

FUNCTIONAL ANALYSIS OF A CASZ1/CHD5 INTERACTION IN CARDIAC
DEVELOPMENT AND DISEASE

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ABSTRACT

STEPHEN P. SOJKA: Functional analysis of a CASZ1/CHD5 interaction in cardiac development and disease
(Under the direction of Frank L. Conlon)

Early cardiac development involves coordination of a number of processes including the specification and differentiation of cardiomyocytes, the ventral migration of cardiac cell populations, the formation of a linear heart tube, and the subsequent morphogenic movements required for cardiac looping and chamber formation. These early processes require the precise spatial and temporal regulation of a number of genetic pathways, which coordinate the activation and repression of various cardiac-specific transcription factors and their targets. These transcription factors are in part regulated by the formation of transcriptional complexes via interaction with regionally expressed cofactors. In this dissertation, I explore the role of protein-protein interactions in regulating the novel para zinc finger transcription factor CASTOR (CASZ1).

We carried out a yeast two-hybrid screen to identify cardiac specific CASZ1 interacting partners and identified congenital heart disease protein 5 (CHD5), a small coiled coil protein expressed within the putative critical region of chromosome 21 associated with congenital heart disease in Down syndrome patients. We observe that CASZ1 and CHD5 co-localize in the nuclei of cardiomyocytes and interact *in vivo*. We demonstrate that the interaction between CASZ1 and CHD5 is necessary for early cardiogenesis in *Xenopus*. We find that the absence of CASZ1 or CHD5 results in a failure of cardiac looping and early chamber formation. We

observe that CASZ1 and CHD5-depleted cardiomyocytes fail to undergo the cell shape changes associated with cardiomyocyte maturation, and that cardiomyocytes display defects in cell adhesion and apical-basal polarity. Thus CASZ1 and CHD5 are collectively required for the maintenance of early cardiomyocyte integrity. Overall this work provides a mechanism by which CASZ1 functions in early cardiac development and implicates CASZ1 and CHD5 collectively as potential causal factors for congenital heart disease in not only Down syndrome patients but in the general population as well.

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LIST OF ABBREVIATIONS

ASD	Atrial septal defect
AVSD	Atrioventricular septal defect
CA-GFP	Cardiac actin EGFP
Cas	<i>Drosophila</i> CASTOR
CASZ1	CASTOR (vertebrate homologue)
CHD	Congenital heart disease
CHD5	Congenital heart disease protein 5
CID	CHD5 interacting domain
COL6A	Collagen type VI protein
DSCAM	Down syndrome cell adhesion molecule
DSCR	Down syndrome critical region
DYRK1A	Dual specificity tyrosine-phosphorylation-regulated kinase 1A
EGFL7	Epidermal growth factor-like protein 7
ER	Endoplasmic reticulum
ES	Embryonic stem cell
H3K4me3	Histone 3 lysine 4 methylation
miR	MicroRNA
MO	Morpholino
NFAT	Nuclear factor of activated T-cells
PDA	Patent ductus arteriosus
SEM	Scanning electron microscopy
Shh	Sonic hedgehog

TEM	Transmission electron microscopy
Tmy	Cardiac tropomyosin
ToF	Tetralogy of Fallot
VSD	Ventricular septal defect
WRB	Tryptophan rich basic protein
ZO	Zona occludens (tight junction) protein

CHAPTER 1: INTRODUCTION

Down syndrome, a genetic disorder characterized by an extra copy of chromosome 21, is the most frequent of live-born chromosomal disorders (Parker et al., 2010). Recent studies estimate that globally 1 out of 1,000 live-births have the condition, which in the vast majority of cases is due to a pre fertilization nondisjunction event resulting in complete trisomy of chromosome 21 (Weijerman et al., 2010; Lana-Elola et al., 2011). Individuals with Down syndrome have a number of physical and mental disabilities owing to the large number of genes affected by this disorder and the resulting myriad of defects in growth and neurological development (Weijerman et al., 2010). The vast range of development defects seen in Down syndrome historically has had a severe impact on life expectancy, but as medicine and surgical techniques have evolved, life expectancy for Down syndrome patients in the U.S. has risen from 12 in the 1940s to 35 in the 1980s to a current average of approximately 60 years (Penrose, 1949; Thase, 1982; Glasson et al., 2002). Still, further insights into the molecular genetics of Down syndrome and its associated disorders are crucial toward continuing to improve the treatment of the underlying pathologies of this condition.

Cardiac defects in Down syndrome patients

Included in the myriad of Down syndrome associated pathologies is congenital heart disease (CHD), which is present in 40-80% of Down syndrome patients depending on the population sampled (Weijerman et al., 2010; Salih, 2011; Roizen et al., 2013). Roughly 65 to 80% Of Down syndrome cases with CHD can be traced to the presence of a single cardiac defect

(Figure 1.1) (Vida et al., 2005; Elmagrpy et al., 2011). The most common CHD phenotypes seen in Down syndrome patients are atrioventricular septal defects (AVSD), ventricular septal defects (VSD), and atrial septal defects (ASD). Intriguingly, there are striking regional patterns of prevalence among these three defects, with AVSD being most prevalent in American (U.S.) and European populations, VSD being most prevalent in Asia, ASD being most prevalent among Latin American countries, and African populations lacking an apparent trend (Tandon and Edwards, 1973; Park et al., 1977; Freeman et al., 1998; de Rubens Figueroa et al., 2003; Vida et al., 2005; Abbag, 2006; Al-Jarallah, 2009; Ali, 2009; Nisli, 2009; Weijerman et al., 2010; Elmagrpy et al., 2011; Salih, 2011; Khan and Muhammad, 2012; Shrestha and Shakya, 2013). All measured Down syndrome populations contain a lower percentage of cases with patent ductus arteriosus (PDA) and Tetralogy of Fallot (ToF), while isolated cases of more rare defects such as bicuspid aortic valve, supra-pulmonary stenosis, and double outlet right ventricle have also been observed (Al-Jarallah, 2009). Interestingly, the common cardiac defect of transposition of great arteries (TGA) is extremely rare in Down syndrome cases (Unolt et al., 2013). A number of non-cardiac pathologies are significantly associated with CHD in Down syndrome. Pulmonary hypertension is significantly increased in Down syndrome patients with CHD, with 50% of advanced cases in total populations represented by people with Down syndrome (Vazquez-Antona et al., 2006; D'Alto and Mahadevan, 2012; Shrestha and Shakya, 2013). A significant number of Down syndrome cases with CHD also have gastrointestinal malformations (Abbag, 2006; Orun et al., 2011) as well as increased incidence of hypothyroidism (Mihci et al., 2010). Down syndrome patients with CHD also exhibit reduced neurodevelopmental skills such as fine motor, cognitive, and language skills compared to Down

syndrome patients without CHD, providing evidence for a link between phenotypes across discrete tissues in Down syndrome populations (Visootsak et al., 2011; Visootsak et al., 2013).

CHD in Down syndrome patients represents a significant challenge in ensuring quality of life for affected individuals, as CHD represents the highest single contributing factor to mortality in Down Syndrome patients (Roizen and Patterson, 2003; Shin et al., 2007; Weijerman et al., 2010). Due to modern advances in corrective surgery, the life expectancy of Down syndrome individuals with CHD has improved (Hijii et al., 1997; Reller and Morris, 1998; Formigari et al., 2004; Ghimire et al., 2013). Early detection of these malformations, often prenatally, is crucial to correcting them when possible, and advances in echocardiography have made early detection of CHD in Down syndrome fetuses easier and more reliable (Bromley et al., 1995; Clur et al., 2011; Mogra et al., 2011). Still the number of Down syndrome individuals with CHD, particularly adults, remains underreported, and developing novel therapeutic options pre- and postnatally for attenuating CHD in Down syndrome infants necessitates furthering the knowledge of the cellular basis for CHD in Down syndrome (Vis et al., 2010). Computer modeling to simulate conditions that result in AVSD seen in Down syndrome has implicated an increase in cardiac cell adhesion, but the number of genes involved in the chromosomal imbalance seen in Down syndrome make identifying causal factors for CHD in Down syndrome individuals difficult (Kurnit et al., 1985). Furthermore, recent work comparing the gene expression profiles of cells from Down syndrome individuals displaying various cardiac defects to those without CHD indicate a surprisingly low overlap of genes with altered expression in individuals with AVSD and individuals with ASD or VSD, indicating that discrete molecular and genetic causes may be responsible to the variable distribution of CHD types observed in different Down syndrome populations globally (Ripoll et al., 2012). Thus the ongoing challenge is to

continue piecing together the complex molecular and genetic interactions responsible for some or all of the typical CHD phenotypes that occur in the background of Down syndrome.

Critical chromosome 21 region for congenital heart disease in Down syndrome

Determining the critical region(s) of chromosome 21 that are necessary and sufficient to induce the various phenotypes of Down syndrome remains an active area of study within the field. Since the vast majority of Down syndrome cases arise from complete trisomy of chromosome 21 and because the penetrance and expressivity of different defects seen in Down syndrome varies on an individual basis, identifying the specific genes that cause congenital heart defects in Down syndrome patients has proven challenging. A number of strategies in recent years have been enlightening towards more directly linking the misexpression of particular regions of chromosome 21 to congenital heart defects.

One approach that has found success is the analysis of Down syndrome cases with rare instances of partial trisomy of chromosome 21, as patients who only misexpress a subset of genes but still demonstrate congenital heart defects have allowed researchers to further narrow the critical region for CHD in Down syndrome. Initial studies determined the likely region to fall within chromosome 21q22 (Figure 1.2) (Korenberg et al., 1992; Delabar et al., 1993; Barlow et al., 2001b). More recent studies have confirmed these findings through analysis of partial chromosome 21 trisomy patients, in one case narrowing down the putative CHD critical region to a 1.77 MB region of 21q22 containing only ten genes (Kosaki et al., 2005; Korbel et al., 2009; Eggermann et al., 2010). However debate remains as to the identity of the individual genes within these regions that are necessary and sufficient to induce CHD, and examination of additional partial trisomy DS cases posits the existence of multiple CHD critical regions within chromosome 21 (Virden et al., 2012).

Another area of study that has been crucial towards understanding the CHD critical region in Down syndrome is the development of Down syndrome mouse models. Prior to establishment of these models, studies in murine ES cells had shown that misexpression of human chromosome 21 within those cells was sufficient for a delay in cardiomyocyte differentiation, demonstrating a sensitivity to proper chromosome 21 gene dosage in developing cardiac cells (Inoue et al., 2000). The subsequent generation of mice duplicating either the entire or a portion of the murine chromosomal regions syntenic to chromosome 21 have allowed for closer examination of the putative critical regions for CHD in Down syndrome. Critically these models have not only replicated the cognitive defects and other phenotypes seen in human Down syndrome patients, but also have elucidated CHD critical regions overlapping orthologous regions found via human study (Amano et al., 2004; Liu et al., 2011; Liu, C. et al., 2013). Again, the multiple identified genes within these regions combined with the potential interactions with genetic pathways beyond chromosome 21 have made identifying critical CHD causal genes problematic.

Critical genes for CHD in Down syndrome

Much work has been performed to identify the causal genes within chromosome 21 whose misregulation leads to CHD in Down syndrome. One such gene that has emerged as a candidate gene is Down syndrome Cell Adhesion Molecule (DSCAM). DSCAM has been identified by several studies within the minimal critical region required for CHD in Down syndrome (Barlow et al., 2001b; Kosaki et al., 2005; Korbelt et al., 2009). Additionally DSCAM is expressed in fetal cardiac tissue as well as neural tissue, implicating it as a critical gene that may be responsible for several Down syndrome related pathologies across multiple cell types (Barlow et al., 2001a; Baumann, 2007). Misexpression of DSCAM has been linked to typical

cardiac defects seen in Down syndrome such as AVSD (Hubert et al., 1997; Yamakawa et al., 1998). However, recent work in mouse models has provided contradicting evidence that the critical region containing DSCAM is insufficient to induce cardiac defects, though it remains to be seen whether this finding is corroborated in human cases (Dunlevy et al., 2010).

The most likely reason for the disparate findings in DSCAM studies is the probable genetic heterogeneity that causes the various phenotypes of Down syndrome including CHD. Supporting this view is the finding that over-expression of both DSCAM and neighboring gene COL6A2 has the cooperative effect of inducing severe cardiac defects seen in Down syndrome such as ASD and hypertrophy, defects that are conserved from *Drosophila* to mouse and do not arise with the over-expression of either gene alone (Grossman et al., 2011). The genetic association with COL6A2 may clarify earlier work demonstrating an association between genetic variation in the COL6A1-COL6A2 gene cluster and CHD in Down syndrome patients (Davies et al., 1995). Additionally overexpression of collagen VI from the COL6A genes via trisomy 21 in humans has been linked to defects in differentiation of the endocardial cushion, leading to characteristic Down syndrome CHD such as AVSD, VSD, and ASD (Gittenberger-de Groot et al., 2003). Intriguingly, proper expression of COL6A2 also appears required for the subsequent expression of additional extracellular matrix components in the context of Down syndrome (Karousou et al., 2013). Collectively these results indicate a genetic association between DSCAM and collagen producing genes that provide evidence for a molecular basis in regulating production of extracellular matrix at the cellular level that may lead to the multi-tissue morphological defects seen in Down syndrome patients including CHD. An endogenous DSCAM promoter was determined sufficient to induce expression in cells both in the CNS and in neural crest tissue (Barlow et al., 2002). A potential role for DSCAM in the neural crest is

promising as a causal factor of Down syndrome related CHD because cells in the neural crest give rise to a number of tissues in both the neural and cardiac lineages (Kirby and Waldo, 1990). The neural crest also gives rise to thymus tissue, important to note as Down syndrome individuals with cardiac defects also have a high incidence of hyperthyroidism as well (Mihci et al., 2010). Recent work provides evidence for aberrant Sonic Hedgehog (Shh) signaling as a potential source for the phenotypes seen in neural crest derived tissue types in Down syndrome (Roper et al., 2009; Ripoll et al., 2012). Strikingly hedgehog and DSCAM both function within the assembly of *Drosophila* visual circuitry, wherein hedgehog controls axon guidance via adhesion molecule expression, and DSCAM regulates the establishment of proper postsynaptic elements, providing further evidence for an association between Shh signaling and DSCAM function (Melnattur and Lee, 2011). The potential role for DSCAM within the Shh pathway during neural crest development may provide not only a potential source for CHD in Down syndrome but for the various phenotypes in other tissue types as well.

Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (Dyrk1a) is a gene on chromosome 21 that like DSCAM is highly expressed in both neural and cardiac tissue during early development (Okui et al., 1999). Interestingly Dyrk1a also appears to have a cooperative relationship with another chromosome 21 gene, regulator of calcineurin 1 (Rcan1)/Down syndrome critical region 1 (Dscr1), also expressed in early cardiac and neural tissues (Casas et al., 2001). The products of these two genes regulate NFAT transcription factors in a synergistic manner by controlling their nuclear localization in a number of tissues, which may partially explain the broad number of diverse phenotypes in Down syndrome (Arron et al., 2006; Kurabayashi and Sanada, 2013). Specifically in the context of heart development DYRK1A overexpression negatively regulates hypertrophy in cultured cardiomyocytes via calcineurin

mediated regulation of NFAT proteins, linking proper expression of DYRK1A to the maintenance of the cardiac growth program (Kuhn et al., 2009). While follow-up *in vivo* studies failed to find an association between DYRK1A overexpression and reduced hypertrophy, it is worth noting these studies were performed in transgenic mice overexpressing DYRK1A in isolation, not within the context of a Down syndrome mouse model (Grebe et al., 2011). Additionally examination of partial trisomy 21 human patients indicate the region including only Rcan1/Dscr1 is insufficient to induce cardiac defects, yet valve and septal defects are seen in Down syndrome mouse models overexpressing RCAN1/DSCR1. (Lange et al., 2004; Eggermann et al., 2010). Collectively these findings contribute to the evidence for the lack of a single causal gene for CHD in Down syndrome patients.

Study of additional genes within the Down syndrome critical regions of chromosome 21 have yielded promising candidates linked to CHD as well as established which genes have no association with cardiac defects. Ripply transcriptional repressor 3 (RIPPLY3)/Down syndrome critical region 6 (DSCR6) is required for proper mesoderm and neural induction and anterior patterning during early *Xenopus* development, implicating it in the early regulation of proper development of a number of tissues (Li et al., 2013). Further work in mice has demonstrated a requirement for RIPPLY3 in the development of the pharyngeal apparatus, which gives rise to multiple tissues including thymus, arteries, and the cardiac outflow tract, by repressing the T-box transcription factor, Tbx1 (Okubo et al., 2011). salt inducible kinase 1 (Sik1)/Snf1lk, a kinase expressed both in cardiac and skeletal muscle progenitors, has been found to be necessary for proper G2/M transition in these cells, implicating the kinase as essential for several mesodermal development programs (Stephenson et al., 2004; Romito et al., 2010). Kcnj6, a gene that encodes G protein mediated potassium channel components, has a demonstrated association with

cardiac conduction, as over-expression of Kcnj6 induces arrhythmic cardiac beating patterns in mice (Lignon et al., 2008). Phosphatidylinositol glycan anchor biosynthesis, class P (PIGP)/Down syndrome critical region 5 (Dscr5) regulates Wnt-mediated convergent extension during early development, providing evidence for a potential role in establishment of proper cardiac morphogenic movements via regulation of Wnt signaling (Shao et al., 2009). On the other hand, though SH3 domain-binding glutamic acid-rich protein (Sh3bgr) was once identified as a promising candidate gene due to its location within the critical region for CHD in Down syndrome patients, later work revealed no association between its overexpression and altered heart morphogenesis (Egeo et al., 2000; Sandri et al., 2004). Overall, much work remains to determine the role or lack thereof for many of these genes in Down syndrome related cardiac pathologies.

Critical microRNAs for CHD in Down syndrome

The genetic complexity associated with the variable phenotypes of Down syndrome have led researchers to look beyond the coding regions of chromosome 21. Studies examining microRNAs, either those expressed from chromosome 21 or those that have a direct effect on putative critical Down syndrome gene expression, have revealed additional insight into how misregulation of certain molecules may lead to CHD in Down syndrome. miR-155-5p is located and expressed from chromosome 21 and has a demonstrated role in regulating expression of the angiotensin type 1 receptor (AT₁R), an important mediator of several cardiovascular related processes via the angiotensin II hormone. As increased expression of miR-155-5p is associated with a reduction in AT₁R expression, proper dosage of the microRNA during development is crucial to avoid cardiac pathologies and may indicate a potential source of these pathologies in Down syndrome (Elton et al., 2013). The miR-99a/let-7c cluster on chromosome 21 has also

been implicated in cardiac development as well. let-7c promotes cardiac specification and differentiation via upregulating several known regulators of cardiac cell fate such as *Brachury*, *Nkx2.5*, and *Tbx5*, while mir-99a represses cardiac differentiation via regulation of *Nodal* and *Smad2* signaling. Unsurprisingly, expression of these known targets of miR-99a and let-7c are perturbed in Down syndrome patient cardiac tissue, implicating the overexpression of this cluster as likely contributor to CHD (Coppola et al., 2013). Finally two microRNAs, miR-1246 and miR-199b, have been independently identified as functional upstream regulators of Dyrk1a and subsequent calcineurin/NFAT signaling. Intriguingly, mir-1246 functions downstream of p53, implicating that pathway as an additional contributor to the various Down syndrome phenotypes (da Costa Martins et al., 2010; Zhang et al., 2011; Liao et al., 2012). The observation of a number of microRNAs expressed on chromosome 21 has led to recent attempts to use comparison of relative amounts of these microRNAs in amniotic fluid between euploid pregnancies and Down syndrome pregnancies as a non-invasive early screening method for the disorder. Although there was no significant difference in levels of microRNA expression, negating the assay's use as an early screening method, following up on this apparent lack of differential expression between euploid and Down syndrome pregnancies may lead to further insight as to the temporal and spatial requirements for these microRNAs to reduce or ablate CHD in Down syndrome (Kotlabova et al., 2013).

Associated genetic variants for CHD in Down syndrome

The consistent over-expression of a crucial set of genes on chromosome 21 in the vast majority of Down syndrome cases can not alone account for the wide variety of penetrance and severity of CHD in people with the disorder. Thus while much focus has been on determining the critical duplicated gene(s) responsible for CHD, efforts have also been undertaken to

understand how genetic variation beyond chromosome 21 may be contributing to Down syndrome related CHD. As an example, *Creld1* is a gene on chromosome 3 that is required for cardiac development, as certain mutations can lead to atrioventricular septal defects (AVSD) in a small percentage of the population (Robinson et al., 2003). These mutations are found in a larger subset of Down syndrome patients with AVSD, implying that misregulation of *CRELD1* can predispose Down syndrome patients to CHD (Maslen et al., 2006). To clarify this finding, a study examined *Creld1* and *Hey2*, another gene implicated in septal defects, in the background of a trisomy 21 mouse model. Crossing either *Creld1*^{-/-} or *Hey2*^{-/-} mice to trisomy 21 mice yielded either *Creld1*^{+/-} or *Hey2*^{+/-} offspring with significantly increased instances of septal defects compared to offspring from similar crosses with euploid mice. Additionally, crossing *Creld1*^{+/-} and *Hey2*^{+/-} mice to obtain compound heterozygotes also yielded offspring with significant septal defects, supporting the notion that trisomy 21 creates a sensitized background that is more susceptible to deleterious variants of particular genes that may lead to cardiac defects not seen in euploid individuals with these same variants (Li et al., 2012). Concurrent work examining *Creld1* associated haplotypes provides additional evidence to this hypothesis by identifying variants associated with AVSD in a significantly higher percentage of Down syndrome cases over control populations (Kusuma et al., 2011; Ghosh et al., 2012; Zhian et al., 2012).

To further examine this hypothesis, many groups are using a variety of approaches to determine other possible genetic variants that lead to CHD in Down syndrome. One such study compared genetic variants in Down syndrome individuals with AVSD to Down syndrome individuals with no CHD and found a number of potentially damaging variants at a significantly higher percentage in the AVSD group. Intriguingly, a number of the most significantly higher variants were associated with genes in the VEGF pathway including *Col6a1*, *Col6a2*, *Creld1*, as

well as Gata5 (Ackerman et al., 2012). VEGF had been previously linked to Down syndrome as an upstream regulator of Dscr1 expression (Yao and Duh, 2004). As the proper functioning of the VEGF pathway is necessary for proper angiogenesis and hence normal development across a broad number of tissues, it stands to reason that rare deleterious variants within the pathway that are sufficient to only cause isolated cases of CHD in normal populations may cause CHD at an exponentially higher rate in the sensitized Down syndrome background.

The folate metabolism pathway has also been examined for possible Down syndrome CHD variants due to the link between folic acid deficiency and CHD. The folate metabolizing enzyme methylenetetrahydrofolate reductase (MTHFR) in particular has been an intense area of study with regard to two polymorphisms, 677C-T and 1298A-C, and the link between maternal or fetal presence of these polymorphisms and CHD. While past studies have found the link inconclusive, more recent work has determined an significantly increased association with maternal Mthfr polymorphisms and CHD, particularly AVSD, in offspring with Down syndrome (Martinez-Frias et al., 2006; van Beynum et al., 2007; Brandalize et al., 2009; Bozovic et al., 2011). Variants in other genes of the folate pathway have also been recently found to associate with AVSD in Down syndrome offspring, further underscoring the requirement for proper folate metabolism in a sensitized trisomy 21 background (Locke et al., 2010). Recent studies continue to identify CHD associated variants in Down syndrome populations in genes such as Col6a3, another collagen VI family gene expressed on chromosome 2, and other chromosome 21 genes whose link to the pathologies of Down syndrome has yet to be determined (Dey et al., 2013; Sailani et al., 2013). Polymorphisms that predispose Down syndrome patients to CHD may also arise from particular chromosomal movements as well. Presence of AVSD has been associated with a low recombination rate of chromosome 21 in maternal gametes, while VSD in Down

syndrome patients has been linked to heterotrismy, in which three distinct chromosome 21 homologues (from three different grandparents) exist within the same gamete (Zittergruen et al., 1995; Baptista et al., 2000), providing additional evidence that the combination of particular variants may be important in contributing to CHD. Further work towards determining additional deleterious variants of particular genes as well as the critical combinations of variants necessary to induce these defects in a trisomy 21 background will be crucial towards providing clarity and potential therapeutic options for CHD in Down syndrome.

