A ZASP Missense Mutation, S196L, Leads to Cytoskeletal and Electrical Abnormalities in a Mouse Model of Cardiomyopathy

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Background—Dilated cardiomyopathy (DCM) is a primary disease of the heart muscle associated with sudden cardiac death secondary to ventricular tachyarrhythmias and asystole. However, the molecular pathways linking DCM to arrhythmias and sudden cardiac death are unknown. We previously identified a S196L mutation in exon 4 of LDB3-encoded ZASP in a family with DCM and sudden cardiac death. These findings led us to hypothesize that this mutation may precipitate both cytoskeletal and conduction abnormalities in vivo. Therefore, we investigated the role of the ZASP4 mutation S196L in cardiac cytoarchitecture and ion channel biology.

Methods and Results—We generated and analyzed transgenic mice with cardiac-restricted expression of the S196L mutation. We also performed cellular electrophysiological analysis on isolated S196L cardiomyocytes and protein-protein interaction studies. Ten month-old S196L mice developed hemodynamic dysfunction consistent with DCM, whereas 3-month-old S196L mice presented with cardiac conduction defects and atrioventricular block. Electrophysiological analysis on isolated S196L cardiomyocytes demonstrated that the L-type Ca\(^{2+}\) currents and Na\(^{+}\) currents were altered. The pull-down assay demonstrated that ZASP4 complexes with both calcium (Ca\(_{1.2}\)) and sodium (Na\(_{1.5}\)) channels.

Conclusions—Our findings provide new insight into the mechanisms by which mutations of a structural/cytoskeletal protein, such as ZASP, lead to cardiac functional and electric abnormalities. This work represents a novel framework to understand the development of conduction defects and arrhythmias in subjects with cardiomyopathies, including DCM. (Circ Arrhythm Electrophysiol. 2010;3:646-656.)

Key Words: ZASP • Ca\(_{1.2}\) • ACTN2 • telethonin • Na\(_{1.5}\) • DCM • arrhythmias • conduction

Dilated cardiomyopathy (DCM) is the most frequent form of primary cardiomyopathy and one of the leading causes of heart failure. DCM is associated with increased risk of sudden cardiac death (SCD), which accounts for approximately 325,000 deaths per year in the United States alone.\(^1\) Overall, DCM accounts for >10% of all SCD cases and has a 1-year mortality rate of up to 10% to 50%, and approximately 30% to 50% of these deaths are ventricular tachyarrhythmia–mediated SCD.\(^1\) However, severe bradyarrhythmias, such as asystole and conduction blocks, also contribute to mortality, especially in patients with advanced left ventricular (LV) dysfunction. Replacement of deranged myocardium by fibrous tissues has been proposed as a likely mechanism of reentrant tachycardia and/or conduction disease, but very little is known about the cellular mechanisms connecting cytoskeletal alterations to arrhythmias in DCM.\(^2\)

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The LDB3-encoded human ZASP is a sarcomeric cytoskeletal protein that maintains the structural integrity of the

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Z-line.\(^3\) Alternative splicing of the LDB3 gene transcript leads to multiple ZASP isoforms, which contain several domains. The PDZ (PDZK1,DlgA, and z-01) domain is common to all ZASP isoforms, whereas the ZASP-like Motif (ZM), encoded by exons 4 and 6/7 (ZM4 and ZM6), and the 3 C-terminal LIM (Lin11, Isl-1 and Mec-3) domains are isoform-specific.\(^3\) ZASP and its murine homolog Cypher present at least 6 isoforms, including ZASP4 in humans and ZASP4 in mice, which contain exon 4.\(^3\) Interestingly, ZASP4 and Cypher1c are highly homologous, cardiac-specific, and abundantly expressed in human and murine hearts, respectively.\(^3\)

Previous research showed that germline and cardiomyocyte-restricted ablation of Cypher precipitated severe DCM, Z-line derangement and premature death within the first weeks of life secondary to both congestive heart failure and arrhythmic SCD.\(^5\) More recently, we demonstrated a family with DCM secondary to a novel missense mutation, S196L, in ZASP4 (S196L).\(^3\) S196 resides in the cardiac-specific exon 4. Intriguingly, the proband presented with severe ventricular dysfunction and LV chamber dilation, whereas a brother died with precardiomypathic SCD. These findings suggest that further investigations of ZASP4 biology will provide key insights on how cytoskeletal alterations lead to arrhythmias in patients with DCM.

We hypothesized that S196L underlies both cardiomyopathy and arrhythmias caused by cytoskeletal perturbation for the former and acquired ion channel dysfunction for the latter. To test our hypothesis in vivo, we generated transgenic mice with the murine cardiomyocyte-specific α-myosin promoter driving the expression of wild-type ZASP4 (WT) or S196L. We reasoned that this approach would be well tolerated because ZASP4 is highly homologous to the murine Cypher isoform (Cypher1c) while maintaining the functional properties of the human protein in vivo. We selected the α-MHC promoter because of its cardiac-restricted expression pattern and to avoid embryonic lethality due to germline ablation of Cypher.\(^5\) Our results show that S196L is associated with hemodynamic impairment and ion channel dysfunction and suggest, for the first time, to our knowledge, that ZASP4 is a key molecule linking cytoskeletal alterations to rhythm disturbances in DCM patients.

