acid signaling. For instance, retinoic acid treatment leads to an increase in the expression levels of the cardiac transcription factor GATA4 in quail and *xenopus* embryos (126, 176). GATA4 is highly expressed in the posterior inflow tract regions and is thought to play a role in posterior heart morphogenesis (126, 152, 218). Similarly, TBX5 is expressed in the cardiac crescent and later in the posterior region of the developing heart (32, 118). Deficiency in the enzyme responsible for retinoic acid synthesis, retinaldehyde dehydrogenase 2 (RALDH2), leads to downregulation of TBX5 in the sinoatrial region where as higher levels of retinoic acid in the heart tube result in increased levels of TBX5 in the outflow tract and right ventricle (176, 242). Moreover, TBX5 deficiency in mice results in severe hypoplasia of the posterior structures of the primary heart tube indicating a major role of TBX5 in posterior heart development (32). Other factors involved in AP patterning include Coup-TFII, the homeobox transcription factor Irx4, and the basic helix-loop-helix factors Hey1 and Hey2 (218).

Upon rightward looping, the heart becomes asymmetrical. Left-right signaling pathways in the developing embryo control this process and they all seem to culminate in the

expression pattern of the homeodomain factor Pitx2 which is asymmetrically expressed in the left side of the visceral organs. Pitx2 is also involved in the anterior-heart forming field. It is expressed in the left region of the SHF where it is regulated by NKX2-5 and the T-box transcription factor TBX1 (118, 218). Just before the onset of looping, after determining the AP patterning (E8-8.5 in mice), dorsoventral patterning is witnessed and is governed by the expression of various genes at the ventral side of the heart tube. These genes include Nppa, Chisel, Hand1, Cited1, Irx3, Irx5, are distinctively expressed at the outer curvature and except for Hand1, mark the commencement of chamber formation (118, 218).

Chamber-specific genes whose expression is regulated by highly conserved transcription factors control chamber development. For instance, expression of the gene for the atrial natriuretic peptide (ANF), Nppa, is regulated by NKX2-5, GATA4, and TBX5 transcription factors to mark the myocardium of the developing chambers (32, 118, 239). These transcription factors are also expressed in the primary myocardium that lacks Nppa expression indicating the involvement of other regulatory factors that act to repress the chamber gene program in non-chamber forming regions of the heart tube. Such factors include TBX2 and TBX3 (102, 118). Moreover, BMP signaling plays a major role in chamber formation and septation. Bmp10 expression is restricted to the working myocardium and, in a process controlled by NKX2-5, mediates normal proliferation and maturation of cardiomyocytes (258). BMP2 regulates the expression of TBX2 and TBX3, and cardiac-specific deletion of Bmp2 in mice results in the expansion of the chamber markers Nppa1, Cx40 and Chisel, thus suggesting a role for BMP2 in restricting the chamber phenotype (190, 279). Additionally, Bmp2 and Bmp4 are both essential for epithelial-meshenchymal transtion in the OFT and AVC which is important for septation and valve

formation. Thus, in addition to their role in cardiac induction, BMPs have a major role at later stages of heart development during chamber formation and septation (118).

iii. Cardiac Transcription Factors

Various genetic and biochemical approaches have been employed to identify genes essential for cardiac formation. Perhaps the most accepted paradigm is that a combinatorial network of transcription factors regulates the genetic program required for proper cardiomyocyte proliferation, differentiation and survival. The importance of protein-protein interactions for normal heart development is evidenced by the findings that point mutations disrupting these interactions result in cardiac malformation in mice and humans and by the fact that these interactions are evolutionary conserved from drosophila to humans. The most intensely studied cardiac transcription factors are GATA4, TBX5, NKX2-5 and MEF2.

a. GATA family

GATA4 belongs to the GATA subfamily of the evolutionary conserved zinc finger transcription factors superfamily. Members of the GATA subfamily exist in fungi, metazoans and plants where they are crucial for cellular growth and differentiation (185). In higher vertebrates, they have indispensable roles in both embryonic and postnatal development (260). GATA proteins all share the ability to bind DNA through their zinc finger DNA binding domain A/C/T GATA A/G. In higher vertebrates, they possess two zinc finger binding domains encoded by two distinct exons. The C-terminal zinc finger shares the highest homology among vertebrates and others species and represents the major DNA binding domain. The N-terminal zinc finger binds DNA albeit with much lower affinity and is thought to play a role in stabilizing the protein-DNA interaction. Both zinc finger domains

are also involved in protein-protein interactions. The N- and C-terminal sequences flanking the zinc fingers are essential for transcriptional function of the protein (99, 236).

Six members of GATA proteins exist in mammals and, based on their tissue distribution and sequence homology, they are subdivided into two families. The first subfamily consists of GATA1, 2 and 3. These are expressed predominantly in the hematopoietic system where they are essential for lineage specification and differentiation. Mice deficient for any of the three genes die by midgestation, pointing to a non-redundant role of the three proteins despite their co-expression. GATA1 is important for terminal differentiation of erythroid cells and megakaryocytes; GATA2 has an essential role in the proliferation of hematopoietic progenitors. GATA3 expression is restricted to T lymphocytes within the hematopoietic system and is crucial for the terminal differentiation of T cells (81, 105, 254, 343). In addition to their roles in hematopoiesis, GATA1, 2 and 3 have important roles in the others organs where they are expressed. For instance, GATA3 is involved in the formation of the dopaminergic neurons and is crucial for kidney development (163). Finally, mutations in GATA1 and 3 have been linked to human diseases, thus underlining the importance of these GATA factors in development (236).

The cardiac subfamily of GATA proteins consists of GATA4, 5 and 6 (figure I.3 and table 1.1). These are expressed early on in development in the extra-embryonic endoderm prior to the end of gastrulation. Their expression is then confined to the mesoderm and the underlying endoderm of the developing embryo. In the heart, they are expressed as early as the cardiac crescent stage. At this stage, GATA4 is found in the myocardial and endocardial layers where as GATA6 is expressed only in the myocardium overlapping with GATA4 there. GATA5 expression in the myocardium of the cardiac crescent is very weak and it is

mainly found in the endocardium, which represents the major site of GATA5 expression. GATA5 overlaps with GATA4 in the endocardial cushion; however, it is expressed only until midgestation after which it rapidly disappears. GATA4, on the other hand, persists in the mesenchymal cushion cells that will form the valves. GATA5 myocardial expression is evident after looping and is restricted to a few atrial cells. It disappears completely at mouse E14.5. Myocardial GATA4 and GATA6 expression is carried on to the adult atria and ventricles. All GATA6 expressing myocardial cells co-express GATA4. Then again, GATA4 is found in myocytes that don't express GATA6, thus suggesting distinct subpopulations of cardiomyocytes (140, 222, 223, 236, 238).

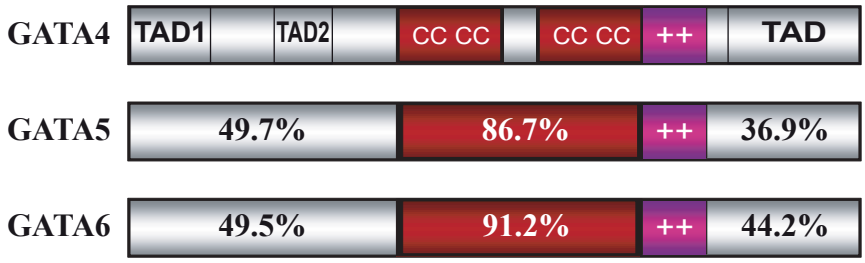

Figure I.3 Schematic diagram of GATA4 protein. Functional domains are shown. GATA4, 5 and 6 are highly homologous in their DNA binding domain but diverge significantly in their N- and C- terminal domains. TAD: transactivation domain. Modified from Temsah and Nemer, 2005 (332).

Table I-1 Spatial and temporal expression of the cardiac GATA factors (adapted from Charron and Nemer, 1999) (42).

	Cardiac Progenitors	Embryonic Heart		Postnatal Heart	
		Myocardium	Endocardium	Myocardium	Endocardium
GATA4	+	+	+	++	+
GATA5	+	+/-	++	-	++
GATA6	+	+	+/-	+	-

GATA4 is also highly expressed in the myocytes of the outflow tract (OFT). As mentioned earlier, myocytes of the OFT and the right ventricle are derived from the SHF (350). Some studies also show that GATA motifs are sufficient to drive the expression of

several cardiac transcription factors to the heart (60, 126, 200). These findings could point to a role of GATA4 in being a transregulator of the SHF. GATA6 is also present in neural crest cells where it is crucial for vascular patterning (236). GATA4 and GATA6 are additionally differentially expressed in the epicardium and a subset of vascular endothelial and smooth muscle cells (236).

Aside from the heart, GATAs 4, 5 and 6 are highly expressed in the endodermal cells of the digestive system (166). GATA4 and 6 are also found in the gonads and the lungs, respectively, where they have important functions: GATA4 is involved in the development of sertoli cells whereas GATA6 plays an important role in lung epithelial cell differentiation (139, 334, 344, 371).

1) Role in embryonic heart development

GATA4, 5 and 6 are critical for various stages of normal heart morphogenesis as evidenced by loss of function studies as well as the fact that GATA4 mutations are linked to congenital heart diseases in humans (236).

Knockout of Gata4 or Gata6 in mice is embryonic lethal. The Gata4 null embryos develop *cardia bifida* and hypoplastic ventricles and die by E8.5-9 (157, 211). In tetraploid complementation assay, where the *cardia bifida* phenotype is rescued, development is halted in the $Gata4^{-/-}$ embryos by E9.5-10. The hearts fail to undergo looping and chamber formation; they display myocardial thinning, lack of proepicardium and absence of endocardial cushion development (354). Gata4 deletion from embryonic cardiomyocytes often results in myocardial thinning, thus, suggesting a role of GATA4 in cardiomyocyte proliferation (42, 272, 380). In addition, targeted deletion of Gata4 from endocardial cells results in hypoplastic valve leaflets suggesting that GATA4 is crucial for proper cushion

formation and cellular differentiation (279). The Gata6 null embryos die by E6.5-7, prior to heart tube formation, due to gastrulation defects. Gata6$^{-/-}$ embryos formed from embryonic stem cells by tetraploid complementation grow until E10.5 with no signs of heart development defects or alterations in cardiac gene expression. This led to the suggestion that heart formation is possible without GATA6 (386). Then again, loss of one copy of each of Gata4 and Gata6 in mice is embryonic lethal pointing to a partial compensation between these two factors (363). In addition, Gata4/6 double knockout mice fail to form a heart indicating that the interaction between these two genes is important for the onset of cardiogenesis (385). Moreover, conditional deletion of Gata6 in vascular smooth muscle or neural crest cells results in perinatal death with a large subset of the null embryos developing aortic arch and outflow tract defects as well as membranous ventricular septal defects (170). These results support a role of GATA6 in smooth muscle cell differentiation and reveal GATA6 as a potential gene for congenital heart disease.

Gata5$^{-/-}$ embryos, on the other hand, are viable albeit recently, they have been found to display a high incidence of bicuspid aortic valves (BAVs). This phenotype is recapitulated in embryos with endocardial-specific Gata5 inactivation confirming a critical role of GATA5 in atrio-ventricular cushion formation (161). These results are in line with the endocardial-specific GATA5 expression and point to GATA5 as a candidate gene for patients with BAVs. Additionally, Gata4$^{+/-}$Gata5$^{+/-}$ and Gata5$^{+/-}$Gata6$^{+/-}$ mice die either embryonically or perinatally mostly due to defects in OFT development, thus pointing to essential genetic interactions between these factors in endocardial cushion formation and OFT morphogenesis (162).

2) Regulation of cardiac GATA factors

Upstream mechanisms regulating GATA factors at the transcriptional and post-translational levels are essential for the specificity of the expression profile of these factors. GATA dosage is crucial for proper cellular differentiation and organ development; thus, identifying these regulatory mechanisms is critical to fully understand their biological function.

One evidence of transcriptional regulation comes from the findings that external stimuli as the growth factors BMP4 and endothelin 1 upregulate GATA4 (216) where as the anticancer cardiotoxic agents adriamycin (7, 257) and Imatinib (Maharsy *et al*, submitted) downregulate Gata4 transcript levels. Another mode of gene expression regulation is alternative splicing and translation initiation. A truncated GATA5 isoform that lacks the whole N-terminal domain as well as the first zinc finger is found in chicken. The isoform maintains the properties of DNA binding and activation of target promoters (192, 238). This process of alternative splicing is evolutionary conserved and is also found in mice (62, 351). A GATA6 isoform is also reported through alternative translation initiation. The new isoform has an elongated N-terminal domain and a higher transcriptional activity (25, 26). The exact targets and functions of the GATA5 and 6 isoforms require further investigation.

At the posttranslational level, GATA proteins are modulated via phosphorylation, acetylation and sumoylation. These modifications affect the transcriptional activity, DNA-binding potential and subcellular localization of the protein. Phosphorylation of GATA4 by the mitogen-activated protein kinase (MAPK) and protein kinase A (PKA) at S105 and S261 respectively leads to enhanced transcriptional activity (43, 333, 341). The C-terminal domain of GATA4 is also targeted by protein kinase C (PKC) (S419,420) increasing its DNA-binding potential (353). MAPK, PKC and PKA are associated with myocyte differentiation

and regulation of cardiac contractility and growth; thus, physiological and pathological cardiac stimuli regulate GATA4 activity (236). Glycogen synthase 3β (GSK3β) also physically interacts with and phosphorylates GATA4 at its N-terminal resulting in a Crm1-dependent nuclear export (221, 263). PKA targets GATA6 as well however leading to its proteolytic cleavage and degradation; thus, activation of PKA in cardiomyocytes seems to have opposing effects on GATA4 and GATA6.

Aside from phosphorylation, GATA4 is also targeted by sumoylation (at Lys36) and acetylation resulting in enhanced transcriptional properties (137, 352). Acetylation of GATA4 is associated with embryonic stem cell differentiation into cardiomyocytes (137). These exquisite regulatory mechanisms of GATA protein expression and activity further emphasize the imperative dose-dependent function of GATA4, and most likely other cardiac GATA factors, at various stages of heart development.

3) Interaction with other DNA-binding transcription factors

GATA proteins mediate their transcriptional activity via multiprotein complex formation with various transcription factors on target promoters. These cell-specific and stimuli-induced combinatory interactions are at the basis of the pleitropic effects of the GATA factors.

The first evidence of these interactions is that GATA4 and the cardiac homeobox factor NKX2-5 cooperatively regulate the Nppa promoter. The GATA4/NKX2-5 interaction is evolutionary conserved and is required early on for cardiomyocyte differentiation and chamber formation (65, 309). It is highly specific for GATA4 and requires the second zinc finger and a part of the C-terminal domain (66). GATA4 and NKX2-5 also cooperate to regulate cardiac α-actin gene expression and other cardiac promoters as Ankrd1 (the gene

encoding CARP, cardiac ankyrin repeat protein); both C- and N-terminal transactivation domains along with the DNA-binding domain of GATA4 are involved in this case (159, 293).

GATA4 also collaborates with MEF2 (myocyte enhancing factor2) and SRF (serum response factor), members of the MADS box family. These interactions are important for proper heart development as well as mediating calcium and hormone signaling (219). Other GATA4 partners include members of the T-box group, TBX5 and TBX20 (317). In fact, TBX5/NKX2-5/GATA4 form an enhanceosome on the Nppa promoter that could be the key for understanding the molecular pathway of atrial septation; mutation in any of these three genes causes septal defects in humans as well as experimental models (32, 87, 236). Members of the basic Helix-loop-helix (bHLH) transcription factors, HAND1/2 and Hey proteins also affect GATA4 activity on target promoters, Nppa and/or the gene encoding the brain natriuretic peptide, Nppb (53, 77, 220). Additional combinatorial interactions include: the Kruppel zinc finger protein YY-1 interactions with GATA4, 5 and 6 proteins through their C-teriminal zinc finger (19, 54) and the newly described cardiac regulator KLF13, member of the kruppel-like family, collaborating with GATA4 through its N- and C-terminal zinc fingers (165).

Aside from their cell-specific cofactors, GATA4, 5 and 6 proteins functionally and physically interact with signal-inducible nuclear factors. Examples of these are the calcium inducible NFAT proteins, the growth factor effectors STATs, and the downstream effectors of the TGF growth/differentiation factors, SMAD proteins. These differential interactions of GATA proteins with signal-inducible factors provide a mechanism for cell-specific effects of extracellular stimuli (236). Moreover, they evidently suggest that GATA factors act as integrators as well as regulators of cell signaling pathways in the heart. GATA4 regulates

BMP4 and is an effector of BMP signaling; GATA4 responds to BMP4 by cooperating with SMAD to activate NKX2-5 expression, which in turn is a cofactor of GATA4 (28, 237) (figure I.4). On a similar note, GATA4 is a crucial nuclear effector of angiotensin and endothelin signaling and at the same regulates the angiotensin receptor and the transcription of endothelin-1 (236). Given the fact that both endothelin-1 and angiotensin II are mediators of mechanical stretch in the heart, it is noteworthy that GATA4 is crucial for mechanical stress-induced cardiac hypertrophy (264).

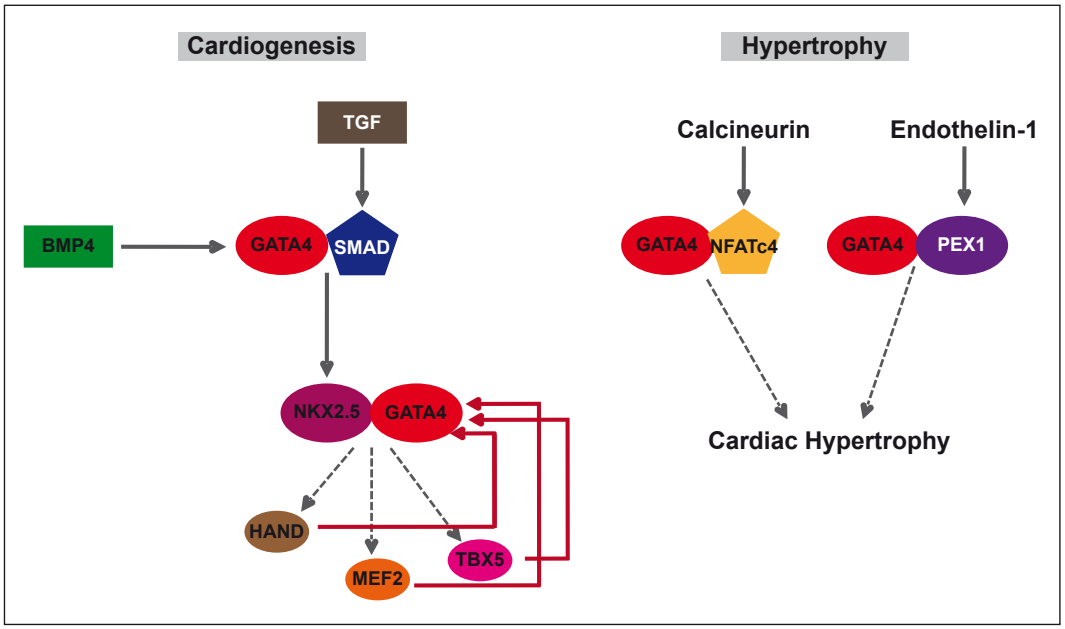

Figure I.4 Cofactors and Effectors of GATA4 in cardiogenesis (left) and hypertrophy (right). In response to the cardiogenic signal BMP4, GATA4 interacts with SMAD (TGF inducible transcription factor) to activate NKX2.5 transcription and mediate the cardiogenic signal. NKX2.5 then interacts with GATA4 to induce the transcription of various downstream targets each of which can further act as cofactors of GATA4 (28, 237). On a similar note, NFATc4 and PEX transcription factors are cofactors of GATA4 in cardiac hypertrophy (150, 212).

4) Interaction with other cofactors

In addition to their DNA-binding partners, GATA factors have been shown to interact with various other cofactors in different processes. For instance, FOG (Friend of GATA) proteins have been shown to interact with the N-terminal zinc finger of GATA factors (335). FOG2/GATA4 interaction is essential for proper heart valve development and coronary

system formation. Transgenic knock in mice harboring a GATA4 mutation that disrupts its interaction with FOG2 die between E11.5 and E13.5 displaying semilunar cardiac valve defects and double outlet right ventricle (DORV) (51, 336). FOG2/GATA4 interaction is also required for normal gonadogenesis (334). GATA4 interacts with the other FOG protein member, FOG1, as well and this interaction seems important for AV valve remodeling (136, 388).

The histone acetyltransferase p300 is another cofactor of GATA4, GATA5 and GATA6 in the heart (54, 132, 347). P300 is recruited to active transcriptional enhancers to activate transcription in a tissue specific manner (388). P300 acetylates GATA4 enhancing its DNA binding and transcriptional activity and this interaction seems critical for the development of cardiac hypertrophy (370). Almost 80% of the chromatin sites bound by p300 are co-occupied by GATA4 as revealed by ChIP-seq experiments in HL1 cells (110). GATA4-p300 interaction is therefore suggested to be important for both proteins to bound chromatin (388). Moreover, GATA4 interacts with the histone deacetylase HDAC2 possibly to regulate embryonic cardiomyocyte proliferation (342).

GATA4 also interacts with the BAF60c subunit of the chromatin remodeling complex SWI/SNF. Chromatin remodeling complexes use energy of ATP hydrolysis to reposition nucleosomes and alter chromatin accessibility. They are classified according to the ATPase subunit, with BRG1 or BRM1 being the ATPase subunit of the SWI/SNF ATPases (388). BAF60c interaction with GATA4 induces the ectopic expression of cardiac gene and the addition of TBX5 stimulates ectopic beating tissue (323). The N-terminal region of GATA4, specifically the region between aa 129-152, is required for the cardiogenic activity of GATA4 and its interaction with BAF60c (83).

In short, ample evidence points to the importance of these cardiac GATA factors for proper heart morphogenesis at various stages. However, the exact mechanisms underlying cardiac defects in patients with defective GATA4, 5 or 6 genes need yet to be fully clarified.

b. T-box family

T-box proteins are a large family of transcription factors important for early cellular commitment, differentiation and organ development. They are characterized by a highly conserved 180 amino acid DNA binding domain, the T-box, which also serves as a conserved protein-protein interacting domain. Members of the family are subdivided according to their degree of homology across this domain. Eighteen T-box factors have been identified so far in mammals; of these, at least six (TBX1, TBX2, TBX3, TBX5, TBX18, TBX20) are required for proper heart development (119, 233, 268).

Tbx1 is expressed in the endoderm and mesoderm of the pharyngeal arches and in the cardiac OFT. It is part of the regulatory network maintaining the balance between a proliferative and differentiated state of second heart field cells (281, 365). The first indication of its role in the heart came from the fact that TBX1 is located in the chromosomal region that is frequently deleted in one allele of DiGeorge syndrome patients (human chromosome 22q11.2). These patients most often present with craniofacial and cardiac abnormalities. The most common congenital heart defects associated with DiGeorge syndrome include tetralogy of Fallot, ventricular septal defects (VSDs), persistent truncus arteriosis (PTA), interruption of the aortic arch, and pulmonary atresia. Mice homozygous for null Tbx1 mutation die at birth and display severe cardiac phenotypes resembling those of DiGeorge syndrome patients, including PTA, VSDs, and mispatterning of the coronary arteries (215, 367).

TBX2 and TBX3 are highly homologous across the entire protein sequence and they function as transcriptional repressors of the chamber-myocardium gene program during heart development (49, 98). Tbx2 and Tbx3 genes are both expressed in the atrioventricular cushion (AVC); Tbx2 is also found in the inflow and outflow tracts and Tbx3 is present in the pharyngeal endoderm and neural crest cells that form the OFT as well as in the sinoatrial node (SAN) primordium where it is required for the development of the SAN and imposing pacemaker function on the atria (44, 102, 119, 206). As Tbx2 expression in the cardiac conduction system begins to decline, Tbx3 starts to upregulate there. This drove researchers to suggest that TBX2 acts to repress chamber-specific genes early on in heart development, whereas TBX3 is required to maintain this repression and delineate the conduction system at later stages of cardiac formation (44, 120). Tbx2 null mice die by E14.5 due to cardiac defects including abnormal OFT septation and misalignment of the pulmonary trunk and aorta. An abnormal expression of chamber-specific genes in the AVC is also seen in these mice (106). In humans, heterozygous TBX3 mutations are associated with ulnar mammary syndrome (UMS), an autosomal dominant disorder characterized by upper limb malformation and hypoplasia of the apocrine and mammary gland (11). Some of these patients also display abnormal development of the ventricular septum, which is also seen in Tbx3-deficient mice. These mice present defective development of the OFT and conduction system as well (9, 57, 206).

TBX5 is a dose-sensitive regulator of heart and limb formation. In humans, TBX5 is the gene mutated in the autosomal dominant disorder Holt-Oram Syndrome (OHS) characterized by forelimb abnormalities and congenital heart defects (figure I.5) (13, 173). Tbx5 expression in the heart is initially observed in the entire heart field with relatively higher levels in the atrium, the left ventricle and the inflow tract and lower to undetectable

levels in the right ventricle. In the atrium, it is found in both the myocardial and endocardial cells. Along with Tbx20 and Nkx2-5, Tbx5 is one of the earliest genes expressed in cardiac precursors (31, 121, 302). In mice, loss of one allele of Tbx5 leads to phenotypic disorders similar to those observed in HOS patients. Of the cardiac defects in these mice we note, atrial septal defects (ASDs), conduction system defects, and abnormalities in ventricular relaxation (32, 390). Loss of both Tbx5 alleles is embryonic lethal by E10.5; the mice display a defective heart tube that fails to loop, the sinoatrial structures and the primitive left ventricle are severely hypoplastic, the atria are underdeveloped, the atrioventricular cushion does not form and the expression of chamber-specific genes (Nppa, MLC2v, Irx4*)* as well as the early cardiac markers (Gata4 and Nkx2-5*)* is greatly reduced (32). This suggests that TBX5 has a major role in chamber differentiation and maturation. It is also required for proper development and function of the cardiac conduction system, for normal development of the epicardium and interventricular septum, as well as for cardiomyocyte proliferation (29, 49, 225).

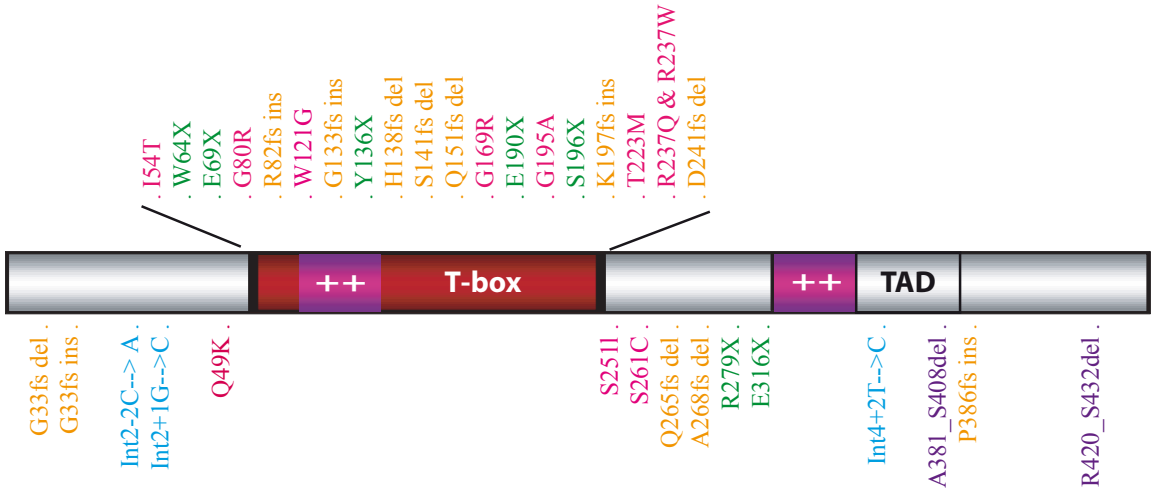

Figure I.5 TBX5 structure showing some of the human mutations associated with Holt-Oram syndrome. Pink: missense; Green: nonsense; Orange: insertions; Blue: intronic mutations; Purple: deletions. TAD: transactivation domain.

Tbx18 expression is observed in the proepicardium, the epicardium as well as both the mesenchymal progenitors and the differentiated myocardium of the sinus venosus (155). At later stages of development, it is found in the left ventricle and the left part of the developing interventricular septum (78). Mice with complete loss of Tbx18 are viable albeit they display an abnormal maturation of the cardiac inflow tract (47). A role for TBX18 in the epicardium was determined from studies in the zebrafish. Unlike adult mammals, the latter have the ability to regenerate up to 20% of ventricular apex resection (271). Researchers noted that in zebrafish, following injury, Tbx18, in response to FGF signaling, is upregulated in the epicardium surrounding the wound and in the subepicardial space within the lesion and the regenerating ventricle (169). On a similar note, using genetic lineage tracing technique in mice, a subset of Tbx18-expressing cells in the proepicardium were shown to migrate to the heart giving rise to myocytes in the interventricular septum as well as ventricular and atrial walls (39). More recently, Zeng *et al*, using immunofluorescence analysis, demonstrated that TBX18 is expressed in cardiomyocytes from E10.5 up to E14.5 (381). These studies thus suggest further insights for the role of the epicardium in cardiac regeneration and a role for TBX18 in the recruitment of epicardial-derived progenitor cells; even though, the epicardial source of cardiomyocytes is debatable (45).

As mentioned earlier, Tbx20 is one of the earliest genes expressed in cardiac precursors. Its expression begins in the anterior lateral plate mesoderm and is further found in the first and second heart fields throughout heart development (156, 324). TBX20 loss of function mutations in humans results in various cardiac defects namely cardiomyopathy, VSDs, abnormal valvulogenesis, and tetralogy of Fallot. Most of the mutations are nonsense or missense mutations in the T-box domain (148, 180). In mice, complete loss of Tbx20 is lethal by E10.5. These embryos display a linear heart tube that fails to elongate and looping

and chamber formation are impaired, thus the requirement of this gene for recruitment of cells from the SHF and for chamber formation. Chamber-specific genes are not activated in these mutants and Tbx2 is ectopically expressed in the linear heart tube (307, 318). A role for Tbx20 in promoting the proliferation and migration of heart valve precursors of the endocardial cushion during embryonic development has also been demonstrated (167, 295).

T-box factors are therefore important for most cardiac cell lineages and are involved in cellular proliferation, migration, differentiation and patterning of the forming heart. They function in accordance with other cardiac transcription factors, as GATA4 and Nkx2-5, to regulate target genes and ensure proper heart development.

c. NKX2-5 transcription factor

NKX2-5 is a member of the class I NK-2 family of homeodomain transcription factors that contain three conserved domains: a homeodomain (HD), a transactivation domain (TD) and an NK-2 specific domain (NK2-SD) (71). The 60-amino acid homeodomain binds DNA through a helix-turn-helix motif of three alpha helices with the 3rd helix ensuring binding specificity and the consensus sites being 5'-TNAAGTG-3' and 5'-TTAATT-3' (71, 179). The homeodomain is also shown to mediate interactions of NKX2-5 with other transcription factors including GATA4, TBX5 and TBX20 (32, 65, 115, 317). Nkx2-*5*, the gene encoding NKX2-5, was first identified as the mouse homolog of the drosophila cardiac regulator *tinman* (151, 179). It is highly expressed during cardiac development and persists in the adult heart. Its expression in the developing heart starts very early in cardiac progenitors and it is found in both the first and secondary heart fields (133, 179, 316), thus pointing to a superior role for NKX2-5 in the hierarchy of cardiac transcriptional regulators. Nkx2-5 knockout mice die *in utero* between E9-10; the mutated

hearts arrest at the looping stage and present with a single underdeveloped primary chamber and a largely truncated and narrowed outflow tract. Nkx2-5 is also important for the formation of the cardiac conduction system, as the null embryos appear to lack atrioventricular node progenitors. Moreover, endocardial cushion formation is absent in these null embryos suggesting a role for NKX2-5 in endocardium development (125, 189, 329). In humans, heterozygous NKX2-5 mutations have been identified in patients with congenital heart disease (CHD). Diverse cardiac malformations have been associated with NKX2.5 mutations most prominent of which are atrial septal defects and atrioventricular conduction block. Other malformations include Tetralogy of Fallot (TOF), ventricular septal defects, Ebstein's anomaly, DORV and hypoplastic left heart syndrome (71, 278, 291). Postnatally, NKX2-5 is thought to act as a regulator of the hypertrophic growth response. NKX2-5 levels increase in response to pathologic hypertrophic stimuli in mice; however, its overexpression is not sufficient to induce hypertrophy (288, 325, 337). Collectively, NKX2-5 arises as a critical modulator of cardiac-specific gene expression controlling the lineage outcomes of various cardiac precursors; its role in the adult heart however needs further characterization.

d. MEF2 family

Myocyte enhancer factor 2 (MEF2) family of transcription factors consists of four members: A, B, C, and D that are critical for regulation of gene expression in a variety of tissues, namely the skeletal muscle and the heart. They act as a "transcriptional switch" where by strongly activating or repressing gene transcription via interactions with numerous cofactors (22). The N-terminal region of MEF2 proteins consists of two highly conserved domains, the MADS (MCM1, AGAMOUS, DEFICIENS, and SRF, serum response factor) and MEF2 domains, both of which are important for dimerization and DNA binding. They

bind an AT-rich DNA sequence found in the promoter of various muscle-specific genes. The MADS domain is generally a 57-amino acid sequence that is also found in other factors like the serum response factor, SRF (122, 269, 301). The C-terminal region, on the other hand, is highly variable as a result of alternative splicing resulting in numerous tissue-specific isoforms (22). It contains a transactivation domain and in the case of MEF2A, C and D, it also contains a nuclear localization signal (22, 24, 374).

In the heart, Mef2 genes are required during embryonic development as well as in the adult stage. Mef2b and Mef2c are co-expressed in the precardiac mesoderm starting from E7.5. Shortly after, at E8.5, Mef2a and Mef2d are detected in the myocardium (67, 214). Mice homozygous for a Mef2c-null mutation die by E9.5. Mutant embryos fail to undergo cardiac looping and the right ventricle does not form. The expression level of various cardiac genes, including Nppa, and the genes encoding cardiac α-actin and α-myosin heavy chain, are severely altered (177). However, conditional deletion of Mef2c in the myocardium results in viable offspring with no apparent phenotype; thus MEF2c seems to play a major role in early cardiac development prior to looping yet is not imperative for later stages (346). On a similar note, loss of Mef2a in mice results in severe abnormalities in heart formation and function; however, the majority of mutants experience sudden perinatal death and display right ventricular dilation, myofiber disorganization and alteration of cardiac gene program characteristic of heart failure. Mutant mice that survive to adulthood have defected mitochondrial biogenesis and are also susceptible to sudden death suggesting a role of MEF2A in regulating mitochondrial content in adult cardiomyocytes (235).

MEF2 proteins have also been associated with cardiac hypertrophic growth. For instance, MEF2 DNA-binding and transactivation potential are enhanced in response to hypertrophic stimuli in postnatal cardiomyocytes (213, 231). Moreover, cardiac-specific

overexpression of MEF2A and MEF2C in mice results in dilated cardiomyopathy and contractile dysfunction and renders the mice more susceptible to pressure overload (366). These findings suggest a role of MEF2 proteins in modulating cardiac dilation rather than the classical hypertrophic growth. Finally, MEF2 factors seem to be important regulators of human cardiac disease response, most probably dilated cardiomyopathy. However, no human mutations have been reported so far and further analyses in this matter will enhance our knowledge of MEF2 actions in the heart (22, 247).

e. KLF13

KLF13 belongs to the Kruppel-like family of transcription factors that were first identified by virtue of their similarity to Sp1, one of the first factors cloned and characterized in mammals. 21 KLF members have been identified in humans so far, of which 17 homologs are found in mice (129). They contain three highly conserved Cys_2/His_2 zinc fingers in the C-terminal region connected by a highly conserved Kruppel-like seven-amino acid sequence [TGEKP(Y/F)X]. The three zinc fingers confer specificity to KLFs to bind the consensus *GC*- and *CACCC* DNA boxes (20, 55). Outside the zinc finger region, the non-DNA binding domain is highly divergent among family members, thus mediating unique transactivation and/or transrepression functions and protein-protein interactions of each factor (55).

KLFs are expressed in various cell types and have been shown to be involved in diverse cellular processes including cardiac remodeling, angiogenesis, hematopoiesis, neoplasia, and moncocyte activation (103). So far, five KLF factors have been shown to play important roles in the heart: KLF2 (158, 362), KLF5 (300), KLF13 (165), KLF15 (76), and KLF10 (274). KLF2 expression is detected in vascular endothelial cells early on during mice embryonic development and its knockout leads to embryonic lethality between E12.5 and

E14.5. The null embryos develop defective blood vessel wall organization and stabilization and KLF2 is later reported to be essential for smooth muscle cell migration (158, 362). A role for KLF5 in the heart comes from its detection in cardiac fibroblasts and fetal vascular smooth muscle cells (VSMCs) (232, 300). Angiotensin II treatment to primary culture of cardiac fibroblasts increases KLF5 expression; KLF5 expression in adult VSMCs is re-induced in response to vascular injury. Moreover, mice heterozygous for Klf5 have an attenuated hypertrophy response to angiotensin II and reduced SMC proliferation following vascular injury. These results together with those implicating KLF5 in activating the expression of various cardiovascular remodeling inducible genes as platelet derived growth factor (PDGF)A/B and vascular endothelial growth factor (VEGF) receptors conclude an important role of KLF5 in cardiovascular remodeling (232, 300). KLF10 has been implicated in the heart due to the finding that male knockout mice only develop signs of pathologic hypertrophy at the age of study (16 months) (274). More research is however required to further characterize its importance for heart function.