Epigenetic and environmental links to CHD in Down syndrome

Even the various deleterious polymorphisms of genes in a sensitized background of trisomy 21 are insufficient to account for the wide-range of CHD seen in Down syndrome. Additional means of gene expression regulation are likely affected as well, and recent studies have indicated that the misregulation of both maternal and fetal epigenetic patterning, particularly methylation, may contribute to not only Down syndrome but the various types of CHD therein. Distinct patterns of methylation have been observed that distinguish Down syndrome patients from euploid individuals, and the development of disorders in Down syndrome patients has been linked to the loss of methylation at certain developmental genes (Jones et al., 2013; Malinge et al., 2013). Specific to cardiac defects, the aforementioned chromosome 21 resident let-7c microRNA functions by promoting the derepression of cardiac transcription factors by increasing levels of H3K4me3 and decreasing H3K27me3 (Coppola et al., 2013). Furthermore, multiple studies have linked maternal hypomethylation to the presence of CHD in Down syndrome offspring (van Driel et al., 2008; Obermann-Borst et al., 2011a).

The source of and the mechanism by which methylation defects lead to Down syndrome and associated CHD remain an active area of study. One theory is that the disruptions in methylation are linked to the misregulation of the folate pathway that is necessary for methyl group metabolism. As noted previously several polymorphisms in folate pathway genes have been linked to CHD in Down syndrome, and folic acid deficiency is associated with CHD in normal and Down syndrome populations alike (Martinez-Frias et al., 2006; van Beynum et al., 2007; Brandalize et al., 2009; Locke et al., 2010; Bozovic et al., 2011). Furthermore studies in recent years have indicated that several genes on chromosome 21 may function in folate or methyl group metabolism (Figure 1.2) (Moscow et al., 1995; Katsanis et al., 1997; Kraus et al., 1999; Solans et al., 2000; Bourc'his et al., 2001). Additionally, a growing hypothesis is that adequate maternal folate metabolism is crucial to maintain necessary centromeric methylation patterns, the absence of which leads to increased risk of nondisjunction (James et al., 1999; Migliore et al., 2009). This theory is backed by work linking hypomethylation to broader chromosomal instability (Eden et al., 2003). Collectively these studies indicate that developing trisomy 21 fetuses are vulnerable to folate pathway mediated CHD from not only deleterious variants or inadequate folate intake from the mother but also from inherited deleterious variants or overexpression of chromosome 21 folate pathway genes. Regional diets and access to sufficient folate may also be a partial source for the variation in Down syndrome related CHD seen throughout global populations and may partially explain the increased or decreased incidence of specific types of CHD such as AVSD versus VSD.

Outside of the folate pathway, there are a number of factors that may alter the epigenetic landscape in a way that increases the risk of CHD. VSD and AVSD in children is positively linked to maternal smoking in the first trimester, which has a demonstrated effect on altering

methylation patterns (Alverson et al., 2011; Joubert et al., 2012; Elliott et al., 2014). Prenatal maternal fever is also associated with AVSD in Down syndrome infants as well, possibly due to epigenetic changes (Oster et al., 2011). Studies have also linked chronic maternal conditions such as diet, diabetes, hypertension, connective tissue disorders, maternal CHD, and maternal age to CHD in offspring (Obermann-Borst et al., 2011b; Liu, S. et al., 2013). While maternal age has an established association with increased Down syndrome risk, many of these conditions have no such association (Antonarakis et al., 2004). Instead it is likely that these conditions function in much the same way as particular genetic variants in that they are associated with a smaller basal rate of CHD seen in offspring that becomes magnified in the sensitized background of trisomy 21. Future research will hopefully further elucidate the environmental factors that can contribute to CHD in Down syndrome and potentially even separate the associated factors from the causal ones.

Although Down syndrome in the vast majority of cases is due to a single chromosomal abnormality, the resulting phenotypes are numerous and variable. All available evidence indicates that a number of factors dictate the number and severity of Down syndrome related disorders such as CHD. As the development of CHD most likely relies on the disruption of multiple genetic pathways, aberrant protein-protein interactions, particular combinations of associated polymorphisms, as well as a number of environmental factors, the specific causal links between Down syndrome and CHD will likely remain elusive. Nonetheless further understanding the underlying genes and other factors contributing to these disorders will serve to understand how to not only manage cardiac pathologies in Down syndrome but in the global population as well.

DISSERTATION GOALS

One potential source of cardiac pathology is the misregulation of cardiac expressed transcription factors and their subsequent downstream targets. The transcription factors often have discrete functions that are regulated by 1) spatial and temporal regulation of their expression and 2) the formation of distinct transcriptional complexes with regionally expressed cofactors. We sought to determine if castor zinc finger 1 protein (CASZ1), a novel para zinc finger transcription factor required for early cardiac cell fate, is regulated by direct protein-protein interactions during early cardiac development. In Chapter 2 we generate a cDNA library from cardiac-enriched tissue during the stages that CASZ1 is known to be required for *Xenopus* cardiac development and then use CASZ1 as bait within a yeast two-hybrid screen to identify potential interacting protein partners encoded by our cDNA library. We identify a number of high priority candidates that, like CASZ1, are either localized within the nucleus or have a conserved role in cardiac development or function. We then develop a series of protocols to thoroughly validate whether these candidates form a functional interaction with CASZ1 within developing cardiomyocytes. In Chapter 3 we describe the characterization of an interaction with CASZ1 of one of our identified candidates, the chromosome 21 expressed protein congenital heart disease 5 protein (CHD5). We describe the functional requirement for this interaction in *Xenopus* during early cardiac looping and morphogenesis, and we demonstrate that CASZ1 and CHD5 are collectively required for proper cardiomyocyte integrity by maintaining cell junctions and apical-basal polarity within cardiomyocytes. Going forward, we will attempt to further characterize this interaction by determining the cardiac specific protein complexes that function together with CASZ1 and CHD5, the downstream genetic targets whose expression is regulated by CASZ1 and CHD5, and the overall pathways that CASZ1 and CHD5 function within to

maintain proper cardiac development. We will also determine the potential association of that this interaction with CHD particularly in the Down syndrome background. Collectively our work will increase our understanding of the important role protein-protein interactions play in cardiac development and disease.

Figure 1.1 Congenital heart disease in Down syndrome.

Common cardiac defects seen in Down syndrome patients include atrioventricular septal defects (AVSD), ventricular septal defects (VSD), atrial septal defects (ASD), patent ductus arteriosus (PDA), and Tetralogy of Fallot (ToF), which is characterized by 1) pulmonary stenosis, 2) overriding aorta, 3) ventricular septal defects, and 4) right ventricular hypertrophy.

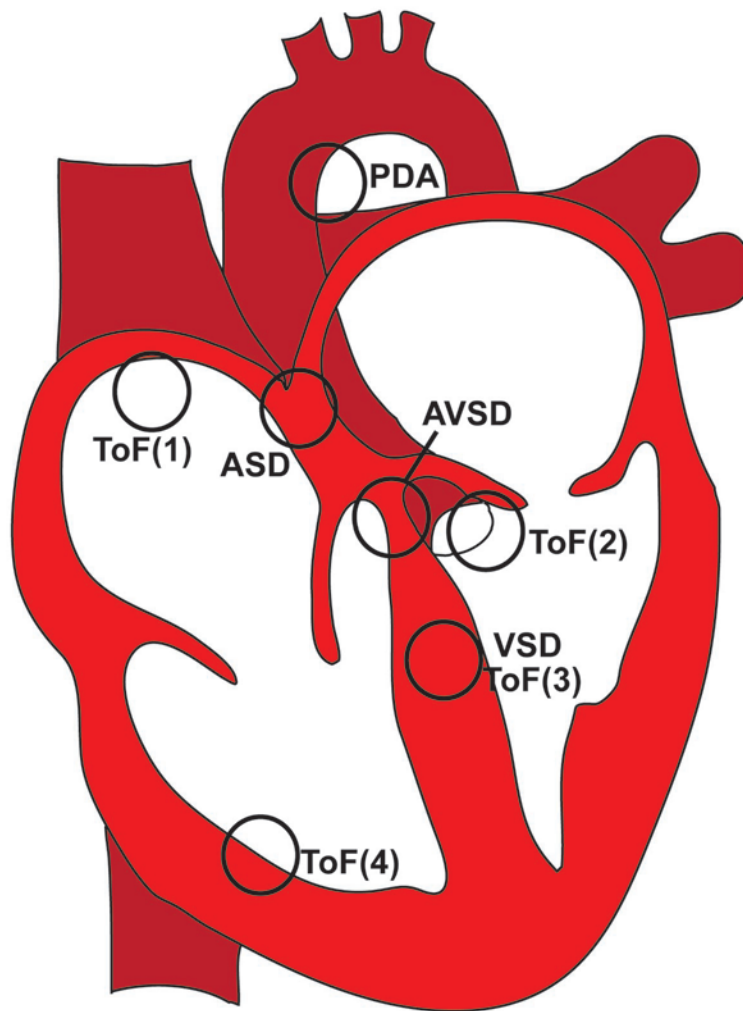
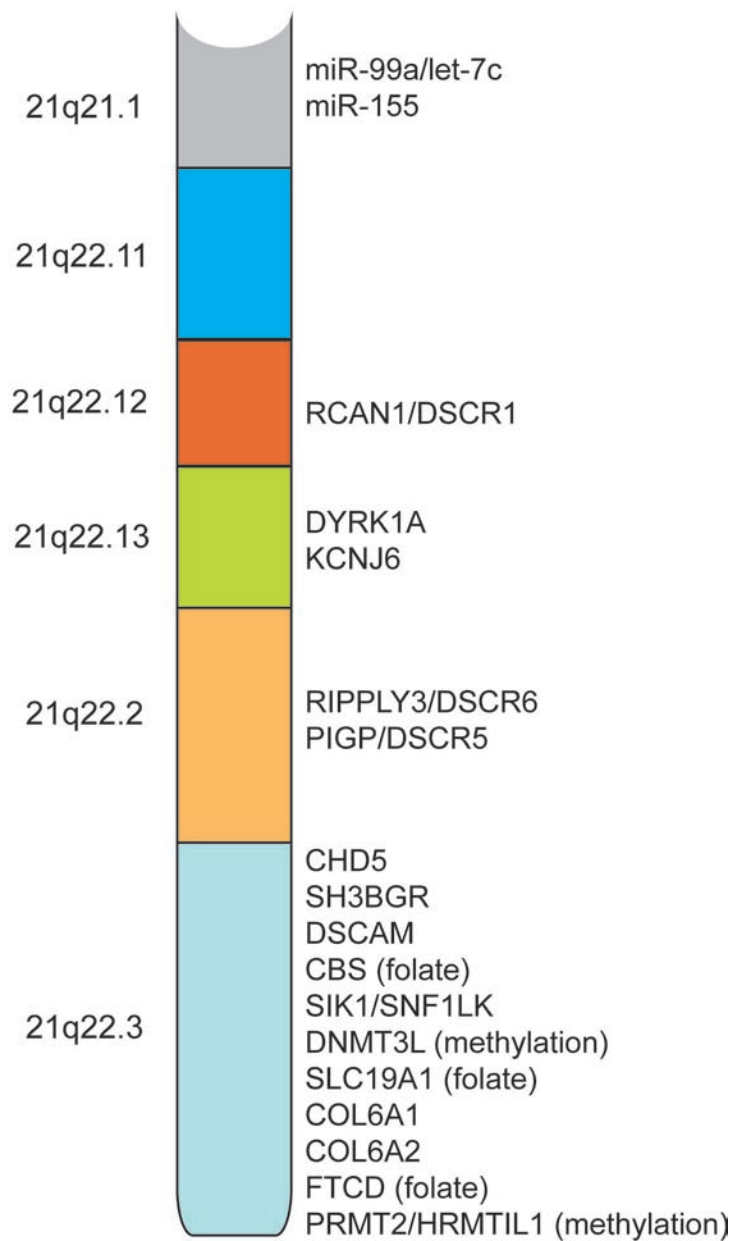


Figure 1.2 Chromosome 21 critical region for CHD in Down syndrome.

A schematic of the critical regions of chromosome 21 that may contribute to congenital heart disease in Down syndrome along with the relative locations of the genes of interest within these regions



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CHAPTER 2: A YEAST TWO-HYBRID SCREEN TO DETERMINE CARDIAC SPECIFIC CASTOR (CASZ1) INTERACTING PROTEINS

ABSTRACT

To further understand the mechanisms by which CASTOR (CASZ1) regulates cardiac development, we performed a yeast two-hybrid screen to identify putative interacting partners. Cardiac-enriched tissue was isolated from *Xenopus* embryos and collected during the stages that require CASZ1 for proper cardiac development. We isolated mRNA from this tissue to generate a cDNA library that was screened in a yeast two-hybrid system using full-length CASZ1 as bait. We identified positive clones by sequencing, and then prioritized them based on established criteria indicative of a probable functional relationship with CASZ1 or a similar role to CASZ1 in cardiac development. High-priority candidates were grouped by function to further assess their potential as CASZ1-interacting partners. Finally, we established protocols to further validate the putative interactions, both *in vitro* and *in vivo*, and subsequently determine any functional relevance for the validated interactions during heart development.

INTRODUCTION

Proper embryonic development requires a precise interplay among complex genetic networks. These networks can function independently to regulate distinct developmental functions but may also cooperate with other networks either redundantly or in a spatially or temporally overlapping manner (MacGrogan et al., 2011; Wynshaw-Boris, 2012; Dawson et al., 2013; Prasetyanti et al., 2013; Li et al., 2014). The regulation of these networks depends heavily on the well-defined control of transcription factors, which, in turn, direct the precisely timed and

tissue-specific expression of downstream genetic targets (Mandel et al., 2010; Afouda and Hoppler, 2011). Many transcription factors have distinct roles during discrete developmental time points, and how exactly a transcription factor is differentially regulated in a context-dependent manner is a major area of study in developmental biology (Goetz et al., 2006; Minguillon et al., 2012; Xu et al., 2013; Nakashima et al., 2014).

The para-zinc finger protein CASTOR (CASZ1) is one such transcription factor that has distinct roles in several tissues during development. Castor family transcription factors were initially identified and characterized in *Drosophila* (Cas) as a transcriptional regulator of neuroblast cell fate (Cui and Doe, 1992; Mellerick et al., 1992). Cas acts as a repressor and functions within a temporal transcriptional cascade that determines whether developing neuroblasts become early-, mid-, or late-born neurons (Kambadur et al., 1998; Grosskortenhaus et al., 2006). Previous work in our laboratory revealed a fundamental requirement for CASZ1 during early vertebrate heart development. Depletion of CASZ1 in *Xenopus* embryos results in the failure of a subset of cardiac progenitor cells at the ventral midline to differentiate into cardiomyocytes. This defect results in varying degrees of cardia bifida, leading to aberrant cardiac morphogenesis, which is characterized by improperly looped hearts with malformed and, at times, duplicated chambers (Christine and Conlon, 2008). To date, there have been limited studies concerning a cardiac-specific role for CASZ1 in mammals. However, *Casz1* is expressed in the primitive heart tube in mice, and *CASZ1* is expressed in adult human heart tissue (Vacalla and Theil, 2002; Liu et al., 2006). Additional work in our laboratory has demonstrated that CASZ1 has an essential, and evolutionarily conserved, role in blood vessel assembly and lumen formation (Charpentier et al., 2013). Consistent with these data, two recent genome-wide association studies

(GWAS) showed a genetic association between the human *CASZ1* locus and both high blood pressure and hypertension, indicating a conserved cardiovascular role for *CASZ1* (Levy et al., 2009).

Despite the well-characterized, essential role for *CASZ1* in various tissues during development, as well as its potential involvement in human disease, little is known about the molecular mechanisms by which *CASZ1* functions. During *Drosophila* neural development, *CASZ1* regulates neuroblast fate through the direct transcriptional repression of POU domain proteins 1 and 2 (*pdm-1 and -2*), which lack a vertebrate homolog and do not appear to be evolutionarily conserved targets (Kambadur et al., 1998; Grosskortenhaus et al., 2006; Tran and Doe, 2008). Recent studies have attempted to provide further insight into neuroblastoma-specific transcriptional targets of human homologue *CASZ1* (Liu et al., 2011a; Liu et al., 2011b). Previously, we identified epidermal growth factor-like domain 7 (*Egfl7*) as a direct transcriptional target of *CASZ1* that is necessary to promote RhoA-mediated blood vessel assembly (Charpentier et al., 2013). However, no cardiac-specific transcriptional targets of *CASZ1* have yet been identified and validated. Intriguingly, work in *Drosophila* has demonstrated a role for *CASZ1* as a transcriptional repressor during early neural development, whereas our studies have implicated *CASZ1* as a transcriptional activator in the context of vascular development (Kambadur et al., 1998; Tran and Doe, 2008; Charpentier et al., 2013). Precisely how *CASZ1* is differentially regulated across various tissues and the molecular mechanisms that dictate its context-dependent activity as a transcriptional activator or repressor remain unclear.

We hypothesized that *CASZ1* interacts with different proteins depending on its spatiotemporal expression, thus forming distinct transcriptional complexes that determine its ability to either activate or repress unique sets of downstream gene targets. To address this

hypothesis, a yeast two-hybrid screen was undertaken to determine interacting protein partners of CASZ1 during early cardiac development. To determine cardiac-specific CASZ1-interacting partners, a cDNA library was generated from *Xenopus* cardiac-enriched tissue during the developmental time span that CASZ1 is expressed and required for proper differentiation of cardiomyocytes. This library was screened using full-length CASZ1 as bait.

MATERIALS AND METHODS

***Xenopus laevis* fertilization**

Xenopus embryos were prepared as previously described and by the following methodology (Showell et al., 2006). *Xenopus* females were injected with 500 units of human chorionic gonadotropin 16 hours prior to fertilization to induce egg laying. Eggs were laid and collected in 1× MMR buffer (100 mM NaCl, 2 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 5 mM HEPES). A *Xenopus* male was euthanized in 2% tricaine solution, and the testes were dissected. Eggs were collected in a beaker, and MMR was replaced with 0.1× MBS (8.8 mM NaCl, 0.1 mM KCl, 0.1 mM MgSO₄, 0.1 mM CaCl₂, 0.5 mM HEPES, 0.25 mM NaHCO₃). Approximately one-third of the minced testes was added to eggs in a minimal volume of 0.1× MBS. Eggs were incubated at room temperature for 30 minutes, followed by washing in 2% L-cysteine hydrochloride to remove the jelly coat to allow for experimental manipulation. Embryos were staged according to Nieuwkoop and Faber's developmental atlas (Nieuwkoop and Faber, 1994).

***Xenopus laevis* tissue collection and isolation**

Xenopus laevis embryos were collected at stages 27 to 29, during the developmental time points that CASZ1 is required for proper heart development (Christine and Conlon, 2008). During these stages, the developing heart field is indistinguishable from the surrounding endoderm and other tissue; thus, tissue containing the differentiating heart field was dissected

based on anatomical landmarks such as the cement gland and brachial arches to minimize the collection of extraneous non-cardiac tissue. Cardiac-enriched tissue was collected, snap frozen, and stored at -80°C. Next, 600 µl of lysis buffer (50 mM Tris HCl [pH 7.5], 50 mM NaCl, 5 mM EDTA, 0.5% sodium dodecyl sulfate [SDS], 250 µg/ml proteinase K) was applied to the frozen tissue, and the mixture was pipetted up and down to homogenize the tissue. The sample was incubated at 45°C for 45 minutes, vortexing for 15 seconds every 15 minutes.

Phenol:chloroform extraction was performed on the sample, followed by precipitation in 100% ethanol at -80°C. The precipitate was spun down at 14,000 rpm at 4°C for 10 minutes, followed by washing with 70% ethanol. The sample was incubated with RQ1 DNase (Promega) and RNAsin (Promega) for 30 minutes at 37°C, and then extraction and precipitation were repeated to remove DNase enzymes and buffer. The sample was resuspended in diethylpyrocarbonate (DEPC)-treated water, and then poly(A) mRNA was purified from the isolated RNA using an Ambion Poly(A) Purist mRNA purification kit.

cDNA library synthesis and amplification

cDNA was synthesized from the isolated mRNA sample using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase and SMART III oligo (5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG-3') and CDS III Oligo (dT) Primer (5'-ATTCTAGAGGCGGAGGCGGCCGACATG-D(T)₃₀VN-3') (Clontech). These primers selectively generate cDNA from poly(A) transcripts and attach 5' and 3' anchors to allow recombination-mediated cloning with the pGADT7-Rec prey vector in yeast. Recombination-mediated cloning allows higher efficiency of in-frame cDNA insertion into a prey vector. First-strand cDNA synthesis and subsequent amplification via long-distance polymerase chain

reaction (PCR) were carried out according to manufacturer's instructions using the Matchmaker Library Construction & Screening Kit (Clontech).

Generation of bait vector and prey library

Competent Y187 and AH109 yeast strains were generated by incubating strains in liquid YPDA medium until OD₆₀₀ reached 0.4-0.5 then pelleted and resuspended in 1.1×TE/LiAc solution. *Xenopus* cDNA was transformed with pGADT7-Rec via homologous recombination into the yeast strain AH109 and selected on -Leu YPD media plates. Transformations were performed by incubating the generated cDNA sample, 3µg pGADT7-Rec and 20µl denatured Herring Carrier DNA with 600µl competent cells in 2.5ml PEG/LiAc solution at 30°C for 45 min. 160µl DMSO was added, sample was heat shocked at 42°C for 20 min, then incubated at 30°C for 90 min before plating. The cloning of full-length CASZ1 into pGBKT7 was performed in two steps owing to size restrictions and lack of common restriction sites. The 5' portion of Casz1 was amplified from Cst-V5 in pCDNA3.1 (Christine and Conlon, 2008) using (For: 5'-GCCACATATGATGAAAGTTGACTAT-3') and (Rev: 5'-AACTGCTGCTGCCTTCAGTG-3'). This fragment along with a 3' Casz1 fragment obtained from 8xhis-FL-Cstβ (unpublished) was then cloned into pGBKT7 via restriction digest with NdeI and SalI. The bait vector was transformed into the yeast strain Y187 as described above and selected on -Trp YPD media plates.

Yeast two-hybrid screen

The Y187 strain, containing full-length CASZ1 in pGBKT7, and the AH109 strain, containing cDNA prey library in pGADT7-Rec, were mated according to the manufacturer's instructions (Clontech). Positive-interacting diploid clones were detected on -Ade/-His/-Leu/-Trp plates containing 3 mM 3-amino-triazole and X-Gal (80 mg/L). The mating efficiency was

determined by spreading 100µl of a 1:10,000, 1:1,000, 1:100, and 1:10 dilution to determine total number of diploid offspring (Clontech). Clones were isolated and restreaked on -Ade/-His/-Leu/-Trp plates containing 3 mM 3-amino-triazole and X-Gal (80 mg/L) to eliminate weak-interacting false positive clones.

Positive clone analysis and DNA isolation and sequencing

Individual yeast clones were grown in 5 ml of -Ade/-His/-Leu/-Trp liquid media. The cultures were pelleted and resuspended in Yeast Lysis Buffer (2% Triton X-100, 2% SDS, 0.1 M NaCl, 0.01 M Tris [pH 8.0], 1 mM EDTA). Approximately 500 µl of acid washed glass beads (425–600 µm) and 200 µl of phenol:CHISMA were added to the samples, which were shaken for 5 minutes at maximum speed in a TOMY microtube mixer. The samples were spun down, and the top layer was collected and added to a tube containing 2 ml of 100% ethanol. The samples were incubated at room temperature for 2 minutes, and then were centrifuged for 10 minutes at 14,000 rpm. The supernatant was discarded, and the pellets were washed with 70% ethanol (chilled at -20°C) and resuspended in 70% ethanol. The cDNA inserts in pGADT7-Rec were then isolated by transformation into *Escherichia coli* under ampicillin selection. The purified plasmids were sequenced using 5' and 3' LD-Insert Screening Amplimers (Clontech). Using blastx analysis of protein databases and translated nucleotide queries, individual clones were identified and prioritized based on criteria discussed in the Results section.

