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Transcriptional regulation of the sodium channel gene (*SCN5A*) by GATA4 in human heart

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ABSTRACT

Aberrant expression of the sodium channel gene (*SCN5A*) has been proposed to disrupt cardiac action potential and cause human cardiac arrhythmias, but the mechanisms of *SCN5A* gene regulation and dysregulation still remain largely unexplored. To gain insight into the transcriptional regulatory networks of *SCN5A*, we surveyed the promoter and first intronic regions of the *SCN5A* gene, predicting the presence of several binding sites for GATA transcription factors (TFs). Consistent with this prediction, chromatin immunoprecipitation (ChIP) and sequential ChIP (*Re*-ChIP) assays show co-occupancy of cardiac GATA TFs GATA4 and GATA5 on promoter and intron 1 *SCN5A* regions in fresh-frozen human left ventricle samples. Gene reporter experiments show GATA4 and GATA5 synergism in the activation of the *SCN5A* promoter, and its dependence on predicted GATA binding sites. *GATA4* and *GATA6* mRNAs are robustly expressed in fresh-frozen human left ventricle samples as measured by highly sensitive droplet digital PCR (ddPCR). *GATA5* mRNA is marginally but still clearly detected in the same samples. Importantly, *GATA4* mRNA levels are strongly and positively correlated with *SCN5A* transcript levels in the human heart. Together, our findings uncover a novel mechanism of GATA TFs in the regulation of the *SCN5A* gene in human heart tissue. Our studies suggest that GATA5 but especially GATA4 are main contributors to *SCN5A* gene expression, thus providing a new paradigm of *SCN5A* expression regulation that may shed new light into the understanding of cardiac disease.

1. Introduction

Sodium channel, voltage-gated, type V alpha subunit ($Na_V1.5$) drives the sodium current that initiates the upstroke of the cardiac action potential [1]. $Na_V1.5$ interacts with several regulatory proteins (β-subunits, Nedd4-2 ubiquitin ligase, calmodulin, among others) that modulate $Na_V1.5$ membrane trafficking and function [2]. Genetic mutations in the *SCN5A* gene, which encodes the $Na_V1.5$ subunit, have been linked to cardiac arrhythmias (Brugada syndrome, long QT syndrome type 3, idiopathic ventricular fibrillation, atrial fibrillation, progressive cardiac conduction defects, congenital sick sinus syndrome, and sudden infant death syndrome) [1,3].

However, recent findings also suggest that aberrant *SCN5A* gene expression may increase susceptibility to arrhythmogenic diseases. For example, low $Na_V1.5$ levels in heterozygous *Scn5a* +/− knockout mice recapitulate cardiac defects found in human individuals carrying disease-associated *SCN5A* mutations, and the severity of these defects is directly correlated with levels of $Na_V1.5$ expression [4]. In addition, certain haplotypes in the *SCN5A* promoter have been associated with aberrant *SCN5A* promoter activity and QRS duration on the electrocardiogram [5]. Finally, a common genetic variant (rs6801957) found in a distal *SCN5A* enhancer region has been associated with abnormal *SCN5A* expression and slow cardiac conductance [6,7]. The rs6801957 variant impairs T-box transcription factor 3 and 5 (TBX3/TBX5) binding

Abbreviations: ChIP, chromatin immunoprecipitation; cRNA, complementary RNA; ddPCR, droplet digital PCR; GATA-BS, GATA binding sites; HEK, human embryonic kidney; *Re*-ChIP, sequential ChIP; $Na_V1.5$, sodium channel, voltage-gated, type V alpha subunit; TSS, transcription start site; TF, transcription factor; WT, wild-type..

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to the enhancer, and has been proposed to promote changes in *SCN5A* expression. Collectively, these few studies suggest that dysregulation of *SCN5A* gene expression may be linked to cardiac disease, and that understanding the mechanisms of *SCN5A* transcriptional regulation may uncover novel determinants of sodium channel-related cardiac diseases.

The core promoter of the human *SCN5A* gene spans from nucleotides −261 to +140 relative to the transcription start site (TSS, +1) [8], and it contains conserved regions with putative regulatory functions. Downstream conserved regions may also include potential *cis*-regulatory elements, between TSS and exon 1 and within intron 1 [9]. In particular, intron 1 contains a predicted GATA1 binding site (BS). Mutation of this site reduces the promoter activity of the *Scn5a* gene in neonatal mouse cardiomyocytes, suggesting a direct role of GATA TFs in the regulation of basal *SCN5A* transcription [8]. The mammalian genome encodes six GATA TFs (GATA1–6). From those, GATA4, GATA5 and GATA6 are expressed in mesoderm- and endoderm-derived tissues, including the heart [10]. GATA-BS are present in *cis*-regulatory regions of multiple cardiac-specific genes and are required for their proper cardiac expression [11]. GATA4 is considered a master regulator of cardiac transcriptional networks, and plays a key role in cardiogenesis and in adult cardiac cells [12]. Mechanistically, GATA4 acts synergistically with other TFs and activates gene expression by promoting H3K27ac deposition [13]. Of note, GATA4 has been described to be involved in the developing atrioventricular cardiac conduction system; accordingly, heterozygous *Gata4* +/− mice display short PR intervals [14]. Mutations in the *GATA4* gene affecting DNA binding or interactions to other TFs have also been linked to heart dysfunction [15,16].

Here, we explored a potential role of GATA TFs on transcriptional regulation of the human *SCN5A* gene. We found that cardiac GATA TFs, mainly GATA4 and also GATA5, regulate the expression of the *SCN5A* gene via a synergistic mechanism. In fresh-frozen human heart samples, we observed that *GATA4* transcript levels positively correlate with *SCN5A* transcript levels. Overall, we suggest that GATA4 plays a major role in the regulation of the *SCN5A* gene in the human heart, which may shed new light into the understanding of human cardiac arrhythmias.

