Zfhx3 Transcription Factor Represses the Expression of SCN5A Gene and Decreases Sodium Current Density (I_{Na})

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Abstract: The ZFHX3 and SCN5A genes encode the zinc finger homeobox 3 (Zfhx3) transcription factor (TF) and the human cardiac Na+ channel (Nav1.5), respectively. The effects of Zfhx3 on the expression of the Nav1.5 channel, and in cardiac excitability, are currently unknown. Additionally, we identified three Zfhx3 variants in probands diagnosed with familial atrial fibrillation (p.M1260T) and Brugada Syndrome (p.V949I and p.Q2564R). Here, we analyzed the effects of native (WT) and mutated Zfhx3 on Na+ current (I_{Na}) recorded in HL-1 cardiomyocytes. ZFHX3 mRNA can be detected in human atrial and ventricular samples. In HL-1 cardiomyocytes, transfection of Zfhx3 strongly reduced peak I_{Na} density, while the silencing of endogenous expression augmented it (from −65.9 ± 8.9 to −104.6 ± 10.8 pA/pF; p ≥ 8, p < 0.05). Zfhx3 significantly reduced the transcriptional activity of human SCN5A, PITX2, TBX5, and NKX25 minimal promoters. Consequently, the mRNA and/or protein expression levels of Nav1.5 and Tbx5 were diminished (p ≥ 8, p < 0.05). Zfhx3 also increased the expression of Nedd4-2 ubiquitin-protein ligase, enhancing Nav1.5 proteosomal degradation. p.V949I, p.M1260T, and p.Q2564R Zfhx3 produced similar effects on I_{Na} density and time- and voltage-dependent properties in WT. WT Zfhx3 inhibits I_{Na} as a result of a direct repressor effect on the SCN5A promoter, the modulation of Tbx5 increasing on the I_{Na}, and the increased expression of Nedd4-2. We propose that this TF participates in the control of cardiac excitability in human adult cardiac tissue.

Keywords: Zfhx3; SCN5A; Nav1.5; Tbx5; Pitx2c; cardiac; sodium current; patch-clamp

1. Introduction

ZFHX3 gene encodes Zfhx3 or the AT motif binding factor (ATBF1), a transcription factor (TF) with multiple homeodomains and zinc finger motifs. Zfhx3 is widely expressed in many tissues [1] and participates in the regulation of myogenic [2] and neuronal differentiation. Zfhx3 was reported to inhibit cell proliferation, negatively regulate c-Myb, and trans-activate the cell cycle and cyclin-dependent kinase inhibitor 1A, thus functioning as...
a tumor suppressor in several cancers [3]. Additionally, it was demonstrated that Zfhx3 participated in some TF networks in the heart. Indeed, Huang and coworkers showed that Zfhx3 positively and reciprocally regulated the expression of PITX2, which encodes the Pitx2c TF [4]. Moreover, both, Zfhx3 and Pitx2c, regulate the expressions of NPPA, TBX5 and NKX25 genes which encode the atrial natriuretic peptide, as well as Tbx5 and Nkx2.5 TFs, respectively [4].

Genome-wide association studies (GWAS) significantly associated atrial fibrillation (AF) with two variants (rs7193343 and rs2106261) in the ZFHX3 gene that appear outside coding regions [5,6]. The rs7193343 variant was also associated with ischemic and cardioembolic stroke [7]. The association of the rs2106261 variant with AF was replicated in an Asian cohort. Moreover, rs2106261 was also associated with coronary disease in an African American cohort [8,9]. Previous studies did not identify a clear association between this SNP and measures of atrial structure [10]. Moreover, an update meta-analysis demonstrated that rs7193343 and rs2106261 were not associated with AF recurrence [11]. More recently, variants in ZFHX3 were also associated with sick sinus syndrome [12].

The SCN5A gene encodes the human cardiac Na$^+$ channel (Nav1.5), which generates the fast Na$^+$ current (I$_{Na}$). The I$_{Na}$ is responsible for the Na$^+$ influx that depolarizes the membrane potential during the atrial and ventricular action potential upstroke. Thus, it plays a critical role in excitability and intracardiac conduction velocity. Recently, it was functionally demonstrated that Tbx5, a TF belonging to the T-box family, promotes the expression of the SCN5A gene, and thus increased I$_{Na}$ in human cardiomyocytes derived from induced pluripotent stem cells (hiPSC-CM) [13]. Furthermore, the Brugada Syndrome (BrS)-associated variant p.F206L Tbx5, lacks this pro-transcriptional effect, thus markedly reducing I$_{Na}$ [13]. On the other hand, Nav1.5 expression decreases in atrial chamber-specific Pitx2 conditional mutants, suggesting that Pitx2c positively modulates Scn5a expression [14]. However, in another report it was demonstrated that Scn5a mRNA expression increases in the left atria of mouse after Pitx21 heterozygous deletion [15].

Here, we decided to analyze the effects of native (WT) Zfhx3 on the I$_{Na}$ magnitude and the expression of the human minimal SCN5A promoter. Moreover, we also tested the effects of three different variants that we identified in unrelated probands diagnosed with familial AF (p.M1260T) and BrS (p.V949I and p.Q2564R). Our results demonstrated that WT Zfhx3 inhibits I$_{Na}$ as a consequence of a direct repressor effect on the SCN5A promoter, by the modulation of Tbx5-increasing effects on the I$_{Na}$, and by increasing the expression of Nedd4.2. Additionally, we described that all three of these variants also inhibit I$_{Na}$ similarly to WT Zfhx3, even when p.Q2564R Zfhx3 lacks the repressor effect at the level of the minimal SCN5A promoter.