RESULTS

Yeast two-hybrid screen to identify cardiac-enriched CASZ1-interacting proteins

To screen for cardiac specific CASZ1 interacting partners, I dissected cardiac enriched tissue from *Xenopus* embryos during the stages CASZ1 is known to be required for cardiac development (Christine and Conlon, 2008). mRNA was isolated and collected from the tissue,

and a cDNA library was generated. Following successful cloning and transformation of the cardiac-enriched *Xenopus* cDNA library into the pGADT7-Rec prey vector within the yeast strain AH109 via homologous recombination, the transformation efficiency was examined via selection on -Leu plates. A total of 1.1×10^6 positive transformants per 3 μ g of mRNA were obtained, exceeding the threshold for sufficient transformation efficiency and complexity (Clontech). After mating the prey library-containing AH109 strain with a Y187 strain containing full length CASZ1 as bait, the mating efficiency was determined via selection on -Leu/-Trp plates and subsequent extrapolation. The mating efficiency was determined to be 1×10^6 diploid offspring screened for interaction, meeting the threshold for sufficient prey and bait containing offspring screened (Clontech). Overall, 237 clonal colonies were obtained on -Leu/-Trp/-His/-Ade plates with an average insert length of approximately 900 bp.

Identification of positive clones by sequencing

Clones were then sequenced and identified via BLAST analysis, scanning for non-redundant protein sequences via blastx, which identifies homologous protein sequences using a translated nucleotide query (Gish and States, 1993). Sequences were not obtained from a subset of 36 clones after repeated purification, so they were deemed low priority and set aside for future analysis. Additionally, 43 clones, comprising 23 distinct candidates, did not align to any known domain or protein within the administered BLAST criteria; they also were set aside for future analysis. Thus, 158 clones were identified that were aligned to a known protein or domain.

Selection of high-priority, CASZ1-interacting protein candidates

Several further steps were taken to refine our list of putative CASZ1-interacting proteins. We assessed whether the remaining identified candidates were cloned in frame to the GAL4 activation domain because BLAST analysis examines all the reading frames of the translated

nucleotide query sequence in an unbiased manner (McGinnis and Madden, 2004). It was also determined whether candidates resided within classes of proteins commonly identified as false-positive interactors within a two-hybrid system. These classes include proteosomal subunits, ribosomal subunits, and heat- and cold-shock proteins related to the stress response, among others (Serebriiskii and Golemis, 2001). Subsequently, literature searches were undertaken to establish whether the potential candidates met any criteria for consideration as a high-priority candidate. These criteria included candidates that are expressed in cardiac tissue, are nuclear localized, have an established role in the regulation of transcription or determination of cell fate, and are associated with known cardiac pathologies. These steps eliminated 121 of the 158 identified clones, leaving 37 clones representing 18 distinct candidates of high interest (Table 2.1). We identified one candidate for further analysis, Congenital heart disease protein 5 (CHD5). The functional characterization of the interaction between CHD5 and CASZ1 is explored in detail in Chapter 3. The following section discusses our other candidates of high interest that will be explored further in the future.

DISCUSSION

The yeast two-hybrid screen generated several putative CASZ1-interacting candidates. Using the above-listed criteria, we successfully identified proteins that were localized to the nucleus, were expressed in cardiac tissue, or possessed both qualities, all crucial qualifiers for identifying likely CASZ1-interacting partners. The resulting culled list of high-priority candidates was then cataloged into groups of proteins featuring similar criteria for identification as high priority (Table 2.1).

Candidates associated with cardiac development and disease

Although CASZ1 has never been linked directly to a particular vertebrate cardiovascular pathology, its probable role as a cardiac- and vascular-expressed transcription factor gives credence to the hypothesis that proper regulation of CASZ1 is necessary to promote proper cardiovascular function. Indeed, misregulation of CASZ1 is likely to be associated with cardiovascular disease, given the established requirement for CASZ1 in the regulation of appropriate cardiac morphogenesis, blood vessel development, and lumen formation in *Xenopus*, as well as its association with both high blood pressure and hypertension in humans. Thus, we selected several proteins as high-priority candidates due to their known, or potential, association with cardiac pathology. One high-priority candidate was congenital heart disease protein 5 (CHD5), also known as tryptophan rich basic protein, which was first cloned out of the region of chromosome 21 linked to congenital heart disease (CHD) in Down syndrome patients (Egeo et al., 1998). CHD5 is nuclear localized in cardiac-derived fibroblasts, and depletion of the protein in medaka fish leads to defects in cardiac morphogenesis (Murata et al., 2009). Furthermore, proper regulation of CHD5 is required for proper cardiac repolarization, demonstrating a role for CHD5 in cardiac conduction (Milan et al., 2009). Another high-priority CASZ1-interacting candidate was the cardiac troponin-interacting kinase TNNI3K, which is another protein that must be regulated tightly during cardiac development. Overexpression of TNNI3K leads to increased cardiac myogenesis and mediates cardiac injury via myocardial infarction (Lai et al., 2008). Intriguingly, other studies have demonstrated that overexpression of TNNI3K, via increased kinase activity, leads to cardiac hypertrophy and cardiac dysfunction—specifically, dysfunction of the left ventricle in a pressure overload mouse model. These data indicate that tight regulation of TNNI3K is necessary for proper cardiac growth and function (Tang et al.,

2013; Wang, H. et al., 2013; Wang, X. et al., 2013). Another kinase identified as a high-priority candidate was Ca/calmodulin-dependent protein kinase (CaMK), which has been linked to cardiac disorders. Like TNNT3, increased signaling of CaMK leads to cardiac myopathy—specifically, in this case, cardiac arrhythmia-induced heart failure (Nakagawa et al., 2006; Sag et al., 2009; Herren et al., 2013). The identification of two kinases, each with a strong link to proper cardiac function, as CASZ1-interacting partners suggests that CASZ1 could also be regulated by post-translational modifications. Finally, multiple clones of the proteases cathepsin L and cathepsin B, as well as the protease inhibitor cystatin B, were independently identified as CASZ1 interactors. Cystatin B and cathepsin B colocalize in the nucleus of some differentiated cell types, whereas lower levels of cathepsin L in cardiomyocytes results in dilated cardiomyopathy in mice (Riccio et al., 2001; Danjo et al., 2007; Spira et al., 2007). These findings point to a potential collective role for cathepsins and cystatins in the regulation of cardiac cell fate, a function that may involve their interaction with CASZ1.

Candidates involved in transcription or regulation of cellular fate

As a zinc finger protein, CASZ1 presumably functions as a DNA-binding transcription factor to regulate cardiac cell fate and subsequent morphogenesis. We classified a number of candidates as high priority due to their known roles in regulating either transcription or cell fate. One such protein, transforming acidic coiled-coil protein 3 (TACC3), also known as Maskin, is a centrosomal protein that was traditionally associated with mitotic spindle assembly. However, TACC3 is also upregulated during differentiation in several tissues, indicating its potential role in cell-fate regulation (Sadek et al., 2003). One study has confirmed this possibility, demonstrating that Maskin regulates GATA-binding protein 1 (GATA-1)-mediated hematopoiesis by binding friend of GATA-1 (FOG-1) and sequestering the GATA-1 cofactor in

the nucleus (Garriga-Canut and Orkin, 2004). Conceivably, Maskin could similarly function to regulate cardiomyocyte fate through the control of localization of CASZ1 in cardiac tissue. Related to the identification of Maskin, we identified two independent clones, each replicated four times, of generic coiled-coil domain-containing proteins of unknown origin. This finding introduces crucial information about the broader protein structural domains of CASZ1-interacting proteins because proteins with coiled-coil domains have a number of relevant biological functions involving gene regulation (Mason and Arndt, 2004). Furthermore, this discovery provides evidence that CASZ1 can bind to proteins with coiled-coil domains, such as Maskin. Another regulator of transcription identified in the screen was Daxx. Daxx serves as a transcriptional repressor in a number of tissues, and it mediates transcription, as well as nuclear localization, of target proteins via post-translation modification, namely SUMOylation (Shih et al., 2007; Puto and Reed, 2008). To date, no study has clarified whether CASZ1 regulation is dependent on post-translational modification. Therefore, this path of research may be worth exploring further.

We also identified several CASZ1-interacting candidates that are involved in general regulation of gene expression, a finding that warrants further examination owing to the noteworthy connections of these CASZ1-interacting candidates to mesodermal development. For example, RuvB-like 2 (RuvBl2) is a DNA helicase that, interestingly, is found in increasing abundance during skeletal-muscle patterning (Jha and Dutta, 2009; Castorena et al., 2011; Flavin et al., 2011). In addition, transcription elongation factor b (P-TEFb), which associates with cyclin-dependent kinase 9 (Cdk9), also mediates transcription in several tissues. The misregulation of P-TEFb is associated with many differentiation defects and pathologies, and P-TEFb directly interacts with GATA-binding protein 4 (GATA4) to promote cardiomyocyte

differentiation (Cabart et al., 2004; Zhou and Yik, 2006; Muniz et al., 2012). Another candidate, the Nck SH2/SH3 adaptor protein, is notable for its role in mediating protein-protein interactions within signaling complexes during development. Furthermore, inactivating Nck results in patterning defects along both the anterior-posterior and dorsal-ventral axes, a finding that can be traced to disruptions in early mesodermal cell fate (Tanaka et al., 1997). Finally, we identified an elongation initiation factor that serves as a regulator of translation and first seemed unrelated to the role of CASZ1 in regulating transcription. However, a particular isoform, eIF4A, is tightly regulated by MyoD during muscle differentiation, demonstrating that transcription and translation can be tightly and simultaneously regulated. Furthermore, the tissue-specific manner of this coupled regulation could be due to the formation of tissue-specific protein complexes (Galicia-Vazquez et al., 2014). Although these proteins mediate gene expression across a number of cell types, their specific roles in mesodermal cell fate suggest that they may function likewise via physical association with proteins linked to cardiomyocyte fate, such as CASZ1.

Candidates involved in Rho-mediated cell adhesion and cytoskeletal organization

During cardiac development, a subpopulation of cardiac precursors located at the ventral midline fail to undergo differentiation into cardiomyocytes in the absence of CASZ1. This differentiation failure results in cardia bifida and subsequent defects in cardiac morphogenesis. Studies have determined that this phenotype is due to a primary defect in cardiomyocyte differentiation and is not due to defects in the underlying endoderm, the extracellular matrix, or cell polarity (Christine and Conlon, 2008). However, the possible contributions of defects in either cell-cell adhesion or cytoskeletal organization have not been examined closely in the context of this aberrant morphogenesis. Strikingly, a number of proteins were identified in this screen that are involved in the integrity of developing tissue, as either facilitators of cell-cell

adhesion or as structural components linked to proper adhesion of cardiomyocytes. For example, we identified partial clones of an unidentified guanine nucleotide-binding protein and an ADP-ribosylation factor. Small G proteins in both of these classes function within the Rho GTPase pathway to regulate a number of processes in cardiomyocytes, including cytoskeletal organization, cell growth, and cell morphology. The misregulation of signaling associated with these proteins leads to cardiac hypertrophy and, in some cases, heart failure (Ren and Fang, 2005). Another identified protein, zona occludens protein 2 (ZO2), functions alongside its related homolog ZO1 and is mediated by Rho signaling to form tight junctions in various developing tissues (McCormack et al., 2013). Additionally, the screen candidate flotillin functions in a Rho-dependent manner and is associated with multiple processes relevant to CASZ1 functions, such as polarization of hematopoietic cells and cytoskeletal rearrangement during differentiation (Langhorst et al., 2008; Rajendran et al., 2009). Finally, myosin and cardiac actin were identified from the screen as well. In addition to validating the enrichment of cardiac-expressed genes in the screen, the presence of these structural genes is also interesting considering that their expression is also Rho mediated (Shi and Wei, 2013; Shi et al., 2013; Lee et al., 2014). The presence of so many identified proteins in this screen that are regulated by Rho proteins is an intriguing finding that warrants further examination and discussion.

Further validation of CASZ1-candidate interaction during cardiac development

All of the high priority candidates have been validated via rescreening of the clones in the presence of the full-length CASZ1 bait in the two-hybrid system. The candidates that have been chosen for further analysis will undergo a series of validations to further demonstrate a true interaction with CASZ1. First, the interaction will be validated *in vivo*. We will inject mRNAs encoding an epitope-tagged CASZ1 and an epitope-tagged prospective protein partner into

fertilized *Xenopus* embryos, then we will perform reciprocal coimmunoprecipitation assays. Alternatively, in the absence of a positive *in vivo* interaction, we will attempt to validate the interaction in an *in vitro* assay, such as a GST pull-down assay. While confirming the interaction of these prospective candidates, concurrent expression analysis will take place. *In situ* hybridization will be performed on *Xenopus* embryos to examine whether the expression pattern of the prospective candidate overlaps with CASZ1 in cardiac tissue, during the stages that CASZ1 is required for heart development. We will also perform immunohistochemistry to determine whether the putative partner and CASZ1 colocalize within the nuclei of cardiomyocytes, either by detection of endogenous proteins with commercially available antibodies, or by detection of an expressed epitope-tagged protein using an appropriate antibody. Validation of the putative interactions outside of the two-hybrid system, and confirmation of the coexpression and colocalization of CASZ1 and its interacting partners in cardiac tissue at the relevant stages of cardiac development, are crucial steps that must be performed prior to pursuing the functional consequences of these interactions.

Next, steps will be taken to examine whether there are functional roles for the interactions between CASZ1 and the identified protein partners. First, the protein partner will be depleted in *Xenopus* embryos using morpholinos, and the resulting embryos will be examined for the presence of cardiac defects that are similar to those observed in CASZ1-depleted embryos. Overexpression of the protein partner will also be attempted to examine whether the cardiac defects will phenocopy those observed in embryos overexpressing CASZ1. To confirm a genetic requirement for the CASZ1/partner interaction, suboptimal doses of morpholinos for each protein will be simultaneously injected into *Xenopus* embryos. Embryos will then be examined to determine whether knock down of both of these proteins will result in phenotypes similar to

full depletion of either protein alone. Finally, we will determine the minimal-interacting domain of CASZ1 for the identified partner. Next, we will determine whether deletion of this minimal region from CASZ1 abrogates the collective functional role for CASZ1 and its partner during heart development.

The examination of several of these high-priority candidates should further elucidate the mechanisms of CASZ1 regulation in cardiac development. Once these candidates have been fully assessed, and either validated or eliminated as candidates, the next logical step will be a closer examination of the unidentified clones that were pulled from the two-hybrid screen. We will first validate these sequences by *in situ* analysis to screen for expression patterns similar to CASZ1, and then attempt to validate their physical interactions with CASZ1. In addition, stored portions of the cDNA library in yeast may be rescreened either to obtain more independent clones of previously identified candidates or locate novel candidates for interaction with CASZ1. Overall, the initial screen has proven to be successful in identifying several strong candidates that may function via physical interaction with CASZ1 to regulate heart development.

Table 2.1 High priority candidates of interest. Potential CASZ1 interacting proteins identified by establishment of nuclear localization, role in transcriptional control, role in cardiac pathology, role in development, and/or role in regulation of cell fate. Proteins identified in multiple clones are designated as (#X). For characterization of cardiac expression and nuclear localization, Y=Yes, N=No, and ND=Not determined.

Putative interacting partner	Expressed in heart	Nuclear localization	Function	References
Associated with cardiac development and disease				
Congenital heart disease protein 5 (CHD5) (2x)	Y	Y	Nuclear localized protein associated with CHD in Down Syndrome patients; Required for proper cardiac morphogenesis and repolarization	(Egeo et al, 1998) (Murata et al, 2009) (Milan et al, 2009)
TNNI3 interacting kinase	Y	N	Cardiac specific kinase that phosphorylates cardiac troponin; overexpression is associated with cardiac hypertrophy and dysfunction	(Lai et al, 2008) (Tang et al, 2013) (Wang, H. et al, 2013) (Wang, X. et al, 2013)
Ca/Calmodulin dependent protein kinase	Y	ND	Kinase whose activity is mediated by HDACs and whose improper regulation is linked to heart failure	(Nakagawa et al, 2006) (Sag et al, 2009) (Herren et al, 2013)
Cystatin B (6x) / Cathepsin B (2x)	Y/Y	Y/Y	Certain cystatins and cathepsins collectively colocalize in nuclei of differentiated tissues and are associated with cardiomyopathy	(Riccio et al, 2001) (Danjo et al, 2007) (Spira et al, 2007)
Associated with transcription and regulation of cell fate				
Maskin/TACC3 (2x)	Y	Y	Mitotic spindle associated protein upregulated in differentiated tissues; Inhibits GATA-1 mediated hematopoietic differentiation via interaction with FOG-1	(Sadek et al, 2002) (Garriga-Canut and Orkin, 2004)
DNA helicase RuvB-like 2 (2x)	Y	Y	Helicase involved in transcription; increased levels associated with proper patterning of skeletal muscle	(Jha and Dutta, 2009) (Castorena et al 2011) (Flavin et al, 2011)
NCK SH2/SH3 adaptor protein	Y	Y	Signaling protein whose misexpression leads to developmental defects associated with improper mesodermal cell migration	(Tanaka et al, 1997)
Daxx	Y	Y	Repressor that mediates transcription and nuclear localization via post-translational modification of various proteins	(Shih et al, 2007) (Puto and Reed, 2008)

Putative interacting partner	Expressed in heart	Nuclear localization	Function	References
Elongation initiation factor (2x)	Y	N	Protein necessary for translation; eIF4A tightly regulated by MyoD during differentiation	(Galicia-Vazquez et al, 2014)
Transcription elongation factor B / CDK9 (3x)	Y	Y	Protein necessary for proper transcription to proceed; Complexes with GATA4 to regulate cardiomyocyte differentiation; Misregulation leads to differentiation defects and various pathologies	(Cabart et al, 2004) (Zhou and Yik, 2006) (Muniz et al, 2012)
Associated with Rho-mediated cell adhesion and cytoskeletal organization				
Guanine nucleotide binding protein (5x) / ADP ribosylation factor	Y/Y	Y/Y	Proteins associated with Rho GTPase pathway linked to cardiac structural integrity and a number of processes linked to proper morphogenesis in many tissues such as regulation of cell fate or proper cell-cell adhesion	(Ren and Fang, 2005) (Langhorst et al, 2008) (Rajendran et al, 2009) (McCormack et al, 2013) (Shi and Wei, 2013) (Shi et al, 2013) (Lee et al, 2014)
ZO-2	Y	N		
Flotillin	Y	N		
Myosin	Y	N		
Actin alpha cardiac muscle	Y	N		
Putative interacting domain of interest				
Coiled coil domain containing 86 (4x-2 independent)	ND	ND	Coiled coil domain of unknown protein that may indicate binding properties of putative CASZ1 functional groups	(Mason and Arndt, 2004)

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CHAPTER 3: CONGENITAL HEART DISEASE PROTEIN 5 ASSOCIATES WITH CASZ1 TO MAINTAIN CARDIOMYOCYTE CELLULAR INTEGRITY¹

ABSTRACT

The identification and characterization of the cellular and molecular pathways involved in the differentiation and morphogenesis of specific cell types of the developing heart are critical to understanding the process of cardiac development and the pathology associated with human congenital heart disease. Here we show that the cardiac transcription factor CASTOR (CASZ1) directly interacts with Congenital Heart Disease 5 protein (CHD5), also known as tryptophan rich basic protein (WRB), a gene located on chromosome 21 in the proposed region responsible for congenital heart disease in Down syndrome patients. We demonstrate that loss of CHD5 in *Xenopus* leads to compromised myocardial integrity, improper deposition of basement membrane, and a resultant failure of hearts to undergo cell movements associated with cardiac formation. We further report that CHD5 is essential for CASZ1 function and the CHD5-CASZ1 interaction is necessary for cardiac morphogenesis. Collectively, these results establish a role for CHD5 and CASZ1 in the early stages of vertebrate cardiac development.

INTRODUCTION

Congenital heart disease is an endemic disorder, occurring in 1% of all live births (Roger et al., 2011). Down syndrome (DS) is the most common cause of human genetic heart defects with an estimated 35-60% of DS patients afflicted with a range of cardiac abnormalities, most frequently including atrioventricular septal defects, atrial and ventricular septal defects,

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Tetralogy of Fallot, and patent ductus arteriosus (Goodship et al., 1998; Chaoui et al., 2005; Abbag, 2006). The diversity of cardiac phenotypes along with the inheritance of varying portions of an extra copy of chromosome 21, as well as additional chromosomal abnormalities in a subset of DS patients, has made identifying the critical gene(s) for heart disease in DS patients problematic (Korbel et al., 2009; Lyle et al., 2009). Although recent studies have utilized mouse models to identify the putative critical region in chromosome 21 associated with heart disease in DS patients, specific genes or groups of genes responsible for any of the cardiac phenotypes have not been identified (Shinohara et al., 2001; Liu, C. et al., 2011). Among the candidate genes is congenital heart disease protein 5 (CHD5), also known as tryptophan rich basic protein (WRB). CHD5, initially identified in a screen for genes within a restricted region of chromosome 21 associated with heart disease in DS patients, was found to be expressed in fetal hearts and shown to localize predominately to the nuclei of cells of cardiac origin (Egeo et al., 1998). CHD5 was also identified in a drug-sensitized screen in zebrafish as a regulator of myocardial repolarization (Milan et al., 2009) and depletion of CHD5 in medaka fish has been reported to be associated with general cardiac defects of unknown etiology (Murata et al., 2009). In addition to these findings, CHD5 has also been reported to be localized to the membrane of the endoplasmic reticulum (ER) where it is proposed to function as a receptor to transport charged proteins across the ER membrane (Ando and Suzuki, 2005; Vilardi et al., 2011). Currently no CHD5 knockout mouse exists, so the cellular requirements and the mechanisms by which CHD5 functions in vertebrates remain to be established.

Studies investigating the transcriptional control of cardiomyocyte differentiation have revealed a fundamental requirement for the para-zinc finger transcription factor CASTOR (CASZ1) in vertebrate heart development. Depletion of CASZ1 in *Xenopus* embryos results in

failure of a subset of progenitor cells to differentiate into cardiomyocytes, resulting in cardiac bifida and aberrant cardiac morphogenesis (Christine and Conlon, 2008). To date there have been limited studies on CASZ1 in mammals, however a partial murine *Casz1* cDNA was shown to be expressed in the primitive heart tube in mice (Vacalla and Theil, 2002) and *Casz1* was shown to be expressed in adult human heart tissue (Liu et al., 2006). Intriguingly two recent genome-wide association studies (GWAS) showed a genetic association between the human *Casz1* locus and high blood pressure and hypertension (Levy et al., 2009; Takeuchi et al., 2010) implicating a potential link between CASZ1 and cardiovascular dysfunction. Consistently, it has been recently demonstrated that CASZ1 has an essential and evolutionarily conserved role in blood vessel assembly and lumen formation (Charpentier et al., 2013b; Charpentier et al., 2013a).

To determine the molecular mechanisms by which CASZ1 functions to control cardiac morphogenesis, we conducted a two hybrid screen and identified CHD5 as a CASZ1 interacting partner. We have gone on to demonstrate that CASZ1 and CHD5 are co-localized in the nuclei of the developing myocardium. We further show that CHD5 is essential for cardiomyocyte adhesion and basement membrane depositions and that loss of CHD5 leads to a failure of cardiomyocytes to undergo the appropriate cell movements associated with the formation of a properly looped heart. Finally, we show that CHD5 is essential for CASZ1 function and the CHD5-CASZ1 interaction is necessary for cardiac morphogenesis. Collectively, these studies establish a role for CASZ1 and CHD5 for maintaining cardiomyocyte integrity.

