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# Mutant Caveolin-3 Induces Persistent Late Sodium Current and Is Associated With Long-QT Syndrome

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***Background***—Congenital long-QT syndrome (LQTS) is a primary arrhythmogenic syndrome stemming from perturbed cardiac repolarization. LQTS, which affects ≈1 in 3000 persons, is 1 of the most common causes of autopsy-negative sudden death in the young. Since the sentinel discovery of cardiac channel gene mutations in LQTS in 1995, hundreds of mutations in 8 LQTS susceptibility genes have been identified. All 8 LQTS genotypes represent primary cardiac channel defects (ie, ion channelopathy) except LQT4, which is a functional channelopathy because of mutations in ankyrin-B. Approximately 25% of LQTS remains unexplained pathogenetically. We have pursued a "final common pathway" hypothesis to elicit novel LQTS-susceptibility genes. With the recent observation that the LQT3-associated, *SCN5A*-encoded cardiac sodium channel localizes in caveolae, which are known membrane microdomains whose major component in the striated muscle is caveolin-3, we hypothesized that mutations in caveolin-3 may represent a novel pathogenetic mechanism for LQTS.

***Methods and Results***—Using polymerase chain reaction, denaturing high-performance liquid chromatography, and direct DNA sequencing, we performed open reading frame/splice site mutational analysis on *CAV3* in 905 unrelated patients referred for LQTS genetic testing. *CAV3* mutations were engineered by site-directed mutagenesis and the molecular phenotype determined by transient heterologous expression into cell lines that stably express the cardiac sodium channel $hNa_v1.5$. We identified 4 novel mutations in *CAV3*-encoded caveolin-3 that were absent in >1000 control alleles. Electrophysiological analysis of sodium current in HEK293 cells stably expressing $hNa_v1.5$ and transiently transfected with wild-type and mutant caveolin-3 demonstrated that mutant caveolin-3 results in a 2- to 3-fold increase in late sodium current compared with wild-type caveolin-3. Our observations are similar to the increased late sodium current associated with LQT3-associated *SCN5A* mutations.

***Conclusions***—The present study reports the first *CAV3* mutations in subjects with LQTS, and we provide functional data demonstrating a gain-of-function increase in late sodium current. **(*Circulation*. 2006;114:2104-2112.)**

**Key Words:** arrhythmia ■ death, sudden ■ ion channels ■ tachyarrhythmias ■ tachycardia ■ torsade de pointes

Congenital long-QT syndrome (LQTS), a potentially fatal, heritable cardiac channelopathy characterized by delayed cardiac repolarization, prolonged QT interval, and lethal ventricular arrhythmias, results in significant risk of syncope, seizures, and sudden cardiac death.[1,2] Genetic investigations demonstrate that mutations in $\alpha$ subunits of cardiac potassium and sodium channels account for ≈75% of congenital LQTS (LQT1, LQT2, and LQT3) and that ≈1% of LQTS are due to mutations involving ankyrin-B, potassium channel $\beta$ subunits, or voltage-dependent L-type calcium channel $\alpha$ 1C subunit (LQT4, LQT5, LQT6, LQT7, and LQT8).[3–9] Thus far, all known LQTS susceptibility genes encode cardiac ion channels (either $\alpha$ pore-forming subunits or auxiliary $\beta$ subunits) except LQT4, caused by *ANK2*-encoded ankyrin-B. This protein, thought to participate in localization of sodium or calcium channels to the sarcolemma and mutations, results in ion channel dysfunction caused by altered localization. The genetic defect in ≈25% of patients with LQTS remains unknown. We hypothesized that mutations in an ion channel–associated protein, which disrupts ion

## Clinical Perspective p 2112

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channel function, similar to the functional mechanism of LQT4, could precipitate LQTS. We further hypothesized that *CAV3*, which encodes caveolin-3, the major scaffolding protein present in caveolae in heart, could be such an LQTS susceptibility gene.

Caveolae are 50- to 100-nm omega-shaped microdomains of the plasmalemma, particularly abundant in cells of the cardiovascular system, including endothelial cells, smooth muscle cells, macrophages, cardiomyocytes, and fibroblasts.[10] Caveolae are involved in vesicular trafficking and serve as a platform to organize and regulate a variety of signal transduction pathways; they also play an important part in cholesterol homeostasis.[10] Caveolins are the principal proteins of caveolae. The 3 isoforms of caveolin are encoded by separate genes. Although caveolin-1 (*CAV1*) and caveolin-2 (*CAV2*) are coexpressed in most cell types, *CAV3*-encoded caveolin-3 is specifically expressed in cardiomyocytes and skeletal muscle. Some cardiac ion channels have been specifically localized to caveolae such as the *SCN5A*-encoded voltage-gated $Na^+$ channel ($hNa_v1.5$),[11] the voltage-dependent $K^+$ channel ($K_v1.5$),[12] the sodium-calcium exchanger,[13] and the L-type $Ca^{2+}$ channel.[14] In the heart, a variety of other signaling molecules have been found in caveolae, including the $\beta_2$-adrenergic receptor and associated proteins of the G-protein/adenylyl cyclase/protein kinase A pathway.[14] Therefore, caveolae can serve both to compartmentalize and to regulate ion channel function and cell signaling factors. A critical role for caveolin-3 and caveolae in cardiac excitability is likely, but so far, no evidence has been provided for their involvement in heritable arrhythmia syndromes.

We therefore sought to investigate whether *CAV3* may be implicated in LQTS. Furthermore, we investigated whether mutations in *CAV3* could alter cardiac repolarization by modulating the cardiac *SCN5A*-encoded sodium channel activity because of the previous association of this channel with caveolae and the established pathogenic link between gain-of-function *SCN5A* mutations and LQT3. We discovered that perturbations in caveolin-3 constitute a novel pathogenic mechanism for LQTS.

