A common cardiac sodium channel variant associated with sudden infant death in African Americans, SCN5A S1103Y

Leigh D. Plant,1 Peter N. Bowers,2 Qianyong Liu,1 Thomas Morgan,2 Tingting Zhang,1 Matthew W. State,2 Weidong Chen,3 Rick A. Kittles,4 and Steve A.N. Goldstein1

1Department of Pediatrics and Institute for Molecular Pediatric Sciences, Pritzker School of Medicine, Biological Sciences Division, University of Chicago, Chicago, Illinois, USA. 2Departments of Pediatrics, Genetics, and Child Study, Yale University School of Medicine, New Haven, Connecticut, USA. 3National Human Genome Center, Howard University, Washington, D.C., USA. 4Comprehensive Cancer Center, Ohio State University, Columbus, Ohio, USA.

Introduction
Sudden infant death syndrome (SIDS) is the leading cause of infant mortality in the US among all racial and ethnic groups, representing nearly one-third of deaths between 1 month and 1 year of age (1). These losses continue despite dramatic improvement with the 1994 launch of the “Back to Sleep” campaign discouraging prone sleep for infants, a known risk factor for SIDS (2) that indicates a role for environmental stressors in the disorder. SIDS is defined as sudden and unanticipated death in an infant with no recognized lethal disorder after an investigation that includes autopsy, examination of the death scene, and review of the history. Suggesting a role for genetic predisposition, there is significant variation in the incidence of SIDS among racial and ethnic groups; rates for African Americans, Native Americans, and Hispanics and Asians are 3 times those for whites and 6 times higher than for Africans (1).

While multiple recognized risk factors suggest that SIDS is more than just a disease, its pathogenesis is multifactorial. The cardiac hypothesis (4, 5) has regained support due to identification in postmortem genetic samples from SIDS cases of mutations in the same ion channel genes that cause inherited and acquired long QT syndrome (LQTS) (6–11), congenital sick sinus syndrome (16), and progressive cardiac conduction defect (Lenegre-Lev disease) (17). Of note, many of these disorders are associated with bradycardia, the only consistent premorbid arrhythmia seen on cardiopulmonary monitors in SIDS cases immediately prior to death (18). Most SCN5A mutations causing arrhythmia produce channels that continue to operate late in the cardiac cycle when normal channels are silent; such late activity is a substrate for chaotic rhythms and sudden death (19–21). In African Americans, the common missense variant SCN5A S1103Y confers an 8-fold increased risk of arrhythmia without marked change in channel function in vitro (22). Genetic modulation of risk by exposure to extrinsic factors is seen in patients who carry potassium channel variants that are well tolerated until drug exposure produces arrhythmia (10, 11) and is consistent with a multi-hit pathogenesis for cardiac arrhythmia (23). To investigate the impact of changes in cardiac ion channel function on the risk of SIDS, samples were screened for genetic variations in SCN5A, and the attributes of variant channels were studied in the presence and absence of stressors and antiarrhythmic medications.

Results
Cohort and genetic analysis. The 224 autopsy-confirmed cases of SIDS that we report in our case-control study reflect the expected distribution of SIDS cases in the US based on sex and ethnicity, with higher numbers of African Americans than whites (African American, 133; white, 86) and males than females (male, 106; female, 79). The control population for the African American SIDS cases consisted of 1,056 African American adults without known health problems. Twenty of the 133 African American SIDS cases had SCN5A variants that changed the channel protein (Supplemental Tables 1 and 2;
supplemental material available online with this article; doi:10.1172/JCI25618DS1). Thirteen cases were heterozygous for a common variant where a C replaces A in the second position of codon 1103 on 1 copy of SCN5A to yield a change from serine to tyrosine in the channel (Figure 1), indicating the genotype S1103/ Y1103 (SY). Three cases (2 female, 1 male) were homozygous for the variant genotype Y1103/Y1103 (YY), and so these infants carried only Y1103 channels. Four other cases had rare variants that changed a residue elsewhere in the channel on 1 SCN5A allele (Supplemental Figure 1). Of the 1,056 African American adult controls, the wild-type genotype S1103/S1103 (SS) was observed 935 times, SY was seen 120 times (similar to the previously reported frequency; refs. 22, 24), and 1 individual was homozygous (YY). This last individual could not be relocated to investigate for evidence of family history of SIDS or cardiac rhythm disturbances; he was 40 years of age and healthy at the time of sample collection.