**Methods**

**Plasmid Construction and Generation of Transgenic Mice**

The full-length ZASP4-WT 2.184 bp cDNA sequence was amplified using human cardiomyocyte cDNA as a template and cloned into a vector containing the mouse α-myosin heavy chain (α-MHC) promoter and conjugated with 6His-V5 tag. The S196L mutation was generated using the following mutagenesis primers: forward, CCT-GCCGGGCTTGAGCCAGG; reverse, CTCGGCTGGCT-CAAGCCCGGCAG; following the Quikchange site-Directed Mutagenesis protocol from Stratagene. The linearized plasmids of both ZASP4-WT and S196L DNA were injected into fertilized oocytes (2-cell blastocyst stage) derived from C57BL/6j mice, and then the oocytes were transferred into the oviducts of pseudopregnant FVB mice. These were performed by the Transgenic and Homologous Recombination Core Facility of Baylor College of Medicine. The α-MHC promoter is the most used and worldwide accepted system to provide cardiac-restricted expression in mice hearts. Transgenic mice were identified by polymerase chain reaction.

**Echocardiography Assessment of Cardiac Function**

A commercial high-resolution ultrasonic system (Vevo 770, VisualSonics, Toronto, Ontario, Canada) equipped with a 30-MHz mechanical probe was used for echocardiography. The mice were placed on a warming platform in a supine position and were anesthetized by mask inhalation of isoflurane, and ECG limb electrodes were placed. The chests were cleaned with a chemical hair remover to minimize ultrasound attenuation. Aquasonic 100 gel (Parker Laboratories) was applied to the thorax surface to optimize visibility of the cardiac chambers. The heart was first imaged in the 2D mode in the parasternal long-axis view. From this view, an M-mode cursor was positioned perpendicular to the interventricular septum and posterior wall of the LV at the level of the papillary muscles, and M-mode images were obtained for off-line measurement of wall thickness and chamber dimensions. IVSDs (interventricular septum diameter in systole), IVSDD (interventricular septum diameter in diastole), LVP-WDs (left ventricular posterior wall diameter in systole), LVPWWd (left ventricular posterior wall diameter in diastole), LVDs (left ventricle diameter in systole), and LVDd (left ventricle diameter in diastole) were measured individually 3 times during different cardiac cycles from M-mode images and the average value of the above indices was obtained. According to the following equations, FS, LV mass, and EF were calculated: FS=(LVVDd−LVDs)/LVDd×100% ; EF=(LVDd3−LVDs3)/LVDd3×100% ; LV mass=1.04[(IVSDD+ \(LVPWdD\dagger−LVDd\dagger)\]

**MRI Assessment of Cardiac Function**

MRI images were acquired using the Bruker Biospin 7T horizontal MR scanner (Ettlingen, Germany). We sedated the animals for the procedure using isoflurane and we monitored heart rate, respiratory rate, and body temperature continuously throughout the study. Both ECG and respiratory-triggered cine gradient echo images were acquired using the short-axis and 4-chamber geometries. Fourteen to 18 images were acquired for each cardiac cycle depending on the heart rate. Images were acquired using a FOV of 3.4 cm\(^2\), slice thickness of 1 mm, and an in-plane resolution of 135 μm. Usually, 10 to 12 images are required for a typical volumetric image set covering both ventricles. Custom designed software for standard planimetry techniques (ImageJ, National Institutes of Health, Bethesda, Md) was used to analyze off-line images. Both right and left ventricular endocardial and epicardial contours were drawn on each of the 10 to 12 short-axis slices, and the contour data were used to calculate the volumes.

The right ventricular thickening was calculated by first drawing 5 radial bisectors from the endocardial to the epicardial contour of the right ventricle. These were drawn in both diastole and systole. The thickening is calculated as the [(thickness (systole)− thickness (diastole))]/thickness (diastole). The thickening reported for each animal is the average thickening of the 5 radii.

**ECG Measurements**

The nontransgenic littermate controls (NTG), WT, and S196L mice underwent telemetric ECG recordings beginning at 4 months of age. A radiotelemetry transmitter (Model EA-F20, Data Sciences International, St Paul, Minn) was surgically inserted into the peritoneal cavity, with one lead subcutaneously placed at the right shoulder and the other placed at the lower left abdomen. Before recording, mice were allowed to recover for 2 to 3 days, followed by 1 week of recording. The ECG signals were acquired and stored on Dell Optiplex 280 computer using Dataquest A.R.T. system (Data Science International). Tracings were analyzed for heart rate, PR and QRS intervals using Igor software (WaveMetrics, Lake Oswego, Ore).

**Cell Isolation and Electrophysiological Analysis**

Mice ventricular myocytes were isolated from 4- to 5-month-old NTG, WT, and S196L mice using a nonperfused cardiomyocyte isolation kit (Cellutron Life Technology, Baltimore, Md). Briefly,
Figure 1. Echocardiogram and cardiac MRI analysis of 10-month-old mice. A, Echocardiographic evaluations of heart function using M-mode echocardiography of 10-month-old mice, showing a significant reduction in LV systolic function in S196L mice compared with WT and NTG mice. B, Echocardiographic measurements demonstrate significant reduction in fractional shortening [FS% (NTG = 46.17, IQR = 49.9; 38.5); WT = 42.72, IQR = 52.7; 39.1); S196L = 24.25, IQR = 27.1; 21.0); *P = 0.000003 for S196L versus all other groups;
hearts were harvested, and, after removal of the atria, the ventricles were subjected to washing buffer and then treated with digesting buffer. The digested cells were plated in a Petri dish for 2 hours to exclude fibroblasts. The remaining nonadherent cardiomyocytes were used for the patch-clamp experiments.

