Genetic modifiers in familial cardiac rhythm disorders
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Chapter 5

Identification of RCAN1 as a Genetic Modifier of Atrio-Ventricular Conduction in the Setting of Cardiac Sodium Channel Disease

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ABSTRACT

Background
We exploited the variability in cardiac conduction disease among patients from an extended kindred harboring the SCN5A mutation 1795insD and a segregating population of mice harboring the homologous mutation (Scn5a1798insD/) to search for novel genetic modifiers of cardiac conduction.

Methods and Results
Association analysis (n=1308 tag-SNPs) in the kindred (100 mutation carriers, 115 non-carriers) of genetic variants within and in flanking regions of 18 candidate genes uncovered linkage (LOD=3.7) and association with PR-interval at the region of chromosome 21 harboring the KCNE1 and KCNE2 candidate genes. The SNP displaying the most significant association within this region (rs2834506, p=9.8e-08), was observed within intron 3 of the RCAN1 (Regulator of Calcineurin 1) gene, located upstream of KCNE1. This association was subsequently validated in an independent set of patients harboring different mutations in SCN5A; this also allowed for the detection of a significant interaction between rs2834506 and SCN5A mutation carriership. As the KCNE1 and KCNE2 genes were considered unlikely modulators of PR-interval, we next sought evidence for a role of RCAN1 in mediating this effect. The involvement of RCAN1 was supported by findings in Scn5a1798insD/ F2 progeny of FVB/N and 129P2 mice displaying variable conduction disease severity. In these mice, a significant correlation was found between ventricular Rcan1 mRNA transcript levels and PR-interval (n=56 mice; r=-0.333, p=0.012). Since RCAN1 is a regulator of the pro-hypertrophic calcineurin/Nfat-pathway, we hypothesized that the Scn5a1798insD/ mutation disrupts intracellular Ca\(^{2+}\)-homeostasis, thereby setting the stage for calcineurin-activation which subsequently impacts on PR-interval. Indeed, elevated intracellular Na\(^{+}\) and diastolic Ca\(^{2+}\) levels were observed in cardiomyocytes of Scn5a1798insD/ mice. Moreover, chronic activation of the calcineurin/Nfat pathway through application of Transverse Aortic Constriction (TAC) elicited extreme AV-dysfunction, AV-block and sudden death in Scn5a1798insD/ mice, which was prevented by treatment with the Nfat-inhibitor cyclosporine-A.

Conclusion
We identify genetic variation within RCAN1, a regulator of the calcineurin/Nfat-signaling pathway, as a modifier of PR-interval duration in the setting of cardiac sodium channel disease. We propose that abnormal Ca\(^{2+}\)-homeostasis as a consequence of sodium channelopathy impacts on atrio-ventricular conduction through the activation of the calcineurin/Nfat pathway.
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**INTRODUCTION**

The genetic basis of the Mendelian cardiac arrhythmia syndromes associated with sudden cardiac death (SCD) has been brought into focus over the last 15 years and a large spectrum of mutations, primarily in genes encoding components of cardiac ion channels, has been reported. Genotype-phenotype studies in these disorders have clearly established that they are not spared from the phenomena of reduced penetrance and variable expression typical of Mendelian disorders. Thus, extensive variability in clinical manifestations is often observed even among family members carrying an identical ion channel gene mutation, with some individuals exhibiting overt abnormalities on the electrocardiogram (ECG) and suffering potentially fatal arrhythmias, whereas other mutation carriers display normal ECGs and remain symptom-free throughout life. While evidence points to a role for genetic background, genetic modifiers of phenotypic variability in arrhythmia syndromes remain largely unknown.

Mutations in \( SCN5A \), which encodes the major sodium channel isoform in heart, are associated with various arrhythmia disorders. Gain-of-function mutations prolong cardiomyocyte repolarization and cause the Long-QT syndrome type 3 (LQT3), while loss-of-function mutations reduce the action potential upstroke velocity and cause Brugada syndrome (BrS) and/or cardiac conduction disease (CCD). On rare occasions, \( SCN5A \) mutations are associated with multiple sodium channel biophysical defects in sodium channel function and lead to clinical manifestations of both gain (LQT3) as well as loss (BrS, CCD) of sodium channel function. The first such “overlap” mutation, \( SCN5A-1795insD \), was described by our group in a large family with clinical manifestations of LQT3, BrS and CCD occurring either in isolation or in combinations thereof. Knock-in mice (\( Scn5a^{1798insD/+} \)) carrying the mouse homolog of this mutation recapitulate the diverse clinical phenotype associated with the mutation in patients. Ventricular cardiomyocytes isolated from \( Scn5a^{1798insD/+} \) mice displayed action potential prolongation as a consequence of an increased tetrodotoxin-sensitive persistent inward \( Na^+ \) current, explaining the Long QT phenotype of mutation carriers. In addition cardiomyocytes from these mice were also characterized by a slower action potential upstroke velocity caused by a reduction in peak sodium current density, which on the other hand explains the conduction disease and Brugada Syndrome features in mutation carriers. While our initial studies in \( Scn5a^{1798insD/+} \) mice focused on mice of the FVB/N genetic background (FVB/N-\( Scn5a^{1798insD/+} \), hereafter referred to as FVB/N-MUT), we subsequently investigated genetic background effects on disease severity by studying mutant mice of the 129P2 strain (129P2-\( Scn5a^{1798insD/+} \), 129P2-MUT). These studies demonstrated that the mutation was associated with greater severity of conduction and repolarization disease in mice of the 129P2 inbred strain as opposed to the FVB/N strain, indicating an important role for genetic background in modulation of disease severity.
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To identify novel genes and pathways influencing the severity of conduction and repolarization disease associated with sodium channelopathy, we here carried out parallel investigations in humans and mice segregating the SCN5A-1795insD mutation. We provide evidence that genetic variation within the RCAN1 gene, encoding a regulator of the calcineurin signaling pathway, modulates the extent of PR-interval prolongation in mutation carriers and that the calcineurin-NFAT pathway modulates atrio-ventricular conduction in the setting of sodium channelopathy.

