Essential Role of the Zinc Finger Transcription Factor Casz1 for Mammalian Cardiac Morphogenesis and Development

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Background: The CASZ1 (castor zinc finger 1) gene localizes to chromosome 1p36, and 1p36 deletion syndrome is related to congenital heart disease (CHD). However, the 1p36 genes that contribute to heart disease have not been clearly delineated. Human CASZ1 gene localizes to 1p36 and encodes a zinc finger transcription factor. Casz1 is required for Xenopus heart ventral midline progenitor cell differentiation. Whether Casz1 plays a role during mammalian heart development is unknown. Our aim is to determine 1p36 gene CASZ1 function at regulating heart development in mammals. We generated a Casz1 knock-out mouse using Casz1-trapped embryonic stem cells. Casz1 deletion in mice resulted in abnormal heart development including hypoplasia of myocardium, ventricular septal defect, and disorganized morphology. Hypoplasia of myocardium was caused by decreased cardiomyocyte proliferation. Comparative genome-wide RNA transcriptome analysis of Casz1 depleted embryonic hearts identifies abnormal expression of genes that are critical for muscular system development and function, such as muscle contraction genes TNNI2, TNN1, and CKM; contractile fiber gene ACTA1; and cardiac arrhythmia associated ion channel coding genes ABCC9 and CACNA1D. The transcriptional regulation of some of these genes by Casz1 was also found in cellular models. Our results showed that loss of Casz1 during mouse development led to heart defect including cardiac noncompaction and ventricular septal defect, which phenocopies 1p36 deletion syndrome related CHD. This suggests that CASZ1 is a novel 1p36 CHD gene and that the abnormal expression of cardiac morphogenesis and contraction genes induced by loss of Casz1 contributes to the heart defect.

Congenital heart disease (CHD) is the most common type of birth defect, affecting over 1% of all live births and accounting for one-third of all major congenital anomalies. A heart developmental program is induced by developmentally regulated signal transduction molecules and mediated by tissue-specific transcription factors. These transcription factors repress lineage inappropriate genes and activate cardiac-specific genes such as those responsible for cardiac morphogenesis and contractility. Disruption of this process affects heart development and function. CHD is associated with chromosome anomalies such as Down syndrome (trisomy 21), deletion 1p36 syndrome, deletion 4p syndrome, and deletion 22q11 spectrum. Many of the genetic mutations associated with CHD encode transcription factors such as GATA4, GATA6, TBX5, TBX20, HAND1, HAND2, MYOC, and NKK2.5. However, the underlying genetic basis of most forms of CHD remains unclear, necessitating further investigation into the genetic basis of CHD.

Human CASZ1 is a 1p36 gene encodes a zinc finger transcription factor. Casz1 was first described as a neural fate determination gene in Drosophila (8–9). An in situ hybridization analysis of Casz1 showing high levels of expression in the heart of developing mouse embryos was the first study to implicate Casz1 in heart development. Our studies confirmed the relatively high levels of Casz1 in murine heart and showed ele-

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4The abbreviations used are: CHD, congenital heart disease; En, embryonic day n; H&E, hematoxylin and eosin; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; IPA, ingenuity pathway analysis; qRT-PCR, quantitative real time PCR; GSEA, gene set enrichment analysis.
TABLE 1

| Genotyping PCR primers | Primer name         | Primer sequence |
|------------------------|--------------------|-----------------|
| mCasz1LWT-1869_s (cDNA) | CAGATAGTGATCCCCAATTTACAA |                |
| mCasz1L-2102_a (cDNA)  | GTGATAGACATGCTGGCTTTCTCT |                |
| mCasz1geo2_a (cDNA)    | ATTGCAGGCGTGCGACATTTGCGGG |                |
| mCasz1WT_s (genomic DNA) | AGCCAAAGGTATGCTGGCTTTA |                |
| mCasz1WT_a (genomic DNA) | CGAGTGGAGTGCTGGCTTTTCT |                |
| mCasz1Trp_a (genomic DNA) | TAAAGTGACCCTCCCCAACGCTT |                |

Here we report that Casz1 is critical for murine heart development. We find that Casz1 deletion leads to abnormal cardiogenic gene expression and causes reduced cardiomyocyte proliferation, a ventricular septal defect and defective cardiogenesis that ultimately led to heart failure and embryonic lethality. Our results demonstrate that Casz1 is required for normal mammalian heart development and function.

MATERIALS AND METHODS

The Generation of Casz1+/geo/geo Mice—The 129 OLA Casz1 trapped murine embryonic stem cells (Sanger CJ0565) were used to establish Casz1 knock-out mouse. The Casz1 gene trap inserted a fgeo reporter after Casz1 exon 9 and was sequence verified. The embryonic stem cells were injected into C57BL6 blastocysts, and the chimeras were bred to C57BL6 wild type to generate mixed 129/C57BL6 mice as described previously (19). The characterization of Casz1 mutant mice was performed by PCR using genomic DNA as template or reverse transcriptase PCR using cDNA as template. The primer sequences were listed in Table 1. All animals and procedures for mouse experiments were approved by the National Cancer Institute Animal Care and Use Committee.

X-Gal Staining—Embryos were fixed with 4% paraformaldehyde/PBS at 4 °C for 5 min. X-gal staining was performed as reported (20). In brief, the embryos were incubated with 1 mg/ml X-gal solution at 37 °C overnight. Postfixation with 4% paraformaldehyde/PBS was performed at room temperature for 25 min, and the embryos were sunk in 15% sucrose/PBS at 4 °C overnight. For sectioning, the embryos were embedded in gelatin and frozen dissected at 8-μm thickness, and then the slides were degelatinized and fixed with 4% paraformaldehyde/PBS. Whole embryo and section images were taken by using the software QCapture.