## Methods

### Patient Demographics

Between 1998 and 2004, 905 (571 female, 63.3%) unrelated patients (364 from Baylor College of Medicine and Texas Children's Hospital, 541 from the Mayo Clinic College of Medicine) were referred to either the Phoebe Willingham Muzzy Molecular Cardiology Laboratory at Baylor College of Medicine/Texas Children's Hospital or Mayo Clinic's Sudden Death Genomics Laboratory for LQTS genetic testing. All patients underwent physical examination, family history, and ECG analysis. The average age at diagnosis was $28\pm9$ years (range, 0 to 81 years), and the resting QTc was $480\pm40$ ms (range, 365 to 759 ms).

### *CAV3* Mutational Analysis

Blood was obtained after informed written consent was given for subjects referred for LQTS genetic testing. Genomic DNA was extracted from peripheral blood lymphocytes and lymphoblastoid cell lines, which were established as an alternative DNA source as previously described.[15] Only a minimal number of patients had DNA extracted from lymphoblastoid cell lines for characterization, and no *CAV3* mutations were identified in those samples. Using polymerase chain reaction (PCR), denaturing high-performance liquid chromatography, and direct DNA sequencing, we performed open reading frame/splice site mutational analysis on *CAV3* (chromosome 3p25, 2 exons). PCR amplification was performed using coding region flanking primers designed in our laboratory with Oligo software. Primer sequences, PCR conditions, and denaturing high-performance liquid chromatography conditions are available on request.

Mutational analyses of the entire coding regions of the LQTS-associated genes—*KCNQ1* (LQT1), *KCNH2* (LQT2), *SCN5A* (LQT3), *KCNE1* (LQT5), *KCNE2* (LQT6), and *KCNJ2* (LQT7 or ATS1)—and targeted analysis of *ANK2* (LQT4) and *RyR2* (CPVT1) were performed previously.[8,15]

All genetic variants regarded as putative LQTS-associated mutations changed a conserved residue or splice site that altered the primary amino acid structure of the encoded protein; this change must have been absent in at least 500 ethnically matched reference alleles. Control genomic DNA was obtained from the Human Genetic Cell Repository sponsored by the National Institute of General Medical Sciences and the Coriell Institute for Medical Research (Camden, NJ).

Synonymous single nucleotide polymorphisms were excluded from consideration.

### *CAV3* Cloning and Mutagenesis

The wild-type (WT) human *CAV3* 456-bp coding sequence was cloned from human cardiac cDNA by PCR using the proofreading *Pfu*$^+$ Turbo DNA polymerase (Stratagene, La Jolla, Calif) to avoid improper nucleotide incorporation using the following primers: 5′-ATGATGGCAGAAGAGCACAC-3′ (sense) and 5′-TGGTGCTGCGGAAGGAGGTC-3′ (antisense). Site-directed mutagenesis was performed with the QuikChange Site-Directed Mutagenesis Kit (Stratagene) using the vector containing the *CAV3*-WT insert as a template. The primers used for mutagenesis are available on request; PCR and bacteria transformation were performed according to manufacturer's instructions.

The mutated F97C-*CV3* and S141R-*CAV3* clones were sequenced to ensure the presence of the mutations and the absence of other substitutions introduced by the DNA polymerase.

### Mammalian Cell Transfection

WT *CAV3* and mutant *CAV3* were subcloned into the mammalian expression vector pcDNA3 (Invitrogen Corp, Carlsbad, Calif) for transient transfection in HEK293 cells stably expressing $hNa_v1.5$. The stable HEK293 cell lines expressing $hNa_v1.5$ were established as previously described.[16] The WT or mutant DNA was transiently cotransfected with green fluorescent protein (GFP) at a ratio of 1:10 into the HEK293 cell line with SuperFect from Qiagen (Valencia, Calif). DNA mixture (sample DNA plus GFP) was diluted to 150 $\mu$L with OptiMEM (Gibco, Invitrogen Corp). The 150 $\mu$L DNA and OptiMEM mixture was mixed with SuperFect at a ratio of 1:5 and incubated for 10 minutes at room temperature. After 10 minutes of incubation, 1 mL MEM-complete media was added to the DNA and SuperFect mixture for 5 hours of transfection under normal growth conditions. After 5 hours of transfection, the transfected cell was washed twice with phosphate-buffered saline (PBS) and returned to normal growth conditions with 3 mL media for 24 hours before macroscopic sodium current was measured.

### Electrophysiological Measurement

Macroscopic sodium current ($I_{Na}$) was measured at 22°C to 24°C with the standard whole-cell patch-clamp method as previously described.[17] Briefly, an Axopatch 200B amplifier and pClamp8.0 software (Axon Instruments, Foster City, Calif) were used with series resistance compensation and a low-pass filter of 5 kHz. The extracellular (bath) solution contained (in mmol/L) 140 NaCl, 4 KCl, 1.8 $CaCl_2$, 0.75 $MgCl_2$, and 5 HEPES. The pipette solution contained (in mmol/L) 120 CsF, 20 CsCl, 2 ethyleneglycoltetraacetic acid, and 5 HEPES. Standard activation, steady-state inactivation, and recov-

