Identification of GATA4 Regulatory Mechanisms of Heart Development and Disease

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Abstract

The development and function of the heart is governed by a conserved set of transcription factors (TFs) that regulate gene expression in a cell-type, time point and stimulus driven manner. Of these core cardiac TFs, the most ubiquitously expressed is the zinc finger protein GATA4. In cardiomyocytes, GATA4 is central to proliferation, differentiation, hypertrophy and induction of pro-survival pathways. In cardiac endothelial cells, it is required for valve and septal development, although the exact mechanisms remain unclear. To regulate such a wide array of functions in a spatially and temporally controlled manner, GATA4 interacts with specific protein partners, the majority of whom have been identified in cardiomyocytes. However, a complete understanding of the protein interactome of GATA4, particularly in cardiac endothelial cells, has not yet been achieved. Using a mass spectrometry-based approach, we have identified a series of novel GATA4 interacting partners in cardiac endothelial cells. 3xFlag GATA4 was stably overexpressed via retroviral transduction in the TC13 cardiac endothelial precursor cell line, immunoprecipitated from nuclear protein extracts and sent for HPLC-ESI-MS/MS. Several novel GATA4 interacting partners were identified including the chaperone protein Heat Shock Protein 70 (HSP70), the inducible orphan nuclear receptor Nerve Growth Factor 1β (NGFIβ, NUR77) and the Drosophila-Binding/Human Splicing protein family members Non-POU Domain Containing Octamer Binding Protein (NONO) and Paraspeckle 1 (PSPC1). Chapter 1 discusses the interaction between GATA4 and HSP70 and its role in cardiomyocyte survival upon exposure to chemotherapeutic agent Doxorubicin (DOX). HSP70 binds directly to GATA4, preventing DOX-mediated cleavage and degradation by Caspase-1, cardiomyocyte cell death and heart failure. Chapter 2 focuses on the cooperative interaction between GATA4 and NUR77 in cardiac microvascular endothelial cells and its central role in myocardial angiogenesis in

response to pressure overload. The GATA4-NUR77 complex transactivates the promoter of Angiopoietin-Like 7 (ANGPTL7), a secreted pro-angiogenic chemotactic factor, triggering endothelial cell proliferation and tube formation in cultured cardiac endothelial cells and increasing myocardial capillary density *in vivo*. Chapter 3 discusses the interaction between GATA4 and the DBHS proteins NONO and PSPC1 in the regulation of cardiac development. These proteins play opposing roles when bound to GATA4 as PSPC1 enhances GATA4 activation of critical cardiac promoter targets and NONO acts as a rheostat to repress GATA4 activity. *In vivo*, loss of NONO results in left ventricular non-compaction consistent with humans with loss-of-function mutations. However, simultaneous *Gata4* haploinsufficiency partially rescues this phenotype. Together, this data identifies multiple novel cell type and time point specific GATA4 protein partners and sheds light on GATA4 regulatory mechanisms in cardiac development and disease.

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AAA	Anomalies of the Aortic Arch
ACD	Acquired Cardiac Disease
ANF	Atrial Natriuretic Factor or Atrial Natriuretic Peptide
AngII	Angiotensin II
ANGPT2	Angiopoeitin 2
ANGPTL7	Angiopoeitin-Like Protein 7
ASD	Atrial Septal Defects
AT1R	Angiotensin II Type I Receptor
ATP	Adenosine Triphosphate
AVN	Atrioventricular Node
BAV	Bicuspid Aortic Valve
BCL2	B-Cell CLL/Lymphoma 2
BCLxl	B-Cell CLL/Lymphoma Extra Large
BNP	Brain Natriuretic Peptide
CAD	Coronary Artery Disease
CDK4	Cyclin Dependent Kinase 4
CHD	Congenital Heart Disease
ChIP	Chromatin Immunoprecipitation
CNP	C-Type Natriuretic Peptide
Col4a1	Collagen 4a1
DBHS	Drosophila Binding/Human Splicing Protein Family
DMEM	Dulbecco's Modified Eagle Medium
DORV	Double-Outlet Right Ventricle
Dox	Doxorubicin
EC	Endothelial Cell
ECM	Extracellular Matrix
EMSA	Electrophoretic Mobility Shift Assays
EMT	Epithelial to Mesenchymal Transition
eNOS	Endothelial Nitric Oxide Synthase
ET-1, Edn1	Endothelin-1
FOG2	Friend of GATA 2
GTEx	Genotype-Tissue Expression Project Portal
GWAS	Genome Wide Association Studies
HEY2	Hes Related Family BHLH Transcription Factor with YRPW Motif 2
HPLC-ESI-MS/MS	High Performance Liquid Chromatography, Electrospray Ionization Tandem Mass Spectrometry
HUVECs	Human Umbilical Vascular Endothelial Cells
IACUC	Institutional Animal Care and Use Committee, uOttawa
ICAM-2	Intercellular Adhesion Molecule 2
IL-1β	Interleukin-1β
IPA	Ingenuity Pathway Analysis
KLF13	Krüppel-Like Factor 13
LA	Left Atrium

LV	Left Ventricle
LVNC	Left Ventricular Noncompaction
MI	Myocardial Infarction
Mib	Mindbomb
MMP1	Matrix Metalloprotease 1
NBRE	NUR Binding Element
NCC	Neural Crest Cell
NFATc	Nuclear Factor of Activated T Cells
NKX2.5	NK2 Transcription Factor Related, Locus 5
NO	Nitric Oxide
NONO	Non POU Domain Containing Octamer Binding Protein
NR4A	Nuclear Receptor Subfamily 4 Group A
NUR77	Nuclear Orphan Receptor 77
OHGS	Ottawa Heart Genomics Study
PECAM	Platelet Endothelial Cell Adhesion Molecule
PEX1	Peroxisomal Biogenesis Factor 1
PH3	Phosphohistone 3
PPI	Protein Protein Interactions
PSPC1	Paraspeckle Component 1
PTA	Persistent Truncus Arteriosus
PTM	Post-Translational Modifications
RA	Retinoic Acid
RA	Right Atrium
RV	Right Ventricle
SAN	Sinoatrial Node
siRNA	Short Interfering RNA
SMAD4	SMAD Family Member 4
SP1	Specificity Protein 1
SRF	Serum Response Factor
TBX5	T-box Transcription Factor 5
TF	Transcription Factors
TNFα	Tumor Necrosis Factor α
ToF	Tetralogy of Fallot
TPM	Transcripts per Kilobase Million
TUNEL	Terminal Deoxynucleotidyltransferase- Mediated dUTP End-Labeling
UTR	Untranslated Region
VCAM	Vascular Endothelial Cell Adhesion Molecule
Vcan	Versican
VEGF	Vascular Endothelial Growth Factor
VSD	Ventricular Septal Defects
VSMC	Vascular Smooth Muscle Cell
vWF	von Willebrand Factor
βMHC	Beta Myosin Heavy Chain

1. Introduction

1.1 The role of the heart

The heart is the first organ to form during development and is central to the survival of all cells throughout the body. Its primary role is to drive blood flow to circulate oxygen, distribute metabolites required for cellular functions and remove waste products. This is accomplished by the coordinated contraction and relaxation of its four chambers. The right atrium (RA) and ventricle (RV) circulate blood returning from the systemic circulation towards the lungs to release carbon dioxide and reabsorb oxygen [1]. Deoxygenated blood returning from the systemic circulation enters the RA via the superior and inferior vena cava. The RA then contracts, forcing blood through the atrioventricular septum via the tricuspid valve and into the RV. The RA then relaxes and the RV contracts, closing the tricuspid valve, opening the pulmonary valve and forcing blood into the pulmonary artery and towards the lungs [1]. The left atrium (LA) and ventricle (LV) drive the systemic circulation. Once blood has returned from the lungs, it enters the LA via the pulmonary vein. The LA contracts forcing blood through the mitral valve into the LV. The LA then relaxes and the LV contracts, closing the mitral valve and opening the aortic valve, forcing blood into the aorta and towards the rest of the body [1]. This synchronized series of two pumps and 4 chambers present in mammals, birds and crocodilians offers a significant advantage over the 3 chambered heart of most reptiles, 2 chambered heart of fish or open circulatory system of insects as it completely separates oxygenated and deoxygenated blood and therefore is a much more efficient system [2,3].

Prior to the 1980s, this mechanical pump action was thought to be the only role of the heart in the body [4]. Although it is still most well known as a pump, we now know that it serves an equally important endocrine function as well [4]. With the use of electron microscopy to study subcellular details of atrial cardiomyocytes in the 1960s, it was demonstrated that these cells have features consistent with cells that secrete peptide hormones [5,6,4]. These features include a multitude of storage granules, Rough Endoplasmic Reticuli and Golgi Apparati. In 1981, de Bold and colleagues published their seminal work indicating for the first time that the heart is an endocrine organ [7]. This paper demonstrated that atrial myocardial extracts exert a strong diuretic and natriuretic effect when injected into rats and led to the discovery of Atrial Natriuretic Factor (ANF, also known as Atrial Natriuretic Peptide or ANP), a small peptide hormone that causes increased excretion of salt and water, vasodilation and increased vascular permeability [7–11]. Soon after, the related peptide hormones Brain Natriuretic Peptide and C-Type Natriuretic Peptide were discovered (BNP and CNP respectively) [12,13]. Of this natriuretic peptide hormone family, only ANF and BNP are secreted from the heart in significant amounts and became known as the cardiac natriuretic peptides [4]. Their critical role in cardiac disease became apparent when Nemer and colleagues found that while at baseline, ANF is primarily secreted by the atrial myocardium, cardiac hypertrophy or heart failure induces a 100-fold increase in ANF secretion from the ventricular myocardium [14,15]. This response was determined to be an attempt to reduce cardiac workload by lessening pressure overload [16]. What's more, BNP expression mimics that of ANF and has now been developed into a highly successful bioassay used to diagnose and stratify patients with heart failure [17–19]. Since the discovery of the cardiac natriuretic peptides, our understanding of cardiac endocrine function has become more complex and involves secretion of hormones including Endothelin-1 (ET-1) and members of the calcitonin gene-related peptide

family [4]. Collectively, this information has led to a greater appreciation of the multifactorial role of the heart and the molecular mechanisms that govern both cardiac mechanical and endocrine function.

1.2 Cardiac Development

Cardiac development consists of a highly complex series of steps that form the 4 cardiac chambers, the outflow tract and the valves and septa that separate them (Figure 1.1). During gastrulation, the embryo is formed of three germ layers that will give rise to all bodily tissues: the ectoderm, the endoderm and the mesoderm [20]. The vast majority of cardiac tissue derives from the mesoderm with a minor ectodermal contribution in the form of neural crest cells [20]. At murine embryonic day E7.5 (E18 in humans), a population of mesodermal cells called the lateral plate mesoderm forms the cardiac crescent, a bilaterally symmetrical structure composed of two distinct fields of cells called the primary and secondary heart fields [20]. The primary heart field will eventually give rise to the left ventricle and contribute to the atria and AV canal. The secondary heart field will give rise to the right ventricle and contribute to the atria and outflow tract[20,21]. At E8 (E20 in humans), cells of the primary heart field then migrate and fuse along the midline to form the linear heart tube. This structure consists of a tube of endocardial cells overlaid by cardiomyocytes in a basic endocardium/myocardium layout [20]. These two tissue types are separated by a primitive extracellular matrix secreted by the myocardium called the cardiac jelly [20,22]. Shortly thereafter, cells from the secondary heart field are added to the arterial and venous poles, elongating the structure and forming distinct regions [23]. At this point, the linear heart tube consists of the truncus arteriosus that will eventually become the outflow tract, the bulbus chordis that will become the right ventricle, a primitive ventricle that will become the left ventricle, a primitive atrium that will contribute to the right and left atria and the sinus

venosus that will contribute to the atria as well as form the sinoatrial node and the coronary sinus [24]. At E8.5, very soon after the initiation of the heartbeat, cardiac looping begins (E24 in humans) [23]. The heart begins by bending into a C-shape and develops a morphologically distinct atrium, ventricle and outflow tract [23,25]. It then continues bending into S-shape such that the arterial and venous poles are aligned on the same plane [21,23,25] . During S-looping, the cardiac jelly thickens in the regions that will become the atrioventricular canal and the outflow tract [23]. These regions develop into the endocardial cushions and are derived from endocardial endothelial cells that have undergone epithelial to mesenchymal transition (EMT) and have migrated into the cardiac jelly [23]. Upon the near completion of cardiac looping, ventricular trabeculation begins via the movement of the cardiac jelly separating the myocardium and endocardium and the formation of endocardial pouches that grow inwards towards the myocardium [23,26]. Trabeculae consist of small outgrowths of myocardial cells lined by endocardium that increase ventricular surface area, cardiac output and wall stiffness [23,26]. These structures serve to increase nutrient and oxygen uptake, buffer the effects of high pressure blood flow on the chamber walls and promote emptying efficiency of the ventricle during systole [23,26–31]. Afterwards, the myocardium begins to compact, forming the mature, thickened ventricular myocardium and contributes to the formation of the ventricular septum [26].

Throughout looping and trabeculation, the heart simultaneously begins forming the valves and septa separating the heart chambers [23]. As discussed above, trabeculation and compaction of the myocardium contribute to the formation of cardiac septa. As well, development of the endocardial cushions consisting of mesenchymal progenitor cells overlaid with endocardial endothelial cells is required [23]. Before finishing their development into mature valves and septa, they already assist with the shunting of blood from the atria to the ventricles

and out the outflow tract [23]. However, once mature, they are a far more effective system used to ensure the unidirectional flow of blood. To finish development, the endocardial cushions found between the atria and ventricles fuse and condense to form the mature tricuspid and mitral valves whereas the endocardial cushions separating the ventricles from the outflow tract remodel to form the semilunar valves (the pulmonary valve and aortic valve) [23].

During development, the heart also forms a layer external to the myocardium referred to as the epicardium [20]. The epicardium is derived from the proepicardium beginning at E11.5 (E34 in humans) that itself derives from the mesodermal cells that lie dorsal to the linear heart tube [20]. As well, these cells also give rise to cardiac fibroblasts, coronary vascular smooth muscle cells, coronary endocardial cells and even a small number of cardiomyocytes, making them an important contributor to cardiac development [20].

These developmental steps are virtually complete prior to birth. All structures are fully formed and with the advent of the first breath, the foramen ovale, an open valve flap created by the septum primum and secundum separating the atria that allows the pulmonary circulation to be circumvented prior to birth is now closed [32]. As well, approximately one week post-birth, cardiomyocytes lose their ability to proliferate [33,34]. Together, these steps create the complex three-dimensional form of the heart and are tightly regulated by a series of molecular pathways to ensure correct cardiac development and function.

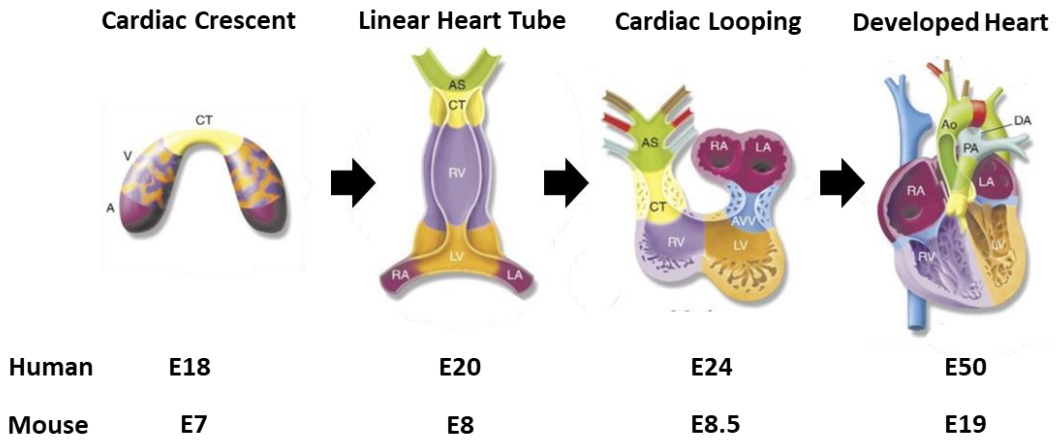

Figure 1.1 The stages of cardiac development. A = atrium, Ao = aorta, AS = aortic sac, CT = conotruncal segment, DA = ductus arteriosus, LA = left atrium, LV = left ventricle, PA = pulmonary artery, RA = right atrium, RV = right ventricle, V = ventricle. Adapted from Srivastava and Olson 2000 [35].

1.3 Cardiac Structure

Once fully developed, the heart is composed of 3 distinct layers: the pericardium, the myocardium and the endocardium (Figure 1.2). The pericardium is the outermost layer of the heart and serves as a protective barrier against mechanical and immunological threat [36]. It also limits maximum distention of the heart, thereby limiting maximum cardiac volume [36]. The pericardium is composed of 2 components. The outermost layer is the parietal pericardium made of a fibrous sac of connective tissue lined with a layer of serosal mesothelial cells [1,37]. The visceral pericardium, otherwise known as the epicardium, adheres directly to the surface of the heart and is composed of cubical serosal mesothelium overlaying a space containing connective, adipose and vascular tissue [38,39]. The visceral pericardium also contributes cells and paracrine signals to the heart during development and after cardiac injury [40–45]. To allow the heart to contract and relax with minimal resistance inside the pericardial sac, the serosal mesothelial cells

of both the parietal and visceral pericardium secrete a mucopolysaccharide fluid into the pericardial cavity separating the two tissues [37,46].

The myocardium lies directly underneath the epicardium and generally refers to the musculature of the heart. The primary role of the myocardium is to generate the contraction and relaxation required to circulate blood throughout the body. This tissue is structurally complex and consists of various types of cardiomyocytes, fibroblasts, fibrous extracellular matrix, arteries, veins, lymphatic vessels and nerves [47]. The myocardium is structured slightly differently in each of the 4 cardiac chambers. As the chambers of the left side of the heart are responsible for the perfusion of the entire body, they are required to generate more force per contraction and as such, are thicker than their right-sided counterparts [1,48]. In particular, the left ventricle free wall is approximately 3 times thicker than the right ventricle free wall [48,49]. Both ventricles contain trabeculae, irregular ridges and protrusions of myocardial tissue overlaid by endocardium that point towards the inner ventricle chamber [1]. The trabeculae of the left ventricle are finer and more intricate than those of the right ventricle although they share the same general morphology [1].

The endocardium is the innermost layer of the heart that lines each cardiac chamber and forms the valves and septa [49–51]. It is comprised of a luminal layer of endothelial cells overlaying a thin layer of connective tissue and a subendocardial layer comprising blood vessels, nerves, connective tissue and Purkinje Fibres [49]. Aside from its numerous roles during cardiac development, the endocardium plays an important hemostatic role [49]. As the endothelial cells are in direct contact with the contents of the cardiac chambers and are antithrombotic, they are required to prevent cells from adhering and forming a clot [52,53]. Structurally, the endocardium lining the chambers is thickest in the atria compared to the ventricles and on the left side of the

heart versus the right side of the heart [49]. The endocardium also comprises 4 valves whose primary role is to ensure the unidirectional flow of blood through the heart [49]. Two atrioventricular valves separate the atria from the ventricles: the tricuspid valve on the right side of the heart and the mitral valve on the left side. The two semilunar valves consist of the pulmonary valve that separates the right ventricle from the pulmonary artery and the aortic valve that separates the left ventricle from the aorta [49]. All but the mitral valve are composed of three leaflets and are deemed tricuspid. The mitral valve only contains 2 leaflets and is therefore bicuspid [49]. These structures do not exert a force by themselves; instead, each valve opens and closes passively due to a pressure differential on opposing sides of the leaflets [54].

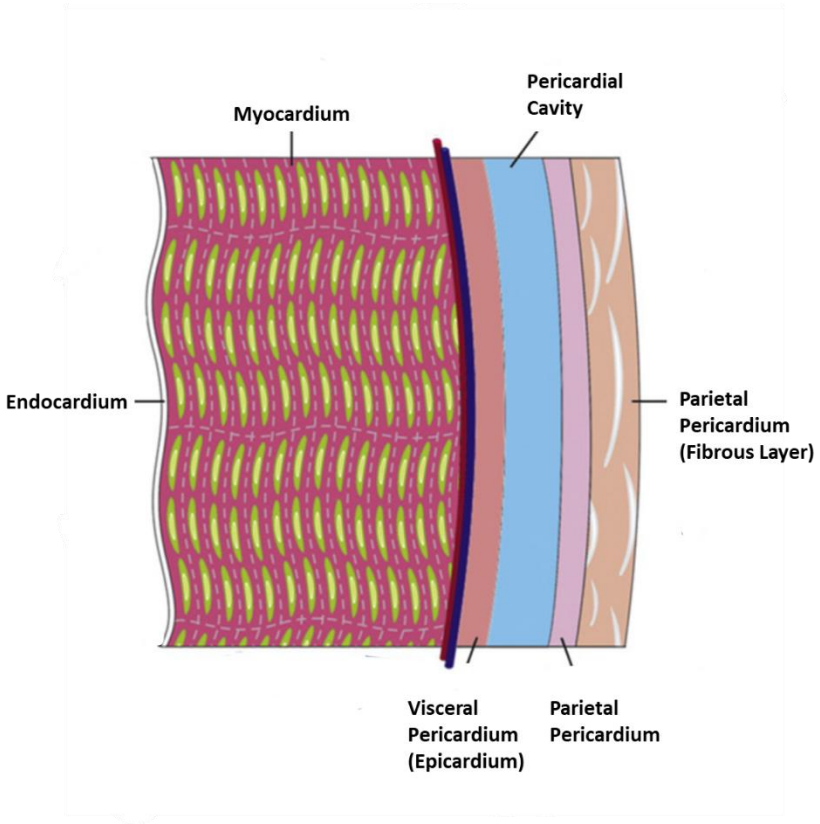

Figure 1.2 Cross section of the heart indicating its different tissue layers. Adapted from Sepantafar *et al* 2016 [55]

1.4 The Cellular Composition of the Heart

As discussed above, the structure of the heart is complex and consists of many tissue layers. Each layer of the heart is composed of multiple cell types that are all required to ensure proper cardiac development and function (Figure 1.3).

1.4.1 Cardiomyocytes:

Cardiomyocytes, the most well-known and well-studied cardiac cell type, are the contractile units of the heart. Although they only make up 25-35% of total cells in the heart, they occupy 70-85% of heart volume [56]. During development, they arise from the lateral plate mesoderm and are first observed in the linear heart tube stage. However, some publications have shown that a small population of cardiomyocytes are also derived from proepicardial cells [20]. Aside from being the largest cells in the heart, they have several other distinguishing morphological differences. These features include the sarcoplasmic reticuli and sarcomeres that together are responsible for the excitation-contraction coupling that generates the heartbeat [57,58]. As well, they are frequently binucleated [59]. Shortly after birth, cardiomyocytes of the ventricles undergo another round of karyokinesis without undergoing cytokinesis, leaving the cell with two nuclei [59]. From this point forward, cardiomyocytes are considered to be non-proliferative, although some studies do show that they do have a very limited regenerative capacity [60,61]. To increase in size in the adult heart, the myocardium grows instead by cardiomyocyte hypertrophy, the process by which the cells increase protein synthesis, reorganize and expand their sarcomeric network and grow in size [62].

There are three main varieties of cardiomyocytes in the heart belonging to the atria, ventricles and conduction system. A complete understanding of the similarities and differences

between these cells (particularly atrial versus ventricular cardiomyocytes) has not yet been achieved. However, many contractile, structural and gene expression differences have been identified [63]. The primary characteristic distinguishing atrial cardiomyocytes from the other two subtypes during development is their requirement for retinoic acid [64]. Overexpression of retinoic acid early in development results in hyperplastic atria and hypoplastic ventricles, suggesting that more myocytes had become committed to the atrial lineage [65]. The primary physiological differences distinguishing atrial from ventricular cardiomyocytes are the absence of transverse tubules, the presence of atrial specific granules with associated Golgi complexes, a higher density of mitochondria and only one elongated nucleus rather than two separate nuclei [66–68]. Conduction system cardiomyocytes can be distinguished from the other subtypes by their locations, morphology, electrophysiology and marker expression [67]. These cells are located in the Sinoatrial Node (SAN), Atrioventricular Node (AV Node), Bundles of His and Purkinje Fibres and their main role is to produce and propagate the electrical impulses that stimulate cardiac contraction [67]. The specialized cardiomyocytes of the two nodes are spindle shaped and either act as the originators of the cardiac current (SAN) or receive the current and have a relatively slow conduction velocity (AV Node) [67]. Cardiomyocytes of the Bundles of His and Purkinje fibres feed the current throughout the right and left ventricles and have a very fast conduction velocity [67]. Collectively, atrial, ventricular and conduction system cardiomyocytes lead to the orchestrated contraction and relaxation of the myocardium and as such, have been the focus of the majority of research on cardiac cells.

1.4.2 Endothelial Cells

Although much smaller than their cardiomyocyte counterparts, endothelial cells play a central role in cardiac development and function and represent the largest population of cells in

the heart (>60% of all non-myocytes) [56]. In total, there are 5 known varieties: endocardial, microvascular, coronary arterial, venous and lymphatic endothelial cells [69]. They all differ from one another in their cellular origins, morphology and roles in the heart [69].

Endocardial cells are the first cardiac endothelial cells to arise during development and like cardiomyocytes, are derived from the lateral plate mesoderm and first appear at the linear heart tube stage. There, they form a primitive endocardium beneath the cardiomyocyte layer [69]. In the adult myocardium, these cells line each cardiac chamber, forming a barrier between the circulating blood and myocardium [70]. They are also the primary constituents of the valves and septa that separate the chambers and great arteries from one another [70]. Aside from these important roles, they also secrete several small molecules, peptides and neurohormones that have a direct impact on cardiac growth, metabolism, contractility and rhythm including Nitric Oxide (NO), ET-1 and members of the Vascular Endothelial Growth Factor family, among others [70]. Morphologically, endocardial cells are among the largest endothelial cells of the body, have well developed organelles and secrete larger amounts of paracrine factors in comparison to other cardiac endothelial cell types [70].

As their names imply, coronary arterial, venous and microvascular endothelial cells line the major arteries, veins and capillaries that supply the heart with blood [69]. Unlike endocardial cells, they derive from the vascular plexus made from pro-epicardial cells that migrate from the sinus venosus [44]. During early stages of development, cell respiration in the myocardium takes place by simple diffusion. As such, these cells are not required to be fully developed and form completed vascular networks until later stages of development when the myocardium has thickened [69]. These cells are critical to cardiac function as they dynamically regulate vascular tone and permeability and, in the case of microvasculature, undergo angiogenesis to dynamically

regulate myocardial perfusion [71,72]. To do so, cardiac vascular endothelial cells respond to a number of factors including cytokines, Tumor Necrosis Factor α (TNFα) and interferons [73]. They also secrete important signaling molecules themselves including Angiopoietins and NO [73,74]. Unfortunately, these cells are frequently involved with cardiac disease. Coronary artery endothelial cells are frequently the site of atherosclerotic plaque development and microvascular capillary density becomes dramatically reduced during heart failure [75,76].

Lastly, the lymphatic vessels of the heart are also lined with endothelial cells and compared to the other cardiac endothelial cells, far less is known about their origins and functions [69]. Currently, it is thought that they derive from lymphangioblasts that immigrate to the heart concurrently with coronary vasculature development and it is likely that they control permeability and tone of the lymphatic vessels [69]. However, the specific roles of these cells in the heart have yet to be determined.

1.4.3 Fibroblasts

Cardiac fibroblasts are connective tissue cells that primarily serve as structural components in the heart [77]. These flat, spindle-shaped cells secrete extracellular matrix (ECM) components such as collagens and fibronectin and like the vast majority of cardiac cells, are mesenchymal in origin [77]. More specifically, these cells originate from the pro-epicardium [77]. Arguably the most important role of cardiac fibroblasts is the formation and management of the ECM network forming the scaffolding for cardiomyocytes, endothelial cells and other cardiac components. This scaffolding is central to the formation of the 3D structure of the heart [77]. The secreted ECM is composed of several components including collagens, fibronectins, proteoglycans and glycoproteins and distributes mechanical forces throughout the myocardium, contributes to signal transduction and interconnects all cardiac cells [77]. Another central role of

fibroblasts is the creation of scar tissue after myocardial damage. As cardiomyocytes are non-proliferative, fibroblasts produce an ECM scar to fill the region where cardiomyocytes are lost. Although the formation of this scar is critical to cardiac function post-injury, it is non-contractile and as such, contributes to myocardial stiffening, reduced cardiac contractility and heart failure [77].

1.4.4 Vascular Smooth Muscle Cells

Vascular smooth muscle cells make up a relatively small percentage of cardiac cells and are primarily present in the coronary vasculature, directly overtop of the endothelial layer [78]. There, they provide support to the vessel and maintain intravascular pressure and tissue perfusion [79]. They are also present in small numbers in the valve leaflets [80]. The origin of these cells is somewhat complicated as they can develop from the splanchnic or somatic lateral plate mesoderm, paraxial mesoderm as well as from neural crest cells [79,81,82]. In the adult heart, they primarily exist in a quiescent, contractile phenotype but can rapidly dedifferentiate into a proliferative one to repair vascular injury [79].

1.4.5 Epicardial Cells

As with cardiac fibroblasts and vascular endothelial cells, epicardial cells derive from the pro-epicardium [83,84]. During development, they migrate over the surface of the heart in what was originally thought to be only a protective layer for the myocardium [83–85]. However, it is now known that the epicardial cells contribute to the proper development of the myocardium and coronary vasculature via the secretion of paracrine factors [83,84,86]. Their other roles include the secretion of a layer of ECM that separates itself from the myocardium and aids in signaling

between the two layers. A small number of epicardial cells also undergo EMT, migrate through the ECM and contribute to the cardiomyocyte lineage too [84,85].

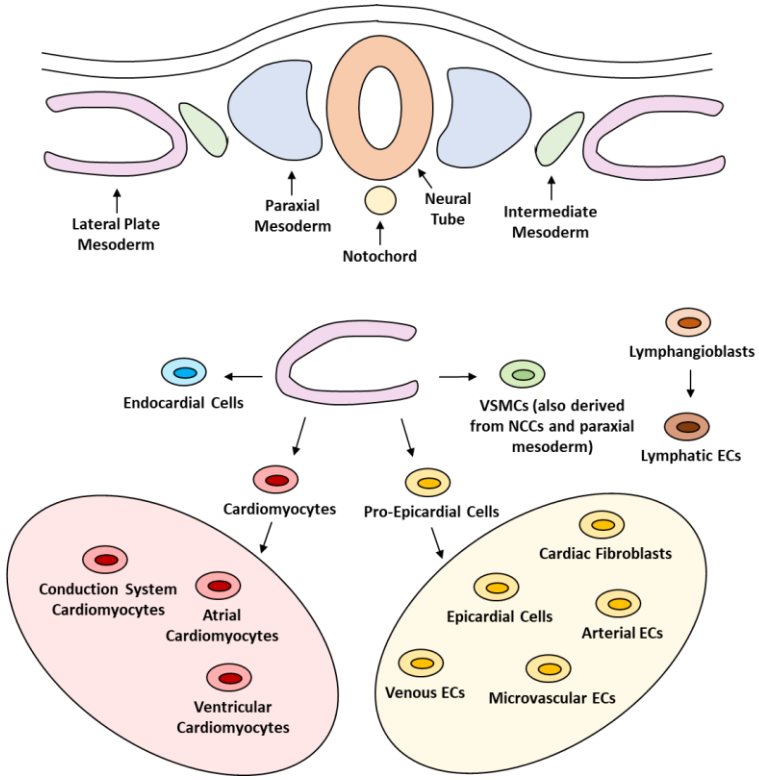

Figure 1.3 The Cellular Composition of the Heart. ECs = endothelial cells, VSMCs = Vascular Smooth Muscle Cells, NCCs= Neural Crest Cells. Adapted from Safadi *et al.* 2009 [87]

1.5 Cardiac Disease

1.5.1 Congenital Heart Disease

The development of the heart is tightly regulated to ensure proper function throughout the lifespan of the organism. Errors in these developmental programs are associated with cardiac structural and functional problems collectively referred to as Congenital Heart Diseases (CHDs). CHDs represent a major healthcare burden worldwide as they are present in over 1% of live births and 10% of aborted fetuses [88]. They are the leading cause of mortality due to birth defects

and often require surgical interventions early on in life [88]. Phenotypically, CHDs are an extremely heterogenous group of defects that can affect the myocardium, endocardium and conduction system. Examples of each have been listed in table 1.1. As well, they are heterogenous in terms of severity. Severe CHDs are often associated with fetal loss during development or require medical intervention within the first year of life [88,89]. Examples include Transposition of the Great Arteries and Hypoplastic Left Heart Syndrome [89,90]. In the past several years, surgical options for those affected by major cardiac defects have improved greatly and have improved longevity and quality of life [88–90]. As such, an increasing proportion of these individuals survive until adulthood [88]. However, severe CHDs remain associated with higher instances of mortality [88]. Other individuals affected by CHD present with milder phenotypes that often include small septal or valve defects [90]. Although these phenotypes are generally not associated with an immediate high risk of mortality, they are associated with adverse cardiac outcomes later in life including stroke, heart failure and arrythmias [91,92]. As they do not present with immediate symptoms suggesting a cardiac malformation, milder cases of CHD frequently remain undetected and as such, improvements to diagnostic strategies are greatly needed [92–94].

CHDs can be caused by environmental factors such as teratogen exposure or mutations in key developmental genes including those outlined in the section entitled "The Regulation of Gene Expression in the Heart" [89]. However, for the majority of CHD cases, no single mutation has been associated with the observed phenotype [89,90]. New DNA sequencing technologies such as whole exome sequencing and genome wide association studies (GWAS) are improving our ability to detect mutations in both established and previously unknown CHD-causing genes and increasing the number of cases for which a genetic cause can be identified [89,90]. However,

research supports that in the majority of cases, CHD are multifactorial in origin and are caused by a combination of both genetic and environmental factors [89,90].

Defect Name	Abbreviation	Localization
Atrial Fibrillation	AF	Conduction System
Atrial Septal Defect	ASD	Endocardium
Atrioventricular Septal Defect	AVSD	Endocardium
Bicuspid Aortic Valve	BAV	Endocardium
Coarctation of the Aorta	CoA	Endocardium
Dilated Cardiomyopathy	DCM	Myocardium
Double Inlet Left Ventricle	DILV	Endocardium
Double Outlet Right Ventricle	DORV	Endocardium
Hypertrophic Cardiomyopathy	HCM	Myocardium
Patent Ductus Arteriosus	PDA	Endocardium
Patent Foramen Ovale	PFO	Endocardium
Tetralogy of Fallot	TOF	Endocardium/Myocardium
Transposition of the Great Arteries	TGA	Endocardium
Ventricular Septal Defect	VSD	Endocardium

Table 1.1 Examples of common CHDs, their standard abbreviation and their general localization in the heart.

1.5.2 Acquired Cardiac Disease

As discussed above, although CHDs are the most common birth defect, they are only present in approximately 1% of live births and in most cases cardiac development proceeds without incident. However, the post-natal heart can be subject to acquired cardiac diseases (ACDs), those that present as a result of an external cardiac stressor rather than a developmental error. Acquired cardiac disease represents a major healthcare burden in Canada and worldwide.

In Canada, it is the second leading cause of death (following cancer) and is currently diagnosed in 2.4 million people [95]. It also represents a massive economic cost, both directly due to hospitalizations, medications, medical appointments and rehabilitation and indirectly due to lost productivity caused by mortality or disability [96]. Worldwide, ACD disease is the leading cause of death, accounting for 17.7 million deaths annually (31% of the total) [97]. Furthermore, the aging population in developed nations such as Canada along with increased global obesity rate, sedentary lifestyles and prevalent smoking and alcohol consumption is predicted to drive the cardiac disease rate higher globally [97,98]. By 2030, it is predicted to cause 23.6 million deaths annually [97].

ACDs present in multiple different forms that can be for the most part classified into 4 groups: ischemic, hypertensive, inflammatory, and rheumatic [99]. Ischemic cardiac disease is by far the most common and involves the inadequate perfusion of the myocardium due to reduced blood flow, increased myocardial oxygen demand or both [100]. The most common cause of ischemic heart disease is Coronary Artery Disease (CAD), a condition whereby the coronary vasculature becomes damaged leading to reduced myocardial blood flow [100,101]. CAD is most frequently by plaque accumulation and a narrowing of the vessel lumen and as it progresses slowly, often over decades, it is difficult to detect in early stages [100,101]. At later stages, it causes angina (chest pain) and dyspnea (shortness of breath) [100,102]. In severe cases, a complete blockage of coronary vasculature referred to as myocardial infarction (MI) can occur and if not immediately fatal, leads to rapid cardiomyocyte death, the formation of a fibrous scar and the eventual progression into heart failure [100,103].

Hypertensive cardiac disease is also a major contributor to cardiac disease rates globally. It is defined as cardiac injury caused by the response of the left ventricular myocardium to

mechanical stress due to elevated arterial pressure [104]. As detailed in the section below, although the myocardium is initially able to compensate for the increased workload and sustain cardiac output, consistent exposure to pressure overload results in cardiac remodeling and eventual heart failure as detailed in the section below on cardiac compensation, decompensation and heart failure [104,105]. Mutations in several genes have been associated with increased susceptibility to hypertension and include members of the Renin-Angiotensin-Aldosterone Pathway as well as renal sodium and water handling genes [106]. However, environmental risks also play a major role as age, sedentary lifestyle and sodium consumption are all known causes of hypertension too [107–109].

Inflammatory and rheumatic cardiac disease are less prevalent in higher income nations; however, in low and middle-income countries, they continue to present a major healthcare challenge [99]. Inflammatory cardiac disease involves the inflammation of the myocardium (myocarditis), pericardium (pericarditis) or endocardium (endocarditis) caused by viral, bacteria, fungal or parasitic infection, environmental toxins or adverse reactions to some medications [110]. Rheumatic heart disease involves damage to the cardiac tissue due the body's autoimmune response to a throat infection by *Streptococcus pyogenes* [111]. Lastly, although not one of the major varieties of cardiac disease globally, the link between ACD and exposure to chemotherapeutic agents is now appreciated [112]. Anthracyclines including Doxorubicin, Tyrosine Kinase Inhibitors including Imatinib and DNA/RNA Synthesis Inhibitors including 5-flurouracil have all been linked to long term cardiac damage [113–115]. Furthermore, the cardiotoxic effect of these substances often presents long after the chemotherapy is complete, making it challenging to identify susceptible individuals [116].

Although ACDs demonstrate a wide range of phenotypes, the common denominator between them is that they will all eventually lead to cardiomyocyte death, reduced cardiac output and heart failure, the inability to pump sufficient blood to provide for the metabolic and oxygen demands of the body [117]. Likewise, ACDs have much in common with CHDs as well. Both ACD and CHD are heterogenous in presentation and most commonly affect the myocardium (for example inherited hypertrophic and dilated cardiomyopathy and myocardial infarction) and endocardium (for example valvular and septal defects, aortic valve stenosis, atherosclerosis causing CAD). Likewise, they are heterogenous in terms of severity and range from fatal to completely asymptomatic. They represent a significant healthcare burden worldwide as CHDs are the most commonly diagnosed birth defect and ACD is the most common cause of death globally [88,99]. As well, both ACD and CHD are multifactorial in origin. Although roughly one third of CHD cases can currently be attributed to genetic mutations, the cause of the remaining two thirds is unclear [118]. Likewise, a genetic predisposition to ACDs such as hypertensive cardiac disease can be identified in some families but in most cases, only environmental factors are identified as the causative mechanism [106]. Recently, it has become appreciated that both CHD and ACD are more complex and that the presence of mutations in genes previously not associated with cardiac development and function, modifier genes that alter the expression of key cardiac genes and their interactions with environmental factors may explain the complex presentation of cardiac diseases in the population [118].

