Isolated left ventricular noncompaction (LVNC) results from excessive trabeculation and impaired myocardial compaction during heart development. The extracellular matrix (ECM) that separates endocardium from myocardium plays a critical but poorly understood role in ventricular trabeculation and compaction. In an attempt to characterize solute carrier family 39 member 8–null (Slc39a8-null) mice, we discovered that homozygous null embryos do not survive embryogenesis and exhibit a cardiac phenotype similar to human LVNC. Slc39a8 encodes a divalent metal cation importer that has been implicated in ECM degradation through the zinc/metal regulatory transcription factor 1 (Zn/MTF1) axis, which promotes the expression of ECM-degrading enzymes, including Adamts metalloproteinases. Here, we have shown that Slc39a8 is expressed by endothelial cells in the developing mouse heart, where it serves to maintain cellular Zn levels. Furthermore, Slc39a8-null hearts exhibited marked ECM accumulation and reduction of several Adamts metalloproteinases. Consistent with the in vivo observations, knockdown of SLC39A8 in HUVECs decreased ADAMTS1 transcription by decreasing cellular Zn uptake and, as a result, MTF1 transcriptional activity. Our study thus identifies a gene underlying ventricular trabeculation and compaction development, and a pathway regulating ECM during myocardial morphogenesis.
Zinc transporter *Slc39a8* is essential for cardiac ventricular compaction

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Isolated left ventricular noncompaction (LVNC) results from excessive trabeculation and impaired myocardial compaction during heart development. The extracellular matrix (ECM) that separates endocardium from myocardium plays a critical but poorly understood role in ventricular trabeculation and compaction. In an attempt to characterize solute carrier family 39 member 8–null (*Slc39a8*-null) mice, we discovered that homozygous null embryos do not survive embryogenesis and exhibit a cardiac phenotype similar to human LVNC. *Slc39a8* encodes a divalent metal cation importer that has been implicated in ECM degradation through the zinc/metal regulatory transcription factor 1 (Zn/MTF1) axis, which promotes the expression of ECM-degrading enzymes, including ADAMTS metalloproteinases. Here, we have shown that *Slc39a8* is expressed by endothelial cells in the developing mouse heart, where it serves to maintain cellular Zn levels. Furthermore, *Slc39a8*-null hearts exhibited marked ECM accumulation and reduction of several Adamts metalloproteinases. Consistent with the in vivo observations, knockdown of *SLC39A8* in HUVECs decreased ADAMTS1 transcription by decreasing cellular Zn uptake and, as a result, MTF1 transcriptional activity. Our study thus identifies a gene underlying ventricular trabeculation and compaction development, and a pathway regulating ECM during myocardial morphogenesis.

Introduction

Cardiac ventricular morphogenesis begins from a tubular structure composed of an outer 1-cell layer of myocardium and an inner 1-cell layer of endocardium separated by extracellular matrix (ECM), or cardiac jelly (1). Trabeculation in mice begins at embryonic day 9 (E9.0), when myocardial cells protrude into cardiac jelly to form projections lined by endocardium: structures named trabeculae. Trabeculae rapidly grow and expand between E9.5 and E13.5. By E14.5, trabeculae are long and thin, with little cardiac jelly between myocardium and endocardium. Concomitant with the development of coronary circulation, trabeculae go through compaction, collapsing into the ventricle wall and becoming part of the compact myocardium. Therefore, the mature ventricle has thick compact myocardium with few trabeculae (2, 3). Endocardium plays unique and critical roles in trabeculation and compaction by secreting signaling molecules that orchestrate cardiomyocyte proliferation and differentiation and enzymes that are involved in ECM production and degradation (1, 4, 5).

