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# Primary Cilia in Cardiac Valve Development and Disease

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by

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## Dedication

I would like to dedicate this work to my family: my mom, dad, sisters Amanda and Lindsey, brother TJ, fiancé Patrick, and dog Luna. Their patience and understanding have been an enormous help in accomplishing this work. To my fiancé Patrick for listening to my practice talks approximately a million times, coming to lab on Saturday mornings at 5 am because I 'just had to do one more cyclohexamide experiment', and for making me laugh when I was so stressed I could cry. To my mom and dad for talking to me every day (sometimes twice a day) as I drove to and from work, for pretending to understand the science I rambled on about, and for teaching to never give up on my dreams. To my sisters Amanda, Lindsey, and Jamie and brother TJ for supporting me through all of this and always being there to talk. To my future mother in law who is already a second mom to me, your love and support have gotten me through the toughest weeks. Thanks also to the members of the #norrislab Kim, Kathrine, Amanda, Diana, Lilong, Kelsey, Janiece, and Rebecca. Without their daily love, support, and emergency coffee/cupcake breaks this would not have been possible. Last but not least I would like to thank the families that participated in these studies. Without their contribution this science could not have been possible.

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MVP-Mitral valve prolapse
BAV-Bicuspid aortic valve
CHD-congenital heart disease
EMT- endothelial to mesenchymal transformation
ECM-extracellular matrix
AV- atrioventricular
OFT-outflow tract
DMP- dorsal mesenchymal protrusion
TGFβ- transforming growth factor β
BMP- bone morphogenetic protein
N-CAM- neural cell adhesion molecule
PECAM-1- platelet endothelial cell adhesion molecule
Nfatc1- nuclear factor of activated T cells-c1
FGFs-fibroblast growth factors
AVC-atrioventricular cushion
EGF- Epidermal growth factor
aSMA- alpha smooth muscle actin
PCNA- proliferating cell nuclear antigen
FLNA- Filamin-A
VIC-valvular interstitial cells
VEC-valvular endothelial cells
MMP- matrix metalloproteases
NCC-neural crest cells
EPDC-epicardially derived cells
TAA- thoracic aortic aneurysm
AA- aortic aneurysm
CAVD-calcific aortic valve disease
TIMP-tissue inhibitiors of MMPs
BD-Barlow disease
FED-fibroblastic deficiency
JBTS- Joubert syndrome
MKS- Meckel syndrome
NPHP- nephronophthesis
BBS- Bardet-Biedl syndrome
PKD- Polycystic kidney disease
IFT-intraflagellar transport
Hh- hedgehog
Ptch- patched
Smo-smoothened
Shh-sonic hedgehog
Ihh-indian hedgehog
Dhh- desert hedgehog
GliA-Gli activator
GliR- Gli repressor

OPN- osteopontin
HSC- hepatic stellate cells
PKA- protein kinase A
RTKs- receptor tyrosine kinases
PDGFR-platelet derived growth factor receptor
EGFR- epidermal growth factor receptor
PDGF-platelet derived growth factor
Pkd1-polycystic kidney disease 1
Pkd2-polycystic kidney disease 2
ADPKD- autosomal dominant kidney disease
NICD- notch intracellular domain
KV- Kuepfer's vesicle
PCD- Primary Cilia Dyskinesia
AVSD- atrioventricular septal defect
Tctn3- Tectonic protein 3
AS- aortic stenosis
aVIC- aortic valve interstitial cell
3D-IHC- 3D-immunohistochemistry
IHC- immunohistochemistry
H and E – Hematoxylin and Eosin
GFP- enhanced green fluorescence protein

**KATELYNN AMBER TOOMER**, Primary Cilia in Cardiac Valve Development and Disease (Under the direction of RUSSELL A. NORRIS)

## ABSTRACT

Cardiac valve disease is a major health burden affecting around 5% of the population. Two of the most common cardiac valve disease include mitral valve prolapse (MVP) and bicuspid aortic valve disease (BAV). Currently treatment options consist of invasive surgeries to either repair or replace the damages valve tissues. Evidence suggests that both MVP and BAV are congenital diseases that present complications in patients later in life. Treatment options for these diseases are lacking due to an incomplete understanding of the molecular causes.

Mitral valve prolapse (MVP) is one of the most common forms of cardiac valve disease and affects ~2-3% of the human population. MVP can lead to secondary complications such as arrhythmias, heart failure, and sudden cardiac death and 1 in 10 patients will require valve surgery. There are no effective nonsurgical treatments for MVP and therapeutic efforts have been hindered by an incomplete understanding of its fundamental causes. One accessible source of such information may come from genetic studies of MVP. We previously reported familial and GWAS studies that identified genetic mutations and/or excellent candidate targets as causal to MVP. Pathway analyses suggested a common cellular and molecular thread between these studies and invoke the primary cilia as potential unifying mechanism. This discovery is further bolstered by our recent identification of a mutation in a cilia gene in a large family with MVP, DZIP1. Our data show genetic haploinsufficiency of primary cilia in cardiac valves leads to a non-syndromic mitral valve disease in mouse models whereas complete genetic ablation

enhances mitral valve phenotype severity and generation of bicuspid aortic valve (BAV). We present, for the first time, a potential common cellular and molecular thread through which MVP and potentially BAV can arise. These studies define the primary cilia as a critical, and previously unrecognized facet of cardiac valve development. Uncovering how valve disease genes regulate downstream signaling cascades will provide key mechanistic insights into MVP and potentially BAV pathogenesis at a cellular and molecular level.

# CHAPTER 1: INTRODUCTION

In the United States it is estimated that 92.1 million adults have at least one type of cardiovascular disease, a number that is projected to grow to around 140 million US adults in 2030 (Benjamin, Blaha et al. 2017). One of the most common cardiac diseases in today's society is heart valve disease with a prevalence of about 5%, causing over 24,000 deaths each year in the United States with no effective non-surgical treatment (Benjamin, Blaha et al. 2017). Heart valve disease can either be acquired or congenital and shows variable expression and severity. Thus, depending on the type of valve disease, heterogeneity and variability in age of clinical presentation is the rule versus the exception. In addition to heart valve disease being a major cause of morbidity and mortality in the aged population, congenital or developmental defects in the cardiovascular system are the most common cause of infant deaths. How these defects occur, the mechanisms that result in the disease progression, and potential treatments options are poorly understood. The overall goal of my research throughout the past 5 years was to take genetic information from patients with common cardiac valve diseases and elucidate the reason that valve diseases occur. Our studies have led to the uncovering of new molecular and cellular mechanisms during valve development. The following chapters will detail how we identified mechanisms underlying valve development and disease, the specific types of valve diseases we studied, and potential to harness this information for new treatments beneficial to patients with valve diseases.

**Cardiac Valves:**

The mammalian heart is composed of four chambers: 2 atria and 2 ventricles. Controlling unidirectional blood flow from the atria into the ventricles and out through the arterial connections are the cardiac valves (Fig 1.1). The atrioventricular mitral and tricuspid valves are positioned at the intersection between the left atrium/ventricle and the right atrium/ventricle, respectively. These sets of valves are critical for regulating proper blood flow that enters the heart and passes through the atria to the ventricles and are thus called "in-flow" valves. During ventricular contraction, these valves shut and blood exits through outlet segments. Blood from the left ventricle exits through the aortic valves, into the aorta and serves to feed the body. The deoxygenated blood subsequently returns to the right side of the heart and, upon right ventricular contraction, is pumped out through the pulmonic valves and into the pulmonary arteries on its way to the lungs (Fig 1.1). Oxygenated blood returns to the left side of the heart, and the cycle continues for the duration of life. Defects in the function of any of these valves results in changes in the proper cycling of blood and can lead to serious health complications and death. Thus, understanding how valve defects occur is critical for improving patient health. Defining how valves form during development will likely provide key insight into how their dysfunction occurs, with the hypothesis that altered construction of these tissues will engender life-long defects that effect morbidity and mortality.

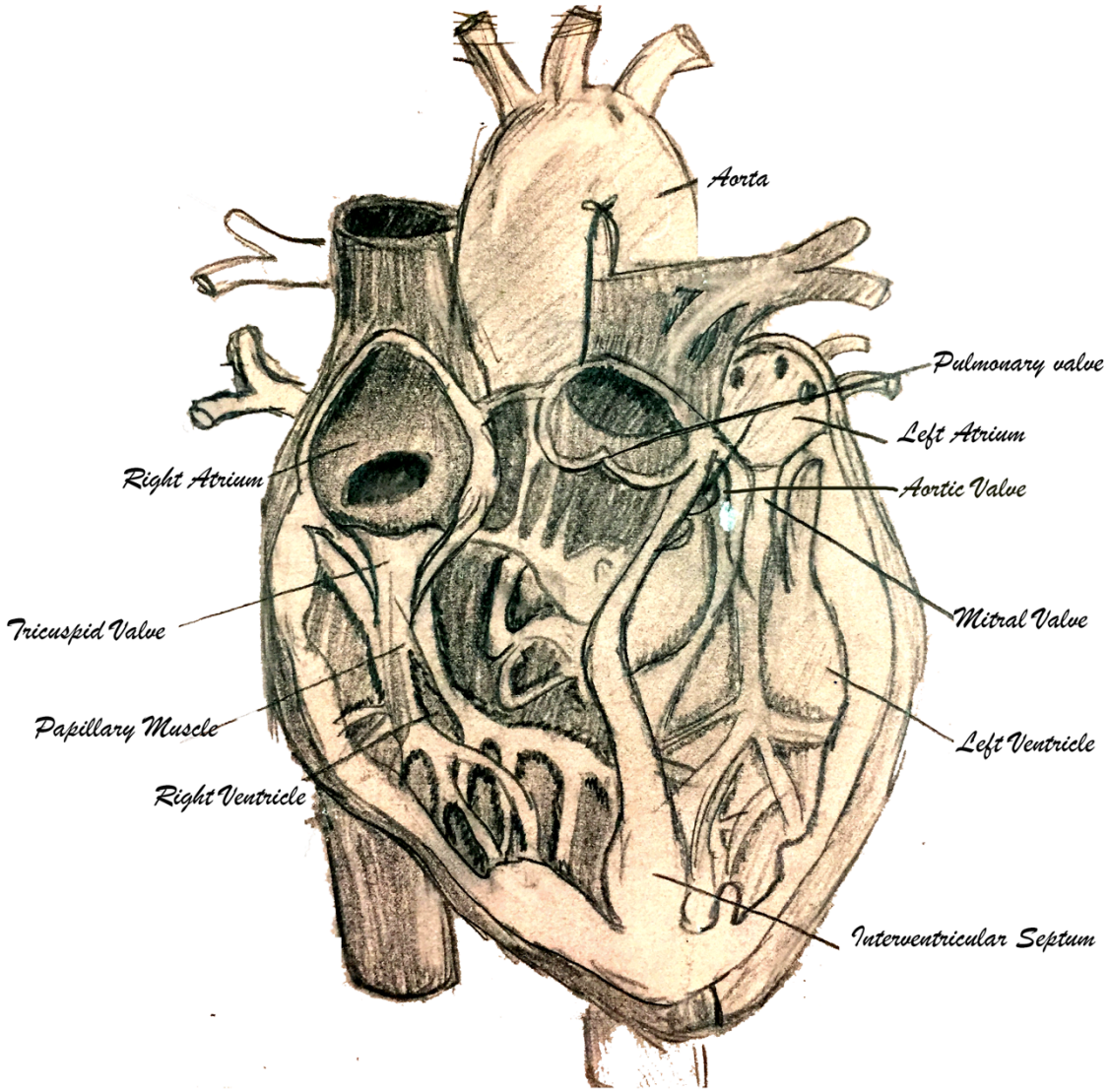


**Figure 1.1 The Human Heart.** Sketch showing the inside of a human heart. Oxygenated blood flows into the left atria from the lungs and travels through the mitral valve into the left ventricle. During contraction blood then moves through the aortic valves into the aorta and to the rest of the body. De-oxygenated blood enters the heart through the superior vena cava into the right atrium through the tricuspid valve and into the right ventricle. De-oxygenated blood then leaves the heart traveling through the pulmonary valve and out the pulmonary artery to the lungs. Drawing by Rebecca Stairley.

**The Developmental Origin of Cardiac Valves:**

During development the heart valves form from rudimentary structures called "endocardial cushions". The outflow tract cushions develop into aortic and pulmonary valves (cusps) while the atrioventricular (inflow) cushions develop into mitral and tricuspid valves (leaflets). Orchestration of how these cushions form and mature into valve leaflets or cusps is complex and requires the integration of biomechanical stimuli, extracellular signals, intracellular signaling cascades and transcriptional regulation of target genes, all occurring in the heart which is continually beating. Alterations in any steps of the developmental process are known to cause defects in valve formation. Based on these previous studies, many valve diseases are known to have a developmental origin and underscore the importance of defining how the integration of molecular, mechanical and biochemical cues provide a road-map for valve development.

The structure and function of the valves are dependent on how they are built during development. Cardiac valve development occurs in multiple stages; formation of the cardiac jelly, migration of endocardial cells into the cardiac jelly, proliferation and matrix deposition of post endothelial to mesenchymal transformation (EMT) cells, matrix compaction and elongation and stratification of valve matrix into distinct layers. Cardiac development begins with the migration of two populations of cells from the primitive streak, forming the primary and secondary heart fields. Around E8.0 in mouse, these two cell populations fuse to form the cardiac crescent (Combs and Yutzey 2009, Garside, Chang et al. 2013). The primary heart field cells give rise to the left ventricle, atrioventricular canal, and part of the right ventricle and atria while the second heart field contributes to the right ventricle, atria outflow tract, and interventricular septum. Around

E8.5, the cardiac crescent fuses, thereby generating a linear heart tube. The heart tube consists of an outer myocardial layer and inner endocardial layer which are separated by a layer of extracellular matrix called the cardiac jelly composed mostly of proteoglycan glycosaminoglycans. This tube loops rightward to form the four-chambered mammalian heart. After looping to the right, the heart tube forms specified structures such as the atrioventricular (AV) junction and ventricular outflow tract (OFT). At this stage, myocardial cells begin secreting ECM. The initial stages of valvulogenesis begin with the formation of the atrioventricular (AV) and outflow tract cushions (OFT). The mural leaflets of the AV valve form through the formation of lateral cushions on the left and right side of the AV junction (Wessels, Markman et al. 1996). The superior and inferior endocardial cushions fuse together to form components of the AV septum which later fuses with the developing ventricular septum, mesenchymal cap and dorsal mesenchymal protrusion (DMP). Together these different structures form the valvuloseptal complex (Perez-Pomares, Phelps et al. 2002). This structure forms the anterior leaflet of the mitral valve and septal leaflet of the tricuspid valve.

The cardiac cushions form when myocardium secretes ECM, called cardiac jelly, into the interstitial space between the endocardium and myocardium. This matrix is composed of collagens (I, III, IV), glycoproteins (fibronectin, laminin, fibrillin), and mucopolysaccharides (glycosaminoglycans like chondroitin sulfate and hyaluranate) (Manasek 1970). As the cushion matures (E9.5-10.5), endocardial cells undergo endothelial to mesenchymal transformation (EMT) and migrate into the cardiac jelly (Markwald, Fitzharris et al. 1977). In order for cells to undergo this process, they must lose cell-cell junctions, cell polarity, transition into mesenchymal cells, and acquire a

migratory phenotype (Garside, Chang et al. 2013). Pioneering work by Markwald and Bernake in 1982 showed that a subset of endocardial cells from explanted AV canals, isolated prior to EMT were capable of transforming into mesenchymal cells with invasive, migratory potential (Bernanke and Markwald 1982). They, and others, also showed that this process is dependent on signals from the myocardium (Runyan and Markwald 1983, Mjaatvedt, Lepera et al. 1987).

Two major players in this EMT process are TGFβ and BMP. These signals are important for embryonic development as deletion of either TGFβ or BMP result in embryonic lethality. In early cushion formation, BMP is required for the activation of the endocardium, formation of the cardiac jelly, and for initiation and formation of the cushions. BMP2 secreted by the myocardium is necessary for AV valve EMT while BMP4 is necessary for OFT cushion growth. Deletion of BMP2 specifically from the myocardium using Nkx2.5-Cre results in failure of cushion formation (Ma, Lu et al. 2005). Mice lacking the BMP receptor ALK2 fail to transform into mesenchymal cells and result in embryonic lethality(Wang, Sridurongrit et al. 2005).

TGFβ also plays a critical role in the EMT process through promotion of mesenchymal invasiveness (MacGrogan, Luxan et al. 2014, Bosada, Devasthali et al. 2016). TGFβ 1 and 2 are expressed during early cushion formation in the cardiac cushion mesenchyme and by the endocardium prior to invasion. TGFβ3 however is expressed after EMT and is not expressed in the endocardium suggesting its role may be in the later remodeling phase of valve development. Experiments show TGFβ is sufficient to induce EMT in chick explants. Treatment of explants with TGFβ1 induces EMT in the absence of the normally required AV myocardium (Potts and Runyan 1989,

Ramsdell and Markwald 1997). Additionally, neutralizing antibodies for TGFβ1 or TGFβ3 prevent the formation and migration of cushion mesenchymal cells.

After receiving signals from the myocardium, endocardial cells then have to disconnect from each other in order to migrate into the cardiac jelly. Notch1 signaling during this time is important for cells undergoing EMT to delaminate from the endocardial layer. In Notch signaling mutants, cells fail to delaminate and migrate into the ECM. It is thought that Notch signaling induces expression of pro-migratory transcription factor Snail which directly represses VE-cadherin promoter activity (Combs and Yutzey 2009). Loss of endocardial cell-cell adhesion during EMT is associated with down regulation of VE-cadherin as well as neural cell adhesion molecule (N-CAM), vascular endothelial (VE)-cadherin, and platelet endothelial cell adhesion molecule 1 (PECAM-1)(Crossin 1991, Baldwin, Shen et al. 1994).

In addition to cells undergoing EMT a subpopulation of endothelial cells remain lining the cushion where they are necessary in forming a tight protective boarder. The endocardial transcription factor, nuclear factor of activated T cells-c1 (Nfatc1), is important in maintaining endothelial phenotype and promoting proliferation of endothelial cells which do not undergo the EMT process (Sugi, Yamamura et al. 2004, Wu, Wang et al. 2011, MacGrogan, Luxan et al. 2014, Bosada, Devasthali et al. 2016, Saxon, Baer et al. 2017).These endothelial cells do not undergo EMT and are maintained as the boundary of cushion development.

After migrating into the cardiac jelly, EMT derived cells undergo a highly proliferative stage in order to populate the forming tissue. Fibroblast growth factor (FGFs) promote post-EMT mesenchymal cell proliferation. Treatment of chick

atrioventricular valve cushion (AVC) explants with FGF results in increased cell proliferation (Sugi, Ito et al. 2003). Epidermal growth factor (EGF), however, has the opposite effect. EGF inhibits BMP signaling which is necessary for activation of SMAD1/5/8. Studies have shown mutations causing reduction in EGF signaling result in increased cell proliferation through activation of BMP (Person, Klewer et al. 2005). Mesenchymal cells during this stage in valve development express twist1, Msx1/2, Sox9 and Tbx20, factors that have been shown to be important for promoting proliferation (de Vlaming, Sauls et al. 2012).

Around E14.5 many cell processes are occurring including cell migration and ECM secretion and remodeling. Cells adopt a fibroblast-like phenotype where they produce fibrillar collagens and become contractile, breaking down and synthesizing matrix. These cells then express alpha smooth muscle actin (aSMA) a marker of activated fibroblasts. These processes are less well defined although some progress has recently been made. During this stage in development, ECM compartments form and ultimately results in the beginning of zonal boundaries demarcated by areas of collagen and proteoglycan secretion. Additionally, between E14.5 and E18.5 cell density increases with condensation occurring first at the atrial side and expanding towards the ventricular side (Kruithof, Krawitz et al. 2007). Valvular interstitial cells increase expression of N-cadherin, proliferating cell nuclear antigen (PCNA) and αSMA. N-cadherin promotes cell adhesion and is necessary for cell alignment and leaflet elongation. Improper alignment of cells within the leaflet results in disorganized matrix production and ultimately valve disease. At this stage cell polarity is important in order to begin properly aligning cells within the tissue.

Deposition and remodeling of matrix through compaction is also occurring at these later stages of valve development (E17.5-postnatal stages) as indicated by increase αSMA and BMP expression (Kruithof, Krawitz et al. 2007). BMP, periostin, and Nfatc1 play important roles in this remodeling process. BMP is important in this stage of development as well because it regulates expression of Tbx2. Mice lacking BMP2 expression fail to express Tbx2 which is necessary for increased ECM deposition (Combs and Yutzey 2009). Periostin, a matricellular protein involved in connecting cell surface to structural ECM components, is also highly expressed. Studies have shown that periostin plays an important role in compaction of the cushion mesenchyme and is important for valve development (Ghatak, Misra et al. 2014). Loss of periostin results in a non-compacted myxomatous-like phenotype (Norris, Borg et al. 2008).

Filamin-A (FLNA) is also very important for the remodeling stages of valve development. FLNA is a cytoskeletal actin-binding protein that when removed causes cardiac valvular dystrophy. Functionally, FLNA cross-links cortical actin filaments into three dimensional structures. Knockout mice have defects in post-EMT cardiac morphogenesis suggesting its role in valve remodeling. Other proteins expressed in the valve during this remodeling phase of development include the small GTPases Rho and Rac. These proteins function to interact with the actin cytoskeleton in order to compact the ECM.

Mesenchymal cells stop expressing Twist1, Tbx20, Msx1, and Msx2 and begin to express markers of more differentiated lineages (Tao, Kotick et al. 2012). Interestingly, these markers often are similar to those found in cartilage and early bone formation (Combs and Yutzey 2009). Condensation of matrix in skeletal development is necessary

for patterning of condensed areas into bone. For this reason, expression of these markers in valve development might be required for ECM patterning into specified matrix regions. In the condensing late embryonic valves, BMP2 signals activate Sox9 transcription factor and expression of aggrecan. In addition, FGF4 activates scleraxis and tenascin, genes that are characteristic of the collagen rich fibrosa layer of the valve.

The valves continue to develop during late gestation and into postnatal times changing to accommodate different hemodynamic forces and molecular signals. Cells in postnatal valve leaflets continue to remodel matrix and begin the elongation phase where proliferation within specified regions and apoptosis in other regions occurs. This is necessary in order to regulate valve growth to form a thin elongated valve. Molecular signals from FGF4 stimulates proliferation in the distal tip, while EGF and TGFβ 2 prevent proliferation at the base of the developing valve (Sugi, Ito et al. 2003, Butcher and Markwald 2007).

Finally, the valve undergoes ECM stratification and development of distinct spongiosa and fibrosa regions composed of mainly collagens and proteoglycans, respectively (Riem Vis, Kluin et al. 2011). Elastin expression is localized to the flow side of the valve while collagens are expressed in the fibrosa layer away from blood flow(Hinton and Yutzey 2011). The developmental and molecular mechanisms regulating valve stratification are currently not known.

**Valve Cells Types**

The postnatal valve is composed of valvular interstitial cells (VIC), which constitute the majority of the valve and are typically fibroblastic, and valvular endothelial

cells (VEC) which cover the valve and provide a tight outer boarder. VICs are believed to have many different characteristics depending on their location within the tissue. In fact, some people believe there are at least 5-10 different phenotypes of a valve interstitial cell (Liu, Joag et al. 2007, Horne, VandeKopple et al. 2015). These cells can range in phenotype from matrix remodeling activated fibroblast that express aSMA to dormant VICs that populate the leaflets. VECs are critical for maintaining homeostasis in the valve through regulations of inflammation and vascular remodeling(Butcher and Nerem 2007). Disruption of the endothelial boarder has been shown to lead to valve disease (Leask, Jain et al. 2003, Butcher and Markwald 2007, El-Hamamsy, Balachandran et al. 2009). During disease, VECs have been shown to undergo EMT and repopulate the valve (Bischoff and Aikawa 2011).

VICs are the cells that populate the valve and are responsible for matrix deposition and maintenance of the tri-layer ECM structure. They have great plasticity and can change back and forth between fibroblasts and smooth muscle cells secreting both matrix and matrix metalloproteases (MMPs). In healthy valves VICs are primarily quiescent however in disease they become activated and can become contractile and migratory(Rabkin-Aikawa, Farber et al. 2004). VICs can respond to biochemical and biomechanical stresses. For example, ex-vivo stretching can alter transcriptional responses and lead to increased expression of inflammatory genes. (Warnock, Nanduri et al. 2011). In addition, in experiments when valves are removed and placed under planar tension they up-regulate expression of $\alpha$-smooth muscle actin. This leads to remodeling of ECM components through activation of RhoA (Gould, Yalcin et al. 2016).

Valve endothelial cells (VECs) also play an important role in valve biology. VECs are responsible for serving as a boundary between VICs and the hemodynamic environment of the heart. Since VECs are in contact with the circulation, they are exposed to shear stress and circulating signaling molecules, cytokines and other risk factors such as cholesterol, lipids and inflammatory cells (Huk, Austin et al. 2016). In this way VECs can also signal to underlying VICs to influence behavior and ECM production. Studies have shown that TGFb1 expressed by VECs can induce expression of Sox9 in developing VICs (Huk, Austin et al. 2016). In aortic valves, it has been shown that the VECs play a role in VIC response to stress, again demonstrating the importance of a proper endothelial border (Butcher and Nerem 2006).

An additional cell type that is sometimes found in the valve are hematopoietic derived cells. This cell type is less well described and are thought to be derived from either infiltrating circulating cells or activation of osteoblastic phenotypes in resident VICs(Liu, Joag et al. 2007, Huk, Austin et al. 2016). In normal tissue and disease, circulating immune cells positive for CD45, infiltrate the valve tissue and have the ability to remodel matrix (Barth, Koster et al. 2005, Hajdu, Romeo et al. 2011). Our group has shown that in both humans and mice with myxomatous valve disease there is an increase in hematopoietic cell engraftment (Sauls, Toomer et al. 2015). Localization of these cells, in disease states, correlates with increased MMP2 expression suggesting that these cells are promoting myxomatous degeneration through a paracrine mechanism with resident VICs.

Although a large majority of the processes during valve development are the same among the different valves, there are some molecular and hemodynamic differences. A

major difference between AV and OFT development is the contribution of different cell types and biomechanical forces. The consequences of these differences are not fully appreciated but are believed to contribute to the unique tissue-specific anatomical functions of each valve tissue.

**Outlet Valves**

The semilunar valves develop from two distinct cell populations, endocardial cells that undergo EMT and contribute to the proximal cushions, and neural crest cells (NCCs) that migrate to the distal cushions from the dorsal neural tube (Kelly, Brown et al. 2001). Early in development, NCCs from the aortic arch migrate to the developing cushion. At the same time, a subpopulation of cells undergo EMT and invade the cushions. Fusion of these two cell populations is essential for the formation of the aortic and pulmonic outlets. Shortly after the cushions are formed the intermediate part of the outflow tract splits into the aorta and the pulmonary trunk. This process is facilitated by migration of cells from the second heart field and fusion of two different populations of neural crest cells (Spicer, Bridgeman et al. 2014).

**Inlet Valves**

In addition to endocardially derived cells, the AV valves are also composed of epicardially derived cells (EPDCs). Epicardial cells expressing WT1 migrate into mainly the mural leaflets but also the septal leaflets beginning around E12 (Wessels, van den Hoff et al. 2012). As the valves continue to develop, between E12 and postnatal day 0, more of the leaflets are populated with EPDCs. In the mural leaflets, which are largely

derived from the lateral atrioventricular cushions, the EPDCs replace a significant amount of endocardially derived cells whereas the septal leaflets have very few EPDCs as a contributing cell population (Wessels, van den Hoff et al. 2012). This process is regulated, in part, by signaling of Bmp2 through its receptor Alk3. Loss of Alk3 in EPDCs results in loss of EPDC migration into the mural or posterior AV valve leading. Loss of this signaling pathway leads to enlarged valves during development and myxomatous valves in adult mice(Lockhart, Boukens et al. 2014).

**Biomechanical Influences**

Biomechanical forces are also a differing factor in the development of AV and OFT valves. It is well established that hemodynamics play a significant role in the development of the cardiac tissues. In the mature heart, blood flows from areas of low pressure regions such as the atria to those of high pressure regions in the ventricles. For this reason, the AV valves experience different pressures than the OFT valves. Studies have shown that the mechanical forces of the beating heart during development influence the formation and matrix composition of the valves (Butcher, McQuinn et al. 2007). Matrix within the valves is organized within specific regions based, at least partly, on the hemodynamic forces the valves are exposed to. In addition, the leaflet shape and constrains felt by the AV and OFT valves are different. In the mitral valve, the leaflets are connected to tendinous chords, which attach to the left ventricular myocardium via the papillary muscle. These fibrous structures provide tension to the mitral leaflets on a beat-by-beat basis and keep the tissues from billowing back into the left atrium. The

outflow tract cusps, alternatively, do not have chordal structures and attach directly to the wall of the aorta.

A study looking at postnatal valve remodeling showed that mitral valve leaflets and aortic cusps behaved differently in response to biomechanical forces. Mitral valve leaflets that underwent sustained increase in systolic load undergo significant lengthening compared to controls while there was no change in aortic valve annulus (Tibayan, Louey et al. 2015). The authors speculated that this change was caused by differences in the support systems of the different valves allowing the aortic valve to be resistant to load-induced dilation. While dilation of the aortic annulus doesn't change in response to biomechanical load, studies have shown that constriction of OFT during chick development results in decreased OFT cushion volume and altered gene expression in the cushions (Menon, Eberth et al. 2015, Menon, Eberth et al. 2017).

**Parallels Between Valve and Limb Development**

A parallel can be drawn between cardiac cushion development and limb bud development. Similar developmental processes occur in each tissue. Expression of cartilage, tendon, and bone are important for heart valve maturation and ECM remodeling. During disease development ECM boundaries are lost resulting in defects in mechanical function of the valve. Often, heart valve disease such as Marfans, Ehlers-Danlos, and Stickler syndrome, occur because manifestation of connective tissue disorders that affect cartilage, tendon, bone and skin (Lincoln, Lange et al. 2006).

Protein expression during limb and valve development are also very similar. Sox9, BMP2, and aggrecan, for example, are all important for differentiation and

remodeling in both tissues. In the developing limb bud BMP induces expression of Sox9 and aggrecan which are necessary for differentiation of chondrocytes into bone (Akiyama, Chaboissier et al. 2002). This pathway is also expressed during valve development. BMP2 is highly expressed by the developing cushion tissue where it also induces Sox9 and aggrecan expression (Lincoln, Kist et al. 2007). Sox9 during late embryonic valve development is necessary for proper valve remodeling.

Signaling mechanisms between the two tissues seems to also be conserved. In the limb bud, distinct zonal boundaries are defined by secreted signals in those locations, one of which is hedgehog signaling. In the valve, these regions coincide with differential gene expression of proteins such as collagens and proteoglycans. Currently the molecular cues that cause zonal boundaries and cellular phenotypic changes during valvulogenesis are unclear. However, one can postulate that similar to the limb bud, these signals most likely will consist of gradient of morphogens and differences in receptor expression (Coutinho and Antunes 2017).

Strong evidence has been provided showing a developmental basis for many valve diseases (Garg, Muth et al. 2005, Hinton and Yutzey 2011, Durst, Sauls et al. 2015). Therefore, it is important to recognize that mutations that occur at conception might be asymptomatic at birth and through childhood but may result in disease phenotypes later in life. Mitral valve prolapse for example has been described in the past as a disease of aging, however recent studies from our lab and others have shown that MVP is in fact a congenital heart disease with defects in valve development resulting in impaired adult valves (Durst, Sauls et al. 2015). This idea, that developmental changes can cause disease later in a patient's life appears to be a reoccurring theme, as our lab and

others have noticed (Hinton, Alfieri et al. 2008, Markwald, Norris et al. 2010, Norris, Moreno-Rodriguez et al. 2010, Lockhart, Boukens et al. 2014, Durst, Sauls et al. 2015, Prakash, Borreguero et al. 2017). Another well studied congenitally based cardiovascular valve disease is bicuspid aortic valve (BAV). Patients with BAV also have developmentally based valve disease that progresses overtime into a myxomatous phenotype. The mechanism by which these diseases occur and progress is poorly understood and currently the only treatment option is surgery for valve repair or replacement. Therefore, understanding these developmental processes is essential for determining disease mechanisms and ultimately therapeutic targets.

**Bicuspid Aortic Valve Disease**

Bicuspid aortic valve (BAV) disease is one of the most common congenital valvular heart defects affecting between 0.5-1.2% of the population(Martin, Kloesel et al. 2015, Martin, Lorca et al. 2017). BAV is just one of many left-sided heart defects such as aortic stenosis, coarctation of the aorta, mitral valve abnormalities and hypoplastic left heart (Martin, Kloesel et al. 2015). BAV is a congenital heart defect that results in defects in the formation of the three aortic cusps, either by failure of one of the cusps to form or fusion of two cusps. Often, BAV occurs with other aortic valve diseases such as aneurism, infective endocarditis, calcific aortic valve, and thrombus formation (Abdulkareem, Smelt et al. 2013). Patients with BAV have a higher occurrence of aortic valve replacement and at a younger age than population norms (Joziasse, Vink et al. 2011). Clinically, BAV is usually non-syndromic but can be diagnosed in patients that have other confounding diseases such as Marfan syndrome, Holt-Oram syndrome,

ventricular noncompaction, adult-onset aortopathy, and Loeys-Dietz syndrome(Martin, Kloesel et al. 2015). The etiology of non-syndromic BAV is still unclear, although evidence suggests some form of genetic inheritance. Familial studies suggest an autosomal dominant method for inheritance, with implications for Notch1 and GATA5 as causal (Huntington, Hunter et al. 1997, Bonachea, Chang et al. 2014).

BAV is diagnosed primarily by transthoracic echocardiography but sometimes is confirmed by transesophageal and 3-dimensional echocardiography. Functionally, BAV causes aortic regurgitation, aortic stenosis, infective endocarditis and dilatation of the thoracic ascending aorta. In addition, BAV patients often have an increased risk of aortic dissection and a high incidence of associated thoracic aortic aneurysm (TAA) formation. Around 50% of individuals with BAV also develop thoracic aortic aneurisms with 50% of those patients affected with aortic dissection and often death (Nadorlik, Bowman et al. 2017).

Patients with BAV often are associated with increased prevalence of aortic aneurysm (AA). AA is described as an increase in aortic size above the normal threshold set for characteristics such as age, gender and body mass index. In general, the growth rate for AA is between 0.1 and 1.0 cm/year (Elefteriades 2010). The prevalence of ascending AA with BAV ranges from 35-80% depending on the study criteria; however, patients with AA have a substantial increase in morality risk once the aortic diameter reaches 6cm (Davies, Gallo et al. 2006).

Multiple factors, such as genetic predisposition, epigenetic modifiers, age, diabetic status, and molecular regulation of MMP's and TGFβ's, are at play regarding the development of BAV and aneurysm. Genes such as ACTA2, MYH11, FLNA, and

SMAD3 have been identified as contributors for non-syndromic AA while FBN1, TGFBR1 and TGFBR2 have been implicated in the development of syndromic AA (Abdulkareem, Smelt et al. 2013). This information may be useful in discovering a mechanism for BAV as no genes have been found that prove to be conclusive for BAV with AA.

One of the most common comorbidities associated with BAV is calcific aortic valve disease (CAVD). In fact, patient with BAV are more likely to develop CAVD with more than 50% of patients having severe aortic stenosis and requiring valve replacement(Peeters, Meex et al. 2017). CAVD develops when the mesenchymal cells of the aortic valve differentiate into bone precursors and start producing excess calcific ECM. During CAVD genes expressed during early valve development such as Sox9 are reactivated along with regulatory proteins that promote the development of cartilage and bone lineages(Wirrig and Yutzey 2014). CAVD occurs in two steps, first endothelial cells are activated or damaged leading to an immune response and second, fibrosis and increased calcification occurs. These changes then lead stiffening of the aorta and valves resulting in improper hemodynamic forces. CAVD is caused by complex interactions between multiple risk factors such as genetic mutations, environment, and biomechanics(Huk, Austin et al. 2016). Currently there is no treatment other than surgery because of the limited knowledge of underlying mechanisms.

