FIBULIN-4A IN ZEBRAFISH DEVELOPMENT

by

Sandeep M. Khatri

B.Eng., Visvesvaraya Technological University, India, 2007

MS, University of Missouri- Saint Louis, 2009

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This dissertation was presented

by

Sandeep Khatri

It was defended on

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and approved by

Dissertation Advisor:
Zsolt Urban, PhD
Associate Professor, Department of Human Genetics
Graduate School of Public Health, University of Pittsburgh

Committee Members:

Beth Roman, PhD
Associate Professor, Department of Human Genetics
Graduate School of Public Health, University of Pittsburgh

Michael Tsang, PhD
Associate Professor, Department of Human Genetics
Graduate School of Public Health, University of Pittsburgh

Catherine Baty, DVM (DACVIM), PhD
Research Assistant Professor, Department of Human Genetics, Department of Medicine, Graduate School of Public Health and School of Medicine
University of Pittsburgh
ABSTRACT

Fibulin-4 is an extracellular matrix protein required for the formation of the elastic fibers. Human mutations in the fibulin-4 gene cause autosomal recessive cutis laxa with widespread systemic involvement and congenital presentation. To study the developmental functions of fibulin-4, I studied one of the two fibulin-4 genes, \textit{fbln4a}. The mRNA for \textit{fbln4a} RNA was expressed in the adaxial cells around the notochord during somite formation, and in the myosepta, head, and heart at later stages. Fbln4a protein was localized to the notochord sheath starting from somite formation suggesting differential expression of \textit{fbln4a} RNA and protein in the midline structures during development. To inactivate \textit{fbln4a} in vivo, we used a retroviral mutant and an antisense morpholino oligonucleotide (MO) for transient knockdown. Embryos homozygous for the \textit{fbln4a} mutation showed a complete loss of the Fbln4a protein, but presented no obvious gross abnormalities. However, \textit{fbln4a} knockdown yielded cardiovascular and musculoskeletal defects at 2 days post fertilization including pooling of blood at the caudal vein plexus, vascular hemorrhage in the head, reduced circulation and heart rate, bent notochord, rounded somites and reduced embryo length. All of these features were rescued by co-injection of \textit{fbln4a} mRNA with the MO. Early cardiac and vascular progenitor markers showed an expansion of the heart field and reduction of the vascular...
fields in knockdown embryos. Homozygous mutants were resistant, whereas heterozygotes were sensitized to the effect of fbln4a MO, protective compensatory mechanisms as a possible reason for phenotypic discrepancy between fbln4a mutant and knockdown animals. Inhibition of transforming growth factor beta (Tgfb) signaling with a small molecule rescued both the cardiovascular and connective tissue anomalies in knockdown embryos. By varying the period of treatment, I identified late gastrulation and early segmentation as the critical periods. I conclude that Fbln4a inhibits Tgfb signals emanating from the notochord and regulates cardiac and vascular progenitor pools. The public health significance of this work is the identification of Tgfb inhibition as a candidate approach for treating of fibulin-4-related cutis laxa, an orphan disease for which no treatment has been available to date.
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ABBREVIATIONS

ALK5: activin receptor-like kinase
ARCL1B: autosomal recessive cutis laxa type 1B
ASO: antisense oligonucleotide
BMP: bone morphogenetic proteins
cbEGF: calcium binding epidermal growth factor
cDNA: complementary DNA
Co-SMAD: common mediator SMAD
Cutis laxa: cutis laxa
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
dpf: days post fertilization
ECM: extracellular matrix
ELN: elastin
ER: endoplasmic reticulum
FBLN1: fibrillin-1
FBLN4: fibulin-4
FBN2: fibrillin-2
FN1: fibronectin-1
GDF: growth and differentiation factors
hpf: hours post fertilization
I-SMAD: inhibitory SMAD
LAP: latency associated peptide
LDS: Loeys-Dietz syndrome
LLC: large latent complex
LM: laminin
LOX: lysyl oxidase
LOXL: lysyl oxidase-like
LTBP: latent transforming growth factor beta binding protein
MBP1: mutant p53 binding protein 1
MFS: marfan syndrome
MO: morpholino
PCR: polymerase chain reaction
PVDF: polyvinylidene difluoride
qPCR: quantitative PCR
R-SMAD: receptor-specific SMAD
RNA: ribonucleic acid
RT-PCR: reverse transcriptase PCR
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLC: small latent complex
SMC: smooth muscle cell
TGFBR: transforming growth factor beta receptor
TGFβ: transforming growth factor beta signaling
WGA: wheat germ agglutinin
WISH: whole mount in situ hybridization
WMIS: whole mount immunostaining
1.0 INTRODUCTION

1.1 EXTRACELLULAR MATRIX

The extracellular matrix (ECM) is a complex network of proteins, glycoproteins and proteoglycans, which provides scaffolding and structural support to cells and organs. The ECM endows tissues with the mechanical strength necessary through life. The ECM is versatile in composition, enabling specialized adaptation to the functional requirements of each particular tissue (Bosman and Stamenkovic 2003). Additionally, it is capable of exchanging information with cells and modulating a whole host of processes including cell migration, attachment, differentiation, repair and survival. Identification of ECM proteins remains a challenging task due to the insoluble and frequently cross-linked nature of these proteins. So far a repertoire of more than 300 proteins has been identified in matrices of mammals and is called the “core matrisome” (Hynes and Naba 2012). The list comprises of almost 300 proteins, including 43 collagen subunits, three dozen proteoglycans and around 200 glycoproteins. In addition, there are numerous proteins that have been found associated with ECM, such as ECM-modifying enzymes, growth factors and cytokines (Hynes and Naba 2012). The main components of ECM include collagens, laminins, fibronectin, proteoglycans, and elastin. Elastin is an insoluble protein which, together with scaffold-forming fibrillin microfibrils makes elastic fibers. These
assemblies provide resilience to connective tissues, and will be further discussed in the next section.

1.2 ELASTIC FIBERS

Elastic fibers are one of the major ECM macromolecular components that are vital for endowing tissues, such as blood vessels, skin and lungs with elastic properties which are essential for their dynamic functions (Sato, Wachi et al. 2007). During evolution, elastic fibers enabled transition from open to the closed circulatory system in vertebrates, which bear high pulsatile pressures (Wagenseil and Mecham 2009). Elastic fibers provide expansion and recoil properties to large arteries, aorta and pulmonary arteries during heart contraction and relaxation, and therefore help maintain blood pressure within the cardiovascular system.

Elastic fibers adopt tissue-specific networks that depend on the elastic requirements of a particular tissue. In blood vessels, where elastic fibers account for up to 50% of dry weight elastin forms layers, known as lamellae in the tunica media (Wagenseil and Mecham, 2009). In the dermis of the skin, elastin is less abundant (2%), but it provides resistance to deformational forces by forming thick interlaced fibers that become thinner towards the surface of the skin (Kielty, Wess et al. 2002). In the lungs, similar to the skin, elastic fibers comprise approximately 2% of the dry weight, forming a thin, highly branched structures located at the tips of the alveolar septa and a highly connected network that links the respiratory tissues and provide expansion and recoil properties to the lungs (Shifren and Mecham 2006). In addition to their structural and
mechanical roles, elastic fibers regulate the bioavailability of growth factors and the 
adhesion of cells. In particular, they regulate activation and signaling of transforming 
growth factor beta (TGFβ) family, and interact with integrins and syndecans, thereby 
directing cell adhesion, cell survival, proliferation and migration (Bax, Mahalingam et al. 
2007).

1.2.1 MAJOR STRUCTURAL COMPONENTS OF ELASTIC FIBRES

Elastic fibers contain two major components distinguishable by electron microscopy: 
elastin, a hydrophobic cross-linked polymer which forms the core of the structure, and 
fibrillin-rich microfibrils which are partly embedded in and partly surround elastin (Kielty, 
Sherratt et al. 2002). Following a more detailed introduction to the structural proteins 
fibrillins and elastin, I will introduce the most relevant of the more than thirty identified 
elastic fibers associated components, including a family of cross-linking enzymes, lysyl 
oxidase (LOX) and LOX-like proteins, and proteins that facilitate elastic fiber assembly, 
namely fibulins and latent transforming growth factor binding proteins (LTBPs).

1.2.1.1 Tropoelastin and elastin

The precursor of polymeric elastin is a 72 KDa soluble protein called tropoelastin. 
Tropoelastin is synthesized by fibroblasts, lung alveolar cells, chondrocytes and vascular 
smooth muscle cells (Martin, Vrhovski et al. 1995), and contains alternating hydrophobic 
and hydrophilic domains. It has a remarkable biophysical property of coacervation, which 
is dependent on concentration, temperature, ionic strength and pH. When a tropoelastin 
solution is warmed at 37°C at pH 7, it undergoes a phase transition, forming an emulsion
of droplets with high protein concentration suspended in a solution of low tropoelastin content (Vrhovski and Weiss 1998). Coacervation is highly reversible and is enabled by interactions between the hydrophobic domains of tropoelastin. Although self-association of tropoelastin through coacervation is thought to be necessary, it is not sufficient for formation of elastic fibers. Tropoelastin aggregates need to be deposited onto microfibrils in an orderly and uniform fashion and have to be crosslinked through their exposed, hydrophilic, lysine-rich crosslinking domains (Clarke, Arnspang et al. 2006). An overview of the process of elastic fiber assembly is shown in section 1.2.2. Elastin is the most abundant ECM protein in the aorta and great arteries where it provides elasticity and recoil. Elastin null mice die perinatally due to severe obstructive vascular disease associated with increased proliferation and disorganization of smooth muscle cells (Li, Brooke et al. 1998), and also fail to develop alveoli in the lungs (Wendel, Taylor et al. 2000). Heterozygous mice, however, survive well into adulthood and exhibit hypertension, associated with increased number of elastic lamellae in the aorta and large arteries, and decreased size of blood vessels at normal blood pressure (Wagenseil, Nerurkar et al. 2005).

1.2.1.2 Fibrillin-1 and microfibrils

Fibrillin-1 is a large ECM glycoprotein (~ 350 KDa) and the major component of the microfibrillar scaffold (Handford, Downing et al. 2000). In elastic tissues, this scaffold is essential for elastin fiber assembly as it acts as a template upon which tropoelastin gets deposited and crosslinked (Kielty, Wess et al. 2002). Mice lacking fibrillin-1 die perinatally due to vascular and pulmonary complications, related to abnormal elastic fibers, emphasizing the importance of fibrillin-1 in elastogenesis (Carta, Pereira et al. 2006).
Microfibril formation is facilitated by the self-assembly of fibrillin-1 via N- and C-terminal interactions, yielding a characteristic beads-on-a-string structure that can be visualized by rotary shadowing electron microscopy. Fibrillinogenesis depends on the pre-existing fibronectin network, without which fibrillin-1 microfibrils cannot be formed by cultured cells (Marson, Rock et al. 2005). Fibrillin-1 binds to latent TGFβ binding proteins LTBPs -1, -3, and -4, which control secretion, bioavailability and activation of latent TGFβ, but also have distinct roles in ECM organization (Todorovic and Rifkin 2012).

1.2.1.3 Lysyl oxidase enzymes

Lysyl oxidase (LOX) is a 30 KDa enzyme, which, together with four other LOX-like proteins (LOXL1-4), comprises of a family of copper-dependent enzymes (Grau-Bove, Ruiz-Trillo et al. 2015). LOX and four LOX-like enzymes (LOXLs) are responsible for crosslinking elastin. LOXs oxidize the epsilon amino groups of lysines to aldehydes, which spontaneously condense to form elastin-specific covalent desmosine or isodesmosine crosslinks (Sato, Wachi et al. 2007). The crosslinking domains in tropoelastin assume an α-helical secondary structure which places two lysines in one chain in close proximity to a pair of lysines on another chain to form the tetrafunctional crosslinks (Brown-Augsburger, Tisdale et al. 1995). This process of crosslinking increases the stability of elastic fibers, preventing their degradation by proteinases (Romero, Tinker et al. 1986). Two distinct knockout mouse lines implicated LOX in the development of the cardiovascular and respiratory systems, and the skin (Maki, Rasanen et al. 2002, Hornstra, Birge et al. 2003, Maki, Sormunen et al. 2005). Desmosine levels were severely reduced indicating diminished crosslinking (~ 60% in aorta) in the mice.
resulting in lethal abnormalities, including ruptured aortic aneurysms. Mice deficient in LOXL1, a member of the LOX family with most similarity to LOX, exhibit cardiovascular and connective tissue defects, although they are viable and show a normal life span (Liu, Zhao et al. 2004).

### 1.2.1.4 Fibulins

Fibulins are a seven member ECM calcium-binding glycoproteins which interact with a range of ECM molecules, such as elastin, fibrillins, LOX, and fibronectin (Timpl, Sasaki et al. 2003, Kobayashi, Kostka et al. 2007). Through interacting with ECM components, fibulins are thought to be a bridge within the ECM, helping in assembly of structures of elastic fibers (de Vega, Iwamoto et al. 2009). Fibulin-1 can be found in the elastin core, fibulin-2 and fibulin-5 at the interface of elastin core and microfibrils, and fibulin-4 within the bundles of microfibrils and all these proteins have been shown to bind to elastin (Kobayashi, Kostka et al. 2007). Despite domain organization similarity amongst the fibulin protein family, knockout mice of fibulin -1 or -2 do not show altered elastogenesis, however knockouts of fibulins -3, -4, -5 have a deleterious effect of elastic fiber deposition (Kostka, Giltay et al. 2001, Nakamura, Lozano et al. 2002, McLaughlin, Chen et al. 2006, McLaughlin, Bakall et al. 2007, Horiguchi, Inoue et al. 2009). Despite having high sequence similarity, fibulin-4 and fibulin-5, have distinct roles in elastogenesis due to differences in their binding characteristics to elastin and LOX. Fibulin-5 binds to tropoelastin with high affinity than fibulin-4 (K\textsubscript{D}=64 nM, K\textsubscript{D}=131 nM, respectively) while LOX binds fibulin-4 strongly in comparison to fibulin-5 (K\textsubscript{D}= nM, K\textsubscript{D}=131 nM, respectively)(Choudhury, McGovern et al. 2009). Fibulin-4 has been shown to facilitate the elastin crosslinking step of elastic fiber assembly, whilst Fibulin-5 regulates the
deposition of elastin globules onto the microfibrillar scaffold (Choudhury, McGovern et al. 2009).