2. Materials and methods

2.1. Cells, antibodies and primers

Cardiac cells derived from embryonic rat ventricle (H9c2 cells) and human embryonic kidney 293 (HEK293) cells were maintained under standard cell culture conditions. Antibodies used in the experiments were: α-GATA4 (sc-1237x), α-GATA5 (sc-9054x), α-GATA6 (sc-7244x) (all from Santa Cruz Biotechnology, Dallas, TX, USA), α-HA (ab1424, Abcam, Cambridge, UK), α-actin (A2066, Sigma, St Louis, MO, USA), and α-FLAG (F1804, Sigma). Secondary HRP antibodies were: α-rabbit (32460), α-goat (31402), and α-mouse (32430) (all from Thermo Scientific, Rockford, IL, USA).

For qPCR gene expression analyses, we used the following rat primers: *Gata4* (QT02350684) and *Scn5a* (QT00186263) from Qiagen (Hilden, Germany); *Nppa* (R_Nppa_1) and *Kcnh2* (R_Kcnh2_1) from Sigma; *β-actin* (Fw 5′AGCCATGTACGTAGCCATCC′, Rv 5′ CTCTCAGCTGTGGTGGTGAA3′). For droplet digital PCR (ddPCR) analyses, we used the following validated expression probes: GATA4-FAM (dHsaCPE5050488), GATA5-FAM (dHsaCPE5036300), GATA6-FAM (dHsaCPE5037510), GATA4-HEX (dHsaCPE5050489), and GADPH-HEX (dHsaCPE5031597) from Bio-Rad (Hercules, CA, USA).

2.2. Plasmids and site directed mutagenesis

Details for plasmids and site directed mutagenesis are provided in Supplementary Materials and Methods.

2.3. Computational analyses

TFSEARCH and MEME bioinformatic tools were used to identify GATA-BS in the *SCN5A* proximal promoter (from positions - 1125 bp to +857 bp from the TSS).

2.4. Human cardiac tissue collection

Human left ventricle samples were collected from fourteen end-stage heart failure patients undergoing cardiac transplantation (Hospital Clínic, Barcelona). Control left ventricle heart samples, not used for transplantation, were obtained from four organ donors (Hospital Clínic, Barcelona). All individuals signed a written consent to participate in the study. Biopsies were collected from the explanted heart, washed and snap-frozen in liquid nitrogen immediately after surgery. To ensure minimum variation due to sample collection, all samples were dissected from the same heart area, the anterior wall of the left ventricle, by the same person. Histologically, samples comprise the three heart layers (epicardium, myocardium and endocardium). All procedures were approved by the ethical committees of the Hospital Dr. Josep Trueta de Girona and the Hospital Clínic de Barcelona and conform the principles outlined in the Declaration of Helsinki.

2.5. Chromatin immunoprecipitation (ChIP) and sequential chromatin immunoprecipitation (Re-ChIP) assays

ChIP and *Re*-ChIP assays were performed using human adult left ventricle samples. ChIP was adapted from Gomes et al., 2006 [17] and *Re*-ChIP from Furlan-Magaril et al., 2009 [18]. An expanded description of these techniques is available in Supplementary Materials and Methods.

2.6. Transient transfection and luciferase assays

PromoterA-luciferase or PromoterB-luciferase (200 ng), EF1αPromoter-Renilla (20 ng) constructs, and GATA expression vectors were transfected into H9c2 cells using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA) following the manufacturer's specifications. We transfected 1200 ng of each GATA expression vectors, except for the dose-response experiment in which we used 600, 1200 and 1800 ng. DNA for each condition was equalized with pcDNA3.1. Cells were harvested 48 h later with passive lysis buffer (Promega, Madison, Wi, USA), and processed for firefly and renilla luciferase activity with Dual Luciferase Reporter Assay System on a GloMax-96 luminometer (Promega).

2.7. RNAi experiments

We transfected H9c2 cells with 25 nM of ON-TARGETplus Non-targeting Pool (D-001810-10) or two different siGENOME siRNA Rat *Gata4* (D-090725-02 and D-090725-03; all from Dharmacon, Thermo Scientific) using Dharmaphect (Thermo Scientific). 48 h post-transfection, we isolated total RNA using the RNeasy Mini Kit (Qiagen) and treated the samples with DNase I to remove genomic DNA contamination. After reverse transcription of 1 μg of RNA (Reverse Transcription kit, Qiagen) we performed qPCR with validated primers for the target genes (*Gata4*, *Scn5a*, *Nppa*, *Kcnh2*, and *β-Actin*) and the Kapa-SYBR Green detection (Kapa Biosystems, Wilmington, MA, USA) on a Mastercycler ep realplex (Eppendorf, Hauppauge, NY, USA).

For the luciferase experiments, H9c2 cells were transfected with 25 nM of control or *Gata4* siRNAs and re-transfected after 24 h with PromoterA-luciferase or PromoterB-luciferase (200 ng) and EF1αPromoter-Renilla (20 ng) constructs. Cells were harvested 24 h later and processed for luciferase assay as described above.

2.8. Co-immunoprecipitation experiments

We co-transfected HEK293 cells with HA/GATA4 (5 μg) and GATA5 (5 μg) expression vectors using Lipofectamine 2000. 48 h after transfection we lysed the cells in IP lysis buffer (50 mmol/L Tris HCl pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP-40 and Protease Inhibitors Cocktail (PI; Roche, Madrid, Spain)) and incubated 1 mg of the total lysate with α-HA antibody. The immunocomplexes were subjected to Western blot with α-GATA5 antibody. 1% of total protein was used as input.

2.9. Sodium current recordings

Xenopus laevis oocytes were obtained as described in the Supplementary Materials and Methods section. In vitro transcription of *SCN5A* gene was done using the mMESSAGE mMACHINE® T7 Transcription Kit (Life Technologies). Synthetic transcript (cRNA) concentration was determined using a NanoDrop spectrophotometer and checked by agarose gel electrophoresis. Different *SCN5A* cRNA dilutions were injected in *Xenopus laevis* oocytes and after 3 days at 18 °C, four batches of oocytes were tested. We measured sodium currents by whole cell patch-clamp technique using two-microelectrode voltage clamp. Peak current was determined at −20 mV and the holding potential was at −120 mV. Measurements were performed using reduced extracellular Na^+ (20 mM).