Cell Culture and Transfection Conditions—Human cardiac fibroblasts from a normal 18-year-old individual were obtained from a commercial source (Innoprot) and cultured as previously described (21, 22). In brief, cells were grown in Iscove’s modified Dulbecco’s medium (Lonza), supplemented with 20% FBS (HyClone), 10 ng/ml basic fibroblast growth factor (R&D), 1% penicillin/streptomycin (Thermo Fisher Scientific), and 1% l-glutamine (Lonza). Cells were passaged 1:3 twice a week, and experiments were performed between passages 4 and 6.

CASZ1b overexpression in human cardiac fibroblasts was achieved by transfection of 5 μg of CASZ1b-GFP plasmid (Origene) or an equivalent amount of control vector using an Amaxa P1 primary cell 4D Nucleofector kit (Lonza). All the analyses were performed using four independent CASZ1b nuleofection experiments in different human cardiac fibroblast cell preparations.

HL-1 cells, a cardiac muscle cell line derived from mouse atrial cardiomyocyte tumor lineage, were maintained as described previously (23). Briefly, cells were cultured on gelatin (0.02%, w/v)/fibronectin (10 μg/ml)-coated flasks or plates. The cells were maintained in Claycomb medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM l-glutamine, 0.1 mM norepinephrine, 100 unit/ml penicillin, and 100 μg/ml streptomycin (Thermo Fisher Scientific).

HL-1 cells were plated in 12-well plates. For knockdown experiments, 65 nm all stars negative control siRNA (Qiagen catalog number SI03650318) or murine Casz1 siRNA (Qiagen catalog number SI00970529) was transfected using Lipofectamine 2000 (Invitrogen) according manufacture’s instruction. For overexpression experiment, 2 μg pCMVTag2A-CASZ1b plasmids (11) or empty vector pCMVTag2A were transfected using Lipofectamine 2000 (Invitrogen). Two days later cells were collected for subsequent experiments. This experiment has been repeated twice.

Primary cardiomyocytes were isolated from E14.5 embryo hearts as reported (24) without preplating to remove the fibroblast cells. Cardiomyocytes were cultured on laminin coated 4-well chambers or 24-well plates with coverslip for 2 days before immunofluorescence staining.

Section Immunofluorescence and H&E Staining—These techniques were performed as previously described (25, 26). Embryos were fixed with 4% paraformaldehyde/PBS at 4C overnight. After fixation, the embryos were either placed in 70% ethanol and then embedded in paraffin compound or sunk in 30% sucrose/PBS at 4 °C and then embedded in OCT compound. 5-μm-thick paraffin sections of embryo hearts were used for H&E staining or immunofluorescence staining. Paraffin embedding and H&E staining were performed by Histoserv Inc. For immunostaining studies, histologic sections were deparaffinized in xylene and rehydrated to PBS. Staining was performed using anti-MF 20 antibody (developmental studies hybridoma bank mouse antibody, 1:30 overnight at 4 °C), pH3 antibody (Sigma-Aldrich H9908, rat antibody, 1:300 overnight at 4 °C), desmin antibody (Millipore MAB3430, mouse antibody, 1:200 overnight at 4 °C), and α-actinin (Abcam ab9465, mouse antibody, 1:100 overnight at 4 °C) to detect their expression. For immunofluorescent detection, either Alexa 488- or Alexa 568-conjugated secondary antibodies (Thermo Fisher Scientific 1:250, 1 h at room temperature) were used. Stained sections were imaged using a Zeiss ApoTome 2 microscope, a
Leica TGS SP5 confocal microscope or a Nikon Eclipse TE300 microscope.

**TUNEL Assays—**Apoptosis (TUNEL) assays were performed on paraffin sections using DeadEnd Fluorometric TUNEL system kit (Promega) and counterstained with DAPI. Stained sections were imaged using a Zeiss ApoTome 2 microscopy. Confocal microscopy was performed using a Leica TGS SP5 confocal microscope or a Nikon Eclipse TE300 microscope.

**Whole Mount Staining of the Embryo Heart—**Whole mount staining was performed as described previously (26, 27). In brief, hearts were dissected from E14.5 embryos, fixed in 4% paraformaldehyde/PBS at 4 °C overnight, and then placed in 70% ethanol at 4 °C for extended storage. Staining was performed using anti-β-galactosidase antibody (Cappel, rabbit antibody 1:40, 20 min at room temperature) to detect heart structure. For immunofluorescent detection, either Alexa 488- or Alexa 568-conjugated secondary antibody (Invitrogen, 1:40, 20 min at room temperature) was used to detect Casz1, and using phalloidin-Alexa 488 (Invitrogen, 1:40, 20 min at room temperature) to detect cytoskeleton. 

**Western Blot—**Western blot was performed as previously described (13, 28). In brief, to extract protein from heart, whole hearts were dissected from E14.5 embryos and dissolved in radiolabeled precipitation assay buffer. Protein was extracted using Bioruptor sonicator with protein extraction beads at high power for 10 cycles (30 s on and 30 s off). Proteins were electrophoresed in a 4–20% polyacrylamide gel (Bio-Rad) and transferred electrophoretically to nitrocellulose membranes (Schleicher & Schuell). Rabbit anti-GAPDH (Santa Cruz, 1:4000) and rabbit anti-Casz1 (Rockland, 1:1000) were used to detect GAPDH and Casz1 protein level in heart. To extract protein from human cardiac fibroblasts, cells were lysed in Laemmlı buffer 5 days after CASZ1b transfection. Forty micrograms of protein lysate was loaded onto gels to assess CASZ1 protein levels. Membrane has been probed with CASZ1 antibody (polyclonal, 1:500 Santa Cruz) and α-tubulin antibody (monoclonal, 1:4000 Cell Signaling).