1.2.1.5 LTBPS

Latent TGFβ binding proteins (LTBP) are secreted glycoproteins which are structurally homologous to fibrillin microfibrils with several conserved domains. Major function of LTBPs is thought to be controlling secretion, bioavailability and activation of TGFβ in the ECM. However, LTBPs -1, -2, -4 have been implicated in elastic fiber assembly, due to their ability to interact with fibrillin-1 microfibril and their co-localization with microfibrils (Isogai, Ono et al. 2003, Hirani, Hanssen et al. 2007). Further, LTBPs -1 and -4 have been shown to be assisting functions of fibulins -4 and -5, by inhibiting their binding to fibrillin-1 (Ono, Sengle et al. 2009) and LTBP-1 has been shown to be interacting with fibulin-4 and fibrilin-1 simultaneously. Mutations in LTBP-4 lead to Urban-Rifkin-Davis syndrome in human, which is characterized by disorganized elastic fibers (Urban, Hutchagowder et al. 2009). Mice lacking LTBP-4 exhibit similar elastic fiber deformation to that observed in mice lacking fibulon-4 or -5, confirming that LTBPs have an additional role in ECM than to controlling TGFβ signaling.

1.2.2 ELASTIC FIBER ASSEMBLY

Elastic fiber assembly is a complex and highly organized hierarchical process that occurs during the late fetal and early postnatal stages of mammalian development (Figure 1). The process of elastinogenesis starts with the production of fibrillin microfibrils which act as a scaffold onto which elastin is deposited (Sato, Wachi et al. 2007). Next, tropoelastin,
forms globular micro-aggregates, presumably through the process of coacervation (Clarke, Arnspong et al. 2006). This process aligns tropoelastin appropriately for crosslinking and increases the affinity of tropoelastin to bind to LOXs (Vrhovski and Weiss 1998). Small covalently cross-linked elastin globules are then deposited on the microfibril template, where they further undergo maturation to produce a stable, continuous elastin core. The merger of several elastic fibres to each other leads to the formation of a network, which endows organs the typical ability to withstand repeated cycles of stretch and recoil. This, so called “macroassembly” of elastic fibers is dynamic, cell-directed process as demonstrated by time-lapse imaging of live cells (Czirok, Zach et al. 2006, Kozel, Rongish et al. 2006)
Figure 1. Elastic fiber assembly model.
Tropoelastin is secreted on the cell surface where it undergoes coacervation, forming droplets which are crosslinked by LOX. Cross-linked elastin is then deposited onto the microfibrillar scaffold where it further gets crosslinked to form mature elastic fibers in the ECM.
1.3 TGFβ SIGNALING PATHWAY

TGFβ is the prototypic member of a large superfamily of evolutionary conserved secreted cytokines, which include TGFβs, activins, inhibins, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs). Each of the TGFβ family members have crucial roles in multiple processes throughout development and in the maintenance of tissue homeostasis in adult life. Therefore, abnormal signaling by TGFβ family members has been implicated in many human disease, including cancer, fibrosis, autoimmune and cardiovascular disorders (Blobe, Schiemann et al. 2000, Morikawa, Derynck et al. 2016).

TGFβ is a potent growth factor for normal epithelial, hematopoetic and immune cells, and plays an important function in tissue homeostasis (Roberts and Sporn 1993). In humans there are three TGFβ proteins (TGFβ1, TGFβ2, TGFβ3) encoded by separate genes (Shi and Massague 2003). These three human proteins share 75% amino acid sequence homology and have demonstrated comparable signaling activities but different expression pattern between cells and tissues (Millan, Denhez et al. 1991). TGFBR1 and TGFBR2 receptors contain intracellular serine-threonine kinase domains and on binding of the active ligands to their extracellular domains form heteromeric complexes. Both TGFBR1 and TGFBR2 are phosphorylated at tyrosine and serine/threonine residues, which enables complex regulatory interactions with a variety of downstream signal transduction pathways.
<table>
<thead>
<tr>
<th>Molecule category</th>
<th>Molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligands</td>
<td>TGFβ1, TGFβ2, TGFβ3</td>
</tr>
<tr>
<td>Type I receptor</td>
<td>TGFB1R1 (ALK5)</td>
</tr>
<tr>
<td>Type II receptor</td>
<td>TGFB2</td>
</tr>
<tr>
<td>Type III receptor</td>
<td>TGFB3</td>
</tr>
<tr>
<td>R-SMADS</td>
<td>SMAD2, SMAD3</td>
</tr>
<tr>
<td>Co-SMAD</td>
<td>SMAD4</td>
</tr>
<tr>
<td>I-SMAD</td>
<td>SMAD6, SMAD7</td>
</tr>
<tr>
<td>TGFβ target genes</td>
<td>Collagen, CTGF, αSMA, Periostin</td>
</tr>
</tbody>
</table>

Abbreviation: ALK: activin receptor like kinase; R-SMAD: receptor-specific SMADs; Co-SMAD: common mediator SMAD; I-SMAD: inhibitory SMAD

Active TGFβs ligands bind with high affinity and specificity to the transmembrane TGFβ type II receptors (TGFB2), which activates the TGFβ type I receptor (TGFB1R1 or ALK5). Activated TGFB1R initiates canonical SMAD signaling by phosphorylation of receptor-associated SMADs (R-SMADs), SMAD2, and SMAD3. Phosphorylated R-SMADs form complex with Co-SMAD (SMAD4), which together translocate to the nucleus and target gene expression of various biological processes (Feng and Derynck 2005) (Figure 2) (Massague and Gomis 2006). In addition to the canonical signaling described above, TGFβs can also signal through non-canonical (non-SMAD) mechanisms, including
PI3 kinase, MAPK, RhoA-Rock pathways. The canonical SMAD pathway is central to the growth inhibitory action of TGFβ (Derynck and Zhang 2003, Moustakas and Heldin 2005).

**Figure 2. TGFβ signaling pathway.**

Signaling by TGFβ through serine/threonine kinase receptors. Downstream signal transduction takes place via SMAD-dependent and SMAD-independent pathways.
1.3.1 TGFβ biology

Mature TGFβ is a covalent 25 KDa homodimer produced after intracellular proteolytic cleavage from its propeptide, also known as the latency associated peptide (LAP). LAP component stays bound to the mature TGFβ non-covalently, and the two components together form the small latent complex (SLC). The LAP confers latency to mature TGFβ by preventing it from binding to its receptor. In the endoplasmic reticulum (ER), SLC generally becomes disulfide bound to (latent TGFβ binding protein) LTBP proteins via the LAP, and together they form the large latent complex (LLC) (Rifkin 2005). Because LTBP's are incorporated into the ECM and TGFβ bound LTBP's are part of LLC, are also targeted to the matrix, wherein TGFβ is stored in latent form for future activation (Taipale, Miyazono et al. 1994, Isogai, Ono et al. 2003). Knock-in mice expressing mutant TGFβ1 which is unable to bind to LTBP exhibit decreased activity of TGFβ, indicating the importance of LLC for normal TGFβ signaling (Yoshinaga, Obata et al. 2008). Various molecules in the ECM are able to release TGFβ from its latent complexes and, thus activate it, including proteases (Yu and Stamenkovic 2000), thrombospondin-1 (Schultz-Cherry and Murphy-Ullrich 1993), reactive oxygen species (ROS) (Barcellos-Hoff and Dix 1996), and integrins (Munger, Huang et al. 1999).
ProTGFβ undergoes a series of steps inside the cell through the endoplasmic reticulum and the Golgi apparatus to attain a mature TGFβ complex (red) attached to LTBP5 and is called large latent complex (LLC). This mature TGFβ complex is secreted into the ECM wherein the LTBP5 interact with fibronectin, fibrillin and heparan sulfate proteoglycans. Proteolytic cleavage of latent form by ECM components or mechanical stretching leads to release of active TGFβ (green). Cells respond to TGFβ through membrane receptors which signal through downstream signaling pathways.
1.3.2 Extracellular control of TGFβ signaling by elastic fibers

The ECM provides a repository for a variety of different growth factors (Schultz and Wysocki 2009). Storage and tightly regulated release of TGFβ family of cytokines by the ECM components is a good example of multi-faceted interactions between ECM and the growth factors (Figure 3). The general understanding has been that matrix binding of latent TGFβ complexes is imperative for proper TGFβ function, and impaired binding to the matrix yields a TGFβ deficient state inside the cells. This hypothesis is supported by mutation in *Tgfb1* gene which results in decreased matrix association of latent TGFβ and yields mice with phenotype associated with decreased TGFβ levels (Yoshinaga, Obata et al. 2008). However, several papers, reported in recent years have shown how defective ECM results in enhanced TGFβ signaling instead of the expected decreased TGFβ state.

Recently, a crucial role of elastic ECM has come to fore in regulating TGFβ bioavailability in the vascular system. Defects in the ECM molecules are thought to be change the physical properties of the vessel walls and thereby compromise of the vasculature. In addition, several lines of evidence established a link between ECM molecules and TGFβ-SMAD signaling at different levels. Elastic fibers sequester TGFβ to play its intracellular role. During normal development or injury, sequestration of TGFβ by elastic fibres may be a negative feedback signal indicating that sufficient amounts and quality of ECM has been produced. This link between elastic fibers components and TGFβ has been established by reports of loss of function mutations in several ECM
proteins (Table 2) showing dysregulated TGFβ signaling and this is discussed in the next section.

**Table 2. Human disorders and animal models associated with elastic fiber components and altered TGFβ signaling**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Human syndrome</th>
<th>Animal models</th>
<th>TGFβ signaling</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBN2 (Fibrillin-2)</td>
<td>Contractural arachnodactyly</td>
<td>KO: bilateral syndactyly</td>
<td>↑↑</td>
</tr>
<tr>
<td>FBLN4 (Fibulin-4) (Fibrillin-4)</td>
<td>Autosomal recessive cutis laxa type 1B</td>
<td>KO: perinatal lethality, arterial narrowing, aortic rupture, Hypomorphic : tortuous aorta, aortic aneurysm</td>
<td>↑↑</td>
</tr>
<tr>
<td>LTBP1</td>
<td>No disease known so far</td>
<td>KO: persistent truncus arteriosus</td>
<td>↓</td>
</tr>
<tr>
<td>LTBP3</td>
<td>No disease known so far</td>
<td>KO: craniofacial malformations, osteosclerosis</td>
<td>↓</td>
</tr>
<tr>
<td>LTBP4</td>
<td>Autosomal recessive cutis laxa type 1C</td>
<td>KO: developmental emphysema, aortic tortuosity, cardiac hypertrophy, reduced skin thickness</td>
<td>↓</td>
</tr>
</tbody>
</table>
1.4 ECM COMPONENTS AND REGULATION OF TGFβ SIGNALING

1.4.1 LTBPs

LTBPs are large ECM molecules which are structurally related to fibrillins (FBNs) and are ECM components that appear to be involved in the secretion and targeting of TGFβ to sites at which it is either stored and/or activated. Since LTBPs are part of the large latent complex (LLC) which is sequestered to the ECM, defective LTBPs should result in increased intracellular TGFβ activity. However, reduced TGFβ signaling was observed in all LTBP mutants studied to date, supporting the notion that LTBPs are not only important for the sequestration of TGFβ but also for its activation.

LTBP-1 deficient mice die shortly after birth from persistent truncus arteriosus and interrupted aortic arch, along with cardiac abnormalities like hypoplastic endocardial cushions during early development and hyperplastic valves during late valvulogenesis, as a result of altered epithelial to mesenchymal transition, a process known to be mediated by TGFβ. The cell populations of the heart in these mice show decreased TGFβ signaling illustrated by reduced pSmad2 staining and a decrease in the expression of TGFβ responsive genes like connective tissue growth factor (CTGF) and periostin (Todorovic, Frendewey et al. 2007, Todorovic, Finnegan et al. 2011). Similarly, mice deficient of LTBP3 show phenotype of bone abnormalities and defective septation of the terminal pulmonary air sacs, phenotypes consistent with loss of TGFβ signaling (Dabovic, Chen et al. 2002, Colarossi, Chen et al. 2005). Unlike LTBP-1 and LTBP-3, LTBP-4 has multiple functions, both as regulator of TGFβ and also a promoter of elastic fiber
assembly. \(LTBP4^{-/-}\) mice display developmental emphysema, cardiac hypertrophy and colorectal prolapse with diminished ECM deposition of TGF\(\beta\)1 and decreased p-Smad2 in lungs and colon (Sterner-Kock, Thorey et al. 2002). In addition to binding TGF\(\beta\)1, LTBP4 facilitates TGF\(\beta\) signaling by binding TGFBR2 and stabilizing the TGFBR receptor complex for sustained signaling. In the absence of LTBP4 the TGFBR receptor complex undergoes ligand-mediated endocytosis and lysosomal degradation (Su, Huang et al. 2015). Similar studies have not been performed to test if LTBP1 and LTBP3 play role in stabilizing TGFBR complex.