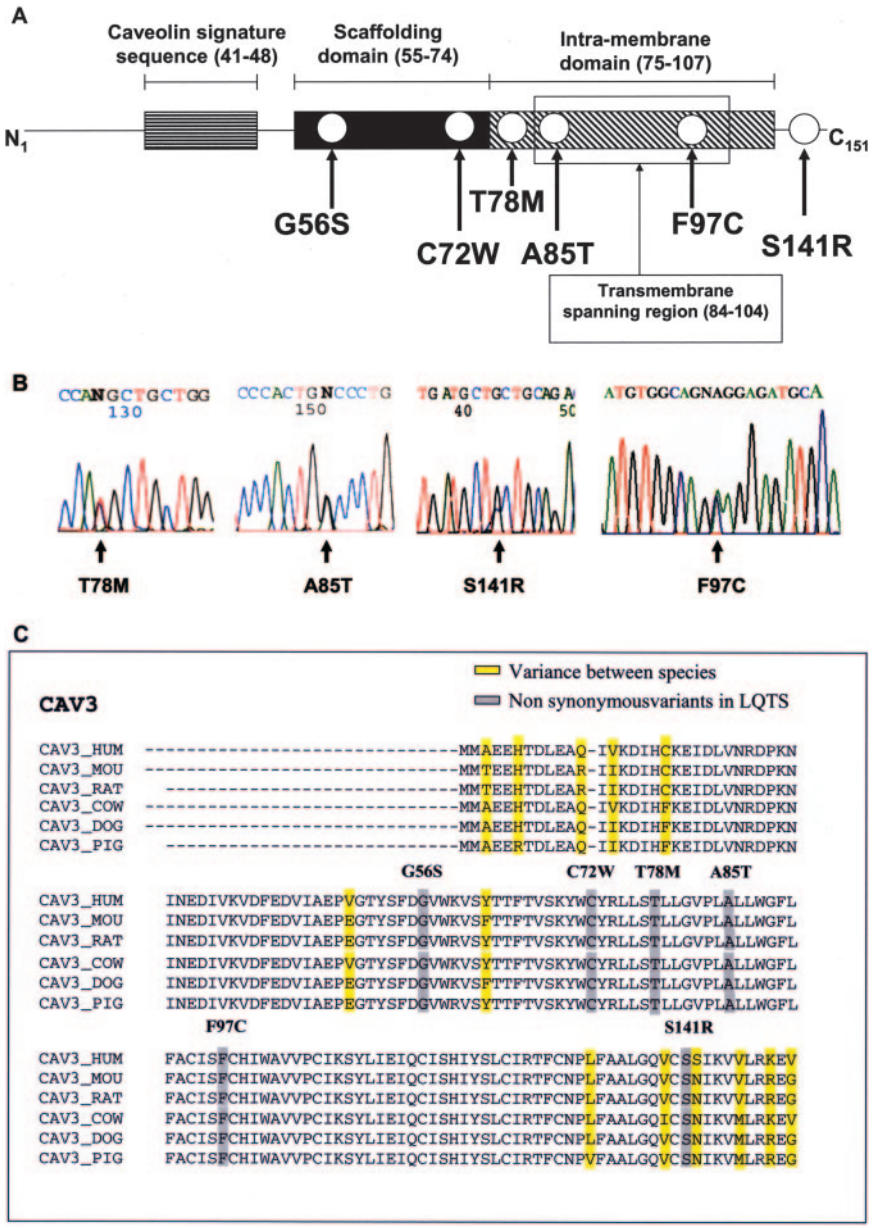


**Figure 1.** *CAV3* mutations in LQTS. Schematic representation of the linear topology of the caveolin-3 protein shows both the location of critical domains and the nonsynonymous single nucleotide polymorphisms (common and rare) identified in LQTS patients (A). Sequencing analysis showed the novel nucleotide variants leading to nonsynonymous changes, which have been identified in each patient (B), and the amino acid conservation analysis identified that all LQTS variants modified highly conserved amino acids in caveolin-3 (C).

ery protocols were performed, and the results were fit to a Boltzmann equation for activation and inactivation and recovery data were fit to an exponential equation with protocol details as previously reported.[17] Late $I_{Na}$ was measured at the end of a 775-ms depolarization from −140 to 0 mV after passive leak subtraction as previously described.[17] We have previously shown that this leak subtraction method is comparable to saxitoxin subtraction methods. Data are reported as mean±SD. The Kruskal-Wallis test was performed to determine statistical significance among multiple groups. Statistical significance was determined by a value of $P<0.05$.

## Immunoprecipitation

WT and mutant *CAV3* constructs were subcloned into a bicistronic vector (pIRESGFP, kind gift from David Johns, Johns Hopkins University) to express the desired *CAV3* construct and GFP under control of a cytomegalovirus promoter.

*SCN5A* was subcloned into pcDNA3, a mammalian expression vector (Invitrogen). Plasmids containing cDNAs encoding WT *SCN5A*, WT *CAV3*, and mutations of *CAV3* were cotransfected in the following combinations: WT *SCN5A*+WT *CAV3*, WT *SCN5A*+*CAV3*-P104L, WT *SCN5A*+*CAV3*-S141R, and WT *SCN5A*+*CAV3*-F97C in HEK293 cells using the transfection reagent FuGENE 6 (Roche Applied Science, Indianapolis, Ind), according to the manufacturer's protocol. Further details of the immunoprecipitation procedures can be found in the online-only Data Supplement.

Immune complexes were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4% to 15% gradient gels, Bio-Rad, Hercules, Calif) and transferred to polyvinylidene difluoride membranes. Nonspecific binding sites were blocked by immersion of membranes at 4°C for 30 minutes in PBS-Tween (0.1%, Tween-20) containing 5% (wt/vol) dried skim milk. Membranes were subsequently probed with an anti-hNa$_v$1.5 antibody (rabbit polyclonal, 1:200, directed against conserved segment in the intracellular III-IV loop, Upstate USA, Inc, Charlottesville, Va) and an anti–caveolin-3 antibody (1:10 000, BD Biosciences, San Diego, Calif).