KLF15 plays a role in the postnatal heart. It is expressed in adult cardiomyocytes and acts as a negative regulator of cardiac hypertrophy (76). Its expression is reduced in response to hypertrophic stimuli; its overexpression on the other hand inhibits the hypertrophic gene expression profile in rat neonatal cardiomyocytes. Klf15 null mice appear normal at baseline albeit, in response to pressure overload, they develop severe eccentric hypertrophy (76). Moreover, promoter analyses studies revealed that KLF15 inhibits the function of two pro-hypertrophic transcriptional activators, GATA4 and MEF2 (76). KLF15 expression is also detected in cardiac fibroblasts and in SMCs (76, 186). It is reported to act as a negative regulator of SMC proliferation. Its expression in VSMCs is greatly reduced following vascular injury and $Klf15^{-/-}$ mice have an exaggerated response to vascular damage (186).

We recently identified KLF13 as a novel regulator of cardiogenesis in our lab (165). First evidence for its role in the heart comes from the finding of a CACCC box on the Nppb (brain natriuretic peptide) promoter that is flanked by a GATA site. Deletion or mutation of this CACCC element leads to a reduction in Nppb promoter activity in postnatal cardiomyocytes; this reduction is more pronounced in atrial myocytes (165). KLF13 is a transactivator of other cardiac promoters as Nppa, cardiac α-actin and β-myosin heavy chain; it not only activates these genes on its own but also collaborates with the cardiac transcription factors GATA4 and GATA6 on these target promoters (165). KLF13 is expressed in the heart and the cephalic mesenchyme of the developing mouse embryo (196). A detailed spatiotemporal expression analysis of KLF13 in the developing mouse heart by Lavalleé *et al* shows that E9.5 embryos have strong expression in the heart and the epidermis. At E10.5, KLF13 is highly detected in the atrial myocardium and the endocardium. By E12.5, its expression is stronger in the atria yet is also highly detected in the ventricular trabeculae. KLF13 is greatly downregulated in the postnatal heart; however, it is still found in the atria and ventricles with highest levels in the interventricular septum and the atrioventricular valves (165). Outside the heart, it is expressed in other tissues such as the brain mesenchyme, the dorsal ganglions, skeletal muscles and the vascular vessel endothelial cells of the liver (165). A similar expression pattern is also observed in the *Xenopus* (165). Knockdown of *xKlf13* in *Xenopus* results in a cardiac phenotype. Early heart formation up to the heart tube stage is normal in these embryos. However, shortly after looping and chamber formation, several defects are observed. The hearts are smaller with no clear morphological demarcations, beating is slower and pericardial edema is observed. Ventricles are smaller with lack of trabeculation, atrioventricular cushion formation and valve maturation is delayed and atrial septal defects are evident (165). Several cardiac markers are depleted in

these knockdown embryos starting at tail bud stage 25 (cardiac crescent fusion occurs at this stage) and stage 30 (when the first phenotypic defects occur). These genes include *Gata4, Gata5, Gata6, Tbx5, Nppa* and *αMlc,* thus indicating a role of KLF13 in cardiomyocyte differentiation. *In vitro* studies in mice showed that KLF13 activates the cyclin D1 promoter in cooperation with GATA4; this finding together with the hypoplastic phenotype of the knock down hearts indicate a role of KLF13 in cardiomyocyte proliferation as well (165). Lavalleé *et al* also show that the cardiac phenotype in the knockdown embryos can be rescued either by Klf13 itself or by injection of Gata4 RNA in a dose-dependent manner (165). As for human genetic studies, heterozygous microdeletions in the chromosomal region 15q13.3 (harboring at least seven genes including KLF13) have been identified in patients with congenital heart disease among other clinical features (168). KLF13 is therefore an early regulator of heart formation and could emerge as a novel candidate gene for human congenital heart disease. Screening for human mutations as well as the development of a mammalian mouse knock out will further our understanding of its mechanism of action in the developing heart.

iv. Congenital Heart Diseases

Congenital heart diseases (CHD) are defects in the structure or function of the heart that occur before birth. They are the leading cause of deaths in infants under 1 year of age. 19-75 of every 1000 live births have some sorts of heart abnormality (30, 117). It has been established through various genetic and epidemiologic studies that defective genes in collaboration with the environment are on the basis of most congenital heart problems. Most CHDs are associated with genetic disorders as Holt-Oram, DiGeorges, Alagille, Noonan and Williams-Beuren syndromes, albeit isolated cases have been reported

a. Types of congenital heart defects

CHDs can be grouped into three main categories (30, 239). The first group is called "cyanotic heart disease." Babies with this type of heart disease appear blue due to mixing of oxygenated and deoxygenated blood. Defects that belong to this category include transposition of the great arteries (TGA) in which the aorta connects to the right ventricle instead of the left and the pulmonary artery arises from the left ventricle instead of the right. This is a very common type of cyanotic defect presenting in newborns and accounts for 10-11% of all CHDs (14, 379). Another cyanotic disease is double outlet right ventricle (DORV). Both the aorta and pulmonary artery align with the right ventricle in this case which occurs as a result of incomplete rotation of the outflow tract (30, 275). Tetralogy of Fallot (TOF) can also contribute to cyanotic heart disease. This consists of four abnormalities: a hole in the interventricular septum termed ventricular septal defect or VSD, narrowing of the pulmonary artery termed pulmonary stenosis (PS) which causes interference with the flow of blood from the right ventricle, an overriding aorta in which the aorta is on top of the VSD instead of being aligned with the left ventricle, and thus receives some blood from the right ventricle, and finally hypertrophy of the right ventricle. TOF occurs in 9 to 14% of children with CHDs (59, 379). Another form of cyanotic disease is persistent truncus arteriosis (PTA). This happens when the tuncus arteriosis fails to separate into the aorta and pulmonary artery during embryonic development (30, 379). Other cyanotic heart defects include Epstein's anomaly (abnormality of the tricuspid valve), pulmonary atresia (underdevelopment of the pulmonary valve) and tricuspid atresia (complete absence of the tricuspid valve) (30).

The second broad category of CHDs is "left-sided obstruction defects." These include hypoplastic left heart syndrome (HLHS) characterized mainly by severe underdevelopment

of the left ventricle. It results primarily from atresia of the aortic valve and represents 1% of all CHD. Infants with HLHS usually die in the first month of age (30, 275, 379). Aortic coarctation is another form of obstruction defect in which the aorta is narrowed resulting in impeding flow of the systemic blood. This accounts for 10% of CHDs. Another left-sided obstructive abnormality is aortic stenosis, which is a narrowing mainly in the aortic valve leading to obstruction of blood flow from the left ventricle. It forms 5 to 10% of all CHDs and is often associated with Williams, Noonan and Turner syndromes (379). Interrupted aortic arch (IAA) (underdevelopment of the aorta) and mitral stenosis (narrowing of the mitral valve) are two other forms of left-sided obstruction defects (30, 379).

The third group of CHDs is sepation defects; these could affect the atrial septum, the ventricular septum, or the atrioventricular structure. Atrial septal defects or ASDs result from incomplete formation of either the septum primum or septum secundum. This creates a shunt between the left and right atria, thus causing mixing of oxygenated and deoxygenated blood. ASDs account for 10% of all CHDs with the secundum ASD being the most common type in children. They are often found in patients with Holt-Oram, Noonan and Ellis-van Creveld syndromes (379). Ventricular septal defects or VSDs occur in 40% of all CHDs, and are thus the most common congenital heart abnormality. These are characterized by a hole in the interventricular septum (IVS) and are classified according their location into: perimembranous, muscular, inlet and subpulmonary (379). A perimembranous VSD affects the membranous and a part of the muscular septum; this is the most common VSD in children. The muscular septum consists of three components: the inlet, the trabecular and the infundibular part. A muscular VSD is that occurring in the trabecular part of the muscular septum. Inlet and subpulmonary occur in the inlet and infundibular parts respectively (207). The hemodynamic significance of a VSD is determined by the size of the defect and the

relative pulmonary and systemic vascular resistances, which control the interventricular shunt (262). VSDs are present in patients with Holt-Oram syndrome as well as others like DiGeorge and Noonan Syndromes and can also be seen as an isolated defect. Some ASDs and VSDs have the potential of closing spontaneously after birth (275, 379). Atrioventricular septal defects (AVSDs) (also referred to as endocardial cushion defects) result from malformation of the atrioventricular canal cushions. One type of endocardial cushion defect is the "Goose-neck deformity" that can be seen on a left ventricular angiography. It is characterized by an abnormal apical positioning of the anterior mitral valve leaflet that encroaches on the left ventricular outflow tract resulting in a narrowing of its lumen and an anterior displacement of the aorta. The distance between the mitral valve and the apex (inlet distance) is abnormally shorter than that between the aortic valve and the apex (outlet distance) due to the deficiency of the IVS (23, 75, 286, 340). In a complete AVSD, communication between both atria and both ventricles is possible due to the presence of an ostium primum ASD, a VSD and a common AV valve. AVSDs account for 5% of all CHDs and are associated mainly with Down Syndrome. Isolated cases are also present as an autosomal dominant trait (275).

Other congenital defects that do not belong to any of the three broad groups include bicuspid aortic valve (BAV) and patent ductus arteriosus (PDA). Bicuspid aortic valve is the most common CHD (the second most common being VSDs), affecting 1-2% of the population with a higher male prevalence. It most likely results from fusion of the right and left valve leaflets during valve development resulting in two abnormally asymmetrical leaflets instead of three symmetrical ones. BAVs occur in isolation or in association with other congenital abnormalities mainly coarctation of the aorta, interrupted aortic arch, and VSDs. They are usually asymptotic until the third or fourth decade of the patient's life where

the valve becomes dysfunctional. The patient often then develops severe complications including valvular stenosis, regurgitation, dilation or rupture of the aorta and becomes at risk of infective endocarditis (79, 375). Throughout fetal life, a blood vessel, ductus arteriosus, connects the aorta and pulmonary artery to bypass the lungs and ensure proper fetal blood circulation. Failure of this vessel to close after birth results in what we call "patent ductus arteriosus" or PDA; thus, communication between the aorta and pulmonary artery persists resulting in mixing of oxygenated and deoxygenated blood. It occurs mostly in premature babies but is also seen in full term ones. If untreated, PDA results in mutli-organ failure including pulmonary edema and eventually congestive heart failure (40, 117).

b. Transcription factors associated with CHDs

Genetic analysis studies and sequencing of candidate genes important for normal cardiac formation in patients with CHDs have helped unravel the genetics of these diseases (figure I.6). The combinatorial interactions of various transcription factors required for their normal function aid in understanding how more than one gene could be responsible for one structural defect.

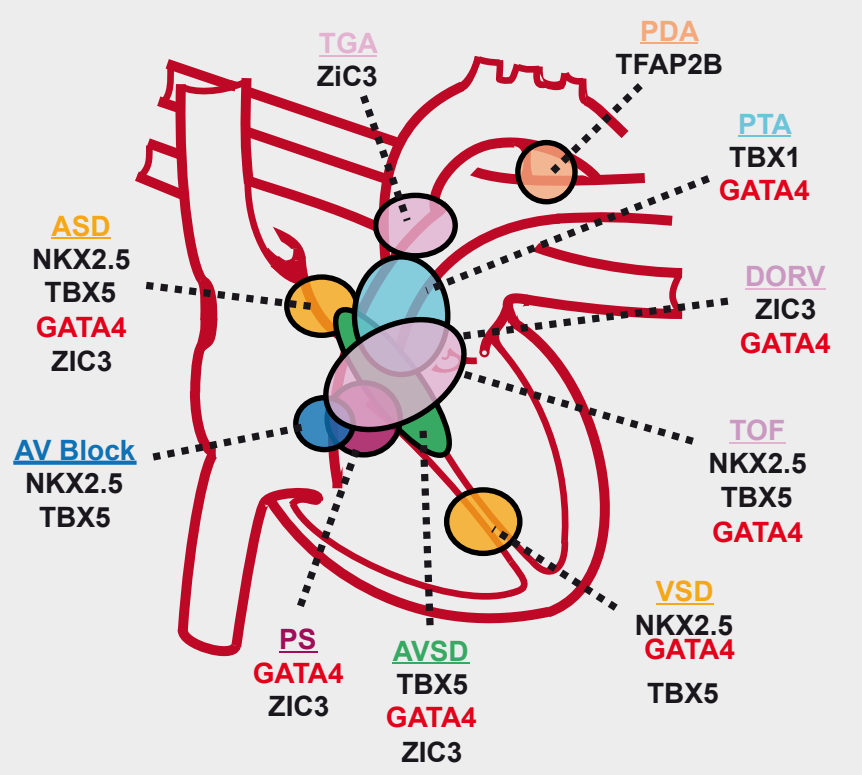

Figure I.6 Heart structural anomaly sites assoicated with transcription factor mutations. PDA: patent dutus arteriosus; PTA: persistent truncus arteriosus; DORV: double outlet right ventricle; TOF: Tetralogy of Fallot; VSD: ventricular septal defect; AVSD: atrioventricular septal defect; PS: pulmonary stenosis; AV Block: atrioventricular block; ASD: atrial septal defect; TGA: transposition of the great arteries (239).

Analyses of interacting partners, upstream regulators and downstream effectors of cardiac transcription factors help identify CHD-causing genes and understand the mechanisms of the defects in CHD patients. TBX5 is the causative gene of Holt Oram Syndrome (HOS), a complex disease characterized by ASDs, VSDs, and conduction defects. TBX5 is an upstream regulator of connexin 40, thus providing a molecular basis of the conduction defects in HOS patients (13, 173, 239). TBX1 is considered as the DiGeorge syndrome-causing gene (178, 205). NKX2.5 and GATA4 mutations have been found in patients with septal defects (16, 87); GATA4, TBX5 and NKX2.5 have thus been suggested to be CHD-causing genes through their combinatorial interactions (30, 239). Signaling defects have been linked to valvular diseases. JAG1 (the gene encoding the transmembrane

ligand of Notch receptors) and more recently NOTCH2 mutations have been linked to Alagille syndrome, characterized by liver problems, pulmonary artery stenosis and less frequently TOF (173, 199, 244). NOTCH1 is thought to be the causative gene in some cases of BAVs, aortic stenosis, HLHS, and TOF (88). Notch signaling is important for epithelial-mesenchymal transformation that is crucial for valve development (338). Noonan syndrome is linked to mutations in a number of genes that encode members of the Ras-MAPK (mitogen activated protein kinase) pathway as SHP2, RAF1, and SOS1. Noonan syndrome involves various cardiac defects as pulmonary valve abnormalities, AVSDs, and hypertrophic cardiomyopathy (89, 253, 277, 280, 331).

Finally, identifying the various regulators of heart development and understanding their mechanism of action will allow the discovery of potential cardiac disease-causing genes. Analyses of the combinatorial communications among these regulators and their interaction with the environment will help elucidate the molecular pathways on the basis of these diseases and possibly prevent their secondary health risks in the postnatal life.

B. The adult heart

The postnatal mammalian heart is a four-chambered muscular organ required to maintain the flow of blood within the pulmonary and systemic circulatory systems (figure I.7). Through constant rhythmic contraction-relaxation movements, it continuously pumps deoxygenated blood to the lungs and oxygenated blood to the rest of the body organs; thus, it is absolutely required for the distribution of oxygen and nutrients throughout the whole body. Its continuous need of high amount of energy makes it rely on fatty acid oxidation for fuel, rather than glucose oxidation, which most other tissues use as a source of energy. Fatty acid oxidation is however entirely dependent on oxygen and therefore the heart switches to

glycolysis under low-oxygen conditions, as is the case with ischemia or cardiac hypertrophy. The heart is also capable of metabolizing other energy sources like lactate and ketone bodies if present in adequate amounts (52). The machinery used by the heart to generate energy is therefore adjusted according to metabolic demands so that this restless organ continuously performs its indispensible function.

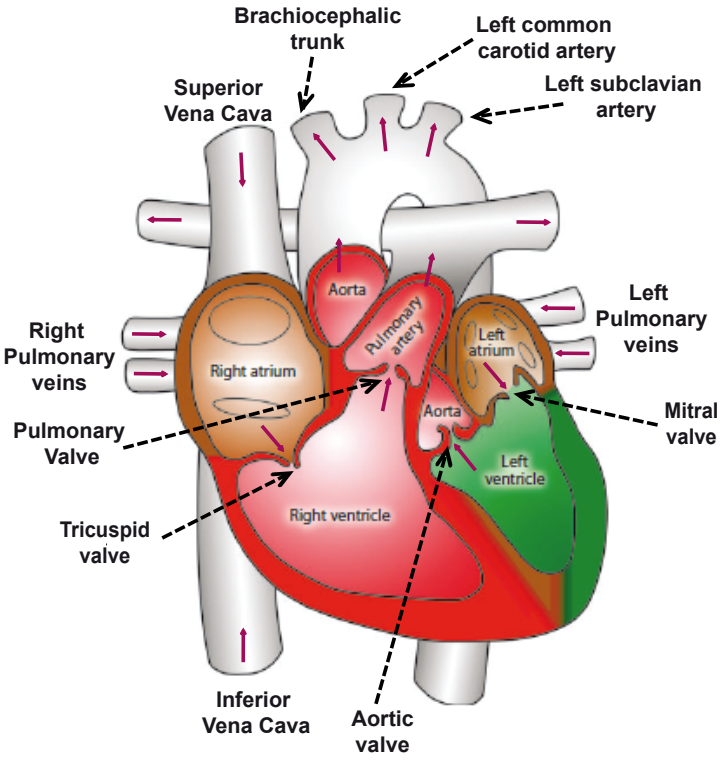

Figure I.7 Structure of the adult mammalian heart. Small purple arrows indicate direction of blood flow. Adapted from Musunuru et al, 2010 (228).

i. Cellular composition and gross anatomy of the postnatal mammalian heart

The heart is a heterogeneous tissue composed of different cell types that act in a well-coordinated dynamic manner to regulate the response of the heart to various stimuli and maintain its electrical and biochemical nature as well as its three dimensional structure. Myocytes (the contractile cells of the heart) and fibroblasts make up the majority of the

cellular composition (~ 56% and 27% by number respectively in the adult murine heart). Fibroblasts play a major role in myocardial repair and the formation of scar tissue following myocardial infarction. Phagocytes are found between blood vessels and fibers to engulf debris. Nerve fibers innervate the heart and act with the conduction system to regulate contraction. Endothelial and vascular smooth muscle cells (7% and 10% respectively in the mouse) are also present in this highly vascularized organ (12, 193).

Aside from the obvious size difference and a few discrepancies in the overall shape, a full-grown mouse and human heart are very similar anatomically, thus rendering the mouse a legitimate model for the human heart. In humans, the average weight of an adult heart is 250-300g with 60-70 beats/min as compared to the 0.2g adult mouse heart of 500-600 beats/min (358). The human heart is pyramidal in shape with a flat dorsal plane since it rests on the diaphragm unlike the mouse heart whose free movement within the pericardial cavity gives it a more ellipsoidal shape. In the mouse, the atria are relatively tiny as opposed to the human strikingly salient atrial structures. Moreover, owing to the longer gestational period from the time the heart completes septation to fetal delivery (7 months), the human heart is almost fully mature at birth where as in the mouse, some of the developmental processes to achieve full maturation are still going on in the neonatal period (308, 358). In overall structure, the adult mammalian heart is composed of four chambers, two atria and two ventricles separated by the interatrial and interventricular septum (IAS and IVS) respectively. The IVS is muscular in nature and is massively thick in humans, almost thicker than the left ventricular wall; where as in mice, it is compact and not as thick. An atrioventricular septum (AVS) is located between the two septae, being a thin fibrous structure in humans and a relatively thick muscular structure in the mouse. Two valves are located at the atrioventricular junctions, a bicupsid mitral valve separating the left atrium from the left ventricle and a

tricuspid valve at the right atrium-right ventricle junction. In humans, four pulmonary veins drain into the left atrium; however, in the mouse heart, the pulmonary veins converge and empty into a single opening in the left atrium (358). The left superior caval vein (LSCV) of the human heart regresses at some point during cardiac morphogenesis and the remaining portion becomes the coronary sinus. In the mouse heart, on the other hand, the LSCV persists in the postnatal heart; persistence of the LSCV in humans is deemed a congenital defect (356, 358).

Finally, despite the differences listed above, the remarkable similarity in the anatomy of the murine and human hearts renders the mouse a very good model for human heart disease. A considerable amount of transgenic mice developed so far have allowed great progress in understanding the genetics of a good number of congenital heart problems. Thorough examination of the minimal structural differences between the two species allows more accurate deductions of the findings obtained from these mouse models.

ii. Cardiac Hypertrophy

a. Types of cardiac hypertrophy

Cardiomyocytes preserve their ability to divide for a short period after birth, after which, up until recently, the heart is considered a terminally differentiated organ. Cardiac growth then occurs mainly by increase in size of almost a limited number of cardiomyocytes and by hyperplasia of nonmuscular cells (250). This response of the heart to relieve temporary stress, for example the stress caused by pregnancy or long-term exercise, is termed physiologic hypertrophy. Under this condition, resting heart rate is decreased at the same time that stroke volume is elevated; thus, the heart is more efficient in pumping blood. This type of hypertrophy is therefore beneficial to the heart (290). Cases of sudden cardiac

death are therefore very rare among athletes as compared to the general population, unless they are occurring as a result of an undiagnosed cardiac defect that gets aggravated with excessive exercise (52, 82). Physiologic hypertrophy is usually characterized by a uniform increase in chamber size accompanying the growth of the ventricular wall and IVS, termed eccentric hypertrophy. Sarcomeres are added in series and in parallel to increase the length and width of the cells (113).

Cardiac hypertrophy can therefore be considered as an adaptive response of the heart to meet the hemodynamic demand; yet again, if the stress persists and becomes acute the heart then undergoes maladaptive changes that could result in cardiac dysfunction and ultimately heart failure, a process termed pathologic hypertrophy (101). Conditions of chronic hypertension and aortic stenosis usually result in concentric hypertrophy in which the ventricular wall and IVS thicken leading to a decreased chamber volume. The cardiomyocytes grow more in width than in length. This is usually accompanied by fibrosis and may or may not lead to cardiac dysfunction. In cases of dilated cardiomyopathy or myocardial infarction on the other hand, the heart undergoes an eccentric form of pathologic hypertrophy complemented with cardiac dilatation. Sarcomeres are predominantly added in series to individual cardiomyocytes. Extensive fibrosis, cardiomyocyte death and cardiac dysfunction accompany this form of hypertrophy that could ultimately lead to heart failure (figure I.8) (113).

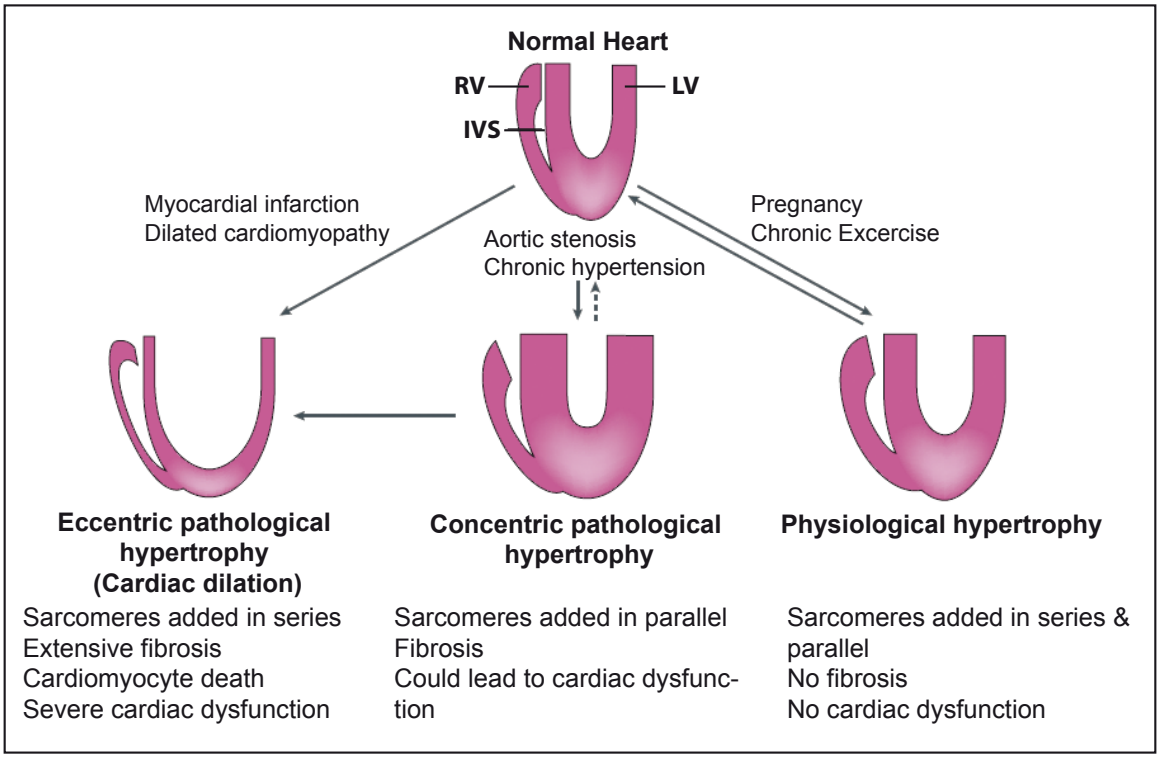

Figure I.8 Types of Cardiac Hypertrophy. RV: right ventricle; LV: left ventricle; IVS: interventricular septum. Adapted from Heineke and Molkentin, 2006 **(113).**

The pathological process of detrimental cardiac hypertrophy can be divided into two stages: a compensation phase and a decompensation phase. In the compensation stage, the cardiomcyotes grow in size and there is an increased deposition of collage and other extracellular matrix components, a process termed fibrosis. The heart walls thus thicken in an attempt of the heart to accommodate the increase in stress. However, as fibrosis progresses and more collagen fibrils are deposited in the interstitial area, the cardiac muscle becomes stiff, impairing the heart function. This represents the decompensation phase of the pathological process. When the heart can no longer pump enough blood to meet the systemic demand, the patient goes into heart failure (52).

b. Molecular mechanism of cardiac hypertrophy

For a full expression of the hypertrophied cardiomyocyte phenotype, the protein synthesis machinery is upregulated, and gene expression profile is reprogrammed (143). Hemodynamic stress first activates the expression of the early response genes, c-jun, c-fos and c-myc, followed by re-induction of the "fetal gene program," Nppa, Nppb, and the genes encoding fetal isoforms of sarcomeric proteins, the thick filament protein β-MHC (myosin heavy chain) and the thin filament protein skeletal α-actin (143, 369). On the contrary, the adult isoform α-MHC and the calcium regulatory protein sarcoplasmic reticulum calcium ATPase2 are down regulated in response to stress. The transcription factors, GATA4, MEF2 and NKX2.5, known to regulate β-Mhc expression during embryonic development, are also upregulated in cardiac hypertrophy (143, 369).

The two main activators of cardiac hypertrophy are biochemical stress and neurohormonal factors (184, 299). Cytoskeletal and sarcomeric proteins and stretch-sensitive ion channels are thought to mediate the biochemical stress; yet, how this is sensed by the cell and transduced into a hypertrophic response remains elusive (113, 184, 299). Neurohormonal factors known to induce cardiac growth include catecholamines, endothelin-1, angiotensin II, and IGF-1 (insulin-like growth factor-1) (113). Several neurohormonal factors activate receptor tyrosine kinases (RTK) to mediate cardiac hypertrophy, albeit, the majority do so through G-protein coupled receptors like $G_{\alpha q}$. The net outcome of the activation of many of these receptors is the mobilization of stored calcium. Increase in intracellular calcium activates mitogen-activated kinases (MAPKs), calcium-dependent protein kinases, or phosphatases. This then results in the activation of ERK1/2 (extracellular-signal regulated kinases 1/2), the CaMKII/HDAC (histone deacetylase) pathway, and the calcineurin-NFAT

(nuclear factor of activated T-cells) pathways (184, 299). Several transcription factors are activated in response to calcium. These include GATA4, NFAT and MEF2 and in turn regulate the expression of various cardiac genes like those encoding ANF, desmin, and SERCA2 (299). $G_{\alpha q}$ coupling proves to be an important step in the pathological hypertrophy process and therefore serves as a therapeutic target for treatment of pathological cardiac hypertrophy (113).

iii. Genetic and acquired cardiomyopathies

Cardiomyopathies are defects of the heart muscle that can either be primary due to a genetic defect or occurring secondary to other cardiovascular diseases. Examples of acquired cardiomyopathies are those occurring as a result of atherosclerosis (or inflammation of the arteries), rheumatic heart disease (leading to fibrosis of the heart valves), hypertension, diabetes and obesity (203). Cardiomyopathies of genetic basis include the previously described congenital heart diseases, hypertrophic cardiomyopathies, dilated cardiomyopathies, restrictive cardiomyopathies and arrhythmogenic cardiomyopathies (306). Hypertrophic cardiomyopathy (HCM) is the most common form of sudden cardiac death in adolescents and children (229). It is characterized by hypertrophied left ventricle with a small lumen, thickened IVS and enlarged atria (229). Diastolic dysfunction is common in HCM and 25% of the patients have OFT obstruction (306). Mutations in several genes encoding contractile proteins have been shown to cause HCM with an autosomal dominant inheritance pattern (229). Dilated cardiomyopathy (DCM), the most common form of cardiomyopathy, is characterized by impaired systolic function and dilation of either only the left ventricle or both ventricles. It can be caused by ischemia and alcohol abuse; albeit, in many cases, the cause is unknown and DCM is then referred to as idiopathic cardiomyopathy

(306). Restrictive cardiomyopathy (RCM), on the other hand, is the least common form of cardiomyopathies. Systolic function is preserved in RCM; yet, the diastolic function is impaired. The ventricular walls are stiffened and the diastolic volume of the LV or both ventricles is reduced. The reduced compliance of the ventricles in RCM can ultimately result in atrial dilatation (306).

Cardiac arrhythmia is a defect in the electrical activity of the heart resulting in a very slow or very fast regular or irregular heart beat. It can arise from the atria, as in the case of atrial fibrillation, or from the ventricles. Aging, hypertension, hyperthyroidism, and valve problems can lead to atrial fibrillation. Once diagnosed, heart rate controlling drugs and anticoagulants are administered to manage the symptoms and prevent stroke (203). Ventricular arrhythmias are associated with heart attacks and are important causes of sudden cardiac death. Electrical defibrillation is needed to restore normal cardiac activity in this case and early diagnosis is crucial (203, 319). Several arrhythmia-susceptibility genes that encode ion channels have been identified so far, thus providing a better knowledge of the molecular pathogenesis of the disease (229).

iv. Echocardiography measurement of cardiac remodeling and function

A large number of genetically modified mice models have been developed so far to mimic human cardiac diseases. Assessing cardiac structure and function in these models is crucial to further characterize these heart abnormalities. Echocardiography is a routinely used technique in humans to assess cardiac physiology. This technique has been scaled down for mouse use and is now the most powerful tool to study cardiac physiologic changes in intact genetically modified mice (322).

This tool allows 2D-guided M-mode measurements of left ventricular (LV) overall structure and function. LV diastolic and systolic dimensions (LVEDD: LV end-diastolic dimension; LVESD: LV end-systolic dimension), LV posterior wall thickness at both diastole and systole (LVPWd and LVPWs respectively), and the interventricular septum thickness at diastole and systole (IVSd and IVSs) are determined. The LV mass is then calculated from these indices using a specific formula that have been demonstrated to correlate well with overall morphometric measurements (86, 322). For assessment of global LV function, indices like fractional shortening (FS), defined as the fraction of diastolic dimension lost during systole, and ejection fraction (EF), which represents the fraction of blood ejected by the LV in systole, are calculated. Additionally, cardiac output (CO), referring to the volume of blood pumped per minute, stroke volume (SV), aortic flow and aortic constriction velocities are obtained using another form of echocardiography, called Doppler echocardiography. This tool is widely used in humans and rodents to determine diastolic function (322).

Echocardiography is currently one of the most important, non-invasive screening techniques used by researchers to assess cardiac structure and function in human cardiac disease mice models.

In short, the heart is a highly complex and perhaps one of the most vital organs in the human body. Its crucial role in supplying blood and nutrients to all body organs is imperative to orchestrate physical and emotional health in the individual. Defects in heart functions cause numerous problems that could ultimately lead to patient death. Unfortunately, even though a large number of cardiac abnormalities are preventable, cardiac defects remain the leading cause of death throughout the world.

C. Cell cycle proteins and the cardiomyocyte cycle

i. Cell cycle proteins

The eukaryotic cell cycle, from the conclusion of one cell division to the start of the next, is divided into four distinct phases: Gap1 phase (G1), S phase (where DNA replication occurs), gap2 phase (G2), and M phase or mitosis (figure I.9). In a typical normally dividing cell, G1 phase takes around 12hrs, S phase is around 6 to 8 hrs, G2 phase is around 3 to 6 hrs, and M phase lasts about 30 min. Cells that are metabolically active but have stopped dividing either temporarily or indefinitely are said to be in a quiescent or resting state termed Go. This state is typical for "terminally" differentiated cells like neurons, cardiomyocytes, and myotubes (294).

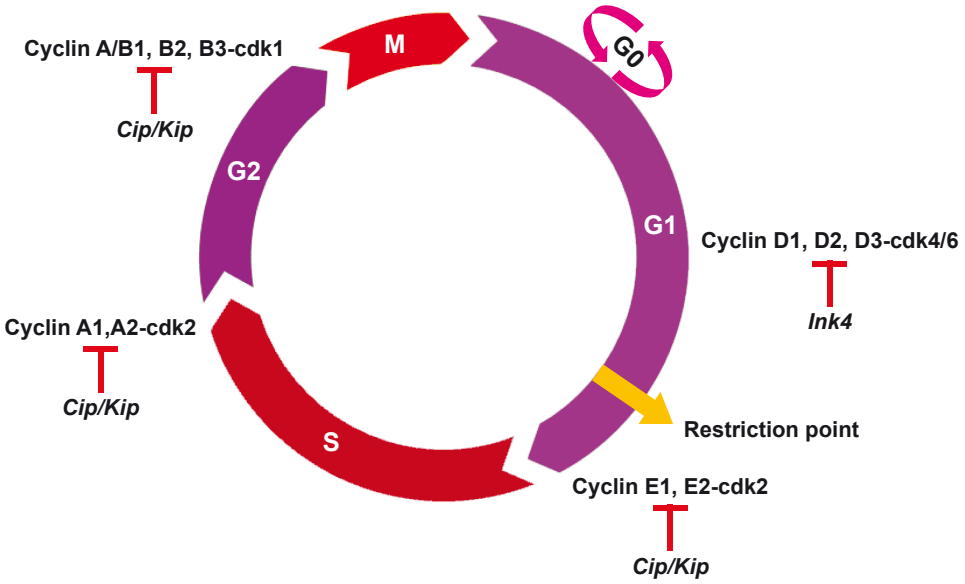

Figure I.9 Mammalian cell cycle. The phase-corresponding cyclins and CDKs are indicated. Cyclin Ds-CDK4/6 are important for the G1 phase progression and G1-S transition along with Cyclins E and A-CDK2. The cell cyclin inhibitors at each phase are also shown.

Various protein complexes consisting of a regulatory subunit called the cyclin and their catalytic partner, the cyclin-dependent kinase or CDK, are differentially expressed at

different stages of the cell cycle. G1 cyclins/CDK complexes respond to extracellular growth stimuli to instigate G1 phase progression and DNA replication. Cyclin Ds along with their catalytic partners CDK4/CDK6 are activated in early G1 and accumulate progressively as cells proceed in G1 phase. Mitogen-stimulated signal transduction pathways activate cyclin D gene transcription and translation, their assembly with their CDK partners, and their import into the nucleus (297). Activated cyclinD/CDK complexes promote cell cycle progression in part by phosphorylating pRb protein. pRb in its hypophosphorylated form binds to and inhibits the transcription initiation factor E2F. As cells progress in G1, pRb is hyperphosphorlated by cyclin D/CDK, thus releasing E2F, which then binds to DP-1 family members to activate the transcription of various S-phase required genes including cyclin E and cyclin A. Cyclin D/CDK complexes allow G1-S phase progression also by sequestering members of the Cip/Kip cell cycle inhibitors including p21 and p27; hence, preventing them from blocking cyclin E/CDK2 function (297, 298).