Whole-cell configuration of the voltage-clamp technique was used to record sodium (\(I_{\text{Na}}\)) and L-type calcium currents (\(I_{\text{Ca,L}}\)) as previously described.\(^9\) The glass pipette electrodes were made from Corning 7056 glass capillaries (Warner Instrument, Hammed, Conn) with a pipette resistance of 2–3 MΩ/2L in the bath solution. Whole-cell recordings were carried out at ambient temperature (22°C–25°C) for \(I_{\text{Na}}\) and at 36°C for \(I_{\text{Ca,L}}\). Step-pulse voltages were generated with Axopatch 200B amplifier using Pclamp 9 software (Molecular Device, Sunnyvale, Calif). The recorded currents were filtered using a built-in 4-pole Bessel low-pass filter (cutoff frequency: 10 kHz for \(I_{\text{Na}}\) and 2 kHz for \(I_{\text{Ca,L}}\)) and then digitized at 20 and 5 kHz, respectively. The pipette solution to record \(I_{\text{Na}}\) contained (mM): 5 NaF, 115 CsF, 20 CsCl, 10 EGTA, and 10 HEPES (pH 7.35 with NaOH). Bath solution contained (mM): 5 NaCl, 1.0 MgCl\(_2\), 130 tetraethylammonium (TEA) chloride, 5 CsCl, 10 HEPES, and 10 glucose (pH 7.35 with NaOH). For the \(I_{\text{Na}}\) recordings, intracellular pipette contained (mM): 85 Aspartate, 20 TEACl\(_2\), 2 MgCl\(_2\), 10 EGTA, 10 HEPES, 5 Mg-ATP, and 5 Na\(_2\)GTP (pH 7.2 with KOH). Extracellular buffer contained (mM): 135 NaCl, 1 MgCl\(_2\), 1.8 CaCl\(_2\), 5.4 KCl, 10 HEPES, and 10 glucose (pH 7.35 with NaOH). Whole-cell currents amplitude and gating kinetics were analyzed using Clampfit (Axon Instruments, Sunnyvale, Calif) and Igor software (WaveMetics, Lake Oswego, Ore).

Cardiomyocyte Contraction Assessment

Cardiomyocytes were isolated from the LV free wall of Langendorff-perfused hearts. Mice were anesthetized with ketamine and xylazine and anaesthetized with heparin (50 U ip). Hearts were excised rapidly, cannulated, and perfused at 37°C with modified Krebs-Henseleit solution. Each heart was perfused at a constant hydrostatic pressure resulting in a steady-state flow rate in the range of 2 to 3 ml/min for 3 to 5 minutes and then perfused with additional collagenase (0.5 mg/ml, Worthington type 2) and 0.05 mmol/L Ca\(^2+\). After a 50% to 100% increase of flow rate, the ventricle was excised and cut into small pieces in the same digestion buffer and swirled gently for 3 to 5 minutes to acquire isolated cardiomyocytes. Whole-cell currents were filtered using high-pass filter INa contained (mmol): 5 NaF, 115 CsF, 20 CsCl, 10 EGTA, and 10 HEPES (pH 7.35 with NaOH). Whole-cell currents amplitude and gating kinetics were analyzed using Clampfit (Axon Instruments, Sunnyvale, Calif) and Igor software (WaveMetics, Lake Oswego, Ore).

Histology and Immunohistochemistry

Mouse hearts were isolated and continuously perfused with cardioplegic solution containing KCl 3.73 g/L, and 10% neutralized formalin (Sigma-Aldrich) or frozen in liquid nitrogen and stored in –80°C. Perfused hearts were paraffin-embedded, and 5-μm sections were stained with hematoxylin and eosin or Masson trichrome. For cryosections (5 μm), mouse hearts were equilibrated in 30% sucrose solution and mounted in O.C.T. (VWR). Sections were fixed with acetone at –20°C for 10 minutes, followed immunofluorescence staining with primary antibodies (anti-V5-ZASP4 and anti-Na\(_{\text{L}}\) 1.5 or Ca\(_{\text{L}}\) 1.2 from Alomone, Jerusalem, Israel) for 1 hour and incubation with corresponding secondary antibodies conjugated with FITC Alexa Flour 488 or Texas Red (Molecular Probes, Invitrogen) for 30 minutes. In addition, heart sections from NTG mice were stained with an anti-ZASP monoclonal antibody generated by Dr Faulkner and recognizing the endogenous Cypher1c homolog, as previously described.\(^4\) Sections were mounted with prolonged gold antifade reagent (Molecular Probes, Invitrogen) and visualized using an Olympus BX51 microscope.

In Vitro Interaction Pull-Down Assay

Interactions between ZASP4 and Na\(_{\text{L}}\) 1.5 as well as between ZASP4 and Ca\(_{\text{L}}\) 1.2 were studied with His-tagged pull-down assays (ProFound pull-down PolyHis Protein: Protein Interaction Kit; Pierce). To purify WT and S196L, protein, Escherichia coli cells were grown and harvested 24 hours after transformation with ZASP4 WT-pcDNA 3.1/V5-His and S196L-pcDNA 3.1/V5-His plasmids. The bacterial pellet was incubated for 30 minutes with ProFound lysis buffer (Pierce) containing protease inhibitor cocktail (Sigma), and centrifuged at 12,000 g for 5 minutes to clarify the crude E. coli lysate. The supernatant (300 μL) containing wt (His) or S196L (His) was then incubated with immobilized cobalt chelate (Pierce) in a spin column at 4°C for 2 hours. After washing 5 times, the immobilized His-tagged ZASP4 WT or S196L protein was incubated at 4°C for 4 hours with the lysates from neonatal rat myocardium. After extensive washing with washing buffer (Pierce), the proteins were eluted in elution buffer (washing buffer with 290 mmol/L imidazole) and the eluted proteins were separated by SDS-PAGE, and stained with antibodies to the voltage gated pan-sodium channel (Sigma-Aldrich, St Louis, Mo) and Ca\(_{\text{L}}\) 1.2 (Alomone, Jerusalem, Israel). The pcDNA3.1/V5-His-Topo/IacZ plasmid (Invitrogen Co) was used as the negative control.