**RNA Isolation and cDNA Analysis by qRT-PCR—**Total RNA was extracted from Casz1$^{+/+}$ and Casz1$^{+/geo}$/Casz1$^{+/geo}$ hearts at E12.5, or human cardiac fibroblast cells, or mouse HL-1 cardiomyocytes using the RNeasy mini kit (Qiagen) as per the manufacturer’s protocol. Quantitative measurements of different gene levels were obtained using Step One Plus real time PCR (Applied Biosystems) as described previously (11, 28). The expression of mouse GAPDH mRNA was used as an internal control for heart or HL-1 cells, and mRNA expression of human GAPDH was used as internal control for human cardiac fibroblast cells. The primer sequences were listed in Table 2. The experiment was repeated at least once in triplicate.

**The Oligonucleotide Microarray—**Affymetrix Mouse MO gene 2.0 ST arrays were used to determine genes regulated by Casz1 in embryonic murine hearts. Total RNA was extracted from three Casz1$^{+/+}$ and three Casz1$^{+/geo}$/Casz1$^{+/geo}$ hearts at E12.5. Among these hearts, one Casz1$^{+/+}$ heart and one Casz1$^{+/geo}$/Casz1$^{+/geo}$ heart were from the same litter as a pair, and hearts from three different litters were used as biological replicates. Array hybridization, chemiluminescence detection, and image acquisition were performed by Laboratory of Molecular Technology of NCI-Frederick core facility. Raw CEL files were uploaded onto Partek Genomics Suite v6.6 for data analysis. A Fisher’s test was applied to identify differentially expressed features by comparing Casz1$^{+/geo}$/Casz1$^{+/geo}$ and Casz1$^{+/+}$/Casz1$^{+/+}$ samples. The filter thresholds for a p value of <0.05 and an absolute fold change of 1.5 were imposed to retrieve a list of significantly differentially expressed genes. The differentially expressed genes were uploaded onto ingenuity pathway analysis (IPA) for further data analysis.

**Statistical Analyses—**Statistical analyses of the data were performed using a t test or CHITEST with p < 0.05 considered

### Table 2

| Primer name | Forward primer sequence | Reverse primer sequence |
|-------------|-------------------------|-------------------------|
| mGAPDH      | AACTTTGAGCAATGTTGGAAGG  | ATGCGAAGAGATGTTCTTGG    |
| mCas1−2935  | CTTCTCCGCAATCTGAGAGAAT  | TGTCTCTGCTGAGCTCTTGC    |
| mABCAN      | CCTTCTGCTGAGCTCTTGCCT   | CAGAAGCCTCAGAACCCTC     |
| mACTA1      | CCAAGCTGAGCCAGAGCAAGA   | AGCCAGCTGAGCCAGAGCAAGA  |
| mTNX2       | TCCGGGCTGACTGAGCTGCTG   | TGGCCTCAGACTGACCTCTCT   |
| mTNIT1      | CCAAGCCGAGGTGGCTCTGGT   | TGGCCTGAGTTCTGTGCTT     |
| mTGTA7      | TCCCTGCTGAGCTGCTCTTCT   | TGGCCTGAGTTCTGTGCTT     |
| mTGTA10     | CCTCTGCTGAGCTGCTCTTCT   | TGGCCTGAGTTCTGTGCTT     |
| mCOL2A1     | CATCTGCTGAGCTGCTCTTCT   | TGGCCTGAGTTCTGTGCTT     |
| mMAG2A      | CCTTCTGCTGAGCTCTTGCCT   | CAGAAGCCTCAGAACCCTC     |
| mMAG2A      | TCCGGGCTGACTGAGCTGCTG   | TGGCCTCAGACTGACCTCTCT   |
| mMAG2A      | CCTTCTGCTGAGCTCTTGCCT   | CAGAAGCCTCAGAACCCTC     |
| mMAG2A      | TCCGGGCTGACTGAGCTGCTG   | TGGCCTCAGACTGACCTCTCT   |
| mMAG2A      | CCTTCTGCTGAGCTCTTGCCT   | CAGAAGCCTCAGAACCCTC     |
| mMAG2A      | TCCGGGCTGACTGAGCTGCTG   | TGGCCTCAGACTGACCTCTCT   |

* Primer names starting with “m” represent mouse genes, and primer names starting with “h” represent human genes.
FIGURE 1. Casz1 expression pattern during mouse embryogenesis. A, cartoon of the Casz1 gene trap that inserted with a βgeo reporter after Casz1 exon 9 resulting in a truncated Casz1. B, genotyping of Casz1-trapped mice by RT-PCR. C, whole mount X-gal staining (blue) of E9.5 Casz1+/−βgeo embryos shows that Casz1 was expressed in the hindbrain, neural tube, and heart, which are further demonstrated by dissected embryo sagittal sections 1, 2, and 3 (100× magnification). Sagittal section 3b (200× magnification) shows that Casz1 was expressed in the cardiomyocytes. D and E, whole mount X-gal staining of E12.5 Casz1+/+, Casz1+/−βgeo, and Casz1βgeo/βgeo embryos showed that Casz1 is expressed in the eye, dorsomedial telencephalon, cranial ganglia, nasal placode, somite, neural tube, and heart. F, real time PCR to detect wild type Casz1 allele and Gata4 mRNA levels in E12.5 hearts. G, the protein levels of Casz1a/Casz1b and GAPDH were visualized by immunoblotting E14.5 whole heart lysate with anti-Casz1 antibody and anti-GAPDH antibody. H, whole mount anti-β-galactosidase staining (red) of E14.5 Casz1+/+, Casz1+/−βgeo, and Casz1βgeo/βgeo hearts.
significant. The values in the graphs are expressed as means ± S.D. or S.E. The statistical tests were two-sided.