Many forms of congenital heart defects result from, or are associated with, abnormal cardiac trabeculation and compaction. For instance, left ventricular noncompaction (LVNC) is a cardiomyopathy caused by arrested compaction. It is characterized by excessive trabeculation with deep intertrabecular recesses and thin compact myocardium (6). It usually affects both ventricles, but is more prominent on the left in humans. In the pediatric population, LVNC is the third most common cardiomyopathy after dilated cardiomyopathy and hypertrophic cardiomyopathy (7). Typical complications of LVNC include heart failure, ventricular arrhythmias, and systemic embolic events (8). Mutations in genes encoding sarcomeric, mitochondrial, cytoskeletal, and nuclear membrane proteins, and Notch signaling pathway components have been identified in LVNC patients (9–13), but the molecular mechanisms remain largely unresolved, reflecting a lack of understanding of the compaction process.

Cardiac jelly serves as the media for the signal exchanges between endocardium and myocardium (14). Perturbation of cardiac jelly production or degradation has been shown to cause abnormal trabeculation and compaction. Two major components of cardiac jelly are hyaluronan, a mucopolysaccharide, and versican, a chondroitin sulfate proteoglycan. Mice lacking hyaluronan synthase-2 or versican do not develop trabeculae (15–17). On the other hand, a substantial amount of cardiac jelly needs to be degraded prior to ventricular compaction. Cardiac jelly degradation is mediated by metalloproteinases including Adams family members. *Adams1*-knockout mice develop hypertrabeculation and *Adams9*-haploinsufficient mice develop ventricular noncompaction accompanied by cardiac jelly accumulation (5, 18). Conversely, mice overexpressing *Adams1* or *Adams5* exhibit sparse trabeculation due to excessive cardiac jelly degradation (5, 19). However, the upstream factors regulating *Adams* expression, and the mechanism by which cardiac jelly regulates trabeculation and compaction are not well understood.

Zinc (Zn) is required for the structure and function of a variety of enzymes and transcription factors (20). Zn deficiency has been shown to result in developmental defects including multiple types of cardiac abnormalities (21, 22). Zn homeostasis is primarily regu-
lated by 10 Zn exporters and 14 Zn importers (23). Solute carrier family 39 member 8 (Slc39a8, which encodes a protein historically termed ZIP8) is a cellular Zn importer (24) and has been shown to play a causal role in the pathogenesis of osteoarthritis by promoting cellular Zn uptake, resulting in increased metal-regulatory transcription factor 1 (Mtf1) activity, increased Adamts expression, and ECM degradation (25). Here, we found that Slc39a8-null mice exhibit typical ventricular noncompaction phenotypes associated with reduced Adamts transcription and reduced cardiac jelly degradation. Our study demonstrates that ZIP8 is crucial for ventricular trabeculation and compaction, reveals a potentially novel regulator of ventricular myocardial development, and provides a valuable model to study ventricular noncompaction.

Results

Slc39a8 is expressed in the developing heart and regulates Zn levels. By quantitative reverse transcription PCR (qRT-PCR) analysis on whole-heart lysates, we found that Slc39a8 was expressed in the developing heart. The expression peaked at E12.5 and then gradually declined to low levels in adult heart (Figure 1A). RNA in situ hybridization further verified the expression of Slc39a8 in E12.5 heart (Figure 1B). Further qRT-PCR analysis on single-cell populations isolated by fluorescence-activated cell sorting (FACS) demonstrated that Slc39a8 was expressed in cardiac endothelial cells of E12.5 hearts (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI96993DS1). To study the role of Slc39a8 in cardiac ventricular morphogenesis, we generated Slc39a8–/– mice by removing the third exon of the Slc39a8 gene (26). Slc39a8 mRNA was efficiently deleted in the Slc39a8–/– hearts (Figure 1C). Consistent with the function of ZIP8 to promote cellular Zn uptake (24), Zn levels were lower in E14.5 Slc39a8–/– hearts compared with Slc39a8+/+ hearts (P = 0.06) (Figure 1D).