2. Results

2.1. Zfhx3 Is Expressed in the Human Myocardium

First, we questioned whether Zfhx3 was expressed in the human adult myocardium. To answer this question, we accessed the Genotype-Tissue Expression (GTEx) project which collects and analyzes multiple human post mortem tissues [16]. GTEx RNA-seq data of ZFHX3 from human atrial ($n$ = 297) and ventricular ($n$ = 303) samples averaged 2.2 ± 0.05 and 1.4 ± 0.04 transcripts per million (TPM), respectively. These data suggest that Zfhx3 is indeed expressed, even though the mRNA expression level, both in the atria and ventricles, is significantly lower ($p < 0.01$) than that of TBX5 (59 ± 1.5 and 12.6 ± 0.5 TPM in atria and ventricles, respectively) and NKX25 (115 ± 3.3 and 106 ± 3.5 TPM). For comparison, mRNA expression levels of PITX2 are significantly ($p < 0.01$) lower than those of ZFHX3 (0.4 ± 0.09 and 0.02 ± 0.002 TPM in atria and ventricles, respectively). Certainly, Pitx2c is almost not expressed in human ventricles, while its low expression in the atria increases in patients with chronic AF [17].
2.2. Zfhx3 Markedly Reduces $I_{Na}$

Figure 1A shows $I_{Na}$ traces recorded in HL-1 cells transfected/not transfected with the plasmid encoding the native form of the human Zfhx3 using the protocol shown in the upper part. In Figure 1B, the $I_{Na}$ density is represented as a function of the membrane potential of the test pulse. In cells transfected with Zfhx3, the $I_{Na}$ density consistently and significantly decreased compared with non-transfected cells at several membrane potentials. In fact, maximum $I_{Na}$ decreased from $-70.6 \pm 6.8$ to $-27.7 \pm 2.8 \mu A/pF$ ($p < 0.05, n \geq 26$). The overexpression of Zfhx3 in HL-1 cells was confirmed by WB (Figure 1E).

![Figure 1](image.png)

**Figure 1.** (A,C). $I_{Na}$ traces recorded by applying the protocol shown at the top in HL-1 cells transfected/not transfected (–) with Zfhx3 WT, (A) or with scrambled or siRNA against Zfhx3 (siZfhx3) (C). (B,D). Mean current-density voltage curves for $I_{Na}$ recorded in HL-1 cells transfected/not transfected with Zfhx3 WT (B) or with scrambled or siZfhx3 (D). (E) Representative WB images showing the expression of Zfhx3 (top) and vinculin (bottom; loading control) in cells transfected/not transfected with Zfhx3 WT. (F,G). Representative WB (top) and total protein gel (bottom) images (F) and the corresponding densitometric analysis (G) showing the expression of Zfhx3 (red arrow) in cells transfected with Scrambled (Scr) or siZfhx3. In (B,D,G) each point/bar is the mean ± SEM of “n” experiments. ** $p < 0.01$ vs. Scrambled. In (B,D), ANOVA followed by Tukey’s test and multilevel mixed-effects model; in (F) un-paired two tailed Student’s $t$-test and multilevel mixed effects model.

In another group of experiments, the expression of Zfhx3 in HL-1 cells decreased when using siRNAs (Figure 1F,G). The specificity of the silencing effects was confirmed by using a scrambled siRNA in “control” cells. Figure 1C,D shows that $I_{Na}$ density was significantly increased in Zfhx3-silenced cells ($p < 0.05, n \geq 9$).
We also analyzed the possible effects of Zfhx3 on the voltage dependence of $I_{\text{Na}}$ activation (Figure 2A,B) and inactivation (Figure 2C,D). Neither transfection nor silencing of Zfhx3 modified either the voltage dependence of activation or inactivation of the $I_{\text{Na}}$. Consequently, the midpoint and slope values of the activation and inactivation curves were not modified under any experimental condition (Table 1) ($p > 0.05$, $n \geq 6$).

Figure 2. (A,B). Voltage-dependence of $I_{\text{Na}}$ activation ($G/G_{\text{max}}$) in HL-1 cells transfected/not transfected (−) with Zfhx3 WT (A) or with scrambled or siRNA against Zfhx3 (siZfhx3) (B). (C,D). Voltage dependence of $I_{\text{Na}}$ inactivation in HL-1 cells transfected/not transfected with Zfhx3 WT (C) or with scrambled or siZfhx3 (D). In (A–D) continuous lines represent the fit of a Boltzmann equation. Each point is the mean ± SEM of “n” experiments.

Time dependence of $I_{\text{Na}}$ activation was quantified by fitting a monoexponential function to the activation phase of the maximum $I_{\text{Na}}$ trace of each experiment [13]. The activation time constant ($\tau_{\text{act}}$) averaged 0.19 ± 0.01 ($n = 43$) and 0.22 ± 0.03 ms ($n = 19$) in cells transfected/not transfected with Zfhx3, respectively, indicating that the TF did not modify the activation kinetics ($p > 0.05$) (Table 1). The inactivation kinetics of maximum $I_{\text{Na}}$ current traces were described by a biexponential function. Figure 3A shows the fast ($\tau_{\text{f}}$) and slow ($\tau_{\text{s}}$) time constants of inactivation of peak $I_{\text{Na}}$ recorded in HL-1 cells transfected/not transfected with Zfhx3. As can be observed, Zfhx3 expression did not modify the inactivation kinetics of the $I_{\text{Na}}$ (Table 1). Consistently with these findings, Zfhx3 silencing did not modify it either (Figure 3B and Table 1). The persistent component of $I_{\text{Na}}$ or late $I_{\text{Na}}$ ($I_{\text{Na,L}}$) was quantified as the percentage of the peak $I_{\text{Na}}$ [13] and plotted in Figure 3C,D. These figures demonstrate that $I_{\text{Na,L}}$ magnitude was not different in HL-1

cells transfected/not transfected with Zfhx3 (Figure 3C) \((p > 0.05, n \geq 14)\) or in cells in which Zfhx3 expression was silenced/not silenced (Figure 3D) \((p > 0.05, n \geq 7)\). Finally, we also analyzed the reactivation kinetics of the \(I_{Na}\) using a double-pulse protocol (upper panel in Figure 3E). The reactivation process was described by the fit of a monoexponential function to the data and, as is shown in Figure 3E and Table 1, Zfhx3 did not significantly modify this \((p > 0.05, n \geq 11)\). The reactivation kinetics were also identical in cells in which the expression of Zfhx3 was silenced/not silenced by means of specific siRNAs \((p > 0.05, n \geq 4)\) (Figure 3F and Table 1).