1.4.2 Fibrillins

Fibrillins are major constituents of microfibrils and bind LTBP4 (Isogai, Ono et al. 2003). Therefore, impairment of microfibril organization due to mutations in fibrillin-1 should affect the sequestration of LLC into the ECM and alter TGF\(\beta\) signaling. Indeed, mutations in fibrillin-1 causes Marfan syndrome (MFS) a systemic connective disorder primarily associated with cardiovascular manifestations in which disruption of the microfibrils leads to decreased incorporation of the LTBP in the ECM but increased TGF\(\beta\) signaling both in patients and in fibrillin-1 deficient mice (Dietz, Cutting et al. 1991, Zilberberg, Todorovic et al. 2012). Consistent with this observation, several phenotypes of MFS, including aortic dilation and dissection, hypertrophic mitral valves and developmental emphysema, are reversed in fibrillin-1 mutant mice by the administration of TGF\(\beta\) neutralizing antibody (Neptune, Frischmeyer et al. 2003, Ng, Cheng et al. 2004). These observations are
consistent with a function of fibrillin-1 in TGFβ signaling sequestration but not in TGFβ activation.

1.4.3 Fibulins

Fibulins comprise a group of eight proteins, among which FBLN -3, -4, -5 play important roles in elastic fiber formation and maintenance. Thus, deficiency in these proteins results in impaired elastogenesis (Nakamura, Lozano et al. 2002, McLaughlin, Chen et al. 2006, McLaughlin, Bakall et al. 2007, Horiguchi, Inoue et al. 2009), but so far none are known to interact with components of TGFβ complex (latent or active) or to play any role in TGFβ sequestration. Nevertheless, evidence for increased canonical TGFβ signaling has been found in both humans and mice deficient in fibulin-4. *Fbln4* deficient mice show enhanced TGFβ signaling by enhanced p-Smad2 levels in the aortic tissues and increased TGFβ2 levels in blood plasma (Hanada, Vermeij et al. 2007, Ramnath, Hawinkels et al. 2015). In another study, smooth muscle-specific conditional knockouts of *Fbln4* showed enhanced TGFβ signaling through non-canonical pathway, as indicated by increased Erk1/2 phosphorylation (Huang, Davis et al. 2010).
1.4.4 Role of TGFβ in vascular development

Blood vessels form by a combination of two processes known as vasculogenesis and angiogenesis, both of which lead to formation of tubes lined by endothelial cells (Adams and Alitalo 2007). Vasculogenesis refers to formation of new vessels from a mass of proliferating cells to form a primary capillary plexus. Angiogenesis refers to sprouting of new capillary networks from pre-existing vessels (Risau 1997). Both of these processes are regulated by growth factors in a highly coordinated manner during embryo development. The critical role of TGFβ family members in vascular development is underscored by the observations that nearly all knock-out mice for TGFβ receptors, ligands and intracellular components lead to death during midgestation due to heart defects and lack of circulation and mutations in the genes encoding TGFβ pathway components are linked to increasing number of human pathologies with vascular dysfunction as shown in Table 3. Mice with null mutation in Tgfb3 result in neonatal lethal lung dysplasia with intrapulmonary vascular dysplasia as well as cleft palate (Kaartinen, Voncken et al. 1995). Null mutation in Tgfb2 results in early embryonic lethality owing to cardiac defects as well as defects in pulmonary lobation (Bartram, Molin et al. 2001). Null mutation in Tgfb1 gene results in lethal postnatal pulmonary inflammation (Shull, Ormsby et al. 1992). The embryos lacking receptors TGFBR1 (ALK5) and TGFBR2 die during mid-gestation due to impaired vascular development, exhibiting hyper-dilated and leaky vessels. The primary target cell for the TGFβ is the endothelial cell (EC) but is also is important for proper differentiation and function of SMCs and pericytes (Goumans and Mummery 2000).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Human syndrome &amp; Symptoms</th>
<th>Animal models</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFB1</td>
<td>Camurati-Engelmann disease – bone dysplasia, muscular weakness, limb pain, fatigability.</td>
<td>KO: embryonic lethal with vascular defects or postnatal lethality from autoimmune disease (phenotype is modifier dependent)</td>
</tr>
<tr>
<td>TGFB2</td>
<td>Familial thoracic aortic aneurysms and dissections</td>
<td>KO: aortic arch defects, cardiac septal defects, perinatal lethality</td>
</tr>
<tr>
<td>TGFB3</td>
<td>Syndrome with low muscle mass, growth retardation, and clinical features like MFS</td>
<td>KO: cleft palate, delayed lung maturation, die shortly after birth</td>
</tr>
<tr>
<td>TGFBR1/ALK5</td>
<td>Loeys-Dietz syndrome – aortic aneurysms and tortuosity, hypertelorism and cleft platate</td>
<td>KO: embryonic lethal, angiogenesis defects.</td>
</tr>
<tr>
<td>TGFBR2</td>
<td>Loeys-Dietz syndrome</td>
<td>KO: embryonic lethal, vascular defects</td>
</tr>
</tbody>
</table>
1.4.5 Interplay between elastic fibers and TGFβ signaling

The interplay between multiple ECM components, latent TGFβ complexes and TGFβ activators determine the levels of active TGFβ. Some of these interactions have been recognized through studies of rare genetic disorders affecting connective tissues, such as in MFS, Loeys-Dietz syndrome (LDS) and cutis laxa. During normal development or injury, sequestration of TGFβ by elastic fibers can be thought of as a negative feedback signal indicating that sufficient amounts and quality of ECM has been produced. Loss of function mutations in elastic fiber genes playing role in TGFβ sequestration like FBN1 result in elevated TGFβ signaling (Kaartinen and Warburton 2003, Dabovic, Chen et al. 2009).

Conversely, TGFβs can up-regulate the expression of many genes necessary for the production of elastic fibers including fibronectin (Ignontz, Endo et al. 1987), LTBPs (Weikkolainen, Keski-Oja et al. 2003), ELN (Kucich, Rosenbloom et al. 1997), LOXs (Kim, Lee et al. 2008), and FBLN5 (Kuang, Joyce-Brady et al. 2006). This regulation by TGFβs occurs at the transcriptional or posttranscriptional level depending on the gene. In an example of post-transcriptional regulation, both ELN and FBN1 are in part suppressed by miR29 family of micro-RNAs by binding to the 3’-untranslated regions of both RNAs (van Rooij, Sutherland et al. 2008). The expression of miR29 is inhibited by TGFβ, thus relieving the repression of the FBN1 and ELN.
1.4.6 Therapeutic targeting of TGFβ signaling pathway

As TGFβ affects wide variety of biological processes, blockage of TGFβ and its signaling pathway provides multiple therapeutic opportunities. There are 4 major types of TGFβ inhibitors use for limiting cancer progression and in some instances vascular abnormalities in humans and animal models which can be classified as 1.) Ligand traps, 2.) antisense oligonucleotide, 3.) small molecule kinase inhibitors, and 4.) peptide aptamers (Figure 4) (de Larco and Todaro 1978, Moses, Branum et al. 1981).

Ligand traps include use of anti-ligand neutralizing antibody raised against individual ligands or against all 3 TGFβ ligands by designing appropriate epitope or soluble receptor proteins that have the ability to compete with TGFβ receptors. Some of the humanized pan-TGFβ monoclonal antibodies are developed by Genzyme for patients including Lerdelimumab (Mead, Wong et al. 2003), Metelimumab (Cordeiro, Gay et al. 1999), and GC-1008 (Fresoliumumab) (Morris, Tan et al. 2014). TGFβ monoclonal antibody, 1D11, developed by Sanofi, which is a pan-neutralizing anti-mouse TGFβ antibody has been shown to bind to all three TGFβ isoforms and reduce the lung cancer metastasis (Nam, Terabe et al. 2008). Antisense oligonucleotides (ASOs) can also be used to reduce bioavailability of TGFβ ligands by blocking TGFβ synthesis post-transcriptionally.

ASOs are single-stranded nucleotide molecules, which hybridize to target mRNA, and thereby inhibiting its translation and/or promoting its synthesis by mRNA degradation of the blocked mRNA (Bennett and Swayze 2010). One of the TGFβ2 targeting ASO is AP12009 (Trabedersen) (Hau, Jachimczak et al. 2007). However, oligonucleotides have
some limitations like RNA binding affinity, and possible non-specific/off target effects and issues with cellular uptake.

Whereas ligands traps and ASOs limit the biosynthesis of the TGFβ ligands, they fail to block receptor signaling. This is where a class of kinase receptor kinase blocking inhibitors have been developed and used to block TGFβ signaling. Glaxosmithkline developed a small molecule inhibitor, SB-431542, which specifically targets TGFβRI and is now widely used in basic research for in in vitro studies (Laping, Grygielko et al. 2002). LY364937, another TGFβRI/ALK5 inhibitor has shown to be reduce early lung and bone metastases in nude mice (Bandyopadhyay, Agyin et al. 2006).
The TGFβ is often elevated in human tumors and vasculature. This has led to development of range of anti-TGFβ drugs. The four major classes of TGFβ inhibitors include ligand traps, antisense oligonucleotides, peptide aptamers and kinase inhibitors.
1.5 FIBULIN-4

Fibulin-4, also known as EFEMP2 (epidermal growth factor domain containing fibulin-like extracellular matrix protein 2) or MBP1 (mutant p53 binding protein 1), is a 54 KDa, 433 amino acid ECM glycoprotein that is rich in negatively charged amino acids, giving it slightly acidic properties (Gallagher, Greene et al. 2001). It is one of the eight members in the fibulin family of ECM glycoproteins involved in ECM organization and function. Throughout this thesis I will use fibulin-4 (FBLN4 for human and Fbln4 for zebrafish) instead of EFEMP2 to emphasize the structural and functional similarities among the members of the fibulin gene family. FBLN4 is highly expressed in the medial layers of arteries and veins and in heart valves, and moderate level of expression is seen in skeletal muscle, placenta, kidneys and pancreas, and low levels in lungs and brain. FBLN4 is also expressed in adult human fibroblasts and articular chondrocytes (Chen, Zhang et al. 2009). Like all the other members of fibulin family of proteins, fibulin-4 contains a characteristic N-terminal domain, an array of calcium-binding epidermal growth factor (EGF) like domains, the first with an additional 28 amino acid insert, and a characteristic fibulin C-terminal (FC) domain. The N-terminus contains a signal peptide sequence, which is required for FBLN4 to be secreted into the extracellular space and also an EGF-like adjoining domain. The gene encoding fibulin-4 has been mapped to human chromosome 11q13, and the protein is encoded by 11 exons (Katsanis, Venable et al. 2000). Fbln4 was first identified as an N-terminally truncated protein lacking a signal peptide and interacting with the intracellular mutant tumour suppressor protein p53 (Gallagher et al., 1999). However, these studies have not been replicated since and all currently annotated FBLN4 transcripts have a signal peptide.
1.6 FUNCTIONS OF FIBULIN-4

Several studies have shown that fibulin-4 is essential for elastogenesis, and for normal composition and functions of tissues abundant in elastic fibers, such as heart, blood vessels, and lungs. Recently, fibulin-4 has been shown to be important in other processes, such as smooth muscle cell differentiation, control of TGFβ bioavailability and bone development (Horiguchi, Inoue et al. 2009, Renard, Holm et al. 2010, Erickson, Opitz et al. 2012). In the ECM fibulin-4 is known to be binding to tropoelastin and LOX, two components that play crucial roles in the elastic fiber assembly (Choudhury, McGovern et al. 2009, Horiguchi, Inoue et al. 2009).

1.6.1 Fibulin-4 is essential for elastic fiber assembly

The importance of fibulin-4 for normal development of tissues rich in elastic fibers was first demonstrated by a knockout mouse model studied by McLaughlin et al. Mice lacking fibulin-4 developed lethal lung and vascular defects, such as emphysema, arterial tortuosity, aneurysm and hemorrhages, resulting in prenatal death. (McLaughlin, Chen et al. 2006). Histological examination of aorta, lung and skin of these mice showed severely fragmented elastic fibers. These characteristics phenotypes and elastic fiber disruption were later found associated with FBLN4 mutations in human patients (Huchtagowder, Sausgruber et al. 2006, Hoyer, Kraus et al. 2009, Renard, Holm et al. 2010, Sawyer, Dicke et al. 2013).

In the fibulin-4 knockout mice, elastin aggregates were observed forming electron-dense rod-like structures instead of solid, continuous elastic lamellae suggesting
improper elastin crosslinking. Indeed, a 94% drop in the levels of desmosine was observed, which is a crosslink product only found in mature elastin. However, at the gene expression level, the synthesis of neither tropoelastin nor LOX were altered in fibulin-4 mutant mice. In summary, fibulin-4 facilitates elastin assembly and crosslinking.

1.6.2 Fibulin-4 regulates the differentiation and function of aortic smooth muscle cells

Although mouse models of elastin, fibulin-5 and fibulin-4 deficiency all exhibit disrupted elastic fibers and similar vascular and lung abnormalities, only absence of fibulin-4 causes lethal ruptured aortic aneurysms. Therefore, it is believed that apart from its role in elastic fiber assembly, fibulin-4 must have another function in the aortic wall. Huang et al (2010) developed a smooth muscle specific (SMC) fibulin-4 deficient mice which exhibited lethal aortic aneurysms and the analysis of its aortic smooth muscle cells revealed several abnormalities. The SMCs showed increased proliferation, decreased differentiation, degenerative changes and reduction in smooth muscle contractile proteins (Huang, Davis et al. 2010). As a result, the function of SMCs was severely impaired, affecting the stability of the aortic wall. The aorta of these deficient mice showed increased pERK1/2 signaling. In another study by Ramnath et al (2015) isolated SMCs from fibulin-4 deficient mice showed reduced growth and upregulated TGFβ signaling by increased SMAD2 phosphorylation. The reduced growth of SMCs in these mice could be reversed by treatment with TGFβ neutralizing antibody (Ramnath, Hawinkels et al. 2015). These findings suggest that fibulin-4 has an indispensable role in the development of the
vascular system through its role both in elastic fiber formation and in differentiation and function of aortic SMCs.

