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DCHS1 Regulated miRNA Processing and its Effects on Valve Endocardium Stabilization

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DCHS1 Regulated miRNA Processing and its Effects on Valve Endocardium Stabilization

By

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A thesis submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Master of Science in Biomedical Sciences in the College of Graduate Studies.

Department of Regenerative Medicine and Cell Biology

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Dedication

This thesis is dedicated to my wonderful family: my parents, Brooke and Dan, my siblings, Lily and Daniel, and my grandparents Kirk and Helen. Thank you for the endless love and support you have given me. Without you I would have never been able to complete my greatest accomplishments. I love you always.

Acknowledgements

I would first like to thank my loving family for supporting me and getting me where I am today. Second, I would like to thank my best friend, for your support and encouragement and allowing me to cry and complain when I needed to. I would also like to thank the Medical University of South Carolina and the Master of Biomedical Science program for the incredible opportunity to further my education. I would also like to thank my committee members. Chip, my mentor and leader, thank you for your support and guidance during this process. I appreciate your patience and leadership. Your guidance and knowledge helped me to become a competent scientist and to feel confident to become a science teacher. I am forever grateful. Dr. Kasman, thank you for keeping me on track and giving me words of encouragement to get me through graduate school. Dr. Kourtidis, thank you for support and guidance as well as use of your lab and materials for the many experiments that did not pan out. Dr. Kubalak, thank you for the direction and advice that you have given me. Dr. Muise-Helmericks, thank you for your uplifting attitude and for telling me what I need to hear, but always in a kind and supportive way. Thank you for all of your hard work and time that you have given me during this journey. Finally, thank you to my lab mates in the Norris lab, Diana, Kelsey, Janiece, Cortney, Tyler, Leon, Rebecca, and Reece, for your guidance, help, support, and encouragement that has undoubtedly helped to get to the finish line.

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LIST OF ABBREVIATIONS

AV-Atrioventricular
BMP- Bone Morphogenetic Protein
CKO- Conditional Knockout
DCHS1-Dachsous Cadherin Related 1
DRE- Dchs1 Rosa NfatC1 Enhancer Cre
DT-Dchs1 Knockout mouse
DZIP1- DAZ Zinc Finger Protein 1
ECM- Extracellular Matrix
EMT- Endothelial to Mesenchymal Transition
GWAS-Genome Wide Association Study
HUVEC- Human Umbilical Vein Endothelial Cell
HuVEC- Human Valve Endothelial Cell
IHC- Immunohistochemistry
KD- Knockdown
KO- Knockout
LIX1L-Lix1 Like
MVP- Mitral Valve Prolapse
OFT- Outflow Tract
PFA- Paraformaldehyde
PKD- Polycystic Kidney Disease
TGFb- Transforming Growth Factor Beta
VEC- Valvular Endothelial Cell
VIC- Valvular Interstitial Cell
ZEB-2- Zinc Finger E-Box-Binding Homeobox 2
GFP- Green Fluorescent Protein

MARY KATHLEEN RUMPH, DCHS1 Regulated miRNA Processing and its Effects on Valve Endocardium Stabilization (Under the direction of RUSSEL A. NORRIS)

ABSTRACT

Mitral valve prolapse (MVP) is one of the most common forms of cardiac valve disease and affects 1 in 40 individuals worldwide. MVP can lead to arrhythmias, heart failure, and sudden cardiac death and 1 in 10 patients will require valve surgery. Surgery for MVP is now the fastest growing cardiovascular intervention in the Western world. As such, MVP carries a significant burden of morbidity and mortality. Our lab was the first to identify a cause for non-syndromic MVP using a combination of linkage analyses, as well as capture sequence of the linkage interval, to identify loss of function mutations in the cadherin gene, *DCHS1*. Two-hybrid screens were undertaken to further understand DCHS1 function and the RNA binding protein, LIX1L was identified as the only interacting protein. LIX1L binds and promotes miRNA processing through interactions as an RNA-binding protein and miRNAs. This leads us to the hypothesis: DCHS1-regulated miRNA processing stabilizes the valve endocardium. Expression studies have corroborated this theory as DCHS1, LIX1L, and the microprocessor proteins are expressed in endothelial cells in the mitral valve. Cell culture data shows that a loss of DCHS1 compromises processing of target miRNAs through the microprocessor leading to a significant decrease in miRNA expression. Expression studies have also shown that loss of Dchs1 reduces valve endocardial stability, which may be caused by the loss of miRNAs. These studies illustrate the importance of DCHS1 effects on valve endocardium stabilization in MVP. Uncovering how these changes lead to clinically significant pathology later in life is crucial to the characterization of MV

CHAPTER 1: INTRODUCTION AND BACKROUD

Introduction

The heart is an essential organ in the human body that is the center of the circulatory system. It is comprised of four chambers: the left atrium, right atrium, left ventricle, and right ventricle. Each chamber is crucial to conducting how blood flows to keep an organism alive. The left and right sides of the heart contain their own pumping systems, but they are able to communicate effectively in order to optimize blood flow. The right atrium receives the deoxygenated blood from the body and pumps it to the right ventricle through the tricuspid valve. Subsequently, the right ventricle pumps the deoxygenated blood to the lungs through the pulmonary valve. The left atrium receives the oxygenated blood from the lungs and pumps it to the left ventricle through the mitral valve. The left ventricle then pumps the oxygen-rich blood through the aortic valve to the rest of the body (Buckberg, Nanda, Nguyen, & Kocica, 2018).

In our lab, we are focused on heart development and how improper heart development can lead to cardiac disease in a patient's life. Specifically, we focus on cardiac valve development. As previously mentioned, there are four valves in the heart: the pulmonary valve, the aortic valve, the tricuspid valve, and the mitral valve. Each valve functions to promote unidirectional blood flow through the heart. If there is any disruption to this process, such as incorrect valve development, this can lead to valve diseases such as mitral valve prolapse. My research aims to establish a mechanism by which mitral valve prolapse is caused. This is important to provide additional information to recent studies because it provides a mechanism for understanding the fundamental molecular and developmental processes that can be applied to the disease. The following

chapters will address our recent developmental, genetic, and molecular findings regarding valvular development and mitral valve prolapse.

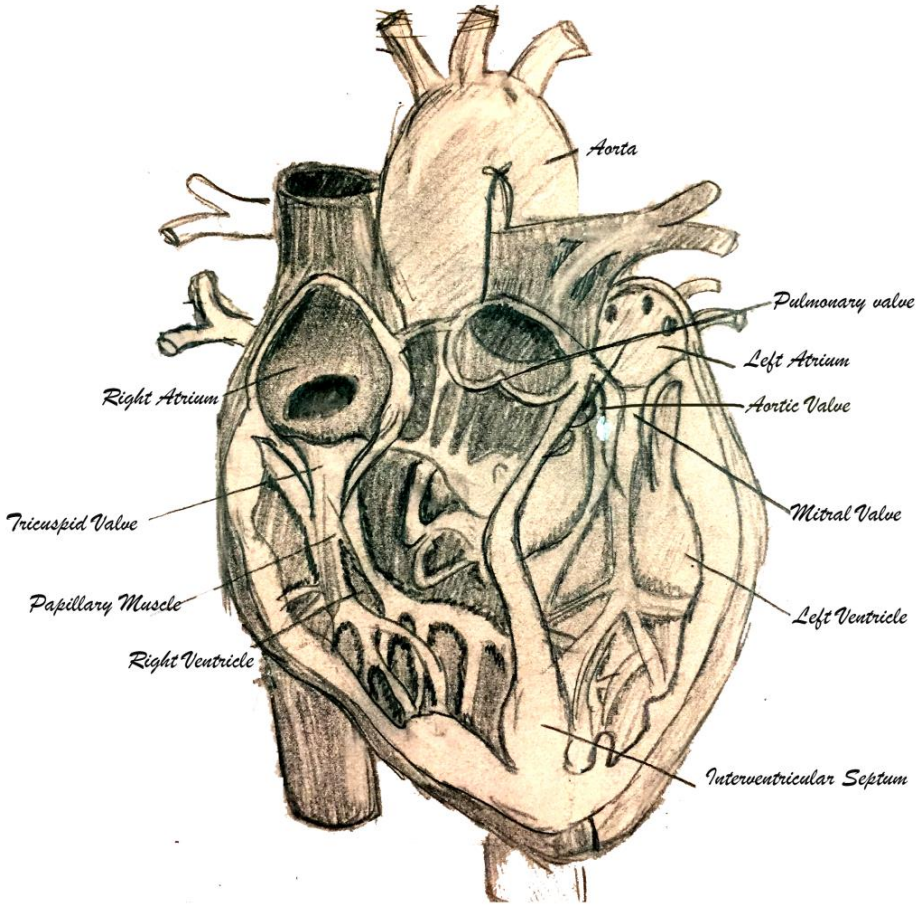

Figure 1.1 The Gross Anatomy of the Human Heart. The human heart is comprised of four chambers: the left atrium, right atrium, left ventricle and the right ventricle. The left and the right side of the heart have their own pumping systems. The right atrium receives deoxygenated blood from the body and pumps it to the right ventricle through the tricuspid valve. The right ventricle then pumps the deoxygenated blood to the lungs through the pulmonary valve. The left atrium receives the oxygenated blood from the lungs and pumps it to the left ventricle through the mitral valve. The left ventricle pumps the oxygen-rich blood through the aortic valve out to the rest of the body (Drawing by Rebecca Stairley, Norris Lab).

Valve Development

The human heart consists of four chambers (right atrium, right ventricle, left atrium, and left ventricle), and four valves (aortic and pulmonic semilunar), mitral and tricuspid valves. For the purpose of this study, we focus on the mitral valve leaflets. The mitral valve leaflets are located between the left atrium and the left ventricle. The structure of the leaflets plays an important role for the proper function of the heart (Figure 1.1). The valves work together to optimize and maintain unidirectional blood flow throughout the heart (Horne et al, 2015). The mitral valve leaflets are enclosed with valvular endothelial cells (VECs), while the remaining volume of the leaflet is comprised of valvular interstitial cells (VICs) (Hinton et al. 2006) (Rabkin-Aikawa, Farber, Aikawa, & Schoen, 2004).

Valve development starts from a cardiac tube that is made of myocardium and endocardium. Between these two layers is a region called the cardiac jelly which is made of space-filling proteoglycans (Person, Klewer, & Runyan, 2005). The myocardium discharges a variety of growth factors that induce the endothelium to undergo endothelial-to-mesenchymal transformation (EMT). This results in the cardiac jelly being invaded by mesenchyme to initiate the first phase of valve development. This process is crucial in defining correct valve formation. As the valves continue to grow and stretch into the left ventricle, the fibrous leaflets become thinner, increasing the extracellular matrix (ECM) deposition and remodeling. It is not until several days after birth of a newborn that highly organized and distinct layers that represent the ECM are seen. The ECM of the valve is composed of three stratified layers that are rich in collagens, proteoglycans and elastin. Both mouse and human valves have these boundaries although there is variation on how many ECM zones exist. The complete valve maturation and remodeling in mammals will continue into juvenile stages (Combs & Yutzey, 2009).