### Table 1. Effects on the time- and voltage-dependent properties of \(I_{Na}\).

| Zfhx3     | \(\tau_{act}\) (ms) | \(V_{hact}\) (mV) | \(k_{act}\) | \(\tau_{sinact}\) (ms) | \(A_{sinact}\) (%) | \(\tau_{sinact}\) (ms) | \(A_{sinact}\) (%) | \(V_{hinact}\) (mV) | \(k_{inact}\) | \(\tau_{react}\) (ms) |
|-----------|----------------------|-------------------|-------------|-------------------------|-------------------|------------------------|----------------------|-----------------|-------------------|----------------------|
| ⋯         | 0.22 ± 0.03          | −45.4 ± 1.9       | 5.1 ± 0.3   | 1.1 ± 0.05              | 12.4 ± 1.6        | −91.8 ± 2.2            | 5.0 ± 0.1            | 14.9 ± 4.3       |
| WT        | 0.19 ± 0.01          | −42.6 ± 0.9       | 5.5 ± 0.2   | 1.2 ± 0.06              | 10.8 ± 1.1        | −91.7 ± 1.8            | 5.2 ± 0.2            | 12.9 ± 1.4       |
| p.V949I     | 0.20 ± 0.02          | −40.8 ± 2.2       | 5.1 ± 0.3   | 1.1 ± 0.07              | 83.3 ± 1.8        | 16.7 ± 1.8             | 5.1 ± 0.2            | 15.0 ± 2.2       |
| p.M1260T    | 0.24 ± 0.04          | −43.6 ± 2.3       | 5.3 ± 0.3   | 1.1 ± 0.01              | 80.9 ± 1.9        | 19.1 ± 1.9             | 5.0 ± 0.2            | 15.7 ± 2.5       |
| p.Q2564R    | 0.19 ± 0.02          | −44.9 ± 1.6       | 5.3 ± 0.2   | 1.2 ± 0.08              | 85.9 ± 2.6        | 14.1 ± 2.6             | 5.2 ± 0.2            | 12.3 ± 1.1       |
| Scrambled  | 0.18 ± 0.01          | −40.4 ± 1.5       | 5.2 ± 0.3   | 1.1 ± 0.1               | 85.0 ± 3.0        | 15.0 ± 3.0             | 5.0 ± 0.1            | 12.7 ± 2.9       |
| siZfhx3    | 0.18 ± 0.02          | −40.0 ± 1.9       | 5.1 ± 0.3   | 1.1 ± 0.05              | 88.1 ± 0.7        | 11.9 ± 0.7             | 5.1 ± 0.3            | 12.4 ± 3.1       |

\(A_{sinact}\) and \(A_{sinact}\) = amplitudes of the fast and slow components of inactivation yielded by the fit of a biexponential function to the peak maximum current decay. \(\tau_{act}\) = time constant of activation yielded by the fit of a monoexponential function to the peak maximum current. \(\tau_{sinact}\) and \(\tau_{sinact}\) = fast and slow time constants of inactivation yielded by the fit of a biexponential function to the peak maximum current decay. \(\tau_{react}\) = time constant of recovery from inactivation for \(I_{Na}\). \(V_{hact}\) and \(k_{act}\) = midpoint and slope values of conductance-voltage curves; \(V_{hinact}\) and \(k_{inact}\) = midpoint and slope values of the inactivation curves. Each value represents mean ± SEM of >6 cells/experiments from at least 3 different dishes in each group. Statistical comparisons were made by using ANOVA followed by Tukey’s test.

**Figure 3.** (A,B). Fast and slow time constants of inactivation obtained by fitting a biexponential function to the maximum \(I_{Na}\) traces in HL-1 cells transfected/not transfected with Zfhx3 WT (A), or with scrambled or siRNA against Zfhx3 (siZfhx3) (B). (C,D). Mean \(I_{Na}\), recorded in HL-1 cells transfected/not transfected with Zfhx3 WT (C), or with scrambled or siZfhx3 (D). (E,F). Time course of the recovery of \(I_{Na}\) inactivation in HL-1 cells transfected/not transfected with Zfhx3 WT (E) or with scrambled or siZfhx3 (F). Each point/bar is the mean ± SEM of “n” experiments.
In conclusion, Zfhx3 markedly decreased $I_{\text{Na}}$ without modifying its voltage- and time-dependent characteristics. These data suggested that Zfhx3 inhibited $I_{\text{Na}}$ just by decreasing the expression of the Nav1.5 proteins. Thus, Western blot (WB) experiments were conducted to test this hypothesis and demonstrated that HL-1 cells transfected with Zfhx3 significantly decreased Nav1.5 protein levels ($p < 0.05$, $n \geq 7$) (Figure 4A,B). Moreover, a concomitant decrease in the Scn5a mRNA expression was detected by Reverse Transcription Quantitative PCR (RT-qPCR) experiments ($p < 0.05$, $n \geq 4$) (Figure 4C). Finally, we tested whether Zfhx3 directly reduced the expression of the human minimal SCN5A promoter using luciferase assays. Indeed, Zfhx3 repressed the expression of the minimal SCN5A promoter, as well as that of the human minimal SCN1B promoter, which encoded the Navβ1 ancillary subunit of the cardiac Na$^+$ channel ($p < 0.05$, $n \geq 3$) (Figure 4D).