Electron Microscopy Analysis

Once anesthetized, mice hearts were excised immediately and cut into small pieces, then fixed in 3% glutaraldehyde solution and processed according to standard protocol in the Integrated Microscopy Core at Baylor College of Medicine. Images were taken using Hitachi H-7500 Transmission Electron Microscope.

Statistics

Comparison of the data were done using the Kruskal-Wallis test or the Mann-Whitney-Wilcoxon test when appropriate, with P<0.05 considered statistically significant. All the data in the text and Figure are expressed as median along with the interquartile range (IQR) unless otherwise stated.

Results

S196L Overexpression Precipitates Hemodynamic Dysfunction

We established 2 separate transgenic mouse lines for both WT and S196L to rule out secondary phenotype effects caused by the random transgene insertion. Expression of the ZASP4-V5 fusion protein generated by the transgene was

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Figure 1 (Continued). \(P=0.94\) for WT versus NTG, and ejection fraction [EF%] [NTG=76.95, IQR=[80.8; 69.6]; WT=74.46, IQR=[84.9; 69.2]; S196L=48.93, IQR=[54.3; 43.6]; \(P=0.00001\) for S196L versus all other groups; \(P=0.83\) for WT versus NTG]. C, Representative short-axis MRI images of 10-month-old NTG, WT and S196L mice (n=3 per group) showing the dilated and thin LV wall in S196L mice. D, MRI measurements demonstrate that S196L mice have significantly increased LV end-diastolic volume (LVEDV) [NTG=48.93, IQR=[54.3; 43.6]; \(P=0.0001\) for S196L versus all other groups; \(P=0.94\) for WT versus NTG] and decreased LV ejection fraction (EF) [NTG=61.65, IQR=[67.9; 52.9]; WT=65.5, IQR=[68.3; 55.6]; S196L=46.4, IQR=[48.9; 42.9]; \(P=0.0022\) for S196L versus all other groups; \(P=0.58\) for WT versus NTG].
Figure 2. Cardiac gross anatomy and histological analysis in S196L mice. A, Hematoxylin and eosin staining from serial myocardial sections from at least 3 mice per group showing gross morphology and 4-chamber coronal section of 10-month-old mouse hearts demonstrates the dilation of the LV. B, LV mass does not show statistical significant difference between the three groups [(NTG=66.45, IQR=(72.9; 62.3); WT=63.4, IQR=(70.6; 60.0); S196L=67.2, IQR=(71.2; 63.2); P=0.57 for S196L versus all other groups]. C, Histological analyses of hearts from ZASP4 transgenic mice show mild cardiac fibrosis in S196L mice. Cardiac sections from 10-month-old NTG, WT, and S196L mice were stained with Masson trichrome (×200 and ×400). Note the mild fibrous tissue present in S196L mice, which was stained blue.
confirmed by immunohistochemistry and Western blot using the anti–V5 antibody, which discriminates the expression of the human ZASP4 isoform from the endogenous Cypher homolog (Supplemental Figure 1). We selected lines WT-41, S196L-93, and S196L-94, in which the mutated transgene expressed 4 to 6 times the amount of WT protein. Supplemental Figure 1 shows the confocal microscopy analysis of immunohistochemistry using the anti-V5 antibody, which demonstrates that the transgene localized at the Z-line and colocalized with the Z-line marker ACTN2.

The mendelian frequency of newborns WT and S196L transgenic male and female mice was normal, and the lifespan of both genotypes was similar. Growth of NTG, WT, and S196L did not differ, although the latter appeared less active.

Echocardiographic analysis of NTG (n=3), WT (n=4), and S196L (n=6) mice was performed at 4, 7, and 10 months. At 4 and 7 months of age, WT and S196L mice showed no significant difference in cardiac size and systolic function compared with NTG (data not shown), whereas at 10 months, S196L mice developed cardiac dilation and decreased fractional shortening (FS) and ejection fraction (EF) compared with WT and NTG (Figure 1A–B). MRI (at 10 months) confirmed that S196L showed LV end-diastolic dilation and decreased LV systolic function (Figure 1C-D). Although WT mice showed a slightly increased LVEF and decreased LVEDV compared with NTG, these differences were not statistically significant (Figure 1C and 1D). These data show that S196L overexpression in mice leads to severe cardiac dysfunction.

Figure 3. Ultrastructural analysis and contractile function in NTG, WT, and S196L mice. A, Electron microscopic images of cardiac sections from NTG, WT, and S196L mice are shown. Note the altered sarcomeric and Z-band structure in S196L mice. Arrows indicate the Z-bands. B, Sarcomeric shortening was performed at both baseline and after isoproterenol infusion showing statistical significant difference in S196L mice cardiomyocytes contractile function compared with NTG and WT myocardial cells after ISO infusion (NTG baseline=9.24, IQR=(11.3; 6.2); NTG ISO=17.9, IQR=(21.2; 15.8); WT baseline=9.28, IQR=(13.5; 6.5); WT ISO=19.65, IQR=(23.75; 14.7); S196L baseline=8.65, IQR=(11.8; 7.2); S196L ISO=16.13, IQR=(20.2; 11.7); P=0.84 for S196L versus NTG and WT at baseline; and P=0.034 for S196L versus WT after ISO treatment).