MATERIALS AND METHODS

SCN5A mutation study populations
The discovery study population consisted of a large Dutch kindred of European decent segregating the SCN5A-1795insD mutation associated with manifestations of Long-QT syndrome type 3 (LQT3), Brugada syndrome (BrS) and cardiac conduction disease (CCD) occurring either in isolation or in combinations thereof. The replication study set consisted of individual probands as well as probands and relatives originating from families segregating mutations in SCN5A (other than SCN5A-1795insD) and presenting clinically with LQT3, BrS/CCD or LQT3+CCD. The replication study set was recruited in the Netherlands, France, Italy and the United Kingdom. All subjects studied were of European descent. Only individuals with available DNA and ECG were included. The study was approved by the institutional review boards of the respective centers and all study participants provided their (written) informed consent.

ECG-phenotyping of the SCN5A-1795insD study population
Heart-rate and ECG indices of conduction and repolarization were measured from ECGs acquired in the absence of anti-arrhythmic drugs. All ECGs were digitalized and analyzed using ImageJ (http://rsb.info.nih.gov/ij/). Only sinus rhythm complexes were analyzed. Measurement of all parameters (heart-rate, PR-interval, QRS-duration and QT-interval) was done manually on-screen, in lead II whenever possible. Parameters were averaged from up to 3 consecutive beats with similar preceding RR-intervals. For QT- and heart-rate corrected QT (QTc), the tangent method with Bazett’s correction was used.

SNP selection, genotyping
Haplotype tagging SNPs (tagSNPs) were selected using Tagger based on HapMap data release #20 / phase II using the March 2006 NCBI B35 genome assembly and dbSNP build 125 data. The following criteria were applied for tagSNP selection: HapMap CEU population, pairwise only tagging with r2 ≥ 0.8 and a minor allele frequency (MAF) ≥ 10%. To account for genetic variation in regions surrounding each gene, we considered up- and downstream genetic data in the tagging procedure. Included regions were defined by
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linkage-disequilibrium (LD), blocks as previously reported. We included at least 50 kb of both up- and downstream information.

SNP genotyping was performed using a custom assay (GoldenGate) on an Illumina-BeadStation500Gx (Illumina Inc., San Diego, USA). Multiple quality control measures were implemented: sample call rate <0.95, and SNP call rate <0.98. Monomorphic SNPs were removed and genotypes were checked for Mendelian errors using the mistyping method in Mendel 8.0 and were removed when inconsistent. A total of 1308 out of 1424 SNPs (92%) passed the quality control criteria and were included in the linkage and association analyses. SNP genotyping in the validation dataset was done using a Taqman assay on a Lightcycler System (Roche).

Linkage and association analysis

Phenotypic data for mutation carriers and non-carriers were normally distributed (Shapiro-Wilk-test, W>0.90). Effects of sex, age and mutation-carrier status on ECG characteristics were analyzed using a linear mixed effects model with correction for family relations (lmekin function) implemented in the Kinship package (1.1.0-22) in R (http://www.r-project.org/).

Genetic distance maps were calculated using the kosambi mapping function (Mendel) and estimation of (multipoint) identity-by-descent and linkage analyses were carried out using SOLAR. Two linkage models were used; 1) with adjustment for age and sex, and 2) model 1 with additional adjustment for SCN5A mutation carrier status. The latter model was used to identify effects of SNPs independent of the (large) effect of the SCN5A-1795insD mutation. All LOD-scores reported were corrected for inaccuracy caused by possible non-normal trait distribution using the empirical LOD-adjustment in SOLAR. Following Landers & Kruglyak, who proposed a LOD-score of >1.9 as suggestive linkage and >3.3 as genome wide significant, and correcting for four different phenotypes, we consider LOD-scores >2.4 (p=4.2e-04) and >3.6 (p=2.2e-05) in the present study as thresholds for suggestive and genome-wide significance, respectively.

Family-based association analyses were performed using linear mixed effect models from the Kinship package. Prior to the main analyses, each SNP-phenotype relationship was investigated for dominance deviation using an additive model with a heterozygosity indicator variable. In case of significant (p<0.1) dominance deviation, either a dominant or recessive genetic model was applied in the subsequent analyses (depending on the direction of the dominance deviation). Otherwise an additive genetic model was applied. Similar to the linkage analysis, two models were used in the main association analyses; 1) with adjustment for age and sex, and 2) model 1 with additional adjustment for SCN5A mutation carrier status.
Based on Nyholt’s method\textsuperscript{44}, which is a simple correction for multiple testing using spectral decomposition on the matrices of pairwise LD between SNPs to determine the number of independent SNPs (tests), the number of independent tests in our data set was 1308. Thus the significance threshold for association was calculated by dividing the nominal $\alpha$ (0.05) by the number of independent tests (1308) for 4 phenotypes and 2 models, resulting in a Bonferroni corrected significance threshold $\alpha^*$ of $4.8e^{-06}$.

Within the validation families, association analyses were performed using a linear mixed effects model with adjustment for age, sex, disease phenotype (and carrier status) with the most appropriate genetic model for the SNP to be validated. The significance threshold applied in the validation study was 0.05 divided by the number of SNPs to be validated. (Since only a single SNP was validated, the significance threshold in the validation sample was $p < 0.05$)

**Generation of Scn5a\textsuperscript{1798insD/+} mice**

Heterozygous Scn5a\textsuperscript{-1798insD} (Scn5a\textsuperscript{1798insD/+}) mice in the two different genetic backgrounds (129P2-Scn5a\textsuperscript{1798insD/+} and FVB/N-Scn5a\textsuperscript{1798insD/+}) were generated as described previously\textsuperscript{5,11}. 129P2-Scn5a\textsuperscript{1798insD/+} mice were maintained in the 129P2 genetic background by crossing them with wild-type 129P2 mice that were invariably purchased from Harlan. FVB/N-Scn5a\textsuperscript{1798insD/+} mice were maintained in the FVB/N genetic background by crossing them with wild-type FVB/N mice that were invariably purchased from Charles River Laboratories. Mice were genotyped as described previously\textsuperscript{11}. Scn5a\textsuperscript{1798insD/+} F2 progeny were generated by crossing FVB/N-Scn5a\textsuperscript{1798insD/+} and 129P2-Scn5a\textsuperscript{1798insD/+} mice as described previously\textsuperscript{19}. All experiments were performed on adult (3 to 5 months old, males and females) Scn5a\textsuperscript{1798insD/+} mice with their wild-type littermates as control, and were in accordance with governmental and institutional guidelines for animal use in research.