Figure 4. (A,B). Representative WB (top) and total protein gel (bottom) images (A) and densitometric analysis (B) of the expression of Nav1.5 (red arrow) in HL-1 cells transfected/not transfected with Zfhx3 (B). (C,D). Scn5a mRNA levels (C), and relative luminescence values generated by human SCN5A and SCN1B minimal promoters (D) measured in HL-1 cells transfected/not transfected with Zfhx3 WT. In (B–D), each bar is the mean $\pm$ SEM of “n” experiments; each dot represents 1 experiment in (B,C) and is the mean value of a technical triplicate in (D). (B,C) Un-paired, two-tailed Student’s t-test and multilevel mixed-effects model. (D) ANOVA followed by Tukey’s test and multilevel mixed-effects model.
2.3. Zfhx3, Tbx5 and Pitx2c Interplay

Previous results suggested that there is a positive reciprocal modulation between ZFHX3 and PITX2C [4]. Additionally, both Zfhx3 and Pitx2c regulate the expression of other cardio-specific TF such as Tbx5 and Nkx2.5 [4]. Thus, we decided to test the effect of Zfhx3 on the expression of these TFs, which also regulated the magnitude of cardiac $I_{Na}$ [13]. Figure 5A,B shows that transfection with Zfhx3 significantly decreased the protein expression of Tbx5 as demonstrated by WB experiments. Moreover, Zfhx3 significantly decreased the TBX5 mRNA expression (Figure 5C) ($p < 0.05$, $n = 6$), an effect that we attributed to the remarkable inhibition produced by Zfhx3 on the expression of the human minimal TBX5 promoter as detected in luciferase assays (Figure 5D) ($p < 0.01$, $n = 6$). Regarding Pitx2c, unfortunately the HL-1 expression of both the protein and the mRNA was negligible; therefore, we were not able to accurately detect them with the antibodies and primers used in WB and RT-qPCR experiments (see Methods in the Supplementary Material). Conversely, in luciferase assays we could demonstrate that Zfhx3 significantly repressed the expression of the human minimal PITX2 promoter (Figure 5D) ($p < 0.01$, $n = 4$). Finally, the results of the luciferase assay demonstrated that Zfhx3 also markedly and significantly decreased the expression of the human minimal NKX25 promoter (Figure 5D) ($p < 0.01$, $n = 3$).

![Figure 5](image-url)

**Figure 5.** (A–D). Representative WB (top) and total protein gel (bottom) images (A); densitometric analysis of Tbx5 expression (red arrow) (B); Tbx5 mRNA levels (C); and relative luminescence values generated by human TBX5, PITX2, and NKX25 minimal promoters (D) measured in HL-1 cells transfected/not transfected with Zfhx3 WT. In (B–D), each bar is the mean ± SEM of “n” experiments; each dot represents 1 experiment in (B,C) and is the mean value of a technical triplicate in (D). (B,C) Un-paired two tailed Student’s t-test and multilevel mixed-effects model. (D) ANOVA followed by Tukey’s test and multilevel mixed-effects model.
2.4. Zfhx3 and Nedd4.2 Expression

It was extensively demonstrated that the ubiquitin protein ligase Nedd4-2 ubiquiti-nates Nav1.5 by binding to the PY motif located at the C-terminus of the channel, and promotes its degradation by the proteasome [18]. Since the expression of Zfhx3 markedly reduced \( I_{Na} \), we questioned whether it promoted in some way, the degradation of Nav1.5 channels. The WB data shown in Figure 6A,B demonstrated that the protein expression of Nedd4.2 significantly increased in HL-1 cells transfected with Zfhx3 \( (p < 0.05, n = 6) \). In accordance, mRNA levels of Nedd4l also significantly increased \( (p < 0.05, n = 7) \) (Figure 6C).

![Figure 6. (A–C). Representative WB (top) and total protein gel (bottom) images (A), densitometric analysis of Nedd4-2 expression (red arrow) (B), and Nedd4l mRNA levels (C) measured in HL-1 cells transfected/not transfected with Zfhx3 WT. In (B,C), each bar is the mean ± SEM of “n” experiments and each dot represents 1 experiment. Un-paired two tailed Student’s t-test and multilevel mixed-effects model.](image)

2.5. p.V949I, p.M1260T, and p.Q2564R Zfhx3 Variants

The first proband (BrS-1; III:1) is a 38-year-old male diagnosed with BrS (Figure 7A,B) who carries an implantable cardioverter defibrillator. His mother (II:3), uncle (II:1), aunt (II:5, mother’s sister), and sister (III:2) died suddenly at the ages of 59, 50, 51, and 32, respectively (Figure 7A). Another proband’s uncle (II:2) suffered a sudden cardiac arrest at age 72, but he was resuscitated. Gene panel sequencing identified that his mother carried a rare variant in KCN2 encoding p.Leu839Pro human ether-a-go-go (hERG) channels that generate the rapid component of the delayed rectifier current \( (I_{Kr}) \). The p.L839P hERG variant was never annotated in the Genome Aggregation Database (gnomAD, https://gnomad.broadinstitute.org/, accession on 29 November 2021) nor related with BrS. The gene panel sequencing demonstrated that the proband did not carry this variant (Table 2). However, it revealed that the patient carried a rare variant in CACNA1B gene encoding p.Arg377Gln Cav2.2 channels that was predicted as deleterious by two out six of the software tools for prediction of the impact of amino acids substitution (Table 2). The p.R377Q Cav2.2 variant, in turn, was not present in the patient’s mother (Figure 7A). No genetic data were available from his aunt and sister. The proband has two cousins (III:3 and III:4; daughters of his deceased aunt), who also carry the p.R377Q Cav2.2 variant and two nephews (IV:1 and IV:2; daughters of his deceased sister) who do not carry KCN2 or CACNA1B variants (Figure 7A). His cousins and nephews exhibit normal ECGs and do not have history of syncope. Furthermore, his cousins and another aunt (II:4) underwent a flecainide test that was negative.