The mechanisms underlying CAVD is still poorly understood, however, evidence suggests that disease progression is driven by Notch, osteoprotegerin, Wnts and BMPs. Calcification has been seen in adult mice with mutations in Notch1 or RBPJ that have been fed a high fat diet (Garg, Muth et al. 2005, Nus, MacGrogan et al. 2011). In

addition, mice with endothelial deletion of the Notch1 ligand Jag1 have valve calcification later in life. It is believed that this process occurs through regulation of Hes and Hey, downstream transcription factors in the Notch pathway that regulate transcription of Runx2. In addition to Notch signaling, regulation of Sox9 may be important for development of calcific valves. Heterozygous loss of Sox9 using a Col2a1 cre results in aortic valve calcification (Lincoln, Kist et al. 2007).

In nearly all cases of severe BAV aortic dilation occur resulting in altered hemodynamic stresses and triggering increased MMP release. There are two theories for why the aortic dilatation occurs; either from hemodynamic stresses felt because of the bicuspid morphology or through disruption of developmental programs(Jain, Engleka et al. 2011, Longobardo, Jain et al. 2016). Often in BAV patients a wrinkling and folding of the cusp can occur causing an abnormality in the blood jet that is formed when compared to normal tricuspid aortic valves (Abdulkareem, Smelt et al. 2013). However, abnormal flow patterns cannot account for all BAV as hemodynamic forces do not correlate with aneurysm size seen in BAV patients (Martin, Kloesel et al. 2015).

The genetic underpinnings of BAV are still unclear although familial studies have provided evidence for a potential autosomal-dominant pattern. Family recurrence rate for patients with BAV is approximately 35% (Huntington, Hunter et al. 1997). Family members of patients with BAV often display BAV as well as other left-sided CHD. In seemingly sporadic cases of BAV, epidemiological studies have identified a 10% increased risk of BAV in siblings and offspring. For this reason, the American Heart Association and American College of Cardiology recommends screening of first-degree relatives of affected adults (Nadorlik, Bowman et al. 2017). Preliminary evidence shows

that children who may be asymptomatic are at risk for increased ascending aortopathies (Nadorlik, Bowman et al. 2017). While there appears to be a genetic link for BAV strong genetic evidence has not been provided to account for a large majority of cases. Although not inclusive, mutations in Notch1 and GATA5 have been identity in some patients with BAV. In a large study, a family with aortic valve disease was screened for candidate genes. Linkage analysis was preformed to identify a single locus on chromosome 9. From this screen, *NOTCH1* was identified and direct sequencing of *NOTCH1* in affected patients identified a mutation in the gene(Garg, Muth et al. 2005).

GATA5 has also been implicated in the development of BAV. Mice with endocardial cushion deletion of GATA5 results in right non-coronary BAV formation(Laforest, Andelfinger et al. 2011). GATA5 signals through both Tbx20 and Notch to regulate cell proliferation and migration of cells within the cushions. Loss of GATA5 results in decreased mesenchymal cells within the cushions. This however was not a very high penetrant phenotype with only 25% of knockout mice developing BAV.

Human studies have also suggested a link between BAV and GATA5 mutations(Padang, Bagnall et al. 2012, Bonachea, Chang et al. 2014). Four rare non-synonymous variations within the GATA5 transcriptional activation domains were found from a large screen of 100 patients with BAV(Padang, Bagnall et al. 2012). An additional screen for mutations in GATA5 was done on 78 patients with BAV(Bonachea, Chang et al. 2014). Sequencing data found two rare sequence variants in the GATA5 gene with an estimated prevalence of 2.6%. These studies provide evidence for GATA5 in the development of BAV however they are not explanatory in all cases. In addition to Notch1 and GATA5, links have been made between BAV and a variety of genes, Tgfβ,

KCNJ2, fibrillin, and nitric oxide synthase. More specifically, Tgfβ signaling has proved to be disrupted in patients with BAV and AA. During valve development, Tgfβ is important for EMT, cell migration and regulation of matrix deposition, processes that are often affected in patients with BAV. Mutations in Tgfβ have been found in humans with Loeys-Diets syndrome, a disease frequently diagnosed in patients with BAV(Loeys, Chen et al. 2005, Martin, Kloesel et al. 2015).

Since BAV is a congenitally based disease, understanding the mechanisms that occur during development are important for understanding disease pathogenesis. Currently, little is understood about how a bicuspid instead of tricuspid valve forms. It is possible that this occurs from fusion of two leaflets or failure of one of the leaflets to form (De Mozzi, Longo et al. 2008). Normally the two leaflets that form are of unequal size with the larger leaflet having a central raphe or ridge that results from fusion of the commissures (Siu and Silversides 2010). The presence and position of the raphe varies among patients and diagnosis of a raphe or tricuspid valve can be contested between surgeon and pathologists (Joziasse, Vink et al. 2011). The fusion of the leaflets can occur in three ways, right-left, right-non-coronary, and left non-coronary(Nadorlik, Bowman et al. 2017). The most common fusion is of the right and left coronary cusps affecting about 70% of BAVs. While right/left fusion is the most common, right non-coronary fusion is associated with BAV in patients. (Siu and Silversides 2010).

The aortic valves form from a multi-step process as described above. Briefly, formation of the valve starts with expression of ECM from myocytes and overlying endocardial cells forming the cardiac jelly, migration of cells into the jelly (EMT of endothelial and neural crest cells), proliferation and finally elongation of the cusps. It is

therefore logical that multiple mechanisms could explain improper formation of the cusps or the etiology of BAV. Improper migration of the neural crest cells as well as disruption in extracellular matrix deposition could be potential etiologies. More specifically, during development, disrupted Notch signaling can lead to disruptions in neural crest cell patterns resulting in BAV(Jain, Engleka et al. 2011).

Maintaining proper balance of ECM is important for aorta and valve biology and disruption of proper ECM maintenance results in disease. Mature aortic valve structure consists of three distinct layers, the fibrosa, spongiosa, and ventricularis. Each layer is composed of specified ECM which functions to facilitate a functional valve. Matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) are secreted by smooth muscle cells from the aortic wall, fibroblasts, and endothelial cells and regulate this balance of ECM. Disruption of this process results in dysfunctional valve cusps. Patients with BAV often have decreased aortic elasticity, reduced fibrillin-1 content and increased MMP activity supporting a role for disruption of ECM boundaries in valve disease (Joziasse, Vink et al. 2011, Abdulkareem, Smelt et al. 2013).

**Mitral Valve Prolapse**

In the 2017 American Heart Association report, mitral valve prolapse (MVP) was reported as the most common valvular disease. Worldwide, MVP has been shown to affect 1 in 40 individuals and for this reason it is a major health care burden (Freed, Benjamin et al. 2002). Originally described in the 1960's, MVP is described as a very common Mendelian cardiovascular disorder characterized by the systolic displacement of the mitral leaflets above the line of the annulus into the left atrium (Barlow and Bosman

1966). Structurally, the leaflets are described as myxomatous, or having increased proteoglycans, elastins, and fragmented collagens. Although often clinically benign, MVP is often associated with mitral regurgitation, bacterial endocarditis, congestive heart failure, atrial fibrillation and even sudden cardiac death (Freed, Acierno et al. 2003). Currently there isn't any non-surgical treatment for MVP most likely due to lack of understanding of the molecular and genetic causes of the disease. This lack of understanding combined with the increased population affected by the disease makes finding alternative treatments imperative.

MVP can be present with other diseases (syndromic) or independently (non-syndromic). Syndromic MVP can occur with diseases such as in Marfan syndrome while non-syndromic MVP is an isolated valve event. Clues from familial studies have shown non-syndromic MVP to be a genetically heterogeneous autosomal dominant trait that has both sex and age-dependent penetrance. (Freed, Acierno et al. 2003). Increasing evidence suggests that congenital valve malformations and disruption of developmentally regulated pathways may lead to MVP or myxomatous valve disease later in life.

MVP is characterized by the billowing of one or both leaflets back into the atria during ventricular systole. MVP patients have significantly elongated leaflets, increased left atrial size and mitral regurgitation(Freed, Benjamin et al. 2002, Freed, Acierno et al. 2003). The Framingham Heart study was pivotal because for the first time MVP diagnostics was described in detail by echocardiography(Freed, Levy et al. 1999). Until recently, published data suggested the prevalence of MVP to be between 5 and 15% even up to 35% in some studies (Freed, Levy et al. 1999). Many patients were misdiagnosed because clinicians did not understand the proper anatomical dimensions of the mitral

annulus. In this study, investigators were able to enroll large numbers of patients with familial MVP in order to determine the prevalence of disease in a way that used the proper anatomical dimensions of the annulus. Through this study the rate of false positive diagnosis has decreased with a prevalence of 1.3% of patients enrolled in the study being diagnosed with MVP. From this study a more concrete understanding of diagnosis was reached. Additionally, they found that complications that were often associated with prolapse such as heart failure, atrial fibrillation, cerebrovascular disease and syncope were not higher among subjects with MVP than among those without MVP.

In healthy mitral valves, the structure of the leaflets plays an important role in their proper function. The valve is composed of three distinct layers composed of collagens, proteoglycans and elastins. Both mouse and human valves appear to have these boundaries although there is slight variation on how many ECM zones exist. Structurally, patients with MVP have valves that have undergone myxomatous degeneration, which is characterized by excess deposition of proteoglycans and collagens with fragmentation of collagens and elastins. In addition to the myxomatous changes that occur in the valve, the endothelial boundary is often perturbed (Barlow and Pocock 1985, Anyanwu and Adams 2007, Bischoff, Casanovas et al. 2016). Structural changes within the leaflet during myxomatous degeneration prevent the leaflets from properly functioning. Over time, the constant mechanical pressures felt by the valve lead to elongated mitral chords resulting in a flailing leaflet. As a whole, changes in ECM, valvular interstitial cells (VIC) activation and valvular endothelial cells (VEC) disruption contribute to the anatomical defects that define the myxomatous valve phenotype.

Two types of myxomatous degeneration in patients with MVP have been described, Barlow's disease (BD) and fibroelastic deficiency (FED). BD valves tend to be thickened with elongated chordae, which sometimes can be thickened fused or even calcified. Structurally, these valves exhibit myxoid infiltration, blurring of the structural boundaries, and collagen alterations(Barlow and Pocock 1985, Anyanwu and Adams 2007, Hjortnaes, Keegan et al. 2016). BD valves are often much larger than normal valves with anterior leaflet size at 38mm or greater(Anyanwu and Adams 2007). Increased proliferation, and high proteolytic enzyme activity are often seen (Hjortnaes, Keegan et al. 2016). The annulus in patients with Barlow's disease is always dilated and often there is sever posterior annular dilation. Calcification of the annulus is sometimes found. In FED however the valve tissue is different. FED valves have connective tissue deficiencies. Instead of enlarged valves as seen in BD, they often have thinned valves as a result of deficiencies in collagen, elastins and proteoglycans with preserved leaflet architecture (Anyanwu and Adams 2007). Chordae in these patients tend to be thicker with increased elastin and collagen(Hjortnaes, Keegan et al. 2016). Molecularly, FED valves have increased elastin, pERK and SMAD2 (Hjortnaes, Keegan et al. 2016). In both diseases, activated VICs with increased a-SMA, vimentin, and MMPs are observed.

**Genetic Discoveries in MVP**

Determining the genetic contribution of MVP has proved to be a difficult undertaking. Prior to the proper diagnosis criteria clinicians based diagnosis on single-dimensional and eventually 2D echocardiography(Barlow and Bosman 1966). Using these parameters, diagnosis for MVP resulted in decreased specificity. It wasn't until the

saddle shaped description of the valve that specificity increased without loss of sensitivity(Freed, Levy et al. 1999). With proper diagnosis implemented and the phenotypic basis for MVP understood, researchers have been able to make advances on the genetic contribution to the disease. The first locus for autosomal dominant MVP, MMVP-1 was mapped in 1999 by Disse *et al*. Through familial screens, they were able to show significant linkage for markers mapping to chromosome 16p(Disse, Abergel et al. 1999). They also found that among the four large families, linkage could be confirmed on only two. This demonstrated the genetic heterogeneity of the disease. In 2003 a study by Freed et al discovered a second locus for MVP (MMVP2) mapping to chromosome 11p15.4 in a large family with inherited MVP(Freed, Acierno et al. 2003). The gene involved in X-linked myxomatous valvular dystrophy (XMVD), Filamin A, was discovered in 2007 by Kyndt et al. This was the first gene described as being responsible for isolated nonsyndromic valvulopathy. With the founding of the first ever transatlantic network to study mitral valve disease in 2006 the leading investigators, including members of our group, in mitral valve disease all over the world came together to find the causal genes involved in non-syndromic MVP. From this collaboration, our group was able to identify the first gene for MVP, *DCHS1*(Durst, Sauls et al. 2015). In this study, three large families with inherited MVP were studied. Deep sequencing and linkage analysis revealed a region on chromosome 11 as potentially causal. Capture sequencing of the linkage interval identified two mutations in the *DCHS1* gene among the three families(Durst, Sauls et al. 2015). In addition, loss of *Dchs1* as being causal was verified using a global heterozygous mouse model. This is the first mouse model for MVP described and is valuable for future studies of diseases mechanisms. These major genetic

studies have proved invaluable in further understanding the mechanism of disease progression and have been the starting point for this thesis work.

**Primary Cilia**

Current evidence from our lab and others in the literature has pointed to an association of mutations in genes involved in primary cilia signaling and function with cardiac diseases. Primary cilia can be thought of as the antenna of the cell. They have been shown to function as mechano-sensors and chemo-sensors to regulate many essential pathways(Pala, Alomari et al. 2017). Defects in either motile or non-motile cilia have been shown to affect almost all parts of the body, therefore illustrating the importance of proper cilia assembly and function. Primary cilia defects can be caused by multiple mechanisms such as defect in cilia formation, signal transduction components or trafficking.

Any defect in cilia structure or function is termed a ciliopathy. A large collection of ciliopathies have been described including Joubert syndrome (JBTS), Meckel syndrome (MKS), nephronophthisis (NPHP) and Bardet-Biedl syndrome (BBS) (Wang, Li et al. 2017). Interestingly these ciliopathies also are associated with valve disease (Karp, Grosse-Wortmann et al. 2012). Polycystic kidney disease (PKD), for example has been associated with MVP and increased prevalence of aortic aneurysm(Smyth, Snyder et al. 2003). Studies have reported that 25% of patients with PKD also have MVP(Lumiaho, Ikaheimo et al. 2001). In patients with JBTS, aortic stenosis and BAV have been

observed (Karp, Grosse-Wortmann et al. 2012). Bardet-Biedl syndrome (BBS) has been linked with both MVP and aortic valve deficiencies(Mukta, Tiwari et al. 2008, Purkait, Roy et al. 2012). The mechanism by which cilia dysfunction can cause valve disease and whether the valve defects in ciliopathy patients is a primary defect of disrupted cilia is not known. Better understanding of this link may provide valuable insight into currently lacking diseases etiology.

The observation of the cilia is as old as the discovery of the microscope, dating back to the times of Antoine van Leeuwenhoek in 1632 (Fuchs 1984). Cilia are microtubule-based structures that are composed of an axoneme, basal body, and transition zone. The axoneme acts as a molecular highway allowing proteins to traffic up and down carrying cargo (Fig 1.2). The basal body, which is originally derived from the centrosome of dividing cells, acts to anchor the axoneme in place. Almost all cells have either a single non-motile, primary cilium, or multiple motile cilia. These classifications help dictate the cilia's role in different tissues.

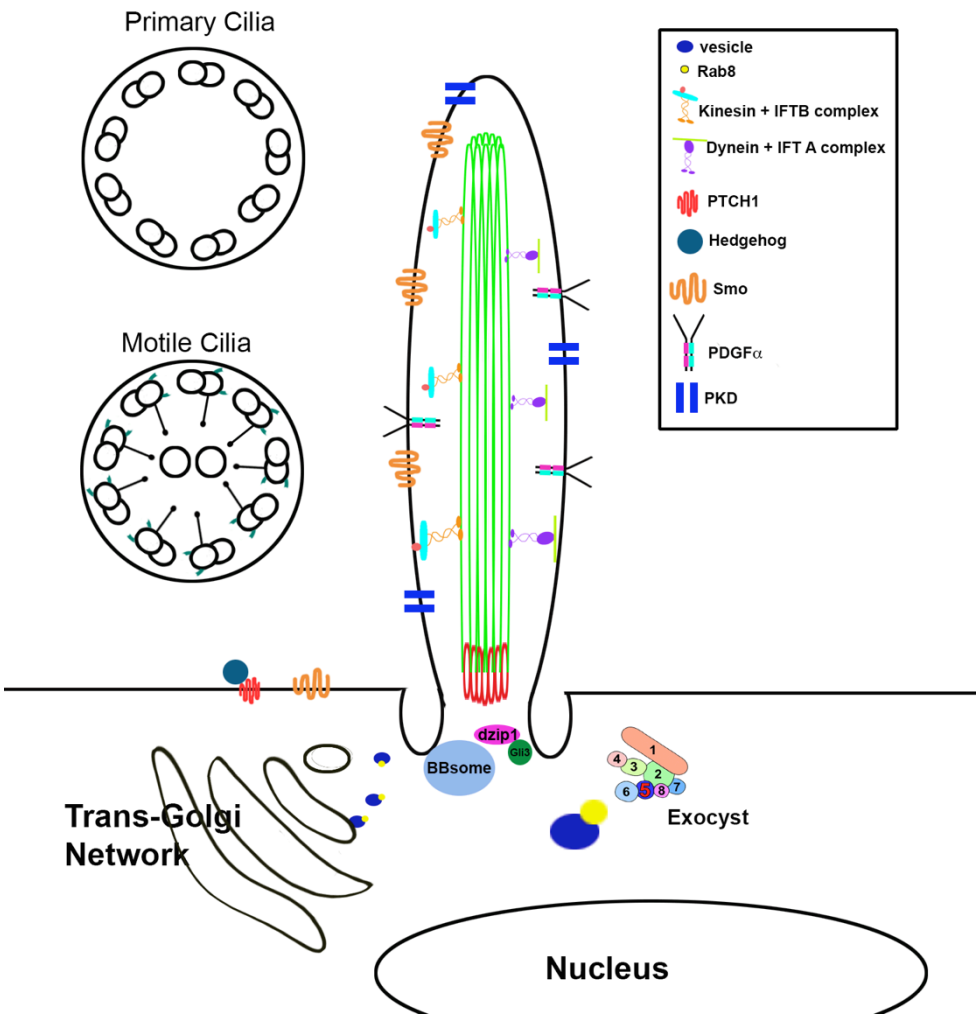


**Figure 1.2 The Primary Cilia is a Signaling Hub for the Cell.** Cartoon of the primary cilia showing the axoneme with microtubule structures in green and the basal body in red. Signaling molecules are found at the base, along the axoneme, and in the transition zone of the cilia. Proteins traffic to the cilia through the trans-golgi network. There they are packaged in vesicles attached to Rab8. Primary cilia are composed of a 9 + 0 outer microtubule doublets structure while motile cilia are composed of a 9 + 2 structure.

Motile cilia, also known as flagella in some cases, can be found as the propelling force of sperm, *Paramecium*, and in the lining of the trachea where they move mucus. They can be found in the developing embryo on nodal cells helping to direct fluid flow and determine left right laterality. Structurally, motile cilia are described as having a 9+2 microtubule doublet structure which connects to a central pair of microtubules to the axonemal inner and outer dynein arms in order to power the cilia and allow it to move. Primary cilia on the other hand are found one per cell and are non-motile. They act as molecular signaling antenna which relay information to the cell. The axoneme or antenna like structure of the primary cilia is surrounded by a lipid bilayer membrane that is continuous with the cell membrane but is enriched with specific proteins involved in signaling pathways. Many major signaling pathways such as Notch1, BMP, TGFB, PDGF, Hedgehog, and PKD, have been shown to signal through the primary cilia. Receptors from these pathways can be found on the axoneme or within the ciliary pocket near the basal body. Loss of the primary cilia structure has been shown to disrupt these signaling pathways and leading to changes in downstream gene expression.

The basal body of the primary cilia has been shown to be the point of localization of multiple proteins associated with cilia formation in addition to centrosomal and pericentriolar proteins. The ciliary membrane is contiguous with the plasma membrane of the cell, so in order to keep signaling compartmentalized, the cilium have a transition zone. The transitions zone acts as a gate allowing only proteins involved in cilia structure and/or function into the primary cilia.(Garcia-Gonzalo and Reiter 2017)

Proper cilia function is dependent on proper formation of the cilia, also known as ciliogenesis. This process is very complex and requires spatial and temporal expression

of a vast repertoire of ciliogenic proteins in a tissue and cell-specific manner. Briefly, ciliogenesis is composed of three steps. First, the mother centriole is modified to become the basal body at which point it attaches to the plasma membrane through its distal appendages. Then the transition zone is formed and finally the rest of the axoneme extends out from the cell body. The extension of the axoneme is constructed and maintained by the intraflagellar transport (IFT) machinery composed of kinesins, dyneins and IFTA and B molecules(Reiter and Leroux 2017). IFT proteins are essential for primary cilia structure and function. IFT-A and IFT-B form large platforms transporting cargo between the base and tip of the cilium. Ift88 is a component of the IFT-B complex along with Ift172, Ift52 and Ift57. Removal of any of these components results in loss of primary cilia and loss of response to Hh ligands. However, genetic ablation of the IFTA complex does not result in total loss of primary cilia, just abnormal formation. These mutants have increased Hh signaling as opposed to the lack of Hh signaling found in IFTB mutants. Loss of any of the IFT proteins result in embryonic lethality, polydactyly, randomization of the left-right body axis, as well as neural tube closure and patterning defects(Murcia, Richards et al. 2000, Haycraft, Zhang et al. 2007).

One very important pathway involved with proper formation of the primary cilia is the exocyst complex. The exocyst is an eight-member protein complex, originally discovered in yeast, whose function is essential for exocytosis and ciliogenesis(Lipschutz and Mostov 2002). To assemble the primary cilia, the centrosomes of the cells have to migrate to the apical membrane. The exocyst helps in this process by assisting in basal body migration, membrane docking and fusion of the ciliary vesicles at the site of cilium outgrowth. The exocyst also moves proteins to and from the primary cilia through

trafficking cargo from the ER-GOLGI membrane. In yeast, mutants of individual exocyst members results in failure of vesicles to dock or fuse with the plasma membrane causing accumulation of secretory vesicles within the cell. Loss of a key component of the exocyst, Sec10, results in loss of ciliogenesis and formation of cysts in kidney cells(Zuo, Guo et al. 2009).

**Ciliary Signaling**

Many signaling pathways have been shown to be reliant on the primary cilia(Pala, Alomari et al. 2017). Various membrane bound receptors, ion channels, transporters and downstream proteins localize to the primary cilia. Thus, cilia function as nodal signaling hubs that can respond to various biochemical and mechanical cues. Well studied pathways such as Notch, TGFβ, hedgehog, and PDGF, for example, rely upon the cilia for signal transduction. The niche environment of the primary cilia allows for a unique signaling opportunity not found elsewhere in the cell. The ciliary membrane is physically restricted and has different protein and lipid composition than the adjacent cell membrane (Nachury, Seeley et al. 2010). Additionally, the primary cilia has more membrane per area than any other area in the cell and for this reason small signaling changes can have a large effect and cell response. Changes in localization of receptors, binding of ligands, influx of calcium, and trafficking up and down the cilia have a large impact on the cell. All of which would not be possible in another organelle.

As shown in Figure 1, trafficking of various proteins to and within the primary cilium has multiple functions. By localizing proteins in a spatial and temporal pattern the primary cilium acts to coordinate signaling through regulating protein localization, gene

expression and protein-protein interactions. Regulation of signaling by the primary cilia can be accomplished by movement up and down the axoneme, restriction at the base or tip, processing by enzymes within and at the base of the primary cilia and post translational modifications. Membrane barriers and the transition zone within the cilia help to regulate these functions. In addition, trafficking of proteins through exosomes to the primary cilium is important for these processes. The primary cilia can facilitate budding of the ciliary membrane at the base in order to release exosomes into the extracellular space. In the kidney, these membrane bound vesicles carry important biomarkers of disease state (Chacon-Heszele, Choi et al. 2014). The exocyst complex is important for the formation of these vesicles along the ciliary axoneme forming cilia-interacting exosomes. In these ways various signals are relayed through the cell by the primary cilia.

**Hedgehog signaling**

One of the best studied cilia dependent signaling pathways is the hedgehog (Hh) pathway. Although cilia are not required for Hh signaling in *Drosophila*, they are required for mammalian Hh signaling. Members of the hedgehog signaling family have been shown to localize to the base of the primary cilia. Receptors patched (Ptch1) and smoothened (smo) traffic up and down the ciliary axoneme in response to any of the hedgehog ligands; sonic (Shh), indian (Ihh) or desert (Dhh). In the presence of hedgehog signal, patched removes repression of smo allowing it to traffic into the ciliary axoneme. Once in the axoneme, smo can then interact with Sufu suppressing its activity and allowing for processing of Gli family members (Gli1,2, and 3). Gli transcription factors

can exist in both full-length active form (GliA) and cleaved repressive form (GliR). In the absence of Hh ligand, Gli transcription factors are proteolytically cleaved into repressor forms.

IFT is required for production of GliA and Gli3R (May, Ashique et al. 2005). Disruption of the IFT proteins disrupts Hh signaling as well (Haycraft, Zhang et al. 2007). In mice with deletion of the IFT proteins, the Shh signaling pathway remains inactive. This is surprising since loss of cilia results in loss of Gli3 processing into its repressive form. However, this suggests that loss of cilia results in insufficient Gli processing. In addition, loss of IFT result in loss of Gli2 activation of the pathway. For the most part, Gli1 is independent of IFT signaling, except in a positive feedback mechanism since Gli1 expression is dependent on hedgehog pathway activation.

Each Gli transcription factor plays a role in a developmental and tissue specific manor. Gli1 is primarily an activator of the Hh pathway. Transcription of Gli1 leads to further activation of the pathway through activation of both Gli2 and Gli3. This feed-forward loop results in increased transcription of Gli1. Gli1 has been shown to regulate downstream transcription of extracellular matrix genes. For example, Gli1 activation leads to increased expression of collagen 1 as shown in pancreatic cancer studies (Duan, Ma et al. 2014). In addition, Gli1 can bind to an enhancer element on osteopontin (OPN) regulating its expression (Pritchett, Harvey et al. 2012).

The other major activator of the Hh pathway is Gli2. Gli2 activator is formed by a cilia and smo dependent process. Upon HH interacting with Ptch1, Ptch1 is removed from the cilia and allows Smo to enter the cilia. In the presence of Hh, Gli2 in complex with Gli3 and SuFu are trafficked to the tip of the cilia where they accumulate. At the tip

of the cilia this complex dissociates allowing Gli2 to become active. In models of fibrosis such as in the liver, Gli2 has been shown to be an important regulator of fibrotic signals. Gli2 is an upstream regulator of Sox9 and through this regulation is responsible for osteopontin expression in hepatic stellate cells (HSC). The HSCs become activated into proliferative myofibroblasts in response to injury and begin migrating into the surrounding parenchymal cells. Much like in a myxomatous valve they then begin to secrete ECM such as collagen 1 which is damaging to the tissue structure (Pritchett, Harvey et al. 2012).

Gli3 is critical for basal suppression of the Hh pathway. Gli3 repressor is formed in a protein kinase A (PKA) dependent manor through proteolysis. When cleaved, N-terminus acts as transcriptional repressor. Gli3 is negatively regulated by GPR161 which, in the absence of Hh signal, activates Gαs, thereby activating adenylyl cyclase increasing levels of cAMP and finally activating PKA. PKA then goes on to phosphorylate sites of Gli3, targeting it for proteolysis by βTrCP/Cul1. Gli3R then moves into the nucleus and suppresses Hh target genes. Binding of Hh to its receptor Ptch1 facilitates the removal of Gpr161 from the cilia thereby preventing further downstream processing of Gli3 and allowing it to remain in its full length active state. TULP3 regulates the localization of Gpr161 in the cilia. (Mukhopadhyay and Rohatgi 2014)

The HH pathway has many proteins which act to further enhance or repress the pathway. For example, Gli3 regulates expression of Gli1, which regulates expression of Ptch1. (Haycraft, Banizs et al. 2005) Loss of Polaris, also known as Ift88, results in increased Gli3A suggesting inefficient processing. Gli3R is able to repress the Hh pathway regardless of the presence or absence of Ift88; however, there is increased Gli3A

in the absence of Ift88. This suggest that small amounts of Gli3R is able to repress the pathway even in the presence of Gli3A. Experiments have shown that smo accumulates at the base of the cilia within 1hr of treatment with Shh. However, localization of smo to the primary cilia is not sufficient for Hh signaling. Experiments with loss of retrograde ciliary trafficking results in Hh insufficient phenotypes (Bangs and Anderson 2017).

**Receptor Tyrosine Kinases**

Reviewed in (Christensen, Clement et al. 2012), receptor tyrosine kinases such as platelet derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF1R), and fibroblast growth factor (FGF) have all been expressed at the axoneme of primary or motile cilia. RTKs can interact with other signaling pathways and have been implicated in cross talk with g-protein coupled receptors to regulate ECM production. Disruption of this process results in many human health problems and diseases that, at least in part, mimic defects observed in patients with known ciliopathies.

Platelet derived growth factor or PDGF is a receptor tyrosine kinase that is upregulated upon starvation and cilia formation (Clement, Mally et al. 2013). PDGFRα localizes to the cilia while PDGFRβ localizes along the cell membrane. PDGFRα signaling has been implicated in different types of cancers and is associated with fibrotic diseases (Christensen, Clement et al. 2012). In some cell types localization to the primary cilia is necessary for receptor dimerization and downstream signaling. PDGF signaling through the cilia can regulate cell cycle and cell migration. Stimulation of PDGFRα leads to activation of AKT and MEK1/2-ERK1/2 resulting in localization of the $Na^+/H^+$

exchanger, NHE1, to the leading edge of the cell which is necessary for cell migration(Clement, Mally et al. 2013). Genetic deletion of PDGFRα and β in mice results in loss of epicardial EMT, increased fibrosis and loss of vascular smooth muscle cell migration during vascular formation (Smith, Baek et al. 2011, Gallini, Lindblom et al. 2016).

**Calcium/PKD/ERK**

Polycystic kidney disease 1 (Pkd1) and 2 (Pkd2) have been well studied for their causal mutations in about 90% of patients with autosomal dominant polycystic kidney disease (Avasthi, Maser et al. 2017). Mutations in either *PKD1* or *PKD2* result in loss of polycystins to the ciliary membrane. Polycystin-2 interacts with polycystin 1 to regulate calcium signaling. Pkd1 regulates G-protein signaling pathway through binding to Gα subunits. Pkd2 functions as a calcium channel that is activated through bending of the primary cilia(Pala, Alomari et al. 2017). Pkd1 and Pkd2 dimerize and together function with ryanodine receptors to mediate transduction of extracellular mechanical stimulus into a calcium signaling response (Nauli, Alenghat et al. 2003). In addition to developing PKD, mice with these mutations have a high penetrance of cardiac malformations (Wu, Markowitz et al. 2000). One study looking at cardiac abnormalities in patients with autosomal dominant polycystic kidney disease (ADPKD) found 25% of the patients with PDK had MVP (Lumiaho, Ikaheimo et al. 2001). In addition to MVP, aortic regurgitation and left ventricular hypertrophy have been documented in patients with ADPKD(Hossack, Leddy et al. 1988, Martinez-Vea, Bardaji et al. 1999). These data suggest a potential role for cilia signaling in patients with MVP.

**Notch**

Notch signaling is very important during development in patterning the embryo. Notch receptors have been shown to signal though the primary cilia by regulating cleavage of Notch (Ezratty, Stokes et al. 2011, Pala, Alomari et al. 2017). Notch signaling occurs when ligands Delta or Jagged bind one of the four Notch receptors (Notch1-4). Upon binding, Notch receptor is proteolytically cleaved via γ-secretase, resulting in the formation of Notch intracellular domain (NICD). NICD translocates to the nuclease and regulates target genes such as Hes and Hey(Tran, Sharma et al. 2014). Evidence for the primary cilia in regulating Notch signaling has previously been shown in skin and cardiac development. In skin development, Notch 3 signals through the primary cilia to regulate epidermal differentiation (Ezratty, Stokes et al. 2011). The primary cilia functions in processing Notch to generate NICD. Presenilin-2, the enzyme responsible for Notch cleavage, localizes to the basal body and its activity has been shown to be dependent on the primary cilium.

Prior to ligand binding Notch receptor undergoes endosomal sorting either for recycling to the plasma membrane or for lysosomal degradation. Disruption of this endosomal sorting complex results in over activation of Notch. Ciliary function is linked to endosomal recycling and exosome formation and studies have shown disruption of Notch recycling leads to increased activation of the pathway. In zebrafish and human cell lines, loss of basal body proteins leads to an increase in expression of Notch targets (Leitch, Lodh et al. 2014). It is thought that Notch trafficking to the cilia is controlled by Bardet-Biedl syndrome (BBS) proteins. Loss of BBS proteins results in aberrant

trafficking of the receptor through late endosomes to lysosomes and impaired recycling of the Notch receptor (Leitch, Lodh et al. 2014).

Notch1 signaling also plays a role in cilia length regulation (Lopes, Lourenco et al. 2010). Cilia length is a critical regulator of cilia function. Reduction of Notch signaling in zebrafish results in shorter cilia while hyperactivation produces longer cilia. In addition, changes in motile cilia length in the zebrafish node/ Kupffer's vesicle (KV) result in laterality defects.

**Cilia in the heart**

Many well studied ciliopathies have been shown to play a role in CHD and cardiovascular disease. Large scale mutagenesis screens showed that cilia play a role in left right patterning of the heart as well as cardiac looping(Li, Klena et al. 2015). In addition to motile cilia, primary cilia have also been implicated in heart development and have been shown to cause CHD when disrupted. Some well-studied CHDs such as atrioventricular septal defects, left- right patterning, heterotaxy, conotruncal defects, and left ventricular obstruction have been linked to defects in cilia function (Klena, Gibbs et al. 2017). Defects in Hh signaling in the second heart field derived structure, the dorsal mesenchymal protrusion (DMP) lead to atrioventricular septal defects (AVSDs)(Briggs, Burns et al. 2016). Primary Cilia Dyskinesia (PCD) for example is a ciliopathy that results in defects of ciliary motility. Predominantly PCD results in difficulty with mucociliary clearance and organ laterality defects(Harrison, Shapiro et al. 2016). Links have been made between PCD and CHD because 50% of patients with PCD have organ laterality defects and 3.5-6% have cardiovascular malformations(Kennedy, Omran et al.

2007). In addition, the tectonic protein 3 (Tctn3) localizes to the transition zone of the cilia and forms a large complex with several ciliopathy-associated proteins. Disruption of this protein results in incorrect heart looping(Wang, Li et al. 2017).

The first description of cilia in the heart was in 1969 by Rash et al. (Rash, Shay et al. 1969). Through electron microscopy screens they were able to identify cilia in chick, rabbit, mouse and lizard embryos in nonmitotic cardiomyocytes. Later in 2008, Slough et al published a more thorough description of cilia in the heart (Slough, Cooney et al. 2008). They show cilia expression in the developing heart at E9.5 in both myocardium and cardiac endothelium. They also show cilia on the endothelium of the developing endocardial cushions although staining is not very clear. Additionally, at E12.5, they show cilia present on the ventricular surface of trabeculations as well as in the developing valves. Global deletion of Kif3a (a critical motor protein needed for driving ciliogenesis) results in failure of cardiac cilia to form and disrupted formation of endocardial cushions with decreased cellularity. These studies however lack data identifying the effects of cilia loss into postnatal valve development, due to embryonic lethality of the global knockout. They also suggest that the cilia function as mechanosensors since they are expressed during the stage of heart development, which rely on sensing blood flow for morphogenesis. They hypothesize that cilia function in different ways during different stages of cardiac development. Although this paper was not a complete description of cilia in the heart at all stages of development they provide the first evidence that primary cilia are present and may be playing a role in cardiac development.

In order to determine the genetics that may be involved in congenital heart disease, large scale ENU screens were completed by Cecilia Lo's lab(Li, Klena et al.