**Electrocardiographic (ECG) measurements in mice**

Mice were anesthetized using isoflurane inhalation (0.8-1.0 volume % in oxygen) and surface ECGs were recorded from subcutaneous 23-gauge needle electrodes attached to each limb using the Powerlab acquisition system (ADInstruments). ECG traces were signal averaged and analysed for heart rate (RR-interval), PR-, QRS- and QT-interval duration using the LabChart7Pro software (ADInstruments). QT-intervals were corrected for heart rate using the formula: QTc=QT/(RR/100)\textsuperscript{1/2} (RR in ms).

**Quantitative RT-PCR**

RNA was isolated from LV samples of F2 mice, and Rcan1 and Kcne1 mRNA expression levels were quantified using the LightCycler system for real-time RT-PCR (Roche Applied Science). Quantitative PCR data was analyzed with the LinRegPCR program. All samples were processed in triplicate and expression levels were normalized to Hprt.
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(Persistent) sodium current measurements

Cell isolation

For the isolation of ventricular myocytes, excised hearts were first perfused in a Langendorff system (37 °C) with normal Tyrode’s solution for 5 minutes (containing in mmol/l: 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 5.5 glucose, 5 HEPES; pH 7.4 (NaOH)). Next, the heart was perfused for 8 minutes with a similar solution in which the calcium concentration was lowered to 1 µM, after which the enzyme Liberase Blendzyme type 4 (Roche; 0.05 mg/ml) and trypsin (Boehringer, 1 µl/ml of 2.5% solution) were added to the low calcium solution for 10 minutes. Digested tissue was gently triturated in the low calcium enzyme solution and isolated cells were washed twice in the low calcium solution and twice in normal calcium Tyrode’s solution, both supplemented with bovine serum albumin (BSA, 50 mg/ml). Quiescent, rod-shaped cells with clear cross-striations and smooth surface were selected for measurements.

Data acquisition

Membrane potentials and currents were recorded in the perforated or ruptured whole-cell configuration of the patch-clamp technique using patch pipettes (1-3 MW, borosilicate glass). Signals for sodium current were low-pass filtered with a cut-off frequency of 5 kHz and digitized at 5-10 kHz; action potential measurements were filtered and digitized at 10 and 40 kHz, respectively. Series resistance was compensated by ≥75%. For voltage control, data acquisition, and analysis, custom-made software was used.

Sodium current properties

Sodium current properties were determined at room temperature using conventional voltage clamp protocols as indicated in Figure X, with a holding potential of -120 mV and a cycle time of 5 seconds. Current density was calculated by dividing whole-cell current amplitude by cell capacitance ($C_m$). Data for voltage-dependence of activation and inactivation were fitted with a Boltzmann function ($y=[1+\exp((V-V_{1/2})/k)]^{-1}$), where $V_{1/2}$ is the half-maximal voltage and $k$ is the slope factor. Data for the recovery from inactivation was fitted by a two-exponential function ($y=y_{\infty}+A_f[1-\exp(-t/\tau_f)]+A_s[1-\exp(-t/\tau_s)]$), where $A_f$ and $A_s$ are fractions of fast and slow inactivating components and $\tau_f$ and $\tau_s$ are the time constants of the fast and slow inactivating components, respectively. Since the current decay could not be reliably fit with a two-exponential function, the time course of inactivation was instead determined by analyzing the time required for 50% of current decay to occur ($t_{50%}$). The bath solution contained (in mmol/l): 7.0 NaCl, 133 CsCl, 1.8 CaCl₂, 1.2 MgCl₂, 11.0 glucose, 5.0 HEPES, and 5 µM nifedipine; pH 7.4 (CsOH). Pipettes were filled with (in mmol/l): 3.0 NaCl, 133 CsCl, 2.0 MgCl₂, 2.0 Na₂ATP, 2.0 TEACl, 10 EGTA, 5.0 HEPES; pH 7.3 (CsOH).
The persistent sodium current was measured at 37ºC as 30 µM TTX-sensitive current during a descending voltage ramp protocol (Figure X, inset). The bath solution contained (in mmol/l) 130 NaCl, 10 CsCl, 1.8 CaCl$_2$, 1.2 MgCl$_2$, 11.0 glucose, 5.0 HEPES, and 5 µM nifedipine; pH 7.4 (CsOH). The pipette solution was identical to that used for the sodium current measurements described above.

**Intracellular calcium and sodium measurements**

Ventricular myocytes were attached to a poly-lysine (0.1 g L$^{-1}$)-treated coverslip on the stage of a microscope (Nikon Diaphot). A perfusable chamber (height 0.4 mm, diameter 10 mm, volume 30 µL), containing two platinum electrodes for field stimulation (8 mm distance, 40 V cm$^{-1}$ bipolar square pulses of 0.5-ms duration), was pressed onto the cover slip. The microscope stage and perfusion chamber were maintained at 37 ºC. The measuring window was adjusted with a rectangular diaphragm to the cellular surface of one quiescent rod-shaped myocyte. For every experimental condition, fluorescence measurements were taken in 3 to 5 myocytes per heart. Intracellular sodium ([Na$^{+}$]$_i$) and calcium ([Ca$^{2+}$]$_i$) concentrations were measured in myocytes loaded with the fluorescent probes sodium-binding benzofuran isophthalate (SBFI) and Indo-1 respectively in HEPES solution without albumin as described previously. SBFI fluorescence was measured in dual wavelength emission mode and [Na$^{+}$]$_i$ was calculated using previously obtained values for $R_{max}$, $R_{min}$, $\beta$ and $k_d$; dual wavelength emission mode provides a more sensitive and sodium specific measurement of [Na$^{+}$]$_i$ than the dual excitation technique. Because [Na$^{+}$]$_i$ does not change on a beat to beat basis, calculated [Na$^{+}$]$_i$ data were averaged over the entire cardiac cycle. Na$^+/K^+$-ATPase activity was inhibited with 100 µmol/l ouabain.