Next-generation sequencing of the proband identified the p.Val949Ile Zfhx3 variant (rs113497421) that was predicted as deleterious (Table 2). The proband does not carry any other variant predicted as pathogenic (according to the guidelines for the interpre-
tation of variants, see Supplementary Material and Methods) [19] in genes linked with BrS. Considering the effects of Zfhx3 on $I_{Na}$, we hypothesized that the p.V949I variant could act as a genetic modifier and we decided to analyze its presence in the relatives available for a genetic test by Sanger sequencing. None of them carried the p.V949I Zfhx3 variant (Figure 7A).

![Figure 7A](image1.png)

**Figure 7.** (A,C,E). Pedigrees of the three families of this study. The arrows indicate the probands; circles and squares represent females and males, respectively, and filled symbols represent the subjects diagnosed with BrS (A,E) or familial AF; (C) + or − indicate the presence or absence of the variants. AF: atrial fibrillation; BrS: Brugada Syndrome; HCM: hypertrophic cardiomyopathy; SD: sudden death. (B,D,F). Representative ECGs of BrS-1 (B), AF-1 (D) and BrS-2 (F) probands.
Table 2. Summary of all nonsynonymous exonic missense variants identified in the probands.

| Proband | Gene   | Genotype | Ancestral Allele /Variant | dbSNP_ID   | MAF       | Aminoacid Substitution | Transcript   | Provean Prediction | SIFT Prediction | Polyphen Prediction | Mutation Taster       | Mutation Assessor | LRT     |
|---------|--------|----------|---------------------------|------------|-----------|------------------------|-------------|-------------------|----------------|-------------------|---------------------|------------------|---------|
| BrS-1   | CACNA1B| HET      | G/A                       | rs774297154| 0.0000041 | R377Q                  | NM_000718.3 | Deleterious       | Damaging        | Benign            | Benign              | Low              | Neutral |
|         | FLNB   | HET      | G/A                       | rs201369608| 0.00007   | R1009Q                 | NM_001164317.1 | Neutral           | Tolerated       | Benign            | Probably deleterious | Medium           | Damaging |
|         | ZFHX3  | HET      | G/A                       | rs113497421| 0.002     | V949I                  | NM_006885.3 | Neutral           | Tolerated       | Probably deleterious | Probably deleterious | Low              | Neutral |
| BrS-2   | ANK2   | HET      | A/G                       | D3126G     | 0.0011484 | Deleterious            | Deleterious             | Probably damaging | Medium           | Deleterious       | Neutral           | Damaging        |
|         | ZFHX3  | HET      | A/G                       | rs141564201| 0.0007    | Q2564R                 | NM_006885.3 | Neutral           | Tolerated       | Unknown           | Probably deleterious | Medium           | Damaging |
| AF-1    | NEURL1 | HET      | T/C                       | F35L       | 0.0042104 | Neutral                | Tolerated           | Benign            |                | Probably deleterious | Low              | Neutral |
|         | ZFHX3  | HET      | A/G                       | rs777360037| 0.000004 | M1260T                 | NM_006885.3 | Neutral           | Tolerated       | Benign            | Probably deleterious | Low              | Damaging |
| AF-2    | ANK2   | HET      | G/A                       | E3016K     | 0.0011484 | Neutral                | Tolerated           | Benign            |                | Probably deleterious | Low              | Neutral |
|         | ZFHX3  | HET      | A/G                       | rs777360037| 0.000004 | M1260T                 | NM_006885.3 | Neutral           | Tolerated       | Benign            | Probably deleterious | Low              | Damaging |

Only non-synonymous exonic missense variants with a coverage >30 and with an occurrence in our local database = 1 were included. AF = atrial fibrillation; BrS = Brugada Syndrome; HET = heterozygous; LRT: likelihood ratio test; MAF = mean minor allele frequency from all ethnic groups where the variant was identified as provided in https://gnomad.broadinstitute.org/, accession on 29 November 2021.
We also identified two 59- and 61-year-old siblings with AF (AF-1 and AF-2) (Figure 7C). AF-1 exhibited paroxysmal AF (Figure 7D) with frequent episodes that were suppressed when the patient underwent catheter ablation. One year later she was ablated to eliminate a right ventricular outflow tract tachycardia. At the present time, she suffers sporadic palpitations of a short duration (<1 min). AF-2 had persistent AF refractory to multiple electrical cardioversions that progressed to permanent AF treated with amiodarone. Additionally, he had hypertrophic cardiomyopathy as demonstrated by echocardiography. Their deceased mother (I:2) also had AF diagnosed when she was 50 years old, and suffered two stroke episodes. The deceased father (I:1) and sister II:1 of the probands also exhibited hypertrophic cardiomyopathy, while II:4 died of brain cancer when she was 55 years old. The two index cases have children and nephews who have not presented with any electrical or structural alterations to date (third generation; Figure 7C). Next-generation sequencing of the probands identified that they shared the p.Met1260Thr Zfhx3 variant, predicted as deleterious by two out of six prediction tools (Table 2). The presence of this variant was analyzed by Sanger sequencing in other members of the family, and was identified in one of the daughters (III:6) of AF-2 (Figure 7C).

The third proband (II:3, BrS-2) was a 59-year-old woman who was diagnosed with BrS by an ECG conducted for pre-surgical purposes (Figure 7E,F). Thereafter, she underwent a flecainide test that was positive. She was and remains asymptomatic; however, her cousin (II:1, Figure 7E) suddenly died in his sleep when he was 49 years old. Her son (III.1) and daughter (III.2) exhibit normal ECG and had negative flecainide tests. Next-generation sequencing of the proband identified that they shared the p.Gln2564RArg Zfhx3 and the p. Asp3126Gly Ankyrin B variants. The latter was predicted as deleterious (Table 2). Furthermore, ANK2 loss-of-function variants were extensively related with inherited cardiac arrhythmias [20], even with BrS [21], although this relationship is currently under debate [22].