Hardy-Weinberg equilibrium. Observed and expected genotypic frequencies, assuming Hardy-Weinberg equilibrium (HWE), for each genotype in African Americans were as follows. In SIDS cases (n = 133): SS 117, 115 expected; SY 13, 17.6 expected; YY 3, 0.68 expected. In controls (n = 1,056): SS 935, 938 expected; SY 120, 115 expected; YY 3, 3.52 expected. Case genotype frequencies were not in HWE (χ² ≈ 9.21; P = 0.002), whereas control frequencies did not significantly deviate from HWE (χ² ≈ 2.04; P = 0.15). In SIDS cases, there was a 4-fold excess of YY genotypes over the expected amount. In the controls, there was 1 YY individual, whereas 3 or 4 were expected. Comparing YY frequency in SIDS cases with controls, 3 of 133 (2.3%) versus 1 of 1,056 (0.1%), gives an odds ratio for SIDS of 24.4 (95% confidence interval, 1.9 to 1,279), indicating an approximately 24-fold increase in risk with the YY genotype. Overrepresentation of YY in SIDS cases was statistically significant, with a P value of 0.005 by Fisher’s exact test. To rule out misattribution in SIDS cases identified as African American, all SIDS cases with SY and YY genotypes were evaluated and confirmed to bear the expected genetic ancestral proportions for Americans of African descent and were unrelated (Supplemental Table 1 and Supplemental Figure 2).

Figure 1
A polymorphism in SCN5A encodes a variant cardiac sodium channel. (A) Denaturing HPLC waveform and direct sequencing of wild-type and S1103Y variant. The chromatogram shows the additional peak resulting from heteroduplex assembly of S1103 and Y1103 amplicons. Forward sequence of homozygous S1103 and Y1103 SIDS cases shows the C3308A change that leads to the substitution of serine (S) by tyrosine (Y). (B) Topology of the cardiac sodium channel encoded by SCN5A shows the cytoplasmic location of the S1103Y missense change (red), the 4 homologous membrane domains (DI–DIV), the pore-forming (P) loops, and the voltage-sensing segments (+).

Y1103 channels inactivate abnormally at low pH. SCN5A encodes a 2016 residue α subunit with 4 similar domains (Figure 1B, DI–DIV) that fold in pseudosymmetric fashion to form a single, central ion permeation pathway lined by 4 pore loops. Position 1103 is in the intracellular linker between DII and DIII. To investigate channel operation, wild-type S1103 or Y1103 channel α subunits were expressed with the β subunit of SCN5A, SCN1B, in mammalian tissue culture cells (HEK-293) (25). Channels were first studied using whole-cell patch-clamp mode so currents passed by many channels were assessed simultaneously. At pH 7.4, S1103 and Y1103 channels were found to function indistinguishably, showing the same midpoint for voltage-dependent activation (Figure 2A), midpoint for voltage-dependent steady-state inactivation (Figure 2B), and time course for recovery from inactivation (Figure 2C). Splawski and colleagues (22) observed subtle changes in gating of Y1103 channels under baseline conditions that were not apparent in our system. Hypothesizing that a more significant change in channel function might result from a challenge experienced in vivo, we assessed the effect of acidosis.

Because neither residue is charged, an amino acid change from S to Y is not expected to produce pH sensitivity a priori. Nonetheless, lowering cytosolic pH (from 7.4 to 7.0 and 6.7) was found to induce progressive shifts in the voltage dependence of steady-state inactivation (V½) for Y1103 channels (Figure 2, bottom row); thus, Y1103 showed V½ to equal –88 ± 1, –84 ± 2, and –77 ± 2 mV at pH 7.4, 7.0, and 6.7, respectively (n = 4–8). Wild-type S1103 channels did not show a similar change (V½ = –87 ± 2, –88 ± 1, and –84 ± 1 mV at pH 7.4, 7.0, and 6.7, respectively; n = 7–21) and so significantly differed with Y1103 at pH 6.7 (P = 0.005). In contrast, external acidification is expected to block peak current and shift steady-state inactivation (27), and these effects were observed for both channels to the same degree (Supplemental Table 3). Internal acidification was also found to speed recovery from inactivation of Y1103 but not wild-type SCN5A channels (Figure 2, bottom row). Changing the internal solution to pH 6.7 led to a small decrease in recovery time for Y1103 channels that reached statistical significance (P < 0.05; n = 11 for each condition). Internal acidification also induced a small shift in the current/voltage relationship for Y1103 (Figure 2A, bottom inset) that did not achieve statistical significance.