MATERIALS AND METHODS

Morpholinos and *Xenopus* injections

Antisense oligonucleotides against CHD5 (CCACGCACAGCTCTCCGCCATGTTT) were injected at 40 ng/embryo at the one-cell stage (Tandon et al., 2012). Embryos develop to

the reported stages (Nieuwkoop and Faber, 1994). Efficiency of the morpholinos was tested by injection at the one-cell stage with 40 ng morpholino and subsequently injected in both blastomeres at the two-cell stage with full-length *Xenopus* CHD5-GFP mRNA (Open Biosystems, Accession: BC094207). Western blot analysis at stage 27 was performed as previously published (Brown et al., 2005) mouse anti-GFP 1:10,000 (JL-8, Clontech), mouse anti-GAPDH as a positive loading control 1:10,000 (Millipore), HRP anti-mouse 1:10,000 (Jackson), and ECL Western Blotting Analysis System (Amersham). Additional antibodies used for immunoblotting include mouse anti-V5 1:5000 (Invitrogen), mouse anti-FLAG 1:1000 (Invitrogen), mouse anti-SHP2 1:2500 (BD trans labs), chick anti-CHD5 1:10,000 (Abcam), and HRP anti-chick 1:30,000 (Jackson). Additional antisense oligonucleotides were designed against the exon 1 donor site (CGATGAATCGCTTACTTACAAGCGA) and the exon 2 donor site of CHD5 (CTGAAACCCGTTACATTACCATGT). Exon 1 donor oligo and exon 2 donor oligo were injected separately at 40 ng per embryo and collectively at a suboptimal combined 40 ng per embryo (20 ng exon 1 donor oligo and 20 ng exon 2 donor oligo).

For rescue experiments *cardiac actin*-GFP (CA-GFP) transgenic *Xenopus* embryos (Latinkic et al., 2002) were injected at the one-cell stage with 80 ng of CASZ1 morpholino (Christine and Conlon, 2008) and co-injected with 0.25 ng of either CASZ1-V5 mRNA or CASZ1 Δ CID-V5 mRNA. Whole-mount immunohistochemistry (IHC) was performed at stage 37. Phenotypic scoring was statistically analyzed using a two-tailed Fisher's exact test for significance (three independent experiments with embryo number >20 per condition). For mis-expression experiments embryos were injected at the one-cell stage with 2 ng CASZ1 CID (amino acids 785-998) mRNA. Embryos were collected at stage 37 and examined as described in text.

RT-PCR

Primers were designed to *X. laevis Chd5* (F: 5' ATGGCGGAGAGCTGTGCGGC 3') and (R: 5' GTTTGTCTGGTCATCTTGTTG 3') and *X. laevis Casz1* (F: 5' CTGGAGAATCACTGAGGCCA) and (R: 3' CTCTGGTAGTTGCACTCAGG) and PCR was performed on cDNA derived from whole embryo RNA at listed stages. *EF1alpha* was a positive control housekeeping gene. Efficiency of *Chd5* splice-blocking morpholinos was tested by RT-PCR from cDNA derived from stage 25 whole embryos. Nested RT-PCR was performed with primers to exon 1 (F, NMA-238: 5' GTGCTTCTCTGCAATCTCTG), intron 2 (R, NMA-239: 5'-GGGATGTTGTTACCTCTTCC) and exon 2 (R, NMA-240: 5'-TGATAGTTGTGCCGTCCTGC). Wild-type cDNA and genomic DNA were used as positive controls for amplification by the designed primers.

cDNA library construction, yeast two-hybrid screen, and construction of CASZ1 mutants

Tissue containing the heart field was dissected from *X. laevis* embryos (stage 27-29) based on anatomical landmarks; i.e. cement gland and branchial arches. mRNA was purified with Ambion Poly(A) Purist mRNA purification kit, and cDNA was generated and cloned as prey into pGADT7-Rec in yeast strain AH109 using Matchmaker Two-Hybrid System (Clontech). Full-length CASZ1 (Christine and Conlon, 2008), (Accession: NM_001110756) was cloned as bait into pGBKT7 in yeast strain Y187. Yeast strains were mated, and positive clones detected on –Ade/-His/-Leu/-Trp plates containing 3mM 3-amino-triazole according to manufacturer's instructions. DNA from individual clones was isolated and sequenced. CASZ1 deletions were made using CASZ1-pGBKT7 bait vector as the parent vector via fusion PCR mutagenesis. Primers and PCR conditions available upon request.

Co-Immunoprecipitation

One-cell *Xenopus* embryos were injected with the following capped mRNAs: 1) 1 ng *Cas21-V5* only, 2) 1 ng *Chd5-eGFP* only and 3) 1 ng each *Cas21-V5* and *Chd5-eGFP*. Embryos were collected at stage 12.5 and de-yolked based (Link et al., 2006). Briefly, culture media was removed from embryos and embryos were pipetted in de-yolking buffer (55 mM NaCl, 1.8 mM KCl, 1.25 mM NaHCO₃), and mechanically disrupted by passing through a P1000 pipette tip inserted into a P200 pipette tip. Lysate was gently rocked at 4°C for 2 minutes, and centrifuged at 300 *g* for 30 seconds to gently pellet cells. Yolk-containing supernatant was discarded and pellet was resuspended in 1 ml HEPES resuspension buffer (20 mM HEPES, 1.2% polyvinylpyrrolidone, pH 7.4, 1X protease inhibitors) and added dropwise to liquid nitrogen. Pellets were thawed and incubated in 5 ml lysis buffer (200mM K-HEPES pH 7.4, 1.1M KOAc, 20mM MgCl₂, 1% Tween-20, 10μM ZnCl₂, 10μM CaCl₂, 500 mM NaCl, 1.0% Triton-X, and 0.5% deoxycholic acid, 1X protease inhibitors). Lysates were homogenized with 40 strokes each in a PTFE tissue grinder (VWR) and nuclei were further lysed with two 15-second pulses of a Polytron (Thermo Fisher Scientific Inc.) at setting 22, with one minute recovery between pulses. Lysates were cleared at 7500 *g* for 10 minutes. Once cleared, lysates were incubated with Dynabeads M-270 Epoxy (Invitrogen) that were coated with anti-GFP (Cristea et al., 2005) antibody. Incubation with antibody-conjugated beads was performed with end-over-end rotation at 4°C for one hour. Beads were washed seven times with lysis buffer and bound proteins were then eluted with 30 μl 1X SDS sample buffer (10% glycerol, 100 mM Tris, pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 10μg/ml bromophenol blue). Western blot analysis was used to confirm proper immunoprecipitation of CAS21-V5 and CHD5-GFP with anti-V5 (46-1157, Invitrogen, 1:5000) and anti-GFP (JL8, Clontech, 1:10000) antibodies, respectively.

Immunohistochemistry

Whole-mount antibody staining was performed as described (Goetz et al., 2006; Langdon et al., 2007; Langdon et al., 2012) with mouse anti-Tropomyosin (Developmental Studies Hybridoma Bank) 1:10 and Alexa 568 anti-mouse, 1:250 IgG (H+L). Embryos were then cleared in 2:1 benzyl benzoate: benzyl alcohol and imaged on a Leica MZ16F fluorescence microscope with a Qimaging Retiga 4000RV Fast1394 camera with QCapture software as well as a Zeiss 700 confocal microscope with MyZen software. Z-stacks taken with MyZen software were imported to Imaris x64 6.1.5 software (Bitplane AG, St. Paul, MN) for 3D rendering and isosurfacing at the University of North Carolina Microscopy Services Lab as previously described (Doherty et al., 2010). Sectioned antibody staining was performed with rabbit anti-CHD5 (Sigma) 1:100, guinea pig anti-CASZ1 1:500 (Amin et al., 2014), mouse anti-ZO1 (Invitrogen) 1:100, rabbit anti-laminin (Sigma) 1:100, rabbit anti-phospho histone H3 (Millipore) 1:100, rabbit anti-caspase-3 (Cell Signaling) 1:50, and Alexa 647 donkey anti-rabbit (Invitrogen) 1:1000 and Alexa 568 donkey anti-mouse (Invitrogen) 1:250, Alexa 488 donkey anti-mouse IgG, H+L, and Cy3 donkey anti guinea pig (Jackson ImmunoResearch). Stained sections were imaged on BX61 fluorescent microscope or Zeiss 700 confocal microscope, and ImageJ was used for analysis and cell counts. Cell counts of total cells positive for CA-GFP and total cells positive for phospho-histone H3 expression were performed on transverse serial sections of control and CHD5-depleted stage 37 hearts ($n \geq 3$). Mitotic index was calculated by dividing total cells positive for CA-GFP and pH3 expression by total cells positive for CA-GFP. Cell counts and mitotic index were compared for statistical significance using a student t-test.

Embryos fixed in 4% paraformaldehyde were embedded in 4% low melt agarose (Promega) and sectioned by a Leica VT1200S vibratome to 100 μ m thickness. Sections were

washed in PBS+1% Triton X-100 (PBS-T), blocked in PBS-T+10% FBS, and incubated overnight at 4° with primary antibodies rabbit anti-Claudin-5 (Santa Cruz sc-28670) 1:500, mouse anti-ZO-1 (Invitrogen 33-9100) 1:100, rabbit anti-Fibronectin (Sigma F3648) 1:100, mouse anti-β1 Integrin (DSHB 8C8) 1:100. The following day, sections were washed in PBS-T then incubated with secondary antibodies (1:1000 Alexa Fluor dyes, Molecular Probes) overnight at 4°. Sections were washed in PBS-T, incubated with 200ng/mL DAPI (Sigma) and mounted. Images were taken with a Zeiss 700 confocal microscope.

Whole-mount in situ hybridization

Whole-mount *in situ* hybridization was conducted as described (Harland, 1991; Brown et al., 2003) using antisense probes of *Nkx2.5* (Tonissen et al., 1994), *Tbx20* (Brown et al., 2003), *Cas21* (Christine and Conlon, 2008), *Chd5* (Accession: BC094207), *Eomes* (Accession: NM_001128652.1) and *Otx2* (Accession: BC077357) cloned in pSC-B-amp/kan (Stratagene).

Electron microscopy imaging

The pericardial cavity membrane was excised in embryos anaesthetized in 0.1% w/v tricane in ice cold 0.1 x MBS before being fixed in 2.5% glutaraldehyde (EM grade, Electron Microscopy Science, PA) as previously reported (Tandon et al., 2013) in 0.1 x MBS pH 7.6, at 4°C overnight. Embryos were washed in 0.1X MBS, dehydrated into 100% ethanol, dried, mounted ventral side up and sputter coated with Gold-Palladium to approximately 40 nm thickness before being scanned with a Zeiss Supra 25 FESEM microscope. Embryos were also processed for sectioning and visualized on a Zeiss EM 910 Transmission Electron Microscope (TEM) (Microscope Services Laboratory, UNC).

RESULTS

CASZ1 interacts with CHD5

It is well established that the molecular mechanisms of early heart development are highly conserved from *Xenopus* to human (Brade et al., 2007; Goetz and Conlon, 2007; Warkman and Krieg, 2007; Bartlett and Weeks, 2008; Afouda and Hoppler, 2009; Gessert and Kuhl, 2009; Evans et al., 2010; Kaltenbrun et al., 2011; Tandon et al., 2013) and studies in *Xenopus* have demonstrated an essential role for the transcription factor CASZ1 in cardiac differentiation and heart morphogenesis (Christine and Conlon, 2008). To ascertain the molecular mechanism by which CASZ1 functions in cardiac development, we undertook a yeast two-hybrid screen, using full-length *Xenopus* CASZ1 as bait to screen a cDNA library which we generated from stage 28 *Xenopus* cardiac enriched tissue, a period that approximately corresponds to E7.5 in mouse and day 18 $\pm$ 1 in human. Thus, the library was created from tissue at the time and place CASZ1 is required (Christine and Conlon, 2008). The library was deemed ‘cardiac enriched’ since at this period of development heart tissue is contiguous with lateral mesoderm and therefore, we cannot state that we isolated heart tissue exclusively. From this screen, we identified the 33 most carboxy-terminal residues (amino acids 137-170) of *Xenopus* CHD5 (Fig. 3.1A, B). CHD5 is a coiled-coil 14kD protein initially cloned and identified from the minimal chromosomal region associated with congenital heart disease in DS patients (Fig. 3.1C). Accordantly, it has been shown that CHD5 is predominantly expressed within the nuclei of cultured human fibroblasts derived from fetal endocardial tissue (Egeo et al., 1998). We identified a putative *Xenopus* CHD5 EST and confirmed the clone to be full-length (Fig. 3.1C, S3.1A, S3.1E). Subsequent analysis by BLAST and Metazome (www.metazome.net) demonstrated the clone to be the homolog of CHD5 from zebrafish, chicken, mouse, and human,

as it has similar exon/intron structure and is syntenic to other CHD5 homologs (Fig. S3.1A-E). Examination of the region of CHD5 that interacts with CASZ1 (amino-acids 137-170) shows CHD5 to be 76% identical and 94% similar with the human CHD5 orthologue (Fig. S3.1B). We further demonstrate that CASZ1 and CHD5 interact via co-immunoprecipitation of epitope-tagged proteins in *Xenopus* embryos confirming CASZ1 and CHD5 can interact *in vivo* (Fig. S3.1F).

RT-PCR revealed CHD5 to be expressed at all stages of development (Fig. 3.1D) and in agreement with CHD5 being identified as an interacting partner of CASZ1, *Chd5* is found to be expressed in cardiac tissue, as well as anterior mesoderm and neural tissue of early *Xenopus* tadpoles (Fig. 3.1E-M, S3.2) consistent with previously published expression of *Casz1* (Christine and Conlon, 2008). We find that CHD5 is expressed ubiquitously throughout the developing heart and that CHD5 and CASZ1 co-localize in the developing myocardium within the nuclei of cardiomyocytes (Fig. 3.1F-M, S3.3). Collectively, these findings demonstrate that CHD5 and CASZ1 are co-expressed within the developing myocardium and physically associate.

CHD5 is required for cardiac morphogenesis

The observation that CHD5 is contained within the critical region of DS patients with congenital heart disease, and the observation that CHD5 interacts with CASZ1 implies a role for CHD5 in heart development. To test this hypothesis, we depleted CHD5 in *Xenopus* embryos using translation blocking as well as splice-blocking antisense morpholinos (MOs) (Fig. S3.4-6). We did not observe any overt phenotypes prior to early tadpole stages (stage 29 and 32) when linear heart tube formation and cardiac looping begins with either splice-blocking or translational-blocking MOs (Fig. S3.7). Consistently, we did not detect alterations in the

expression of the cardiac markers *Nkx2.5* and *Tbx20* collectively indicating that CHD5 is not essential for cardiac specification (Fig. S3.7A-X).

By cardiac looping stages (stage 37) (Newman and Krieg, 1998; Kolker et al., 2000; Mohun et al., 2000), we observed overt alterations in CHD5-depleted hearts relative to controls. Examination of cardiac tissue by Tropomyosin expression showed CHD5-depleted cardiomyocytes initiate differentiation but fail to complete the processes of cardiac looping and chamber formation (Fig. 3.2A-B). Consistently, transverse sections of *cardiac actin*-EGFP transgenic embryos (CA-TG) (Latinkic et al., 2002) showed that in comparison to the distinct cardiac chambers of control embryos, CHD5-depleted embryos display an underdeveloped and thickened tubular linear heart tube (Fig. 3.2C-D). The cardiac defects do not appear to be secondary to broader defects in anterior patterning since we do not note discernible differences in expression of anterior markers *Otx2* (Pannese et al., 1995) and *Eomes* (Ryan et al., 1998) between control and CHD5-depleted embryos (Fig. S3.8).

To gain insight into the underlying cellular defects of CHD5-depleted hearts, we performed 3D reconstruction on Z-stack images of Tropomyosin-stained hearts (Fig. 3.2E-M). In contrast to control hearts (Fig. 3.2E-G), CHD5-depleted hearts either arrested cell movements prior to fusion of the cardiac fields at the midline (Fig. 3.2H-J) or disrupted cell movements prior to chamber formation (Fig. 3.2K-M) ($n > 25$ per condition, $n \geq 2$ biological replicates, Fig. 3.2N). Ultrastructural analysis performed by scanning electron microscopy (SEM) on hearts from cardiac looping stages (stage 37, $n = 10$ per condition, $n \geq 2$ biological replicates) revealed regardless of the class of phenotype the majority of CHD5-depleted embryos contained cardiomyocytes that failed to undergo normal cell shape changes associated with development and maturation of the linear heart tube (Fig. 3.3A-D). We further observe a failure of looping and

chamber formation in CASZ1-depleted embryos (Fig. 3.3E-F). We further observe that the CHD5-depleted hearts have a more severe phenotype than those derived from CASZ1-depletion suggesting that CHD5 may function by interacting with other cardiac proteins. However, we find that in both CHD5- and CASZ1-depleted hearts the cardiomyocytes remain as a loose collection of cells versus controls (Fig. 3.3A,B,E).

Further examination showed the alteration in cardiac morphology in CHD5-depleted hearts is associated with a decrease in cardiomyocyte cell number (mean=1,817±130 vs. 1,499±184, $n \geq 3$) and a lower mitotic index than stage matched controls (mean=2.98±0.68 vs. 1.07±0.42, $n \geq 3$) but not an increase in cell death (Fig. S3.9). Thus, CHD5-depleted cardiomyocytes remain viable and undergo cell division albeit at a slower rate.

CHD5 and CASZ1 are required for cardiomyocyte adhesion and deposition of basement membrane

Our findings that CHD5 and CASZ1-depleted hearts appear as a loose group of cardiomyocytes versus the smooth surface of control hearts led us to investigate the tight junction markers zonula occludens-1 (ZO-1) which is expressed at cell-cell contacts in cardiomyocytes at cardiac looping stages (stage 37). In stark contrast to control embryos, ZO-1 expression is either diffuse or absent in CHD5 and CASZ1-depleted cardiomyocytes. (Fig. 3.4A-L). These findings are corroborated by transmission electron microscopy (TEM) which showed that at the cardiac looping stage (stage 37) depletion of either CHD5 or CASZ1 is associated with large gaps between cell boundaries and accompanying breaks in the basement membrane of the myocardium (Fig. 3.4M-O, 3.5A-C). We further observe that the basement membrane protein laminin is ectopically deposited in the deeper layers of the myocardium in CHD5-depleted and in CASZ1-depleted hearts (Fig. 3.5D-I). Thus, CHD5 and CASZ1 function appear to establish or maintain cardiomyocyte integrity.

To determine the onset of these cellular defects, we examined the expression of ZO-1 and another tight junction marker Claudin-5 at stage 33, a stage prior to any overt cardiac defect. We observed at these stages that ZO-1 (Fig. 3.6A-L) and Claudin-5 (Fig. 3.6M-X) are expressed at low levels and in a diffuse pattern in CHD5 and CASZ1-depleted embryos relative to controls (Fig. 3.6A-X). We further note that we did not detect any alterations in β 1-integrin expression between control and CASZ1- or CHD5-depleted cardiac tissue suggesting that the cellular defects we observe are due to perturbations in tight junction formation and not a general loss of extracellular matrix-cytoskeletal junctions (Fig. S3.10). Taken together these results suggest that proper tight junction formation and deposition of basement membrane between cardiomyocytes are dependent on CASZ1 and CHD5 at the period when cardiomyocytes approach and fuse at the ventral midline.

CASZ1 activity requires interaction with CHD5

Given the requirement for CASZ1 and CHD5 in cardiac development, we sought to further determine the relationship between CASZ1 and CHD5. We therefore assessed the requirement for CHD5 on expression of CASZ1. We observed that *CasZ1* expression appears unperturbed in CHD5-depleted embryos (Fig. S3.11, data not shown) and found that CASZ1 nuclear localization in cardiomyocytes is maintained in CHD5-depleted hearts (Fig. S3.11). Taken together these results imply that CASZ1 does not function downstream of CHD5 and that CHD5 is not essential for CASZ1 nuclear localization.

To determine the relevance of the CASZ1-CHD5 interaction, we generated a deletion series of CASZ1 and find that CHD5 interacts within a previously uncharacterized region of the carboxy terminus of CASZ1 (a.a. 785-998) (Fig. 3.7A, B). Our studies further show that this

region, henceforth referred to as the CHD5 Interacting Domain (CID), is both necessary and sufficient for the CASZ1-CHD5 interaction (Fig. 3.7A, B).

To test if CHD5 is required for the CASZ1-CHD5 interaction in the heart we took advantage of previous findings that depletion of CASZ1 leads to cardiac defects manifested by failure of the heart to fuse across the midline, undergo correct looping, and properly form cardiac chambers (Fig. 3.7C-H, 3.7O-Q) (Christine and Conlon, 2008). We further found that that these defects can at the least be partially rescued by mis-expression of full-length CASZ1 mRNA (Fig. 3.7I-K, 3.7O-Q). However, mis-expression of mRNA encoding *Casz1* with a deletion of the CID, though expressed as determined by western blot analyses of embryos injected with *Casz1* or *Casz1* Δ *CID* mRNA (Fig. S3.12), fails to rescue any aspect of heart development in CASZ1-depleted embryos (Fig. 3.7L-N, 3.7O-Q, Table S3.1). Taken together these results imply that the CHD5-CASZ1 interaction is essential for CASZ1 function and heart morphogenesis.

To further assess the role of the CID domain of CASZ1 we utilized the observation that the global mis-expression of CASZ1 results in a cardiac phenotype similar to that of CHD5-depletion (Fig. 3.8A-H). One explanation for this observation is CASZ1 may bind and titrate out endogenous CHD5. To test this possibility, we mis-expressed the CID domain of CASZ1 and found resulting embryos had phenotypes similar to that of mis-expression of full-length CASZ1; cardiac looping and morphogenesis defects (CASZ1, 65%, n=23; CID, 30%, n=29, N $\geq$ 2 biological replicates) (Figure 3.8E-L). Collectively, these findings suggest that the CID acts in a dominant negative manner and further support a role for the CASZ1-CHD5 interaction in heart development.

DISCUSSION

Our studies demonstrate a cooperative role for CHD5 and CASZ1 during cardiac development. We identify CHD5 as a CASZ1 interacting partner and establish that this functional interaction occurs via a novel uncharacterized region of CASZ1 that is necessary for proper cardiogenesis to proceed. We demonstrate that this interaction is required for cardiac morphogenesis during the early stages of cardiac looping and chamber formation, and that these defects are driven by a failure to establish proper cardiomyocyte adhesion and basement membrane.

CASZ1 and context-dependent transcriptional regulation

CASZ1 was initially identified and characterized in *Drosophila* (dCas) as a transcriptional regulator of cellular fate within the developing central nervous system (Cui and Doe, 1992; Mellerick et al., 1992). dCas acts as a repressor and functions within a transcriptional cascade through the direct repression of *pdm* (Kambadur et al., 1998), which lacks a vertebrate homologue and does not appear to be an evolutionarily conserved target. Recent studies have provided further insight into neuronal specific transcriptional targets of human CASZ1 (Liu, Z. et al., 2011a; Liu, Z. et al., 2011b). In regards to cardiovascular development, recent studies have shown that in *Xenopus* and human epithelial cells CASZ1 functions to directly regulate *Epidermal Growth Factor-Like Domain 7 (Egfl7)* which in turn acts to promote RhoA-mediated vertebrate vascular development. Taken together with the collective work in *Drosophila*, these studies imply that CASZ1 transcriptional activity is context-dependent. However, the transcriptional mechanisms by which CASZ1 functions and the role of CHD5 in this regard remain to be established.

CHD5 has distinct dual functions during development

CHD5 was first identified as a nuclear localized cardiac expressed gene contained in a restricted region of chromosome 21 triplicated in DS patients (Egeo et al., 1998) and later found to localize to the nuclei of medaka fish cardiomyocytes (Murata et al., 2009). Interestingly, work on the yeast homolog of CHD5, MDM39, identified synthetic interactions with Spf1p, a P-type ATPase involved in protein transport of charged molecules across the ER membrane (Ando and Suzuki, 2005). These findings are supported by work demonstrating that in mammalian cells CHD5 can function as an ER membrane receptor responsible for insertion of proteins into the ER membrane through a direct physical interaction with TRC40/Asna1 (Vilardi et al., 2011). Our studies have demonstrated that CHD5 is nuclear localized along with CASZ1 in the developing myocardium, suggesting a cardiac-specific role in transcriptional regulation. Combined with past studies, our work raises the possibility that CHD5 has dual cellular functions, one CASZ1-dependent within the nucleus and the other CASZ1-independent in the ER membrane. Dual function of a protein in the nucleus as well as the ER membrane is not unprecedented in biology, as the transcription factor sterol regulatory element binding protein (SREBP) has well-characterized roles in both the ER membrane and the nucleus during the processes of sterol synthesis (Espenshade and Hughes, 2007). CHD5 most likely interacts with distinct protein complexes depending on its differing cellular roles. Future studies will determine the precise mechanisms that regulate the decision of CHD5 to localize in either the ER membrane or the nucleus during development as well as whether CHD5 and CASZ1 interact and function within a larger transcriptional complex.