S196L and Abnormal Cardiac Morphology and Histology

Histological studies using hematoxylin and eosin staining from serial myocardial sections demonstrated abnormal gross anatomy in 10-month-old S196L mice with LV dilation (Figure 2A), with no statistical difference in cardiac mass measurements acquired by MRI in NTG, WT, and S196L mice (Figure 2B). Masson trichrome staining denoted mild focal fibrosis in ventricular myocardium of the10-month-old S196L mice compared with NTG and WT mice (Figure 2C). These data suggest that ZASP4 mutations are responsible for altering the cytoarchitecture of the working myocardium.
**S196L Impairs Cardiomyocyte Sarcomeric Contraction**

Electron microscopy analysis revealed severe sarcomere derangement and Z-band disorganization in the myocardium of the 10-month-old S196L mice (Figure 3A). We reasoned that contractile function may be impaired in these mice and assessed it by studying isolated cardiomyocytes from 10-month-old NTG, WT, and S196L mice at baseline and on stimulation by isoproterenol, as previously published. The percentage of sarcomere shortening was reduced significantly in S196L cardiomyocytes compared with NTG and WT, suggesting an impaired contractile function. This abnormality was not compensated by sympathetic stimulation, as assessed by isoproterenol infusion (Figure 3B). Our data provide an ultrastructural basis for the abnormal morphology and histology associated with ZASP4 mutation and demonstrates that these defects are severe enough to lead to functional abnormalities in affected hearts. In addition, these findings suggest that the functional cardiac derangements associated with mutation of ZASP4 are persistent in different pathophysiological conditions.

**ECG Abnormalities in S196L Mice Precede Hemodynamic Changes**

The ECG limb electrodes, used when we performed the echocardiogram on the 10-month-old NTG, WT, and S196L mice, detected an altered ECG pattern in S196L mice when compared with WT animals (Supplemental Figure 2). Therefore, we reasoned that ZASP may represent an important biological factor linking cardiac functional and electric derangements and that ECG abnormalities may also occur before the development of overt hemodynamic dysfunction. To test this hypothesis, we performed ECG studies on conscious 4-month-old NTG, WT, and S196L mice (n=6 per group) (Figure 4A and Table 1). The S196L mice demonstrated slower heart rate and prolonged PR and QRS intervals compared with the NTG and WT mice (Figure 4A and Table 1). These abnormalities represent a common finding in patients with DCM, and represent a likely substrate for the development of severe tachyarrhythmias and bradyarrhythmias in this disease. These data show that ZASP is a key factor connecting cytoskeletal and electric myocardial derangements in the working heart.

**S196L Alters Both L-Type Calcium Currents and Sodium Currents in Murine Cardiomyocytes**

To investigate how the S196L mutation leads to conduction disturbances, we focused our electrophysiological studies on L-type calcium currents (I_{Ca,L}) and sodium currents (I_{Na}) because they represent the major currents involved in cardiac conduction and depolarization (I_{Ca,L} and I_{Na}) and excitation-contraction coupling (I_{Ca,L}). In addition, previous reports...
demonstrate that the ZASP-binding proteins α-actinin-2 (ACNT2) directly binds and modulates Cav1.2,10 whereas Na1.5 binds to and is regulated by the Z-line protein Telethonin/T-Cap.9 Patch-clamp studies were carried out on both ICa,L and INa currents in isolated cardiomyocytes from 4- to 5-month-old NTG, WT, and S196L mouse hearts before the onset of hemodynamic dysfunction. Figure 4B-a shows that ZASP-binding proteins both ICa,L and INa currents in isolated cardiomyocytes from 4- to 5-month-old NTG, WT, and S196L mouse hearts demonstrated that both WT and S196L shifted the onset and peak currents of ICa,L amplitude obtained from the cardiomyocytes of WT or S196L mice. Although the peak current amplitude were similar between WT and S196L, S196L shifted the onset and peak currents of ICa,L. Table 2 summarized the biophysical parameters of ICa,L obtained from at least 5 animals per line.

Because INa has been reported to play an important role in cardiac conduction, characteristics of INa were also studied in cardiomyocytes. The threshold and peak voltage of the I-V relationships were rightward shifted by approximately 15 mV (Figure 4B-b), leading to reduced calcium channel (Cav1.2) function. Figure 4B-c demonstrates that S196L rightward-shifted the voltage dependency of the steady-state activation and inactivation of ICa,L. Table 2 summarized the biophysical parameters of ICa,L obtained from at least 5 animals per line.

Table 1. ECG Parameters Observed in NTG, WT, and S196L Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT (n=10)</th>
<th>S196L (n=11)</th>
<th>NTG (n=6)</th>
<th>P Value</th>
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<tr>
<td>PR, ms</td>
<td>35.0 [32.0–37.0]</td>
<td>40.5 [37.0–53.0]†</td>
<td>33.0 [32.0–34.0]</td>
<td>0.00049</td>
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<td>QRS, ms</td>
<td>16.0 [15.0–17.0]</td>
<td>25.5 [23.0–32.0]¶</td>
<td>16.0 [15.0–17.0]</td>
<td>0.000002</td>
</tr>
<tr>
<td>RR, ms</td>
<td>111.0 [95.0–114.0]</td>
<td>116.0 [105.0–152.0]</td>
<td>114.0 [84.0–116.0]</td>
<td>0.128</td>
</tr>
</tbody>
</table>

Data are presented as median with interquartile range; numbers in parentheses represent the number of animals.