These findings suggest Y1103 channels exposed to low internal pH might operate at times during the cardiac cycle when wild-type channels remain silent. To assess this hypothesis, whole-cell currents were recorded from groups of cells expressing S1103 channels with internal pH 7.4 or 6.7 (Figure 3, A and B; n = 10
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cells for each condition). As expected, currents rapidly activated and inactivated to baseline, a double exponential fit revealed time constants of 1.8 ± 0.04 and 2.0 ± 1.8 ms and 1.9 ± 0.01 and 1.8 ± 0.8 ms at normal and low pH, respectively. Conversely, Y1103 channels produced sustained whole-cell currents at low internal pH that did not return to baseline and showed altered inactivation kinetics (Figure 3, A and B). Late current was 5.23% ± 0.85% of peak, and time constants for recovery from inactivation were 2.5 ± 0.01 and 1.9 ± 3.4 ms and 2.6 ± 0.01 and 79 ± 0.6 ms at normal and low pH, respectively (n = 10 cells, 50 sweeps/cell).

These changes in the voltage dependence of steady-state inactivation (11 mV) and sustained current (5%) with lowered pH were comparable to 2–6 mV shifts and 1–3% sustained currents previously reported for SCNSA-dependent arrhythmia by others (22, 28, 29). To confirm that late currents observed with low internal pH were carried by SCN5A rather than by other channel, transport, or leak pathways, single-channel function was studied.

Single Y1103 channels show abnormal late reopenings. Single wild-type cardiac sodium channels are closed at rest, open briefly with membrane depolarization, and then rapidly inactivate, staying silent despite maintained depolarization; the channels stay inactivated until the membrane repolarizes to resting potential, at which point they return to the closed state in preparation for the next heartbeat. A shift in the voltage dependence of inactivation with low pH suggests that single Y1103 channels may be active after depolarization, when wild-type channels are inactivated. As expected, single wild-type S1103 channels in inside-out patches opened just once on depolarization and then remained inactive at both pH 7.4 and pH 6.7 (Figure 3A); conversely, Y1103 channels operated normally at pH 7.4 but showed abnormal reopenings during depolarization at pH 6.7. Repetitive cycles of stimulation of single channels averaged in cumulative fashion revealed that both the Y1103 change and low internal pH were required to induce sustained (late) current (Figure 3B).

Mexiletine suppresses pathological late reopenings. Bennett and colleagues demonstrated that SCNSA mutations associated with familial LQTS type 3 produce late channel reopenings without application of external stressors (8). These reopenings are blocked 5–10 times more effectively than initial openings (i.e., those required for normal cardiac function) by the channel blocker mexiletine, an antiarrhythmic used in adults and children (30). To assess drug suppression of Y1103 channels, a repetitive activation protocol approximating a heart rate of 150 bpm was used (sodium channel block is sensitive to rate due to altered drug affinity in closed, open, and inactivated states). First, block of initial (peak) currents in whole-cell mode at pH 7.4 and 6.7 was studied. At pH 7.4, S1103 and Y1103 channels were inhibited similarly by propranolol and amiodarone (Supplemental Table 4), whereas Y1103 channels were somewhat more sensitive to mexiletine (Figure 5A, S1103

K_{1/2} = 23.2 ± 1.1 µM [where K_{1/2} is the drug concentration yielding half maximal blockade], Y1103 K_{1/2} = 13.7 ± 1.3 µM; n = 8). At pH 6.7, peak currents of Y1103 channels similarly remained sensitive to blockade (K_{1/2} = 16.2 ± 1.2 µM; n = 8). To assess block of abnormal late reopenings, single Y1103 channels were studied at pH 6.7. As expected, application of 5 µM mexiletine produced no significant suppression of initial activation (the null trace frequency was unaltered; Figure 5B), while nearly complete suppression of pathological late activity seen in the absence of drug (Figure 4B) was achieved (Figure 5B).