## Immunohistochemistry

To evaluate the localization of *CAV3*-encoded caveolin-3 and *SCN5A*-encoded hNa$_v$1.5 in human heart, 5-$\mu$m sections from frozen right myocardial ventricles were stained with a goat-raised anti–caveolin-3 antibody and a rabbit-raised anti-hNa$_v$1.5 antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif). Each primary antibody

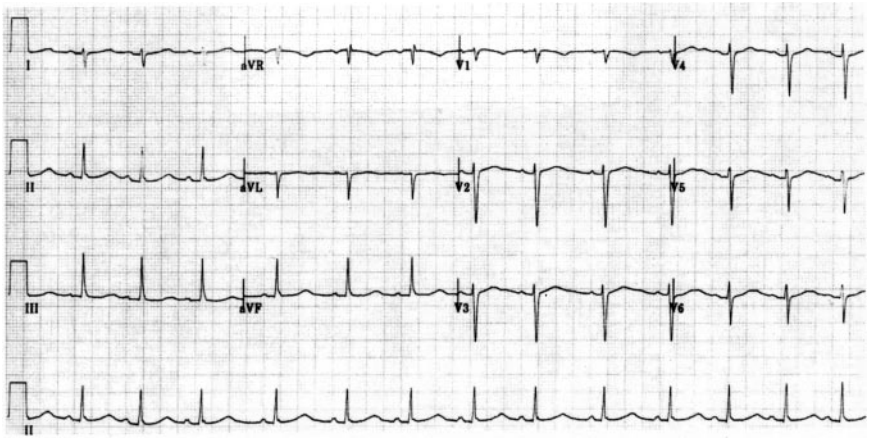


**Figure 2.** ECG of patient with the LQTS-associated mutation F97C-*CAV3*. Note the prolonged QT interval in case 1 bearing the F97C mutation in *CAV3*.

was diluted 1:500 in PBS and incubated with the sections for 1 hour at room temperature. After 3 washes in 1× PBS, the slides were incubated for 30 minutes at room temperature with the following secondary antibodies: a fluorescein isothiocyanate–conjugated anti-goat antibody to detect caveolin-3 and the Texas red–conjugated anti-rabbit antibody to identify $hNa_v1.5$ (Molecular Probes, Carlsbad, Calif), diluted 1:500, and then mounted as previously described.[18]

The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

## Results

### Clinical Evaluation and Mutation Analysis

Mutational analysis of *CAV3* in 905 unrelated LQTS subjects identified 6 nonsynonymous single nucleotide polymorphisms (Figure 1A) in 17 patients (1.9%). Sequencing analysis identified the single nucleotide polymorphisms as heterozygous missense mutations (Figure 1B) leading to amino acid changes in residues conserved across several species (Figure 1C).

The novel mutation F97C was observed in a 13-year-old female asthmatic patient presenting with shortness of breath and chest pain. The ECG demonstrated marked QT prolongation (QTc, 532 ms; Figure 2 and Table 1, case 5). Notably, this prolongation was present only but reproducibly on β-agonist inhaler therapy for her asthma. Her family history was unremarkable, and screening ECGs among all first-degree relatives revealed normal QTc (data not shown). Genetic analysis revealed a de novo 290T>C mutation (Figure 1B), leading to F97C, involving a highly conserved residue (Figure 1C) in the transmembrane domain (Figure 1A).

The 423C>G mutation, resulting in the missense mutation S141R, was discovered in a 16-year-old white male patient who presented with nonexertional syncope and a QTc of 480 ms (Table 1, case 6). The mutation localizes to the functional C-terminal domain of caveolin-3 and modifies a conserved residue (Figure 1A through 1C). Comprehensive open reading frame/splice site mutational analysis of all other known LQTS-associated genes was negative. Consistent with the negative family history and normal screening ECGs among first-degree relatives, genetic testing confirmed that S141R was also a spontaneous de novo mutation.

The other novel *CAV3* mutations (T78M and A85T), which alter highly conserved residues, were identified in 4 patients (Figure 1 and Table 1) and, like F97C and S141R, were absent in >1000 control alleles. T78M was noted in 3 probands with a family history from small kindreds (cases 1 through 3), with 1 proband also possessing a published LQT2-associated KCNH2 mutation (A913V). The A85T mutation was elucidated as a de novo mutation in 1 case (Table 1, case 4).

**TABLE 1. Summary of Putative *CAV3* Mutations Found in Patients With LQTS (Absent in ≈1000 control alleles)**

| Case | Sex | Age at Diagnosis, y | Race | Nucleotide Change | *CAV3* Variant | Other LQTS Mutations | Presenting Symptom | QTc, ms | Other ECG Abnormalities | Family History | Other Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | F | 14 | W | 233C>T | T78M* | A913V-KCNH2 (LQT2) | Nonexertional syncope | 405 | U waves, sinus bradycardia | Pos | Seizure-like presentation |
| 2 | M | 8 | B | 233C>T | T78M* | ... | Nonexertional syncope | 433 | Marked sinus bradycardia | Pos | ... |
| 3 | M | 40 | W | 233C>T | T78M* | ... | Asymptomatic | 456 | ... | Pos | ... |
| 4 | F | 36 | W | 253G>A | A85T* | ... | Cardiac arrest in sleep | NA | NA | Neg | ... |
| 5 | F | 10 | W | 290T>G | F97C* | ... | Shortness of breath, chest pain | 532† | Neg | Neg | ... |
| 6 | M | 16 | W | 423C>G | S141R* | ... | Nonexertional syncope | 480 | NA | Neg | |

W indicates white; Pos, positive; B, black; and Neg, Negative.

*Novel variant.

†QTc of 532 ms recorded in the setting of albuterol metered-dose inhaler therapy for asthma.