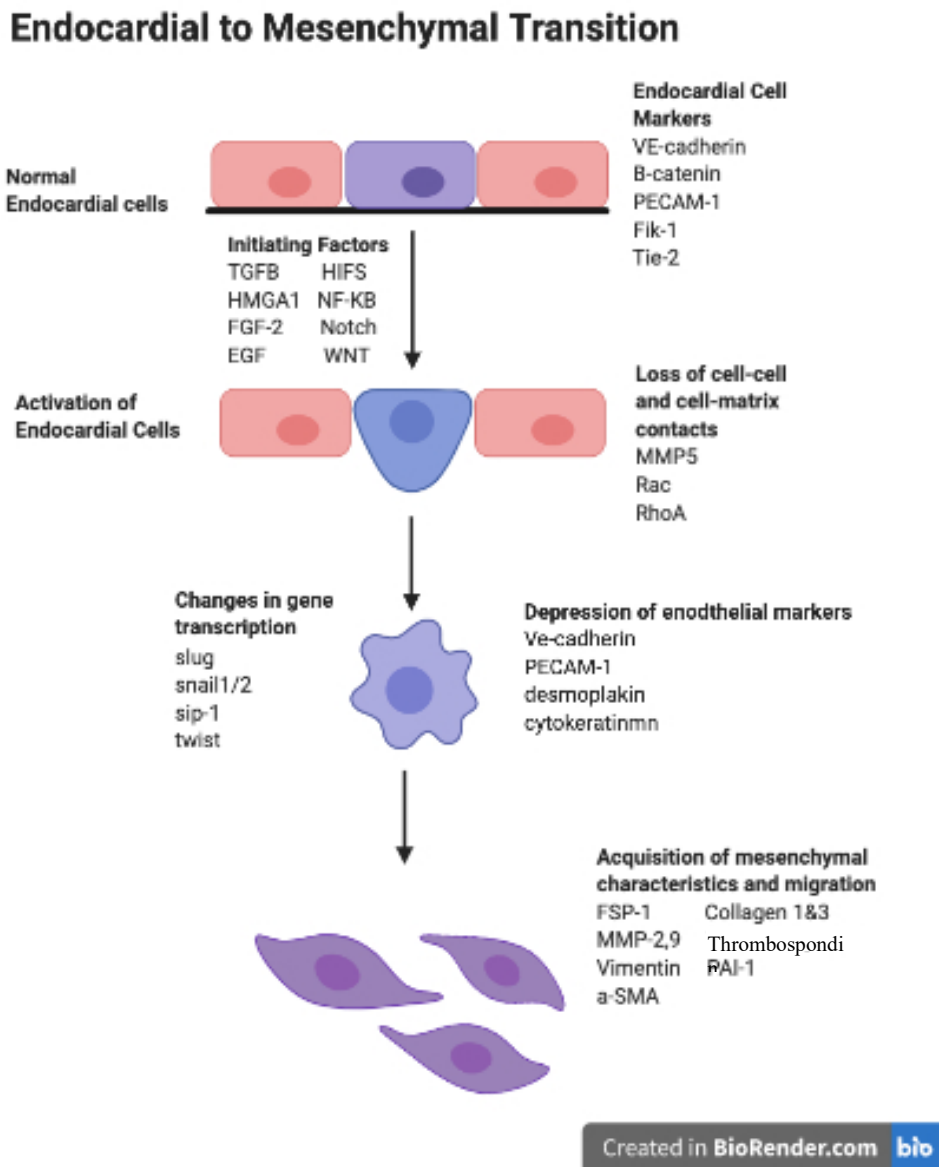

Figure 1.2 Endocardial to Mesenchymal Transition: Endothelial-to-mesenchymal transition describes the process by which endothelial cells transform into mesenchymal cells. It is associated with a loss of endocardial cell markers such as VE-cadherin, B-catenin, PECAM-1, Fik-1, and Tie-2 and the acquisition of mesenchymal cell markers such as FSP-1, MMP-2,9 vimentin, a-SMA, collagen 1 and 3, thromobospondin, and PAI-1. Endothelial cells those their apical basal polarity and mesenchymal cells gain new migratory properties (Corre & Clere. 2019).

There have been abundant studies that have included the similarities between animal models and human anatomy. This information has been used to use make new discoveries in translational science. Therefore, the comparison of adult aortic valve leaflet structure and composition demonstrates similar stratification in humans, sheep, chickens, rabbits and mice (Combs & Yutzey, 2009). The process of valve formation is universal, accommodating the species in which they reside. As an example, we see conserved valve cell regulatory mechanisms in the smallest species such as *Drosophila* (Lammers et al. 2017).

As we examine valve formation on a molecular level, we look to evaluate the endocardial cushion composition and how the study of valve development includes different signaling mechanisms. The first studies that identified EMT in the heart were through pioneering work from the laboratory of Roger Markwald (Markwald, Fitzharris, & Manasek, 1977). When chick cardiac explants were placed on collagen lattice, the endocardium was seen adopting migratory and invasive characteristics. However, when the myocardium was removed from these cultures, the endocardium failed to change its phenotype. This was the first evidence demonstrating that myocardial-secreted factors could induce an endothelial-to-mesenchymal transformation. From these seminal studies, concerted efforts have focused on identifying the myocardial initiating signals (Runyan & Markwald, 1983). Since then, major growth factors have been identified that regulate this EMT process, such as transforming growth factor beta (TGFb) and bone morphogenetic protein (BMP). Notch signaling, active in the endocardium, has also been defined as playing a critical role in EMT progression and can synergize with TGFb and BMP ligands (Garside, Chang, Karsan, & Hoodless, 2013). The endocardial-to-mesenchymal transition process occurs when subsets of atrioventricular (AV) and outflow tract (OFT) endocardial cells transform into a mesenchymal phenotype, which determines the anatomical placement where the valves will form within the primary heart tube (de Vlaming et al. 2012)

The process of valve development occurs in multiple steps. These steps include: the formation of the ECM, the migration of endocardial cells into the ECM, the event of cardiac

looping that induces endocardial cushion swellings, triggering the overpopulation of endocardial cushions (Markwald, Ftizharris, Bolender, & Bernanke, 1979) (Eisenberg & Markwald, 1995) and the formation of the stratified layers (proteoglycans, elastin and collagen) of the ECM. The cause of the enlargement results from the myocardium of specific regions of the primary heart tube, the AV junction and the OFT upregulate the secretion of the ECM. This process allows for cushion morphogenesis that induces a subset of AV and OFT endocardial cells to lose their junctions and adopt a mesenchymal-like phenotype as they migrate into the cardiac jelly (Person et al, 2005). Additionally, there have been studies that demonstrate the role that both the myocardial and endocardial derived signaling pathways affect endocardial cushion EMT and proliferation of valve progenitor cells (de Lange et al., 2004). This eventually forms the differences between the myocardium and the endocardium.

Cell Types in the Mature Mitral Valve

Through the events of valve development, the valve becomes mature and has its own different cell types. There are two main cell types that make up the valves: the valvular interstitial cells (VICs) and the valvular endothelial cells (VECs). The VEC's reside on the outer rim of the leaflets. It has been shown that any infiltration or breakage of this border can lead to valve disease (Butcher & Nerem, 2007), (Leask, Jain, & Butany, 2003), (Butcher & Markwald 2007) (El-Hamamsy et al., 2009).

The majority of each leaflet is composed of VIC's. Their fibroblastic consistency aids in several different characteristics throughout the valve due to their various placements within the leaflets (Horne et a., 2015). The VIC's essentially act as first responders: if given enough stress they have the capability of migrating and contracting rather than staying in their resting state. Their main role is to mediate and maintain the ECM structure, throughout the maturation of valve development. Any disturbances in the roles of either of these cell types can lead to unfavorable consequences that can affect the biochemical functions leading to diseases. The overall progression of valve development has many evolving steps and if there is a misstep at any event during the process there will be severe complications.

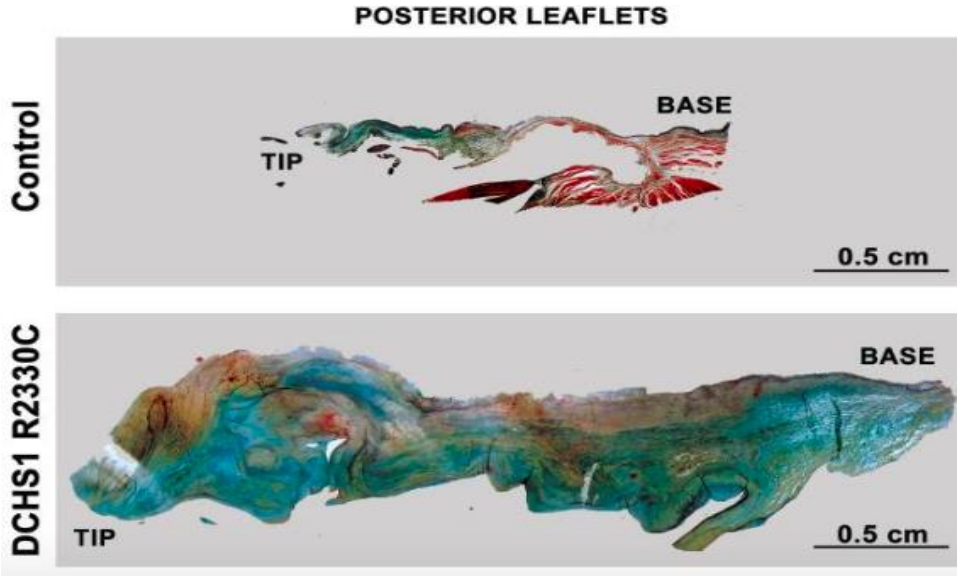

Figure 1.3 Myxomatous Degeneration in Valves. Human posterior leaflets of control and DCHS1 p. R2330C were isolated, fixed and stained with Movat's Pentachrome. Leaflet thickening, elongation and myxomatous degeneration is observed in the DCHS1 p. R2330C leaflet compared to the control. Expansion of the proteoglycan layer (blue) and the disruption of the normal stratification of matrix boundaries is observed in DCHS1 p. R2330C leaflets, because the cell types become mixed and disorganized. Blue=proteoglycan, Black=elastin, Red=Fibrin or cardiac muscle. Scale bars= 0.5cm (Durst et al 2015).

Biochemical Influences

As the valve is undergoing maturation, addition factors can play a role on how the valves are able to perform their job accurately, for example biomechanical forces. In a fully developed heart, the act of blood flowing from the atrium to the ventricle represents blood going from an area with low pressure to an area that is high in pressure. Previous studies have stated that the biomechanical forces behind effectively beating of the heart during valve development influence the formation and matrix composition of the valves (Butcher, Mcquinn, Sedmera, Turner, & Markwald, 2007). During isovolumetric relaxation, an interval in the cardiac cycle, the pressure difference between the left atrium and the left ventricle causes the MV leaflets to open and allow blood to flow from the left atrium into the left ventricle during diastole (Giovanni et al., 2007). During this time, the left ventricle stays in a relaxed state, allowing it to maintain a positive transmitral pressure which aids in filling the ventricle without complications. Soon after active ventricular relaxation, the fluid begins its deceleration and the MV closes, preventing any backflow of blood into the atrium (Saige et al. 1994).

In addition, the leaflets of the mitral valve are connected to tendinous chords that attach to the left ventricular myocardium through the papillary muscle. These fibrous structures provide tension to the mitral leaflets. This along with several other mechanical systems keep the tissues from ballooning upward into the left atrium during ventricular systole. Furthermore, in vitro models have suggested that vortices, the region of blood that is fixed around an axis line, is crucial to the closing of the valve. The vortices are generated by ventricular filling and aid the partial closure of the MV following early

diastole and are essential for the closing of the valve. Without these vortices, the valve would not be able to close during ventricular contraction (Bellhouse & Reid, 1969).

Developmental Basis of Disease

To start investigating valve disease, researchers have designed many studies surrounding genetic disorders found in large families and from previous work published. Researchers have found similarities in disease like Alzheimer's and diabetes because of the same genetic anomalies surrounding these diseases and valve disease (Reitz et al., 2007) In each instance there are gene mutations, these mutations are present at conception, which leads to inheritable traits passed from generation to generation in families and the diagnosis of these diseases appearing later in life. Identifying the genes and their function in familial disease is vital in understanding how mutations of these genes lead to disease. With mitral dystrophies, individuals in a family can show a high degree of variation in when they are diagnosed with the disease, despite the fact the affected individuals all carry the same mutation. It has been found that children with mitral valve prolapse (MVP) have been diagnosed as young as six years of age (Kyndt et al. 1998). Furthermore, this knowledge proposes that a patient's genetic makeup could dictate the severity of the valve disease. This is key in investigating the complexity behind valve disease, as it could open the door for new therapeutic aids and the discovery of associated pathways that are required for proper valve development.

Mitral Valve Prolapse

MVP is the most common form of cardiac valve disease and is a major health burden. The disease is characterized by the abnormal billowing of one or both of the mitral valve leaflets. Structurally the disease causes myxomatous degeneration and the valves experience an increase in proteoglycan accumulation, fragmented collagen and hyperplasia (Figure 1.3). This phenotype prevents the valve from functioning properly. In addition, this causes an imbalance of biomechanical forces that affects the correct positioning of the leaflets as well.

This disease affects 1 in 40 people worldwide, making its prevalence rate between 2-3%. Based on this information, MVP is expected to afflict approximately 176 million people worldwide (Devereux et al., 1987). These staggering numbers have brought much needed attention to this disease, yet there are many factors surrounding MVP that are unknown. MVP can be either syndromic and recognized as a symptom of a recognized syndrome, like Marfan syndrome, or it can be non-syndromic occurring in isolation.

The term, MVP was coined by Barlow in 1963 (Barlow & Pocock, 1963) when he detected mitral regurgitation in one of his patients when examining their heart through an angiography. MVP carries a significant burden of morbidity and mortality. There are no effective nonsurgical treatments for MVP and an incomplete understanding of its fundamental causes has hindered therapeutic efforts. While surgical techniques continue to improve, the number of surgical cases and associated mortality rates are increasing. Surgeries for degenerative mitral valve disease increased by more than 44% from 2011-2016 and currently >90,000 mitral valve surgeries occur each year making it the fastest growing cardiovascular intervention in the United States (Coutinho & Antunes, 2017).