Discussion

The SCNSA Y1103 allele has been associated with arrhythmia and sudden death in 3 situations. African Americans with a single copy of the allele show an 8-fold increase in risk for arrhythmia as they reach middle age (22). Three siblings in a white kindred carrying a single allele have syncope, ventricular tachycardia, ventricular fibrillation, and sudden death (31). Here, homozygous African Americans infants carrying 2 Y1103 alleles showed a 24-fold increased risk for SIDS

Discussion
cases out of HWE. We hypothesize that the allele is a substrate for cardiac QT interval prolongation and ventricular arrhythmia in the setting of a secondary challenge that is tolerated by infants with wild-type channels. We previously proposed a similar mechanism to mediate some cases of drug-induced arrhythmia where variant ion channel activity or was markedly increased or did not come to light until the stress of drug action (10, 11). In the case of Y1103 sodium channels, acidosis was seen to produce gain-of-function late reopenings, abnormal behavior associated with dangerous arrhythmia in patients with familial LQTS due to SCN5A mutations. The operation of Y1103 channels under baseline conditions was normal; this suggests that homozygous S1103Y infants will have a normal electrocardiogram in the absence of stressors and that this test would therefore not identify these genetically at-risk infants. Lastly, while the present report focuses on SCN5A S1103Y, the multifactorial nature of SIDS argues for mutation screening of other cardiac and noncardiac genes.

In this context, it is notable that many of the known risk factors for SIDS are stressors that can lead to prolonged apnea and respiratory acidosis — a common event in normal infants that may be exacerbated by diminished respiratory drive in situations including sleep, respiratory infection, multiple siblings (increasing infectious exposures), premature birth, and exposure to secondhand cigarette smoke. We speculate that increased risk for arrhythmia in aging African Americans with the Y1103 allele (22) may result from a similar mechanism, that is, low intracellular pH due to metabolic acidosis in poorly perfused cardiac muscle fed by an atherosclerotic coronary bed. Importantly, the internal pH levels of 7.0 and 6.7 used here to challenge Y1103 channels in vitro are relevant to cardiac physiology. While normal arterial pH is approximately 7.4, intracellular pH of cardiac myocytes is much lower. Cardiomyocytes in anesthetized, ventilated rats, rabbits, dogs, sheep, and ferrets have internal pH levels between 6.9 and 7.1 at baseline (32, 33). Internal pH decreases to 6.6–6.8 with respiratory acidosis and/or hypoxia (34, 35). Furthermore, acid loads dissipate slowly in myocardium, suggesting that basal microheterogeneity in intracellular pH will be pronounced under stress (32, 36).

It should be noted explicitly that suppression of abnormal function of Y1103 channels in vitro does not indicate in vivo effectiveness and that therapy with antiarrhythmic medication carries risk; conversely, mexiletine shortens the QT interval in animal models and adults with LQTS type 3 (37), and 2 infants with LQTS type 3 have been treated with mexiletine and shown both tolerance of therapy and shortened QT (38, 39). These studies and our results suggest a strategy for prophylactic therapy, that is, use of state-dependent blockers that target late reopenings. The results do not indicate that mexiletine should be administered to YY infants to prevent SIDS at the present time. Should future work support our conclusion that YY infants are at significant risk, a study of prophylactic therapy is first required to assess benefits and risks. Moreover, it is important to note that the composition and environment of the channels we studied in cultured cells only approximate cardiac cells from SIDS patients; indeed, the identity of accessory

Figure 3
Cells expressing Y1103 channels show sustained current at a low pH. Whole-cell currents were recorded in HEK-293 cells expressing S1103 or Y1103 channels as indicated with pipette and bath solutions (see Methods) with cells held at −100 mV and stepped to −30 mV for 100 ms at a rate of 0.5 Hz. Scale bars represent 300 pA and 20 ms. (A) Representative currents recorded from cells at the indicated pH. (B) Ensemble averages of 500 sweeps recorded from 10 cells with the indicated channel and pH. Late current was 5.23% ± 0.85% of peak (n = 10 cells). Peak currents were not significantly different and were −1311 ± 143 pA and 1252 ± 139 pA, for S1103 at normal and low pH, respectively, and 1267 ± 173 pA and 1195 ± 245 pA for Y1103 at normal and low pH, respectively (n = 10 cells per condition).