*P<0.001 versus NTG; †P<0.05 versus WT; ¶P<0.001 versus WT and NTG.

Table 2. Gating Kinetic Parameters of Cav1.2 and Na1.5

<table>
<thead>
<tr>
<th>L-Type Ca2+ Channel</th>
<th>WT (n=11)</th>
<th>S196L (n=13)</th>
<th>NTG (n=9)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak current density, pA/pF</td>
<td>–7.7 [–9.0 to –7.2]</td>
<td>–5.2 [–7.0 to –4.7]*</td>
<td>–10.2 [–10.6 to –9.6]</td>
<td>0.00022</td>
</tr>
<tr>
<td>Activation (n=11)</td>
<td>–15.0 [–22.7 to –6.0]</td>
<td>–5.8 [–9.3 to –6.4]*</td>
<td>–15.9 [–17.1 to –11.3]</td>
<td>0.001</td>
</tr>
<tr>
<td>Vp, mV</td>
<td>5.8 [5.4–7.2]</td>
<td>7.6 [6.5–8.3]†</td>
<td>5.8 [4.8–6.2]</td>
<td>0.007</td>
</tr>
<tr>
<td>Vp, mV</td>
<td>5.8 [5.5–6.7]</td>
<td>6.0 [5.8–6.2]</td>
<td>6.8 [5.8–7.8]</td>
<td>0.486</td>
</tr>
<tr>
<td>Na2+ channel</td>
<td>WT (n=20)</td>
<td>S196L (n=17)</td>
<td>NTG (n=9)</td>
<td></td>
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<tr>
<td>Peak current density, pA/pF</td>
<td>–19.7 [–23.4 to –16.8]</td>
<td>–19.2 [–20.3 to –18.3]</td>
<td>–19.0 [–19.9 to –16.5]</td>
<td>0.534</td>
</tr>
<tr>
<td>Activation (n=9)</td>
<td>–55.3 [–56.4 to –54.9]</td>
<td>–49.2 [–51.7 to –44.9]¶</td>
<td>–53.9 [–63.7 to –52.4]</td>
<td>0.001</td>
</tr>
<tr>
<td>Vp, mV</td>
<td>3.9 [3.1–4.3]</td>
<td>5.0 [4.2–5.3]†</td>
<td>4.6 [4.3–4.9]</td>
<td>0.125</td>
</tr>
<tr>
<td>Inactivation (n=7)</td>
<td>–88.6 [–98.6 to –86.3]</td>
<td>–89.6 [–93.4 to –84.3]</td>
<td>–89.7 [–91.1 to –86.8]</td>
<td>0.879</td>
</tr>
</tbody>
</table>

Data are presented as median with interquartile range; Vp represents midpoint voltage of maximal conductance/currents; k, slope factor; numbers in parentheses indicates number of patches from >5 animals per each group.

*P<0.01 versus WT and NTG; †P<0.05 versus NTG; ¶P<0.001 versus WT and NTG.
S196L remained colocalized with Cav1.2 and Nav1.5 and that the expression of mutant ZASP did not influence either Cav1.2 or Nav1.5 localization (Figure 6A–B). These data provide a new structural basis to understand the effects of ZASP mutations on conduction disturbances in patients with DCM. Taken together, these studies demonstrate that mutation of ZASP targets 2 key ion currents in the myocardium and suggest that abnormalities of the ZASP protein underlie arrhythmias in patients with DCM.

Discussion

Defects in cytoskeletal proteins are a well-established cause of DCM, and altered ZASP represents a recent addition to the genetic heterogeneity of DCM and the complex regulation and maintenance of the contractile apparatus in striated cardiac muscle. In the present study, we used a mouse model to characterize the functional implications of the novel DCM-causing mutation in ZASP (S196L) previously identified in human subjects.3 We showed that the expression of S196L in a cardiac-restricted manner recapitulates the DCM phenotype, consistent with the clinical presentation of the proband and affected relatives we previously described.3 ECG abnormalities, such as conduction disturbances and AV block, appeared as early as 4 months, thus preceding functionally significant myocardial remodeling. We investigated the nature of these electrographic modifications and observed that S196L cardiomyocytes exhibited significant reduction in calcium influx from the L-type calcium channel Cav1.2 and a 10 mV rightward shift of the threshold and the peak voltage in the sodium channel I-V relationships. Although the Cav1.2 plays a small role in the propagation of normal cardiac tissues, its effects might increase significantly in diseased hearts when the Nav1.5 function is impaired.9,17 It is reasonable to speculate that the concomitant reduction of Ca2+ currents and impaired Nav1.5 function might explain the cardiac conduction defects of the S196L mice. More detailed

Figure 5. ZASP4 complexes with both Cav1.2 and Na1.5 in myocardial cells. Pull-down assay was performed to study the interaction between the His-tagged WT and S196L with Cav1.2 (A) and Na1.5 (B). The control was performed using E coli transformed with pcDNA3.1/V5-His-TOPO/lacZ. The expressed WT and S196L proteins extracted and purified from transformed E coli have been mixed with cell lysate of neonatal rat cardiomyocytes and detected after Western blot using antibodies recognizing the V5 (ZASP4), Cav1.2, and pan-sodium channel proteins.