MVP can lead to arrhythmias, heart failure, and even sudden cardiac death, and 1 in 10 patients will require valve surgery. Through various studies, it was found that MVP could be inheritable or a naturally occurring mutation, but the initial diagnosis of MVP left many patients undetected.

The Framingham Heart study detailed vital information found in the echocardiography of MVP patients. It was found that echocardiographers misinterpreted the phenotype because the position of the echo probe did not relay proper mitral valve geometry (Freed et al., 1999). Initially physicians were doing an apical view to obtain the correct orientation of the valve. When the probe was turned there was a representation of the myxomatous valve. Through this study physicians were able to develop a more efficient and precise way to diagnose MVP patients and also detect accompanying health defects with this disease like heart failure and atrial fibrillation.

Recent studies by our group have provided new hope in identifying MVP disease mechanisms, as we were the first to describe genetic causes for non-syndromic MVP (Durst et al., 2015) (Dina et al. 2015) (Table 1.1). Our group has studied the cilia gene, *DZIP1*, a gene that regulates ciliogenesis (Toomer, 2019). If a deleterious missense mutation of *DZIP1* occurs in humans, it can cause autosomal dominant non-syndromic MVP. This was further confirmed in a mouse model that confirmed the pathogenicity of this mutation and revealed impaired ciliogenesis during development, which progressed to adult myxomatous valve disease and functional MVP. Through genetic studies our group has linked the cadherin protein DCHS1 with MVP (Durst et al. 2015) (Dina et al., 2015).

Table 1.1 Genetic associations with mitral valve prolapse in humans

	Gene or chromosome	Defect localization/mechanism/Phenotype
Non-syndromic MVP		
Filamin A-MVD	*FLNA*	Mechanotransduction/ Cardiac valvular dystrophy
Myxomatous disease (MMVP2)	*DCHS1*	Cell Migration and polarity/ *Dchs1* mutations = MVP
Myxomatous disease (MMVP1-3)	*Chr 16-13*	--
Frequent Variants GWAS	*LMCD1*	Transcription factor/ Knockdown of LMCD1 in mice= valve regurgitation
	TNS1	Focal adhesion protein/cytoskeleton organization/*Tns1*$^{-/-}$ mice= myxomatous degeneration in posterior leaflet

Table 1.1: Genes associated with MVP. Genetic anomalies associated with mitral valve prolapse in humans (Durst et al., 2015) (Dina et al. 2015) (Kyndt et al. 1998) (Le Tourneau et al. 2018).

DCHS1 as a Focus

We have chosen to focus on uncovering its function in the mechanism of disease in patients with MVP. Using a combination of linkage analyses as well as exome capture sequencing, our lab has identified the loss of function mutations in the cadherin gene, *DCHS1*, that segregates with MVP in multiple families. Patients with *DCHS1* missense mutations displayed autosomal dominant inheritance, which we have shown in multiple model systems to cause haploinsufficiency, or gene expression that is half the normal level, and disease phenotype (Durst et al. 2015). Disease inception was traced back to defects in valve morphogenesis. Recent strides by other groups have confirmed these findings and identified putatively damaging *DCHS1* variants in as many as 24% of MVP patients (Clemenceau et al. 2018). These data revealed that *DCHS1* mutations are prominently involved in MVP and underscore the importance for defining its role(s) in cardiac development and disease. DCHS1 expression becomes restricted to VEC's after birth suggesting a putative role for this cadherin in stabilizing the valve endocardium. These data are provocative and imply unique roles for DCHS1 in VECs as the endocardial cells begin to arrest EMT.

To gain an understanding of DCHS1 function in the mitral valves, we undertook an unbiased two-hybrid screen of a human heart cDNA library. After screening 110 million clones with the cytoplasmic tail of human DCHS1 protein, we identified the RNA binding protein LIX1L (LIX1-Like) as the only interacting protein. These findings were bolstered by additional reports showing that LIX1L interacts with DCHS1 in other model systems (Bando et al. 2011) (Mao, Kucuk, & Irvine, 2009). Although not much is known

about LIX1L function, previous work has shown its ability to bind to miRNAs and regulates miRNA expression (Nakamura et al. 2015).

Cell Junctions and RNAi

It is well-established that cadherins stabilize cell-cell junctions and propagate downstream signaling cascades through their cytoplasmic domains to regulate tissue morphogenesis (Maitre & Heisenberg, 2013). Recent reports have shown that cells stabilize their junctions and suppress EMT through a mechanism that involves cadherin proteins recruiting miRNA processing complexes to the membrane (Kourtidis et al, 2017) (Kourtidis & Anastasiadis, 2016) (Kourtidis et al., 2015). RNA localization has evolved as a mechanism of energy conservation and efficient regulation of protein signaling to enable rapid molecular and cellular adaptations to changes in environmental conditions (Niessing, Jansen, Pohlmann, & Feldbrugge, 2018) (Jansen, Niessing, Baumann, & Feldbrugge, 2014).

The microprocessor catalyzes the first step of miRNA biogenesis through its core members DROSHA and DGCR8 (Gregory et al. 2004) (Ha & Kim, 2014) (Figure 1.4). DROSHA is a double stranded RNA-specific endoribonuclease and it is the enzymatic component of the microprocessor. DGCR8 is essential for delivering miRNAs to the complex. The complex then processes the initial miRNA transcripts called primary miRNAs (pri-miRNAs) into precursor miRNAs (pre-miRNAs), which are then fed into the Dicer-Ago2 complex to produce the mature miRNA products. Until recently the microprocessor and several accessory components of the RNAi machinery were thought to localize solely in the nucleus (Ha & Kim 2014). However, this dogma was challenged

by recent work, which revealed a functional microprocessor that is recruited by cadherin complexes to epithelial cell junctions (Kourtidis et al. 2015) (Kourtidis & Anastasiadis, 2016). In the study we have used this information to develop testable hypotheses that will uncover key mechanistic insights into MVP pathogenesis at a cellular and molecular level.

All of this information taken together leads to the hypothesis: DCHS1-regulated miRNA processing stabilizes the valve endocardium.

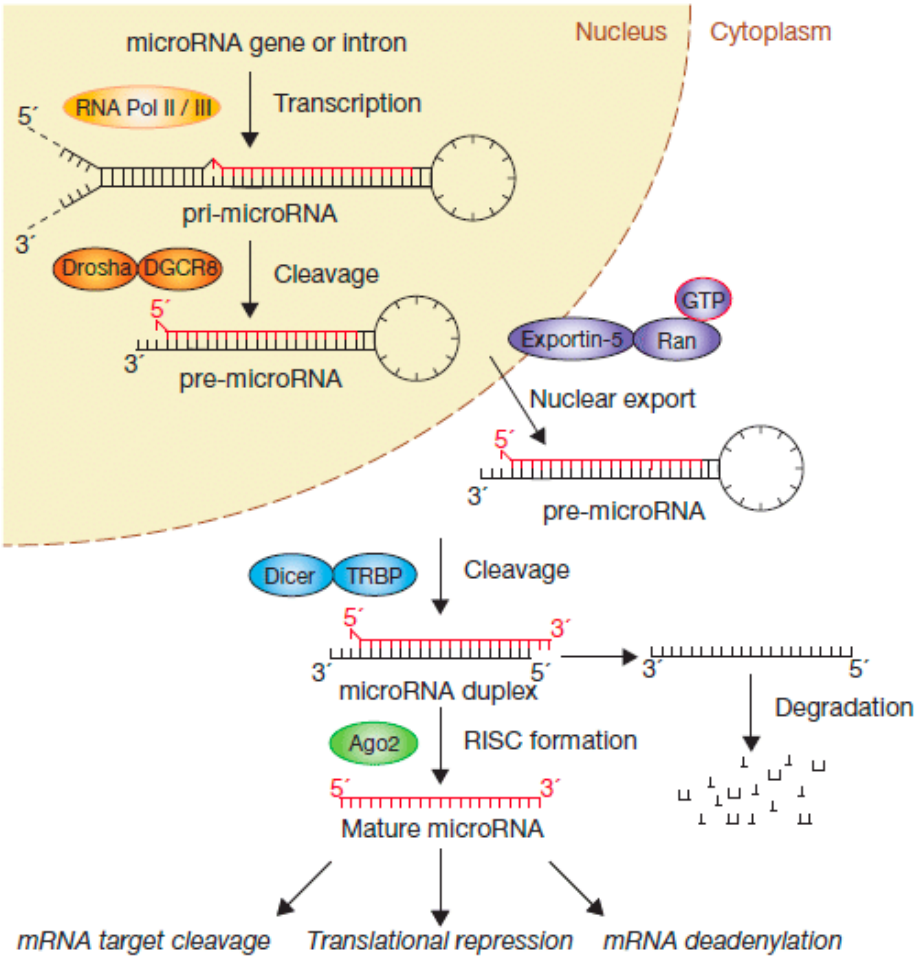

Figure 1.4 Overview of miRNA processing. miRNA maturation includes the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8 in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5–Ran- GTP. In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaut (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation, whereas the passenger strand (black) is degraded. (Tran et al. 2013)

CHAPTER 2: INTERACTIONS AND LOCALIZATION OF A NOVEL MEMBRANE TETHERED MIRNA PROCESSING COMPLEX

Introduction

MVP is one of the most common cardiac diseases and it is the most common cardiac valvular disease. MVP can occur as part of a recognized syndrome such as Marfan syndrome, characterizing it as syndromic MVP, or it can occur in isolation in which case it is called non-syndromic MVP. Although genetic causes for some syndromic forms of MVP are known, the genetic underpinnings of the more common non-syndromic form of MVP, which affects 2-3% of the population, have remained elusive. MVP occurs when the mitral valve leaflets disrupt steady blood flow from the left atrium to the left ventricle. This happens when either one or both of the leaflets (posterior and anterior) balloon upward into the left atrium.

Through the findings revealed in the Framingham Heart study, the diagnosis of MVP was fully understood, and scientists took a closer look into the genetics behind the disease. In 1999, researchers performed familial screens that identified the first locus associated with autosomal dominant MVP, MMVP-1. The locus is on chromosome 16p (Disse et al., 1999) and by performing several more screens that verified the heterogeneity of the disease, linkage analysis mapped to two loci. This was followed by more studies linking MMVP2 located on chromosome 11p15.4.

Additionally, in 2007, the gene Filamin A was found to be linked to MVP because the of its involvement in X-linked myxomatous valvular dystrophy (Le Tourneau et al., 2018; Toomer et al., 2019). These studies ignited researchers to delve more into this finding and search for more components that are responsible for MVP in patients. We now have compelling genetic and functional evidence that significantly advance our understanding of MVP pathogenesis. The gene *DCHS1* (Durst et al., 2015) is well-known

for its encoding of calcium-dependent cell-cell adhesion molecules. Previous studies within our lab found that mutations in DCHS1 cause MVP (Durst et al. 2015). Additionally, a genome-wide association study has led scientists to observe genetic variants within these MVP patients (Dina et al., 2015). However, patients with rare syndromic diseases that stem from alterations in the structure and function of primary cilia ("ciliopathies") have a higher prevalence of MVP, suggesting that primary cilia may play a broad role in disease etiology (Toomer et al, 2019).

Results

Genetic Studies

To date, there has been a short list of genes that are implicated in MVP, some of which are: *DCHS1, TNS1, LMCD1,* and *Filamin A-MVD*. Specifically, *DCHS1* mutations causes myxomatous valves. Furthermore, we studied the pedigree of three families with non-syndromic MVP to begin to identify crucial players involved with disease. Using a combination of linkage analyses as well as exome capture sequencing, our lab has identified the loss of function mutations in the cadherin gene, *DCHS1*, that segregates with MVP in multiple families (Figure 2.1). Patients with *DCHS1* missense mutations displayed autosomal dominant inheritance, which we have shown in multiple model systems to cause haploinsufficiency and disease phenotype (Durst et al. 2015). Disease inception was traced back to defects in valve morphogenesis.