2015). Screening of 87,355 chemically mutagenized mice was done to reveal 218 CHD mouse models. From this screen 34 cilia related genes were discovered. The mutations were in genes that regulated ciliogenesis, ciliary trafficking, and cilia signaling. Of these genes some were found to be mutated in mice with laterality defects suggesting motile cilia while others were found to be mutated in mice without laterality defects suggesting non-motile primary cilia. Comparably, genes found in this screen were found to be similar to those found in a study of human patients with CHD(Zaidi, Choi et al. 2013).

Of the mutations recovered, 12 were found in genes that are in cilia transduced signaling pathways suggesting that ciliary signaling in the heart is important for proper development. Major signaling pathway genes were found also found such as genes mediating Hh, TGFβ, and Wnt signaling. The discovery of mutation in genes regulating vesicular trafficking is also of note since vesicular trafficking and receptor recycling play important roles in regulation of cell signaling. Mutations were found in Dynamin 2, Ap2b1, Ap1b1 and others which have been shown to mediate endocytic receptor recycling(Li, Klena et al. 2015, Klena, Gibbs et al. 2017).

Strong evidence from the literature suggests primary cilia as a common link among valve diseases. Genetic evidence from our lab and others suggests that defects in primary cilia structure and/or function result in valve disease. This thesis work aims to better understand the relationship between primary cilia signaling and valve disease. Starting with a hypothesis that primary cilia are important for valve development and using this information to inform patient-based studies, we were able to discover a novel gene for MVP and potential mechanism for BAV. The following chapters are an illustration of this work.

# CHAPTER 2: LOSS OF PRIMARY CILIA RESULT IN BAV IN MICE

## Introduction

BAV is one of the most common cardiac diseases affecting 0.5-2% of the population. It is the most common congenital heart disease responsible for increased morbidity and mortality in infants. Currently the only treatment option is surgery to replace the damaged tissue. Complications of BAV are severe, ranging from aortic stenosis to aortic rupture and calcific aortic valve. Familial studies have provided evidence that BAV might be linked with autosomal dominant inheritance however the mechanistic underpinnings are unclear.

BAV occurs when only two of the three aortic cusps form properly during embryonic development. Often this occurs in the presence of a raphe, or a fusion line between what would normally be two cusps. Developmentally, the aortic cusps form from anterior second heart field, endocardially derived and neural crest cells which migrate into the cardiac jelly early during development. Signaling cues from the surrounding myocardium as well as endocardial cells help to orchestrate this process. Patients with BAV often have myxomatous aortic valves and are likely to present with other diseases such as aortic aneurism which is caused by defects in ECM structure of the aorta. Therefore, it is likely that defects in cell migration or extracellular matrix production is the causal factor in the development of BAV.

To date, only a few genes have been identified in pedigrees and it is clear that no single gene-model explains BAV inheritance, thus supporting a complex genetic network of interacting genes. However, patients with rare syndromic diseases that stem from alterations in the structure and function of primary cilia ("ciliopathies") exhibit BAV and aortic stenosis (AS) as frequent cardiovascular findings, suggesting that primary cilia

may play a broad role in disease etiology. This contention is supported by our data, which demonstrate that primary cilia are expressed on undifferentiated aortic valve mesenchymal cells during embryonic development and are lost as these cells differentiate into collagen-secreting fibroblastic-like cells. This suggested a role for primary cilia in restraining aortic valve interstitial cell (aVIC) differentiation, which was tested by genetically ablating cilia through Cre-mediated excision of *Ift88*, a critical ciliogenic gene. Loss of *Ift88* resulted in abrogation of primary cilia and increased fibrogenic ECM production. Consequentially, stratification of zonal ECM boundaries normally present in the aortic valve were lost and a highly penetrant BAV phenotype was evident at birth. These data support cilia as critical for restraining ECM production during aortic valve development and broadly implicate these structures in the etiology of BAV disease in humans.

**Results**

Temporal-Spatial Analyses of Primary Cilia During Aortic Valve Development

In order to determine if cilia are expressed in the aortic valve a developmental expression panel was done. Primary cilia expression was determined by immunohistochemical stains for ciliary markers which were then imaged using high-resolution confocal microscopy. From this study we showed that primary cilia are expressed throughout development on the developing VICs. Cilia are expressed on nearly all valve cells early in development (E11.5) at which they are short protrusions averaging around 0.5-1.0μm in length (Fig2.1). As the valve begins to develop further (E13.5-E15.5) cilia are largely absent form VECs but remain on almost all VICs. By late developmental and postnatal time points the expression of the primary cilia appears to be localize to specific regions of the

valve largely the tip and atrialis regions. At this stage, the cilia length appears to have peaked between 2-2.5μm in length. At adult time points the majority of the primary cilia are completely absent from the valve (Fig2.1).

The localization of the primary cilia to specific regions of the valve during development could give insight into their role. During early development, when nearly all cells express cilia, the ECM is composed mainly of proteoglycans. As the valve develops it begins to stratify into different layers and express collagens. This division of matrix matches what we see with expression of the primary cilia. As shown in Figure 2, primary cilia localize to areas high in proteoglycans. Early in development (E11.5) when the valve is composed mainly of proteoglycans such as versican, primary cilia re-expressed on nearly every interstitial cell. When the valve begins to elongate and mature (E17.5 and P0) the cilia are still present in areas highly expressing proteoglycans but are largely devoid in areas high in collagen expression (Fig 2.2). Quantification of cilia length at postnatal day 0 shows increased cilia length in versican rich regions when compared to collagen rich regions. This expression pattern could be explained in two ways, either the matrix is negatively regulating the expression of the cilia or the cilia are dictating the expression patterns of the surrounding ECM.

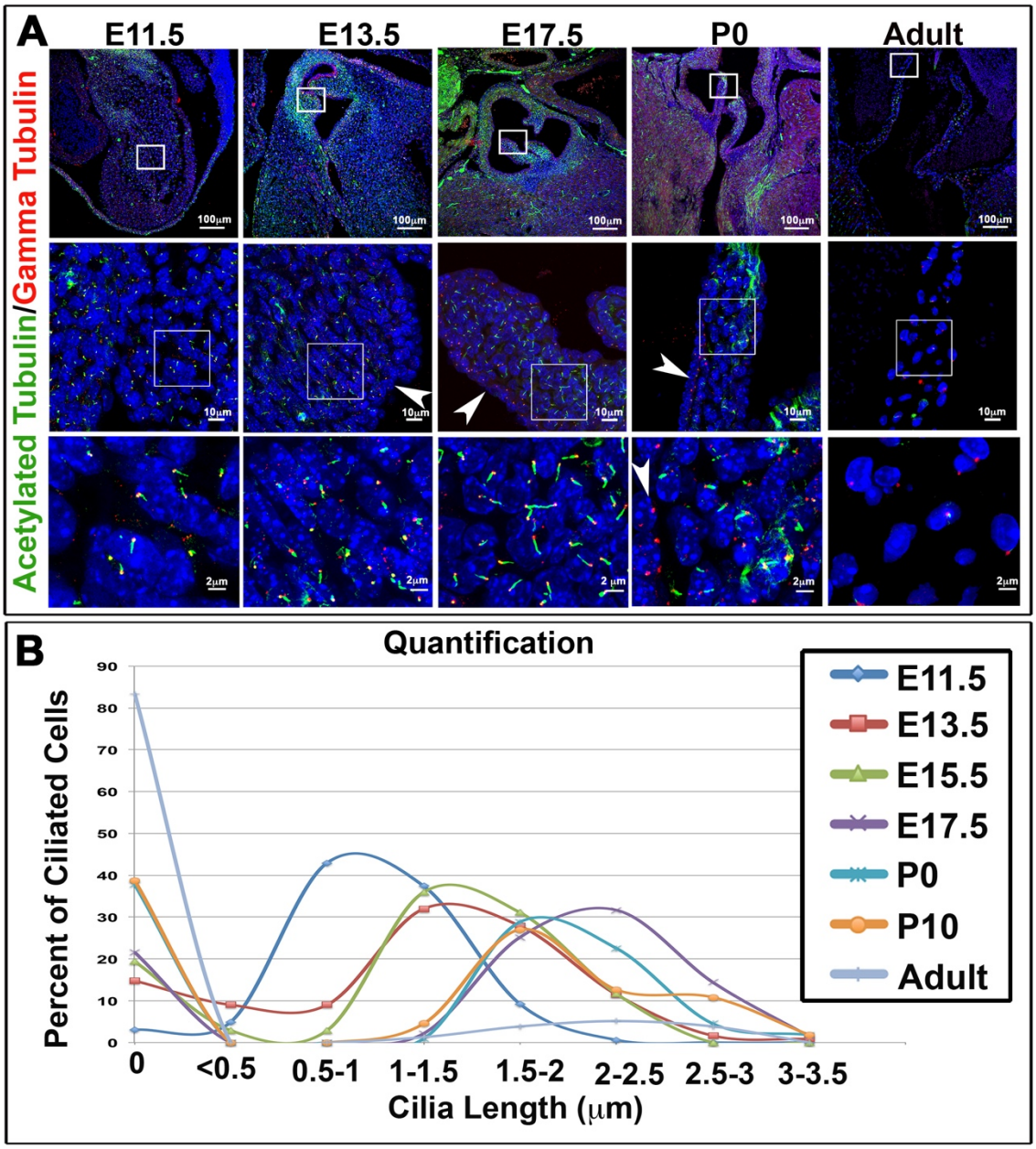


**Figure 2.1: Spatial and Temporal Expression of Primary Cilia during Aortic Valve Development.**

(A) IHC for the ciliary axoneme (green), basal body (red), and nuclei (blue) show expression of cilia throughout valve development. Cilia appear shorter during early embryonic stages and longer right before and after birth. Arrow indicate valvular endothelial cells devoid of cilia (B) Quantification of cilia length showing increased expression of cilia during embryonic development and decreased expression during postnatal development and adult life. Cilia length increases with development.

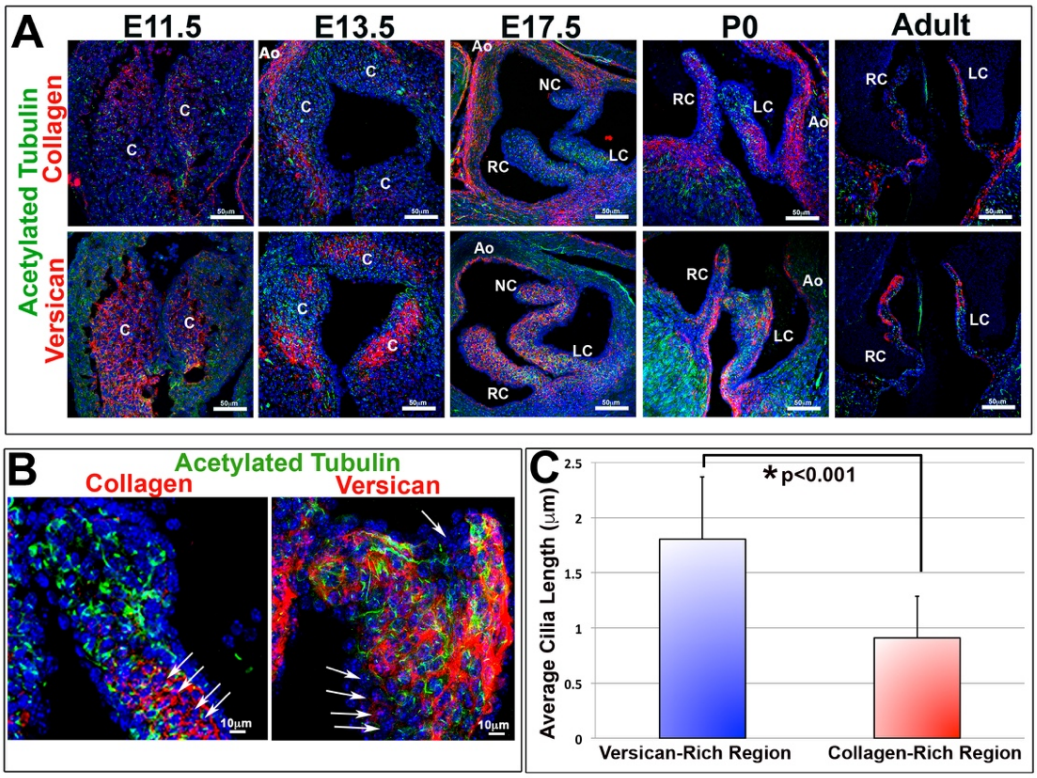


**Figure 2.2 Primary Cilia Co-localize with Proteoglycans During Development**

(A) IHC for the ciliary axoneme (green), collagen (red, top) versican (red, bottom), and nuclei (blue) show expression of cilia throughout development. Cilia appear to be expressed highly in areas rich in proteoglycans and less in areas high in collagen. C= conal cushions, RC= right coronary, LC= left coronary, NC= non-coronary, Ao= aorta. (B) High magnification images of axonemes (green) and collagen/versican (red). Arrows depict collagen rich regions expressing shortened cilia. (C) Quantification of cilia length in both versican and collagen rich regions shows decreased average cilia length in collagen rich regions when compared with versican rich regions, $p<0.001$ Students t-test.

This differential expression pattern could be indicative of a potential role for cilia during development. Previous research has shown that biomechanical forces are important for proper valve formation. It is therefore possible that the cilia present on the VICs are sensing the changes in interstitial fluid flow within the tissue and acting as mechanosensors to orient the proper formation of the valve. Although the function of primary cilia as mechanosensors on non-epithelial cells is poorly known, prior work has shown this as a possibility(Nauli, Jin et al. 2013, Nguyen and Jacobs 2013). In addition, cilia have also been shown to function as chemosensors, which respond to growth factors such as Notch, Wnt, Pdgf, and Hedgehog. One study on hedgehog signaling in the valve showed normal cushion development in sonic hedgehog and smoothened Tie2 conditional knockout mice (Goddeeris, Rho et al. 2008). This is not surprising as previous research has shown sonic (Shh) and indian (Ihh) hedgehog are not expressed in the developing valves (Bitgood and McMahon 1995). They do show however that desert hedgehog (Dhh) is expressed in the developing endocardium of the cushion. Therefore, hedgehog signaling may still be playing a role in the development of the cushions through Dhh and non-canonical hedgehog signaling. To determine if hedgehog signaling was playing a role we began by preforming RT-PCR on cushion mesenchyme, looking for expression of pathway members (Fig 2.3). Both receptors for the pathway, patched and smoothened as well as downstream transcription factors, Glis, were expressed by mouse atrioventricular cushion cells. Notably, the ligands for the pathway Shh, Ihh and Dhh were not expressed. This was to be expected as previous research shows no expression of Shh and Ihh in the valves and expression of Dhh only in the VECs(Bitgood and McMahon 1995)

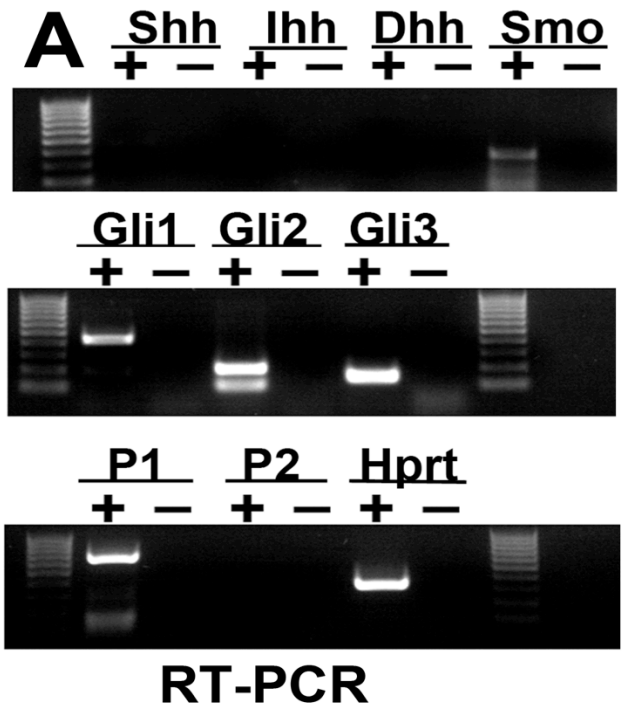


**Figure 2.3. Hedgehog Signaling Components are Expressed in VICs.** RT-PCR done on mouse AVC cells. RNA was extracted from AVC cells and reverse transcription was done to get DNA. Gels show expression of Hh receptors smoothened and patched. Expression of the downstream Gli transcription factors are also observed. No expression of Hh ligands Shh, Ihh, or Dhh is observed. (-) represents negative control, no DNA.

Expression of smoothened was confirmed by 3D-immunohistochemistry (3D-IHC) on aortic valve interstitial cells during development. Data show smoothened on the axoneme of the primary cilia which is indicative of active Hh signaling (Fig 2.4 A). In the absence of Hh ligand, patched represses smoothened and prevents its entry into the primary cilium. In the presence of Hh ligand, during active signaling, patched relieves its repression and smoothened is allowed to enter the primary cilia. In addition, IHC staining shows nuclear expression of downstream Gli3 transcription factor at postnatal day 0 (Fig 2.4 B). These data suggest that active Hh signaling is occurring in the aortic valves through crosstalk between endothelial secreting Dhh and VICs expressing primary cilia.

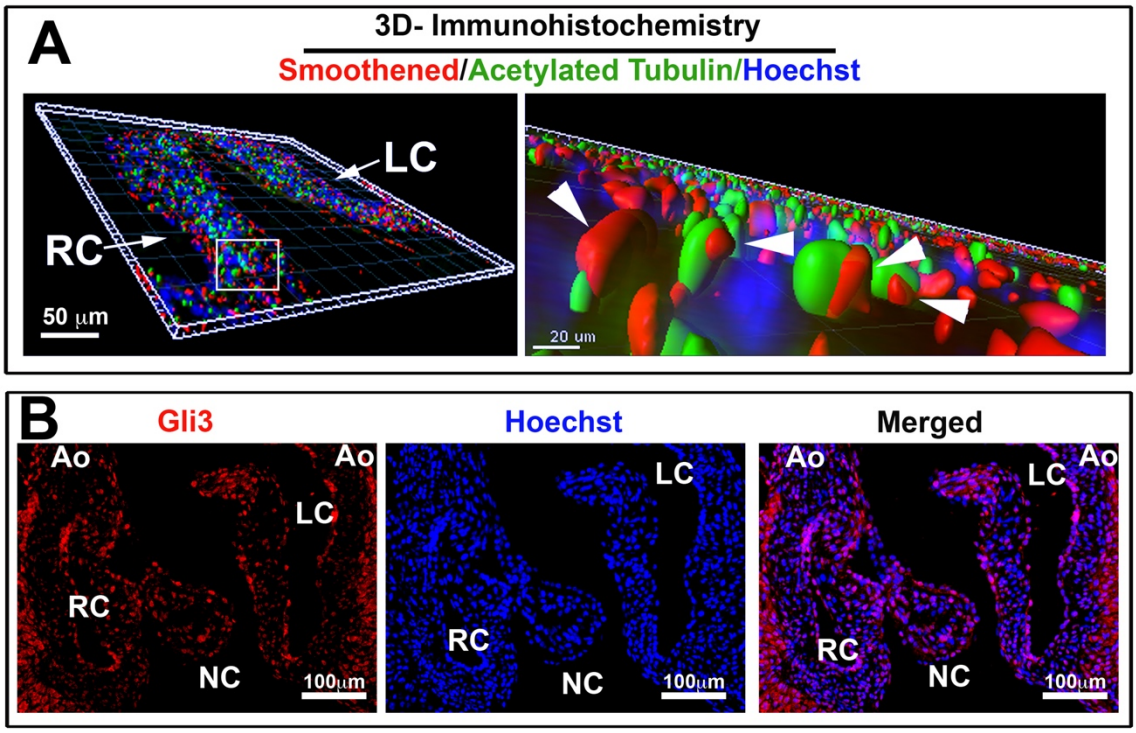


**Figure 2.4 Active Hedgehog Signaling Occurs in the Developing Aortic Valves.** A. 3D-IHC showing expression of smoothened (red) on the axoneme (green) of the primary cilia. Arrow heads show activated smoothened. B. IHC showing nuclear localization of Gli3 (red) in P0 aortic valves. RC=right coronary, LC=left coronary, NC=non-coronary, AO=aorta. LC=left coronary, NC=non-coronary, AO=aorta.

To determine if cilia are functionally important a mouse model with valve specific cilia deletion was used. One model for cilia ablation is the Ift88 conditional mouse created by Brad Yoder's lab (Haycraft, Zhang et al. 2007). Ift88 is an intraflagellar transport protein that acts as a scaffold for vesicle transport up and down the ciliary axoneme. It has been shown to be essential for the formation and maintenance of primary cilia. Loss of Ift88 results in either total deletion or very short ciliary axonemes and global deletion is early embryonic lethal (Pazour, Dickert et al. 2000, Veland, Awan et al. 2009). Since global deletion of primary cilia is embryonic lethal, the cardiac specific *Nfatc1* cre mouse was used to breed with the conditional *Ift88* mouse. The aortic valves are composed of anterior second heart field progenitors, endocardial and endocardial derived cells as well as a small population of migrating neural crest cells (Leung, Liu et al. 2015, Koenig, Lincoln et al. 2017). Therefore, the Nfatc1 cre mouse was used in order to produce a valve specific deletion of Ift88. To confirm cilia expression was gone in the conditional knockout animals, immunohistochemical stains for primary cilia were performed. Stains show loss or very short axonemes in conditional knockout animals and normal cilia structure in the wild type animals (Fig 2.5).

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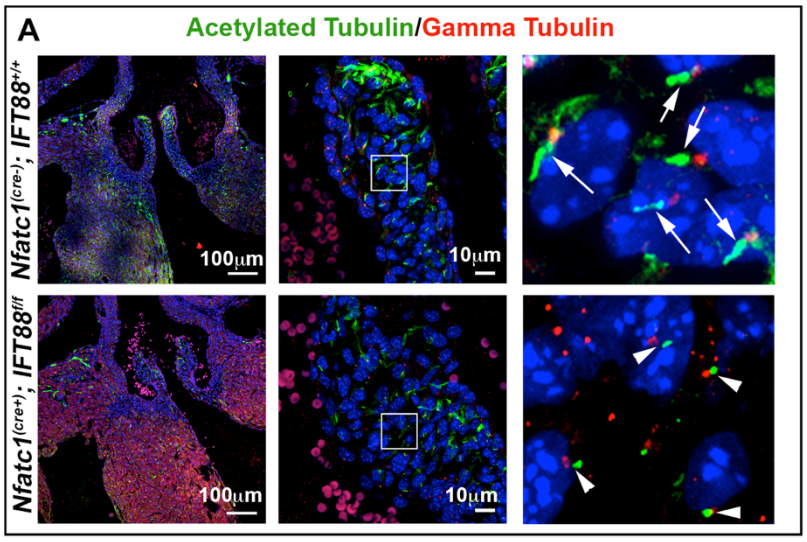



**Figure 2.5 Cilia Expression is Lost in Ift88$^{f/f}$ Nfatc1$^{cre+}$ Mice**. IHC showing loss of cilia expression in conditional knockout animals. Axonemes in green and basal bodies in red. Arrows pointing to cilia expression; arrow heads pointing to loss or shortened cilia.

Structural defects were observed in conditional knockout valves. Histology showed merging of hinge regions between the right and non-coronary leaflets indicative of a bicuspid valve (Fig 2.6). Three-dimensional reconstruction showed the formation of two instead of three cusps in the conditional knockout when compared to litter mate controls. This bicuspid phenotype was observed in a high percentage of all knockouts examined, about 68 percent, with about ten percent showing a raphe like fusion. In addition, volume measurements were taken and the right coronary leaflet of the conditional knockout was significantly larger when compared to wild type controls.

Since increased extracellular matrix is one of the hallmarks of diseased valves, we tested whether alterations in ECM production were evident in the fused NfatC1/Ift88 aortic cusps. Immunofluorescence staining shows increased expression of ECM protein. At postnatal day 0 conditional knockouts have increased expression of versican, and collagen1$\alpha$1 (Fig 2.7).

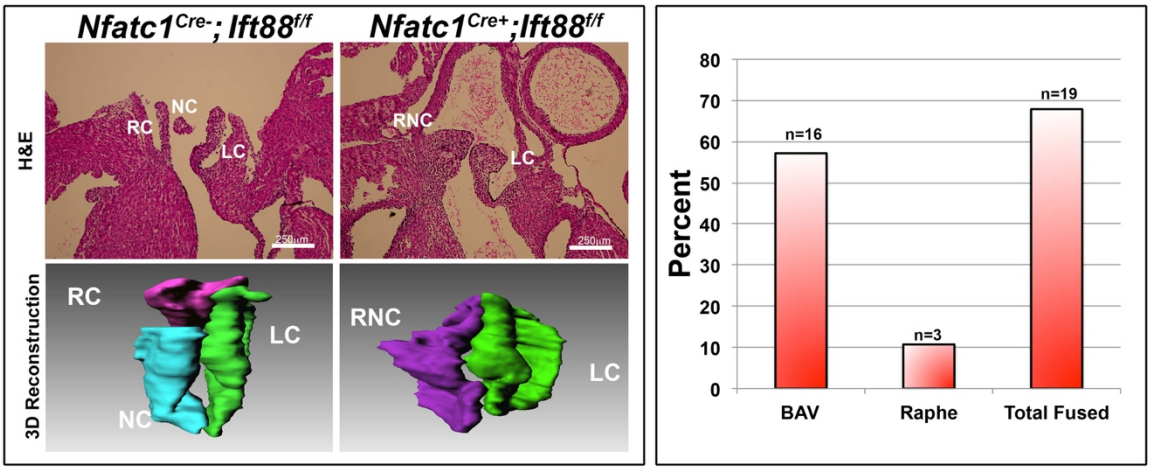



**Figure 2.6 Conditional deletion of Ift88 results in BAV.** H and E staining showing fusion of right coronary (RC) and non-coronary (NC) leaflets in the conditional knockout. LC=left coronary. 3D reconstructions show BAV phenotype in conditional knockouts. Graphs depicts percentage of BAV in conditional knockouts.

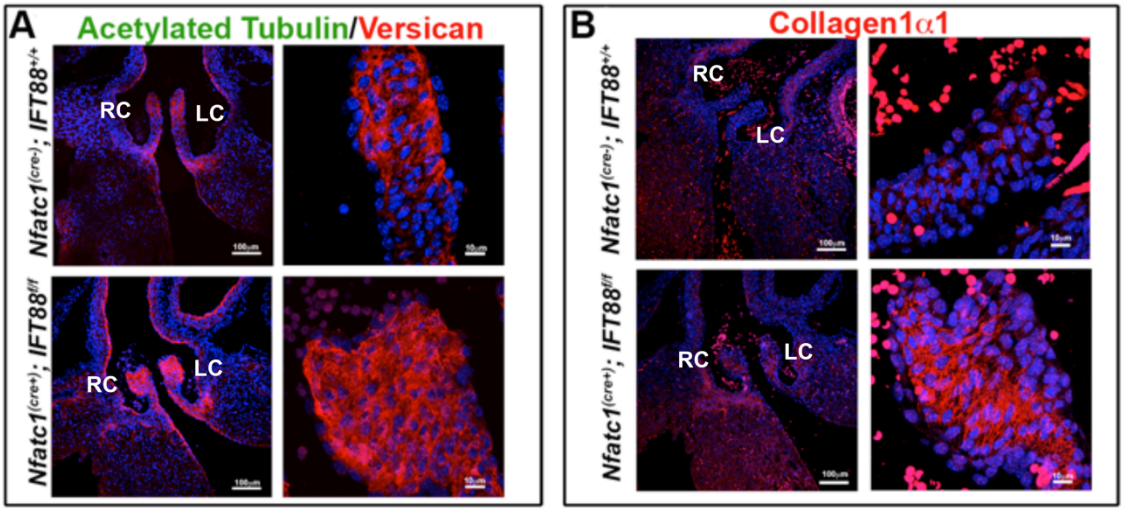



**Figure 2.7 Ift88 Conditional Knockout Aortic Valves Have Increased ECM Expression.** (A) IHC for versican showing increased expression in the conditional knockout. (B) IHC for collagen1a1 showing increased expression in the conditional knockout. RC= Right coronary cusp, LC= Left coronary cusp.

Additionally, this bicuspid phenotype did not appear to be caused by increased proliferation since there was no significant difference in Ki67 or Ph3 positive cells in conditional knockouts when compared to controls (Fig 2.8). When total cell number was counted, there was also no significant difference between conditional knockout and wild type. These data suggest that loss of primary cilia in the aortic valve does not result in increased proliferation or migration of cells into the leaflet.

In addition to seeing increased extracellular matrix, we observed decreased cell density in the Nfatc1$^{cre+}$; Ift88$^{f/f}$ mice. Observations from H and E staining showed less cells within specific regions suggesting that the enlarged aortic valve could be caused by increased matrix (Fig 2.9). Nuclei from a specified region within the valve were counted in order to determine nuclear density within the tissue. These data indicate that the valve ECM is expanding and this change can explain the increased valve size seen in the conditional knockouts.

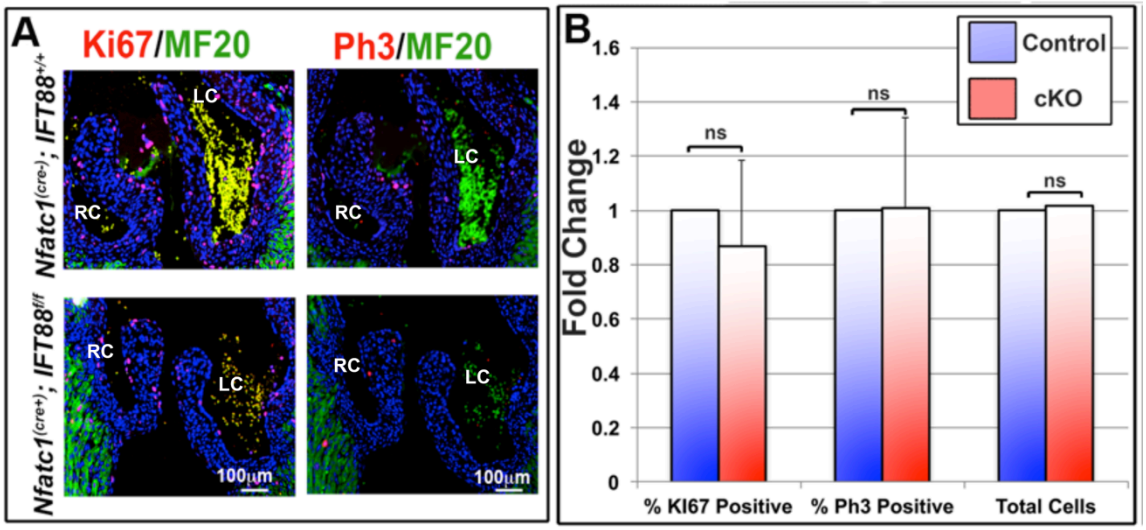



**Figure 2.8 Loss of Primary Cilia does not Affect Proliferation or Infiltration of Cells.** A. IHC for Ki67 and phosphohistone H3 showing proliferating cells (red) and Mf20 marking the myocardium (green). B. Quantification of A showing no significant difference in proliferating cells or total cell number. Students t test was used to determine significance $p>0.05$. RC= right coronary, LC= left coronary.

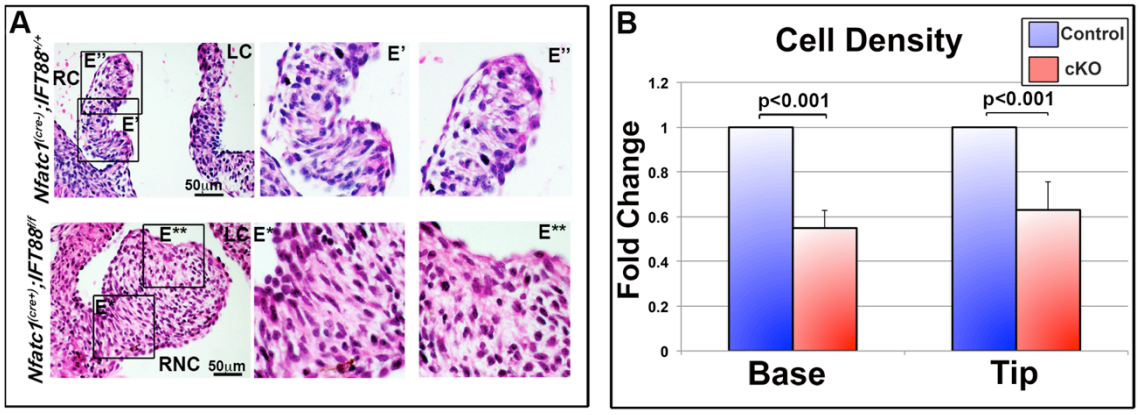



**Figure 2.9 Conditional Ablation of Primary Cilia Results in Decreased Cell Density.** A. H and E of P0 aortic leaflets show decreased nuclei density at both the base and tip of the right, right non-coronary leaflet (RNC). B. Quantification of A showing decreased cell density between conditional knockout and control at the base and tip of the leaflet.

Increased proteoglycans and fragmented collagen is indicative of diseased valves so we next looked at the adult mice to determine if loss of primary cilia leads to adult aortic disease. Staining's showed similar trends to that observed during postnatal time points. Nfatc1$^{cre+}$;Ift88$^{f/f}$ mice had increased expression of collagen and versican with enlarged myxomatous cusps (Fig 2.10). Quantification of cell density also showed decreased cell density in conditional knockout cusps (Fig 2.11). Cells within a specified area of the valve were quantified and conditional knockout valves has increased ECM expression and decreased cell density.

Next, we wanted to look at the type of collagen produced in these valves to determine if collagen synthesis was being affected. To do this herovici stains were done on adult conditional knockout and wild-type control valves. Data show increased expression of newly synthesized collagen and decreased expression of full length mature collagen (Fig 2.11).

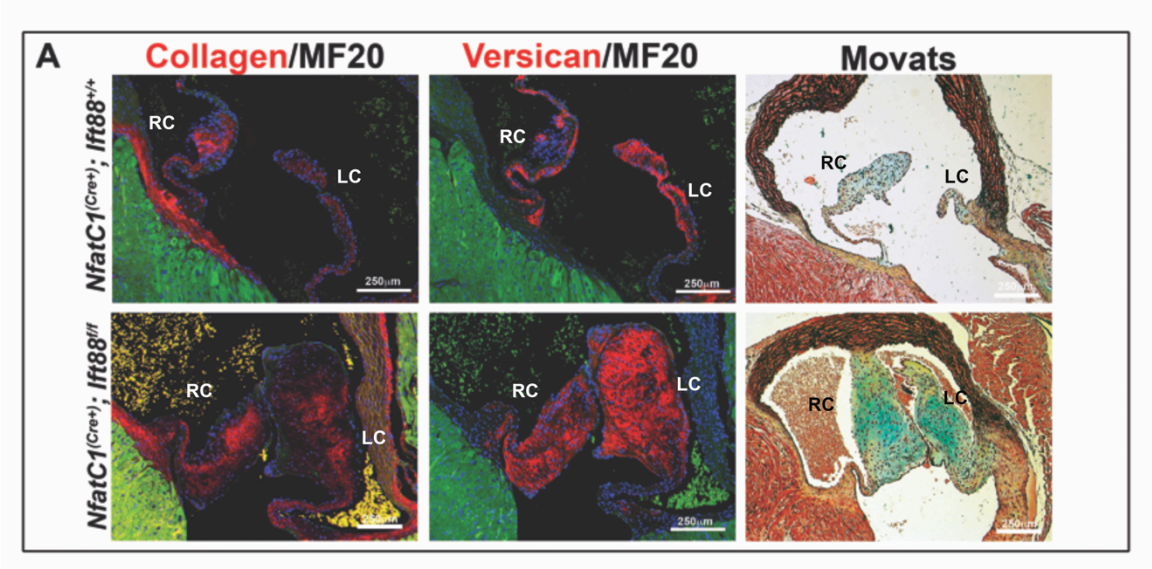



**Figure 2.10 Loss of Primary Cilia results in myxomatous adult BAV.** IHC, left and middle panel, show collagen and versican staining in red and mf20 myocardial staining in green. Movat's pentachrome stain, right panel, shows mixing of ECM boundaries with increased proteoglycan (blue) and collagen (yellow) in the conditional knockout cusps.