Three cyclin D members exist in mammals (D1, D2 and D3) and are differentially expressed in different cell types. Individual cyclin D knockout mice are viable albeit they present with focal abnormalities (294, 298). Cyclin D1 null mice are smaller in size and present with neuronal defects, hypoplastic retina and abnormal breast tissue development during pregnancy (74, 305). Female cyclin D2 knockout mice are sterile and the males have hypoplastic testes. Postnatal development of the cerebellum and expansion of B-cells are also affected as a result of cyclin D2 inactivation (123, 164, 304). Loss of cyclin D3 in turn compromises early maturation of lymphocytes in the thymus (303). Once DNA replication starts, cyclin D/CDK complexes are no longer required until the cell completes the first round of division and re-enters G1 phase. Removal of the mitogenic signal halts cyclin D synthesis and leads to their destabilization and rapid degradation. If this occurs in early G1,

proliferation stops and the cells go into G0 phase. However, if this happens after a certain point in G1 phase called the G1 restriction point, then the cell cycle progression occurs despite the external growth factors withdrawal. This restriction point is thought to be lost in a lot of human cancers (256, 294).

Following cyclin D expression, cyclin E proteins start to appear in the nucleus in early G1 and their expression reaches maximum at the G1/S phase and declines thereafter. Two cyclin E isoforms exist in mammals (E1 and E2) and they form a complex with their catalytic partner, CDK2 (63, 149). Cyclin E/CDK2 complexes are ubiquitously expressed in all cell types and they have similar functions to cyclin D/CDK in phosphorylating Rb, albeit at preferentially different sites, and targeting p27 (298). However, they inhibit p27 by phosphorylating it on a single residue (Thr 187) leading to its targeting for ubiquitination and subsequent proteasomal degradation rather than by sequestering it (345). Cyclin D and E are thought to act in cooperation to inhibit pRb (187). Then again, cells that lack pRb function still require cyclin E to proceed into S phase, thus demonstrating a role of cyclin E proteins that differs from that of cyclin Ds (245). Moreover, cyclin E/CDK2 complexes are not only important for G1 regulation, but are also reported to phosphorylate another set of targets that have a direct role in cell duplication (373).

Cyclin E/CDK2 activity declines in early S phase as a result of cyclin E degradation whereby cyclin E gets phosphorylated by CDK2 itself and GSK-3β there by targeting it for ubiquitination and proteasomal degradation (48, 357, 360). During this phase, cyclin A, whose expression is low in G1, increases steadily from S to G2 phase, and finally gets degraded in M phase (208, 265). Cyclin A is thought to play a role in G1-S transition, S phase progression via its association with CDK2, and G2-M transition by forming a complex with cdc2 (CDK1) (73, 265, 284). G2/M transition and M phase entry are regulated by cyclin

B/cdc2 complexes. Cyclin B expression begins in S phase, peaks throughout G2 with the highest associated activity seen at G2/M boundary and gets rapidly degraded at anaphase. Three cyclin B isoforms exist in mammals (B1, B2 and B3) (267). Cyclin B1 and B2 are cytoplasmic during interphase and get translocated to the nucleus at the G2/M shift right before breakdown of the nuclear envelope. Cyclin B3 is nuclear all through interphase, associates with both CDK2 and cdc2 and is structurally similar to cyclin A (84, 85, 266).

Cyclin levels are mainly regulated by two posttranslational modifications: phosphorylation and ubiquitination. For instance, as the cells enter S-phase, GSK-3β enters the nucleus and phosphorylates cyclin D1 at the conserved Thr-286 amino acid in the C-terminal. Thr-286 phosphorylated cyclin D1 is recognized by the nuclear exportin Crm1, which translocates the cyclin D1-CDK4 complex to the cytoplasm where it gets degraded by a ubiquitin-proteasome mechanism (4, 91). A similar process was also observed in cardiomyocytes during cardiac hypertrophy (104). More recently, micro RNAs (miRNA) were reported to control cyclin levels by targeting their mRNAs for degradation. Cyclin D1 is a direct downstream target of miR-34a (321); miR-34a arrests proliferation and regulates the G1-S transition of the cell cycle (111, 330). Cyclin D2 is a downstream target of miR-133a and has therefore been suggested to be an effector of miR-133a in the mouse heart. Loss of both miR-133a1 and miR-133a2 in mice is lethal embryonically or perinatally due to ventricular septal defects with 50% penetrance (181). Cyclin protein levels are therefore tightly regulated to ensure proper progression of the cell cycle.

In case of damage, the cell has the ability to stop proliferation in G1, S or G2 phase via surveillance pathways called checkpoints. These are required to ensure the proper completion of one phase of the cell cycle before proceeding to the next (70, 108). They function by delaying the activation of a specific cyclin/CDK complex at a certain stage

during the cell cycle to allow for damage repair before progressing to the subsequent phase. In the alternative case where the damage cannot be repaired, the cell can then undergo apoptosis that may or may not be accompanied by growth arrest or it could go into a permanent G0-like phase, senescence (70, 107).

ii. Cycling mechanism of the cardiomyocyte

During fetal life, cardiomyocytes divide to increase in number; yet, soon after birth their replication potential slows down dramatically and the heart then responds to growth signals by increase in size of almost a fixed number of cells. Rodent cardiomyocytes exit the cell cycle in the first few days after birth. Human cardiomyocytes, on the other hand, continue to proliferate for the first few months after birth (160). Before they complete cell cycle withdrawal, cardiomyocytes undergo a final round of incomplete cell division. DNA replication followed by nuclear division occurs without cytokinesis (acytokinetic mitosis), resulting in binucleated cells with dipoloid (2n) DNA content. The amount of binucleated cardiomyocytes is estimated to be 85-90% in rodents (171, 312) and 25% in humans (248). Most human cardiomyocytes, on the other hand, go through a final round of DNA synthesis that is not followed by nuclear division or cytokinesis resulting mostly in mononucleated cells with tetraploid (4*n*) DNA content or sometimes even higher as in the case of hypertrophy (2, 248). Some non-mammalian species like the zebrafish can fully regenerate their heart after amputation of up to 20% of their ventricle (271). The regenerated cardiac cells arise from differentiated cardiomyocytes that undergo limited dedifferentiation followed by proliferation (128). Moreover, a population of cardiomyocytes in the zebrafish ventricular wall appear to contribute to cardiac muscle regeneration by inducing expression of the cardiac transcription factor, GATA4 (146).

Genes encoding cell cycle proteins (cyclin Ds, A, B1 and E), cyclin dependent kinases (cdc2, CDK2, CDK4, and CDK6) as well as those required for DNA replication like PCNA (proliferating cell nuclear antigen) are highly expressed in the developing heart (3). Cyclin D expression in mice hearts starts very early on at embryonic day E10.5 (234, 282). Individual cyclin D knockout mice are viable and do not develop heart abnormalities; however, those lacking all three D-type cyclins die by mid-gestation due to heart defects and hematopoietic problems. Hearts from mutant embryos display thin ventricular walls as well as large VSDs (153). Consistently, $Cdk2^{-/-}$.$Cdk4^{-/-}$ double knockout mice exhibit reduced global size, enlarged atria and thin ventricular walls and die by embryonic day 15. These mutants have reduced proliferation in some regions of the heart (18). The results from these studies point to an essential role of cyclinD/CDK4 in normal heart development (3).

Cardiomyocyte exit from the cell cycle is accompanied by a decrease in the expression levels of cyclins and Cdks and the upregulation of CDK inhibitors, like p16 and p21 (35, 130). Moreover, the retinoblastoma protein Rb is expressed at very low levels in embryonic cardiomyocytes. It is upregulated in the neonatal heart and becomes the predominant pocket protein in adult cardiomyocytes (127, 191). Embryonic stem cells lacking Rb exhibit hindered expression of cardiac-specific transcription factors accompanied by delayed cardiomyocyte differentiation. Another pocket protein member, p130 is also expressed in the adult myocardium. Mice lacking both Rb and p130 display elevated heart to body weight ratio with an increased number of mitotic nuclei in the heart, thus pointing to a role of these proteins in cardiomyocyte cell cycle exit and differentiation (3, 191). Other gene expression profiles are altered as cardiomyocytes exit the cell cycle and undergo "terminal" differentiation. A switch from fetal gene isoforms to adult ones occurs as in the

case of β-myosin heavy chain and skeletal actin that are replaced by the adult forms α-MHC and cardiac actin, respectively (3, 320).

Increased cyclin D expression is reported following cardiac hypertophy (36, 172, 243, 327). A transient upregulation of cyclin D2, cyclin D3, CDK4 and CDK6 protein levels was present in the hearts of rats at the first stages of pressure-overload induced hypertrophy (172). In another study where Angiotensin II was used to elicit hypertrophy, augmented cyclin D-CDK levels were accompanied by increased Rb phosphorylation; overexpressing the cdk inihibtors p21 and p16 inhibited the hypertrophy response (243). Increased transcription of tRNA genes by RNA polymerase III (Pol-III) is usually associated with cardiac hypertrophy. The CD-cdk-Rb pathway regulates Pol-III transcription. During G0-G1 phase, hypophosphorylated Rb binds to and represses TFIIIB (Pol-III specific transcription factor); phosphorylation of RB by cyclin D-CDK relieves this repression (292). Moreover, phosphorylation of Rb by cyclin D2 was important to derepress the Pol-III gene and allow the hypertrophy response to transaortic constriction (6). These studies point to an important role of cyclin D-CDK in regulating the growth of postnatal cardiomyocyte and the development of cardiac hypertrophy.

Several transcription factors have been shown to regulate the cardiomyocyte cell cycle. Loss of Tbx5 in *Xenopus* causes reduced cardiomyocyte proliferation with a G1/S phase cell cycle arrest; overexpression of Tbx5 on the other hand increases the index of cardiomyocyte mitosis (92). Moreover, a report from our lab indicates an alternative splicing regulation of Tbx5 generating a long isoform involved in inducing proliferation and cardiac growth and a shorter isoform associated with growth arrest (90). Other transcription factors associated with cardiomyocyte proliferation include GATA4 (described earlier), Myc

transcription factors (members of the basic helix-loop-helix-leucine zipper bHLHZ), and the hypoxia-inducible-factor-1α (HIF-1α) (3).

In short, postnatal cardiomyocytes have very limited regenerative capacity. More research is required to understand the basis of their "terminal differentiation" in order to open more doors for potential cardiac therapy.

iii. Heart Regeneration

a. Self-renewal potential of adult cardiomyocytes

Not so long ago, the notion that mammalian cardiomyocytes are terminally differentiated cells has been challenged. Several reports suggest that cardiac mass increase in pathologically hypertrophied human hearts (more than 450g) is accompanied by a linear increase in cardiomyocyte nuclear number (94, 160). Bergmann *et al* calculated the age of cardiomyocytes in humans using DNA integration of ^{14}C produced during nuclear bomb testing in the Cold War. They suggest that cardiomyocytes renew yearly at a rate of ~1% in a 20-year old individual and 0.3% in a 75-year old; thus, over a human lifespan, around 45% of cardiomyocytes are renewed under normal conditions (17). A second report states that cardiomyocytes are renewed at a rate of 22% per year. In this study, Kajstura *et al* used postmortem hearts from cancer patients treated with iododeoxyuridine (IdU), a thymidine analogue that incorporates into nascent DNA and sensitizes cells to radiation therapy (131). The rate of cardiomyocyte proliferation reported in this study is as high as that of non-cardiomyocytes and contradicts the fact that most human cardiomyocytes are polyploidy by stating that 80% are diploid; thus, it is probably overestimated (160). Several groups have also stated that one or more populations of cardiac progenitor cells (CPCs) exist in the adult heart. Based on their expression of the surface markers c-kit or sca-1, or on their

cardiosphere-forming property or their fluorescent dye efflux ability, various populations were isolated (160). More recently, Porrello *et al* reported that the mammalian cardiomyocytes retain the ability to proliferate and regenerate the heart following partial surgical resection for a short period after birth (270).

These reports clearly show that the adult heart retains some degree of regeneration. However, be it from cardiac progenitor cells or from other sources, any injury to the adult heart results in a stiffer, less contractile heart that could ultimately go into failure. Thus, the intrinsic activity of the adult mammalian heart is not sufficient to restore normal function of diseased hearts as observed in the zebrafish; thus, various approaches to replace dead myocytes are being studied.

b. Therapeutic approaches to drive myocardial repair

Over the last ten years or more, researches geared their attention to interventional approaches for cardiac repair. Cellular reprogramming, stem cell therapy, inducing differentiated cardiomyocytes to proliferate, and tissue engineering are various strategies used by scientists in attempts to repair damaged hearts.

Investigators make use of the cardiac progenitor cell population(s) existing in the adult mammalian heart for stem cell therapy attempts. Approaches include mobilizing endogenous progenitors to the site of injury or expanding them *in vitro* and their transplanting into the damaged myocardium. Better understandings of the signals important to induce these progenitors to proliferate and migrate and whether these grafts will successfully fuse with the existing myocardium are required to get the best out of these trials (5, 160, 310). Other stem cell sources include bone-marrow-derived cells and marrow-derived stromal cells. These are reported to induce tissue repair via a paracrine mechanism

(194, 209, 227, 251). Embryonic stem cells (ESCs) and the induced pluripotent stem cells (iPSCs) are also a potential indefinite supply of cardiomyocytes. Better knowledge of the signaling and transcriptional pathways of cardiac differentiation will allow optimal uses of these sources for cardiac therapy (5, 160). More recently, the epicardium has arisen as a source of progenitor cells in the heart and a potential target for cardiac regeneration; yet, this still requires further investigation (39, 45, 387).

Alternative approach to stem cell therapy is the induction of mature cardiomyocytes to proliferate and repopulate the damaged tissue. Recent evidence shows that postnatal cardiomyocytes could be driven to proliferate (37, 72, 174, 191, 328, 361, 389). Tamamori-Adachi *et al* reports that nuclear import of cyclin D1/cdk4 promotes post-mitotic cardiomyocytes to enter the cell cycle and divide (328). Cultured cardiomyocytes are induced to proliferate upon adenoviral delivery of cyclin D2. Inhibition of the signaling molecule p38 mitogen-activated-protein (MAP) kinase promotes post-mitotic cardiomyocyte proliferation (72). Moreover, compound heterozygous mice for a null and a dominant negative allele of the tyrosine kinase receptor for stem cell factor, c-kit, exhibit cardiomyocyte hyperplasia and improved left ventricular function when subject to pressure overload (174). Cardiac-specific overexpression of both cyclin A2 and cyclin D2 enhances cardiac function following injury with a reduction of infarct size as a sign of regeneration in the case of cyclin D2 (109, 259, 361). Hence, cell cycle approaches to drive myocardial repair could provide great hope for heart failure therapy (389).

A different attempt for cardiac therapy is the reprogramming of fibroblasts, particularly cardiac fibroblasts, to cardiomyocytes. No master gene regulator of the cardiac muscle has been identified so far. Very recently, Srivastava's and Olson's groups reported a combination of three critical regulators of heart development (MEF2C, GATA4, and TBX5)

capable of inducing the reprogramming of cardiac fibroblasts into cardiomyocyte-like cells. The induced cells have a gene expression profile very close to that of cardiomyocytes and possess cardiomyocyte functional properties such as beating spontaneously. In Olson's s study, HAND2 transcription factor was also required (124, 311). The reprogramming is a direct event and does not pass through a dedifferentiated precursor state. Moreover, GATA4 and TBX5 are also involved in direct differentiation of mouse mesoderm into cardiomyocytes (323); thus, a similar mechanism could exist between these two processes. On the other hand, another group reported an alternative method to reprogram mouse embryonic fibroblasts into beating cardiomyocytes using the "Yamanaka factors" KLF4, OCT4, c-MYC, and SOX2 (68). Fibroblasts are involved in scar-formation following cardiac injury. Reprogramming them into functional cardiomyocytes is challenging yet provides an amazing potential regenerative approach for cardiac therapy following ischemia.

Final approach to cardiac therapy is tissue engineering; though, this strategy is less extensively studied. One group engineered heart tissue from rat neonatal cardiomyocytes and implanted them on infracted hearts of immune-suppressed rats. One month after implantation, hearts receiving the engineered tissue showed better contractile function and improved conduction velocities across the infarct, suggesting successful connection of the contractile graft to the surrounding native myocardium (391). Another study indicates that cell sheets generated from cardiac progenitor cells and transplanted onto infracted myocardium enhance cardiac function by promoting neovascularization and inducting cardiogenesis (378).

The research of cardiac therapy has witnessed considerable advances in a short period of time bringing great hope to patients with heart problems. However, a lot is yet to be learned before heart regeneration becomes clinically possible.

D. Research Plan

i. Objective

The main objective of this thesis is to identify proteins that interact with GATA4 and can cooperate with it in cardiac cell proliferation.

ii. Rationale and hypothesis

The complex process of heart development involves coordinated differentiation and proliferation achieved through combinatorial interactions between transcription factors and cell cycle proteins. Deregulation of either process leads to cardiac disease. GATA4 is a central regulator of cardiac development and plays important roles in the myocardium and the endocardium. We hypothesize that GATA4 cooperates with distinct cofactors to regulate specific genetic programs of proliferation or differentiation. We also hypothesize that interaction between cell cycle proteins and GATA4 underlie physiologic heart growth. Most of the work so far dealt with the role of GATA4 in differentiation. GATA4 also has a critical role in cellular proliferation and hypertrophy but the molecular pathways and effectors underlying these GATA4-dependent processes are still poorly understood.

iii. Specific aims and methodology

We will use combination mouse genetics and cell and molecular biology techniques to:

a- Study the relationship between GATA4 and cyclin D2 in cardiomyocytes.

b- Test the role of KLF13, a GATA4 cofactor, in cardiac cell proliferation.

In the first two chapters, we report a novel cross talk between cyclin D2 and GATA4, and in the third, we document the role of KLF13 in endocardial cell proliferation. The data

presented in this thesis identify novel insights for understanding cell specific effects of cyclin Ds and uncover a new role for KLF transcription factors in heart formation.

II. Chapter I: Cyclin D2 Rescues Size and Function of GATA4 Haploinsufficient Hearts

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Author Contribution:

In the present manuscript, I contributed to almost all of the analyses of the GATA4$^{+/}$.CD2 mice (figures II.4 till II.6) as well as the work generating figures II.1F, II.2D, II.2E, and II.3B. The echocardiography results were performed by my colleagues in the lab. The cyclin D2 transgenic mice were generated by previous members of the lab. I also wrote the manuscript.

Cyclin D2 Rescues Size and Function of GATA4 Haploinsufficient Hearts

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Running Head: CD2 rescue of GATA4 haploinsufficient hearts

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A. Abstract

Transcription factor GATA4 is a key regulator of cardiomyocyte growth and differentiation and 50% reduction in GATA4 levels results in hypoplastic hearts. Search for GATA4 targets/effectors revealed cyclin D2 (CD2), a member of the D-type cyclins (D1, D2, and D3) that play a vital role in cell growth and differentiation, as a direct transcriptional target and a mediator of GATA4 growth in postnatal cardiomyocytes. GATA4 associates with the CD2 promoter in cardiomyocytes and is sufficient to induce endogenous CD2 transcription and to dose-dependently activate the CD2 promoter in heterologous cells. Cardiomyocyte specific overexpression of CD2 results in enhanced postnatal cardiac growth due to increased cardiomyocyte proliferation. When these transgenic mice are crossed with Gata4 heterozygote mice, they rescue the hypoplastic cardiac phenotype of $Gata4^{+/-}$ mice and enhance cardiomyocyte survival and heart function. The data uncover a role for CD2 in the postnatal heart as an effector of GATA4 in myocyte growth and survival. The finding that postnatal upregulation of a cell cycle gene in GATA4 haploinsufficient hearts may be protective opens new avenues for maintaining or restoring cardiac function in GATA4-dependent cardiac disease.

Keywords: Hypertrophy, apoptosis, cardiomyocyte proliferation, transgenic mice

B. Introduction

D-type cyclins (Cyclins D1, D2, and D3) are G1 cyclins that play a vital role in differentiation and cell cycle regulation (298). In response to mitogenic signals, cyclin Ds were shown to associate with the cyclin dependant kinases cdk4 and cdk6, phosphorylate the tumor suppressor retinoblastoma protein Rb along with the family members p107 and p130, and control cell cycle progression (135, 298). However and despite compelling evidence from *in vitro* studies on the role of D-type cyclins in the cell cycle, *in vivo* work indicated that during embryonic development, CD-cdk4 and CD-cdk6 complexes are not required for proliferation of the majority of cell types (153). Mice embryos lacking all three D-type cyclins are viable and relatively normal at embryonic day 13.5 but later develop cardiac abnormalities including severely thinned walls and ventricular septal defects as well as severe anemia leading to embryonic death (153, 154). These observations together with the data from the cdk4/cdk6 double knockout mice led to the conclusion that in certain compartments such as the myocardium and the hematopoietic system, D-type cyclins are indispensible for cell proliferation (154). Consistent with a cell specific function, D-type cyclins have distinct tissue- and cell- specific expression patterns (27, 154, 364), the transcription mechanisms underlying this spatial specificity remain incompletely understood.

During embryogenesis, heart development involves coordinated cardiomyocyte differentiation and proliferation. Myocyte proliferation drastically decreases soon after birth and postnatal heart growth occurs mostly thru enlargement of cardiomyocytes size, a process termed cardiac hypertrophy (204). Additionally, evidence suggests that the adult mammalian heart can be induced to regenerate (204). For example, inhibition of p38 MAP Kinase

enables proliferation of adult cardiomyocytes (72). Similarly combined deletion of retinoblastoma (Rb) plus p130 genes enhances myocytes proliferation in part thru upregulations of G1 dependent kinase activities (191). A direct role of G1 cyclins in promoting postnatal cardiomyocyte proliferation has been reported (285). Nuclear import of cyclin D1/cdk4 enables post-mitotic cardiomyocytes to enter the cell cycle and divide (328) whereas, cardiac specific overexpression of cyclin D2 enhances post-ischemic heart repair (109, 259). More recently, it was shown that neonate mammalian cardiomyocytes could be induced to proliferate following partial surgical resection leading to transient regenerative potential of neonate hearts (270). Interestingly, in Zebrafish which can regenerate their heart after amputation of up to 20% of the ventricle, the regenerated cardiac cells were shown to arise from differentiated cardiomyocytes that undergo limited dedifferentiation followed by proliferation (128); a population of cardiomyocytes in the zebrafish ventricular wall appear to contribute to cardiac muscle regeneration by inducing expression of transcription factor GATA4, a critical regulator of cardiogenesis (146).

Initially identified as an upstream regulator of the cardiac natriuretic peptide genes *NPPA* and *NPPB*, GATA4 regulates a plethora of cardiac genes involved in several cellular processes including differentiation, proliferation and survival (236, 239). GATA4 deletion from embryonic cardiomyocytes consistently leads to myocardial thinning, supporting a role for GATA4 in myocyte proliferation (246, 272, 380). Although many direct transcription targets have been identified, the effectors of GATA4 actions are incompletely understood. Interestingly, CD2 was shown to be regulated by GATA4 in the anterior heart field of developing embryos (282); GATA4 was also shown to cooperate with KLF13 in activating CD1 (165). Whether CD2 (or CD1) activation mediates GATA4-dependend cardiomyocyte

proliferation has not yet been established. In addition to its critical role for embryonic heart development, GATA4 plays an important role in the postnatal heart where it is required for cardiomyocyte survival and adaptive response. A number of stimuli that induce cardiac hypertrophy were shown to increase GATA4 levels, transcriptional activity and/or DNA binding and upregulation of GATA4 is sufficient to induce hypertophic growth of neonate cardiomyocytes (43, 175). Loss of one Gata4 allele results in hypoplastic hearts, increased cardiomyocyte apoptosis and impaired adaptive response (7). Surprisingly, targeted upregulation of GATA4 specifically in adult hearts either through adenovirus mediated delivery to rat hearts or inducible transgenesis in mice is cardioprotective but not associated with myocyte proliferation suggesting that these terminally differentiated cells may lack critical GATA4 cofactors and/or effectors for proliferative growth (112, 287).

Search for GATA4 targets/effectors in neonatal cardiomyocytes revealed that Cyclin D2 (CD2) levels were exquisitely sensitive to GATA4 and chromatin immunoprecipitation confirmed *in vivo* GATA4 occupancy of the CD2 promoters in these cells. We therefore tested whether CD2 may mediate GATA4 growth effects in cardiomyocytes. We report that when crossed with $Gata4^{+/-}$ mice, transgenic mice with myocardial specific expression of CD2 are able to rescue $Gata4^{+/-}$ hypoplastic hearts and restore cardiac function to control wild type level. In $Gata4^{+/-}$ mice, CD2 rescued basal as well as doxorubicin induced cardiomyocyte apoptosis and promoted cardiomyocyte proliferation as evidenced by the increased number of $Ki67^{+}$ cells. Together, the data support a role for cyclin D2 as an effector of GATA4 in cardiomyocyte proliferation. They also unravel potential new protective function for CD2 in adult hearts.

C. Materials and Methods

Plasmids. Cyclin D2-luciferase was generated by subcloning the rat Cyclin D2 promoter in the PxP1 vector. GATA4 expression vectors were previously described (7, 65). All constructs were confirmed by sequencing.

Cell cultures and transfections. Primary cardiomyocytes, NIH3T3 and 293T cells were maintained in culture and transfected as described previously (7, 90). Atrial and ventricular cardiomyocyte primary cultures were prepared from 4- to 5-day-old Sprague-Dawley rats (Charles River). Neonatal rats were sacrificed by decapitation. Atrial and ventricular tissues were aseptically removed and washed with Joklik's modified Eagle's medium (GIBCO). The tissues were then minced and subjected to three sequential digestions of 30, 20, and 10 min each in 0.1% collagenase (Cooper Biomedicals). To stop the enzymatic digestion, we added cold fetal calf serum to a final concentration of 28.5%. Undigested tissue remnants were removed by filtration through a nylon mesh (pore size, 100 p,m). The cell-containing filtrate was then centrifuged, and the resulting cell pellet was resuspended in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum (FCS). To eliminate fibroblasts, we preplated the cells for 20- to 30-min periods, after which the unattached cardiomyocyte-enriched cells were collected. The cardiac cells were plated in Primaria (Falcon) plates. The experiments were done at least twice in duplicate with different DNA preparations.

RT-PCR. The oligonucleotides used for amplification of Cyclin D2 were 5'-CGT CTA GAA TGG AGC TGCT GTG CTG CGA GGT GG-3' (forward) and 5'- GGG GTA CCT CAC AGG TCA ACA TCC CGC ACG TC-3' (reverse). RT-PCR was performed as described previously (58).

Chromatin immunoprecipitation assays and QPCR analysis. ChIP assay was performed as previously described (353). The primers used were: 5'-AAAGTTTCCGCACGAGGGTCAT-3' (sense) and 5'-CCCTGAGGCTTAGACTCCTGATAACT-3' (anti-sense) for the GATA-site; 5'-TTTGAAGTTTGGTCAGGCCAGC-3' (sense) and 5'-GCAAGCTGGAAGGGCAGTTAGAT-3' (anti-sense) for the GATA-like site.

Real-Time PCR analysis. Total RNA was isolated from mouse tissues using TRIZOL reagent (Invitrogen). Transcript levels of the various markers were determined by real-time PCR as previously described (58).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from cardiomyocytes in primary cultures. Binding reactions were done at room temperature using 1ug of poly(dI-dC). The probes used for binding are: GATA-like: 5'-GAG GGA AAG ATT GAA AGG AG-3' (sense) and 5'- CTC CTT TCA ATC TTT CCC TC-3' (anti-sense); mutated GATA-like: 5'-GAG GGA AAG gTT GAA AGG AG-3' (sense) and 5'- CTC CTT TCA AcC TTT CCC TC-3' (anti-sense); GATA-site: 5'- TCA GAA AGG ATA ATC AAT AG-3' (sense) and 5'- CTA TTG ATT ATC CTT TCT GA-3' (anti-sense); mutated GATA-site: 5'- TCA GAA AGG gTg ATC AAT AG-3' (sense) and 5'- CTA TTG ATc AcC CTT TCT GA-3' (anti-sense). The ANF probe was previously described (7).

Transgenic Mice. All animal experimentations were carried out in accordance with institutional guidelines for animal care. Experiments were approved by IACUC of the University of Ottawa, which conforms to that of the US National Institute of Health (NIH) (Assurance number for University of Ottawa: A5043-01). At end points, mice were

anesthetized with 2.2 µl/g i.p KXA cocktail (Ketamine 42.86 mg/ml, Xylazine 8.57 mg/ml, and Acepromazine 1.43 mg/ml) and sacrificed. For Euthanasia, CO2 and cervical dislocation (CD) was used. CD without prior anesthesia is required for embryo collection in mice. The $Gata4^{+/-}$ mice were previously described (7). To overexpress Cyclin D2 in the heart, mouse *Cyclin D2* cDNA under the control of the alpha MHC promoter was subcloned in the SV40 expression vector (255). Cardiac specific HA-CD2 expression in transgenic mice was validated using western blot. For Masson Trichrome Staining, hearts were processed and stained as previously described (7, 90). For echocardiography analysis, mice were anesthetized (2.0% isoflurane, 80 ml/min 100% O_2) and two-dimensional guided M-mode echocardiography was performed using a Visual-Sonics VEVO 700 and a 30-MHz linear array transducer as described by Aries *et al.*, 2005. Doxorubicin (Dox) treatment was done as previously described (7). Briefly, mice were injected with 15mg/kg Dox intraperitoneally and sacrificed 7 days post-injection.

Immunohistochemistry. Immunohistochemistry on heart sections from each mouse group was performed as previously described (7). The antibodies used were: GATA4 (7), ANF (Rabbit anti-Atrial Natriuretic Factor, Peninsula Laboratories T-4014), Cyclin D2 (mouse monoclonal, abcam, ab3087), Ki67 (Clone SP6, Rabbit Monoclonal, Thermo Scientific RM-9106-S0), phospho histone H3 (Ser 10, Rabbit polyclonal, Millipore 06-570), sarcomeric α-actinin (mouse monoclonal, Sigma A7811), and DAPI for nuclei staining (molecular probe D3571).

TUNEL assay was carried out using apoptag kit (Chemicon, S7100) according to the apoptag protocol.

Cell number and surface area. Total cell number was determined by calculating the number of cells within a μm^2 area and multiplying by the surface area of the left ventricle wall and the interventricular septum. Surface area was determined using "ImageJ" and the "AxioVision LE" software.

Statistics. The data are presented as mean ± SEM; $p<0.05$ by student t-test is considered statistically significant.

D. Results

i. Cyclin D2 is a direct GATA4 transcriptional target.

DNA chip analysis using RNA from neonate cardiomyocytes infected with adenoviruses expressing LacZ, or sense or antisense Gata4 transcripts, identified Cyclin D2 (CD2) as a GATA4 inducible transcript. PCR analysis confirmed that CD2 mRNA levels were increased in cells overexpressing GATA4 and decreased in cells with antisense mediated knockdown of GATA4 (figure II.1A). Bioinformatics examination of the cyclin D2 (CD2) promoter revealed two evolutionary conserved GATA binding sites, a proximal GATA-like site at -352 bp and a distal (-801 bp) GATA consensus site (figure II.1B). Electromobilty shift assays using cardiomyocytes nuclear extracts and probes designed to correspond to the distal and proximal GATA sites of the CD2 promoter, confirmed that both sites bound GATA4 with high affinity. Addition of anti-GATA4 antibody confirmed that the bound protein is GATA4 and not any other member of the GATA family (upper arrow in figure II.1C). Next, we tested whether GATA4 was able to directly activate the CD2 promoter; we co-transfected the CD2-luc reporter with various doses of a GATA4 expression vector. As shown in figure II.1D, GATA4 was able to robustly activate the CD2 promoter in a dose dependant manner. To determine whether GATA4 associates with the CD2 promoter *in vivo*, we performed chromatin immunoprecipitation (ChIP) assays using neonate primary cardiomyocyte cultures. As shown in figure II.1E, GATA4 was enriched 9 and 5 fold at the distal and proximal domains of the CD2 promoter respectively. Consistent with a role for GATA4 in CD2 regulation, Q-PCR analysis revealed a significant decrease in CD2 transcript levels in Gata4 heterozygote embryonic hearts (figure II.1F). This decrease was not observed

for CD1. As positive control, Nppa and Gata4 transcript levels were measured and found to be similarly decreased in Gata4 heterozygote hearts relative to age matched wild type littermates. Immunostaining on heart sections of E15.5 wild type and $Gata4^{+/-}$ embryos showed that CD2 protein levels were greatly reduced in the ventricles of $Gata4^{+/-}$ embryos in line with the Q-PCR data (data not shown). Together, these findings confirm that cyclin D2 is a direct target of transcription factor GATA4 in postnatal cardiomyocytes.

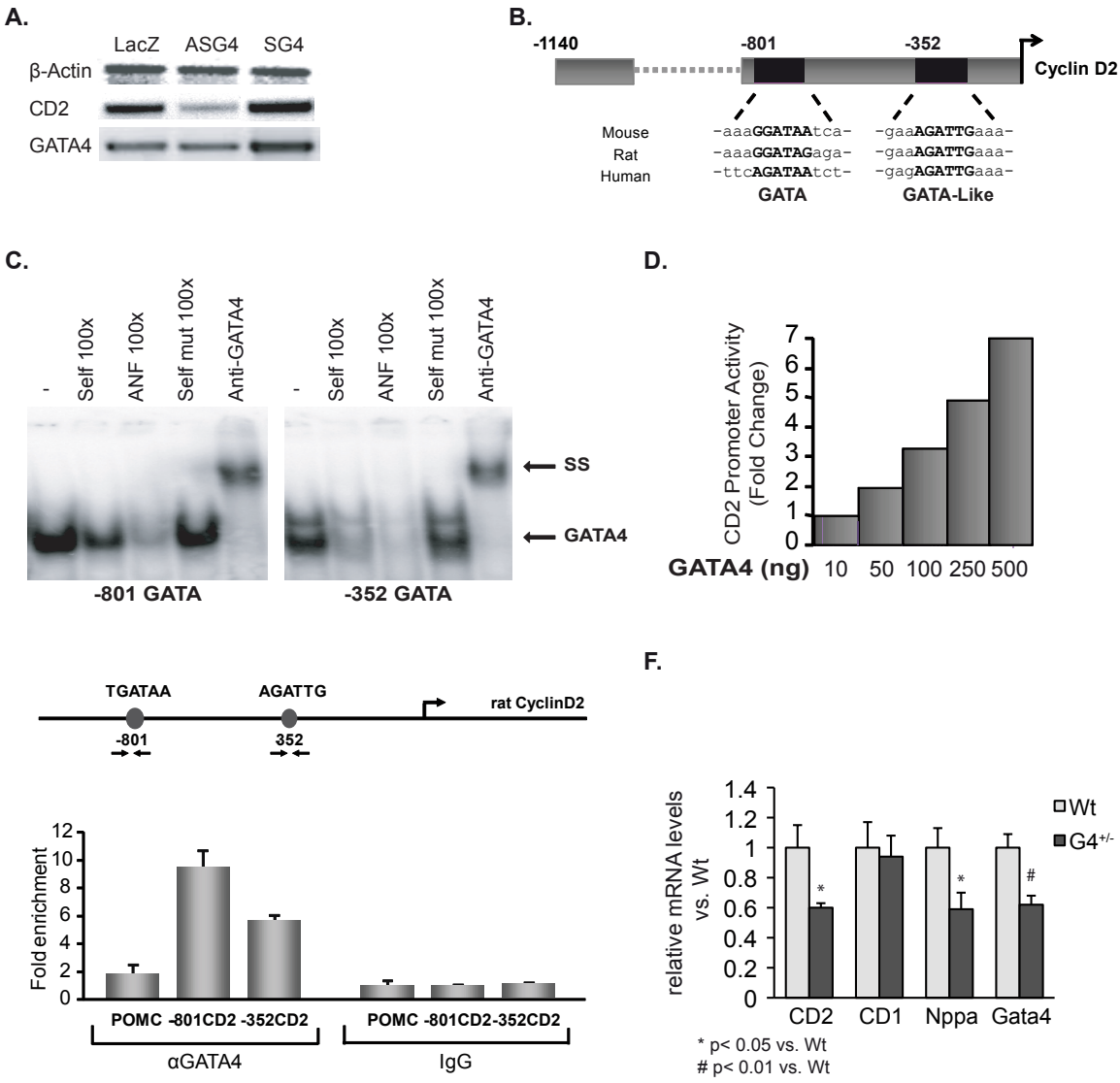

Figure II.1 Cyclin D2 is a direct GATA4 target. A. RT-PCR analysis of changes in cyclin D2 mRNA levels in cardiomyocytes infected with adeno-lac Z, sense- or antisense GATA4. GATA4 mRNA levels are shown and B-actin is used as an internal control. B. Schematic representation of the cyclin D2 promoter showing the location and evolutionary conservation of proximal GATA-like and the distal GATA consensus sites. C. Electromobility shift assays were performed using nuclear extracts from rat cardiomyocytes and primers corresponding to the two putative GATA binding sites on the cyclin D2 promoter. Supershifts (SS) were performed using anti-GATA4 antibody. Note how the addition of a cold self probe or the consensus ANF GATA site competes with the binding but not the addition of a self mutant. D. Dose dependent activation of cyclin D2 promoter by GATA4. Transient co-transfections were carried out in NIH-3T3 cells using increasing doses of rat GATA4 expression vectors and the cyclin D2-luc reporter. The data are the mean of n=4 E. Enrichment of GATA4 on the endogenous cyclin D2 promoter as revealed by ChIP. The results are from one representative carried out in duplicates on primary cardiomyocytes. F. Q-PCR analysis of transcript levels in total RNA from E15.5 wild type (Wt) and Gata4$^{+/-}$ (G4$^{+/-}$) embryos.

ii. Enhanced cardiac growth in myocardial CD2 transgenic mice.

