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The multiple EGF-like domain protein ITGBL1 is involved in heart development and modulates cellular response to collagen

Author
Dumas, Kevin

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The Multiple EGF-like Domain Protein ITGBL1 is Involved in Heart Development and Modulates Cellular Response to Collagen

by

Kevin Dumas

DISSERTATION

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“If I have seen further,

it is by standing on the shoulders of giants.”

-Sir Isaac Newton

This work is dedicated to my family,

whose unwavering support

and selflessness made my education possible.

And

To all the individuals who have mentored me,

that I may pass on to others the curiosity

and knowledge they shared with me.

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Contributions to thesis work

Chapter 2 is adapted from a manuscript in preparation. The work was done under the supervision of Joseph Shieh. Selena Martinez performed the breakpoint-spanning qRT-PCR experiment. Zebrafish lines were provided by the Stanier Lab at UCSF, with assistance from Xiaoyan Ge. Kevin Dumas performed all other experiments described in this dissertation.

Abstract

Congenital heart disease (CHD) is the most common form of congenital malformation and is observed in ~1% of the population. The genetics underlying congenital heart disease are in need of further characterization. A goal of our work in the Shieh Lab is to identify new genes involved in heart development and congenital heart disease and to provide data that sheds light on the function of identified genes at the cellular and organismal levels.

In this thesis, we begin with an introduction reviewing literature relevant to our investigation. To aid reader's understanding of the topics covered in this thesis, we provide a brief review of the following subjects: the genetics of congenital heart disease, left ventricular outflow tract disorders, integrin and ECM interactions and their influence on heart development, valve development and the TGF-β pathway. We also comprehensively review the known data on *ITGBL1*. We conclude by raising a series of questions that will be answered by experimental data in Chapter 2.

In Chapter 2, we describe the analysis of the poorly studied ITGBL1 gene and its role in cardiac development and cell behavior. ITGBL1 was identified as a gene with high expression in the aorta during embryonic development in mice, with particularly high expression noted in the endothelial cell layer of the aorta and the aortic valve. Our investigation identified that knockdown of itgbl1 in zebrafish leads to incomplete cardiac looping and increased ventricular width. Additionally, we identify an individual with a large, rare copy number duplication of the 5' end of ITGBL1 with hypoplastic left heart

disease. We identify that this duplication is transcriptionally active and creates a novel transcript across the duplication breakpoint.

We observe that overexpressing ITGBL1 in cells leads to a diminished ability to adhere to specific members of the extracellular matrix, suggesting that ITGBL1 can modulate cell interaction or response to its environment. Additionally, we see that overexpressing ITGBL1 decreases endothelial response to collagen, demonstrating ITGBL1's capability to modulate ECM-dependent cell behavior. We then identify that overexpression of ITGBL1 can influence signaling activity within the TGF-β pathway. Lastly, we discuss our findings and the relevance of our research.

In Chapter 3, we examine the implications of our research and discuss future directions that might yield further insight into the function and role of ITGBL1 in cardiac development.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

Introduction

Overview of cardiac genetics

With around 40,000 cases per year, congenital heart disease (CHD) is the most prevalent birth defect in the United States ("Data and Statistics | Congenital Heart Defects | NCBDDD | CDC," n.d.). While CHD is observed in about 0.8-1% of births (Hoffman and Kaplan, 2002; Montaña et al., 1996), the underlying cause is often unknown. Epidemiological studies provide strong evidence in favor of genetics playing a role in CHD, though the importance of environmental effects should not be overlooked. A national cohort study in Denmark found that first-degree family members had a 3 to 80 fold increased risk of having CHD compared to baseline population levels (Øyen et al., 2009). Importantly, the different heart abnormalities that comprise CHD differ in familial occurrence (Øyen et al., 2009; Peyvandi et al., 2014). Identification of multi-generation families in which predicted-pathogenic mutations associate with disease further increase confidence in the role of genetics in CHD (Blue et al., 2014; Perera et al., 2014; Xiang et al., 2013).

About 70-80% of congenital heart disease cases are observed outside of an identified syndrome and have no known etiological basis (Gelb and Chung, 2014; Nora, 1968). The genetics of isolated CHD has largely focused on single gene mutations, with insight coming from family data, animal model investigations, genetic screens, exome sequencing and epigenetic analyses. Single genes with roles in cardiac development and human disease include FBN1 (Dietz et al., 1991), TBX5 (Basson1 et al., 1997),

NOTCH1 (Garg et al., 2005), PTPN11 (Tartaglia et al., 2001) and others (Cowan and Ware, 2015).

Many genes with studies supporting a role in cardiac development are transcription factors (Clark et al., 2006) and signaling effectors of the major developmental pathways (Cowan and Ware, 2015). The signals provided by these proteins act in concert to differentiate cells within the heart and specify function. The complex web of interactions in the heart creates multiple opportunities for dysregulation and subsequent pathogenesis. A multifactorial model – in which multiple different genetic mutations, in concert with environmental insults, contribute to CHD – was proposed in the 1960s (Nora, 1968). As the scientific literature surrounding the genetics of congenital heart disease and heart development has matured, scientists have increasingly begun to appreciate the heterogeneous nature of CHD (Barron et al., 2009; Cowan and Ware, 2015; McBride et al., 2005). In addition to evidence from familial studies, strong support for a complex genetic etiology is presented in recent publications of broad genetic analyses in which numerous genes, many previously unassociated with heart development and CHD, were identified (Homsy et al., 2015; Y. Li et al., 2015; Zaidi et al., 2013).

Left ventricular outflow tract obstructions

Left ventricular outflow tract abnormalities (LVOTO) are a set of structural developmental malformations containing a wide spectrum of clinical severity and affecting the left side of the heart (McBride et al., 2005). Support for the role of genetics

in LVOTO comes from a variety of sources: First, epidemiological studies have shown that forms of LVOTO are highly heritable (Cripe et al., 2004; Hinton et al., 2007). Additionally, genome wide investigations have discerned that individuals with left-sided congenital heart defects may have an increased burden of rare structural variation within their genomes (Hitz et al., 2012; Payne et al., 2012; White et al., 2014). Exome sequence studies have hypothesized that individuals with LVOTO have a higher than expected burden of predicted damaging de novo mutations (Homsy et al., 2015). Intriguingly, the observation that family members of individuals with severe LVOTO have an increased disposition for other heart defects involving the left side of the heart – including aortic root dilation, aortic coarctation, transposition of the great arteries and atrioventricular septal defects – has led to the hypothesis that LVOTO is a spectrum of clinical abnormalities with a shared genetic etiology (Brenner and Kuehl, 2011; Hinton et al., 2007; Loffredo et al., 2004).

The most severe form of LVOTO is hypoplastic left heart disease (HLH). HLH is characterized by underdevelopment of 4 structures on the left side of the heart: the mitral valve, the left ventricle, the aorta and the aortic valve. Together, the underdevelopment of these 4 structures leads to a significant loss in blood flow to the body following birth, and HLH is fatal without surgical intervention (Barron et al., 2009; Fruitman, 2000). HLH has a reported incidence of ~0.02% (Brenner and Kuehl, 2011; Hinton et al., 2007; Morris et al., 1990). The genetic etiology of HLH is poorly understood. One gene explored functionally in HLH is NOTCH1 (Garg et al., 2005; McBride et al., 2008). Genes associated with HLH through targeted sequencing and other methods include FOXL1, FOXC2 (Iascone et al., 2012), GJA1 (Dasgupta et al.,

2001), ERBB4 (McBride et al., 2011), NKX2.5 (Elliott et al., 2003), RBFOX2 (Homsy et al., 2015), and others (Lahm et al., 2015).

Integral to the investigation of HLH is the etiological relationship between the four phenotypes of which it is comprised. A current debate exists over whether certain phenotypes are primary to others. More specifically, previous investigations in lamb, chicken and zebrafish models have demonstrated that reduced or abnormal blow flow through the outflow tract and atrioventricular junction can lead to abnormal development of distal structures within the heart (Hove et al., 2003), including the left ventricle (Fishman et al., 1978; Harh et al., 1973). These findings support the possibility of the ventricular and mitral valve phenotypes observed in HLH being secondary to abnormal development of the aorta or aortic valve. These findings are all the more interesting given the known role of NOTCH1 – a gene with convincing functional data supporting a casual role in the development of HLH – in aortic valve development (Garg et al., 2005). Further analysis of the proteins and signals present in and around the developing aorta and aortic valve are needed to better understand the etiology of HLH and LVOTO.

Extracellular matrix and integrins

The extracellular matrix (ECM) is a dynamic set of proteins, proteogylcans and other organic molecules that constitute the environment of cells. Cells secrete and receive proteins and signaling factors to and from the ECM. This complex signaling process changes during development and in response to various environmental stimuli. Changes in the ECM can influence cell behaviors including proliferation, migration,

adhesion and differentiation. The relevance of the extracellular matrix (ECM) in heart development can be seen from anatomical analyses of mouse knock out models. Mutations in genes such as hyaluronan (Camenisch et al., 2001), elastin (Hinton et al., 2010), fibrillin1 (Ng et al., 2004), fibulin-5 (Hanada et al., 2007), periostin (Snider et al., 2008), collagen1a1 (Hinton and Yutzey, 2011), and collagen 3a1 (Liu et al., 1997) all lead to abnormal valve phenotypes in mice. These findings demonstrate the necessity for environmental signals during development. More research is needed to better understand the signals controlling cell growth, migration, differentiation and survival during aortic and cardiac development.

Integrins are integral, membrane-spanning proteins that serve to coordinate cell interaction with their environment, including ECM proteins. Integrins are comprised of two heterodimer family sets: the α-integrins and the β-integrins. In vertebrates, 18 α subunits and 8 β subunits are present in the genome. Each β subunit will non-covalently bond to an α subunit to create a heterodimer capable of mediating cell-cell and cell-extracellular matrix interactions. β integrin subunits are composed of a cytoplasmic domain responsible for binding actin adapter proteins, a transmembrane domain, 4 EGF-like domains, a PSI domain, a hybrid domain and a β propeller domain responsible for binding to ligands in the extracellular matrix. Currently, the function of the EGF-like domains are thought to be structural - serving to separate the ligand binding domain from the cell membrane (Fu et al., 2012).

The necessity for integrins in cardiac and vascular development can be observed though hypomorphic and null allele mouse models. Itga5 loss of function mutations lead to abnormal blood vessel and aortic morphology (Yang et al., 1993). Itga4 null alleles

lead to epicardial abnormalities and cardiac hemmorage (Yang et al., 1995). Conditional Itgb1 knockout mice display abnormal ventricular morphology and dilated cardiomyopathy (Shai et al., 2002). These findings complement the observations from knockout models involving proteins that form major components of the ECM and demonstrate the rich interplay between integrins and the proteins that comprise the cell environment.

Valve development

Development and maturation of the aortic valve is a complex and incompletely understood process. Growth begins with the formation of the endocardial cushion from the endothelial layer of the outflow tract (de Lange et al., 2004). Within the endocardial cushion, signals from the TGF- β, NOTCH, WNT and BMP signaling pathways promote an endothelial to mesenchymal transition (EndMT) program, prompting an invasive and proliferative phenotype characterized by a diffuse extracellular matrix (ECM) structure (Hinton and Yutzey, 2011; Lincoln and Garg, 2014; Molin et al., 2003). Cells undergoing EndMT downregulate cell-cell adhesion molecules such as cadherins and degrade the basement membrane. This is followed by the appearance of migratory appendages such as lamellipodia and a migration into the ECM surrounding the endocardial layer ("Heart Development and Regeneration, 1st Edition | Nadia Rosenthal, Richard Harvey | ISBN 9780123813329," n.d.). In the later stages of valve development, valve leaflets thin and undergo significant ECM remodeling, ultimately forming the 3 layers observed

in normal, mature valves that are characterized by high in elastin, proteoglycans and collagen (Gross and Kugel, 1931; Kruithof et al., 2007; Schoen, 2008).

TGF-β signaling in valve development

The TGF-β signaling is a powerful developmental pathway capable of controlling cell proliferation, differentiation, apoptosis and other complex cellular behaviors. For all its potential, TGF-β has been shown to have highly context-dependent effects depending on the cell type and environment in which is it is studied (Massagué, 2012). While TGF-β has a demonstrated potential to influence cell behavior through several downstream effector proteins (Derynck et al., 2014), the background presented here will focus on canonical signaling.