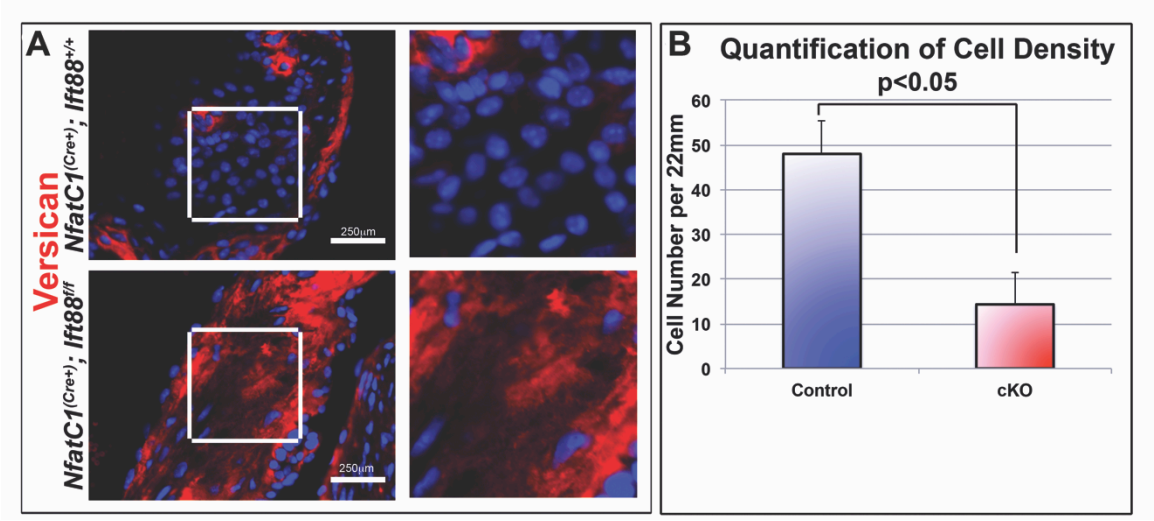



**Figure 2.11 Loss of Primary Cilia Leads to Decreased Cell Density in Adult Aortic Valves.** A. IHC for versican (red) and nuclei (blue) show less nuclei in the conditional knockout compared with control. B. Quantification of A showing significant difference between cell density in conditional knockout valve when compared to control.

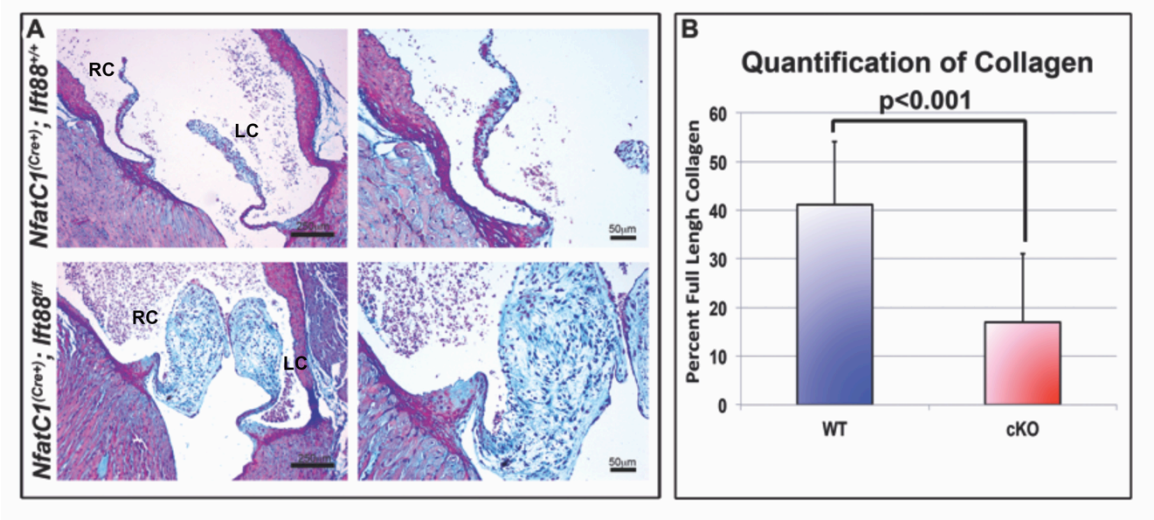



**Figure 2.12 Loss of Primary Cilia results in decreased mature collagen expression.** A. Herovici's stains showing mature collagen in red and immature collagen in blue. Conditional knockout valves have decreased full length collagen when compared to controls. B. Quantification of A, there is a statistically significant decrease in full length collagen in the conditional knockout valves when compared to controls. Students T-test, $p<0.001$.

This, along with the previous data, suggest that primary cilia are needed during development to regulate cell maturation and extracellular matrix production. The Herovici staining showed decreased expression of full length collagen suggesting that loss of primary cilia in valve cells during development results in a high turnover or fragmentation of collagen.

**Discussion**

The genetic underpinnings of BAV are currently not well understood. These studies are the first description of primary cilia expression in the aortic valves during development. These data show that primary cilia are important for proper valve development and may play a prominent role in the development of BAV. Primary cilia act to promote VIC differentiation and therefore proper ECM production. In this way the primary cilia act to direct valve architecture during development allowing for proper function. When primary cilia or signaling through the primary cilia is prematurely lost, accelerated differentiation occurs. This results in VICs that are overproducing ECM, which ultimately results in myxomatous BAV.

Future studies are needed to determine the molecular mechanisms that underlie this phenotype although evidence suggest that primary cilia regulate ECM production though activation of the dessert hedgehog signaling pathway. Previous research shows Gli transcription factors play a role in regulating ECM gene expression such as collagen (Bolanos, Milla et al. 2012, Amano, Densmore et al. 2014, Duan, Ma et al. 2014). To better understand the role primary cilia may be playing in valve development and disease

we could perform RNA seq on isolated valve leaflets both wildtype and conditional knockout mice at early embryonic times such as E13.5 and at postnatal time points such as P0. These data could be informative as to when this change in matrix is occurring and if the genes in the hedgehog pathway are involved. Additional experiments looking at target genes of Gli transcription factors could give insight into what genes the Gli factors are regulating in the aortic valves. Chip-Seq experiments with pulldown of Gli3 and sequencing of its targets would show what genes Gli3 is regulating. In addition, looking at non-canonical hedgehog signaling may be informative. Studies have shown hedgehog ligands signaling through other pathways independent of Gli transcription factors (Fei, Sanchez-Mejias et al. 2012).

In order to further investigate the hedgehog signaling pathway other conditional knockout mice could be used. Analysis of the smoothened conditional knockout mouse would help to determine if the hedgehog signaling pathway plays a role in this valve phenotype. If smoothened conditional mice develop BAV then it would provide evidence that this pathway plays a role. In addition, some in vitro studies could be performed by treating mouse atrioventricular cushion cells with Dhh to see if Gli processing is affected. Culturing of VICs from conditional knockout and control valves could be used also for treatment with Dhh followed by western blots looking at Gli processing. Cyclopamine, an inhibitor of the Hh signaling pathway could be used to treat the control valve tissue in order to show that inhibition of the Hh signaling pathway is regulating this change in the Ift88 conditional mice.

Additional pathways may also play a role in this process. Cilia have been shown to regulate many important developmental pathways such as Wnt, TGFβ, PDGF and

Notch. Since Notch gene mutations have been implemented in the development of BAV this pathway might be good to investigate. Histological stains for cleaved notch in knockout and wildtype mice would show if notch cleavage is affected in this model. The PDGFR pathway may also play a role in the BAV phenotype we observed. PDGFα localizes to the primary cilia and can play a role in ERK signaling. In addition, PDGF is regulated by TGFβ which plays an important role in valve development and the progression of myxomatous disease (Narine, De Wever et al. 2006, Maredia, Greenway et al. 2017, Peng, Yan et al. 2017). There is also a strong association in pregnant mothers of circulating lymphatic PDGF with offspring that had congenital heart disease(Steurer, Norton et al. 2017). To test this hypothesis, leaflets from P0 Ift88 conditional knockout and wildtype valves would be dissected and western blots would be done probing for PDGFα and phosphorylated PDGFα as well as other members of the pathway such as AKT, ERK and p-ERK.

# CHAPTER 3: A DEVELOPMENTAL BASIS FOR MITRAL VALVE PROLAPSE AS A CILIOPATHY

## Introduction

Until recently, primary cilia were thought to be vestigial evolutionary remnants with no persisting function. Recent evidence however has shown that this is not true. Genetic discoveries have linked mutations in cilia genes to a spectrum of rare syndromic diseases known as ciliopathies. There is a wide range of organs affected by these ciliopathies such as heart, kidney, skeletal or neurological tissues. One particularly interesting finding shows a 25% comorbidity of autosomal dominant polycystic kidney disease ADPKD and MVP. In addition, these diseases have a very similar characterization of increased proteoglycan deposition as well as disorganization and fragmentation of the collagen and ECM. This co-morbidity of ADPKD and MVP, as well as their similar pathological features suggests that perhaps MVP could be a disease of altered primary cilia function.

To begin to understand the relationship between mitral valve development and disease we started by asking the question, "Are primary cilia expressed during development on mitral valve tissues?". Previous reports showing primary cilia in the mitral valve have been vague and unclear. In order to determine if primary cilia were expressed throughout development immunohistochemical stains for the axoneme and basal body were done.

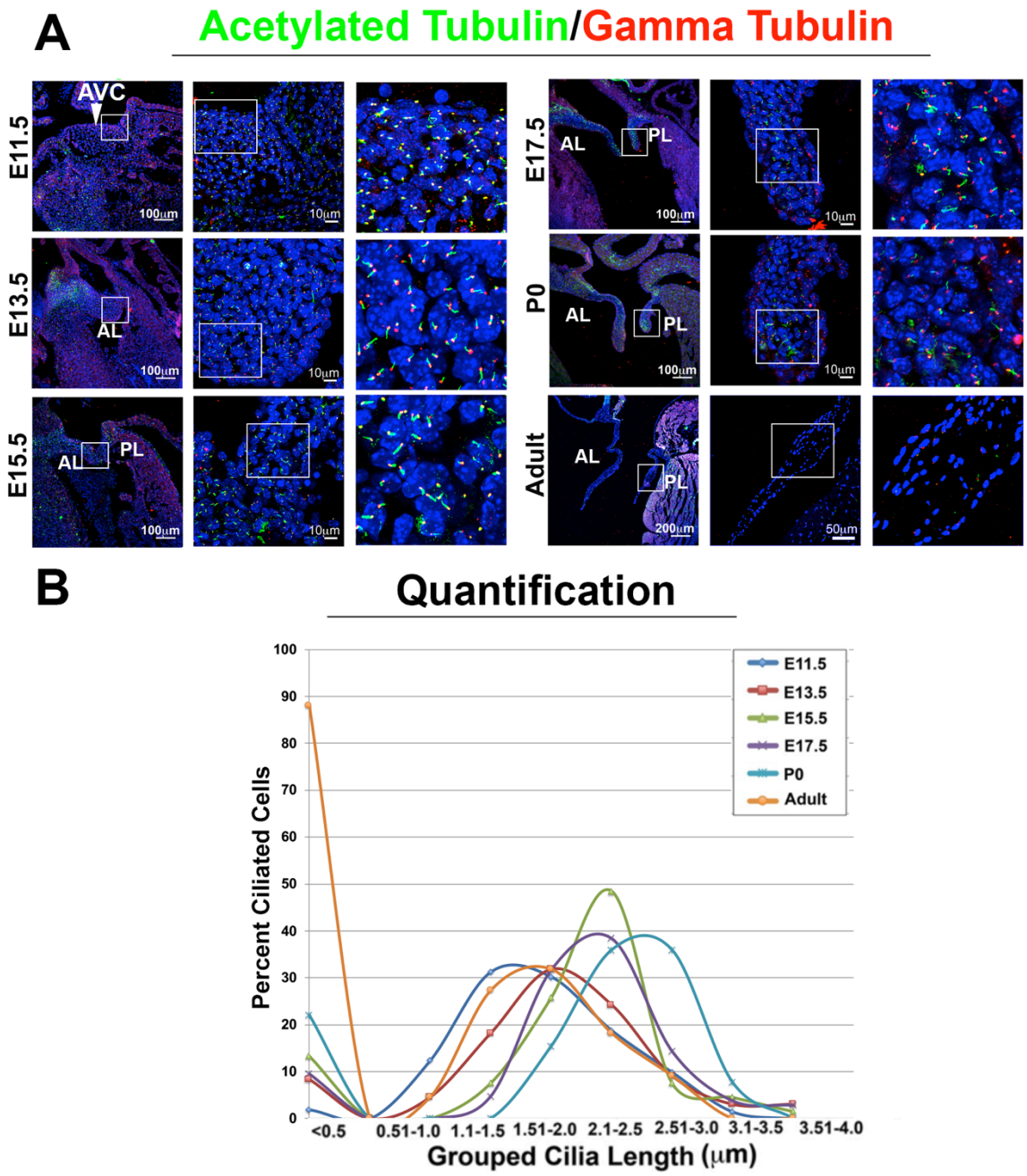



**Figure 3.1 Primary Cilia are Expressed During Mitral Valve Development.** A. IHC for primary cilia staining axonemes (green) basal bodies (red) and nuclei (blue) show expression of primary cilia on VICs. Cilia expression is lost at adult stages. B. Quantification of grouped cilia length shows ciliary length increases during development while percent ciliated cells decreases.

Expression patterns showed multiple interesting things. Cilia are expressed on nearly every cell very early in development from E9.5-E11.5 (Fig 2.1A). They are expressed on nearly all VICs and some VECs and are short in length averaging between 0.5 and 1μm in length (Fig 3.1). As the cushion enters its differentiation and proliferation stages, around E13.5-15.5 the cilia are expressed solely on VICs with about 85 percent of cells expressing cilia. In addition, cilia at these stages are longer with their grouped cilia length peaking around 1.5μm. As the valve continues to develop during later embryonic development and postnatal time points its cilia reach peak length at around 2μm (Fig 3.1B). At adult stages, cilia expression is almost completely gone. Cilia length has been shown to change during development in the kidney and is necessary for signaling during development. Interestingly, ciliary expression at theses time points appear to be localized to specific regions within the valve. Cilia length has been shown to vary by cell type suggesting a necessity for different lengths during development(Avasthi, Maser et al. 2017). Ciliary length is regulated by the localization and quantity of IFT protein as well as other proteins necessary for cilia assembly (Avasthi, Maser et al. 2017). Kinases have been shown to regulate cilia length as well, acting as sensors to regulate this process. In the kidney, cilia length increases during development (Saraga-Babic, Vukojevic et al. 2012). Data show that embryonic cilia in the collecting ducts are around 0.55μm long, while those from postnatal cells were significantly longer at 1.23μm (Saraga-Babic, Vukojevic et al. 2012). This change in length is also observed in the nephron, with length changing from 0.81μm to 3.01μm (Saraga-Babic, Vukojevic et al. 2012). This is similar

to what was observed in the mitral valve during development (Fig 3.1). This change in cilia length provides support for cilia playing a role during embryonic development.

To show that cilia from VECs were not being broken off or missed during sectioning, other endothelial cell types were examined. As shown in figure 3.2, ventricular and interventricular septum endocardial cells express primary cilia. These data suggest that primary cilia are expressed on the ventricular and septal endocardial cells but not valvular endocardial cells during development.

To determine if the cilia expression found in mice also was found in humans, fetal human hearts were examined. Immunohistochemical stains for primary cilia were performed on 10 weeks and 13 weeks fetal human hearts. Staining shows primary cilia present on the interstitial cells at both stages (Fig 3.3). Analysis of adult human mitral valve tissues also follows a similar pattern as in mice with very few if any cilia observed (data not shown).

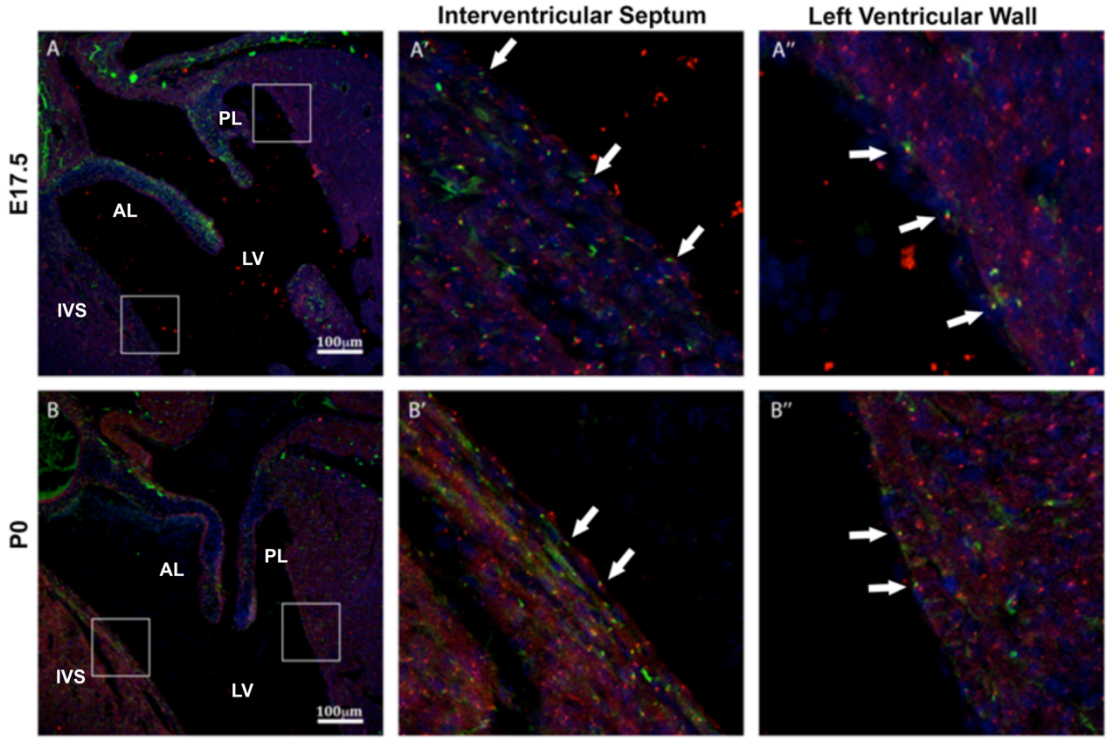



**Figure 3.2. Primary Cilia are Expressed on Ventricular and Septal Endothelial Cells.** IHC showing expression of axonemes (green) and basal bodies (red) on endothelial cells of the interventricular septum (A) and left ventricular wall (B) in developing E17.5 (A) and P0 (B) hearts. E17.5 high magnification images of the interventricular septum (A') and (B') and P0 high magnification images of the left ventricular wall (A'') and (B''). Arrows point to primary cilia expression.

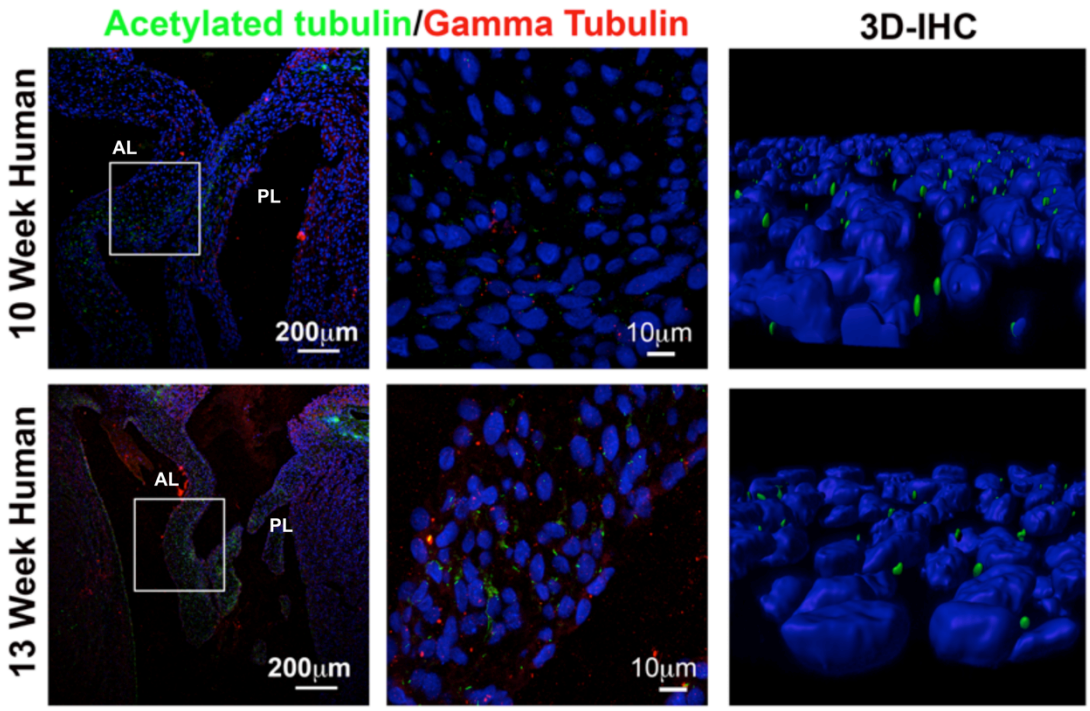



**Figure 3.3. Primary cilia are Expressed on Fetal Human Valves.** IHC (left and middle panel) shows expression of acetylated tubulin (green) and gamma tubulin (red) on 10 week and 13-week fetal valve tissue. 3D reconstructions of IHC (right panel) shows ciliary axonemes (green) on VICs. AL= anterior leaflet, PL= posterior leaflet.

To better understand if primary cilia correlate with regions of ECM stratification, IHC was preformed using antibodies against proteins that demarcate specific ECM boundaries within the mitral valve. ECM expression during development stratifies into specific regions of the valve that appeared to be localized with the primary cilia. To determine if ECM and cilia were co-localizing to specific regions within the valve we stained for two well documented ECM proteins, versican and collagen1a1.

Early during development, the AV cushions are composed mainly of proteoglycans with little collagen expression. At this stage as shown in figure 3.1, the cilia are short but expressed on nearly every cell. As the valve ECM begins to mature the cilia appear to localize to specific regions in the valve mainly expressing versican. At postnatal day 0 cilia are highly expressed in proteoglycan (versican) rich regions and are notably absent in collagen expressing regions (Fig 3.4C). When quantified, the percentage of ciliated cells in versican rich regions were significantly higher than the percentage of ciliated cells in the collagen rich regions (Fig 3.4C). In addition, cilia that are expressed in versican regions are significantly longer than those expressed in collagen regions (Fig 3.4D). These data suggest that cilia size and number promote organization of the valve extracellular matrix into zonal boundaries characteristic of normal mitral valve histoarchitecture.

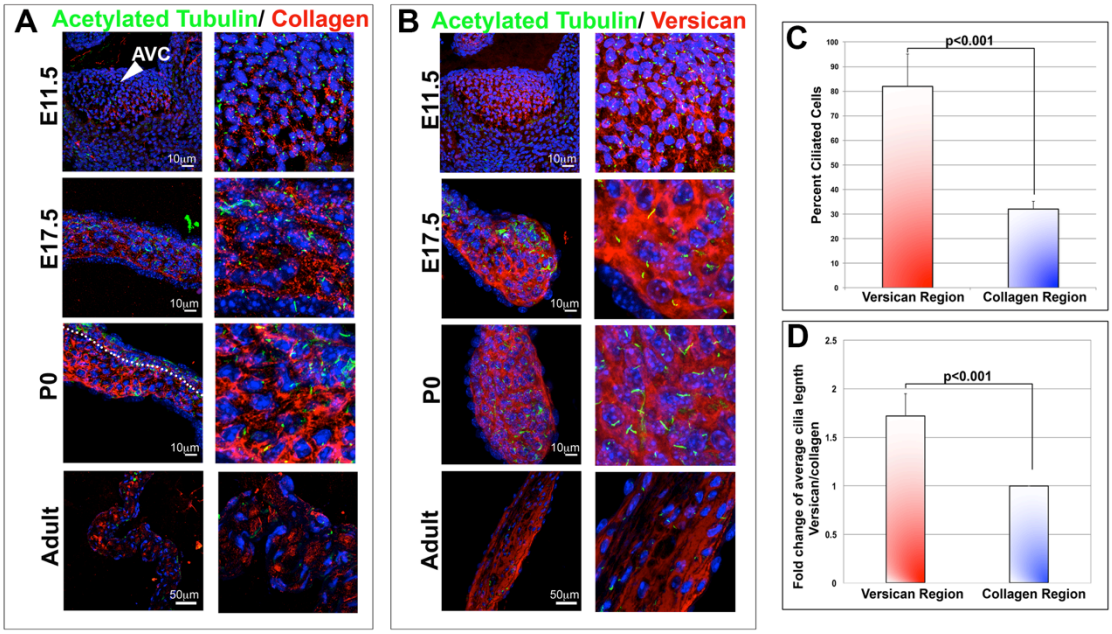



**Figure 3.4. Primary Cilia are Expressed in Areas Rich in Versican. (A)** IHC of posterior leaflets show primary cilia marked with acetylated tubulin (green) co-stained with collagen (red) show few cilia within this region. **(B)** Immunohistochemical stains of primary cilia marked with acetylated tubulin (green) co-stained with versican (red) show increased expression of acetylated tubulin in areas of high versican expression. **(C, D)** Quantification of IHC stains at P0 showing a 70% decrease in cilia length and a 250% reduction in the number in collagen-rich regions. AVC=atrioventricular cushion. Dotted line demarcates high collagen vs. low-collagen boundary.

To determine if primary cilia are necessary for mitral valve development we took a mouse genetics approach. As previously mentioned, *Ift88* is an intraflagellar transport protein necessary for primary cilia formation. Conditional removal of *Ift88* in the valves using the NfatC1$^{cre}$ driver was used to assess the role of primary cilia in the mitral valve. To confirm that cilia were lost using this system, IHC was performed on conditional wildtype, heterozygous and knockout animals. Staining showed partial loss of primary cilia in heterozygous animals and complete loss or very shortened cilia in knockout animals (Fig 3.5A). Histologically, the conditional knockout and heterozygous mice have enlarged anterior leaflets (Fig 3.5B). 3D reconstruction of H and E staining showed a trend towards significance of conditional heterozygous and a significant increase in anterior leaflet volume of the conditional knockout when compared to wildtype littermate controls (Fig 3.4C).

Primary cilia play a role in sensing fluid movements in surrounding tissues. Motile cilia are used to propel molecules and objects across the surface of some tissues. In addition, many studies have shown that during valve development the biomechanical forces that are present play a major role in how the valves are formed. Therefore, one would hypothesize that primary cilia would be expressed on the surface of the valves. However, this is not what we observe. Very early during valve development (E9.5) we see some VEC primordia expressing primary cilia however the percentage is very small. Therefore, we hypothesized that the primary cilia expressed on the VICs are what is regulating valve development. To further address this question, we conditionally deleted Ift88 in multiple cre models during development.

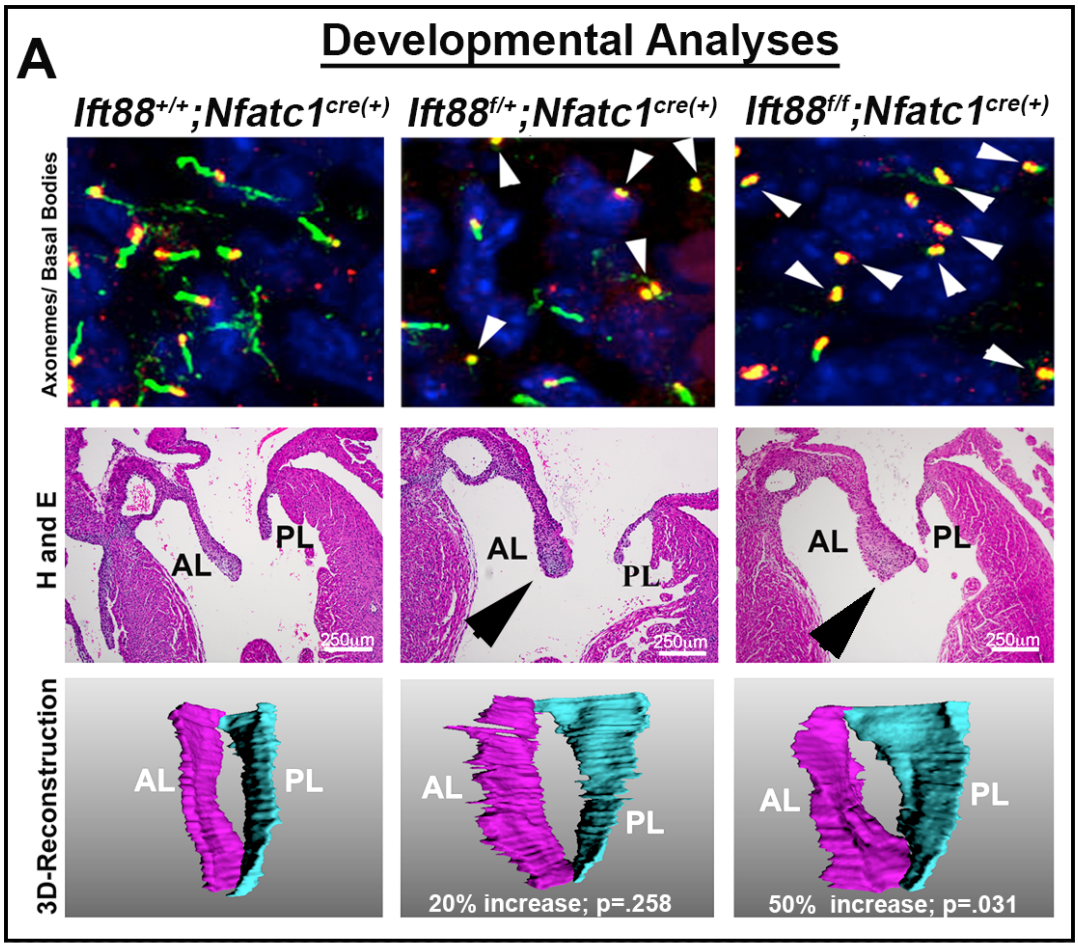



**Figure 3.5 Loss of *Ift88* Impairs Ciliogenesis During Development and Leads to Enlarged Postnatal Mitral Valve.** (A) IHC at postnatal day 0 show gene dosage effect for *Ift88* on axoneme (green) formation in the mitral valves (white arrow heads). (B) H and E staining's show that cHET and cKO have enlarged mitral leaflets. *Student t-test* of valve volumes obtained from 3D-reconstructions (C) show a trend towards significance in the cHET and a significant increase in valve volume in the cKO. AL=Anterior leaflet, PL= Posterior Leaflet.

| Cre-line | Gene knockout | Cell-Type Specificity | Expected Valve Defects |
|---|---|---|---|
| *NfatC1*$^{Cre}$ | *Ift88*$^{f/f}$ | VEC + VIC | Pan Valve Defects |
| *Periostin*$^{Cre}$ | *Ift88*$^{f/f}$ | VIC | Pan Valve Defects |
| *Nfatc1*$^{en\text{-}Cre}$ | *Ift88*$^{f/f}$ | VEC | No Defects |
| *WT1*$^{Cre}$ | *Ift88*$^{f/f}$ | Epicardium and EPDCs | Mural AV Valve Defects |

**Table 3.1 A list of Cre-lines and expected outcomes.** VIC=valve interstitial cells, VEC= valve endothelial cells.

As shown in Table 3.1, conditional deletion of Ift88 using the Nfatc1$^{en\text{-}Cre}$ would ablate cilia expression in only endocardial cells. We hypothesized that conditional loss of Ift88, with cre models that are expressed in VICs (periostin and NfatC1), will result in an enlarged valve phenotype. In addition, if ciliated VICs are regulating valve development then conditional loss of *Ift88* in VECs, using the enhancer cre, will not have a phenotype.

Data show that conditional loss of primary cilia in VICs using the periostin cre driver results in an enlarged valve phenotype (Fig 3.6). This change trends towards significant but is not statistically significant(anterior leaflet p=0.31, posterior leaflet p=0.27). The anterior leaflet of these mice appear to be enlarged in specific regions and not uniformly large (Fig 3.6). Further analysis looking at length and width measurements might be helpful in fully characterizing this phenotype.

Surprisingly conditional loss of Ift88 with the enhancer cre driver resulted in significantly larger posterior leaflet and an anterior leaflet that was enlarged although not significantly so, when compared to wild type littermates (anterior leaflet p=0.03; posterior leaflet p=0.16) (Fig 3.6). There are two possibilities for why this phenotype may be occurring. First since we see very little to no primary cilia expression in the endocardial cells of the valve, loss of Ift88 may be playing a cilia independent roll in valve development. It has been shown that Ift88 can play a role in microtubule dynamics and cell migration independent of primary cilia (Borovina and Ciruna 2013, Boehlke, Janusch et al. 2015). There are, however, some endocardial cilia on the valves very early during development (E9.5-10.5). The other possible explanation is that loss of primary cilia on the endocardial cells during early valve development is enough to result in enlarged mitral valves.

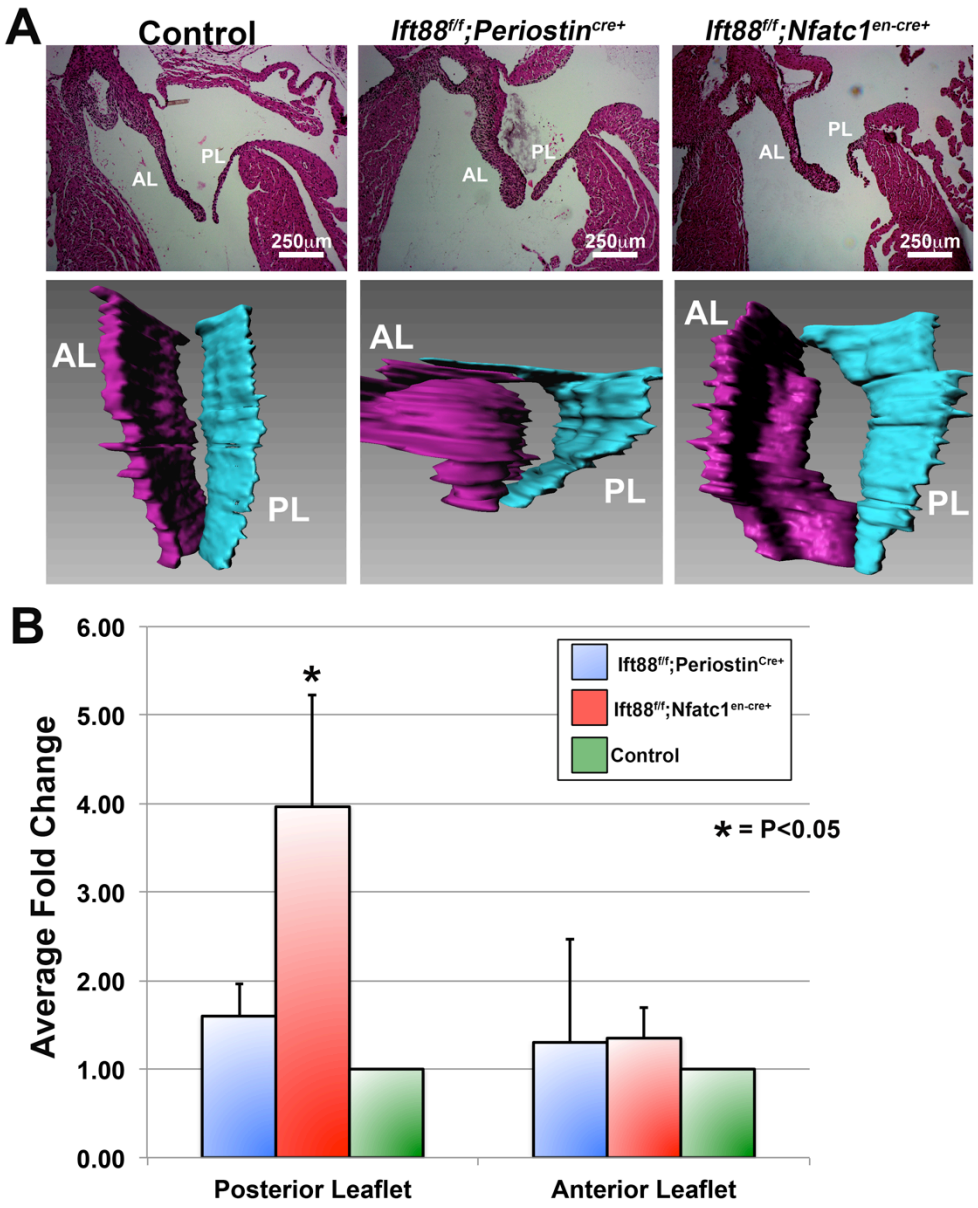



**Figure 3.6 Deletion of Ift88 using Periostin and Enhancer Cre models results in enlarged mitral valves**. A. H and E and 3D reconstruction of Ift88 periostin and enhancer cre conditional knockout mice showed enlarged anterior leaflets when compared to control. B. Quantification shows a trend towards a significant increase in valve volume is seen in periostin cre posterior and anterior leaflets while a significant increase in volume is seen in the enhancer cre posterior leaflet.