**Transverse aortic constriction (TAC)**

Mice were anaesthetized by isoflurane (mean 2.5% in oxygen), intubated with a 20 G polyethylene catheter, and ventilated (200 µL, 160 strokes/min) with a rodent ventilator (Minivent, Hugo Sachs Electronics, Germany). The thoracic cavity was accessed through a small incision at the left upper sternal border in the second intercostal space. A 7-0 silk suture was passed around the aorta between the right innominate and left common carotid arteries. Constriction of the transverse aorta was performed by tying against a 27 G needle, which was subsequently removed. The same procedure was followed in sham animals, except for the constriction. A gradient of approximately 50mmHg across the aortic valve was confirmed by Doppler echocardiography. In a subset of mice, a radiotelemetry transmitter (Data Sciences International) was surgically inserted into the peritoneal cavity. After 2 days of recovery, ECG signals were measured continuously until mice were sacrificed (2 weeks after TAC).
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Electrophysiological assessments in Langendorff-perfused hearts

Two weeks after TAC- or sham-surgery, surface ECG analysis was performed and hearts were excised and perfused in a Langendorff set-up. Wenckebach periodicity (WBP) was determined by >2 sec pacing at the left atrium. Starting at 150 ms, the cycle length was reduced in steps of 10 ms until a single stimulation failed to activate the ventricles. For the AV nodal refractory period (AVNRP), the same protocol was used for the ERP. Atrio-ventricular delay (AV-delay) was determined by calculating the difference between the time of LA stimulation and the onset of ventricular activation.

Statistical analysis

Data are presented as mean ± SEM, unless otherwise specified. Differences between groups were analyzed by unpaired Student’s t-test, nonparametric test, or ANOVA as appropriate. The level of statistical significance was set to \( p < 0.05 \), unless otherwise mentioned. The degree of association between PR-interval and LV transcript levels was determined by Pearson’s correlation (\( r \)).

RESULTS

Phenotypic variability among carriers of the SCN5A-1795insD mutation

An extensive genealogical search allowed us to trace the SCN5A-1795insD family back to the eighteenth century, enabling the construction of a highly extended pedigree (Figure 1A). DNA and 12-lead ECGs were available for 215 individuals (100 mutation carriers) from the last 4 generations of the family. As expected, ECG conduction (PR-interval, QRS-duration) and repolarization (QTc-interval) parameters were significantly prolonged in SCN5A-1795insD carriers versus non-carriers (Supplementary Table S1). There was a striking variability in all ECG parameters, both among mutation carriers and non-carriers (Figure 1B). In mutation carriers, while some of the variance in ECG parameters was accounted for by effects of age and sex (Table 1), 53%-89% of the variance in these traits remained unexplained, providing a rationale for searching for genetic modifiers of disease expression in this family.


<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Females vs. males</th>
<th>Age (per year)</th>
<th>Carriers vs. Non carriers</th>
<th>R²</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>4.14 ± 2.38</td>
<td>-0.31 ± 0.06</td>
<td>0.25 ± 2.46</td>
<td>0.08</td>
<td>4.5e-07</td>
</tr>
<tr>
<td>PR-interval (ms)</td>
<td>-7.81 ± 4.00</td>
<td>0.76 ± 0.10</td>
<td>27.58 ± 3.76</td>
<td>0.052</td>
<td>9.0e-14</td>
</tr>
<tr>
<td>QRS-duration (ms)</td>
<td>-8.65 ± 2.17</td>
<td>0.24 ± 0.06</td>
<td>13.72 ± 2.10</td>
<td>9.3e-05</td>
<td>3.82e-05 (*)</td>
</tr>
<tr>
<td>QTc-interval (ms)</td>
<td>1.95 ± 3.35</td>
<td>0.16 ± 0.14</td>
<td>57.76 ± 3.77</td>
<td>0.72</td>
<td>0.23</td>
</tr>
</tbody>
</table>

R²: Explained variance by sex, age and SCN5A-1795insD mutation carriership (*and interaction if p<0.01). SE: standard error. *Depicts a significant interaction between age and carrier ship.
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Figure 1  | (A) The pedigree of the large family carrying the SCN5A-1795insD founder mutation. (B) Correlation between ECG parameters in mutation carriers and non-carriers. PR-interval vs. QRS-duration and QTc-interval vs. heart rate. (C) Bar graph showing the mean and standard deviation of PR-interval between the different genotype groups for rs2834506 in SCN5A-1795insD carriers and non-carriers. (D) Regional association plot for SNPs at the chromosome 21q22.12 region with PR-interval in the family. (Figure generated using SNAP47. Recombination fraction is according to HapMap CEU data. The correlation ($r^2$) of the interrogated SNPs with rs2834506 is represented by red shading, with the strongest red representing the strongest correlation.)
SNP rs2834506 on chromosome 21 is associated with PR-interval in the SCN5A-1795insD family

To identify genetic variants modulating disease severity in SCN5A-1795insD mutation carriers, we investigated single nucleotide polymorphisms (SNPs) located in or around 18 candidate genes for effects on heart rate and ECG parameters of conduction and repolarization by linkage and association analyses. Genes investigated were selected on the basis of either being disease-causing genes in cardiac arrhythmia syndromes, functionally important subunits of such genes, or genes significantly associated with the QTc-interval in the general population based on the results of genome-wide association studies (Supplementary Table 2). Haplotype tagging SNPs (tagSNPs) were selected as described previously (details also provided in the Methods section). Besides the systematic analysis of each gene, the selected tagSNPs permitted the investigation of flanking regions as tag-SNPs capturing haplotypes at least 50 kb up- and downstream of the candidate genes tested were also included. A total of 1,424 SNPs were identified in this way, of which 1308 passed the quality control criteria (see methods for details) and were used for linkage and association analyses. A complete list of the 1308 SNPs tested is presented in Supplementary Table 3.

As expected, due to the effects of the SCN5A-1795insD mutation, linkage analysis using age and sex as covariates (model 1) revealed high LOD scores for conduction (PR, QRS) and repolarization (QTc) parameters at the region of SCN5A on chromosome 3 (LOD scores of 12.9, 6.3 and 19.5, respectively). Using a model with additional correction for SCN5A-1795insD mutation carriership (model 2), uncovered linkage that was not detected in the previous analysis. The chromosome 21 locus in the region of the KCNE1 and KCNE2 candidate genes now showed significant linkage to PR-interval (LOD=3.7), while the chromosome 3 region harboring the genes SCN5A, GPD1L and CAV3 displayed suggestive linkage with PR-interval (LOD=3.1).