RESULTS

Expression Pattern of Casz1 during Embryonic Development—Casz1 gene encodes two major isoforms: full-length Casz1α and a shorter isoform Casz1β, with both isoforms sharing the same 5′-proximal "promoter" region and the first 16 exons. In the Casz1 knock-out mouse, the Casz1 gene trap inserts a βgeo reporter after Casz1 exon 9 (Fig. 1A), resulting in a truncated Casz1 allele in which 10 of the 11 zinc fingers of full-length Casz1 are lost. The zinc fingers are critical domains for Casz1 function (14). Using primers specific for the WT (Casz1+/+) allele and the trapped allele, we found that Casz1+/+ mice only have the unaltered Casz1 allele, whereas Casz1 heterozygous (Casz1+/βgeo) mice have both the WT allele and the trapped allele (Fig. 1B). Among 183 progeny of male Casz1+/βgeo and female Casz1+/+ mice, 63 (35.4%) mice are WT (Casz1+/+) and 120 (64.6%) mice are heterozygous (Casz1+/βgeo), but none are homozygous (Casz1βgeo/βgeo). This indicates that Casz1 mutation in both alleles results in embryonic lethality. Timed mating results showed that the embryonic lethality occurred at E17.5; no live Casz1βgeo/βgeo embryos were observed after E17.5.

To determine the role of Casz1 during embryogenesis, we first investigated the expression pattern of Casz1 during embryonic development. In Casz1 trapped embryos, the βgeo reporter was under the control of endogenous Casz1, and therefore X-gal staining or anti-β-galactosidase staining could be used to characterize the pattern of Casz1 expression during embryonic development. Whole mount X-gal staining of E9.5 embryos showed that Casz1 was expressed in the neural tube and heart (Fig. 1C, left panel), which was consistent with previously reported in situ hybridization analysis results (10). Transverse sectioning showed that Casz1 was expressed in the hindbrain and dorsal aspect of the spinal cord, as well as in cardiomyocytes of the heart (Fig. 1C, right panel). Whole mount X-gal staining of E12.5 embryos showed that Casz1 was expressed in the eye, dorsomedial telencephalon, cranial ganglia, nasal placode, somite, neural tube, and heart (Fig. 1, D and E). To confirm the expression of Casz1 in the heart, total RNA was extracted from the entire heart of E12.5 embryos, and real time PCR was performed using primers that only recognize the wild type allele but not the trapped allele. Consistent with X-gal staining, real time PCR results showed that Casz1 mRNA was expressed in the heart (Fig. 1F). Endogenous Casz1 mRNA was detected in the heart of Casz1+/+ embryos, a half amount of Casz1 mRNA level was detected in the heart of the Casz1+/βgeo embryo, and the Casz1 mRNA level was undetectable in the heart of the Casz1βgeo/βgeo embryo. Gata4, an early cardiac marker, was expressed at similar levels in these hearts (Fig. 1F). Western blot analysis of E14.5 hearts using an anti-Casz1 antibody demonstrated the protein expression of both Casz1α and Casz1β isoforms in WT hearts but not in Casz1βgeo/βgeo heart, confirming the deficiency of Casz1 in the knock-out mouse (Fig. 1G). Whole mount staining of the E14.5 hearts using anti-β-galactosidase antibody showed that Casz1 was expressed in the ventricles and atriums of the heart (Fig. 1H).

Loss of Casz1 in the Embryos Leads to an Abnormal Myocardium Development—The Casz1βgeo/βgeo embryos at E16.5 showed a swollen phenotype with accumulation of fluid in subcutaneous regions, which is often associated with heart failure in embryos (Fig. 2A). The E16.5 Casz1βgeo/βgeo heart is morphologically different in shape, with the ventricle apex being spherical and not the normal triangular in shape (Fig. 2B).

Edema was detected in Casz1βgeo/βgeo embryos at E15.5 but not seen in WT or Casz1+/+ embryos (Fig. 2A). E15.5 embryos were transverse dissected, and H&E staining was performed to investigate heart development. Unlike the WT heart or Casz1+/+ heart, the compact myocardium (free wall) of both ventricles and the septum in the Casz1βgeo/βgeo heart was hypoplastic (Fig. 3, B–D, column 1). At higher magnification (Fig. 3, B–D, columns 2 and 4), it was clear that E15.5 Casz1βgeo/βgeo heart has abnormal myocardium with significantly thinning and less compact of walls in both the left and right ventricles, and the septum was also thinner and less compact (Fig. 3, B–D, column 3). Moreover, more blood cells accumulated in the liver of the Casz1βgeo/βgeo embryo when compared with the livers of the Casz1+/+ and Casz1+/βgeo embryos (Fig. 3E), a finding also consistent with functional defects in the Casz1βgeo/βgeo heart.

We further focused on E14.5 embryos heart, at a time when the ventricle septation is complete. Edema was also detected in some of the E14.5 Casz1βgeo/βgeo embryos, but not as clear as in E15.5 embryos. Indeed, the skin on the back of the Casz1βgeo/βgeo embryos exhibits an enlarged lymphatic network, although sprouting lymphangiogenesis appeared to occur normally at the lymphatic vascular front (Fig. 4). This lymphatic phenotype may
be a secondary defect of loss of Casz1 because β-galactosidase staining did not show the expression of Casz1 in lymphatic endothelial cells.

The Casz1\textsuperscript{geo/geo} heart was morphologically distinct from the Casz1\textsuperscript{+/geo} or Casz1\textsuperscript{+/-} heart, with the left ventricle apex being spherical and not the normal triangular in shape (Fig. 5A). Consecutive sectioning of the E14.5 embryos showed that three of three Casz1\textsuperscript{geo/geo} hearts had four chambers, four valves, and normal outflow tract alignment, but the ventricular septum was abnormal, with ventricle septal defects (Fig. 5B). The compact myocardium of both ventricles and the septum of the E14.5 Casz1\textsuperscript{geo/geo} heart were hypoplastic, although they were not as obvious as the E15.5 Casz1\textsuperscript{geo/geo} hearts (Fig. 5B, top panels). Higher magnifications clearly showed the ventricular septal defect in Casz1\textsuperscript{geo/geo} heart but not in Casz1\textsuperscript{+/-} heart (Fig. 5B, bottom panels).