1.6 Cardiac Compensation, Decompensation and Heart Failure

Despite the multitude of diseases that can affect it, the heart is relatively resilient and able to adapt to most cardiac stressors on a short-term basis. Cardiac compensation refers to the heart's ability to maintain adequate cardiac output despite changes in physiological,

pharmacological or pathological stimuli and is accomplished through the modification of stroke volume and/or heart rate [119]. Acute changes in blood flow require only short-term modifications to either of those two variables. Chronic cardiac stress leads to lasting physical adaptations to the heart itself, one of the most effective being cardiac hypertrophy [120]. This process can be divided into two categories: physiological hypertrophy and pathological hypertrophy. Physiological hypertrophy is characterized by normal cardiac morphology, no loss of cardiomyocytes, sufficient myocardial capillary density and normal or enhanced cardiac function [121,122]. Known physiological hypertrophy-inducing stimuli include normal post-natal growth, pregnancy and exercise training [120,123]. As cardiac growth must occur in proportion to the growth of the developing organism, a certain degree of cardiomyocyte hypertrophy is required throughout the progression from infancy to adulthood [123]. Similarly, physiological hypertrophy is also required to support the natural volume overload characteristic of pregnancy [124]. Exercise training, particularly aerobic training, is also capable of inducing physiological hypertrophy which can also be referred to as the "athlete's heart" [123]. This increase in cardiac mass is required to support the physiological demands of exercise training on the body including rapid consumption of oxygen, increased generation of waste products and greater requirement for the blood-borne substrates to produce adenosine triphosphate (ATP) [123,125].

In contrast to physiological hypertrophy, pathological hypertrophy is defined as an increase in cardiac size caused by myocardial hypertrophy that, while initially compensatory, is ultimately characterized by aberrant cardiac morphology and deteriorating function [120,123,126,127]. Diseases causing pathological hypertrophy are becoming more and more commonplace among the population and include hypertension, atherosclerosis, myocardial damage due to infarction and aortic stenosis [123]. Morphologically, hearts undergoing pathological hypertrophy generally

grow in a concentric manner leading to a significant decrease in chamber size in proportion to wall thickness whereas physiological hypertrophy generally presents with an eccentric geometry [126]. As well, unlike physiological hypertrophy, pathological hypertrophy is stimulated by neurohumoral activation as well as mechanical stress [127]. Direct stimulation of cardiomyocytes by norepinephrine of the sympathetic nervous system and Angiotensin II of the Renin-Angiotensin-Aldosterone system triggers downstream signaling pathways that activate cardiomyocyte pro-hypertrophic genes to initially maintain function but ultimately contribute to the progression to heart failure [127].

Although both physiological and pathological hypertrophy both cause an increase in myocyte size, sarcomeric reorganization and an initial increase in cardiac output, there are several cardiac characteristics unique to pathological hypertrophy that help explain why it ultimately leads to HF [120,123,126,127]. The induction of pathological hypertrophy and not physiological hypertrophy is associated with the activation of genes required during cardiac development. Collectively, the expression of these genes is referred to as the fetal gene program and includes transcription factors (TFs) of the GATA, MEF2, SRF and NK2 families, natriuretic factors ANF and BNP and structural proteins including Beta Myosin Heavy Chain (βMHC) [127–129]. Activation of developmental TFs serves to activate downstream fetal genes and the activation of natriuretic peptides alleviates strain on the heart via diuresis, natriuresis and vasodilation [130]. The switch from αMHC to βMHC involves switching from a more rapid contractile phenotype to a more economical phenotype in terms of force generation [127,131,132]. This switch from αMHC to the fetal βMHC is associated with poorer cardiac performance and outcomes [131,132].

Another hallmark of pathological hypertrophy is cardiomyocyte cell death. If the disease-causing stimulus continues to cause pressure or volume overload on the heart, cardiomyocytes

are eventually unable to withstand their increased workload and as such, begin to die [127]. This point in disease progression is referred to as decompensation. At the same time, capillary density decreases throughout the myocardium via unknown mechanisms, creating an ischemic environment that places further strain upon those that remain [127,133,134]. These conditions result in a feed-forward cycle that ultimately results in increasing cardiomyocyte death [127]. At this point during disease progression, fibrous lesions form in the regions of the ventricles left vacant due to myocyte death [120,123,126,127]. As they are a non-contractile tissue, they further limit cardiac function. The combined effect of the activation of the fetal gene program, decreased ventricular capillary density, increased cardiomyocyte death and formation of fibrotic lesions result in the decrease in systolic and/or diastolic cardiac function and are unfortunately non-reversible [127]. Once the heart is unable to provide adequate blood flow to meet the metabolic needs of the body, the patient is said to be in heart failure [135].

1.7 The Regulation of Gene Expression in the Heart

As described in the previous section, the heart is a complex three-dimensional organ required to perform consistently from the earliest stages of development throughout the entire lifespan of the organism. The many cell types that make up the heart that are detailed in the previous section must proliferate and differentiate properly as well as interact with one another and respond to environmental cues [136]. To do so, each cell activates a highly specialized gene expression program that is specific to its cell type, time point, and stimuli detected. The proper regulation of these gene expression programs is central to cardiac cell fates and is therefore critical to cardiac development and function. The activation of gene expression programs is induced by upstream signaling cascades initiated by chemical signals detected on the cell surface [136]. These signaling cascades terminate with the activation of transcription factors (TFs), DNA

binding proteins that bind to specific recognition motifs located on the promoters and enhancers of target genes [136,137]. Once bound, TFs either induce or repress gene expression to regulate cardiac cell fates [136].

Given that gene expression programs in all cells are regulated by TF binding, locating recognition motifs on the promoters of important cardiac genes lends insight onto which TFs play central roles in the heart [138]. The first studies using this approach took place in the developing heart and since then, several important cardiac TFs have been identified [136]. However, the recognition motifs of a select few were found on the promoters of virtually all cardiac developmental genes and as such were determined to be key developmental regulators [136]. The TFs, members of the GATA, MEF2, NK2, TBX and HAND families, have been highly conserved throughout evolution and are now considered "core cardiac TFs" [136,139]. These TFs are well expressed in the heart, particularly during development, and activate diverse pathways in cardiomyocytes and endocardial cells [136,139]. Furthermore, by binding to recognition motifs on the promoters of their own genes, they self-sustain their high levels of expression during development [136]. At a protein level, core cardiac TFs are also well known to complex with one another to synergistically activate target promoters to enhance their expression [140–142].

The importance of these core cardiac TFs is best illustrated by transgenic animal models and human mutations linked to CHDs. Full body knockouts of *Gata4*, *Gata6*, *Tbx5*, *Nkx2.5*, *Mef2a*, *Mef2c*, *Hand1* and *Hand2* are embryonic lethal at various points of gestation or die shortly after birth [143–152]. Mice haploinsufficient or overexpressing these factors present with cardiac defects that are often exacerbated by neurohumoral or mechanical stressors [129,147,149,151–158]. In each case, expression of each TFs downstream gene targets is altered leading to downstream phenotypic changes in cardiac cells and tissues. As well, several mutations of these core TFs have been

associated with human cases of CHDs that vary widely in presentation and severity [141,159–165]. This *in vivo* evidence demonstrates that the proper development of the heart hinges on the correct expression and function of these core cardiac TFs.

Cardiac development is, for the most part, completed during embryonic gestation. Once born, the heart must grow in size to parallel the overall growth of the organism but does not undergo gross structural or morphological changes [123]. As such, birth signals a rapid switch in the gene expression programs occurring in cardiac cells to reflect the change from a developmental to a post-natal phenotype [166]. These changes include a decrease in natriuretic peptide expression, isoform switches between structural and contractile proteins (for example, expression of αMHC increases and expression of βMHC decreases) and activation of fatty acid metabolic genes [131,132,166]. Upon normal physiological conditions, expression of the core TFs remains stable in the post-natal heart. However, upon exposure to pathological cardiac stressors, their expression increases to re-activate fetal genes and preserve cardiac function [166–168]. Upon detection of cardiac stressors, for example the binding of neurohumoral ligands to cell surface receptors or the sensing of mechanical stress from pressure or volume overload, upstream signaling cascades trigger the core cardiac TFs to induce expression of target genes [167–169]. These genes include natriuretic peptides, fetal isoforms of structural and contractile proteins and genes involved with glucose metabolism [166–169]. The core cardiac TFs also activate pathways that are unique to the post-natal heart including the induction of cardiomyocyte hypertrophy, the activation of pro-survival pathways and the secretion of pro-angiogenic chemotactic factors [128,129,134,168,170].

The re-expression of the core cardiac TFs and the fetal gene program is thought to be a compensatory response used by cardiomyocytes in an attempt to preserve heart function despite

exposure to stress [171]. The importance of these TFs in this context is best illustrated by animal models. Cardiomyocyte-specific *Gata4*-null mice exposed to transaortic banding to induce pressure overload presented with cardiac dilatation, increased cardiomyocyte death and worsened cardiac function compared to wild type mice who presented with a compensated hypertrophy response [172]. Conversely, cardiomyocyte-specific overexpression of GATA4 induced hypertrophy in the absence of any pro-hypertrophic stimuli [128]. Both the *Gata4* knockout and overexpressing mice showed dysregulated pro-hypertrophic target gene expression [128,172]. Similar studies have been completed on mice with increased or reduced GATA6, MEF2A, MEF2C and MEF2D expression with comparable results [128,153,156,157,173]. However, the preservation of cardiac function by the core developmental cardiac TFs and the fetal gene program is considered to be, at best, a temporary solution. It is associated with poor cardiac outcomes in both murine and human studies, suggesting that although beneficial short term, its expression is eventually associated with maladaptive cardiac changes over time [166,167,169].

The past several years of research have shed light on the cardiac gene regulatory programs that mediate cell-type, time-point and stimulus-induced cell fates. For example, we are now capable of re-programming fibroblasts and stem cells into cardiomyocyte-like cells via the ectopic overexpression of specific TFs [174,175]. In a post-natal context, we can induce a hypertrophic phenotype in both cultured cardiomyocytes and *in vivo* via the overexpression of specific cardiac genes, even in the absence of hypertrophy-inducing stimuli [128,156]. However, our understanding of the regulation of cardiac cell fates, although vastly improved, remains incomplete. Despite our ability to reprogram cells into beating cardiomyocytes, our understanding of the pathways that produce conduction system, atrial and ventricular cardiomyocytes remains unclear [64,176,177]. As well, we do not completely understand the

mechanisms distinguishing physiological hypertrophy associated with sustained or improved cardiac function versus pathological hypertrophy associated with poor cardiac function [178]. As such, there remains much to learn to fully understand the TF-governed pathways that regulate cardiac developmental and post-natal cell fates. Furthermore, we understand far less about cardiac non-myocyte cell fates compared to cardiomyocytes. Despite their large relative size and importance in the heart, cardiomyocytes make up only 25-35% of all cardiac cells [56]. Other cells that play vital roles in the heart include a variety of endothelial cells, smooth muscle cells and fibroblasts that have been described in the section on cardiac structure. The importance of other cardiac cell types is best illustrated by the multiple diseases that centre around them including valve and septal congenital heart defects, decreased capillary density and the formation of fibrotic lesions during heart failure and aortic dissection and rupture. An improved understanding of the TF-mediated regulatory mechanisms governing gene expression programs of cardiac non-myocytes will lead to improved understanding of how multiple cell types cooperatively ensure proper cardiac development and post-natal function and how they are altered leading to disease.

1.8 The GATA Family of Transcription Factors

GATA proteins are a family of ancient TFs that play central roles in the regulation of gene expression in development, differentiation and homeostasis [179]. This family has been highly conserved throughout evolution and members are found in plants, animals and fungi in varying numbers. For example, the plant *Arabidopsis thaliana* contains 29 separate homologues, fungi encode 35 and vertebrates encode 6 [179,180]. The vertebrate factors have been subdivided into two groups. GATA1, 2 and 3 are expressed in mesoderm and ectoderm-derived cell lineages and are primarily located in hematopoetic cell lineages [181]. GATA4, 5 and 6 are expressed in mesoderm and endoderm derived cell lineages and display their highest expression in cardiac tissues [181].

However, all 6 GATA factors play significant and non-redundant roles in other organs and structures that are outlined in table 1.2.

Vertebrate GATA factors all share a similar structure. Each contains an N-terminal transactivation domain required for gene activation or repression, a DNA binding domain and a C-terminal domain containing a nuclear localization sequence. The DNA binding domains consist of two zinc fingers of the structure Cys-X2-Cys-X17-Cys-X2-Cys that each coordinate a zinc ion. The C-terminal zinc finger is over 70% conserved between all 6 GATA homologues whereas the N and C-termini have diverged considerably [181,182]. GATA factors were all named for their DNA recognition motif, 5'-WGATAR-3' and bind it directly via their C-terminal zinc finger, with the N-terminal zinc finger aiding to stabilize the interaction [179]. Once bound, GATA factors directly activate or repress the transcription of target genes, thereby regulating their expression. By regulating groups of target genes in a specific cell type, time point or stimulus dependent manner, GATA factors directly control the expression of cell fates and as such, are vital to the proper development and functioning of the body.

GATA FACTOR	CELL/TISSUE EXPRESSION	KNOWN ROLES	KNOCKOUT VIABILITY
GATA1	- Erythrocytes - Mast cells - Megakaryocytes - Testes	- Hematopoietic differentiation	Embryonic lethal at E11.5-E12.5
GATA2	- Erythrocytes - Mast cells - Megakaryocytes - CNS - Vasculature	- Hematopoietic stem cell proliferation and differentiation - Neuronal development - Endothelial cell differentiation and homeostasis	Embryonic lethal at E12.5
GATA3	- T-cells - Erythrocytes - CNS - Kidney - Breast - Vasculature	- T-cell, neuronal and mammary epithelial cell differentiation - Hematopoietic stem cell proliferation - Endothelial cell homeostasis	Embryonic lethal at E11-E12
GATA4	- Heart - Testes - Ovaries - Lung - CNS - Stomach - Liver - Pancreas - Intestines	- Cardiomyocyte proliferation, differentiation and hypertrophy - Endocardial cell proliferation - Gonadogenesis - Pulmonary development - Astrocyte hemostasis - Gastric epithelial development - Hepatocyte differentiation - Pancreatic endoderm specification - Jejunal-ileal homeostasis	Embryonic lethal at E7-E9.5
GATA5	- Heart - Vasculature - Ovaries - Stomach - Liver - Pancreas - Intestine	- Endocardial, stomach gland epithelial and liver precursor cell development - Vascular endothelial homeostasis - Pancreatic specification	Viable and Fertile
GATA6	- Heart - Testes - Ovaries - CNS - Lung - Pancreas - Intestines - Stomach	- Cardiac morphogenesis - Outflow tract development - Cardiac hypertrophy - Gonadal morphogenesis - Astrocyte homeostasis - Lung epithelial differentiation - Pancreatic endoderm specification - Intestinal epithelial differentiation - Gastric differentiation	Embryonic lethal E5.5-E7

Table 1.2 Localization and roles of the 6 vertebrate GATA factors as well as the viability of their full body knockouts[143,144,181,183–206].

1.9 Cardiac Roles of the GATA Factors

Via the use of *in vitro* experiments, cell culture studies and *in vivo* knockout and overexpression models, several cardiac specific roles of the GATA factors have been identified. The hematopoietic GATA factors play lesser known (but still important) roles in cardiac formation and function whereas GATA4, 5 and 6 have been extensively studied and are known as central regulators of cardiac gene expression. Of the three cardiac GATA factors, the most well-studied is GATA4 which will be discussed at the end of this section. However, it is important to note that the proper expression and function of all cardiac-expressed GATA factors is central to heart development and post-natal function and that changes in their expression levels are associated with disease.

1.9.1 GATA1

GATA1 was the first GATA factor to be identified in 1989 and is located on the X chromosome in both mouse and human[207]. It is well expressed in several hematopoietic cell lineages where it plays central roles in development and differentiation. However, its expression in cardiac tissues is virtually undetectable[208]. In fact, its lack of expression is central to cardiac development. The TF NKX2.5, one of the earliest markers of cardiac cell lineages, directly represses GATA1 expression to supress erythroid cell fates during cardiac development. *In vivo*, mesodermal cells of *Nkx2.5* knockout (KO) embryos show a marked increase in the erythroid/hematopoietic gene expression profile compared to wild type embryos that is governed by an increase in GATA1 expression[208]. Furthermore, addition of exogenous GATA1 can partially rescue expression of the erythroid/hematopoietic gene expression program inhibited by NKX2.5 overexpression[208]. As such, although GATA1 does not play a central role in cardiac

development per se, the correct expression and function of cardiac vs hematopoietic TFs is central to proper development of mesoderm-derived tissues, including the heart.

1.9.2 GATA2

The second GATA factor to be discovered, aptly named *Gata2*, was discovered by cDNA cloning and cross-hybridization screening to identify genes related to *Gata1* [209]. Located on chromosome 3 in human and on chromosome 6 in mouse, its expression is more widespread in the body compared to GATA1. In cardiac tissues, its expression is extremely low but can be detected in the aortic endothelium and smooth muscle cells [210]. The main role of GATA2 in the endothelium is to activate and maintain proper endothelial gene regulatory programs and directly affects expression of endothelial nitric-oxide synthase (eNOS), von Willebrand Factor (VWF), Intercellular Adhesion Molecule 2 (ICAM-2) and Vascular Endothelial Cell Adhesion Molecule (VCAM) 3-1 [211–218]. *In vivo*, lack of GATA2 expression results in edema and haemorrhage due to incomplete abscission of lymphatic and venous endothelium[219]. GATA2 also plays a role in vascular remodeling in CAD [210,220]. Its expression, stability and promoter binding are increased in the arteries of newly grafted organs susceptible to plaque formation and several *GATA2* mutations have been correlated with familially-linked early onset CAD in humans [210,220]. As such, this TF does appear to play a central role in the vascular endothelium but as GATA factor expression in vascular endothelial cells is variable, the expression of GATA2 in other cardiac endothelial cells remains unknown [218].

1.9.3 GATA3

The third hematopoetic GATA factor is named *Gata3* and is found on chromosome 2 in mouse and chromosome 10 in human. Like GATA2, it is expressed in a relatively wide array of

tissues of ectodermal and mesodermal origin including cells of the hematopoietic, T-cell and nervous system [187,191,192,218]. GATA3 is the only hematopoietic GATA family member with detectable expression in the heart during development. It is expressed in the endocardium under the control of an endocardial-specific enhancer region [221]. Cardiac GATA3 expression can be detected as early as E9.5 and by E11.5, it is strongly expressed in the endocardial ridges and endothelium of the outflow tract [222]. It is also expressed, albeit to a lower extent, in the atrioventricular canal [222]. This expression pattern persists until E15.5-E17.5, when it can also be detected in the right atrium (RA) but then becomes undetectable in the adult heart [222]. The expression of GATA3 in the developing heart is critical to proper endocardial and outflow tract development as evidenced by mouse models with reduced GATA3 expression. These mice present with ventricular septal defects (VSDs), double-outlet right ventricle (DORV), anomalies of the aortic arch (AAA) and persistent truncus arteriosus (PTA) [222].

Like GATA2, GATA3 also plays important roles in the development and function of the vasculature by activating key downstream targets including VWF and the tyrosine kinase receptor TIE2 [183,214,223]. In vascular endothelial cells, GATA3-mediated activation of TIE2 leads to increased cell migration, survival and tube formation [183]. This pathway is central to the induction of angiogenesis and anti-inflammatory pathways as demonstrated by the impaired angiogenesis and increased monocyte adhesion induced by GATA3 knockdown in Human Umbilical Vascular Endothelial Cells (HUVECs) [183]. As noted above, the expression of specific GATA factors is variable in different regions of the vascular endothelium but, given its expression in other cardiac endothelial tissues, it is possible that GATA3 may play a role in the development and function of the cardiac vasculature as well.

1.9.4 GATA5

Gata5 is located on chromosome 2 in mice and chromosome 20 in humans and is the only GATA family member whose full body knockout is viable and fertile [224]. In the heart, its major site of expression is the endocardium. It is first detected in the endothelial cells of the linear heart tube and remains well expressed until mid-gestation [225]. In the myocardium, GATA5 is expressed at much lower levels and can only be detected in a small number of cardiomyocytes adjacent to the endocardium and endocardial cushions [225,226]. This weak myocardial expression is undetectable by E14.5 and its function remains unknown [225].

Because the whole body *Gata5* knockout is viable, studies on the complete loss of its expression have been helpful in determining its role in cardiac development. These knockouts present with hypoplastic hearts and have an increased incidence of bicuspid aortic valve (BAV), a condition upon which only two leaflets of the aortic valve are present rather than the usual three [199]. Adults present with aortic stenosis that worsens over time as well as increased incidence of left ventricular hypertrophy, likely due to the mechanical stress placed on this chamber by the valve deformity [199]. The loss of *Gata5* causes these phenotypes by impairing differentiation of endocardial cells of the endocardial cushions [199]. This effect has also been observed in cell culture, where the loss of GATA5 impairs differentiation of endothelial-endocardial cells via inhibition of its interaction with its TF interacting partner Nuclear Factor of Activated T Cells (NFATc) [227]. *In vivo*, loss of GATA5 expression was associated with reduced Notch pathway signaling and endocardial gene expression [199]. Taken together, these data suggest that the requirement for GATA5 is not specific to one single endocardial gene expression pathway but rather that it is a central regulator of multiple gene regulatory networks in this tissue. Furthermore, as mice with specific knockout of *Gata5* in the endocardium recapitulate the

cardiac phenotypes observed in the whole body knockout mice, these effects appear to be governed specifically by endocardially-expressed GATA5 [199].

The central role of GATA5 in the endocardium and, in particular, the outflow tract, requires its interaction with the other two cardiac GATA factors, GATA4 and GATA6 [224]. Compound *Gata5-Gata4* heterozygote mice exhibit reduced viability compared to single heterozygotes due to endocardial defects including DORV, complete atrioventricular canal defects, tricuspid and mitral valve hypertrophy and atrial and ventricular septal defects (ASDs and VSDs) [224]. All can be attributed to abnormal development of the endocardial cushions and demonstrate that GATA4 and GATA5 interact *in vivo* to regulate proper endocardial cell development [224]. Similarly, compound *Gata5-Gata6* heterozygote mice exhibit DORV and VSDs that is likely due to defective retinoic acid signaling, further emphasizing how GATA5 expression is central to multiple endocardial gene regulatory pathways [224]. The effects of reduced expression of GATA5 can also be observed in humans. Several *GATA5* mutations have been identified in individuals with endocardially-based CHDs including BAV, DORV, pulmonary stenosis, ASDs and VSDs [224,228–231]. Interestingly, a number of *GATA5* mutations have also been linked to dilated cardiomyopathy, suggesting that although its expression may be very low and transient, GATA5 may play an important role in the development and function of the myocardium [232]. However, whether these effects are mediated by GATA5 expression in the myocardium or through paracrine effects from the endocardium remains unknown.

Like GATA2 and GATA3, GATA5 plays an important role in the vascular endothelium where it plays important roles in the activation of the nitric oxide and PKA pathways [184]. *Gata5* knockout mice exhibit endothelial dysfunction and hypertension, and as with the cardiac phenotypes observed with these mice, are recapitulated completely by the endothelial-specific

Gata5 knockout animals [184]. Furthermore, *GATA5* was also identified as a susceptibility gene for human hypertension as two *GATA5* variants have been associated with the administration of anti-hypertensive medication by the Action in Diabetes and Vascular Disease: Peterax and Diamicron MR Controlled Evaluation (ADVANCE) and the Ottawa Heart Genomics Study (OHGS) clinical trials [184]. These data, along with those showing the role of GATA5 in the endocardium, demonstrate that GATA5 plays a central role in the endothelium of the entire cardiovascular system.

1.9.5 GATA6

Gata6 is located on chromosome 18 in both mouse and human and its primary sites of expression in the heart are the myocardium and outflow tract [225]. Myocardial expression of GATA6 begins during the formation of the cardiac crescent and overlaps with its family member GATA4. In the linear heart tube, this expression is strongest towards the peripheral end that will eventually form the atria and sinus venosus [225]. The expression of GATA6 in cardiomyocytes continues throughout development and is detectable in the myocardium of both atria and both ventricles; however, not all myocytes in these tissues are GATA6 positive, suggesting that at least two populations of cardiomyocytes are required for proper myocardial formation and function [225,233]. The outflow tract is the other major site of GATA6 expression. Its expression begins at E9.5 and persists throughout development of this structure [233]. There, GATA6 expression can be detected in multiple cell types including neural crest cells, vascular smooth muscle cells, endothelial cells and secondary heart field cells, suggesting that it plays a multifactorial role in outflow development [233–235]. Further supporting this hypothesis, multiple human mutations of *GATA6* have been directly implicated in a wide variety of outflow tract

development defects including Persistent Truncus Arteriosus (PTA), Tetralogy of Fallot (ToF) and BAV [235–237].

Mouse models of *Gata6* haploinsufficiency and complete loss-of-function have shed light onto its crucial role in cardiac development. The whole body *Gata6* knockout mouse is embryonic lethal at E6.5 due to extraembryonic defects and problems with visceral endoderm function [143,234]. These mice do not possess any cardiac tissue at all, demonstrating that early expression of GATA6 is central to the first stages of cardiac development [143,234]. These findings support research in cell culture that demonstrate that a lack of GATA6 expression results in impaired embryonic stem cell differentiation into beating embryoid bodies [238]. Originally, it was though that *Gata6* heterozygote mice do not present with any observable cardiac defects but recent work has shown that they are prone to RL-type BAV, the most common BAV variant observed in humans [235]. This phenotype is attributed to the requirement of GATA6 in the secondary heart field for valve remodelling and correct ECM composition during OFT development [235]. As well, when deleted from vascular smooth muscle or neural crest cells, outflow tract defects including PTA, aortic arch patterning defects, DORV and VSDs are observed [233]. In the myocardium, an NKX2.5-Cre-mediated *Gata6* knockout that inhibits GATA6 expression post-E14.5 results in perinatal lethality from VSDs, irregular septal thickness and loss of trabeculae [153].

GATA6 directly interacts with its other cardiac GATA family members to exert its multiple roles in myocardial and outflow tract development. Given that both GATA4 and GATA6 are both widely expressed in the myocardium, it is unsurprising that *Gata4-Gata6* compound heterozygotes are embryonic lethal at E13.5. These mice present with a wide array of defects including hypoplastic myocardium, VSDs, outflow tract defects and abnormal smooth

muscle cell development [234]. These malformations also coincide with decreased MEF2c and β-myosin heavy chain (βMHC) expression, two proteins that are fundamental to proper cardiac structure and function [234]. Others have attributed GATA6 function in myocardial development to its interaction with another core cardiac TF, TBX5 [239]. As well, as discussed previously in the section on GATA5, compound *Gata5-Gata6* heterozygote mice exhibit DORVs and VSDs partially attributed to defective retinoic acid signaling [224]. As such, it is apparent that in cardiac development, GATA6 function requires interactions with several TFs to activate specific downstream cardiac gene programs.

GATA6 continues to play an important role in the heart throughout adulthood, particularly in the activation of cardiomyocyte hypertrophy [153]. This pro-hypertrophic phenotype was first observed by Liang *et al* (2001) who noted that the overexpression of GATA6 in primary cardiomyocytes is sufficient to induce hypertrophy in the absence of other pro-hypertrophic stimuli [128]. To study the effect of GATA6 *in vivo*, conditional knockout lines were developed that delete its expression at later embryonic stages, allowing for proper cardiac development and the generation of viable and fertile mice [153]. βMHC-Cre promoter-driven *Gata6* deletion results in mice whose cardiac development proceeds relatively normally aside from a slight decrease in overall size along with an increase in average cardiomyocyte length and total area [153]. These mice also have impaired cardiomyocyte proliferation [153]. However, when subjected to pressure overload by trans-aortic constriction (TAC), they show an impaired hypertrophic response characterized by decreased cardiomyocyte size, decreased fractional shortening and impaired induction of the fetal gene program [153]. Furthermore, these mice are more susceptible to heart failure as over time, they exhibit poor cardiac contractility, peripheral edema and cardiac dilation [153]. Cumulatively, these results demonstrate that GATA6 is required

for the induction of cardiac hypertrophy and when impaired, leads to impaired compensatory hypertrophic responses and poor cardiac outcomes.

1.9.6 GATA4

The final member of the vertebrate GATA family, *Gata4*, is located on chromosome 14 in mouse and chromosome 8 in human. This TF is unique among its family members as it is the only one to be ubiquitously expressed in all cardiac tissues (myocardium, endocardium and epicardium) in both the developing and adult heart [144,154,172,239–243]. GATA4 expression during development begins at E7-7.5 in the precardiac mesoderm and is detectable in the endocardium, myocardium and other embryonic structures by E8 [145,244]. As the heart continues to develop, GATA4 expression remains widespread and is found in the myocardium including all cardiomyocytes, the epicardium and endocardium including all valves, septa and the outflow tract [144,154,172,224,239–243]. This high level of expression persists until approximately one week after birth but can be re-induced upon exposure to cardiac stressors such as exercise, pressure overload or myocardial infarction [134,154,172,245].

As with the other GATA factors, much of our information regarding GATA4 function in the heart has been obtained through animal models. Full body *Gata4*-null mice are embryonic lethal at E8.5 due to defects in ventral foregut closure and cardia bifida, demonstrating its importance at the very earliest stages of cardiac development [144,145,181]. The corresponding heterozygote mice were initially thought to develop normally but are now known to be at increased risk of developing hypoplastic ventricular myocardium, common atrioventricular valve, DORV and septal defects [144,145,224,246]. Due to the embryonic lethality of the *Gata4*-null mouse, the study of GATA4 function at later stages of cardiac development, in the adult heart or in specific cardiac tissues has been done using conditional knockout and overexpression studies.

The majority of this work has made use of the Cre-loxP recombination technique but it is worth noting that the addition of loxP sites to the *Gata4* allele already results in a 20% reduction in GATA protein level even before Cre excision. This demonstrates that even subtle changes to the *Gata4* sequence can have effects on the heart [246]. *Gata4* excision driven by Nkx2.5-Cre removes myocardial GATA4 expression by E9.5 and is embryonic lethal by E11.5 due to myocardial thinning, hypoplastic right ventricle and a lack of mesenchyme cells in the endocardial cushions [247]. βMHC-driven *Gata4* deletion occurs by E17.5 and results in viable and fertile offspring that later develop progressively decreasing left ventricular function and ventricular dilation [172]. Similarly, αMHC-driven *Gata4* knockout lowers GATA4 expression by 70% by 8 weeks of age and gives rise to the same phenotype observed with the βMHC-mediated knockout that presents at a later age [172]. αMHC-mediated GATA4 overexpression, on the other hand leads to increasing heart/body weight ratio, activation of fetal gene program and physiological changes associated with hypertrophy such as fibrosis [128]. Far fewer *in vivo* studies have been done on endocardially-expressed GATA4 but Rivera-Feliciano *et al* (2006) have shown that a Tie2-Cre driven *Gata4* deletion results in embryonic lethality by E12.5 due to impaired atrioventricular epithelial to mesenchymal transition leading to hypocellular cushions [240]. As well, *Gata4* haploinsufficient mice have also been crossed with mice with limited expression of other key transcription factors. Aside from the *Gata4*/*Gata5* and *Gata4*/*Gata6* compound heterozygote mice that have been discussed in the previous two sections, *Gata4* heterozygote mice have also been crossed with *Tbx5* heterozygotes resulting in embryonic lethality from atrial and atrioventricular septal defects as well as myocardial thinning [239,242].

Animal models have also lent insight into the role of GATA4 in cardiac disease. In mice exposed to cardiac stressors including myocardial infarction or pressure overload, GATA4

becomes upregulated in the myocardium to mediate pro-hypertrophic and pro-survival mechanisms. As such, when GATA4 expression is inhibited, cardiac outcomes worsen [134,154,172,248]. *Gata4*-null mice show increased susceptibility to heart failure caused by chemotherapy, specifically caused by Doxorubicin or Imatinib [170,249,250]. As well, a myocardial-specific *Gata4* knockout subjected to pressure overload exhibited impaired cardiomyocyte hypertrophy, increased cardiomyocyte death and increased hypoxia due to loss of capillary density [134,172]. On the other hand, increased GATA4 expression is cardioprotective. In the week following birth, GATA4 expression levels normally decrease as the heart switches from a developmental to a post-natal phenotype. When GATA4 levels are preserved, neonate mice subjected to cryoinjury show improved regenerative capacity, decreased scar formation and improved outcomes [245]. Likewise, GATA4 overexpression in the myocardium improves ejection fraction, increases capillary density and decreases both infarct size and cardiomyocyte death caused by myocardial infarction [248]. The role of GATA4 in cardiac disease has also been studied without direct overexpression or knockout of its transcript. A 35% reduction in GATA4 expression is observed in the db/db mouse model of type two diabetes that is susceptible to ischemic injury, fibrosis, collagen accumulation, apoptosis, and left ventricular dysfunction that causes decreased transactivation of its gene targets *Nppa*, *Nppb* and *Myh6* [251]. Collectively, these findings all demonstrate that the maintenance of GATA4 expression is fundamental to cardiac health and that its inhibition is central to the pathogenesis of several cardiac diseases.

Like animal models, human mutations of *GATA4* have also offered insight into its role in the heart and how its dysregulation leads to cardiac defects. Currently, well over 100 mutations have been published that span the entire length of the *GATA4* sequence (Figure 1.4). Rather than simply being associated with one or two cardiac phenotypes, the cardiac diseases associated with

GATA4 mutations are heterogeneous and include defects of the myocardium, the endocardium and the conduction system. These phenotypes range from cardiomyopathies, valve and septal defects, atrial fibrillation, outflow tract defects and conditions that encompass multiple defects such as ToF. However, it is of note that the majority of *GATA4* mutations are associated with endocardial defects. The mechanisms linking the mutated genotype to the defective phenotype remain poorly understood in all but a few cases. Mice homozygous for the V217G mutation are embryonic lethal at E12.5 due to inhibition of the interaction between GATA4 and Friend of Gata 2 (FOG2) [252]. These mice lack coronary vessels, have a thinned myocardium and exhibit a number of outflow tract and valve defects [252]. Mice homozygous for G296S (G295S in mouse) are embryonic lethal at E11.5 and heterozygotes recapitulate the septal defects observed in humans with the same mutation [239]. This phenotype could be attributed to impaired GATA4 transcriptional activation of the *Ccnd2* gene leading to reduced cardiomyocyte proliferation [239]. Lastly, GATA4 transgenic mice with the ASD-causing M310V mutation show impaired GATA4-mediated activation of its target αMHC [253]. However, for the majority of other *GATA4* mutations, we have only a limited understanding of the biochemical pathways linking the mutated protein to the cardiac defect. As such, an improved understanding of the mechanisms underlying GATA4 function is required to better understand its role in cardiac disease.

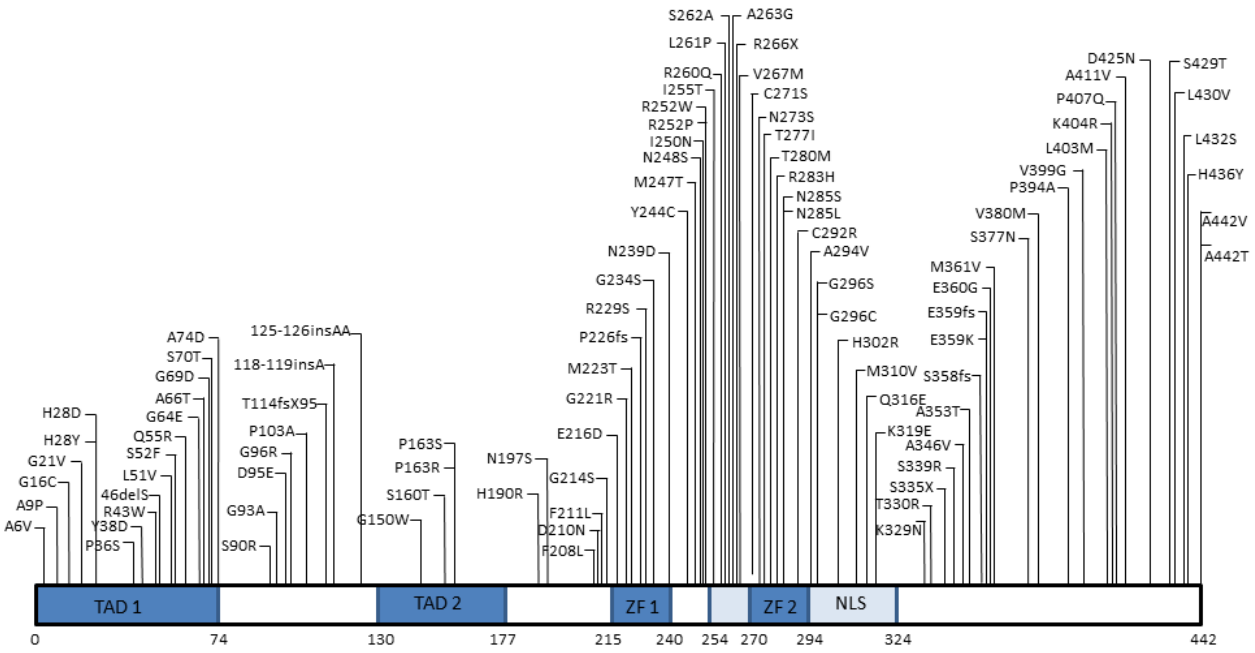

Figure 1.4 Schematic of the GATA4 protein with labelled CHD-associated human mutations.

1.10 The Cell-Specific Roles of GATA4 in the Heart

The diversity of phenotypes observed in GATA4 mouse models and in humans with *GATA4* mutations is indicative of its involvement in a wide array of processes central to cardiac development and function. Since these phenotypes affect all cardiac tissues and developmental time points, it is apparent that GATA4 plays distinct roles unique to each. To better understand these GATA4-regulated mechanisms, its involvement in each cellular and temporal context has been studied. Much of this work has focused on cardiomyocytes. During development, GATA4 is directly involved in cardiomyocyte proliferation and differentiation, two processes that must occur in a coordinated manner to generate the functioning myocardium [254]. The direct involvement of GATA4 in proliferation was first identified in mouse models of *Gata4* knockout, haploinsufficiency or mutation that result in a thinned, hypoplastic myocardium [144,145,224,246,247,252]. Since then, the mechanisms underlying this effect have been identified and

generally centre around its activation of cell-cycle genes, particularly members of the Cyclin and Cyclin-Dependent Kinase family [254–258]. In mice heterozygous for the *Gata4*-null allele in the second heart field (the region that gives rise to the right ventricle and intraventricular septum), embryonic lethality occurs at E13.5 due to impaired GATA4 activation of Cyclin D2, Cyclin A2 and CDK4 causing impaired myocardial proliferation [256]. Later, it was shown that GATA4 activates both Cyclin D2 and CDK4 directly throughout the entire myocardium and that transgenic overexpression of Cyclin D2 in the myocardium is sufficient to rescue the hypoplastic phenotype observed in *Gata4* heterozygote mice [254,258]. The role of GATA4 in cardiomyocyte differentiation was first observed in cell culture models of cardiogenesis. Knockdown of GATA4 in P19 cells interrupts differentiation at the cardioblast stage whereas overexpression of GATA4 in the same model leads to a 10-fold increase in beating cardiomyocytes [259]. *In vivo*, GATA4 was shown to direct expression of cardiogenic factors like Bone Morphogenic Protein 4 (BMP4) in cardiac precursors and cells of the adjacent endoderm [260]. Soon after, the ectopic overexpression of GATA4 alone was shown to be sufficient to induce differentiation of *Xenopus* ectoderm explants into beating cardiac tissue [261]. Since then, multiple groups have differentiated several cell types into beating cardiomyocytes via the expression of a few cardiac factors that always include GATA4 [174,175,262,263]. These cell types include differentiated mouse mesodermal cells, adult cardiac and dermal fibroblasts, adipose tissue-derived mesenchymal stem cells and embryonic stem cells [174,175,262,263]. Collectively, this research supports the hypothesis that expression of GATA4 is central to both cardiomyocyte proliferation and differentiation and explains why impaired expression of this TF frequently results in myocardial defects.