In association analysis with correction for age and sex effects, all ECG traits i.e. heart rate, PR-interval, QRS-duration and QTc-interval were, as expected, found to be highly associated with SNPs in and around the SCN5A gene on chromosome 3 (data not shown). After additional correction for SCN5A-1795insD mutation carriership, only a single association exceeded our pre-specified Bonferroni-corrected threshold for statistical significance of 4.8×10⁻⁶ (Supplemental Table 3). This SNP, rs2834506, on chromosome 21, was found to be associated with PR-interval (p=9.8×10⁻⁶). The best fitting genetic model for the association of this SNP with PR-interval was a dominant genetic model. Adding SNP rs2834506 to the linkage model for PR-interval resulted in a disappearance of the
linkage signal on chromosome 21, indicating that this SNP underlies the linkage signal at this locus in the linkage analysis. SNP rs2834506 accounted for 8% of the variance in PR-interval among mutation carriers, bringing the total explained variance to 55%.

The G-allele of rs2834506 was associated with increased PR-interval (Figure 1C) and although the effect of the G-allele appeared stronger in mutation carriers as compared to non-carriers, no significant interaction was detected when a model including an interaction term between rs2834506 and SCN5A-1795insD carriership was used ($p_{interaction}=0.33$; $\beta$ ($\pm$SE)=5.5 $\pm$ 5.6 ms). Moreover, no effect of rs2834506 on PR-interval was detected in a large sample of the general population ($n=5370$, $p>0.05$), European descent) pointing to a possible SCN5A mutation-specific effect of the variant.

Association between rs28934506 and PR-interval in carriers of SCN5A mutations other than SCN5A-1795insD

We next explored the effect of rs2834506 in probands and small families harboring SCN5A mutations other than SCN5A-1795insD. Since the SCN5A-1798insD mutation is associated with disease features of both gain (LQT3) as well as loss of sodium channel function (CCD, BrS) these additional individuals were classified in 3 categories: (i) isolated LQT3, i.e. in the absence of CCD ($n=118$; 61 mutation carriers), (ii) LQT3 with CCD ($n=55$ carriers) or (iii) isolated BrS and/or CCD, i.e. in the absence of QTc-prolongation ($n=320$, 164 carriers).

Analyzing the rs2834506 effect in the carriers and non-carriers within these probands and families uncovered a significant SNP*carriership interaction: the effect of rs2834506 was more pronounced in the carriers compared to the non-carriers ($b = 10 \pm 5$ ms, $p=0.052$). When we combined the data from the SCN5A-1795insD family with the new probands and families, the SNP*carriership interaction effect improved ($p=0.045$). In this analysis, combining the 1795insD family and the additional sets, carriers with the AG/GG genotype had on average a PR-interval that was 12 $\pm$ 3 ms longer than individuals with the AA-genotype, whereas in non-carriers the AG/GG-genotype was associated with an increase of only 5 $\pm$ 2 ms.

Considering mutation carriers only from the additional probands and small families ($n=280$), 123 patients carried at least one G-allele at rs2834506 and these patients showed, on average, 7 $\pm$ 4 ms longer PR-interval compared to SCN5A carriers with the AA-genotype ($p=0.06$, correction for sex, age and disease category). When we considered the two larger phenotypic groups with the distinct/pure phenotypes, that is, isolated LQT3 or isolated CCD/BrS, the effect of the polymorphism appeared stronger in the LQT3 group
suggesting that the interaction may be stemming from the gain-of-function properties of the LQT3-causing SCN5A mutations (Figure 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>Beta</th>
<th>SE</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1795insD</td>
<td>19.74</td>
<td>3.96</td>
<td>100</td>
</tr>
<tr>
<td>LQT</td>
<td>11.73</td>
<td>5.55</td>
<td>61</td>
</tr>
<tr>
<td>LQT+CCD</td>
<td>6.13</td>
<td>8.49</td>
<td>55</td>
</tr>
<tr>
<td>BrS/CCD</td>
<td>6.75</td>
<td>5.79</td>
<td>164</td>
</tr>
</tbody>
</table>

FIGURE 2  | Forrest plot of the effect (Beta ± SE) in ms of rs2834506 on PR-interval in the the SCN5A-1795insD family and the validation sets of SCN5A mutation carriers with a LQT3, LQT3+CCD, or CCD/BrS phenotype.

SNP rs2834506 is located within RCAN1, encoding the regulator of calcineurin 1.

In our candidate gene approach, rs2834506 was included in the association analysis by virtue of its location upstream of the KCNE1 gene which encodes MinK, a regulatory subunit of the repolarizing potassium current \( I_{Ks} \). However, examination of the exact location of rs2834506 showed that it is in fact located within intron 1 of the RCAN1 gene, situated upstream of KCNE1 (Figure 1D). RCAN1 (previously called MCIP and DSCR1) encodes ‘Regulator of Calcineurin 1’ which is highly expressed in heart and regulates calcineurin, a calcium-activated phosphatase that promotes hypertrophic growth of the heart\(^{15}\). Since transgenic mice overexpressing constitutively active calcineurin display premature sudden death and profoundly prolonged PR-intervals\(^{16-18}\), we hypothesized that the effect observed at this locus may in fact be mediated by RCAN1 through the calcineurin pathway. To investigate this possibility, we assessed whether we could detect a correlation between cardiac Rcan1 transcript levels and PR-interval in mutant F2 progeny generated by crossing Scn5a\(^{1795insD/}\) (MUT) mice of the two distinct inbred strains (FVB/N-MUT and 129P2-MUT) displaying different severity of PR-interval prolongation\(^{5,19}\). Rcan1 mRNA expression levels (corrected for the house-keeping gene Hprt) were significantly correlated with PR-interval \((n=56\text{ mice}; r=-0.333, p=0.012)\), whereas no significant correlation was found between Kcne1 mRNA levels and PR-interval \((n=56\text{ mice}; r=0.113, p=0.405)\). This data provides support to the idea that RCAN1 may modify the PR-interval in the setting of sodium channelopathy through effects on and calcineurin signaling.
The $\text{Scn5a}^{1798\text{insD}/+}$ mutation causes abnormal intracellular sodium and calcium homeostasis