In order to determine which action of GATA4 is mediated by CD2 in cardiomyocytes, we developed a mouse line with myocardial specific CD2 overexpression using the mouse αMHC promoter (figure II.2A). This promoter drives expression of transgenes specifically to the heart and can deliver genes that promote various cell fates in postnatal ventricles including cardiomyocyte hypertrophy (90, 255). Tissue-specific transgene expression was monitored at the protein level using western blot. As expected, Cyclin D2 expression was restricted to the ventricles and atria. No expression of the transgene was detected in the lungs, liver, kidney, or spleen (figure II.2B). Western blots as well as immunohistochemistry showed that exogenous CD2 was upregulated in both nuclear and cytoplasmic compartments (Figure II.3A and data not shown). The transgenic mice were viable and phenotypically appeared normal. Two independent transgenic lines out of 6 obtained were used to analyze the effect of CD2 overexpression on the heart and both produced identical results. Analyses of hearts of CD2 transgenic mice showed a statistically significant increase in heart weight corrected by femur length as compared to the wild type littermate hearts (figure II.2C left panel). Echocardiography on wild type and transgenic mice showed normal ejection fraction (EF) and other cardiac functions in CD2 transgenics as compared to their wild type littermates (figure II.2C right panel and data not shown). Masson Trichrome staining of adult heart sections confirmed a thicker left ventricular wall in CD2 transgenic mice as compared to wild type (Wt) littermates. Consistent with the functional data, higher magnification of trichrome stained section revealed no pathologic cardiac remodeling; there was no sign of fibrosis or cardiomyocyte hypertrophy (figure II.2D and figure 4A). A time course analysis of cardiac changes revealed that heart mass was similar in

neonate Wt and transgenic mice and that increased left ventricular mass to body weight (LV/BW) ratio was detectable in 30d and older CD2 transgenics. These findings which are consistent with postnatal activation of the αMHC promoter suggest enhanced postnatal cardiac growth in CD2 transgenics. QPCR analysis using mRNA from hearts of CD2 transgenic mice and their wild type littermates revealed no upregulation of genes associated with pathologic hypertrophy such as Nppa and Nppb (the genes encoding the cardiac hormones ANF and BNP) as well as skeletal actin (ACTA1) and βMHC. In fact, a consistent significant decrease in Nppb was detected (figure II.2E). Immunostaining on ventricular sections from mice confirmed that ANF was not increased in the ventricles of CD2 transgenics (Figure II.3A). Given the above, we tested whether increased cardiomyocyte proliferation accounts for enhanced postnatal cardiac growth. Immunostaining for two proliferation markers, Ki67 and the mitosis marker phosphohistone H3 (phH3), showed a three to four fold increase in the percent of proliferation in the hearts of adult CD2 transgenic mice as compared to their wild type littermates (figure II.3B). Consistent with this, the number of cardiomyocytes per surface area was significantly increased in adult CD2 transgenic ventricles (figure II.4D). These results suggest that cyclin D2 overexpression promotes postnatal cardiac growth with maintained function, mainly through enhanced cardiomyocyte proliferation.

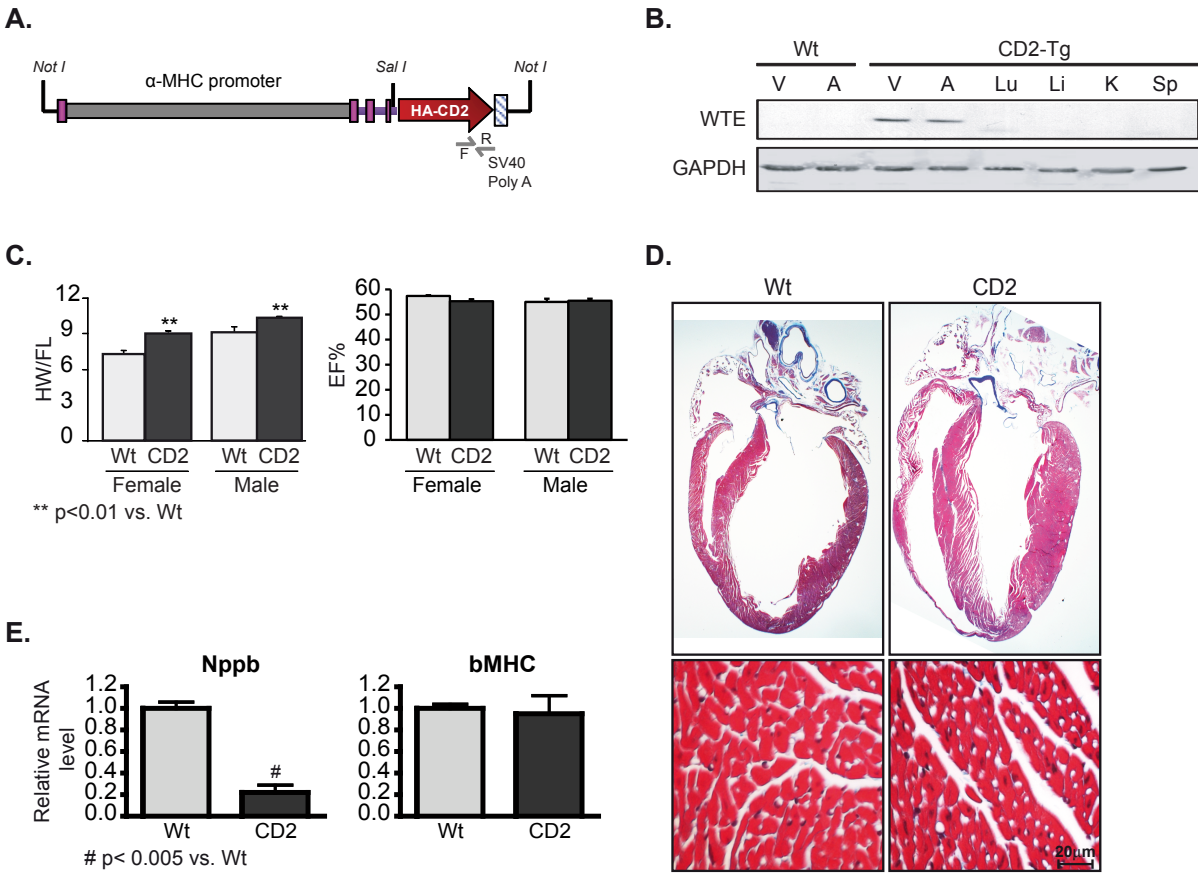

Figure II.2 Myocardial overexpression of cyclin D2 in transgenic mice. A. Schematic representation of the α-MHC-HA-CD2 transgene. "F" and "R" indicate the forward and reverse primers used for screening. **B.** Cardiac specific expression of the CD2 transgene as determined by western blot using HA antibody. **C.** Measurements of the heart weight corrected by the femur length (HW/FL) and the ejection fraction (EF%) in young (~80d old) wild type and transgenic female and male mice. n=16-23 for each group. The results shown are the means +/- standard deviations. **D.** Masson trichrome staining of whole heart sections from adult (170d old) wild type and CD2 transgenic mice. Note the thicker left ventricular wall of the transgenic heart. **E.** Measurement of Nppb and βMHC mRNA levels by QPCR analysis using RNA from heart tissues of CD2 transgenic mice (CD2) and their wild type (Wt) littermates.

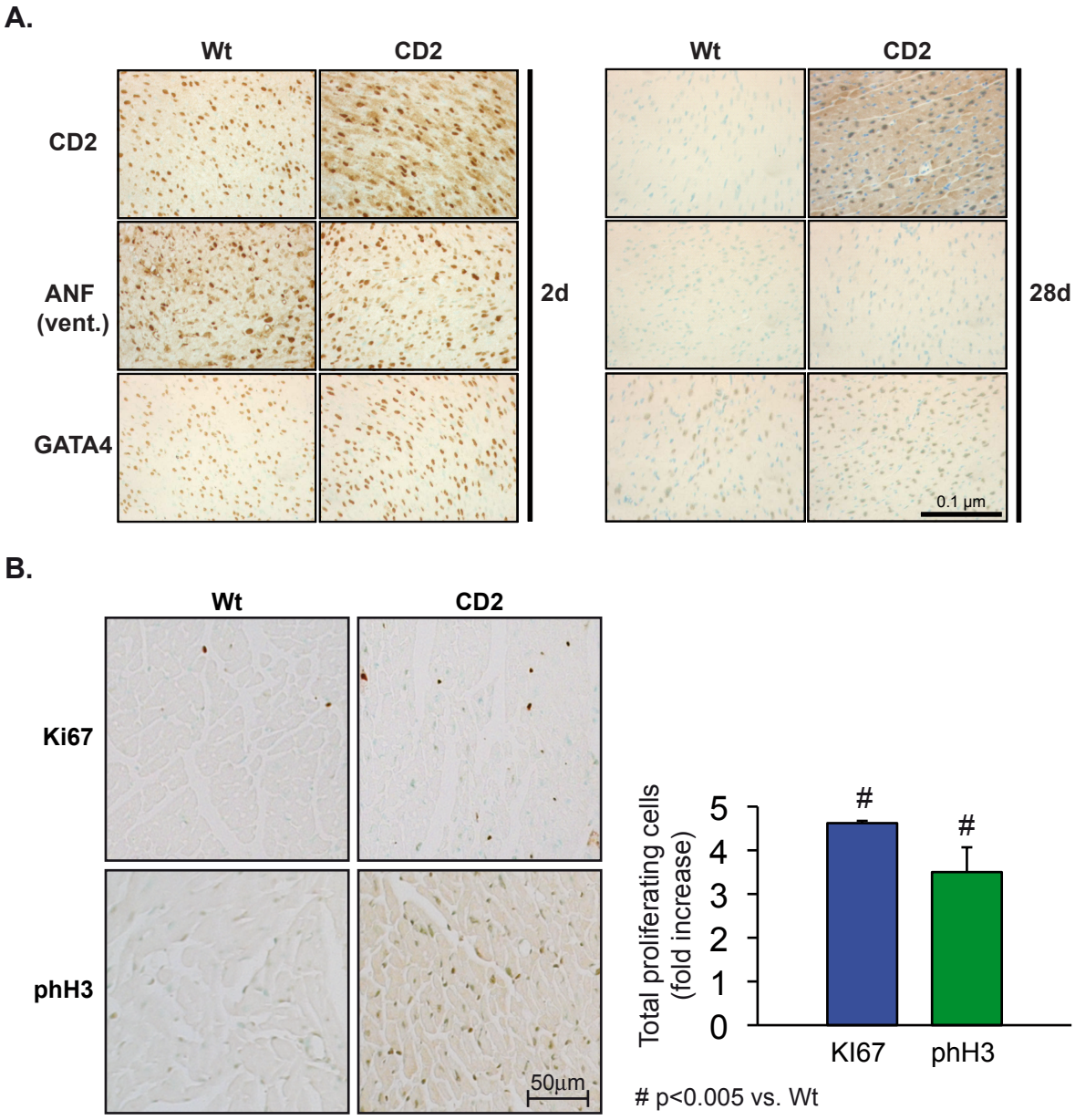

Figure II.3 A. Immunohistochemical analysis on ventricular heart sections from neonate (2d left panel; 28d right panel) wild type (Wt) and CD2 transgenics. CD2 = cyclin D2. Note how ANF staining is similar in Wt and CD2 hearts **B.** Immunohistochemical staining for proliferation markers Ki67 and phosphohistone H3 (phH3) on adult heart sections (~170d old) from wild type and CD2 transgenics (Tg). The histogram represents quantification of 24 fields from 2 different hearts for each group. The results are expressed as the average percentage of positive cells.

iii. Cyclin D2 rescues the hypoplastic cardiac phenotype of Gata4$^{+/-}$ mice

As stated earlier, GATA4 haploinsufficiency results in hypoplastic hearts. To determine if Cyclin D2 may be a GATA4 effector in cardiomyocyte proliferation, we tested whether myocardial specific overexpression could rescue cardiac growth in Gata4$^{+/-}$ mice.

For this, CD2 transgenic mice were crossed with Gata4 heterozygote mice. Masson Trichrome staining of heart sections showed that the CD2.Gata4$^{+/-}$ (CD2.G4$^{+/-}$) mice had normal hearts in contrast to the hypoplastic hearts of Gata4$^{+/-}$ mice; higher magnification of the Trichrome stained sections also showed increased interstitial collagen deposition in Gata4$^{+/-}$ but not in CD2 Gata4$^{+/-}$ hearts. Staining of heart sections with Sirius red confirmed these observations. (figure II.4A middle and lower panels). Echocardiography revealed that the ejection fraction and LV/BW of the CD2.G4$^{+/-}$ mice were almost restored to normal levels (figure II.4B). Cell surface area measurements revealed no significant difference among the four genotypes (figure II.4C). Interestingly, the number of cardiomyocytes per surface area was significantly increased in adult CD2 transgenic as well as in CD2.G4+/- ventricles as compared to wild type and Gata4$^{+/-}$ hearts respectively (figure II.4D).

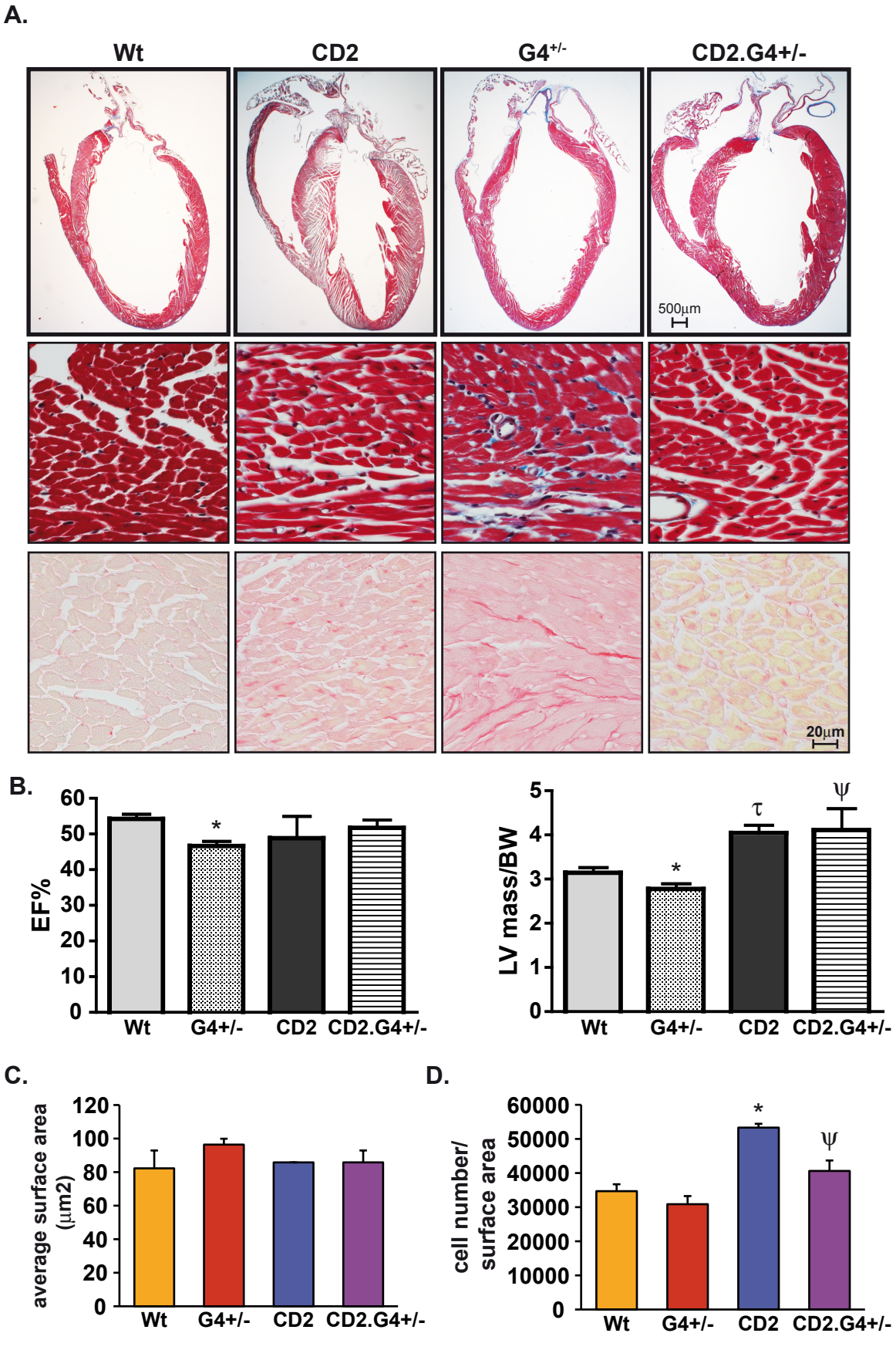
A.
Wt
CD2
G4+/-
CD2.G4+/-
500µm
20µm
B.
EF%
60
50
40
30
20
10
0
*
Wt
G4+/-
CD2
CD2.G4+/-
LV mass/BW
5
4
3
2
1
0
*
τ
ψ
Wt
G4+/-
CD2
CD2.G4+/-
C.
average surface area (µm2)
120
100
80
60
40
20
0
Wt
G4+/-
CD2
CD2.G4+/-
D.
cell number/ surface area
60000
50000
40000
30000
20000
10000
0
*
ψ
Wt
G4+/-
CD2
CD2.G4+/-

Figure II.4 CD2 rescues Gata4$^{+/-}$ haploinsufficient hearts. A. Trichrome staining (upper and middle panel) and Sirius red staining (lower panel) of whole heart sections from adult wild type, HA-CD2 transgenic, Gata4$^{+/-}$ (G4$^{+/-}$), and CD2.G4$^{+/-}$ mice (170d old). Note the hypoplastic heart of Gata4$^{+/-}$ mice and the larger left ventricle of the CD2 mice. Note as well the collagen deposition (blue in Trichrome and red in Sirius red staining) in the G4$^{+/-}$ heart as evidence of fibrosis. **B.** Echocardiography analysis showing the left ventricular mass measurement corrected by the body weight (LV/BW) and the ejection fraction (EF%) of the different mouse genotypes. N=5-6 for each group. * $p<0.05$ vs. Wt; τ $p<0.005$ vs. Wt; Ψ $p<0.05$ vs. G4$^{+/-}$. **C.** Average cell surface area calculated from ventricular sections of adult wild type, CD2 transgenic, Gata4$^{+/-}$ (G4$^{+/-}$), and CD2.G4$^{+/-}$ mice (170d old). The histogram represents quantification of 5 fields from 2 different hearts for each group. The results presented are the mean +/- SEM. **D.** Total cell number in ventricular and interventricular septum sections of adult wild type, CD2 transgenic, Gata4$^{+/-}$ (G4$^{+/-}$), and CD2.G4$^{+/-}$ mice (170d old). The results presented are the mean +/- SEM. n=3 for each group. * $p<0.05$ vs. Wt; Ψ $p<0.05$ vs. G4$^{+/-}$.

The ability of CD2 to rescue GATA4 deficiency was further assessed at the gene expression level by QPCR analysis on mRNA from postnatal (30-40d) and adult (170-190d) hearts. Two sets of genes were measured. On the one hand, those that are biomarkers of pathologic cardiac stress and hypertrophy – including natriuretic peptide genes and skeletal actin – which are upregulated in Gata4$^{+/-}$ mice; on the other, those that are essential for normal heart function such as the sarcosplasmic calcium pump (SERCA) and the potassium channel kv4.2. As shown in figure II.5, CD2 upregulation largely normalized gene expression pattern of postnatal Gata4$^{+/-}$ hearts (II.5A). This normalization was maintained in adult hearts (II.5B).

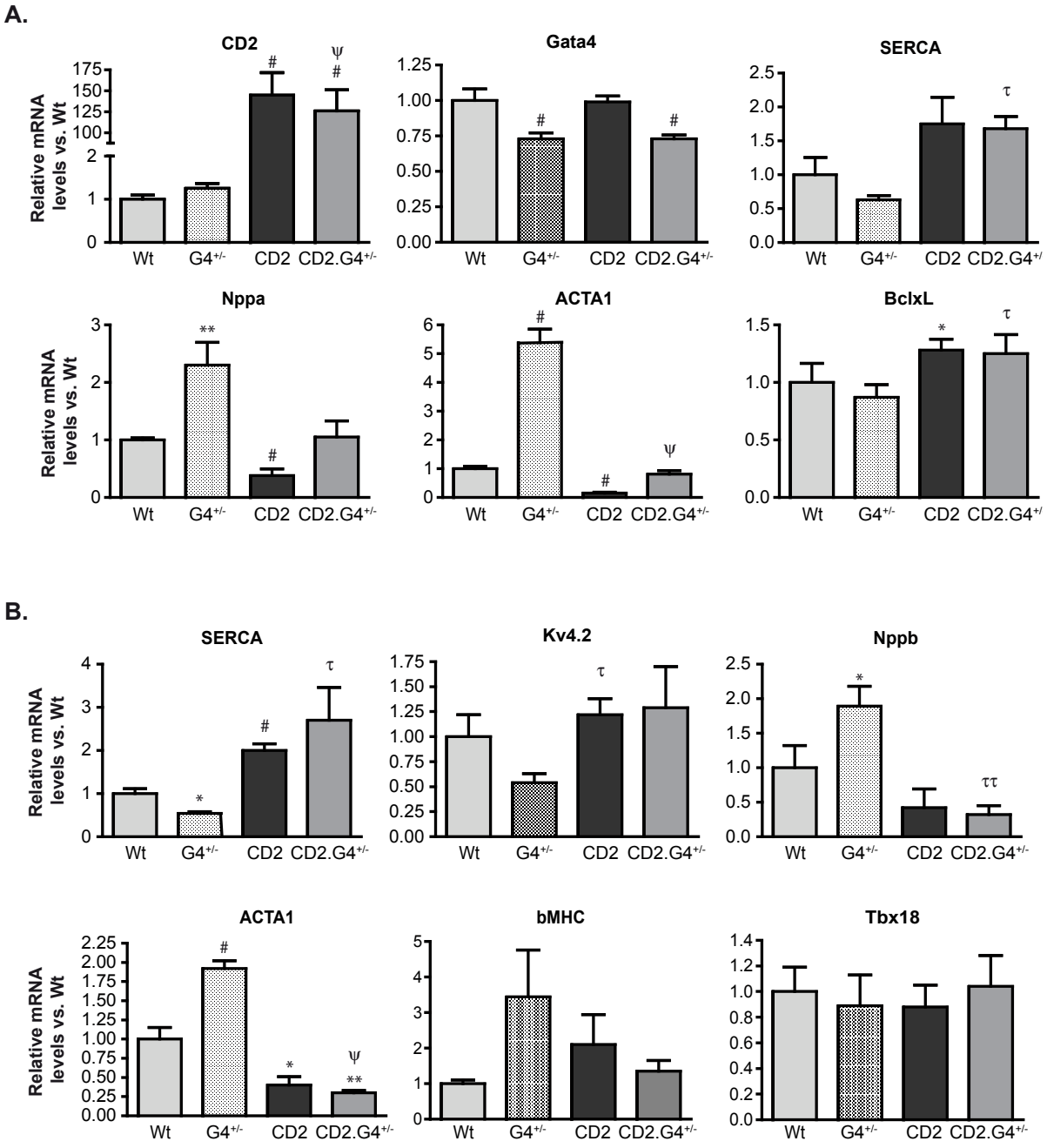

Figure II.5 Gene expression changes in CD2 expressing hearts. A. QPCR analysis using RNA from heart tissues of 30 to 40-day-old mice (n=4-5 for each group). Note how Nppa and ACTA1 levels are normalized in the CD2.G4$^{+/-}$ mice vs. G4$^{+/-}$. **B.** QPCR analysis using RNA from adult heart tissues (170d) (n=3 for each group). Note how most markers are restored back to normal in the CD2.G4$^{+/-}$ mice. * $p<0.05$ vs. wt; ** $p<0.01$ vs. wt; # $p\leq0.005$ vs. wt; τ $p<0.05$ vs. G4$^{+/-}$; ττ $p\leq0.01$ vs. G4$^{+/-}$; ψ $p<0.005$ vs. G4$^{+/-}$.

Next, we analyzed cardiomyocyte proliferation and survival in CD2.G4$^{+/-}$ mice and their control groups. Immunofluorescence was carried out using Ki67 or phH3 as a marker

for proliferation (red) and Actinin co-staining (green) to label cardiomyocytes (figure II.6A top panel). Quantification of the fluorescent signals showed increased proliferation in the hearts of CD2 and CD2.G4$^{+/-}$ mice (figure II.6A bottom graphs). Gata4$^{+/-}$ mice have increased cardiomyocyte apoptosis (7); TUNEL assays unexpectedly revealed that CD2 overexpression was able to restore the percent of apoptosis in Gata4$^{+/-}$ mice to the level observed in Wt hearts (figure II.6B). Given this result, we tested whether CD2 overexpression was able to rescue the exaggerated Doxorubicin-induced apoptosis in Gata4 haploinsufficient mice. As seen in figure II.6C, CD2 restored drug-induced apoptosis of Gata4$^{+/-}$ mice to Wt levels.

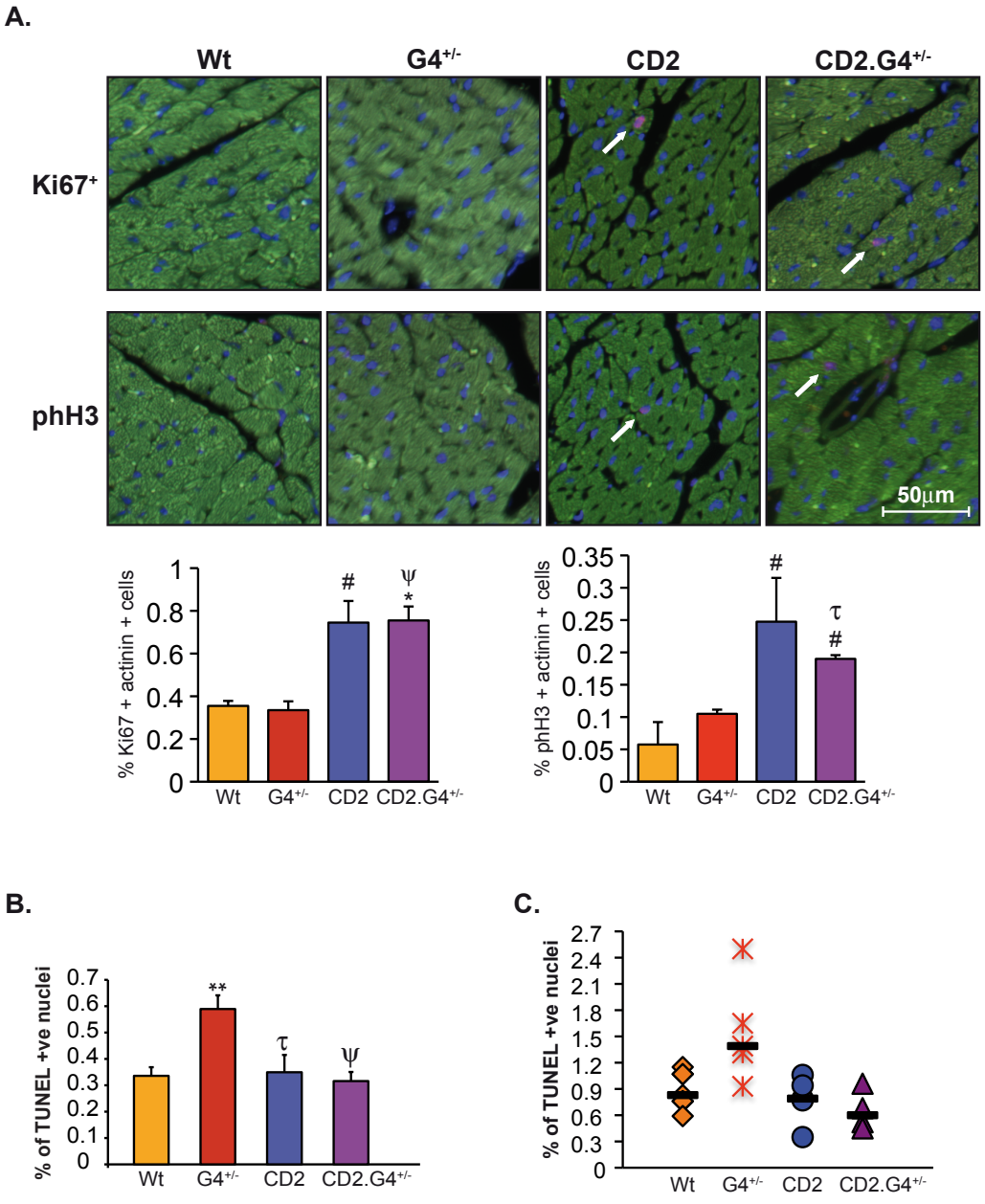

Figure II.6 Analysis of cardiomyocyte proliferation and survival. A. Top panel. Immunofluorescent analysis of Ki67 and phH3 expression on adult heart sections. Green is Actinin staining to mark cardiomyocytes; red is for Ki67 or phH3 staining as indicated; blue marks nuclei (DAPI). White arrows indicate positive nuclei. **Bottom panel.** Percent of Ki67/actinin and phH3/actinin positive nuclei vs. wild type is shown. Note the significant increase in the level of proliferation in the CD2 and CD2.G4$^{+/-}$ mice. The results presented are the mean +/- SEM. (n=4 for each mouse group). # $p< 0.05$ vs. Wt; * $p< 0.005$ vs Wt; τ $p< 0.05$ vs. G4$^{+/-}$; ψ $p< 0.005$ vs. G4$^{+/-}$. **B.** Quantification of TUNEL assays on adult heart sections (170d) showing the percent of TUNEL positive nuclei. Ten regions from each heart section were counted. The results shown are the mean +/- SEM (n=3 for each group). Note the statistically significant increase in the percent of apoptotic nuclei in the Gata4$^{+/-}$ mice, which was completely restored back to Wt level in the CD2/G4$^{+/-}$ mice. # $p< 0.005$ vs. Wt; ψ $p< 0.005$ vs. G4$^{+/-}$. **C.** Scatter plot showing the % of TUNEL positive cardiomyocytes in heart sections from adult (180d) Doxorubicin (Dox) treated mice. Sixteen regions from each heart section were taken. (n= 4-7 for each group).

Together, these results show that myocardial CD2 upregulation is able to restore, at least in part, the genetic and growth alterations caused by GATA4 haploinsufficiency and suggest that CD2 is a GATA4 effector in cardiomyocyte growth.

E. Discussion

It is now well established that the zinc finger transcription factor GATA4 is crucial for proper heart development and maturation. Mutations in GATA4 cause various congenital heart problems reflecting its pleitropic role in cardiac cell proliferation and differentiation. However, the effectors of GATA4 actions are incompletely understood. In the presented work, we provide evidence that Cyclin D2 is not only a direct target of GATA4 but is also an effector of GATA4 in postnatal cardiomyocytes growth. Remarkably, upregulation of CD2 in cardiomyocytes rescues growth and adaptive stress response of GATA4 haploinsufficient hearts. These findings provide a new paradigm that may help protect cardiac function of GATA4 haploinsufficient hearts.

Cardiac specific overexpression of cyclin D2 caused an increase in cardiac mass with maintained normal cardiac function as evidenced from echocardiography and biomarker analysis. The fact that these transgenic mice were viable with normal cardiac function throughout adult life (up to 300d) suggests that enhanced cardiac expression of cyclin D2 may have beneficiary effects on the postnatal heart, possibly through limited regenerative capacity. Indeed, our results show increased cardiomyocyte proliferation in the hearts of adult CD2 transgenic mice. These results are generally consistent with those reported for a similar αMHC overexpressing mouse line (259).

Crossing the CD2 transgenic mice with the $Gata4^{+/-}$ mice which have hypoplastic hearts and increased sensitivity to drug-induced cardiotoxicity (7, 246) revealed that Cyclin D2 can compensate – at least in part - for GATA4 haploinsufficiency. Indeed CD2 rescued

heart size and adaptive response to drug-induced apoptosis of Gata4 heterozygote mice. $Gata4^{+/-}$ mice develop stress-induced pathological hypertrophy, a likely consequence of a lower number of myocytes and decreased myocyte survival and contractile function (7). We found that CD2 upregulation was sufficient to restore cardiac mass thru increased myocyte proliferation not hypertrophy. CD2 also improved heart function as evidenced from biomarker analysis. It is interesting to note that cardiac growth in CD2 transgenics on either Wt or $Gata4^{+/-}$ background occurred mostly thru postnatal cardiomyocyte proliferation not hypertrophy suggesting that GATA4 effectors in hypertrophy and proliferation may be distinct.

The finding that CD2 rescued basal and drug-induced survival of cardiomyocytes with GATA4 haploinsufficiency was unexpected and noteworthy. While a more direct effect of CD2 on myocyte survival cannot be excluded, it is possible that by normalizing cardiac size and function CD2 reduced basal oxidative stress level and enhanced adaptive response. CD2 has been suggested to play an important role in biomechanical stress response of the heart based on the attenuated response to transaortic constriction of CD2 null mice (6). Be it as it may, the finding that postnatal upregulation of CD2 is beneficial to GATA4 hypoplastic hearts may open new avenues for cardioprotection for individuals with heterozygous mutations in *GATA4* or GATA4 dependent cardiac dysfunction.

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Disclosures

Conflict of interest: None declared.

Author Contributions

M. Nemer designed research; A. Yamak, R. Temsah, W. Maharsy, S. Caron, P. Paradis, and A. Aries performed research. A. Yamak and M. Nemer analyzed data. A. Yamak and M. Nemer wrote the paper.

III. Chapter II: Cyclin D2 is a GATA4 Cofactor in Cardiogenesis

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2- School of Biosciences, Cardiff University, Cardiff, United Kingdom

Author contribution

I performed 95% of the work in the present chapter. Our collaborator, Dr. Branko Latinkić, performed the cardiogenesis work in the *Xenopus*. My honor student at the time, Rola Dali, prepared the CycD2-C2C12 stable cell line.

A. Abstract

D-type cyclins (Cyclins D1, D2, and D3) are G1 cyclins that play a vital role in differentiation and cell cycle regulation. The mechanisms underlying their cell specific effects are incompletely understood. Here we show that transcription factor GATA4, a key regulator of cardiomyocyte growth and differentiation, recruits cyclin D2 (CycD2) to its target promoters. GATA4 and CycD2 interaction results in synergistic activation of GATA dependent transcription. This effect is specific to CycD2 and CycD1 was unable to potentiate GATA4 activation. GST pull-down and co-immunoprecipitation assays show that GATA4 is able to physically interact with CycD2 and with its cyclin dependent kinase CDK4. CDK4 is able to phosphorylate GATA4 *in vitro,* and inhibition of cdk4 reduces GATA4 activity. Chromatin immunoprecipitation assay showed that CycD2 is bound *in vivo* to GATA-target promoters and cardiogenesis assays in *Xenopus* confirmed that CycD2 enhances the cardiogenic function of GATA4 *in vivo*. Together, our data uncover a novel role for CycD2 as a coactivator of GATA4 in the developing heart. Moreover, the GATA-CycD2 interaction may represent a paradigm that helps explain cell specific effects of cyclin Ds.

B. Introduction

D-type cyclins (Cyclins D1, D2, and D3) are G1 cyclins that play a vital role in differentiation, regulation of the cell cycle and tumor formation (298). They are deemed to connect the cell cycle machinery with the extracellular environment. In response to mitogenic signals, cyclin Ds associate with cyclin dependant kinases CDK4 and CDK6, phosphorylate the tumor suppressor retinoblastoma protein Rb along with the family members p107 and p130, and control the G1-S phase transition in the cell cycle together with Cyclin E and A/CDK2 complexes (135, 298). Cyclins and CDKs bind the consensus sequences RXL and SP respectively and cyclin/cdk substrates were often shown to contain the consensus sequence S/T-P-X-R/K (138, 224). As opposed to other cyclins, D-type cyclins have distinctive tissue- and cell- specific expression patterns (27, 197, 296, 364). In addition to the Rb protein family, CyclinD-CDK4 complex was found to phosphorylate and inhibit the transcription factor SMAD3 and the myb like transcription factor, DMP1 (114, 198). Cyclin D1-CDK4 was also shown to bind to and inhibit the transcriptional activity of MyoD (276, 382). Moreover, cyclin D1 binds to the activation domain of STAT3 and inhibits its transcriptional activity in a cdk4 independent mechanism (21). On the other hand, cyclin D3 was shown to physically interact with and potentiate the transcriptional activity of human activating transcription factor 5 (182). In the heart, cyclin D/CDK4 is imperative for cellular proliferation and normal morphogenesis. Triple cyclin D knockout in mice results in cardiac abnormalities and severe anemia leading to embryonic lethality (153). $Cdk2^{-/-}$.$Cdk4^{-/-}$ double knockout mice develop cardiac defects and die by embryonic day E15.5 (18).