TGF-β is secreted into the ECM in a latent form (Gentry et al., 1988; Lawrence et al., 1984), pre-associated with a latency associated peptide (LAP) (Lyons et al., 1988) and latent TGF-β binding proteins (LTBPs) (Saharinen and Keski-Oja, 2000). This latent complex integrates into the ECM to prevent unintentional activation. During activation, TGF-β is released from its latent form and processed (Dubois et al., 1995; Gentry et al., 1988; Lyons et al., 1988) into an active form capable of binding TGF-β receptors. After binding of TGF-β to the type-II receptor, the type-II receptor activates the type-I receptor (Wrana et al., 1994, 1992). This in turn phosphorylates Smad proteins which localize to the nucleus to initiate gene expression further directing cell behavior and function (Moustakas et al., 2001).

During development of the heart, TGFB1 and TGFB2 first appear in the endocardium before spreading to the endothelial cells in the endocardial cushions, while TGFB3 is present in the ascending aorta at later stages of development (Akhurst et al., 1990; Molin et al., 2003). Exogenous addition of TGFB3 to endothelial cells in collagen culture leads to cellular hypertrophy, migration and expression of markers of EndMT such as αSMA (Nakajima et al., 1999, 1997). TGFB2 deficient mice show hypoplastic aortic development as well as a double outlet right ventricle phenotype (Sanford et al., 1997), demonstrating its necessity for proper cardiac and outflow tract development. The relevance of proper TGF-β signaling in aortic development can also be ascertained through observation of diseases such as Marfan Syndrome, which is associated with high TGF-β signaling due to mutations in the ECM scaffold protein FBN1; and Loeys-Dietz syndrome, which is associated with mutations in the TGF-β receptor proteins TGFBR1 and TGFBR2 (Benke et al., 2013). While TGF-β signaling has been extensively studied, new context-dependent roles and signaling modifiers are still in need of characterization.

Introduction to ITGBL1

ITGBL1 “Integrin beta like 1” is a poorly characterized integrin-like protein. The protein was first reported in 1999 by Berg et. al (Berg et al., 1999). First identified after identification of two clones baring homology to integrin subunits in osteoclastoma and fetal lung cDNA libraries, ITGBL1 was cloned out in its entirety and found to be 2493 nucleotides long. This sequence includes 1485 nucleotides of open reading encoding

494 amino acid residues. The first 23 amino acids comprise residues typical of a signal peptide sequence. This is followed by 471 amino acids containing 10, tandem EGF-like cysteine-rich repeats (Berg et al., 1999). While initially described as containing one incomplete EGF-like repeat, follow up by Takagi et al. revealed that the 10^{th} EGF-like domain was complete (Takagi et al., 2001). The protein is predicted to have an N-linked glycosylation site at Asn 405.

Initial alignment by Berg, and more comprehensive analysis by Takagi, show that ITGBL1 bears significant homology to the EGF-like domains found in β integrins (Takagi et al., 2001). This relationship was reinforced by the presence of 8 cysteines per subunit, rather than the 6 cysteines found in other EGF-like domains. Intriguingly, other members of the ECM known to be important in cardiac development, such as laminin, fibrillin and latent TGF- β binding protein, also contain 8 cysteines, but the spacing of these residues does not mirror the spacing observed in ITGBL1 or β integrins. Of further interest to the biology of this protein is that no RGD sequence – a known integrin binding site – is present in ITGBL1 (Berg et al., 1999).

Expression analysis of the ITGBL1 transcript was performed via dot blot in the initial paper in which ITGBL1 was described (Berg et al., 1999). While evidence provided in the figure did not include a control blot, the authors state in their results section that transcript was only identified in the aorta.

More recently, ITGBL1 has been investigated in the context of cancer. A study in 2015 investigated the role of ITGBL1 in the context of bone cancer and metastasis. Investigation of gene expression datasets revealed that ITGBL1 expression is

correlated with higher risk of bone cancer metastasis. Furthermore, ITGBL1 expressing cancer cells injected into healthy mice were shown to decrease markers of lung metastasis while increasing the volume of osteolytic legions. Promoter mutation analyses demonstrated that ITGBL1 could be activated by RUNX2. Further research demonstrated that ITGBL1 promoted migration of cancer cells towards osteoblasts in a TGF-β dependent manner. This data was supplemented with relatively weak – but still intriguing – evidence that ITGBL1 could increase levels of TGFB1 and TGFB3, increase nuclear SMAD2 and increase Smad luciferase activity compared to controls (X.-Q. Li et al., 2015).

Within the context of non-small cell lung cancer, ITGBL1 has been shown to be downregulated in malignant tissues. These low levels of expression were also shown to correlate with reduced survival probability in cancer patients. Knockdown of ITGBL1 in cell culture was shown to promote cancer cell migration in scratch wound and trans-well invasion assays. Conversely, addition of ITGBL1 was shown to inhibit cancer cell migration and invasion (Gan et al., 2016).

Completing the current knowledge base on ITGBL1 are papers demonstrating the following: ITGBL1 was shown to be upregulated in adherent hematopoteic stem cell fractions compared to non-adherent fractions (Alakel et al., 2009). ITGBL1 was found to be expressed at significantly higher levels in adult human corneal endothelial cells compared to pediatric samples (Frausto et al., 2014). ITGBL1 was shown to be significantly upregulated in placental tissue during mid-gestation (Uusküla et al., 2012). ITGBL1 was found to be upregulated in an isoproterenol-induced model of cardiomyopathy and coincides with the development of fibrotic tissue (Galindo et al.,

2009). Lastly, ITGBL1 was also found to be part of a 6 gene signature that predicted cirrhosis risk in chronic Hepatitis B patients.

Conclusion

The high prevalence of congenital heart disease, coupled with high heterogeneity observed in CHD cases, demonstrates the need for identification of new genes and strategies for CHD. While many works have sought to identify new genes through unbiased, genome or exome-wide investigations, candidate genes are rarely subjected to little, if any, functional follow up to validate the true importance of candidate genes or suspected pathogenicity of candidate variants. This trend is related in part to the higher time and financial costs related to functional follow up. However, functional follow up is necessary to better understand the relationship between rare variants and their role in genetic disease and to shed new light on mechanisms of disease within CHD.

These observations prompted our investigation into the identification and functional analysis of a rare CNV identified in an individual with HLH. Specifically, our investigation centers on the functional analysis of the integrin-like protein ITGBL1, which was previously shown to have high expression in the aorta. This observation prompted the following questions regarding the role of ITGBL1 in heart development:

1) Is ITGBL1 expressed in the heart during embryonic development?

2) Does modulation of ITGBL1 dosage lead to abnormal cardiac development?

3) Does modulation of ITGBL1 dosage lead to changes in cell/ECM interactions?

4) Can ITGBL1 affect any signaling pathways known to influence cardiac development?

As a whole, the research described in this thesis demonstrates the significance of ITGBL1 during development. By testing the impact that modulation of ITGBL1 dosage can have at the cellular and organismal level, this work lays a foundation for future research exploring ITGBL1 in development and provides a model by which other investigators might follow up other intriguing CNVs identified in congenital heart disease and other disorders.

Chapter 2: The Multiple EGF-like Domain Protein ITGBL1 is Involved in Heart Development and Modulates Cellular Response to Collagen

Abstract

The genes encoding extracellular matrix components and adhesion-related signaling molecules serve important roles in the heart, as evidenced by human genetic disorders affecting the aorta and aortic valve, such as Marfan, Williams, Loeys-Dietz, and Weill-Marchesani syndromes. Here, we identify new functions of the multiple EGF-like domain protein ITGBL1 given its human aortic expression and integrin sequence homology. Our results indicate ITGBL1 is a highly-conserved protein expressed in multiple species with homology to the beta-integrin stalk region. Itgbl1 knockdown consistently affected zebrafish ventricular size while *itgbl1* RNA introduction ameliorated the abnormality, suggesting a role of *itgbl1* in zebrafish cardiac development. To identify potential molecular functions, human ITGBL1 expression and cell-based assays were performed. Expression data analysis for potential *ITGBL1*-associated pathways demonstrated significant enrichment for extracellular matrix, collagen and adhesion functional processes. We found that cells engineered to express ITGBL1 demonstrated selective differential adhesion to collagen compared to fibronectin. Furthermore, collagen-dependent vascular endothelial cell network formation was inhibited by ITGBL1, independent of cell number. ITGBL1-expressing cells decreased Smad reporter activity, suggesting ITGBL1 may influence the TGF-beta pathway and affect extracellular matrix-dependent signaling. To evaluate the potential influence of ITGBL1 on human outflow tract abnormalities, we assessed genomic structural variation in individuals with hypoplastic left heart for 13q33.1 variation. We show that *ITGBL1* is copy number variable in a hypoplastic left heart-affected individual and that the resulting truncated protein yields a continuing ITGBL1 function when tested in cell adhesion. These data

suggest that the multiple EGF-like protein ITGBL1 is important in the heart and functions by altering interaction with the extracellular matrix. To our knowledge, these results are the first description of *ITGBL1* potential roles in this highly-conserved organ.

Introduction

Congenital heart malformations can demonstrate various genetic contributions as evidenced by familial aggregation or identification of novel changes in candidate genes. Several recent studies have examined genes highly expressed in the heart as candidate genes, and these candidates may be important to investigate along with their molecular pathways (Homsy et al., 2015; Zaidi et al., 2013). Among left ventricular outflow tract obstructive heart lesions (LVOTO), a severe form is hypoplastic left heart, where the left ventricle is under-developed and the left-sided valves develop abnormally. LVOTO lesions may result from obstructed blood flow during cardiac development (Fishman et al., 1978; Harh et al., 1973; Hove et al., 2003), suggesting aortic valve formation is important to consider as a determinant of blood outflow and potentially of ventricular development. Interestingly, family members of hypoplastic left heart patients may have an increased predisposition for aortic valve abnormalities (Brenner and Kuehl, 2011; Hinton et al., 2007; Loffredo et al., 2004). Such observations have directed focus to molecular processes affecting the cardiac outflow tract.

The primordial valve develops from the endocardial cushion at the endothelial layer of the cardiac outflow tract (de Lange et al., 2004). Within the endocardial cushion, signaling from conserved pathways including TGF-β promote an endothelial to mesenchymal transition (EndMT) program, prompting a cellular phenotype characterized by a diffuse extracellular matrix (ECM) secretion (Hinton and Yutzey, 2011; Lincoln and Garg, 2014; Molin et al., 2003). Valve leaflets also undergo significant ECM remodeling, forming the layers of mature valves that are characterized by structural ECM components such as elastin and collagen (Gross and Kugel, 1931;

Kruithof et al., 2007; Schoen, 2008). Collagen is known to be important in human cardiac structures, as highlighted by valve abnormalities in individuals with mutations in collagen and select ECM modifying genes (Combs and Yutzey, 2009; Dagoneau et al., 2004; Deak et al., 1991; Hortop et al., 1986; Kuivaniemi et al., 1997). Collagens are key structural components of the elastic aortic wall, with collagen I, III, and IV and fibrillin making microfibrils that are important in function. The precise mechanisms by which cells and ECM interact in the development of the aorta and outflow tract are important and maintenance of these structures may also be related to developmental integrity. Recent studies of congenital heart disease have found a number of sequence variants in genes highly expressed in the heart, particularly in individuals with LVOTO (Hitz et al., 2012; Homsy et al., 2015). Since previous studies in lamb, chick and zebrafish models have shown that abnormal blood flow through the outflow tract can lead to alterations heart development (Fishman et al., 1978; Harh et al., 1973; Hove et al., 2003), we have focused on largely uncharacterized genes with high expression in the developing outflow tract. Here we describe *ITGBL1* and its functions and demonstrate its potential role in heart development and disease.

Integrin beta-like 1 (ITGBL1) is an integrin-like protein containing ten, tandem EGF-like domains, which bear significant homology to EGF-like domains found in β-integrins (Takagi et al., 2001). ITGBL1 lacks the transmembrane and cytoplasmic domains for integrin signal transduction and lacks an integrin ligand-binding domain. These features make ITGBL1 unique compared to other members of the β-integrin family and provide impetus for experiments helping to solve ITGBL1 mechanisms to date. Interestingly, ITGBL1 is a conserved protein with high expression in the human outflow tract (Berg et

al., 1999), suggesting a possible role in heart function. We here determine how ITGBL1 influences cellular behavior and examine ITGBL1's potential role in the heart and development.