The calcineurin/Nfat pathway is activated by increased intracellular levels of calcium ($[\text{Ca}^{2+}]_i$). Since the association of the $\text{RCAN1}$ SNP rs2834506 with PR-interval was restricted to $\text{SCN5A}$ mutation carriers, we thus hypothesized that the mutation causes abnormal intracellular $\text{Ca}^{2+}$ homeostasis with subsequent calcineurin/Nfat-pathway activation, thereby providing a target for the regulatory effects of $\text{RCAN1}$. $[\text{Ca}^{2+}]_i$ may be increased secondary to increased intracellular sodium levels ($[\text{Na}^+]_i$) as a consequence of the sustained inward sodium current ($I_{\text{Na,sus}}$) associated with this mutation\textsuperscript{11}. We therefore investigated the magnitude of $I_{\text{Na,sus}}$, $[\text{Ca}^{2+}]_i$, and $[\text{Na}^+]_i$ in ventricular cardiomyocytes isolated from 3-month old wild-type (WT) and MUT mice of both strains.

As 129P2-MUT mice presented with a more severe phenotype compared to FVB/N-MUT mice, we first explored whether this was also reflected in a greater $I_{\text{Na,sus}}$ as a difference in $I_{\text{Na,sus}}$ between the two MUT mouse strains would allow us to establish whether a dose-dependent relationship existed between the magnitude of $I_{\text{Na,sus}}$ and the extent of increase in $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$. Indeed, while 129P2-MUT and FVB/N-MUT mice displayed a similar reduction in peak $I_{\text{Na},\text{rest}}$, $I_{\text{Na,sus}}$ was significantly greater in 129P2-MUT compared to FVB/N-MUT (Figure 3A-F). Moreover, a gradation was observed in the magnitude of $I_{\text{Na,sus}}$ among the 4 groups of mice studied, with FVB/N-WT mice being the least affected and 129P2-MUT mice the most severely affected. Of note, FVB/N-MUT mice displayed an $I_{\text{Na,sus}}$ that was comparable to 129P2-WT. As expected, the graded severity observed in $I_{\text{Na,sus}}$ across the 4 line of mice, was reflected in the amplitude of steady-state $[\text{Na}^+]_i$, diastolic $[\text{Ca}^{2+}]_i$, and $\text{Ca}^{2+}$ transient amplitude with the 129P2-MUT (which has the largest $I_{\text{Na,sus}}$) displaying the greatest increase in these parameters (Figure 3G-J). This data demonstrates that the $I_{\text{Na,sus}}$ associated with this mutation disrupts intracellular $\text{Ca}^{2+}$ homeostasis, thereby setting the stage for activation of the calcineurin/Nfat-signaling pathway and a potential regulatory effect of $\text{RCAN1}$.
Identification of RCAN1

Figure 3  |  (A) Representative example of peak sodium current measurement assessed with conventional voltage clamp (protocol shown as inset); (B) Average current-voltage relationships for sodium current in ventricular myocytes from FVB/N-WT (n=10), FVB/N-MUT (n=11), 129P2-WT (n=14), and 129P2-MUT (n=11) mice; (C) Average peak sodium current (mean±SEM, pA/pF) measured at a holding potential of -40 mV; (D) Representative example of sustained inward sodium (I\textsubscript{Na,sus}) current measurement assessed with a ramp protocol (see top panel); (E) Average current-voltage relationships for I\textsubscript{Na,sus} in ventricular myocytes from FVB/N-WT (n=9), FVB/N-MUT (n=9), 129P2-WT (n=10), and 129P2-MUT (n=10) mice; (F) Average I\textsubscript{Na,sus} (mean±SEM, pA/pF) measured at a holding potential of -20 mV; (G) Representative examples for intracellular calcium transients in isolated ventricular myocytes; (H) Average intracellular sodium ([Na\textsuperscript{+}]) concentrations measured at a stimulation frequency of 6Hz in FVB/N-WT (n=13), FVB/N-MUT (n=22), 129P2-WT (n=15), and 129P2-MUT (n=16) isolated ventricular myocytes; (I-J) Average intracellular diastolic calcium concentrations ([Ca\textsuperscript{2+}]) and calcium transient amplitudes measured at a stimulation frequency of 6 Hz in FVB/N-WT (n=16), FVB/N-MUT (n=26), 129P2-WT (n=16), and 129P2-MUT (n=16) isolated ventricular myocytes.
Figure 4  | (A) Average heart weight to tibia length ratios for WT-sham, WT-TAC, MUT-sham and MUT-TAC mice (untreated or treated with cyclosporine-A, CsA); (B) Survival curve for WT and MUT mice after TAC; (C) Telemetric ECG recordings indicating normal sinus rhythm in WT-TAC and sinus bradycardia and AV-block in MUT-TAC mice; (D) Representative examples of AV-mapping traces in isolated Langendorff-perfused hearts from WT-TAC and MUT-TAC mice, indicating prolonged AV-conduction delay in MUT-TAC; (E) Average values for AV-conduction delay during sinus rhythm in WT-sham, WT-TAC, MUT-sham and MUT-TAC hearts (untreated or treated with cyclosporine-A, CsA); (F) Increased AV-delay in MUT-TAC hearts at shorter coupling intervals; (G) Average values for AV-nodal effective refractory period (ERP) in WT-sham, WT-TAC, MUT-sham and MUT-TAC hearts (untreated or treated with cyclosporine-A, CsA); (H) Average values for Wenckebach periodicity in WT-sham, WT-TAC, MUT-sham and MUT-TAC hearts (untreated or treated with cyclosporine-A, CsA). * indicates p<0.01 versus WT sham, # denotes p<0.01 versus MUT sham.
Identification of RCAN1

Chronic activation of the calcineurin/Nfat pathway elicits AV-block and sudden death in Scn5a1798insD/+ mice