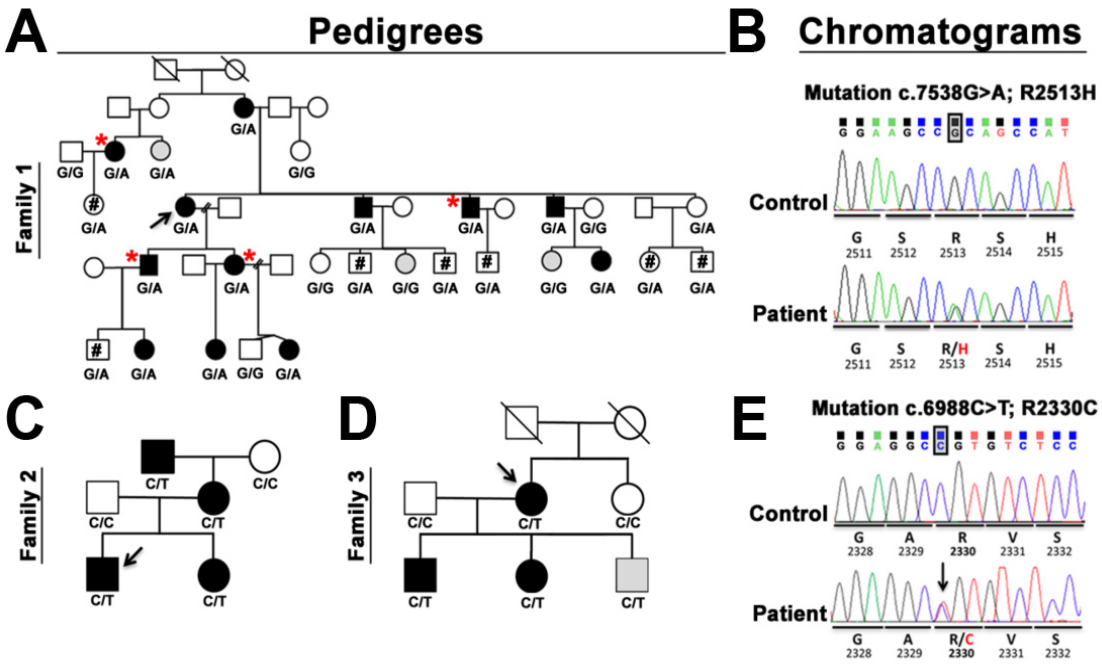

Figure 2.1 Pedigrees and mutation identification. (A,C,D) 3 families with inherited autosomal dominant non-syndromic MVP. Black shading=MVP in all pedigrees. Probands are indicated with an arrow. Mutations in *DCHS1* were identified in each of these families. *DCHS1* Genotypes: Family 1- c.7538G>A (p. R2513H), Family 2&3- c.6988C>T (p.R2330C). No genotype =individual was unavailable for study. **(B,E)** DNA sequence showing c.7538G>A or c.6988C.T *DCHS1* mutations

Two-Hybrid Screening

To gain an understanding of DCHS1 function in the mitral valves, we undertook an unbiased two-hybrid screen of a human heart cDNA library, using the company Hybrigenics. The two-hybrid screen was designed to establish direct protein-protein interactions by a simple bait-prey concept that examines the physical interactions of the protein in question. This technology can uncover various protein as well as DNA interactions that can lead to a network of integrated components. We used the DCHS1 protein as the "bait" to find interactions with the molecule. After screening 110 million clones with the cytoplasmic tail of human DCHS1 protein, we identified the RNA binding protein LIX1L (LIX1-Like) as the only interacting protein (Figure 2.2). Follow up co-IPs and additional two-hybrid screens defined LIX1L as interacting with a small region of the cytoplasmic tail of DCHS1. These finding were bolstered by additional reports showing that LIX1L interacts with DCHS1 in other model systems (Bando et al. 2011) (Mao, Kucuk, & Irvine, 2009).

Lix1L is an RNA-binding protein that has shown its ability to promote RNA expression (Nakamura, 2015). We hypothesize this occurs through an interaction with the microprocessor components DROSHA and DGCR8 (Kourtidis, 2017). DROSHA is a double-stranded RNA-specific endoribonuclease and is the enzymatic component of the microprocessor. DGCR8 is essential for delivering miRNAs to the complex.The complex then processes the initial miRNA transcripts called primary miRNAs (pri-miRNAs) into precursor miRNAs (pre-miRNAs), which are then fed into the Dicer-Ago2 complex to produce the mature miRNA products. Until recently, the microprocessor and several accessory components of the RNAi machinery were thought to localize solely in the

nucleus. However, this dogma was challenged by recent work, which revealed a functional microprocessor that is recruited by cadherin complexes to epithelial cell junctions (Kourtidis, 2017). This led us to our hypothesis that DCHS1-LIX1L interactions promote membrane tethering of the microprocessor (DROSHA and DGCR8), which clips pri-miRNA to generate pre- miRNA. pre-miRNA is further processed into mature miRNAs (Figure 2.3)

A

Protien Name	# of clones	Region	Confidence	Function
LIX1L	3	29-224	A	RNA BINDING
LIX1L	2	14-148	A	RNA BINDING
LIX1L	1	19-177	A	RNA BINDING
LIX1L	1	36-109	A	RNA BINDING

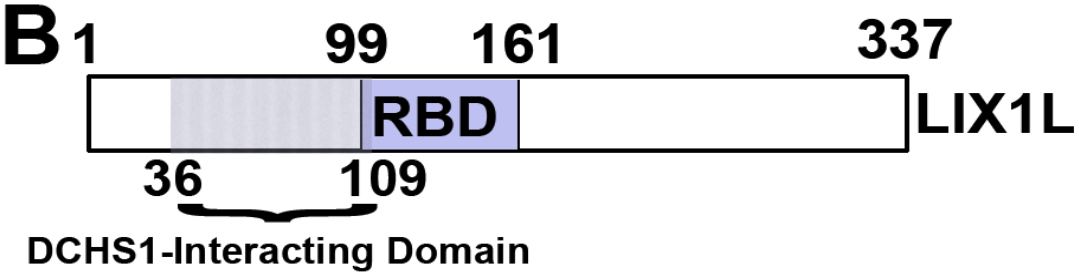

Figure 2.2 DCHS1 Interaction with LIX1L. Based on the information provided from the Hybrigenics two-hybrid screen **(A)** Number of LIX1L interacting clones identified and their regions (represented in amino acids). **(B)** Schematic depicting LIX1L, its RNA binding domain (RBD) and interaction domain with DCHS1 (amino acids 36-109, grey region)

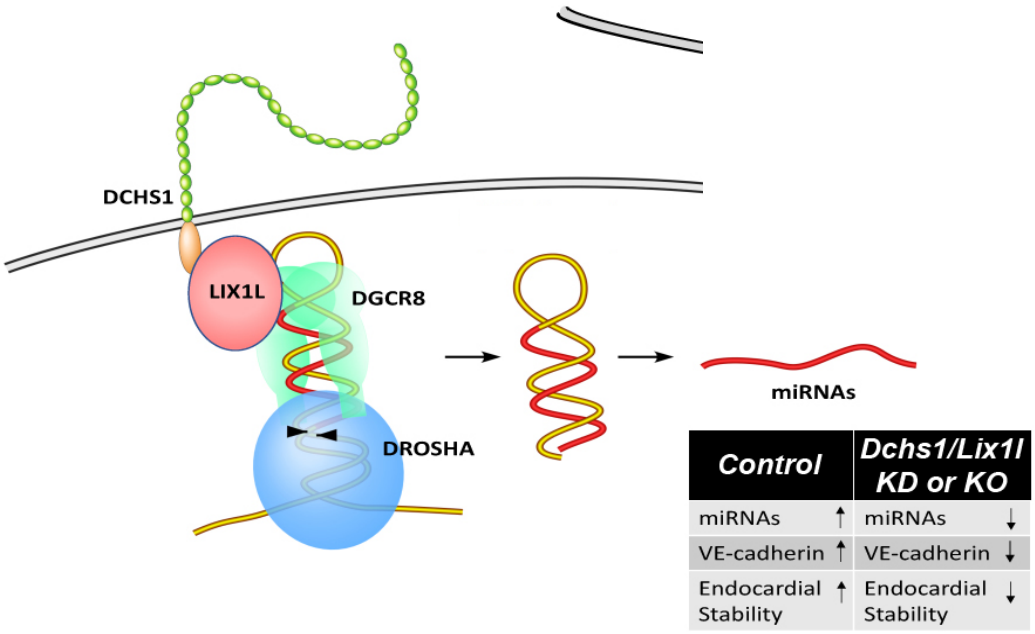

Figure 2.3 Working model of Dchs1-microprocessor interaction. Dchs1-Lix1L interactions promote membrane tethering of the microprocessor (DROSHA and DGCR8), which clips pri-mRNA (arrowheads) to generate pre-miRNA. pre-miRNA is further processed into mature miRNAs. We hypothesize that with a loss of DCHS or LIX1L, there will be a decrease in miRNAs associated with EMT suppression, as well as VE-cadherin, which leads to a decrease in endocardial stability.

Dchs1 and Lix1L

Expression studies, using immunohistochemistry (IHC), showing co-expression of Dchs1 with Lix1L in valve endocardium post-EMT morphogenesis were conducted. They were able to support our findings as DCHS1 and LIX1L are both only expressed in the VECs (Figure 2.4). Although not much is known about Lix1L function, previous work has shown its ability to bind to RNA and regulate miRNAs (Nakamura et al. 2015).

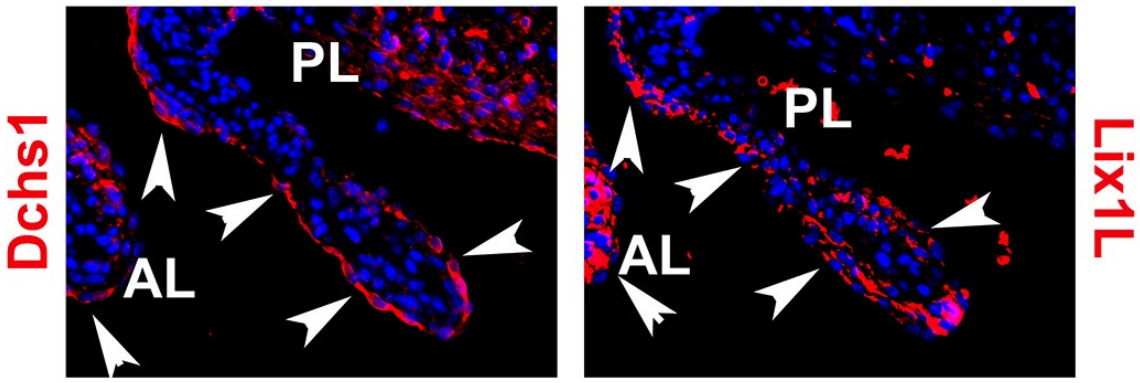

Figure 2.4 Expression of Dchs1 and Lix1L in mitral valves at P0. Dchs1 and Lix1L show significant overlap in their expression in the valve endocardium AL, PL= anterior and posterior mitral leaflets.

To further identify the relationship between DCHS1 and LIX1L we created a knockout mouse model, *Dchs1* Rosa NfatC1 enhancer cre (DRE), to study the effect of the deletion of Dchs1 on Lix1L. We found that with the deletion of DCHS1, LIX1L was also removed from the valve (Figure 2.5). In the wild type mice, DCHS1 and LIX1L were both expressed in the immunohistochemistry stains in the valve endothelial cells. They then are downregulated in the *Dchs1* knockout mice. This reinforces our findings from the two-hybrid screen that stated that DCHS1 and LIX1L interact because LIX1L is downregulated when DCHS1 is lost.