Figure 6. ZASP colocalization analysis with Cav1.2 and Na1.5 in mice myocardium. A, Immunohistochemistry using antibodies against Cav1.2 (red) and V5 (green), which identifies either WT or S196L, shows the typical striated pattern of cardiac cells, whereas the merged images demonstrate their colocalization at the Z-band. B, Immunohistochemistry shows that WT and S196L colocalize also with the cardiac sodium channel Na1.5 as demonstrated in the merged image.
in silico analyses are warranted to link our biophysical data to the ECG phenotypes.

Although S196L alters $I_{\text{Na}}$ and $I_{\text{Ca-L}}$ in our mouse model, other ion channels might be affected and contribute to the observed conduction defects. For example, a recent elegant study by Ueda et al\(^{18}\) suggests that the hyperpolarization-activated channel 4 (HCN4), previously associated with sinus node dysfunction and preferentially expressed in the cells controlling the heart rate, is also expressed in the working myocardium. Genetic mutations of this channel can cause channel dysfunction, leading to conduction disturbances and ventricular arrhythmia.

Although we expressed the S196L mutation using the most significant cardiac-specific ZASP isoform background (ZASP4)\(^{3–6}\) both mice and humans express other ZASP isoforms containing exon 4, which would therefore include the S196L mutation.\(^{3–6}\) Thus, the S196L mutation in the ZASP4 isoform represents one of the mutant ZASP splice isoforms that could be affected and our findings may represent only the “tip of the iceberg” of the effects of ZASP mutations in the heart. This may explain why, contrary to what occurs in humans with DCM, we did not detect SCD in our mouse model.

To study the cardiac-specific effects of exon 4 mutation, we opted to generate transgenic mice overexpressing human ZASP4 cDNA because this transgene allows better conclusions about the role of the mutated human ZASP4 protein in vivo. In fact, despite a high degree of homology between ZASP4 and Cypher\(^1\), there are significant differences in the protein sequence and structure of the murine and human ZM4 domains. Transgenic animals have been widely used to study the effects of genetic mutations in vivo, but knock-in mice containing the mutation in the conserved residue may provide a more physiological approach and will require additional future studies.

Our data indicate that altered calcium and sodium currents precede cardiac hemodynamic and structural changes and suggest that the ECG abnormalities may result directly from the effect of S196L on a cascade pathway involving Ca\(_{\text{1.2}}\) and Na\(_{\text{1.5}}\) as the ultimate targets. These findings are consistent with the observation that several patients with “compensated” heart failure are clinically stable with no evidence of symptoms such as fluid retention and pulmonary edema. Therefore, it is plausible that the S196L mouse hearts present ECG anomalies resulting from altered ion channels during the first months of life before obvious hemodynamic alterations. These findings might also imply that the ECG may represent an early diagnostic parameter in DCM subjects carrying this ZASP mutation.

We did not find evidence that the ZASP protein and the calcium and sodium channel proteins interact directly, but our data provide the first evidence that ZASP4 is part of a protein complex with them. In addition, S196L colocalized and complexed with Ca\(_{\text{1.2}}\) and Na\(_{\text{1.5}}\), in agreement with what occurs for mutant caveolin-3 (CAV3)\(^{12,13}\) α-1-syntrophin (SNTA1)\(^{14,15}\) and T-Cap.\(^{11}\) ZASP4 could perturb Ca\(_{\text{1.2}}\) and Na\(_{\text{1.5}}\) function through other Channel Interacting Proteins (ChIPs) such as the ZASP-binding proteins ACTN2, which modulate Ca\(_{\text{1.2}}\)\(^{10,20}\) and Telethonin/T-Cap, which regulates Na\(_{\text{1.5}}\).\(^9\) We speculate that the effects of ZASP-S196L on the functions of Ca\(_{\text{1.2}}\) and Na\(_{\text{1.5}}\) are mediated by the ZASP-binding partner that physically interacts with these ion channels. This hypothesis is consistent with recent studies showing that perturbations of ChIPs may underlie the acquired disturbances of ion channels in cardiomyopathies and channelopathies.\(^{10–15,19,20}\)

In the present study, we describe that the ZASP-S196L mutation affects the function of the ion channels. These findings may partly explain how loss-of-function mutations in SCN5A precipitate the development of conduction defects in DCM.\(^{21–22}\) However, further studies dissecting the detailed mechanism of the EP abnormality in S196L cardiomyocytes are warranted.

Although we have identified ZASP-S196L in DCM, other groups observed the same mutation in hypertrophic cardiomyopathy\(^23\) and isolated noncompaction of the left ventricle.\(^24\) It has been reported that patients with LV noncompaction present with an “undulating” phenotype from dilated to hypertrophic cardiomyopathy.\(^24\) Therefore, it is possible that, in addition to the S196L mutation, other unknown genetic factors predispose human subjects to the clinical variability in their phenotypic presentation.

In conclusion, we present the first in vivo model of a ZASP-induced DCM recapitulating the clinical phenotype observed in human subjects. We present evidence that ZASP4 is part of a macromolecular complex including the ion channels Ca\(_{\text{1.2}}\) and Na\(_{\text{1.5}}\). This complex probably represents the structural basis of the electric disturbances of this DCM mouse model. Although the exact mechanism through which mutant ZASP4 alters calcium and sodium current remains elusive, we present evidence that cardiac-restricted expression of S196L is associated with cardiomyopathy and ECG abnormalities consistent with high risk of SCD.