2.6. Effects of the p.V949I, p.M1260T, or p.Q2564R Zfhx3 on the \( I_{Na} \)

To functionally test the effects of these variants on \( I_{Na} \) we used HL-1 cells transfected/not transfected with p.V949I, p.M1260T, or p.Q2564R Zfhx3. As can be observed in Supplementary Figure S1, all these residues are very conserved in different species. The ZFHX3 mRNA expression is not different upon the transfection of cells with the cDNA encoding WT or mutated Zfhx3, as demonstrated by qPCR experiments (Cycle to threshold = 25.9 ± 0.3, 25.7 ± 0.3, 26.1 ± 0.2, and 25.6 ± 0.3 for WT, p.V949I, p.M1260T, and p.Q2564R Zfhx3, \( n = 7, p > 0.05 \)) (Supplementary Figure S2). The \( I_{Na} \) density recorded in cells transfected with these mutants was not statistically different than that recorded in cells transfected with the WT form (\( p > 0.05, n \geq 17 \)) (Figure 8A). Therefore, as in the presence of WT Zfhx3, maximum \( I_{Na} \) density was markedly and significantly decreased in cells transfected with Zfhx3 p.V949I, p.M1260T, or p.Q2564R compared with non-transfected cells (\( p > 0.01, n \geq 17 \)) (Figure 8B). As was the case with the WT form, none of the variants significantly modified any time- or voltage-dependent characteristics of the \( I_{Na} \) (Table 1).

Considering these results, we assumed that all three variants retained their repressor activity in the human minimal SCN5A and SCN1B promoters. Figure 8C shows that p.V949I and p.M1260T Zfhx3 significantly repressed SCN5A promoter expression (\( p < 0.01, n = 3 \)), whereas p.Q2564R produced no such effect. Conversely, Figure 8D shows that all three variants significantly repressed the expression of the SCN1B promoter (\( p < 0.01, n = 3 \)).

Since p.Q2564R Zfhx3 did not repress the expression of the SCN5A promoter, the question arose of how this variant also reduced the \( I_{Na} \) density. To answer it we conducted luciferase assays with all three Zfhx3 variants to test their effects on the expression of the human minimal TBX5, PITX2 and NKX25 promoters. Figure 9A shows that all three variants, including p.Q2564R, significantly repressed the expression of the minimal TBX5 promoter (\( p < 0.01, n \geq 3 \), p.V949I and p.M1260T also significantly repressed the expression of the minimal PITX2 and NKX25 promoters (Figure 9B,C) (\( p < 0.01, n \geq 3 \)). Conversely,
p.Q2564R Zfhx3 failed to repress the expressions of both PITX2 and NKX25 promoters (Figure 9B,C) \( p < 0.01, n \geq 3 \).

**Figure 8.** (A,B). Current-density voltage curves (A) and mean peak density (B) for \( I_{\text{Na}} \) recorded in HL-1 cells transfected with the indicated constructs. (C,D). Mean luminescence values measured in HL-1 cells transfected with human SCN5A (C) or SCN1B (D) minimal promoters together with the indicated Zfhx3 constructs. Each point/bar is the mean ± SEM of “\( n \)” experiments; in (B), each dot represents 1 experiment and, in (C,D), is the mean value of a technical triplicate. ANOVA followed by Tukey’s test and multilevel mixed-effects model.

**Figure 9.** (A-C). Mean luminescence values measured in HL-1 cells transfected with human minimal TBX5 (A), PITX2 (B) or NKX25 (C) promoters together with the indicated Zfhx3 constructs. Each bar is the mean ± SEM of “\( n \)” experiments and each dot represents the mean value of a technical triplicate. ANOVA followed by Tukey’s test and multilevel mixed-effects model.
3. Discussion

Our results demonstrate that Zfhx3 inhibits $I_{Na}$ as a result of a direct repressor effect on the SCN5A promoter, as well as the increased expression of Nedd4-2, and the modulation of Tbx5-increasing effects on the $I_{Na}$. Thus, we provide a novel and complex mechanism by which this TF could modulate cardiac excitability.

The overexpression of the cDNA encoding Zfhx3 in HL-1 cells markedly reduced $I_{Na}$ density, while Zfhx3 silencing led to the opposite result. Neither expression nor silencing modified the time- and voltage-dependent properties of the current or the $I_{Na,L}$, suggesting that the factor affected mostly the expressions of channel proteins, rather than gating. Our luciferase experiments demonstrated that Zfhx3 markedly decreases transcriptional activity of human SCN5A and SCN1B minimal promoters. Zfhx3 regulates transcription via direct interactions with predicted adenine and thymine-rich (AT) motifs [23] and these motifs are present in the promoter regions of both SCN5A and SCN1B genes (see Supplementary Figure S3). The effects on the SCN5A promoter were correlated with a reduction in mRNA and the protein levels of Nav1.5 channels. With regard to Navβ1, its effects on $I_{Na}$ properties depend on the expression system used [24]. Nevertheless, it is generally accepted that it increases the Nav1.5 cell surface expression [25]. Thus, it is possible that the decrease in Navβ1 levels produced by Zfhx3 contributes to $I_{Na}$ inhibition. Zfhx3 controls development, tumorigenesis and other biological processes that require a fast and dynamic protein turnover [26]. Therefore, we hypothesized that Zfhx3 could affect Nav1.5 channel degradation, in addition to synthesis. Indeed, we demonstrated that Zfhx3 increased the Nedd4-2 expression, an effect that favoured the proteasomal degradation of the channels [18] and would contribute to the net Zfhx3-induced $I_{Na}$ inhibition.

To the best of our knowledge, there are no previous data demonstrating a regulatory role of Zfhx3 on Nav1.5 channels. Conversely, data showing its effects on the expression and function of cardiac $K^+$ channels are more abundant [4,27,28]. It was shown that Zfhx3 silencing in HL-1 cells increased the expressions of Kv1.4, Kv1.5 and Kir3.4 channels, which resulted in augmented ultra-rapid delayed rectifier ($I_{Kur}$), transient outward ($I_{to}$) and acetylcholine-sensitive potassium ($I_{KAc}$) currents and the shortening of the action potential duration [27]. More recently, Lkhagva et al. described that Zfhx3 silencing in HL-1 cells led to a significant increase in the ATP-sensitive K current ($I_{KATP}$) [28]. There are also data suggesting that Zfhx3 regulates intracellular calcium handling, since Zfhx3 knockdown in HL-1 cells increased the ryanodine receptor (RYR2), RyR2 p2808, and SERCA2a (ATP2A2) mRNA expression [27].