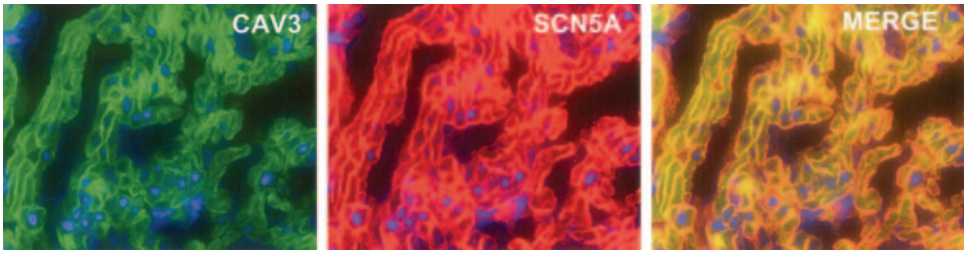


**Figure 3.** Caveolin-3 and hNa$_v$1.5 localization in human myocardium. Immunohistochemical analysis on human tissue from right ventricular free wall performed with anti–caveolin-3 and anti-hNa$_v$1.5 antibodies demonstrates that both caveolin-3 (green) and hNa$_v$1.5 (red) show sarcolemmal localization. Colocalization is confirmed in the merge panel, along with the cell nuclei (blue).

In addition, we identified the G56S and C72W common polymorphisms, localized to the caveolin-3 scaffolding domain (Figure 1A).[19,20] In the present study, we compared the frequency of the G56S polymorphism among healthy black versus healthy white controls using the Fisher exact test. G56S was identified in 25% of blacks and 0% of white controls ($P<10^{-7}$), indicating that G56S is a relatively black-specific genetic variant. With a prevalence similar to that found in the black controls, 8 black cases had the G56S polymorphism, and 5 of these 8 black cases also had mutations in other established LQTS-causing channels. Similarly, C72W was observed in 3 white LQTS cases, 0% of white controls, and 2% of black controls.

## *CAV3* and hNa$_v$1.5 Localization in Human Myocardium

Although colocalization of the cardiac sodium channel and caveolin-3 at the sarcolemma of cells in myocardial tissue has been already established in rat ventricular cardiomyocytes,[11] we sought to examine whether caveolin-3 and hNa$_v$1.5 also colocalize in human myocardium. Sections from human right ventricular biopsies were used for immunofluorescence labeling of caveolin-3 and hNa$_v$1.5. Figure 3 demonstrates that both caveolin-3 and hNa$_v$1.5 colocalize at the plasma membrane of human cardiomyocytes, suggesting that in human heart, caveolin-3 and hNa$_v$1.5 may be part of the same macromolecular protein complex, which substantiates the possible pathological effect of mutant caveolin-3 on the cardiac sodium channel hNa$_v$1.5. Although cardiac expression of caveolin-3 has previously been established in human subjects,[21] to the best of our knowledge, this is the first report demonstrating caveolin-3 and hNa$_v$1.5 colocalization in human myocardium by immunohistochemical analysis.

## Cellular Electrophysiology

Based on their absence in >1000 reference alleles, the nature of amino acid substitution and conservation across species, sporadic de novo status, and the lack of any other LQTS-associated mutations, F97C and S141R represented the strongest candidate mutations for the pathogenesis of LQTS and were functionally characterized. WT *CAV3* (Figure 4B) and mutant *CAV3* (Figure 4C and 4D) were transfected into a stable hNa$_v$1.5-expressing HEK293 cell line, as well as an empty vector control (Figure 4A), and robust $I_{Na}$ was present in all cases. Summary data for peak current density and kinetic parameters of activation, and inactivation also showed no differences (Table 2). For recovery parameters, the caveolin-3 mutations tended to have a smaller recovery time constant for the slow component of recovery by about half, but this did not reach statistical significance for the F97C mutant (Table 2). A small amount of $I_{Na}$ continued to flow after the initial rapid inactivation, and inspection of the traces in Figure 4 suggests that this late $I_{Na}$ may be increased in the mutants, especially F97C. When this was rigorously exam-

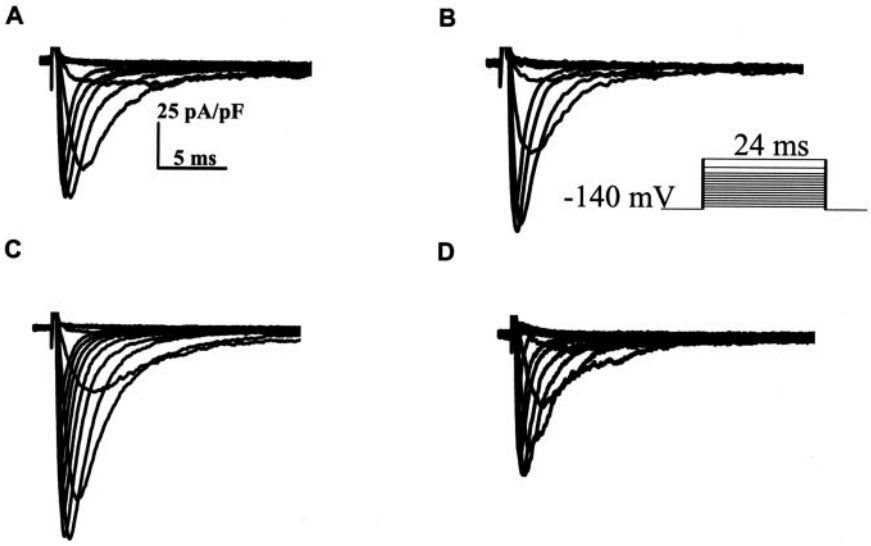


**Figure 4.** Effect of WT and mutant *CAV3* expression on $I_{Na}$ in stable hNa$_v$1.5-expressing HEK293 Cells. Whole-cell $I_{Na}$ traces were recorded with test potentials of 24-ms duration from −120 to 60 mV from a holding potential of −140 mV. Representative $I_{Na}$ traces were recorded from pcDNA3 (A), WT *CAV3* (B), F97C-*CAV3* (C), and S141R-*CAV3* (D) transiently expressed in stable hNa$_v$1.5 cell lines.