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**Disclosures**

Dr Ackerman is a consultant for PGxHealth. Intellectual property derived from Dr Ackerman’s research program resulted in license agreements in 2004 between Mayo Clinic Health Solutions (formerly Mayo Medical Ventures) and PGxHealth (formerly Genaissance Pharmaceuticals).

**References**

Dilated cardiomyopathies are often genetic and associated with arrhythmias and sudden cardiac death. The links between genetic mutations causing DCM and arrhythmias are not well defined. We studied a mouse model for the S196L mutation of Cypher, a PDZ-LIM domain Z-line protein, associated with arrhythmias. This mutation is found in a family with dilated cardiomyopathy and sudden cardiac death. In isolated cells, the ZASP4-S196L mutation was found to affect L-type Ca2+ currents and Na+ currents. Furthermore, ZASP can form a protein complex including both calcium (Cav1.2) and sodium (Nav1.5) channels via alpha-actinin2. These findings suggest that primary mutations in any of the components of such complexes could lead to disturbances in the intertwined currents and Na+ current disruption.
A ZASP Missense Mutation, S196L, Leads to Cytoskeletal and Electrical Abnormalities in a Mouse Model of Cardiomyopathy

Zhaohui Li, Tomohiko Ai, Kaveh Samani, Yutao Xi, Huei-Ping Tzeng, Mingxing Xie, Shan Wu, Shuping Ge, Michael D. Taylor, Jian-Wen Dong, Jie Cheng, Michael J. Ackerman, Akinori Kimura, Gianfranco Sinagra, Luca Brunelli, Georgine Faulkner and Matteo Vatta

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**Supplemental methods**

**Co-Immunoprecipitation (Co-IP)**

Full-length WT and ZASP4-S196L as well as the ZM4 domain were cloned by PCR with an artificial in-frame ATG-containing primer into a V5-TOPO vector as previously described. Site-directed mutagenesis of the S196L mutation in the ZM4 motif was performed as previously described. HEK293 cells were transiently transfected with either ZM4-WT or ZM4-S196L along with full-length ACTN2-GFP or telethonin/T-cap-GFP. Twenty-four hours after the transfection, the cells were rinsed with Tris-buffered saline (TBS; 150 mM NaCl, 25 mM Tris-HCL, 5 mM EDTA, pH 7.4), and scraped in ice cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% Nonidet P-40, 1 mM EDTA with the protease and phosphatase inhibitors with final concentrations: 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin.). Gently vortex the tube for 30 seconds, rotate 30 min in cold room. The lysates were centrifuged at 10,000g for 15 min to remove insoluble debris and collection the soluble supernatant. Two mg of total lysate (450 μl) from each sample was incubated for 3 hour at 4°C with anti-GFP antibody (1 μg) then 30 μl of protein A-Agarose (Santa Cruz Biotechnology, Inc.) was added to the samples and further incubated for 2 hours at 4°C. Beads were washed 3 times with RIPA buffer and protease inhibitors and bound proteins were eluted with SDS-PAGE sample buffer by boiling for 5 min. Immune complexes were analyzed by SDS-PAGE (4-12% gradient gels, Bio-Rad) and transferred to nitrocellulose.
membranes. Nonspecific binding sites were blocked by immersion of membranes at 4°C for 30 min in PBS-Tween (0.1%, Tween-20) containing 5% (w/v) dried skim milk. Membranes were probed subsequently with anti-V5 or anti-GFP antibodies (Mouse monoclonal, Sigma,).

**Mammalian two-hybrid system**

Mammalian two-hybrid was performed following the instructions from Stratagene. (Cat No. 211344, CA, USA). Roughly, ZASP4 gene was cloned into pCMV-BD vector and SCN5A was cloned into pCMV-AD vector. The two plasmids and pFR-Luc reporter plasmid (0.25 ug) were co-transfected into HEK293 cells using lipofectamine 2000 at different concentration (0.01 ug, 0.1 ug, 0.5 ug, respectively). pBD-p53 +pAD-SV40T+ pFR-Luc reporter plasmid were used for interaction positive control. pBD-p53 +pAD-TRAF+ pFR-Luc reporter plasmid were used for interaction negative control. pBD-NF-kB+ pFR-Luc reporter plasmid were used for reporter gene activity positive control. Cells were incubated for 24 h, and then lysed by cell lysis buffer provided in the kit. Cell lysate was mixed with assay buffer and luciferase activity was measured using a luminometer. Luciferase activity is expressed in relative light units as detected by the luminometer from the sample.
Supplemental Figure legends

Supplemental Figure 1: Expression of the ZASP4-V5 transgenic product in engineered mice. Expression of the ZASP4-V5 fusion protein generated by the transgene was performed by IHC and Western Blot (WB) using an anti-V5 antibody to detect the exogenous product and discriminate it from the endogenous Cypher homolog.

Supplemental Figure 2: ECG analysis in NTG, WT and S196L mice. The ECG limb electrodes recorded electrical activity simultaneously to the echocardiogram. The ECG pattern differs in the 10 month old S196L mice when compared to WT animals.

Supplemental Figure 3: Immunohistochemistry on isolated adult murine cardiomyocytes. The immunostaining was performed on isolated cardiomyocytes from adult non-transgenic (NTG) mice to detect Cypher/ZASP, Ca_{1.2} and the cardiac sodium channel.
Supplemental Figure 2

A

WT

S196L