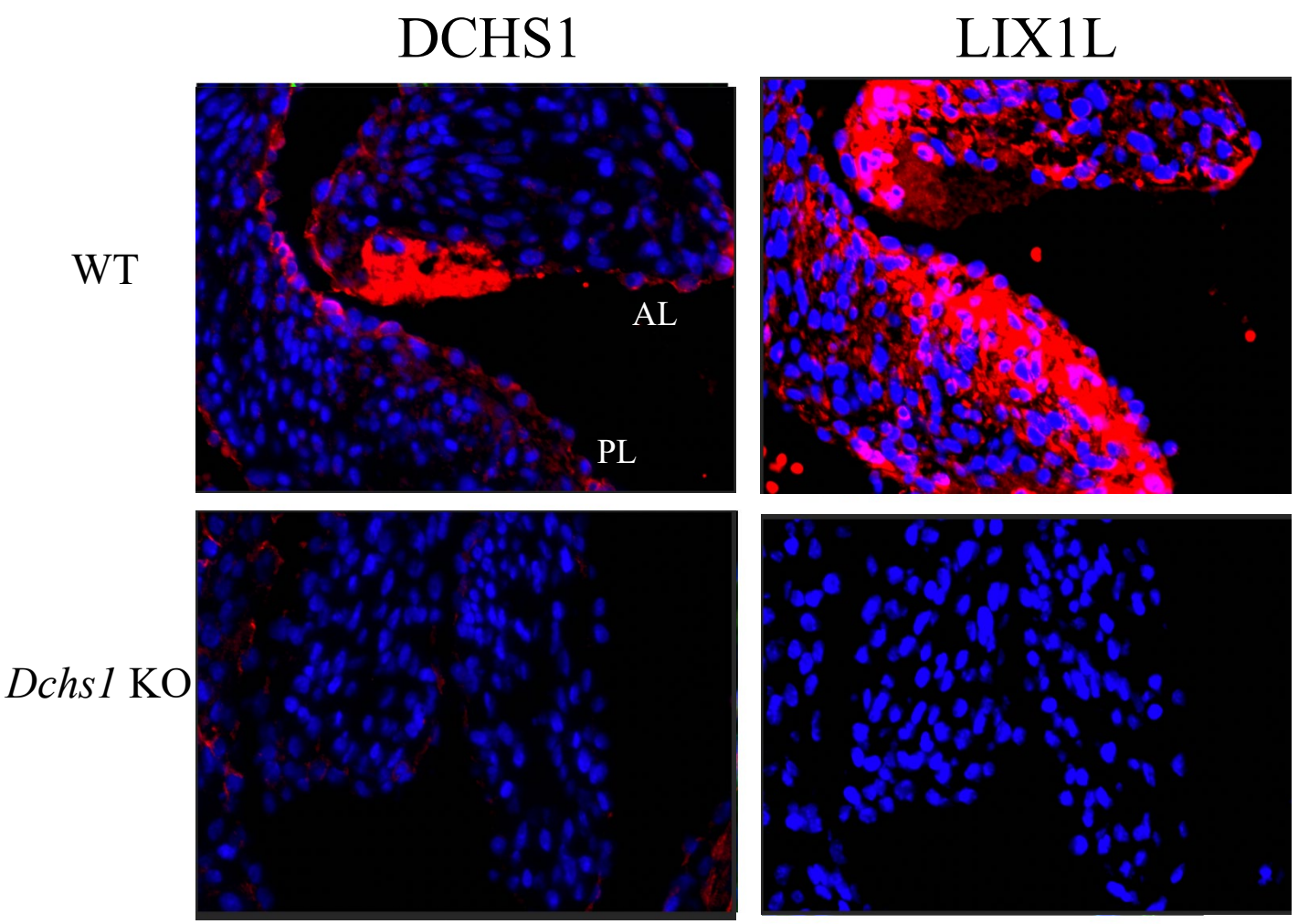

Figure 2.5: Expression of Dchs1 and Lix1L in mitral valves at P0 in wildtype and Dchs1 knockout mice. Expression of DCHS1 (red) in the first column is lost in the *Dchs1* KO mouse model in comparison to the wild-type. Expression of LIX1L (red) in the second column is lost in the *Dchs1* KO mouse model in comparison to the wild-type model. These results further confirm the results of the two-hybrid screen. nucleus (Blue) was also stained to confirm the results in endothelial cells. Images were taken at 40x.

DCHS1, LIX1L, and the Microprocessor

As previously stated, our hypothesis is DCHS1 binds to LIX1L, which binds to the microprocessor (DROSHA and DGCR8). To support this theory, DGCR8 and DROSHA were stained in wild-type E14 and P0 mice. We observe both DROSHA and DGCR8 concentrated at the valve endocardial cell membrane with very little expression evident within nuclei of VECs or VICs during fetal or neonatal timepoints (Figure 2.6). This pattern of expression mirrors that of DCHS1 and LIX1L at P0 (compares Figure 2.4 and 2.6).

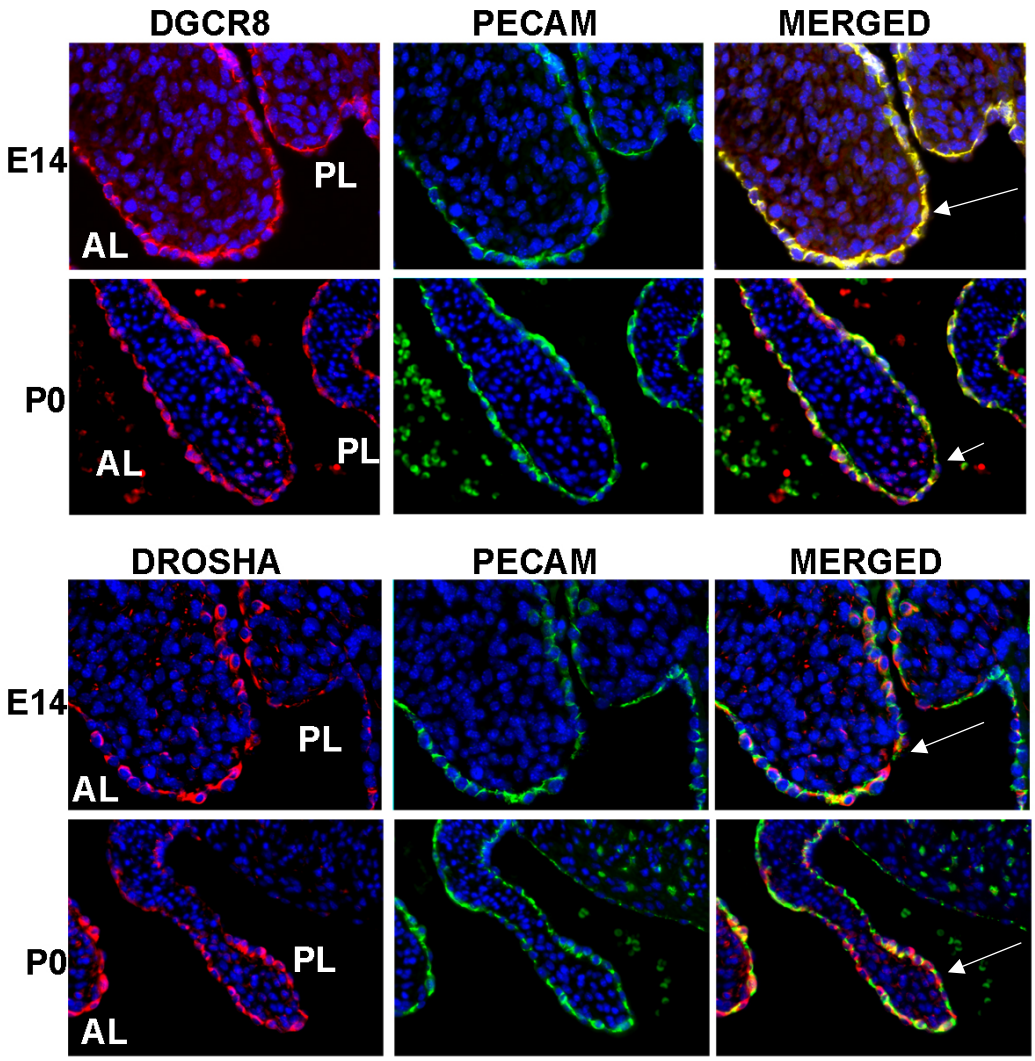

Figure 2.6 Expression of the microprocessor components in the mitral valve. IHC for DGCR8 DROSHA and PECAM at E14.5 and P0. Significant overlap of membrane stained PECAM with the microprocessor in the valve endocardium is observed (arrows). Scant or no expression for this complex was observed in the valve interstitial cells.

Discussion

Though the genetics behind MVP are still developing, the beginning of fully understanding which genes cause this disease are examined through our studies. The mechanism by which *DCHS1* causes MVP is still not completely understood but as we take a closer look at DCHS1, LIX1L, and the microprocessor we begin to form a broad hypothesis that mechanisms from *DCHS1* mutations result in MVP are directly related to LIX1L and the microprocessor.

These studies illustrate that interactions between DCHS1, LIX1L and the microprocessor are all present in the VECs in the wild-type mice, which leads us to believe that there may be interaction between them. With a mutation that results in a loss of DCHS1, LIX1L is lost as well, and this gives us a broad idea of how they are interacting, and that the mutation is significantly affecting the stability of LIX1L. We believe that the cadherin protein DCHS1, interacts with RNA binding protein LIX1L, which then interacts with the microprocessor components DROSHA and DGCR8, which affects endocardial stability.

Further experiments are needed to firmly understand these interactions. In particular, studies where *Lix1L* is knocked down in both a mouse model and a cell model should be conducted to confirm that loss of LIX1L wouldn't result in a loss of DCHS1 should be conducted. Further studies looking at the localization of the microprocessor components DROSHA and DGCR8 after the loss of DCHS1 to determine if a DCHS1 mutation leads to loss of the microprocessor should also be conducted. Cell culture studies using VECs would also be beneficial to this research to be able to confirm the location of these proteins.

CHAPTER 3: DCHS1 REGULATES MIRNA PROCESSING AND STABILIZES THE ENDOCARDIUM THROUGH MIRNA PROCESSING

Introduction

Processing of pri-miRNAs to pre-miRNAs is an essential step in miRNA biogenesis and is mediated by the microprocessor (Figure 2.3). Impaired function of the microprocessor results in attenuated processing of pri-miRNAs and in decreased levels of pre-miRNAs and their mature miRNA products. We have identified a set of downregulated mature miRNAs – miR-200c, let7e, let7g, and miR-24 – based on information from miRbase that showed that these miRs are involved in EMT suppression, in both the DCHS1 and LIX1L knockdown endothelial cells. We propose that DCHS1 interacts with the microprocessor through LIX1L to regulate the processing of a subset of miRNAs because: (I) there is a molecular link between DCHS1 and LIX1L; and (II) LIX1L is a RNA-binding protein that regulates miRNAs (Nakamura et al. 2015).

miR-200c belongs to the miR-200 family, which is believed to play an essential role in tumor suppression by inhibiting EMT (Korpal and Kang, 2008). If we are to incorporate this miR into our hypothesis it is likely that a decrease in DCHS1 leads to a decrease in LIX1L which leads to a decrease in the mislocalization of microprocessor components Drosha and Dgcr8, which would lead to decreased mir-200c, leading to increased EMT and therefore decreased endocardial stability.

miR-Let7e and miR-Let7g have been shown to be downregulated in congenital heart disease and to target HMGA, which promotes EMT (Lee and Dutta, 2007). If we incorporate these miRs into our hypothesis it is likely that a decrease in DCHS1 leads to a decrease in LIX1L which leads to a mislocalization microprocessor components Drosha and Dgcr8, which leads to a decrease in miR-Let7e and miR-Let7e, which then increases HGMA, leading to increased EMT and decreased endocardial stability.

miR-24 has been found to be suppressed by TGF-B signaling (Sun et al., 2015). TGF-B can induce EMT, and therefore it is likely that this miR would decrease in the case of aberrant EMT . If we are to incorporate this miR into our hypothesis it is likely that a decrease in DCHS1 leads to a decrease in LIX1L which leads to a mislocalization components DROSHA and DGCR8, which would lead to decreased mir-24, leading to increased EMT and therefore decreased endocardial stability.

Results

Expression Study

An expression study was conducted to compare the DGCR8 and DROSHA expression in wild-type and *Dchs1* knockout mice. The results from this study show that a mutation in the *Dchs1* gene leads to disrupted polarity in the VEC cells as well as diffuse CD31 expression, which is indicative of junctional defects (Figure 3.1). There is a near total loss of membrane polarization of the microprocessor components, meaning they dissociate from the membrane. The results also indicate mislocalization of the microprocessor and potentially decreased functionality of the complex. This supports our theory that a mutation in *Dchs1* leads to impaired function of the miRNA processor.