GATA4 also plays important roles in post-natal cardiomyocytes, particularly in the induction of hypertrophy, survival and angiogenesis. The pro-hypertrophic role of GATA4 was

first suggested by the identification of a conserved GATA motif on a region of the *Myh7* promoter required to induce both pressure overload and α1-adrenergic agonist-induced hypertrophy [264]. In cultured cardiomyocytes, overexpression of GATA4 is sufficient to induce sarcomeric reorganization, increase surface area and increase total protein accumulation [128]. On the other hand, expression of a dominant-negative GATA4 transcript in the same model inhibits hypertrophy in response to GATA4 expression or stimulation by phenylephrine [128]. These results are recapitulated in vivo as a cardiomyocyte-specific 2.5-fold overexpression of GATA4 induces a hypertrophic response whereas cardiomyocyte-specific knockout of *Gata4* impairs hypertrophy and worsens cardiac function in response to trans-aortic construction (TAC) [128,172]. The induction of GATA4-mediated cardiomyocyte hypertrophy can be induced via several upstream pathways including those induced by pressure overload as well as treatment by neurohumoral factors including phenylephrine, isoproterenol, endothelin-1 and Angiotensin II [128,265–267]. Once induced, GATA4 regulates the expression of several gene families including structural (*Myh7*, *Acta1*, *Ctnt*), calcium handling (*Serca2*, *Ncx1*) and natriuretic (*Nppa*, *Nppb*) genes that allow cells to increase in surface area and improve contractile function [128,264,268–272].

GATA4 also plays a key role in the induction of pro-survival pathways, an important role considering that cardiomyocytes are replication deficient and as such, all cardiomyocytes lost from the myocardium cannot be replaced. The role of GATA4 in survival was first observed in cardiomyocytes treated with the cardiotoxic chemotherapy drug Doxorubicin (Dox). Treatment with Dox leads directly to decreased GATA4 expression and increased cardiomyocyte death both in cultured cardiomyocytes and *in vivo* [170]. As well, *Gata4* heterozygous mice are more prone to cardiomyocyte death and worsened cardiac function in response to Dox treatment compared to wild type animals [170]. Conversely, GATA4 overexpression in culture and *in vivo* is directly

correlated to decreased cardiomyocyte death and improved cardiac function post-myocardial infarction or Dox treatment [167,247]. The pro-survival effects of GATA4 are attributed to its ability to directly activate members of the BCL family. Upon direct GATA4 binding and transactivation of the *bclx* and *bcl2* promoters, the resulting proteins repress stress-induced apoptotic pathways protecting cardiomyocytes and maintaining cardiac function[170,273]. The pro-angiogenic effect of GATA4 in cardiomyocytes can also be considered cardioprotective as the formation and maintenance of capillaries throughout the myocardium is critical to the prevention of hypoxia-induced cell death. In co-cultures of human umbilical vascular endothelial cells (HUVECs) with cardiomyocytes overexpressing or inhibiting GATA4, tube formation was either increased or decreased, respectively[134]. *In vivo*, overexpression of GATA4 in the myocardium results in an increase in capillary density and cardiac function at baseline and in response to myocardial infarction[131,247]. On the other hand, knockout of GATA4 in the myocardium represses pressure overload or cryoinjury-induced increases in LV capillary density and impairs cardiac contractility[134,245]. Currently, our understanding of the mechanisms underlying GATA4-mediated angiogenesis in the myocardium involve its direct binding and activation of the Vascular Endothelial Growth Factor A (*Vegfa*) promoter, a potent pro-angiogenic growth factor[134]. Indeed, inhibition of VEGF-A by a receptor agonist blocks GATA4-induced tube formation in cardiomyocyte/HUVEC co-cultures[134]. Conversely, overexpression of VEGF-A via adenoviral vector in the myocardium can rescue capillary density in myocardial *Gata4*-null mice[134].

In contrast to the roles of GATA4 in cardiomyocytes, its roles in cardiac endothelial cells are far less understood. However, as the majority of human mutations lead to congenital heart defects in the endocardium (valve, septal and outflow tract defects primarily), it is apparent that GATA4 plays several roles in endocardial cells as well. GATA4 is well expressed in both

endocardial progenitors and throughout the developing and post-natal endocardium [227,260]. Deletion of *Gata4* in the endocardium via the Tie2-Cre transgene impairs GATA4 activation of the Erbb3-Erk pathway required for epithelial to mesenchymal transition (EMT) in the endocardial cushions [240]. As such, these animals exhibit defects in their atrioventricular canals [240]. Simultaneous expression of a *Gata4* mutant that cannot interact with FOG cofactors (GATA4 V217G) rescues the EMT phenotype but mesenchymal cells cannot proliferate properly and as such, these mice have hypocellular cushions that cannot remodel into the atrioventricular septum properly [240,252]. A role for GATA4 in endocardial cell survival has also been observed as the cotransfection of TBX5 and GATA4 synergistically activates the *bclx* promoter in TC13 endocardial precursor cells [242]. However, aside from this, little is known about the role of GATA4 in the endocardium and its role in the vascular endothelium of the heart remains completely unknown.

Over the past 3 decades, there have been major advances in our understanding of the GATA4-mediated mechanisms that underlie cardiac cell fates. This information has allowed us to better understand cardiac development, function and disease. However, there remain many unanswered questions on the role of this TF in the heart that would allow us to improve diagnostics and treatment of both congenital and acquired cardiac diseases. In developing cardiomyocytes, we have deduced several pro-proliferative and pro-differentiative GATA4-regulated pathways but remain unable to activate them to repair myocardial damage post ischemic injury. In post-natal cardiomyocytes, we understand that GATA4 regulation of cell fates is central to the induction of hypertrophy, pro-survival pathways and angiogenesis required to maintain cardiac function in the face of stress. However, we are unable to maintain these GATA4-mediated compensatory responses long term and as such, cannot prevent progression to

heart failure, a disease that affects millions of people for which we have no cure. Continuing study of the role of GATA4 in these cells will no doubt answer many of these questions and improve patient outcomes in the future.

If there are still unanswered questions on the role of GATA4 in cardiomyocytes, it is unsurprising that the knowledge gap in our understanding of its roles in other cardiac cells is even more stark. The cardiac endothelium is central to proper cardiac development and function and the absence of proper GATA4 expression results in a multitude of endocardial defects observed in both mice and humans. As described in the previous section on cardiac structure, there are several different varieties of endothelial cells in the heart that are all required for cardiac development and post-natal function. Aside from a modest number of studies linking GATA4 to their endocardial cell fates during development, nothing is known about GATA4 in other cardiac endothelial cell types, particularly the cells that line the vasculature of the heart. For example, the endothelium that lines the myocardial microvasculature that is required to prevent myocardial ischemia during hypertrophy and is atrophied during heart failure via unknown mechanisms [134,248,274]. As well, the endothelium lining coronary arteries and veins provide for the high metabolic demands of the heart and are blocked during myocardial infarction [275,276]. Lastly, the endothelium of coronary lymphatic vessels is required for immune surveillance and tissue fluid homeostasis required to avoid infection and cardiac edema [277]. As such, an improved understanding of the roles of GATA4 in cardiac endothelial cells will improve our understanding of endocardial development and congenital heart diseases but will also shed light on the molecular mechanisms governing several acquired cardiac diseases that are traditionally thought of through a myocardial lens.

1.11 The Regulation of GATA4 Expression and Function

To better understand how GATA4 controls cell fates, an in depth understanding of its regulation in specific cellular environments is needed. Over the past several years, this topic has been the focus of many publications that have centred around three primary mechanisms: covalent addition of post-translational modifications (PTMs), inhibition by micro-RNA and interactions with protein partners.

1.11.1 PTMs:

PTMs involve the covalent enzymatic modification of a protein via the addition of chemical moieties to specific amino acids [278]. These chemical moieties vary widely in structure but the most commonly reported are phosphorylation, acetylation, glycosylation, amidation, methylation and ubiquitination [279]. Although diverse in structure, PTMs all share a common role: to regulate protein activity/function [278]. Several GATA4 PTMs have been identified that modulate its activity to give rise to specific cell fates, the majority of which are phosphorylation sites (Figure 1.5). GATA4 phosphorylation is triggered by several signal transduction pathways including the Mitogen Activated Protein Kinase (MAPK), Cyclic Guanosine Monophosphate (cGMP) and Protein Kinase C pathways [268,280–285]. Upon stimulation by erythropoietin (EPO), phenylephrine (PE) or pressure overload, GATA4 is phosphorylated at S105 by the MEK1-ERK1/2 pathway, a branch of the MAPK signal transduction pathway, to increase GATA4 stability, DNA binding and activation of pro-hypertrophic and pro-survival promoters [268,280–283]. GATA4 S105A knock-in mice exhibit impaired cardiomyocyte hypertrophy, increased cell death and a more rapid progression to heart failure in response to pressure overload, PE stimulation or crossing with a MEK1 overexpressing mouse line [268]. However, this serine is completely dispensable for cardiogenesis [281]. GATA4 is also phosphorylated at S261 upon hypertrophic

stimulation by 90 kDa ribosomal S6 kinase (RSK), a terminal effector kinase of the MAPK pathway, as well as Protein Kinase G (PKG), the terminal kinase of the cGMP pathway [282,284]. Phosphorylation at S261 increases GATA4 binding onto gene targets and increases its interactions with its cofactors NKX2.5 and p300 [282,284]. This site may also play a role in cardiac development as human congenital heart disease-linked mutations interfere with phosphorylation at this site [284]. Other kinases known to phosphorylate GATA4 are Protein Kinase C (PKC), an Angiotensin II-induced kinase that increases GATA4 binding of gene targets and Glycogen Synthase Kinase 3β (GSK3β), a negative modulator of GATA4 that decreases GATA4 nuclear localization and transactivation of target genes in the context of β-adrenergic signaling [285,286]. However, the exact amino acid target residues of both PKC and GSK3β remain unknown [285,286]. As with phosphorylation at S105 and S261, these sites are both important for GATA4 regulation during hypertrophy [285,286].

Aside from phosphorylation, GATA4 is also modified by acetylation, methylation and SUMOylation. Acetylation of GATA4 is completed by the histone acetyltransferase p300 and is important for both cardiogenesis and post-natal cardiac hypertrophy [283,287–289]. Acetylation by p300 promotes the differentiation of embryonic stem cells into cardiomyocytes and increases GATA4 transactivation of developmental and post-natal gene targets including *Nppa*, *Myh6*, *Myh7* and *Edn1* [283,287–289]. In hypertrophy, p300 acetylates lysines at amino acids 311, 318, 320 and 322 and increases transactivation of the *Nppa* and *Edn1* promoters [289]. Conversely, when these sites are mutated to non-acetylatable residues, PE-induced hypertrophy is impaired in primary cardiomyocytes [289]. These acetylations may be dependent on other PTMs as phosphorylation at S105 by ERK1/2 increases p300 binding and acetylation of GATA4 during EPO-induced hypertrophy [283]. It is also worth noting that although these specific acetylation sites

are required for cardiomyocyte hypertrophy, it is quite likely that p300 acetylates different sites on the GATA4 protein in the context of cardiogenesis. Other PTMs of GATA4 that modulate its function include methylation by Polycomb Repressive Complex 2 (PRC2) at lysine 299 (K299) that impairs the GATA4-p300 interaction and is required for cardiac morphogenesis [290]. As well, SUMOylation by Signal Transducer and Activator of Transcription 1 increases binding of target promoters in the small intestine [291]. Cumulatively, these GATA4 PTMs serve to modulate its subcellular localization, its ability to interact with specific protein partners and target specific downstream genes and as such, they serve to diversify the activity/function of GATA4 and allowing it to control cell, time point and stimulus-specific cell fates.

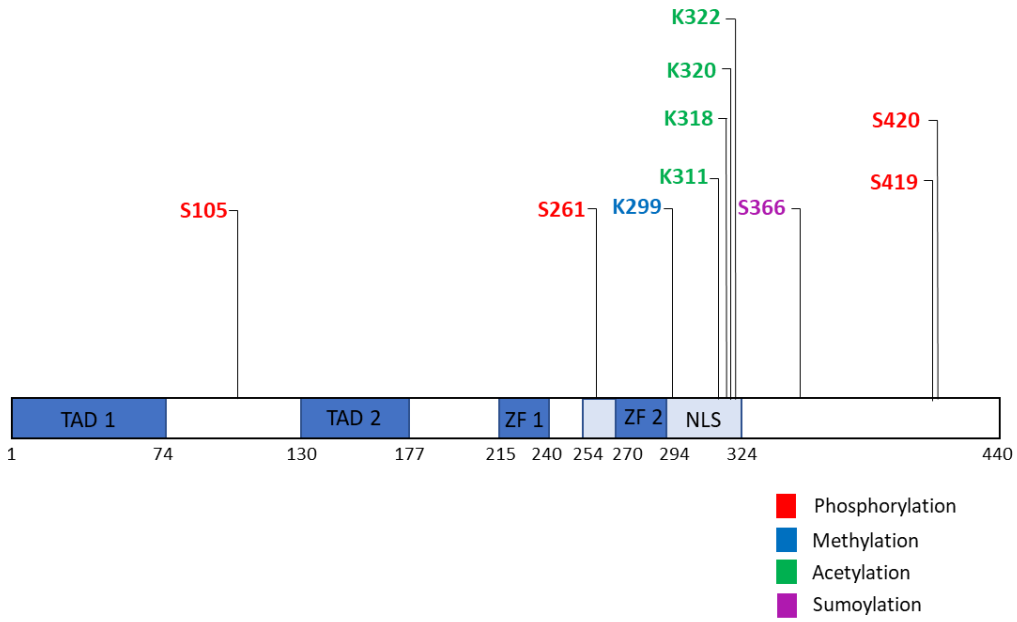

Figure 1.5 Known GATA4 Post-Translational Modifications

1.11.2 RNA Interference

Micro RNAs (miRNAs) are a family of small non-coding RNAs of approximately 20 base pairs that regulate gene expression by binding to complimentary sequences on the 3' untranslated regions (3' UTRs) of target genes. This binding triggers degradation of the mRNA, thus inhibiting translation and protein expression [292–294]. MiRNAs are important regulators of many cellular functions and now have known roles in both the development and post-natal function of the heart [294]. *Gata4* mRNA is targeted by several miRNAs that modulate its expression, primarily during hypertrophy (Table 1.3) [292,293,295,296]. miR-26a and b both target *Gata4* directly to repress cardiomyocyte hypertrophy [293,296]. Upon stimulation by TAC or AngII, miR-26a expression is repressed, allowing GATA4 expression to increase [296]. These changes are accompanied by increased GATA4 target gene expression and cardiomyocyte hypertrophy both in primary cultures and *in vivo* [296]. Similar results were observed when studying miR-1 that also targets *Gata4* mRNA to suppress pro-hypertrophic gene expression both in culture and *in vivo* [297]. As well, miR-208a targets *Gata4* to inhibit both hypertrophic and pro-survival gene expression. Located in an intron of *Myh6,* its expression is associated with the regulation of hypertrophic and survival mechanisms in the heart and has a putative recognition sequence on the 3' untranslated region (UTR) of *Gata4* [292,295]. *In vitro*, miR-208a represses luciferase expression of a Gata4-luc construct and *in vivo,* is sufficient to induce cardiac hypertrophy and arrhythmias. MiR-208a knockout triggers an increase in GATA4 protein expression and target gene expression [292]. This miRNA also appears to play a role in Doxorubicin cardiotoxicity as it is upregulated during Dox treatment while simultaneously decreasing GATA4 expression, increasing cardiomyocyte death and decreasing cardiac function [295]. Pre-treatment with a miR-208a antagomir rescues GATA4 and BCL2 expression while improving cardiac function [295].

Interestingly, human data also supports experimental evidence linking miRNA regulation of GATA4 to cardiac development and function. Several CHD-associated *GATA4* variants located in the 3'UTR are found in predicted miRNA binding sites, suggesting that these mutations could interfere with *Gata4* post-transcriptional regulation during development [298]. Although these miRNA binding sites have not been confirmed, it is highly likely that as yet unknown miRNA-mediated regulation of GATA4 is required for its spatial, temporal and stimulus-specific expression and function.

Name	Localization	Role
miR-1	Myocardium	Inhibits Cardiomyocyte Hypertrophy
miR-26a	Myocardium	Inhibits Cardiomyocyte Hypertrophy
miR-26b	Myocardium	Inhibits Cardiomyocyte Hypertrophy
miR-208a	Myocardium	Inhibits Cardiomyocyte Hypertrophy and Survival Pathways

Table 1.3 Published miRNAs targeting *Gata4* in the heart.

1.11.3 Protein-Protein Interactions

Protein-protein interactions (PPIs) are physical complexes formed between two or more proteins that regulate their activity and function [299]. They are central to all biological processes as proteins rarely (if ever) exert cellular functions by themselves [299]. Instead, proteins interact with one another to increase, diversify and lend specificity to their cellular functions. For example, a single protein may interact with a protein partner present at only one developmental time point, in one cell type or induced by a particular external stimulus (e.g. mechanical stretch). The same protein may then interact with a second protein partner under another set of circumstances, thus allowing it to regulate multiple cell fates.

PPIs involving TFs such as GATA4 are very common and modulate their activity in several ways (Table 1.4). These include facilitating recruitment to target promoters, controlling access to target genes by nuclear-cytoplasmic shuttling and modulating function by enzymatic modifications (e.g. addition of PTMs) [286,300,301]. GATA4 plays specific roles in all cardiac cell types and at all developmental time points and as such, virtually all GATA4-mediated functions require interactions with protein partners. PPIs also help explain how mutations found throughout the entire length of the GATA4 protein lead to congenital heart defects, not just in regions with known functions (e.g. zinc fingers, nuclear localization sequence and transactivation domains). Given that they require specific amino acid residues and correct 3D structure to mediate direct contact between each component protein, any change in binding sites or protein conformation may inhibit an important PPI and impair cardiac development [302]. As such, given the importance of GATA4 in the regulation of cardiac cell fates, a comprehensive knowledge of cardiac development and function requires a thorough understanding of the GATA4 interactome.

As with our understanding of GATA4 regulation by PTMs and miRNAs, the majority of our information on GATA4 PPIs has been obtained in cardiomyocytes. In the developing heart, GATA4 PPIs play central roles in both cardiomyocyte proliferation and differentiation. At the earliest stages of cell commitment to the cardiac lineage, both the homeobox TF NKX2.5 and GATA4 are well-expressed and interact with one another to induce the cardiac gene program [140]. By physically interacting with one another via the C-terminal zinc finger and C-terminus of GATA4 and the C-terminal homeodomain of NKX2.5, they transactivate target genes central to cardiac development including *Nppa* and *Actc1* [140,303,304]. These two central regulators of cardiogenesis are also able to auto-regulate their expression *in vivo* by binding to conserved GATA binding elements on the cardiac specific *Nkx2.5* enhancer and NKX2.5 binding elements

on the *Gata4* promoter [305,306]. Despite its important role in cardiogenesis, this PPI is insufficient to induce the cardiac gene program by itself [144–146,259,307–309]. Instead, other GATA4 PPIs are able to directly reprogram cells into the cardiac lineage and include TBX5 and MEF2c. GATA4 interacts directly with both of these TFs to activate cardiac genes including *Nppa*, *Nppb*, *Actc1*, *Myh6* and *Myh7* [141,300]. As with the interaction between GATA4 and NKX2.5, these interactions are mediated by the DNA binding domains of each TF, suggesting that these complexes can easily assemble on target promoter binding regions [303]. The combined transfection of GATA4, TBX5 and MEF2c in post-natal cardiac fibroblasts, dermal fibroblasts or murine embryonic stem cells induces cellular contraction, expression of a gene profile and activation of cardiomyocyte-specific markers [175,263]. Similarly, direct interaction between GATA4 and TBX5 and the SWI/SNF BAF chromatin remodelling complex BAF60c differentiates mouse non-cardiac mesodermal or adipose tissue into beating cells that express cardiomyocyte specific markers and repress noncardiac mesodermal genes [174,262,281]. Aside from these core cardiac TFs, GATA4 also interacts directly with its family member GATA6 to transactivate the *Nppa, Nppb, Mef2c* and *Myh7* promoters required for cardiomyocyte differentiation *in vivo* [234,310]. Interestingly, mice haploninsufficent for both *Gata4* and *Gata6* also exhibit decreased cardiomyocyte proliferation suggesting that the GATA4/6 complex plays a pro-proliferative role as well [234]. Other GATA4 PPIs that play a role in cardiomyocyte proliferation include Cyclin D2, Cyclin Dependent Kinase 4 (CDK4) and Krüppel-Like Factor 13 (KLF13) [254–256,258]. Unlike the majority of GATA4 interacting partners, these proteins interact with GATA4 via its N-terminal transactivation domain (Cyclin D2), C-terminus (CDK4) and N-terminal zinc finger (KLF13) showing that protein complexes can assemble anywhere along the GATA4 protein sequence and not just with the C-terminal zinc finger [254,255,311]. Together, these data support an important role for GATA4

PPIs in cardiomyocyte proliferation and differentiation and helps to explain how point mutations found throughout the length of the GATA4 protein lead to CHD.

GATA4 PPIs also play important roles in post-natal cardiomyocytes. Upon sensing of mechanical or neurohumoral stimuli, the myocardium undergoes structural and functional changes that are activated in part by the re-expression of the fetal gene program. These same stimuli reactivate myocardial GATA4 expression that is normally reduced post-development. Given that GATA4 PPIs regulate the fetal gene program during development, it is unsurprising that the same PPIs play central roles in the induction of hypertrophy and survival gene programs as well. As discussed above, the GATA4-MEF2 interaction plays an important role in cardiomyocyte development and is also central to the induction of cardiomyocyte hypertrophy [300,312]. This interaction is induced by upstream signals originating from endothelin-1 and calcium/calmodulin-dependent protein kinase IIδ3, that allow for the nuclear import of both MEF2c and GATA4 so that they can synergize on the pro-hypertrophic Calcineurin-Aβ promoter and likely many others [312]. The GATA4-NKX2.5 interaction is also key to the induction of hypertrophy and activates the *Nppb* promoter in response to mechanical stretch [313]. This interaction has even been the target of small molecule inhibitors that are cardioprotective against both ischemic injury and pressure overload [314]. As well, other key developmental regulatory proteins such as GATA6, TBX5 and BAF60c have also been shown to be necessary for post-natal function and although they have not been shown to interact with GATA4 directly in this context, it would not be entirely unexpected if they did [20,128,315]. Likewise, several GATA4 pro-hypertrophic protein partners are also well expressed throughout development, suggesting that they could interact with GATA4 in this context as well. For example, Serum Response Factor (SRF), a MADS-box TF that when deleted from cardiomyocytes causes embryonic lethality due

to myocardial defects, interacts with GATA4 in the post-natal heart in response to endothelin-1 signaling [316–318]. When overexpressed, both TFs increase myocyte size and induce structural reorganization [128,316,319]. Together, they coactivate several important hypertrophy genes including *Actc1*, *Acta1* and *Fos* and are induced by many of the same signals including the calcineurin signaling pathway [316,320–322]. This pathway also facilitates other GATA4 PPIs as once dephosphorylated by calcineurin, NFATc is translocated to the nucleus where it can directly interact with both SRF and GATA4 [320]. The GATA4-NFAT3 complex can then synergize on the *Nppb* promoter and induce hypertrophy and eventual dysfunction reminiscent of human cardiac remodelling *in vivo* [320]. Other protein partners of GATA4 required for the induction of cardiomyocyte hypertrophy include p300, HEY2, cFOS, SP1 and PEX1 [266,323–326]. These complexes can also induce multiple cell fates simultaneously as the interaction between GATA4 and PEX1 (ZFP260) induces both pro-hypertrophic and pro-survival gene expression required to protect the non-proliferative myocytes from cell death [327].

Unlike GATA4 PPIs required for cardiomyocyte development and function, relatively little is known about those found in cardiac endothelial cells. GATA4 expression is strong throughout the endocardium and endothelial layers of the microvasculature, major coronary arteries and veins [241]. Likewise, endothelial-specific *Gata4* knockout mice have demonstrated the requirement for GATA4 expression and function in these cells [240]. Compared to the myocardium, the cardiac endothelium has not been as thoroughly studied and as such, few endothelial GATA4 PPIs have been identified. However, there are a few that have been described and of them, the first to be identified is aptly named Friend of GATA 2 (FOG2) [252,328,329]. The C-terminal region containing zinc fingers 5 to 8 of FOG2 interacts directly with the N-terminal zinc finger of GATA4 and either activates or represses its activity in a cell specific manner [328,329]. The FOG2-

GATA4 PPI is dependent on the valine at GATA4 position 217 and when mutated to alanine *in vivo*, results in embryonic lethality at E12.5 due to valvular and outflow tract defects [252]. There are currently no cases of CHDs where patients harbour mutations of this residue but mutations surrounding this site including G214S, E215D and M223T all result in endocardial defects and could possibly interfere with this PPI [330,331]. Similarly, other human mutations have helped to identify other endothelial GATA4 protein partners. G303E and G296S both lead to atrioventricular septal and valvular defects as a result of impaired GATA4-SMAD Family Member 4 (SMAD4) interaction [332]. Inhibition of this PPI causes impaired activation of the DNA-binding Protein Inhibitor *Id2* promoter and defective endocardial cushion formation [332]. Indeed, compound haploinsufficieny of endothelially-expressed *Gata4* and *Smad4* recapitulates the atrioventricular septal defects observed in humans harbouring the *GATA4-SMAD4* inhibiting mutations [332]. Likewise, the G296S mutation also inhibits the GATA4-TBX5 interaction [141]. In endocardial cells, GATA4 and TBX5 synergise on the promoter that encodes eNOS, a protein central to proper endothelial development throughout the endocardium and vasculature [242]. As well, compound haploinsufficiency of *Gata4* and endothelially-expressed *Tbx5* results in reduced viability due to severe ASDs [242]. As discussed above, this complex plays a central role in cardiomyocyte development as well and as such, it must be differentially regulated depending on cell type (either via the addition of other protein partners or PTMs).

Name	Localization	Role
Cyclin D2	Myocardium	Cardiomyocyte Proliferation
CDK4	Myocardium	Cardiomyocyte Proliferation
FOG2	Endocardium	Endocardial Development
GATA6	Myocardium	Cardiomyocyte Differentiation
HEY2	Myocardium	Cardiomyocyte Hypertrophy
KLF13	Myocardium	Cardiomyocyte Proliferation
MEF2c	Myocardium	Cardiomyocyte Differentiation and Hypertrophy
NFAT3	Myocardium	Cardiomyocyte Hypertrophy
NKX2.5	Myocardium	Cardiogenesis, Cardiomyocyte Hypertrophy
P300	Myocardium	Cardiomyocyte Hypertrophy
PEX1	Myocardium	Cardiomyocyte Hypertrophy and Survival
SMAD4	Endocardium	Endocardial Development
Sp1	Myocardium	Cardiomyocyte Hypertrophy
SRF	Myocardium	Cardiomyocyte Hypertrophy
TBX5	Myocardium/ Endocardium	Cardiomyocyte Differentiation, Endocardial Development

Table 1.4 List of known GATA4 interacting partners, their localization in the heart and their known roles

Cell culture and *in vivo* overexpression and knockout models as well as human mutations linked to CHD have all demonstrated the central requirement for GATA4 in cardiac development and function. The effects of this protein are found in all cardiac cell types, at all developmental time points and in response to multiple external stimuli. As such, GATA4 expression and function must be tightly regulated. GATA4 regulatory mechanisms in the heart centre on PTMs, miRNAs and PPIs. They are extremely complex and allow GATA4 to activate specific gene expression programs that control cardiac cell phenotypes. It is important to note that these regulatory mechanisms do not act independently. Instead, all regulatory mechanisms act in

concert, allowing for more complex regulation of GATA4 function. For example, phosphorylation of GATA4 induced by upstream signaling pathways like the MEK-ERK pathway may allow GATA4 to interact with pro-hypertrophic protein partners and together, activate pro-hypertrophic genes such as myosins, troponins and natriuretic peptides. Likewise, the covalent addition or removal of PTMs requires the direct interaction between GATA4 and enzyme protein partners including kinases and histone acetyltransferases. Furthermore, expression of miRNAs that repress expression of GATA4 or its protein partners can directly inhibit complex formation and activation of specific target genes. Our understanding of these processes has improved considerably over the past several years and has primarily focused on GATA4 regulation in cardiomyocytes. However, a great deal remains to be learned in those cells as well as in the cardiac endothelium as both are significantly affected by both congenital and acquired cardiac disease. An improved understanding of cell-specific GATA4 functions in cardiomyocytes and cardiac endothelial cells will improve our understanding of heart development and function and offer new insights on diagnosis and treatment options for disease.

1.12 Objective and Hypothesis

Given the information discussed above, the objective of this project is to identify novel GATA4 protein partners and downstream gene targets involved with cell fate determination. I hypothesize that GATA4 controls important cardiac cell-specific pathways via interactions with as yet undiscovered protein-protein interactions. Interactions with these partners modify GATA4's ability to activate specific target promoters and as such, establish the downstream gene programs required to control cell fates. Inhibition of GATA4 interactions with its protein partners or transactivation of its target genes contributes to cardiac disease.

Work presented in this thesis has:

1) Identified HSP70 as a GATA4 interacting partner that can inhibit its Doxorubicin-induced degradation by Caspase-1,
2) Demonstrated that the interaction between GATA4 and NUR77 mediates ANGPTL7 secretion and angiogenesis in the left ventricular microvasculature during pressure overload and
3) Identified NONO and PSPC1 as GATA4 regulators that modulate endothelial gene expression required for endocardial development.

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2. Chapter I: Caspase-1 cleavage of transcription factor GATA4 and regulation of cardiac cell fate

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2.1 Statement of the Manuscript

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2.2 Contribution Statement

AA designed and performed experiments (Figures 1, 2 A,C,D and 5D, E), analyzed data and interpreted results, JW designed and performed experiments (Figures 2B, E, F and G, 3 and 6) analyzed data, interpreted results and helped write the manuscript, WS performed experiments (Figure 4) and provided technical support, HK designed experiments, analyzed data, interpreted results and helped write the manuscript, MS analyzed data, interpreted results and edited the manuscript and MN conceived the project, designed experiments, interpreted results and helped write the manuscript.

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2.5 Disclosures

None

2.6 Abstract

Caspase-1 or Interleukin-1β (IL-1β) converting enzyme is a pro-inflammatory member of the caspase family. An IL-1β independent role for caspase-1 in cardiomyocyte cell death and heart failure has emerged but the mechanisms underlying these effects are incompletely understood. Here we report that transcription factor GATA4, a key regulator of cardiomyocyte survival and adaptive stress response is an *in vivo* and *in vitro* substrate for caspase-1. Caspase-1 mediated cleavage of GATA4 generates a truncated protein that retains the ability to bind DNA but lacks transcriptional activation domains and acts as a dominant negative regulator of GATA4. We show that caspase-1 is rapidly activated in cardiomyocyte nuclei treated with the cell death inducing drug Doxorubicin. We also find that inhibition of caspase-1 alone is as effective as complete caspase inhibition at rescuing GATA4 degradation and myocyte cell death. Caspase-1 inhibition of GATA4 transcriptional activity is rescued by HSP70 which binds directly to GATA4 and masks the caspase recognition motif. The data identify a caspase-1 nuclear substrate and suggest a direct role for caspase-1 in transcriptional regulation. This mechanism may underlie the inflammation independent action of caspase-1 in other organs.

2.7 Introduction

Caspase-1 is best known for its role in inflammation through the processing of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18 [1]. Mice lacking caspase-1 (*Casp1*$^{-/-}$) are viable but fail to activate and secrete IL-1β [2]. In addition to its function in inflammation, caspase-1 plays a role in programmed cell death in myeloid cells, lymphocytes as well as in the heart and brain [3,4]. Whereas *Casp1*$^{-/-}$ mice have no developmental programmed cell death defects, they are protected against ischemic brain injury and heart failure [3,4]. In both neurons and cardiomyocytes, a direct role for caspase-1 in promoting cell death in the absence of inflammation has been demonstrated using *in vitro* cell cultures and *in vivo* models. For example, myocardial specific overexpression of caspase-1 induces a massive increase in cardiomyocyte death in young mice without any increase in tissue or plasma levels of IL-1β, IL-18 or other inflammatory mediators; conversely, *Casp1*$^{-/-}$ mice show a lesser degree of cell death after induction of myocardial infarction [4]. Similarly, expression of caspase-1 in neonate rat cardiomyocyte cultures increases cell death by 4-5 fold [4]. Because postnatal cardiomyocytes have limited regenerative capacity, their loss as occurs following myocardial infarction or chemotherapy leads to heart remodeling, loss of contractility and ultimately heart failure [4]. Indeed, cardiomyocyte death is increased in human heart failure and induction of cell death in experimental models is sufficient to cause heart failure [5]. Together, the data suggest that caspase-1 inactivates key molecules and pathways that promote cardiomyocyte survival.

Transcription factor GATA4, a member of the zinc finger GATA family, has emerged as a key cardiomyocyte survival factor and an essential regulator of the postnatal cardiomyocyte stress response. Cardiomyocytes with downregulated GATA4 levels have increased rates of cell death at basal levels and in response to cardiotoxic drugs such as Doxorubicin (Dox) or tyrosine

kinase inhibitors [6,7]. These cells also fail to mount any adaptive response to mechanical or neuroendocrine stress [8–10]. GATA4 is also a potent cardiogenic factor essential for cardiomyocyte commitment and differentiation [11]. We now report that GATA4 is cleaved by caspase-1 *in vitro* and in cardiomyocytes. The resulting cleaved protein acts as a dominant negative isoform unable to maintain the genetic program required for myocyte survival. The data identify a target for caspase-1 in the nucleus and a pathway to explain its cardiac action.

2.8 Materials and Methods

Plasmids

GATA4 and all luciferase reporters used were previously described [6,11,12]. GATA4 point mutations were subcloned into the pGEX vector and the N-terminal flag-tagged Caspase-1 constructs were produced by PCR from rat cDNA and was subcloned into the pcDNA3 vector. The pcDNA3.1-F-HSP70-GFP construct was a kind gift from Dr. Stephen Lee (University of Ottawa) [13]. All constructs were verified by sequencing.

Cell Culture and transfections

Cardiomyocytes from 4 day old Sprague-Dawley rats (Charles River) were harvested, cultured and manipulated as previously described [6]. Myocytes were treated with Doxorubicin (SIGMA) at 300 nM for the indicated time in the presence or absence of a protease inhibitor (MG-132, 10 μM in DMSO, CalBioChem 474790) or caspase inhibitors: caspase-1 inhibitor (YVAD-CHO, 10 μM in DMSO, CalBioChem 400011) or pan-caspase inhibitor (zVAD-FMK, 10 μM in DMSO, CalBioChem 219007). Inhibitors were added to cardiomyocytes 30 minutes before the

addition of Dox. NIH3T3, HL1, TC13 and AD293 cells were maintained and manipulated as previously reported [14,15]. Luciferase assays were carried out as described previously [16].

Western blot

Western blots of nuclear extracts from cardiac myocytes or other cell lines overexpressing various GATA4 proteins were performed as previously described [8]. Western blots of nuclear extracts from cardiac myocytes or other cell lines overexpressing various GATA4 proteins were performed as previously described [8]. Anti-HA (Santa Cruz, sc-805) anti-Flag (Sigma, F1804), anti-p300 (Santa Cruz, SC-585X) and anti-nucleolin (Santa Cruz, sc-55486) were all used at a dilution of 1/500. Anti-caspase-1 (Cell Signaling, 2225), antiBclxL (Cell Signaling, 2762), anti-GAPDH (Abcam, ab8245) and anti-GATA4 (sc-25310) were used at 1/1000 dilution. Homemade rabbit GATA4 and GATA6 antibodies were used at a dilution of 1/2000 and 1/500, respectively [17].

Electrophoretic mobility shift assays (EMSA)

DNA binding of GATA4 mutants was assessed using nuclear extracts from AD293 cells and the proximal GATA site from the rat Nppa promoter as described previously [8].

Coimmunoprecipitation

AD293 cells were transfected with pCGN-HA-GATA4 and/or Flag- pcDNA3.1-F-HSP70-GFP using Effectene transfection reagent (Qiagen) according to the manufacturer's guidelines. Nuclear extracts were incubated with anti-Flag M2 coupled magnetic beads (Sigma) overnight as described by Morin *et al* [16]. Bound proteins were revealed with anti-HA or anti-Flag antibodies by western blot.

Terminal Deoxynucleotidyltransferase- Mediated dUTP End-Labeling (TUNEL) assay for apoptosis

Apoptosis was detected by the TUNEL technique as recommended in the Apoptag kit (Chemicon). An average of 10 random fields with 100 nuclei per field was analyzed.

Immunofluorescence

Immunofluorescence experiments were carried out as described previously [8]. Anti-Caspase-1 (Abcam, ab-1872) was used at a dilution of 1/200 and Alexa Fluor® 546 Goat Anti-Rabbit IgG (Molecular Probe, A-11035) was used at a dilution of 1/500. Hoechst (Molecular Probe, H-1398) was used at a dilution of 1/5000. Images acquisition was completed using the Zeiss AxioObserver D1 microscope.

Immunohistochemistry

Immunohistochemistry was completed as previously described [6]. Rabbit anti-caspase-1 antibody (Abcam ab-1872) was used at a dilution of 1/200. A homemade rabbit anti-GATA4 antibody was used at a dilution of 1/500.

In vitro Translation and Pull down assays

In vitro translation and pull down assays were carried out as described previously [8]. ^{35}S-labelled *in vitro* translated proteins were produced using the T7 Quick-Coupled Transcription/Translation System (Promega) according to the procedures provided by the manufacturer. Pull down assays were carried out as described previously [8]. Briefly, recombinant GST-fused proteins were produced in BL-21 *E. coli* and purified on sepharose beads. *In vitro*

translated proteins were incubated with GST fusion proteins overnight at 4°C. Bound proteins were detected by autoradiography.

Caspase Cleavage Assays

Cleavage of the *in vitro* transcribed and translated ^{35}S-labeled substrates was performed in a 20 μl reaction containing 2 μl of *in vitro* transcribed and translated ^{35}S-labeled substrates by incubation at 37 °C for 4 h in the presence or absence of purified human recombinant caspase-1 or caspase-3 (170 ng) in CHEG buffer (with 10 mM dithiothreitol freshly added). The cleavage reaction was terminated by the addition of Laemmli SDS loading buffer and resolved by SDS-PAGE. The gel was fixed in 10% acetic acid and 40% ethanol for 0.5 h; the signal was then amplified by incubating the gel with NAMP 100V amplifying solution (Amersham Biosciences) for 30 min. The gel was placed on a Whatman paper, dried at 70 °C for 1 h, and exposed at -80 °C, and the signal was viewed by autoradiography [18].

QPCR

RNA was extracted using Trizol and then reverse transcribed with the Omniscript reverse transcriptase (Qiagen). QPCR analyses were used to measure change in GATA4 and ribosomal protein S16 mRNA levels using the Quantitech SYBR green (Qiagen).

Animal Models

C57/B6 mice were treated with Dox as previously described [6]. Casp1$^{-/-}$ mice have been previously described [19]. For Dox and YVAD-CHO experiments, animals were injected i.p. with 5 mg/kg YVAD-CHO and 20 mg/kg Dox. Injections were separated by 1 hour. After one week,

animals were sacrificed by cervical dislocation and the heart was cryopreserved. All experiments were approved by the University of Ottawa and McGill University animal care committees and were carried out as per institutional guidelines for animal care. Mason trichrome staining was completed as previously described [20].

FAM-FLICA assay

The FAM-FLICA assay is specific to active caspase-1 and measures binding of caspase-1 to cognate sites. The assays were done as per the manufacturer's instructions (ImmunoChemistry Technologies, catalog number 97). Briefly, cardiomyocytes plated on glass coverslips were incubated with FAM-FLICA reagent diluted in serum free media for 1 hour at 37ºC. Cells were then washed 3 times for 5 minutes in media and fixed with 4% PFA. Cells were then washed 3 times in PBS and mounted with Prolong Gold (Life Technologies, P36930). Fluorescence image acquisition was completed using the Zeiss AxioObserver D1 microscope.