Results

ITGBL1 is highly conserved between species

Comparison of the protein sequence of ITGBL1 by alignment of human (13q33.1), mouse (14qE5) and (zebrafish chr9) indicates a high degree of conservation (Fig 1A). Notably, all 80 cysteine residues that comprise the EGF-like domains within the protein are conserved from zebrafish to humans. The cysteine residues within the EGF-like domains of β-integrins, which closely resemble those of ITGBL1, are important for proper protein folding and function (Takagi et al., 2001), hence conservation of cysteine residues within ITGBL1 suggests an evolutionarily conserved structure and potential function of ITGBL1 even though ITGBL1 is only comprised of EGF-like domains. Given incomplete annotation for this conserved and potentially cardiac-expressed gene, we examined the locus in several species. Depiction of the *itgbl1* locus in zebrafish using the UCSC genome browser [assembly Zv9/danRer7] showed two, shorter transcripts with incomplete homology to the full length *ITGBL1* transcripts observed in humans and mice (Fig 1B). We performed RT-PCR analysis of *itgbl1* isoforms using zebrafish cDNA and identified a full length transcript (Fig 1C) homologous to mammalian transcripts. The presence of a full length transcript with alignment to the mammalian homolog suggests that *ITGBL1* has a common transcript across several vertebrate species and suggests further studies in these systems could help determine gene function.

ITGBL1 is expressed during heart development

Given the previous report of *ITGBL1* expression in the human aorta by dot blot (Berg et al., 1999), we examined *Itgbl1* expression in embryonic mouse heart by dissecting portions of the heart at E14.5, when the major cardiac structures are present. *Itgbl1* transcript was enriched in the cardiac chambers of the heart compared to control tissues such as the lung and liver. Higher relative levels of expression were identified in the outflow tract (aorta and pulmonary artery) (Fig 2A). We also examined ITGBL1 transcript data from the GTEx project (human) and the mouse Gene Expression Atlas (GTEx Consortium, 2013; Petryszak et al., 2014). Both datasets indicate ITGBL1 expression is highest in aortic samples compared to all other tissue and cell types assayed (Fig S1). ITGBL1 protein expression was also assessed by immunohistochemical (IHC) staining of mouse heart tissue sections. The aorta and pulmonary artery demonstrated marked staining, and the mouse aortic valve also demonstrated notable ITGBL1 signal (Fig 2B). Further protein assays using Western blotting also verified protein expression in human *ITGBL1*-cDNA transfected cells, suggesting antibody targets ITGBL1, and in human cardiac tissue (data not shown). Therefore, multiple lines of evidence support cardiac expression.

Itgbl1 is required for normal heart development in zebrafish

To test a potential role for *itgbl1* in heart development, we used Tg(cmlc2:rasGFP); Tg(cmlc2:dsred) cardiac fluorescent reporter zebrafish embryos and morpholino (MO) injection. *itgbl1* translation-blocking morpholino-injected embryos demonstrated increased ventricular size, decreased cardiac looping and cardiac edema compared to controls at 2 and 3 dpf, without other gross disturbance in embryo morphology. This

phenotype was consistently observed in a majority of embryos (Figs 3A and 3B). Cardiac phenotypes observed among zebrafish were enumerated with normal, mild, moderate and severe phenotypes (Methods), and *itgbl1* MO-injected embryos showed a marked increase of mild and moderate cardiac abnormality (Figs 3C and 3D). Importantly, these phenotypes were attenuated when *itgbl1* RNA was injected, demonstrating that *itgbl1* transcript largely rescues the knockdown phenotype.

We imaged control and *itgbl1*-morpholino fish hearts using confocal microscopy and compared cardiac dimensions. Knockdown of *itgbl1* led to an increase in the size of the ventricle (Figs 4A and 4B) and width of the atrioventricular junction, whereas the diameter of the outflow tract was unaffected (Figs 4A and 4B). Additionally, a modest increase in cardiomyocyte length was observed (Fig 4C), whereas the number of cardiomyocytes was not significantly affected (Fig 4D). These data suggest *itgbl1* is important in cardiac morphology and development in zebrafish and argue against a pure non-specific effect, as can sometimes be seen. Additionally, *itgbl1* morpholino injection into vascular-labeled Tg(FLK:GFP) zebrafish was performed and did not lead to gross vascular disruption compared to controls, but did lead to a modest reduction in intersomitic blood vessel length compared to dorsal vein length at 1dpf (data not shown). Therefore, the *itgbl1* MO had a more notable impact on zebrafish heart development without gross disturbance of the embryo, furthermore the RNA injection support that the effect is due to an effect on itgbl1, although further data would be interesting.

ITGBL1 **is copy number variable in an individual with hypoplastic left heart**

Since ITGBL1 is expressed in the human heart and is potentially important in development, we sought to determine whether the *ITGBL1* gene locus might be altered in individuals with congenital heart disease. Microarray analysis was performed on blood samples of nine individuals with hypoplastic left heart (HLH). Microarray analysis by comparative genomic hybridization originally revealed one individual with ~ 590 kb duplication in the 13q33.1 region involving *ITGBL1* and two other transcripts: *NALCN* and *NALCN-AS* (Figs 5A and 5C). This duplication was not present in microarray controls or in data from the 1000 genomes project. Similar sized overlapping duplications were only observed 3 times in data from intellectual disability case and control cohorts containing over 40,000 samples visualized in DBVar (Coe et al., 2014), suggesting this is not a common variant. This cytolocation was previously found to harbor copy-number variation in individuals with congenital heart disease (Lalani et al., 2013; Silversides et al., 2012; Xie et al., 2014). The 13q33.1 duplication we identified was further validated via SNP microarray we performed (Fig 5A) and by genomic DNA qPCR to confirm copy number (data not shown). We also utilized PCR and identified a DNA breakpoint whose sequence jointly maps to *ITGBL1* (intronic DNA between the 7th and 8th exons of *ITGBL1*) and intergenic DNA on the reference genome, confirming the duplication (Fig 5B). We found the duplication included the first 7 exons of *ITGBL1*, the candidate gene; *NALCN*, a gene whose function is seemingly not required for heart development (Aoyagi et al., 2015; Chong et al., 2015; Fukai et al., 2016; Lu et al., 2007); and NALCN-AS, a poorly studied non-coding transcript (Figs 5B and 5C). This finding suggested the possibility of a novel transcript that creates a truncated form of ITGBL1 if there is transcription across the duplication breakpoint. We tested this by RT-

PCR analysis, using tissue from the affected individual, and found the presence of a novel transcript extending from the duplicated 7th exon of *ITGBL1* into typically intergenic DNA (Fig 5D). This confirmed that the duplicated copy of *ITGBL1* was transcriptionally active (Fig 5C). This novel transcript was validated with Sanger sequencing of patient cDNA (Fig 5E) and subsequently by RNA-Seq from available patient tissue, demonstrating reads spanning the duplication breakpoint (data not shown). These data suggested that ITGBL1 can be affected by structural or copy number variation interrupting ITGBL1. The observation of a heart malformation and potential involvement of ITGBL1 should be further explored, as one candidate gene could lead to further understanding of a more complex molecular process.

ITGBL1 and its coexpressed genes are enriched for extracellular processes

To determine whether *ITGBL1* expression might be part of established gene expression programs, we sought to identify and analyze genes coexpressed with *ITGBL1*. We used gene coexpression analysis tools (Obayashi et al., 2008) to examine gene expression microarray data from multiple species and tissue types. We identified the top ~100 genes whose expression had the highest correlation with *ITGBL1* expression (Table S1). Functional enrichment analysis performed on the top coexpressed genes using ConsensusPathDB (Kamburov et al., 2011) revealed *ITGBL1* and coexpressed genes were highly enriched for ECM proteins and cell-environment interactions and also for developmental processes including cardiovascular and circulatory development (Table 1).

Table 1. *ITGBL1* and coexpressed genes are enriched for certain biological processes

Term Name*	Term GO ID	p-value	q-value
proteinaceous extracellular matrix	GO:0005578	1.73E-49	6.75E-48
extracellular matrix organization	GO:0030198	3.80E-35	1.14E-32
extracellular matrix structural constituent	GO:0005201	3.11E-20	7.47E-19
extracellular space	GO:0005615	1.67E-16	3.26E-15
skeletal system development	GO:0001501	2.53E-16	3.81E-14
cell adhesion	GO:0007155	1.69E-15	1.05E-13
multicellular organismal macromolecule metabolic process	GO:0044259	1.91E-15	1.92E-13
extracellular matrix disassembly	GO:0022617	2.71E-15	2.04E-13
collagen catabolic process	GO:0030574	3.86E-15	2.33E-13
multicellular organismal catabolic process	GO:0044243	1.19E-14	5.97E-13
sulfur compound binding	GO:1901681	1.32E-14	1.58E-13
collagen binding	GO:0005518	1.42E-14	4.68E-13
basement membrane	GO:0005604	1.81E-12	2.35E-11
integrin binding	GO:0005178	6.30E-12	1.04E-10
fibrillar collagen trimer	GO:0005583	2.83E-11	2.76E-10
cardiovascular system development	GO:0072358	5.21E-11	1.96E-09
circulatory system development	GO:0072359	5.21E-11	1.96E-09
vasculature development	GO:0001944	6.57E-11	2.20E-09
extracellular matrix binding	GO:0050840	1.52E-10	1.21E-09

*top20 categories from enriched processes list

Upregulation of *ITGBL1* expression leads to a substrate-specific decrease in cell adhesion

To identify potential functions of *ITGBL1* and determine whether it has a potential role in cell adhesion, we created expression constructs expressing wildtype *ITGBL1* or a shortened version of *ITGBL1* that models the novel transcript identified in the congenital heart patient. 293T cells were transfected with plasmids expressing *ITGBL1* (ITGBL1 FL), a truncated protein variant of ITGBL1 (Tr, containing the first six EGF-like domains or seven exons of human *ITGBL1*) or EGFP-vector (control), all with EGFP C-terminal tags. After verification of construct expression by blotting of transfected cell lysates (data not shown), we then plated transfected cells on ECM substrates to test *ITGBL1*

influence on cell adhesion. Cells transfected with *ITGBL1* showed a selective decreased adhesion when tested on collagens I and IV but not on fibronectin, suggesting specificity of ITGBL1 in affecting cellular interaction with members of the extracellular matrix. Transfection of the truncated *ITGBL1* demonstrated further inhibition in adhesion to collagen substrates compared to the full-length *ITGBL1,* suggesting that the truncated *ITGBL1* present in the patient might retain functional activity despite shortened protein length. Transfection of both the wildtype and short *ITGBL1* constructs exhibited the highest levels of inhibition to adhesion on collagen substrates. Adhesion to fibronectin was maintained across all transfection groups, suggesting the novel, truncated *ITGBL1* isoform may have additional influence on cell adhesion on collagen substrates (Fig 6).

***ITGBL1* expression affects collagen-dependent endothelial cell network formation**

Since we previously noted ITGBL1 protein staining in the endothelial layer of developing murine aorta – a collagen containing tissue – and ENCODE data support endothelial cell expression, we hypothesized that one site of action of ITGBL1 may be in endothelial cells. To determine whether *ITGBL1* might play a role in collagen-driven endothelial cell behaviors, we measured the influence of *ITGBL1* on the formation of endothelial cell networks that typically result in response to collagen I. Addition of collagen substrate normally leads to endothelial cell network formation akin to organization of endothelial cells for primitive vasculature (Davis and Senger, 2005). HUV-EC-C (human vein endothelial) cells were electroporated with *ITGBL1*-expressing or control plasmids and monitored for their capacity to form tube-like networks with collagen substrate. HUVECs with *ITGBL1* showed a diminished capability to form tube-like networks compared to

control vector electroporated cells (Figs 7A and 7B). This decrease in cellular network formation was not due to changes in live cell numbers (Fig 7C). These data suggest *ITGBL1* can influence endothelial cell behavior with collagen as the extracellular matrix, and this potentially could serve as a model for differences in endothelial/vascular development. We also performed qRT-PCR on *ITGBL1* and control electroporated HUVECs and noted expression of ITGBL1 led to decreases in *ITGA11* and *FBN1* transcripts, which encode a collagen-binding integrin subunit and ECM component, respectively (data not shown).