**TABLE 2. Kinetic Parameters for $Na_v1.5$ Alone, With WT *CAV3*, and With Mutant *CAV3***

| | pcDNA3 | *CAV3* | F97C | S141R |
|---|---|---|---|---|
| $I_{Na}$ density | | | | |
| pA/pF | −360±89 | −348±139 | −416±120 | −409±77 |
| Activation | | | | |
| V1/2, mV | −44±8 | −43±9 | −49±4 | −52±3 |
| Slope factor | 5 | 5 | 5 | 5 |
| Inactivation | | | | |
| V1/2, mV | −82±3 | −84±2 | −85±3 | −83±2 |
| Recovery | | | | |
| τf, ms | 2±0.2 | 3±0.4 | 2±0.8 | 2±0.7 |
| τs, ms | 52±12 | 51±11 | 28±9* | 38±6 |
| As, % | 88±4 | 78±5 | 82±1 | 79±1 |
| n | 8 | 9 | 10 | 9 |

pA/pF indicates current density; V1/2, voltage of half-maximal activation/inactivation; τf, current fast inactivation; and τs, current slow inactivation.

The fitted kinetic parameters and $I_{Na}$ density from n experiments were averaged and are reported as mean±SD. pcDNA3 (empty vector), WT-*CAV3*, and 2 *CAV3* mutants (F97C and S141R) were transiently expressed in $hNa_v1.5$-stable cell line. All parameters were analyzed by Kruskal-Wallis test across pcDNA3, WT *CAV3*, and 2 *CAV3* mutants. There is no statistically significant difference for $I_{Na}$ density, activation, inactivation from recovery, rate of recovery, and time constant for slow component when pcDNA3, WT *CAV3*, or 2 *CAV3* mutants were expressed in $hNa_v1.5$-stable cell lines.

ined by prolonged depolarization and careful leak subtraction, late $I_{Na}$ was clearly increased in the presence of each of the caveolin-3 mutations, F97C and S141R (Figure 5A). Summary data (Figure 5B) show a 4-fold increase in late $I_{Na}$ for F97C and a 3-fold increase for S141R mutants. This degree of accentuated late $I_{Na}$ is equal to that observed in patients established to have LQT3-associated mutations in *SCN5A*.[22]

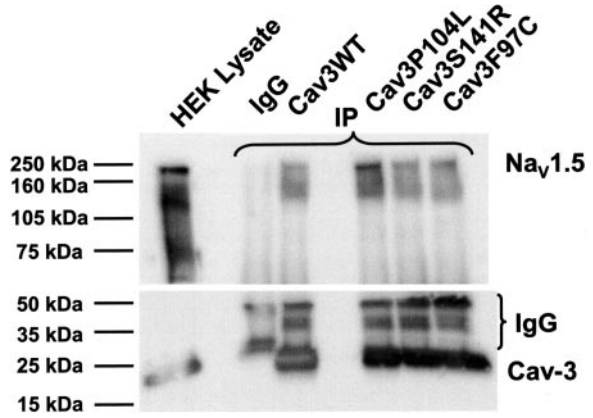


**Figure 6.** $hNa_v1.5$ and either WT or mutant caveolin-3 coimmunoprecipitate. HEK293 cells were transfected with pcDNA3-WT $hNa_v1.5$+WT *CAV3*, pcDNA3-$hNa_v1.5$+P104L-*CAV3*, pcDNA3-WT $hNa_v1.5$+S141R-*CAV3*, or pcDNA3-WT $hNa_v1.5$+F97C-*CAV3*. The lysates were subjected to immunoprecipitation using anti–caveolin-3 antibody and were analyzed by immunoblotting. $hNa_v1.5$, WT caveolin-3, and mutant caveolin-3 are detected in the immunoprecipitates, whereas control immunoglobulin G does not immunoprecipitate the proteins.

## Immunoprecipitation of $hNa_v1.5$ and Caveolin-3

Given that a previous study has suggested that $hNa_v1.5$ and *CAV3* are associated on the basis of their coimmunoprecipitation from cardiac muscle lysates,[11] we sought to determine whether the increase in late $I_{Na}$ induced by the caveolin-3 mutants was conferred by a loss of the association between normal $hNa_v1.5$ and mutant caveolin-3. HEK293 cells were transfected with WT *SCN5A*+WT *CAV3*, WT *SCN5A*+P104L-*CAV3*, and WT *SCN5A*+S141R-*CAV3*, *SCN5A*+F97C-*CAV3*, and these cell lysates were solubilized in Triton X-100 and n-octyl-D-glucoside containing buffer and subjected to immunoprecipitation with anti–caveolin-3 or control mouse immunoglobulin G. Immune complexes were analyzed with Western blotting. In Figure 6, anti-