Slc39a8 deletion results in hypertrabeculation and noncompaction. Slc39a8–/– mice appeared phenotypically indistinguishable from Slc39a8+/+ mice. Slc39a8–/– mice were fertile and had normal life spans. Genotyping of Slc39a8–/– breeding progenies at different embryonic time points and at weaning revealed that Slc39a8–/– mice were embryonic lethal by E16.5 (Supplemental Table 1). Histological analysis revealed that compared with Slc39a8+/+ hearts, Slc39a8–/– hearts exhibited hypertrabeculation and noncompaction phenotypes including excessive trabeculae (+24.9%, P < 0.01 for E12.5 and +44.2%, P < 0.05 for E14.5) and thin compact myocardium (−53.9%, P < 0.001 for E12.5 and −72.7%, P < 0.001 for E14.5), which are the hallmarks of LVNC (Figure 2A and Supplemental Figure 2). These phenotypes were evident at E12.5 and prominent at E14.5, and Slc39a8–/– embryos that survived until E16.5 had an even stronger phenotype (Supplemental Figure 3). We also observed ventricular septal defects (VSDs) in Slc39a8–/– hearts (Figure 2A). Some E14.5 Slc39a8–/– embryos exhibited body edema, suggesting that cardiac muscle function was compromised. Further analysis by both in situ hybridization and qRT-PCR demonstrated that Slc39a8–/– hearts had elevated expression of Bmp10, a cytokine that is an established trabecular marker, and which is known to promote cell proliferation during trabeculation (Figure 2, B and C, and ref. 4). In agreement with this, BrdU immunostaining demonstrated that cardiomyocyte proliferation was significantly increased in E12.5 Slc39a8–/– hearts compared with that of Slc39a8+/+ hearts (Figure 2D), while cell death was similar between groups at the same stage (Supplemental Figure 4).

Slc39a8 deletion leads to decreased expression of Adamts metalloproteinases and impaired cardiac ECM degradation. To understand the potential molecular mechanisms by which Slc39a8 regulates ventricular trabeculation and compaction, we performed a microarray analysis with E12.5 Slc39a8+/+ and Slc39a8–/– hearts. Interestingly, multiple Adamts metalloproteinases, including Adamts5, -7, -15, and -19, were found to be down-regulated in Slc39a8+/+ hearts (Supplemental Figure 5A). These findings were further verified by qRT-PCR analysis, which showed a 31% decrease of Adamts1 (P = 0.001), 50% decrease...
of Adams5 (P < 0.001), 30% decrease of Adams7 (P = 0.013), 40% decrease of Adams15 (P = 0.003), and 48% decrease of Adams19 (P < 0.001) in Slc39a8<sup>−/−</sup> hearts compared with Slc39a8<sup>+/+</sup> hearts (Figure 3A). Adams metalloproteinases are key proteinases that mediate cardiac ECM degradation (5, 19). Consistent with this, the ECM pathway was the top downregulated pathway in our GO analysis of the microarray results (Supplemental Figure 5B). We therefore examined ECM by Alcian blue staining, which detects major cardiac ECM components including hyaluronan and versican (18, 19). Consistent with decreased expression of Adams metalloproteinases, the intensity of Alcian blue staining was substantially increased in E12.5 Slc39a8<sup>−/−</sup> hearts compared with Slc39a8<sup>+/+</sup> hearts (Figure 3B). Furthermore, immunofluorescence (IF) staining demonstrated...
SLC39A8 knockdown in HUVECs results in decreased ADAMTS1 expression and MTF1 transcriptional activity. ADAMTS metalloproteinases are primarily expressed in endocardial cells in the developing heart, where ZIP8 is also expressed. To further explore the potential molecular mechanism by which ZIP8 regulates ADAMTS expression, we knocked down SLC39A8 in human umbilical vein endothelial cells (HUVECs) using siRNA. Western blot analysis confirmed effective knockdown of SLC39A8 (Figure 4A). Consistent with decreased Adamts in embryonic Slc39a8–/– hearts, SLC39A8 knockdown resulted in decreased ADAMTS1 expression in HUVECs (~49%, P < 0.001) (Figure 4C). It has been reported that ZIP8 regulates Adamts expression by modulating cellular Zn uptake and subsequently Mtf1 transcriptional activity during osteoarthritis (25). Consistent with this model and the in vivo observation of reduced Zn in Slc39a8–/– embryonic hearts, SLC39A8 knockdown led to reduced cellular Zn uptake (~38%, P = 0.0002) (Figure 4B).
Ventricular noncompaction is a cardiomyopathy that can develop severe complications over time. Excessive cardiac jelly degradation leads to hypotrabeculation, while impaired cardiac jelly degradation results in hypertrabeculation and noncompaction. While the amount of cardiac jelly is clearly both important and under dynamic temporal control, it is not well understood how its degradation is regulated as myocardial morphogenesis progresses. In this study, we demonstrated that \textit{Slc39a8} \textit{–/–} mice developed ventricular hypertrabeculation and noncompaction and impaired cardiac jelly degradation as a result of decreased \textit{Adamts} expression. Consistently, knocking down \textit{SLC39A8} in HUVECs decreased \textit{ADAMTS1} expression by reducing cellular Zn uptake and MTF1 transcriptional activity.