In the present study, we aim to further understand cell-specific effects of cyclin Ds through interaction of Cyclin D2 with the tissue specific transcription factor, GATA4. GATA4 is a critical regulator of cardiogenesis and is predominantly expressed at all stages of heart development. It has an indispensible role in the embryonic and postnatal hearts as well as in cardiomyocyte survival (237). Our results indicate that GATA4 recruits cyclin D2 to its target promoters and that cyclin D2 interacts physically and functionally with GATA4 to enhance its cardiogenic function. The current data provide a mechanism to explain cell specificity of cyclin Ds and a rationale for targeting cell cycle proteins for cardiac repair.

C. Materials and Methods

Plasmids. The luciferase constructs and most of the GATA expression vectors were previously described (7, 65, 238). Cyclin D2-luciferase was generated by subcloning the rat Cyclin D2 promoter in the PxP1 vector. The Cyclin D2 and CDK4 expression vectors were obtained by PCR mediated amplification of mouse cDNA using oligonucleotides harboring the start codon (forward) and stop codon (reverse) and subcloning into the pCGN vector in phase with the hemagglutinin (HA) epitope in case of HA-CycD2 construct, into the pCDNA3-3XFlag vector in phase with the 3XFlag in the case of Flag-CycD2, or in pcDNA3 vector in case of cdk4. N-terminal deletions and site-directed mutagenesis of GATA4 were generated using PCR-mediated mutagenesis. All constructs were confirmed by sequencing.

Cell cultures and transfections. NIH3T3, 293T, and C2C12 cells were maintained in culture and transfected as described previously (7, 90). For cotransfection assays, the total amount of DNA was maintained constant by adding the appropriate amount of empty DNA vector. For treatment with cdk4 inhibitors, 2uM of the indicated inhibitor was added directly to the media the following morning post transfection and cells were lysed 18 hrs after. Cdki was obtained from Calbiochem (Cat. No: 219476) and RO506220 was obtained from Roche and was previously described by Burgess *et al* (34). The experiments were done at least twice in duplicate with different DNA preparations.

Chromatin immunoprecipitation assays and QPCR analysis. ChIP assay was performed as previously described (353). The primers used were: 5'-GGGATCGTGTTTGAAGTTTG-3' (sense) and 5'-AGATCTCCTCCCCCTCTCAG-3' (anti-sense) for the GATA-site on

cyclin D2 promoter and 5'-TCCTCCCCATCTGTGTCATC-3' (sense) and 5'-GGATCCATCACCATCAATAACC-3' (anti-sense) for the *Dessert* gene.

Real-Time PCR analysis. Total RNA was isolated from heart and liver tissues of 2-4 day old mice pups using TRIZOL reagent (Invitrogen). Transcript levels of the various markers were determined by real-time PCR as previously described (58).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from 293T cells. Binding reactions were done at room temperature using 1ug of poly (dI-dC). The ANF probe was previously described (7).

Transgenic Mice. All animal experimentations were carried out in accordance with institutional guidelines for animal care. Experiments were approved by the institutional Animal Ethics Committee which conforms to that of the US National Institute of Health (NIH) (Assurance number for University of Ottawa: A5043-01). At end points, mice were anesthetized with 2.2 µl/g i.p KXA cocktail (Ketamine 42.86 mg/ml, Xylazine 8.57 mg/ml, and Acepromazine 1.43 mg/ml) and sacrificed. For Euthanasia, CO2 was used. To overexpress Cyclin D2 in the heart, mouse *Cyclin D2* cDNA under the control of the alpha MHC promoter was subcloned in the SV40 expression vector (255). Cardiac specific HA-CycD2 expression in transgenic mice was validated using western blot (data not shown). The GATA-luc mice harbor a 3X-GATA-luc construct. Expression of this reporter is detected in GATA expressing organs as the heart, liver, lung and brain (data not shown).

Co-immunoprecipitations and Western Blots. Co-immunoprecipitations assays were carried out using nuclear extracts from 293T cells overexpressing the appropriate protein as previously described (353). Western blots were done following Co-IPs or on nuclear extracts

from 293T cells overexpressing the relevant GATA4 constructs as previously described (353).

***In vitro* pull down and kinase assays.** The recombinant GST-GATA4 protein constructs were produced as previously described (43). Pull down and kinase assays were carried out as previously described (43, 150). Cdk4 and CycD2 were *in vitro* translated using the Promega TNT T7 Quick Coupled Transcription/Translation Systems kit (Cat. No: L1170) according to the manufacturers' protocol. The cdk4/CycD1 kinase was obtained from Cell Signaling (Cat. No: 7530). All experiments were repeated at least twice with different protein preps.

Statistics. The data are presented as mean ± SEM; $p<0.05$ by student t-test is considered statistically significant.

D. Results and Discussion

i. Cyclin D2 interacts specifically with GATA4 and enhances its transcriptional activity

Co-transfection of cyclin D2 and GATA4 in NIH-3T3 cells showed that cyclin D2 greatly enhances GATA4 activity on its target promoters, ANF and cyclin D2 (282, 368) (figure III.1A, left and middle panels); this effect was also observed on the minimal GATA-dependent promoter suggesting that the synergy requires only GATA-binding sites (figure III.1A, right panel). These results suggest that GATA4 could recruit cyclin D2 (CycD2) to a subset of its target promoters. To test this possibility, we performed co-immunoprecipitation assays on AD-293 cells expressing Flag-GATA4 and HA-CycD2 alone or in combination. As shown in figure III.1B, cyclin D2 was able to co-immunoprecipitate with GATA4 *in vivo* as revealed on the western blot following the co-immunoprecipitation. To further determine if cyclin D2 is able to directly interact with GATA4, *in vitro* pull-down assays were performed with GST- produced N- and C-terminal GATA-4 fusion proteins and *in vitro* translated cyclin D2. Figure III.1C shows that cyclin D2 is able to physically interact with N-terminal GATA4. We then tested whether GATA4/CycD2 interaction could also be observed with the related cyclin D1 (CycD1). Luciferase assays performed on NIH3T3 cells co-transfected with GATA4 and/or CycD1 and GATA-luc showed that CycD1 does not synergize with GATA4 on GATA-dependent promoter suggesting that this GATA/CycD synergy is specific to CycD2 (figure III.1D). This result is consistent with a recent paper showing that cardiac specific CycD1 overexpression in mice reduced GATA4 protein levels and inhibited cardiomyocyte differentiation (234). Furthermore, to check if the

CycD2/GATA interaction is a more general paradigm relevant to other cells than cardiomyocytes, we tested the ability of CycD2 to synergize with the hematopoietic GATA members, GATA 1,2 and 3 as well as the other two cardiac members, GATA5 and GATA6. Figure 1E shows that CycD2 does not synergize with any of the hematopoietic members nor with GATA5 or GATA6. This suggests that CycD2/GATA4 interaction is very specific.

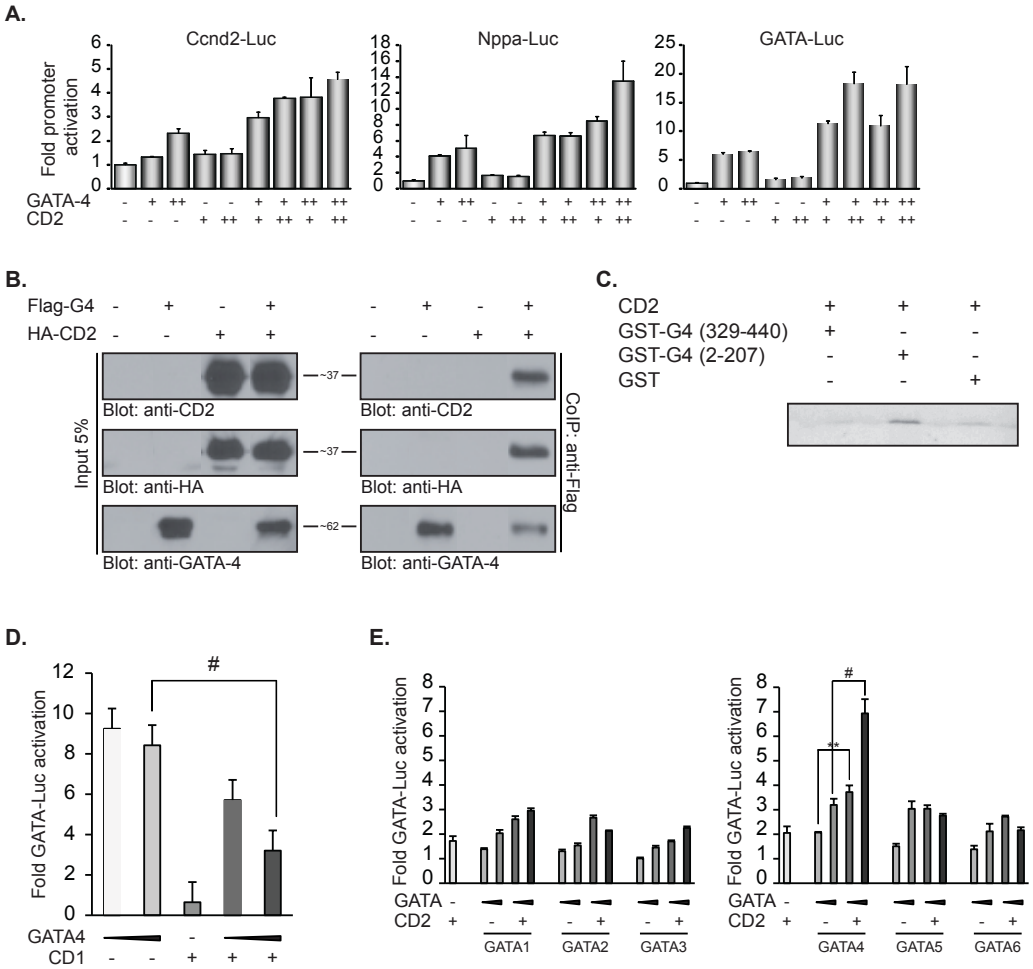


Figure III.1 GATA4 and Cyclin D interaction. A. Transient transactivation of cyclin D2, Nppa and GATA-dependent promoters by GATA4 and CycD2 in NIH3T3 cells. 250ng and 500ng of GATA4 expression vector and 3ug and 4ug of CycD2 expression vectors were used. **B.** CycD2 co-immunoprecipitates with GATA4 *in vivo*. Nuclear extracts from 293T cells transfected with Flag-GATA4 and/or HA-CycD2 expression vectors were immunoprecipitated using an anti-flag antibody, separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluride (PVDF) membranes and subjected to immunoblotting using anti-HA, anti-CycD2 or anti-GATA4 antibodies. The results here are from one representative experiment of three. **C.** CycD2 physically interacts with N-terminal GATA4 *in vitro*. *In vitro* translated radiolabeled CycD2 protein was incubated with glutathione sepharose beads containing GST alone, GST-N-terminal GATA4 or GST-C-terminal GATA4 fusion proteins. The bound proteins were then resolved by SDS-PAGE and revealed by autoradiography. Note that CycD2 binds to N-terminal GATA4 (2nd lane). The experiment is one representative of two. **D.** Transient transactivation of GATA-dependent promoter by GATA4 and CycD1 in NIH3T3 cells. 250ng and 500ng of GATA4 and 3ug and 4ug of CycD1 expression vectors were used. Note that CycD1 does not synergize but rather inhibits GATA4 activity. #p<0.005 vs. GATA4 at the specified dose. **E.** Transient transactivation of GATA-dependent promoter by GATA1,2, or 3 (left) or GATA4,5, or 6 (right) and CycD2 in NIH3T3 cells. 50ng and 100ng of GATA and 1ug of CycD2 expression vectors were used in case of GATA1,2,3; 5ng and 10ng of GATA and 0.5ug of CycD2 expression vectors were used in case of GATA4,5,6. Note a statistically significant synergy of CycD2 with GATA4 only. No synergy was seen with the other GATA members even at different doses. ** p< 0.01; # p<0.005 vs. GATA4.

ii. A discrete region in the GATA4 N-terminal is required for CycD2 synergy

In order to determine which region of GATA4 is required for its interaction with CycD2, we carried out a structure-function analysis of GATA4. Mutant proteins that harbored various C-terminal or N-terminal deletions were used. All mutants were tested for their ability to be expressed in the nucleus at similar levels and bind DNA using western blot analysis and electrophoretic mobility shift assays (EMSA) (figures III.2A and III.2B and previously described). When tested in cotransfections, N- and C-terminal GATA4 deletions reduced transcriptional activity consistent with previous reports. When the same constructs were tested for synergy with CycD2, removal of the C-terminal had very minimal effect on the synergy. Removal of the first 145 amino acids reduced but did not abrogate the synergy. However, removal of the first 174 amino acids almost completely abolished the synergy suggesting that amino acids (aa) 145-174 on the GATA4 protein are involved in the interaction with CycD2 (figure III.2C). The 145-174 aa region of GATA4 is conserved across GATA4 proteins from different species from zebrafish to human but is not present in other members of the GATA family.

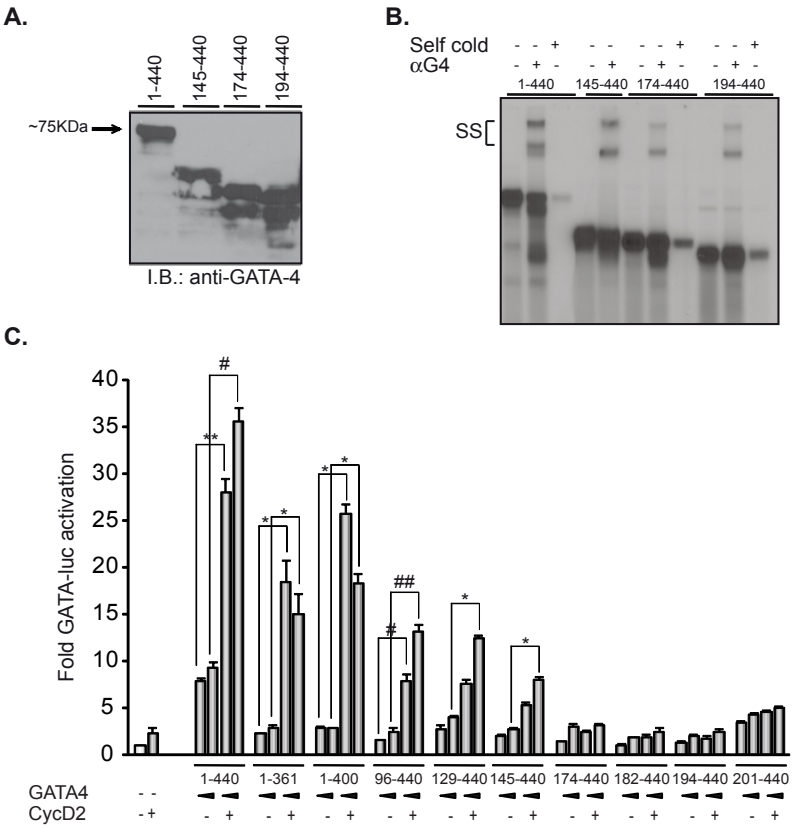


Figure III.2 Structure-function analysis. A. & B. Western blot analysis and Electromobility shift assay (EMSA) showing the expression levels and DNA-binding of the three indicated N-terminal-deleted HA-tagged recombinant GATA4 proteins. EMSA was done on nuclear extracts from 293T cells overexpressing each of the indicated GATA4 expression vectors. Supershift was performed using anti-GATA4 antibody. **C.** Structure function analysis of GATA4/CycD2 activation of the GATA-dependent promoter. Transient transfections were carried out in NIH3T3 cells using the indicated GATA4 and/or CycD2 expression vectors and the GATA-luc reporter. The results shown are those of one representative experiment of three carried out in duplicates with the standard deviation of the mean. * $p<0.05$; ** $p<0.01$; # $p<0.005$; ## $p<0.0001$ vs. GATA4.

iii. CDK4 kinase activity is essential for GATA4 transcriptional function

A schematic representation of GATA4 protein with the amino acid sequence between 145-174 is shown in figure III.3A and reveals a highly evolutionary conservation. Of interest, two conserved amino acids in this region, S160 and P163, have been reported mutated in humans with heart defects. P163S was found in patients with tetralogy of Fallot (261, 384), P163R was reported in a group of patients with atrial septal defects or ventricular septal defects (38); very recently, S160T was identified in patients with atrial fibrillation and was associated with a significant decrease in GATA4 transcriptional activity (372).

Bioinformatics analysis using the Motif Scan website (http://scansite.mit.edu/motifscan_seq.phtml) suggested that S160 may be a putative cdk phosphorylation site. To further determine if these amino acids, P163 and S160 are crucial for the CycD2/GATA4 interaction, we mutated both P163 and S160 sites on the GATA4 (129-440) protein. When co-transfected with CycD2 in NIH3T3 cells, the synergy of P163 and S160 mutants with CycD2 on the GATA-target promoter (Nppa-luc) as compared to their wild type GATA4 (129-440) was greatly reduced (figure III.3B). Mutants were tested for their ability to be expressed in the nucleus and bind DNA using western blot analysis and EMSA, respectively (figures III.3C and III.3D). Since S160 is a putative CDK phosphorylation site, this led us to suggest that phosphorylation of GATA4 at S160 may be required for its interaction with CycD2. We therefore tested if the catalytic partner of CycD2, CDK4, is able to regulate GATA4 activity. Luciferase assays performed on NIH3T3 cells co-transfected with increasing doses of GATA4 protein and GATA-Luc promoter and treated with CDK4 inhibitor showed reduced GATA4 activation of its target promoter (figure III.3E). Moreover, the effect of this inhibitor was less pronounced on the S160 mutant than on its wild type GATA4 (figure III.3F). These results suggest that CDK4 kinase activity is required for the full transcriptional function of GATA4.

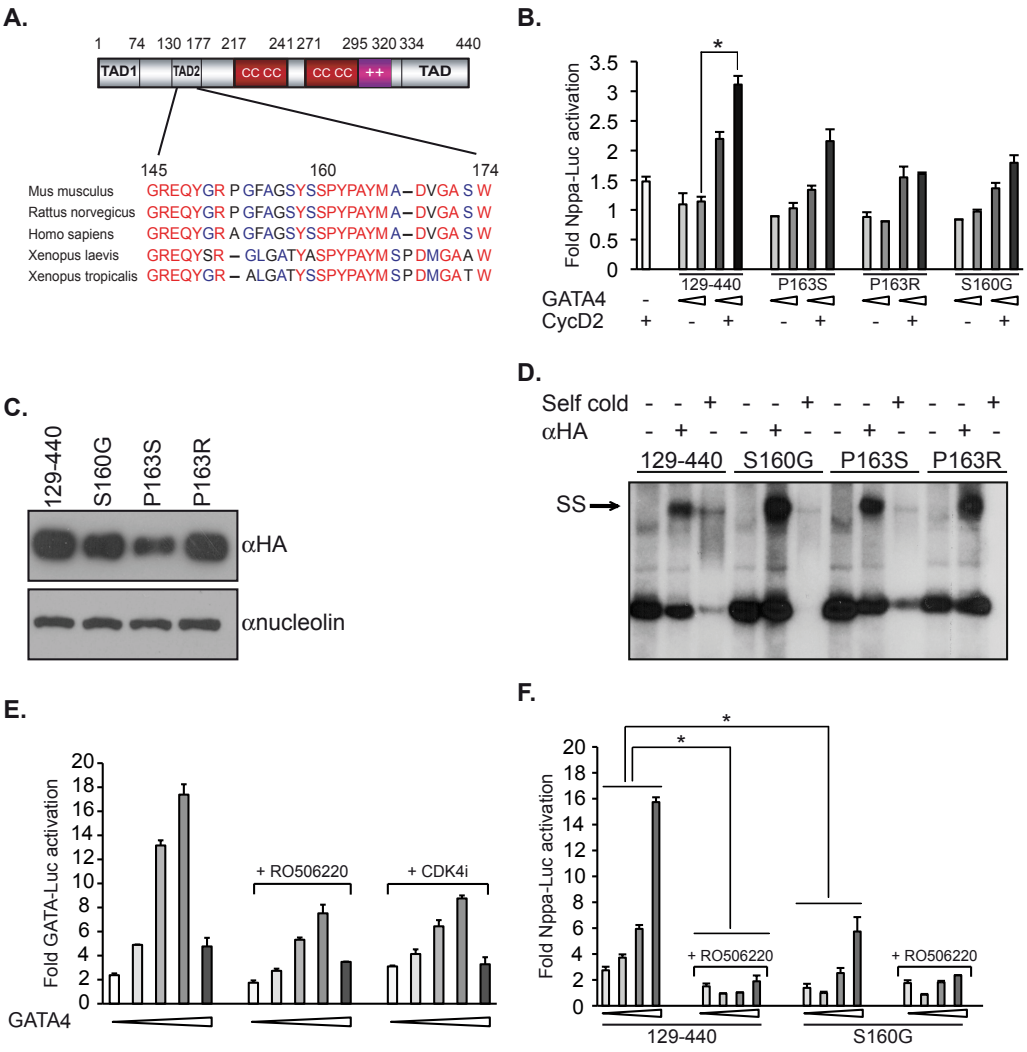


Figure III.3 A. Schematic representation of GATA4 protein. Note that region 145-174 amino acid and particularly amino acid S160 is highly conserved among the mouse, rat, human and xenopus. **B.** S160 and P163 amino acids are required for synergy with CycD2. NIH3T3 cells were transiently transfected with Nppa-Luc, CycD2, and/or the indicated GATA4 expression vectors. S160G, P163S and P163R GATA4 mutants were prepared by PCR-mediated mutagenesis. 4ug of CycD2 and 250ng and 500ng of the GATA4 expression vectors were used. Note that S160G and the human mutations P163S and P163R synergy with CycD2 are significantly reduced. * $p<0.05$ vs. 129-440. **C. & D.** Western blot analysis and Electromobility shift assay (EMSA) showing the expression levels and DNA-binding of the three indicated mutant HA-tagged recombinant GATA4 proteins. EMSA was done on nuclear extracts from 293T cells overexpressing each of the indicated GATA4 expression vectors. Supershift was performed using anti-HA antibody. **E.** Inhibition of CDK4 reduces GATA4 activity. NIH3T3 cells were transiently transfected with the GATA-dependent promoter and increasing doses of GATA4 expression vector (5, 10, 50, 100 and 500ng) with or without treatment with CDK4 inhibitor. 2uM of the indicated CDK4 inhibitors was used. The cells were treated the following day after transfection and kept for 18hrs. **F.** Effect of the CDK4 inhibitor RO506220 on S160G GATA4 mutant. NIH3T3 cells were transiently transfected with Nppa-luc and increasing doses of the indicated GATA4 expression vector (10, 50, 100 and 250ng) with or without treatment with CDK4 inhibitor as above. Note S160G activity is affected less prominently by CDK4 inhibitor RO506220 than its wild type. * $p<0.05$ vs. 129-440.

iv. CDK4 is a positive regulator of GATA4 through phosphorylation

In order to check the mechanism by which CDK4 regulates GATA4, we first tested the ability of CDK4 to physically interact with GATA4. Co-immunoprecipitation assays on AD293 cells expressing Flag-GATA4 and CDK4 followed by western blot analysis revealed that CDK4 was able to co-immunoprecipitate with GATA4 *in vivo* (figure III.4A). *In vitro* pull-down assays using GST- produced N- and C- terminal GATA4 fusion proteins and *in vitro* translated CDK4 showed that CDK4 directly interacts with C-terminal GATA4 (figure III.4B). When tested for the ability of CDK4 to phosphorylate GATA-4 using *in vitro* kinase assay -where active CDK4 kinase was incubated with N- and C-terminal GST GATA4 fusion proteins and ^{32}P- cdk4 was found to phosphorylate N-terminal GATA4. Incubation of CDK4 kinase with GST-N-terminal GATA4 harboring the S160 mutation greatly reduced this phosphorylation suggesting that S160 is a major CDK4 phosphorylation site on GATA4 protein (figure III.4C). To further elaborate the effect of CDK4 on GATA4 function, we tested its synergistic activity with GATA4 on GATA-target promoters. Luciferase assays on NIH3T3 cells co-transfected with GATA4 and CDK4 revealed that CDK4 enhances GATA4 activity on its target promoter (GATA-Luc) (figure III.4D). Cyclin Ds were shown to interact with their catalytic partners CDK4/6 through a lysine residue in the cyclin box (50). To check if interaction of CycD2 with GATA4 requires the CDK4 binding site on CycD2, we generated a K112 CycD2 mutant. When co-transfected with GATA4 in NIH3T3 cells, this mutant was able to synergize with GATA4 on GATA-target promoter in a manner similar to wild type CycD2 (figure III.4E). Moreover, no triple synergy was seen between CycD2, CDK4 and GATA4 on activation of GATA-luc promoter (figure III.4F). These results suggest that CDK4 positively regulates GATA4 transcriptional activity through

phosphorylation. However, the interaction of CycD2 with its catalytic partner CDK4 is not required for the CycD2/GATA4 synergy.

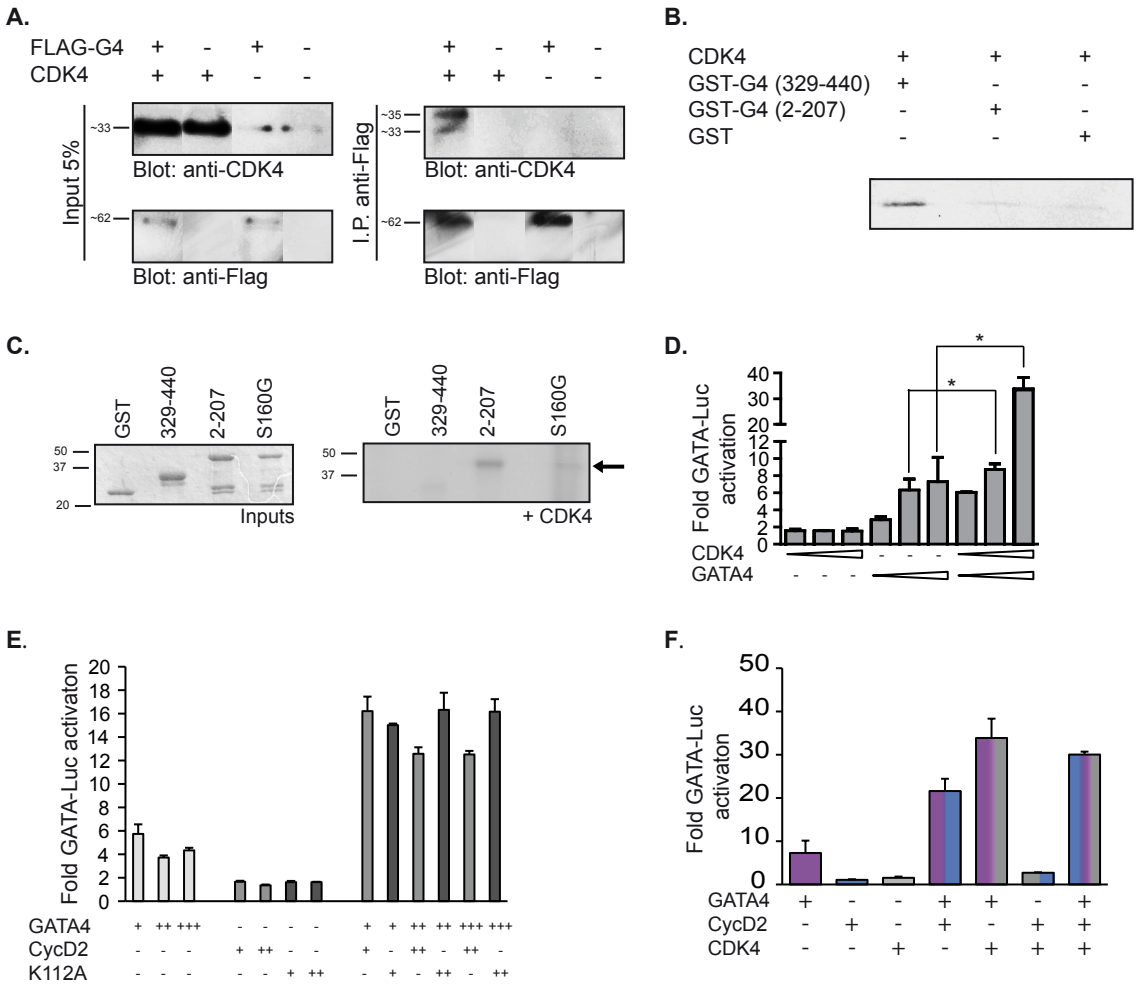


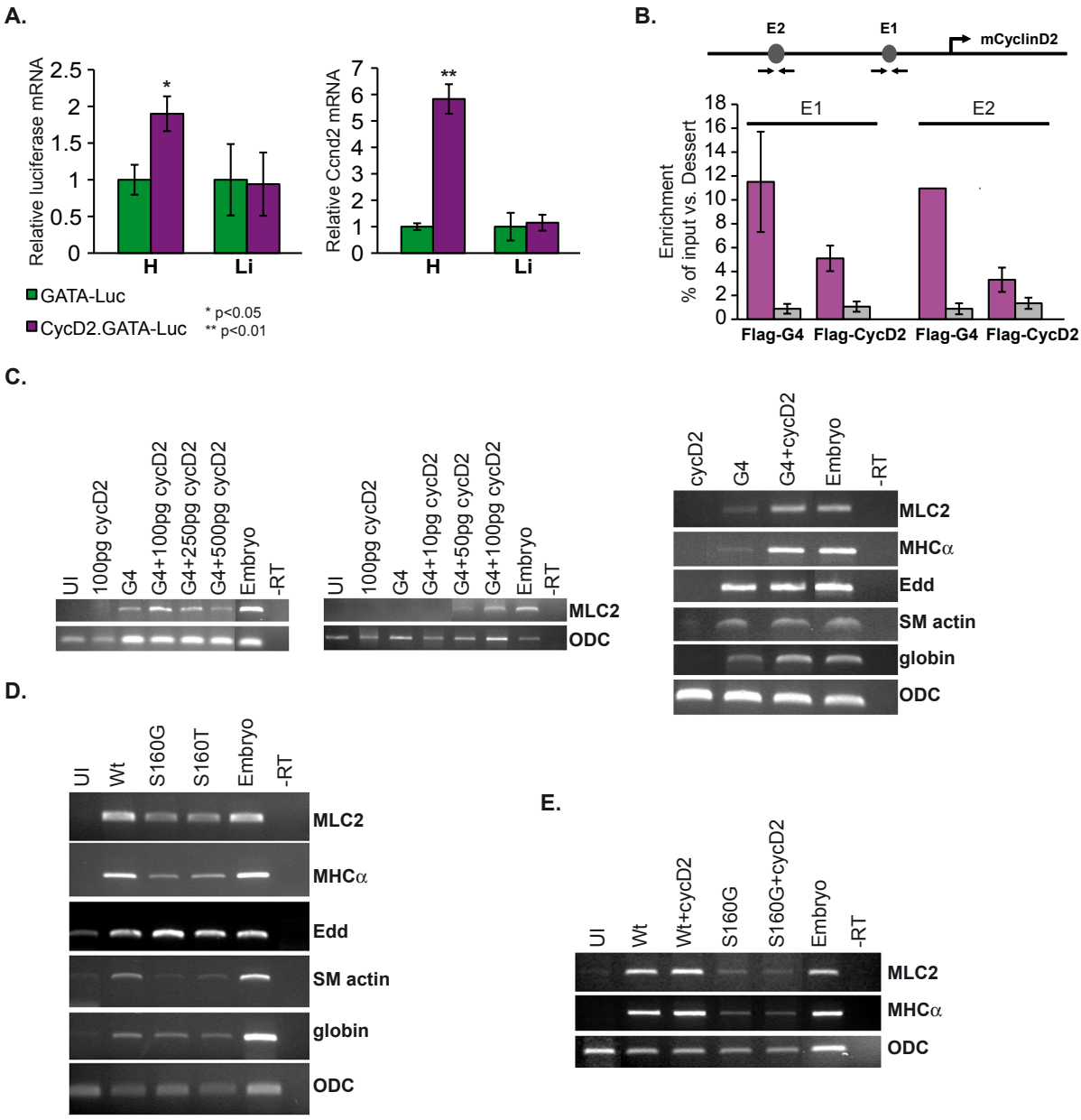
Figure III.4 GATA4 and CDK4 interaction. A. CDK4 co-immunoprecipitates with GATA4 *in vivo*. Nuclear extracts from 293T cells transfected with Flag-GATA4 and/or CDK4 expression vectors were immunoprecipitated using an anti-flag antibody, separated on SDS-PAGE transferred to PVDF membranes and subjected to immunoblotting using anti-CDK4 or anti-flag antibodies. The experiment is one representative of two. **B.** CDK4 physically interacts with C-terminal GATA4 *in vitro*. *In vitro* translated radiolabeled CDK4 protein was incubated with glutathione sepharose beads containing GST alone, GST-N-terminal GATA4 or GST-C-terminal GATA4 fusion proteins. The bound proteins were then resolved by SDS-PAGE and revealed by autoradiography. Note that CDK4 binds to C-terminal GATA4 (1st lane). The experiment is one representative of two. **C.** *In vitro* CDK4 phosphoryaltion of GST-GATA4 fusion proteins. Active CDK4 kinase was able to phosphorylate N-terminal GST-GATA4 (2-207) fusion protein (black arrow) but not GST- alone, nor C-terminal GST-GATA4 (329-440) nor N-terminal GATA4 harboring the S160G mutation. The experiment is one representative of three **D.** CDK4 enhances GATA4 transcriptional activity on GATA-dependent promoter. NIH3T3 cells were transiently transfected with GATA-Luc, CDK4 (5, 10 or 50ng), and/or GATA4 (5, 10 or 25ng) expression vectors. * $p<0.05$ vs. GATA4. **E.** CycD2/GATA4 synergy does not require cdk- binding site. NIH3T3 cells were transiently transfected with GATA-Luc, GATA4, and/or CycD2 expression vectors. K112A CycD2 mutant was prepared by PCR-mediated mutagenesis. 50ng, 250ng and 500ng of GATA4 expression vector and 1ug and 4ug of CycD2 and K112A expression vectors were used. **F.** Lack of triple synergy between CycD2, CDK4 and GATA4. NIH3T3 cells were transiently transfected with GATA-luc, CDK4 (50ng) and/or GATA4 (25ng) and/or CycD2 (50ng) expression vectors.

v. CycD2 associates with GATA4 *in vivo* and enhances its cardiogenic function

To test for an *in vivo* association of GATA4 with CycD2, GATA-luc transgenic mice were crossed with mice overexpressing cyclin D2 specifically in the heart (under the control of α-MHC promoter). QPCR analysis revealed that GATA-luc mice with a cardiac specific cyclin D2 overexpression have a two-fold increase in the level of luciferase transcript specifically in the heart relative to the GATA-luc mice (figure III.5A, right panel). This increase was not observed in the liver. To further confirm that CycD2 associates with GATA binding sites on target GATA promoters, we performed chromatin immunoprecipitation (ChIP) assays on the endogenous ccnd2 gene using C2C12 cell lines with stable overexpression of Flag-CycD2. Previously, we showed that GATA4 is enriched on the Ccnd2 promoter (368). We tested whether the same fragments are associated with CycD2. As shown in figure III.5B, flag-CycD2 was enriched 5.1 and 3.3 folds on the -99 and -299 GATA sites respectively (-355 and -554 from ATG respectively) on cyclin D2 promoter with respect to the negative gene *Dessert* as compared to 1.06 and 1.34 folds of IgG enrichment, respectively. Consistent with our previous results, GATA4 is enriched at both sites on the cyclin D2 promoter as well.

To check the biological consequences of CycD2/GATA4 interaction on cardiogenesis, we examined the ability of cycD2 to alter the activity of GATA4 in ectodermal (animal cap) explants from *Xenopus* embryos. GATA4 is known to induce cardiogenesis in pluripotent animal cap explants, providing a powerful/useful assay/platform for modulators of this activity (83). To enhance detection of anticipated positive effect of cycD2 on activity of GATA4, we used suboptimal amount of GATA4 mRNA (300pg;

optimal range is 400-1000pg) together with a range of cycD2 mRNA concentrations from 10-500pg. As shown in figure III.5C (right panel), cycD2 mRNA specifically stimulated the cardiogenic activity of GATA4 as evident by the induction of the cardiomyocyte specific markers, MLC2 and αMHC but not the endodermal marker endodermin (Edd), the smooth muscle marker (SM actin) or the hematopoietic marker (globin). Moreover, and consistent with its reduced transcriptional activity (figure III.3B and F), GATA4 S160 mutation that abrogates cdk4 phosphorylation and CycD2 synergy decreased GATA4-dependent induction of cardiac markers (figure III.5D) and abrogated the ability of cycD2 to potentiate GATA4 cardiogenesis (figure III.5E).