Casz1 Deficiency in Heart Leads to the Decrease of Cardiomyocyte Proliferation.—To investigate whether the hypoplasia of Casz1\textsuperscript{geo/geo} compact myocardium was caused by reduced cardiomyocyte proliferation, we stained the transverse
sections of E14.5 Casz1+/+ and Casz1geo/geo heart for the cardiomyocyte marker MF-20 (the heavy chain of striated myosin II) and for phosphorylated histone H3 (pH3), a marker of the M phase of the cell cycle (Fig. 5C, left panels). Loss of Casz1 led to a more than 3-fold decrease in the fraction of pH3 positive cardiomyocytes compared with Casz1+/+ hearts (Fig. 5C, right panel), which is consistent with a decrease in cell proliferation contributing to the hypoplasia in the Casz1geo/geo hearts. To determine whether apoptosis also contributes to the hypoplasia of the compact myocardium in Casz1geo/geo heart, we performed a tunnel assay and found that there was no increase in the number of apoptotic cells in Casz1geo/geo heart compared with Casz1+/+ hearts at the time evaluated (Fig. 5D).

Casz1geo/geo Heart Exhibits Disorganized Myofiber Orientation and Cell Alignment—Because the Casz1geo/geo heart was morphologically distinct from the Casz1+/+ or Casz1geo/geo heart (Fig. 5A), we next investigated whether loss of Casz1 affects myofiber organization and cellular alignment. E14.5 whole mount hearts were stained with phalloidin-Alexa 488 to visualize F-actin and the arrangement of the thin filaments. The immunofluorescence analysis revealed that the myocytes within the heart wall of E14.5 Casz1+/+ heart were well aligned; however, disruption of the fiber orientation and cell alignment were observed in both the left ventricle and the right ventricle of Casz1geo/geo heart; representative images of the left ventricles are shown in Fig. 6.

Casz1geo/geo Heart Has an Abnormal Gene Expression Pattern—To understand the underlying mechanisms contributing to the Casz1geo/geo heart defect, we analyzed the transcriptome of the E12.5 Casz1+/+ and Casz1geo/geo hearts. Genome-wide RNA expression-profiling analysis of Casz1geo/geo heart versus Casz1+/+ heart showed that the expression level of 187 Affymetrix probe set IDs were significantly changed (decreased or increased greater than 1.5-fold, \( p < 0.05 \)). Among these probe set IDs, 148 mapped to known gene ID with 53 genes being down-regulated and 95 genes up-regulated in Casz1-deficient heart (Fig. 7A and supplemental Table S1).

Gene ontology and IPA assays showed that three groups of altered genes might contribute to the Casz1geo/geo heart abnormalities. These include 1) cell adhesion molecules, 2) genes involved in muscular system development and function, and 3) ion channel genes.

Partek gene ontology analysis of these 148 genes showed that “biological adhesion” genes are one of the most significantly enriched group of genes with enrichment score of 4.5 and enrichment \( p \) value of 0.01 (Fig. 7B). These genes include ACAN, aggrecan; ITGA7, integrin \( \alpha 7 \); ITGA10, integrin \( \alpha 10 \); ITGB8, integrin \( \beta 8 \); CDH6, cadherin 6; COL2A1, collagen, type II \( \alpha 1 \); and COL8A1, collagen type VIII \( \alpha 1 \). We validated the microarray findings by using quantitative real time PCR (qRT-PCR) to assay some of these cell adhesion genes with known cardiac expression. The qRT-PCR results showed that ACAN, ITGA7, ITGA10, and COL2A1 mRNA levels were significantly increased in Casz1geo/geo heart (Fig. 7D). These genes are known to be important for cell organization and tissue morphogenesis.

IPA analysis of Casz1 target genes showed that in the physiological system development and function categories, genes involved in skeletal and muscular system development and function are the most significantly enriched genes (Fig. 7C, left panel). These genes include TGF\( \beta 3 \), transforming growth factor, \( \beta 3 \); CKM, creatine kinase, muscle; ACTA1, skeletal actin \( \alpha 1 \); CASQ1, calsequestrin 1, fast-switch skeletal muscle; TNNT1, troponin I type 2, skeletal, fast; and TNNT3, troponin T type 1, skeletal, slow. These are muscle development and/or muscle contraction genes or genes encoding cell structure proteins. Consistent with the microarray results, the qRT-PCR results showed that representative gene CKM, ACTA1, TNNI2, and TNNI3 mRNA levels were significantly decreased in Casz1geo/geo heart (Fig. 7D).