To test whether reduced Zn influx contributes to the reduction of \textit{ADAMTS1} expression by \textit{SLC39A8} knockdown, we treated HUVECs with TPEN, a Zn-specific metal ion chelator (27). Interestingly, TPEN treatment also resulted in reduced \textit{ADAMTS1} expression (–28%, \(P = 0.009\)). Conversely, ZnCl\textsubscript{2} treatment increased \textit{ADAMTS1} expression (+43%, \(P = 0.003\)) (Figure 4C). Lastly, we sought to understand whether \textit{SLC39A8} regulates the expression of \textit{ADAMTS1} through modulating MTF1 transcriptional activity by Zn influx. We examined MTF1 transcriptional activity using a reporter construct in which firefly luciferase reporter gene expression is under the control of an MTF1 transcriptional response element (ref. 27 and Figure 4D). Both \textit{SLC39A8} knockdown and TPEN treatment resulted in significantly decreased MTF1 transcriptional activity (–39%, \(P = 0.05\) and –74%, \(P = 0.001\)), while ZnCl\textsubscript{2} treatment greatly enhanced MTF1 transcriptional activity (4.37-fold, \(P = 0.05\)) (Figure 4E). Taken together, these results strongly suggest that \textit{SLC39A8} regulates \textit{ADAMTS1} expression by modulating cellular Zn uptake and consequently MTF1 transcriptional activity.

**Discussion**

Ventricular noncompaction is a cardiomyopathy that can develop severe complications over time. Excessive cardiac jelly degradation leads to hypotrabeculation, while impaired cardiac jelly degradation results in hypertrabeculation and noncompaction. While the amount of cardiac jelly is clearly both important and under dynamic temporal control, it is not well understood how its degradation is regulated as myocardial morphogenesis progresses. In this study, we demonstrated that \textit{Slc39a8}\textsuperscript{–/–} mice developed ventricular hypertrabeculation and noncompaction and impaired cardiac jelly degradation as a result of decreased \textit{Adams} metalloproteinase expression. Consistently, knocking down \textit{SLC39A8} in HUVECs decreased \textit{ADAMTS1} expression by reducing cellular Zn uptake and MTF1 transcriptional activity.

\textit{Slc39a8} hypomorphic mice have been reported to exhibit diminished Zn uptake, multiple-organ hypoplasia, anemia, and perinatal death, although it was not reported whether there were cardiac defects in those mice (28). There were quite a few simi-
larities in phenotypes between our Slc39a8−/− mice and the Slc39a8 hypomorphic mice, such as early embryonic/neonatal lethality and reduced cellular Zn uptake. However, it does appear that the phenotypes of Slc39a8−/− mice are more severe than those of the Slc39a8 hypomorphic mice, which is likely a reflection of the degree of the loss of gene function in these 2 different lines of mice.