1.6.3 Fibulin-4 and control of TGFβ bioavailability

Patients suffering from fibulin-4-associated cutis laxa and mice lacking fibulin-4 exhibit increased TGFβ signaling (Hanada, Vermeij et al. 2007, Renard, Holm et al. 2010). TGFβ signaling is upregulated in aortas of adult hypomorphic mice (*FBLN4R/R*) mice and postnatal treatment with losartan, an angiotensin (Ang) II type 1 receptor antagonist and an indirect inhibitor of TGFβ signaling improves lifespan of *FBLN4R/R* mice, however does not affect aortic vessel wall structure. Prenatal treatment of these mice with losartan prevents elastic fiber fragmentation in the aorta, strongly indicating that altered TGFβ is associated with disturbed elastic fiber structure which is consistent with the view that elasin is required during late prenatal to early postnatal periods of development (Moltzer, te Riet et al. 2011). TGFβ pathway which is essential for controlling cell proliferation and differentiation, and its perturbation has also been implicated in numerous other cardiovascular diseases, including Marfan syndrome and LDS as mentioned earlier (Neptune et al., 2003) (Loeys, Chen et al. 2005). The reasons for dysregulated TGFβ signaling are not known but one possible explanation can be fragmentation of elastic fibers in absence of fibulin-4 may be leading to impaired sequestration of TGFβ. Recently it has been shown that there is a direct functional and biochemical interaction between LTBP-4 long form and fibulin-4 (Bultmann-Mellin, Essers et al. 2016) providing an explanation for altered TGFβ sequestration in fibulin-4 deficient tissues. In summary,
increased TGFβ signaling been suggested by multiple lines of evidence as one of the contributing pathological mechanisms leading to the phenotype observed in patients suffering from cutis laxa caused by mutations in fibulin-4.

1.6.4 Fibulin-4 deficiency and mutations

Biallelic loss of function mutations in the FBLN4 gene cause autosomal recessive cutis laxa 1B (ARCL1B) which is a rare disease with manifestations including sagging skin, arterial tortuosity, aortic aneurysms, pulmonary hypertension, developmental emphysema, bone fragility, arachondactyly, joint laxity and diaphragmatic and inguinal hernias. The first mutation in the FBLN4 gene was reported in a 2 yr old girl, a recessive missense mutation (p.E57K) (Huchtagowder, Sausgruber et al. 2006). Since then a total of 35 patients have been reported worldwide with ARCL1B, and 26 of them died either at birth or early childhood (Dasouki, Markova et al. 2007, Hoyer, Kraus et al. 2009, Renard, Holm et al. 2010, Al-Hassnan, Almesned et al. 2012, Erickson, Opitz et al. 2012, Iascone, Sana et al. 2012, Kappanayil, Nampoorthiri et al. 2012, Sawyer, Dicke et al. 2013, Hebson, Coleman et al. 2014). The 12 pathogenic mutations include 9 missense and 3 frameshift mutations which are uniformly distributed within the FBLN4 gene (Figure 5). Parents, who are heterozygous carriers of disease-causing FBLN4 mutations are asymptomatic and commonly consanguineous. Reports of FBLN4 mutations resulting in disease are rare, and due to frequent neonatal lethality, ARCL1B is likely to be underdiagnosed. Diagnosis of autosomal recessive cutis laxa caused by fibulin-4 mutations can be
challenging due to large phenotypic overlap with other subtypes of cutis laxa and arterial tortuosity syndromes, and due to clinical heterogeneity shown by patients.

![Figure 5. Fibulin-4 mutations in ARCL1B.](image)

A signal peptide at the N-terminus (orange), EGF-like adjoining domain (purple), followed by a modified cbEGF-like domain (green) with a 28 amino acid insert (yellow), five cbEGF-like domains (blue) and the C-terminal FC domain (red hexagon). Tie lines indicate the locations of ARCL1B mutations identified to date.

### 1.6.5 ZEBRAFISH AS A MODEL FOR STUDIES ON FBLN4

The zebrafish has become a central model system to investigate vertebrate development by both forward and reverse genetics (Lawson and Wolfe 2011). Early foundational studies utilized zebrafish in large-scale forward genetic screens, based simply on observing embryonic morphology resulted discovery of many genes which play role in different aspects of embryonic development including gastrulation, cardiovascular morphogenesis, and hematopoiesis (Hammerschmidt, Pelegri et al. 1996, Ransom, Haffter et al. 1996, Stainier, Fouquet et al. 1996, Goessling and North 2014). Zebrafish presents advantages to its users by having a rapid external development, with gastrulation starting by 6 hpf, heart beating by 24 hpf and circulation through head and tail of the embryo by 30 hpf (Isogai, Horiguchi et al. 2001). Zebrafish adults lay large
clutch sizes of transparent embryos which facilitate mapping of causative mutant genes during embryogenesis (Lawson and Wolfe 2011). Zebrafish is amenable to genetic manipulation and permanent knockout lines are generated using CRISPR/Cas9 or TALEN technology, while transient gene knockdown studies are performed using antisense oligonucleotide (Bedell, Wang et al. 2012, Hruscha, Krawitz et al. 2013). In addition to genetic studies, zebrafish also supports pharmacological approaches. Addition of previously characterized small molecules to fish water allows researchers to manipulate specific biochemical pathways of interest, and also allows for identification of novel small molecules that perturb particular signaling pathways or developmental processes by use of chemical screens (Goessling and North 2014).

1.6.6 Zebrafish Fibulin-4a

In addition to its role in elastic fiber assembly during late fetal periods, zebrafish Fbln4a is required for gastrulation process during embryogenesis. It facilitates cell migration for the anterior convergent movements of the prechordal plate progenitors downstream of Wnt/βcaternin/Stat3 pathway. Knockdown of Stat3 and Fbln4a separately showed abnormal anterior migration during zebrafish gastrulation, resulting in a mispositioned and shortened anterior-posterior (AP) axis (Zhang, Yin et al. 2014). However, cell specification of gastrulation progenitors was not affected by knockdown of fbln4a. In in vitro and in vivo experiments, Fbln4a protein was secreted into the ECM and promoted for formation of fibronectin (FN) and laminin (LM) matrices. Increase or decrease in wild type fbln4a expression or overexpression of mutant fbln4a was able to disrupt FN and LM matrix identifying a previously unrecognized function of Fbln4a.
1.7 DISSEMENT AIM

Fibulin-4 is an ECM glycoprotein that is essential for elastogenesis and hence for appropriate elastic recoil functions of elastic fiber-rich tissues such as aorta, elastic arteries, lungs and skin. Mouse studies by various research groups have extensively focused on understanding postnatal role of the *FBLN4* and have shown its role in elastic fiber assembly and integrity of aortic structure. Moreover, previous human and mouse studies have shown abnormal transforming growth factor-beta (TGFβ) signaling in FBLN4 mutant tissues.

However, not much is known about the embryonic role of fibulin-4 and the pathological molecular mechanisms that underlie the developmental disease caused by loss of fibulin-4. This is where zebrafish model proves advantageous as the transparent embryo develops rapidly, outside of mother's body, and has fully developed organs (or counterparts to mammalian organs) including a functioning cardiovascular system within 2 days post fertilization. A recent zebrafish transient knockdown study showed that Fbln4a is required for the organization of the extracellular matrix (ECM) and the convergent extension movements of the prechordal plate progenitors during gastrulation (Zhang, Yin et al. 2014), however, it remains unclear how these developmental changes are related to phenotypes observed in patients with permanent FBLN4 mutations, and what signaling pathways are activated as a consequence of Fbln4a deficiency.

Based on the above discussed published knowledge, I hypothesized that Fbln4a deficiency alters the differentiation of cardiac and vascular progenitors through the dysregulation of TGFβ signaling at early embryonic stages to which contributes to the
cardiovascular malformations observed after organogenesis. To test this hypothesis I pursued 3 specific aims:

Aim 1. Characterize the gene expression pattern of \textit{fbln4a} during early development to identify the tissues wherein \textit{fbln4a} mRNA and protein are expressed.

Aim 2. Perform loss of function studies for \textit{fbln4a} using a retroviral mutant and also by generating and validating transient knockdown reagents of \textit{fbln4a}. Compare the loss of function effects between germline mutant and transient knockdown with a focus on cardiovascular development.

Aim 3. Test if altered TGF\(\beta\) signaling mediates the cardiovascular lesions in Fbln4a deficient embryos using small molecule inhibitors, and identify the critical period of TGF\(\beta\) action.

According to National Institutes Health Office of Rare disease research estimated 30 million people living in the United States have some type of rare disease of which there are more than 7000 types. More than 80\% of rare diseases are genetic and can be life threatening and 30\% of rare disease patients die before the age 5 deeply impacting patients and their families with physical and mental distress (Moisan, Ricketts et al. 1999, Bavisetty, Grody et al. 2013). Since majority of rare genetic diseases are monogenic and are transmitted from parents to offspring; it makes them excellent targets for study in animal models like mouse and zebrafish. Animal models of human disease lay foundation for drug discovery as they allow for identification of novel pharmacologics while connecting gene function with specific pathogenesis. Several novel small molecules with potential to treat human pathologies have been discovered using high throughput
zebrafish drug screens and since then have entered into clinical trials (Cutler, Multani et al. 2013, Zon 2014, Li, Lahvic et al. 2015).
2.0 MATERIALS AND METHODS

2.1 ZEBRAFISH MAINTENANCE AND CARE

Adult zebrafish (*Danio rerio*) were maintained according to standard husbandry protocols and experiments were performed in accordance with NIH guidelines. Embryos were grown at 28.5°C in 30% Danieau [17mM NaCl, 2mM KCl, 0.12mM MgSO₄, 1.8 mM Ca(NO₃)₂, 1.5mM HEPES]. For imaging, embryo medium was supplemented with 0.0003% phenylthiourea (Sigma, St. Louis, MO, USA) at ~ 24 hours post-fertilization (hpf) to prevent melanin synthesis. Wildtype AB* zebrafish embryos were used for injection purposes. Embryos were staged following published guidelines (Kimmel, Ballard et al. 1995)

2.2 PROTEIN SEQUENCE ANALYSIS

Amino acid sequences of human and zebrafish FBLN4 were obtained from the GenBank database. The accession code for human FBLN4 - NP058634, zebrafish Fbln4a - NP001008587 and Fbln4b - NP001153830. Protein sequence alignment was performed using multiple sequence alignment feature of CLC Main Workbench 6.9.1.
2.3 MORPHOLINO-MEDIATED KNOCKDOWN

Antisense morpholinos (MO) were purchased from Gene Tools, Philomath, Oregon, USA. Splice blocking MOs were designed following the recommendations of the manufacturer to target the exon 3/intron 3 junction of \( fbln4a \) using (MO-1: 5’-TGAACGCTGTTTTACCTTTGCAGTG -3’). To target \( fbln4b \) gene we used (MO-2: 5’-CACTCATTATGTCTGCGTACAATA-3’) against the exon 4/intron 4 junction. The morpholinos were diluted in distilled water and pressure-injected into the yolk cells of 1-to 2-cell stage embryos using Eppendorf FemtoJet Micromanipulator 5171. Phenol red (1x) (Sigma) was co-injected as a dye tracer. MO concentrations were determined spectrophotometrically and 5 nl was injected into embryos at increasing doses (1-10ng) to determine optimal concentrations. The optimal dose for both MO-1 and MO-2 both was selected as 5ng based on phenotypic effect. The control morpholino (5’-CCTTTACCTCAGTTACAATTATA-3’) targeting human beta-globin gene (GenBank accession number - AH001475.2) was injected as the same concentration as the experimental morpholino (5ng) for each experiment.

2.4 RNA FOR MICRO-INJECTION

A full-length 1.4kb \( fbln4a \) c-DNA was cloned into pCRII-TOPO plasmid vector. The vector containing \( fbln4a \) cDNA cassette was double digested with HindIII and EcoRI restriction enzyme (New England Biolabs) and the cassette was gel eluted and ligated into pOX oocyte expression vector for making \( fbln4a \) mRNA. For in vitro transcription, 10\( \mu \)g of
fbln4a-pOX plasmid DNA was digested with EcoRI for linearization and finally 1µg was used as template for T3 in vitro transcription using mMessage Kit (Invitrogen). MCherry mRNA provided by Dr. Michael Tsang (University of Pittsburgh) was used as control RNA. 1 µl of RNA was micro-injected into the cytoplasm of 1 cell stage embryos.

### 2.5 DNA ISOLATION, PCR AND DNA SEQUENCING

For genotyping, genomic DNA was isolated from whole embryos or adult tail biopsies using DNeasy Blood and Tissue Kit (QIAGEN) and 30ng of DNA for used for each PCR reaction. Validation of retroviral mutant was performed by designing a genotype assay to amplify intronic and exonic regions at the site of retroviral insertion in fbln4a gene. Primers were designed using Design primer feature of CLC Main workbench version 6 (Table 4).

### 2.6 HEART RATE MEASUREMENT

Embryos were kept in individual dishes and individually removed from the 28°C incubator immediately prior to measurement to minimize the effect of temperature on heart rate. Heart rates were measure directly by observing the embryos under a stereomicroscope and counting heart beats for 1 min.
2.7 RNA ISOLATION AND RT-PCR

Total RNA isolation, total RNA was extracted using 500μl TRIzol reagent (Invitrogen) from a clutch of 30-40 embryos at different developmental stages. The samples were homogenized using a plastic homogenizer pestle (USA Scientific). TRIzol separates the lysates, on addition of phase separator bromochloropropene (BCP) into 3 phases: a lower red organic phase, an interphase containing DNA, and a colorless, upper aqueous phase with RNA. After 10min storage at room temperature, the suspension was centrifuged at 10,000g for 15 min at 4°C. The upper phase was transferred into a new tube and mixed with RNase-free 70% ethanol. The samples were next transferred to RNeasy Minelute spin column as part of the RNA Mini Kit (QIAGEN). Next the samples washed twice with 500μl 80% ethanol a couple of times. Finally, RNA was eluted in 15 μl RNase-free water.