2.9 Results

GATA4 is an immediate early target of Doxorubicin (Dox) in the heart, affecting both transcriptional and post-translational mechanisms. Depletion of GATA4 dose dependently induces cell death, a process that can be rescued by exogenous GATA4 [6]. Time course analysis of Dox effects revealed that the GATA4 protein was markedly depleted after 3 hrs of treatment (the earliest point studied) in the absence of any significant decrease in transcript levels (**Figure 2.1 A Left Panel, and B**). GATA6 protein levels remained unchanged (**Figure 2.1 A, Middle Panel**). The decrease in the native GATA4 immunoreactive band was accompanied by the concomitant appearance of a 20KDa band. GATA4 degradation was independent of the proteasome as shown

by the inability of a proteasome inhibitor to prevent the Dox-dependent decrease of GATA4 protein (**Figure 2.1 C**). To confirm whether these changes occur at post-translational stages, a CMV-driven HA-GATA4 expression vector was transfected into the cardiomyocyte cell line HL-1 and treated with Dox. As shown in figure 2.1 D, Dox treated extracts had significantly less intact exogenous GATA4 as revealed with the HA and GATA4 antibodies which recognize N and C-terminal epitopes respectively [21]. A GATA4 protein deleted of its entire N-terminal domain (amino acids 201-440) was then transfected into HL1 cells and exposed to Dox. In Dox treated cells, the C-terminal GATA4 antibody detected a doublet suggesting that a cleavage site lies within this domain. This doublet was not recognized by the N-terminal HA tag implicating cleavage at the N-terminus of the protein. The difference in size between the two bands suggested cleavage between amino acids 225-230.

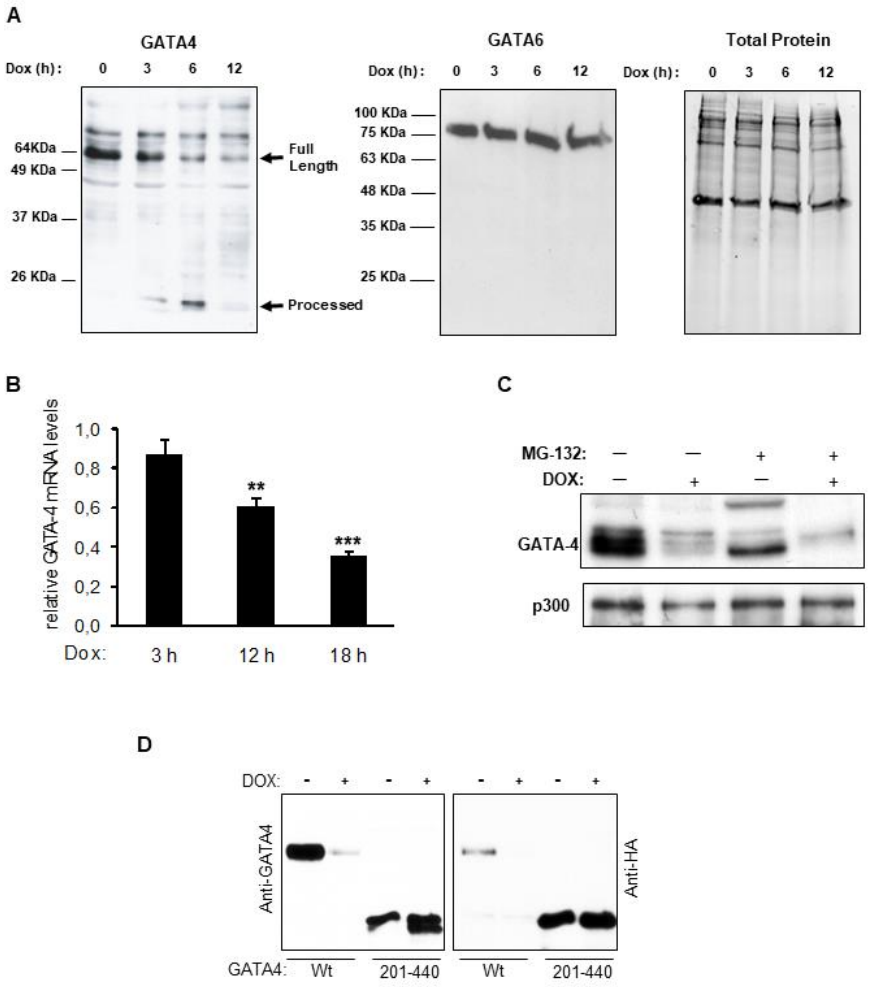

Figure 2.1

Figure 2.1 Dox-induced GATA4 depletion is independent of the ubiquitin-proteasome pathway. (**A**) Effect of time course treatment of Doxorubicin (Dox) on GATA4 (left panel), GATA6 (middle panel) and total protein (right panel) levels. Nuclear extracts were prepared from primary cardiomyocyte cultures treated for the indicated times with Dox (300 nM) and subjected to western blot analyses. (**B**) Depletion of GATA4 transcripts after 12 h of Dox treatment. Cardiomyocytes were treated for the indicated times with Dox. RNA was subjected to Real Time PCR. GATA4 mRNA levels were normalized to S16 mRNA. The results are shown as mean ± SEM and analyzed by one way ANOVA with Bonferroni post-test relative to the 3 h Dox treatment (n = 3). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. (**C**) Depletion of GATA4 protein by Dox is not prevented by a proteasome inhibitor. Cardiomyocytes were treated with Dox for 12h in the presence or absence of 10 μM a proteasome inhibitor MG132. Nuclear extracts were subjected to western blot to detect GATA4 protein. P300 was used as a control. (**D**) A putative cleavage site in the N-terminal region of GATA4. Transient transfection was carried out in HL-1 atrial cardiomyocytes using GATA4 WT and a GATA4 N-terminal deletion (201-440) mutant. Nuclear extracts were subjected to western blot analysis using anti-HA and anti-GATA4 antibodies to detect N- and C-terminal fragments respectively.

We tested whether GATA4 degradation was caspase dependent. Co-treatment of cardiomyocytes with Dox and a pan caspase inhibitor abrogated GATA4 depletion (**Figure 2.2 A**) and significantly attenuated cardiomyocyte death (**Figure 2.2 C**). Next, we determined which caspase was responsible for GATA4 depletion and cardiomyocyte death. Cardiomyocytes were treated with Dox in the presence or absence of YVAD-CHO, a selective caspase-1 inhibitor. Co-treatment with Dox and YVAD-CHO prevented GATA4 depletion, demonstrating that inhibition of caspase-1 protects against Dox-induced GATA4 degradation (**Figure 2.2 B**). In contrast, caspase-3 inhibition had only a modest effect on GATA4 levels and on Dox-induced cardiomyocyte apoptosis (data not shown). The caspase-1 inhibitor was also as effective as the pan-caspase inhibitor at reducing cell death in response to Dox treatment (**Figure 2.2 C**). These results are indicative of an important role for caspase-1 in Dox-induced cardiotoxicity. We examined whether Dox treatment was associated with caspase-1 activation by both western blot and FLICA assay which measures active caspase-1 binding to cognate sites. Western blot analysis of nuclear extracts revealed the presence of cleaved caspase-1 in Dox-treated cardiomyocytes at 3 and 12 hrs post-treatment (**Figure 2.2 D**). Similarly, FLICA assays confirmed increased caspase-1 activation (4-fold) in Dox-treated cells (**Figure 2.2 E**). Immunofluoresence staining of caspase-1 in the cardiac HL1 cell-line (**Figure 2.2 F**) and in primary cardiomyocytes (**Figure 2.2 G**) showed caspase-1 localization to the nucleus in Dox-treated cells.

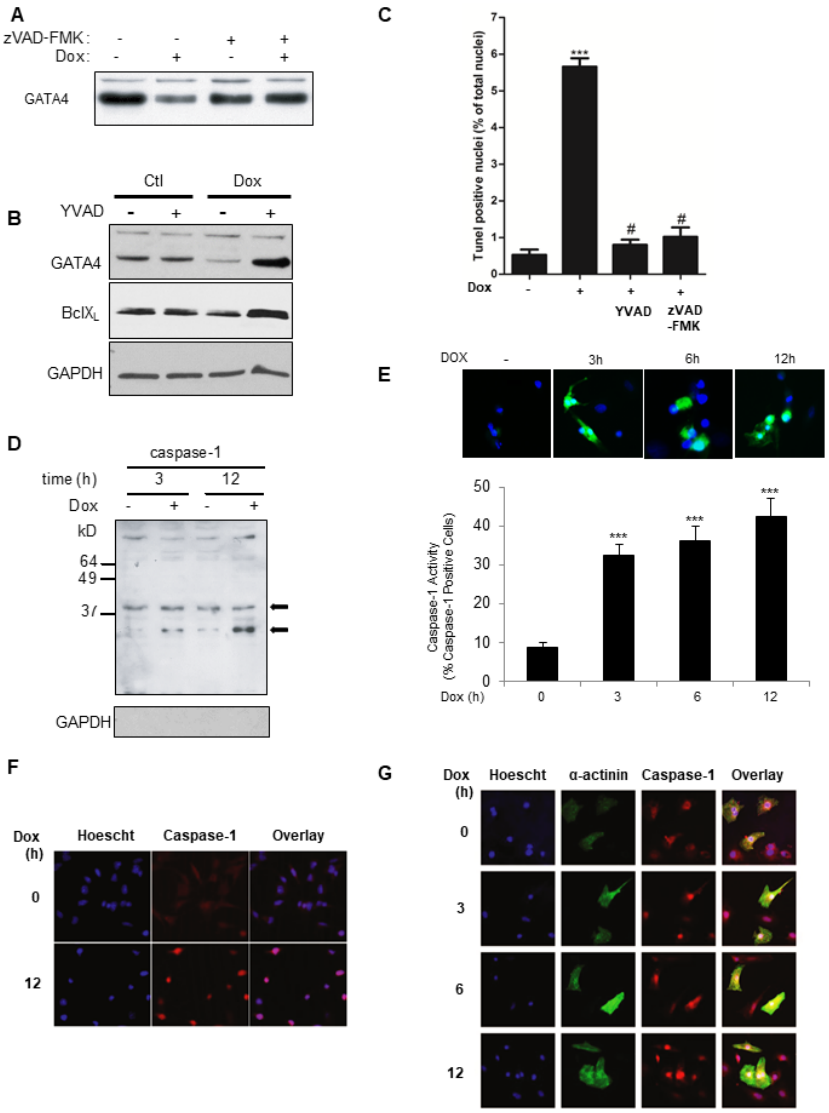

Figure 2.2

Figure 2.2 Dox-induced GATA4 depletion is caspase-1 dependent. (**A**) Pan-caspase inhibitor restored GATA4 expression. Cardiomyocytes were treated with Dox in the presence or absence of pan-caspase inhibitor (zVAD-FMK) for 12 hours and analyzed by western blot. (**B**) Caspase-1 inhibitor prevented Dox-dependent GATA4 depletion. Cardiomyocytes were treated in presence or absence of Dox with a caspase-1 inhibitor (YVAD-CHO). Western blots were carried out to detect GATA4 and its downstream target BclxL. GAPDH was used as a loading control. Note how changes in BclxL levels parallel those of GATA4. (**C**) Effect of caspase inhibition on cardiomyocyte apoptosis. Quantification of TUNEL assays in primary cardiomyocytes treated with the indicated inhibitors. The results are shown as mean ± SEM and analyzed by one way ANOVA with Bonferroni post-test relative to the control (*) or to the Dox treatment alone ($^{\#}$). ***$P \leq 0.001$, $^{\#}P \leq 0.001$. Note how caspase-1 inhibition is as effective as the pan-caspase inhibitor at abrogating Dox-induced apoptosis. D-G. Increased activation and nuclear localization of caspase-1 in Dox-treated cardiomyocytes. (**D**) Western blots of nuclear cardiomyocyte extracts. Notice how caspase-1 is activated (lower band) after 3 and 12 hours of Dox treatment. GAPDH staining was used to control for cytoplasmic contamination. (**E**) Representative images (top panel) and quantification (lower panel) of a FAM-FLICA assay measuring caspase-1 activity in control and Dox-treated cardiomyocytes. Results are shown as percent of caspase-1 positive cells. ***$P \leq 0.0001$. In the top panel, green is active caspase-1 and blue is Hoechst staining. F and G. Immunofluorescence of HL1 cells (**F**) and primary cardiomyocytes (**G**) treated with Dox for the indicated time. Caspase-1 is labeled in red, α-actinin is labeled in green and Hoechst staining is labeled in blue.

Caspase-1 nuclear localization in response to Dox was also observed *in vivo*. Wild type mice treated with Dox show stronger nuclear caspase-1 staining in comparison to control mice and a concomitant decrease in GATA4 nuclear staining (**Figure 2.3 A**). To determine the effect of caspase-1 inhibition on cardiomyocyte cell death and cardiac remodeling, TUNEL assays and trichrome staining were carried out on heart tissue sections of wild type mice treated with Dox in the presence or absence of the caspase-1 inhibitor YVAD-CHO (**Figure 2.3 B and C**). Treatment with YVAD-CHO significantly reduced the number of TUNEL positive nuclei and fibrotic lesions, consistent with a role for caspase-1 in Dox-induced cardiotoxicity. $Casp1^{-/-}$ mice treated with Dox showed an attenuated response compared to similarly treated wild type mice as measured by cell death and the presence of fibrosis (**Figure 2.3 B and C**). These results suggest that reduction of caspase-1 activity *in vivo* is protective against Dox cardiotoxicity.

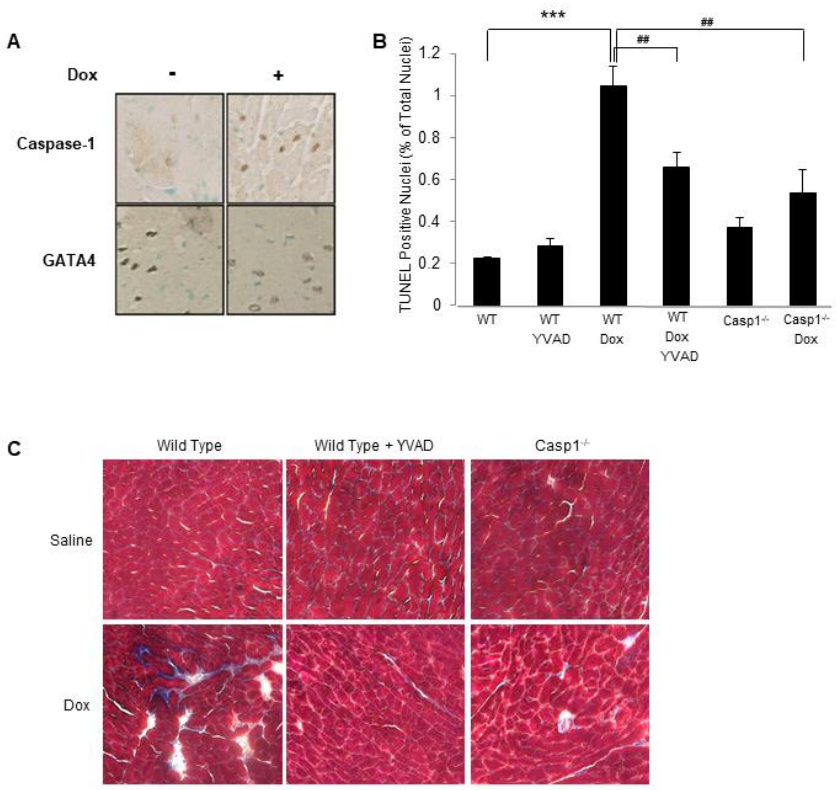

Figure 2.3

Figure 2.3 Caspase-1 inhibition is protective against Dox cardiotoxicity *in vivo*. (**A**) Dox induces an increase in caspase-1 and a decrease in GATA4 staining *in vivo*. Immunohistochemistry of ventricular tissue sections from wild type mice treated with Dox or vehicle. Caspase-1 staining is shown in the top panels and GATA4 staining in the bottom panels. (**B**) Caspase-1 inhibition or loss attenuates cardiomyocyte cell death *in vivo*. Quantification of TUNEL assays of wild type mice treated with Dox and YVAD-CHO as well as $Casp1^{-/-}$ mice treated with Dox. The results are shown as the mean ± SEM and analyzed by Student's T-Test of wild type control mice (*) or of wild type Dox-treated mice (#). ***$P \leq 0.0001$, $^{\#\#}P \leq 0.001$. (**C**) Effect of caspase-1 inhibition or loss on Dox induced cardiac fibrotic cardiac lesions *in vivo*. Trichrome staining of transverse sections of left ventricular tissue of wild type mice treated with Dox and YVAD-CHO or $Casp1^{-/-}$ mice treated with Dox. Blue staining represents fibrotic lesions.

To determine whether GATA4 is a direct substrate of caspase-1 or -3, we searched for putative caspase recognition motifs on the GATA4 protein. Caspase-3 preferably cleaves at DEVD sequences whereas the preferred sites of caspase-1 contain a bulky and hydrophobic amino acid at the P4 position such as tryptophan and tyrosine (e.g. W/YxxD) [22,23]. Two putative caspase-1 sites

that fit these criteria are present on the GATA4 protein and are evolutionary conserved in human, mouse and rat: YMAD[168] within the major transcription activation domain and WRRD[230] within the first zinc finger (**Figure 2.4 A**). Another conserved motif DMFD[208] may correspond to a low affinity caspase-3 recognition site. Figure 2.4 B depicts the possible polypeptides resulting from caspase cleavage. Incubation of *in vitro* translated GATA4 with active caspase-1 produced 3 fragments around 18, 26 and 32KDa. In contrast, no caspase-3 cleavage products were detected (**Figure 2.4 C**). The fragments obtained from the caspase-1 digestion are consistent with processing cleavage at D168 and D230. To confirm that these are caspase-1 cleavage sites, we prepared a series of mutant proteins in which these residues alone or in combination are converted into alanine effectively eliminating the caspase motif. As shown in figure 2.4 D, mutation of both D168 and D230 render GATA4 completely resistant to caspase-1 cleavage. These results confirm that GATA4 is a direct caspase-1 substrate and that caspase-1 processes GATA4 at two specific cleavage sites. Of note, cleavage at either position would result in a truncated nuclear GATA4 protein capable of binding DNA as shown in figure 2.5 A, but missing the N-terminal transactivation domains. As well, cleavage at D230 would lead to loss of the N-terminal zinc finger, a region critical to protein-protein interactions [24,25]. As expected, the deletion mutants that would result from cleavage at D168 and D230 had reduced transcriptional activation (**Figure 2.5 B**) and when co-expressed with native GATA4, reduced its activity on target promoters (**Figure 2.5 C**).

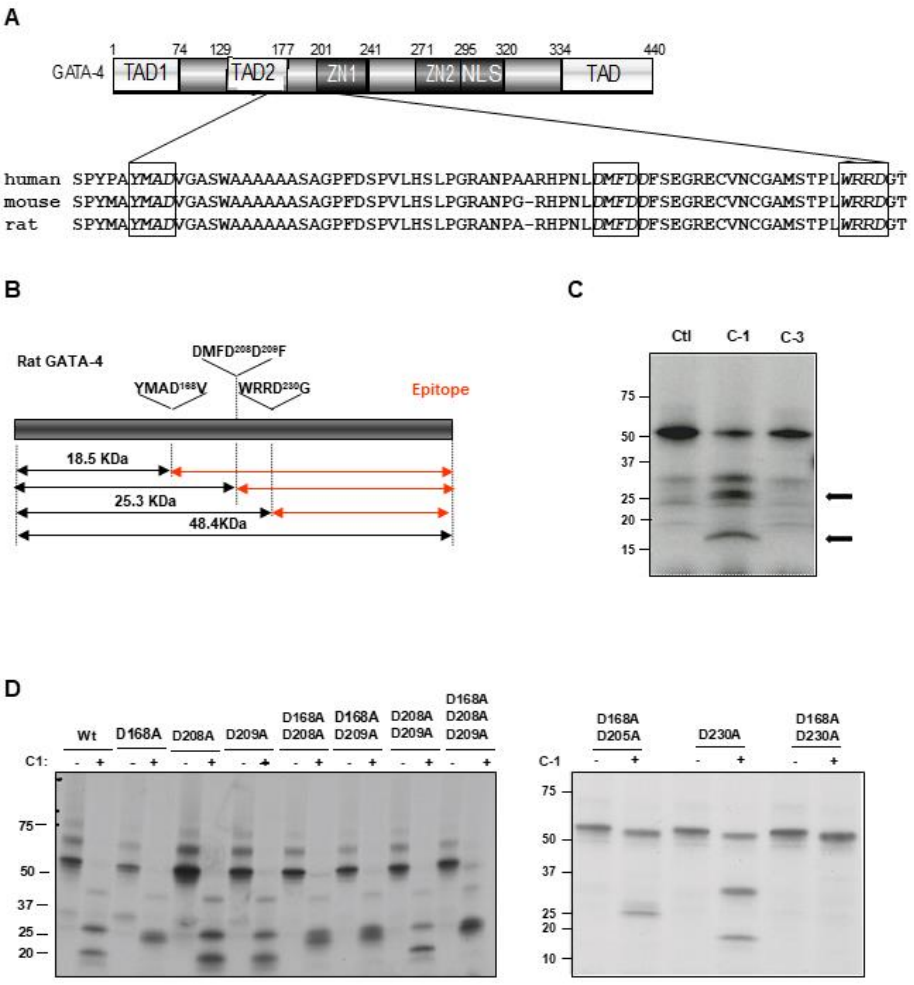

Figure 2.4

Figure 2.4 GATA4 is a direct substrate for caspase-1. (**A, B**). Schematic representation of rat GATA4 (accession number P46152). Alignment of GATA4 from different species shows that the putative cleavage sites YMAD168, DMFD208 and WRRD230 in boxes are conserved in human, mouse and rat. (**B**) Predicted size of GATA4 fragments cleaved by caspase-1. The depicted red fragments can be detected by the GATA4 antibody (epitope). (**C**) *In vitro* caspase cleavage assays. *In vitro* translated radiolabelled GATA4 was exposed to purified caspase-1 (C-1) and caspase-3 (C-3). Arrows indicate cleavage products by caspase-1 but not by caspase-3. (**D**) Caspase-1 cleavage of GATA4 mutants identifies D168 and D230 as cleavage sites. *In vitro* cleavage assays using purified caspase-1 and *in vitro* translated GATA4 WT and GATA4 mutants (single or double mutations as indicated in panel D). Note how double mutation of D168 and D230 prevents the cleavage by caspase-1.

Next, we tested the effect of caspase-1 on GATA4 activity *ex vivo*. NIH3T3 cells were co-transfected with GATA4 and a GATA dependent reporter in the presence or absence of caspase-1. As shown in figure 2.5 D, caspase-1 dose dependently inhibited GATA4 transcriptional activity of the reporter. A similar effect was also observed on the ANF promoter, a well-known GATA4 target. In contrast, the activity of a caspase-1 resistant GATA4 mutant (D168A/D230A) was not significantly affected by caspase-1. These results indicate that GATA4 is a caspase-1 substrate and that caspase-1 is a negative regulator of GATA4.

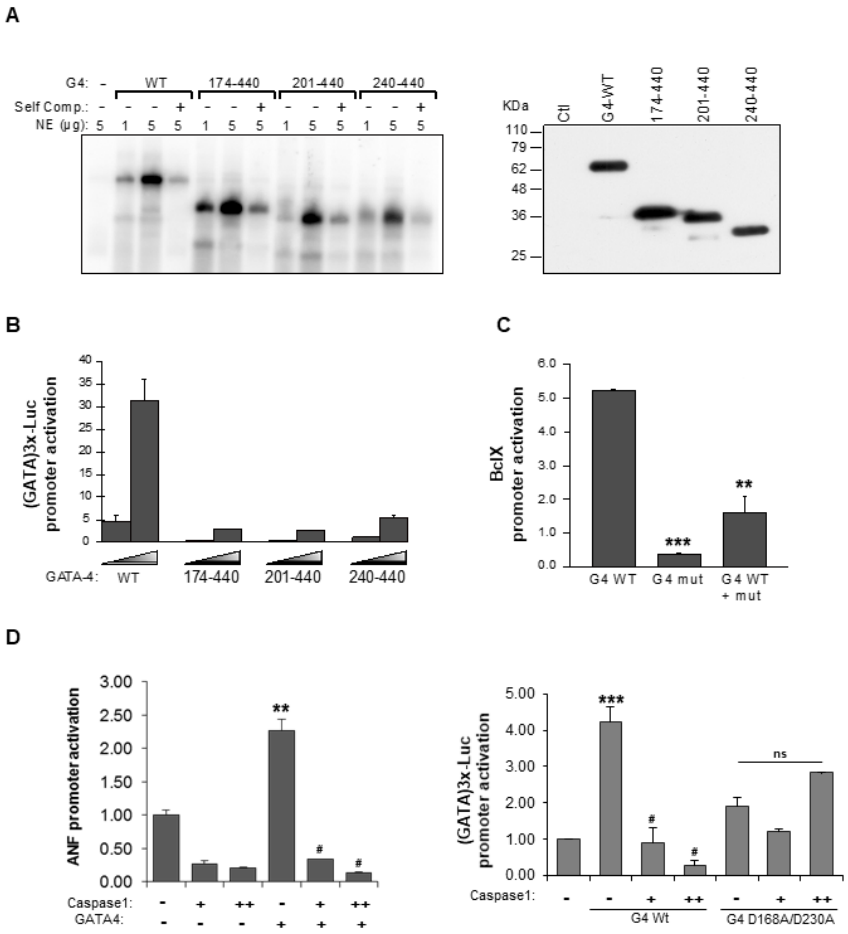

Figure 2.5

Figure 2.5 Caspase-1 is a negative regulator of GATA4 transcriptional activity. (**A**) Left panel. N-terminal truncated GATA4 proteins bind DNA. Nuclear extracts from AD293 cells transfected with the indicated GATA4 constructs were tested for their ability to bind GATA elements using EMSA assays. NE: nuclear extracts, self comp: self competition with cold probe. The right panel is a western blot showing equivalent protein expression levels for all constructs. (**B**) Dose response for wild type and truncated GATA4 protein co-transfected with the (GATA)3x-Luc promoter. (**C**) Caspase-1 cleaved GATA4 acts as dominant negative. GATA4 WT and GATA4 mut (aa 174-440) were co-transfected with the BclxL promoter. The results are shown as mean ± SEM and analyzed by one way ANOVA with Bonferroni post-test relative to the GATA4 WT treatment. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. (**D**) Effect of caspase-1 on GATA4 dependent transcription. Left Panel. (GATA)3x–luc reporter (1 μg) was co-transfected with 100 ng of native or mutant GATA4 expression vector with or without 50 and 500 ng of caspase-1 expression vector. The results are shown as mean ± SEM and analyzed by one way ANOVA with Bonferroni post-test relative to the control (*) or GATA4 WT treatment ($^{\#}$). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, $^{\#}$P ≤ 0.001. Right Panel. Effect of caspase-1 on the ANF promoter in response to GATA4. The amount of plasmid DNA used is the same as in left panel. The data are the mean ± SEM of two experiments carried out in duplicate. The results are shown as mean ± SEM and analyzed by one way ANOVA with Bonferroni post-test relative to the control (*), GATA4 WT treatment ($^{\#}$) or mutant GATA4 treatment. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, $^{\#}$P ≤ 0.001, ns = not significant. Note how caspase-1 completely abrogates GATA4 activation and how mutation of the 2 major caspase-1 cleavage sites renders GATA4 resistant to this effect.

Inhibition of GATA4 - a cardiomyocyte survival factor – by caspase-1 is consistent with the reported involvement of caspase-1 in myocyte cell death and heart failure. We asked whether interaction of GATA4 with other cofactors might serve to mask the caspase-1 recognition motifs and protect GATA4 from caspase-1 cleavage. We focused on HSP70 because it was identified by mass spectrometry as a component of nuclear GATA4 complexes in cardiogenic TC13 cells (our unpublished data) and because HSP70 is cardioprotective [26]. Co-immunoprecipitation of transfected GATA4 and HSP70 confirmed that the two proteins interact in cell nuclei (**Figure 2.6 A and B**). Pull down assays using GST-GATA4 proteins (**Figure 2.6 C**) and *in vitro* translated HSP70 were carried out to identify the HSP70 interacting domain on GATA4. As shown in figure 2.6 D, HSP70 bound mainly the N-terminal domain of GATA4 and a 40 amino acid fragment spanning GATA4 amino acids 130-170 was sufficient to retain HSP70. To determine the effect of HSP70 and Caspase-1 on GATA4 protein expression, nuclear extracts from NIH3T3 cells transfected with GATA4, Caspase-1 and HSP70 were analyzed by western blot (**Figure 2.6 E**). Compared to transfection with GATA4 alone, co-transfection of caspase-1 and GATA4 yielded

lower levels of GATA4 protein. However, GATA4 protein levels were rescued by concomitant HSP70 expression. The relevance of this interaction on GATA4 transcriptional activity was examined by luciferase assay. We co-transfected a GATA-luciferase reporter with a GATA4 expression vector in the presence or absence of caspase-1 and HSP70 (**Figure 2.6 F**). HSP70 prevented the caspase-1 mediated reduction of GATA4 transcriptional activation, maintaining GATA4 activity to a similar level as observed in the absence of caspase-1. Together the data indicate that GATA4 is a caspase-1 substrate and suggest that physical interaction with HSP70 may protect GATA4 from caspase-1 processing and inactivation.

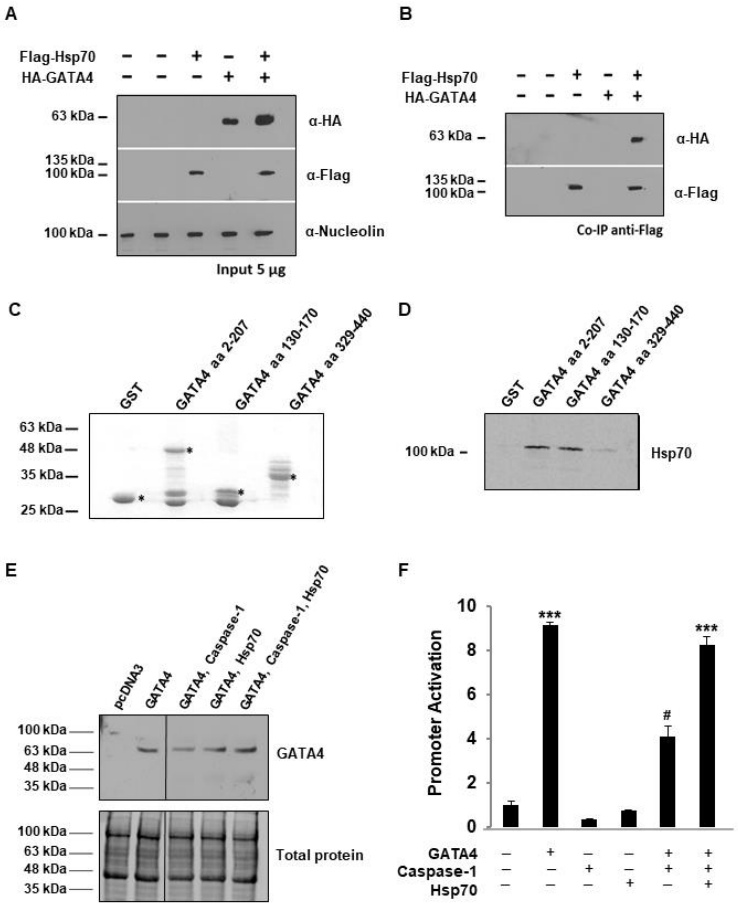

Figure 2.6

Figure 2.6 HSP70 physically interacts with GATA4 and rescues caspase-1 inhibition. (**A, B**) HSP70 co-immunoprecipitates with GATA4. Nuclear extracts from AD293 cells transfected with HA-GATA4 and/or Flag-HSP70-GFP were immunoprecipitated with anti-Flag antibody, separated on a 10% (vol/vol) SDS-PAGE and immunoblotted with anti-HA, anti-Flag and anti-Nucleolin antibodies. (**C, D**) HSP70 interacts directly with the N-terminal of GATA4. GST and GST bound GATA4 aa 2 – 207, aa 130 – 170 and aa 329 – 440 fusion proteins were incubated with *in vitro* translated HSP70. Bound proteins were resolved using SDS-PAGE (12% vol/vol) and revealed using autoradiography. Fusion protein inputs (C) were resolved using SDS-PAGE (12% vol/vol) and stained using coomassie blue. Astrices indicate fusion protein bands. (**E**) HSP70 prevents caspase-1 mediated GATA4 processing. NIH 3T3 cells were transfected with expression vectors for GATA4, caspase-1 and/or HSP70. Nuclear extracts were analyzed by western blot. (**F**) Hsp70 rescues caspase-1 mediated inhibition of GATA4 transcriptional activity. Transfection into NIH3T3 cells of the GATA dependent Nppb promoter –Luc reporter along with the indicated expression vectors for GATA4, caspase-1 and HSP70 and activations thereof. The results are shown as mean ± SEM and analyzed by one way ANOVA with Bonferroni post-test relative to the control (*) or GATA4 treatment ($^{\#}$). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, $^{\#}$P ≤ 0.001 (n = 3). Note how caspase-1 inhibits GATA4 transactivation in the absence but not in the presence of HSP70.

2.10 Discussion

Transcription factor GATA4 is a critical survival factor for cardiomyocytes and an angiogenic factor of the infarcted heart [6,9,27,28]. Decreased levels of GATA4 promote cardiomyocyte death and sensitize myocytes to drug induced cell death. The data presented here reveal that GATA4 is inactivated by caspase-1 cleavage which leads to transcriptional downregulation of cell survival pathways (e.g. Bcl-xL) and irreversible cardiac damage. This negative feedback loop would amplify the deleterious effects of cardiotoxic insults and is consistent with the degenerative nature of some cardiac disease such as heart failure. Conversely, the finding that HSP70 interacts with GATA4 to prevent caspase-1-dependent inhibitory effects might explain – at least in part – the cardioprotective effects of HSP70. For example, erythropoietin has been shown to have cardioprotective effects against ischemic or non-ischemic heart disease including Dox-induced cardiotoxicity [29]. Erythropoietin prevents Dox-mediated GATA4 depletion and also increases HSP70 expression which may serve as the first control against GATA4 depletion [30]. The mechanisms by which erythropoietin may exert its cardioprotective role via induction of HSP70 and stabilization of GATA4 would be reminiscent of its mechanism of action in erythropoiesis where it induces HSP70 to protect against caspase-3 cleavage of GATA1 [31].

Other cardioprotective inducers such as exercise, CaMKII and preconditioning also increase HSP70 [26,32–34]. It is therefore tempting to speculate that HSP70 cardioprotection in these instances also involves preventing caspase-1-mediated GATA4 degradation.

Caspase-1 is best known for its role in the NLRP3 inflammasome where it cleaves and processes IL-1β and IL-18 [1]. The involvement of the NLRP3 inflammasome has been documented in several cardiac contexts including acute myocardial infarction, heart failure and myocardial contractile dysfunction due to sepsis [35–37]. Furthermore, Dox has also been shown to induce the NLRP3/caspase-1/IL-1β pathway in the context of macrophages and dendritic cells [38]. However, our findings demonstrate that in cardiac tissue, Dox-induced caspase-1 activation is involved with other non-canonical pathways as well. This is particularly interesting given that transgenic mice overexpressing caspase-1 show an increase in cardiomyocyte cell death without a concomitant increase in IL-1β and IL-18 secretion [4]. This suggests the involvement of a non-inflammatory mechanism such as cleavage of GATA4 and subsequent dysregulation of cardiomyocyte survival pathways.

Few validated caspase-1 substrates are known besides IL-1β and IL-18. Using a proteomic approach, 41 proteins were identified that can be cleaved by caspase-1; they include translation machinery, chaperones and cytoskeletal proteins as well as several enzymes of the glycolysis pathway [18]. No nuclear targets for caspase-1 have yet been identified despite the fact that caspase-1 expression is observed in the nucleus [39]. This is in contrast to caspase-3 which has been reported to cleave several transcription factors including GATA1 in hematopoietic cells and MEF2 in neuronal cells [40,41]. The identification of GATA4 as a nuclear substrate for caspase-1 suggests a direct role for this caspase in transcriptional regulation. Interestingly, sequence analysis reveals that the D230 recognition site is conserved in all 6 members of the GATA family which, in addition

to the heart, play a critical role in immune cells, neurons and the gut. This is noteworthy given the role of caspase-1 in inflammation, neuronal survival and, more recently, in triglyceride metabolism [42,43]. Whether caspase-1 targets additional GATA proteins or other transcription factors in cardiac and extra cardiac tissues will be worth investigating.

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3. Chapter II: Endothelially Expressed GATA4 Regulates Myocardial Angiogenesis via Activation of Angiopoietin-Like 7

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3.1 Statement of the Manuscript

The manuscript "Endothelially Expressed GATA4 Regulates Myocardial Angiogenesis via Activation of Angiopoietin-Like 7" is ready to be submitted (September 28th, 2018)

3.2 Contribution Statement

In this manuscript, JW performed almost all experiments (Figs 1, 2A, C-F, 3, 4, 5B-C, 6, 7, 8, 9B and Supplementary Figs 1-3), analyzed/interpreted results and wrote/edited the manuscript. MX and HW performed initial in vivo experiments and analyzed data, HK interpreted results and edited the manuscript and MN designed experiments, interpreted results and edited the manuscript.

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3.5 Disclosures

None

3.6 Abstract

In response to chronic pressure overload, the left ventricular myocardium hypertrophies to maintain cardiac output despite its increased hemodynamic workload. This adaptive response requires the simultaneous activation of microvascular angiogenesis to prevent ischemia in the remodelling tissue. In cardiomyocytes, pro-hypertrophic, survival and angiogenic pathways are activated by the zinc finger transcription factor GATA4 whose expression increases significantly in the compensating myocardium and is reduced during end-stage heart failure. However, the role of GATA4 in the microvascular endothelium actively undergoing angiogenesis remains unknown. In this work, the role of GATA4 in the cardiac endothelium was investigated in the TC13 mouse endocardial progenitor cell line as well as human cardiac microvascular endothelial cells. There, our results show that GATA4 directly activates endothelial cell proliferation and differentiation in response to pro-angiogenic stimuli. This response is mediated via the activation of the GATA4 target Angiopoietin-Like 7 (ANGPTL7), a secreted extracellular matrix-modifying protein homologous to the Angiopoietin family of vascular growth factors. GATA4, in combination with the immediate early gene Nuclear Orphan Receptor 77 (NUR77), binds to and activates the *angptl7* promoter to increase ECM gene expression required for endothelial cell migration. *In vivo*, stimulation with the vasoconstrictive peptide Angiotensin II lead to increased left ventricular capillary density and ANGPTL7 expression. However, in *Gata4* haploinsufficient mice, this response was impaired. This work has identified a novel pro-angiogenic role for GATA4 in the heart and demonstrates that both endothelial and myocardial GATA4 functions are central to cardiac compensation during pressure overload.

3.7 Introduction

Chronic pressure overload represents a significant healthcare burden in Canada. Its most common cause, hypertension, affects over 1 in 5 Canadians over the age of 20, is the most common ailment for which medication is prescribed and costs the healthcare system billions of dollars annually [1,2]. Other causes including aortic stenosis are also placing increased strain on the medical system due to their increasing prevalence in our aging population [3]. Initially, chronic pressure overload induces an adaptive cardiac response characterized by cardiomyocyte hypertrophy, activation of pro-survival biochemical pathways and re-activation of the fetal gene program [4–8]. These changes, collectively referred to as compensated hypertrophy, allow for the maintenance of cardiac function despite the presence of sustained stress. The induction of compensated hypertrophy hinges on the simultaneous activation of angiogenesis throughout the left ventricular myocardium [5]. This newly formed microvasculature allows for the adequate oxygen delivery, waste removal and metabolite supply required by hypertrophying cardiomyocytes. However, many patients presenting with compensated hypertrophy eventually decompensate into heart failure [9]. This condition characterized by rapidly decreasing left ventricular angiogenesis and capillary density results in cardiomyocyte death, LV dilatation, decreased cardiac output and eventual death [4,5,10]. The pathways underlying cardiac microvascular angiogenesis in the hypertrophying myocardium have so far focused on the role of the secretion of pro-angiogenic chemotactic factors such as VEGF-A from ischemic cardiomyocytes [11–13]. However, the pathways underlying the endothelial cell activation that governs angiogenesis remain poorly understood. Moreover, we do not understand how these mechanisms are impaired leading to the transition from compensation to decompensation. A better understanding of these pathways will allow us to more accurately diagnose and effectively

treat patients to delay or reverse maladaptive cardiac changes and avoid progression into decompensation and heart failure.