Figure 4
Single Y1103 channels show abnormal late reopenings. Single channels studied inside-out, off-cell patches excised from HEK-293 cells. Currents were stimulated every 2.5 seconds by a 50-ms depolarizing pulse to −30 mV from a holding potential of −120 mV. Data were recorded at filter and sampling frequencies of 5 and 50 kHz, respectively. Pipettes were filled with bath solution described in Methods. Cells were perfused with the pipette solution described in Methods. For each cell, null sweeps (with no channel activity) were identified, averaged offline, and subtracted from data sweeps before analysis. For display purposes, data were refiltered offline using a 2-kHz Bessel filter. (A) With depolarization, single S1103 channels opened, inactivated rapidly, and did not reopen. This behavior was unaltered when internal pH was lowered from 7.4 to 6.7. Null traces at pH 7.4 and 6.7 were 50% ± 2.7% (n = 15 patches, 1,367 sweeps) and 51% ± 3.4% (n = 12 patches, 1,028 sweeps), respectively. In contrast, Y1103 channels behaved like S1103 channels at pH 7.4 but showed late reopenings at pH 6.7. Null traces were 51% ± 4.5% at pH 7.4 (n = 8 patches, 742 sweeps) and 51.7 ± 2.5% (n = 10 patches, 942 sweeps) when pH was reduced to 6.7. (B) Ensemble average traces (n = 100–150 sweeps) for the indicated channels and conditions. Y1103 channels failed to remain in the inactivated state when exposed to internal pH 6.7. Scale bars: 0.5 pA; 10 ms.
subunits in human sodium channels are still a subject requiring investigation (see ref. 40).

As with any association study, our findings will be need to be replicated in a separate cohort of African Americans. Even in relatively homogeneous genetic isolates, population stratification remains a potential explanation for false positive associations (41). However, the allele has been associated with cardiac arrhythmia in adults (22), and changes in function we observed here with low pH provide support for a plausible disease mechanism. The frequency of SIDS associated with changes in SCN5A channels in African Americans in our cohort was approximately 5%. Thus, 7 of 133 SIDS cases were homzygous for YY or heterozygous for other residue changes in SCN5A channels. Changes at sites other than 1103 altered channel function at normal pH (S524Y, 2 cases; R689H and E1107K, 1 case each; Q. Liu and S.A.N. Goldstein, unpublished observations). Like individuals with inherited LQTS type 3, these infants had SCN5A gain in function in a single mutant allele. In contrast, African Americans homzygous for Y1103 were at increased risk for SIDS despite channels that appeared to be normal at baseline. The apparent etiology is that late reopenings of variant channels were fully suppressed by 5 µM mexiletine. Single Y1103 channels studied in inside-out off-cell patches showed that late reopenings of variant channels were fully suppressed by 5 µM mexiletine. Null traces in the absence of drug (see Figure 3B) at pH 7.4 and 6.7 were 51% ± 4.5% (n = 8 patches, 742 sweeps) and 52 ± 2.5% (n = 10 patches, 942 sweeps), respectively; null traces at pH 6.7 with drug were 55% ± 3.5% (n = 6 patches, 561 sweeps). The therapeutic blood level of mexiletine is 0.8–2.0 µg/ml (3.7–9.3 µM). Scale bars: 2 ms, 200 pA (A); 0.3 pA, 10 ms (B).