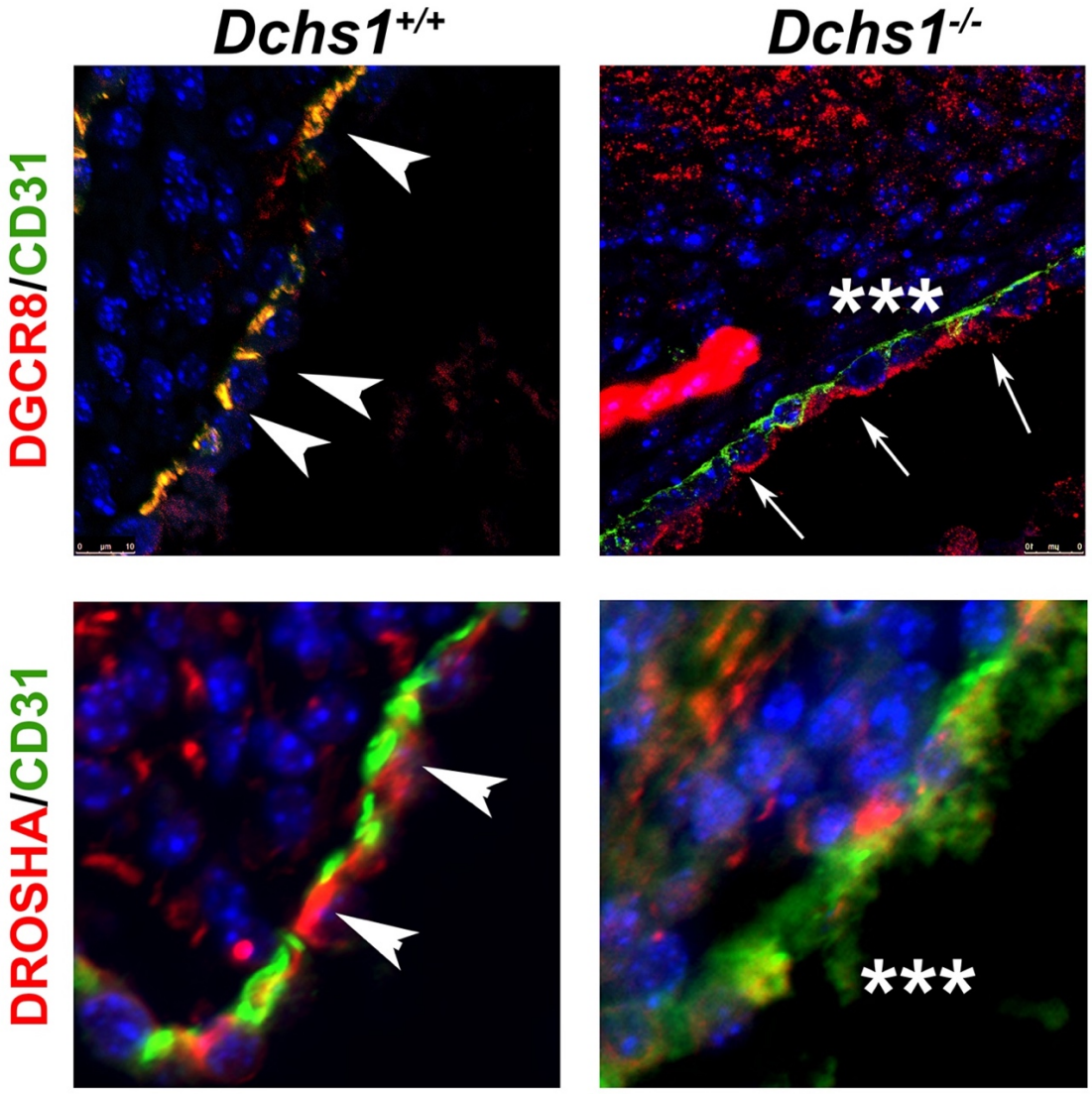

Figure 3.1 DCHS1 Regulates localization of RNAi proteins at E15.5. IHC of mitral valves from control (*Dchs1*$^{+/+}$) and KO (*Dchs1*$^{-/-}$) mice for DROSHA and DGCR8. In KO mitral valves, DGCR8 and DROSHA polarity is disrupted (arrows). There is near total loss of membrane polarization of the microprocessor. Asterisks show diffuse CD31 expression (green), indicative of junctional defects. Hoescht (blue) is the nuclear stain.

qPCR

To confirm our hypothesis that miR-200c, miR-Let7g, miR-Let7e, and miR-24 were affected by a mutation in *Dchs1*, qPCRs where conducted to show the difference in amount of these miRs in controls versus *Dchs1* knockdowns and *Lix1L* knockdowns in human umbilical vein endothelial cells (HUVECs) (Figure 3.2 and 3.3). The results show significant differences between the control to the *Dchs1* knockdown and an even greater decrease from the control to the *Lix1L* KD. This supports the hypothesis that *Dchs1* mutations lead to mislocalization of the microprocessor and therefore potentially decreased functionality of the complex.

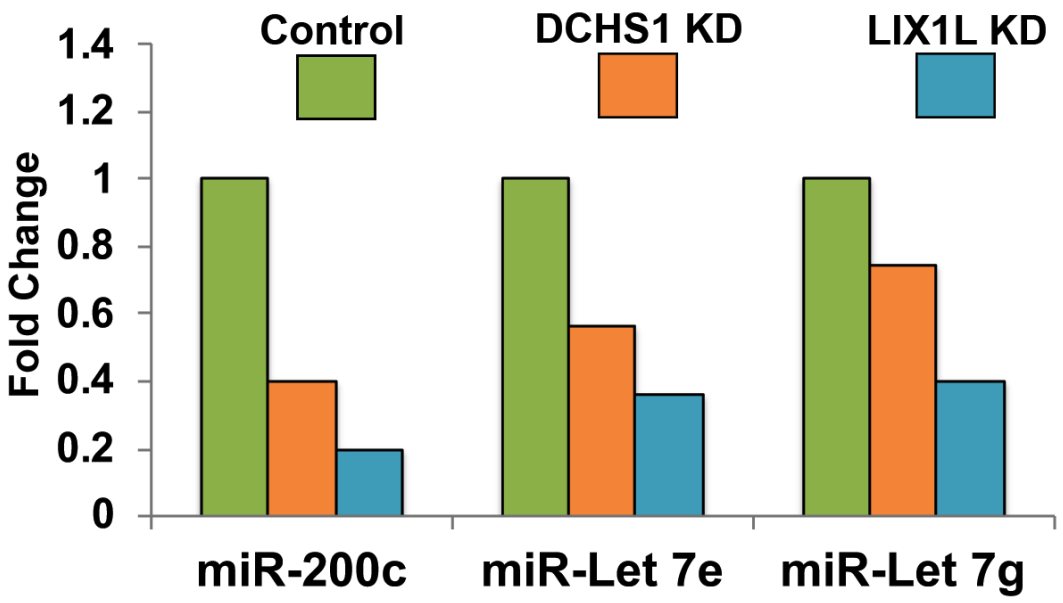

Figure 3.2: DCHS1 and LIX1L regulate miRNA expression of miR-200c, miR-Let7e, and miR- Let7g. qRT-PCR showing miR-Let7e, miR-Let7g and miR-200c are downregulated in *Dchs1* and *Lix1L* KD endothelial cells. There is a % fold decrease from the control to the *Dchs1* KD and an even greater decrease from the control to the *Lix1L* KD. This supports the hypothesis that *Dchs1* mutations lead to disruptions in miRNA-processing and the hypothesis that LIX1L binds to the microprocessor and promotes miRNA processing

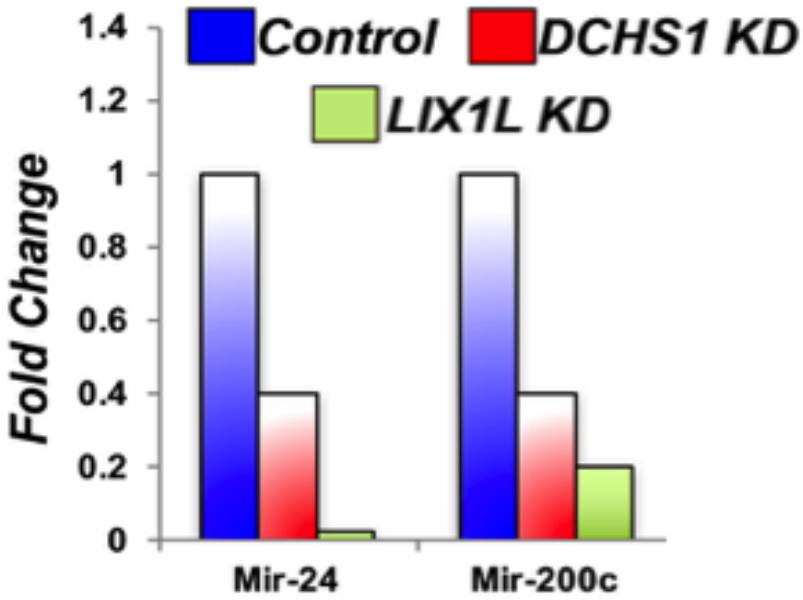

Figure 3.3 DCHS1 and LIX1L regulate miRNA expression of miR-200c and miR-24

qRT-PCR showing miR-24 and miR-200c are downregulated in *Dchs1* and *Lix1L* KD endothelial cells. There is a % fold decrease from the control to the *Dchs1* KD and an even greater decrease from the control to the *Lix1L* KD. This supports the hypothesis that Dchs1 mutations lead to disruptions in miRNA-processing and the hypothesis that LIX1L binds to the microprocessor and promotes miRNA processing.

VEC stability through miRNAs

Mechanisms regulating valve endocardial stability post-EMT have previously been unknown. Failure to stabilize the endocardial epithelium can cause increased EMT and permeability of the endothelial barrier, or the ability to allow molecules in and out of the cell, which leads to defects that are associated with valvular heart disease (Lamouille, Xu, Derynck, 2014). This hypothesis is supported by our previously explained data (I) DCHS1, LIX1L and the microprocessor are primarily expressed by VECs during neonatal and adult timepoints. (II) We have identified miR-200c and miR-24 levels to be significantly decreased upon loss of DCHS1 and/or LIX1L (Figures 3.2 and 3.3). (III) Reduced expression of these miRNAs was anticipated as resulting in an increase in its main target, ZEB-2, a master regulator of EMT and a transcriptional repressor of VE-cadherin.

Western Blot

A western blot was conducted to determine if mutations in Dchs1 and Lix1L resulted in an increase to ZEB2, a master regulator of EMT and a transcriptional repressor of VE-cadherin (Figure 3.4). Array data from our *Dchs1* KO mice and our *Dchs1* KD cells revealed an increase in ZEB-2 coincident with *Dchs1* and *Lix1L* knockdown VECs showing reduced VE-cadherin and increased ZEB2. This supports the hypothesis that reduced expression of DCHS1 and LIX1L result in an increase in ZEB2 and a decrease in VE-Cadherin, and therefore an increase in EMT, and a decrease in endocardial stability

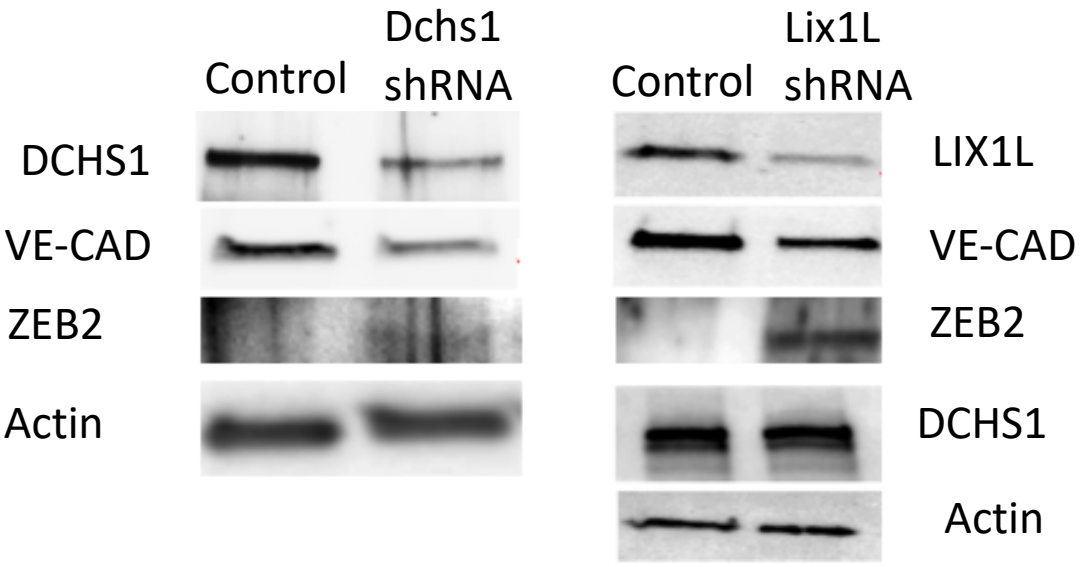

Figure 3.4 Knockdown of DCHS1 and LIX1L: Human VECs were infected with DCHS1 **(A)** or LIX1L **(B)** shRNA lentiviruses and put under selection. Western analyses show that stable cell lines exhibit a reduction in VE-Cadherin and diminished expression of DCHS1 and LIX1L. The transcriptional repressor of VE-cadherin, ZEB2 is increased in both the *Dchs1* and *Lix1L* KD cells. *Lix1L* knockdown has no effect on DCHS1 expression.