The concentration of the RNA was measured by UV spectrophotometry using a Nanodrop 2000 instrument (Thermo Fisher Scientific). RT-PCR analysis determine efficacy of splice morpholinos was carried out on total RNA isolated from zebrafish embryos or adult tail biopsies using Trizol (Invitrogen), and 1μg of RNA was reverse-transcribed with Superscript IV First Strand Synthesis System (Thermo Fisher Scientific) to produce complementary DNA (cDNA), following the manufactures instructions. To each RNA sample (1μl), random hexamers (1μl, 50ng/μl), dNTPs, and DEPC-treated water (up to 10μl) were added before a 5 min incubation at 65°C. Before PCR, 10x RT Buffer (2 μl), MgCl2 (4μl, 25nM), dithiothreitol (DTT, 2μl, 0.1M), RNase OUT (1μl, 40U/μl), SuperScript III Reverse transcriptase (200U/μl; 1μl) were added to the mix. After incubation for 10min at 25°C, 50 min at 50°C and 5 min at 85°C, the reactions were chilled on the ice, briefly centrifuged, 1μl RNase H was added to each sample and the reactions were incubated
at 37 °C for 20 min. The cDNA reactions were stored in -20°C and 0.5 μl of this reaction was used in polymerase chain reaction (PCR). For direct visualization of amplification products, cDNA was amplified using standard PCR conditions and appropriate primer pairs of different exons (Table 4).

### 2.8 WHOLE MOUNT IMMUNOSTAINING AND WGA STAINING

Whole-mount embryos were fixed in 4% paraformaldehyde (PFA) at 4°C overnight. After fixation, the embryos were washed 2 times 5 min each in PBS containing 0.1% Triton X-100, followed by 1h wash in PBS containing 0.5% Triton X-100 at room temperature. After the wash, the embryos were blocked for 3 h at room temperature in blocking buffer containing PBS in 1% DMSO, 1% Triton-X 100, 0.2% BSA and 5% normal donkey serum. Embryos were incubated overnight at 4°C in a 1:250 dilution of Fbln4a primary antibody, and washed 4-6 times 30 min each in PBS containing 0.1% Triton X-100 (PBST). The embryos were again blocked in blocking buffer for 1h and followed by 3h incubation with Alexa 568 anti rabbit IgG secondary antibody at 1:250 dilution. Whole embryos were washed 4 times 15 min each in PBST at room temperature. For wheat germ agglutinin (WGA) staining embryos were washed 2 times with PBST containing Rhodamine labeled-WGA (Vector Laboratories, Catalog number - RL1022) at 1: 1000 dilution for 10 min each. Next, embryos were washed 2 times with PBST 10 min each and placed in PBS and imaged using an Olympus BX51 microscope and DP71 camera.
Table 4. Primers used for morpholino and genotyping validation

<table>
<thead>
<tr>
<th>RT-PCR for morpholino resulted exon splicing</th>
<th>Primer and Sequence</th>
<th>Tm, Extension time</th>
</tr>
</thead>
</table>
| **fbln4a c-DNA** | (forward fbln4a:exon1) 5’ CTCAGAAAGAGATCCACTG 3’  
(reverse fbln4a:exon4) 5’ CAGGCTTCAGTGATTGTC 3’ | 50°C, 30 sec |
| **fbln4b c-DNA** | (forward fbln4b:exon2) 5’ TGTGTATGTGACAGTGTTG 3’  
(reverse fbln4b:exon6) 5’ AAGAGAAAGATCTCTGGCA 3’ | 50°C, 30 sec |
| **actin c-DNA** | (forward actin) 5’ AATGAGCGTTTCCGTTGC 3’  
(reverse actin ) 5’ GGACAGGGGAGCGGCAAGAT 3’ | 50°C, 30 sec |
| **Genotyping of mutant line** | | |
| **fbln4a** | (forward fbln4a:intron1) 5’ GATATTTAGGAACCGTGTGA 3’  
(reverse fbln4a:exon3) 5’ CTTTGAGTGCTGTCTGTG 3’  
(reverse retrovirus) 5’ TCGGACAGACACAGATAA 3’ | 50°C, 60 sec |
| **RT-PCR for mutant line** | | |
| **fbln4a c-DNA** | (forward fbln4a:exon1) 5’ CTCAGAAAGAGATCCACTG 3’  
(reverse fbln4a:exon3) 5’ CTTTGAGTGCTGTCTGTG 3’  
(forward fbln4a:exon4) 5’ GACAATCACTGAAGCCTG 3’  
(reverse fbln4a:exon6) 5’ GACATTAACACAGCGATG 3’  
(forward fbln4a:exon8) 5’ CTACCTGTGCAATATCA 3’  
(reverse fbln4a:exon10) 5’ TCCATTGTCATCCAGA 3’ | 50°C, 30 sec |
2.9 PROTEIN EXTRACTION AND IMMUNOBLOTTING

Embryos were dechorionated by pronase and deyolked in PBS supplemented with a protease inhibitor cocktail (P1860, Sigma) by vigorous pipetting followed by a short centrifugation step. Protein lysate was extracted using a lysis buffer containing 20 mM Tris/Cl pH 7.5, 150mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% TritonX-100 and protease inhibitor cocktail (Sigma). Embryos were homogenized using a pestle homogenizer for 30-45 seconds and lysate was incubated on ice for 5 min. Supernatants were clarified by centrifugation at 14000rpm for 5 min in a microcentrifuge at 4°C. Protein concentration was measured using Bradford assay and samples were stored in -80°C. A total of 15µg of protein was loaded on a 4-15% SDS-PAGE gradient gel. Before loading samples were incubated at 95°C for 5 min in Laemmli Sample buffer (4x, Bio-Rad) and supplemented with 5% β-mercaptoethanol. The SDS-PAGE gel was subjected to electrophoresis at 100V for 75 min to 90 min. For transfer, the gel was positioned between packing pads, filter papers and polyvinylidene difluoride (PVDF) membrane and then placed into a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell for 120 min at 80 V on ice. Nonspecific binding sites on the membrane were blocked by incubation in Odyssey Blocking Buffer (Li-cor) for 1 h. Antibodies were diluted in Odyssey Blocking Buffer, containing 0.2% Tween 20 (Fisher). The incubation with primary antibodies was overnight at 4°C. The incubation with secondary antibodies was for 1h at room temperature. Blots were scanned using Li-cor Odyssey CLx Infrared imaging system, and band intensity was quantified and normalized using the Image Studio software. Primary antibodies used were anti-Fbln4a (anti-rabbit antiserum raised against the unique peptide sequence in the 1st calcium binding EGF domain) (1:1000), anti-Actin (C-11) (sc-1615, Santa Cruz)
Secondary antibody used for immunoblotting were IRDye 800CW donkey anti-rabbit IgG (H+L) (Li-cor) (1:10000), and IRDye 680LT donkey anti goat (H+L) (Li-cor) (1:10000).

2.10 IN SITU HYBRIDIZATION

Embryos were collected at different developmental stages: 30% epiboly, shield, 75% epiboly, tailbud, 2 somites, 4 somites, 10 somites, 24hpf, 30hpf, 48hpf, 72hpf. All the embryos were fixed in 4% paraformaldehyde/PBS for 2 days at 4°C. If necessary, the embryos were dechorionated with fine point tweezers in PBS and washed in methanol for 10 min at room temperature. Methanol wash was followed by dehydration in methanol, and stored at -20°C for in situ hybridization. Digoxigenin-labeled riboprobes were generated according to the manufacturer’s protocol (Roche, Indianapolis, IN, USA). Whole mount in situ hybridization was performed in an InSituPro VSi liquid handler (Intavis Inc, Chicago, IL, USA). Embryos stored in -20°C were subjected to pre-chilled acetone treatment at -20°C for 10 min. Acetone was replaced by methanol and embryos were incubated at room temperature for 10 min. Embryos were then washed in methanol twice at room temperature. Embryos were then rehydrated in steps starting with 50% methanol/PBS (5 min), 30% methanol/PBS (5 min), PBS (2 x 5 min), and PBTw (PBS + 1 gram of bovine serum albumin + 0.1% Tween-20) (2 x 5 min). Embryos were then hybridized at 65°C for 12-14h with riboprobe diluted 1:500 in hybridization buffer (50% formamide, 2x SSC, 0.1% tween-20, 5mM EDTA, 0.1% CHAPS). Embryos were then washed with 50% formamide, 2x SSC/0.3% CHAPS (2 x 30 min, 65°C), 2x SSC/0.3%
Specimens were then blocked with (5 % sheep serum (Sigma), 2% blocking reagent (Roche)/MAB) and incubated with embryo-adsorbed anti-digoxigenin, fab fragments antibodies coupled to alkaline phosphatase (Roche), 1:2000 in 5% sheep serum/PBT. Embryos were then washed 9-11 x 20 min with PBTw. The embryos were washed with staining buffer (100mM Tris-HCl pH 9.5, 100mM NaCl, 50mM MgCl2, 0.1% Tween 20) twice for 5 min at room temperature. Embryos were next placed in 6 well plates and incubated with substrate BM Purple overnight at 4°C (Roche Inc.). Stained embryos were fixed in 4% PFA and photographed using BX51 compound microscope with UPLFLN 20x/0.5 objective and DP71 camera (Olympus). All figures represent embryos that were simultaneously processed for fixation and staining. The following antisense riboprobes were generated scl (Liao, Paw et al. 1998), hand2 (Yelon, Ticho et al. 2000), nkx2.5 and gata4 (Chen and Fishman 1996).

2.11 TGFβR1 INHIBITOR TREATMENT

Tgfbr1 inhibitor LY-364947 (Calbiochem) was solubilized in pure DMSO to 20mM final concentration. For treatment of morpholino injected embryos LY-364947 was diluted in zebrafish fish water containing 1mM Tris-HCL pH 7.4, 1% DMSO, 1x 30% Danieau buffer, 0.003% 1-phenyl-2-thiourea (PTU) to have working concentration of 10µM. From the 10µM working concentration further dilutions were made and embryos were exposed to (0µM-1µM in 1% DMSO) LY-364947 beginning at different time regimes starting with 6 hpf and examined at 48hpf. Treatments were carried out in 6 well-plates.
3.0 RESULTS

3.1 ZEBRAFISH FIBULIN-4 GENES

Zebrafish have two paralogous genes for fibulin-4, fbln4a and fbln4b. Comparative analysis of the fbln4 gene structure, the transcripts and the amino acid sequences of the human and zebrafish fibulin-4s shows a relatively high level of conservation. All three genes (human FBLN4 and zebrafish fbln4a and fbln4b) have 11 exons where the first is a non-coding exon. The three genes differ mostly in the sizes of the introns and the length of the 3’-untranslated region (Figure 6A). The pairwise amino acid sequence identity among the three fibulin-4 proteins is high (65-73%, Figure 6B), with the exception of a unique insert within the first calcium-binding epidermal growth factor-like (cbEGF) domain highlighted in the multiple alignment (Figure 7A). These unique peptides have been used as antigens to generate antibodies against the Fbln4a and Fbln4b proteins. Given that Fbln4a was slightly more similar (68%) to the human FBLN4 protein than Fbln4b (65%), it is more likely to be the ortholog of human FBLN4 and therefore I focused my subsequent studies on Fbln4a.
Figure 6. The structure of zebrafish fibulin-4 genes.

(A) The human and zebrafish fibulin-4 genes drawn to scale (cartoons) and key characteristics of each gene (table). The following transcripts with Ensembl (and RefSeq) identification numbers are represented: *Dr. fbln4a*: ENSDART00000134098.1 (NM_001008587); *Dr. fbln4b*: ENSDART00000139460.2; *Hs. FBLN4*: ENST00000307998.6 (NM_016938). Horizontal lines: introns; long vertical bars: exons; short vertical bars: non-coding exons; *Dr: Danio rerio; Hs: Homo sapien*; kb: 1000 (kilo) base pairs; aa: amino acid residues. (B) Percent identity of the human and zebrafish fibulin-4 proteins compared by multiple sequence alignment.
Figure 7. Fbln4a protein structure and sequence alignment.

(A) Domain structure of the fibulin-4 protein. (B) Multiple alignment of the human and zebrafish fibulin-4 proteins, with protein accession numbers listed next to the protein name. Non-conserved amino acids are highlighted in pink and represented by stars in the consensus sequence. The peptide sequences used as antigens for antibodies against Fbln4a and Fbln4b are in blue boxes. Dashes show missing amino acids. Colored bars underneath the consensus sequence indicate fibulin-4 domains using the same color scheme as in panel A.
3.1.1 Developmental expression of zebrafish fibulin-4 RNA

To understand the role of fibulin-4 in embryogenesis, we first investigated how early the zebrafish *fbln4* genes are expressed. We performed semi-quantitative reverse transcription PCR (RT-PCR) for both zebrafish *fbln4* genes starting from one-cell stage to germ ring stage by using beta actin expression as a control (Figure 8). The reason we selected these stages is because the zygotic transcription starts by germ ring stage. Thus, one can differentiate between maternal transcripts and zygotic transcripts. The results for the RT-PCR showed that both *fbln4a* and *fbln4b* mRNA were present throughout this early developmental period. Because zygotic gene expression in zebrafish does not start until the mid-blastula transition (between the 1000 cell and germ ring stages), our results show that both *fbln4* genes produce maternal transcripts.