Since there was so much variation in the phenotypes that were observed we wanted to look at cre expression in these animals. To do this we used the Rosa26 green fluorescence protein (GFP) reporter mouse crossed with the NfatC1, periostin, and NfatC1-enhancer cre mice. This allows GFP expression in all cells that cre recombinase is active.

Data show that the periostin and enhancer cre models are not as robust as originally thought (Fig 3.7). GFP expression shows limited periostin cre activity in VICs as shown in figure 3.7. and the enhancer cre mouse also showed limited expression. NfatC1 enhancer cre expression was seen on the ventricularius aspect of the valve with limited expression on the atrialus. Taken together, these data suggest that partial loss of primary cilia within the valve is sufficient to result in a phenotype. Therefore, to remain consistent the NfatC1 cre was used for the following experiments.

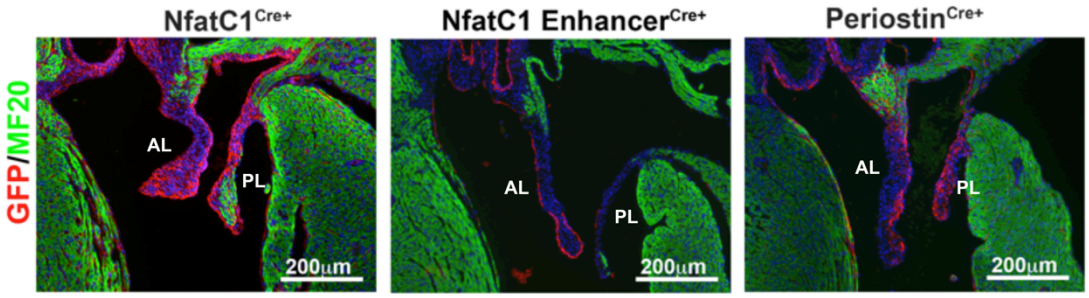



**Figure 3.7. Cre Recombinase Expression Patterns.** IHC for GFP shows Cre activity under specific drivers. GFP expression in NfatC1 mice is nearly all valve cells. NfatC1 enhancer expression is localized mainly to the ventricularus aspect and is absent in the majority of artrial endothelial cells. Periostin cre expression is found in VICs within the anterior and posterior leaflet but is not expressed by all VICs. AL= anterior leaflet, PL=posterior leaflet.

One of the common themes among models with MVP is increased proliferation and since primary cilia regulate cell cycle we began by looking for changes in proliferation. We looked at proliferation of the *Ift88 Nfatc1*$^{cre}$ mice as a way of explaining the increase in valve volume. Data show no significant difference in proliferating cells in conditional heterozygous or knockout animals when compared to wild type littermates (Fig 3.8). To determine if the increase in valve size could be explained by increase infiltration or migration of cells, total cell numbers in each leaflet were also counted (Fig 3.8C). No difference was observed in total cell number of wildtype or conditional heterozygous and knockout animals. These data suggest that the increase in valve volume cannot be explained by increased proliferation or migration of cells in the conditional knockout and heterozygous leaflets.

Another hallmark of myxomatous valve diseases is increased ECM so the increase in valve volume that was observed could be explained by increase in ECM. To determine if there is more ECM in Ift88 conditional mice cell density was examined. Decreases in cell density, but no change in total cell number, would be explained by changes in ECM production. Decreased VIC density was observed in the anterior leaflet of conditional knockout animals (Fig 3.9). These data were then confirmed by whole transcriptome analyses (RNAseq) analysis.

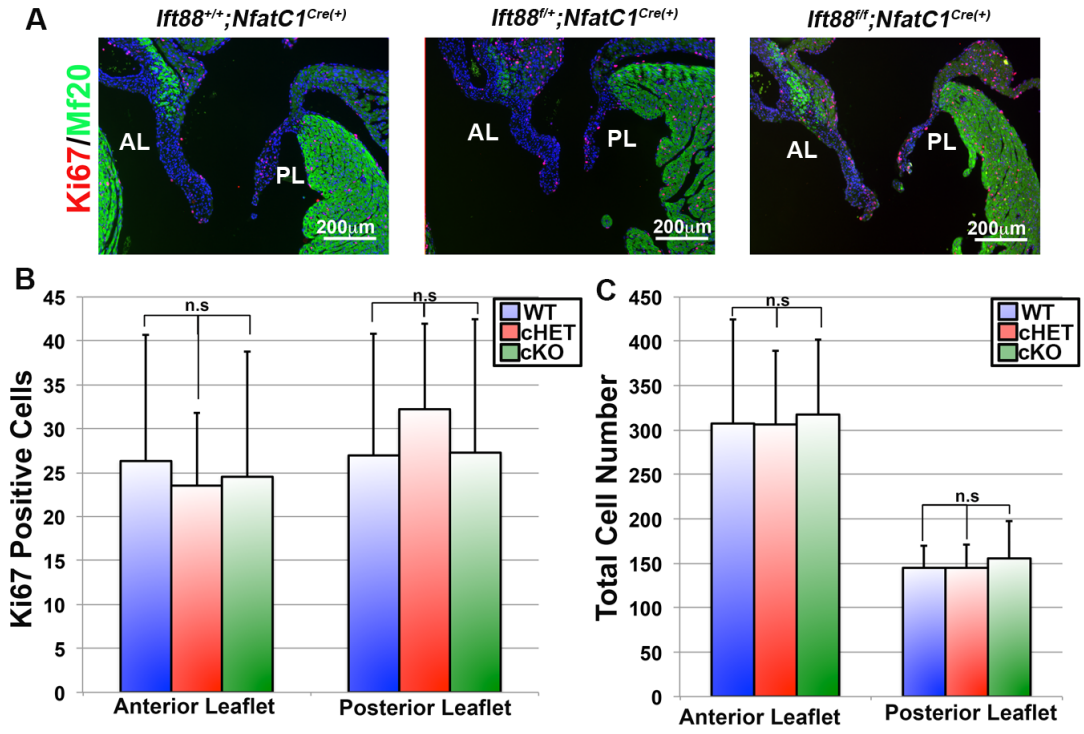



**Figure 3.8 Loss of Primary Cilia have no Effect on Proliferation. (A)** IHC for proliferating cells stained with ki67 (red) and myocardium stained with mf20 (green). **(B)** Quantification of the stains from A showing no significant difference in wildtype, conditional heterozygous and knockout leaflets. **(C)** Quantification of total cells in both anterior and posterior leaflets showed no significant difference in wildtype, conditional heterozygous and knockout leaflets. AL= Anterior Leaflet, PL= Posterior Leaflet

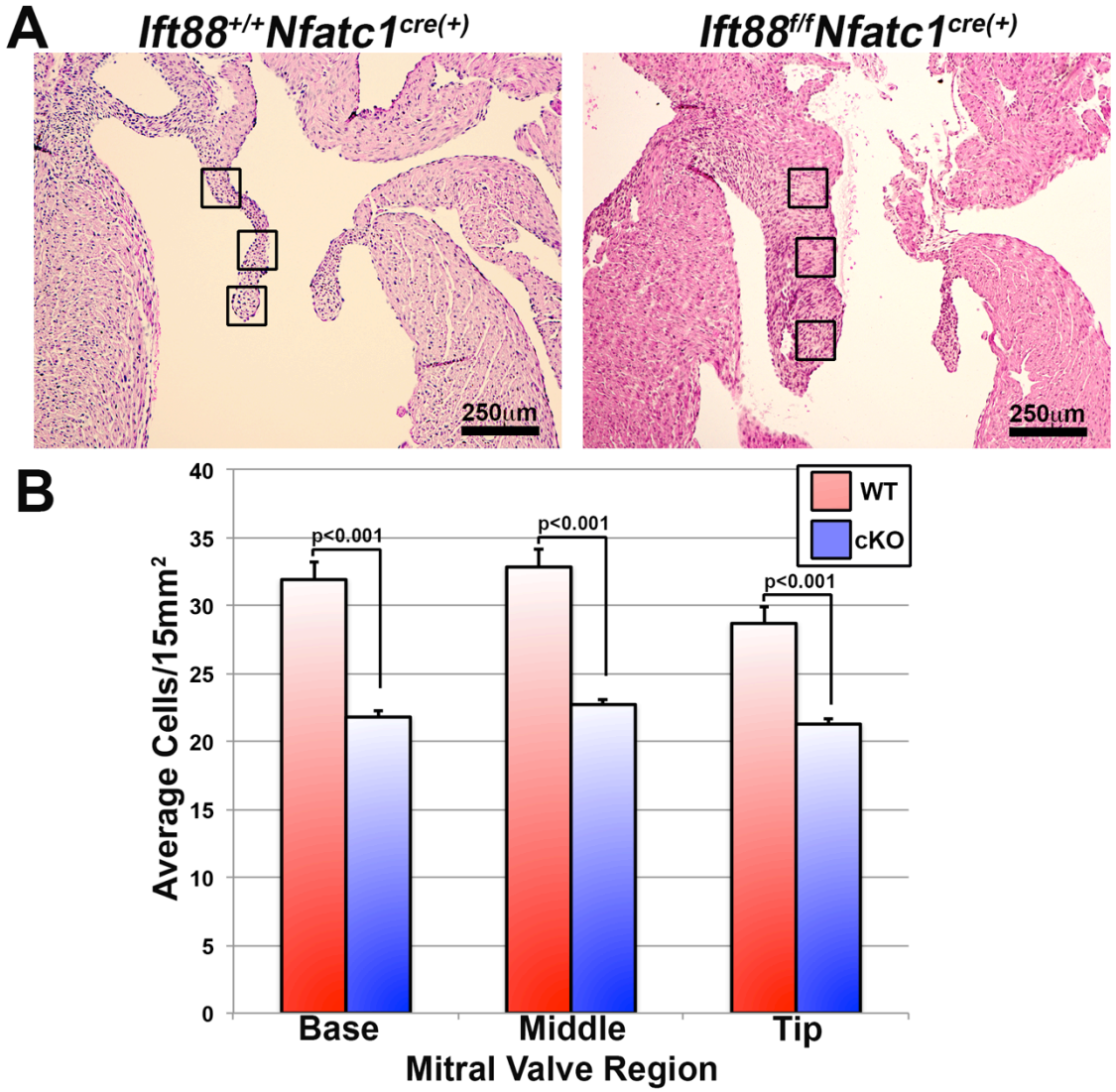



**Figure 3.9 Loss of Primary Cilia Results in Decreased Cell Density. (A)** H and E showing areas of decreased cell density in the conditional knockout anterior leaflet. Boxes show representative area where cell counts were performed at the base, middle and tip of the leaflet. **(B)** Quantification of areas represented in A, at the base, middle and tip of the anterior leaflet. There is a significant decrease in the number of cells per area at the base, middle and tip of the anterior leaflet of the conditional knockout. Students t-test p-value <0.001.

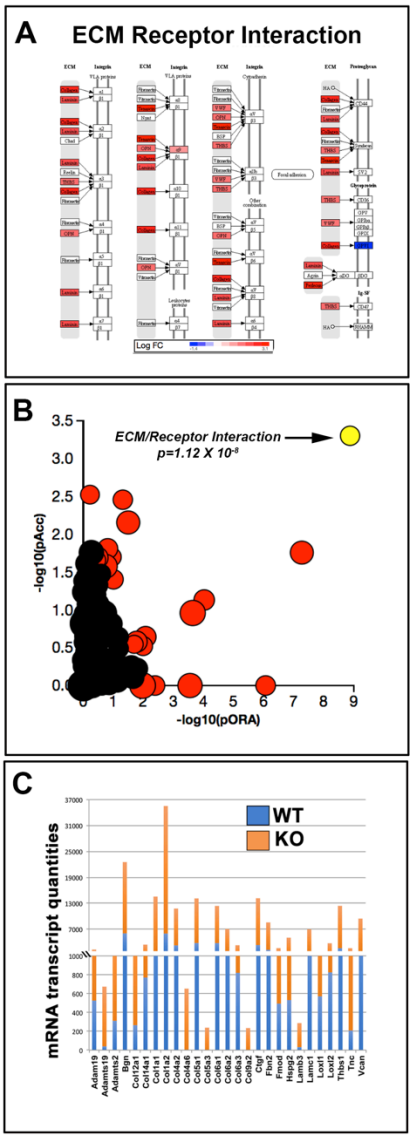



**Figure 3.10: Loss of Primary Cilia Results in Increased Expression of ECM Pathways.** A. Perturbation vs. over-representation plot**.** The most significant change observed in RNAseq datasets was ECM/Receptor interactions (arrow and yellow circle) with a p-value= $1.12X10^{-8}$ B. mRNA transcript quantities of differentially expressed ECM genes. Graphical representation of ECM genes that are significantly upregulated in the Ift88 conditional knockout mitral valves. Genes were selected based on $p<.05$. C. Pathway analyses of Differentially Expressed Genes. Pathway representation showing genes are increased in the Ift88 conditional knockout (red) or decreased (blue) and their intersection with receptor-mediated pathways.

Individual leaflets from wildtype and knockout animals were dissected and extracted RNA was analyzed. Of note markers of extracellular matrix were significantly increased in the conditional knockouts. These data confirmed that loss of primary cilia result in robust activation of fibrogenic and proteoglycan ECM gene programs that is consistent with early stages of myxomatous degeneration.

Further analysis was preformed to determine if the endothelial cell number was changed in these mice. Increases in valve volume should be associated with increases in endothelial cell number to compensate for the increased valve sizes. However, when we counted total endothelial cells we found that there was no change in cell number between conditional knockouts and wild-type litter mates (Fig 3.11). This might be explained by changes in proliferation among the endothelial cells. To address this question, stains were done looking at proliferating endothelial cells. Again, no difference was observed between conditional knockout and wildtype valves in both anterior and posterior leaflets (Fig 3.11). These data suggest that loss of primary cilia results in increased valve volume which cause endothelial cell stretching.

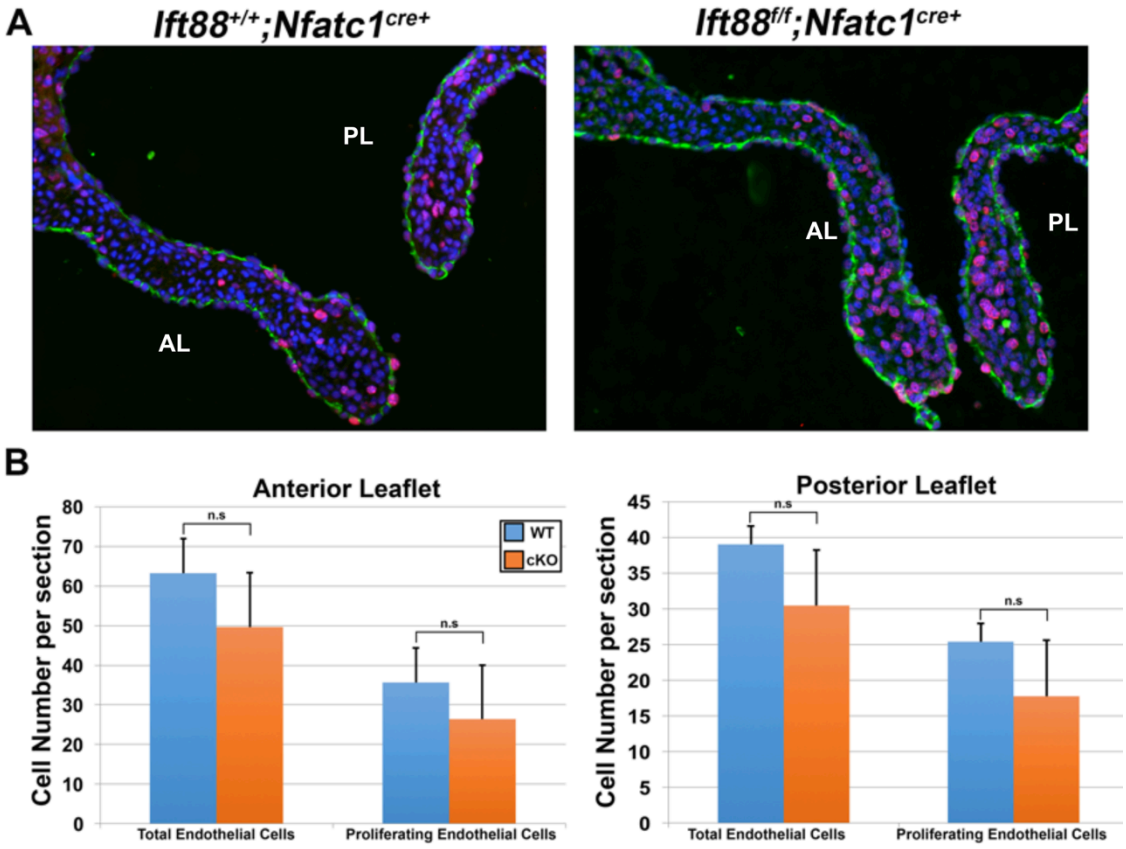



**Figure 3.11. Loss of Primary Cilia Results in Endothelial Cell Stretching. A.** IHC showing stains for pcam (green) marking the endothelial cells, ki67 (red) marking the proliferating cells, and dapi (blue) staining the nuclei. **B.** Quantification shows no significant difference in total endothelial cell number or proliferation of endothelial cells in both anterior and posterior leaflets. AL= anterior leaflet, PL= posterior leaflet

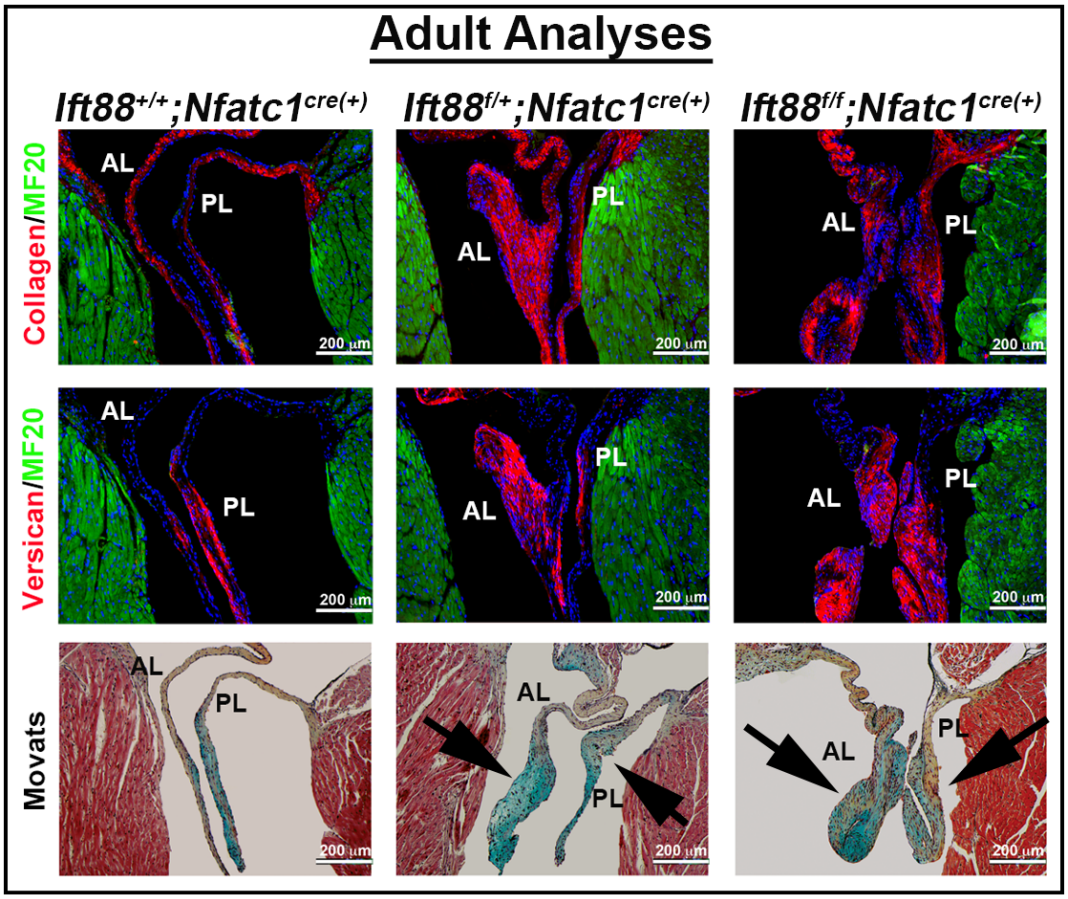



**Figure 3.12. Loss of Primary Cilia Leads to Myxomatous Mitral Valve Disease.** IHC (top and middle) and Movat's staining (bottom) of 4-6 month old *Ift88* deficient mice show loss of normal versican and collagen boundaries and a myxomatous phenotype in conditional heterozygous and knockout animals (black arrows). Movats staining shows increased proteoglycans (blue), and collagens (yellow). AL, PL = Anterior and Posterior Leaflets, respectively.

To determine if these developmental changes result in adult myxomatous mitral valve degeneration adult Ift88 conditional mice were analyzed. Histologically these mice develop myxomatous degeneration as shown by proteoglycan and collagen accumulation (Fig 3.12). H and E and Movat's pentachrome stains show loss of normally stratified valves composed of distinct zones of elastin, collagen and proteoglycan in conditional heterozygous and knockout animals (Fig 3.12). Patients with myxomatous valve disease and MVP have disrupted matrix boundaries and increased production of collagens as seen in figure 3.13. The Ift88 conditional mice phenocopy the patients, having significantly increased collagen production and disruption of matrix boundaries as marked by increased blue staining in Herovici's stain (Fig 3.13). Thus, loss of primary cilia causes developmental defects that are characterized by expansion of extracellular matrix and disrupted histological architecture that progress to an adult myxomatous valve pathology, similar to that observed in patients with MVP.

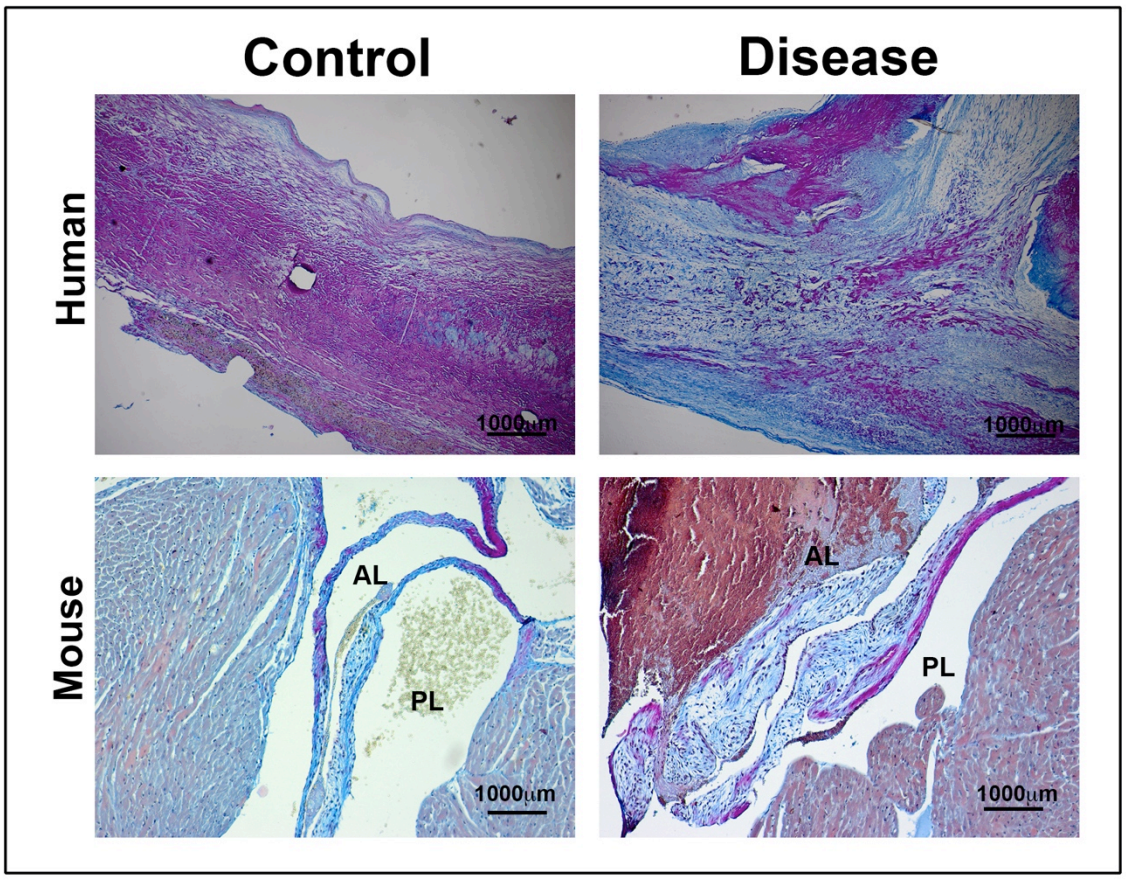



**Figure 3.13 Ift88 Conditional Mouse Models Phenocopy Matrix Disruption and Increased Collagen Deposition Found in Human Patient Valves.** Herovici's stains showing immature or newly synthesized collagen in blue and mature collagen in red. Both diseased human and mouse valves have blurring of mature and immature collagen as well as increased production of new collagen.

**Genetic association of Primary Cilia and MVP**

Based on these findings as well as evidence from the literature showing a 25% comorbidity of ADPKD and MVP, we hypothesized that primary cilia may play a broad role in mitral valve disease in humans. To test this hypothesis, we wanted to evaluate the link between the current MVP genes (DCHS1 and FLNA) and ciliogenesis. IHC staining for cilia in these mice showed significant decrease in cilia length. Dchs1 heterozygous mice had significant decrease in the number of ciliated cells at E17.5 (Fig 3.14). 3D reconstruction of primary cilia in these valves show significant loss of axonemes. Quantification showed a significant decrease in the number of ciliated cells (Fig 3.14). In addition, the Filamin A mouse model of MVP also had significant cilia loss. At postnatal day 0 of both Dchs1 knockout and filamin A conditional knockout showed significant decrease in average cilia length when compared to controls (Fig 3.15). These data suggest that primacy cilia may play a role in the development of MVP regardless of the gene involved.

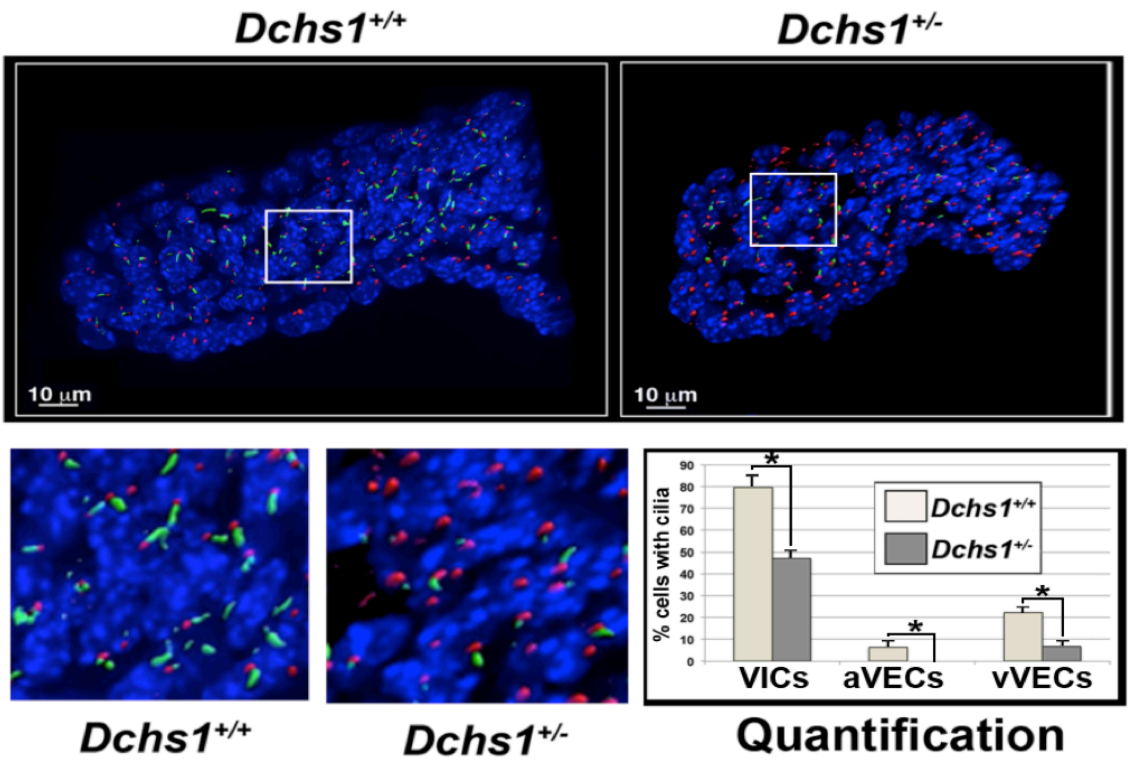



**Figure 3.14 Dchs1 Heterozygous Mice have a Primary Cilia Defect.** 3D reconstruction of cilia from E17.5 posterior leaflets show loss of axonemes (green) in Dchs1+/- mice. Red=Basal Bodies. Magnification of boxed area shown. >50% loss of cilia in VICs and VECs (aVECs, vVecs= atrialis and ventricularis valve endothelial cells. *p<0.01.

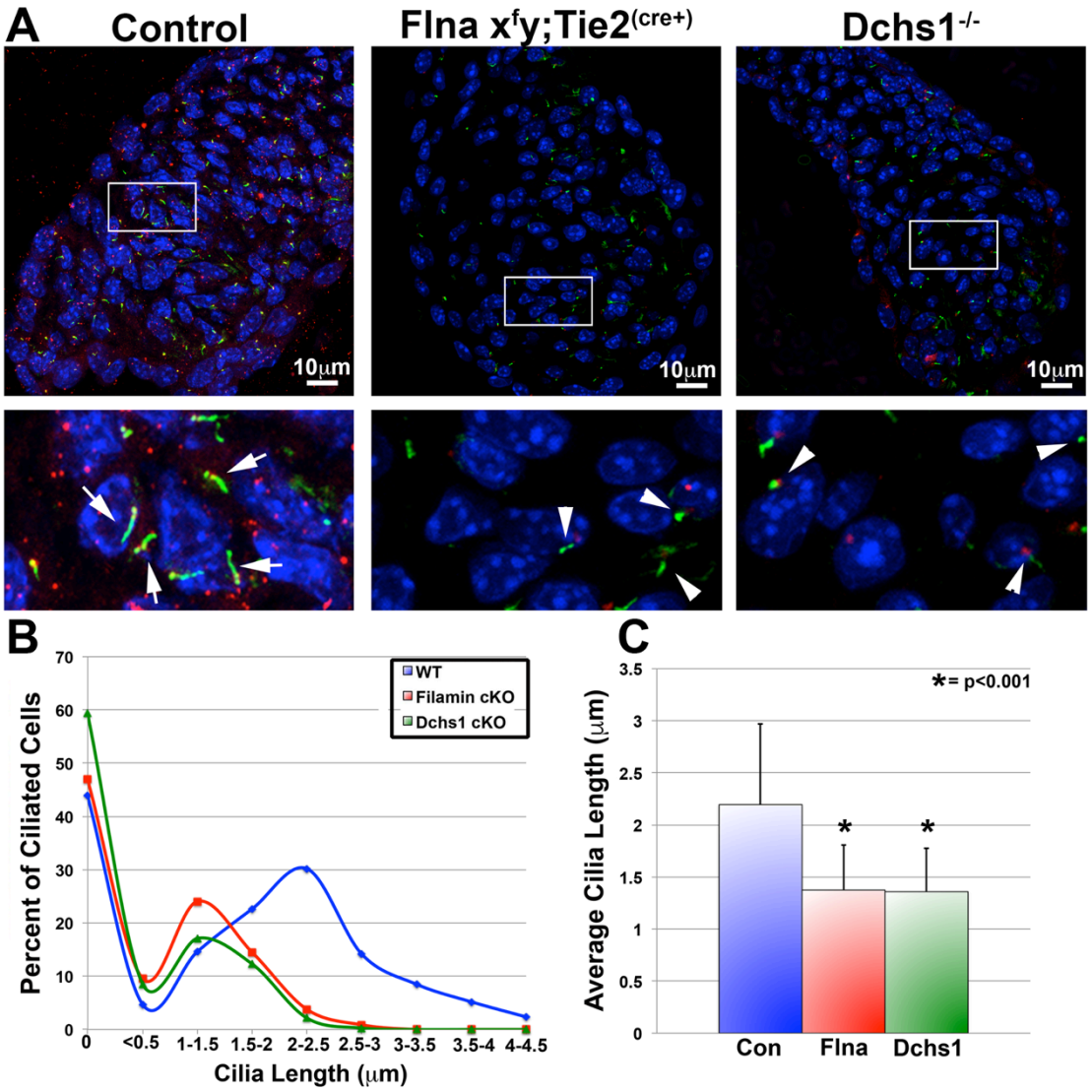



**Figure 3.15 Decreased cilia expression in knockouts associated with MVP. (A)** IHC of P0 Dchs1 knockout and filamin-A conditional knockout mitral leaflets showing axonemes (acetylated tubulin-green) basal bodies (gamma tubulin-red). **(B, C)** Grouped cilia length of Dchs1 knockout and filamin-A conditional knockout mice show a shift in grouped cilia length with the majority of cilia ~1.4μm in length while control P0 mice have the majority of cilia ~2.25 μm in length, which represents a 40% decrease in cilia length in the knockout mice with p<.001.

We further evaluated broad involvement of the cilia pathway in MVP by analyzing GWAS data from 1,412 MVP cases and 2,439 control samples to test for global enrichment with significant association among a list of 278 different genes known to be involved in primary cilia biology, mainly as harboring rare mutations in a diverse panel of ciliopathies.

We performed SNP test ratio method on several GWAS where phenotypes of cases and controls were permuted to generate 1000 simulated GWAS. Then, according to a pre-defined *p-value* threshold, the method consisted of counting the number of times the pathway tested included a higher ratio of significant to non-significant SNPs, compared to the 1000 simulated GWAS (Table 3.1). As recommended for this method, we used the KEGG pathways catalog in addition to our customized list of cilia genes. Given the limited number of highly associated SNPs in the current GWAS, we set significance at $P<0.05$ to allow more SNPs to contribute the analyses. Among nominally associated SNPs in the GWAS, we found that SNPs that map in the cilia genes list are more likely ($P=0.03$) to be enriched for associated SNPs with MVP.

| Ratio of significant to non-significant SNPs higher in simulated data | Total number of analyses | Empirical p-value | Description of the pathway |
|---|---|---|---|
| | | | |
| 1 | 1001 | 0.001 | Phagosome |
| 1 | 1001 | 0.001 | Viral myocarditis |
| 2 | 1001 | 0.002 | Hypertrophic cardiomyopathy (HCM) |
| 2 | 1001 | 0.002 | Dilated cardiomyopathy |
| 3 | 1001 | 0.003 | Oxidative phosphorylation |
| 3 | 1001 | 0.003 | Primary immunodeficiency |
| 4 | 1001 | 0.004 | Glycolysis / Gluconeogenesis |
| 4 | 1001 | 0.004 | Antigen processing and presentation |
| 5 | 1001 | 0.005 | Fructose and mannose metabolism |
| 5 | 1001 | 0.005 | Cardiac muscle contraction |
| . | . | . | . |
| . | . | . | . |
| . | . | . | . |
| 34 | 1001 | 0.034 | Manually curated cilia gene list |

**Table 3.1: GWAS Phenotypes of Cases and Controls were Permuted to Generate 1000 Simulated GWAS.** Among nominally associated SNPs in the GWAS (SNPs<0.05), we found that SNPs that map in the cilia genes list are more likely (trend P=0.034) to be enriched for associated SNPs with MVP. Of note, this analysis highlighted at the top 10 enriched pathways consistent cardiac enriched pathways including hypertrophic cardiomyopathy, dilated cardiomyopathy and cardiac muscle contraction

## Discussion

These data show for the first time that primary cilia are necessary for proper mitral valve development. Thorough these studies, we have shown that primary cilia are expressed both spatially and temporally on valvular interstitial cells but not endothelial cells as previously described. Similar to studies from the kidney, primary cilia in the heart increase in length with development, a phenomenon which is likely important in regulation of developmental processes occurring at the different stages. These primary cilia localize to areas of increased proteoglycan accumulation which has strong implications for a chemosensory roll. Further studies are needed to determine if primary cilia regulate ECM expression or if localization of cells within a specific ECM regulates the expression and length of a primary cilia.