Expression of ITGBL1 alters Smad activation

Because TGF-β signaling is intimately related to cell interaction with the extracellular matrix and has been implicated in human diseases involving the aorta, such as Loeys-Dietz and Marfan syndromes, we also assessed the influence of ITGBL1 expression on TGF-β pathway. To assess the potential influence of *ITGBL1* on the TGF-β pathway activity in culture, we used pathway-responsive NMuMG cells with control or *ITGBL1*-expressing plasmids, as well as reporter plasmids expressing Smad-responsive luciferase and constitutively-expressed renilla luciferase as transfection control. A significant reduction of Smad reporter luminescence was observed when *ITGBL1* was present. These data show ITGBL1 is potentially capable of influencing the downstream signaling effectors of the TGF-β pathway and suggest a possible means through which expression of *ITGBL1* could influence behavior of these cells. Given these initial studies on ITGBL1 functions, further studies may determine how ITGBL1 influences both more complex behavior of cell types and cell-matrix interactions.

Discussion

Here, our results support a role of the highly conserved protein ITGBL1 in the heart. The robust expression of ITGBL1 we observed in the mammalian outflow tract complements data from human tissue (GTEx) (GTEx Consortium, 2013) and the mouse Expression Atlas (Petryszak et al., 2014) showing *ITGBL1* is expressed highly in the aorta. Given the high level of expression of ITGBL1, and its ability to influence cell/ECM interactions, we propose that ITGBL1 may be involved in outflow tract development or ongoing aortic physiology and maintenance.

Our data suggest ITGBL1 may affect interaction with the extracellular matrix, which is important during development and later ages. In *ITGBL1* gene coexpression analysis, we noted that ECM genes were highly enriched. This may mean that ITGBL1 is part of an ECM expression program governing cell behavior in the aorta. The integrity and structure of the outflow tract are intimately tied to ECM proteins, as seen in several aorta and valve phenotypes observed in mouse models with altered ECM components (Combs and Yutzey, 2009). Furthermore, mutations in ECM component in humans can lead to abnormalities of the outflow tract, as seen in individuals with Williams syndrome. It is possible that ITGBL1 may be a novel ECM component important in the aorta.

The ten tandem EGF-like domains that comprise ITGBL1 have significant homology to the EGF-like domains found in beta integrins, which is intriguing given our results regarding *ITGBL1*'s influence on adhesion. The conservation of cysteine domains within the protein suggests a conserved structure for ITGBL1. The role of these domains in traditional beta integrins is thought to be structural, serving to separate the distal, ligand-binding domains of beta integrin proteins from the cellular membrane and

mediate conformational changes related to integrin activation. ITGBL1, which lacks domains other than the EGF-like domains, could potentially serve a different role.

We found ITGBL1 alters cellular adhesion on specific members of the ECM and is capable of influencing endothelial cell behavior on vascular-expressed collagens. ITGBL1 is predicted to be secreted and is observed in cell supernatants by western blot (data not shown). It is possible that ITGBL1 is a member of the ECM and modulates adhesive signals to cells bordering collagen substrate. It might compete for collagen receptors without providing the tension-related signals created by collagen, thus causing decreased adhesion. Interestingly, we found that between E12.5 and E17.5, ITGBL1 and several collagens are upregulated in murine valve tissue (our analysis of cardiac expression data by Chakraborty et al.) (Chakraborty et al., 2010). It is thus possible that ITGBL1 might influence cell/ECM interactions during valve maturation. In keeping with a potential cell-ECM role for ITGBL1, Gan et al. recently examined *ITGBL1* in lung cancer cells and found that recombinant ITGBL1 reduced invasion and migration, both potentially ECM-dependent behaviors (Gan et al., 2016). In all, the findings suggest ITGBL1 and other coexpressed proteins may be part of an ECM expression program with the potential to influence substrate-driven cell behavior.

Our results indicate that *ITGBL1* can influence Smad signaling, an important component of the TGF-β pathway. Interestingly, Li et al. reported in breast cancer cell studies that *ITGBL1* affects TGF-β signaling (X.-Q. Li et al., 2015). It is possible that ITGBL1 has a role in modulating the TGF-β pathway and may be involved in EndMT - a TGF-β dependent process adopted by endothelial cells during the early stages of valve development. Given the expression of ITGBL1 in the aorta and these results, it is

interesting that mutations in genes related to the TGF-β pathway are linked to abnormal outflow tract phenotypes, such as in Marfan syndrome and Loeys-Dietz syndrome. Interestingly, RBFOX2, which was recently associated with hypoplastic left heart is involved in EMT (Braeutigam et al., 2014; Homsy et al., 2015). With a growing body of associations between genetic abnormalities in the TGF-β pathway and abnormal cardiac or aorta development (Table S3), it will be interesting to see if *ITGBL1* variation may play a further role in this system.

Outflow tract function relies on the formation of the aorta and anatomy of the aortic valve more proximally. The classic experimental results in chicken and lamb (Fishman et al., 1978; Harh et al., 1973) that demonstrate restriction of the aorta can lead to ventricular hypertrophy and, if done during development, can lead to left ventricular hypoplasia are interesting. We identified a rare copy number variant affecting *ITGBL1* in a hypoplastic left heart patient, and it is possible that alterations in *ITGBL1* have contributed to this lesion in the outflow tract, however further studies are needed given the rarity of this copy number variant. Further study into this gene and its coexpressed partners could also provide insight into the role in outflow tract development.

Materials and Methods

Mouse RNA analysis

Total RNA was isolated from dissected mouse organs (E14.5) using phenol/chloroform extraction and precipitation. cDNA was prepared using SuperScript III reverse transcriptase (Life Technologies) following manufacturer's instructions. qRT-PCR was performed using Taqman (Life Technologies) primers for *Itgbl1* (Mm00520935_m1 and Mm01200043_m1) and for *Gapdh* (Mm99999915_g1). Relative expression was calculated using *Gapdh* as a control and then plotted relative to kidney (lowest value observed).

Mouse Immunohistochemistry

E14.5 mouse heart sections were rehydrated and heated in citrate solution. Slides were blocked in MOM blocking reagent (abcam, ab127055) for 1 hour and exposed to anti-ITGBL1 antibody (Sigma, AV42196) at a 1:200 dilution for 1 hour. Visualization was performed using anti-rabbit IgG secondary antibody conjugated to HRP and DAB reagent (Vector Laboratories, SK-4100).

Zebrafish Phenotyping

Zebrafish Tg(cmlc2:rasGFP), Tg(cmcl2:dsnuclearRed) and flk1:EGFP strains were used for fluorescent visualization of cardiac and vascular structures and were housed at 28°C following standard procedures. Antisense morpholino oligonucleotides (MO) were designed against zebrafish ITGBL1 (GeneTools). Morpholino designed against target sequence, gcatgtttagatccccaccagaaac, overlaps the start codon and 5' UTR of the transcript. MO was dissolved in water for a 1mM stock solution and diluted to 0.2mM prior to injection. 30ng/ul of ITGBL1 RNA was added to morpholino injection solutions

for rescue experiments. Morpholino, rescue or water control solutions were injected into embryos at the one cell stage.

For RNA coinjection experiments, RNA was collected from 3dpf zebrafish embryos using Trizol (Life Technologies). cDNA was prepared using SuperScript III reverse transcriptase (Life Technologies) following manufacturer's instructions. Full length ITGBL1 RNA was amplified using primers (Table S2) and cloned into pCS2+ for expression. Plasmid was linearized using Not1 and transcribed using mMESSAGE mMACHINE (Life Technologies) capped transcription assembly. RNA was precipitated and purified using RNeasy Mini Kit (Qiagen) and injected into zebrafish embryos at 1 cell stage. Embryos were phenotyped using light, fluorescent and confocal microscopy.

Zebrafish RNA analysis

RNA was collected from 3dpf zebrafish embryos using Trizol (Life Technologies). cDNA was prepared using SuperScript III reverse transcriptase (Life Technologies) following manufacturer's instructions. ITGBL1 transcript was amplified using primers (Table S2).

Confocal microscopy

For visualization via confocal microscopy, injected Tg(cmlc2:rasGFP) or Tg(cmcl2:dsnuclearRed) embryos were transferred to aqueous tricaine solution for 10 minutes. Fish were washed in PBS and incubated in 4% PFA for 90 minutes to fix tissue. Fish were then positioned in 1% low melt agarose solution ventral side up. Confocal images were obtained using a Leica SP5 confocal microscope. Captured image stacks were visualized and analyzed in FIJI. Nuclei were enumerated and cell length was measured along the longest axis of the cell. Fluorescent imaging of

zebrafish vasculature was done with using a Leica MZ16 F dissecting scope using an EFC310 FX digital camera.

Copy number variant analyses

Informed consent was obtained under a human subjects research protocol as approved by the University of California San Francisco. Human CNV analysis was performed as follows. DNA samples were isolated from HLH patient blood and assayed using the Agilent 244K array. CNVs were called using platform software. Duplication was validated using Affymetrix 6.0 SNP array, as well as with qPCR (data not shown).

Duplication transcript analysis

RNA from the hypoplastic left heart individual thymic tissue was isolated using Trizol (Life Technologies). cDNA was prepared using SuperScript III reverse transcriptase (Life Technologies) following manufacturer's instructions. qRT-PCR was performed on patient RNA and control pooled thymic RNA (Clontech 636586).

Informatics

Coexpression analysis was used to identify the top genes whose expression were most positively correlated with *ITGBL1* using CoXpressDB (coxpresdb.jp). Coexpressed genes were analyzed with ConsensusPathDB (cpdb.molgen.mpg.de) to identify enriched pathways and processes associated with *ITGBL1* and the top 99 most coexpressed genes.

Cell culture

All cells were cultured at 37°C, according to standard culture protocols. HEK 293T cells were cultured in DMEM with 10% FBS (JR Scientific, 43603-500) and penicillin/streptomycin. HUV-EC-C (CRL-1730) cells were obtained from American Type

Culture Collection and cultured in F12K medium supplemented with 10%FBS, 0.1mg/ml Heparin (Sigma, H3149), 0.04mg/ml endothelial cell growth supplement (Sigma, E2759), and penicillin/streptomycin, according to ATCC instructions. NMuMG cells were cultured in DMEM with 10% FBS supplemented with 10ug/ml insulin (Sigma, I0516).

Plasmid constructs and cloning

Human *ITGBL1* cDNA was obtained from OpenBiosystems MGC collection (MHS1010-9204684), amplified with high fidelity polymerase, cloned into pEGFP-N1 expression vector (Clontech), and sequenced for verification (Full length *ITGBL1*). The first 7 exons of *ITGBL1* (truncated *ITGBL1*) were also amplified separately as a model for the index patient duplication. Empty pEGFP-N1 vector was used as a control during functional cell culture experiments.

Adhesion assay

ITGBL1 expressing plasmids were transfected into HEK 293T cells with fluorescently tagged full length *ITGBL1*, truncated *ITGBL1* or empty expression vector using Lipofectamine 2000 (Life Technologies) and cells were seeded at 2.5e6 cells/well into 6 well plates to allow for expression. After 48 hours, cells were gently trypsinized and plated in 96-well plates at 8e5 cells/ well with ECM coatings: 1.6ug fibronectin (Sigma, F1141), 3.2ug collagen I (Sigma C8919) or 3.2ug collagen IV (Sigma C5533) and previously blocked with 1% BSA. Cells were incubated for adhesion for 2 hours then washed 3 times, fixed in 4% PFA for 15 minutes and stained with 0.1% crystal violet diluted in methanol. Cells were counted per well using light microscopy.

Network analysis

Human vein endothelial cells, HUV-EC-C were transfected using the Nucleofector V kit (Lonza, VCA-1003) following manufacturer's instructions. Transfected cells were seeded at 3e5 cells/ml and plated into 24 well plates. After 24 hours, 3.0mg/ml collagen I (Corning, 354249) diluted in media was overlaid onto cells. Cells were observed for morphology at 1, 2 and 3 days post transfection by photography of middle and quandrants of each well. Network formation was quantified by measuring the area vacated by cells after collagen overlay using the Fiji Selection tool, followed by the Analyze function. HUV-EC-C viability was determined using PrestoBlue Cell Viability Reagent (Life Technologies, A13261) after 2 hour incubation following manufacturer's instructions.