In addition to the alterations in expression of the cell adhesion and muscle development genes in Casz1geo/geo heart, we found dysregulation of multiple ion channel genes in Casz1geo/geo heart (Fig. 7C, right panel). Some of these genes, especially the potassium and calcium channel genes, have the potential to affect the conduction system function in the heart, whereas some of them have been reported to affect cardiac arrhythmia. These genes include ABCC9, ATP-binding cassette, subfamily C, member 9 (part of an ATP-dependent potassium channel); ATP1A3, ATPase, Na\(^+\)/K\(^+\) transporting, \( \alpha 3 \) polypeptide; CACNA1D, calcium channel, voltage-dependent, L type, \( \alpha 1D \) subunit; KCNK3, potassium channel, subfamily K,
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FIGURE 5. Decreased proliferation in Casz1<sup>geo/geo</sup> heart. A, the E14.5 Casz1<sup>+/+</sup> hearts were morphologically different compared with the Casz1<sup>geo/geo</sup> or wild type heart (compare the green arrow targeted region). B, lower magnification of H&E staining of transverse sections from E14.5 embryos (upper panels). The lower panels represent a magnification of the regions that are depicted in the blue squares in the upper panel. The green arrow denotes the septal defect apparent in the serial sectioning of the Casz1<sup>geo/geo</sup> heart compared with the Casz1<sup>+/+</sup> heart. C, paraffin sections from E14.5 hearts from Casz1<sup>+/+</sup> and Casz1<sup>geo/geo</sup> hearts were immunostained with antibodies for MF20 (red) and the mitosis marker phosphohistone H3 (pH3, green). The nuclei were stained with DAPI (blue; lower panels, high magnification). In the high magnification images, arrows indicate pH3<sup>-</sup> cells. The graph (right panel) represents the relative percentage of pH3 positive cells compared with the total number of DAPI positive Casz1<sup>geo/geo</sup> or Casz1<sup>+/+</sup> cardiomyocytes from at least five sections from each embryo. The Casz1<sup>geo/geo</sup> cardiomyocytes proliferate significantly slower than Casz1<sup>+/+</sup> cardiomyocytes (p < 0.00001). D, tunnel staining (green) was performed using E14.5 heart paraffin sections (upper panels), and nuclei were stained with DAPI (blue). The lower panels represent magnified areas delineated in the red squares in the upper panels. There was no significant difference in the number of apoptotic cells observed in Casz1<sup>geo/geo</sup> cardiomyocytes compared with Casz1<sup>+/+</sup> cardiomyocytes.

member 3; and KCND2, potassium voltage-gated channel, shal-related subfamily, member 2. Consistent with the microarray results, qRT-PCR results showed that representative gene CACNA1D and ATP1A3 mRNA levels were significantly increased, whereas ABCC9 and KCND2 mRNA levels were significantly decreased in Casz1<sup>geo/geo</sup> heart (Fig. 7D).

Because loss of Casz1 led to a decrease of cardiomyocyte proliferation, we assessed whether Casz1 affects the expression of genes controlling cell cycle and cell proliferation. IPA analysis of Casz1 target gene showed that genes involved in cell cycle and cellular growth were significantly enriched (Fig. 8A). These genes include EDN1, endothelin-1; NEFL, neurofilament, light polypeptide; TGFβ3, transforming growth factor β3; DCC, deleted in colorectal carcinoma; and SJK1, salt-inducible kinase 1. Consistent with the microarray results, the changes in the expression of these genes were confirmed by qRT-PCR (Fig. 8B). In the microarray data, we found that TP63, tumor protein 63, a classic cell cycle and growth regulation gene, was up-regulated by 1.4-fold in Casz1 knock-out heart, which was not included in the initial analysis because the cut-off was set as > or <1.5-fold. However, qRT-PCR results confirmed the up-regulation of TP63 in Casz1-deficient heart (Fig. 8B). Thus the dysregulation of genes involved in cell cycle and cell growth regulation in Casz1-deficient heart may contribute to a decrease in cardiomyocyte proliferation.

Validation of Casz1 Target Genes in Cellular Models—To provide additional evidence for Casz1-dependent gene expression in heart, we performed gain and loss of function studies in cellular models. For overexpression expression experiments we used the human CASZ1b cDNA construct because it is the most evolutionarily conserved isoform (11, 14). First, we used human cardiac fibroblasts whose level of Casz1 expression is not detectable by routine qRT-PCR. After overexpression of Casz1 in cardiac fibroblast cells, the muscular system developmental gene TNNT1, CKM, and the contract fiber gene ACTA1 mRNA were significantly up-regulated, whereas the cell adhe-
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To visualize thin filaments, Casz1+/+ and Casz1Hgeo//Hgeo E14.5 hearts were stained with phalloidin-488 (green) to detect F-Actin and co-stained with TOPRO3 to show cell nuclei. The left panels show the co-staining results of the left ventricles of the hearts. The right panels represent magnified areas delineated in the red squares in the left panels. Enlarged images showed complete disruption of the fiber orientation/cell alignment in the left ventricle (LV) of Casz1Hgeo//Hgeo heart.

DISCUSSION

In this study, we find that Casz1 is critical for murine heart development. Although Casz1 has been shown to be important in heart development in Xenopus (17), our study indicates that loss of Casz1 in murine hearts has a different functional impact than was shown in Xenopus. In this study, we demonstrated that Casz1 deletion in mice leads to abnormal heart development including abnormal gene expression, hypoplasia of myocardium, a ventricular septal defect, and aberrant heart morphology.

Casz1 deletion also led to the embryonic lethality occurred at E17.5. Although heart failure may lead to this embryonic lethality, we cannot rule out the possibility that aberrant development of other organ such as the nervous system may be involved, because Casz1 is highly expressed in the nervous system during embryogenesis (Fig. 1, C and D). Future studies will focus on how loss of Casz1 affects nervous system development and embryonic lethality.
Casz1 deficiency in the murine embryonic heart led to hypoplasia of the left and right ventricles, as well as the septum, and was associated with decreased cell proliferation (Figs. 3 and 5). This contrasts with the finding that knockdown of Casz1 in Xenopus heart led to overproliferation of ventral midline cells (17). One possibility that may account for the different phenotypes in these two species is that in the Xenopus knockdown utilized a morpholino technique, which may not be as fully penetrant as the

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**Diagram A**

- Casz1+/+ vs Casz1<sup>[geo]</sup>/geo
- Casz1 represented genes (63)
- Casz1<sup>[geo]</sup> replicated genes (63)