A.
Relative luciferase mRNA
2.5
2
1.5
1
0.5
0
*
H
Li
GATA-Luc
CycD2.GATA-Luc
* p<0.05
** p<0.01
Relative Ccnd2 mRNA
7
6
5
4
3
2
1
0
**
H
Li
B.
E2
E1
mCyclinD2
E1
E2
Enrichment
% of input vs. Dessert
18
16
14
12
10
8
6
4
2
0
Flag-G4
Flag-CycD2
Flag-G4
Flag-CycD2
C.
UI
100pg cycD2
G4
G4+100pg cycD2
G4+250pg cycD2
G4+500pg cycD2
Embryo
-RT
UI
100pg cycD2
G4
G4+10pg cycD2
G4+50pg cycD2
G4+100pg cycD2
Embryo
-RT
MLC2
ODC
cycD2
G4
G4+cycD2
Embryo
-RT
MLC2
MHCα
Edd
SM actin
globin
ODC
D.
UI
Wt
S160G
S160T
Embryo
-RT
MLC2
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SM actin
globin
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Wt+cycD2
S160G
S160G+cycD2
Embryo
-RT
MLC2
MHCα
ODC

Figure III.5 GATA4 associates with CycD2 *in vivo.* **A.** GATA-luc transgenic mice were crossed with HA-CycD2 mice. Total RNA was isolated from heart and liver tissues of P3-day-old mice using QIAzol lysis reagent (Qiagen). Transcript levels of the various markers were determined by real-time PCR as previously described. Note the increase of luciferase mRNA levels of CycD2.GATA-Luc mice relative to those of GATA-luc mice specifically in the heart (right panel). Left panel is a control to show the overexpression of cyclin D2 in the hearts of CycD2.GATA-luc mice but not in the liver. The results are shown as the mean +/- SEM (n=3). **B.** Enrichment of CycD2 and GATA4 on cyclin D2 promoter as revealed by ChIP. *Dessert* is used as a negative gene. IgG is a negative control. Flag-GATA4 and Flag-CycD2 C2C12 stable cell lines were used. The results are the mean +/- SEM (n=3). Oligos used: *Cyclin D2* E1: 5'- GGGATCGTGTTTGAAGTTTG-3' (sense) and 5'-AGATCTCCTCCCCCTCTCAG-3' (antisense); *Cyclin D2 E2:* 5'-CTTTATGCCCCCATGGTATG-3' (sense) and 5'-GGCCTGACCAAACTTCAAAC-3' (antisense); *Dessert:* 5'- TCCTCCCCATCTGTGTCATC-3' (sense) and 5'- GGATCCATCACCATCAATAACC-3' (antisense). **C. Left and middle panels:** CycD2 stimulates cardiogenic activity of rGATA4 in dose dependent manner. Suboptimal dose of rGATA4 (300 pg) was co-injected with a high range (left panel) or low range (middle panel) of cyclin D2 mRNA. Animal cap explants were cultured until st. 34 and expression of cardiomyocyte marker MLC2 and ubiquitously expressed ODC were determined by RT-PCR. **Right panel:** Stimulation of GATA4 activity by CycD2 is restricted to induction of cardiac but not other cell fates. Animal cap explants injected with 100pg CycD2 mRNA, 300pg GATA4 mRNA, or the two combined, were analyzed for expression of indicated markers at st. 34. MLC2 and MHCa are exclusively expressed in cardiac myocytes, endodermin (Edd) is an endodermal marker, SM actin marks smooth muscle and globin is a marker of blood. **D.** S160 is important for GATA4 activity. 300pg of the different GATA constructs were injected. Animal explants were cutlutred as abovel and expression of indicated markers were determined by RT-PCR. **E.** S160 is important for stimulation of GATA4 activity by CycD2. Animal explants were injected with 100pg CycD2 mRNA with/without 300pg of the indicated GATA4 construct. Expression of MLC2, MHCα and ODC were determined by RT-PCR.

Collectively these results indicate that cyclin D2 but not cyclin D1 is a coactivator of GATA4 in cardiogenesis. Different interacting partners have been reported for CycD1 and CycD3 in different tissues. For instance, CycD1 has been shown to interact with the histone acetyl transferase P/CAF (p300/CREB-binding protein-associated protein), thus fascilitating the interaction of P/CAF with the estrogen resceptor (ER) and possibly providing a mechanism for CycD1 stimlated ER transcriptional activity and oncogenic potential in breast cancer (202, 241). The zinc finger transcription factor INSM1 has also been shown to bind to CycD1 leading to cell cycle arrest and inhibition of proliferation thereby suggesting a possible mechanism for pancreatic endocrine cell differentiation where INSM1 plays an important role (383). CycD3 was shown to be a coactivator of the nuclear receptor PPARγ (peroxisome proliferator-activated receptor gamma) in adipogenesis (289). On the other hand, CycD1 has been reported to inhibit PPARγ-mediated adipogenesis via HDAC recruitment (80). CycD1/CDK4 was also shown to phosphorylate and inhibit the DNA binding ability of BRCA1 in breast cancer cells (138). Our results therefore provide a mechanism for cell specific effects of members of the cyclin D family.

Co-transfection of CycD2 and GATA4 in NIH3T3 cells revealed that both proteins synergize to activate various GATA4 target promoters. Our data show that CycD2 interacts physically with the 2nd N-terminal TAD of GATA4 that spans aa 130-177 (83). CycD2 contacts the region between amino acids 145 and 174, a region highly conserved across species. Recently, we identified a 24 aa region (aa 129-152) within this TAD essential for the cardiogenic function of GATA4 and for its physical interaction and synergistic transcriptional activity with BAF60c. CycD2/GATA4 interaction requires phosphorylation of

GATA4 by the CycD2 catalytic partner CDK4 at the amino acid S160. S160 is highly evolutionary conserved and its mutation has been linked to congenital heart disease in humans (372). This raises the intriguing possibility that reduced CycD2 interaction may be the molecular basis understanding the link between S160 mutation and CHD.

Finally, our results identify a GATA-CycD2 feedback loop where CycD2 is a direct transcriptional target (368) and coactivator of GATA4 in myocyte growth.

IV. Chapter III: Essential Role for KLF13 in Heart Development

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2- Emory University, School of Medicine, Atlanta, USA
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4- Institut de Recherches Cliniques de Montréal (IRCM), Montréal (Québec), Canada

Author Contribution

In the present manuscript, I performed all the cellular and molecular analyses of the *Klf13*$^{-/-}$ embryos and neonates. The *Klf13*$^{-/-}$ knockout mice were previously generated by other members of the lab. I also wrote the manuscript.

A. Abstract

KLF13 is a member of the Krüppel-like transcription factors that are important regulators of cell proliferation and differentiation. KLF13 is highly enriched in the developing heart where it is found in both myocardial and endocardial cells. In myocytes, it interacts with GATA4 and regulates the A- and B-type natriuretic peptide genes, Nppa and Nppb. Knock down of *Klf13* in *Xenopus* causes developmental heart defects, suggestive of an important role in heart morphogenesis. To test whether this role is evolutionary conserved in the mammalian heart, we deleted the Klf13 gene in transgenic mice using homologous recombination. Mice lacking both Klf13 alleles are born at reduced frequency owing to severe heart defects. Variable cardiac phenotypes are observed in Klf13 null mice and most are endocardial cushion defects including "Goose-neck" deformity and atrioventricular (AV) valvular abnormalities. Epithelial-mesenchymal transformation (EMT) is altered in these mice with reduced endothelial cell proliferation and enhanced mesenchymal differentiation in the AV cushion. Surviving Klf13 null mice have several structural cardiac anomalies including cardiomyopathies and aortic valve stenosis; expression levels of several cardiac genes are also altered. Our data uncover a role for a new class of transcription factors in heart formation and point to KLF13 as a potential congenital heart disease causing gene in human.

B. Introduction

Congenital heart diseases (CHD) account for the loss of around 25% of human embryos and around 1-2% of newborns have some form of cardiac malformations (30, 239). Despite the significant advances of the past two decades that have shed more light on our understanding of cardiac morphogenesis, few CHD-causing genes have been identified to date and the genetic basis of the majority of familial congenital heart diseases remain unelucidated.

Cardiac morphogenesis is a complex process where by multiple lineages contribute to a properly developed heart. The first or primary heart field (PHF) gives rise mainly to the left ventricle and most of the atria; the secondary or anterior heart field (AHF) contributes to the right ventricle, outflow tract (OFT) and parts of the atria (283, 314). One important step that takes place during heart development is epithelial-mesenchymal transformation (EMT). This is crucial for the formation of the cardiac cushions that ultimately give rise to the cardiac valves. An extracellular matrix or cardiac jelly (composed of elastin, collagen, and glycosaminoglycans) separates the endocardial from the myocardial layer in the primary heart tube. A subpopulation of cells from the endocardial layer in the atrioventricular canal (AVC) and OFT, in response to inductive signals from the overlying myocardium, undergoes EMT, thus, cellularizing the cardiac jelly and forming the endocardial cushions (EC) (195). Various signaling molecules govern this process of cardiac valve formation including VEGF, Notch, wnt/β-catenin, TGF-β, BMP and hyaluronic acid signaling (8). Malformation of the atrioventricular canal cushions result in atrioventricular septal defects (AVSDs) or endocardial cushion defects. One type of these defects is “Goose-neck deformity” that can be seen on a left ventricular angiography. It is characterized by an abnormal apical positioning

of the anterior mitral valve leaflet that encroaches on the left ventricular outflow tract resulting in a narrowing of its lumen and an anterior displacement of the aorta. The distance between the mitral valve and the apex is abnormally shorter than that between the aortic valve and the apex due to the deficiency of the IVS (23, 75, 340). Non-membranous VSDs and AV valve defects can also be observed in association with "Goose-neck deformity." The molecular basis underlying this deformity is unclear and no gene has been linked with this valvular abnormality. AVSDs account for 5% of all CHDs and are associated mainly with Down syndrome. Isolated cases are also present as an autosomal dominant trait (275, 379).

KLF13 has recently been identified as a novel regulator of cardiogenesis (165). It belongs to the Kruppel-like family of transcription factors that were first identified by virtue of their similarity to Sp1, one of the first factors cloned and characterized in mammals. 21 KLF members have been identified in humans so far, of which 17 homologs are found in mice (129). They contain three highly conserved Cys_2/His_2 zinc fingers in the C-terminal region connected by a highly conserved Kruppel-link seven-amino acid sequence [TGEKP(Y/F)X]. The three zinc fingers confer specificity to KLFs to bind the consensus *GC*- and *CACCC* DNA boxes (20, 55). KLF13 is expressed in the heart and the cephalic mesenchyme of the developing mouse embryo (196). A detailed spatiotemporal expression analysis of KLF13 in the developing mouse heart by Lavalleé *et al* shows that E9.5 embryos have strong expression in the heart and the epidermis. At E10.5, KLF13 is highly detected in the atrial myocardium and the endocardium. By E12.5, its expression is stronger in the atria yet is also highly detected in the ventricular trabeculae. KLF13 is downregulated in the postnatal heart where the highest levels are found in the interventricular septum and the atrioventricular valves (165). A similar expression pattern is also observed in the *Xenopus*

(165). Knockdown of *xKlf13* in *Xenopus* results in a cardiac phenotype. Although early heart formation up to the heart tube stage appears normal in these embryos, several defects are observed shortly after looping and chamber formation. Among others, the single ventricle is often hypoplastic, atrioventricular cushion formation and valve maturation is delayed and several cardiac markers are altered in these knock down embryos (165); these findings are suggestive of a role of KLF13 in amphibian heart morphogenesis (165).

In this study, we tested the role of KLF13 in mammalian cardiogenesis. We show that Klf13 null mice have defective heart morphogenesis and postnatal viability. Endocardial cushion defects including "Goose-neck" deformity and AV valvular anomalies are observed. The mechanism underlying these defects involves altered EMT with reduced endothelial cell proliferation and excessive mesenchymal differentiation in the AV cushion. Surviving $Klf13^{-/-}$ mice have several structural cardiac abnormalities and the expression of several genes involved in EMT is altered. Our data point to a novel role of KLF13 in endocardial cell proliferation and heart formation and identify KLF13 as a potential CHD causing gene.

C. Materials and Methods

Animals. All animal experimentations were carried out in accordance with institutional guidelines for animal care. Experiments were approved by the International Animal Care and Use Committee (IACUC) of the University of Ottawa, which conforms to that of the US National Institute of Health (NIH) (Assurance number for University of Ottawa: A5043-01). At end points, mice were anesthetized with 2.2µl/g i.p KXA cocktail (Ketamine 42.86 mg/ml, Xylazine 8.57 mg/ml, and Acepromazine 1.43 mg/ml) and sacrificed. For Euthanasia, CO2 and cervical dislocation (CD) was used. CD without prior anesthesia is required for embryo collection in mice.

Generation of Klf13 knockout mice. The knockout mice were generated by targeted deletion of the 2nd exon. A schematic representation of the knockout strategy is described in the results. The mice were kept on the C57/BL6 background. Pregnant mothers or newborn litters were sacrificed at various embryonic and postnatal time points. The morning a vaginal plug was observed was considered as embryonic day (E) 0.5.

Histology. Adult heart tissues or staged embryos were fixed in 4% paraformaldehyde at 4^{o}C overnight, processed, paraffin embedded and sectioned at 4µm intervals as previously described (237). Masson Trichrome and Alcian blue staining were done as previously described (90, 161).

Echocardiography anaylsis. Mice were anesthetized (2.0% isoflurane, 80 ml/min 100% O_2) and two-dimensional guided M-mode echocardiography was performed using a Visual-

Sonics VEVO 700 and a 30-MHz linear array transducer as described by Aries *et al.*, 2004 (7).

Real-Time PCR analysis. Total RNA was isolated from mouse tissues using TRIZOL reagent (Invitrogen). Transcript levels of the various markers were determined by real-time PCR as previously described (58).

Immunohistochemistry. Immunohistochemistry on heart sections from each mouse group was performed as previously described (7). The antibodies used were: Ki67 (Clone SP6, Rabbit Monoclonal, Thermo Scientific RM-9106-S0), phospho histone H3 (Ser 10, Rabbit polyclonal, Millipore 06-570).

Cell count. Image J software was used to count the endocardial and mesenchymal cushion cells.

Statistics. The data are presented as mean ± SEM; $p<0.05$ by student two-tailed *t*-test is considered statistically significant.

D. Results

i. Klf13$^{-/-}$ mice have a cardiac phenotype.

The mouse Klf13 gene consists of two exons. We generated the Klf13$^{-/-}$ mice through targeted deletion of exon 2, which encodes the 2nd and 3rd zinc fingers and the entire C-terminus. The targeting vector was produced through homologous recombination in ES cells (figure IV.1A). Southern blot analysis confirmed the presence of the targeted allele in ES cells (figure IV.1B and data not shown) and PCR analysis verified the existence of the wild type (Wt) and mutant alleles (data not shown). Klf13 heterozygous mice were intercrossed to generate Klf13$^{-/-}$ mice and were kept on a C57BL/6 genetic background. Quantitative PCR analysis (QPCR) confirmed the deletion of exon 2 in the knockout mice. Transcript levels from the first coding exon were reduced by 80% (figure IV.1C).

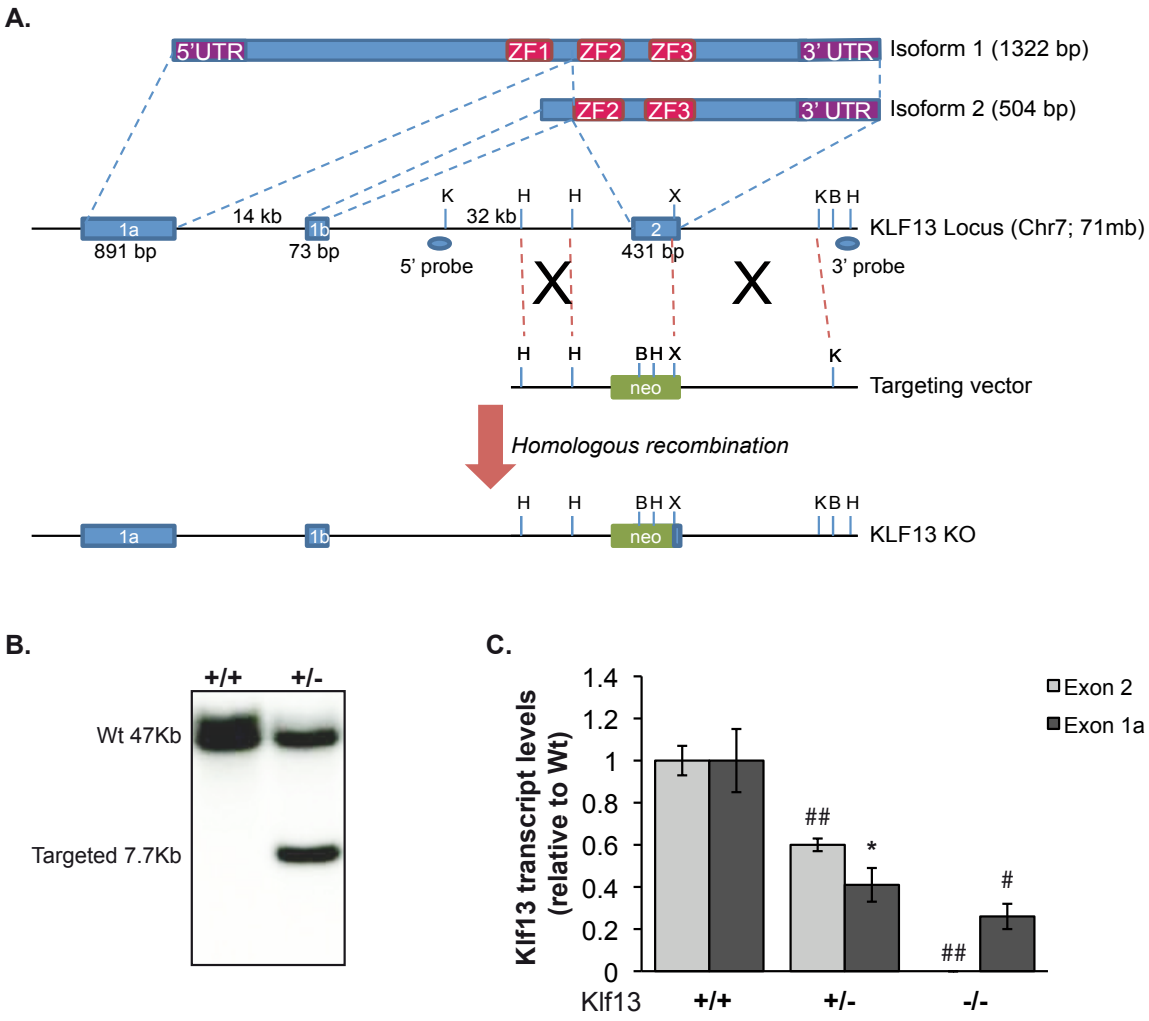


Figure IV.1 Generation of Klf13-null allele. A. Klf13 locus and targeting strategy. Positions of the 5' and 3' probes used in southern blot are shown. Homologous recombination deletes most of exon2 replacing it with a neo cassette. ZF: zinc finger; UTR: untranslated region; Wt: wild type. **B.** Southern blot analysis of targeted ES cells. Genomic DNA was digested with Hpa1 and hybridized to the 3' probe. **C.** QPCR analysis of Klf13 transcripts in hearts of adult mice. Results confirm complete absence of exon 2 and 80% reduction of exon 1a in Klf13$^{-/-}$ mice. TBP was used as an internal control. * $p<0.05$; # $p<0.005$; ## $p<0.0001$ vs. *Klf13*$^{+/+}$.

First, we examined the survival of the Klf13 null mice. Even though they were viable, knockout mice were born at almost half the expected Mendelian ratios. Three to five litters were analyzed at each stage (figure IV.2A-C). Anatomical examination revealed a cardiac phenotype in a subset of the surviving mutant mice: right ventricular dilation and left ventricular hypertrophy were observed (figure IV.2D). Quantitative PCR (QPCR) analysis on RNA from adult hearts showed that the expression levels of various cardiac genes were

altered in Klf13$^{-/-}$ mice as compared to wild types: ACTA1, SERCA, HAND2 and GATA4 transcript levels were significantly increased (figure IV.2E) (n=5 for Wt; n=6 for Klf13$^{+/-}$ and Klf13$^{-/-}$). Further examination by echocardiography analysis revealed a statistically significant increase in the mean pressure gradient across the aortic valve in ~ 60% of the mutant mice (figure IV.3F). Other cardiac functions appeared normal (figure IV.3A-E) (n=6 for Wt, n=7 for Klf13$^{-/-}$). These results suggest that a subset of the Klf13 null mice suffer from aortic valve disease and/or left ventricular dysfunction.

A.

Wt/KO X KO/KO

E18.5	+/-	-/-
Expected	50%	50%
Observed	48.6%	51.4%

B.

Wt/KO X Wt/KO

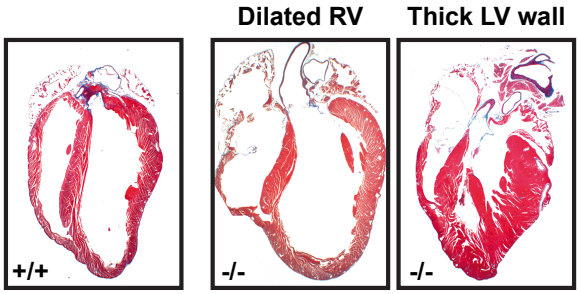
P0	+/+	+/-	-/-
Expected	25%	50%	25%
Observed	43.3%	40%	16.7%

C.

Wt/KO X Wt/KO

Postnatal	+/+	+/-	-/-
Expected	25%	50%	25%
Observed	31.6%	54.5%	14.0%

D.



E.

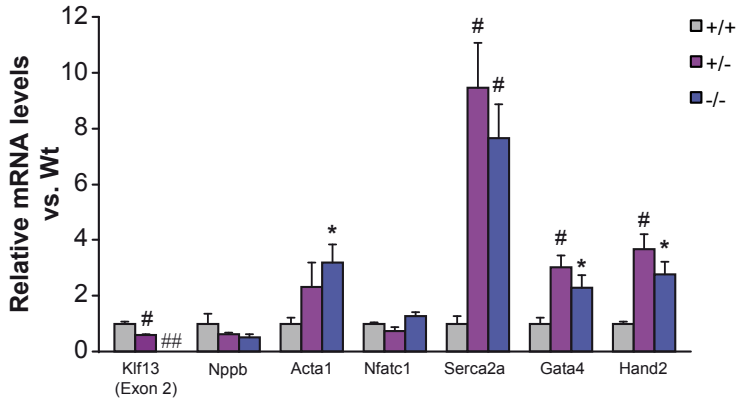


Figure IV.2 Postnatal death in Klf13$^{-/-}$ mice. A-C. Expected Mendelian ratios of Klf13 genotypes at E18.5, P0 and postnatally. Three to five litters were used at each stage. Note the lower than expected ratios of Klf13$^{-/-}$ at P0 and postnatally. **D.** Trichrome staining of whole heart frontal sections of Klf13$^{+/+}$ and Klf13$^{-/-}$ mice. Dilation of the right ventricle and thickening of the left ventricular wall were seen in a subset of the analyzed Klf13$^{-/-}$ mice. RV: right ventricle; LV: left ventricle. **E.** QPCR analysis using RNA from heart tissue of ~100-day-old mice (n=5-6 for each group). Note the statistically significant change in the transcript levels of ACTA1, SERCA2A, GATA4 and HAND2. *p<0.05; #p<0.005; ##p<0.0001 vs. Klf13$^{+/+}$.

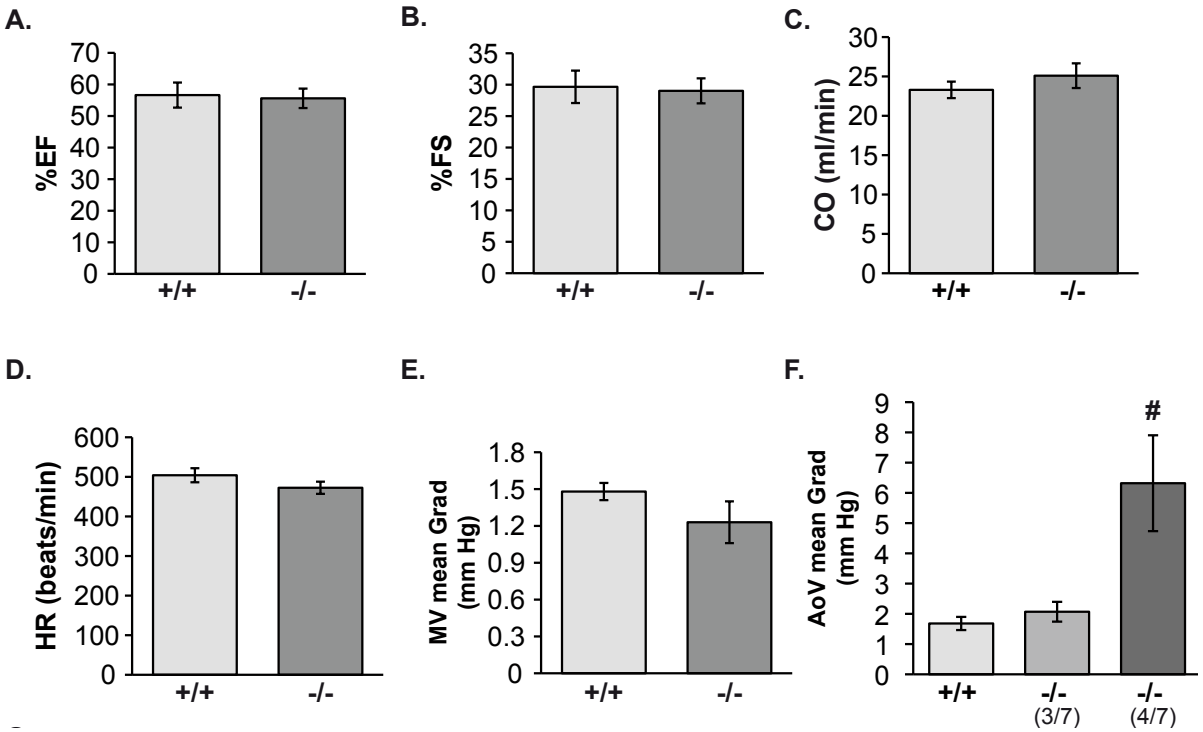


Figure IV.3 Aortic valve defects in Klf13$^{-/-}$ mice A-F Echocardiography analysis of 265-day-old Klf13$^{+/+}$ and Klf13$^{-/-}$ mice (n=6 Wt; n=7 KO). Note the statistically significant increase in the mean pressure gradient across the aortic valve of a subset of the Klf13$^{-/-}$ mice. # $p<0.005$ vs. Klf13$^{+/+}$. EF: ejection fraction; FS: fractional shortening; CO: cardiac output; HR: heart rate; MV: mitral valve; AoV: aortic valve; Grad: gradient.

ii. AVC and myocardial defects in Klf13$^{-/-}$ neonates.

As mentioned earlier, up till embryonic day 18.5 (E18.5), Klf13$^{-/-}$ mice were obtained at normal Mendelian ratios. Around 35% of the mice die shortly after birth at postnatal day 0.5 (P0.5) and only half of them survive to adulthood. Consequently, we analyzed the hearts at P0; hence, including in our analysis those that don't make it to adulthood. Anatomical examination revealed variable penetrance of myocardial and atrioventricular cushion (AVC) defects in these mice with higher incidences of AVC defects (n=4 for Wt; n=27 for Klf13$^{-/-}$) (refer to table 1 for percentages of incidence). Serial sections were thoroughly examined to confirm the observed phenotypes. Among the myocardial defects seen were ventricular hypertrophy (figure IV.4E-F); right ventricular dilation (figure IV.4I), right atrial dilation and right atrial hypertrophy (figure IV.4K-L). The AVC defects observed were thick AV

valves (figure IV.4D) and "Goose-neck" deformity (figure IV.4G) which was sometimes accompanied by a non-membranous or AV type VSD (AVSD) (figure IV.4H). Ventricular hypertrophy and right atrial dilation were also observed at earlier stages of development E13.5-14.5 (figure IV.5B and IV.5D) (n=6 for Wt; n=14 for $Klf13^{-/-}$). These results point to a defective endocardial and/or myocardial development in these mice. Note that the dilation of the right atria in some cases and its hypertrophy in others point to dysregulated proliferation in the hearts of these mice. No cardiac defects were observed in $Klf13^{+/-}$ neonates (n=6) (data not shown).

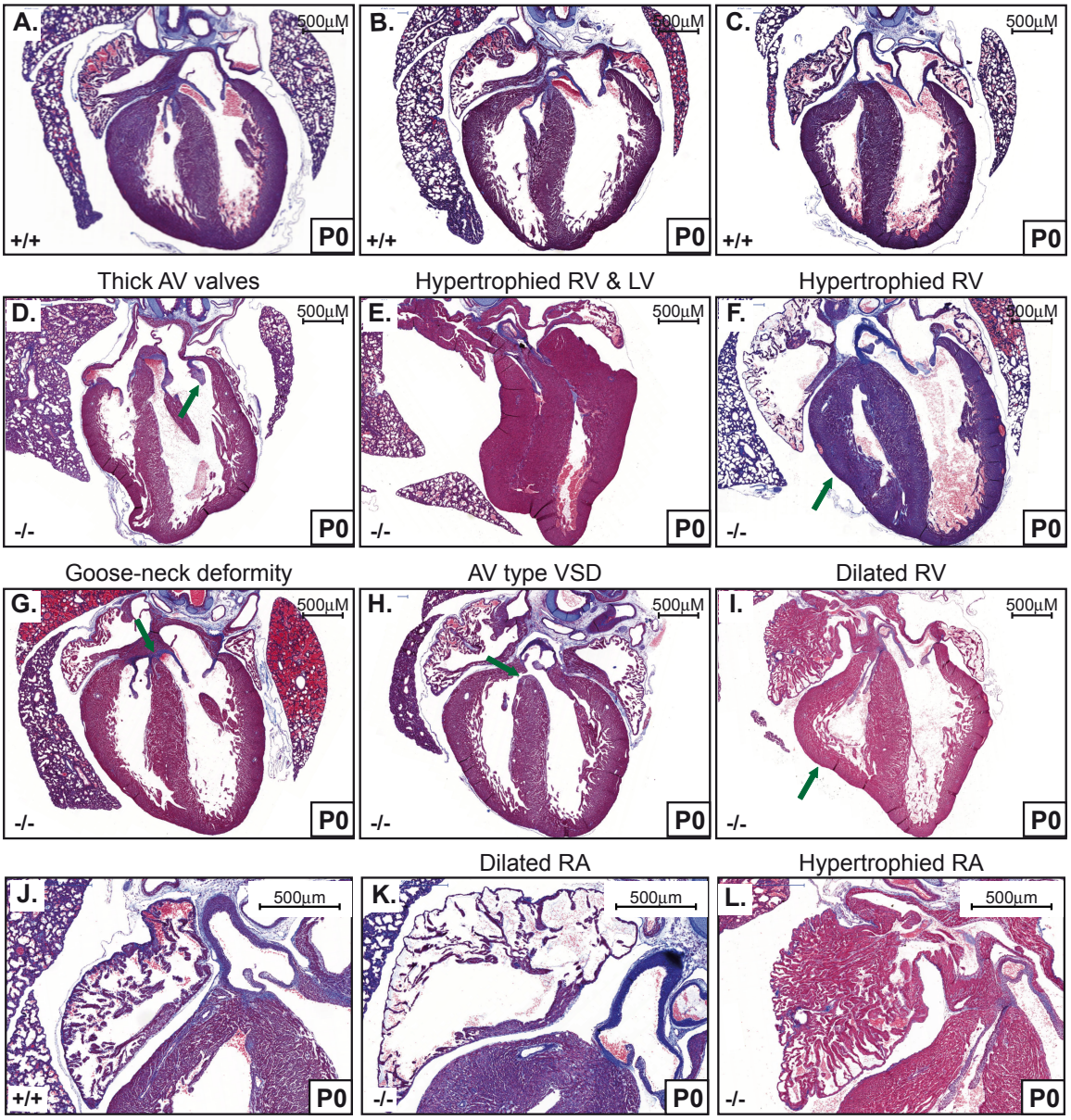


Figure IV.4 Variable cardiac morphogenetic defects in Klf13$^{-/-}$ neonates. A-L Trichrome staining of frontal heart sections of Klf13$^{+/+}$ and Klf13$^{-/-}$ P0 mice. Note the variable cardiac defects in the Klf13$^{-/-}$ neonates (D-L). AV: atrioventricular; VSD: ventricular septal defect; RA: right atrium.

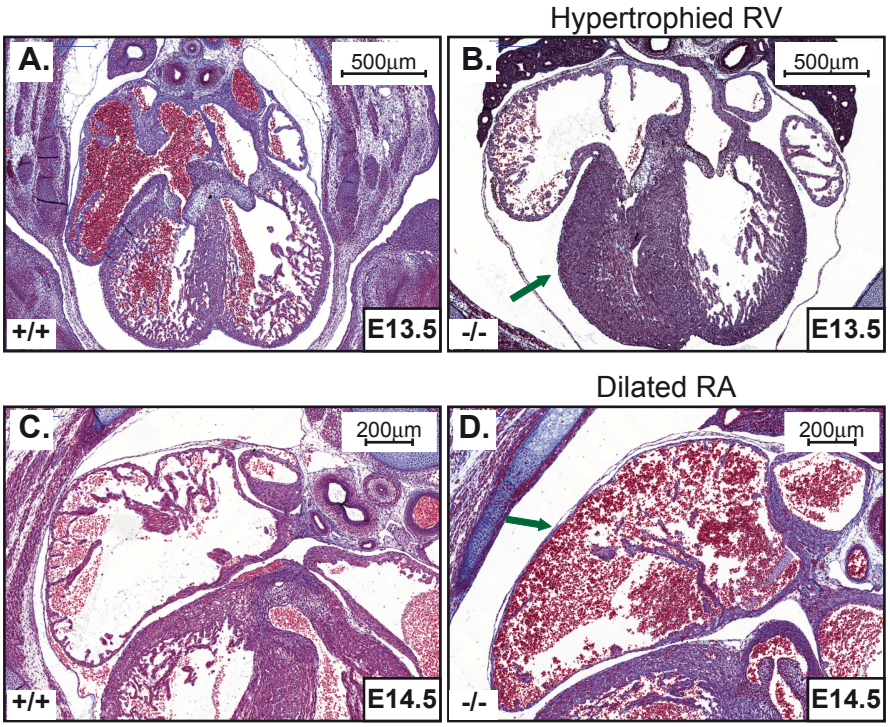
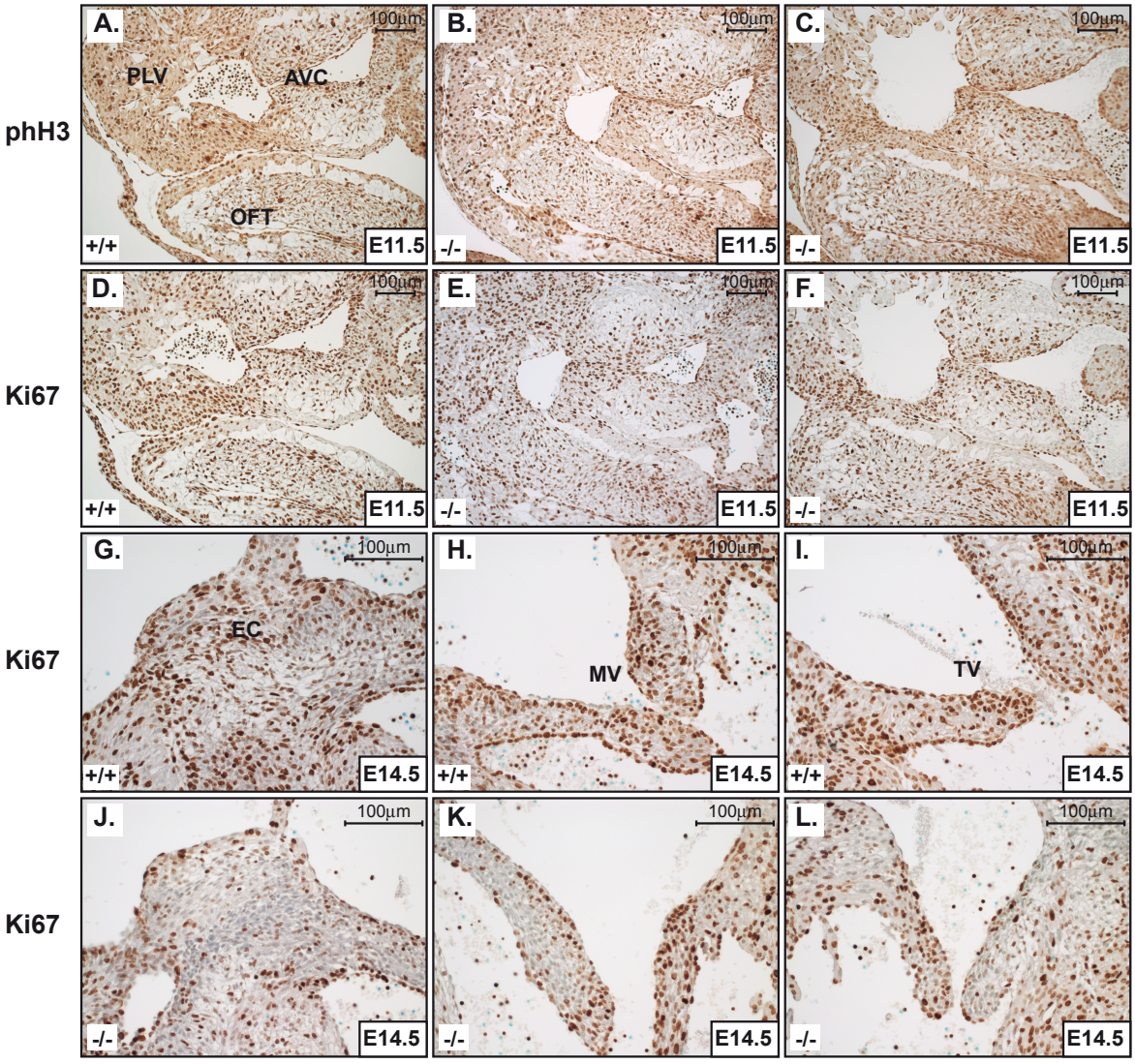


Figure IV.5 Anatomical analysis of Klf13$^{+/+}$ and Klf13$^{-/-}$ at E13.5-14.5. A-D Trichrome staining of frontal sections of Klf13$^{+/+}$ and Klf13$^{-/-}$ E13.5 (A-B) and E14.5 (C-D) embryos. Note the hypertrophied right ventricle (B) and the dilated right atria (D) in the knockout embryos.

iii. Reduced proliferation and early differentiation in Klf13$^{-/-}$ AVC.