Smad activation analysis

NMuMG cells from murine mammary tissue were transfected with a combination of: 0.46ug of control or *ITGBL1* expressing plasmids, 0.23ug of 4x Smad-Binding-Element Luciferase expressing plasmid and 0.05ug of *Renilla* luciferase expressing plasmid using Lipofectamine 2000 (Life Technologies). Cells were seeded at a density of 4e5 cells/ml and plated into 24 well plates. After 48 hours, cells were harvested and lysates measured using reagents from the Dual-Luciferase Reporter Assay System (Promega) and Mithras LB940 plate reader (Berthold Technologies).

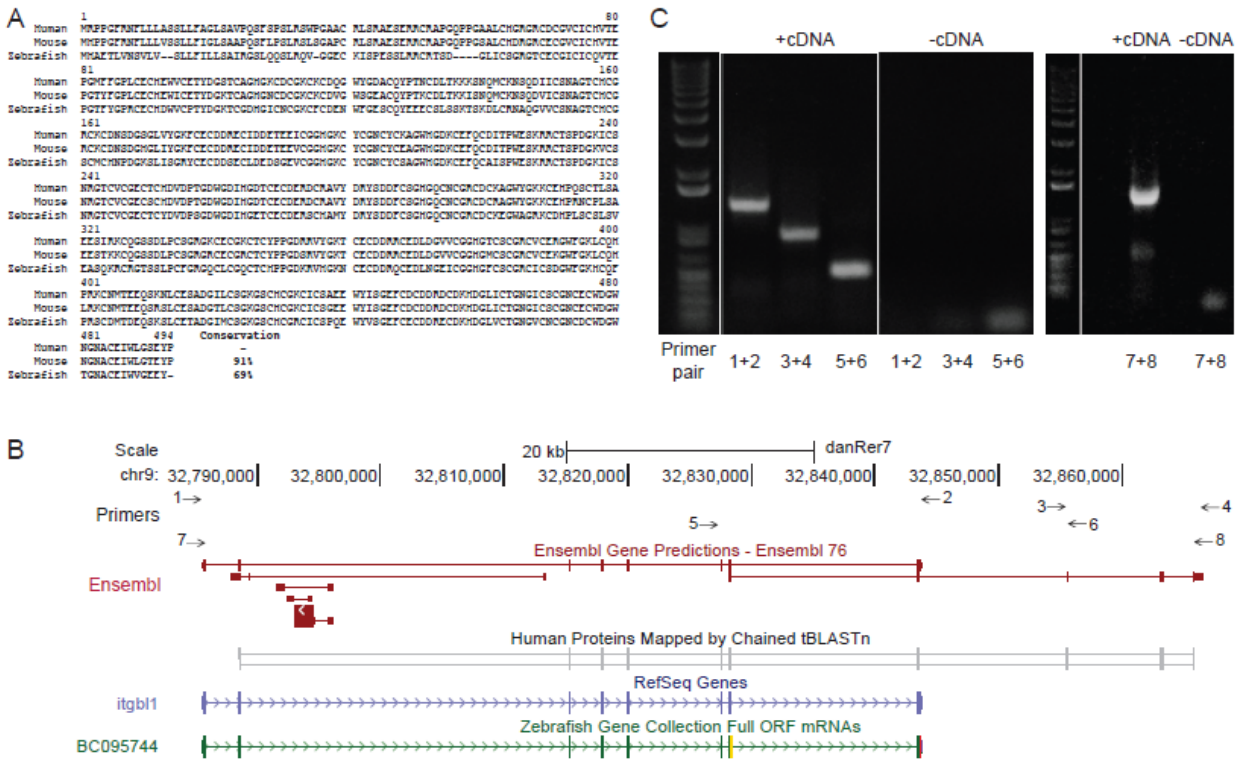

Fig 1. Evolutionary conservation of ITGBL1

(A) Alignment of ITGBL1 amino acid sequences by species. (B) *itgbl1* transcript annotation in the UCSC genome browser. Zebrafish *itgbl1* annotation in Zv9/danRer7 Ensembl prediction and RefSeq does not include the full-length transcript predicted in human and mouse. Primer numbers used for transcript analysis by RT-PCR are shown above Ensembl track. (C) RT-PCR amplification of partial and full-length (~1600bp) *itgbl1* transcripts from zebrafish embryos.

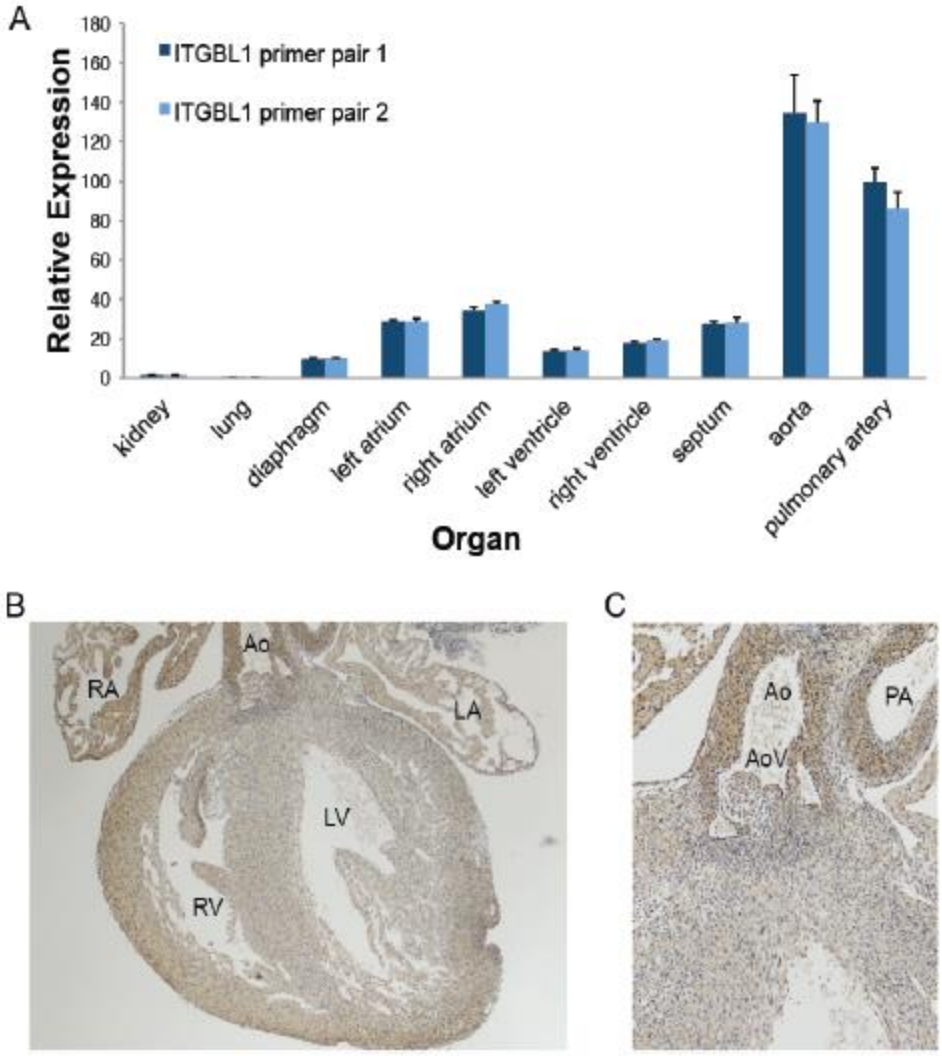

Fig 2. ITGBL1 expression in the heart

(A) qRT-PCR from E14.5 mouse tissues demonstrates expression of *Itgbl1*. The aorta and pulmonary artery have relatively high levels of expression. Two different exon spanning primer pairs shown (Methods). (B and C) Immunohistochemistry for ITGBL1 performed on E14.5 mouse sections: The heart shows staining in the aorta (Ao), aortic valve (AoV), pulmonary

artery (PA) and right atria (RA) with lower levels in the right ventricle (RV) and left atria (LA).

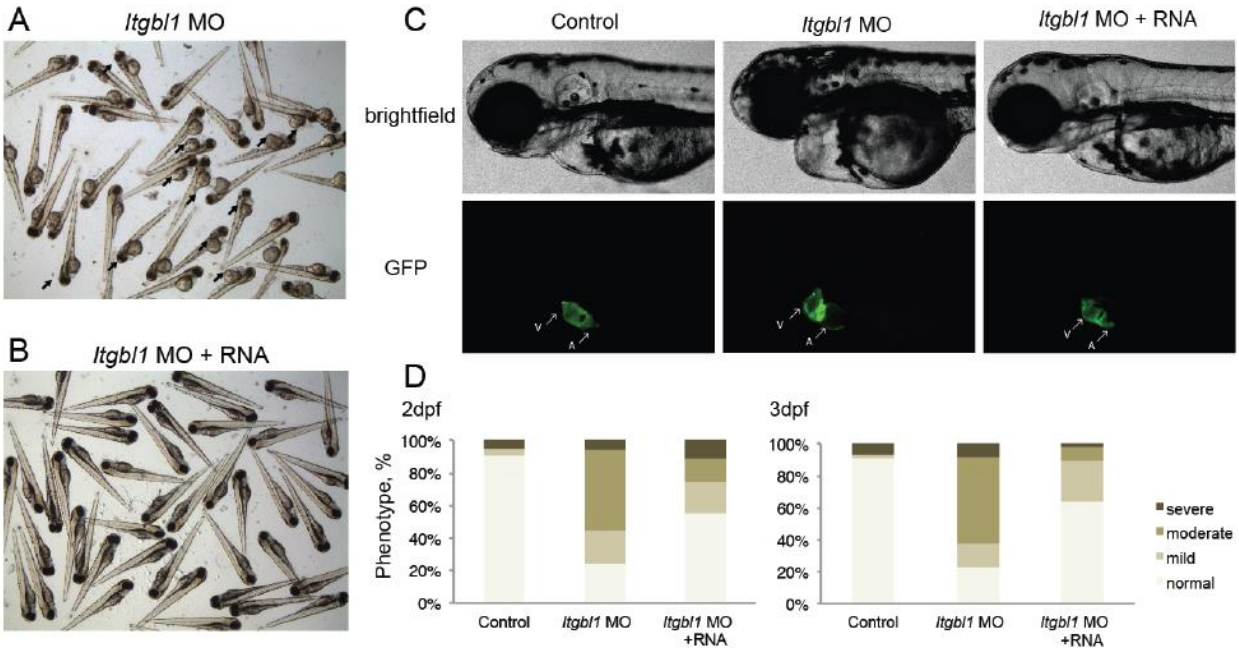

Fig 3. *itgbl1* knockdown and rescue in zebrafish

(A) Phenotype of *itgbl1* antisense morpholino (MO) injected zebrafish embryos. Cardiac edema and increased heart size (arrows) are observed, shown at 3 days post fertilization (dpf). (B) Effects of *itgbl1* antisense morpholino are ameliorated by coinjection of *itgbl1* RNA. (C) Injection of *itgbl1* RNA reduces severity of cardiac phenotype of*itgbl1* morpholino (3 dpf shown). (D) Quantification of zebrafish phenotypes at 2 and 3dpf. Chi Squared test, $p<7.37E-36$, $p<2.81E-43$, n=120-225 fish per column per day.