Primary cilia are necessary for proper valve development and when disrupted result in myxomatous valve disease. Our studies using the Ift88 conditional mouse model show loss of primary cilia prematurely results in increased postnatal valve volume. This increase in valve volume is not caused by increased proliferation, decreased apoptosis, increased infiltration of extra-cardiac cells or increased EMT. The increase in valve volume is caused by increased ECM expression as shown by decreased cell density and increased ECM expression. Since fibroblasts in the valve organize matrix, this increase in matrix could also be a loss of compaction issue. To determine this, further studies would be needed. Matrix compaction assays could be done by embedding VICs from Ift88 control and conditional knockout mice in collagen gels and determining if the compaction of collagen is affected more in either genotype.

These changes shown during valve development change the structure and stratification of ECM expression which results in myxomatous adult valves. Conditional deletion of Ift88, and thereby primary cilia, result in myxomatous adult mitral valves. In this model we see blurring of matrix boundaries and increased collagen and versican. Additionally, conditional knockouts have increased collagen production indicating that matrix turnover is affected. More work could be done to determine if these mice do develop MVP. Echocardiography of aged mice to look for prolapse could be done.

Additionally, Dhh has been shown in the literature to be important for endothelial boundary function and endothelial integrity. (Chapouly, Yao et al. 2016) Interestingly, in our Ift88 conditional knockout animals we saw no difference in total endothelial cell number. An increase in valve size but no increase in endothelial cell number suggest that the endothelial cells within the valve would have to stretch to compensate. This stretching may be leading to cytokine release which signals to circulating macrophages that the tissue was damaged. In other disease models and in human patients with myxomatous mitral valve disease, infiltration of immune cells is common.

Human data further supports the hypothesis that primary cilia are important for proper valve function. GWAS analyses were performed on patients with MVP. Data show, increased association of ciliary genes with MVP patients. These studies suggest that primary cilia may play a broad role in the development of MVP. Previously identified MVP genes, Dchs1 and FlmnA, show decreases in primary cilia length as well. This novel finding provides further support for the role of primary cilia in valve disease.

These studies show MVP is a congenitally based disease of the primary cilia that results in changes in ECM remodeling resulting in weakened valve leaflets. These leaflets

over time become myxomatous. The increase in valve size leads to strain on endothelial boundary formation ultimately leading to releases of cytokines and infiltration of macrophages as seen in Filamin A adult mice and human patients. This process occurs over many years in the patient's life and therefore MVP patients are often asymptomatic early on in life.

# CHAPTER 4: MUTATIONS IN DZIP1 CAUSE MITRAL VALVE PROLAPSE

## Introduction

Currently little is known about the etiology of MVP. Familial studies have provided evidence for a genetic association but until recently a causal gene has not been identified. Informative studies from our lab has shown an association between primary cilia and valve disease. Published literature shows a 25% comorbidity between MVP and an established ciliopathy ADPKD. In addition, our data from Ift88 cilia knockout mice show that primary cilia are important for valve development and cause myxomatous valve disease when disrupted. These data suggest that primary cilia are important for proper valve development and when disrupted play a role in the development of MVP.

In order to further evaluate the role of primary cilia in MVP, we examined our patient data to determine if cilia genes were associated with the linked region. Analysis was done on a multi-generational family with inherited autosomal dominion MVP previously linked to chromosome 13 (Fig 4.1). Out of 43 individuals enrolled in the original study, nine patients met the full clinical diagnostic criteria for MVP, with two individuals having moderate to severe MR and one with ruptured chordae that required surgical intervention. Additionally, six were coded as minimal MVP, and two had prodromal pre-clinical forms, which have recently been reported to progress and have a familial association. None of the individuals in the pedigree exhibited extracardiac manifestations, and all MVP-affected individuals were deemed as “non-syndromic”.

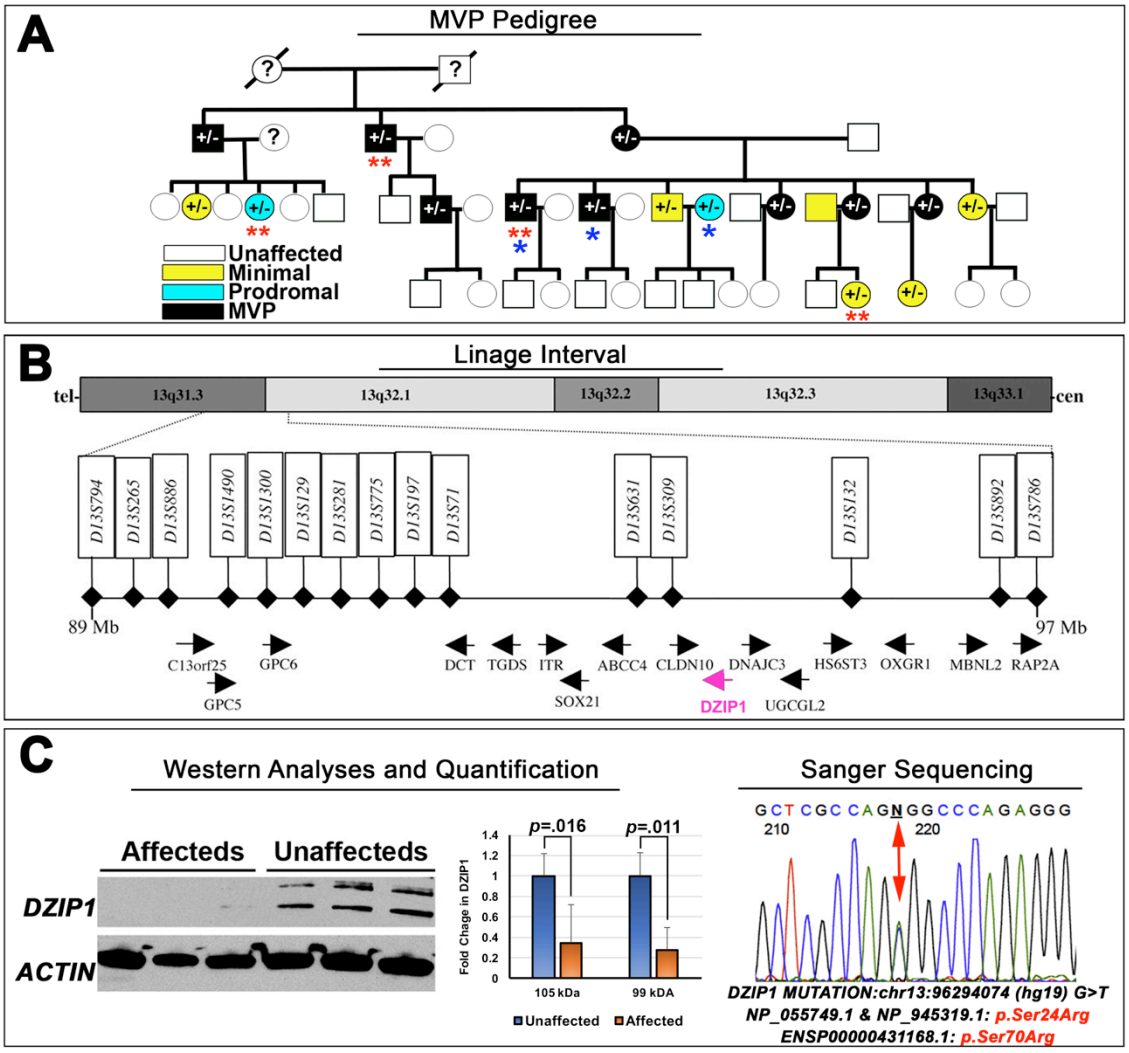



**Figure 4.1 Identification of DZIP1 as an MVP gene**. **(A)** Multigenerational family with inherited, autosomal-dominant, non-syndromic MVP. ?= unknown phenotype. **(B)** Human transcript and marker maps of the linkage interval on chromosome 13. Candidate region is within 13q31.3-32.1, and all *RefSeq* genes and their orientations are shown within the 8.2-Mb interval. *DZIP1*, the only cilia gene within the locus is highlighted in pink. **(C)** Western analyses and statistical analyses (*t-test*) for DZIP1 showing that each of the 3-affecteds analyzed had a significant reduction (*p=.016, p=.011*) in DZIP1 protein expression. Sanger sequencing identified a single missense mutation within exon 5 of *DZIP1* resulting in a serine to arginine change. +/- denotes DZIP1 variant in affected individuals.

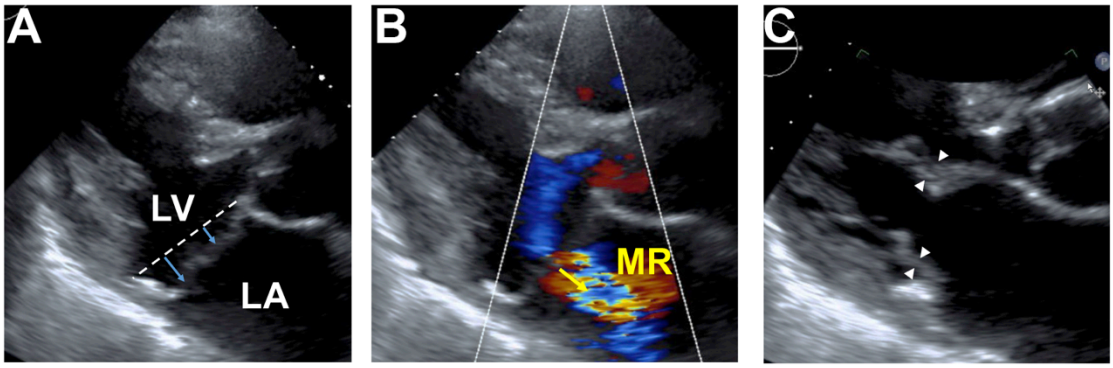



**Figure 4.2 Echocardiography of Proband from Chromosome 13 family. (A)** Parasternal long-axis view of proband from US family showing prolapse (blue arrows) above the annulus during ventricular systole (dotted line). **(B)** Significant MR is observed in this patient (yellow arrow). **(C)** Thickened, myxomatous appearing mitral leaflets (arrowheads).

The proband of this family displayed significant bileaflet prolapse coincident with mitral regurgitation (Fig 4.2). The linkage peak spanned 8.2 Mb and included 14 genes (Fig 4.1). In order to define the putative MVP gene(s) in this family, we used a candidate-based approach. *DZIP1* was the only gene within the linkage region that had previously been shown in other model systems to regulate ciliogenesis and/or cilia signaling.

What is Dzip1?

DZIP or DAZ-interacting protein was originally identified in zebrafish where it was shown to interact with DAZ (Moore, Jaruzelska et al. 2004). DAZ and DAZ-like proteins (DAZL) are expressed in the developing testies and ovary where they function in early germ cell development. Their expression is gone by early somatic cell generation but DZIP expression is maintained and expressed by neuronal cells. Dzip1 is a centrosomal protein that is important for ciliogenesis, vesicle transport to the primary cilium, and downstream Hh signaling. Loss of Dzip1 results in loss of primary cilia. In zebrafish, the gene igu encodes for Dzip1 which is shown to localizes to lysosomes and endosomes(Sekimizu, Nishioka et al. 2004). For this reason, Dzip1 has been implicated shuttling proteins between the nucleus and cytoplasm.

Dzip1 is an important regulator of Hh signaling. In igu mutants, Hh signaling is impaired with decreased Gli activator formation and increased Gli repressor suggesting that Igu/Dzip1 function is required for full activation of Gli proteins in response to Hh signals(Sekimizu, Nishioka et al. 2004). In addition, Dzip1 is the bridge between IFT88 and Gli3. It has been shown to bind Gli3, but not Gli1 or Gli2, preventing Gli3 protein from entering the nucleus. Loss of Dzip1 in mice leads to accumulation of Gli3 in the nucleus(Wang, Low et al. 2013).

Vesicle transport also plays a very important role in the formation of the primary cilia. In mouse embryonic fibroblasts (MEFs), Dzip1 localizes to the basal body and shuttles IFT88 to the distal appendages of the mother centrioles during cilia formation(Wang, Low et al. 2013). Loss of Dzip1 results in loss of primary cilia. Dzip1 localizes to the centrosome during cell division and promotes the release of $Rab8^{GDP}$. Rab8 is the core mediator of vesicle trafficking to the cilium and loss of Rab8 localization or inhibition of the formation of $Rab8^{GDP}$ leads to improper cilia formation(Zhang, Zhang et al. 2015). In addition, the BBSome proteins are essential for regulating vesicle transport and localization of proteins to the primary cilia for assembly. First discovered in patients with Bardet-Biedl syndrome, the BBSome consists of multiple proteins which assemble to bind and target membrane vesicles by reading signals on vesicles destined for the ciliary membrane(Jin, White et al. 2010). Dzip1 plays a role in this process through its binding of PCM1 which mediates the recruitment of the BBSome to the centrosome(Zhang, Wang et al. 2017).

**Results**

We began our experiments by examining if Dzip1 is expressed in the developing mitral valve. RNA *in situ* and IHC experiments revealed robust expression of Dzip1 in the developing anterior and posterior mitral leaflets, with protein expression being localized to basal bodies within mitral valve interstitial cells (Fig 4.3).

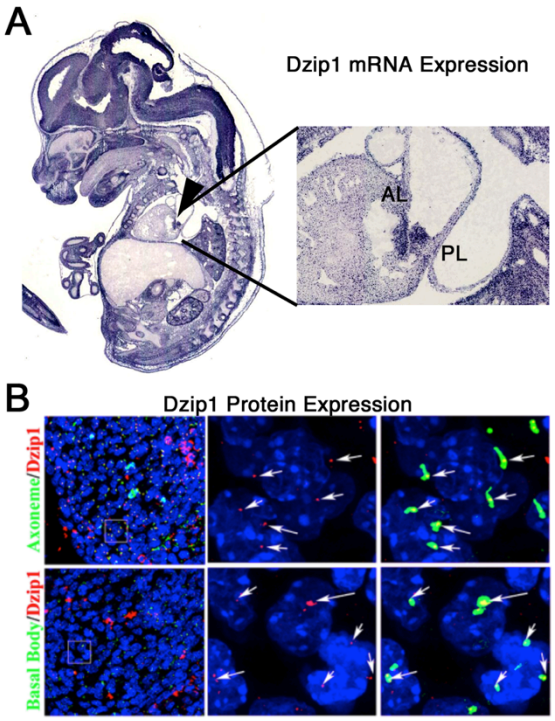



**Figure 4.3 Expression of Murine Dzip1. (A)** Section in situ of Dzip1 at E14.5 showing robust mRNA in the anterior and posterior mitral leaflets (AL, PL, respectively). **(B)** IHC for Dzip1 (red) and axonemes (green) or basal bodies (green) showing expression of Dzip1 at the basal body in E10.5 AV cushion mesenchyme.

Western analyses of lymphoblasts from affected and unaffected family members revealed significant down-regulation of DZIP1 protein expression (Fig 4.1). Sanger sequencing of DZIP1 revealed a proline-to-serine change in two known DZIP1 isoforms (p.S70R, p.S24R). Whole exome sequencing (WES) confirmed that no other coding changes were present within the linkage interval. Sanger sequencing of all members of the family confirmed segregation of the DZIP1 variant. A second, three-generation, family of European descent was identified with autosomal dominant MVP, and WES followed by Sanger sequencing identified the same P.S70R, p.S24R change in DZIP1 affected individuals (Fig 4.4). Minor allele frequency analyses in ExAC/gnomAD revealed that this variant is exceedingly rare, and was never observed in 122,000 exomes, and only once in 15,500 genomes. To test whether Dzip1 plays a significant role in ciliogenesis and MVP, conditional ablation of *Dzip1* (with $NfatC1^{cre}$) was performed in the mitral leaflets.

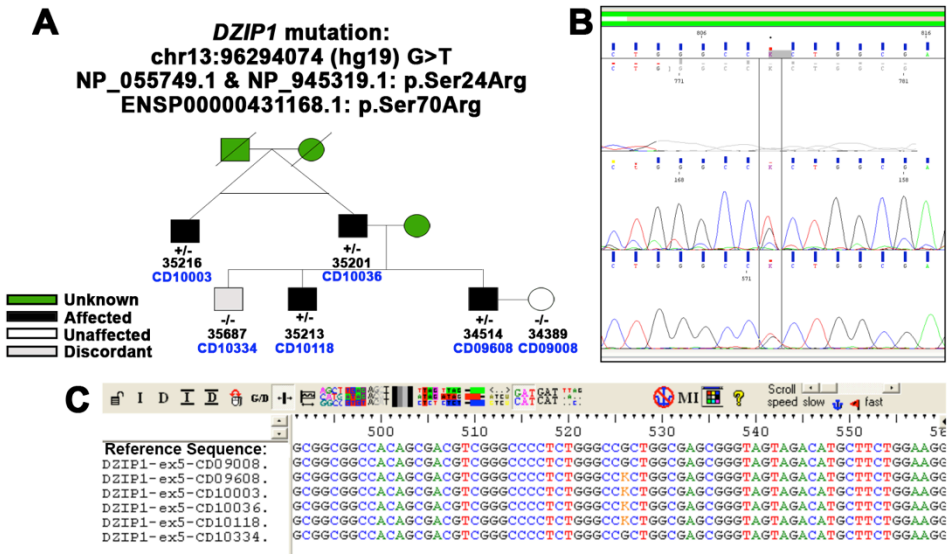



**Figure 4.4 Identification of additional MVP family with a DZIP1 mutation. (A)** Pedigree of family showing inherited, autosomal dominant MVP. += DZIP1 mutation carrier with the same mutation (p.S24R, p.S70R) as the original family. Grey box is an individual that lacks a DZIP1 mutation but has MVP. This individual has a different disease presentation with more symmetrical prolapse and altered anterior displacement of the coaptation point, suggesting a discordant phenotype unrelated to DZIP1 variants. **(B, C)** Sanger sequencing showing DZIP1 mutation and reference sequences for each of the family members.

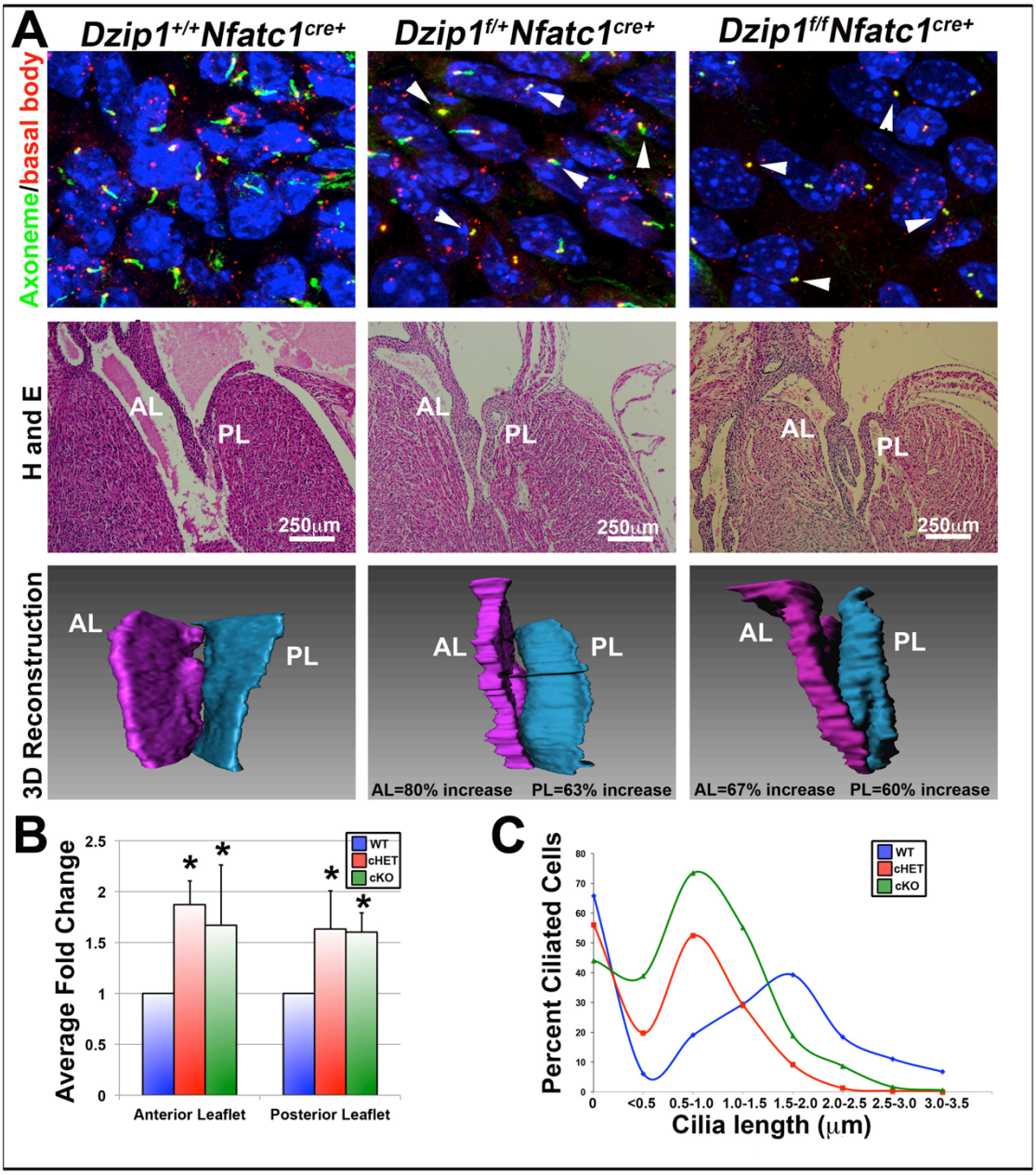



**Figure 4.5 Conditional deletion of Dzip1 results in increased postnatal valve size. (A)** IHC, H & E, and 3D reconstructions show loss of primary cilia (acetylated tubulin-green) and increased valve size in conditional heterozygous and knockout mice at P0. Basal bodies (gamma tubulin-red) and nuclei (Hoechst-blue). **(B)** Quantification of 3D reconstructions in A (*=p<.005). **(C)** Quantification of grouped cilia length in WT, conditional heterozygous (cHET) and conditional knockout (cKO) anterior leaflets showing decreased cilia length in cHET and cKO.

IHC showed loss of primary cilia in conditional knockout animals and partial loss in conditional heterozygous animals (Fig 4.5). The cilia that were present in these leaflets were significantly shorter than their wild type littermate controls. Histologically, conditional heterozygous and knockout animals had enlarged valve size as shown from H and E staining as well as 3D reconstruction (Fig 4.5). Both anterior and posterior leaflet volume of both heterozygous and knockout animals were significantly larger when compared to wild type littermates.

Previous research has shown Dzip1 to sequester Gli3 in the cytoplasm promoting its cleavage and preventing its localization in the nucleus (Wang, Low et al. 2013). Therefore, we wanted to asses Gli3 processing in the Dzip1 conditional mice. Protein was taken from single anterior leaflets of Dzip1 conditional heterozygous, knockout and control animals. Western blots were run and probed with Gli3 antibody. Data show no change in Gli3 cleavage between conditional heterozygous, knockout and control animals (Fig 4.6).

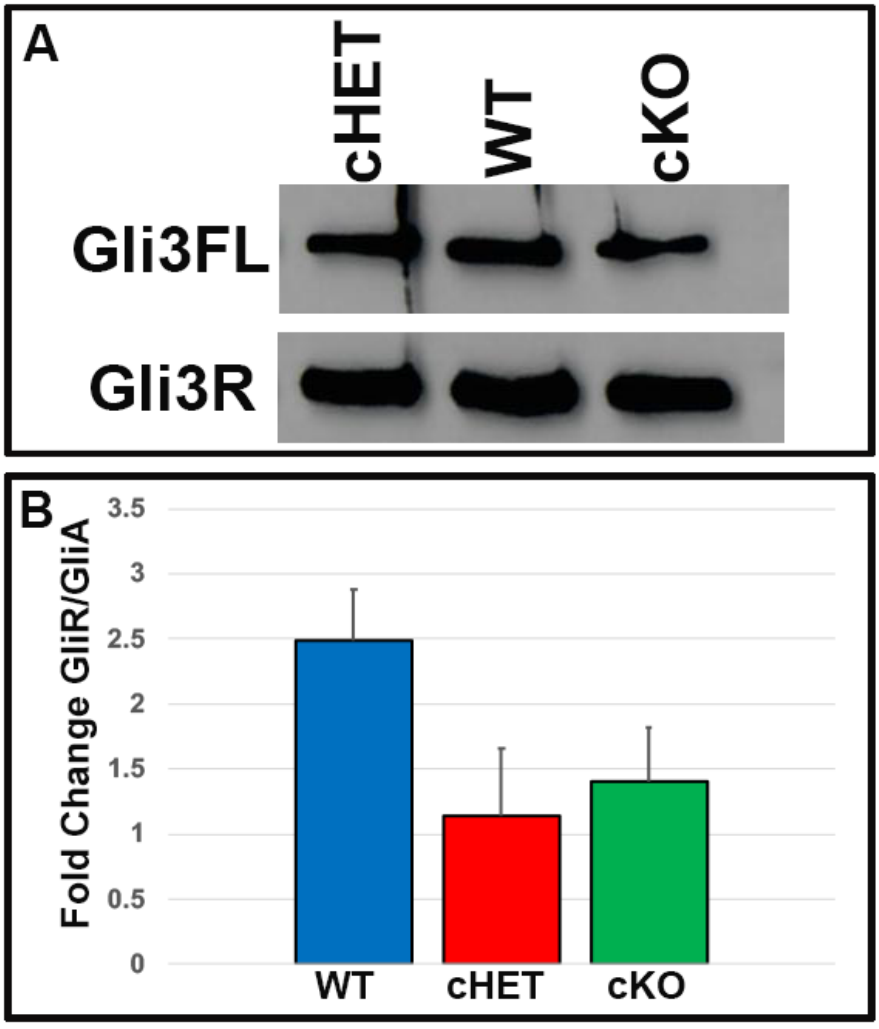



**Figure 4.6 Conditional Loss of Dzip1 Does Not Change Gli3 Processing.** Western blot of mouse AV cushion cell lysate probed for Gli3 shows no change in levels of Gli3R/Fl. B. Quantification of Gli3 western blot showing no significant difference in levels of repressor (Gli3R) and full-length activator (Gli3FL).

To determine if loss of Dzip1 and primary cilia play a role in the development of mitral valve prolapse, adult mice were assessed. Additional analyses of adult *Dzip1* conditional knockout mice (using either $NfatC1^{Cre}$ or $Tie2^{Cre}$) revealed a myxomatous phenotype with increased proteoglycans and collagen and loss of the normal ECM zonal boundaries, observed in control mitral leaflets (Fig 4.7).

Functional echocardiographic assessment revealed that these structurally altered mitral valves exhibited prolapse and leaflet elongation in conditional heterozygotes (2 out of 4) and knockout animals (4 out of 5) (Fig 4.7). MVP was never observed in control animals and there were no statistically significant alterations in ventricular contractile function between the conditional Dzip1 heterozygote, knockout and control animals (Fig 4.8). Interestingly, we noted a gene dosage affect in which loss of one allele of Dzip1 was sufficient to perturb ciliogenesis during development and engender a mitral valve phenotype in the adult. These data support our finding of a rare, damaging DZIP1 missense mutation in two families with MVP, which support the proposed hypothesis that MVP is also a form of a ciliopathy.

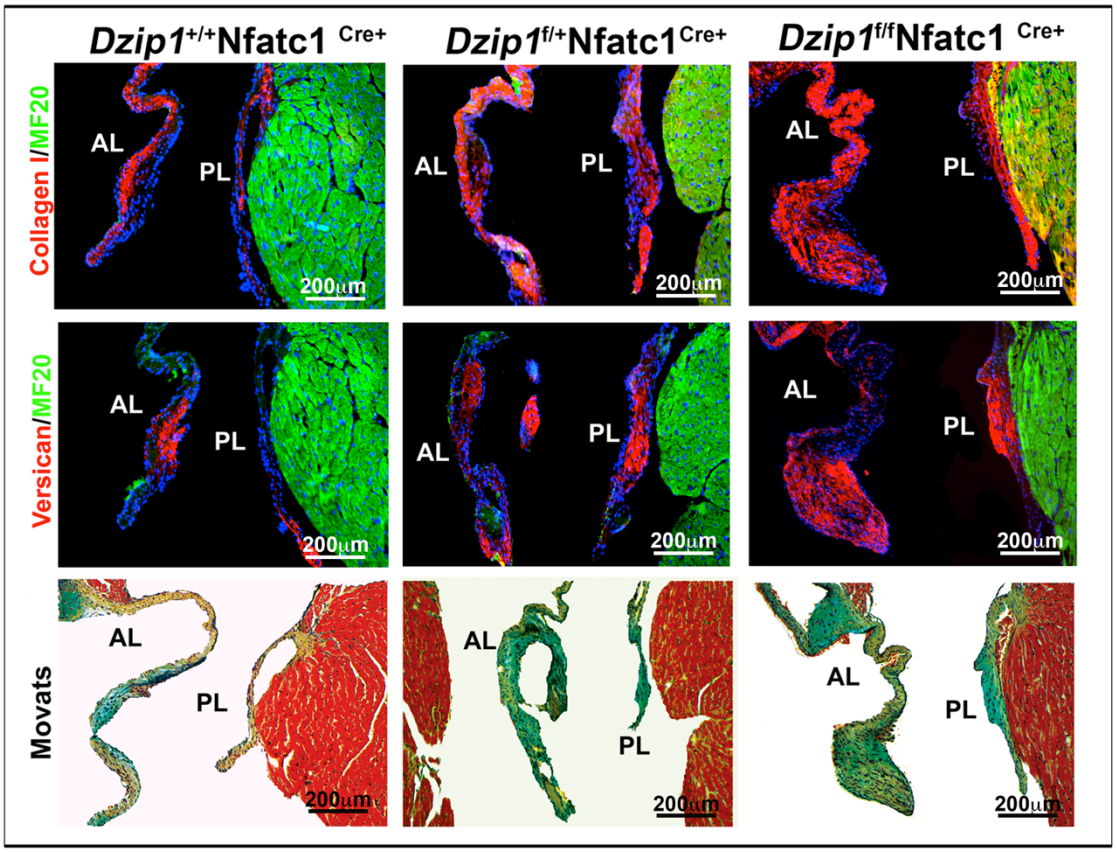
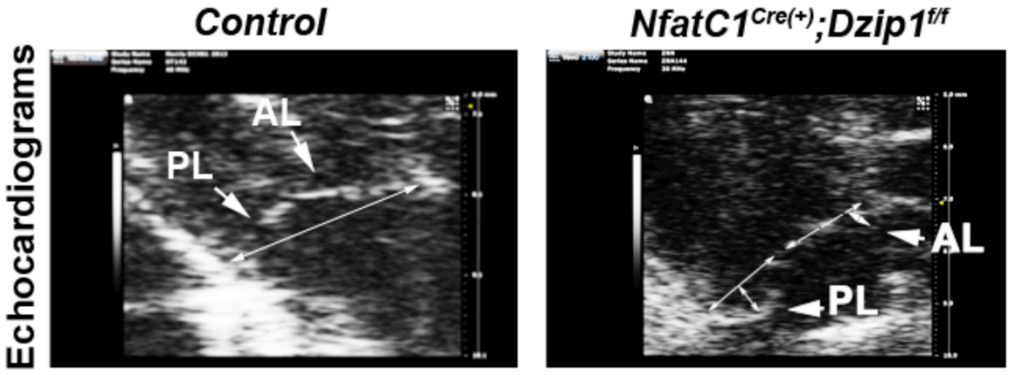



**Figure 4.7 Loss of Dzip1 Results in Myxomatous Adult Mitral Valves**. IHC and Movat's stains in adult Dzip1 conditional wildtype heterozygous and knockout animals show significant enlargement in valve size and blurring of matrix boundaries. Dzip1 conditional heterozygous and knockout animals have increased collagen (top) and versican expression (middle) when compared to wildtype littermates.




**Echocardiographic Analyses**

| | Wild-type | Heterozygous | Homozygous |
|---|---|---|---|
| Age (weeks) | 29.1±3.0 | 27.7±2.6 | 29.1±4.5 |
| Body weight (g) | 38.1±4.8 | 37.3±4.8 | 39.6±9.3 |
| LV End-diastolic Dimension (mm) | 4.05±0.49 | 3.73±0.31 | 4.18±0.42 |
| LV Wall Thickness (mm) | 0.97±0.09 | 1.02±0.08 | 1.03±0.12 |
| Fractional Shortening (%) | 35.0±6.2 | 35.8±6.3 | 40.8±7.1 |
| LV End-diastolic Volume (μL) | 68.7±14.7 | 67.4±12.2 | 69.1±17.6 |
| LV Ejection fraction (%) | 67.1±4.8 | 63.8±7.0 | 70.2±8.1 |
| LV Mass (mg) | 128±8 | 118±14 | 148±39 |
| Sample size (n) | 4 | 5 | 3 |

Values presented as Mean±SEM

**Figure 4.8 Dzip1 Deletion Results in MVP.** Parasternal long-axis views of Dzip1 deficient mitral leaflets shows leaflet elongation and bileaflet prolapse during ventricular systole. AL, PL= anterior and posterior leaflet respectively. Ao=Aorta

## Discussion

These data are the first to show cilia are important for the proper development of the mitral valve. Disruption of cilia through deletion of Dzip1 results in enlarged postnatal mitral leaflet that develop into myxomatous adult valves. Loss of Dzip1 results in MVP in adult mice as shown by echocardiography and this phenotype is what is seen in patients with Dzip1 mutations. Further studies need to be done to identify a mechanism by which Dzip1 mutations result in decreased cilia length. Preliminary data in the lab suggest that this mutation may disrupt binding to important molecules involved in ciliogenesis. Generation of the point mutation mouse has provided insight into the biology of the patients with Dzip1 mutations. Preliminary data from these Dzip1 point mutation knockin mice show enlarged postnatal leaflets as well as myxomatous adult leaflets.

In order to determine the mechanism by which Dzip1 deletion results in MVP further experiments are needed. Loss of Dzip1 in mouse models results in myxomatous valve phenotype similar to the Ift88 conditional mice. Increases in ECM is seen in both models. To further evaluate these changes RNA seq could be done at a time point prior to valve enlargement, such as E13, to compare changes between Ift88 and Dzip1 conditional mice. Further studies looking at hedgehog signaling may also prove valuable. Since Dzip1 has been previously implicated in sequestering Gli3 in the cytoplasm but we see no change in Gli3 processing IHC looking at Gli3 localization in conditional knockout mice could be interesting.

# CHAPTER 5: OVERALL DISCUSSION

Valve disease is one of the most common cardiac diseases affecting between 0.5-2% of individuals. MVP and BAV are two of the most common valve diseases and currently the only treatment option is surgical replacement or repair. The etiology of these diseases are poorly understood however evidence has shown a developmental origin. Better understanding of these developmental processes and signaling pathways has provided insight into the mechanism by which primary cilia regulate ECM production. Future work is still needed to identify which signaling pathway is responsible for this regulation although some evidence suggests non-canonical hedgehog signaling is playing a role.