To determine the origin of the AVC defects in these mice, we analyzed embryos at E11.5. Immunohistochemistry for the proliferation marker Ki67 and the mitosis marker phH3 was done on wild type and knockout sections. Less staining of both markers was observed in the AVC of the Klf13 null embryos (figure IV.6B, C, E, F). This decrease was also seen at E14.5 in the endocardial cushion (EC), mitral valve (MV) and tricuspid valve (TV) (figure IV.6J-L). We then counted the numbers of Ki67 and phH3 positive cells in the AVC of three wild type and eight knockout E11.5 embryos. Cell counts indicated reduced levels of both endocardial and mesenchymal Ki67 and phH3 positive cells in the Klf13$^{-/-}$ AVC. Proliferation of the endocardial cells seemed to be affected more dramatically (figure IV.6M).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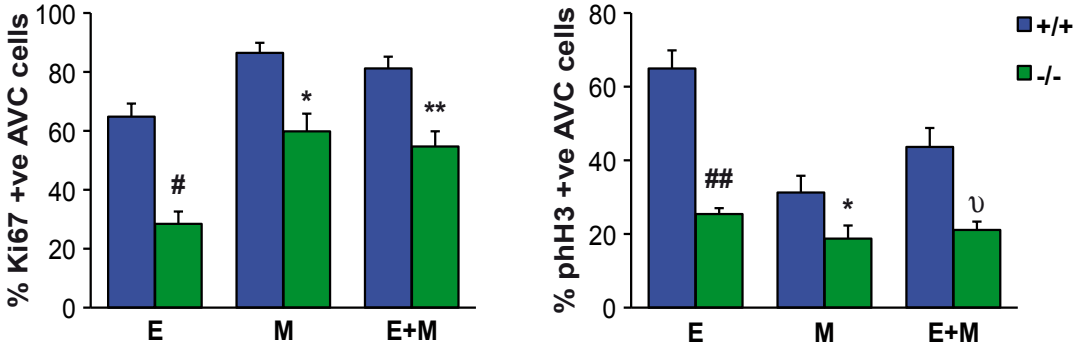
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Figure IV.6 Reduced proliferation in the AVC of Klf13$^{-/-}$ embryos. A-F Immunohistochemistry analysis of Ki67 and phH3 proliferation markers in E11.5 Klf13$^{+/+}$ and Klf13$^{-/-}$ mice. Note the reduced level of positive nuclei in the AVC of the knockout embryos (B-C and E-F). **G-L** Immunostaining of Ki67 on Klf13$^{+/+}$ and Klf13$^{-/-}$ embryos at E14.5. Note decreased staining in the EC (J), the mitral valve (K) and the tricuspid valve (L) of the knockout embryos. **M-N** Percent of Ki67 and phH3 positive endocardial and mesenchymal cells in the AVC of E11.5 Klf13$^{+/+}$ and Klf13$^{-/-}$ embryos (n=3 for Wt; n=8 for KO). Note the significant decrease in the percent of proliferating mesenchymal and endocardial cells. * $p<0.05$; ** $p<0.01$; υ $p<0.001$; # $p<0.0005$; ## $p<0.0001$ vs. Klf13$^{+/+}$. PLV: primitive left ventricle; AVC: atrioventricular cushion; OFT: outflow tract; EC: endocardial cushion; MV: mitral valve; TV: tricuspid valve; E: endocardial; M: mesenchymal.

Next, we verified if the formation of the cardiac jelly was altered in these mice. The cardiac jelly is critical for development of the EC. It arises from epithelial-mesenchymal (EMT) transformation of a subset of endocardial cells that transform into mesenchymal cells, invade and cellularize the extracellular matrix (ECM). Sections from $Klf13^{+/+}$ and $Klf13^{-/-}$ E11.5 embryos were stained with alcian blue. Alcian blue stains acid glycoseaminoglycans components of the ECM; thus, it marks EMT. Increased staining was observed in the ECs of Klf13 null embryos as compared to the wild types (figure IV.7C-D). This indicates increased EMT in the cushions of these mice. To further confirm this, we counted the numbers of endocardial and mesenchymal cells in the AVC of E11.5 Klf13 null embryos. Twelve wild type and twenty-four knockout sections were counted. Decreased numbers of endocardial cells and increased numbers of mesenchymal cells were observed in the AVC of the mutant embryos as compared to the wild types (figure IV.7E). These results, together with the finding of decreased proliferation in the AVC of the knockout embryos, suggest defective ECM and early differentiation in the AVC of Klf13 knockouts.

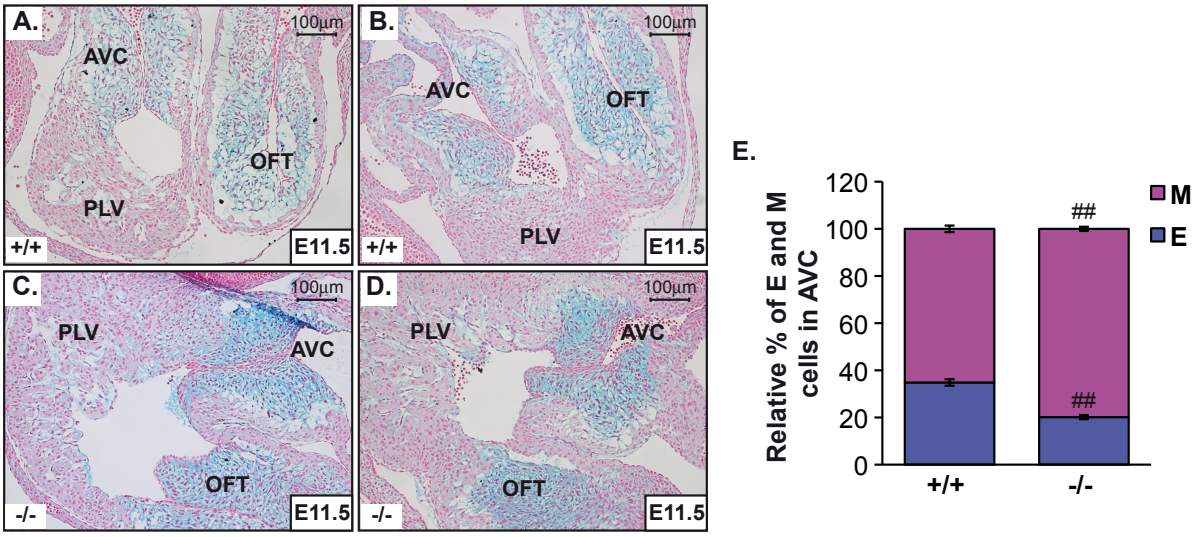

Figure IV.7 Increased EMT in the AVC of Klf13$^{-/-}$ embryos. A-D. Alcian blue staining of Klf13$^{+/+}$ and Klf13$^{-/-}$ E11.5 sections. Increased blue staining is observed in the AVC and OFT of the Klf13$^{-/-}$ sections (C-D). **E.** Percent of endocardial and mesenchymal cells in the AVC of Klf13$^{+/+}$ and Klf13$^{-/-}$ embryos at E11.5. Note the increase in the percent of mesenchymal cells and the decrease in that of endocardial cells in the knockout mice. ## $p<0.0001$ vs. Klf13$^{+/+}$.

Delamination of endocardial cells from the endocardium and their transformation into mesenchymal cells is a complex process governed by various signaling molecules and involves a large subset of genes. QPCR analysis on RNA from P0 hearts revealed altered expression levels of several EMT regulating genes (figure IV.8). VEGF, CTGF, Hif1α, and Adamts2 transcript levels were significantly reduced in the knockout hearts as compared to wild types. These results further confirm defective EMT in the hearts of the knockout mice. Moreover, other cardiac markers were altered in the mutant hearts. Nppa (gene encoding atrial natriuretic peptide ANF) and SERCA transcript levels were significantly reduced. The latter suggests heart failure in a subset of the knockout mice. On the other hand, ACTA1 levels were significantly increased consistent with the adult hearts (n=5 for each genotype).

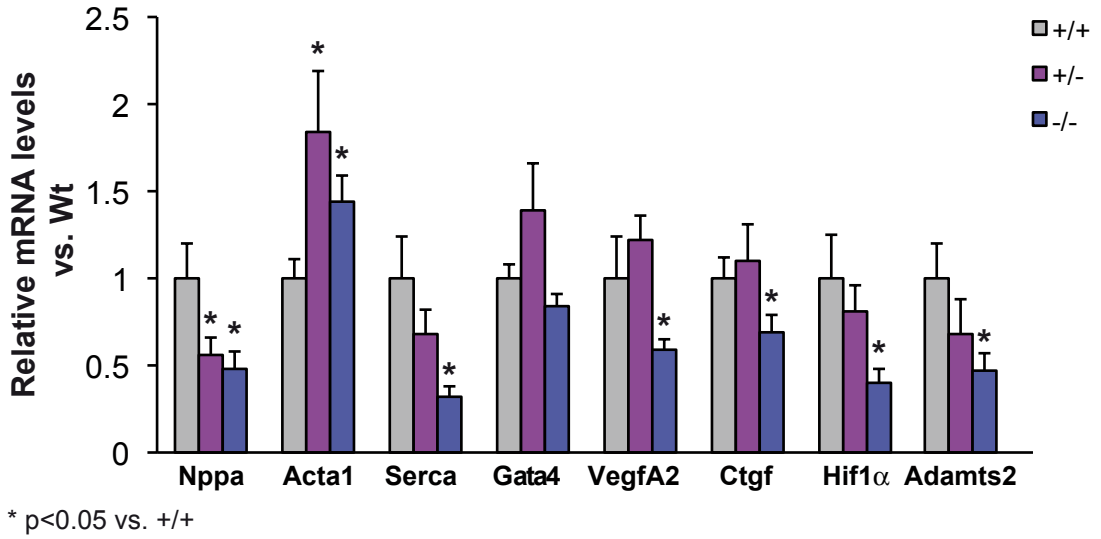

Figure IV.8 Altered cardiac gene markers in Klf13$^{-/-}$ neonates. QPCR analysis using RNA from heart tissue of P0 day old mice (n=5 for each group). Note the statistically significant change in the transcript levels of the analyzed markers in the knockout mice. *p<0.05 vs. Klf13$^{+/+}$.

Collectively, these results point to an important role of KLF13 in mammalian heart development and endocardial cell proliferation. They indicate that KLF13 is essential for proper EMT and AV cushion development. Moreover, the data suggest that loss of KLF13 in mice results in AV cushion defects and in 50% of these cases causes perinatal death.

E. Discussion

In this study, we show that KLF13 is a novel regulator of the EMT and of endocardial cell proliferation. KLF13 is a member of the KLF family of DNA binding proteins that contain three Cys_2His_2 residues. The first evidence for their role in cardiovascular development came from gene disruption studies that revealed the essential role of KLF2 in blood vessel integrity (158). KLF2 was later reported to be important for vascular smooth muscle cell migration (362). Another KLF member, KLF5, was also detected in developing blood vessels albeit it plays a distinct role from that of KLF2 therein. $Klf5^{+/-}$ mice showed attenuated cardiac remodeling and decreased levels of arterial-wall thickening in response to external stress (300). Other KLF members involved in the heart include KLF10 and KLF15. Klf10 null mice developed signs of pathologic hypertrophy (274). KLF15 was reported to act as a negative regulator of cardiac hypertrophy and was later shown to inhibit smooth muscle cell proliferation; $Klf15^{-/-}$ mice had an exaggerated response to vascular damage (76, 186).

An early report by Lavalleé *et al* showed that KLF13 is predominantly expressed in the developing mouse heart and knocking it down in *Xenopus* revealed its critical role in heart development (165). The Klf13 null embryos developed ventricular hypotrabeculation, atrial septal defects (ASDs) and delayed AV cushion formation pointing to KLF13 function in both the endocardium and the myocardium (165). In the present manuscript, we used mouse genetics to determine the role of KLF13 in the mammalian heart. $Klf13^{-/-}$ mice were viable; however, postnatal death was observed and the mice were obtained at almost half the expected Mendelian ratio. Dilatation of the right ventricle or hypertrophy of the left ventricular wall was seen in a subset of the surviving Klf13 null adult mice. Increased aortic

valve mean pressure gradient and left ventricular mass was observed in 50-60% of the analyzed hearts. When examined at P0, myocardial and AV cushion defects were observed with variable penetrance; higher percentages were seen in the AV cushion defects. Among the AV cushion defects was "Goose-neck deformity." This is usually accompanied with anterior displacement of the aorta, non-membranous VSDs, and valvular defects (23, 75, 340), which were also seen in the analyzed hearts. One outcome of AV cushion defects is left ventricular outflow tract obstruction in the adults (15), which could explain the increased aortic valve mean pressure gradient observed in the surviving mutant mice. This in turn could lead to the ventricular hypertrophy seen in these mice. The complex process of cardiac cushion formation starts at E9.5 in the mouse and involves interaction between the myocardium and the endocardium. The presence of both myocardial and cardiac cushion defects in the Klf13 knockout hearts reflect cross talk between the endocardium and the myocardium. However, it could also point to a distinct role of KLF13 in both the myocardium and the endocardium.

Formation of the endocardial cushion starts with the deposition of extracellular matrix (ECM) components. This is then followed by EMT to cellularize the cushions, leading to their maturation and remodeling. Defective ECM organization has been linked to endocardial cushion defects. Various proteases, including the ADAMTS family of metalloproteases, are required for degradation of ECM molecules, a significant process for ventricular trabeculation and endocardial cushion maturation (144, 145, 315). Two ECM components that have been reported to be important for heart development include hyaluronan (HA) and versican. Versican is cleaved by matrix metalloproteinases (MMPs) and members of the ADAMTs family of metalloproteinases (144, 145). Decreased Adamts2

transcript levels were detected in the hearts of Klf13 mutant neonates. Reduced ADAMTS levels could affect cleavage of versican, which is also able to bind to and stabilize hyaluronan.

Alcian blue stain binds to acidic glycosaminoglycans components of the endocardial cushion, and thus marks ECM deposition and EMT. Increased alcian blue staining was noticed in the cushions of the Klf13 knockout hearts, thus, providing further evidence for accumulation of versican and hyaluronic acid in the ECs. Moreover, immunostaining for the proliferation markers Ki67 and phH3 showed decreased proliferation of both the endocardial and mesenchymal cells in the AVC of the KLF13 mutants; the proliferation of endocardial cells was more dramatically affected. These results reflect increased EMT and premature differentiation in the AVC of Klf13 null hearts. Indeed, VEGF transcript levels were decreased in the hearts of the Klf13 null neonates. VEGF is reported to be a negative regulator of EMT (61). Moreover, there was a lower percentage of endocardial cells and higher percentage of mesenchymal cells in the AVC of Klf13 null hearts as compared to the wild types at E11.5; these observations are consistent with reduced proliferation and early differentiation in the AVC of *Klf13* knockouts. Other EMT markers, such as hypoxia inducible factor Hif1α and connective tissue growth factor CTGF were also reduced providing more proof of an abnormal EMT in the mutant hearts. Collectively, these results indicate defective ECM and EMT in the Klf13 null hearts and point to a novel role of KLF13 in the organization of the ECM and in the proliferation of endocardial cells.

The natriuretic peptide genes, Nppa and Nppb, encoding ANF and BNP respectively, are known *in vitro* targets of Klf13 (165) and were down-regulated in the Klf13 null hearts. On the other hand, transcript levels of the cytoplasmic calcium pump, SERCA, were

downregulated in the P0 mutant hearts suggesting cardiac stress. SERCA levels were, on the other hand, upregulated in the surviving adult Klf13 null hearts. Thus, the absence of KLF13 also has profound effects on adult heart homeostasis.

Altogether, these results point to KLF13 as a novel regulator of mammalian cardiac development and more specifically in the development of the AV cushion and in endocardial cell proliferation. Based on these data, it is tempting to speculate on the role of KLF13 as a novel congenital heart disease causing or modifier gene in humans.

V. General Discussion

Cardiovascular disease remains the leading cause of death in industrializes countries and accounts for more than 17 million deaths per year world wide. Understanding the molecular basis of disease requires a better knowledge of the genes and pathways needed for normal cardiovascular development and homeostasis. GATA4 is one of the most critical and intensely studied cardiac transcription factors. It has a crucial role in proper heart development and maturation and postnatally, it is important for cardiomyocyte survival and adaptive response. Its pleitropic role in cardiac cell proliferation and differentiation is well reflected in the fact that GATA4 mutations cause a wide range of various congenital heart problems. However, the effectors of GATA4 actions are incompletely understood. In the present thesis, we identified two effectors/cofactors of GATA4 in cardiac cell proliferation, a growth inducible protein cyclin D2 (CycD2), and a cell specific transcription factor KLF13. We not only proved that CycD2 is a direct target of GATA4, but we also showed that it is an effector of GATA4 in postnatal cardiomyocytes. Moreover, we provided evidence for a cross-regulatory loop between the two proteins in which CycD2 enhances transcriptional activity through GATA dependent recruitment to GATA4 target promoters *in vivo*. Finally, we identified KLF13 as a novel regulator of endocardial cell proliferation and epithelial-mesenchymal transformation in the heart.

CycD2 is a GATA4 effector in postnatal cardiomyocyte growth and survival

Evidence for the function of GATA4 in the postnatal heart first came from the identification of GATA4 as an upstream regulator of the cardiac natriuretic genes *Nppa* and

Nppb and was later established when GATA4 was found essential for the activation of various cardiac promoters in the adult myocardium (96, 201, 210). GATA4 is highly expressed in postnatal cardiomyocytes and its knockdown has deleterious effects on the expression levels of various cardiac transcription factors (42). Overexpression of GATA4 induces hypertrophic growth of neonatal cardiomyocyte cultures and induction of cardiac hypertrophy by various stimuli enhances GATA4 levels, its transcriptional activity and/or DNA binding capacity (43, 175). Furthermore, GATA4 was identified as a key regulator of cardiomyocyte survival. First indication for its role in cardiomyocyte survival came from studies done in P19 cells. Knockdown of GATA4 in these cells blocks their differentiation at the cardioblast stage and leads to extensive apoptosis (97). Loss of one Gata4 allele in mice results in hypoplastic hearts and increased drug-induced cardiotoxicity (7) whereas cardiomyocyte specific overexpression of GATA4 is cardioprotective (112, 287).

Even though many direct GATA4 transcription targets have been identified, little is known about the effectors of GATA4 actions. In this thesis, we identified cyclin D2 as a direct transcriptional target and effector of GATA4 in the adult heart. Cyclin D2 (CycD2) is a member of the cell cycle proteins that play vital role in cell growth and differentiation. GATA4 was able to associate with the Ccnd2 promoter and activate it in a dose-dependent manner. To test whether CycD2 was an effector of GATA4 in proliferative or hypertrophic growth, we generated transgenic mice with cardiomyocyte specific overexpression of Ccnd2 (gene encoding cyclin D2). Ccnd2 transgene was driven by the αMHC promoter that directs expression specifically to the adult myocardium (100, 188). Increased left ventricular mass with enhanced cardiomyocyte proliferation was observed in the hearts of these mice, while hypertrophy markers were not affected. The transgenic mice had normal cardiac functions and were viable up till the analyzed stage (300d) indicating that Ccnd2 overexpression is

beneficial to the heart. Our results were generally consistent with a similar αMHC expressing model where Ccnd2 overexpression was able to reduce infarct size in mice 170d post induction of the myocardial infarct (109, 259). Loren Field's group generated a similar model where they overexpressed Ccnd1 specifically in the heart. In their mice, enhanced DNA synthesis and multinucleation was observed (313). Nuclei positive for tritiated thymidine incorporation were often observed as two opposite nuclei in close proximity with lower amount of silver grains per nuclei which is an indication of a binucleated cell (313). Our Ccnd2-transgenic hearts had increased overall heart size and cardiomyocyte number; thus, unlike the MHC-Ccnd1 mice, our MHC-Ccnd2 mice seem to have sustained cardiomyocyte cell cycle activity and cytokinesis. Similarly, another report by Loren Field's suggested that Ccnd2-cardiomyocyte specific overexpression but not Ccnd1 had sustained cardiomyocyte cell cycle activity following myocardial injury (259).

Overexpression of GATA4 in the heart was not associated with cardiomyocyte proliferation (112, 287) suggesting that these more or less terminally differentiated cells lack cofactors and/or effectors of GATA4 in proliferative growth. As mentioned above, Gata4 heterozygote mice have hypoplastic hearts and increased myocyte apoptosis (7). When crossed with the cyclin D2 transgenic mice, heart size and function were normalized in the resulting mice. Interestingly, cyclin D2 was able to rescue both basal cardiomyocyte survival and drug-induced cardiotoxicity of the Gata4 haploinsufficient mice. These results suggest that Ccnd2 upregulation can –at least in part- compensate for Gata4 haploinsufficiency.

The data presented here are quite interesting. Cyclin D2 could be playing a direct effect on cardiomyocyte survival. Alternatively, it is possible that the adaptive response of the heart is indirectly enhanced through normalized cardiac size and function. Moreover, the fact that cardiac growth in the Ccnd2 transgenics on both the wild type and the $Gata4^{+/-}$

background occurred mostly through cardiomyocyte proliferation strongly supports a role of cyclin D2 as a mediator of GATA4 action in cardiomyocyte proliferation. It also indicates that GATA4 effectors in proliferation and hypertrophy are distinct. Extensive research on postnatal cardiomyocyte proliferation showed that the adult heart retains an intrinsic ability to divide; yet; this regenerative capacity is clearly not adequate to individuals with an injured myocardium. The current results further suggest that the adult myocardium can indeed be induced to regenerate to an extent that reverses the diseased state.

CycD2 is a cofactor of GATA4 in cardiogenesis

The finding that CycD2 can compensate for GATA4 haploinsufficiency led us to further investigate the GATA4-CycD2 pathway and to test whether CycD2 has a direct effect on GATA4 activity. Co-transfection of CycD2 and GATA4 in NIH3T3 cells revealed that both proteins synergize to activate various GATA4 target promoters. Furthermore, *in vitro* GST pull down and *in vivo* co-immunoprecipitation assays revealed a direct physical interaction between the two proteins, specifically through the N-terminal region of GATA4. This interaction was specific to GATA4 and not the other GATA members. When tested for a similar function of CycD2, cyclin D1 (CycD1) was not able to synergize with GATA4. On the contrary, an inhibition of GATA4 activity was noticed (figure 1D). CycD1-CDK4 was recently reported to decrease GATA4 protein levels and target it for proteasomal degradation (234). GATA4 protein has two trans-activation domains in the N-terminal and one in the C-terminal. Structure function analysis revealed that the synergy between CycD2 and GATA4 requires the 2nd N-terminal trans-activation domain of GATA4, more specifically a region between amino acids 145 and 174 that is highly conserved among species. Furthermore, we found through *in silico* analysis that this region contains a potential CDK phosphorylation site around the motif aa160 (224). This led us to test whether GATA4 was targeted by

CDK4. Interestingly, CDK4 was able to physically interact with GATA4 and enhance its transcriptional activity; inhibition of CDK4 greatly affected GATA4 activity. Moreover, using *in vitro* phosphorylation assay, we found that an active CDK4 kinase phosphorylates GATA4 at amino acid S160. This phosphorylation seemed to be important for the CycD2-GATA4 synergy.

We then aimed to demonstrate an *in vivo* relevance of the GATA4-CycD2 interaction. We crossed the αMHC-Ccnd2 mice (described above) with a transgenic mouse line that expresses the luciferase gene driven by a GATA-dependent promoter. The resulting mice had an increase in the luciferase transcript levels specifically in the heart reflecting a CycD2-GATA interaction. Since we proved earlier that CycD2 interacts specifically with GATA4, it is safe to suggest that this cardiac GATA member is indeed GATA4 (and not GATA6). Moreover, using chromatin immunoprecipitation assay, we found that CycD2 is bound *in vivo* to GATA-target promoter. Lastly, using cardiogenic assays in *Xenopus*, we were able to demonstrate that CycD2 enhanced cardiogenic effects of GATA4. This effect was abolished when an S160 GATA4 mutant was used. Altogether, these findings suggest that GATA4 tethers CycD2-CDK4 to its target promoters on which CycD2 acts as a GATA4 coactivator. We propose that this process occurs in two steps. CDK4/CycD2 active kinase is recruited to C-terminal of GATA4 and phosphorylate the N-terminal TAD at S160. Phosphorylated GATA4 can now synergize with CycD2, thus, enhancing the transcriptional activity and cardiogenic function of GATA4 (figure V.1).

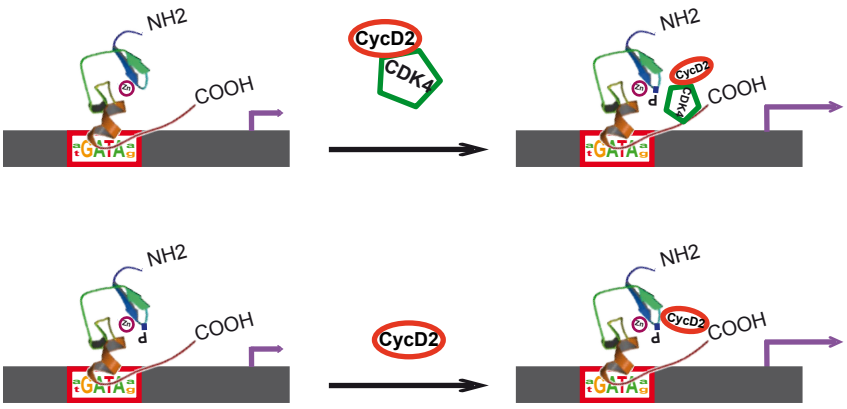

Figure V.1 Proposed model for CycD2 recruitment to GATA4 target promoters. Active CDK4 kinase (CDK4/CycD2) is recruited first. It phosphorylates GATA4 at S160 and enhances its activity. Phosphorylated GATA4 then recruits CycD2. CycD2 binds N-terminal GATA4, increases its transcriptional activity and enhances its cardiogenic function. GATA structure is that of GATA2 taken from the UCSC genome web browser.

The recent report by Nakajima *et al*, 2011 showed that GATA4 is a CDK4 substrate. However, in their article, they found that CDK4 phosphorylates GATA4 at S105- a previously reported MAPK phosphorylation site (34, 268)- targeting it for degradation. Our data presented here indicate that, at least *in vitro*, S160 is the major phosphorylation site of CDK4 on GATA4 and that this leads to an enhancement in its biochemical and biological activity. The CycD2-GATA4 interacting domain is of high significance as it includes three GATA4 human mutations found in cardiac patients (38, 261, 273, 372, 384). Particularly, mutated S160 was reported in patients with familiar atrial fibrillation and was associated with reduced GATA4 activity (372) re-enforcing our data that this amino acid is important for an enhanced GATA4 function. Generating an S160 knock-in mouse model in the future will be useful to further characterize the significance of this amino acid for GATA4 function in the heart.

The present data uncover a cross-regulatory loop between GATA4 and CycD2, in which CycD2 is both a GATA4 effector and coactivator. GATA4 enhances Ccnd2 transcription, which in turn cooperates with GATA4 to enhance its function. Finally, our data uncover a role of CycD2 in the developing and postnatal heart. They provide novel insight for understanding cell specific effects of cyclin Ds (through their differential interactions with tissue-specific transcription factors) and the possibility for targeting the cell cycle in cardiac therapy.

KLF13 is a regulator of endocardial cell proliferation and epithelial-mesenchymal transformation (EMT)

In addition to its role in the myocardium, GATA4 is critical for the development of the endocardium where it has been reported to collaborate with numerous transcription factors (140, 162, 226, 230, 317). We have identified in our lab the transcription factor KLF13 as yet another GATA4 cofactor in early heart development (165). KLF13 is a member of the Krüppel-like transcription factors that are important regulators of cell proliferation and differentiation. Several KLF members are expressed in the heart in a spatial and temporal specific manner (76, 158, 186, 274, 300, 315). KLF13 expression in the heart starts as early as the heart tube stage and is later found in both myocardial and endocardial cells, thus, displaying a similar expression pattern as that of GATA4 in the heart. Klf13 physically interacts with N-terminal GATA4 to co-regulate the A- and B-type natriuretic peptide genes, *NPPA* and *NPPB*, as well as other cardiac promoters namely cardiac actin and β-MHC (165). In *Xenopus*, knock down of Klf13 causes developmental heart defects namely ventricular hypotrabeculation, atrial septal defects and delayed EC formation; suggesting that it plays a role in the endocardial and/or myocardial development (165). We used mouse genetics to determine the role of Klf13 in the mammalian heart. Targeted deletion of exon 2

was done using homologous recombination. In a previously reported Klf13$^{-/-}$ mouse model, researchers demonstrate a role of KLF13 in regulation of erythropoiesis and in the normal development of B and T cells at various stages (93, 252). In this model, there was a lower number of mice at 3 weeks of age than would be expected by Mendelian inheritance (93). The mutant mice were reported to have enlarged spleens and thymi (93, 252). Gordan *et al* also indicate in their report that the mice had enlarged hearts but no detailed explanation of the heart phenotype was reported (93). Although the heart phenotype was not extensively analyzed, it is noteworthy to mention that this model was generated by targeted deletion of exon 1. In a project going on in our lab, we were able to demonstrate that two isoforms of KLF13 exist in the heart (Darwich *et al*, manuscript in preparation). The 2nd isoform has an alternatively spliced exon 1 (denoted as exon 1b in our knockout strategy, figure 1A). This could suggest that in the previous Klf13 knockout model by Gordan *et al*, the 2nd isoform is still produced, thus, interfering with the appearance of a clear heart phenotype. In our model, we deleted exon 2, the common exon between the two isoforms, ensuring the deletion of both isoforms. It would be useful in the future to develop a 2nd isoform specific knockout to distinguish between the functions of the two isoforms.

Our Klf13$^{-/-}$ mice were born at 50% reduced frequency. Ventricular and valvular defects were observed in the surviving adult mice; a large subset of the mice had increased mean blood pressure gradient across the aortic valve and an increase in the left ventricular mass. When analyzed at P0, variable cardiac phenotypes were observed in these knockouts mainly endocardial cushion defects including “Goose-neck” deformity and atrioventricular (AV) valvular abnormalities. Epithelial-mesenchymal transformation (EMT) seemed to be affected in these mice and they had reduced cellular proliferation in their AV cushion. Components of the ECM were affected pointing to a role of KLF13 in organization of the

ECM. Additionally, myocardial defects were observed primarily right atrial dilatation and ventricular hypertrophy. These results may suggest that KLF13 plays distinct roles in the myocardium and the endocardium. However, it could also be that the myocardial defects are a consequence of the endocardial defects. To better understand this distinction, it would be useful in the future to develop a conditional knockout in which Klf13 is deleted specifically in the myocardium. If these knockouts develop the same myocardial defects we observe in this report, then this proves a specific role of KLF13 in the myocardium. However, in the case where no myocardial defects are observed, this suggests that the myocardial abnormalities in our mutants would be a consequence of the defective endocardium.

Our results point to a clear role of KLF13 in the proper development of the endocardium, where its expression overlaps with that of GATA4 therein. Several studies have demonstrated a critical role for GATA4 in the endocardial development. Inactivation of *Gata4* in endothelial cells was embryonic lethal by E12.5 due to failure of the EC to form properly (279). Moreover, Gata4 haploinsufficiency has been associated with endocardial defects mainly common atrioventricular canal and double-outlet left ventricle (DORV) (272) and GATA4 mutations have been linked to patients with atrial septal defects (ASDs) (183). It would be interesting to check if Klf13 and Gata4 genetically interact for a proper endocardial formation. We currently generated in our lab a $Klf13^{+/-}$.$Gata4^{+/-}$ mice to address this issue.

VI. Summary and Future Perspectives

Congenital heart diseases (CHDs) are the foremost cause of infant mortality and nearly 1-2% of live births have some sort of cardiac abnormality (30, 239). Proper heart morphogenesis requires a balance between cellular proliferation and differentiation and it is clear by now that dysregulated cardiac cell proliferation causes congenital heart defects. GATA4 is a central regulator of both processes and has a well-established role in myocardial and endocardial cell proliferation. Extensive studies aimed at understanding GATA4 function in differentiation; yet, not much is known about the processes underlying the role of GATA4 in cellular proliferation. The data presented in this thesis demonstrate a role of CycD2 as an effector and a cofactor of GATA4 in myocardial cell proliferation and suggest that KLF13 is a GATA4 cofactor in endocardial cell proliferation. Collectively, the results may represent a new model by which GATA4 regulates cardiac cell division. The three proteins could be part of a common pathway whereby CycD2 and KLF13 enhance GATA4 activity on its target promoters in a cellular specific manner (figure V.2). Support from this comes from the observation that cyclin D1 is a common target for KLF13 and GATA4 and that GATA4 feeds back to activate its own promoter (41, 240).

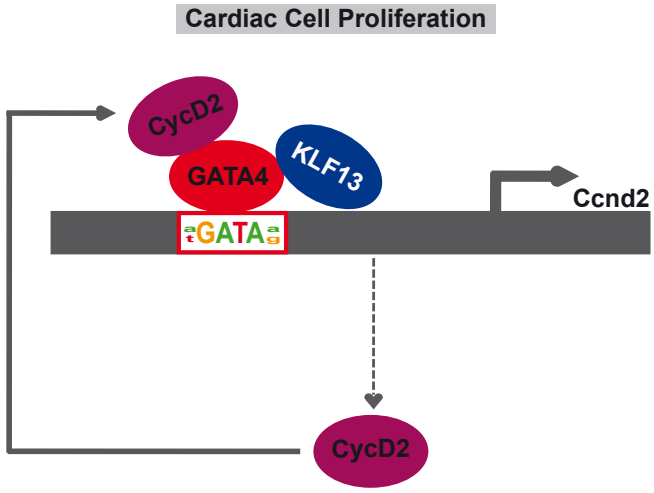

Figure VI.1 Proposed model of GATA4 function in cardiac cell proliferation. GATA4 recruits cyclin D2 (CycD2) and KLF13 to Ccnd2 promoter. CycD2 then positively feeds back to enhance GATA4 function.

To further examine this model, it could be interesting to identify chromatin bound GATA4 containing complexes using the "modified chromatin immunoprecipitation" assay (mChIP) combined with mass spectrometry (MS) and sequence analysis. We have generated in our lab endocardial cells that stably overexpress GATA4 protein. mChIP/MS on GATA4 complexes immunoprecipitated from these cells will identify chromatin bound GATA4 partners in the endocardial cells. Moreover, isolated cardiomyocytes could be infected with GATA4 adenovirus followed by mChIP/MS to identify GATA4 complexes in myocardial cells. Atrial HL1 cell line with retroviral insertion of GATA4 could also be used. Comparing the partners obtained in each case will help identify cell specific partners of GATA4. They could also allow us to determine whether GATA4, CycD2 and KLF13 are part of one complex or appear as separate complexes in myocardial vs. endocardial cells. Moreover, "high performance liquid chromatography" coupled to mass spectrometry (HPLC-ESI-MS/MS) analysis could be used to determine *in vivo* phosphorylation state of GATA. Again endocardial (TC13), proliferating myocardial (HL1) as well as primary myocardial cells could be used.