CHD5 and Down syndrome

CHD in Down syndrome patients encompasses a number of distinct disorders from

Tetralogy of Fallot to atrioventricular septal defects (Abbag, 2006). A recent clinical investigation determined that the disorder of chromosome 21 monosomy also results in cardiac defects including hypoplastic and hypertrophied left ventricle and atrial as well as ventricular septal defects (Fisher et al., 2013). Recent work in mice investigating congenital heart disease in DS exclude *Chd5* from the minimal region that induces congenital heart disease upon duplication (Liu, C. et al., 2011). In contrast, a recent screen in zebrafish identified CHD5 as a regulator of myocardial repolarization (Milan et al., 2009), and subsequent work in the medaka fish demonstrated that CHD5-depleted hearts had general looping and chamber defects (Murata et al., 2009). Our results for the first time demonstrate a mechanism by which CHD5 functions during cardiac development via interaction with a cardiac transcription factor and demonstrate cardiac defects in CHD5-depleted embryos. Furthermore, we induce cardiac defects in *Xenopus* embryos by misexpressing the minimal CID, effectively interfering with the proper level of interaction between endogenous CHD5 and CASZ1. Collectively these studies suggest that 1) the range of cardiac defect type and severity is due to a number of contributing genes and 2) that proper dosage of these genes is critical to maintaining proper cardiac development. We demonstrate that proper CHD5 levels are critical during development for the maintenance of proper cardiac morphogenesis and raise new questions about a possible link between CHD5, CASZ1 and congenital heart disease. CHD5 most likely possesses overlapping functional roles with other developmental genes within the chromosome 21 region that collectively are necessary at the correct dosage for proper cardiac development to proceed. Continuing to characterize the link between CHD5, CASZ1, and cardiovascular disease will be crucial to understanding how this disease manifests not only in DS patients, but in the human population as a whole.

Figure 3.1. CASZ1 interacts with CHD5.

A-B, Full-length CASZ1 (bait) interacts with a partial CHD5 protein (prey) in a yeast two-hybrid assay. Cells placed under –His/-Ade selection. **C**, Schematic of CHD5 depicting predicted domains. **D**, RT-PCR on cDNA derived from whole *Xenopus* embryo lysate. *Ef1a* was used as a loading control. **E**, Expression of *Chd5* RNA by *in situ* analysis in early tadpole *Xenopus* embryos (stage 37) heart (h), pronephros (pr), eye (e), pharyngeal arches (pa). **F-M**, Co-localization of CHD5 and CASZ1 protein in the nuclei of the developing *Xenopus* (stage 40) myocardium (m). White boxes in F-I correspond to magnified regions in J-M. Scale bars: (A-C)=100 μ m, (E)=500 μ m, (F-I)=50 μ m, (J-M)=5 μ m

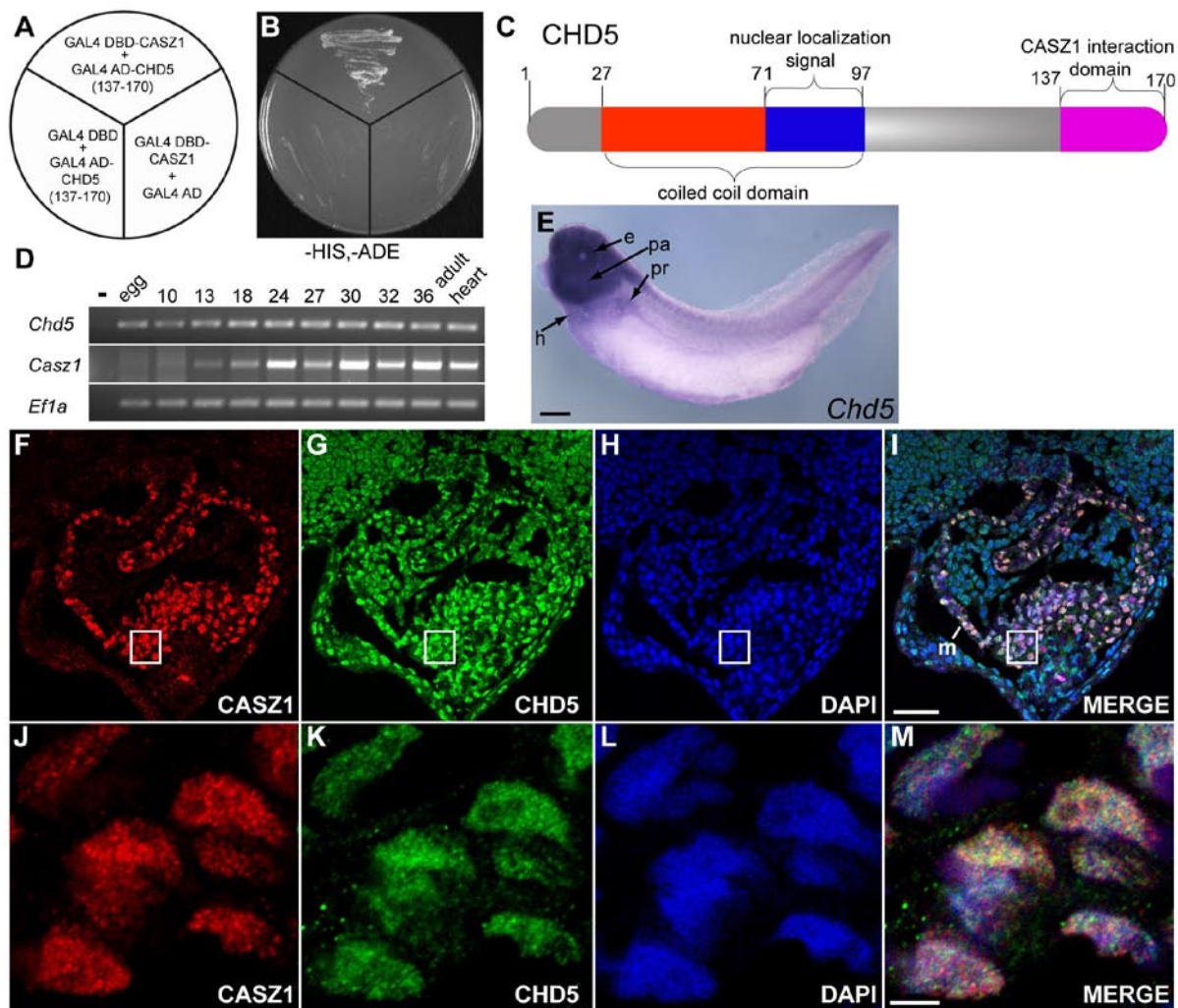


Figure 3.2. CHD5 is required for cardiac morphogenesis.

A-B, CHD5-depleted embryos (stage 37) exhibit smaller, improperly looped hearts that fail to undergo chamber formation. Lateral views of whole-mount antibody staining with anti-Tropomyosin antibody. **C-D**, CHD5-depleted hearts fail to develop fully formed chambers and have a thicker myocardial layer. Transverse sections of CA-GFP transgenic *Xenopus* embryos (stage 37). **E-N**, 3D reconstructions of early tadpole *Xenopus* embryos (stage 37) stained with anti-Tropomyosin antibody viewed laterally and posteriorly. CHD5-depleted hearts fail to complete migration and fusion (**H-J**) or fail to undergo proper looping and chamber formation (**K-M**); ventricle (v), outflow tract (o), and inflow tract (i). D: dorsal, A: anterior, V: ventral, P: posterior. **N**, Frequency of cardiac abnormalities between control and CHD5-depleted embryos. Scale bars: (A-D)=100 μ m, (E-M)=50 μ m

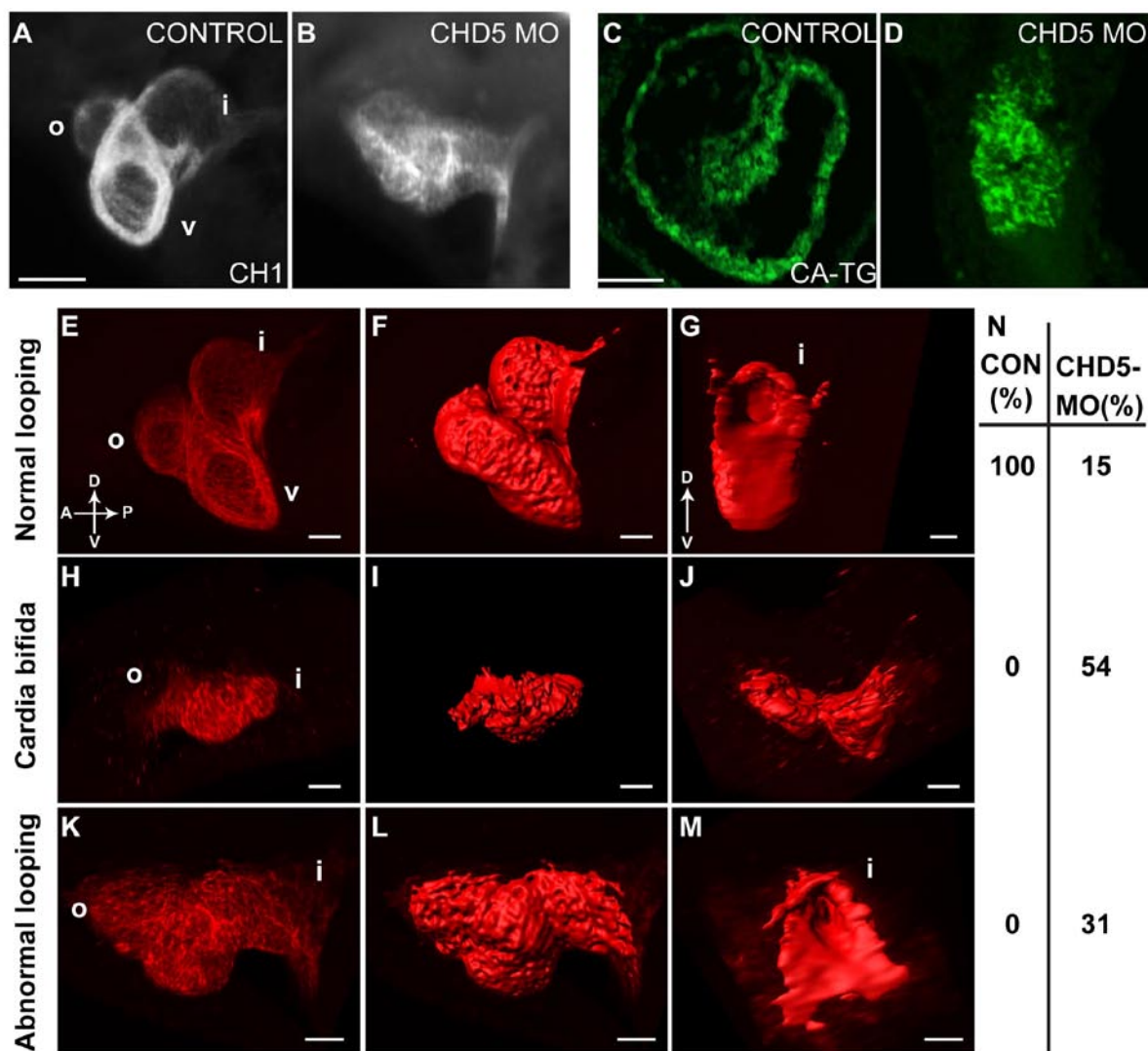


Figure 3.3. CHD5 is required for cardiac morphogenesis.

A-D, Scanning electron microcopy (SEM) of *Xenopus* embryos at early tadpole stage (stage 37) in **(A)** control and **(B-D)** CHD5-depleted embryos. CHD5-depleted embryos fail to complete looping and begin chamber formation and fail to undergo normal cell shape changes associated with development and maturation of the linear heart tube. **E-F**, CASZ1-depleted embryos exhibit similar defects in cardiac looping and cardiac cell shape changes. Scale bar: (A-F)=50 μm

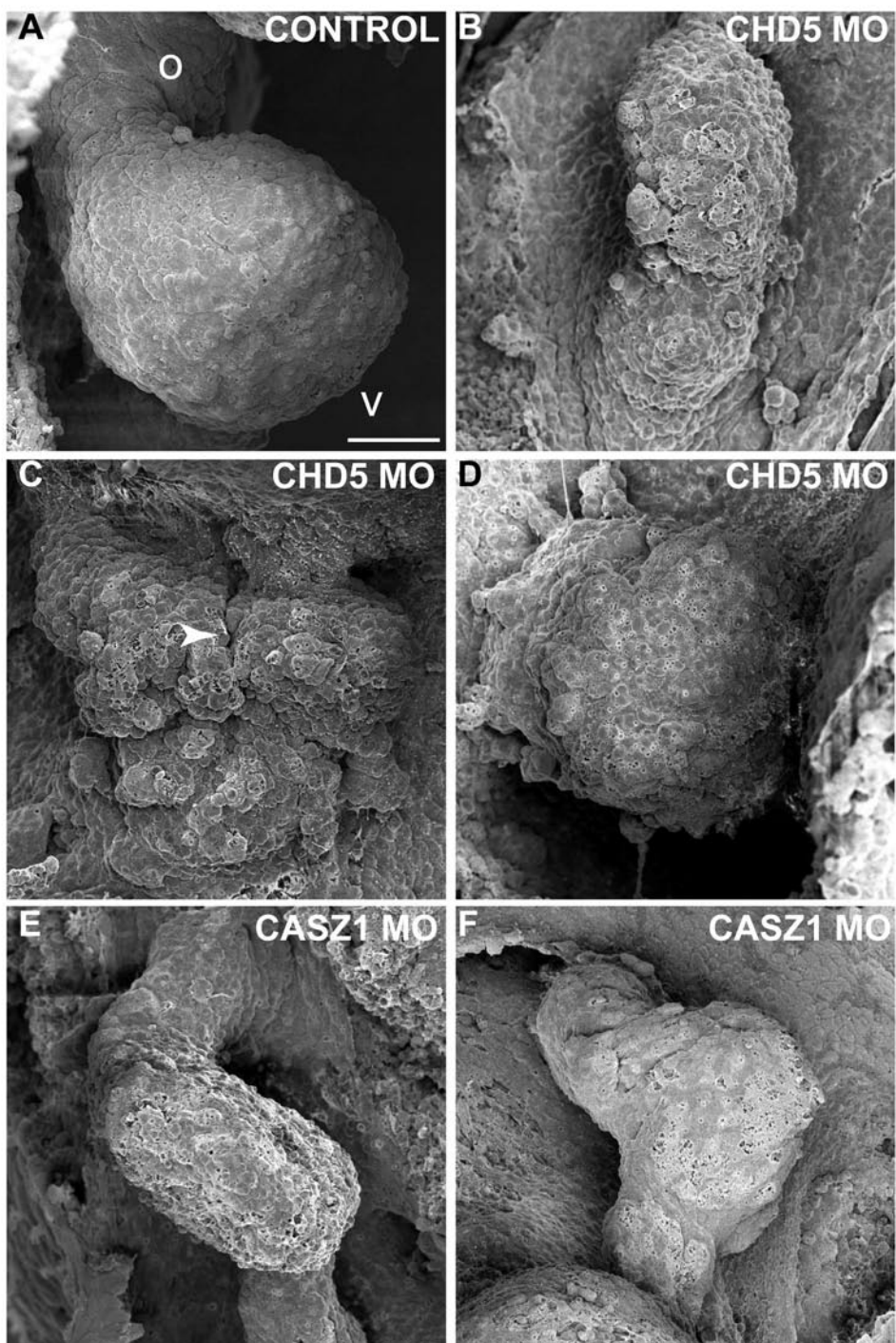


Figure 3.4. CHD5 and CASZ1 are required for cardiomyocyte adhesion.

A-L, Transverse sections of CHD5 and CASZ1-depleted CA-GFP embryos (stage 37) display diffuse or reduced expression of ZO-1 indicating defects in tight junction formation. Phenotypes represented across multiple sampled embryos ($n \geq 4$, from 2 biological replicates). Boxes in A-F represent magnified images in G-L. Merge represents ZO-1 (red), DAPI (blue), and CA-GFP (green). **M-O**, TEM imaging of transverse sections of control, CHD5-depleted, and CASZ1-depleted myocardium (stage 37) reveal large intercellular gaps between cardiomyocytes (arrowheads) adjacent to inner myocardial chamber (ic) in both CHD5-depleted and CASZ1-depleted embryos compared to control embryos ($n \geq 2$, from 2 biological replicates). Scale bars: (A-F)=100 μm , (G-L)=20 μm , (M-O)=5 μm

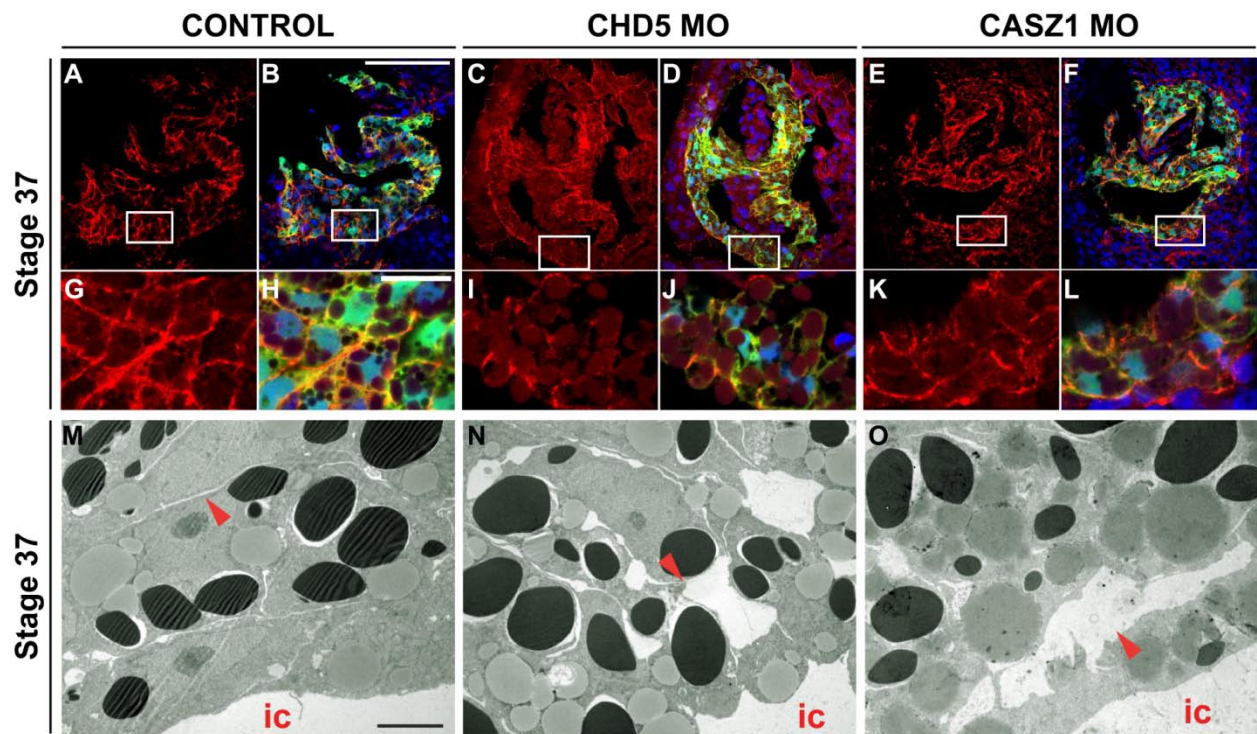


Figure 3.5. CHD5 and CASZ1 are required for deposition of myocardial basement membrane.

A-C, The basement membrane (bm) forms as a discontinuous membrane on the basal surface of the myocardium in CHD5 and CASZ1-depleted embryos as shown by TEM imaging (stage 37) (n=2). **D-I**, CHD5 and CASZ1-depleted CA-GFP hearts (stage 37) exhibit areas of depleted or improperly localized laminin (yellow arrows). Phenotypes represented across multiple sampled embryos (n ≥ 4, from 2 biological replicates). Merge represents laminin (red), DAPI (blue), and CA-GFP (green). Scale bars: (A-C)=0.2 μm, (D-I)=100 μm

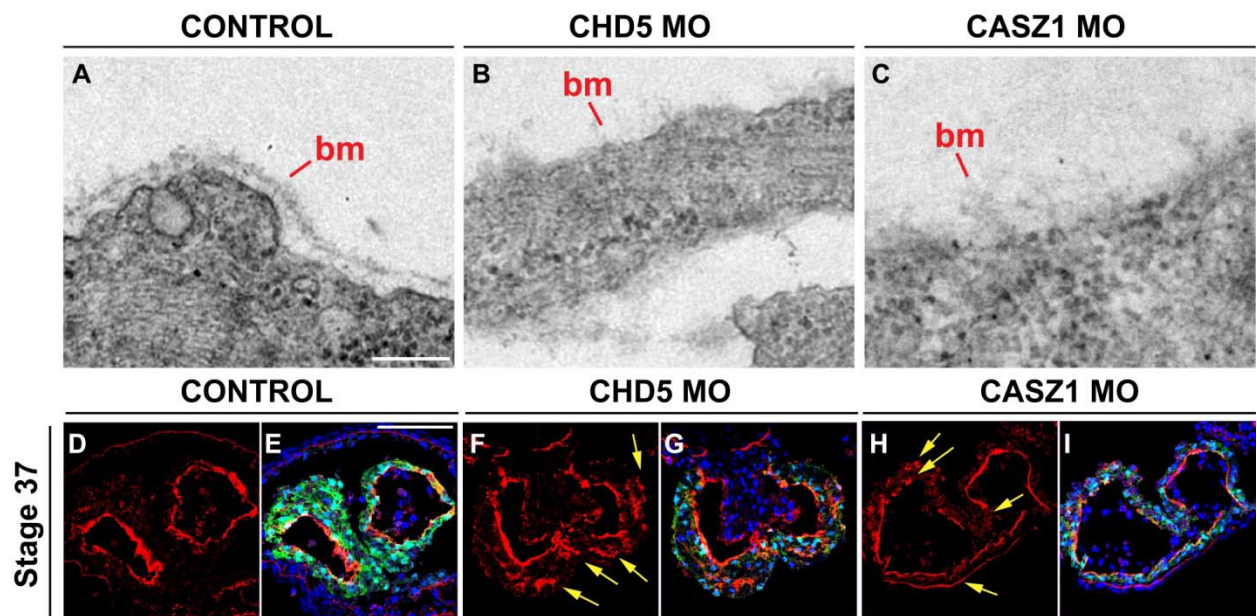


Figure 3.6. CASZ1 and CHD5 are required for cardiomyocyte adhesion prior to cardiac looping and chamber formation.

A-X, Control, CHD5-depleted, and CASZ1-depleted CA-GFP embryos were examined for expression of the tight junction markers ZO-1 (**A-L**) and Claudin-5 (**M-X**). We note that expression of both ZO-1 and Claudin-5 expression are reduced and appear diffuse and unorganized in transverse sections of CHD5-depleted and CASZ1-depleted hearts (stage 33). Phenotypes represented across multiple sampled embryos ($n \geq 4$). White boxes in **A,B,E,F,I,J,M,N,Q,R,V** correspond to magnified images in **C,D,G,H,K,L,O,P,S,T,W,X** respectively. Merge represents ZO-1 or Claudin-5 (red), DAPI (blue), and CA-GFP (green). Scale bar: (**A,B,E,F,I,J,M,N,Q,R,V**)=100 μ m, (**C,D,G,H,K,L,O,P,S,T,W,X**)=20 μ m.