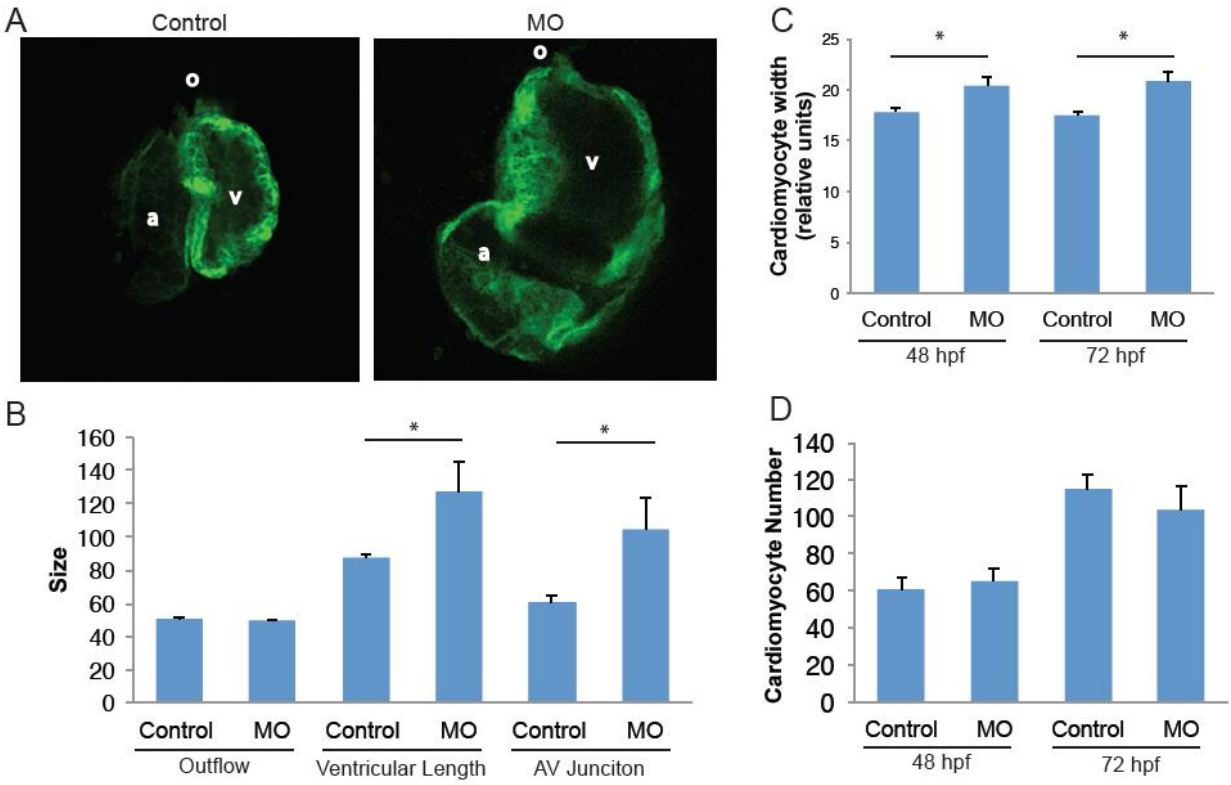

Fig 4. *itgbl1* knockdown cardiac phenotype

(A) *itgbl1* antisense morpholino alters cardiac morphology, as shown by confocal section of cmlc2:GFP transgenic hearts from ventral aspect. (B) Size of ventricle and atrioventricular junction are increased in *itgbl1* morpholino-injected fish compared to controls. There is an increase in the size (C) but not number (D) of cardiomyocytes. Structures listed are atria (a), ventricle (v) and outflow (o). Data are represented as mean +/- SEM. * = t-test, unpaired, $p<.05$

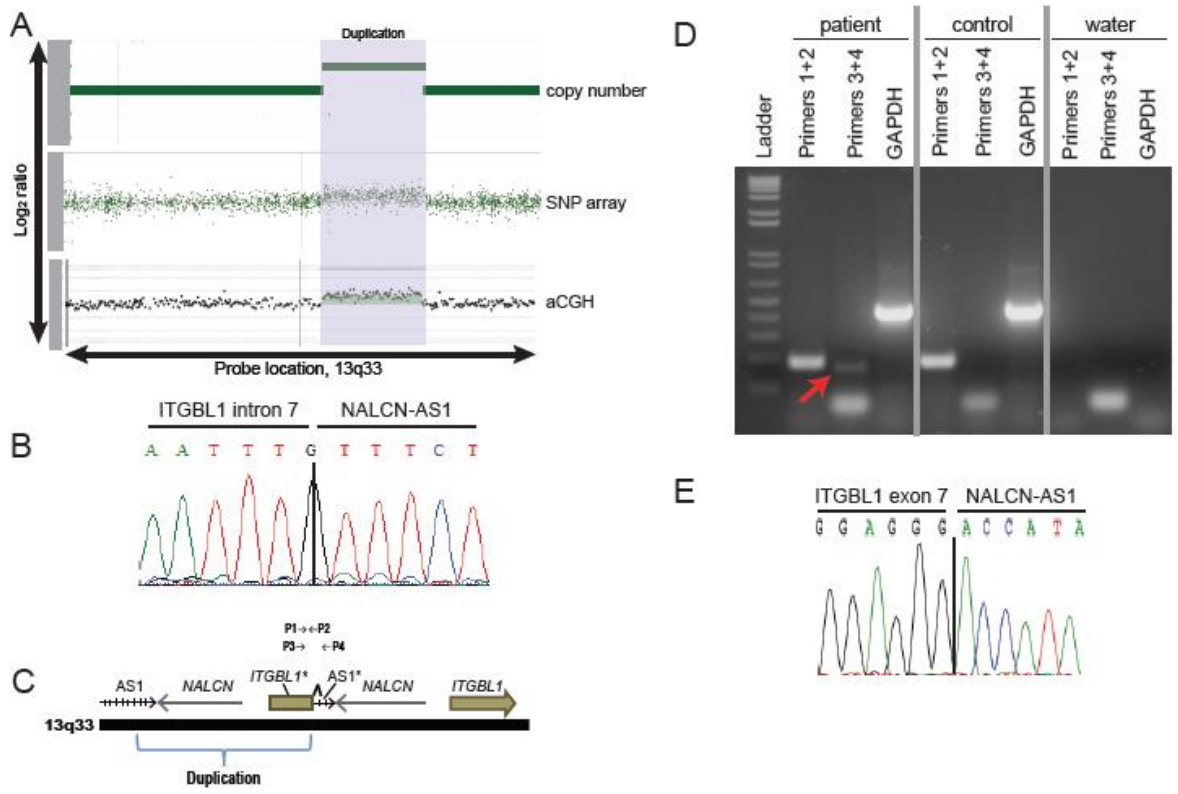

Fig 5. A 13q33.1 copy number variant in a congenital heart disease patient includes *ITGBL1*

(A) A rare 587kb duplication (highlighted region) was identified via array comparative genomic hybridization (aCGH) and validated (SNP array). (B) Genomic DNA duplication junction was identified by tiled Sanger sequencing. (C) Schematic of mutated patient allele. Potential for unique transcript, *ITGBL1*-NALCN-AS1**, shown. Location of amplification primers used for RT-PCR, P1-P4, shown at top. (D) Transcription of the duplicated *ITGBL1* region as shown by amplification of a unique junction-specific PCR product from cDNA from the affected patient thymus and control thymus.

(E) Sequence electropherogram of cDNA junction spanning from *ITGBL1* exon 7 to *NALCN-AS1*, indicating expression of duplicated *ITGBL1*.

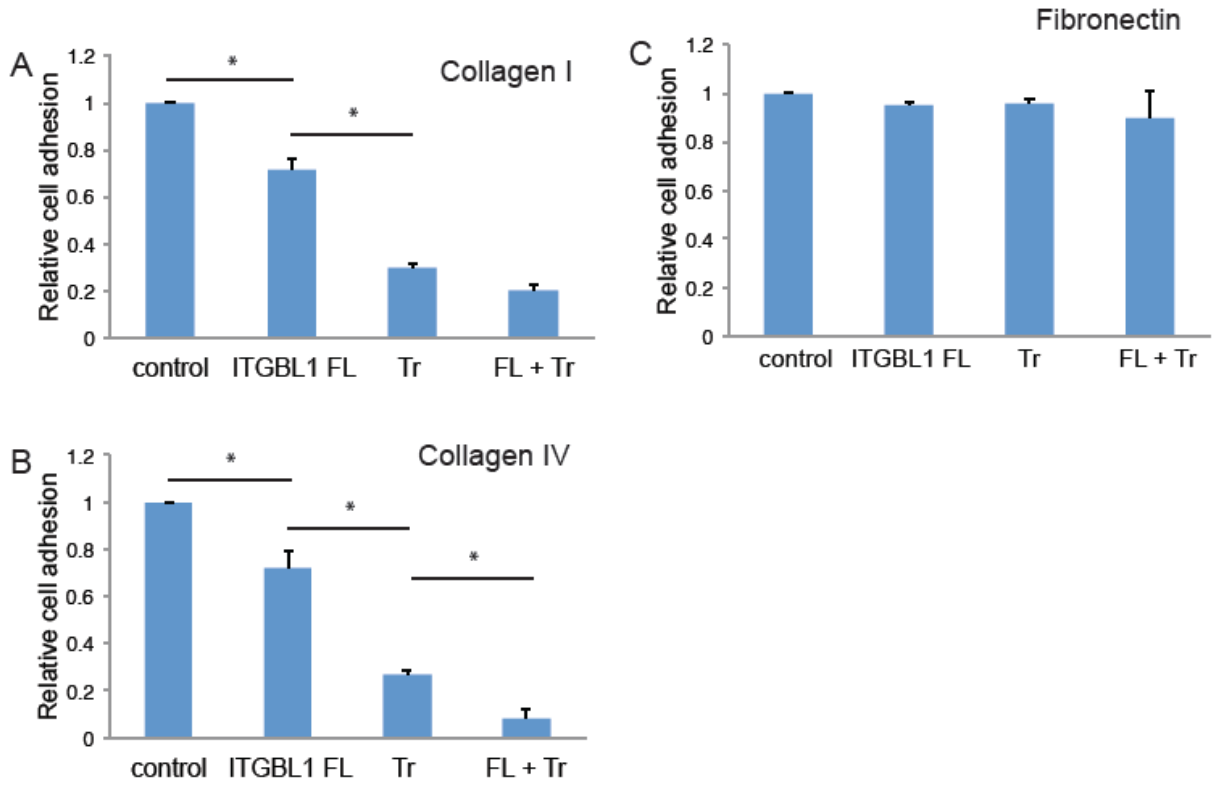

Fig 6. *ITGBL1* expression in 293t cells alters adhesion in a substrate-specific manner

(A-C) Differential adhesion of 293T cells transfected with *ITGBL1* constructs. Control plasmid, full length ITGBL1 (ITGBL1 FL), truncated ITGBL1 (Tr) or both (FL+Tr) transfected 293T cells show differential adhesion to collagen I (A) and collagen IV (B), but not fibronectin (C). Data are represented as mean +/- SEM. * = t-test, unpaired, $p<.05$

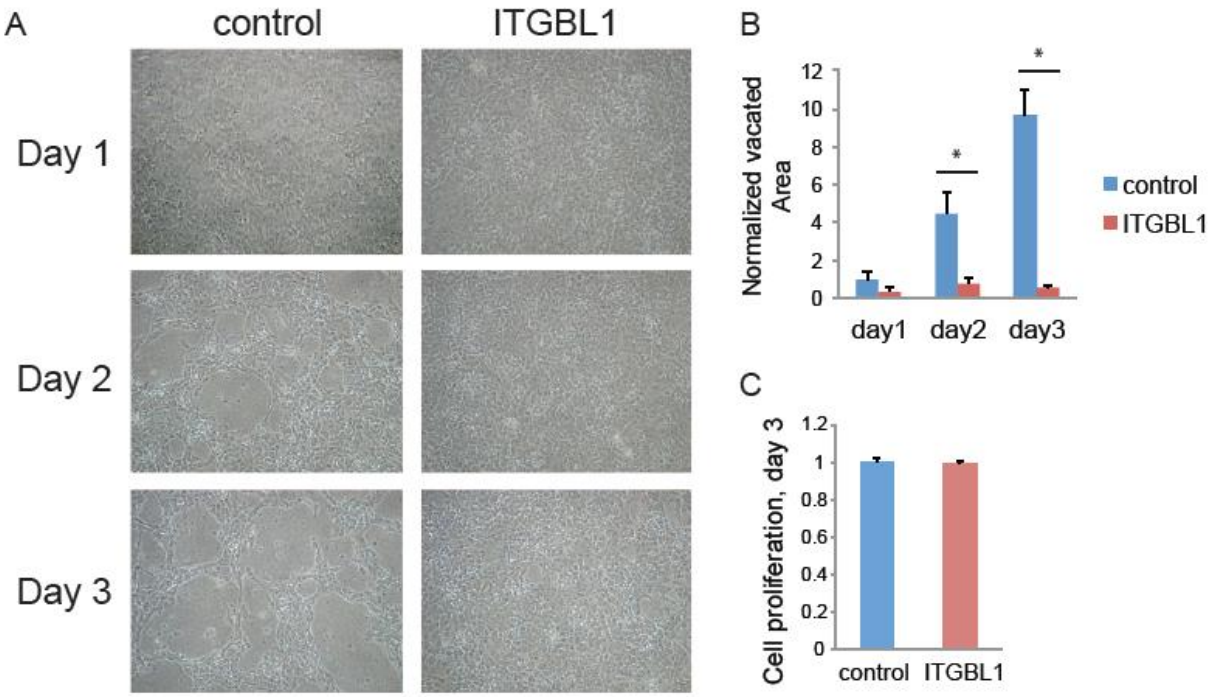

Fig 7. *ITGBL1* expression in HUVECs inhibits network formation

(A) Control transfected HUVECs respond to collagen matrix by forming networks (days 2 and 3), while *ITGBL1* expressing HUVECs do not. (B) Quantification of network formation in response to *ITGBL1* overexpression. (C) *ITGBL1* expression did not affect numbers of viable cells. Data are represented as mean +/- SEM. * = t-test, unpaired, $p<.05$