This study leads us to consider genetic screening in African Americans for SCN5A S1103Y in at least 3 situations: infants with acute life-threatening events, siblings of SIDS victims, and couples that experience infertility or fetal demise (42). Given the apparent increased risk, a comprehensive study of postmortem molecular analysis of approximately 2,000 SIDS cases (the number reported each year in the US) is recommended. If replicated, prospective screening in a large cohort of African American infants should be considered. Finally, it is imperative to continue to support effective public health efforts to decrease known environmental risk factors (e.g., prone sleeping position). SIDS is argued by this work to result, like many common disorders, from a genetic predisposition that yields poor tolerance of common challenges to physiological homeostasis.

**Methods**

SIDS probands. Approval for human study protocols was obtained from the Human Investigations Committee of Yale University. Postmortem tissue was collected from 224 unrelated SIDS cases from 1985 to 1992. Medical information was limited to sex, ethnicity, age at death, time of autopsy, and a brief history. Neither parental DNA nor family history were available. While cases were diagnosed as SIDS, it is not certain that all infants in our cohort died of SIDS because it is a diagnosis of exclusion with no definitive test. Apparently healthy adult African Americans served as controls; they were not screened for a family history of sudden death or SIDS.

Genomic DNA preparation, amplification, and mutational analysis. Genomic DNA was extracted from frozen heart, brain, or kidney blocks (~1 cm). Tissue was macerated on ice, and DNA was harvested by phenol/chloroform extraction, ethanol precipitation, and suspension in Tris-EDTA. The coding regions of SCN5A were amplified using primers and PCR conditions as shown in Supplemental Table 5. Denaturing HPLC heteroduplex analysis (WAVE; Transgenomic Corp.) was performed in the presence or absence of wild-type DNA, and variant samples were directly sequenced. For SCN5A S1103Y, all African American SIDS cases and controls were both screened by denaturing HPLC and directly sequenced. S1103Y was previously called S1102Y by Splawski et al. (22), whereas current mutation nomenclature for SCN5A now uses the long isoform NM_198056.1 (43).

Electrophysiology. Y1103 was introduced into SCN5A (NM_198056.1), the less abundant and longer isoform (43) carried in pcdNA1, using Pfu (Stratagene). Constructs were transiently cotransfected with GFP-tagged SCN1B (the β subunit of cardiac SCN5A channel, isoform b; NM_001037) in HEK-293 cells using lipofectamine (Invitrogen Corp.). After 24–60 hours, whole-cell recording was performed using a 200B amplifier and pCLAMP software (version 8.0; Axon Instruments). Voltage errors were minimized using 80% series resistance compensation. Whole-cell recording was performed using internal (pipette) and bath solutions, as previously described (22): internal, 60 mM CsCl, 80 mM CsF, 10 mM EGTA, 1 mM CaCl2, 1 mM MgCl2, 5 mM Na2ATP, and 10 mM HEPES (pH adjusted to indicate levels with CsOH); bath, 130 mM NaCl, 5 mM CsCl, 2 mM CaCl2, 1.2 mM MgCl2, 10 mM HEPES, and 5 mM glucose (pH 7.4 with NaOH). Inside-out patch recording was performed using the same solutions, with the internal solution in the bath and the bath solution in the pipette. Mexiletine, propranolol, and amiodarone were from Sigma-Aldrich. Stock solutions were made in bath solution (mexiletine) or dimethyl sulfoxide (propranolol and amiodarone). Single channels were recorded in inside-out excised patches using borosilicate pipettes (Clark) coated with Sylgard (Dow Corning). All experiments were performed between 20–22 ºC.

Statistics. In addition to estimation of odds ratios and exact 95% confidence limits, P values were computed using Fisher’s exact test, with a
P value less than 0.05 being considered statistically significant. HWE predictions were reported as expected versus observed for SIDS cases and controls using EpiInfo 2000 software (version 1.1.2; http://www.cdc.gov/epiinfo/). Electrophysiology data are reported as mean ± SEM with ANOVA unless otherwise noted.

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Address correspondence to: Steve A.N. Goldstein, Department of Pediatrics and Institute for Molecular Pediatric Sciences, University of Chicago, Pritzker School of Medicine, 5721 South Maryland Avenue, Chicago, Illinois 60637, USA. Phone: (773) 702-6205; Fax: (773) 702-4523; E-mail: sangoldstein@uchicago.edu.

Peter N. Bowers, Leigh D. Plant, and Qianying Liu contributed equally to this work.