Currently the only treatment option for both MVP and BAV is invasive surgery to either repair or replace the damaged valve. For patients with BAV and aortic stenosis, the gold standard of treatment is surgical aortic valve replacement (SAVR) which has proven to be both symptomatic and provide survival benefits (Al Kindi, Salhab et al. 2014). Over the past decade these procedures have improved greatly and inventions such as the MitraClip, a trans catheter mitral valve repair device designed to reduce MR, has helped patients live longer healthier lives (Velazquez, Samad et al. 2015). However, there is still room for improvements. Secondary complications caused by valve disease cause damage to the heart in other ways resulting in long term cardiovascular disease and current treatment options are often invasive, risky and not always suitable for the elderly population most often effected by these diseases. Devices such as the MitraClip help improve the 30-day mortality of 7.7% of MitraClip patients compared to what is

predicted had mitral valve surgery been attempted. However, these devices are not perfect. The Endovascular Valve Edge-to-Edge Repair Study (EVEREST II) study showed mitral valve surgery was more effective in reducing MR than the MitraClip (George, Varghese et al. 2011, Velazquez, Samad et al. 2015). For these reasons a better understanding of the developmental origins and molecular mechanisms for these diseases will allow clinicians to move forward with less invasive more effective treatment options.

Structural changes that occur in the valves of patients with MVP and BAV include myxomatous degeneration. A myxomatous valve has increased accumulation of proteoglycans, fragmented collagens, and hyperplasia. Over time, these changes in the histoarchitecture of the valve result in mechanically weakened structure which become elongated and, in the case of MVP, are susceptible to prolapse. Little is known about how the valves become myxomatous although evidence points to a genetic origin.

Our studies suggest that primary cilia play a major role in this process. Through spatial and temporal regulation of valve differentiation and matrix deposition, the primary cilia act as molecular clocks. In this way the primary cilia relay information to the cell telling it what stage in development the tissue is undergoing and allowing the cells to differentiate producing the correct ratio of ECM at each stage of development. Loss of this signal results in improper valve differentiation and matrix deposition. Structural changes during development then progress to adult valve disease because the tissue is compromised at an early age. Over the course of the patients life, enlarged postnatal leaflets cause endothelial boarders to stretch and weaken which potentially allows for cytokine release and infiltration of extracardiac cells. Previous models of myomatous valve disease show infiltration of hematopoetic derived CD45 positive cells. This

infiltration leads to increased MMP secretion and further matrix degradation. At this stage in a patient's life they may become symptomatic while early in life when the disease is still occurring they would be considered non-symptomatic. For these reasons, understanding the processes involved in mitral and aortic valve development are essential in determining a molecular mechanism for valve disease.

In our studies we noticed the development of BAV in our Ift88 conditional knockout mice but not in our Dzip1 conditional knockout mice. If both genes affect primary cilia, how can this be the case? The severity of cilia loss may be an explanation. Conditional deletion of Ift88 to remove primary cilia is the most severe level of cilia loss while deletion of Dzip1 deletion is a less severe. Ift88 conditional knockout mice develop both MVP and BAV while conditional heterozygous only develop MVP. In the Dzip1 conditional knockout mice we observed MVP but not BAV. The Ift88 conditional knockout mice have almost complete cilia loss with a few very short cilia while the Dzip1 conditional knockout mice have shorter cilia.

An additional explanation could be differences in tissue expression of the molecules involved in Dzip1 signaling. The "ciliome" constitutes ~2-3% of all mammalian genes and it's likely that components of the ciliome are expressed in unique spatial, temporal, and tissue-specific manners, such that the mitral and aortic valves differ in presence/expression of subsets of cilia genes. It is possible that Dzip1 signaling is more important for mitral valve development than aortic valve development. To test this stains for various pathway members could be done in both mitral and aortic valves to see if there was variation in expression. IHC showing differences for any of the Gli transcription factors, members of the non-canonical HH pathway such as Rac and Rho, or

PDGFRα signaling pathway such as AKT, or PI3K could explain differences in phenotypes.

More work is needed to determine a signaling mechanism for these changes. In both aortic and mitral valves, loss of primary cilia with conditional deletion of Ift88 resulted in increased ECM production and decreased cell density. Determining how loss of primary cilia results in increased matrix proved to be complicated but we can speculate about what pathways may be involved. Since loss of Dhh resulted in a valve phenotype similar to that seen with deletion of Ift88 it is possible that the HH signaling pathway is involved. No change was observed in processing of Gli3 which could mean that Gli2 is playing a role or that Dhh is signaling in a non-canonical way. To further evaluate these pathways multiple experiments could be done. Western blots using tissue from Dzip1 and Ift88 conditional knockout and wild type tissue could be probed for PERK, pAKT, PI3K or Gli transcription factors. To determine if non-canonical HH signaling is occurring a Rac/Rho activity assay can be done. In this assay, Rac/Rho in its active form, bound to GTP, is pulled down using a specific antibody. The protein is then eluted off the beads and can be run in on a western blot to look at relative abundance.

From the data collected we propose the following working model. Inhibition of ciliogenesis, reception of signal by the cilia, or deletion of ligand needed for cilia signaling results in improper valve development ultimately leading to valve disease. Disruption of cilia structure through deletion of Ift88 results in myxomatous mitral and aortic valves with a high penetrance of BAV. These mice develop enlarged mitral valves with increased expression of versican and collagen. A similar phenotype is observed in the Dzip1 conditional knockouts. Conditional loss of Dzip1results in enlarged

myxomatous mitral valves that ultimately results in MVP. Disruption of the HH ligand Dhh also causes enlarged postnatal valves although the mechanism by which this is occurring is still under investigation.

In the future this work has the potential to influence therapies for these diseases. One common thread between multiple models of valve disease is the disruption of the ECM boundaries. If a therapy could target this disruption we could potentially stop further degradation of the matrix and maintain a functional valve leaflet. Therapies using Nano particles are appealing for this function. Using antibody targeted against fragmentation of collagen or elastin tethered to nanoparticles which are filled with drugs we could possibly target the damaged tissue before further issues could arise. One potential drug option would be something that repairs fragmented elastin or collagen. Another potential option could be a crisper molecule that could repair the mutation that is seen in the patients with the DZIP1 mutations.

More work is still needed to further elucidate the molecular mechanisms involved in valve disease, however, substantial progress has been made. The concept of valve disease as a ciliopathy is a novel idea and opens up the potential for further research which is likely to help undercover the ethology of these diseases. Here we present genetic, cellular and molecular data to broadly support primary cilia defects as a cause of MVP and BAV, which help to define the molecular and developmental basis for these common diseases.

# CHAPTER 6: MATERIALS AND METHODS

## Mouse Studies

All mouse experiments were performed under protocols previously approved by the Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina. Prior to cardiac resection, mice were euthanized in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996.) Comparisons between sexes were evaluated and no significant differences were observed. As such, data is comprised of pooled sexes for all experiments. Mating strategies were as follows. Female mice heterozygous for the conditional "floxed" allele of each respective gene (Ift88, Dzip1, and Dhh) were bred with male transgenic mice who were heterozygous for the conditional "floxed" allele as well as expressing Cre under the control of the respective promoters (NfatC1, NfatC1- enhancer, Periostin, or Tie2). Mice resulting from these mating would be Cre negative or positive and had the potential to be wildtype, heterozygous or homozygous for the floxed allele. Filamin A conditional mice were bred similarly but male mice were conditional knockouts as FilaminA is X chromosome specific. Dchs1 global mice were generated using a heterozygous female with a heterozygous male. All mice were outbred.

## IFT88 conditional mice

*Ift88* conditional mice and genotyping were previously described(Haycraft, Zhang et al. 2007). Histology was performed on embryonic and adult wild-type ($NfatC1^{Cre-};Ift88^{f/f}$) conditional heterozygous ($NfatC1^{Cre-};Ift88^{f/+}$) and conditional knockout ($NfatC1^{Cre+};Ift88^{f/f}$) hearts on mixed background.

**Dzip1 conditional mice**

Conditional knockout mice were generated by a targeted homologous recombination approach using a Dzip1 targeting construct (PG00125_Z_2_E04) purchased from the KOMP mouse repository. The conditional knockout would delete exons 8 and 9 (Dzip1-202 transcript) and cause a reading frame shift and premature translational termination. The conditional mutant allele was genotyped using forward primer: 5'-GCCAAAGTGGTTTGCCTGACA -3' and reverse primer: 5'-GCAGGTTAAACACTCATATAGC-3' (210bp for wt and 290bp for mutant). Histology was performed on embryonic and adult wild-type ($NfatC1^{Cre-};Dzip1^{+/+}$) conditional heterozygous ($NfatC1^{Cre+-}:Dzip1^{f/+}$) and conditional knockout ($NfatC1^{Cre+};Dzip1^{f/f}$) hearts on mixed background. Additional wild-type ($Tie2^{Cre+};Dzip1^{+/+}$) and conditional knockout ($Tie2^{Cre+};Dzip1^{f/f}$) adult hearts were examined as well. Data collected from the *NfatC1* and *Tie2* Cre lines were comparable.

**Histology, Immunohistochimistry/Immunofluorescence**

Embryonic and adult tissue were processed for hematoxylin and eosin (H and E) staining, Herovici's collagen stain and, immunohistochemistry/immunofluorescence (IHC) as previously described. IHC of cilia stains to look at expression and measure cilia length were done on 15 μm thick sections to insure measurement of full cilia length. Histology and IHC were preformed using 5μm thick sections from E11,13,15,17, P0, and Adult (4-8month) aortic valves. H and E stains were performed as previously described. Herovci stains were preformed using American MasterTech Herovici's Collagen Stain Kit

Procedure (cat#KTHER). For immunohistochemistry (IHC): Antigen retrieval was performed for 1 minute using antigen unmasking solution (Vector Laboratories, Burlingame, CA, USA, Cat#H-3300) by pressure cooker (Cuisinart, Stamford, CT, USA). The following are the antibodies and their dilutions; Acetylated Tubulin (Sigma, Cat#T6793, 1:500), Gamma Tubulin (Abcam, Cambridge, MA, USA, Cat#ab11317, 1:1000), Versican (gift from Stan Hoffman, Medical University of South Carolina, 1: 250), Collagen (gift from Stan Hoffman, Medical University of South Carolina, 1: 250), MF20 (DSHB, Iowa City, IA, USA, Concentrate, I:50), Ki67 (Abcam, Cat#ab16667, 1:250), Phospho-histone H3 (EMD Milipore, Darmstadt Germany, Cat#06-570, 1:250), Smoothened (LSBio, Seattle WA, Cat#LS-A2666, 1:250), Gli3 (Origene, Rockville MD, Cat#TA337186, 1:250). Primary antibody was detected using fluorescent secondary antibody, Goat anti-Mouse IgG Alexa fluro 488 (Cat#A-11029, 1:100), Goat anti-Rabbit Alexa fluor 488 (Cat#A-11034, 1:100) anti-Mouse Alexa fluor 568 (Cat#A-11004, 1:100), anti-Rabbit Alexa fluor 568 (Cat#A-11036, 1:100) Cy5 goat anti-Mouse (Cat#A-10524, 1:100) and Cy5 goat anti-Rabbit (Cat#A-10523, 1:100) (Life Technologies, Rockville, MD, US). Nuclei were counterstained with Hoechst (Life Technologies, Cat #H3569, 1:10,000) for 10 minutes and slides were coverslipped with SlowFade mounting medium (Life Technologies, Cat#S36937). Fluorescence imaging was preformed using Zeiss Axioimager M2 and Leica TCS SP5 AOBS Confocal Microscope System.

**Confocal Microscopy**

Images were acquired using the Leica TCS SP5 AOBS Confocal Microscope System ( Leica Microsystems, Inc., 410 Eagleview Boulevard, Suite 107, Exton, PA 19341). Z-

stacks were set by finding the highest and lowest depth with visible fluorescence and using the system optimized setting to determine steps. Z-stacks were then compiled to form maximum projection images.

**3D reconstruction**

3D reconstructions of H and E images were preformed to generate volumetric measurements of postnatal day 0 right, left, and non-coronary leaflets, similarly to previous reports. Briefly, 5μm sections throughout the width of the aortic valve were H and E stained and imaged using the Olympus BX40 bright field microscope. Images were then aligned using ImageJ FIJI and imported into Imaris 8.0. Manual reconstruction was performed by tracing each individual cusp on every section and combining all traces to create a 3D structure. Additionally, 3D reconstructions of immunohistochemistry were performed by importing Z-stack images of 15μm sections into Imaris and creating surface renderings based on intensity of the stains.

**Quantifications**

Cilia length measurements were performed using Z-stack images of right coronary cusps (Chapter 2) or posterior leaflets (Chapter 3 and 4) stained with acetylated alpha tubulin, gamma tubulin and counterstained with Hoechst. Z-stack images were then imported into Imaris software and measurements were taken from the base to the tip of the axoneme. All cilia in the field of view were measured, n=3 per developmental time point or genotype. Measurements were then ranked and grouped; zero microns represents absence of cilia. Quantification of cilia length in versican and collagen regions was performed on

left coronary leaflets in the same way, n=3. Average cilia length was then calculated and Student's t-test was done ($p<0.001$).

Cell density quantifications were measured by counting all nuclei in a specified area, 25.4mm$^2$ for post-natal measurement and 22 mm$^2$ for adult measurements. Postnatal measurements were taken at the base and tip of the right coronary leaflets, n=3. Adult measurements were taken at the tip of right coronary leaflets, n=3. Measurements were compared to wild-type data to generate fold change and statistical significance was calculated using a Student's *t*-test ($p<0.001$).

Quantification of Collagen was preformed using Herovici stained right coronary leaflets. Surface area of red staining was measured in Image J and percentages were calculated based on total leaflet surface area. n=3, with three areas per animal measured.

**RNA Seq analysis**

Mitral leaflets were dissected from P0 *NfatC1*$^{Cre(+)}$*; Ift88*$^{f/f}$ and P0 *NfatC1*$^{Cre(+)}$*, Ift88*$^{+/+}$. Total RNA was isolated using MicroRNeasy (Qiagen). Purity and quantification was determined by Bioanalyzer. The library preparation was made using the SMART-Seq® v4 RNA-seq kit(Clontech Laboratories, Mountain View, CA) following the manufacturer's instructions.

The analysis was carried out on an OnRamp Bioinformatics Genomics Research Platform (OnRamp Bioinformatics, San Diego, CA). "OnRamp's advanced Genomics Analysis Engine utilized an automated RNAseq workflow to process the data, including (1) data validation and quality control, (2) read alignment to the mouse genome (mm10) using STAR RNA-seq aligner, (3) generation of gene-level count data with HTSeq, and (4)

differential expression analysis with DEseq2, which enabled the inference of differential signals with robust statistical power(Dobin, Davis et al. 2013, Love, Huber et al. 2014, Davis-Turak, Courtney et al. 2017). Transcript count data from DESeq2 analysis of the samples were sorted according to their adjusted p-value or q-value, which is the smallest false discovery rate (FDR) at which a transcript is called significant. FDR is the expected fraction of false positive tests among significant tests and was calculated using the Benjamini-Hochberg multiple testing adjustment procedure(Benjamini and Hochberg 1995). The DE list was them submitted to the iPathway Guide tool from Advaita® Bioinformatics, this tool uses a systems biology approach in order to identify pathways that are significantly impacted in any condition - from high-throughput gene expression data. The impact analysis incorporates the classical probabilistic component of the magnitude of the expression changes of each gene, the position of the differentially expressed genes on the given pathways, the topology of the pathway that describes how these genes interact, and the type of signaling interactions between them(Draghici, Khatri et al. 2007).

**Echocardiography**

Mice were anesthetized with 3–5% isoflurane vapor in an anesthesia chamber and then placed on a biofeedback warming station with nose cone anesthesia of 1.5–2.5% isoflurane, which was regulated to maintain a heart rate between 500 to 600 beats/min, while providing anesthesia (abolition of the toe pinch reflex). The hair over the chest was removed using a commercially available depilatory cream. Ultrasound gel was placed on the chest, and echocardiography measurements were performed using a 40-MHz probe

with a spatial resolution of 30 μm (Vevo2100; Visualsonics). Two-dimensional and M-mode echo images were obtained in the parasternal short- and long-axis views. LV volumes and ejection fractions were computed from the parasternal long-axis recordings and LV mass was computed from the short axis measurements (Mani, Balasubramanian et al. 2009, Kholmukhamedov, Logdon et al. 2014, Zavadzkas, Stroud et al. 2014). For terminal studies, heart harvest was performed following this procedure. The entire echocardiography procedure took ~20-30 min per mouse.

**Human Studies**

Fetal hearts (12 and 20 weeks) were obtained from elective terminations that were lawfully performed at Brighman and Women's Hospital, Boston, MA under IRB approval (2010P001333). Samples were stored in RNA later and maintained at the Harvard BioBank. Tissues were washed and refixed in formalin followed by tissue processing, embedding and sectioning as we have previously described (Durst, Sauls et al. 2015). MVP was diagnosed in two-dimensional long-axis echocardiographic views by ≥2mm leaflet displacement superior to the annulus hinge points (Levine, Stathogiannis et al. 1988, Levine, Handschumacher et al. 1989). Prodromal morphology was noted based on leaflet coaptation abnormally displaced >40% anterior relative to the mitral annulus, and minimal superior displacement was based on <2mm displacement (Nesta, Leyne et al. 2005, Delling, Gona et al. 2014). These morphologies associate with progressive prolapse (Delling, Rong et al. 2016)and link to the haplotype of affecteds with fully diagnostic displacement (Nesta, Leyne et al. 2005).

**Genetic Studies:**

Complete details on the family in Figure 3A and the original linkage analysis can be found in (Nesta, Leyne et al. 2005). Epstein Barr Virus (EBV) transformed lymphoblast cell lines were created on all members of the family available for study. Protein was isolated from cultures for the individuals marked with a "*" in Figure 3A and Western analysis performed using a DZIP1 rabbit monoclonal antibody (epitomics) Sanger sequencing using primers for each exon of DZIP1 was performed for all members of the pedigree. Exome sequencing was performed on four individuals carrying the linked haplotype and all genes in the candidate interval were analyzed for coding sequence mutations.

**Family 2 Genetics:**

**PCR reactions & High-Resolution Melting (HRM) Analysis**

PCR for HRM assay were performed in "LightCycler® 480 Multiwell Plate 96, white" reaction plates. 12.5 ng of genomic DNA and 0.25µM final concentration of each primer were used per reaction (10µL final volume).

| **Reagent** | **Volume per well (µL)** |
|---|---|
| Water, PCR Grade | 2.5 |
| Primer Forward (10µM) | 0.25 |
| Primer Reverse (10µM) | 0.25 |
| LightCycler® 480 Probes Master, 2x concentrated | 5 |
| LightCycler® 480 ResoLight Dye, 20x concentrated | 0.75 |
| DNA sample (10ng/µL) or Negative control (Water) | 1.25 |

The PCR and HRM assays were performed on the "LightCycler® 480 Instrument" (Roche). Run and analysis are performed with the "LightCycler® 480 Software Version 1.5" and the "LightCycler® 480 Gene Scanning module" (Roche).

For "ResoLight Dye" detection, the filter "SYBR Green I / HRM Dye (465-510 nm)" was selected (Integration Time Mode: Dynamic).

Initial activation step was set at 95°C for 10min (Ramp rate 4.4°C/sec), followed by a 55-cycle program [denaturation, 95°C for 15sec (Ramp rate 4.4°C/sec); annealing, touchdown 68°C to 55°C (with 1°C step size and 1 cycle step delay) for 15sec (Ramp rate 2.2°C/sec); and elongation, 72°C for 20sec (Ramp rate 4.4°C/sec); acquisition mode, "Single"]. The subsequent melting program included three steps: denaturation at 95°C for 1 sec (Ramp rate 4.4°C/sec), renaturation at 40°C for 30sec (Ramp rate 2.2°C/sec), and then melting from 65°C to 97°C (Ramp rate 2.2°C/sec) with 25 acquisitions per °C (Ramp rate 0.02°C/sec); acquisition mode, "Continuous") and a final cooling step was set at 50°C for 30sec (Ramp rate 2.2°C/sec). Finally, after the run analysis, real-time PCR products were directly sequenced in order to obtain the clusters genotype (few samples from each curve cluster). Sanger sequencing was performed on the same PCR amplicons and primers, using standard conditions.

**Genome wide association (GWAS) study on Cilia**

Using our previous GWAS on MVP (Dina, Bouatia-Naji et al. 2015), we tested for a global enrichment for significant association among a large list including 278 different genes reported as involved in cilia biology, mainly as harboring rare mutations in a diverse panel of ciliopathies. We used the SNP test ratio method as described in (O'Dushlaine, Kenny et al. 2009). In this analysis we first performed several GWAS where phenotypes of cases and controls were permuted to generate 1000 simulated GWAS. Then, according to a pre-defined p-value threshold, the method consists in counting the number of times the pathway tested included a higher ratio of significant to non significant SNPs, compared to the 1000 simulated GWAS. As recommended for this method, we used the KEGG pathways catalog, in addition to our customized list of cilia

genes. Given the limited number of highly associated SNPs in the current GWAS that includes ~1500 cases and 2500 controls, we set significance at P<0.05 to allow more SNPs to contribute the analyses. Among nominally associated SNPs in the GWAS (SNPs<0.05), we found that SNPs that map in the cilia genes list are more likely to be associated with MVP (P=0.03).

**Statistics**

Statistical significance was determined using a student's t-test (two tailed, type 2) with significance ($p<0.05$). Statistical data are presented as standard deviations from the mean.

REFERENCES:


Abdulkareem, N., J. Smelt and M. Jahangiri (2013). "Bicuspid aortic valve aortopathy: genetics, pathophysiology and medical therapy." Interact Cardiovasc Thorac Surg **17**(3): 554-559.

Akiyama, H., M. C. Chaboissier, J. F. Martin, A. Schedl and B. de Crombrugghe (2002). "The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6." Genes Dev **16**(21): 2813-2828.

Al Kindi, A. H., K. F. Salhab, E. E. Roselli, S. Kapadia, E. M. Tuzcu and L. G. Svensson (2014). "Alternative access options for transcatheter aortic valve replacement in patients with no conventional access and chest pathology." J Thorac Cardiovasc Surg **147**(2): 644-651.

Amano, K., M. Densmore, R. Nishimura and B. Lanske (2014). "Indian hedgehog signaling regulates transcription and expression of collagen type X via Runx2/Smads interactions." J Biol Chem **289**(36): 24898-24910.

Anyanwu, A. C. and D. H. Adams (2007). "Etiologic classification of degenerative mitral valve disease: Barlow's disease and fibroelastic deficiency." Semin Thorac Cardiovasc Surg **19**(2): 90-96.

Avasthi, P., R. L. Maser and P. V. Tran (2017). "Primary Cilia in Cystic Kidney Disease." Results Probl Cell Differ **60**: 281-321.

Baldwin, H. S., H. M. Shen, H. C. Yan, H. M. DeLisser, A. Chung, C. Mickanin, T. Trask, N. E. Kirschbaum, P. J. Newman, S. M. Albelda and et al. (1994). "Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31): alternatively spliced, functionally distinct isoforms expressed during mammalian cardiovascular development." Development **120**(9): 2539-2553.

Bangs, F. and K. V. Anderson (2017). "Primary Cilia and Mammalian Hedgehog Signaling." Cold Spring Harb Perspect Biol **9**(5).

Barlow, J. B. and C. K. Bosman (1966). "Aneurysmal protrusion of the posterior leaflet of the mitral valve. An auscultatory-electrocardiographic syndrome." Am Heart J **71**(2): 166-178.

Barlow, J. B. and W. A. Pocock (1985). "Billowing, floppy, prolapsed or flail mitral valves?" Am J Cardiol **55**(4): 501-502.

Barth, P. J., H. Koster and R. Moosdorf (2005). "CD34+ fibrocytes in normal mitral valves and myxomatous mitral valve degeneration." Pathol Res Pract **201**(4): 301-304.

Benjamin, E. J., M. J. Blaha, S. E. Chiuve, M. Cushman, S. R. Das, R. Deo, S. D. de Ferranti, J. Floyd, M. Fornage, C. Gillespie, C. R. Isasi, M. C. Jimenez, L. C. Jordan, S. E. Judd, D. Lackland, J. H. Lichtman, L. Lisabeth, S. Liu, C. T. Longenecker, R. H. Mackey, K. Matsushita, D. Mozaffarian, M. E. Mussolino, K. Nasir, R. W. Neumar, L. Palaniappan, D. K. Pandey, R. R. Thiagarajan, M. J. Reeves, M. Ritchey, C. J. Rodriguez, G. A. Roth, W. D. Rosamond, C. Sasson, A. Towfighi, C. W. Tsao, M. B. Turner, S. S. Virani, J. H. Voeks, J. Z. Willey, J. T. Wilkins, J. H. Wu, H. M. Alger, S. S. Wong, P. Muntner, C. American Heart Association Statistics and S. Stroke Statistics

(2017). "Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association." Circulation **135**(10): e146-e603.
Benjamini, Y. and Y. Hochberg (1995). "Controlling the false discovery rate: a practical and powerful approach to multiple testing." Journal of the royal statistical society. Series B (Methodological): 289-300.
Bernanke, D. H. and R. R. Markwald (1982). "Migratory behavior of cardiac cushion tissue cells in a collagen-lattice culture system." Dev Biol **91**(2): 235-245.
Bischoff, J. and E. Aikawa (2011). "Progenitor cells confer plasticity to cardiac valve endothelium." J Cardiovasc Transl Res **4**(6): 710-719.
Bischoff, J., G. Casanovas, J. Wylie-Sears, D. H. Kim, P. E. Bartko, J. L. Guerrero, J. P. Dal-Bianco, J. Beaudoin, M. L. Garcia, S. M. Sullivan, M. M. Seybolt, B. A. Morris, J. Keegan, W. S. Irvin, E. Aikawa and R. A. Levine (2016). "CD45 Expression in Mitral Valve Endothelial Cells After Myocardial Infarction." Circ Res **119**(11): 1215-1225.
Bitgood, M. J. and A. P. McMahon (1995). "Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo." Dev Biol **172**(1): 126-138.
Boehlke, C., H. Janusch, C. Hamann, C. Powelske, M. Mergen, H. Herbst, F. Kotsis, R. Nitschke and E. W. Kuehn (2015). "A Cilia Independent Role of Ift88/Polaris during Cell Migration." PLoS One **10**(10): e0140378.
Bolanos, A. L., C. M. Milla, J. C. Lira, R. Ramirez, M. Checa, L. Barrera, J. Garcia-Alvarez, V. Carbajal, C. Becerril, M. Gaxiola, A. Pardo and M. Selman (2012). "Role of Sonic Hedgehog in idiopathic pulmonary fibrosis." Am J Physiol Lung Cell Mol Physiol **303**(11): L978-990.
Bonachea, E. M., S. W. Chang, G. Zender, S. LaHaye, S. Fitzgerald-Butt, K. L. McBride and V. Garg (2014). "Rare GATA5 sequence variants identified in individuals with bicuspid aortic valve." Pediatr Res **76**(2): 211-216.
Borovina, A. and B. Ciruna (2013). "IFT88 plays a cilia- and PCP-independent role in controlling oriented cell divisions during vertebrate embryonic development." Cell Rep **5**(1): 37-43.
Bosada, F. M., V. Devasthali, K. A. Jones and K. Stankunas (2016). "Wnt/beta-catenin signaling enables developmental transitions during valvulogenesis." Development **143**(6): 1041-1054.
Briggs, L. E., T. A. Burns, M. M. Lockhart, A. L. Phelps, M. J. Van den Hoff and A. Wessels (2016). "Wnt/beta-catenin and sonic hedgehog pathways interact in the regulation of the development of the dorsal mesenchymal protrusion." Dev Dyn **245**(2): 103-113.
Butcher, J. T. and R. R. Markwald (2007). "Valvulogenesis: the moving target." Philos Trans R Soc Lond B Biol Sci **362**(1484): 1489-1503.
Butcher, J. T., T. C. McQuinn, D. Sedmera, D. Turner and R. R. Markwald (2007). "Transitions in early embryonic atrioventricular valvular function correspond with changes in cushion biomechanics that are predictable by tissue composition." Circ Res **100**(10): 1503-1511.
Butcher, J. T. and R. M. Nerem (2006). "Valvular endothelial cells regulate the phenotype of interstitial cells in co-culture: effects of steady shear stress." Tissue Eng **12**(4): 905-915.

Butcher, J. T. and R. M. Nerem (2007). "Valvular endothelial cells and the mechanoregulation of valvular pathology." Philos Trans R Soc Lond B Biol Sci **362**(1484): 1445-1457.
Chacon-Heszele, M. F., S. Y. Choi, X. Zuo, J. I. Baek, C. Ward and J. H. Lipschutz (2014). "The exocyst and regulatory GTPases in urinary exosomes." Physiol Rep **2**(8).
Chapouly, C., Q. Yao, S. Vandierdonck, F. Larrieu-Lahargue, J. N. Mariani, A. P. Gadeau and M. A. Renault (2016). "Impaired Hedgehog signalling-induced endothelial dysfunction is sufficient to induce neuropathy: implication in diabetes." Cardiovasc Res **109**(2): 217-227.
Christensen, S. T., C. A. Clement, P. Satir and L. B. Pedersen (2012). "Primary cilia and coordination of receptor tyrosine kinase (RTK) signalling." J Pathol **226**(2): 172-184.
Clement, D. L., S. Mally, C. Stock, M. Lethan, P. Satir, A. Schwab, S. F. Pedersen and S. T. Christensen (2013). "PDGFRalpha signaling in the primary cilium regulates NHE1-dependent fibroblast migration via coordinated differential activity of MEK1/2-ERK1/2-p90RSK and AKT signaling pathways." J Cell Sci **126**(Pt 4): 953-965.
Combs, M. D. and K. E. Yutzey (2009). "Heart valve development: regulatory networks in development and disease." Circ Res **105**(5): 408-421.
Coutinho, G. F. and M. J. Antunes (2017). "Mitral valve repair for degenerative mitral valve disease: surgical approach, patient selection and long-term outcomes." Heart.
Crossin, K. L. (1991). "Cell adhesion molecules in embryogenesis and disease." Ann N Y Acad Sci **615**: 172-186.
Davies, R. R., A. Gallo, M. A. Coady, G. Tellides, D. M. Botta, B. Burke, M. P. Coe, G. S. Kopf and J. A. Elefteriades (2006). "Novel measurement of relative aortic size predicts rupture of thoracic aortic aneurysms." Ann Thorac Surg **81**(1): 169-177.
Davis-Turak, J., S. M. Courtney, E. S. Hazard, W. B. Glen, Jr., W. A. da Silveira, T. Wesselman, L. P. Harbin, B. J. Wolf, D. Chung and G. Hardiman (2017). "Genomics pipelines and data integration: challenges and opportunities in the research setting." Expert Rev Mol Diagn **17**(3): 225-237.
De Mozzi, P., U. G. Longo, G. Galanti and N. Maffulli (2008). "Bicuspid aortic valve: a literature review and its impact on sport activity." Br Med Bull **85**: 63-85.
de Vlaming, A., K. Sauls, Z. Hajdu, R. P. Visconti, A. N. Mehesz, R. A. Levine, S. A. Slaugenhaupt, A. Hagege, A. H. Chester, R. R. Markwald and R. A. Norris (2012). "Atrioventricular valve development: new perspectives on an old theme." Differentiation **84**(1): 103-116.
Delling, F. N., P. Gona, M. G. Larson, B. Lehman, W. J. Manning, R. A. Levine, E. J. Benjamin and R. S. Vasan (2014). "Mild expression of mitral valve prolapse in the Framingham offspring: expanding the phenotypic spectrum." J Am Soc Echocardiogr **27**(1): 17-23.
Delling, F. N., J. Rong, M. G. Larson, B. Lehman, D. Fuller, E. Osypiuk, P. Stantchev, B. Hackman, W. J. Manning, E. J. Benjamin, R. A. Levine and R. S. Vasan (2016). "Evolution of Mitral Valve Prolapse: Insights From the Framingham Heart Study." Circulation **133**(17): 1688-1695.
Dina, C., N. Bouatia-Naji, N. Tucker, F. N. Delling, K. Toomer, R. Durst, M. Perrocheau, L. Fernandez-Friera, J. Solis, P. investigators, T. Le Tourneau, M. H. Chen, V. Probst, Y. Bosse, P. Pibarot, D. Zelenika, M. Lathrop, S. Hercberg, R. Roussel, E. J. Benjamin, F. Bonnet, S. H. Lo, E. Dolmatova, F. Simonet, S. Lecointe, F. Kyndt, R. Redon, H. Le

Marec, P. Froguel, P. T. Ellinor, R. S. Vasan, P. Bruneval, R. R. Markwald, R. A. Norris, D. J. Milan, S. A. Slaugenhaupt, R. A. Levine, J. J. Schott, A. A. Hagege, M. V. P. France, X. Jeunemaitre and M. N. Leducq Transatlantic (2015). "Genetic association analyses highlight biological pathways underlying mitral valve prolapse." Nat Genet **47**(10): 1206-1211.
Disse, S., E. Abergel, A. Berrebi, A. M. Houot, J. Y. Le Heuzey, B. Diebold, L. Guize, A. Carpentier, P. Corvol and X. Jeunemaitre (1999). "Mapping of a first locus for autosomal dominant myxomatous mitral-valve prolapse to chromosome 16p11.2-p12.1." Am J Hum Genet **65**(5): 1242-1251.
Dobin, A., C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson and T. R. Gingeras (2013). "STAR: ultrafast universal RNA-seq aligner." Bioinformatics **29**(1): 15-21.
Draghici, S., P. Khatri, A. L. Tarca, K. Amin, A. Done, C. Voichita, C. Georgescu and R. Romero (2007). "A systems biology approach for pathway level analysis." Genome Res **17**(10): 1537-1545.
Duan, W., J. Ma, Q. Ma, Q. Xu, J. Lei, L. Han, X. Li, Z. Wang, Z. Wu, S. Lv, Z. Ma, M. Liu, F. Wang and E. Wu (2014). "The Activation of beta1-integrin by Type I Collagen Coupling with the Hedgehog Pathway Promotes the Epithelial-Mesenchymal Transition in Pancreatic Cancer." Curr Cancer Drug Targets **14**(5): 446-457.
Durst, R., K. Sauls, D. S. Peal, A. deVlaming, K. Toomer, M. Leyne, M. Salani, M. E. Talkowski, H. Brand, M. Perrocheau, C. Simpson, C. Jett, M. R. Stone, F. Charles, C. Chiang, S. N. Lynch, N. Bouatia-Naji, F. N. Delling, L. A. Freed, C. Tribouilloy, T. Le Tourneau, H. LeMarec, L. Fernandez-Friera, J. Solis, D. Trujillano, S. Ossowski, X. Estivill, C. Dina, P. Bruneval, A. Chester, J. J. Schott, K. D. Irvine, Y. Mao, A. Wessels, T. Motiwala, M. Puceat, Y. Tsukasaki, D. R. Menick, H. Kasiganesan, X. Nie, A. M. Broome, K. Williams, A. Johnson, R. R. Markwald, X. Jeunemaitre, A. Hagege, R. A. Levine, D. J. Milan, R. A. Norris and S. A. Slaugenhaupt (2015). "Mutations in DCHS1 cause mitral valve prolapse." Nature **525**(7567): 109-113.
El-Hamamsy, I., K. Balachandran, M. H. Yacoub, L. M. Stevens, P. Sarathchandra, P. M. Taylor, A. P. Yoganathan and A. H. Chester (2009). "Endothelium-dependent regulation of the mechanical properties of aortic valve cusps." J Am Coll Cardiol **53**(16): 1448-1455.
Elefteriades, J. A. (2010). "Indications for aortic replacement." J Thorac Cardiovasc Surg **140**(6 Suppl): S5-9; discussion S45-51.
Ezratty, E. J., N. Stokes, S. Chai, A. S. Shah, S. E. Williams and E. Fuchs (2011). "A role for the primary cilium in Notch signaling and epidermal differentiation during skin development." Cell **145**(7): 1129-1141.
Fei, D. L., A. Sanchez-Mejias, Z. Wang, C. Flaveny, J. Long, S. Singh, J. Rodriguez-Blanco, R. Tokhunts, C. Giambelli, K. J. Briegel, W. A. Schulz, A. J. Gandolfi, M. Karagas, T. A. Zimmers, M. Jorda, P. Bejarano, A. J. Capobianco and D. J. Robbins (2012). "Hedgehog signaling regulates bladder cancer growth and tumorigenicity." Cancer Res **72**(17): 4449-4458.
Freed, L. A., J. S. Acierno, Jr., D. Dai, M. Leyne, J. E. Marshall, F. Nesta, R. A. Levine and S. A. Slaugenhaupt (2003). "A locus for autosomal dominant mitral valve prolapse on chromosome 11p15.4." Am J Hum Genet **72**(6): 1551-1559.