GATA4 is a zinc finger transcription factor that is a central regulator of multiple processes critical to the developing and post-natal heart [14]. Expressed in all cardiac cell types, it regulates the expression of a wide range of downstream genes such as the natriuretic factors Atrial and Brain Natriuretic Peptides (ANP and BNP), structural proteins such as Troponins and α and β Myosin Heavy Chain and survival factors such as BCL2 and BCLXL [14–16]. In the developing heart, the importance of GATA4 is best illustrated by point mutations that are associated with congenital heart diseases, the majority of whom cause endocardial defects [17–20]. Furthermore, in the post-natal heart, our lab and others have demonstrated that sustained GATA4 expression is associated with cardiac compensation and maintained capillary density whereas low levels correspond to decreased capillary density, cardiomyocyte death and decompensation [11,12,21–23]. The roles of GATA4 in the heart have been most thoroughly studied in cardiomyocytes where it has been shown to play central roles in proliferation and differentiation during development as well as hypertrophy, activation of pro-survival pathways and secretion of pro-angiogenic chemotactic factors during adulthood [11,12,21,24–28]. These diverse roles are mediated by interactions between GATA4 and specific protein interacting partners that are specified for precise cellular and temporal contexts. The role of GATA4 in the cardiac endothelial cells, however, is much less understood. However, given that GATA4 its known roles in cardiomyocytes via protein-protein interactions, it is likely to mediate its roles in the cardiac endothelial cells in a similar manner. In this paper, we demonstrate that GATA4 expressed in cardiac microvascular endothelial cells is required to mediate angiogenesis during hypertrophy via the interaction with a novel protein partner, the inducible transcription factor NUR77 and

their combined activation of the *angptl7* promoter. The GATA4-NUR77-mediated secretion of ANGPTL7 facilitates angiogenesis via changes to ECM gene expression.

3.8 Methods:

Plasmids

The ANF-pXP2, 3xFlag GATA-pXP2 (cloned from the BNP promoter), rat GATA4-pcgn and rat GST-GATA4-constructs were previously described [29–31]. The pCMV and 3xFlag-rat NUR77-pCMV plasmids were a kind gift from Dr. Jacques Drouin (Institut de Recherches Cliniques de Montréal). To produce the control pLNCX2 plasmid, the 3xFlag sequence was subcloned into the pLNCX2 vector (Clontech, 631503) at the BglII/BamHI sites. A V5His tag was also subcloned between the BamHI and HindIII sites. To produce 3xFlag GATA4 and 3xFlag NUR77 pLNCX2 plasmids, the complete rat GATA4 or NUR77 sequences were subcloned from the GATA4-pcgn and NUR77-pCMV plasmids and subcloned into the BamHI in frame with the 3xFlag and V5-His constructs. The Angptl7 promoter from mouse gDNA between -900 and -1 bp from the transcription start site using the primers sense 5'-CGCGGATCCATGATTTCAGAAGAG-3' and antisense 5'-CGCGGATCCTGAATACCCCACCAA-3' and subcloned into the pXP2 plasmid at the BamHI site. All constructs were confirmed by sequencing.

Cell Culture, Transfections, Transductions and Differentiations

NIH 3T3 were maintained in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin. 24 hours prior to calcium phosphate transfection, cells were plated at a confluency of 50,000 cells/well in 12-well dishes and transfected as previously described [31]. Luciferase assays were completed as previously described [32].

TC13 cells were maintained in DMEM supplemented with 10% horse serum and penicillin/streptomycin as previously described [33]. Differentiation of TC13s was completed by plating 25,000 cells/well in 12 well dishes coated with undiluted Matrigel (Corning) and treated with 5 x 10^{-4} M Retinoic Acid (Sigma, R2625). Cells were shielded from light and monitored over a 72-hour period. To produce the TC13 pLNCX2, 3xFlag GATA4 and 3xFlag NUR77 stable cell lines, the 3xFlag pLNCX2, 3xFlag GATA4 pLNCX2 and 3xFlag NUR77 pLNCX2 plasmids were transfected via calcium phosphate into AD293 cells. 48 hours post-transfection, media containing retroviral particles was collected and added to 80% confluent TC13 cells in the presence of 10 uM hexadimethrine bromide (Sigma, TR-1003-G). 48 hours post-transduction, media was replaced with DMEM supplemented with 10% Horse Serum and 500 ug/ml G418 and was replaced every 48 hours (Sigma G8168). Overexpression of the 3xFlag-tagged construct was confirmed one week post-transduction by QPCR, western blot and immunofluorescence.

Human Cardiac Microvascular Endothelial Cells (Promocell, C-14029) were maintained on 10 cm Primaria culture dishes (Corning, C353803) in Endothelial Cell Growth Medium with MV2 growth supplement (Promocell C-22022) and penicillin/streptomycin. Differentiation into endothelial cell tubes was completed by plating 10,000 cells/well in Primaria 96-well dishes coated with undiluted Matrigel (Corning, C356234). Differentiation was complete 8 hours after plating. Transfection with scramble and Angptl7 (Sigma MISSION siRNA, SIC002, SASI_Hs01_00060514 and SASI_Hs01_00060515) was completed using Lipofectamine 3000 (Thermo-Fisher, L3000015). Transduction of scramble non-mammalian shRNA control and Gata4 shRNA (Sigma, SHC202 and TRCN0000020424) was completed as previously described [34].

Immunoprecipitation and Mass Spectrometry Analysis

To obtain 3xFlag GATA4-coupled complexes, nuclear extracts were obtained from the 3xFlag GATA4 and pLNCX2 TC13 cell lines and stored at -80^{o}C until use. Immunoprecipitations were completed by incubating 25 mg of nuclear extracts in IP Buffer (120 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 8, 0.5% NP-40) with 200 ul of flag or IgG coupled protein A/G magnetic beads (Sigma M8823, Abcam ab18413, Millipore LSKMAGAG10) overnight at 4^{o}C with rotation. Beads were washed 3 times in IP buffer and 10 ul were reserved for verification by western blot. Beads were boiled for 5 minutes in western blot sample buffer (ensuring that it does not contain beta-mercaptoethanol) and separated by SDS-PAGE on a 12% polyacrylamide gel. Gels were silver stained, bands were excised and sent for HPLC-ESI-MS/MS analysis by the Proteomics Resource Centre at the Ottawa Institute of Systems Biology (University of Ottawa).

Microarray

Microarray analysis of the 3xFlag GATA4, 3xFlag NUR77 and respective control cell lines was completed as previously described [34]. Functional analysis was completed using Ingenuity Pathway Analysis (IPA) by Qiagen (www.qiagen.com/ingenuity).

Immunofluorescence and Phosphohistone H3 Quantification

TC13 and HCMEC cells were plated in chambers (Millicell EZ Slide, Millipore PEZGS0416) at a confluency of 25,000 cells/well. 24 hours post-plating, cells were fixed in 1% PFA for 15 minutes and washed 3 times in cold PBS. Immuonfluorescence was completed as previously described [35] with Flag (Sigma F1804) and Phosphohistone-H3 (Millipore 06-570) antibodies.

Angiogenesis Assays

HCMEC cells were differentiated on Matrigel as described in the "Cell Culture, Transfections, Transductions and Differentiations" section. Tube formation was quantified as Total Master Segment Length quantified by the Angiogenesis Analyzer plugin for ImageJ (http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ) [36]. 10 fields were quantified per condition unless otherwise stated.

Co-immunoprecipitation

Co-immunoprecipitations were completed as previously described [37]. Briefly, flag-coupled complexes were immunoprecipitated from nuclear extracts of NIH 3T3 cells transfected with GATA4-pcgn and 3xFlag NUR77-pCMX or relevant controls. Western blots were revealed with anti-GATA4 (homemade antibody) and anti-Flag (Sigma F1804) antibodies [38].

Pull Down Assays

Pull down assays were completed as previously described [31]. Briefly, the 3xFlag NUR77-pCMX plasmid was in vitro translated and labelled with ^{35}S-Met using the TnT Quick Coupled Transcription/Translation System (Promega) and incubated with agarose beads with bound GST alone, GST-GATA4 aa 2-254 or GST-GATA4 aa 271-440. Bound protein was resolved on 12% SDS-PAGE gels, dried and exposed with film.

Western Blot

Western blots were completed as previously described [31]. Antibodies were used at the following concentrations: Anti-Flag (Sigma F1804) 1/1000, anti-NUR77 (Abcam ab153914) 1/1000, anti-ANGPTL7 (LSBio LS-C354136) 1/1000, anti-GAPDH (Abcam ab8245) 1/2000

and anti-Nucleolin (Cell Signaling 14574) 1/2000. Homemade anti-GATA4 antibody was used at a concentration of 1/1000 [38].

Chromatin Immunoprecipitation

ChIP assays were completed as previously described [24,39]. The primers used are sense 5'-TCCTCCCCATCTGTGTCATC-3' and antisense 5'-GGATCCATCACCATCAATAACC-3' for the Chromosome 15 Gene Desert negative control, sense 5'-AGAGGGAATCGAGCCTTCTG-3' and antisense 5'-GGGTTGGTCATCCACACTTC-3' for the distal Angptl7 promoter GATA binding site (-776 to -649 bp from TSS), sense 5'-GAATGATGGCCAGACAAAGC-3' and antisense 5'-TTGGACTGGAGACTGCTGAG-3' for the middle Angptl7 promoter GATA binding site (-477 to -360 bp from TSS) and sense 5'-TCCAGTCCAACTCTTTCTTGC-3' and antisense 5'-GCCACTGGCTCAGCTCTATC-3' for the proximal Angptl7 promoter GATA binding site (-369 to -197 bp from TSS). 3 ug of antibody was used per 25 ug of chromatin. The antibodies used are anti-IgG mouse (Abcam ab18413) and anti-GATA4 (Santa Cruz SC-25310X).

QPCR

Total RNA was isolated from cells using Trizol reagent (Invitrogen). QPCR was completed as previously described [24,40]. Primers are available upon request.

Immunohistochemistry

Immunohistochemistry was completed as previously described [21]. Anti-CD31 (Abcam ab28364) was used at a concentration of 1/150 and anti-ANGPTL7 (LSBio LS-C354136) was used at a concentration of 1/400.

In Vivo Studies

All animal experiments were completed in accordance to University of Ottawa guidelines for animal care and were approved by Institutional Animal Care and Use Committee (IACUC). 120 day old wild type and *Gata4*$^{+/-}$ mice (previously described in Molkentin et al (1997) [41] were treated with 1 ug/kg/min of Angiotensin II or a saline solution for 2 weeks by Alzet microosmotic pump (Model 1002 Durect Corporation, Cupertino, CA). At end points, mice were perfused in 4% paraformaldehyde, embedded and sectioned for immunohistochemistry as previously described [42,43].

3.9 Results:

GATA4 regulates cardiac endothelial cell activation

To begin to assess the requirement for GATA4 in cardiac endothelial cells, a stable GATA4-overexpressing cell line was produced in TC13 cardiac endothelial precursor cells via retroviral transduction of a 3xFlag GATA4-pLNCX2 construct (**Figures 3.1 A-B**). Upon differentiation with Retinoic Acid (RA) on Matrigel-coated plates, the TC13 cell line exhibits an endothelial morphology of elongated cells interconnected to form vascular-like networks (**Supplementary Figure 3.1 A**) [33]. Moreover, they exhibit a gene expression profile indicative of cardiac endothelial cells including increased expression of *Gata5, Nfatc1* and *Tbx20* (**Supplementary Figure 3.1 B**) [33]. Stable overexpression of 3xFlag GATA4 resulted in a 1.67-fold increase in total GATA4 protein, indicating that while the exogenous tagged construct is well expressed, it is not present at such a high level that our data is not physiologically relevant (**Figure 3.1 B**). In response to pro-angiogenic stimuli, endothelial cells undergo a dynamic change in phenotype such that the quiescent cells of an existing vessel become activated and

branch off to form a new capillary [44–46]. One cell, referred to as the tip cell, acquires a migratory phenotype and moves against a gradient of angiogenic factors including Vascular Endothelial Growth Factors (VEGFs) and Angiopoietin 2 (ANGPT2) towards the region of ischemia [44–46]. Neighbouring endothelial cells of the pre-existing vessel (referred to as stalk cells) follow behind the tip cell, dividing and elongating to form the endothelial layer of the developing vessel [44–46]. As such, endothelial cell proliferation and differentiation into vascular networks is critical to the induction of angiogenesis, including that that occurs during compensated myocardial hypertrophy. To determine if GATA4 plays a role in endothelial cell proliferation and differentiation, 3xFlag-GATA4 overexpressing TC13 cells (henceforth referred to as 3xFlag-GATA4-TC13s) and control cells transduced with viruses produced with the empty pLNCX2 construct (pLNCX2-TC13s) were stained with the mitosis-specific marker Phosphohistone H3 (PH3) and were differentiated into vascular networks using RA and Matrigel (**Figures 3.1 C-D**). 3xFlag-GATA4-TC13s showed a 3-fold increase in PH3 staining compared to pLNCX2-TC13 controls (**Figure 3.1 C**). Likewise, upon differentiation with RA and Matrigel, 3xFlag-GATA4-TC13 cells expressed significantly higher levels of the endothelial specific markers Platelet Endothelial Cell Adhesion Molecule (*Pecam1*), Nuclear Factor of Activated T Cells 1 (*Nfatc1*), Von Willebrand Factor (*Vwf*) and Endothelin 1 (*Edn1*, ET-1) (**Figure 3.1 D**). These results suggest that GATA4 could play a role in endothelial cell activation during the onset of angiogenesis.

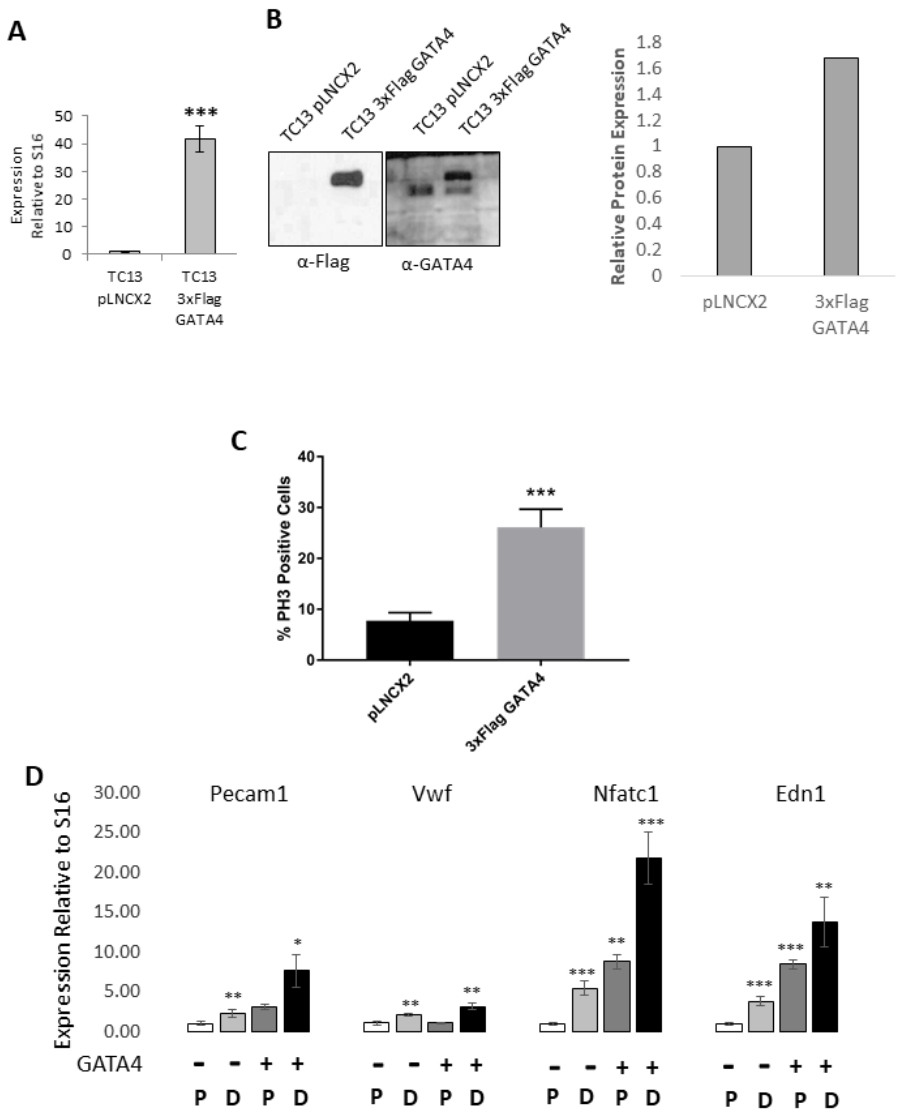

Figure 3.1

Figure 3.1 GATA4 regulates endothelial cell proliferation and differentiation. (**A**) Relative expression of Gata4 in 3xFlag GATA4 TC13 cells compared to control pLNCX2 TC13 cells as measured by qPCR (n=4). The results are reported as the mean ± sem, ***P ≥ 0.001. (**B**) Western blot and quantification of GATA4 and Flag expression in control TC13 pLNCX2 and TC13 3xFlag GATA4 cells. (**C**) Quantification of phosphohistone H3 (PH3) staining in TC13 pLNCX2 and 3xFlag GATA4 expressing stable lines. 3 wells per treatment were quantified, 10 fields per well. The results are reported as the mean ± sem, ***P ≥ 0.0001. (**D**) Relative expression of cardiac endothelial gene markers in proliferating and differentiating 3xFlag GATA4 and control pLNCX2 TC13 cells (n=6). The results are reported as the mean ± sem, *P ≥ 0.05, **P ≥ 0.01, ***P ≥ 0.001.

Although all ECs throughout the body share many structural, phenotypic and functional similarities, different populations diverge in their response to external stimuli and gene expression profiles [47]. These differences are largely attributable to varying expression of transcription factors as they are the major regulators of cell fates. GATA transcription factors are expressed in multiple cell lineages including endothelial cells where they are required for differentiation, homeostasis, blood pressure maintenance, angiogenesis and survival [33,34,48–51]. These roles are specific to distinct populations of endothelial cells and as GATA factors function in a dosage sensitive manner, these roles are likely dependent on their relative expression in each endothelial cell type. In the heart, GATA4 is the most ubiquitously expressed GATA factor and can be found in virtually all cell types including the microvascular endothelial cells [52]. This is the endothelial cell population that undergoes angiogenesis in the myocardium in response to pressure overload. As such, we wished to determine the relative expression levels of GATA4 and the other mammalian GATA factors in different cardiac and non-cardiac endothelial cell populations. qPCR was used to determine the relative quantities of the three cardiac GATA factors in TC13 cells as well as various human primary endothelial cell lines. In TC13s that phenotypically resemble cardiac microvascular endothelial cells, expression of Gata4 is significantly higher than the expression of either Gata5 or Gata6 (**Figure 3.2 A**). This effect was consistent in both mice and humans as Gata4 expression is highest in cardiac microvascular endothelial cells compared to other populations of endothelial cells (**Figure 3.2 B**). Collectively, this suggests that GATA4 plays a role specific to this population. This data also suggests that other Gata factors play endothelial cell population-specific roles as their expression also varied among human endothelial cell lines (**Figure 3.2 B**). To further examine the role of GATA4 in these specific endothelial cells, lentiviral-mediated shRNA delivery was used to knock down its

expression (**Figure 3.2 C**). Gata4 transcript levels were reduced by 45%, thereby producing a visible phenotype compared to control cells transduced with scramble shRNA but avoiding the likely loss of viability that would result from a greater level of knockdown. Gata4 shRNA treated HCMECs exhibited reduced PH3 staining compared to controls, suggesting that the loss of Gata4 directly inhibits proliferation (**Figure 3.2 D**). As well, as HCMECs differentiate to form vascular-like networks when plated on Matrigel in the same manner as the TC13 cell line, we determined whether the loss of GATA4 would affect this process. Cells transduced with Gata4 shRNA containing lentivirus exhibited produced poorly developed vascular-like networks compared to controls, as quantified using the program Angiogenesis Analyzer available for ImageJ (http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ) (**Figure 3.2 E-F**). As such, cardiac endothelial cell proliferation and differentiation appear to be governed in part by endothelially-expressed GATA4.

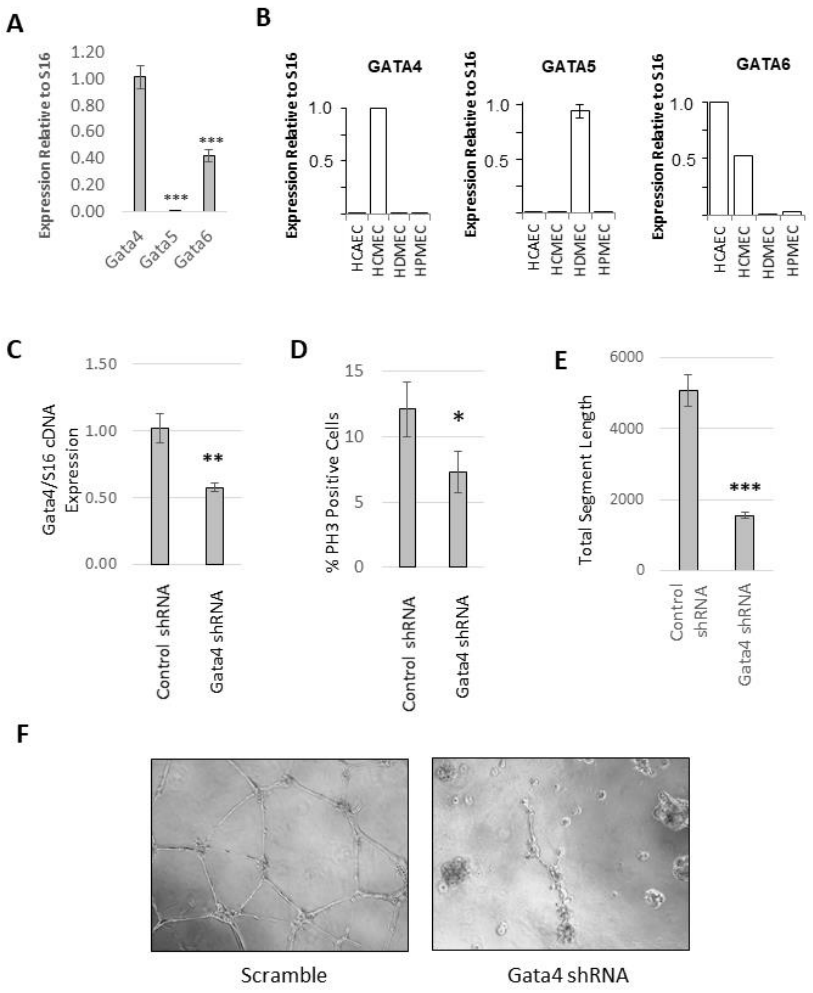

Figure 3.2

Figure 3.2 GATA4 is selectively expressed in cardiac microvascular endothelial cells to regulate proliferation and differentiation. (**A**) Relative expression of Gata4, Gata5 and Gata6 in TC13 cells as measured by qPCR (n=4). The results are reported as the mean ± sem, ***P ≥ 0.001. (**B**) Relative Expression of Gata4, 5 and 6 in Human Carotid Artery Endothelial Cells (HCAECs), Human Cardiac Microvascular Endothelial Cells (HCMECs), Human Dermal Microvascular Endothelial Cells (HDMECs) and Human Pulmonary Microvascular Endothelial Cells (HPMECs) as measured by qPCR. (**C**) Relative qPCR expression of Gata4 compared to S16 in HCMEC stable lines expressing scramble (control) or Gata4 shRNA (n=4 per condition). The results are reported as the mean ± sem, **P ≤ 0.01. (**D**) Quantification of phosphohistone H3 (PH3) staining in HCMEC stable lines expressing scramble (control) or Gata4 shRNA. 4 wells per treatment were quantified, 6 fields per well. The results are reported as the mean ± sem, *P ≥ 0.05. (**E**) Quantification of total master segment length of tubes formed by HCMEC stable lines expressing scramble (control) or Gata4 shRNA (n=10 per condition). The results are reported as the mean ± sem, ***P ≥ 0.001. (**F**) Representative images of tubes formed by HCMEC stable lines expressing scramble (control) or Gata4 shRNA.

GATA4 mediates cardiac endothelial cell activation via transactivation of the secreted protein ANGPTL7

To identify potential downstream gene targets of GATA4 required to activate cardiac endothelial cell proliferation and differentiation, transcriptomic analysis of the TC13 pLNCX2 and TC13 3xFlag GATA4 cell lines was completed using the Affymetrix GeneChip Mouse Gene 2.0 ST Array (**Figure 3.3 A**). Transcriptomic analysis identified 1219 genes (687 increased and 532 decreased, Log2 fold change ≥0.6 or ≤-0.6 and P≤0.05, Mann–Whitney test with P value adjusted—Benjamini and Hochberg—for multiple comparisons) with altered expression between the TC13 pLNCX2 and TC13 3xFlag GATA4 cell lines (**Figure 3.3 B, Supplementary Table 3.1**). Functional analysis completed by the Ingenuity Pathway Analysis (IPA) Software (QIAGEN) identified multiple pathways enriched by GATA4 overexpression that are central to cardiac endothelial function including "Angiogenesis", "Vasculogenesis", "Movement of Endothelial Cells" and "Proliferation of Endothelial Cells" ($P=8.44 \times 10^{-20}$, 7.75×10^{-16}, 3.74×10^{-11} and 1.48×10^{-9} respectively, Fisher's Exact Test) (**Supplementary Table 3.1**). Enrichment of several genes in these pathways, several of whom were not previously known to be targets of GATA4, was confirmed by qPCR and was consistent with our microarray results (**Figure 3.3 C**). Of these genes, a select few demonstrated extremely robust enrichment including Angiopoietin-Like 7, (ANGPTL7, 63.42-fold enrichment compared to control), a secreted protein named for its homology to the Angiopoietin family of pro-angiogenic chemotactic factors (**Figure 3.3 C**). Although little is known about ANGPTL7, it has been shown to be specifically upregulated in response to hypoxia where it promotes endothelial cell proliferation, migration, tube formation and invasiveness in a colorectal cancer model [53]. It has also been shown to be a regulator of ECM component genes known to be important during angiogenesis in a mouse model of

glaucoma [54]. In cardiac endothelial cells, expression of ANGPTL7 is highly correlated to GATA4 expression levels as it is significantly upregulated in response to overexpression in TC13s and reduced by half in response to Gata4 shRNA knockdown in HCMECs (**Figures 3.3 D-E**). As such, we wished to explore the possibility that ANGPTL7 is a downstream target of GATA4 in cardiac endothelial cells whose expression is required for cardiac microvascular endothelial cell activation during angiogenesis.

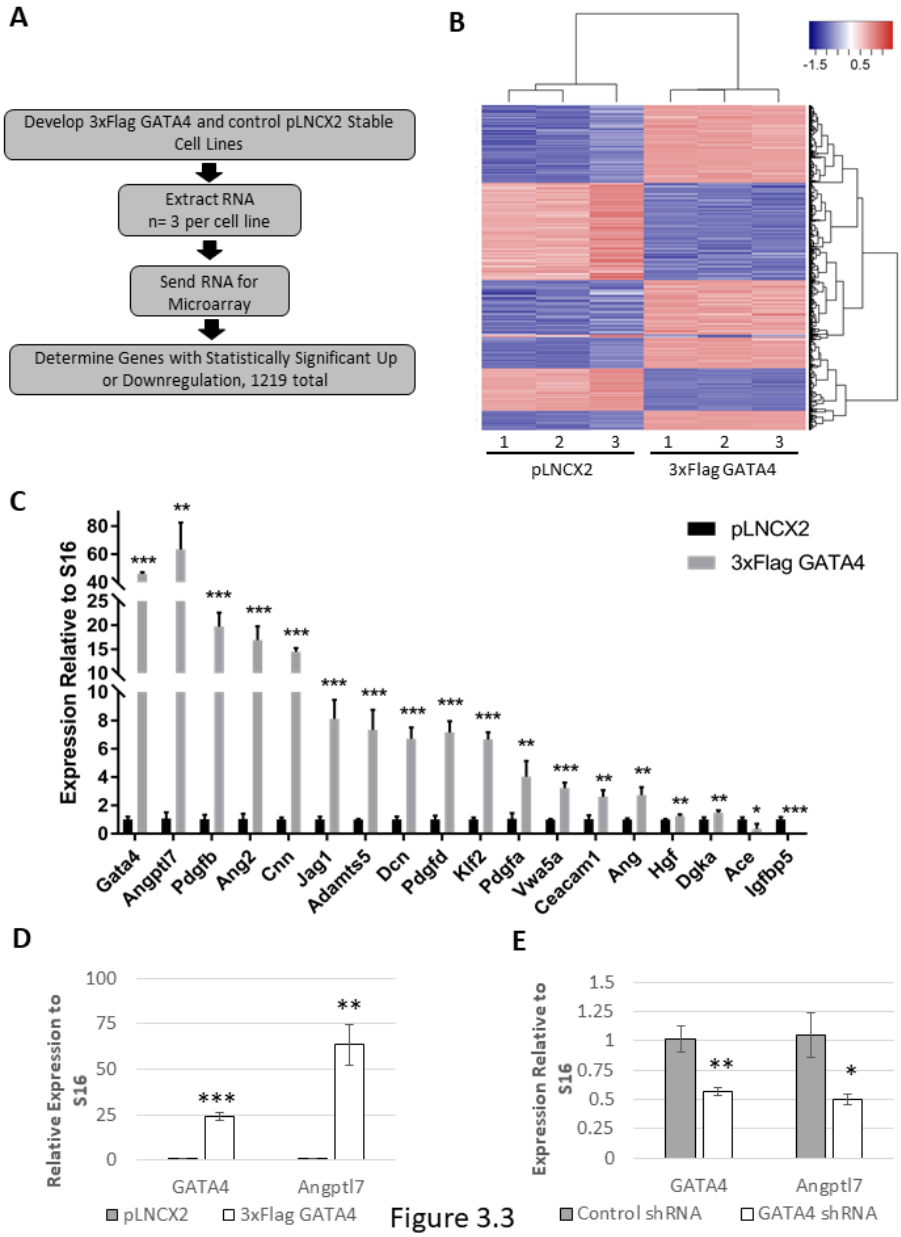

Figure 3.3

Figure 3.3 GATA4 regulates multiple pathways in cardiac endothelial cells. (**A**) Schematic of microarray analysis workflow used to determine differential gene expression in control pLNCX2 and 3xFlag GATA4 TC13 cell lines. (**B**) Heatmap representing the differentially regulated genes between the TC13 pLNCX2 and TC13 3xFlag GATA4 cell lines. Colour is representative of the Log2 RMA (Affimetrix Mouse ST 2.0 Microarray, n=3 per group). (**C**) qPCR validation of selected genes showing differential expression in TC13 pLNCX2 vs. TC13 3xFlag GATA4 cell lines as observed by microarray. The results are reported as the mean ± sem of 3 replicates, *P ≥ 0.05, **P ≥ 0.01, ***P ≥ 0.001. (**D**) Relative expression of Gata4 and Angptl7 in 3xFlag GATA4 TC13 cells compared to control pLNCX2 TC13 cells as measured by qPCR (n=3). The results are reported as the mean ± sem, **P ≥ 0.01, ***P ≥ 0.001. (**E**) Relative qPCR expression of Gata4 and Angptl7 compared to S16 in HCMEC stable lines expressing scramble (control) or Gata4 shRNA (n=4 per condition). The results are reported as the mean ± sem, *P ≤ 0.05.

If ANGPTL7 is a downstream effector of GATA4 in cardiac microvascular endothelial cells, the knockdown of either protein should yield a similar phenotype in cardiac microvascular endothelial cells. As the reduction of Gata4 expression had resulted in a decrease in both proliferation and tube formation in cardiac endothelial cells, we wished to determine whether a reduction in Angptl7 expression would do the same. Transfection of two separate Angptl7-targeting siRNAs (as well as a scrambled negative control) into the HCMEC cell line resulted in a reduction in Angptl7 expression by 40% and 82% respectively, as assessed by western blot (**Figure 3.4 A**). Proliferation and differentiation were then assessed by PH3 staining and Matrigel tube formation assays (**Figure 3.4 B-D**). Angptl7 knockdown resulted in a dose-dependent decrease in PH3 staining indicative of a decrease in proliferation directly associated with Angptl7 expression (**Figure 3.4 B**). Likewise, Angptl7 knockdown repressed tube formation in a dose dependent manner as well (**Figure 3.4 C-D**). As the phenotype of HCMECs treated with Angptl7 siRNA mirrored that of the cells treated with Gata4 shRNA, our results suggest that GATA4 mediates its pro-proliferative and differentiation effects via activation of ANGPTL7. As such, we wished to determine whether the *angptl7* promoter is a direct target of GATA4. Bioinformatics analysis of the *angptl7* promoter by Genomatix (genomatix.de) located 3 conserved GATA motifs within the first 1000 base pairs from the transcription start site

labelled the distal, middle and proximal sites (**Figure 3.4 E, yellow circles**). Chromatin immunoprecipitation of endogenous GATA4 in the TC13 cell line demonstrated that GATA4 can bind to both the distal and proximal GATA motifs, demonstrating that it is a direct target of GATA4 in cardiac endothelial cells (**Figure 3.4 F**).

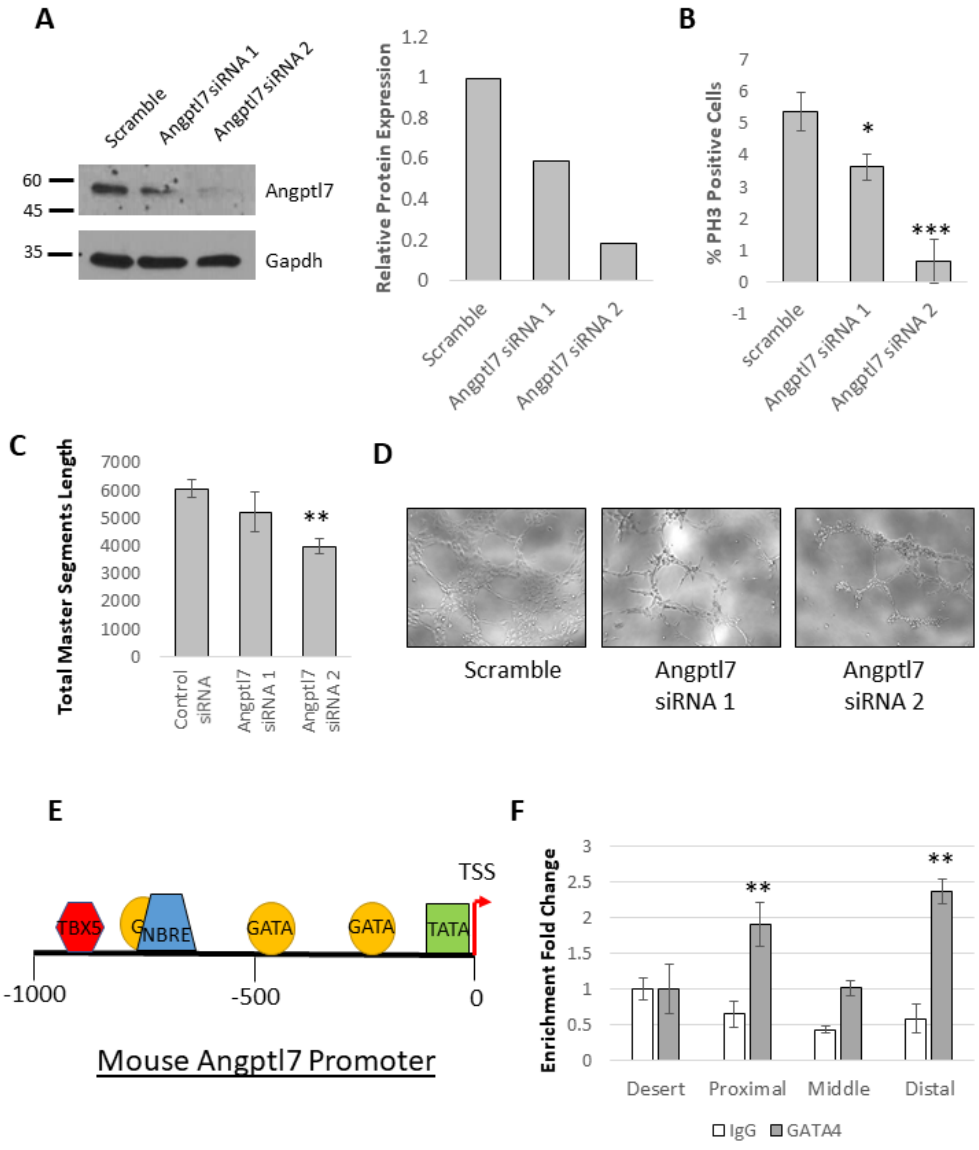

Figure 3.4

Figure 3.4 GATA4 controls expression of the pro-angiogenic factor ANGPTL7. (**A**) Western blot representation and quantification of Angptl7 and Gapdh expression in HCMECs transfected with two separate Angptl7-targetting siRNAs (Angptl7 siRNA 1 and Angptl7 siRNA 2). (**B**) Quantification of phosphohistone H3 (PH3) staining in HCMECs transfected with scramble (control) or Angptl7 siRNA. 4 wells per treatment were quantified, 10 fields per well. The results are reported as the mean ± sem, *P ≥ 0.05. (**C**) Quantification of total master segment length of tubes formed by HCMECs transfected with scramble (control) or Angptl7 siRNA (n=20 per condition). The results are reported as the mean ± sem, **P ≥ 0.01. (**D**) Representative images of tubes formed by HCMEC stable lines expressing scramble (control), or one of two Angptl7 shRNAs. (**E**) Schematic of the 1000 base pairs proximal to the mouse Angptl7 transcription start site with GATA and TBX5 binding sites as well as the TATA box and transcription start site (TSS). (**F**) Chromatin immunoprecipitation (ChIP) of GATA4 occupancy on the distal GATA binding site on the Angptl7 promoter. A chromosome 15 gene desert was used as a negative gene and an IgG antibody as an immunoprecipitation negative control. The results are reported as the mean ± sem, **P ≥ 0.01 compared to IgG.

NUR77 is a pro-angiogenic GATA4 interacting partner in cardiac endothelial cells

As GATA4 mediates the majority of its functions in cardiac cells via interactions with protein partners, we sought to determine whether other transcription factor binding sites lie near the distal or proximal GATA binding motifs. One such site was a NUR Binding Element (NBRE) that is recognized by the 3 members of the NUR family of zinc finger transcription factors (**Figure 3.4 E, Blue Trapezoid**). Of the three proteins, Nuclear Receptor Subfamily 4 Group A1, 2 and 3 (NR4A1, 2 and 3), NR4A1, otherwise known as Nuclear Orphan Receptor 77 (NUR77) is the most strongly expressed in the left ventricle (GTEx Portal, data not shown). Moreover, NUR77 plays an important role in the induction of angiogenesis in endothelial cells as it is a direct downstream effector of Vascular Endothelial Growth Factor A (VEGF-A) [55]. To determine whether NUR77 can complex with GATA4, co-immunoprecipitation and pull-down assays were performed and indicated that the two proteins can interact directly with one another via the second zinc finger and C-terminus of GATA4 (**Figures 3.5 A-B**). To determine whether NUR77 can modulate GATA4-mediated activation of target promoters, both sequences were cotransfected into NIH3T3 cells along with a reporter construct composed of either a synthetic 3xGATA or Atrial Natriuretic Factor (ANF) promoter coupled to a luciferase reporter (**Figure**

3.5 C). NUR77 strongly potentiated GATA4-mediated activation of both promoters, demonstrating that these two proteins can interact together to transactivate gene targets.