Expression Study

An expression study was conducted to determine if *Dchs1* knockout E15.5 mice have diffuse expression of CD31 and therefore decreased stability in the endocardium (Figure 3.5). The results show *Dchs1* knockout mice have diffuse expression of CD31, which is consistent with altered endocardial structure and stability, and poorly established junctional connections. It is also apparent that CD31 is observed within the *Dchs1* knockout fetal valve interstitium which indicates aberrant EMT.

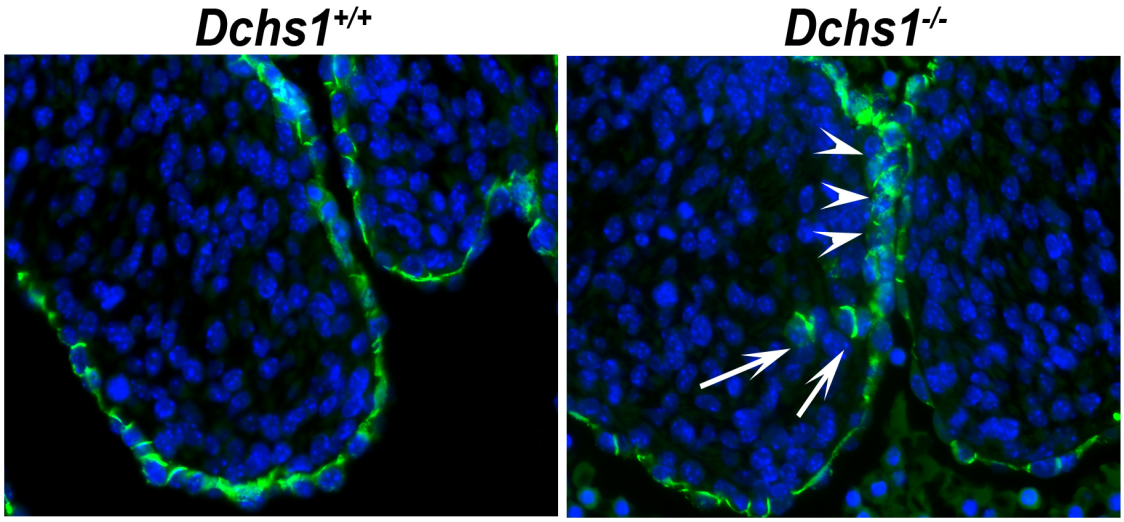

Figure 3.5 Evidence for increased EMT in DCHS1 valves: Invading PECAM positive cells were observed in the *Dchs1*$^{-/-}$ (arrows) valves at E15.5, indicative of aberrant EMT. PECAM staining appears disorganized (arrowheads), indicative of poorly established junctional connections.

Discussion

With each of our findings, we come closer to understanding the role that DCHS1 and LIX1L play in the disease genotype of MVP. Our significant findings indicate that loss of DCHS1 and LIX1L likely affect the microprocessor, and therefore miRNA biogenesis. Our studies show that losses of DCHS1 and LIX1L lead to a decrease in miRNAs that suppress EMT. This supports our hypothesis that loss of DCHS1 leads to loss of endocardial stability through an increase of aberrant EMT. Further studies should be completed to show that the decrease in miRNAs related to EMT suppression is greater than that of miRNAs that are unrelated to EMT. We were able to mark aberrant EMT in our knockout *Dchs1* mice valves, however, lineage tracing using GFP and NfatC1-enhancer cre, a cre line that only affects endocardial cells that do not undergo EMT, should be conducted to confirm the aberrant EMT. Though there is more work to do, our studies have laid the groundwork for future experiments and expanded the knowledge regarding the mechanisms behind *DCHS1* in MVP patients.

CHAPTER 4: OVERALL DISCUSSION

Overall Discussion

Mitral valve prolapse is one of the most common forms of cardiac disease, affecting approximately 1 in 40 people worldwide. Prolapsed valves result in regurgitation, arrhythmias and even sudden cardiac death in serve cases. The phenotype of a mitral valve prolapse, includes vales with myxomatous degeneration, that results in an increase in proteoglycans and fragmented collagen and hyperplasia

Currently, the only treatment for mitral valve prolapse is invasive surgery to replace or repair the valve. This can be both dangerous and expensive for the patient. While the disease is not completely understood, studies from our lab suggest that the cadherin protein DCHS1 plays a critical role in MVP.

Our genetic studies have revealed temporal-spatial expression patterns for the gene, Dchs1, in relationship to MVP. Using linkage and capture sequence of the linkage interval, our lab has identified loss of function mutations in the cadherin gene, *DCHS1*, which segregates with MVP in several families. Patients with *DCHS1* missense mutations displayed autosomal dominant inheritance, which causes haploinsufficiency and disease phenotype (Durst et al. 2015). The disease origin was traced back to defects in valve morphogenesis. This data revealed that *DCHS1* mutations are involved in MVP and emphasize the importance for defining its role(s) in cardiac development and disease. DCHS1 expression becomes restricted to VEC's after birth suggesting a supposed role for this protein in stabilizing the valve endocardium. These data are interesting and imply unique roles for DCHS1 in VECs as the endocardial cells begin to end EMT.

To gain an understanding of DCHS1 function in the mitral valves, we undertook a two-hybrid screen of a human heart cDNA library. After screening 110 million clones

with the cytoplasmic tail of human DCHS1 protein, we identified the RNA binding protein LIX1L as the only interacting protein. We used this information to conduct our studies testing for the interaction of these two proteins.

Our studies have supported the data showing that DCHS1 and LIX1L interact. Through immunofluorescence studies we see that DCHS1 and LIX1L are expressed in only the valve endothelial cells. We also found that with a loss of DCHS1 in our *Dchs1* knockout mice, there was a loss of LIX1L as well. These data help to suggest the link between DCHS and LIX1L. We also studied the link between the genes Dchs1 and *Lix1L* with the microprocessor components DROSHA and DGCR8, since LIX1L has been confirmed to bind to RNA and regulate miRNA levels. Our expression study showed that expression of the microprocessor is restricted to the VECs which is consistent with the expression of DCHS1 and LIX1K. This, as well as localization of to the membrane provides evidence for a link between DCHS1, LIX1L, and the microprocessor.

It has also been well-established that cadherins stabilize cell-cell junctions and propagate downstream signaling cascades through their cytoplasmic domains to regulate tissue morphogenesis (Maitre & Heisenberg, 2013). Recent reports have shown that cells stabilize their junctions and suppress EMT through a mechanism that involves cadherin proteins recruiting miRNA processing complexes to the membrane (Kourtidis et al, 2017) (Kourtidis & Anastasiadis, 2016) (Kourtidis et al., 2015). RNA localization as a method of energy conservation and regulation of protein signaling has changed to allow molecular and cellular changes to modifications. (Niessing, Jansen, Pohlmann, & Feldbrugge, 2018) (Jansen, Niessing, Baumann, & Feldbrugge, 2014).

The microprocessor catalyzes the first step of miRNA biogenesis through its core members DROSHA and DGCR8 (Gregory et al. 2004) (Ha & Kim, 2014). DROSHA is a double stranded RNA-specific endoribonuclease and it is the enzymatic component of the microprocessor. DGCR8 is essential for delivering miRNAs to the complex. The complex then processes the initial miRNA transcripts called primary miRNAs (pri-miRNAs) into precursor miRNAs (pre-miRNAs), which are then fed into the Dicer-Ago2 complex to produce the mature miRNA products. Until recently the microprocessor and several accessory components of the RNAi machinery were thought to localize solely in the nucleus (Ha & Kim 2014). However, this dogma was challenged by recent work, which revealed a functional microprocessor that is recruited by cadherin complexes to epithelial cell junctions (Kourtidis et al. 2015) (Kourtidis & Anastasiadis, 2016).

We used this information to develop experiments to test DCHS1 regulation of miRNA processing and stabilization of the endocardium through miRNA processing. Using an expression study, we showed that loss of DCHS1 in mice leads to disrupted polarity and junctional defects, supporting our theories. We then conducted qPCR to determine if loss of DCHS1 and LIX1L leads to a decrease of miRs related to EMT suppression. We saw that each of our selected miRs had a decrease between the control and the *Dchs1* knockout models. There was an even greater decrease from control to the *Lix1L* knockout model. These provides information to support the idea that a mutation that results in a loss of Dchs1 affects both miRNA processing and EMT.

Finally, we developed experiments to test VEC stability through miRNAs. Failure to stabilize the endocardial epithelium can cause increased EMT and permeability of the endothelial barrier, defects that have been associated with valvular heart diseases.

Reduced expression of these miRNAs was anticipated as resulting in an increase in its main target, ZEB2, a master regulator of EMT and a transcriptional repressor of VE-cadherin.

We conducted a western blot to determine if there was a change in ZEB2 with the losses of DCHS1 and LIX1L in a knockout model. We saw an increase in ZEB2 and a decrease in VE-cadherin, which was consistent with our hypothesis, and a possible cause for increased EMT in the cells, and therefore decreased cell stability leading to MVP. An expression study to determine if *Dchs1* knockout mice at E1.5.5 have diffuse expression of CD31 and therefore decreased stability in the endocardium was conducted. The results showed diffuse expression of CD31, which is consistent with altered endocardial structure, stability, and poorly established junction.

Future studies should be conducted to further define the mechanism by which *Dchs1* mutations cause MVP. The hope is that this information can be used in the future to develop therapies to treat this common but life threatening condition. The data provided gives a better understanding of the cellular and molecular cause of MVP as it is related to DCHS1, LIX1L, and the microprocessor.

CHAPTER 5: FUTURE DIRECTIONS

Interactions and localization of a novel membrane tethered- miRNA processing complex in vitro in HuVECs.

We tested the hypothesis that DCHS1 is required for membrane tethering of the RNAi microprocessor components DROSHA and DGCR8 through its interaction with LIX1L using an in vitro approach. Unfortunately, we were unable to achieve this data with human valve endothelial cells (HuVECs) as they proved difficult to grow, maintain, and infect, based on our unsuccessful trials as the cells are primary. We were unable to achieve knockdown cells. In the future, this experiment should be attempted again. Western blot analyses of membrane and total cell preparations from each cell line should be used to quantify the subcellular levels of microprocessor proteins (DROSHA and DGCR8). Additionally, confocal microscopy should be paired with 3D immunocytochemistry (ICC) to identify whether apical/basal polarity of the complex is altered in the knockdown cells. Confocal data will be used to calculate the *Manders coefficient* for co-localization of microprocessor complex at WGA-stained membranes as we've shown previously. To validate a direct effect of the DCHS1-LIX1L complex on localization of the microprocessor, rescue experiments should be performed. Electroporation of an epitope-tagged mouse full-length DCHS1 construct (which will not be downregulated in the DCHS1-shRNA stable cells) or a construct that lacks the Lix1L interaction motif into DCHS1 KD VECs should occur. Reciprocal experiments should be performed by electroporating an epitope-tagged full length murine LIX1L construct or one that lack the DCHS1 interaction domain into our human LIX1L KD VECs. Both constructs will have an iRES-eGFP insert for ease of visualizing transfectants. Quantification of western analyses from membrane preparations and ICC should determine whether re-expression of

these mutant proteins (or control full length proteins) can rescue expression levels and membrane localization of the microprocessor.

Co-immunoprecipitation

If the binding of DCHS1 to LIX1L is required for interaction with the microprocessor, then immunoprecipitating DCHS1 should also "pull-down" the microprocessor complex and be visualized by Western blotting. To ensure specificity, we should perform reversible cross-linking of the complex. This should initially be tested in our control human VECs to prove the interaction occurs. Following successful interaction results, we should pull down DCHS1 in LIX1L KD cells as well as in peptide treated cells and probe for the complex. If DCHS1 requires LIX1L as an intermediate to interact with the microprocessor, the interaction between DCHS1 and the complex should be impaired/lost in the KD or peptide treated cells. Rescue experiments (described above), should be performed once we confirm these interactions.