Freed, L. A., E. J. Benjamin, D. Levy, M. G. Larson, J. C. Evans, D. L. Fuller, B. Lehman and R. A. Levine (2002). "Mitral valve prolapse in the general population: the benign nature of echocardiographic features in the Framingham Heart Study." J Am Coll Cardiol **40**(7): 1298-1304.
Freed, L. A., D. Levy, R. A. Levine, M. G. Larson, J. C. Evans, D. L. Fuller, B. Lehman and E. J. Benjamin (1999). "Prevalence and clinical outcome of mitral-valve prolapse." N Engl J Med **341**(1): 1-7.
Fuchs, A. (1984). "Fifty years Antonie van Leeuwenhoek. Its history and its impact." Antonie Van Leeuwenhoek **50**(5-6): 425-432.
Gallini, R., P. Lindblom, C. Bondjers, C. Betsholtz and J. Andrae (2016). "PDGF-A and PDGF-B induces cardiac fibrosis in transgenic mice." Exp Cell Res **349**(2): 282-290.
Garcia-Gonzalo, F. R. and J. F. Reiter (2017). "Open Sesame: How Transition Fibers and the Transition Zone Control Ciliary Composition." Cold Spring Harb Perspect Biol **9**(2).
Garg, V., A. N. Muth, J. F. Ransom, M. K. Schluterman, R. Barnes, I. N. King, P. D. Grossfeld and D. Srivastava (2005). "Mutations in NOTCH1 cause aortic valve disease." Nature **437**(7056): 270-274.
Garside, V. C., A. C. Chang, A. Karsan and P. A. Hoodless (2013). "Co-ordinating Notch, BMP, and TGF-beta signaling during heart valve development." Cell Mol Life Sci **70**(16): 2899-2917.
George, J. C., V. Varghese, G. Dangas and T. E. Feldman (2011). "Percutaneous mitral valve repair: lessons from the EVEREST II (Endovascular Valve Edge-to-Edge REpair Study) and beyond." JACC Cardiovasc Interv **4**(7): 825-827.
Ghatak, S., S. Misra, R. A. Norris, R. A. Moreno-Rodriguez, S. Hoffman, R. A. Levine, V. C. Hascall and R. R. Markwald (2014). "Periostin induces intracellular cross-talk between kinases and hyaluronan in atrioventricular valvulogenesis." J Biol Chem **289**(12): 8545-8561.
Goddeeris, M. M., S. Rho, A. Petiet, C. L. Davenport, G. A. Johnson, E. N. Meyers and J. Klingensmith (2008). "Intracardiac septation requires hedgehog-dependent cellular contributions from outside the heart." Development **135**(10): 1887-1895.
Gould, R. A., H. C. Yalcin, J. L. MacKay, K. Sauls, R. Norris, S. Kumar and J. T. Butcher (2016). "Cyclic Mechanical Loading Is Essential for Rac1-Mediated Elongation and Remodeling of the Embryonic Mitral Valve." Curr Biol **26**(1): 27-37.
Hajdu, Z., S. J. Romeo, P. A. Fleming, R. R. Markwald, R. P. Visconti and C. J. Drake (2011). "Recruitment of bone marrow-derived valve interstitial cells is a normal homeostatic process." J Mol Cell Cardiol **51**(6): 955-965.
Harrison, M. J., A. J. Shapiro and M. P. Kennedy (2016). "Congenital Heart Disease and Primary Ciliary Dyskinesia." Paediatr Respir Rev **18**: 25-32.
Haycraft, C. J., B. Banizs, Y. Aydin-Son, Q. Zhang, E. J. Michaud and B. K. Yoder (2005). "Gli2 and Gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function." PLoS Genet **1**(4): e53.
Haycraft, C. J., Q. Zhang, B. Song, W. S. Jackson, P. J. Detloff, R. Serra and B. K. Yoder (2007). "Intraflagellar transport is essential for endochondral bone formation." Development **134**(2): 307-316.
Hinton, R. B., Jr., C. M. Alfieri, S. A. Witt, B. J. Glascock, P. R. Khoury, D. W. Benson and K. E. Yutzey (2008). "Mouse heart valve structure and function: echocardiographic

and morphometric analyses from the fetus through the aged adult." Am J Physiol Heart Circ Physiol **294**(6): H2480-2488.
Hinton, R. B. and K. E. Yutzey (2011). "Heart valve structure and function in development and disease." Annu Rev Physiol **73**: 29-46.
Hjortnaes, J., J. Keegan, P. Bruneval, E. Schwartz, F. J. Schoen, A. Carpentier, R. A. Levine, A. Hagege and E. Aikawa (2016). "Comparative Histopathological Analysis of Mitral Valves in Barlow Disease and Fibroelastic Deficiency." Semin Thorac Cardiovasc Surg **28**(4): 757-767.
Horne, T. E., M. VandeKopple, K. Sauls, S. N. Koenig, L. J. Anstine, V. Garg, R. A. Norris and J. Lincoln (2015). "Dynamic Heterogeneity of the Heart Valve Interstitial Cell Population in Mitral Valve Health and Disease." J Cardiovasc Dev Dis **2**(3): 214-232.
Hossack, K. F., C. L. Leddy, A. M. Johnson, R. W. Schrier and P. A. Gabow (1988). "Echocardiographic findings in autosomal dominant polycystic kidney disease." N Engl J Med **319**(14): 907-912.
Huk, D. J., B. F. Austin, T. E. Horne, R. B. Hinton, W. C. Ray, D. D. Heistad and J. Lincoln (2016). "Valve Endothelial Cell-Derived Tgfbeta1 Signaling Promotes Nuclear Localization of Sox9 in Interstitial Cells Associated With Attenuated Calcification." Arterioscler Thromb Vasc Biol **36**(2): 328-338.
Huntington, K., A. G. Hunter and K. L. Chan (1997). "A prospective study to assess the frequency of familial clustering of congenital bicuspid aortic valve." J Am Coll Cardiol **30**(7): 1809-1812.
Jain, R., K. A. Engleka, S. L. Rentschler, L. J. Manderfield, L. Li, L. Yuan and J. A. Epstein (2011). "Cardiac neural crest orchestrates remodeling and functional maturation of mouse semilunar valves." J Clin Invest **121**(1): 422-430.
Jin, H., S. R. White, T. Shida, S. Schulz, M. Aguiar, S. P. Gygi, J. F. Bazan and M. V. Nachury (2010). "The conserved Bardet-Biedl syndrome proteins assemble a coat that traffics membrane proteins to cilia." Cell **141**(7): 1208-1219.
Joziasse, I. C., A. Vink, M. J. Cramer, M. F. van Oosterhout, L. A. van Herwerden, R. Heijmen, G. T. Sieswerda, B. J. Mulder and P. A. Doevendans (2011). "Bicuspid stenotic aortic valves: clinical characteristics and morphological assessment using MRI and echocardiography." Neth Heart J **19**(3): 119-125.
Karp, N., L. Grosse-Wortmann and S. Bowdin (2012). "Severe aortic stenosis, bicuspid aortic valve and atrial septal defect in a child with Joubert Syndrome and Related Disorders (JSRD) - a case report and review of congenital heart defects reported in the human ciliopathies." Eur J Med Genet **55**(11): 605-610.
Kelly, R. G., N. A. Brown and M. E. Buckingham (2001). "The arterial pole of the mouse heart forms from Fgf10-expressing cells in pharyngeal mesoderm." Dev Cell **1**(3): 435-440.
Kennedy, M. P., H. Omran, M. W. Leigh, S. Dell, L. Morgan, P. L. Molina, B. V. Robinson, S. L. Minnix, H. Olbrich, T. Severin, P. Ahrens, L. Lange, H. N. Morillas, P. G. Noone, M. A. Zariwala and M. R. Knowles (2007). "Congenital heart disease and other heterotaxic defects in a large cohort of patients with primary ciliary dyskinesia." Circulation **115**(22): 2814-2821.
Kholmukhamedov, A., C. Logdon, J. Hu, R. A. McKinney, F. G. Spinale, J. J. Lemasters and R. Mukherjee (2014). "Cyclosporin A in left ventricular remodeling after myocardial infarction." Am J Physiol Heart Circ Physiol **306**(1): H53-59.

Klena, N. T., B. C. Gibbs and C. W. Lo (2017). "Cilia and Ciliopathies in Congenital Heart Disease." Cold Spring Harb Perspect Biol **9**(8).
Koenig, S. N., J. Lincoln and V. Garg (2017). "Genetic basis of aortic valvular disease." Curr Opin Cardiol.
Kruithof, B. P., S. A. Krawitz and V. Gaussin (2007). "Atrioventricular valve development during late embryonic and postnatal stages involves condensation and extracellular matrix remodeling." Dev Biol **302**(1): 208-217.
Laforest, B., G. Andelfinger and M. Nemer (2011). "Loss of Gata5 in mice leads to bicuspid aortic valve." J Clin Invest **121**(7): 2876-2887.
Leask, R. L., N. Jain and J. Butany (2003). "Endothelium and valvular diseases of the heart." Microsc Res Tech **60**(2): 129-137.
Leitch, C. C., S. Lodh, V. Prieto-Echague, J. L. Badano and N. A. Zaghloul (2014). "Basal body proteins regulate Notch signaling through endosomal trafficking." J Cell Sci **127**(Pt 11): 2407-2419.
Leung, C., Y. Liu, X. Lu, M. Kim, T. A. Drysdale and Q. Feng (2015). "Rac1 Signaling Is Required for Anterior Second Heart Field Cellular Organization and Cardiac Outflow Tract Development." J Am Heart Assoc **5**(1).
Levine, R. A., M. D. Handschumacher, A. J. Sanfilippo, A. A. Hagege, P. Harrigan, J. E. Marshall and A. E. Weyman (1989). "Three-dimensional echocardiographic reconstruction of the mitral valve, with implications for the diagnosis of mitral valve prolapse." Circulation **80**(3): 589-598.
Levine, R. A., E. Stathogiannis, J. B. Newell, P. Harrigan and A. E. Weyman (1988). "Reconsideration of echocardiographic standards for mitral valve prolapse: lack of association between leaflet displacement isolated to the apical four chamber view and independent echocardiographic evidence of abnormality." J Am Coll Cardiol **11**(5): 1010-1019.
Li, Y., N. T. Klena, G. C. Gabriel, X. Liu, A. J. Kim, K. Lemke, Y. Chen, B. Chatterjee, W. Devine, R. R. Damerla, C. Chang, H. Yagi, J. T. San Agustin, M. Thahir, S. Anderton, C. Lawhead, A. Vescovi, H. Pratt, J. Morgan, L. Haynes, C. L. Smith, J. T. Eppig, L. Reinholdt, R. Francis, L. Leatherbury, M. K. Ganapathiraju, K. Tobita, G. J. Pazour and C. W. Lo (2015). "Global genetic analysis in mice unveils central role for cilia in congenital heart disease." Nature **521**(7553): 520-524.
Lincoln, J., R. Kist, G. Scherer and K. E. Yutzey (2007). "Sox9 is required for precursor cell expansion and extracellular matrix organization during mouse heart valve development." Dev Biol **305**(1): 120-132.
Lincoln, J., A. W. Lange and K. E. Yutzey (2006). "Hearts and bones: shared regulatory mechanisms in heart valve, cartilage, tendon, and bone development." Dev Biol **294**(2): 292-302.
Lipschutz, J. H. and K. E. Mostov (2002). "Exocytosis: the many masters of the exocyst." Curr Biol **12**(6): R212-214.
Liu, A. C., V. R. Joag and A. I. Gotlieb (2007). "The emerging role of valve interstitial cell phenotypes in regulating heart valve pathobiology." Am J Pathol **171**(5): 1407-1418.
Lockhart, M. M., B. J. Boukens, A. L. Phelps, C. L. Brown, K. A. Toomer, T. A. Burns, R. D. Mukherjee, R. A. Norris, T. C. Trusk, M. J. van den Hoff and A. Wessels (2014). "Alk3 mediated Bmp signaling controls the contribution of epicardially derived cells to the tissues of the atrioventricular junction." Dev Biol **396**(1): 8-18.

Loeys, B. L., J. Chen, E. R. Neptune, D. P. Judge, M. Podowski, T. Holm, J. Meyers, C. C. Leitch, N. Katsanis, N. Sharifi, F. L. Xu, L. A. Myers, P. J. Spevak, D. E. Cameron, J. De Backer, J. Hellemans, Y. Chen, E. C. Davis, C. L. Webb, W. Kress, P. Coucke, D. B. Rifkin, A. M. De Paepe and H. C. Dietz (2005). "A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2." Nat Genet **37**(3): 275-281.
Longobardo, L., R. Jain, S. Carerj, C. Zito and B. K. Khandheria (2016). "Bicuspid Aortic Valve: Unlocking the Morphogenetic Puzzle." Am J Med **129**(8): 796-805.
Lopes, S. S., R. Lourenco, L. Pacheco, N. Moreno, J. Kreiling and L. Saude (2010). "Notch signalling regulates left-right asymmetry through ciliary length control." Development **137**(21): 3625-3632.
Love, M. I., W. Huber and S. Anders (2014). "Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2." Genome Biol **15**(12): 550.
Lumiaho, A., R. Ikaheimo, R. Miettinen, L. Niemitukia, T. Laitinen, A. Rantala, E. Lampainen, M. Laakso and J. Hartikainen (2001). "Mitral valve prolapse and mitral regurgitation are common in patients with polycystic kidney disease type 1." Am J Kidney Dis **38**(6): 1208-1216.
Ma, L., M. F. Lu, R. J. Schwartz and J. F. Martin (2005). "Bmp2 is essential for cardiac cushion epithelial-mesenchymal transition and myocardial patterning." Development **132**(24): 5601-5611.
MacGrogan, D., G. Luxan, A. Driessen-Mol, C. Bouten, F. Baaijens and J. L. de la Pompa (2014). "How to make a heart valve: from embryonic development to bioengineering of living valve substitutes." Cold Spring Harb Perspect Med **4**(11): a013912.
Manasek, F. J. (1970). "Sulfated extracellular matrix production in the embryonic heart and adjacent tissues." J Exp Zool **174**(4): 415-439.
Mani, S. K., S. Balasubramanian, J. A. Zavadzkas, L. B. Jeffords, W. T. Rivers, M. R. Zile, R. Mukherjee, F. G. Spinale and D. Kuppuswamy (2009). "Calpain inhibition preserves myocardial structure and function following myocardial infarction." Am J Physiol Heart Circ Physiol **297**(5): H1744-1751.
Maredia, A. K., S. C. Greenway, S. Verma and P. W. M. Fedak (2017). "Bicuspid aortic valve-associated aortopathy: update on biomarkers." Curr Opin Cardiol.
Markwald, R. R., T. P. Fitzharris and F. J. Manasek (1977). "Structural development of endocardial cushions." Am J Anat **148**(1): 85-119.
Markwald, R. R., R. A. Norris, R. Moreno-Rodriguez and R. A. Levine (2010). "Developmental basis of adult cardiovascular diseases: valvular heart diseases." Ann N Y Acad Sci **1188**: 177-183.
Martin, M., R. Lorca, J. Rozado, R. Alvarez-Cabo, J. Calvo, I. Pascual, H. Cigarran, I. Rodriguez and C. Moris (2017). "Bicuspid aortic valve syndrome: a multidisciplinary approach for a complex entity." J Thorac Dis **9**(Suppl 6): S454-S464.
Martin, P. S., B. Kloesel, R. A. Norris, M. Lindsay, D. Milan and S. C. Body (2015). "Embryonic Development of the Bicuspid Aortic Valve." J Cardiovasc Dev Dis **2**(4): 248-272.
Martinez-Vea, A., A. Bardaji, C. Gutierrez, C. Garcia, C. Peralta, J. Aguilera, P. Sanchez, J. Vidiella, P. Angelet, T. Compte, C. Richart and J. A. Oliver (1999).

"Echocardiographic evaluation in patients with autosomal dominant polycystic kidney disease and end-stage renal disease." Am J Kidney Dis **34**(2): 264-272.
May, S. R., A. M. Ashique, M. Karlen, B. Wang, Y. Shen, K. Zarbalis, J. Reiter, J. Ericson and A. S. Peterson (2005). "Loss of the retrograde motor for IFT disrupts localization of Smo to cilia and prevents the expression of both activator and repressor functions of Gli." Dev Biol **287**(2): 378-389.
Menon, V., J. F. Eberth, R. L. Goodwin and J. D. Potts (2015). "Altered Hemodynamics in the Embryonic Heart Affects Outflow Valve Development." J Cardiovasc Dev Dis **2**(2): 108-124.
Menon, V., J. F. Eberth, L. Junor, A. J. Potts, M. Belhaj, D. J. Dipette, M. W. Jenkins and J. D. Potts (2017). "Removing vessel constriction on the embryonic heart results in changes in valve gene expression, morphology, and hemodynamics." Dev Dyn.
Mjaatvedt, C. H., R. C. Lepera and R. R. Markwald (1987). "Myocardial specificity for initiating endothelial-mesenchymal cell transition in embryonic chick heart correlates with a particulate distribution of fibronectin." Dev Biol **119**(1): 59-67.
Moore, F. L., J. Jaruzelska, D. M. Dorfman and R. A. Reijo-Pera (2004). "Identification of a novel gene, DZIP (DAZ-interacting protein), that encodes a protein that interacts with DAZ (deleted in azoospermia) and is expressed in embryonic stem cells and germ cells." Genomics **83**(5): 834-843.
Mukhopadhyay, S., H. B. Badgandi, S. H. Hwang, B. Somatilaka, I. S. Shimada and K. Pal (2017). "Trafficking to the primary cilium membrane." Mol Biol Cell **28**(2): 233-239.
Mukhopadhyay, S. and R. Rohatgi (2014). "G-protein-coupled receptors, Hedgehog signaling and primary cilia." Semin Cell Dev Biol **33**: 63-72.
Mukta, V., S. Tiwari and M. Kalirattiname (2008). "Bardet-Biedl syndrome with rheumatic aortic regurgitation." J Assoc Physicians India **56**: 729-731.
Murcia, N. S., W. G. Richards, B. K. Yoder, M. L. Mucenski, J. R. Dunlap and R. P. Woychik (2000). "The Oak Ridge Polycystic Kidney (orpk) disease gene is required for left-right axis determination." Development **127**(11): 2347-2355.
Nachury, M. V., E. S. Seeley and H. Jin (2010). "Trafficking to the ciliary membrane: how to get across the periciliary diffusion barrier?" Annu Rev Cell Dev Biol **26**: 59-87.
Nadorlik, H., J. L. Bowman, S. Fitzgerald-Butt, M. L. Mah, K. L. McBride, J. P. Kovalchin and V. Garg (2017). "Abnormal Longitudinal Growth of the Aorta in Children with Familial Bicuspid Aortic Valve." Pediatr Cardiol **38**(8): 1709-1715.
Narine, K., O. De Wever, D. Van Valckenborgh, K. Francois, M. Bracke, S. DeSmet, M. Mareel and G. Van Nooten (2006). "Growth factor modulation of fibroblast proliferation, differentiation, and invasion: implications for tissue valve engineering." Tissue Eng **12**(10): 2707-2716.
Nauli, S. M., F. J. Alenghat, Y. Luo, E. Williams, P. Vassilev, X. Li, A. E. Elia, W. Lu, E. M. Brown, S. J. Quinn, D. E. Ingber and J. Zhou (2003). "Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells." Nat Genet **33**(2): 129-137.
Nauli, S. M., X. Jin, W. A. AbouAlaiwi, W. El-Jouni, X. Su and J. Zhou (2013). "Non-motile primary cilia as fluid shear stress mechanosensors." Methods Enzymol **525**: 1-20.
Nesta, F., M. Leyne, C. Yosefy, C. Simpson, D. Dai, J. E. Marshall, J. Hung, S. A. Slaugenhaupt and R. A. Levine (2005). "New locus for autosomal dominant mitral valve prolapse on chromosome 13: clinical insights from genetic studies." Circulation **112**(13): 2022-2030.

Nguyen, A. M. and C. R. Jacobs (2013). "Emerging role of primary cilia as mechanosensors in osteocytes." Bone **54**(2): 196-204.
Norris, R. A., T. K. Borg, J. T. Butcher, T. A. Baudino, I. Banerjee and R. R. Markwald (2008). "Neonatal and adult cardiovascular pathophysiological remodeling and repair: developmental role of periostin." Ann N Y Acad Sci **1123**: 30-40.
Norris, R. A., R. Moreno-Rodriguez, A. Wessels, J. Merot, P. Bruneval, A. H. Chester, M. H. Yacoub, A. Hagege, S. A. Slaugenhaupt, E. Aikawa, J. J. Schott, A. Lardeux, B. S. Harris, L. K. Williams, A. Richards, R. A. Levine and R. R. Markwald (2010). "Expression of the familial cardiac valvular dystrophy gene, filamin-A, during heart morphogenesis." Dev Dyn **239**(7): 2118-2127.
Nus, M., D. MacGrogan, B. Martinez-Poveda, Y. Benito, J. C. Casanova, F. Fernandez-Aviles, J. Bermejo and J. L. de la Pompa (2011). "Diet-induced aortic valve disease in mice haploinsufficient for the Notch pathway effector RBPJK/CSL." Arterioscler Thromb Vasc Biol **31**(7): 1580-1588.
O'Dushlaine, C., E. Kenny, E. A. Heron, R. Segurado, M. Gill, D. W. Morris and A. Corvin (2009). "The SNP ratio test: pathway analysis of genome-wide association datasets." Bioinformatics **25**(20): 2762-2763.
Padang, R., R. D. Bagnall, D. R. Richmond, P. G. Bannon and C. Semsarian (2012). "Rare non-synonymous variations in the transcriptional activation domains of GATA5 in bicuspid aortic valve disease." J Mol Cell Cardiol **53**(2): 277-281.
Pala, R., N. Alomari and S. M. Nauli (2017). "Primary Cilium-Dependent Signaling Mechanisms." Int J Mol Sci **18**(11).
Pazour, G. J., B. L. Dickert, Y. Vucica, E. S. Seeley, J. L. Rosenbaum, G. B. Witman and D. G. Cole (2000). "Chlamydomonas IFT88 and its mouse homologue, polycystic kidney disease gene tg737, are required for assembly of cilia and flagella." J Cell Biol **151**(3): 709-718.
Peeters, F., S. J. R. Meex, M. R. Dweck, E. Aikawa, H. Crijns, L. J. Schurgers and B. Kietselaer (2017). "Calcific aortic valve stenosis: hard disease in the heart: A biomolecular approach towards diagnosis and treatment." Eur Heart J.
Peng, Y., S. Yan, D. Chen, X. Cui and K. Jiao (2017). "Pdgfrb is a direct regulatory target of TGFbeta signaling in atrioventricular cushion mesenchymal cells." PLoS One **12**(4): e0175791.
Perez-Pomares, J. M., A. Phelps, M. Sedmerova, R. Carmona, M. Gonzalez-Iriarte, R. Munoz-Chapuli and A. Wessels (2002). "Experimental studies on the spatiotemporal expression of WT1 and RALDH2 in the embryonic avian heart: a model for the regulation of myocardial and valvuloseptal development by epicardially derived cells (EPDCs)." Dev Biol **247**(2): 307-326.
Person, A. D., S. E. Klewer and R. B. Runyan (2005). "Cell biology of cardiac cushion development." Int Rev Cytol **243**: 287-335.
Potts, J. D. and R. B. Runyan (1989). "Epithelial-mesenchymal cell transformation in the embryonic heart can be mediated, in part, by transforming growth factor beta." Dev Biol **134**(2): 392-401.
Prakash, S., L. J. J. Borreguero, M. Sylva, L. Flores Ruiz, F. Rezai, Q. D. Gunst, J. L. de la Pompa, J. M. Ruijter and M. J. B. van den Hoff (2017). "Deletion of Fstl1 (Follistatin-Like 1) From the Endocardial/Endothelial Lineage Causes Mitral Valve Disease." Arterioscler Thromb Vasc Biol **37**(9): e116-e130.

Pritchett, J., E. Harvey, V. Athwal, A. Berry, C. Rowe, F. Oakley, A. Moles, D. A. Mann, N. Bobola, A. D. Sharrocks, B. J. Thomson, A. M. Zaitoun, W. L. Irving, I. N. Guha, N. A. Hanley and K. P. Hanley (2012). "Osteopontin is a novel downstream target of SOX9 with diagnostic implications for progression of liver fibrosis in humans." Hepatology **56**(3): 1108-1116.
Purkait, R., B. N. Roy, T. Samanta, A. K. Mallick and T. Sinhamahapatra (2012). "Rheumatic valvular insufficiency in Bardet-Biedl syndrome: a case report." J Indian Med Assoc **110**(9): 651-652.
Rabkin-Aikawa, E., M. Farber, M. Aikawa and F. J. Schoen (2004). "Dynamic and reversible changes of interstitial cell phenotype during remodeling of cardiac valves." J Heart Valve Dis **13**(5): 841-847.
Ramsdell, A. F. and R. R. Markwald (1997). "Induction of endocardial cushion tissue in the avian heart is regulated, in part, by TGFbeta-3-mediated autocrine signaling." Dev Biol **188**(1): 64-74.
Rash, J. E., J. W. Shay and J. J. Biesele (1969). "Cilia in cardiac differentiation." J Ultrastruct Res **29**(5): 470-484.
Reiter, J. F. and M. R. Leroux (2017). "Genes and molecular pathways underpinning ciliopathies." Nat Rev Mol Cell Biol **18**(9): 533-547.
Riem Vis, P. W., J. Kluin, J. P. Sluijter, L. A. van Herwerden and C. V. Bouten (2011). "Environmental regulation of valvulogenesis: implications for tissue engineering." Eur J Cardiothorac Surg **39**(1): 8-17.
Runyan, R. B. and R. R. Markwald (1983). "Invasion of mesenchyme into three-dimensional collagen gels: a regional and temporal analysis of interaction in embryonic heart tissue." Dev Biol **95**(1): 108-114.
Saraga-Babic, M., K. Vukojevic, I. Bocina, K. Drnasin and M. Saraga (2012). "Ciliogenesis in normal human kidney development and post-natal life." Pediatr Nephrol **27**(1): 55-63.
Sauls, K., K. Toomer, K. Williams, A. J. Johnson, R. R. Markwald, Z. Hajdu and R. A. Norris (2015). "Increased Infiltration of Extra-Cardiac Cells in Myxomatous Valve Disease." J Cardiovasc Dev Dis **2**(3): 200-213.
Saxon, J. G., D. R. Baer, J. A. Barton, T. Hawkins, B. Wu, T. C. Trusk, S. E. Harris, B. Zhou, Y. Mishina and Y. Sugi (2017). "BMP2 expression in the endocardial lineage is required for AV endocardial cushion maturation and remodeling." Dev Biol **430**(1): 113-128.
Sekimizu, K., N. Nishioka, H. Sasaki, H. Takeda, R. O. Karlstrom and A. Kawakami (2004). "The zebrafish iguana locus encodes Dzip1, a novel zinc-finger protein required for proper regulation of Hedgehog signaling." Development **131**(11): 2521-2532.
Siu, S. C. and C. K. Silversides (2010). "Bicuspid aortic valve disease." J Am Coll Cardiol **55**(25): 2789-2800.
Slough, J., L. Cooney and M. Brueckner (2008). "Monocilia in the embryonic mouse heart suggest a direct role for cilia in cardiac morphogenesis." Dev Dyn **237**(9): 2304-2314.
Smith, C. L., S. T. Baek, C. Y. Sung and M. D. Tallquist (2011). "Epicardial-derived cell epithelial-to-mesenchymal transition and fate specification require PDGF receptor signaling." Circ Res **108**(12): e15-26.

Smyth, B. J., R. W. Snyder, D. F. Balkovetz and J. H. Lipschutz (2003). "Recent advances in the cell biology of polycystic kidney disease." Int Rev Cytol **231**: 51-89.
Spicer, D. E., J. M. Bridgeman, N. A. Brown, T. J. Mohun and R. H. Anderson (2014). "The anatomy and development of the cardiac valves." Cardiol Young **24**(6): 1008-1022.
Steurer, M. A., M. E. Norton, R. J. Baer, G. M. Shaw, S. Keating, A. J. Moon-Grady, C. D. Chambers and L. L. Jelliffe-Pawlowski (2017). "The association of maternal lymphatic markers and critical congenital heart defects in the fetus-A population based case-control study." Am J Med Genet A **173**(5): 1231-1236.
Sugi, Y., N. Ito, G. Szebenyi, K. Myers, J. F. Fallon, T. Mikawa and R. R. Markwald (2003). "Fibroblast growth factor (FGF)-4 can induce proliferation of cardiac cushion mesenchymal cells during early valve leaflet formation." Dev Biol **258**(2): 252-263.
Sugi, Y., H. Yamamura, H. Okagawa and R. R. Markwald (2004). "Bone morphogenetic protein-2 can mediate myocardial regulation of atrioventricular cushion mesenchymal cell formation in mice." Dev Biol **269**(2): 505-518.
Tao, G., J. D. Kotick and J. Lincoln (2012). "Heart valve development, maintenance, and disease: the role of endothelial cells." Curr Top Dev Biol **100**: 203-232.
Thanassoulis, G., J. W. Yip, K. Filion, M. Jamorski, G. Webb, S. C. Siu and J. Therrien (2008). "Retrospective study to identify predictors of the presence and rapid progression of aortic dilatation in patients with bicuspid aortic valves." Nat Clin Pract Cardiovasc Med **5**(12): 821-828.
Tibayan, F. A., S. Louey, S. Jonker, H. Espinoza, N. Chattergoon, F. You, K. L. Thornburg and G. Giraud (2015). "Increased systolic load causes adverse remodeling of fetal aortic and mitral valves." Am J Physiol Regul Integr Comp Physiol **309**(12): R1490-1498.
Tran, P. V., M. Sharma, X. Li and J. P. Calvet (2014). "Developmental signaling: does it bridge the gap between cilia dysfunction and renal cystogenesis?" Birth Defects Res C Embryo Today **102**(2): 159-173.
Veland, I. R., A. Awan, L. B. Pedersen, B. K. Yoder and S. T. Christensen (2009). "Primary cilia and signaling pathways in mammalian development, health and disease." Nephron Physiol **111**(3): p39-53.
Velazquez, E. J., Z. Samad, H. R. Al-Khalidi, C. Sangli, P. A. Grayburn, J. M. Massaro, S. R. Stevens, T. E. Feldman and M. W. Krucoff (2015). "The MitraClip and survival in patients with mitral regurgitation at high risk for surgery: A propensity-matched comparison." Am Heart J **170**(5): 1050-1059 e1053.
Wang, C., J. Li, Q. Meng and B. Wang (2017). "Three Tctn proteins are functionally conserved in the regulation of neural tube patterning and Gli3 processing but not ciliogenesis and Hedgehog signaling in the mouse." Dev Biol **430**(1): 156-165.
Wang, C., W. C. Low, A. Liu and B. Wang (2013). "Centrosomal protein DZIP1 regulates Hedgehog signaling by promoting cytoplasmic retention of transcription factor GLI3 and affecting ciliogenesis." J Biol Chem **288**(41): 29518-29529.
Wang, J., S. Sridurongrit, M. Dudas, P. Thomas, A. Nagy, M. D. Schneider, J. A. Epstein and V. Kaartinen (2005). "Atrioventricular cushion transformation is mediated by ALK2 in the developing mouse heart." Dev Biol **286**(1): 299-310.
Warnock, J. N., B. Nanduri, C. A. Pregonero Gamez, J. Tang, D. Koback, W. M. Muir and S. C. Burgess (2011). "Gene Profiling of Aortic Valve Interstitial Cells under

Elevated Pressure Conditions: Modulation of Inflammatory Gene Networks." Int J Inflam **2011**: 176412.
Wessels, A., M. W. Markman, J. L. Vermeulen, R. H. Anderson, A. F. Moorman and W. H. Lamers (1996). "The development of the atrioventricular junction in the human heart." Circ Res **78**(1): 110-117.
Wessels, A., M. J. van den Hoff, R. F. Adamo, A. L. Phelps, M. M. Lockhart, K. Sauls, L. E. Briggs, R. A. Norris, B. van Wijk, J. M. Perez-Pomares, R. W. Dettman and J. B. Burch (2012). "Epicardially derived fibroblasts preferentially contribute to the parietal leaflets of the atrioventricular valves in the murine heart." Dev Biol **366**(2): 111-124.
Wirrig, E. E. and K. E. Yutzey (2014). "Conserved transcriptional regulatory mechanisms in aortic valve development and disease." Arterioscler Thromb Vasc Biol **34**(4): 737-741.
Wu, B., Y. Wang, W. Lui, M. Langworthy, K. L. Tompkins, A. K. Hatzopoulos, H. S. Baldwin and B. Zhou (2011). "Nfatc1 coordinates valve endocardial cell lineage development required for heart valve formation." Circ Res **109**(2): 183-192.
Wu, G., G. S. Markowitz, L. Li, V. D. D'Agati, S. M. Factor, L. Geng, S. Tibara, J. Tuchman, Y. Cai, J. H. Park, J. van Adelsberg, H. Hou, Jr., R. Kucherlapati, W. Edelmann and S. Somlo (2000). "Cardiac defects and renal failure in mice with targeted mutations in Pkd2." Nat Genet **24**(1): 75-78.
Zaidi, S., M. Choi, H. Wakimoto, L. Ma, J. Jiang, J. D. Overton, A. Romano-Adesman, R. D. Bjornson, R. E. Breitbart, K. K. Brown, N. J. Carriero, Y. H. Cheung, J. Deanfield, S. DePalma, K. A. Fakhro, J. Glessner, H. Hakonarson, M. J. Italia, J. R. Kaltman, J. Kaski, R. Kim, J. K. Kline, T. Lee, J. Leipzig, A. Lopez, S. M. Mane, L. E. Mitchell, J. W. Newburger, M. Parfenov, I. Pe'er, G. Porter, A. E. Roberts, R. Sachidanandam, S. J. Sanders, H. S. Seiden, M. W. State, S. Subramanian, I. R. Tikhonova, W. Wang, D. Warburton, P. S. White, I. A. Williams, H. Zhao, J. G. Seidman, M. Brueckner, W. K. Chung, B. D. Gelb, E. Goldmuntz, C. E. Seidman and R. P. Lifton (2013). "De novo mutations in histone-modifying genes in congenital heart disease." Nature **498**(7453): 220-223.
Zavadzkas, J. A., R. E. Stroud, S. Bouges, R. Mukherjee, J. R. Jones, R. K. Patel, P. J. McDermott and F. G. Spinale (2014). "Targeted overexpression of tissue inhibitor of matrix metalloproteinase-4 modifies post-myocardial infarction remodeling in mice." Circ Res **114**(9): 1435-1445.
Zhang, B., G. Wang, X. Xu, S. Yang, T. Zhuang, G. Wang, H. Ren, S. Y. Cheng, Q. Jiang and C. Zhang (2017). "DAZ-interacting Protein 1 (Dzip1) Phosphorylation by Polo-like Kinase 1 (Plk1) Regulates the Centriolar Satellite Localization of the BBSome Protein during the Cell Cycle." J Biol Chem **292**(4): 1351-1360.
Zhang, B., T. Zhang, G. Wang, G. Wang, W. Chi, Q. Jiang and C. Zhang (2015). "GSK3beta-Dzip1-Rab8 cascade regulates ciliogenesis after mitosis." PLoS Biol **13**(4): e1002129.
Zuo, X., W. Guo and J. H. Lipschutz (2009). "The exocyst protein Sec10 is necessary for primary ciliogenesis and cystogenesis in vitro." Mol Biol Cell **20**(10): 2522-2529.