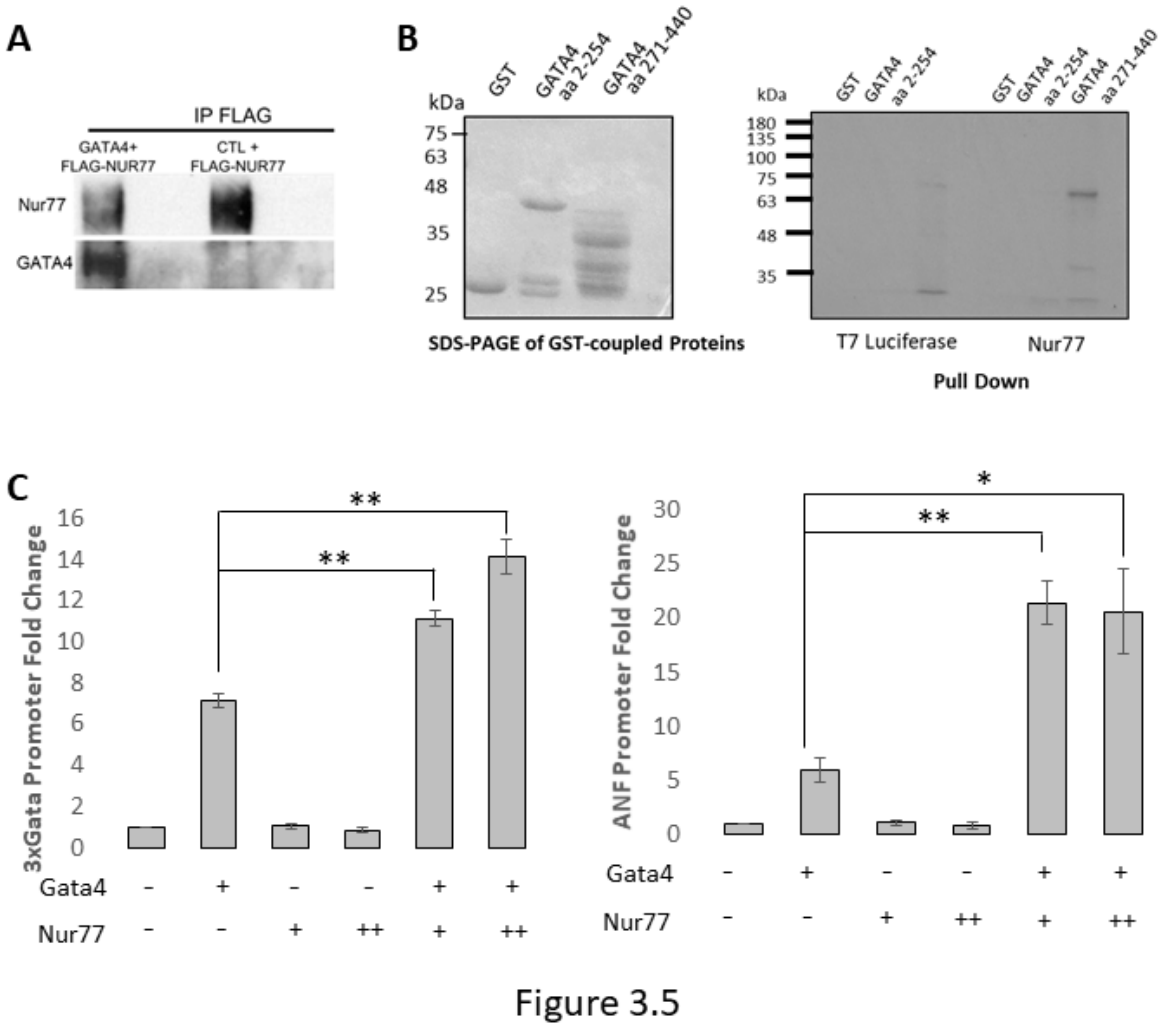

Figure 3.5

Figure 3.5 GATA4 complexes with the inducible factor NUR77 to transactivate target promoters. (**A**) GATA4 and Flag-NUR77 were cotransfected into AD293. Flag complexes were immunoprecipitated from nuclear extracts. Western blot indicates that GATA4 and NUR77 complex together. (**B**) Pull down of radiolabelled NUR77 with GST alone (negative control) or GST-coupled N-terminal (aa 2-254) or C-terminal (aa 271-440) GATA4. Left panel: SDS-PAGE of 5 ug of GST proteins used for pull down assay. Right panel: pull down assay. (**C**) Measure of GATA4 and NUR77 transcriptional activation of the Atrial Natriuretic Factor (ANF) and 3xGATA promoters as measured by luciferase assay. The results are reported as the mean ± sem, *p ≥ 0.05, **P ≥ 0.01.

As NUR77 is an interacting partner of GATA4 that may also bind to the *angptl7* promoter, we sought to determine whether it plays an important role in cardiac endothelial cells. As we had done to study the role of GATA4 in cardiac endothelial cells, we produced a stable NUR77-overexpressing cell line via retroviral transduction of a 3xFlag NUR77-pLNCX2 construct into the TC13 cell line (**Figure 3.6 A-B**). Stable overexpression of 3xFlag NUR77 resulted in a 1.6-fold increase in total NUR77 protein (**Figure 3.6 B**). As with the 3xFlag GATA4 cell line, this level of overexpression is observable but is not very far above physiological levels and as such, remains relevant to the endogenous mechanisms taking place in these cells (**Figure 3.6 B**). Upon production of this cell line, an immediate and easily observable increase in cell proliferation was noticed in the 3xFlag NUR77 TC13s compared to control pLNCX2-TC13s. Quantification of mitotic cells by fluorescent PH3 staining resulted in a 2-fold increase in PH3 positive cells compared to pLNCX2-TC13s (**Figure 3.6 C**). As well, upon differentiation of these cell lines on Matrigel-coated plates to produce vascular-like EC tubes, the 3xFlag NUR77 cells expressed higher levels of endothelial cell markers including *Vwf*, *Nfatc1* and *Edn1* (**Figure 3.6 D**). This phenotype produced by overexpression of 3xFlag NUR77 and 3xFlag GATA4 was strikingly similar and suggested that both proteins play complimentary roles in cardiac endothelial cells, potentially by activating the same pathways regulating EC activation during angiogenesis.

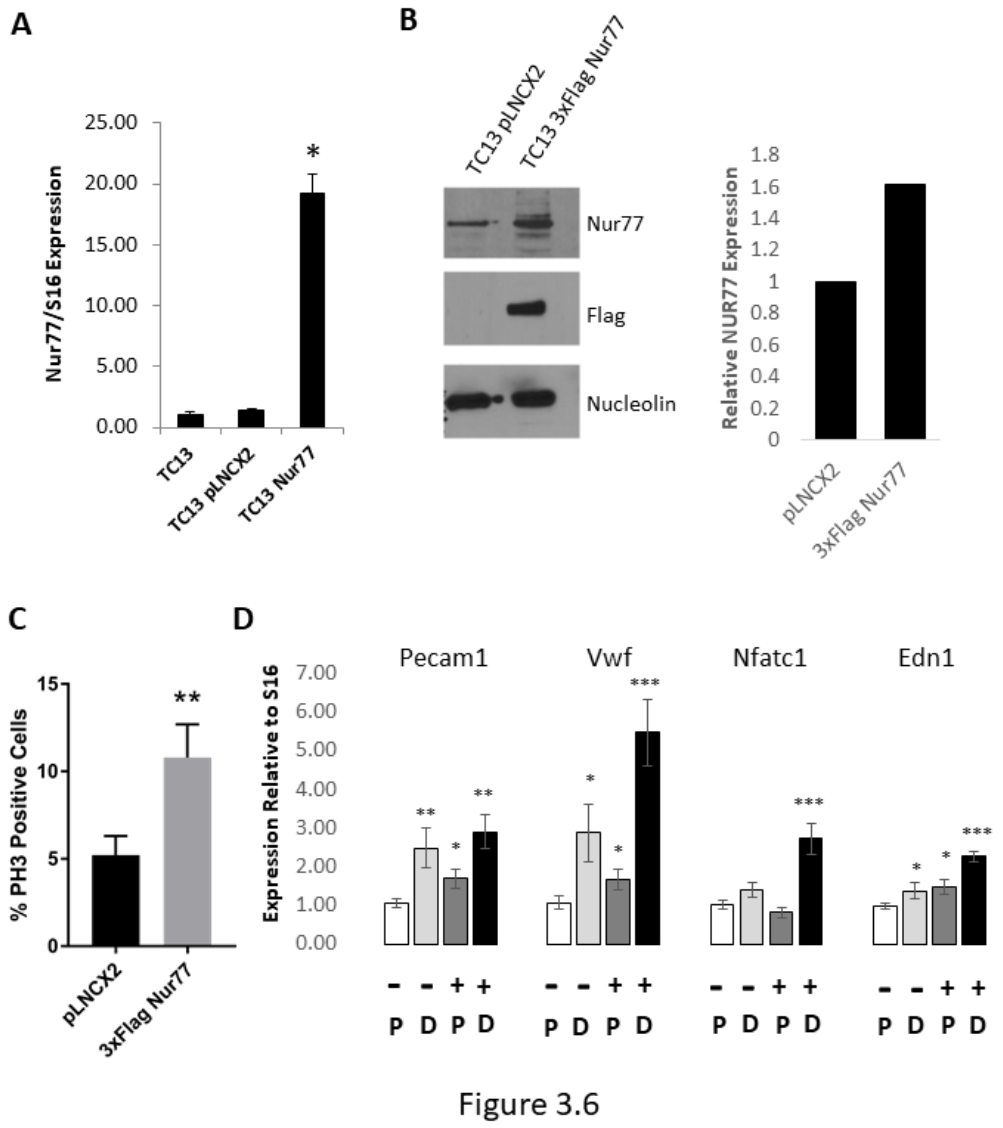

Figure 3.6

Figure 3.6 NUR77 regulates endothelial cell proliferation and differentiation. (**A**) qPCR of the relative expression of NUR77 compared to S16 in the 3xFlag NUR77 TC13 stable line and control pLNCX2 cell line. (**B**) Western blot image and quantification showing the 3xFlag Nur77 expression in the 3xFlag NUR77 TC13 cell line. (**C**) Quantification of phosphohistone H3 (PH3) staining in TC13 pLNCX2 and 3xFlag GATA4 expressing stable lines. 3 wells per treatment were quantified, 10 fields per well. The results are reported as the mean ± sem, **P ≥ 0.01. (**D**) qPCR of the relative expression of cardiac endothelial gene markers in proliferating and differentiating 3xFlag NUR77 and control pLNCX2 cells. The results are reported as the mean ± sem *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

NUR77 and GATA4 cooperatively activate ANGPTL7 expression

As the phenotypes of TC13 cell overexpressing 3xFlag GATA4 and 3xFlag NUR77 were so similar, we wished to determine whether NUR77 overexpression alters the expression of a similar set of gene targets. As such, transcriptomic analysis of the TC13 pLNCX2 and TC13 3xFlag NUR77 cell lines was completed using the Affymetrix GeneChip Mouse Gene 2.0 ST Array (**Figure 3.7 A**). Transcriptomic analysis identified 73 genes (62 increased and 11 decreased, Log2 fold change $\geq$0.6 or $\leq$-0.6 and P$\leq$0.05, Mann–Whitney test with P value adjusted—Benjamini and Hochberg—for multiple comparisons) with altered expression between the TC13 pLNCX2 and TC13 3xFlag NUR77 cell lines (**Figure 3.7 B, Supplementary Table 3.2**). Functional analysis completed by the Ingenuity Pathway Analysis (IPA) Software (QIAGEN) identified pathways enriched by NUR77 overexpression that are central to cardiac endothelial function including "Vasculogenesis", "Movement of Endothelial Cells" and "Interaction of Endothelial Cells" ($P=6.99 \times 10^{-5}$, 5.52×10^{-4} and 1.99×10^{-3} respectively, Fisher's Exact Test) (**Supplementary Table 3.2**). Interestingly, many similar pathways were also significantly modified by 3xFlag GATA4 expression (**Supplementary Table 3.1**). Likewise, of the 73 genes whose expression was modified by 3xFlag NUR77 overexpression, 54 were also modified by 3xFlag GATA4 overexpression, suggesting that both proteins converge on the same genes involved with EC function and angiogenesis (**Supplementary Table 3.2**). Enrichment of several of these commonly regulated genes were confirmed by qPCR (**Figure 3.7 C**). As with 3xFlag GATA4 overexpression, the gene showing the strongest enrichment in response to 3xFlag NUR77 overexpression was ANGPTL7 (12-fold increase compared to control pLNCX2 TC13 cells) (**Figure 3.7 C**). Together, this data suggests that GATA4 and

NUR77 can cooperatively regulate ANGPTL7 expression in cardiac endothelial cells and this interaction may contribute to cardiac endothelial cell activation during angiogenesis.

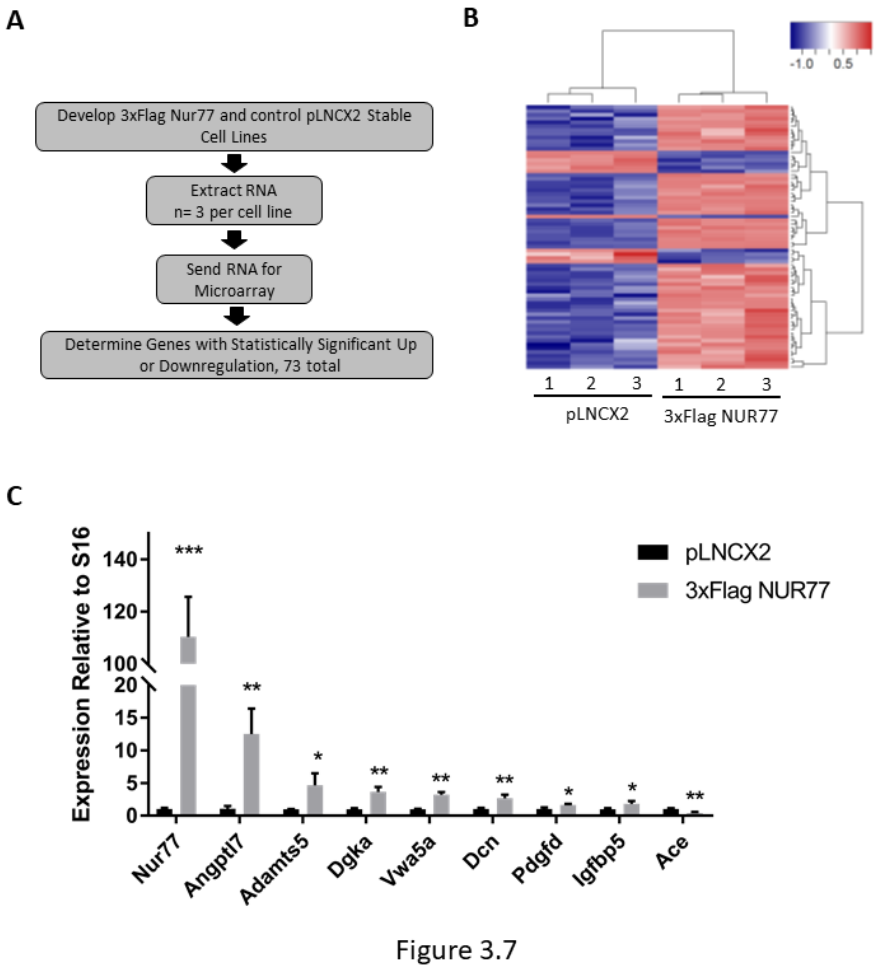

Figure 3.7

Figure 3.7 NUR77 regulates expression of several cardiac endothelial genes including Angptl7. (**A**) Schematic of microarray analysis workflow used to determine differential gene expression in control pLNCX2 and 3xFlag NUR77 TC13 cell lines. (**B**) Heatmap representing the differentially regulated genes between the TC13 pLNCX2 and TC13 3xFlag NUR77 cell lines. Colour is representative of the Log2 RMA (Affimetrix Mouse ST 2.0 Microarray, n=3 per group). (**C**) qPCR validation of selected genes showing differential expression in TC13 pLNCX2 vs. TC13 3xFlag NUR77 cell lines as observed by microarray. The results are reported as the mean ± sem of 3 replicates, *P ≥ 0.05, **P ≥ 0.01, ***P ≥ 0.001. Note that Angptl7 expression shows the strongest level of dysregulation upon NUR77 overexpression.

To determine if this is indeed the case, luciferase assays were used to determine whether both proteins, both alone or in tandem, can activate Angptl7 promoter (**Figure 3.8 A**). As the

binding sites for GATA4 and NUR77 are located within 900 base pairs of the transcription start site of Angptl7, this region was cloned upstream of the luciferase reporter gene of the pXP2 plasmid. GATA4 was able to activate this promoter in a dose dependent manner but NUR77 by itself was insufficient to do so (**Figure 3.8 A**). However, when co-transfected together, NUR77 doubles GATA4-mediated promoter activation, suggesting that this complex is able to activate the ANGPTL7 promoter and that this interaction requires GATA4 recruitment of NUR77 (**Figure 3.8 B**). As we had now established that GATA4 and NUR77 activate expression of ANGPTL7, we wished to determine what occurs downstream of this interaction. Although very little is known about the function of ANGPTL7 in endothelial cells, in primary human trabecular meshwork cells of the eye, it mediates the expression of extracellular matrix (ECM) proteins including versicans, collagens and matrix metalloproteases [54]. These proteins play central roles in endothelial cell activation during angiogenesis as they must be extensively remodelled to promote proliferation and migration of the ECs in the direction of hypoxia. In HCMECs treated with Gata4 shRNA, Angptl7 expression is reduced by half (**Figure 3.3 E**). In this model, qPCR analysis demonstrated that the ECM genes known to be downstream of ANGPTL7 are dysregulated including Matrix Metalloprotease 1 (*Mmp1*), Versican (*Vcan*) and Collagen 4a1 (*Col4a1*), suggesting that these same proteins modulated by ANGPTL7 in the eye may also be regulated by this protein in cardiac endothelial cells as well (**Figure 3.8 C**). Likewise, we also sought to determine what events occur upstream of GATA4-NUR77 mediated activation of Angptl7 and the induction of angiogenesis in cardiac endothelial cells. Endothelial cells of the vasculature remain quiescent in the absence of pro-angiogenic stimuli. However, in the presence of chemotactic factors including VEGFs, ANGPTs and growth factors, they are activated and proliferate and migrate in the direction of hypoxia [44–46]. GATA4 expression in HCMECs remains

stable regardless of whether the cells are differentiating into vascular networks or not. However, as NUR77 is an inducible protein whose expression is generally triggered by a specific stimulus, we wished to determine if the induction of angiogenesis coincides with the induction of NUR77 expression (**Figure 3.8 D**). As pro-angiogenic chemotactic factors including VEGF-A, ANGPT2 and other growth factors are contained in the serum of HCMECs, varying levels of serum exposure can be used to control pro-angiogenic stimulation. Western blot of HCMECs exposed to half or full serum conditions shows that cells exposed to a full serum environment express 5 times more NUR77 than those exposed to a reduced serum environment, suggesting that upon binding of pro-angiogenic ligands on the surface of endothelial cells, NUR77 is induced and is able to interact with GATA4. This complex would then be available to activate the Angptl7 promoter (**Figure 3.8 D**).

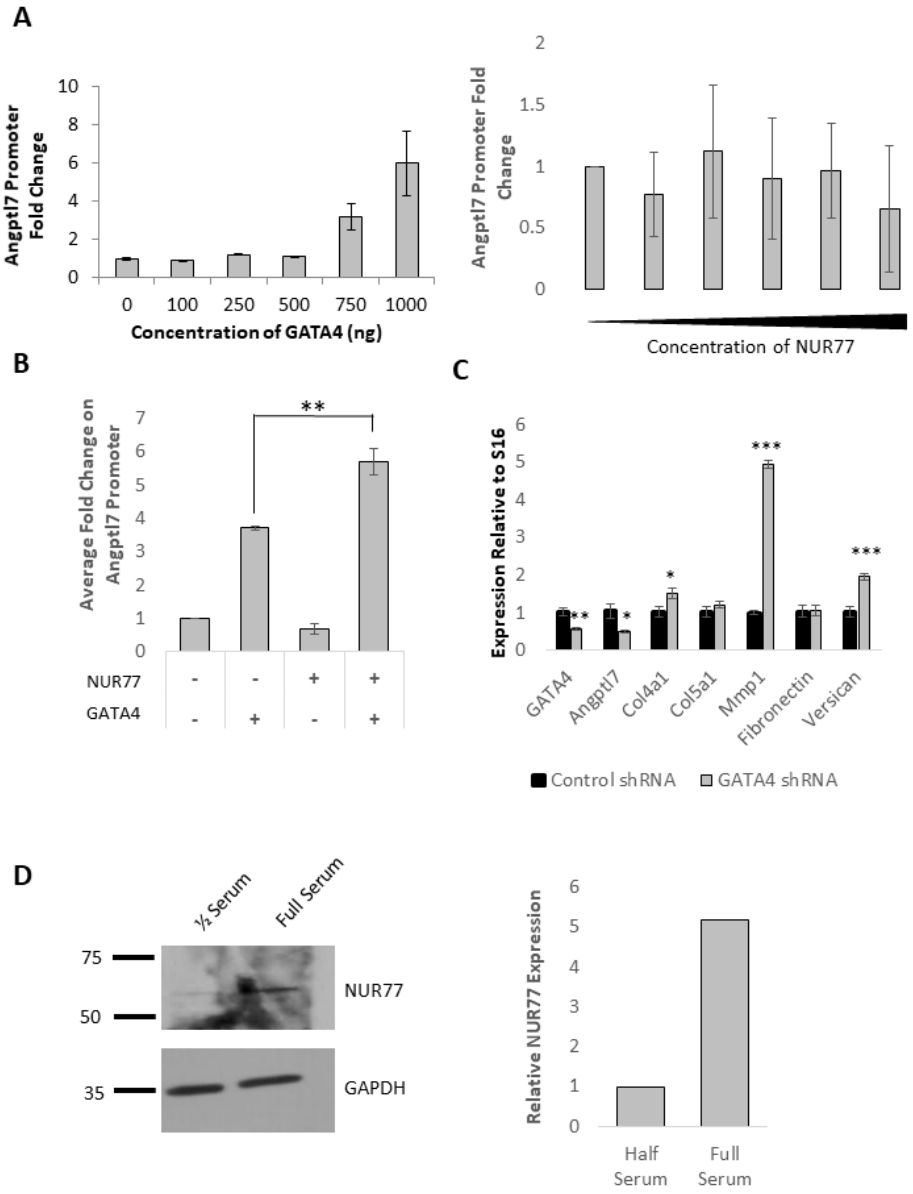

Figure 3.8

Figure 3.8 The GATA4-NUR77 complex activates ANGPTL7 expression in response to pro-angiogenic stimuli. (**A**) Luciferase activity of NIH 3T3 cells transiently transfected with the Angptl7-luc pXP2 plasmid as well as increasing doses of the Gata4-pcgn (left panel) or Nur77-pCMX (right panel) plasmid (0, 100, 250, 500, 750 and 1000 ng, respectively). Representative image of 4 replicates shown. (**B**) Measure of combined GATA4 and NUR77 transcriptional activation on the Angptl7 promoter as measured by luciferase assay. Graphs are representative images of 3 replicates. (**C**) Expression of extracellular matrix genes relative to S16 in HCMEC stable lines expressing scramble (control) or Gata4 shRNA. The results are reported as the mean ± sem, *P ≥ 0.05, ***P ≥ 0.001. (**D**) Western blot images and quantification of NUR77 and GAPDH in HCMECs exposed for 48 hours to half or full serum pro-angiogenic media TC13 pLNCX2 and TC13 3xFlag GATA4 cells.

ANGPTL7 regulates angiogenesis in response to pressure overload in vivo in a GATA4-dependent manner

As we had determined that activation of ANGPTL7 by the GATA4-NUR77 complex is central to endothelial cell activation in cell culture, we now wished to determine if this mechanism occurs in cardiac microvascular endothelial cells *in vivo* in response to pressure overload. To do so, we used a well-established model of treatment with Angiotensin II (AngII), a peptide hormone that binds to the Angiotensin II Type I Receptor (AT1R) on the surface of both cardiomyocytes and endothelial cells to induce myocardial hypertrophy in response to pressure overload. Treatment of wild type C57B6 mice with 1 ug/kg/min of AngII for 14 days led to a significant increase in myocardial hypertrophy observable by trichrome staining (**Figure 3.9 A**). However, in Gata4 heterozygote mice that have an impaired hypertrophy response when exposed to pressure overload, the hearts increase significantly in size and the left ventricle dilates leading to reduced cardiac function and higher mortality (**Figure 3.9 A**) [21,23]. Immunohistological staining of cardiac serial sections taken from these mice demonstrated that in response to AngII treatment, wild type mice show a considerable induction of CD31 staining throughout the LV myocardium in a salt and pepper distribution, demonstrating that the microvasculature of the heart is actively undergoing angiogenesis to prevent ischemia of the newly hypertrophied tissue (**Figure 3.9 B, Left Panel**). However, the Gata4 haploinsufficient mice have a considerably lower degree of CD31 staining indicating that the angiogenic response in the heart after AngII exposure is impaired (**Figure 3.9 B, Left Panel**). The same pattern of expression was observed with ANGPTL7 expression (**Figure 3.9 B, Right Panel**). Although not expressed in larger vessels throughout the heart that do not remodel in response to AngII exposure, it is well expressed in the same salt and pepper distribution throughout the myocardium of wild type mice

treated with AngII, suggesting that it is being expressed in the remodeling microvasculature of the LV. However, as with the CD31 staining, ANGPTL7 expression is markedly reduced in the Gata4$^{+/-}$ mice, demonstrating that when GATA4 expression is reduced *in vivo*, both ANGPTL7 secretion and angiogenesis are impaired (**Figure 3.9 B, Right Panel**). As such, we conclude that angiogenesis in the cardiac microvasculature hinges on GATA4-mediated induction of ANGPTL7 and that when GATA4 expression is reduced, an ANGPTL7-mediated angiogenic response cannot be mounted upon exposure to pressure-overload.

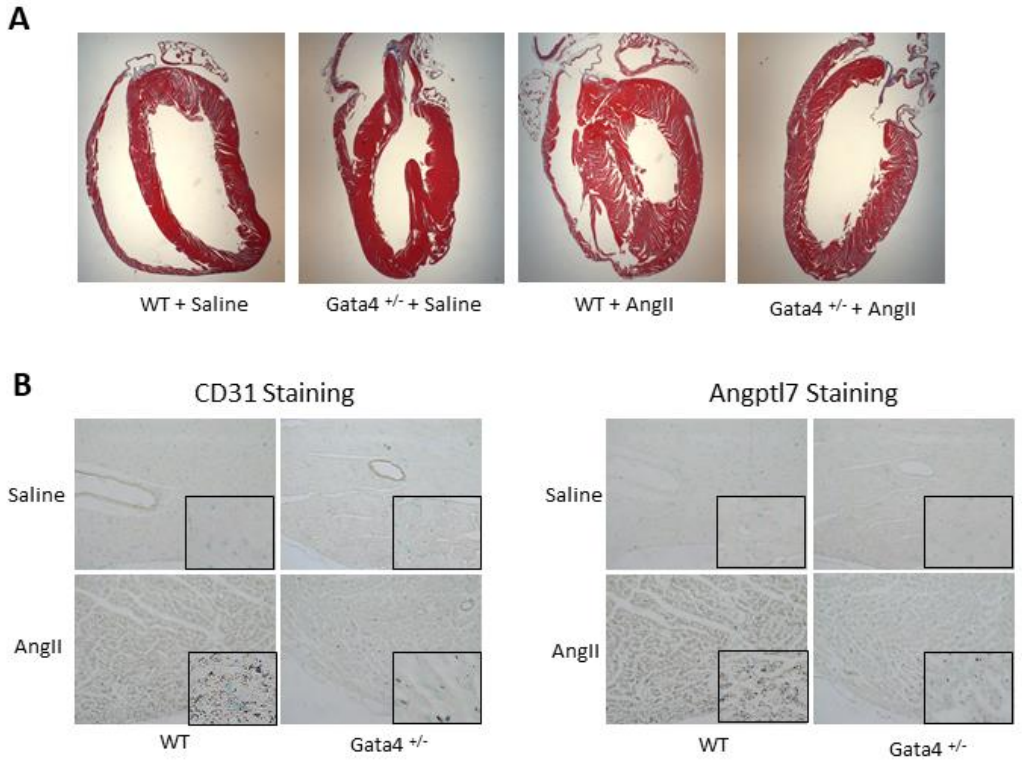

Figure 3.9

Figure 3.9 GATA4 haploinsufficiency impairs left ventricular ANGPTL7 expression and angiogenesis in response to Angiotensin II stimulation. (**A**) Masson's Trichrome staining of frontal whole heart sections from adult wild type (WT) and Gata4$^{+/-}$ mice treated with saline or Angiotensin II (AngII). (**B**) 40x imaging of CD31 (left panel) and Angptl7 (right panel) staining of left ventricular sections of adult wild type (WT) and Gata4$^{+/-}$ mice treated with saline or Angiotensin II (AngII), n=4 per condition.

3.10 Discussion

In this report, we are the first to our knowledge to identify a cardiac endothelial cell-specific mechanism required for the induction of angiogenesis in the myocardium. The central cardiac regulator GATA4 interacts directly with inducible transcription factor NUR77, whose expression is prompted by pro-angiogenic stimuli. Together, they transactivate the promoter of the secreted protein ANGPTL7, leading to the endothelial cell proliferation and tube formation required to form new capillaries in ischemic regions of the myocardium. This pathway is central to the increase in myocardial capillary density observed upon pressure overload-induced hypertrophy, as observed in wild type mice treated with the vasoconstrictive peptide hormone AngII. Previously published work by our group and others has demonstrated that Gata4 heterozygote mice are unable to mount an effective compensatory response when exposed to various pro-hypertrophic stimuli [12,21–23]. Now, we have now shown that these mice are also unable to effectively transactivate the expression of ANGPTL7 needed to maintain myocardial capillary density leading to worsening cardiac outcomes. These results imply the interesting possibility that the inhibition of the GATA4-ANGPTL7-angiogenesis pathway may be a hallmark of the switch between compensated hypertrophy to decompensation. It has been previously established that GATA4 levels are significantly increased both in culture and *in vivo* by a multitude of pro-hypertrophic stimuli ranging from trans-aortic constriction to treatment with various neurohumoral stimuli [21–23]. This increase can be correlated with an increase in myocardial capillary density [11,12]. Conversely, it has also been established that both GATA4 expression and capillary density are markedly reduced in the failing myocardium [21,22]. Given that cardiac decompensation is attributed to a loss of contractile function due to cardiomyocyte death, it is possible that the decrease in GATA4 expression and the resulting impairment of

angiogenesis may contribute to cardiomyocyte death by increasing hypoxia in the left ventricular myocardium. In the future, it would be interesting to assess whether transgenic overexpression of GATA4 or ANGPTL7 specifically in the cardiac endothelium is protective against the effects of AngII, trans-aortic constriction or other hypertrophy-inducing stimuli.

Although the GATA4 expression is known to be pervasive throughout the heart, this report is among the first to describe a role for this protein specifically in the cardiac microvascular endothelial cells. Our findings have uncovered a specific mechanism whereby GATA4, its protein partner NUR77 and its downstream effector ANGPTL7 activate endothelial cell activation required for angiogenesis. As well, Maliken et al (2018) have recently demonstrated that GATA4 expression is required for endothelial cell differentiation essential for both angiogenesis and myocardial capillary network integrity using an endothelial-specific Gata4 knockout model [52]. However, there are likely many more pathways in cardiac endothelial cells that are governed by GATA4 expression. Early in development, GATA4 is central to the development of the pro-epicardium and some of these cells will eventually differentiate into the endothelial cells of the vascular plexus [56]. Indeed, it has been shown that the loss of GATA4 in the epicardium completely prevents vascular plexus formation, demonstrating its importance in the cardiac microvascular endothelial cells from the earliest developmental stages [57]. Even in the adult heart, our microarray results demonstrate that the overexpression of GATA4 directly affects the expression of several well established pro-angiogenic genes including Platelet Derived Growth Factors, Angiogenins and other growth factors. As well, the almost complete knockdown of Angptl7 in human cardiac microvascular endothelial cells could not completely recapitulate the phenotype of cells with only a modest reduction of GATA4 as both proliferation and tube formation, while impaired, were not as severely impacted. This implies that while

ANGPTL7 is an important effector of GATA4 during angiogenesis, it is almost certainly not the only one. Furthermore, the paracrine effects of GATA4 on cardiac microvascular endothelial cells via its expression in cardiomyocytes have also been well described. Heineke et al (2007) were the first to describe a pro-angiogenic role for GATA4 in cardiomyocytes via the secretion of pro-angiogenic factors including Vascular Endothelial Growth Factor-A (VEGF-A) both in cell cultures and *in vivo* [11]. Since then, other groups have also reported on the reported similar findings [13,58]. Collectively, these data conclusively show that the role of GATA4 in cardiac microvascular endothelial cells and in angiogenesis is multifactorial, an unsurprising finding as its roles in cardiomyocytes are diverse as well.

The identification of a GATA4-mediated pro-angiogenic pathway in the myocardium that involves a secreted protein raises the interesting possibility that it could be used as a biomarker for cardiac disease. Our data demonstrates that during compensation, ANGPTL7 levels in the myocardium increase significantly but in Gata4 haploinsufficient mice who have an impaired hypertrophic response, ANGPTL7 levels are blunted. Given that ANGPTL7 is secreted by endothelial cells in direct contact with the flow of blood, this suggests that in the earliest stages of compensated hypertrophy (or indeed perhaps in physiological hypertrophy), serum levels of ANGPTL7 may increase proportionately. As well, these levels may then drop upon decompensation, allowing physicians to mark the time point marking the switch from compensation to later stages of heart failure. Serum levels of ANGPTL7 are readily detected by ELISA and were recently shown to be increased in obese patients by Abu Farha et al. (2017) [59]. Of course this would not represent the first GATA4 downstream target used as an effective biomarker for cardiac disease. BNP and its residual cleavage product N-terminal-proBNP are both used widely throughout hospitals to effectively diagnose and stratify patients with heart

failure [60]. The addition of a new biomarker that may be sensitive to even the earliest stages of cardiac stress would offer another diagnostic and prognostic tool to improve patient outcomes in the future.

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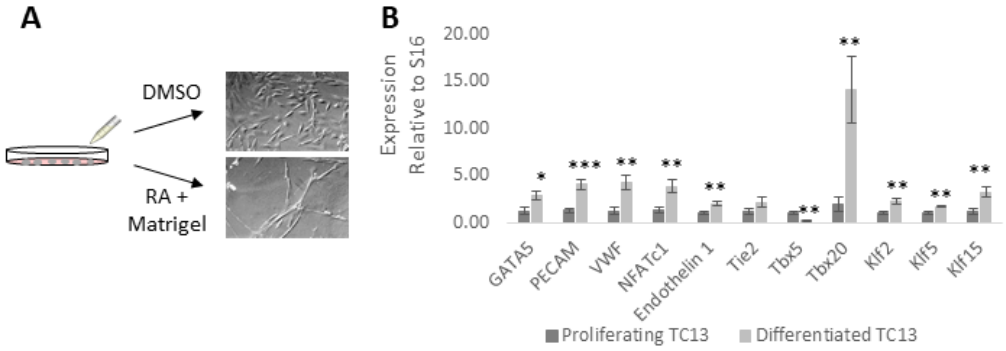
3.12 Supplementary Figures and Tables



Supplementary Figure 3.1

Supplementary Figure 3.1 Differentiated TC13 cells form tubular networks and express markers of the cardiac endothelium. (**A**) Schematic of TC13 cardiac endothelial precursor cell differentiation by treatment with Retinoic Acid and plating on Matrigel. (**B**) Expression of endothelial/endocardial specific markers in proliferating and differentiated TC13 cells by qPCR (n=6 per condition). The results are reported as the mean ± sem, *P ≥ 0.05, **P ≥ 0.01, ***P ≥ 0.001.

A

Pathways Affected by 3xFlag Overexpression in TC13 Cells	p value	Number of Genes Affected	Examples of Affected Genes
Development of the Vasculature	$2.36x10^{-21}$	181	Cysltr1, Fgf5, Edn1, Bmp6, Pik3cb
Angiogenesis	$8.44x10^{-20}$	162	**Angptl7,** Fgf18, Pdgfd, Egln3, Bmp2, Jag1, Ang, Ang2
Vasculogenesis	$7.75x10^{-16}$	130	Wnt5a, Fgfr1, Ptx3, Mmp9, Fermt2
Movement of Endothelial Cells	$3.74x10^{-11}$	66	**Angptl7**, Igfbp3, Klf2, Fgf5, Edn1, Pparg, Igf2
Migration of Endothelial Cells	$3.57x10^{-10}$	60	Ptk2b, Fgf13, Sema3e, Pdgfb, Tgfb2
Endothelial Cell Development	$8.03x10^{-10}$	62	Hipk2, Fgf13, Pdg2a, Fgfr1, Tfpi
Development of Endothelial Tissue	$8.64x10^{-10}$	63	Ngf, Wnt5a, Klf2, Bmper, Itga1/a6/ a11/b8
Proliferation of Endothelial Cells	$1.48x10^{-9}$	55	Cdk5/9/18, Cdkn3/1a/2a, Pdgfb
Tubulation of Endothelial Cells	$4.12x10^{-6}$	22	Btc, Nab2, Cyr61, Hgf, Pdgfa

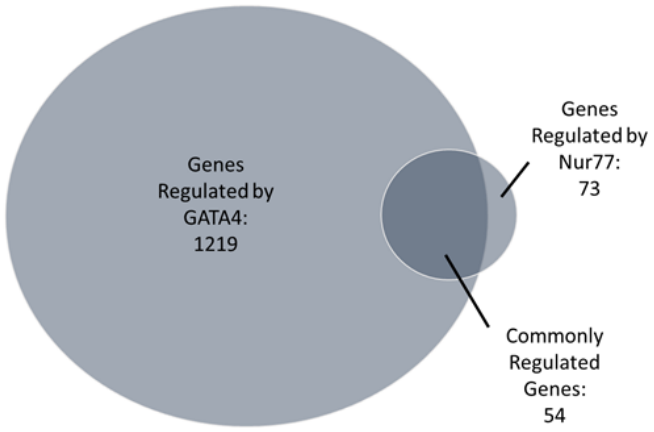
Supplementary Table 3.1

Supplementary Table 3.1 GATA4 regulates multiple pro-angiogenic gene networks in TC13 cells. (**A**) Functional analysis of differentially regulated pathways between control pLNCX2 and 3xFlag GATA4 TC13 cell lines as determined by Ingenuity Pathway Analysis (Qiagen) and literature review. Dysregulated pathways were considered statistically significant if $P \leq 0.05$, right-tailed Fisher's Exact Test. Individual genes were considered significantly regulated if Log2 fold change ≥ 0.6 or ≤ -0.6, $P \leq 0.05$ (Mann–Whitney test with P value adjusted—Benjamini and Hochberg—for multiple comparisons).

A

Pathways Affected by 3xFlag Nur77 Overexpression in TC13 Cells	p value	Number of Molecules Affected	Examples of Affected Genes
Development of Vasculature	4.63×10^{-6}	19	Ace, Adamts1, Ceacam1, Cnn1, Dlx5, Tll1
Vasculogenesis	6.99×10^{-5}	14	Adamts1, Apls2, Egln3, Fbln5, Tf, Gdnf
Movement of Endothelial Cells	5.52×10^{-4}	8	**Angptl7**, Adamts1, Ceacam1, Col11a1, Dcn1, Dgka, Olr1, Vwa5a
Interaction of Endothelial Cells	1.99×10^{-3}	5	Dcn, Flbn5, Olr1, Ptk2b
Endothelial Cell Development	1.00×10^{-2}	6	Adamts1, Ceacam1, Dgka, Ptk2b, Sulf1

B



Supplementary Table 3.2

Supplementary Table 3.2 NUR77 regulates multiple pro-angiogenic gene networks in TC13 cells. (**A**) Functional analysis of differentially regulated pathways between control pLNCX2 and 3xFlag NUR77 TC13 cell lines as determined by Ingenuity Pathway Analysis (Qiagen) and by literature review. Dysregulated pathways were considered statistically significant if $P \leq 0.05$, right-tailed Fisher's Exact Test. Individual genes were considered significantly regulated if Log2 fold change ≥ 0.6 or ≤ -0.6, $P \leq 0.05$ (Mann–Whitney test with P value adjusted—Benjamini and Hochberg—for multiple comparisons). (**B**) Schematic of microarray-identified genes whose expressions are altered by 3xFlag GATA4 overexpression (left circle), 3xFlag NUR77 overexpression (right circle) or both (overlapping regions of circles).