There is evidence showing that Zfhx3 establishes gene regulatory networks with other cardio-enriched TFs such as Tbx5 and Pitx2c [29], suggesting that these TFs could regulate the expression of genes relevant to cardiac electrical activity in a coordinated manner. Indeed, Huang and coworkers demonstrated that, in HCT116 cells, Pitx2c negatively regulates the expression of miR-1, which reduced the expression of ZFHX3, resulting in a positive regulation of ZFHX3 by Pitx2c. Zfhx3, in turn, positively regulates expression of PITX2, resulting in a cyclic loop of cross-regulation between ZFHX3 and PITX2 [4]. Both, Zfhx3 and Pitx2c, regulate the expressions of NPPA, TBX5 and NKX25 genes which encoded the atrial natriuretic peptide, and Tbx5 and Nkx2.5 TF, respectively. Additionally, Tbx5 may also regulate the expression of Zfhx3 [30]. Furthermore, other TFs could also be involved. Indeed, a gene interaction network dominated by Nkx2-5, Tbx3, Zfhx3, and Synpo2l was recently identified in the human left atria [31].

Our luciferase assays, RT-qPCR and WB experiments confirmed the results of Tbx5, since the expression of Zfhx3 reduces the activity of the minimal promoter and the mRNA and protein levels of Tbx5. On the other hand, we recently demonstrated that Tbx5 increases Nav1.5 channel expression and $I_{Na}$ density in hiPSC cardiomyocytes by means of a pro-transcriptional effect on the gene promoter [13]. Thus, we assume that the repression of the TBX5 gene transcription produced by Zfhx3 contributes to the $I_{Na}$ inhibition produced by Zfhx3.
Conversely, under our experimental conditions, the expression of Zfhx3 did not increase, but decreased the luminescence generated by the human PITX2 minimal promoter. Unfortunately, in HL-1 cells, we were not able to detect measurable mRNA and protein levels of Pitx2c to confirm these results, most likely due to a limited expression of this factor. The reasons underlying the discrepancy between our results and those of Huang and coworkers [4] are unknown, although the use of different cellular models could play a role. The biology and expression levels of Zfhx3 and Pitx2c in HL-1 cells, which are of murine cardiac origin, and HCT116 cells, which are of human colon cancer origin, are likely very different.

Contradictory results were obtained regarding the effects of Pitx2c and the expression of the 

\( \text{Scn5a} \) gene. Previous reports demonstrated that Nav1.5 expression decreased in atrial-chamber-specific Pitx2 conditional mutants [14], while it increased in mouse left atria after Pitx2 heterozygous deletion [15]. Thus, we cannot predict the net effect on the \( I_{\text{Na}} \) magnitude produced by the repressor effects of Zfhx3 on the expression of the \( \text{PITX2} \) gene.

We also showed that Zfhx3 decreased the luciferase activity generated by the human N\( \text{NKX25} \) promoter. Our results suggest that Zfhx3, Tbx5, Pitx2c, and N\( \text{kx2-5} \) TFs reciprocally interact with each other in a complex way to control the expression of Nav1.5 channels, and thus cardiac conduction velocity and excitability. Furthermore, changes in the expression levels of these TFs would affect the \( I_{\text{Na}} \) magnitude. In this sense, it was proposed that noncoding variations in regulatory sequences affect the expression of genes encoding transcriptional regulators and/or the function of regulatory elements of given target genes, altering gene expression and conferring disease susceptibility [29]. Should this occur, changes in Zfhx3 levels produced by the SNPs identified in GWAS studies disrupted the equilibrium within the transcriptional networks, leading to important consequences on cardiac excitability.

We also analyzed the consequences of three \( \text{ZFHX3} \) variants found in probands that did not carry any variant in the genes so far associated with inherited arrhythmogenic syndromes. Our results show that the three variants produced a similar reduction in the \( I_{\text{Na}} \) density as the WT form, suggesting that they do not cause familial AF or BrS through differential effects on \( I_{\text{Na}} \). It is possible that these variants contribute to the phenotype of the carriers through different mechanisms not explored here, such as affecting intracellular calcium-handling proteins [27], changing the expression of \( \text{ZFHX3} \) or other genes, or acting as genetic modulators of another still unidentified causative variant [32]. Moreover, as described in Table 2, all three patients also carry other nonsynonymous exonic missense variants predicted as potentially deleterious. It is possible, that the phenotype of the patients is only apparent when, in addition to the variant in \( \text{ZFHX3} \), the other variants of which they are carriers are present. Unfortunately, our experiments in HL-1 cells cannot rule out this hypothesis since it would be necessary to generate cardiomyocytes derived from iPSC from each patient and to conduct the sequential correction of each variant with CRISPR-Cas9 technology. Tsai et al. identified four missense variants (p.E460Q, p.V777A, p.M1476I, and p.S3513G) in the coding region of the \( \text{ZFHX3} \) gene in patients with AF, although they did not functionally analyze the consequences of the variants [33]. To compare the predicted risk of these AF-associated variants with the variants identified here, we obtained the Combined Annotation Dependent Depletion (CADD) score (https://cadd.gs.washington.edu/, accession on 29 November 2021). CADD considers a wide range of functional categories, effect sizes and genetic architectures, and can be used to prioritize causal variations [34]. The score predicts that the risk of p.V949I and p.Q2564R is slightly higher than p.E460Q and much higher than the rest of the variants (Supplemental Table S1). Our luciferase assays showed that the p.Q2564R variant exhibited a different transcriptional profile from WT and the other variants analyzed here, since it did not reduce the luciferase activity generated by \( \text{SCN5A} \) and \( \text{PITX2} \) promoters. The reason for these differences could be related to the position of the variant. Both p.V949I and p.M1260T are within the eighth and twelfth zinc finger domains, while p.Q2564R is the closest to a DNA-binding domain, being in a reasonable proximity to the third homeodomain of Zfhx3.
Although the p.Q2564R variant led to a similar reduction in \( I_{Na} \) density than WT and the rest of the variants, our data suggest that it reduced \( I_{Na} \) density by suppressing the Tbx5-increasing effects, without producing a direct effect on SCN5A gene transcription.