Stage 33

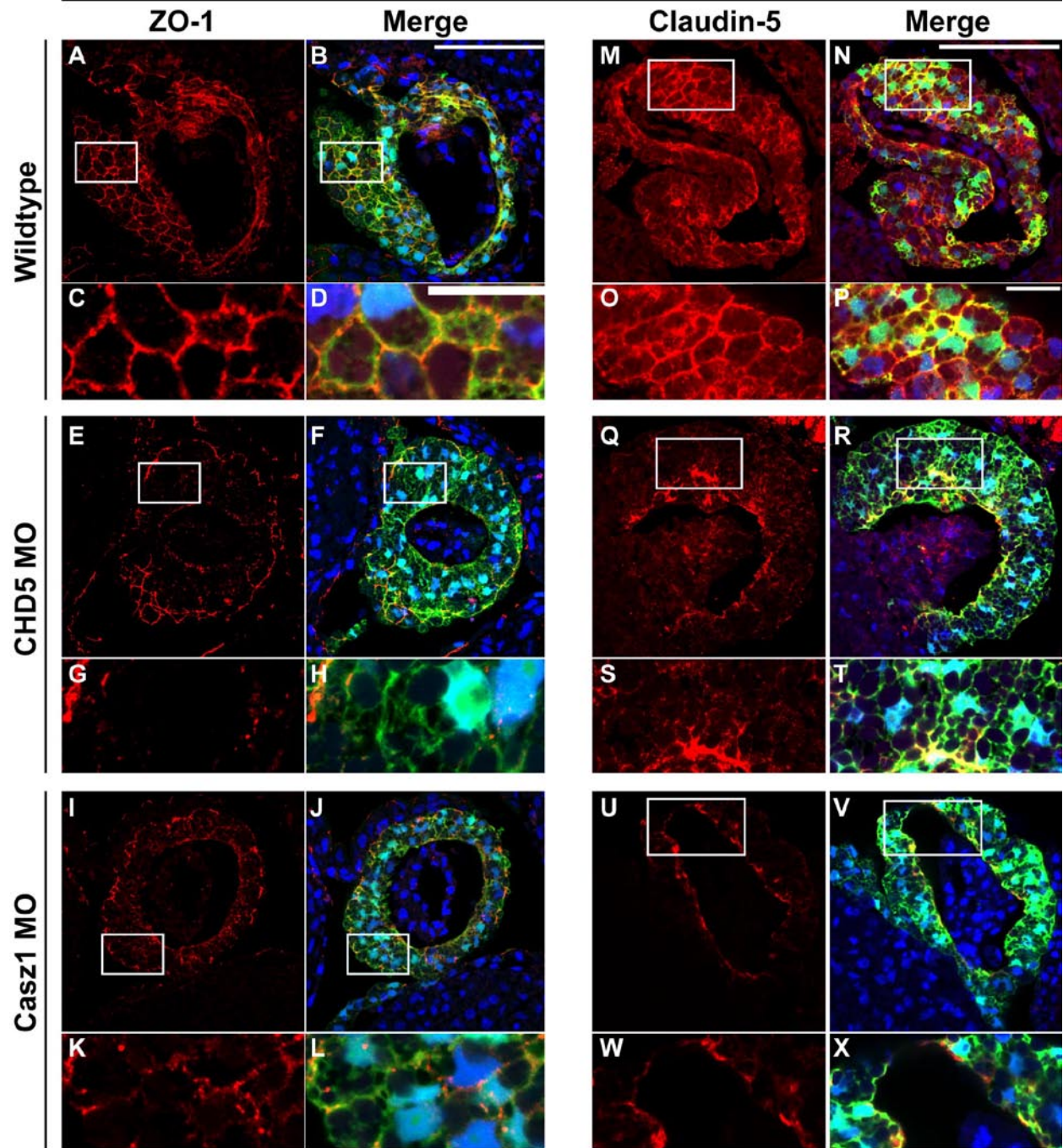


Figure 3.7. CHD5 is critical for CASZ1 function.

A-B, The region of CASZ1 containing the minimal CHD5 interacting domain residues a.a. 785-998 was identified using a yeast two-hybrid assay. **C-N**, Control (C-E) and CASZ1-depleted *Xenopus* embryos (F-H) compared to CASZ1-depleted embryos co-injected with mRNA encoding either full-length CASZ1 (I-K) or CASZ1 Δ CID (L-N) at early tadpole stage (stage 37). Full-length CASZ1 partially rescued cardiac looping and chamber formation (v), outflow tract (o), and inflow tract (i) while CASZ1 Δ CID failed to rescue any aspect of cardiogenesis. **O-Q**, Three independent experiments (n>20 per condition) of embryos were scored for cardia bifida (O), degree of proper cardiac looping (P), and chamber formation (Q) via whole-mount staining with anti-Tropomyosin antibody. Fisher's exact test was performed for significance, NS-not significant *-p=0.003, ***-p<0.0001 Scale bars: (C,D,I,J,F,G,L,M)=500 μ m, (E,H,K,N)=100 μ m

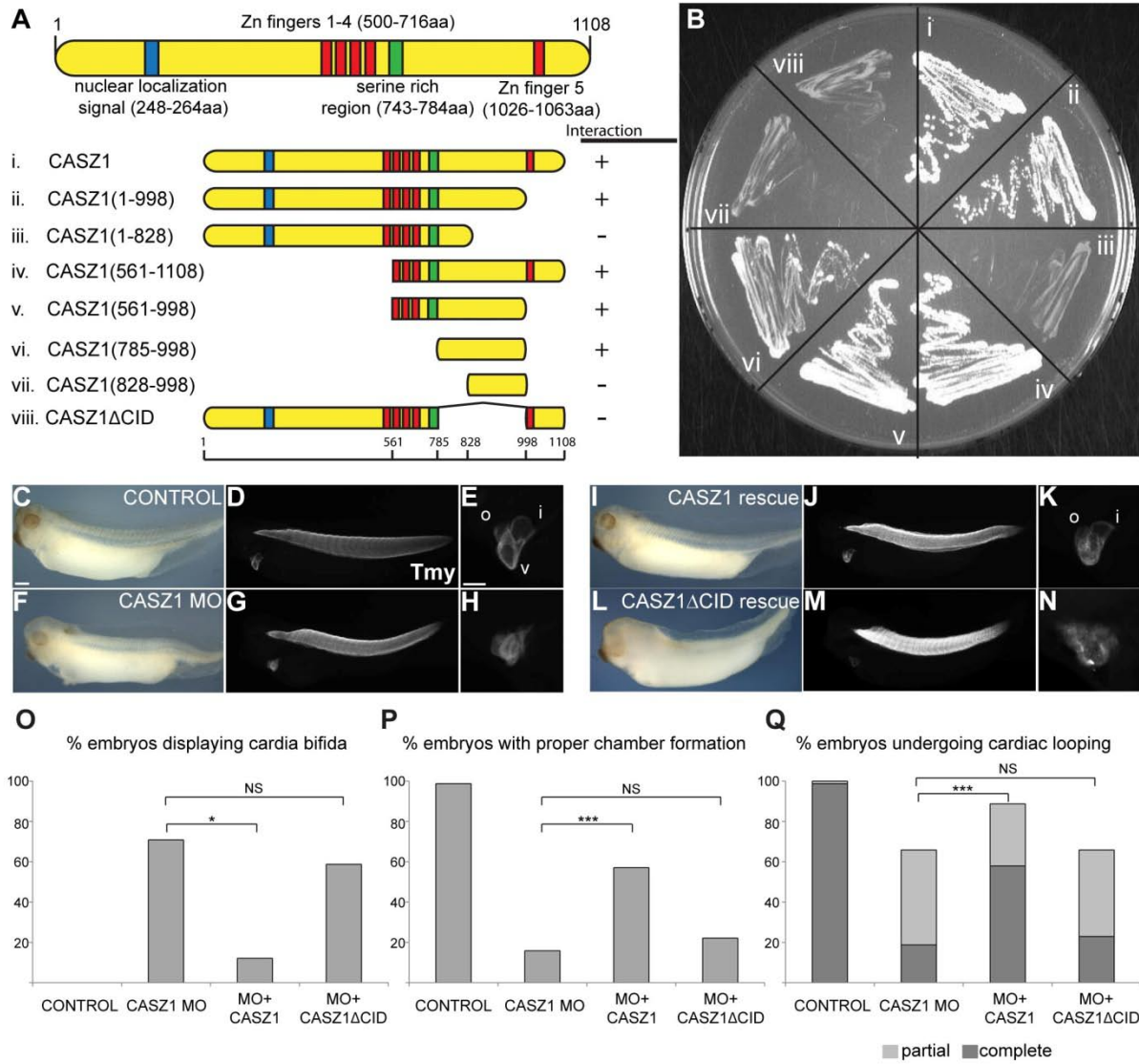


Figure 3.8. The CASZ1 CID domain can function as a dominant negative version of CHD5.

A-D, Control embryos at early tadpole stage (stage 37) exhibit looping and formation of the cardiac chambers; ventricle (v), outflow tract (o), and inflow tract (i). **E-L**, CASZ1 and CASZ1 CID misexpressing embryos display incomplete convergence of the outflow and inflow tract as seen in dorsal views (**I, K**) (arrowheads) and a partially closed inflow tract as seen in posterior views (**H, L**) (arrowheads). Scale bars: (A-L)=100 μ m

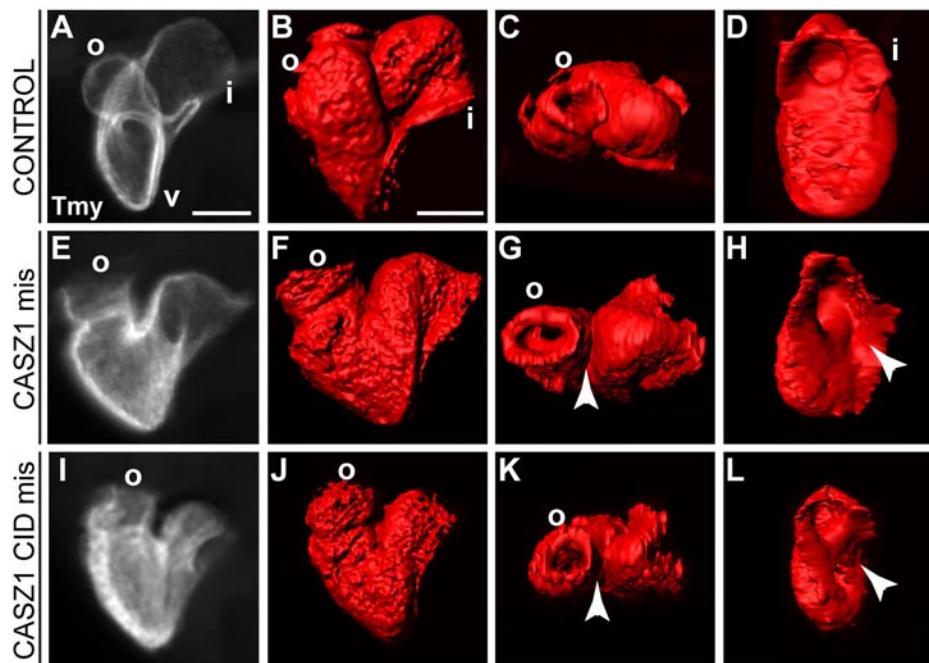


Figure S3.1. Characterization of *Xenopus* CHD5.

A, Protein sequence alignment of *Xenopus* CHD5 cDNA via BLAST (GeneDoc) compared to CHD5 orthologues. Red line underlines predicted coiled coil domain, blue line predicted nuclear localization signal and green line the sequence derived from the minimum cDNA clone identified in a yeast two-hybrid screen to interact with CASZ1. **B,** Percent identity and similarity between CHD5 orthologues. **C,** Predicted genomic locus structure of *Xenopus* CHD5 (5' to 3', not to scale). Exons are shown in boxes with the corresponding size given in basepairs. **D,** A synteny comparison of *Xenopus* CHD5 and other vertebrate homologues, demonstrating strong conservation in its chromosomal position across vertebrate species (Metazome). **E,** Western analysis of a FLAG-tagged protein product of a putative *Xenopus* CHD5 homologue indicated protein runs at the predicted molecular weight, 19 kDa, as recognized by both anti-FLAG and anti-CHD5 antibodies. **F,** Western analysis demonstrating *in vivo* co-immunoprecipitation of CASZ1-V5 and CHD5-GFP in *Xenopus* embryos.

A

```
X. laevis : MAESCA---WLLVLSAVLLCNICKALLPTFSSSIISRLQLQKDAEQEYQMRSDIQYMRQ : 54
Human    : MSSAAADHWAWLLVLSFVFVCNVLRIILLPSFSSSFMSRVLQKDAEQESQMRAEIQDMKQ : 58
Mouse    : MSASETRDWAFLVLSFVFVCNILIRILLPSISSFIISRVLQKDAEQESQMRAEIQQGMKQ : 58
Chick    : -----ISKLLQKDAEQESQMRAEIQNMMKQ : 24
X. trop  : MAESCA---AWLLVLSAVFLCNICKIILLPSFSSSIVSRLQLQDAEQEYEMRSDIQTMRQ : 55
Fish     : MAAG----FNWFLVLSSVFLCNIVKTFLPSSIFLSKIFHKDADQEMEMRTEIQNMKM : 54
         m      w lvls v   cn       lp      ss 6S46lqkDAeQE 2MR IQ M4g
                                     predicted coiled coil domain
        60          *              80          *              100          *
X. laevis : ELSNISMMDEFARYARLERKINKMTDKLKTHVKSRTAQLSKIKWVISIVFYVLQAALM : 112
Human    : ELSTVNMMDEFARYARLERKINKMTDKLKTHVKARTAQIAKIKWVISVAFYVLQAALM : 116
Mouse    : ELSTVNMMDEFARYARLERKINKMTDKLKTHVKARTAQIAKIKWFISVAFYFILQAALM : 116
Chick    : ELSTIISMDEFARYARLERKINKMTDKLKTHVKARTAQIAKIKWINIVFYFILQAALM : 82
X. trop  : ELSNISMMDEFARYARLERKINKMTDKLKTHVKSRTAQLSKIKWVISIVFYVLQAALM : 113
Fish     : ELSTIISMDEFARYARLERKINKMTDCLTKTIKVSRTAQAQAKMKWIVNIIFYILQAALM : 112
         ELS 6 MMDEFA4YARLERKINKMTDkLKThvK RTAQl K6KW 6 6 FY6LQAALM
                                   predicted nuclear localization signal
        120          *              140          *              160          *
X. laevis : VSLINKYYSEPVTVLPSPKWIAPLERLVAFTPICISGGVGITCWLVVCKNVVAIMLYPIS : 170
Human    : ISLINKYYSPVAVVPSPKWITPLDRLVAFPTRVAGGVGITCWILVCNKVVAIVLHPFS : 174
Mouse    : ISLINKYYSPVAVVPSPKWITPLDRLVAFPTRVAGGIGITCWILVCNKVVAIVLHPFS : 174
Chick    : ISLINKYYSEPVTVLPSPKWIAPLERLVAFTPGVAGGVGITCWLVVCKNVVAIMLHPFS : 140
X. trop  : VSLINKYYSEPVTVLPSPKWIAPLERLVAFTPIAGGVGITCWLVVCKNVVAIMLYPIS : 171
Fish     : ISLIINKYYALPVTVVPSPKWIAPLERLVAFPSCVAGGVGITCWLVVCKNVVALIIQAVS : 170
         6SLIWKYYS PV V6PSKW6 PL RLVAFP3 6aGG6GITCW66VCNKVVA66L p S
                                   CASZ1 interacting domain
```

B

X. laevis CHD5 homologues

	Human	Mouse	Chick	<i>X.tropicalis</i>	Fish
% identity	74	72	72	94	69
% similarity	89	87	80	98	88

C

134 166 68 115 233

4,111 20,695 18,937 1,278

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
Human	White	Yellow	Dark Blue	White	Green	Dark Grey	Light Blue	Pink	Orange	White	Red												
Mouse	Purple	Yellow	Dark Blue	White	Green	Dark Grey	Light Blue	Pink	Orange	White	White												
Rat	Purple	Yellow	Dark Blue	White	Green	Dark Grey	Light Blue	Pink	Orange	White	Red												
Chicken	White	White	Yellow	Dark Blue	Green	Dark Grey	Light Blue	Pink	Orange	Red	White												
Frog	White	White	Yellow	Dark Blue	Green	Dark Grey	Pink	Orange	White	White	White												

ETS domain containing transcription factor
 Down Syndrome critical region protein
 WDR protein, form B
 High mobility group nucleosomal binding domain 14
 Congenital heart disease 5 protein (CHD5)
 C21orf13 protein
 SH3 domain-binding glutamic acid-rich protein
 Beta-1,3-galactosyltransferase 5
 IGSF5 protein
 non-synthetic

E

	Flag-Chd5 EST	
	-	+
anti-Flag		
anti-CHD5		

F

CASZ1-V5	+	+	-
CHD5-GFP	-	+	+
	IP: CHD5 (GFP)		

CASZ1 (V5)

CHD5 (GFP)

Figure S3.2. Chd5 is expressed in the heart and anterior tissue.

A-B, *in situ* images of late tailbud *Xenopus* embryos (stage 29 and 34) demonstrating *Chd5* expression in the developing heart (h) as well as in other anterior structures such as pronephros (pr), eye (e), and pharyngeal arches (pa). Scale bar: (A-B)=100 μ m

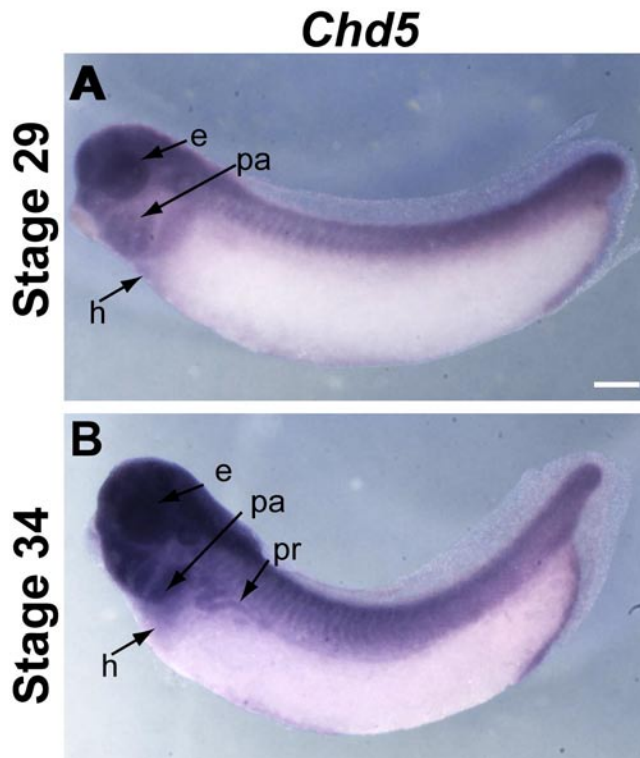


Figure S3.3. CASZ1 and CHD5 co-localize in the nuclei of cardiomyocytes.

A-H, CASZ1 and CHD5 co-localize in nuclei of developing cardiomyocytes of *Xenopus* tadpoles (stage 40) as demonstrated by CASZ1 and CHD5 specific antibody staining; staining with secondary antibody alone shown to control for the specificity of the CHD5 antibody across multiple tissues (**B versus F**). Scale bar: (A-H)=50 μ m

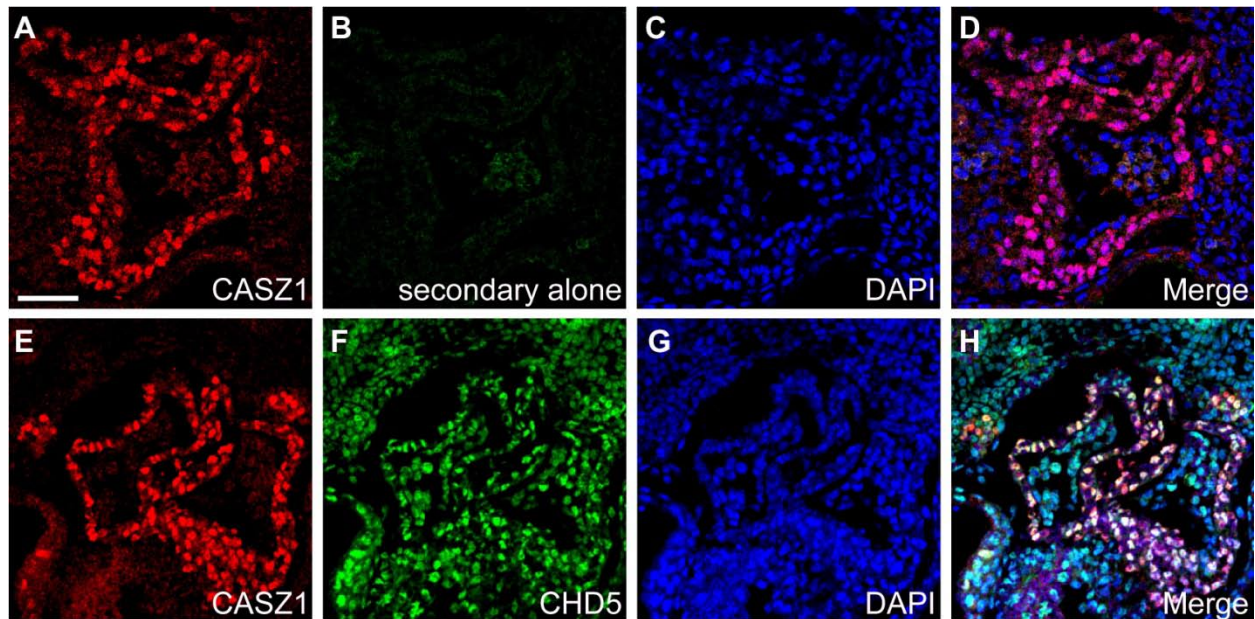


Figure S3.4. Efficacy of translation blocking CHD5 morpholinos.

A, Schematic depicting sequential injections of CHD5 MO targeting 5' UTR and CHD5-GFP. **B**, Schematic of *Chd5* and the *Chd5-GFP* fusion protein generated. **C**, CHD5 MO efficiently depletes CHD5-GFP by blocking translation of injected mRNA in *Xenopus* embryos (stage 27) as assessed by western blot analysis (WB) with anti-GFP antibody. GAPDH was used as a loading control.

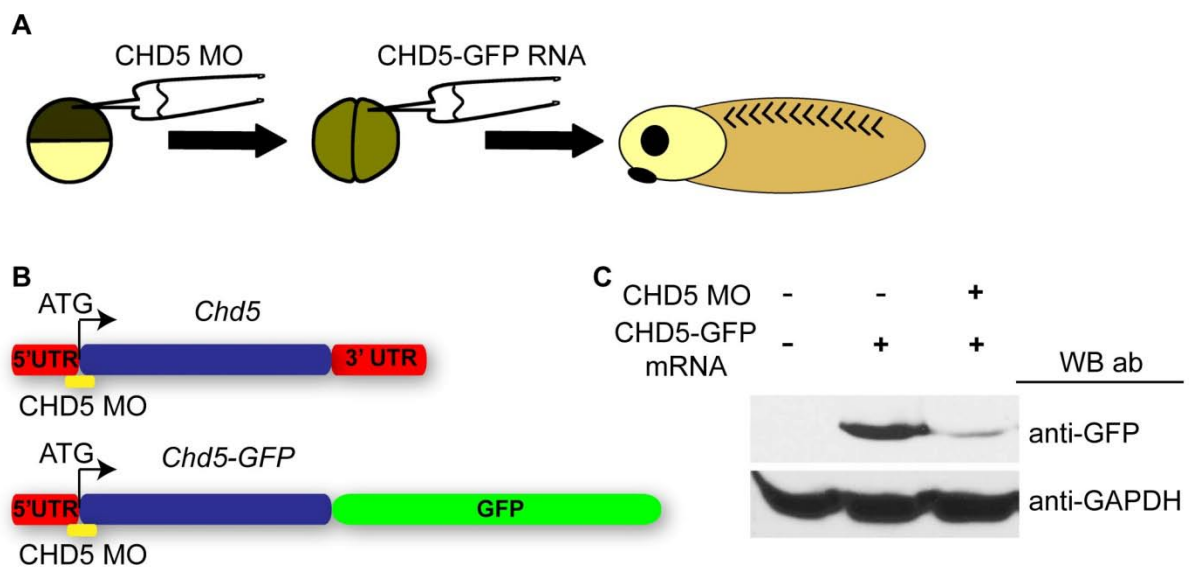


Figure S3.5. Efficacy of splice blocking CHD5 morpholinos.