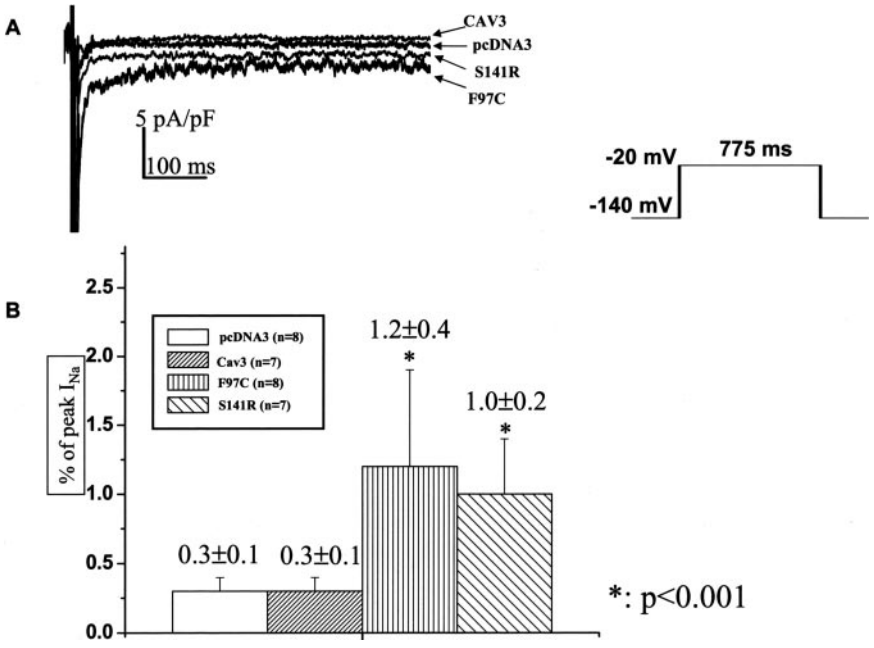


**Figure 5.** F97C- and S141R-*CAV3* increase late sodium current. $I_{Na}$ traces in response to a step to −20 mV for 700-ms duration from a holding potential of −140 mV (see protocol inset) are shown with peak current off-scale to better show the late currents (A). The cell capacitance is as follows: pcDNA3=11 pF, *CAV3*=1 2 pF, F97C=12 pF, and S141R=12 pF. Summary data for late $I_{Na}$ represented as percent of peak $I_{Na}$ were significantly increased when F97C and S141R were expressed (B). *Statistically significant differences between mutant *CAV3* and experiments with WT *CAV3* and without *CAV3* (pcDNA3) ($P>0.001$).

$hNa_v1.5$ antibody detected an immunoreactive band for the $hNa_v1.5$ channel at 240 kDa in the lysate lane and anti–caveolin-3 immunoprecipitation lanes, demonstrating that $hNa_v1.5$ coimmunoprecipitates with WT caveolin-3 in HEK293 cells, similar to the result in cardiac muscle. In the bottom part of the blot, caveolin-3 protein was detected in the lysate and the immunoprecipitation lanes at 18 kDa. Neither protein immunoprecipitated with control mouse immunoglobulin G. Furthermore, the LQTS-associated mutant F97C-caveolin-3 and S141R-caveolin-3 and the limb girdle muscular dystrophy–associated P104L-caveolin-3 immunoprecipitated with $hNa_v1.5$, similar to WT caveolin-3. These results suggest that these disease mutations in *CAV3* do not result in the loss of association between $hNa_v1.5$ and caveolin-3 as a basis for the increase in late $I_{Na}$. Subtle changes in the interactions between the associated proteins would not be detected by this assay, however.

## Discussion

The pathogenetic basis for LQTS has focused principally on ion channels. Only recently, the finding of LQTS-causing mutations in ankyrin-B (LQT4) as a nonchannel protein has led to further exploration of secondary disruptions in ion channel metabolism (expression, posttranslational modification, trafficking, and electrophysiology).

Caveolae, within the plasmalemma, are compartments involved in the scaffolding of ion channels such as $hNa_v1.5$[23]; their activity is critical in the development of ventricular action potential. Colocalization of $hNa_v1.5$ and caveolin-3 has been previously demonstrated at the sarcolemma of rat cardiomyocytes[11] but not in human myocardium.

Here, we demonstrate that both caveolin-3 and $hNa_v1.5$ colocalize at the plasma membrane of human cardiomyocytes, suggesting that caveolin-3 and $hNa_v1.5$ are part of a macromolecular complex important in cardiac function across species, thus substantiating the possible pathological effect of mutant caveolin-3 on $hNa_v1.5$.

In addition, caveolin-3 modulates the sympathetic response in the heart by colocalizing key signaling molecules of the $\beta_2$-adrenergic receptor cascade, including G proteins, adenyl cyclase, and protein kinase A, which can directly phosphorylate $hNa_v1.5$.[24] It is intriguing to note that the adolescent female hosting the de novo F97C–caveolin-3 mutation displayed pronounced QT prolongation only during albuterol inhaler therapy for her asthma. This may suggest a role of F97C–caveolin-3 in altered sympathetic response, with a direct modulator effect on $hNa_v1.5$ or an effect that is mediated by G protein–coupled receptors and other signaling molecules such as protein kinase A. However, the $\beta_2$-adrenergic receptor stimulation also may regulate other sensitive channels, notably $Ca_v1.2$ calcium channels, which also are associated with caveolae and may be involved in the response.[14,25,26]

Caveolin-3 abnormalities have been implicated previously in limb girdle muscular dystrophy, rippling muscle disease, hyper-CKemia, and distal myopathy, but no cardiac manifestations resulting from these mutations have been clearly described.[19] A distinct *CAV3* mutation (T63S) was identified in siblings with hypertrophic cardiomyopathy, although no clear mechanism has been defined.[27] However, no signs of skeletal muscular involvement or primary cardiomyopathy were present in our patients.

Our findings demonstrate for the first time that caveolin-3 directly modifies $hNa_v1.5$ kinetics and subserves a novel pathogenic substrate for LQTS. We identified 6 nonsynonymous genetic variants in 905 unrelated patients referred for LQTS genetic testing, including the 2 common polymorphisms G56S and C72W.[19]

The 4 novel LQTS-associated mutations were absent in >1000 reference alleles and altered highly conserved residues predicted to affect the protein secondary structure using in silico models (data not shown), suggesting perturbation of normal caveolin-3 function.