4. Chapter III: NONO and PSPC1: Novel Endocardial GATA4 Interacting Partners Required for Cardiac Development

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4.1 Statement of the Manuscript

The manuscript "NONO and PSPC1: Novel Endocardial GATA4 Interacting Partners Required for Cardiac Development" is ready to be submitted (September 28th, 2018)

4.2 Contribution Statement

In this manuscript, JW performed all experiments, analyzed/interpreted results and wrote/edited the manuscript. LG performed initial stages of *in vivo* experiments and analyzed data, HK interpreted results and edited the manuscript and MN designed experiments, interpreted results and edited the manuscript.

4.3 Acknowledgements

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4.5 Disclosures

None

4.6 Abstract

The zinc finger transcription factor GATA4 is widely expressed throughout heart development where it acts as an important regulator of cell fates. In cardiomyocytes, GATA4 governs proliferation and differentiation pathways but its roles in other cardiac cells and tissues remain unclear. GATA4 is also well expressed in the endocardium, and loss of function *in vivo* has been linked to valve and septal defects. Likewise, human *GATA4* mutations disproportionately affect endocardial tissues. To activate downstream pathways, GATA4 interacts with a wide variety of protein partners that modify its function. These modifiers either enhance or inhibit GATA4 activation of target promoters in a cell and time-point specific manner. To determine how GATA4 governs endocardial cell functions, we aim to identify novel modifiers of GATA4, the downstream pathways they regulate and how mutations to pathway components lead to cardiac defects. To do so, 3xFlag GATA4 was stably overexpressed in the TC13 cardiac endothelial precursor cell line via retroviral transduction and immunoprecipitated from nuclear protein extracts. The resulting protein complexes were analyzed by HPLC-ESI-MS/MS and identified Non POU Domain Containing Octamer Binding Protein (NONO) and Paraspeckle Component 1 (PSPC1) as novel GATA4 interacting partners. These proteins act in an opposing manner to either activate (PSPC1) or repress (NONO) GATA4 activation of target promoters in cardiac endothelial cells. *In vivo,* loss of *Nono* expression results in left ventricular noncompaction and recapitulates the phenotype observed in children with loss-of-function *NONO* mutations. However, simultaneous *Gata4* haploinsufficiency rescues this phenotype. This data shows that a balance between activating and repressing GATA4 modifiers is required to regulate endocardial downstream pathways and to ensure correct cardiac development.

4.7 Introduction

Congenital Heart Disease (CHD) represents a spectrum of cardiac developmental abnormalities present at birth and is the leading cause of infant mortality in western nations [1]. In Canada, a CHD is diagnosed in approximately 1% of all live births and, with the advent of improved imaging and other diagnostic techniques, this number is increasing [2,3]. CHDs are an extremely heterogenous group of defects that affect the endocardium, myocardium and conduction system [1]. They also vary widely in severity as some require surgical intervention within the first year of life and significantly shorten life expectancy while others cause relatively minor defects [3]. However, even less severe phenotypes remain problematic as they can significantly increase risk of acquired cardiovascular disease [4,5]. As such, a significant amount of research on their origins, heterogenicity and diagnosis has occurred but has not yet yielded a complete understanding of the mechanisms underlying CHD.

CHDs are largely attributed to genetic mutations to key cardiac genes. These mutations then interfere with downstream pathways required during development. In rare cases, a direct relationship between mutations in one specific disease-causing gene can be attributed to a singular clinical presentation (for example *TBX5* and Holt-Oram Syndrome) [6]. However, in the majority of cases, a direct relationship between genotype and phenotype has not been ascertained, suggesting a more complex form of inheritance than simple Mendelian genetics. Furthering this hypothesis is the fact that for the majority of CHDs, mutations in multiple genes have been associated with a single phenotype. For example, mutations in *GATA4*, *NKX2.5* and *MEF2C* among others have all been linked to ventricular septal defects (VSDs) [7–9]. Conversely, individuals with the same mutation may present with different phenotypes, even among members of the same family. To begin to understand these complex methods of inheritance, a large

number of animal models with reduced expression of genes associated with CHDs have been developed [10–12]. Interestingly, even among animals with the same genetic background, observed CHD phenotypes are both heterogenous and partially penetrant. Collectively, these effects in humans and in mice are often attributed to the involvement of modifier genes, genes whose expression alters the phenotypic expression of another locus [13–15]. These mutations can negatively impact the expression of important cardiac developmental genes, leading to observable phenotypes. Conversely, modifier gene mutations resulting in a gain of function can explain why animal models and humans harbouring mutations in disease-causing genes may not present with cardiac defects at all [16]. As such, to better understand how and why CHDs develop with varying penetrance and severity, a complete understanding of the network of cardiac developmental genes and their modifiers is required.

The zinc finger transcription factor GATA4 is a central regulator of cardiac development and function and is well expressed throughout all cardiac tissues. Whole body *Gata4*-null mice are embryonic lethal between embryonic day 7-9.5 and lack a linear heart tube [17]. Heterozygote mice (*Gata4* $^{+/-}$), while viable, present with multiple developmental abnormalities including smaller hearts, myocardial thinning, septal defects and valvular problems [10,18]. In cardiomyocytes, GATA4 is required for both proliferation and differentiation during development as well as hypertrophy and survival in the adult [19–25]. Far less is known about its roles in the endocardium. However, endocardial-specific knockout studies present with septal and valvular problems [26]. Furthermore, over 100 mutations along the length of the *GATA4* gene have been linked to CHDs, the majority of whom present with endocardial problems suggesting that it plays a central role in this tissue as well [27]. To play such wide-ranging roles in the heart, its expression and function is controlled by several factors including interactions with protein

partners. In cardiomyocytes, several GATA4 partners have been identified that modify its function and allow it to control cell, time point and stimulus-specific pathways (as outlined in table 1.4 in the thesis introduction) [28–32]. In contrast, far fewer protein interacting partners have been identified in endocardial cells. Using an unbiased mass-spectrometry based approach, we have identified two novel GATA4 interacting partners in endocardial cells, Non-POU Domain Containing Octamer Binding Protein (NONO) and Paraspeckle Component 1 (PSPC1). NONO and PSPC1 are multifunctional DNA and RNA binding proteins involved with mRNA modification, DNA repair, alternative splicing and transcriptional regulation [33–35]. Their roles in the body include modulation of circadian rhythms, androgen receptor signaling and in the case of NONO, wound healing [36–39]. Our results show that when interacting with GATA4, they play opposing roles, with PSPC1 enhancing GATA4 transcriptional activation of target promoters and NONO repressing it. NONO repression of GATA4 was particularly evident on important promoters including *vegfa*, *nos3* the *notch1* required for endocardial/endothelial cell functions during development. *In vivo*, loss of *Nono* expression results in increased endothelial gene expression and left ventricular noncompaction (LVNC), a phenotype observed in humans with *NONO* loss of function mutations. However, these phenotypes can be partially rescued when crossed with *Gata4* haploinsufficient mice. Collectively, this data has identified two novel protein partners of GATA4 without previously known roles in the heart and has contributed to our understanding of the complexity of cardiac development. This information may provide valuable insights into potential therapeutic targets for GATA4-related disorders in the future.

4.8 Methods

Plasmids

The luciferase reporter constructs ANF-pXP2, 3xFlag GATA-pXP2 (cloned from the BNP promoter), Vegfa-pXP2 and Bclx-pXP2 were previously described [25,40–43]. The 1.6 kbp Nos3-pGL2 promoter was a kind gift from Dr. W. Sessa [44,45] and the Notch1-pGL4 promoter was a kind gift from Dr. Gian-Paolo Dotto [46]. The mammalian expression vector Gata4-pCGN was previously described and the Nono-pCDNA3 was a kind gift from Dr. Benjamin Blencowe [40,47]. Pspc1-pCGN and pCDNA3 were produced by subcloning from murine genomic DNA using the 5'- CGCGGATCCATGTTAAGAGGAAAC -3' and 5'- CGCGGATCCTTAATATCTCCGACG -3' primers and by inserting the sequence into the plasmids using the BamHI site. The 3xFlag-GATA4-pLNCX2 retroviral expression vector was produced as previously described [48].

Cell Culture

NIH 3T3 were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin [49]. TC13 cells were cultured in DMEM supplemented with 10% horse serum and 1% penicillin/streptomycin as previously described [50].

Generation of 3xFlag GATA4 TC13 Stable Line

The TC13 cell line overexpressing the 3xFlag GATA4 or control pLNCX2 constructs was made as previously described [48]. Briefly, 3xFlag GATA4-pLNCX2 and empty pLNCX2 constructs were transfected into AD293 cells using the calcium phosphate method. After 48 hours, retroviral particle containing media was collected and added to 80% confluent TC13s. Positive cells were selected for using 500 ug/ml G418 (Sigma G8168). Overexpression of the

3xFlag-tagged construct was confirmed one week post-transduction by QPCR, western blot and immunofluorescence [48].

Immunoprecipitations and Mass Spectrometry

3xFlag-GATA4 coupled complexes were obtained by immunoprecipitating Flag-bound complexes from nuclear extracts of the 3xFlag-GATA4 and pLNCX2 TC13 cell lines as previously described [48]. Briefly, nuclear extracts were obtained from the 3xFlag GATA4 and pLNCX2 TC13 cell lines as previously described [29] and immunoprecipitated with flag-specific antibody or IgG coupled protein A/G magnetic beads (Sigma M8823, Abcam ab18413, Millipore LSKMAGAG10) overnight at 4^{o}C. Complexes were dissociated from magnetic beads by boiling for 5 minutes and were separated by SDS-PAGE on a 12% polyacrylamide gel. Gels were silver stained, bands were excised and sent for HPLC-ESI-MS/MS analysis by the Proteomics Resource Centre at the Ottawa Institute of Systems Biology (University of Ottawa).

Ca_2PO_4Transfections and Luciferase Assays

NIH 3T3 cells were seeded at a confluency of 50,000 cells per well in 12-well dishes as previously described [28]. 24 hours later, cells were transfected with luciferase reporter and mammalian expression plasmids by calcium phosphate as previously described [28]. Cells were lysed 48 hours post-transfection and 100 ul was added to white 96-well plates (BRANDplates 781602) plates along with 100 ul of luciferase buffer (100 mM Tris-HCl pH 8 and 1x luciferine mix, 10x consists of 5 mM Luciferine, 1 mM Lithium CoA, 2 mM Dithiothreitol, 50 mM $MgCl_2$ and 25 mM ATP). Luciferase activity was read using the GLOMAX 96 Microplate Luminometer (Promega).

siRNA Knockdown Experiments

TC13 cells were plated at a confluency of 100,000 cells per well in 6-well plates. 24 hours post-seeding, cells were transfected with 10 nM of Nono targeting siRNA or scramble (Sigma MISSION siRNA, SASI_Mm01_00076443, SIC002) using Lipofectamine RNAiMax as directed by the manufacturer (Thermo-Fisher, 13778030). RNA was collected using TRIZOL reagent (Invitrogen, 15596026) and converted to cDNA using the QuantiTect Reverse Transcription Kit (QIAGEN 205313).

Pull Down Assays

Pull down assays were completed as previously described [49]. Briefly, using the TnT Quick Coupled Transcription/Translation System (Promega), the NONO and PSPC1 pcDNA3 plasmids were in vitro translated and labelled with ^{35}S-Met. They were then incubated with agarose beads with bound GST alone, GST-GATA4 aa 2-254 or GST-GATA4 aa 271-440. Bound protein was resolved on 12% SDS-PAGE gels, dried and exposed with film.

QPCR

QPCR was completed as previously described [51]. Primers are available upon request.

Animal Models

All animal experiments were completed in accordance to University of Ottawa guidelines for animal care and were approved by Institutional Animal Care and Use Committee (IACUC). The *Nono* knockout mouse line on the C57B6 background was a kind gift from Dr. Stephen Brown at the University of Zurich and was produced as described [37]. *Gata4* heterozygous mice on a C57B6 background (*Gata4*$^{+/-}$ mice) are as previously described [17]. To collect neonate (P0) pups, animals were sacrificed and fixed in 4% paraformaldehyde. They were then processed,

embedded in paraffin and sectioned at 4 μm thick for Masson's Trichrome staining by the uOttawa Histology Core.

4.9 Results

NONO and PSPC1 are novel endocardial GATA4 interacting proteins

To identify novel protein partners that modify GATA4 function in the endocardium, we employed an unbiased immunoprecipitation and mass-spectrometry approach using the TC13 cardiac endothelial cell line as previously described [48]. TC13s, upon exposure to retinoic acid (RA) on Matrigel-coated plates, differentiate into elongated endothelial cells that robustly express markers of the endocardium including Gata5, Vwf and Nfatc1 [48,50]. To facilitate the identification of GATA4 interacting partners in this cell line, GATA4 was stably overexpressed via retroviral transduction of a 3xFlag GATA4 construct (**Supplementary Figure 4.1**). The resulting Flag-tagged complexes were immunoprecipitated from nuclear protein extracts and identified using high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) (**Supplementary Figure 4.1**) [48]. This method offers several advantages over the traditional candidate-based approach used to identify the majority of known GATA4 interacting proteins. Candidate based partner identification requires previous or suspected knowledge of a protein's role in the cell type and molecular pathways in question whereas mass spectrometry-based identification requires no previous knowledge of GATA4-interacting partners at all. As such, it is possible to identify entirely unknown or unsuspected partners of GATA4 as well as completely novel roles for these complexes in the heart. Furthermore, our use of the TC13 cell line also facilitates identification of endocardial GATA4 complexes as obtaining *in vivo* samples of endocardium is difficult to do without myocardial contamination and in large enough quantities to confidently identify low abundance proteins

such as transcription factors. As the TC13 cell line actively proliferates, we are able to generate large quantities of protein from an endocardial model to identify GATA4 protein complexes. Using this approach, we have identified multiple potential GATA4 interacting partners in the endocardium including other transcription factors, enzymes and chaperones. Several of the identified GATA4 partners have been previously described by our group and others including Heat Shock Protein 70, Nuclear Orphan Receptor 77 and Histone Deacetylase 2 [25,48,52]. Many others, however, were entirely unknown to interact with GATA4. Of this group, the most consistently identified protein was the Drosophila Binding/Human Splicing (DBHS) family member Non-POU Domain Containing Octamer Binding Protein (NONO) and Paraspeckle-1 (PSPC1) (**Figure 4.1 A**). Interestingly, NONO plays important roles in aortic endothelial cells that share many functional and structural similarities with endocardial cells. There, it represses the expression of the important endothelial/endocardial gene endothelial Nitric Oxide Synthase (eNOS), and its reduced expression is correlated with increased incidence of aortic dissection in humans [53–55]. Although nothing is known about PSPC1 in endothelial or endocardial tissues, it frequently heterodimerizes with NONO to exert cellular functions [33,35]. As such, we wished to determine whether interactions between GATA4, NONO and PSPC1 play important roles in the development and function of the endocardium. Bioinformatics analysis using the Genotype-Tissue Expression Project Portal (GTEx Portal, Broad Institute, MIT and Harvard, https://gtexportal.org) confirmed that both NONO and PSPC1 are robustly expressed throughout the body including in cardiac tissues (**Figure 4.1 B**) [56]. To confirm that they can interact directly with GATA4, pull down assays were performed. Interactions between GATA4 and both NONO and PSPC1 were confirmed and were found to be mediated by the second zinc finger and C-

terminus of GATA4 (**Figure 4.1 C**). This region of the protein mediates the majority of its protein-protein interactions.

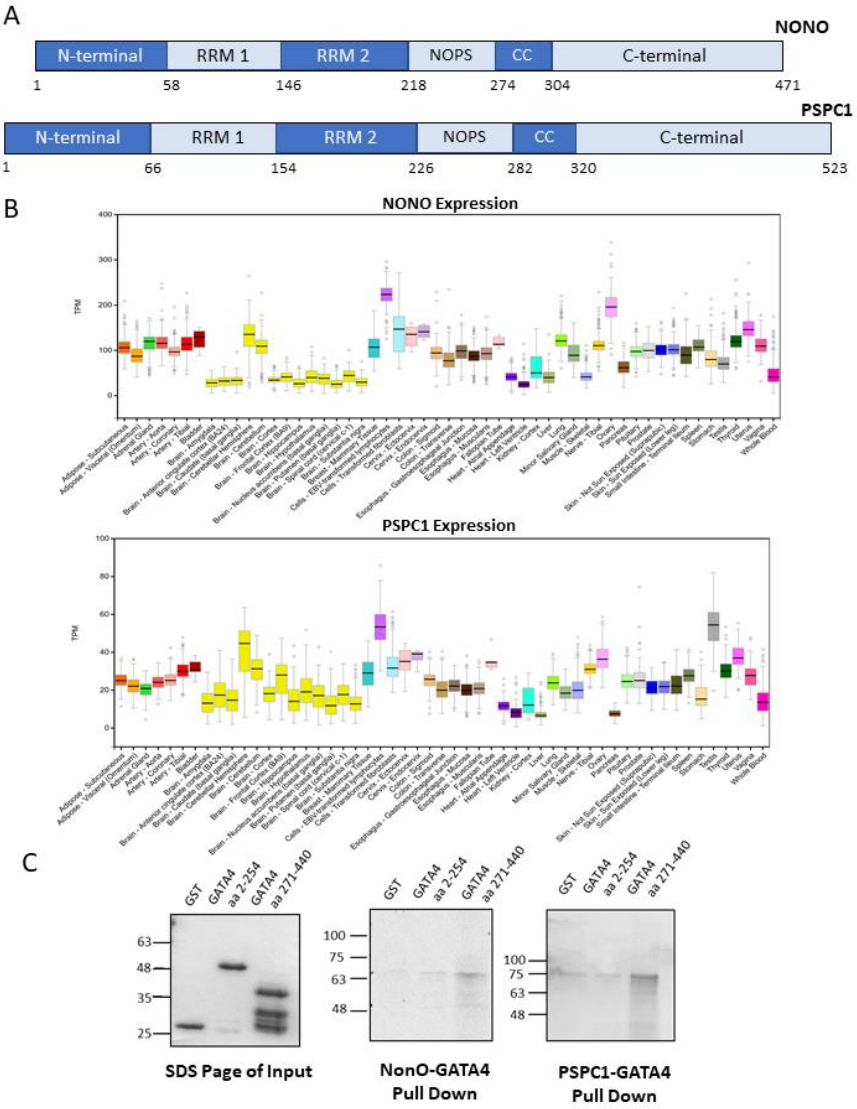

Figure 4.1

Figure 4.1 GATA4 physically interacts with NONO and PSPC1. (A) Schematic of the structure of the DBHS protein family members NONO and PSPC1. RRM = RNA Recognition Motif, NOPS = NONA Paraspeckle Domain, CC = Coiled Coil Domain. **(B)** Level of NONO (left) and PSPC1 (right) expression throughout the body obtained via the Genotype-Tissue Expression Project Portal (GTEx Portal, Broad Institute, MIT and Harvard, https://gtexportal.org). Y axis represents the transcripts per kilobase million (TPM) from RNA-sequencing (RNA-seq) data from human samples. **(C)** NONO and PSPC1 interact with the second zinc finger and C-terminus of GATA4. GST alone or coupled to GATA4 aa 2-254 (N-terminus and 1st zinc finger) or GATA4 aa 271-440 (2nd zinc finger and C-terminus) were incubated with in vitro translated and ^{35}S-Met-labelled NONO, PSPC1 or control luciferase protein. Proteins were separated by SDS-PAGE and revealed by autoradiography. Left panel: SDS-PAGE

of GST fusion proteins. Middle panel: pull down assay of GATA4 and NONO. Right panel: pull down assay of GATA4 and PSPC1.

NONO inhibits GATA4/PSPC1-mediated promoter activation and endothelial gene expression

The main cellular function of GATA4 is to bind to consensus 5'-WGATAR-3' sequences on the promoters of target genes to control their expression. GATA4 selectively transactivates a wide variety of cardiac genes in a cell, time point and external stimulus-driven manner, acting as a major regulator of cardiac cell fates. Although their roles in the heart are unclear, both NONO and PSPC1 are known to modify gene transcription, mRNA processing and protein function of their targets [57]. As such, we wanted to determine whether NONO and PSPC1 can modify GATA4 function by either interfering or enhancing its ability to transactivate target promoters. Luciferase assays were completed whereby Gata4, Nono and Pspc1 mammalian expression plasmids were cotransfected with reporter plasmids of several known GATA4 promoter targets into NIH 3T3 cells (**Figure 4.2**). The GATA4 target promoters tested included one of its best-characterized targets Atrial Natriuretic Factor promoter (*Nppa*), a minimal BNP promoter driven by 3 GATA consensus sites (*3xGATA*) as well as two promoters affecting the function of endocardial cells, the Vascular Endothelial Growth Factor A (*Vegfa*) and B-cell Lymphoma Extra Large (*Bclx*) promoters [25,40,58–60]. On all targets tested, NONO repressed GATA4-mediated transactivation (**Figure 4.2 A**). Although NONO has been shown to transactivate some promoter targets, our results are consistent with previous findings by other groups where NONO is frequently associated with target gene repression [36–39,54,61,62]. PSPC1, on the other hand, enhanced GATA4-mediated transactivation of all GATA4 target promoters tested (**Figure 4.2 B**). This suggests to us that while they may heterodimerize and cooperate together in some

contexts, NONO and PSPC1 likely do not do so when modifying GATA4 activation of target promoters [63–65].

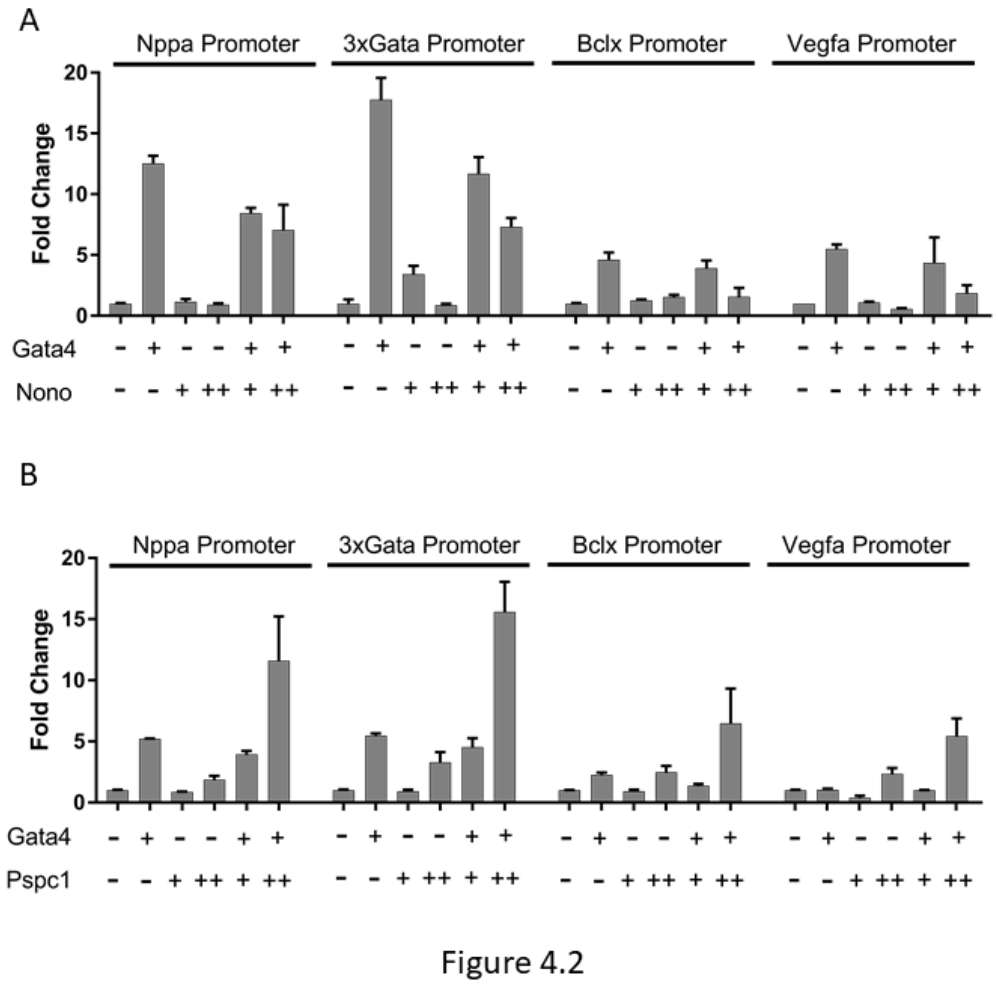

Figure 4.2

Figure 4.2 NONO and PSPC1 Modulate GATA4 Transcriptional Activity. (A) NONO inhibits GATA4-mediated transcriptional activity. NIH 3T3 cells were transfected with 500 ng of *Nppa, 3xGata, Bclx* or *Vegfa* promoters coupled to a luciferase reporter gene along with 100 ng Gata4 or 100 or 500 ng of Nono mammalian expression vectors. Note that the combined transfection of Gata4 and Nono has an overall inhibitory effect on known GATA4 target promoters. Results represent the mean ± SEM of the fold change relative to cells transfected with empty vectors alone. Representative figures shown, n = 3 **(B)** PSPC1 enhances GATA4-mediated transcriptional activity. NIH 3T3 cells were transfected with 500 ng of *Nppa, 3xGata, Bclx* or *Vegfa* promoters coupled to a luciferase reporter gene along with 100 ng Gata4 or 100 or 500 ng of Pspc1 mammalian expression vectors. Note that the combined transfection of Gata4 and Pspc1 enhances fold-activation of GATA4 target promoters. Results represent the mean ± SEM of the fold change relative to cells transfected with empty vectors alone. Representative figures shown, n = 3

As NONO and PSPC1 both physically interact with GATA4 and affect its function, we wanted to determine whether these complexes play a role in cardiac endothelial cells. Short interfering RNA (siRNA) targeting Nono was transfected into TC13 cells and changes in endothelial gene expression were measured by QPCR (**Figure 4.3 A, black and dark grey bars**). Nono expression was reduced by 60%; however, no significant changes in known GATA4 target genes expressed in endocardial cells were observed. Simultaneous overexpression of GATA4 using the 3xFlag GATA4 TC13 cell line induced a significant enhancement of pro-proliferative, survival and endothelial signaling genes, furthering our hypothesis that NONO represses GATA4 function (**Figure 4.3 A, white bars**). Interestingly, PSPC1 expression was enhanced by GATA4 upregulation and NONO knockdown, suggesting that not only does it interact with GATA4 to enhance promoter transactivation, its expression may also be induced by GATA4-mediated pathways as well. Most notably, eNOS and NOTCH1 expression were strongly increased by the concomitant increase in GATA4 and decrease in NONO expression (5.5 and 4.3-fold respectively). Similarly, VEGFA transcript levels were also increased significantly (1.5-fold). eNOS is the main regulator of nitric oxide signaling in the heart and is required for vasodilation, septal development, activation of survival pathways, regulation of metabolism and valve development and function [44,66–68]. NOTCH1 is central to valve and septal development, endocardial/myocardial signaling during trabeculation and coronary vessel morphogenesis [69]. VEGFA controls a wide array of functions including the regulation of endocardial/myocardial cell fates during development, vascular development and function and epithelial to mesenchymal transition during valve formation [70–72]. All three factors are expressed throughout the endocardium [68,69,71]. Although GATA4 is known to play important roles in this tissue, little is known about the specific genes it transactivates. As such, using luciferase assays,

we determined whether these three promoters can be transactivated by GATA4 (**Figure 4.3 B**). Consistent with previous work by our lab, GATA4 was able to dose-dependently transactivate the *vegfa* and *nos3* (eNOS) promoters (**Figure 4.3 B**) [44,60]. However, GATA4 was also able to strongly activate transcription of the *notch1* promoter, a previously unknown GATA4 target gene (**Figure 4.3 B**). Together, these results show that GATA4 is required to transactivate important endocardial/endothelial genes; however, in a cardiac endothelial cell model, its function is inhibited by NONO, strongly suggesting that NONO acts as a rheostat to temper GATA4 function in endocardial/endothelial cells. Given that GATA4 was shown to activate many of its target genes with PSPC1, we wanted to know whether NONO can repress GATA4/PSPC1-mediated promoter activation. To test this hypothesis, we performed a luciferase whereby GATA4, PSPC1, and the 3xGATA reporter constructs were transfected into NIH 3T3 cells in the presence or absence of NONO (**Figure 4.3 C**). As observed previously, GATA4 and PSPC1 synergistically activated the 3xGATA promoter. However, the co-expression of NONO abrogated this effect suggesting that it is a negative modifier of GATA4 activity.

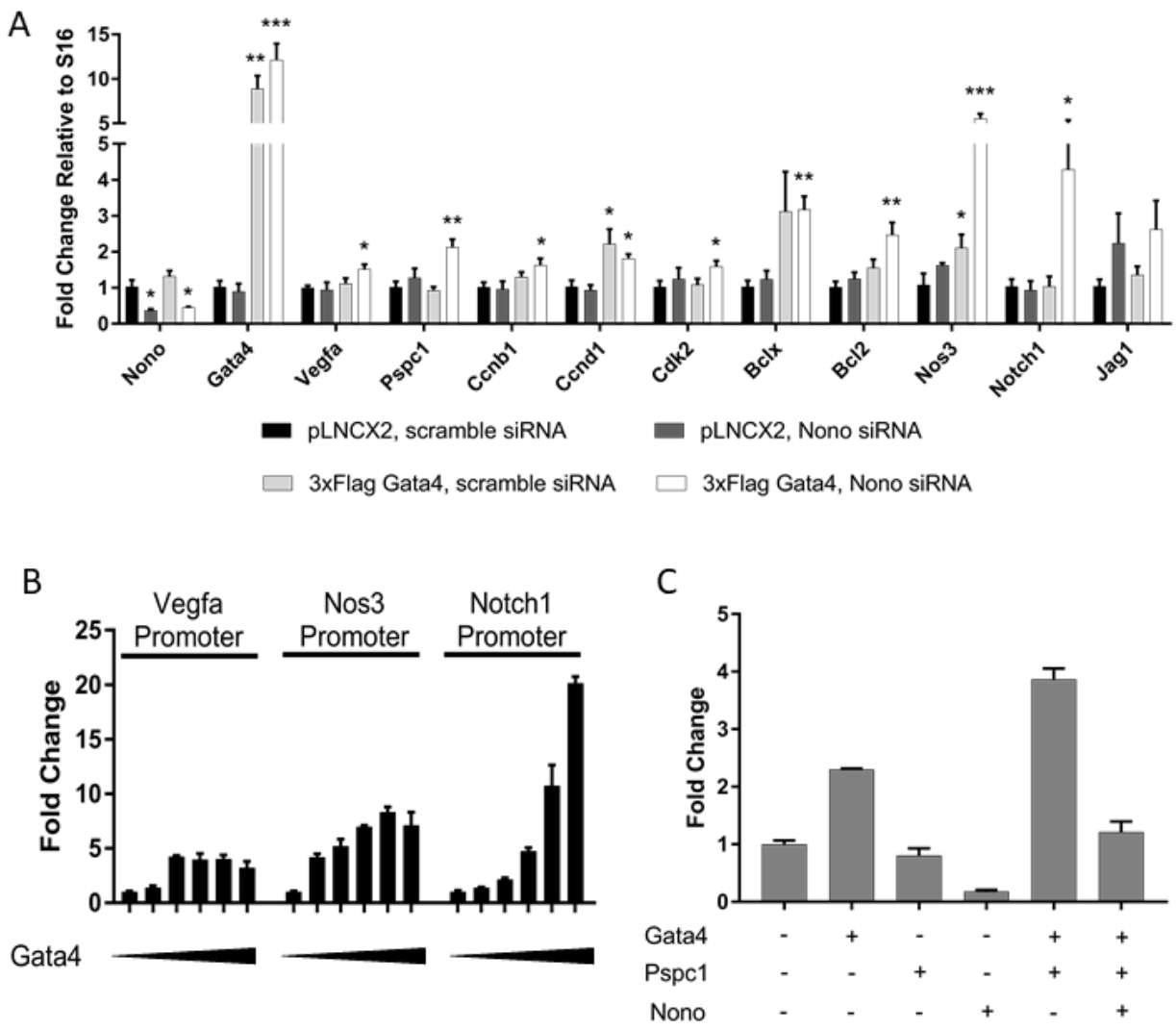

Figure 4.3

Figure 4.3 NONO acts as a rheostat to modulate GATA4-mediated endothelial gene expression. (A) Simultaneous GATA4 overexpression and Nono repression induces significant changes to cardiac endothelial cell gene expression. TC13 cardiac endothelial precursor cells stably overexpressing a pLNCX2 or 3xFlag GATA4 construct were transiently transfected with 10 ng of siRNA targeting Nono mRNA. qPCR analysis was completed on cDNA obtained 48 hours post-transfection. The results are reported as the mean ± sem, *P ≥ 0.05, **P ≥ 0.01, ***P ≥ 0.001. **(B)** GATA4 dose dependently activates the *Vegfa, Nos3* and *Notch1* promoters. NIH 3T3 cells were transfected with 500 ng of *Vegfa, Nos3* or *Notch1* promoters coupled to a luciferase reporter gene along with 0-1000 ng Gata4 mammalian expression vectors. Results represent the mean ± SEM of the fold change relative to cells transfected with empty vectors alone. Representative figures shown, n = 3. **(C)** NONO represses GATA4/PSPC1-mediated transcriptional activation. 50 ng of Gata4, 100 ng of Pspc1 and 500 ng of Nono mammalian expression vectors were transfected into NIH 3T3 cells co-expressing 500 ng of a *3xGata* luciferase reporter plasmid. Note that the simultaneous transfection of Nono abolishes Gata4/Pspc1 synergy. Results represent the mean ± SEM of the fold change relative to cells transfected with empty vectors alone. Representative figure shown, n = 3.

Nono loss of function in vivo is associated with left ventricular noncompaction (LVNC)

Following our identification of NONO as a GATA4 binding partner and modifier, three loss of function *NONO* mutations were published in humans with congenital heart defects (CHDs) [73]. Prior to their identification, *NONO* mutations had only been associated with global developmental delay and neurological phenotypes including intellectual disability, macrocephaly, thickened corpus callosa and small cerebellum [74]. *NONO* is located on the X chromosome. The three males hemizygous for *NONO* loss of function mutations produce no detectable NONO protein [73,74]. All three children presented with left ventricular noncompaction (LVNC), a condition consisting of abnormal and prominent trabecular myocardium with deep recesses overlaying a smaller compact myocardium [75]. This combination of phenotypes gives the myocardium a spongy appearance. Although trabeculation and compaction appear to involve only the myocardium, these processes rely heavily on endocardial cell functions. Trabecular development begins upon the migration of regions of the endocardium in the direction of the epicardial wall while it simultaneously secretes paracrine factors that induce cardiomyocyte proliferation, differentiation and migration into the newly forming projections. This process is largely complete by embryonic day 14.5 and compaction of the ventricular myocardium begins as cardiomyocytes collapse towards the epicardial wall to thicken the myocardium [76]. These two processes are highly intertwined as hypotrabeculation is frequently accompanied by compact myocardium defects (hypoplastic myocardium), whereas hypertrabeculation is associated with noncompaction [77]. The signaling events governing trabeculation that begin in endocardial cells are centered around the transmembrane receptor NOTCH1 [76,78,79]. During trabeculation, NOTCH1 activates signaling pathways in the endocardium that secrete paracrine factors to the myocardium through the cardiac jelly separating the two tissue layers [76,79]. These pathways

trigger cardiomyocyte proliferation, differentiation and migration into the trabecular ridges [76,78,79]. Knockdown and overexpression mouse models of *Notch1*, its modifiers and its downstream targets in both endocardial cells and cardiomyocytes have been associated with either LVNC or a hypoplastic myocardium accompanied by hypotrabeculation [77,80–89]. Likewise, mutations in NOTCH1 target genes Mindbomb 1 and 2 (*MIB1* and *2*) have been linked to LVNC in humans [89,90]. Currently, a role for NONO anywhere in the heart has not been discovered but our data has shown that it represses GATA4 activation of *Notch1* in an endocardial/endothelial cell model. Moreover, given the consistent presence of LVNC among these children with *NONO* mutations, we wished to determine whether it plays an important role in trabeculation and compaction. To begin, we obtained *Nono*-deficient mice as a kind gift from Dr. Stephen Brown at the University of Zurich [37]. These mice were produced by gene-trapping the intron preceding the *Nono* transcription start to produce a whole-body knockout. Detectable levels of *Nono* transcript cannot be found throughout the body, including in the heart [37]. Animals from this line are viable, fertile and born at approximately Mendelian ratios, suggesting that no massive deleterious effects are incurred by the loss of *Nono* expression (**Figure 4.4 A**). However, upon examination of frontal sections obtained from neonatal mice (P0), several cardiac defects were apparent. Loss of *Nono* expression was associated with a hypoplastic, enlarged right atrium (RA), an increased propensity towards hypertrophy of both ventricles (bilateral hypertrophy) as well as an enlarged heart (**Figures 4.4 B, C**). Most noticeably, *Nono* heterozygote and null mice recapitulated the LVNC phenotype observed in humans with loss of function *NONO* mutations (**Figures 4.4 B-E**). Examination of Masson's Trichrome stained cardiac sections demonstrated that *Nono* haploinsufficent and null mice have an increased number of trabeculae throughout the left ventricle (LV) separated by more significant recesses as compared to wild type littermates

(**Figure 4.4 B, D**). As well, the level of compaction observed in haploinsufficient and null mice decreased in a dose-dependent manner between wild types, heterozygotes and null animals as measured by the ratio between compact and total myocardium (**Figure 4.4 D, E**). These results suggest that NONO plays a significant role in trabeculation and compaction. Given our previous results, these effects may be caused by the loss of NONO repression of GATA4 activity in the endocardium.