To further investigate the idea that the Scn5a1798insD/+ mutation stimulates the calcineurin/Nfat pathway and thereby impacts on atrio-ventricular conduction, we subjected FVB/N-MUT and FVB/N-WT mice to Transverse Aortic Constriction (TAC). This surgical intervention leads to chronic pressure overload in the left ventricle, activation of the calcineurin/Nfat pathway, and ultimately development of cardiac hypertrophy. We hypothesized that if the mutation indeed impacts on atrio-ventricular conduction by activation of this signaling pathway, then the setting of increased calcineurin/Nfat signaling during TAC should result in an increased atrio-ventricular slowing in mutant mice as opposed to sham-operated mutant mice. After TAC, WT and MUT mice developed similar extent of cardiac hypertrophy (Figure 4A). However, 8 out of 19 MUT-TAC mice (42%) died suddenly between day 5 and day 14 day post-TAC-surgery, whereas all WT-sham, WT-TAC and MUT-sham mice survived the 2-week TAC period (Figure 4B). Continuous 24-hour telemetry recording revealed that the MUT-TAC mice that died prematurely developed progressively increased PR-intervals and bradycardia, ultimately culminating in (complete) AV-block and death (Figure 4C). In surviving MUT-TAC animals, PR-intervals on surface ECGs were not significantly increased as compared to MUT-sham (data not shown). However, electrophysiological measurements in isolated Langendorff-perfused hearts revealed profound AV-conduction delay in surviving MUT-TAC mice as compared to WT-TAC, WT-sham or MUT-sham during baseline atrial stimulation at 120 ms, and this effect was further exacerbated at shorter coupling-intervals (Figure 4D,E). Similarly, both the AV-nodal refractory period and the Wenckenbach periodicity were significantly prolonged in MUT-TAC hearts compared to the other groups, indicating the development of substantial atrio-ventricular dysfunction secondary to TAC in MUT mice only (Figure 4F,G). Treatment with the calcineurin-inhibitor cyclosporin-A (CsA; 15 mg/kg i.p. twice daily) prevented the development of cardiac hypertrophy in both WT-TAC and MUT-TAC to an equal extent (Figure 4A). However, while CsA had no effect on electrophysiological properties in WT-TAC, WT-sham or MUT-sham hearts, it completely prevented sudden death and normalized atrio-ventricular conduction properties in MUT-TAC mice (Fig. 4D-G). Thus, the secondary effects of the mutation on intracellular Na+ and Ca2+ homeostasis makes Scn5a1798insD/+ hearts more susceptible to the deleterious effects of calcineurin/Nfat-pathway activation on atrio-ventricular conduction and provide a target for the regulatory effects of RCAN1.

**Discussion**

Through linkage and association analysis in an extended family with cardiac sodium channelopathy caused by the SCN5A-1795insD mutation, we identified genetic variation within the RCAN1 gene as a modifier of the PR-interval. Further association analysis in a set of patients harboring other SCN5A mutations, confirmed this association and...
established an interaction between the SNP and SCN5A mutation carriership. Studies in mice carrying the homologous mutation (Scn5a<sup>1798insD/+</sup>) support the concept that intracellular Ca<sup>2+</sup> homeostasis is disrupted as a consequence of the increased sustained sodium current characteristic for this mutation, which makes the hearts of mutation carriers more susceptible to the deleterious effects of calcineurin/Nfat-pathway activation on atrio-ventricular conduction and provides a target for the regulatory effects of RCAN1.

Like many Mendelian disorders, inherited arrhythmia syndromes typically display reduced penetrance and variable disease expression, which have been attributed to effects of environmental or genetic modifiers. Some important modifiers such as age<sup>20</sup>, gender<sup>21</sup>,<sup>22</sup>, heart-rate<sup>23</sup> and drug treatment<sup>10, 24</sup> are already recognized. However, while evidence points to a role for genetic modifiers of disease severity<sup>5, 7, 25</sup>, these remain largely unknown<sup>3</sup>. Recent genome-wide association studies in the general population have uncovered a number of SNPs in various genes associated with heart rate, and ECG indices of conduction (PR, QRS) and repolarization (QTc) (reviewed in Kolder et al. 2012<sup>26</sup>). As for most biological traits, the loci identified display small effect sizes and in aggregate explain only a small fraction of the total heritability for a given trait, leaving a large portion of heritability remains unexplained. A complementary approach to such population studies in the identification of genetic variants impacting on these traits comprises family-based studies which although requiring appreciable effort and resources for recruitment of family members, offer distinct advantages<sup>10</sup> USA. laird@hsph.harvard.edu</Refman>They are robust against population admixture and stratification, and allow both linkage and association to be tested. The employment of families such as the one studied in the current paper additionally provides a genetically sensitized setting which may favour the identification of genetic modifiers in a modest sample size. Moreover, they allow for the identification of SNP*mutation interactions, which is not possible in the general population.

In the present study, the large size of the SCN5A-1795insD pedigree coupled to the variable disease severity and pleiotropic effects of the mutation make it a powerful model for uncovering genetic modifiers. Indeed, our results provide strong evidence in support for a role of genetic variation on chromosome 21q22.12 in modulation of PR-interval. The most significant SNP (rs2834506) within this region displayed a p-value (p=9.8e-08) that not only passed our pre-determined threshold for significance (p<4.8e-06) but was even borderline significant when one considers the commonly used genome-wide significance p-value cut-off of p<5.0e-8 corresponding to Bonferroni adjustment for 1 million independent tests<sup>28</sup>.
A role for the 21q22.12 chromosomal region, harboring KCNE1 and KCNE2, in regulation of atrio-ventricular conduction was at first glance rather unexpected. KCNE1 and KCNE2 encode b-subunits, respectively MinK and MiRP-1, of the major repolarization currents I_{Ks} and I_{Kr}. Thus, genetic variation at these genes would be expected to affect cardiac repolarization rather than conduction. Inspection of the association signals within this region showed that the most highly associated SNP, rs2834506, which we selected for genotyping by virtue of the fact that it lies upstream of the KCNE1 gene, actually lies within intron 3 of the RCAN1 gene (previously called MCIP) encoding ‘Regulator of Calcineurin 1’. RCAN1 is highly expressed in heart and regulates calcineurin, a calcium-activated phosphatase that promotes hypertrophic growth of the heart. The availability of transgenic mice carrying the knock-in mutation Scn5a^{1798insD/+} allowed for further investigation of the relevance of the observed association. Scn5a^{1798insD/+} mice recapitulate the diverse clinical phenotype of the patients and moreover, Scn5a^{1798insD/+} mice of two separate genetic backgrounds display varying severity of conduction and repolarization disease. In mutant F2 progeny generated by crossing Scn5a^{1798insD/+} mice of these two distinct inbred strains, we found a significant correlation between cardiac Rcan1 (but not Kcne1) transcript levels and PR-interval, thereby extending the observed clinical association between RCAN1 and PR-interval in human SCN5A-1795insD mutation carriers to the cardiac gene expression level.