To further investigate the temporal and spatial expression patterns of *fbln4a* mRNA we collected wildtype zebrafish embryos at different stages and detected *fbln4a* expression by using whole mount in situ hybridization (WISH). We analyzed *fbln4a* expression during embryonic development from 30% epiboly to 2 days post fertilization (dpf) in wildtype embryos (Figure 9). WISH shows that *fbln4a* is a maternal transcript and localized to entire epiblast. At 75% epiboly, the expression is seen in the margins of the epiblast and hypoblast. At tailbud stage, the expression becomes prominent in the head regions and adaxial cells (Figure 9C-D). By 10 somites, the gene expression intensity increases in the adaxial cells adjacent to the notochord and the expression is seen along the entire length of the notochord (Figure 9G-J). At 24hpf, the *fbln4a* expression is seen in the head and in the myosepta, the connective tissue located between the muscle segments called myomeres (Figure 9K-L). By 30hpf, the expression in the head remains,
whereas the expression in the myosepta gradually becomes restricted to the tail (Figure 9M-N). Finally, at 48hpf, expression was observed in the head and the whole heart (Figure 9O-P). The presence of *fbln4a* transcripts in the adaxial cells close to the notochord suggests that the *fbln4a* gene is regulated by signals emanating from the notochord, perhaps the same signals that specify adaxial cells, such as sonic hedgehog (Shh) and echidna hedgehog (Ehh) (Currie and Ingham 1996).

**Figure 8. Developmental expression of the fibulin-4 mRNA.**

RT PCR analysis of the expression of *fbln4a* and *fbln4b* in unfertilized eggs and embryos up to the germ ring stage. RNA was pooled from at least 30 embryos at each time point, and beta actin was used as a loading control. 1k=1000
Figure 9. Developmental localization of \textit{fbln4a} mRNA.

Whole mount \textit{in situ} hybridization shows \textit{fbln4a} expression at various stages of development. (A) At 30\% epiboly \textit{fbln4a} mRNA is present throughout entire epiblast. (B) At 75\% epiboly the expression is concentrated on the margins of the epiblast (blue arrow) and hypoblast (red arrow). (C-F) At tailbud stage, the expression becomes prominently in the head (blue arrow) and in adaxial cells (red arrow). (G-J) During somitogenesis, expression remains in head (blue arrow) but is abundant in the adaxial cells (red arrow) throughout the length of the mesoderm up to the tail bud. (K-L) By 24hpf, the expression becomes dominant in the head (blue arrow) and posteriorly in the tail with higher expression in the vertical myosepta (red arrow). (M-N) At 30hpf the expression fades away in the head and moves further posteriorly in the tail. (O-P) By 48hpf there is no expression in tail but abundant in the head (blue arrow) and the heart (red arrow).
3.1.2 Developmental expression of zebrafish fibulin-4a protein

We further investigated the developmental expression of the Fbln4a protein using whole mount immunostaining (WMIS) to study and compare protein expression to the mRNA expression (Figure 10). WMIS of the wild type zebrafish embryos using anti-Fbln4a antibody revealed dynamic expression pattern of this protein throughout the development and delineated its presence during heart morphogenesis. At 30% epiboly, Fbln4a is expressed in the entire mesendoderm and matches completely the expression of the mRNA (Figure 10A). At tailbud stage Fbln4a is seen expressed in the axial mesoderm from the polster to the tail bud (Figure 10E-F). During segmentation, Fbln4a expression is maintained in the axial mesoderm and the notochord with more prominent signal in the polster (an anterior and lateral part of prechordal plate that underlies the forebrain during somitogenesis and gives rise to hatching gland) and tailbud cells than in the notochord (Figure 10G-L). Dorsal and dorsolateral views of the embryo show brightly stained cells with localization consistent with the polster, which surrounds the forebrain in an arc (Figure 10M-P). Whether the Fbln4a protein expression is intracellular or extracellular was not determined conclusively although testing with DAPI staining suggested that Fbln4a is deposited in extracellular puncta surrounding the polster cells (data not shown). During somitogenesis Fbln4a is localized to the notochord during development. At 24 hpf Fbln4a is found over the entire notochord sheath (Figure 12). Thus, I found Fbln4 protein to be localized to structures that express it with the two notable exceptions of the polster cells, which appear to sequester Fbln4 around them, and the notochord to which Fbln4 is bound following secretion by the adaxial cells.
Figure 10. Developmental expression of the Fbln4a protein.

Whole mount immunofluorescent staining (A, C, E, G, I, K and M-P) shows Fbln4a expression at various stages of development, with bright field images of the same embryos shown side-by-side for orientation (B, D, F, H, J and L) in lateral view with anterior to the left. (A, B) At 30% epiboly diffuse Fbln4a signal is seen throughout the epiblast. (C, D) By 75% epiboly Fbln4a is concentrated to the epiblast margins (red arrows) and the hypoblast of the head (blue arrow) to tail region. (E, F) At the tailbud stage the mesendoderm from head to tail is positive for Fbln4a. (G-L), During early segmentation, 2 to 10 somite (som) stages, Fbln4 is primarily localized to the head (blue arrow) and tail (red arrow) mesoderm. Dorsal (M-O) and dorsolateral (P) views of the head region show strong punctate labeling spreading posteriorly in an arc consistent with the polster (white dotted lines).
3.1.3 Validation of Fbln4a mutant embryos

To generate a vertebrate model for FBLN4-related cutis laxa in which we could analyze both embryogenesis and later stages of development, we obtained a zebrafish mutant containing a retroviral insertion in exon 2 of fbln4a gene (Figure 11A). The insertion breakpoints were validated by PCR amplifications across each insertion breakpoint and DNA sequencing (Figure 11A). Based on these data, a 3-primer genotyping assay was designed which accurately and robustly distinguished the mutant and wild type alleles in all three genotypes (Figure 11B).

RT-PCR of fbln4a across the insertion site produced no product in homozygous mutant embryos (Figure 11C,D), however, RT PCR products covering exons downstream of the insertion site showed equal intensity in WT and mutant samples (Figure 11C,E,F), suggesting that the retroviral insertion did not affect the stability of the mutant RNA. Nevertheless, immunoblotting of the homozygous mutant embryos at 24hpf and 48hpf indicated that protein expression was completely lost in this line herein referred to as fbln4a-/- (Figure 11G). A likely explanation for the lack of Fbln4a protein in our mutant animals is that the signal peptide is absent from the predicted mutant protein (Figure 11H). Without the signal peptide Fbln4a cannot enter the endoplasmic reticulum (ER) and will likely be incorrectly folded and unstable in the absence of the required chaperones and redox molecules residing in the ER. Alternatively, the mutant mRNA may be poorly translated, as it lacks a sufficiently strong match to the Kozak sequence (gccgccRccAUGG) (Kozak 1987) required for the recognition of the translation initiation site by ribosomes.
We further validated the \textit{fbln4a^{-/-}} mutants by performing WMIS on the embryos obtained from heterozygous parents. WMIS followed by confocal microscopy showed colocalization of Fbln4a with a notochord marker, wheat germ agglutinin in control embryos at 24hpf, but Fbln4a was lost from the notochord sheath in \textit{fbln4a^{-/-}} animals Figure 12. In summary, the zebrafish retroviral mutant line lacks any functional Fbln4a protein, and thus is a null mutant.
Figure 11. Validation of fbln4a retroviral insertion mutant.

(A) A diagram showing the location of the retroviral insertion downstream of the fbln4a start codon. Primers used for genotyping zebrafish embryos are indicated.  

(B) Genotyping of fbln4a +/+, +/-, -/- embryos. 

(C) Location of primer pairs used for RT-PCR on a diagram of the fbln4a transcript. 

RT-PCR products amplified between exons 1-3 (D), exons 4-6 (E), and exons 8-10 (F) from fbln4a+/+, fbln4a+-, and fbln4a-/- embryos. 

(G) Western blotting of fbln4a+/+ and fbln4a-/- zebrafish embryos. 

(H) A schematic view of the predicted protein products of the wildtype and mutant alleles.
Figure 12. Validation of fbln4a retroviral insertion mutant by whole mount immunostaining.

Confocal image of the whole mount immunostained embryos for Fbln4a antibody (green) which shows localization of the protein in the notochord sheath at 24hpf. Wheat germ agglutinin which stains the notochord was used as control (red).

3.1.4 fbln4a mutants does not exhibit any obvious phenotype

Homozygous fbln4a<sup>-/-</sup> mutant embryos obtained by breeding heterozygous parents displayed no noticeable morphological abnormalities compared to wildtype up to at least 9 days post fertilization (Figure 13). Furthermore, as a quantitative variable relevant to cardiovascular health we measured the heart rates of all the embryos obtained from the heterozygous breeding and later genotyped the embryos. We did not observe any significant difference in the heart rates between fbln4a<sup>-/-</sup> embryos or the fbln4a<sup>+/+</sup> embryos (Figure 13B). At 6dpf, the mutants developed normal swim bladder just like the wildtype equivalent embryos. Moreover, the offspring from heterozygous parents were raised to adulthood and tail-clipped for genotyping at 3 months and we observed no statistically significant deviation from the expected Mendelian ratios (Figure 13C). The adult
homozygous fish were in-crossed to obtain all homozygous offspring and we found that all the embryos were viable and fertile up to 1 year, suggesting that \textit{fbln4a} mutant embryos have complete gene compensation for the loss of \textit{fbln4a} (data not shown).

**Figure 13.** Zebrafish \textit{fbln4a} mutant embryos do not exhibit any obvious phenotype.  

(A) There are no obvious morphological differences between \textit{fbln4a} homozygous embryos and wildtype embryos up to 9 days and beyond up to adulthood (not shown). (B) No significant difference in the heart rates was observed between \textit{fbln4a}+/+ and \textit{fbln4a} -/- offsprings obtained from heterozygous parents at 48hpf (n=20, unpaired t-test). (C) At 4 months, offspring from heterozygous parents maintained mendelian ratios. The $\chi^2$ statistic has been calculated to assess significant difference between expected and observed Mendelian ratios of wildtype, heterozygous and homozygous embryos, observed no significant difference (P = 0.1884).
3.1.5 The protein product of the maternal \textit{fbln4a} mRNA is present up to the tailbud stage

To investigate the persistence of the protein product of the maternal \textit{fbln4a} mRNA, we compared embryos derived from heterozygous breeding to ones derived from homozygous breeding by performing WMIS for Fbln4a. We found that homozygous embryos derived from heterozygous breeding had maternal protein present up to the tailbud stage, but no Fbln4a was seen in embryos derived from homozygous breeding Figure 14. At 10 somite stage specific staining in the notochord was absent in the \textit{fbln4a}^{-/-} embryos obtained from both breeding schemes. Thus, we conclude that maternal Fbln4a may contribute to zebrafish development up to the tailbud stage, but zygotic Fbln4a is abundantly present during segmentation.
**Figure 14. Maternal Fbln4a is present up to tail bud stage.**

*fbln4a*<sup>+</sup> embryos obtained by breeding heterozygous parents show presence of maternal Fbln4a protein up to tailbud stage (blue and red arrows). *fbln4a*<sup>−</sup> embryos obtained by breeding homozygous parents do not show expression of Fbln4a protein at any stage of development.
3.1.6 Validation of fbln4a morpholino knockdown

To further investigate the developmental consequences of Fbln4a deficiency, we performed transient loss-of-function experiments using antisense morpholino oligonucleotide-mediated gene knockdown. We designed splice-blocking morpholino oligonucleotides to target the intron 3 donor site of fbln4a and the donor site of intron 4 of fbln4b as a negative control (Figure 15A). RT-PCR analysis and DNA sequencing of morphant embryos demonstrated complete skipping of exon 3 of fbln4a up to 24 hpf and partial skipping at 48hpf (Figure 15B). Exon3 skipping results in a frameshift and premature termination of translation yielding a premature stop codon in exon 4 resulting in truncated protein which would likely be dysfunctional. Immunoblotting for Fbln4a in morphant embryos showed that there is a 96% reduction in protein expression at 24 hpf and 89% reduction at 48 hpf in comparison to control morpholino injected embryos (Figure 15C). We further validated the knockdown effect by performing WMIS in morphants and we found that the Fbln4a protein was completely absent from the notochordl sheath (Figure 16).
Figure 15. Validation of morpholino knockdown of fbln4a by RT-PCR and immunoblotting.

(A) Design of the exon skipping strategy used to target the zebrafish fbln4a and fbln4b genes by injecting antisense morpholino oligonucleotides (MO) targeting splice sites. (B) RT-PCR analysis of fbln4a and fbln4b MO embryos showed a smaller product than controls (ConMO) as a result of exon skipping. Beta actin was used as a loading control. (C) Immunoblotting confirmed the knockdown at the protein level, with beta actin as a loading control. Relative protein Fbln4a normalized to actin and the control samples are shown below the immunoblot image.
Figure 16. Validation of morpholino knockdown of *fbln4a* by whole mount immunostaining.

Epifluorescent images of control morpholino injected embryos (A-B) show colocalization of Fbln4a immunostaining (A) and wheat germ agglutinin (WGA) staining (B) in the notochord sheath. In contrast, *fbln4a* morpholino injected embryos lack Fbln4a in the notochord (C), which, however, does stain with WGA (D) Non-specific staining is seen in the yolk sac in all images. Scale bars: A-B 1 mm; C-D 2 mm. Presence or absence of notochord staining marked by blue arrow
3.1.7 Transient knockdown of fbln4a exhibit cardiovascular defects

Based on pilot dose-response studies we found that the injection of 5 ng of fbln4a MO into wildtype zygotes produced a consistent phenotype characterized by a variety of cardiovascular abnormalities at 2 days post fertilization (dpf) (Figure 17D). Injection of the same amount of non-target control MO produced no phenotypes (Figure 17A). In addition, the fbln4a MO treated embryos suffered from reduced circulation, pooling of blood in the caudal vein plexus or around the heart, cardiac and hindbrain edema, vascular hemorrhage in the head, and underdeveloped segmental vessels by 48 hpf. The morphant embryos had a significantly (p< 0.0001) reduced average heart rate of 80-85 beats per minute compared to control MO injected embryos, which had average heart rate of 125 beats per minute (Figure 17G). Musculoskeletal anomalies included reduced embryo length, curved tail and rounded somites Figure 18. Knockdown of fbln4a results in rounded myomers. Despite a multitude of developmental anomalies, the survival of the morphant embryos was normal (Figure 17H).