A

Cross: Nono$^{WT/Y}$ x Nono$^{WT/KO}$

Progeny (50 total)	**Nono$^{WT/WT}$**	**Nono$^{WT/KO}$**	**Nono$^{WT/Y}$**	**Nono$^{KO/Y}$**
Expected	25%	25%	25%	25%
Observed	19 (25.33%)	22 (29.33%)	19 (25.33%)	15 (20%)

B

Phenotype	**Nono WT/WT or WT/Y**	**Nono WT/KO**	**Nono KO/KO or KO/Y**
LVNC	0/4	4/6	5/6
Enlarged/ Hypocellular RA	0/4	2/6	4/6
Enlarged Heart	0/4	1/6	2/6
Bilateral Hypertrophy	0/4	1/6	3/6
VSD	0/4	1/6	1/6

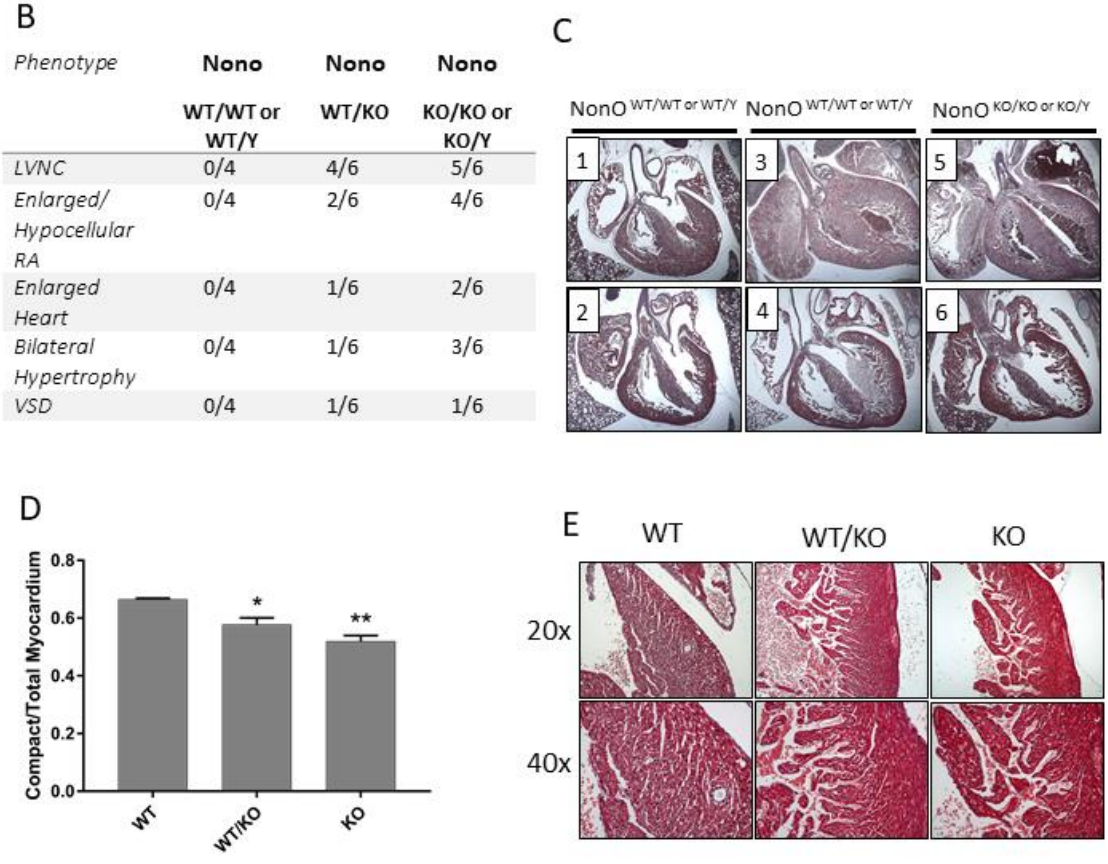

Figure 4.4

Figure 4.4 Nono loss of function *in vivo* causes left ventricular noncompaction. (A) Frequency of genotypes expected and observed in mice obtained by crossing wild type C57B6 mice with Nono$^{+/-}$ C57B6 mice. **(B)** Summary of phenotypes observed in Nono wild type, haploinsufficient and null mice observed at P0 (at birth). LVNC, left ventricular noncompaction. RA, right atrium. VSD, ventricular septal defect. **(C)** 5x images of trichrome stained frontal sections of Nono wild type (1,2), haploinsufficient (3,4) and knockout (5,6) P0 mice. **(D)** Measurement of the ratio between compact and total myocardium observed in Nono wild type, haploinsufficient and null mice. Results represent the mean ± SEM per group. n = 3-6 animals per group, 6 fields quantified per animal. Statistics calculated by one-way ANOVA with Dunnett's multiple comparison test with reference to Nono wild type animals. * $p \geq 0.05$, ** $p \geq 0.01$ **(E)** 20x (upper panel) and 40x (lower panel) of trichromed stained frontal sections of Nono wild type, haploinsufficient and null mice demonstrating lack of compaction and hypertrabeculation observed among haploinsufficient and null mice.

Gata4 haploinsufficiency can partially rescue LVNC associated with Nono loss of function

Our previous findings demonstrated that NONO acts as a negative modifier of GATA4 function, inhibiting its transactivation of several target promoters (**Figures 4.1-4.3**). Levels of mRNA and protein expression must be tightly controlled during cardiac development- too much or too little of any one signaling molecule can dramatically alter downstream pathways and lead to CHD. Therefore, we hypothesized that the loss of NONO may lead to enhanced GATA4 transactivation of target promoters and lead to LVNC. As such, if GATA4 levels are simultaneously reduced *in vivo*, an improvement in LVNC would be observed. To test this hypothesis, we crossed *Gata4* heterozygous mice with our *Nono* loss of function mouse line. *Gata4* null mice were not able to be used as they are embryonic lethal early in gestation due to cardiac defects [17]. *Gata4* heterozygotes, however, are viable and display CHD phenotypes including septal defects, valve defects and hypoplastic myocardium [10,17,18]. Interestingly, myocardial hypoplasia has been previously associated with errors in trabeculation and compaction [85]. The progeny from these crosses were genotyped to determine whether the compound reduction of GATA4 and NONO alters Mendelian ratios. *Nono* wild type, haploinsufficient or null animals were born at roughly expected ratios when expressing both wild type alleles for *Gata4* (**Figure 4.5 A**). This is consistent with our previous observations when

genotyping the *Nono* mouse line (**Figures 4.4 A**). However, when both male and female progeny were simultaneously haploinsufficient for *Gata4* and *Nono*-KO, they were born at higher than expected frequencies compared to *Gata4* heterozygotes who were also *Nono w*ild types or heterozygotes (**Figure 4.5 A**). This suggests that the combined reduction of *Gata4* and *Nono* expression may be beneficial in some way in comparison to reduced *Nono* expression alone. Frontal Masson's Trichrome stained sections from P0 animals demonstrated that the frequency of LVNC is reduced significantly in *Gata4* heterozygous *Nono* null (*Gata4*$^{+/-}$ *Nono*$^{-/-or\ -/Y}$) mice compared to *Nono* null (Gata4$^{+/+}$ Nono$^{-/-\ or\ -/Y}$) mice (**Figure 4.5 B, C, E**). Likewise, measurement of the ratio of compact to total myocardium is also partially recovered (**Figure 4.5 D-E**). Together, this data suggests that the regulation of GATA4 function by activating and repressing protein partners is central to trabeculation and compaction signaling *in vivo*. More specifically, we suggest that NONO represses GATA4 activation of endothelial target promoters and that when absent, leads to the overactivation of these targets and a propensity towards LVNC. Conversely, the reduced Gata4 signaling in Gata4 $^{+/-}$ mice leads to a reduction in activation of downstream pathways and a hypoplastic myocardium. However, the compound reduction of GATA4 and NONO partially restores the level of GATA4 signaling and restores ventricular chamber development.

A

Males	*Cross: Gata4$^{WT/WT}$ Nono$^{WT/Y}$ x Gata4$^{WT/KO}$ Nono$^{WT/KO}$*			
Progeny (27 total)	**Gata4$^{WT/WT}$ Nono$^{WT/Y}$**	**Gata4$^{WT/WT}$ Nono$^{KO/Y}$**	**Gata4$^{WT/KO}$ Nono$^{WT/Y}$**	**Gata4$^{WT/KO}$ Nono$^{KO/Y}$**
Expected	25%	25%	25%	25%
Observed	11 (41%)	7 (26%)	0 (0%)	9 (33%)

Females	*Cross: Gata4$^{WT/WT}$ Nono$^{WT/Y}$ x Gata4$^{WT/KO}$ Nono$^{WT/KO}$*			
Progeny (21 total)	**Gata4$^{WT/WT}$ Nono$^{WT/KO}$**	**Gata4$^{WT/WT}$ Nono$^{KO/KO}$**	**Gata4$^{WT/KO}$ Nono$^{WT/KO}$**	**Gata4$^{WT/KO}$ Nono$^{KO/KO}$**
Expected	25%	25%	25%	25%
Observed	6 (29%)	6 (29%)	3 (14%)	6 (29%)

B

Phenotype	Gata4 WT Nono WT	Gata4 WT/KO Nono WT	Gata4 WT Nono WT/KO	Gata4 WT Nono KO	Gata4 WT/KO Nono WT/KO	Gata4 WT/KO Nono KO
LVNC	0/7	1/7	6/10	5/6	2/5	3/8
Enlarged RA	0/7	0/7	2/10	4/6	1/5	4/8
Hypocellular RA	0/7	0/7	5/10	4/6	2/5	6/8
Smaller Heart	2/7	5/7	4/10	1/6	4/5	5/8
Enlarged Heart	0/7	0/7	1/10	2/6	0/5	0/8
Bilateral Hypertrophy	0/7	0/7	1/10	2/6	0/5	1/8
VSD	0/7	0/7	0/10	1/6	0/5	0/8

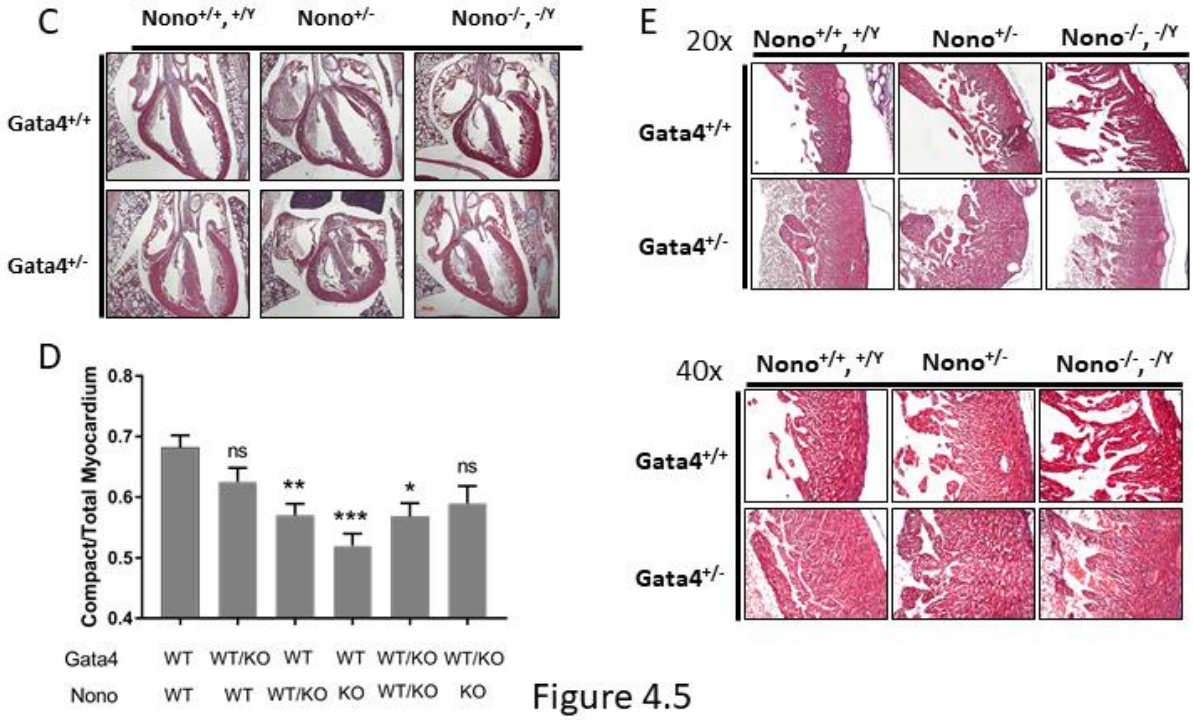

Figure 4.5

Figure 4.5 Gata4 haploinsufficiency partially rescues LVNC associated with Nono loss of function. (A) Frequency of genotypes expected and observed in mice obtained by male Gata4+/+ Nono -/Y mice with female $Gata4^{+/-}$ $Nono^{+/-}$ mice. Note that $Gata4^{+/+}$ $Nono^{+/-\text{ , -/- or -/Y}}$ mice are born at reduced frequencies in comparison to $Gata4^{+/-}$ $Nono^{+/-\text{ ,-/- or -/Y}}$ mice. Results are split between male (upper panel) and female (lower panel) progeny **(B)** Summary of phenotypes observed in Gata4 heterozygote Nono heterozygote or null mice observed at P0. LVNC, left ventricular noncompaction. RA, right atrium. VSD, ventricular septal defect. **(C)** 5x images of trichrome stained frontal sections of wild type (upper left), $Nono^{+/-}$ (upper middle), $Nono^{-/-\text{ or -/Y}}$ (upper right), $Gata4^{+/-}$ (bottom left), $Gata4^{+/-}$ $Nono^{+/-}$ (bottom middle) and $Gata4^{+/-}$ $Nono^{-/-\text{ or -/Y}}$ (bottom right) P0 mice. **(D)** Measurement of the ratio between compact and total myocardium observed in $Gata4^{+/-}$ $Nono^{+/-}$ and $Gata4^{+/-}$ $Nono^{-/-\text{ or -/Y}}$ mice compared to wild type or single heterozygote or null mice. Results represent the mean ± SEM per group. n = 5-10 animals per group, 6 fields quantified per animal. Statistics calculated by one-way ANOVA with Dunnett's multiple comparison test with reference to wild type animals. ns = not significant, * $p \geq 0.05$, ** $p \geq 0.01$, *** $p \geq 0.001$. **(E)** 20x (upper panel) and 40x (lower panel) of trichromed stained frontal sections of wild type (upper left), $Nono^{+/-}$ (upper middle), $Nono^{-/-\text{ or -/Y}}$ (upper right), $Gata4^{+/-}$ (bottom left), $Gata4^{+/-}$ $Nono^{+/-}$ (bottom middle) and $Gata4^{+/-}$ $Nono^{-/-\text{ or -/Y}}$ (bottom right) P0 mice. Note that trabeculation and compaction are improved in Gata4 heterozygote Nono null mice.

4.10 Discussion

Over the past 25 years, our lab and others have identified GATA4 as a central regulator of many cardiac developmental pathways. This research has largely focused on the role of GATA4 in cardiomyocytes. However, GATA4 is also central to endocardial/endothelial cell functions during the formation of valves, septa and coronary vasculature. Its importance in these tissues is best illustrated by the large number of *GATA4* human mutations associated with endocardial CHDs. However, these mutations also demonstrate the complex nature of GATA4 function in this tissue. Phenotypes observed among individuals with *GATA4* mutations are often partially penetrant and heterogenous, even among family members with the exact same mutation. A large number of people present with CHDs for whom no *GATA4* mutation (or a mutation in any other known CHD-associated gene) can be found. As such, identifying modifiers of important cardiac genes such as GATA4 such as NONO and PSPC1 can shed light on the regulatory mechanisms governing cardiac development and how mutations in a wider variety of genes can cause CHDs. A balance between inhibitory modifiers like NONO and activating modifiers like PSPC1 is required because GATA4 function is dosage sensitive and even small

increases or decreases in its level of expression can lead to dysregulation of developmental pathways and CHD. This is readily observed in *Gata4* haploinsufficient and *Nono*-null mice. Reduced expression of GATA4 leads to a decrease in GATA4 target promoter activation and, among other phenotypes, to a hypoplastic myocardium [18]. Conversely, reduced expression NONO, an inhibitor of GATA4, leads to enhanced GATA4 activation of target promoters and LVNC. By simultaneously reducing both GATA4 and NONO expression, we have restored the proper level of GATA4 signaling in endocardial cells and have partially rescued the LVNC and the hypoplastic myocardium phenotypes.

A better understanding of the many pathways that contribute to cardiac development will also improve our ability to find causative mutations in patients with CHDs. Currently, a complete picture of all the molecular pathways cooperating with one another during cardiac development, while improved significantly over the past several years, is far from complete. As such, when looking for clinically significant mutations in patients and families with LVNC, we are limited by our current understanding of these pathways. This is because frequently only genes known to be associated with a particular phenotype are sequenced [91]. Therefore, it is not surprising that we can only find a clinically significant mutation in 35-40% of patients tested [75]. These mutations are primarily located in sarcomeric and cytoskeletal genes but also include genes encoding contractile, mitochondrial and calcium handing proteins [75]. They are also largely expressed in the myocardium. Mutations in endocardial genes important to trabeculation and compaction have not been identified despite their importance in this process. This work has identified a new pathway in endocardial cells that is important to trabeculation and compaction that when altered leads to LVNC. LVNC-causing mutations in both *NONO* and *GATA4* had been previously identified by whole exome sequencing but a relationship between the mutated genotype and the CHD

phenotype had been to date unknown [73,92,93]. Additionally, these data shed light on their downstream target genes *NOTCH1*, *NOS3* and *VEGFA* as potential sequencing sites for patients with LVNC. Even though human mutations in these genes have not been currently linked to LVNC, it is quite likely that they exist but have not yet been identified as their association with LVNC was previously unknown. Mutations to other NOTCH1 pathway genes in trabeculation *MIB1* and *2* have been associated with LVNC already and its likely more will be found as patient sequencing becomes less expensive and more widespread [89,90]. A better understanding of this and other developmental pathways, and how they lead to the correct development of the myocardium, will identify more genes that should be screened, increasing our likelihood of finding a causative mutation in more cases.

The identification of GATA4 modifiers may also have implications in the treatment of post-natal cardiac disease. As well as being a central regulator of cardiac development, GATA4 is also important to post-natal cardiac functions including hypertrophy, secretion of pro-angiogenic factors and the activation of pro-survival pathways [24,25,60,94]. Decreased GATA4 expression is associated with cardiotoxicity seen upon treatment with the chemotherapeutic drug Doxorubicin [25]. It is also associated with pressure overload induced by aortic banding and is correlated with increased cardiomyocyte death, fibrosis and decreased cardiac function [25,95]. These changes can be attributed in part to a decrease in GATA4-mediated activation of pro-survival genes such as *Bcl2* and *Bclxl* [25]. As well, CHD is an independent risk factor for the development of post-natal cardiac disease and is often linked to mutations in *GATA4* that inhibit its function [96,97]. Therefore, the identification of a negative modifier of GATA4 activity could offer a potential therapeutic target. It has previously been proposed to develop a pharmaceutical that enhances GATA4 function, thereby increasing cardiac cell survival during exposure to

stressors such as chemotherapy, chronic hypertension or a pre-existing risk factor such as a CHD [25]. However, traditionally, it has been easier to inhibit rather than enhance protein function with medication. It may be possible to identify a pharmaceutical target that interferes with NONO activity and as such, enhances GATA4 activity, and increases expression of pro-survival targets including BCL2 and XL. A drug with this functionality would have a positive impact on the millions of patients living with heart failure and highlights why a thorough knowledge of modifier genes is required to develop new treatment strategies for those affected by cardiac disease.

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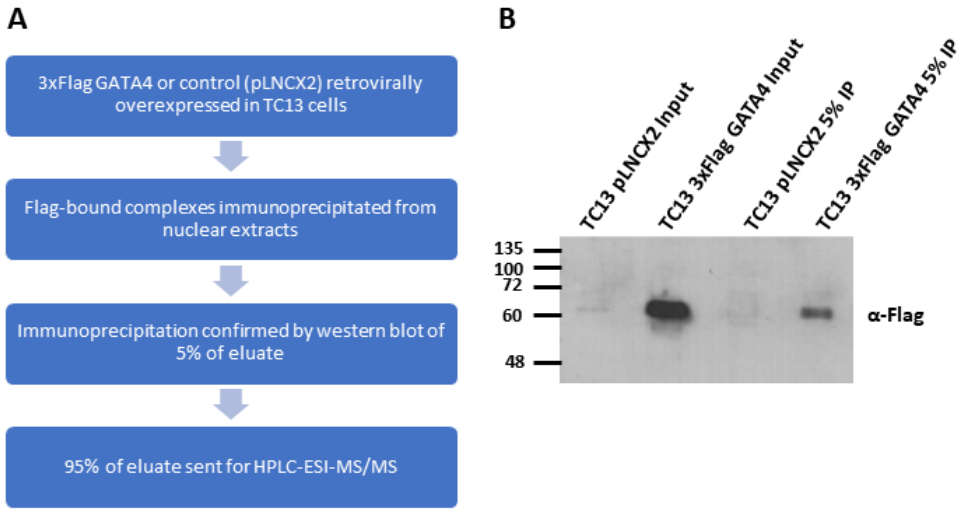
4.12 Supplementary Figures



Supplementary Figure 4.1

Supplementary Figure 4.1 Isolation of GATA4 complexes from the TC13 pLNCX2 and 3xFlag GATA4 cell lines. (A) Schematic of workflow used to immunoprecipitate 3xFlag GATA4 complexes from 3xFlag GATA4 TC13 (and control pLNCX2-TC13) cells. **(B)** Western blot of a 5% input and immunoprecipitation of flag-coupled complexes from nuclear protein extracts of the pLNCX2 and 3xFlag GATA4 TC13 cells.

5. General Discussion

The transcription factor GATA4 is a dosage sensitive regulator of heart development and post-natal cardiac function. During development, Gata4 mutations are linked to congenital heart defects of the myocardium and endocardium. In the post-natal heart, GATA4 expression is required for compensatory responses to cardiac stressors including chemotherapy and pressure overload. Although this transcription factor has been the focus of a great deal of research over the past 25 years, the mechanisms underlying its multiple roles in the various cardiac cell types remain incompletely understood. The research presented in this thesis has identified 3 novel GATA4-centred pathways in cardiac cells that regulate cardiomyocyte survival during Doxorubicin treatment, angiogenesis in the left ventricular myocardium and endocardial development. These results provide novel insights into the molecular function and regulation of GATA4 in myocardial and endothelial cells and have potential clinical implications for the diagnosis and treatment of heart disease.

5.1 GATA4 is a central regulator of cardiac endothelial cell development and function

The heart relies on the development and cooperative interaction of many different cell types. These include ventricular, atrial and conduction system cardiomyocytes, fibroblasts, epicardial cells and 5 distinct populations of endothelial cells. Each plays an indispensable role in cardiac function. Endocardial cells serve as a protective layer between the myocardium and the circulation, ensure unidirectional blood flow via valves and septa and secrete paracrine signals that regulate myocardial development [1,2]. Vascular endothelial cells release and respond to vasoactive substances, regulate vascular permeability and interact with immune cells [3]. In addition, microvascular endothelial cells control remodelling of the myocardial capillary network

based on this tissue's oxygen requirements [4]. Lymphatic endothelial cells are required for tissue-fluid homeostasis, lipid transport and immune surveillance [5]. Each is also affected by congenital and/or acquired cardiac disease. CHD disproportionately affects the endocardium as both septal defects (e.g. ASDs and VSDs) and valve problems (e.g. BAV) are some of the most commonly diagnosed phenotypes [6]. Even defects of the myocardium such as left ventricular noncompaction can originate with errors in the development and function of the overlying endocardium [7]. Likewise, in the adult, diseases often thought of through a myocardial lens often have a significant involvement of the cardiac endothelium. For example, myocardial infarction causing cardiomyocyte death and loss of contractility occurs often as a result of plaque accumulation on the endothelial layer of coronary arteries [8]. Similarly, heart failure is attributed to the loss of cardiac contractility but the loss of cardiomyocytes can be preceded by a substantial reduction in myocardial capillary density [9,10]. Often, our approaches to identifying new diagnosis and treatment strategies also centre around the myocardium (for example, searching for causative mutations in myocardial genes, stem cell renewal of lost cardiomyocytes post-MI and enhancing cardiomyocyte survival pathways). These approaches are extremely valuable. However, as the causes and progression of cardiac disease involves the cardiac endothelium as well, targeting these cells and tissues offers a promising approach for the discovery of new diagnostics and treatments.

Previous work by our lab and others shows that GATA4 plays an important role in cardiac endothelial cells [4,11]. However, the specifics of how it does so remain unclear. Whole body *Gata4* heterozygous mice as well as human *GATA4* mutations lead to septal and valvular defects including ASDs, VSDs and BAV [12–15]. Similarly, endothelial-specific deletion of *Gata4* results in embryonic lethality at E12.5 due to endocardial cushion defects [12]. In cardiac vascular

endothelial cells, GATA4 expression is required for the formation of the initial vascular plexus that gives rise to the coronary circulation [16]. As well, it mediates the secretion of its target VEGF-A from cardiomyocytes to induce angiogenesis and increase myocardial capillary density [4,17]. A more direct role for GATA4 in vascular endothelial cells was first identified in TC13s where it is well-expressed and the inhibition of its downstream target VEGF-A impairs differentiation into angiogenic tubes [11]. The work presented in this thesis has elaborated on these endocardial GATA4 signaling pathways. We show that endothelially-expressed GATA4 regulates the expression of several vital endocardial/endothelial genes including *Vegfa*, *Nos3*, *Notch1*, *Angptl7* and many more. In microvascular endothelial cells, we show that GATA4 activates both proliferation and differentiation required for the induction of angiogenesis in the post-natal heart. Additionally, we show that correct expression of both GATA4 and its negative modifier NONO is required for proper trabeculation and compaction of the myocardium- this process relies heavily on signals originating from the overlying endocardium. This work has contributed significantly to our understanding of the molecular mechanisms taking place in endocardial/endothelial cells, has identified multiple new GATA4 regulatory mechanisms in the heart and highlights the importance of the cardiac endothelium in heart development and function.

5.2 GATA4 modifiers influence penetrance and heterogenicity of CHDs

As previously discussed in the introduction and in Chapter 4, CHDs have complex origins because cardiac phenotypes do not arise via the actions of one single gene. Instead, a network of genes and their corresponding proteins regulate each aspect of cardiac development. As such, small changes to a single factor can lead to dysregulation of downstream pathways and cause cardiac defects. There have been several genes (including *GATA4*, outlined in figure 1.4 of

the introduction) whose mutations are associated with human CHDs [18]. However, mutations to these genes are found in relatively few cases [19]. As such, the identification of modifiers that affect the expression of important cardiac proteins like GATA4 can help us obtain a clearer understanding of the genetic origins of CHD. This has already been observed in knockdowns or mutations to other known GATA4 protein interacting partners that modify its function (listed in table 1.4). For example, KLF13 and GATA4 interact to induce cardiomyocyte proliferation via activation of the *Nppb* and *Ccnd1* promoters [20]. Knockdown of either gene impairs their interaction and leads to a hypoplastic myocardium [20]. In the endocardium, knockout of the GATA4 partner FOG2 or a *Gata4* mutation that abrogates their interaction (V217G) leads to a host of valve, septal and vascular defects [15,21]. This work has identified 4 new GATA4 modifiers: HSP70, NUR77, NONO and PSPC1. Each affects GATA4 function in a specific pathway including those that regulate cardiomyocyte survival, angiogenesis and endocardial development. If mutations were to occur in any of these 4 genes, downstream pathways would be altered and defects in these 4 functions could be observed. So far, only mutations to *NONO* have been identified in individuals with CHDs [22]. However, with the advent of more cost-effective high throughput sequencing available to clinicians, mutations to HSP70, NUR77 and PSPC1 may be identified as well.

Mutations in modifiers such as *HSP70*, *NUR77*, *NONO* and *PSPC1* can help us understand why mutations in CHD-associated genes like *GATA4* lead to partially penetrant and heterogenous phenotypes. As discussed previously, GATA4 functions are dosage sensitive and even small changes to its expression are associated with increased prevalence of cardiac defects [23]. Therefore, mutations in modifier genes that cause even minor changes to GATA4 expression and function can affect downstream signaling and cause cardiac defects. This effect is also

readily observed between mice produced on different genetic backbones. For example, Winston et al (2010) observed that among three animal models of *Nkx2.5* haploinsufficiency, the animals produced on a C57B6 background had a higher incidence of CHD compared to those produced on FVB/N or A/J backgrounds [24]. These strains would all likely possess different polymorphisms in Gata4 modifiers that would alter Gata4 expression and function differently and lead to discrepancies in CHD frequency and presentation.

It must also be noted that although mutations to Gata4 modifiers are often associated with a loss of function and cardiac defects, gain of function mutations in modifier genes can also occur. These mutations may be able to compensate for a deleterious mutation in their target and suppress the defective phenotype. Our findings show that mice that are simultaneously haploinsufficient for *Gata4* and *Nono*-null do not present with the myocardial thinning or left ventricular noncompaction observed in *Gata4* heterozygotes or *Nono*-null animals individually. This suggests that the two mutations can compensate for one another to some extent. Other examples of this effect can be found in the literature. For example, *TBX5* mutations are autosomal dominant for the development of Holt Oram Syndrome, a condition consisting of several heart and limb defects [25]. However, some individuals with mutations present with very mild or even nonexistent cardiac defects, even when family members with the same mutation have severe CHDs [25]. This is likely due to polymorphisms in modifiers that can compensate for the deleterious mutation in *TBX5* and partially or completely suppress the formation of cardiac defects.

Lastly, some of the heterogenicity of CHD phenotypes linked to *GATA4* mutations can also be explained by its involvement in a wide range of developmental pathways occurring in different cell types. In each pathway, GATA4 interacts with a different set of protein partners

that affect its ability to activate target promoters. These interactions are mediated by different residues along the GATA4 protein. In this thesis, we have observed that the N-terminal zinc finger of GATA4 interacts with HSP70 in cardiomyocytes to promote cardioprotection. The C-terminal zinc finger and C-terminus of GATA4, on the other hand, interacts with NUR77 in microvascular endothelial cells to promote angiogenesis and with NONO and PSPC1 in endocardial cells to regulate their development and function. As such, if a mutation were to occur in the N-terminal zinc finger of GATA4, it may abrogate GATA4-HSP70 interactions in cardiomyocytes but have no effect on GATA4 interactions with NUR77, NONO and PSPC1 in microvascular endothelial and endocardial cells. However, a patient with a mutation in the C-terminal zinc finger or C-terminus of GATA4 may have normal GATA4-HSP70 interactions but be unable to interact with NUR77, NONO and/or PSPC1. As such, different mutations to *GATA4* (or its modifiers) can result in a wide range of potential phenotypes in cardiac cells and tissues. This information demonstrates that an improved understanding of how GATA4 interacts with protein partners that modify its function will allow us to get a clearer picture of the genetic basis of CHDs.

5.3 GATA4 pathways as diagnostic and therapeutic targets for cardiac disease

In this work, we have identified how novel GATA4 modifiers and downstream targets play important roles in cardiac development and in post-natal function. Furthermore, we show that alterations to these pathways lead to both developmental defects as well as impaired adaptation to cardiac stressors in the adult heart. This information can be harnessed when trying to identify novel diagnostics and treatment strategies for congenital and adult cardiac disease. As discussed in the last paragraph and throughout this thesis, CHD has a high hereditary component. However, the underlying causative mutations often remain unknown. Our data identifies new

associations between CHD and GATA4 modifiers and downstream targets without previously known roles in cardiac development. As such, these modifiers and targets can be added to the list of genes screened in patients with CHD to identify the genetic causes of their disease. In addition, it will allow help us identify at-risk family members who may be affected. It is also important to note that these developmental genes frequently play roles in the function of the post-natal heart, explaining why individuals with CHD are at increased risk for the development of adult cardiovascular disease. Therefore, information presented in this thesis may also be harnessed in the prevention of secondary risks in affected individuals and their families.

The work presented in this thesis has also identified new diagnostics strategies for acquired cardiac disease. Biomarkers for heart failure are highly sought after because they are non-invasive and often cost-effective means of diagnosing, stratifying and guiding treatment [26]. Although increased GATA4 expression is correlated with cardiac compensation and vice versa, it is not readily tested for as it is not secreted into circulating fluids. Therefore, it would require a biopsy of left ventricular myocardium to detect its expression in heart failure. Alternatively, the identification of circulating GATA4 targets could provide a better means of identifying and prognosticating people who are at risk of heart failure. For instance, our data suggests that ANGPTL7 should go up in patients who are in the earliest stages of cardiac remodelling and would present before the onset of symptoms. This would allow us to catch cardiac disease earlier and prevent its progression. However, in later stages of heart failure, myocardial capillary density decreases. If, as we hypothesize, ANGPTL7 expression goes down with decreasing capillary density in late stage heart failure, it could help us prognosticate and guide more aggressive treatment strategies.

Our identification of novel GATA4 modifiers and downstream pathways in the heart can also be used to develop new therapies for cardiac disease. As mentioned above, high levels of cardiac GATA4 expression are associated with preserved cardiac function whereas low levels are associated with decompensation [4,27]. As such, therapeutic strategies that preserve GATA4 function despite exposure to cardiac stressors could dramatically improve patient outcomes. Our work has identified several possible modifiers of GATA4 that could be targeted to enhance its function. Increased induction of HSP70 could protect GATA4 from degradation and maintain expression of pro-survival downstream targets such as *Bcl2* and *Bclxl*. This would increase cardiomyocyte survival and ward off decompensation. Similarly, increased nuclear expression of NUR77 in cardiac microvascular endothelial cells could maintain GATA4-regulated angiogenic pathways and prevent ischemia, further reducing stress on cardiomyocytes. Furthermore, as mentioned in the above section, developmental cardiac genes such as *Gata4* often play important roles in the adult heart. Aside from its developmental roles, GATA4 also controls hypertrophy, angiogenic and pro-survival pathways to maintain cardiac homeostasis upon exposure to cardiac stressors. Therefore, identification of GATA4 modifiers important during cardiac development may also be relevant to post-natal disease. Our findings show that PSPC1 enhances and NONO reduces expression of several GATA4 targets known to play important roles in acquired cardiac disease. These genes include the pro-survival factor BCLx, the pro-angiogenic factor VEGF-A and the NO-modulating eNOS. As such, enhancing PSPC1 or inhibiting NONO functions may prove to be good therapeutic strategies as well.

5.4 Contributions to Knowledge and Future Studies

The pathways underlying cardiac development and post-natal function are complex and remain incompletely understood. This leads to limitations in our ability to detect and treat

cardiac disease. Via the detection of novel GATA4 modifiers and downstream gene targets, the work presented in this thesis has identified novel mechanisms governing cell and stage-specific pathways in the heart. This work has also offered insights into potential diagnostic and treatment strategies for congenital and acquired cardiac defects. However, it has also left interesting questions that remain to be answered.

1) Further insights from our mass spectrometry approach:

Our unbiased mass spectrometry-based approach to identify GATA4 interactors identified over 100 potential protein partners of which only 4 were studied in detail. Even so, several novel insights into the regulation of GATA4 function were obtained. Therefore, this list of GATA4 interacting partners offers a wealth of data that will be studied to identify other mechanisms required for cardiac development and function. We will begin by studying their roles in different cardiac cells in culture and identify their downstream targets via unbiased approaches like microarray or RNA sequencing. The cell-specific roles of these partners in cardiomyocytes and cardiac endothelial cells can then be explored *in vivo.* This would be accomplished via tissue-specific knockout models driven by Cre under the control of the *myh6* or *tie2* promoters [28,29]. As well, our mass spectrometry results also identified novel GATA4 phosphorylation sites not elaborated on in this thesis. As discussed in the introduction, PTMs have the power to modulate GATA4 expression by controlling its subcellular localization and interactions with protein partners. As such, the roles of these phosphorylation sites in GATA4 function including the kinases involved, the protein interactions they facilitate and the downstream mechanisms they activate will be studied in greater detail.

2) Testing the linkage between GATA4 interactors/targets and CHD

Given that our work has identified many potential new GATA4 protein interacting partners and downstream targets, we would like to determine whether mutations therein are associated with CHD. Often, when looking for mutations associated with CHD, only a restricted number of genes previously associated with cardiac defects are sequenced and as such, mutations in as-yet unknown CHD-associated genes are missed. However, with the advent of high throughout sequencing that is rapidly becoming less expensive and more accessible, we are able to sequence entire genomes of CHD patients [30]. This will allow us to search for causative mutations in newly discovered GATA4 modifiers and targets, even retroactively. Although high throughput sequencing of CHD patients is still relatively rare, we are still able to study genotype-phenotype relationships in cardiac disease via animal knockout models of GATA4 modifiers and downstream targets. Collectively, this information will lend valuable insights on how GATA4-centred pathways govern the development and function of the heart and will clarify the relationship between genotype and phenotype in cases of CHD.

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Curriculum Vitae

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Objective

I am a bilingual PhD candidate working with Dr. Mona Nemer at the University of Ottawa. I aim to submit and defend my doctoral thesis in the Fall 2018 term on GATA4 regulatory mechanisms in cardiac development and disease.

Education

01/2013- present:	PhD Candidate, Biochemistry, University of Ottawa, Supervisor: Dr. Mona Nemer
09/2011- 12/2012:	MSc Candidate, Biochemistry, University of Ottawa, Supervisor: Dr. Mona Nemer. Successfully passed transfer exam to move directly into the PhD program.
09/2007- 05/2011:	BSc, Honours, Biology, Acadia University, Supervisor: Dr. David Kristie

Publications

Manuscript in Progress:	"Endothelially-expressed GATA4 regulates myocardial angiogenesis in response to pressure overload," **Whitcomb J.**, Zhang M., Wang H., Komati H., Nemer M.
Manuscript in Progress:	"NonO and PSPC1: Novel endothelial GATA4 interacting partners required for cardiac development," **Whitcomb J.**, Gharibeh L., Komati H., Nemer M.
Manuscript in Progress:	"GATA4: a novel nuclear substrate of AKT necessary for its prosurvival function in cardiomyocytes," Komati H. and Maharsy W., **Whitcomb J.**, Beauregard J., Nemer M.
12/2014:	"Caspase-1 cleavage of transcription factor GATA4 and regulation of cardiac cell fate," Aries A and **Whitcomb J**, Shao W, Komati H, Saleh M, Nemer M., Cell Death and Disease, Pubmed ID: 25501827

National/International Conference Presentations

28/05/2018- 01/06/2018:	"GATA4 is an upstream activator of Angiopoietin-like 7 in endothelial cells", **Whitcomb, J.** Komati, H. and Nemer, M. Oral Presentation, IUBMB Focused Meeting on GATA Transcription Factors, Crete, Greece
26/04/2018- 27/04/2018:	"Say NonO to Congenital Heart Disease: Identification of Novel GATA4 Interacting Partners Central to Cardiac Development", **Whitcomb, J.** Komati, H. and Nemer, M. Poster Presentation, Ottawa Heart Research Conference, Ottawa, Ontario
07/08/2016- 12/08/2016:	"Differential Interactions Between Zinc Finger Proteins Regulate Endothelial Cell Fates", **Whitcomb, J.** Komati, H. and Nemer, M. Poster Presentation, KLF and Sp Transcription Factors in Disease and Regenerative Medicine. Snowmass, Colorado
01/06/2015- 04/06/2015:	"Identification of Regulatory Mechanisms Governing GATA4 Functional Diversity in the Heart**"**, **Whitcomb, J.** Aries, A. Shao, W. Komati, H. Saleh, M. and Nemer, M. Poster Presentation, Canadian Student Health Research Forum, Winnipeg, Manitoba
24/04/2015- 25/04/2015:	"Hsp70, a novel GATA4 interacting-partner necessary for cardiomyocyte survival", **Whitcomb, J.** Aries, A. Shao, W. Komati, H. Saleh, M. and Nemer, M. Poster Presentation, 3rd Annual Ottawa Heart Research Conference, Ottawa, Ontario

Scholarships and Awards

28/05/2018- 01/06/2018:	Travel Award, IUBMB Focused Meeting on GATA Transcription Factors, Crete, Greece
03/2017:	Second Place PhD Presentation Biochemistry, University of Ottawa Biochemistry, Microbiology and Immunology Poster Day 2017

09/2016- 08/2017: 09/2011- 08/2012:	Ontario Graduate Scholarship
12/05/2016:	Best PhD Presentation in Biochemistry, University of Ottawa Biochemistry, Microbiology and Immunology Poster Day 2016
02/12/2015:	Graduate Studies Leadership Award, Faculty of Medicine Awards in Education
05/05/2015:	Servier Award for Best Basic Science Oral Presentation, Ottawa Heart Institute (UOHI)'s 28th Annual Research Day
25/04/2015:	Best Poster Presentation, Ottawa Heart Research Conference
10/03/2015:	Best PhD Presentation in Biochemistry, University of Ottawa Biochemistry, Microbiology and Immunology Seminar Day 2015
02/12/2015:	Graduate Studies Leadership Award, Department of Biochemistry, Microbiology and Immunology, University of Ottawa
15/05/2014:	Second Place PhD Presentation in Biochemistry, University of Ottawa Biochemistry, Microbiology and Immunology Poster Day 2014
09/2013- 08/2016:	Canadian Institutes for Health Research Doctoral Award
09/2012- 08/2013:	Queen Elizabeth II Graduate Scholarship in Science and Technology

Leadership

13/05/2017- 21/05/2017:	Participant, 6th International Summer School in Medical and Biosciences Research and Management, Athens and Neo Itilo, Laconia, Greece
09/2016- present:	Student Representative, Biochemistry Graduate Program Committee, University of Ottawa
05/2014- 04/2016:	Vice President Internal, Biochemistry, Microbiology and Immunology Graduate Students Association
05/2013- 04/2014:	Vice President Communications, Biochemistry, Microbiology and Immunology Graduate Students Association

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