**Limitations of the Study**

We did not analyze the effects of Zfhx3 expression on \( I_{Na} \) recorded in cultured native cardiomyocytes. To this end, we would need to include the cDNA of Zfhx3 within some viral vector. Unfortunately, the size of ZFHX3 (17,669 bp) prevents its inclusion in the vast majority of available viral vectors. We used HL-1 cells that were of a murine cardiac origin and were widely used for these purposes as a good model of cardiomyocytes in culture [35]. However, since HL-1 cells are not differentiated enough and do not display lateral membranes and intercalated disks, as is the case for adult cardiomyocytes, we were not able to analyze the possible effects of Zfhx3 on Nav1.5 localization. We surmise that Zfhx3 impacts the expression of the channels within the whole cardiomyocyte. However, we cannot rule out the differential effects on Nav1.5 targeted to specific sub-domains, since Zfhx3 may affect the expression/function of some of the multiple proteins that define distinct pools of Nav1.5 channels in cardiomyocytes [36,37]. Furthermore, the \( I_{Na} \) inhibition exceeds the decrease in the total expression of Nav1.5 protein produced by Zfhx3. This would suggest that Zfhx3 ultimately decreases the presence of Nav1.5 channels in the cell membrane (not explored here) by additional translational or post-translational mechanisms that are currently unknown. We did not demonstrate the binding of Zfhx3 to its target promoters by EMSA. Again, the huge size of the Zfhx3 protein (404 kDa) hampers its synthesis via conventional systems based on reticulocytes [13].

**4. Material and Methods**

**4.1. Study Approval**

The Investigation Committees of the University Hospitals La Paz, 12 de Octubre and Puerta de Hierro (ITACA study) approved the clinical evaluation of probands and all family members. Studies conformed to the principles outlined in the Declaration of Helsinki. Each patient gave written informed consent.

**4.2. DNA Sequencing**

DNA was extracted from whole blood, and whole-exome sequencing was performed at NIMGenetics (Madrid, Spain) [13,32]. Supplementary Table S2 describes the genes selected for the bioinformatics analysis performed after whole exome sequencing. The presence of the variants was also confirmed in the probands and some family members by Sanger sequencing.

**4.3. Access to Public Human Cardiac RNA-Seq Data**

GTEx RNA-seq data (RSEMv1.2.22 (v7) version) from human atria and ventricles and the resulting TPM values for ZFHX3, TBX5, PITX2, and NKX25 were accessed through the Human Protein Atlas database (https://www.proteinatlas.org/, accession on 29 November 2021) [13].

**4.4. Cell Culture and Transfection**

HL-1 cells were cultured and transiently transfected with the cDNA-encoding human WT or mutant Zfhx3 by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as previously described [13,17,32].

**4.5. Patch-Clamp Recordings**

\( I_{Na} \) was recorded at room temperature using the whole-cell, patch-clamp technique [13,17,32,38]. Micropipette resistance was kept below 1.5 MΩ when filled with the internal solution and immersed in the external solution (see composition in Supplementary Methods). To mini-
mize the influence of the expression variability, each construct was tested in a large number of cells obtained from at least 3 different HL-1 batches. In all cases, the expression of WT or mutated Zfhx3 was identified by the green fluorescent signal under fluorescent microscopy.

4.6. Analysis of the mRNA Expression (RT-qPCR)

mRNA expression of ZFHX3, SCN5A, SCN1B, NEDD4L, TBX5, PITX2, and NKX25 was measured by RT-qPCR using TaqMan Gene Expression Assays [17,32].

4.7. Western Blot Analysis and Zfhx3 Silencing

Nav1.5, Nedd4-2, and Tbx5 proteins were detected by WB in HL-1 cells transfected/not transected with WT or mutated Zfhx3 following procedures previously described. For Zfhx3 silencing, HL-1 cells were transfected with ON-TARGETplus mouse Zfhx3 siRNA SMARTpool or with siRNA Universal Negative Control (scrambled) by using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions [13,17,32,36,38].

4.8. Luciferase Assays

HL-1 cells were transfected with pLightSwitch_Prom [Active Motif, Carlsbad) vectors carrying the minimal promoters of human SCN5A, SCN1B, TBX5, PITX2, and NKX25 genes and luminescence was measured as described [13,17,32].

4.9. Statistical Analyses

Throughout the paper results were expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). To compare data from ≥3 experimental groups, one-way ANOVA followed by Tukey’s test was used, while unpaired two-sided t-test was chosen when comparing data from two experimental groups. In small size samples (n < 15), statistical significance was confirmed by using non-parametric tests (two-sided Wilcoxon’s test). To take into account repeated sample assessments, data were analyzed with multilevel mixed-effects models. Normality assumption was verified using the Shapiro–Wilk test. Variance was comparable between groups throughout the manuscript. We chose the appropriate tests according to the data distributions. A value of p < 0.05 was considered significant. Additional methodological details are included in Supplementary Materials.

5. Conclusions

Zfhx3 inhibits I
\(_{Na}\) by a complex mechanism involving the reduction in Nav1.5 channel expression, the modulation of the Tbx5-increasing effects, and the increase in Nav1.5 channel degradation by the proteasome. We propose that this TF could participate in the control of cardiac excitability in human adult cardiac tissue.

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