2.10. Gene expression analysis in human heart tissue by droplet digital PCR

We disrupted 30 mg of human left ventricle samples with a glass dounce homogenizer in RLT buffer (Qiagen), and purified the total RNA using the RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's instructions. To avoid contamination with genomic DNA we included a step of DNase I treatment. Reverse transcription was performed as described above.

Quantification by ddPCR was carried out in 20 μL reactions containing 1× ddPCR Supermix for Probes (No dUTP), 250 nM of each commercial probe, 900 nM specific commercial primers, and 1 μL or diluted cDNA according to manufacturer's recommendations (PCR conditions: 10 min at 95 °C, 40 cycles of 30 s at 94 °C and 30 s at 55 °C, and 10 min at 98 °C). Droplet generation and absolute droplet quantification was performed in a QX200 Droplet Digital PCR System (Bio-Rad) using the QuantaSoft Software (Bio-Rad), and analysis was performed using Excel (Microsoft) and Prism (GraphPad). Quantification references: human GAPDH-HEX and GATA4-HEX.

2.11. Statistical analyses

Data are reported as mean ± SEM. Statistical analysis was conducted with SPSS or GraphPad Prism 6 version for Mac (GraphPad). For two-groups-only comparisons, we conducted two-tailed *t*-test. For analysis of multiple groups, we conducted one-way ANOVA, multiple comparisons comparing the mean of each group to the mean of every other group. Differences were considered significant at $p \le 0.05$: $p \le 0.05$ (*), $p \le 0.01$ (**), and $p \le 0.001$ (***).

3. Results

3.1. GATA4 and GATA5 co-occupy the SCN5A proximal promoter in human heart

To explore the potential role of GATA TFs in the regulation of the human *SCN5A* gene, we surveyed the *SCN5A* promoter for the presence of GATA-BS in the region comprised between −1125 and +857 bp of the *SCN5A* gene (hereafter referred to as *SCN5A* proximal promoter; +1 referring to the TSS). This in silico analysis predicted multiple GATA-BS in the *SCN5A* promoter and intron 1 (Supplementary Fig. S1).

Cardiac TFs GATA4, GATA5 and GATA6 are 85% identical in their amino acid sequence within the DNA binding domain, thus the three bind virtually to the same predicted GATA-BS [10]. We examined the binding of GATA4, GATA5 and GATA6 across the *SCN5A* promoter and first intronic regions in fresh-frozen human heart samples by ChIP analysis. Soluble chromatin of human adult left ventricles was prepared and incubated with GATA4, GATA5 or GATA6 antibodies (Fig. 1A). In agreement with our computational prediction, qPCR analysis of the immunoprecipitated material showed robust binding of GATA4 and GATA5 near to the TSS (−198 bp) and intron 1 (+505 bp) regions of the *SCN5A* gene. We did not detect binding of these TFs to the distal regions analyzed (−8803 and −5916 bp), which did not contain predicted GATA-BS. We observed some level of GATA6 binding on the same regions as GATA4 and GATA5, but ChIP enrichment was not statistically significant relative to control (Fig. 1A).

To examine whether GATA4 and GATA5 co-occupy the same DNA molecules of the *SCN5A* promoter or whether instead both occupy different DNA molecules, we next performed *Re*-ChIP analyses in fresh-frozen human heart samples. Soluble chromatin fragments of the *SCN5A* gene first immunoprecipitated with GATA4 antibodies were efficiently re-immunoprecipitated with GATA5 antibodies (Fig. 1B). These results suggest that GATA4 and GATA5 bind to the same DNA molecules of the *SCN5A* promoter and intron 1 regions. In support of our ChIP analysis, GATA6 did not show significant co-enrichment (Fig. 1B).

3.2. GATA TFs regulate SCN5A gene transcription

To evaluate the functional relevance of GATA TF binding to the *SCN5A* promoter and intron 1 regions, we next performed reporter assays in rat cardiac H9c2 cells. These cells derive from rat embryonic ventricle, and are a well-established "cardiac-like" cellular model to study gene expression [19]. We first examined whether endogenous rat GATA4 regulates rat *Scn5a* expression. Efficient RNAi-based *Gata4* knockdown (80–90%) with two independent siRNAs induced 40–50% reduction in rat *Scn5a* mRNA expression (Fig. 2A; decrease of GATA4 protein levels in *Gata4*-siRNA transfected cells shown in Supplementary Fig. S2). As expected, mRNA levels of atrial natriuretic factor (*Nppa*), a well-known GATA4 target [20,21], were similarly reduced upon *Gata4* siRNA transfection, but we did not observe the same effect on mRNA expression of the non-GATA4 target *Kcnh2* gene, which encodes a potassium channel involved in the repolarization phase of the cardiac action potential. These results support the specificity of our findings, and suggest that GATA4 positively regulates *SCN5A* gene expression.

Next, we determined whether GATA TF regulatory effects on the *SCN5A* gene occur via binding to the promoter and first intronic regions, as suggested by the ChIP assays in human cardiac tissue. We performed reporter assays using a PromoterA-luciferase construct, containing the luciferase gene under control of the fragment −713 to +282 bp (relative to the TSS) of the human *SCN5A* gene. We transfected H9c2 cells with the PromoterA-luciferase construct and increasing amounts of GATA4, GATA5 or GATA6 expression vectors (analysis by Western blot confirmed correct expression of these three proteins; Fig. 2B, right panel). GATA4 stimulated *SCN5A* promoter activity in a dose-dependent manner (Fig. 2B, left panel). GATA5 also stimulated *SCN5A* promoter activity, although to a lesser extent than GATA4. GATA6 overexpression led to marginal, but still significant activation at high concentrations. To exclude the possibility that overexpressed GATA6 was not fully transcriptionally active, we also tested the effect of GATA6 overexpression on the pancreatic and duodenal homeobox 1 (*Pdx1*) promoter, a known target for both GATA4 and GATA6 [22]. We observed that GATA6 overexpression activates the reporter gene through the *Pdx1* promoter (Supplementary Fig. S3).