To test the specificity of the observed phenotypes, we performed overexpression and rescue studies by injecting fbln4a and control mRNA into fertilized eggs. Injection of 100 ng fbln4a mRNA produced strikingly similar phenotypes to fbln4a MO, including blood pooling in the tail, pericardiac and hindbrain edema, and curved tail (Figure 17E), but did not affect survival. The injection of 100 ng mCherry (control) mRNA did not affect normal development (Figure 17B). However, co-injection of fbln4a mRNA with the MO rescued the morphant phenotype yielding 70% normal embryos (Figure 17E). These experiments provide two lines of evidence to support the specificity of the fbln4a morphant phenotype: (1) the ability to rescue the effect of MO knockdown by mRNA injection, and (2) the
similarity of the knockdown and overexpression phenotypes. Indeed, similar phenotypes have been observed for gain and loss of function mutations in both humans (Zweier, Sticht et al. 2007) and zebrafish (Kabashi, Lin et al. 2010).

**Figure 17. Knockdown and rescue of embryos injected with fbln4a MO.**

Injection of 5 ng control (Con) MO (A), 100 pg of control mRNA (B), or the combination of the two (C) did not affect the development zebrafish embryos. In contrast both injection of 5 ng fbln4a MO (D) and fbln4a mRNA (E) results in embryos with a range of cardiovascular and connective tissue abnormalities including blood pooling (arrow head) hindbrain edema (arrow head) and curved tail. In contrast the co-injection of fbln4a MO and mRNA rescued the embryos (F). In all panels, head faces left and dorsal is up. Scale bars: 1 mm. The dose and the composition of the mRNA and morpholinos are indicated on the images. (G) The heart rate of control (Con) and fbln4a MO injected embryos were recorded in beats per minute (bpm) units. ****p=0.0001 (H) Quantification of embryos in percentages with normal, abnormal and dead categorization. The number of embryos assessed in each group is shown below each bar. All data were collected at 48hpf.
Based on the early expression of \textit{fbln4a} in relevant tissues, such as the paraxial and head mesoderm, we hypothesized that the observation of late stage cardiovascular phenotype might be a consequence of impaired specification of cardiovascular progenitors located in the lateral plate mesoderm whose expression is initiated during somitogenesis. In the \textit{fbln4a} morphant embryos the expression of \textit{hand2}, which marks the entire zebrafish heart forming region (HFR) (Yelon et al. 2000) is expanded both in the anterior and posterior directions (Figure 19C-D). The expression of the heart-specific transcription factors such as \textit{nkx2.5} and \textit{gata4} were increased (Figure 19A-B, G-H). In contrast, expression of the \textit{scl}, a marker of hematopoietic and vascular progenitors (Liao et al. 1998) showed decreased and disorganized expression pattern (Figure 19E-F). Our findings suggest that Fbln4a limits cardiac and enhances vascular cell fates.

\textbf{Figure 18. Knockdown of fbln4a results in rounded myomeres.}
Figure 19. Knockdown of *fbln4a* disrupts the fate decision of cardiovascular progenitors during somitogenesis.

The expression of *nkh2.5* (A, B) *hand2* (C, D) *scl* (E, F) and *gata4* (G, H) were assessed by *in situ* hybridization in embryos injected with 5 ng of control (Con) or *fbln4a* MO. The embryos are oriented to show the anterior lateral plate mesoderm in dorsal view. Overlapping parts of the heart field are marked by *nkh2.5*, *hand2*, and *gata4*, and show expansion in morphant embryos, whereas *scl* marks the blood and vessel progenitors which are reduced and abnormally positioned in morphants. The proportion of embryos showing the indicated changes are shown in the bottom right corner of each image.
3.1.8 Fbln4a heterozygous embryos are sensitized to, but homozygous mutants are resistant to MO treatment

To further investigate the molecular basis of the lack of phenotype observed in fbln4a mutants, and provide additional evidence for the specificity of the morphant phenotype we injected fbln4a MO into fbln4a+/+, fbln4a+-, and fbln4a-- embryos and assessed the consequent phenotypes at 48hpf. Wildtype embryos injected with low dose (3ng) of fbln4a MO did not exhibit any noticeable gross abnormalities (Figure 20B), whereas we observed characteristic cardiovascular and connective tissue defects in fbln4a+/+ embryos injected with the optimal dose of 5ng fbln4a MO (Figure 20C), similar to earlier results. Unlike in wildtype embryos, 3ng MO treatment led to the development of anomalies in 55% of fbln4a+- heterozygotes (Figure 20E) including shortened body axis, pericardiac and caudal blood pooling and hindbrain edema (Figure 20L-N) whereas 5ng MO dose resulted in anomalies of similar severity and frequency of as in fbln4a+/+ embryos (Figure 20F). Homozygous embryos obtained by breeding homozygous adult parents did not show any abnormalities at any morpholino concentration (Figure 20G-I). Quantitative and statistical analysis showed highly significant (p<0.0001) differences between phenotype frequencies in fbln4a+- embryos treated with 3 ng MO compared to either fbln4a+/+ or fbln4a-- fish with the same treatment (Figure 20O), as well as fbln4a+- fish injected with 5ng MO compared to fbln4a+/+ and fbln4a+- embryos injected with the same dose of MO.
In summary, we identified an optimal dose of the *fbln4a* MO, which had no effect on *fbln4a* mutant embryos but caused clear cardiovascular defects in the WT, indicating that the morphant phenotypes were specific and not due to off-target effects, which are expected to be detected in *fbln4a*⁻/⁻ animals. The observation of a phenotype at low doses in the heterozygous embryos which contain half as much *fbln4a* further supports the specificity of *fbln4a* MO. The lack of MO phenotype in *fbln4a*⁻/⁻ embryos suggest that the discrepancy in mutant to morphant phenotype can be due to activation of compensatory developmental genes or pathways in homozygous mutants. Thus, the results validate the specificity of both the mutation and the MO reagent.
Figure 20. Effect of *fbln4a* morpholino on wildtype, heterozygous and homozygous embryos.

Gross morphology of *fbln4a*+/+ (WT) (A-C), *fbln4a*+/− heterozygous (D-F), and *fbln4a*−/− homozygous (G-I) embryos injected with 0 (A, D, G), 3 (B, E, H) and 5 ng (C, F, I) of *fbln4a* MO. Higher magnification images of the head (J, L) and tail (K, N) illustrating normal morphology in WT embryos (J, K) and cardiac and hindbrain edema and tail blood pooling (arrowheads) in heterozygous embryos injected with 3 ng of *fbln4a* MO (L, N). In all panels, head faces left and dorsal is up. Scale bars represent 250µm in panels A-I, and 100µm in panels J-N. All the embryos at 50hpf. Quantitatvie analysis of the frequency of developmental anomalies in untreated and MO treated fish with different genetic backgrounds (O). Pairwise χ² tests were used to compare counts of abnormal embryos between the groups (***p< 0.0001, NS- not significant). The total number of embryos assessed in each group is shown below each bar.
3.1.9 Rescue of fbln4a morphants by Tgfbr1 inhibition

Because elevated TGFβ signaling has been shown in aortic and lung tissues of human patients with FBLN4 recessive mutations (Renard, Holm et al. 2010), we adopted a pharmacological approach to evaluate the effect of zebrafish fbln4a knockdown on Smad dependent canonical TGFβ pathway. For this we decided to inhibit TGF receptor 1 (Tgfbr1) kinase activity using a small molecule inhibitor, LY-364947 through which the canonical signaling takes place. We chose this chemical genetic approach because there are 2 tgfbr1 genes in zebrafish, tgfbr1a and tgfbr1b. With the use of LY-364947 we are able to inhibit both paralogs with 1 drug as kinase domains of both proteins is highly conserved. We used LY-364947 because it specifically targets the Tgrbr1 kinase function and which is, in contrast to other Tgfbr1 inhibitors, much less potent against related kinases such as Tgfbr2 (Singh, Chuaqui et al. 2003, Li, Wang et al. 2006) and has been previously shown to be effective in targeting canonical TGFβ signaling in zebrafish embryos (Zhou, Cashman et al. 2011). We employed an LY-364947 dilution series ranging from 0 to 1µM to ConMO to asses toxic effects or to detect any phenotypes at this range of concentration. Treatment of ConMO injected embryos with 0.25µM, 0.5µM, 0.75µM and 1µM of LY-364947 from 6hpf up to 48hpf had no effect on normal morphology of injected embryos as expected at 2dpf (Figure 21). This is consistent with our previously published dose-response studies on LY-364947 treatment in zebrafish, which showed no phenotypic consequences with treatment concentrations up to 10µM (Willaert, Khatri et al. 2012). However, fbln4a morphants treated with both 0.5 µM, 0.75 µM and 1µM LY-364947 showed gross morphological rescue of embryo length, blood circulation, no
caudal and heart blood pooling and normal head development when examined at 2dpf (Figure 21). The dose-response of the rescue experiments indicated 0.5 µM as the optimal concentration of LY-364947 required to reverse the morphological defects seen as a result of fbln4a loss.

Figure 21. Tgfbr1 inhibition rescues fbln4a morphants.  
A minimum of 50 ConMO (A) and fbln4aMO (B, C) injected embryos were used for each drug treatment. A dilution series of LY364947 was used for the experiment to determine the optimal dose of the drug. Morphant embryos were rescued by 0.5-1µM LY364947. Scale bar = 250µm.  
(D) The proportions of embryos with normal physiology shown as percentages at each drug concentration.
3.1.10 Identification of critical time regime for LY-364947 rescue

In order to determine the critical time period during which manipulation of TGFβ signaling rescues \textit{fbln4a} morphants, I performed drug treatment experiments for various periods of time (Figure 22). As described above, treatment of \textit{fbln4a} MO injected embryos starting from 6 hpf up to 48 hpf rescued 70% of the \textit{fbln4a} morphants (Figure 21D). Later, we divided the treatment periods into 2: 6-18 hpf and 18-48 hpf. The 6-18hpf treatment showed similar rescue percentage as 6-48 hpf while 18-48 hpf treatment did not show any rescue of \textit{fbln4a} gross phenotype (Figure 23A,B). Next, the time period of 6-14 hpf was again split into 2 four-hour treatments (6-10 hpf and 10-14 hpf) and 2 eight hour treatments (6-14 hpf or 10-18 hpf). Out of all the 4 combinations only 6-14 hpf treatment showed rescue of \textit{fbln4a} morphants (Figure 23C-F) which span gastrulation and somitogenesis stages of development. These results suggest that role of \textit{fbln4a} in cardiovascular development via TGFβ signaling is stage dependent between gastrulation and segmentation. Further, testing of normalization of the abnormal cardiovascular markers is required to confirm direct relation between altered TGFβ signaling and cardiovascular development lesions in \textit{fbln4a} morphants.
Figure 22. The critical period for rescuing *fbln4a* morphants by Tgfbr1 inhibition.

The time scale showing different time windows used for drug experiment as indicated in hours post fertilization. Green bars indicate rescue and red bars show no rescue.
Figure 23. Quantification of LY-64947 treated fbln4a morphants.

Graphical representation of the percentage of embryonic zebrafish with fbln4a specific abnormalities when treated by LY364947 drug was at 0.5µM and 1µM concentrations for 6-18 hpf (A), 18-48 hpf (B) 10-18hpf (C) 6-10 hpf (D) 10-14 hpf (E) and 6-14 hpf (F). The number of animals in each group is shown below the bars. Pairwise $\chi^2$-tests were performed to compare groups (**p < 0.001). Monster: embryos with severe, non-specific anomalies.
3.2 DISCUSSION

In this study we describe for the first time the characterization of a stable fbln4a mutant zebrafish. We find that, contrary to transient knockdown studies performed in this project showing role of fbln4a in cardiovascular development and studies performed by Zhang et. al 2015 showing role of fbln4a in convergent extension, homozygous fbln4a mutant embryos display no discernible convergent extension defects or cardiovascular abnormalities, survive into adulthood and are fertile. These embryos have a straight body axis and no overt morphological abnormalities. In contrast, transient knockdown embryos of fbln4a exhibit clear cardiovascular defects at 2 dpf including defects in blood circulation with reduced heartbeat, pooling of blood in the caudal vein plexus, and shortened body axis.

3.2.1 Fbln4a and tendon development

The expression of fbln4a in the myosepta as early as 24 hpf is remarkable. Vertical myosepta are tendons between the myomeres. A critical early differentiation marker of tendon cells is scleraxis-a (scxa) which starts to be expressed in the myosepta at 36 hpf. Therefore, fbln4a marks tendon progenitors prior to their differentiation to tenocytes. Interestingly, fbln4a morphants develop rounded myomeres by 48 hpf instead of the straight chevron-shaped muscle segments observed in wildtype fish (Chen and Galloway 2014). These observations are consistent with the requirement of Fbln4a for tendon development. In situ hybridization for key markers of tendon development (scxa, xirp2a) at the appropriate stages (24, 36, 48, 72 hpf) in morphant and control embryos will need
to be performed to define the precise developmental stage at which Fbln4a is required for the formation of tendons. Consistent with our results implicating Fbln4a in tendon development, both people and mice with Fbln4 mutations develop joint laxity and contractures associated with the disorganization of tenocytes and abnormal collagen fibrillogenesis (Markova, Pan et al. 2016).