To further understand the role of Klf13 in endocardial cell proliferation, it could be interesting to generate cell specific knockouts of Klf13 mice using the Cre-loxP technology. Klf13 floxed mice could be generated and crossed with Tie2-Cre or Nkx2.5-Cre mice to delete the Klf13 gene specifically in the endocardium and the myocardium, respectively. The resulting knockout mice could then be analyzed in a similar manner as done in this thesis to determine the phenotypes. This would allow further identification of the specific role of Klf13 during heart development and determining whether the myocardial defects observed in our whole body knockout mice are primary or secondary to the endocardial cushion defects. Furthermore, to understand the genetic interaction between Klf13 and other endocardial transcription factors, the Klf13 knockout mice could be crossed with mice having one allele of Gata4, Gata5, Tbx5, or Nfatc.

KLF13 seems to arise as a novel CHD causing gene. As such, screening human patients for mutations in the KLF13 gene is of great importance. Several reported human mutations have already linked GATA4 to heart problems. Yet, incomplete penetrance and phenotype variability are common in CHDs supporting the existence of genetic modifiers. The results of this thesis provide a further evidence of this notion. We have determined in our lab the region required for interaction of KLF13 with GATA4 (56). Testing for GATA4 and KLF13 mutations that disrupt this interaction could help unravel the genetics underlying various congenital heart diseases.

Collectively, our results may open new cardio-protection avenues for individuals with GATA4 dependent cardiac dysfunction and identify KLF13 as a potential CHD causing gene. Studying the GATA4 pathway and identifying its partners have helped unravel the molecular basis of some CHD causing genes. Future discovery of more cardiac regulators

and understanding the molecular basis of CHDs is essential for preventions of these defects and possible development of therapeutic approaches for myocardial repair.

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Appendix: Biochemical Identification of newly characterized Tbx5 isoforms

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Author Contribution

The isoforms were detected and cloned by my colleague in the lab. I characterized the newly identified isoforms (figures 3 and 4) and wrote the manuscript.

Abstract

Tbx5 is a member of the T-box family of transcription factors and a dosage sensitive regulator of heart and limb development. *TBX5* is the gene mutated in the complex heart and limb disorder, Holt-Oram syndrome. We recently reported that Tbx5 is regulated through alternative splicing and characterized a C-terminal truncated isoform of Tbx5. In this manuscript, we identify two additional isoforms (Tbx5c and Tbx5d) that differ in their C-terminal region. Both isoforms retain the capacity to bind DNA. However, their cellular localization and interaction with cofactors is altered. These results provide novel insights for post-transcriptional regulation of Tbx5 and may help analyze genotype-phenotype correlations in Holt Oram syndrome patients, especially for those who don't carry any of the reported *TBX5* mutations.

Introduction

Holt-Oram syndrome (HOS) is an autosomal dominant disorder characterized by upper limb and cardiac defects (14). The most common structural heart abnormalities include atrial septal defects (ASDs) and ventricular septal defects (VSDs). Conduction defects have also been commonly seen and they mostly involve atrioventricular blocks. Hypoplastic left ventricle, mitral valve problem and endocardial cushion defects have also been reported in HOS patients (2, 13). Genetic linkage analysis have mapped the disease to the chromosomal locus where TBX5 is located and mutations in TBX5 have been found in patients with HOS. Moreover, Tbx5 expression pattern in the upper limb, atria and left ventricle along with mouse genetics studies have strengthened the causative link between TBX5 and HOS (4). Over than 50 mutations in the TBX5 locus have been identified so far in HOS patients (11).

Tbx5 is a member of the large family of T-box transcription factors critical for early cellular commitment, differentiation and organ development. They bind specific DNA motifs, called TBE or T-box binding elements, located in their T-box domain. Tbx5 activates the atrial natriuretic factor (ANF) and connexin 40 (cx40) promoter and is reported to cooperate with other transcription factors, mainly GATA4 and Nkx2.5, to synergistically regulate downstream targets (4, 8, 12). Tbx5 has two nuclear localization signals (one in the N-terminal of the T-box and one in the C-terminal outside the T-box domain) and to date only one transactivation domain has been described in the C-terminal region next to the nuclear localization domain (5, 17).

Most of the Tbx5 mutations have been reported to alter protein production; albeit others, especially those located in the T-box domain, lead to the production of a functionally impaired protein with altered DNA binding ability, transcriptional activity, and/or interaction with cofactors (7, 8, 10). These findings led to the suggestion of haploinsufficiency as the mechanism of pathogenesis. Mutations in the coding as well as the intronic regions have been identified in HOS patients. However, the detection rate in familial as well as sporadic cases is relatively low, 35% (3); thus, suggesting the existence of yet another unidentified HOS-causing locus. An alternative explanation would be that unscreened mutations within untranscribed or untranslated regions of Tbx5 account for this low detection rate. We recently reported the existence of a novel alternatively spliced Tbx5 isoform, resulting in the production of a shorter protein. The two proteins were produced in a growth-factor regulated manner and they had distinct biochemical properties; thus, providing evidence that Tbx5 dosage may be developmentally regulated through the production of alternatively spliced isoforms of diverse functions (9).

In the present work, we report the existence of two additional alternatively spliced Tbx5 isoforms. We used mouse embryonic limb RNA to isolate the new Tbx5 cDNAs and cloned the two new isoforms in expression vectors. We show that the new isoforms are able to bind DNA with high efficiency. Their transcriptional activity and interaction with known Tbx5 cofactors is however altered. The results presented are important for a better understanding of the mechanism of action of Tbx5 and will aid in mutation screening for HOS patients.

Material and Methods

Plasmids and RT-PCR. Tbx5 isoforms were amplified by reverse transcription (RT-PCR) using an oligonucleotide in the first codon of the T-box and an oligonucleotide in the poly A tail. The isoforms were then subcloned in pcDNA3 vector in phase with Kozak-triple flag epitope. Oligos used were: 5'-GGAGGTACCGCCGATACAGATGAGGGCTTTG-3' (forward for Tbx5a, Tbx5c, Tbx5d and Tbx5e); 5'-GCAGGTACCGAAGGAATCAAGGTGTTTCTTCATG-3' forward for Tbx5b; 5'-CCGGAATTCTTAGCTATTCTCACTCCACTCTGG-3' reverse for Tbx5a and Tbx5b; 5'-GCACTCGAGCTAATGAAAGGATGGTGAGAGAG-3' reverse for Tbx5c; 5'-CGCGAATTCCTATATTTCTGTGCCACTTACTT-3' reverse for Tbx5d; 5'-CCCCTCGAGCTAAAGCAGAGGCCTTTGCATCCGAG-3' reverse for Tbx5e. GATA4 and Nkx2.5 expression vectors as well as the ANF-luciferase construct were previously described (6, 9).

Cell culture and transfections. NIH3T3, and 293T cells were maintained in culture and transfected as described previously (1, 9). For cotransfection assays, the total amount of DNA was maintained constant by adding the appropriate amount of empty DNA vector.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from 293T cells. Binding reactions were done at room temperature using 1ug of poly (dI-dC). The probe used was previously described (9).

Western Blots. Western blots were done on nuclear extracts from 293T cells overexpressing the relevant Tbx5 constructs as previously described (16). Tbx5 antibody used was previously described (9).

Immunocytochemistry. Immunocytochemistry was done on 293T cells overexpressing the relevant Flag-Tbx5 constructs as previously described (9). Antibody used was mouse anti-Flag M2 (Sigma F1804).

Results and Discussion

In the course of our analysis of the previously identified short Tbx5 isoform, western blot analysis using the N-terminal Tbx5 antibody detected an intermediate band that co-migrated with a Tbx5 protein truncated of the last 118 amino acids (aa) (1-400). To determine if these bands represent novel Tbx5 isoforms, we isolated cDNA from mouse heart and limb RNA using 5' oligonucleotides located in the first codon of the T-box and a 3' oligonucleotide amplifying the poly A tail. With this strategy, we discovered the existence of new Tbx5 cDNAs sequences two of which produced two novel Tbx5 isoforms of 404 and 376 aa termed Tbx5c and Tbx5d respectively (figure 1A). Once the additional exons were sequenced, we cloned the novel isoforms from mouse embryonic atria (Tbx5c) and forelimb

(Tbx5d) cDNA using oligos in the 1st codon (ATG) and the stop codon of the different isoforms. The two sequences result from either an inclusion of an alternate exon 9 leading to an in frame addition of 77 aa or from the use of an alternate exon 9 donor splice site respectively leading to a 77aa divergent region in case of Tbx5c and 49aa divergent region in case of Tbx5d right after amino acid 327 (figure 1B and C). Moreover, sequence analysis of the genomic Tbx5 locus revealed the possibility of a fifth Tbx5 isoform, Tbx5b. This isoform would be a result of an alternatively spliced exon1 leading to a truncation of 50 aa from the N-terminal region (figure 1A and C). Further confirmation of the *in vivo* existence of this isoform is required.

We then used the previously characterized N-terminal Tbx5 antibody to analyze the profile of Tbx5 in cardiac cells (9). TC13 are endocardial cells and have previously been shown to express Tbx5 (15). Western blot analysis on nuclear extracts from these cells revealed the existence of multiple isoforms (figure 2A). The band around 48KDa would co-migrate with the two novel isoforms, Tbx5c and Tbx5d. We then analyzed Tbx5 complexes in postnatal hearts. Nuclear extracts were obtained with 300mM NaCl elution and dialyzed to 100mM salt. Chromatin binding proteins were enriched with phosphocellulose IEX. Fractions of moderately chromatin bound proteins (300mM salt) and tightly chromatin bound proteins (700mM) were obtained. The 300 and 700mM protein fractions were then size fractionated under non-denaturing conditions using a gel filtration Superose 6 column. Fractions were then processed for western blot using the N-terminal Tbx5 antibody. The 300mM fraction contained bands that correspond to Tbx5a (~64-80KDa) and the previously identified Tbx5e (~30KDa) (previously referred to as Tbx5b in Georges *et al*). A putative Tbx5c/d band could also be detected (~40-48KDa) (figure 2B, top). The 700mM fractions contained immunoreactive bands that correspond to Tbx5a in the relatively smaller

complexes and to Tbx5e in the larger complexes (figure 2B, bottom). These results suggest that different Tbx5 complexes involve distinct isoforms with different chromatin binding properties.

We further investigated the biochemical properties of the newly identified Tbx5 isoforms. All isoforms were subcloned in pcDNA3 expression vectors and tagged with a triple Flag tag. Western blot analysis revealed that all isoforms were more or less expressed at similar levels (figure 3A). Immunocytochemistry on 293T cells transfected with the various isoforms revealed that both Tbx5a and Tbx5b were completely nuclear indicating that removal of the first 50 amino acids does not affect nuclear localization. Tbx5e was almost entirely cytoplasmic. Tbx5c and Tbx5d, in addition to Tbx5 1-327 i.e. Tbx5a truncated of the last 191 aa were detected in both the nucleus and the cytoplasm (figure 3B). These results are consistent with our previous results and with a previous report indicating the presence of a nuclear localization signal between aa 325 and 340 (5, 9). Electrophoretic mobility shift assay (EMSA) revealed that all isoforms were able to bind the TBE site with similar capacity (figure 3C).

Transcriptional activity assay on NIH3T3 cells transfected with ANF-luciferase (ANF-luc) reporter and the various Tbx5 isoforms revealed that Tbx5c, Tbx5d, as well as Tbx5a truncated of the last 191 aa were much weaker activators of the ANF promoter. As per our previous work, Tbx5e activation of the ANF promoter was also very weak. Removal of the first 50 amino acids (Tbx5b) did not have a dramatic effect on the activity. These results are consistent with our previous findings of two TADs in the C-terminal region (9). They could also suggest that Tbx5c and Tbx5d target genes may be distinct from those of Tbx5a. Interestingly, different Tbx5 isoforms seem to have distinct cofactors as well. Tbx5c was still able to synergize with GATA4 on the ANF promoter in a similar manner to Tbx5a. On the

other hand, Tbx5d synergistic effect with GATA4 was more modest (figure 4B). Additionally, Tbx5c was able to synergize weakly with Nkx2.5, another known Tbx5a cofactor (figure 4C) while Tbx5d ability to cooperate with Nkx2.5 was abrogated. Of note, Tbx5 1-327 maintained minimal synergistic ability with both GATA4 and Nkx2.5 (figure 4B and C). These results suggest that the different Tbx5 isoforms may recruit distinct cofactors raising the possibility that they may be involved in distinct cellular processes and target differential downstream genes.

TBX5 is a dosage-sensitive regulator of heart and limb development and is the causative gene of Holt-Oram syndrome (HOS). Although the mechanism of disease is assumed to be haploinsufficiency, not much is known about the mechanism of regulation of Tbx5 levels and activity. In this report, we provide further evidence that Tbx5 is highly regulated through alternative splicing. Together with the previously reported isoform, the present data reveal that Tbx5 can exist as at least five distinct isoforms with distinctive biochemical properties. Our results provide further evidence that the C-terminal domain of Tbx5a includes a TAD and is important for interaction of Tbx5 with its known cofactors, namely GATA4 and Nkx2.5. Loss of this domain led to reduced transcriptional activity as well as aberrant interaction with cofactors. Moreover, the nuclear localization of the protein was also affected consistent with the previous finding of a nuclear localization signal between aa 325 and 340 (5, 9).

Genotype-phenotype correlations are a major clinical challenge in congenital heart disease in general, and in HOS patients in particular mainly due to the wide range of mutations and the variability in disease-expressivity within family members. Based on our finding, mutations in the C-terminal region of Tbx5 would affect only Tbx5a; since the other isoforms would be intact, only a subset of Tbx5 target genes may be affected providing an

explanation for less severe phenotype in these patients. Moreover, for nearly 30% of HOS patients, no mutation has been found in the known TBX5 exons. Our findings thus provide additional sequences within the *TBX5* locus for gene mutation screening in patients with HOS and other heart and/or limb abnormalities. Finally, regulation of *TBX5* by alternate splicing would affect the level at which a certain isoform is expressed and could therefore provide a mechanism for the variability in the severity of the phenotype as a result of Tbx5 haploinsufficiency. Consequently, our findings will help better understand the mechanisms by which TBX5 mutations cause pathogenesis and could shed more light on genotype-phenotype correlations in HOS and possibly other CHDs.

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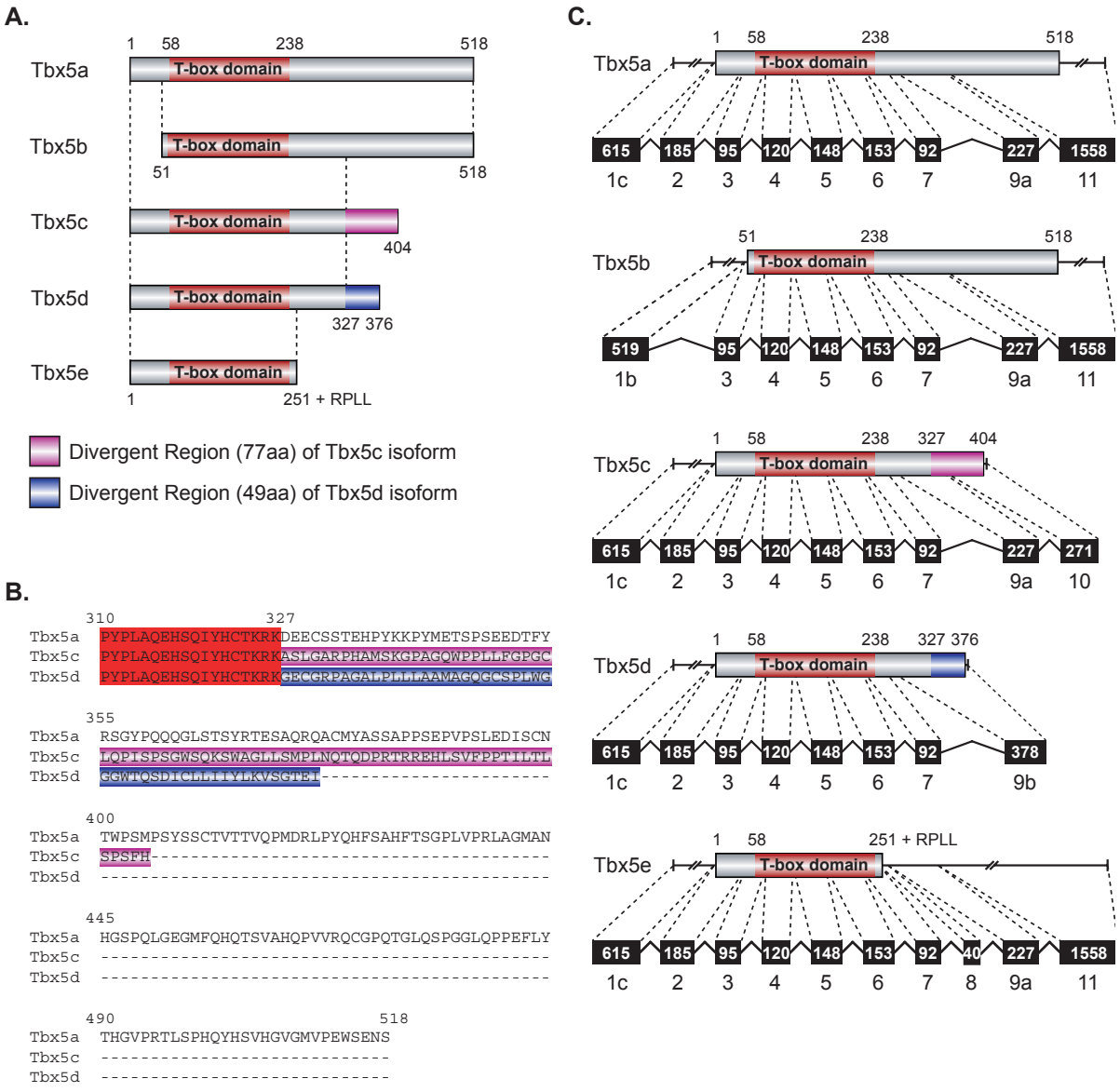

Figure 1 A. Schematic representation of *Mus musculus* Tbx5 isoforms. At least 5 isoforms are translated from the different splice variants. The red box is the DNA-binding T-box domain of the protein. The purple and blue boxes are the regions that are divergent in the Tbx5c and Tbx5d, respectively. **B.** Multiple sequence alignment of Tbx5a, Tbx5c and Tbx5d showing the divergence in the C-terminal region of the three Mm Tbx5 isoforms. **C.** Schematic representation of the exons alternatively spliced to produce the different protein isoforms in *Mus musculus.*

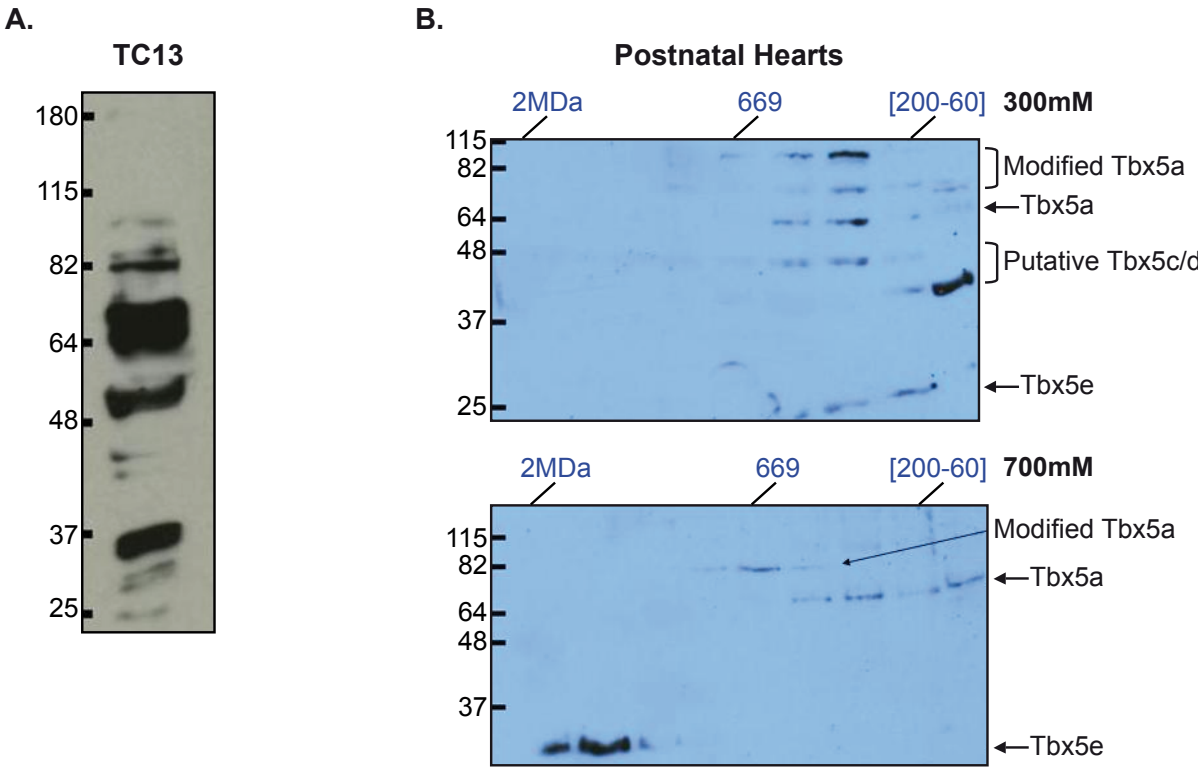

Figure 2 Western blot analysis of Tbx5 in cardiac cells. A. Western blot with Tbx5 antibody on TC13 nuclear extracts. Note the different isoforms present. **B.** Tbx5 complexes in postnatal mammalian heart. Mouse adult heart nuclear extracts were extracted with 200mM NaCl and dialyzes to 100mM salt. Chromatin binding proteins were enriched with phosphocellulose IEX. This leads to two fractions extracted at 300 or 700 mM salt, corresponding to moderate and strong chromatin binding protein fractions respectively. The 300 and 700 mM fractions (above and below respectively) were then passed through a gel filtration Superose 6 column and eluted according to protein complex size. Fractions were then processed for western blotting. Note how different Tbx5 complexes involving distinct isoforms with different chromatin binding behaviour can be observed.

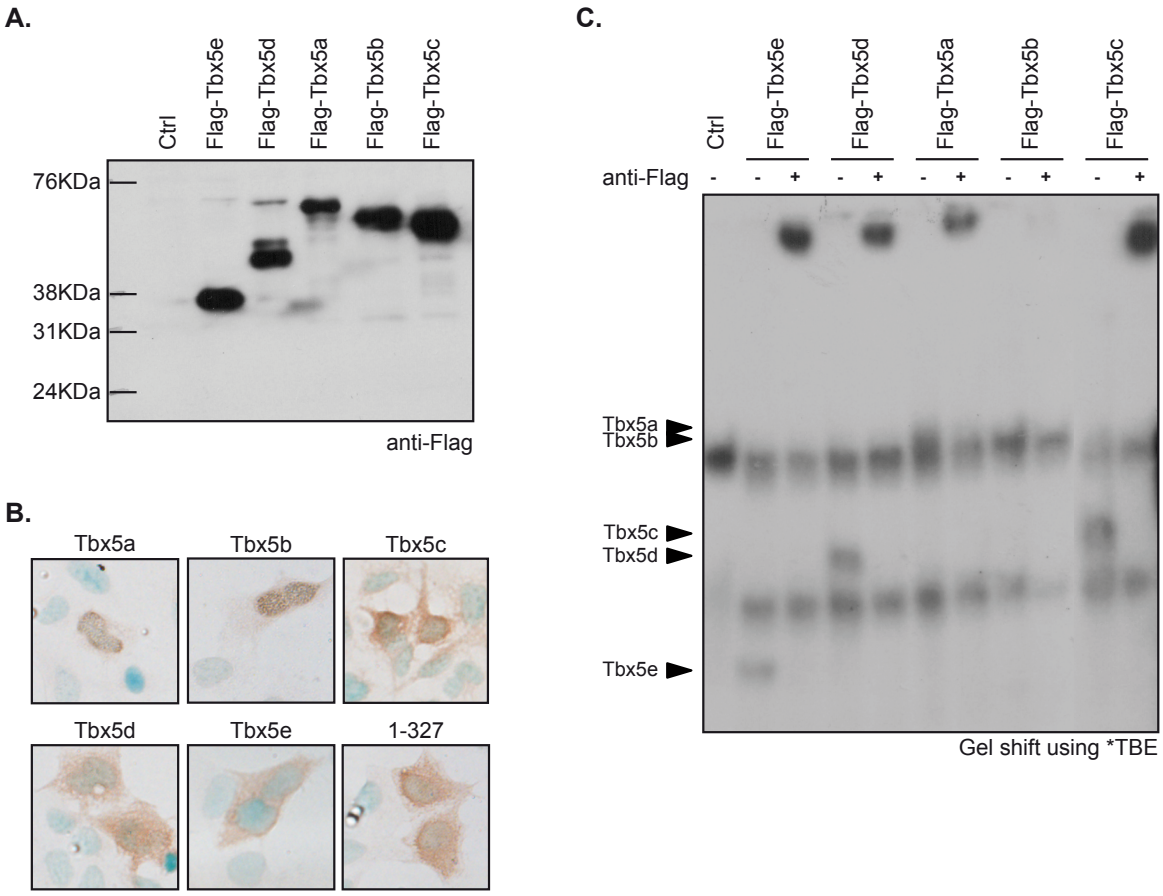

Figure 3 A. Expression of Tbx5 isoforms by western blot. Western blot analysis on 293T cells overexpressing the indicated Tbx5 isoform was done using Flag-M2 antibody. Note how all isoforms have similar expression levels. **B.** Immunocytochemical localization of Tbx5 isoforms. Immunocytochemistry was performed on 293T cells overexpressing the indicated Tbx5 protein using Flag-M2 antibody. Note the complete nuclear localization of Tbx5a and Tbx5b, the cytoplasmic localization of Tbx5e and the nuclear and cytoplasmic localization of Tbx5c and Tbx5d. **C.** DNA binding properties of Tbx5 isoforms. Electrophoretic mobility shift assay was performed using nuclear extracts from 293T cells overexpressing the indicated Tbx5 isoform. Supershift/blocking was done with anti-Flag M2 antibody. Ctrl: control.

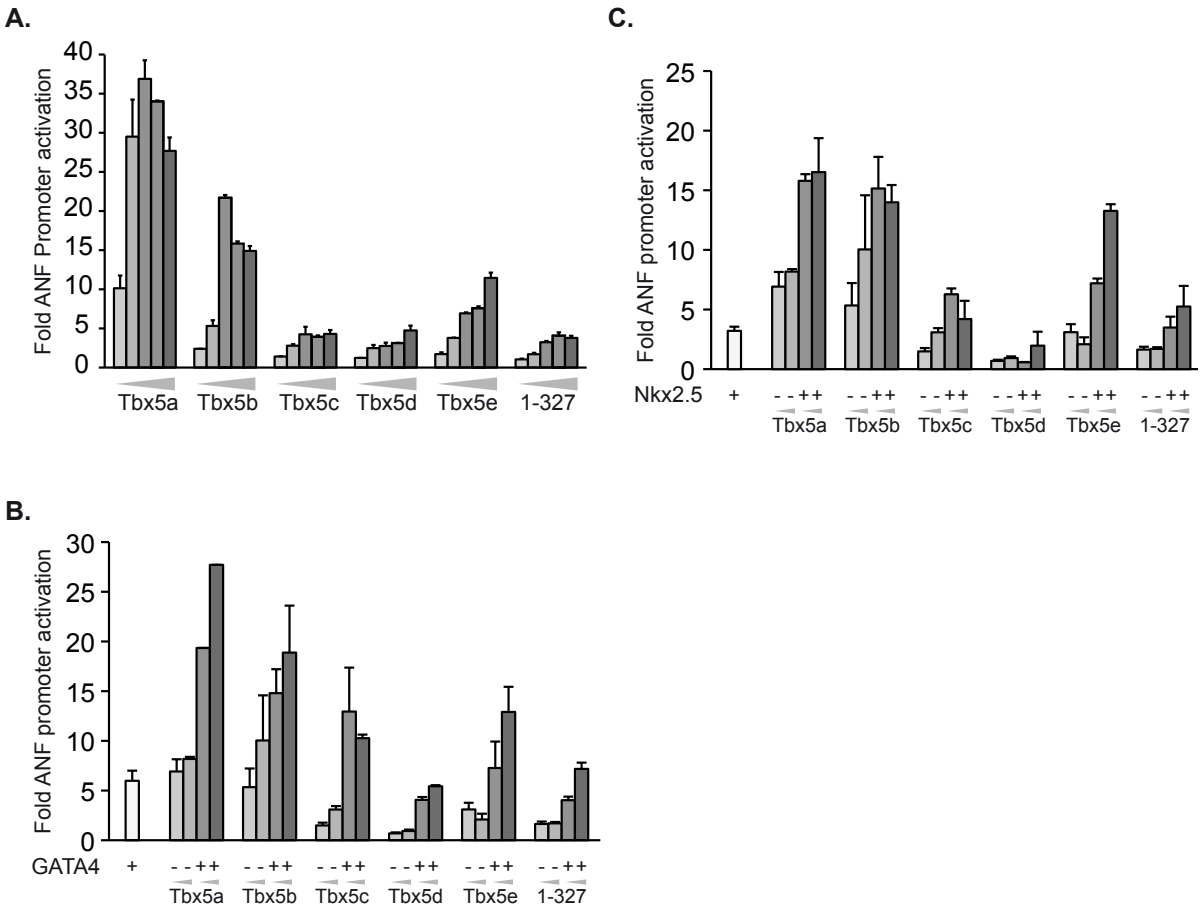

Figure 4 Differential abilities of the Tbx5 isoforms to activate transcription and interact with cofactors. **A.** Transcriptional activity of Tbx5 isoforms in NIH3T3 cells co-expressing ANF-luc and increasing doses of the indicated Tbx5 protein. Note the weak transcriptional activities of Tbx5c and Tbx5d. **B. & C.** Synergistic activity of the various Tbx5 isoforms with GATA4 (B) or Nkx2.5 (C) on ANF promoter. Note the weak/absent synergy of Tbx5c and Tbx5d with GATA4 and Nkx2.5. The results shown are those of one representative experiment of at least two carried out in duplicates with the standard deviation of the mean

Curriculum Vitae

Abir Yamak

Education

2007-present	**PhD. Candidate, Human and Molecular Genetics** University of Ottawa, Ottawa, Canada McGill University, Montréal, Canada
2004-2006	**M.Sc. In Biochemistry** American University of Beirut (AUB), Beirut, Lebanon
2000-2004	**B.Sc. In Biochemistry** Beirut Arab University (BAU), Beirut, Lebanon
1984-1999	**Lebanese Baccalaureate in Experimental Science** Tripoli Evangelical School, Tripoli, Lebanon

Skills

- Fluent in English and Arabic Languages. Intermediate level in French Language.
- Lab techniques:
  - Cell culture
  - RNA and DNA analysis including RNA and DNA extraction, RT-PCR, QPCR, DNA cloning, PCR and gel electrophoresis.
  - Protein analysis including western blot, electro mobility shift assay (EMSA), immunohistochemistry, immunofluorescence, immunocytochemistry, immunoprecipitation and chromatin immunoprecipitation (ChIP).
  - Mouse handling including IP injection, perfusion, embryo dissection and analyses of embryonic hearts at various stages.
- Computer skills including Mac OS X, Windows, Microsoft Office, Adobe Photoshop, Adobe illustrator, End Note, Prism and Internet browsing (including PubMed).
- Basic Bioinformatics knowledge (NCBI, UCSC Genome browser, Multialin, MultiTF, motifscan).
- Highly committed towards success.
- Great writing and presentation skills.
- Great communication skills and teamwork spirit.

Experience

Sept. 2008- now University of Ottawa Ottawa, Canada

PhD candidate.

I work on cardiac development and differentiation as well as supervise and co-supervise honours and masters students.

Sept. 2007- Aug. 2008 IRCM Montréal, Canada

Research on my PhD project at the Institut de Recherches Cliniques de Montréal (IRCM).

June 1, 2007-Aug. 31, 2007 AUB Beirut. Lebanon

Research Assistant at the American University of Beirut (AUB).

Jan. 2007- April 2007 IRCM Montréal, Canada

Training at the IRCM, cardiac development lab.

July 2005- July 2006 AUB Beirut, Lebanon

Graduate Assistant at The American University of Beirut (AUB).

I worked on "primary carnitine deficiency" in Lebanese patients.

I also worked on a mouse model of "congenital diaphragmatic hernia."

Sept. 2005- Oct. 2006 BAU Beirut, Lebanon

Lab instructor at Beirut Arab University (BAU).

I was a lab instructor for 3rd and 4th year biology and biochemistry students.

Sept. 1, 2003- Sept. 13, 2003 National Four Mills Tripoli, Lebanon

Training at the lab.

I worked on analysis of flour and wheat.

June 27, 2002- July 27, 2002 Hôpital De La Paix Tripoli, Lebanon

Training at the medical lab.

I conducted blood, urine and stool tests on patients.

Summer 2000, Fall 2001, Fall & Spring 2005. Tripoli and Beirut, Lebanon

Private Tutor

I gave private lessons to intermediate, high school and freshman students in chemistry, biology, mathematics and English.

Publications

- Cyclin D2 rescues size and function of GATA4 Haploinsufficient Hearts. **Abir Yamak**, Rana Temsah, Wael Maharsy, Sophie Caron, Pierre Paradis, Anne Aries, Mona Nemer. Am J Physiol Heart Circ Physiol. 2012 Aug 24. [Epub ahead of print]
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- Klf13 is a novel regulator of cardiogenesis in mammals. **Abir Yamak**, Salim Hayek, Wael Maharsy, Rami Darwich, Hiba Komati, Gregor Andelfinger, Mona Nemer. Manuscript ready for submission.
- Biochemical characterization of newly identified Tbx5 isoforms. **Abir Yamak**, Romain

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Abstracts

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- Deletion of Klf13 in mice leads to endocardial cushion defects**. A. Yamak**, S. Hayek, W. Maharsy, R. Darwich, H. Komati, G. Andelfinger, and M. Nemer. UOHI Research day. Ottawa, 2012.
- Cyclin D2 is a GATA-4 cofactor involved in cardiac growth. **Yamak A.**, Temsah R., Aries A., Caron S., Nemer M. University of Ottawa Poster Day. Ottawa 2011.
- Essential role for KLF13 in heart development. S. Hayek, **A. Yamak**, R. Darwich, W. Maharsy, H. Komati, G. Andelfinger, and M. Nemer. Weinstein Cardiovascular Development Conference. Cincinnati, 2011.
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- Cyclin D2 association with GATA4, a cardiac specific transcription factor. **Yamak AS. Abir**, Temsah Rana, Caron Sophie, Aries Anne, Dali Rola, Mona Nemer. BMI seminar symposium. Ottawa, 2011.
- Cyclin D2 is a GATA-4 cofactor involved in cardiac growth. **Abir Yamak**, Rana Temsah, Anne Aries, Sophie Caron, Mona Nemer. Weinstein Cardiovascular Development Conference. Amsterdam, 2010.
- Essential role of Gata4 in cellular proliferation and Differentiation. **Abir Yamak**, Mona Nemer. BMI seminar symposium. Ottawa, 2010.
- Cycline D2, une cible transcriptionelle directed de GATA-4. **Abir Yamak**, Rana Temsah, Sophie Caron, Mona Nemer. ACFAS, Ottawa, 2009.
- Cyclin D2 rescues postnatal heart development in Gata4 heterozygote mice. **Abir Yamak**, Rana Temsah, Sophie Caron, Mona Nemer. Weinstein Cardiovascular Development Conference. San Francisco, 2009.
- Cyclin D2, a direct transcriptional target of GATA4. **Abir Yamak**, Rana Temsah, Pierre Paradis, Mona Nemer. University of Ottawa Poster Day. Ottawa 2008.

Volunteer work and Activities

- Member of “Let’s Talk Science.”
- Member of “Scientists Without Borders.”
- Raised money for “Run for the Cure” to support breast cancer research.
- Member of “Dance Studios” salsa club.
- Member of “Ottawa Researchers Squash Team.”
- Previous member of TES volleyball and basketball teams and SNAP volleyball team.

Interests

Salsa dancing, squash, biking, swimming, skating, skiing, and reading.

Awards

- Biochemistry Graduate Program University of Ottawa Travel Award, 2012.
- BMI Poster Day University of Ottawa Prize, 1st place, May 2011.
- Faculty of Graduate Studies University of Ottawa Travel Grant, 2011.
- Biochemistry Graduate Program University of Ottawa Travel Award, 2011.
- Differential scholarship at University of Ottawa, 2009-2011.
- BMI Poster Day University of Ottawa Prize, 3rd place, May 2008.

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