GATA4 and GATA6 synergistically activate the natriuretic peptide *Nppa* and *Nppb* genes [20], as well as the pancreatic *Pdx1* gene [22]. To test for GATA TF synergies in the control of the *SCN5A* gene, we co-transfected different combinations of GATA4, GATA5 and GATA6

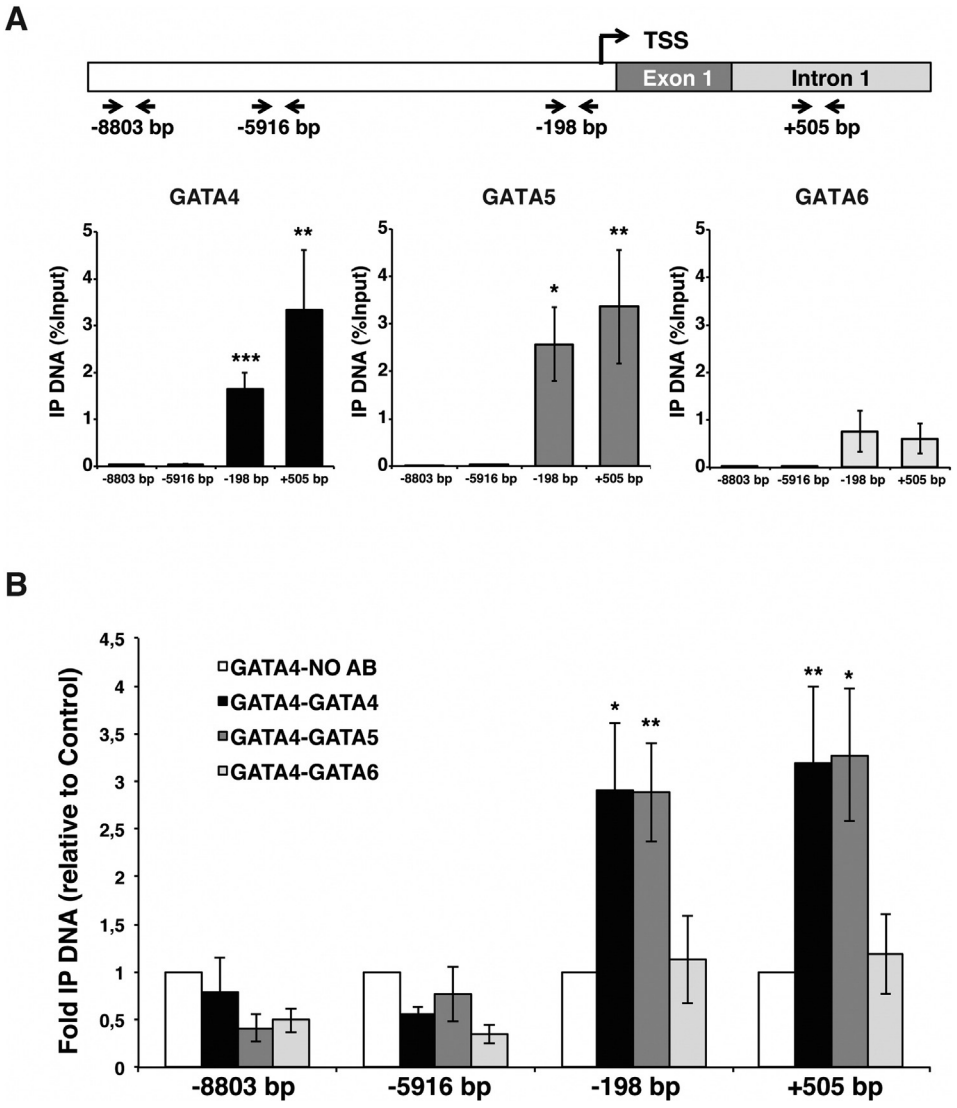

Fig. 1. GATA4 and GATA5 bind to the *SCN5A* proximal promoter in the human heart. A. ChIP assays from human left ventricle samples using antibodies against GATA4, GATA5 and GATA6, followed by qPCR. Numbers indicate central base pairs of amplicons. Results are shown as percentage of input (mean ± SEM, $n = 6$). Significance was examined by the *t*-test relative to negative control (−8803 bp) region. B. *Re*-ChIP assays from human left ventricle samples. Chromatin was incubated with α-GATA4 antibody, and then immunocomplexes were subjected to a second round of immunoprecipitation using antibodies against GATA4, GATA5, GATA6 or without antibody (control). Results are shown as fold immunoprecipitated DNA relative to the control condition (mean ± SEM, $n = 6$). Significance was examined by the *t*-test relative to no antibody control. $^{*}p \le 0.05$, $^{**}p \le 0.01$, and $^{***}p \le 0.001$.

expression vectors in H9c2 cells. We observed a synergistic effect on expression of the reporter gene when GATA4 and GATA5 were co-transfected (Fig. 2C). However, we did not see evidence of synergistic effects upon co-transfection of GATA4 and GATA6, or GATA5 and GATA6. Notably, when GATA4 and GATA6 were co-transfected, we detected a similar level of activation to the observed after overexpression of GATA4 alone. In a parallel experiment, we tested the effect of GATA4 and GATA6 co-expression on the *Pdx1* promoter. In this case, we observed synergistic activation of GATA4 and GATA6, as previously reported [22] (Supplementary Fig. S3). Altogether, these results suggest a major role of GATA4/5 synergism in the activation of the *SCN5A* promoter.

3.3. GATA4/GATA5 synergism is decreased in mutated intron 1 GATA-BS

Our ChIP and *Re*-ChIP experiments show that, in addition to the region upstream TSS, GATA TFs bind to the intronic 1 region (+505 bp). We therefore tested the effects of GATA TFs on a second construct, PromoterB-luciferase, that contains the luciferase gene under control of the fragment −260 to +612 bp from the *SCN5A* gene (Supplementary Fig. S1). PromoterB showed 3-fold higher transcriptional activity on the reporter gene than PromoterA (Fig. 3A), which supported previous findings suggesting that intron 1 contains additional *cis*-positive regulatory elements [8]. In addition, GATA4/5 synergism was greater on PromoterB than on PromoterA (Supplementary Fig. S4), but we observed a similar (~40–60%) reduction in *SCN5A* promoter activity associated to *Gata4* knockdown in both promoter constructs (Fig. 3B). These data suggest that the effect of *Gata4* knockdown on *Scn5a* mRNA levels in Fig. 2A is the result of a promoter-dependent activity, although we cannot completely exclude the possibility that *Gata4* siRNAs could have other indirect effects.