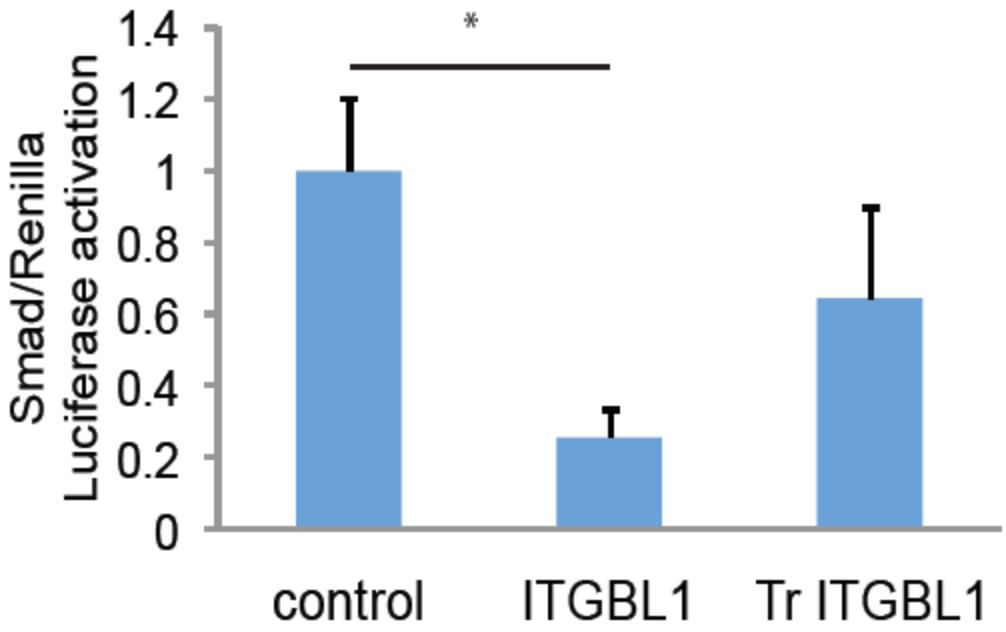

Fig 8. *ITGBL1* expression alters Smad activation

Normalized Smad luciferase reporter activity in NMuMG cells transfected with *ITGBL1* or the short form of ITGBL1 (Tr ITGBL1). Values depicted represent Smad luciferase activity normalized by renilla luciferase activity. Data are represented as mean +/- SEM. * = t-test, unpaired, $p<.05$

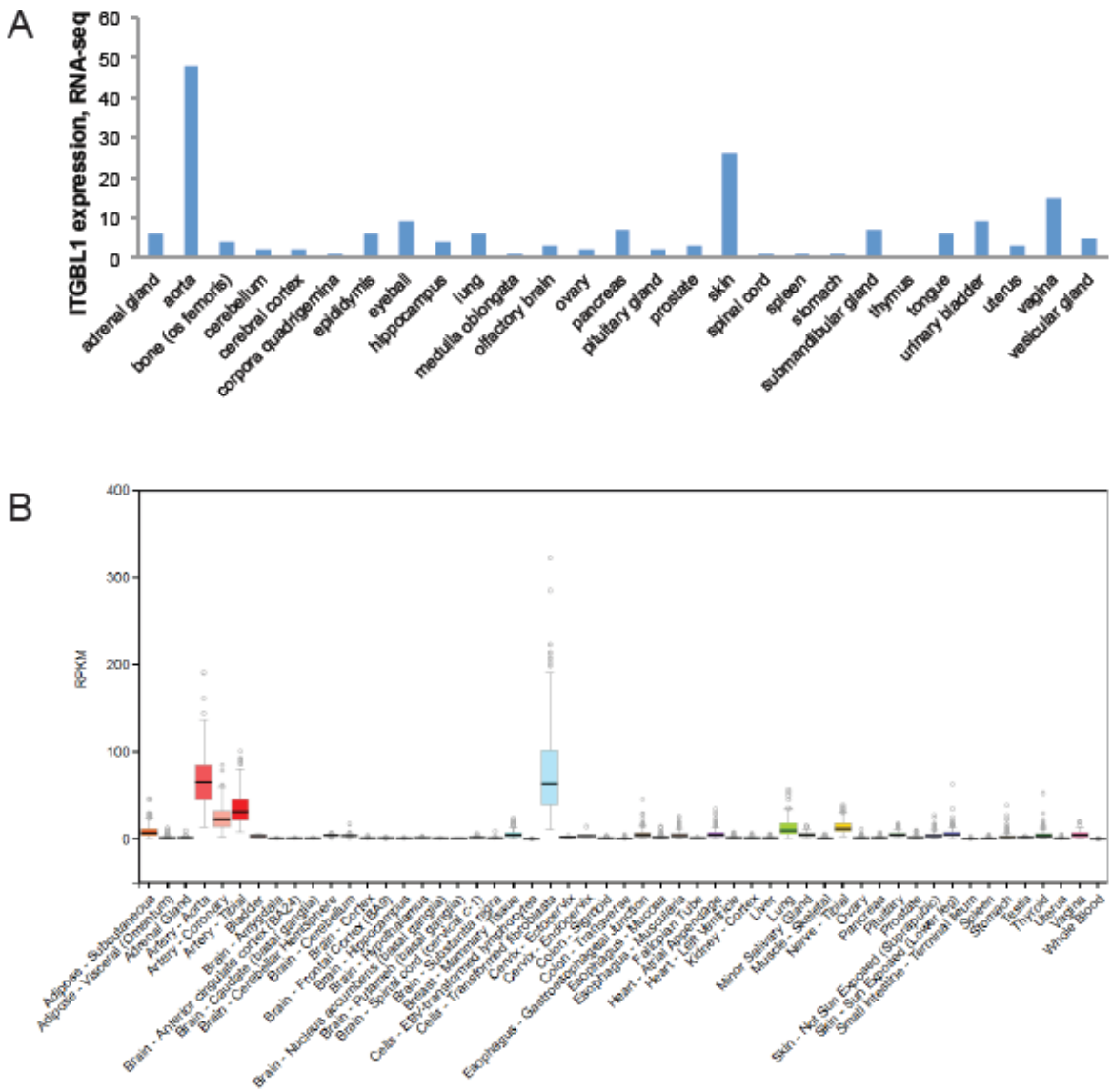

Figure S1. *ITGBL1* expression by tissue type from Gene Expression Atlas and GTEx

(A) Mouse gene expression atlas enriched in the aorta. (B) Human ITGBL1 expression data from GTEx demonstrates enrichment in aorta.

Table S1. Top ITGBL1 Coexpressed Genes

RANK	SYMBOL	NAME	Entrez Gene ID
0	ITGBL1	integrin, beta-like 1 (with EGF-like repeat domains)	9358
1	SFRP4	secreted frizzled-related protein 4	6424
2	COMP	cartilage oligomeric matrix protein	1311
3	FBN1	fibrillin 1	2200
4	ASPN	asporin	54829
5	NALCN	sodium leak channel, non-selective	259232
6	COL10A1	collagen, type X, alpha 1	1300
7	LTBP2	latent transforming growth factor beta binding protein 2	4053
8	OMD	osteomodulin	4958
9	FAP	fibroblast activation protein, alpha	2191
10	MFAP5	microfibrillar associated protein 5	8076
11	COL8A1	collagen, type VIII, alpha 1	1295
12	THBS2	thrombospondin 2	7058
13	DCN	decorin	1634
14	FNDC1	fibronectin type III domain containing 1	84624
15	LRRC15	leucine rich repeat containing 15	131578
16	CCDC80	coiled-coil domain containing 80	151887
17	SFRP2	secreted frizzled-related protein 2	6423
18	COL8A2	collagen, type VIII, alpha 2	1296
19	ITGA11	integrin, alpha 11	22801
20	BGN	biglycan	633
21	CDH11	cadherin 11, type 2, OB-cadherin (osteoblast)	1009
22	GLT8D2	glycosyltransferase 8 domain containing 2	83468
23	COL11A1	collagen, type XI, alpha 1	1301
24	POSTN	periostin, osteoblast specific factor	10631
25	ISLR	immunoglobulin superfamily containing leucine-rich repeat	3671
26	MATN3	matrilin 3	4148
27	LOXL1	lysyl oxidase-like 1	4016
28	SULF1	sulfatase 1	23213
29	FGF14	fibroblast growth factor 14	2259
30	AEBP1	AE binding protein 1	165

RANK	SYMBOL	NAME	Entrez Gene ID
31	PRELP	proline/arginine-rich end leucine-rich repeat protein	5549
32	FAM26E	family with sequence similarity 26, member E	254228
33	TMEM119	transmembrane protein 119	338773
34	ITGB5	integrin, beta 5	3693
35	COL12A1	collagen, type XII, alpha 1	1303
36	COL1A2	collagen, type I, alpha 2	1278
37	TIMP3	TIMP metallopeptidase inhibitor 3	7078
38	PTGIS	prostaglandin I2 (prostacyclin) synthase	5740
39	CORIN	corin, serine peptidase	10699
40	PRRX1	paired related homeobox 1	5396
41	ECM2	extracellular matrix protein 2, female organ and adipocyte specific	1842
42	FIBIN	fin bud initiation factor homolog (zebrafish)	387758
43	LOX	lysyl oxidase	4015
44	NOX4	NADPH oxidase 4	50507
45	COL5A1	collagen, type V, alpha 1	1289
46	EDIL3	EGF-like repeats and discoidin I-like domains 3	10085
47	HMCN1	hemicentin 1	83872
48	COPZ2	coatomer protein complex, subunit zeta 2	51226
49	CTSK	cathepsin K	1513
50	INHBA	inhibin, beta A	3624
51	MXRA8	matrix-remodelling associated 8	54587
52	COL5A2	collagen, type V, alpha 2	1290
53	COL6A3	collagen, type VI, alpha 3	1293
54	LUM	lumican	4060
55	COL3A1	collagen, type III, alpha 1	1281
56	XG	Xg blood group	7499
57	VGLL3	vestigial like 3 (Drosophila)	389136
58	OLFML2B	olfactomedin-like 2B	25903
59	DACT1	dapper, antagonist of beta-catenin, homolog 1	51339