Dchs1 regulation of miRNA processing in vitro

The hypothesis is that DCHS1 interacts with the microprocessor through LIX1L to regulate the processing of a subset of miRNAs. This will be conducted *in vitro*, with HuVECs to determine the effects of the Dchs1 regulation of miRNA processing on cell to cell interactions. To establish that the processing of these miRNAs is indeed impaired at the level of the microprocessor, we should examine the levels of the pri-miRNA and the pre-miRNA of miRNAs: miR-24 and miR-200c by qPCR in DCHS1 and LIX1L KD VECs, DCHS1 peptide treated VECs and in KD VECs that have been rescued by Lix1l or

Dchs1 constructs. RNA-Crosslinking and Immuno-Precipitation (RNA-CLIP) experiment will also be performed. Using this approach, control VECs should be fixed in low Paraformaldehyde (PFA) dilution to covalently, but reversibly, crosslink RNA-protein complexes, which will then be immunoprecipitated with a DCHS1-specific antibody under stringent conditions. This experiment should: (I) confirm binding of DCHS1-regulated pri-miRNAs (miR-24, miR200c) to a DCHS1-LIX1L-microprocessor complex; and (II) identify the entire DCHS1-RNA target repertoire. DCHS1 RNA-CLIP and DROSHA RNA-CLIP will also be performed in control, LIX1L KD and peptide treated cells to determine whether DCHS1-LIX1L interactions are critical for recruitment of these miRNAs to DCHS1 and/or DROSHA to enable miRNA processing.

Dchs1 regulation of miRNA processing in vivo

In the future, tests to determine DCHS1 regulation of miRNA processing in vivo will be conducted. The hypothesis is that DCHS1 interacts with the microprocessor through LIX1L to regulate the processing of a subset of miRNAs. This would be conducted by isolating RNA from mitral valve leaflets to further determine if loss of endocardial DCHS1 and/or LIX1L results in dysmorphic valves and that there is a decrease in miRs related to EMT. Those miRNAs that are identified as being mis-processed will be validated *in vivo* by isolating RNA from anterior mitral valve leaflets at E12.5, the likely start of EMT, and/or P0 and performing qPCR for each specific target. The models to be used initially will be the global DCHS1 and LIX1L KO, heterozygote and compound mutant mice. However, we expect that the microprocessor complex is primarily restricted to valve endocardium after EMT, at which point we will observe mislocalization of DROSHA and DGCR8 in the *Dchs1* KO mice. miRNA processing defects in the valve endocardium will

be assayed in conditional het, KO and compound *Dchs1*/*Lix1L* mice using the Nfatc1-Enhancer-Cre, which is only active in Post-EMT endocardial cells. Laser capture of valve endocardium will be performed via standard methods, and qPCR of our previously selected miRNAs (mir-200c, Let-7e, Let7g, and miR-24) will be performed with controls being 5S rRNA and U6 snRNA. Quantification of mitral valve geometry (volume, surface area, length and width) will be performed to determine if loss of endocardial *Dchs1* and/or *Lix1L* results in dysmorphic valves as anticipated. Quantification of aberrant EMT can be investigated in vivo by generating lineage trace models NfatC1-enchancer-cre Rosa-GFP mice, in the conditional *Dcsh1* and *Lix1L* floxxed mice at P0 as well as by performing EMT assays. Once we identify that a compromised endocardium, our studies will evolve to include these studies. Additionally, experiments aimed at identifying direct or indirect roles of ZEB2 will be developed if we demonstrate involvement of this transcriptional repressor in endocardium stability.

DCHS1 stabilizes the endocardium through miRNA processing

The goal of this experiment should be to identify whether loss of DCHS1-LIX1L interactions reduce VEC stability through miRNAs. This hypothesis is supported by these findings: DCHS1, LIX1L and the microprocessor are primarily expressed by VECs during neonatal and adult timepoints, our previously identified miRs, reduced expression of these miRNAs, DCHS1 KO mice diffuse expression of CD31, and CD31 observed on cells within the *Dchs1* KD fetal valve interstitium, indicating aberrant EMT. We suspect this is a result in an increase in its main target, ZEB2, a master regulator of EMT and a transcriptional repressor of VE-cadherin. This can be tested using proteins collected from

mouse valve samples and a western blot to examine if there is increased expression of ZEB2 in the in vivo model.

To test whether the VEC monolayer is compromised, we should initially perform trans-endocardial resistance (TER) on control, DCHS1 and LIX1L knockdown VEC lines using the ECIS® (Electric Cell-substrate Impedance Sensing) system. Rescue experiments should be performed with the goal of restoring levels of miRNAs using miR mimics as well as wildtype constructs for DCHS1 and/or LIX1L in both knockdown cell lines. Anti-miRs (a-miRs) targeting miR-24 and miR-200c in wild-type VECs should also be tested to examine whether these phenocopy the potential TER defects in DCHS1 and LIX1L KD cells. Our selected miR levels will be quantified by qPCR and VE-cadherin, CD31 and ZEB2, will be quantified by qPCR, Western and ICC to determine if the impedance measurements are a function of reduced VE-cadherin and CD31 driven by the transcriptional repressor ZEB2 in a DCHS1, LIX1L, miR-dependent mechanism.

Additional controls for our studies should test if transfection of mutant DCHS1 constructs that impair LIX1L interactions or the DCHS1 peptide, which targets the LIX1L-DCHS1 interaction fail to rescue potential differences in TER. This control experiment should test the function of the DCHS1 cytoplasmic tail in regulating endocardial stability through miRs while maintaining extracellular DCHS1 interactions.

CHAPTER 6: MATERIALS AND METHODS

Mouse studies

All mouse studies 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

Dchs1 knockout mice (DT mice)

To acquire knock out DT mice, a male heterozygote and a female heterozygote, both for Dchs1 were bred, from our vivarium. This breeding occurred for several generation to achieve wildtype, heterozygous, and knock out Dchs1 mice.

Dchs1 Rosa nfatc1 enhancer-cre mice (DRE mice)

To acquire DRE mice, a doubly floxxed Dchs1female mouse was bred with a male Rosa positive NfatC1 enchancer-cre positive male mouse. The mice were bred for several generations to achieve wild type, heterozygous, and conditional knockouts.

Histology and Immunohistochemistry

Embryonic and neonatal tissues were processed for immunohistochemistry (IHC) from D and DRE (wildtype, heterozygous, and homozygous). IHC staining was performed on 5 um thick sections from stages: E15.5, E19, and P0 mitral valves. For 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 primary antibodies and their dilutions: Novus Dchs1 1:500, Novus Lix1L, 1:100, abcam Drosha 1:100, Sigma Actin 1:500, Dianova Pecam 1:50, abcam GFP 1:500, DSHB MF20 1:100, Novus Zeb1 1:500, Thermofisher Ve-Cadherin 1:100, and Sigma Dgcr8 1:100. Primary antibody was detected using the following fluorescent secondary antibody at 1:100 dilution: goat anti-mouse 568, goat anti-rabbit 568, goat anti-chicken 568, donkey anti-rat 488, and donkey anti-mouse 488. Nuclei were counterstained with Hoescht (Life Technologies, Cat #H3569, 1:10,000) for 10 minutes and slides were cover-slipped with SlowFade mounting medium (Life Technologies, Cat#S36937). Fluorescence imaging was performed using Ziess Axio Imager.

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.

Cell Culture

HUVEC (human umbilical vein endothelial cells) were acquired from the Kourtidis Lab. HuVEC (Human Valve endothelial cells) were shared by the Bischoff Lab at Boston Children's Hospital. Both cell lines were cultured in a 37 dress celcius, 5% CO2

incubator, in their respective media. HUVEC media was EGM-2 from Lonza and HuVEC media was an EGM-2 Endothelial Cell Growth Medium-2 Bullet Kit from Lonza, with 10% heat-inactivated fetal bovine serum, and 1% GPS. The Bullet Kit includes EGM-2 and Singlequots of bFGF, VEGF, EGF, IGF-1, and ascorbic acid. The media was filtered before the addition of the Singlequots Hydrocortisone was removed from the bullet kit. The HuVEC media was aliquoted into 50 mL tubes and frozen to keep the media fresh. Media on the cells were changed every 2-3 days and cells were passaged by using EDTA trypsin when plates were approximately 80% confluent.

ShRNA infection

To begin shRNA infection, the cells were plated in 60 mm plates. The cells were approximately 40% confluent the day of the infection. There was an extra plate to use as the non-infected control. Once the cells were 40% confluent, medium was replaced in each place with 2 mls of fresh medium. Polybrene was added at 8ug/ml final (1:1000 from a 8mg/ul stock). Plates were swirled to mix polybrene and leave in the hood for 3-5 minutes. 0.5 ml virus was added and incubated O/N at 37°C. 24 hours post infection media was changed, and the cells were split if they were reaching 80-90% confluency. 48 hours post infection, the antibiotic selection with puromycin began, the amount determined by a kill curve. The cells were split again if they were approaching 80-90% confluency. The old plates were bleached if the cells were split. The cells were cultured until puromycin killed all of the cells in the non-infected control. The antibiotic dose was switched to 50% lower for a maintenance dose.

Protein Extraction

RIPA buffer was used for protein isolation. 400 ul of RIPA were used for 100 mm plate and 250 ul were used for a 60 mm plate. To create RIPA cocktail a 1:100 dilute of protease inhibitor cocktail and 1:100 of phosphatase inhibitor to the RIPA buffer was added. Ice was ready, and plates were washed with ice cold PBS twice. The RIPA was added to the plates and a cell scraper was used to scare cells (used 1 scraper per plate). Protein lysate was added to syringe and the plunger was used to pass the cells through the needle to homogenize further. The cells were kept on ice. The tubes were spun at 15000 rpm for 5 minutes at 4°C. The supernatant (minus pellet) was collected into fresh tubes and stored at -20°C or immediately used for BCA assay.

BCA Assay

The equations for the BCA assay are as follows: (9 stds + (number of assays+1)) x 2 x 0.2 ml=X and X/51=Y. Y ul of solution B of the BCA kit is added to (X-Y) of A solution to makeup the BCA solution. 10 ul of standard in a row and duplicated in another were added to a 96 well plate. 10 ul of each sample were added in duplicate. 200 ul of BCA assay solution was added and the plate was kept in the dark at 37°C for 30 minutes. Plates were read at 594 nm on a plate reader.

Western Blot

The protein concentration was calculated based on the BCA results. A water was set up and 80 ul of protein lysate and 20 ul of 10x Laemmli Buffer were mixed in a separate tube. Samples were boiled for 5 minutes, spun down and left at room temperature for

loading gels. A 4-20% gradient readymade BIORAD Gel was used. 1x electrophoresis buffer was made from 10x stock and Millipore water. The ladder was thawed on ice. The gel was loaded in the cassette and the green tape was removed. The buffer was poured into the cassette until it reached the line of the appropriate number gels. The samples and ladder were loaded into the wells. The samples were loaded according to the BCA assay. The gel was run at 200 V for approximately 30-40 minutes or until the ladder just runs out of the gel. The gels were transferred using the TURBO transfer unit using transfer buffer, transfer membrane kit, and the mid-molecular weight settings. The membrane was then blocked using 3% milk in TBST for 30 minutes at room temperature on a shaker. Primary and secondary antibody were in 3% milk in TBST. The primary antibody solution was added to the membrane and incubated at 4°C overnight while rotating. The next day the membrane was washed 3 times in TBST for 15 minutes each. The secondary antibody was then added and incubated at room temperature for 1 hour. The membrane was then washed 3 times using TBST for 15 minutes each. The 1:1 ECL solution was used and stained for approximately 5 minutes. The membrane was then viewed on the ChemiDoc.

RNA extraction

A Purelink RNA isolation kit from Thermo Fisher that contained all of the necessary materials was used to conduct this experiment. Trizol was kept at room temperature for 10-15 minutes. The media was aspirated quickly off the cell plates. 1 ml of trizol was added for 60-100 mm plates. The trizol was evenly spread and left on for 5 minutes. The mixture was pipetted up and down several times to homogenize the cells. The cells were

collected and kept at -80°C or used to proceed to the RNA extraction kit. 200 ul of chloroform were added per every 1 ml of trizol. The mixture was shaken vigorously and kept at room temperature for 5 minutes. The mixture was then centrifuged at 6°C and 15000 rpm for 15 minutes. The top phase was collected and moved to a fresh tube. Equal volumes of EtOH was added and then mixed by inverting. Half of the solution was transferred to a spin down column. The sample was spun at max speed for 1 minute and the flow through was discarded. This step was repeated until all of the sample is used. DNAase mixture was prepared for 80 ul per sample using 10x DNAase buffer, resuspended DNAase, and RNA free water. 350 ul of Wash 1 buffer was added to the tube and spun at 12000 rom for 15 seconds and the flow through was discarded. 80 ul of DNAase mixture was added to the column and incubated at room temperature for 15 minutes. 350 ul of Wash 1 buffer was added to the column and spun down at 12000 rpm for 15 seconds and the flow through was discarded. 500 ul of wash II was added to the column and spun down at 12000 rpm for 15 seconds two times. The tube was spun down again at 15000 rpm for 2 minutes and rid of any flow through. Add RNAase free water and incubate at room temperature for 5 minutes. Spin down and collect in new tube to get the final RNA sample.

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