We also tested whether the regulatory roles of GATA4 and GATA5 on the *SCN5A* promoter were dependent on the presence of specific GATA-BS. For this purpose, we generated two new versions of PromoterB-luciferase construct containing mutations in one or two predicted GATA-BS (Fig. 3C and Supplementary Fig. S1B). These predicted elements, located within the intron 1 region, are highly conserved among mammals (Supplementary Fig. S5). Luciferase assays showed that activation of the *SCN5A* promoter by GATA4 decreased with the number of mutated GATA-BS. Similarly, GATA4 and GATA5 synergism was significantly reduced in GATA-BS mutant constructs (Fig. 3C). These

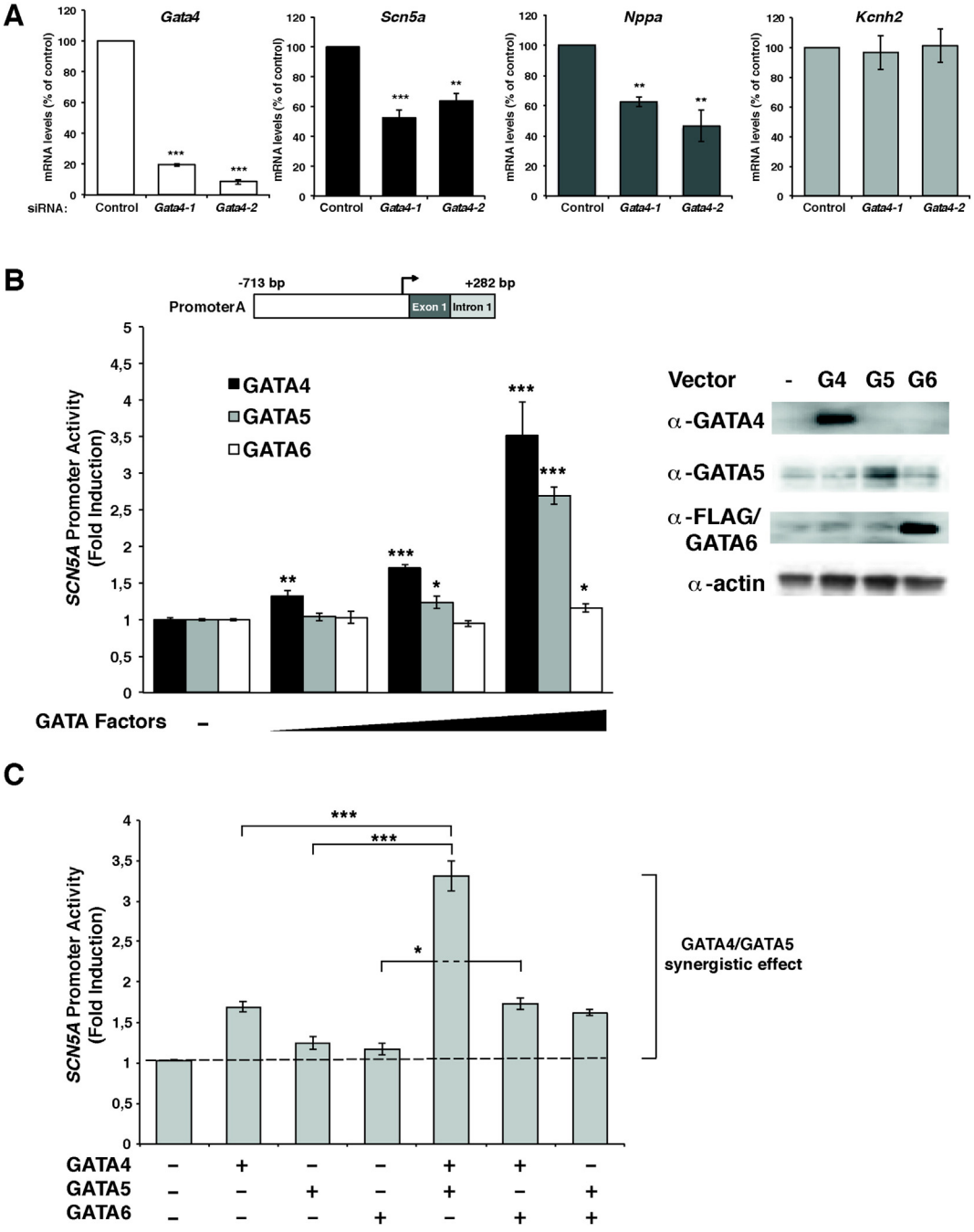

Fig. 2. GATA TFs activate the *SCN5A* promoter. A. *Gata4* siRNAs downregulate *Scn5a* expression. qPCR analysis of *Gata4*, *Scn5a*, *Nppa* and *Kcnh2* mRNA levels from H9c2 cells transfected with control or *Gata4* siRNAs (1 and 2). Transcript levels were normalized to *β-Actin* mRNA and reported as percentage relative to control siRNA (mean ± SEM, $n = 8$). Significance was examined by the *t*-test relative to control siRNA. B. Left panel. Luciferase experiments in H9c2 cells transfected with PromoterA-luciferase construct and increasing amounts (600, 1200 and 1800 ng) of the indicated GATA TF expression vectors. Luciferase values were normalized to renilla and are shown as fold induction relative to non-overexpressing control conditions (mean ± SEM, $n = 6$). Significance was examined by the *t*-test relative to control. Right panel. Western blot of transfected samples shows correct expression of GATA TFs. C. Luciferase experiments in H9c2 cells transfected with PromoterA-luciferase and the indicated GATA factor (1200 ng). Luciferase values were normalized with renilla and are shown as fold induction relative to control (mean ± SEM, $n = 6$). Multiple group comparison was performed using the ANOVA test. Statistical significance of *SCN5A* promoter activity in the presence of each GATA TF relative to control conditions is not shown in Fig. 2C for clarity, and was as indicated in Fig. 2B. $^{*}p \le 0.05$, $^{**}p \le 0.01$, and $^{***}p \le 0.001$.

findings further support that GATA4 and GATA5 mediate their regulatory actions on *SCN5A* transcription via GATA-BS. However, those sites present in intron 1 would not be completely indispensable, since transcriptional activity of a promoter construct without these sites (PromoterA) is also increased upon GATA TFs overexpression (Fig. 2B and C).