RANK	SYMBOL	NAME	Entrez Gene ID
60	SPOCK1	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1	6695
61	FST	follistatin	10468
62	PDGFRB	platelet-derived growth factor receptor, beta polypeptide	5159
63	PODN	podocan	127435
64	MXRA5	matrix-remodelling associated 5	25878
65	C7orf10	chromosome 7 open reading frame 10	79783
66	KCNE4	potassium voltage-gated channel, Isk-related family, member 4	23704
67	EPYC	epiphycan	1833
68	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	6678
69	THY1	Thy-1 cell surface antigen	7070
70	MOXD1	monooxygenase, DBH-like 1	26002
71	TMEM200A	transmembrane protein 200A	114801
72	SSPN	sarcospan	8082
73	F2RL2	coagulation factor II (thrombin) receptor-like 2	2151
74	DPT	dermatopontin	1805
75	GPX8	glutathione peroxidase 8 (putative)	493869
76	SRPX2	sushi-repeat containing protein, X-linked 2	27286
77	NOV	nephroblastoma overexpressed	4856
78	PCOLCE	procollagen C-endopeptidase enhancer	5118
79	DKK3	dickkopf 3 homolog (Xenopus laevis)	27122
80	PDGFRL	platelet-derived growth factor receptor-like	5157
81	LINC00312	long intergenic non-protein coding RNA 312	29931
82	CTGF	connective tissue growth factor	1490
83	CTHRC1	collagen triple helix repeat containing 1	115908
84	LOC100128178	uncharacterized LOC100128178	100128178
85	SMOC2	SPARC related modular calcium binding 2	64094
86	COL6A2	collagen, type VI, alpha 2	1292

RANK	SYMBOL	NAME	Entrez Gene ID
87	SGCD	sarcoglycan, delta (35kDa dystrophin-associated glycoprotein)	6444
88	WISP2	WNT1 inducible signaling pathway protein 2	8839
89	SLIT2	slit homolog 2 (Drosophila)	9353
90	MIR100HG	mir-100-let-7a-2 cluster host gene (non-protein coding)	399959
91	FBLN2	fibulin 2	2199
92	NEGR1	neuronal growth regulator 1	257194
93	CILP	cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	8483
94	ADAMTS12	ADAM metallopeptidase with thrombospondin type 1 motif, 12	81792
95	PRSS23	protease, serine, 23	11098
96	KIAA1462	KIAA1462	57608
97	PRKG1	protein kinase, cGMP-dependent, type I	5592
98	EFEMP1	EGF containing fibulin-like extracellular matrix protein 1	2202
99	NNMT	nicotinamide N-methyltransferase	4837

Table S2. Primers

zebrafish primers	sequence (5'--3')
1	gcattcatatggagtgcgctgtt
2	acctccacagatctctccgttc
3	ctgcagtgatggatggtttg
4	aattgggtttaggggtgagc
5	gtgcacctgctatgatgtgg
6	cacaggctcttgctctgctc
7	*gtaatcgatcgccacc*atgcatgcagagactttggtg*
8	*gaatctgagcta*atattcaacccagatttcacaggcg*
	*italics used for cloning purposes

human primers	sequence
p1	atggggacacctgtgaatgt
p2	gcacttcctgatgctctcct
p3	caggctggtatgggaagaag
p4	gtcacgcttcttgctctgtg
genomic fwd	tctcatagttctttaaatgaggaggaa
genomic rev	tgcctcttctcctccaaatg

Table S3. TGFB-associated variants in cardiac/aortic phenotypes

gene	variant	phenotype
SMAD2	de novo splice	ASPLENIA SYNDROME;ATRIAL SEPTAL DEFECT, SECUNDUM;DEXTROCARDIA;DOUBLE OUTLET RIGHT VENTRICLE;ILL;PULMONARY STENOSIS, VALVAR;TRANSPOSITION D-LOOP;UNBALANCED COMPLETE COMMON ATRIOVENTRICULAR CANAL
SMAD2	de novo missense	ABDOMINAL HETEROTAXY;ALL;DEXTROCARDIA;DORV + AVSD;HETEROTAXY;HYPOPLASTIC LEFT VENTRICLE;HYPOPLASTIC MAIN PULMONARY ARTERY;HYPOPLASTIC PULMONARY ANNULUS;INFERIOR VENA CAVA ANOMALY;INTESTINAL MALROTATION;LEFT AORTIC ARCH WITH NORMAL BRANCHING PATTERN;LEFT SUPERIOR VENA CAVA ENTERING LEFT ATRIUM;PARTIALLY ANOMALOUS PULMONARY VEINS;PULMONARY STENOSIS, SUBVALVAR;RIGHT SUPERIOR VENA CAVA ABSENT;SITUS AMBIGUOUS;UNBALANCED COMPLETE ATRIOVENTRICULAR CANAL, RIGHT DOMINANT
SMAD4	de novo missense	AORTIC ARCH HYPOPLASIA;COARCTATION
PITX2	de novo missense	ABNORMAL AORTIC ARCH;COARCTATION
FBN2	de novo missense	TETRALOGY OF FALLOT
FBN1	de novo missense	AORTIC STENOSIS - SUBVALVAR;HYPOPLASTIC RIGHT VENTRICLE;LEFT AORTIC ARCH WITH NORMAL BRANCHING PATTERN;MALPOSITIONED ATRIAL SEPTUM;MITRAL ATRESIA;STRADDLING TRICUSPID VALVE;TRANSPOSITION D-LOOP WITH VENTRICULAR SEPTAL

		DEFECT;TUBULAR HYPOPLASIA OF AORTA + COARCTATION;USUAL CORONARY ARTERIES IN D-LOOP TGA;VENTRICULAR SEPTAL DEFECT, ECD TYPE;VENTRICULAR SEPTAL DEFECT, MALALIGNMENT RESTRICTIVE;VENTRICULAR SEPTAL DEFECT, SINGLE
FBN1	de novo missense	AORTIC STENOSIS, SUBVALVAR, DISCRETE, MEMBRANEOUS;MITRAL STENOSIS ARCADE

*Homsy J, Zaidi S, Shen Y, Ware JS, Samocha KE, Karczewski KJ, et al. De novo mutations in congenital heart disease with neurodevelopmental and other congenital anomalies. Science. 2015;350: 1262–1266. doi:10.1126/science.aac9396

Chapter 3: Implications and Future Directions

Implications and Future Directions

The findings presented here are the first descriptions of the roles of *ITGBL1* in the heart and in development. We probe the function of a poorly studied protein in cells and in an *in vivo* model. These findings are bridged in part by observations of ITGBL1 coexpressed genes being enriched in members of the ECM, cell adhesion and cardiovascular development processes. These data provide a foundation upon which others might launch more detailed questions about ITGBL1 biology.

ITGBL1 is composed of ten, tandem EGF-like domains. These domains bear homology to domains observed in integrins which are thought to be structural in nature. ITGBL1 is also observed to be coexpressed with other structural members of the ECM such as LTBP2, FBN1, COMP, and numerous collagens. It is possible that ITGBL1 is a structural protein in the ECM. The high expression in the aorta, coupled with the hypothesized rigidity of the integrin EGF-like domain (Takagi et al., 2001), could point to ITGBL1 being a structural component that helps modulate the unique hydrodynamic forces observed in the aorta.

The data we present on the function of *ITGBL1* in cells suggests that ITGBL1 has a capacity to influence specific cell/ECM interactions. Additionally, eleven collagens are found within the top 100 genes coexpressed with ITGBL1 (Table1). This finding could point to cells co-regulating various collagens along with ITGBL1 to coordinate cell behavior. Such findings are intriguing given multiple collagens are observed to be upregulated during valve development (Chakraborty et al., 2010) and multiple collagen mutations have been associated with cardiovascular abnormalities (Hinton and Yutzey,

2011; Kuivaniemi et al., 1997). This expression program could be related to ECM-mediated signaling, though further research is needed to better understand ITGBL1's potential role in this process.

Understanding into ITGBL1's mechanism of action and physical role is limited due to the lack of information on the ITGBL1 protein. In our experiments, western blot analyses of ITGBL1 from primary tissue and cell lines has revealed protein bands not only at the expected size but also bands indicating proteins of smaller sizes. These bands were not observed in controls. Possible explanations for the different protein bands are alternative transcript splicing or post-translational protein modification or processing. Investigation into the processing of ITGBL1 could be examined if evidence emerges to suggest other forms of the protein are relevant. Such insights might be helpful in elucidating the ways through which ITGBL1 can influence cell behavior.

Additional information on ITGBL1 function and role in cell/ECM interactions could come through the identification of molecular binding partners for ITGBL1, or perhaps a receptor for which ITGBL1 is a ligand. Targeted experiments by Co-IP could be considered. A more unbiased approach could be provided through a protein pull-down of ITGBL1 followed by mass-spectrometry. The ITGBL1-EGFP fusion construct we created could be helpful in this capacity, as EGFP purification columns are presently available. Identification of a binding partner, especially if any are binding proteins or ECM members, could help understand the influence on cellular behavior and launch further research.

The conservation of ITGBL1 among vertebrates supports a potential relevance in development. Our zebrafish-based investigation of the role of *itgbl1* in development suggests *itgbl1* is required for correct heart development. We observed cardiac morphology change and edema that was highly prevalent and we were able to rescue with ITGBL1 RNA. The differences in ventricular structure we observe when *itgbl1* was knocked down are intriguing and further studies would be helpful. We would also propose that further experiments to disrupt *itgbl1* by knockout could be useful, as this could point to the effects of having null dosage *itgbl1*. While we have done an efficient first step for investigating function, follow-up testing of *itgbl1* in zebrafish development could be accomplished through the use of a knockout using CRISPR. Alternatively, investigation into the role of *Itgbl1* in the development of a 4-chambered, mammalian heart could be accomplished through the use of CRISPR or recombinant DNA technology in the murine system. To focus analysis on specific tissues, creation of a conditional allele, coupled with the use of tissue-specific CRE, could yield targeted analysis on the role of *Itgbl1* during cardiac or outflow tract development. Using such a model, experiments designed to investigate the time-course of *Itgbl1* expression in the developing aorta and valve, the influence of *Itgbl1* dosage on aortic and valve morphology, and the influence of *Itgbl1* dosage on ECM protein expression could all help to further identify *Itgbl1*'s role in development.

Our results show that when we express ITGBL1 in cell culture, we can alter downstream activation of the TGF-β pathway. This finding is intriguing given the strong body of literature relating TGF-β to EndMT and valve development. Further investigation of the potential relationship could provide further evidence for role the ITGBL1 in development.

Altering *ITGBL1* in an organism or in a valve model, followed by analysis of markers of EndMT – including Slug, Snai1, Twist, αSMA and Vimentin – in the developing endocardial cushions/early aortic valve, would all be a logical extension of this work. Investigation of these same markers in vascular endothelial cells cultured with or without knockdown/out of *ITGBL1*, and with or without exogenous TGF-β could also provide complementary data. Lastly, modulating levels of *ITGBL1* in vascular endothelial cells and measuring cell migration or invasion in to ECM/collagen substrates could also determine whether or not *ITGBL1* also plays a role in the invasive cell phenotype observed during valvulogenesis.

Our preliminary studies of other human CNVs in the *ITGBL1* region using data from HLH patients shows that the rare *ITGBL1* copy number duplication identified in this family is uncommon even amongst HLH/LVOTO individuals. This finding is not surprising given the well documented heterogeneity among CHD cases (Homsy et al., 2015; Zaidi et al., 2013). Intriguingly, many other proteins in the canonical TGF-β signaling pathway – including Smad2 and Smad4 – and the extended TGF-β superfamily – including BMPR1a, BMPR1b and BMPR2 – have been implicated in congenital heart disease. Additionally, mouse models of CHD showing incomplete penetrance of LVOTO phenotypes were demonstrated to have abnormal expression of transcripts within the TGF-β pathway as measured by RNA-seq (personal correspondence with other cardiac researcher). These findings provide interesting parallels to our results and suggest that additional members of the TGF- β signaling pathway, in addition to canonical members, should be considered for investigation in new cases of HLH and LVOTO.

This work provides functional testing for a rare CNV encountered when screening CHD. We hope that the work in this thesis might encourage others to pursue in-depth *in vitro* and *in vivo* functional follow up of rare variants identified from large genome/exome studies, given their importance in understanding pathogenesis. Recent studies have used strategies such as filtering genes by high expression in the heart during development (Hitz et al., 2012; Zaidi et al., 2013) or confirmation of candidate genes in secondary cohorts (Dina et al., 2015) to help prioritize candidates for functional follow-up. The presence of large genetic databases such as ExAC might also prove useful in filtering variants. Importantly, data from the congenital heart cohorts could allow for analysis of both CNV and SNV mutations, which will allow scientists to investigate a more comprehensive pool of variants to better determine pathogenic mutation burden, epistatic interactions and likelihoods for disease. Further datasets incorporating gene expression, epigenetic modification and other data sources may need to be incorporated to fully understand the genetic etiology of CHD.

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