On the basis of the aforementioned characteristics and the lack of any other LQTS-associated mutations, F97C and S141R represented the most compelling molecular candidates for LQTS pathogenesis. Electrophysiological analysis of F97C and S141R in a cell line with stable expression of $hNa_v1.5$ showed dramatic increases in late $I_{Na}$ compared with $hNa_v1.5$ alone or WT *CAV3*. Interestingly, expression of WT *CAV3* compared with empty plasmid in the HEK293 cells did not alter $I_{Na}$ in any measurable way, suggesting that WT *CAV3* does not modulate $hNa_v1.5$ function; however, the related protein flotillin-1 endogenously expressed in HEK293 cells may serve a role comparable to that of caveolin-3 in maintaining basal $hNa_v1.5$ function, as suggested previously for HCN4 channels expressed in this model system.[28] Nevertheless, the mutant F97C-*CAV3* and S141R-*CAV3* clearly demonstrated a gain-of-function effect on late $I_{Na}$ which is quantitatively similar to that reported in LQT3,[22] suggesting that this may be 1 substrate for the prolonged QT interval and associated arrhythmias.

The molecular mechanisms linking these *CAV3* mutations to altered $hNa_v1.5$ function are not fully defined, however. The immunoprecipitation data suggest that *CAV3* mutations do not alter the overall association between caveolin-3 and cardiac sodium channels, so more subtle molecular interactions or channel regulation may be altered. For none of the previously identified modulators of late $I_{Na}$, including heart failure, ischemia, and cytoskeletal disruption, have the molecular mechanisms been defined.[29] Thus, for the *CAV3* mutations, significant future research is necessary to identify the precise mechanisms directly responsible for the increase in late $I_{Na}$.

Although we did not study the electrophysiological properties of the T78M-*CAV3* mutation, it is important to note that both cases 1 and 2 presented with sinus bradycardia (Table 1). In 2003, Veldkamp and colleagues[30] investigated in an elegant study the role of the 1795insD $hNa_v1.5$ mutant on sinoatrial node electrical activity. They demonstrated that 1795insD presented a persistent inward current and a negative shift in voltage dependence of inactivation, which causes a reduction in sinus rate, leading to bradycardia. This suggests that T78M could modulate the $I_{Na}$ mimicking the 1795insD $hNa_v1.5$ mutant, consistent with the clinical features characterizing the LQT3 phenotype.[30]

Caveolin-3 is the second non–ion channel protein (after ankyrin-B) implicated in the pathogenesis of congenital

LQTS. Consistent with the final common pathway hypothesis,[31] genes encoding cardiac channel–interacting proteins, which secondarily disrupt ion channel function, may confer genetic susceptibility for LQTS. Caveolin-3 is one of a potentially large group of such proteins. In addition, $hNa_v1.5$ appears to be disrupted in a variety of arrhythmia-associated disorders, including LQTS and Brugada syndrome,[32] both possible substrates of sudden infant death syndrome[33] and atrioventricular block.[34]

### Study Limitations

In this report, although we evaluated a large cohort of patients with LQTS, we identified primarily de novo mutations in *CAV3*. The few small kindreds with T78M-*CAV3* declined further genotyping. Thus, we did not have access to large multigenerational families with complete or nearly complete penetrance of the clinical LQTS phenotype, which could have provided cosegregation data further supporting the causality of *CAV3* mutations in LQTS. In addition, the clinical data sets were not complete for all patients in the cohort, as is often the case for sudden death cases in young patients. Therefore, further analysis of certain mutations is needed to completely define the clinical phenotypes.

In addition, although mutations in the coding sequence and exon/intron boundaries[35] of the candidate genes are the most obvious target for a first screening, defects other than in the coding sequence have indeed been discovered and associated with primary arrhythmias. In particular, a large DNA duplication in the gene coding for KCNH2[36] and a common sodium channel promoter haplotype associated with conduction defects among Asians[37] have been described.

Therefore, although we focused on the coding sequence of *CAV3* in this study, we believe that further investigation, including genomic rearrangements and noncoding sequence variants, may play a significant role in the orphan form of LQTS.

Finally, although we provide strong evidence for a mechanistic link between *CAV3* mutations and LQTS mediated by an increase in late $I_{Na}$, we anticipate that modulation of other ion channels or signaling pathways regulating ionic currents also may contribute to the pathophysiology, given the localization of multiple ion channels and signaling proteins to caveolae in ventricular cardiomyocytes. Despite these limitations, we have identified novel mutations in *CAV3* that increase the late sodium current and are specifically associated with LQTS.

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## Disclosures

None.

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## CLINICAL PERSPECTIVE

Long-QT syndrome (LQTS) is a potentially lethal, heritable arrhythmia syndrome affecting ≈1 in 3000 persons. Since the sentinel discovery of cardiac channel mutations as the pathogenic basis for LQTS in 1995, LQTS has been viewed as a "cardiac channelopathy." To date, 8 LQTS-susceptibility genes have been discovered; 7 of these genes encode critical ion channel subunits. The other gene (*ANK2*) encodes an adapter protein, ankyrin-B. Mutations in these 8 genes account for ≈75% of LQTS. The search continues for novel pathogenic mechanisms. In the present study, we provide the first evidence that the *CAV3*-encoded scaffolding protein caveolin-3 constitutes the ninth LQTS-susceptibility gene. Caveolin-3 is expressed specifically in striated muscle and localizes to specialized membrane microdomains (caveolae), little caves in which certain ion channels reside together with specific cellular signaling molecules. Importantly, the LQTS-associated mutations in caveolin-3 alter the properties of the caveolar-localized cardiac sodium channel ($Na_v1.5$) and confer a gain-of-function phenotype on the structurally intact sodium channel. Our findings are likely to expand the perspective of the genetic underpinnings of the cardiac channelopathies. Although scaffolding proteins play critical roles in modulating ion channel function, the concept that inherited arrhythmia syndromes like LQTS may originate from defects in scaffolding or adaptor proteins rather than the channel itself opens the field to an entire class of candidate genes to consider for the remnant of LQTS (25%) that awaits a pathogenic explanation. In addition, these observations suggest that treatments aimed at scaffolding proteins rather than specific ion channels may represent an alternative antiarrhythmic strategy.