Since GATA4 and GATA5 co-regulate the *SCN5A* promoter, we also tested whether GATA4 and GATA5 interact. We transfected HEK293 cells with HA-tagged GATA4 and GATA5 expression vectors and immunoprecipitated GATA4 from the lysates using *anti*-HA antibodies. GATA5 was detected in the immunoprecipitated material by Western blot, suggesting that GATA4 and GATA5 interact, directly or indirectly (Fig. 3D). Collectively, our data suggest that GATA4 and GATA5 form a complex and synergistically activate *SCN5A* transcription via GATA-BS, including, but not exclusively, the most conserved GATA-BS in intron 1.

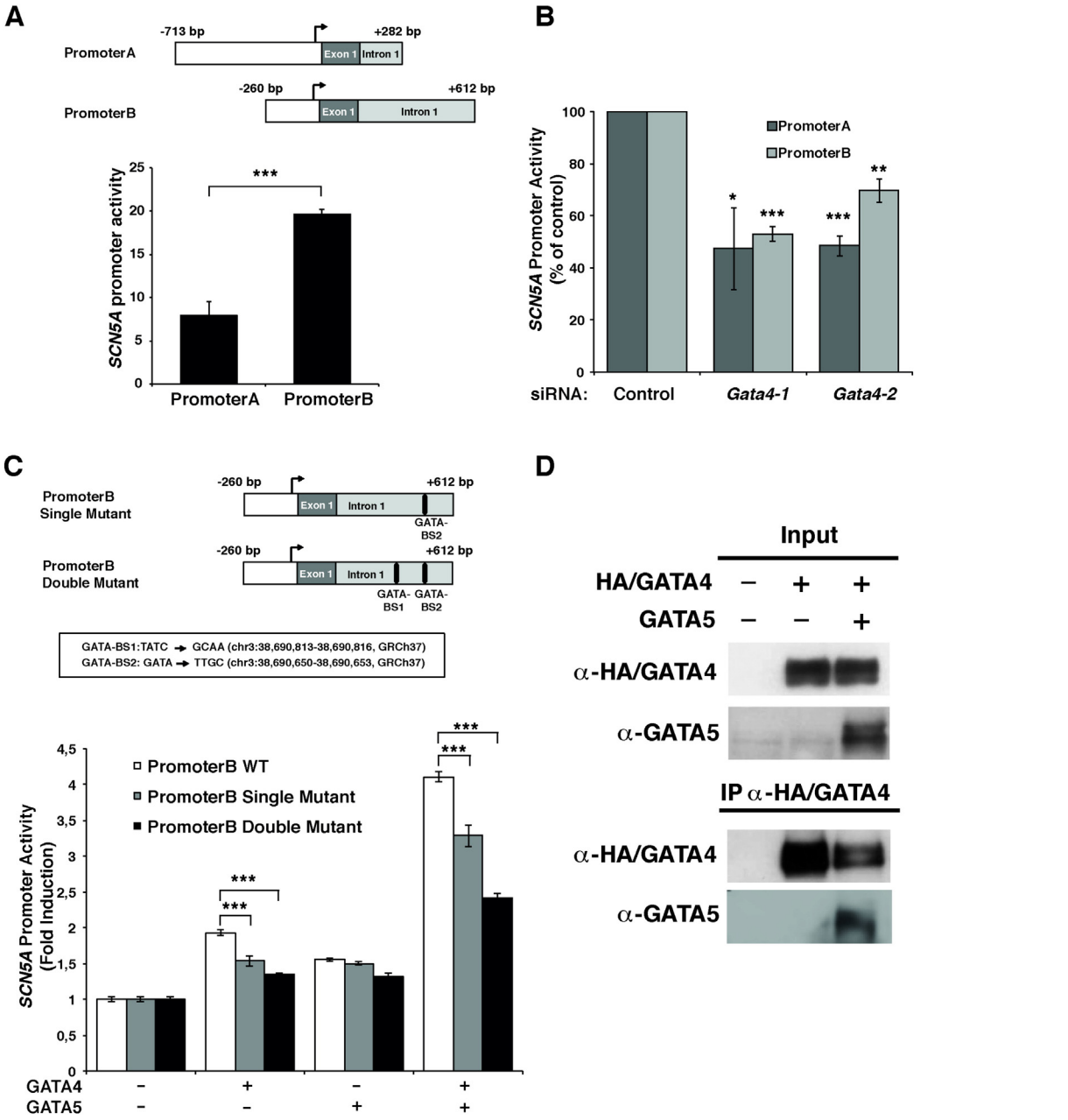

Fig. 3. GATA4/GATA5 synergism is decreased in mutated intron 1 GATA-BS. A. Luciferase assays in H9c2 cells transiently transfected with PromoterA-luciferase or PromoterB-luciferase (200 ng) and EF1αPromoter-Renilla (20 ng). Luciferase values were normalized with renilla (mean ± SEM, $n = 5$). Significance was examined by the *t*-test. B. Luciferase experiments in H9c2 cells transfected with PromoterA-luciferase or PromoterB-luciferase, and control or *Gata4* siRNAs. Luciferase values were normalized with renilla and are shown as percentage over control (mean ± SEM, $n = 3$). Significance was examined by the *t*-test relative to control siRNA. C. Top. Schematic representation of the single and double mutant PromoterB constructs. The numbers indicate the initial and the end position from the TSS (represented by an arrow) and the black lines indicate the mutated GATA-BS. Nucleotide changes for GATA-BS1 and GATA-BS2 and their genomic position according to GRCh37 are also shown. Bottom. Luciferase experiments in H9c2 cells transfected with PromoterB-luciferase WT or mutated constructs and the indicated GATA factor expression vectors. Luciferase values were normalized with renilla and are shown as fold induction relative to control (mean ± SEM, $n = 3$). Significance was examined by the *t*-test assay relative to the WT construct. D. Total lysates from HEK293 cells transfected with GATA4-HA and GATA5 expression vectors were immunoprecipitated with α-HA antibody. Western blot of input and immunoprecipitated samples is shown. Images are representative of two independent experiments. $^{*}p \le 0.05$, $^{**}p \le 0.01$, and $^{***}p \le 0.001$.