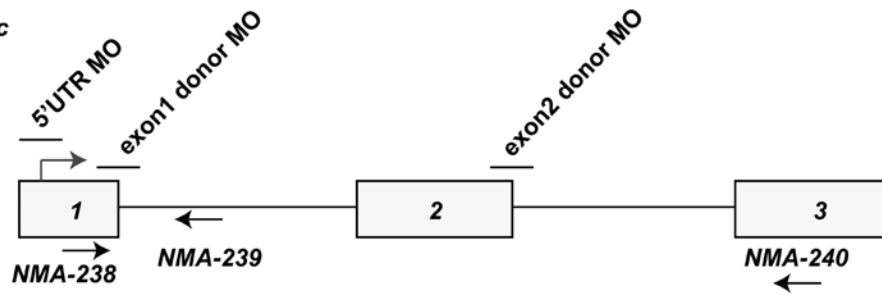
A, Schematic demonstrating location of splice junction morpholinos generated to block splicing between *Chd5* exon donor sites 1 and 2 and relative location of primers (NMA-238, 239, 240) used. **B,** RT-PCR demonstrating efficiency of splice junction morpholinos. Lack of expected PCR product in cDNA samples derived from morpholino injected samples (likely due to nonsense-mediated decay of aberrantly spliced mRNA). *gapdh* specific primers used as control.

A

Chd5 genomic
sequence:

Morpholinos:

Primers:



B

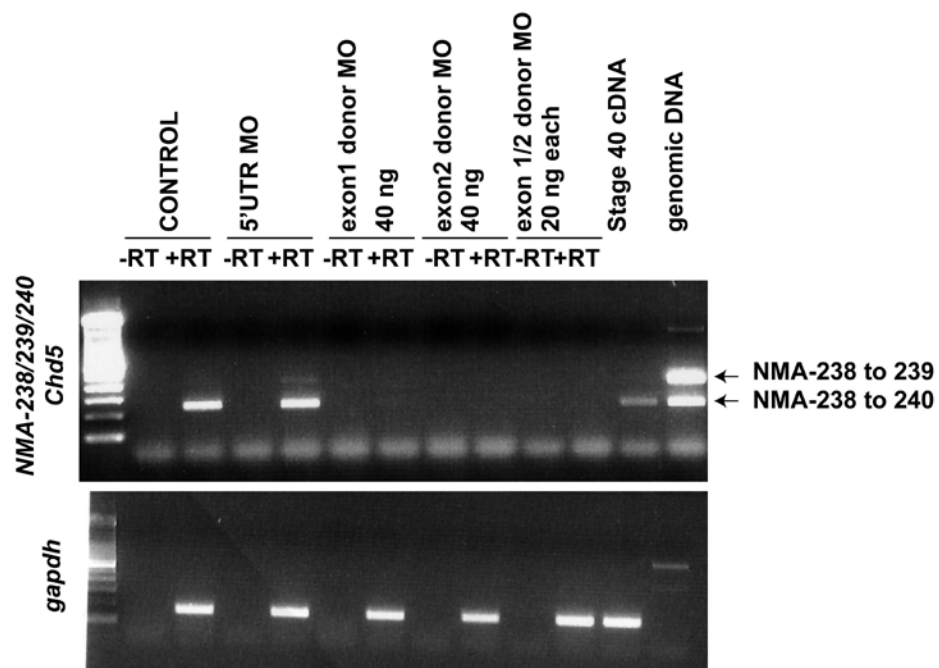
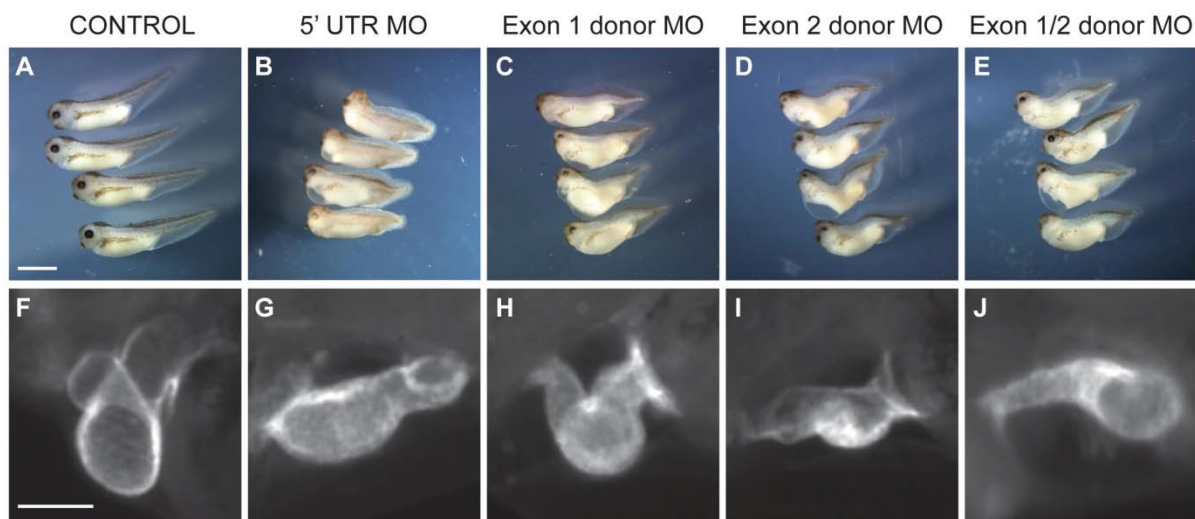


Figure S3.6. CHD5 morpholinos induce cardiac defects.

A-E, Brightfield images of early tadpole control, 5' UTR, and splice junction MO injected embryos reveal similar morphological defects with either set of CHD5 MOs. **F-J**, Whole-mount IHC images of representative looping defects seen in embryos under all MO conditions visualized with anti-Tropomyosin staining. **K**, Quantification of cardiac defects in control and all MO conditions, total of two independent experiments (n>20 embryos per condition). Scale bars: (A-E)=2.5 mm, (F-J)=100 μ m



K

	CONTROL	5' UTR MO	Exon 1 donor MO	Exon 2 donor MO	Exon 1/2 donor MO
cardia bifida	0/45 0%	21/43 49%	16/46 35%	15/39 38%	2/44 5%
abnormal looping	1/45 2%	18/43 42%	17/46 37%	16/39 41%	14/44 32%

embryos totaled from two independent experiments

Figure S3.7. CHD5 is not required for early cardiac specification or movement of cardiac cells to the ventral midline.

A-L, CHD5-depleted embryos undergo proper early cardiac patterning as demonstrated by lateral views of *in situ* analysis with the early cardiac markers *Nkx2.5* (A-F) and *Tbx20* (G-L). Ventral views of CHD5-depleted embryos demonstrate proper movement of cardiac cells to the ventral side of the embryo as visualized by *in situ* hybridization of early cardiac markers *Nkx2.5* (M,N,Q,R,U,V) and *Tbx20* (O,P,S,T,W,X). Scale bars: (A-L)=500 μ m, (M-X)=500 μ m

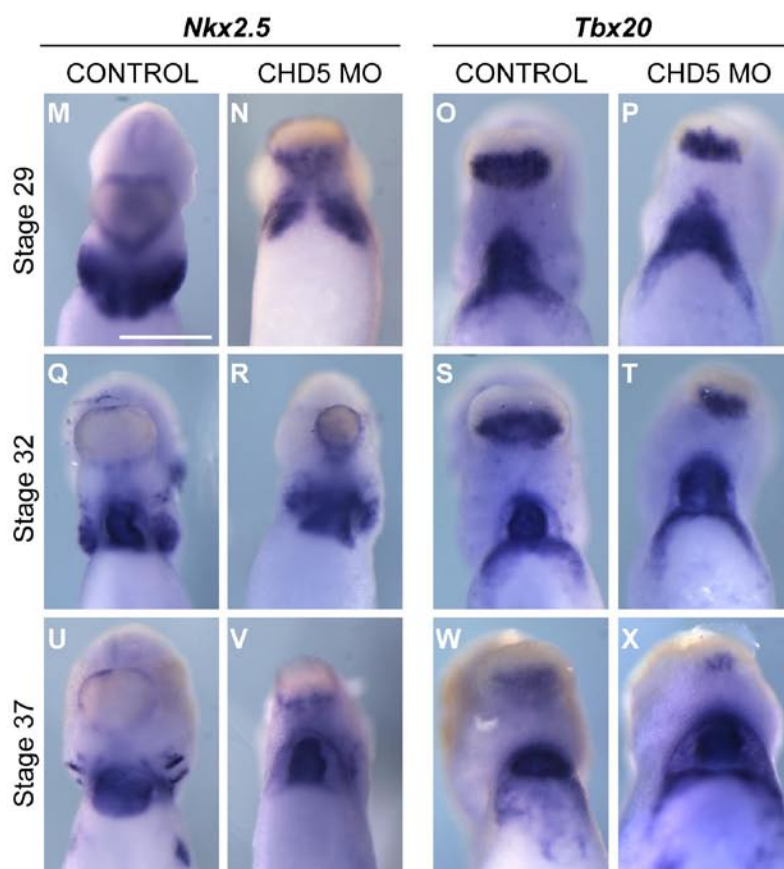
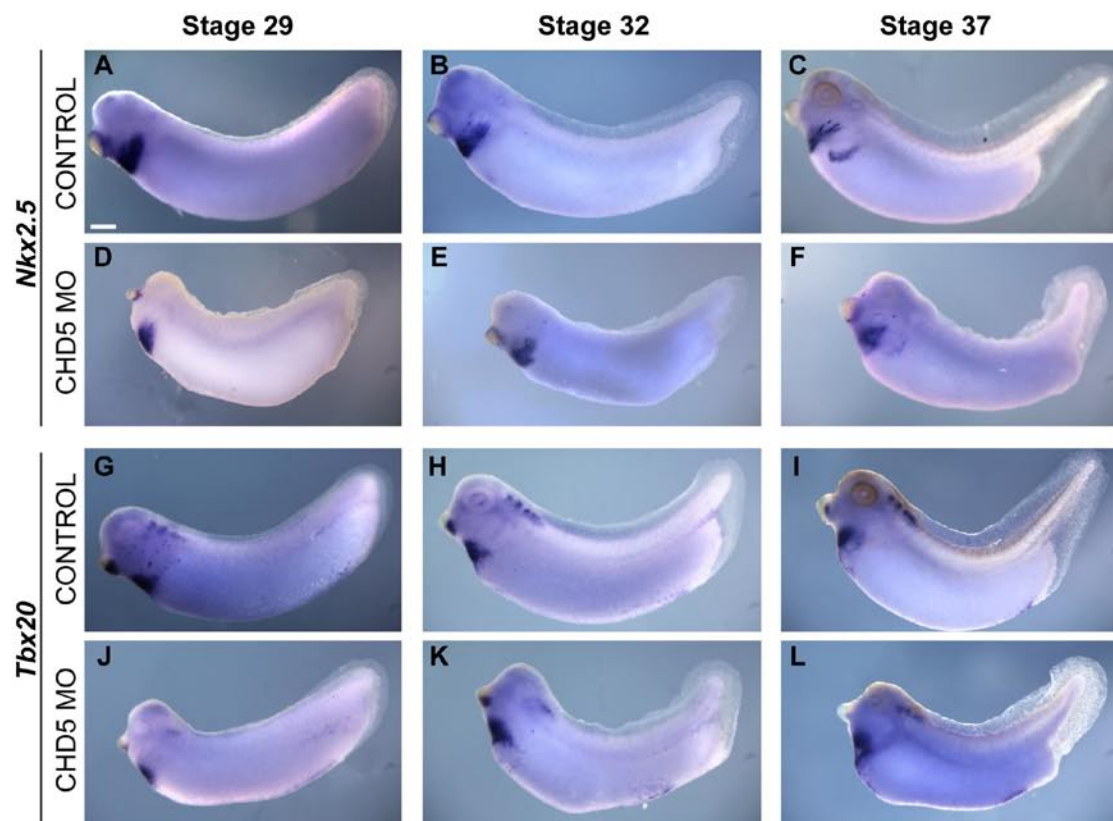


Figure S3.8. CHD5 is not required for anterior patterning.

A-D, CHD5-depleted embryos (stage 37) properly express the anterior markers *Otx2* and *Eomes* as examined by *in situ* analysis. Scale bars: (A-D)=500 μ m

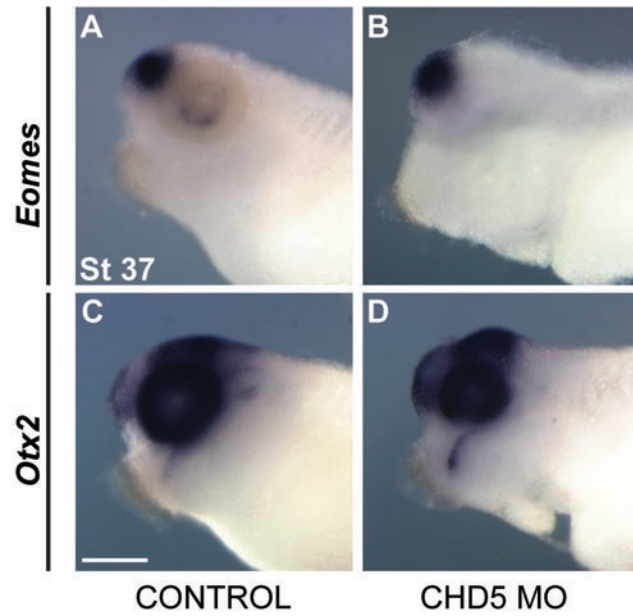


Figure S3.9. CHD5-depletion reduces cardiomyocyte number but is not associated with programmed cell death.

A-G, CHD5-depleted embryos (stage 37) display a reduced number of cardiomyocytes compared and a reduced mitotic index as revealed by phospho-histone H3 (red) expression (*= $p < 0.05$). Mitotic index was calculated by dividing number of cells positive for phospho-histone H3 and cardiac actin-GFP by total number of cardiac actin-GFP positive cells **H-M**, Neither control nor CHD5-depleted embryos exhibit programmed cell death as marked by cleaved caspase-3 (red; transverse sections of CA-GFP embryos show cell death in surrounding mesenchyme but not in cardiomyocytes), n=2 per condition, 1 biological replicate). Scale bar: (A-F, H-M)=100 μ m

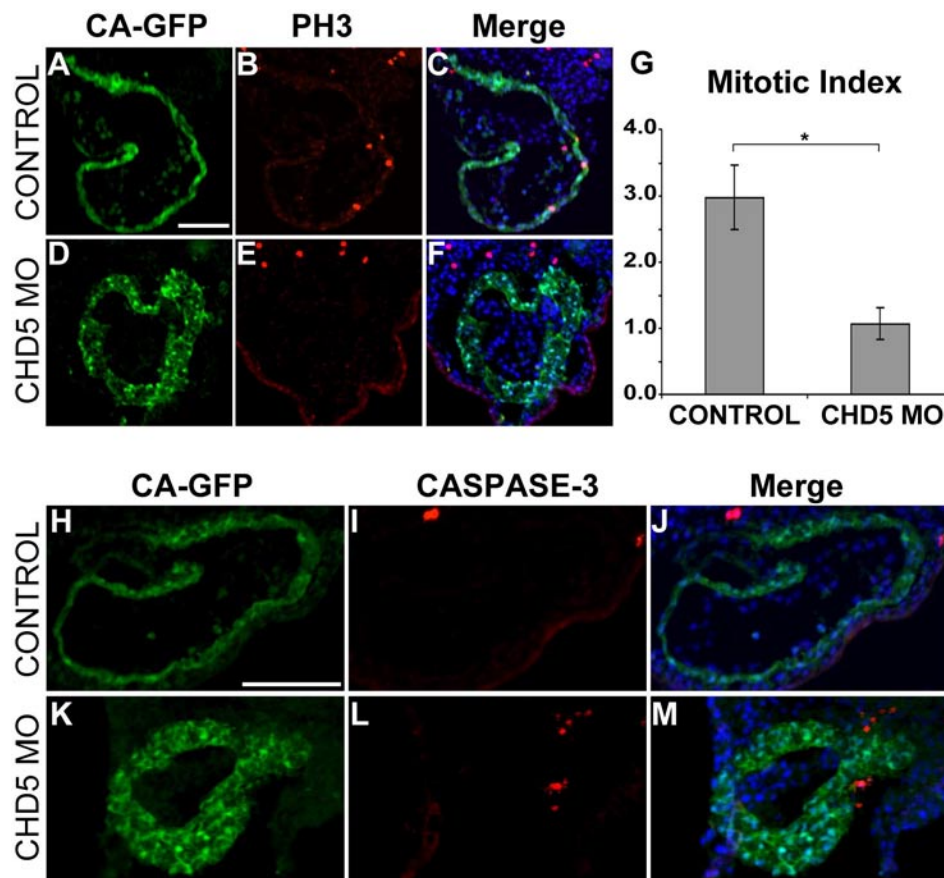


Figure S3.10. β 1-integrin expression is not altered in CHD5 and CASZ1-depleted embryos.

A-L, Control, CHD5-depleted, and CASZ1-depleted CA-GFP embryos were stained for β 1-integrin to mark extracellular matrix-cytoskeletal junctions. The overall structure of cardiomyocytes remained unchanged among all three conditions. White boxes in A,B,E,F,I,J correspond to magnified images in C,D,G,H,K,L. Merge represents β 1-intgerin (red), DAPI (blue), and CA-GFP (green). Scale bar: (A,B,E,F,I,J)=100 μ m, (C,D,G,H,K,L)=20 μ m

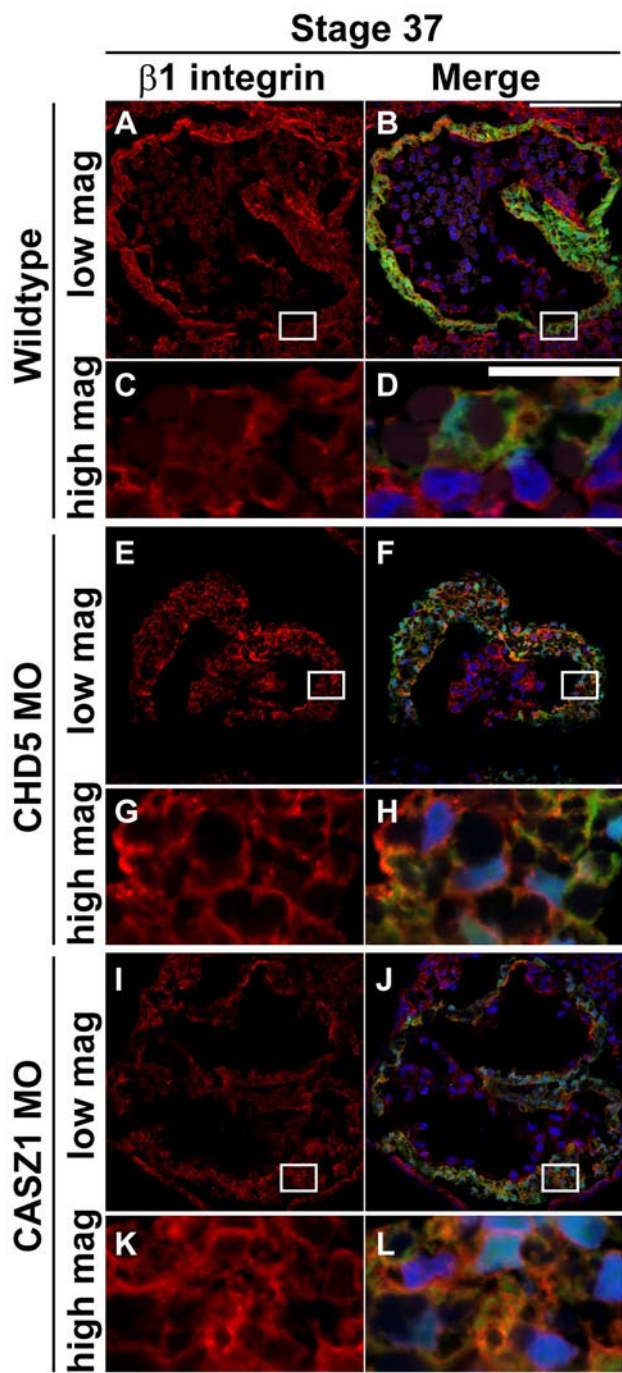


Figure S3.11. CHD5 is not required for CASZ1 nuclear localization.

A-H, CASZ1 remains localized to the nucleus of cardiomyocytes in CHD5-depleted embryos as visualized by immunostaining for CASZ1, Tropomyosin and DAPI on transverse sections of stage 37 embryos. Scale bar: (A-H)=50 μ m

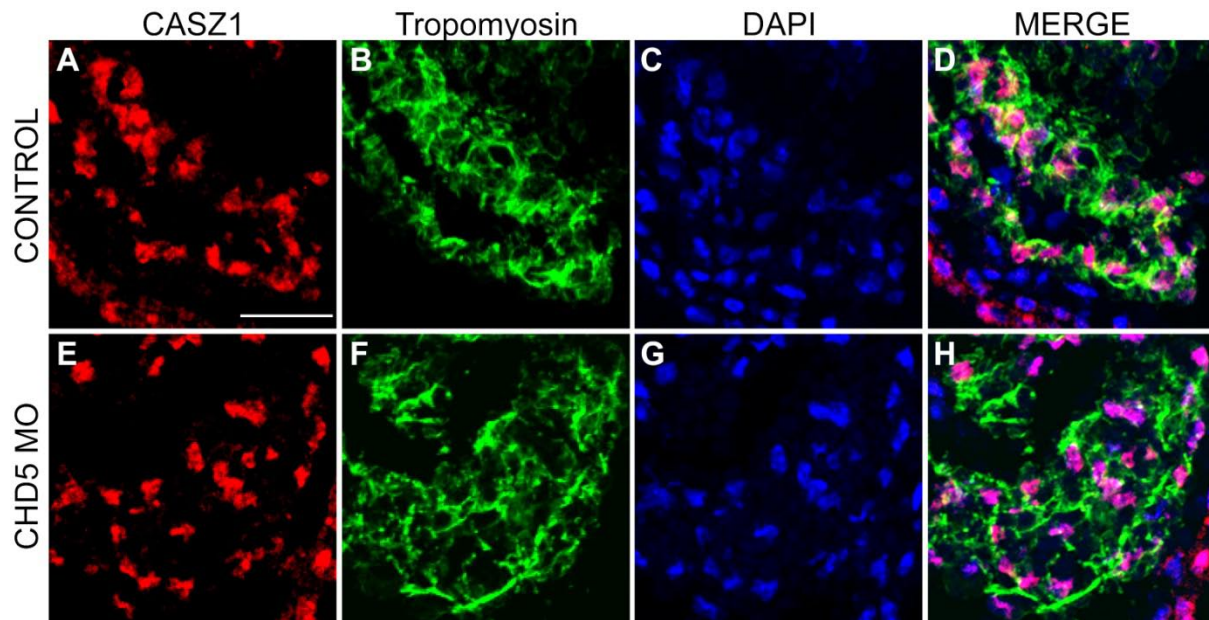
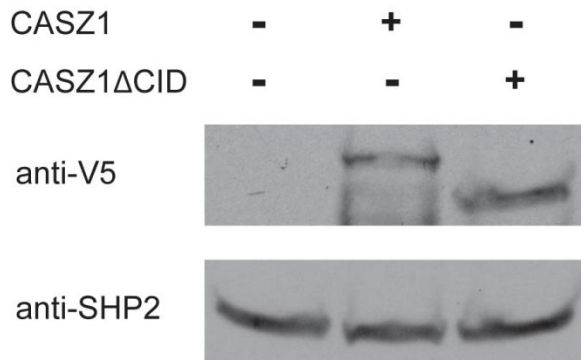


Figure S3.12. CASZ1-V5 and CASZ1 Δ CID-V5 are efficiently translated in *Xenopus*.

mRNA encoding CASZ1-V5 and CASZ1 Δ CID-V5 was injected into *Xenopus* embryos at the one-cell stage and embryos were collected at stage 37. Western analysis with a V5-specific antibody confirmed the presence of the correct size molecular weight proteins. Anti-SHP2 was used as a loading control.