One key finding of our study is that deletion of Slc39a8, a Zn transporter, in mouse leads to ventricular noncompaction. LVNC is classified by the American Heart Association as a primary genetic cardiomyopathy (29). Familial occurrence is frequent, and autosomal dominant, autosomal recessive, X-linked, and maternal transmissions have been observed (30). Multiple genetic mutations have been identified in LVNC patients, but the molecular mechanism underlying LVNC remains elusive (30). Our results support the hypothesis that LVNC is a distinct genetic cardiomyopathy with a developmental basis. It establishes the causal relationship between Slc39a8 loss-of-function and LVNC in mouse and provides insight into the mechanism underlying the disease. Our study suggests that SLC39A8 might be considered as a candidate gene when screening LVNC patients for gene mutations. The clinical features of LVNC are often heterogeneous: with or without association with other heart defects or cardiomyopathy (31). Based on our current Slc39a8 genetic deletion findings, we expect that patients carrying SLC39A8 loss-of-function mutations might present typical ventricular noncompaction features, accompanied by other heart defects such as VSD and cardiomyopathy, though the severity and spectrum of the clinical presentation is likely to be affected by the nature of the SLC39A8 mutations and epigenetic factors. Our study also suggests that Zn deficiency (regardless of its cause) may be involved in the pathogenesis of LVNC. This is in agreement with previous studies showing that maternal Zn deficiency resulted in multiple forms of cardiac abnormalities including thin ventricular wall and irregular trabeculae (21, 22). Genetic testing for SLC39A8 mutations in cases of LVNC may be worth consideration.

Another key finding of our study is that the Zn/ZIP8/MTF1 axis regulates the genes encoding multiple ADAMTS metalloproteinases, and consequently impacts cardiac jelly degradation during trabeculation and compaction. It is known that Adamts metalloproteinases are repressed at the initial stage of trabeculation when cardiac jelly is required (5, 19). However, it is unknown how they are de-repressed at the end of the trabeculation stage to degrade cardiac jelly and to terminate trabeculation. In chondrocytes, the Zn/ZIP8/MTF1 axis has been reported to upregulate matrix-degrading enzymes including Adamts5 during osteoarthritis (25). We demonstrated that this regulation is conserved in endothelial cells and that it plays a potentially novel and important role in the regulation of Adams, cardiac jelly degradation, and myocardial morphogenesis. An interesting observation is that Slc39a8 expression peaked at E12.5, which matched very well with the initiation of Adamts5 de-repression, cardiac jelly degradation, and trabeculation termination (5).

Various mouse models have established a critical role of cardiac jelly in trabeculation and compaction. Although the mechanism is not completely understood, one hypothesis is that cardiac jelly defects can disrupt signaling transduction between endocardium and myocardium. Our observation of elevated Notch signaling and BMP10 in Slc39a8−/− heart provide support for this hypothesis. Further studies are required to elucidate the mechanism underlying the interaction between cardiac jelly and signaling transduction and the impact it has on myocardial morphogenesis.

Collectively, our study revealed a potentially novel gene underlying ventricular noncompaction and a regulatory pathway mediating ECM degradation during myocardial morphogenesis. It suggests that SLC39A8 may be added to the list of genes to be screened in patients with ventricular noncompaction or other diseases involving dysregulation of ECM degradation.

**Methods**

**Mice.** Mice harboring an Slc39a8 constitutive knockout allele, C57BL/6-Slc39a8tml.2 mrl, were generated and provided by Merck. Details of the design of the Slc39a8−/− mice can be found at http://www.taconic.com/mouse-model/slc39a8-ko. The constitutive knockout allele was detected using genotyping primers 2476_27 (S′-CAGGGTTTCTCAGTGTAACAGG-3′) and 2476_32 (5′-CCAATATGCCCATACACGATAGG-3′). The R26R EYFP mice, which have a loxP-flanked STOP sequence followed by the enhanced yellow fluorescent protein (EYFP) gene inserted into the Gt(Rosa)26Sn locus, were purchased from the Jackson Laboratory (stock 006148) and crossed with Tie2-Cre transgenic mice (stock 004128), also obtained from the Jackson Laboratory.

**Cell culture.** HUVECs (Lanza) were cultured in VascuLife EnGS medium (Lifeline, catalog LL-0002). To knock down SLC39A8, cells were plated at a density of 2.5 × 10^4 per well in a 12-well plate. The next day (at ~70% confluence), cells were transfected either with 10 nM Ambion Silencer Select control siRNA (Life Technologies, catalog 4390843) or SLC39A8 siRNA (Life Technologies, catalog 4392420) using RNAiMax (Life Technologies, catalog 13778075). Cells were used for experiment 8 hours after siRNA treatment. To chelate Zn, cells were treated with 1 μM N,N,N′,N′-tetakis(2-pyridylmethyl) ethylenediamine (TPEN) (Sigma-Aldrich, catalog P4413) for 24 hours. To load cells with Zn, cells were treated with ZnCl2 (25 μM) for 4 hours.