RCAN1 is highly expressed in heart and regulates calcineurin, a calcium-activated phosphatase that promotes hypertrophic growth of the heart. RCAN1 directly binds to the catalytic domain of calcineurin and has been suggested to act as a feedback inhibitor of calcineurin, although activation of calcineurin by RCAN1 has also been described. Interestingly, transgenic mice over-expressing constitutively active calcineurin display premature sudden death and profoundly prolonged PR-intervals. Action potential (AP) recordings in neonatal cells from these mouse hearts before the development of hypertrophy showed decreased peak sodium current and decreased sodium channel availability, suggesting that alterations in this current are directly and independently linked to the same calcineurin signaling pathway as myocardial hypertrophy. Decreased Scn5a mRNA and Na_{1.5} protein levels have also been reported in hearts of calcineurin-overexpressing mice. In response to sustained elevated intracellular Ca^{2+}, calcineurin becomes activated in the cytoplasm where it dephosphorylates its transcriptional effector, NFAT. This results in translocation of NFAT to the nucleus, interaction with other transcription factors, and induction of hypertrophic gene expression. Here, we have shown that the Scn5a^{1798insD/+} mutation causes enhanced sustained inward sodium (I_{Na,sus}) current in ventricular cardiomyocytes, with subsequent increased intracellular Na^{+} and Ca^{2+}-levels, setting the stage for calcineurin-Nfat pathway activation. Notably, mice of the 129P2 inbred strain showed more pronounced increase of I_{Na,sus}, intracellular Na^{+}
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and Ca\(^{2+}\), and atrio-ventricular conduction delay secondary to the Scn5a\(^{1798\text{insD}+/+}\) mutation, indicating a dose-dependent effect. Furthermore, Scn5a\(^{1798\text{insD}+/+}\) mice developed severe atrio-ventricular conduction abnormalities and sudden death after TAC, in the setting of chronic enhanced calcineurin/Nfat-activation. These TAC-induced effects were prevented by the calcineurin-inhibitor cyclosporin-A, confirming the functional interaction between calcineurin/Nfat pathway activation and atrio-ventricular conduction in the setting of the Scn5a\(^{1798\text{insD}+/+}\) mutation. Interestingly the only three carriers within this family that have died suddenly although they were implanted with a pacemaker were homozygous for the G-allele of rs2834506.

SCN5A mutations are associated with a wide range of clinical symptoms and syndromes, including long QT syndrome type 3 (LQT3), Brugada syndrome, sinus node dysfunction, (atrio-)ventricular conduction disease, atrial standstill, dilated cardiomyopathy and atrial fibrillation. From a biophysical point of view, SCN5A mutations causing Brugada syndrome and conduction disease are typically loss-of-function mutations associated with reduced peak sodium current and/or sodium channel availability. In contrast, LQT3 mutations generally cause an increase in non-inactivating, persistent (sustained) inward sodium \((I_{\text{Na,sus}})\), leading to continuous Na\(^{+}\)-influx during the entire duration of the action potential and subsequent prolongation of action potential and QT-interval. In some instances, as is the case for SCN5A-1795insD, one single mutation can cause both gain- and loss-of-function biophysical alterations in combination with a clinical overlap syndrome of LQT3, Brugada syndrome and/or conduction disease. Our current findings demonstrate that enhanced \(I_{\text{Na,sus}}\) in Scn5a\(^{1798\text{insD}+/+}\) mice leads to increased intracellular Ca\(^{2+}\) levels, thus setting the stage for the calcineurin/Nfat-pathway and its regulator RCAN1. Since the RCAN1 SNP had only an effect in mutation carriers and displayed no effect in a large sample of the general population, enhanced \(I_{\text{Na,sus}}\) and subsequent calcineurin/Nfat-activation appears essential for the effects of RCAN1 on atrio-ventricular conduction to occur. This is further corroborated by the finding that the association between the RCAN1 polymorphism and PR-interval appeared stronger in carriers of other SCN5A mutations presenting with a LQT3 phenotype, as compared to those presenting with Brugada syndrome and/or conduction disease.

In this study we also report suggestive linkage of chromosome 3 in the region of the CAV3, GPD1L and SCN5A genes in modulation of heart rate and PR-interval. SCN5A encodes the pore-forming α-subunit of the cardiac sodium channel, while CAV3 and GPD1L encode respectively caveolin-3 and glycerol-3-phosphate dehydrogenase 1-like protein, both interacting with the sodium channel α-subunit. This effect was detected in the analysis...
correcting for carriership of the SCN5A mutation, suggesting that genetic variability within this region, separate from the causal mutation in this family, also impacts on heart rate and atrio-ventricular conduction.

In recently published GWAS meta-analysis studies for QT-interval in the general population\textsuperscript{34,35}, genetic variation within or upstream of the *NOS1AP* gene was consistently the most significant association with this trait. In both of these studies rs12143842 emerged as the most strongly associated SNP in this region. Although in our study, this SNP was not genotyped directly, it was captured by SNP rs16847548 with an $r^2$ of 0.82 (HapMap CEU) which was in turn significantly associated with QTc in our data ($p=4.0\text{e}{-04}$). The SNP most strongly associated with QTc in our data was however rs7539281 ($p=4.0\text{e}{-05}$).

In conclusion, we have applied a multi-faceted approach in both humans and mice segregating the same mutation in the SCN5A gene and have identified *RCAN1* as a genetic modifier of phenotype severity in cardiac sodium channelopathy. Moreover, our findings point to abnormal intracellular Na\textsuperscript{+} and Ca\textsuperscript{2+} homeostasis in mediating this effect through the activation of calcineurin/Nfat signaling.

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REFERENCE LIST

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43. Ref Type: Computer Program