3.2.2 Morphant and mutant phenotypic discrepancies

There can be several possible explanations for the discrepancy between morphant and mutant phenotype, a phenomenon that is increasingly being recognized and observed by various research groups for different genes (e.g. pak4, megamind, ift88, flt4) in targeted mutants (Kok, Shin et al. 2015). The discrepancies between mutant and knockdown approaches are not particular to zebrafish, but have also been seen in Drosophila and Arabidopsis (Yamamoto, Jaiswal et al. 2014, Gao, Zhang et al. 2015). The possible explanations for discrepancy include an unexpectedly non-pathogenic lesion, maternal contribution, gene compensation, and MO off-target effects. We have probed some of these possibilities in the fbln4a project.

To address the issue of non-pathogenic lesion we have extensively validated the fbln4a retroviral mutant. First, we identified retroviral genomic insertion site by sequencing the region of retroviral insertion. Next, we validated the gene disruption of fbln4a at the RNA level by amplification of the exons through the region of retroviral and confirmed the loss of transcription exons 1 and 2 which code for the signal peptide, the Fbln4a protein. Without the signal peptide, the Fbln4a protein would not be secreted into the ECM. WMIS confirmed that in fbln4a retroviral mutant the Fbln4a protein was not
detectable either as a result of inefficient translation or reduced protein stability. This finding rules out the possibility of residual protein function that can arise due to alternative splicing or any unanticipated functions of truncated proteins. Thus, by thorough validation we have convinced ourselves that this retroviral mutant is a complete loss of function or null mutant. Therefore, the lack of phenotype observed in mutants can either due to maternal transcript of \textit{fbln4a} or due to compensatory effects as yet unidentified.

To test the possible protective effect of Fbln4a maternal protein in rescuing the null mutants from any morphological defects, we tested the embryos for presence of Fbln4a protein by WMIS during early developmental stages. The results showed presence of the Fbln4a protein up to tailbud stage which suggests that if Fbln4a had an important role during gastrulation as suggested by Zhang et. al, the maternal protein would compensate for it and help in normal gastrulation processes in the mutant embryos. However, we were able to in-cross homozygous adults to obtain all normal homozygous offspring and grow them upto adulthood suggesting that compensatory mechanisms in the \textit{fbln4a} mutant overshadow the maternal effect of Fbln4a protein.

Gene compensation has been documented to be one of the reasons for phenotypic discrepancies between morphants and mutants. An example of such a scenario is study of \textit{egfl7} gene, where a null mutation in humans causes a mild partially penetrant defect in vascular stability, while wild type zebrafish embryos injected with \textit{egfl7} morpholino exhibit vascular morphogenesis defects. However, \textit{egfl7} mutant embryos are protected from the effects of \textit{egfl7} MO, due to compensatory effect by upregulated adhesion molecules only in the mutants due to the genetic loss of \textit{egfl7} (Rossi, Kontarakis et al. 2015). A similar scenario may exist for \textit{fbln4a} retroviral mutant embryos but further
experimental work is needed to identify the reasons for lack of any cardiovascular phenotype by identifying the type of compensation for \textit{fbln4a}. One possible way of compensation could be redundancy of \textit{fbln4a} gene with \textit{fbln4b}, the other isoform of zebrafish \textit{fbln4}. This could be done by checking the expression levels of \textit{fbln4b} gene in homozygous embryos quantitatively by qPCR and by immunoblotting. Alternatively, we could try inject \textit{fbln4b} morpholino at different doses into \textit{fbln4a} homozygous retroviral mutant and check if we observe any phenotype at lower doses. Also other compensatory genes or pathways which may potentially be playing protective role could be identified by performing and comparing transcriptome profiling of the knockdown embryos and homozygous mutant embryos to determine the possible explanation for phenotypic discrepancies.

In forward mutagenesis screens, adopted for zebrafish have been successful, with focus on cardiovascular phenotypes, compensatory mechanisms do not appear to come to be activated in many mutants (Stainier, Fouquet et al. 1996). However, it must also be noted that in certain large-scale zebrafish mutagenesis studies only 6\% of disruptive mutations in protein-coding genes have induced an observable phenotype (Kettleborough, Busch-Nentwich et al. 2013).

3.2.3 Specificity of MO effects

While MOs have been considered a landmark advance for their ease of usage in model organisms, problems with their application in zebrafish embryos emerged around 2010/2011. Most notably, MOs induce p53-dependent (tumor suppressor) apoptosis in a sequence-independent manner (Robu, Larson et al. 2007). However, not much is known
concerning the mechanism by which MOs induce p53 activation. Consequently, some researchers have started injecting p53 MOs to suppress the cell death effect but then the results interpreted are not in wild type background but on a background lacking p53. For our *fbln4a* project we have adopted an alternative approach to address this issue of possible off target effects of the *fbln4a* MO. We decided to utilize our non-phenotypic *fbln4a* mutant embryos by injecting optimal doses of *fbln4a* MO into them and comparing the MO effect between WT and homozygous backgrounds. We found that even at optimal dose of MO the homozygous embryos exhibit no cardiovascular defects as seen in the wildtype background, suggesting we do not activate p-53 inducing off target effects by use of MO and the effect is specific in the wildtype background. Conversely, the heterozygous embryos showed more susceptibility to the MO effects as they exhibit cardiovascular defects even at lower dosage with half as much genetic material further establishing the specificity of the MO reagent. In summary, we have established that morpholino effects we observe in heterozygous and wildtype embryos are specific for the loss of *fbln4a* function. These experiments also implicate molecular compensatory mechanisms as the most likely explanation for the lack of phenotype in the *fbln4a* mutant fish.

### 3.2.4 Fbln4a and TGFβ signaling

The range of phenotypes seen in *fbln4a* morphants such as defective blood circulation, blood pooling in the caudal vein and vascular hemorrhage could be reversed by using TGFBRI inhibition. This suggests that in the *fbln4a* morphant embryos there is an increase in TGFβ signaling. The rescue of the *fbln4a* morphants is stage dependent and
takes place only when TGFBR1 inhibition treatment is administered in the time window of 6hpf-14hpf spanning the gastrulation and somitogenesis stages in the zebrafish embryo. The heart and vascular morphogenesis begins at the end of gastrulation. The gene expression studies for fbln4a showed its expression in the prechordal plate at tailbud stage, in the adaxial cells adjacent to notochord during somitogenesis and in the heart at 24hpf-48hpf. The tgfb2 gene is expressed in the prechordal plate at tailbud stage and in the notochord during the somitogenesis which exactly matches the expression pattern of fbln4a gene expression supporting the hypothesis that fbln4a may directly or indirectly regulate TGFβ ligands. The tgfb3 gene is not detectable during gastrulation, but is active in the notochord during somitogenesis (1-13 somites) and in both the heart and notochord from at 24hpf (Cheah, Jabs et al. 2005).

Expression of all the TGFβ1/2/3 R-smads (Smad2, Smad3) and Smad4 transcripts through which TGFβ signaling takes place is seen since blastula as a consequence of maternal origin (Dick, Mayr et al. 2000). However, during gastrulation, they are either expressed at low levels or not detectable (smad3a and smad3b), while from tail bud stages the mRNA production increases. The expression smad2 and smad4 is seen throughout the entire embryo, but particularly in the head and tail (Hsu, Lin et al. 2011). Overlapping gene expression pattern of fbln4a and tgfb2 and tgfb3 ligand genes suggest possible genetic interaction between these components and further experiments need to be performed to establish the link.
3.2.5 Evolutionary fate of fibulin-4 paralogs

Many human genes have two paralogs in zebrafish as a result of a genome duplication that occurred in the common ancestor of teleosts approximately 320-350 million years ago (Glasauer and Neuhauss 2014). Given the extensive evolutionary time that elapsed since this genome duplication event, all non-essential gene duplicates have been lost. The remaining gene duplicates mostly evolved through subfunctionalization to undertake parts of the original gene function by assuming complementary expression patterns (Glasauer and Neuhauss 2014). However, some of the gene duplicates diverged by one of the two assuming a new function (neofunctionalization) occasionally by acquiring new protein domains (Kassahn, Dang et al. 2009). In some cases, both copies are retained with the same expression patterns and function to ensure correct expression level (dosage selection). Certain gene ontology (GO) functional groups have preferentially retained both paralogs, including genes involved in integrin signaling and growth factor activity (Kassahn, Dang et al. 2009). Given that fibulin-4 is involved in growth factor regulation, it is not surprising that both paralogs have been retained in zebrafish.
4.0 CONCLUSION

In summary, our work shows that even in the face of clear evidence of a potentially disruptive loss of function mutation of \textit{fbln4a} in zebrafish mutants, it is difficult to be unearth the role of this gene during embryonic development due to either maternal contribution or compensatory genes. However, the non-phenotypic retroviral mutant can act as a resource to test and establish the specificity of \textit{fbln4a} morpholino since it lacks the target for the morpholino. Transient knockdown of \textit{fbln4a} by morpholino exhibits specific cardiovascular defects in wildtype embryos which can be reversed by stage dependent inhibition of TGF\(\beta\) signaling using TGFBR1 receptor inhibitor drug suggesting increased TGF\(\beta\) signaling in knockdown embryos. However, further molecular testing of the TGF\(\beta\) ligand, receptor and downstream target genes is required to prove the detailed mechanisms of TGF\(\beta\) regulation by Fbln4a.
4.1 FUTURE WORK

4.1.1 Quantitative PCR analysis of TGFβ related genes in morphants and mutants

Based upon the observation that Tgfbr1 inhibitor drug treatment of fbln4a knockdown embryos at 0.5 μM or 1μM concentration rescues the morphants of their gross cardiovascular phenotype we would check for change in TGFβ receptors, ligands and target genes by quantitative PCR (qPCR).

Endpoints: qPCR for (1.) TGFβ receptor genes: transforming growth factor beta2 (tgfbr2), transforming growth factor beta 1a (tgfbr1a), transforming growth factor beta 1b (tgfbr1b). (2.) TGFβ target genes: col10a1, matn1, shox2, ctsh, smad6 and smad7 (3.) TGFβ ligand genes: transforming growth factor beta 1a (tgfb1a), transforming growth factor beta 2 (tgfb2), transforming growth factor beta 3 (tgfb3).

Method: The optimal dose of morpholino (5ng/embryo) would be injected into wild type embryos for transient knockdown. I would collect 50-60 embryos of fbln4a morphants at tailbud and 10 somite stage for RNA extraction. Following house keeping genes would be used: elf, gapdh, and bactin1. 3 biological replicates would be used for the experiment.

Expected results: We expect to see change in the gene expression for at least some of the components of the TGFβ pathway at the mRNA level.
4.1.2 RNA profiling analysis to identify the pathways activated by \textit{fbln4a} deficiency & developmental compensation

The aim of this study would be to compare the transcriptome profile of \textit{fbln4a} morphant and mutant embryos at 3 different developmental stages using RNA sequencing to identify gene expression profile. Also we would compare the embryos with and without the TGF\(\beta\)R1 inhibition to be able to compare the how the \textit{fbln4a} morphant embryos are affected by TGF\(\beta\) inhibition at the molecular level. Since RNA Sequencing does not rely on predefined probes, it allows for an unbiased discovery of new transcripts in the experiment of choice. This will allow us to identify the molecules underlying the different phenotypes observed in mutants versus the morphants at the same time explain the role of TGF\(\beta\) inhibition in \textit{fbln4a} morphant embryos.

**Endpoints:** Analysis of RNASeq results obtained after sending total RNA samples.

**Method:** The optimal dose of morpholino (5ng/embryo) will be injected into wild type embryos for injection. I would collect 50-60 embryos of \textit{fbln4a} morphants in 2 sets for RNA extraction at tailbud, 10 somite, and 48hpf stage. The 2 sets would include one with TGF\(\beta\)R1 inhibition and one without TGF\(\beta\)R1 inhibition. Also I would collect corresponding 50-60 \textit{fbln4a}\(^{-}\) embryos from homozygous parents at tailbud, 10 somites and 48hpf stage to know which genes or pathways compensate in an event of loss of \textit{fbln4a}. RNA sequencing will be performed by sending collected RNA samples to Genomic Research Core at the University of Pittsburgh.

**Expected results:** The results would better explain how does TGF\(\beta\)R1 inhibition rescues \textit{fbln4a} morphant embryos at the molecular level. Also the RNASeq experiment would
help us identify the molecules or genes that play role in compensation for loss of \textit{fbln4a} in the retroviral mutant for it to not show any phenotype.

\textbf{4.1.3 Testing cardiovascular markers in LY-364947 treated \textit{fbln4a} morphants}

In light of the observation that knockdown of \textit{fbln4a} gene in embryos exhibit change in early cardiovascular progenitors like \textit{hand2}, \textit{scl} and that drug treatment of ALK5 inhibitor in \textit{fbln4a} morphants rescues the embryos of their gross cardiovascular phenotype it would be logical to check if Fbln4a serves as a link between TGF\textsubscript{\beta} signaling and cardiovascular development. In order to answer this question, I would have to check for normalization of cardiovascular markers in \textit{fbln4a} knockdown embryos treated with LY-364947.

\textbf{Endpoints:} WISH for \textit{hand2}, \textit{scl}, \textit{nkx2.5} for \textit{fbln4a} morphant embryos at 10 somites (14hpf) treated with LY-364847 between 6hpf-14hpf.

\textbf{Method:} The optimal dose of morpholino (5ng/embryo) will be injected into wild type embryos for transient knockdown. Apply LY-364947 drug treatment starting from 6hpf-14hpf and fix 50-60 \textit{fbln4a} morphant embryos using 4\% PFA at 10 somite (14hpf) stage for WISH.

\textbf{Expected results:} If TGF\textsubscript{\beta} plays role in regulating cardiovascular progenitors in \textit{fbln4a} morphants then we would expect normalization of \textit{scl} and \textit{hand2} markers upon LY-364947 treatment in comparison to untreated morphants.


selectivity versus transforming growth factor-b eta type II receptor kinase and mixed lineage kinase-7." J Med Chem 49(6): 2138-2142.