For the MTF1 reporter assay (Qiagen, catalog CCS-5033L) (25), cells were transfected with a mixture of a MTF1-responsive firefly luciferase construct and a constitutive Renilla luciferase construct (40:1) by reverse transfection using Lipofectamine 3000 (Thermo Fisher Scientific, catalog L3000015). Cells (1 × 10⁵), 120 ng reporter construct mixture, 0.12 μL P3000, and 0.2 μL Lipofectamine 3000 were used per well of a 96-well plate. To study the effect of SLC39A8 knockdown on MTF1 reporter activity, cells were transfected with MTF1 reporter constructs for 24 hours and then treated with 20 nM control siRNA or SLC39A8 siRNA using RNAiMax for 8 hours before being harvested for the MTF1 reporter assay. To study the effect of Zn chelation, cells were treated with TPEN (1 μM) 24 hours after transfection of the reporter construct and the MTF1 reporter assay was performed 24 hours later. To study the effect of Zn overload, cells were treated with ZnCl2 (25 μM) 24 hours after transfection of the reporter construct and the MTF1 reporter assay was performed 4 hours later. The MTF1 reporter assay was performed using Dual-Luciferase Reporter Assay System (Promega, catalog E1910).

**Histology, IF staining, RNA in situ hybridization, and Alcian blue staining.** Histology, IF staining, and Alcian blue staining were performed as was previously described (19). Embryos were fixed in 2% paraformaldehyde overnight, dehydrated using graded ethanol, and embedded in paraffin. Eight-micrometer-thick sections were collected. The antibodies used for IF staining were rat anti–PECAM-1 (1:500, BD PharMingen, catalog 553370), rabbit anti-versican (1:200, Millipore, catalog AB1033), rabbit anti-DPEAAE (1:200, Pierce, catalog PAI-1748A), rat anti-BrDU

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rnaseq. Total RNA was isolated using an RNeasy Mini kit. Microarray analysis was performed using Affymetrix Mouse Gene 2.0 ST chips, as previously described (33). Functional annotation was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.7 (34). The data set can be accessed via the NCBI’s gene expression omnibus (GEO GSE103673).

cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, catalog 4368814). qPCR was performed using Fast SYBR Green Mastermix (Life Technologies, catalog 4385614). A list of SYBR primers used can be found in Supplemental Table 2.

Western blot. Embryonic mouse hearts were homogenized in 100 μl PBS with protease and phosphatase inhibitors. Protein (15 μg) was separated via a NuPage SDS Page system (Invitrogen, catalog NP0322BOX). Primary antibodies used were anti-NICD1 (1:1,000, Cell Signaling Technology, catalog 4147), anti-DPEAAE (1:2,500, Thermo Fisher Scientific, catalog PA1-1748A), and anti-β-actin (1:1,000, Santa Cruz Biotechnology, catalog sc-81178). HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (1:2,500, GE Healthcare Life Sciences, catalog NA934V and NA931V) and SuperSignal West Femto Maximum Sensitivity Substrate (Life Technologies, catalog 34094) were used to visualize the signal.

Statistics. Two-tailed Student’s t tests were used to detect differences between 2 groups in all experiments unless otherwise noted. The χ2 test was performed to compare the observed genotype distribution of Slc39a8−/− breeding progenies with expected Mendelian ratios. All data represent the mean ± SD unless otherwise noted. P less than 0.05 was considered to be significant.

Study approval. All mouse experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania following guidelines described in the NIH Guide for the Care and Use of Laboratory Animals.

Author contributions WL, DL, JAE, and DJR designed experiments, analyzed the data, and wrote the manuscript. WL and DL performed the experiments. NJH supervised experiments, analyzed data, and edited the manuscript. LC, LL, and FL provided technical help.

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