Supplemental Table 3.1. Cumulative results from three independent experiments of *Xenopus* embryos scored for presence of cardia bifida defects, cardiac looping defects, and chamber formation defects.

Supplemental Table 1: Embryo statistics for CASZ1 morpholino rescue

	expression of markers of cardiac differentiation	cardia bifida	proper looping		proper chamber formation
			partial	complete	
CONTROL	100/100 100%	0/100 0%	1/100 1%	99/100 99%	99/100 99%
CASZ1 MO	94/94 100%	67/94 71%	44/94 47%	18/94 19%	15/94 16%
CASZ1 MO + CASZ1	108/108 100%	13/108 12%	34/108 31%	63/108 58%	62/108 57%
CASZ1 MO + CASZ1ΔCID	82/82 100%	35/82 59%	35/82 43%	19/82 23%	18/82 22%

embryos totaled from three independent experiments

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CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

Proper cardiac development is regulated by the precise spatial and temporal input of a number of discrete genetic pathways. These complex genetic interactions make establishing causal factors for congenital heart disease a challenge, particularly in the background of broader genetic defects such as those seen in Down syndrome patients. To further understand the relevant pathways in cardiac development and disease, we performed a yeast-two hybrid screen for interacting protein partners of CASTOR (CASZ1), a transcription factor previously characterized in our lab as a regulator of cardiomyocyte differentiation. We identified and confirmed congenital heart disease protein 5 (CHD5), encoded from chromosome 21, as a CASZ1 interacting partner. We established that this interaction is necessary for proper cardiac morphogenesis, and that knock down of either CASZ1 or CHD5 interferes with cardiomyocyte adhesion and apical-basal polarity during the early stages for cardiac looping. Collectively the studies in this dissertation promote a potential functional role for these two proteins in Down syndrome related cardiac pathology.

CASZ1 and CHD5 interact to regulate cardiac morphogenesis

In Chapter 2 we described the design, implementation, and results of a screen to identify potential interacting protein partners of CASZ1. Previous studies examining CASZ1 function have focused on its role during early neural development. Interestingly none of these prior studies have identified a protein that interacts with CASZ1, making our screen the first attempt to identify a functional CASZ1 interacting partner in the context not only of cardiac tissue but of any CASZ1-expressing tissue. While several of the candidates identified from the screen have

established broad developmental roles in a number of tissues, none of the candidates found had a primary established role in the context of neural development, underscoring the specificity of the cDNA library derived from cardiac-enriched tissue as well as the potential tissue specificity within which these proteins are interacting with CASZ1.

Previous work in our lab has demonstrated CASZ1 depleted *Xenopus* embryos exhibit cardia bifida, the failure of the migrating cardiac fields to fuse at the ventral midline prior to early cardiac tube formation. This cardia bifida is due to the failure of a subset of cardiac precursors to undergo differentiation (Christine and Conlon, 2008). Interestingly we also observe cardia bifida within a subset of CHD5 depleted *Xenopus* embryos. However we have not yet determined whether the primary cause for this phenotype in the absence of CHD5 is a defect in differentiation or an underlying defect in the migration or fusion of these precursor populations. While it remains possible that CASZ1 and CHD5 function collectively to regulate regional cardiomyocyte patterning, they may collectively be required only for proper cardiac adhesion and polarity associated with the migratory movements of cardiac precursors. CASZ1 function then may be mediated by alternative transcriptional complexes not including CHD5. Future analysis of earlier stages in the absence of either protein will be crucial towards answering these questions.

CASZ1 and CHD5 maintain cardiomyocyte integrity

We used a number of approaches to better understand the cellular basis for the defects in cardiac morphogenesis observed in CHD5 and CASZ1 depleted embryos. Examination of transverse sections of control, CHD5 depleted, and CASZ1 depleted hearts using transmission electron microscopy (TEM) revealed that in the absence of either protein, cardiomyocytes failed to maintain their tissue integrity, displaying large gaps between individual cells within the

myocardial layer. Immunostaining for zona occludens 1 protein (ZO1) and claudin-5 (CLDN5), markers of focal adhesion, revealed diffuse staining in the absence of CHD5 or CASZ1.

Collectively these studies indicate that CHD5 and CASZ1 are required for cardiomyocyte integrity via maintenance of proper tight junctions in cardiomyocytes and may play an additional role in mediating cellular adhesion.

This finding is novel but unsurprising, as previous studies have already established a role for CASZ1 in regulating adhesion in other cell types. CASZ1 expression in neuroblastoma (NB) cells leads to upregulation of cell-cell adhesion molecules, and increased CASZ1 levels are associated with increased adherence and limited movement in NB cell populations (Liu et al., 2011b). Recent work in our lab has established a requirement for CASZ1 in proper vessel assembly. Endothelial cells depleted of CASZ1 exhibit defects in adhesion and contractility (Charpentier et al., 2013b). While the pathways by which CASZ1 regulates neural cell adhesion remain unclear, and it remains unknown how CASZ and CHD5 function to regulate cell adhesion within developing cardiomyocytes, our lab has previously found that CASZ1 mediates endothelial cell adhesion via the direct regulation of epidermal growth factor-like domain 7 (*Egfl7*), which in turn facilitates RhoA signaling to carry out the cellular processes necessary for vessel formation (Charpentier et al., 2013b). Strikingly a number of proteins involved within the Rho signaling pathway were identified in our screen as CASZ1 interacting proteins, providing evidence that CASZ1 may activate RhoA signaling to coordinate cardiomyocyte adhesion during early heart morphogenesis in much the same way that these processes are regulated within endothelial cells during blood vessel formation (Charpentier et al., 2013a). It remains to be determined what, if any, functional relevance these potential CASZ1 interactors have in the context of regulation of cardiomyocyte adhesion and integrity. Since several of the potential

interactors are end products directly involved in cell adhesion or cardiomyocyte structure such as ZO2 and cardiac actin, it is possible that they interact with CASZ1 in a negative feedback manner when their expression is no longer required. The involvement of CASZ1 in the regulation of cell adhesion across multiple tissues further implicates the transcription factor in Down syndrome related pathologies, as many of the cellular defects observed in Down syndrome patient cells have been linked to an imbalance of chromosome 21 resident genes involved in cell adhesion (Barlow et al., 2001b; Antonarakis et al., 2004; Maslen et al., 2006; Grossman et al., 2011; Jones et al., 2013). As separate depletion of CHD5 or CASZ1 is sufficient to induce adhesion defects in cardiomyocytes, it is likely that the increased dosage of CHD5 observed in Down syndrome may abrogate proper CASZ1 regulation of RhoA-mediated cell adhesion in not only cardiomyocytes but in other tissues as well.

Our studies also revealed a failure of inner myocardial basal membrane formation in the absence of either CHD5 or CASZ1 by both TEM and immunostaining for the basal membrane component laminin. While consistently observed, these defects were not as pronounced as the defects in adhesion, indicating that they may be a secondary defect to the disruptions in focal adhesion and overall cardiomyocyte integrity. This defect in basal membrane formation may also indicate a misregulation of extracellular components within the membrane. Intriguingly, type VI collagen proteins have been identified as important components of basal lamina. Depletion of Collagen VI induces perturbations of adhesion in epithelial cells as well as misregulation of Rho signaling dependent modifications of extracellular matrix components (Yao and Duh, 2004; Groulx et al., 2011). In chapter 1 we examined the possible link between the misexpression of multiple type VI collagen encoding genes on chromosome 21 and Down syndrome related CHD. This provides evidence for a possible mechanism where CHD5 is

required to regulate CASZ function and associated downstream Rho signaling to facilitate cellular adhesion and extracellular matrix component production and stability. Overexpression of CHD5 in Down syndrome could disrupt these processes, resulting in a similar phenotypic effect as seen from the direct overexpression of chromosome 21 collagen VI genes.

A potential functional role for CASZ1 and CHD5 in neural development

Within our studies, we observe CHD5 expression in a number of anterior structures in addition to the developing heart, including the developing hindbrain. Intriguingly, CASZ1 is expressed in the developing hindbrain as well, indicating that CHD5 may also regulate CASZ1 function during neural development as well. The requirement for Castor family members during early neural development has been well studied in *Drosophila*. The *Drosophila* homologue Castor (Cas) has been determined to function within a temporal cascade required to establish neuroblast identity. Specifically, Cas functions to specify late-born neuronal identity in multiple neuroblast lineages within a temporal window between similar timepoints characterized by the expression of Pdm and grainy head (Grh), which also specify late-born neurons as well. To establish its window, Cas must repress Pdm expression, and the resulting sequential expression of Pdm, then Pdm and Cas together, then Cas alone allows for the generation of diversity among these late-born neurons (Isshiki et al., 2001; Grosskortenhaus et al., 2006; Tran and Doe, 2008; Li et al., 2013). Strikingly the expression of Cas is regulated within various segmented regions of neuroblast populations by a number of upstream factors (Park et al., 2009; Ahn et al., 2010; Kim and Yoo, 2014). Furthermore Cas within these discrete populations controls neuronal cell fate by spatially and temporally regulating a number of different downstream targets (Kambadur et al., 1998; Hitier et al., 2001; Baumgardt et al., 2007; Maurange et al., 2008; Baumgardt et al., 2009; Fontana and Crews, 2012; Chai et al., 2013). This complex spatial and temporal

regulation of Cas and its targets is facilitated by at least seven identified enhancers, some of which are directly regulated by Cas itself (Kuzin et al., 2012). Surprisingly little is known about the role of CASZ1 in vertebrate neural development outside of isolate studies in neural crest derived neuroblastoma (NB) cell lines examining CASZ1's potential tumor suppressor role. Within this system, CASZ1 has been demonstrated to promote NB differentiation and enhance cellular adhesion (Liu et al., 2011b; Liu et al., 2013). The polycomb complex histone methyltransferase EZH2 and retinoic acid have been identified as upstream regulators of CASZ1 expression in NB cells, and CASZ1 has been shown to regulate cell cycle progression through activation of pRB (Liu et al., 2011a; Wang et al., 2012; Liu et al., 2013). Overall, it remains unclear whether the pathways that Cas functions in *Drosophila* during neural development are conserved in vertebrates. A closer examination of the expression of *Chd5* and its subsequent localization could clarify whether CHD5 plays a role in neural stem cell fate via a direct interaction with CASZ1, and whether this interaction has any specific spatial or temporal requirement for determining a particular neuronal identity.

A tissue specific role for CHD5

Owing to its location on chromosome 21, expression in cardiac tissue, and demonstrated links between its misregulation and cardiac defects, CHD5 has long been suspected to be involved in cardiac development (Egeo et al., 1998; Milan et al., 2009; Murata et al., 2009). We demonstrate for the first time a mechanistic role for CHD5 in cardiogenesis via direct interaction with CASZ1. Other previous work has identified CHD5 as an endoplasmic reticulum (ER) membrane associated protein that recruits tail-anchored proteins for insertion into the ER membrane (Ando and Suzuki, 2005; Vilardi et al., 2011; Yamamoto and Sakisaka, 2012). In contrast we observe colocalization of CHD5 and CASZ1 in myocardial nuclei during early

Xenopus cardiac looping and morphogenesis. CHD5 is more broadly expressed than CASZ1 and appears to localize to the nucleus in other cell types as well. These discrete results raise new questions about how CHD5 functions across various tissues. It may globally regulate tail-anchored protein insertion within the ER membrane but also localize to the nucleus via direct interaction with tissue-specific partners such as CASZ1. We have not yet examined whether CHD5 remains nuclear localized in cardiomyocytes in the absence of CASZ1. Co-immunoprecipitation of CHD5 in cardiac cells in both the presence and absence of CASZ1 followed by comparison and analysis of CHD5-associated complexes via mass spectrometry may give insight as to whether the interaction of CHD5 with CASZ1 facilitates the formation of unique protein complexes that facilitate cardiac-specific functions.

Sonic hedgehog signaling and the neural crest: a candidate pathway

Our expectation in carrying out a two-hybrid screen for CASZ1 interacting partners was to identify regionally expressed proteins that would explain the previously identified differential effects of CASZ1 depletion in differing areas of the heart (Christine and Conlon, 2008). Though CASZ1 function is most likely regulated in different tissues by distinct interacting complexes or upstream signals, the broad expression of CHD5 in a number of cell types including neural structures indicates that CASZ1 in part may be required to carry out identical processes (such as cellular adhesion) within the same pathway across multiple tissues.

The hedgehog signaling pathway is a candidate pathway within which CASZ1 and CHD5 may play a role. Hedgehog signaling is required for the induction of neural crest derived neural tissue as well as cardiac tissue (Wada et al., 2005; Washington Smoak et al., 2005; Calloni et al., 2007), and altered Hedgehog signaling has been identified as a potential source for cardiac defects in Down syndrome patients (Ripoll et al., 2012). Interestingly, *Drosophila* Castor has

been previously linked to the hedgehog signaling pathway in non-cardiac tissues; however while *Castor* functions downstream of Hedgehog signaling in the maintenance of follicle stem cells, the transcription factor functions upstream of Hedgehog signaling to regulate neuroblast cell cycle exit (Chai et al., 2013; Chang et al., 2013). One challenge going forward will be determining if and how *CASZ1* and *CHD5* function with hedgehog signaling to regulate cardiogenesis, as *CASZ1* and *CHD5* could function globally upstream of hedgehog signaling to facilitate the patterning of neural crest derived cell types, or *CASZ1* and *CHD5* could be activated downstream of hedgehog signaling within those cell types to facilitate tissue specific functions.

A potential role for *CASZ1* and *CHD5* in pathology of Down syndrome

A direct role for *CASZ1* in the pathology of any known disease has never been established. However recent genome wide association studies have linked *CASZ1* to hypertension, implying a potential role in cardiovascular disease (Levy et al., 2009; Takeuchi et al., 2010). Additionally *CASZ1* is located on chromosome 1 within a 25 megabase region whose partial deletion has been recently associated with a number of cancers. Specifically heterozygous deletion of this region results in diminished tumor suppressor capacity (Henrich et al., 2012). While this study indicates a potential role for *CASZ1* as a tumor suppressor gene, it also demonstrates that proper dosage of *CASZ1* is necessary to prevent aberrant cell growth and development. Strikingly, our studies for the first time implicate *CASZ1* as a potential associated gene for Down syndrome related pathologies. *Casz1* is expressed in a number of developing tissues including neural, endothelial, and cardiac tissues. Improper regulation of *CASZ1* could potentially lead to the defects that are collectively observed in Down syndrome such as cognitive defects, CHD, and hypertension (Vazquez-Antona et al., 2006; Visootsak et al., 2011; D'Alto and Mahadevan, 2012; Shrestha and Shakya, 2013). *CASZ1* also has an established role in

regulating cellular adhesion in multiple tissues, a phenotype also observed in the trisomy 21 condition (Barlow et al., 2001b; Antonarakis et al., 2004; Maslen et al., 2006; Grossman et al., 2011; Jones et al., 2013). *Dscam*, one of the genes explored in Chapter 1 linked to CHD in Down syndrome, has a notably similar expression profile to *Casz1*, as they are expressed in developing neural and cardiac tissue (Barlow et al., 2001b; Barlow et al., 2001a; Barlow et al., 2002; Baumann, 2007). However unlike *Casz1*, *Dscam* resides within chromosome 21, thus the potential mechanism that links *Dscam* to Down syndrome related defects most likely comes directly from its trisomy mediated altered dosage. Crucially the establishment of chromosome 21 resident CHD5 as a functional interactor of CASZ1 provides a potential mechanism that could link the misregulation of CASZ1 to one or more Down syndrome related pathologies. Within our studies the overexpression of CASZ1 as well as the minimal CASZ1 CHD5 interacting domain (CID) are both sufficient to induce cardiac defects in *Xenopus*. Intriguingly these defects are only seen in a subset of embryos overexpressing these protein products, much like CHD is observed in a subset of Down syndrome patients, implying that additional factors may contribute to these pathologies on an individual basis. The precise mechanism by which CASZ1 is regulated by its interaction with CHD5 has yet to be determined. Our studies have revealed that CASZ1 localization does not appear affected by the absence of CHD5, but we have not examined the localization of CASZ1 in the context of overexpressing CHD5. In fact our studies in general were unable to address whether cardiac development proceeds normally when CHD5 is overexpressed. Within the *Xenopus* system, even small increases in CHD5 dosage lead to early pre-neurulation lethality, preventing an examination within *Xenopus* of the CHD5 dosage changes seen in Down syndrome. This preliminary finding indicates that a further exploration of other CHD5-binding partners may provide additional insight into the phenotypes generated by its

misregulation. Another putative candidate identified in our Chapter 2 screen that may clarify a potential role for CASZ1 in Down syndrome is Ca/calmodulin-dependent protein kinase (CaMK), which functions within the calcineurin signaling pathway in both neural and cardiac development (MacDonnell et al., 2009; Dong et al., 2010). Two critical genes on chromosome 21, *Dyrk1a* and *Dscr1*, have an established cooperative role in regulating calcineurin mediated NFATc signaling in multiple tissues, providing evidence that CASZ1 may function within that pathway as well via its association with CHD5 (Okui et al., 1999; Arron et al., 2006; da Costa Martins et al., 2010; Kurabayashi and Sanada, 2013). Going forward, determining the precise effect that overexpression of CHD5 has on CASZ1 activity will be crucial towards determining what role, if any, CASZ1 may have in the pathologies of Down syndrome.

CASZ1 and CHD5 as therapeutic targets for Down syndrome related CHD

Our studies provide preliminary evidence for a collective role for CHD5 and CASZ1 in the pathology of Down syndrome, identifying this interaction as a potential target for mitigating one or more of the defects associated with Down syndrome. Our studies have determined a minimal region of CASZ1 required for interaction with CHD5. This finding may be useful for designing small therapeutic peptides that could competitively bind with CHD5. Treating trisomy 21 embryos with this peptide could restore normal levels of the interaction between endogenous CHD5-CASZ1 by squelching excess CHD5 via binding by this peptide. Quantification of the increased levels of CHD5 in trisomy 21 cells would be critical to determining the ideal dose of this peptide that could restore normal CASZ1-CHD5 mediated gene function. An alternative potential Down syndrome related CHD therapy could focus on the consequences of misregulating CASZ1 via overexpression of CHD5 by determining the downstream targets that CASZ1 regulates. Our lab is currently utilizing RNAseq to identify cardiac specific gene targets

of CASZ1. Coupling this data with matched samples in a trisomy 21 background could identify CASZ1 downstream genetic targets that are misregulated during Down syndrome, allowing for more targeted therapies for specific pathologies observed in the disorder. Barring a corrective *in utero* therapeutic option, further understanding the potential role for CASZ1 in Down syndrome related CHD may allow for improved medical treatment of Down syndrome individuals. Studies examining the causal factors for CHD in Down syndrome have posited the hypothesis that these defects are not only due to the increased dosage of a number of critical genes via trisomy 21 but also due to the presence of preexisting genetic variants of genes on other chromosomes that become deleterious in the sensitized Down syndrome background (Maslen et al., 2006; Li et al., 2012). Furthermore studies have been able to link particular variants of specific genes with individual defects (Robinson et al., 2003; Maslen et al., 2006; Kusuma et al., 2011; Ghosh et al., 2012; Li et al., 2012; Zhian et al., 2012). Using a genomic approach we could identify polymorphisms associated with putative regulatory elements and nearby noncoding regions to the gene encoding CASZ1. Then we could examine the genomes of Down syndrome patients with various cardiac defects and determine whether any of these defects are significantly associated with a particular CASZ1 polymorphism. This information could then be used in early genetic screens to determine probable outcomes of Down syndrome fetuses and result in more informed decisions on potential future corrective surgery and other treatments later in life.

Future Directions

Overall our work has provided a mechanism by which early cardiomyocyte integrity during early morphogenesis is maintained by CASZ1 via a direct interaction with CHD5. The challenge going forward will be determining the broader context in which this interaction functions to regulate these processes. Our lab has developed two tools that are crucial towards

continuing our work with CASZ1, 1) a *CasZ1* conditional knockout mouse that has been crossed to a Nkx2.5-cre mouse to generate CASZ1 null cardiac precursors and cardiomyocytes and 2) a construct encoding CASZ1 fused to the Avi-tag epitope, which takes advantage of the highly efficient and strong affinity interaction between biotin and streptavidin.

To understand how CASZ1 and CHD5 function collectively in cardiac development, we are first ascertaining whether these two proteins function within a larger transcriptional complex. Recently our lab has begun utilizing the immunoprecipitation of proteins of interest from cryolysed tissue samples coupled with tandem mass spectrometry to analyze the entire complexes associated with these proteins, not just the direct interactors. We will utilize this technology by misexpressing CASZ1-Avi within 293 cells, which endogenously express CHD5, to determine the proteins that make up the putative CASZ1-CHD5 transcriptional complex. We will then misexpress CASZ1-Avi with the CHD5 interacting domain deleted (CASZ1 Δ CID) to determine whether CHD5 is required to facilitate the formation of this complex. Following the identification of a transcriptional complex within which CASZ1 and CHD5 interact, we will then perform identical assays in embryonic stem cell (ES) derived cardiac cell lines to determine whether the identified complex forms within cardiomyocytes or if distinct cardiac-expressed components are instead required to facilitate tissue-specific functions. These assays will also be attempted in isolated *Xenopus* cardiac tissue misexpressing either CASZ1-Avi or CASZ1 Δ CID-Avi. Immunoprecipitations will also be performed on mouse cardiac tissue using a commercially available CASZ1 antibody to determine the conservation of these complexes across vertebrate species.

Another objective towards understanding the cardiac role for CASZ1 and CHD5 is to identify the cardiac specific downstream genetic targets regulated by the CASZ1-CHD5

interaction. While several Cas targets have been identified in *Drosophila* neuroblasts, and previous work in our lab has identified *Egfl7* as a downstream CASZ1 target during blood vessel assembly, no cardiac specific CASZ1 transcriptional target has yet been identified and validated. To address this we will first isolate hearts from wildtype and CASZ1 depleted *Xenopus* embryos and perform RNAseq to assay for genes with significantly altered expression in the absence of CASZ1. We will then perform the same RNAseq analysis comparing wildtype cardiac tissue and cardiac tissue from our conditional CASZ1 knockout mouse. Examining the altered gene expression conserved between vertebrate species would provide valuable insight into the cardiac specific genetic pathways that CASZ1, along with CHD5, may be functioning within.

In addition to these studies, we will also revisit our yeast two-hybrid screen to examine other potential high priority candidates. Maskin in particular is a candidate of high interest that may function to regulate CASZ1 nuclear localization in the same way that it functions within the hematopoietic lineage by restricting FOG-1 from the nucleus (Garriga-Canut and Orkin, 2004). Candidates will also be validated that are involved in pathways such as calcineurin signaling and Rho mediated cell adhesion that have been linked to CHD in Down syndrome. The validation of other CASZ1-interacting proteins may provide additional valuable insight into its cardiac function.

Beyond determining the potential functional pathways for CASZ1, further exploring the extent of its role in mammalian cardiac development is a crucial step towards establishing a potential link for CASZ1 to cardiovascular disease in humans. To that end we have utilized the aforementioned CASZ1 conditional knockout mouse to examine the cardiac requirement for CASZ1 in mammals. Interestingly, mouse embryos with CASZ1 null cardiomyocytes display cardiac hypoplasia and ventral septal defects. Additional data implicates CASZ1 as a potential

regulator of the cardiac cell cycle as well as being necessary for the maintenance of the boundaries of the primary and secondary heart fields. Overall the evidence thus far only strengthens the prospect of CASZ1 being a potential causal gene for cardiac pathologies, and it will be interesting to determine within a mammalian system whether these cardiac defects are also linked to a CASZ1- CHD5. Crossing the CASZ1 conditional knockout mouse with a similar CHD5 conditional knockout mouse or an established Down syndrome mouse model could provide valuable insight into the link between these proteins and the phenotypes of the disorder. Collectively these future studies will serve to clarify the potential role for the CASZ1-CHD5 interaction in the emergence of Down syndrome associated CHD.

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