3.4. Correlation of GATA4 and SCN5A mRNA levels in human adult heart

To examine whether changes in *SCN5A* RNA directly correlate with sodium currents, we took advantage of *Xenopus* oocytes, which lack endogenous *SCN5A* and are a common model system in electrophysiological studies [23]. We injected increasing amounts of synthetic complementary RNA (cRNA) encoding human *SCN5A* in *Xenopus* oocytes and measured the corresponding $Na_V1.5$ current using whole cell patch-clamp. We observed a positive and linear correlation between peak $Na_V1.5$ current and injected levels of *SCN5A* cRNA ($r^2 = 0.9962$; Fig. 4A). Thus, the relative levels of *SCN5A* mRNA may have direct, linear biological effects.

To investigate a direct relationship between levels of *SCN5A* mRNA and GATA TFs in human heart tissue, we next applied the high sensitive and accurate droplet digital (ddPCR) approach. In fresh-frozen left ventricle samples (four samples from healthy donors and fourteen samples from end-stage heart failure patients), we detected robust *GATA4*, *GATA6*, and *SCN5A* mRNA expression, as well as marginal but still clear

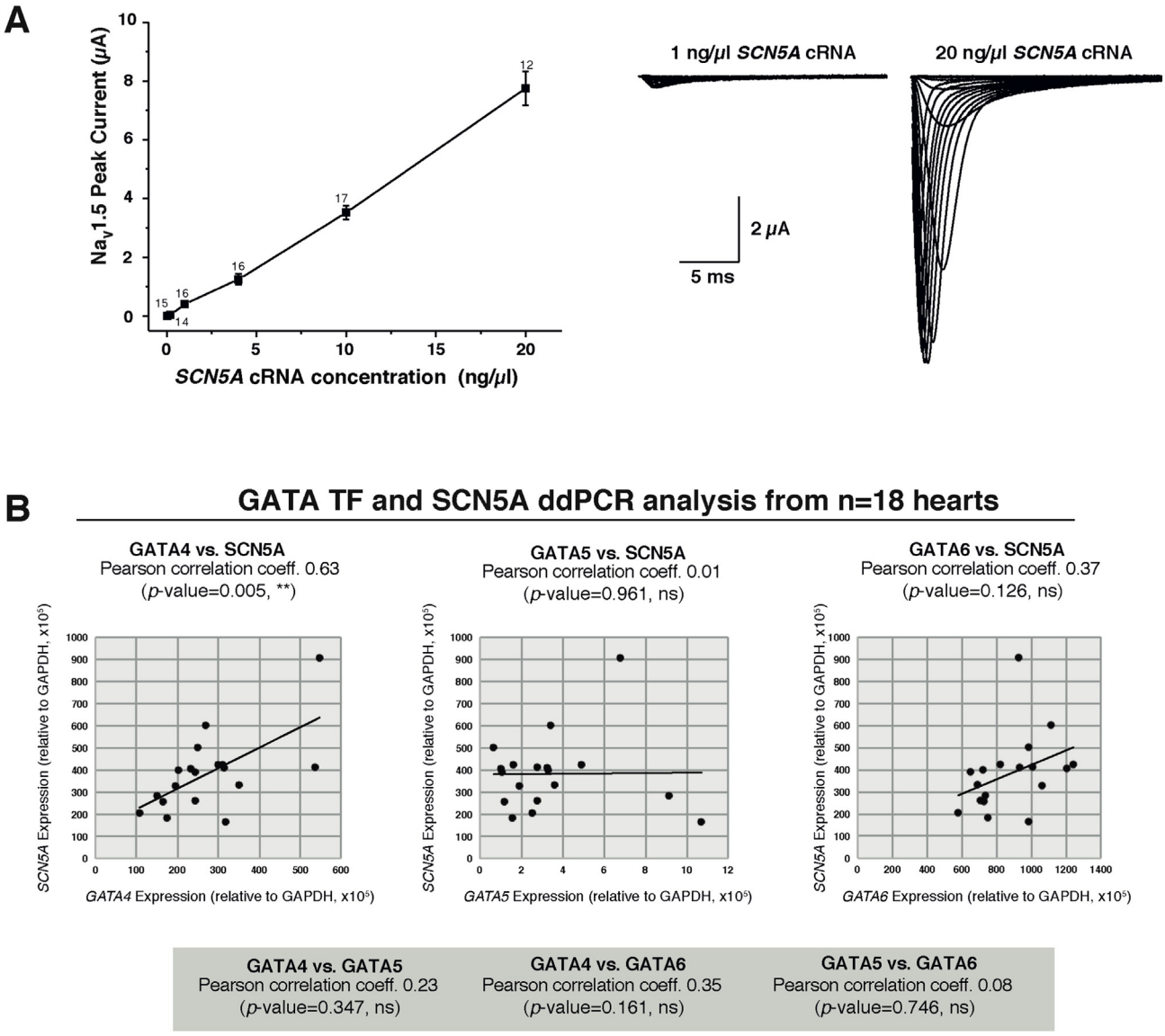

Fig. 4. A. Left. Peak Na$_V$1.5 current measurements of *Xenopus* oocytes injected with the indicated *SCN5A* cRNA concentrations. Numbers mean the measurements done in each condition. Right. Representative whole cell sodium current traces recorded from *Xenopus* oocytes injected with 1 and 20 ng/μl of *SCN5A* cRNA. B. Droplet digital PCR analysis of GATA TF and *SCN5A* mRNA levels in $n = 18$ human left ventricle samples. Data are displayed in scatter plots to compare each GATA TF with *SCN5A* mRNA levels, and Pearson correlation analysis was performed to determine statistical significant correlation. $^*p \le 0.05$, $^{**}p \le 0.01$, and $^{***}p \le 0.001$.

signal over background for *GATA5* mRNA (relative levels were as follows: *GATA4* levels were 76-fold higher than *GATA5* levels, and *GATA6* levels were 2.6-fold higher than *GATA4* levels; Fig. 4B). Similarly, we detected the three GATA TFs by Western blot in a fresh-frozen human left ventricle sample (Supplementary Fig. S6), although an accurate measurement of relative expression was not possible since each antibody intrinsically displays a particular affinity for its antigen.