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**Diagram B**

- GO Enrichment Score of Biological Process
  - 1. Signaling
  - *2. Biological adhesion* (p=0.01)
  - 3. Establishment of localization
  - 4. Response to stimulus (p=0.04)
- * PERP, COL2A1, ITGA7, ACAN, ITGA10, ITGB8, COL8A1, FREM2, CDH6, NRP2

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**Diagram C**

1. **Physiological System Development and Function**
   - 1. Skeletal and Muscular System Development and Function
   - 2. Tissue Development
   - 3. Behavior
   - 4. Nervous System Development and Function
   - 5. Organismal Functions
   - 6. Organ Development

   - *TNNT1, ENPP1, CKM, TNNT2, ACAN, XYLT1, COL2A1, MYOZ1, MYPN, ITGA10, MRVI1, MYOF, HTR1B, EDN1, FGF18, CASQ1, MYH3, TGFB3, TNRFSF1B, ABCC9, ACTA1*

2. **Molecular and Cellular Function**
   - 1. Cell-To-Cell Signaling and Interaction
   - *2. Molecular Transport* (p=0.03)
   - 3. Carbohydrate Metabolism
   - 4. Lipid Metabolism
   - 5. Small Molecule Biochemistry
   - 6. Cell Morphology

   - *ABCC9, ATP1A3, CACNA1D, EDN1, GABRB1, GRIN3A, KCND2, KCNK3, RAMP1, SIK1, SLC24A2, TRPC5*

**Note:** only ion transport genes were listed here

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**Diagram D**

- Relative mRNA level
  - Casz1
  - ACAN
  - ITGA7
  - ITGA10
  - COL2A1
  - CKM
  - ACTA1
  - TNNT2
  - TNNT1
  - CACNA1D
  - ABC9
  - KCND2
  - ATP1A3

**Legend**

- Casz1+/+
- Casz1<sup>[geo]</sup>/geo

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**Legend**

**Cell adhesion**

- Casz1
- ACAN
- ITGA7
- ITGA10
- COL2A1

**Muscle development and contraction**

- CKM
- ACTA1
- TNNT2
- TNNT1

**Ion channel**

- CACNA1D
- ABCC9
- KCND2
- ATP1A3
Casz1- gene trapped murine model. However, it is also possible that the differences in the heart development defects noted between *Xenopus* and mice are due to the major differences in the *Casz1* gene in these species. In *Xenopus*, the *Casz1* gene encodes two isoforms Cstα (1108 amino acids) and Cstβ (1087 amino acids), and both have five zinc fingers (17). However, the murine *Casz1* locus, like the human, encodes two major isoforms; the short isoform Casz1b is similar to *Xenopus Casz1*, but the full-length isoform Casz1a has 1762 amino acids with 11 zinc fingers (12). The evolutionarily acquired six extra zinc fingers in mouse Casz1a may play certain roles during heart development through differential DNA binding or protein-protein interactions. Consistent with this hypothesis, microarray analysis of neuroblastoma cells transfected with either CASZ1a or CASZ1b showed that 16% genes were specifically regulated by either Casz1a or Casz1b (data not shown), indicating that there are some isoform-specific targets, at least in some cell types.

The murine *Casz1*-deficient hearts exhibit morphology anomalies, a ventricular septal defect and hypoplasia of myocardium (Figs. 3 and 5), which may be caused by abnormal gene expression. Cardiac morphogenesis is a complex process that is coordinately regulated by many transcription factors such as *Nkx2.5*, *TBX5*, *GATA4*, and *GATA6* (3–5, 7). *Casz1* is a novel transcription factor highly expressed in heart, and we found that abnormal expression of cell adhesion genes are significantly enriched in *Casz1*-deficient heart (Fig. 7B). Cell adhesion molecules play critical role for embryo and tissue morphogenesis (29, 30). *Casz1* regulates cadherin family protein (*CDH6*), collagens (*COL2A1* and *COL8A1*), and integrins (*ITGA7*, *ITGA10*, and *ITGB8*), which are known to be important for tissue morphogenesis (31–33). Although it has not been reported that these genes specifically affect cardiac morphogenesis, the abnormal expression of such cell adhesion genes may contribute to the disorganized morphology in *Casz1*-deficient heart. The dysregulation of genes involved in cell cycle and cell growth regulation in *Casz1*-deficient heart (Fig. 8) may lead to hypoplasia of myocardium. For example, both TGFβ3 and TP63 are able to induce cell cycle arrest and inhibit cell proliferation (34–37), so the up-regulation of these genes in *Casz1*-deficient heart may lead to the decrease of cardiomyocyte proliferation.

**FIGURE 7. Abnormal gene expression in *Casz1*+/geo+/geo heart.** A, microarray analysis of RNA from three E12.5 *Casz1*+/+/ hearts and three E12.5 *Casz1*+/geo+/geo hearts. The heat map was generated using Partek software. Two-thirds of these genes are aberrantly up-regulated, and one-third are aberrantly down-regulated in *Casz1*+/geo+/geo hearts. B, Partek gene ontology analysis showed that the top two categories of enriched genes are “signaling” and the process of biological adhesion. Biological adhesion genes in *Casz1*+/geo+/geo hearts that are up-regulated (red) or down-regulated (blue) are listed below. C, IPA assays showed gene enrichment in the categories of physiological system development and function (panel 1) with a key subcategory being skeletal and muscular system development and function genes that are up-regulated (red) or down-regulated (blue) listed below. D, verification of microarray results. The mRNA levels of representative genes encoding cell adhesion molecules, muscle contraction, and muscular development proteins and ion channels were evaluated by real time PCR and normalized to GAPDH gene. The mRNA levels of these genes in *Casz1*+/geo+/geo hearts were significantly different compared with their levels in *Casz1*+/+ hearts. The bar graph represents means ± S.D. (all *p < 0.005*).
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The abnormal expression of muscle development and function genes, as well as the genes encoding contractile filament proteins in the Casz1<sup>+/geo</sup>/<sup>−geo</sup> heart (Figs. 7 and 10A), may contribute to the heart abnormalities. Among muscle development and function genes, CASQ1, TNNI1, and TNNI2 are genes required for muscle contraction (38–41). Gene ontology assay using GSEA tool showed that 50% of the contractile fiber genes were negatively enriched in Casz1 deficient heart. As a contractile fiber gene, ACTA1 is important for the organization of the sarcomeres (39). Consistent with the finding of dysregulation of contractile fiber gene expression, we found that Casz1-deficient cardiomyocytes displayed abnormal Z line formation (Fig. 11). Because cardiac Z line plays an important role in cardiomyocyte signal transduction and disease and sarcomeres play a critical role in cardiac pump functions (39, 42–45), it suggests dysregulation of contractile fiber genes and muscle contraction genes in Casz1-deficient heart contributes to the heart failure.