Notably, we observed a positive and significant (Pearson coefficient 0.63) correlation between *GATA4* and *SCN5A* transcripts in human adult cardiac tissue (Fig. 4B). No significant correlation was detected between *GATA5* or *GATA6* and *SCN5A* mRNA levels, or among GATA TFs (Fig. 4B). Altogether, these results further support that GATA4 plays a major role in the regulation of *SCN5A* mRNA levels in human adult heart.

4. Discussion

This study reveals that GATA4 synergizes with GATA5 in the activation of the *SCN5A* gene via binding to GATA-BS within the *SCN5A* proximal promoter and intron 1 regions. This conclusion is supported by experiments performed in human adult left ventricle tissue, as well as in a cell line derived from rat embryonic ventricle. Our ddPCR analysis also reveals a positive and significant correlation between *GATA4* (but not *GATA5* or *GATA6*) and *SCN5A* mRNA levels in human cardiac samples, thus supporting a direct role of GATA4 in regulating *SCN5A* gene expression in the human heart. We should note, however, the limitation of this ddPCR analysis, since heart samples included the three heart layers (epicardium, myocardium and endocardium). Thus, the relative abundance of each layer in each sample may affect the overall measured levels of GATA factors and *SCN5A*, since *SCN5A* expression has been reported to vary across the human cardiac ventricular wall [24].

Previous reports showed synergistic effects of GATA4 with other TFs, including GATA6 [20], Nkx2-5 [25], TBX5 [16], and MEF2 [26]. Consistent with these effects, recent ChIP-seq studies in mouse cardiomyocytes identified cardiac enhancers co-occupied by multiple TFs, including GATA4 [27–29]. Notably, GATA4 was shown to co-locate with TBX3, TBX5, and NKX2-5 on two enhancers, included within the mouse *Scn10A* gene and downstream of the *Scn5a* gene [6,30]. These two enhancers activate *Scn5a* transcription by looping with the *Scn5a*

promoter, as it was shown by 4C-seq [7]. Here, therefore, we favor a mechanism by which GATA4 may function with GATA5 and other TFs, yet to be determined, to regulate the *SCN5A* gene. Although the role of GATA5 in the human adult heart remains unclear [10,11,31–34], our results favor a model in which GATA4 and GATA5 co-regulate the *SCN5A* gene, and we speculate that part of the controversy about GATA5 expression in the adult heart can be explained by divergent mRNA and protein GATA5 levels.

Our ChIP and *Re*-ChIP experiments suggest that GATA4 and GATA5 bind to the *SCN5A* promoter and intron 1 regions in the human adult heart, while no binding is detected in far upstream regions (−8803 bp and −5916 bp). These observations are in agreement with recent findings showing that, in mouse fetal heart, ~80% of GATA4 sites are distal to TSS regions; however, in mouse adult heart, GATA4 shifts to TSS-proximal locations, and only ~45% of GATA4 sites are distally located [13]. In this study, He et al. detected GATA4 ChIP-seq signal at the mouse *Scn5a* promoter, but not at intron 1 [13]. Data from the Mouse ENCODE Project revealed that *cis*-regulatory elements have undergone important evolutionary changes between human and mouse, but the regulatory networks are highly conserved between orthologous cell types of both species [35]. Consistently, we speculate that, although GATA4-BS differ between human and mouse, GATA4 function as regulator of the *SCN5A* gene is preserved. This possibility is supported by the observation of substantial reduction in H3K27 acetylation, a histone mark associated with active transcription, on the *Scn5a* promoter in *Gata4* knockout mouse embryos, thus confirming the importance of GATA4 in *SCN5A* gene expression activation [13].

The identification of GATA4 and GATA5 as novel regulators of *SCN5A* transcription raises the possibility that these factors may be associated with arrhythmogenic diseases. Genetic variants in *GATA4* (or *GATA5*) genes could be related to arrhythmogenic diseases via dysregulation of *SCN5A* expression. The effect of mutations on GATA4 (and GATA5) function could determine the levels of $Na_V1.5$ channel expression, which could explain the variability of phenotypes associated with *SCN5A*-related arrhythmias. In support of this idea, *Gata4* +/− mice, although viable, display short PR intervals [14].

Our data also raises the intriguing question whether genetic variation in GATA-BS could lead to arrhythmogenic diseases. Single nucleotide variants (SNVs), insertions, and deletions affecting GATA-BS could impair GATA TF regulatory functions on the *SCN5A* gene, thereby modulating *SCN5A* expression or even protecting from cardiac arrhythmias. The 1000 Genomes database includes two SNVs in the GATA-BS regions studied here, rs552565807 and rs115996915, but direct studies would be needed to identify their potential effects in *SCN5A* expression. In any case, the possibility that genetic variations in GATA-BS could lead to arrhythmias is already supported by genome-wide association (GWAS) studies indicating that most disease-associated variants lie within regulatory regions [36]. Indeed, de novo discovery of mutations in nonexonic regions (versus exonic regions) holds the promise that uncovering the transcriptional regulation of cardiac genes can lead to a better understanding of heart diseases.

5. Conclusions

In this study, we report the regulatory role of GATA4 on the *SCN5A* gene in human heart. Our work provides evidences that: 1) GATA4 and GATA5 co-occupy the *SCN5A* promoter and the first intronic regions, interact, and synergize to regulate the expression of the *SCN5A* gene in human heart tissue; 2) two GATA-BS found in intron 1 are important but not the sole contributor elements for this regulatory role; and, 3) *GATA4* and *SCN5A* mRNA levels positively correlate in adult human hearts. Overall, we anticipate that the study of $Na_V1.5$-related arrhythmias will need to extend to GATA TFs and GATA-BS.

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Disclosures

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2016.10.013.

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