The cause of heart defects in Casz1 deletion embryos is likely due to altered expression of multiple Casz1 downstream targets acting in combination, because many Casz1 target genes are reported to be important for heart function and/or have been implicated in CHD. For example, a mutation in the muscle contraction gene ACTA1 was found in a case of nonfatal hypertrophic cardiomyopathy (46), and TNNI2 mutations were found in hypertrophic cardiomyopathy (47); mutations in developmental gene TGFβ3 were found in right ventricular cardiomyopathy disease (48, 49). Ion channels such as potassium channels and calcium channels are important for cardiac conduction (50–53), and they were transcriptionally regulated by key cardiac transcriptional factors (54). Mutations in ABCC9 a potassium channel gene were identified in human dilated cardiomyopathy (55). The involvement of many of these genes in heart function raises the possibility that these genes work in combination to mediate Casz1 regulatory functions during heart development.

In fact, key cardiac transcription factors control heart development via regulation of sets of genes that mediate cardiac morphogenesis and contractility (3–6). Casz1 targets overlap with targets of known cardiac transcription factors such as MYOCD, MEF2C, and GATA4. For example, ACTA1, CACNA1D, COL2A1, TAGLN, and LMOD1 are regulated by Casz1, as well as MYOCD. ACTA1, CKM, MYOZ1, and COL2A1 are regulated by Casz1, as well as MEF2C. Although
these genes are both regulated by Casz1 and MYOCD or MEF2C, they were not regulated in the same direction (up or down), which suggests that Casz1 is not downstream or upstream of MYOCD or MEF2C. Casz1 may cooperate with them to ensure that these genes are expressed at the appropriate level or time during heart development. It is unclear at present whether these genes are direct targets of Casz1 because of a lack of a ChIP grade Casz1 antibody, but our future plan will include the identification of Casz1 direct targets in heart. Nevertheless, using two different in vitro cellular models, we confirmed that many of the muscle development genes, contract fiber genes, and cell adhesion genes regulated by loss of Casz1 in the heart in vivo were similarly regulated by Casz1 in these cellular models in vitro (Fig. 9).

Our finding that Casz1 is critical for mammalian heart development and Casz1 target genes are related to the pathogenesis of CHD suggests that Casz1 may be a novel CHD gene. Casz1 homozygous deletion in mice led to heart failure and embryonic lethality, although the Casz1 heterozygous deletion did not show apparent abnormalities in mice. This is not unusual because some CHD causative genes cause severe heart defect and embryonic lethality in the homozygous gene knock-outs.

**FIGURE 10. Sarcomeric organization in Casz1<sup>geo/geo</sup> heart.** A, GSEA assay indicated the negative enrichment of genes encoding contractile fiber proteins (panels a and b), as well as genes encoding contractile fiber part proteins (panel c). NES, normalized enrichment score; Nom, nominal; FDR, false discovery rate. B, sections containing the trabecular regions of E14.5 hearts were labeled for desmin (red), and nuclei were stained with DAPI (blue). There is a striated pattern for desmin labeling in both wild type and Casz1-deficient hearts, but the striated patterns are not as uniform or apparent in the Casz1-deficient heart.
but no apparent heart anomalies in heterozygous mice (56–58). CASZ1 resides on chromosome 1p36.22. Chr1p36 deletion is one of the most common terminal deletions observed in humans, and monosomy 1p36 is associated with many diseases such as central nervous system malformations (88%), skeletal anomalies, and heart defects (71–75%), but the 1p36 genes that contribute to heart disease have not been clearly delineated (2, 16, 59). Interestingly, 1p36 deletion-related CHD includes cardiac noncompaction and ventricular septal defect (2, 16), which was also seen in our Casz1-deficient heart (Figs. 5 and 5). Although no mutations of CASZ1 have been reported in CHD patients, loss of heterozygosity of CASZ1 occurs in those patients with 1p36 deletion syndrome-related CHD. Recent exome sequencing results discovered de novo mutations in histone-modifying genes in CHD that contribute to ~10% of severe CHD, in which the key developmental genes expression were dysregulated (60). We have shown that one allele of CASZ1 may be lost by 1p36LOH in neuroblastoma tumors, and the other allele may be epigenetically silenced by PRC2 complex-mediated histone modifications (61). This raises the possibility that CASZ1 expression may be dysregulated in CHD with de novo mutations in histone modifying genes. Importantly, our previous study has shown that HDAC inhibitor treatment of neuroblastoma cells leads to an increase of CASZ1 expression (61), suggesting a potential therapeutic modality.

Our data clearly demonstrate that CASZ1 is a novel cardiac transcription factor that plays an important role in cardiac development and morphogenesis in mammals. CASZ1 is a candidate CHD associated gene, and genetic or epigenetic alteration on the CASZ1 gene may contribute to CHD.

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